

# The Amino Acid Sequence of an Extracellular Nuclease of *Staphylococcus aureus*

## I. LINEAR ORDER OF THE FRAGMENTS PRODUCED BY CLEAVAGE WITH CYANOGEN BROMIDE\*

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### SUMMARY

An extracellular nuclease of *Staphylococcus aureus*, strain V8, was purified to a state of sufficient homogeneity for the study of its covalent structure. The purified enzyme exhibits both ribonuclease and deoxyribonuclease activities. The protein contains alanine and glutamine as the amino- and carboxyl-terminal residues, respectively. Neither cysteine nor cystine is present. Cyanogen bromide digestion of the nuclease yielded five fragments, designated A, B, C, D, and E. These fragments have been purified and analyzed for amino acid composition. Fragments A, B, and C contain amino-terminal alanine, tyrosine, and threonine, respectively. Both D and E have valine as the amino-terminal residue. Homoserine was found in A, B, C, and D, whereas the carboxyl-terminal residue of E was glutamine. Tryptic peptides of each of fragments A, C, D, and E have been separated and analyzed for amino acid composition and amino-terminal residues. The tryptic peptides of the nuclease containing methionine have also been isolated and similarly examined. By a consideration of these results and the total amino acid composition of the nuclease, together with examination of tryptic peptide maps, the minimum molecular weight of the nuclease has been calculated to be approximately 17,000, and the cyanogen bromide fragments have been assigned the order A-B-C-D-E.

An extracellular nuclease produced during the growth of *Staphylococcus aureus* has been shown to catalyze the hydrolysis of phosphodiester bonds in both ribonucleic and deoxyribonucleic acids (3, 4). A constant ratio of specific activities has been observed during the purification procedure, and preparations which appear to be homogeneous on the basis of both chemical and physical tests retain this dual specificity. Chemical studies on the amino acid sequence were undertaken because of interest in the structural basis of this two-fold function and

because, as is discussed in this communication, the protein has chemical features which may make it of special value in the study of side chain interactions that determine its tertiary structure. The polypeptide is devoid of sulfhydryl groups and disulfide bonds, undergoes a sharp reversible "melting" of native conformation over a narrow range of temperature, and contains a considerable amount of helical structure on the basis of its optical rotatory properties.

The polypeptide chain of the highly purified nuclease was subjected to cleavage at its four methionyl bonds with cyanogen bromide. The resulting five fragments were separated and, on the basis of amino acid analysis, digestion with trypsin, and end group analyses, were arranged in their proper order along the chain.

### EXPERIMENTAL PROCEDURE

**Materials**—The filter cake used as the starting material for purification was prepared as described previously (4). Trypsin (Worthington, twice crystallized) was treated with diisopropyl fluorophosphate as described by Potts *et al.* (5). Diisopropyl fluorophosphate-treated carboxypeptidase A was donated by Dr. J. Potts (5). Samples of calf thymus DNA were gifts of Dr. D. MacD. Green and Dr. M. Nirenberg. Thymine-methyl-<sup>3</sup>H DNA from *Escherichia coli*, K<sub>12</sub>λ, was given by Dr. A. Weissbach (6). High molecular weight yeast RNA was obtained from Sigma. Protein crystals made from the purified nuclease described below were provided by Drs. T. Hazen and A. Cotton.

Carboxymethyl cellulose (medium) was obtained from Sigma. Phosphocellulose (Whatman, column chromedia, p11 fibrous powder) was obtained from Angel (7.4 meq per g). Sephadex G-50 (medium) was obtained from Pharmacia (water regain = 5.1 ± 0.3 g per g; particle size, 100 to 270 mesh). Dowex AG 50W-X2 (200 to 400 mesh) was obtained from Calbiochem. All of these ion exchange materials were washed with distilled water, 1 N NaOH, distilled water, 3 N HCl, and distilled water. Other chemical reagents were analytical grade.

Cellophane dialysis tubing (Visking) was heated at 80° for 72 hours or boiled in 1% NaHCO<sub>3</sub> for 10 min<sup>1</sup> to decrease pore size.

**Assay of Enzymic Activity**—The methods for the determination of ribonuclease and deoxyribonuclease activities, and the def-

\* Preliminary reports of this work have appeared (1, 2).

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<sup>1</sup> R. Suriano, personal communication.

initiation of units, were described previously (4). The concentration of protein was determined by measuring absorbance at 280 m $\mu$ . The activity of the nuclease is expressed in RNase units unless otherwise specified. One absorbance unit at 280 m $\mu$  was assumed to be 1 mg of protein per ml for the calculation of specific

activities. DNase activity with DNA-<sup>3</sup>H as substrate was determined according to the method of Weissbach and Korn (6). Yeast RNA was used as inhibitor of DNase activity, and calf thymus DNA was added as unlabeled carrier DNA after the incubation.

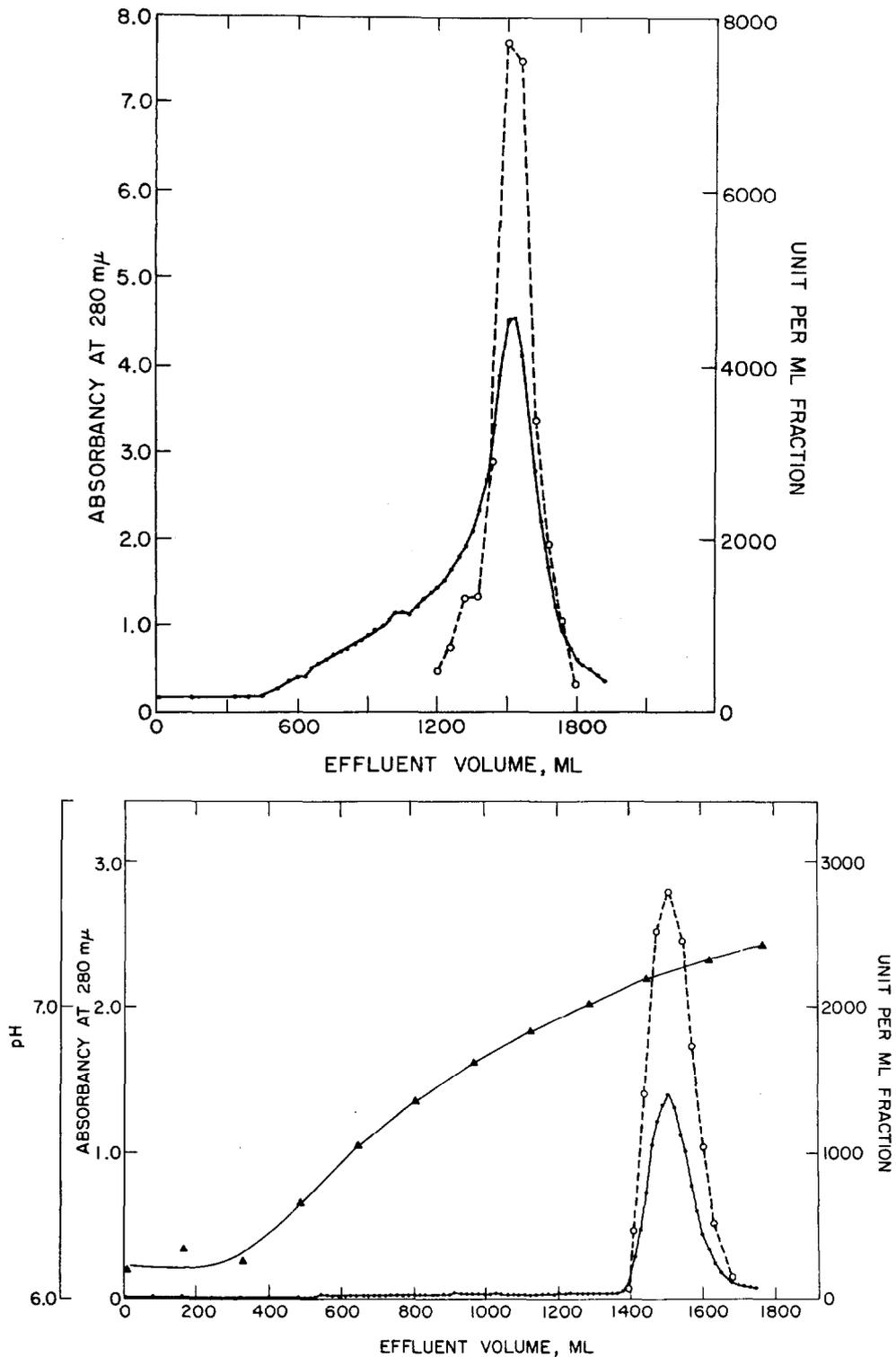


FIG. 1. Chromatographic purification of the nuclease on carboxymethyl cellulose in the first fractionation (*upper*) and the second fractionation (*lower*). The crude extract obtained from 100 g of filter cake (see Table I) was applied to the column in the first fractionation. In the second fractionation the fraction containing the nuclease (410,000 units) was applied. ●—●, absorbance at 280 m $\mu$ ; ○---○, RNase activity; ▲—▲, pH.

**Purification of Nuclease**—All procedures were performed at 4°. One hundred grams of filter cake were routinely used for each preparation. The cake was dissolved in 200 ml of 0.05 M sodium phosphate buffer, pH 6.1, and the solution was dialyzed against 6 liters of the same buffer for 36 hours with four changes. The activity lost through the cellophane tubing during dialysis was about 1.5% of the total activity. The dialysis solution was centrifuged in a Servall Superspeed centrifuge at 4000 rpm for 10 min. The supernatant solution (crude extract) was applied to a carboxymethyl cellulose column. The unadsorbed activity was 7% of the total activity applied. The procedure for the first fractionation on the carboxymethyl cellulose column (4 × 30 cm) was essentially as described previously (4) except that a two-chamber Varigrad was used to produce a linear gradient, with 1 liter of 0.05 M sodium phosphate, pH 6.1, and 1 liter of 0.15 M potassium phosphate, pH 7.5, as the first and second buffers, respectively. The elution was carried out at a flow rate of 45 to

60 ml per hour. Fractions were collected every 10 to 15 min. A typical elution diagram is shown in Fig. 1A. The fractions giving specific activities of more than 900 units per mg were pooled. After adjustment to pH 6 by the addition of phosphoric acid, the pooled fraction was diluted with distilled water to 2.5 times its volume (approximate final concentration, 0.05 M phosphate). The acidified solution was applied to the second carboxymethyl cellulose column (2.2 × 75 cm) which was equilibrated with 0.05 M sodium phosphate buffer, pH 6.1, and gradient elution was carried out as with the first column. Fractions were collected every 30 min at a flow rate of 16 ml per hour. A typical diagram of the second fractionation is shown in Fig. 1B. The fractions with constant specific activity were combined. The combined solution (approximately 100 ml) was dialyzed against 6 liters of distilled water with two changes for a total of 20 hours. After lyophilization, the dried material was dissolved in 20 ml of distilled water. The concentrated solution was again dialyzed against 6 liters of distilled water with four changes for 36 hours to remove the last traces of buffer salt. The dried material obtained by lyophilization was used in the studies described below. Table I summarizes the results of this purification procedure. Fig. 2 shows the ultraviolet absorption spectrum of the purified nuclease.

TABLE I

Purification of nuclease from *S. aureus* V8<sup>a</sup>

Enzyme fraction	Total volume ml	Activity units per ml	Absorbance at 280 m $\mu$	Specific activity	Total units	A <sub>290</sub> : A <sub>280</sub>
Crude extracts . . . . .	670	1,160	39.7	2.9	776,000 <sup>b</sup>	1.04
First carboxymethyl cellulose fraction . . . . .	530	1,650	1.82	920	877,000	1.65
Second carboxymethyl cellulose fraction . . . . .	103	4,750	2.37	2,000	489,000	1.70

<sup>a</sup> One hundred grams of the filter cake were used as starting material.

<sup>b</sup> The total activity in the crude extracts was variable, presumably due to the extent of removal of inhibitors by dialysis. The total number of units obtained from 100 g of the filter cake was occasionally as high as 2 million.

**Determination of Ultraviolet Absorbance**—A sample of the purified nuclease was dried over P<sub>2</sub>O<sub>5</sub> under reduced pressure at 25° for 3 days to constant weight. An aqueous solution of the dried protein, containing 1.0 mg per ml, showed an absorbance at 280 m $\mu$  of 1.16.

**Electrophoresis and Ultracentrifugal Analysis**—The procedures employed were essentially the same as those described by Craven, Steers, and Anfinsen (7). Glycine-HCl buffer of 0.1 ionic strength, for electrophoresis, was made according to the method of Miller and Golder (8).

**Cyanogen Bromide Digestion**—The following modification by Steers *et al.* (9) of the Gross and Witkop procedure (10) was utilized. Protein preparations were dissolved in 70% formic acid

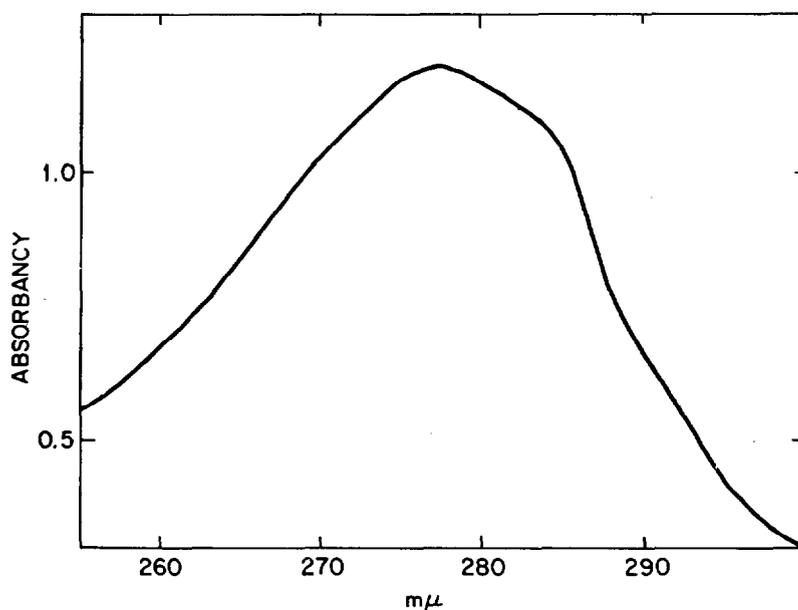


FIG. 2. The ultraviolet absorption spectrum of the nuclease in 0.15 M sodium phosphate buffer, pH 7.2 (see the text).

to make a 1% solution. A 30-fold molar excess of  $\text{CNBr}^2$  per methionine residue was added. The reaction mixture was kept at 25° for 20 hours. Distilled water was then added (2 volumes), and the diluted solution was lyophilized. The reaction proceeded to the extent of more than 90% as judged by amino acid analysis.

