Bovine Thymus Poly(adenosine Diphosphate Ribose) Polymerase*

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About 1,300-fold purification of poly(adenosine diphosphate ribose) polymerase has been achieved from the extract of bovine thymus with a recovery of 10 to 20%. The final preparation has a purity of 99%, and the enzyme is composed of a single peptide with a molecular weight of 130,000.

The purified enzyme required NAD⁺, Mg²⁺, a thiol compound, DNA, and histones for full activity. Whereas DNA is essential for activation of the enzyme, histones are not. The observed stimulation of the reaction by histones is shown to be due to masking of the inhibitory effect of contaminating denatured DNA in native DNA preparation.

The concentration of DNA required for half-maximal enzyme activity (apparent Kₚ for DNA) is proportional to the concentration of enzyme in the reaction mixture. The minimum estimation of the number of nucleotide pairs of DNA required for half-maximal activation of one enzyme molecule is 220 to 240 for bulk of calf thymus DNA, while the value is 10 for a calf thymus DNA fraction, "active DNA," which was separated from the enzyme fraction in a stage of the purification. These results suggest that the enzyme is activated by binding to a specific site on calf thymus DNA.

The apparent Kₚ for NAD⁺ and the maximum velocity of the enzyme are estimated to be 60 μM and 0.91 μmol per min per mg, respectively.

Poly(adenosine diphosphate ribose) polymerase, an enzyme which is localized in the nucleus of eukaryotic cells (1-3) and tightly bound to chromatin (4), catalyzes the formation of a homopolymer of repeating ADP-ribose units from NAD⁺. The structure of this unique polymer, poly(ADP-ribose), has been determined by other investigators (5-7). The polymerized products synthesized in vitro using isolated nuclei or chromatin are found to have various chain length from monomer to polymers and to be bound to various nuclear proteins including histones (8), non-histone chromosomal proteins (9), nuclear enzymes such as (Ca²⁺, Mg²⁺)-dependent endonuclease (10), and RNA polymerase I (11), and protamine (12), probably by a covalent linkage.

Although the physiological function of this enzyme has not been elucidated yet, its involvement in DNA synthesis (13, 14), cell proliferation (15, 16), cell differentiation (17), control of the expression of genes (18), and mechanisms of nuclear enzymes (10, 11), modification of chromatin structure (18), and DNA repair (19) is suggested.

In spite of widespread interest in the possible biological roles of this enzyme, efforts at extensive purification have been limited, the purification has remained partial (20-23), and the reaction mechanism is not satisfactorily understood.

With partially purified polymerase, we (20) and Yamada et al. (21) have established that the enzyme reaction is completely dependent on DNA, although the role of histones or so-called ADP-ribose-accepting proteins remained unclear, mainly due to the unsatisfactory purity of the enzyme preparation.

In this report we will describe a purification method and the physical and functional properties of an essentially homogeneous poly(ADP-ribose) polymerase from bovine thymus, as well as discuss the role of DNA and histones in the enzyme reaction.

MATERIALS AND METHODS

Materials—[Adenine-2,8-³H]NAD⁺ was purchased from New England Nuclear, Boston. NAD⁺ (grade 5), calf thymus whole histones (type 2), and calf thymus lysine-rich histone (type 3-S) were products of Sigma. Snake venom phosphodiesterase (EC 3.1.4.1) was obtained from Worthington Biochemical Corp., N. J. Poly(d(A)) and poly(d(T)) were obtained from Miles Laboratories, Inc., Ind.

DNA-cellulose was prepared according to the method of Alberts and Herrick (24). The prepared DNA-cellulose contained approximately 2 mg of DNA per ml of packed volume.

Hydroxyapatite was prepared by the method of Siegelman et al. (25).

The thymus of 2- to 3-year old castrated oxen was obtained from Osaka Municipal Slaughterhouse, Osaka, Japan.

pH of Buffers—The pH of all buffers was adjusted at 25°C.

Assay for Protein and DNA—Protein and DNA were estimated by the methods of Lowry et al. (26) and Burton (27), respectively.

DNAs Used for Enzyme Assay—DNAs were dissolved in 0.1× SSC (standard saline/sodium citrate: 0.15 M NaCl, 0.015 M sodium citrate. pH 7.2).

