POLY ADENOSINE DIPHOSPHATE RIBOSE SYNTHESIS 
AND NICOTINAMIDE ADENINE DINUCLEOTIDE 
TRANSGLYCOSIDASES

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1. Enzymic Synthesis of Poly ADP-ribose from NAD

Recent findings from several laboratories including our own indicate that an 
enzyme fraction obtained from mammalian nuclei catalyzes a direct polymerization 
of the ADP-ribose portion of NAD with the simultaneous release of nicotinamide 
(1–6). In a typical experiment shown in Table 1, when NAD was incubated with

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount of incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD-[adenine-8]-\textsuperscript{14}C</td>
<td>4.50</td>
</tr>
<tr>
<td>NAD-[adenosine-U]-\textsuperscript{14}H</td>
<td>4.75</td>
</tr>
<tr>
<td>NAD-[AMP phosphate]-\textsuperscript{32P}</td>
<td>4.94</td>
</tr>
<tr>
<td>NAD-[both phosphates]-\textsuperscript{32P}</td>
<td>4.86</td>
</tr>
<tr>
<td>NAD-[NMM phosphate]-\textsuperscript{32P}</td>
<td>4.73</td>
</tr>
<tr>
<td>NAD-[ribose-U in NMM]-\textsuperscript{14}C</td>
<td>4.84</td>
</tr>
<tr>
<td>NAD-[nicotinamide-7]-\textsuperscript{14}C</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The reaction mixture contained 50 \textmu m moles of NAD indicated, 7.5 \textmu m moles of MgCl_2, 
1 \textmu m mole of NaF, 1 \textmu m mole of 2-mercaptoethanol, 15 \textmu m moles of KCl. 25 \textmu m moles of 
Tris-HCl buffer, pH 7.4 and the enzyme preparation in a total volume of 0.25 ml. 
After incubation for 15 minutes at 37\degree, the reaction was stopped by the addition 
of 5 ml of 5% trichloroacetic acid. The mixture was transferred to a Millipore 
filter (pore size, 0.45 \mu); the filter was washed five times with 10 ml aliquots of 5% 
trichloroacetic acid, pasted on an aluminum planchet, and dried. The radioactivity 
determined with a Geiger-Muller gas flow counter (Nuclear-Chicago). \textsuperscript{3}H-samples 
were determined with a Packard Tri-Carb liquid scintillation spectrometer. The 
methods of preparation of these substrate were described previously [5].

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\textsuperscript{5} Recipient of a Postgraduate Fellowship from the Sigma Chemical Company.
a nuclear enzyme preparation, equimolar quantities of all components except the nicotinamide moiety of NAD were incorporated into acid-precipitable material. Comparative studies on this reaction and the nicotinamide mononucleotide-dependent ATP polymerization, originally described by Chambon, Weill and Mandel (7) and later confirmed partly by Fujimura and Sugimura (8), have revealed that both reactions are fundamentally identical and that NAD but not ATP is the immediate substrate for the polymerization reaction (5). As already described by Dr. Mandel and Dr. Sugimura in this symposium, the product is made acid-soluble to produce a single compound upon treatment with venom phosphodiesterase. In addition, experiments with various double-labeled NADs as substrates indicate that ratios of the radioactivities of various portions of NAD in the original substrates are maintained in the acid-precipitable material as well as in the phosphodiesterase product (Table 2). The results indicate that the phosphodiesterase product is an isomer of ADP-ribose which is derived directly from the substrate, NAD. By

<table>
<thead>
<tr>
<th>Radioactive NAD (double labeled)</th>
<th>Ratio of</th>
<th>Ratio of radioactivities in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate</td>
<td>Acid-insoluble product</td>
</tr>
<tr>
<td>NAD-[adenine-8]-^{14}C and-[AMP]-^{32}P</td>
<td>^{14}C : ^{32}P</td>
<td>6.34</td>
</tr>
<tr>
<td>NAD-[adenine-8]-^{14}C and-[NMN]-^{32}P</td>
<td>^{14}C : ^{32}P</td>
<td>1.88</td>
</tr>
<tr>
<td>NAD-[adenosine]-^{3}H and-[ribose-U in NMN]-^{14}C</td>
<td>^{3}H : ^{14}C</td>
<td>2.00</td>
</tr>
</tbody>
</table>

The reaction mixture (2.5 ml) contained 300 µmoles of doubly labeled NAD as indicated, 75 µmoles of MgCl₂, 10 µmoles of NaF, 10 µmoles of 2-mercaptoethanol, 150 µmoles of KCl, 250 µmoles of Tris-HCl buffer, pH 7.4, approximately 4 mg of the nuclear enzyme protein. After the mixture was incubated for 20 minutes, the reaction was stopped by the addition of 20 ml of 2% perchloric acid. The precipitate was collected by centrifugation, and was washed several times at 0-4°C until no more radioactivity was found in the washings. The precipitate was suspended in 5 ml of water and neutralized with 2 N KOH, and the suspension was dialyzed overnight. The product was then digested with purified venom phosphodiesterase and the resulting acid-soluble materials were separated by Dowex 1 formate column chromatography.

