Ikehara, M., Tazawa, I., & Fukui, T. (1969) *Biochemistry 8*, 736–743.
Ikehara, M., Limn, W., & Fukui, T. (1977) *Chem. Pharm. Bull. 25*, 2702–2707.
Ikehara, M., Limn, W., & Uesugi, S. (1978) *J. Carbohydr., Nucleosides, Nucleotides 5*, 163–185.
Krakauer, H., & Sturtevant, J. M. (1968) *Biopolymers 6*, 491–512.
Limn, W., Uesugi, S., Ikehara, M., & Miles, H. T. (1983) *Biochemistry 22*, 4217–4222.
Manning, G. S. (1972) *Biopolymers 11*, 937–949.
Manning, G. S. (1978) *Q. Rev. Biophys. 11*, 179–246.
Mathelier, H. D., Howard, F. B., & Miles, H. T. (1979) *Biopolymers 18*, 709–722.
Maxam, A. M., & Gilbert, W. *Methods Enzymol. 65*, 499–560.
Michelson, A. M., Monny, C., & Kapuler, A. V. (1970) *Biochim. Biophys. Acta 217*, 7–15.
Moffat, J., & Khorana, H. G. (1961) *J. Am. Chem. Soc. 83*, 649–658.
Muraoka, M., Miles, H. T., & Howard, F. B. (1980) *Biochemistry 19*, 2429–2439.
Pauling, L. *The Nature of the Chemical Bond*, p 260, Cornell University Press, Ithaca, NY.
Powell, J. I., Fico, R., Jennings, W. H., O'Bryan, E. R., & Schultz, A. R. (1980) *Proc. IEEE Comput. Soc., Int. Conf., 21st*, 185–190.
Ross, P. D., & Scruggs, R. L. (1969) *J. Mol. Biol. 45*, 567–569.
Ross, P. D., Scruggs, R. L., Howard, F. B., & Miles, H. T. (1971) *J. Mol. Biol. 61*, 727–733.
Record, M. T., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.
Sakore, T. D., & Sobell, H. M. (1969) *J. Mol. Biol. 43*, 77–87.
Silverton, J. V., Limn, W., & Miles, H. T. (1982) *J. Am. Chem. Soc. 104*, 1081–1087.
Sundaralingam, M. (1973) *Jerusalem Symp. Quantum Chem. Biochem. 5*, 417–455.
Tavale, S. S., & Sobell, H. M. (1970) *J. Mol. Biol. 48*, 109–123.
Wang, A. H. J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.
Zimmerman, S. B. (1982) *Annu. Rev. Biochem. 51*, 395–427.

Nonenzymic Adenosine 5′-Diphosphate Ribosylation of Poly(adenosine diphosphate ribose)†

Miyoko Ikejima* and D. Michael Gill
Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111
Received January 4, 1985

ABSTRACT: Poly(adenosine 5′-diphosphate ribose) [poly(ADP-ribose)] is spontaneously ADP-ribosylated when it is incubated with nicotinamide adenine dinucleotide, especially in 0.5 M NaCl and at an alkaline pH. The ADP-ribose residues are monomeric and are attached to the middle of polymer chains. The linkage is similar to, and may be identical with, that of the branch points that are created in cells. RNA is also spontaneously ADP-ribosylated, but not DNA.

Poly(adenosine 5′-diphosphate ribose) [poly(ADP-ribose)] is a nuclear macromolecule that is synthesized at DNA breaks and may play some role in their repair (Juarez-Salinas et al., 1979; Benjamin & Gill, 1980a; Berger et al., 1980; Durkacz et al., 1980; Shall, 1983; Ikejima et al., 1983). It is rapidly degraded in situ, and the newly synthesized material has a complete range of sizes up to at least several hundred residues (Tanaka et al., 1978; Benjamin & Gill, 1980a,b; Ikejima et al., 1983). For the most part, the polymer consists of ADP-ribose residues joined in series by 1″→2′ glycosidic bonds between ribose residues, but there are branches involving 1‴→2″ ribose→ribose bonds approximately every 50–100 residues (Miwa et al., 1979; Juarez-Salinas et al., 1982; Kanai et al., 1982; Sugimura & Miwa, 1982). The structure of a branch point is shown in Figure 1A. After digestion by snake venom phosphodiesterase, backbone residues yield phosphoribosyl-AMP (PR-AMP)[1] while the branch points yield di-(phosphoribosyl)-AMP [(PR)2-AMP] (Figure 1).

We have found that pure poly(ADP-ribose) itself becomes radioactive when it is incubated with radioactive NAD. The product consists of radioactive ADP-ribose residues attached to internal sites along poly(ADP-ribose) chains. By several criteria, these attachments are indistinguishable from the poly(ADP-ribose) branch points formed in cellular environments. Although the rate is slow under the conditions we have used, the reaction may illuminate the mechanism whereby poly(ADP-ribose) polymerase introduces branch points, and we discuss whether the natural branching mechanism may include a nonenzymic step.

Experimental Procedures

Poly(ADP-ribose) composed of chains of 10–30 ADP-ribose residues was a gift from Dr. Masanao Miwa, National Cancer Center Research Institute, Tokyo, Japan. After digestion with phosphodiesterase, nucleotide analysis using high-performance

† This work was supported by Grant GM28482 from the National Institutes of Health.

[1] Abbreviations: PR-AMP, 2′-(5″-phosphoribosyl)adenosine 5′-phosphate, 2′-ribosyladenosine 5′,5″-bis(phosphate), or *O*-α-D-ribofuranosyl(1″→2′)adenosine 5′,5″-bis(phosphate); (PR)2-AMP, 2′-[2″-(5‴-phosphoribosyl)-5″-phosphoribosyl]adenosine 5′-phosphate, 2′-[2″-(1‴-ribosyl)-1″-ribosyl]adenosine 5′,5″,5‴-tris(phosphate), or *O*-α-D-ribofuranosyl(1‴→2″)-*O*-α-D-ribofuranosyl(1″→2′)adenosine 5′,5″,5‴-tris(phosphate).

