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SUBSTRATE AND INHIBITOR INDUCED CHANGES IN THE OPTICAL ROTATORY DISPERSION OF ASPARTATE TRANSCARBAMYLASE

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One of the main homeostatic control processes in cells is the modulation of the activity of certain enzymes by small metabolites and catabolites. Allosteric enzymes[1] show large changes in catalytic activity in the presence of effectors (inhibitors or activators) that are often stereochemically unrelated to the substrate or product of the enzyme of interest. A model for the allosteric enzyme regulation mechanism, based largely on kinetic evidence, has been proposed by Monod, Changeux and Wyman[2]. This model proposes that small molecule effectors alter enzyme catalytic acitvity by bringing about conformational changes in the enzyme structure. Independent evidence for or against the alteration of protein conformation by allosteric effectors is important for assessing the correctness of the viewpoint of this model. The work presented here reports on some optical rotatory dispersion (ORD) results that demonstrate changes in the conformation of the enzyme aspartate transcarbamylase caused by substrate and by an allosteric inhibitor.

Aspartate transcarbamylase (ATCase)[3] is one of the best characterized examples of an allosteric enzyme and is suitable for physical study because it can be highly purified. ATCase is the first enzyme in the pyrimidine biosynthetic pathway. Aspartic acid and carbamyl phosphate

are coupled by ATCase to form carbamylaspartate, which is transformed by a series of enzymatic reactions to the pyrimidine nucleotides, uridine triphosphate (UTP) and cytidine triphosphate (CTP). As first shown by Yates and Pardee[4], cytidine derivatives exert a regulatory influence upon the activity of the enzyme in whole cells and in cell extracts. Kinetic measurements on purified ATCase show that CTP binds reversibly at a site distinct from the catalytic site, and inhibits the enzyme by reducing its affinity for the substrate aspartate[5]. The level of the ultimate product, CTP, is controlled by feedback inhibition of ATCase.

ATCase has a sigmoidal substrate saturation curve for aspartate at pH's greater than 6.1, indicating a cooperative (homotropic[2]) interaction between catalytic sites, i.e., ATCase increases its aspartate affinity with increasing aspartate concentration. Figure 1a shows the effect of CTP and pH on the substrate saturation curve of ATCase. Cooperative interactions between catalytic sites and the modification of this interaction by CTP (heterotropic interaction[2]) are examples of indirect interactions between binding sites that are characteristic of allosteric enzymes. The cooperative substrate site interaction allows nonreactive substrate analogs that are simple competitive inhibitors at high concentrations to activate the enzyme at low concentrations. Figure 1b shows the activation of ATCase by a substrate analog at low concentrations, and the competitive inhibition at high concentrations. Several substrate analogs show this effect.

The kinetic effects, in summary, are as follows: CTP promotes a decrease in ATCase's affinity for aspartate while substrates, substrate analogs, and protons increase enzyme affinity for aspartate. At a given aspartate

concentration, the aspartate affinity of the enzyme determines the catalytic activity (reaction velocity) of ATCase. The activity changes at a fixed aspartate concentration are represented in Figure 1a by the arrows showing the direction of the activity shift upon addition of CTP and H^+.

Materials and Methods. All chemicals were obtained commercially. The sodium salt of poly-L-glutamic acid, supplied by Mann Research, Inc., had an average degree of polymerization of about 180, estimated by the intrinsic viscosity in 0.1 M NaCl. The poly-L-glutamic acid concentration was estimated from the dry weight, assuming one water of hydration per residue. ATCase was a gift from Dr. John Gerhart. The enzyme assay[5] and method of preparation[21] were previously described. All enzyme solutions contained 2 mM mercaptoethanol and 0.2 mM EDTA. Potassium phosphate was used for the pH 7.0 and pH 6.1 buffers, while Tris HCl was used for pH 8.0. The buffers were 40 mM for the kinetic measurements and 4 mM for the optical measurements below 240 mμ. The enzyme concentrations were determined from the absorption at 279 mμ, using $A^{1\,cm}_{1\,mg/ml} = 0.59$. The average residue weight ($\bar{M}$) of ATCase was estimated, from a preliminary amino acid analysis[17], to be 120.

