

A Restriction Enzyme from *Hemophilus influenzae*

I. Purification and General Properties

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Extracts of *Hemophilus influenzae* strain Rd contain an endonuclease activity which produces a rapid decrease in the specific viscosity of a variety of foreign native DNA's; the specific viscosity of *H. influenzae* DNA is not altered under the same conditions. This "restriction" endonuclease activity has been purified approximately 200-fold. The purified enzyme contains no detectable exo- or endonucleolytic activity against *H. influenzae* DNA. However, with native phage T7 DNA as substrate, it produces about 40 double-strand 5'-phosphoryl, 3'-hydroxyl cleavages. The limit product has an average length of about 1000 nucleotide pairs and contains no single-strand breaks. The enzyme is inactive on denatured DNA and it requires no special co-factors other than magnesium ions.

1. Introduction

A number of bacteria are capable of recognizing and degrading ("restricting") foreign DNA, such as the DNA of a virus grown on another bacterial strain. The DNA of the host is protected by a "host-controlled modification" (Arber, 1965). Recently, Meselson & Yuan (1968) have purified a restriction endonuclease from *Escherichia coli* K12. The enzyme has the interesting properties: (1) that it is site-specific in action, producing only a limited number of double-strand breaks in unmodified DNA, and (2) that it requires adenosine triphosphate and *S*-adenosyl methionine in addition to magnesium ions.

We have made the chance discovery of what appears to be a similar type of enzyme in *Hemophilus influenzae*, strain Rd. In the course of some experiments in which competent *H. influenzae* cells were incubated with radioactively labeled DNA from the *Salmonella* phage P22, we found that this DNA was apparently degraded since it could not be recovered in cesium chloride density gradients. It seemed likely that the effect was one of restriction. We were able to show the presence in crude extracts of an endonuclease activity which produced a rapid decrease in viscosity of foreign DNA preparations and which was without effect on the *H. influenzae* DNA. We describe in this report the purification and properties of the endonuclease. As with the *E. coli* restriction enzyme, our enzyme produces double-strand breaks in a limited number of specific sites. The enzyme requires only magnesium ions as a co-factor, unlike the *E. coli* enzyme. A preliminary report has been published (Smith & Wilcox, 1969).

2. Materials and Methods

(a) Bacterial and phage strains

H. influenzae strain Rd was obtained from Dr Roger Herriott as a frozen culture. The phage P22 c_2 clear plaque mutant (Levine, 1957) grown on *S. typhimurium* LT2 was used as a source of phage P22 DNA. Phage T7 and its host *E. coli* B were obtained from Dr Bernard Weiss.

(b) Enzymes and standards

Bacterial alkaline phosphatase was obtained from Worthington Biochemical Corp. Polynucleotide kinase, 5000 units/ml. (Richardson, 1965) and rechromatographed bacterial alkaline phosphatase, 20 units/ml. (Weiss, Live & Richardson, 1968) for use in the ^{32}P terminal labeling procedure were kindly supplied by Dr Bernard Weiss.

Bovine serum albumin and sperm whale myoglobin with molecular weights of 67,000 and 17,800, respectively, were obtained in a molecular weight marker kit from Mann Research Laboratories.

(c) Nucleic acids and nucleotides

Phage P22 was purified from L broth lysates (Levine, 1957) by differential centrifugation and banding in a CsCl step gradient (Thomas & Abelson, 1966). The DNA was extracted with 1 vol. of cold, redistilled phenol and then precipitated with 2 vol. of cold ethanol and redissolved in 1 vol. of NaCl-Tris buffer (0.05 M-NaCl, 0.01 M-Tris-HCl, pH 7.4, formerly ST buffer). The precipitation was repeated and the DNA was finally redissolved in the above buffer at 1.30 mg/ml. This DNA stock was used for the viscometry assays (see below).

Unlabeled phage T7 DNA was similarly prepared from phage grown on *E. coli* B. Phage T7 labeled with ^{32}P was prepared from phage grown in synthetic medium containing 2 μg phosphorus/ml. (Smith, 1968) and 5 μC of carrier-free [^{32}P]orthophosphate/ml. (New England Nuclear Corp.). The labeled phage were purified as described above and then extracted once with cold phenol. The DNA-containing aqueous phase was removed and dialysed for 20 hr against three 500-ml. changes of NaCl-Tris buffer.

Unlabeled *H. influenzae* DNA was extracted by the procedure of Marmur (1961) from a culture grown to saturation in Difco brain-heart infusion supplemented with NAD, 2 $\mu\text{g}/\text{ml}$., and hemin (Eastman), 10 $\mu\text{g}/\text{ml}$. *H. influenzae* DNA labeled with [^3H]thymidine was prepared from cells grown in a synthetic medium as described by Carmody & Herriott (1970).

[γ - ^{32}P]ATP was obtained from Dr Bernard Weiss. The method of preparation has been described (Weiss *et al.*, 1968).

