Poly(ADP-ribose) synthetase of rat liver was found to catalyze automodification on multiple sites. When the synthetase was incubated with 2.4 μM NAD for 20 s in the presence of DNA, more than 90% of ADP ribose incorporated into acid-insoluble material co-migrated with the synthetase (Mr = 108,000) upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Incubation for longer times or at higher NAD concentrations led to the increase in the apparent molecular weight, up to >500,000, of the poly(ADP-ribose) enzyme. The enzyme-bound polymers had branch structures at a frequency of once every 50 ADP-ribose residues. After correction for branching, the enzyme was estimated to bind as many as 15 polymers of the average chain length of 80 or more ADP-ribose units. The poly(ADP-ribose)-enzyme enzyme was labile in 0.1 M NaOH and 2 M NH₄OH (pH 7.0) at 25 °C, indicating a similar type of ester bond as in ADP-ribose histones. The automodified enzyme was less active than the unmodified enzyme but not the production of free polymers. Whether or not the automodified enzyme serves as an intermediate in poly(ADP-ribose)ylation of other proteins has not been determined.

In order to investigate these problems, we analyzed the automodification of the synthetase purified from rat liver. We report here that the synthetase catalyzes branching of poly(ADP-ribose) and that, after correction for branching, automodification takes place at multiple sites on the enzyme molecule. Evidence is also presented that the automodified synthetase does not produce free polymers, nor does it serve as an intermediate in poly(ADP-ribose)ylation of other proteins.

### MATERIALS AND METHODS

**Chemicals and Enzymes**—[AMP-32P]NAD and [Aden-2,8-3H]NAD were obtained from New England Nuclear; [Aden-5,5'-3H]NAD and nucleotide-[U-14C]glucose were from the Radiochemical Centre, Amersham; NAD and calf thymus DNA were obtained from Sigma; proteinase K was from Merck; Solunene 380 was from Packard; Escherichia coli alkaline phosphatase was from Worthington. [ribosyl(NMN)-3H]NAD was prepared from 5'-[3H]glucose as described previously (19). *Crotalus adamanteus* venom phosphodiesterase was obtained from Sigma and further purified by the method of Iga et al. (20). Calf thymus histone H1 was prepared by the method of Johns (21). Poly(ADP-ribose) synthetase was purified to apparent homogeneity by a slight modification of the method previously reported (10). The purified synthetase exhibited a single protein band at the position of M, = 108,000 upon SDS-polyacrylamide gel electrophoresis and had the specific activity of 400 nmol/min/mg of protein as assayed under the conditions described below.

Poly(ADP-ribose)ylation of Synthetase—The standard reaction mixture contained 100 mM Tris/Cl (pH 8.0), 10 mM MgCl₂, 1.25 mM dithiothreitol, 100 μM [Aden-5'-3H]NAD (10 cpm/μmol), 50 μg/ml of DNA, and poly(ADP-ribose) synthetase in a total volume of 0.2 ml. The reaction was carried out for 10 min at 37 °C and terminated by the addition of 20% Cl₃CCOOH. An aliquot of the mixture was analyzed for acid-insoluble radioactivity using a Millipore filter (19). The other aliquot was centrifuged for 20 min at 15,000 × g, and the precipitate, washed twice with 10% Cl₃CCOOH and once with ethyl ether, was subjected to the following analyses.

**Proteinase K Treatment of Poly(ADP-ribose)ated Material and Polyacrylamide Gel Electrophoresis**—Poly(ADP-ribose)ated material was dissolved in 90 μl of 15 mM sodium phosphate (pH 7.2) and catalyzed automodification in the presence of DNA and in the absence of other acceptors. These authors also suggested a possible production of free polymers from the automodified synthetase, on the basis of pulse-chase experiments (15) and analysis of the number of polymers produced per enzyme molecule (15, 16). Our preliminary experiments have confirmed automodification of the enzyme but not the production of free polymers (18). Whether or not the automodified enzyme serves as an intermediate in poly(ADP-ribose)ylation of other proteins has not been determined.

### RESULTS

**Poly(ADP-ribose) synthetase** is a chromatin-bound enzyme which synthesizes a protein-bound homopolymer, poly(ADP-ribose), from NAD (1–3). Various nuclear proteins, including histones and nonhistone proteins, have been reported as acceptors of this polymer in isolated nuclei (1–7) and in vivo (8, 9). Recently, a branch structure of poly(ADP-ribose) was demonstrated by Miwa et al. (9). The enzyme responsible for the branching has not yet been identified.

