

STUDIES OF THE AROMATIC CIRCULAR DICHROISM OF STAPHYLOCOCCAL NUCLEASE

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Abstract.—Specific contributions of tyrosyl and of tryptophanyl residues can be distinguished in the near-ultraviolet circular dichroic spectrum of staphylococcal nuclease. Upon binding of the inhibitor deoxythymidine 3',5'-diphosphate in the presence of Ca^{++} , a significant change in the circular dichroic spectrum results which has been used to characterize the interaction of ligand and enzyme. The data suggest that the asymmetric environment of certain tyrosyl residues is altered by binding of the nucleotide inhibitor.

The optical activity of side-chain chromophores contributes to the ultraviolet circular dichroism and optical rotatory dispersion spectra of proteins.¹⁻³ Circular dichroism has the advantage of being confined to the wavelength region of absorption, which facilitates resolution of overlapping optically active bands. Nevertheless, in most proteins the interpretation of specific spectral features is complicated by overlapping of tryptophanyl, tyrosyl, cystinyl, and peptide bond transitions.

The extracellular nuclease of *Staphylococcus aureus*⁵ is favorably suited for characterization of its near-ultraviolet circular dichroism spectrum. This enzyme lacks disulfide bridges. Its single tryptophan residue is inaccessible to solvent, and the fluorescence and ultraviolet spectral properties of the tryptophan are unaffected by ligand binding.⁶⁻⁸

In the native enzyme, 2 of the 7 tyrosyl residues appear to be inaccessible to solvent and chemical reagents; upon binding of the competitive inhibitor, deoxythymidine-3',5'-diphosphate,⁴ 2 or 3 additional tyrosines become masked.⁶⁻⁹ Chemical modification studies indicate that the tyrosyl residues at positions 85, 115, and 27 are located in the substrate-binding region of the enzyme.⁸⁻¹⁰ The process of binding ligand is probably accompanied by a subtle, localized conformational change.^{7, 8} Nuclear magnetic resonance¹¹ and X-ray crystallographic¹² studies also indicate that tyrosine residues play a major role in the binding process.

This paper describes the circular dichroism spectra of nuclease and of a performic acid-oxidized derivative. Circular dichroism measurements of the interaction of deoxythymidine-3',5'-diphosphate with nuclease in the presence of Ca^{++} confirm the 1:1 stoichiometric binding of this substrate analog and illustrate the usefulness of difference circular dichroism spectra.

Materials.—Nuclease was prepared from culture media of *Staphylococcus aureus*, Foggi strain, by the method of Moravek *et al.*¹³ Performic acid oxidation of nuclease was carried out with 10-fold excess of reagent at 0°C for 2 hr.^{14, 15}

Methods.—Determination of enzyme concentration was made by quantitative amino acid analysis of acid-hydrolyzed samples,¹⁶ as well as by measurement of ultraviolet ab-

sorbance. For nuclease, $E_{277}^{0.1\%}$ is 0.97; for performic acid-oxidized nuclease, $E_{247}^{0.1\%}$ is 0.70.

Circular dichroism. Circular dichroism was measured with a Cary model 60 recording spectropolarimeter equipped with a 6001 circular dichroism attachment, including a Pockels cell. Molecular ellipticity $[\theta]$, with dimensions as degree $\text{cm}^2/\text{decimole}$, was calculated² from the observed ellipticity θ in degrees. The mean residue weights were calculated from the known sequence and from the chemical modifications introduced in the performic acid-oxidized derivative.¹⁵ The buffer used for measurements of ultraviolet absorption and circular dichroism was 0.05 M Tris-HCl, pH 8.0, prepared with deionized water (Hydro Service & Supply, Inc.) and subjected to Millipore filtration. The sample solution, in a 3.1 ml quartz cuvette of 1 cm path length or in a 2.6 ml cuvette of 0.1 cm path length, was placed in the cell holder and equilibrated at 27°C for at least 15 min before measurements were taken. To measure the near-ultraviolet circular dichroism spectra, the 0.02° range was used, with slow scanning speed (2–3 nm/min) and with signal/noise ratio at maxima or minima at least 6:1 for studies with nuclease and deoxythymidine-3',5'-diphosphate. All spectra, including buffer runs, were measured in duplicate or triplicate, with good reproducibility and without hysteresis. Dynode voltage was kept within the range of 0.22 to 0.36 kv, and slit width within 0.5–1.0 nm.