**Digestion with Trypsin**—Nuclease and separated cyanogen bromide peptides were incubated with trypsin (1% by weight of substrate) at 37° for 3 hours. The pH during digestion was adjusted to 8.0 with  $\text{NH}_4\text{OH}$ . For amounts of substrate less than 1 mg, 0.05 M ammonium bicarbonate, pH 8.0, was used as buffer.

**Subtilisin Digestion**—One hundred micrograms of peptide were dissolved in 15  $\mu\text{l}$  of 0.05 M ammonium bicarbonate, and the solution was adjusted to pH 8 (using phenol red) by the addition of 15  $\mu\text{l}$  of 0.01 M  $\text{NH}_4\text{OH}$  if necessary. Subtilisin (Nagarse) (1 mg in 5  $\mu\text{l}$  of 0.05 M ammonium bicarbonate) was added. The reaction mixture was incubated at 37° for 3 hours and lyophilized.

**Peptide Mapping**—Peptide maps were performed as described by Katz, Dreyer, and Anfinsen (11). A small drop of phenol red was added at the origin as a reference standard in the chromatographic dimension as used by Canfield (12). Free lysine which was released from the nuclease or cyanogen bromide peptides served as a reference standard for the electrophoretic dimension. The peptides were located by spraying with 0.25% ninhydrin in ethyl alcohol. Tryptophan-, arginine-, histidine- and tyrosine-, and methionine-containing peptides were located on the map with Ehrlich, Sakaguchi, Pauli, and platinum iodide reagents, respectively (13).

For the preparation of certain purified peptides, aqueous solutions of the trypsin digest, containing 0.1 to 0.2  $\mu\text{mole}$  of each peptide, were applied on Whatman 3MM filter paper. After location by light staining with 0.025% ninhydrin solution at 25°, spots were cut out and peptides were eluted with 50% aqueous pyridine at 25°. The eluates (5 to 10 ml for each spot) were lyophilized. The dried materials obtained were dissolved or suspended in 0.6 ml of distilled water and frozen.

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis was performed at 4–6° with 7.5% “standard gel” and 15% “4.3 gel” as described in the manual supplied by the Canal Industrial Corporation. The direction of the electrical current was reversed when necessary. Samples of 0.1 to 0.3 mg of protein (or peptides) were applied and run at a constant current of 3 to 4 ma per tube for 3 to 5 hours.

**Paper Electrophoresis**—Pyridinium acetate buffers, pH 3.6 and 6.5 (11), were used on Whatman No. 3MM paper. Applied voltage was 2500 volts for 80 min.

**Preparative Electrophoresis**—A Brinkman continuous flow electrophoretic separator was employed for preparative electrophoresis. The samples to be applied were dissolved in 0.01 M ammonium bicarbonate, pH 8.7, to make 1 to 2% solutions. The same buffer was utilized for making the buffer curtain. Temperature was maintained at 6°. The applied voltage was 2000 volts, and the current was 120 ma. The minimum rate of dosage of the sample was used. Phenol red was added to the sample solution as an internal electrophoretic reference material.

**Gel Filtration of Cyanogen Bromide Peptides**—All procedures were carried out at 25°. A column of Sephadex G-50 (3 × 260 cm) was poured from a thin slurry, which was allowed to settle

<sup>2</sup> The abbreviations used are:  $\text{CNBr}$ , cyanogen bromide; DNP-, 2,4-dinitrophenyl-; DFB, 1-fluoro-2,4-dinitrobenzene.

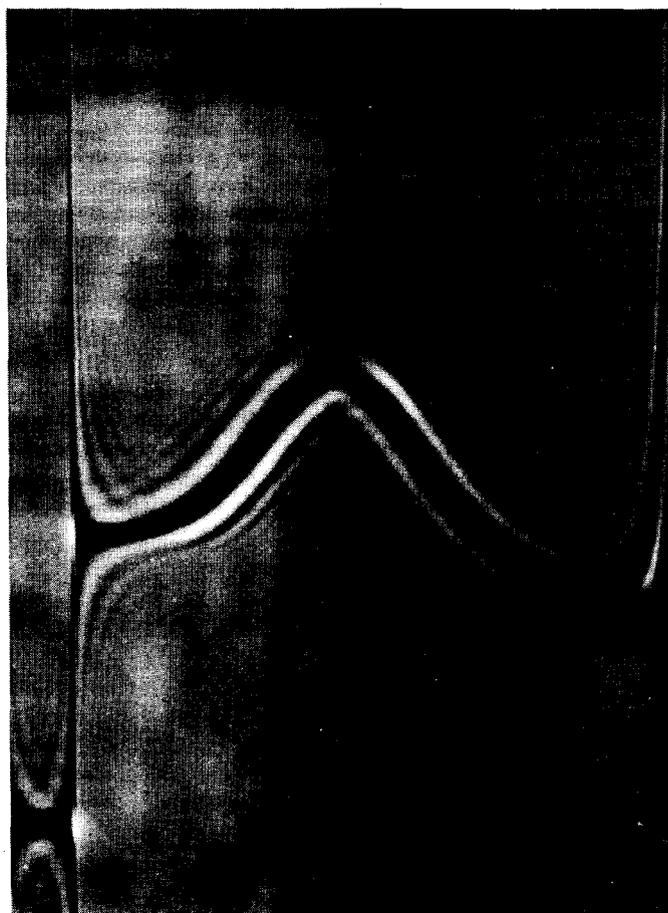


FIG. 3. Schlieren pattern of the purified nuclease obtained in the Spinco model E ultracentrifuge. The photograph was taken 200 min after reaching a speed of 59,780. The enzyme had been previously dialyzed against 0.05 M Tris-HCl buffer, pH 6.9, containing 0.1 M NaCl and 0.001 M ethylenediaminetetraacetic acid. Protein concentration was 6.1 mg per ml.

without extra packing pressure. The column was equilibrated with 0.01 N acetic acid containing 0.1% ammonium acetate.<sup>3</sup> The sample, an 8% solution of the peptide mixture in the same buffer, was applied to the column and washed in with three 1-ml applications of the buffer. Elution was carried out with the same buffer at a flow rate of 9.6 ml per hour. Fractions were collected every 30 min and assayed by measuring absorbance at 280 m $\mu$ . Suitable aliquots (15 to 100  $\mu\text{l}$ ) were subjected to alkaline hydrolysis, and hydrolysates were examined by the ninhydrin color reaction according to the method of Hirs, Moore, and Stein (14, 15).

**Peptide Separation on Dowex 50**—The procedures described by Canfield and Anfinsen (12, 16) have been employed. A Dowex AG 50W-X2 column, 0.9 × 93 cm, equilibrated at pH 3.8, was used. Gradient elution was performed at a flow rate of 23 ml per hour with a Sigma motor pump, model T8. The buffer volume in each chamber of the Varigrad was 150 ml. Fractions were collected every 10 min. Fractions comprising each peak were pooled and lyophilized. The dried materials obtained were dissolved in 0.6 ml of distilled water and frozen.

<sup>3</sup> L. C. Craig, Rockefeller University, personal communication.

*Further Purification of Peptides*—All of the tryptic peptides that separated and the subtilisin peptides derived from one of them were examined for purity by two-dimensional mapping as described above. Larger aliquots of the fractions that contained more than one tryptic peptide were separated by paper electrophoresis at pH 3.6 or chromatography of wide bands on Whatman 3MM. The purified peptide components were eluted with 50% aqueous pyridine and lyophilized.

Aliquots of the pooled fractions of the cyanogen bromide peptide mixture were examined by paper electrophoresis (2500 volts) at pH 6.5 for 90 min. Larger aliquots of each cyanogen bromide

peptide fraction were applied as wide bands. After location by staining of a side strip with ninhydrin the components were eluted with 50% aqueous pyridine and lyophilized.

*Amino Acid Analysis*—Samples were hydrolyzed in 0.5 ml of constant boiling HCl in evacuated, sealed tubes at 110° for 20 hours. After removal of HCl by rapid evacuation over NaOH pellets, amino acid analyses were performed by the method of Spackman, Moore, and Stein (17) on a Spineo model 120 amino acid analyzer equipped with an Infotronics model CRA-10A integrator. The recorder of the analyzer was equipped with a 4- to 5-mv resistor card (Minneapolis-Honeywell, kit part No.

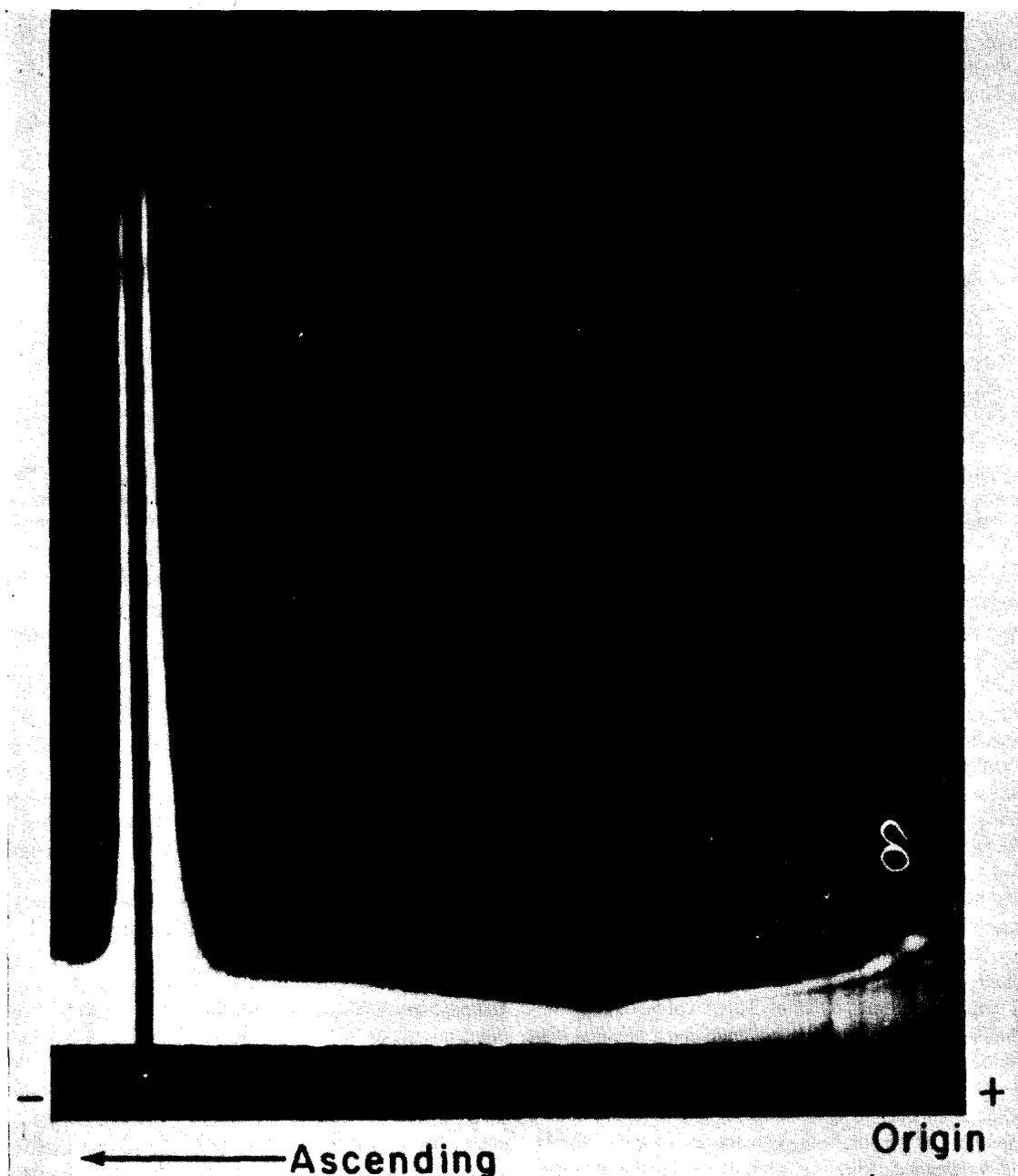


FIG. 4. Moving boundary electrophoresis of the purified nuclease, obtained in the Perkin-Elmer model 238 electrophoresis apparatus. Protein concentration was 6.1 mg per ml, in glycine-HCl buffer (0.1 ionic strength), pH 3.51 (see "Experimental Procedure"); conductivity, 6.2 mmhos. The photograph was taken after 115 min at a constant voltage of 100 volts. A similar symmetrical schlieren pattern was obtained with free electrophoresis of the purified nuclease in 0.05 M citrate, pH 7.5, which was kindly carried out by Dr. William R. Carroll.

