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# A CIRCULAR DICHROISM STUDY OF CONFORMATIONAL CHANGES INDUCED IN POLYPEPTIDES AND PROTEINS BY TEMPERATURE, ACIDS, SOLVENTS, AND SALTS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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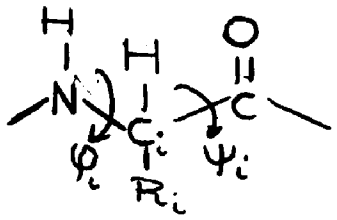
## Abbreviations

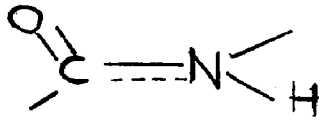
| | |
|---|---|
| $A_1$ | Eylar's basic protein |
| ATGEE | acetyl tetra glycine ethyl ester |
| C. A. T. | computer-of-average-transients |
| CD | circular dichroism |
| CE | chloroethanol |
| DMA | N, N-dimethylacetamide |
| DNA | deoxyribonucleic acid |
| EH | extended helix |
| GuHCl | guanidine hydrochloride |
| HFPD | 1,1,1,3,3,3,-hexa fluoropropan-2,2-diol |
| NMA | N-methylacetamide |
| PBLA | poly-$\gamma$- benzyl-L-asparate |
| PBLG | poly-$\gamma$-benzyl-L-glutamate |
| PL | poly-L-lysine |
| PMLG | poly-$\beta$-methyl-L-glutamate |
| PO | poly-L-ornithine |
| PP I | poly-L-proline, form I |
| PP II | poly-L-proline, form II |
| TFE | trifluoroethanol |
| TFA | trifluoroacetic acid |
| SDS | sodium dodecyl sulfate |
| $[\alpha]_\lambda$ | specific rotation at wave length $\lambda$. |
| $\Delta\varepsilon$ | difference in molar extinction for left circularly and right circularly polarized light |
| $[\eta]$ | intrinsic viscosity |
| $[\Theta]$ | ellipticity |
| $\varphi, \psi$ | torsion angles defining configuration in structrual unit of polypeptide chain |

# Chapter I · Introduction

## A. Macromolecular Conformations of Proteins.

A protein is a polypeptide. The structural unit may be taken as:



where $R_i$ is one of the sides chains of a naturally occurring L amino acid, and,  , the peptide unit, is planar and trans. This is based on knowledge of small peptide structures which are known from x-ray diffraction (Pauling, Corey, and Branson, 1951). An exception for the preference of trans over cis can occur when a carbon substitutent replaces the hydrogen atom on the nitrogen of the residue (e.g. a prolyl residue). An L-prolyl residue situated among residues of the kind —NH—CHR—CO— should be expected to occur preferentially in the trans form however (Flory, 1969).

The conformation of the polypeptide is defined by specifying each set of $\varphi_i$ and $\psi_i$ angles in successive structural units. In an ordered conformation the rotational angles about successive bonds have a definite pattern. Many globular proteins have an arrangement of the component amino acid residues which is quite complicated but nevertheless fixed and subject only to thermal vibrations. When the rotational angles have no definite repeat a disordered structure will result.

There are restrictions on the possible arrangements that a real molecule can take. The minimum free energy state

will result from the balance of several forces. It is disappointing, however, that models available at the present time do not predict that the native state should be the stable form for a given protein (Tanford, 1970). The main difficulty is connected with the fact that there are strong forces favoring the native state, and about equally strong forces favoring the denatured state.

Experimentally we are obtaining detailed knowledge of the crystalline structure of some native globular proteins from x-ray diffraction studies. Since the crystals are bathed in liquid in order to be stable, we can expect the structure of the molecules when in solution to be similar in most respects to that deduced from the solid state. Many modified proteins can also be studied by x-ray diffraction, but the disordered molecules cannot be so investigated. We must rely on solution methods.

Many different techniques can be used to study the molecule in solution. Some give information on the overall shape of the molecule [e.g. Simha's treatment of viscosity, Zimm's treatment of light scattering, measurement of the diffusion coefficient, concentration dependence of osmotic pressure, x-ray scattering and polarizability (see, Tanford, 1961)]. Other techniques give information at the interatomic level (e.g. infrared, Raman, nuclear magnetic resonance, u.v. absorption, difference spectra and fluorescence spectra). None of these techniques has the versatility or overall sensitivity of circular dichroism when the problem involves changes in the

optically active synthetic polypeptides and proteins. The companion method of optical rotatory dispersion is sometimes useful if the solvents employed are opaque in the spectral region of interest. The measurement of optical rotation, however, at one wave length only can be very misleading. Thus the same $\alpha_D$ can be obtained when poly-L-proline is partially in form I and partially in form II as when it is in the random form.

The overall shape of any long chain molecule will depend on the affinity of the segments of the macromolecule for one another, the affinity of the solvent molecules for each other, and the affinity of the chain segments for the solvent molecules. Tanford (1957) has discussed the three extreme situations which may arise. Random coils result when many possible conformations have the same energy; then many spatial conformations occur with equal probability. Helical rods will occur if a specific attraction between a particular part of each segment of a chain molecule for another specific part of another segment is the predominant attractive force. The third form is the compact, rigid, impenetrable sphere shape that can result when there are some kinds of segments in the molecule that have a strong segment-segment interaction, while other segments have a stronger segment-solvent interaction.

Minimum potential energy of a globular protein is mainly achieved by adopting a compact configuration in which hydrophobic groups are placed in the interior, and polar groups are placed in contact with the surrounding aqueous solvent. Effective hydrophobic interactions and electrostatic interactions

both require close contact between the interacting groups. The antipathy for partial packing accounts for the "all or none" character in the transition from the native state of a globular protein to a more open structure, the so-called "random" state.

Synthetic poly-L-peptides may be used as models to study the mechanisms involved in stabilizing the extended and helical states of proteins, but they will have to have the exact sequence of hydrophobic and hydrophilic amino acid residues as a globular protein to be useful in understanding the compact arrangements.

B. Configurations of Synthetic Polypeptides

Early work on the structure of synthetic polypeptides centered around the use of x-ray diffraction and infrared spectroscopy of films and fibers (Ambrose and Elliott, 1951; Bamford, Hanby and Happey, 1951). About the same time Pauling and coworkers (Pauling, Corey and Branson, 1951; Pauling and Corey, 1951) were proving the planarity of the peptide unit and were developing the theory of the alpha helix and the beta pleated sheet as preferred forms of the polypeptide. From 1954 to 1957 Doty, Blout and coworkers began the study of the synthetic polypeptide conformations in solution. Optical rotatory dispersion, sedimentation, viscosity, light scattering and infrared were combined as tools. Polybenzyl-L-glutamate (PBLG) was found to undergo a transition by changing the solvent. A rigid rod (radius of gyration proportional to molecular weight and with length per residue of 1.5 Å) defined the structure

when the solvent was chloroform plus 0.5% formamide. This confirmed that the helical state found in the solid persists in solution. When the solvent was more polar, such as dichloroacetic acid, the conformation was taken to be a solvated random coil. The basis for this decision was the slope of a plot of the log of specific viscosity vs the log of the molecular weight. The value of the slope was 0.85 (Doty, Bradbury and Holtzer, 1956). This value is typical of a randomly coiled polymer having a rather extended average configuration which is usually caused by considerable steric hindrance to rotation or by a high degree of solvation (Tanford, 1961). Strong solvation is expected in the acid. In the case of gelatin, in water, a more contracted average configuration should, and is, found by the viscosity chain length relationship (Boedtker and Doty, 1954) . Due to strong absorption by the acid, the infrared spectra could not be recorded in the region of 1650 $cm^{-1}$. With poly-L-glutamic acid (PGA), a transition could be followed by infrared using $D_2O$- dioxane as the solvent. The conformational change could be obtained from partially "random" to completely "random" by raising the temperature from 25° to 90° in a solution made by mixing 0.2 M NaCl in $D_2O$ with dioxane, (2:1), at pH 6.2 (Doty et al., 1957). Two bands appear in the 1650 $cm^{-1}$ region. One occurs at 1645 $cm^{-1}$ at 25° and shifts to 1650 $cm^{-1}$ and weakens as the temperature is raised. It is attributed to a weakened C–O..D–N vibration in the portion of the sample still having the helical configuration. The stronger band remains constant in position at 1664 $cm^{-1}$,

and increases in intensity with temperature. Solvated forms of model amides (Richards and Thompson, 1947) and low molecular weight synthetic polypeptides (Blout and Asadourian, 1965) absorb in the 1670-1680 $cm^{-1}$ region. A 15 $cm^{-1}$ decrease is expected in this frequency upon deuteration since deuteration lowers the amide I of poly- DL- alanine by this amount (Elliott, 1954). Therefore the 1664 $cm^{-1}$ band was assigned to a solvated nonhelical form of PGA.

C. Circular Dichroism of Polypeptides.

Circular dichroism (CD) studies on polypeptides were first reported by Holtzwarth, Gratzer and Doty (1962). In the last few years there has been extensive use of CD as equipment has become available. For reviews see, for example: Beychok (1967), Bovey (1968, 1969), Timasheff et al.(1967), Urry (1968) and Tinoco and Cantor (1970).

The CD of a right-handed helical L-amino acid polypeptide can be accepted as well documented. It consists of negative bands at 222 nm and 208 nm, and a positive band at 192 nm. There is some variation in the relative intensities of these bands with solvent: in alcohols, the 208 nm band is deeper than the 222 nm band, but the reverse is true in water (Quadrifoglio and Urry, 1968a).

The left-handed $\alpha$ helix of poly-L-amino acids has been found to occur in poly-$\beta$-methyl-L-aspartate and in poly-$\beta$-benzyl-L-aspartate. The CD pattern is given in Urry's review (1968). A slightly different spectrum is reported in this thesis (see Fig.7-14 a). Since the left-handed helix is not

the mirror image of the right-handed helix, neither is the CD. (The left-handed $\alpha$ helix of the D-amino acid polymer is the mirror image of the right-handed $\alpha$ helix of the L-amino polymer.) Calculations in terms of torsional, nonbonded, electrostatic and hydrogen-bonding energies predict the change in helix sense for the two aspartate derivatives (Ooi, et al. 1967). By varying the effective radii of the various atoms, especially hydrogen atoms, Lotan, et al.(1969) have been able to explain the change in preferred sense of several polyaminoacid helices from right- to left-handed as the temperature is increased.

The CD spectra of polypeptides in the beta configuration show a single negative band at 217 nm in silk and in poly-L-lysine and 222 nm in poly-L-serine, and a single positive band at 197 nm. Temperature and solvent effects change the relative and absolute magnitudes of the observed bands, (e.g. poly-L-serine, Quadrifoglio and Urry, 1968b). Also the similarity of computed CD spectra for paralled and antiparallel pleated sheets makes it difficult to assign the experimental beta structures (Pysh, 1970).

The early 1962 work on the CD of ionized poly-L-glutamic acid (PGA) showed a strong negative rotatory band at 212 nm (Holtzwarth et al., 1962). By 1965, improved technique showed a small positive band near 220 nm, in addition to the large negative band (Holtzwarth and Doty, 1965; Velluz and Legrand, 1965). Later, a third band near 235 nm and negative in sign was found by Carver, et al.(1966). The origin of the bands was said not to be elucidated, but the bands were accepted to represent the random state.

Stevens, et al. (1968) accepted the above analysis for synthetic polypeptides, but suggested that perhaps it does not hold for globular "random" proteins since they cannot undergo the structural fluctuations of a random coil in solution. They considered as a better model the CD spectrum of a dried down film of ionized PGA. This suggestion is repeated in a recent paper from the same laboratory (Fasman, et al., 1970). Strangely they relate kinetic fluctuations to the "strong sharp signals" in ionized PGA solutions.

Using optical rotatory dispersion, Rosenheck (1968) decided that the action of $H_2SO_4$ on polypeptides results in disordered structures. Since then Steigman, et al. (1969), Peggion, et al. (1970) and Balasubramanian and Roche (1971) have discussed the CD of several polypeptides in $H_2SO_4$ and in methanesulfonic acid, $CH_3SO_3H$. The spectra have been reported with a small positive band in the 220nm region and a larger negative band near 200 nm. It has been accepted by all the above authors that the chains are in a random coil configuration.

A similiar CD spectrum to that of ionized PGA has been reported for poly-[$N^5$-(2-hydroxy-ethyl)-L-glutamine] in water (Adler et al., 1968). Likewise, several polypeptides in the gem-diols show a small 220 nm band and a larger negative 200 nm band (Balasubramanian and Roche, 1971).

Poly-L-proline is known to exist in two distinct forms in both the solid state and in solution. In water and organic acids it stabilizes in a levorotatory form, designated as

PP II. As normally prepared by polymerization of the N-carboxy anhydride and precipitation from pyridine, it exists in a different form called PP I, which is dextrorotatory at 589 nm. This form is stable in aliphatic alcohols but unstable in water and organic acids. From x-ray work it was found that Form I is a right-handed helix with a residue translation of 1.85 Å and with the peptide bonds in the cis conformation (Traub and Shmueli, 1963). Form II, on the other hand, is left-handed and has a residue repeat of 3.12 Å and has the peptide bonds in the trans conformation (Cowan and McGavin, 1955). The CD of PP II was first given by Carver et al. (1966), and the transformation of I into II in trifluoroethanol was reported by Bovey and Hood (1967). Transition in water, of a low molecular weight sample, from Form I to II was given by Tiffany and Krimm (1968b).

Traub and Yonath (1967) described two structures for $(Gly\text{-}Pro\text{-}Ala)_n$ in the solid state: a collagen type supercoiled triple helix and, in films grown from aqueous solution, a non-supercoiled structure of three helices similiar to the collagen I and II structures of Rich and Crick (1961). Brown et al. (1969) have now done the CD of this polytripeptide. At $-112^0$ in ethylene glycol-water solution (2:1, v/v) the CD is similiar to collagen. The structure transforms, without a sharp melting point, to give a CD similiar to denatured collagen. The lack of a sharp melting point may be related to the low molecular weight of the sample (14,500 ± 500) compared to collagen of molecular weight 300,000.

Rosenheck and Sommer (1967) have questioned the belief that the fully ionized polypeptide chain can be representative of a random-coil state and have suggested that the negative CD band can be interpreted as arising from the lower energy exciton band of a single extended-chain conformation. According to their calculations the two exciton components of this structure should be located at about 203 and 179 nm.

D. Statement of the Problem

It is clear that, while the CD spectra of certain regular structures (e.g. $\alpha$ helix, $\beta$ chain, polyprolines I and II, collagen) are well-defined, no clear-cut assignment of the CD of a "random" polypeptide chain has been made by previous workers. In fact, the correlation of a single spectrum with a "random coil" reveals the restricted conception which has existed of the possible range of unordered structures.

It is the goal of this thesis to establish the form of the CD spectrum to be associated with unordered polypeptide chain conformations. We will show that the previously assigned spectrum is incorrect. Furthermore the details of the CD are expected to be dependent on the disordering agent, and this factor will be discussed in some detail for agents such as temperature, pH, salts, alcohols, urea, guanidine hydrochloride and strong acids.

Generalizations will be attempted from recording the ways in which different proteins and synthetic polypeptides respond, and we will look for correlations in terms of known structures.

# Chapter 2 Experimental Methods and Materials.

## A. Circular Dichroism

The electric field of a plane polarized light wave may be represented by a vector oscillating in a plane as a function of time. At any given time, the vector may be considered to be the resultant of two vectors of equal length, $E_L$ and $E_R$, rotating in opposite directions about the axis of propagation of the wave with a frequency $\nu$. Thus a plane polarized wave may be considered as two circularly polarized waves, left- and right-handed, whose planes of polarization rotate continuously in opposite sense about the axis of propagation of the wave.

In the vicinity of an absorption band it is possible that these components are not equally absorbed. As a result, $E_L$ and $E_R$ will no longer be of equal length, and their resultant will now trace out an ellipse rather than a plane. The resultant light is said to be elliptically polarized. The absorption of one circularly polarized wave in preference to the other is called circular dichroism.

## B. Parameters Uses to Describe Circular Dichroism

The intensity of light of each polarized component after passing through a solution can be given in two forms:

$$I = I_0\, 10^{-\varepsilon_i c d} = I_0\, e^{-4\pi k \frac{d}{\lambda}}$$

where $\varepsilon_i$ = molar extinction coefficient, c = concentration in gram moles (or mean residue weight in gram moles per l in the case of proteins and polypeptides), $\lambda$ = wave length

in cm, d = path length in cm and $k_i$ = index of extinction ( i = L for left-handed rotating light, i = R for right-handed rotating light).

thus $\Delta\varepsilon = \varepsilon_L - \varepsilon_R = [4\pi(k_L - k_R) \log e] / c\lambda$

The ellipticity of the wave which results after passing through the sample is defined as the angle ($\varphi$) whose tangent is the ratio of the minor to the major axis of the ellipse of the E vector that is traced out in time.

Let the two component amplitudes of the left and right E vectors be:

$$E_L = E_0\, e^{-2\pi k_L\, d/\lambda} \qquad \text{and } E_R = E_0\, e^{-2\pi k_R d/\lambda}$$

Then the major axis is $E_L + E_R$, and the minor axis is $E_L - E_R$, and the ellipticity is:

$$\tan\varphi = \frac{E_L - E_R}{E_L + E_R} = \frac{e^{-2\pi k_L d/\lambda} - e^{-2\pi k_R d/\lambda}}{e^{-2\pi k_L d/\lambda} + e^{-2\pi k_R d/\lambda}} = \tanh \pi(k_L - k_R)\, d/\lambda$$

Since $\varphi$ is a small angle, to a good approximation:

$\varphi = \pi(k_L - k_R)d/\lambda$ in radians, or $180\,(k_L - k_R)d/\lambda$ in degrees. Molar ellipticity is defined by:

$$[\theta] = \frac{100\,\varphi}{c\,d} = \frac{(100)\,(180)\,(k_L - k_R)}{c\,\lambda}$$

therefore

$$[\theta] = \frac{(100)\,(180)\,\Delta\varepsilon}{4 \log e\, \pi} = 3300\,\Delta\varepsilon$$

**(The units of $[\theta]$ are reported in deg. cm$^2$/ decimole to conform with the literature. This is achieved through the conversion factor 100 in the above equations.)**

Experimentally, circular dichroism spectra were recorded in this laboratory using a Durrum-Jasco Model 5 Optical Rotatory Dispersion Recorder, with CD attachment. Our machine has been increased in sensitivity by Sproul Scientific Co. The deflection, in centimeters, measured from a base line (given by cell plus solvent) gives, when multiplied by a scale factor, the optical density difference (O.D.) between the left and right circularly polarized components at a given wave length.

Thus $\Delta\varepsilon = (O.D.)/cd$

and $[\theta] = 3300\,\Delta\varepsilon$

Jacketed temperature cells 0.1 mm, 1 mm and 5 mm (Opticel, Bellsville, Md.) were used for most of the experiments.

Mean residue weights were used from analysis in the literature. If unknown, 115 was used for proteins. Most concentrations were determined from the dry weight of samples used. If lypholized, 10% of the dry weight was assumed to be moisture. In all cases where absorption coefficients of an aromatic band were known, absorption was used to calculate the concentration of protein.

C. <u>Basis for the Use of Circular Dichroism to Study Conformation</u>

The interaction of light with a molecule effects a momentary change in distribution of charges, i.e., a change in the relative positions of positive nuclei and electrons. If the molecule is dissymmetric and contains neither a plane nor a center of symmetry, it interacts preferentially with one of

the circularly polarized components of a plane polarized light wave. After going through the sample, the resultant light has its plane of polarization rotated. The measurement of the angle of rotation when plotted as a function of wave length is the optical rotatory dispersion curve (ORD), while the difference absorbance of the circularly polarized beams results in a circular dichroism curve (CD). To measure CD, incident linear polarized light may be converted first to left and then to right circularly polarized light by making use of the Pockels effect. In this effect, a Z-cut uniaxial crystal becomes biaxial when it is placed in an electric field. Thus there is a certain voltage that will make the crystal act as a quarter-wave plate for a given wave length. This voltage is approximately a linear function of the wave-length. By applying an ac electric field of the proper voltage to the crystal, the CD of a sample will be directly related to the ac component of the current recorded by a photomultiplier in the detector.

Two factors are necessary to produce a CD band: a chromophore and a source of asymmetry. Asymmetric chromophores in the normally used wave length region are rare. Hexahelicene is the best known example. Usually the chromophores have high symmetry, but become optically active by interaction with an asymmetric group. An example of a symmetric chromophore (one that has a mirror image) is the planar peptide moiety in poly-L-alanine. Here, optical activity is provided by the dissymmetrically placed neighboring groups; i.e., the methyl, the CH, and other peptide groups in the polypeptide.

The quantum mechanics of optical activity was first published by Rosenfeld (1928). He showed that the rotational strength of any molecule depends on the matrix elements of the electric and magnetic dipole moment operators. For a transition from the state o to the state a the rotational strength was shown to be:

$$R_{oa} = \mathrm{Im}\, \underset{\sim}{\mu}_{oa} \cdot \underset{\sim}{m}_{ao}$$

Where Im means the imaginary part,

and $$\underset{\sim}{\mu}_{oa} = e\int \psi_o \sum_i \underset{\sim}{r}_i \psi_a^* \, d\tau$$

$$\underset{\sim}{m}_{ao} = \frac{e}{2mc}\int \psi_a \sum_i (\underset{\sim}{r}_i \times \underset{\sim}{p}_i)\, \psi_o^* \, d\tau$$

where $\psi_o$, $\psi_a$ are the electronic wave functions for the molecule in the state o and the state a, $\underset{\sim}{r}$ and $\underset{\sim}{p}$ are the position and momentum vectors of electron i, m is the mass, and e is the charge of the electron.

Experimentally, R for each resolved band of the CD spectrum is:

$$R = (22.98 \times 10^{-40}) \int_{\text{band}} (\varepsilon_L - \varepsilon_R)/\lambda \, d\lambda$$

$(\varepsilon_L - \varepsilon_R)$ in liters/mole cm, and $\lambda$ in cm (Moffitt and Moscowitz, 1959).

A general expression for the rotational strength of an electronic transition has been given and discussed by Tinoco (1962). In a polymer of identical residues, the contributions to the rotational strength arise from three types of terms. An exciton contribution arises from the resonant interaction of the collection of degenerate energy levels. A coupled

oscillator contribution arises from the simultaneous motion of electrons in two interacting groups, and a one electron contribution from interactions within a group in the static field of the other groups.

An extensive calculation of the rotatory properties of an $\alpha$ helix and $3_{10}$ helix of right-handed and left-handed poly-L-alanine was made by Woody and Tinoco (1967) and extended by Woody (1968). Recent calculations by Pysh (1970) include the $\alpha$ helix, poly-L-proline helices, and parallel and anti-parallel pleated sheets. Another paper by Aebersold and Pysh (1970) calculates optical properties, including CD, of amorphous polypeptides. Theoretical results on poly-L-proline and on amorphous polypeptides are poor, if one compares the calculated curves to the ones taken as experimentally representing the states. One difficulty is that rotational strengths of bands can show significant dependence as $\varphi$ and $\psi$ are varied by only a few degrees (Vournakis et al., 1968). Another, is that the effect of solvents has not been considered.

It must be concluded, therefore, that while theoretical calculations show the dependence of CD on chain conformation, and have given reasonable agreement for some structures, no fully consistent theory yet exists. The problem of correlating conformation with CD spectrum is further complicated by the non-uniqueness which must presently be assumed; that is it seems possible that more than one structure can give rise to the same spectrum.

D. Materials.

Most of the polypeptides and proteins used in this thesis were obtained commercially. Exceptions are $A_1$ protein which was given by Dr Eylar, of Salk. Feather keratin was obtained from sea-gull feathers collected at the seashore by the author and by Professor Krimm's father. Collagen was obtained from the tails of young rats that were used in other experiments by others in the laboratory. Silk protein was prepared from some silk net found at home.

Bovine serum albumin, Sigma (MRW 107), $E^{1\%}_{1\ cm} = 6.67_{278nm}$

Deoxyribonuclease I, bovine, salt free, type II, 6x recryst., Sigma.

Chymotrypsinogen A, bovine, salt free, 6 x recrystallized (MRW 105), Sigma.

Chymotrypsin, salt free, bovine, cryst. and lyophilized, $E^{1\%}_{1\ cm} = 20_{280\ nm}$.

Deoxyribonuclease I, bovine, 1 x cryst. and lyophilized, Sigma.

Insulin, crystalline, (MRW 113), $E^{1\%}_{1\ cm} = 10.4_{277\ nm}$, Sigma.

Lactoglobulin, (MRW 113), $E^{1\%}_{1\ cm} = 9,6_{278\ nm}$, Sigma.

Lysozyme, 3 x cryst., dialized and lyophilized, (MRW 111) $E^{1\%}_{1\ cm} = 26.9_{280\ nm}$, Sigma.

Myoglobin, sperm whale, (MRW 112), $E^{1\%}_{1\ cm} = 100_{409\ nm}$, CAB.

Ovalbumin, (MRW = 115).

Pepsin, 2 x crystallized and lyophilized, Sigma.

Ribonuclease A, bovine, 5 x crystallized, salt free, (MRW 110), $E^{1\%}_{1\ cm} = 7.16$ 277.6 nm , Sigma.

Soybean Trypsin Inhibitor, crystallized, (MRW 110), $E^{1\%}_{1\ cm} = 10.1$ at 280 nm.

All polypeptides were obtained from Pilot except samples of poly-L-proline which were obtained from Sigma, and Miles.

To prepare feather keratin, feathers were washed and the medulla removed from the rachis. The rachis was then dissolved in 10 M urea - 0.1 M $NaHSO_3$ at room temperature or 40° for 24 hours. This was followed by dialysis against several changes of distilled water. If an oxidized sample was wanted, cleaned rachis was treated with 2 % peracetic acid and then removed and dissolved in dilute $NH_4OH$ and then the solution was dialized against distilled water. MRW was taken as 101, and $E^{1\%}_{1\ cm} = 7_{277nm}$

Collagen was prepared from fat tail tendons by dissolving in 2 % acetic acid. MRW was taken as 100.

Silk net was dissolved in conc. LiBr and then dialized; MRW 73, $E^{1\%}_{1cm} = 11.3$ 280 nm .

## Chapter 3 Preliminary Experiments

The first experiments using CD in this laboratory turned out to be the basis on which this thesis is built.

### A. Feather Keratin

Prior to starting CD experiments, several years work was done by the author on modifying the x-ray diffraction pattern of feather keratin in order to get indications of where S-S bonds reside in the native fibrous state (Tiffany and Krimm, 1969b). The uniqueness of the diffraction pattern (Astbury, 1931) is an indication of the complexity of the native fibrous structure. The interesting question was asked "Is there any special structure to the molecule in solution?". Earlier ORD work in the near uv and visible region of the spectrum had indicated that the structure was random (Westover, Krimm and Tiffany, 1962). This had been confirmed by Harrap and Woods (1964). CD was not much more revealing (Tiffany and Krimm, 1969a). Many different experimental conditions for getting the molecule into solution were tried (e.g. oxidation of the inter S-S bonds by peracetic acid, followed by solution in dilute $NH_4OH$, or reduction of the S-S bonds in urea-bisulfite solutions). None of the methods gave any CD dichroism pattern other than the uninteresting monotonically decreasing curve with a negative maximum in the 200 nm region (Fig. 3-1). Films from water solution do have the typical $\beta$ pattern with the negative peak at 217 nm. X-ray diffraction patterns of dried samples differ markedly from the native protein however (Rougvie and Burke, 1967). Recent work (this thesis, fig. 3-2) indicates that some local

order can develop even though the main part of the molecule is disordered. Thus in fig. 3-2, we observe a negative CD band in the near uv at 275 nm. The particular sample of oxidized feather keratin had been prepared several weeks before this spectrum was run. No band was present in this region initially on running the sample immediately after 24 hour dialysis to remove the $NH_4OH$ used to dissolve the peracetic oxidized feather. We will see a similiar development of dichroism with time at 275 nm in some proteins (fig.6-18) following fluoroethanol and ethanol treatment. Fig. 3-2 shows that 5 M urea increased the intensity of the band in feather keratin, but on raising the temperature to $50^{o}$ the band weakened, and in water completely disappeared. The band may be due to S-S bond reformation, or to tyrosines that somehow have settled in an assymetric environment.

B. Collagen

Denatured collagen would seem a likely example of a randomly coiled protein. X-ray diffraction studies of the native fibrous collagen have led to the interpretation of the structure as a three-fold left-hand triple helix with a slight super twist existing between the three strands. It is not certain that the coiled coil of the solid form is carried over into solution. For a review, see Carver and Blout (1968). The CD of collagen in solution was first reported by Tiffany and Krimm (1967). The native form was found to have a CD spectrum very similiar to that of poly-L-proline II (PP II), only shifted 6 nm to the blue. The relative intensity of the

two bands in the different systems is, to a first approximation, the same. The shift in position of the CD bands corresponds to a similiar shift in the absorption bands from which the dichroism bands arise. The relative intensities of the bands of collagen compared to PP II are now in dispute, one laboratory quoting PP II as the more intense (Timasheff, et al., 1967) and another laboratory quoting just the reverse (Brown, et al., 1969). The methods used to determine concentration may be at fault. Madison and Schellman (1970) found ichthyocol collagen to have the same dichroism as the guinea pig skin collagen that was reported by Brown et al. (1969). A higher dichroism for collagen may be a result of the more rigid structure. Pysh (1967) has made theoretical calculations on poly-L-prolines I and II as well as collagen. He thinks the similarity of the dichroism of collagen and PP II is accidental and assigns the small positive band in PP II as a $\pi\pi^*$ while the small positive band in collagen is thought to be an $n\pi^*$. We, however, find similar solvent shifts for the two bands: when alcohols are substituted for water both positive bands shift to the blue. In PP II the intensity doubles when the solvent is 90% alcohol and 10% water over that found in water alone. The collagen band increases in intensity only slightly. This may imply that it is much more difficult to increase the dichroism of a structure that is already rigid compared to the possibility of stiffening the floppy single chain helix of PP II. This effect of alcohols on the structure of PP II does not seem to have been noted before. See fig. 3-3. Similar

solvent shifts strongly suggest that the bands should have the same assignments in collagen and PP II.

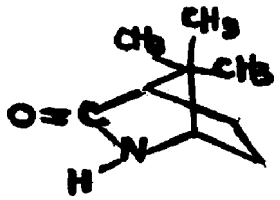
On denaturing rat-tail collagen by heating above 39°, or by the addition of urea or $CaCl_2$ or sodium dodecyl sulfate (SDS), the positive band at 221 nm disappears and the large negative band near 197 nm weakens to approximately 1/3rd its original intensity and shifts, about 3 nm to the red (Fig. 3-4 as first reported by Tiffany and Krimm, 1967). It is to be noted that there is no noticeable solvent effect due to salt: the same band results on heating in water or on introducing $CaCl_2$. (We will note effects due to specific salts on collagen as well as other proteins, but they will occur at higher concentrations than that required to unravel the triple helix (fig. 8-12). A definite temperature effect on the denatured state is evident when the temperature is raised above that needed to cause the chains to separate. The dichroism is seen to decrease in magnitude. Most interesting is the hint of a positive band in the far u.v. in the denatured state. Thus one should not assume that there is only a negative band near 200 nm. Also to be pointed out is the fact that films formed at 60° give the same CD spectrum as solutions at 60°. This is different from the case of ionized PGA (Fasman, et al. 1970). We conclude, contrary to Fasman et al. that the dichroism is not being influenced by "constant motion of the coil" in the solution form of the random state. The similarity of the shape of the denatured collagen CD spectrum to that of feather keratin should not go unnoticed.

C. Poly-L-proline (PP I and PP II)

Poly-L-proline was studied in the cis (I) and the trans (II) forms, using several different molecular weight samples and solvents. By taking CD spectra every few minutes the transition from I to II was easily followed in water solutions for the low molecular weight samples (M. W. 1500) and in trifluoroethanol for the higher molecular weight samples (M. W. 13,500) (Tiffany and Krimm, 1968b). The reverse transition could be followed in the low molecular weight sample by introducing methanol or propanol into the water or trifluoroethanol solutions. At the time of these studies it was found that the Sigma sample, of M. W. 13,500, was not completely soluble in water on initial mixing. It was not until later that it was decided to look at the cloudy solutions and at the filtrates. It was found that the cloudy solutions contained aggregates that have a "form" optical activity. There are no examples of this kind of optical activity of aggregates in the literature, as far as we know. It was later found possible to make these aggregates as well as similiar types of super structures from collagen as well as poly-L-proline. See Appendix I.

The effect of salts on the conformation of PP in Form I or II was studied next. The important fact emerged that in the presence of concentrated $CaCl_2$ the C. D. spectrum taken immediately, (figs. 3-5), was not that of an intermediate between I and II but instead showed strong similarity, in form, to that of denatured collagen (Fig. 3-4). It was already

known that the $[\alpha]_D$ of PP II in saturated $CaCl_2$ decreases in magnitude from -540° to -233° (Harrington and Sela, 1958) and that the specific viscosity is also drastically reduced (Steinberg et al., 1960), and because of the similarity of the CD of PP in $CaCl_2$ to that of denatured collagen it was inferred (Tiffany and Krimm, 1968b) that the regular structure of PP I and II is lost in strong solutions of this salt. That this is a conformation change and not a change "loosely classified as solvent effect" is strongly reinforced by the fact in 6 M $CaCl_2$ the CD spectrum of the rigid model compound:




is the same intensity and shape as the CD of the compound in water alone, but with a 3nm blue shift (Goodman, 1970).

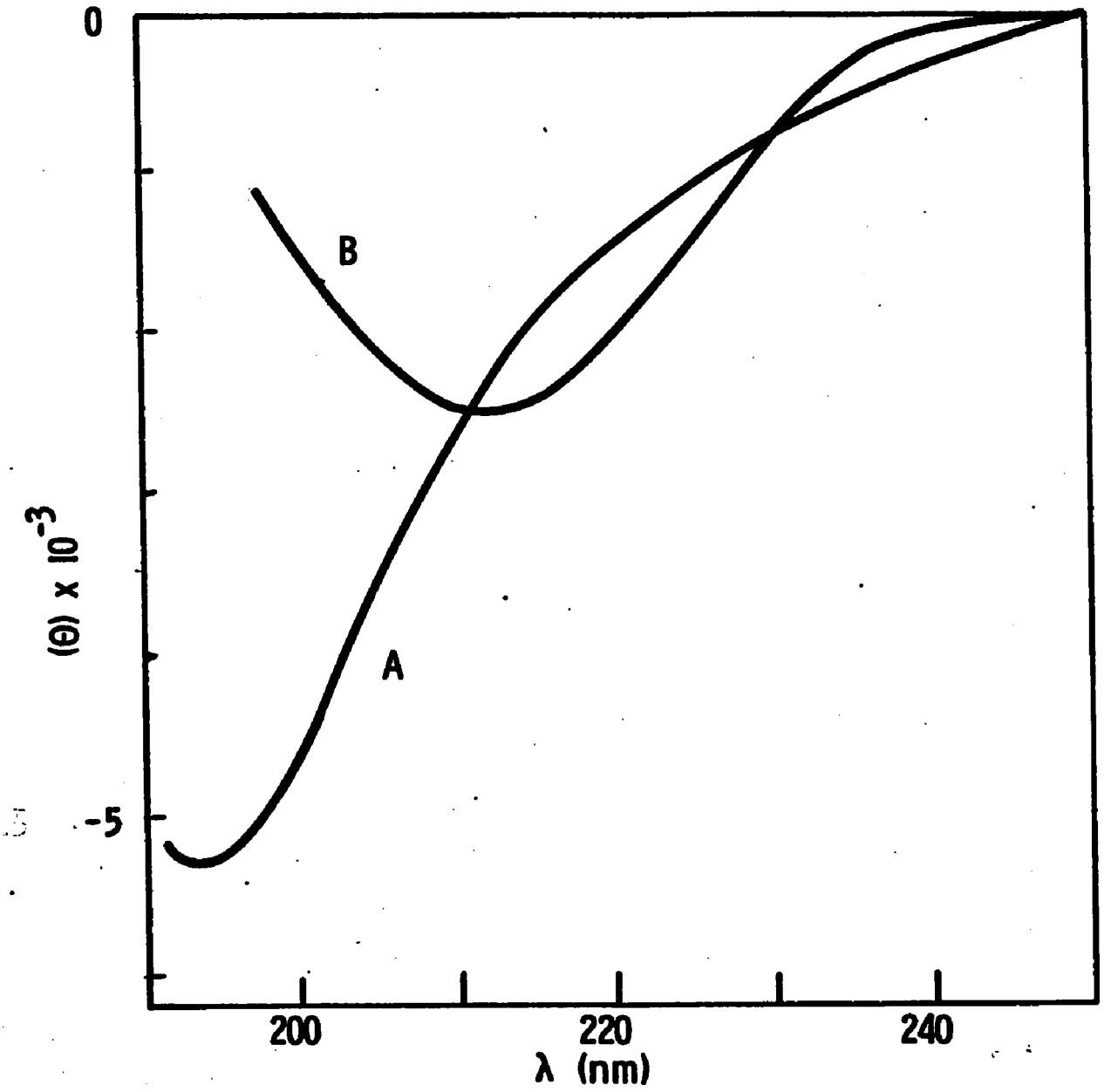



Fig. 3-1. CD of feather keratin, prepared by oxidation of S-S bonds by 2% peracetic acid, 12 hr., followed by dissolution in 0.2 N $NH_4OH$, then dialized against $H_2O$: (A)Solution; (B) film made by drying solution down on quartz window.

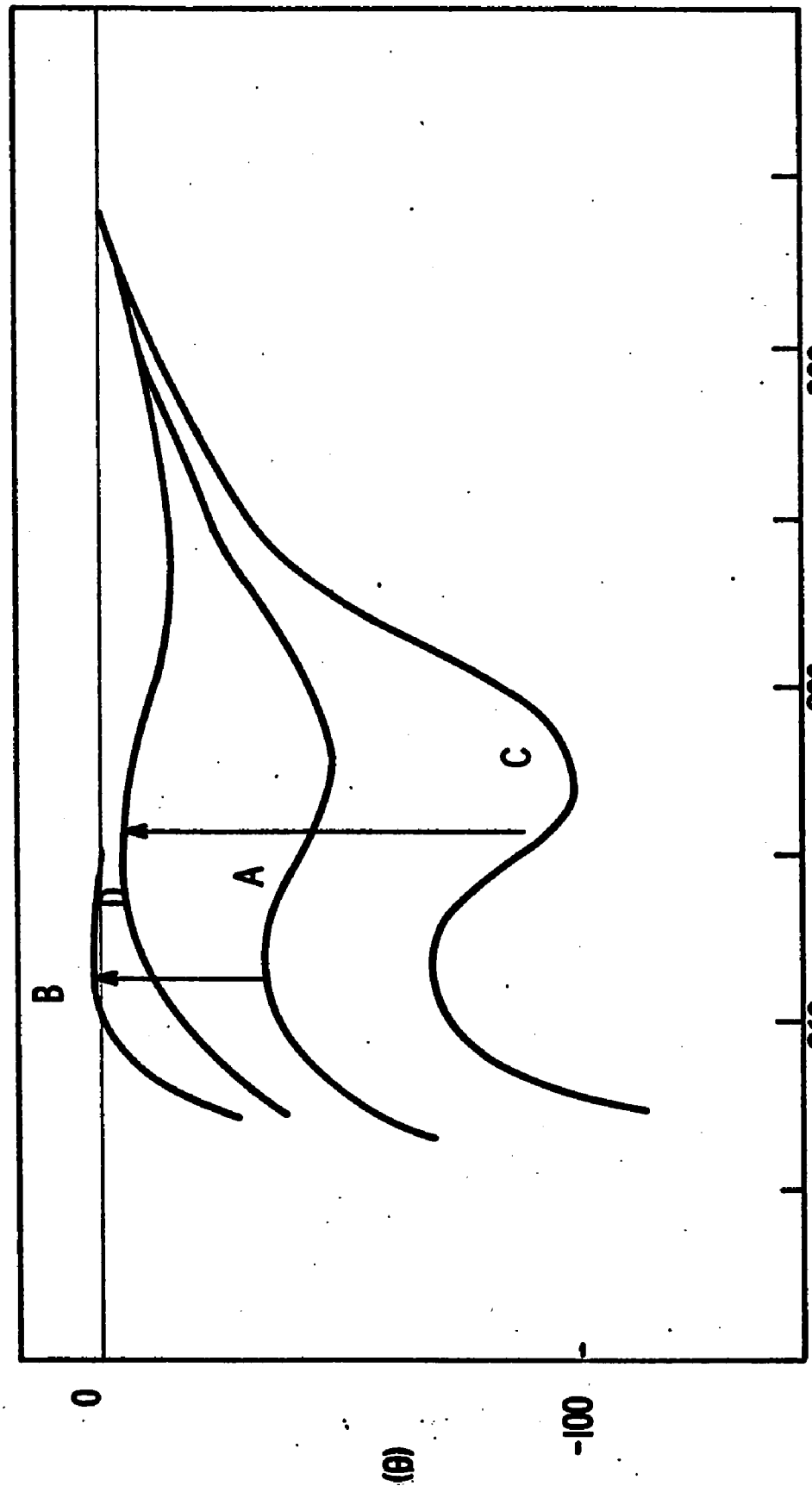



Fig. 3-2. Near uv CD of feather keratin: A, CD spectrum which has developed at 25° on storage (water solution); B, effect of temp.(50°); C, effect of 5 M urea (25°) → D (50°)

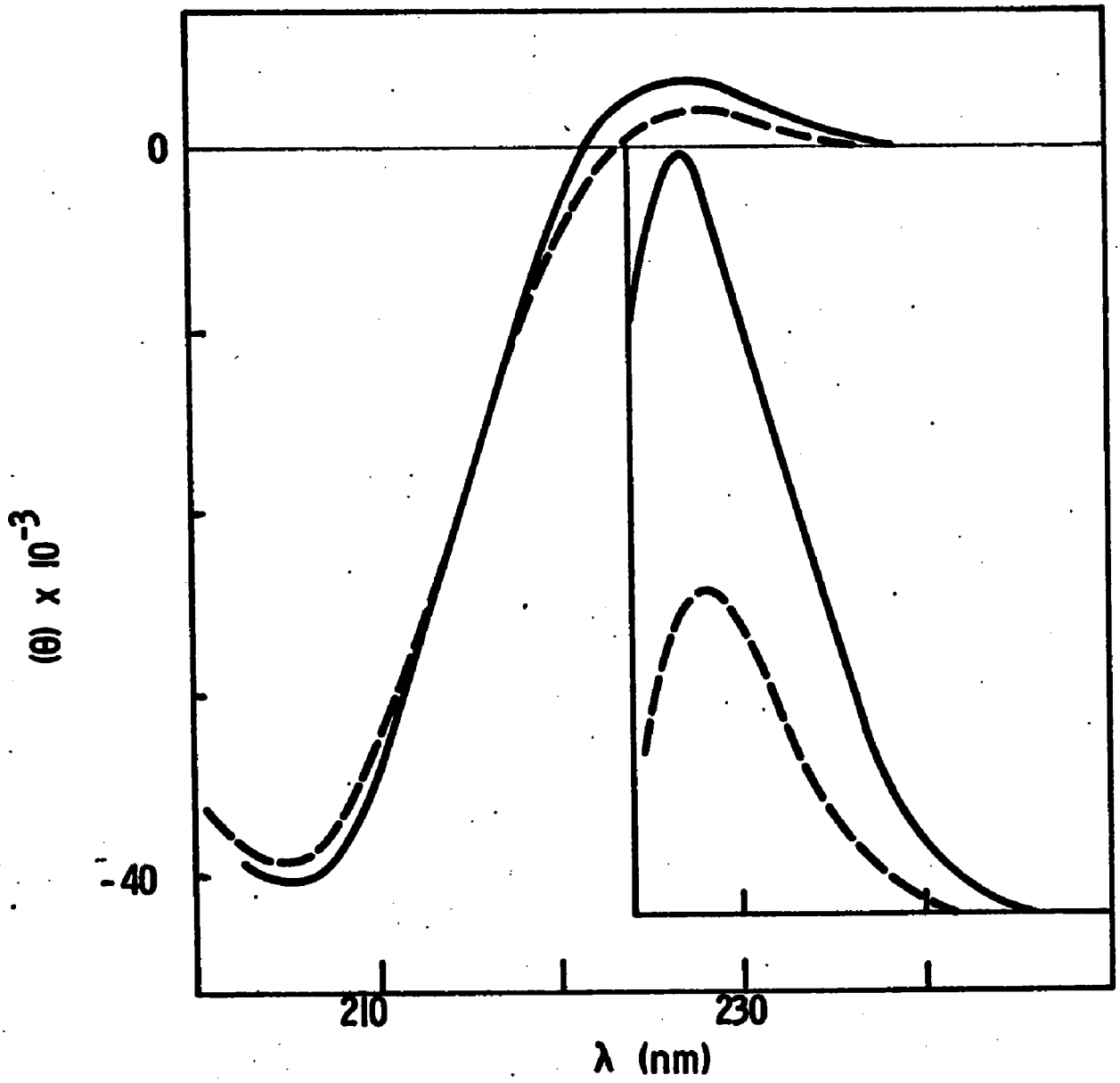



Fig. 3-3. Solvent interaction in poly-L-proline II. CD spectra obtained after immediate dilution with different alcohols. The effect is immediate and independent of the particular alcohol or its ability to produce the II to I transition. (——)in $H_2O$.(– – –) in ethanol, methanol, trifluoroethanol or ethylene glycol.

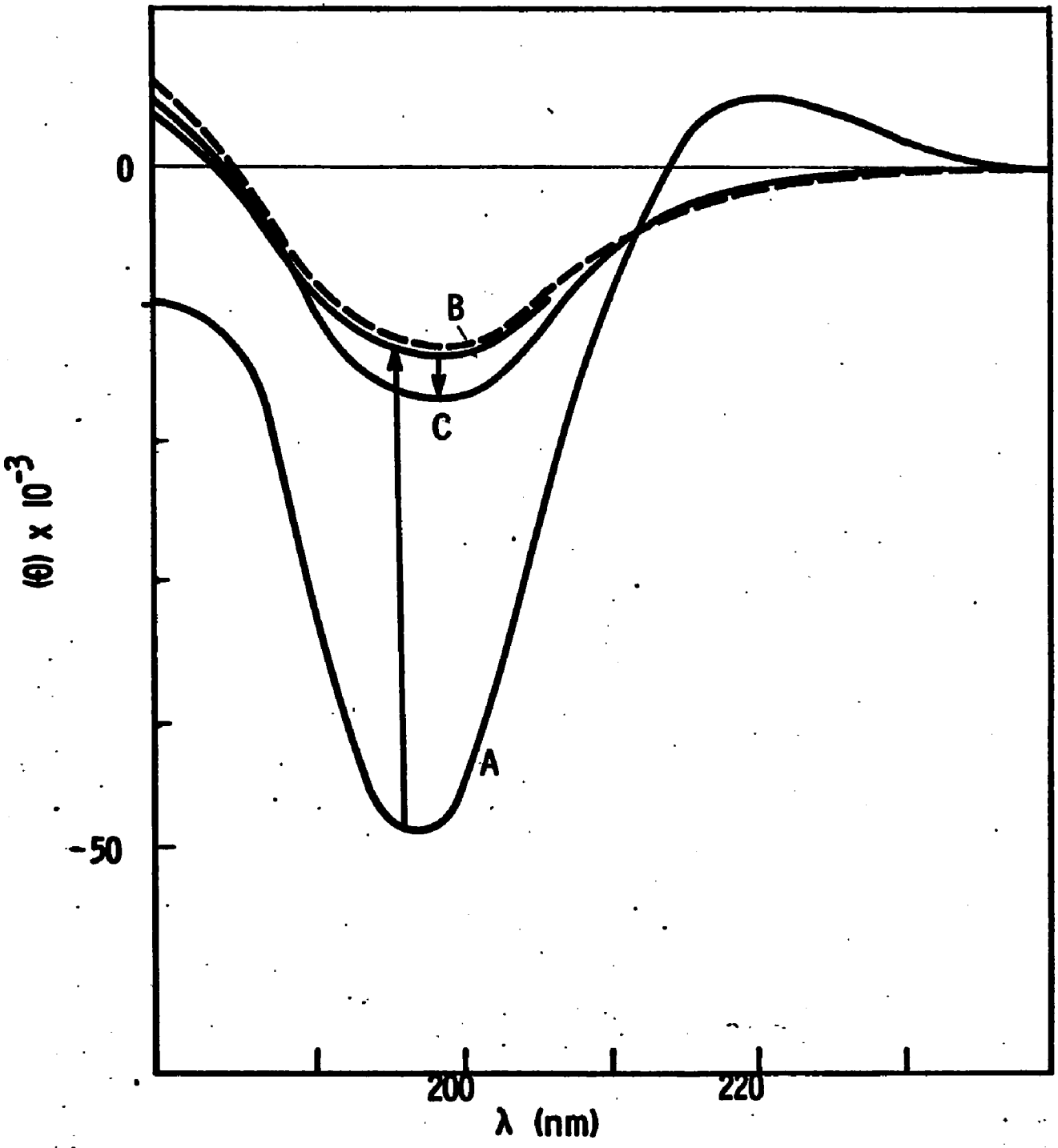



Fig. 3-4. CD of rat-tail collagen:

(A) native; (B) heat-denatured at 60°; (C) heat denatured cooled to 25° and run immediately; (---) film from heat denatured.

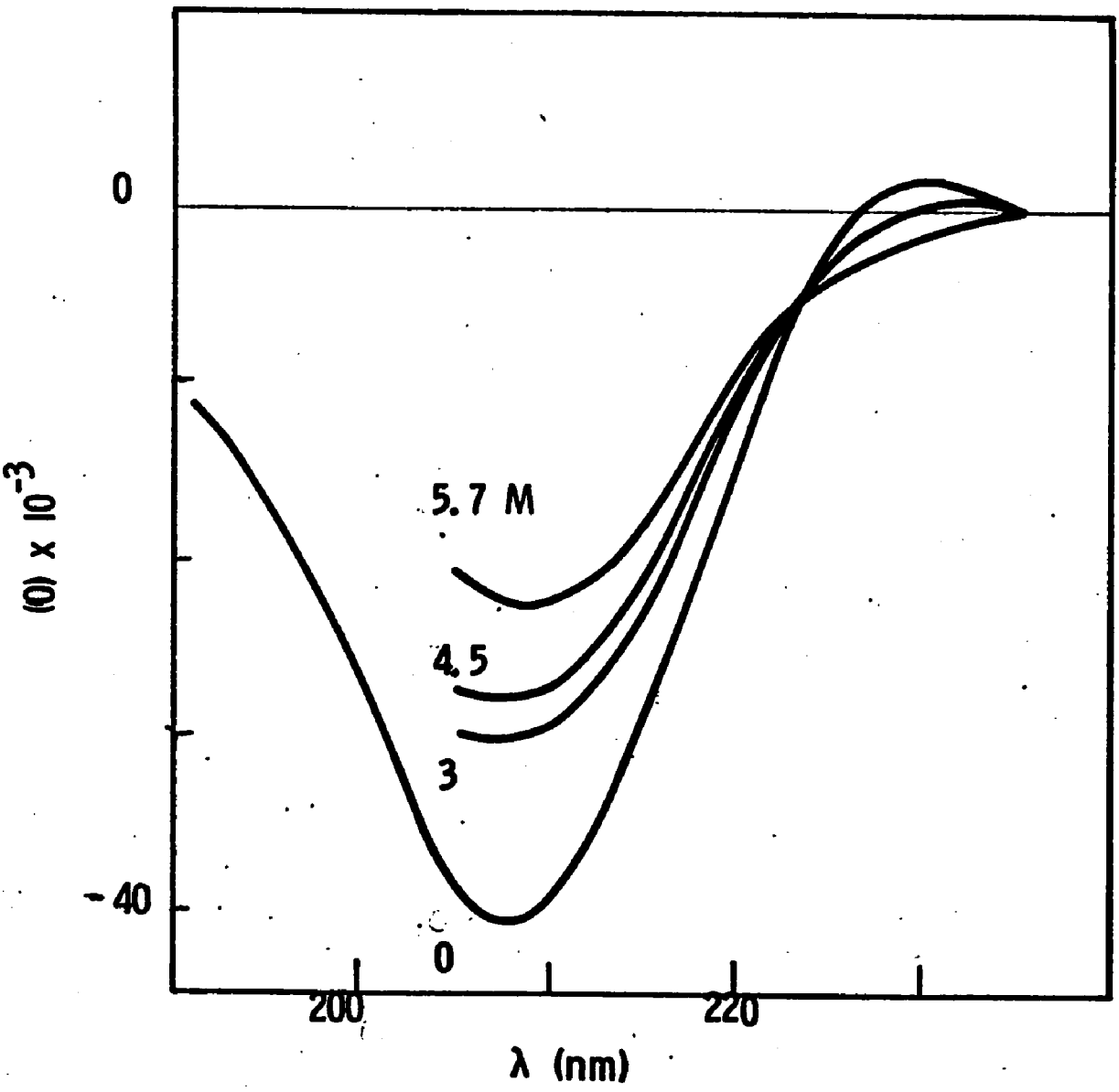



Fig. 3-5 CD of poly-L-proline, mol. wt. 7500
Effect of $CaCl_2$ on form II. Immediate recordings.

## Chapter 4 Structures of Polyelectrolyte-polypeptides. The "Extended Helix" when Ionized.

If, as indicated in the previous chapter, the CD spectrum of an unordered polypeptide chain is characterized in the observable uv region essentially by a single negative band near 200 nm, then the spectra of ionized polypeptides cannot be taken (as assumed heretofore) as representative of a random state. Such spectra as for example that of ionized PGA were considered (Carver, et al., 1966) to consist of a weak negative band at 235 nm, a stronger positive band at 218 nm, and a very strong negative band at 195 nm.

Experiments (Tiffany and Krimm, 1968a) showed that PGA and poly-L-lysine (PL) as well as poly-L-ornithine (PO), when completely ionized and free from buffer, do not have any noticable negative band in the 235 nm region. See figure 4-1 for a typical spectrum. It was noted that the solutions must be dilute enough that neighboring chains do not interfere. With relative freedom from counter-ions, then maximum repulsion between the charges should extend the chain. It is well known that the viscosity increases greatly under these conditions, but that it is difficult to treat the data theoretically (Morawetz, 1965). However, the electrostatic interactions will tend to favor a helical rather than a random structure. This follows since symmetry requires that interactions of a charge with its neighbors be independent of the charge being considered, and, as pointed out by Krimm and Mark (1968) small departures from helical symmetry will raise the energy since increase in

repulsion energy between approaching charges is always greater than the decrease in energy of charges moving apart. Simple Coulomb summation of interaction energies coupled with the limitations of steric hindrance suggests, that the rotation between units, for a minimum in potential energy, would be near 140° and that the helix would be left-handed. Detailed calculations have been made by Krimm and Mark (1968). This is exciting, since it now makes sense that the CD spectra of these charged polypeptides resembles that of PP II which is known to have an approximately 3 X left-handed helical structure at least in the solid state from interpretation of the fibrous x-ray diffraction pattern (Cowan and McGavin , 1955).

Very important evidence that the ionized polypeptides are tending to take an extended helical structure is that there is a dramatic increase in the strength of the CD bands representative of the structure when the temperature is lowered toward 0° (fig. 4-1, 4-2 and 4-3). One would expect a more rigid form would be more possible under these conditions. It is also known that the rigid model compounds have much more intense CD bands than the flexible model compounds (Goodman et al., 1969). There is no sharp isodichroic point in the family of CD spectra of an ionized PGA solution taken at different temperatures. Instead there is a gradual shift of the cross-over point from 204 to 208 nm as the temperature is raised from 5° to 55°. This could be due to more randomness being introduced at the higher temperatures. The possibility of some rigidity in the structure of ionized PL at low temperatures is also suggested from n.m.r. (Joubert, et al., (1969).

Poly-L-aspartic acid should now be added to the polypeptides with charged side-chains that can take an extended conformation due to charge repulsion. Figure 4-3 shows that by reducing the temperature to 5° the CD spectrum of poly-L-aspartic acid in water resembles the spectra obtained for PGA, PL and poly-L-ornithine. This CD spectrum of poly-L-aspartic acid is very sensitive to its environment, and the usual curve obtained from samples dialized against distilled water showed the 216 nm peak as a shoulder on the larger negative dichroism.

It is a well known fact that it is difficult to obtain poly-L-aspartic acid in a good $\alpha$ helical form, and that the helical form is very sensitive to interfering substances. Thus Brahms and Spach (1963) found the negative CD band near 222 nm to be only 1/5 as intense as the corresponding band in PGA under similar conditions of negative pH, and also found that the band for poly-L-aspartic acid was eliminated by the addition of 0.3 M acetate ion. Yan et al. (1968) have calculated that right-handed-$\alpha$-helices have the lowest energy for both PGA and poly-L-aspartic acid, but that the energy difference between the right- and left-handed conformations of minimum energy is 1.47 kcal/mole for the first case and only 0.23 kcal/mole for the poly-L-aspartic acid.

## Discussion

It is interesting to note some adverse comments that have appeared in the literature since our first presentation

of the local extended helix for the ionized state of PGA, PL, and PO. Thus Meyer (1969) did a simple repeat of our reported experiments and found the same results. He states his opinion, however, that the CD of ionized PGA and Pl is that of the "true random, disordered form", since he does not like our comparsion to PP II spectra since the bands are not at the same wave length nor of exactly the same intensity. He thus overlooks the fact that the CD bands are related to absorption bands which also have approximately the same spectral shifts as the CD bands. As far as comparing the intensities of the CD bands, we have shown that the relative intensity in both ionized polypeptides and PP II can vary over a wide range. In the case of ionized polypeptides, increasing temperature or adding salt weakens the CD bands of the extended state. A smaller, but similiar temperature effect is also noted for PP II (Fig. 4-4). The PP II bands are also strongly influenced by the interaction of the polymer with the solvent (Fig. 3-3). Most interesting, urea, probably through hydrogen bonding to the backbone carbonyl, intensifies both the positive bands of ionized polypeptides (Figs. 5-1, 5-3) and PP II (Fig. 5-5). An increase in the intrinsic viscosity of PP II by urea (Blout and Fasman, 1958) indicates a stiffening of the coil therefore the observed increase in intensity of the CD bands is not a "solvent" effect.but, instead, an actual conformational effect.

Fasman, Hoving, and Timasheff (1970) have objected to our hypothesis that ionized PGA and PL ( Tiffany and Krimm, 1968c) have a local extended structure. They say this, even after

admitting that a highly kinked chain containing short sequences of regular helices would behave hydrodynamically as a coil; they quote the authors of three papers on the general overall shape of a random coil (Brant and Flory, 1965; Flory and Miller, 1966; and Miller, Brant and Flory, 1967) in their rejection of our concepts. Local order, however, cannot be tested by a few hydrodynamic parameters. In the paper by Fasman et al. (1970) we are badly misquoted as having said that PGA exists in an extended structure in dilute salt. We, of course, said that salt collapses the extended state. Nor have we inferred, as implied by Fasman et al. (1970), that the extended state cannot be taken by other polypeptides due to the action of other forces besides charge repulsion.

The paper by Fasman et al. (1970) is pertinent to this thesis, however, since it discusses the random state of proteins. They present the CD of a film made from solution of ionized PGA as a suitable model for the random coil part of a globular protein. It is suggested that the CD of a film made from a solution is different from that of the solution itself because the chain is no longer capable of random flight motions of a "true statistical coil." This of course implies that the CD of the solution is influenced by the constant motion of the coil but it is well known that electronic transitions are relatively instantaneous with respect to Brownian motion. They have not considered the fact that when a solution of an ionized polypeptide is dried down to a film, the extended state is destabilized since the counterions must move in close to the chain

and tend to neutralize the charges on the chain and therefore cause the conformation to go toward α-helical. On further drying, an aggregated state would be expected. Fasman et al. (1970), however, says that removal of water from PGA leads to a random conformation. We see no indication that this happens. If a film is dried down rapidly the structure might be caught in an intermediate semi-extended state. This can certainly be representative of many proteins which are not high in α-helicity or β-structure. It should be noted, in passing, that a gross error appears in Figure 6 of their article. The actual dichroism of ionized PL is at least 2½ times as great as that indicated. This invalidates their main conclusion that the dichroism of many "unordered" proteins cannot be made up, in part, of a component CD similar to that of ionized PL.

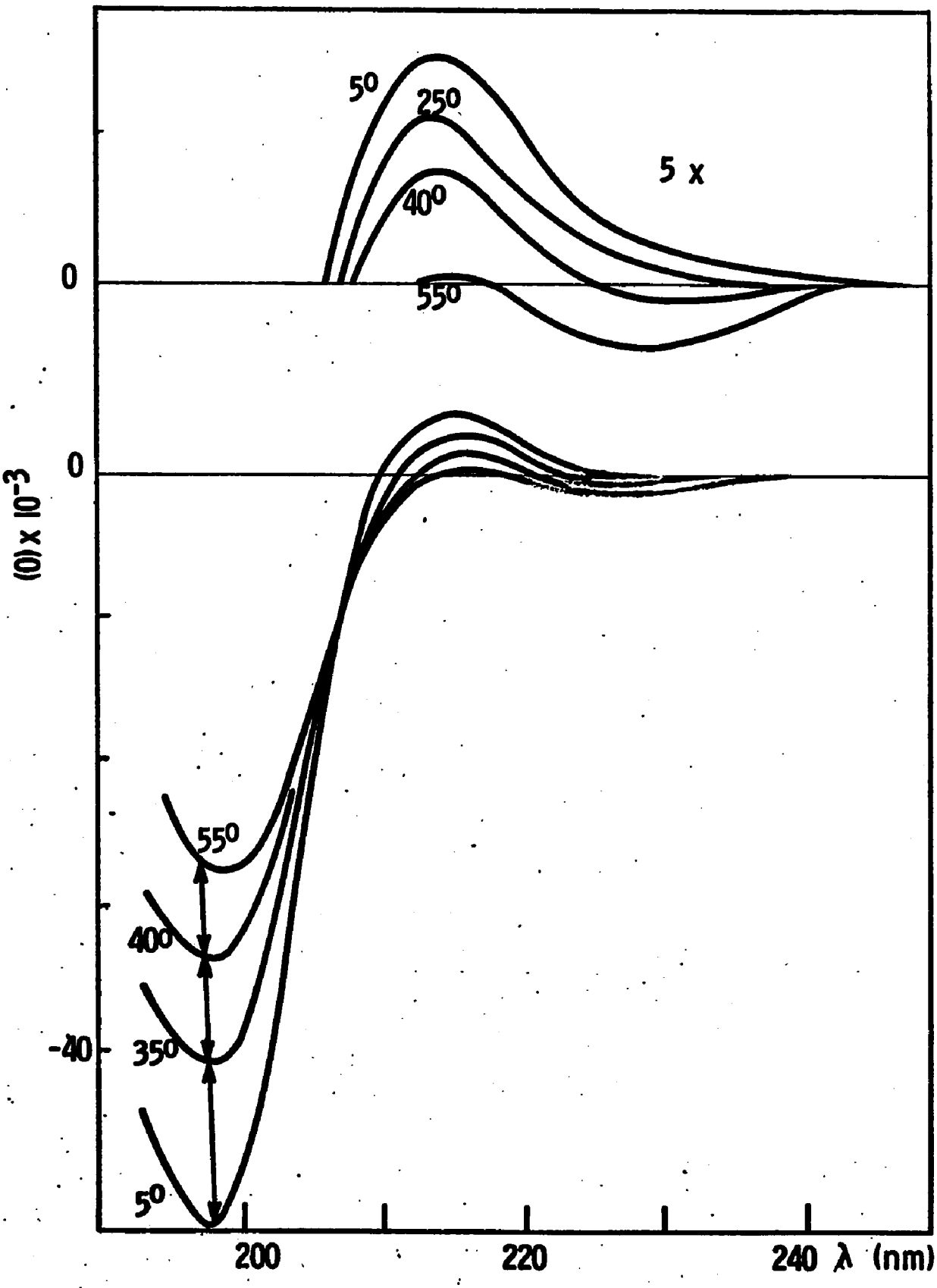



Fig. 4-1. CD poly-L-glutamic acid (pH 7). Effect of temp.

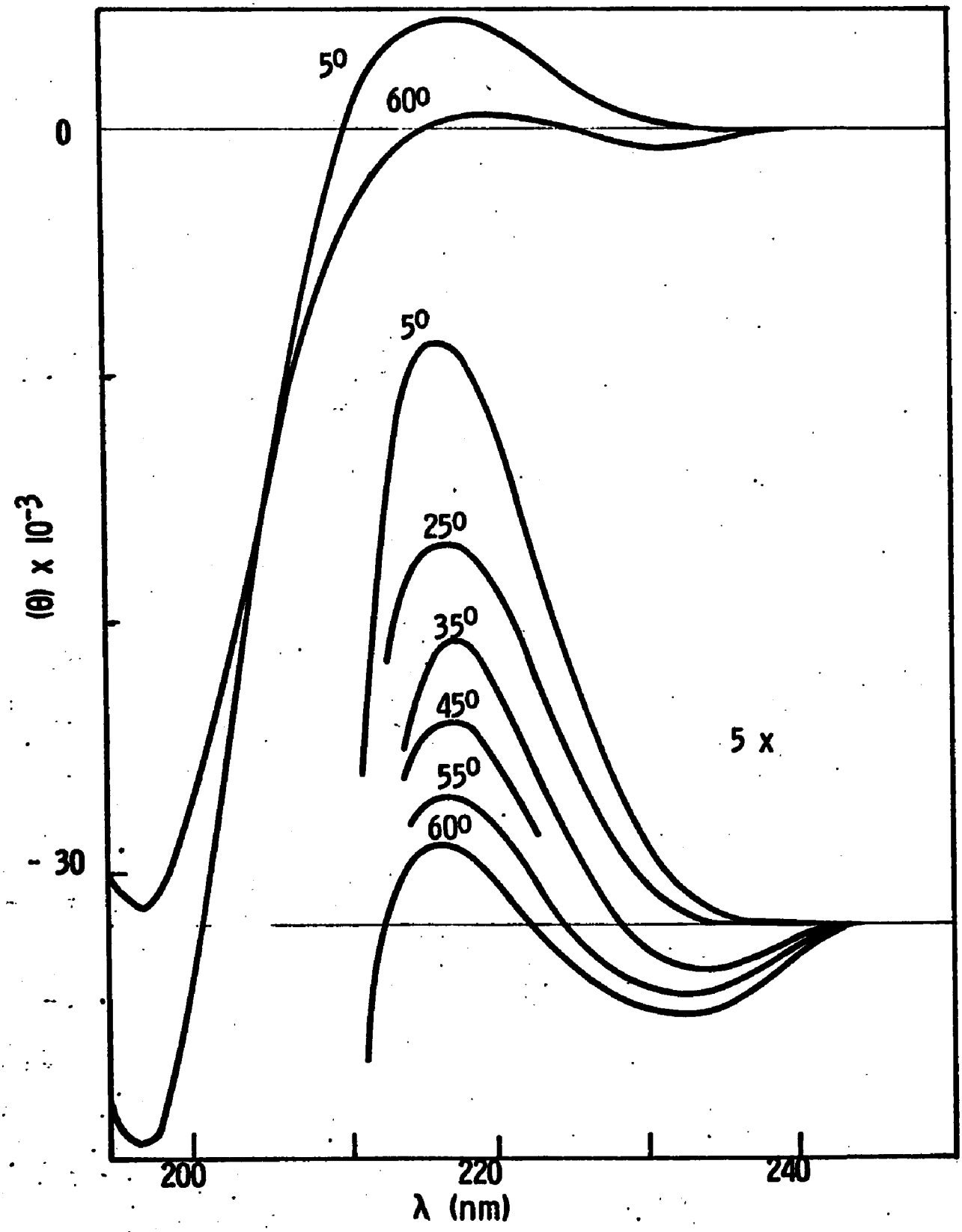



Fig. 4-2. CD of poly-L-lysine (pH 7). Effect of temperature.

