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DISCLAIMER

A Spectrometer for the Measurement of Magnetic and Natural Circular Dichroism*

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ABSTRACT

An instrument employing several novel features has been assembled for the measurement of magnetic and natural circular dichroism (MCD and CD) in the visible, ultraviolet and near infrared spectral regions. The instrument is fitted with two magnets: an electromagnet for routine use and a superconducting magnet for use when higher performance is required. The contributions of CD and MCD are separated by measuring the net spectrum twice; first with the magnetic field parallel to the Poynting vector of the optical beam and then with it antiparallel. The value of the net optical activity for each wavelength increment is converted to digital form and the manipulations required to separate CD and MCD and signal averaging are performed by a computer. The data can be plotted in a variety of formats on a Cal Comp Plotter. The instrument is equipped with a Cary Model 14 prism-grating monochromator which, unlike double quartz prism monochromators, has good wavelength dispersion from the uv to the near ir spectral regions. Performance in the ultraviolet has been considerably improved by replacing the previously used electro-optical modulator (Pockels cell) with a quartz strain-birefringence modulator. The capabilities of this instrument are illustrated by spectra for the natural CD of ribulose-1,5-diphosphate carboxylase at 22° from 195-245 nm, the MCD of metmyoglobin cyanide at -196° and 22° from 340-470 nm, and the MCD of oxyhemoglobin at 4° from 700-1100 nm.

INTRODUCTION

Our spectrometer, unlike most other instruments used to measure magnetic circular dichroism (MCD), was not derived by mounting a magnet in an existing, commercially available spectropolarimeter.[1] The instrument is intended to be used to investigate the CD and MCD of biological materials in the ultraviolet, visible and near-infrared. By assembling an instrument from discrete components, we were able to incorporate several features which greatly facilitate this work. This report will concentrate on these unique aspects of our spectrometer.

INSTRUMENTATION

A schematic plan view of the optical train and a block diagram of the electronics logic are shown in Fig. 1. Light from a 450-watt Xenon arc is collimated by an off axis ellipsoidal mirror and dispersed by a prism-grating double monochromator (Cary Instruments Div., Varian Associates, Palo Alto, California). The monochromatic beam passes through a Rochon polarizer (Carl Lambrecht) and the ordinary component is alternately rendered right- and left-circularly polarized by a piezo-optical modulator driven at 50 kHz (Morvue Electronic Systems, Tigard, Oregon), which is programmed to operate as a quarter-wave plate. The modulated beam then passes through the sample and falls on the photomultiplier tube. If MCD is being measured, the sample is located in the magnetic field of either an iron-core electromagnet or a superconducting magnet. If only CD is being measured, the sample is placed in a third position which allows ample space for controlled temperature sample holders, cryogenic dewars, and actinic illumination equipment. Both magnets and the "CD only" sample area are located

on an aluminum table which can roll relative to the stationary optical bench on bronze roller bearings. The table is moved laterally to position the sample in the light beam by a hand-operated lead screw.

The photomultiplier current is amplified and the a.c. component sent to a phase-sensitive detector while the difference between the d.c. component and a fixed reference voltage is used to control the high voltage power supply of the photomultiplier so that the d.c. photocurrent remains essentially constant. The output of the phase-sensitive detector, which is proportional to the net circular dichroism is displayed locally on an x-y recorder and is converted to digital form and transmitted to a laboratory-wide data collection system.[3] Averaged results from multiple passes are also displayed locally on a Tektronix, Inc., Type 611 storage display unit equipped with a Tektronix 4601 hard copy unit for rapid analysis of results.

One of the novel features revealed in Fig. 1 is the presence of two magnets, either of which may be positioned in the optical beam. The two magnets represent different trade-offs between performance on the one hand and ease of use and economy on the other. The magnitude of the circular dichroism induced by a magnetic field is directly proportional to the magnitude of the field. Thus, when high sensitivity is required, the sample is placed in the superconducting magnet where the magnetic field intensity can be as high as 6.6 Tesla (66,000 Gauss). Use of the superconducting magnet is indicated either when the magnetic anisotropy ($\Delta\varepsilon_{MCD}/\varepsilon$, see eqs. 1 and 5 below) is small in an absolute sense or when the magnetic anisotropy is small compared to the natural anisotropy ($\Delta\varepsilon_{CD}/\varepsilon$).

The magnetic field of the electromagnet is only about 1.4 T when it is fitted with pole pieces which accommodate 1 cm path cuvettes. However, it is energized simply by throwing a switch and turning a knob and thus requires considerably less effort to operate than does the superconducting magnet. Further, operation of the electromagnet does not involve the high operating expenses incurred by the superconducting magnet (principally for liquid helium).

The electromagnet has one additional limitation. The light beam enters and leaves the sample via holes bored in the pole pieces. To insure a reasonably homogeneous field the diameter of these holes was restricted to 1 cm. The light beam at its greatest extent is larger than the size of the hole so that about 50% of the available light must be masked off to prevent unwanted scattering from the inside of the magnet. Loss of this extra intensity becomes a critical factor in the far uv and near ir where light intensity and detector sensitivity, respectively, are critical factors in determining the instruments signal-to-noise ratio. Because of the inhomogeneity of the magnetic field we have found the use of a standard sample superior to a Hall probe gaussmeter for determining field strength upon repositioning of the magnet. In this manner the light beam can be used to measure the average magnetic flux across that part of the reference and sample which is illuminated. Solutions of potassium ferricyanide, prepared gravimetrically or assuming $\epsilon_{420}^{mM} = 1{,}020$, were routinely utilized. For temperatures near 22°C, $\Delta\epsilon_{422}/H = 3.0\ (M \cdot cm \cdot Tesla)^{-1}$. The superconducting magnet has a bore 2.6 cm in diameter, with a more homogenous field, and passes the entire beam freely.