Poly(ADP-ribose) synthetase has been purified to apparent homogeneity from various sources (10–15). Yosihara et al. (15) and others (15, 17) reported that the purified synthetase catalyzed automodification in the presence of DNA and in the absence of other acceptors. These authors also suggested a possible production of free polymers from the automodified synthetase, on the basis of pulse-chase experiments (15) and analysis of the number of polymers produced per enzyme molecule (15, 16). Our preliminary experiments have confirmed automodification of the enzyme but not the production of free polymers (18). Whether or not the automodified enzyme serves as an intermediate in poly(ADP-ribose)ylation of other proteins has not been determined.

**Poly(ADP-ribose) Synthetase**—The standard reaction mixture contained 100 mM Tris/Cl (pH 8.0), 10 mM MgCl₂, 1.25 mM dithiothreitol, 100 μM [Aden-5'-3H]NAD (10 cpm/μmol), 50 μg/ml of DNA, and poly(ADP-ribose) synthetase in a total volume of 0.2 ml. The reaction was carried out for 10 min at 37 °C and terminated by the addition of 20% Cl₃CCOOH. An aliquot of the mixture was analyzed for acid-insoluble radioactivity using a Millipore filter (19). The other aliquot was centrifuged for 20 min at 15,000 × g, and the precipitate, washed twice with 10% Cl₃CCOOH and once with ethyl ether, was subjected to the following analyses.

**Proteinase K Treatment of Poly(ADP-ribose)ated Material and Polyacrylamide Gel Electrophoresis**—Poly(ADP-ribose)ated material was dissolved in 90 μl of 15 mM sodium phosphate (pH 7.2) and catalyzed automodification in the presence of DNA and in the absence of other acceptors. These authors also suggested a possible production of free polymers from the automodified synthetase, on the basis of pulse-chase experiments (15) and analysis of the number of polymers produced per enzyme molecule (15, 16). Our preliminary experiments have confirmed automodification of the enzyme but not the production of free polymers (18). Whether or not the automodified enzyme serves as an intermediate in poly(ADP-ribose)ylation of other proteins has not been determined.

The term "automodification" does not necessarily imply that the reaction is definitively intramolecular.

The abbreviations used are: SDS, sodium dodecyl sulfate; isoADP-ribose, 2'-[5'-phosphoribosyl]-5'-AMP; phosphoribosyl-isoADP-ribose, 2'-[1'-ribosyl-2'-[1'-ribosyl]]adenosine-5',5',5'-trisphosphate.
containing 3% SDS. After incubation for 1.5 h at 25 °C, two 40-μl samples were removed; to each sample was added 5 μl of either a solution of proteinase K (6 mg/ml) or water. Both samples were incubated for 4 h at 25 °C and then mixed with a solution (20 μl) containing 0.032% bromphenol blue, 3.2% 2-mercaptoethanol, and 65% glycerol. The samples were subjected to electrophoresis in cylindrical SDS (0.1%)-polyacrylamide (7.5%) gels (0.5 x 7 cm) as described by Weber and Osborn (22). After electrophoresis, each gel was cut longitudinally in half; one-half was stained with Coomassie blue, and the other half was sliced into 2-mm pieces. The pieces were treated with 0.5 ml of Soluene 350 for 3 h at 50 °C and examined for radioactivity.

**Phosphodiesterase Digestion of Poly(ADP-ribose) and Paper Chromatography of the Digest—Poly(ADP-ribose)ylated material was dissolved in 100 μl of 1 N NaOH. The mixture was incubated for 30 min at 35 °C to release poly(ADP-ribose) from acceptors and then lyophilized. The residue was dissolved in a solution (60 μl) containing 50 mM potassium phosphate (pH 7.5), 10 mM MgCl₂, 2.5 mM AMP, 2 μg of trypsin, and 0.3 μl of snake venom phosphodiesterase. The mixture was incubated for 6 h at 37 °C. The digest was applied to Whatman 3MM filter paper and chromatographed in a solvent system of isobutyric acid/concentrated NH₄OH/water/0.1 M EDTA Na₂ (68:13:2:1). Strips 1 cm wide were cut out and examined for radioactivity.

