Synthesis of Polyadenosine Diphosphate Ribose by Isolated Nuclei of Swine Aortic Tissue†

K. Janakidevi* and Choon Koh

ABSTRACT: Properties of nuclear poly(ADPR) synthesizing enzyme from intima plus media of swine aortic tissue are described. The synthesis of poly(ADPR) by isolated nuclei is stimulated by the addition of various polynucleotides, the most effective being the synthetic polymer, poly[d(A-T)], and native calf thymus DNA. Although high concentrations of pancreatic DNase inhibit this reaction, lower nuclease concentrations exert a significant stimulatory effect on the incorporation of NAD. The DNase treatment or addition of exogenous polynucleotides appears to effect the elongation of the poly(ADPR). The inhibition of the stimulated activity by histones, specifically the lysine-rich histones, seems to indicate that regions of DNA rich in adenine and thymine are essential for the activity. A role for poly(ADPR) polymerase in regulating DNA synthesis could be envisaged as involving competition for DNA.

Poly(ADPR)[1] polymerase has been reported from several kinds of eukaryote nuclei (Chambon *et al.*, 1963; Nishizuka *et al.*, 1967; Fujimura *et al.*, 1967), and catalyzes the polymerization of ADPR moieties of NAD to form a homopolymer that is covalently linked to nuclear proteins (Nishizuka *et al.*, 1969). In addition to an acceptor protein that it ribosylates, the enzyme also requires DNA for optimum activity. Although the precise role of the enzyme and its product is not clearly understood, it has been shown to be related to DNA synthesis and to the maintenance of chromatin structure (Hilz and Kittler, 1971; Sugimura, 1973). Burzio and Koide (1970, 1971) have reported that nuclei preincubated with NAD showed a significant inhibition of DNA polymerase activity. Smulson *et al.* (1971), using synchronized HeLa cells, showed an inverse relationship between activities of DNA polymerase and poly(ADPR) polymerase during the cell cycle. Recent findings suggest, however, that there may be a positive correlation between poly(ADPR) synthesis and DNA synthesis (Colyer *et al.*, 1973). These observations, as well as reports that nicotinamide nucleotide concentrations are low in a variety of transplantable and induced tumors (Jedeikin and Weinhouse, 1955; Briggs, 1960; Clark *et al.*, 1966), prompted us to study poly(ADPR) polymerase in aortic smooth muscle cells, in order to understand the role of this enzyme in the control of DNA synthesis and cell proliferation of smooth muscle cells during atherosclerosis.

Materials and Methods

ATP, calf thymus DNA, histones (type IV and V), nicotinamide, NMN, NAD, and thymidine were purchased from Sigma Chemical Co., St. Louis, Mo. Poly[d(A-T)] and poly(dG)·poly(dC) were obtained from Miles Laboratories, Elkhart, Ind. Pancreatic DNase, venom phosphodiesterase, and DPNase (=NADase) were bought from Worthington Chemical Co., Freehold, N.J.; Spectrafluor, Triton X-100, and [*adenine*-U-^{14}C]NAD were purchased from Amersham/Searle, Chicago, Ill.

Nuclei were prepared from intima plus media of swine aorta as previously described (Janakidevi, 1972). Activated DNA was prepared according to Loeb (1969). The standard assay system contained 15 μmol of $MgCl_2$, 2.5 μmol of dithiothreitol, 15 μmol of KCl, 2 μmol of NaF, 50 μmol of Tris-HCl buffer (pH 8.0), 0.1 μCi of [^{14}C]NAD (specific activity 136 Ci/mol), and nuclear suspension containing 100–200 μg of protein, in a total volume of 0.5 ml.

