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SPIN STATES OF HEMEPROTEINS BY MAGNETIC CIRCULAR DICHROISM

Larry E. Vickery

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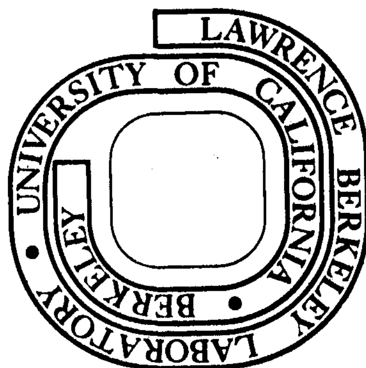

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SPIN STATES OF HEMEPROTEINS BY MAGNETIC CIRCULAR DICHROISM

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Spin States of Hemeproteins by MCD

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Introduction

The optical spectra of hemeproteins have been known for some time to be sensitive to the spin state of the heme iron, but no quantitative analysis of either absorption band intensity or wavelength position has proved generally applicable for their assignment. Recent findings on the magneto-optical activity of hemeproteins, however, suggest that this technique can provide a relatively straightforward spectroscopic method for determining spin states. The sensitivity of the method to both ferrous and ferric redox states and the ability to obtain measurements at physiological as well as cryogenic temperatures complements the more commonly used electron paramagnetic resonance technique. In addition measurements can be obtained on dilute solutions (micromolar range) in the presence of other paramagnetic species, offering advantages over direct susceptibility measurements, the Mössbauer effect and the Evans nuclear magnetic resonance shift method.

The phenomenon of magneto-optical activity was discovered over one hundred years ago by Michael Faraday who found that the presence of a magnetic field applied parallel to the direction of the measuring light beam could induce optical rotation. In contrast to natural optical activity this property is exhibited by all matter and does not require either an inherent molecular asymmetry or the presence of an asymmetric environment. Considerable progress toward understanding the theoretical basis of the origin of the Faraday effect has been achieved recently and several reviews on the subject are available.[1-3] The magnetically induced optical activity can be expressed and determined either as magnetic optical rotatory dispersion (MORD) or as magnetic circular dichroism (MCD). MORD measures the difference

in refractive index between left and right circularly polarized light (L and RCPL), while MCD measures the difference in absorption. Just as in natural optical activity the two phenomena can be related by the Kramers-Kronig dispersion relations; only MCD will be discussed here because it leads to less complex band shape and overlap and the transitions of interest occur in an optically accessible region. General discussions of biochemical applications of both MORD[4] and MCD[5] have appeared in this series.

Three types of magneto-optical effects can be distinguished according to their origin and each gives rise to a characteristic spectroscopic band shape (see Figure 1). Faraday A terms arise from transitions to orbitally degenerate excited states when the applied magnetic field splits the energy levels into components of opposite angular momentum (Zeeman effect); these then selectively absorb either L or RCPL because of the opposite angular momentum associated with the polarized photons. This effect can only occur in chromophores possessing sufficient symmetry to have molecular orbitals of similar energy, and since most metalloporphyrins have approximate D_{4h} symmetry, A terms are expected for the heme π-π^* transitions. The shape of A terms in an MCD spectrum resembles the derivative of the absorption band, and their intensity is a sensitive function of the excited state orbital angular momentum and the transition bandwidth and oscillator strength.

Faraday B terms arise from a magnetic field induced mixing of transitions. These occur in all compounds and are most intense for close-lying, orthogonally polarized transitions. They are in general weak for symmetric a, b and c type hemes but dominate the spectra of d heme and other porphyrin complexes of D_{2h} symmetry in which the x and y directions are not equivalent. Except for the change in sign these B terms resemble the absorption bands in shape.

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Finally, Faraday C terms occur in paramagnetic materials which have spin degenerate ground states. As with A terms a Zeeman splitting of the energy levels leads to a shift in the absorption of L and RCPL, but in this case the two initial levels will be differently populated according to a Boltzmann distribution. This gives rise to different absorption intensities for L and RCPL which are temperature dependent. For bands whose width is greater than the splitting induced (generally a few wave numbers) the net difference in absorption between L and RCPL yields an MCD shape resembling the absorption curve. The intensity of the MCD is directly proportional to the population difference of the two ground states and therefore to the reciprocal of the absolute temperature. All heme complexes with the exception of diamagnetic low spin ferrous forms have unpaired electrons and hence can be expected to exhibit C terms. This paramagnetic C type MCD is not restricted to optical transitions directly involving the iron atom (porphyrin-to-iron change transfer or iron d-d transitions) but is also observed for the predominantly π-π^* porphyrin bands in the visible and nearUV Soret regions due to (iron) spin-(porphyrin) orbit coupling.[6-8] It is this sensitivity of the MCD spectrum to the spin of the iron which makes the technique a useful probe of the hemeprotein active center. This article will describe methods of measuring the temperature dependence of the MCD which are necessary to establish the paramagnetic origin of C terms and investigate spin states in hemeproteins.

Examples of the types of MCD curves observed with hemeproteins in different redox and spin states are illustrated by the spectra of the myoglobin complexes shown in Figure 2. These curves are all taken from the room temperature results and hence by themselves do not distinguish C terms, but dramatic changes in the spectra with changes in the paramagnetism of the iron are evident. In the ferrous case the diamagnetic carbonmonoxide complex exhibits

only simple A terms typical of many hemochromes,[10] while the MCD of the deoxy form is extremely temperature dependent allowing one to distinguish between the low (S=0) and high (S=2) spin states. Both ferric forms are paramagnetic but the much more intense C terms of the low spin (S=½) when compared with the high spin (S=5/2) complex allow one to determine the amount of hemichrome present. A more detailed discussion of these observations will follow the sections on experimental procedures.

Instrumentation

Commercially available CD spectrophotometers can be adapted for MCD measurements simply by placing a magnet in the sample compartment so that the magnetic field is parallel to the measuring light beam. In the case of electromagnets this means that holes must be located through the pole pieces, and for superconducting magnets the bore must go through the solenoid. Superconducting magnets which attain fields of 50,000 Gauss or greater provide high sensitivity since MCD is directly proportional to field strength; this is advantageous when signals are weak in an absolute sense due either to a small magnetic anisotropy ratio ($\Delta\varepsilon_{MCD}/\varepsilon$) or to low concentrations and when the magnetic anisotropy is small relative to the natural anisotropy ($\Delta\varepsilon_{CD}/\varepsilon$). Electromagnets capable of generating near 15,000 Gauss, however, offer an important advantage in providing a more open and accessible sample region for large or complexly shaped cells, temperature control accessories, and side illumination. Sensitivity problems can be met by time averaging; in addition, they are less expensive to purchase and operate. The relatively intense signals of hemes together with the need for sample manipulations weighs in favor of the electromagnet for experiments on hemeproteins--all of the spectra reported here were obtained at fields from 9,000 to 15,000

Gauss--and the few modified procedures for working with superconducting magnets will not be discussed.