**FIG. 1** Polymerization of NAD and Structure of Poly ADP-ribose
chemical, physical and enzymic analyses we have reached to the same conclusion that the reaction product is a new macrohomopolymer which is composed of repeating ADP-ribose units (Fig. 1). Since the identification of structure as well as the properties of this macrohomopolymer have been discussed in great detail by the preceding speakers, the nature of this enzyme will be briefly described here.

2. Association of Poly ADP-ribose Synthesis with Deoxyribonucleoprotein

The enzymic activity responsible for the poly ADP-ribose synthesis is essentially localized in the nucleus of various mammalian and other vertebrate tissues and organs. In addition, the activity appears to be tightly associated with DNA. Microorganisms thus far tested do not possess the activity including Escherichia coli, Pseudomonas fluorescens, Lactobacillus casei and Neurospora crassa. When rat liver nuclei were fractionated into various components by a slight modification of the method described by Marushige and Bonner (9), more than 95% of the total activity was recovered in the deoxyribonucleoprotein complex as shown in Table 3. When the complex was mixed with a Cs₂SO₄ solution and was centrifuged for 72 hours, a major portion of DNA appeared as a single peak at a density of 1.34 which carried less dissociable protein and was slightly lighter than pure DNA (Fig. 2). The enzymic activity appeared along with the main peak of this gradient and was not detected in a protein fraction. All RNA's were precipitated to the bottom under these conditions, and denatured protein was floating as a skin at the top of the gradient.

![Table 3](Intranuclear Distribution of Enzymic Activity of Poly ADP-ribose Synthesis)

<table>
<thead>
<tr>
<th>Nuclear fraction</th>
<th>Enzymic activity per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble fraction</td>
<td>0</td>
</tr>
<tr>
<td>Ribosomal fraction</td>
<td>3</td>
</tr>
<tr>
<td>Deoxyribonucleoprotein fraction</td>
<td>95</td>
</tr>
<tr>
<td>Nucleonemata (^a)</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Ribonucleoprotein network and membrane fraction.

Fig. 3 shows further an association of the enzyme with DNA. The deoxyribonucleoprotein complex was chromatographed on a Sephadex G-200 column in the presence of a high salt concentration as described by Georgiev et al. (10). Ammonium sulfate was employed as a dissociating agent of histone from DNA because of its profitable properties to be described later. As shown in Fig. 3, the activity of poly ADP-ribose synthesis was recovered exclusively in the void volume consisting mainly of DNA and not detected in the dissociated protein fraction. In addition, the polymer prepared in vitro appeared to be tightly associated with DNA as judged by gel filtration technique as well as by sucrose density gradient analysis. Further studies of this association have revealed that the polymer was not covalently linked to DNA, however, but was bound to DNA through some protein molecule since treatment with either sodium lauryl sulfate or proteinase prompted the release of polymer from DNA. These results suggest that the polymer synthesis is closely related to deoxyribonucleoprotein complex especially with DNA. In
The poly ADP-ribose synthetic activity was assayed with NAD-(adenine-8)-$^{14}$C under the conditions described in Table 1. •—•, enzymic activity; —, optical density at 260 m$\mu$; ... protein.

In fact, the activity of polymer synthesis was almost completely abolished by a prior treatment with pancreatic DNase but not with RNase. The exact mechanism of DNA participated in this reaction must be explored by further investigations. Although the activity appeared to be tightly associated with deoxyribonucleoprotein complex, the preparative method employed here did not distinguish the nucleolar and extranucleolar chromatins. Indeed, the NAD pyrophosphorylase, which has been generally accepted to be a nucleolar enzyme (11), was recovered consistently in this fraction. Dr. Mandel has proposed in this symposium that the activity is mainly localized in the nucleoli. The exact localization of this enzyme within the nucleus has remained to be established.

It has been reported that ammonium sulfate increases the RNA synthesis by isolated mammalian nuclei (12) and also by a DNA-RNA polymerase complex (13). The stimulation has been tentatively ascribed to the removal of histone from DNA, which is exposed to RNA polymerase as a template. Using crude deoxyribonucleoprotein complex obtained from rat liver, the activities of poly ADP-ribose and
RNA syntheses were assayed in the presence of an increasing amount of ammonium sulfate. As shown in Fig. 4, the template activity for RNA synthesis is stimulated markedly by lower concentrations of ammonium sulfate. In contrast, the polymer synthesis is strongly inhibited under these conditions, but again increases at higher salt concentrations with the maximum at 1.6 M, and an inverse relation is observed between RNA and polymer synthesis. The result suggests that the polymer synthesis is closely related to the structure of deoxyribonucleoprotein complex. The decrease of poly ADP-ribose synthesis at lower ionic strength and stimulation of RNA synthesis were observed with many other salts, but the increase of polymer synthesis at higher ionic strength was observed only with sulfate and phosphate ions irrespective of pairing cations. Detailed properties as well as the mechanism of this salt effect will be described elsewhere.