(d) Zone sedimentation of DNA in sucrose density-gradients

Native DNA was sedimented in linear gradients of 5 to 20% (w/v) sucrose in NaCl-Tris buffer. Denatured DNA was centrifuged in similar gradients containing 0.1 M-NaOH. The DNA was denatured by addition of 0.1 vol. of 1 N-NaOH for 5 min at room temperature followed by neutralization with 0.1 vol. of 1.1 N-HCl, 0.2 M-Tris as described by Studier (1965). Centrifugation was carried out in an SW50 rotor at 4°C in a Spinco model L centrifuge. Approximately 30 ten-drop fractions were collected from the bottom of the tube directly into scintillation vials containing 15 ml. of scintillation medium (2-methoxy-ethanol, 373 ml.; PPO, 9.3 g; dimethyl-POPOP, 30 mg; toluene to 1 liter) and counted in a Packard scintillation spectrometer.

(e) Assay of the enzymic activity by DNA viscometry

DNA viscosity measurements were performed at 30°C in an Ostwald viscometer having a flow-time for water of approximately 60 sec. The viscometer was filled with 3.5 ml. of phage P22 DNA solution, 40 $\mu\text{g}/\text{ml}$. in Tris-Mg-mercaptoethanol buffer (6.6 mM each of Tris buffer, pH 7.4, MgCl_2 , and mercaptoethanol). Several flow-time measurements were taken after the DNA solution had reached thermal equilibrium and these were generally repro-

ducible to within 0.1 sec. Five to 50 μ l. of extract or purified enzyme was then introduced into the reservoir bulb of the viscometer and rapidly mixed by blowing air retrograde into the bulb. Flow-time measurements were taken as rapidly as was practical. The DNA viscosity was expressed as specific viscosity, $\eta_{sp} = (t/t_D) - 1$, where t_D represents the flow time at the end of the experiment, 5 min after addition of 50 μ g of pancreatic DNase (this corresponded to the flow-time for pure solvent within the accuracy of the measurements). Specific viscosity measurements were plotted against time on semi-logarithm paper as fractional values of the zero-time value. One unit of enzyme activity is defined as that amount which produces a decrease in the DNA specific viscosity of 25% in 1 min under the conditions described above. It should be pointed out that the viscometric assay is valid even on crude extracts since, as will be shown in the Results section, no activity is found in crude extracts against homologous DNA.

(f) *Purification of endonuclease R*

H. influenzae cells were grown in 12 l. of brain-heart infusion, supplemented with 10 μ g hemin/ml. and 2 μ g NAD/ml. to o.d.₆₅₀ = 0.7, harvested by centrifugation, washed once, and resuspended in 20 ml. of 0.05 M-Tris (pH 7.4), 0.001 M-glutathione. The cells were disrupted by sonication for 4 min at 8 A output on a Bronson sonicator while being cooled in an ice-salt water bath. All subsequent operations were carried out at 0 to 4°C. Cellular debris was removed by centrifugation for 30 min at 100,000 g. The supernatant (27 ml.) was brought to 1 M-NaCl by addition of 1.58 g of NaCl and layered onto a 2.5 cm \times 49 cm Bio-Gel A 0.5 M (200 to 400 mesh) column prewashed with 10 vol. of 1 M-NaCl, 0.02 M-Tris-HCl, pH 7.4, 0.01 M-mercaptoethanol. Elution was carried out at 1.2 ml./min using the same buffer solution and 6-ml. fractions were collected. Fractions 18 to 28, containing nearly all the activity and only 10% of the o.d.₂₆₀ absorbing material, were pooled. The pooled fractions (65 ml.) were diluted with 140 ml. of 0.02 M-Tris, pH 7.4, and stirred in an ice bath. Ammonium sulfate, 64.3 g, was added slowly over a 30-min period. The precipitate (0 to 50%) was removed by centrifugation. The supernatant solution was precipitated with ammonium sulfate, 15.8 g, to obtain a 50 to 60% precipitate. The supernatant was again reprecipitated with 16.8 g of ammonium sulfate and this precipitate (60 to 70%) was combined with the 50 to 60% precipitate and dissolved in 20 ml. of 0.05 M-NaCl, 0.02 M-Tris (pH 7.4), 0.001 M-mercaptoethanol.

Part of the 50 to 70% ammonium sulfate fraction was further purified by column chromatography. A phosphocellulose (Whatman, P11) column, 0.5 cm \times 9.5 cm, was equilibrated with 100 ml. of 0.01 M-potassium phosphate buffer, pH 7.4. 6 ml. of the ammonium sulfate fraction, containing 60 mg. of protein, was diluted with 54 ml. of 0.01 M-phosphate buffer, pH 7.4, and loaded onto the column at a flow rate of 5 ml./hr at 4°C. Protein was then eluted stepwise with 5-ml. portions of 0.01 M-potassium phosphate buffer, pH 7.4, containing increasing molarities of KCl as follows: 0.0, 0.1, 0.2, 0.3 and 0.4 M. Fractions of 1 ml. were collected. The bulk of the activity was eluted at 0.2 M-KCl. The first two fractions at 0.3 M-KCl contained a small amount of activity and were combined with the 0.2 M-KCl fractions.