Optical rotatory dispersion measurements utilized a Cary Model 60 recording spectropolarimeter. The rotations were independent of slit width and the specific rotations were independent of the (concentration) (path length) product over a ten-fold range. Lamp position adjustment and slit width adjustment were made for minimum noise level at the wavelength region of interest. The cells, made by Pyrocell, Inc., were selected for minimum birefringence and were cleaned inside and out by

soaking in concentrated HNO_3-H_2SO_4 (50/50 by volume) and by a one-minute exposure to MeOH-3 M KOH (50/50 by volume). When properly cleaned, the cells could be removed and replaced in the polarimeter with no observable change in the rotation. Cells of 0.1, 1.0 and 10 mm pathlength were used. All measurements were performed at 4.5 ± 1.0°C. The specific rotation measurements for different samples at the same solution conditions varied 1 to 2%. The variation is probably due to volumetric errors in sample preparation. This variation of specific rotation from sample to sample was too large to permit a satisfactory determination of the absolute specific rotations of the enzyme in the presence and absence of effectors. However, we could determine the relative rotation in the presence and absence of effector by a titration method. For the titration experiments, 2.5 ml of enzyme solution was pipetted into a 0.1 or 1.0 mm cell and the ORD spectrum measured. Ten- to twenty-lambda aliquots of concentrated effector solutions, previously adjusted to the appropriate pH, were added with a micropipet. The enzyme solution was mixed by slow inversion of the cell and the ORD spectrum measured. Corrections for dilution and CTP rotation were applied. The magnitude of rotation at selected wavelengths was most precisely determined by chart recording of the rotation at each fixed wavelength for extended periods (ca. 10 min) with a 10-or 30-second pen time constant. After running a spectrum the peak and trough wavelength points were repeated and the rotations were unchanged within the noise level in all cases. The specific residue rotation [R'] was calculated according to the relation:

$$[\Phi'] = \frac{3}{n^2 + 2} \quad \frac{\alpha_\lambda}{l\ c} \quad \frac{\bar{M}}{100}$$, where

α_λ = observed rotation in degrees, l = pathlength in decimeters, c = concentration in grams/ml, $\bar{M}$ = average residue weight, and n = refractive index of the solution at wavelength of measurement. The refractive index of water at 231 mμ and 20°C was used for all wavelengths. The Lorentz factor, $3/(n^2 + 2)$, for H_2O at 231 mμ is 0.7635.

Absorption measurements utilized a N_2-purged Cary Model 15 spectrophotometer. Absorption spectra were taken relative to a buffer solution of identical concentration. The phosphate buffer used for the far UV absorption measurements had a maximum absorption of one at 180 mμ in a 0.1 mm cell.

Helix content of ATCase. Optical rotatory dispersion (ORD) in the far ultraviolet region of peptide bond absorption is sensitive to protein secondary structure[7,8]. Figure 2 shows the UV ORD of a model polypeptide, poly-L-glutamic acid (PLGA), in the helical (2a) and random coil (2b) conformations. Similar curves, differing slightly in magnitude, have previously been published by other workers[8]. Figure 2c shows the UV ORD of ATCase, the spectrum is strikingly similar in shape to that of helical PLGA, but the magnitude/residue is much smaller. The ORD curve of ATCase can best be fit with about 37% of the PLGA helix specific residue rotation (Fig. 2a) with no contribution of the nonhelical residues. To a first approximation we conclude that about 37% of the residues of ATCase are in the helical conformation. Since there may be many short regions of helix in the molecule, the actual helix content may be somewhat higher than 37% due to an expected chain length dependence of the rotation/residue

of a polypeptide helix[9]. The only appreciable deviation of the fit of a 37% PLGA helix specific residue rotation to the ATCase ORD occurs in the region between 220 and 205 mμ. The small deviation of the fit has a rather complicated wavelength dependence, and is thought to be due to an almost complete cancellation of opposing contributions of the Cotton effects of the various nonhelical residues. In a study of the far UV optical activity of myoglobin, using circular dichroism, Holzwarth and Doty[10] have found a similar cancellation of the contributions of the nonhelical residues. In the case of myoglobin, the circular dichroism can best be fit by 70% model polypeptide helix dichroism with no contribution from the nonhelical residues[11].