**Enzyme Treatments of Phosphorylsyl-isoADP-ribose and DEAE-Cellulose Column Chromatography—Putative phosphorylsyl-isoADP-ribose eluted was from a paper chromatogram with 550 μl of water. Three samples (175 μl each) were removed from the eluate and lyophilized. One sample was dissolved in a solution (40 μl) containing 25 mM Tris/HCl (pH 8.0) and 0.054 unit of alkaline phosphatase. Another sample was dissolved in 40 μl of 25 mM potassium phosphate (pH 7.5) containing 10 mM MgCl₂, 5 mM AMP, and 0.5 unit of phosphodiesterase. Both mixtures were incubated for 2 h at 37 °C and then lyophilized. The two samples thus treated and the sample untreated were dissolved in 0.2 ml each of 7 M urea. The solutions were separately applied to DEAE-cellulose columns (0.5 x 15 cm) equilibrated with 20 mM Tris/HCl (pH 7.8) containing 7 M urea (23). Each column was eluted with a linear gradient (total 120 ml) of 0 to 0.2 M NaCl in the same buffer.

**Gel Filtration of Poly(ADP-ribose)ylated Synthetase—Poly(ADP-ribose)ylation reaction carried out under specified conditions was terminated by the addition of potassium acetate (final 0.5 M, pH 5.5). The mixture was applied to a Bio-Gel P-200 column (1.5 x 28 cm) equilibrated with 20 mM Tris/HCl (pH 7.5) containing 0.15 M KCl, 10% glycerol, and 1 mM diithiothreitol, and the column was eluted with the same buffer. HClO₄ Extraction of Histone H1—Poly(ADP-ribose)ylation reaction carried out with histone H1 was terminated by the addition of 5% (final) HClO₄. After standing for 30 min at 0 °C, the mixture was centrifuged for 20 min at 15,000 x g. The supernatant fraction was referred to as the HClO₄ extract.

**Protein Determination—Protein was assayed by the method of Lowry et al. (24) with bovine serum albumin as the standard.

**RESULTS**

**Multiple Autopoly(ADP-ribose)ylation**

**Automodification of Poly(ADP-ribose) Synthetase—**When purified poly(ADP-ribose) synthetase was incubated for 20 s with 2.4 μM [³²⁵]NAD in the presence of DNA and acid (20% Cl₂COOH)-insoluble products were analyzed by electrophoresis in SDS-polyacrylamide gels, a major radioactivity peak co-migrated with the enzyme protein band (Fig. 1A). After extensive treatment with proteinase K, this radioactivity peak moved broadly to the position of the tracking dye where free mono- and oligo(ADP-ribose) migrated (25) (Fig. 1B). These profiles indicated that most of poly(ADP-ribose) molecules were bound to the synthetase protein. A longer incubation, for 2 min, resulted in a slight decrease in the mobility of the radioactivity peak and spreading of the protein band (Fig. 1C). The proteinase K digest migrated more slowly than the digest of 20-s incubation products (Fig. 1D). When the synthetase was incubated for 10 min with 100 μM NAD, both the radioactivity and the protein band remained at the top of the gel (Fig. 1E). The proteinase K digest was distributed broadly between the tracking dye and the top of the gel (Fig. 1F), showing that the enzyme-bound polymers were much larger on an average and heterogeneous in size.

The chain number and the average chain length of the enzyme-bound polymers, as estimated by phosphodiesterase digestion (4) and corrected for the frequency of branching (see below), were 0.5 mol/mol of enzyme (10 ADP-ribose units), 1.1 mol/mol (20 ADP-ribose units), and 9 mol/mol (48 ADP-ribose units) under the conditions of Fig. 1A (B, C, and D), and E (F), respectively. When the synthetase was incubated with 100 μM NAD for 60 min, the molecular weight of the automodified enzyme was estimated to exceed 500,000 by electrophoresis in lower concentration (3 to 5%) gels (data not shown).

The amounts of free polymers were estimated from the radioactivity moving faster than the enzyme (Fig. 1A, C, and E) as less than 5%, 10%, and 20% of the enzyme-bound polymers under the above respective conditions. Presumably, these free polymers were released nonenzymically from the synthetase under slightly alkaline conditions of incubation (pH 8.0), solubilization in the SDS solution (pH 7.2), and electrophoresis (pH 7.3) (see below).

These results indicated that essentially all polymers produced by the purified poly(ADP-ribose) synthetase in the absence of other acceptors were bound to the enzyme itself, and that longer incubations at higher NAD concentrations resulted in very extensive automodification at multiple sites.