0006-2960/85/0424-5039$01.50/0

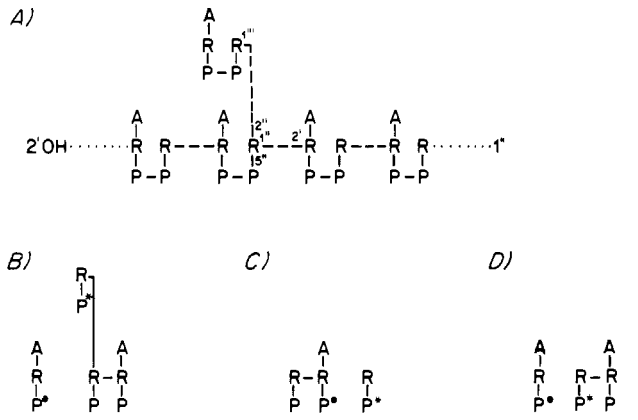


FIGURE 1: (A) Representation of a portion of a poly(ADP-ribose) chain with one branch: A = adenine; R = ribose; P = phosphate. The glycosidic 1″ → 2′ bonds between ADP-ribose residues are dashed. Some of the ribosyl carbon atoms are numbered. The termini of the backbone chain are unsubstituted at the 2′- or 1″-positions. (B–D) The radioactive products expected to be formed by digestion with phosphodiesterase (B) by branch addition, (C) by 1″-extension, and (D) by 2′-extension. (*) phosphate labeled in [$^{32}P_2$]NAD only; (●) phosphate labeled in [$^{32}P_2$]NAD and in [*adenylate*-^{32}P]NAD.

liquid chromatography showed little contamination (Figure 7C), and further purification was not attempted. It contained 5–10 branch residues per 1000 residues (Dr. M. Miwa, personal communication). It had been lightly labeled with ^{14}C at the adenine residues (0.46 μCi/mmol). The ^{14}C radioactivity was usually negligible but had to be considered in the experiments involving [$^{32}P_2$]NAD (Figure 7D).

Other Chemicals. [$^{32}P_2$]NAD (initially 420 mCi/mmol) was isolated from the liver of a mouse injected with [^{32}P]-orthophosphate, as described by Benjamin & Gill (1980a). Approximately 56% of its radioactivity was in the nicotinamide-proximal phosphate. [*adenylate*-^{32}P]NAD (100–200 Ci/mmol) was obtained from ICN, Irvine, CA.

[*adenylate*-^{32}P]ADP-ribose was generated from it by incubation with porcine brain NAD glycohydrolase (EC 3.2.2.5) (Sigma Chemical Co., St. Louis, MO), which was then removed by ultrafiltration with an Amicon micropartition system equipped with a YMT membrane. Snake venom phosphodiesterase (EC 3.1.4.1) was purchased from Worthington Biochemical Corp., Freefold, NJ, and was further purified by chromatography on a column of Blue Sepharose (Pharmacia Fine Chemicals, Pincataway, NJ) to remove any contaminating nonspecific phosphatase and 5′-nucleotidase (Oka et al., 1978). One unit hydrolyzes 1 μmol of *p*-nitrophenyl-TMP in 1 min at 37 °C. The plasmid pBR322 was digested with *Hae*III (New England BioLabs, Beverley, MA) as described by Benjamin & Gill (1980b). RNA from bakers' yeast type III (mostly tRNA) was purchased from Sigma and was further purified by phenol extraction and ethanol precipitation.

Incubation of Poly(ADP-ribose) with [^{32}P]NAD. The incubation mixture usually consisted of 0.9 mM (with respect to residues) poly(ADP-ribose), about 1 μM [*adenylate*-^{32}P]-NAD, 0.5 M NaCl, and 40 mM glycine–NaOH buffer, pH 9.5. The mixture was incubated in a 0.5-mL Eppendorf tube at 37 °C for about 16 h and then precipitated with ethanol (LeGrice & Sonenshein, 1982), 2 μg of yeast RNA being used as a carrier. The recovery of poly(ADP-ribose) was estimated at 72% by analysis on gels before and after precipitation, but the distribution among the different-sized bands was not changed.

Phosphodiesterase Digestion. When the product was to be digested, the incubation was scaled up by 2- or 3-fold. The NAD concentration was increased in proportion when its specific activity was low, as detailed in the figure legends.

Poly(ADP-ribose) was precipitated from the incubation medium with ethanol, dried, and dissolved in 100 μL of 6 M guanidine hydrochloride with incubation for 1 h at 37 °C. The solution was mixed with 0.1 nmol of unlabeled NAD and applied to a Bio-Gel P4 column (12 mm × 150 mm) equilibrated with 0.4 M LiCl and 5 mM Tris-HCl, pH 8.0, in order to remove unincorporated [^{32}P]NAD. The material eluting at the void volume was collected, precipitated with ethanol, and dissolved in 40 μL of water. A portion of this sample was analyzed by thin-layer chromatography. The remainder, containing about 120 μg of poly(ADP-ribose), was digested with 0.05 unit of snake venom phosphodiesterase in the presence of 10 mM magnesium acetate at 37 °C for 3 h. The digest was analyzed by thin-layer chromatography and by high-performance liquid chromatography.

Gel Electrophoresis, Staining, and Autoradiography. Dry samples for electrophoresis were boiled for 2 min in gel-solubilizing buffer that contained 1% sodium dodecyl sulfate. They were fractionated on 10–18% polyacrylamide slabs in the presence of 0.1% sodium dodecyl sulfate (Benjamin & Gill, 1980a) and then stained with silver nitrate (Morrisey, 1981). We found that the silver stain revealed polynucleotides as well as proteins (Beidler et al., 1982) and could reveal 0.5 μg of DNA, 1 μg of RNA, or 1 μg of poly(ADP-ribose). Dried gels were exposed to Kodak XAR-5 X-ray film for 4–11 days without intensifying screens.

RESULTS

Formation of Radioactive Poly(ADP-ribose). Poly(ADP-ribose) becomes radioactive if it is incubated with [^{32}P]NAD. Covalent bonding is implied by the following: (a) the incorporated label is stable in 6 M guanidine hydrochloride and in 1% sodium dodecyl sulfate at 100 °C; (b) it remains associated with the polymer upon precipitation with ethanol or trichloroacetic acid; (c) it cochromatographs with poly(ADP-ribose) on dihydroboryl-Bio-Rex 70 (Wielckens et al., 1981); (d) each rung of the ladder is labeled of poly(ADP-ribose) species of different sizes that forms when the heterogeneous polymer is fractionated by polyacrylamide gel electrophoresis (Figure 2, lanes b–e; but note Polyacrylamide Gel Analysis).

