The Relationship between Human Serum and Human Pancreatic DNase I*

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Deoxyribonuclease (DNase) activities have been partially purified from human serum and pancreas. Several of their physical and enzymatic characteristics were determined and compared in order to evaluate their relatedness.

Human serum deoxyribonuclease has an isoelectric point in the range of 3.9 to 4.3 and a molecular weight of 33,000 to 38,000. Optimal enzymatic activity at pH 7.9 was dependent on both Mg²⁺ and Ca²⁺, whereas a pH optimum of from 5.5 to 5.8 was observed in the presence of Mg²⁺ and ethylene glycol bis(β-aminoethyl ether) N,N',N'-tetraacetic acid (EGTA). The proportion of single strand or double strand breakage products at early stages of DNA digestion were variable functions of the composition of the buffers employed for the reactions. Single strand breakage was predominant under all reaction conditions. Double strand breakage occurred with greatest frequency under neutral conditions in the presence of Mg²⁺ and Ca²⁺, was inhibited by the inclusion of 0.15 M NaCl, and did not occur at pH 5.8 in the presence of Mg²⁺, EGTA, and 0.15 M NaCl.

Human pancreas deoxyribonuclease exhibited essentially the same physical properties and enzymatic characteristics as those of the human serum enzyme. Thus, human serum deoxyribonuclease may originate in the pancreas.

Strong and Hewitt (1) have observed at least five neutral, cofactor-dependent isozymes of deoxyribonuclease (DNase) activity in human serum and plasma which are distinguishable by their electrophoretic mobilities in DNA-polyacrylamide gels (2). The activities were demonstrated to be endoDNases by virtue of their ability to react with covalently closed circular PM2 DNA included in the gels as substrate. Preliminary characterization of the cofactor dependence and the pH optimum of the activities indicated similarities with those previously reported for the activity in unfractionated serum (3) or partially purified from human serum (4, 5).

Because of the possible utility of these DNase isozymes as genetic and/or diagnostic markers, our ultimate objectives are to determine the physical basis of the electrophoretic differences between the serum DNase isozymes and identify the tissue(s) of origin of serum DNase. To this end, we have partially purified and characterized the major electrophoretic form of serum DNase and the major DNase from human pancreas. The similarity in properties of these enzymes indicates that the pancreas could be a tissue of origin for one of the serum DNase isozymes.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade purity. The following markers were standards for calibration of the column used to determine molecular weight. Blue dextran and egg white ovalbumin were products of Pharmacia. Escherichia coli alkaline phosphatase and bovine erythrocyte carbonic anhydrase were purchased from Worthington. Bovine serum albumin (grade A) was obtained from Pentex and sperm whale myoglobin from Mann Research Laboratories. The amphyoles for isoelectric focusing were purchased from Bio-Rad Laboratories. Agarose (electrophoresis purity) was obtained from Bausch and Lomb or Bio-Rad. Acrylamide, N,N'-methylenebisacrylamide (bis), N,N',N'-tetramethylethylene diamine (Temed), and ammonium persulfate were products of Canvax.

PM2 DNA Preparation

Covalently closed circular DNA of the marine bacteriophage PM2 used in all DNase assays was prepared by the method of Espejo and Canelo (6, 7) as modified by Strong and Hewitt (1). Purified covalently closed circular PM2 DNA was dialyzed against SSC (0.15 M NaCl, 0.015 M Na citrate) and stored frozen. DNA stored in this manner is usable for at least 6 months.

PM2 DNA Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was performed essentially under conditions established by Davis (8) and modified by Strong and Hewitt (1) and Grdina et al. (9) on 8-cm polyacrylamide gels (inside diameter, 6 mm; 7% acrylamide) containing 10 μg/ml of closed circular PM2 DNA, 0.025 M Tris, pH 9.3 (4°C), and 0.06% Temed. Electrophoresis buffer was 0.005 M Tris, 0.005 M glycine. A constant voltage of 200 V (5 to 7 mA) was applied to a set of 12 gels and electrophoresis was terminated after a 3- to 4-hr run at 4°C.