In most of our experiments, we used a protein concentration averaging about 143 μg with a standard deviation of 21. In any particular experiment the protein concentrations are the same and the variations are between different nuclear preparations. NAD incorporations in these various nuclear preparations fall within the standard deviations given in the tables. Generally the incorporation is a function of nuclear protein concentration up to about 100 μg of protein after which a plateau is observed and above 200-μg levels there is usually a small drop probably due to increase in hydrolases. Variations from this standard assay system are indicated in the appropriate tables or figures. The mixture was incubated at 37° and the reaction terminated with ice-cold 10% Cl_3CCOOH containing 0.04 M sodium pyrophosphate. The precipitate collected by centrifugation was repeatedly washed with ice-cold 5% Cl_3CCOOH to remove all acid-soluble radioactivity. The final pellet was dissolved in 0.2 N NaOH. One aliquot was diluted to 1.0 ml with water, 10 ml of a cocktail (Triton–toluene–Spectrofluor (521:1000:42)) was added, and the sample was counted in Nuclear-Chicago Isocap 300. Protein was determined in a second aliquot by the method of Lowry *et al.* (1951). Specific activity was expressed as pmol of NAD incorporated/mg of protein. All assays were done in triplicate and the values are presented as means and standard deviations.

Results

As shown in Table I, incubation of aortic nuclei with NAD labeled in the adenine moiety results in the incorporation of radioactivity into an acid-insoluble fraction. This incorporation can be virtually eliminated by using a nuclear preparation heated for 5 min at 100°. Mg^{2+} and a sulfhydryl compound (dithiothreitol) are essential for the activity. Thymidine

† From the Department of Pathology and Specialized Center of Research in Atherosclerosis, Albany Medical College, Albany, New York 12208. *Received October 25, 1973.* Supported by U. S. Public Health Service Grant HL 14177.

[1] Abbreviations used are: ADPR, adenosine diphosphate ribose; DNase, pancreatic deoxyribonuclease; NAD, nicotinamide adenine dinucleotide.

TABLE I: Requirements for Poly(ADPR) Synthesis.[a]

Conditions	Specific Activity
Complete	159 ± 28
Minus $MgCl_2$	15 ± 3.1
Minus dithiothreitol	25 ± 5.2
Boiled enzyme blank	0
Zero time blank	0
Plus nicotinamide	
2.5 mM	5 ± 0.75
5.0 mM	3 ± 1.2
10 and 20 mM	0
Plus thymidine	
2.5 mM	7.1 ± 1.8
5.0 mM	4.2 ± 1.5
10 and 20 mM	0
Plus NADase (0.025 unit)	5.2 ± 0.51

[a] Aortic nuclei were incubated for 30 min at 37°.

and nicotinamide are potent inhibitors, and concentrations as low as 2.5 mM result in a 90–95% inhibition of NAD incorporation. The inclusion of NADase in the assay medium abolishes the activity almost totally.

Figure 1 illustrates the time course of poly(ADPR) synthesis by isolated aortic nuclei determined with two different concentrations of the substrate. The net incorporation of NAD continues for a longer period of time than was reported for rat liver nuclei irrespective of the NAD concentrations (Nishizuka *et al.*, 1967). After the maximum is reached, the amount of poly(ADPR) synthesized declines rather slowly. In the presence of 2 M ammonium sulfate the amount of poly(ADPR) synthesized decreased considerably but the time course has not changed. However, as shown by Nishizuka *et al.* (1968), low ammonium sulfate concentrations are more inhibitory than 2 M salt.

Yamada *et al.* (1971) have shown that a partially purified enzyme from rat liver nuclei shows an absolute requirement for DNA. The dependence on DNA was further established by the sensitivity of the reaction to pancreatic DNase. In the present study pancreatic DNase has a biphasic effect on the incorporation of NAD. At lower concentrations, up to about 50 μg of the nuclease (Figure 2), NAD incorporation is stimulated two- to threefold over the controls and then begins to drop, although still higher than the controls. To show a significant inhibition due to DNase, concentrations of the nuclease as high as 400 μg are required. In contrast to the DNase effect, the inclusion of venom phosphodiesterase in the incubations (Figure 2) yields a progressive inhibition with increasing concentrations of the diesterase. This is in agreement with the observations made from other tissues and due to the hydrolysis of the phosphodiester bond.