The dissociation constant (K_i) for the nuclease-deoxythymidine-3',5'-diphosphate complex was estimated from the deviation from linearity of the plot of difference in ellipticity versus molar ratio of deoxythymidine-3',5'-diphosphate to enzyme, as described with the difference absorption spectra.⁶ All circular dichroism studies of nucleotide binding were performed at pH 8.0, 0.05 M Tris-HCl and 10 mM Ca^{++} , conditions of optimal nucleotide binding.

Results.—Spectral features of the CD of nuclease and of performic acid-oxidized nuclease: Native nuclease at neutral pH exhibits a large negative ellipticity band centered at 220 nm, with $[\theta]_{220} -9950$ deg. $\text{cm}^2/\text{decimole}$ (Fig. 1). By contrast, performic acid-oxidized nuclease has a featureless, negative ellipticity curve which is consistent with unstructured polypeptide chains.¹⁷

In the near-ultraviolet region (Fig. 2), the nuclease circular dichroism spectrum has a small positive band at 296 nm, attributable to tryptophan, and a much larger negative band centered at 277 nm, attributable to the tyrosyl residues; $[\theta]_{296} = +9$, and $[\theta]_{277} = -75$ deg. $\text{cm}^2/\text{decimole}$. The circular dichroism band of tryptophan may be affected by the more intense tyrosyl band of the opposite sign. Addition of Ca^{++} (10 mM) does not affect these bands. The molecular ellipticity of the band at 277 nm is about 0.8 per cent of that at 220 nm. In contrast, performic acid-oxidized nuclease has no positive band in the 296 nm region, consistent with the destruction of tryptophan in this derivative.¹⁵ Furthermore, only a shallow negative ellipticity is observed in the 275–284 nm range, corresponding to -13 deg. $\text{cm}^2/\text{decimole}$. Although the tyrosyl residues are intact in the performic acid-oxidized nuclease, the loss of conformation upon oxidation results in diminution and broadening of the circular dichroism. Performic acid-oxidized nuclease, which has been used as a "control" for native nuclease in the present studies, appears to be denatured as judged by measurements of ultraviolet absorption, optical rotatory dispersion, and circular dichroism, yet it retains about 8 per cent of the specific activity of nuclease and has been shown by several techniques to bind deoxythymidine-3',5'-diphosphate in the presence of Ca^{++} .¹⁵

CD of the ligand, deoxythymidine-3',5'-diphosphate: Although the wavelength of maximal absorption of deoxythymidine-3',5'-diphosphate is 267 nm, the peak

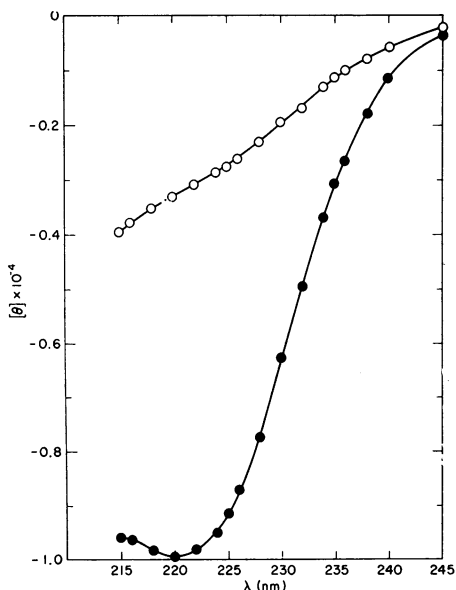


FIG. 1.—Circular dichroism in the 215–245 nm range of 0.1% nuclease (●—●) and performic acid-oxidized nuclease (○—○) in 0.05 *M* Tris-HCl, pH 8.0. The light path length was 0.1 cm.

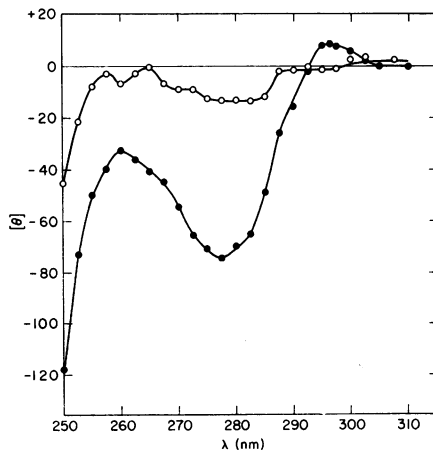


FIG. 2.—CD in 250–310 nm range of nuclease (●—●) and performic acid-oxidized nuclease (○—○). The conditions were the same as those described in Fig. 1, except that the light path length was 1 cm.