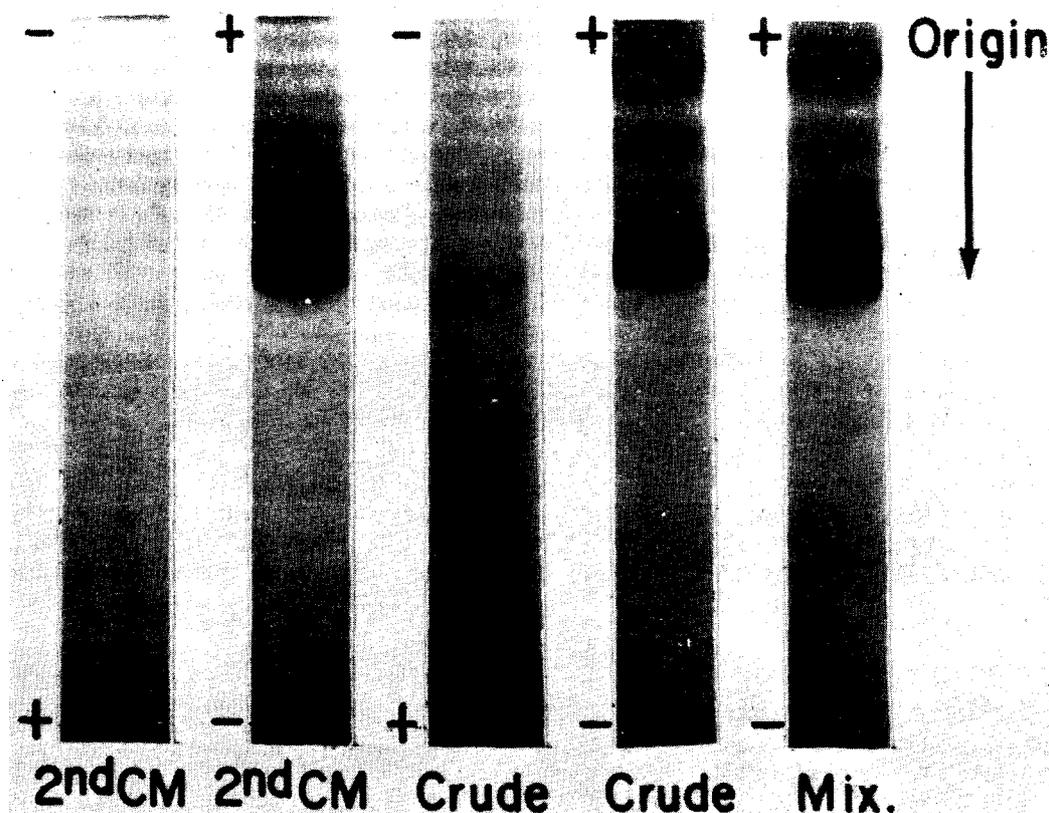


Fig. 5. Comparison of purity of the crude extracts (*Crude*), the purified nuclease (*2nd CM*), and a mixture of these. The total number of nuclease units applied to gels was much higher with the purified nuclease than with the crude extracts so that separable impurities, if any, could be readily seen. The gels used here were standard gels (see the text) with both normal and reversed polarities.

365928-999). A series of analyses on the acid hydrolysates of the nuclease containing between 0.01 and 0.1  $\mu$ mole of each amino acid showed that analyses at these low levels were reproducible to within 10%. The analytical values shown (see Table V and Reference 7) have, therefore, been reported to three significant figures in many cases. The procedure of Dreyer and Bynum (18) was also employed for amino acid analysis in some cases as reported by Canfield (19). A voltage gradient of 25 volts per cm was applied for 2 hours at 44–48°.

**Determination of Cysteine and Cystine**—The nuclease, oxidized with HCOOOH according to the method of Hirs (20), was analyzed for cysteic acid. Reduction and subsequent alkylation with iodoacetate- $^{14}$ C (21) was also applied to the nuclease preparation. Radioactive iodoacetate (specific activity 16,500 cpm per  $\mu$ mole; New England Nuclear) was employed. The reaction mixture contained, in a total volume of 10 ml, 4.8 g of urea, approximately 17 mg of nuclease, 50  $\mu$ l of 2-mercaptoethanol, and 0.1 M Tris buffer, pH 8.0. One drop of phenol red was added to furnish internal monitoring of pH.

**Tryptophan**—The spectrophotometric methods of Goodwin and Morton (22) and Bencze and Schmid (23) were used to determine the content of tryptophan, with the use of 0.1 N NaOH solutions of the nuclease. Alkaline hydrolysis of the nuclease was performed in evacuated, sealed tubes at 110° for various lengths of time by a modification of the method of Lugg (24). The hydrolysate was analyzed with the amino acid analyzer. The tryptic peptide containing tryptophan was detected on two-dimensional peptide maps with Ehrlich's reagent. The Ehrlich-

positive tryptic peptide was eluted as described above and digested further by subtilisin. The resulting fragments were separated on two-dimensional peptide maps and located by staining with Ehrlich's reagent.

**Total Amide Nitrogen Determination**—The amide ammonia of the nuclease was determined according to the method of Dr. Robert Hill.<sup>4</sup> Dried nuclease, 6.6 mg, was dissolved in 0.60 ml of constant boiling HCl. Aliquots of 0.10 ml each were placed in hydrolysis tubes, and 0.20 ml of distilled water was added to each tube. Acid hydrolysis was performed in evacuated, sealed tubes at 110° for various lengths of time. Hydrolysis was stopped by immersing tubes in powdered Dry Ice. As a reference, a 0.10-ml aliquot was subjected to complete hydrolysis by the method described above and analyzed on the amino acid analyzer.

**NH<sub>2</sub>-terminal Residues by Dinitrophenylation**—The NH<sub>2</sub>-terminal residues of the nuclease and of the cyanogen bromide peptides were quantitatively determined by the modified Levy procedure (25). An unpublished modification, kindly made available by E. Bynum, W. Dreyer, and C. Bennett, was applied with tryptic peptides as follows: samples of 0.05 to 0.1  $\mu$ mole, dissolved in 20  $\mu$ l of water, were placed in test tubes (1.5 × 15 cm), and 200  $\mu$ l of 1 M trimethylamine acetate, pH 9.7, were added. One volume of 1-fluoro-2,4-dinitrobenzene was dissolved in 20 volumes of anhydrous ethyl alcohol. Aliquots of 5  $\mu$ l of the fluorodinitrobenzene solution were mixed with the sample solutions. The

<sup>4</sup> R. L. Hill, Duke University, personal communication.

reaction mixtures were incubated for 3 hours at 37° and lyophilized. The dried mixtures were put in a vacuum oven over NaOH pellet, and sublimation was performed at 60° for 4 hours. The residues were extracted with ether and again placed in a vacuum oven at 60° for 3 hours. The dried materials were dissolved in 0.5 ml of constant boiling HCl and hydrolyzed in evacuated sealed tubes at 110° for 16 hours. The hydrolysates were subjected to two-dimensional chromatography by the modified Levy procedure. All manipulations were performed in a darkened laboratory. The two-dimensional chromatographic separation of ether-soluble DNP-amino acids was achieved with the *tert*-amyl alcohol-1 M NH<sub>4</sub>OH (4:1) system in the first direction and 1.5 M potassium phosphate, pH 6.0, in the second (19). The water-soluble DNP-amino acids were extracted with *n*-butyl alcohol and examined by paper electrophoresis at pH 6.5.

*Edman Degradation*—The Edman procedure modified by Margoliash (26) was employed for the determination of the NH<sub>2</sub>-terminal residue of the nuclease. Phenylthiohydantoin of amino acids were identified by thin layer chromatograms with two different solvent systems; chloroform-methanol (9:1) and

chloroform-formic acid (20:1). The chromatographic plates were made with MN-cellulose powder 300 (Machery, Nagel and Company). The spots were located with iodine azide spray (27). The NH<sub>2</sub>-terminal residues of tryptic peptides and of a small cyanogen bromide fragment of the nuclease were also determined by the following modified Edman degradation (28-30). The reaction mixtures consisted of 0.2 to 0.5 μmole of peptide in 50 μl of distilled water, 50 μl of 2% phenylisothiocyanate in pyridine, and 5 μl of 25% aqueous trimethylamine. The mixture was placed in a conical 8-ml glass tube fitted with a standard taper glass plug which had two openings, one stoppered with a rubber vaccine port and one with a ground glass stopper. After the tubes were flushed with O<sub>2</sub>-free nitrogen through a hypodermic needle for 2 min, the vessel was sealed and incubated at 40° for 4 hours. The mixture was extracted five times with 0.5 ml of thiophene-free benzene under nitrogen. The upper layers were discarded following each extraction after centrifugation in an International clinical centrifuge. The residual solutions were rapidly taken to dryness under reduced pressure. Cyclization of the phenylthiocarbonyl derivatives was performed by adding

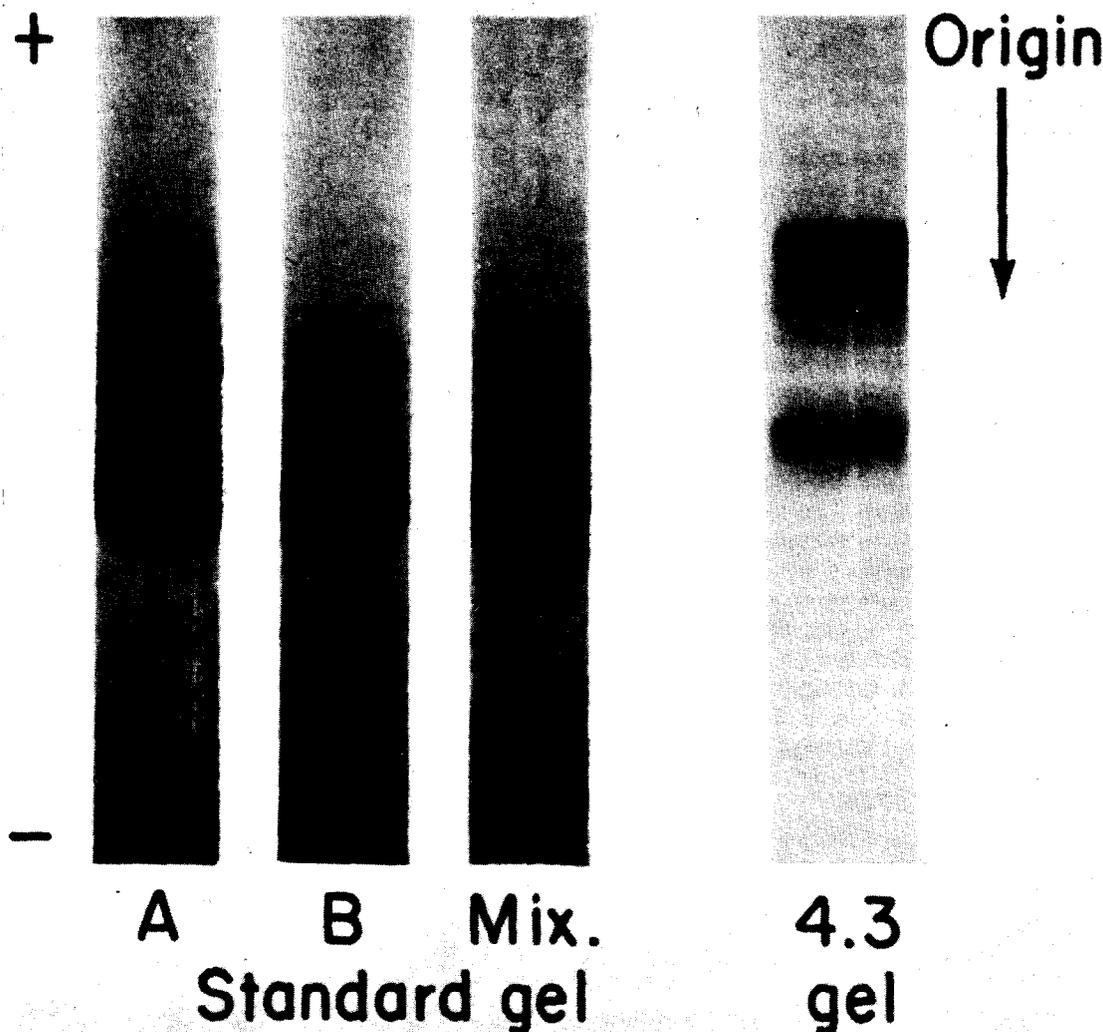


FIG. 6. Electrophoretic pattern of crystalline nuclease on acrylamide gel electrophoresis (standard gel). A, needles; B, hexagonal crystals; Mix, a mixture of both samples. Electrophoretic behavior of the purified nuclease at pH 4.3 (see the text) is labeled "4.3 gel."

0.3 ml of trifluoroacetic acid and incubating the resulting solution at 40° for 15 min under nitrogen. After having been dried under reduced pressure, the residue was extracted four times with 0.5 ml of ethylene chloride. The solvent was removed by Pasteur pipette. The extracts and the residual material were taken to dryness under reduced pressure over NaOH pellets at 25°. The dried extracts were dissolved in 0.5 ml of 30% aqueous alcohol, adjusted to pH 1 by the addition of concentrated HCl, and incubated at 80° for 1 hour for the cyclization of the thiazolinone derivative, and the solutions were then lyophilized. The dried materials were dissolved in 0.3 ml of 0.1 N NaOH and hydrolyzed in the sealed, evacuated tubes at 110° for 16 hours to form the free amino acids from phenylthiohydantoin derivatives. The dry residual material, containing the protein or tryptides in which the original NH<sub>2</sub> terminals had been removed, was dissolved in 200  $\mu$ l of 50% aqueous pyridine. Two aliquots of 10  $\mu$ l were removed and placed in a hydrolysis tube. The aliquots and the residual solutions were lyophilized. The aliquots were then hydrolyzed in evacuated, sealed tubes with 0.5 ml of constant boiling HCl at 110° for 16 hours. Amino acid analyses of the hydrolysates were performed with the automatic amino acid analyzer, and by paper electrophoresis by the method of Dreyer and Bynum as described above.

