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Evidence for Adenosine Diphosphate Ribosylation of Ca^{2+}, Mg^{2+}-dependent Endonuclease

(DNA synthesis/NAD)

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ABSTRACT **The molecular basis for the inhibition of the Ca^{2+},Mg^{2+}-dependent endonuclease resulting from the formation of poly(adenosine diphosphate ribose) (ADP-Rib) was studied in a simplified system containing purified rat liver or bull semen endonuclease, purified rat liver poly(ADP-Rib) synthetase, [^{3}H]NAD^+, and DNA. Poly(ADP-Rib) synthetase activity was stimulated when Ca^{2+}, Mg^{2+}-dependent endonuclease was added to the reaction mixture in place of histones, suggesting that the endonuclease can act as an acceptor for ADP-Rib. Evidence was presented to show that the ADP-Rib moiety of [^{3}H]NAD^+ was incorporated in the endonuclease fraction. The [^{3}H]ADP-Rib bound to the endonuclease was in the form of monomers and oligomers and not long chain polymers. The present results suggest that the Ca^{2+},Mg^{2+}-dependent endonuclease was ADP-ribosylated when the endonuclease was incubated with poly(ADP-Rib) synthetase and NAD^+.**

Poly(ADP-Rib) synthetase is located in the nucleus of eukaryotic cells and transfers the ADP-Rib moiety of NAD^+ to nuclear proteins to form a polymer (1, 2). The function of poly(ADP-Rib) in chromatin and ADP-ribosylation of nuclear protein has not been established. Studies on the synthetase activity and on the formation of the polymer in cells undergoing replication suggest that the enzyme might play a regulatory role in DNA synthesis (3–8).

Evidence was presented in previous reports (8, 9) to show that the inhibition of DNA synthesis induced by poly(ADP-Rib) formation can result from releasing DNA polymerase from chromatin and by blocking the activity of Ca^{2+}, Mg^{2+}-dependent endonuclease. Endonucleases possess the ability to activate the template (primer activity) of chromatin or nuclei for DNA synthesis (8, 10, 11), and might play a role in DNA synthesis of eukaryotic cells.

In the present paper, evidence will be presented to show that the Ca^{2+},Mg^{2+}-dependent endonuclease is directly ADP-ribosylated when it is incubated with poly(ADP-Rib) synthetase and NAD^+.

MATERIALS AND METHODS

NAD^+ (grade V) was purchased from Sigma Chemical Co., St. Louis, Mo.; [*adenosine-2-*^{3}H]NAD^+ was a gift of Dr. S. Shall, Sussex, England. [*Adenosine U-*^{14}C]NAD^+ and [*methyl-*^{3}H]thymidine were purchased from commercial sources. Rat liver DNA was prepared by the method of Kay *et al.* (12). Histones were prepared from isolated rat liver nuclei by extraction with 0.25 N HCl. DNA-cellulose was prepared with calf thymus DNA as described by Alberts and Herrick (13). It contained 570 μg of DNA per ml of packed cellulose.

Protein and DNA were determined as described by Lowry *et al.* (14) and Burton (15), respectively. Preparation of highly polymerized [^{3}H]DNA from *Escherichia coli* and [^{3}H]DNA gel, and the assays for poly(ADP-Rib) synthetase, preparation of chromatin, and the purification of rat liver poly(ADP-Rib) synthetase were described in previous reports (9, 16, 17). The specific activity of the synthetase was 208 units/μg of protein.