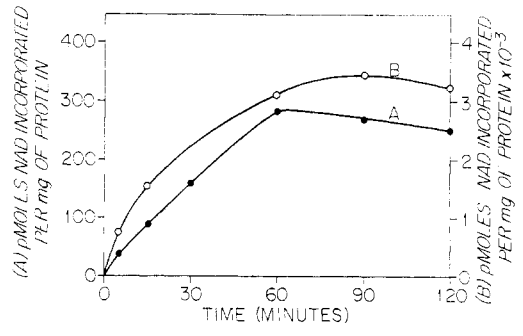

FIGURE 1: Time course of poly(ADPR) synthesis at two different substrate concentrations: (A) 1.5 nM NAD; (B) 15.8 nM NAD.

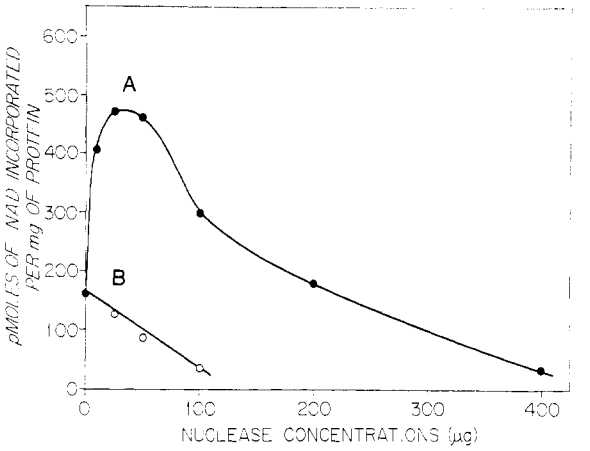

FIGURE 2: Effect of varying concentrations of nuclease on poly(ADPR) synthesis by isolated aortic nuclei. Incubations were carried out for 30 min at 37° with 0.1 μCi of [^{14}C]NAD (specific activity 136 Ci/mol): (A) pancreatic DNase; (B) venom phosphodiesterase.

The observation by Green and Dobrjansky (1972) that pancreatic DNase is a potent inhibitor of NADase has indicated that the stimulatory effect observed in the present experiment may be due to the inhibition of the NAD splitting enzyme, which would then make more substrate available for the synthesis of the polymer. We therefore tested the effect of DNase in a system with a tenfold higher concentration of NAD. This resulted in a considerably higher incorporation of NAD into poly(ADPR), but did not alter the stimulatory effect of DNase. At 25 and 50 μg of nuclease levels (Table II, expt I), the activity doubled over the control levels, a finding in keeping with the results shown in Figure 2. The stimulatory effect remains unchanged even when the nuclei were preincubated with DNase. In expt II of Table II, nuclei were preincubated for 15 min at 37° with 25 μg of the nuclease and the

TABLE II: Effect of Substrate Concentrations on the Stimulation of Poly(ADPR) Synthesis by DNase.[a]

Expt	Substrate Concn (nM)	Additions[b] None	Additions[b] 25 μg of DNase
I	1.5	182 ± 24.8	375 ± 38
	15.8	2600 ± 176	5091 ± 493
			4704 ± 148[c]
II	1.5	232 ± 24	567 ± 23
	15.8	1984 ± 364	4493 ± 186
	51.5	2502 ± 382	13086 ± 1246
	101.6	2346 ± 367	20814 ± 1497

[a] Assay mixtures are as described in the text. In expt I reaction mixtures were preincubated for 5 min without the enzyme and the reaction was started by the addition of the enzyme as a nuclear suspension. In expt II, the assay mixtures containing the nuclei but without the substrate were preincubated for 15 min at 37° with or without the nuclease and the reaction was started by the addition of the substrate. All assays were incubated for 30 min at 37°. [b] The numbers under this column represent specific activity. [c] 50 μg of DNase present instead of the 25 μg.

