Purification, Modification during Purification, and Characterization of a Deoxyribonuclease from Pseudomonas aeruginosa*

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SUMMARY

The only major deoxyribonuclease forming acid-soluble fragments from radioactively labeled DNA was isolated from cell-free extracts of Pseudomonas aeruginosa. The enzyme was purified 325-fold, yielding two bands on polyacrylamide gel electrophoresis, the lesser of which was a distinct ribonuclease activity. Extensive modification of the deoxyribonuclease during purification to produce multiple species could be avoided by the use of phenylmethylsulfonyl fluoride and the destruction of endogenous nucleic acids. Marked instability of the enzyme at all stages of purification was overcome by the use of 30% glycerol and 2-mercaptoethanol; the purified preparation had a pH optimum of 7.8. The enzyme closely resembled exonuclease III of Escherichia coli in several respects; the products were 5'-mononucleotides and inorganic phosphate; phosphate release was dependent on the presence of 3'-phosphoryl end groups in DNA; hydrolysis was initiated at the 3'-terminus; native DNA was degraded three to four times faster than heat-denatured DNA. Unlike exonuclease III the entire substrate could be hydrolyzed providing there were several readitions of enzyme during the incubation. It was optimally stimulated by 2.5 mM magnesium and to a lesser extent by manganese. The P. aeruginosa exonuclease was inhibited by ionic strength above 0.05 M Tris because of a reduced binding affinity for the DNA substrate; both the exonuclease and phosphatase functions demonstrated almost identical inhibition by p-chloromercuribenzoate and ethylenediaminetetraacetate. The molecular weight was estimated to be 42,500 by gel filtration.

EXPERIMENTAL PROCEDURE

Materials

The following materials were from commercial source: DEAE-cellulose and hydroxylapatite from Bio-Rad Laboratories; Sephadex gels from Pharmacia; reagents for polyacrylamide gel electrophoresis and 2-mercaptoethanol from Eastman Chemicals; glycero1 and ammonium sulfate from Fisher; pepsin (2500 units per mg), soybean trypsin inhibitor, E. coli alkaline phosphatase (20 units per mg), bovine pancreas RNase (3000 units per mg), p-chloromercuribenzoate, ethylenediaminetetraacetate, sodium chloride, sodium phosphate, sodium acetate, sodium pyrophosphate, sodium orthophosphate, sodium fluoride, sodium carbonate, sodium hydroxide, sodium sulfate, and sodium chloride.

There has been no adequate description of deoxyribonuclease activity in the genus Pseudomonas and in particular for Pseudomonas aeruginosa. An extracellular enzyme was detected by Streifield et al. (1) and partly described by Guschlbauer and Halleck (2). Except for the preliminary investigation by the latter two authors no attention has been paid to intracellular enzymes. The work of Guschlbauer and Halleck (2) suggested some curious properties of P. aeruginosa DNases including a requirement for both citrate and Mg²⁺ ions for maximal activity in a partly purified preparation.

The intracellular enzymes are of interest, in our opinion, because the low levels of total DNase activity present in the strain we investigated may facilitate the detection of other DNase functions present in small quantities but of special significance, e.g. restriction enzymes. Holloway (3) has demonstrated an active restriction mechanism in P. aeruginosa. The organisms are also extremely sensitive to ionizing radiation (4) and thus repair and excision enzymes may be of interest.

Furthermore, P. aeruginosa is a member of an ubiquitous group of microorganisms which because of their widespread nature are of more environmental interest than the much more restricted but better characterized Escherichia coli. P. aeruginosa MAC 264 differs from E. coli in several respects including the failure to incorporate exogenous thymidine,¹ and preliminary experiments suggested differences in the DNases present.

Thus the present work was undertaken to define the basic DNase system in a typical strain of P. aeruginosa and provide the background for subsequent investigations of the problems referred to above. This report deals with the purification and partial characterization of the single quantitatively significant DNase present and with the severe modification problems which occurred during that purification.

¹ L. E. Bryan, unpublished experiments.
per mg) heated to 80° before use, and DNase (2000 units per mg) from Worthington Biochemical Corp.; micrococcal nuclease (1.0 x 10^4 units per mg) from Miles Laboratories; p-nitrophenyl thymidine 3'-phosphate from Raylo Chemicals, Edmonton, Alberta; 32P labeled phosphoric acid and γ labeled ATP from New England Nuclear; polyethyleneimine cellulose thin layer plates from J. T. Baker Chemical Co.; all other reagents were obtained from Sigma and were reagent grade. Micrococcus sordonensis nuclease (electrophoretically pure and free of 3'-nucleotidase activity) and polynucleotide kinase were the kind gifts, respectively, of Miss Cecily Mills, Department of Microbiology, and Dr. V. Petkau, Department of Biochemistry, University of Alberta.

All bacterial strains were obtained from the Department of Microbiology, University of Alberta.

Methods

Growth of P. aeruginosa

The organism was grown in 10-liter New Brunswick Micro-Ferm fermentors (air flow, 4 to 8 liters per min; stirring, 200 to 400 rpm) at 30°. The medium was identical with that of von Tiggesstrom and Razzell (5) except that 0.5% glucose replaced ethanol. Synthetic medium, used only in growth studies, was that of Norris and Campbell (6) except that MgSO4, 7H2O was reduced to 500 mg per liter.

Enzyme Assays

Standard DNase Assay—[3H]DNA was prepared with E. coli 5275 thymine as described by Weissbach and Korn (7) and the DNA isolated by the method of Marmur (8). Unlabeled DNA was prepared in an identical manner with an E. coli strain and a medium containing no thymine.

The assay was modified somewhat from that of Weissbach and Korn (7). Reaction mixtures were reduced to microproportions. Each contained in a volume of 0.1 ml, 50 µg per ml of [3H]DNA (2.8 x 10^6 dpm per nmole), 0.05 M Tris-HCl pH 7.8, 2.5 mM MgCl2, 0.01 M 2-mercaptoethanol, and sufficient enzyme to produce 0 to 32% solubilization of the DNA substrate. Assays were terminated with 100 µl of cold 6% perchloric acid; carrier was bovine serum albumin (50 µl of 10 mg per ml). The supernatant obtained after centrifugation for 1 min in a Beckman Microfuge (200 µl) was counted in 5 ml of Bray's scintillation fluid (9) containing 10 µl of 5 N KOH at 25° in a Nuclear-Chicago Actigraph III) spots cut out and counted in a toluene-based scintillation fluid (Omnifluor, Nuclear-Chicago). Activity was determined by the method of Richardson and Kornberg (11) using 3'.phosphoryl DNA and the production of one A360 unit of acid-soluble material in 1 min.