Purification of Endonucleases. The preparation of endonucleases from rat liver chromatin was described in a previous report (16). The acid Mg^{2+}-dependent endonuclease was extracted from chromatin with a buffer containing 5% glycerol (v/v), 50 mM Tris·HCl (pH 7.4), 2 mM EDTA, 0.1 M NaCl. The Ca^{2+},Mg^{2+}-dependent alkaline endonuclease was extracted from the remaining chromatin with the same buffer containing 0.35 M NaCl. For each mg of DNA in chromatin, 0.25 ml to 0.5 ml of buffer was used. The extracts containing the Ca^{2+},Mg^{2+}-dependent endonuclease obtained from crude chromatin (200 mg of DNA) were precipitated with ammonium sulfate. The material precipitated between 50 and 85% saturation was collected. The pellet was suspended in 3 ml of Medium A [20% glycerol, 50 mM Tris·HCl (pH 7.4), 2 mM EDTA, 2 mM 2-mercaptoethanol] and dialyzed against Medium A for 4 hr at 0°. The retentate was centrifuged at 2000 × *g* for 10 min. The supernatant was placed on a carboxymethyl-cellulose column (0.8 × 4-cm) equilibrated with Medium A. The column was washed with 10 ml of Medium A containing 0.8 M NaCl. The enzyme was eluted with Medium A containing 0.3 M NaCl. Fractions of 1 ml were collected and the active enzyme fractions were pooled. The pH of the pooled fraction was adjusted to 5.1 by the addition of 1.5 volume of 1 M sodium acetate buffer (pH 5.0). The mixture was incubated at 37° for 10 min. Following this incubation more than 95% of poly(ADP-Rib) synthetase activity was destroyed without affecting the endonuclease activity. The enzyme preparation was dialyzed against 1 liter of Medium A overnight and stored at −70°. The recovery of the enzyme from chromatin ranged from 10 to 29% in five separate experiments. A purification of 370-fold from chromatin was achieved. The specific

Abbreviations: ADP-Rib, adenosine diphosphate ribose; DNase, deoxyribonuclease; PRib-AMP, 2′-(5″-phosphoribosyl)-5′-AMP.

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TABLE 1. *Effect of nucleases and histones on rat liver poly(ADP-Rib) synthetase activity*

Proteins added	Uptake of [^{3}H]ADP-Rib (cpm)
Control	1245
Ca^{2+},Mg^{2+}-dependent endonuclease, rat liver	
0.14 μg (6 units)	2320
0.28 μg (12 units)	3353
0.70 μg (30 units)	4455
Mg^{2+}-dependent endonuclease, rat liver	
2.0 μg (10 units)	858
5.2 μg (25 units)	806
Histones, rat liver	
1.0 μg	2537
2.0 μg	3299
Control	
Ca^{2+},Mg^{2+}-endonuclease, bull semen	
0.6 μg (20 units)	1653
1.2 μg (40 units)	2562

The control system contained 10 μM[^{3}H]NAD (75,000 cpm/nmol), 10 mM $MgCl_2$, 50 mM Tris·HCl buffer (pH 8.0), 1 mM EDTA, and 2 μg of rat liver DNA in a total volume of 0.2 ml. The mixture was incubated for 10 min at 25°. The reaction was stopped by the addition of 2.5 ml of 10% ice-cold trichloroacetic acid. The amount of radioactivity in the precipitated material was determined.

activity of the purified enzyme was 22 units/μg of protein.

The Mg^{2+}-dependent acid endonuclease was purified from an 0.1 M NaCl extract of rat liver chromatin as described in a previous report (16) by precipitation with ammonium sulfate and by chromatography on a carboxymethyl cellulose column. A purification of 190-fold from chromatin was achieved. The specific activity of the purified enzyme was 4.8 units/μg of protein.

Ca^{2+},Mg^{2+}-dependent alkaline endonuclease was purified from 4 ml of bull semen which was obtained from Eastern Artificial Insemination Cooperative, Inc., Ithaca, N.Y. The seminal plasma was separated from sperm by centrifuging at 2000 × *g* for 10 min. To 3 ml of seminal plasma containing 240 mg of protein with endonuclease activity of 28 × 10^4 units, 12 ml of Medium A containing 0.1 M NaCl were added. The mixture was centrifuged at 105,000 × *g* for 60 min. The supernatant was chromatographed on a carboxymethylcellulose column and the column was eluted as described above for the rat liver enzyme. The eluted enzyme fraction was dialyzed against Medium A and stored at −70°. The recovery of enzymic activity was about 10% starting with seminal plasma. A purification of 17-fold from seminal plasma was achieved. The specific activity of the purified enzyme was 16.7 units/μg of protein.

Analysis of [^{3}H]ADP-ribosylated Endonuclease by Gel Filtration on Sephadex G-100 Column. A mixture containing appropriate amounts of Ca^{2+},Mg^{2+}-dependent endonuclease, 50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 10 μM [^{3}H]NAD^+ (8 × 10^7 cpm/20 nmol), 20 μg of rat liver DNA, and 50 units of poly(ADP-Rib) synthetase in a total volume of 1.5 ml was incubated at 25° for 10 min. The reaction was terminated by the addition of 2 ml of a solution containing 2 M NaCl, 10% glycerol, 0.2 M sodium acetate buffer (pH 5.0), 4 mM EDTA. The mixture (3.5 ml) was placed on a Sephadex G-100 column (2.8 × 34-cm) which was equilibrated with a medium containing 50 mM sodium acetate buffer (pH 5.0), 5% glycerol, 2 mM EDTA, 1 M NaCl. The column was eluted with the same medium. Fractions of 4 ml were collected.