Gel DNase Analysis

After electrophoresis, whole gels were removed from their glass tubes into reaction buffer (0.1 M Tris-HCl, pH 8.1, 37°C, 10 mM Mg²⁺, 1 mM Ca²⁺) at 4°C for 30 min. Gels were then transferred into test tubes containing prewarmed buffer and incubated at 37°C for 20 to
60 min to allow enzymatic activity. The reaction was stopped by placing gels in ice cold ethidium bromide staining buffer (0.01 M Tris-HCl, pH 8.1, 4°C, 0.02 M NaCl, 5 mM EDTA, 10 μg/ml of ethidium bromide). After shaking overnight (16 h) in staining buffer, the gels were examined for fluorescence in a spectrophotofluorometer (Amicon-Bowman) equipped with a gel scanner device (excitation, 510 nm; emission, 590 nm). EndoDNase activity is identified by regions of enhanced fluorescence wherever DNA breakage occurs, by virtue of the greater capacity of nicked circular and linear DNA to bind ethidium (1, 2).

**EndoDNase Assay**

For each assay, a total reaction mixture (0.2 ml) containing 10 mM buffer adjusted to the desired pH and indicated concentrations of cofactors, and 10 μg of covalently closed circular PM2 DNA was warmed at 30°C for 5 min. To this mixture, a 10-μl sample was added, mixed, and incubated (30°C) for the desired time period (30 min). At the end of incubation, 3.0 ml of denaturation buffer were added, and the solution was blended on a Vortex mixer. The denaturation buffer was prepared by adjusting the pH of 0.13 M Na2PO4, 0.03 M Na3EDTA (1% of the total volume) to 12.1 (22°C) with 0.15 M NaOH, and the solution was brought to a final volume with 0.15 M NaCl. Following the addition of denaturation buffer, 0.1 ml of ethidium bromide (200 μg/ml) was added and the solution was again blended overnight (16 h) on a Vortex mixer. Fluorescence was measured in a 1-cm' cuvette using a spectrophotofluorometer (Aminco-Bowman; excitation, 510 nm; emission, 590 nm). A unit of activity was defined as: unit = - ln (F0 - Fincubation)/Fmax - fn (%) where F0 = fluorescence remaining after 30-min incubation, Fmax = fluorescence of 10 μg of DNA not incubated with enzyme, and fn = fluorescence of reaction buffer not containing DNA. One unit of activity is that amount which introduces an average of one DNA strand break/PM2 molecule in the assay in 30 min.

**Exonuclease Assay**

For this study, highly degraded (2°C) DNA was used as a substrate. The exonuclease assay was performed under optimum conditions for endoDNase activity as described below. After 30 min at 37°C, the reaction was terminated by adding EDTA and chilling in an ice bath. Approximately 7,506 cpm of DNA were layered onto a column (0.9 x 10 cm) of Sephadex G-25 and chromatographed. Exonuclease activity was detected by the appearance of radioactivity in the region in which cold thymine was eluted, as demonstrated with snake venom phosphodiesterase I (EC 3.1.4.1).

**Isoelectric Focusing**

Isoelectric focusing was performed as described in Bio-Rad Laboratories Technical Bulletin 1030. The isoelectric points were estimated using wide range ampholyte (Bio-Lyte ?, Bio-Rad Laboratories). Gel slices (0.5 cm) were placed in degassed water (4°C) overnight to determine the pH gradient. EndoDNase activity was determined using the solution assay described above, following overnight elution of the enzyme from gel slices in 0.01 M Pipes buffer (pH adjusted to 7.0 at 22°C) with a 1 M solution of Triton X.

**Determination of K0**

The K0 was experimentally determined under constant enzyme concentration by varying the DNA concentration. In order to vary the DNA concentration used in the solution assay, one must also vary the concentration of ethidium bromide added. This is true because fluorescence is not proportional to closed circular DNA concentration unless a saturating ethidium concentration is maintained (9, 10). The saturating ethidium concentration was determined and used; concentration of DNA used was and equal to twice the DNA concentration. The linear equations were found by polynomial regression.

**Agarose Gel Electrophoresis**

The gel and electrophoresis buffers were 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3. Agarose (0.9 g/100 ml buffer) was heated in a water bath until dissolved, then cooled to 55° to 60°C before pouring 8-cm gels. The gels were inverted onto a nylon mesh. Electrophoresis was at 100 V for 4.0 to 4.5 h at room temperature. Gels were stained overnight in a 0.5 μg/ml-solution of ethidium bromide in gel buffer and scanned in a spectrophotofluorometer (excitation, 510 nm; emission, 590 nm). The area under each fluorescence peak was determined by plotting with a polar planimeter (Dietzgen).