The photomultipliers are mounted on a base which can be moved both parallel and perpendicular to the optical axis and locked in position at any point within its range of travel. The perpendicular motion permits the rapid and reproducible interchange of detectors. Presently the instrument is fitted with photomultipliers with S-1 and S-20 photocathodes for near ir and visible-uv operation, respectively. The parallel motion is used to bring the detectors close to the sample cuvette when the CD of a highly scattering sample is being measured. When MCD is being measured the detectors are moved away from the magnets.

A prism-grating monochromator was chosen because the original version of the instrument was built to study the CD and MCD of chlorophyll in the near ir.[2] The monochromator has nearly constant dispersion in this region and is thus superior to the quartz prism monochromators of commercial instruments whose dispersion drops sharply at long wavelengths. The original instrument[1] was fitted with an electro-optical modulator (Pockels cell) which in combination with the prism-grating monochromator prevented operation in the uv below about 250 nm as a result of low light intensity. This situation has been corrected by replacing the Pockels cell with a photoelastic modulator. The wider acceptance angle of this modulator drastically reduced the effective f number of the optical train.

CALCULATIONS

One of the greatest experimental difficulties involved in measuring the MCD of a biological material is that the sample is likely to exhibit natural CD. The observed circular dichroism is thus the sum of these two effects: i.e.,

$$\Delta A \equiv A_L - A_R = \Delta A_{CD} + H\Delta A_{MCD} \qquad (1)$$

where A_L and A_R are the absorbance of left and right circularly polarized light and H is the magnetic field intensity.[4] The standard procedure for extracting ΔA_{MCD} is to measure ΔA first with the magnetic field on and then with it off.[7] We use a modification of this procedure which doubles the amount of time spent recording in the presence of the magnetic field and thus improves the MCD signal-to-noise ratio by $\sqrt{2}$. First we measure the net CD (i.e., ΔA) with the magnetic field parallel to the light beam. Then we reverse the direction of the field and repeat the measurement. The two measured spectra are given by

$$\Delta A_+ = \Delta A_{CD} + |H|\ \Delta A_{MCD}$$

and (2)

$$\Delta A_- = \Delta A_{CD} - |H|\ \Delta A_{MCD}$$

where the absolute value sign indicates that the sign of the magnetic field is now written explicitly. Addition of the two spectra effectively cancels the MCD contribution since reversal of the direction of the magnetic field simply changes the sign of the MCD and

$$\Delta A_{CD} = \frac{\Delta A_+ + \Delta A_-}{2} \quad . \tag{3}$$

Thus the CD signal-to-noise ratio is also increased by a factor of $\sqrt{2}$. Subtraction of the two curves removes the natural CD contribution and yields

$$\Delta A_{MCD} = \frac{\Delta A_+ - \Delta A_-}{2} \quad . \tag{4}$$

If the base line is known to be flat, the same two sets of data also suffice to determine ΔA_{CD}. Otherwise, a solvent-only reference must be run and the data subtracted from Eq. (2). At higher detection sensitivities or in instances of inadequate shielding, the baseline may be slightly dependent on

the magnetic field. In these cases the MCD of the reference is measured for both orientations of the magnetic field and subtracted from the corresponding sample spectra. Conversion from the units of absorbance to units of molar extinction is accomplished using relations of the form

$$\Delta\epsilon = \frac{\Delta A}{\underline{c}\,\underline{\ell}} \tag{5}$$

where $\underline{c}$ is the molar concentration and $\underline{\ell}$ is the optical path through the sample in cm.

The direction of the magnetic field in the electromagnet is reversed by reversing the direction of current flow in the magnet's coils. This simple approach is impractical with the superconducting magnet since 10 to 15 minutes are required to charge the magnet to its full field and also because charging and discharging the magnet greatly increases the rate of helium loss. Our solution to the problem of reversing the direction of the field was to mount the magnet on a base which rotates through 180°. The limits of rotation are determined precisely by adjustable stops which insure reproducible alignment.

RESULTS

We have previously reported MCD spectra of cytochrome c in the visible and near ir spectral regions obtained using the 6.6 T superconducting magnet.[9] The spectra were redrawn from a computer output and did not illustrate the signal-to-noise ratios attainable with the instrument as do the data reported herein. Presently, data are transmitted directly to a Scientific Data Systems Model Sigma 2 computer equipped with a Cal Comp plotter,[3] and the experimental results are plotted in a manner suitable for photocopying.

The raw data obtained in an MCD experiment on oxyhemoglobin are given in the photograph shown in Fig. 2. For this sample and in this spectral region the MCD greatly exceeds the CD, and the two curves for the opposite field directions are practically symmetrical about the basline. The use of Eqn. 4 in the calculations, however, removes any CD contribution, and it is not necessary to scan the sample at zero field. Since all measurements are made in the presence of the magnetic field, the MCD signal-to-noise ratio is increased. This is especially advantageous when investigating small changes in difference MCD spectra.