The enzyme was finally concentrated by precipitation of the combined phosphocellulose fractions (7 ml.) with 3.3 g of ammonium sulfate. The precipitate was redissolved in 1.5 ml. of 0.20 M-NaCl, 0.02 M-Tris (pH 7.4) containing bovine serum albumin, 0.3%, at a final activity of 16 units/ml. The activity is stable at 4°C in this solution for at least 8 months. Table 1 summarizes the purification procedure. The significant increase in total enzyme units observed following ammonium sulfate precipitation is not explained, but could be due to removal of interfering activities.

(g) ³²P-labeling of the 5'-end of the DNA

The 5'-phosphoryl ends of DNA were first dephosphorylated by incubation with alkaline phosphatase and then rephosphorylated with [γ -³²P]ATP in the polynucleotide kinase reaction as described by Weiss *et al.* (1968). The reactions were carried out as follows: DNA, 20 m μ moles, in 0.26 ml. 0.1 M-Tris-HCl, pH 8.0, was incubated with 5 μ l. of bacterial alkaline phosphatase (20 units/ml.) at 37°C for 30 min to remove the terminal 5'-phos-

phoryl groups. A 0.035-ml. volume of a kinase reaction mixture (containing 0.3 M-MgCl₂; 0.03 M-potassium phosphate buffer, pH 7.4; [γ -³²P]ATP, 1 μ mole/ml., 2.2×10^8 cts/min/ μ mole; and 1.0 M-dithiothrietol, in a ratio of 2 : 2 : 2 : 1 by vol.), and 5 μ l. of polynucleotide kinase, 5000 units/ml., were then added and the reaction mixture was incubated for 30 min at 37°C. The terminally labeled DNA was precipitated at 0°C by adding 0.3 ml. of 0.1 M-sodium pyrophosphate followed by 2.5 ml. of cold 6% trichloroacetic acid containing 0.01 M-sodium pyrophosphate. The precipitate was collected on a glass filter, washed with nine 2-ml. portions of 6% trichloroacetic acid containing pyrophosphate, and two 2-ml. portions of ethanol. After drying, the filters were counted in a scintillation counter. Under these conditions labeling takes place only at the ends of native DNA. Internal single-strand breaks (nicks) are not labeled because at 37°C the phosphatase is inactive at the nicks (Weiss *et al.*, 1968). If the DNA is denatured with alkali before labeling, then the nicks are exposed and can be labeled by the above procedure.

(h) *Molecular weight estimation by gel filtration*

The molecular weight of purified enzyme was estimated by filtration through a 0.8 cm \times 17.2 cm column of superfine Sephadex G200 (Pharmacia Fine Chemicals). The column was washed with several volumes of NaCl-Tris buffer at a flow rate of 0.5 ml./hr under 10 cm of hydrostatic pressure at 4°C. A mixture containing 250 μ g of bovine serum albumin, 250 μ g of myoglobin, 0.5 optical density unit (at a wavelength of 650 nm) of dextran blue 2000 (Pharmacia Fine Chemicals), and 25 μ l. of purified *H. influenzae* enzyme (16 units/ml.) in a volume of 0.1 ml. was layered onto the column and eluted with NaCl-Tris buffer under the above conditions. Forty 10-drop fractions (0.23 ml.) were collected. Dextran blue was measured by absorbance at 650 nm and the bovine serum albumin and myoglobin by absorbance at 230 nm. The enzyme activity was measured in the following way. Ten μ l. of each fraction was incubated for 30 min at 37°C with 25 μ l. of ³²P-labeled T7 DNA (28 μ g/ml., 6.5×10^8 cts/min/ml.), 1 μ l. of bacterial alkaline phosphatase, 57 units/ml., and 0.2 ml. of Tris-Mg-mercaptoethanol buffer containing 0.05 M-NaCl. The reaction tubes were chilled and 0.1 ml. vol. of salmon sperm DNA, 500 μ g/ml. was added as carrier. The DNA was precipitated with 0.3 ml. of chilled 10% trichloroacetic acid. After 5 min the tubes were centrifuged at 4000 g for 10 min and 0.5 ml. of each supernatant was removed and counted in scintillation medium. This assay procedure gives results similar to the more cumbersome viscometry method but can only be used with the purified enzyme. The phosphatase serves to cleave off ³²P groups exposed by the enzyme digestion and these then appear as trichloroacetic acid-soluble radioactivity.

3. Results

(a) *Detection of an H. influenzae nuclease specific for foreign DNA*

H. influenzae extracts appear to contain no detectable endonuclease activity against native *H. influenzae* DNA by the viscometric assay (see Materials and Methods). Addition of 20 μ l. of an extract from sonicated cells (containing 16 mg protein/ml.) to a viscometer containing *H. influenzae* DNA caused no decrease in η_{sp} during 60 minutes at 30°C (Fig. 1). However, the η_{sp} of phage P22 DNA was significantly decreased by as little as 10 μ l. of extract under the same conditions. The extract thus apparently contains a nuclease with specificity toward the foreign DNA. Addition of 50 μ l. of extract produced approximately a fivefold greater rate of fall of the η_{sp} of the phage DNA. Fractional decrease in η_{sp} proceeded logarithmically with time until the value was below 0.7, after which the decrease became less rapid. The proportionality between initial rate of decrease in η_{sp} and the amount of extract added provides a quantitative assay. A unit of the enzyme activity can be defined as that amount which produces a 25% decrease in η_{sp} in one minute.