Calf thymus DNA, two lots of highly polymerized calf thymus DNA (type 1, Lot 26 c 9560 and Lot 93 c 9501-95) were obtained from Sigma. "Active DNA," a DNA fraction separated from poly(ADP-ribose) polymerase during hydroxyapatite column chromatography (see under "Purification of Enzyme" in the text) was purified by extraction with a phenol mixture according to the method described by Parish (28) and by CaCl₂ equilibrium density gradient centrifugation. The DNA has an approximate molecular weight of 200,000 and showed a buoyant density of 1.704 in CsCl density gradient. Analyzes of the base composition of the DNA has revealed that the DNA is double-stranded and has a 43% GC content. Neither RNA nor proteins were detectable in the DNA preparation. Since this DNA fraction had a much higher efficiency (approximately 20-fold) in stimulating poly(ADP-ribose) polymerase than bulk of calf thymus DNA, it was designated "active DNA" in this paper. The details of the properties of this DNA will be described elsewhere.²

Poly(d(A))-poly(d(T)), the double-stranded synthetic polymer,
Purification and Properties

was prepared by annealing single-stranded poly(d(A)) and poly(d(T)) as described previously (29).

Denatured calf thymus DNA, calf thymus DNA (Lot 93 c 9501-95, 0.2 mg/ml in 0.2 x SSC), was heated at 100°C for 10 min and rapidly cooled. The net increase in the absorbance at 260 nm of the sample was 0.08.

Partially denatured calf thymus DNA, calf thymus DNA (Lot 93 c 9501-95, 0.2 mg/ml in distilled water), was incubated at 25°C for 10 min. During the treatment partial denaturation of DNA occurs, probably due to the low concentration at low ionic strength, as reported by Inman and Jordan (30). At the end of the incubation concentrated SSC was added to the sample to give the final concentration of 0.1 x SSC. Since the net increase in the absorbance at 260 nm of the sample was 8.8%, the extent of denaturation of the DNA is estimated to be approximately one-fourth of the total DNA.

Assay for Sugars—Sugars were measured by the anthrone reaction described by Ashwell (31).

Assay for Poly(ADP-ribose) Polymerase—The standard reaction mixture contained 25 mM Tris- HCl buffer, pH 8.0, 10 mM MgCl₂, 0.5 mM dithiothreitol, 10 μM [adenine-2,8-'H]NAD⁺ (95 cpm/pmol), 2 μg each of calf thymus DNA (Lot 26 c 9560) and calf thymus whole histones, and an appropriate amount of the enzyme in a total volume of 0.2 ml. The mixture was incubated at 25°C for 10 min, and the reaction was terminated by the addition of 2 ml of ice-cold 10% trichloroacetic acid. The acid-insoluble material was collected on a glass fiber filter, and the radioactivity was counted by a liquid scintillation spectrometer as described previously (32).

One unit of the enzyme activity was defined as being equivalent to 1 nmol of ADP-ribose incorporated into acid-insoluble material per min under the described conditions.

In some experiments the composition of the reaction mixture, scale of the reaction, and the incubation time were changed as indicated.

SDS-Polyacrylamide Gel Electrophoresis—The gel electrophoresis and staining of protein bands with Amido black were performed according to the method of Hayashi and Ohba (33). Marker proteins for molecular weight determination were obtained from Boehringer Mannheim, West Germany. Densitometric tracing of the stained gels at 606 nm was performed with a Toyo Densitrol DMU-2.

Product Analysis—Poly(ADP-ribose) synthesis was carried out using 0.3 μg of purified enzyme (2.3 pmol) in the standard reaction mixture in a total volume of 2.0 ml, where [adenine-2,8-'H]NAD⁺ (45 cpm/pmol) was used as substrate. After 20 min incubation at 25°C, the reaction was terminated by the addition of 0.5 ml of ice-cold 40% trichloroacetic acid. The acid-insoluble material was collected on a glass fiber filter, and radioactivity was counted as described previously (32). Purification and properties of this DNA-competition step (DNA-cellulose fraction) was separated from the enzyme activity by this step. The DNA eluted at 0.15 M NaCl, no other peak of enzyme activity was observed through the elution steps. The flow rate was 30 ml per h. As shown in Fig. 1, the enzyme eluted at approximately 30 mM NaCl. As shown in Table I, the enzyme activity applied remained fixed on the column. The enzyme was eluted stepwise by applying 300 ml of Buffer B containing 0.2 M NaCl. The enzyme was eluted at the same buffer at a flow rate of 5 ml/min. The fractions containing the bulk of the enzyme activity were collected, and, after the first centrifugation to eliminate denatured protein, the sample was applied to a DNA-cellulose column (100 ml, 3 x 15 cm, containing approximately 200 mg of fixed DNA), which had been washed and equilibrated with Buffer B containing 0.2 M NaCl. After washing the column with 150 ml of the buffer, more than 85% of the enzyme activity applied remained fixed on the column. The enzyme was eluted stepwise by applying 300 ml of Buffer B containing 1 M NaCl. The enzyme activity was eluted at this concentration of NaCl with a recovery of 80 to 90%. No activity was observed on further washing of the column with the buffer containing 2 M NaCl. The active fractions were collected and combined (70 to 120 ml, DNA-cellulose fraction).

