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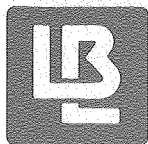
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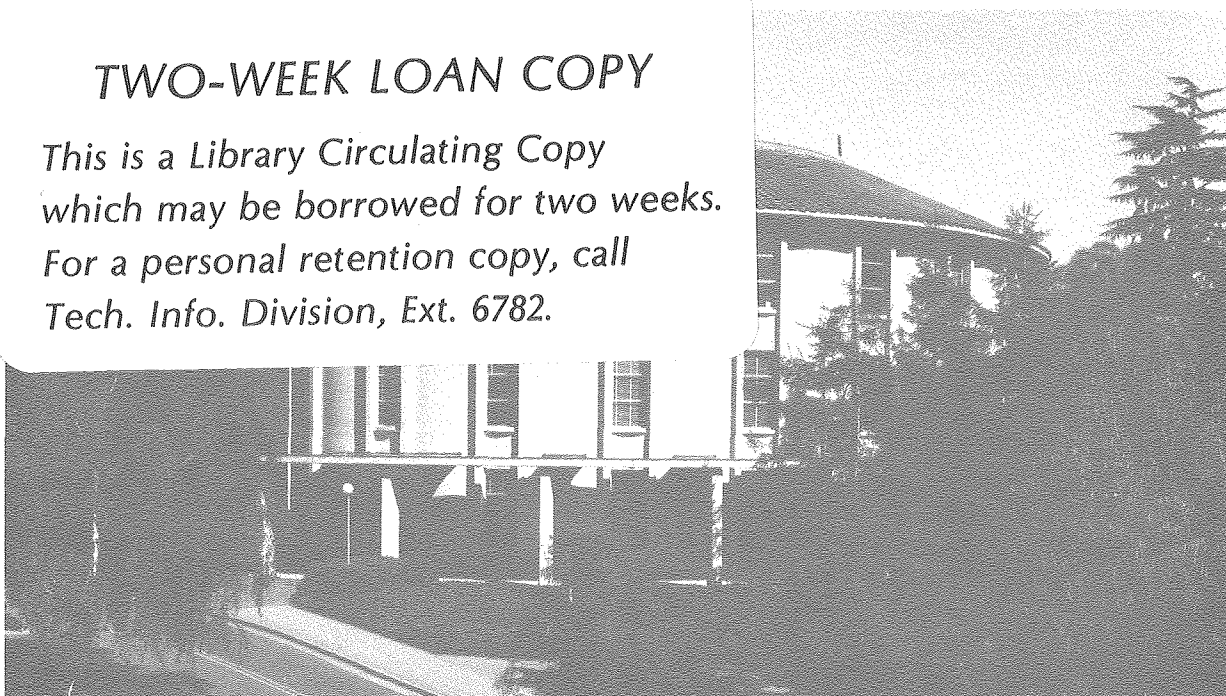
Charles Reich and Ignacio Tinoco, Jr.

October 1979

DISCLAIMER

Fluorescence Detected Circular Dichroism of

Dinucleoside Phosphates.

A Study of Solution Conformations and the Two-State Model

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Synopsis

Three fluorescent dinucleoside phosphates containing $1,N^6$-ethenoadenosine (ϵA), $\epsilon A_p \epsilon A$, $\epsilon A_p \epsilon C$, and $\epsilon A_p U$ were studied using fluorescence detected circular dichroism (FDCD), circular dichroism (CD) and absorption measurements. The FDCD data indicate that $\epsilon A_p \epsilon C$ and $\epsilon A_p U$ can be described as two state systems consisting of a fluorescent species and a stacked, nonfluorescent species. Thermodynamic stacking parameters are calculated for these molecules using the van't Hoff equation. $\epsilon A_p \epsilon A$ is found to be a more complicated system with a fluorescent CD which is different in shape, but comparable in magnitude, to the conventional CD of the dimer. This molecule, unlike the other two dinucleoside phosphates, cannot be characterized as a two state system; it is described as consisting of at least three states at temperatures above 35°C.

The CD data were subjected to a linear analysis in order to determine the minimum number of states present. In agreement with the FDCD data, $\epsilon A_p \epsilon C$ and $\epsilon A_p U$ are found to consist of a minimum of two states, while $\epsilon A_p \epsilon A$ is indicated to have at least three. The more complicated behavior of the latter dimer is also indicated by the values of the unstacked CD obtained in the van't Hoff analysis.

INTRODUCTION

Since the dinucleoside phosphates represent the smallest possible polynucleotides, their solution conformations and thermodynamic properties have been extensively examined[1] using

such techniques as NMR,[2-6] absorption, CD and optical rotatory dispersion (ORD).[7-10] The ORD of these dimers can be used to predict the ORD of polynucleotides and single stranded RNA;[1,11,12] therefore the bases must have approximately the same relative orientation in the polymers as in the dimers. Thus a detailed knowledge of the properties of the dinucleoside phosphates is helpful in understanding the properties of more complicated structures.

One important model used extensively in interpreting the temperature dependence of the optical properties of dinucleoside phosphates is the two-state hypothesis. The dimers are proposed to exist in two forms

$$STACKED \rightleftharpoons UNSTACKED$$

in equilibrium with each other. Such a model allows one to use the van't Hoff method for calculating the thermodynamic parameters of stacking if the values of the experimental property under study are known for the stacked and unstacked states. There has been much concern, however, about the validity of applying a two-state hypothesis to these types of systems. Davis[9] has shown that absorbance and ORD data yield different thermodynamic values for A_pA. Glaubiger et al.[13] showed that the ORD data for A_pA could be explained equally well with a static two-state model or a dynamic oscillator model. Powell et al.[10] examined this question in some detail and concluded that although multi-state models could not be ruled out, evidence existed that the two-state model could yield meaningful parameters.

One spectroscopic method which could be potentially useful in determining the details of the stacking process is that of fluorescence. Unfortunately, the naturally occurring nucleic acid bases are only weakly luminescent, and thus fluorescence studies are usually carried out in glasses at low temperature (see for example Eisinger et al.[14]). For studies under normal solution conditions it is necessary to use fluorescent analogs of the nucleic acid bases. One of the most useful of these[15,16] is that produced by the chloroacetaldehyde modification of adenosine to yield the $1,N^6$-ethenoadenosine (ϵA). This molecule combines the advantage of a large quantum yield and a long fluorescence lifetime, with the ability to function as a substitute for adenine in many enzyme systems. Tolman et al.[16] have examined the dynamics of base stacking in ϵA containing dinucleoside phosphates using fluorescence techniques while Baker et al.[17] have performed a detailed fluorescence study of $\epsilon A_p \epsilon A$.

