Title

CIRCULAR DICHROISM (CD) AND FLUORESCENCE DETECTED CIRCULAR DICHROISM (FDCD) OF DNA AND POLY d(A-C):d(G-T) IN ETHANOLIC SOLUTIONS - A NEW METHOD FOR ESTIMATING CD DIFFERENTIAL SCATTERING

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DISCLAIMER

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by

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CIRCULAR DICHROISM (CD) AND FLUORESCENCE DETECTED CIRCULAR DICHROISM (FDCD) OF DNA AND POLY [(dA-C)·d(G-T)] IN ETHANOLIC SOLUTIONS - A NEW METHOD FOR ESTIMATING CD DIFFERENTIAL SCATTERING

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ABSTRACT

A method is presented for determining the circular dichroism of systems whose CD spectra contain contributions from CD differential scattering. The technique is shown to detect light over 4π steradians, and, thus, for the first time, a complete correction for scattering is possible. The method is applied to ethanol-condensed DNA and poly [(dA-C)·d(G-T)]. From the results obtained, the former are proposed to have an A type secondary structure. This is significant in view of proposals in the literature concerning the structure of DNA during transcription. The condensed polynucleotide particles are shown to exhibit behavior similar to that of cholesteric liquid crystals. CD difference spectra, obtained from the scattering corrections and showing the contributions to different sections of the scattering envelope, are displayed. It is asserted that these scattering patterns contain information about the tertiary structure of the condensed DNA particles studied.

Circular dichroism (CD) has proven to be a useful and sensitive tool in probing the structure of biological macromolecules. Many systems which have been studied with this technique, such as bacteriophage, membrane incorporated proteins, DNA in chromosomes, etc., are intense light scatterers (Cantor and Hearst, 1969, Glaser and Singer 1971, Ji and Urry, 1969, Schneider, Schneider, and Rosenheck, 1970, Urry and Krivacic, 1970, Dorman and Maestre, 1973, Dorman and Hearst, 1973). Since commercial CD spectrometers are ratio measuring devices (Velluz, Legrand, and Grosjean, 1965), structures which scatter both senses of circularly polarized light with equal efficiency will cause no change in the measured ellipticity. Many light scattering suspensions, however, scatter left and right circularly polarized light to differing extents; i.e., they exhibit differential light scattering. This is measured by the CD spectrometer as if it were differential absorption and can lead to major distortions in the recorded spectra. The presence of such scattering can often be evidenced by observed CD signal in regions where the sample does not absorb light. Distorted spectra of this type can, of course, lead to great difficulties in interpretation of the measured signal.

The most common experimental method for correction of differential scattering is to increase the solid angle of detection in order to collect more scattered light. This has been accomplished by increasing the size of the detector, bringing it closer to the sample cell, or both (Dorman and Maestre, 1973, Dorman, Hearst, and Maestre, 1973). Configurations of this type can collect light scattered by as much as 90 degrees from the incident direction. Probably the most effective

scatter correction method to date is the use of the fluorscat technique Dorman and Maestre, 1973, Dorman, Hearst, and Maestre, 1973). This method is effective even for scattering angles greater than 90 degrees and has been successfully used in many applications (Dorman and Maestre, 1973, Dorman, Hearst, and Maestre, 1973, Girod, Johnson, Huntington, and Maestre, 1973). Unfortunately, in none of the above experimental methods can the light detector be placed between the sample and the incident beam. Thus, one cannot correct for backscattering by the sample. In this paper we present a method, using fluorescence detected circular dichroism (FDCD), in which a non-optically active fluorescent substance can be used as a CD detector having a solid angle of detection of 4π steradians. Thus, back-scattering by the particles can be detected. The CD spectra of DNA and poly [d(A-C)·d(G-T)] particles in ethanol solution will be presented using the above mentioned techniques for correction of differential scattering. In addition, FDCD spectra of these particles in solution with the fluorescent compound α-napthylamine will be displayed and shown to introduce further corrections in the measured CD.

FDCD CORRECTION OF DIFFERENTIAL SCATTERING

In fluorescence detected circular dichroism, the total fluorescence of a molecule is measured after excitation by right and left circularly polarized light of varying wavelength (Turner, Maestre, and Tinoco, 1974, Turner, Tinoco, and Maestre, 1975, Tinoco and Turner, 1976, Tinoco, Ehrenberg, and Steinberg, 1977, White, Pao, and Tung, 1975, Turner, 1978). For a system consisting of one fluorescent substance and one

or more optically active compounds, the signal measured is

$$(1) \qquad \theta_F = -28.65\left(\frac{\Delta\epsilon_F}{2\epsilon_F} - R\right)$$

where $\Delta\epsilon_F$ and ϵ_F are the CD and extinction coefficient of the fluorophore, and

$$(2) \qquad R = \frac{\Delta A}{2A} - \frac{2.303\ \Delta A 10^{-A}}{2(1-10^{-A})}$$

In expression (2), ΔA is the <u>total</u> differential absorbance of the sample, and A is the <u>total</u> absorbance. Thus, although only emission from the fluorophore is being directly measured, the signal is a function of the optical activity of all the components of the sample. This can be understood if one considers that the relative amount of left and right circularly polarized light reaching a fluorophore at any point in a solution is mediated by the differential absorbance of the medium in front of the fluorescer.

We now consider a case where the sample to be measured consists of a mixture of a nonoptically active fluorescent substance and an optically active differential scatterer. In this instance, $\Delta\epsilon_F/\epsilon_F$ equals zero, and equation (1) can be rearranged to

$$\Delta A = \frac{2A(10^A - 1)\theta_F}{28.65(10^A - 2.303A - 1)} \qquad (3)$$

Thus, the CD of the differential scatterer can be measured through the use of a non-optically active fluorophore. In effect what is happening is that the scattering particles are surrounded on all sides by fluorescent detectors. These detectors intercept the scattered light in addition

to the incident radiation and, thus, yield a signal which is a function of all the scattered and transmitted light.

In using the FDCD method to correct for differential scattering, it should be noted that the assumption is being made that the average pathlength of the scattered light is the same as that of the transmitted radiation. This is obviously true for light scattered at an angle of 0^{o} and 180^{o} and, for a one cm cell, 90^{o}. Other directions lead to either a greater or lesser average optical path than for the aforementioned angles. The exact mean pathlength thus depends on the details of the scattering pattern. In the most extreme cases the deviation from equality of mean pathlength is about 6%, while for isotropic scattering this reduces to 2%. Thus, the assumption of equal average optical paths is substantially correct. This does not guarantee that one will obtain a complete correction for scattering, however. A correction based on equality of average pathlength assumes a linear relationship between signal and optical path. Fluorescence intensity, however, bears an exponential relationship to the absorption pathlength. Thus, for solutions having a relatively high total optical density, the FDCD results may deviate from complete correction . The sign and extent of this deviation is a function of the details of the scattering pattern, and the error can become particularly important for those systems in which there is an unusually large amount of differential scattering at angles close to 180°. In this case it may become necessary to use solutions of less than 0.1 total OD since at these concentrations the relationship between optical path and fluorescence intensity is essentially linear. In practice, then,

one determines the FDCD correction for solutions having several different fluorophore concentrations. If there is no real change in the measured $\Delta\varepsilon$ at different OD's, then one can use a solution concentration which yields an advantageous signal to noise ratio. If there are significant changes, however, it will be necessary to determine a corrected $\Delta\varepsilon$ at low total OD.