The following observations indicate that the donor is NAD and not a contaminant. (a) Prior incubation of the NAD with an NAD glycohydrolase to form [*adenylate*-^{32}P]ADP-ribose prevents the incorporation (Figure 2, lane f). We see instead the labeling of some proteins (lane h), presumably via Schiff-base intermediates (Kun et al., 1976); the same proteins are labeled by the [^{32}P]ADP-ribose formed by the alkaline hydrolysis of [*adenylate*-^{32}P]NAD (lane g). (b) The incorporation into poly(ADP-ribose) was not given by the radioactive contaminants that remained unbound when the NAD preparation was passed over a column of dihydroboryl-Bio-Rex 70. (c) NAD labeled in both of its phosphatase gave similar results to [*adenylate*-^{32}P]NAD although it contained only one-sixth of the impurities of the latter.

Conditions for the Optimal Labeling of Poly(ADP-ribose). Nonenzymic modification occurs under the conditions of salt concentration and pH generally used for studying the enzymic synthesis of poly(ADP-ribose). However, the amount of labeling can be increased by 2–3-fold by providing an alkaline environment (Figure 3). The nature of the buffer is not particularly important except that borate buffer reduces the labeling, probably because it binds the riboses of NAD and poly(ADP-ribose). Labeling is also increased by up to 0.5 M of NaCl (Table I), but we found no further increase with more

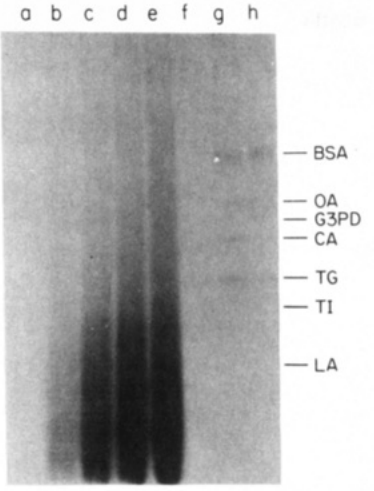


FIGURE 2: Autoradiogram of poly(ADP-ribose) after incubation with 5 μM [*adenylate*-^{32}P]NAD (a–e) or [*adenylate*-^{32}P]ADP-ribose (f). Each reaction mixture of 50 μL contained 0.9 mM poly(ADP-ribose), 0.5 M NaCl, and 40 mM glycine–NaOH, pH 9.5. Samples were incubated at 37 °C for 0 (a), 4 (b), 8 (c), 12 (d), or 16 h (e and f). A mixture of proteins (molecular weight marker set from Sigma Chemical Co.) was also incubated for 16 h with [*adenylate*-^{32}P]NAD (g) and [*adenylate*-^{32}P]ADP-ribose (h). This mixture contained 0.7 μg each of α-lactalbumin (LA), soybean trypsin inhibitor (TI), trypsinogen (TG), carbonic anhydrase (CA), glyceraldehyde-3-phosphate dehydrogenase (G3PD), ovalbumin (OA), and bovine serum albumin (BSA). Their positions are marked with bars.

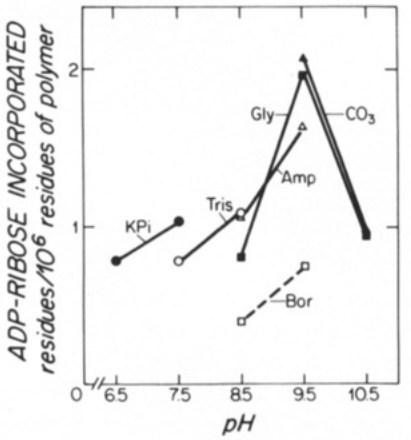


FIGURE 3: Effect of pH. Poly(ADP-ribose), 0.9 mM, was incubated for 15 h at 37 °C with 5 μM [*adenylate*-^{32}P]NAD, 0.5 M NaCl, and 40 mM of the buffer indicated: KP_i, K_2HPO_4–KH_2PO_4; Tris, Tris-HCl; Amp, 3-[*N*-(α,α-dimethylhydroxyethyl)amino]-2-hydroxypropanesulfonic acid (Ampso)–NaOH; Gly, glycine–NaOH; CO_3, Na_2CO_3–$NaHCO_3$; Bor, $Na_2B_4O_7$–HCl or $Na_2B_4O_7$–NaOH.

concentrated NaCl. Accordingly, we have used a pH of 9.5 (glycine buffer) in the presence of 0.5 M NaCl as our standard condition. Magnesium is only slightly beneficial in the absence of NaCl and is inhibitory in the presence of NaCl, while EDTA and ATP have small or no effects (Table I).

Dependence on Reactant Concentrations. While maintaining a constant 0.5 μM radioactive NAD, we supplied various concentrations of nonradioactive NAD (Figure 4). The extent of modification of poly(ADP-ribose) increased with the NAD concentration, but less rapidly than expected. The slope of the double-logarithmic plot in Figure 4B is about 0.64 whereas a slope of 1.0 (as illustrated by the dashed line in the figure) is expected for a first-order event. A possible explanation is that the NAD may associate intermolecularly and thus decrease the amount available to react with poly(ADP-ribose) (Reddy et al., 1981). The dependence on the concentration of poly(ADP-ribose) is first order (Figure 4).

Table I: Incorporation of ADP-ribose into Poly(ADP-ribose)

incubation mixture[a]	ADP-ribose incorporated (fmol)	
	no NaCl	0.5 M NaCl
control	8.9	52.4
no poly(ADP-ribose)	1.9	2.7
+$Mg(OCOCH_3)_2$, 10 mM	14.9	27.9
+EDTA, 10 mM	14.8	48.7
+ATP, 1 mM	10.2	43.4

[a] Incubation mixtures of 50 μL contained the concentration of NaCl shown, 40 mM glycine–NaOH, pH 9.5, 0.9 mM poly(ADP-ribose), and 0.8 μM [*adenylate*-^{32}P]NAD at a specific activity of 30 381 cpm/pmol.