In this paper we study ϵA-containing dinucleoside phosphates using the luminescence-related technique of fluorescence detected circular dichroism (FDCD).[18-23] In this method, the sample is excited with circularly polarized light of varying wavelength, and the total emitted light is detected for the two senses of polarization of the exciting light. Thus, the technique can be used to obtain the CD of a fluorescent chromophore in the particular molecular environment of the system under study. Three dinucleoside phosphates, $\epsilon A_p \epsilon A$, $\epsilon A_p \epsilon C$, and $\epsilon A_p U$ are examined. It is shown that FDCD provides spectra which are

unobtainable using conventional CD techniques and that new and basic information concerning solution conformations and the dynamics of base stacking can be obtained. A preliminary report has appeared in the proceedings of a NATO Advanced Study Institute on Optical Activity and Chiral Discrimination.[24]

MATERIALS AND METHODS

A_pA, A_pC, A_pU and 5' AMP were purchased from Sigma Chemical Co. The εA derivatives were synthesized according to the method of Tolman et al.[16] and purified two to three times using paper chromatography. The eluent used was a 75% ethanol, 1M ammonium acetate solution. All buffer salts were of reagent grade and all solutions were made up using double-distilled water. After purification, solutions of the dimers were prepared in buffers consisting of 0.01 M sodium cacodylate, 0.01 M sodium chloride, pH 7.0. Alpha napthylamine was purchased from Sigma and re-crystallized from an ethanol-water mixture until white needles were obtained. The compound slowly turns red upon exposure to air; it was therefore stored in a vacuum desiccator at 4°C. Solutions of alpha napthylamine were prepared in the above cacodylate buffer. It should be noted that alpha napthylamine is on the OSHA list of regulated carcinogens that have require-ments for special handling in laboratories; appropriate pre-cautions must be taken.

All absorption spectra were measured using a Cary 14 spectro-photometer. The CD and FDCD experiments were performed using the modified Cary 6001 spectrometer described previously.[18,25]

A specially selected Hamamatsu R376 end-window photomultiplier tube with a 1-1/8" diameter photocathode was employed in making CD measurements, while FDCD spectra were recorded using a Hamamatsu R375 2" end-window photomultiplier. The CD spectrometer was calibrated using a +-10-camphorsulfonic acid solution whose ellipticity was checked in the spectrometers of two laboratories in addition to our own. A 1 mg/ml solution in a 1 cm cell was assumed to have an ellipticity of 0.312 at λ_{max}. The Pockel's cell orientation was also carefully adjusted in order to avoid absorption artifacts. All data were digitally recorded by an on-line computer,[26] and the output was subsequently smoothed and processed. The FDCD spectra were scanned 5 to 9 times, depending upon temperature, and the signals averaged in order to increase the accuracy of the measurements. At the end of a series of scans, those solutions whose spectra were measured at higher temperatures were also scanned at room temperature in order to check for sample decomposition. A new solution was run for each temperature above 50°. $\epsilon A_p \epsilon C$ measurements were made using a rectangular fluorescence cuvette having a 1 cm pathlength, while the spectra of the other two molecules were measured in a 3 mm pathlength cell having 1 cm width. Solution concentrations were adjusted so that the highest optical density over the wavelength scanned was less than one. This procedure was followed in order to insure that the photomultiplier was detecting emission equally from all parts of the sample cell. Extinction coefficients used for the dinucleoside phosphates were obtained from the paper by Tolman et al.[16] The concentrations employed were 5.7×10^{-5} M for $\epsilon A_p \epsilon A$, 5.8×10^{-5} M for $\epsilon A_p U$, and 2.4×10^{-5} M for $\epsilon A_p \epsilon C$. Some FDCD measurements were recorded with a

linear polarizer in front of the photomultiplier in order to ascertain whether the spectra contained contributions from photo-selection effects.[23] None were observed. All FDCD baselines were measured using α-napthylamine. The large quantum yield of about 0.4,[27] the long fluorescence lifetime, and the convenient absorption range of this molecule have made it very useful for recording baselines despite the inconvenience of using a substance on the OSHA list of regulated carcinogens. Finally, all FDCD measurements were performed using a Schott KV380 inter-ference filter which passes light of wavelength greater than 380 nm.

Using the CD data, van't Hoff stacking parameters were calculated.[1] The temperature range employed, 2° to 76°, was not great enough to directly measure the CD of the fully stacked state or the fully unstacked state. Therefore, two different methods were used to obtain thermodynamic values. In the first the assumption was made that the ellipticity of the unstacked state was equal to the sum of the ellipticities of the con-stituent nucleotides. The CD of the stacked state was then assumed to be that value which afforded the best least squares fit of the CD data to the van't Hoff equation. In the second method, taken from Powell et al.,[10] no assumption was made concern-ing the ellipticity of the unstacked state, but instead all of the parameters in the van't Hoff relation were varied until the best least squares fit of the data was obtained. These two techniques will be referred to in the text as methods I and II, respectively.

Results

The CD spectra of $\varepsilon A_p \varepsilon A$, $\varepsilon A_p U$ and $\varepsilon A_p \varepsilon C$ at various temperatures are shown in Figure 1. In general, twelve different temperatures were employed for each compound, five of which are presented in each illustration. The CD is of course considerably changed from that of the unmodified dimers. In each case a broad negative band appears at wavelengths longer than 300 nm corresponding to the first broad εA absorption band. Clear isodichroic points, implying the presence of a two state system, exist for $\varepsilon A_p \varepsilon A$ at 276 nm and 230 nm. One isodichroic point is observed for $\varepsilon A_p U$ at 290 nm, while $\varepsilon A_p \varepsilon C$ exhibits two isodichroic points at 300 nm and 243 nm. The εA and εC modifications have the effect of greatly increasing the observed CD signal. Thus, the $\Delta \varepsilon$ per monomer of $A_p A$ has a maximum at room temperature of about 6, while the corresponding $\varepsilon A_p \varepsilon A$ has a maximum at room temperature of about 10. The observed CD is in all cases much more intense and greatly different in shape from the CD of the constituent nucleotides, implying the existence of a great deal of base stacking.[1]

Figures 2 and 3 display some of the fluorescent anisotropies, $\Delta \varepsilon_F / \varepsilon_F$, obtained for the three dinucleoside phosphates at various temperatures. Since the CD spectrometer measures ratios of intensities reaching the photomultiplier, the relationship between FDCD signal and CD is[2,1]

$$\frac{\Delta \varepsilon_F}{\varepsilon_F} = -\frac{\theta_F}{14.32} + 2R \qquad (1)$$

where $\Delta\varepsilon_F$ and ε_F are the CD and extinction coefficient of the fluorescent species, θ_F is the FDCD signal, and

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

In the above expression ΔA is the total, or conventional CD of the sample, while A is the total absorbance. Thus, the fluorescent anisotropies are obtained through combining measurements of FDCD, CD and absorbance. Ninety five per cent confidence limits were calculated for all points in the spectra in Figures 2 and 3. For $\varepsilon A_p \varepsilon A$, these ranged from about $\pm 0.04 \times 10^{-3}$ near the absorption maxima at 300 nm and 275 nm, to $\pm 0.1 \times 10^{-3}$ near the CD minimum at 241 nm. Uncertainties for the other two dimers were about 20% lower because of the larger quantum efficiencies of these compounds.[16] The confidence limits remained fairly constant with increasing temperature, despite an observed decrease in fluorescence, because of the increasing number of scans recorded at higher temperatures.

