Immunochemistry of Staphylococcal Nuclease. I. Physical, Enzymatic, and Immunological Studies of Chemically Modified Derivatives*

Gilbert S. Omenn,† David A. Ontjes,‡ and Christian B. Anfinsen

ABSTRACT: Chemically modified derivatives were prepared and characterized for use in studies of the antigenicity of staphylococcal nuclease. Performic acid oxidized nuclease appeared to be denatured by spectroscopic methods, yet retained about 8% of the deoxyribonuclease and ribonuclease activity, as well as the ability to bind the inhibitor deoxythymidine 3′,5′-diphosphate. This derivative cross-reacts fully with native nuclease, when tested against antibody elicited by either antigen.

A series of acetylated and trifluoroacetylated derivatives exhibited relatively less alteration of conformation than did the performic acid oxidized nuclease. The fluorescence emission of the intact tryptophan residue was especially sensitive to the conformation of this set of derivatives. These derivatives had an average of 11, 18, and 23 of the 23 ε-amino groups modified, drastically changing the surface charge of the protein. With increasing extent of substitution, enzymatic and antigenic activities decreased in parallel. The antigenic cross-reactivity and enzymatic properties of the performic acid oxidized derivative are consistent with the view that staphylococcal nuclease, lacking disulfide bridges and having low helix content, is a highly motile or flexible polypeptide chain.

Staphylococcal nuclease is a well-characterized extracellular enzyme (Taniuchi and Anfinsen, 1968; Cuatrecasas *et al.*, 1968b), consisting of a single polypeptide chain of 149 amino acid residues and lacking any disulfide bridges. Fuchs *et al.* (1969) have reported the production in rabbits of antisera against nuclease that both inhibit the enzymatic function and precipitate with nuclease.

In their recent review, Sela *et al.* (1967) emphasized the generalization from early immunochemical studies (Landsteiner, 1945) that denatured globular proteins react poorly or not at all with antibodies to the native protein. Furthermore, fragments of the amino acid sequence usually are poor inhibitors of the reaction between the native protein and its antibody, leading to the conclusion that protein conformation is essential for antibody recognition and binding. With several well-characterized protein antigens, such as pancreatic ribonuclease (Brown *et al.*, 1959), lysozyme (Arnon and Sela, 1969), and myoglobin (Crumpton and Wilkinson, 1965; Atassi and Saplin, 1968), immunochemical studies have led to inferences about the relative contribution of conformation to antigenicity and about linear sequences which may contain antigenic determinants.

In characterizing the antigenicity of nuclease, we have attempted to distinguish the roles of conformation and of linear sequence determinants and then to identify antigenic regions in the sequence. This paper compares the physical, enzymatic, and immunological features of native nuclease with nuclease derivatives greatly modified in surface charge without marked change in conformation, and with a performic acid oxidized derivative that lacks the conformational characteristics of the native enzyme, but has relatively little alteration of amino acid residues. The companion paper reports studies which implicate specific portions of the linear sequence as antigenic (Omenn *et al.*, 1970).

Materials and Methods

Materials. Highly purified nuclease was prepared as previously described (Mofavek *et al.*, 1969). Formic acid (99%) was obtained from Eastman, hydrogen peroxide (30%) from Baker and Adamson, ethyl trifluorothioacetate from Pierce, and *N*-acetylimidazole from Aldrich.

Performic Acid Oxidation of Nuclease. Using the method of Hirs (1967), 5 volumes of 30% hydrogen peroxide and 95 volumes of 99% formic acid were allowed to react at room temperature for 120 min. Nuclease (20 mg) was dissolved in 0.5 ml of 99% formic acid and 0.1 ml of methanol at 0°, and 0.3 ml of the performic acid solution (estimated to be a tenfold excess based on methionine) was added. After standing at 0° for 2 hr, the protein solution was diluted with 40 ml of ice water and lyophilized twice to give 21 mg of white powder.

Acetylation of Nuclease. Nuclease (30 mg) was dissolved in 15 ml of 0.01 M Tris-HCl (pH 7.5), and 0.4 ml of 0.5 M acetylimidazole (freshly dissolved in the same buffer) was added to give an estimated 100-fold molar excess (Sokolovsky *et al.*, 1966; Cuatrecasas *et al.*, 1968a). The reaction at 25° was followed by assay of aliquots for enzymatic activity, which decreased to 11% at 30 min, to 2% at 60 min, and to 0.2% at 90 min. Upon dialysis against water at 5°, the nuclease derivative precipitated. About 30% of the acetylated product was suspended in water, and titrated to pH 11.5 to remove any tyrosyl-*O*-acetyl groups. Enzymatic activity was increased slightly to 2% of the initial activity after standing at this pH for 4 hr.

* From the Laboratory of Chemical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received September 24, 1969.*

† Present address: Departments of Genetics and Medicine, University of Washington, Seattle, Wash.

‡ Present address: Department of Medicine, University of North Carolina, Chapel Hill, N. C.

TABLE I: Amino Acid Composition of Derivatives of Nuclease.[a]

Amino Acid	Amino Acid Composition[b] (residues/mole)			
	Theoretical[c]	Performic Acid Oxidized Nuclease	Heavily Acetylated Nuclease	Trifluoroacetylated Nuclease
Lys	23	26.5	24.0	28
His	4	4.9	3.8	4.2
Arg	5	5.3	4.7	4.9
Asp	14	14.8	14.9	13.7
Thr	10	9.7	9.8	10.0
Ser	5	4.9	4.7	5.0
Glu	18	17.2	18.3	17.8
Pro	6	6.0	5.9	6.6
Gly	10	10.2	10.1	9.2
Ala	14	12.4	13.6	12.0
Cys (1/2)	0	0	0	0
Val	9	6.7	7.7	7.6
Met	4	0.2	5.8	3.4
Ile	5	5.8	5.0	4.9
Leu	11	11.2	11.5	10.6
Tyr	7	6.9	6.9	6.2
Phe	3	3.0	3.4	3.1
Trp[d]	1	0	1	1
Met-sulfone	0	3.3	0	0

[a] Results obtained on 20-hr acid hydrolysates. [b] By the method of Spackman *et al.* (1958). [c] Taniuchi *et al.* (1967a); Cusumano *et al.* (1968). [d] Estimated by fluorescence emission and by extinction coefficient.