The results for a case where the CD magnitude is comparable to the MCD are illustrated in Fig. 3. The plus and minus field curves are not mirror images of one another and indicate the presence of a relatively intense natural CD. The optical activity in the absence of the field is seen to be the average of spectra obtained for opposite field directions (cf., Eqn. 3).

The computer plotted results obtained with three different biological samples, in different spectral regions and at varying temperatures, are given in Figs. 4-6. The natural CD of the enzyme, ribulose-1,5-diphosphate carboxylase, in the protein intrinsic, or peptide bond absorption region, is shown in Fig. 4. The noise level obtained in the region of interest for determination of polypeptide conformation, viz., ca. 210 nm, is approximately $\Delta A \approx 2 \times 10^{-5}$.

Fig. 5 shows the MCD of the cyanide complex of ferri- or metmyoglobin at 22° and at -196°. The increased MCD observed at liquid nitrogen temperature is due to C type MCD terms resulting from the degenerate ground state of the Fe(III) heme.[10] For the study of low temperature effects on the MCD of samples a narrow dewar is inserted into the 2.8 cm gap between

magnet pole pieces providing a field of 0.97 T, and either a cold inert gas blown over the sample or liquid nitrogen added directly. The options of plotting the results in units of differential molar extinction rather than differential absorption and in terms of energy rather than wavelength were chosen in contrast to Fig. 4. Noise levels in this spectral region are generally about $\Delta A = 2 \times 10^{-5}$ for a single pass when a sample of O.D. = 1 and a 0.3 sec time constant are used (see also Fig. 3).

The MCD spectrum of oxyhemoglobin in the near ir was obtained using an S-1 photomultiplier (Fig. 6) and the electromagnet fitted with longer pole pieces yielding a field of 1.43 T. This MCD spectrum is very similar to the spectrum of equine oxyhemoglobin reported by Sutherland et al.[11] Sample temperature was maintained with a water jacketed cell. Further extensions into the ir spectral region will be possible shortly with the incorporation of an InAs detector of the type described by Stephens et al.[12] and ancilliary servos.

Assembled, as opposed to purchased, instruments offer the advantage of flexibility and make it easier to tailor the instrument for specific applications as well as offering protection against obsolescence. With the recent appearance of a commercial photo-elastic modulator and its associated electronics, all of the basic components of a CD or MCD instrument can now be purchased separately. Thus, this approach to instrument development should become increasingly attractive.

REFERENCES

* Research sponsored, in part, by the U. S. Atomic Energy Commission.

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‡ Postdoctoral research fellow of the National Institute of General Medical Sciences, National Institutes of Health.

[1] The present instrument has evolved from an instrument originally constructed by Dratz and Klein. A detailed description of this precursor was given by Dratz.[2]

[2] E. A. Dratz, Ph.D. Thesis, 1966, University of California, Berkeley.

[3] J. A. Despotakis, R. L. Fink, M. S. Itzkowitz, and M. P. Klein, presented at the Pacific Conference on Chemistry and Spectroscopy, San Francisco, 1970, UCRL Preprint #20132. A detailed description of the Laboratory of Chemical Biodynamics data acquisition system, ACQUIRE, for publication, is in preparation.

[4] Exceptions occur for exceedingly narrow absorption lines, magnetic fields in excess of those available for normal laboratory use or in samples exhibiting cooperative effects. See references 5 and 6 for recent reviews on MCD theory and applications.

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[7] A. Abu-Shumays et al.[8] reported an instrument which eliminates the effects of CD by modulating the magnetic field rather than the polarization of the light beam. Two measurements are still required for an MCD measurement (sample and baseline) but the baseline is generally flat in contrast to the present situation in which wavelength dependent

variations in the "baseline" may be greater than the contribution of the MCD. The limitation of this elegant method is the difficulty of modulating a powerful magnet at the required frequency. The magnet which Abu-Shumays et al. used had a peak field of only 0.35 Tesla.

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[12] G. A. Osborne, J. C. Cheng, and P. J. Stephens, Rev. Sci. Inst. 44, 10 (1973).

FIGURE LEGENDS

Fig. 1. Schematic plan view of the optical components and electronics layout. The heavy arrows indicate movement of components.

Fig. 2. MCD spectra of human oxyhemoglobin. The sample concentration was 7.7×10^{-5} M in heme with a maximum absorption of 1.12 at 577 nm in a 1 cm cell. Solvent: 0.1 M HEPES, pH 7.0, 10^{-3} M EDTA, 10^{-3} M inositol hexaphosphate; temperature: 4°; scan rate: 5 Å/sec; time constant: 0.1 sec; slit width: 0.4 mm; magnetic field: 1.43 T; two passes each with the magnetic field parallel (+H) and antiparallel (-H) to the Poynting vector of the measuring beam are shown.

Fig. 3. CD and MCD spectra of human methemoglobin. The heme concentration was 7.0×10^{-6} M yielding a maximum absorption of 1.26 at 405 nm in a 1 cm cell. Solvent: 0.05 M HEPES, pH 6.1; temperature: ambient (22°); scan rate: 5 Å/sec; field: 1.23 T; single passes were obtained on an x-y recorder with the sample in positive field (+H), negative field (-H), zero field (CD), and a solvent-only baseline (Ref.).