**Purification Technique**

**Human Serum DNase**

Serum from clotted blood (4 to 8 h) was removed by decantation, pooled (10 to 30 sera), and cooled during centrifugation (3,000 x g, 5 min, 4°C). A white insoluble material, which floated on the top of the sample and contained very little DNase activity, was removed and discarded. Cleared serum was stored at 4°C until used (up to 2 weeks) or immediately dialyzed overnight (16 h) against Buffer A (0.04 M Tris/sucinate buffer, pH 8.3, 4°C, 1 mM Na3EDTA). The molarity of this and all other Tris/sucinate buffers refers to the concentration of Tris. A precipitate, which formed during dialysis and contained very little DNase activity, was removed by centrifugation (20,000 x g, 10 min, 4°C) and discarded.

**Chromatography on DEAE-agarose—DEAE Bio-Gel A (100 to 500 mesh, Bio-Rad Laboratories) was suspended in a concentrated solution (10x) of Buffer A and a column (1.5 x 60 cm) was packed under 150 cm of pressure and equilibrated with 5 to 8 volumes of Buffer A. A sample (25 to 50 ml) was loaded onto a column and washed with 120 ml of Buffer A at a flow rate of 24 to 30 ml/h. Enzymatic activity was then eluted by gradually decreasing the pH from 8.3 to 4.6 by a linear gradient of 450 ml each of starting Buffer A and limit Buffer B (0.03 M Tris/sucinate buffer, pH 4.5, 4°C, 1 mM Na3EDTA). When 90 to 100 ml of eluent remained, an elution buffer (20 ml of Buffer A and 20 ml of Buffer B) was added and elution was continued. After 24 to 30 ml/h, enzymatic activity was observed and verified by measuring absorbance at 280 nm, except for alkaline phosphatase, which was determined spectrophotometrically. Approximately 3.0 ml-fractions were collected. Active fractions were pooled and pooled activity was determined by the method of Lowry et al. (11). Pooled activity was concentrated by Diaflo ultrafiltration (Amicon), using a PM-10 membrane (75-mm diameter).

**Chromatography on Sephadex G-25—Sephadex G-150 (40 to 120 μm, Pharmacia) was prepared by hydrating in H2O and fines were removed three to four times by decantation. A column (2.5 x 60 cm) was packed under 100 cm of pressure and equilibrated in Buffer C (0.01 M Tris/sucinate, pH 8.3, 4°C, 1 mM Na3EDTA) at a flow rate of 20 ml/h. The 20- to 25-ml concentrate from ultrafiltration was applied and eluted, eluting at a flow rate of 12 to 15 ml/h. Fractions of 1.4 to 1.7 ml were collected.

**Chromatography on DEAE-Agarose II—A column of DEAE-Bio-Gel A (0.9 x 16 cm) was packed in Buffer C under 110 cm of pressure at a flow rate of 60 ml/h. The pool from the Sephadex G-150 column (65 to 75 ml), which was equilibrated in Buffer C during gel filtration, was subjected to a sodium chloride gradient with Buffer C (30 ml) at a flow rate of 12 ml/h. The bound protein, containing endoDNase activity, was then eluted with a linear gradient of 0 to 0.2 M NaCl in Buffer C (50 ml of each solution). Fractions of 1.0 to 1.4 ml were collected, assayed, and analyzed on DNA gels. Enzymatic activity eluted at a low sodium concentration (0.065 to 0.090 M) as determined by conductivity measurements. Pooled activity was concentrated by ultrafiltration (PM-10, 25-mm diameter).

**Chromatography on Polyanionamide Gel Beads—Bio-Gel P-100 (50 to 150 mesh, Bio-Rad Laboratories) was hydrated in Buffer C overnight with slow stirring and a column (2.5 x 91.5 cm, 449 ml) was packed under 47 cm of pressure at 36 ml/h. The column was calibrated with the following protein markers: 1) alkaline phosphatase, 80,000 daltons; 2) carbonic anhydrase, 31,000 daltons; 3) ovalbumin, 45,000 daltons; 4) myoglobin, 17,800 daltons. The void volume was measured by the use of blue dextran. Markers were dissolved in Buffer C and 3.0 ml of each marker or serum enzyme were individually applied to the column, and eluted at a flow rate of 36 ml/h. The protein in each fraction (1.8 ml) was determined by reading the absorbance at 280 nm, except for alkaline phosphatase, the activity of which was determined by the method of Goren and Levinthal (12), and serum DNase, the activity of which was measured by the solution DNase assay. The relationship between K0 and molecular weight (determined by the least squares) was:

\[ K_0 = 1.9123 - 0.3839 \log (M) \]