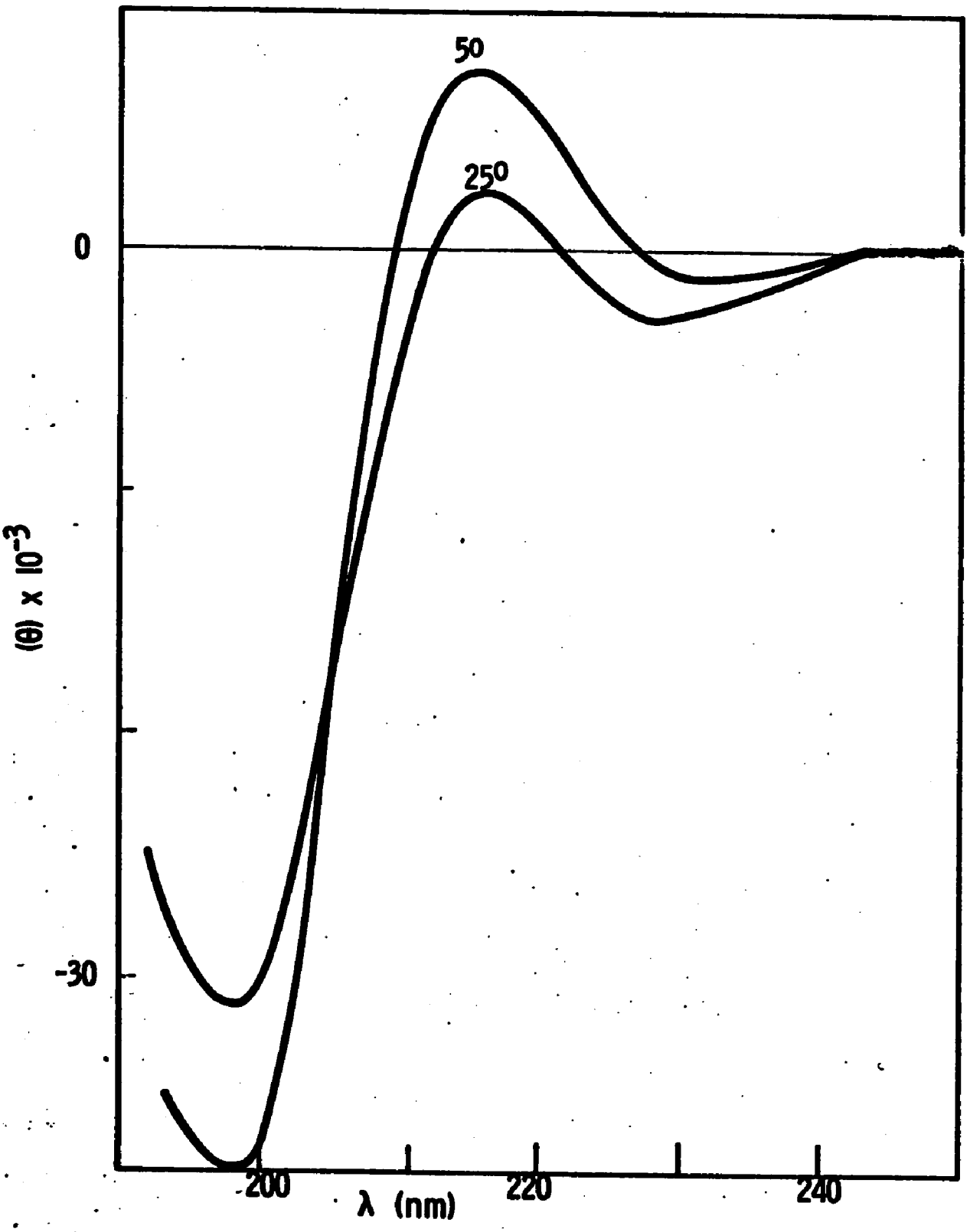



Fig. 4-3. CD of poly-L-aspartic acid (pH 7). Effect of temp.

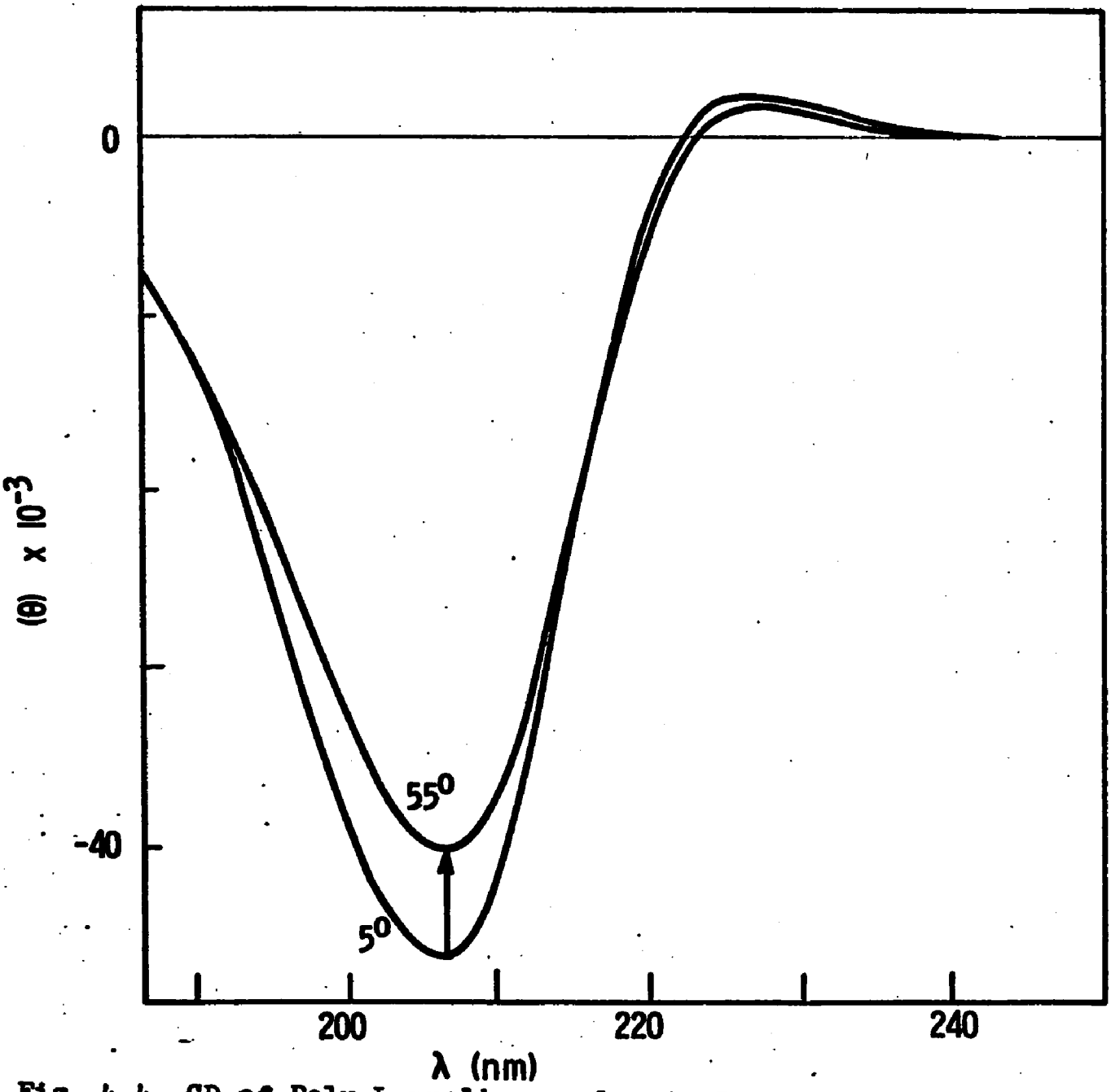



Fig. 4-4. CD of Poly-L-proline, mol. wt. 35,000. Effect of temperature. $5^{0} \rightarrow 55^{o}$.

## Chapter 5

## Extended States due to Urea and Guanidine Hydrochloride.

It has been stated that it is difficult to find a suitable technique for demonstrating the involvement of urea and guanidine hydrochloride (GuHCl) in the structure of proteins and polypeptides at the molecular level. Nemethy (1967) said the mechanism of the interaction of urea remains one of the unsolved problems.

It was once widely assumed that urea denatured proteins through an ability to break hydrogen bonds (Mirsky and Pauling, 1936). Schellman (1955), however, in a study of the thermodynamics of the hydrogen bond in water, concluded that hydrogen bonds give only a marginal stability to ordered structures.

It is possible that urea is interacting with the sidechains of the polypeptide. Thus it is known that urea increases the solubility of many nonpolar compounds in aqueous solution. This solubility increases with temperature. Denaturation of proteins by urea is usually aided by raising the temperature. Taking this into account, it was suggested that urea contributes to the denaturation of proteins through a hydrophobic effect (Kauzmann, 1959). By favoring exposure of the nonpolar groups to the solvent the hydrophobic bonds will be broken. Substitution of alkyl groups for hydrogen or an increase in chain length of alkyl substitutents should increase the effectiveness of denaturation by this mechanism (see Robinson and Jencks, 1965, for discussion).

The possibility exists of the involvement of urea and GuHCl directly interacting with the carbonyl and amide groups along the backbone. Thus Robinson and Jencks (1965) used the model compound acetyltetraglycine ethyl ester (ATGEE):

$$CH_3-CO-NH-CH_2-CO-NH-CH_2-CO-NH-CH_2-CO-NH-CH_2-CO$$
$$\qquad\qquad\qquad

used was impermeable to protein. They found, in 8 M urea, 1 urea molecule bound for every 3.5 amino acid residues in folded (native) unreduced lysozyme. This increased to 1 urea molecule being bound per 2.8 amino acid residues of lysozyme that was unfolded by the simultaneous action of urea plus reducing agent. (Similarly, by dry weight measurements on protein solution and dialyzate, Kielley and Harrington (1960) showed GuHCl binds to ribonuclease and to myosin (0.05 g/g of protein), and Hade and Tanford (1967), working with aldolase, lactoglobulin, lysozyme, ovalbumin, ribonuclease, and bovine serum albumin obtained preferential binding of GuHCl of about 0 to 0.11 g/g protein.)

In order to interpret the CD spectra of proteins and synthetic polypeptides in urea and guanidine HCl, we must consider how the reagents affect bicyclic amides. Goodman et al. (1969) found the dichroism of bicyclic amides to be of the same order of magnitude as that occurring in helical polypeptides, in sharp contrast to the intensity of bands in the monocyclic compounds which were only 20% as intense. The enhancement, as they pointed out, is undoubtedly a function of the rigidity of the bicyclic compounds. When the bicyclic compounds were dissolved in 8 M urea there was little difference in the optical activity. This very important experiment shows that urea is not having a direct effect on the chromophore. Therefore, in the case of proteins and polypeptides, we can consider changes observed in urea as being due to conformational transformations.

1. Poly-L-lysine (PL)

When urea is added to a water solution of PL so that the concentration of urea is 8 M the 218 nm positive band is intensified. This is very noticable at low temperatures (Fig. 5-1).

A series of experiments using different amounts of urea on PL in 0.01 N NaOH showed that the alpha helical form of PL is easily disrupted by low concentrations of urea. At high concentrations or urea we see development of a positive CD band near 218 nm which we relate to an extended state (Fig. 5-2).

Our results are interesting in view of the work done by Dearborn and Wetlaufer (1970), which included the effect of 8 M urea and 6 M GuHCl on PL. At pH 6 they say the CD of PL is the same in 6 M GuHCl as in 0.01 M NaCl. On the basis of this result they concluded that the ionized form of PL cannot be electrostatically stabilized, and gave this as strong argument against the extended-helix of Tiffany and Krimm (1968a and 1969a). They apparently did not do the companion experiment in 8 M urea on the ionized PL. They did not notice that the overriding influence of either urea of GuHCl is, under the conditions of low temperature, to extend the "Extended-helix" more than occurs in water alone. The mechanism is probably hydrogen bonding to the backbone carbonyl groups increasing the rigidity. An ordinary salt, acting only through electrostatic interference, does knock down the positive 218 nm CD band, as we originally pointed out (Tiffany and Krimm, 1968a).

2. Poly-L-Glutamic Acid (PGA)

Ionized PGA in 8 M urea, similar to PL, gives a sharp increase in the intensity of the 218 nm band over that given by the aqueous solution at the same temperature. Again, lowering the temperature to 5° greatly increases the intensity of the band (Fig. 5-3). Our results are to be contrasted with the results of Hermans (1966). He found the optical rotation in 8 M urea, 0.1 M KCl, at pH 7 not to be dependent on temperature. The temperature range studied was not given. The effect, if due to binding of urea to the peptide carbonyl, by analogy with the model compound ATGEE should increase at low temperature. It is important to note that since the intensity of the band increases in water alone when the temperature is lowered we can be sure it is a locally ordered state that is forming and not a random one, say, due to more urea binding.

PGA at pH 3.89 is in the helical form. If the sample is made up to be in 8 M urea at pH 3.89, however, the helical form is seen to be destabilized. The CD curve is only 1/3 the depth (Fig. 5-3). Again our results are to be contrasted with those of Hermans (1966). He found little destabilization of the $\alpha$ helix in 8 M urea at pH 3.5, at room temperature. His sample gave a less negative $[\phi]_{230}$ as the temperature was raised from 10° to 70°, going from -12,000° to -4,000°. Our sample went, instead, toward $\alpha$ helix when the temperature was raised. That is, the CD curve moved down. The simple explanation is that our sample was already past the random state and was already partially extended in 8 M urea at room temperature. Thus

raising the temperature, in our case, as well as in Hermans' case, made the samples go toward random form.

If urea is added to a PGA sample that is in an aggregated form due to low pH, the main interaction that is noted by CD is the removal of the aggregated state, as seen by the CD spectra returning more to the normal $\alpha$ helix form (Fig. 5-4). This type of interaction would explain the statements by Fasman et al. (1964) and Hermans (1966), that urea was ineffective on $\alpha$ PGA.

3. Poly-L-proline I and II.

In urea, PP I transforms to PP II. Blout and Fasman (1967) noted, however, that the intrinsic viscosity and the negative optical rotation of PP II were increased. We see, from CD, that the 226 nm band of PP II is intensified by urea (Fig. 5-5). A similar intensification of the CD bands is seen in 5.7 M GuHCl (Fig. 5-6). It was noted that lowering the temperature made no further increase in the band intensity. The structure is probably fully extended.

We can conclude that an increase in the positive band in ionized PGA, ionized PL, and in the extended form of PP, PP II, indicates that the relatively loosly coiled structures are made more rigid and better formed by interaction with urea. The plausible mechanism is through hydrogen bonding to the carbonyl groups along the backbone of the polypeptide chain.

4. Eylar's Basic Protein ($A_1$).

Of all the proteins tested, $A_1$, Eylar's basic protein, responds to perturbants in a manner similar to the simple

polypeptide PL. $A_1$ actually is a highly basic protein with an isoionic point exceeding 12. It is also rather extended in its native state. Thus Eylar and Thompson (1969) give the intrinsic viscosity as 9.27 ml/g and the viscosity increment as 12.9 allowing the axial ratio to be calculated as approximately 10: 1.

The action of urea on $A_1$ (Fig. 5-7) does not change the CD pattern very drastically. Instead, similar to ionized PL, there is an increase in the + 218 nm region. Lowering the temperature dramatically increases the intensity of the band. The action is very analogous to the action of urea on PGA, PL and PP II, and presumably is due to the same mechanism of urea binding to the peptide carbonyl groups stabilizing the extended state.

5. Soybean Trypsin Inhibitor (STI).

STI is a typical globular protein by hydrodynamic criteria, having an intrinsic viscosity of about 3 ml/g. It is said to be denatured by heat, changes of pH, or by the addition of urea (Tanford, 1968 review). The CD of STI is reported by Jirgensons et al. (1969) and Jirgensons and Capetillo (1970). CD bands in the far uv occur at 200 nm [$(\Theta) = -16,200$] and at 226 nm [$(\Theta) = +1060$]. In addition there are bands in tne near uv. These have been studied in detail by Ishida et al. (1969) and Baba et al. (1969).

The simplest explanation of the CD spectrum of this molecule is that there are regions of extended helices dominating the far uv. The conformation cannot be a random coil,

as Jirgensons points out, for then we would not expect to see denaturation. Nor can it be due to a complicated twisted configuration whose overall CD just happens to look like the extended helices, since we can show that by perturbing the molecule the dichroism changes in a similar manner to that seen in the extended structures already discussed. Thus 9 M urea increases the positive band occurring near 230 nm. Similarly this band is increased in intensity by lowering the temperature (Fig. 5-8). We can follow an increase in the negative band at 200 nm also with decreasing temperature, and by using high concentration we see that the negative band near 275 nm increases in the same proportion. On raising the temperature to 50° a sudden change occurs in CD. The positive 230 nm band disappears and the 200 nm band decreases rapidly in intensity (Fig. 9-2). This is thus seen to be a cooperative opening of the molecule. S-S bonds have been implicated in the stabilization of the STI native conformation. Thus Jirgensons et al. (1969) have shown that the structure is lost when the S-S bonds are reduced. Raising the temperature to 50° would not break S-S bonds, however; therefore we can assume that S-S bonds are a stabilizing factor but a great deal of randomization can still be achieved with S-S bonds intact. It would be interesting to test the CD of reduced STI at low temperature. It may be that only the temperature of transition is higher when S-S bonds are present. Any similarity of the CD of native STI to oxidized glutathione as observed by Jirgensons et al. (1969) is probably accidental. From Fig. 5-8

we see the temperature of transition is lowered by 9 M urea from 50° to 45°. This is probably due to the effect of urea on other parts of the globular molecule than that part which is stabilized by the urea. Baba et al. (1969) did not look for, and therefore did not find the effect of urea at room temperature, nor the effect of lowering the temperature below room conditions.

6. Lysozyme.

Lysozyme has been extensively studied by many physical techniques. Thus its x-ray diffraction pattern has been interpreted (Phillips, 1967), the sequence of amino acids is known, and there is a suggested mechanism for its catalytic activity. Dickerson and Geis (1969) give a vivid review on lysozyme's structure and mechanism of action. CD studies have been made by many researchers [Beychok, 1965 and 1967; Sarker and Doty, 1966; Yatani et al., 1968; Straus et al., 1969; Tamburro et al., 1970; Timasheff, 1970].

It has been noted that lysozyme can interact with 3 to 9 M urea without significant change in optical rotatory properties or changes in intrinsic viscosity (Warren and Gordon, 1970). Similarly, we see little influence of 8 M urea on the CD spectra in the region governed by the conformation of the chain. The influence of the urea is, however, readily shown, by the greater instability of the molecule to temperature (Fig. 5-9). In the near uv CD spectra (Fig. 5-10) the positive band, near 190 nm, is found to double in intensity when 8 M urea is used in place of water. On adding a reducing agent the intensity

of the band further increased. But on heating to 55° in the urea-reducing agent solution there is an abrupt decrease to zero intensity throughout the near uv CD region. The molecule has opened up in a cooperative manner. On recooling to 25° the loosened chain further expands (Fig. 5-9). This particular sample was not cooled below room temperature, but we would expect to see still further expansion and, hopefully, an indication of a positive peak in the 220 nm region.

The increase in intensity of the 290 nm band cannot be considered to be due to not taking into account the Lorentz correction for index of refraction in the urea solution. Fasman (1963) gives the index of refraction of 8 M urea as 1.4572 at $\lambda$ equal 289.5 nm. Thus $3/(n^2 + 2)$ is approximately 0.73. If this factor is applied to the CD measurements, the readings would still not account for the higher intensity measured in the 290 nm band, and would lead to completely inconsistent results in the far uv bands since the overall structure is known not to be much affected on the basis of optical rotation and viscosity measurements. It is better to explain the initial changes as due to an attack on the amino acids which are on the outside of the globular molecule. The band at 250 nm which appears while the molecule is being acted on by the urea-reducing agent should be noted. We will see it again when lysozyme is treated with salts (fig. 8-27). Yatani et al. (1968) give a CD spectrum of reduced lysozyme in 8 M urea. Their sample apparently was not completely reduced, however, since they show a residual (+) band at 290 nm.

7. Insulin.

Insulin is a rather small globular molecule (M.W. 6,000) whose x-ray diffraction analysis has only recently appeared (Adams et al., 1969). It has two inter-chain S-S bridges and one S-S loop in one of its two chains. CD studies have been done by Beychok (1965), Timasheff et al. (1967), Frank and Veros (1968), Morris et al. (1968) and Menendez and Hersko-vits (1970).

We only studied the effect of 9 M urea on unreduced insulin (Fig. 5-11). Here as in all proteins studied, there is indication of a somewhat expanded denatured state since the CD spectra show a shoulder in the 220 nm region which rises closer to the base line as the temperature is lowered to 5°. The S-S bonds apparently keep the structure from taking a good extended form.

8. Myoglobin.

Sperm whale myoglobin was the first protein to have its structure revealed by x-ray diffraction (Kendrew et al., 1961). It is known to be approximately 60-70% $\alpha$-helical from x-ray work as well as from ORD and CD analysis (Urnes et al., 1961; Beychok and Blout, 1961; Samejima and Yang, 1964; Breslow et al., 1965; Harrison and Blout, 1965; Schechter and Epstein, 1968; Hermans et al.,,1969; Strass et al., 1969).

Since myoglobin has no S-S cross-links it is a good protein to use in studying denatured states. Sperm whale myoglobin is more resistant than horse myoglobin to urea (Schechter and Epstein, 1968). Using sperm whale myoglobin,

it is interesting to see the effect on the CD spectrum of increasing the temperature to 50° in different concentrations of urea (Fig. 5-12). Thus a larger change is made by changing the temperature in the 9 M than in the 8 M than in the 6 M urea. But, up to 50° the temperature changes are reversible. When, however, the temperature is first raised to 95° for 5 minutes and then the sample allowed to cool to room temperature, an effect, irreversible to temperature, is observed. Now the molecule is in a state to fully interact with the urea, and by cooling to 5°, we see the 220 nm band develop.

9. Pepsin.

Pepsin is an interesting molecule since CD spectra indicate that it occurs in a native state in the β structure (Fig. 5-13)(Jirgensons, 1970). Perlmann (1958 and 1967) has found pepsin to be "very resistant" to urea and GuHCl, and found little effect on $[\alpha]_{366\ nm}$ in 8 M urea, the highest concentration used. Our first experiment met with immediate success, however, since we used 9 M urea at room temperature. All signs of structure are lost. We did not pursue the experiments further to try to get better indications of extension.

10. Ribonuclease (RNase).

Ribonuclease has been well characterized structurally. Thus its x-ray diffraction pattern has been interpreted by Kartha et al. (1967) and many researchers have reported on the CD of the molecule (Hashizume et al., 1967; Simons and Glazer, 1967; Tamburro et al., 1968; Schellman and Lowe, 1968;

Herskovits and Menendez, 1968; Isemura et al., 1968; Beaven and Gratzer, 1968; Simons et al., 1969; and Horwitz et al., 1968).

In 1957 Sela and Anfinsen, using optical activity as a tool, showed that there was considerable disruption of RNase by 8 M urea and that 0.15 M $PO_4$ restored the structure to normal. (This might explain Anfinsen's findings (1961) that RNase was fully active in 8 M urea against RNA.) Pflumm and Beychok (1969) noted that the pH must not be on the acid side in order to get the return to close to the native CD spectrum in both the near and far uv when 0.15 M phosphate is present. The CD spectrum of RNase in 8 M urea, without phosphate, has been given both by Hashizume et al. (1967) and by Pflumm and Beychok (1969). Neither report any positive band in the 220 nm region. Our results show that RNase in 8 M urea is very sensitive to temperature (Fig. 5-14). As expected, 8 M urea disrupts the native structure at room temperature. However, by lowering the temperature we find that a positive 225 nm band develops. When the temperature was raised to 60°, we see that the dichroism resembles that of a random coil. This can be due to removal of urea from its binding position on the carbonyl and also due to temperature causing destabilization of the extended state.

Using guanidine HCl, we find the same effect on RNase as with urea (Fig. 5-15).

11. Silk.

Silk is an atypical protein in that normal silk is composed of a very high percentage of glycine. Thus Bombyx mori

is quoted to have 43% glycine, 31% alanine and 10% serine. In the solid state, the diffraction pattern of Bombyx mori has been analyzed to be an antiparallel-chain pleated sheet structure (Marsh, Corey and Pauling, 1955). In solution it is also easy to get β conformation, especially by adding less polar solvents than water such as methanol or dioxane (Iizuka and Yang, 1968). In water solutions, prepared from dialyzing out the LiBr that is used to stabilize, silk is often found to be in a rather disordered state. On adding 8 M urea, Iizuka and Yang, report a positive CD band near 220 nm, and an increase in intrinsic viscosity from 0.33 dl/g in 0.2 M NaCl to 0.63 in 8 M urea. Since silk is not cross-linked by S-S this is what we would expect on the basis of experiments with other proteins and polypeptides.

Discussion.

Through observation of the CD spectra, and through co[illegible]ations of other experimental measurements, we have reached the conclusion that most polypeptides and proteins tend to bind urea and guanidine hydrochloride to the backbone carbonyl and that at high concentrations of the reagent and at low temperatures this binding leads to ordered extended states. When the temperature is raised the ordering and the binding are disrupted and the structure tends to randomize. The evidence for ordering is the similarity of the CD spectra of proteins in concentrated urea and guandidine HCl at low temperature to the CD spectrum of PP II which is known to have a 3X left-handed helical structure. Also in favor of order is the

better formation of this spectrum with lowering of the temperature. The evidence for the chain being extended is (1) this form of PP II, which gives a similar CD spectrum is extended, (2) all acidic and basic side-chains are exposed to the solvent and titrate normally (Tanford, 1968 review), (3) the specific viscosity of proteins in urea and guanidine HCl is very high compared to the specific viscosity of the native globular forms and increases in value on lowering the temperature (e.g. Hamaguchi and Kurono, 1963). Direct binding experiments have given evidence that urea and guanidine HCl are binding to proteins (e.g. Gordon and Warren, 1968 and 1970). From (1) the temperature coefficient with increased binding at low temperatures and (2) from consideration of the only plausible mechanism of similar interaction with PP II, the position of the binding must be at the backbone peptide carbonyl.

Other mechanisms of interaction of urea and guanidine HCl are not ruled out. Thus we can make use of the data of Herskovits et al. (1970). The denaturing ability of the urea derivatives is given by them in terms of the concentration of the denaturant required to produce a 50% change in any physical or macromolecular parameter that can be used to follow the unfolding process of the protein under investigation. In terms of this parameter they found the effectiveness of the ureas as a class of protein denaturants to increase with hydrocarbon content and alkyl substitution of the urea. In terms of possible interaction with the side-chains this points to a predominantly hydrophobic mechanism of protein denaturation,

if we mean the initial destabilization of the globular form. Actually, an analysis of their data in terms of the ability of the different reagents to effectively change the optical rotatory dispersion of myoglobin would order the substituted ureas in an exact reverse series, with the unsubstituted urea being the most effective. This is most likely due to better binding ability to the backbone when the denaturant can fit in close to the chain.

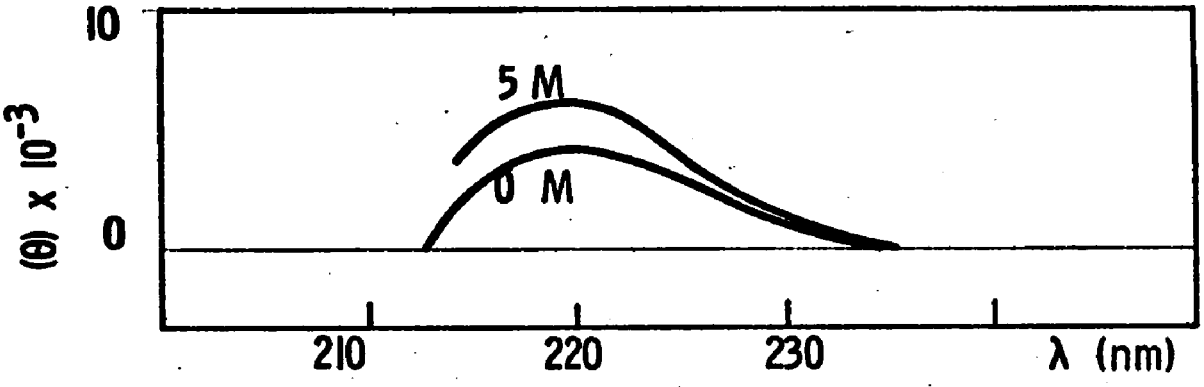



Fig. 5-1. CD of poly-L-lysine(pH 7), 25°, Effect of 5 M urea.

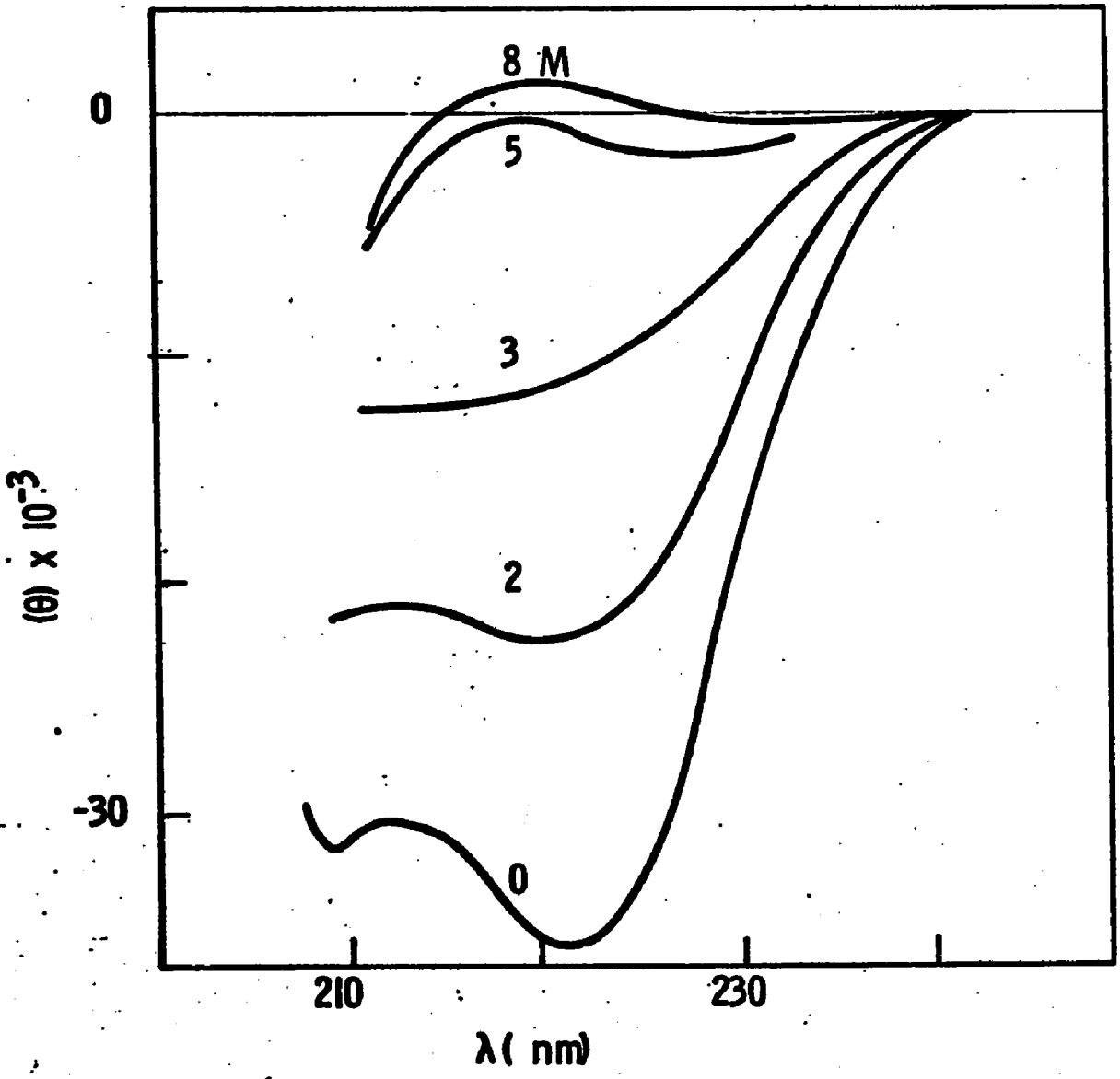



Fig. 5-2. CD of poly-L-lysine in 0.01 N NaOH plus added urea.

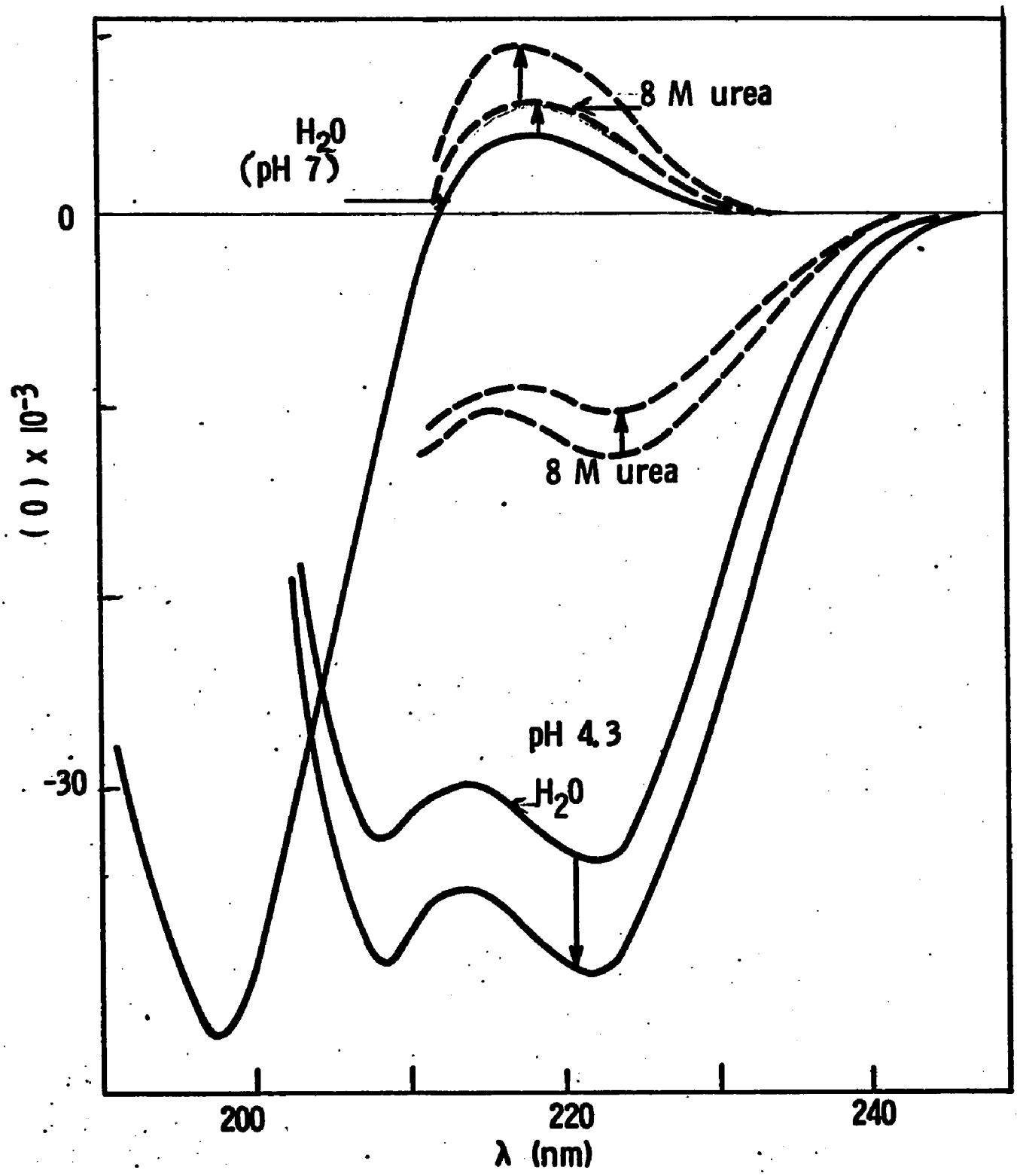



Fig. 5-3. Poly-L-glutamic acid: effect of 8 M urea on ionized and on neutralized polymer, and changes with temperature. Arrows ⟶ indicate a lowering of temperature from 25° to 5°.

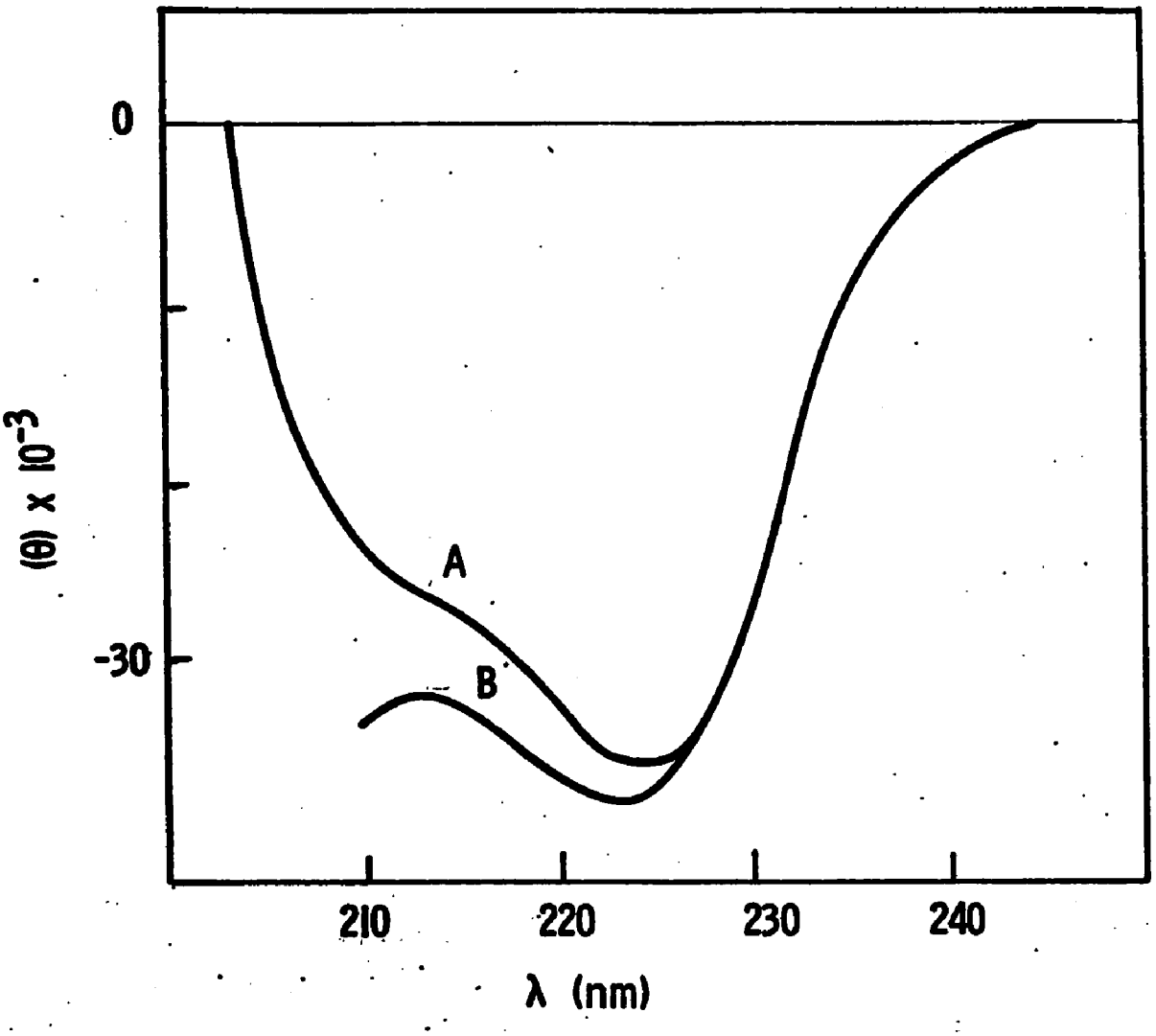
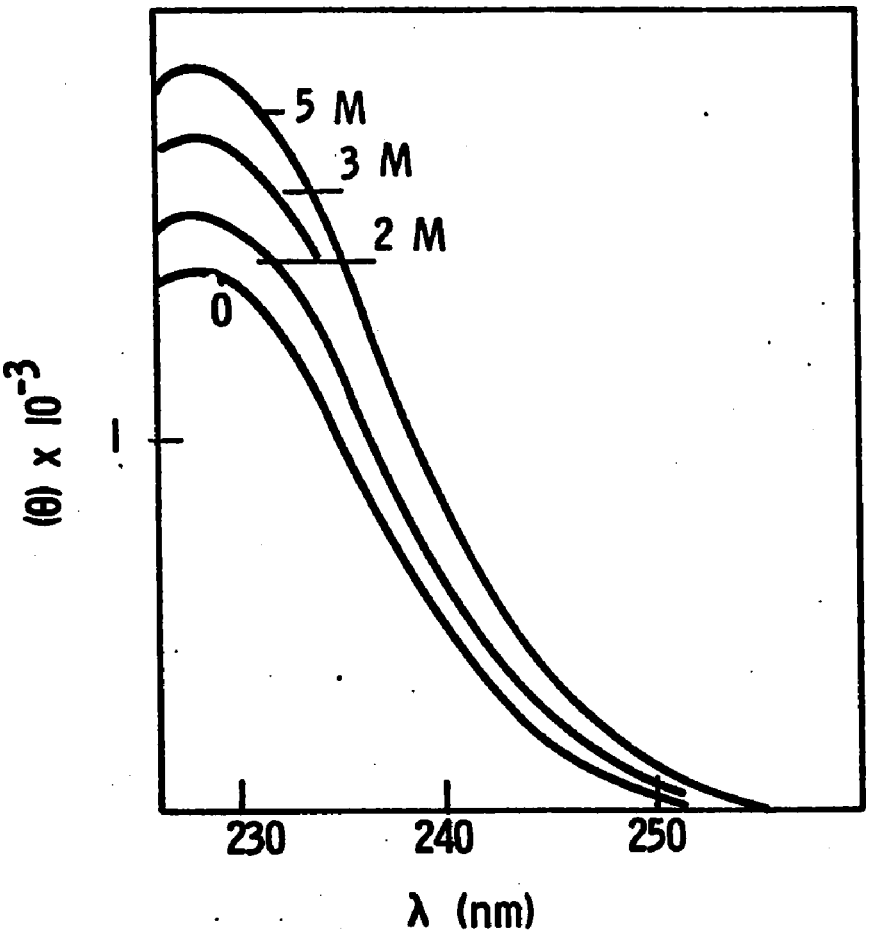



Fig. 5-4. CD of highly aggregated sample of poly-L-glutamic acid (A) is disaggregated by 5 M urea (B).



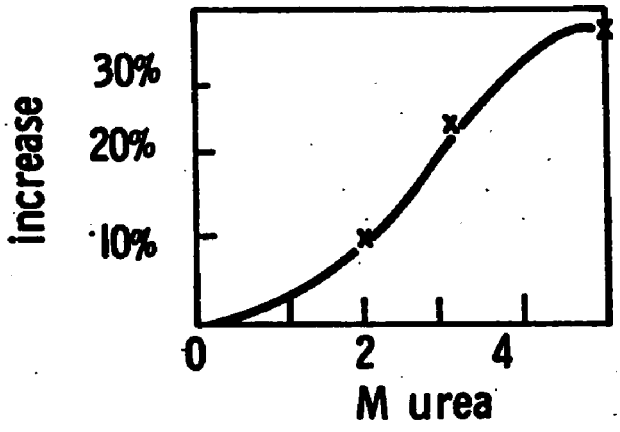




Fig. 5-5.

Effect of urea on the positive CD band of poly-L-proline, mol. wt. 1550.

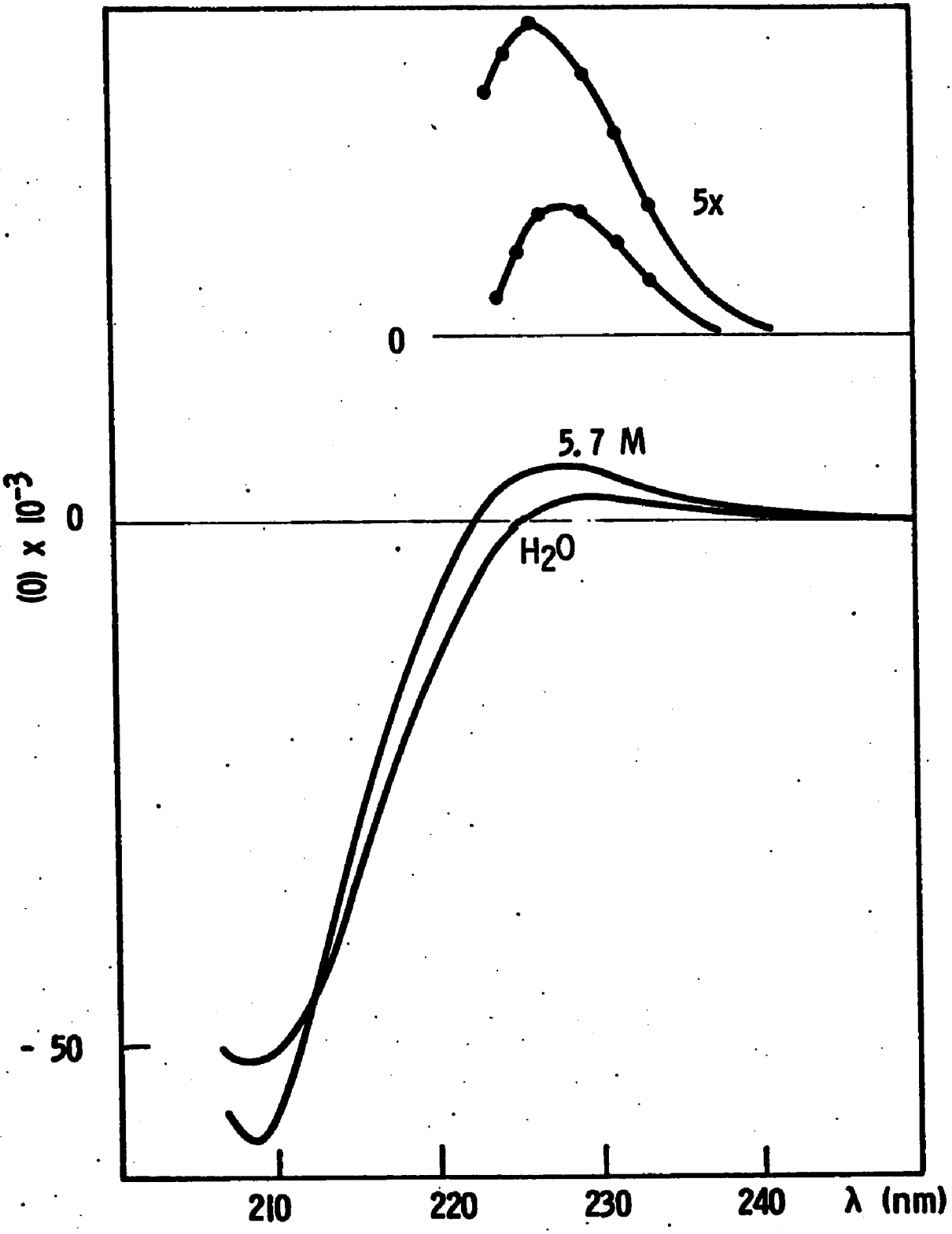



Fig. 5-6. Effect of ultra-pure guanidine hydrochloride on poly-L-proline, mol. wt. 11,500, form II.

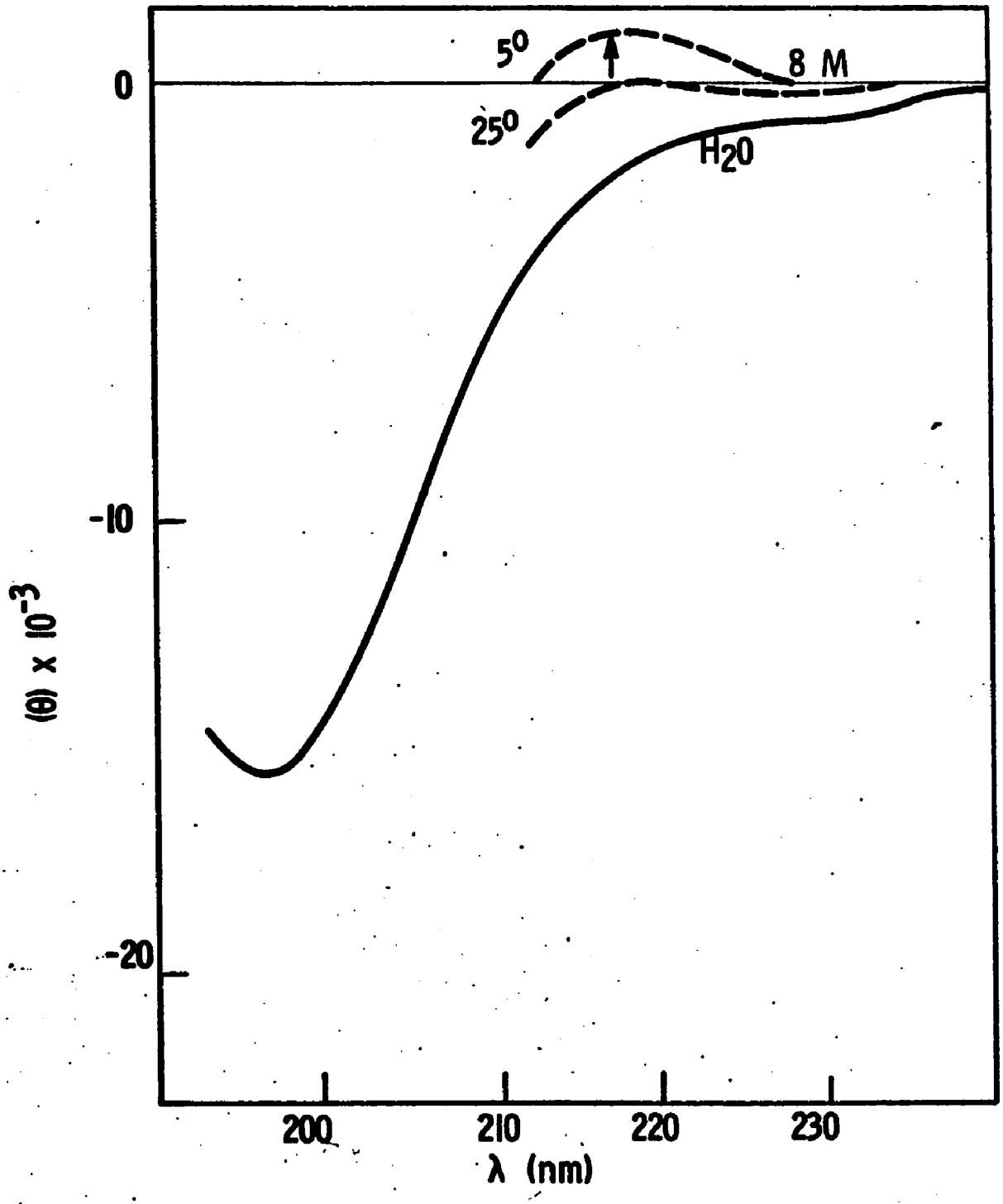



Fig. 5-7. CD of $A_1$ protein of Eylar: effect of 8 M urea: —— native, in $H_2O$, — — denatured in 8 M urea 25° ⟶ 5°

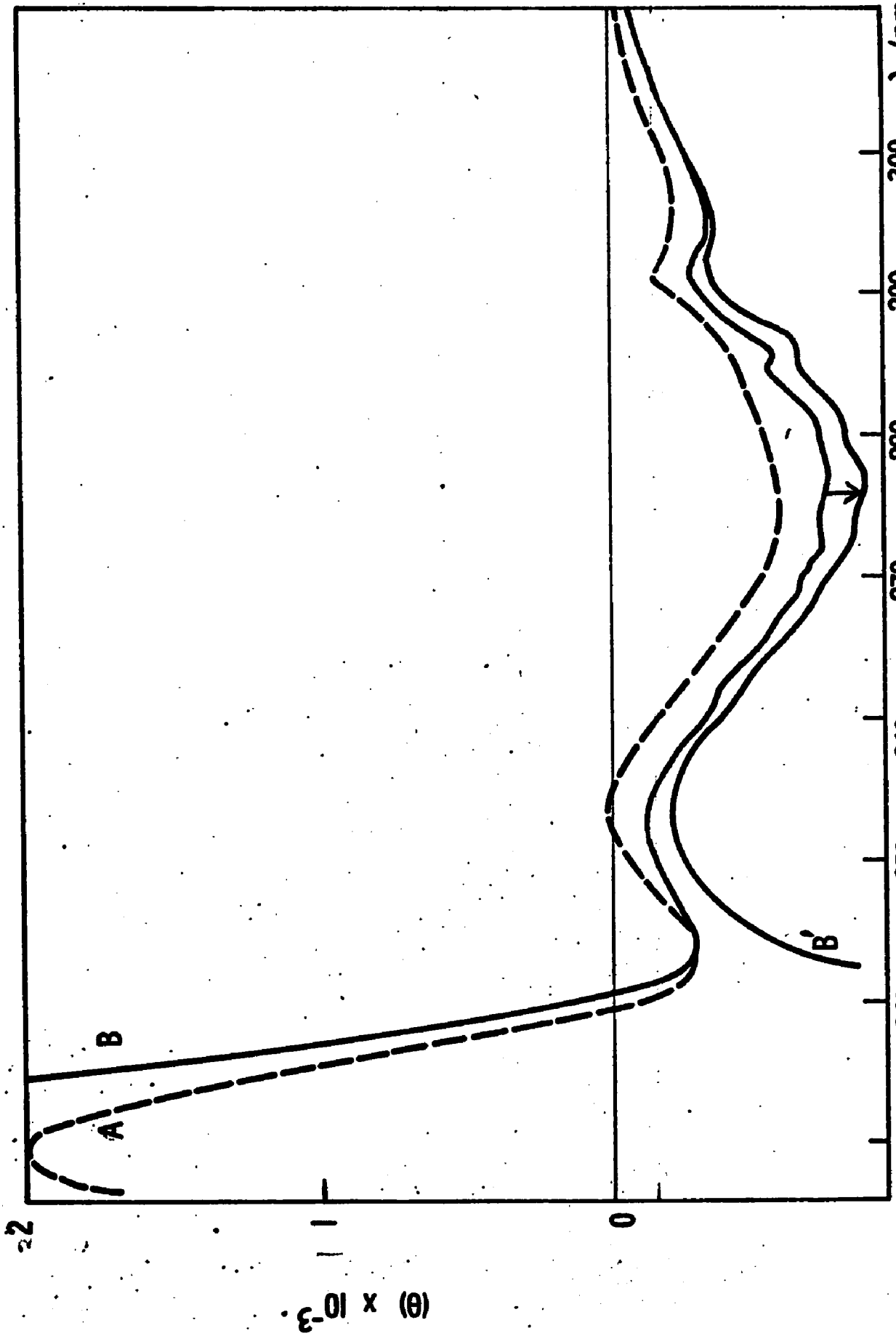



Fig. 5-8. CD of soybean trypsin inhibitor: (A) — in $H_2O$, (B) in 9 M urea at 350 (B') at 45°.

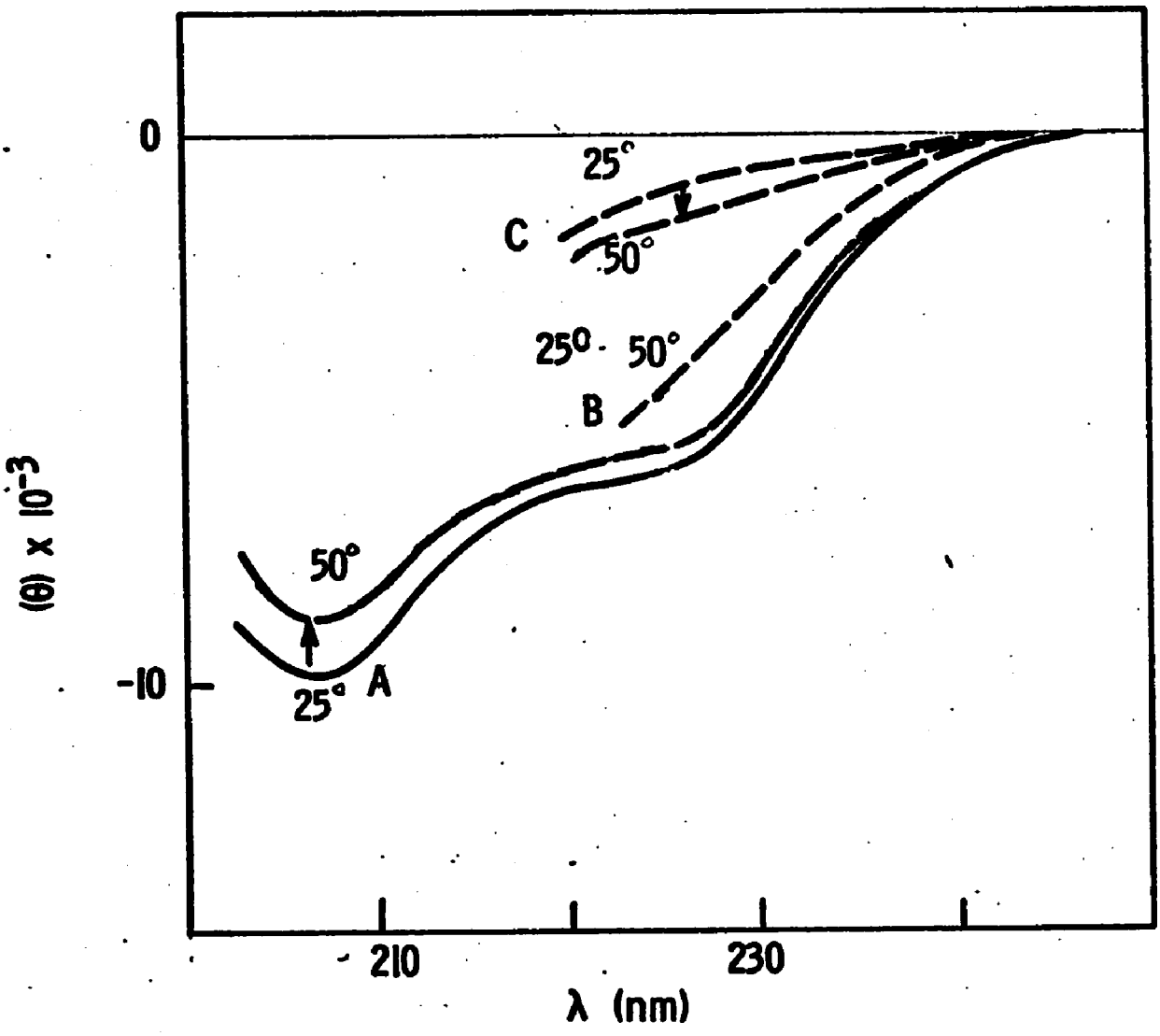



Fig. 5-9. CD of lysozyme: effect of 8 M urea with and without reducing agent (1.26 g/l $Na_2SO_5$). (A) in $H_2O$ , $25^{\circ} \rightarrow 50^{\circ}$, (B) in 8 M urea there is little effect until raise the temp. to $50^{\circ}$, (C) a much larger effect in 8 M urea plus reducing agent $25^{\circ} \rightarrow 50^{\circ}$.

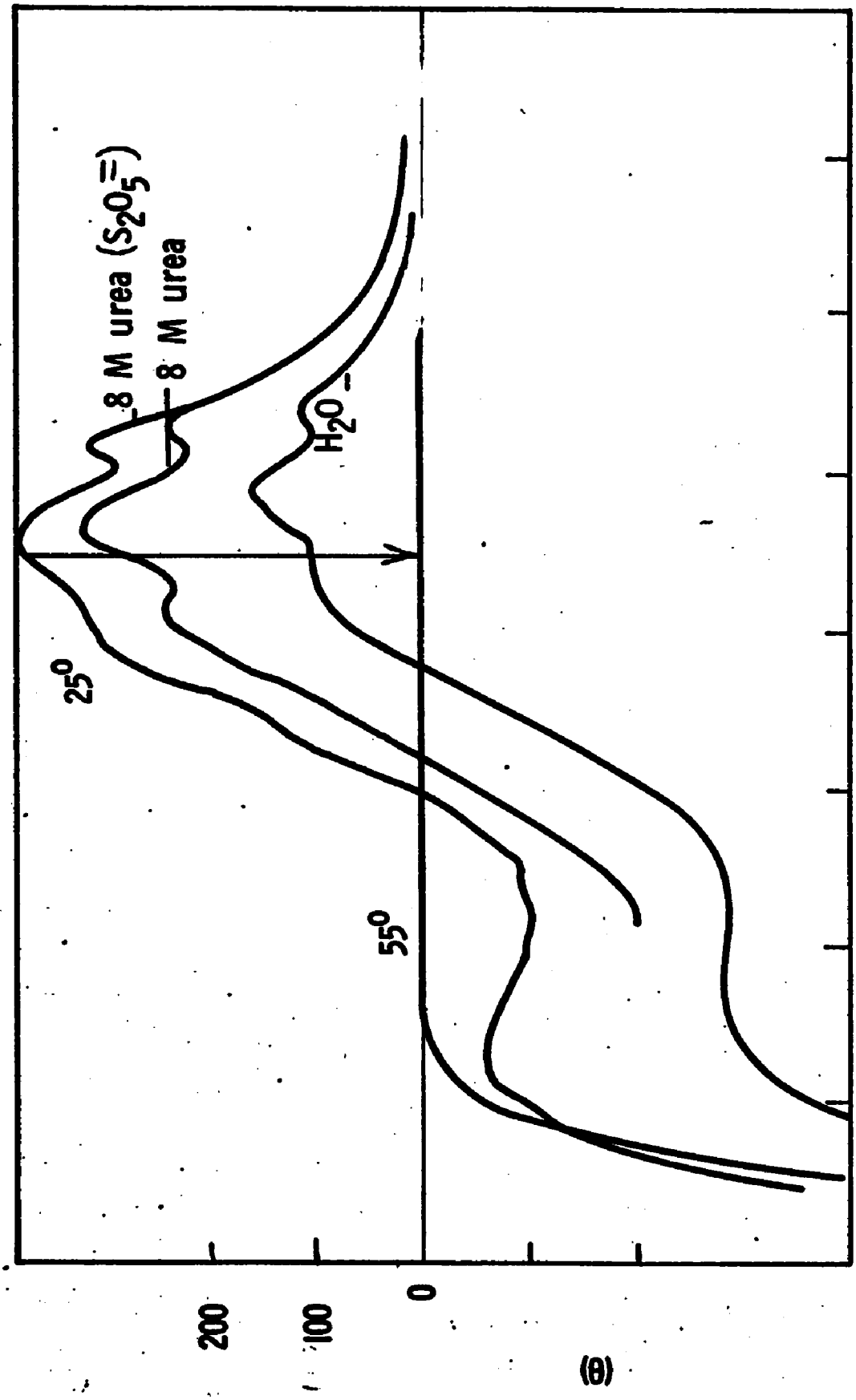



Fig. 5-10. Near uv CD of lysozyme: effect of 8 M urea plus reducing agent. An increase in the intensity of the bands is followed by complete elimination.

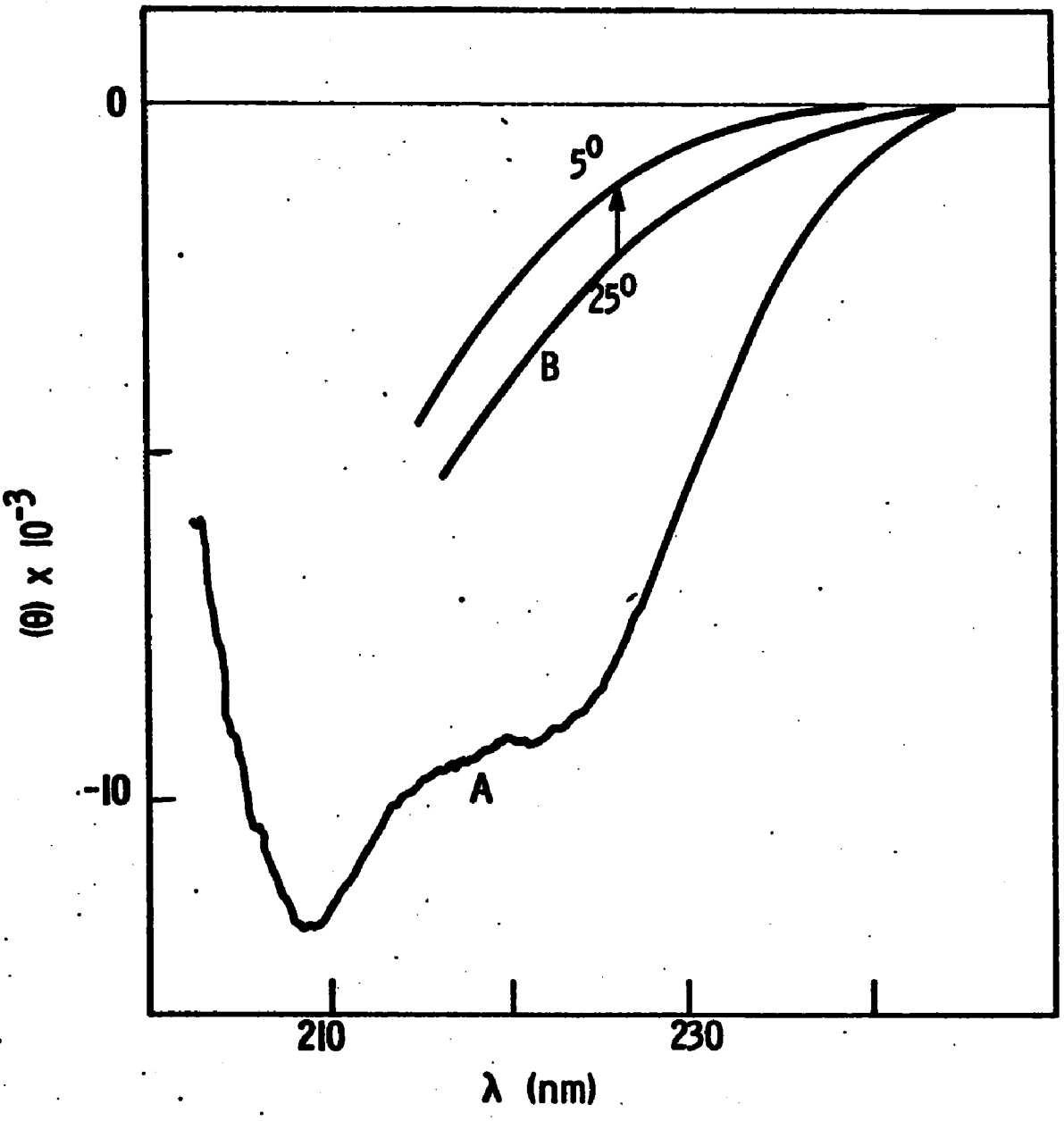



Fig. 5-11. CD of insulin, pH 1.5: effect of 9 M urea: (A) in $H_2O$, pH 1.5, (B) plus 9M urea $25^0 \rightarrow 5^0$.