MATERIALS AND METHODS

Calf thymus DNA was purchased from Miles Laboratories, while E. coli DNA was purchased from Worthington Biochemical Corporation. The DNA samples were dissolved in sodium phosphate buffer, pH 7.0, and dialyzed twice against 0.01 M EDTA, 0.002 M sodium phosphate; twice against 0.01 M NaCl, 0.002 M sodium phosphate; and finally four times against 0.002 M sodium phosphate. All solutions were at pH 7.0. The extinction coefficient used for calf thymus DNA was 6450 liters/mole-cm, while the E. coli extinction coefficient was 6540 liters/mole-cm (Felsenfeld and Hirshman, 1968). Poly [d(A-C)·d(G-T)] was kindly supplied by Dr. Robert L. Ratliff of the Los Alamos National Laboratory and was prepared as previously described (Gray and Ratliff, 1975). The polymer was dialyzed in a fashion similar to the DNA samples except that a 0.01 M sodium phosphate solution, pH 7.0, was used as a buffer.

Ethanol particles of DNA were prepared by adjusting the sodium concentration in the aqueous solution and then adding sufficient ethanol to produce a solution containing 80% ethanol by weight. The alcohol was added slowly with constant stirring over the course of a half hour.

Ethanol-condensed calf thymus DNA in 60% ethanol by weight was prepared by adding first ethanol to the DNA and then adding concentrated NaCl to raise the NA^+ concentration to 20 mM.

Poly [d(A-C)•d(G-T)] condensates were produced by preparing a solution containing 50% ethanol by weight and then adjusting the NaCl concentration to 50 mM.

α-Napthylamine was purchased from Sigma and recrystallized from an ethanol-water mixture until white needles were obtained. The compound turns red slowly upon exposure to air and, thus, was stored in a vacuum desiccator at 4^oC. α-Napthylamine at pH 7.0 was found to have an emission maximum at 444 nm (excitation at 315 nm). Solutions for baselines were prepared by dissolving the fluorescer in the same buffers used for the DNA and polynucleotide particles.

All absorption spectra were measured using a Cary 14 spectrophotometer. Fluorescence spectra were run on a Perkin Elmer MPF-2A spectrophotometer. The CD and FDCD experiments were performed using the modified Cary 6001 spectrometer described previously (Dorman, Hearst, and Maestre, 1973, Turner, Maestre, and Tinoco, 1974). A Dumont no. KM2703 end-window photomultiplier tube (PMT) with a 2 inch diameter photocathode was used in the different types of CD measurements, while a Hammamatsu R375 end-window photomultiplier was employed in the FDCD experiments. All data were digitally recorded by an on-line computer (Tomlinson, 1968), and the output was then smoothed and processed using a CDC 7600 computer. Some of the FDCD and fluorscat scans were run up to nine times and averaged in order to increase the accuracy of the measurements. All CD spectra were run using a one cm pathlength cell. The FDCD experiments

were performed with rectangular cuvettes having either a one cm pathlength or else a three mm pathlength and a one cm width. All the FDCD spectra were measured using a Schott KV 418 interference filter which effectively cuts off all light with wavelengths below 400 nm.

In accordance with the methodology described previously, FDCD measurements were performed on solutions of α-napthylamine and DNA or polymer having very low optical densities. The results obtained, within experimental error, were similar to those observed with more concentrated solutions, and, thus, the latter were employed in making final measurements. These final solutions were prepared by adjusting the DNA or polynucleotide concentration at 260 nm to about 0.3 OD and then adding solid α-napthylamine until the OD at 305 nm was also approximately 0.3. Thus a solution was obtained having a maximum OD of about 0.3 at 305 nm and 0.6 at 260 nm. The absorbance ratios of the sample components in this solution represent a compromise between the desire to obtain a large amount of fluorescence in order to reduce noise, and the desire to have as large a θ_F as possible. The latter quantity, as can be seen in expression (2), decreases with increasing fluorescer concentration.

Another question to be settled before considering experimental results is the suitability of α-napthylamine as a CD detector. First of all, the α-napthylamine must yield average, non-directional $\Delta\varepsilon$'s. To determine if this were true FDCD spectra of fluorescer solutions were measured with a linear polarizer in front of the photomultiplier. The results obtained were the same whether the polarizer was in a parallel or perpendicular orientation. This indicates that fluorescence lifetime

of α-napthylamine is long enough so that the directional effects of photoselection are averaged out (Tinoco et al., 1977).

It is also important to make certain that α-napthylamine does not interact with the scattering particles. Thus, CD and fluorescence measurements were made on aqueous solutions of calf thymus DNA before and after addition of α-napthylamine. No change in the spectra was observed. Absorption experiments also showed no indication of fluorescer-DNA interaction. Finally, FDCD measurements of the uncondensed DNA solutions were made. The dissymmetry factor, $\Delta\varepsilon_F/\varepsilon_F$, was found to be zero, thus indicating that no CD was induced in the fluorophore upon addition to DNA. It was thus concluded, on the basis of the foregoing evidence, that α-napthylamine does not interact with DNA and is consequently a suitable CD detector for the macromolecular systems studied in this paper.

Results

Figure 1 shows the CD and FDCD of calf thymus DNA particles in 80% ethanol. Spectra similar to the first three curves in the illustration have been reported previously (Girod, Johnson, Huntington, and Maestre, 1973). The first curve, labeled far, was measured with an experimental configuration having a small detector angle, and thus little correction for scattering is expected. This curve differs greatly from the normal B type spectrum of calf thymus DNA in aqueous solution and is more reminiscent of DNA in an A form. As one can see, the CD signal is strong at long wavelengths where the sample does not absorb light. The magnitude of this apparent signal is indicative of intense differential

scattering. Some correction to the scattering is observed when the detector angle of acceptance is increased by bringing the PMT closer to the sample cell, but the long wavelength region remains essentially the same. A larger degree of correction is observed when the fluorscat is utilized. In this case, the 305 nm band is greatly reduced in intensity, thus eliminating most of the long wavelength signal. This last spectrum represents the greatest degree of scattering correction previously obtainable. The FDCD correction method, with its larger angle of detection should produce further improvement in the removal of scattering effects, and it is gratifying to observe that this is so. In the last curve in Figure 1, it is seen that the first negative band has now essentially disappeared, the 270 nm maximum has been reduced in intensity, and a new negative band has appeared at about 240 nm. Since the fluorscat technique probably corrects for all but back scattering, the unique corrections observed utilizing FDCD are attributed to collection of this last portion of scattered light.