Fig. 4. CD spectrum of spinach ribulose-1,5-diphosphate carboxylase. The sample contained 0.4 mg protein/ml (7×10^{-7} M in enzyme). Solvent: 0.1 M Tris-HCl, pH 7.6, 0.05 M magnesium chloride, 0.05 M sodium bicarbonate; path: 0.02 cm; temperature: ambient (22°); scan rate: 2.5 Å/sec; time contant: 1 sec; slit width: 2 mm; photomultiplier: EMI 9559 QB; ten passes were averaged.

Fig. 5. MCD spectra of sperm whale metmyoglobin cyanide. The sample concentration was 6.7×10^{-5} M with a maximum absorption of 1.47 at 423 nm in a path length of 0.2 cm. Solvent: potassium glycerophosphate-glycerol-0.1 M sodium phosphate, in equal volumes, pH 6.8; scan rate: 5 Å/sec; time constant: 0.3 sec; slit width: 0.4 mm; field: 0.97 T; photomultiplier: EMI 9559 QB; a total of four passes were averaged.

Fig. 6. MCD and absorption spectra of human oxyhemoglobin. The sample was 1.6×10^{-3} M in heme and had a maximum absorption at 930 nm of 0.48 in a 1 cm cell. Solvent: 0.1 M HEPES, pH 7.0, 10^{-3} M EDTA; temperature: 4°; scan rate: 5 Å/sec; time constant: 1 sec; slit width: 2 mm; field: 1.43 T; four passes were averaged. A Dumont model 6911 S-1 type phototube was used for the MCD measurement and a Cary Model 14R spectrophotometer for the absorption spectrum.