The product was then dialyzed exhaustively against water and lyophilized to yield 12 mg. Indirect determination of the number of acetylated lysines by the nitrous acid deamination method (Cooke *et al.*, 1963) indicated that an average of 18 of the 23 lysine residues in the product has been acetylated. This derivative is designated "heavily acetylated" nuclease.

In order to prepare a "lightly acetylated" nuclease derivative, 20 mg of nuclease was dissolved in 10 ml of 0.01 Tris (pH 7.5), and reacted with 0.1 ml of 0.5 M acetylimidazole (a 40-fold excess) at 25°. Enzymatic activity fell more slowly, reaching a minimum of 40% after 60 min. The reaction was stopped by dialysis against water. Incubation at pH 11.5 for 1 hr failed to generate higher activity. Analysis of the product by deamination indicated an average of 11 acetylated lysine residues/molecule.

Trifluoroacetylation of Nuclease. By the method of Goldberger and Anfinsen (1962), 30 mg of nuclease was dissolved in 7 ml of water and brought to pH 10.0 with 1 M KOH in a pH-Stat apparatus. Ethyl trifluorothiolacetate (0.4 ml) was added, and pH was maintained at 9.9–10.0 by addition of KOH solution while stirring at 25° for 1 hr. Addition of several drops of 1 M acetic acid brought the pH to 5 and produced a bulky precipitate. The suspension was dialyzed against four changes of 0.05 M acetic acid and then lyophilized, with a yield of 28 mg. All 23 of the lysine residues were trifluoroacetylated, as determined by the deamination procedure.

Antisera. Antibodies to nuclease were elicited in four rabbits by subcutaneous injection of nuclease (1%) in saline emulsified with an equal volume of Freund's complete adjuvant. After repeat immunizations on day 21 and on day 28, the rabbits were bled repeatedly between day 31 and day 43. Serum from each animal was pooled separately, and a γ-globulin fraction was isolated by repeated precipitation in 40% saturated ammonium sulfate, followed by dialysis against 0.9% saline and dilution to the original volume of antiserum. The γ-globulin from one animal (γG 14) was used.

Antibodies against performic acid oxidized nuclease were elicited in two rabbits by an identical immunization schedule, except that the performic acid oxidized derivative was used as the immunogen. The γ-globulin fractions were isolated as above, and the fraction obtained from one animal (γG 15) was used.

Spectroscopic Methods. Ultraviolet absorption spectra were obtained with a Cary 15 recording spectrophotometer, and single-wavelength absorbance readings with a Zeiss PMQII spectrophotometer. Fluorescence studies were performed with an Aminco-Bowman spectrofluorometer, using a 0.3-ml quartz cuvet at 25°. The instrument was equipped with an RCA 1P28 photomultiplier tube and a grating blazed at 300 mμ, giving an essentially linear response over the 280–400-mμ range (Chen, 1967). The excitation beam was passed through a horizontally polarizing filter to minimize scatter, and the unfiltered emission was recorded by an American Instrument Co. X-Y recorder.

Optical rotatory dispersion and circular dichroism were measured with a Cary 60 recording spectropolarimeter equipped with a 6001 circular dichroism attachment. The mean residue weights were calculated on the basis of the sequence and the chemical modifications introduced. All studies were carried out in distilled water (pH 6–7), at 27°. The sample solu-

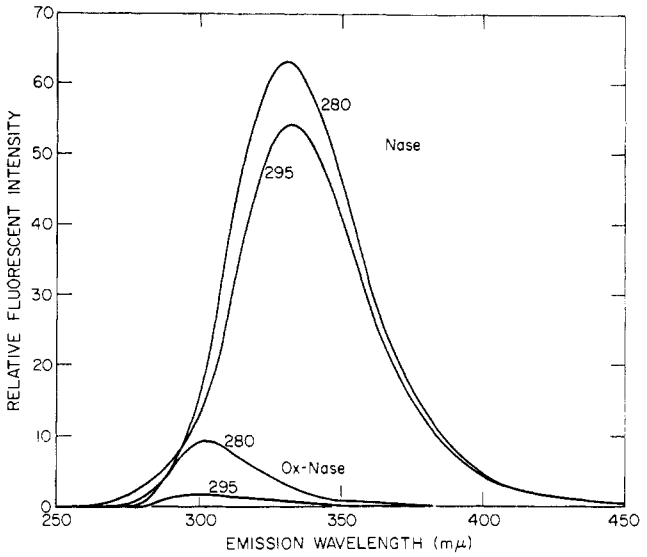

FIGURE 1: Fluorescence emission spectra of nuclease (Nase) and of performic acid oxidized nuclease (OX-nase) upon excitation at 280 mμ or at 295 mμ, as indicated. Concentrations were 58 μM in 0.05 M Tris-HCl, pH 8.0.

tion, in a 3.1-ml quartz cuvet of 10-mm or a 2.6-ml cuvet of 1-mm path length, was placed in the cell holder and equilibrated for about 15 min before measurements. Observed rotation or ellipticity, in degrees, was converted into mean residue rotation or to molecular ellipticity (in deg cm²/dmole) by standard methods (Beychok, 1967).

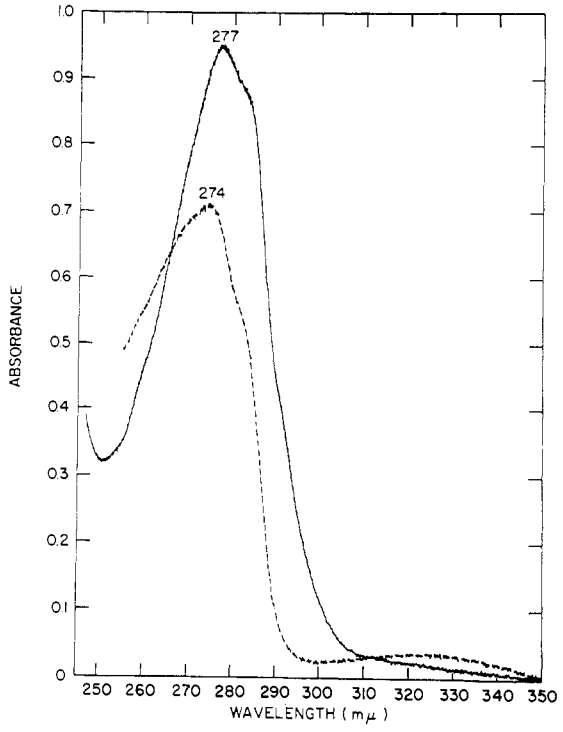

FIGURE 2: Ultraviolet absorption spectra of nuclease (—) and of performic acid oxidized nuclease (– – –) at neutral pH, 58 μM, with maxima at 277 mμ for nuclease and at 274 mμ for the derivative.