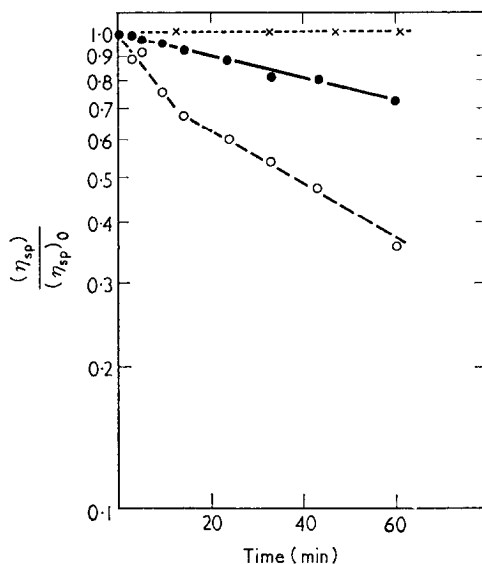


FIG. 1. Effect of a sonicated cell extract from *H. influenzae* on the specific viscosity of phage P22 and *H. influenzae* DNA.

Two viscometers containing 3.5 ml. of phage P22 DNA at a concentration of 40 $\mu\text{g}/\text{ml}$. in Tris-Mg-mercaptoethanol buffer were equilibrated at 30°C. At zero time, 10 μl . (—●—●—) of sonicated cell extract from *H. influenzae*, containing 16 mg protein/ml. was added to the first viscometer and 50 μl . (—○—○—) was added to the second. Measurements of the specific viscosity (η_{sp}) were then taken at intervals and were plotted as fractional values of the zero time specific viscosity (η_{sp})₀. Control measurements were obtained from a viscometer containing 3.5 ml. of *H. influenzae* DNA at a concentration of 40 $\mu\text{g}/\text{ml}$. in the same buffer to which 20 μl . (—×—×—) of cell extract was added at zero time. (The initial specific viscosity of the *H. influenzae* DNA solution was comparable to that of the phage P22 DNA solution.)

TABLE I
Purification procedure

	Total (units)	Total (mg)	Spec. act. (units/mg)
100,000 g supernatant	135	1350	0.10
Bio-Gel column	185	650	0.28
Ammonium sulfate (50 to 70% ppt)	455	200	2.2
Phosphocellulose column†	144	5.5	26.0

† Entries are calculated assuming that all of the ammonium sulfate fraction was purified through the phosphocellulose step.

(b) *Properties of the purified nuclease*

A preparation of the *H. influenzae* nuclease approximately 200-fold purified from the sonicated cell extract was obtained as described in Materials and Methods.

(i) *Magnesium ion requirements*

Enzymic activity is dependent on the presence of Mg^{2+} ions (Table 2). The purified enzyme was optimally active when assayed at 6.6×10^{-3} M- Mg^{2+} in 0.06 M-NaCl. The

activity was decreased by a factor of about 50 when assayed at 10^{-4} M-Mg²⁺ and was unmeasurable at 10^{-5} M-Mg²⁺. About 40% of the maximum activity was obtained at 10^{-3} M-Mg²⁺.

TABLE 2

Effects of sodium chloride and magnesium ion concentration on the nuclease activity

Mg ²⁺ (M)	NaCl (M)	Relative activity
6.6×10^{-3}	0.00	1.0
6.6×10^{-3}	0.02	2.3
6.6×10^{-3}	0.04	2.9
6.6×10^{-3}	0.06	3.4
6.6×10^{-3}	0.08	3.2
6.6×10^{-3}	0.10	2.2
10^{-5}	0.06	< 0.01
10^{-4}	0.06	0.06
10^{-3}	0.06	1.3

DNA viscosity measurements were carried out with phage P22 DNA, 40 μ g/ml. in 3.5 ml. of solvent containing 6.6 mM-Tris-HCl, pH 7.4, and 6.6 mM-mercaptoethanol at the various listed NaCl and Mg²⁺ concentrations. Activities are expressed relative to that obtained under standard assay conditions in Tris-Mg-mercaptoethanol solvent.

(ii) Salt requirements

Optimum activity was obtained in 0.06 M-NaCl (Table 2). At this molarity an approximately threefold increase was obtained over that found under the standard assay conditions in Tris-Mg-mercaptoethanol buffer.

(iii) Molecular weight estimate

In the agarose column purification step the nuclease activity was recovered in fractions corresponding to an approximate molecular weight of 80,000. Gel filtration

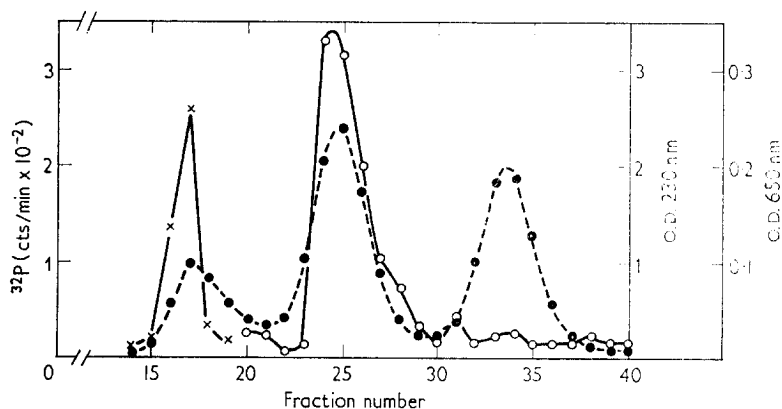


FIG. 2. Gel filtration of the purified nuclease on a Sephadex G200 column.