**Carboxyl-terminal Residue Determination**—A solution of carboxypeptidase A, prepared as described by Potts *et al.* (5), was employed. When serial carboxypeptidase digestion was performed to obtain information of the carboxyl-terminal sequence, 0.1 to 0.5  $\mu$ mole of the nuclease or peptide was dissolved in distilled water to make a 2% solution (w/v). A one-tenth volume of carboxypeptidase A solution was added. The incubations were performed at 25° and 37°. Aliquots of 0.1 volume of the reaction mixture were removed at timed intervals. These were immediately acidified with 5 N acetic acid to yield 0.3 N acetic acid and left at 25° for 30 min. Five microliters of the acidified aliquots were removed for assay of enzymic activity, and the residual solutions were lyophilized to be analyzed for released amino acids with the amino acid analyzer. Ascending chromatograms at 25° on Whatman No. 1, with 80% aqueous pyridine as solvent, were also used for qualitative analysis.

**Determination of Sugar Content**—The phenol-sulfuric acid method of Dubois *et al.* (31) was used to examine the nuclease for carbohydrate, with D-glucose as a standard.

**Determination of Radioactivity**—A Packard Tri-Carb liquid scintillation spectrometer, equipped with an automatic sample changer, was utilized to determine the radioactivity of samples dissolved in Bray's solution (32). The radioactivity of 1 absorbance unit of DNA-<sup>3</sup>H was found to be  $14 \times 10^4$  cpm.

## RESULTS

**Purity of Nuclease Preparation**—The purified nuclease described above appeared to be homogeneous on the basis of ultracentrifugation,<sup>5</sup> free boundary electrophoresis,<sup>5</sup> and acrylamide gel electrophoresis (Figs. 3, 4, and 5). The two small components previously observed during free boundary electrophoresis (4) had been removed. When the preparation was examined by electrophoresis on polyacrylamide gel, two bands were occasionally observed, even with crystalline preparations, as shown in Fig. 6. The fast running band was dominant on standard gel

and the slow one on 4.3 gel. To examine the nature of this phenomenon, a nuclease preparation which showed such heterogeneity was studied in the Brinkmann preparative electrophoresis apparatus (Fig. 7). Aliquots taken across the effluent curtain were subjected to polyacrylamide gel electrophoresis. Partial separation of the two components was achieved. The almost homogeneous materials in tubes 12 and 24 retained their electrophoretic behavior when rerun on standard gel (Fig. 8), but both yielded a mixture of the fast and slow components when run on the 4.3 gel. Chromatography of material from tube 12 on carboxymethyl cellulose columns (as described under "Experimental Procedure") also produced both the fast and slow components in proportion to that shown by the original nuclease preparation. Thus the two forms of the nuclease appear to be interconvertible. The basis for this interconversion is unknown at present.

The two forms were examined by amino acid and carbohydrate analysis (Table II), peptide mapping of trypsin digests, polyacrylamide gel electrophoresis of CNBr digests, and measurements of specific activities against RNA and DNA. None of these procedures showed significant differences. Together with the end group analyses, which indicate single NH<sub>2</sub>- and COOH-terminal residues, and the partial sequence analysis described below, these data indicate that the nuclease preparation is homogeneous in the covalent sense and adequate for structural studies.

**RNase and DNase Activities**—As illustrated in Fig. 9, the linear ranges of activity with respect to enzyme concentration are limited for both RNase and DNase activity, particularly the latter. Furthermore, the activities are sensitive to low levels of certain anions such as citrate and phosphate (Table III). Comparisons of the ratios of the two activities during purification therefore required estimations at several levels of enzyme concentration under carefully standardized conditions. The constant ratios of the activities previously found during the purification procedure (2) suggested that both RNA and DNA serve as substrates for the nuclease, and this conclusion is supported by the data shown in Fig. 10 where constant relative activities were observed across the entire effluent peak in a typical

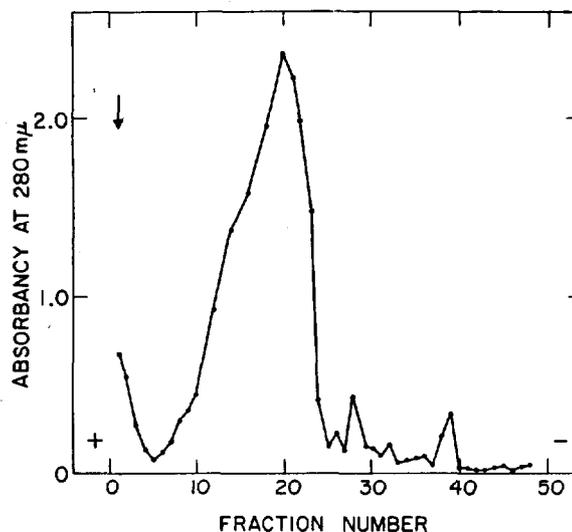


FIG. 7. Preparative electrophoresis of the purified nuclease. Twenty milliliters of the nuclease solution (460 mg of protein) were applied at the port indicated by the arrow. Other details are given in "Experimental Procedure."

<sup>5</sup> These experiments were performed with the cooperation of Dr. R. Suriano.

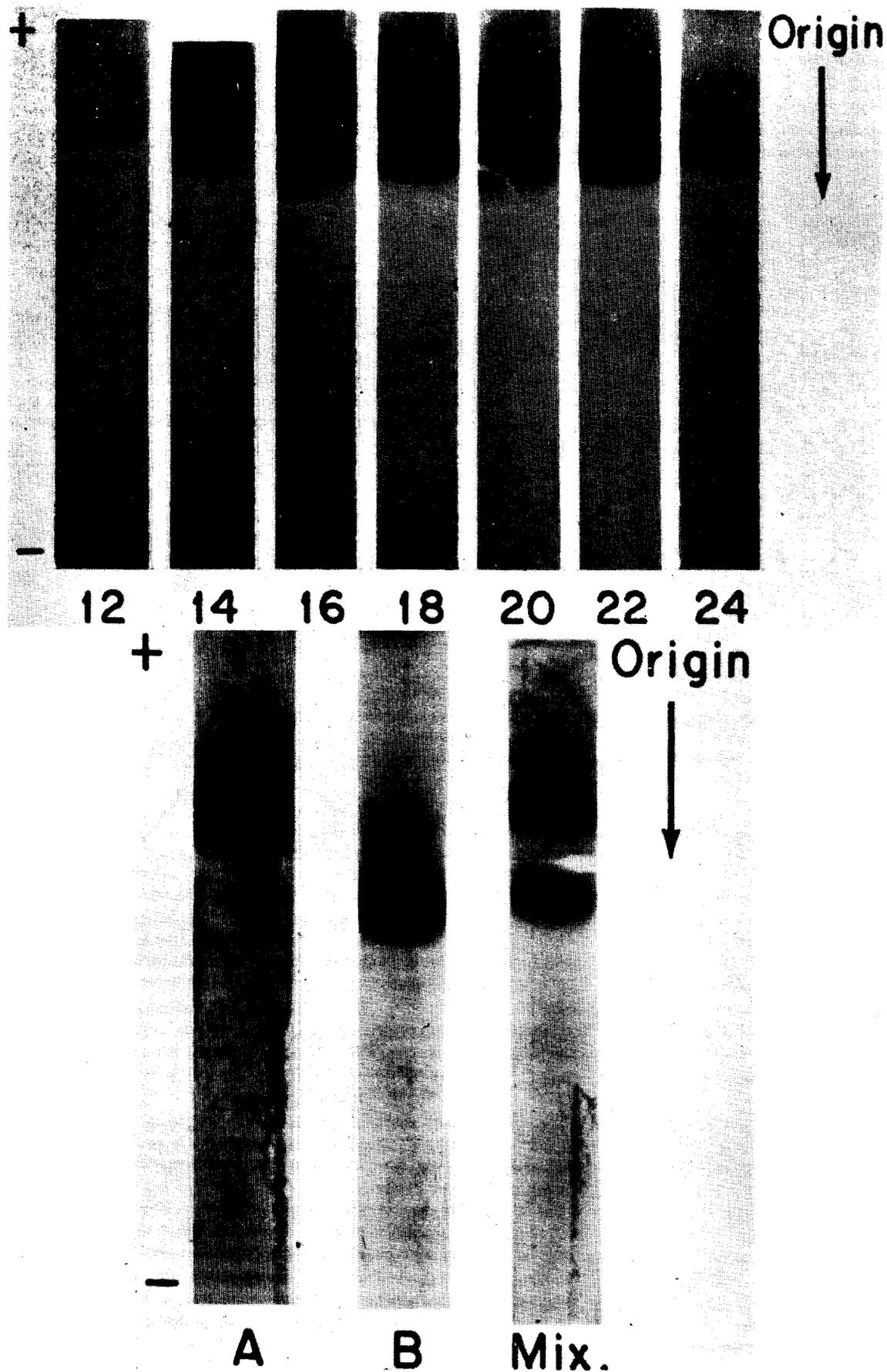


FIG. 8. *Upper*, polyacrylamide gel electrophoresis pattern (standard gel) of fractions from preparative electrophoresis (Fig. 7). Suitable aliquots from alternate tubes were lyophilized to be used for the electrophoresis. *Lower*, electrophoretic behavior on polyacrylamide gel of two forms of the nuclease separated by preparative electrophoresis. *A* and *B* show the mobilities on standard gel, of samples obtained from tubes 12 and 24, respectively (see Fig. 7); and *Mix* represents the pattern obtained from a mixture of the two.

TABLE II

Comparison of amino acid compositions and sugar contents of two components obtained electrophoretically from nuclease preparation

	Component I <sup>a</sup>		Component II <sup>a</sup>	
	$\mu\text{mole}$	$\mu\text{mole } \%$	$\mu\text{mole}$	$\mu\text{mole } \%$
Lys	0.270	15.7	0.303	15.6
His	0.030	1.7	0.040	2.0
Arg	0.055	3.2	0.065	3.4
Asp	0.166	9.7	0.187	9.6
Thr	0.122	7.1	0.127	6.5
Ser	0.062	3.6	0.062	3.2
Glu	0.197	11.5	0.240	12.4
Pro	0.071	4.1	0.087	4.5
Gly	0.113	6.6	0.138	7.1
Ala	0.173	10.1	0.185	9.5
Cys	0.000	0.0	0.000	0.0
Val	0.116	6.7	0.121	6.2
Met	0.026	1.5	0.036	1.9
Ile	0.070	4.1	0.065	3.4
Leu	0.132	7.7	0.155	8.0
Tyr	0.076	4.4	0.088	4.5
Phe	0.040	2.3	0.041	2.1
	<i>mole/mole protein</i>		<i>mole/mole protein</i>	
Glucose equivalents <sup>b</sup>	0.23 <sup>c</sup>		0.66 <sup>c</sup>	

<sup>a</sup> The fast and slow running components on standard acrylamide gel are designated as Components I and II and were obtained from tubes 12 and 24 in Fig. 7, respectively.

<sup>b</sup> See "Experimental Procedure."

<sup>c</sup> The amounts of protein used for analysis were 0.080 and 0.12  $\mu\text{mole}$  for Components I and II, respectively.

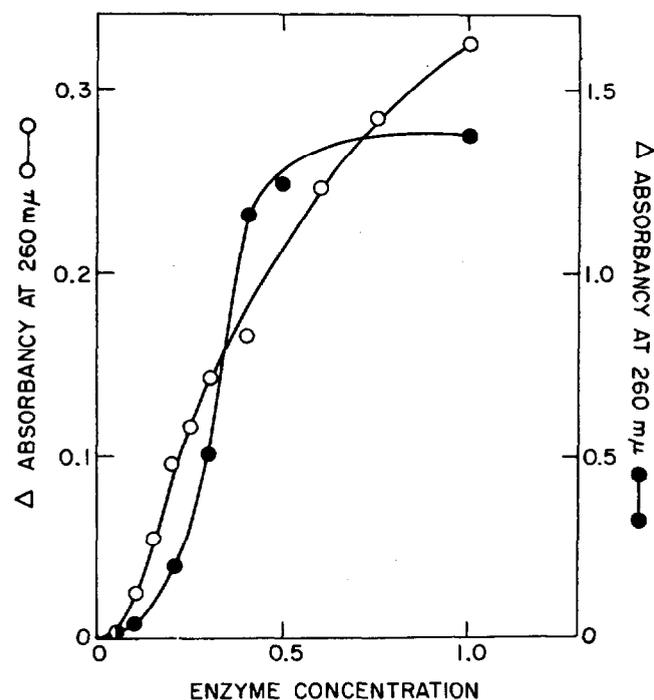


FIG. 9. RNase and DNase activities of the nuclease as a function of enzyme concentration. The methods of assay are described in "Experimental Procedure." ○—○, RNase; ●—●, DNase.

TABLE III

Effect of phosphate and citrate ions on RNase and DNase activity

Addition	Activity	
	DNase	RNase
	% of control	
None <sup>a</sup>	100	100
Phosphate, $5 \times 10^{-4}$ M	117	105
Phosphate, $5 \times 10^{-3}$ M	228	
Citrate, $5 \times 10^{-4}$ M	337	239
Citrate, $5 \times 10^{-3}$ M	542	184

<sup>a</sup> The concentrations of contaminating phosphate ions from the original enzyme solution were  $1.2 \times 10^{-5}$  M and  $5 \times 10^{-6}$  M in the reaction mixtures for the DNase and RNase assays, respectively.