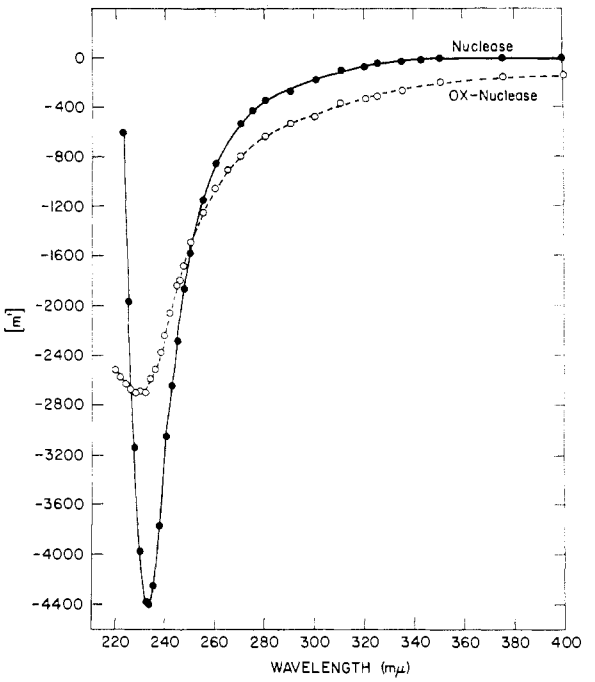

FIGURE 3: Optical rotatory dispersion spectra of nuclease (●—●) and of performic acid oxidized nuclease (OX-nuclease, ○—○), pH 7.

Immunochemical Methods. γ Globulin fractions were evaluated by double immunodiffusion (Ouchterlony, 1949) in Hyland immunoplates, and by quantitative precipitin reactions. The latter were performed by incubating 0.15–0.3-ml aliquots of γ-globulin solution with increasing amounts of antigen in 0.145 M NaCl containing 0.05 M Tris-HCl (pH 7.5), in a total volume of 1.0 ml. After incubation for 1 hr at 37° and 24 hr at 4°, the precipitates were centrifuged, washed three times with ice-chilled buffer solution, and dissolved in 0.1 N NaOH. The optical densities of the dissolved precipitates were measured at 280 mμ.

Enzymatic Assays. Nuclease and derivatives were tested for enzyme activity with DNA and RNA as substrates in the spectrophotometric assay of Cuatrecasas *et al.* (1967a).

Results

Physical and Chemical Characterization of the Modified Derivatives. The spectral data for native nuclease are in general agreement with previously published observations, including the estimate of 18% helix content by optical rotatory dispersion and circular dichroism (Taniuchi and Anfinsen, 1968) and the postulation of a buried, hydrophobic environment for the single tryptophan residue from fluorescence and ultraviolet absorption studies (Taniuchi and Anfinsen, 1969; Cuatrecasas *et al.*, 1967b).

In the performic acid oxidized derivative, the four methionine residues were converted nearly quantitatively into the sulfones (Table I). The value of 0.2 mole of methionine/mole of protein represents an average of results of 0.0 and 0.4 obtained for two different preparations which gave identical conformational, enzymatic, and immunochemical results. There was no loss of tyrosyl or other residues, based on the results

TABLE II: Summary of Physical, Enzymatic, and Immunological Properties of Nuclease and Its Derivatives.

Derivative	Ultraviolet Absorption		Tryptophan Fluorescence				Enzymatic Act. (%)	Immunological Reactivity	
	λ_{max}	$E^{0.1\%}_{\lambda max}$	λ_{max}	Relative Intensity (%)	$[m']_{233}$	$[\theta]_{220}$		Immunodiffusion in Agar	Maximal Precipitin Reaction (%)
Nuclease	277	0.97	331	100	−4400	−9950	100	++	100
Performic acid oxidized nuclease	274	0.70	None	None	−2640	−3300	6–8	++	96
Lightly acetylated nuclease	277	0.97	331	100	−3860	−8000	~30	++	50
Heavily acetylated nuclease	277	0.97	336	66	−3580	−6980	2	+	<10
Trifluoroacetylated nuclease	277	0.90	341	22	−3980	−6900	0	0	0

of amino acid analysis. The single tryptophan chromophore was quantitatively oxidized. Fluorescence emission spectra (Figure 1) upon excitation at 280 mμ showed only a small tyrosyl fluorescence, which gave a smoothly gaussian peak centered at 303 mμ when recorded at higher photomultiplier gain. Excitation at 295 mμ, to exclude the contribution of tyrosyl residues, gave no detectable fluorescence, even at a gain twenty times that shown in Figure 1.