This fraction contained a small amount of DNA (30 to 60 μg/ml), and the stimulatory effect of exogenous DNA on the enzyme reaction was still partial (about 2-fold stimulation).

Hydroyxylapatite Column Chromatography—DNA-cellulose fraction of the enzyme (80 to 120 mg of protein) was directly applied to a hydroyxylapatite column (2 x 10 cm, 30 ml), which had been washed and equilibrated with Buffer A containing 0.2 M NaCl. After washing the column with 20 ml each of Buffer A and Buffer B containing 1 M NaCl, at a flow rate of 30 ml per h. After washing the column with 20 ml each of Buffer A and Buffer B containing 0.5, 1.0, and 2.0 M KCl, successively, elution was carried out by applying two linear gradients (0 to 0.05 M and 0.05 to 0.30 M potassium phosphate buffer, pH 7.4) in Buffer A containing 2 M KCl with a total volume of 400 ml for each step. The flow rate was 30 ml per h. As shown in Fig. 1, the enzyme eluted at approximately 30 mM potassium phosphate buffer as a single sharp peak with a coincident protein peak. No other peak of enzyme activity was observed through the elution steps.

DNA contaminating the enzyme fraction in the previous step (DNA-cellulose fraction) was separated from the enzyme activity by this step. The DNA eluted at 0.15 M potassium phosphate (observed as the last absorbance peak in Fig. 1). The DNA preparation was designated "active DNA" in this paper since the DNA had a very high efficiency in stimulating the enzyme reaction. Purification and properties of this DNA are briefly summarized under "Materials and Methods."

The enzyme fraction (hydroyxylapatite fraction, 70 to 90 ml,
Complete DNA dependency. 10 to 20 mg of protein was free of DNA and showed a calibrated with the same buffer. The enzyme sample was then applied to a small hydroxylapatite column (1.2 ml) previously washed with 10 ml each of potassium phosphate buffer, pH 7.4 in Buffer A containing 1 mM KCl. The enzyme was eluted with 20 ml of Buffer A containing 1 mM KCl and 0.5 M potassium phosphate buffer, pH 7.4. The flow rate was 2 to 3 ml per h, and 1-ml fractions were collected. About 60 to 80% of the applied enzyme activity was recovered in a total volume of 4 ml. A 20-fold concentration of the enzyme sample can be easily achieved by the hydroxylapatite procedure. This concentrating procedure was used through this study when needed.

The purified enzyme (0.5 to 2 mg of protein/ml) was stored at -70°C with additional 2 mM dithiothreitol. The activity remained stable for more than 3 months. Estimated by SDS-polyacrylamide gel electrophoresis followed by Amido black staining of the gel as described under “Materials and Methods.” The enzyme preparation from the first chromatography on a Sephadex G-200 column showed a single main band on the gel, although it was still contaminated with 2 minor bands. The purity of the enzyme at this stage was estimated to be 95% from the density of each band. The purity of the final enzyme preparation, rechromatographed on a Sephadex G-200 column, was 99% by the same analysis.

**Estimation of Sugar Content**—When the sugar content of the purified enzyme (184 μg of protein) was measured by the anthrone reaction, no sugars were detected in the preparation. This method is capable of detecting 4 μg of glucose exogenously added to the purified enzyme. Thus the sugar content in this enzyme preparation was less than 2% of the amount of protein on a weight basis.

**Ultraviolet Absorption Spectrum**—The ultraviolet absorption spectrum of the purified enzyme solution (0.74 mg/ml in Buffer A) was measured with a Hitachi spectrophotometer (model 200-10). The spectrum has a maximum at 280 nm. The absorbance at 280 nm was 0.738. The ratio of $E_{280}$ to $E_{260}$ is 1.69. From this ratio the content of nucleic acids in the enzyme preparation can be estimated (36) to be less than 0.25% of the amount of protein on a weight basis. The value corresponds to less than one nucleotide per enzyme molecule.

**Molecular Weight of Enzyme**—When the molecular weight of the enzyme was determined from its elution volume in a Sephadex G-200 column calibrated with proteins of known molecular weights, a value of 150,000 to 160,000 was obtained.

The purified enzyme migrated as a single band on 5 and 7.5% polyacrylamide gel columns containing 0.1% SDS at pH 7.2 in sodium phosphate buffer. When calibrated with marker proteins of known molecular weights, a molecular weight of 130,000 was obtained.