The range of molecular weight equaled the antilog of (M ± 0.0171), where 0.0171 was the standard error of the estimate.
Human Pancreatic DNase

The homogenate was produced in a Waring Blender at pH 5.0 in Buffer D (0.01 M Tris succinate, 40 mM CaCl₂, 1.0 mM NaH₂PO₄) by adding approximately 100 g of debrided tissue to 300 ml of buffer and homogenizing repetitively for 20 s. The homogenate was strained through four to six layers of sterile gauze and the strained fibrous material was returned to the Waring Blender and the procedure was repeated once. The strained solutions were combined and brought to 1.0% in streptomycin sulfate by the dropwise addition of a fresh solution of streptomycin sulfate (5%) over 30 min with continuous stirring in an ice bath. The mixture was allowed to stir an additional 1 h. The precipitate was removed by centrifugation (100,000 × g) and the supernatant was dialyzed against Buffer D. A second precipitate, formed during dialysis, was collected by centrifugation at 10,000 × g. The soluble fraction was then collected and dialyzed against the 37°C water bath for 4 h. The bulky precipitate which formed was removed by centrifugation at 10,000 × g. The soluble fraction was then collected and dialyzed against the 37°C water bath and incubated overnight.

Ammonium Sulfate Procedure—The heat-treated preparation was then extracted with ammonium sulfate. The salt was added slowly over 30 min with stirring in an ice bath while the mixture was maintained at pH 5.0. In preliminary steps, DNase activity was precipitated over the range of salt saturation from 40 to 80% of maximum solubility. All precipitates were collected by centrifugation and resuspended in 40 ml of Buffer D. The precipitate formed at 20% saturation contained very little DNase and was, therefore, discarded. Protein precipitated between 20 to 60% saturation was solubilized and re-extracted, whereas the precipitate from 60 to 80% saturation was stored. The re-extraction procedure consisted of precipitation at 50% ammonium sulfate saturation and collection, with the resulting supernatant fraction being brought to 80% saturation. The precipitates at 80% saturation were continuously saved. The precipitate formed at up to 50% salt saturation was again solubilized, and the re-extraction procedure was continued for two more cycles. All protein precipitated at 80% ammonium sulfate saturation was combined, resuspended in Buffer D, and further purified.

Chromatography on Phosphocellulose—The resin (phosphocellulose, Sigma) was cycled in base and acid until free of ammonium. The prepared resin was then poured into a column (2.5 × 90 cm) and packed under 50 cm of water pressure. Packaged resin was then equilibrated with starting Buffer D. The ammonium sulfate fraction was loaded onto the column and eluted with Buffer D at a flow rate of 60 ml/h. Approximately 15-ml fractions were collected. The activity eluted between 1 and 2 column volumes. Active fractions were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane. The concentrate was pressure-dialyzed against 0.02 M potassium phosphate buffer, pH 7.0.

Chromatography on Hydroxylapatite—One part of hydroxylapatite resin (Bio-Gel HTP, Bio-Rad Laboratories) was mixed into three parts of 5% streptomycin sulfate by the dropwise addition of a fresh solution of streptomycin sulfate (5%) over 30 min with continuous stirring in an ice bath. The mixture was allowed to stir an additional 1 h. The precipitate was removed by centrifugation (100,000 × g) and the supernatant was dialyzed against Buffer D. A second precipitate, formed during dialysis, was collected by centrifugation at 10,000 × g. The soluble fraction was then collected and dialyzed against the 37°C water bath and incubated overnight.

Protein concentration is below the sensitivity of the method of Lowry (11).

RESULTS

Quantitative DNase Assay—In order to aid the enzyme purification and make quantitative measurements of DNase activity in different samples, we developed a new fluorometric DNase assay that exploits the differential stabilities to pH denaturation between nicked circular or linear DNA and covalently closed circular DNA. In contrast, unbroken PM2 DNA is more stable to pH and denatures gradually between pH values of 11.8 and 13.0; it retains about 80% double-stranded structure at pH 12.1. Thus, residual enhanced fluorescence is proportional to residual unbroken PM2 DNA after incubation with DNase, adjustment to pH 12.1, and addition of ethidium bromide. Measurements are reliable with as little as 2.0% survival of closed circular DNA, which is equivalent to 3.9 breaks/DNA.