Disorganization of the conformation of the native enzyme occurred in the production of the oxidized derivative. The blue shift of the ultraviolet absorbance maximum to 274 mμ parallels the effect of acid denaturation upon native nuclease absorbance (Figure 2). The decrease in the absorption coefficient of the oxidized derivative reflects the proportionate role of the single tryptophanyl residue in the ultraviolet absorbance of nuclease (Table II). The optical rotatory dispersion spec-

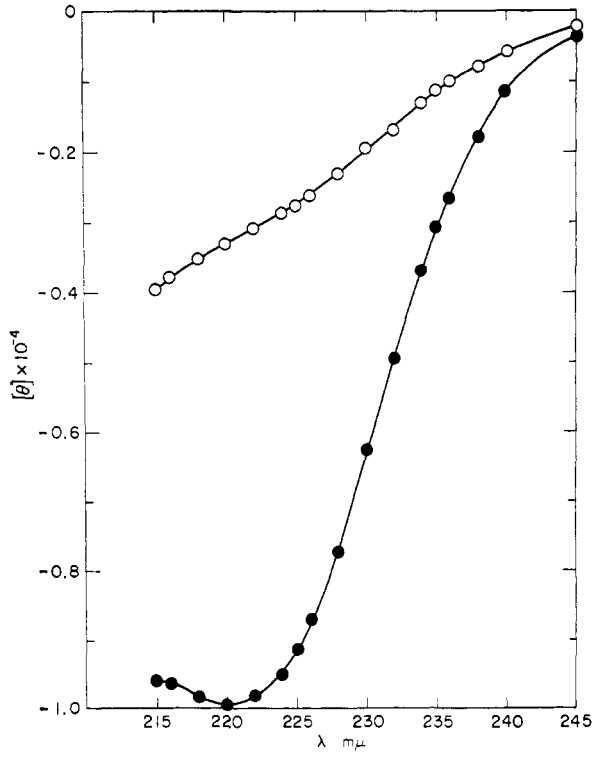

FIGURE 4: Ultraviolet circular dichroism spectra in the 245–215-mμ range of nuclease (●—●) and of performic acid oxidized nuclease (○—○), pH 7.

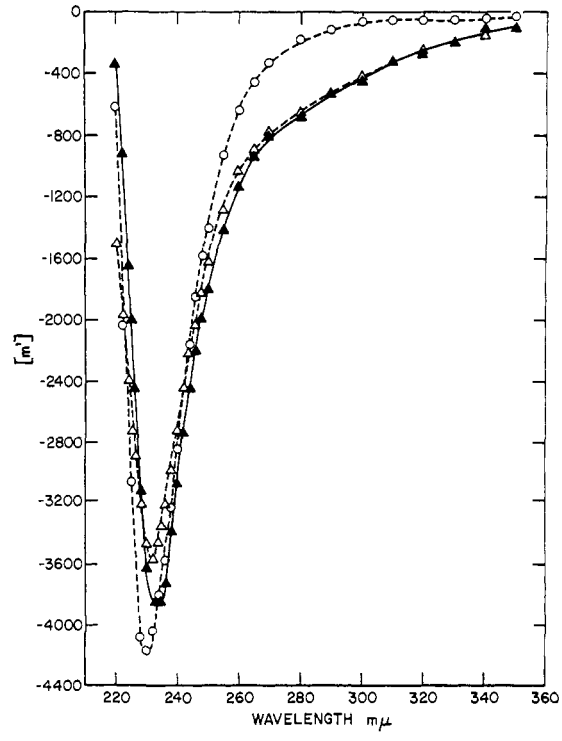

FIGURE 5: Optical rotatory dispersion spectra of "lightly acetylated" nuclease (▲—▲), "heavily acetylated" nuclease (△—△), and trifluoroacetylated nuclease (○—○), pH 7.

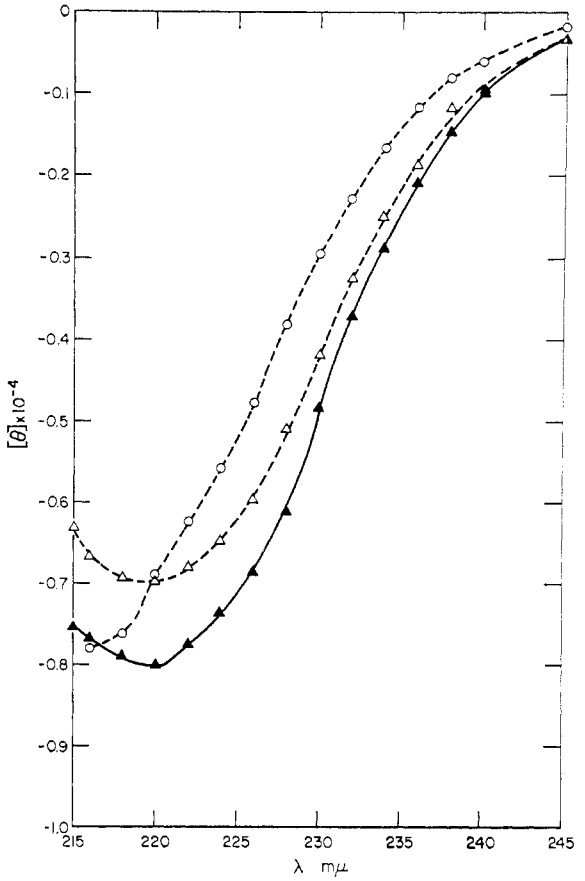

FIGURE 6: Ultraviolet circular dichroism spectra in the 245–215-mμ range of "lightly acetylated" nuclease (▲—▲), "heavily acetylated" nuclease (△—△), and trifluoroacetylated nuclease (○—○), pH 7.

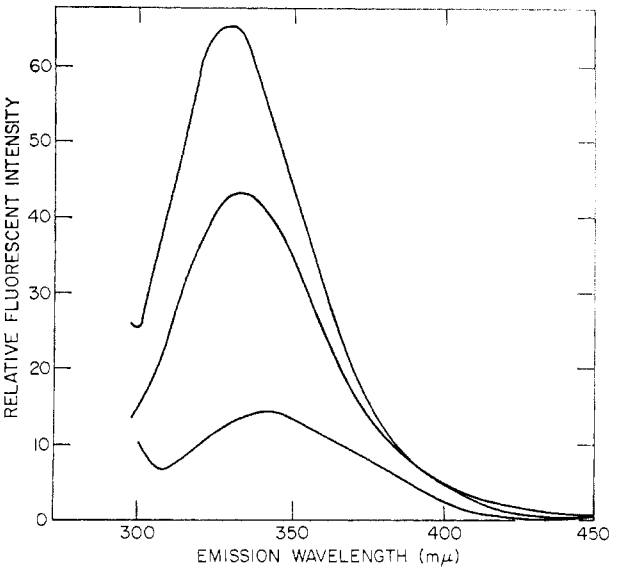

FIGURE 7: Fluorescence emission spectra upon excitation at 295 mμ of "lightly acetylated" nuclease (top curve), "heavily acetylated" nuclease (middle curve), and trifluoroacetylated nuclease (bottom curve), pH 7, 58 μM.

trum (Figure 3) gives evidence of little more peptide-bond transition optical activity than that of random synthetic polypeptides. In the circular dichroism spectrum (Figure 4) the characteristic trough at about 220 mμ is lost entirely, with a gradually increasing negative ellipticity in the same range and of the same magnitude as that observed with the structureless nuclease fragments P_2 and P_3 (Taniuchi and Anfinsen, 1968). The aromatic circular dichroism band centered at 277 mμ is also lost in this derivative (Omenn *et al.*, 1970).