The data in Figures 2 and 3 are presented in terms of $\Delta\varepsilon_F / \varepsilon_F$. If the extinction coefficients of the fluorescent species were known, one could obtain the CD of these species from the fluorescent anisotropy. For purposes of comparison, the assumption is made that the extinction coefficient of the fluorescent conformations equals that of the εA monomer, as neither U nor εC are fluorescent under our conditions. For reasons which will be apparent later, this is probably not too bad an approximation for $\varepsilon A_p \varepsilon C$ and $\varepsilon A_p U$, while for $\varepsilon A_p \varepsilon A$ it can introduce an error of, at worst, 20-30%. Since qualitative comparisons are sufficient

for most of our purposes, this is an acceptable error. Figures
4 and 5 show the resultant fluorescent CD of the three dimers
at 24° compared to their respective total CD at the same tempera-
ture. Although both chromophores in the εA dimer are fluorescent,
it can be seen in Figure 5 that the fluorescent CD of $εA_pεA$ is
very different from its total CD with the former curve being
almost the mirror image of the latter. The reason for the
difference is that FDCD directly detects only those conformations
which are fluorescent and in the case of the εA dimers, the stacked
conformations are quenched and make no contribution to the
fluorescent CD. This can be seen by the observation that $εA_pεA$
has only 7% of the quantum yield of 5' εAMP.[16] A further check
was made by comparing $Δε_F$ of 5' εAMP to its $Δε$. Since of course
stacking interactions are negligible for the monomer, the two
CD's should be the same. Within experimental error, this was
found to be the case. FDCD thus provides an opportunity to
observe a unique CD of the "non-stacked" conformations of
dinucleoside phosphates. The fluorescent CD's in Figures 4a and
4b are also quite unlike their total CD's. In this case the
result is a combination of the fact that U and εC are not
fluorescent, and thus do not contribute to $Δε_F$, and the fact
that the stacked conformations of these dimers are also quenched.

DISCUSSION

$εA_pεC$ and $εA_pU$ Stacking

The data in the previous section allow one to answer some
of the questions concerning the stacking properties and parameters

of the dinucleoside phosphates. First of all, the FDCD results
provide a unique opportunity for determining whether a
particular molecule exists in only two discrete states. This is
because FDCD detects only fluorescent species, and the stacked
states of the three εA dimers studied are quenched. Thus if
these molecules exist in a two-state system, there will be only
one fluorescent species, and the fluorescent anisotropies, which
are not a function of concentration, should not exhibit any
change with respect to temperature. On the other hand if more
than one fluorescent state exists, then the fluorescent anisotropy
is

$$\frac{\Delta\varepsilon_F}{\varepsilon_F} = \frac{\sum_i \phi_i c_i \Delta\varepsilon_i}{\sum_i \phi_i c_i \varepsilon_i}$$

where ϕ_i refers to the quantum yield of species i, and the sum-
mation is over all fluorescent states present. In a sample
containing more than one fluorescent species, therefore, the
measured anisotropy is a quantum yield weighted average and should
change with temperature.

Figure 2 displays the fluorescent anisotropy of $\varepsilon A_p \varepsilon C$. Within
experimental error, this quantity does not change with changing
temperature, and thus, one can conclude that $\varepsilon A_p \varepsilon C$ is indeed a
two-state system. This is further illustrated in Figure 4a in
which the striking difference between the fluorescent CD and the
total CD of this molecule is displayed. The very large CD of
the dinucleoside phosphate disappears almost completely in the
fluorescent curve leaving a very small CD greatly changed in

shape from the first spectrum. If the unstacking of the dimer were due to a dynamic or multistate process, one would expect to see a fluorescent CD which was intermediate between dimer and monomer in shape and intensity. Instead, as can be seen in Figure 6, the fluorescent CD has the same shape as that of 5' εAMP, although it has a somewhat greater intensity. We therefore conclude that the $\varepsilon A_p \varepsilon C$ dimer is a two-state system and that the second, fluorescent state is almost completely unstacked with very little interaction between the εA and εC residues.

The two-state behavior exhibited by $\varepsilon A_p \varepsilon C$ is also manifested in the $\varepsilon A_p U$ system. This is shown in Figure 4b where the fluorescent anisotropy is shown to be constant, within experimental error, with changing temperature. In Figure 6 the fluorescent CD is compared to that of 5' εAMP. While the intensities are comparable, the shape of the monomer and dimer curves are different, indicating some residual intramolecular interaction for the fluorescent species of the molecule. It is interesting to note the CD trends at 240 nm. At this wavelength, $\Delta\varepsilon$ per mole of dinucleoside phosphate increases in magnitude from -2.9 at 2° to -3.3 at 76°, while one would expect an opposite trend if the CD were truly approaching that of the constituent monomers. In Figure 4b, $\Delta\varepsilon_F$ is seen to be greater than $\Delta\varepsilon$ at 240 nm. This suggests the conclusion, arrived at above, that the stacked state of $\varepsilon A_p U$ is in equilibrium with a fluorescent state which is not fully unstacked.

Since $\varepsilon A_p \varepsilon C$ and $\varepsilon A_p U$ have been indicated to be two state systems, it is possible to use the observed circular dichroism in a van't Hoff type calculation with some confidence in the meaningfulness of the results. The thermodynamic stacking parameters obtained, both from method I and the Powell method are listed in Table I. For both $\varepsilon A_p U$ and $\varepsilon A_p \varepsilon C$, the greatest signal to noise ratio is at about 280 nm so that use of this wavelength yields the most reliable parameters. The other parameters listed, however, are reasonably consistent with the 280 nm values considering the differing signal to noise ratios. The ΔH of stacking is about -5 kcal/mole for both dimers, with $\varepsilon A_p \varepsilon C$ exhibiting the greater degree of stacking. The latter finding is in agreement with the results of reference 16. In addition, the free energies were determined to be small at room temperature in accord with the results found for the naturally occurring nucleic acid bases.[9]

$\varepsilon A_p \varepsilon A$ Stacking

The van't Hoff method was also applied to

$\varepsilon A_p \varepsilon A$. In this case, as can be seen in Table I, the different methods of treating the data give different results. For method I, if one assumes $\Delta \varepsilon$ (unstacked) is equal to the monomer value of -0.65 at 243 nm, then ΔH of stacking is computed to be about -8 kcal/mole. If one uses the Powell method, however, a much better least squares fit is obtained and a very different ΔH of -4 kcal/mole is calculated. The $\Delta \varepsilon$'s (unstacked) obtained for 243 nm and 224 nm with this method turn out to be -4.5 and -3.7 which are much greater in

magnitude than the CD of the ϵA monomer. These results suggest that the $\epsilon A_p \epsilon A$ system is very different from that of the previous two dinucleoside phosphates. Such a conclusion is confirmed in Figure 3 which displays the fluorescent anisotropies of $\epsilon A_p \epsilon A$ at various temperatures. The magnitudes of $\Delta \epsilon_F / \epsilon_F$ are much greater than those measured for $\epsilon A_p U$ and $\epsilon A_p \epsilon C$ and, in addition, are not constant with temperature. This is further illustrated in Figure 7 where the fluorescent anisotropy of $\epsilon A_p \epsilon A$ at 243 nm is displayed for 10 different temperatures. The dissymmetry is unchanging, within experimental error, below 35° but begins to decrease above this temperature. The data are thus consistent with a two state model below 35°, but at higher temperatures at least one additional state begins to appear.