We use the molecular weight of 130,000 for various calculations in this study.

**Stability of Enzyme**—When the purified enzyme was stored at a protein concentration of 0.5 to 1.5 mg/ml in Buffer A, the enzyme was quite stable, and no appreciable loss of the catalytic activity was observed during the storage for 3 months at -70°C. On storage at 0°C, the loss of the enzyme activity was 0 to 10% in a month. Thus the enzyme is fairly stable when it is stored at a relatively high concentration in Buffer A at 0°C or -70°C. However, after dilution to less than 20 μg/ml with Buffer A, the enzyme activity rapidly decreased during storage at 0°C. About 55 and 75% of the enzyme activity was lost when samples of 20 and 4 μg/ml were stored at 0°C for 4 h, while the loss of activity by the 500- and 100-μg/ml samples was not noticeable on storage. The addition of

**TABLE I**

<table>
<thead>
<tr>
<th>Purification of poly(ADP-ribose) polymerase</th>
<th>Protein content and enzyme activity were assayed as described under “Materials and Methods.” The purification began with 800 g of bovine thymus.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification step</td>
<td>Protein (mg)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>28,400</td>
</tr>
<tr>
<td>Ammonium sulfate (40-80%)</td>
<td>8,850</td>
</tr>
<tr>
<td>DNA-cellulose fraction</td>
<td>113</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>11.2</td>
</tr>
<tr>
<td>Sephadex G-200 (I)</td>
<td>6.8</td>
</tr>
<tr>
<td>Sephadex G-200 (II)</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Protein in the sample (200 to 500 μg) was precipitated with 10% trichloroacetic acid, collected by centrifugation, and dissolved in 0.1 N NaOH.*
bovine serum albumin (0.1 to 5.0 mg/ml) to the dilution buffer (Buffer A) could not prevent the decrease in enzyme activity.

**Time Course of Reaction**—When a relatively small amount of the enzyme (0.01 to 0.03 µg) was incubated in the standard reaction mixture, the time course of the reaction was approximately linear for the first 2 min, then the reaction proceeded at a continuously decreasing rate for about 30 min; finally the reaction rate became constant again and was maintained at the level of about 20% of the initial velocity for at least 1 h (Fig. 2). Thus, when the initial velocity must be measured exactly, an incubation time of less than 2 min was used. Ten-minute incubations were used throughout this study, unless otherwise indicated, since the amount of product synthesized during a certain incubation time is directly related to the amount of enzyme used, for incubation times of up to 90 min, despite the complicated time course of the reaction.

**Requirements of the Enzyme Reaction**—As shown in Table II, the purified enzyme requires DNA, histones, Mg²⁺, and a thiol compound, such as dithiothreitol, for its full activity. These requirements are essentially identical with those previously reported by us (20) and other investigators (21), using rather crude enzyme preparations from rat liver.

Omission of DNA from the reaction mixture resulted in nearly complete (>99.5%) loss of the enzyme activity. However, omission of histones suppressed the reaction only partly, a fact contrary to a previous suggestion that, for initiation of poly(ADP-ribose) synthesis, the enzyme requires an acceptor protein like histones (8). The histone-dependent stimulation of the reaction varied from 8 to 400%, depending on the nature of DNA added to support the enzyme activity, as described later.

The effect of dithiothreitol seems to be slight in these experiments with freshly prepared enzyme. However, when an old enzyme preparation, which had been stored for about 2 months at 0°C, was examined, the enzyme activity decreased to about 50% when dithiothreitol was omitted from the standard reaction mixture. This, combined with the observation that the polymerase activity is suppressed by about 90% by adding 0.5 mM N-ethylmaleimide to the reaction mixture in place of dithiothreitol (data not shown), indicates that the observed differences in the stimulatory effect of dithiothreitol may be dependent on the extent of oxidation of the active —SH group(s) in the enzyme protein.

**Effect of Temperature and pH on Enzyme Activity**—The optimum pH for the enzyme reaction is approximately 8.5 in Tris-HCl buffer and 9.0 in glycine/NaOH buffer. In the standard assay, the pH was kept at 8.0 with Tris-HCl buffer. Under these conditions, the enzyme activity is 85% of the activity observed at pH 8.5 in Tris-HCl buffer.

The highest incorporation of precursor during 10-min incubation in the standard reaction mixture takes place at 25°C (Fig. 3). At 10°C, the reaction proceeds more linearly but with a lower reaction rate (about 50% of the rate at 25°C). At 37°C and 42°C, the incorporation for 10 min was 70 and 30% of that at 25°C, respectively. On prolonged incubation at these higher temperatures, the reaction rate further decreased, probably due to heat inactivation of the enzyme.