RNase Assay—Each reaction mixture contained in a volume of 0.1 ml, 1 mg per ml of yeast RNA, 0.1 M Tris-HCl, pH 7.8, 5 mM EDTA, and 0.01 M 2-mercaptoethanol. The procedure was otherwise that described by Nestle and Roberts (10) except that centrifugation was as described for the DNase assay. One unit is the production of 1 nmole of acid-soluble nucleotide equivalent of DNA in 12 min.

DNA Phosphatase Assay—The assay was performed by the method of Richardson and Kornberg (11) using 3'-phosphoryl [32P]DNA in a reaction mixture identical with that of the standard DNase assay. A unit is defined as the production of 1 nmole of phosphate in 12 min. Assays of DNase activity with [32P]DNA required a correction for the phosphate released by the phosphatase function inherent in the P. aeruginosa DNase. This was done by subtracting the 32P measured by the phosphatase assay from the total 32P solubilized in the DNase assay in identical time periods.

Proteolytic Assays—Casein hydrolysis was measured by the method of Kunitz (12). In order to overcome endogenous changes in materials absorbing at 280 nm in crude extracts, preparations to be assayed were in some cases adjusted to pH 3 with 1 N HCl at 25° for 5 min and then assayed. A unit of activity is the production of one A280 unit of acid-soluble material in 20 min. Hydrolysis of benzoyl L-arginine ethyl ester was assayed by the method of Schwerk and Takemada (13) with a Gilford recording spectrophotometer, model 2400. One unit is the formation of 1 µmole of benzoyl L-arginine in 1 min.

Other Assays—Assays were performed as previously described for M. sordonensis nuclease (14), phosphomonoesterase activity (15) modified to contain 1 mM p-nitrophenyl phosphate and 2.5 mM MgCl2, and phosphodiesterase I and II activity (16). In the latter case the assays were modified in some instances to meet the requirements of the P. aeruginosa exonuclease. Tris-HCl (0.05 M) at pH 7.5 or 9.3 was used to assay phosphodiesterase II, and in addition to standard assay conditions for phosphodiesterase I, Tris-HCl (0.05 M), pH 7.5, was used to assay for that enzymatic function. DNA polymerase was assayed essentially as described by Richardson et al. (17) by means of [3H]dATP (specific activity, 1.5 x 10^6 dpm per nmole) with a concentration of 0.2 mM for each of the four deoxynucleoside triphosphates. Aliquots (50 µl) were sampled at 10 min intervals for 40 min to Whatman No. 1MM filter discs (previously treated with 50 µl of 0.01 M EDTA and a 1:10 dilution of saturated sodium pyrophosphate). Filters were washed (18), dried, and counted in a toluene-based scintillation fluid (Nuclear-Chicago, New England Nuclear). Micrococcal nuclease was assayed exactly as the DNase assay except that the reaction mixture contained 0.01 M CaCl2, 0.05 M glycine, pH 9.3, in place of MgCl2 and Tris-HCl. 5'-Nucleotidase assay reaction mixtures contained in a final volume of 100 µl, 5'-ribo- or deoxyribo-nucleoside monophosphoryl DNA, 0.05 M Tris-HCl, pH 7.8, 2.5 mM MgCl2, 0.01 M 2-mercaptoethanol, and 20 µl of enzyme solution. Assays were incubated at 37° for 2 or more hours, terminated by 100 µl of 6% perchloric acid, and the Pi released determined (19).

Modification of DNA was carried out as previously described to produce denatured DNA (7) (hyperchromicity, 36 to 39%), 32P-labeled DNA (21) with an initial specific activity of 5.8 x 10^6 dpm per nmole of nucleotide equivalent, 3'-phosphoryl DNA
from $^3$H]DNA or $^{32}$P]DNA (11), 5'-phosphoryl DNA (11) except that 1 ng of pancreatic DNase per ml was used, and $^{32}$P-labeled 5'-phosphoryl DNA end groups (29) using DNA labeled throughout with tritium and which had a final specific activity for $^{32}$P of 626 dpm/5 µg of DNA. (Specific activity for tritium was 2.8 x 10$^6$ dpm per n mole.) 3'-Hydroxyl DNA was prepared by treating 3'-phosphoryl $^{32}$P]DNA (250 µg per ml) with E. coli alkaline phosphatase (2 units per ml) at 37° until phosphate release (detected by the DNA phosphatase assay) was maximal. The alkaline phosphatase contained no detectable DNase activity. Specific activity was 5.2 x 10$^6$ dpm per n mole. The final concentration of all DNA preparations was 250 µg per ml except for the $^{32}$P-labeled 5'-phosphoryl end groups in [H]DNA which contained 125 µg per ml.

Chromatography

Chromatography Materials—DEAE-cellulose was washed in a solution of 1 M NaCl and 0.1 M NaOH and subsequently equilibrated with Buffer A (0.03 M Tris-HCl, pH 7.5, 0.01 M 2-mercaptoethanol, and 1 M EDTA) containing 30% glycerol (v/v) and 5% phenylmethylsulfonyl fluoride. Phenylmethylsulfonyl fluoride was deleted in those cases where the effect of proteolytic modification on the elution profile of DEAE-cellulose was being examined. It was also deleted in the second DEAE-cellulose column in the purification sequence. Hydroxylapatite was prepared by washing the commercial product in 0.2 M potassium phosphate, pH 7.5, containing 0.01 M 2-mercaptoethanol. It was packed as a thin slurry at a flow rate of 4 ml per hour in a column (2.5 x 20 cm). The column was equilibrated with 30% glycerol in 0.01 M potassium phosphate and 0.01 M 2-mercaptoethanol. Sephadex G-200 and G-75 gels were hydrated as suggested by Pharmacia in Buffer A and deactivated by suction. Columns, specified in the figure legends, were packed in the respective buffer minus glycerol and after packing were re-equilibrated with 30% glycerol in the respective buffer. Dextran blue was used to determine the void volumes and NaCl the total volume of Sephacryl columns.