of its positive ellipticity band is located at 273 nm (Fig. 3). The observed ellipticity was proportional to the deoxythymidine-3',5'-diphosphate concentration over the range of deoxythymidine-3',5'-diphosphate concentration that was added to nuclease solutions to give molar ratios of 0.5, 1.0, 2.0, and 3.0 to 1, deoxythymidine-3',5'-diphosphate/nuclease. The molecular ellipticity of deoxythymidine-3',5'-diphosphate is +4100 deg. cm²/decimole. For comparison, the equivalent molar ellipticity of nuclease (149 residues) is -11,200 deg. cm²/decimole, or -1600 deg. cm²/decimole tyrosine, averaging over the 7 tyrosyl residues.

Interaction of deoxythymidine-3',5'-diphosphate with nuclease: Figure 4 shows the circular dichroism spectra observed upon addition of several concentrations of deoxythymidine-3',5'-diphosphate to nuclease in the presence of Ca⁺⁺. Interpretation of these spectra is complicated by the overlap of the positive band of deoxythymidine-3',5'-diphosphate (Fig. 3) and the negative band of nuclease (Fig. 2). Difference circular dichroism spectra were, therefore, computed by calculating the corresponding algebraic sum of the independent spectra of nuclease and of deoxythymidine-3',5'-diphosphate in the same buffer, and subtracting these from the observed spectra (Fig. 5). A positive ellipticity difference in the 260–300 nm range clearly results from the interaction of nuclease with deoxythymidine-3',5'-diphosphate.

A plot of the difference in ellipticity at a given wavelength ($\Delta\theta_{280}$) as a function of the molar ratio of ligand to enzyme demonstrates that equimolar concentra-

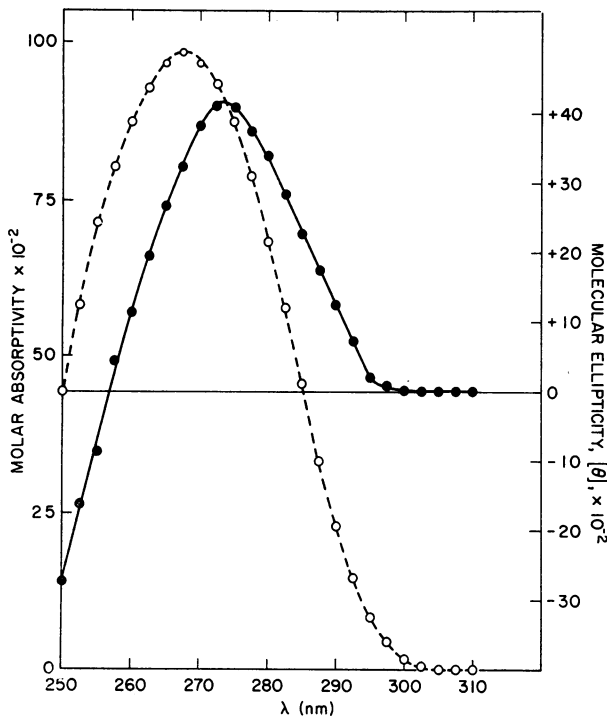


FIG. 3.—Ultraviolet absorption (○--○) and circular dichroism (●--●) of deoxythymidine-3',5'-diphosphate in 0.05 M Tris-HCl, pH 8.0.

tions of the deoxythymidine-3',5'-diphosphate produce 88 per cent of the total $\Delta\theta_{280}$ (Fig. 6). Assuming 1:1 stoichiometry of binding, the dissociation constant is estimated to be 10^{-6} M, in the same range as the values estimated from inhibition of enzymatic activity,¹⁸ gel filtration,¹⁹ quenching of tyrosyl fluorescence,⁷ and difference ultraviolet spectroscopy.⁶

As indicated by other methods, binding of deoxythymidine-3',5'-diphosphate to nuclease is dependent upon Ca^{++} concentrations and is subject to the complex influences of pH upon tyrosyl ionization, deoxythymidine-3',5'-diphosphate ionization, and enzyme conformation.⁵⁻⁷ With CD, a 1:1 deoxythymidine-3',5'-diphosphate/nuclease mixture in the absence of added Ca^{++} gave a $\Delta\theta_{280}$ nearly as large as that described above in the presence of added Ca^{++} . However, addition of 2×10^{-3} M EDTA reduced this difference by 50% and subsequent addition of Ca^{++} to final concentration of 10 mM fully restored the difference circular dichroism spectrum to that observed in the absence of EDTA (Fig. 7).