**Effect of DNA on Enzyme Activity**—As previously reported by us (20) and Yamada et al. (21), poly(ADP-ribose) polymerase activity is dependent on DNA, and DNA can be replaced by various synthetic deoxypolyribonucleotides (21, 29). Yamada and Sugimura (35), using a partially purified rat liver enzyme, observed that DNA also could be replaced by polyanions such as heparin and poly(vinyl sulfate) in the presence of an excess amount of histone H1 relative to these polyanions. With our purified enzyme, however, neither these polyanions nor yeast RNA (2 µg/assay, added in place of DNA) could stimulate the enzyme reaction at all even in the presence of an excess amount of calf thymus histone H1 (0 to 50 µg/assay).

In order to compare the effect of different kinds of DNA on poly(ADP-ribose) polymerase activity, saturation curves for the enzyme activity as a function of DNA concentration were prepared using three DNAs: calf thymus DNA (Lot 93 c 9501-95), poly(d(A))-poly(d(T)), and “active DNA” (DNA separated from the crude enzyme preparation as described under “Materials and Methods”). As shown in Fig. 4A, these three DNAs were different both in their concentration required for saturating the enzyme activity and in the saturated levels of enzyme activity with these DNAs. Since the double reciprocal plots (Fig. 4B) from the saturation curves were linear within the range where the reaction rate increased with increasing concentration of DNA, the apparent Kₘ for these DNAs and

![Fig. 2. Time course of poly(ADP-ribose) synthesis. Various amounts of enzyme (A, 0.01 µg; B, 0.02 µg; C, 0.03 µg) were incubated in the standard assay mixture for the indicated times. Incorporation of ADP-ribose was measured as described under “Materials and Methods.”](image-url)

![Fig. 3. Effect of temperature on the enzyme reaction. Incorporation of ADP-ribose into acid-insoluble material was measured as described under “Materials and Methods” except that the temperature of incubation and the incubation time were changed as indicated. An amount of 0.04 µg of the enzyme was used for each assay. The tubes were incubated at 10°C, 20°C, 25°C, 30°C, 37°C, 42°C, and 50°C, respectively.](image-url)
the $V_{\text{max}}$ were obtained from the plots. As shown in the lower part of Table III, the apparent $K_m$ for DNA is doubled by doubling the concentration of enzyme, and the ratio of the $K_m$ for DNA to enzyme concentration is constant at any enzyme concentration. Thus the DNA/enzyme ratio required for half-saturation of the enzyme activity was 220 to 240, 40, and 10 base pairs per enzyme molecule for calf thymus DNA, poly(d(A)) - poly(d(T)), and "active DNA," respectively (Table III).

Despite similar $V_{\text{max}}$ values (Table III) obtained from double reciprocal plots for three DNAs, the plateau levels of enzyme activity reached (Fig. 3A) were fairly different. The decrease in the enzyme activity at plateau level was observed when a DNA preparation contained a relatively small part of denatured DNA. For example, the plateau level decreased by approximately 80 and 50% by the partial denaturation (approximately 25%) of calf thymus DNA and by the addition of heat-denatured calf thymus DNA (0.1 µg) to "active DNA" (1.0 µg), respectively (Table IV).

Effect of Histones—The rates of poly(ADP-ribose) synthesis as a function of histone concentration are presented in Fig. 5A. In the experiment the reactions were supported by various DNAs at fixed concentrations. The extent of stimulation of the reaction by histones varied markedly depending on the source, the lot of preparation, and the physical state of the DNA added. With "active DNA," poly(d(A)) - poly(d(T)), and a calf thymus DNA (Lot 93 c 9501-95), the reaction rates are near maximum even without histones, and the histone-dependent-stimulation is only 8 to 25%, while the stimulations are as great as 100% and 400% for the reactions with a calf thymus DNA (Lot 36 c 9560) and partially denatured DNA, respectively (Fig. 5A). In spite of the remarkable histone-dependent stimulation of the reaction with the latter two DNAs, however, the enzyme activities saturated with histones seem to be similar to, or slightly higher than, the activity attained by using the former three DNAs as an activator without adding histones. Thus, various DNAs show similar activity in stimulating the reaction at the saturating concentration (1 or 2 µg/0.2 ml) when histones are present in the reaction mixture. No appreciable histone-dependent stimulation was observed for the reaction with heat-denatured DNA.