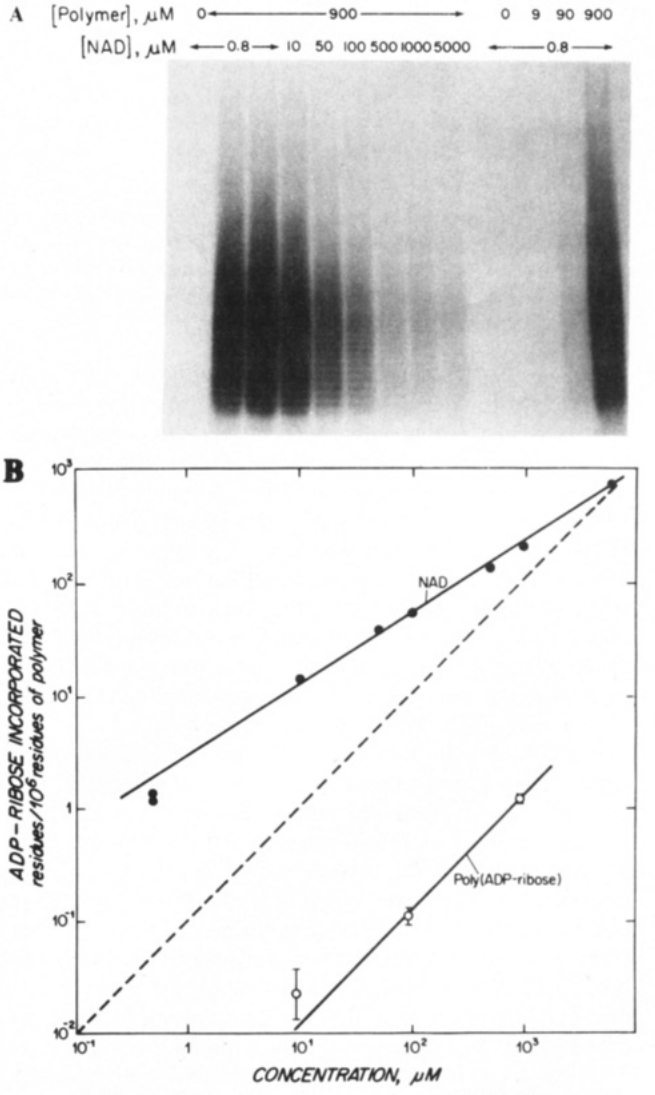


FIGURE 4: Dependence of the incorporation of ADP-ribose on the concentrations of NAD and poly(ADP-ribose). Samples contained 0.5 M NaCl, 40 mM glycine–NaOH, pH 9.5, the amount of poly(ADP-ribose) marked, 0.5 μM [*adenylate*-^{32}P]NAD, and various concentrations of unlabeled NAD. (A) Autoradiogram. (B) Relationships between the incorporated ADP-ribose and the NAD (●) or poly(ADP-ribose) (○) concentration. The incorporation was estimated by counting each lane of the dried gel, correcting for the appropriate blank (first or tenth lanes from the left). The dotted line shows the slope expected for a simple two-substrate reaction.

Polyacrylamide Gel Analysis. Since only a minority of the poly(ADP-ribose) chains become modified, the distribution of the bulk of the poly(ADP-ribose) chains visualized by gel electrophoresis and silver staining is not changed. By carefully aligning the stained gel with its autoradiogram, however, it becomes evident that the radioactive product is displaced by

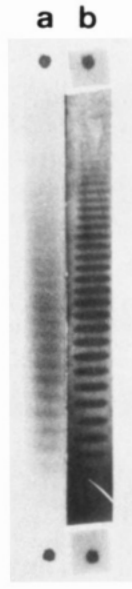
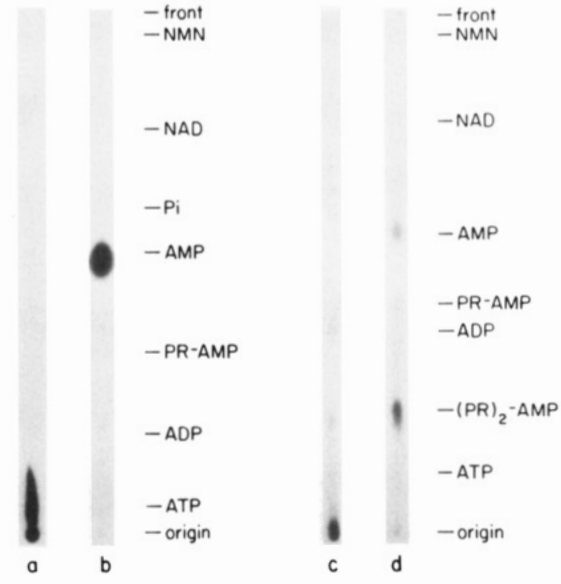


FIGURE 5: Comparison of the migration distances in a polyacrylamide gel of stained (bulk) and radioactively modified poly(ADP-ribose). The piece of stained gel with registration dots added on the top and bottom (b) and its autoradiogram (a) were photographed side by side in precise register.



[Adenylate-^{32}P]NAD [Both phosphates-^{32}P]NAD

FIGURE 6: Phosphodiesterase digestion products analyzed by PEI–cellulose thin-layer chromatography. Poly(ADP-ribose) was incubated with [*adenylate*-^{32}P]NAD (a and b) and with [$^{32}P_2$]NAD (c and d). It was analyzed before (a and c) and after (b and d) incubation with snake venom phosphodiesterase. Samples of 1 μL were applied repetitively to plastic sheets coated with PEI–cellulose F (MCB, Cincinnati, OH) that had been prewashed with 10% NaCl and were developed with 1 M LiCl (Randerath & Randerath, 1968). Since the R_f values varied with the amounts of salt in the sample, temperature, etc., each run included a set of standards (NAD, AMP, ADP, and ATP), which was added both as a separate track and mixed with a portion of the unknown sample. Authentic (PR)2-AMP was also included in sample d. The standards were detected by ultraviolet light. The positions of NMN, PR-AMP, and (PR)2-AMP were also determined from standards run in parallel. PR-AMP, (PR)2-AMP, and ADP-ribose give trailing spots, but the simpler nucleotides give discrete spots. The autoradiograms were exposed with intensifying screens for 1 (a and b) or 10 days (c and d).

about half of the band to band distance to the heavier side (Figure 5). Under Discussion we show that this is best explained as a branching addition of ADP-ribose, an explanation that is supported by the structural analysis described next.