Since one can assume a two state model below 35°, the lower temperature CD data can be used in a method I type of calculation to obtain the stacking parameters between the stacked and fluorescent states. In this case the CD of the "unstacked" state is not taken from the monomer CD but from the $\Delta \epsilon_F$ curve displayed in Figure 5. The results of this analysis are listed in Table I. Unlike the previous method I analysis, the ΔH of stacking has now been reduced to about the same value as the ΔH's of $\epsilon A_p U$ and $\epsilon A_p \epsilon C$. The magnitude of $\Delta \epsilon_F$ in Figure 5 indicates, however, a considerable amount of intramolecular interaction still exists in the fluorescent conformations of $\epsilon A_p \epsilon A$. It is difficult to propose a conformation for this fluorescent state without detailed calculations. Certainly the near reversal in shape of the $\Delta \epsilon$ and $\Delta \epsilon_F$ curves in Figure 5 indicate that the stacked and fluorescent conformations are very different.

Johnson et al.[28] have calculated the effects of changing
dinucleotide conformation on CD, but a reversal of sign was not
considered. It was asserted in this paper that opening up the
bases while maintaining the same relative orientation reduced
the CD without changing its shape. On the other hand, sliding
the bases past each other did result in a shape change. It is
this latter type of conformational change which is probably
occurring in $\varepsilon A_p \varepsilon A$. Using the equilibrium constant obtained
from the van't Hoff analysis together with the measured CD of
FDCD data, one can also obtain the $\Delta\varepsilon$ of the stacked state. The
calculated resultant curve is illustrated in Figure 5.

The next question to be considered is the nature of the
unstacking process of the εA dimer above 35°. The simplest model
to assume is that a second, virtually unstacked, fluorescent
species of $\varepsilon A_p \varepsilon A$ begins to appear at higher temperatures. Note
that the FDCD data cannot be used to obtain the stacking
parameters between the two fluorescent states directly since,
from expression (3), a knowledge of the relative quantum yields
of the two species is required. The parameters from the two
state analysis of the εA dimer below 35° can be used, however,
in a three state analysis to get at least a rough idea of the
stacking parameters at higher temperatues. The equilibrium
constants calculated between the two fluorescent states as a
result of this analysis are small and vary widely depending upon
the exact values of the parameters used. It is thus difficult
to draw conclusions from the values of the equilibrium constants.
Small K's, indicating low concentrations of the second fluorescent

state, are not inconsistent, however, with the large changes in
anisotropy in Figure 3. The second, presumably unstacked,
fluorescent state would be expected to have a larger quantum
yield than the first fluorescent state. Since fluorescent
anisotropies obtained from a system containing more than one
fluorescent species represent a quantum yield weighted average,
appearance of even a small amount of an unstacked $\varepsilon A_p \varepsilon A$ species
could have a very large effect on $\Delta \varepsilon_F / \varepsilon_F$. We therefore assume
the simplest model in explaining the FDCD data; i.e., below 35°
the stacked state of $\varepsilon A_p \varepsilon A$ is in equilibrium with a single, at
least partially stacked, fluorescent species. Above 35°, a
second unstacked fluorescent species begins to appear. It should
be noted that such an assumption does not mean that the data
cannot be explained by a model consisting of more than three
states. We are merely stating that a three state hypothesis is
the simplest consistent with the FDCD results.

Linear Analysis of CD Data

A linear analysis of the CD data was done in order to
determine how many linearly independent basis curves were
necessary to recreate a set of CD spectra measured at different
temperatures. The assumption is made that the CD of any
particular dinucleoside phosphate conformation does not change
with temperature so that the number of basis curves found can

be identified with the number of distinguishable conformations in the system.

The method used for the above analysis was that of Lloyd.[29] This method uses a least squares minimization to construct from the experimental spectra the minimum set of basis curves necessary to fit all of the observed data. The technique is superior to the similar method of matrix rank analysis,[30],[31] in that the latter procedure requires the choice of certain reference spectra. This is adequate for curves having large magnitudes, but when components are present whose signal is only an order of magnitude larger than the experimental error, matrix rank analysis ceases to be satisfactory. The present method of analysis gives equal weight to all the experimental spectra and, therefore, is a more sensitive technique.

The Lloyd method, as described above, was applied to all the CD data measured for each of the three dinucleoside phosphates studied in this work. The minimum number of basis curves necessary to reproduce the spectra, within experimental error, was determined by calculating the variance as each new basis curve was added, and applying a statistical F test to the results. Since only one average variance for an entire spectrum was obtained, the spectra were divided up into sections of equal noise in order to make the F test meaningful. The results indicated that the $\epsilon A_p U$ and $\epsilon A_p \epsilon C$ data could be adequately described with two basis curves. For $\epsilon A_p \epsilon A$, on the other hand, although two basis curves were almost adequate, the F test indicated addition of a third curve gave a satistically significant improvement. It should be pointed out that linear analysis methods

cannot distinguish between components having identical temperature dependencies. Thus, although one cannot say that a system does not contain more distinct states than the number of basis spectra calculated for it, one can certainly say that it is not likely to contain less. Bearing this in mind, we conclude that the results of the linear analysis of the spectra are in agreement with those obtained by FDCD.

CONCLUSION

The data presented in this paper indicate that $\epsilon A_p U$ and $\epsilon A_p \epsilon C$ can be described as two state systems. This might seem surprising since, intuitively, one might expect a non-cooperative, many-state type of behavior from such systems. However the bulk of the evidence, especially the lack of change of the fluorescent anisotropies with changing temperature, clearly supports a two-state model. This type of behavior is especially evident in the case of $\epsilon A_p \epsilon C$. The very large difference between the conventional and fluorescent CD of this molecule is striking, and there appears to be little evidence in the $\Delta \epsilon_F$ curve for the contributions from intermediate states that one might expect from a multistate or torsional oscillator[13] type of system. It should be noted that in speaking of a two-state model, it is probably more nearly correct to speak of a set of closely spaced microstates, spectroscopically indistinguishable, separated in terms of thermodynamic properties from a second set of micro-states. It is in this respect that $\epsilon A_p U$ and $\epsilon A_p \epsilon C$ are considered to exhibit two-state behavior. Since these two dinucleoside

phosphates have been shown to exist in two states,   it
may be that many, or even most, of the naturally occurring
dinucleotides also can be described in terms of two sets of species,
thus making meaningful the van't Hoff parameters calculated for
these molecules.