At least three types of improvements over commercial spectrometers can be made in laboratory designed instruments. The first of these involves the use of a piezo-optical (stress-plate) modulator rather than the conventional electro-optical modulator (Pockels cell) as a programmable quater wave plate. The quartz photoelastic device usually operates at frequencies near 50 KHz making kinetic experiments possible and can be driven at amplitudes sufficient to work in the near IR. Stephens and co-workers have assembled an MCD instrument which operates to 2 microns utilizing this and a solid state detector[11]; the spectrometer described by Sutherland et al. performs well to about 1.2 microns using a photomultiplier tube having an S-1 type photo-cathode.[12] A second type of improvement has been achieved by incorporating monochrometers with better dispersion in the visible and near IR regions.[11,12] While the double prism monochrometers in most commercial CD instruments function well in the UV, prism-grating combinations make it easier to resolve the often sharp heme bands which occur in the visible region and provide increased intensity and wavelength accuracy. Finally, interfacing a computer to any spectrometer system can greatly facilitate the large amount of data processing necessary to extract the MCD curves by making the calculations necessary to correct for the presence of natural CD and baselines as described below. In addition the computer can be used for signal averaging or smoothing, to convert raw data to the units desired and to accurately calculate even very small difference spectra.

Methods of Measurement

Units. In recording a spectrum what is actually measured is the difference in absorption of L and RCPL

$$\Delta A = A_L - A_R \qquad [1]$$

This value can be expressed in terms of molar absorptivity (or extinction) difference and placed on a unit magnetic field basis using the expression

$$\Delta\varepsilon/_H = \frac{\Delta A}{M \cdot l \cdot H} \quad \text{with the units } (M \cdot cm \cdot T)^{-1} \qquad [2]$$

where M = molar concentration, l = sample cell pathlength in cm, and H = magnetic field strength in Tesla where one T = 10,000 Gauss. The sign convention is such that $\Delta\varepsilon/H$ is positive when the field is parallel to the direction of propagation of the measuring beam and negative when antiparallel. These units are readily comparable to absorption $\varepsilon(M \cdot cm)^{-1}$ units and generally give $\Delta\varepsilon/H$ values for hemeproteins from 1-100 $(M \cdot cm \cdot T)^{-1}$. As an extension of early optical rotatory studies some results are reported in units of degrees ellipticity which are related to the absorption difference by

$$\Theta \text{ (deg.)} = 33\ \Delta A. \qquad [3]$$

the molar ellipicity is then often defined on a unit magnetic field basis of one Gauss

$$[\Theta]_m = \frac{100 \cdot \Theta}{M \cdot l \cdot H} \quad (\text{deg} \cdot \text{cm}^2 \cdot \text{decimole}^{-1} \cdot G^{-1}). \qquad [4]$$

The two MCD expressions are thus related by

$$[\Theta]_m/\text{Gauss} = 0.33\ \Delta\varepsilon/_{\text{Tesla}}.$$

<u>Calibration</u>. The sensitivity of the spectrometer can be checked using D-camphor sulfonic acid, preferably recrystallized several times from benzene or ethyl acetate and well dried. Reported values of the CD of this compound vary from $\Delta\varepsilon_{290}$ = 2.20 to 2.49 $(M \cdot cm)^{-1}$ (see Ref. 13), but most work has been reported on the basis of the lower value so that at a concentration of 1 mg/ml, $\Delta A_{290} = 9.3 \times 10^{-3}\ (cm)^{-1}$.

Magnetic field strength can be measured directly with a Hall probe or Gaussmeter or can be determined with chemical standards. Since field inhomogeneity

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can result from having holes in the tapered pole pieces, calibration with solutions has the advantage of averaging the magnetic flux over the volume actually measured providing similar path lengths and slit or beam widths are used. Potassium ferricyanide exhibits no natural CD but has an easily measurable MCD in the visible with $\Delta\varepsilon_{422}/H = 3.0\ (M\cdot cm\cdot T)^{-1}$ at room temperature, so that a 10^{-3}M solution in a 1 cm cell will exhibit a ΔA of 3×10^{-3}/10,000 Gauss field.[12] Fresh samples should be used and can be prepared gravimetrically or assuming $\varepsilon_{420} = 1{,}040\ (M\cdot cm)^{-1}$, and since the MCD signal arises from a C term the sample should be within a few degrees of 20°C. Cobaltous sulfate, $\Delta\varepsilon_{510}/H = 1.86 \times 10^{-2}(M\cdot cm\cdot T)^{1}$, has also been recommended for this purpose.[14]

Calculations. When analog results are read directly from charts, the MCD is most simply determined as the difference between the field-on ($\Delta A_{+} = \Delta A_{MCD} + A_{CD}$) and field-off ($\Delta A_{CD}$ only) spectra, a solvent baseline being necessary only if CD results are desired. Alternatively, the two spectra can both be recorded with the field on but reversed in direction. This changes the sign of the MCD without affecting the CD. The MCD is then calculated as one half the difference between the two curves

$$\Delta A_{MCD} = \frac{\Delta A_{+} - \Delta A_{-}}{2} \qquad [6]$$

When digital data storage and a computer are available it is advantageous to use the latter method since the field is on in both scans and the MCD signal-to-noise ratio is improved by a factor of $\sqrt{2}$; in addition, the CD, also signal averaged, can be calculated from the same experiment

$$\Delta A_{CD} = \frac{\Delta A_{+} + \Delta A_{-}}{2} \qquad [7]$$

With high quality digital data, weak CD (or MCD) spectra can be obtained by this method even in the presence of an intense MCD (or CD). Figure 3

shows the raw data and computer-generated MCD and CD plots of such an example. In this case no baseline spectra were recorded but precaution must be taken to insure a flat instrumental baseline and to provide proper shielding of the photomultiplier (mu metal is convenient) to prevent field lines of force from affecting the dynode chain amplification.

<u>Recording Spectra</u>. Certain aspects of MCD spectroscopy make it necessary to pay more careful attention to operating parameters than is usually necessary in absorption spectroscopy. One problem, common to CD as well, is that because very small differences in absorption of L and RCPL (generally one part in 10^3 to 10^5) are measured large numbers of photons are needed to obtain good statistics. Consequently, a high intensity light source and wide slit widths are generally used to improve signal-to-noise levels. However, care must be taken to maintain an instrumental bandpass less than 1/5 of the natural spectral band width to resolve narrow transitions; the slit width required can be determined from the dispersion curve provided with the instrument. The best procedure is to determine the slit width, response time constant and scan rate parameters for a given band empirically by decreasing each until no further sharpening or increase in intensity is observed. It is also recommended that multiple scans always be recorded even when signal averaging is not used to insure reproducibility and as a check for sample stability during the measurement.