The lightly acetylated and heavily acetylated derivatives of nuclease were found to have normal extinction coefficients and fluorescence intensities, confirming the expectation that treatment with base removed any tyrosyl-*O*-acetyl groups. Both derivatives gave ultraviolet absorption spectra indistinguishable from nuclease. Some change in conformation is apparent, however, in the lightly acetylated nuclease, with significant diminution of $[m']_{233}$ and of $[\theta]_{220}$ in optical rotatory dispersion and circular dichroism spectra, respectively (Figures 5 and 6). Nevertheless, the fluorescence spectrum (Figure 7) closely resembles that of nuclease, and the main features of the spectra are qualitatively maintained. Heavily acetylated nuclease shows more marked conformational alteration in both optical rotatory and circular dichroism spectra, and the decreased quantum yield and red shift of the tryptophan fluorescence to 336 mμ serves as an especially sensitive marker of conformational change.

The ultraviolet spectrum of the completely trifluoroacetylated nuclease was unchanged from that of the acetylated derivatives and of native nuclease. The small decrease in extinction coefficient (Table II) is accounted for almost entirely by the increase in molecular weight, so that the molar absorptivity is approximately the same. The tryptophanyl fluorescence emission is decreased greatly in intensity and shifted further to 341 mμ (Figure 7). In free tryptophan and in the nuclease fragment P_3, in which tryptophan is freely exposed to aqueous solvent (Taniuchi and Anfinsen, 1969), the tryptophan emission maximum occurs at 348 mμ. Thus, the tryptophan residue in trifluoroacetylated nuclease may still be incompletely accessible to solvent. The optical rotatory dispersion and circular dichroism spectra support the view that considerable organized structure and conformational asymmetry persist in this derivative, despite the drastic alteration of surface charge. The $[m']_{233}$ is reduced from that of nuclease by only 10%, and the trough is shifted to 230 mμ. In the rather anomalous circular dichroism spectrum, negative ellipticity equivalent to that of the heavily acetylated nuclease at 220 mμ develops, but the form of the curve is clearly different, suggesting that asymmetry may have resulted from unusual conformational properties.

Enzymatic Activity of the Modified Derivatives. With the acetylated and trifluoroacetylated derivatives, DNase activity declined progressively with increasing degree of substitution. With an average of 11 lysyl ε-amino groups acetylated, activity fell to about 30%, with 18 acetylated to 2%, and with all 23 trifluoroacetylated no activity could be detected. By contrast, the far more drastic alteration of conformation of the performic acid oxidized nuclease still permitted a residual enzyme activity of 6–10%, in both the DNA and RNA assays.

The oxidized derivative resembled native nuclease in its stability to heating at 100° for 15 min or to exposure to 0.1 M HCl for the same time. The derivative was inhibited to a comparable degree by 10^{-4} M deoxythymidine 3′,5′-diphos-

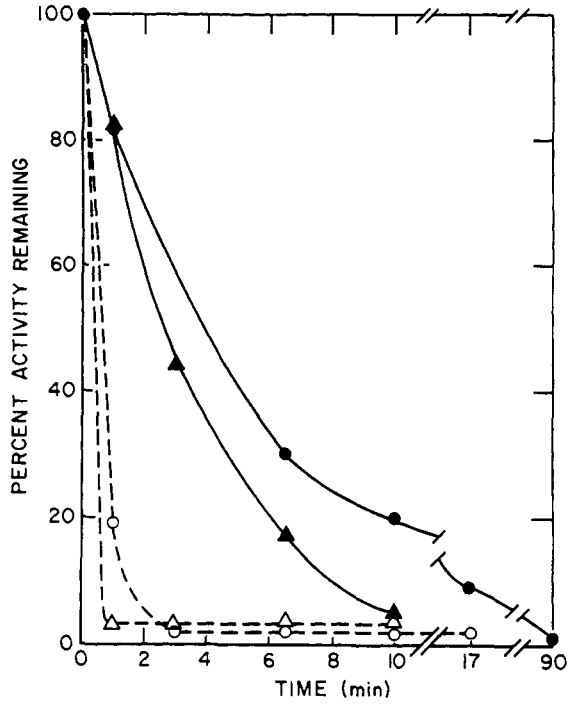

FIGURE 8: Inactivation of DNase activity of performic acid oxidized nuclease by tryptic digestion. Weight ratios of 10% trypsin in the absence (△—△) and presence of 10^{-4} M deoxythymidine 3′,5′-diphosphate and 10^{-2} M $CaCl_2$ (▲—▲) and of 1% trypsin in the absence (○—○) and presence (●—●) of these ligands were used. The buffer was 0.05 M Tris-HCl, pH 8.0, at 25°.