RESULTS AND DISCUSSION

In a previous report (16) evidence was presented demonstrating that the Ca^{2+},Mg^{2+}-dependent endonuclease obtained from rat liver and bull seminal plasma was inhibited on incubation with NAD^+ and poly(ADP-Rib) synthetase. To establish that a direct ADP-ribosylation of the endonuclease did take place, partially purified Ca^{2+},Mg^{2+}-dependent endonuclease was incubated with poly(ADP-Rib) synthetase and [^{3}H]NAD^+ (Table 1). Since the ADP-Rib moiety of NAD^+ is linked covalently to nuclear proteins (1, 2) and histones must be present in the synthetase system for full activity (17), the endonuclease was tested for its ability to act as an acceptor protein for ADP-Rib. The addition of 1 and 2 μg of rat liver whole histones to the poly(ADP-Rib) synthetase assay system resulted in a stimulation of the enzymic activity of 2.5 to 3 times the control value (Table 1). With larger amounts of histones the stimulation was less dramatic. When rat liver or bull semen Ca^{2+}, Mg^{2+}-dependent endonuclease was added to the synthetase assay system in place of histones, the incorporation of [^{3}H]-ADP-Rib was markedly increased (Table 1). The synthetase activity increased in proportion to the amount of endonuclease added to the reaction mixture and the stimulatory potency of the endonuclease preparation was far greater than that of histones (Table 1). Hence, it is unlikely that the stimulatory capacity of the enzyme preparation can be attributed to histones as contaminants. The present results suggest that Ca^{2+},Mg^{2+}-dependent endonuclease can act as an acceptor for [^{3}H]ADP-Rib and replace histones in the assay system. On the other hand, the incorporation of [^{3}H]-ADP-Rib was not influenced when equivalent units of pancreatic DNase I or micrococcal endonuclease were added to the synthetase assay system. With Mg^{2+}-dependent endonuclease the incorporation of [^{3}H]ADP-Rib was less than the control values. The basis for this decrease in the incorporation is not clear.

An alternate explanation for the finding is that the endonuclease hydrolyzed the DNA into multiple small fragments, which in turn stimulated synthetase activity, since poly(ADP-Rib) synthetase requires DNA for activity (17). It was reported that treatment of isolated nuclei with appropriate amounts of DNase stimulated poly(ADP-Rib) synthesizing capacity (18), probably by increasing the formation of free DNA fragments. In the present study equivalent units of endonuclease and DNases were added to the assay system. Under these conditions a substantial amount of fragmented DNA will be produced by DNase I. However, only a slight amount will be formed by Ca^{2+},Mg^{2+}-dependent endonuclease because with EDTA present in the incubation medium, the endonuclease will be inactive. Furthermore, the addition of DNase I to the reaction mixture did not affect the synthetase activity. These findings do not support the argument that the observed stimulation was due to the production of DNA fragments by the Ca^{2+},Mg^{2+}-dependent endonuclease. It was noted, however, that when large amounts of deoxyribonuclease I (1200 units) or micrococcal nuclease (3200 units) were added to the assay system, the synthetase activity increased