<u>Sample Handling</u>. Room temperature MCD measurements can be obtained with the normal rectangular or cylindrical cuvettes used for absorption spectroscopy. Since MCD arises only from absorption and not refractive differences, strain or imperfections in the optical windows is not important and homemade cells of pyrex or lucite are quite satisfactory in the visible

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spectral region. Sample concentrations should be adjusted so that at least 5% of the light is transmitted (O.D.<1.3) at all wavelengths; optimal signal-to-noise ratio is obtained at an O.D. near 0.8 to 0.9, and at O.D. values above 2.0 (<1% transmittance) not only is the noise level unnecessarily high but there is a danger of non-linear dynode gain. For a typical hemeprotein this mears that a total heme concentration of about 10 μM is desirable for measurements in the Soret region (350-470 nm), 50-100 μM for the visible bands (470-670 nm) and approximately 1 mM for the near-IR transitions (0.7-1.7 microns) if a 1 cm cell is to be used. Short path cells for samples of high absorbance can be accommodated in most magnets, but the actual pole piece gap may restrict the length of longer path cells.

For measurements in the 0-37° range or higher jacketed cells or cell holders can easily be placed in the electromagnet gap with a stream of dry nitrogen flushed over the window to prevent condensation in the lower range. If the temperature needs to be maintained only slightly below the freezing point of water, 0° to -10°, to stabilize some reactive compounds, freezing point depression solvents of the type recommended by Douzou[15] can be used in connection with refrigerated circulators. For determination of C terms in the MCD spectra, however, a much wider range of temperature variation is preferred and this requires both special glass-forming solvent mixtures and cryostats.

The solvent system of choice for C term determination depends on the lower temperature limit desired and compatability with the sample to be studied. Solutions of glycerol in water or buffer (75% glycerol) have proved convenient for a number of heme-proteins and usually allow measurements to temperatures as low as -100° to -150°C before cracking and scattering the

incident beam. Solutions of ethylene glycol also remain sufficiently fluid below 0°C for low temperature mixing experiments,[15] and a 50% aqueous mixture will form a clear glass down to about -140°C. Potassium glycerophosphate, glycerol and buffer in equal volume amounts yield a glass stable to liquid nitrogen temperature (-196°C), but not all proteins are stable in this high ionic strength mixture. Care must also be taken to neutralize the alkaline glycerophosphate, and it is recommended that all glycol solvents be added slowly with sufficient mixing to prevent high local concentrations. The lowest temperature measurements, down to 4°K, have been obtained using saturated solutions of sucrose. All of the solvent systems seem to form stable glasses best in short path length cells (1-5 mm). This may serve to minimize the strain induced by small volume changes; sample cells with thin, flexible lucite windows can be easily made and used.

Vacuum dewars of almost any geometry can be constructed from glass, quartz, or metal with windows for use down to liquid nitrogen temperature. The main restriction is a short total optical path to fit between the magnet pole pieces, a gap which may be just greater than 1 cm. We have found it convenient to have interchangable pole pieces to provide wider gaps of 1 to 2 inches at the expense of some field strength in order to accommodate cryostats and other long path cells. Temperature control can be achieved by regulating the flow rate of dry nitrogen through coils immersed in liquid nitrogen before flowing into the dewar and over the sample. Figure 4 shows a sketch of one way to set this up. Flow rate and heat regained in the transfer from the coils to the sample dewar determine the temperature obtained and an evacuated transfer tube is recommended to approach -170°; liquid nitrogen can also be added directly into the sample dewar. A copper-constantan

thermocouple embedded into the sample cell is useful over the 30° to -196° range. An even less expensive variation of this approach would be to encase the magnet gap with styrofoam in place of a dewar. This might prove advantageous in experiments in which high scattering samples (such as turbid membrane preparations) are being used since a light pipe could then be placed directly against the sample to collect a greater percentage of the scattered light.

Measurements of MCD from room temperature to liquid nitrogen temperature are sufficient for establishing the paramagnetic origin of intense C terms since this changes the Boltzmann distribution of ground state populations and MCD intensity by a factor of 3.8. However, for very weak C term signals or cases in which spin exchange coupling is suspected, it is advantageous to obtain measurements at lower temperatures. Commercial cryostats using liquid helium transfer or closed cycle heat exchange capable of temperatures of 2°K and 10°K, respectively, are marketed by Air Products and Chemicals, Inc.

Several types of side effects can complicate the interpretation of low temperature MCD measurements. Small increases in MCD intensity arise from solvent contraction on cooling, but for the above mentioned glycol systems this results only in a volume change of 10-15% on going to -196°C and has generally been ignored. Changes in bandwidths of transitions, however, can lead to much larger effects. While the Soret band of most hemeproteins is not appreciably narrowed at low temperature, the Q bands can become much sharper and more intense: examples in which the MCD peak intensity of a diamagnetic reduced cytochrome increased due to narrowing almost as much as would be expected for a C term has been reported.[10,16] Such effects can be checked by recording the absorption and/or CD spectra at low temperature and by monitoring bandwidth or peak-to-trough splitting

of the MCD curve. Further effects can also be observed with systems which are photosensitive, such as the carbon monoxide complex of hemeproteins, since photodissociation may not be reversible at low temperatures or photostationary states may be established at high light intensities. Reversible conformational changes in hemeproteins can also occur as the temperature is lowered.[7]

Results

The first measurements of the temperature dependence of the MCD of a hemeprotein establishing the presence of paramagnetic MCD effects were described by Briat *et al*. for cytochrome $\underline{b}_2$.[17] Since then other reports have appeared on hemoglobin,[7,18,19] myoglobin,[9,19] cytochromes *c*,[10,18] $\underline{b}_5$[10] and *P*-450,[20] cytochrome *c* oxidase,[21] and some isolated heme derivatives.[22,23] Typical results for both ferrous and ferric forms of hemeproteins are discussed below.

Ferrous case. The MCD spectra of reduced, Fe(II) hemeproteins are the most straightforward to interpret since the low spin (S = 0) form is diamagnetic and only the high spin (S = 2) form can yield temperature dependent *C* terms. Reduced cytochrome *c* for example exhibits no change in its Soret MCD spectrum from 22° to -132°C and the visible region shows only effects due to band narrowing which are also apparent in the absorption spectrum.[10] High spin ferrous forms of the above mentioned hemeproteins on the other hand exhibit MCD spectra in the Soret region which are extremely temperature sensitive and must be composed predominantly of *C* terms. The shape of the MCD curve of deoxyhemoglobin is very similar to that of deoxymyoglobin shown in Figure 2, but the cytochrome oxidase case is more complex since this enzyme contains both high and low spin heme *a*. Figure 5 illustrates

how the high spin cytochrome $\underline{a}_3$ (S = 2) portion of the spectrum can be resolved by a temperature dependence study: the difference spectrum between any two temperatures yields only the C term contribution of that chromophore while the low spin cytochrome a (S = 0) component spectrum is un-affected. The shape of the room temperature MCD spectrum of high spin reduced horseradish peroxidase[24] is also similar to these curves consistent with a C term origin, but reduced cytochrome P-450 exhibits a Soret region MCD spectrum which is very different[20,25,26] even though it is thought to exist in an S = 2 state. The reason for the unusual MCD band shape in ferrous P-450 is not known but the difference from other systems emphasizes the importance of temperature dependence measurements for assignment of the S = 0 and S= 2 spin states. Triplet (S = 1) states are not generally considered to be populated in hemoproteins, but evidence for this spin state has been obtained for Fe(II) phthalocyanin in certain solvents (See Ref. 27). The paramagnetism of this spin state would also be expected to yield Faraday C terms.