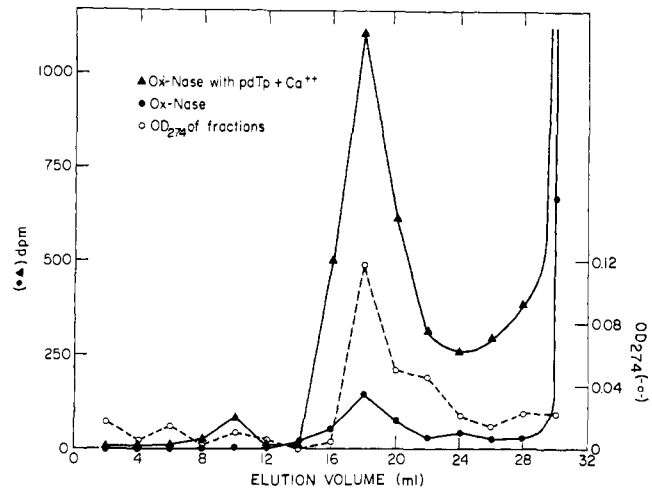

FIGURE 9: Gel filtration of tritiated performic acid oxidized nuclease (0.8 mg) in the absence (●—●) and in the presence (▲—▲) of 6×10^{-4} M deoxythymidine 3′,5′-diphosphate and 10^{-2} M $CaCl_2$–0.05 M Tris-HCl, (pH 8.1) on a Sephadex G-25 column (2 × 15 cm). The elution of protein is indicated by the absorbance at 274 mμ (○- - -○). Flow rates were 1 ml/min.

phate. To rule out activity due simply to contamination of the derivative with native nuclease, several further studies were carried out.

Affinity Chromatography of Performic Acid Oxidized Nuclease on Deoxythymidine 3′,5′-Diphosphate–Sepharose. Cuatrecasas *et al.* (1968c) have described the reversible adsorption of nuclease to a column of Sepharose to which the ligand deoxythymidine 3′,5′-diphosphate has been covalently bound. Certain inactivated nuclease derivatives do not bind to such a column, affording a means of separating active and inactive forms of the enzyme (Cuatrecasas *et al.*, 1969). To a 0.5 × 5 cm deoxythymidine 3′,5′-diphosphate–Sepharose column was applied 1.6 mg of performic acid oxidized nuclease in 1.0 ml of 0.05 M borate buffer (pH 8.0) and 0.01 M $CaCl_2$. Upon elution with buffer, no protein was recovered. All of the applied protein was recovered promptly by subsequent elution with (pH 11) NH_4OH solution. The specific activity of the recovered material was the same as that originally applied. Since the oxidized derivative clearly binds to the affinity column, however, these results cannot rule out contamination with native enzyme.

Protection by Ligands against Trypsin Digestion of Performic Acid Oxidized Nuclease. Susceptibility to digestion by trypsin was tested by adding trypsin to solutions of performic acid-oxidized nuclease in the presence and absence of Ca ions and deoxythymidine 3′,5′-diphosphate (Figure 8). Weight ratios of trypsin to protein of 10 and 1% were used. The ligands afforded striking protection to the nuclease derivative at both the high and low ratios of trypsin addition. It should be noted, however, that after 20 hr the oxidized derivative was completely inactivated, even in the presence of ligands. With native nuclease, 8–10% of the original activity remains after a similar incubation (Taniuchi *et al.*, 1967b). These results are consistent with a more flexible structure for the liganded performic acid oxidized derivative than for the comparable native complex.

Tritium Exchange of Performic Acid Oxidized Nuclease. The oxidized derivative was tritium labeled by equilibration at 4° for 5 days with 1.0 mCi of tritiated water as described by Schechter *et al.* (1968). With exactly the same conditions and column separation method described for nuclease, performic

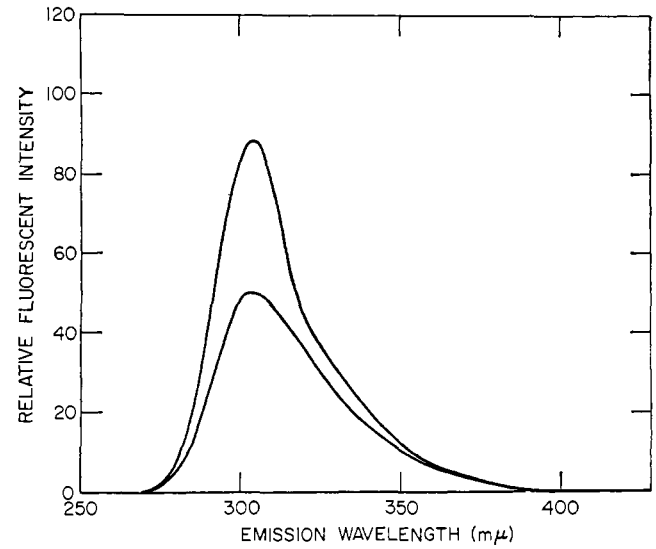

FIGURE 10: Fluorescence emission spectra upon excitation at 280 mμ of performic acid oxidized nuclease (58 μM) in the absence (top curve) and in the presence (bottom curve) of 170 μM deoxythymidine 3′,5′-diphosphate in 0.5 M Tris-HCl (pH 8.0)–0.01 M $CaCl_2$. The fluorescent intensity was corrected for absorbance by the nucleotide.

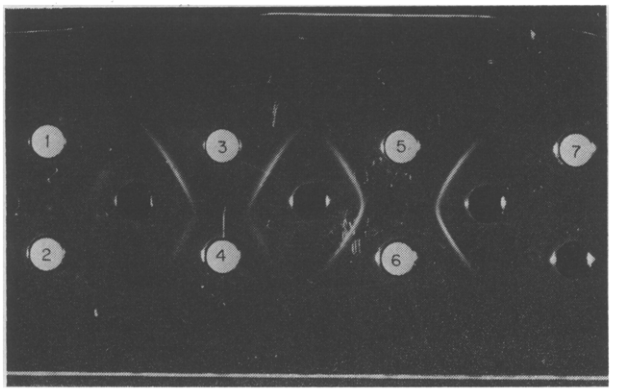

FIGURE 11: Agar immuno-double-diffusion experiments at 25° with antinuclease γ-globulin preparation in center wells and with (1) "heavily acetylated" nuclease, (2) trifluoroacetylated nuclease, (3) native nuclease, (4) performic acid oxidized nuclease, (5) "lightly acetylated" nuclease, (6) native nuclease again, and (7) a cyanogen bromide digest of nuclease, all in the concentration range 200–500 μg/ml.