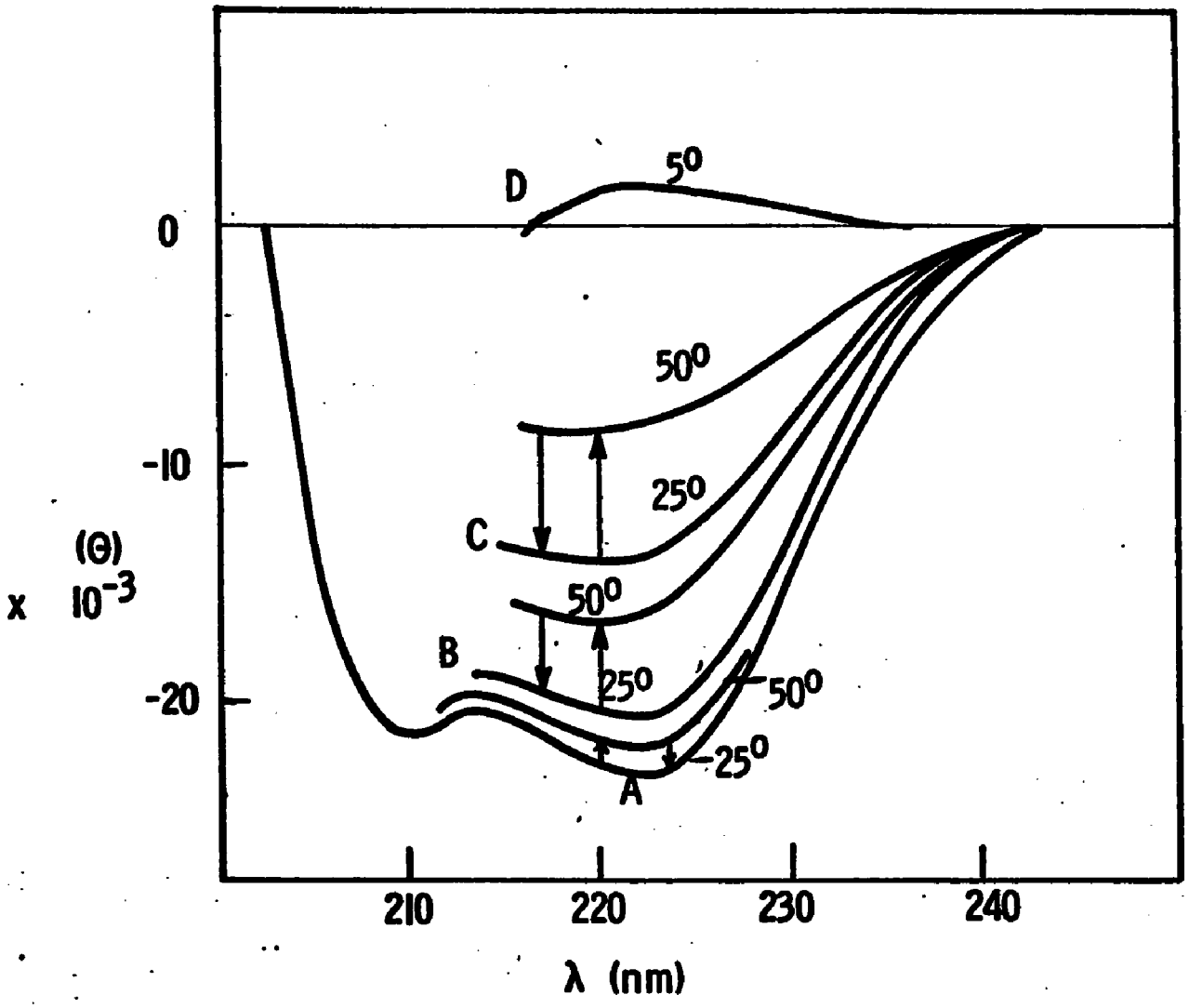



Fig. 5-12. CD of myoglobin (sperm whale): effect of heating in different concentrations of urea: (A) equilibrated in 0 to 6 M urea; (B) equilibrated in 8 M urea; (C) equilibrated in 9 M urea; (D) sample heated to 95° for 5 min. then cooled to 5° in 9 M urea.

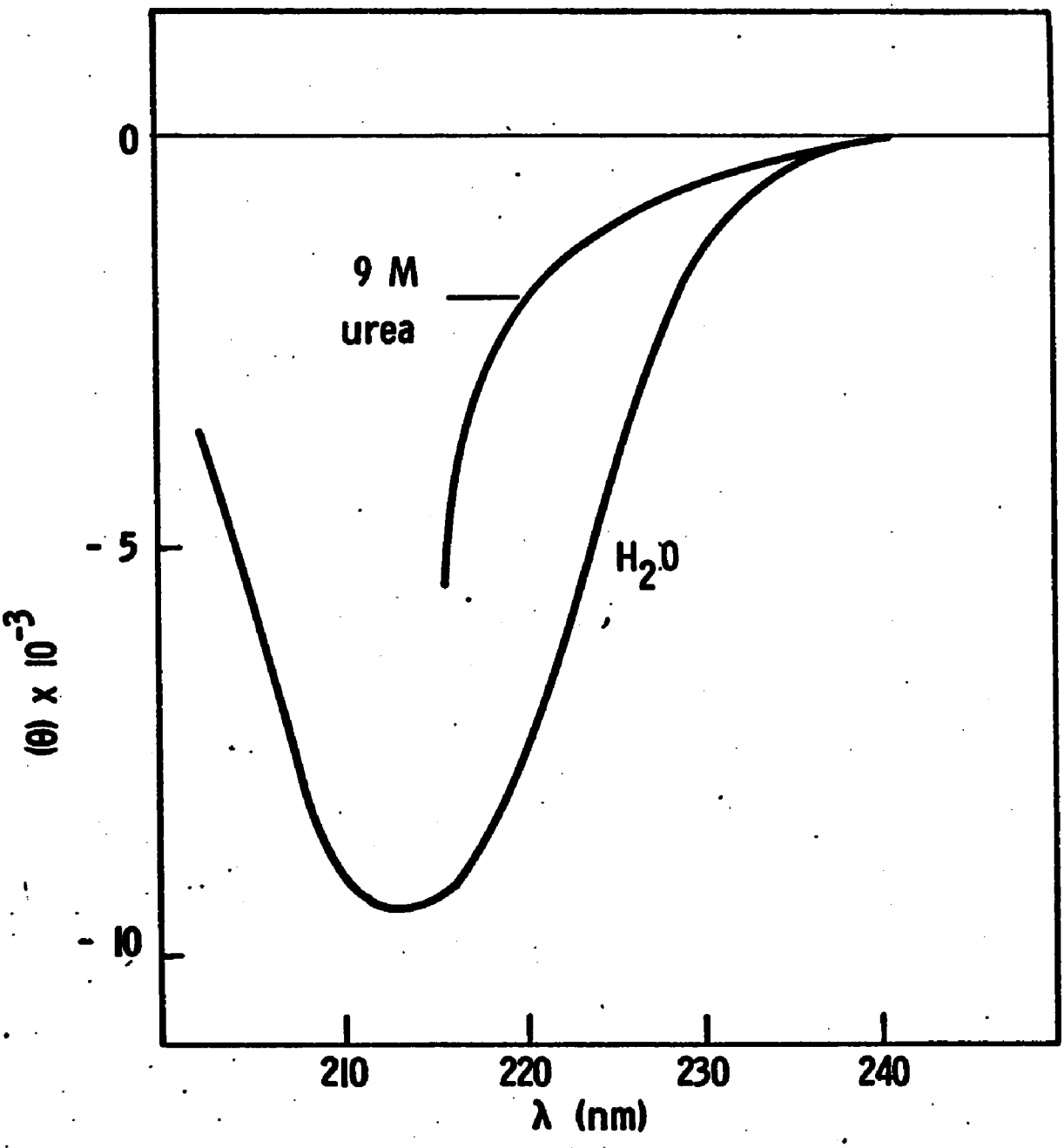



Fig. 5-13. CD of pepsin: effect of 9 M urea at 25°.

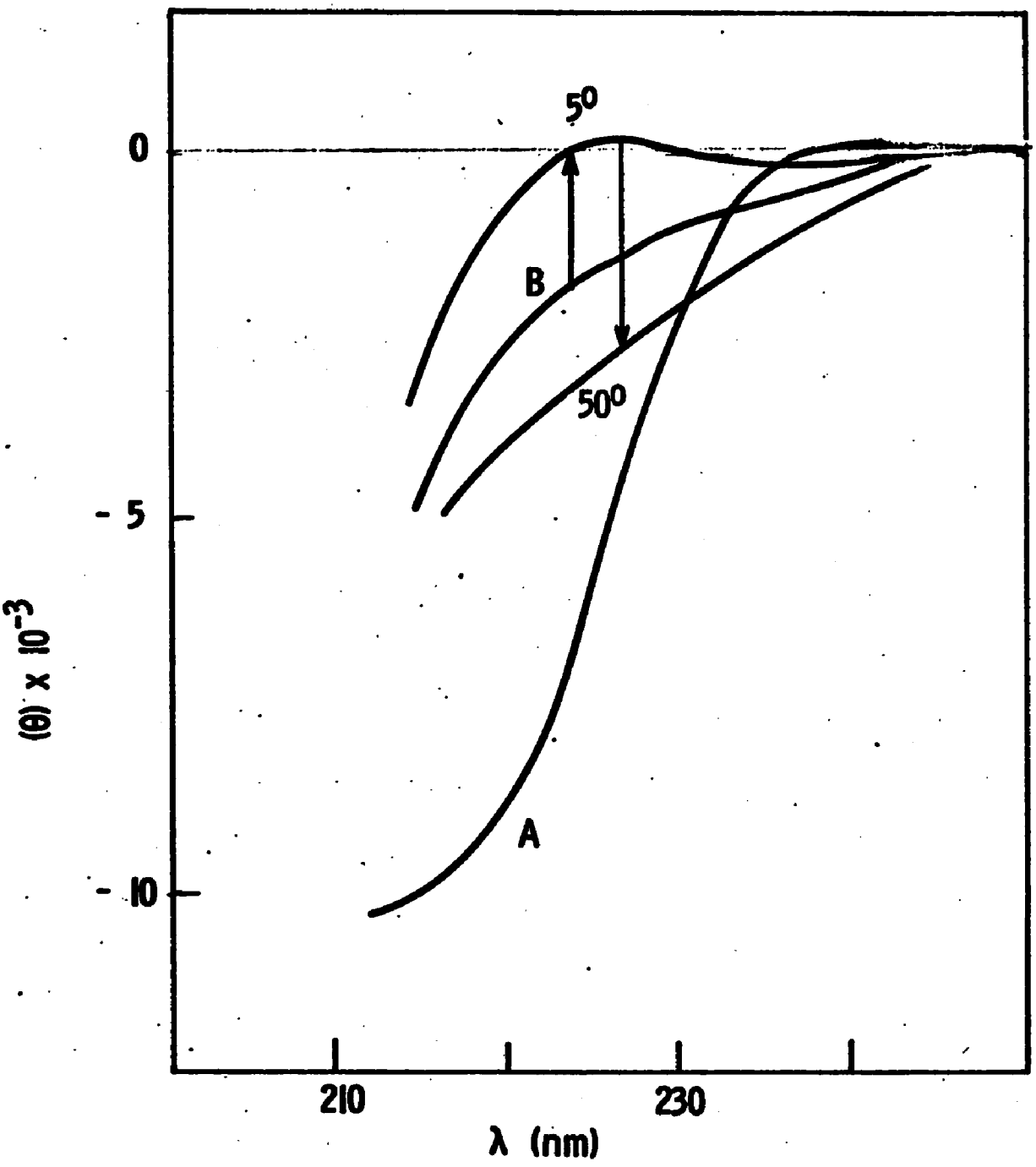



Fig. 5-14. CD of ribonuclease: effect of temperature in 8 M urea. (A) in $H_2O$, (B) in 8 M urea, initially at 25°, then temperature lowered to 5°, then warmed to 50°.

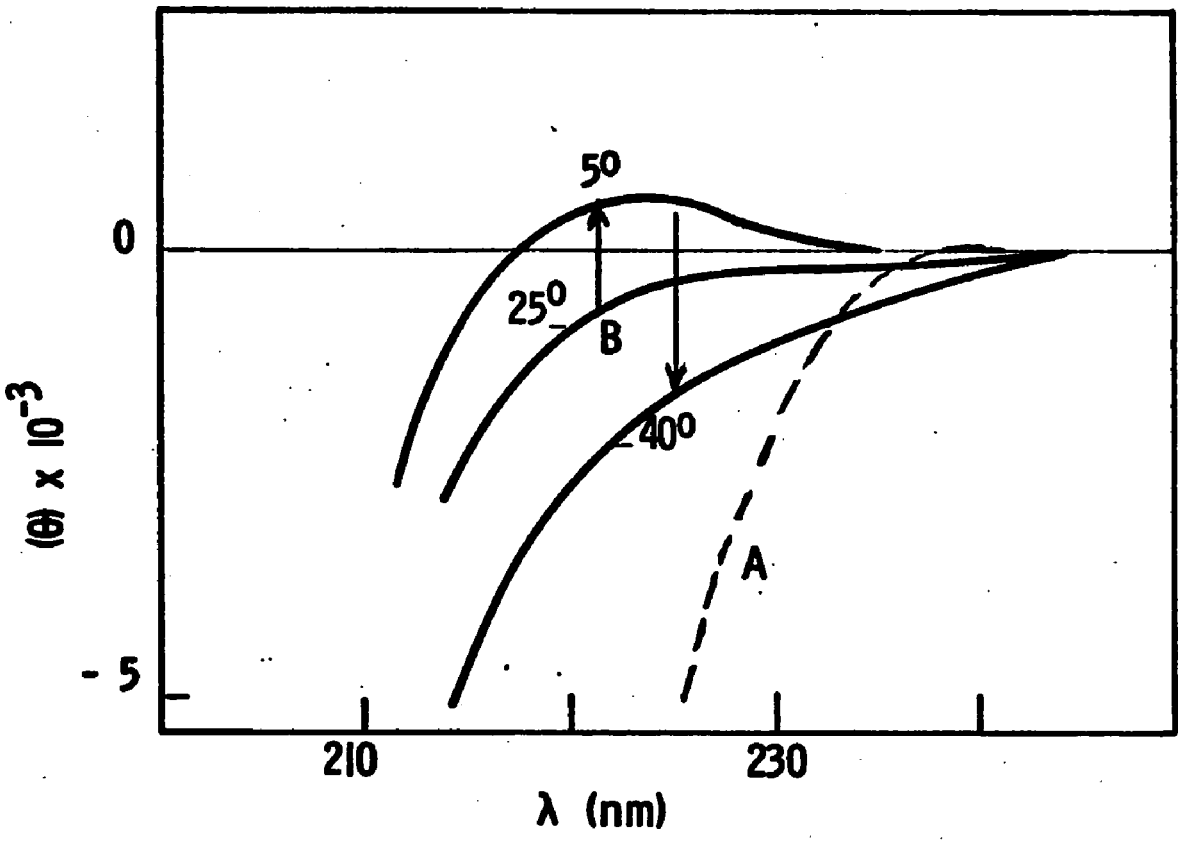



Fig. 5-15. CD of ribonuclease: effect of temperature in 5.7 M solution of ultra-pure guanidine hydrochloride: (A) in $H_2O$; (B) in 5.7 M guanidine hydrochloride $25^{\circ} \rightarrow 5^{\circ} \rightarrow 40^{\circ}$

## Chapter 6 Solvent Interaction

### A. Alcohols.

One method of disrupting a globular protein is through the use of alcohols and other nonpolar solvents. Doty introduced the use of chloroethanol (Doty, 1960), and found that it increased the negative $b_0$ values obtained from optical rotatory dispersion for tropomysin, insulin, bovine serum albumin, ovalbumin, lysozyme, pepsin, histone, ribonuclease and globin (H). This was taken to indicate an increase in right-hand alpha helicity over that obtained in water solutions. He suggested this was due to a shift in the balance between intermolecular hydrogen bonding from the protein to water to intramolecular hydrogen bonding within the molecule. The gradual addition of chloroethanol (CE) to a water solution of ribonuclease was reported by Weber and Tanford (1959) to cause a two stage transition. The first was postulated to be an unfolding or partial unfolding of the native structure and the second was said to be a refolding into a new conformation at high concentrations of CE. Similar results were reported for diisopropiphosphoryl chymotrypsin (Martin and Bhatnagar, 1967) in CE-$H_2O$, and for β-lactoglobulin in dioxane-$H_2O$ (Tanford et al., 1960). Other alcohols can be used to produce effects similar to CE if some acid is present. Thus Kurono and Hamaguchi (1964) showed that aggregation caused by methanol could be prevented by the addition of HCl. The acid is added to remove the protein from being near its isoelectric point. Tanford et al. (1960) stressed that the alcohols provide a

hydrophobic environment for the formation and stability of the hydrogen bonded α helix. Sun (1969) suggested that in the case of bovine serum albumin, in the acid region, 33% alcohols and dioxane seem to be altering the ionic interaction through change in pK of carboxyl groups by lowering the dielectric constants of the media. However, it should be noted that the dielectric constants of the simple monohydric alcohols is a function of chain length, decreasing as the number of carbons increases from one to four. Thus, Harrap (1969), studying the decrease in the denaturation temperature of collagen, decided that the lowering of the denaturation temperature was a function of the number of carbons in the straight-chain alcohols. And, already in 1962, Schrier et al. had pointed out that the effectiveness of alcohols in lowering the transition temperature of ribonuclease increased with chain-length. Most interesting Schrier et al. (1965) found that the transition enthalpies were the same for all alcohols and averaged to 137 kcal./mole. This is the same value obtained in water alone. Thus the lowering of the transition temperatures by alcohol was said to have an entropic origin. This was related to hydrophobic bonding, and the suggestion was made that this takes place by mirror image, or slot positioning of the nonpolar group on the alcohol to a side-chain on the polypeptide. Chauduri and Yang (1968) said that alcohols, in general (1) change the pK of ionization of the side-chain ionizable groups, reducing the repulsion between like charges, (2) lower the water activity thus decreasing the competition of water for hydrogen bonding

to the amide linkages, and (3) lower the hydrophobic bonding between side-chains so that stabilization by side-chain interaction becomes less important. Krimm, Mark and Tiffany (1969) noted that the extended helix, in the charged polypeptides, should and does collapse as the dielectric constant is decreased by the substitution of alcohol for water. This was discussed in terms of the counterions being attracted to the side-chain charges, thus balancing out the repulsive forces of the side-chains for each other and allowing steric forces to give an $\alpha$ helix.

That the conformation taken by most proteins when exposed to high concentration of alcohols is an $\alpha$ helix has been shown by the appearance of the typical $\alpha$ helix ORD or CD spectrum [e.g. histones, casein, chymotrypsinogen, soybean trypsin inhibitor in n-propanol (Jirgensons, 1967), histones in CE (Inoue and Timasheff, 1968), insulin in acidic methanol, ethanol, propanol or in CE (Menendez and Herskovitz, 1970), ribonuclease in CE (Hashizume et al., 1967), myoglobin in acidic ethanol (Hermans et al., 1969)]. Reduced residue rotation at 233 nm also has been used to indicate $\alpha$ helicity.

## Experimental

In order to further understand the effect of alcohols on protein and polypeptide structure, we have looked at several different proteins in trifluoroethanol (TFE), using different concentrations of reagent and varying the temperature in some cases. Fig. 6-1 shows the effect of TFE on collagen. Only a small amount of TFE is needed to disrupt the native structure.

Higher concentrations (60% to 90%) seem to further randomize the separated single strands. $A_1$ protein (Fig. 6-2) in different concentrations of TFE forms a family of curves, with an isodichroic point at 202 nm. An initial rapid change in conformation with increase in alcohol is followed by a slower change (in the concentration of TFE range 40% to 80%), but a large change then ensues, particularily in the range of 90% to 99% TFE and the system reaches approximately 70% $\alpha$ helicity. Bovine serum albumin (Fig. 6-3), under our conditions, approximately doubles its helicity. Lowering the temperature to 5° tended, at all concentrations of alcohol, to give greater helicity. Lysozyme (Fig. 6-4) in TFE gave very similiar results to lysozyme in CE (Timasheff, 1970). 90% iso-propanol gave a similiar CD to that produced by 90% n-propanol $[(\Theta)_{210nm} = -18,000]$. 90% methanol gave $[\Theta] = -19,300$, while that of 90% TFE was -22,000. Pepsin (Fig. 6-5) opened up rather easily with 30% TFE, then slowly transformed as the concentration of TFE was increased to 60%. Between 90 and 99% there was a further drastic change. This pattern of transformation... seems typical of all proteins which have initially only a small percentage, or none, of $\alpha$ helicity. In the case of ribonuclease (Fig. 6-6) the effect of varying the temperature was tried on a sample in 30% TFE and on a sample in 90% TFE. Lowering the temperature decreased the negative dichroism in the 30% TFE sample but increased the negative dichroism in the 90% sample. This suggests that a random state in TFE-$H_2O$ may exist for an intermediate range of concentrations in this case. Whereas only a modest

change was recorded ( $-(m)_{233}$ from a value of 3 $x10^{-3}$ in the native state at pH 2.8 to 4 $x10^{-3}$ in 8 M/1, pH 2.8) for chymotrypsinogen in propanol (Herskovits et al. 1970a) a large effect was found by us for chymotrypsinogen in 95% TFE (Fig. 6-7). It is possible that the small change seen by Herskovitz was due to the protein forming an aggregated state such as in our case of acidic 90% methanol. The interesting shape of the CD of the native state of chymotrypsinogen should be noted. The 225 nm + peak on the negative slope is evident in the work of McConn et al. (1969), but we have carried the spectrum further into the uv. We see that a similar + band appears in the CD of chymotrypsin and that it increases in dichroism (becomes more +) while the stronger negative band near 200 nm also increases (i.e. becomes more negative) when the temperature is dropped from room temperature to 8° (Fig. 6-8). That these bands may be related to part of the molecule that is extended might be inferred from our work on the extended state. X-ray diffraction studies of Freer et al. (1970) also indicate that the backbone chain is more or less fully extended. It is said to double back on itself to form sections of distorted anti-parallel pleated sheets. No band was noted by Fasman et al. (1966) in the 225 nm region of the CD spectrum of chymotrypsinogen, nor was the near uv CD correctly detailed. It is interesting that chymotrypsinogen and chymotrysin have similiar near uv CD

(Fig. 6-9). Myoglobin, in different alcohols, has been extensively studied by Herskovits et al. (1970a). They concluded that alcohols as a class of denaturing agents act primarily through their effects on hydrophobic bonding within the protein, and that the effectiveness of the alcohols increased with chain length of hydrocarbon content of the alcohol. Denaturing ability was related to the relative lowering of the solvent denaturation midpoint (i.e. the concentration or volume fraction of denaturing agent required to produce a 50% change in the particular parameter employed to follow the unfolding or denaturation transition). Like Hermans et al. (1969), they note an initial decrease in helicity at low concentration of alcohol (i.e. up to 40% ethanol) then an increase in helicity with increasing concentration of alcohol. It is interesting that methanol, ethanol, 1-propanol and 2-choloroethanol, when used in concentrations of 99% v/v, in 0.01 N HCl, all achieved approximately the same end result in making the molecule close to 100% helical. Herskovits et al. (1970a) did not indicate the fact that the initial decrease in helicity is due to the indirect cause of removal of the heme group and not to be expected in the general case. That the heme is removed by ethanol was shown by Hermans et al. (1969) by centrifuging the solutions. A precipitate of hemin was obtained at the bottom of a yellowish solution. In our own experiments, we show the effect of increasing amounts of TFE. Our results are what would be expected on the basis of the work of Herskovits and Hermans (Fig. 6-10). It is interesting

to note the almost complete elimination of dichroism in the near uv (Fig. 6-11) in this molecule that lacks S-S bonds. In the more general case of proteins that possess some S-S bonds, we obtain a negative dichroic band in the 260 to 280 nm region of the CD spectrum when the solvent is 90% TFE (Fig. 6-12). One particular molecule, in which the S-S bond is present in large quantities, is insulin. Menendez and Herskovits (1970) have reported CD data using acidified (0.01N HCl) 99% alcohol solutions. The alcohols used were methanol, ethanol, propanol and ethylene glycol as well as 2-chloroethanol. Using the CD parameters of Greenfield and Fasman (1969) they found that the formal estimates of helix content change from 27% to 30% for the native protein in aqueous solutions to 40% to 80% in acidic 99% methanol, ethanol, or propanol. They found less negative $b_o$ and CD parameters at 209 and 222 nm in acidic ethylene glycol solutions than in water solutions. To explain this, they suggested that the insulin dimer unit is dissociated in this solvent. Our data (Fig. 6-13) suggest a different interpretation. Using different percentages of TFE we obtain a family of CD curves. In 99% TFE there is a doubling of the depth of the 209 nm band and 1.6x increase in depth of the 222 nm band over that found in the native insulin in water. The large relative difference in the depths of the two bands could be an indication of the inability in this molecule to form long stretches of $\alpha$ helices. In 90% ethylene glycol (Fig. 6-13) we find, contrary to Menendez and Herskovits (1970), that the CD curve indicates a more random

conformation than the native. The dichroism is deeper in the 209 nm region and more shallow in the 222 nm region than in the native case. It should be noted that the CD curves of insulin in 20-40% TFE are of similar shape to those obtained using 90% ethylene glycol, so it may be that the second solvent is just less effective in causing the conformational change. It is also obvious from the family of curves in different percentages of TFE that intermediate concentrations of TFE do not give states whose dichroism can be made up by summing part of the native and part of the 99% TFE CD curves. Therefore an intermediate state occurs. This is probably due to an initial loosening of the helical part of the molecule in the native state. This phenomenon is not obvious in bovine serum albumin or apomyoglobin (Figs. 6-2 and 6-10). The next protein molecule we wish to discuss is silk. Ordinary silk (Bombyx mori) has a large percentage content of the amino acid glycine (43%) and of the amino acid alanine (31%) and 10% is serine. Iizuka and Yang (1968) have reported the CD of silk in water, salt, urea, alcohol, and dioxane solutions. They concluded that silk fibroin in aqueous media has a disordered conformation, but that the molecule is less extended than would be true of a random coil. They described a transformation of coil-to-$\beta$ conformation of silk in methanol-water and dioxane-water solutions. Most interesting, they found that salt (0.1M NaCl) prevents a 1:1 v/v solution of dioxane-water from initiating in silk the $\beta$ conformation, which forms if the salt is omitted. In the review article on "The Properties of Proteins

in Nonaqueous Solvents," Singer (1962) points out that salt may increase the solubility of proteins in weakly protic solvents. Since the β state is an aggregated form, addition of salt may, therefore, keep it from forming. In 90% TFE there was a slight tendency to form a β structure (Fig. 6-14). Lowering the temperature aids the transformation. Soybean trypsin inhibitor was also tested with TFE (Fig. 6-15). The results could almost be predicted. Thus after a resistant start, 10% TFE having no effect, the molecule comes apart at 20% TFE, then slowly forms an α-helix as the alcohol concentration is increased.

Discussion

The interaction of alcohols with polypeptides and proteins is complicated by the possibility of several simultaneously operating mechanisms.

(1) Interaction by "apolar bonding" with the side-chains to disrupt the energy balance has been stressed by Scheraga and coworkers (Schrier et al., 1965). This seems to be the predominant mechanism for most proteins. In favor of this interpretation we have the evidence from Schrier et al. (1965) that in the case of ribonuclease the length of the chain of the alcohol and lack of branching are important factors in whether or not an alcohol is a good denaturant. Most important, however, is the necessity that the transition is entropy dependent since $\Delta H$, with and without alcohols, is the same. That hydrogen bonding to the backbone carbonyl is not to be considered as important follows from (1) the fact that the

conformation taken by the proteins (with the exception of silk and collagen) seems to be an $\alpha$-helix, and not the more open extended structures that occur when urea, guanidine HCl and monovalent cations bind to the carbonyl, and (ii) in the case of poly-L-proline it had been shown by infrared that n-butanol and ethanol are bound less strongly to the carbonyl than is trifluoroethanol (Strassmair et al., 1969 a and b). The alcohols, n-butanol and ethanol favor the more closely packed I conformation of poly-L-proline whereas TFE stabilizes the more open II form. Still, in the case of the simple proteins all the alcohols seem to lead to the same $\alpha$-helical state. The amount of helix that develops will depend on the particular sequence of amino acids in the protein and residual crosslinks that have not been broken by the alcohol. The high reversibility to revert immediately to native form on diluting out the alcohol points to the lack of breakage of S-S bonds in the proteins tested. The near uv negative band in the 270-280 nm region of these proteins in alcohol could be due to S-S optical activity remaining under these conditions. Ordinary silk cannot form an $\alpha$ helix, probably due to its high glycine content, and in alcohol (including TFE) it tends to interact with itself, and not the solvent, and takes a $\beta$ structure. Collagen cannot form an $\alpha$ helix because of its pyrrolidine content, and if it is denatured by alcohols can only become a random coil. The interactions leading to reformation of a triple helix after denaturation in the presence of alcohol must be weakened. Collagen is complicated by having polar and

apolar regions along the chain of a fiber. Russell and Cooper (1969) choose to consider alcohols as directly binding to the backbone of collagen chains. Any hydrophobic effects are considered to be indirect and to apply only by influencing the immediate aqueous environment which stabilizes the hydrogen-bond formation at neighboring polar groups. This rather begs the issue since they choose to ignore inter-chain bonding outside of hydrogen-bonding. Also they consider direct hydrogen-bonding of alcohols "to promote rotational freedom about the main bonds of the polypeptide chain" in the case of poly-L-proline as a causative in the transformation II to I. But the alcohols that lead poly-L-proline to transform to I are those which do not bond to the carbonyl as well as water itself. Since this mechanism does not work for poly-L-proline it should not be suggested for other molecules where it is equally unlikely.

(2) In the case of poly-L-proline we have a simple model compound where only binding to the backbone carbonyl by the solvent need be seriously considered. Here, binding to the backbone carbonyl does lead to II, the form which is more open and available to solvent interaction. Thus solvents which cannot donate hydrogen or are poor hydrogen bond agents lead to form I. Take for example: dioxane, methanol, ethanol and n- and isopropanol, and butonal. The fact that PP stabilizes in form II in TFE only tells us that TFE is better at hydrogen bonding to the backbone carbonyl than non-fluorinated alcohols but does not tell us if it is better or poorer than water which also leads to form II.

(3) In special cases where electrostatic forces are dominant in determining the stability of a conformation, such as the ionized polyelectrolytes and some proteins which may be strongly stabilized by charge interaction, the solvents with the low dielectric constants will be most important in producing change, but not necessarily toward helicity.

B. Dioxane

Dioxane is not like an alcohol in its interaction with a polypeptide. Thus it has no possibility of hydrogen bonding, nor is its shape suitable for the "slot" mechanism of interaction with linear side-chains. Its most outstanding property seems to be its low dielectric constant ($\epsilon = 2.2$). It therefore has the possibility of interferring with charge interactions. We find that it denatures proteins but does not give the same intermediate conformational states that occur when alcohols are used. Thus CD spectra indicate that dioxane leads to random structures for ribonuclease (Fig. 6-16) and lysozyme (Fig. 6-17). In keeping with our CD results, Hamaguchi and Kurono (1963) reported that $b_0$ of lysozyme decreased in magnitude for concentrations of dioxane greater than 50% v/v. We have some indications that the amount of dioxane needed to destabilize a protein is very sensitive to the pH or charge on the molecule, so different laboratories may report different effects of this denaturant. Thus, Weber and Tanford (1959) reported that dioxane-water was quite similar to the system chloroethanol-water on ribonuclease and suggested that it was causing a partial conversion of ribonuclease to the helical

form. They did not report any data, however. In the case of β-lactoglobulin, at pH 3 and ionic strength 0.02, Tanford et al. (1960), found $b_o$ approaching -400° for 75% dioxane from the initial value of approximately -20° in water solutions. This is strong indication that the conformation was going toward α-helix.

No broad generalizations as to the final state can be made. The inability of dioxane to hydrogen bond to the carbonyl backbone, however, is probably the reason we have found that PP goes from form II to I in 70% v/v water solutions. Higher concentrations of dioxane caused precipitation.

C. Gem-diols

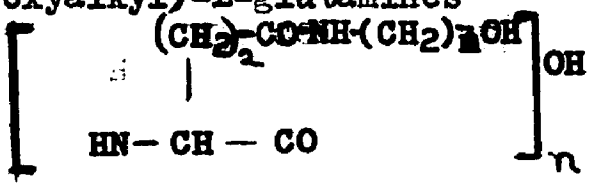
Hexafluoropropan-2, 2-diol (HFPD) is a gem diol and was described by Longworth (1964) to be a helix breaker for polypeptides. Goodman and Rosen (1964) found that a 27.8 molar solution of HFPD was helicogenic for poly-γ-methyl-L-glutamate (PMLG). Middleton and Lindsey (1964) have shown that the highly fluorinated gem-diols form much stronger hydrogen bonds with tetrahydrofuran than does trifluoroethanol (TFE) or hexafluoropropan-2-ol (HFID). We can, therefore, expect that HFPD hydrogen bonds to the peptide chain. Again, maximum interaction is achieved when the chain is extended, not randomly coiled. Thus, we are not surprised that a positive peak at 218 nm was found when HFPD was the dominant solvent (i.e. 52% HFPD: 48% HFIP (##) or (1 mole HPPD to 0.54 moles $H_2O$) for the polypeptide PMLG using CD to monitor the structural changes resulting from varying the solvent composition (Balasurbramanian

amd Roche, 1970). A transition was also observed when the polymer was poly-L-alanine, but the CD is not given in Balasubramanian and Roche's paper; therefore, we do not know if a 220 nm peak is possible when the side-chain is short.

D. Water

Water is known to hydrogen bond to amides and peptides. Thus formamide was shown by cryoscopic measurements to form a complex with one molecule of water per molecule of formamide (English and Turner, 1915). Polyglycine in the solid state was found to bind up to half a molecule of water per peptide group (Mellon et al., 1948). Crystalline myoglobin may have one water molecule each for the CO- and NH- groups on the surface of the protein molecule (Kendrew, 1962).

The configuration taken by a polypeptide is influenced by all the forces acting on the system. Side-chain interactions can have a profound influence on thermodynamic stability of polypeptide-chain conformations. In aqueous medium, hydrophobic (or nonpolar) bonding seems to be the main factor in shaping the stable form. Lotan et al. (1966) demonstrated the extent to which nonpolar interaction between aliphatic regions within the side-chains of amino acid residues stabilize the α-helical conformation in the homologous series of polymers of the $N^5$ -(ω-hydroxyalkyl)-L-glutamines




where m = 2,3 and 4. The influence of the elongation of the hydrocarbon section within the side-chains on helix stability

in the presence of water was clearly shown. (In TFE the length of the side-chains was unimportant; all were α helical at 24°.) The polymer with m equal 2 (PHEG) was shown by Adler *et al.* (1968) to have similar CD to ionized PGA when dissolved in water and to be α helical in methanol. If we accept the appearance of a positive peak at 220 nm in the CD spectrum to indicate an extended state, PHEG in water is an example of an extended structure due to steric interference of the side-chain (which is still bulky at m = 2), and with hydrogen bonding to water in place of internal hydrogen bonding. Lotan *et al.* (1966) plotted $b_o$ v.s. T for the series m = 2, 3, and 4. In the case of m = 2, $b_o$ went from a +50 to 0 when the temperature was raised from 0° to 75°. Probably the extended form was being randomized. It would be interesting to find other water soluble, nonpolar polypeptides with shorter side-chains in order to study the relative importance of bulky side-chains on extension per se.

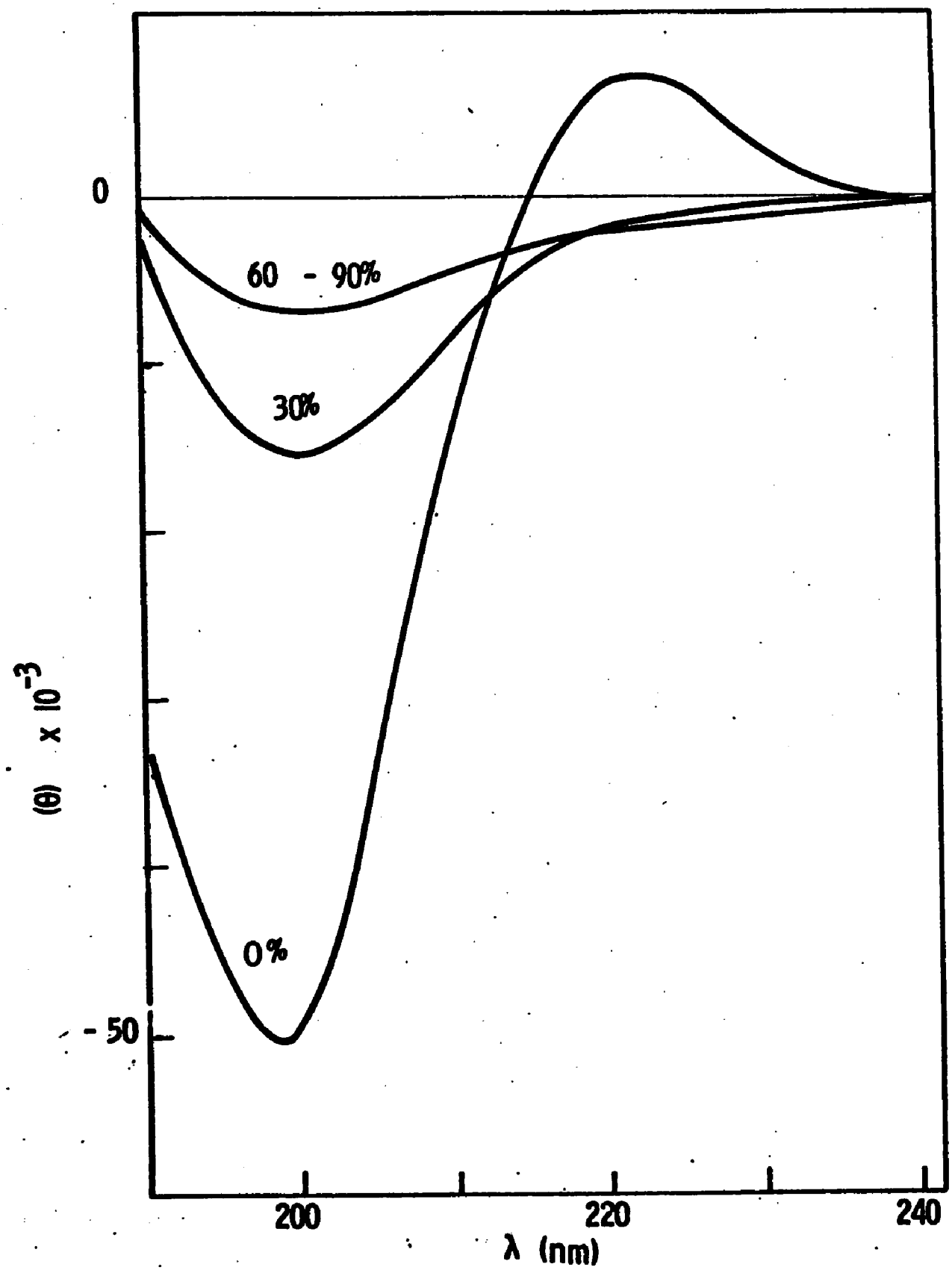



Fig. 6-1. CD of collagen: effect of trifluoroethanol.

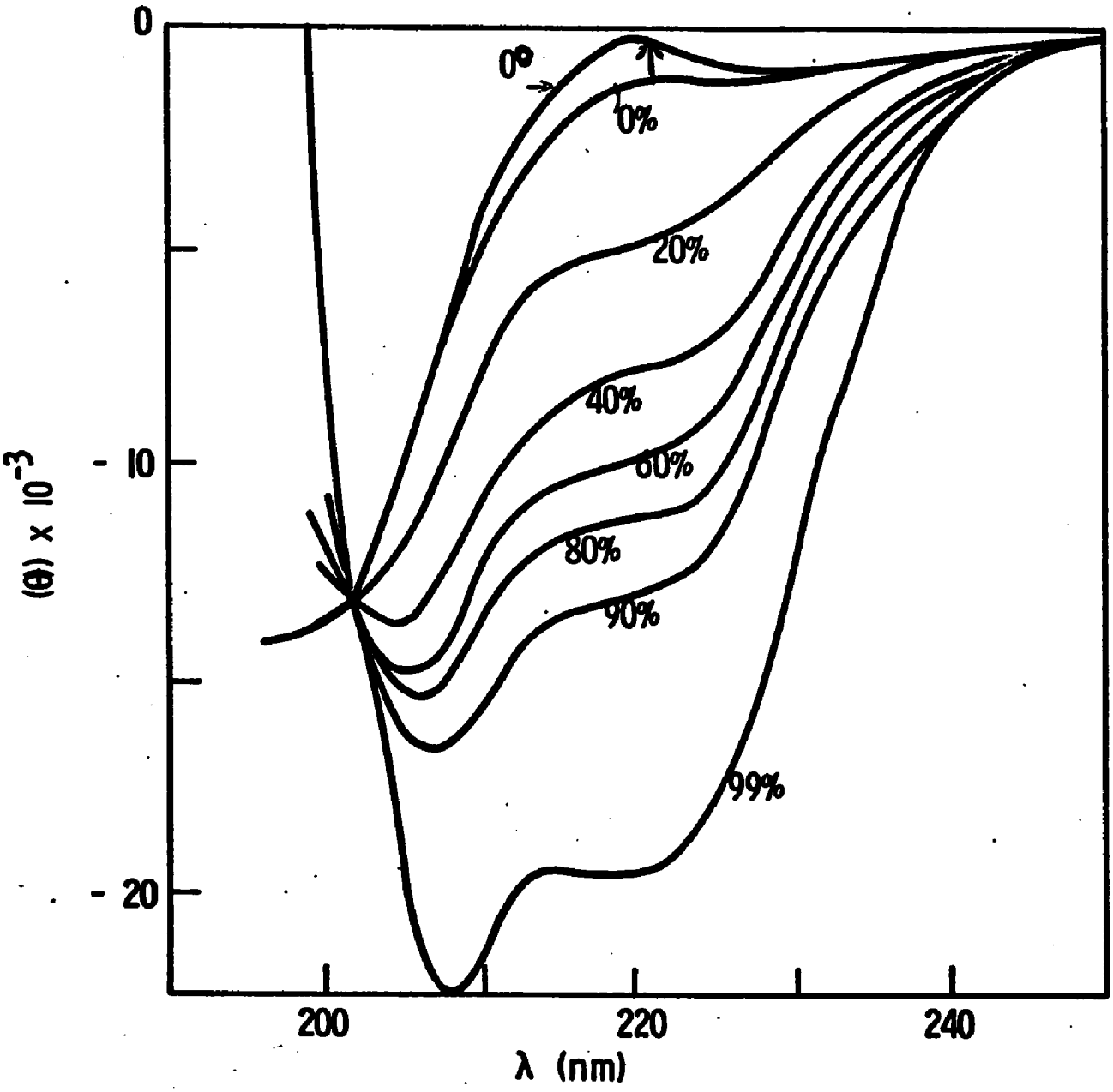



Fig. 6-2. $A_1$ protein of Eylar, 0.0354 g/l, 25°: effect of varying trifluoroethanol content.

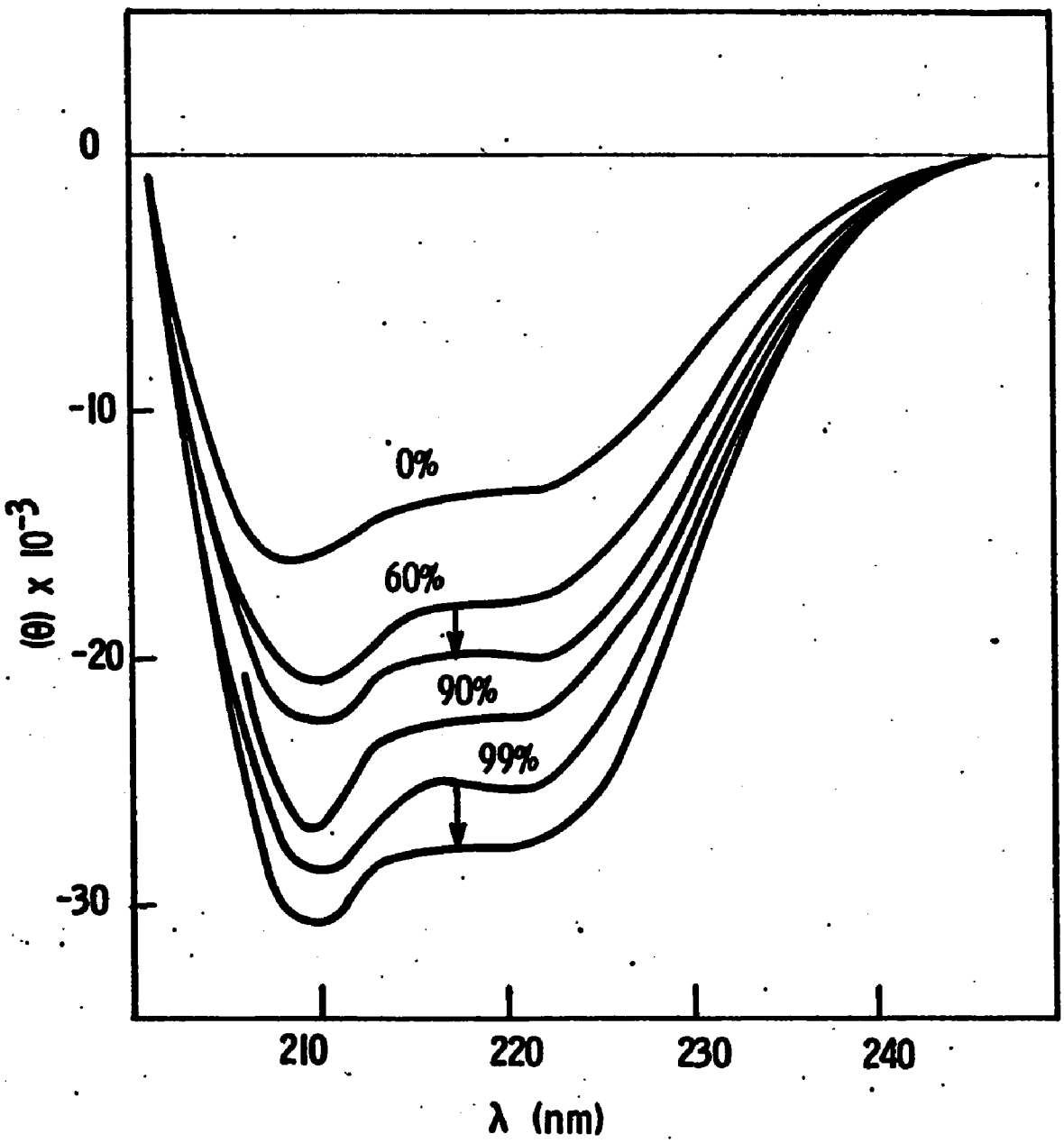



Fig. 6-3. CD of bovine serum albumin: effect of trifluoroethanol at 25° and 5°. (25° → 5°)

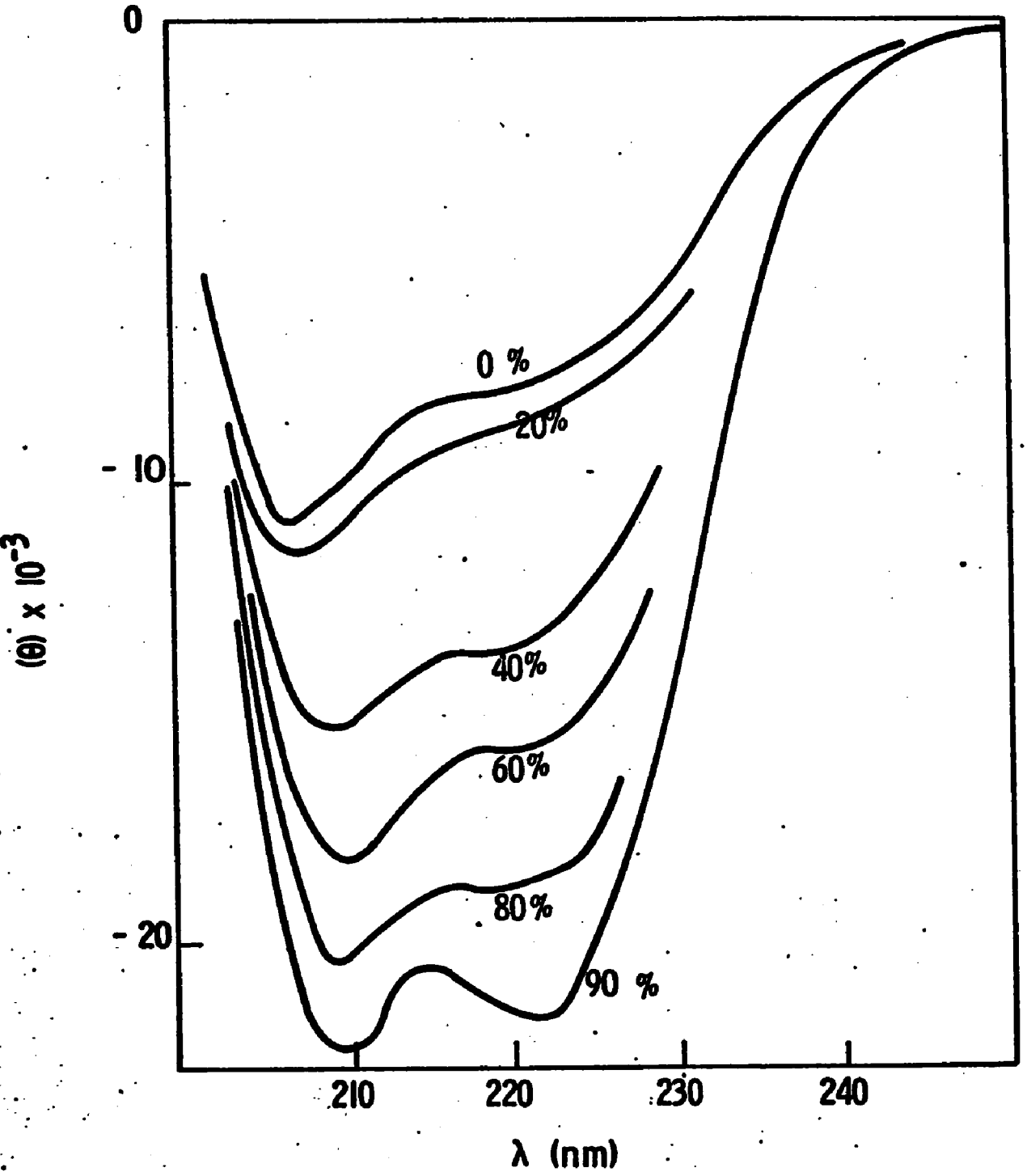



Fig. 6-4. CD of lysozyme: effect of trifluoroethanol.

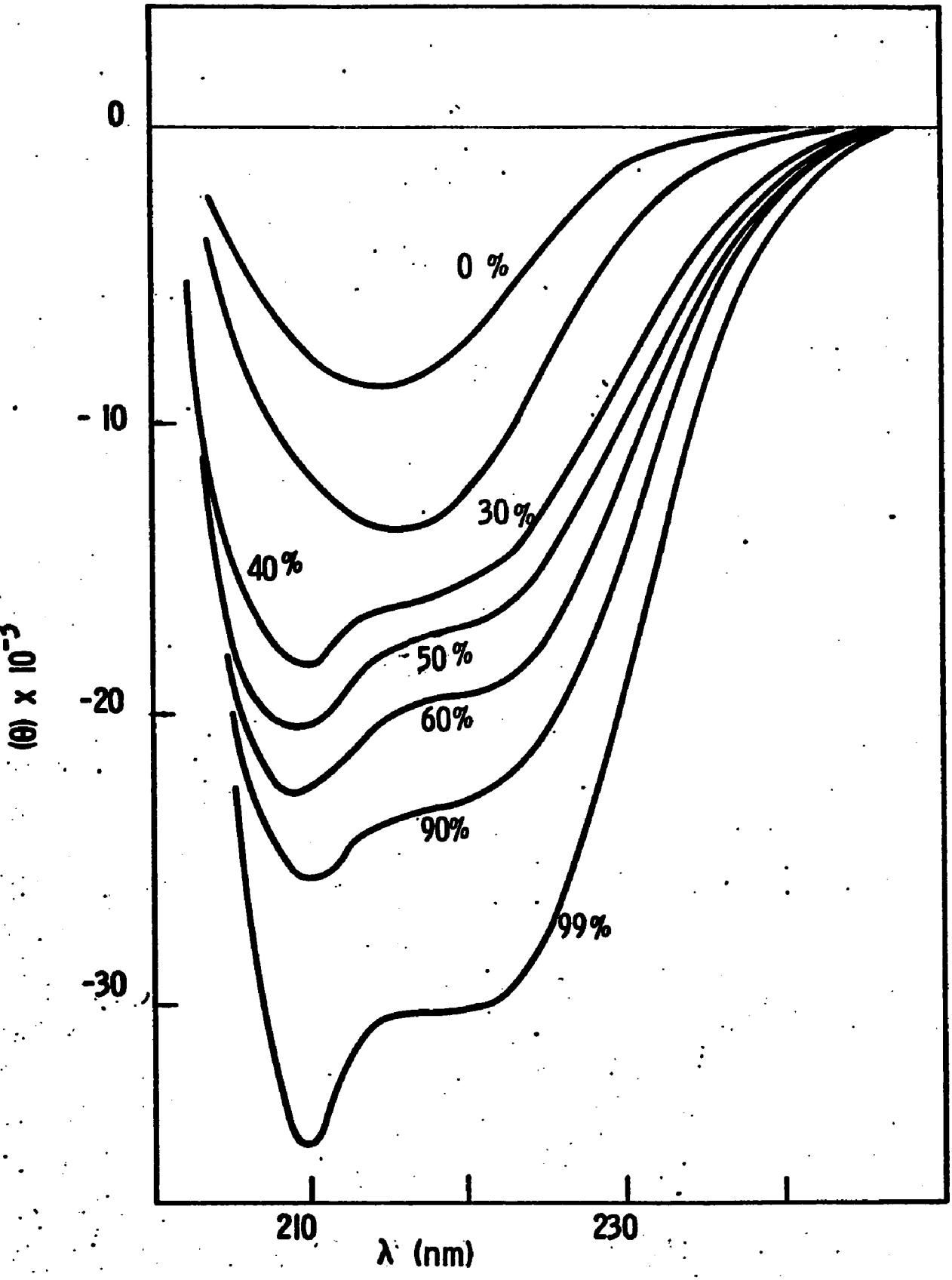



Fig. 6-5. CD of pepsin: effect of trifluoroethanol.

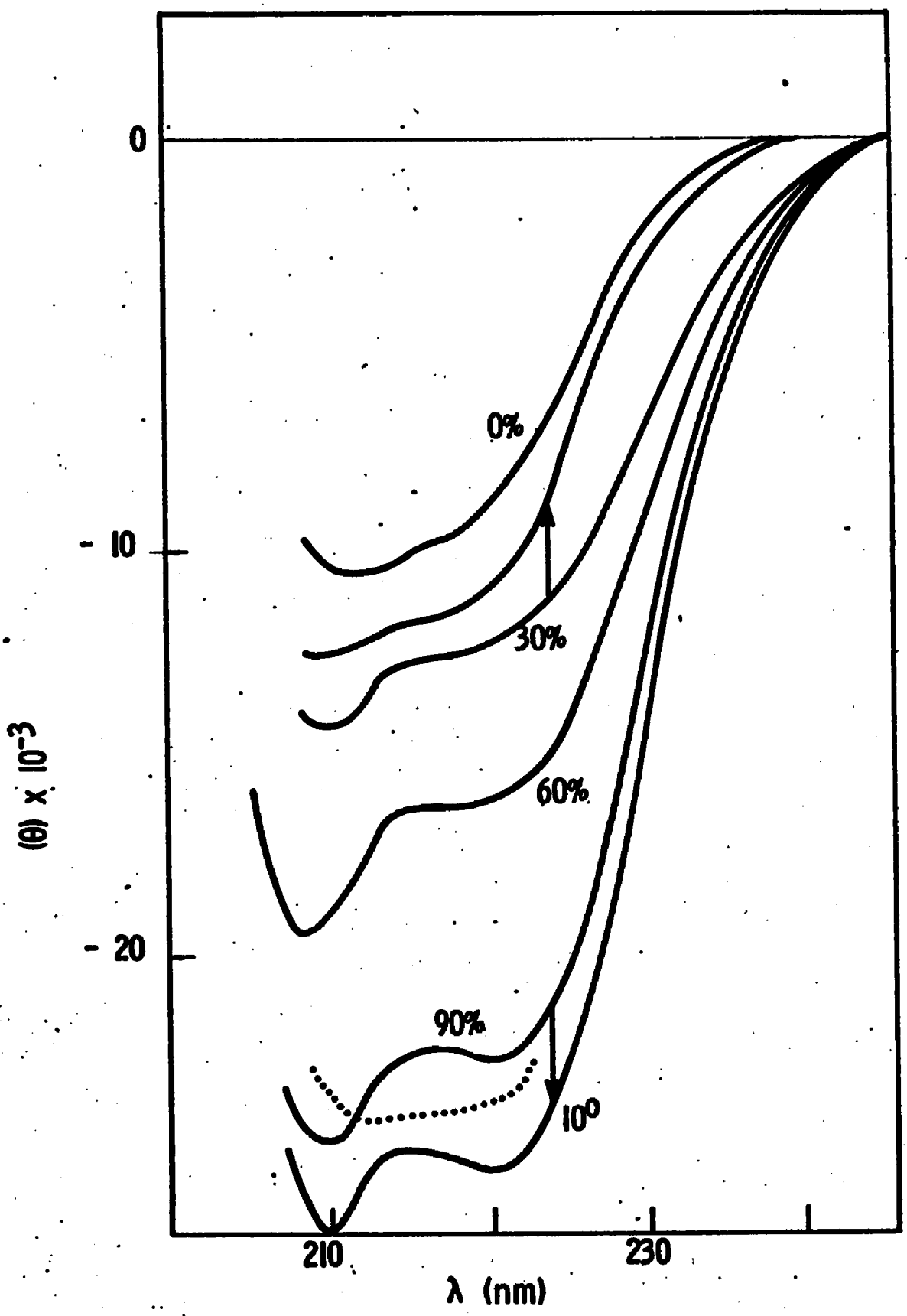



Fig. 6-6. CD of ribonuclease: effect of trifluoroethanol(—); effect of 90% ethanol(....). (25° → 10°).

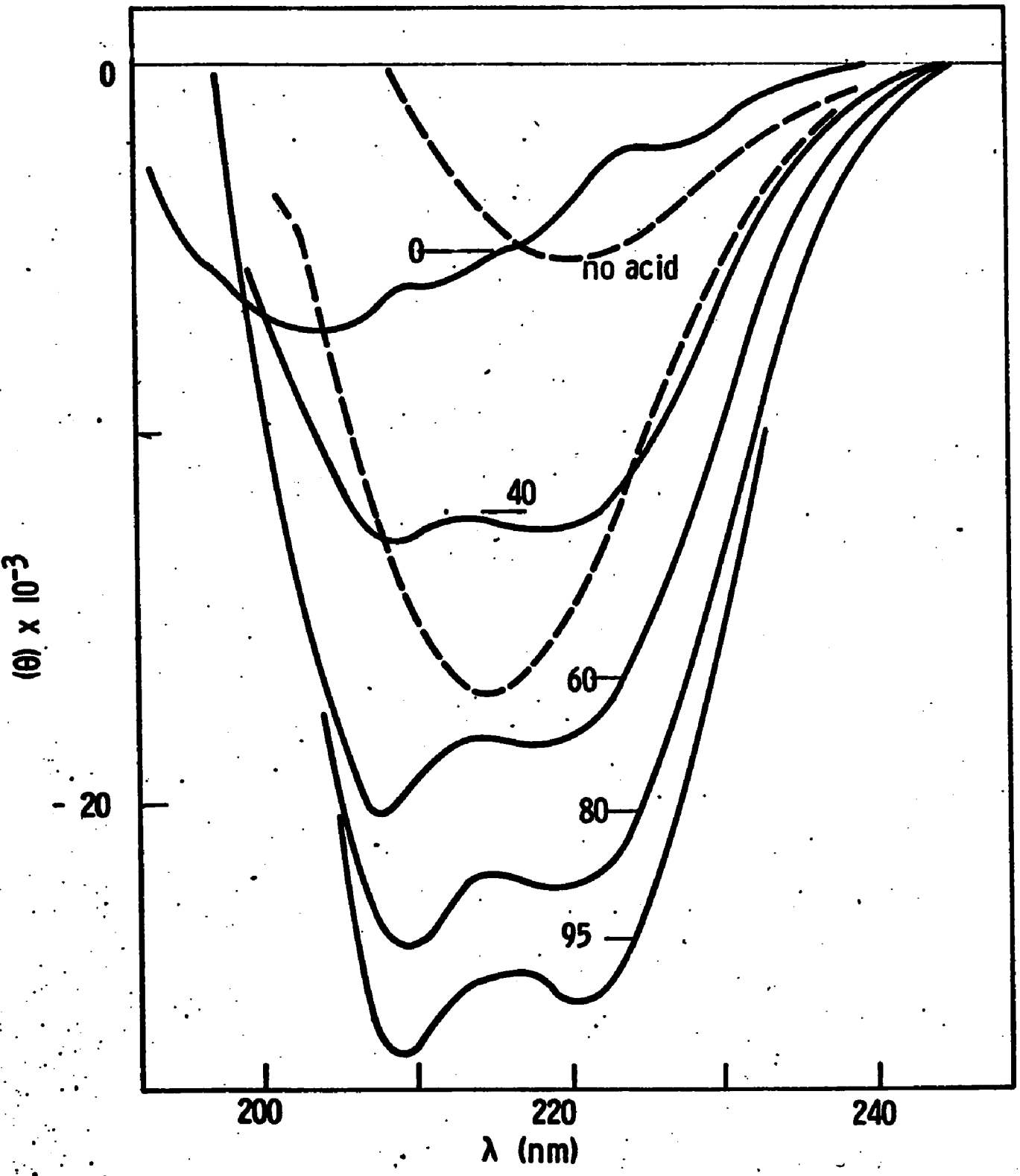



Fig. 6-7. α-Chymotrypsinogen: effect of trifluoroethanol (——); 90% methanol, with and without added acid (— — —)

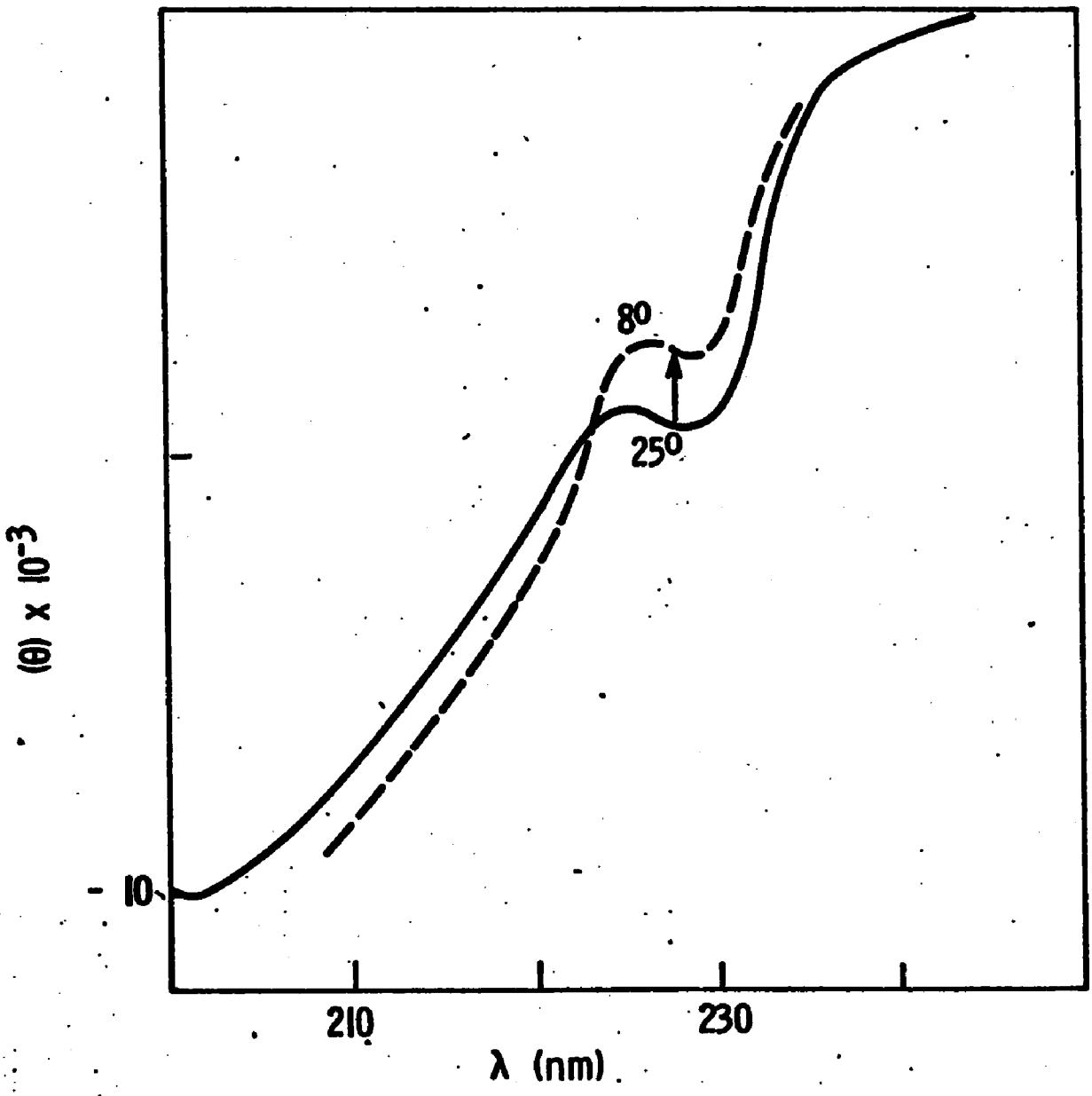



Fig. 6-8. CD of chymotrypsin: effect of temperature.

