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Magnetic Circular Dichroism of Cytochrome c *

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ABSTRACT

We have measured the magnetic circular dichroism of ferrocytochrome c from 350 to 1000 nm and the MCD of ferricytochrome c from 350 to 750 nm. We present expressions for the shapes of MCD and absorption spectra in the presence of a magnetic field of randomly oriented molecules for the general case in which the absorption band is split in zero field and the two components of the split band have different intensities. After correcting for the effects of unresolved zero-field splitting, we find that the "angular momentum" of the Q_{00} band of Fe(II) cyt c is 7.9 Bohr magnetons. We show that the 23-fold decrease in the MCD of the Q_{00} band upon oxidation of Fe(II) to Fe(III) is due to about a 5-fold increased band width while the oscillator strength and angular momentum remain essentially unchanged. The increase in band width may reflect greater mixing of the porphyrin orbitals with the d levels of the iron ion in the oxidized state. The MCD of Fe(II) cyt c in the near infrared (600-1000 nm) can be decomposed using essentially the same components previously used to decompose the CD if some components are presumed degenerate. The angular momenta of the degenerate components are in the range expected for d→d transitions. The CD and MCD of the 695 nm band of Fe(III) cyt c are both relatively weak and suggest respectively that the transition is electric dipole allowed and nondegenerate.

INTRODUCTION

Cytochrome c is a nearly ubiquitous constituent of the respiratory systems of biological organisms. By shuttling from the reduced, Fe(II), to the oxidized, Fe(III), state, cytochrome c and the various other cytochromes act as redox carriers to couple dehydrogenases with terminal acceptors such as molecular oxygen. Thus, differences in the properties of the protein in its oxidized and reduced states are of critical importance in understanding its biological function. Cytochrome c can be studied by a great variety of spectroscopic methods because of its prosthetic group, a heme moiety which is linked directly to the peptide chain by cysteine residues at the vinyl groups. The heme moiety accounts for all of the absorption of the protein from 300 nm in the UV through the visible and into the near infrared. Furthermore, large changes occur in the visible and near infrared regions when the protein is oxidized from Fe(II) to Fe(III). It is not surprising therefore that optical spectroscopy has been one of the principal physical tools used to study the cytochromes. (See, for example, the review by Margoliash and Schejter.[1]) Indeed, MacMunn discovered the cytochromes in 1886 by observing their absorption spectra in tissues.

Most optical studies have involved measurement of absorption spectra either <u>in situ</u> or in solution. However, during the past decade more powerful optical techniques have been used to probe cytochrome c. Among these are circular dichroism (CD), and its Kramers-Kronig transform, optical rotary dispersion (ORD),[2] and single crystal polarized absorption.[3]

Cytochrome c became the first biological material to which the modern techniques of magnetic optical activity were applied when Shashoua[4]

reported the magnetic ORD (MORD) of the oxidized and reduced protein in the visible and Soret regions (See Fig. 1). Shortly thereafter Dratz[5] measured the MCD of the same spectral regions. Both Shashoua and Dratz found a very large difference between the magnetic optical activity of the oxidized and reduced states in the visible regions. Since the biological activity of cytochrome c requires it to shuttle from the oxidized to the reduced state, the spectral changes which accompany this transition are of great interest. Magnetic optical activity appears to be the optical technique most sensitive to this transition. Therefore we investigated the MCD of the visible and Soret regions using a spectrometer with a signal-to-noise ratio about six times greater than the instrument used by Dratz. We discovered considerable new structure in the visible bands of the oxidized protein. We show further that the large decrease in the MCD of the Q_{00} band upon oxidation is related to the greater width of the absorption band compared to the reduced states.

Eaton and Charney[6] have concluded from CD measurements that the very weak, near infrared absorption bands of ferrocytochrome c contain d→d transitions. These transitions are particularly important because native cytochrome c is a diamagnetic low-spin compound which has six paired electrons and is thus not amenable to study by electron spin resonance or other techniques which require paramagnetic molecules. We measured the MCD of the near infrared bands and found that the tentative resolution proposed on the basis of CD[6] is tenable if certain components are presumed degenerate. The angular momenta of these degenerate transitions are of the correct magnitude for d→d transitions.

Finally, we measured the MCD and CD of the absorption band of ferricytochrome c at 695 nm. The absorption at 695 nm, together with

other unresolved transitions at slightly shorter wavelengths, disappears when the protein is treated with 4 M urea or similar reagents which remove the methionine sulfur as the iron ion's fifth ligand.[24] The MCD and CD of these bands suggest respectively that the 695 nm band is nondegenerate and electric-dipole allowed. These results are consistent with the proposals of Eaton and Hochstrasser[3] that the 695 nm absorption is a charge transfer transition from the A_{2u} (π) orbital of the porphyrin ring to the d_{z^2} level of the iron ion, but do not exclude charge transfer transition involving the axial ligands.

EXPERIMENTAL

Materials

Lyophilized samples of horse heart cytochrome c (Sigma Chemical Co., grade VI) were stored at 0°C until shortly before use. SDS polyacrylamide gel electrophoresis indicated that a few percent of the protein molecules were in dimers, trimers or higher aggregates and gave a molecular weight of 12,400 for the monomer, which is close to the accepted value.[1] In most experiments the lyophilized material was used without further purification. For one 77°K absorption spectrum in the 600 to 1000 nm region, we purified a sample by ion exchange chromatography.[7] All room temperature measurements were made with the protein dissolved in .1 M potassium phosphate buffer at pH 7.0. In the low temperature experiments the protein was dissolved in the same buffer and then diluted with an equal volume of glycerol. As supplied, most of the protein molecules were in the oxidized state, but a small fraction was present in the reduced form. The presence of this small fraction of the reduced protein was easily detected in the MCD at 550 nm even when the absorption spectrum gave no indication of its presence. To complete the oxidation

a small volume of $K_3Fe(CN)_6$ was added to achieve a final molar ratio of one-tenth that of the protein. Reduction was achieved by adding a slight molar excess of sodium dithionite or sodium ascorbate. Results were independent of the means of reduction. Concentrations were determined from published values of molar extinction coefficients.[1]

Instrumentation

The MCD spectrometer has evolved from an instrument previously described by Dratz.[5] Recent modifications include replacing the 1.25 T electromagnetic by a 6.6 T superconducting solenoid. To separate natural CD from MCD we measure each spectral region twice: once with the magnetic field parallel to the direction of the light beam and then with it anti-parallel. The direction of the field of the superconducting solenoid is reversed by rotating the magnet through 180° about an axis perpendicular to the light beam. The signal proportional to the net circular dichroism (CD plus MCD) was converted to digital form by a Vidar Corp. model 260 voltage to frequency converter and stored in a Nuclear Data Corp. model 180 multichannel analyzer. Spectra with low signal-to-noise ratios were scanned repeatedly; each scan was summed with the results of previous scans in the multichannel analyzer. The summed spectra for both directions of the magnetic field were transferred to a digital computer via punched paper tape. The computer separated the contributions of CD and MCD, converted the raw data into units of molar extinction (M^{-1} cm^{-1}) or molar extinction per unit magnetic field (M^{-1} cm^{-1} T^{-1}), converted the wavelength of each point to wavenumber (expressed in cm^{-1} or $(\mu m)^{-1}$; $1(\mu m)^{-1} = 10{,}000$ cm^{-1}), and plotted the results. The instrument was calibrated with a 1.0 cm pathlength cell containing a 1.0 mg

per ml aqueous solution of d-10-camphorsulfonic acid. At 290 nm this solution has a CD (A_l-A_r) of + 9.3 x 10^{-3} optical density units.

The CD of the native protein bands of Fe(II) cyt c was also measured with a Cary Instruments Corp. Model 60 spectropolarimeter equipped with a model 6001 CD attachment. Absorption spectra were measured with a Cary Instruments Corp. Model 14R recording spectrophotometer. Absorption spectra were also digitized and processed and plotted by the computer. Liquid nitrogen temperature absorption spectra were recorded by placing the sample in a 2 mm pathlength cell which was in thermal contact with a reservoir of liquid nitrogen. For low-temperature MCD measurements the sample was contained in a 1 cm path cell which was inserted into the bore of the room temperature access which passes through the solenoid. The sample was cooled by flowing chilled nitrogen gas through the hollow walls of the cell. With the water-glycerol solvent system, the sample developed cracks at 77°K, so measurements were restricted to the temperature of dry ice.

Decomposition of the experimental spectra into sums of Gaussian or Lorentzian curves plus their derivatives was accomplished by interactive computer routines. The data and proposed resolutions were viewed on a Tektronix Type 611 storage display oscilloscope. The operator could change the position, width and amplitude of each band. When a satisfactory fit was achieved, the data, net fitting curve and the individual fitting components were plotted by a California Computer Products Model 565 digital incremental plotter.

The measurement of MCD requires two arbitrary sign conventions. First we measure the difference in absorption of left and right circularly

polarized light (A_L-A_R). The definition of left circular polarization is such that the tip of the electric vector of a left circularly polarized wave traverses a right-handed helical path. Second, the direction of the magnetic field is considered to be "positive" when it is parallel to the Poynting vector of the light and "negative" when it is antiparallel.

RESULTS AND DISCUSSION

We have investigated the MCD in six distinct spectral regions. The visible, Soret and near infrared regions of ferrocytochrome c and the visible, Soret and red or 695 nm regions of ferricytochrome c as shown in Fig. 1. The degree of resolution of the different spectra varied drastically. The visible bands of ferrocytochrome c are well resolved even in absorption and thus the MCD could be analyzed with a considerable degree of sophistication. At the other extreme are the near infrared bands of ferrocytochrome c which show almost no structure in absorption. Here CD and MCD become of paramount importance in decomposing and interpreting the spectra.