Paper Chromatography—Paper chromatography for separation of nucleotides and phosphate was performed for 7 hours in a descending system (23). Spots were located by ultraviolet light or $^{32}$P radioactivity (Nuclear-Chicago Actigraph III). $R_f$ values were $P_r$, 0.95, dCMP, 0.63, TMP, 0.47, dGMP, 0.37, and dAMP, 0.19.

Polyethyleneimine Cellulose Thin Layer Chromatography—This was performed according to Randerath and Randerath (24) using 1 N acetic acid and 0.3 M LiCl. Spots were located by ultraviolet light (standards) and by radioactivity of 0.5-inch slices counted in Omnifluor.

DEAE cellulose 7 x Urea Chromatography—This was carried out by the method of Tomlinson and Tener (25) using DEAE-cellulose columns (1 x 22 cm) equilibrated with 0.02 M Tris-HCl, pH 7.8, and 7 M urea (urea-buffer). The eluting buffer gradient was performed according to Randerath and Randerath (24) US-

RESULTS

Preparation of Cell-free Extract—Cells were collected by continuous centrifugation at 2° and resuspended at 1 g wet weight per 5 ml of Buffer A. The cell suspension (300 ml volumes) was subjected to ultrasonic treatment (Bronwill Biosonik III, with a ³-inch probe, maximal power) for 6 min. The temperature during this procedure did not exceed 12°. The sonic extract was centrifuged at 4° at 100,000 x g for 90 min. The supernatant solution was collected.

Stabilization during Purification The DNase activity proved to be very labile after the cell-free extract stage. Further fractionation resulted in loss of 50 to 95% of the activity after another one or more steps. 2-Mercaptoethanol, particularly in purified preparations, aided stabilization of the DNase when used in concentrations of up to 0.1 M. Glycerol was determined except that one had been incubated at 37° with 1 µg of pancreatic DNase for 30 min.

Use of Phenylmethylsulfonyl Fluoride

Phenylmethylsulfonyl fluoride was prepared as a 0.04 M or 0.01 M solution in 95% ethanol and diluted 1 part to 19 parts of cell-free extract or the appropriate buffer to obtain a final concentration of 2 or 5 mM. Under these conditions there was no problem with solubility of the phenylmethylsulfonyl fluoride.

Gel Electrophoresis

This was performed by the method of Davis (26). Gels were stained with 1% Amido schwarz for 1 hour. Electrophoresis performed for the recovery of DNase or RNase was carried out at 4° with all buffers previously cooled to that temperature. No sample gel was used; instead, 0.2 ml of 40% sucrose was used as the first step in gel formation as described by Davis (26). After polymerization the sucrose was removed and samples containing 30% glycerol were applied to the tubes which had been positioned in the electrophoresis apparatus (containing buffers). At the termination of the run, gels were placed on glass (4°) overlying measured paper and sliced into 15 equal sections. Each section was placed into 200 µl of buffer (2.5 mM potassium phosphate, pH 7.5, 0.01 M 2-mercaptoethanol, 30% glycerol) and kept for 24 hours at 4°. Samples (50 µl) were assayed for DNase, RNase, and phosphatase.

Deoxyribose and Ribose

These sugars were determined by the methods of Steele et al. (27) and Mejbaum (28), respectively.

Molecular Weight Estimation

Molecular weight was estimated by the method of Andrews (29) except that 30% glycerol was present in the equilibration and eluting buffers (0.05 M potassium phosphate, pH 7.4, 0.01 M 2-mercaptoethanol) and in the samples applied to the columns. Column specifications of the G-75 and G-200 columns, respectively, were 2.5 x 37.5 cm and 2.5 x 35 cm, void volumes, 63 ml, and total volumes, 184 and 166 ml. Fractions of 1 ml were collected at a flow rate of 6 ml per hour. Sample (0.5-ml volumes) protein concentration was 4 mg per ml except for the P. aeruginosa exonuclease which was 0.25 mg per ml. The latter was detected in fractions by the DNase assay and the standards by absorbance at 260 nm.

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to be essential to achieve purification beyond the cell-free extract stage. This was particularly so if the cell-free extract was incubated with MgCl₂ and pancreatic RNase (autoactivation step) in order to destroy contaminating nucleic acid prior to further fractionation procedures. Table I illustrates the effect on stability of various glycerol concentrations. Thirty per cent was selected because of the similar stabilizing effect to 40% but with a less marked increment in viscosity. An even more marked effect of glycerol, however, was to stabilize the enzyme while on the various chromatography columns in the purification sequence. The recovery from the initial DEAE-cellulose column was less than 5% in the absence of 30% glycerol. The autoactivation step (see below) in the absence of glycerol also led to a marked decline in stability both to storage at 4° and -20° and to further fractionation.

**Enzyme Modification during Purification**—It was observed during the early purification sequences that DNase activity existed in multiple forms. Such multiplicity was seen during either gel filtration or ion exchange chromatography. Two factors appeared responsible for these results; one was association with nucleic acid fragments and the other proteolytic attack by an endogenous protease.

**Association with Nucleic Acid**—The use of gel filtration demonstrated that the DNase activity of the cell-free extract eluted in at least two positions on Sephadex G-200. Fig. 1 demonstrates that the bulk of DNase activity eluted in the void volume of a Sephadex G-200 column and about 15% in the internal volume. As shown, essentially all of the detectable DNase activity could be made to elute in the internal volume by incubating the cell-free extract for 90 to 150 min at 37° with 2.5 or 5 mM MgCl₂ and 20 μg per ml of pancreatic RNase (autoactivation). Phenylmethylsulfonyl fluoride was included in the incubated mixture in order to inhibit proteolysis (see “Proteolysis during Purification” below); thus, the observed shift in elution pattern is attributed to the destruction of associations between the enzyme and nucleic acid molecules. The elution profile obtained with the incubated enzyme corresponded closely to that obtained for the purified enzyme.