FIG. 4  Effects of Ammonium Sulfate Concentrations on RNA and Poly ADP-ribose Syntheses with Crude Deoxyribonucleoprotein Complex

The enzymic activities were assayed in the presence of salt as indicated. Poly ADP-ribose synthesis was determined as described in Table 1. RNA synthesis was determined as described by Widnell and Tata (12). Relative viscosity was measured using a Ostwald viscosimeter. Solubility was determined by measuring the optical density of supernatant solutions at 260 mµ after centrifugation for 10 minutes at 20,000 × g in the presence of salt as indicated. ○—○, poly ADP-ribose synthesis; ○⋯○, RNA synthesis; —, viscosity of reaction mixture; ⋯, solubility of deoxyribonucleoprotein complex.

3. Comparison of Poly ADP-ribose Synthesis and NAD Transglycosidases

Little attention has been paid to the fact that nicotinamide ribose linkage of NAD is a high energy bond which contained approximately 8 kilocalories (14). This has been recognized first by Kaplan and his coworkers, who have also established that a single protein of mammalian NADase shows two different catalytic
activities: NAD-transglycosidase and NAD-hydrolase activities (14,15). As shown in Fig. 5, the enzyme reacts with NAD to produce an ADP-ribose-enzyme complex as an intermediate with a concomitant release of nicotinamide. A subsequent transfer of the ADP-ribose grouping to some other pyridine derivatives such as acetyl-pyridine, thionicotinamide, nicotinate and nicotinamide itself results in the formation of NAD analogues (NAD-transglycosidase activity). This reaction has been referred also to as an exchange reaction. When the ADP-ribose portion is transferred to water, a simple hydrolysis of NAD will be observed (NAD-hydrolase activity).

![Diagram of NAD and ADP-ribose reactions](image)

**Kaplan et al., 1953**

*Fig. 5 Reactions Catalyzed by Mammalian NADase*

When the rat liver was fractionated into various subcellular components, a major portion, more than 95%, of the total cellular NADase activity is localized in the endoplasmic membrane. This NADase will be referred to as Cytoplasmic NADase. The deoxyribonucleoprotein complex mentioned above contained approximately 2-3% of the total NADase activity. The NADase associated with the complex will be referred tentatively to as Nuclear NADase. The poly ADP-ribose synthesis may be related to an NADase type reaction, since nicotinamide is released during the polymerization and an ADP-ribose unit is transferred successively to the adenosine-ribose of another ADP-ribose unit (Fig. 1). Subsequent studies were undertaken, therefore, to compare the polymer synthesis with cytoplasmic as well as nuclear NADase reactions, and to explore further the mechanism of poly ADP-ribose synthesis.

The activity of polymer synthesis is strongly inhibited by various pyridine derivatives which are well known as inhibitors of NADase. For examples, nicotinamide and isonicotinic acid hydrazide inhibit these reactions as shown in Fig. 6. The effects of inhibitors on these reactions are very similar, although a more profound effect of nicotinamide on the polymer synthesis is observed. The inhibition of NADase by nicotinamide is due to an exchange of the nicotinamide portion of NAD with exogeneously added nicotinamide (14). In fact, as shown in Fig. 7, both cytoplasmic and nuclear NADases can catalyze the exchange reaction and
FIG. 6 Effects of Nicotinamide and Isonicotinic Acid Hydrazide on Cytoplasmic and Nuclear NADases and Poly ADP-ribose Synthesis

Poly ADP-ribose synthesis was assayed with NAD-(adenine-8)-14C as described in Table 1. The NADase activity was assayed enzymically by measuring the decrease of NAD with alcohol dehydrogenase. These activities were determined in the presence of either nicotinamide or isonicotinic acid hydrazide as indicated. INAH represents isonicotinic acid hydrazide.

FIG. 7 Exchange Reaction of the Nicotinamide Portion of NAD with Exogeneously Added Nicotinamide

The reaction mixture (0.25 ml) contained 50 μmoles of non-radioactive NAD, 25 μmoles of Tris-HCl buffer (pH 7.4 with nuclear NADase, and pH 6.5 with cytoplasmic NADase), the enzyme preparation, and either nicotinamide-7-14C or nicotinic acid-7-14C as indicated. After incubation for 15 minutes at 37°, the reaction was stopped by heating for one minute.
in a boiling water bath. The mixture was cooled, centrifuged, and an aliquot of the supernatant was chromatographed on paper with 1 M ammonium acetate, pH 5.0-ethanol (3:7) as solvent. The radioactivity incorporated into NAD or deamido-NAD was determined by direct paper strip counting with a Packard Tri-Carb liquid scintillation spectrometer.