Ferric case. For Fe(III) hemeproteins both the low spin (S = 1/2) and high spin (S = 5/2) states are paramagnetic and give rise to C terms. Experimentally it has been found, however, that in the Soret spectral region the MCD intensity of the low spin form is much greater than that of the high spin form. Figure 2 shows the MCD spectra of the completely low spin cyanide derivative of ferrimyoglobin and of the completely high spin fluoride form, and the intensity of the latter is seen to be neglible by comparison. In addition, it was found that other complexes of myoglobin which produce thermal spin state mixtures give rise to MCD spectra whose intensities are approximately linearly proportional to the amount of low spin form present in the equilibrium.[9] This is presumably due to the difference in

the extent of spin-orbit coupling in the planar low spin vs. the out-of-plane high spin iron. Several other completely low spin hemeproteins differing in their axial coordination and even heme type (cytochromes b, b_5, c, c_1 and f and hemopexin complexes with proto- and deuteroheme) also have MCD intensities at 20°C similar to the ferrimyoglobin cyanide complex. This suggests that as an initial approximation of spin state one can compare the room temperature spectrum of a given ferrihemeprotein with myoblobin cyanide assuming that a long wavelength extermum value of $\Delta\varepsilon/H$ = -90 to -100 $(M{\cdot}cm{\cdot}T)^{1}$ and a short wavelength peak value of about +70 $(M\ cm\ T)^{-1}$ corresponds to the 100% low spin state. The existence of an immediate spin state (S = 3/2) has been proposed for cytochrome c′,[28] but the MCD behavior of such systems has not been investigated.

This simplified procedure of comparing single temperature measurements is often sufficient for establishing spin state differences within a single system, e.g. changing solvent conditions or addition of enzyme substrates, effector molecules, axial ligands, and denaturants, but should not be relied upon for accurate determinations of absolute spin states. Cytochrome oxidase and cytochrome P-450 are examples of systems where the myoglobin results cannot be applied directly. The low spin ferric forms of each of these enzymes exhibit much weaker Soret MCD intensities than would be expected on the basis of the above arguments. In the case of cytochrome oxidase it was found that ferriheme a in a completely low spin complex does not yield the same Soret C term intensity as heme b or c and hence a different standard must be used for a-type heme complexes; the cytochrome P-450 difference is not understood but like a number of other unusual spectroscopic properties of the protein it may be a result of mercaptide coordination.

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The MCD quantity that would be most valuable to correlate with spin state is the actual C term intensity expressed as the ratio of the MCD intensity to the absorption intensity and determined from the change in the MCD spectrum with temperature. This may not be possible due to effects of temperature on spin equilibria (see below), but an example of measurements on a purely low spin hemeprotein is present in Figure 6. MCD spectra of cytochrome b_5 were recorded at six temperatures from 22° to -196°C and the trough and peak extrema intensities, as well as the CD trough, are plotted against the reciprocal of the absolute temperature (ideally we would compare the integrated intensity changes with temperature). The slope of this plot represents the C term contribution to the MCD spectrum. That weaker A and, or B terms also contribute somewhat to the spectrum is evidenced by the fact that the data at 419 nm do not extrapolate to zero at infinite temperature where C terms disappear; comparison of the intensity change between any two temperatures also shows that the effect is greater than would be expected on the basis of Boltzmann ground state populations indicating a temperature independent component of opposite sign at this wavelength.

Spin state equilibria. Because of the large differences in shape, intensity and temperature dependence of the MCD of hemeproteins in different spin states, the technique can be used to investigate spin equilibria. In the ferrous state most hemeproteins seem to be exclusively high or low spin with no clear examples of a $(S = 2)\rightleftharpoons(S = 0)$ thermal spin equilibrium. In the ferric state, however, the high and low spin forms often lie close in energy with the equilibrium a sensitive function of temperature as well as axial ligand field strength. By analysis of the temperature dependence

of the MCD intensity of ferric spin state mixtures one can extract the thermodynamic parameters for the interconversion between the two forms. Figure 7 presents hypothetical examples in which $(S = 5/2) \rightleftharpoons (S = 1/2)$ spin equilibria are poised at an equal mixture of both forms near room temperature. The broken lines represent typical MCD intensities for pure low spin (upper curve) and high spin (lower curve) ferric heme complexes; a linear temperature dependence which extrapolates to zero at infinite temperature (i.e. no $\underline{A}$ or $\underline{B}$ terms) has been assumed. The presence of a thermal spin equilibrium leads to nonlinearity and a failure of the curve to extrapolate to near zero at $1/T = 0$. Equilibria having an $S = 1/2$ ground state would approach the low spin limit asymptotically, the curvature being most easily recognized in cases with a large negative enthalpic change; spin mixtures with an $S = 5/2$ ground state would actually exhibit an initial decrease in MCD intensity as the temperature is lowered. The actual ΔH and ΔS values for an experimental system can be determined by curve fitting.

Spin Coupling. Some hemeproteins possess more than one heme and or other paramagnetic centers which may be close enough for magnetic exchange to occur. The interaction may involve direct overlap of the orbitals containing the unpaired electrons or may occur through a superexchange mechanism involving orbitals of bridging diamagnetic species. A parallel allignment of spin results in a ferromagnetic interaction and an anti-parallel allignment leads to antiferromagnetism; either of these states can exist in a thermal equilibrium with the non-interacting state, and the temperature dependence of the susceptibility can be used to determine the strength of the exchange interaction. The reader is referred to reference 29 for a quantitative treatment of the magnetic properties of polynuclear transition metal complexes.

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The effect spin coupling has on the MCD hemeproteins has not been explored in any detail as of yet, but the sensitivity of MCD to the heme spin state suggests that the technique will prove useful for exploring the behavior of such systems in the future. In particular, the ability to obtain magnetic data over a broad temperature range may make it possible to investigate states lying at higher energies than is possible by EPR. This is illustrated by some results obtained for cytochrome oxidase,[21] a heme enzyme which contains two molecules of heme a and two g-atoms of copper per mole. In the fully oxidized enzyme a strong antiferromagnetic coupling between one cupric (S = 1/2) center and ferricytochrome a_3 (S = 5/2) to yield an S = 2 system has been proposed to account for the lack of an observable EPR signal for either species and for the linearity of the magnetic susceptibility (see reference 30). The MCD of the oxidized enzyme, however, does not show any paramagnetic effects attributable to the S = 2 state over the temperature range 20° to -145°, i.e. the MCD of cytochrome a_3 appears to be quite weak as expected for the non-interacting S = 5/2 state. The addition of cyanide to the enzyme converts cytochrome a_3 to the low spin (S = 1/2) state but does not produce any new EPR signals suggesting that cytochrome a_3 and the EPR-undetectable copper are still spin coupled at low temperature. The room temperature MCD spectrum, however, exhibits an intensity expected for the S = 1/2 cyanide complex in an uncoupled system. While it is possible that a similar MCD curve could arise from the ferromagnetic S = 1 coupled state, the results are most simply interpreted in terms of a weak exchange interaction, J, so that spin coupling is manifest only in the lower temperature range of the EPR measurements (the lack of an observable Cu(II) S = 1/2 EPR signal at higher temperatures could result from cross relaxation effects with the iron S = 1/2 state without strong

ferromagnetic coupling).[21] A temperature depencence study of the MCD of this derivative should provide valuable insight into the mechanism of metal-metal interactions in this enzyme.