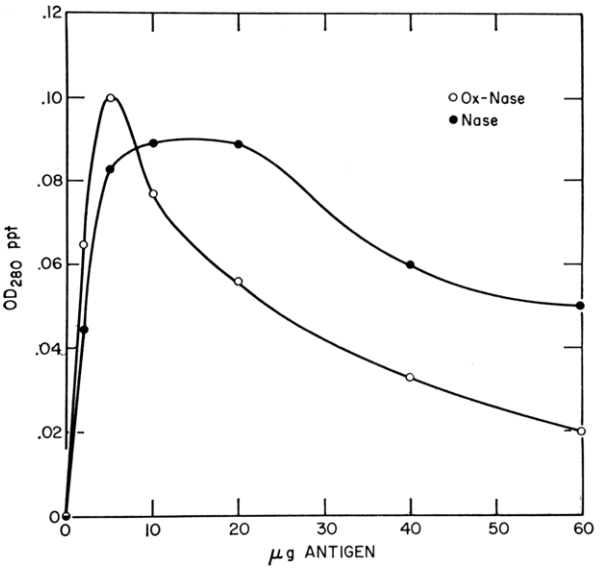

FIGURE 13: Quantitative precipitin reactions of nuclease (●—●) and of performic acid oxidized nuclease (○—○) with antibody elicited by the performic acid oxidized nuclease, γG15, 0.15 ml in a total volume of 0.5 ml of buffered saline (pH 7.5, 4°). Precipitates were dissolved in 0.1 N NaOH.

acid oxidized nuclease was found to retain an average of 1 tritium atom/molecule of protein in the absence of ligands and 9 tritium atoms/molecule upon addition of deoxythymidine 3′,5′-diphosphate and Ca to the eluting buffer (Figure 9). Under these conditions nuclease retains 22 and 57 tritium atoms, respectively. Again, the increment of 8 tritium atoms molecule of the derivative cannot be accounted for by a contamination by 8% native nuclease.

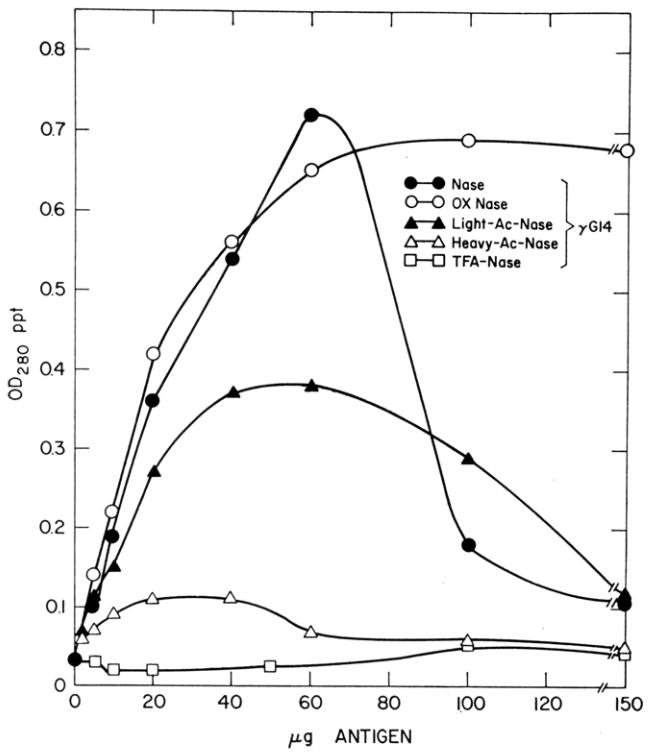

FIGURE 12: Quantitative precipitin reactions of nuclease (●—●), performic acid oxidized nuclease (○—○), "lightly acetylated" nuclease (▲—▲), "heavily acetylated" nuclease (△—△), and trifluoroacetylated nuclease (□—□) with 0.15 ml of antinuclease γG14 in total volume of 0.6 ml of buffered saline (pH 7.5, 4°). Precipitates were dissolved in 0.1 N NaOH.

Quenching of Fluorescence of Performic Acid Oxidized Nuclease. A 3:1 molar excess of deoxythymidine 3′,5′-diphosphate to performic acid oxidized nuclease gave quenching of the fluorescent intensity by about the same proportion as reported for quenching of 295-mμ emission of nuclease (Cuatrecasas *et al.*, 1967b). Such a study was simplified with performic acid oxidized nuclease, since all the fluorescence is due to tyrosyl groups and quenching could be measured for the full-emission spectrum, with maximum at 303 mμ (Figure 10).

Immunological Reactivity of the Modified Derivatives. All of the derivatives were tested together with nuclease in immuno-double-diffusion experiments on agar plates using the γ-globulin fractions of antisera against nuclease (Figure 11). The lightly acetylated nuclease gave a sharp line of identity with nuclease; heavily acetylated nuclease gave only a faint precipitin line; and trifluoroacetylated nuclease gave none at all, even when used in great excess. Performic acid oxidized nuclease gave a somewhat broadened precipitin line, but no spurring could be detected between its precipitin line and that of nuclease.

Quantitative precipitin reactions with γ-globulin 14 are shown in Figure 12, using 0.15 ml of γ-globulin solution in each tube. The performic acid oxidized nuclease gave nearly the same maximal amount of precipitate, but at a higher concentration of antigen, and its precipitation failed to be inhibited significantly by further excess of antigen, usually a sign of heterogeneous binding reactions. Lightly acetylated nuclease and heavily acetylated nuclease resembled native nuclease, but gave much less precipitate and reached an equivalence point at a lesser concentration of antigen. Trifluoroacetylated nuclease gave no precipitation at all, nor did several unrelated antigens.

TABLE III: Inhibition of Enzymatic Activity by Antibody.

10 μg of Nuclease γ/ml Assayed[a]		100 μg of Performic Acid Oxidized Nuclease γ/ml Assayed[a]		
ΔOD_{260}/min per ml	Rel Act. (%)	γG Added (μl)	ΔOD_{260}/min per ml	Rel Act. (%)
14.8	100	0	11.9	100
15.0	100	Control γG (100)	11.9	100
10.4	70	γG 14 (0.5)		
3.8	26	γG 14 (5)	9.1	76
2.4	16	γG 14 (10)	6.6	55
0.1	1	γG 14 (100)	0.1	2
15.0	100	γG 15 (10)		
12.9	86	γG 15 (30)	6.2	52
12.3	82	γG 15 (100)	1.7	14
9.8	65	γG 15 (200)	1.3	11

[a] Aliquots (10 μl) of each enzyme solution assayed after γG solution added to DNA substrate in cuvets in Gilford spectrophotometer. Note: performic acid oxidized nuclease is added at ten times the concentration of nuclease, so results with tenfold greater volume of γG solutions should be compared.

