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Demonstration of high molecular weight poly (adenosine diphosphate ribose)

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ABSTRACT

An electrophoretic system was established that resolves poly-(adenosine diphosphate ribose), enzymatically synthesized polymer from NAD^+, by size difference of one residue on polyacrylamide gel. The existence of a polymer of at least 65 residues was demonstrated by band counting in this system. The polymer showed a heterogeneous size distribution on the electrophoregram, and the molecular weight of the largest polymer was deduced to be more than 4.5×10^5 daltons. The discrepancy between the size, estimated by electrophoresis, and the chain length, determined by the ratio of total radioactivity to that derived from the terminus, suggests that the polymer has a branched structure.

INTRODUCTION

Poly(ADP-Rib) is synthesized from NAD^+ in eukaryotic cell nuclei (1-3). This polymer has been suggested to bind covalently to histone and to have a regulatory role in DNA synthesis and mitosis (4-6). The average chain length of the polymer was reported to be 1-30 ADP-Rib units (4-6). In previous work the size of the polymer was determined by analysis of the products of enzymic digestion, and the chain length was calculated as the ratio of total radioactivity to that derived from the *A terminus*, the terminus having the AMP portion of the ADP-Rib unit of poly-(ADP-Rib)(7). However, this method provided information only on the average chain length, and not on the size distribution.

Polyacrylamide gel electrophoresis is an excellent method for separation of polynucleotides of various sizes, and in previous work we obtained a clear separation of poly(ADP-Rib)s with 4-10 residues by this method (7). Recently, single-stranded DNA molecules in the size range of 2-100 nucleotides have also been separated into discrete bands by polyacrylamide gel electro-

phoresis (8). Therefore, to determine the size distribution of poly(ADP-Rib), we tried to fractionate it by modifying our previous method (7), finding that poly(ADP-Rib)s with up to 65 residues were able to be resolved into discrete bands in this way. We also demonstrated the existence of much larger poly(ADP-Rib). Moreover, a comparison of the sizes of discrete bands with their chain lengths, determined by a conventional method, suggested that poly-(ADP-Rib)s with more than 30 residues have a branched structure with several *A termini*.

MATERIALS AND METHODS

Preparation and Fractionation of Poly(ADP-Rib) Poly(ADP-Rib) was prepared from [adenine-8-^{14}C]ATP and NMN using isolated rat liver nuclei, or from [adenine-U-^{14}C]NAD^+ using calf thymus nuclei, and purified as described by Sugimura et al. (9). The purified poly(ADP-Rib) was then separated into four fractions by hydroxylapatite column chromatography (Fig. 1): fraction I was not adsorbed to the column with 0.10 M sodium phosphate buffer (pH 6.8); fractions II, III and IV were eluted with 0.10-0.25 M, 0.25-0.33 M and 0.33-0.50 M sodium phosphate buffer (pH 6.8), respectively. Poly(ADP-Rib) of fractions III and IV contained no oligonucleotides and contained less than 1% amino acid residues after hydrochloric acid hydrolysis (10). Each fraction was dialyzed against 0.1 mM EDTA (pH 7), lyophilyzed, dissolved in 0.1 mM EDTA (pH 7) and stored at -20°C.

Polyacrylamide Gel Electrophoresis The buffer system used was as described previously (7), except that the ratio of N,N'-methylenebisacrylamide to acrylamide was lowered to 1:30 and the concentration of acrylamide was 20%. A vertical slab gel electrophoresis system (model SE520, Hoefer Scientific Instruments Inc.) was used, the dimensions of the gel being 0.75 × 170 × 270 mm. Pre-electrophoresis was performed at 270 V (10 V/cm) for 6 hr, and electrophoresis was performed at the same voltage for the times indicated in the text. Samples were mixed with an equal volume of 50% glycerol containing 0.1% BPB and 0.1% XC before electrophoresis, and volumes of 5 to 10 µl were introduced into slots in the slab gel. Relative mobility (Rm) was calculated with BPB as a reference.