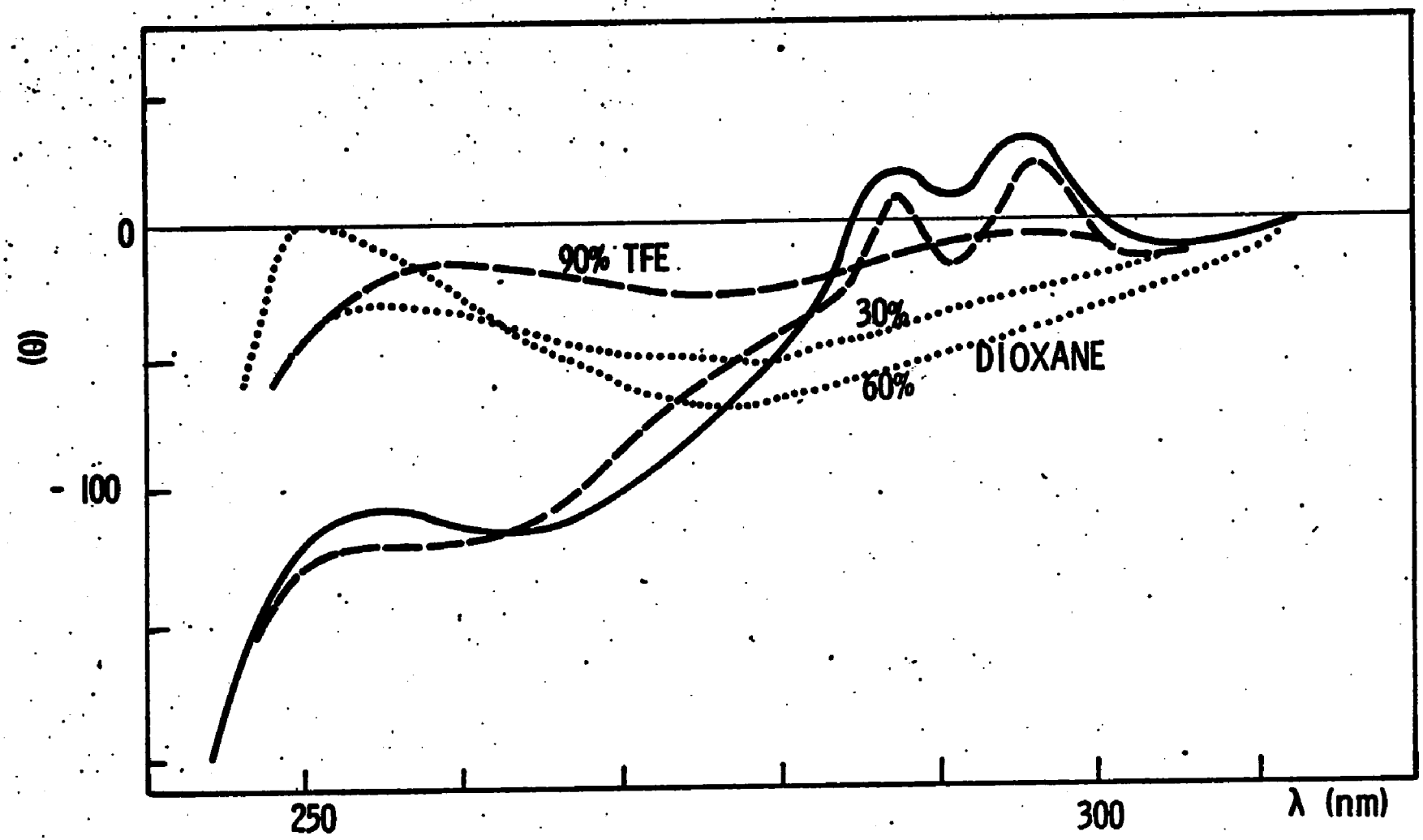



Fig. 6-9. Near uv CD: (——) chymotrypsin in water; (— —) chymotrypsinogen in water; —— —— chymotrypsinogen in 90% trifluoroethanol; ····chymotrypsinogen in dioxane.

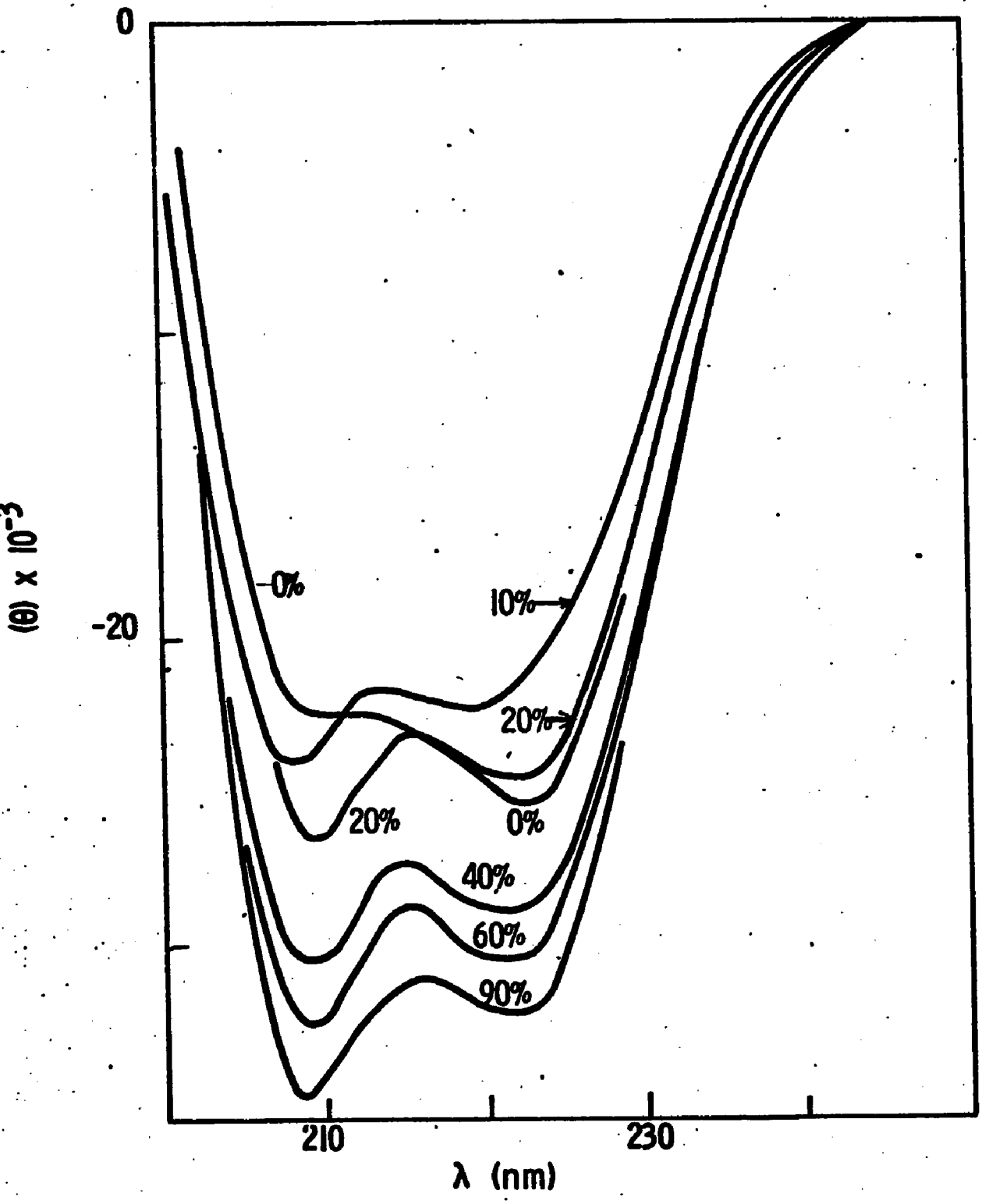



Fig. 6-10. CD of myoglobin: effect of different percentages of trifluoroethanol.

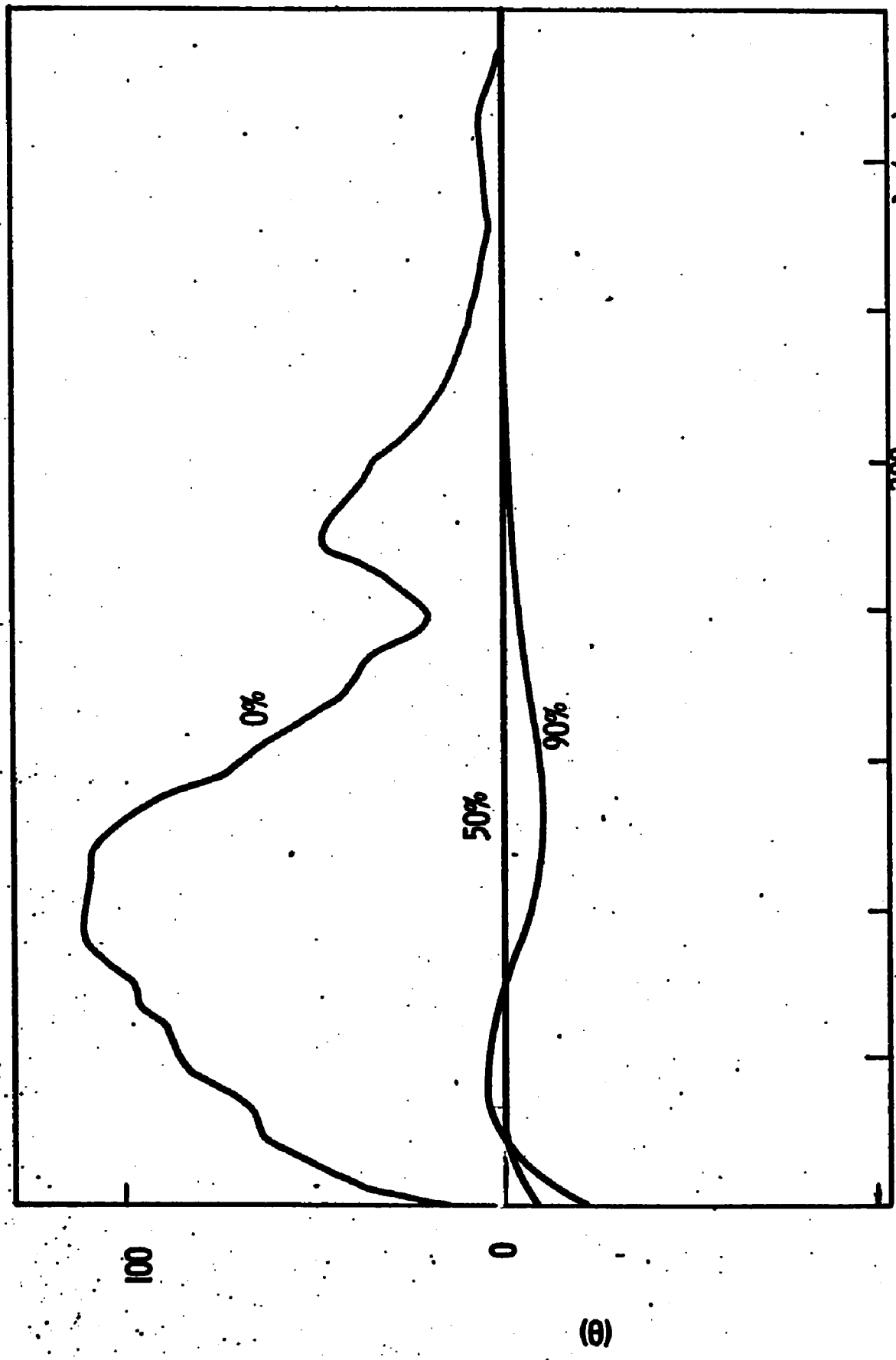



Fig. 6-11. Near uv CD of myoglobin: effect of trifluoroethanol.

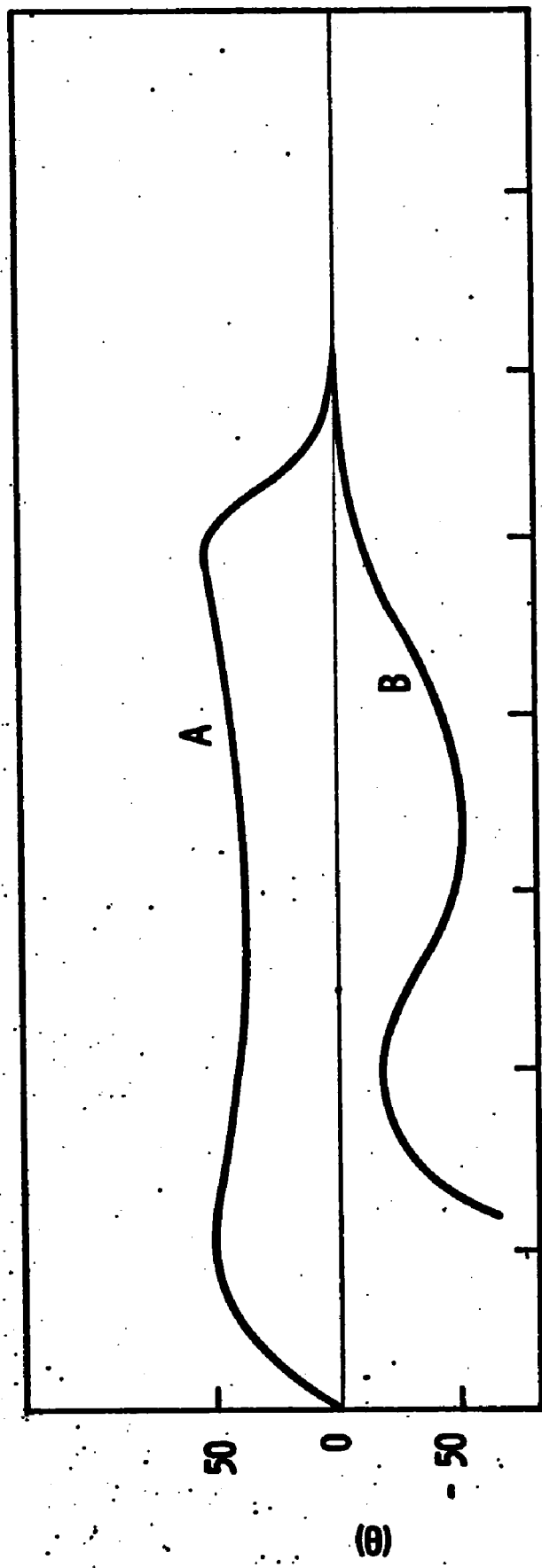



Fig. 6-12. Near uv CD of pepsin: effect of 90% trifluoroethanol. (A) in $H_2O$, (B) in alcohol.

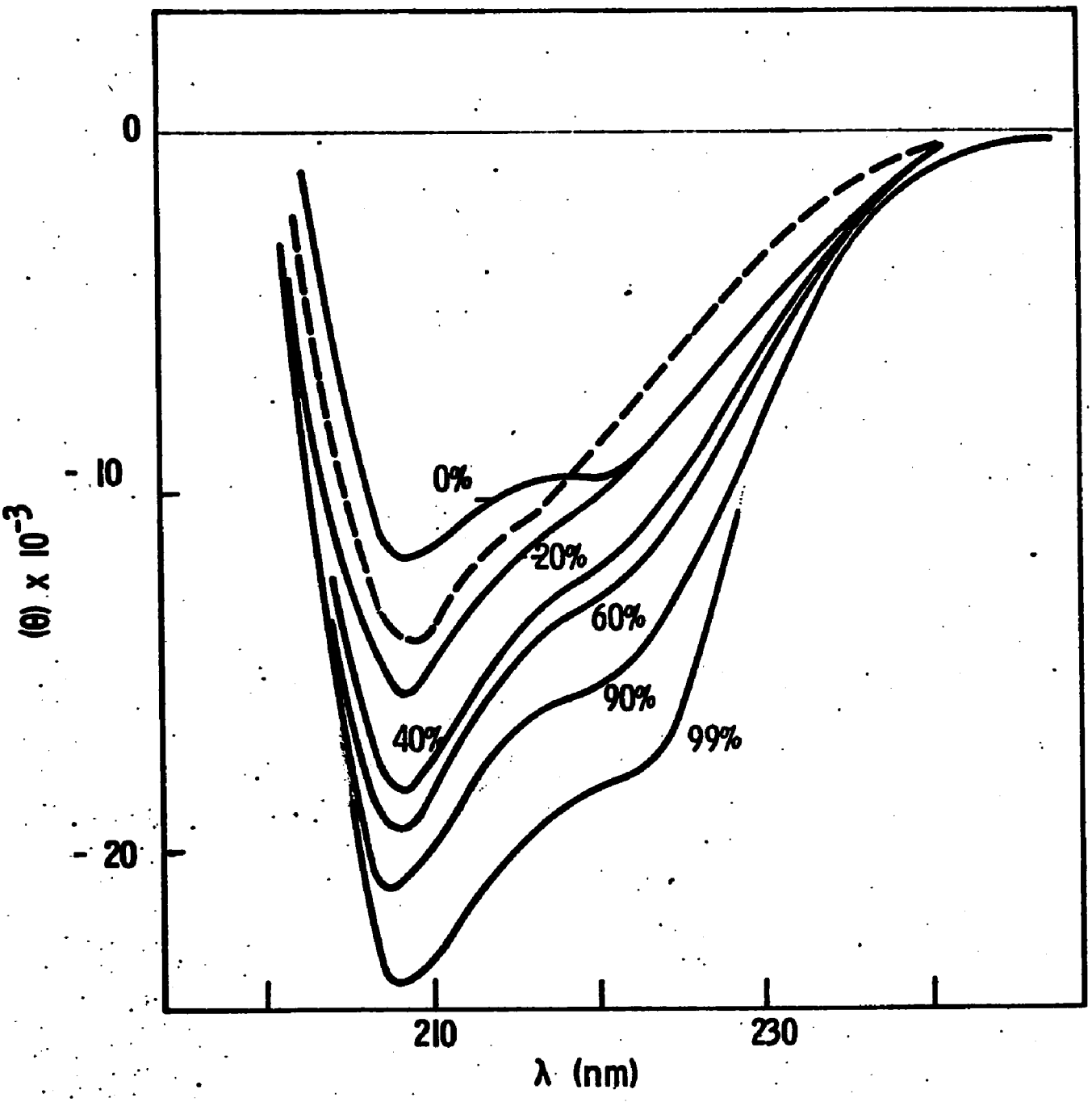



Fig. 6-13. CD of insulin: —— effect of trifluoroethanol; —— effect of 90% ethylene glycol.

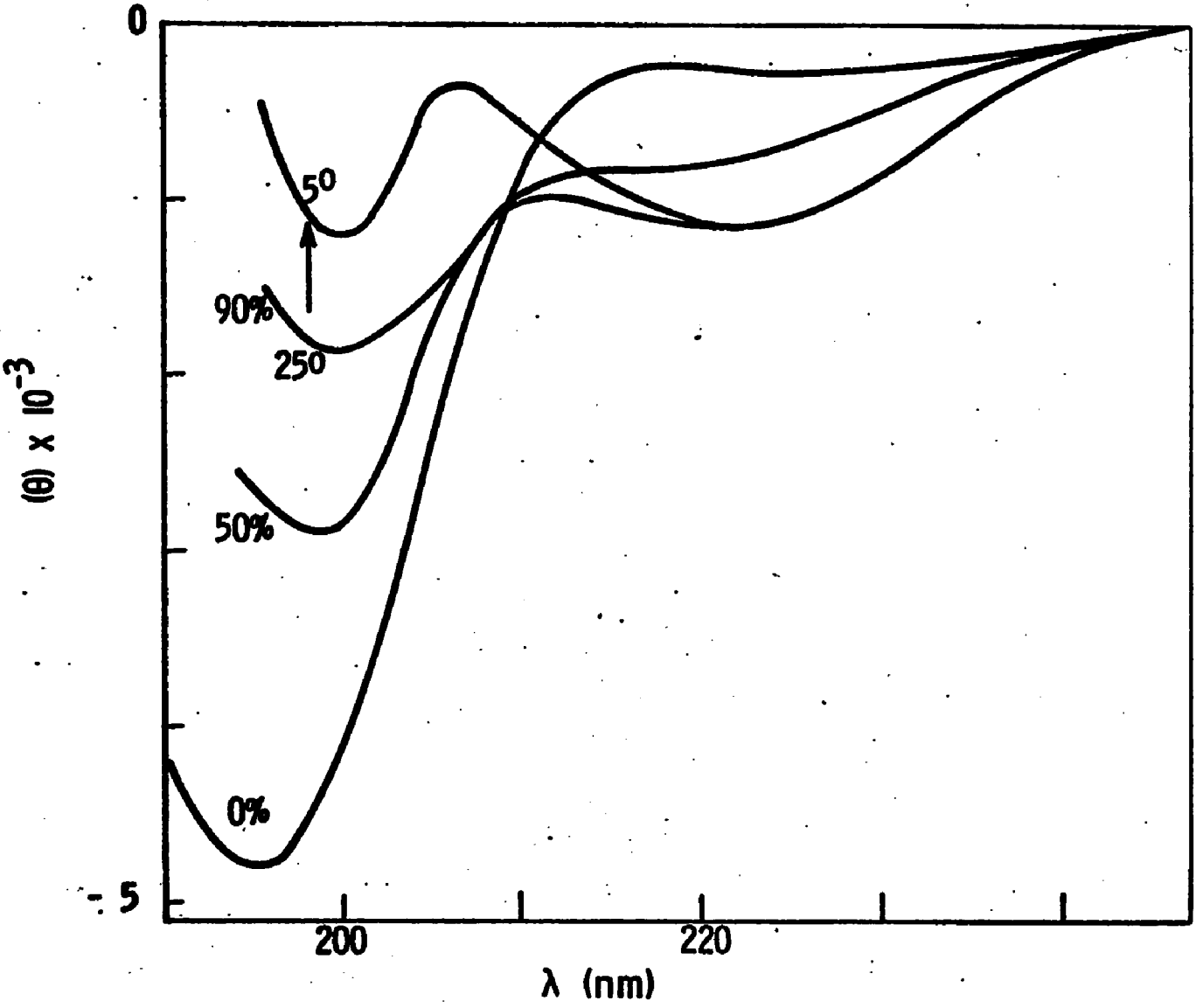



Fig. 6-14. CD of silk; effect of trifluoroethanol. ($25^{\circ} \rightarrow 5^{\circ}$

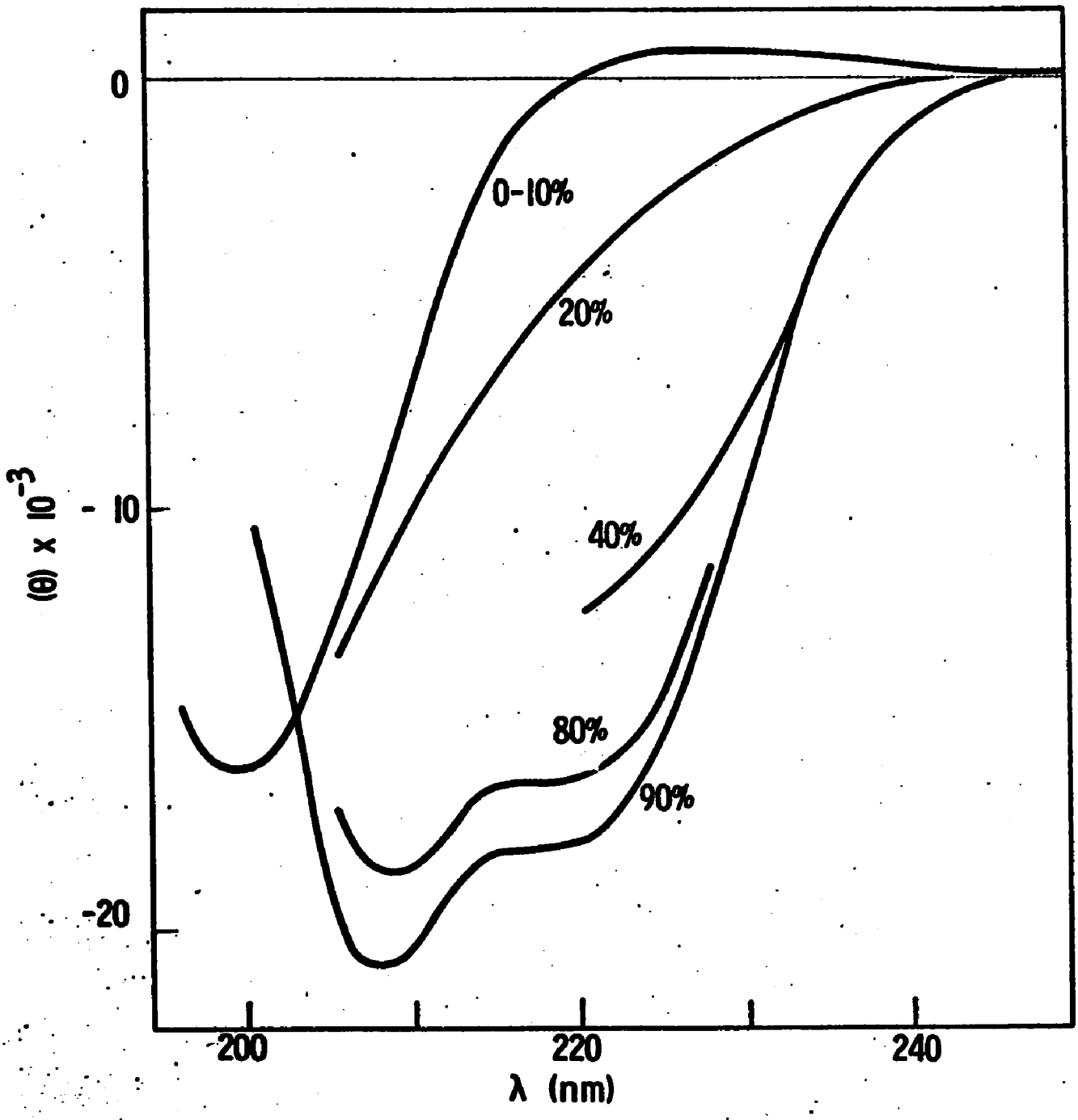



Fig. 6-15. CD of soybean trypsin inhibitor: effect of trifluoroethanol.

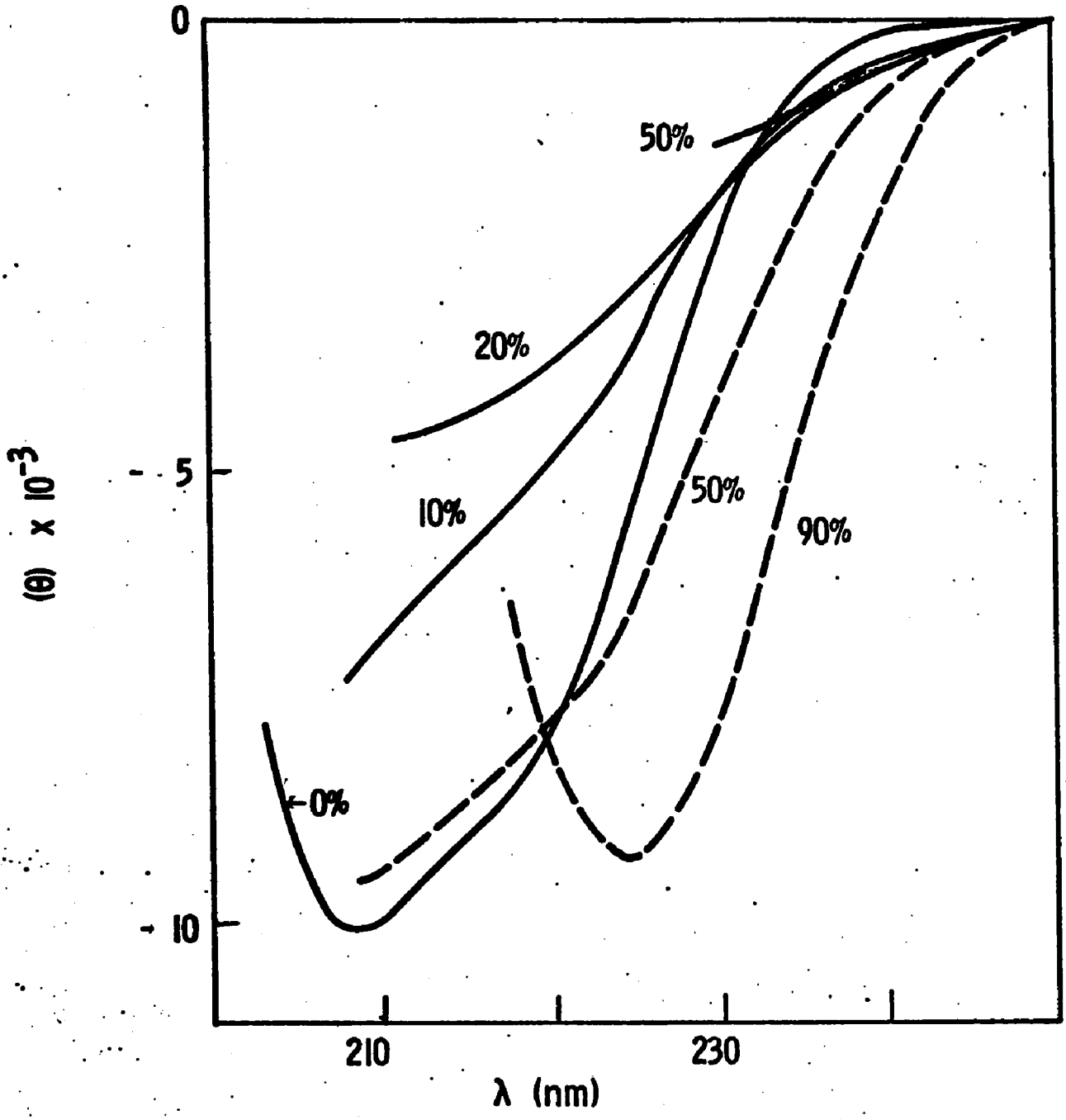



Fig. 6-16. CD of ribonuclease: effect of dioxane (——); effect of trimethyl phosphate (— —).

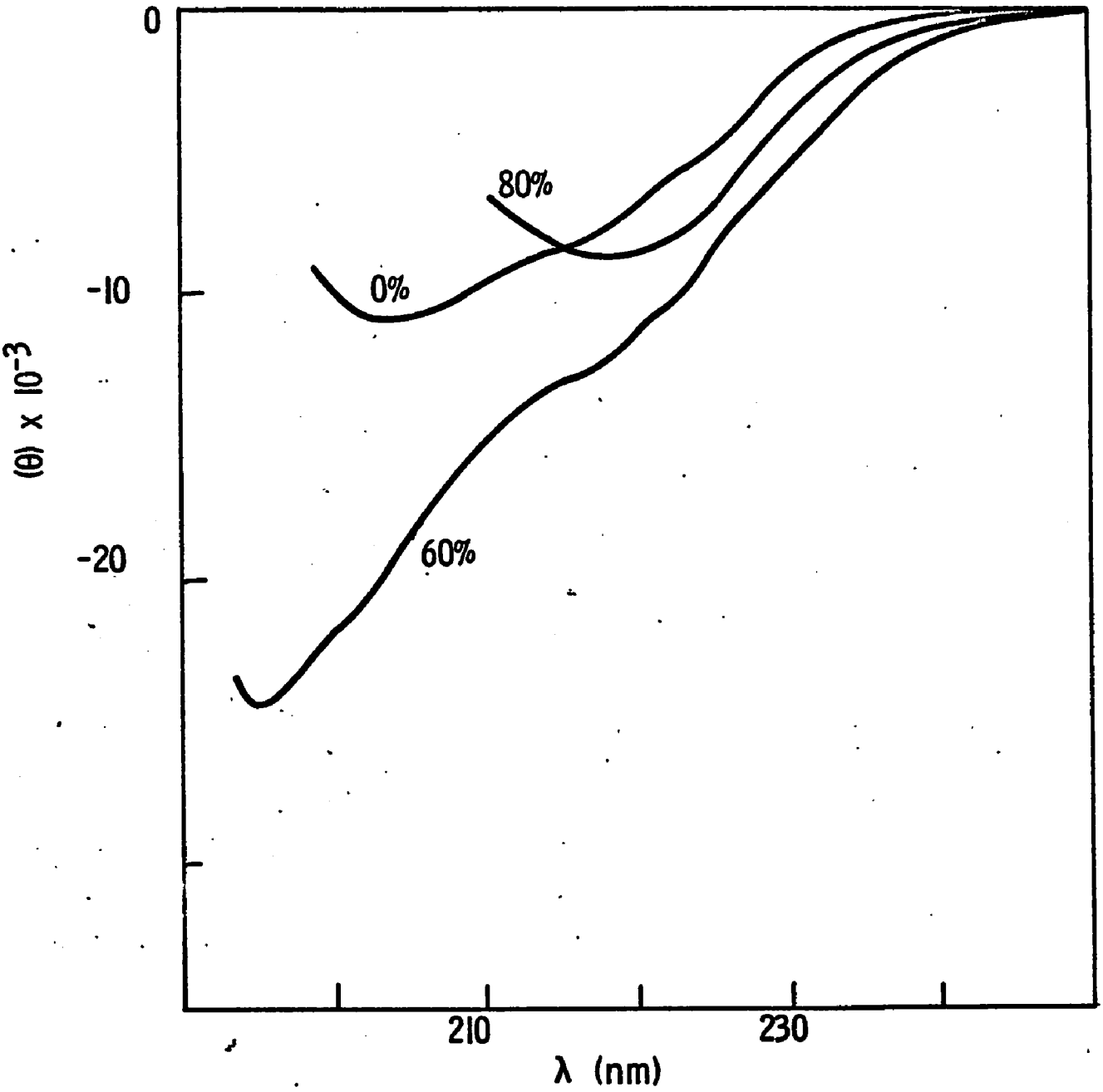



Fig. 6-17. CD of lysozyme: effect of dioxane.

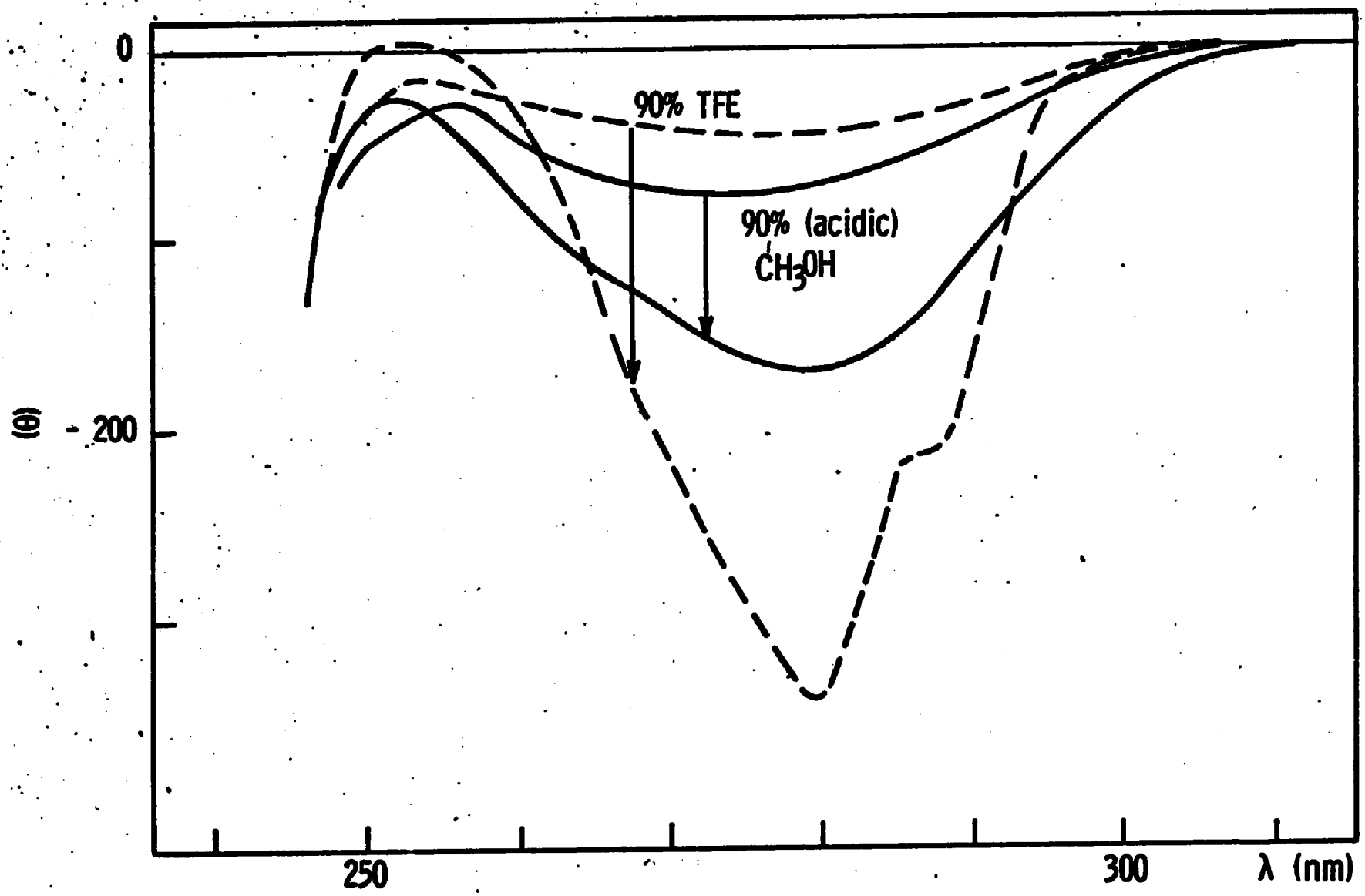



Fig. 6-18. Near uv CD of lysozyme: in 90% trifluoroethanol (– – –) showing change with time; in 90% methanol (acidic) (——) showing change with time (→) 1 hr.

Chapter 7

## Effect of Strong Acids on the Conformation of the Polypeptide Chain

The mechanism of the interaction of a polypeptide with an acid to cause a transition from a helical state is still in question.

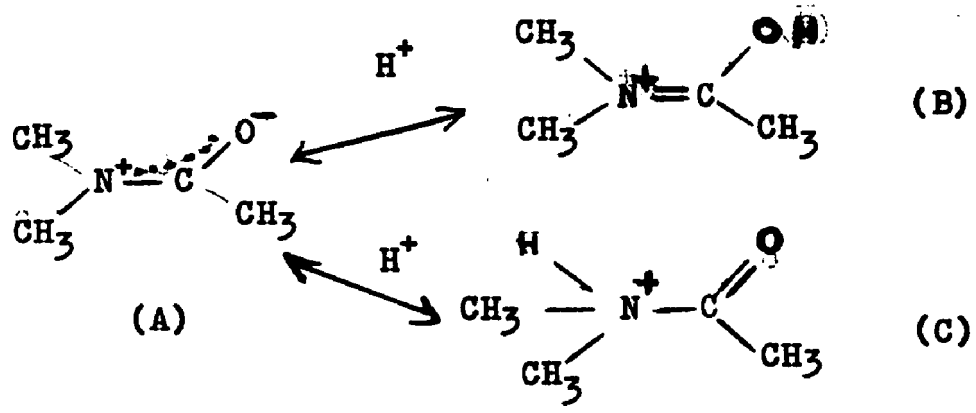
Most methods of studying model compounds have given indications that more than one reaction occurs on increasing the acid concentrations. Thus Steigman et al. (1969) showed a complicated series of changes in the uv absorption spectra of N-benzyl-glycine n-propylamide on increasing the $H_2SO_4$ concentration. The band in the 220 nm region shifts to the visible, at first diminishing in intensity and then increasing in intensity as the acid concentration is increased. Bovey (1968) discussed the rigid model compound D-oxolupanine in $CHCl_3$-acid systems. As the concentration of trifluoroacetic acid (TFA) is increased the positive CD band in the 230 nm region is found to first increase and then decrease in intensity and shift to the blue as the acid strength is increased. In concentrated $H_2SO_4$ the band almost disappears. In still another example, in the infrared region, Hanlon and Klotz (1965) reported a new band in the spectrum of poly-L-leucine at 1.48 M that appears when the acid strength is increased in the solvent system TFA-chloroform. It is at maximum intensity for intermediate acid concentrations. In 1970, Nawrot and Veis studied the interaction of formic acid with some model amides. They concluded that hydrogen bonding is prevalent over the entire

mixing range, that protonation of amides occurs at high acid concentration, and that ion pair formation is present at intermediate acid concentrations. There has been some discussion that polypeptides do not interact in the same way as model molecules N-methylacetamide (NMA) and N, N-dimethylacetamide (DMA) in their interaction with TFA since there is a marked deshielding of the TFA protons for NMA and DMA whereas there is an upfield shift of the TFA protons when the acid strength is increased to destabilize the helical state of the polypeptide (Stewart et al., 1967). This has recently been discussed by Liu and Lagnowski (1970). They find that the chemical shift of TFA protons decreases as the TFA content increases at constant polymer concentration because the relative polymeric effect on the chemical shift of TFA decreases whether the polymer is in helical or nonhelical state. But during the transition there is a need for TFA molecules to be used to interact with the peptide carbonyl groups that were engaged in intramolecular association in the helix form. Therefore the observed relative chemical shift increases while the transition is taking place. This analysis, while it does not reveal the mechanism, at least makes it likely that the same kind of interaction with acids can take place in the model compounds, NMA and DMA and polypeptides.

We would like to use CD to study conformational changes induced by acids in the structure of proteins and polypeptides. Most of the early studies were done on systems which are opaque in the uv and measurement of optical activity changes were

limited. Two of the few attempts to record the near uv changes by CD were done on the systems poly-γ-benzyl-L-glutamate (PBLG) and poly-β-benzyl-L-asparate (PBLA) in chloroform-TFA solutions (Bovey, 1968 and 1969). The far uv cut-off was at approximately 220 nm, so they could not show the shape of the bands that are related to conformation, but could indicate that PMLA is left-handed, while PBLG is right-handed when helical, as expected from $[\alpha]_D$ and $b_o$ values. A different approach to the problem was introduced by Rosenheck (1968) who made use of the transparency of $H_2SO_4$ to study the effect of a strong acid on polypeptide chains. He was limited to ORD and uv absorption studies. From these he suggested that a more truly random coil results in strong acid than is caused by ionization of the polyelectrolytes, poly-L-glutamic acid (PGA) and poly-L-lysine (PL), since the 200 nm Cotton effect is weaker in the acid. Some indication of protonation is given by the blue shift and decrease in intensity of the absorption maxima since the same occurs, only to a larger extent, in the model compound, N, N-dimethylacetamide (DMA) at the same acidity. Rosenheck suggested as a possibility that the helix-coil transition results as a consequence of electrostatic interactions between protonated peptide units, and pointed out that protonation is well accepted to be on the carbonyl oxygen (Fraenkel and Franconi, 1959 and 1960) and therefore stabilization of the barrier to rotation around the amide C-N bond would occur.

Actually, protonation is not a simple process (for review, see Steigman et al., 1969). This is strongly indicated from NMR studies on model compounds. Thus, Phillips et al. (1955), using DMA, showed collapse of the doublet of the non-equivalent N-methyl protons of the normal molecule as free rotation about the double bond sets in at intermediate acid concentrations ( pH 1). Berger et al. (1959) noted, however, that the doublet reappears under strongly acidic conditions. Since the peaks are not symmetrical, spin-spin interaction caused by protonation, could not be the cause of the doublet. The free rotation and collapse of the doublet is said to be due to protonation on the nitrogen (species C).




Only a small amount of this state is present since there is no splitting of the $N^+CH_3$ resonances by coupling with the N-H proton, as pointed out by Temussi and Bellofiore (1969). And in the very strong acid the dominant species is seen to be B to account for the reappearance of the doublet. Even with TFA and formic acid, Nawrot and Veis (1970) have been able to show the collapse of the N-methyl doublet of DMA. This strongly suggests that even these acids can, under suitable conditions,

cause protonation. Hydrochloric acid was used by Fraenkel and Franconi (1960) on DMA to obtain the reappearance of the N-methyl doublet at low temperatures. The same effect was accomplished at room temperature by Berger et al. (1959) by adding dioxane to perchloric acid solutions of DMA. The dioxane is added to slow down the rate of exchange of the proton so the doublet can be seen.

Because of our previous work on the effect of ionized side-chains on the configuration of the polyelectrolyte polypeptides, we expected to see an effect of protonation of the backbone also. In the general case, simple considerations of charge repulsion, should lead to a planar zig-zag structure. This would be the maximum extended structure. However, this does not correspond to a very favorable position on the ($\varphi$,$\psi$) map (Schellman and Schellman, 1964) and we should not be surprised if the real structures deviate slightly from this hypothetical form. Poly-L-proline and derivitives cannot open up at all due to the constriction of the pyrrolidine ring. Form II of PP is already the most extended structure.

## Experimental

### A. Poly-L-Lysine (PL)

PL is a polymer of particular interest in its interaction with acid. Under these conditions the side-chains are already ionized and the polymer is in the so-called "random" state which we have seen to be EH. When $H_2SO_4$ is added this structure is destabilized (Fig. 7-1). There are definite

indications of development of some $\alpha$ helicity at intermediate acid concentration that are hardly to be classified as "solvent effect" of the acid. Peggion *et al.* (1970) have also experimented on the effect of $H_2SO_4$ on PL. Their results, as far as reported, are similar to ours. They did not notice the interesting detail of the changes in the 220 nm region, and the development of helicity. In the case of $HClO_4$ in its interaction with PL there is no question that the anion $ClO_4^-$ binds to the side-chain. The charge neutralization makes the polymer go completely helical, even at low pH. The binding is a very sensitive function of temperature (Fig. 7-2), and occurs for all perchlorate salts as well as the acid. Adding excess $HClO_4$ over that needed to make the polymer helical tends to aggregate PL, especially at low temperatures (Fig. 7-3). Then additional $HClO_4$ causes soluble extended states as the acid apparently interacts with the backbone. The viscosity data (Fig. 7-4) on PL at different concentrations of $H_2SO_4$ fits in well with our interpretation of partial helical intermediate states, which would decrease the overall size of the molecule and therefore the viscosity. In high acid concentrations the viscosity again increases. It is interesting that $[\eta]$ in 100% acid is greater than $[\eta]$ in water. The charges, of course, due to protonation of the peptide group can be closer together than in the simple charged side-chain. PL in water, and the overall structure could tend to be more expanded. It is impossible to see the effect of counter-ions in strong acid. Thus salts can be added without producing an

effect on the CD spectrum; the $H_2SO_4$ molecule is too much self-dissociated. The CD of PL in the strong acid consists of two bands, close in wave length and sign to those occurring in PL in water, but having weaker intensity (Fig. 7-1).

B. Poly-L-alanine.

In poly-L-alanine it was found exceptionally difficult to develop a strong positive CD band in the 220 nm region. Only by going to low temperature and high dilution of the polymer (0.02 g/l) could we get indications that a 220 nm band could form in 90% $H_2SO_4$ (Fig. 7-5). This may be related to the lack of a strong steric requirement for development of an extended helix in this case due to the shortness of the side-chain.

C. Poly-L-glutamic acid.

Poly-L-glutamic acid could not be followed through a complete range of $H_2SO_4$ concentrations due to development of precipitation in the 40 to 50% acid range (Fig. 7-6) On the acid side, however, the polymer was again soluble and developed the typical +220 nm band. It is interesting to note that there is little indication of partical helicity in the CD bands in the 57 to 76% acid range, and the curves seem to be best described as partial random and partial extended.

D. Poly-L-proline (PP).

Changes in the PP CD spectra with acid are most helpful in attempting to understand mechanisms of interaction of strong acids with a polypeptide chain. First, by noting that

the PP II structure is not the stable form in the strong acid (Figs. 7-7 and 7-8), we immediately see that charge repulsion alone is not the only consideration to be used in determining the resultant structure, since the three-fold left-handed helix, PP II, is the most extended form of the polypeptide under the constraint of the pyrrolidine ring. Hydrogen bonding to the carbonyl is ruled out, because that would stabilize the open form II. The resultant spectra in different concentrations of acid do not go through an isodichroic point so we do not expect one simple mechanism. It is interesting that PP I in n-propanol solution, on having 10% of the solution replaced by $H_2SO_4$ (95.6%), immediately gives a CD spectrum that we have come to interpret as a random coil (Fig. 7-9). On dilution, after five minutes interval, with n-propanol we see that most of the cis form, PP I, is still present. Under these conditions, we can say that not much cis-trans isomerism has taken place. A random coil CD spectrum can apparently exist independent of the cis-trans isomerism. This implies that rotation may be occurring about the $C^{\alpha}-C'$ bond or that the double bond character of the C-N bond has not been loosened enough to allow a complete flip of the pyrrolidine ring. All that is needed for a random coil CD is many different configurations with equal possibility of existence. When PP I is treated with more concentrated $H_2SO_4$, say 90%, and immediately diluted (1:9) with n-propanol or water, the resultant CD spectra tend to be still random, so presumably there are no long stretches of cis or trans existing before or after dilution. This is

interesting since if PP II is treated with 90% $H_2SO_4$ and diluted back with in fifteen minutes, again, 1:9, the conformation is seen to be Form II. Thus we can deduce that the trans configuration is the favored one in the strong acid. If the cis configuration is presented with the strong acid, trans isomerism occurs at random along the chain under the influence of the acid but is not necessarily the reason for the loss of Form I in the acid.

Similar to propanol solutions, TFE solutions of PP require much less acid to produce a random coil CD (Fig. 7-10). We will see that salts also, are more effective in their interaction with the backbone of polypeptides and proteins when used in an alcohol solution (Chapter 8). If protonation occurs under the influence of strong acid, we would not expect a decrease in viscosity such as occurs in strong salt solutions (Blout and Fasman, 1958; Harrington and Sela, 1958: Ciferri and Orofino, 1966 and Mattice and Mandelkern, 1970), since repulsion would keep the overall structure from collapsing. Under conditions that favor the trans configuration, PP II, such as in acetic acid, strong acids have been noted to act as a catalyst to speed up the I to II change (Steinberg, et al., 1960). Here we must expect that the loosening of the peptide double bond due to some protonation on the nitrogen has allowed the ring to flip to the more desired trans position. Diluting back solutions that have equilibrated in strong acid we find gives PP II, no matter whether the diluent is alcohol or water. This is in keeping with work on the model compounds,

however, since most of the protonation is suppose to be on the oxygen. Once a bond has flipped to trans it should tend to remain there, if the double bond character of the N - C improves in the very strong acid.

It may be possible to tell the effect of acid on the cis-trans proportions by the use of nmr. Studies on PP have already been reported by Conti et al. (1969), Deber et al. (1970) and Torchia and Bovey (1971). Resonances at 5.6τ and 5.3τ have been assigned to the α-cis and α-trans protons, respectively. A good system to study the influence of strong acid may very well be 32% HCl - PP, since HCl lacks the high viscosity of $H_2SO_4$. A survey experiment shows (Fig. 7-11) that 32% HCl gives a CD spectrum similar to $H_2SO_4$, only the negative CD band was not shifted to the red, nor was the absorption spectrum shifted to the blue, as occurs in the case of $H_2SO_4$. After being in the 32% HCl for 30 minutes samples returned to normal PP II on dilution.

E. Collagen

Collagen is destabilized by low concentrations of $H_2SO_4$. Thus 5% acid is sufficient to pull the triple helix apart and cause a CD spectrum similiar to that given by heat denaturation (Fig. 7-12). Additional acid results in further decrease in the intensity of the single negative CD band. In this case the individual chains cannot elongate due to charge repulsion of the protonated backbone because of the high content of proline. The high percentage of glycine (approx. 30%),

and the lack of large side-chains, could also hinder the stabilization of an extended structure, of the separated chains.

F. Poly-β-benzyl-L-asparate

Poly-β-benzyl-L-asparate (PBLA) is known to be in a left-handed α helical state in chloroform, but this structure is considerably less stable than the right-handed α helical form of poly-γ-benzyl-L-glutamate (PBLG) (Bradbury et al., 1960). The CD spectra of both of these molecules, in $CDCl_3$ with varying percentages of TFA are given by Bovey (1968), but only in the uv to 224 nm. We easily record a complete CD spectrum to 190 nm of PBLA in 90% trifluoroethanol 10% $H_2SO_4$ (Fig. 7-13). It is most interesting. Only 10% $H_2SO_4$ is sufficient to unorder the left-hand helix and produce an extended structure that appears to be right-handed. Thus we find the small negative band at 230 nm replacing the positive 220 nm band seen in most other polymers and proteins. The large positive band at 209 nm replaces the 195 nm negative band ordinarily recorded and a whole new band (negative, in this case) comes into view at 195 nm. Urry (1968) discusses the red shift which occurs in the helical spectrum of poly-β-methyl-L-asparate. He suggests that it may arise from interaction of the amide group with the ester group. We expect the same interaction for PBLA.

In order to record the left-handed α helical state of PBLA a sample was dissolved in chloroform. The solution was then diluted with 2 chloroethanol and the CD spectrum run

immediately (Fig. 7-14). It was found possible to record in the uv to 205 nm when a 0.1 mm cell was used. The spectrum is similiar to that given by Bovey (1968) for PBLA in chloroform where, however, the cut-off due to solvent absorption was near 224 nm. No resolution of an $n\pi^*$ is visible. The conformation was not stable in this solvent medium and at 5° and at 25° slowly transformed into another conformation that is not clearly identifiable. It is apparent that the asparates are very sensitive to their environment and will require further study.

G. Poly-L-aspartic Acid.

Poly-L-aspartic acid was found by Brahms and Spach (1963) to give indications of being right-handed at pH 4.3 since the recorded CD spectrum to 220 nm was negative. The value of the ellipticity was only 1/5th that of unionized poly-L-lysine. They remarked that 0.3 M acetate ion probably causes another conformation. Our experiments show that the right-handed helical form certainly is unstable. Thus a pH 3.4 the poorly formed right-had helix (i.e. the CD spectrum looks like that of partically titrated poly-L-lysine or poly-L-glutamic acid).is unstable at 25° and at 5° and apparently switches to a right-handed extended form.(Fig. 7-16). The same form of CD spectra results in strong $H_2SO_4$ solutions (Fig. 7-15).

H. Poly-γ-methyl-L-glutamate (PMLG).

PMLG is a typical polypeptide and in concentrated $H_2SO_4$ gives a CD spectrum that we identify as left-handed and

an extended helix. On decreasing the temperature from 25° to 5° the 220 nm band doubles in intensity (Fig. 7-17). Since Steigman et al. (1969) reported that the ethyl ester has a more intense band at 222 nm in 83.6% methansulfonic acid than in 99% methanesulfonic acid it seemed desirable to test the methyl ester. (The ethyl ester was not available to us to see if we could duplicate their results.) Our experiments using methane sulfonic acid gave no indication that weaker acid concentration would produce a more intense positive band (Fig. 7-18) than the 100% solution. (In all our experiments with strong acid we found it necessary to add the acid slowly to a sample packed in an ice bath in order to keep the temperature down to prevent secondary effects of the acid.) Incidentally, PMLG dissolved directly in 70% $HClO_4$ was in the $\alpha$ helical state.

Discussion

We have observed the CD spectra of many polypeptides and proteins in strong acids. The spectra of poly-L-alanine (Fig. 7-5), poly-L-lysine (Fig. 7-1), poly-L-glutamic acid (Fig. 7-6), poly-$\gamma$-methyl-L-glutamate (Fig. 7-12), bovine serum albumin (Fig. 7-19), chymotrypsin (Fig. 7-20), feather keratin (Fig. 7-21), insulin (Fig. 7-22), lysozyme (Fig. 7-23), myoglobin (Fig. 7-24), soybean trypsin inhibitor (Fig. 7-25), silk (Fig. 7-26) in 85 to 90% $H_2SO_4$ are given for examples. In all of these a small + CD band occurs in the 220 nm region. It always increases in intensity when the temperature is lowered from 25° to 5° and usually shifts to the blue. A larger negative band is found near 195 nm. It is not noticed to be

as temperature sensitive. By comparing the CD of the examples given, we are led to the conclusion that there is a strong indication that the same conformation is reached in all polypeptides and proteins, that are not high in proline content, when thay interact with strong acids. A truly random state, however, is not indicated from theoretical considerations nor from the experimental results. Thus from the work on the effect of strong acids on model compounds we expect some protonation of the backbone of the polypeptides. A charging of the chain results. The repulsion of the charges on the backbone itself will tend to make the chain expand. Simple considerations of a rod model indicate that a planar zig-zag structure would result if there are no pyrrolidine rings to restrain the expansion. Packing considerations of real atoms must, however, be considered. The coincidence of the dichroic bands near the same wave length as occurs in the charged side-chain polypeptides and in extended polypeptides that are not protonated may not be accidental. The simple three-fold LH helix may be the most sterically acceptable form of the extended chain.

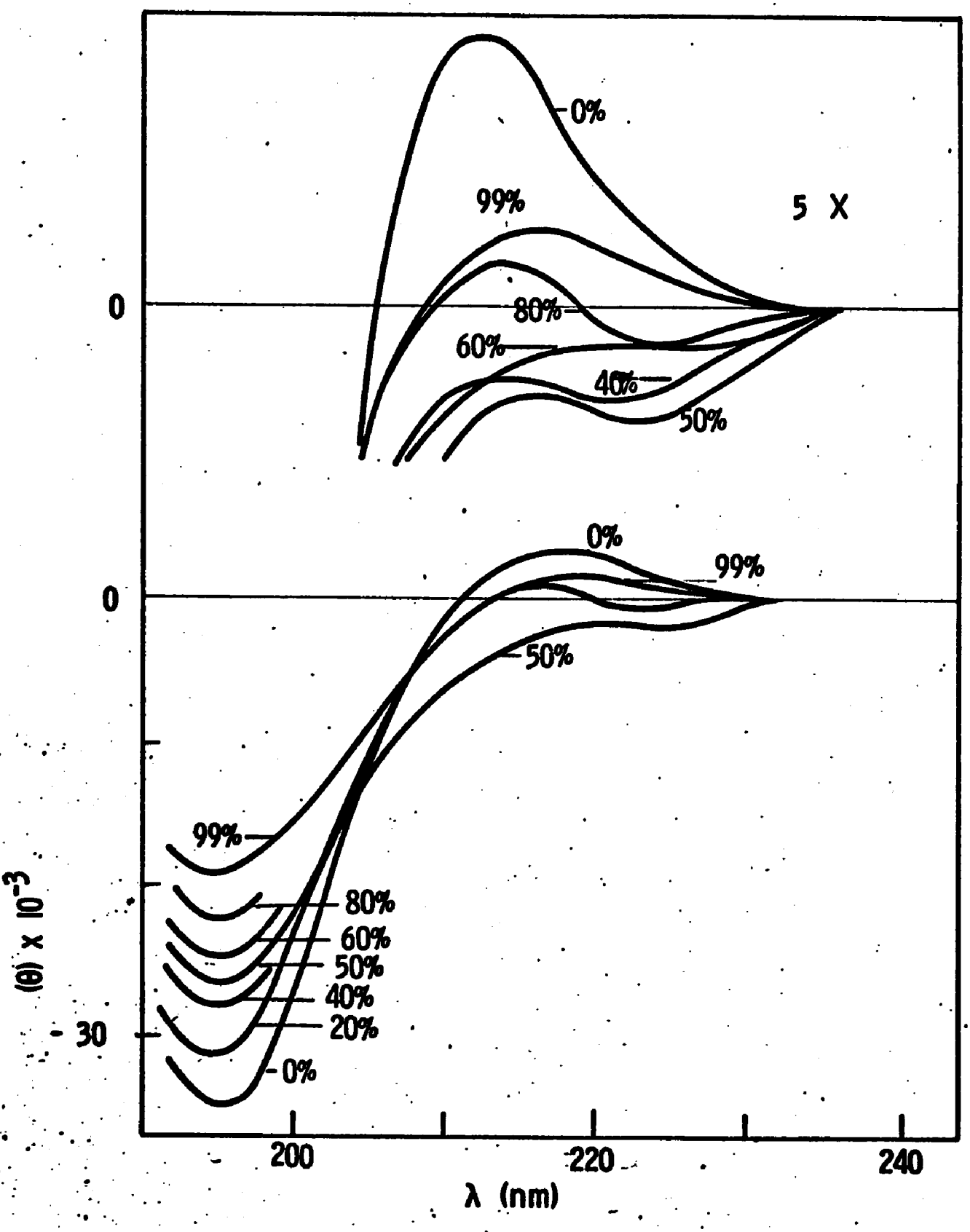



Fig. 7-1. CD of poly-L-lysine: effect of $H_2SO_4$.

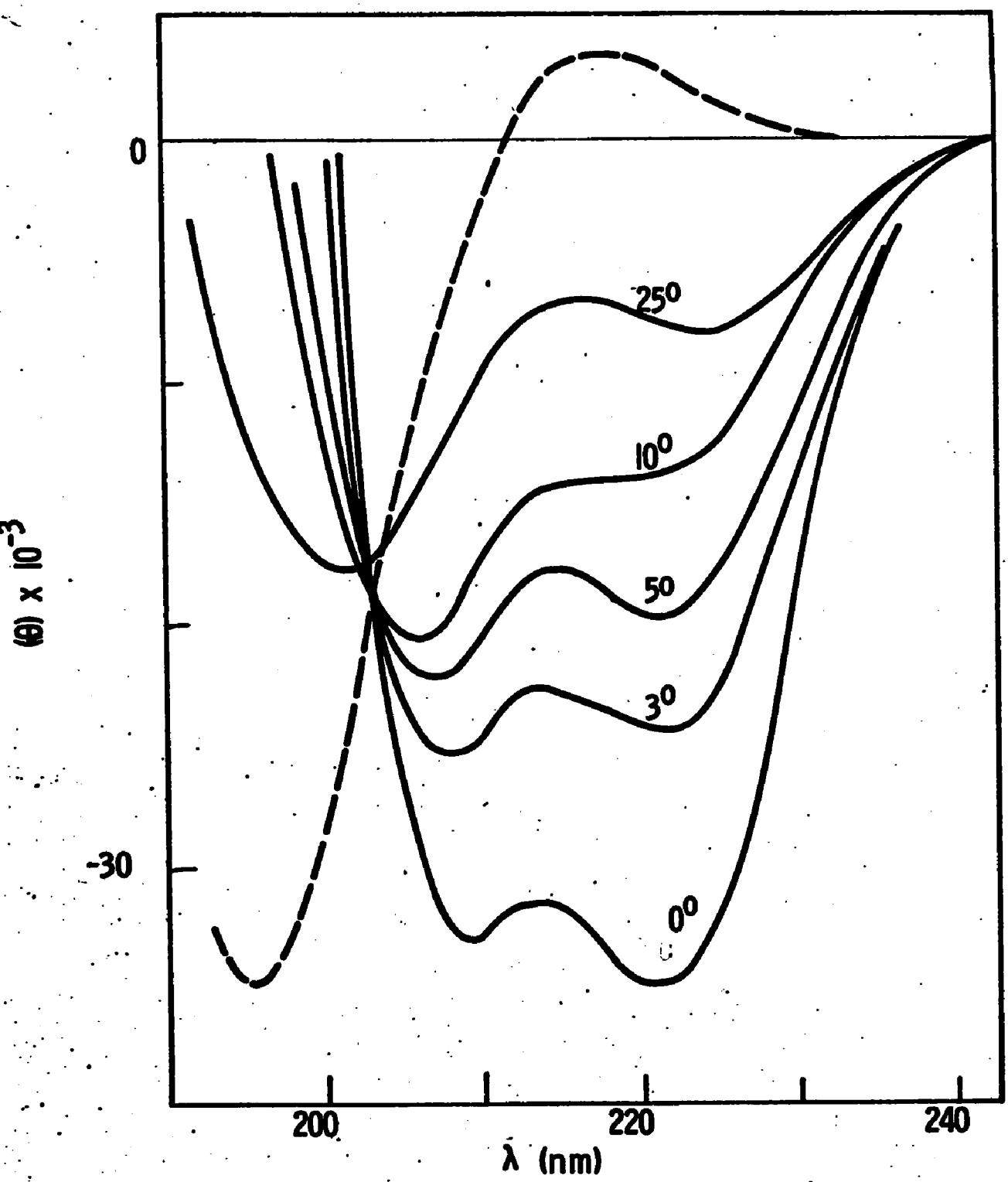



Fig. 7-2. CD of poly-L-lysine, 0.137 g/l; in 0.7% $HClO_4$ at different temperatures; (——) in $H_2O$ at 25°, for comparsion.

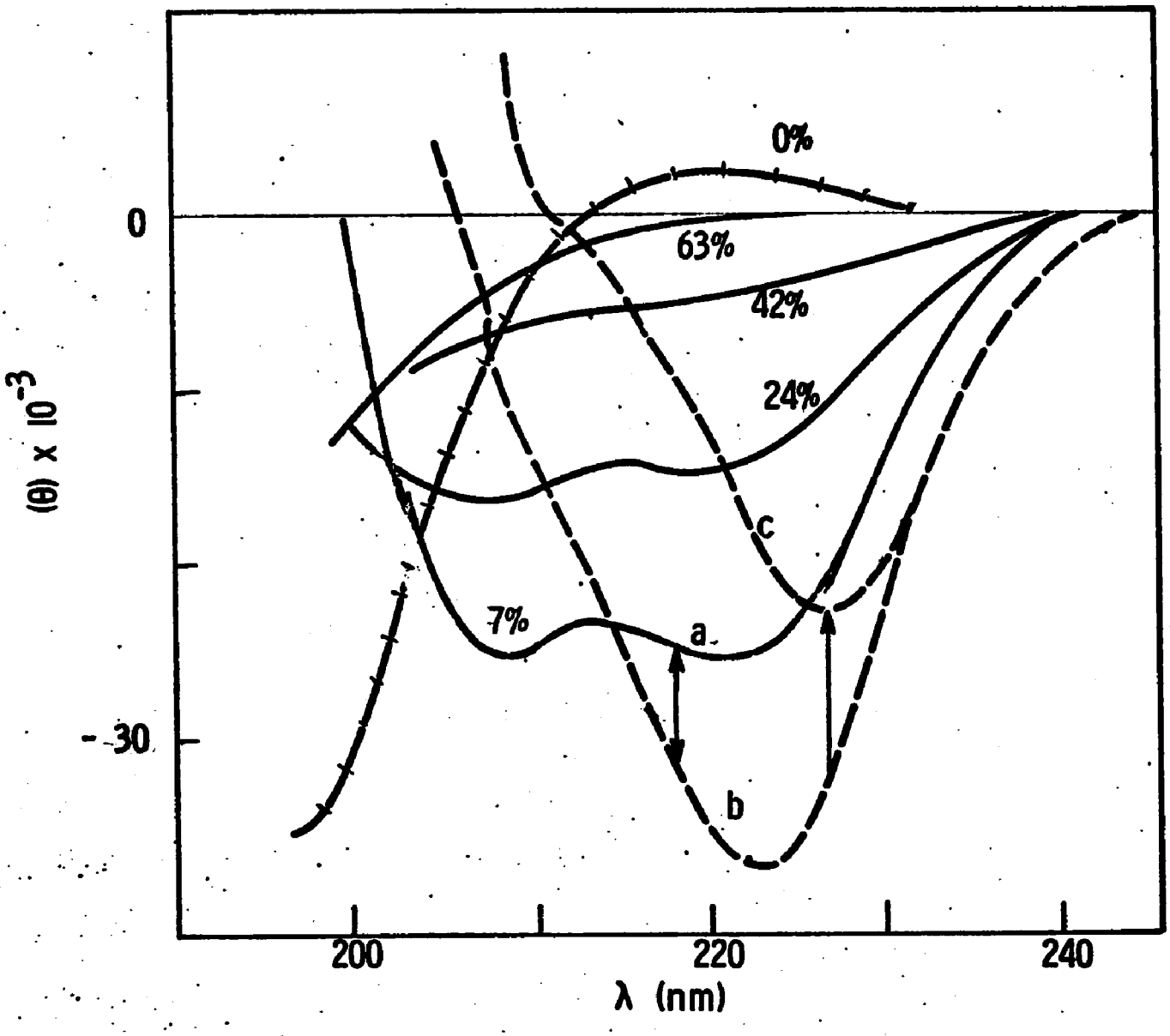



Fig. 7-3. CD of poly-L-lysine: (—·—·—) in $H_2O$; at 25°; in 7 % $HClO_4$ [(a) at 25°, (b) at 5° in 5 min. followed by (c) if left at 5° for 20 minutes, but back to (a) if rewarmed to 25°]; other concentrations of $HClO_4$ as indicated, at 25°. Curves b and c show aggregation occurring at 5° in 7% $HClO_4$.