Addition of deoxythymidine-3',5'-diphosphate to performic acid-oxidized nuclease in 1:1 and 3:1 molar ratios produced only a very small difference ellipticity. Higher ratios could not be used because of the absorbancy of the nucleotide. Similar concentrations of deoxythymidine-3',5'-diphosphate (about 10^{-4} M) have been shown to bind to performic acid-oxidized nuclease by several methods.¹⁵

Solvent perturbation of the CD of deoxythymidine-3',5'-diphosphate: The peak ellipticity was enhanced by 40 per cent dioxane (v/v), 20 per cent ethylene glycol (v/v), and 40 per cent ethanol (v/v) of deoxythymidine-3',5'-diphosphate by 5-20

per cent (Fig. 8), but none of these solvents shifted the peak or altered its shape significantly, and none gave a difference spectrum in tandem, paired-cell, ultraviolet-absorption studies.

Discussion.—The finding, in these studies of the circular dichroic properties of staphylococcal nuclease, that the tyrosyl residues generate significant optical activity with a molecular ellipticity at 277 nm of -1600 deg. $\text{cm}^2/\text{decimole}$ tyrosine, correlates with the abundant evidence that the properties of certain tyrosyl residues depend upon the uniquely ordered structure of the native protein conformation. Presumably this asymmetric environment is almost completely lacking in the performic acid-oxidized derivative, the circular dichroism spectrum of which has only a broad, shallow, negative ellipticity in the same wavelength range.

Negative ellipticity bands at neutral pH in the 270–280 nm range, presumably originating from tyrosyl residues, have been reported for several proteins, including RNase,^{1, 20–21} human carbonic anhydrase B,²² and β -lactoglobulin.²³ Positive dichroism at 275 nm was observed for L-tyrosine and helical polytyrosine,²⁴ and for phenolic diketopiperazines.²⁵

FIG. 5.—Difference CD spectra resulting from the interaction of 58 μM nuclease with 10 mM Ca^{++} and pdTp added in a 0.5 (∇ --- ∇), 1-(\bullet — \bullet), 2-(Δ --- Δ), and 3-(\circ — \circ) fold molar excess to nuclease. These curves are calculated from the observed spectra shown in Fig. 4 by subtracting the algebraic sum of the individual spectra of nuclease and of pdTp obtained under the same conditions.

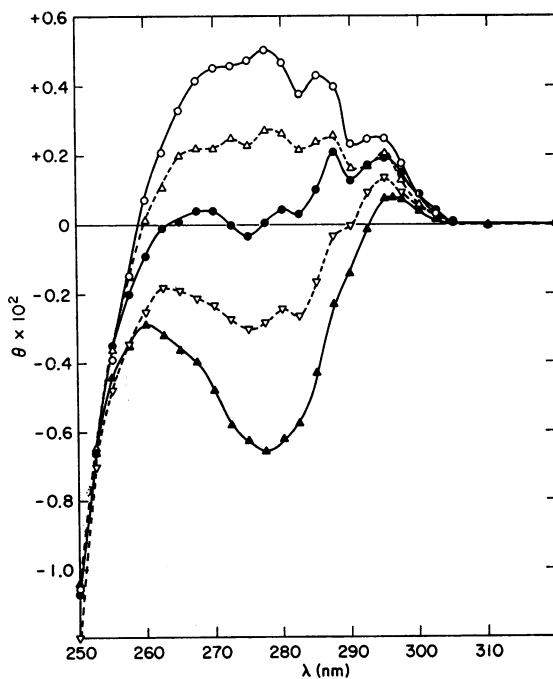
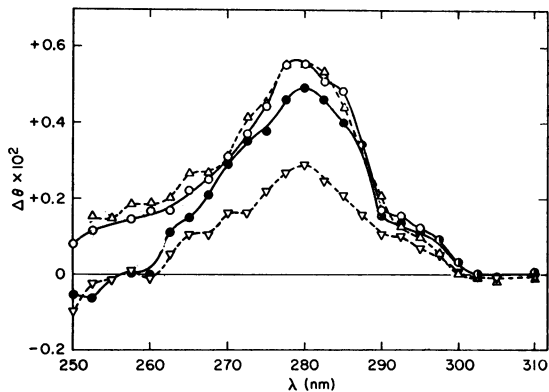


FIG. 4.—Observed ellipticity, θ , of 58 μM nuclease (\blacktriangle — \blacktriangle) in 0.05 M Tris-HCl pH 8.0, 0.01 M Ca^{++} , and upon addition of pdTp in molar ratios 0.5:1 (∇ --- ∇), 1:1 (\bullet — \bullet), 2:1 (Δ --- Δ) and 3:1 (\circ — \circ).