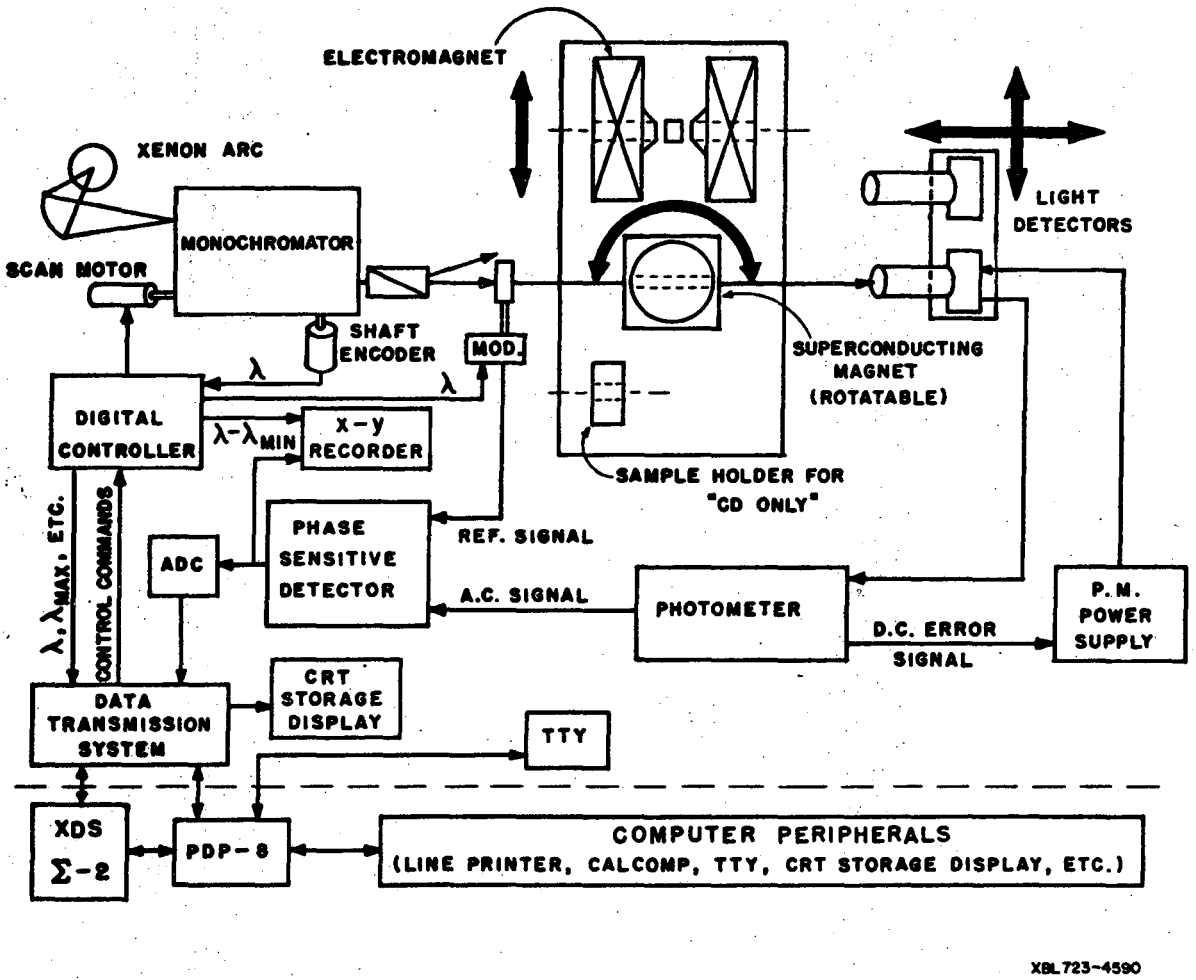

ELECTROMAGNET
XENON ARC
MONOCHROMATOR
SCAN MOTOR
SHAFT ENCODER
MOD.
LIGHT DETECTORS
SUPERCONDUCTING MAGNET (ROTATABLE)
SAMPLE HOLDER FOR "CD ONLY"
DIGITAL CONTROLLER
λ-λMIN
X-Y RECORDER
λ, λMAX, ETC.
CONTROL COMMANDS
PHASE SENSITIVE DETECTOR
REF. SIGNAL
ADC
A.C. SIGNAL
PHOTOMETER
D.C. ERROR SIGNAL
P.M. POWER SUPPLY
DATA TRANSMISSION SYSTEM
CRT STORAGE DISPLAY
TTY
XDS Σ-2
PDP-8
COMPUTER PERIPHERALS
(LINE PRINTER, CALCOMP, TTY, CRT STORAGE DISPLAY, ETC.)
XBL723-4590

Fig. 1.