Several other methods are available for helix content estimation from ORD data[12,13]. The other treatments of this problem rely on the monotonic visible and near UV data, and we feel that the most reliable method should be the direct observation of the peptide helix Cotton effects as done here. The exact absolute value of helix content of ATCase is not of prime importance in our work and, furthermore, we did not want to use the large amounts of material required for precise visible measurements. None of the methods of helix content estimation are really satisfactory for proteins with low helix content since local charge and dipole fields[14] of regions of the protein wrapped around the helical segments are not present in the reference polypeptides. It should be mentioned that PLGA may not be the ideal reference material for helix content estimation, and these problems have been discussed by Yang and McCabe[15].

The helix content of proteins can also be estimated from the hypochromic effect that the helical conformation exerts on the ultraviolet peptide bond absorption[16]. This method agrees well with ORD in the case of simple polypeptides and some proteins. The use of this method for proteins is complicated by the contribution of aromatic amino acid side chains, as well as arginine, methionine, cystine, amide, and carboxylate groups to the absorption in the region of peptide bond absorption. The method is attractive because it requires small amounts of material and we felt that more cases should be tried to fully explore its possible usefulness. The side chain contribution to the absorption of ATCase was estimated from an amino acid analysis[17] and the amino acid side chain extinction coefficients given by Rosenheck and Doty[16]. The absorption spectrum of ATCase is given in Figure 2d. The helix content of ATCase was estimated by this method to be 95% at 190 mμ, 51% at 197 mμ, and 48% at 205 mμ. The 190 mμ point has the largest relative correction for side chain absorption and is least trustworthy. The 197 mμ point has the smallest relative correction, and Rosenheck and Doty[16] believe 197 mμ to be the most reliable.

The peptide hypochromism gives a value of helix content for ATCase larger than that predicted by ORD. If the 190 mμ point is neglected, the peptide bond hypochromism indicates about 50% helix. This is acceptable agreement with the ORD value of 37%. We consider the hypochromism value as the less reliable since the side chain as well as the peptide bond absorption may be considerably perturbed by the environment inside the protein.

Substrate and inhibitor-induced changes in ORD. The entire ORD curve of ATCase changes in magnitude when CTP, substrate analogs, or protons are added to the enzyme. The observed changes are small (about 5-6% maximum) but can be measured over most of the wavelength range of interest. Table I shows the specific rotation/residue of ATCase at the trough of the 235 mμ Cotton effect in the presence of various substances that affect its kinetic behavior. We use 235 mμ as a convenient wavelength for tabulating the ORD change because the relative errors are lowest at this wavelength. These measurements were repeated several times and always showed the trends indicated in Table I; however, the variations in the observed differences were large enough to make the magnitude of the change uncertain. We found that titration of the change in optical rotation shows the rotational changes clearly (see the Materials and Methods section).

Figure 3a shows the change in ATCase rotation observed upon addition of small aliquots of a concentrated mixture of carbamyl phosphate and succinate, a nonreactive substrate analog that is bound to the enzyme. The rotation decreases with increasing substrate concentration, while the catalytic activity increases. The role of carbamyl phosphate is important to note here, in that carbamyl phosphate is required for the rotatory change, yet causes no change by itself (within experimental error). Perhaps carbamyl phosphate is necessary for the binding of succinate. Figure 3b shows the increase in rotation of ATCase as the CTP concentration increases. The catalytic activity decreases with increasing CTP concentration. The effect of added protons on the enzyme shows increased rotation (Table I) with decreased activity (Fig. 1a).

All measurements consistently show an inverse correlation between the magnitude of the rotation and the catalytic activity of ATCase.

The changes in rotation clearly show that there is a change in the relationships of amide residues in the protein accompanying changes in the catalytic activity. Figure 4 shows the maximum change in the ORD of ATCase caused by the addition of substrate analog. The shape of the difference ORD curve is very close to that of the polypeptide helix, indicating that, on the face of it, some residues of the protein have undergone a coil-to-helix transition. The noise level is relatively high in the region of 200 mμ and below, and it is difficult to follow the details of the change; however, it is clear that the maximum change in this region relative to the maximum change at 233 mμ is consistent with a change in helix content.

Care must be taken in assigning such small changes to any particular structural alteration, and we must examine alternative explanations carefully.