reaction was initiated by the addition of the labeled substrate. The preincubation step did not change the stimulatory effect and, furthermore, the incorporation of NAD increased linearly with rising substrate concentrations in the nuclease-treated system. However, in the absence of the nuclease, the concentrations of the substrate that are required for maximum incorporation are much lower and fall between 15 and 50 nM. These results demonstrate that the influence of DNase is not due to any protective effect on NAD but may be ascribed to either the availability of free DNA, or an acceptor protein, or a combination of both of these factors.

The stimulatory effect observed with pancreatic DNase led us to test the effect of exogenous polynucleotides on the poyl-(ADPR) polymerase activity. All the polynucleotides tested showed a stimulatory effect, although some are more efficient than others (Table III, expt I). Generally the order of efficiency is native DNA > poly[d(A-T)] = activated DNA > denatured DNA > poly(dG)·poly(dC). Figure 3 shows the effect of increasing concentrations of native calf thymus DNA on poly(ADPR) synthesis. As shown here, DNA concentrations of up to 50 μg produced a steady increase in the incorporation of NAD, after which the activity seemed to reach a plateau.

The stimulated activities due to DNase or exogenous polynucleotides are also strongly inhibited to the same extent as untreated nuclei by nicotinamide, thymidine, and NADase. The products of the stimulated and unstimulated activities could be hydrolyzed by venom phosphodiesterase.

Contrary to our expectations, when nuclei pretreated with DNase or nuclei to which exogenous DNA has been added were assayed for poly(ADPR) synthesis in the presence of histones (Table III, expt II), the stimulatory effect was reversed. Lysine-rich histones were more effective than arginine-rich histones. On the other hand, when histones were added to unstimulated nuclei, there was no inhibitory effect.

TABLE III: Effect of Various Polynucleotides and Histones[a] on the Activity of Poly(ADPR) Polymerase.

Expt	Additions	Specific Activity
I	None	174 ± 21
	100 μg of calf thymus DNA	
	Native	820 ± 20
	Denatured	630 ± 68
	Activated	738 ± 36
	0.5 unit of poly[d(A-T)]	743 ± 35.35
	0.5 unit of poly(dG)·poly(dC)	391.5 ± 20.5
II	None	142.5 ± 38.9
	100 μg of F_1 histone	134 ± 20
	25 μg of DNase[b]	375.5 ± 12
	25 μg of DNase plus 50 μg of F_1 histone	187.5 ± 3.5
	25 μg of DNase plus 50 μg of F_3 histone	271 ± 10
	100 μg of native calf thymus DNA	435.5 ± 19
	100 μg of native DNA plus 100 μg of F_1 histone	124 ± 19.8
	100 μg of native DNA plus 100 μg of F_3 histone	273 ± 8.5

[a] F_1 histone is lysine rich and F_3 histone is arginine rich. [b] When DNase was added, the nuclei were first preincubated with it for 15 min at 37°, and the reaction started by the addition of the labeled substrate.

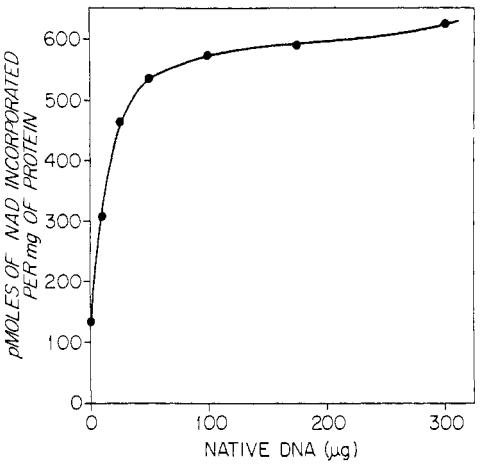

FIGURE 3: Effect of exogenous calf thymus native DNA on poly-(ADPR) synthesis. Assay mixture is as described in the text.