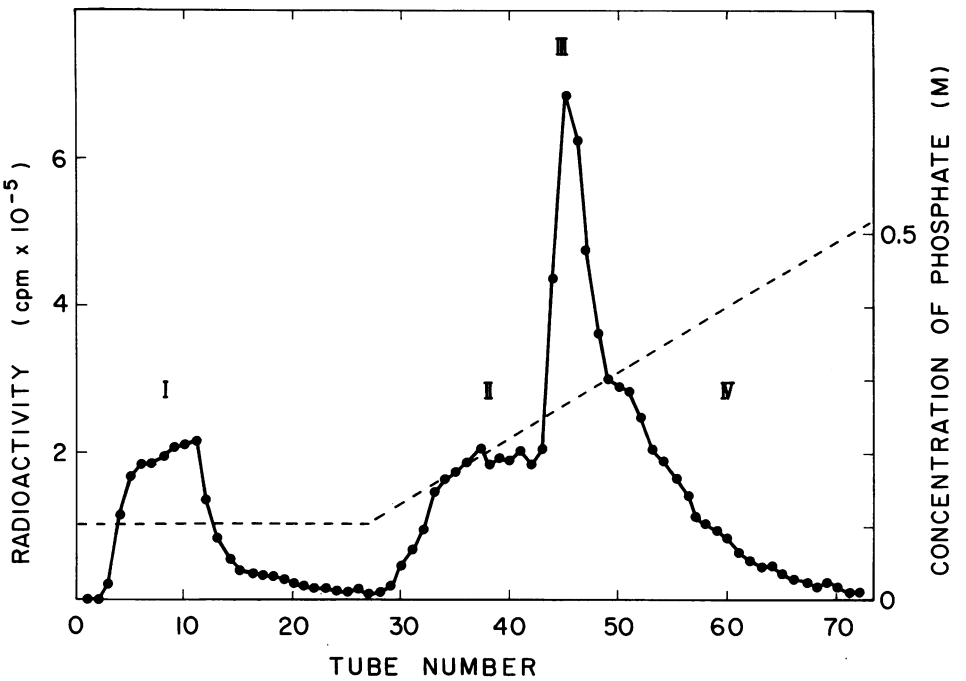

FIGURE 1. Hydroxylapatite column chromatography of poly(ADP-Rib). The radioactivity and phosphate ion concentrations are indicated as ●——● and ---, respectively.

Autoradiography After electrophoresis, the gel was dried and exposed to Fuji KX Medical film (Fuji Photo Film Co. Ltd., Tokyo, Japan) for 1 to 2 weeks.

Staining Gels were stained with Stains-all (Eastman Kodak Co., Rochester, N.Y.) as described by Dahlberg et al.(11). Poly-(ADP-Rib) stained blue, and about 0.8 μg of poly(ADP-Rib) per band could be detected with this stain. Staining of low molecular weight material was unsuccessful, and thus fraction I, which contained poly(ADP-Rib)s with less than 10 residues, could not be stained.

Extraction of Radioactivity from the Gel Gels were cut into 1 mm slices and each slice was soaked in distilled water containing 0.02% NaN_3 overnight at room temperature. Under these conditions, about 70% of the radioactivity was recovered from the gel.

Determination of Chain Length Chain length was determined

as reported previously (7). Briefly, poly(ADP-Rib) was digested with alkaline phosphomonoesterase from Escherichia coli, the products were extracted with phenol and digested with snake venom phosphodiesterase, and the final products were analyzed by paper chromatography. The chain length was calculated as a ratio of total radioactivity to that of AMP and ribosyl-AMP derived from the *A terminus*.

Hap Fragments of fd DNA DNA fragments of bacteriophage fd obtained by digestion with the restriction enzyme from Haemophilus aphirophilus (Hap) (12) were a gift from Prof. M. Takanami, Kyoto University.