FIG. 1. Reaction kinetics of serum DNase. Serum samples were incubated in standard buffer (0.01 M Pipes, pH 7.0, 10 mM MgCl₂, 1 mM CaCl₂) for the times indicated. The remaining fluorescence (relative to background) was measured using the Aminco-Bowman fluorometer (excitation, 510 nm; emission, 590 nm) at the following doses of samples: 0 µl (○—○), 0.25 µl (□—□), 0.5 µl (△—△), 0.75 µl (■—■), 1.0 µl (▲—▲). Inset, relates the slopes of the above curves to the amount of serum analyzed.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Purification of a DNase from human serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Pooled donor serum</td>
</tr>
<tr>
<td></td>
<td>Total activity</td>
</tr>
<tr>
<td></td>
<td>units</td>
</tr>
<tr>
<td>3,000 × g spin</td>
<td>33,100</td>
</tr>
<tr>
<td>105,000 × g spin</td>
<td>33,100</td>
</tr>
<tr>
<td>DEAE-agarose (pH elution)</td>
<td>44,500</td>
</tr>
<tr>
<td>Diaflo concentration</td>
<td>28,000</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>17,600</td>
</tr>
<tr>
<td>DEAE-agarose (NaCl elution)</td>
<td>9,800</td>
</tr>
<tr>
<td>Diaflo concentration</td>
<td>7,600</td>
</tr>
<tr>
<td>Bio-Gel P-100</td>
<td>2,900</td>
</tr>
</tbody>
</table>

*This value represents only the activity pooled for further purification (49% of total activity).

Protein concentration is below the sensitivity of the method of Lowry (11).
molecule. The average number of breaks/PM2 DNA molecule is determined on the basis of a Poisson distribution of breakage events. In this manner, an average of one break/DNA molecule has occurred when 63% of the closed circular molecules are broken. Fig. 1 demonstrates the relationship between the logarithm of relative fluorescence and time, the slope of which is linearly dependent on the quantity of enzyme activity. Thus, under the conditions used in these studies, the activity measurement can be used to determine enzyme concentration. The assay is rapid, accurate, and reproducible with a standard deviation of ±11%.

**Purification of Human Serum EndoDNase I**—Table I summarizes the yields of protein and DNase activity at each stage of purification from a pooled serum sample and serum obtained from an individual donor. This procedure provides purification factors of from 700 to 1000 with yields of 9 to 10% of the initial activity. A reproducible increase in the total amount of activity occurred after chromatography on DEAE-agarose, which may indicate removal of a DNase inhibitor known to be present in human blood (13).

The protein concentration of the Bio-Gel P-100 fraction was below 1 µg/ml, which precluded determination of the purity of this fraction. This fraction contained only one electrophoretic band of endoDNase activity representing the fastest electrophoretic form present in unfractionated serum. No exoDNase activity was detectable in this fraction, which was used in most of the physicochemical and enzymatic analyses performed.

**Purification of Human Pancreatic EndoDNase I**—Table II summarizes the yields of protein and DNase activity at each stage of purification. This procedure provides a purification factor of about 300 with a yield of 15% of the activity present in the crude homogenate. A 160% increase in activity was obtained in the ammonium sulfate fraction, possibly indicating removal of DNase inhibitor at this stage of purification.

The purified activities from both human serum and human pancreatic DNases were analyzed by DNA-gel electrophoresis for similarities in electrophoretic mobility (Fig. 2). Gel scans a and b, 2 and 4 units, respectively, of untreated serum were analyzed. In gel c, 10 units of crude pancreatic homogenate were analyzed. In gel d, 0.8 unit of pancreatic DNase from the Bio-Gel HTP column was analyzed. In gel e, 0.5 unit of serum DNase from the Bio-Gel P-100 column was analyzed.