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Figure Legends

Figure 1. Origins of the different Faraday terms in magnetic optical activity.

Figure 2. MCD and absorption spectra of myoglobin complexes in various redox and spin states (data replotted from Reference 9).

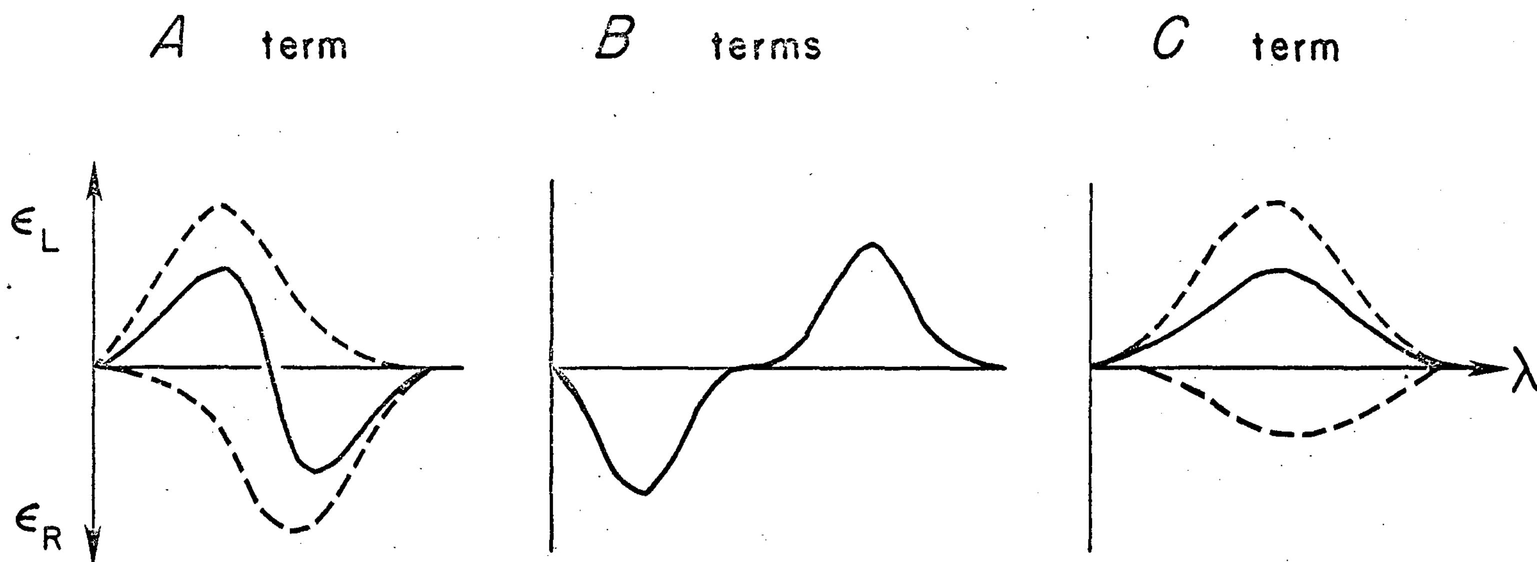
Figure 3. MCD and CD spectra of oxyhemoglobin: Left panel, chart showing original traces, four passes were recorded for each field direction; center panel, signal averaged MCD, equation (6); right panel, signal averaged CD, equation (7), assuming no baseline correction. The sample contained 72 μM hemoglobin A_o in 0.1 M HEPES, 1mM EDTA, 1 mM inositol hexaphosphate, pH 7.0, 4°C, path = 1 cm, field = 1.42 T.

Figure 4. Experimental apparatus for variable temperature MCD measurements (20° to -196°C).

Figure 5. Temperature dependence of the Soret MCD spectrum of reduced cytochrome oxidase.[21]

Figure 6. Temperature dependence of the Soret MCD spectrum of oxidized cytochrome b_5.[10]

Figure 7. Temperature dependence of the Soret MCD intensity for hypothetical cases of Fe(III) low spin/high spin equilibria.