Visible Bands

Fe(II)

The absorption and MCD spectra of ferrocytochrome c are shown in the lower part of Fig. 2. The narrow absorption band at 1.82 $(\mu m)^{-1}$ arises from transitions from the zero vibrational level of the porphyrin ground state to the zero vibrational level of the lowest porphyrin singlet excited state and is called the Q_{00} transition.[8] Between 1.86 and 2.0 $(\mu m)^{-1}$ there are several bands arising from transitions to higher vibrational levels of the excited state of the porphyrin and known collectively

as the Q_{01} band. The MCD of the Q_{00} band resembles the derivative of the absorption spectrum and thus indicates that either the initial or final state is (approximately) degenerate.[10] Indeed, it is well known that the ground state is nondegenerate (symmetry A_{1g}) and the first excited state is doubly degenerate (symmetry E_g).[9]

We will represent the wavefunction of the nondegenerate ground state by $|g\rangle$ and the wavefunctions for the two lowest energy singlet states as $|x\rangle$ and $|y\rangle$. The transition dipoles for $|g\rangle \rightarrow |x\rangle$ and $|g\rangle \rightarrow |y\rangle$ are perpendicular to each other and define the x and y axes of the molecule.[9] The z axis is perpendicular to the plane of the porphyrin ring system. The energies of states $|x\rangle$, $|y\rangle$ and $|g\rangle$ are respectively E_x, E_y and E_g.

Recently we showed[13] that an external magnetic field will couple states $|x\rangle$ and $|y\rangle$ to produce two new states

$$|1\rangle = \alpha|x\rangle + i\gamma|y\rangle$$

and

$$|2\rangle = \gamma|x\rangle - i\alpha|y\rangle \qquad (1)$$

where

$$\alpha = (\Delta E_T + \Delta E_{ZF})/D$$

$$\gamma = (\beta M_z H \cos\Theta)/D$$

$$D = \{[\Delta E_T + \Delta E_{ZF}]^2 + [\beta M_z H \cos\Theta]^2\}^{1/2}$$

$$\Delta E_{ZF} = (E_y - E_x)/2$$

$$\Delta E_T = \{\Delta E_{ZF}^2 + [\beta M_z H \cos\Theta]^2\}^{1/2}$$

$$M_z = -(i/\hbar)\langle x|L_z|y\rangle .$$

H is the strength of the external magnetic field, β is the Bohr magneton and Θ is the angle between the z axis of the molecule and H. The energies of the new states are E_1 and E_2 where $E_2 - E_1 = 2\Delta E_T$, as shown in Fig. 3. The wavelength-dependent molar extinction coefficients for the absorption

of right and left circularly polarized light by the transitions $|g\rangle \rightarrow |1\rangle$ and $|g\rangle \rightarrow |2\rangle$ are given by:

$$\varepsilon_{L/R,1}(\bar{\nu}) = 3/16 \int_0^{\pi} f(\bar{\nu},\bar{\nu}_1)[(1+\cos^2\Theta)(\alpha^2\varepsilon_x+\gamma^2\varepsilon_y) \mp 4\alpha\gamma\,\{\varepsilon_x\varepsilon_y\}^{1/2}\cos\Theta]\sin\Theta\, d\Theta$$

and (2)

$$\varepsilon_{L/R,2}(\bar{\nu}) = 3/16 \int_0^{\pi} f(\bar{\nu},\bar{\nu}_2)[(1+\cos^2\Theta)(\gamma^2\varepsilon_x+\alpha^2\varepsilon_y) \pm 4\alpha\gamma\,\{\varepsilon_x\varepsilon_y\}^{1/2}\cos\Theta]\sin\Theta\, d\Theta$$

where in $\pm$ signs the upper sign goes with the L subscript and the lower sign goes with the R subscript. We assume that the shape of the absorption bands for the transitions to $|x\rangle$ and $|y\rangle$ can be described by

$$\varepsilon_x(\bar{\nu}) = \varepsilon_x\, f(\bar{\nu},\bar{\nu}_x)$$

and (3)

$$\varepsilon_y(\bar{\nu}) = \varepsilon_y\, f(\bar{\nu},\bar{\nu}_y)$$

<u>i.e.</u>, ε_x and ε_y are the maximum values of $\varepsilon_x(\bar{\nu})$ and $\varepsilon_y(\bar{\nu})$ and $f(\bar{\nu},\bar{\nu}_i)$ is an absorption envelope whose maximum amplitude is unity at $\bar{\nu}_i$. The parameters $\bar{\nu}_x$, $\bar{\nu}_y$, $\bar{\nu}_1$, $\bar{\nu}_2$, $\bar{\nu}_{ZF}$, and $\Delta\bar{\nu}_T$ are the exact analogies of E_x, E_y, E_1, E_2, ΔE_{ZF}, and ΔE_T expressed in units of wavenumber instead of energy. Thus, according to the definitions of $\bar{\nu}_1$ and $\bar{\nu}_2$, $f(\bar{\nu},\bar{\nu}_1)$ and $f(\bar{\nu},\bar{\nu}_2)$ are functions of Θ and must remain inside the integral in eqn. (2). In Ref. 13 we assumed that $f(\bar{\nu},\bar{\nu}_i)$ was a Gaussian function and evaluated the integral over Θ numerically. However, it is more instructive to perform the integration analytically without placing any special restrictions on the shape of $f(\bar{\nu},\bar{\nu}_i)$ [14] other than that it must be a non-negative function with its single maximum value of unity at $\bar{\nu}_i$, that it and all of its derivatives are continuous functions of $\bar{\nu}$, that both $\varepsilon_x(\bar{\nu})$ and $\varepsilon_y(\bar{\nu})$ have the same absorption envelope, and that the magnetic field shifts the envelope rigidly. We expand $f(\bar{\nu},\bar{\nu}_i)$ in a Taylor series about $\bar{\nu}_0$ where $\bar{\nu}_0 = (\bar{\nu}_1+\bar{\nu}_2)/2$, <u>i.e.</u>,

$$f(\bar{\nu},\bar{\nu}_{1/2}) = \sum_{n=o}^{\infty}[(\pm\Delta\bar{\nu}_T)^n/n!]\ f^{(n)}(\bar{\nu},\bar{\nu}_o) \tag{4}$$

where $f^{(n)}(\bar{\nu},\bar{\nu}_o) = d^n f(\bar{\nu},\bar{\nu}_o)/d\bar{\nu}^n$.

The upper signs are for state 1 and the lower for state 2. Eqn. (4) is substituted into eqn. (2), and we calculate the total absorption of left and right circularly polarized light

$$\varepsilon_{L/R}(\bar{\nu}) = \varepsilon_{L/R,1}(\bar{\nu}) + \varepsilon_{L/R,2}(\bar{\nu}) \tag{5}$$

Every term in the resulting series can be integrated with respect to Θ by virtue of three identities which insure that $\Delta\bar{\nu}_T$ is raised only to even powers. The identities are:

$$\alpha^2 + \gamma^2 = 1 \tag{6a}$$

and

$$\alpha^2 - \gamma^2 = \Delta\bar{\nu}_{ZF}/\Delta\bar{\nu}_T \tag{6b}$$

$$\alpha\gamma = (\not{\beta} M_z H \cos\Theta)/2\Delta\bar{\nu}_T \tag{6c}$$

where $\not{\beta}(= \beta/hc)$ is the Bohr magneton expressed in units of wavenumber per Tesla. We took the value of $\not{\beta}$ to be 46.7 $m^{-1}T^{-1}$.

We expand the various powers of $\Delta\bar{\nu}_T$ by means of the binomial theorem and perform the integration over Θ with the result

$$\begin{aligned}\varepsilon_{L/R}(\bar{\nu}) = {} & (3\varepsilon_o/2)\sum_{k=o}^{\infty}(f^{(2k)}(\bar{\nu},\bar{\nu}_o)/(2k)!)\sum_{i=o}^{k}\binom{k}{i}(\Delta\bar{\nu}_{ZF})^{2(k-i)}(\not{\beta}HM_z)^{2i}(i+1)/(2i+1)(2i+3) \\ & + 3\Delta\varepsilon\Delta\bar{\nu}_{ZF}\sum_{k=o}^{\infty}(f^{(2k+1)}(\bar{\nu},\bar{\nu}_o)/(2k+1)!)\sum_{i=o}^{k}\binom{k}{i}(\Delta\bar{\nu}_{ZF})^{2(k-i)}(\not{\beta}HM_z)^{2i}(i+1)/(2i+1)(2i+3) \\ & \mp 3\ \not{\beta}HM_z(\varepsilon_o/4)\{1-[2\Delta\varepsilon/\varepsilon_o]^2\}^{1/2}\sum_{k=o}^{\infty}(f^{(2k+1)}(\bar{\nu},\bar{\nu}_o)/(2k+1)!)\sum_{i=o}^{k}\binom{k}{i}(\Delta\bar{\nu}_{ZF})^{2(k-i)}(\not{\beta}HM_z)^{2i}/(2i+3)\end{aligned} \tag{7}$$

where $\varepsilon_o = \varepsilon_x + \varepsilon_y$ and $\Delta\varepsilon = (\varepsilon_x - \varepsilon_y)/2$.