Identification of Phosphodiesterase Digestion Products. We purified the radioactive product by chromatography on Bio-Gel P4 to remove, in particular, low molecular weight species. This was successful, for chromatography on poly(ethylenimine) thin-layer plates resolved only one radioactive spot. This spot tailed from the origin in a distribution identical with that of the starting polymer (visualized by ultraviolet light) (Figure 6, lanes a and c). There was no detectable NAD, NMN, AMP, ADP, ADP-ribose, or phosphate.

We digested the radioactive product to completion with snake venom phosphodiesterase and analyzed the digests by thin-layer chromatography and by high-performance liquid chromatography using an anion-exchange column. Phosphodiesterase digestion caused the UV-absorbing poly(ADP-ribose) to move from the origin of the thin-layer plate to the position of PR-AMP. When the precursor NAD had been labeled in the adenine-proximal phosphate, the radioactive product of phosphodiesterase digestion moved to the position of AMP (Figure 6, lane b). Its identity as [^{32}P]AMP was confirmed by high-performance liquid chromatography (Figure 7A,B). The finding that AMP is the sole radioactive digestion product is inconsistent with an addition of ADP-ribose at the 1″-terminus of the polymer, for this would yield PR-AMP as the only radioactive digestion product (Figure 1C), but is consistent with an addition at the 2′-terminus or at branch positions along the chain (panel D or B of Figure 1).

To distinguish addition at 2′-termini from addition at branch positions, it was necessary to use as the precursor NAD labeled in both of its phosphates. This precursor, being prepared from the liver of a mouse injected with [^{32}P]orthophosphate, has a much lower specific activity than the available [*adenylate*-^{32}P]NAD and necessitated a scaleup from the previous protocol. Poly(ADP-ribose) that had been incubated with [$^{32}P_2$]NAD was digested with snake venom phosphodiesterase, and the digest was analyzed by thin-layer chromatography (Figure 6, lane d) and high-performance liquid chromatography (Figure 7C,D). This time the [^{32}P]AMP in the digest was accompanied by about the same amount of material with the chromatographic properties of [^{32}P](PR)2-AMP, which is the product expected from branch points. Since none of this material had been radioactive when [*adenylate*-^{32}P]NAD was used as the precursor, it must contain the phosphate only from the NMN half of NAD, again as expected for branch structures (see Figure 1B). It is significant that the digest contained no [^{32}P]NMN for this eliminated the possibility that any NAD was incorporated intact.

Glycohydrolase Digestion of the Modified Poly(ADP-ribose). Poly(ADP-ribose) glycohydrolase is known to hydrolyze all of the ribose–ribose linkages of poly(ADP-ribose), both those in the backbone and those at naturally-occurring branch points. Complete digestion thus yields solely ADP-ribose (Miwa et al., 1974, 1981). Portions of the radioactive polymers prepared from [*adenylate*-^{32}P]NAD or [$^{32}P_2$]NAD were incubated with the glycohydrolase, and the digests were analyzed by thin-layer chromatography (data not shown) and in the former case by high-performance liquid chromatography (Figure 8). In every case the only radioactive product was [^{32}P]ADP-ribose, which suggests again that the ADP-ribose residue of NAD is added to poly(ADP-ribose) by new ribose–ribose linkages.

Nonenzymic ADP-Ribosylation of Other Polynucleotides. [^{32}P]NAD was incubated with defined DNA (a *Hae*III digest of pBR322) and with yeast RNA (mostly tRNA) (Figure 9). The DNA was not labeled, but there was detectable labeling