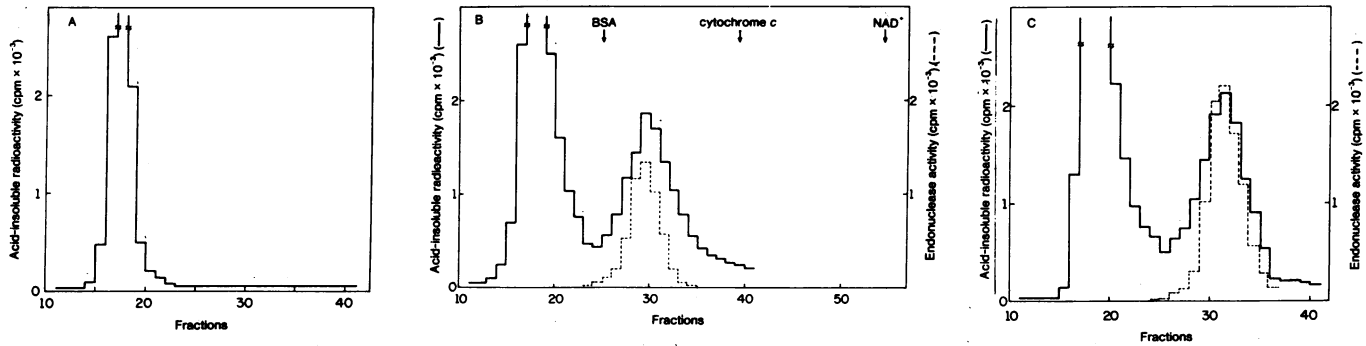

FIG. 1A–C. Sephadex G-100 gel filtration of reaction mixtures which contained poly(ADP-Rib) synthetase, [^{3}H]NAD$^+$, and Ca^{2+},Mg^{2+}-dependent endonuclease. (A) control (endonuclease omitted), (B) rat liver Ca^{2+},Mg^{2+}-dependent endonuclease, (C) bull semen Ca^{2+},Mg^{2+}-dependent endonuclease. Elution of bovine serum albumin (BSA) and other molecular weight markers is indicated in B.

by 1.5- and 2-fold, respectively (unpublished data). Similar stimulation of the synthetase activity was demonstrated by others (18, 19), when large amounts of DNase were used.

To establish that the ADP-Rib formed was associated or linked covalently to the endonuclease, the reaction mixtures containing purified poly(ADP-Rib) synthetase, [^{3}H]NAD$^+$ and endonuclease were subjected to gel filtration through a Sephadex G-100 column. With purified poly(ADP-Rib) synthetase and [^{3}H]NAD$^+$ in the reaction mixture, the void volume (fractions 5–20; Fig. 1A) contained all of the acid-insoluble radioactive material. This radioactive material was not hydrolyzed with 0.1 N NaOH and was found to consist of polymers of ADP-Rib. With the addition of rat liver or bull semen Ca^{2+},Mg^{2+}-dependent endonuclease to the reaction mixture, a second peak of radioactive material was eluted (Fig. 1B or C). The latter peak of radioactivity corresponded to those fractions containing endonuclease activity, which was determined by running a parallel experiment with untreated endonuclease (Fig. 1B). [^{14}C]NAD$^+$ used in place of [^{3}H]NAD$^+$ gave identical results. To determine whether or not NADase was able to transfer the ADP-Rib moiety of NAD$^+$ to the endonuclease, bull semen Ca^{2+},Mg^{2+}-dependent endonuclease was incubated with [^{3}H]NAD$^+$ and *Neurospora* NADase. Although NADase was capable of hydrolyzing 20% of the NAD$^+$ to [^{3}H]ADP-Rib, no radioactive material was incorporated into the endonuclease fraction. These results suggest that free ADP-Rib does not interact or bind with endonuclease.

Another finding which supports the contention that the radioactivity was associated with the endonuclease is that when fractions 26–33 from the Sephadex column (Fig. 1B), which contained the [^{3}H]NAD$^+$-treated endonuclease, were pooled and subjected to chromatography on a DNA-cellulose column (Fig. 2), the radioactive material was located in the fractions that contained the endonuclease. To establish that the radioactive material was linked to the enzyme, fractions containing [^{3}H]NAD$^+$-treated endonuclease obtained from the DNA-cellulose column were pooled. The enzyme was precipitated by the addition of trichloroacetic acid and the precipitate was collected. The radioactivity was released when the precipitate was treated with trypsin and Pronase but not with DNase or RNase, suggesting that [^{3}H]ADP-Rib was attached to proteins. The products of the proteolytic hydrolysis were completely acid-soluble, suggesting that the incorporated ADP-Rib was composed of monomers and oligomers rather than long chain polymers, which are acid-insoluble.