The stimulation of the reaction in response to histones was also examined using varying concentrations of calf thymus DNA. As seen in Fig. 5B, the concentration of histones required for optimal enzyme activity show a parallel increase with the concentration of DNA in the reaction mixture, even at a fixed concentration of enzyme. This is in sharp contrast to the response caused by DNA, where the concentration of DNA did not seem to be related to the efficiency of stimulation.

**Table IV**

<table>
<thead>
<tr>
<th>DNA used</th>
<th>Concentration of DNA (µg/0.2 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus DNA</td>
<td>0.00</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>0.02</td>
</tr>
<tr>
<td>Native</td>
<td>0.52</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>1.00</td>
</tr>
<tr>
<td>Lot 93 c 9501-95</td>
<td>2.00</td>
</tr>
<tr>
<td>Native</td>
<td>0.81</td>
</tr>
<tr>
<td>Partially denatured (about 25% denaturation)</td>
<td>2.00</td>
</tr>
<tr>
<td>Heat denatured</td>
<td>0.08</td>
</tr>
<tr>
<td>Active DNA (1 µg) + denatured DNA (0.1 µg)</td>
<td>0.67</td>
</tr>
<tr>
<td>Active DNA</td>
<td>1.09</td>
</tr>
</tbody>
</table>

"The enzyme activity measured in the standard reaction condition described under "Materials and Methods" was set at 1.00 for comparison of the ability of various DNAs to stimulate the reaction.

**Table III**

<table>
<thead>
<tr>
<th>Enzyme concentration (µg/0.2 ml)</th>
<th>DNA used</th>
<th>$V_{\text{max}}$ (nmol/min)</th>
<th>$K_m$ for DNA (µM)</th>
<th>$K_m$ for DNA enzyme concentration (µg/µmol)</th>
<th>DNA base pair/enzyme molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Active DNA</td>
<td>0.023</td>
<td>0.005</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>0.1</td>
<td>Poly(d(A)) - poly(d(T))</td>
<td>0.022</td>
<td>0.02</td>
<td>0.2</td>
<td>40</td>
</tr>
<tr>
<td>0.1</td>
<td>Calf thymus DNA (Lot 93 c 9501-95)</td>
<td>0.022</td>
<td>0.11</td>
<td>1.1</td>
<td>240</td>
</tr>
<tr>
<td>0.5</td>
<td>93 c 9501-95</td>
<td>0.125</td>
<td>0.50</td>
<td>1.0</td>
<td>220</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>1.25</td>
<td>4.8</td>
<td>1.0</td>
<td>220</td>
</tr>
</tbody>
</table>

"Since this value was estimated at a limited concentration of NAD$^+$ (10 µM) with varying concentration of DNA, the value is lower than the $V_{\text{max}}$ estimated with varying concentration of NAD$^+$ in the standard reaction mixture.

"This estimation is a minimum since the calculation was made on the assumption that all enzyme molecules were active."
formed as described under "Materials and Methods" except that the concentration of calf thymus DNA (Lot 93 c 9501-95) and calf thymus whole histones were varied as indicated. An amount of 0.1 pg of the enzyme and the following concentrations of DNA were used: 0.5 pg of actively DNA (H), 1 pg of partially denatured calf thymus DNA (W), and 2 pg of heat denatured calf thymus DNA (A-A). Preparation of these DNAs were described under "Materials and Methods." B, effect of histones on the enzyme reaction supported by varied concentration of DNA. The enzyme activity was measured as described under "Materials and Methods." The results show that approximately 98% of the product of the enzyme to chromatin in situ may be very different from that found in purified chromatin and reconstituted enzyme-DNA complexes.

The enzyme, when released from chromatin, is quite unstable, probably owing to both the oxidation of the functional thiol group of the enzyme and proteolytic activity in the crude preparation. The activity is partly stabilized by the combination of thiol compounds, NaCN, EDTA, and NaHSO₄, during the purification.

The molecular weight of the enzyme was estimated to be 130,000 and 150,000 to 160,000 by SDS-polyacrylamide gel electrophoresis and gel filtration, respectively. Although there is some difference between the two estimations, the results indicate that the enzyme consists of a single peptide. These estimations are comparable to those of 120,000 to 150,000 for rat liver enzyme by Yamada et al. (21) obtained by sedimentation in sucrose density gradients. Recent sedimentation studies with our enzyme preparation also revealed a comparable value. The small sedimentation coefficient obtained by velocity centrifugation may be due to a significant deviation of the enzyme molecule from a globular shape.