It is now possible to obtain the scattering contributions to the CD for various sectors of the scattering sphere. Thus subtraction of the close spectrum form the far yields the forward scattering contribution, close minus fluorscat furnishes the side contributions, and fluorscat minus FDCD gives the back scattering contributions. These relationships are illustrated in Figure 2, while Figure 3 presents the CD difference spectra obtained for calf thymus DNA.

The results show that most of the scattering is at the side angles, since the close minus fluorscat difference CD is the largest of the differences corresponding to the three main spherical sectors.

Various CD curves for E. coli DNA condensed in 80% ethanol are displayed in Figure 4. The differential scattering of these particular E. coli condensates is greater than that of the calf thymus particles, although the correction trends are the same. CD difference spectra for E. coli are presented in Figure 5. It is important to note that in E. coli and calf thymus the value of the CD at 260 nm is less than 10 (liter $M^{-1}cm^{-1}$).

The most dramatic changes observed in CD occurred upon molecular condensation of the synthetic poly [(dA-C)·d(G-T)] samples. Figure 6 displays the spectra of the polymer in 50% ethanol, low salt solutions. These curves appear to indicate that the double helix is approaching a C type geometry. Upon increasing the sodium ion concentration to 50 mM, however, condensation occurs, and the spectrum changes shape radically. Accompanying this is a 20-fold increase in signal intensity. The new spectrum is illustrated in Figure 7. As in the case of the natural DNA condensates, differential scattering is indicated by apparent CD signal in regions where the sample does not absorb light. Bringing the photomultiplier closer to the sample cell does little to change the observed ellipticity. On the other hand, spectra obtained from fluorscat measurements show virtually complete obliteration of the signal at non-absorbing frequencies, but instead of a diminution of the intense positive band, as expected from the results with the natural DNA's in 80% ethanol, the 268 nm maximum is nearly doubled in intensity. Further increases in the magnitude of this band are obtained from FDCD measurements, while at the same time some lowering of intensity is observed in the 230 nm region. The spectra for poly [d(A-T)·d(G-T)] are displayed in Figure 8, while Figure 9 shows various CD difference curves. Here

as with the condensed natural DNA in 80% ethanol, most of the scattering is at the side angle ; i.e. at right angles to the incoming light beam. The close minus fluorscat difference CD is about 5 times greater for the condensed calf thymus DNA in 60% ethanol. When this sample was corrected by FDCD, the major positive band near 270 nm was also almost doubled in intensity, but the absolute magnitude ($\Delta\varepsilon$=14. ℓ./M-cm) was much less than that seen for the corrected poly [d(A-C)·d(G-T)] spectrum.

DISCUSSION

The corrected CD curves obtained for the calf thymus and E. coli DNA particles are very similar to those observed for A form DNA. This was pointed out by Girod et al. (1973) for the fluorscat spectra, but at that time the authors were reluctant to make an assignment because a complete correction for differential scattering was not available. The FDCD corrected CD spectra obtained in this work include hitherto unavailable corrections for back scattering and still resemble A form curves. Of particular interest in these spectra is the fact that the FDCD corrected results show a new minimum below 240 nm which does not show up with the other types of scatter correction and which is approximately where the first minimum for A form DNA is observed. This fact, together with the observed shapes and intensities of the FDCD spectra, causes us to conclude that the DNA particles considered in this paper have an A type secondary structure. The fact that a condensed form of DNA can assume such a structure is particularly interesting in view of the suggestions in the literature that a B → A transition occurs for DNA during transcription (Hamilton, 1968, Arnott et al, 1968;

Florientiev and Ivanov, 1970).

However, it cannot be ruled out that the positive band at 260-270 nm, which is characteristic of the A conformation, contains a significant contribution from long range order, seen in DNA condensed in 50-60% ethanol, as discussed below. This could be possible since salt was added first, then ethanol to form condensed DNA in 80% ethanol, and some of the DNA may have begun to precipitate at ethanol concentrations of 60% or below.

Examination of the poly [(dA-C)·d(G-T)] spectra does not produce nearly so straightforward an assignment of secondary structure. Although the FDCD correction eliminates the CD signal in those regions where the polymer does not absorb light, the intensity maximum in light absorbing regions becomes greater than 80. As was stated previously, the $\Delta\varepsilon$ remains the same at OD's less than 0.1 so that one cannot propose that the corrected spectra are artifacts resulting from an unusual amount of back scattering. On the other hand, there are no known secondary structures which have $\Delta\varepsilon$'s of so large a magnitude, and it is difficult to see how the signal could be caused by tertiary structure. The solution to this question lies in the fact that we have been assuming the observed differential scattering has been occurring in addition to normal absorption. On the other hand, it has long been known that at certain wavelengths properly oriented cholesteric liquid crystals will scatter one of the senses of circularly polarized light almost completely (Chandrashekar, 1977). If this resonance type of scattering is occurring with the polynucleotide condensates, then the apparent CD signal is a result of light scattered into the photomultiplier tube, and collection of more of this scattered

light with the FDCD method will only lead to an increase in the CD signal. This is exactly what has been observed. We have also noted behavior of this type with DNA-polylysine and DNA-nucleohistone particles and also DNA condensed in 60 to 80% ethanol produced in a different fashion than that described in this paper. For these systems, we have evidence that the particles are indeed behaving like cholesteric liquid crystals and will describe both the experiments and the applications to tertiary structure determination in a later paper (Maestre and Reich, 1979).

Conclusions

The FDCD scattering correction method has been shown to provide a technique for observation of differential scattering over a complete solid angle of 4π steradians. This method, as expected, has furnished greater corrections to the CD of macromolecular particles than was previously possible. CD difference spectra representing back-scattering patterns have been presented for the first time. It has been pointed out that the CD difference spectra of systems exhibiting liquid crystal type behavior contain information about the long range ordered asymmetry, or tertiary structure of the system (Holzwarth, Gordon, McGinness, Dorman, and Maestre, 1974). It is our intention to collect CD difference spectra for a large variety of scattering systems and eventually correlate these curves with long-range molecular structure. The FDCD method may provide a means of not only accurately assessing secondary structure in many of the above and similar scattering systems but may also prove to be a useful and powerful tool in determining the tertiary structure of macromolecular assemblies, once resonance scattering properties are understood.

Finally, we have shown in the case of ethanol-condensed DNA that the appearance of a large positive CD band near 270 nm, usually attributed to the A conformation, may not actually be due to an A conformation if there is some condensation at ethanolic concentrations of 60% w/w or less. This peak may be a strong scattering contribution. The above statement agrees with recently published work on DNA-spermine complexes, in which it was shown by wide angle x-ray scattering that positive psi-type CD spectra may occur for samples containing DNA in the B secondary conformation (Damaschun et al, 1978).

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FIGURE CAPTIONS

Figure 1. The CD of calf-thymus DNA-EtOH condensate as measured by techniques having varying solid angles of light detection.

Figure 2. A diagrammatic representation of the CD scattering envelope of a hypothetical particle. Different sectors are measured by computing the proper differences between measuring methods. Thus, far minus close gives the forward scattering component, close minus fluorscat gives the side lobes and fluorscat minus CD(FDCD) gives the back scattering component. CD(FDCD) means CD obtained from the FDCD measurement as described in the text.

