Cellular Site in *Bacillus subtilis* of a Nuclease Which Preferentially Degrades Single-Stranded Nucleic Acids

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ABSTRACT

BIRNBOIM, H. C. (Albert Einstein College of Medicine, New York, N.Y.). Cellular site in *Bacillus subtilis* of a nuclease which preferentially degrades single-stranded nucleic acids. J. Bacteriol. 91:1004–1011. 1966.—A nuclease, identified by a marked preference for single-stranded nucleic acids, has been demonstrated in extracts of *Bacillus subtilis*. The enzyme was associated with the cell wall-membrane fraction of mechanically disrupted cells and was released from cells which had been converted to protoplasts by lysozyme. The nuclease activity prepared by the latter procedure was found to be activated and solubilized by treatment with trypsin. The enzyme had about 2% activity on native deoxyribonucleic acid (DNA) as compared with denatured DNA. By use of CsCl analytical density gradient ultracentrifugation, this preparation was shown to degrade denatured DNA selectively in mixtures of native and denatured DNA.

The cellular location of certain enzymes in bacteria has been the subject of several recent investigations. In *Escherichia coli*, several phosphatases and nucleases (6, 10, 11, 12) have been shown to be released from bacterial cells when converted to spheroplasts. The localization of these enzymes at or near the cell surface has been proposed. In *Bacillus licheniformis* (previously classified as *B. subtilis*), a penicillinase has been found attached to the protoplast membrane (3, 4). In *B. subtilis*, Momose et al. (8) demonstrated a 5'-nucleotidase, the major portion of which could be released from cells upon conversion to protoplasts with lysozyme. In strains of *Mycoplasma*, several enzymes, including a ribonuclease and a deoxyribonuclease have been found associated with cell membranes (13).

In this paper, we present evidence for the existence in *B. subtilis* of a nuclease characterized by a marked preference for single-stranded deoxyribonucleic acid (DNA), as compared with native, double-stranded DNA, as a substrate. In mechanically disrupted cells, about one-half of the enzyme was found associated with the "cell wall-membrane" pellet. When cells were converted to protoplasts by treatment with lysozyme, the enzyme was released and recovered in the supernatant fraction. The association of the nuclease with the cell wall-membrane of *B. subtilis* was studied, and the partially purified enzyme was characterized with respect to substrate and requirements for optimal activity.

MATERIALS AND METHODS

Bacterial strain. *B. subtilis* 168-2, a transformable strain requiring tryptophan and leucine, was used for studies on enzyme distribution and purification. *B. subtilis* 168T, which requires thymine and tryptophan, was used for the preparation of radioactive DNA.

Media and growth conditions. V-Y broth [Difco Veal Infusion broth, 2.5% (w/v); Difco yeast extract, 0.5% (w/v)] was the routine medium used for growing *B. subtilis* for enzyme isolation. Volumes of 1 liter were inoculated from a single colony and incubated in 2-liter flasks at 37°C in a New Brunswick gyrotary floor shaker for 16 hr. Under these conditions, the culture reached the early stationary phase of growth, about 3 g (wet weight) of bacteria per liter.

Reagents. Lysozyme (egg white), three-times crystallized, B grade, was a product of Calbiochem. Tris(hydroxymethyl)aminomethane (Tris) was Trizma of the Sigma Chemical Co., St. Louis, Mo. Analytical reagent-grade sucrose was obtained from Mallinckrodt Chemical Works, New York, N.Y. Trypsin (Worthington Biochemical Corp., Freehold, N.J.) was used without further purification. Diethylaminoethyl (DEAE) cellulose (Carl Schleicher and Schuell Co., Keene, N.H.) was prepared for use by washing with 1 N NaOH, water, 1 N HCl, 1 N NaOH, and water, and

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was finally suspended in 0.05 M Tris-HCl (pH 8). Columns were packed under 10 psi of pressure and washed with the starting buffer. Sephadex G-100 was purchased from Pharmacia, Uppsalna, Sweden. Carboxy-4000 (polyethylene glycol) was a product of Union Carbide Chemical Co., New York, N.Y. All other chemicals were reagent grade. SSC refers to a solution of 0.15 M NaCl and 0.015 M sodium citrate at pH 7; 0.1 × SSC refers to a 1 in 10 dilution of it.