### Table II

**Purification of a DNase from human pancreas**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>ml</th>
<th>units</th>
<th>mg</th>
<th>units/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>360</td>
<td>15,500</td>
<td>3,360</td>
<td>4</td>
<td></td>
<td>15,500</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>37°C for 24 h</td>
<td>360</td>
<td>12,900</td>
<td>2,790</td>
<td>4</td>
<td></td>
<td>12,900</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>35</td>
<td>25,000</td>
<td>220</td>
<td>112</td>
<td></td>
<td>25,000</td>
<td></td>
<td>112</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>134</td>
<td>3,300</td>
<td>36</td>
<td>91</td>
<td></td>
<td>3,300</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>Bio-Gel HTP</td>
<td>2.6</td>
<td>2,300</td>
<td>1.7</td>
<td>1,340</td>
<td></td>
<td>2,300</td>
<td></td>
<td>1,340</td>
</tr>
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</table>

### Table III

**Comparison of human DNase properties**

<table>
<thead>
<tr>
<th>Property</th>
<th>Serum</th>
<th>Pancreatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mₙ</td>
<td>33,000-38,000</td>
<td>35,000-38,000</td>
</tr>
<tr>
<td>pI</td>
<td>3.9-4.3</td>
<td>3.9-4.3</td>
</tr>
<tr>
<td>Kₘ (mM)</td>
<td>0.222 DNA-phosphorus</td>
<td>0.378 DNA-phosphorus</td>
</tr>
<tr>
<td>pHₜₜ (magnesium ECTA)</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>pHₜₜ (magnesium-calcium)</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td>50% inhibition by:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn²⁺, pH 7.0</td>
<td>0.1 mM</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Zn²⁺, pH 6.8</td>
<td>0.009 mM</td>
<td></td>
</tr>
<tr>
<td>Na⁺, pH 7.0</td>
<td>80 mM</td>
<td>80 mM</td>
</tr>
<tr>
<td>Na⁺, pH 5.8</td>
<td>165 mM</td>
<td>170 mM</td>
</tr>
<tr>
<td>Inhibited by EDTA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Inhibited by 2-mercaptoethanol:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated in Ca²⁺</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Treated in ECTA</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

### Fig. 2. Electrophoretic mobilities of human serum and human pancreatic DNases.

DNA-polyacrylamide gel electrophoresis was performed as described under "Materials and Methods." Electrophoresis was from left to right and the numerals indicate the positions of serum DNase 1, Forms 1 through 5. In gel scans a and b, the serum and pancreatic activities. All of the properties determined were virtually identical for the serum and pancreatic activities.

**Fluorometric Detection of DNase on DNA-Polyacrylamide Gels**—For identification, the electrophoretic isozymes are numbered 1 through 5 from the fastest to the slowest migrating. The sharp peak at the far right in Fig. 2 marks the progression of the glycine, bromphenol blue front. This peak was also observed following incubation of gels containing only lasing solution. As little as 2 µl (approximately 2 units) of serum developed a complete gel pattern during 20 min of incubation, showing five electrophoretically distinct isozymes. With increasing incubation times, a decrease in resolution of activity peaks was observed as fluorescence enhancement reached its maximum.

The purified activities from both human serum and the pancreas were analyzed by DNA-gel electrophoresis for similarities in electrophoretic mobility (Fig. 2). Gel scans a and b show the typical multiple form pattern in normal serum. Gel scan e shows the purified DNase Form 1 from serum. The crude homogenate from the pancreas contained the forms illustrated in scan c. There were large fluorescent peaks at the top of the gel and at the progression front, probably due to nucleic acids present at crude stages of purification. These regions of the gel no longer showed fluorescent peaks after
commercially purified sample of bovine pancreatic DNase I contained in two slices that had a pI range of 3.9 to 4.3. A lyte (i/o), a single peak of activity was observed in gel slices samples were focused for 7 h. Using the broad range ampho-
sulted in constant pI values for standard proteins. Therefore,
Pipes, pH 5.6 to 7.0; or Tris-HCl, pH 7.1 to 9.0. Sodium ion concen-
pH was adjusted by the addition of HCl or Tris base to the indicated
activity, and only 60% of the response with MgCl₂ was obtained. As
the assay was increased up to pH 8.0, the concentration of MgCl₂ required to stimulate maximum activity decrease. Maximum activity was obtained at neutral pH with 25 mM MnCl₂ or at pH 8.0 with 10 mM MnCl₂, and amounted to 95% of that activity with MgCl₂ plus CaCl₂.

Inhibition—Several known inhibitors of bovine pancreatic DNase were tested against the purified serum and pancreatic enzymes. The reaction was inhibited 50% by 10⁻⁴ M zinc and was completely inhibited at 10⁻³ M under otherwise optimal neutral pH conditions. EDTA inhibited the reaction 50% at 0.3 mM at pH 5.5 and 1.8 mM inhibited 50% activity at pH 7.0. All activity was eliminated by EDTA at a concentration equivalent to that of the metal ions at either pH.