Data obtained from autoactivation studies revealed several significant points. Autoactivation resulted in a 2- to 3-fold increase in total DNase activity (Table II) which was maximal with Mg²⁺; Mn²⁺ caused a 1.5-fold increase whereas other cations (Co²⁺, Ca²⁺, Fe²⁺, Ni²⁺, Cu²⁺, and Zn²⁺) failed to cause any increase. RNase alone did not replace Mg²⁺ (Table II). The cations successful in increasing total DNase activity were those which activate the DNase (see cation requirements). The incubation with 5 mM MgCl₂ resulted in an increase of acid-soluble deoxyribose and material absorbing at 260 nm; only those conditions producing the former resulted in a DNase increase. The decrease in total DNase was not considered to be due to activation of additional enzyme(s) as the DNase measured under various assay conditions (standard assay, pH 9.0; standard assay with 2.5 mM CaCl₂ in place of MgCl₂; standard assay using denatured DNA as substrate; assay at pH 5.2, 5 mM EDTA, no MgCl₂) retained a constant relationship to the standard assay throughout the autoactivation procedure. This was also true

| Preparation | A₉₆₀ | Ribose | Deoxyribose | DNase |
|-------------|------|--------|-------------|-------|
| 1. +5 mM MgCl₂ | 18   | 0.03   | 0.25        | +195  |
| 2. +5 mM MgCl₂ + pancreatic RNase (20 μg per ml) | 55   | 0.18   | 0.19        | +95   |
| 3. +5 mM EDTA | 0     | 0.00   | 0.00        | -40   |
| 4. +5 mM EDTA + pancreatic RNase (20 μg per ml) | 45   | 0.25   | 0.0         | +15   |
| 5. No additives | 25   | 0.055  | 0.15        | +45   |

**FIG. 1.** Effects of incubation with MgCl₂ and pancreatic RNase on Sephadex G-200 chromatography of cell-free extracts. One milliliter of crude cell-free extract containing 84 mg of protein (O—O) was applied to a G-200 column (2.5 × 35 cm) which had been equilibrated in 5 mM potassium phosphate, pH 7.4, 0.01 M dithiothreitol, 30% glycerol (v/v), and 5 mM phenylmethyl sulfonyl fluoride. One-milliliter fractions were collected at a flow rate of 6 ml per hour using the same buffer for elution. Fractions were assayed by the standard DNase assay. To compare the effect of incubation with RNase and MgCl₂, the cell-free extract was first incubated at 37° in the presence of 20 μg per ml of RNase, 5 mM MgCl₂, and 5 mM phenylmethylsulfonyl fluoride for 90 min. One milliliter of that preparation containing 35 mg per ml of protein was applied to the same G-200 column after it had been washed with 2 column volumes of the buffer above and the column was then operated in an identical manner (Δ—Δ).
The accumulated evidence suggests that the enzyme is released upon cell breakdown in association with DNA which interferes with the assay and with the migration of the enzyme on gel filtration. Upon destruction of the DNA by the active component of the endogenous DNase these problems are overcome. However, DNA removal creates new problems. The enzyme activity becomes less stable to storage, to chromatography, and also to proteolytic modification (see below). After destruction of the endogenous DNA by the autoactivation step, addition of calf thymus DNA at a concentration of 0.2 mg per ml produced a 20 to 40% increase in enzyme stability. The association of the enzyme with nucleic acid had to be defined clearly in order to evaluate the second apparent cause of modification of enzyme behavior, that is, proteolytic digestion.

The effects of including phenylmethylsulfonyl fluoride in the preparation was added: MgCl₂ (5 mm), RNase (20 μg per ml) and the mixture incubated for 90 min at 37°. B, cell-free extract stored and treated as above, but including 5 mm phenylmethylsulfonyl fluoride. Phenylmethylsulfonyl fluoride was included in the wash and gradient solutions used to develop the column. C, cell-free extract frozen overnight, thawed rapidly, and additions of glycerol, Tris, mercaptoethanol, MgCl₂, RNase, and phenylmethylsulfonyl fluoride made as above. Following incubation for 90 min at 37°, the mixture was applied and eluted in the presence of phenylmethylsulfonyl fluoride. In each case, 2.5 g of protein in 100 ml of pretreated enzyme preparation was applied to the column, washed in with Buffer A containing 30% glycerol and 2 mm phenylmethylsulfonyl fluoride, and eluted with a linear gradient of 550 ml of Buffer A with the same additions and 500 ml of the same buffer containing 0.5 M NaCl. The flow rate was 30 ml per hour, and the fraction volume was 20 ml. DNase (ΔΔΔ); A₃₅₀ (O--O); molarity (---).

The effects of including phenylmethylsulfonyl fluoride in the cell-free extract during the autoactivation with MgCl₂ and pancreatic RNase as well as in all buffers of DEAE-cellulose chromatography are shown in Fig. 2B. There are five fewer peaks eluted from the DEAE-cellulose column, all but one small initial peak eluting at higher ionic strength than the unmodified enzyme in Fig. 2C. One of the possible causes of this distribution of enzyme was proteolytic alteration which had been suggested by the occasional observation of a peak of DNase eluted from the G-200 column in mercaptoethanol. Prior aging conditions or autoactivation as noted. Eight different peaks eluted from the DEAE-cellulose column, all but one small initial peak eluting at higher ionic strength than the unmodified enzyme in Fig. 2C. Even storage of the cell-free extract at 4° for several days led to greater heterogeneity in ion exchange, presumably by the same mechanism which functioned in a shorter time at the autoactivation temperature of 37°.