**Competent cells.** B. subtilis 168-2 was made competent by a method similar to that of Anagnostopoulos and Spizizen (1).

**Addition of radioactive DNA.** B. subtilis 168T was grown in 400 ml of the minimal salt medium of Spizizen (15) containing the following: DL-tryptophan, 100 μg/ml; casein hydrolysate (Difco), 0.05% (w/v); yeast extract (Difco), 0.1% (w/v); glucose, 1.25% (w/v); nonradioactive thymine, 0.5 μg/ml; thymine-2-C14, 35 μg, specific activity 14.8 mc/m mole (Calbiochem). After 15 hr of growth at 37°C, the cells were harvested by centrifugation, and DNA was prepared by the method of Marmur (7), except that the final isopropanol precipitation step was omitted. The yield of DNA was approximately 3 mg. It had a specific radioactivity of 2,500 counts per min per μg, counted on a gas-flow counter. None of the radioactivity was in ribonucleic acid (RNA), as judged by the absence of acid-soluble counts after treatment with pancreatic ribonuclease or with 0.3 N KOH at 37°C for 15 hr. The DNA, dissolved in 0.1 × SSC, was stored at 4°C over chloroform.

**Assay for nuclease activity with denatured DNA as substrate.** B. subtilis DNA-thymine-2-C14 (114 μg/ml in 0.1 × SSC) was denatured by heating at 100°C for 10 min, then chilled quickly in an ice-water bath. An assay mixture was prepared by combining 0.1 ml of denatured DNA, 0.1 ml of CaCl2 (0.1 M), and 0.8 ml of buffer [either 0.1 M sodium glycyglycinate (pH 8.4) or 0.1 M sodium glycinate (pH 9.2)]. A 0.1-ml amount of this was combined with 0.01 ml of enzyme (0.2 to 2 units) and incubated at 37°C for 30 min. The reaction was stopped by chilling on ice, and 0.1 ml of cold carrier DNA (described below) was added followed by 0.5 ml of cold ethyl alcohol-HCl (1 vol of 95% ethyl alcohol-1 vol of 2 N HCl). A flocculent precipitate formed which was removed by centrifugation at 10,000 × g for 5 min at 2°C. Samples of 0.5 ml of the clear supernatant fluid were pipetted and placed on stainless-steel planchets under a heat lamp. The dried samples were counted in a Nuclear Chicago gas-flow low-background counter.

**Assay for nuclease activity with native DNA as substrate.** The method used was similar to the above except that unheated (native) DNA was added instead of denatured DNA.

Blanks incubated as above but containing no enzyme and either denatured or native DNA contained about 2 and 0.75%, respectively, of the total counts added.

The use of ethyl alcohol-hydrochloric acid as precipitant permitted the counting of acid-soluble C14 nucleotides with minimal self-absorption losses on a gas-flow counter. However, it required a larger amount of carrier than usual and, for the purpose, a crude nucleic acid preparation of E. coli was used. It was prepared by the method of Marmur (7) up to the first ethyl alcohol precipitation, after which the pooled nucleic acid was dissolved in 0.1 × SSC, and the absorbance at 260 μm was adjusted to approximately 100. The preparation was heated at 80°C for 10 min to decrease any nuclease activity that might be associated with the carrier.

**Definition of unit.** One enzyme unit was defined as the amount of enzyme which, under the conditions described above, converted 1 μmole of denatured DNA to a form soluble in ethyl alcohol-HCl. Enzyme activity was proportional to enzyme concentration in the range of 0.2 to 2 units. For studies on the distribution of enzyme in fractionated bacterial cells, assays were carried out at pH 8.4. For studies on the purification and properties of the enzyme, the assay was performed at pH 9.2. Specific activity was defined as enzyme units per milligram of protein.