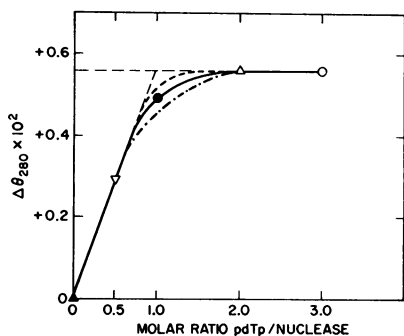


FIG. 6.—Plot of the difference in ellipticity at 280 nm vs. the molar ratio of pdTp to nuclease. The dashed straight lines indicate the line of theoretical linearity for 1:1 stoichiometry of binding and the maximal $\Delta\theta_{280}$ obtained upon titration of the enzyme with ligand. The estimated K_I (see text) is $10^{-6} M$. The value for molar ratio 1.0 is the average of 9 determinations, with a range of 0.46 to 0.52×10^{-2} degree for $\Delta\theta_{280}$. This range is bounded by the dashed curves corresponding to K_I values of $0.3 \times 10^{-6} M$ (---) and $3 \times 10^{-6} M$ (-·-·-). The nuclease concentration was $58 \mu M$.

A positive ellipticity band, probably reflecting the dichroic activity of the tryptophan residue, is observed at 296 nm in the circular dichroism spectrum of native nuclease. Although previous studies indicate that the fluorescence and ultraviolet-absorbing properties of the single, buried, tryptophan residue of nuclease are unaffected by nucleotide binding, the small increase in ellipticity in the 290–296 nm region may represent a minor change in the environment of the tryptophan residue which is undetectable by the other methods. The positive ellipticity band observed in the native enzyme at 296 nm is absent in performic acid-oxidized nuclease. Preliminary circular dichroism studies¹⁵ with H_2O_2 -treated nuclease, in which the methionines are oxidized to sulfoxide and in which the tryptophan appears to be intact, indicate that disorganization of the conformation alone can eliminate the 296-nm band. The assignment of the 296-nm band of native nuclease to tryptophan is in keeping with the observations of a 296-nm band in human carbonic anhydrase B²² and of bands at 285 and 293 nm, plus a shoulder at longer wavelength, in β -lactoglobulin.²³

Specific binding of deoxythymidine-3',5'-diphosphate to the active site of nuclease produces a large and easily measured difference in the near-ultraviolet circular dichroism spectrum, but no detectable difference in the far-ultraviolet peptide band; this agrees with the view that the nucleotide interacts with tyrosyl residues without a major conformational change in the protein.^{5, 7, 8} Because of the overlap of the nucleotide and protein circular dichroism bands, it is not clear whether the near-ultraviolet, positive-difference circular dichroism resulting from the interaction reflects elimination of the negative ellipticity of the aromatic residues of the enzyme or marked enhancement of the positive ellipticity of the nucleotide, or both. Nearly 90 per cent of the maximal difference ellipticity can be observed with a 1:1 ratio of deoxythymidine-3',5'-diphosphate/nuclease (Fig. 5). At the concentrations used, the individually observed ellipticities at 280 nm are $+0.18 \times 10^{-2}$ deg. for deoxythymidine-3',5'-diphosphate and -0.63×10^{-2} deg. for the nuclease. The $\Delta\theta_{280}$ is $+0.49 \times 10^{-2}$ deg., which could be accounted for by a 78 per cent decrease in the value of the nuclease ellipticity but which would require a 272 per cent enhancement of the nucleotide ellipticity. It is reasonable to suspect that perturbations of the tyrosyl residues of nuclease contribute to the observed ellipticity difference, since 2 or 3 of them interact directly with the nucleotide.^{6, 7}

Changes in the ultraviolet spectrum of deoxythymidine-3',5'-diphosphate

upon binding to nuclease were interpreted to indicate that the nucleotide chromophore enters a more hydrophobic environment during binding.⁶ However, decreased environmental polarity of the nucleotide in the enzyme-inhibitor complex cannot explain the observed ellipticity difference, since solvents of low dielectric constant caused only minor changes in the circular dichroism spectrum of deoxythymidine-3',5'-diphosphate (Fig. 8). In recent work with model diketopiperazines containing tyrosine,²⁶ nonpolar solvents caused a blue shift in the absorption and circular dichroism spectra, but no change in sign or magnitude of the ellipticity. Evidence from the emerging X-ray picture¹² indicates that the rings of the thymidine and of a tyrosine residue lie in parallel planes, compatible with π - π interaction, rather than simply in a hydrophobic environment.