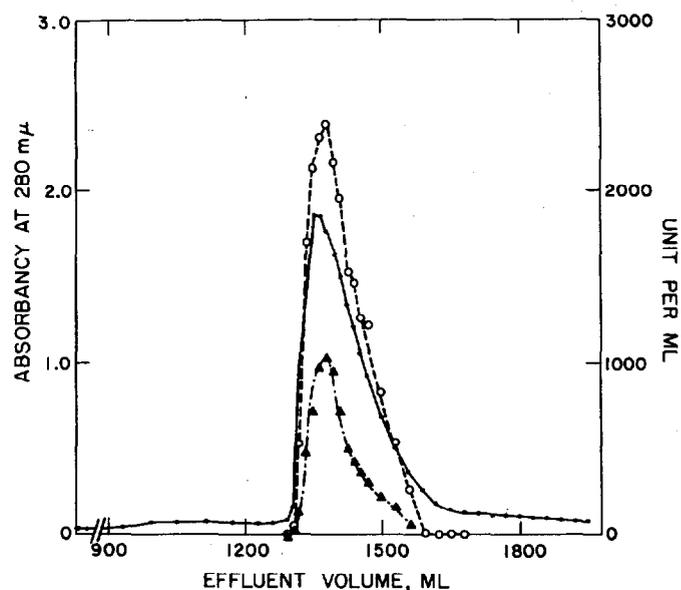


FIG. 10. RNase and DNase activities of chromatographic fractions of the nuclease from carboxymethyl cellulose. ●—●, absorbance at 280  $m\mu$ ; ○—○, RNase; ▲—▲, DNase. The procedure for chromatography was the same as that described for the second fractionation on carboxymethyl cellulose in "Experimental Procedure." Approximately 250 mg of the protein were applied. Aliquots of the fractions comprising the peak in absorbance at 280  $m\mu$  were diluted 100-fold with 0.05 M sodium phosphate buffer, pH 6.1. From each diluted solution, 5- and 10- $\mu\text{l}$  aliquots were incubated for assay of DNase and RNase activities, respectively. The values obtained were plotted without correction for the effect of enzyme concentration (see Fig. 9). The low specific RNase activity is probably due to the nature of the RNA substrate used in this experiment.

chromatographic experiment. These observations, together with the evidence for the purity of the nuclease discussed above, strongly suggest that a single enzyme is responsible for both enzymic activities. Competitive inhibition tests of DNase activity in the presence of added RNA gave results consistent with this conclusion (Table IV) and confirm the results found with another staphylococcal nuclease preparation by Alexander, Heppel, and Hurwitz (3).

*Amino Acid Composition*—The results of analyses of timed hydrolysates are summarized in Table V. The half-cystine content, previously reported to be zero, was checked by anal-

TABLE IV  
Effect of RNA on DNase activity

Concentration of RNA	Concentration of DNA- <sup>3</sup> H	DNase activity
<i>absorbance units/ml</i>		<i>cpm</i>
0	0.25	3535
0	0.75	9700
90	0.25	1387
90	0.75	4222

value of 7 residues of tyrosine per molecule (Table VII). This was checked further by staining peptide maps of trypsin and cyanogen bromide digests with Ehrlich's reagent. Both of the digests showed only a single Ehrlich-positive component. The Ehrlich-positive tryptic peptide was isolated and subjected to further digestion with subtilisin as described above, giving two fragments. Only one fragment, with a high  $R_F$  value on the paper chromatogram, showed a positive reaction with Ehrlich reagent. These results indicate that 1 residue of tryptophan is present per molecule of the nuclease.

TABLE V  
Amino acid composition of nuclease

Residue	Amino acid content after acid hydrolysis for <sup>a</sup>						Average	Calculated moles per 3 moles of phenylalanine <sup>b</sup>
	17 hrs		34 hrs		57 hrs			
	$\mu\text{mole}$		$\mu\text{mole}$		$\mu\text{mole}$			
Lys.....	0.148	0.144	0.148	0.143	0.138	0.150	0.147	18.4 (21.4) <sup>c</sup>
His.....	0.024	0.022	0.024	0.022	0.023	0.025	0.024	3.0
Arg.....	0.039	0.038	0.039	0.036	0.038	0.039	0.038	4.8
Asp.....	0.113	0.113	0.112	0.112	0.105	0.108	0.117	14.6
Thr.....	0.077	0.075	0.078	0.076	0.073	0.075	0.077 <sup>d</sup>	9.6
Ser.....	0.040	0.037	0.038	0.039	0.032	0.033	0.041 <sup>d</sup>	5.1
Glu.....	0.145	0.148	0.154	0.144	0.144	0.148	0.146	18.3
Pro.....	0.041	0.041	0.040	0.036	0.039	0.043	0.041	5.1
Gly.....	0.064	0.064	0.064	0.062	0.063	0.063	0.063	7.9
Ala.....	0.113	0.116	0.112	0.112	0.110	0.114	0.115	14.4
Cys.....	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.0
Val.....	0.069	0.068	0.076	0.071	0.071	0.077	0.074	9.2
Met.....	0.026	0.028	0.029	0.027	0.026	0.028	0.028	3.5
Ile.....	0.038	0.038	0.042	0.038	0.040	0.040	0.040	5.0
Leu.....	0.091	0.091	0.098	0.088	0.090	0.094	0.093	11.6
Tyr.....	0.049	0.049	0.054	0.049	0.052	0.054	0.053	6.6
Phe.....	0.023	0.018	0.025	0.021	0.025	0.023	0.024	(3.0)
Trp.....	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1 <sup>e</sup>

<sup>a</sup> The results of duplicate analyses are given (see the text).

<sup>b</sup> Three tryptic peptides containing phenylalanine were obtained from tryptic digests of cyanogen bromide fragments by separation on Dowex 50 columns (see the text).

<sup>c</sup> The lysine content calculated in this series of analyses was lower than the usual observed value, since the standard lysine gave an unusually high constant. The value shown in parentheses, which was obtained from analyses of 20-hour hydrolysates of several different samples of the nuclease, is considered a better value.

<sup>d</sup> Corrected for slight destruction during hydrolysis.

<sup>e</sup> See the text.

ysis for cysteic acid after performic acid oxidation and by radioactive measurements after alkylation with <sup>14</sup>C-iodoacetic acid. Cysteic acid levels were less than 0.08 mole per mole of nuclease (Table VI). Although approximately 1 mole of iodoacetic acid-1-<sup>14</sup>C was bound per mole of nuclease, subsequent experiments demonstrating slight alkylation of  $\epsilon$ -amino groups of lysine and imidazole nitrogens of histidine residues appear to account for this radioactivity. Furthermore, the direct analysis and sequence determination of tryptic fragments<sup>6</sup> of the nuclease, accounting for the total amino acid content of the protein, indicate the complete absence of half-cystine residues in the polypeptide chain.

Both spectrophotometric methods described under "Experimental Procedure" indicated the presence of approximately 1 mole of tryptophan per mole (Table VII), based on an assumed

<sup>6</sup> To be published.

The amide-NH<sub>2</sub> content, determined as described above, was shown to be 11 moles per mole of nuclease, based on an assumed value of 3 residues of phenylalanine per mole (Table V).

The amino acid analyses permit the calculation of a minimum molecular weight for the protein of approximately 17,000.<sup>7</sup> Preliminary studies on the amino acid content of the separated tryptic peptides are in good agreement with this figure, as described below.

**NH<sub>2</sub>-terminal Residue**—The DFB method yielded alanine exclusively as the NH<sub>2</sub>-terminal residue in a yield of 80 to 90%. The phenylthiohydantoin derivative of alanine, obtained in a yield of 90% by the Edman procedure, was identified in two dif-

<sup>7</sup> The results of high speed equilibrium studies in the ultracentrifuge, together with determinations of partial specific volume in density gradients, yield a molecular weight value in agreement with this number within experimental error; J. Heins, R. Suriano, H. Taniuchi, and C. Anfinsen, unpublished results.

ferent solvent systems on thin layer chromatograms. Alkaline hydrolysis of the intermediate thiazolinone, formed by cyclization of the original phenylthiocarbonyl nuclease, yielded alanine, with a trace amount of glycine upon amino acid analysis.

**COOH-terminal Residue**—Treatment with carboxypeptidase A liberated glutamine in a yield of approximately 80% after incubation at both 25° and 37° for 24 hours. Glutamine was identified qualitatively on ascending chromatograms with 80% pyridine as solvent ( $R_F$ , 0.21) (asparagine,  $R_F$ , 0.14; serine, 0.35). No other amino acids were detected either on the automatic amino acid analyzer or on paper chromatograms. After 24 hours of incubation with the enzyme at 25° and 37°, respectively, 85 and 78% of the specific enzymic activity of the nuclease remained.

**Peptide Maps**—Fig. 11 shows a peptide map obtained with a mixture of peptides produced by trypsin digestion of the nuclease at 37° for 3 hours. Major spots, numbered F2 to F25, were highly reproducible from preparation to preparation. Only F25 gave a positive Ehrlich reaction for tryptophan. Components F15, F17, F18, and F19 were clearly bleached by the platinum iodide test for methionine. F17 and F19 also gave a positive Sakaguchi test for arginine. F2 produced the pink color characteristic of histidine residues when treated with the Pauli reagent. F4 occupied the same position as free lysine as determined by a mixed peptide map of an eluted sample of the peptide with this amino acid.

The components numbered F15, F17, F18, and F19 were eluted from eight peptide maps after location by light staining with ninhydrin, the individual eluates were pooled, and samples were hydrolyzed for automatic quantitative amino acid analysis. Aliquots were also submitted to end group analysis by the DFB technique as described under "Experimental Procedure." These results are shown in Table VIII. The four peptides that gave positive reactions with the platinum iodide method contained methionine as expected and account for the 4 residues of this amino acid indicated by the amino acid analyses shown in Table V. When, as described below, peptide maps were prepared with trypsin digests of CNBr-treated nuclease, the four methionine peptides had disappeared and several new peptides were found as expected.

Amino acid analysis of acid hydrolysates of samples F2 and F4 eluted from the paper by the same method as above indicated the compositions (His, Pro, Lys) and (Lys), respectively.

**Cyanogen Bromide Fragments**—If the nuclease contains 4 residues of methionine, five fragments should be formed after cleavage with cyanogen bromide. End group analysis by the DFB method of CNBr digests showed the presence of 2 moles of  $\text{NH}_2$ -terminal valine and 1 each of alanine, threonine, and tyrosine per mole of nuclease (Table IX). The yields of the latter two DNP-amino acids were unaccountably low in this experiment but have approached 1 residue per molecule in others. Among a variety of methods tried, the most successful for the separation of the cyanogen bromide cleavage products was gel filtration on Sephadex G-50, the results of which are summarized in Fig. 12. The over-all recovery of material applied to the column (based on absorbance at 280  $m\mu$ ) was 65%. An aliquot of every sixth effluent sample was examined by paper electrophoresis at pH 6.5. On the basis of these qualitative tests, fractions were pooled as shown in Table X and lyophilized. These pooled fractions were again examined by paper and polyacrylamide gel electrophoreses and by end group analysis (Table X). Fractions III, VII, and VIII, which were relatively homogeneous on the basis of these

TABLE VI  
Amino acid analysis of nuclease after performic acid oxidation

	Amino acid content	Calculated moles per 3 moles of phenylalanine
	$\mu\text{mole}$	
Lys.....	0.825	22.2
His.....	0.100	2.7
Arg.....	0.175	4.7
Cysteic acid.....	0.003	0.1
Methionine sulfone.....	0.135	3.6
Asp.....	0.502	13.6
Thr.....	0.341	9.2
Ser.....	0.166	4.5
Glu.....	0.664	17.9
Pro.....	0.217	5.9
Gly.....	0.360	9.7
Ala.....	0.511	13.8
Cys.....	0.000	0.0
Val.....	0.325	8.8
Met.....	0.000	0.0
Ile.....	0.175	4.7
Leu.....	0.426	11.5
Tyr.....	0.196	5.3
Phe.....	0.111	(3)

TABLE VII  
Spectrophotometric analysis of mole ratio of tyrosine to tryptophan

Method of analysis	Ratio of tyrosine to tryptophan
Method of Benzene and Schmid (23).....	5
Method of Goodwin and Morton (22).....	6.6
Amino acid analysis after alkaline hydrolysis.....	8.7 <sup>a</sup>

<sup>a</sup> This value was obtained from a sample subjected to 44 hours of hydrolysis, which gave the maximum yield of tryptophan in the series of 20-, 44-, and 68-hour hydrolysates.

tests, were further purified by preparative paper electrophoresis at pH 6.5. The purified components after elution were hydrolyzed and subjected to amino acid analysis.

Fraction V, which contained both  $\text{NH}_2$ -terminal threonine and alanine, was subjected to preparative electrophoresis in the Brinkmann apparatus (Fig. 13) as described in "Experimental Procedure." Aliquots of the effluent fractions were examined by gel electrophoresis, with standard gel. On the basis of these tests, Fractions 27 to 31 (Fraction Va) and 38 to 40 (Fraction Vb) were pooled and examined for  $\text{NH}_2$ -terminal end groups and amino acid composition.

Fraction I, as indicated in Table X, contained all of the amino-terminal residues of the entire CNBr digest and produced a peptide map after further digestion with trypsin which indicated the presence of all the components of the CNBr digest. This fraction was not examined further since it appeared to consist of an aggregated mixture of all the peptide components.