The behavior of $\varepsilon A_p \varepsilon A$ was found to be more complicated than
the above, and this molecule is clearly not a two-state entity
over the entire temperature range examined. This conclusion is
in accord with the fluorescence data of Baker et al.[17] who,
however, proposed that the unstacking of this dimer was a
continuous process. Although this possibility cannot be ruled
out, in view of the FDCD results, it does not seem likely. The
bases in the εA dimer are undergoing a relative motion which
results in a shape change in the CD curves upon going from stacked
state to fluorescent state. For a continuous process, as the
temperature increases and the system becomes more unstacked, this
CD shape change should continue and be reflected in a change in
shape of the fluorescent anisotropies. Since these latter
quantities do not change shape it is difficult to understand how
the unstacking process could be continuous. Baker et al. indicated
that their data could also be explained by a discrete multistate
hypothesis, and it is this model which   is   suggested
by our results. The magnitude of the fluorescent CD of $\varepsilon A_p \varepsilon A$
at 35° is such that a considerable amount of intramolecular
interaction is indicated in the fluorescent species, and the fraction
of unstacked species at physiological temperatures is
small. However,   it is clear that the mechanism of the

unstacking process does not have to be exactly the same for each dinucleoside phosphate, and each dimer must be considered individually.

It has been shown that the multistate behavior of $\epsilon A_p \epsilon A$, which was determined from the anisotropy data, was also indicated by the Powell and linear spectra analyses. It may be that these methods can be used to indicate multistate behavior in other, perhaps nonfluorescent, dimers.

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TABLE I

Thermodynamic Parameters of Stacking

λ(nm)	Method[1]	$\Delta H°$ (kcal/mole)	$\Delta S°$(e.u.)	$\Delta\varepsilon_u$	$\Delta\varepsilon_s$	K (24°)
			$\varepsilon A_p U$			
280	II	−4.9	−17.6	0.38	6.32	0.52
260	II	−5.1	−17.0	0.53	2.49	1.07
240	II	−6.3	−20.4	−1.82	− 1.35	1.44
280	I	−5.7	−20.2	−	5.2	0.55
			$\varepsilon A_p \varepsilon C$			
280	II	−5.0	−17.0	−0.44	11.5	0.94
280	I	−5.3	−17.8	−	11.2	1.03
270	I	−4.6	−16.0	−	8.8	0.84
			$\varepsilon A_p \varepsilon A$			
243	II	−4.1	−15.1	−4.5	34.5	0.48
224	II	−3.6	−13.7	−3.7	−56.5	0.43
224	I	−7.1	−24.4	−	−28.2	0.78
243	I	−8.2	−27.7	−	17.4	1.01
243	I[2]	−5.5	−17.4	−7.6	17.9	1.67

[1] In method I, $\Delta\varepsilon_s$ (stacked) is varied to obtain the best least squares fit to the data. In the Powell method, Method II, $\Delta\varepsilon_u$ (unstacked) and $\Delta\varepsilon_s$ (stacked) are varied to obtain the best least squares fit. See text.

[2] In this case 8 points between 2° and 35° were used along with $\Delta\varepsilon_u$ obtained from the fluorescent anisotropy. See text.

Figure Legends

Figure 1.   CD per monomer unit of three dinucleoside phosphates:
εA is 1,N^6 ethenoadenosine; εC is 3,N^4 ethenocytidine; U is
uridine.   All molecules were in 0.01 M sodium cacodylate,
0.1 M sodium chloride, pH 7.0.

Figure 2.   The Kuhn dissymmetry factor vs. temperature for
εA$_p$εC and εA$_p$U.   These curves were derived from FDCD, CD and
absorption measurements using equation (1).

Figure 3.   The Kuhn dissymmetry factor vs. temperature for εA$_p$εA.

Figure 4.   Conventional CD and fluorescent CD at 24°C for (a)
εA$_p$εC and (b) εA$_p$U.   The values of Δε and Δε$_F$ are per mole
of dinucleoside phosphate.

Figure 5.   The circular dichroism per monomer unit of εA$_p$εA
compared to 5' εAMP.   The curve labeled Δε is the conventional
CD of εA$_p$εA measured at 24°; Δε$_F$ is the fluorescent CD
obtained by multiplying the dissymmetry at 24° by the
extinction coefficients of 5' εAMP; the stacked CD is derived
using the results of a van't Hoff analysis (see text).

Figure 6.   Fluorescent CD per mole of dinucleoside phosphate of
εA$_p$U and εA$_p$εC compared to the CD of 5' εAMP.   The εA$_p$U and
εA$_p$εC curves were obtained by multiplying the respective
dissymmetries at 24° by the extinction coefficients of 5'
εAMP.   T = 24°.