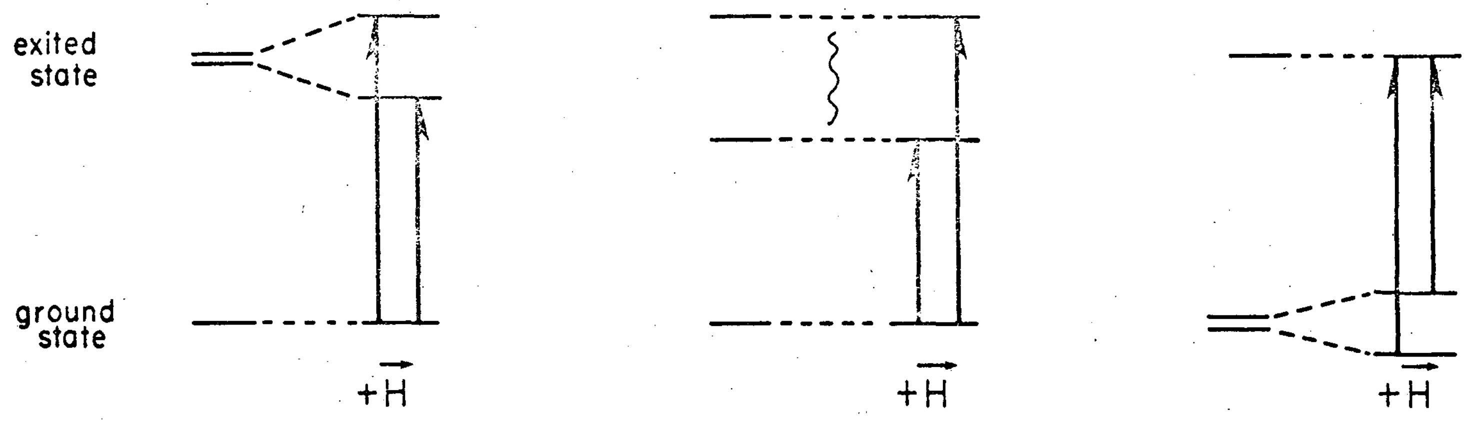


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Figure 1

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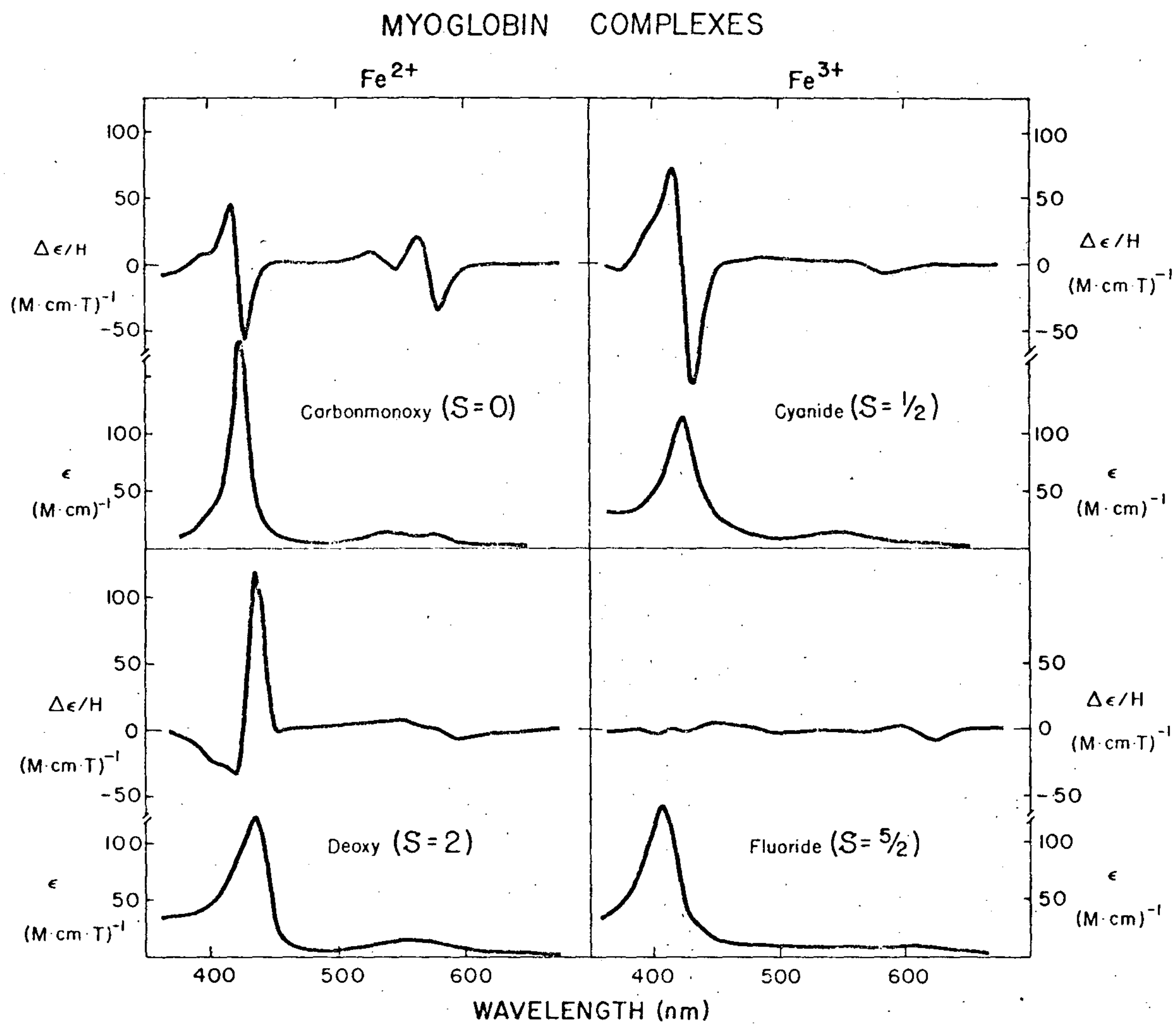