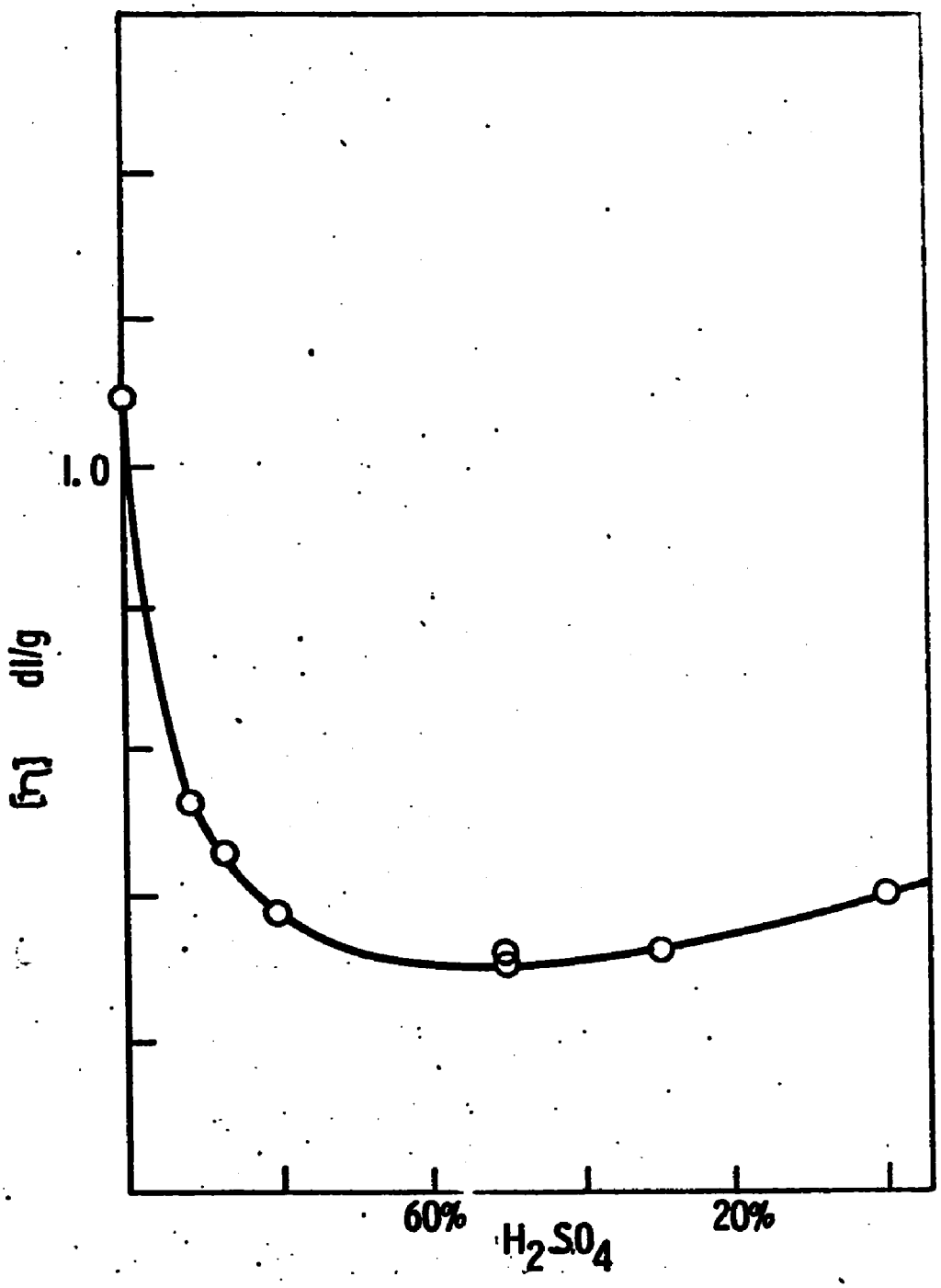



**Fig. 7-4. Intrinsic viscosity of poly-L-lysine in $H_2O$-$H_2SO_4$ (Peggion, et al. , 1970).**

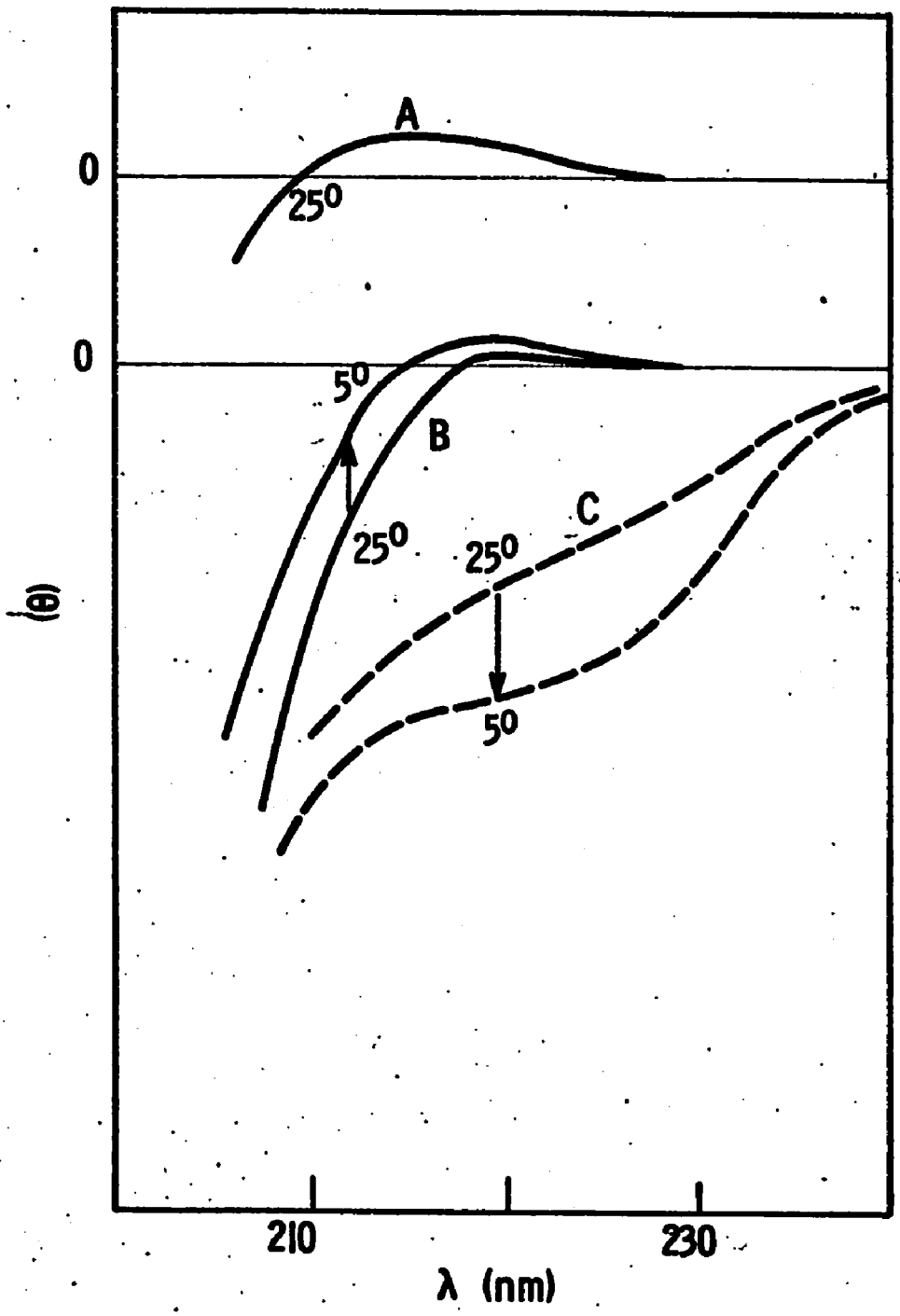



Fig. 7-5. CD of poly-L-alanine: effect of $H_2SO_4$ [(A) 0.01 g/l in 95% $H_2SO_4$ ; (B) 0.02 g/l in 95% $H_2SO_4$; (C) 0.02 g/l in 40% $H_2SO_4$].

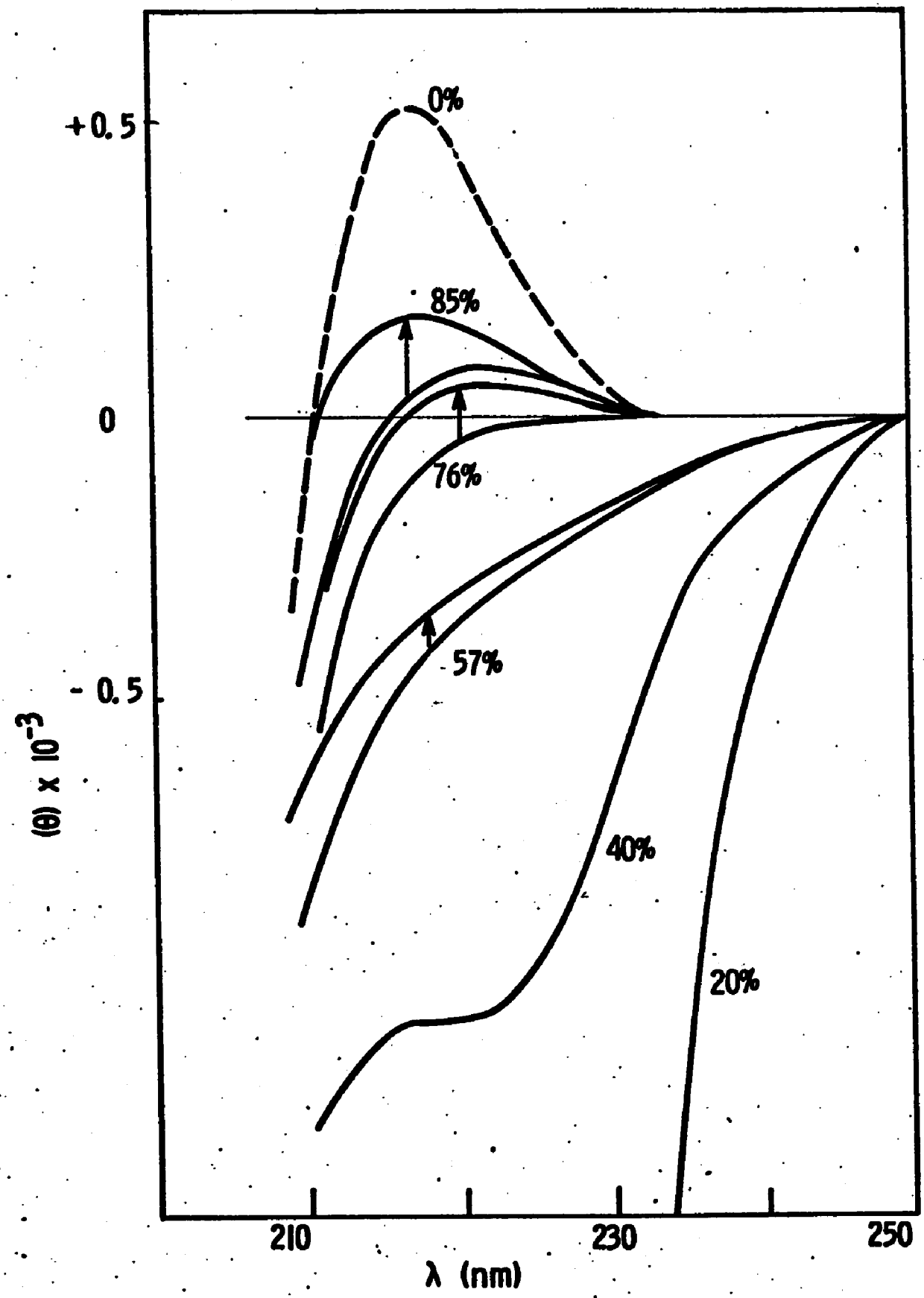



Fig. 7-6. CD of poly-L-glutamic acid: effect of $H_2SO_4$. ($25^o \rightarrow 5^o$). (Temperature at $25^o$ unless indicated).

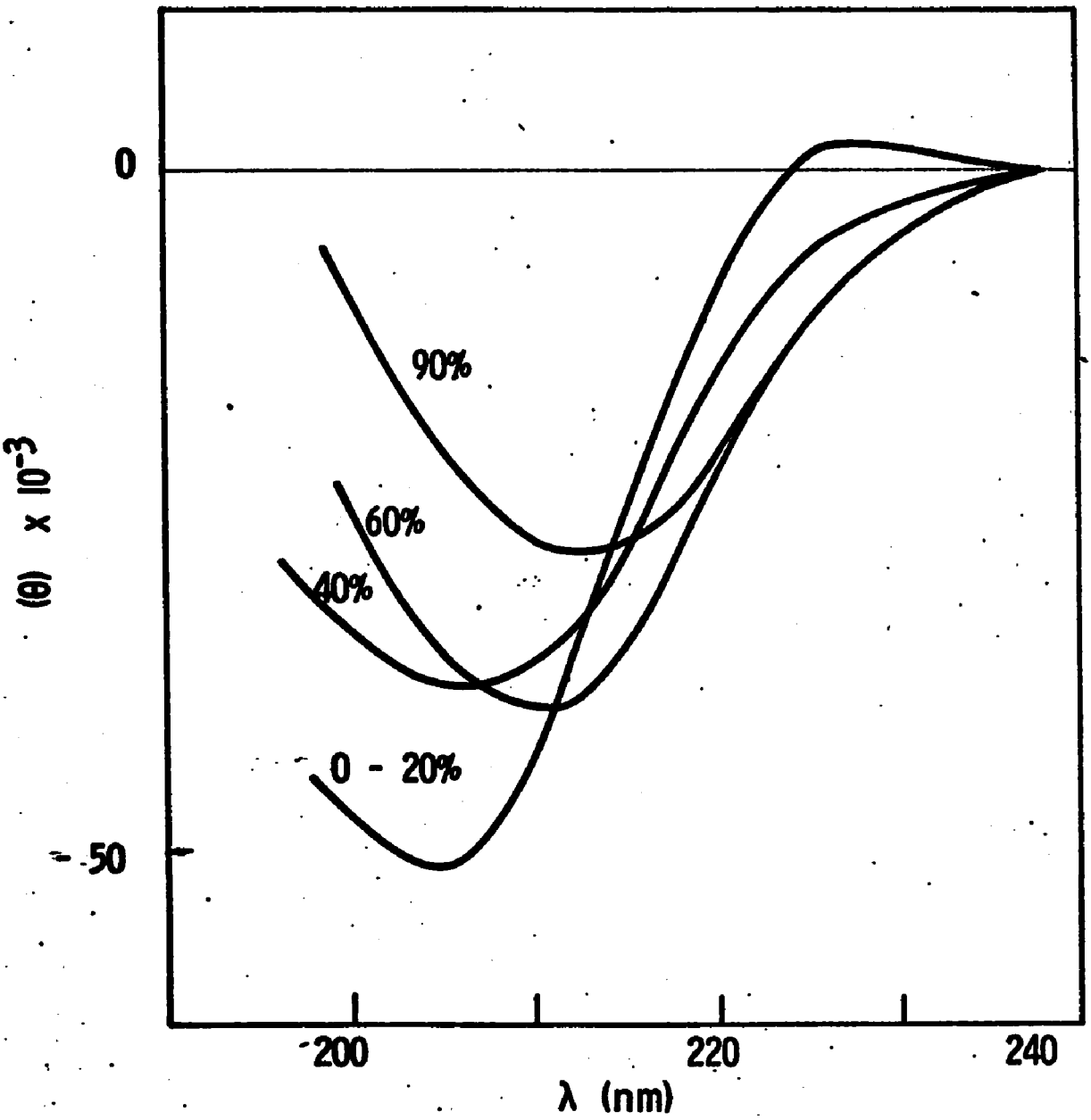



Fig. 7-7. CD of poly-L-proline, mol. wt 35,000, 0.03g/l, 5 mm cell: effect of $H_2SO_4$ on $H_2O$ solution.

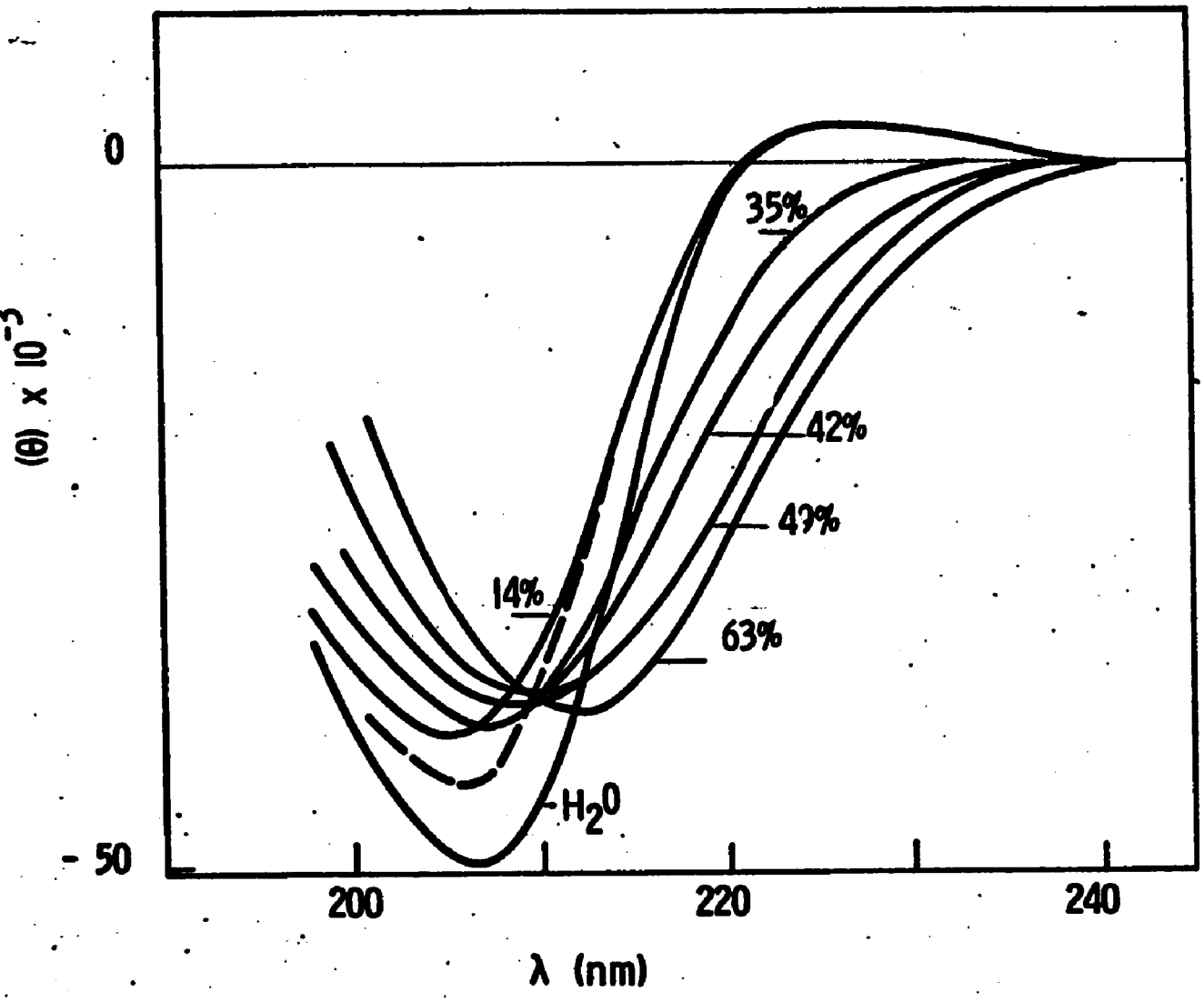



Fig. 7-8. CD of poly-L-proline, mol. wt 7,500, 0.03 g/l, 5 mm cell: effect of $HClO_4$ on $H_2O$ solution. The 63% acid solution was diluted after being in the acid for 15 min. The CD returned to that expected for a 12.6% solution(— —).

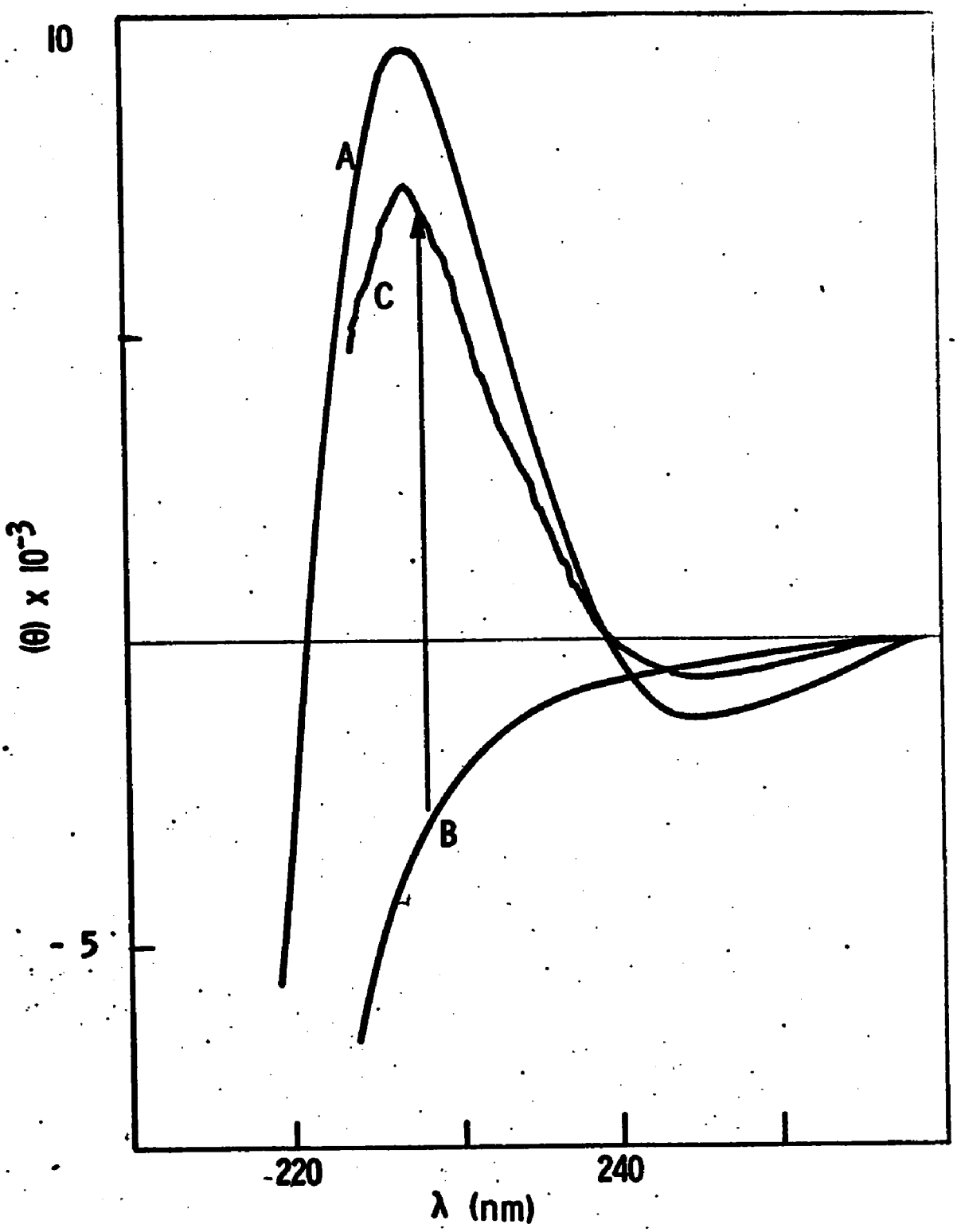



Fig. 7-9. Poly-L-proline, mol. wt. 1,500, in (80% n-propanol: 20% $H_2O$) (A); in (80% n-propanol; 10% $H_2O$; 10% $H_2SO_4$) (B). CD curves run immediately after mixing. On diluting (B)(1:4 n-propanol (C) results showing little change to CD of(A ).

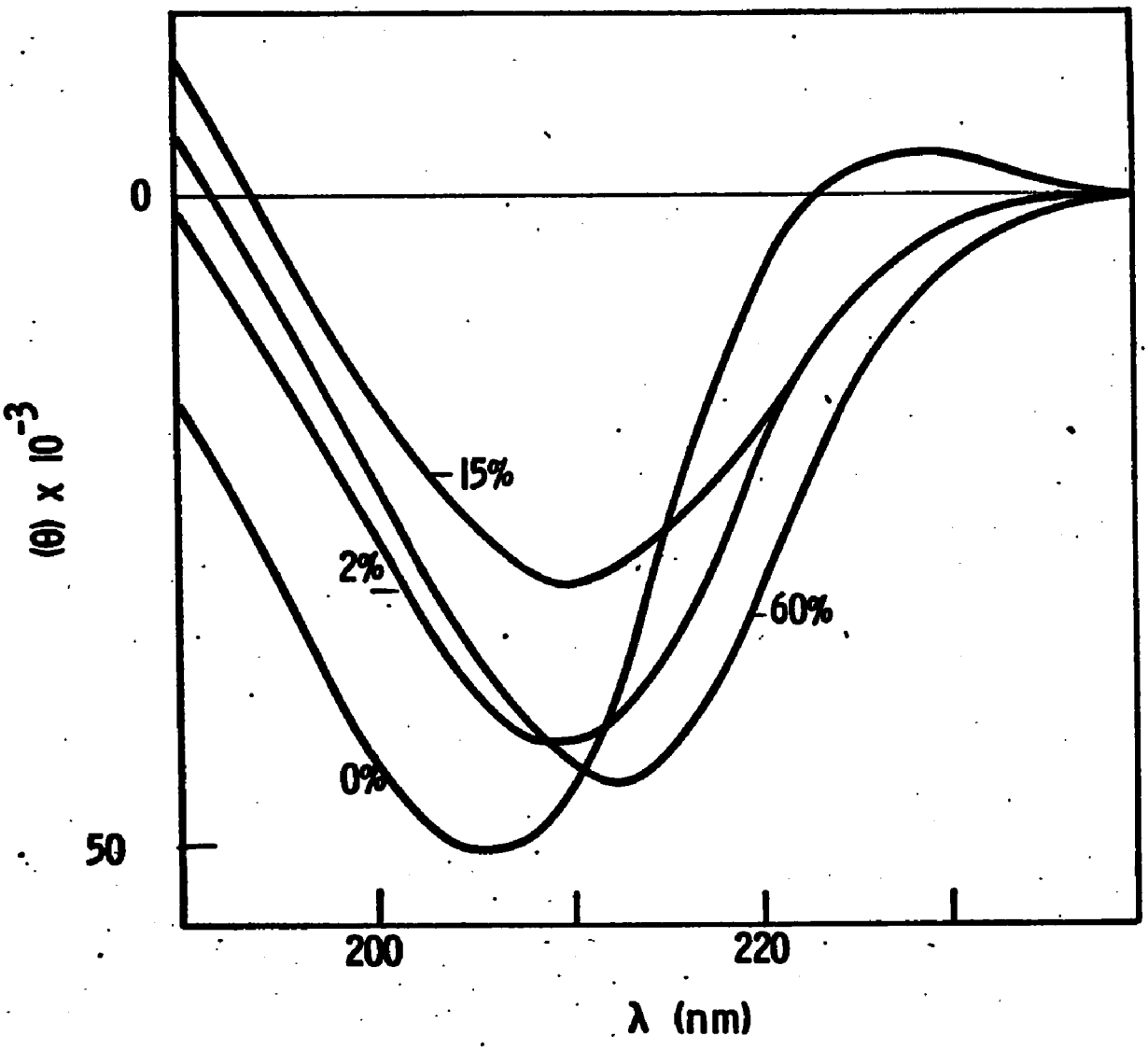



Fig. 7-10. CD of poly-L-proline, mol. wt. 35,000, 0.63g/l: effect of $H_2SO_4$ on trifluoroethanol solutions, showing that only a small amount of acid is necessary, in this case, to destabilize Form II.

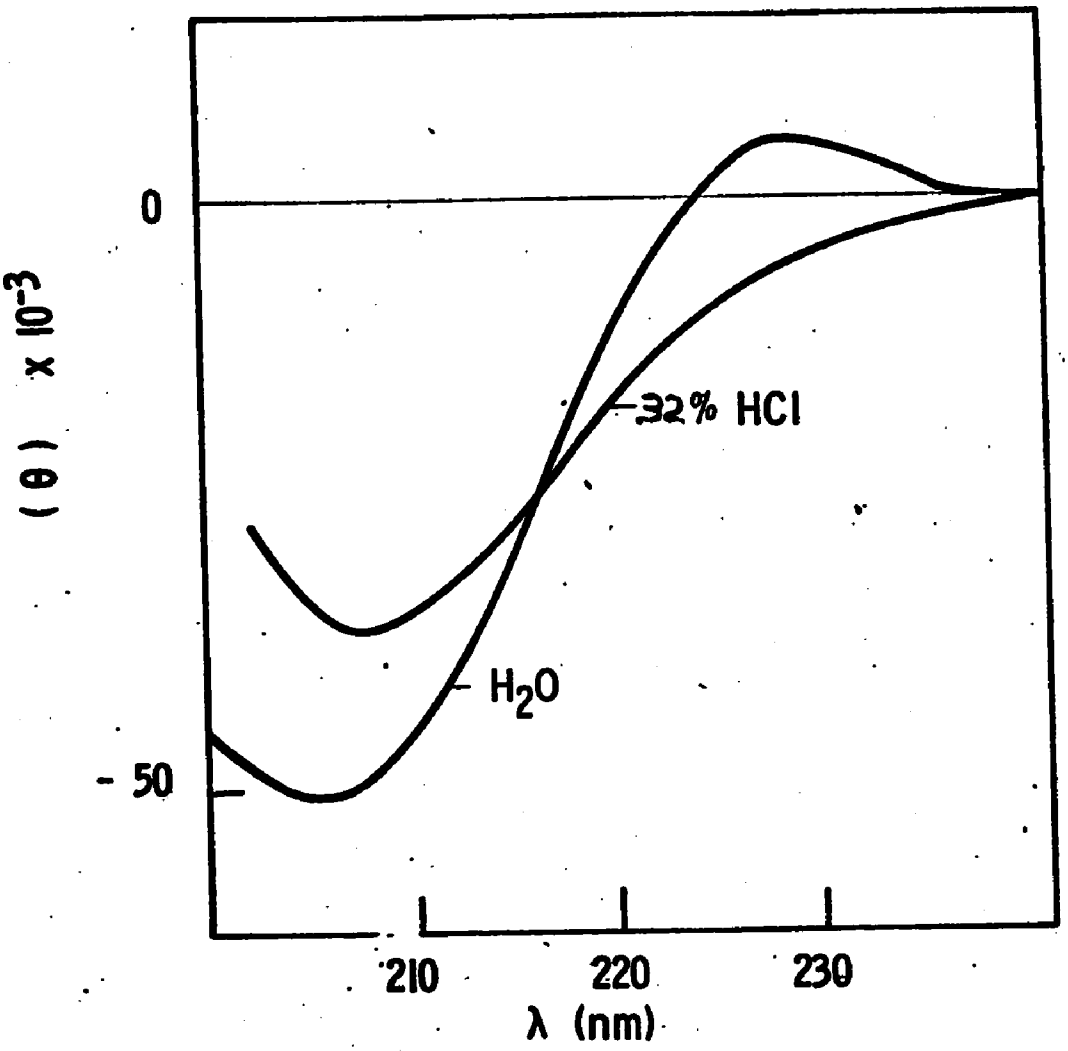



Fig. 7-11. CD of poly-L-proline, mol. wt. 11,500, 1g/l, 0.1 mm cell: effect of 32% HCl on $H_2O$ solution of Form II. (Immediate return to Form II spectra was obtained on diluting 1:50 with $H_2O$ after 1/2 hr.)

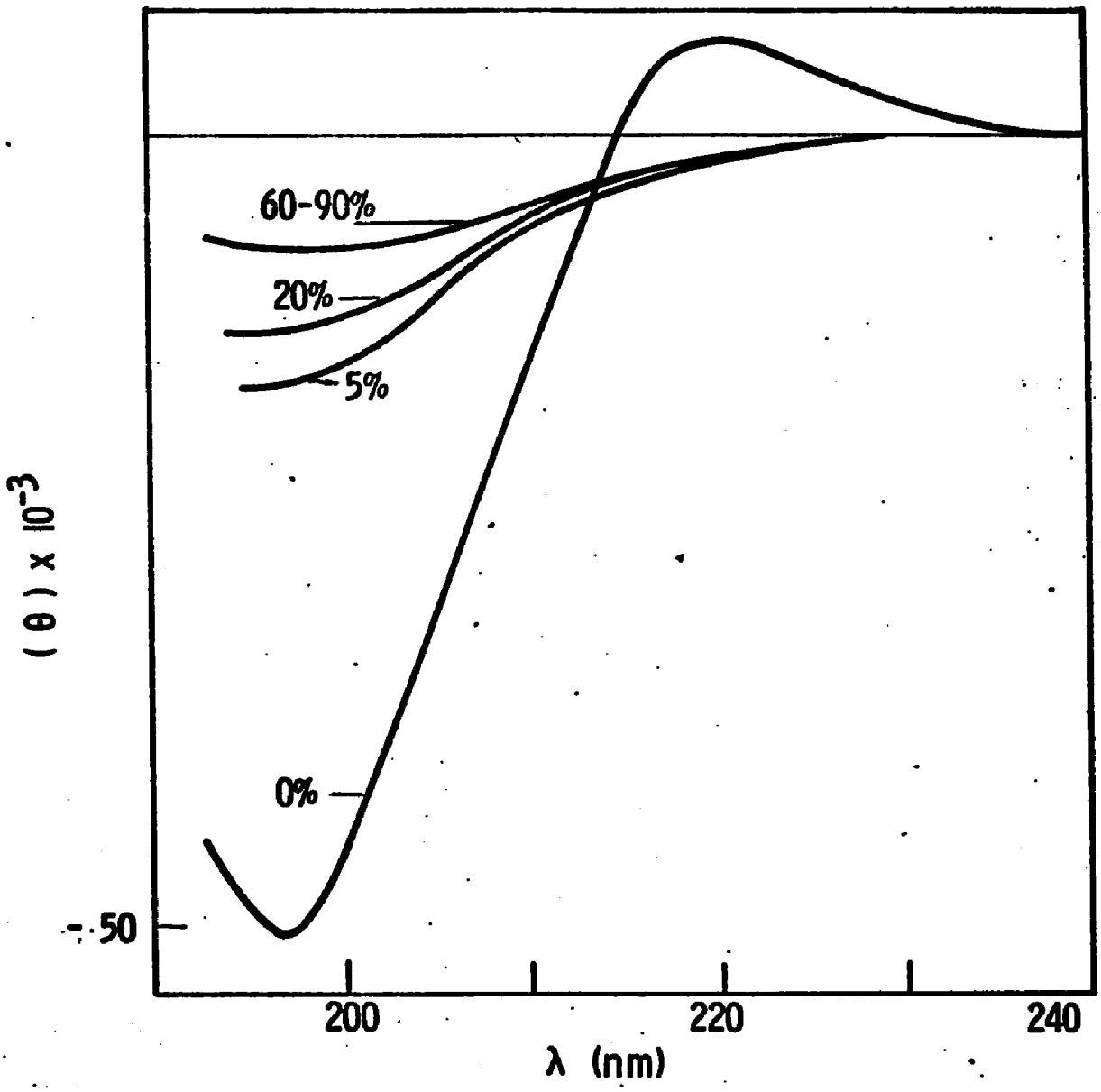



Fig. 7-12. CD of collagen: effect of $H_2SO_4$

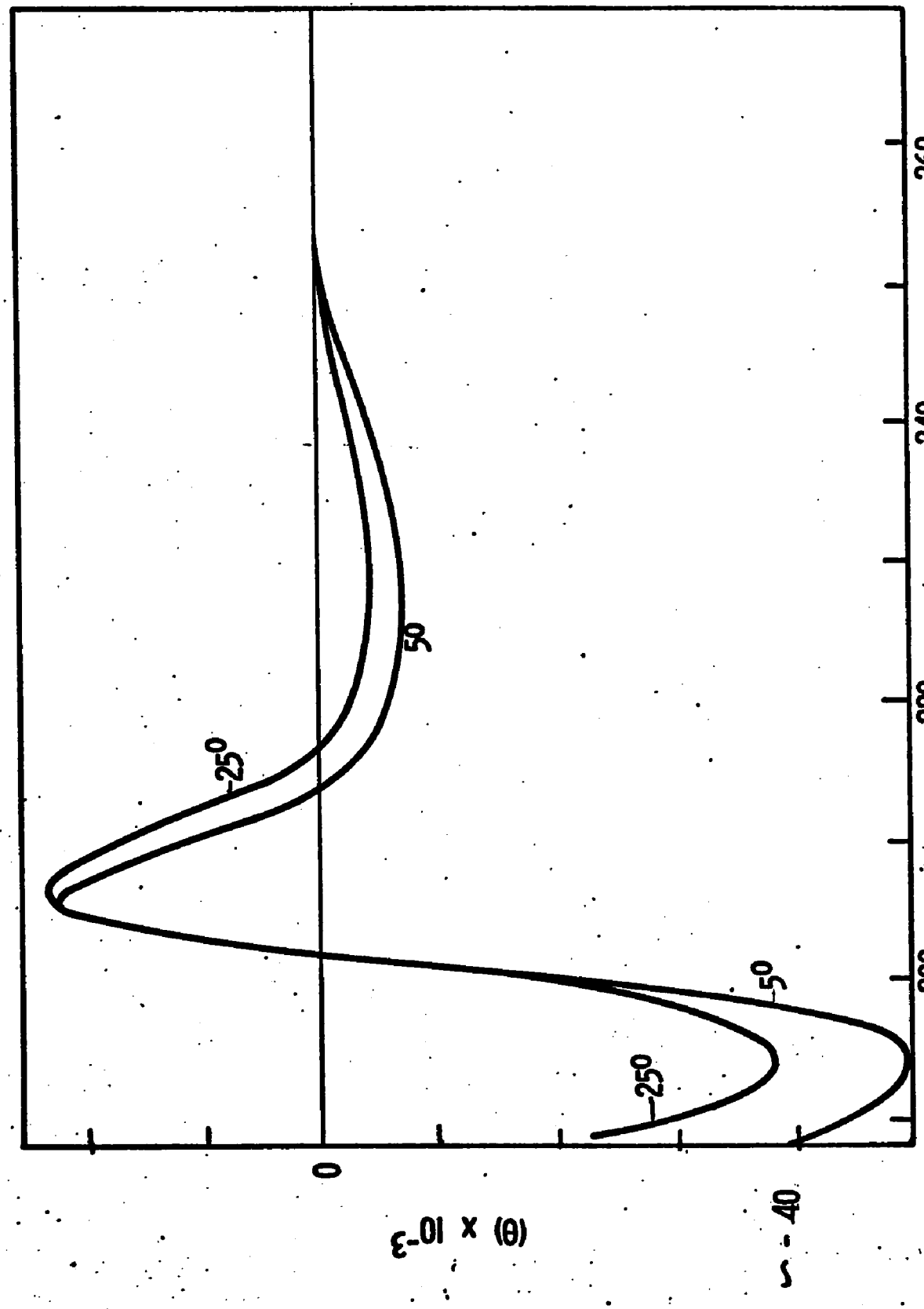



Fig. 7-13.
CD of poly-γ-benzyl-L-asparate in 10% $H_2SO_4$: 90% trifluoroethanol solution.

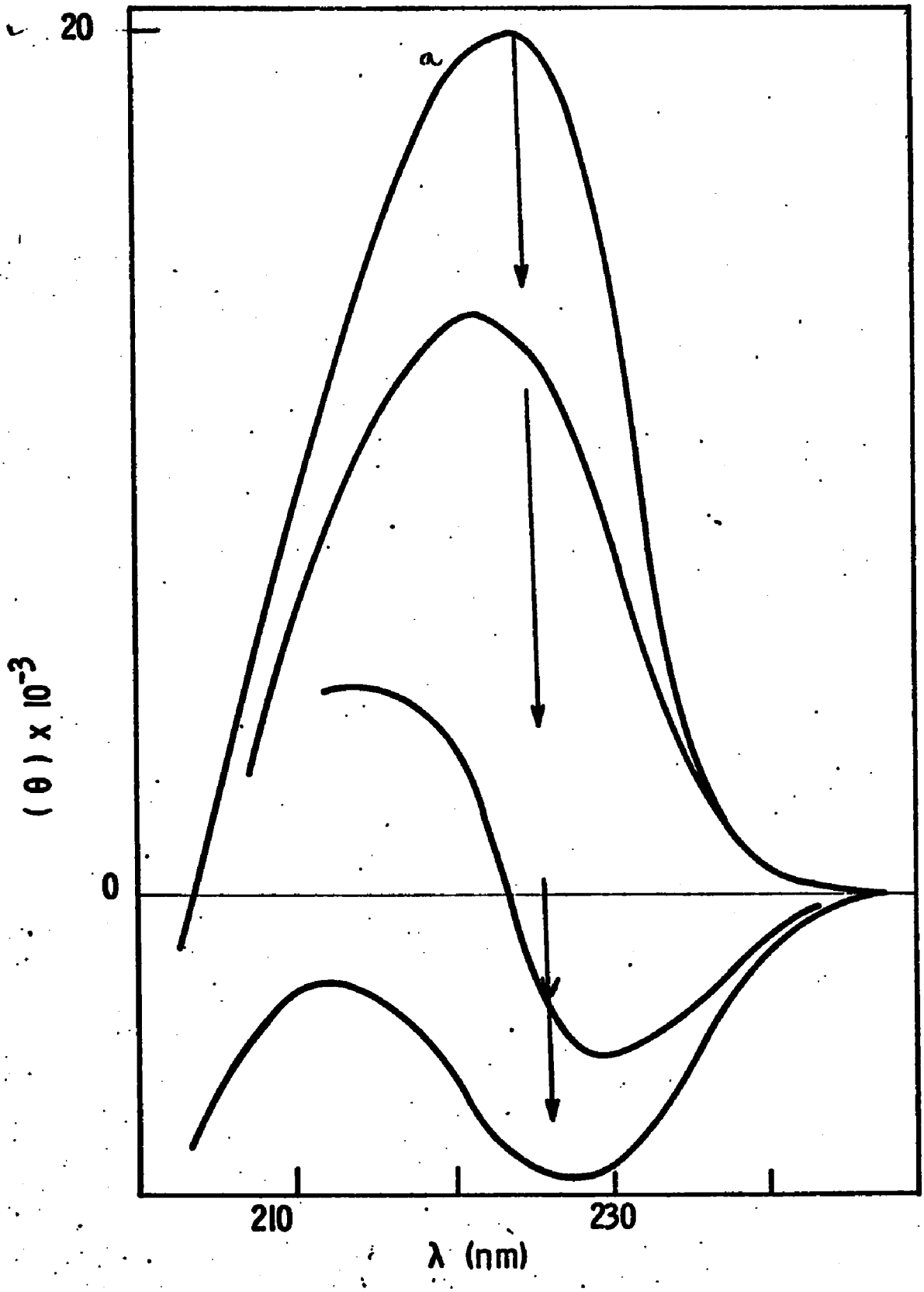



Fig. 7-14. CD of poly-β-benzyl-L-asparate in 10% $CHCl_3$: 90% dichloroethanol. CD changing with time at 5°, spectra run at approximately 30 min. intervals.

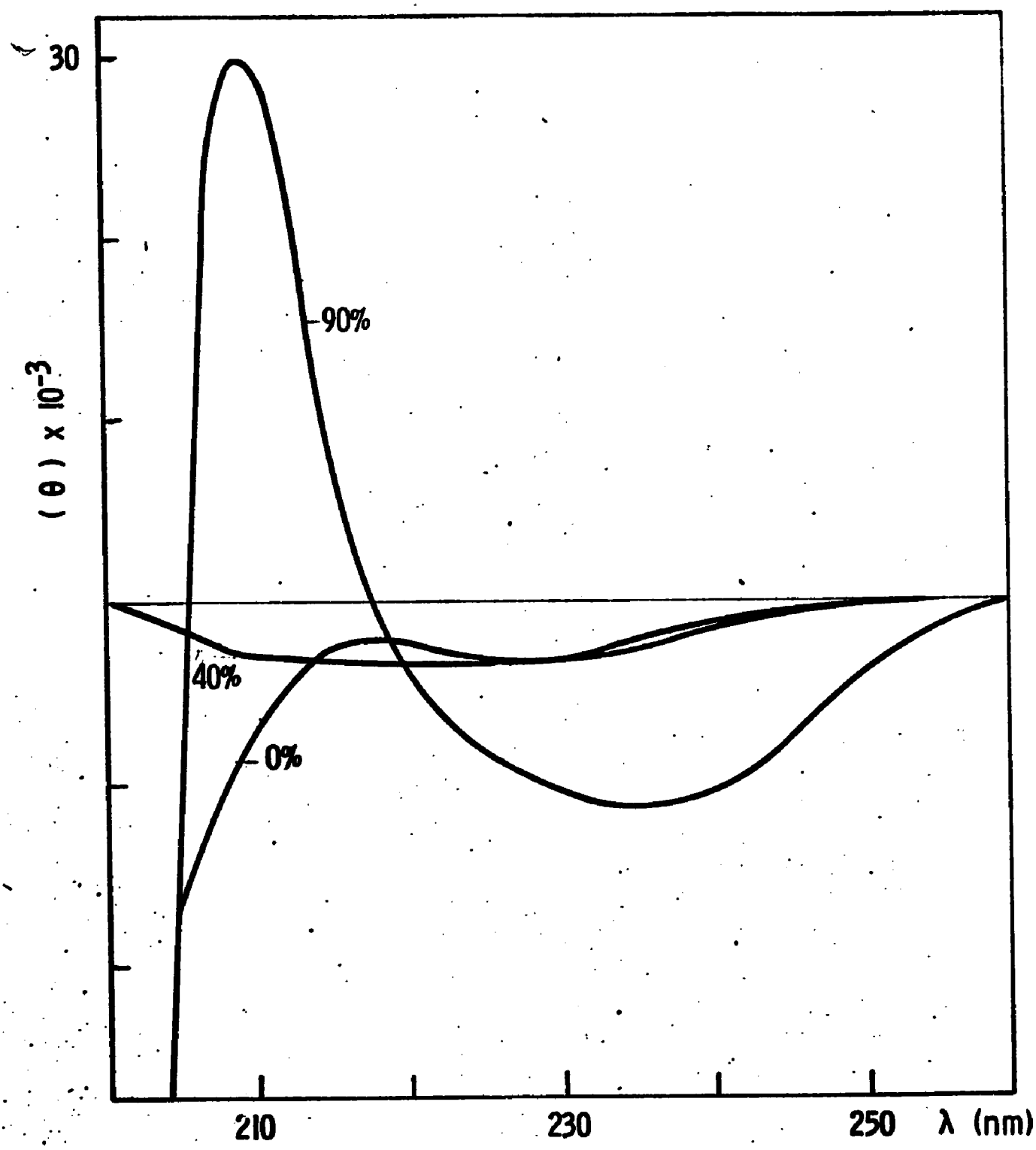



Fig. 7-15. CD of poly-L-aspartic acid: effect of $H_2SO_4$.

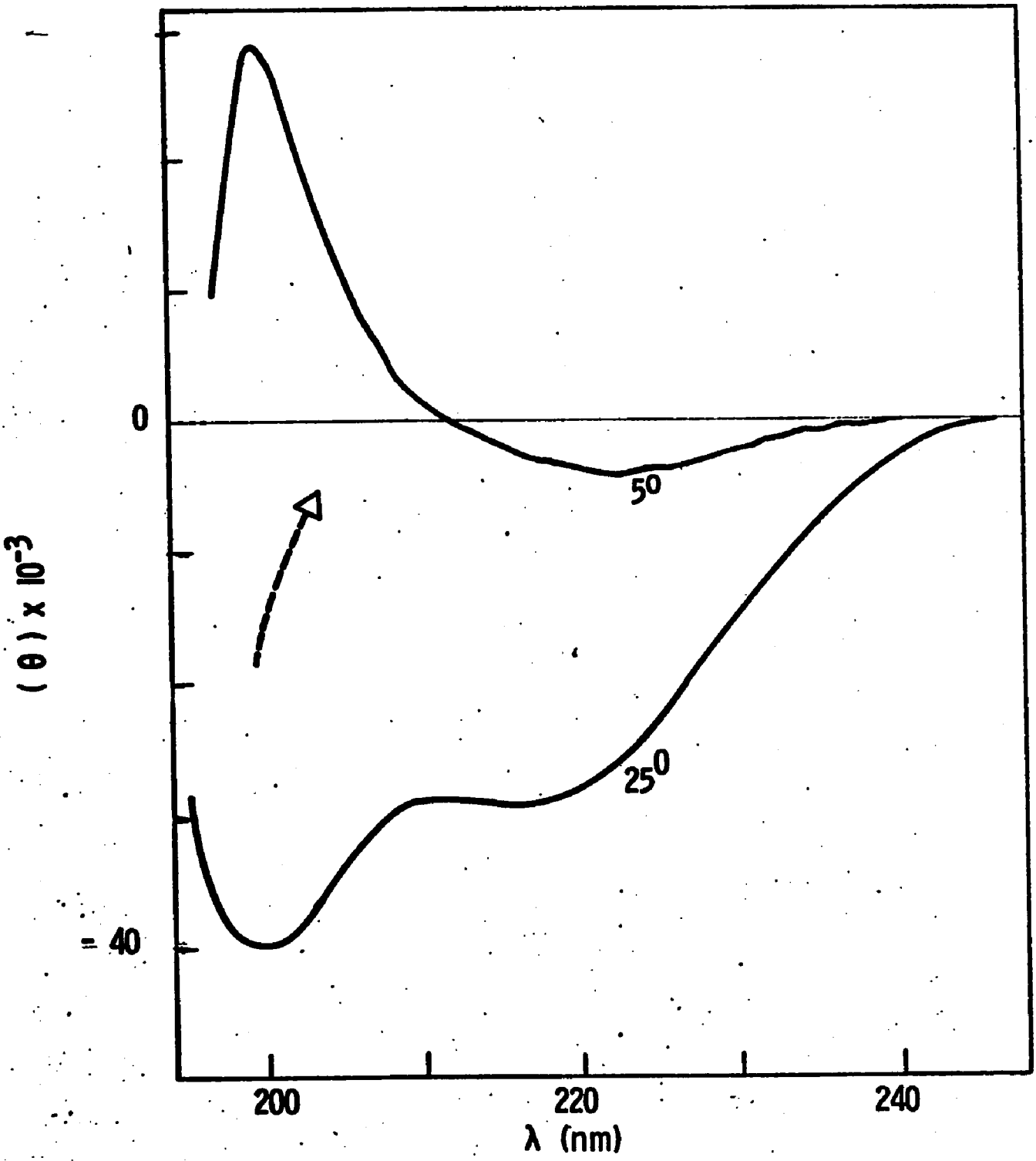



Fig. 7-16. CD of poly-L-aspartic acid, 0.5 g/l, 1 mm cell; effect of temperature at pH 3.4 (sample in trace of $H_2SO_4$

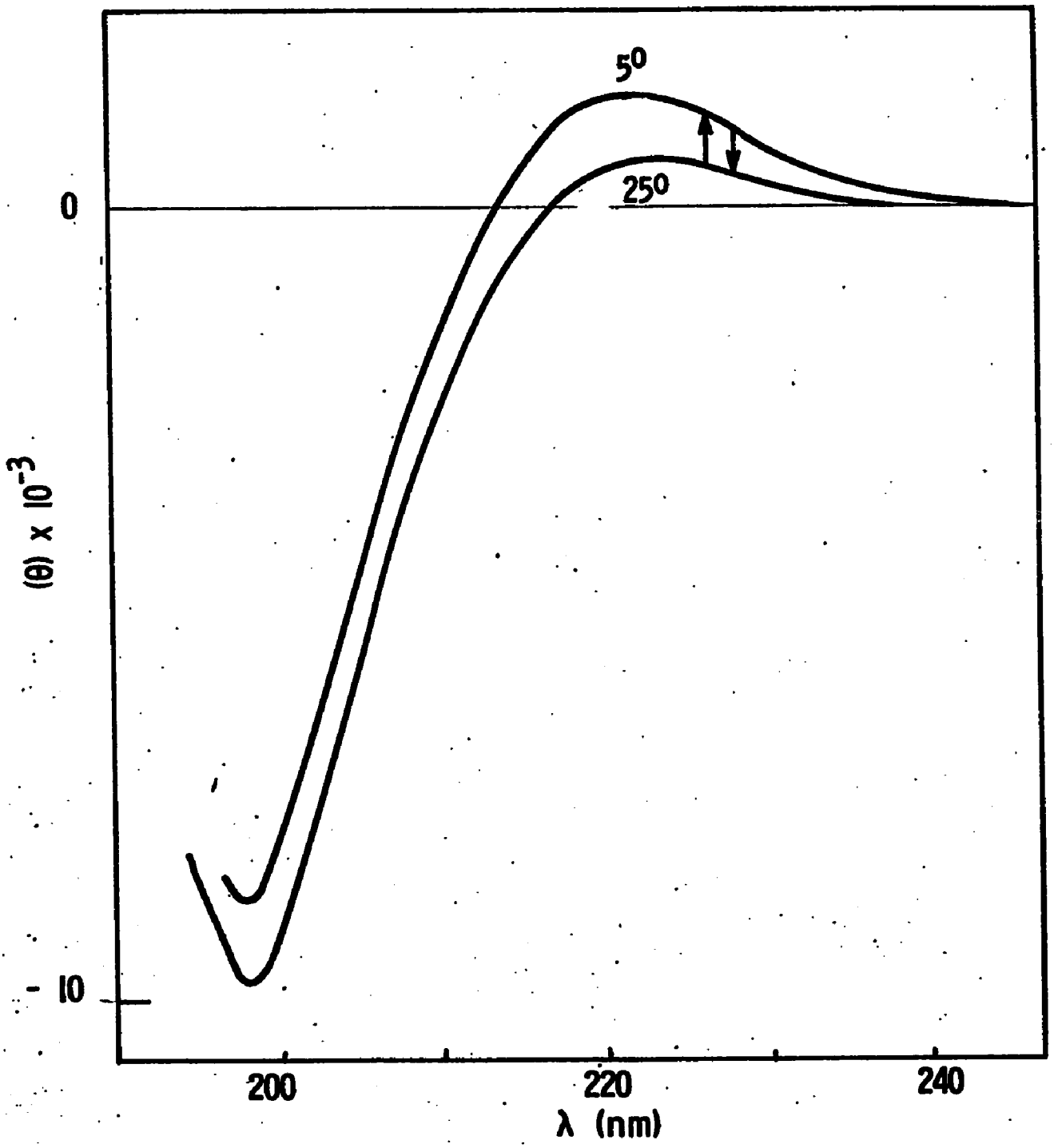



Fig. 7-17. CD of poly-α-methyl-L-glutamate in 95.5% $H_2SO_4$.

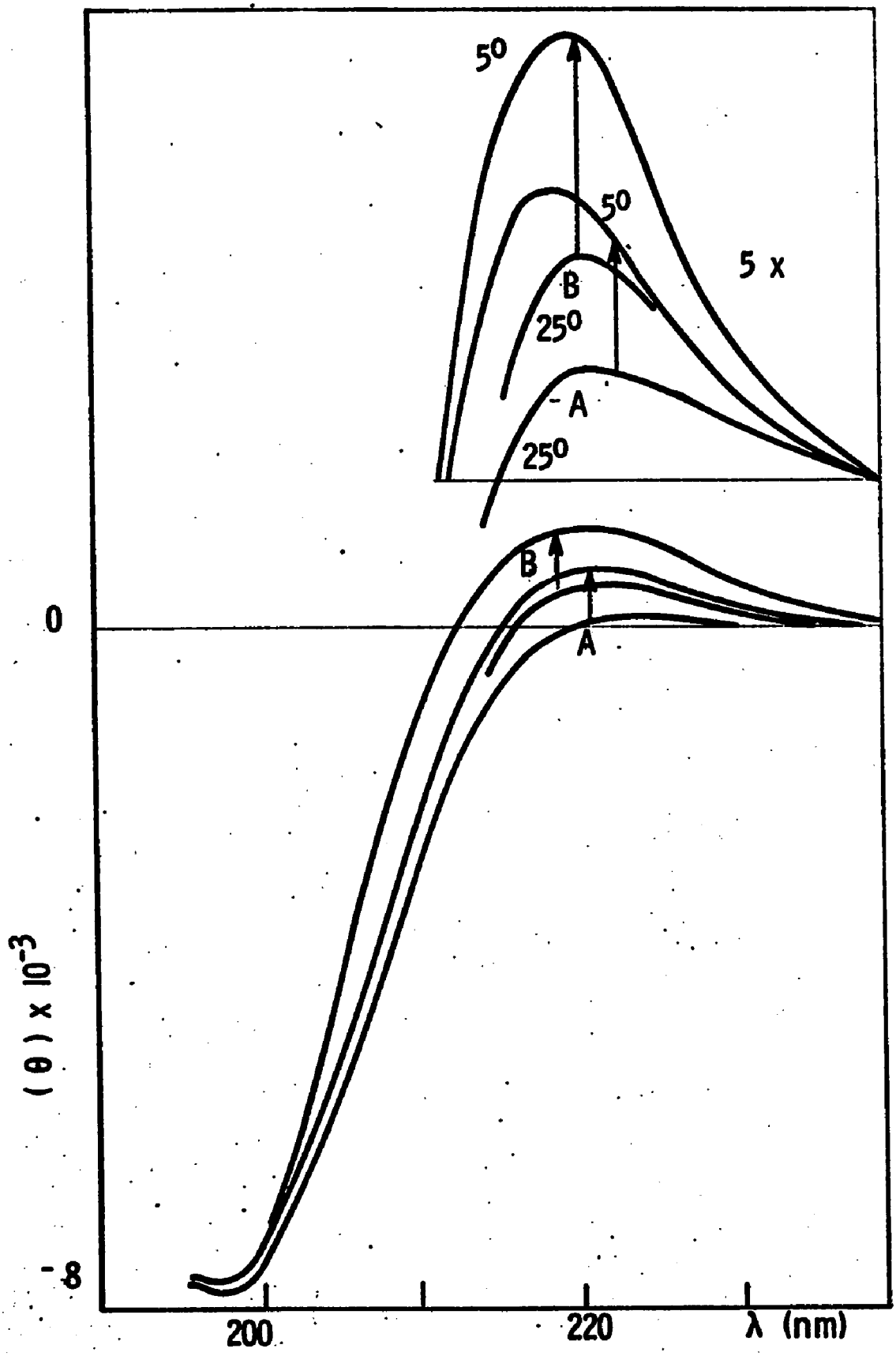



Fig. 7-18. CD of poly-γ- methyl-L-glutamate: effect of temp. in 90 % (A) and 100 % $H_2SO_4$ (B) ($25^{\circ} \rightarrow 5^{\circ}$).

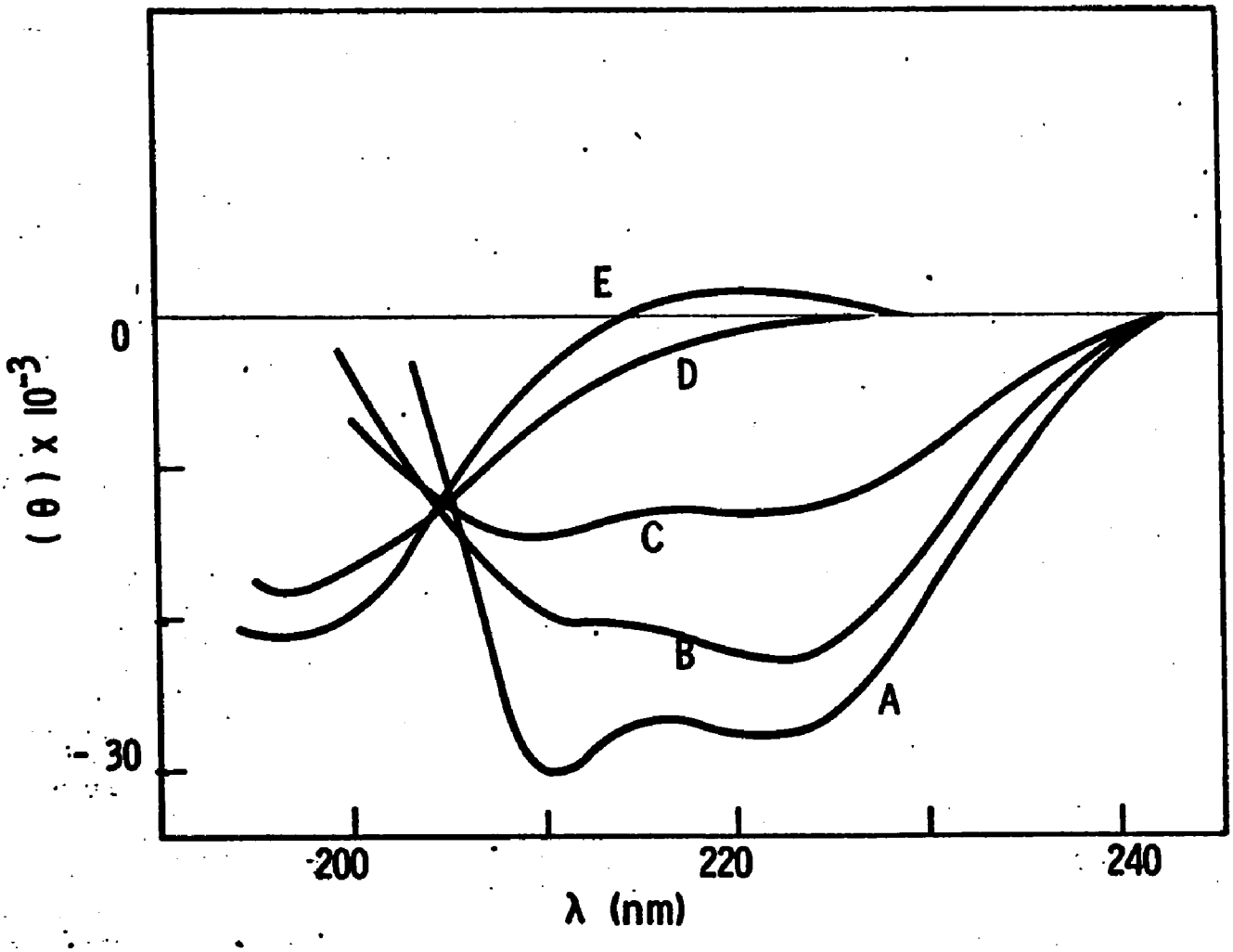



Fig. 7-19. CD of bovine serum albumin: effect of $H_2SO_4$. (A) zero $H_2SO_4$, (B) 9.5 % $H_2SO_4$, (C) 28.5 % $H_2SO_4$; (D) 57% $H_2SO_4$; (E) 85 % $H_2SO_4$. (25° temperature).

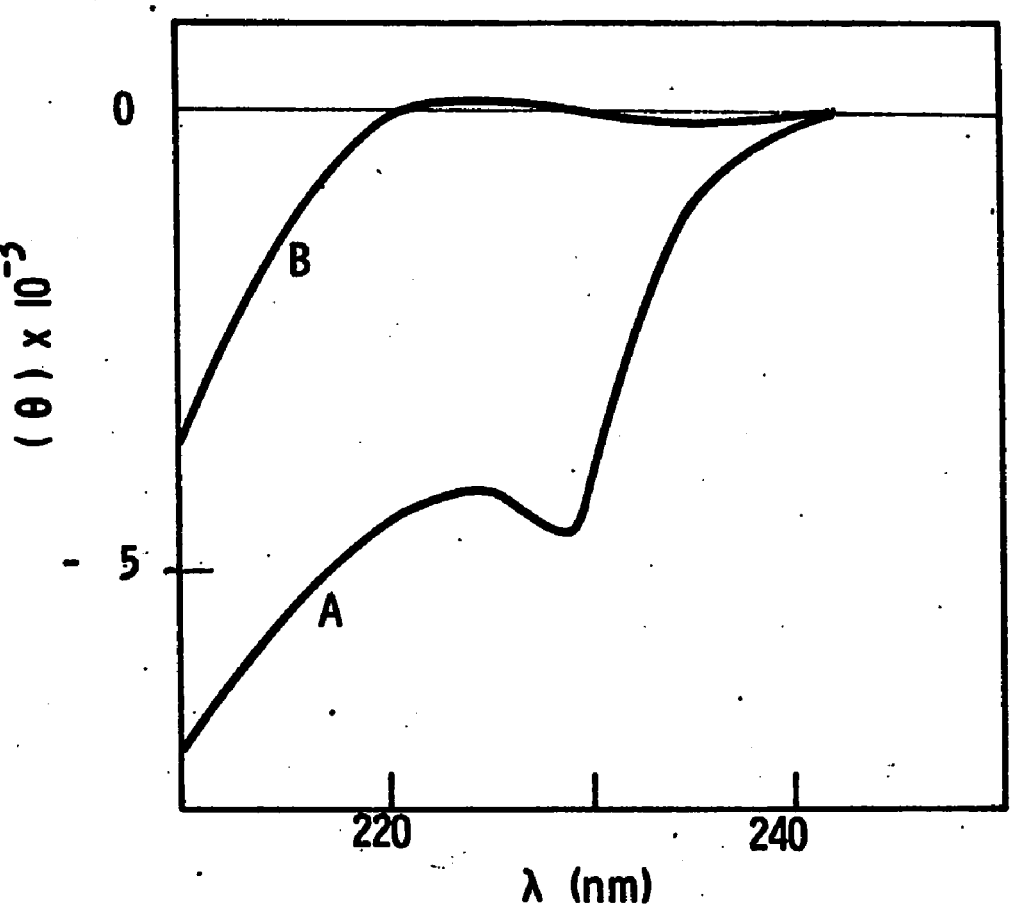



Fig. 7-20 CD of chymotrypsin, 1g/l, 1 mm cell, in $H_2O$ (A), and in 85.5 % $H_2SO_4$ (B).

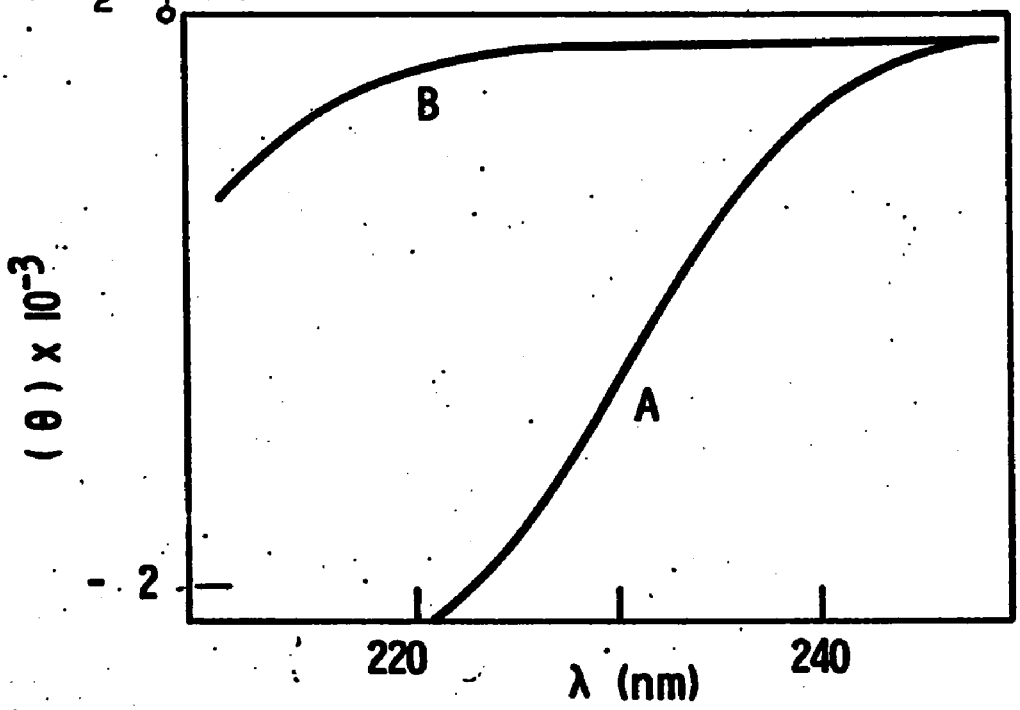



Fig. 7-21. CD of feather keratin, in $H_2O$ (A) and in 85.5% $H_2SO_4$ (B).