Figure 3. Difference CD obtained as described in Fig. 2 for the DNA EtOH condensate described in Fig. 1. These are the differential CD (correctable scattering components).

Figure 4. CD of E. coli DNA EtOH condensed particle as a function of measuring geometry. These condensates have obvious scattering components in the long wavelength regions. Even when corrected by FDCD methods this condensed DNA shows a CD spectrum different from the conservative CD of DNA in the {B or C} geometries (see Fig. 6 this paper).

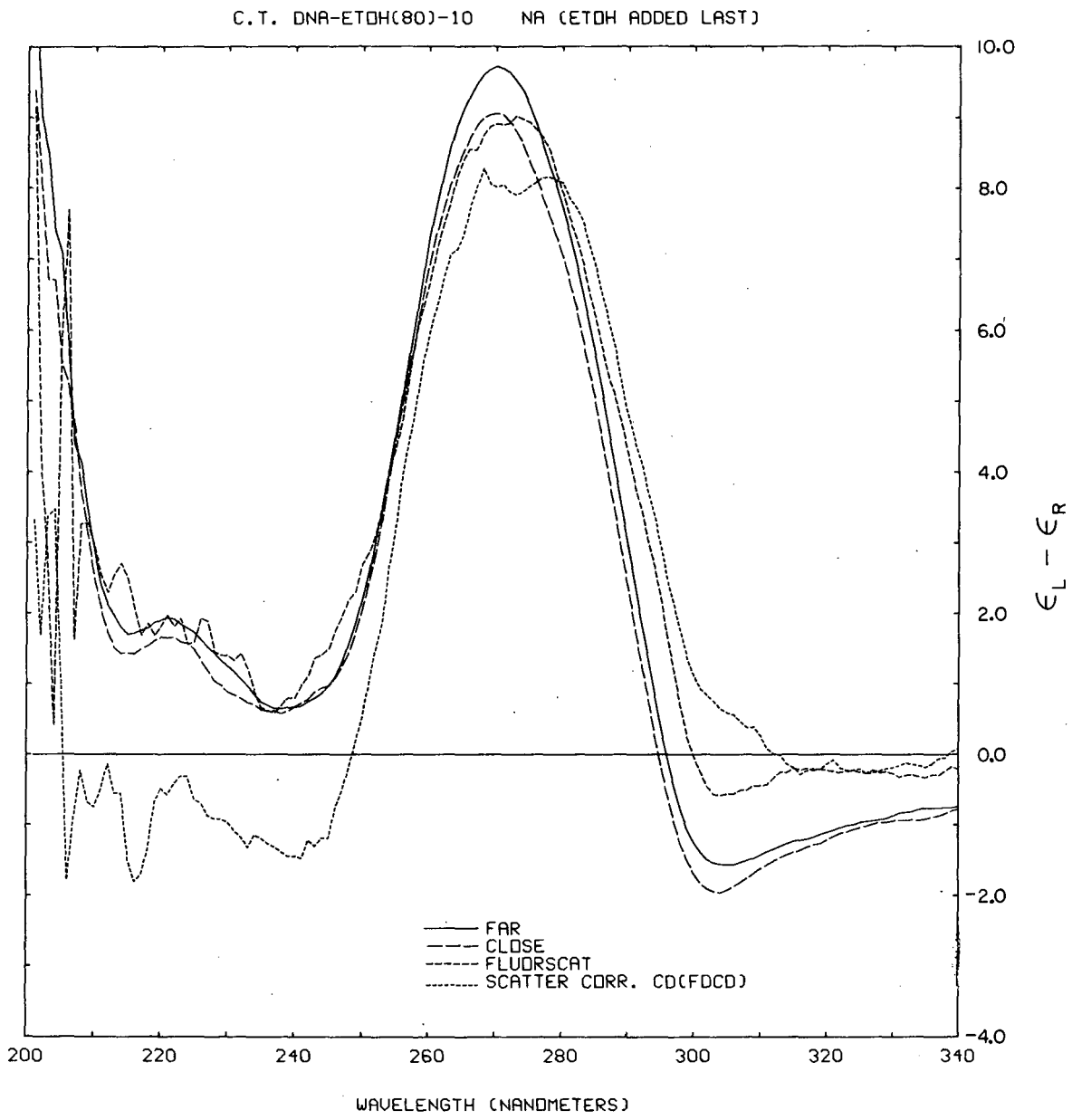
Figure 5. Difference CD curves giving the scattering components as a function of sectors in the scattering envelope. Note that the largest components are in the close minus fluorscat sector; i.e. at right angles to the light beam.

Figure 6. CD of poly [d(A-C)·d(G-T)] in 50% EtOH, 2mM Na and 20 mM Na. This is the so-called conservative CD of DNA reflecting a B or C secondary geometry.

Figure 7. The CD of polymer in Fig. 6 when the salt concentration is increased to 50 mM Na^+. The signal increases by a factor of 40 and differential scattering components appear in the long wavelength region.

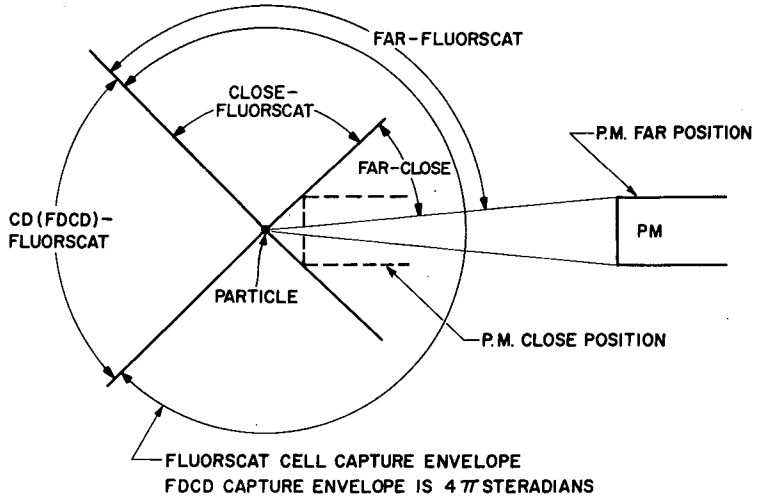
Figure 8. Correction by FDCD methods. The CD (FDCD scatt.) corrected actually increases the value of the signal.

Figure 9. Scattering components for the different sectors of the scattering envelope for the DNA in Figs. 7 and 8.