Protein was estimated by the method of Lowry et al. (5) with bovine serum albumin as standard.

**RESULTS**

**Distribution of nuclease activity in bacterial cells (with denatured DNA as substrate).** Bacterial cultures, harvested at the beginning of the stationary phase of growth, were used for studies on the cellular distribution of nuclease activity. In the three separate experiments reported in Table 1, the nuclease activity of the culture supernatant fluid and the bacterial cells was determined. The bacterial cells were fractionated either by conversion to protoplasts with lysozyme or by disruption in a French pressure cell.

**Protoplasts.** One-half of a washed bacterial pellet was suspended at a concentration of 2 g (wet weight) per 10 ml of 0.5 M sucrose containing 0.05 M Tris-HCl (pH 8.2), 0.01 M divalent cation (shown in Table 1), and 0.5 mg/ml of lysozyme. After 30 to 45 min of incubation at 37°C (at which time microscopic examination showed all bacteria to be spherical), the suspension was centrifuged at 39,000 × g for 30 min. The supernatant fluid was designated "protoplast supernatant fraction." The pellet was suspended in an equal volume of 0.05 M Tris-HCl (pH 8.2) containing 0.01 M divalent cation. This fraction was designated "protoplast lysate."

The distribution of nuclease activity in these fractions is shown in Table 1. In experiments 1 and 2 of Table 1, the majority of the nuclease was released into the 39,000 × g supernatant fluid during the preparation of protoplasts. The attendant 10-fold increase in specific activity suggests that the release of the enzyme was selective and not simply the result of lysis of a part of the cells.

**Pressure cell disruption.** The second half of a bacterial pellet was suspended at a concentration
of 2 g (wet weight) per 10 ml of cold 0.05 M Tris-HCl (pH 8.2), 0.01 M divalent cation (shown in Table 1). The suspension was chilled on ice, and then was passed through a cold French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 20,000 psi. The crushed cells were centrifuged at low speed (8,000 × g, 5 min) to remove unbroken bacteria, and the turbid supernatant fluid was removed and centrifuged at 39,000 × g for 30 min. The clear supernatant fluid was designated "soluble extract." The pellet appeared to contain three layers analogous to those described by Salton (14). The top, brown layer was designated "membrane fragments." The intermediate, whitish layer (cell wall-membrane fraction) was washed once by suspension in 0.05 M Tris-HCl (pH 8.2), and centrifugation at 23,000 × g for 15 min (CW-M wash). The washed pellet (CW-M) was treated with lysozyme (0.5 mg/ml) for 30 min at 37°C (lysed CW-M of Table 1). The bottom layer of the 39,000 × g pellet (intact bacteria) was combined with the 8,000 × g pellet and suspended in the same Tris buffer. This suspension was treated with lysozyme (0.5 mg/ml) for 30 min at 37°C, and the nuclease activity of the lysate was determined (Bacterial pellet of Table 1). Intact bacteria and the cell wall-membrane fraction were identified on the basis of phase-contrast microscopic examination.

The distribution of nuclease activity (assayed at pH 8.4) in these fractions is shown in Table 1. In these experiments, the cells were harvested at the early stationary phase of growth (16 hr). As much enzyme was found to be associated with the cells as was found in the broth supernatant fluid. It may be seen that roughly one-half of the total enzyme of mechanically disrupted cells was recovered in the 39,000 × g pellet. Treatment of the CW-M fraction with lysozyme led to a marked increase in nuclease activity (CW-M, as compared with lysed CW-M, Table 1) as well as a release of about one-half of the enzyme into a 100,000 × g, 30 min, supernatant fraction (data not shown).

The CW-M and lysed CW-M fractions of experiment 3, Table 1, were also tested for nuclease activity with native DNA as substrate at the same pH. The rate of activity was about 10% that seen when denatured DNA was used as substrate, and it also increased after lysozyme treatment.