RESULTS

Poly(ADP-Rib) prepared by in vitro incubation was separated into four fractions I-IV, with average chain lengths of 4.5, 12-16, 22 and 28, respectively, by hydroxylapatite column chromatography as reported previously (7,9) (Fig. 1). Fig. 2 shows the autoradiograms of the four fractions after electrophoresis for 16 hr. With fractions I to III the mobility was dependent upon the average chain length.

As shown in Fig. 2, poly(ADP-Rib) was clearly separated into many bands. Since polyacrylamide gel electrophoresis separates polynucleotides by size, the size of the poly(ADP-Rib) in each band was examined by determining the chain length. After electrophoresis of fractions I and II, several bands were extracted from the gel and the chain length of each band was determined (Table 1). A clear integer order was observed from band number 2 to 15 with minor experimental fluctuation, showing that polyacrylamide gel electrophoresis could separate poly(ADP-Rib)s on the basis of a size difference of one residue. As shown in Fig. 2, $(ADP\text{-}Rib)_8$ had the same mobility as BPB, while $(ADP\text{-}Rib)_{20}$ had the same mobility as XC. (ADP-Rib)s of up to $(ADP\text{-}Rib)_{35}$ were resolved as bands by electrophoresis for 16 hr (Fig. 2), and electrophoresis for 48 hr yielded much finer separation of the components of fractions III and IV (Fig. 3). Since XC moves with $(ADP\text{-}Rib)_{20}$, poly(ADP-Rib)s of up to $(ADP\text{-}Rib)_{65}$ were able to be counted as discrete single bands. $(ADP\text{-}Rib)_{65}$ is the largest polymer ever reported (4-6, 13).

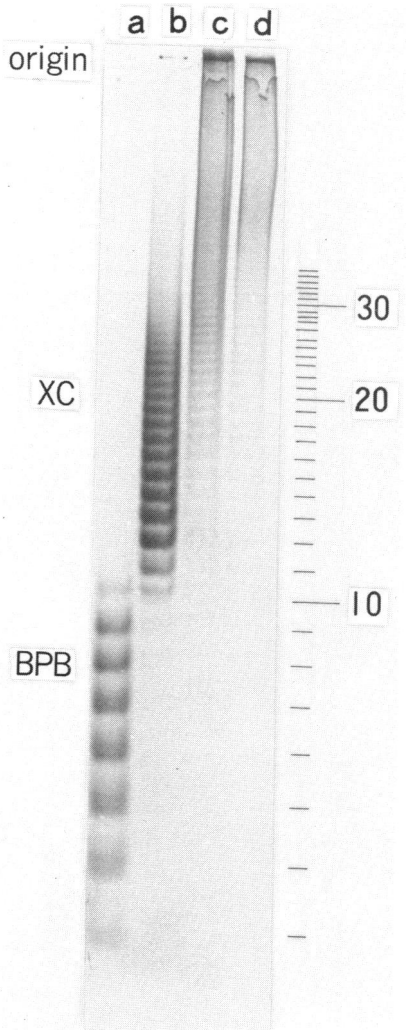

FIGURE 2. Autoradiograms of [^{14}C]poly(ADP-Rib) after 16 hr electrophoresis. The samples are as follows: slot a, fraction I 2.3 × 10^4 cpm; slot b, fraction II 4.2 × 10^4 cpm; slot c, fraction III 3.3 × 10^4 cpm; slot d, fraction IV 2.2 × 10^4 cpm.

The logarithm of the number of residues was plotted against the electrophoretic mobility (Fig. 4). A smooth curve was obtained, as observed with oligo- and polynucleotides in denatured conditions (14,15).