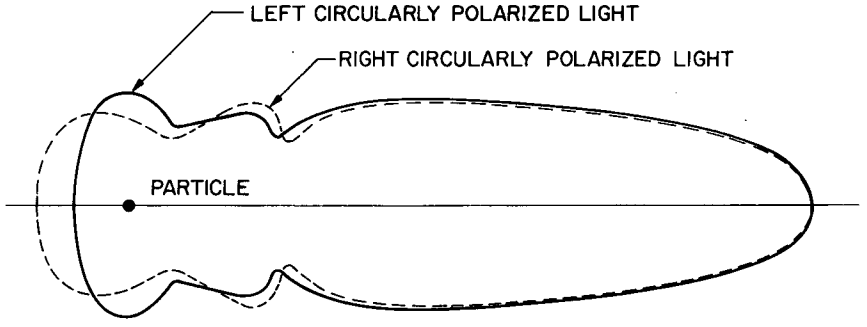


XBL 796-10246

CD SCATTERING ENVELOPE DETERMINATION



SCATTERING PATTERN ENVELOPE



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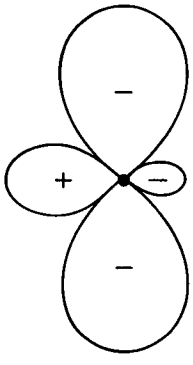
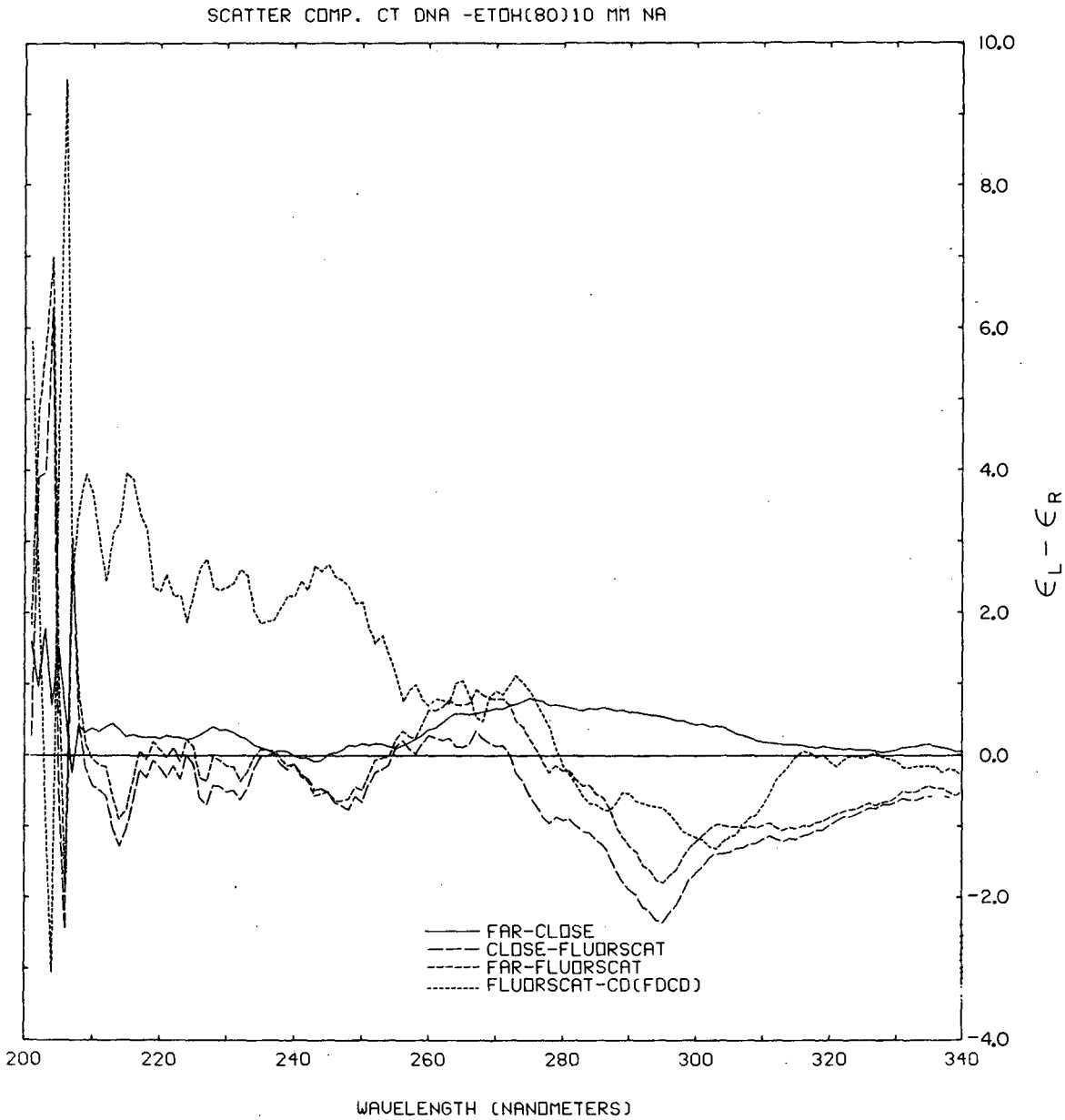


$$\text{SIGNAL} \sim \frac{S_L - S_R}{S_L + S_R}$$

XBL 796-10129



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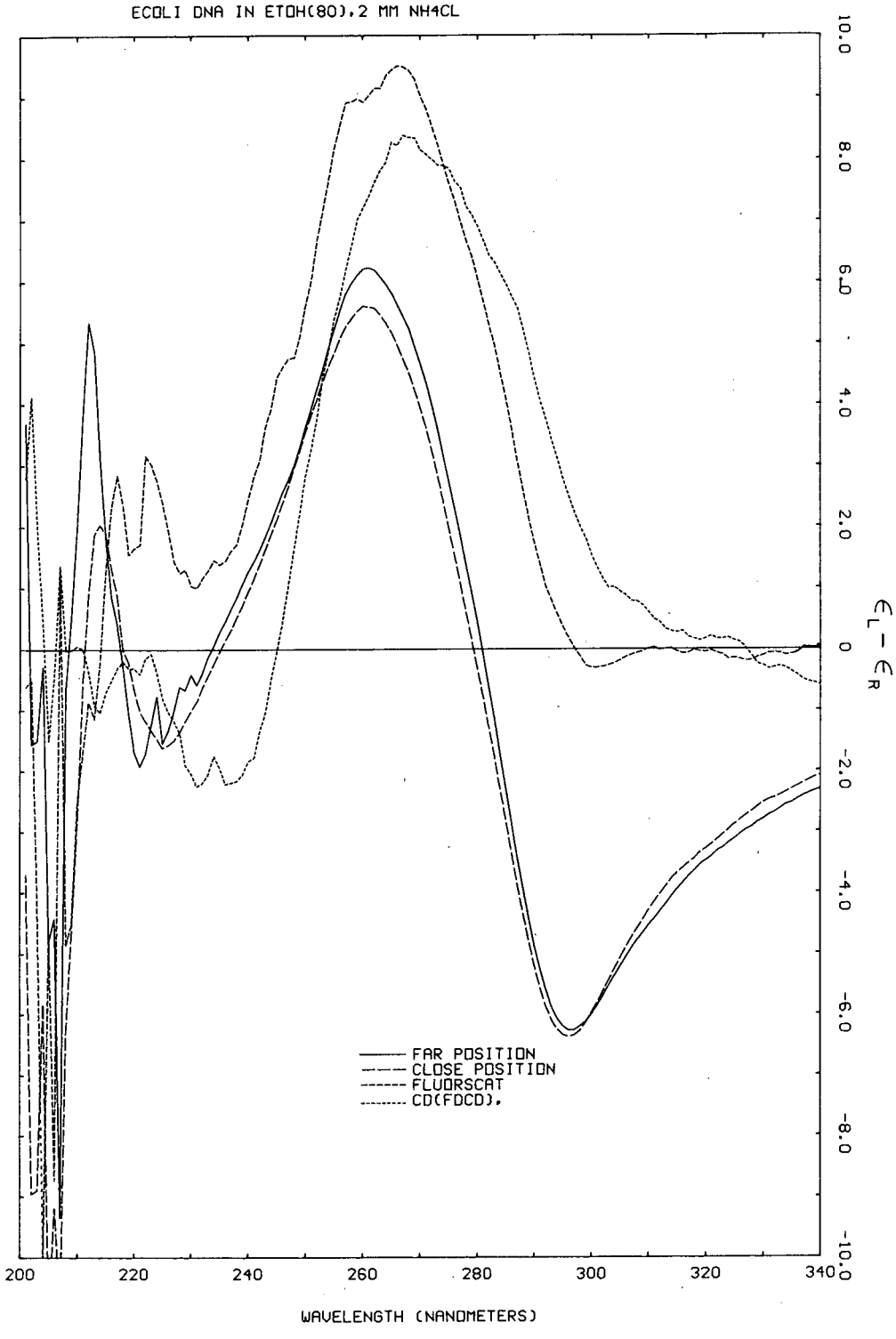