The purified enzyme absolutely required DNA for the reaction. When an appropriate concentration of "active DNA" or a calf thymus DNA (Lot 93 c 9501-95) is added, the enzyme reaction seems to proceed at an appreciably high rate, even

![Graph](image)

**TABLE V**

<table>
<thead>
<tr>
<th>Items analyzed</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme used</td>
<td>2.3 pmol*</td>
</tr>
<tr>
<td>Total ADP-ribose incorporated</td>
<td>700 pmol</td>
</tr>
<tr>
<td>Hydrolysate of the product by snake venom phosphodiesterase, recovered as</td>
<td></td>
</tr>
<tr>
<td>5'-AMP</td>
<td>62 pmol*</td>
</tr>
<tr>
<td>Phosphoribosyl-AMP</td>
<td>623 pmol*</td>
</tr>
<tr>
<td>Undetermined</td>
<td>14 pmol*</td>
</tr>
<tr>
<td>Average chain length</td>
<td>11 ADP-ribose units</td>
</tr>
</tbody>
</table>

*The value was calculated from the amount of enzyme used and the molecular weight of 130,000.

The value is corrected from the total recovery of radioactivity on paper chromatography.

The average chain length is calculated by the method of Fujimura and Sugimura (37), and is the ratio (5'-AMP + phosphoribosyl-AMP)/5'-AMP.

DNA required for optimal enzyme activity parallels the concentration of enzyme (see the previous section). Excess amounts of histones relative to DNA are inhibitory to the reaction (Fig. 5B).

**Product Analysis**—To confirm that the product of the purified enzyme is poly(ADP-ribose) and to determine its average chain length, the product was prepared and hydrolyzed with snake venom phosphodiesterase, and the hydrolysates were analyzed as described under "Materials and Methods." The results show that approximately 98% of the product analyzed is recovered as 5'-AMP (8.8%) and phosphoribosyl-AMP (89%) (Table V). The appearance of these two compounds in the hydrolysates of poly(ADP-ribose) is known to be a unique property of this polymer (5-7). The average chain length was estimated to be 11 ADP-ribose units from the ratio of (5'-AMP + phosphoribosyl-AMP)/5'-AMP according to the method described by Fujimura and Sugimura (37). The total number of chains synthesized, calculated from the ratio of (ADP-ribose incorporated)/(average chain length), was 64 pmol. Since the amount of the enzyme used in the reaction is 2.3 pmol, one enzyme molecule is estimated to have synthesized an average of 28 molecules of poly(ADP-ribose) during 20 min incubation in the standard reaction mixture (this estimation is a minimum since the calculation was made assuming that all enzyme molecules in our preparation were active).

The molecular weight of the enzyme was estimated to be 130,000 and 150,000 to 160,000 by SDS-polyacrylamide gel electrophoresis and gel filtration, respectively. Although there is some difference between the two estimations, the results indicate that the enzyme consists of a single peptide. These estimations are comparable to those of 120,000 to 150,000 for rat liver enzyme by Gill (39) and 150,000 to 160,000 for rat liver enzyme by us (40), using Sephadex G-200 column chromatography. However, they are significantly larger than the estimation of 78,000 for rat liver enzyme by Yamada et al. (21) obtained by sedimentation in sucrose density gradients. Recent sedimentation studies with our enzyme preparation also revealed a comparable value. The small sedimentation coefficient obtained by velocity centrifugation may be due to a significant deviation of the enzyme molecule from a globular shape.

The purified enzyme absolutely required DNA for the reaction. When an appropriate concentration of "active DNA" or a calf thymus DNA (Lot 93 c 9501-95) is added, the enzyme reaction seems to proceed at an appreciably high rate, even
without histones or so-called acceptor proteins. In spite of the relatively small difference between the plateau levels of the enzyme activity with saturating concentration of these two DNAs, the DNAs showed a remarkable difference in the concentration required for half-maximal enzyme activity (apparent $K_m$ for DNA). The apparent $K_m$ for DNA increased with increasing concentration of the enzyme, and the ratio of the apparent $K_m$ for DNA to the enzyme concentration was constant at any enzyme concentration. The same situation is found in the interaction of RNA polymerase and template DNA. Therefore, the apparent $K_m$ for DNA cannot be interpreted as a Michaelis constant or as a measure of enzyme-DNA affinity. The saturation curves of Fig. 4A are essentially titrations of the amount of enzyme present with DNA. When the assumption is made that most of the enzyme molecules in the preparation are active, the DNA/enzyme ratio at half-maximal enzyme activity (220 to 240 base pairs per enzyme molecule for calf thymus DNA and “active DNA,” respectively) can be considered to be minimum estimations of the half-length of these DNAs required for activation of one enzyme molecule. Moreover, if the quality of the effective enzyme-binding sites on the DNAs are the same, the differences in apparent $K_m$ for these DNAs, and thus the DNA/enzyme ratios, are a reflection of the site density on these DNAs. In “active DNA,” this hypothetical site density is 1 per 20 base pairs. This is 20 times more dense than that of the bulk of calf thymus DNA, indicating that the “active DNA” is derived from a part of calf thymus DNA where poly(ADP-ribose) polymerase is richly condensed. In this regard, Mullins et al. (41) have recently shown that the distribution of poly(ADP-ribose) in chromatin is not random, but that the enzyme is localized primarily in transcriptionally active chromatin.