When serum DNase was treated with 2-mercaptoethanol in the presence of CaCl₂, no loss of activity was observed under any reaction conditions. However, when 2-mercaptoethanol was added to the DNase in solution containing excess EGTA, activity was progressively lost. Inactivated DNase did not regain activity by incubation in buffer containing excess CaCl₂. Like serum Form 1 DNase, human pancreatic DNase was not inactivated by 2-mercaptoethanol treatment in buffer containing excess CaCl₂. Pancreatic DNase activity was gradually inactivated by 2-mercaptoethanol treatment in excess EGTA and subsequent addition of CaCl₂ to treated DNase did not reactivate the enzyme.

The serum and the pancreatic enzymes were inhibited by sodium chloride. Serum DNase was inhibited 50% by 80 mM NaCl at pH 5.8 and by 165 mM NaCl at pH 7.0. Similarly, human pancreatic DNase activity was diminished 50% at pH 5.8 by 80 mM NaCl and by 170 mM NaCl at pH 7.0. Both activities were inhibited 95 to 100% by 400 to 500 mM NaCl.

Effect of Reaction Conditions on DNA Breakage—The kinetics of single or double strand breakage by the serum or pancreatic DNase were investigated by the use of agarose gel electrophoresis to analyze reaction products. When serum DNase activity was assayed in MgCl₂ and CaCl₂ at pH 7.0, both single and double strand breaks were produced (Fig. 4A). Both types of strand breaks were similarly observed when the reaction occurred in the presence of MgCl₂ and EGTA at pH 5.8 (Fig. 4B). However, the magnesium-EGTA-activated DNase produced double strand breaks at a lower rate and at pH 5.8. When calcium was added to the magnesium-acti-
vated DNase, the pH of optimum activity was shifted to between 7.0 and 7.5. This cofactor-dependency was observed to co-purify and to have the same molecular weight and isoelectric point, and the same electrophoretic mobility on DNA-polyacrylamide gels. Thus, these pH optimum character-
istics are considered properties of one enzyme.

Optimal Cofactor Concentrations—Similar to studies of DNases in other organisms, the activity of human serum DNase was stimulated to a maximum and then was inhibited as divalent metal ion concentration was increased. When 10⁻⁴ M EGTA was added to the reaction mixture to exclude calcium, maximum stimulation of activity occurred at pH 5.8 with 25 mM MgCl₂ (data not shown). Two concentrations of MgCl₂ were tested at pH 7.0 to determine the concentration of CaCl₂ that yielded maximum activity. A concentration of 1 mM CaCl₂ caused maximum stimulation at both 10 and 25 mM MgCl₂. These studies indicated that 10 mM MgCl₂ plus 1 mM CaCl₂ at pH 7.0 were optimal and, thus, these reaction conditions were established for routine measurements of neutral, cofactor-dependent endonuclease in serum.

Manganese was determined to be an adequate substitute for MgCl₂ at pH 5.8 (assayed with 10⁻⁴ EGTA) or MgCl₂ plus CaCl₂ at pH 7.0 (data not shown). At pH 5.8, a much higher concentration of MnCl₂ (50 mM) was required to stimulate activity, and only 60% of the response with MgCl₂ was obtained. As the pH of the assay was increased up to pH 8.0, the concentration of MnCl₂ required to stimulate maximum activity decreased. Maximum activity was obtained at neutral pH with 25 mM MnCl₂ or at pH 8.0 with 10 mM MnCl₂, and amounted to 95% of that activity with MgCl₂ plus CaCl₂.

the ammonium sulfate fractionation step. The pancreatic ac-
activity which was most highly purified (Fig. 2) had the same
electrophoretic mobility as serum DNase Form 1. The pI values for pancreati-
exhibited a reaction plateau of approximately 20% double strand scissions (Fig. 4B). Simultaneous quantitative assays demonstrated that there was an average of six breaks/molecule after 4 h. Random distribution of six breaks in a PM2 molecule would space them an average distance of 1,600 bases apart and should not result in separation of strands in almost all cases. Therefore, the linear increase in Form III DNA was probably the result of either simultaneous cleavage of both strands as essentially a single event or a strong specificity of the DNase for the complementary strand of, or single strandiness of, regions containing a nick.