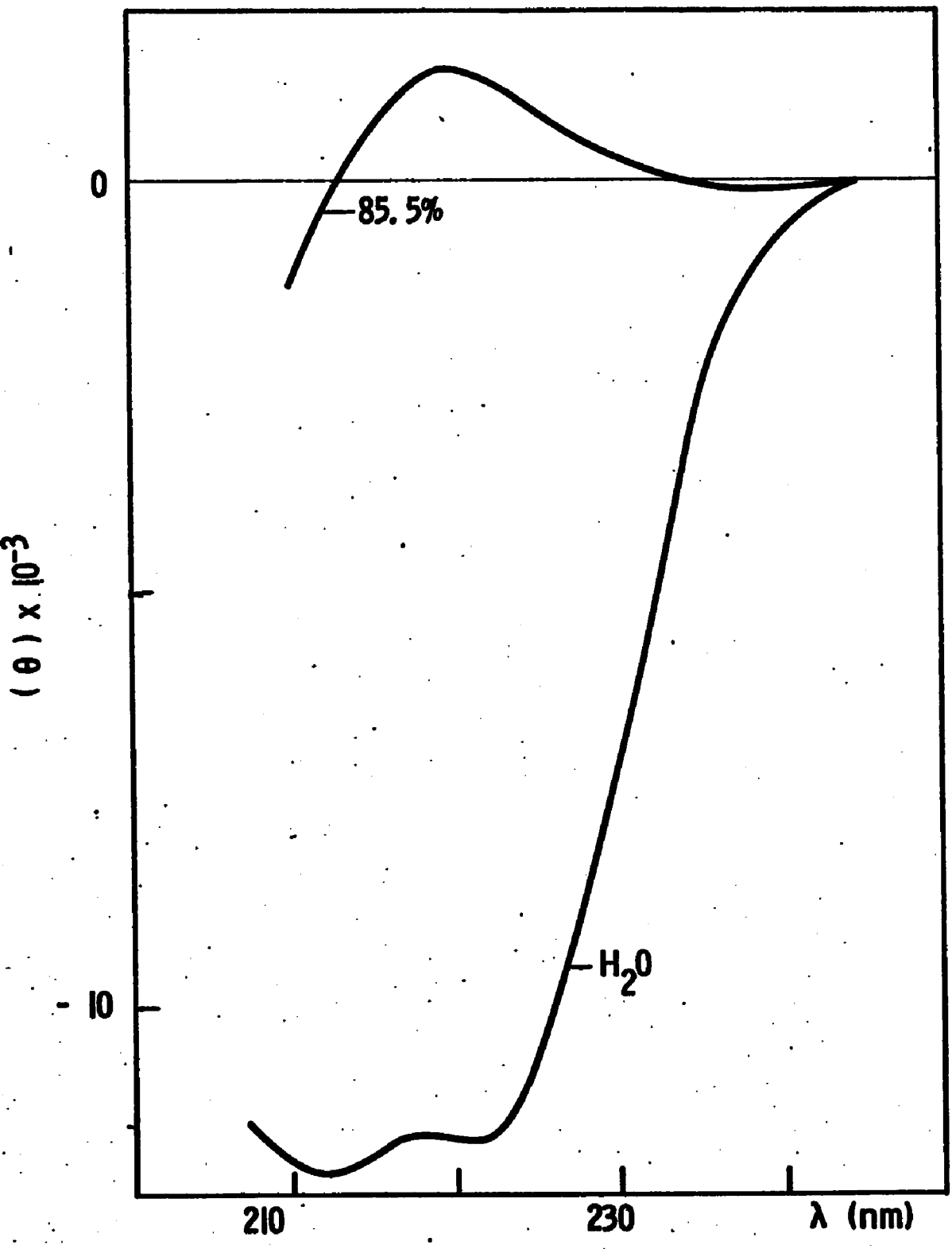



Fig. 7-22. CD of insulin in $H_2O$ and in 85.5% $H_2SO_4$ at 25°.

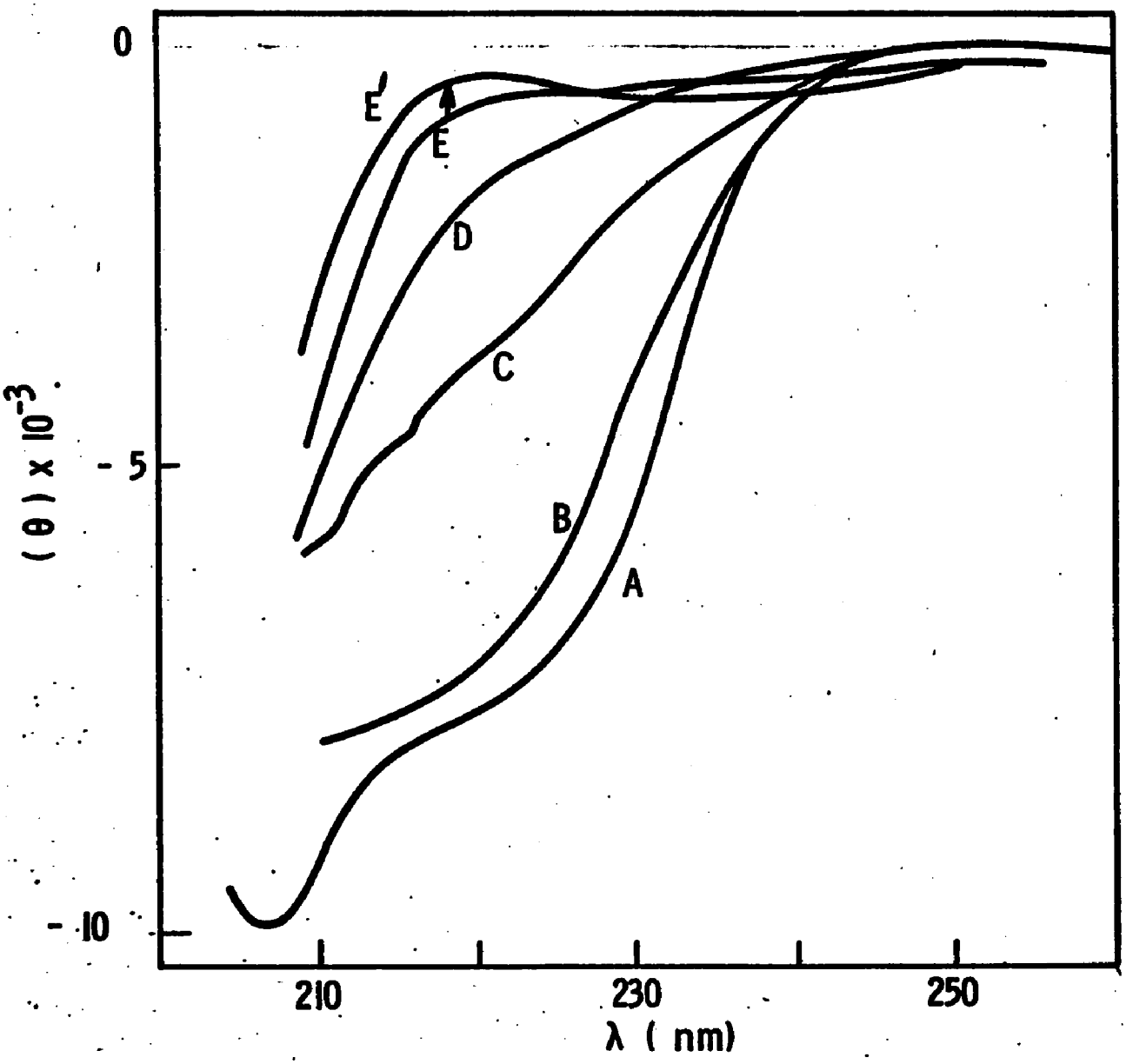



Fig. 7-23. CD of lysozyme, 1 g/l, 1 mm cell: (A) in $H_2O$; (B) in 28.5 % $H_2SO_4$ (25°); (C) in 47.5 % $H_2SO_4$ (25°); (D) in 66.4 % $H_2SO_4$ (25°); (E) in 85.5 % $H_2SO_4$ (25°); (E′) in 85.5 % $H_2SO_4$ ( 5°).

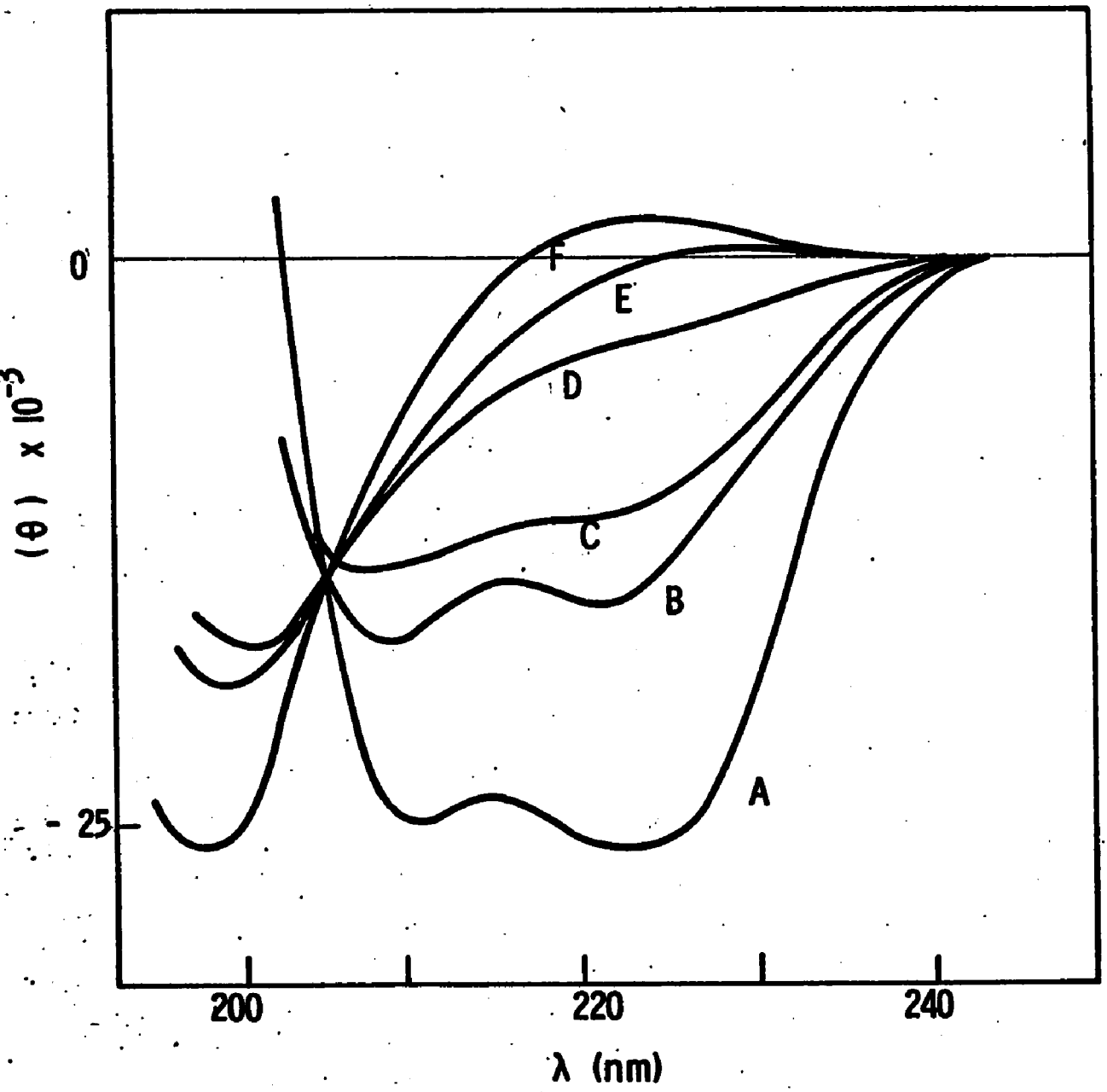



Fig. 7-24. CD of myoglobin, 0.45 g/l, 5 mm cell; at 25°, in $H_2SO_4$: (A) zero %, (B) 4.7%, (C) 22%, (D) 47.5 %, (E) 66.8%, (F) 85.5%.

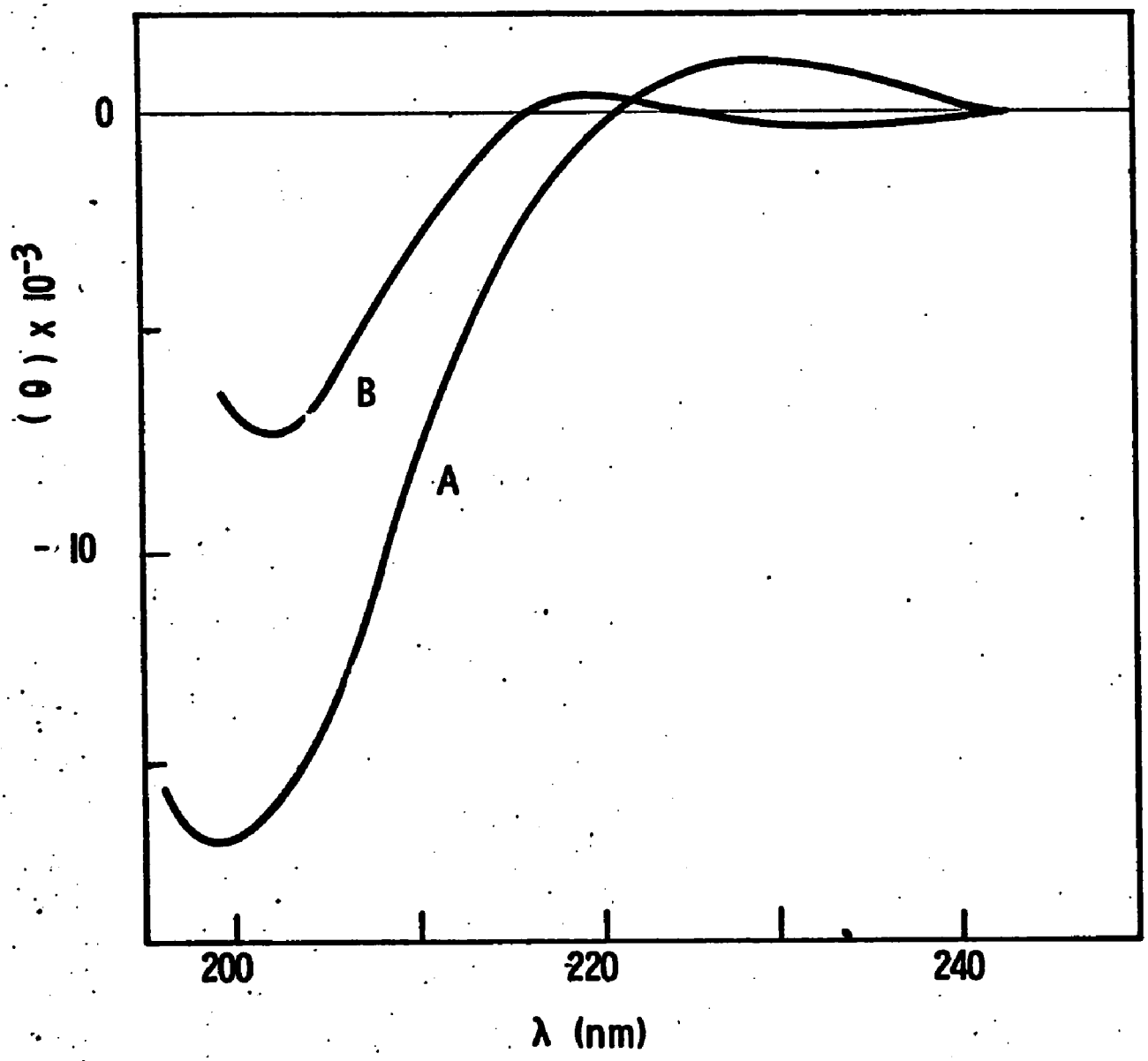



Fig. 7-25. CD of soybean trypsin inhibitor: (A) in $H_2O$, (B) in 90% $H_2SO_4$.

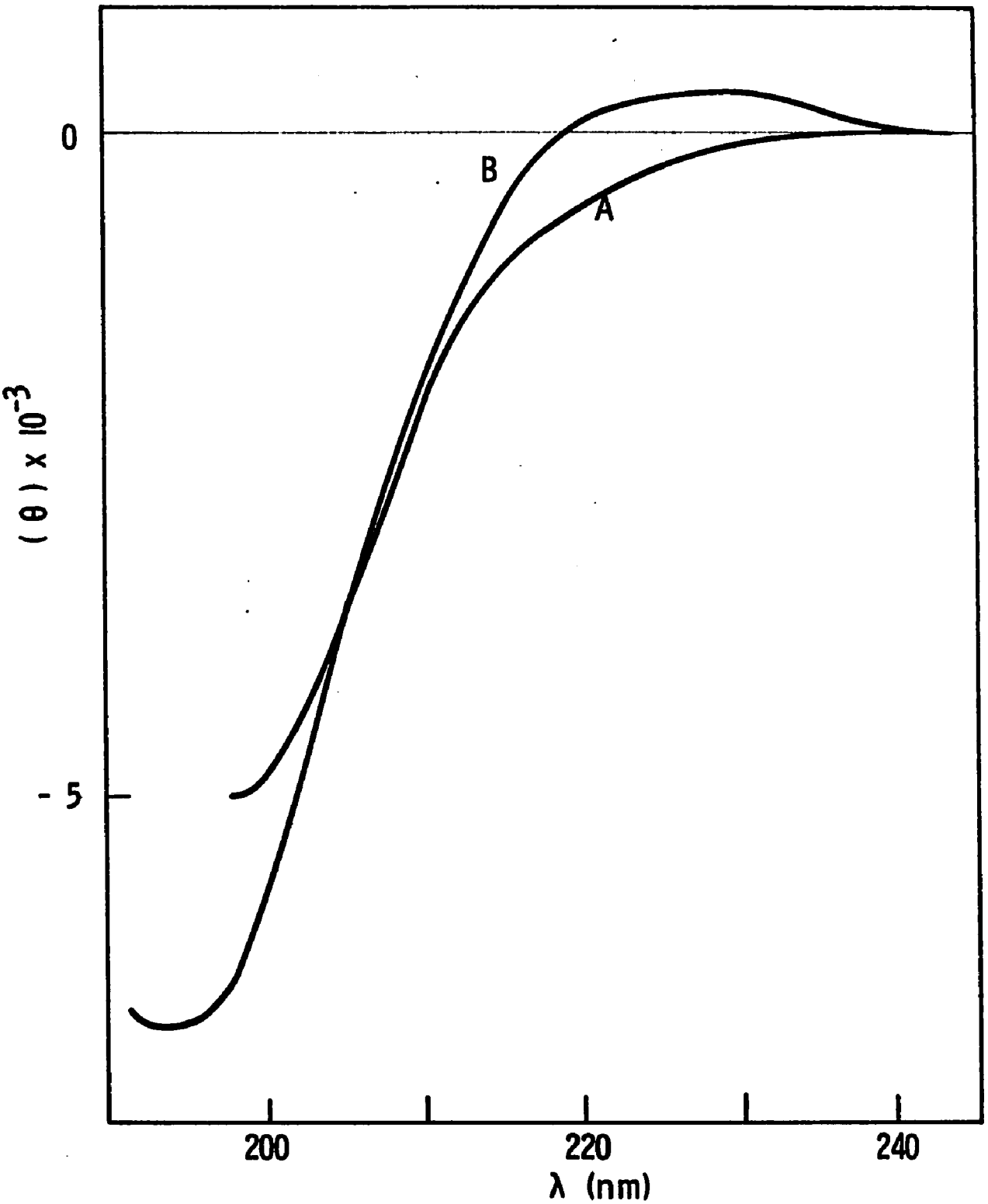



Fig. 7-26. CD of silk: (A) in $H_2O$, (B) in 85.5 % $H_2SO_4$.

Chapter 8

# Interaction of Polypeptides and Proteins with Salts

## A. Introduction

Salts are able to produce dramatic changes in conformation of polypeptides and proteins. Several mechanisms may be operating simultaneously, complicating the analysis, but we will show that some basic conclusions can be reached through consideration of the effects on model compounds, polypeptides and proteins.

Many experiments that have been published on the effects of salts on the conformations of proteins and polypeptides in solution have been limited to techniques which do not give insight into the mechanisms involved. Viscosity is often recorded, and optical rotation at a particular wave length, or over a span of wave lengths with a $b_0$ calculated therefrom. Most recently bovine serum albumin (Noelken, 1970) and poly-L-proline (Schleich and Von Hippel, 1969) have been so studied. An interesting discussion on the degree of denaturation achieved by salts interacting with proteins is given by Tanford (1968). The whole field of the effects of neutral salts on the structure and conformational stability of macromolecules in solution is reviewed by Von Hippel and Schleich (1969).

Circular dichroism (CD) has the possibility of revealing the conformation of the molecule in the salt solution. It is obvious that very different conformations can give the same $b_0$ value and not much confidence can be placed in $b_0$

values to reveal differences in conformation. Thus poly-L-proline when partially transformed from the I state to the II state can have the same $b_o$ value as is obtained when the molecule is in a random form (Tiffany and Krimm, 1968b). Also, changes in the small 220 nm band are lost in the crude interpretation of structure from $b_o$ values.

No far uv CD spectra of proteins in salts seem to have been reported except for our own preliminary work (Tiffany and Krimm, 1967 and 1969). Only a few papers have appeared discussing the CD of polypeptides in salts. Our two papers on the polyelectrolytes, poly-L-lysine (PL) and poly-L-glutamic acid (PGA), (Tiffany and Krimm, 1968a), and on poly-L-proline (PP) (Tiffany and Krimm, 1968b) have been followed by papers by Meyer (1969), by Dearborn and Wetlaufer (1970) and by Mattice and Mandelkern (1970 a and b). These papers were written to continue or dispute our work. Thus Meyer reproduced some of our experimental results on PL and PGA (see Chapter 4) but, nevertheless, disputed our conclusions that the ionized polyelectrolytes had some order due to charge repulsion. Dearborn and Wetlaufer worried whether a salt-treated protein could be random when a protein in guanidine hydrochloride (GuHCl) did not give a similiar CD spectrum. Mattice and Mandelkern (1970a) repeated our CD on PP in $CaCl_2$. They also did viscosity measurements of PP of different molecular weights in solutions of varying amounts of $CaCl_2$ and concluded that the random state was being produced. Independent of our work Quadrifoglio and Urry (1969) gave the CD spectrum of poly-L-serine in 8 M LiCl, and suggested it was due to an unordered form.

Other tools can and will be used to study the conformations that occur due to salt interaction with proteins and polypeptides. Only a start has been made. Thus the Raman spectrum of PP precipitated from $CaCl_2$ solution by ether is reported by Rippon *et al.*, (1970). They were unable, however, to obtain convincing Raman spectra of the solution form. Wallach *et al.*'s method (1970) of scanning the solvent and solution alternately and subtracting the solvent spectra from the solution spectra by the C.A.T. (photon counter with computer-of-average-transients) may help the experimental problem of high background readings. Infrared, of course, also can be used. In the system, poly-L-tyrosine-8M LiCl-methanol, Shiraki and Imahori, (1966) found the amide I mode as a broad band at 1660 $cm^{-1}$, whereas in methanol it was a sharp band at $1650^{-1}$. The LiCl was suggested to be randomizing the conformation of the polypeptide. Nuclear magnetic resonance (nmr) should be helpful in clarifying if ionic absorption on the polypeptide backbone influences the magnetic field felt by the proton on the $C_\alpha$ carbon. The possibility that salts can actually disrupt the trans configuration of PP has been explored by Torchia and Bovey (1971), who concluded that both trans and cis peptide bonds can be randomly distributed along the polymer chain due to the effect of 5 M KI, 4.5 M NaSCN, 9M LiBr and 4 M $CaCl_2$. The possibility that ions are acting indirectly by changing the properties of the solvent water has also been explored by nmr. Thus ribonuclease, like DNA, was shown to denature (melt) in various concentrations of

aqueous $NaClO_4$ at a constant (sodium corrected) value for the water-proton chemical shift (Schleich and Von Hippel, 1970). The conclusion was reached that the $ClO_4^-$ anion does affect macromolecular stability through water structure perturbation. Other anions are suggested to have a $ClO_4^-$-like component in their effect on macromolecular stability. Another use of nmr has recently been made. Thus Rose, (1971) added a paramagnetic ion, $Co^{++}$, to gelatin, at pD 5, and showed that the difference spectrum was due to the shifting out of the scanned field of the resonances due to aspartyl and glutamyl groups because of chelation to the $Co^{++}$ ion.

B. Mechanisms of Salt Interaction with Polypeptides and Proteins.

Before an interpretation of the CD spectra resulting from the effects of salts on polypeptides and proteins can be attempted, we must be aware of the different mechanisms through which salts can change the structure.

(1) One mechanism that is common to all salts is the ability to shield charges residing on the side-chains of the polypeptide or protein. In the case of the polyelectrolytes this shielding can lead to the collapse of the extended state that exists because of charge repulsion of the ionized side-chains (Tiffany and Krimm, 1968; Krimm, Mark and Tiffany, 1969).

(2) Another direct result of the shielding of charged side-chains is revealed in the polyelectrolyte polypeptides when they are in a helical form, or close to helical form in

spite of having some residual charge on the side-chains. Under these conditions the addition of salt reduces the amount of work that must be done against the residual charges when the chain becomes further ionized due to a change in pH. This reduces the stability of the helical form. An example is the interaction of NaCl with PGA as shown by Wada (1960), and Nagasawa and Holtzer (1964).

(3) Some ions specifically bind to a charged side-chain. A good example is $ClO_4^-$, which in very small amounts, can make poly-L-lysine and poly-L-arginine go $\alpha$ helical at pH 6 (Rifkind, 1969). (We have shown that $HClO_4$ can make poly-L-lysine helical even under very acidic conditions (Fig. 7-2). Small amounts of $NaClO_4$ (0.003 M) have also been shown to make poly-L-methionine-S-methyl sulfonium perchlorate (0.0057 M/1) go $\alpha$-helical (Makino, et al., 1968). As far back as 1957, Gregor and Gold reported a very strong polyion-perchlorate interaction using poly-N-methylimidazole hydroxide.

(4) The interaction of $ClO_4^-$ with the structure of DNA (Gordon et al., 1965) and with ribonuclease (Schleich and Von Hippel, 1970) has been interpreted in terms of a change in water structure by the ion. These results should perhaps be reviewed in view of the known binding properties of the $ClO_4^-$ ion. There may be little meaning to the liquid "structure temperature" attainable by either simple heating or adding isothermally the $ClO_4^-$ ion, since our results will show that a different state of the protein ribonuclease results under these different conditions. This does not rule out that some salts may act indirectly though changing the solvent structure.

(5) A main consideration in the mechanism of many salts must be their interaction with the back-bone amide and carbonyl groups. Poly-L-proline, lacking the ability of charges to interact with the pyrrolidine ring, makes a good model compound to study the fundamental interaction along the chain by salts that may occur in all polypeptides and proteins. We will consider it, therefore, in some detail. The fact that it lacks a hydrogen on the backbone nitrogen should not interfere with using it as a general model. The possibility of finding or not finding an effect by salt on the cis-trans isomerism exists for this special protein, and would be useful information in attempting a mechanism of salt interaction with the peptide backbone.

C. Effect of Neutral Salts on the Configuration of Poly-L-Proline.

The initial experiment showing the effect of a lyotropic salt on poly-L-proline II (PP II) was done by Blout and Fasman (1958). They used 2 M and 4 M KSCN and reported drastic changes in viscosity and optical rotation. Thus specific viscosity $[\eta]$ changes from 0.67 in water to 0.32 in 2M salt and to 0.17 in 4 M salt, while optical rotation at 546 nm, $[\alpha]_b$, changed from $-694^\circ$ to $-406^\circ$ and $-402^\circ$ in 2 M and 4 M salt. It was suggested that the effect of KSCN was due to its large polarizability promoting intra-chain contacts leading to contraction of the polymer.

Harrington and Sela (1958) reported $[\alpha]_b$ fell from $-540^\circ$ in water to around $-250^\circ$ in 12.9 M LiBr, or in saturated

$CaCl_2$, or in saturated $LiClO_4$. They also noted the same dramatic change in $[\alpha]_D$ when pyridine saturated with LiBr was used. The effect of salt was shown to be completely reversible. Simple dilution returned $[\alpha]_D$ to its value in water. Sedimentation experiments showed that the effect of LiBr was not a result of aggregation. The specific viscosity in 8 M LiBr was 0.07, a marked drop from its value, 0.23, in water. They concluded that the only possible mechanism was that salt was disrupting the regularity of the structure by causing trans-cis isomerism. A different origin for the effect was later suggested by the same authors. Thus Steinberg, Harrington, Berger, Sela and Katchalski (1960) suggested isomerization about the $C^{\alpha}$-carbonyl carbon bond. This decision was based on (1) the immediated effect of the salts on the value of $[\alpha]_D$ of PP II with an immediate return of $[\alpha]_D$ to normal on dilution at room temperature, and (2) the fact that free rotation was not initiated in N,N-dimethylacetamide by LiBr or KSCN from conclusions based on nmr experiments. They were not perturbed that at a temperature of 1.7° there was an immediate change in $[\alpha]_D$ on dilution, followed by a slow continued change.

That there can be large changes in viscosity without corresponding changes in $[\alpha]_D$ should be noted from the data of Cifferi and Orofino (1966). Thus the effect of 0.7 M KCl was to reduce the $[\eta]$ value of PP II from 0.69 in water to only 0.39 in the salt, while $[\alpha]_D$ would be changed only from -660° to -650° if we use the data of Schleich and Von Hippel (1969).

A slow change in $[\alpha]_D$ was noted by Kurtz and Harrington (1966) when 3M LiBr-propionic acid was used as a solvent for PP I, whereas the change to the same final $[\alpha]_D$ was fast if the same solvent was used on PP II. They also noted, in keeping with the early work on pyridine-LiBr solutions, that 3 M LiBr in anhydrous acetic acid was as effective as 10 M LiBr in water in reducing the value of $[\eta]$ and $-[\alpha]_D$. Several polypeptides and gelatin were found to bind ~~LiBr~~ when precipitated from aqueous solutions by acetone. Polyglycine bound up to 1 mole of LiBr per 1.6 moles of peptide residue. In the case of PP there was found 1 $Li^+$ per 4 or 5 proline residues and, therefore, the suggestion was made that LiBr modified the resonance within the peptide group to allow a multi-residue complex to form.

In 1968 Tiffany and Krimm reported that a CD spectrum, which they related to randomness, was obtained immediately when form I PP was dissolved in 4 M $CaCl_2$. The state of the cis to trans isomerism could be monitered however, by diluting the samples after different periods of time following mixing. In this way, it was concluded that "<u>randomness</u>" was not related to cis - trans isomerism but occurred independently.

Schleich and Von Hippel (1969), using $[\alpha]_D$ as a monitor, concluded that the major specific affectors of salt induced rotatory changes are the anions. The ions tested were ordered: $SCN^- > ClO_4^- > I^- > Br^- > NO_3^- > Cl^-$. They stated, without supplying data, that selected viscosity experiments were done

and that the observed effects on specific levorotation have a structural as well as an optical basis.

## Experimental

### Poly-L-proline (PP)

Since the early experiments reported by Tiffany and Krimm (1968a), more detailed studies have been made using several different salts.

Lithium salts have been reported to be effective denaturants for proteins and polypeptides, but we find LiCl (8 M) an ineffective agent to change the CD spectrum of PP. Thus the 228 nm band of PP II is only reduced in intensity to 0.6 of the same band of PP II in water (Fig. 8-1) whereas the band is approximately at zero relative intensity in 4 M $CaCl_2$ (Fig. 3-5). The family of curves of PP II in different concentrations of LiCl seems to have an isodirhroic point near the zero dichroism line, and therefore, higher concentrations of LiCl would still have positive dichroism in the 228 nm region.

Similarily, $LiClO_4$ is found to be ineffective in transforming the CD spectrum of PP II. In Fig. 8-2 we see, that water solutions of $LiClO_4$ at 5.4 M have made only small changes in the spectra. $NaClO_4$, at the same molarity, seems to have reduced the intensity of the 205 nm band, but the samples were unstable and became cloudy with time. A simultaneous loss of CD intensity resulted. When we go to methanol solutions of the same salts, we again find, at the highest concentrations of both $LiClO_4$ and $NaClO_4$ (Fig. 8-3) no noticeable effect of the salts.

We have already reported the effect of $CaCl_2$ on PP, (Tiffany and Krimm, 1968a), but include one family of curves of PP in $CaCl_2$ at different concentrations of salt (Fig. 3-5). There is a strong indication of an isodichroic point as we initially reported, in spite of the disagreement claimed by Mattice and Mandelkern, (1970).

Water solutions of $Ca(ClO_4)_2$ were also tested (Fig. 8-4). In the most concentrated solutions, approximately 4 M, we find a dramatic shift of the negative band from 207 nm to 215 nm with a decrease of intensity to one half. The positive 228 nm band is lost. Most interesting is the positive dichroism in the 190 nm region, showing the presence of a new band. Both $Ca(ClO_4)_2$ and $Mg(ClO_4)_2$ in methanol solutions proved to be good in disrupting PP II structure (Fig. 8-3). A small continuing change with time occurs.

Another salt, $LaCl_3$, was found to be very good at causing a transformation in PP II. This salt does not seem to have been used previously by any workers in conformation studies, and so its effects should be particularily noted so that it can be applied to other problems in protein structure (Fig. 8-5). One sample of PP II in $LaCl_3$ was heated to 55°. The negative band decreased in intensity. On cooling it remained at the same position. The dichroism in the 225 nm region became more negative with the increase in temperature, but seemed to revert on lowering the temperature. The isodichroic point of the curves remained (Fig. 8-6).

Several experiments were done on diluting the high concentrations of salt solutions to study the reversibility of the salt-polymer interaction. In general, samples were diluted one part to nine of water and the CD spectra were run in a cell with a path length 10 X that used in the undiluted sample. It was always found that the return to form II seemed more rapid than could be recorded under the present techniques. However, by continuous recording at one wave length, a small continued change with time to form II was noted. (We did not get the large effects recorded by Steinberg et al. (1960) and by Kurtz and Harrington (1963) who used LiBr and KSCN and recorded $[\alpha]_D$.)

Many of the salts that have been implicated as effective denaturants are very absorbing in the far uv (e.g. KI, NaSCN, and LiBr). These salts apparently interact mainly through their anions (Schleich and Von Hippel, 1969). The salts of the anion $ClO_4^-$, however, are quite transparent in the uv and we have used them extensively. They are also very soluble in water and alcohol, and $ClO_4^-$ has been classified next best to $SCN^-$ in changing $[\alpha]_D$ of PP II. Therefore, we were quite surprised to find the perchlorates as a class to be ineffective in changing the CD of PP II. In our experiments we can relate the ability of the perchlorates to their cation. Only the di- and tri-valent salts produced dramatic changes. When we work, however, with the proteins, we will see specific effects of the anion $ClO_4^-$ on conformation. These will be related to

interaction with charged side-chains instead of with the backbone. In PP we do not have interaction with side-chains, of course.

Torchia and Bovey (1971) have concluded from their nmr studies that $CaCl_2$ (2 to 4 M), LiBr (2.5 - 9 M) and KI (1.5 - 5 M) convert some trans peptide bonds of PP II (M. W. 2500) to the cis configuration. They suggest that this occurs at random along the chain. If this happens, our dilution experiments would not pick up any indication of PP I on dilution since this would require several cis units bunched together. It would probably just slow down the return to normal structure. It is surprising that 2 M $CaCl_2$ produces the same amount of cis nmr signal as does 4 M $CaCl_2$, however. Also surprising, the half-life from I to II with no salt is given as 90 minutes, and the same half-life is given for Form I to "random" in the presence of salt. Certainly more work needs to be done to explain the anomalies. Steinberg et al. (1960) found an immediate jump in $[\alpha]_D$ followed by a slow change to more negative rotation when a PP LiBr sample was diluted. This, and our work, where we see a slow change following the immediate change of the CD spectra on adding the salt (Fig. 8-3), can be explained in terms of most bonds being disordered by having the accessible range of $C^{\alpha}-C'(\psi)$ angle distorted by the interaction with salt, but allowing some $\psi$'s to be so greatly distorted that some bonds can actually flip from trans to cis or vice-versa. It is also possible there is a direct analogy with the mechanism of protonation. Thus some interaction on

the N by the cation could loosen up the backbone, whereas bonding to the C = O would tend to make the double bond character of the peptide link stronger and prevent the isomerism. The nmr experiments need to be done with higher molecular weight samples to study the effect of the salt on the ends of the polymers,,since it is quite possible that the interaction of the salt would be different on the ends than in the middle of the chain.

The Polyelectrolytes.

Poly-L-glutamic acid (PGA) and poly-L-lysine (PL), as examples of polypeptides that can have charged side-chains, have been previously studied (Tiffany and Krimm, 1968a and 1969a). The interaction with salts can be quite complicated and specific. There can be dramatic changes in pH as salt is added (Fig. 8-7; 8-8). Apparently preferential binding to the side-chain can occur. Only at high concentrations of salts do we see effects that may be related to interaction with the backbone (Fig. 8-8). High concentrations of salt can lead to complex interaction, however. Thus LiCl on PGA is seen to produce a new CD spectrum that may be related to chelating by the $Li^+$ ion or to an optically active aggregate. Nonspecific interaction through charge shielding has already been dealt with (Tiffany and Krimm, 1968a and and Krimm, Mark and Tiffany, 1968. See Chapter 4).

The Proteins.

In order to get some understanding of the mechanism of interaction of salts with proteins, we have surveyed the effect of the salts that are transparent in the uv on the CD spectra of a selected number of proteins. Some generalizations are readily apparent. Thus, LiCl in water or methanol solutions when used in sufficient concentration past that needed to get cooperative opening up of the individual globular proteins, always leads to an expanded state. This is to be contrasted with the multi-valent salts that give collapsed states. The evidence for the extended form in the case of LiCl treatment is the high shoulder on the CD spectrum in the 220 nm region. This shoulder can always be made even higher by lowering the temperature. See [$A_1$ (Fig. 8-9); bovine serum albumin (Fig. 8-10, 8-11); collagen (Fig. 8-12); lysozyme (Fig. 8-13); ribonuclease (Fig. 8-14) and soybean trypsin inhibitor (Fig. 8-15)]. The shape of the CD spectra of proteins denatured by $CaCl_2$ or by $LaCl_3$ is always only a monotonically decreasing curve in the 250 to 205 nm region. Examples are: $A_1$ (Fig. 8-9); bovine serum albumin (Fig. 8-11 and 8-16); chymotrypsinogen (Fig. 8-17); collagen (Fig. 8-12); deoxyribonuclease (Fig. 8-18); lysozyme (Fig. 8-13); myoglobin (Figs. 8-19 and 8-20); ribonuclease, (Fig. 8-21); and soybean trypsin inhibitor (Fig. 8-15). In the case of myoglobin we see an initial effect that may be electrostatic in origin that occurs at low concentrations of the salt, then an abrupt change is seen when the concentration is increased past 5 M.

This is very similiar to the effect of $CaCl_2$ on poly-L-lysine (Fig. 8-8). Changing the temperature does not change the shape of the CD spectrum.

A specific effect of the salts of perchlorates is found. In keeping with the now well known interaction with poly-L-lysine (e.g. Fig. 7-2), we see indications of development of some $\alpha$-helicity in the case of $A_1$ protein of Eylar (Fig. 8-9); chymotrypsinogen (Fig. 8-23); collagen (Fig. 8-12); and ribonuclease (Fig. 8-24). This is rather surprising, but very apparent, and is most probably due to specific binding at positively charged side-chains.

The last generalization that we can make is that all salts are more effective when they are used in methanol solution. Thus, compare the effect of LiCl on bovine serum albumin in methanol (90%) and in water in Figs. 8-11 and 8-10.

It is interesting to explore in more depth the effect of the addition of small amounts of salts to proteins which are already denatured and in high concentrations of urea. Thus 0.1 M of various salts were effective in making noticeable changes in the CD of ribonuclease in 8 M urea at room temperature (Fig. 8-25). The sulfates and the phosphates tend to return the CD spectra to normal.

The near uv CD of proteins in various salts has been studied by many workers. We would just like to point out that the dichroism of many proteins in effective lyotropic salts seem to be similar to each other in all having a shoulder or positive band near 250 nm and a negative band near

270 nm (e.g. Fig. 8-26). We can assume that the proteins are in a random state since even in the presence of high concentrations of LiCl there is evidence of much disorder since the 220 region is only present as a shoulder, not as a positive band. The most likely interpretation of the near uv bands is that they are due to S-S bonds which retain their handedness in high salts. The ready reversibility of most proteins to revert to the native form when salt is diluted out is in favor of this interpretation. If a protein is made up of multichains, such as collagen, we will not get immediate refolding to the native form, nor if a co-factor is removed by the salt will we expect rapid reformation.

Discussion.

Direct CD studies of the effect of salts on conformation of proteins and polypeptides have necessarily been limited to those salts that are transparent enough in the uv when used in high enough concentrations to produce change in the CD bands related to backbone conformation. This has made it impossible to study the salts of the thiocynates and the iodides as well as the bromides at effective concentrations. Thus we have made a one-sided attack on the problem since most of the salts that have proved useful in CD studies seem to be interacting by having effective cations, but whose anions are of neutral ability to denature. Within these limitations, however, we can reach some very interesting conclusions. Thus $Li^+$ is probably binding to only one carbonyl, and not achieving

its effect by chelation. This follows since LiCl is ineffective in disrupting PP II structure. This would be the expected result if $Li^+$ only replaces a hydrogen bonded water molecule. The multivalent cations very likely try to bind to more than one carbonyl and distort the PP II structure in so doing. Calcium and lanthanum chlorides are thus very good denaturants for PP II as well as proteins. LiCl, while it cannot distort the PP II structure can, nevertheless, destroy protein structures by leading to disruption of intrachain H bonding involving the peptide carbonyls. It is interesting that LiCl in its interaction with a protein always produces a CD spectrum that has a high shoulder in the 220 nm region, whereas the di- and tri-valent cation salts produce spectra that have no indication of this shoulder; instead only simple CD curves result with a negative CD band near 200 nm. A simple explanation of this is that the monovalent $Li^+$ binds to one carbonyl whereas the di- and trivalent ions try to interact with more than one carbonyl along the polypeptide chain. In the first case, the chain can expand for any one of a number of reasons such as bulky side-chains or repulsion of charges due to ion binding. A 220 nm positive dichroic band has been found in all cases where we have reason to believe a polypeptide chain of L amino acids exists in an extended state (e.g. at low temperature, PP II, collagen, PGA, PL, poly ornithine, poly-L-aspartic acid, protonated polypeptides that do not have restriction due to pyrrolidine rings, and urea and GuHCl treated proteins and polypeptides, when not

restricted by S-S bonds). Similiarly, when we have reason to believe that the chain is collapsed and with no particular conformation preferred in the sterically available space, the CD spectra have only the negative band near 200 nm with a positive band in the far uv. The only salts studied that apparently are effective due to their anion have been the perchlorates. Here, we have run into the problem of specific binding to positively charged sidechains in the case of the proteins and have found little effect of the perchlorates of sodium or potassium on PP II structure. The ability to relate the changes in structure to binding, however, rules out that the perchlorates are interacting through change in water structure.

Many interactions of salts on the optical rotation of proteins have been categorized as "solvent effect", which has not yet been explained but which is not supposed to be a reflection of a change in conformation of the protein (Tanford, 1968 review, p. 184). An example in question is the decrease in $[\alpha]_D$ (after an increase during the cooperative unfolding) of ribonuclease by LiBr (Bigelow, 1964). We find, however, evidence that some conformational change, induced by salts, is possible both before the cooperative unfolding and following the abrupt loss of the stable globular form. Initial changes may be due to electrostatic interference with charged side-chains as well as due to interaction with exposed backbone peptide units. Similiar interaction continues to occur in the

molecule following the cooperative transition from the globular state. Certainly, different salts produce different effects. In the case of LiCl we have been lucky to have been dealing with a salt which produces its effect through interaction of the monovalent cation, and in the cases of $CaCl_2$ and $LaCl_3$ which apparently are effective through their multivalent cations. Thus we can relate the CD spectra of proteins in high concentrations of LiCl to partially extended states and similarly, in high concentrations of $CaCl_2$ or $LaCl_3$ to collapsed random states.

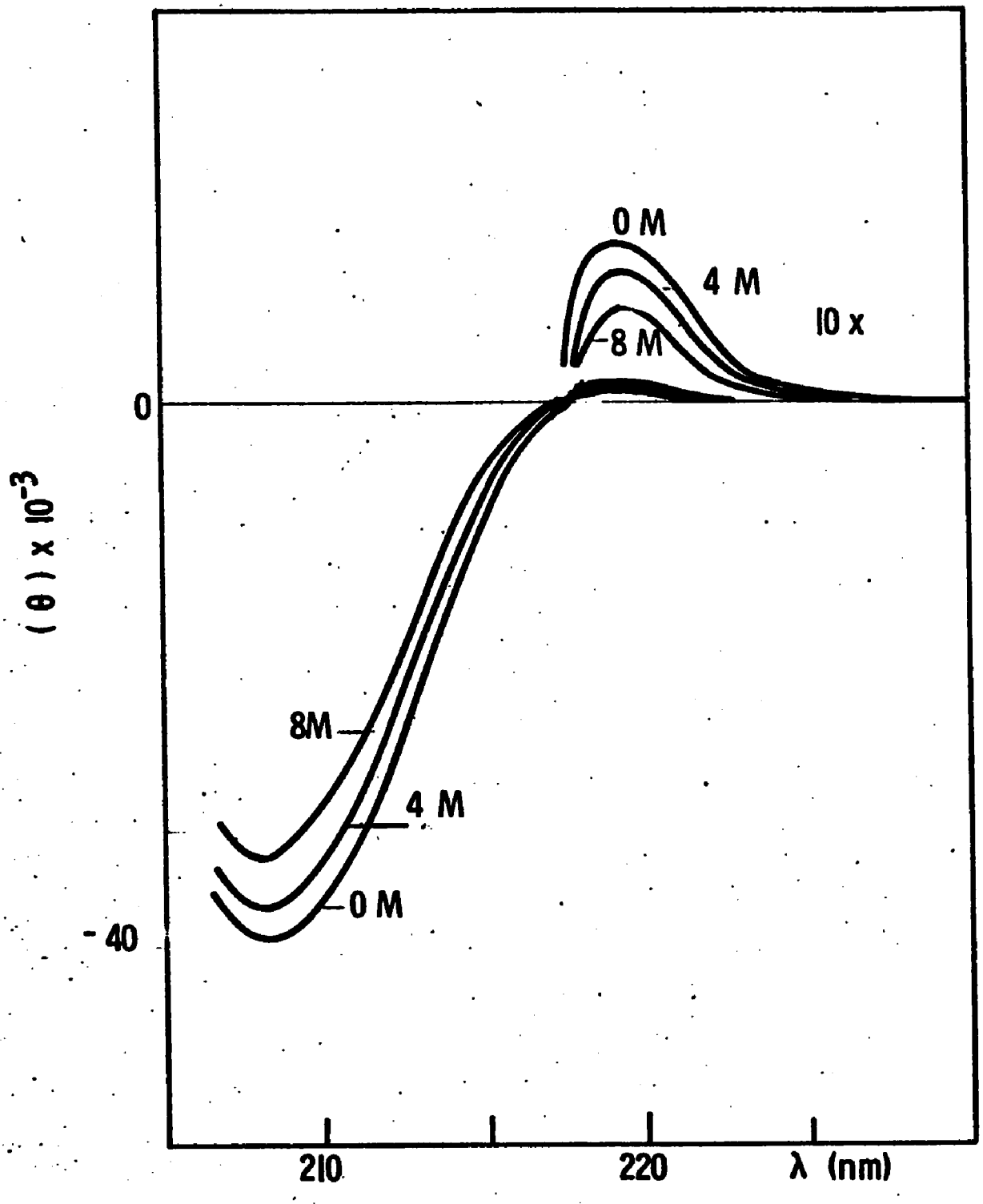



Fig. 8-1. CD of poly-L-proline, mol. wt. 7,500, form II plus various amounts of LiCl.

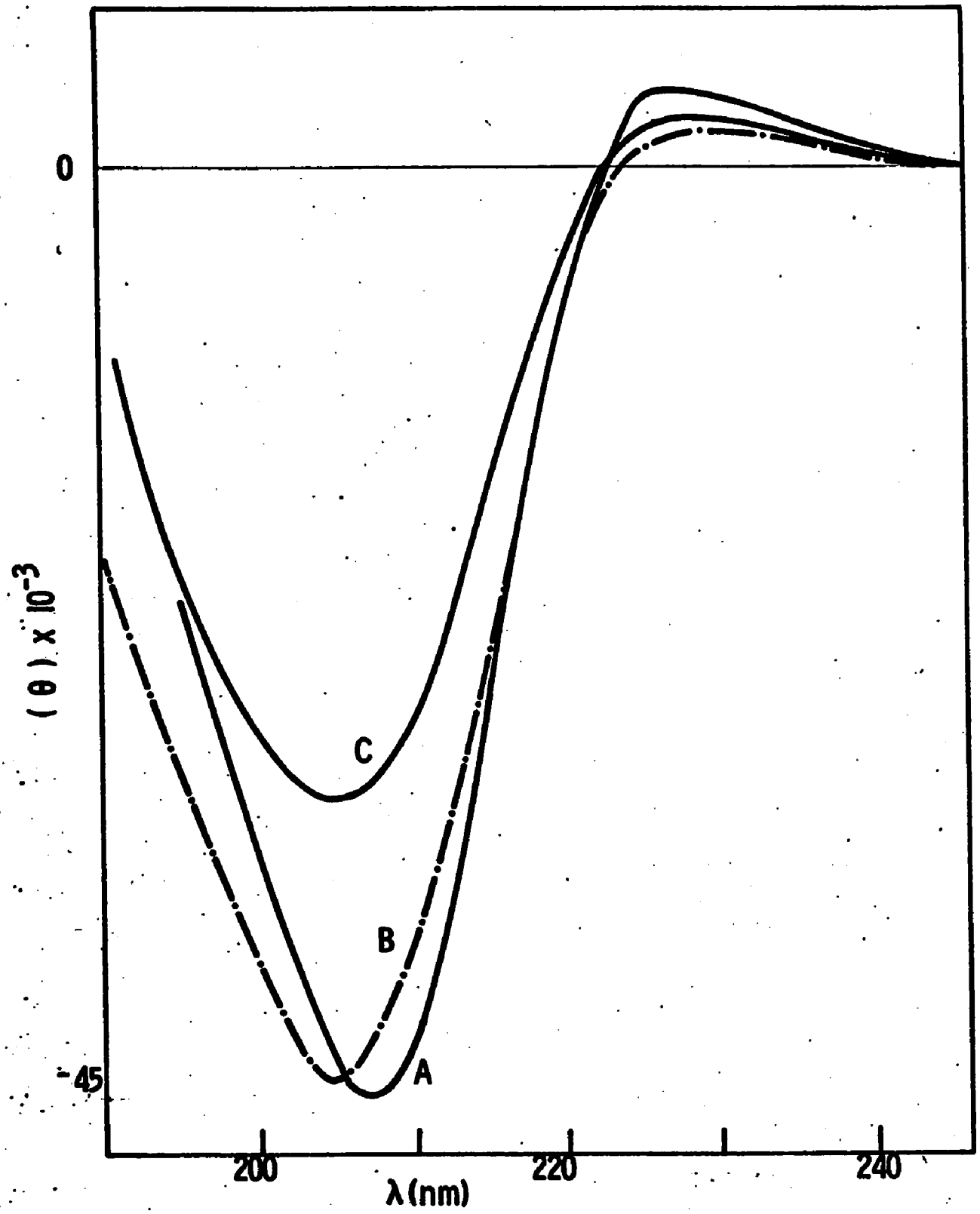



Fig. 8-2. CD of poly-L-proline, mol. wt. 13,500; form II in water (A); form II plus 5.4 M $LiClO_4$ (B); form II plus 5.4 M $NaClO_4$ (C).

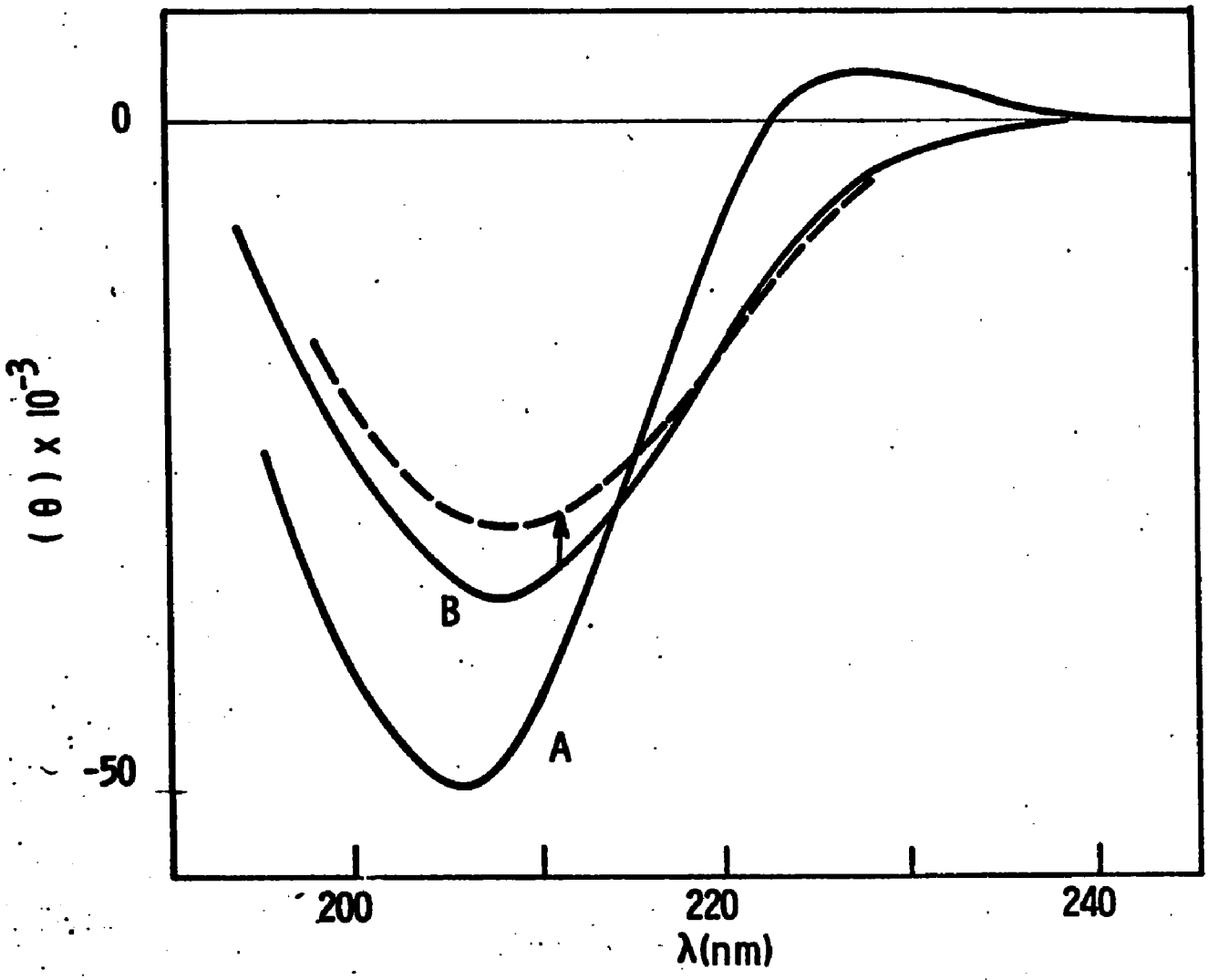



Fig. 8-3. Poly-L-proline, mol. wt. 43,000. Initial sample equilibrated in water so in form II. On making solutions (A) 2.25 M in $LiClO_4$ or in 2.25 M $NaClO_4$ by diluting one part of initial sample with nine parts of 2.5 M $LiClO_4$ or $NaClO_4$ methanol solns there was little change in CD. On diluting with the divalent salts $Ca(ClO_4)_2$ or $Mg(ClO_4)_2$ methanol solutions the CD spectrum is initially (B) . A small change continued for one hour at room temperature. (→)

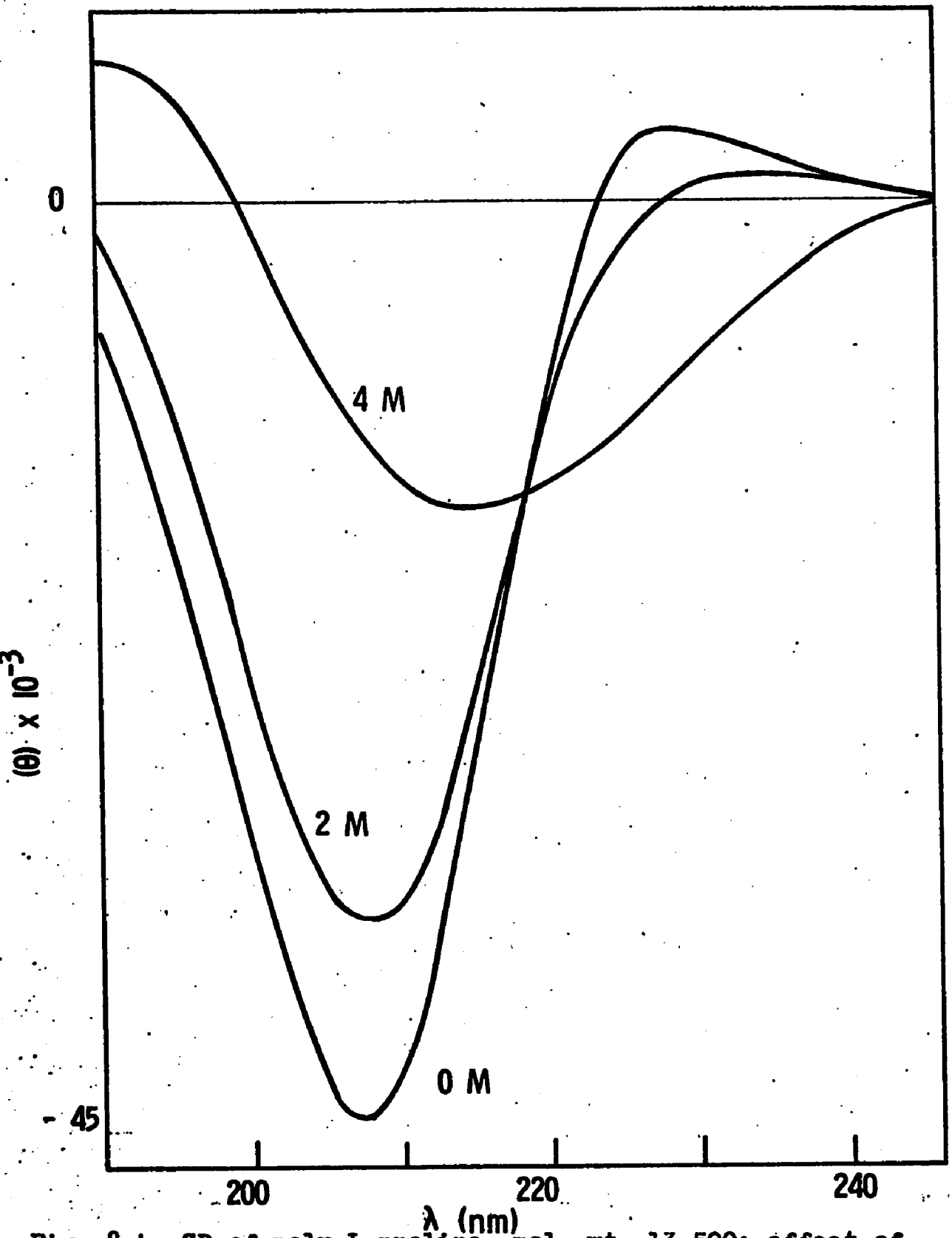



Fig. 8-4. CD of poly-L-proline, mol. wt. 13,500: effect of adding $Ca(ClO_4)_2$ to form II. A positive CD band is shown in the far uv spectrum in 4 M salt.

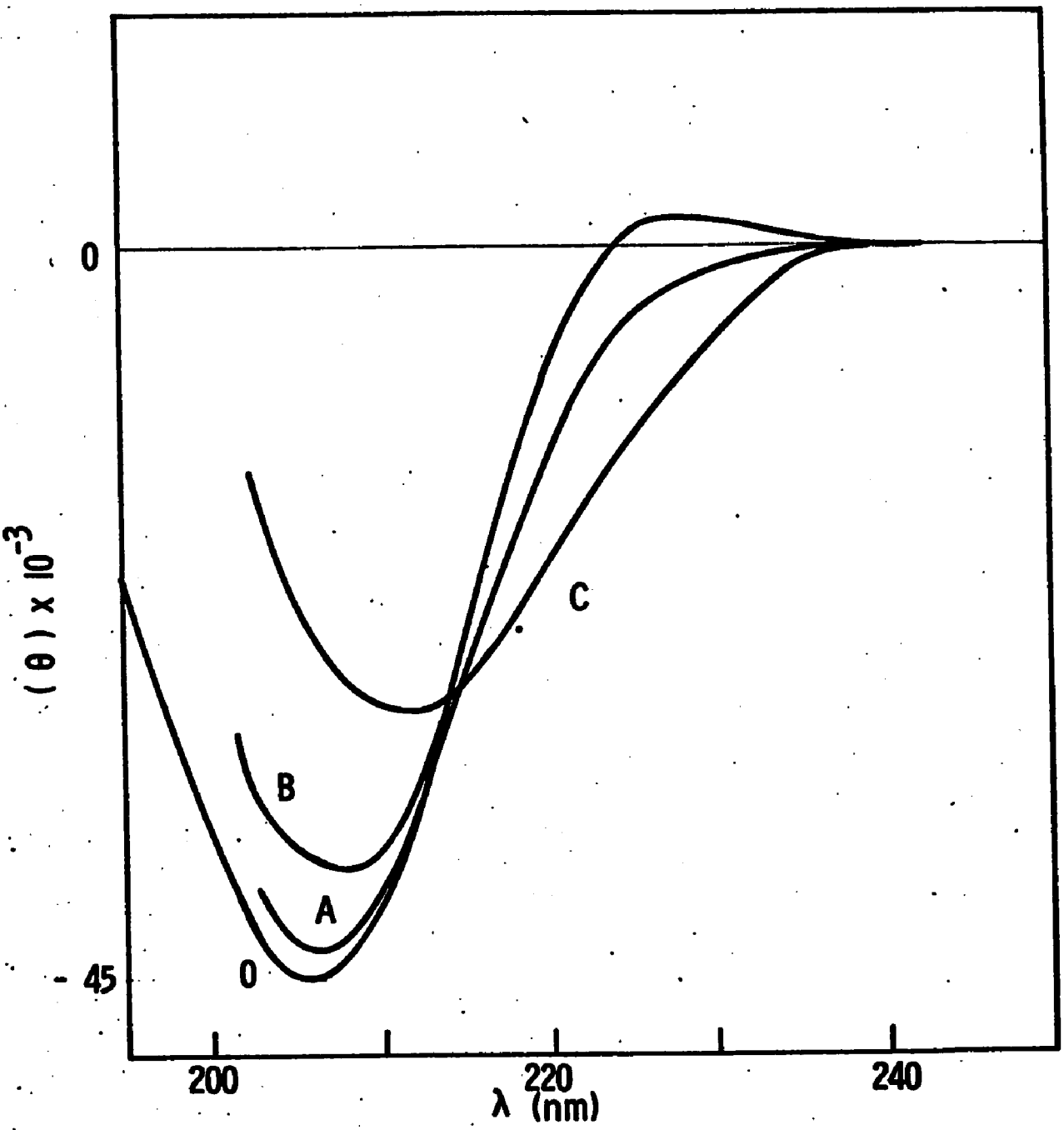



Fig. 8-5. CD of poly-L-proline, mol. wt. 11,500: effect of adding $LaCl_3$ to form II, (A) 2.8 M, (B) 3.2 M, (C) 3.6 M.

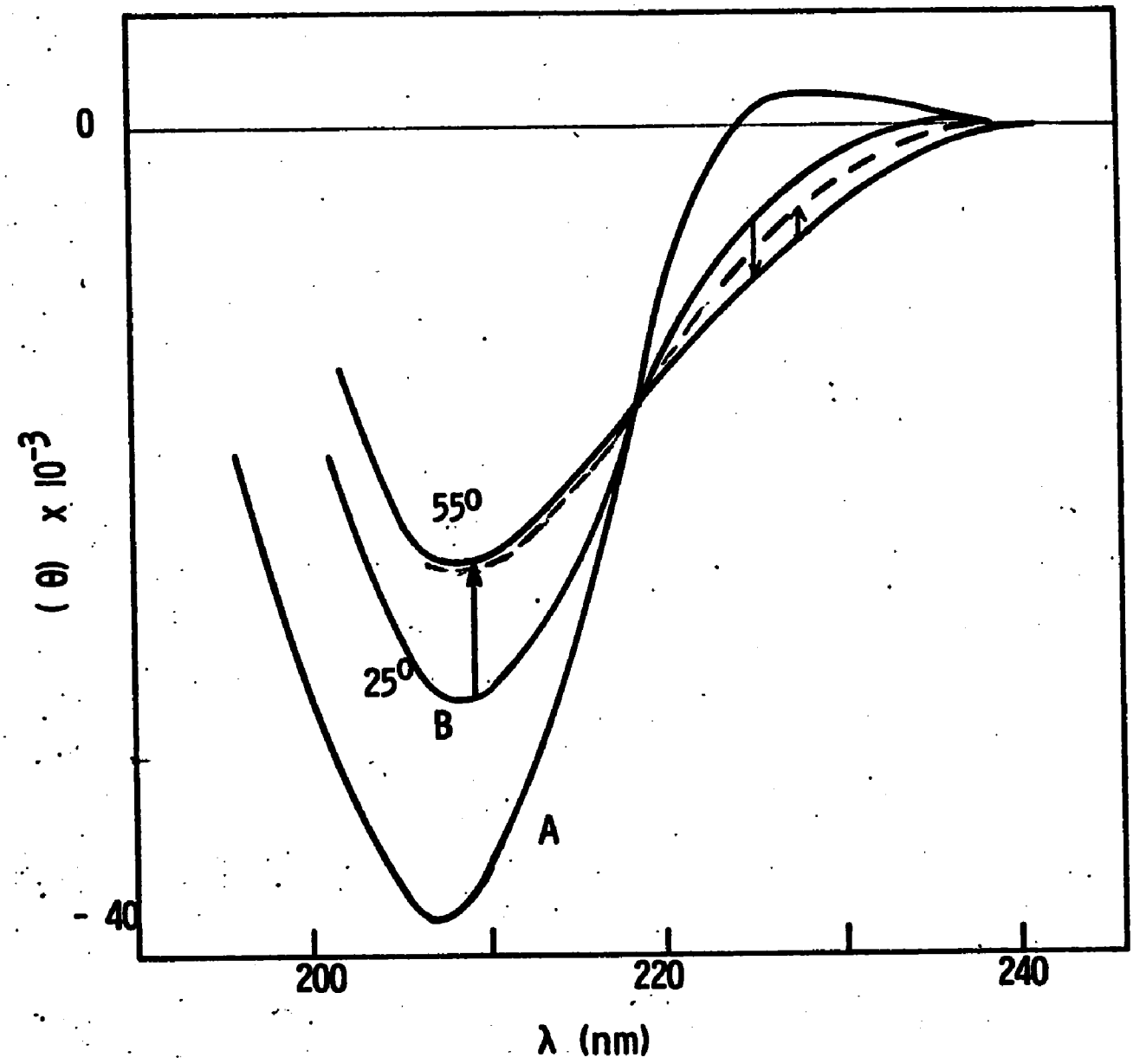



Fig. 8-6. CD of poly-L-proline, mol. wt. 7,500: equilibrated water solution, form II (A); Form II mixed with 3.6 M $LaCl_3$ $25^{\circ} \rightarrow 55^{\circ}$ (B); on returning temperature to $25^{\circ}$ the CD is given by the(– –)curve.