A summary of the properties of the five large fragments produced by CNBr and their designations is given in Table XI. As shown, homoserine is present in Fractions Va and Vb, VII, and VIII (Peptides A, C, D, and B, respectively). Fraction III (Peptide E), lacking this residue, must be COOH-terminal in the nuclease; and Fraction Va (Peptide A), which contains an  $\text{NH}_2$ -

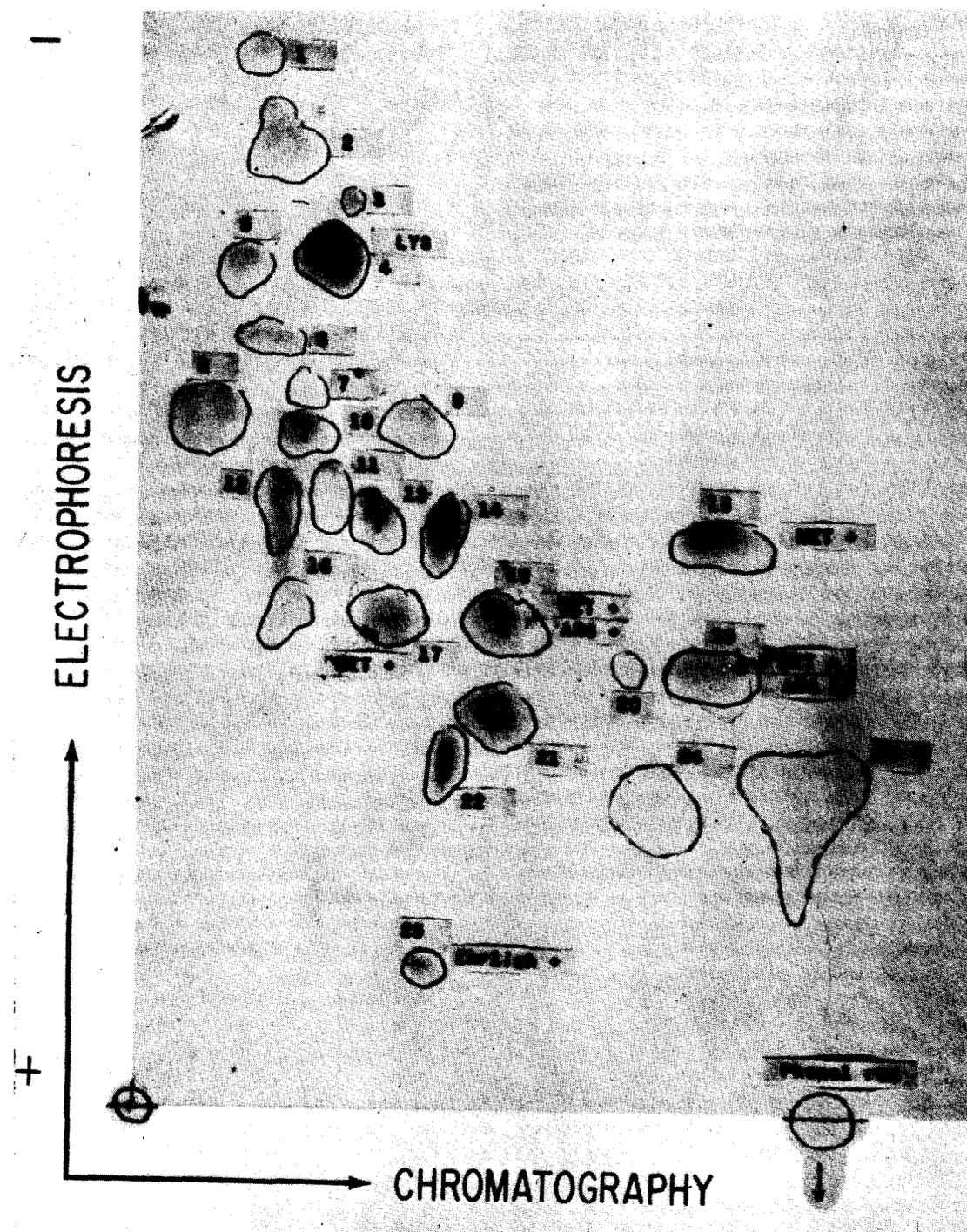


FIG. 11. Peptide map obtained from a trypsin digest of the nuclease. The digest, containing 1 mg of protein, was applied to Whatman No. 3MM paper for two-dimensional separation. Electrophoresis was carried out at 2500 volts for 80 min. The peptides giving a positive platinum iodide reaction are indicated as *MET*. The spots indicated by *Y* gave yellow color upon staining with ninhydrin-alcohol solution. *Ehrlich* indicates the spot with a positive Ehrlich reaction. Among those spots giving a positive Sakaguchi reaction, only spots clearly identified on the original map are shown (as *Arg*).

terminal alanine residue, must be the  $\text{NH}_2$ -terminal peptide. Only Fraction III (Peptide E) gave a positive test for tryptophan and, as discussed below, yielded a fragment upon trypsin digestion which corresponded to the single Ehrlich-positive components on the peptide map. The sum of the residues determined for the individual CNBr fragments corresponds well with the total amino acid content presented in Table V above.

*Trypsin Digestion of CNBr Fragments*—Samples of cyanogen bromide peptide Fractions III, V, and VII were digested with trypsin, and the resulting mixtures of peptides were subjected to separation on Dowex 50-X2 columns as described under "Experimental Procedure." The chromatographic patterns are shown in Fig. 14. The purity of the separated peptides was examined by two-dimensional peptide mapping, amino acid analysis, and

end group determinations by both the DFB and the Edman procedures. Further purification, when necessary, was performed by either paper electrophoresis or paper chromatography as described in "Experimental Procedure." Table XII summarizes

TABLE VIII

*Amino acid compositions and amino-terminal residues of tryptic peptides giving positive reaction for methionine*

The values reported are in micromoles, and the assumed number of residues is given in parentheses. Where no numbers are given, the values obtained were equal to or less than 0.002  $\mu$ mole.

	Designation on peptide map			
	F-15	F-17	F-18	F-19
Lys.....	0.012 (1)	0.025 (1)	0.003 (0)	
His.....		0.003 (0)		
Arg.....		0.004 (0)	0.015 (1)	0.015 (1)
Asp.....		0.023 (1)	0.005 (0)	0.020 (1)
Thr.....		0.003 (0)	0.016 (1)	
Ser.....	0.003 (0)	0.009 (0)	0.004 (0)	0.007 (0)
Glu.....		0.028 (1)	0.015 (1)	0.017 (1)
Pro.....		0.007 (0)	0.015 (1)	
Gly.....	0.004 (0)	0.011 (0)	0.013 (1)	0.006 (0)
Ala.....		0.023 (1)	0.004 (0)	0.018 (1)
Cys.....				
Val.....		0.021 (1)		0.029 (2)
Met.....	0.010 (1)	0.013 (1)	0.012 (1)	0.007 (1)
Ile.....				
Leu.....	0.009 (1)			0.017 (1)
Tyr.....	0.007 (1)			
Phe.....		0.004 (0)	0.015 (1)	
NH <sub>2</sub> -terminal residue <sup>a</sup> .....	Leucine	Methionine	Glycine	Methionine

<sup>a</sup> The analysis was qualitative, but only a single ether-soluble DNP-amino acid was found for each peptide.

the data obtained on these tryptic fragments. This table contains only the data on peptides which were obtained in significant amounts as judged from the amino acid analyses. The calculated yields of these peptides after chromatography on Dowex 50 ranged from 30 to 90%, except those peptides derived from the contaminated fragment described below.

The trypsin digest of purified Peptide C (obtained by preparative electrophoresis of Fraction V; see Table XI) was subjected to peptide mapping, and the position of each component was used to assign, by difference, the map positions of the tryptic fragments of Peptide A, the other CNBr component in Fraction V. Trypsin digests of Fraction VII contained, as minor components, tryptic Peptides TVII-1 and VII-4b (in yields of less than 10% of each peptide). These could be assigned to CNBr Peptide A, which contaminates Fraction VII. They did not

TABLE IX

*Amino terminal analysis of cyanogen bromide fragments of nuclease with DNP-method*

Sample 1, intact nuclease; Sample 2, nuclease treated with 70% HCOOH (without CNBr); Sample 3, nuclease treated with CNBr in 70% HCOOH. Each sample contained 0.12  $\mu$ mole of the nuclease.

Sample	DNP-amino acid found <sup>a</sup>			
	Alanine	Valine	Threonine	Tyrosine
	$\mu$ mole	$\mu$ mole	$\mu$ mole	$\mu$ mole
1	0.10			
2	0.11			
3	0.12	0.24	0.01 <sup>b</sup>	0.02 <sup>b</sup>

<sup>a</sup> No water-soluble DNP-amino acids, other than  $\epsilon$ -DNP-lysine, could be detected by paper electrophoresis in pyridinium acetate, pH 6.5.

<sup>b</sup> The low recovery of these end groups in the present experiment is discussed in the text.

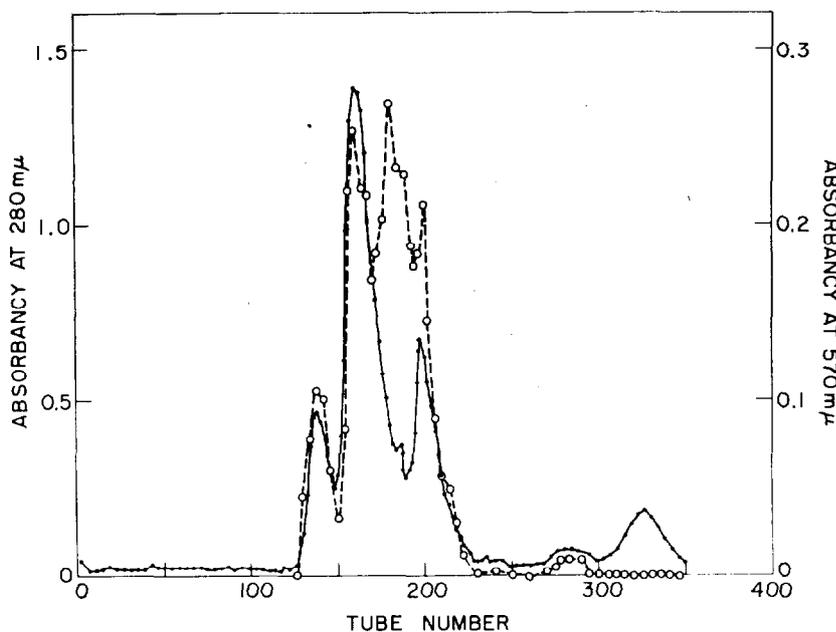


FIG. 12. Gel filtration pattern of cyanogen bromide peptides prepared from the nuclease. The column was loaded with 360 mg of cyanogen bromide digest. Other details are given in "Experimental Procedure." ●—●, absorbance at 280 m $\mu$ ; ○--○, absorbance at 570 m $\mu$ .

appear in the peptide maps of Peptide C. Cyanogen bromide Fragment B (obtained from Fraction VIII of the Sephadex G-50 separation) did not fragment further upon trypsin as tested by peptide mapping. The composition of the CNBr fragments is compared with the sum of the amino acid compositions of the trypsin fragments assigned to them in Table XIII. The total amino acid analysis of the original nuclease is included in this table for over-all comparison.

TABLE X

Qualitative summary of several sets of quantitative analyses for amino-terminal residues of cyanogen bromide fragments prepared by Sephadex G-50 gel filtration

Fraction	Pooled tubes <sup>a</sup>	DNP-amino acid found <sup>b</sup>			
		Alanine	Valine	Threonine	Tyrosine
I	128-145	+	+	+	+
III	151-168	-	+++	-	-
IV	169-173	+	++	+	+
V	174-191	+++	Trace	+++	Trace
VI	192-195	+	++	+	-
VII	196-213	-	+++	-	-
VIII	272-295	-	-	-	+++

<sup>a</sup> See Fig. 10.

<sup>b</sup> Only ether-soluble DNP-amino acids were analyzed (see Table IX). The relative intensities of the DNP-amino acids are qualitatively indicated by plus signs. Ether-soluble DNP-amino acids, other than those shown in this table, were not found.

*Reconstruction of Tryptic Peptide Map*—The map positions of separated tryptic peptides of each cyanogen bromide peptide were determined on two-dimensional peptide maps. A reconstructed peptide map was prepared as shown in Fig. 15. The tryptic

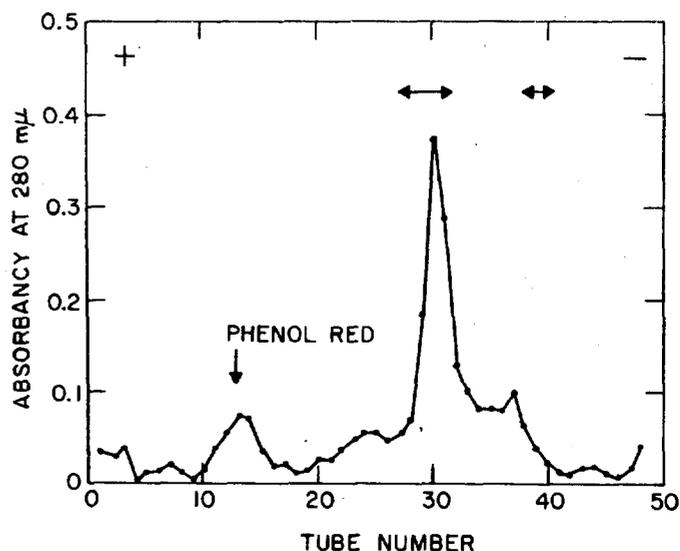


FIG. 13. Preparative electrophoresis of cyanogen bromide fragment Fraction V, obtained by gel filtration. Approximately 10  $\mu$ moles of peptides, dissolved in 0.5 ml of 0.05 M  $\text{NH}_4\text{HCO}_3$ , were applied through the entry port above tube 30. Other details of the procedures are described in "Experimental Procedure." Pooled fractions are indicated by horizontal arrows.

TABLE XI

Approximate amino acid composition of cyanogen bromide fragments

Values are reported in micromoles. The values in parentheses represent assumed number of residues.