The presence of relatively small amounts of denatured DNA in native DNA preparations greatly affects the efficiency of the DNA to support the enzyme reaction (Table IV). The effect of denatured DNA is mainly manifested on the DNA saturation curve as a decrease in the saturation level of the enzyme activity. These results can be interpreted as showing that denatured DNA is not only ineffective in enzyme activation, but also that it is inhibitory to the enzyme activity, probably due to its higher binding affinity with the enzyme and the formation of an abortive enzyme-denatured DNA complex.

As has been reported by us (20) and other investigators (21), the partially purified poly(ADP-ribose) polymerase reaction was stimulated 2- to 3-fold further by exogenously added histones in the presence of an appropriate concentration of DNA. In addition, many reports have shown that histones are ADP-ribosylated on incubation of cell nuclei or chromatin with NAD+ (42-45). These observations suggest that histones serve as an acceptor of ADP-ribose in the reaction and predict that the extensive purification of the enzyme may develop complete dependency of the reaction on histones as well as DNA. Contrary to this expectation, the purified enzyme could synthesize poly(ADP-ribose) at high reaction rate without histones, especially when an appropriate DNA, such as “active DNA” or a calf thymus DNA (Lot 93 c 9501-98), was used in the reaction. A high stimulation by histones was seen only when a DNA with low enzyme-activating ability was used (see Fig. 5A). Since the lowered activity of DNA at the saturating concentration is shown to be due to relatively small amounts of denatured DNA which contaminates native DNA preparations (see Table IV), the observed histone effect can be interpreted as the tight binding of histones with denatured DNA (46) masking its inhibitory activity to the enzyme. Our several unsuccessful trials in demonstrating ADP ribosylation of histones by the purified enzyme (data not shown) lend further support to the interpretation.

The presence of so-called acceptor proteins (8) in our enzyme preparation or in the DNA fraction used is unlikely since the enzyme preparation has a purity of 99%, and DNAs, such as “active DNA” and calf thymus DNAs, do not contain any detectable amount of protein as determined by protein staining after the gel electrophoresis (data not shown). As for initiation of poly(ADP-ribose) synthesis by the purified enzyme, we recently showed that the very early reaction product, oligo(ADP-ribose) with an average chain length of 2.3, is tightly bound to enzyme protein itself, probably by a covalent linkage judging from the SDS-polyacrylamide gel electrophoresis of the early reaction product, and that the enzyme-bound oligomer is elongated on prolonged incubation (34). From the results we surmise that the enzyme molecule has a site providing acceptor-like function in addition to a catalytic site. Contrary to these findings, Okayama et al. (47) recently indicated the presence of a non-protein acceptor, which co-purified with their enzyme preparation from rat liver, and was separated, after the reaction, as a product-bound form from the enzyme on SDS-polyacrylamide gel electrophoresis. This type of acceptor was not detected in our enzyme preparation.

The molecular basis of the very high efficiency of “active DNA” in stimulating the enzyme reaction has not been clarified yet. Apparently its high efficiency is not due to its small molecular size since neither the shearing, the sonication, nor the DNase I treatment of bulk of calf thymus DNA significantly affects the increase of the enzyme-supporting activity. The possibility that a specific terminal structure of “active DNA” is related to the high activity is also negated because a covalently closed circular plasmid DNA (Col El-RSF2124) was also as efficient as bulk of calf thymus DNA. The presence of a significant amount of poly- or oligo(ADP-ribose) in “active DNA” preparation also can be ruled out since poly(ADP-ribose) has been shown to have a much higher buoyant density than DNA (6), and our recent study has shown that the number of adenine residues in “active DNA” is approximately equal to that of thymine as well as guanine is equal to cytosine. Recently Ueda and co-workers reported that their highly purified polymerase from rat liver (48) could not ADP-ribosylate any of the histones, but could elongate the ADP-ribose chain artificially bound to histone (49).

During the preparation of this manuscript, Mandel et al. (50) reported a purification method of calf thymus poly(ADP-ribose) polymerase.

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Purification and Properties

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