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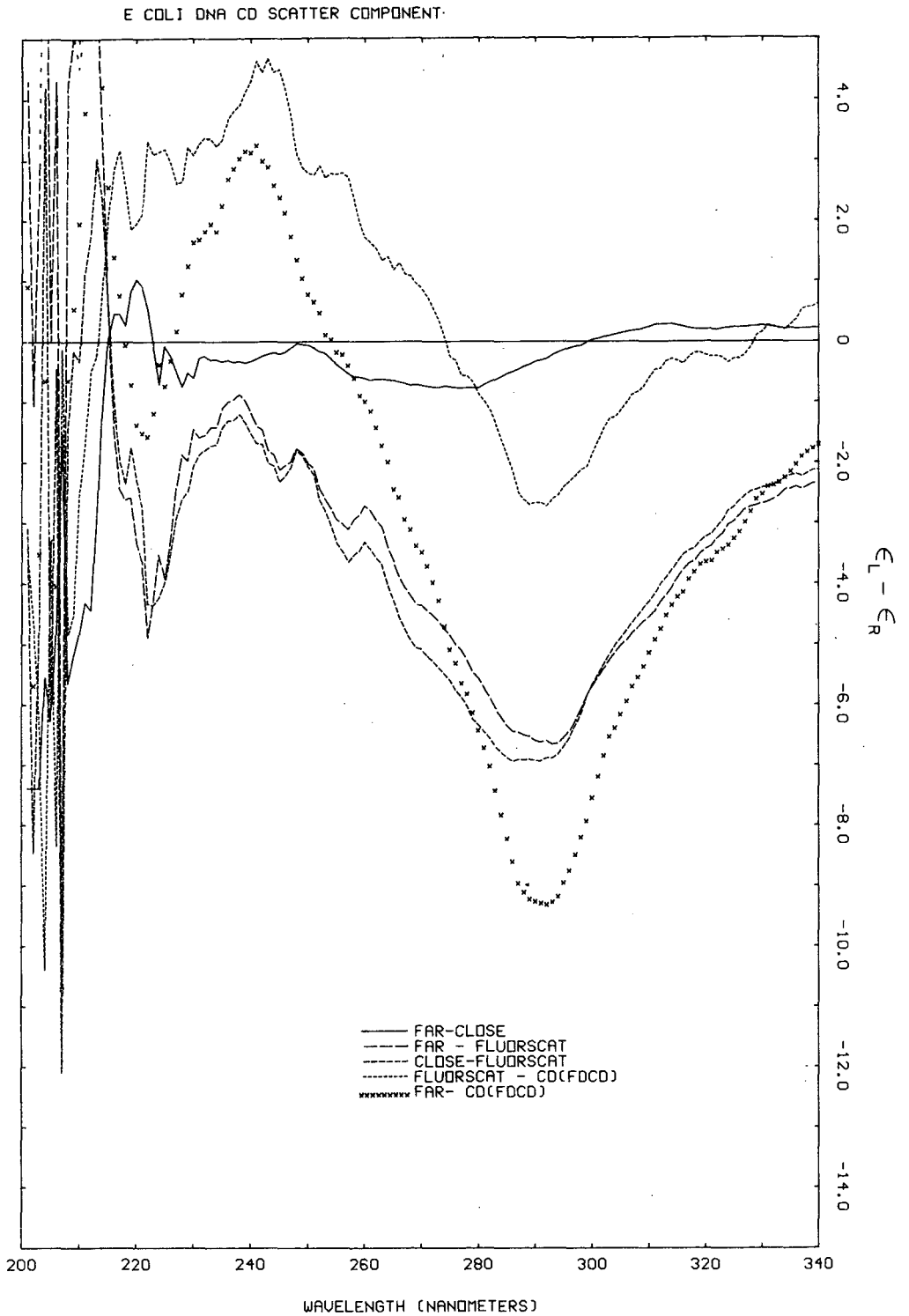
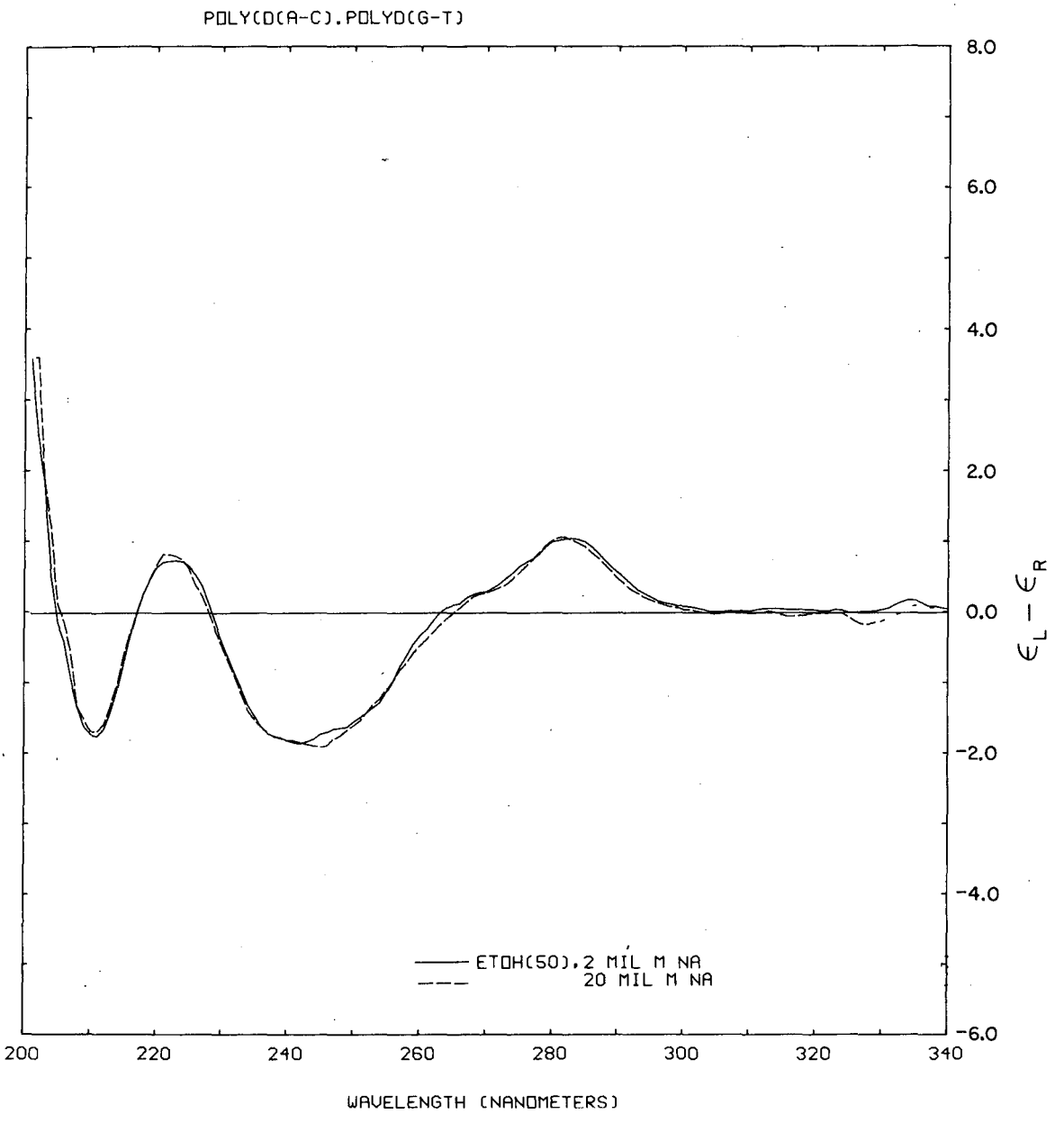