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Figure 2

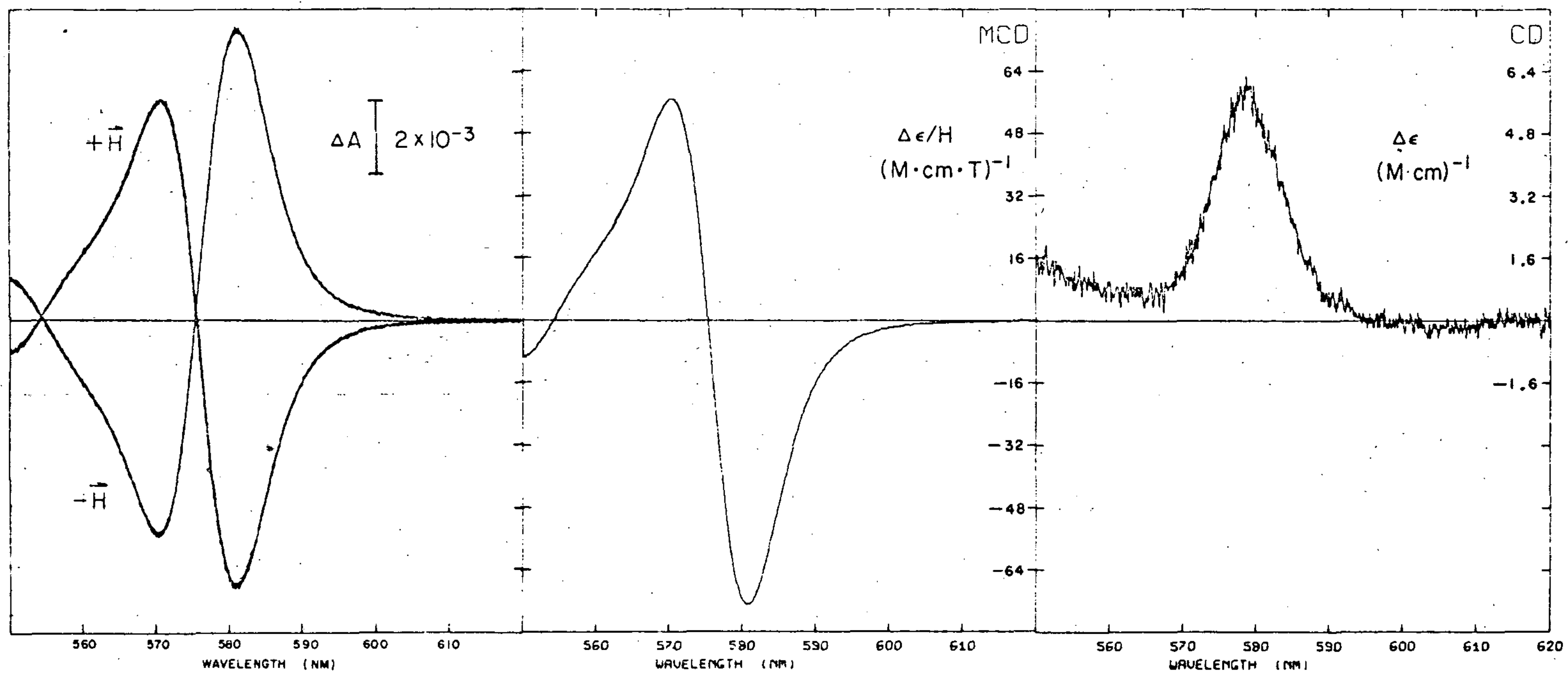

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Figure 3

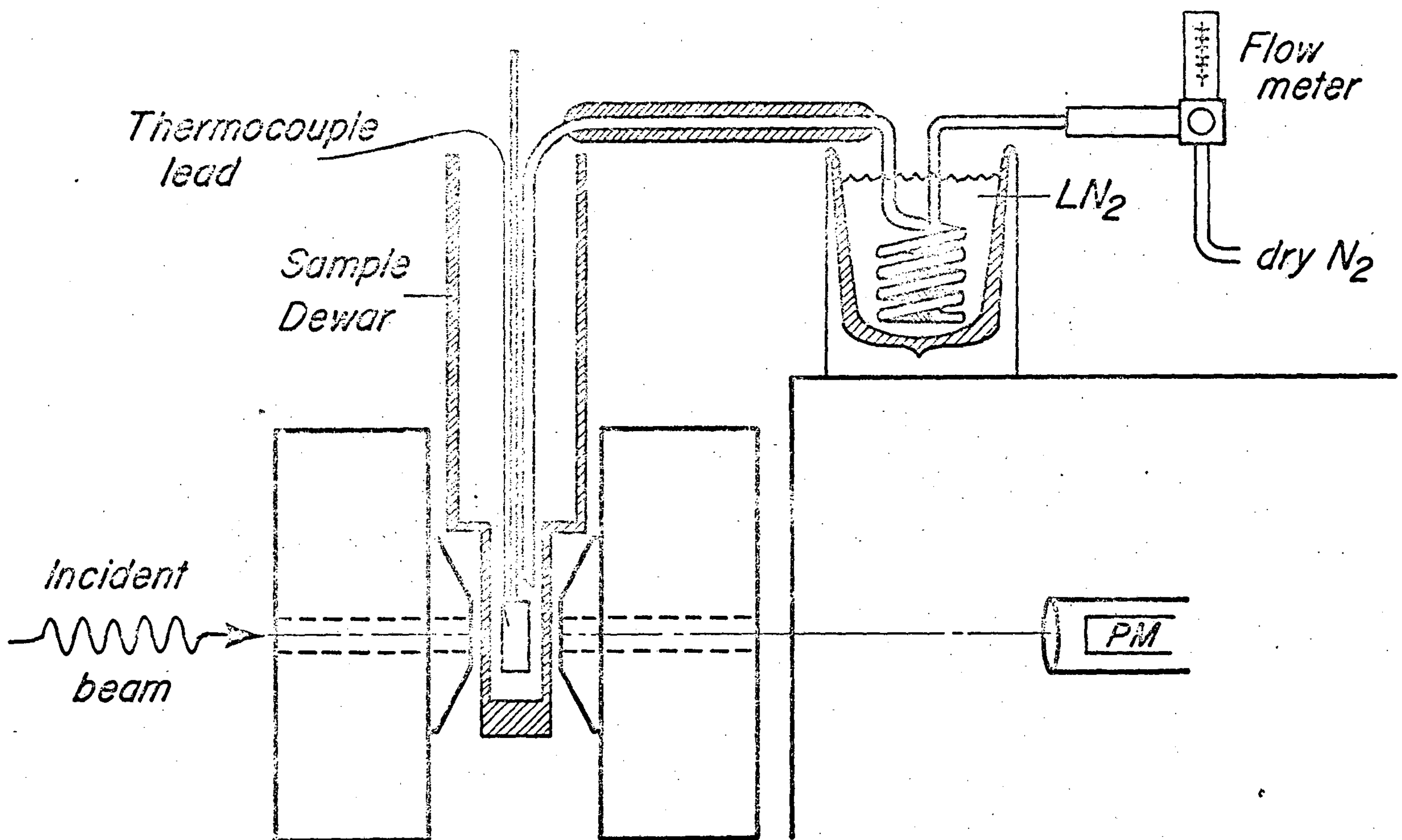

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Figure 4

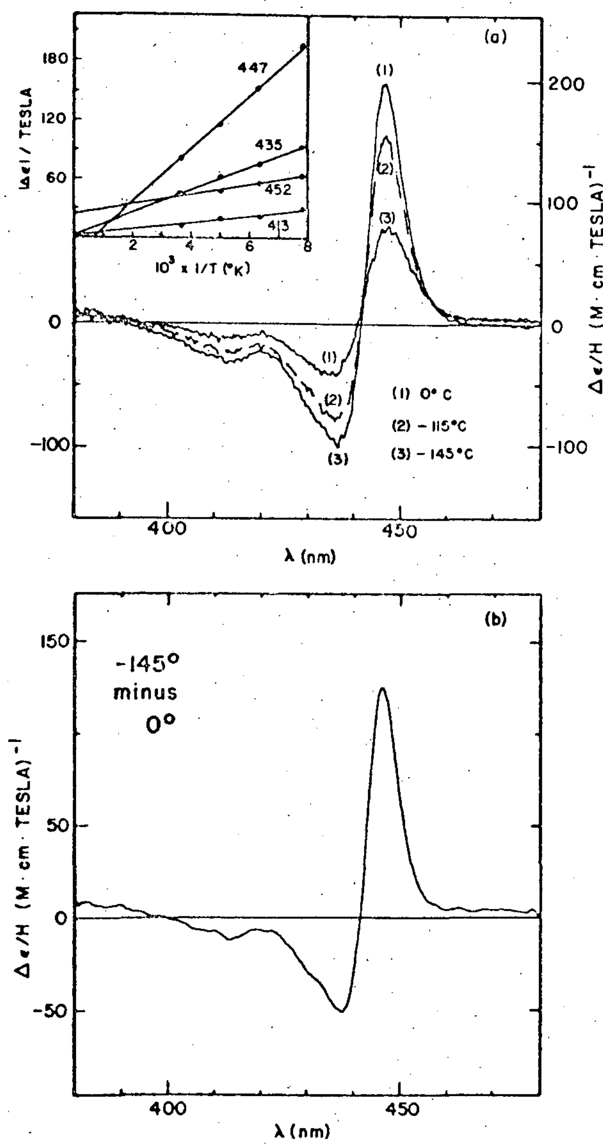

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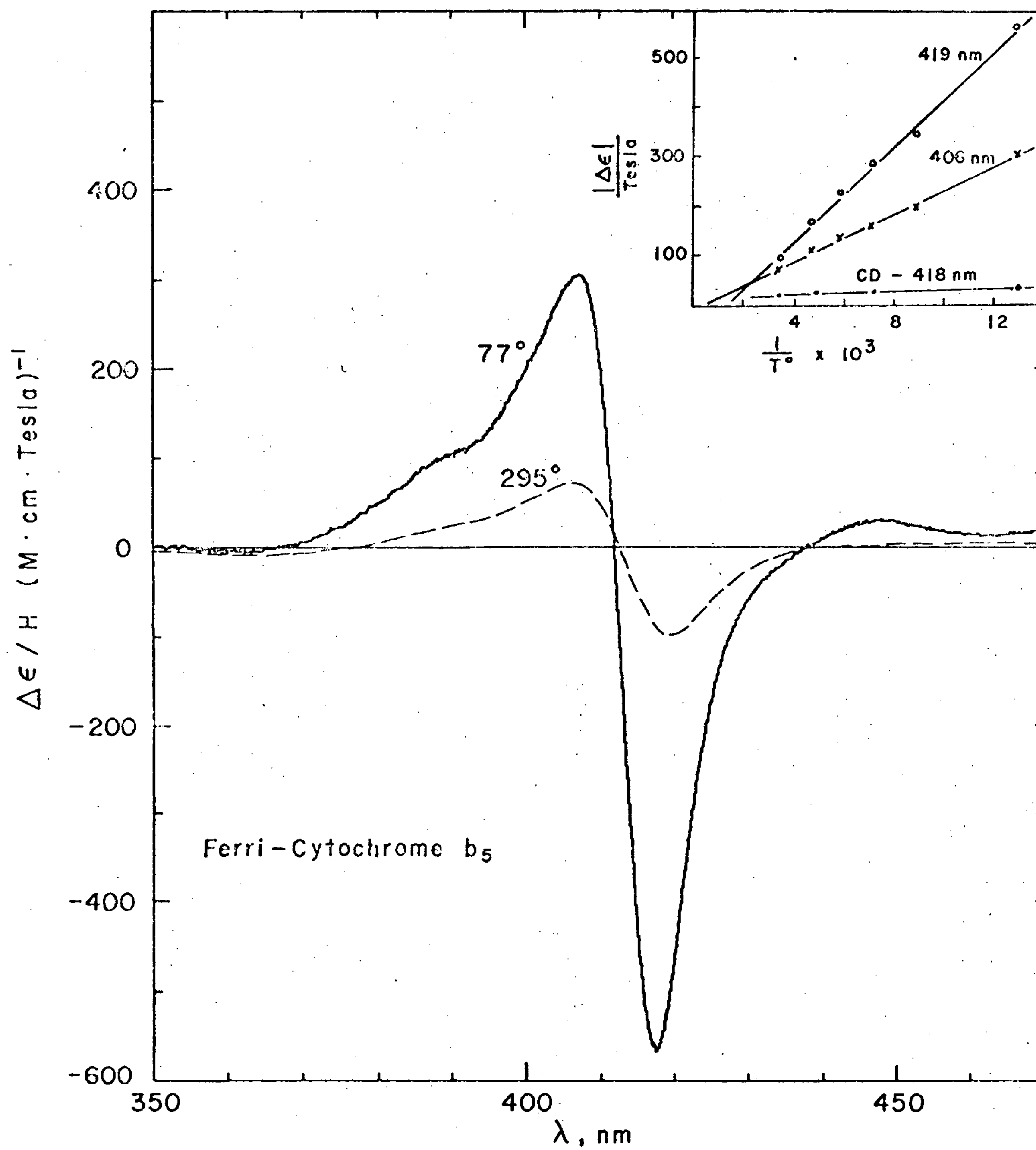