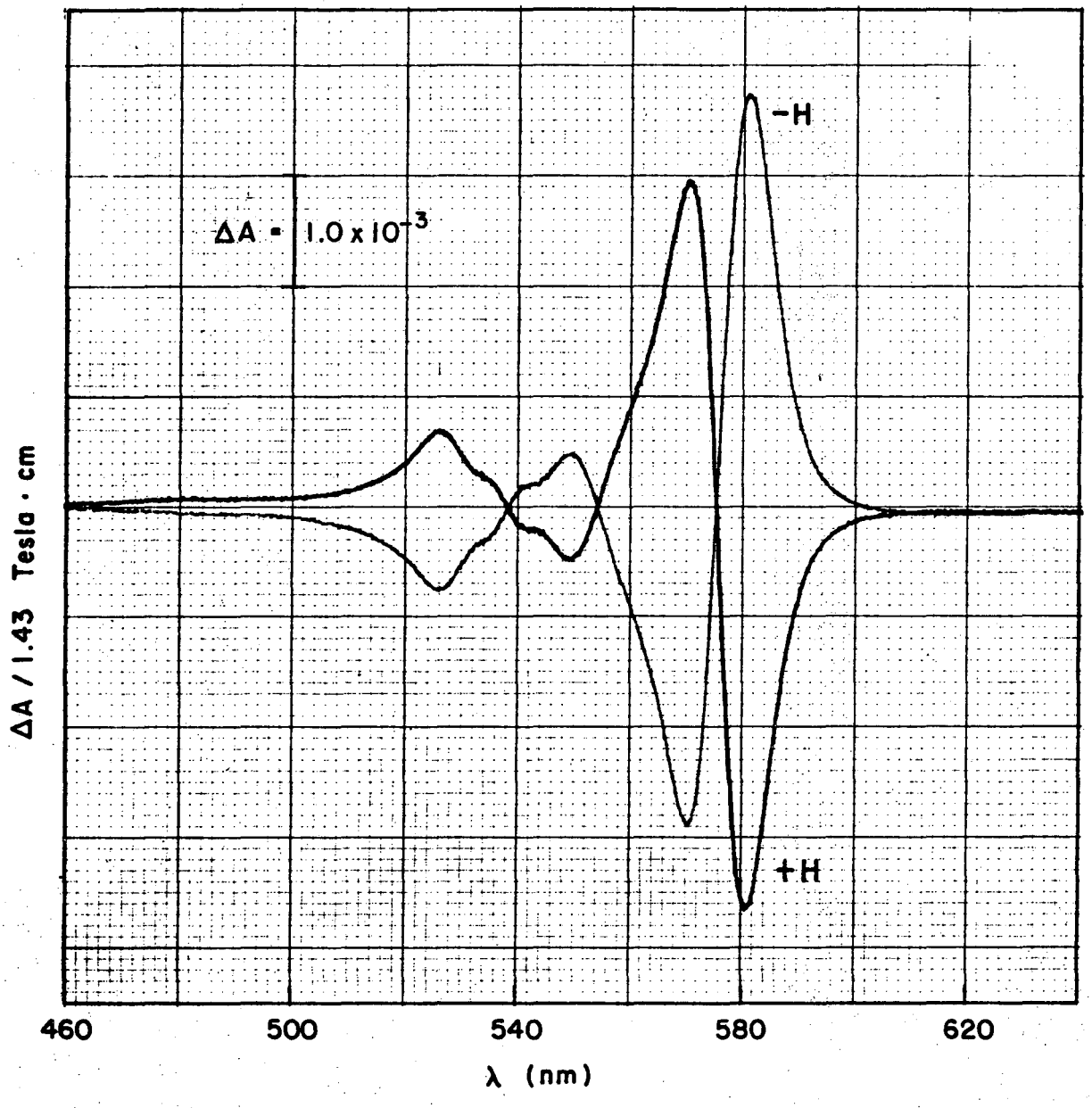
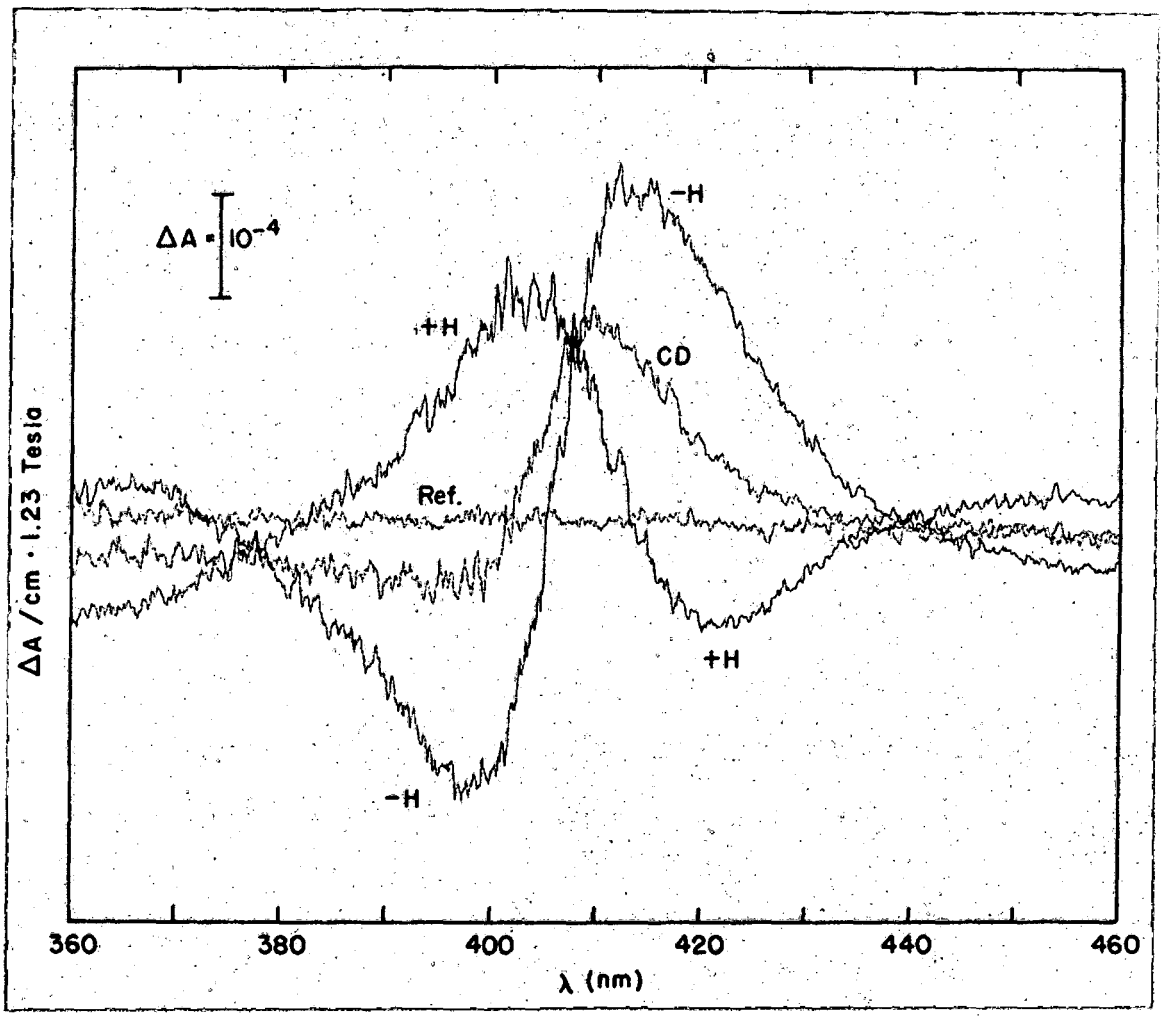