Fig. 8 Effect of Substrate Concentration on the Velocity of Poly ADP-ribose Synthesis and NADase Reactions

The NADase activity was determined by measuring the formation of radioactive nicotinamide with NAD-(nicotinamide-7)-14C as substrate. The radioactive nicotinamide was isolated by paper chromatography and was determined as described in Fig. 7.

Fig. 8 shows also the similarities of the polymer synthesis and NADase activities. It shows effects of concentration of the substrate on the velocity of the reactions. The results indicate that both polymer synthesis and nuclear NADase reactions show a similar affinity for the substrate. The Km value for the nuclear reaction is approximately $2.5 \times 10^{-4} \, M$ and is slightly higher than that for the cytoplasmic NADase reaction ($1.7 \times 10^{-4} \, M$). Cytoplasmic NADase shows the maximal activity at pH 6.4 in contrast to nuclear NADase which is most active at pH 7.4.

Table 4 shows substrate specificities for these reactions. The cytoplasmic NADase splits NADP as well as NAD and its analogues. The nuclear NADase, however, does not react with NADP, which is also inactive for the polymer synthesis. Acetyl pyridine-NAD shows only negligible activity for the nuclear reactions.

These results indicate that at least three different enzymes with NADase activity can be distinguished in rat liver: one is localized in the cytoplasm, and the others are in the nucleus. The cytoplasmic enzyme catalyzes the exchange as well as hydrolytic reactions, as already described by Kaplan and his coworkers (14,15).
FIG. 9. Various Reactions Catalyzed by Mammalian NADases

TABLE 4

Substrate Specificities for Poly ADP-ribose Synthesis and NADase Reactions

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Poly ADP-ribose synthesis</th>
<th>Nuclear NADase</th>
<th>Cytoplasmic NADase</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NADP</td>
<td>0</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>Deamido-NAD</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetylpyridine-NAD</td>
<td>4</td>
<td>4</td>
<td>35</td>
</tr>
<tr>
<td>Thionicotinamide-NAD</td>
<td>50</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

The poly ADP-ribose synthesis was assayed under the conditions described in Table 1, except 25 μmoles of the radioactive substrate indicated. NADase activity was assayed, enzymically by measuring the decrease of substrate employed with either alcohol dehydrogenase (with NAD, acetylpyridine-NAD and thionicotinamide-NAD as substrates) or glucose 6-phosphate dehydrogenase (with NADP as substrate). With deamido-NAD-(nicotinate-7)\(^{14}\)C as substrate the activity was determined by measuring the release of radioactive nicotinate. NADP-(adenine-8)\(^{14}\)C was prepared from NAD-(adenine-8)\(^{14}\)C with NAD kinase purified from pigeon liver. Acetylpyridine-NAD-(adenine-8)\(^{14}\)C and thionicotinamide-NAD-(adenine-8)\(^{14}\)C were prepared from NAD-(adenine-8)\(^{14}\)C by exchange reaction with beef spleen NADase. The detailed procedures of these preparations will be described elsewhere.

The second enzyme is localized in the nuclei and is rather tightly associated with deoxyribonucleoprotein complex. The latter enzyme catalyzes the hydrolysis and exchange reaction. However, it shows slightly different properties from the cytoplasmic enzyme. It does not react with NADP, and has a slightly lower affinity for NAD and the maximum activity at higher pH. The third enzyme is also associated with the deoxyribonucleoprotein complex and catalyzes the new polymer synthesis with a concomitant release of nicotinamide. Comparative studies have revealed that nuclear NADase and poly ADP-ribose synthesis show very similar properties. In fact, the possibility might be considered whether a single enzyme is responsible for the polymer synthesis in addition to NAD hydrolysis and exchange reaction, and whether the polymer synthesis might be one functional aspect of NAD-transglycosidase activity of NADase present in the nucleus. More purified enzyme preparations will be used to examine the mechanism of the polymer synthesis and to elucidate the precise role of nuclear NADase played in this reaction.
Addendum — Bock, K.W., Kronau, R., Grunicke, H., and Holzer, H. have made a similar observation that an NADase obtained from rat liver nuclei by differential centrifugation does not hydrolyze NADP. We are grateful to Dr. Hans Grunicke for making the abstract available to us which presented at the Federation Meeting of the European Biochemical Society held at Oslo, July, 1967. During the course of this study, Roemer and his associates (16) have also distinguished two different NADases: one is in the cytoplasm and the other is in the nucleus.

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REFERENCES