**Branch Structure of Enzyme-bound Poly(ADP-ribose)—**When the enzyme-bound poly(¹⁴C)ADP-ribose was digested

**FIG. 1. SDS-polyacrylamide gel electrophoresis of poly(ADP-ribose)ylated material treated or untreated with proteinase K.** The standard mixture (1.6 ml), modified to contain 2.4 μM (400 cpm/pmol) (A, B, C, and D) or 100 μM (4.9 cpm/pmol) (E and F) [Ado-³²⁵]NAD and 2.4 μg of poly(ADP-ribose) synthetase, was incubated at 37 °C for 20 s (A and B), 2 min (C and D), or 10 min (E and F). Acid-insoluble products were treated (B, D, and F) or not treated (A, C, and E) with proteinase K and subjected to gel electrophoresis as described under "Materials and Methods." Insets show the Coomassie blue staining of the gel. BPB, bromphenol blue.
with phosphodiesterase and the digest analyzed by paper chromatography, three radioactive products were separated in addition to incompletely digested polymers remaining at the origin (Fig. 2). The two products with \( R_F = 0.38 \) and 0.55 were identified as isoADP-ribose and AMP, respectively. The other product with \( R_F = 0.18 \) was tentatively identified as phosphoribosyl-isoADP-ribose \((2'\cdot[1''\-ribosyl-2''\-([1''\-ribosyl]adenosine-5',5'',5'''\-tris(phosphate))\), that was the product from branching points of the polymer (9), on the basis of the following observations. 1) This product had a net negative charge of \(-5 \text{ to } -6\) at pH 7.6 as judged by the elution position from a DEAE-cellulose column in the presence of 7 M urea (Fig. 3A) (23); 2) further treatment with phosphodiesterase did not alter the elution position (data not shown), while the treatment with alkaline phosphatase made this product pass through the column (Fig. 3B), suggesting a loss of the negative charge; 3) an apparently identical product obtained from the incubation with \([\text{Ade-}^3\text{H, Rib(NMN)-}^{14}\text{C}]\text{NAD}\) contained two \([^{14}\text{C}]\)riboses per \([^3\text{H}]\)adenine (Fig. 4). The occurrence of phosphoribosyl-isoADP-ribose was an indication that the polymers had branch structures. The yield of phosphoribosyl-isoADP-ribose was about 2\% of the total ADP-ribose residues, suggesting that the branching occurred at a frequency of once every 50 ADP-ribose residues.

A branched poly(ADP-ribose) has more than one adenosine terminus, and the average chain size (mean ADP-ribose residues per polymer molecule) is calculated from the phosphodiesterase digests as follows:

\[
\text{Average chain size} = \frac{\text{cpm(AMP)} + \text{cpm(isoADP-ribose)} + \text{cpm(phosphoribosyl-isoADP-ribose)}}{\text{cpm(AMP)} - \text{cpm(phosphoribosyl-isoADP-ribose)}}
\]

The number of polymers is obtained as the quotient of total ADP-ribose residues divided by the average of chain size.

**Multiple Acceptor Sites on Synthetase**—The automodification appeared to proceed in a quasidistributive manner; 2.2 pmol of the synthetase produced, at 0 °C in 1 min, about 3 times as much (7 pmol) poly(ADP-ribose) with the average chain size of 2.5 ADP-ribose units (Fig. 5A), and the number of polymers and the average chain size increased almost in parallel for about 10 min. At 37 °C (Fig. 5B), the number of polymers reached a maximum (about 15 mol/mol of enzyme) in 20 min and remained constant thereafter. On the other hand, the chain size as well as ADP-ribose incorporation continued to increase for at least 60 min. These results indicated that each enzyme molecule had about 15 acceptor sites.
and that the polymers did not turn over on the synthetase to produce free polymers. Both at 0 °C and 37 °C, the production of phosphoribosyl-isoADP-ribose was proportional, at a level of 2%, to the total ADP-ribose incorporation. Thus, the frequency of branching was once every 50 ADP-ribose residues, irrespective of the chain size. After 60 min at 37 °C, about 6.5-fold, which was compatible with the observed increase in the molecular weight described above.