For zero-field splittings which are unresolved in absorption the series converges rapidly. In our computations we retained terms through the fourth derivative, i.e.,

$$\varepsilon_{L/R}(\bar{\nu})=(\varepsilon_o/2)[f(\bar{\nu},\bar{\nu}_o)+f^{(2)}(\bar{\nu},\bar{\nu}_o)\{(\Delta\bar{\nu}_{ZF}^2/2)+(\beta^2H^2M_z^2/5)\}+f^{(4)}(\bar{\nu},\bar{\nu}_o)\{(\Delta\bar{\nu}_{ZF}^4/24)+(\Delta\bar{\nu}_{ZF}^2\ \beta^2H^2M_z^2/30)+(3\beta^4H^4M_z^4/280)\}]$$

$$+\ \Delta\varepsilon\Delta\bar{\nu}_{ZF}[f^{(1)}(\bar{\nu},\bar{\nu}_o)+f^{(3)}(\bar{\nu},\bar{\nu}_o)\{(\Delta\bar{\nu}_{ZF}^2/6)+(\beta^2H^2M_z^2/15)\}]$$

$$\mp\ \beta HM_z(\varepsilon_o/4)\{1-[2\Delta\varepsilon/\varepsilon_o]^2\}^{1/2}[f^{(1)}(\bar{\nu},\bar{\nu}_o)+f^{(3)}(\bar{\nu},\bar{\nu}_o)\{(\Delta\bar{\nu}_{ZF}^2/6)+(\beta^2H^2M_z^2/10)\}]. \quad (7b)$$

Note that the expressions for $\varepsilon_L(\bar{\nu})$ and $\varepsilon_R(\bar{\nu})$ are each the sum of three series: the first depends on the total intensity of the composite absorption band and contains only even derivatives; the second depends on the product of the intensity imbalance and the zero-field splitting and contains only odd derivatives, and the third depends on the product of ε_x and ε_y and also contains only odd derivatives.[15] Only the third term contributes to the MCD, which we define as

$$\Delta\varepsilon_M(\bar{\nu}) = (\varepsilon_L(\bar{\nu}) - \varepsilon_R(\bar{\nu}))/H\ . \quad (8)$$

It is also straightforward to calculate the total absorption spectrum from eqn. (7) in both the presence and absence of a magnetic field, i.e.,

$$\varepsilon(\bar{\nu}) = \varepsilon_L(\bar{\nu}) + \varepsilon_R(\bar{\nu}). \quad (9)$$

Note that the magnetic field does not produce any first order changes in $\varepsilon(\bar{\nu})$ but does produce first order effects in both $\varepsilon_L(\bar{\nu})$ and $\varepsilon_R(\bar{\nu})$.

M_z, which in the absence of zero field splitting is the angular momentum quantum number of the degenerate excited states, is a sensitive function of $|x\rangle$ and $|y\rangle$ and is an important physical parameter. We can test the validity of excited state wave functions calculated by means of molecular orbital theory by comparing calculated and experimentally determined values of M_z. We can derive M_z by comparing the magnitude of the observed MCD to eqn. (8) if we know $\Delta\bar{\nu}_{ZF}$, $2\Delta\varepsilon/\varepsilon_o$, and the correct

absorption envelop $f(\bar{\nu},\bar{\nu}_.)$. To determine these parameters we fit the absorption band (measured in the absence of a magnetic field) by

$$\varepsilon(\bar{\nu}) = \varepsilon_x f(\bar{\nu},\bar{\nu}_x) + \varepsilon_y f(\bar{\nu},\bar{\nu}_y) \qquad (10)$$

The fitting procedure is particularly easy for cytochrome c because the 77°K absorption spectrum shows the $|g\rangle \rightarrow |x\rangle$ and $|g\rangle \rightarrow |y\rangle$ transitions clearly resolved.[16] We analyzed the 77°K absorption spectrum with the interactive computer program and found that $\Delta\bar{\nu}_{ZF} = 60 \pm 5\ cm^{-1}$ and $2\Delta\varepsilon/\varepsilon_o = .20$. We also found that Lorentzian functions of the form $f(\bar{\nu},\bar{\nu}_i) = (1 + [(\bar{\nu}-\bar{\nu}_i)/\Delta]^2)^{-1}$ gave good fits to the individual components. Using Lorentzian functions and the 77°K values of $\Delta\bar{\nu}_{ZF}$ and $2\Delta\varepsilon/\varepsilon_o$ as initial estimates we then analyzed the room temperature absorption spectrum with the interactive program and found $\Delta\bar{\nu}_{ZF} = 50 \pm 5\ cm^{-1}$, $2\Delta\varepsilon/\varepsilon_o = .16$ (see Fig. 4b). Next we used a similar program to fit eqn. (8) to the experimental MCD using the room temperature values of $\varepsilon_o, \Delta\bar{\nu}_n$ and $\Delta\varepsilon$. The best fit was found for $M_z = 7.9$ as shown in Fig. 4a. Finally we computed $\varepsilon_L(\bar{\nu})$ and $\varepsilon_R(\bar{\nu})$ by means of eqn. (7). These results are shown in Fig. 4c.

Other workers have calculated M_z for the Q_{00} band of Fe cytochrome c and other metalloporphyrins. In all such cases the excited states were assumed to be exactly degenerate. How do these values of M_z compare with the results of the more elaborate procedure which we used? Malley et al.[17] attempted to measure the value of M_z of the Q_{00} bands of zinc and magnesium coproporphyrin by the difference in wavenumber of the peak values of $\varepsilon_L(\bar{\nu})$ and $\varepsilon_R(\bar{\nu})$. We shall call this type of measurement the integral form of MCD. In reference 13 we showed that the separation of the integral MCD absorption peaks give unreliable estimates of M_z. For the values of $\Delta\varepsilon$ and $\Delta\bar{\nu}_{ZF}$ typically observed in metalloporphyrins, the integral MCD peak separation

substantially overestimates M_z. Indeed, the calculated integral MCD shown in Fig. 4c yields a value of 12.2 ± 1.2 for M_z by this method.

Dratz[5] and Stephens et al.[18] calculated M_z by comparing the differential MCD with the derivative of the (zero-field) absorption spectrum. In reference 13 we also argued on qualitative grounds that this procedure would give fairly good estimates of the correct value of M_z. Now we can assess quantitatively the accuracy of this method. The method is based on the assumption that

$$\Delta\varepsilon_M(\bar{\nu}) = -\beta^* M_z \varepsilon^{(1)}(\bar{\nu})/2 \qquad (11)$$

where $^*M_z$, the apparent angular momentum, is determined by the method of least squares, which requires

$$(\partial/\partial^* M_z) \int [\Delta\varepsilon_M(\bar{\nu}) - \beta^* M_z\, \varepsilon^{(1)}(\bar{\nu})/2]^2\, d\nu = 0 \qquad (12)$$

For $\Delta\varepsilon_M(\bar{\nu})$ we substitute the right-hand side of eqns. (7) and (8) and we calculate $\varepsilon^{(1)}(\bar{\nu})$ from eqns. (7) and (9) with H=0. After performing the operations indicated in eqn. (12), we find

$$^*M_z = M_z \cdot \{1-[2\Delta\varepsilon/\varepsilon_0]^2\}^{1/2}\ (J/I) \qquad (13)$$

where

$$I = \int [f^{(1)}(\bar{\nu},\bar{\nu}_0) + 2\Delta\varepsilon\Delta\bar{\nu}_{ZF}\, f^{(2)}(\bar{\nu},\bar{\nu}_0)/\varepsilon_0 + \ldots]^2\, d\bar{\nu} \qquad (14)$$

and

$$J = \int [f^{(1)}(\bar{\nu},\bar{\nu}_0) + 2\Delta\varepsilon\Delta\bar{\nu}_{ZF}\, f^{(2)}(\bar{\nu},\bar{\nu}_0)/\varepsilon_0 + \ldots][f^{(1)}(\bar{\nu},\bar{\nu}_0) + f^{(3)}(\bar{\nu},\bar{\nu}_0)(\Delta\bar{\nu}_{ZF}^2/6 + \beta^2 H^2, {}^2_z/1 + \cdots]\, d\bar{\nu}.$$

I is the integral of the square of the derivative of the normalized absorption envelope while J is the integral of the product of the derivative of the absorption envelope and the (normalized) MCD envelope. The two functions whose product appears in J have similar shapes and amplitudes but are shifted along the $\bar{\nu}$ axis with respect to each other because of intensity imbalance and zero field splitting. Near $\bar{\nu}_0$ there is a small

region where their product is negative so we can expect that J/I will be slightly less than unity for unresolved zero-field splittings. The square root terms in eqn. (13) will also be very slightly less than unity. Thus, we conclude that the procedure used by Dratz[5] and Stephens et al.[18] gives good estimates of M_z which will tend to be very slightly less than the actual value. These theoretical conclusions agree with our experimental results, since we found that M_z for the Q_{00} band of Fe(II) cyt c was 7.9 while Dratz found a value of 7.8 $\pm$ .5. While the differential MCD gives a better estimate of M_z than does the separation of the peaks of the integral MCD, it provides no obvious indication of the existence of zero-field splitting as does integral MCD.

Fe(III)

The MCD and absorption spectra of ferricytochrome c are also shown in Fig. 2. The scales have been changed because both peak absorption and peak MCD have decreased in comparison to Fe(II). For the MCD the decrease is greater than a factor of twenty. These drastic changes in the spectra of the porphyrin ring system must somehow reflect the removal of an electron from the iron ion.

The Q_{00} band appears as a shoulder in the absorption spectrum at 1.78 $(\mu m)^{-1}$. We interpret the MCD in this region as the sum of a normal A and a negative B term. The A term is responsible for the peak at 1.82 $(\mu m)^{-1}$ and the trough at 1.77 $(\mu m)^{-1}$, while the B term accounts for the greater amplitude of the trough. We interpret the trough at 1.88 $(\mu m)^{-1}$ as a negative B term associated with the Q_{01} bands. The apparent lack of A terms in the Q_{01} region is not surprising since in the Fe(II)

spectrum the MCD of the Q_{01} bands is much less than the Q_{00} band. The slight red shift of the MCD relative to the Q_{01} absorption peak may be due to unresolved A components or may reflect the composite nature of the Q_{01} bands. We assign B rather than C terms to the Q_{00} and Q_{01} bands since the MCD measured at 194°K was the same as the room temperature MCD shown in Fig. 2, when allowance was made for increased density of the sample at the lower temperature.

The MCD of the Q_{00} and Q_{01} bands is superimposed on a broad, positive background. The MCD also reveals a well defined band between 2.1 and 2.4 $(\mu m)^{-1}$ (c.f., Fig. 5).

The negative B terms in MCD of the Q_{00} and Q_{01} bands may be due to interactions with the weak "background" bands since the MCD of these bands is positive. An alternate hypothesis is that these B terms were present in the Fe(II) cyt c spectrum but were masked by the much larger A terms.