Figure 4 shows that poly(ADPR) synthesis is very sensitive to pH of the medium, reaching a sharp optimum at pH 8 and then reducing by nearly 85–95% with a shift of 0.5 pH unit on either side.

Discussion

Some of the conditions under which isolated aortic nuclei incorporate NAD to form a homopolymer are described. The enzymatic product is undoubtedly poly(ADPR), as shown by the sensitivity of the reaction toward nicotinamide, NADase, and venom phosphodiesterase. Further, the hydrolyzed product, when chromatographed on Dowex-1-formate, runs parallel to ADPR and 5′-AMP, the major peak being the ADPR moiety. The system is essentially similar to the one reported from rat liver with few variations. Several workers have reported that the net synthesis of poly(ADPR) ceases within a few minutes and with increasing incubation times is reduced significantly unless high salt concentrations are present to inhibit the hydrolases that affect the product. In the aortic system, however, it was found that the net incorporation of NAD goes on for nearly an hour and the product seems more stable. Concentrations of ammonium sulfate which stimulate rat liver nuclear or chromatin preparations have a significant inhibitory effect in the present system. These differences are probably due to the level or nature of the hydrolyzing enzymes that affect the substrate and the product.

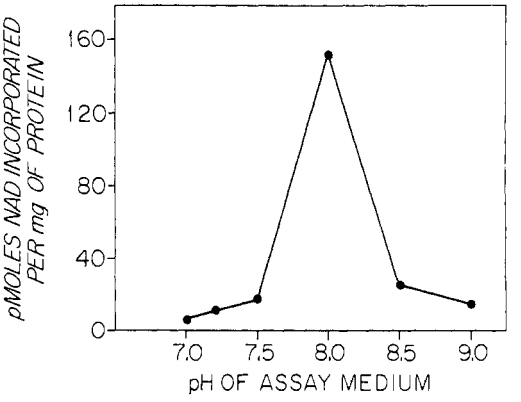

FIGURE 4: pH–activity curve of the reaction. The reaction medium is the same as given in the text except for buffer changes to obtain the different oHs. Substrate concentrations as in Figure 2 and the incubation for 30 min at 37°.

Nishizuka *et al.* (1967, 1969) have suggested that poly(ADPR) synthesis is dependent on DNA and have shown that the prior treatment of chromatin with DNase results in the formation of polymers of decreasing chain length. An absolute dependency of poly(ADPR) synthesis on DNA has been demonstrated by Yoshihara (1972). On the other hand, Fujimura *et al.* (1967) reported a stimulation in the incorporation of NAD into poly(ADPR) in a reaction where the enzymatic system was preincubated with DNase. In the present study we have demonstrated a consistent and very significant stimulation in the incorporation of NAD by nuclei pretreated or assayed with DNase. We believe that the stimulatory effect of DNase is due mainly to the availability of DNA, probably of low molecular weight, produced by the nicking action of the nuclease. In this context, it is pertinent to mention that Gill (1972), using soluble enzyme preparations, has reported that the poly(ADPR) polymerase synthesizing the polymer is associated with a low molecular weight fraction of DNA in the nucleoprotein complex.