Fig. 2A shows the results of a gradient elution of enzyme and protein from a cell-free extract stored for 7 days at 4° prior to autoactivation as noted. Eight different peaks eluted from the DEAE-cellulose column, all but one small initial peak eluting at higher ionic strength than the unmodified enzyme in Fig. 2C. One of the possible causes of this distribution of enzyme was proteolytic alteration which had been suggested by the occasional observation of a peak of DNase eluted from the G-200 column even later than that shown in Fig. 1 (in spite of the use of an ionic strength of 0.1 or greater in eluting buffers to prevent retardation by electrostatic effects). Table III shows that low levels of endogenous proteolysis associated with cell-free extract could be demonstrated by the hydrolysis of casein and benzoyl L-arginine ethyl ester. It was possible to inhibit the proteolytic activity by using the serine esterase inhibitor phenylmethylsulfonyl fluoride (30) at high concentrations (2 to 5 mM) and with incubation at 37°. Inhibition of endogenous proteolytic activity was negligible at 4°.

The effects of including phenylmethylsulfonyl fluoride in the cell-free extract during the autoactivation with MgCl₂ and pancreatic RNase as well as in all buffers of DEAE-cellulose chromatography are shown in Fig. 2B. There are five fewer peaks eluted after incubation with phenylmethylsulfonyl fluoride as opposed to incubation without phenylmethylsulfonyl fluoride of the same preparation aged for 7 days at 4°. When phenylmethylsulfonyl fluoride was added immediately after thawing of cell-free extract stored overnight at -20° only a single DNase peak eluted (Fig. 2C). It thus seems that two enzyme peaks in

**Table III**

Proteolytic activity in cell-free extract and its inhibition

| Preparation                     | Activity | Inhibition |
|---------------------------------|----------|------------|
| Casein hydrolysis assay         |          |            |
| - Phenylmethylsulfonyl fluoride | 0.27     | 100        |
| + Phenylmethylsulfonyl fluoride | <0.005   | 100        |
| Benzoyl L-arginine ethyl ester  | 1.05 × 10⁻² | 100     |
| Hydrolysis                      | <1 × 10⁻⁴ | 100        |

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addition to the original peak are generated by storage at 4° and four more on further incubation at 37° in the absence of phenylmethylsulfonyl fluoride.

The avoidance of storage of extracts at 4° and the use of the incubation step to eliminate nucleic acid in the presence of phenylmethylsulfonyl fluoride to prevent endogenous proteolysis allowed subsequent purification of the DNase activity as a single eluting peak in all subsequent purification steps.

The possibility that the additional peaks eluted from the DEAE-cellulose might be other enzymes was considered highly unlikely because of the treatments which lead to their appearance and because the fractions demonstrated a marked similarity in catalytic requirements (3- to 4-fold preference for native versus denatured DNA, optimal DNase activity with Mg++, complete inhibition by 5 mM EDTA, and almost identical pH profiles from pH 6 to 9.5).

Purification of Enzyme—The results of a typical purification sequence are shown in Table IV (carried out at 4° except as noted).

Autoactivation of Cell-free Extract—Cell-free extract was prepared and frozen at −20° overnight. The following day it was thawed rapidly, adjusted to 30% glycerol, 5 mM phenylmethylsulfonyl fluoride, 0.01 M 2-mercaptoethanol, 0.03 M Tris-HCl, pH 7.5, and 2.5 mM MgCl₂. Pancreatic RNase, while not necessary to produce activation was added at a concentration of 20 μg per ml to hydrolyze contaminating RNA. The preparation was incubated at 37° for 90 min. Monitoring of DNase assays, acid solubility at 200 nm, and hydrolysis of benzoyl L-arginine ethyl ester was carried out at 0, 30, 60, and 90 min to ensure that the maximal increase in DNase activity, greater than 75% proteolytic inhibition by 30 min and the maximal increase in acid-soluble materials had occurred. A preliminary trial on 5 ml of each batch was performed to assess these points.

First DEAE-cellulose Chromatography—From the autoactivation step, 2.3 g of protein in a 100-ml volume was added to a DEAE-cellulose column (2.5 x 60 cm) prepared as described under "Methods," and washed in with 100 ml of Buffer B (Buffer A with 2 mM phenylmethylsulfonyl fluoride and 30% glycerol). A linear gradient of 550 ml each of Buffer B and Buffer A containing 0.5 M NaCl was applied and 20 ml fractions collected (flow rate, 30 to 40 ml per hour). All tubes were examined for absorbance at 280 nm and DNase activity. Linearity of the gradient was confirmed by examining samples of every third tube with a radiometer conductivity meter. The results of a typical column are seen in Fig. 2C. The enzyme eluted at 0.07 M NaCl.

Precipitation of Fraction Material at pH 4.2—Active fractions from the above step were combined and concentrated to 0.2 of original volume using an Amicon UM-10 filter at 40 psi. Sodium acetate (5.0 M, pH 5.2) was added to produce a final concentration of 0.05 M in the DEAE-cellulose concentrate. The pH was adjusted to 5.2 in a stepwise manner with 2 N acetic acid. This procedure was carried out with constant stirring and continuous pH monitoring on a Beckman Expandomatic pH meter. When the pH reached 5.5, 10-min stirring periods were allowed between acetic acid additions. After reaching pH 5.2 the mixture was stirred for 20 min and centrifuged at 27,000 x g for 30 min in the cold. The pH of the supernatant solution was adjusted to 7.0 with 2 M Tris (free base) before the next step.

Ammonium Sulfate Fractionation—Solid ammonium sulfate was added gradually in the proportion of 313 g per liter. After being stirred in an ice bath for 20 min, the preparation was centrifuget at 27,000 x g for 20 min. To the supernatant solution a further 70 g per liter of (NH₄)₂SO₄ was added with stirring to produce saturation in the glycerol buffer system (about 60% saturation in water). The mixture was centrifuged at 27,000 x g for 20 min, and the supernatant solution was dialyzed against 100 volumes of buffer containing 30% glycerol, 0.01 M 2-mercaptoethanol, and 0.01 M potassium phosphate, pH 7.5, for 16 hours. The preparation was concentrated by UM-10 ultrafiltration to 30 ml.