XBL 796-10253



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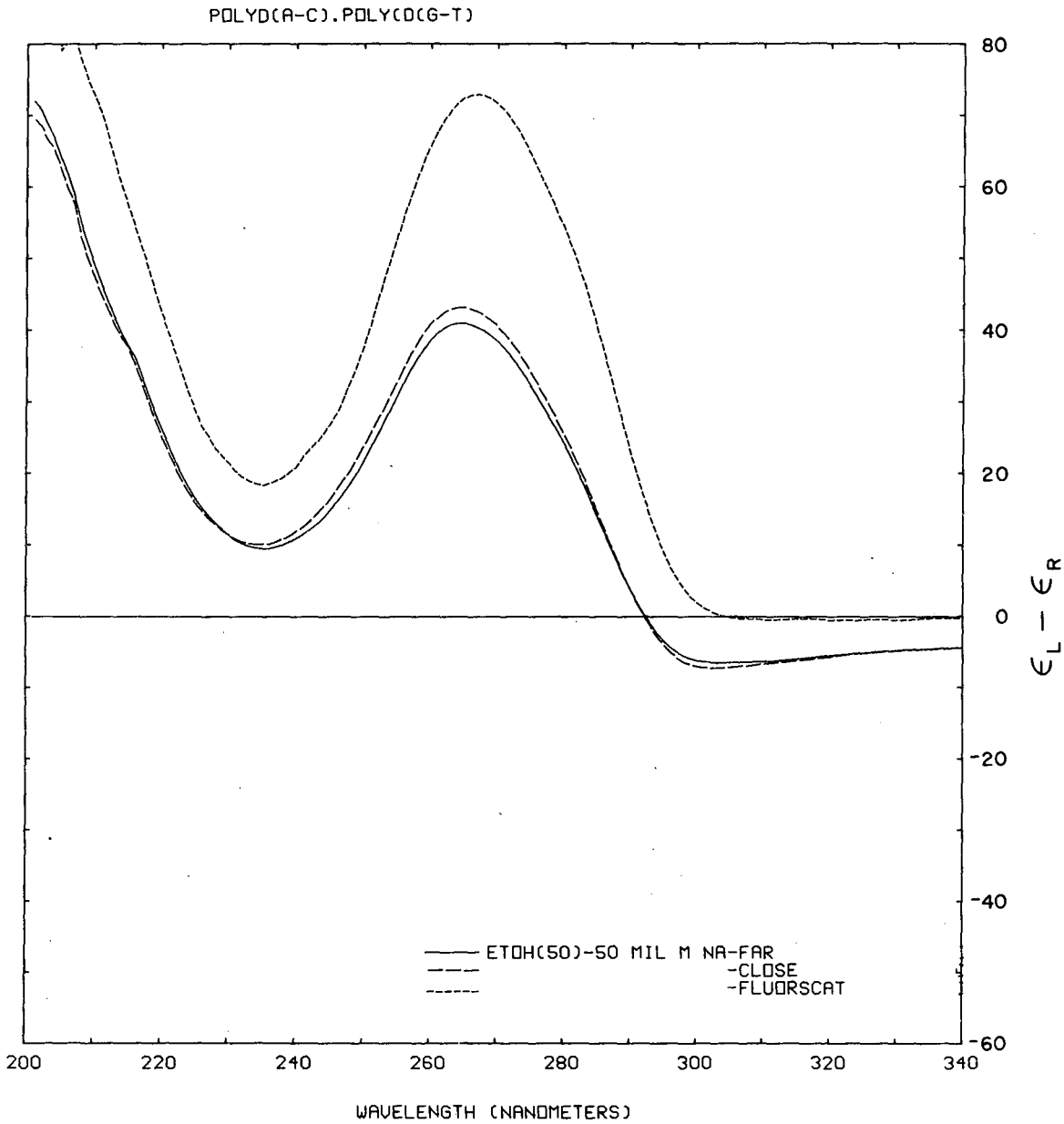