Nuclease activity of intact bacterial cells. The experiment reported in Table 2 was designed to show that part of the nuclease associated with washed cells was accessible to denatured DNA, even though the cells remained "intact." At the times indicated, a 10-ml sample was removed from a culture growing in V-Y broth, and the cells were washed once by centrifugation and suspended in 2.0 ml of 0.05 M imidazole buffer (pH 7.0) containing 0.001 M MnCl₂. A 0.01-ml amount of the suspension was added directly to an assay mixture (pH 8.4; see Materials and Methods) containing denatured C⁴-DNA ("Incubated cell suspension," Table 2). As an estimate of the maximal amount of lysis of the cells that might have occurred during incubation with the radioactive DNA, another sample of cells was concomitantly incubated under similar conditions (no DNA) for an equal length of time. These cells were then chilled and centrifuged at 10,000 × g for 5 min, and the nuclease activity of the supernatant fluid was assayed ("Incubated cell supernatant fraction," Table 2). To determine the total cellular nuclease activity, a third sample of cells, similarly washed, was treated with lysozyme (0.5 mg/ml) for 30 min at 37°C before assay for nuclease activity ("Lysed cells"). The data of Table 2 show that the "Intact cells" manifested

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Broth supernatant fluid</th>
<th>Cell wash</th>
<th>Protoplast supernatant fraction</th>
<th>Protoplasm lysate</th>
<th>Soluble extract</th>
<th>Membrane fragments and CW-M wash</th>
<th>CW-M</th>
<th>Lysed CW-M</th>
<th>Bacterial pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40,200</td>
<td>676</td>
<td>52,400 (887)</td>
<td>24,700 (74)</td>
<td>22,200 (79)</td>
<td>10,800</td>
<td>18,200 (397)</td>
<td>28,500 (597)</td>
<td>16,900</td>
</tr>
<tr>
<td>2</td>
<td>31,700</td>
<td>210</td>
<td>80,300 (561)</td>
<td>9,650</td>
<td>19,750 (74)</td>
<td>4,600</td>
<td>25,500</td>
<td>--</td>
<td>16,700</td>
</tr>
<tr>
<td>3</td>
<td>28,000</td>
<td>495</td>
<td>---</td>
<td>---</td>
<td>13,230 (52.4)</td>
<td>5,400</td>
<td>5,830 (276)</td>
<td>11,800 (561)</td>
<td>6,390</td>
</tr>
</tbody>
</table>

* Total units of nuclease activity (assayed at pH 8.4 with denatured DNA as substrate, as described in the text) recovered from 1 liter of broth supernatant fluid or cells, fractionated either as protoplasts or disrupted in the French press. Numbers in parentheses refer to the corresponding specific activity.

† The media for fractionating cells contained, in addition to the buffer described in the text, the following divalent cations at a concentration of 0.01 M: experiments 1 and 2, Mn++; experiment 3, Mg++. (in each case as the chloride salt).
10 to 40% of the nuclease activity of corresponding cells which had been lysed with lysozyme. The results suggest that part of the cell-bound nuclease was located at or near the cell surface. Nuclease activity in the "incubated cell supernatant fraction" was presumably a reflection of lysis of some of the cells during the incubation, although possibly it could have represented contamination by enzyme from the broth supernatant fluid or excretion of enzyme by the cell, particularly in the case of the 50-hr sample.

Table 2 also shows that up to 12 hr of growth the enzyme associated with the cells was greater than that found in the broth supernatant fluid; between 12 and 50 hr, the broth supernatant enzyme increased considerably, and the cell-bound enzyme changed very little.

**Further purification of the protoplast supernatant nuclease.** The nuclease recovered in the 39,000 × g supernatant fraction after conversion of early stationary phase cells to protoplasts was examined further. Additional centrifugation at 100,000 × g for 30 min did not sediment the enzyme, but attempts at purification by ammonium sulfate fractionation showed that, after its precipitation, the enzyme was associated with a turbid suspension and could not be brought into solution. Because trypsin was reported to have solubilized penicillinase from *B. licheniformis* membranes (3, 4), its action on the insoluble *B. subtilis* nuclease preparation was studied (Table 3).