**Properties of Automodified Synthetase**

**Stability of Poly(ADP-ribose)-Enzyme Linkage**—The bond between poly(ADP-ribose) and the synthetase was stable in 0.1 N HCl and fairly stable at pH 7.0 or pH 8.0 in 0.2 M Tris/HC1; 15% or 30% was split in 60 min at 25 °C in these solutions, respectively (Fig. 6). The bond was, however, very unstable in 0.1 N NaOH or 2 M NH2OH (pH 7.0); 75% or 65% was cleaved under respective conditions in 10 min at 25 °C. These stabilities suggested that approximately 70% of the bonds was of a similar type of carboxyl ester as in ADP-riboseyl histones (26-29). About 30% of the bonds was, however, NH2OH resistant, and about 15% was alkali resistant. Such heterogeneity of the linkages has also been reported for ADP-riboseyl histones (30, 31).

**Catalytic Parameters of Automodified Synthetase**—The automodification of poly(ADP-ribose) synthetase was accompanied by lowering of the catalytic activity. When variously automodified synthetase was prepared and isolated by gel filtration, the $K_m$ value for NAD was found to increase, while...
Poly(ADP-ribose) synthetase (7.5 μg) was incubated for 1 min at 37°C in the standard mixture (1.0 ml) containing 10 μM [32P]NAD (3200 cpm/pmol) and 250 μg/ml of DNA. By this incubation, the enzyme was modified by 92 pmol (≈1.3 mol/mol of enzyme) of poly(ADP-ribose) with the average chain size of 12 ADP-ribose units. The automodified enzyme was separated from remaining NAD by gel filtration as described under "Materials and Methods." Aliquots (0.33 μg (experiment I) or 0.21 μg of enzyme (experiment II)) were incubated for 10 min at 37°C in the standard mixture (1.0 ml) containing, where indicated, 50 μg of histone H1 and 100 μM NAD, either 32P-labeled (170 cpm/pmol) or unlabeled. Acid (20% Cl, C(COOH))-insoluble radioactivity was determined in the total mixture or in the 5% HClO4 extract.

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>ADP-ribose on the synthetase incubated</th>
<th>ADP-ribose newly incorporated</th>
<th>ADP-ribose extractable with 5% HClO4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm</td>
<td>pmol</td>
<td>cpm</td>
</tr>
<tr>
<td>I</td>
<td>None</td>
<td>148,000</td>
<td>46.7</td>
<td>905</td>
</tr>
<tr>
<td></td>
<td>Histone H1</td>
<td>148,000</td>
<td>46.7</td>
<td>655,000</td>
</tr>
<tr>
<td></td>
<td>Histone H1 + [32P]NAD</td>
<td>148,000</td>
<td>46.7</td>
<td>872</td>
</tr>
<tr>
<td>II</td>
<td>None</td>
<td>96,400</td>
<td>30.4</td>
<td>1,200</td>
</tr>
<tr>
<td></td>
<td>Histone H1 + unlabeled NAD</td>
<td>96,400</td>
<td>30.4</td>
<td></td>
</tr>
</tbody>
</table>

Vₚₚ decreased as the number and the length of polymers on the enzyme increased (Table I). The Kₘ for NAD of the synthetase modified with about 4 molecules of the polymer with the average chain size of 28 ADP-ribose units was 5-fold higher and Vₚₚ was about one-half compared with the values for the unmodified enzyme. These parameters of the enzyme prepared by gel filtration without incubation or after incubation without NAD were essentially the same as those of the untreated enzyme. Free poly(ADP-ribose) (average chain size = 23 ADP-ribose units) showed no inhibitory effect on the synthetase at concentrations up to 6.7 μM (≈3000 polymers/enzyme molecule), suggesting that the lowering of the enzyme activity was not caused simply by polymers but their attachment to the synthetase.

**No Production of Free Poly(ADP-ribose)**

The low yield of free polymers (Fig. 1) and no increase in the number of polymers in prolonged incubations (Fig. 5B) suggested that free polymers were not produced enzymically in acid-insoluble fraction. The possibility that the synthetase might catalyze production of acid-soluble oligomers was excluded by analysis of the acid-soluble fraction with DEAE-cellulose column chromatography (Fig. 7). Essentially no radioactivity was eluted from the column at the NaCl concentrations higher than 0.13 M, indicating that free oligomers ranging in size from a dimer up to a 20-mer were not produced under the present conditions which allowed extensive automodification. This result, together with those in Figs. 1 and 5B, appears to support the view that the automodified enzyme did not serve as an intermediate in free polymer production.