Some portions of poly(ADP-Rib) in fractions III and IV remaining near the origin were not separated by electrophoresis

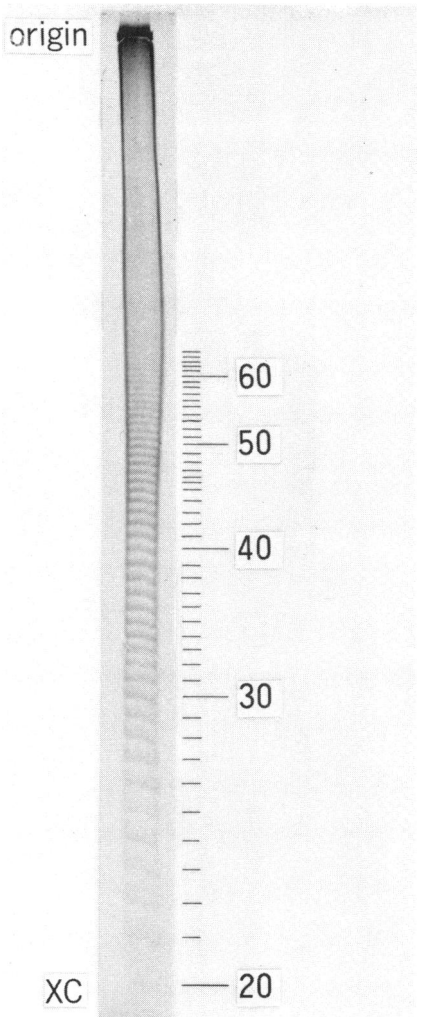

FIGURE 3. Autoradiogram of [^{14}C]poly(ADP-Rib) after 48 hr electrophoresis. The sample is fraction IV 2.2 × 10^4 cpm.

for 72 hr (data not shown); even after incubation in 0.1 N NaOH at 37°C for 1 hr, or in the presence of 7 M urea or 0.1% SDS, or on re-electrophoresis, they remained near the origin. Thus these components near the origin have intrinsically high molecular weights. When poly(ADP-Rib) of fraction III was subjected to electrophoresis for 3.5 hr on disc gel (5 × 90 mm) and then extracted from the gel, 50% of the radioactivity was found near the origin (Rm: 0-0.20). The sizes of the components near the