Protoplast supernatant fluid was brought to 65% saturation with solid ammonium sulfate at 0 C. The precipitate which formed did not redissolve in buffer (0.05 M Tris-HCl (pH 7.5), 0.01 M CaCl₂, 0.001 M MnCl₂), and less than 40% of the nuclease activity was recovered (Table 3 and 4). When this suspension was centrifuged at 100,000 × g for 30 min, about one-half of the nuclease activity remained in the supernatant fluid. However, as is shown in Table 3 and 4, treatment of the ammonium sulfate precipitate with 1 mg/ml of trypsin for 50 min at 37 C resulted in a threefold increase in nuclease activity, the majority of which was soluble after centrifugation at 100,000 × g for 30 min. Trypsin alone had almost no deoxyribonuclease activity, as measured under the same conditions of salt and pH as the *B. subtilis* nuclease. Incubation of the ammonium sulfate precipitate without trypsin for an equal length of time resulted in a definite increase in activity but less than that seen with trypsin treatment.

Preincubation of the protoplast supernatant fluid for 30 min at 37 C (without first precipitating with ammonium sulfate) resulted in a variable, slight increase in activity. As is shown in Table 3, preincubation of protoplast supernatant fluid with trypsin (1 mg/ml) for 50 min at 37 C resulted in a threefold increase in activity, as did trypsin treatment of the ammonium sulfate precipitate.

A purification procedure for nuclease from protoplast supernatant fluid is shown in Table 4. Fractions were assayed for nuclease activity with denatured DNA as substrate at pH 9.2, as described in Materials and Methods.

An amount of 10 g (wet weight) of cells was suspended in 50 ml of a medium containing 0.5 M sucrose, 0.05 M Tris-HCl (pH 7.4), 0.05 M NaCl, 0.01 M MgCl₂, and 0.5 mg/ml of lysozyme, and
TABLE 3. Effect of trypsin on protoplast supernatant fluid nuclease

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>(a) Protoplast supernatant fluid nuclease</td>
<td>100</td>
</tr>
<tr>
<td>(b) (a) 50 min, 37 °C, trypsin</td>
<td></td>
</tr>
<tr>
<td>(c) Ammonium sulfate precipitate of (a)</td>
<td>12</td>
</tr>
<tr>
<td>(d) (c) 50 min, 37 °C, no trypsin</td>
<td>21</td>
</tr>
<tr>
<td>(e) (c) 50 min, 37 °C, trypsin</td>
<td>42</td>
</tr>
<tr>
<td>(f) 100,000 × g supernatant fraction of (e)</td>
<td>31</td>
</tr>
<tr>
<td>(g) Trypsin†</td>
<td></td>
</tr>
</tbody>
</table>

* Assays were carried out at pH 9.2, with denatured DNA as substrate, as described in Materials and Methods.
† The nuclease activity of trypsin was assayed under the same conditions as above; the final concentration in the assay was 100 times greater than in (b), (e), or (f).

TABLE 4. Purification of Bacillus subtilis nuclease

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Units of nuclease* (X 10^{-4})</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplast supernatant fraction</td>
<td>120</td>
<td>56</td>
<td>467</td>
</tr>
<tr>
<td>Protoplast lysate</td>
<td>535</td>
<td>40</td>
<td>75</td>
</tr>
<tr>
<td>Ammonium sulfate (A.S.) precipitate</td>
<td>23.2</td>
<td>17</td>
<td>732</td>
</tr>
<tr>
<td>Trypsin-treated A.S. precipitate</td>
<td>29.2†</td>
<td>66</td>
<td>2,260</td>
</tr>
<tr>
<td>100,000 × g</td>
<td>25.2†</td>
<td>53</td>
<td>2,100</td>
</tr>
<tr>
<td>Concentrated DEAE cellulose fractions</td>
<td>0.5†</td>
<td>23</td>
<td>46,000</td>
</tr>
</tbody>
</table>