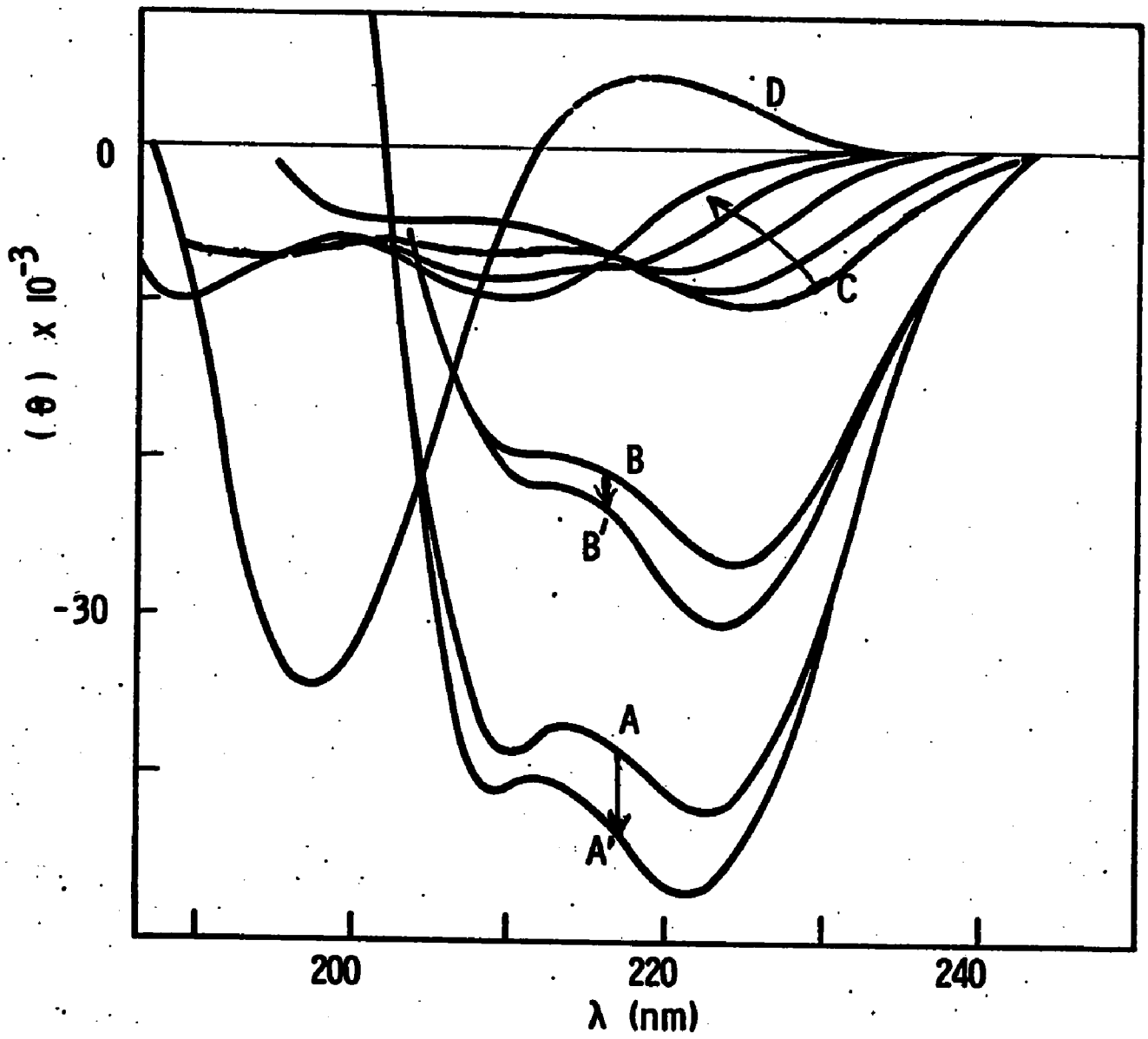



Fig. 8-7. CD of poly-L-glutamic acid: (A) in $H_2O$ at pH 4.6 with HCl at 25°; (A′) at 10°; (B) in 5 M LiCl at pH 3 at 25°, then lowering the temperature to 10° (B′); (C) in 7 M LiCl at 25° with arrow following change with time; (D) in water at pH 6.3, 25°, for comparsion.

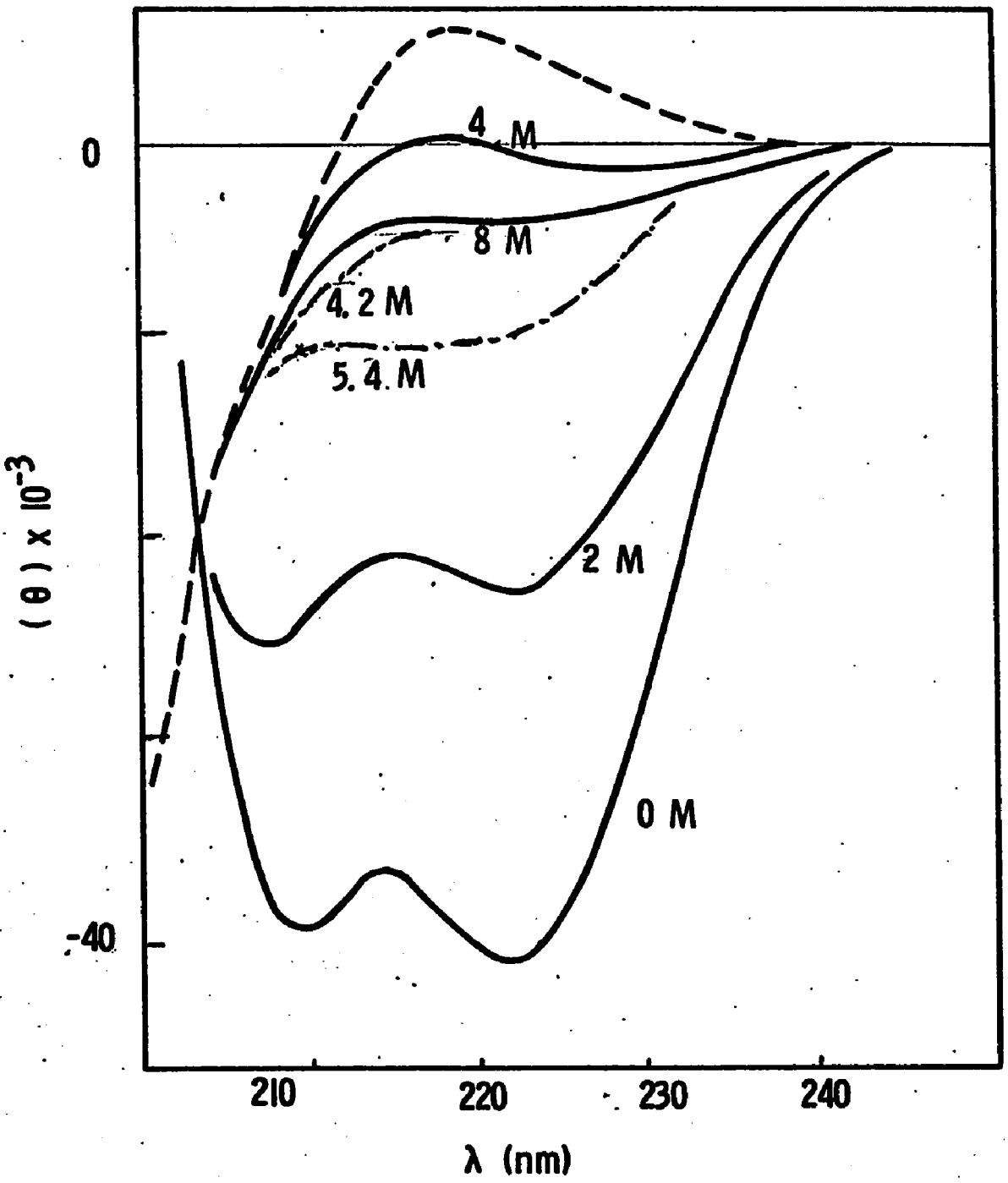



Fig. 8-8. (——) Poly-L-lysine CD in 0.01 M NaOH plus various amounts of LiCl as indicated; (– – –) poly-L-lysine in $H_2O$ alone. (—·—·-) poly-L-lysine in 0.01 N NaOH plus various amounts of $CaCl_2$ as indicated.

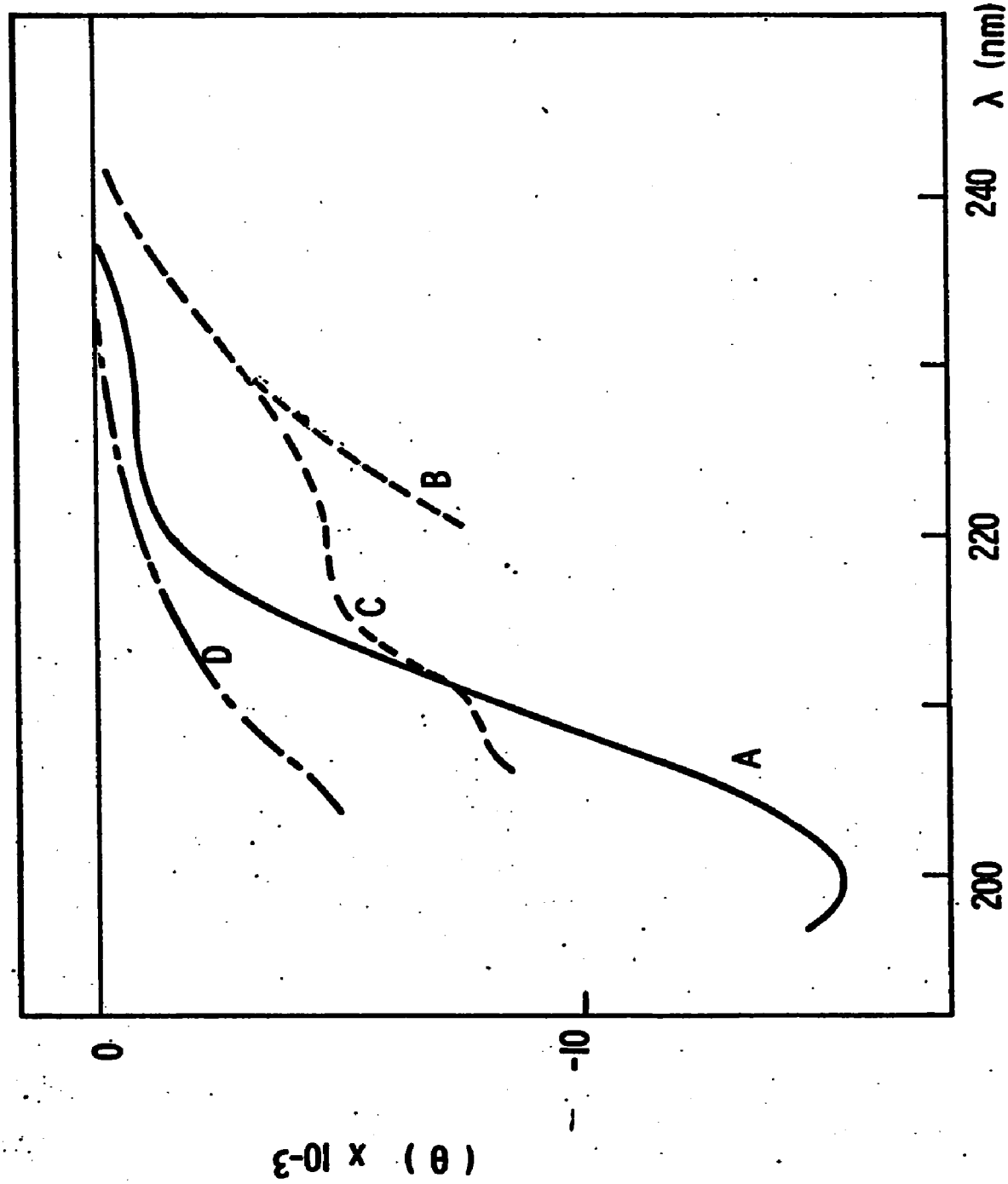



Fig. 8-9.
CD of $A_1$ protein: (A) in $H_2O$, (B) in 5.4 M $CaCl_2$; (C) in 5.4 M $NaClO_4$ ; (D) s.LiCl-90% methanol.

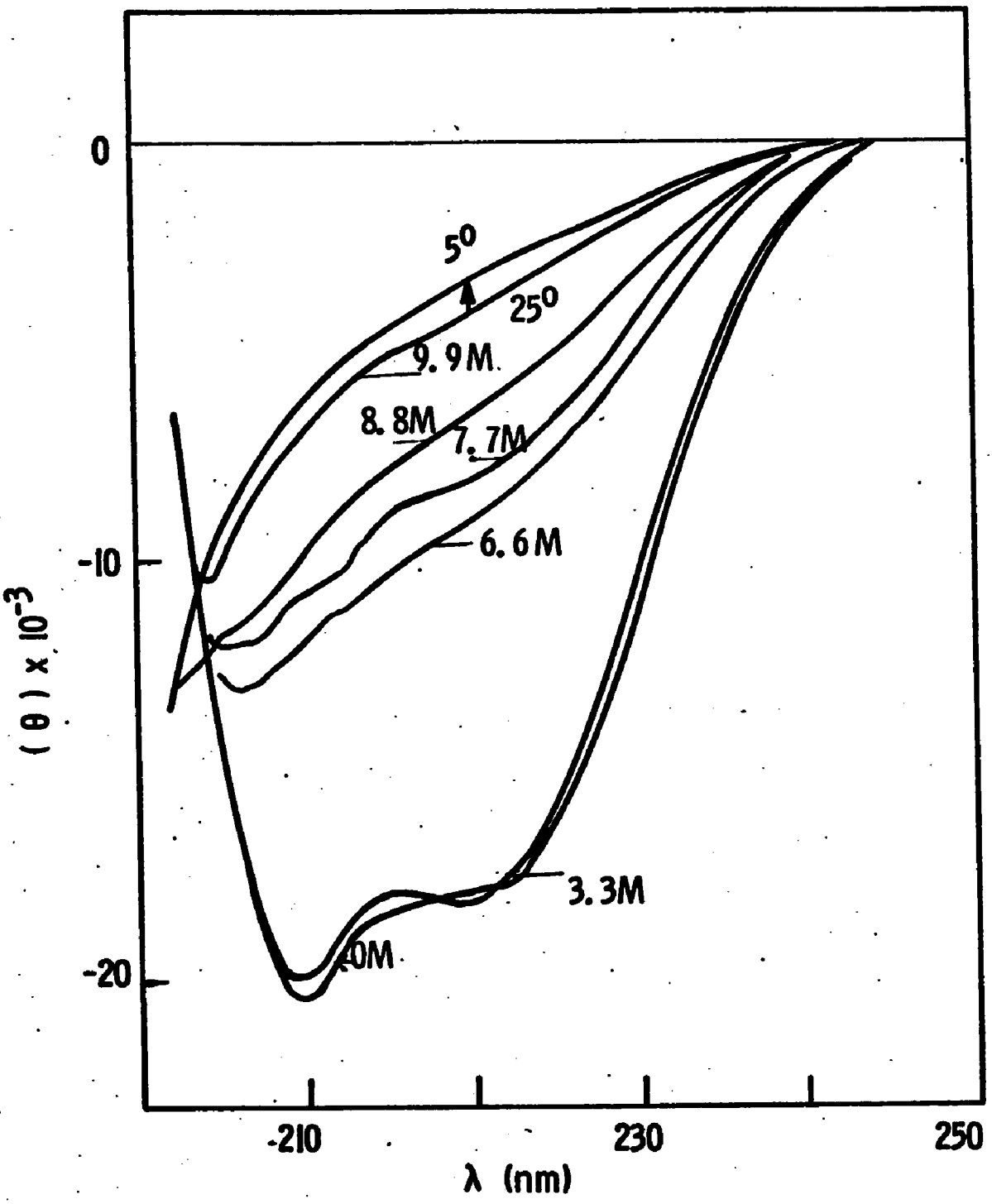



Fig. 8-10. CD of bovine serum albumin: effect of LiCl.

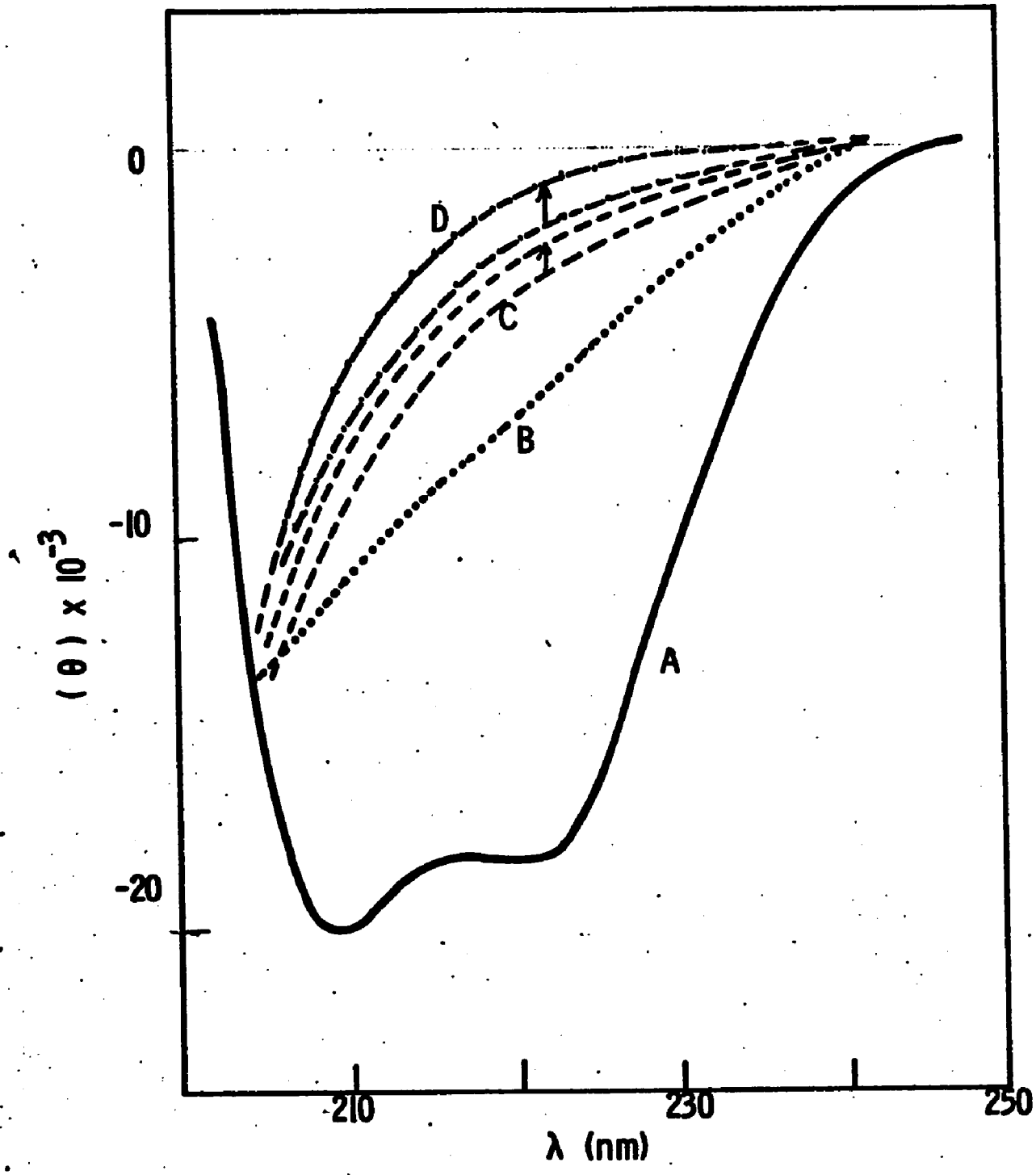



Fig. 8-11. CD of bovine serum albumin: (A) in $H_2O$; (B) in 1.8 M $CaCl_2$ - 90% methanol; (C) in 3.2 M LiCl- 90% methanol; (D) in 5.4 M LiCl - 90% methanol. Arrow points to lowering the temperature from 25° to 5°.

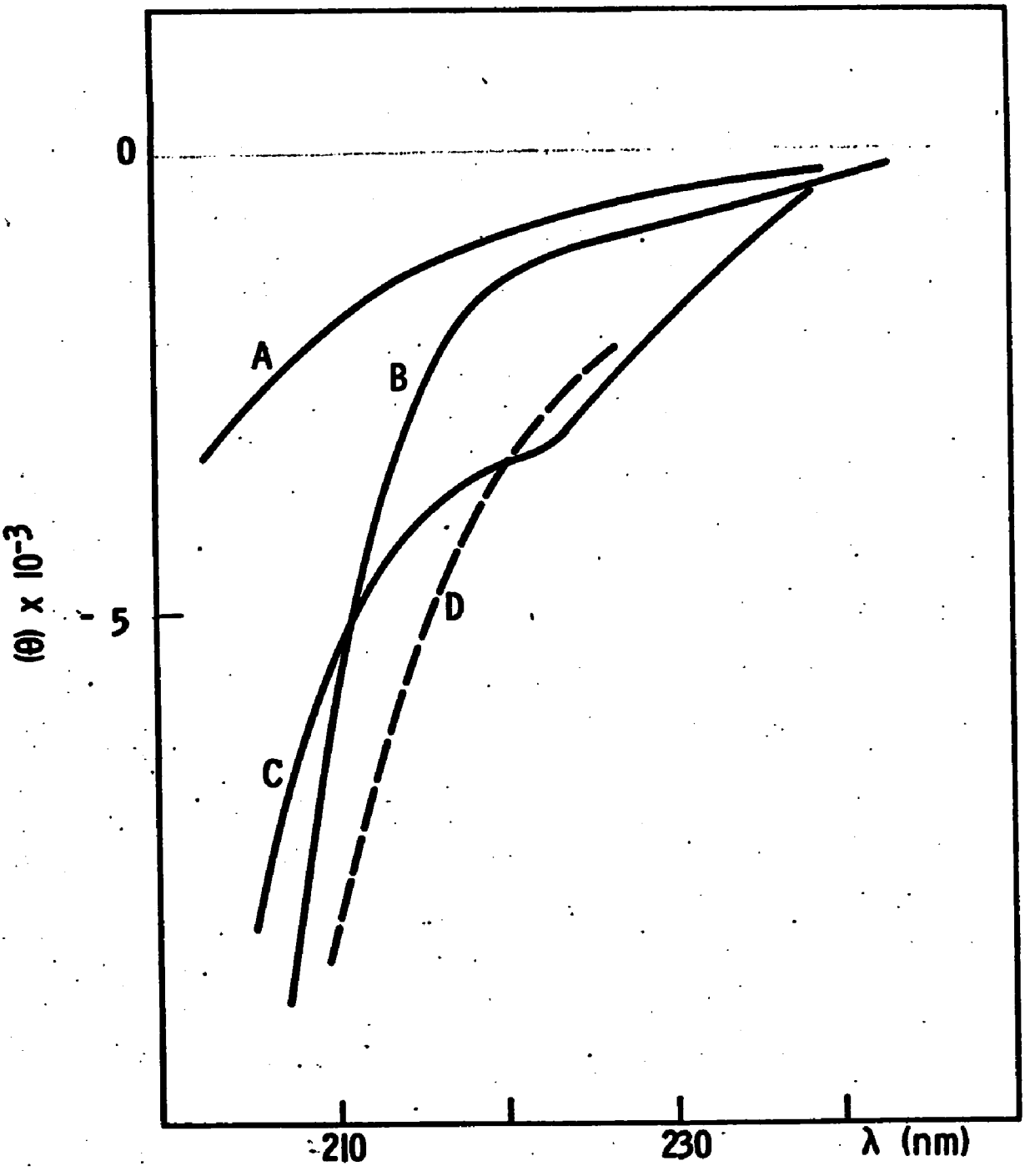



Fig. 8-12. CD rat tail collagen: denatured by 5 M LiCl( A); heat 60°, denatured (B); denatured by 5M $LiClO_4$ (C); denat by 2.5 M $CaCl_2$. (D).

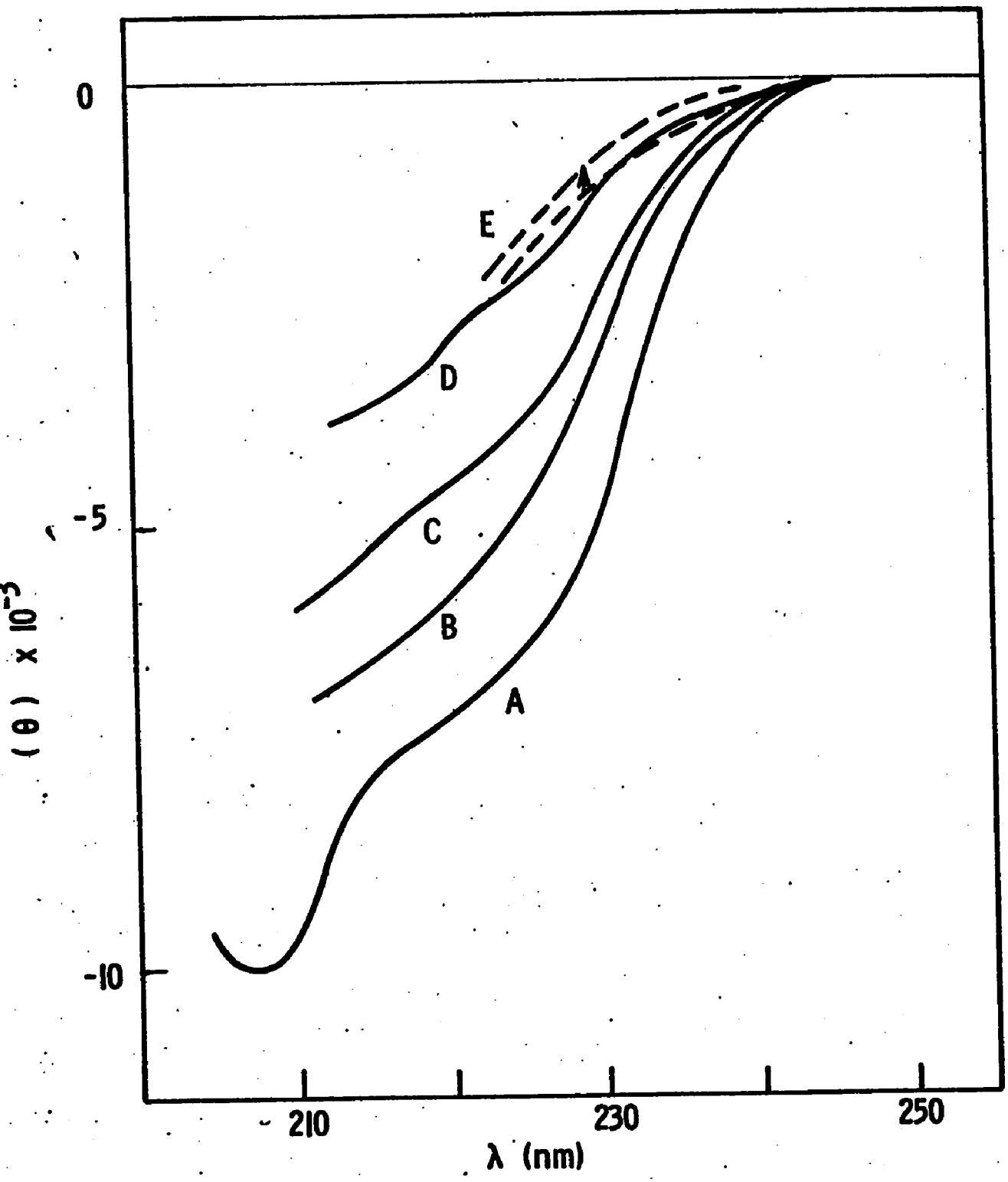



Fig. 8-13. CD of lysozyme: (A) in $H_2O$ and in 7 M LiCl; (B) in 5.8 M $CaCl_2$; (C) in 4.8 M $CaCl_2$; (D) in 9 M LiCl; (E) in 4.2 M LiCl: 90% methanol ( arrow points to change in temp. $25^{\circ} \rightarrow 5^{\circ}$).

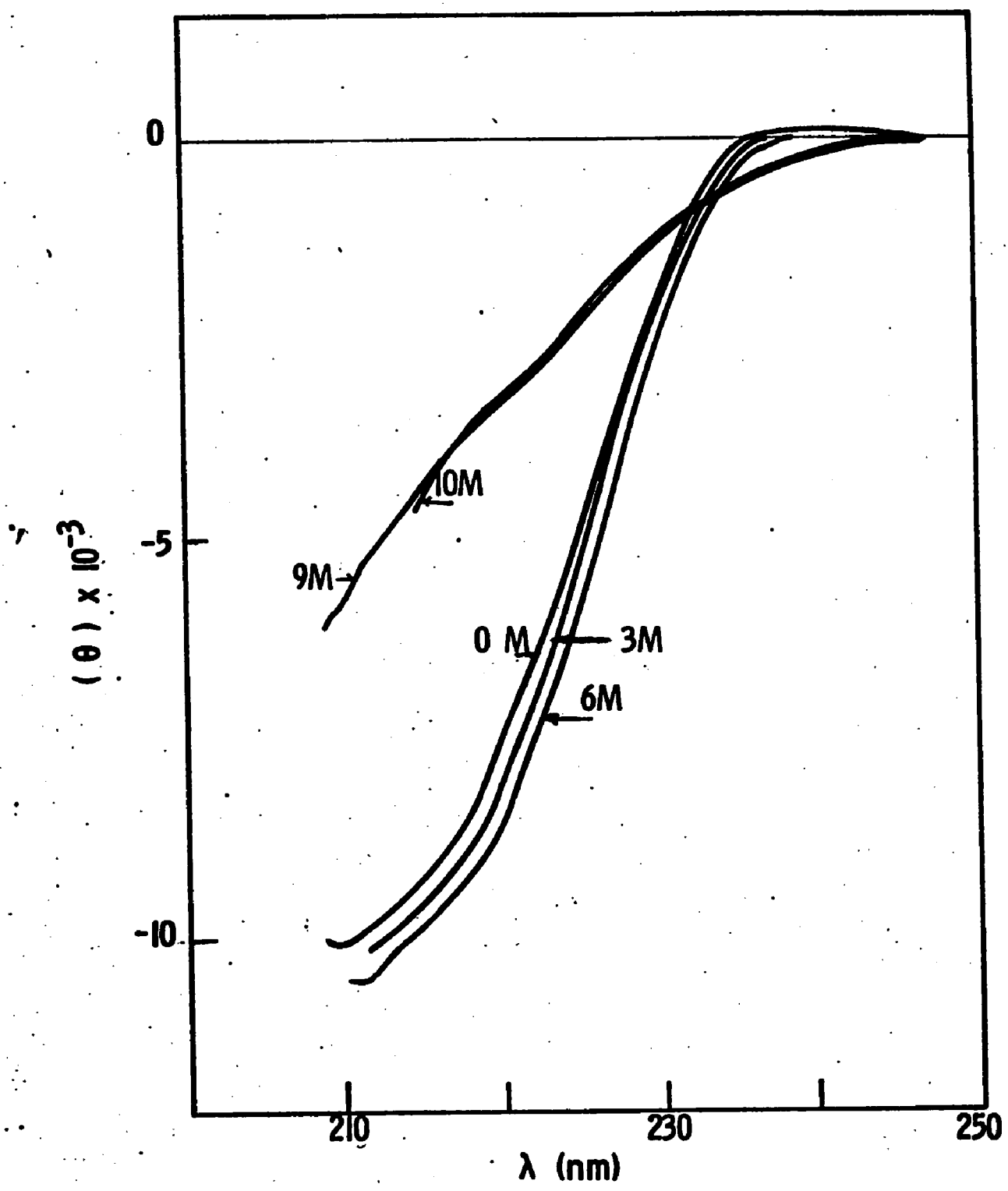



Fig. 8-14. CD of ribonuclease: effect of LiCl

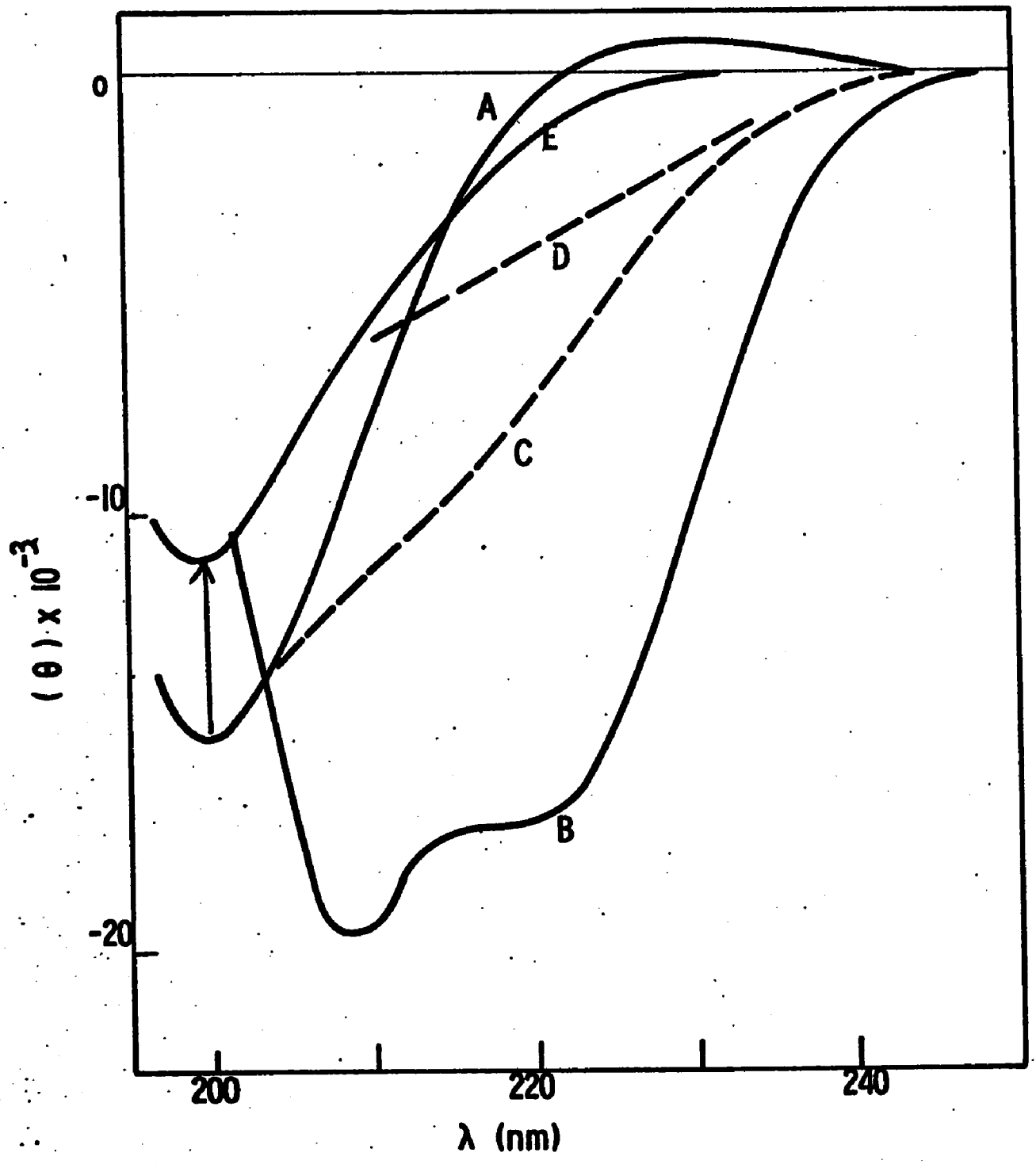



Fig. 8-15. CD of soybean trypsin inhibitor: (A) in $H_2O$; (B) in 90% trifluoroethanol, (C)— —in 5.8 M $CaCl_2$; (D) — —in 9.9 M LiCl; (E) in $H_2O$ at 55°.

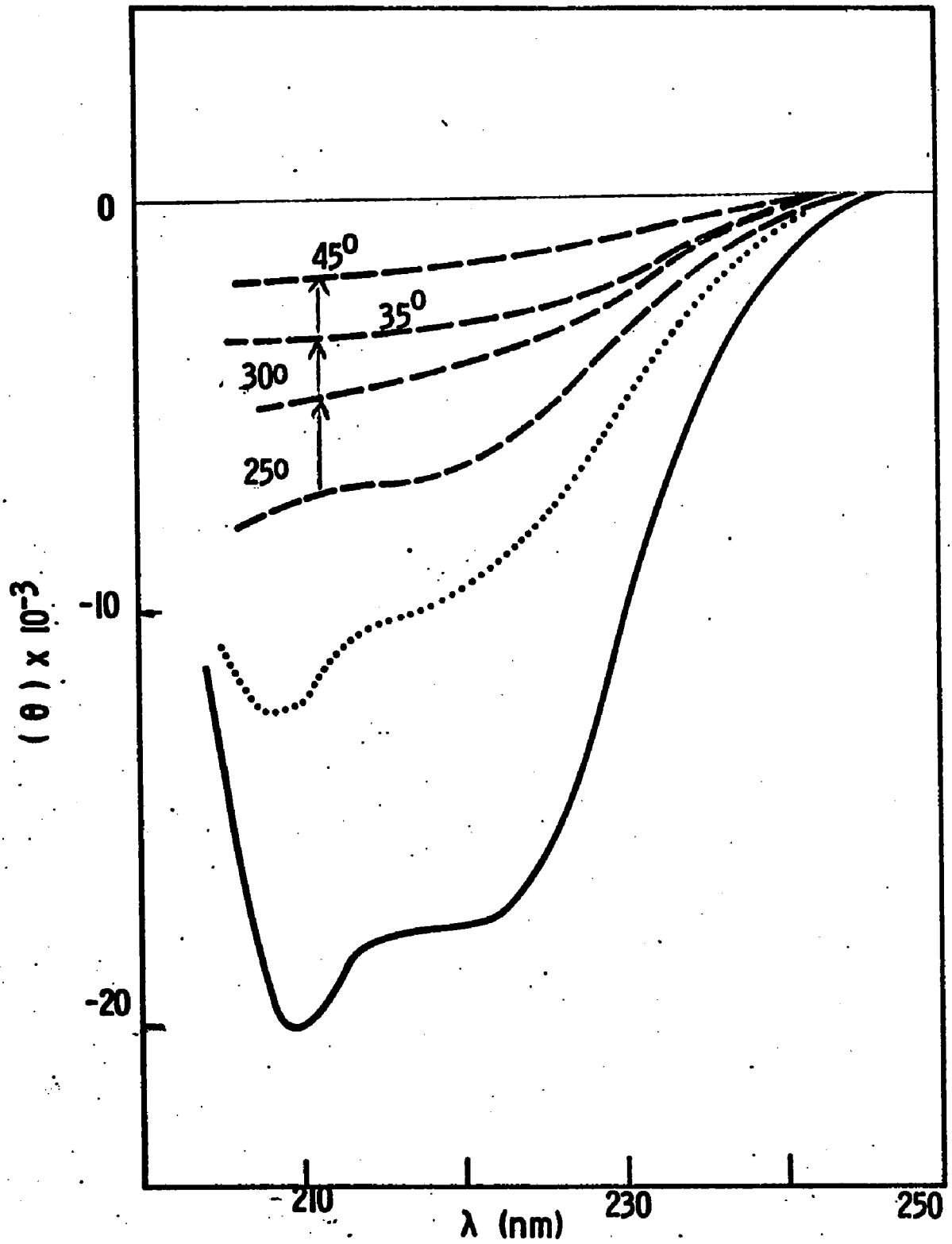



Fig. 8-16. CD of bovine serum albumin: effect of 4.8 M $CaCl_2$ ....., effect of saturated $CaCl_2$ with temp. change — —; in $H_2O$ at 25° ——.

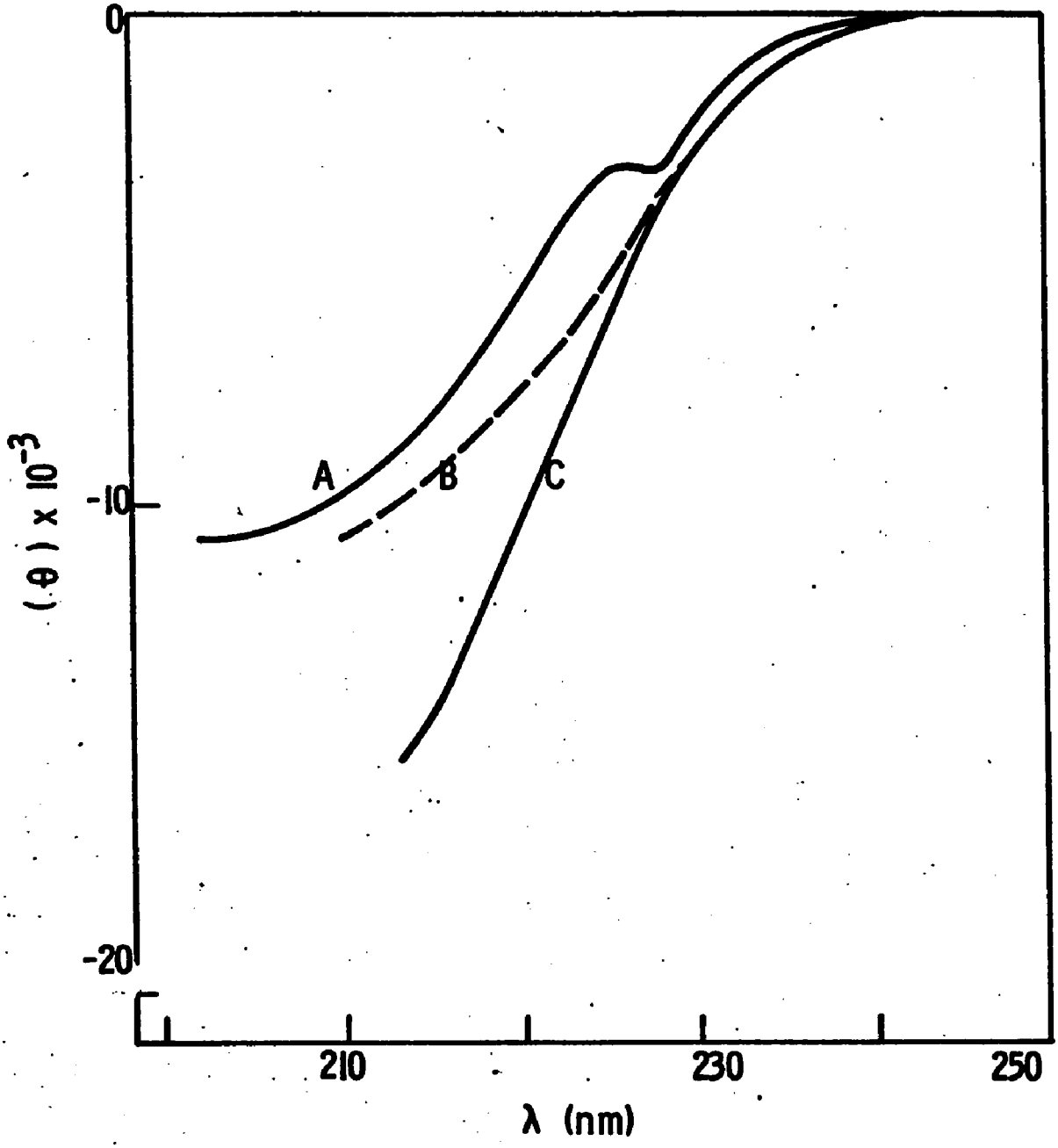



Fig. 8-17. CD of α chymotrypsinogen: (A) in $H_2O$, (B) in 3.2 M $CaCl_2$, (C) in 5.4 M $CaCl_2$.

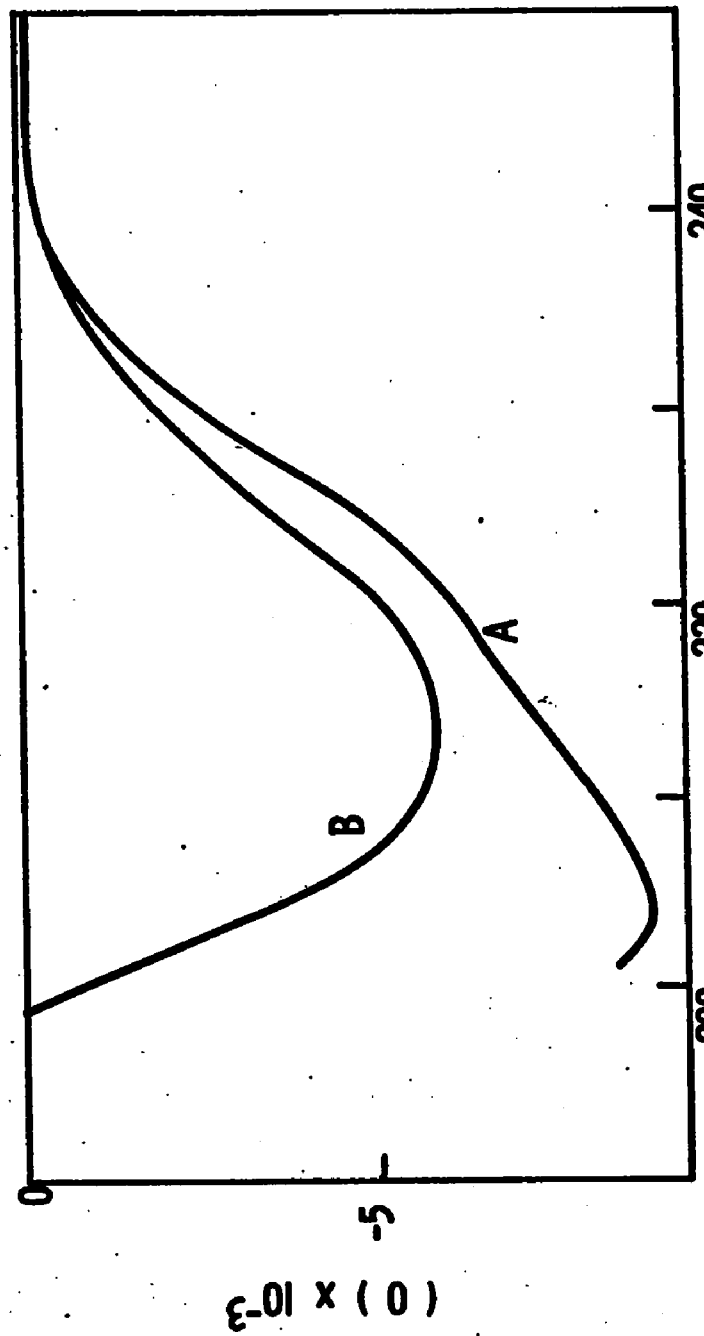



Fig. 8-18. CD of deoxyribonuclease: denaturation by 0.9M $LaCl_3$: 90% methanol.(A); native in water (B).

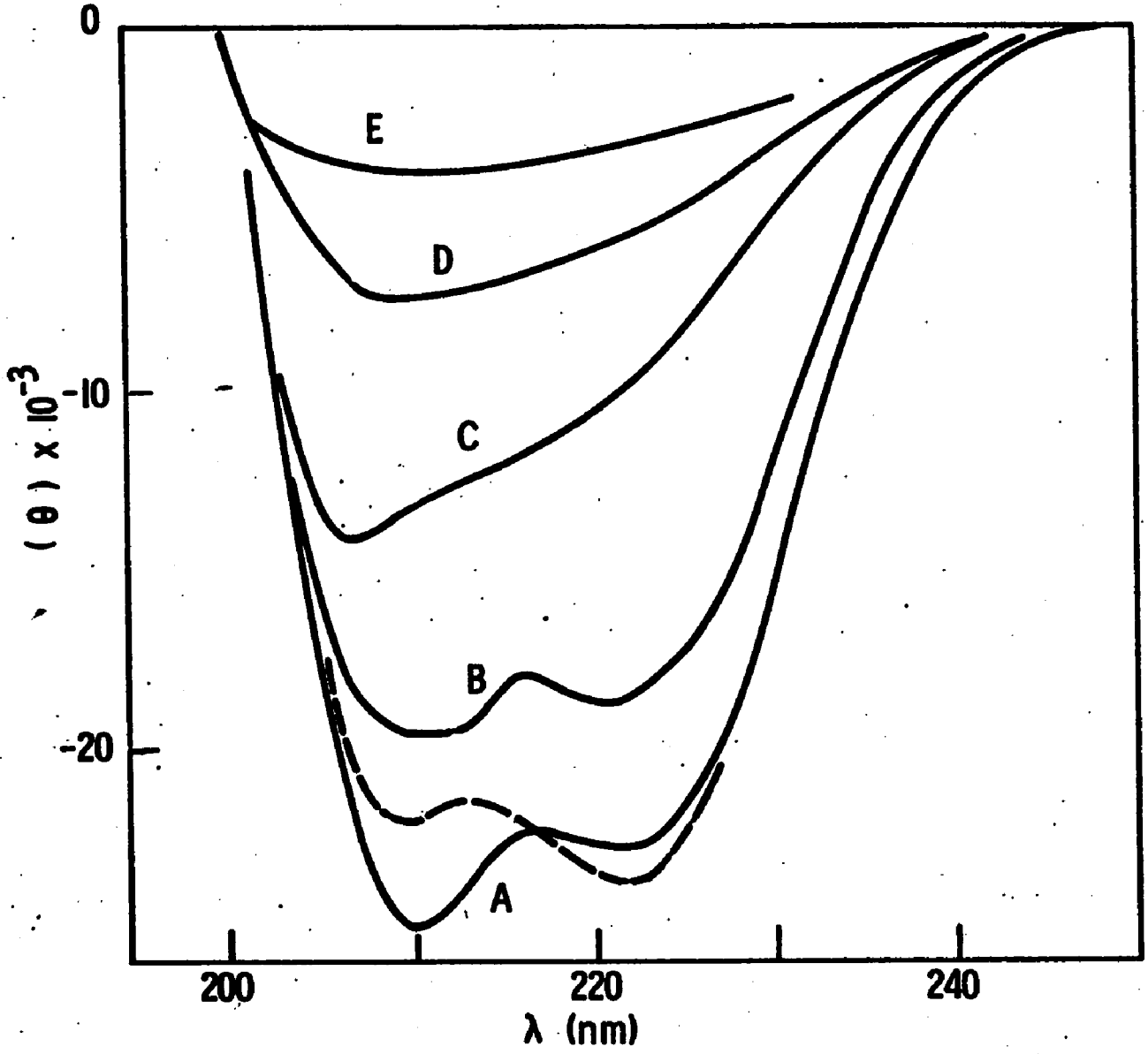



Fig. 8-19. CD of myoglobin: — — in $H_2O$; ——: in 90% methanol [(A) zero $LaCl_3$, (B) 1/3 sat. $LaCl_3$, (C) 1/2 sat. $LaCl_3$, (D) 7/10 sat. $LaCl_3$. (E) sat. $LaCl_3$].

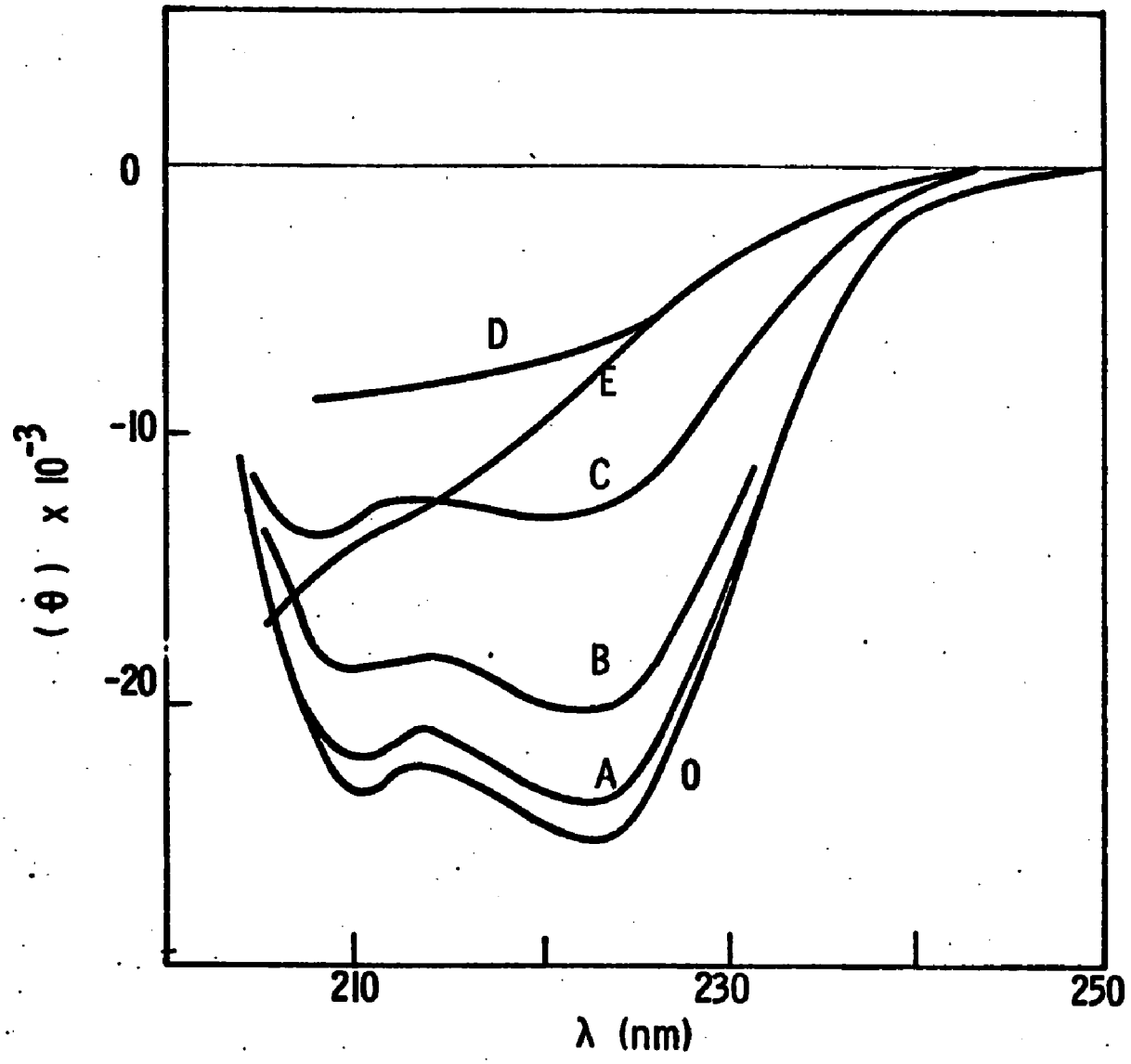



Fig. 8-20. CD of myoglobin (sperm whale): effect of $CaCl_2$ [(O) zero, (A) 0.9 M $CaCl_2$, (B) 1.8 M $CaCl_2$, (C) 3.6 M $CaCl_2$, (D)5.4 M $CaCl_2$, (E) 7.2 M $CaCl_2$].

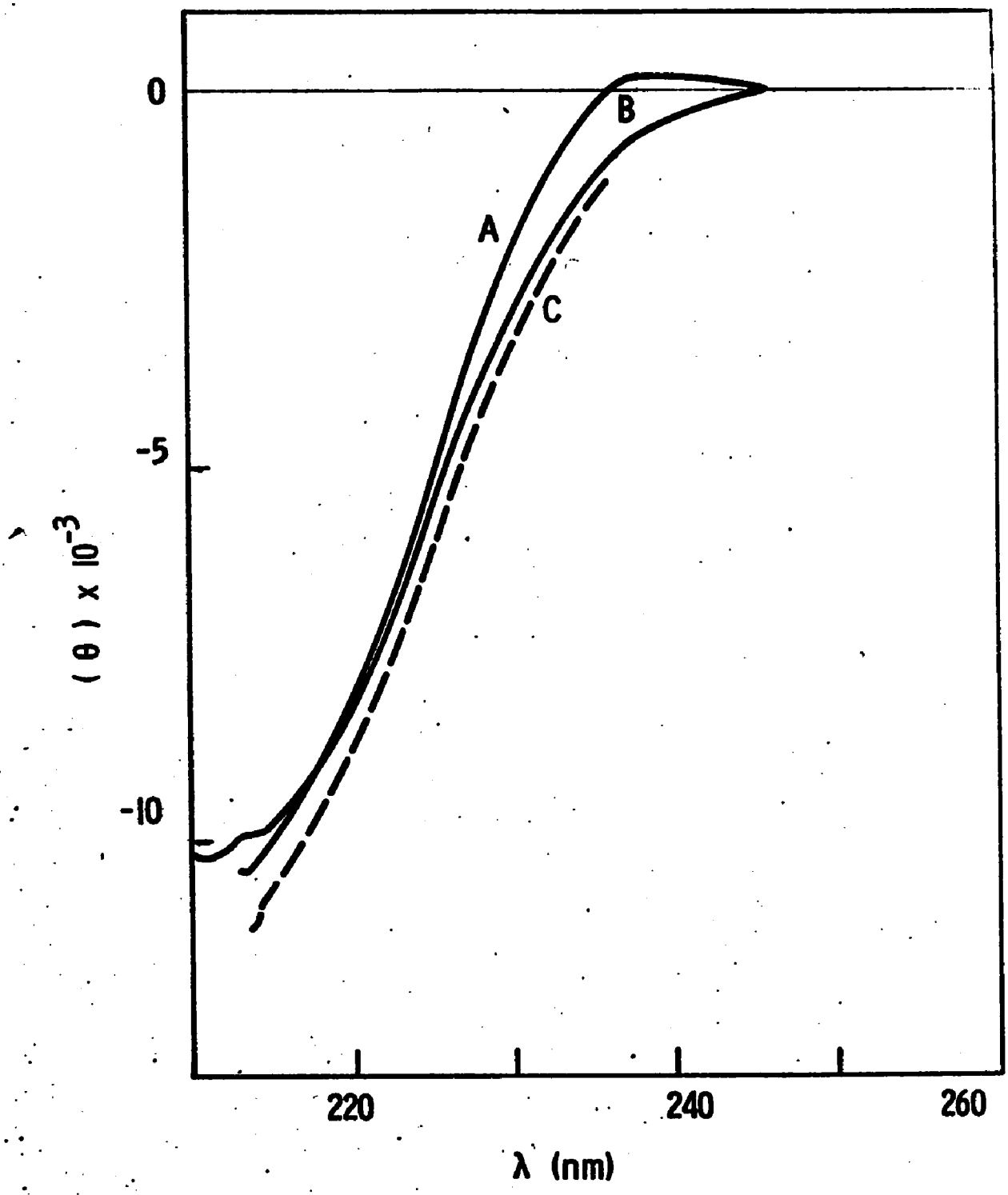



Fig. 8-21. CD of ribonuclease: (A) in $H_2O$, (B) in 5.4 M $CaCl_2$, (C) in 90% methanol sat. with $LaCl_3$.

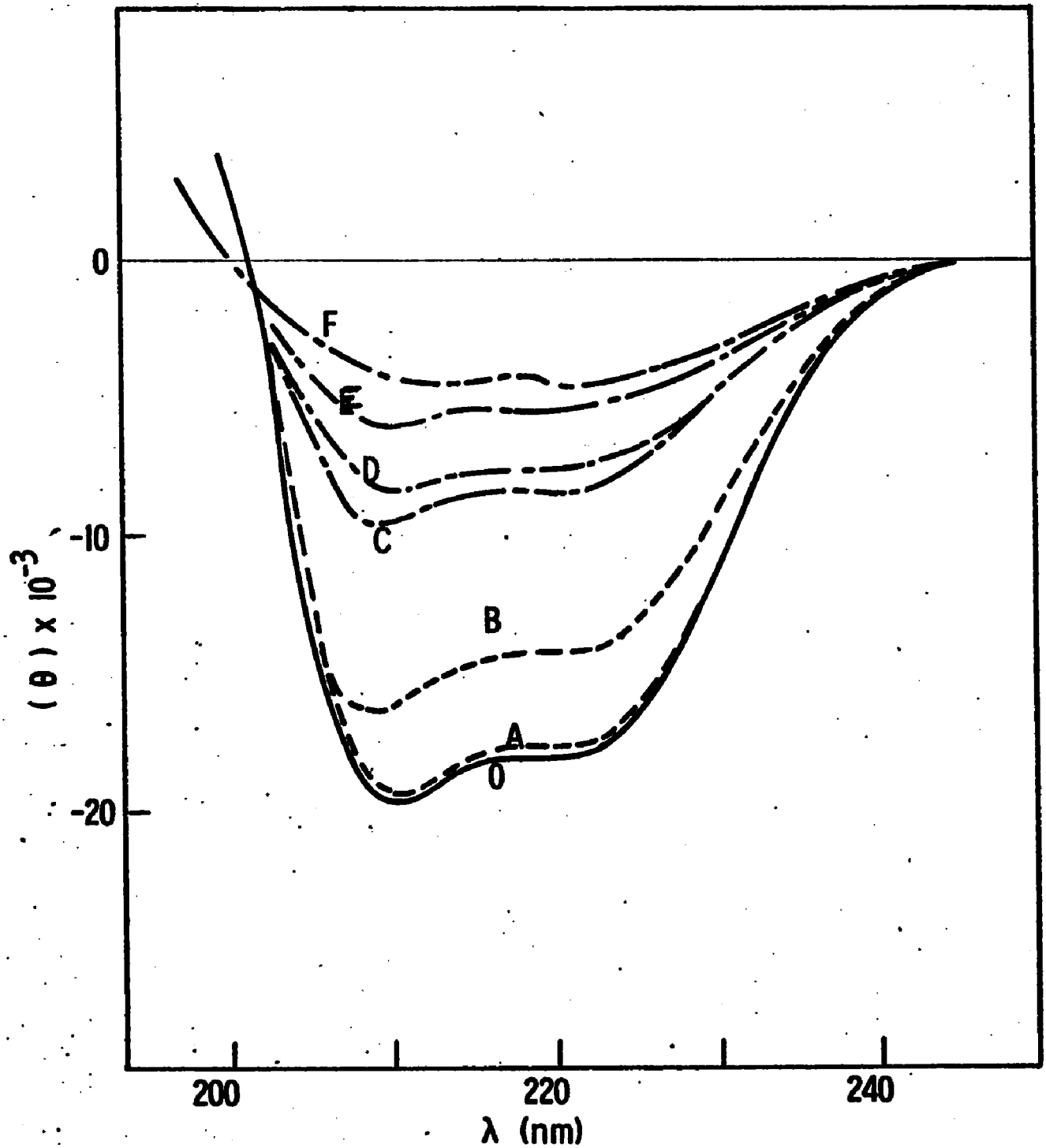



Fig. 8-22. CD of bovine serum albumin: (O) in $H_2O$, (A) in 1 M LiCl, (B) in 5 M LiCl, (C) in 1 M $NaClO_4$, (D) in 2 M $NaClO_4$, (E) in 4M $LiClO_4$, (F) in 5 M $LiClO_4$.

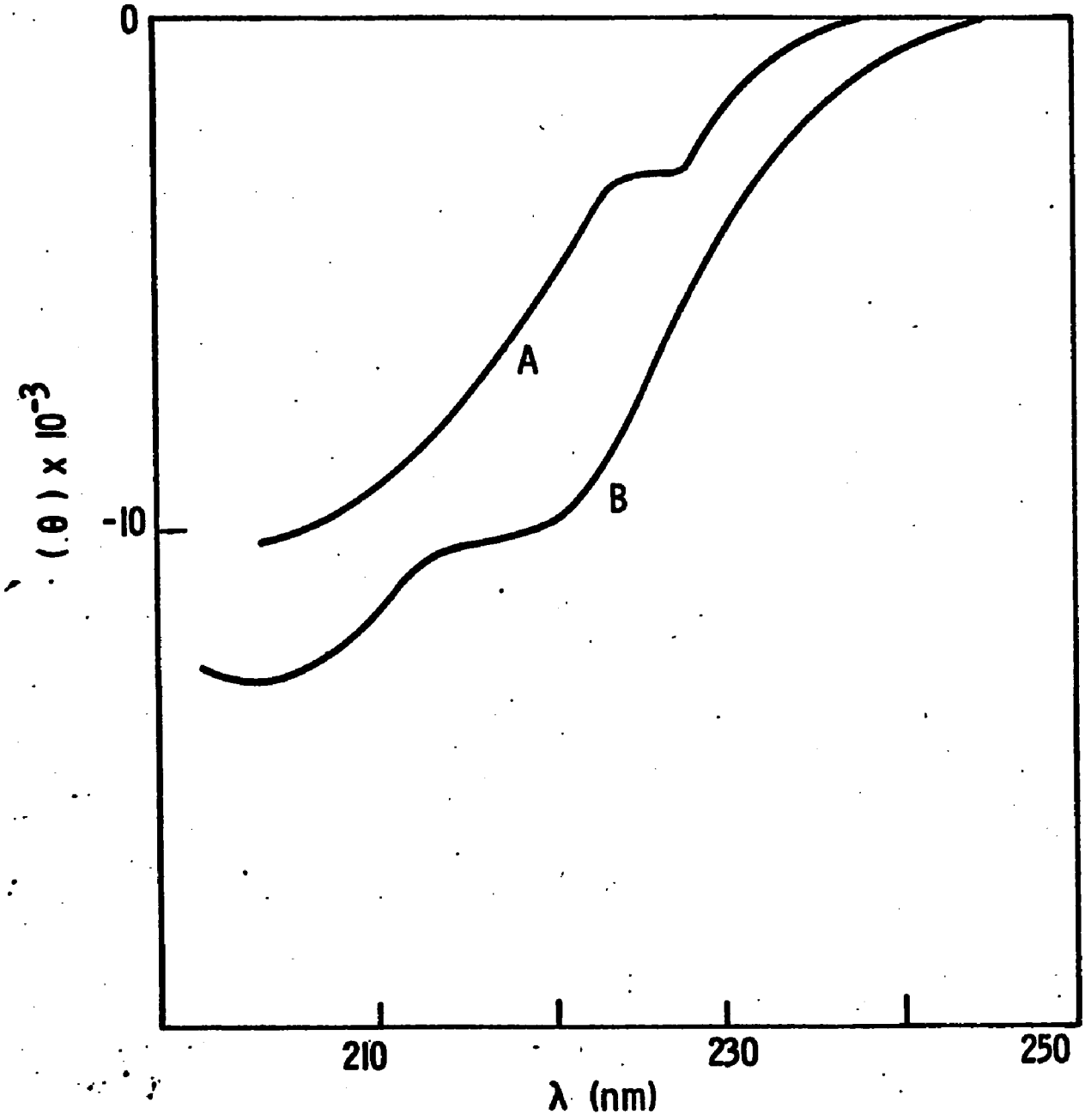



Fig. 8-23. CD of chymotrypsinogen: (A) in $H_2O$ , (B) in 5.4M $NaClO_4$.