Figure 7.   Temperature dependence of the dissymmetry of εA$_p$εA
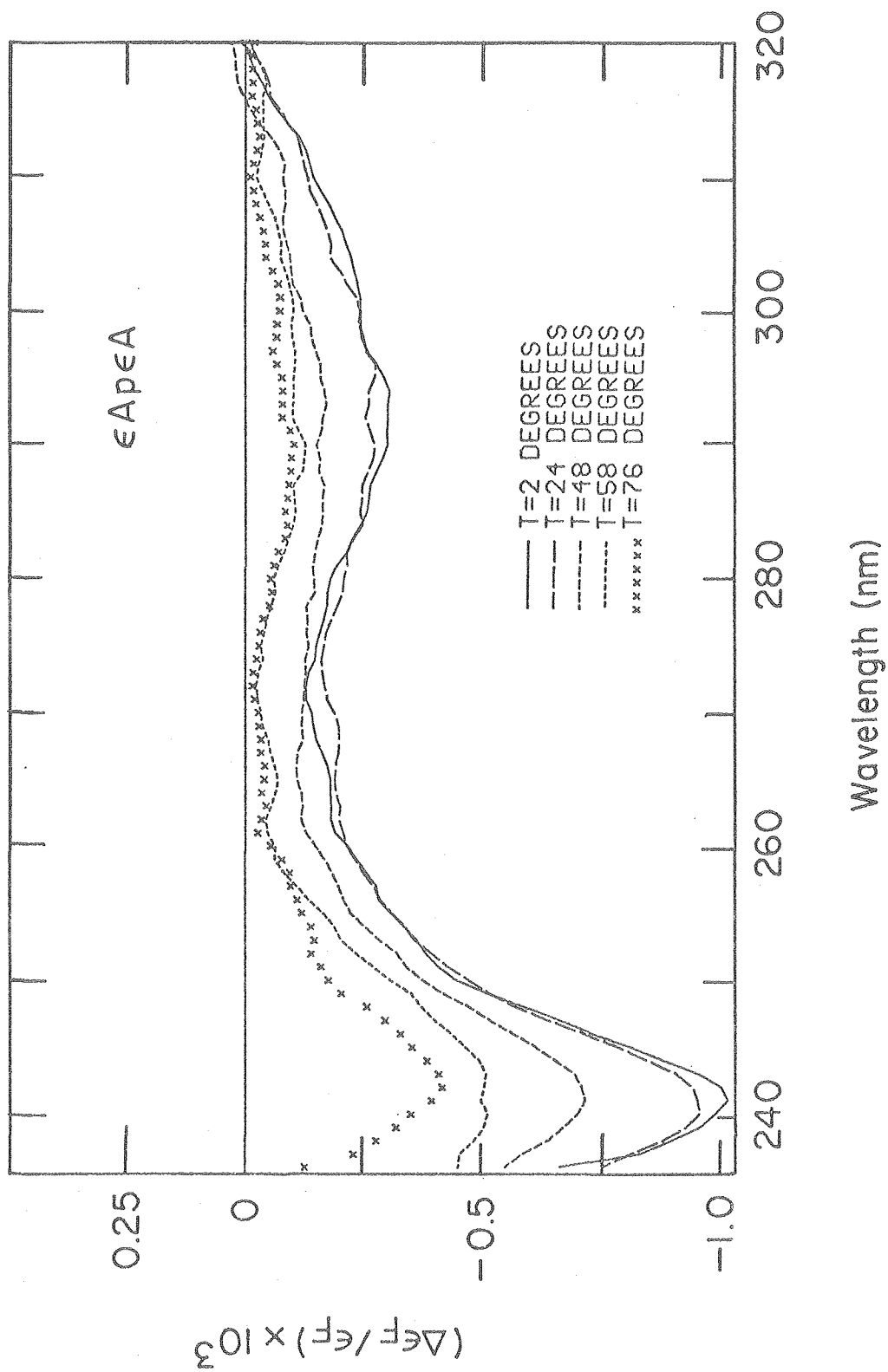at 243 nm; the error bars represent 95% confidence limits.
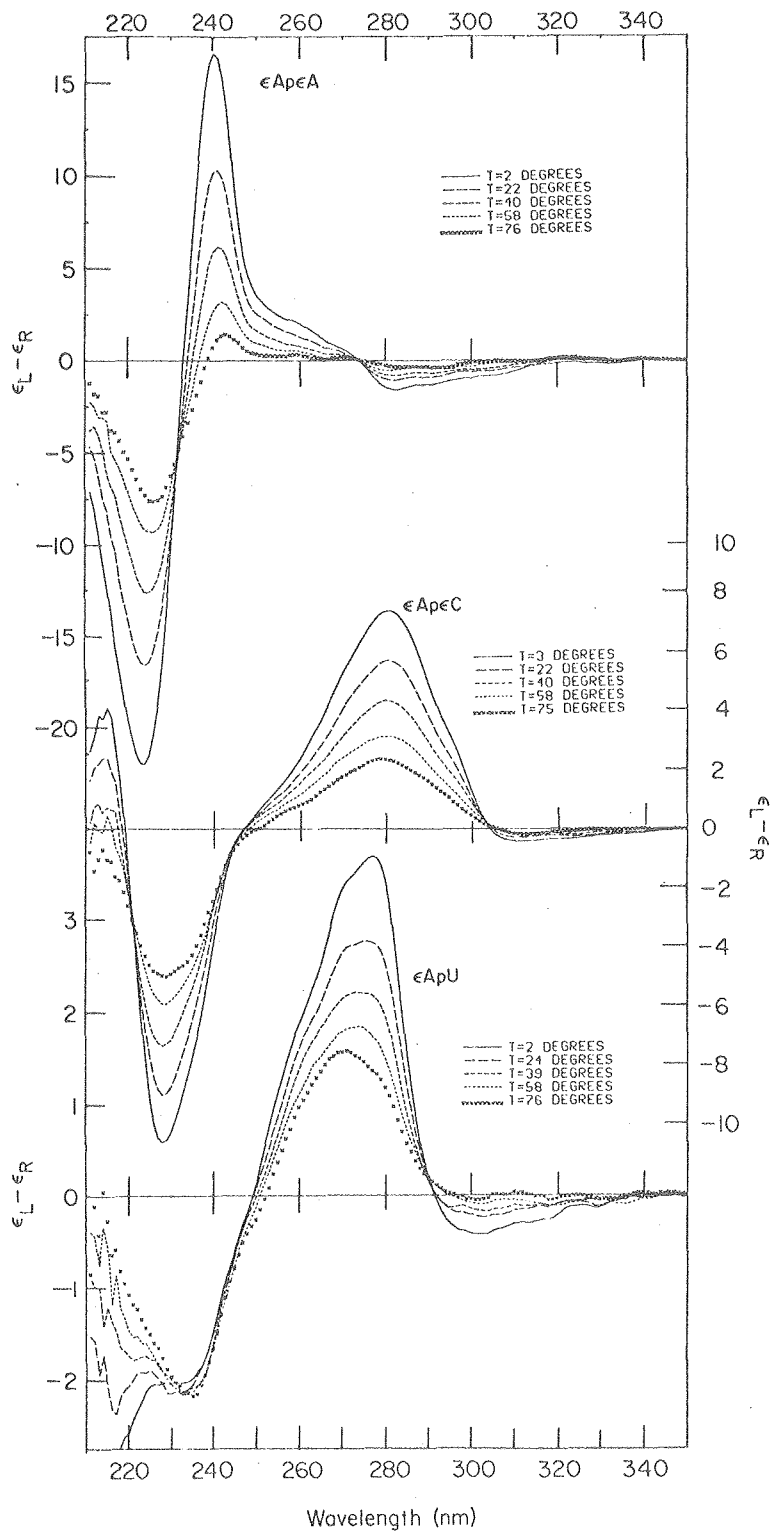
Figure 1.