(1) Small molecules can acquire optical activity when bound to an optically active large molecule[18,19]. If the ORD changes observed were due to induced optical activity in the bound effectors, specific rotations in excess of 10^6 degrees/mole of bound substrate analog would be required to explain the observed change in rotation (even if as many as 10 substrate analogs/mole of enzyme were bound). Specific rotations of this magnitude are much larger than can reasonably be expected. Furthermore, induced Cotton effects will be centered very close to the absorption maxima of the bound molecules. CTP has a weak ORD

spectrum with a Cotton effect centered at 270 mμ. An induced Cotton effect in CTP would show up in the 270 mμ region and could not give rise to an ORD change with the wavelength dependence observed. Substrate analogs, succinate and malate, have somewhat different absorption spectra and both produce an ORD spectrum change of the same shape. Since the same ORD changes are caused by substances with different absorption spectra (CTP, succinate, malate) we can rule out Cotton effects induced in the bound molecules as the source of the changes.

(2) Effector-induced alterations in the nonhelical regions of the protein structure could change the charge and dipole environment around the helix and give rise to an altered helix ORD. Changes in the charge and dipole fields would be expected to have the largest effect on the helix ORD in the electronically forbidden $n - \pi^*$ region around 225 mμ[9,10,14] and to be less important in the electronically allowed $N - V$ peptide bond transition around 190 mμ[10,14]. It is very unlikely that alterations in the static fields would produce the same percentage change in the ORD at 200 mμ and at 233 mμ as is observed. We can conclude with some confidence that the change in ORD is not due to alterations in the nonhelical regions of the protein. (The nonhelical regions may well change under the influence of the allosteric effectors, but this is not the source of the ORD changes we observe.)

(3) In principle, one other possible explanation for a change in ORD with the wavelength dependence of the helix-coil type is a change in the local refractive index due to swelling or shrinking (change in internal hydration), thereby altering the local electric field of the

light inside the protein and leading to an altered ORD amplitude. If we assume a Lorentz local field, a very large change in refractive increment for the protein (of the order of 6% maximum) would be required to explain the observed effect. A change in refractive increment of this magnitude is extremely unlikely.

In summary, the addition of allosteric effectors causes a physical change in the protein structure. The interrelationships of the amide residues clearly change, and, although static field and hydration effects are not strictly excluded, the allosteric transition is most probably accompanied by a change in helix content of the enzyme.

ATCase appears to be composed of six subunits, four regulatory and two catalytic, the interaction of which controls the catalytic activity[20,21]. The helix-coil transition that has been found could change the geometry of the subunits and thereby affect the subunit interaction. It has been observed that subsgrate analogs induce a small change in the sedimentation coefficient of ATCase[17]. The magnitude of the change in ORD and sedimentation are both small. The molecular weight of ATCase[21] is about 3×10^5, which corresponds to about 2.6×10^3 residues, approximately 8.3×10^2 (37%) of which are in a helical environment. The number of residues involved in the allosteric helix-coil transition can be estimated to be about 40-50 residues from the maximum change in rotation of about 5-6%. This small alteration of the enzyme structure occurs during a change of substrate affinity of two orders of magnitude or more. It is not surprising, however, that small alterations of enzyme structure could lead to large changes in activity by affecting the shape, polar environment, or charge on the catalytic site. The allosteric enzyme acts like

a biochemical amplifier[22] in that a small signal causes a large effect.

The estimate of the number of residues involved is a lower limit. The net observed effect could be the result of opposite transitions, one helix-to-coil and the other coil-to-helix, the difference in the number of residues would be seen by our experiments. It is also possible that a large number of residues modify their helix dimensions as compared to a smaller number undergoing a complete helix-to-coil transition. The former point of view is unfavorable because helix-coil transitions tend to be cooperative.

The model for allosteric proteins put forward by Monod et al.[2] supposes that there are two forms of the enzyme in equilibrium. The two forms are assumed to have different catalytic activities and that the binding of substrate or inhibitor shifts the equilibrium between the two forms. The model predicts that the physical effect of the inhibitor is equivalent but opposite to that of the activator. Another possibilitiy, contrary to the model under consideration, is that the inhibitor and activator cause the same kind of physical change but do so in different parts of the molecule. Our results show that the change occurring upon addition of inhibitor is opposite (and approximately equal to) the effect of activator. In terms of Monod, Wyman and Changeux's model, the ORD evidence would imply that the catalytically more active form is the less helical form.