The drastic decrease in MCD upon oxidation is associated with the drop in peak absorption and the increase in band width, seen in both MCD and absorption. If we assume that the integrated absorption intensity, which is proportional to the product $\varepsilon_0\Delta$(Δ is a measure of the width of the band), is constant, then the maximum absorption is proportional to Δ^{-1} while the maximum MCD goes roughly as Δ^{-2}.

This prediction is in reasonable agreement with the experimental data, since upon oxidation the Q_{00} absorption drops by a factor of between 4 and 5 while the MCD decreases by a factor of 23. We computed the ratio of the MCD from the peak-to-trough differences for both Fe(II) and Fe(III). This procedure tends to eliminate the influence of B terms.

The Electronic Structure of Fe(II) and Fe(III) Cyt c

The MCD of the Q_{00} bands of Fe(II) and Fe(III) cyt c implies properties of their electronic states. The value of M_z is a measure of the degree of freedom with which electrons can circulate through the molecule. For completely "free" electrons we would expect $M_z = 9$. Even after making liberal allowance for experimental errors, we conclude that for Fe(II) cyt c, M_z is equal to or greater than 7. The pi electrons of the porphyrin ring must thus be regarded as "nearly free". Intuitively we may think of Fe(II) cyt c as a metal disc embedded in an insulating sphere. This is an attractive model for a molecule whose biological function is to provide temporary storage for an electronic charge.

The high value of M_z and the resulting "free electron" nature of the porphyrin ring are likely related to the axial coordination of the iron ion. For example, Dratz[5] found that the M_z of iron protoporphyrin increased between 50 and 100% when two pyridine molecules substituted for a water molecule and a hydroxyl ion at the 5 and 6 positions of the iron. A theoretical explanation of this effect is clearly desirable. While the effects of the central metal ion have been discussed,[5,25] the best calculations of M_z to date[19] have not explicitly included the orbitals of the central metal ion and its axial ligands.

Our data on Fe(III) cyt c yield the important result that the 23-fold decrease in MCD upon oxidation is not due to a corresponding decrease in M_z but rather to a four- to fivefold broadening of the absorption band. Hochstrasser[23] has recently pointed out that the diffuseness of porphyrin transitions is related to the mixing of the π orbitals of the porphyrin ring and the d-orbitals of the metal atom. Thus in Fe(II) cyt c, we can think of the porphyrin π electrons and the

filled d_{xz} and d_{yz} orbitals as forming distinct systems, while for the Fe(III) molecule, the two systems are strongly mixed. This effect is more apparent in the Q_{00} band than in the Q_{01} or Soret bands, because these bands are much broader even in the reduced protein.

Soret Bands

We can explain some features of the spectra of the Soret bands in terms of the four-orbital theory. However, it is clear that other configurations must be included in the excited states responsible for the Soret bands.

The absorption spectra for ferro- and ferricytochrome c in the Soret region are shown in Fig. 5. The integrated absorption intensities are nearly equal, but, as in the Q bands, Fe(III) has a broader envelope than has the Fe(II). The MCD reveals greater differences. For Fe(II) the MCD is the sum of a normal A term and a positive B term. The positive B component cannot be due to magnetic mixing with the Q band since it would show up as a negative B term of magnitude comparable to the observed A term of that band. Thus, the +B term in the Soret band must be due to mixing with higher energy excited states or to unresolved spectral overlap with another transition. For Fe^{+3} there is less ambiguity since the shoulder in the MCD at 2.6 $(\mu m)^{-1}$ reveals the presence of a third transition.

Fig. 1 shows that the visible bands of Fe(III) are red shifted about 300 cm^{-1} while the Soret band is blue shifted by the same amount with respect to the corresponding bands of Fe(II). These shifts can be interpreted on the basis of the four orbital model as indicating an increase in configuration interaction in the oxidized states.

Near-infrared Bands - Fe(II)

Fig. 6 shows the absorption, CD and MCD of ferrocytochrome c in the near-infrared. The CD is very similar to that recently reported by Eaton and Charney.[6] Both the CD and MCD show that there is more than one band in the region between 1.0 and 1.3 $(\mu m)^{-1}$ which in absorption appears structureless. The large anisotropy factor (the ratio of CD to absorption), compared to the Q and Soret bands, led Eaton and Charney to conclude that the near-infrared spectrum is due in part to d→d transitions. The lowest energy d→d transition is from an A_{1g} ground state to an E_g excited state (the orbital promotions are from d_{xz} and d_{yz} to d_{z^2}). Eaton and Charney also proposed a decomposition of the CD into Gaussian components (i.e., $f(\bar{\nu},\bar{\nu}_i) = \exp - [(\bar{\nu}-\bar{\nu}_i)/\Gamma]^2$. In the low energy region they propose two bands at almost the same energy: a broad band (Γ = 1020 cm^{-1} centered at 1.17 $(\mu m)^{-1}$ with a positive CD, and one narrow band (Γ = 470 cm^{-1}) at 1.165 $(\mu m)^{-1}$ with a negative CD. Eaton and Charney suggest that these two bands may be the two components of the $A_{1g} \rightarrow E_g$ transition. It is quite difficult to reconcile the MCD with this proposal since if the 1.165 and 1.17 $(\mu m)^{-1}$ bands are each non-degenerate, then the MCD would be the sum of just two B terms with the corresponding centers and half-widths, and therefore symmetric. However, the MCD in this region is not symmetric with respect to 1.17. We can, however, rationalize the MCD with Eaton and Charney's bands if the broad band is degenerate. The MCD then corresponds to the sum of a normal A term and a negative B term for the broad band plus a negative B term for the narrow band, as shown in Fig. 7.

From 1.3 to 1.4 $(\mu m)^{-1}$ we can fit the MCD with the sum of a normal A and a positive B term using a band at 1.365 and a halfwidth of 800 cm^{-1}. The CD fitting procedure[6] found a band at this position but with a slightly

larger halfwidth. The remainder of the MCD was difficult to fit using the one remaining CD band at 1.585 $(\mu m)^{-1}$ and normal A terms. This may be due to the influence of the red edge of the Q_{00} band which is exceedingly strong in the MCD but relatively weak in CD. Curve fitting of the absorption spectrum[6] gave the maximum molar extinction coefficient of the 1.365 $(\mu m)^{-1}$ band as 200. The amplitude of the A term is .025 for this band and thus, if we assume Gaussian bands, the angular momentum is about .5 according to eqn. (11). If we assume ε_0 of the wide band at 1.7 $(\mu m)^{-1}$ is 50 $(M^{-1}\ cm^{-1})$, then the angular momentum of that transition is about 1.0 Bohr magnetons. As with any spectral decomposition, however, there is no guarantee of a unique solution. For example, the region from 1.0 to 1.3 $(\mu m)^{-1}$ could also contain three nondegenerate bands at 1.1, 1.17 and 1.23 $(\mu m)^{-1}$. Indeed, 77°K absorption spectra seem to give some support to this notion.[20]

695 nm Band - (Fe(III))

Ferricytochrome c has weak absorption bands to the red of the main absorption in the visible region. Besides the well resolved peak at 1.44 $(\ m)^{-1}$ there is a broad shoulder between 1.5 and 1.6 $(\ m)^{-1}$ (see Fig. 8). The existence of these bands was first reported by Theorell and Akesson.[21] These bands are particularly important because various treatments which destroy the biological activity of the protein cause them to disappear without affecting the other visible transitions.[22] It appears[24] that the existence of the 695 band is absolutely dependent on one of the iron ion's axial ligands being sulfur.

Eaton and Hochstrasser[3] concluded from the single crystal polarized absorption spectrum that the transition dipoles of the native protein

bands are perpendicular to the plane of the porphyrin ring. From the polarization and the sensitivity to ligation they suggested that the 695 nm bands are charge transfer transitions involving the iron ion and either the porphyrin ring or a residue of the polypeptide. In the former case the promotion would be porphyrin $a_{2u}(\pi)$ to iron a_{1g} (d_{z^2}) and the sensitivity to changes in the axial ligand would be due to the sensitivity of the energy of the d_{z^2} orbital to the axial coordination of the iron.

There is evidence of structure in both the CD and MCD of the native protein region. A large portion of the MCD is probably associated with the red edge of the broad positive MCD band which extends through the visible region (c.f., Fig. 2) and which is presumably associated with the porphyrin moiety. The dotted line from 1.39 to 1.51 $(\mu m)^{-1}$ in Fig. 8 is an attempt to separate the MCD associated with the native protein bands from that of the porphyrin. The ratio of the maximum value of the MCD of the native protein bands to their maximum absorption is much lower than the corresponding ratios in the visible and Soret regions of the protein in either oxidation state and of the near-infrared bands of the reduced protein. This suggests that the ground and excited states involved in the native protein bands are either nondegenerate or highly quenched.

Some of the CD to the red of 1.65 $(\mu m)^{-1}$ is also probably due to the red tail of the porphyrin bands but the trough at 1.44 and the shoulder at 1.54 $(\mu m)^{-1}$ appear to be associated with the native protein bands. The ratio of the maximum CD to maximum absorbance is $4 \pm 2 \times 10^4$ (the uncertainty is due to the unresolved contribution of the porphyrin). This anisotrophy is smaller than that of the near-infrared bands of the

reduced protein where the maximum value is 6.5×10^{-3} [6] and suggests that the transitions are Laporte (i.e., symmetry) allowed.