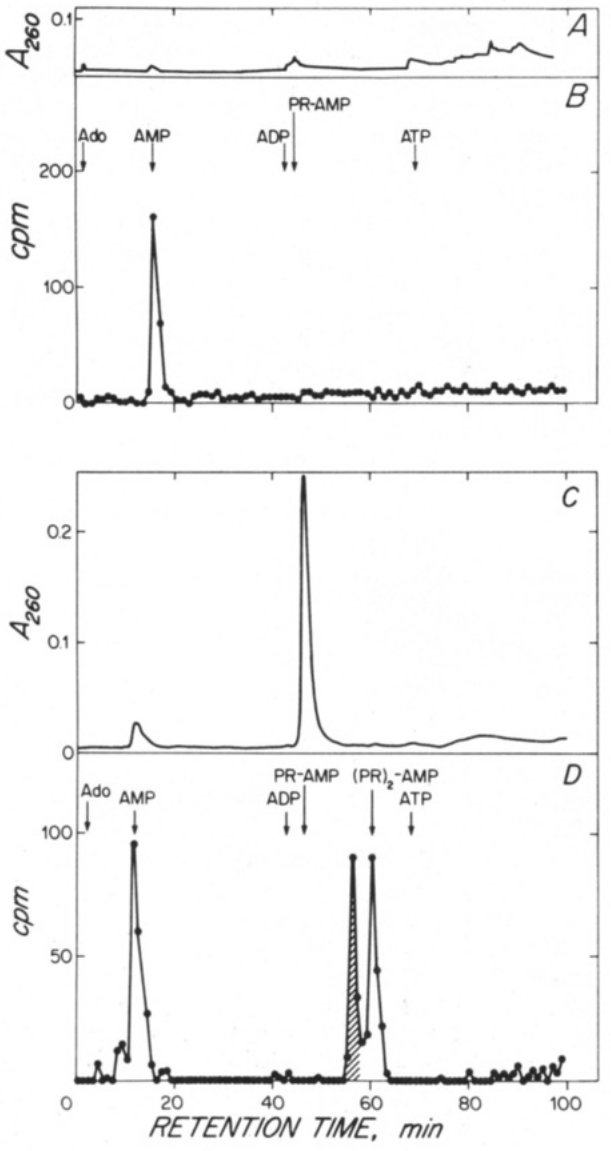


FIGURE 7: Phosphodiesterase digestion products analyzed by high-performance liquid chromatography: (A and B) poly(ADP-ribose), 6.3 nmol, 6 cpm as ^{14}C, was incubated with [*adenylate*-^{32}P]NAD; (C and D) poly(ADP-ribose), 82 nmol, 74 cpm as ^{14}C, was incubated with [$^{32}P_2$]NAD. The products were digested with phosphodiesterase and ultracentrifuged through an Amicon YMT membrane before application of 20 or 50 μL to a Du Pont Zorbax anion-exchange column (4.6 mm × 250 mm) on an Altex 330 liquid chromatograph. Elution was conducted by a method modified from Hartwick & Brown (1975) using the following concentrations of potassium phosphate (low absorbance grade from MCB Manufacturing Chemists, Cincinnati, OH) for 20 min each: 10, 100, 200, 300, and 300 mM plus 500 mM NaCl. This procedure gave good separation of the phosphodiesterase digestion products. Typical retention times were as follows: adenosine, 1.8 min; NMN, 3.4 min; AMP, 15 min; ADP, 37 min; PR-AMP, 39 min; (PR)2-AMP, 53 min; ATP, 60 min. To stabilize the column, we loaded and eluted a dummy sample before each run. Daily variations in the retention times were compensated for by running a standard marker set (adenosine, AMP, ADP, ATP) immediately after the running of the unknown samples. PR-AMP was also identified by its UV absorbancy. It is the main digestion product. (A and C) Absorbance at 260 nm; (B and D) radioactivity of 2.35-mL fractions mixed with Aquassure LSC cocktail (New England Nuclear, Boston, MA) corrected for the slight ^{14}C radioactivity of the PR-AMP. The PR-AMP had zero Cerenkow radiation, confirming its lack of ^{32}P. The hatched peak in (D) is derived from a minor contaminant, which seemed to associate adventitiously with poly(ADP-ribose) during purification. The same peak was found in a phosphodiesterase digest of the precursor NAD alone but was absent in a second preparation of [$^{32}P_2$]NAD and its polymeric derivative.

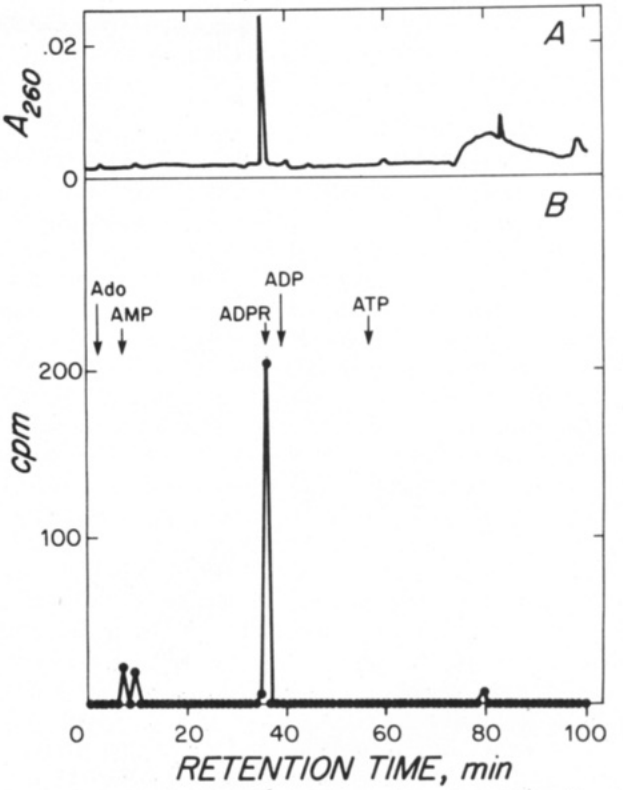


FIGURE 8: Analysis of glycohydrolytic products. Poly(ADP-ribose), 2.16 nmol, 2 cpm as ^{14}C, that had been incubated with [*adenylate*-^{32}P]NAD was digested overnight with 0.01 unit of partially purified poly(ADP-ribose) glycohydrolase (Miwa et al., 1974; a gift from these authors) in the presence of 10 mM dithiothreitol and 0.02% NaN_3 at 37 °C. The digest was ultracentrifuged, and the soluble portion was fractionated by HPLC as described for Figure 7. (A) A_{260}; (B) radioactivity.

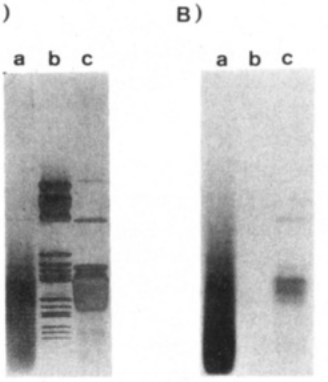


FIGURE 9: Radioactive macromolecules formed nonenzymically from NAD. Sample of 25 μg of poly(ADP-ribose) (a), 25 μg of pBR322 plasmid DNA digested with *Hae*III (b), and 25 μg of yeast RNA (c) were incubated with 0.6 μM [*adenylate*-^{32}P]NAD, 0.5 M NaCl, and 40 mM glycine–NaOH, pH 9.5, at 37 °C for 16.5 h and electrophoresed without prior boiling. (A) Silver-stained gel; (B) autoradiogram.

of the RNA. This suggests that the 2′-hydroxyl group of ribose may be a major site of nonenzymic addition.

Lack of ADP-Ribosylation of ADP-ribose. We searched for a similar reaction involving monomeric ADP-ribose by incubating [^{32}P]NAD with an excess of unlabeled ADP-ribose at pH 9.5, 0.5 M NaCl, and analyzing the whole reaction mixture by DEAE-cellulose column chromatography. The column resolves oligomers of ADP-ribose (Kawaichi et al., 1981). Most of the radioactivity was eluted at the positions of NAD, ADP-ribose, ADP, and AMP. There was a trace amount of radioactivity around the position expected for a

dimer of ADP-ribose, but none of this was in fact a dimer because it was all sensitive to alkaline phosphatase and therefore possessed one or more terminal phosphates.

Discussion

When labeled NAD is incubated with poly(ADP-ribose), radioactive material is transfered nonenzymically to covalent linkage with the polymer. Snake venom phosphodiesterase releases [^{32}P]AMP but no NMN (Figures 6 and 7) while poly(ADP-ribose) glycohydrolase releases [^{32}P]ADP-ribose. The simplest interpretation, therefore, is that ADP-ribose residues are transferred to sites on the polymer from the energy of hydrolysis of NAD's glycosidic bond.