XBL 788-10404

Figure 2.
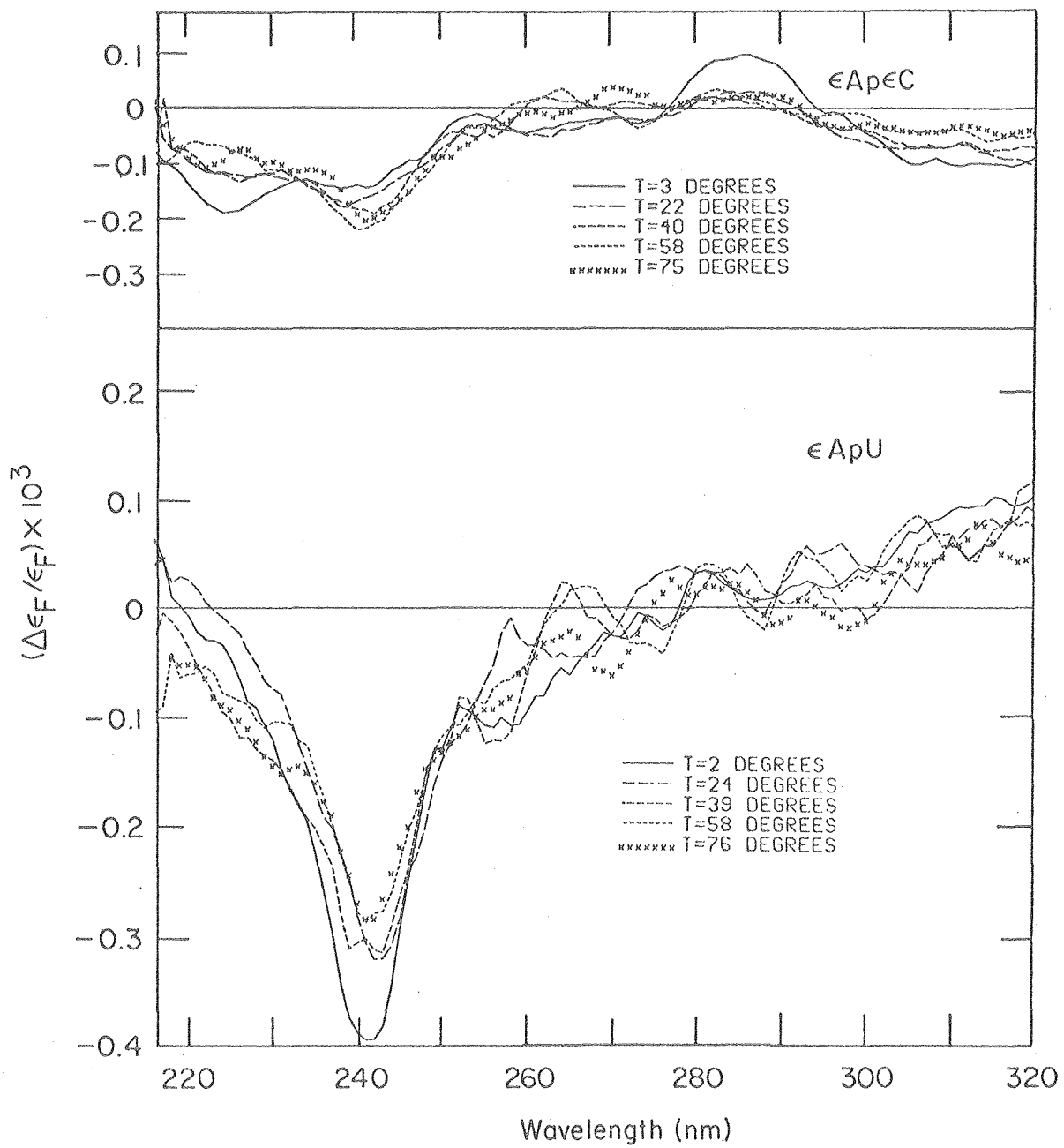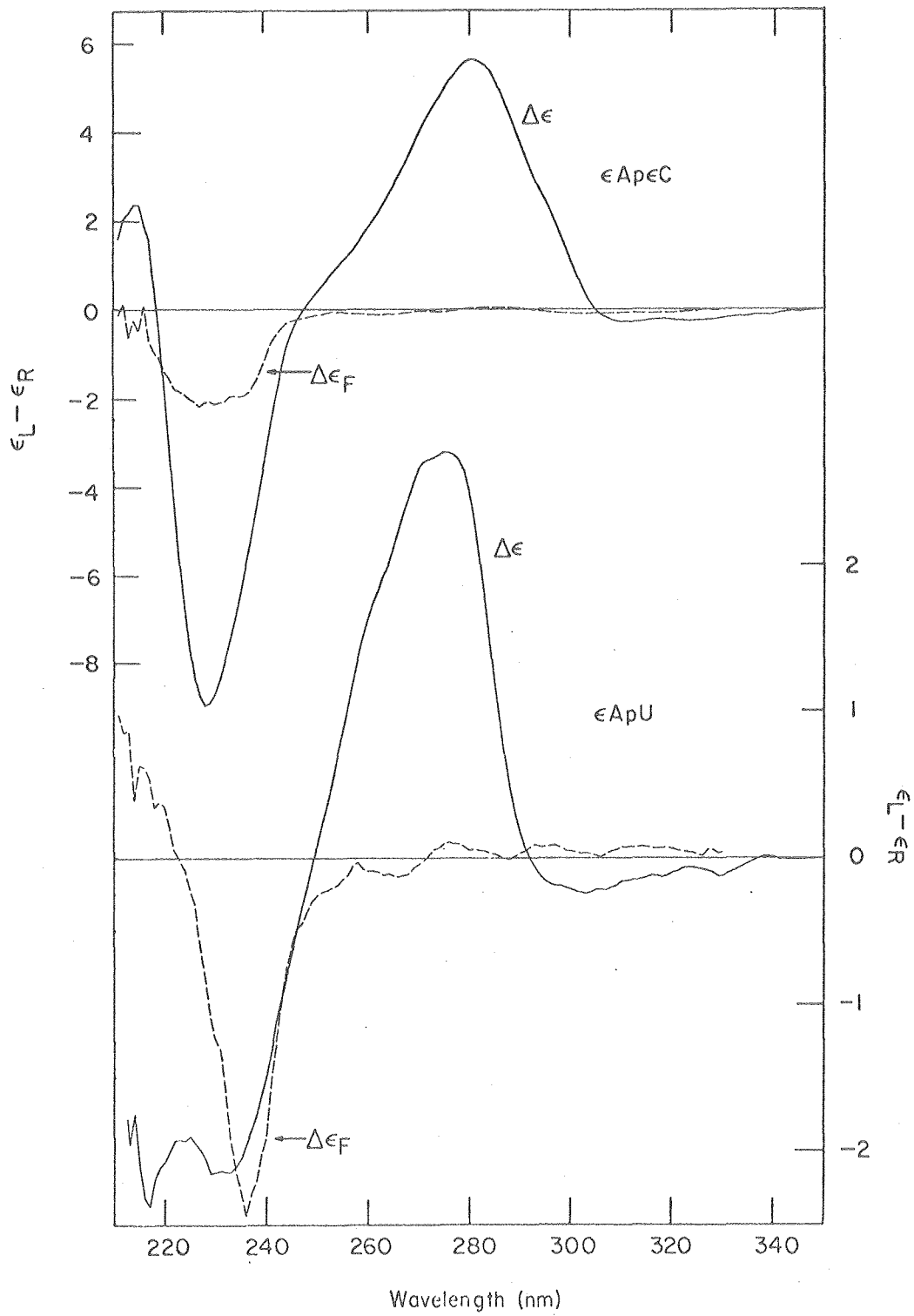
XBL 788-10401