Our results are consistent with the proposal of Eaton and Hochstrasser[3] that the 1.44 $(\mu m)^{-1}$ bands are a_{2u} (π) to a_{1g} (d_{z^2}) charge transfer promotions. The MCD suggests that both initial and final states may be nondegenerate; both a_{2u} and a_{1g} are nondegenerate. The CD suggests that the transition is electric dipole allowed; the direct product of a_{1g} and a_{2u} is a_{2u} which is the representation of the electric dipole operator of the z axis of the molecule in D_{4H} symmetry. The single crystal polarization spectrum shows that the transition dipole of the 1.44 $(\mu m)^{-1}$ band is parallel to the z axis of the molecule. The absorption intensity is low for an electric dipole transition. This presumably is due to the small overlap of the wavefunctions of the initial and final states and reflects the charge-transfer character of the transition. Of course, our data cannot exclude charge transfer transitions involving the axial ligands.

CONCLUSIONS

1. The angular momentum of the excited state responsible for the Q_{00} band in Fe(II) cyt c is about 7.9 Bohr magnetons. This value is essentially identical to that found when no correction was made for the effects of zero-field splitting but significantly larger than the value predicted by the most recent molecular orbital calculations.

2. The values of M_z computed from differential MCD, assuming no zero-field splitting, are reasonable estimates of the true value. However, the differential form of MCD gives no clear indication of the existance of zero-field splitting, while the integral MCD does. The integral form of MCD would grossly overestimate M_z.

3. The drastic decrease in the magnetic optical activity of the Q_{00} band in Fe(III) cyt c compared to Fe(II) cyt c can be explained largely on the basis of broadening of the absorption band rather than any fundamental change in the angular momentum or oscillator strength of the transition. The broadening, which may be accompanied by a substantial increase in zero-field splitting, reflects increased mixing of the e_u porphyrin orbitals with the d_{xz} and d_{yz} metal orbitals in the Fe(III) state.

4. MCD indicates the presence of more than two transitions in the Soret region.

5. The MCD of the near-infrared transitions of Fe(II) cyt c can be decomposed using basically the same band positions and shapes as were used to fit the CD spectrum if two bands are presumed to be degenerate. The angular momenta of both of the degenerate components are of the correct magnitude to be the $A_{1g} \rightarrow E_g$ d→d promotion. We emphasize, however, that there is no uniqueness theorem in curve-fitting.

6. The band at 695 nm in Fe(III) cyt c appears to be nondegenerate and electric dipole allowed. These are properties expected of the a_{2u} (π) $\rightarrow a_{1g}$ (d_{z^2}) porphyrin to iron charge transfer transition.

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[10]This is called an "A" type MCD spectrum. "B" and "C" type MCD spectra resemble the shape of the corresponding absorption spectrum. "B" terms are due to the magnetic coupling of states which are non-degenerate with each other while "C" terms are due to the unequal population of the two components of a degenerate ground state when it is split by a magnetic field. For reviews of magnetic optical activity see Refs. 11 and 12. Our nomenclature for "C" terms follows Ref. 11. The "A", "B", and "C" term nomenclature is sometimes ambiguous. For example, the 280 nm absorption band of tryptophan contains two closely spaced transitions. The MCD of this band qualitatively resembles an "A" term, but can equally well be thought of as two closely spaced "B" terms of opposite sign. The approach to MCD developed in this paper and Ref. 13 avoids this ambiguity.

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[14] In the appendix of Ref. 13 we performed the integration over for the first two terms in the series expansion of the MCD. The equations leading to eqn. 2 are given in detail in Ref. 13. Note that the right hand side of eqns. A-9 and A-10 of Ref. 13 should be increased by a factor of 2 (i.e., $\varepsilon_x(\bar{\nu}) = 2R_x^2 F(\bar{\nu},\bar{\nu}_x)/3$) because $\varepsilon_x(\bar{\nu})$ is the molar extinction coefficient for $|g\rangle \rightarrow |x\rangle$ observed in solution and not the x polarized component of this absorption.

[15] According to the four-orbital model,[9] intensity inbalance is a direct consequence of zero-field splitting. The effect of "B" and "C" type terms is to add odd derivatives to the first term and even derivatives to the second and third terms of eqns. (7).

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TABLE I.

The solid curve in Fig. 7 is calculated from the equation

$$\Delta\varepsilon_M/\bar{\nu} = \sum_i [(\sqrt{2}e^{1/2}(\bar{\nu}-\bar{\nu}_i)A_i/\Gamma_i)+B_i]e^{-[(\bar{\nu}-\bar{\nu}_i)/\Gamma_i]^2}$$

The values of A_i, B_i, $\bar{\nu}_i$ and Γ_i for each band are given below. The names of the three components, Ia, Ib and II are from Ref. 6. The figures in parentheses are the values of $\bar{\nu}_i$ and Γ_i used in Ref. 6 in those cases where they differ from the values we used.

	A	B	$\bar{\nu}_i$ $(\mu m)^{-1}$	Γ_i $(\mu m)^{-1}$
Ia	0	-.045	1.160 (1.165)	.047
Ib	+.01	-.025	1.165 (1.170)	.102
II	+.025	+.065	1.36	.08 (.092)

i

APPENDIX

In a previous paper[13] we calculated the probability of absorption of left and right circularly polarized light from the ground state to states $|1\rangle$ and $|2\rangle$ (c.f., Fig. 3). The four calculated probabilities were expressed as functions of the probability of absorptions to the zero-field states $|x\rangle$ and $|y\rangle$ and also the coefficients α and γ given in eqn. 1. The results of these calculations were given in eqn. 9 and also eqns. 12 of ref. 13. These expressions are much easier to understand, however, if the coefficients α and γ are replaced by functions of the zero-field splitting, $\Delta\bar{\nu}_{ZF}$, and magnetic interaction energy, $\beta M_z H \cos\theta$. The identities required for this transformation are just those given in eqns. 6. If we let ${}^{i}\varepsilon_{L,1}$ stand for the integrated absorption intensity of LCPL for the transition to $|1\rangle$, ${}^{i}\varepsilon_{o}$ for the integrated absorption intensity for the zero-field absorption band and ${}^{i}\Delta\varepsilon$ for the integrated intensity imbalance, then eqns. 12 of ref. 13 become:

$$
\begin{aligned}
{}^{i}\varepsilon_{L,1} &= ({}^{i}\varepsilon_{o}/4) - ({}^{i}\varepsilon_{o}/4)[(\beta M_z H \cos\theta)/\Delta\bar{\nu}_T]\left\{1-[2\,{}^{i}\Delta\varepsilon/{}^{i}\varepsilon_{o}]^2\right\}^{1/2} + ({}^{i}\Delta\varepsilon/2)(\Delta\bar{\nu}_{ZF}/\Delta\bar{\nu}_T) \\
{}^{i}\varepsilon_{R,1} &= \quad '' \quad + \qquad '' \qquad + \qquad '' \\
{}^{i}\varepsilon_{L,2} &= \quad '' \quad + \qquad '' \qquad - \qquad '' \\
{}^{i}\varepsilon_{R,2} &= \quad '' \quad - \qquad '' \qquad - \qquad ''
\end{aligned}
$$

By means of eqns. 15 it is much easier to follow the arguments which lead to Fig. 5 of ref. 13.

FIGURE LEGENDS

Fig. 1. The molar extinction coefficient of Fe(II) and Fe(III) cyt c in the near-ultraviolet, visible and near-infrared regions.

Fig. 2. MCD and absorption spectra of the visible bands of ferri- and ferrocytochrome c. Upon oxidation both the MCD and absorption decrease in magnitude and are broadened while the integrated absorption intensity appears to remain constant. The vertical scales have been changed to retain resolution. Absorption and MCD are plotted in units of $M^{-1}cm^{-1}$ and $M^{-1}cm^{-1}T^{-1}$ respectively.

Fig. 3. Energy levels of the nearly degenerate excited state in the absence and presence of an external magnetic field.

Fig. 4. a) The calculated (——) and observed (···) differential MCD. The calculation used the values of $\bar{\nu}_0, \Delta\bar{\nu}_{ZF}$, ε_0, $\Delta\varepsilon$ and the Lorentzian envelope which fit the zero-field absorption spectrum and was adjusted to fit the experimental data by proper choice of M_z. b) The calculated (——) and observed (···) zero-field absorption spectrum. The calculated spectrum is the sum of two Lorentzian components. No attempt was made to fit the vibrational bands which lie above 1.835 $(\mu m)^{-1}$. c) The integral MCD calculated from the parameters determined above for a magnetic field of 10 T. The absorption band for left circularly polarized light is wider and has a smaller peak amplitude than that for right circular polarization. The two peaks are separated by 57 ± 5 cm^{-1}, corresponding to an apparent angular momentum of 12.2 ± 1.2 Bohr magnetons.