In the above column chromatography, two radioactivity peaks were noted. The major peak eluting at 0.08 M NaCl coincided with ADP-ribose, whereas the minor peak eluting at 0.10 M NaCl did not coincide with ADP-ribose, ADP-ribose dimer, AMP, or isoADP-ribose; its identification is currently under investigation. The amount of ADP-ribose enzymically produced was as much as 25% of ADP-ribose incorporated into acid-insoluble material, suggesting an abortive NAD glycohydrolase activity of the synthetase. In support of this observation, the release of nicotinamide from NAD was ex- 

**Fig. 7. DEAE-cellulose column chromatography of acid-soluble products.** The standard mixture (0.6 ml) containing 0.6 μg of (A) or no poly(ADP-ribose) synthetase (B) was incubated for 20 min at 37°C. The reaction was terminated by the addition of 20% (final) Cl, C(COOH) and the mixture centrifuged for 20 min at 15,000 × g. The supernatant was washed with ethyl ether until the pH became neutral and lyophilized. The lyophilized residue was dissolved in 0.2 M urea, and the solution was subjected to chromatography on a DEAE-cellulose column (0.7 × 25 cm) as described under "Materials and Methods" except that the column was eluted with a linear gradient (total 250 ml) of NaCl from 0 to 0.3 M followed by 1 M NaCl in the described buffer. Arrows indicate elution positions of authentic markers.

**No Transfer of Polymers from Automated Enzyme to Histone**

Whether the automodified synthetase serves as an intermediate in poly(ADP-ribose)lation of other proteins was examined by the following experiments. First, the synthetase was automodified with [32P]NAD and isolated by gel filtration. The isolated enzyme was then incubated with histone H1 in the presence or absence of NAD, and the radioactivity extractable with 5% HClO₄ was quantified; this acid is known to solubilize histone H1 but not the synthetase (33). As shown in Table II, essentially no increase in the amount of HClO₄-extractable ADP-ribose was observed upon the addition of histone H1 alone (experiment I) or together with NAD (experiment II), suggesting that the enzyme-bound poly(ADP-ribose) was not transferred to the histone. This was in contrast to the fact that the automodified synthetase retained the activity to poly(ADP-ribose)late histone H1 using NAD; upon incubation of the automodified enzyme with histone H1 and [32P]NAD, about 25% of ADP-ribose newly incorporated was extractable with HClO₄ (experiment I).
Automodification of Poly(ADP-ribose) Synthetase

**Fig. 8. Pulse-chase of poly(ADP-ribose) bound to the synthetase.** Poly(ADP-ribose) synthetase (2 μg) was incubated at 37 °C in the standard mixture (1.4 ml) modified to contain 0.47 μM [³²P]NAD (39,000 cpm/pmol) (A) or nonradioactive NAD (B). After 1 min (arrow), an aliquot (300 μl) was removed, and the number (3.2 pmol, =0.18 mol/mol of enzyme) and the average chain size (2.8 ADP-ribose units) of the polymers were determined. To the remaining solution were added 60 μl of 1 mg/ml of histone H1 and 40 μl of 0.23 mM nonradioactive NAD (A) or [³²P]NAD (2.4 cpmp/mmol) (B), and the incubation was continued at 37 °C. Aliquots (200 μl each) removed at indicated times were examined for 20% CI:COOH-insoluble radioactivity in the total products (–) or in 5% HClO₄ extracts (△—△).

Second, a pulse-chase experiment was carried out, in which poly(ADP-ribose) synthetase was briefly incubated with [³²P]NAD, and polymers bound to the enzyme were chased by adding an excess amount of nonradioactive NAD and histone H1 (Fig. 8A). In the chase period for 20 min, the HClO₄-extractable radioactivity increased up to 5% of the total radioactivity incorporated into acid-insoluble material. This increase appeared to be explained by poly(ADP-ribosyl)ation of histone H1 using the [³²P]NAD diluted by nonradioactive NAD, because about the same amount of radioactivity was incorporated by incubation of the automodified enzyme with histone H1 and [³²P]NAD diluted to the same specific activity as above (Fig. 8B).

These results indicated that the automodified enzyme did not serve as an intermediate in poly(ADP-ribosyl) histone synthesis.