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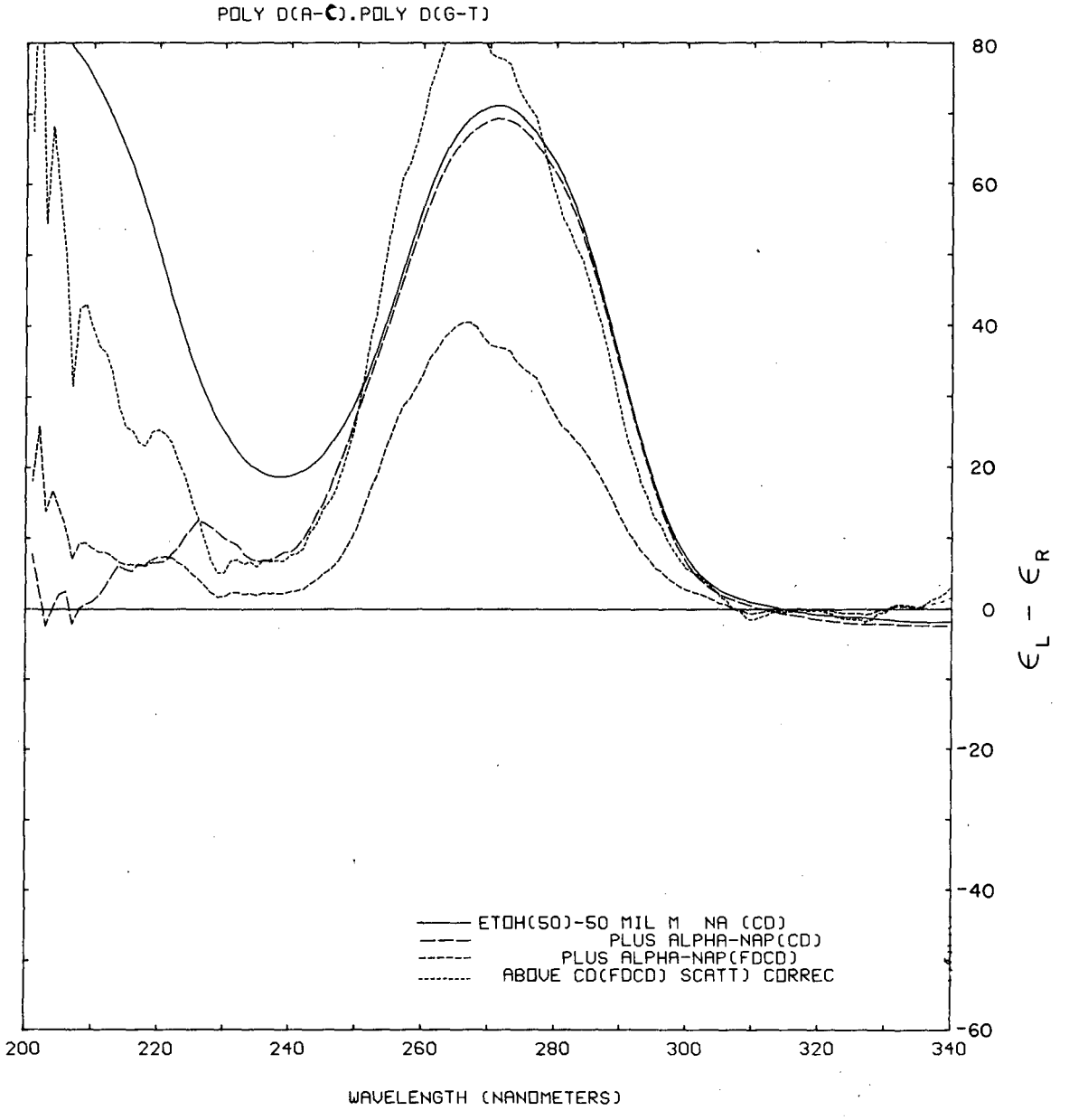

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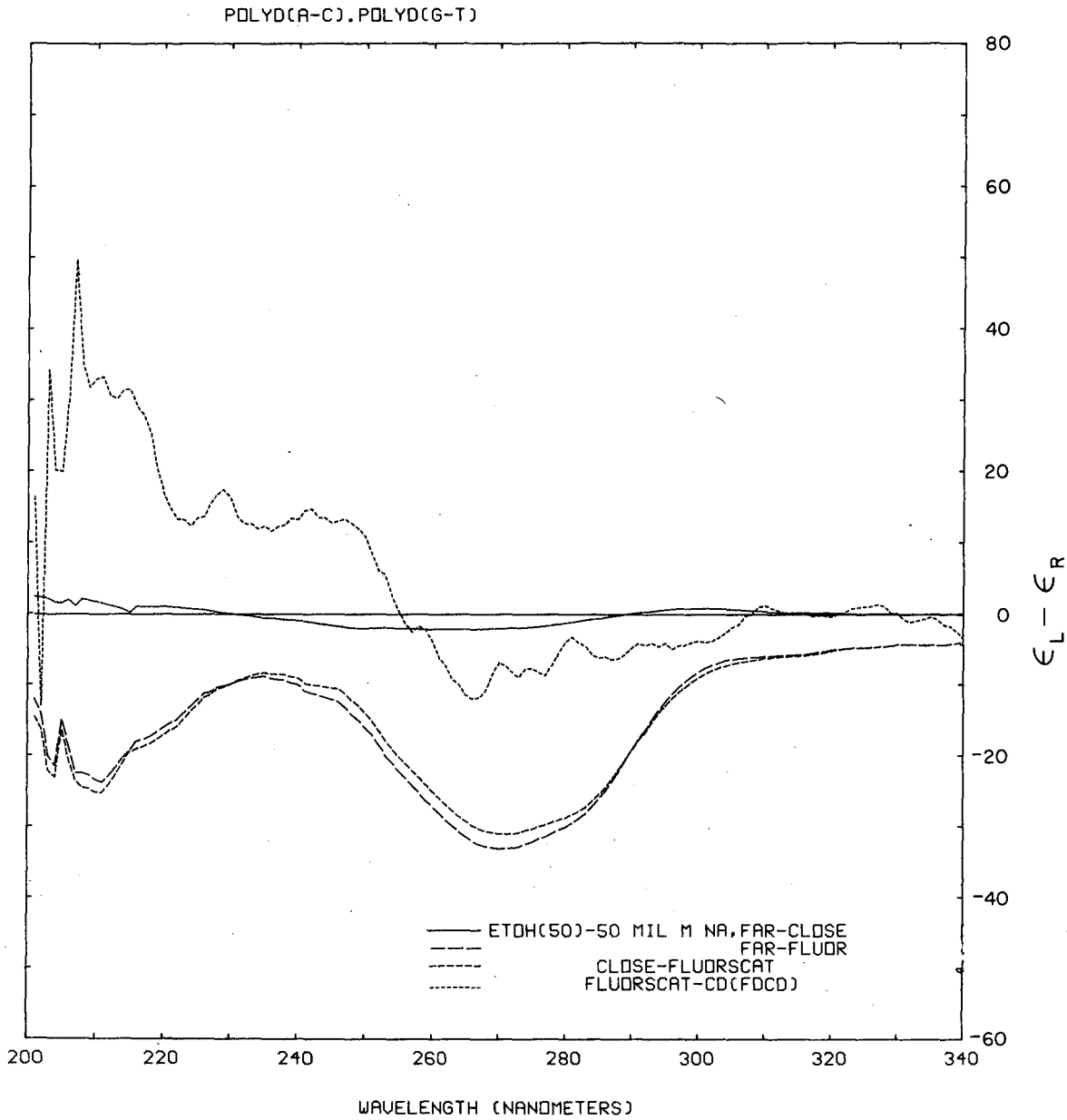

XBL 796-10250

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