To identify the radioactive fragments attached to the enzyme, purified endonuclease incubated with [^{3}H]NAD$^+$ and poly(ADP-Rib) synthetase was subjected to acid and alkali treatment (Table 2), since poly(ADP-Rib) can be detached from histones when incubated in an alkaline medium (1). The finding that the radioactivity in [^{3}H]NAD$^+$-treated endonuclease was not released under acid conditions (Table 2) suggests that ADP-Rib might be linked covalently to endonuclease, as was established for histones (1, 2). On the other hand, about 95% of the radioactivity was found in the acid-soluble fraction when [^{3}H]NAD$^+$-treated endonuclease was incubated in 0.1 N NAOH at 25° for 20 min (Table 2). To avoid excessive degradation of ADP-Rib the [^{3}H]NAD$^+$-treated endonuclease was dissolved in 0.1 N KOH and left standing at 0° for 20 min. Under these milder conditions the degradation of [^{3}H]ADP-Rib was less than 3%. The radioactive materials released from [^{3}H]NAD$^+$-treated endonuclease after alkali treatment were analyzed by paper chromatography (Fig. 3). Oligomers and monomers of ADP-

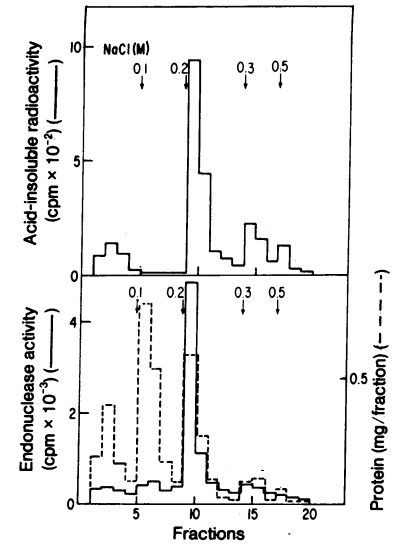

FIG. 2. Chromatography of [^{3}H]NAD$^+$-treated Ca^{2+},Mg^{2+}-dependent endonuclease on DNA-cellulose. Fractions 26–33 from the Sephadex column (Fig. 1B) were pooled and dialyzed against a solution containing 50 mM NaCl, 10% glycerol, and 20 mM Tris·HCl (pH 7.4). This enzyme preparation was adsorbed on a column consisting of about 5 ml of packed DNA-cellulose (see *Methods*). The column was washed with the above buffer and eluted stepwise with the indicated concentrations of NaCl in the same buffer. Aliquots of each fraction were used to measure trichloroacetic-acid-precipitable radioactivity, endonuclease activity, and proteins.

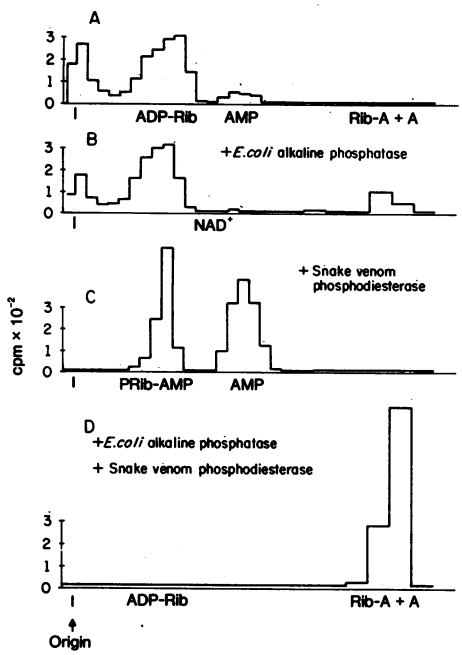

FIG. 3. Paper chromatograms of the radioactive material bound to [^{3}H]NAD-treated Ca^{2+},Mg^{2+}-dependent endonuclease. To 10 ml of [^{3}H]NAD-treated Ca^{2+}-Mg^{2+}-dependent endonuclease fraction (combined fractions 26–34 as shown in Fig. 1B) trichloroacetic acid was added to give a final concentration of 10%. The precipitate was collected on glass fiber filter. The filter was placed in 1 ml of 0.1 N KOH for 30 min at 0°. The alkaline solution was diluted with 5 ml of distilled water and centrifuged at 2000 × *g* for 5 min to sediment the fine glass fibers. The supernatant was collected, neutralized with 0.1 N perchloric acid, and centrifuged. The supernatant was concentrated by flash evaporation. Samples containing 2300 cpm, equivalent to 31 pmol of [^{3}H]ADP-Rib, were analyzed directly (control), or treated with *E. coli* alkaline phosphatase and/or snake venom phosphodiesterase and chromatographed using as a solvent system a mixture of isobutyric acid:NH_4OH:H_2O (66:1:33 by volume) according to a standard procedure (20). The positions of authentic markers are indicated under each figure. Rib-A + A is ribosyladenosine and adenosine. (A) untreated sample (control), (B) treated with *E. coli* alkaline phosphatase (20 μg of protein per 0.1 ml) in 50 mM Tris·HCl (pH 8.0) at 37° for 10 min, (C) treated with snake venom phosphodiesterase (10 μg/0.1 ml) in 50 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, at 37° for 10 min, (D) treated with both snake venom phosphodiesterase (10 μg/0.1 ml) and *E. coli* alkaline phosphatase (20 μg/0.1 ml) in 50 mM Tris·HCl, (pH 8.0), 5 mM $MgCl_2$, at 37° for 10 min.