Dextran blue was measured by absorbancy at 650 nm (—x—x—). Bovine serum albumin and myoglobin were measured by absorbancy at 230 nm (---●---●---). The nuclease activity was measured as released ³²P activity as described in Materials and Methods (—○—○—○—).

of the purified enzyme on Sephadex G200 was carried out with bovine serum albumin and myoglobin as known molecular weight markers. Dextran blue 2000 was used to measure the exclusion volume. The nuclease activity was found in a peak closely associated with the albumin (67,000 molecular weight). The enzyme is thus approximately the same molecular weight (Fig. 2).

(iv) *Foreign DNA versus H. influenzae DNA as substrate*

A mixture of native [^3H]thymidine-labeled *H. influenzae* DNA and native ^{32}P -labeled phage T7 DNA was incubated at 37°C for 30 minutes with an excess of enzyme. Samples were removed from the reaction mixture at zero time (before enzyme addition), five minutes and 30 minutes for assay of trichloroacetic acid-soluble radioactivity and additional fractions were removed for sucrose gradient analysis. One-half of each of the latter fractions was zone sedimented on a neutral sucrose density-gradient and the other half was first denatured in alkali and then sedimented on an alkaline sucrose gradient. At zero time both phage and bacterial DNA sedimented approximately to mid-position in the neutral gradient tube (Fig. 3). The bacterial DNA was more heterogeneous in size and produced a broad band in comparison to the phage DNA. In the alkaline gradient the phage DNA showed a trailing shoulder of smaller pieces but appeared greater than 50% intact. The bacterial peak was slightly broader than in the neutral gradient. After five minutes of treatment with the *H. influenzae* nuclease, the bacterial DNA peaks in both neutral and alkaline gradients were unaltered but the phage DNA was degraded to an average molecular weight of 1.45×10^6 in the neutral gradient and a molecular weight of 0.77×10^6 in the alkaline gradient (calculated according to Studier, 1965) as compared to a molecular weight of 26.4×10^6 for the intact molecule (Studier, 1965). No apparent decrease in size over that found at five minutes was obtained after 30 minutes. The ^{32}P -labeled trichloroacetic acid-soluble radioactivity was $<0.1\%$, $<0.1\%$ and 0.26% at 0, 5 and 30 minutes, respectively. No trichloroacetic acid-soluble ^3H radioactivity was detectable. The nuclease is thus inactive on homologous native DNA, producing neither double- nor single-strand breaks. On heterologous native DNA it appears to act by making a limited number of double-strand breaks. Since the denatured product is approximately one-half the molecular size of the native product, no nicks appear to be produced over and above the double-strand cleavages. The enzyme will be referred to in the remainder of this report as endonuclease R.

(v) *Absence of exonucleolytic activity*

In the above experiment, little, if any, ^{32}P radioactivity was released as trichloroacetic acid-soluble material during the digestion with endonuclease R. Therefore it appears unlikely that the enzyme itself has an exonuclease activity or that it is contaminated with a significant exonuclease activity. In order to substantiate this further the digest was examined for released nucleotides. A solution of ^{32}P -labeled phage T7 DNA was extensively digested with an excess of endonuclease R. A sample was then mixed with unlabeled marker nucleotides and chromatographed in two dimensions. As seen in Table 3, essentially no activity was found associated with the nucleotide spots.