Difference ellipticity measurements can be used as a quantitative parameter of nuclease binding of deoxythymidine-3',5'-diphosphate giving an estimate of the K_I of about 10^{-6} M, in good agreement with other methods. Clearly, however, technical and arithmetical difficulties make this method less attractive than either quenching of tyrosyl fluorescence,⁷ where the nucleotide makes no contribution of its own, or ultraviolet difference absorption,⁶ where the tandem-cell technique allows direct measurement of difference spectra. Other nucleotides with less overlap of circular dichroism bands bind so weakly to nuclease that they could not be used in sufficient concentration because of excessive ultraviolet absorption. As noted in NMR studies of nuclease,¹¹ the present studies detected interaction

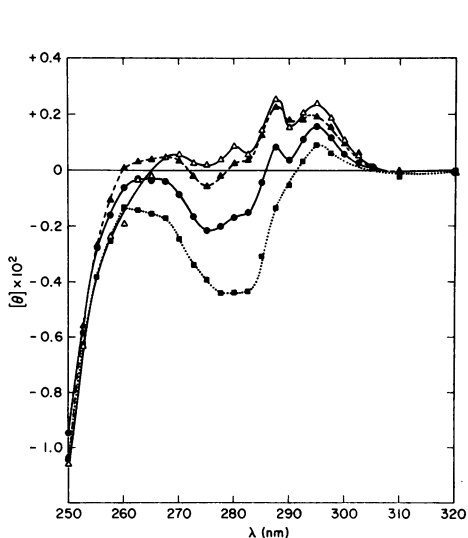


FIG. 7.—Observed ellipticity for nuclease-pdTp complex in 1:1 molar ratio without added Ca^{++} (▲---▲), upon addition of EDTA, 2×10^{-3} M (●---●), and after addition of 0.01 M CaCl_2 (Δ---Δ). For reference, the calculated algebraic sum of the individual CD spectra of pdTp and nuclease in concentrations is given (■...■).

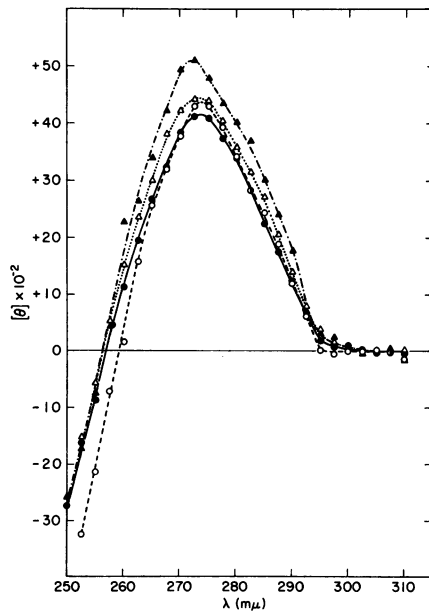


FIG. 8.—Molecular ellipticity of pdTp in 0.05 M Tris-HCl , pH 8.0 (●---●) and in mixtures of Tris buffer with 40% (v/v) dioxane (○---○), 20% (v/v) ethylene glycol (Δ...Δ), and 40% (v/v) ethanol (▲---▲).

of nucleotide with nuclease in the absence of added Ca^{++} , a cation thought to be essential to the binding process.⁵ However, these effects result from the presence of Ca^{++} in the reagents, as demonstrated by the reversal of the circular dichroism effects upon addition of EDTA (Fig. 7). Similar effects of EDTA were observed in tritium exchange experiments of the interaction of deoxythymidine-3',5'-diphosphate with nuclease.²⁶ Ca^{++} does not affect the circular dichroism of either nuclease or deoxythymidine-3',5'-diphosphate alone.

The measurement of circular dichroism has not hitherto been a sensitive indication of the effect of binding of ligands upon specific types of residues. Binding of biotin by avidin (containing 14 tryptophanyl and 4 tyrosyl residues) produced no difference in circular dichroism,²⁷ and addition of 0.25 M *N*-acetylglucosamine to lysozyme simply increased the positive ellipticity of 3 overlapping peaks between 280 and 282 nm.²⁸

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