Rib and a small amount of AMP were found (Fig. 3A). The R_F of the major radioactive peak coincided with that of the ADP-Rib marker. Oligomers of ADP-Rib remained at the origin with the chromatographic system used. The migrations of ADP-Rib and 2′-(5″-phosphoribosyl)-5′-AMP (PRib-AMP) were very close. Hence, to differentiate them, PRib-AMP was dephosphorylated by treatment with *E. coli* alkaline phosphatase. The product of this treatment, ribosyladenosine, migrated in the present chromatographic system like adenosine and moved very close to the solvent front (20). On the other hand, ADP-Rib was not affected by the treatment with alkaline phosphatase and migrated to the same position (Fig. 3B). The small peak of radioactivity found at the position of AMP, after treatment with alkaline phosphatase, moved close to the solvent front together with adenosine (Fig. 3B). PRib-AMP and AMP were formed when the alkali-released material from [^{3}H]NAD$^+$-treated endonuclease was incubated with venom phosphodiesterase (Fig. 3C); whereas ribosyladenosine and adenosine were formed when the materials were treated simultaneously with alkaline phosphatase and phosphodiesterase (Fig. 3D). After this treatment all the radioactivity moved close to the solvent front. The present results are in agreement with the evidence presented by Sugimura (2) for the elucidation of the structure of poly(ADP-Rib) and establish the fact that the radioactivity in [^{3}H]NAD$^+$-treated endonuclease was due to incorporated [^{3}H]ADP-Rib. Moreover, this contention is supported by the finding that [^{3}H]ADP-Rib was the major compound liberated on treatment of the alkali-released materials with rat testis glycohydrolase (unpublished data).

The average chain length of the polymers attached to the endonuclease can be calculated from the relative amounts of PRib-AMP formed on hydrolysis with phosphodiesterase (2). The mean length was estimated to be about 1.8 to 2.0 residues per chain, suggesting that the ADP-Rib incorporated in [^{3}H]NAD$^+$-treated endonuclease was in the form of monomers and short chains.

To date, the enzyme system capable of hydrolyzing the initial ADP-Rib attached to protein has not been discovered. Although rat testis poly(ADP-Rib) glycohydrolase can completely hydrolyze free polymers, it was capable of hydrolyzing only 45–50% of the [^{3}H]ADP-Rib incorporated in [^{3}H]-NAD$^+$-treated Ca^{2+},Mg^{2+}-dependent endonuclease (unpublished data). Furthermore, the glycohydrolase-treated endonuclease remained inhibited, suggesting that ADP-ribosylation of the enzyme or attachment of single residues of ADP-Rib to the endonuclease can account for the inhibitory effect and that formation of long chains of polymers is not a prerequisite for the blocking phenomenon.

Since the radioactivity incorporated into [^{3}H]NAD-treated endonuclease was released when the enzyme was incubated in alkaline medium (pH 10–10.5, Table 2), NAD$^+$-treated endonuclease (0.35 N NaCl extract of rat liver chromatin treated with NAD$^+$) was incubated in a medium (pH 10.3) containing 30% glycerol for 17 hr at 0° (Table 3). Under this experimental condition the activity of the untreated control enzyme remained unchanged and polymers of ADP-Rib were stable.