**DISCUSSION**

Automodification of poly(ADP-ribose) synthetase has been reported with purified enzymes of bovine thymus (16), calf thymus (17), and HeLa cells (15). Our present study showed that poly(ADP-ribose) synthetase of rat liver also catalyzed automodification, and, furthermore, that the modification took place at about 15 sites on the enzyme molecule, to each of which poly(ADP-ribose) as large as 80 ADP-ribose units long could be bound.

The stability studies of the linkage between poly(ADP-ribose) and the synthetase revealed that about 70% of the linkage was acid soluble but alkali labile and NH₂OH sensitive as was reported for the linkage in ADP-ribosyl histones previously (26-29). However, approximately 30% was shown to be NH₂OH resistant, and about 15% was resistant to alkaline cleavage. Heterogeneous NH₂OH sensitivity was first reported from this laboratory for ADP-ribosyl histones prepared with chromatin (30). Recently, further distinction has been made between alkali labile and stable bonds among the NH₂OH-resistant species (31). The origin of such heterogeneity is not known and has been tacitly assumed to be due to heterogeneity of either the acceptor (or acceptor site) or the enzyme or both. The present study using an apparently homogeneous preparation of poly(ADP-ribose) synthetase showed that the ADP-ribose-synthetase linkages were also heterogeneous. The chemical nature of these linkages remains to be elucidated.

Whether this automodification is an intramolecular or intermolecular event is not clear at present. We have obtained a preliminary result that a heat (60 °C, 5 min)-inactivated synthetase accepts as many polymers as the active enzyme does. This may suggest that the modification is, at least in part, intermolecular. As the automodification of the synthetase proceeded, Kₐ for NAD increased and Vₐₐₚ decreased. This partial inhibition by automodification appears to explain the nonlinearity of poly(ADP-ribose) synthesis observed with purified enzymes (13 and Fig. 5).

Yoshihara et al. (16) and Jump and Smulson (15) suggested that poly(ADP-ribose) turned over on the synthetase to produce free polymers. This suggestion was based on the finding that the number of polymers synthesized far exceeded that of the enzyme, while the average chain length remained constant upon long incubations. This estimation, however, needs re-examination because the chain length of branched poly(ADP-ribose) might be underestimated and the number overestimated by the conventional method they used (9). Our results demonstrated that the polymers produced on the synthetase had branch structures, and the number of polymers, corrected for branching, remained constant during the incubation from 20 to 60 min, while the chain size increased steadily. Another evidence presented by Jump and Smulson (15) for the turnover of poly(ADP-ribose) was a rapid loss of pulse-labeled polymers from the synthetase during the chase with unlabeled NAD. This observation also requires reinvestigation; they measured the radioactivity only in the gel region where unmodified synthetase electrophoresed. It seems possible that they missed the automodified enzyme which had an increased molecular weight and moved to other regions of the gels. Our preliminary experiments have suggested that poly(ADP-ribose) synthetases of calf thymus and HeLa cells undergo, like the rat liver enzyme, a remarkable increase in the molecular weight during automodification. These results, together with our failure to detect enzymic production of free polymers to significant extents, argue against the view that the automodified synthetase serves as an intermediate in the formation of free poly(ADP-ribose). Synthesis of free polymers observed in isolated nuclei (34) appears to be ascribable to nonenzymic hydrolysis or enzymic cleavage of poly(ADP-ribose) proteins by ADP-ribose histone hydrolase (18, 35) or unknown enzyme(s).

Purified poly(ADP-ribose) synthetase has been shown to catalyze initiation as well as elongation of poly(ADP-ribose) on histones and nonhistone proteins in in vitro reconstituted systems (25, 33). The present data showed no transfer of polymers from the automodified enzyme to histone H1, suggesting that the automodification is not an intermediate step in poly(ADP-ribose)ylation of other proteins. This feature may be comparable with that of rabbit muscle cAMP-dependent protein kinase; this enzyme autophosphorylates its own catalytic subunit, and the phosphate residue is not transferred to other protein substrates, including histone H1 (36).

Automodification of purified poly(ADP-ribose) synthetase does not appear to be an in vitro artifact. Our recent study (37) suggested that the synthetase was predominantly poly(ADP-ribose)ylated, probably at multiple sites, in human lymphocytes permeabilized to NAD. This study (37) also suggested a close correlation between the automodification and DNA repair. It is possible that poly(ADP-ribose) synthesis plays some crucial roles in the regulation of chromatin functions, not only as a catalyst modifying other proteins but also as a structural element in a poly(ADP-ribose)ylated form.
REFERENCES