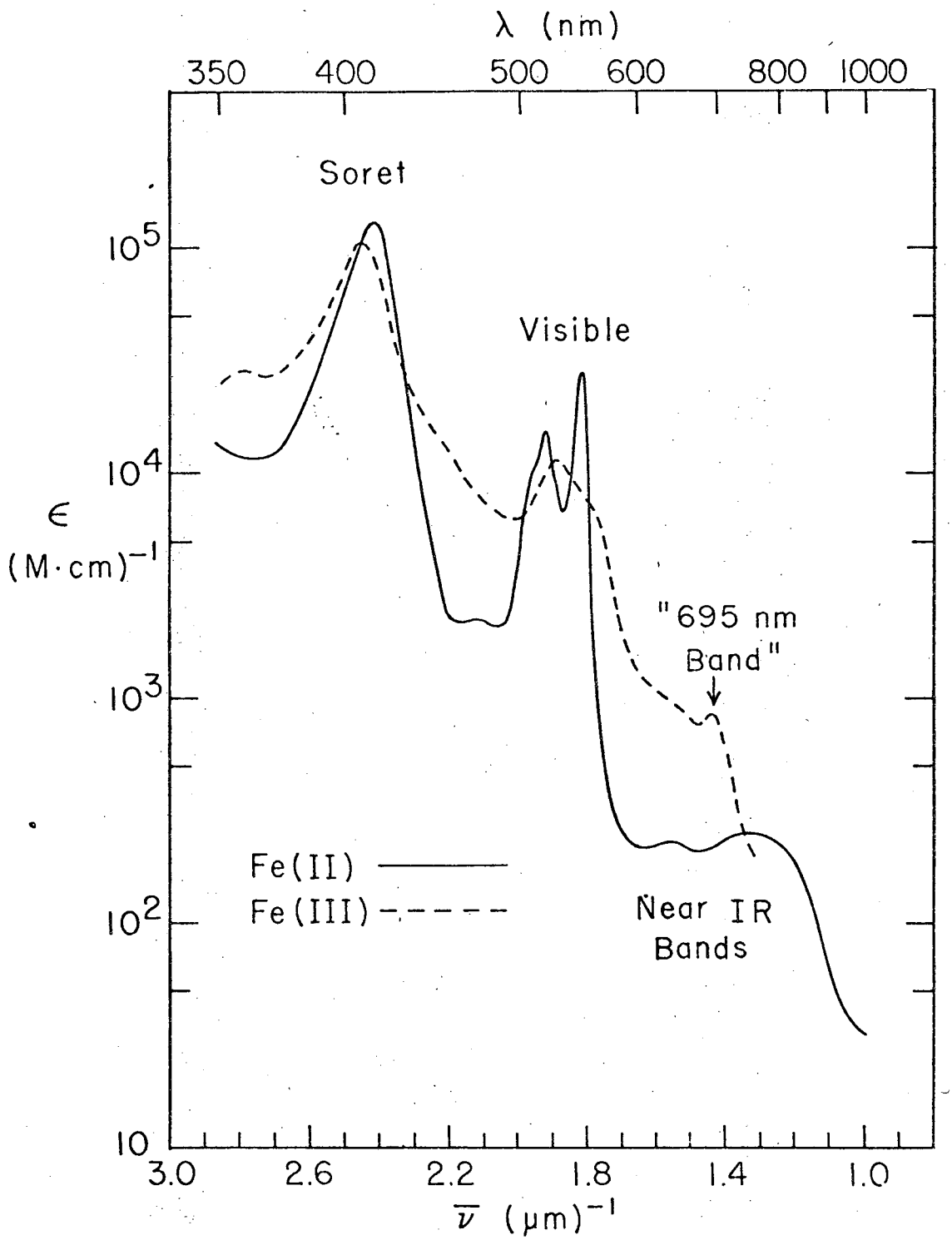
Fig. 5. Absorption and MCD of the Soret bands. The solid lines are for Fe(II) and the dashed lines are for Fe(III).

FIGURE LEGENDS (Cont.)

Fig. 6. Absorption, CD and MCD of ferrocytochrome c in the near-infrared. Both CD and MCD indicate the presence of structure between 1.0 and 1.3 $(\mu m)^{-1}$ which is not resolved in absorption.

Fig. 7. A possible decomposition of the MCD of Fe(II) cyt c using bands similar to those which resolved the CD.[6] The calculated curve (solid line) is the sum of Gaussian components and their derivatives (dashed lines). The circles are the experimental data.

Fig. 8. Absorption, CD and MCD of the 695 nm band of ferricytochrome c. The dotted line is an attempt to separate the MCD of the 695 nm band from that of the porphyrin ring.



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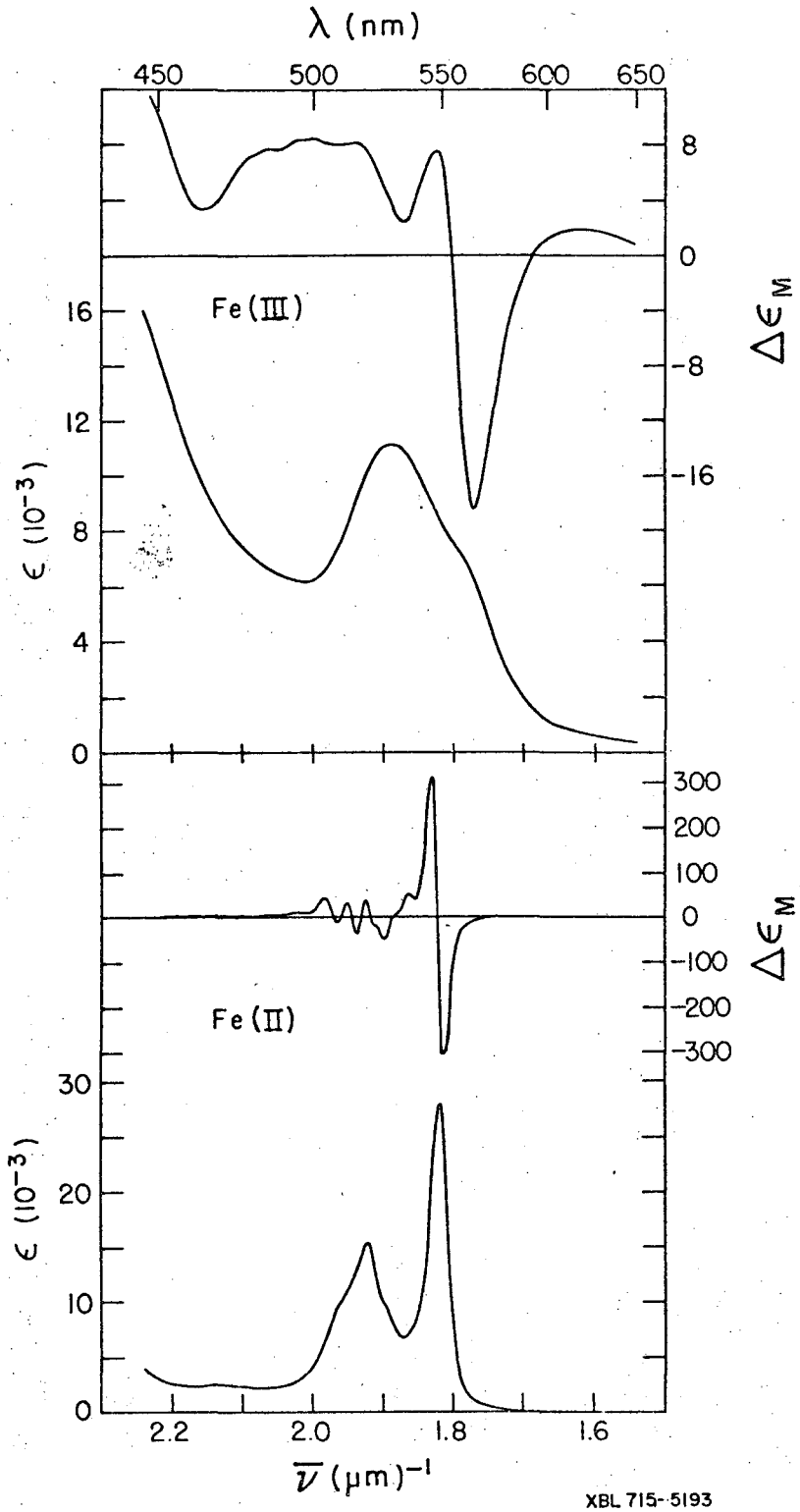
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200
100
0
-100
-200
-300
ΔεM
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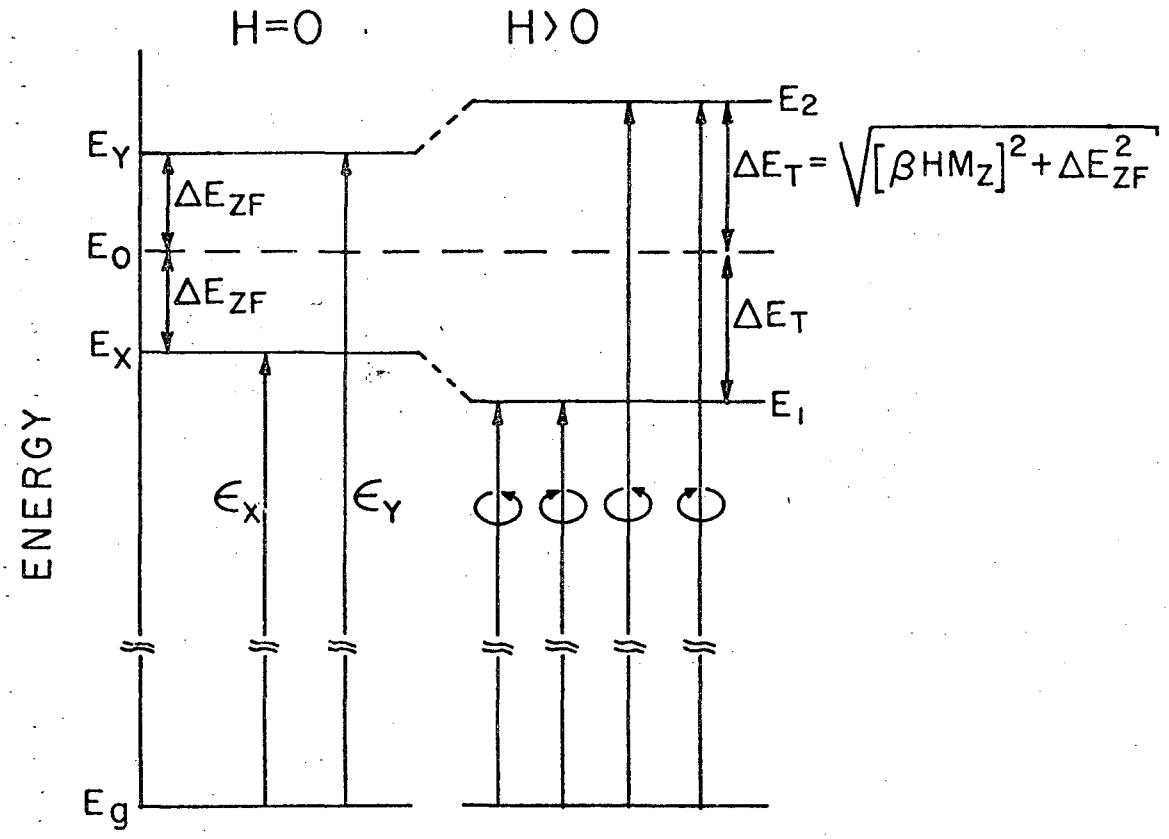
ν̄ (μm)⁻¹
XBL 715-5193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E_0
E_X
E_g
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ΔE_{ZF}
ΔE_{ZF}
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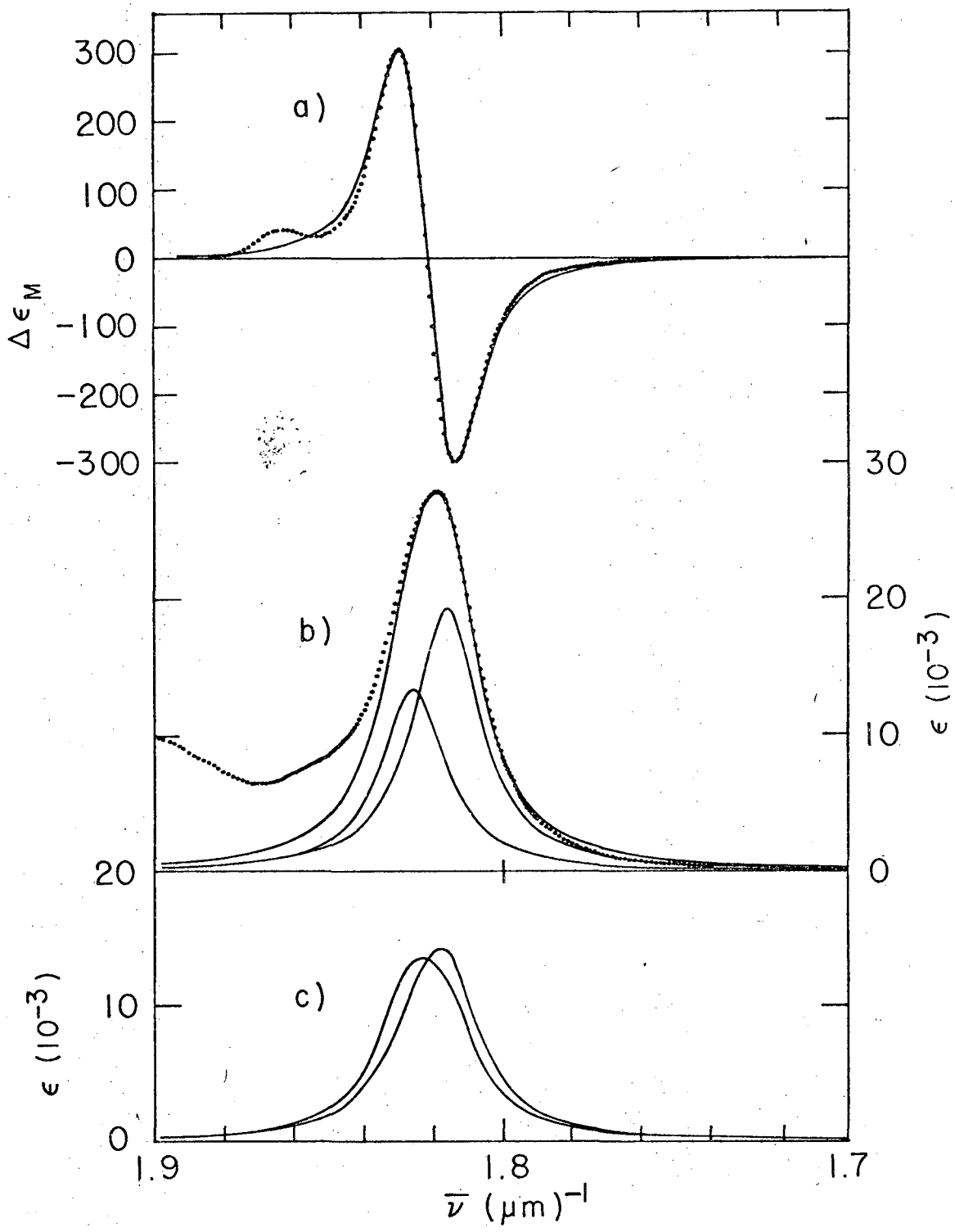
ΔE_T
ϵ_X
ϵ_Y