The transfer seems to be of single ADP-ribose residues. The addition of several ADP-riboses at the same site to form short chains is excluded by the absence of [^{32}P]PR-AMP in the phosphodiesterase digest of the product (Figures 6 and 7).

On polyacrylamide gels the ^{32}P-labeled polymer is retarded by about half of the interval between adjacent oligomers (Figure 5). Terminal addition of an ADP-ribose would result in retardation by a whole interval while less retardation is expected from a branch addition, which would be a smaller hindrance to the polymer's gel migration. This behavior therefore suggests that the transferred residues are mainly attached at internal sites along the poly(ADP-ribose) chain rather than at terminal positions. In confirmation, phosphodiesterase digests contained material indistinguishable by TLC and HPLC from the natural branch-point digest product, [^{32}P](PR)2-AMP (Figure 6, lane d; Figure 7D).

These analytical methods may not distinguish authentic (PR)2-AMP, in which the ribose–ribose bond is 1‴ → 2″, from the 1‴ → 3″ isomer, and the latter structure remains formally possible. We have two reasons for preferring the 1‴ → 2″ bond. First, the [^{32}P]ADP-ribose residues are released by poly(ADP-ribose) glycohydrolase, which is known to hydrolyse the 1‴ → 2″ bond (its effect on 1‴ → 3″ bonds has not been reported). Second, RNA becomes significantly modified under the conditions used for poly(ADP-ribose) while DNA does not (Figure 9), implying that the 2′-OH group is important.

There are other methods of distinguishing 1‴ → 2″ from 1‴ → 3″ structures, but they were not applicable in the present case. The radioactive branch points are much less numerous than the preexisting (unlabeled) branch points, so it is not informative to use techniques such as methylation or periodate oxidation that address the total material rather than just the radioactive portion. We hoped to get further information by comparing the extent of formation of the dimer ([^{32}P]ADP-ribosyl ADP-ribose vs. [^{32}P]ADP-ribosyl 2′-deoxy-ADP-ribose) from the incubation of [^{32}P]NAD with ADP-ribose and with 2′-deoxy-ADP-ribose. Unfortunately, we could not find any dimer formation in either case: the nonenzymic event under study seems to require a macromolecular recipient.

The salt requirement for ADP-ribosylating the polymer suggests further that a particular secondary structure of the poly(ADP-ribose) favors the reaction. It has been reported that the 280/260 absorbancy ratio of poly(ADP-ribose) is increased by a high ionic strength (Minaga & Kun, 1983).

The formation of ribose–ribose bonds is generally a base-catalyzed event, which no doubt explains the alkaline pH optimum we observe. At even higher pHs, the hydrolysis of NAD may dominate and reduce the ultimate yield of modified polymer: NAD has a half-life of 10.6 h at pH 9.5 and 3.5 h at pH 10.5.

Although the nonenzymic event described here is too slow to account by itself for the branch points that exist naturally, it may be a model for one stage in the normal formation of branches. When it is activated by broken DNA, a poly(ADP-ribose) polymerase molecule becomes itself heavily labeled with poly(ADP-ribose) chains and also generates a local pool of free chains that are soon degraded to monomers by poly(ADP-ribose) glycohydrolase (Benjamin & Gill, 1980a; Jump & Smulson, 1980; Ogata et al., 1980; Ferro & Olivera, 1982; Ikejima et al., 1983). One possibility, therefore, is that the enzyme assists branch formation by generating a local concentration of intermediates and that the actual bond formation is nonenzymic. Indeed, some such mechanism is needed to explain how the enzyme's single active site that extends chains linearly (Ueda et al., 1979; Tanigawa et al., 1984) can also engage in such a different chemistry as branch formation (Ueda et al., 1982).

Acknowledgments

We thank Dr. Masanao Miwa for advice and help and for providing us with reagents.

Registry No. Poly(ADP-ribose), 26656-46-2; ADP-ribose, 20762-30-5; NAD, 53-84-9.

References

Beidler, J. L., Hilliard, P. R., & Rill, R. L. (1982) *Anal. Biochem. 126*, 374–380.

Benjamin, R. C., & Gill, D. M. (1980a) *J. Biol. Chem. 255*, 10493–10501.

Benjamin, R. C., & Gill, D. M. (1980b) *J. Biol. Chem. 255*, 10502–10508.

Berger, N. A., Sikorski, G., Petzold, S. J., & Kurohara, K. K. (1980) *Biochemistry 19*, 289–293.

Durkacz, B. W., Omidiji, Q., Gray, D. A., & Shall, S. (1980) *Nature (London) 283*, 593–596.

Ferro, A. M., & Olivera, B. M. (1982) *J. Biol. Chem. 257*, 7808–7813.

Hartwick, R. A., & Brown, P. R. (1975) *J. Chromatogr. 112*, 651–662.

Ikejima, M., Sawada, Y., Dang, A. Q., Suhadolnik, R. J., & Gill, D. M. (1983) in *ADP-Ribosylation, DNA Repair and Cancer* (Miwa, M., Hayaishi, O., Shall, S., Smulson, M., & Sugimura, T., Eds.) pp 129–140, Japan Scientific Societies Press, Tokyo, and VNU Science Press, Utrecht, The Netherlands.

Juarez-Salinas, H., Sims, J. L., & Jacobson, M. K. (1979) *Nature (London) 282*, 740–741.

Juarez-Salinas, H., Levi, V., Jacobson, E. L., & Jacobson, M. K. (1982) *J. Biol. Chem. 257*, 607–609.

Jump, D. B., & Smulson, M. (1980) *Biochemistry 19*, 1024–1030.

Kanai, M., Miwa, M., Kuchino, Y., & Sugimura, T. (1982) *J. Biol. Chem. 257*, 6217–6223.

Kawaichi, M., Oka, J., Ueda, K., & Hayaishi, O. (1981) *Biochem. Biophys. Res. Commun. 101*, 672–679.

Kun, E., Chang, A. C. Y., Sharma, M. L., Ferro, A. M., & Nitecki, D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3131–3135.