In order to explore further the cross-reactivity of nuclease and performic acid oxidized nuclease, both antigens were tested in quantitative precipitin reactions with antiserum elicited by the performic acid oxidized derivative (Figure 13). The titers of antibody were relatively low in both animals immunized with this derivative. The precipitin curves show a reversed relationship between the two antigens in comparison with their reactions with antinuclease (Figure 12). In Figure 13, the homologous antigen, performic acid oxidized nuclease, gives the sharper precipitation peak and subsequent inhibition by excess, while nuclease achieves nearly the same maximal precipitation with a slower fall in precipitate in the region of antigen excess.

Since antinuclease antisera have been shown previously by Fuchs *et al.* (1969) to inhibit the enzymatic activity of nuclease, both anti-nuclease (γ-globulin 14) and anti-performic acid oxidized nuclease (γ-globulin 15) were tested for their ability to inhibit the enzymatic activity of the two antigens. Aliquots of the antibody solutions were added to cuvets containing DNA substrate solution. Then either 1.0 μg of performic acid oxidized or 0.1 μg of native nuclease was added. In the absence of added antibody, these quantities gave equivalent activities. However, the presence of sufficient antibody could completely inhibit the activities of both. γ-Globulin 14 inhibited both the native and oxidized forms of the enzyme to a similar extent (Table III). γ-Globulin 15 also inhibited both, but was relatively more effective against the homologous antigen, performic acid oxidized nuclease.

Discussion

The extensive cross-reactivity of nuclease and performic acid oxidized nuclease when tested with antisera elicited by either antigen is in striking contrast to the lack of cross-reactivity between pancreatic ribonuclease and its performic acid oxidized derivative (Brown *et al.*, 1959). In ribonuclease, performic acid oxidation has the additional effect of disrupting the secondary structure through conversion of half-cystines into cysteic acid. In further studies, May and Brown (1967) have shown that antibodies elicited by performic acid oxidized or carboxymethylated ribonuclease cross-react well with a variety of derivatives in which methionine has been altered, but less well with derivatives having alterations in the cysteine residues. These derivatives were not tested against antibody elicited by native ribonuclease. In other proteins such as sperm whale myoglobin (Atassi, 1967) and bovine parathyroid hormone (Tashjian *et al.*, 1964), oxidation of methionine residues has had little or no effect upon antigenicity.

Performic acid oxidized nuclease appears to be almost fully denatured, as judged by measurements of ultraviolet absorption, optical rotatory dispersion, and circular dichroism, both in the 220-mμ region and in the aromatic region (Omenn *et al.*, 1969). Its strong cross-reactivity with native nuclease suggests that the antigenicity of nuclease is due in large part to linear sequence determinants. This inference is examined further in the following communication, where the antigenicity of peptide fragments is reported. A possibility that cannot be ruled out is that antibody against active nuclease is able to induce or maintain the proper refolding of the oxidized derivative, and hence to cross-react fully with it.

The performic acid oxidized derivative, though apparently denatured, still exhibits 8% of native enzymic activity, and is clearly able to bind ligands, as shown by adsorption to the deoxythymidine 3′,5′-diphosphate–Sepharose column, and by the effects of ligands upon the rate of trypsin digestion, the exchange of tritium, and the tyrosine fluorescence emission spectrum. Methionine and tryptophan, though contributing to the stability of the tertiary structure, apparently are not essential participants in substrate binding or catalysis. Other chemically modified nuclease derivatives prepared in this laboratory indicate that methionine plays an important structural role. A hydrogen peroxide oxidized derivative, in which methionines were oxidized to the sulfoxides, also

retained 8–10% DNase and RNase activity and was identical with the performic acid oxidized derivative on agar immunodiffusion against anti-nuclease antibodies. The peroxide-oxidized derivative contained intact tryptophan, as judged by its ultraviolet absorption and fluorescence spectra. Its fluorescence emission upon excitation at 295 mμ was maximal at 337 mμ, with 29% of the maximal intensity of the tryptophan fluorescence of nuclease. The circular dichroism spectra of the performic acid oxidized and peroxide-oxidized derivatives were entirely superimposable. It is reasonable to expect that the change from hydrophobic methionine residues to the hydrophilic sulfoxides or sulfones can markedly alter the "inside-outside" relationships that are maintained in the native conformation.

The acetylated and trifluoroacetylated derivatives retained much of the ordered structure of native nuclease, on the basis of spectral evidence. In the fully trifluoroacetylated derivative, the blue shift of the trough in the optical rotatory dispersion spectrum and the lack of a trough in the sloping negative ellipticity region of the circular dichroism spectrum may be consistent with ordered structure due to a mixture of helical, β structure, and other factors. The acetylated derivatives lost both enzymatic and antigenic activity together in proportion to the extent of alteration of charged lysine residues in the sequence. Cuatrecasas *et al.* (1968a) have shown that as many as seven lysines may be acetylated without loss of enzymatic activity.

Tyrosyl residues have been modified in other immunological experiments with nuclease. Fuchs *et al.* (1969) reported that nitrotyrosine 85-nuclease and nitrotyrosine 115-nuclease each reacted identically with nuclease against antinuclease globulins.

We conclude, from the present experiments, that antibodies elicited by nuclease are directed to a significant extent against linear sequences and that considerable modification of individual residues, including the four methionines, the single tryptophan, and certain tyrosines does not greatly affect reactivity with antibody. The antigenic reactivity of denatured nuclease is consistent with a relatively flexible or motile conformational state for native nuclease (Schechter *et al.*, 1968), which differs from a number of other globular protein antigens that have been studied by its lack of disulfide bridges and low helix content.

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