An investigation of the responses of the separated regulatory and catalytic subunits[21] to the allosteric effectors would be desirable to separate their rotatory contributions. Correlation of the binding of

effectors with the physical change produced by the effectors is central to the understanding of the mechanism of this enzyme.

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Table I

Values of specific residue rotation of ATCase at 233 mμ in various solutions from a typical experiment. The Δ values are the differences caused by solution alteration. All experiments show the same trends of effector-induced changes in the rotation. The errors shown in the table are the observed noise level in the ORD spectrum.

Sample	$[R']_{233\ m\mu}$		
pH = 6.1	5,240 ± 20	Δ = 90	Δ = 350
pH = 6.1 + 10^{-4} M CTP	5,330 ± 20		
pH = 8.5	5,590 ± 20	Δ = 220	
pH = 8.5 + 5 mM succinate and 3 mM carb. PO_4	5,380 ± 20		

Figure Captions

Fig. 1a. The effect of CTP and pH on the reaction velocity substrate dependence of ATCase. 3.6 mM carbamyl phosphate and 40 mM buffer present in all samples. pH 6.1, ——; pH 7.0 or pH 6.1 plus $2 \cdot 10^{-4}$ M CTP, - - - -; pH 8.6, — · — · . All of the curves (except the pH 6.1 plus $2 \cdot 10^{-4}$ M CTP curve) are taken from Ref. 3. The arrows on the graph are explained in the text.

Fig. 1b. The effect of the substrate analog, maleate, on the reaction velocity of ATCase. 1 mM aspartate, 3.6 mM carbamyl phosphate, and 40 mM potassium phosphate; pH 7. (Curve taken from Ref. 3).

Fig. 2. Optical rotatory dispersion (ORD) and absorption.

a) ORD of Poly-L-Glutamic Acid, pH 4.3 (no added salt), helix form.

b) ORD of Poly-L-Glutamic Acid, pH 7, random coil form.

c) ORD of ATCase, pH 6.1; the average residue weight is taken as 120 g/mole. The dotted portion of the curve is in a region of relatively high noise level and is less reliable than the other data.

d) Absorption of ATCase, 2.08 mg/ml, pH 6.1 in a 0.1 mm cell measured vs. buffer.

Fig. 3. The dependence of the rotation of ATCase, at the trough of the 233 mμ Cotton effect, on the concentration of CTP and substrate analog, succinate.

a) Enzyme (1.75 mg./ml.) at pH 8.6, and aliquots of a mixture of 150 mM potassium succinate and 130 mM carbamyl phosphate (pH 8.6) added.

b) Enzyme (2.08 mg./ml.) at pH 6.1, and aliquots of 20 mM CTP (pH 6.1) added.

Figure Captions (Cont.)

Fig. 4. The change in specific residue rotation of ATCase at pH 8.6 brought about by 4 mM potassium succinate and 4 mM carbamyl phosphate. The difference is presented as (enzyme + effector)-(free enzyme).