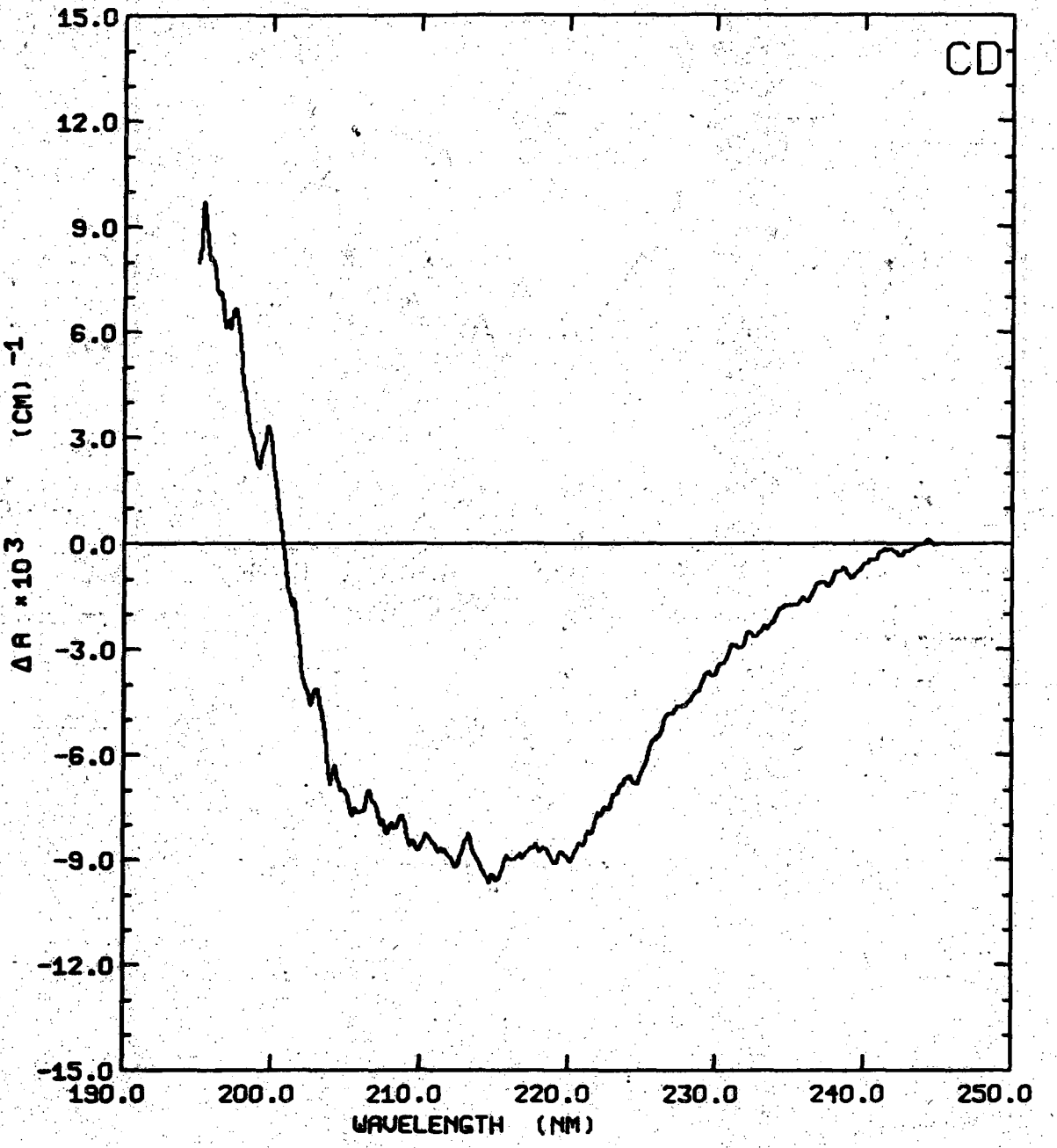