* Assayed at pH 9.2, with denatured DNA as substrate, as described in Materials and Methods.
† Includes the added trypsin.
‡ Concentrated DEAE cellulose fractions were contaminated with traces of Carbowax, used in the concentration step. Carbowax interfered with the assay of protein by the method of Lowry et al. (5), so the protein shown was that assayed before the concentration step.

was incubated at 37 °C with gentle shaking. When protoplast formation was nearly complete (45 min, as judged microscopically), the suspension was chilled on ice and centrifuged at 29,000 × g for 30 min. The supernatant fluid was brought to 65% saturation with solid ammonium sulfate, and after 15 min at 0 °C the precipitate which formed was collected by centrifugation. The precipitate was suspended in 3 ml of 0.05 M Tris-HCl (pH 7.4), 0.01 M CaCl₂, and 0.001 M MnCl₂, and was incubated with trypsin (1 mg/ml) at 37 °C for 50 min; the mixture was then centrifuged at 100,000 × g for 20 min at 2 °C.

This supernatant fluid was diluted to 20 ml with the Tris buffer and run onto a DEAE cellulose column (1.2 by 13 cm) equilibrated with the same buffer. The column was washed with 80 ml of the buffer followed by an equal volume of 0.05 M sodium succinate (pH 6.0), 0.005 M CaCl₂, 0.005 M MnCl₂, and 0.02 M NaCl. No enzyme was eluted from the column by either washing, but a large amount of material absorbing at 280 nm was removed. This was followed by a 400-ml linear NaCl gradient, running from 0.02 to 0.1 M, in 0.05 M sodium succinate (pH 6.0), 0.005 M CaCl₂, and 0.005 M MnCl₂. The flow rate was 40 ml/hr at 5 °C. Forty 10-ml fractions were collected. The enzyme was eluted in a fairly sharp peak at the middle of the gradient, with very little material absorbing at 280 nm. The peak tubes were pooled, and the solution was transferred to a dialysis bag and concentrated by contact with dry Carbowax at 4 °C for about 8 hr. The concentrated nuclease degraded native DNA at 2% of the rate at which denatured DNA was degraded, as measured under the same conditions of pH and salt. In other experiments, the nuclease of the protoplast supernatant fluid degraded native DNA at 10 to 15% of the rate observed with denatured DNA; after purification as described above, the corresponding rate was reduced to 2 to 5%. The enzyme preparation having 2% activity on native DNA was used for studies of its properties.

Omission of the trypsin treatment of the ammonium sulfate precipitate affected markedly the chromatographic behavior of the enzyme. It was eluted from DEAE-cellulose at pH 8.0, at about 0.2 M NaCl, as compared with the elution of trypsin-prepared enzyme at pH 6.0, with 0.05 M NaCl.

Divalent cation requirement of the partially purified enzyme. The effect of varying concentrations of CaCl₂ on enzyme rate is shown in Fig. 1. When assayed at pH 9.2, maximal stimulation was observed with a Ca²⁺ concentration above 0.001 M. MgCl₂ and CuSO₄ were tried at concentrations from 0.002 to 0.01 M, and were found to support activity at less than 10% the rate of Ca²⁺.

Optimal pH of the partially purified enzyme. In 0.05 M glycylglycinate-0.05 M glycinate buffers adjusted to the appropriate pH with 1 N
NaOH, maximal activity was observed between pH 9.0 and 9.6 (Fig. 2).

Evidence for exonucleolytic mode of attack by the partially purified enzyme. The *B. subtilis* nuclease was characterized as an exonuclease by means of gel filtration (Birnboim, in press). When denatured DNA was partially digested by a known exonuclease and then chromatographed on Sephadex G-100, the undigested fragments of DNA were eluted from the column as a peak after the void volume, and the mononucleotides formed by the digestion were eluted as a second distinct peak. When denatured DNA was partially digested by a known endonuclease, the polynucleotide products were eluted from a Sephadex G-100 column as a single, broad peak. When the action of the *B. subtilis* nuclease was examined (Fig. 3), the presence of a DNA peak and mononucleotide peak strongly suggested that the enzyme was an exonuclease.