Hydroxylapatite Chromatography—From the ultrafiltrate 200 mg of protein in a 15-ml volume was added to a hydroxylapatite column and washed in with 80 ml of 30% glycerol in 0.01 M potassium phosphate and 0.01 M 2-mercaptoethanol (Buffer C). The column size was 2.5 x 20 cm. A linear gradient of 300 ml each of Buffer C and of 0.2 M potassium phosphate (pH 7.5) with 30% glycerol and 0.01 M 2-mercaptoethanol was applied, and 12-ml fractions were collected. Every second tube was examined for DNase and absorbance at 280 nm, and gradient linearity was confirmed as described. The active fractions which eluted at 0.05 M phosphate were collected, combined, and concentrated to 3 ml by UM-10 ultrafiltration.

Second DEAE-cellulose Chromatography—The 3 ml of concentrate containing 30 mg of protein was eluted at 18 ml in 30% glycerol containing 0.01 M 2-mercaptoethanol. That preparation was applied to a column (1.5 x 25 cm) of DEAE-cellulose and washed in with 15 ml of Buffer C. A linear gradient of 75 ml each of Buffer C and Buffer C modified to contain 0.2 M potassium phosphate was applied, and 1-ml fractions were collected at a flow rate of 25 ml per hour. Absorbance at 280 nm, DNase, and molarity were examined as before. DNase activity eluted at 0.06 M phosphate and was concentrated to 1 ml by UM-10 ultrafiltration.

Sephadex G-75 Filtration—G-75 gel filtration was carried out with 30% glycerol in 0.01 M 2-mercaptoethanol and 0.05 M potassium phosphate, pH 7.5. The column used was as specified in Fig. 3. It had a void volume of 66 ml and a total volume of 184 ml. The DNase eluted consistently in the fractions seen in Fig. 3.

Purity—The purified enzyme was contaminated with a single band detectable on polyacrylamide gel electrophoresis at pH 8.3 or pH 4.0 as well as by assaying evidence of RNase activity. The RNase was considered to be a contaminant for sev-

| Preparation | Total protein (mg) | Total units | Specific activity | Recovery |
|--------------|-------------------|-------------|------------------|----------|
| 1. Cell-free extract | 2,300 | 12,900 | 5.6 | 100 |
| 2. First DEAE-cellulose | 910 | 52,800 | 18 | 930 |
| 3. pH 5.2 supernatant | 770 | 53,000 | 69 | 410 |
| 4. Ammonium sulfate supernatant | 405 | 27,000 | 68 | 211 |
| 5. Hydroxylapatite | 59.5 | 8,000 | 135 | 62 |
| 6. Second DEAE-cellulose | 2.0 | 9,000 | 1,450 | 22.5 |
| 7. Sephadex G-75 | 0.37 | 760 | 1,810 | 5.9 |

Average values. Total recoveries at this step varied from 203 to 217%.
Pseudomonas aeruginosa DNase

Fig. 3. Elution profile of DNase and RNase activity from Sephadex G-75. One milligram of protein in 1 ml from the second DEAE-cellulose column was added to a G-75 column (2.5 X 37.5 cm) prepared as given under "Methods." RNase activity (—□—□) and DNase (Δ—Δ) were eluted with 30% glycerol-0.01 M 2-mercaptoethanol-0.05 M potassium phosphate buffer, pH 7.5. Fractions of 3 ml were collected at 6 ml per hour flow rate. A\textsubscript{280} was measured on selected tubes (O—O).

Fig. 4. The effect of additional enzyme and substrate on the activity of Pseudomonas aeruginosa exonuclease. Multiple standard DNase assays were prepared in duplicate each containing 1.8 units of exonuclease. The assays were terminated at various times without further additions (O—O), with the addition of 5 μg of [3H]DNA after 2 hours of incubation (A—A), and with the addition of 1.8 units of fresh enzyme at 2 hours (w—w).

Table V

| Type of DNA           | K\textsubscript{m} (μg/ml) | V\textsubscript{max} (units/ml) |
|-----------------------|-----------------------------|-------------------------------|
| Unmodified native     | 38.8                        | 955                           |
| 3'-Phosphorylated DNA | 38.8                        | 955                           |
| 3'-Hydroxylated DNA   | 38.8                        | 955                           |
| Unmodified denatured  | 35.0                        | 65.0                          |
| Unmodified native with 0.07 M NaCl in assay | 140                         | 400                           |

General reasons; it eluted only from the more positively charged gel bands which did not contain DNase (pH 8.3); in addition, the RNase, unlike the DNase, was stimulated by 5 mM EDTA to produce a 2-fold rise in activity and showed no significant loss of activity when acidified to pH 3.0 prior to assay. The RNase is not the pancreatic RNase added during the DNase purification, however, as a trial purification in the absence of added RNase contained RNase in the final purification product. Finally, it can be seen in Fig. 3 that the RNase and DNase activities elute with separate profiles from a Sephadex G-75 column; the two RNase peaks presumably represent the added pancreatic RNase and the Pseudomonas RNase.

Stability of Purified Enzyme—The enzyme was stable to freezing at -20° in the cell-free extract in the absence of glycerol. Thereafter, increasing activity loss occurred unless 30% glycerol was included. In the presence of glycerol 95 to 100% activity could be maintained for a minimum of 8 weeks at -20° for all purification stages. If the purified enzyme was dialyzed free of glycerol and frozen at -20° in 2.5 mM potassium phosphate, pH 7, with or without 2-mercaptoethanol, 60% of activity was lost in a single freeze. The half-life at 37° in the same buffer with 0.01 M 2-mercaptoethanol was 7.5 min. The purified enzyme could be stored at 4° in 30% glycerol in buffer for 14 days with a 5 to 15% loss in activity.

Catalytic Conditions—The pH optimum was 7.8 to 8.0 (Tris-HCl or Tris-maleate). Sulfhydryl reagents were essential to maintain activity in the 37° assay. 2-Mercaptoethanol (0.01 M) or 5 to 10 mM dithiothreitol produced maximal activity and maintained a linear reaction (with time) for at least 12 to 18 min. DNase activity was proportional to the protein concentration per assay over a range of 0.5 to 2.25 μg (6 to 32% substrate solubilization under usual assay conditions). Mg\textsuperscript{2+} (2.5 mM; cations were used as chlorides) produced optimal activation of the DNase; Mn\textsuperscript{2+} and Co\textsuperscript{2+} at the same concentration were 65% and 23%, respectively, as effective. In the absence of added cations activity was 10% of maximal. Other cations (Zn\textsuperscript{2+}, Ca\textsuperscript{2+}, Fe\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}) were 10% or less as effective as Mg\textsuperscript{2+}. The temperature optimum was determined to be 37°.