TABLE 2. *Effect of pH on the release of radioactivity from rat liver Ca^{2+},Mg^{2+}-dependent endonuclease treated with [^{3}H]NAD*

Incubation media	pH	Acid-insoluble radioactivity (cpm)
Control	—	1764
0.1 N HCl	—	1228
acetate	5.0	1230
potassium phosphate	7.0	1165
Tris·HCl	8.0	1003
Tris·HCl	9.0	922
Glycine·NaOH	10.0	395
Glycine·NaOH	10.5	340
0.1 N NaOH	—	72

[^{3}H]NAD$^+$-treated Ca^{2+},Mg^{2+}-dependent endonuclease purified by filtration through Sephadex G-100 (Fig. 1B) was incubated in the respective media for 20 min. The amount of radioactivity in the material precipitated with trichloroacetic acid was measured.

TABLE 3. *Reactivation of NAD$^+$-treated Ca^{2+},Mg^{2+}-dependent endonuclease*

Incubation media	NAD$^+$ Treatment	Endonuclease activity (cpm) Initial	Endonuclease activity (cpm) After 17 hr	Acid-insoluble radioactivity* (cpm) Initial	Acid-insoluble radioactivity* (cpm) After 17 hr
Glycine·NaOH (pH 10.3)	−	6517	6410	1945	2013
	+	740	2067	2183	1948
Tris·HCl (pH 7.4)	−	4882	4820	—	—
	+	760	772	—	—

The reaction mixtures containing [^{3}H]NAD-treated endonuclease were incubated at 0° for 17 hr in the designated media. The values are from a representative run of four separate experiments. All media contained 30% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol.

* As a control to test their stability, polymers of [^{14}C]ADP-Rib were used in place of [^{3}H]NAD-treated endonuclease.

The activity of the alkali-treated endonuclease was at least 3-fold greater than that of the inhibited enzyme and equivalent to about 30–40% of the original control enzymic activity (Table 3). The present results suggest that the observed reactivation of the NAD$^+$-treated endonuclease is due to a dissociation of ADP-Rib from the enzyme and not due to a hydrolysis of polymers of ADP-Rib or to a shortening of polymer chains bound to the enzyme. Although extensive studies were carried out to restore the enzymic activity, the maximum recovery achieved was about 40% of the original control activity.

Yamada *et al.* (21) reported that polymers of ADP-Rib inhibited deoxyribonuclease activity. However, the quantities of polymers used in their study were in excess and other homopolymers were more potent inhibitors of Ca^{2+},Mg^{2+}-dependent endonuclease than poly(ADP-Rib) (16).

The inhibitory effect of poly(ADP-Rib) on the activity of DNA synthesis is variable and depends on the tissue under study. The template activity of Novikoff hepatoma cell nuclei for DNA synthesis was not affected when the nuclei were incubated with NAD$^+$ (22). Furthermore, Roberts *et al.* (23) observed that on incubation of HeLa cell nuclei with NAD$^+$ the template activity was stimulated.

The evidence presented in this report strongly suggests that the ADP-Rib moiety of NAD$^+$ is transferred to Ca^{2+},Mg^{2+}-dependent endonuclease when the enzyme is incubated with NAD$^+$ and poly(ADP-Rib) synthetase. It could be argued that the endonuclease obtained from rat liver chromatin contained some impurities which were ADP-ribosylated and that these complexes inhibited the endonuclease. A premise of such a hypothesis is that the same impurities must exist in bull semen endonuclease preparation, since this enzyme was also ADP-ribosylated and its activity inhibited. We are of the opinion that the evidence presented supports the former contention that direct ADP-ribosylation of the Ca^{2+},Mg^{2+}-dependent endonuclease occurred with a concomitant inhibition of the enzyme activity. With a homogeneous preparation of the bull seminal plasma endonuclease, the problem of impurities might be resolved definitively.

It can be concluded from the present study that the ADP-Rib moiety of NAD$^+$ is transferred to Ca^{2+},Mg^{2+}-dependent endonuclease when the enzyme is incubated with NAD$^+$ and poly(ADP-Rib) synthetase. ADP-ribosylation might be a general mechanism for the regulation of enzymic activities. Diphtheria toxin catalyzes the transfer of the ADP-Rib moiety of NAD$^+$ to elongation factor 2 or transferase II (24, 25). As a consequence of ADP-ribosylation the enzyme is inactivated, which could account for the inhibition of protein synthesis by diphtheria toxin (24, 25). Recently, two separate groups (26, 27) showed that after infection of *E. coli* with bacteriophage T_4 the DNA-dependent RNA polymerase was modified by ADP-ribosylation of the two α-subunits.

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