XBL719-5365



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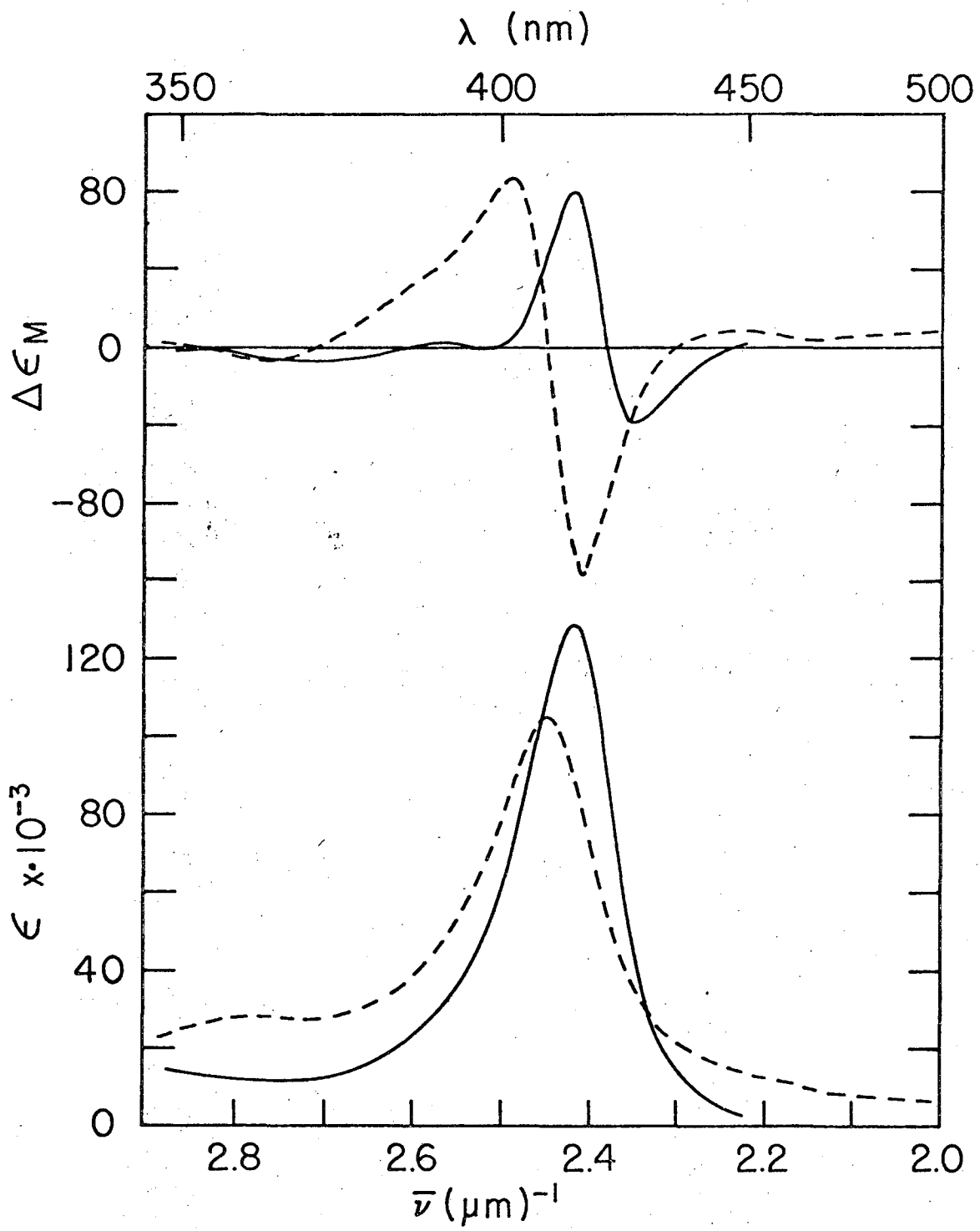
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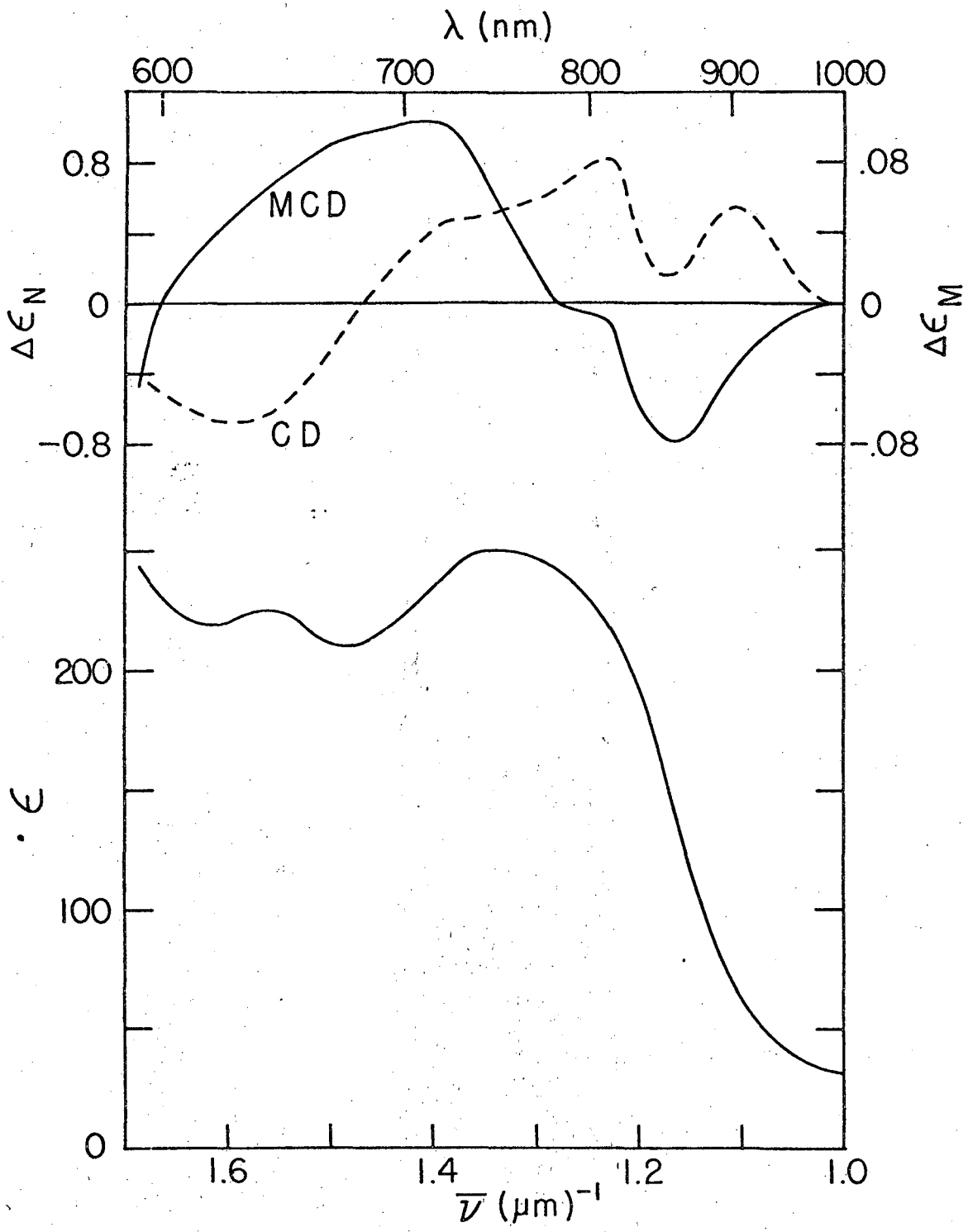
2.0
ν̄ (μm)⁻¹