Substrate Specificity—The DNase demonstrated an approximate 3- to 4-fold preference for native DNA in initial reaction rates. All of the native and 65% of the denatured DNA substrate could be solubilized with five readditions of 1.2 units of DNase to a standard assay incubated at 37° for 45 hours; a single addition solubilized a maximum of 48%. The results seen in Fig. 4 suggest the maximum of 48% was due to both enzymatic inactivation and a relative resistance to further hydrolysis of the partially digested substrate.

The effect of modification of the substrate DNA on apparent K\textsubscript{m} and V\textsubscript{max} values is shown in Table V. The K\textsubscript{m} does not significantly change for native, denatured, 3'-phosphoryl, or 3'-hydroxyl DNA. The V\textsubscript{max} is, however, more than doubled by the introduction of increased numbers of 3'-phosphoryl or hydroxyl groups. The very close relationship of these two V\textsubscript{max} values indicates that the increase in V\textsubscript{max} is not dependent on the 3'-phosphoryl group but rather on the presence of increased numbers of new terminal in general. The profound decrease in V\textsubscript{max} for denatured DNA suggests that the diminished rate is not due to deficient binding but to a less effective hydrolytic mechanism.

Product Analysis—The purified DNase was shown to have only exonuclease activity on DNA, because the products of limited DNA digestion did not include molecules intermediate between mononucleotides and polymer similar to the original DNA.
Fig. 5. DEAE-cellulose chromatography in 7 M urea of the Pseudomonas aeruginosa exonuclease digests of 14H]DNA. Three identical columns were prepared and operated as given under "Methods." 1.2 ml containing 125 µmoles of each of 4 deoxyribonucleotides standards were mixed with 1.2 ml of 7 M urea in 0.02 M Tris-HCl, pH 7.8 (urea-buffer), and applied and eluted from one of the columns (— — —). The position of each of the standards is indicated. A 1.8-ml volume digest ( ▲ — ■) was prepared containing Escherichia coli DNA (0.5 mg per ml); Tris-HCl (pH 7.8, 0.03 M); MgCl₂ (9.5 mM); 2-mercaptoethanol (0.01 M); E. coli [14H]DNA (12.5 µg per ml); and 28 units of the exonuclease per ml. It was allowed to incubate at 37°C for 120 min. At that time it was diluted with an equal volume of urea-buffer and eluted from the second column. An identical control digest except for the absence of the P. aeruginosa exonuclease was incubated, applied, and eluted from the third column in an identical manner (● — ●).

Thus, a comparison of the elution profile of a control digest without enzyme versus a test digest containing DNase from P. aeruginosa together with a mixture of deoxyribonucleotide standards (Fig. 5) shows that the only products of the enzyme reaction are mononucleotides represented by the elution peaks associated with mononucleotide standards.1

In addition to the coincident chromatography of the early peaks with the mononucleotides standards, they were identified by the following procedures. The presence of the 14H label in thymine allowed identification of TMP in Peak 2 of Fig. 2; the absorbance spectrum of Peaks 3 and 4 was consistent with dAMP and dGMP, respectively; rechromatography on DEAE-cellulose columns of the pooled tubes of Peaks 2, 3, and 4 separately (31) to remove urea and subsequent analysis by paper chromatography (see "Methods") allowed the identification of each peak as given in Fig. 2.

The experiment and results documented in Fig. 6 establish that the mononucleotide products are susceptible to the 5'-nucleotidase function of M. sodonensis nuclease described by Berry and Campbell (14) and are, thus, 5'-mononucleotides.

The DNase was also found to have phosphatase activity which could be demonstrated by the phosphatase assay. That assay was shown by paper chromatography (see "Methods") and Sephadex G-10 and G-15 gel filtration (ionic strength of eluting buffers >0.2) to be specific for Pi. Such activity was dependent on the presence of 3'-phosphoryl end groups in DNA (Table VI). The failure of the enzyme to demonstrate phosphatase activity on unmodified DNA or DNA modified to produce 5'-phosphoryl termini as illustrated in Table VI strongly suggests that the only site of phosphatase activity is the 3'-phosphoryl terminus.

The phosphatase assay under the conditions given in under "Methods" had the following characteristics. Proportionality between phosphate release and enzyme concentration existed over a range of 10 to 50% of the total 3'-phosphate present; the assay could be demonstrated by the phosphatase assay. That assay was shown by paper chromatography (see "Methods") and Sephadex G-10 and G-15 gel filtration (ionic strength of eluting buffers >0.2) to be specific for Pi. Such activity was dependent on the presence of 3'-phosphoryl end groups in DNA (Table VI). The failure of the enzyme to demonstrate phosphatase activity on unmodified DNA or DNA modified to produce 5'-phosphoryl termini as illustrated in Table VI strongly suggests that the only site of phosphatase activity is the 3'-phosphoryl terminus in DNA.

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Activation is obtained by 1 mM magnesium, the relative activity respectively, (Fig. 8) with an average of 42,500.

Is linear with time at a DNA concentration of 50 ng per ml for 15 min or longer; the pH optimum is 7.5 (Tris-HCl); optimal activation is obtained by 1 mM magnesium, the relative activity shown with manganese (1 mM) and cobalt is identical with that present.

Inhibition of Other DNase Activity in P. aeruginosa-The only enzyme present in this preparation but was, as previously noted, felt to be a separate phosphatase function. RNAase contaminated the final exonuclease preparation but was, as previously noted, felt to be a separate phosphatase function. RNAase contaminated the final exonuclease preparation but was, as previously noted, felt to be a separate phosphatase function. RNAase contaminated the final exonuclease preparation but was, as previously noted, felt to be a separate phosphatase function. RNAase contaminated the final exonuclease preparation but was, as previously noted, felt to be a separate phosphatase function. RNAase contaminated the final exonuclease preparation but was, as previously noted, felt to be a separate phosphatase function. RNAase contaminated the final exonuclease preparation but was, as previously noted, felt to be a separate phosphatase function. 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FIG. 8. Molecular weight estimation of Pseudomonas aeruginosa exonuclease by Sephadex G-200 and G-75 filtration. The columns were prepared and operated as given under "Methods." 1, ovalbumin; 2, pepsin; 3, soybean trypsin inhibitor; 4, cytochrome c; and 5, P. aeruginosa exonuclease. \(V_e\), elution volume; \(V_t\), total volume; \(V_o\), void volume. (G-200 has larger \(K_m\) numbers and cytochrome c omitted.)