-H
ΔA = 1.0 x 10⁻³
+H
ΔA / 1.43 Tesla · cm
460
500
540
580
620
λ (nm)
XBL743-5083

Fig. 2.



CBB 743-1718

Fig. 3.



XBL742-5068

Fig. 4.

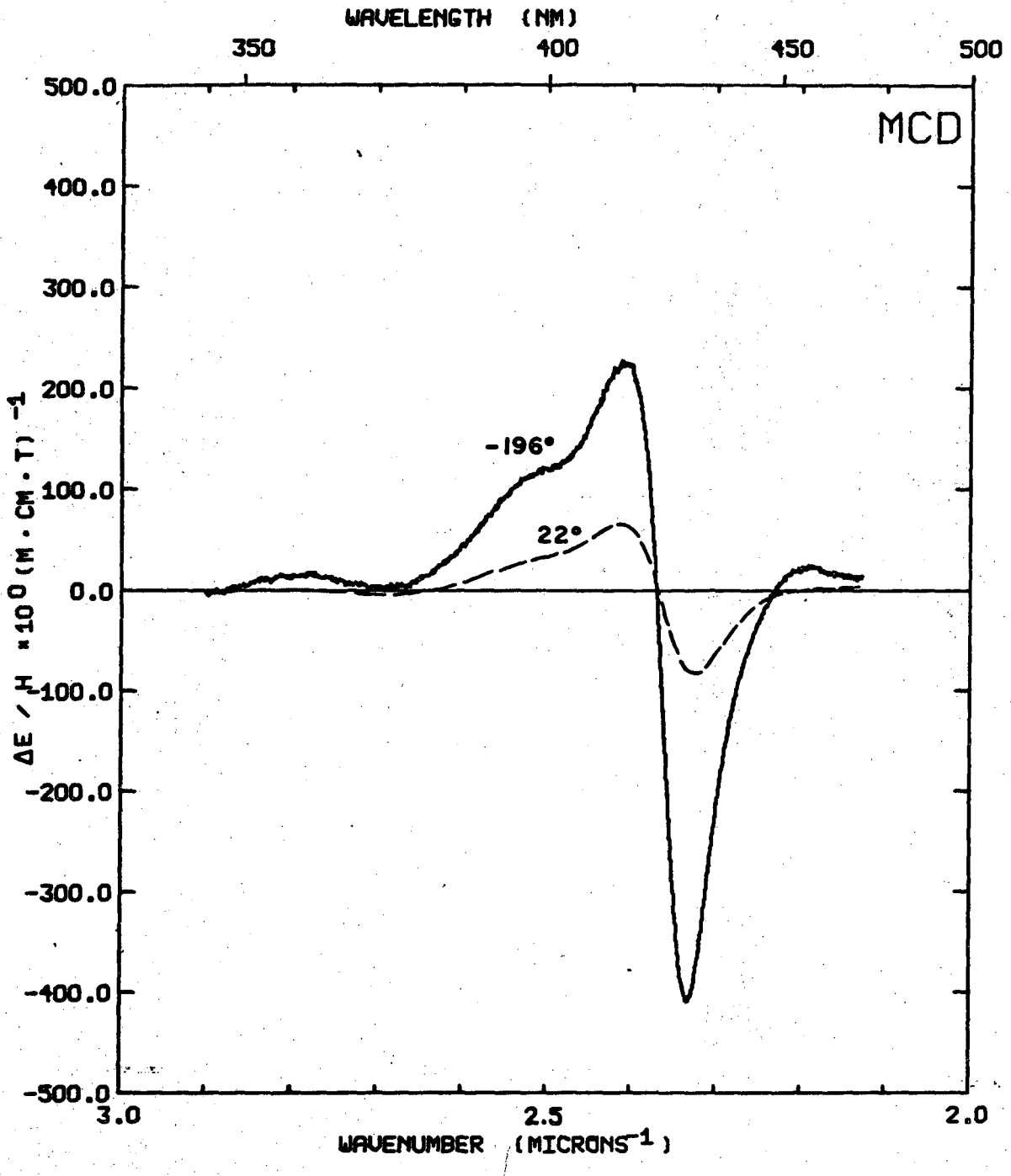

WAVELENGTH (NM)
350
400
450
500
MCD
500.0
400.0
300.0
200.0
100.0
0.0
-100.0
-200.0
-300.0
-400.0
-500.0
-196°
22°
ΔE / H x10^0 (M·CM·T)^-1
3.0
2.5
2.0
WAVENUMBER (MICRONS^-1)
XBL742-5067

Fig. 5.

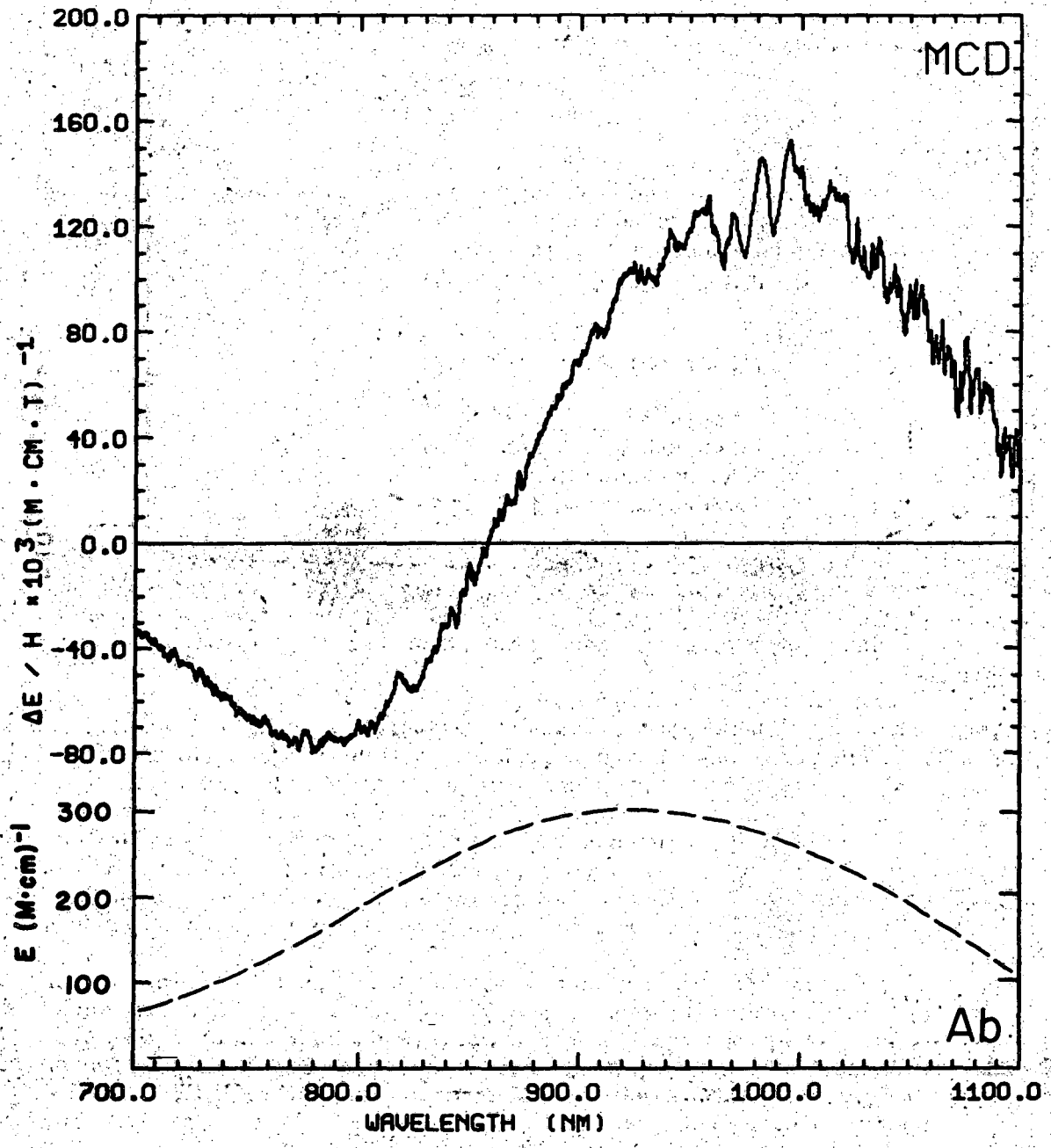

MCD
Ab
200.0
160.0
120.0
80.0
40.0
0.0
-40.0
-80.0
300
200
100
700.0
800.0
900.0
1000.0
1100.0
WAVELENGTH (NM)
ΔE / H x10³ (M·CM·T)⁻¹
E (M·cm)⁻¹
XBL742-5066

Fig. 6.

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