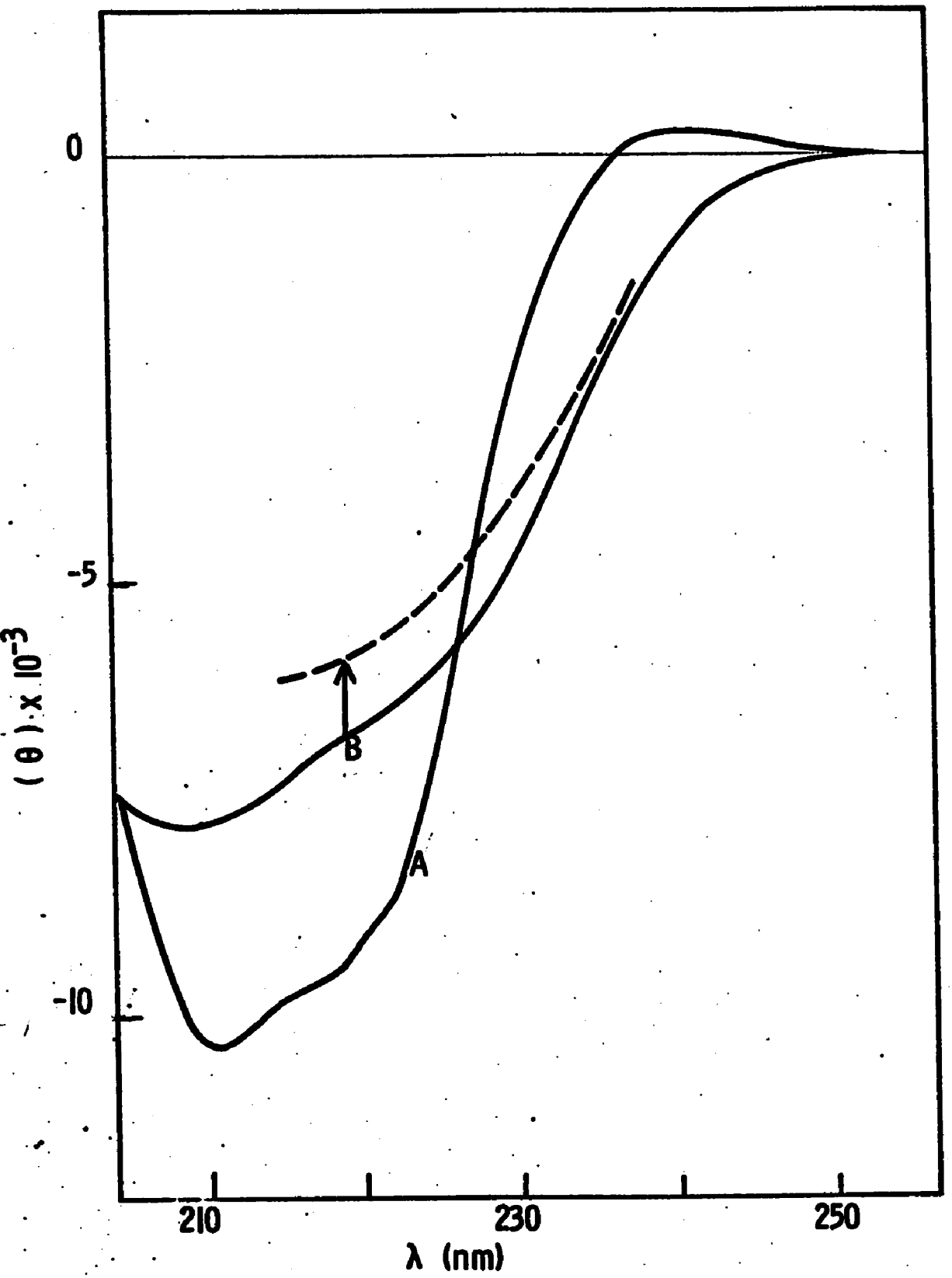



Fig. 8-24. CD of ribonuclease: (A) in $H_2O$, (B) in 4.8 M $LiClO_4$ arrow points to raising the temperature from $25^{o}$ to $55^{o}$.

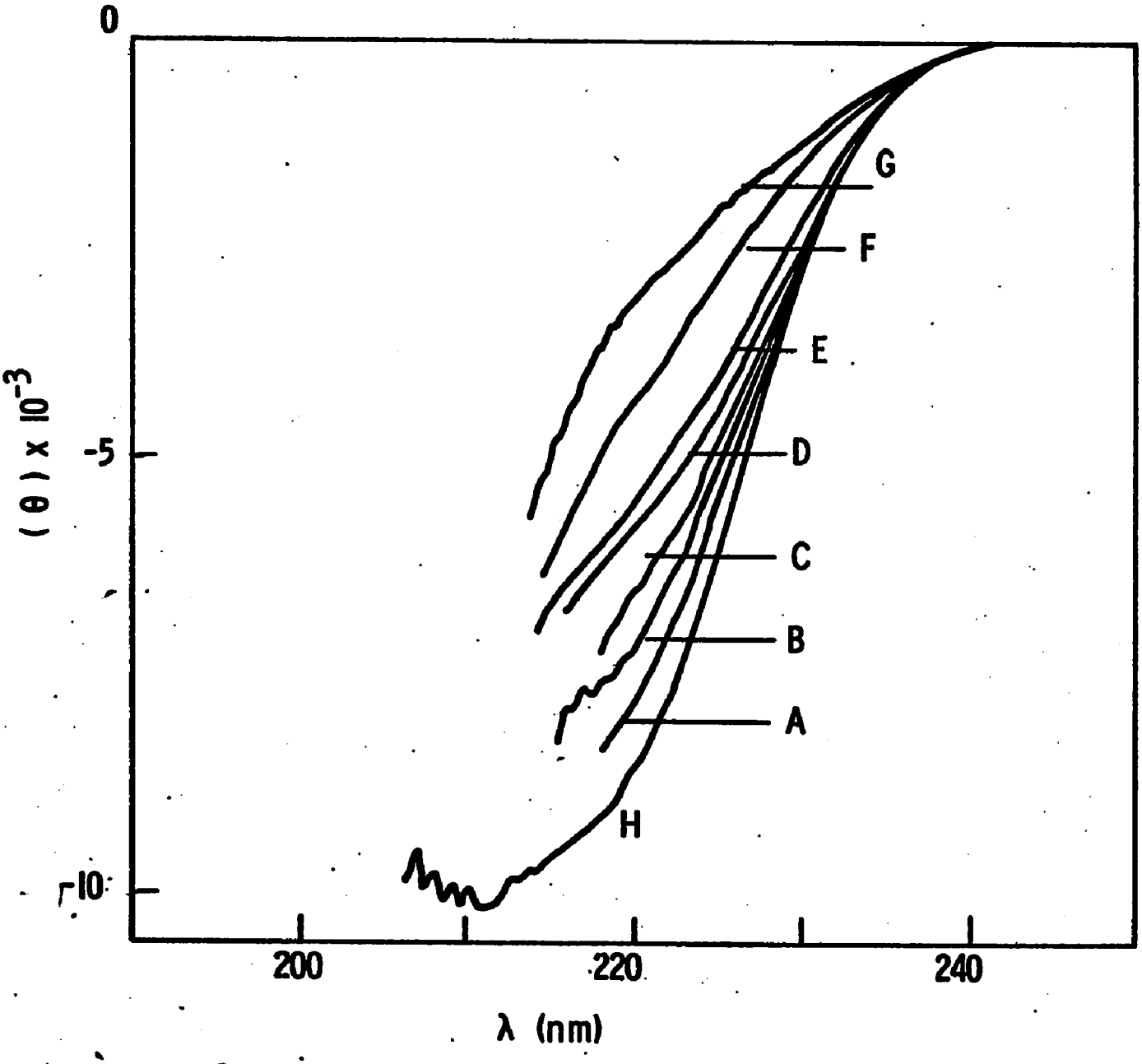



Fig. 8-25. CD of ribonuclease in 8 M urea at room temperature: effect of adding 0.1 M of salts (A) $Na_2HPO_4$, (B) $KH_2PO_4$, (C) $Na_2SO_4$ (D) zero salts, (E) $LiClO_4$, (F) $CaCl_2$ , (G) $Mg\ ClO_4$; sample in $H_2O$ alone, for comparsion (H).

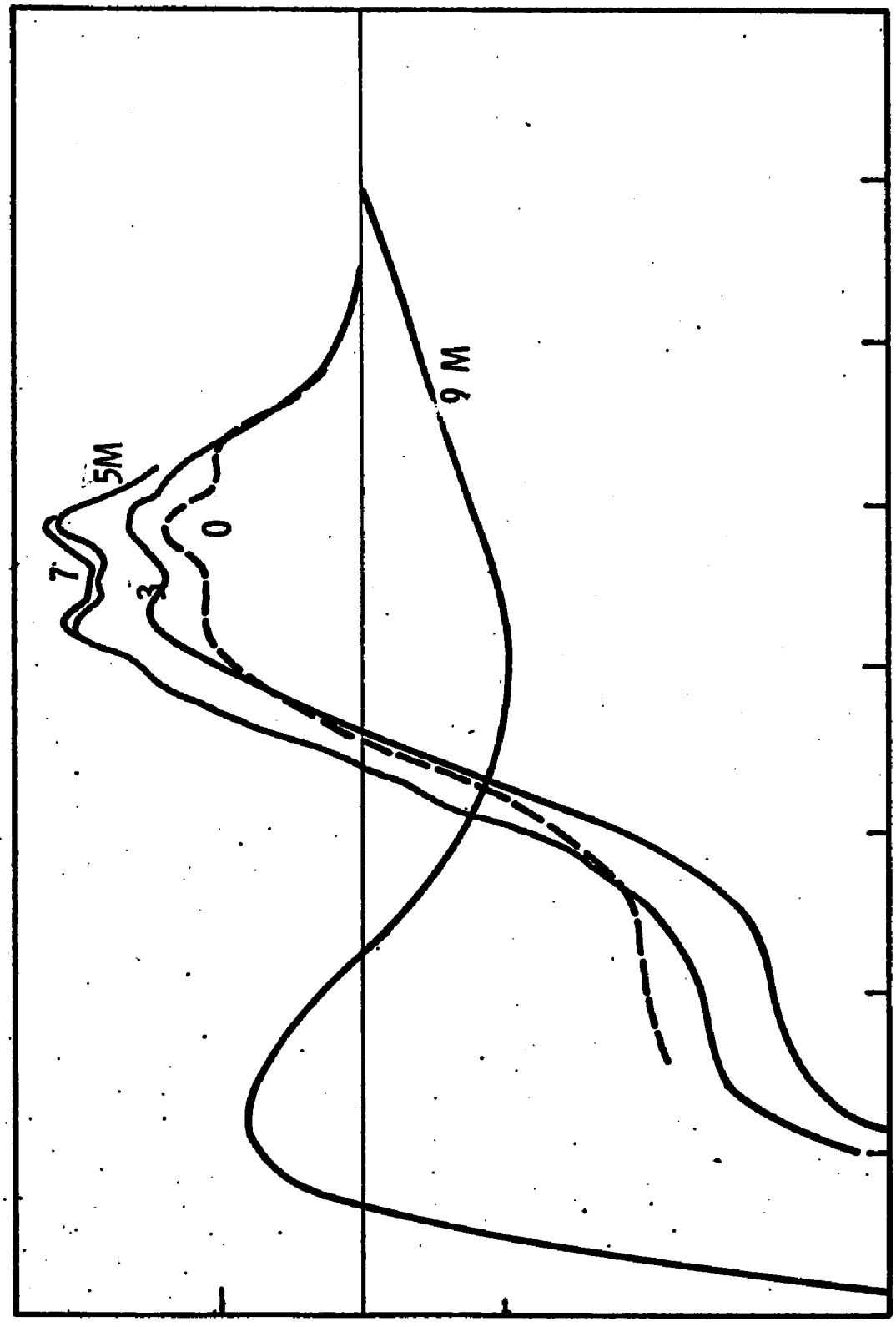



Fig. 8-26. Near uv CD of lysozyme:- - -in $H_2O$; 3M, 5M, 7M and 9M LiCl as marked.

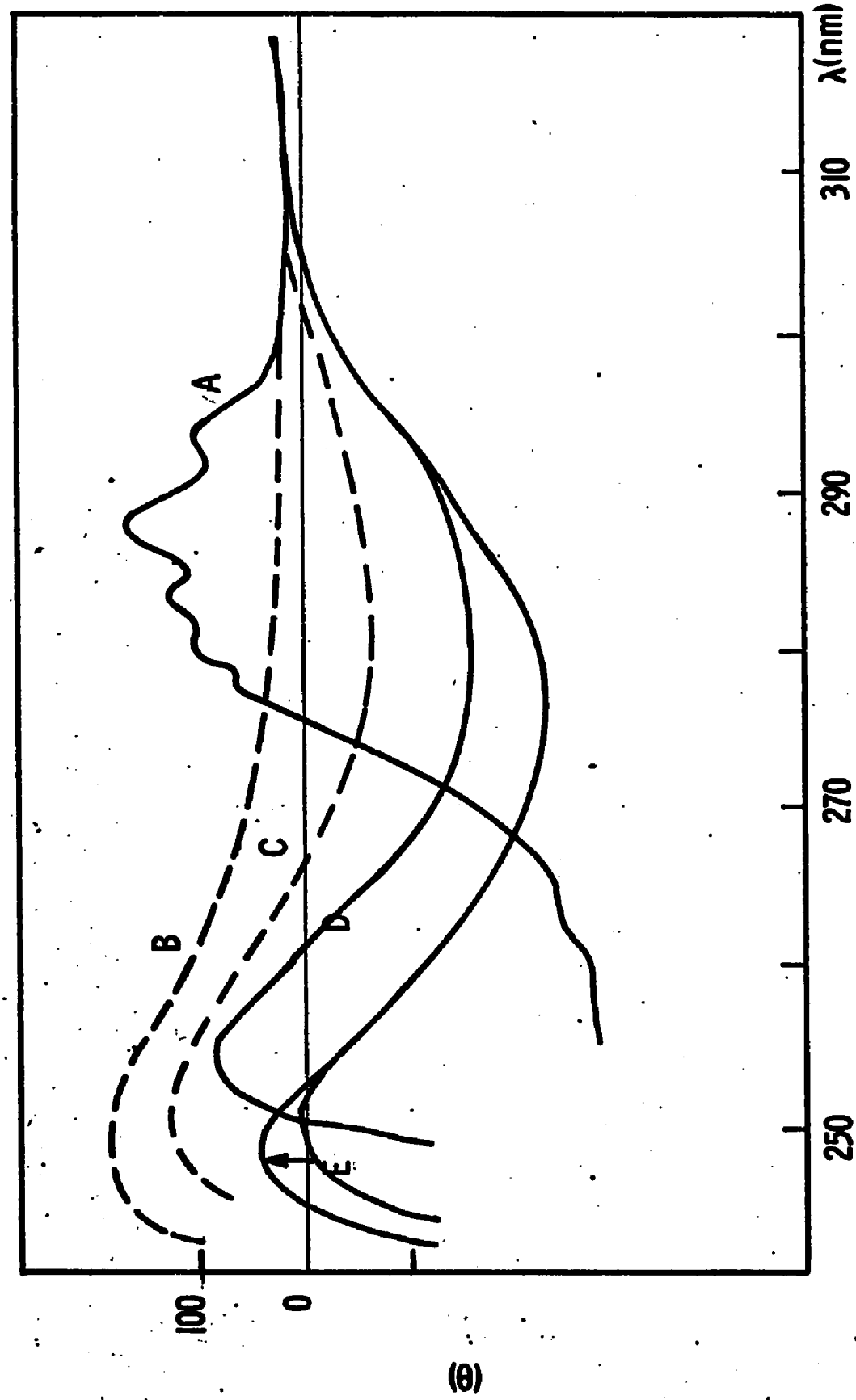



Fig. 8-27. Near uv CD of lysozyme: (A) in $H_2O$, (B) in 4.5 M $CaCl_2$, (C) in 5.8 M $CaCl_2$, (D) in 4.5 M $LiClO_4$, (E) 5.8 M LiCl: 90% methanol ($25^\circ \rightarrow 5^\circ$)

## Chapter 9-Collapsed Random States

In our initial experiments on collagen (Tiffany and Krimm, 1967), we offered as a model for a random coil protein, collagen which was denatured by heat or $CaCl_2$. A simple monotonically decreasing CD curve, with a minimum in the 200 nm region, seems typical of the CD of many proteins when they are denatured under conditions that do not impose new forces leading to preferred structures. When mild conditions of denaturation are used, we run, of course, the risk of not having removed all the order present in the native molecule. Thus most proteins denature at least partially when the temperature is raised. Some regions of the molecule may be more resistant to disruption. Sometimes small changes in the pH are enough to disrupt a globular molecule that has a large number of side chains which can be charged. Such charges can disorder the original detailed coiling without necessarily introducing overwhelming repulsive forces.

### Experimental.

All proteins that were tested by CD after mild denaturation give indications that our first presentation of an experimental CD spectrum for a disordered protein is correct. Thus ribonuclease on being heated loses its negative band that occurs near 210 nm in the native state and a more shallow negative band results near 200 nm (Fig. 9-1). On heating soybean trypsin inhibitor to 50° (Fig. 9-2) the positive band in the 230 nm region is lost and the resultant CD

spectrum looks very similiar to that of denatured collagen (Fig. 3-4). If insulin (Fig. 9-3) is heated at pH 1.5 the characteristic dichroism is lost and there results a monotonically decreasing region in the CD spectrum from 210 to 250 nm. The negative band at 210 nm in the native protein, again shifts to the blue. Similiarly by heating pepsin in 0.50 N NaOH (Fig. 9-4) a simple CD spectrum results with the negative band maximum past 205 nm. Both bovine serum albumin (Fig. 9-5) and ovalbumin (Fig. 9-6) after being heated in dilute $NH_4OH$ lose any indication of $\alpha$ helicity and have only the single CD band near 205 nm. There are many examples in the published literature of other unordered proteins. For example, immunoglobulin at pH 2 (Ooi and Jirgensons, 1970), $\beta$ lacto globulin A at pH 11 (Timasheff et al., 1967). Simple reduction and blockage of S-S bonds does not necessarily lead to a random coil structure for a protein; thus carbamidomethyl lysozyme is seen to have residual structure at 2° (Tamburro et.al., 1970). If a protein that contains S-S bonds, is randomized without reduction or oxidation of the S-S bonds we always see a negative CD band near 270 nm remaining in the near uv. This is probably to be related to dichroism due to the S-S bond itself.

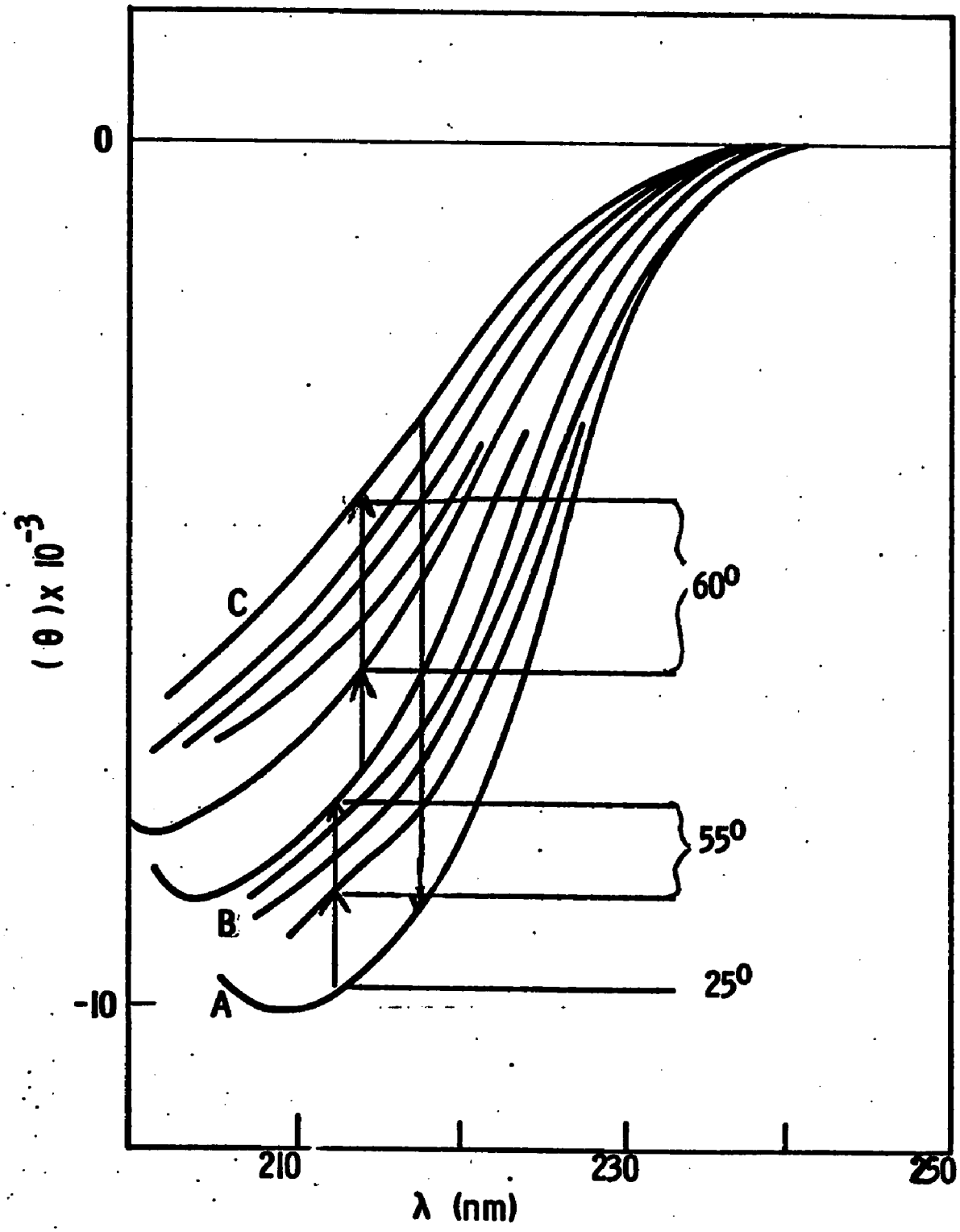



Fig. 9-1. CD of ribonuclease,(1g/l) ; effect of raising the temperature (A) spectrum at 25°, (B) spectra at 5 min. intervals after raising the temp. to 55°, (C) 5 min. intervals at 60°. No cooperative transition is apparent. Effect is reversible.

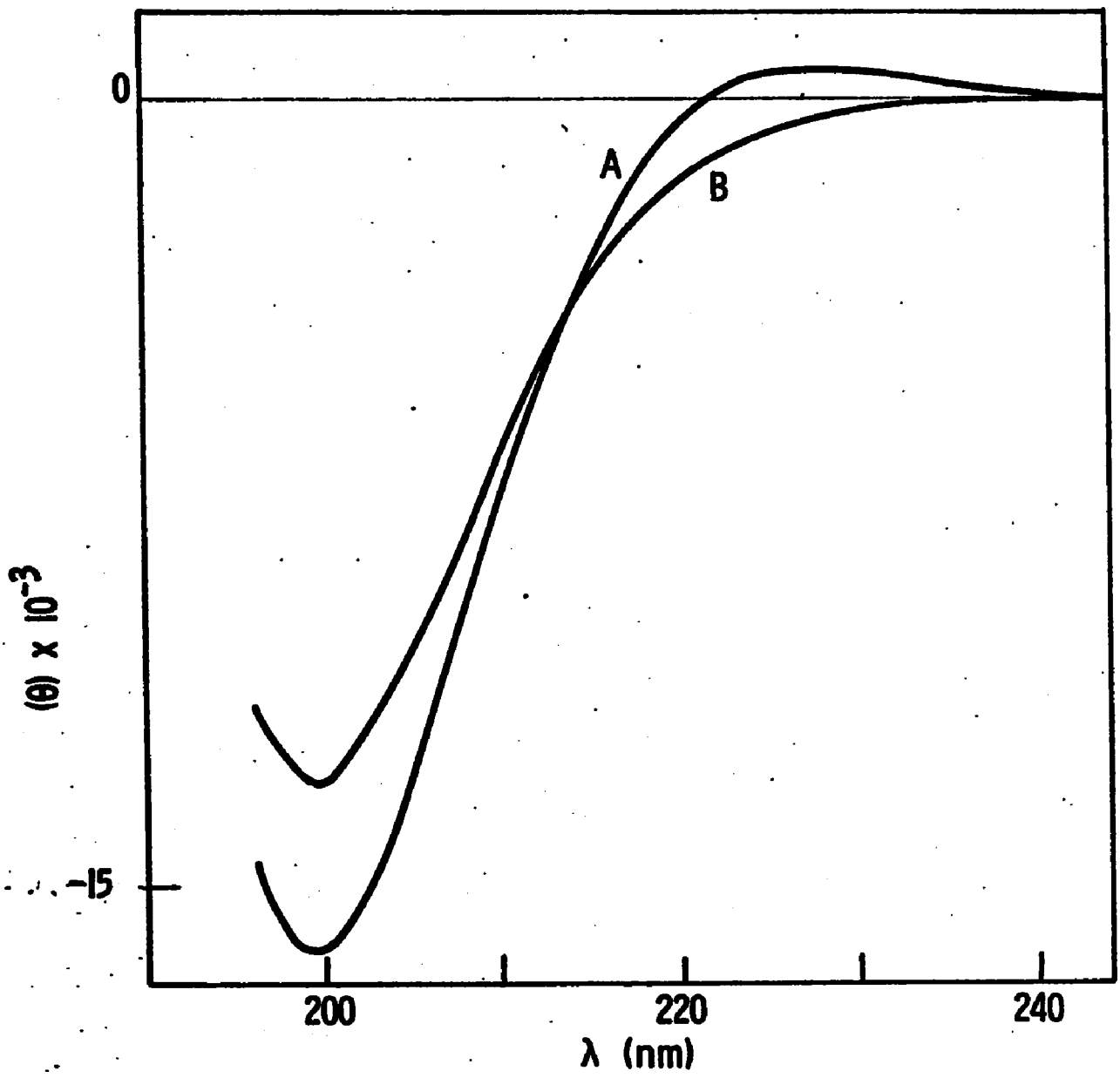



Fig. 9-2. CD spectra of soybean trypsin inhibitor: (A) at 25° in native state, (B) sudden change in CD at 50° . ( Note CD now looks like denatured collagen.)

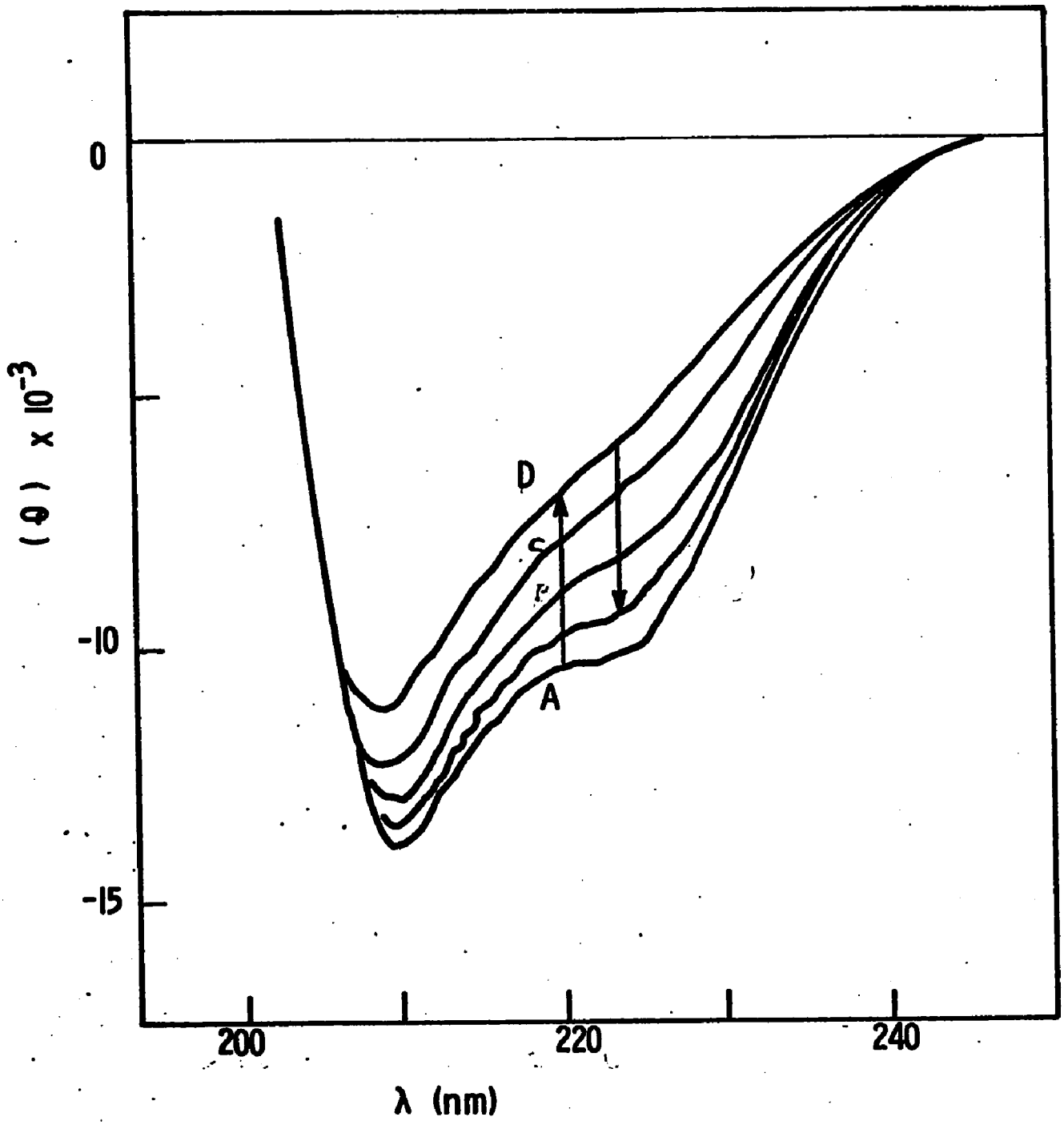



Fig. 9-3. CD of insulin; effect of temperature, (A) 25°, (B) 35°, (C) 50°, (D) 60°. ($D^1$) return to room temperature and spectrum recorded within 10 minutes.

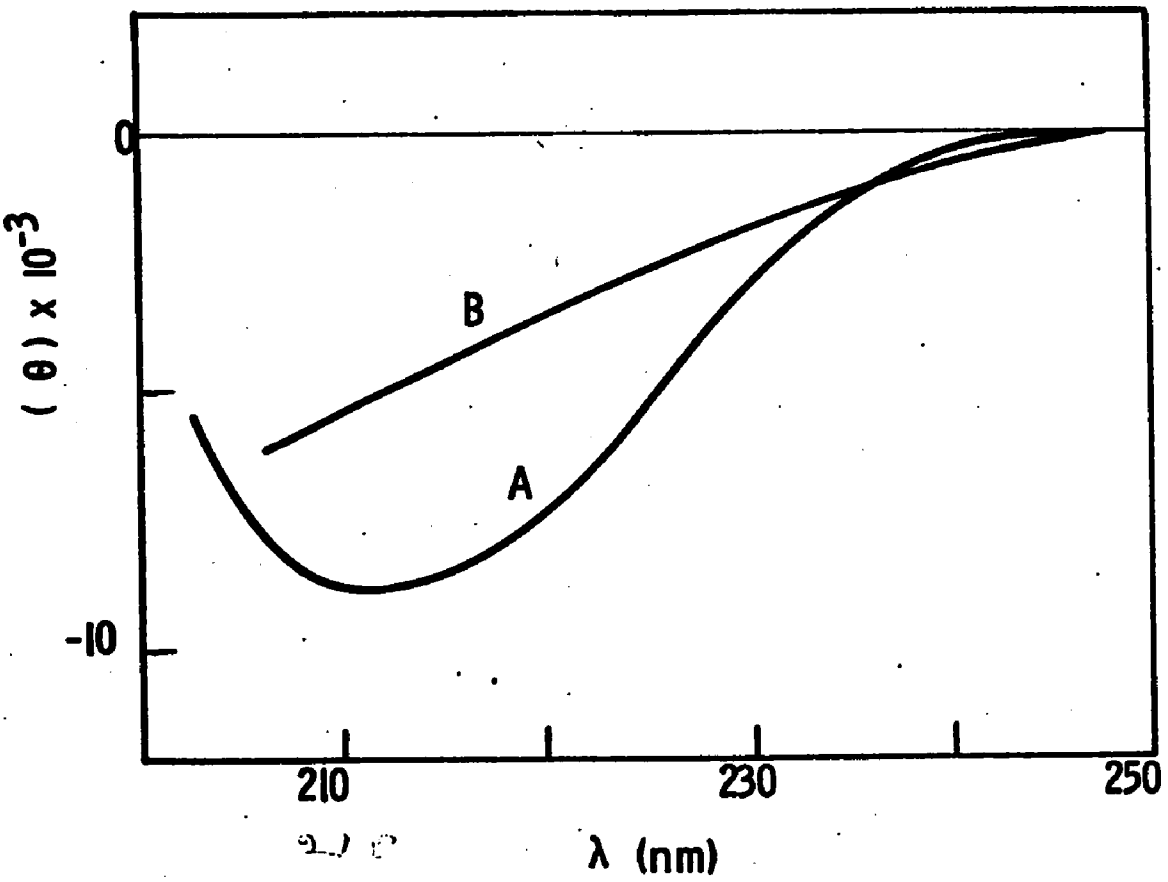



Fig. 9-4. CD of pepsin: (A) native (B) alkali denatured by 0.5 N NaOH.

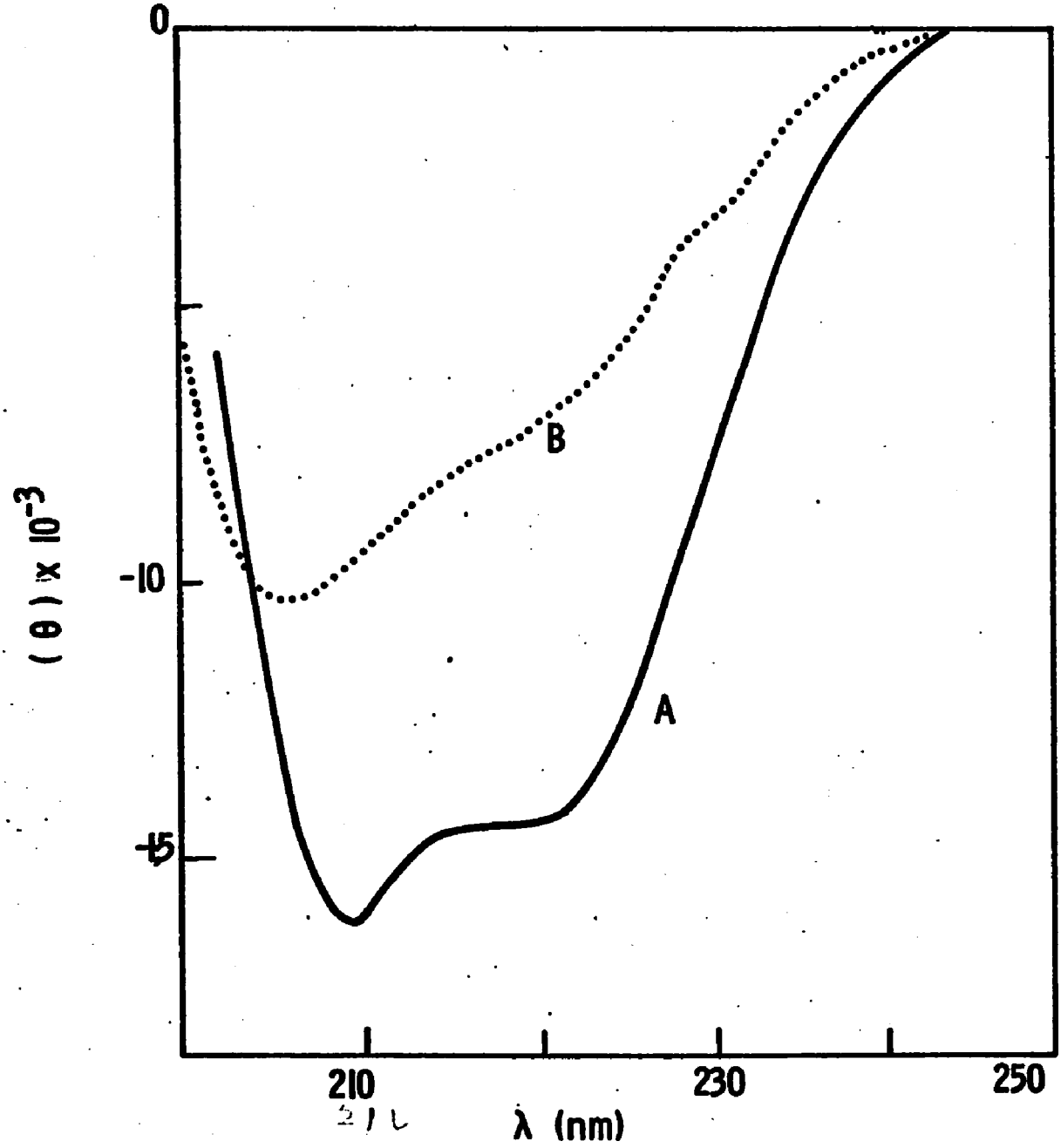



Fig. 9-5. CD of bovine serum albumin: (A) native, in $H_2O$; (B) (....) heat denatured (110°) for 1 hr. in aq. $NH_4OH$ then cooled to 25°.

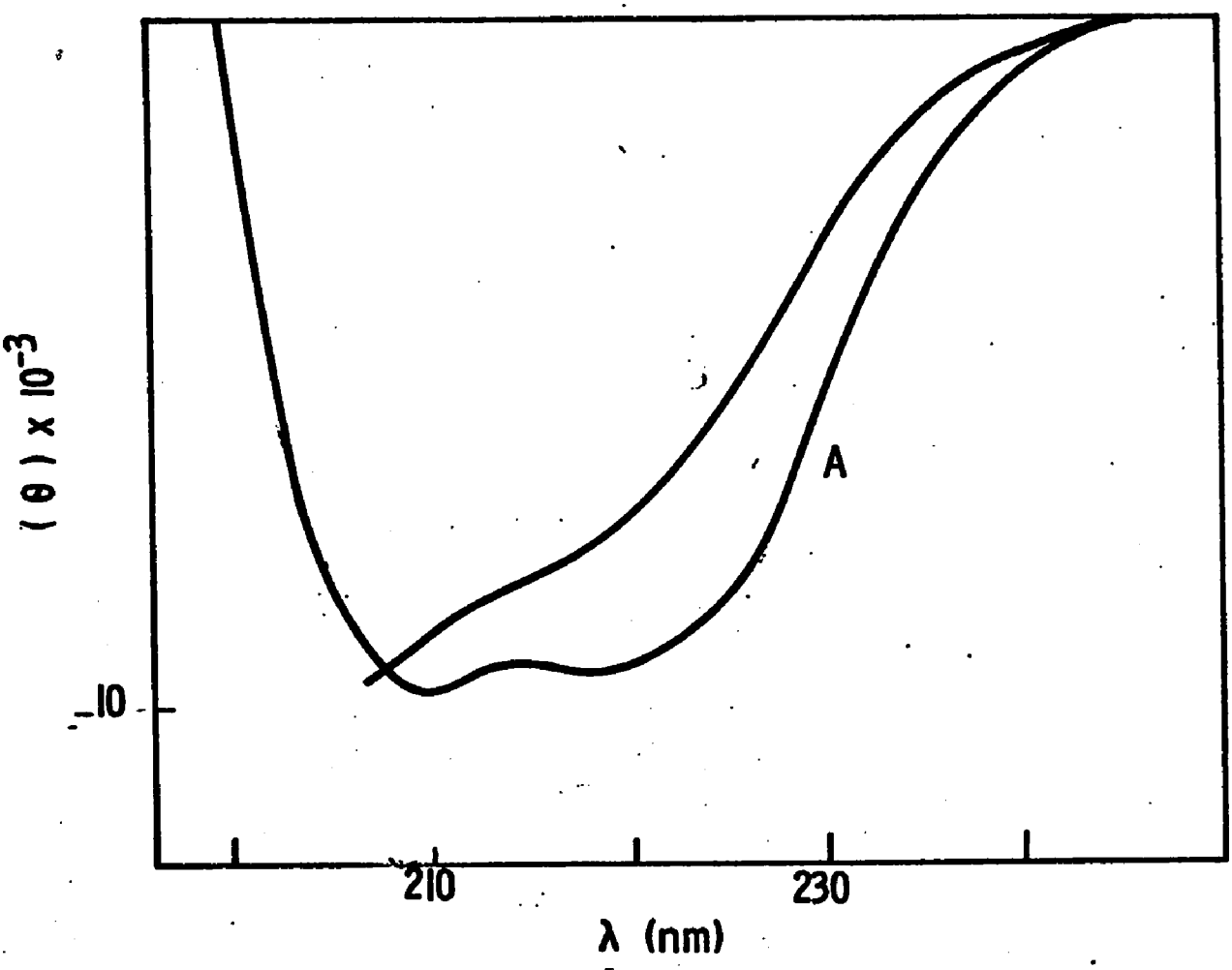



Fig. 9-6. CD of ovalbumin: (A) native at 25°, (B) denatured by heating to 110° , 30 min. and then cooling to 25°.

## Chapter 10 - Conclusions

We have shown that there are many facets to the so-called random state of a polypeptide or protein. Thus the chain can be collapsed with no overall preferred local ordering of the peptide units or else can extend and show local order for any of a number of reasons.

A very good example of the collapsed state is the single chain of one of the collagen units after the triple helix of the native state is made unstable by heat, acids, detergents, or salts. Other proteins are seen to also be in this state when the native form is destabilized, but no overwhelming force is present to impose new overall order. We must of course be careful that remnants of the orginal native configuration have been removed. Globular molecules usually unfold in a cooperative manner. This first stage of denaturation results in molecules that have an intrinsic viscosity larger than when they have a globular form, but less than when they are more fully extended. Many of the side-chains will not be well exposed to the solvent. Neither criteria of intermediate viscosity values or lack of total possibility of side-chains to titrate normally should be considered as evidence of incomplete denaturation.

Many forces that destabilize a native configuration may lead to conditions for a new ordered state to form. The best example of this is the effect of acidic alcohols. Thus in TFE we have seen all normal proteins and polypeptides (we exclude polypeptides of high glycine and proline content) take an helix conformation. Local apolar binding of side-chains to

each other has been disrupted by the alcohol, and the peptide will tend to the minimum energy configuration of the helix under these new conditions. The side-chains should be well available to solvent and titrate normally.

We have shown that if the perturbant has a strong interaction with the carbonyls of the polypeptide backbone the stable form can now be an extended structure. Thus a CO on the backbone cannot be involved with intra-chain hydrogen bonding if it is engaged in external hydrogen or other cation bonding. If the denaturant is urea or guanidine hydrochloride or a lithium salt the resultant CD indicates an extended conformation can form at low temperatures. This extended form has local order probably close to a three - fold left-hand helical structure, and makes probable an interaction of one denaturant ion or molecule with one CO group and not several. It is to be noted that the perturbant must be able to get in close to the backbone and therefore substituted ureas are not as effective as the simple molecule to cause extension although the methyl substituted urea may tend to be better able to disrupt the native protein by interacting with the side chains.

The effective multivalent cations apparently attempt to interact with several CO groups at one time and disrupt ordered structures without leading to extended states. There are indications from analysis of many multivalent cation-protein interactions and through comparision with the CD of

the model system, poly-L-proline and salt, that a collapsed unordered state results.

The general shape of the CD curve that can be accepted for a randomly coiled polypeptide enters into all calculations for the per cent helicity of native globular proteins. Especially serious, it would seem, is the false conception, based on the wrong model, that influences the ideas of the theoretician. Aebersold and Pysh (1970) have presented ionized PGA as a model of a random coil polypeptide, and then have tried to match it with theoretical calculations. Calculations have also been made by Tonelli (1969) and Ronish (1970). It may be that such calculations should not attempted until the CD of the simple state of poly-L-proline II can be reproduced. It is not at present possible (Pysh, 1970).

We see no conflict in any of our conclusions on the "Extended Helix" structure with any of the hydrodynamic experimental results of others on ionized polypeptides. The dramatic increases of the intensity of the CD bands related to this conformation achieved by lowering the temperature, point to a loosely jointed structure that is easily perturbed by raising the temperature. (It is to be noted that most hydrodynamic experiments have been done in the presence of salts which also would collapse the ionized structures, e.g. Brant and Flory, 1965).

There have been many indications that others beside ourselves have noted and worried about the possibility that the ionized polypeptides can have a conformation similiar to PP II. Thus, in 1964, Schellman and Schellman, remarked "one can speculate that the high levorotaion of the random polypeptide chain of PGA might arise from the considerable probability for the orientation angles associated with the collagen fold." Our results make this conclusion plausible.

# Optical Active Aggregated Forms of Poly-L-Proline and of Collagen. A New Phenomenon.

**Synopsis**

Poly-L-proline in the cis form (PP I) as well as collagen, are shown to be able to exist under certain conditions, as superstructures that have very strong circular dichroism bands in addition to the normal bands of the unaggregated state. These bands, in position and intensity, are very sensitive to lyotropic salts and to urea. The bands change as the aggregate grows or as it disappears.

That poly-L-proline I (PP I) can exist in an aggregated form was first noticed during the course of an attempted dissolution in water of a sample of M.W. 13,500, obtained from Sigma Co. The solution was initially cloudy, and would not pass through a micropore filter. The circular dichroism (CD) of the suspension turned out to be extremely interesting. Many new very strong bands are superimposed on the spectrum of the unaggregated state of PP I. The same sample, dissolves rapidly in trifluoroethanol and gives only the normal PP I CD bands.

As the transition from I to II takes place over a period of several hours, the bands shift to the blue and decrease in intensity. The samples become clear, (Fig. A-1).

The complex nature of the bands is seen by their reaction to the addition of lyotropic salts and urea. These reagents have an immediate specific effect on the aggregate spectrum, see Fig. A-2 and table 1.

That the action of the salt is reversible is shown by diluting one part of solution to four parts water and recording the CD in a sample cell five times as long as the one used in the concentrated salt. The CD spectrum is such as would have resulted from using the weaker salt solution initially.

Optically active aggregates can also be formed from solution of PP II in dichloroethanol by reversing the II to I stability with the addition of nine parts of n-propanol to one of the chloroethanol solution, using the sample of molecular weight 13,500. Samples of lower molecular weight are too soluble to form the aggregated state. Higher molecular weight samples tend to come out of solution, in the I form, and so can not be tested for the formation. (See Fig. A-3).

An optically active aggregated form of collagen was found to form on the addition of NaF or $NH_4F$ (0.9 to 1 M) (Fig. A-4). New bands, of variable position and of increasing intensity, are seen to grow with time as aggregation takes place, over a period of a few hours. On dilution with 2% acetic acid, the collagen returned to the normal CD pattern, whereas dilution with water did not dissolve the aggregates. This is interesting since a form similar to the native fibrous

state may have resulted from the salt aggregation. This should be checked by electron microscopy.

The optical activity discussed above is very different from that due to optically active absorption bands. It appears to be due to a form factor. A somewhat similar phenomenon occurs in liquid crystals (Fergason, 1966). It is probable that it will be observed in other aggregating systems, such as actin, which has been reported to occur in a highly optically active form (Mommaerts, 1967).

The high sensitivity to environment may make this form of optical activity a very useful tool in studying the bonds important in association.

A note of caution should also be made in interpreting CD patterns only in terms of transitions to excited states, since we see that other causes of activity may need to be considered.

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Table 1.

Initially recorded Position of CD Bands due to Form Circular Dichroism in Poly-L-proline I samples of MW 13,500.

| solvent | wave length in nm | | | |
|---|---|---|---|---|
| water | 250 (+) | 280 (-) | 370 (+) | 650 (-) |
| 4 M NaCl | 247 (+) | 280 (-) | 365 (+) | 650 (-) |
| 4 M $LiClO_4$ | 247 (+) | 270 (-) | 340 (+) | |
| 4 M $NaClO_4$ | 248 (+) | 270 (-) | 350 (+) | |
| 5 M LiBr | 230 (+) | 245 (-) | 290 (+) | |
| 5 M $CaCl_2$ | 230 (+) | 245 (-) | 275 (+) | |
| 5 M urea | | 245 (-) | 295 (+) | 550 (-) |
| 10 M urea | | 230 (-) | 255 (+) | 350 (-) |

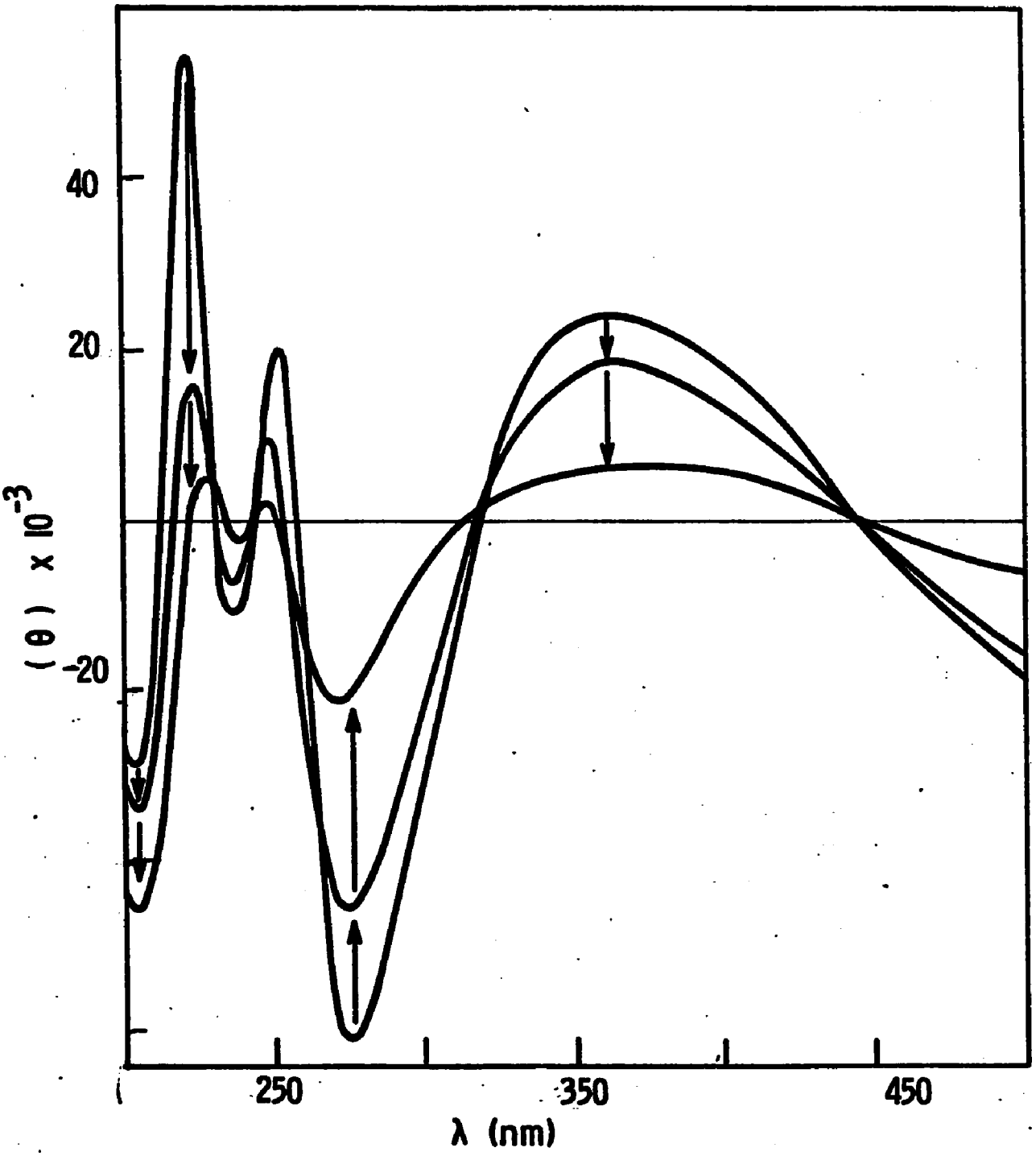



Fig. A-I: CD of poly-L-proline, mol. wt. 13,500, 2 g/l, As cloudy aggregate dissolves CD spectrum changes with time. Bands decrease in intensity and shift slightly to shorter wave lengths.

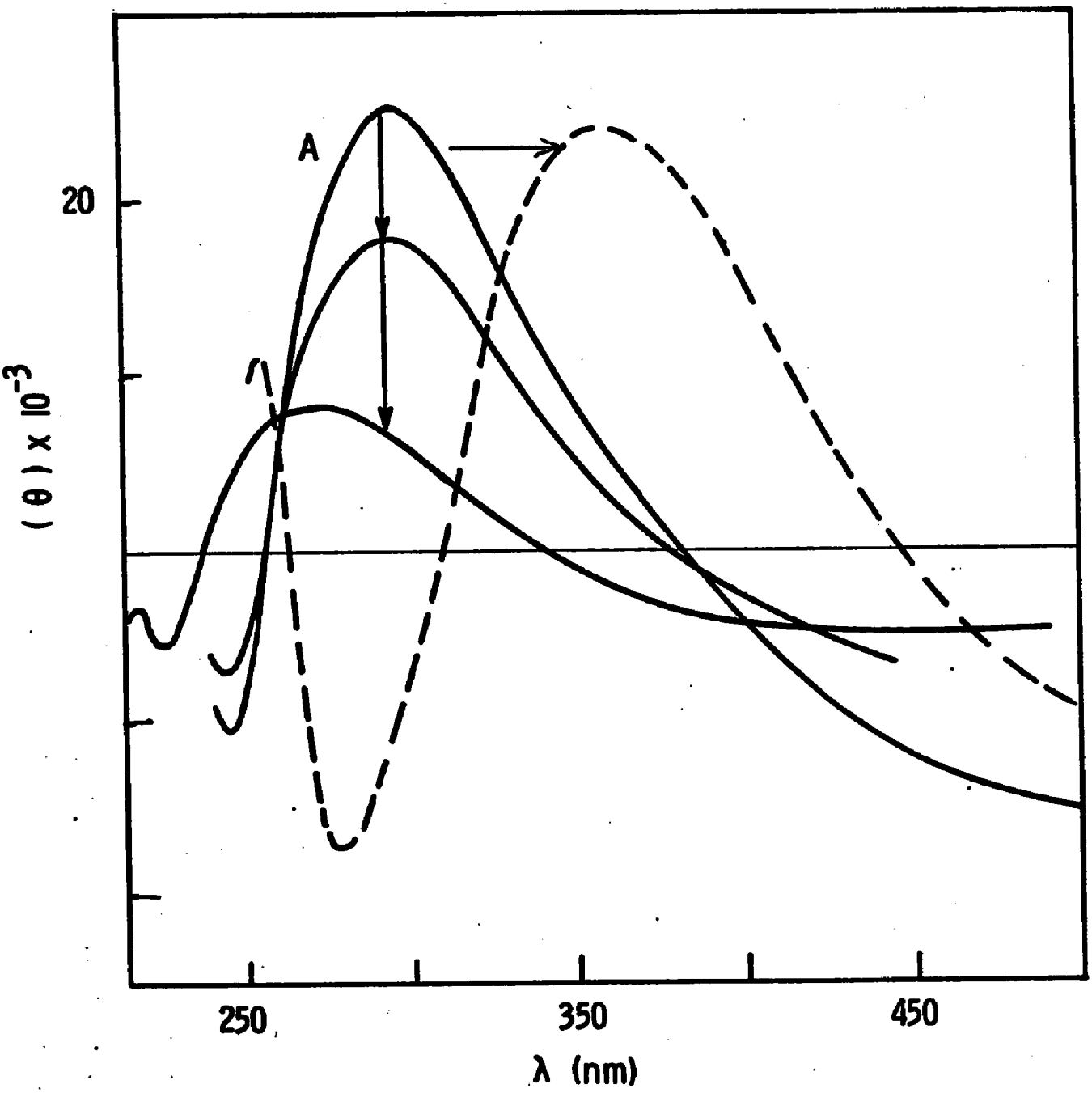



Fig. A-2. CD of poly-L-proline, mol. wt. 13,500. Sample was mixed in 5 M $CaCl_2$. Solution slowly took place. An immediate (A) initial interaction with the salt is observed, followed by a slow change with time. If sample in $CaCl_2$ is diluted with 4x $H_2O$ the CD registered is similar to that of the polymer in $H_2O$, showing the reversible interaction of salt in the aggregate. (— —) Indicates CD of some of sample A diluted with 4X $H_2O$ and run immediately.

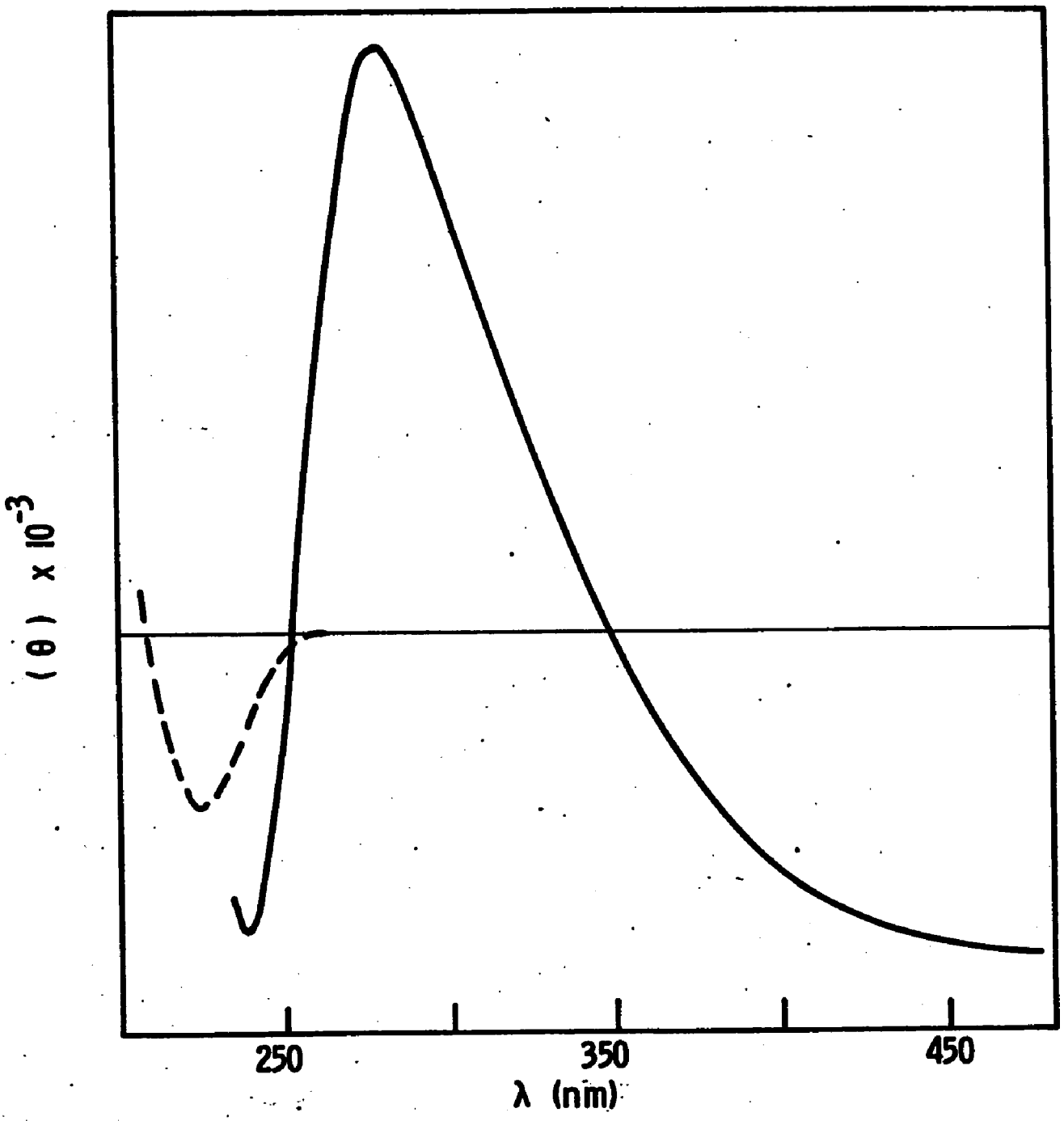



Fig. A-3.: CD of poly-L-proline, mol. wt. 13,500, 1 g/l.

—— Sample equilibrated in N-propanol.

– – – Micropore filtrate of same sample.

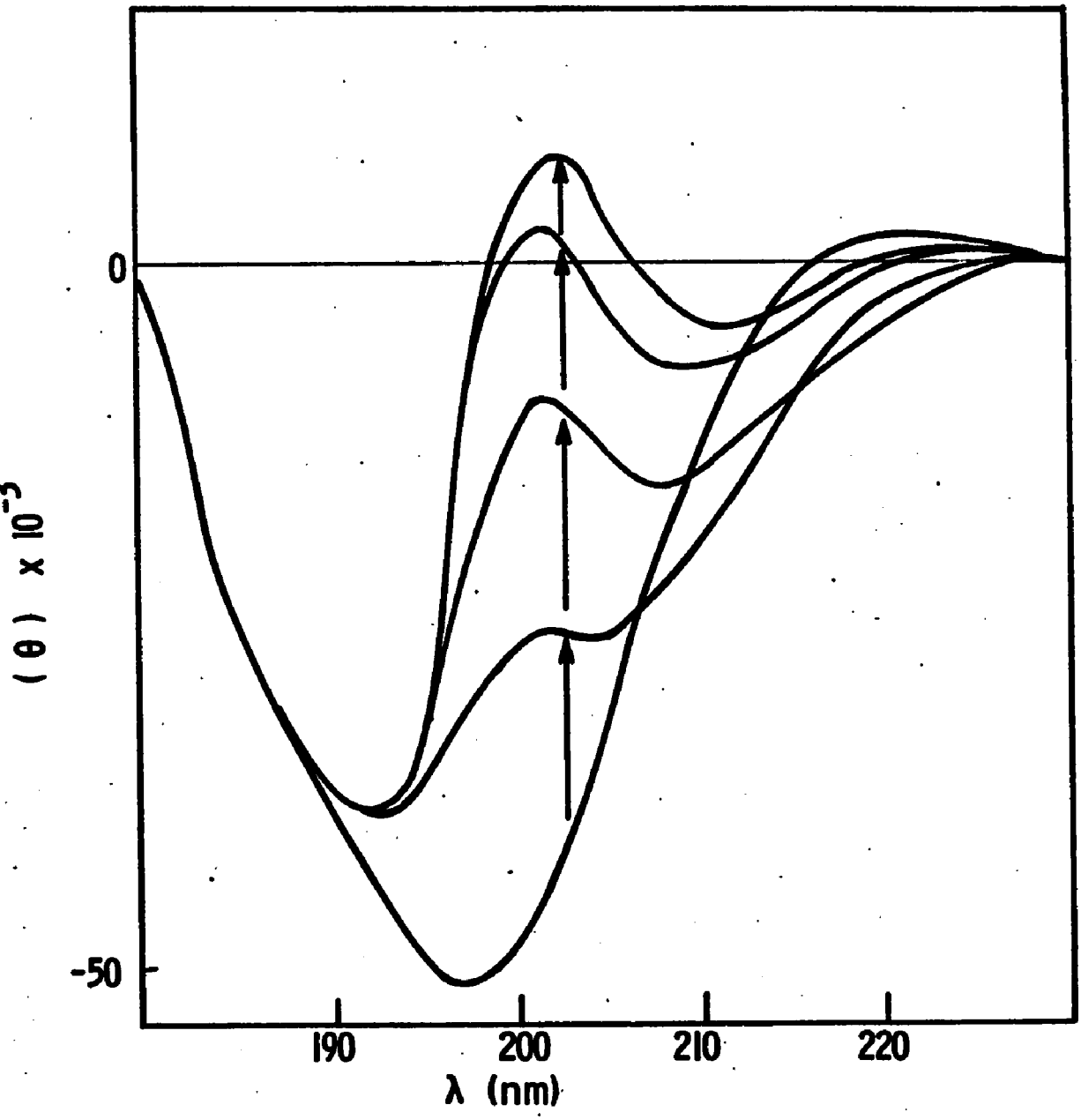



Fig. A-4. CD of collagen showing formation of optically active aggregate in 0.9 M NaF solution. Time interval between curves, approximately 20 min.

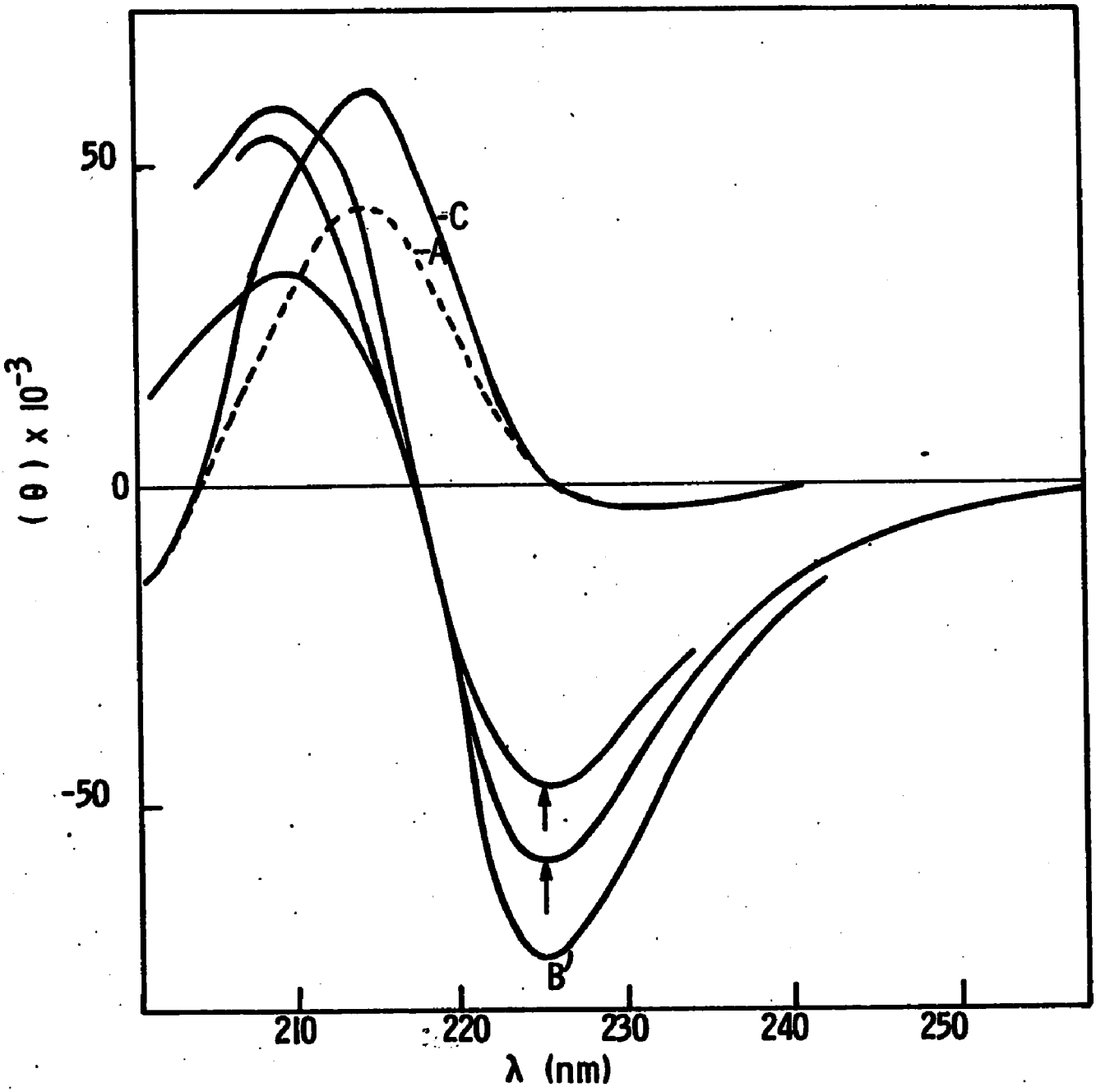



Fig. A-5. Poly-L-proline ( Mann): (A)sample equilibrated in propanol; to this sample is added 9 parts of 6 M $NaClO_4$ water solution to give (B) which is unstable with time. If sample (A) is diluted with 9 parts of water the CD spectrum is given immediately by (C).

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