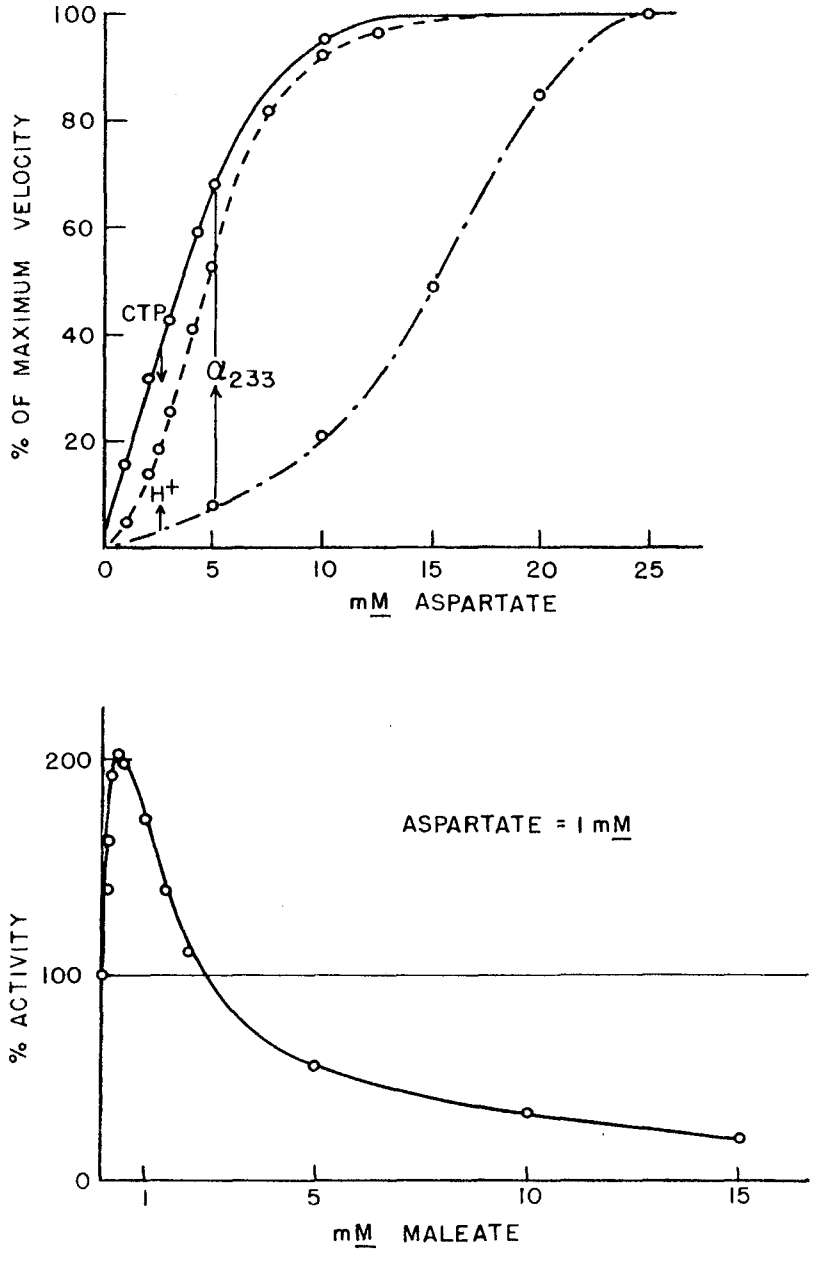

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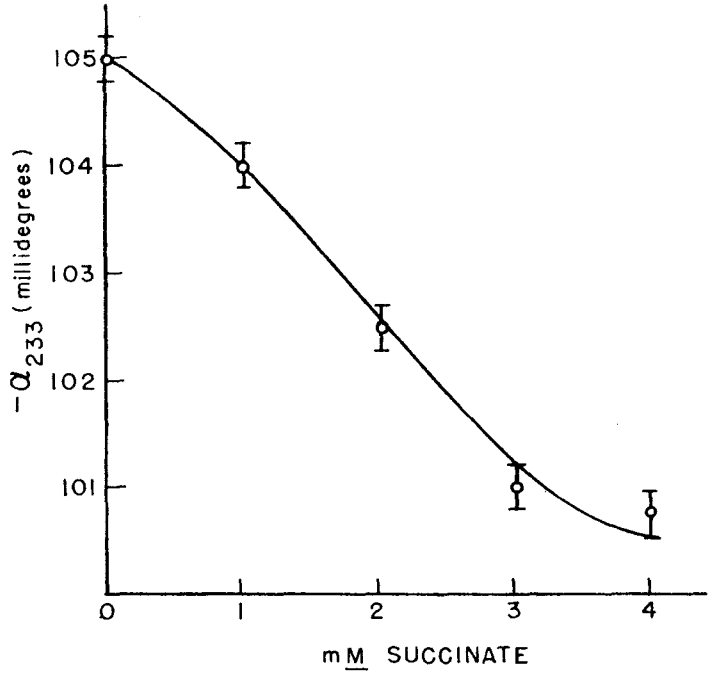
Fig. 1