The activity stimulated by exogenous DNA or DNase appears to be due to the DNA effecting a lengthening of the ADPR polymer chains rather than initiating fresh chains. Preliminary results on the determination of the chain length of poly(ADPR) support this hypothesis. A role for DNA in elongation of the polymer has in fact been suggested (Sugimura, 1973). The fact that histones reverse the stimulatory effect further strengthens our hypothesis that the stimulation is primarily due to elongation rather than initiation of fresh chains, for if the latter were the case one should obtain a further increase in the incorporation of NAD in the presence of an acceptor protein like the histone, rather than observed inhibition. Since histones alone had no effect on the unstimulated system, the inhibition could not be due to any binding of the substrate to the histones. There have been suggestions that histones as a whole preferentially bind to (A + T)-rich regions in DNA (Fredericq, 1971). The inhibitory effect of histones, specifically the F_1 histones, thus suggests that probably the (A + T) rich regions in the DNA are involved in the synthesis of poly(ADPR). Yoshihara and Koide (1973) have stated that poly(ADPR) polymerase activity may be regulated or dependent on areas of DNA rich in dA and dT. In the present system, the limiting factor seems to be free DNA, and conditions that make this available thus stimulate the enzyme activity. Our data with various substrate concentrations exclude the possibility that the stimulatory effect of DNase is related to its known inhibitory effect on nuclear NADase.

It has been suggested strongly that poly(ADPR) synthesis is involved with DNA synthesis, although the mechanism of its involvement is not clearly understood. There have been various reports dealing with this aspect and both a competitive role and a parallel increase of the enzyme activity along with DNA synthesis have been shown. In view of the apparent strong affinity for DNA demonstrated for the poly(ADPR) enzyme, it seems plausible to expect that it competes with DNA polymerase, and that this is at least part of the reason for its apparent "regulatory" role. Experiments to test this conjecture are now under way.

Acknowledgments

The authors are indebted to Professor John M. Reiner, Dr. Theodore Peters, Jr., and Professor Wilbur A. Thomas for helpful discussions and encouragement.

References

Briggs, M. H. (1960), *Nature (London) 187*, 249.
Burzio, L., and Koide, S. S. (1970), *Biochem. Biophys. Res. Commun. 40*, 1013.
Burzio, L., and Koide, S. S. (1971), *Biochem. Biophys. Res. Commun. 42*, 1185.
Chambon, P., Weill, J. D., and Mandel, P. (1963), *Biochem. Biophys. Res. Commun. 11*, 39.
Clark, J. B., Greenbaum, A. L., and McLean, P. (1966), *Biochem. J. 98*, 546.
Colyer, R. A., Burdette, K. E., and Kidwell, W. R. (1973), *Biochem. Biophys. Res. Commun. 53*, 960.
Fredericq, E. (1971), *in* Histones and Nucleohistones, Phillips, D. M. P., Ed., New York, N. Y., Plenum Press, p 135.
Fujimura, S., Hasegawa, S., Shimuzu, Y., and Sugimura, T. (1967), *Biochim. Biophys. Acta 145*, 247.
Gill, D. M. (1972), *J. Biol. Chem. 247*, 5964.
Green, S., and Dobrjansky, A. (1972), *Biochemistry 11*, 4108.
Hilz, H., and Kittler, M. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1693.
Janakidevi, K. (1972), *Biochemistry 11*, 1442.
Jedeikin, L. A., and Weinhouse, S. J. (1955), *J. Biol. Chem. 213*, 271.
Loeb, L. A. (1969), *J. Biol. Chem. 244*, 1672.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Nishizuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O. (1967), *J. Biol. Chem. 242*, 3164.
Nishizuka, Y., Ueda, K., Nakazawa, K., Reeder, R. H., Honjo, T., and Hayaishi, O. (1968), *J. Vitaminol. 14*, 143.
Nishizuka, Y., Ueda, K., Nakazawa, K., Reeder, R. H., Honjo, T., and Hayaishi, O. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 781.
Smulson, M., Henriksen, O., and Rideau, C. (1971), *Biochem. Biophys. Res. Commun. 43*, 1266.
Sugimura, T. (1973), *Progr. Nucl. Acid Res. Mol. Biol. 13*, 127.
Yamada, M., Miwa, M., and Sugimura, T. (1971), *Arch. Biochem. Biophys. 146*, 579.
Yoshihara, K. (1972), *Biochem. Biophys. Res. Commun. 47*, 119.
Yoshihara, K., and Koide, S. S. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 30*, 261.