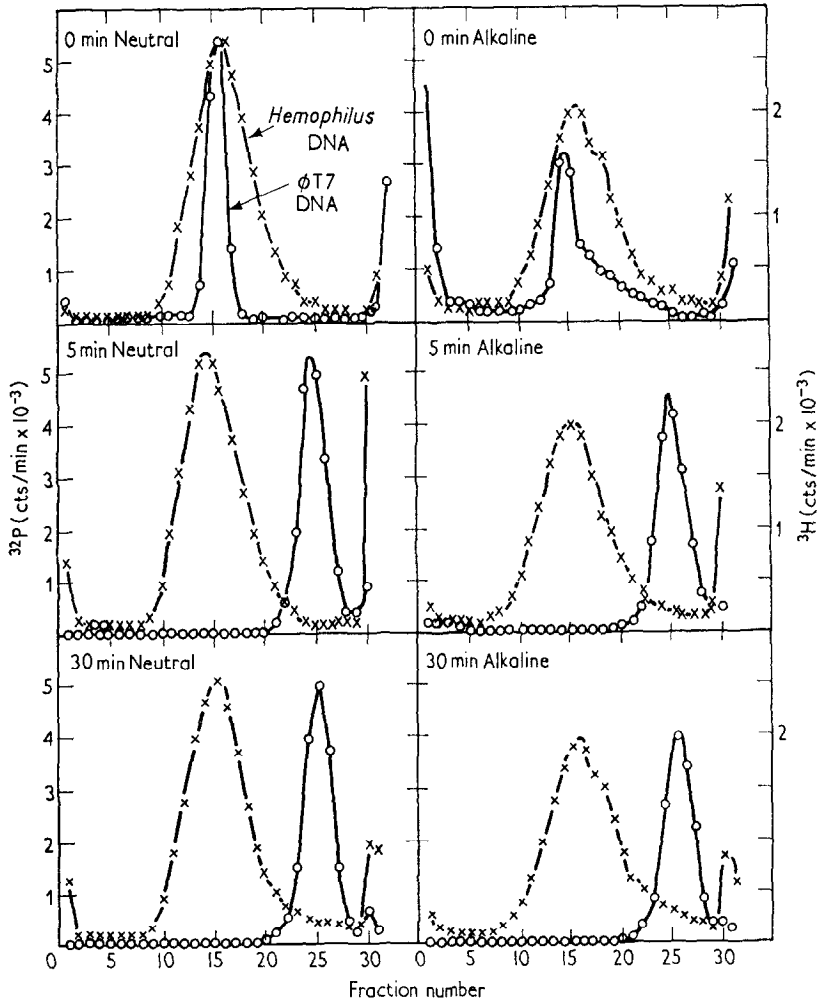


FIG. 3. Zone sedimentation in neutral and alkaline sucrose gradients of the products of the *H. influenzae* nuclease digestion of phage T7 DNA.

The reaction mixture (0.975 ml.) contained ^3H -labeled *H. influenzae* DNA, 17.1 μg , 8.7×10^4 cts/min; ^{32}P -labeled phage T7 DNA, 0.94 μg , 6.4×10^4 cts/min; 50 mM-NaCl; 6.6 mM-Tris-HCl, pH 7.4; 6.6 mM-MgCl₂; 6.6 mM-mercaptoethanol. At zero time 0.1 ml. was removed for trichloroacetic acid precipitation and 0.15 ml. was pipetted into 0.15 ml. of 0.1 M-EDTA, pH 8.0, on ice for sucrose gradient analysis. Purified *H. influenzae* nuclease, 5 μl ., was then added and the reaction mixture was incubated at 37°C. Additional samples for trichloroacetic acid precipitation (0.1 ml.) and sucrose analysis (0.15 ml.) were removed at 5 and 30 min. A 0.1-ml. portion of each of the samples that had been taken for sucrose gradient analysis was layered on a neutral gradient and centrifuged at 50,000 rev./min for 2 hr at 4°C. The remaining 0.2 ml., was alkali-denatured by addition of 10 μl . of 4 M-NaOH and 0.1 ml. was then centrifuged at 50,000 rev./min for 2.5 hr. Liquid fractions collected drop wise from the bottom of the centrifuge tubes were counted directly in scintillation medium. (—x—x—), ^3H radioactivity; (—o—o—), ^{32}P radioactivity. Trichloroacetic acid precipitation was carried out by addition of 0.2 ml. of salmon sperm DNA (500 $\mu\text{g}/\text{ml}$.) and 0.3 ml. of 10% trichloroacetic acid on ice. The samples were centrifuged at 4500 g for 10 min and 0.3 ml. of the supernatant was counted in scintillation medium. The results are reported in the text.

TABLE 3

Release of nucleotides by endonuclease R digestion

Nucleotide species	Counts/50 min	%
dAMP	38	0.0004
dTMP	121	0.001
dGMP	0	0
dCMP	0	0
origin	1.04×10^7	100

A reaction mixture (0.1 ml.) containing ^{32}P -labeled phage T7 DNA 78 μmoles , 5.5×10^4 cts/min/ μmole in Tris-Mg-mercaptoethanol buffer was incubated with 5 $\mu\text{l.}$ of endonuclease R for 120 min at 37°C and for 42 min with an additional 5 $\mu\text{l.}$ of enzyme. Five $\mu\text{l.}$ were then removed, mixed with the 4 standard 5'-nucleotides, spotted on the corner of a thin layer PEI cellulose square and chromatographed in the first dimension with 1 M-formic acid followed by a second dimension with 1 M-LiCl using methods described by Kelly & Smith (1970). The marker spots were located with a shortwave ultraviolet mineralight, cut out and counted in toluene scintillation medium. The origin including the surrounding 1 cm of thin layer material was also counted. The results are presented as 50-min counts corrected for background.

(vi) *Native versus denatured DNA as substrate*

Endonuclease R is active only on native DNA. No decrease in the size of denatured T7 DNA was demonstrable by zone sedimentation on alkaline sucrose gradients after incubation with enzyme (Fig. 4(a) and (b)), whereas native DNA was degraded (Fig. 4(c)).