Effect of the partially purified enzyme on transforming DNA. Figure 4 shows the effect of the partially purified nuclease (2% residual activity on radioactively labeled native DNA as measured by acid solubility) on native *B. subtilis* transforming DNA. Inactivation of transforming DNA is a sensitive test for endonuclease activity. For comparison, the conversion of radioactive, denatured DNA to an acid-soluble form was measured under the same conditions. It may be seen in Fig. 4 that at 10 min 70% of the denatured DNA was converted to an acid-soluble form, at a time that the transforming activity of native DNA was decreased about 30%.

Specificity of *B. subtilis* nuclease with density gradient centrifugation. Since native and denatured DNA may be readily resolved by analytical ultracentrifugation in CsCl, the effect of the nuclease on a mixture of native and denatured *B. subtilis* DNA was studied. Figure 5 shows microdensitometer tracings of ultraviolet-absorption photographs taken in the Spinco model E analytical ultracentrifuge. The upper tracing shows native *B. subtilis* DNA (left peak), denatured *B. subtilis* DNA (center peak), and reference density standard, phage 2C (ρ = 1.742 g/cm³, based on *E. coli* DNA, taken as 1.710 g/cm³).

The lower tracing is the same mixture of native DNA.
and denatured DNA which was incubated with B. subtilis nuclease. The center (denatured) peak has largely disappeared as a consequence of the nuclease digestion, but the native DNA peak is unchanged.

DISCUSSION

Although B. subtilis has been widely used for other studies, its cellular nucleases have not as yet been characterized. The present report describes a nuclease in B. subtilis which degrades denatured DNA at a much faster rate than native DNA, and suggests that the nuclease may be located at or near the cell surface. This suggestion is based upon three observations: (i) the association of the nuclease with a cell wall-membrane fraction when cells were mechanically disrupted; (ii) the release of the nuclease from cells which had been converted to protoplasts by treatment with lysozyme; (iii) the demonstration that 10 to 40% of the total nuclease activity (with denatured DNA as substrate) could be detected in washed cells that remained intact during the assay. In the experiment, the only criterion of “intactness” of the cell was that, after the period of incubation, the enzyme was still sedimentable at low speeds. This could of course be compatible with marked changes in the cell wall.

The location of the enzyme near the cell surface may be related to its excretion, since an extracellular nuclease has been described (2, 9) which resembles in several respects the enzyme described in this report. Since B. subtilis 168-2 is a transformable strain, the location of the nuclease near the cell surface might be expected to inhibit the uptake of denatured DNA if the enzyme is present at high levels during the competent state. This has not as yet been examined.

The cellular location of penicillinase in a related microorganism, B. licheniformis, was studied by Kushner and Pollack (3) and by Lampen (4). Like the B. subtilis nuclease, a large part of the penicillinase was sedimentable after mechanical disruption of the cells. The B. licheniformis penicillinase was released from membrane fragments by the action of trypsin; the B. subtilis nuclease was released from what may be a membrane structure by the same treatment. After exposure to trypsin, the penicillinase was shown to migrate differently during gel electrophoresis (4), whereas the nuclease was found to exhibit a different
chromatographic behavior on DEAE-cellulose. The mechanism of the activation of the *B. subtilis* nuclease by trypsin is unknown, but the phenomenon was observed consistently (see Tables 3 and 4).

Some properties of the *B. subtilis* nuclease have been studied, and it would appear that it will be a useful tool, as has been discussed by Kerr et al. (2). It has been shown that denatured DNA can be selectively degraded in mixtures of native and denatured DNA, but the degradation of, for example, incompletely reannealed DNA has not yet been investigated. Some preliminary evidence from this laboratory suggests that the residual transforming activity of heat-denatured DNA was resistant to inactivation by the enzyme, and therefore possibly due to native molecules of DNA. RNA was also degraded by the enzyme, but this was not studied extensively.

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**LITERATURE CITED**