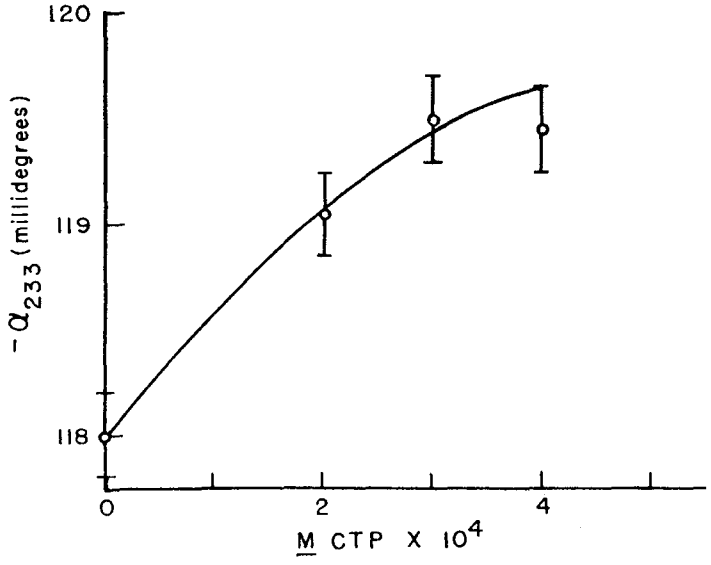


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Fig. 2

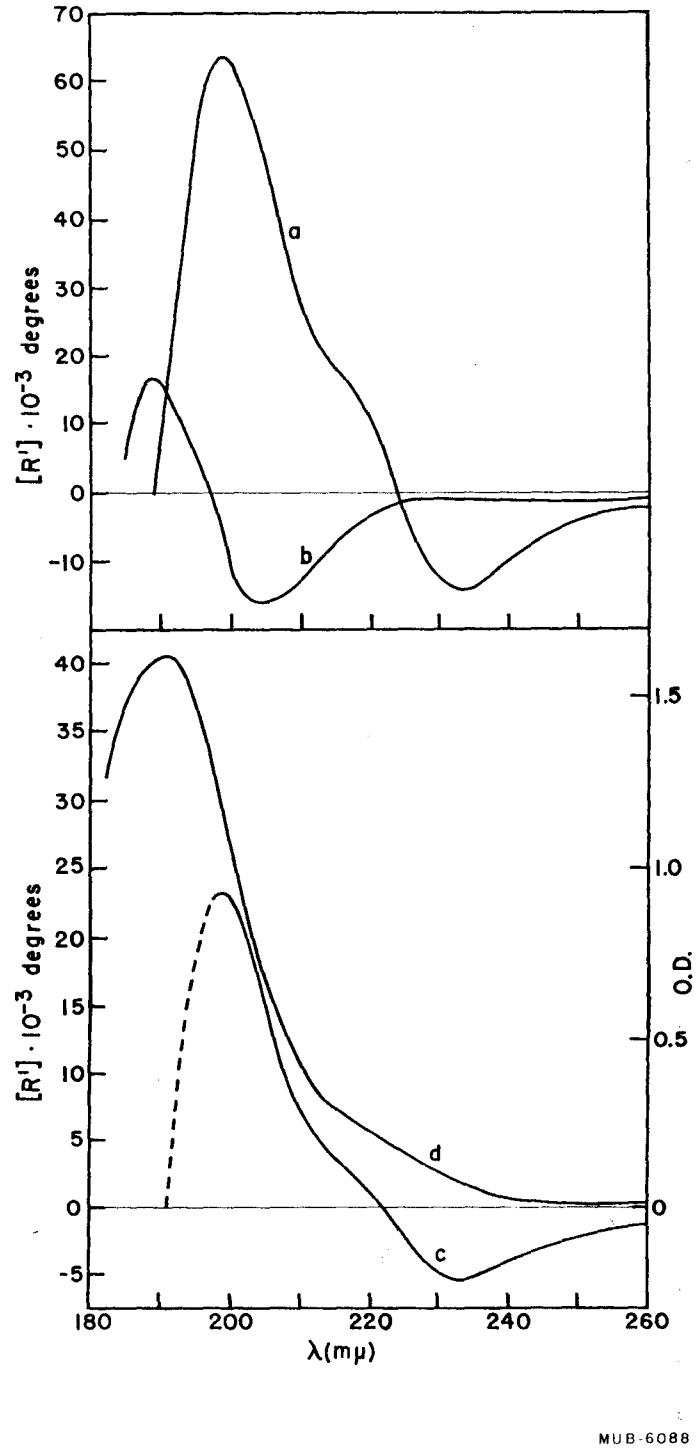
70
60
50
40
30
20
10
0
-10
[R'] · 10^-3 degrees
a
b
40
35
30
25
20
15
10
5
0
-5
[R'] · 10^-3 degrees
1.5
1.0
0.5
0
O.D.
d
c
180
200
220
240
260
λ(mμ)
MUB-6088