	Amino acid content					Total number of residues
	Fraction III	Fraction Va	Fraction Vb	Fraction VII	Fraction VIII	
Lys. ....	0.223 (5)	0.236 (4-5)	0.151 (5-6)	0.081 (4-5)	0.039 (1)	(19-22)
His. ....	0.030 (1)	0.041 (1)	0.019 (1)	0.000 (0)	0.000 (0)	(3)
Arg. ....	0.071 (2)	0.064 (0)	0.021 (1)	0.029 (1-2)	0.000 (0)	(3-5)
Asp. ....	0.276 (6-7)	0.089 (2)	0.031 (1)	0.071 (3-4)	0.002 (0)	(12-14)
Thr. ....	0.042 (1)	0.154 (3-4)	0.086 (3)	0.020 (1)	0.000 (0)	(8-9)
Ser. ....	0.105 (2-3)	0.043 (1)	0.028 (1)	0.011 (0)	0.002 (0)	(4-5)
Glu. ....	0.372 (9)	0.052 (1)	0.077 (3)	0.075 (4)	0.052 (1)	(18)
Pro. ....	0.046 (1)	0.053 (1)	0.077 (3)	0.005 (0)	0.018 (1)	(6)
Gly. ....	0.086 (2)	0.057 (1)	0.059 (2)	0.075 (4)	0.042 (1)	(10)
Ala. ....	0.241 (6)	0.119 (2-3)	0.055 (2)	0.057 (3)	0.000 (0)	(13)
Cys. ....	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	(0)
Val. ....	0.146 (3)	0.053 (1)	0.041 (2)	0.038 (2)	0.000 (0)	(8)
Met. ....	0.000 (0)	0.000 (0)	0.002 (0)	0.000 (0)	0.000 (0)	(0)
Ile. ....	0.038 (1)	0.088 (2)	0.012 (1)	0.029 (1-2)	0.000 (0)	(5-6)
Leu. ....	0.198 (5)	0.131 (3)	0.068 (2-3)	0.023 (1)	0.000 (0)	(11-12)
Tyr. ....	0.000 (2) <sup>a</sup>	0.005 (0) <sup>a</sup>	0.001 (1) <sup>a</sup>	0.000 (3) <sup>a</sup>	0.031 (1)	(7)
Phe. ....	0.003 (0)	0.007 (0)	0.034 (1-2)	0.016 (1)	0.000 (0)	(2-3)
Trp. ....	— (1) <sup>b</sup>	— (0)	— (0)	— (0)	— (0)	(1)
Homoserine. ....	0.000 (0)	0.054 (1)	0.032 (1)	0.021 (1)	0.041 (1)	(4)
NH <sub>2</sub> -terminal residue.	Valine	Alanine	Threonine	Valine	Tyrosine	
Designation of peptide.	E	A	C	D	B	

<sup>a</sup> The values obtained from analysis of samples before purification by paper electrophoresis (see the text) were used to correct for destruction of tyrosine.

<sup>b</sup> Determined by staining with Ehrlich's reagent (see the text).

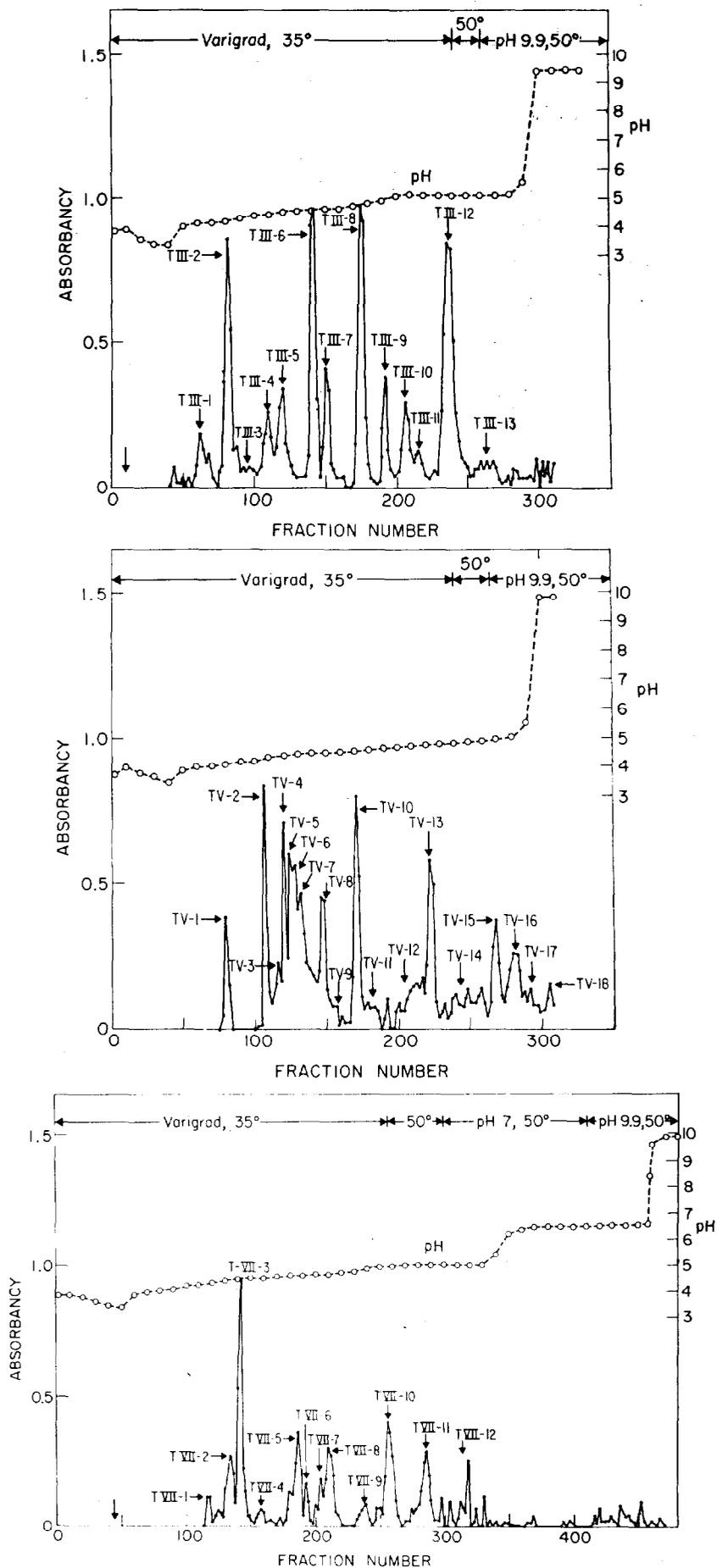


FIG. 14. Chromatography of tryptic peptides prepared from cyanogen bromide Fragments III (*upper*), V (*middle*), and VII (*lower*), on Dowex 50-X2. Samples equivalent to approximately 4, 2, and 4  $\mu$ moles of Fragments III, V, and VII, respectively, were applied.

TABLE XII

Summary of amino acid analyses of acid hydrolysates of tryptic peptides prepared from cyanogen bromide fragment

The values reported are in micromoles, and the assumed number of residues is given in parentheses. Where no numbers are given, the values obtained were equal to or less than 0.002  $\mu$ mole. A positive Ehrlich reaction for tryptophan is shown by a plus sign. The dashes indicate that quantitative analyses were not performed. Peptides derived from Sephadex G-50 Fractions III, V, and VII (see Table X) are summarized in Parts A, B, and C of the table, respectively. As described in the text and in Table V, Fractions III and VII were essentially homogeneous samples of CNBr Fragments E and D. Fraction V was further separated by curtain electrophoresis to yield CNBr Fragments A and C. Peptide maps of trypsin digests of these purified fragments were used to assign the tryptic peptides obtained from Fraction V to either CNBr Fragment A or C. Dowex 50 Fractions TIII-10, TIII-11, TV-3, TV-4, TV-14, TV-16, TV-18, TVII-6, TVII-9, and TVII-12 corresponded to minor spots on peptide maps, and their compositions are not reported here. A sample of Peptide TIII-2 was digested with subtilisin. The two fragments formed were separated by paper chromatography as described in "Experimental Procedure." These fragments were designated TIII-2-S-1 and TIII-2-S-2.

Table XIII

	TIII-1c	TIII-2	TIII-2-S-1	TIII-2-S-2	TIII-4c	TIII-4e	TIII-5b	TIII-5c	TIII-6	TIII-7a	TIII-7b	TIII-8b	TIII-9c	TIII-12
Lys.....	0.021 (1)					0.009 (1)	0.012 (1)	0.012 (1)		0.017 (1)	0.029 (1)	0.054 (2)	0.034 (2)	0.029 (1)
His.....														0.020 (1)
Arg.....									0.032 (1)					0.026 (1)
Asp.....		0.072 (4)	0.119 (3)	0.024 (1)			0.006 (0)		0.025 (1)					0.042 (2)
Thr.....														0.018 (1)
Ser.....		0.031 (2)	0.064 (2)			0.007 (1)						0.016 (1)		
Glu.....	0.027 (1)	0.044 (2)	0.086 (2)			0.016 (2)	0.014 (1)		0.030 (1)	0.019 (1)	0.031 (1)	0.070 (2)	0.023 (1)	0.048 (2)
Pro.....														0.033 (1)
Gly.....	0.025 (1)	0.022 (1)	0.040 (1)				0.003 (0)		0.003 (0)	0.017 (1)	0.028 (1)			0.003 (0)
Ala.....	0.026 (1)	0.022 (1)	0.043 (1)		0.004 (1)	0.016 (2)			0.030 (1)	0.019 (1)	0.034 (1)	0.071 (2)		0.010 (0)
Cys.....														
Val.....					0.003 (1)									0.031 (1)
Met.....									0.057 (2)					
Ile.....		0.019 (1)		0.024 (1)										0.003 (0)
Leu.....	0.026 (1)	0.021 (1)		0.023 (1)					0.032 (1)	0.019 (1)	0.038 (1)			0.050 (2)
Tyr.....					0.003 (1)									0.026 (1)
Phe.....														
Trp.....	—	+ (1) <sup>a</sup>	—	+ (1)	—	—	—	—	—	—	—	—	—	—
Homoserine.....														
NH <sub>2</sub> -terminal residue <sup>b</sup>		Leucine	Serine	Leucine	Valine	Serine	Glutamic acid	Lysine	Valine	Glutamic acid	Glutamic acid	Lysine	Lysine	Valine
Assigned to cyanogen bromide peptide.....	E	E	E	E	E	E	E	E	E	E	E	E	E	E
Inclusion in summation (Table XIII) indicated by +.....	— <sup>c</sup>	+	— <sup>d</sup>	— <sup>d</sup>	+	— <sup>e</sup>	— <sup>f</sup>	— <sup>g</sup>	+	— <sup>c</sup>	+	+	+	+

Table XIIB

	TV-1	TV-2	TV-5b	TV-6	TV-7b	TV-8a	TV-10	TV-13	TV-15a
Lys.....		0.015 (1)	0.011 (1)			0.010 (1)	0.018 (1)	0.028 (2)	0.029 (2)
His.....						0.004 (1)			
Arg.....									0.010 (1)
Asp.....		0.030 (2)	0.010 (1)						
Thr.....		0.016 (1)	0.012 (1)			0.006 (1)	0.031 (2)	0.014 (1)	0.007 (1)
Ser.....				0.009 (1)		0.013 (1)			
Glu.....				0.012 (1)	0.012 (1)	0.011 (1)	0.013 (1)	0.015 (1)	
Pro.....				0.010 (1)	0.006 (1)				
Gly.....		0.013 (1)		0.011 (1)			0.003 (0)	0.013 (1)	
Ala.....		0.014 (1)		0.021 (2)	0.011 (1)	0.013 (1)			
Cys.....									
Val.....		0.016 (1)	0.007 (1)					0.016 (1)	
Met.....									
Ile.....		0.014 (1)			0.008 (1)				
Leu.....	0.017 (1)		0.035 (3)		0.010 (1)	0.010 (1)			
Tyr.....				0.010 (1)					
Phe.....				0.006 (1)					0.016 (1)
Trp.....	—	—	—	—	—	—	—	—	—
Homoserine.....	0.017 (1)								
NH <sub>2</sub> -terminal residue.....	Leucine	Alanine	Leucine	Tyrosine	Glutamic acid	Alanine	Threonine	Lysine	Threonine
Assigned to cyanogen bromide peptide.....	A	A	C	C	A	A	C	C	C
Inclusion in summation.....	+	+	+	+	+ <sup>h</sup>	+	+ <sup>i</sup>	+	+

Table XIIC

	TVII-1	TVII-2c	TVII-3b	TVII-3c	TVII-4b	TVII-5a	TVII-8a	TVII-8b	TVII-11a
Lys.....	0.009 (1)	0.022 (1)	0.004 (0)	0.027 (1)	0.012 (1)	0.031 (1)	0.020 (2)	0.003 (0)	
His.....									0.050 (1-2) <sup>i</sup>
Arg.....								0.035 (1)	
Asp.....	0.015 (2)	0.023 (1)	0.005 (0)	0.033 (1)		0.035 (1)	0.012 (1)		
Thr.....	0.003 (1)	0.014 (1)			0.012 (2)				
Ser.....					0.006 (1)	0.004 (0)	0.004 (0)	0.003 (0)	
Glu.....			0.011 (0)	0.036 (1)			0.028 (2)	0.055 (1)	
Pro.....									
Gly.....	0.008 (1)	0.003 (0)	0.021 (1)		0.003 (0)	0.029 (1)	0.005 (0)	0.036 (1)	0.024 (1)
Ala.....	0.005 (1)		0.033 (1)	0.033 (1)	0.008 (1)	0.029 (1)			
Cys.....									
Val.....	0.006 (1)			0.033 (1)			0.010 (1)		
Met.....									
Ile.....	0.008 (1)		0.003 (0)			0.030 (1)	0.010 (1)		
Leu.....			0.033 (1)						
Tyr.....			0.027 (1)			0.034 (1)			0.014 (1)
Phe.....			0.005 (0)				0.017 (1)		
Trp.....	—	—	—	—	—	—	—	—	—
Homoserine.....						0.004 (0)			
NH <sub>2</sub> terminal residue.....	Alanine	Threonine	Glycine	Valine		Isoleucine	Lysine	Glycine	Tyrosine
Assigned to cyanogen bromide peptide.....	A	D	D	D	A	D	D	D	D
Inclusion in summation.....	— <sup>k</sup>	+	+	+	— <sup>k</sup>	+	+	+	+

<sup>a</sup> The presence of tryptophan was also confirmed by digestion with leucine aminopeptidase (Worthington) by the method of Canfield (18). Ammonium bicarbonate buffer (0.05 M) containing 0.01 M MgCl<sub>2</sub> was used. Tryptophan was determined quantitatively on the amino acid analyzer.