TABLE VIII
Comparison of relative activity under various assay conditions of DNase activity in cell-free extract and in Sephadex G-75 preparations

| Conditions of assay          | Relative activity |
|------------------------------|-------------------|
|                              | Cell-free extract | G-75 preparation |
| \(\text{MgCl}_2, 2.5 \text{mM}\) | 1.0               | 1.0               |
| \(\text{CaCl}_2, 2.5 \text{mM}\) | 0.1               | 0.1               |
| \(\text{EDTA, 2.5 mM (no MgCl}_2)\) | 0.0-0.2           | 0.0               |
| \(\text{pH optimum (Tris-HCl)}\) | 7.8-8.2           | 7.8-8.0           |
| \(\text{Uranil acetate, 10\% trichloroacetic acid:}\) |                  |
| 10\% trichloroacetic acid    | 0.85-0.95         | 0.95-1.0          |
| \(\text{pH 9.0 (Tris-HCl)}\) | 0.65              | 0.65              |
| \(\text{pH 5.2, 2.5 mM EDTA (no MgCl}_2)\) | 0.01              | 0.0               |
| \(\text{Stabilizing effect of 30\% glycerol} \) | Marked            | Marked            |
| \(\text{NaCl, 0.1 M} \)     | 0.2-0.3           | 0.2-0.3           |

DISCUSSION
The enzyme described in this study is a member of a group of exonuclease-phosphatases (exonuclease III, E. coli (11, 35); Diplococcus pneumoniae (23)) but does possess some distinctions. The pneumococcal enzyme is not stimulated by 2-mercaptoethanol; exonuclease III appears to be active only on double stranded DNA as it is able to solubilize only up to 50\% of the substrate (11); exonuclease III exhibits relative phosphatase-exonuclease rates which are identical after an initial lag in exonuclease activity (in the P. aeruginosa enzyme the relative rates are 0.2:1), and it does not appear to undergo an increase in \(V_{max}\) as the result of modification of the DNA substrate to produce 3'-phosphoryl or hydroxyl end groups. The latter behavior of the P. aeruginosa enzyme coupled with a failure to change the \(K_m\) under those conditions suggests it conforms to the catalytic model proposed by Lacks and Greenberg (23).

The exonuclease-phosphatase appears to be the only DNase detected in significant amounts of P. aeruginosa by our assay procedure but at very low activity.

other DNase detected was present as a peak which emerged in the wash fraction of the first DEAE-cellulose column, provided the autoactivation step was not carried out. To ensure that other DNases were not failing to elute from the cellulose, column trials were performed identically with that of Fig. 2C except that no autoactivation step was carried out. However, even though the eluting gradient was followed by 1 M NaCl, no additional activities were detected. The fractions from the initial wash of the column accounted for only 1\% of the original DNase measured and were extremely labile. As a result, only rudimentary characterization was performed in order to distinguish that activity from the exonuclease eluting at 0.07 M NaCl. The early (wash) DNase could be differentiated in two ways. Uranil acetate in trichloroacetic acid was about twice as effective a precipitant (in the DNase assay) as trichloroacetic acid alone (34) whereas the exonuclease demonstrated equal activity using either precipitant; that enzyme was 3 times more active on denatured than native DNA unlike the exonuclease, for which the reverse was true. The characteristics of the DNase measured in the cell-free extract can be seen from Table VIII to be essentially identical with those obtained with the purified exonuclease, further supporting the presence of only one significant DNase in P. aeruginosa.

DNase Content of Cells during Growth—Fig. 9 illustrates that the exonuclease is produced throughout the exponential growth phase of the cells but that the specific activity declines in both complex and defined media during that period and into stationary phase.
levels and could be an extracellular enzyme contaminating our preparations. The absence of other detectable DNases in our system and the detection of an enzyme found in several different bacterial genera strongly suggests that this enzyme is an essential component of the cells’ DNA repair, exclusion, or replication systems. The failure to detect other DNases does not exclude their presence as specific additives (e.g. ATP (38)), or substrate modification (e.g. alkylation (39, 40)) or specialized assay procedures (41) might be necessary to confirm their presence (or absence).

Stabilization, endogenous proteolysis, and association with nucleic acid in the cell extract made this an exceedingly difficult enzyme to purify. Proteolytic modification of enzymes during their purification to produce diminished stability or multiple molecular forms has been noted in several enzyme systems recently (42-46). Yeast hokkotriase has in particular received considerable attention (43) in this regard. The influence of macromolecules in crude enzyme preparations on chromatography and other purification systems has, however, received very little attention.

The apparent purification of the exonuclease-phosphatase is only 325-fold, but there are reasons to believe that this is an underestimation. Support for this supposition is advanced by the reduction from several protein bands in gel electrophoreograms in the second to last purification step to only two in the last step and by the marked decrease in intensity of the RNase band relative to that of the exonuclease in the same two steps. The change in specific activity in these same steps, however, was only 1.25 which is not in keeping with the above observations. It seems probable that inactivated exonuclease (which may have occurred at any step, even the apparent activation process) was purified through the sequence and diluted the final specific activity.

The effect of ionic strength on the apparent binding affinity for the substrate DNA explains the inhibitory effect exhibited on DNase activity and may be the general reason for this widespread finding with other nucleases.

The molecular weight estimation by gel filtration while open to considerable attention (43) in this regard. The influence of molecular forms has been noted in several enzyme systems recently (42-46). Yeast hexokinase has in particular received considerable attention (43) in this regard. The influence of macromolecules in crude enzyme preparations on chromatography and other purification systems has, however, received very little attention.

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