Fig. 3

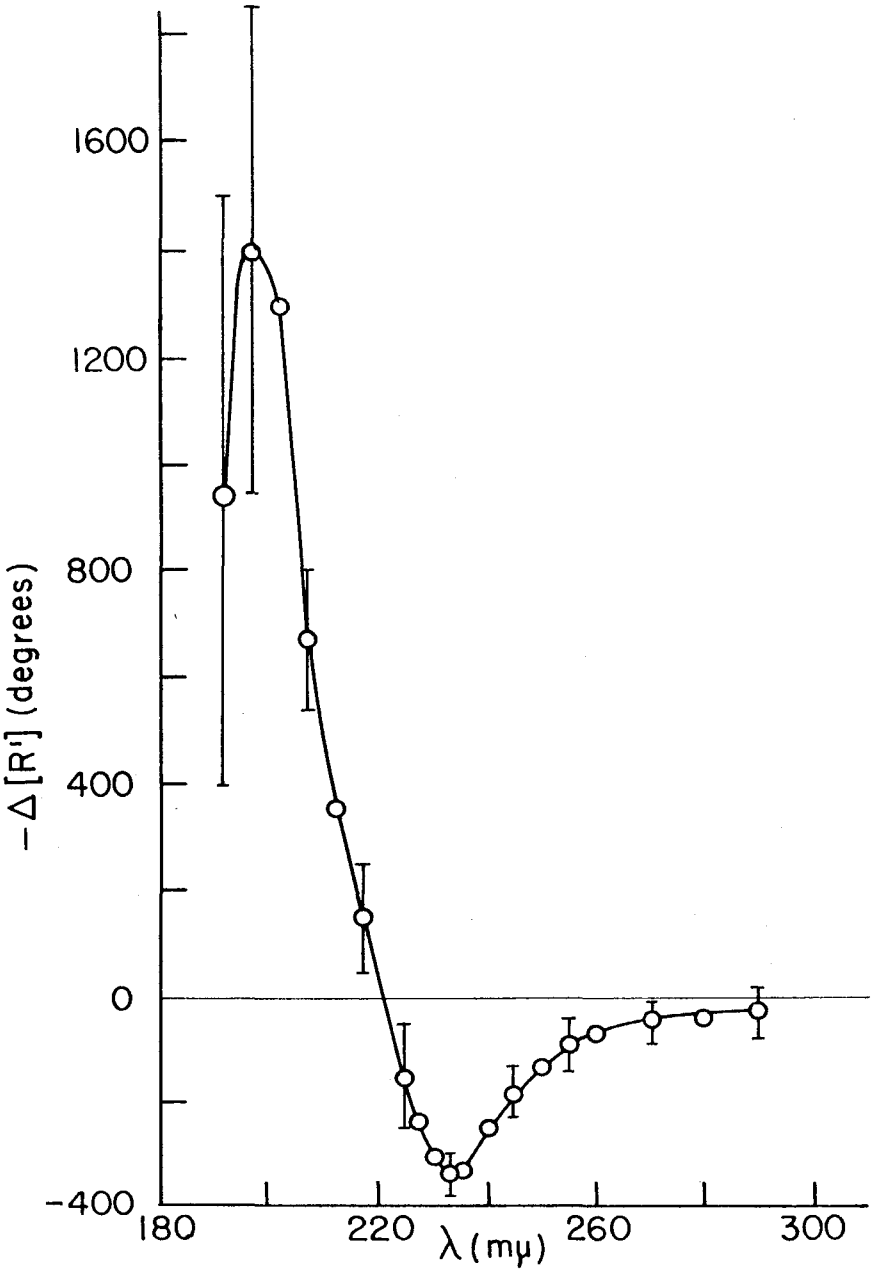

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Fig. 4

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