XBL716-5236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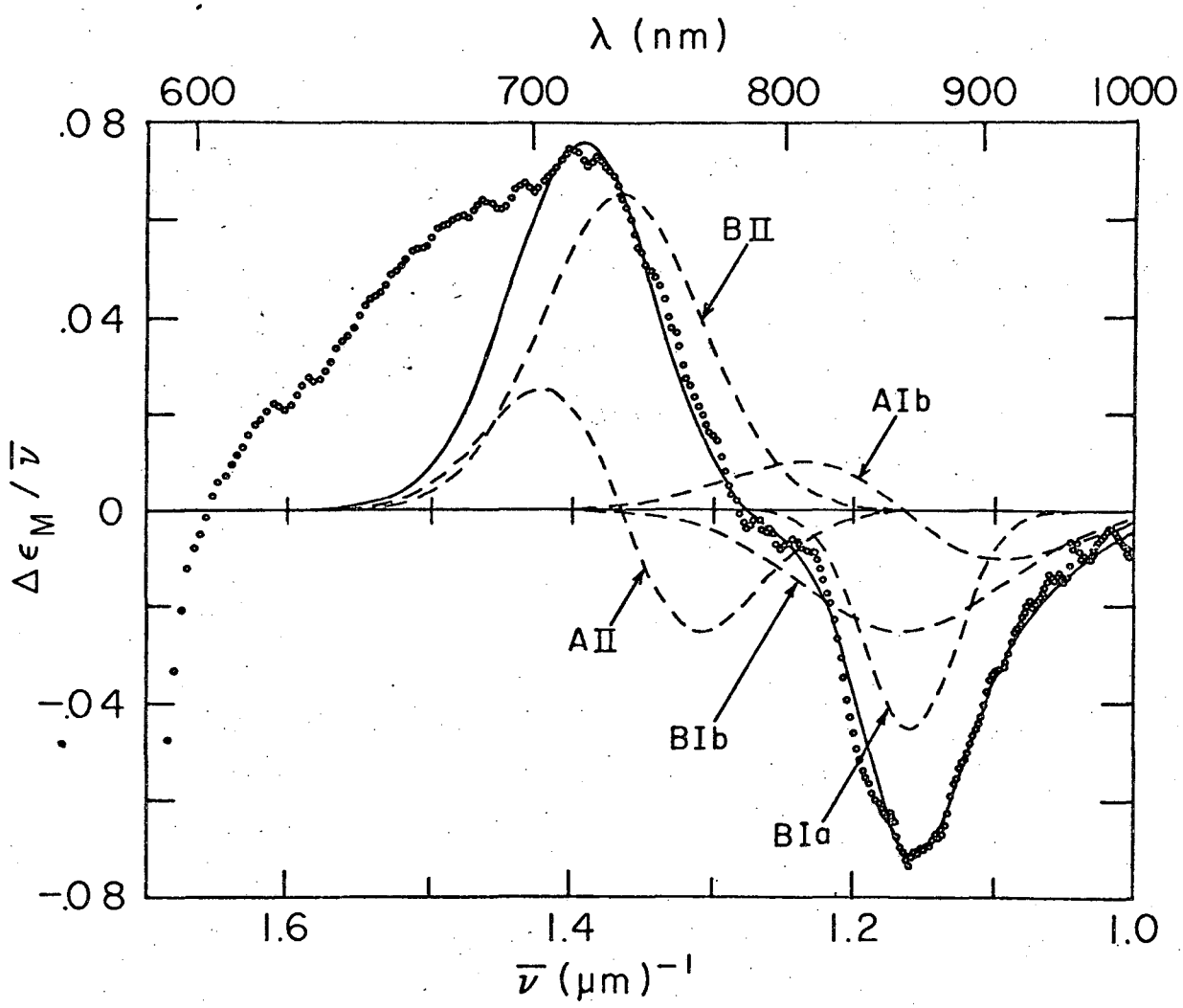
XBL716-5203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$\Delta\epsilon_M / \bar{\nu}$
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BIa
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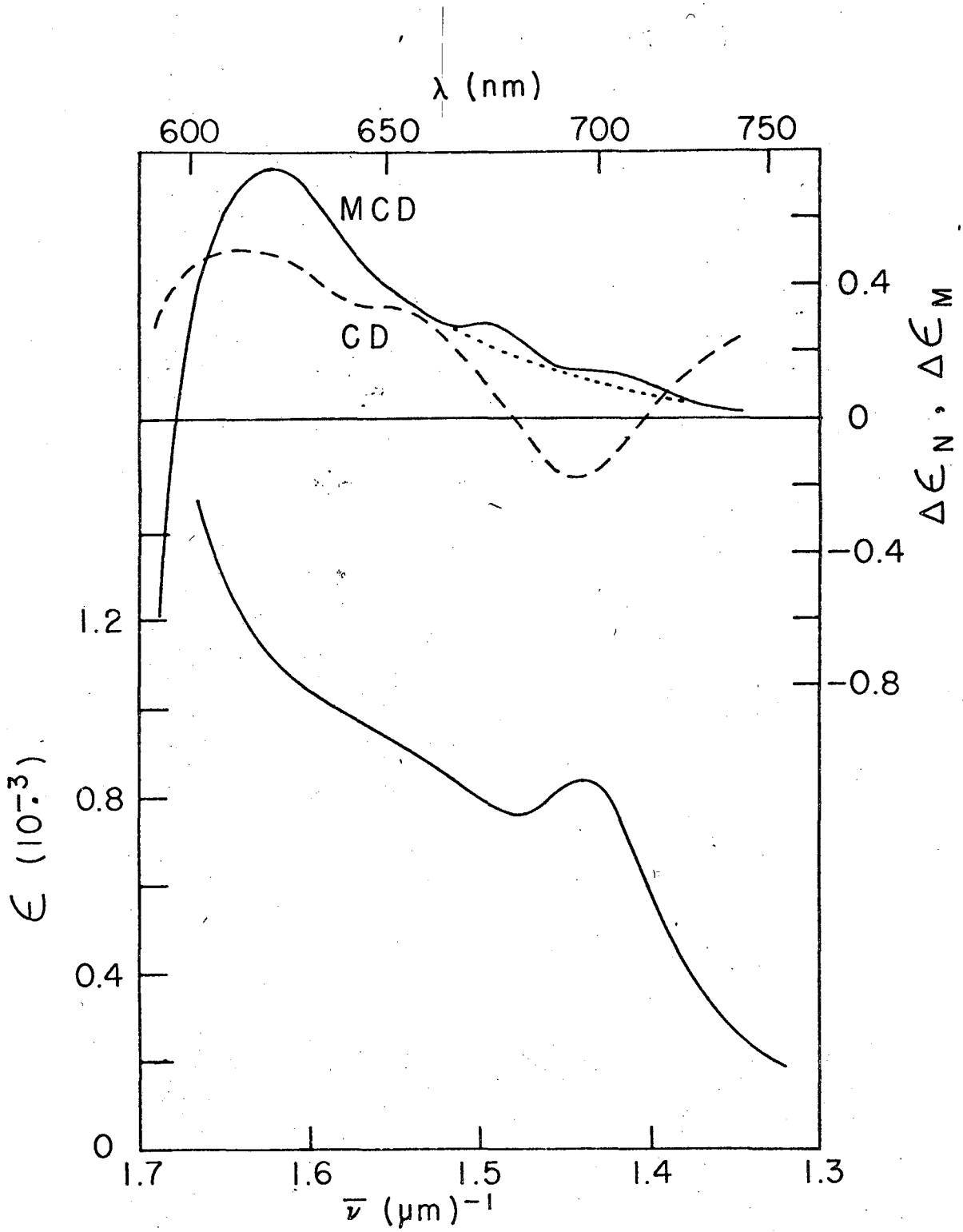
$\bar{\nu}$ (μm)$^{-1}$

XBL 717-5283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0.4
0
ε (10⁻³)
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1.6
1.5
1.4
1.3
ν̄ (μm)⁻¹

XBL717-5281

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