(vii) *3'-Hydroxyl, 5'-phosphoryl cleavage*

Polynucleotide kinase catalyses the transfer of a ^{32}P -labeled phosphoryl group from [γ - ^{32}P]ATP to the 5' terminus of the polynucleotide chain. This reaction is known to depend upon the presence of a free hydroxyl group at the 5' terminus of the substrate and will not occur if a 5'-phosphoryl group is present (Weiss *et al.*, 1968). It follows that if pretreatment of the substrate with bacterial alkaline phosphatase is required in order to obtain transfer then the 5' termini of the substrate must be phosphorylated. As seen in Table 4, phage P22 DNA which has been digested to completion with endonuclease R, incubated with phosphatase, and then with kinase and [γ - ^{32}P]ATP, incorporated 3740 cts/min/20 μmole of phage DNA whereas omission of the phosphatase treatment results in only 136 cts/min incorporation. Thus, endonuclease R produces a 3'-hydroxyl, 5'-phosphoryl cleavage. Without endonuclease R treatment only the ends of the intact DNA molecules are labeled. Subtracting the blank of 66 cts/min obtained when kinase is omitted, 114 cts/min are incorporated per 20 μmole of phage DNA. Using this and the known specific activity of the [γ - ^{32}P]ATP, the molecular weight of the phage DNA is calculated as 26×10^6 (see legend of Table 3 for method of calculation). This figure agrees well with the measured molecular weight of 26.3×10^6 obtained by Rhoades, MacHattie & Thomas (1968). The average calculated molecular weight of the limit product DNA fragments is 0.81×10^6 . Approximately 32 breaks are produced per phage P22 DNA molecule by the enzyme.

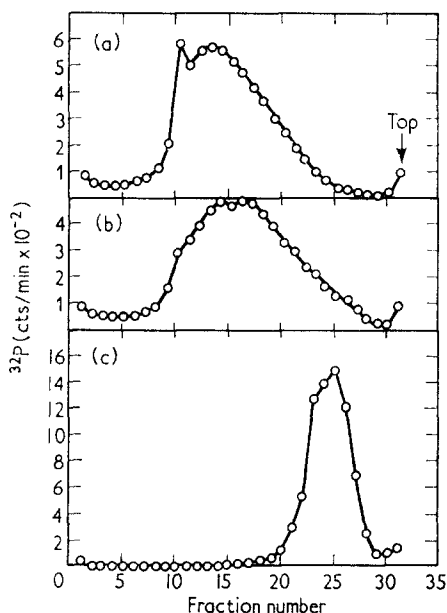


FIG. 4. Nuclease activity on denatured and native DNA.

Three reaction mixtures (a), (b) and (c) were set up in the following way. A control reaction mixture (a) contained 10 μ l. of alkali-denatured 32 P-labeled phage T7 DNA, 1.15×10^4 cts/min/ $m\mu$ mole, 100 $m\mu$ mole/ml. and 50 μ l. of Tris-Mg-mercaptoethanol buffer containing 0.04 M-NaCl. Reaction mixture (b) contained 5 μ l. of purified endonuclease R in addition to the above ingredients. Reaction mixture (c) was the same as in (b) except that 10 μ l. of native 32 P-labeled T7 DNA was used. The three reaction mixtures were incubated for 15 min at 37°C after which 5 μ l. of 0.5 M-EDTA was added and then each was alkali-denatured. Each mixture was then layered onto an alkaline sucrose gradient and centrifuged for 2.5 hr at 50,000 rev./min. Fractions were collected and counted as in Fig. 2. (The 32 P-labeled T7 DNA used in this experiment had suffered considerable radiation damage due to storage, and this accounts for the broad peaks obtained.)

TABLE 4

32 P-labeling of the 5'-end of endonuclease R-treated phage P22 DNA

DNA treatment	Cts/min	Calc. mol. wt
+ Bacterial alkaline phosphatase + kinase	180	26×10^6
Endonuclease + Bacterial alkaline phosphatase	66	
Endonuclease + kinase	136	
Endonuclease + Bacterial alkaline phosphatase + kinase	3740	0.81×10^6

The reaction mixture for endonuclease R digestion (0.45 ml.) contained 180 $m\mu$ moles of phage P22 DNA, 50 mM-NaCl, and 6.6 mM each of Tris-HCl (pH 7.4), $MgCl_2$, and mercaptoethanol. At zero time, endonuclease R (5 μ l.) was added and the mixture was incubated at 37°C for 30 min. Samples of 0.05 ml. were removed at zero time (before addition of enzyme) and after the completion of digestion and treated with the various listed combinations of phosphatase or polynucleotide kinase. The [γ - 32 P]ATP used in the kinase labeling reaction had a specific activity of 2.25×10^5 cts/min/ $m\mu$ mole. The amount of 32 P incorporated terminally was measured as trichloroacetic acid-precipitable counts.

Molecular weight = $660ma/(c-b)$, where m is the $m\mu$ moles of DNA, c is the incorporated terminal 32 P radioactivity, b is the blank in which kinase was absent, a is the specific activity of [γ - 32 P]ATP, and the molecular weight of the base pair is 660.

(viii) Mechanism of double-strand cleavage

The sucrose gradient experiments indicate that native foreign DNA possesses a limited number of substrate sites which are subject to double-strand cleavage by endonuclease R. The question arises as to whether the introduction of the second single-strand break within a site is independent of the first. If so, then a limited digestion with endonuclease R should result in a large excess of nicks over duplex breaks. On the other hand, if the introduction of the second nick is coupled to that of the first, then duplex breaks should appear in significant amount early in the reaction.