Figure 3.
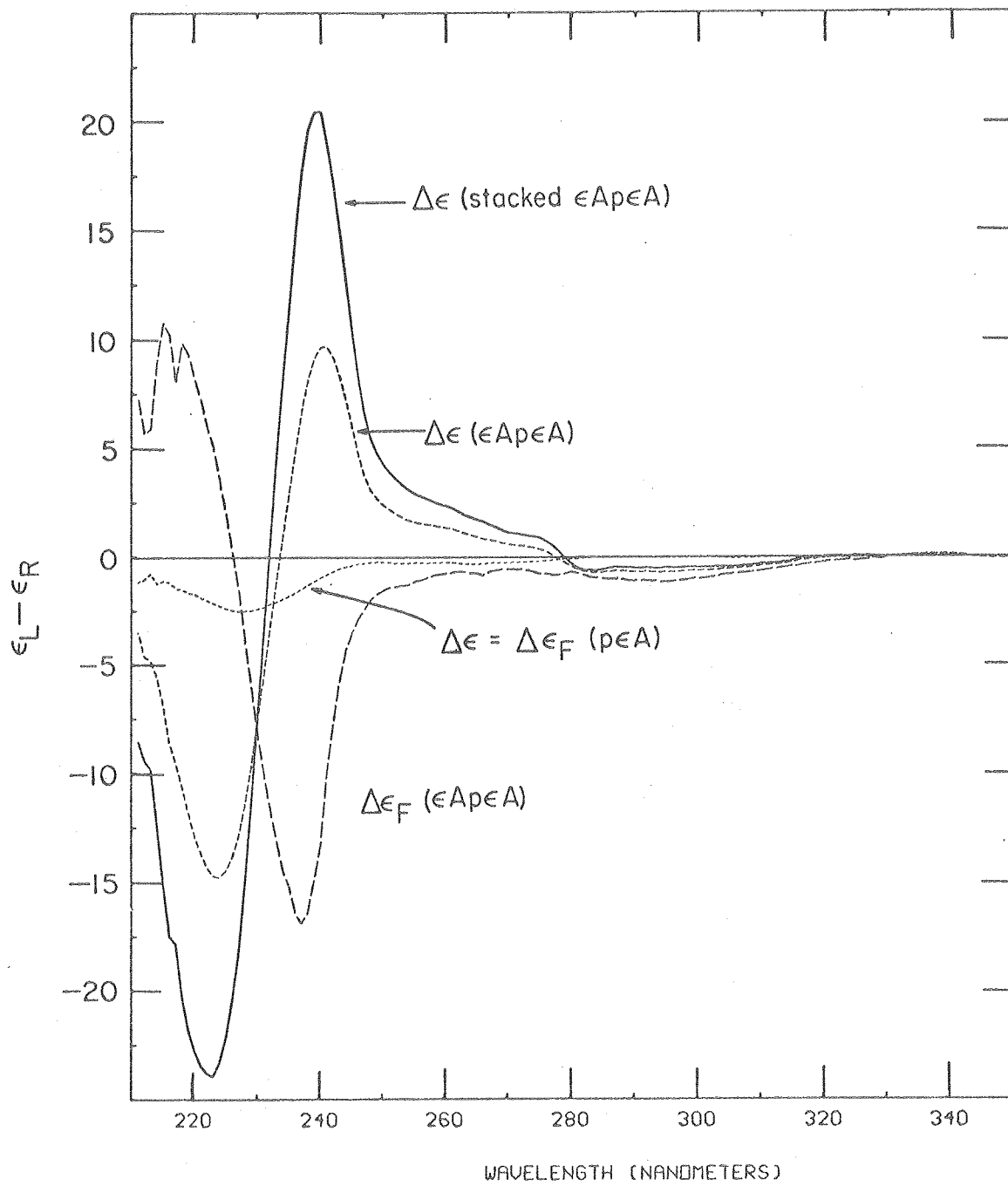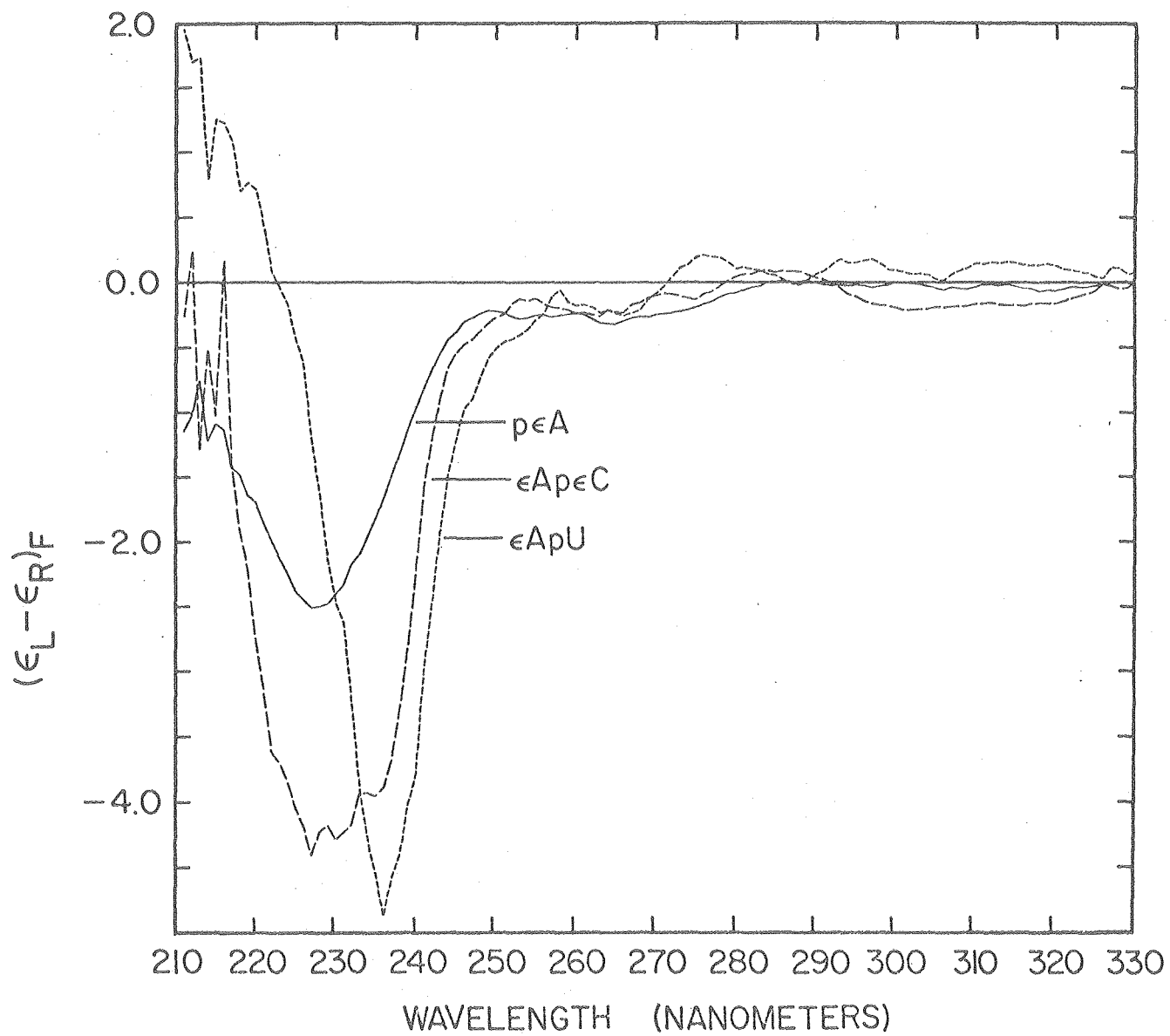
XBL 788-10394

XBL 788-10403

Figure 4.

Figure 5.

XBL 788-10402

Figure 6.

XBL 797-10650