LeGrice, S. F. J., & Sonenshein, A. L. (1982) *J. Mol. Biol. 162*, 551–564.

Minaga, T., & Kun, E. (1983) *J. Biol. Chem. 258*, 725–730.

Miwa, M., Tanaka, M., Matsushima, T., & Sugimura, T. (1974) *J. Biol. Chem. 249*, 3475–3482.

Miwa, M., Saikawa, N., Yamaizumi, Z., Nishimura, S., & Sugimura, T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 595–599.

Miwa, M., Ishihara, M., Takishima, S., Takasuka, N., Maeda, M., Yamaizumi, Z., Sugimura, T., Yokoyama, S., & Mi-

yazawa, T. (1981) *J. Biol. Chem. 256*, 2916–2921.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Ogata, N., Kawaichi, M., Ueda, K., & Hayaishi, O. (1980) *Biochem. Int. 1*, 229–236.
Oka, J., Ueda, K., & Hayaishi, O. (1978) *Biochem. Biophys. Res. Commun. 80*, 841–848.
Randerath, K., & Randerath, E. (1968) *Methods Enzymol. 12*, 323–347.
Reddy, B. S., Saenger, W., Muelegger, K., & Weimann, G. (1981) *J. Am. Chem. Soc. 103*, 907–914.
Shall, S. (1982) in *ADP-Ribosylation Reactions* (Hayaishi, O., & Ueda, K., Eds.) pp 478–520, Academic Press, New York.
Sugimura, T., & Miwa, M. (1982) in *ADP-Ribosylation Reactions* (Hayaishi, O., & Ueda, K., Eds.) pp 43–63, Academic Press, New York.
Tanaka, M., Hayashi, K., Sakura, H., Miwa, M., Matsushima, T., & Sugimura, T. (1978) *Nucleic Acids Res. 5*, 3183–3194.
Tanigawa, Y., Tsuchiya, M., Imai, Y., & Shimoyama, M. (1984) *J. Biol. Chem. 259*, 2022–2029.
Ueda, K., Kawaichi, M., Okayama, H., & Hayaishi, O. (1979) *J. Biol. Chem. 254*, 679–687.
Ueda, K., Kawaichi, M., & Hayaishi, O. (1982) in *ADP-Ribosylation Reactions* (Hayaishi, O., & Ueda, K., Eds.) pp 118–156, Academic Press, New York.
Wielckens, K., Bredehorst, R., Adamietz, P., & Hilz, H. (1981) *Eur. J. Biochem. 117*, 69–74.

Covalent Binding of Benzo[*a*]pyrenediol Epoxides to Polynucleotides†

Fu-Ming Chen
Department of Chemistry, Tennessee State University, Nashville, Tennessee 37203
Received October 4, 1984

ABSTRACT: Spectroscopic studies on the *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene- (*anti*-BPDE-) modified synthetic polynucleotide solutions reveal interesting sequence-dependent stereoselective covalent binding of *anti*-BPDE to DNA. Absorption spectral results indicate that the G·C polymers are much more reactive than the A·T polymers toward this metabolite and the homopolymer suffers higher modification than its corresponding alternating polynucleotide. The covalently attached *anti*-BPDE exhibits only a 2–3-nm red shift in the guanine-containing polynucleotide and native DNA solutions as opposed to the 8-nm red shift in poly(G) and none in the A·T polymers. Distinct stereoselectivities are exhibited by poly(dG-dC)·poly(dG-dC) vs. poly(dG)·poly(dC) as suggested by the oppositely signed CD in the pyrene spectral region. Comparison with the *syn*-BPDE modified polynucleotides reveals some interesting differences with its anti diastereomer. Significant contributions from the intercalated *syn*-BPDE are apparent in the modified guanine-containing polynucleotides as indicated by the appearance of 10-nm red-shifted shoulders. In contrast to the strong dependence on polynucleotides for *anti*-BPDE, the rate of hydrolysis of *syn*-BPDE appears to be insensitive to their presence in the solution. *anti*-BPDE modification on the 50 μM hexaamminecobalt-induced Z-form poly(dG-dC)·poly(dG-dC) is much less extensive than its corresponding B form, possibly the consequence of both structural and ionic strength factors. The spectral characteristics of *anti*-BPDE bonded to these two forms are distinctly different, with the Z form resembling more closely those of A·T polymers. Salt titration of the *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) suggests that the "externally" bound moiety becomes intercalated under high-salt condition as judged by the CD spectral red shift and concomitant intensity enhancement.

Polycyclic aromatic hydrocarbons (PAHs) are prevalent in our polluted environment, and some are known to be carcinogenic as well as mutagenic. These relatively inert compounds exert such activities through enzymatic conversion to reactive metabolites which then chemically bind to cellular macromolecules such as DNA (Harvey, 1981). The most widely studied PAH has been benzo[*a*]pyrene (BP), and there is strong evidence to suggest that the most important ultimate carcinogenic metabolites are the bay region epoxides *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE).

There are two diastereomeric forms of BPDE designated as anti and syn in which the benzylic hydroxyl group at C-7 is trans and cis to the epoxide oxygen, respectively (see Figure 1). Although the syn diastereomer is chemically more reactive (Yagi et al., 1977), the anti isomer is much more carcinogenic (Slaga et al., 1977). The weaker potency of the syn isomer has been attributed to the rapid hydrolysis suffered by this isomer as a consequence of intramolecular hydrogen bonding between the C-7 hydroxyl and the 9,10-epoxy group. Of the two enantiomers of *anti*-BPDE, the (+) isomer is a more potent tumor initiator than the (−) variety (Buening et al., 1978; Slaga et al., 1979) and correlates well with the DNA binding ability of these two enantiomers in vitro (Meehan & Straub, 1979).

anti-BPDE has been shown to preferentially form covalent bonds to the exocyclic amino group of guanine (Weinstein et al., ,1976; Jeffrey et al., 1977). Experiments with natural DNA have shown that such covalent binding is quite stereoselective for the duplex DNA but is devoid of such effect for the single-stranded form. Such specificity is not present for

† Research supported by U.S. PHS Grant CA29817 and in part by MBRS Grant S06RR0892.

0006-2960/85/0424-5045$01.50/0