Human pancreatic DNase responded to the presence of Ca2+ in the same manner as human serum DNase. Double strand breaks accumulated more rapidly in the presence of Ca2+ (Fig. 4C) and reached a plateau in the absence of Ca2+ (Fig. 4D). Inclusion of 0.15 M NaCl in the Mg2+-EGTA reaction buffer greatly inhibited the formation of linear DNA which reached a plateau at only 5% of the total DNA (data not shown). The reaction rate at high salt concentrations was biphasic during the time required to eliminate all of the Form I DNA. Addition of 0.15 M NaCl inhibited the reaction rate to 50% of that observed in low salt at pH 7.0. The breakage in 0.15 M NaCl was linear for the first 60 min and then abruptly slowed to 3% of the rate in low salt for at least 3 more h. The addition of 0.15 M NaCl to pH 5.8 reaction buffers also produced biphasic reaction kinetics with a 50% rate during the first 30 min and only 3% for at least 3½ h longer.

**DISCUSSION**

A major underlying objective of our studies of human serum DNase is to determine the tissue(s) of origin of the serum activities. Previous studies have demonstrated that several pathological conditions of the pancreas influence the level of DNase I-type activity detected in blood serum (16 to 18). Acute pancreatitis resulted in an increase in serum DNase I activity (16, 17), most likely associated with blockage of the pancreatic duct.

The results reported in this paper show that the pancreas could be a tissue of origin of the major electrophoretic form of endoDNase type I activity of human serum, in view of the nearly identical physical and enzymatic characteristics of our partially purified serum and pancreatic activities. Further, the adult human pancreas can be estimated to contain from 5 to 6 times more DNase activity than the total blood volume. Although it has been suggested previously by others (13, 22, 23) that serum DNase is derived from blood platelets, our preliminary studies indicate that there is no electrophoretic identity between DNase activities in serum and platelets.

The physical and enzymatic characteristics of serum and pancreatic DNases reported here are for the most part very similar to those known for bovine pancreatic DNase. A notable difference was observed in the ability of Ca2+ to reactivate activity after inactivation by mercaptoethanol; bovine pancreatic DNase activity can be restored by Ca2+ (24), whereas the human activities cannot. In addition, Price (24) has shown that bovine pancreatic DNase exhibits activity at neutral pH values with only Ca2+ present as cofactor, whereas the human DNases are inactive at all pH values without Mg2+. The pH optima of bovine (24) and human DNases exhibit dependence on cofactors, with an acid optimum observed in Mg2+ only and a neutral optimum in Mg2+ plus Ca2+. The human DNases exhibited the same maximum activity at either acid or neutral optima (Fig. 3), whereas bovine DNase exhibited 3-fold less activity in Mg2+ only at the acid optimum than that observed at the optimal neutral pH in Mg2+ plus Ca2+. It appears bovine and human DNases exhibit similar cofactor-dependent characteristics of DNA strand cleavage. Melgar and Goldthwait (25) have argued that single- and double-hit enzyme kinetics of bovine DNase are associated with different activating cofactors, Mg2+ plus Ca2+ and Mg2+, respectively. Single hit refers to the simultaneous cleavage of both strands of duplex DNA giving rise to chain separation. Double hit means that only one strand is broken at a time, giving rise to a nick, and implies that a second distinct enzymatic cleavage must occur in the same region of the DNA to cause chain separation. Single-hit kinetics was demonstrated by the rapid formation of double strand breaks, whereas double-hit kinetics was associated with a lag time in their assay. Using this method, Melgar and Goldthwait (26) demonstrated that bovine DNase exhibits single-hit kinetics when Ca2+ is present and double-hit kinetics when only Mg2+ is present. We observed that human DNases exhibited predominantly double-hit kinetics under all reaction conditions. Like bovine DNase, human DNase formed double strand breaks more rapidly in the presence of Ca2+ than in its absence.

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[2] The concentration of serum DNase is 2.2 x 10^6 units/ml. Assuming a total blood volume of from 4 to 6 liters (19), about half of which is serum, the total amount of DNase in blood is between 4.4 and 0.6 x 10^6 units. The concentration of pancreatic DNase is estimated to be 0.5 x 10^6 units/gm. Assuming a typical adult pancreas weighs 60 to 90 g (20, 21), the total pancreatic content of DNase is between 30 and 45 x 10^6 units.
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