Figure 5



Vickery
Figure 6

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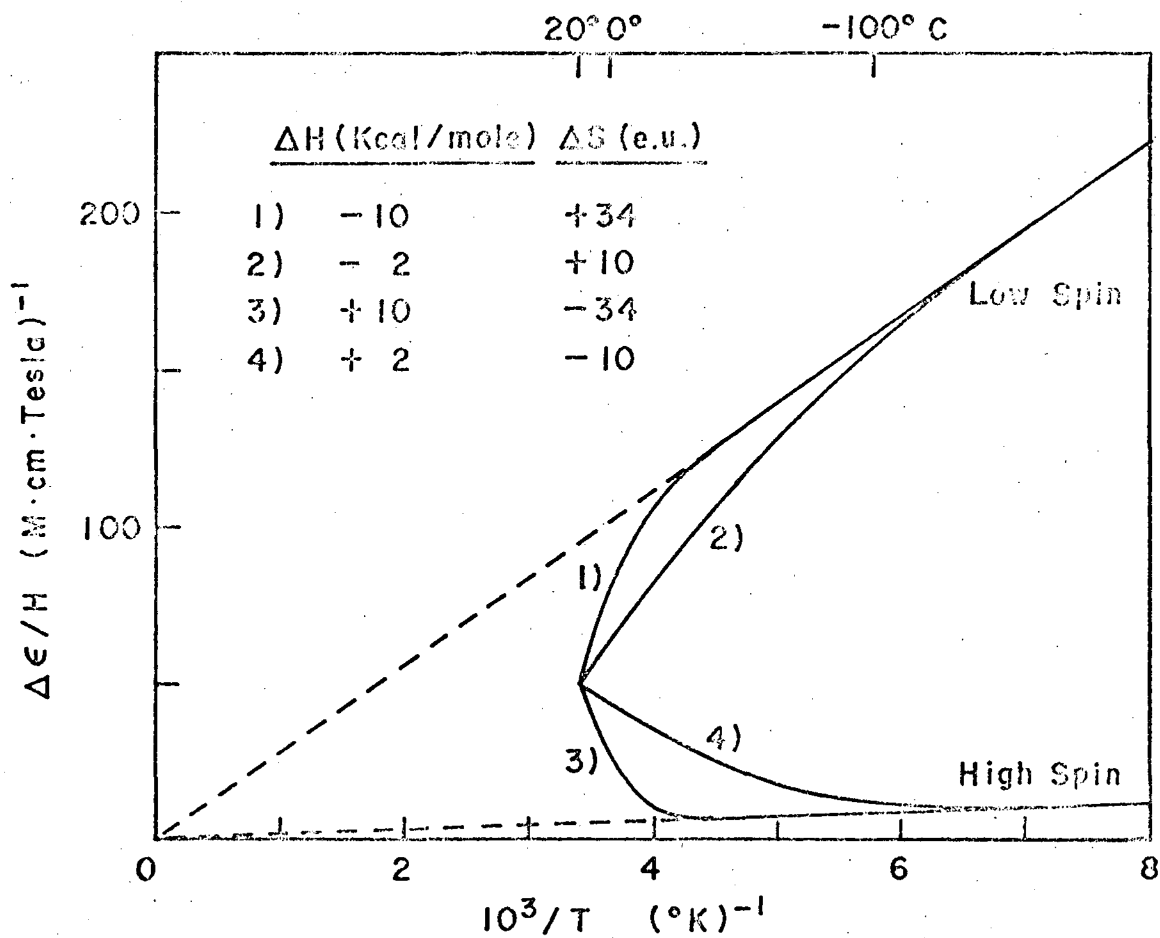

Vickery
Figure 7

This report was done with support from the United States Energy Research and Development Administration. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the United States Energy Research and Development Administration.

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