<sup>b</sup> Aliquots of fractions obtained by chromatography on Dowex 50 were used for amino-terminal residue determination by both dinitrophenylation and Edman degradation. When a peptide was contaminated with a second peptide, the amino-terminal residue could be assigned on the basis of the amino acid composition of the purified peptide.

<sup>c</sup> TIII-1e and TIII-7a have the same amino acid composition as TIII-7b, presumably due to the formation of pyrrolidone carboxylic acid from a terminal glutamine residue.

<sup>d</sup> TIII-2-S-1 and TIII-2-S-2 are constituents of TIII-2.

<sup>e</sup> Peptide TIII-4e is derived from TIII-8b, by loss of a single lysine residue.

<sup>f</sup> Peptide TIII-5b is assumed to be derived from TIII-9c by loss of the NH<sub>2</sub>-terminal lysine residue. This relationship is now under reexamination.

<sup>g</sup> Only free lysine was detected.

<sup>h</sup> The basis of this assignment was obtained from qualitative amino acid analysis of tryptic Peptide TVII-11b, which seemed to include the amino acid components of TV-7b. This peptide is presumably derived from cyanogen bromide Fragment A which contaminated Fragment D as a trailing fraction during the gel filtration on Sephadex G-50 (see the text).

<sup>i</sup> Further study with successive Edman degradation suggests that the glutamic acid residue is not part of this peptide. The nature of the contamination is not clear at present.

<sup>j</sup> In the summation in Table XIII, 1 residue of arginine has tentatively been assigned to this peptide. The presence of a possible 2nd arginine residue is under investigation.

<sup>k</sup> Amino acid compositions of TVII-1 and TVII-4b are the same as those of TV-2 and TV-8a, respectively (see Footnote h and the text).

TABLE XIII

Amino acid composition of cyanogen bromide fragments based on analysis of tryptic peptides derived from each

The following 24 tryptic peptides (identified in the bottom line of Table XII), together with CNBr Fragment B (Table XI), account closely for the amino acid composition of the nuclease. Peptide A: TV-1, TV-2, TV-7b, TV-8a; Peptide C: TV-5b, TV-6, TV-10, TV-13, TV-15a, F-2; Peptide D: TVII-2c, TVII-3b, TVII-3c, TVII-5a, TVII-8a, TVII-8b, TVII-11a; Peptide E: TIII-2, TIII-6, TIII-7b, TIII-8b, TIII-9c, TIII-12. The numbers in parentheses are taken from Table XI. The amino acid composition of the nuclease is taken from Table V. Where no numbers are given, the values obtained were zero.

	Amino acid content in cyanogen bromide fragment					Sum of tryptic peptides <sup>b</sup>	Amino acid composition of the nuclease
	A	B	C <sup>a</sup>	D	E		
Lys.....	3 (5)	(1)	6 (5-6)	5 (4-5)	6 (5-6)	21	21.4
His.....	1 (1)		1 (1)		1 (1)	3	3.0
Arg.....			1 (1)	2 (1-2)	2 (2)	5	4.8
Asp.....	2 (2)		1 (1)	4 (3-4)	7 (6-7)	14	14.6
Thr.....	4 (3-4)		3 (3)	1 (1)	1 (1)	9	9.6
Ser.....	1 (1)		1 (1)		3 (2-3)	5	5.1
Glu.....	1 (1)	(1)	3 (3)	4 (4)	9 (9)	18	18.3
Pro.....	1 (1)	(1)	3 (3)		1 (1)	6	5.1
Gly.....	1 (1)	(1)	2 (2)	4 (4)	2 (2)	10	7.9
Ala.....	3 (2-3)		2 (2)	3 (3)	6 (6)	14	14.4
Cys							
Val.....	1 (1)		2 (2)	2 (2)	4 (4)	9	9.2
Met.....							3.5
Ile.....	2 (2)		(1)	2 (1-2)	1 (1)	5	5.0
Leu.....	2 (3)		3 (2-3)	1 (1)	5 (5)	11	11.6
Tyr.....		(1)	1 (1)	3 (3)	2 (2)	7	6.6
Phe.....			2 (1-2)	1 (1)		3	3.0
Trp.....					1 (1)	1	1
Homoserine	1 (1)	(1)	(1)	(1)		2	

<sup>a</sup> Tryptic peptide F-2 (His, Pro, Lys) (see the text) which was not recovered among the purified tryptic peptides obtained from cyanogen bromide fragments, is assigned to Peptide C on the basis of histidine content.

<sup>b</sup> The estimated number of residues in Peptide B is included.

digest of the nuclease preparation was also subjected to mapping, and major spots were cut and eluted as described in "Experimental Procedure." The resulting peptides were subjected to amino acid analysis after acid hydrolysis. By comparison of position on the peptide map and amino acid composition, almost all of the major spots of the nuclease digest could be found on the reconstructed peptide map, as indicated in Fig. 15, with the exception of those peptides containing methionine. Of the major trypsin fragments studied, only peptide F2 of the original peptide map (Fig. 9) was not accounted for by the CNBr fragments.

**Linear Arrangement of CNBr Fragments**—On the basis of NH<sub>2</sub>-terminal end group data alone, Peptide A can be assigned to the NH<sub>2</sub>-terminal position of the chain. Furthermore, both of the alanine-terminal peptides isolated from trypsin digests may be assigned to this cyanogen bromide fragment (tryptic Peptides TV-2 and TV-8a). Peptide E must occupy the COOH-terminal position since it lacks homoserine and yields the trypsin Fragment TIII-2 which contains neither lysine nor arginine and from

which is liberated glutamine in 60% yield upon 2-hour incubation with carboxypeptidase A at 37° as described under "Experimental Procedure."

The remaining CNBr fragments can be aligned on the basis of the methionine content of the nuclease (4 per molecule) and the composition of the four methionine-containing peptides isolated

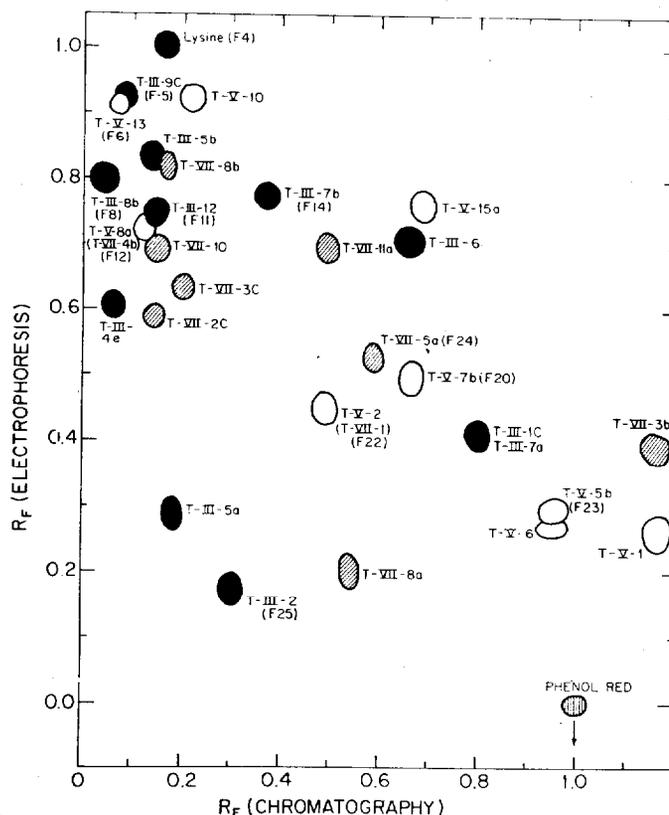


FIG. 15. Reconstructed tryptic peptide map. Phenol red and lysine served as reference standards in the chromatographic and electrophoretic dimensions, respectively. The significance of the designations shown in parentheses is described in the text. On a qualitative basis, the following additional assignments of peptides can be made on the map. Component F-16 on the "experimental" peptide map (see Fig. 11) contains a mixture of peptides, some incompletely cleaved by trypsin, including TVII-2c, and TIII-4e. Similarly, F-21 contains TV-6 and TV-10. F-24 corresponds to the addition of TVII-3b and TVII-5a, and F-14 contains TVII-11a in addition to TIII-7b.

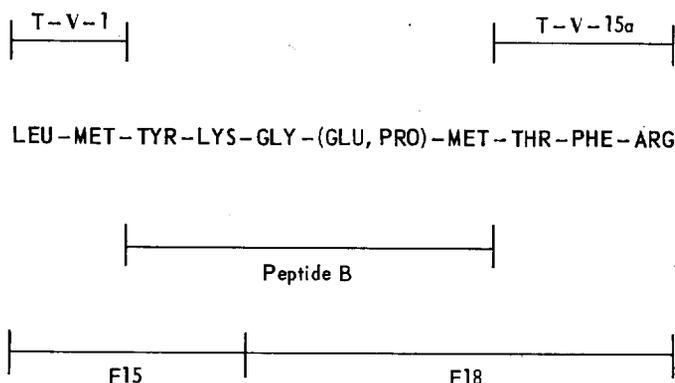


FIG. 16. The linear arrangement of cyanogen bromide Fragments A, B, and C. Peptide B corresponds to Fragment B.

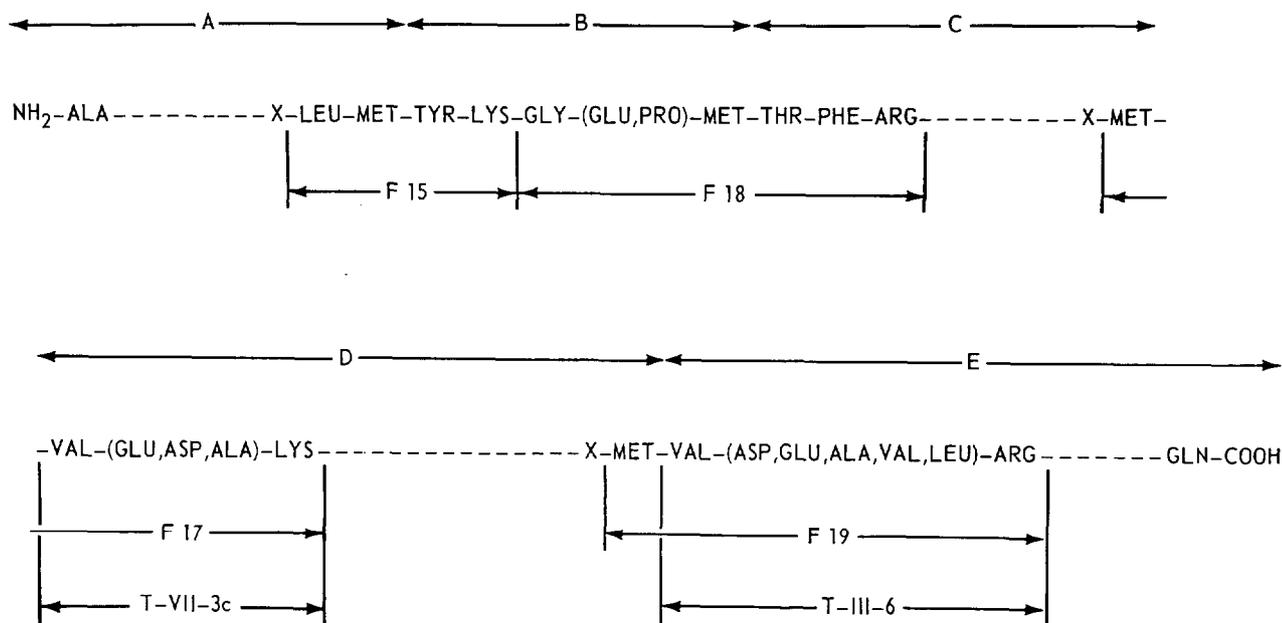


FIG. 17. The linear arrangement of cyanogen bromide fragments derived from the nuclease. The residues indicated as X are presumed to be lysine or arginine. Subsequent studies<sup>6</sup> have indeed shown that these residues are lysine.

from peptide maps. If we compare the amino acid composition and NH<sub>2</sub>-terminal residue of Peptide B with those of trypsin Fragments F-15 and F-18 which contain methionine, the arrangement shown in Fig. 16 can be assigned. If this arrangement is correct, Leu-homoserine and Thr-Phe-Arg should be found among the trypsin fragments of the neighboring CNBr peptides. Both of these were found only in the trypsin fragments obtained from Fraction V which contains Peptides A and C. Thus, Peptide TV-1 is derived from Peptide A and TV-15a from Peptide C. These observations serve to establish the order of the five CNBr fragments as A-B-C-D-E. The amino acid compositions of the two NH<sub>2</sub>-terminal methionine-containing trypsin fragments of the nuclease, F-17 and F-19, are the same as the compositions of the terminal peptides of CNBr Fragments D and E when methionine is added to each. This consistency gives further confirmation to the arrangement shown in Fig. 17.

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