To determine by which mechanism endonuclease R acts, ^{32}P -5' end-labeling techniques were again used. It is possible, as described in Materials and Methods, to discriminate nicks from ends by this procedure taking advantage of the fact that nicks will be labeled only if the DNA is denatured before phosphatase treatment. Separate reaction mixtures were set up containing phage T7 DNA and various concentrations of endonuclease R ranging from an amount capable of producing relatively very few breaks during the incubation to an amount which was saturating. After incubation with the enzyme the DNA was labeled at the 5'-termini with ^{32}P either before or after denaturation (Fig. 5). The data show that with incomplete digestion, more label

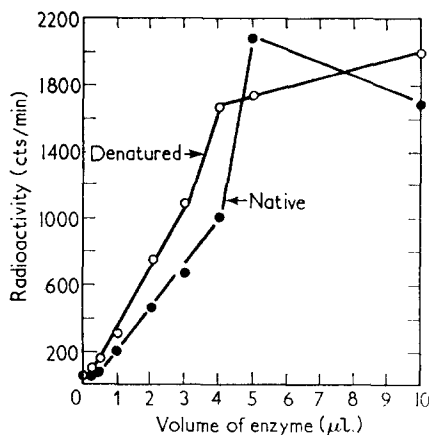


FIG. 5. Production of nicks and double-strand breaks in phage T7 DNA.

The reaction mixtures for endonuclease R digestion each contained unlabeled phage T7 DNA, 66 μmole s and ^3H -labeled *H. influenzae* DNA, 0.23 μmole , 1.5×10^4 cts/min/ μmole in a 0.15-ml. vol of Tris-Mg-mercaptoethanol buffer containing 0.06 M-NaCl. To each reaction mixture the amount of enzyme shown in the Figure was added and incubation was carried out for 20 min at 37°C. Endonuclease R was then inactivated by treatment at 65°C for 10 min. The DNA in each tube was precipitated by 2 ml. of 70% ethanol and washed with 2 ml. of acetone. The DNA was redissolved in 0.15 ml. of NaCl-Tris buffer. An 0.5 ml. sample from each digest was then terminally labeled by the kinase reaction with or without alkali denaturation preceding the phosphatase step. The ^3H -labeled DNA was used to monitor recovery from the precipitation step. Recovery averaged about 60% and corrections were made to 100% recovery.

was incorporated into the DNA after denaturation than before, indicating that some nicks are produced first. However, the early appearance of a significant proportion of breaks clearly suggests an association between the two nicking events which result in a duplex break. This association might be explained by either of two mechanisms. Either the enzyme binds to a site and breaks first one strand and then the other without becoming detached, or else it introduces nicks in separate binding events with the probability for rebinding being much greater following the first nick. In the

latter case we might imagine that the presence of the first nick makes the other chain more accessible.

An additional result of the experiments is that the DNA fragments produced as a limit product with high levels of enzyme contained no nicks, within the limits of error of the terminal labeling procedure, since both curves reached a plateau together at an average of about 1875 cts/min incorporated terminally. Since about 45 cts/min were incorporated into the undigested intact DNA, about 40 to 45 breaks were produced per phage T7 molecule.

4. Discussion

Endonuclease R produces double-strand, 5'-phosphoryl, 3'-hydroxyl cleavages at a limited number of sites on foreign native DNA. In addition to phage T7 and phage P22 DNA, we have tested *S. typhimurium* DNA, salmon sperm DNA and *Bacillus subtilis* DNA. All are degraded to about the same extent. The fragments produced in the limit digest of phage T7 DNA contain no excess of nicks, and essentially no trichloroacetic acid-soluble products or nucleotides are released by the digestion. The enzyme has no demonstrable activity on native *H. influenzae* DNA or on denatured foreign DNA. There are no special co-factor requirements for activity other than magnesium ions.

The similarity of endonuclease R to the restriction enzyme purified from *E. coli* by Meselson & Yuan (1968) is considerable. Both enzymes produce a small number of specific double-strand breaks in foreign DNA and are inactive on DNA of the bacterial strain from which they have been purified. An interesting feature of the *E. coli* enzyme is that it requires *S*-adenosyl methionine and adenosine triphosphate as co-factors in addition to magnesium ions. This suggests a connection between the restriction activity and the DNA modification activity which is known to protect the host DNA from cleavage. Arber (1968) has identified the DNA modification as methylation of adenine to form 6-methyl amino purine and recently Kühnlein, Linn & Arber (1969) have demonstrated modification *in vitro*. We have not as yet investigated DNA modification in *H. influenzae*.

We have been particularly interested in the ability of endonuclease R to "recognize" only a few specific sites on rather large foreign DNA molecules. It appears likely that this recognition specificity resides in the base sequence of the sites. An estimate of the size of the base sequence can be made. For phage T7 DNA we observed 40 to 45 breaks per molecule. Since T7 DNA is about 40,000 base pairs in length, the average fragment is about 1000 base pairs in length. For phage P22 DNA, the average fragment is approximately 1300 base pairs in length. To attain this degree of specificity a site would have to be five to six bases in length providing that the enzyme recognizes a completely unique sequence. In the accompanying paper (Kelly & Smith, 1970) the base sequence recognized by endonuclease R is completely identified and provides confirmation of this estimate.

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