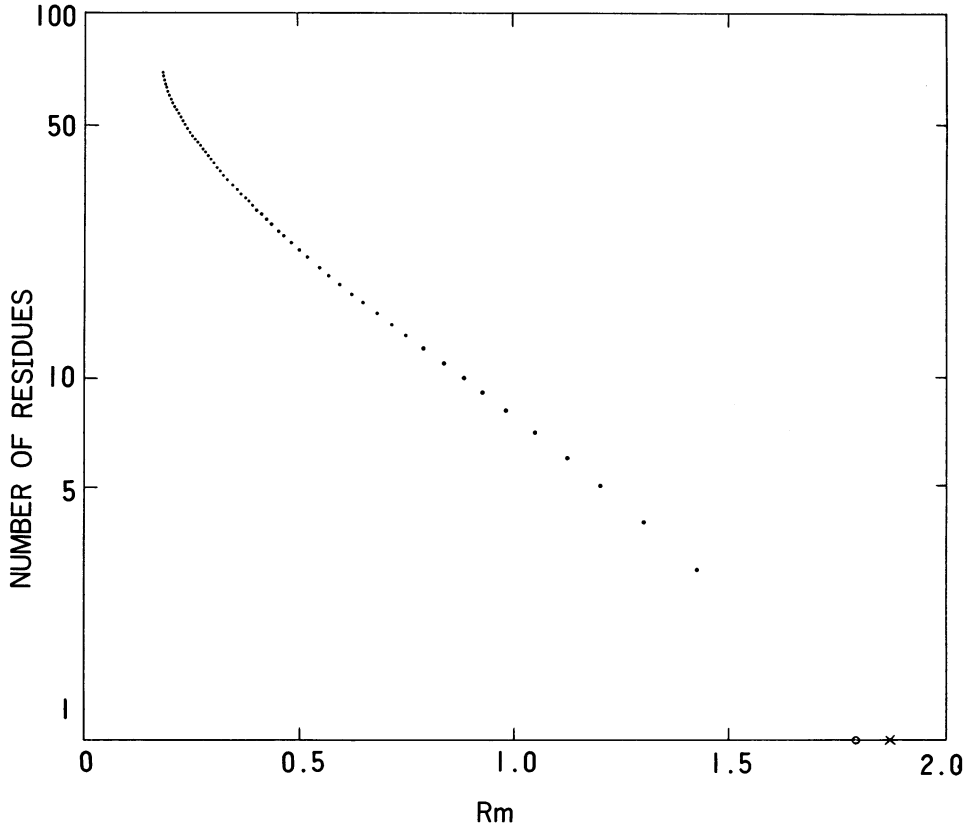

FIGURE 4. Relationship between the number of residues and relative mobility of poly(ADP-Rib). The number of residues (●) is deduced from the number of the band shown in Figs 2 and 3, assuming that $(ADP\text{-}Rib)_8$ has the same mobility as BPB (Rm=1). The mobilities of ADP-Rib and 2'-(5"-phosphoribosyl)-5'AMP are indicated as x and o, respectively.

origin were compared with those of single-stranded DNA by 5% polyacrylamide gel electrophoresis in the presence of 7 M urea (Fig. 5). Some of the poly(ADP-Rib) was again observed near the origin. By comparison of the mobilities with those of Hap digests of fd DNA, fraction III was deduced to contain molecules larger than A fragment (4.5×10^5 daltons)(Fig. 5).

The estimated average chain lengths of poly(ADP-Rib) of fractions III and IV (22 and 28, respectively) seemed to be too small, considering the presence of significant amounts of poly-(ADP-Rib)s larger than $(ADP\text{-}Rib)_{30}$ in these fractions. There-

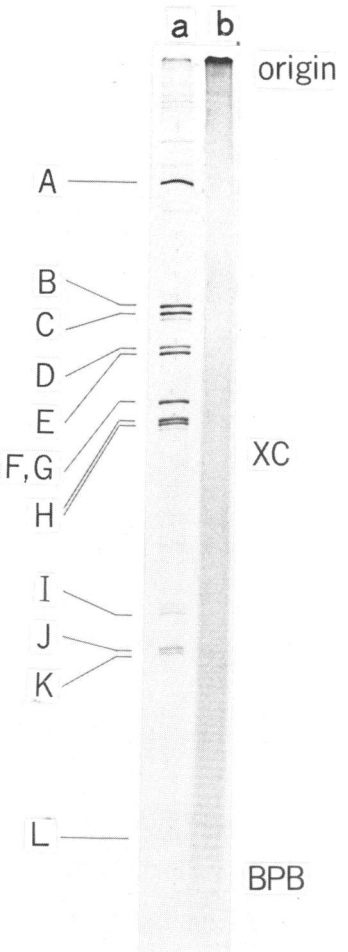

FIGURE 5. Electrophoregrams in 5% gel of poly(ADP-Rib) and Hap digests of fd DNA. Electrophoresis was performed in the presence of 7 M urea. The gel was stained with Stains-all. The samples are as follows: slot a, Hap digest of fd DNA 0.2 A_{260} unit; slot b, poly(ADP-Rib) of fraction III, 0.45 A_{260} unit (20 μg). The restricted fragments of fd DNA (A-L) are named according to the paper of Takanami et al. (12).

fore, we examined the chain length of poly(ADP-Rib) with low mobility by the conventional method. Unexpectedly, the chain lengths of the four bands with Rm values of 0.40, 0.30, 0.27 and 0.19 were 29.1, 28.7, 23.9 and 22.3, respectively, while on electrophoresis the numbers of residues in these bands were estimated as 30, 40, 45 and 65, respectively. Moreover, the

poly(ADP-Rib) remaining near the origin on electrophoresis was found to have a chain length of 23.0 (Table 1). The possibility that this discrepancy was due to aggregation was excluded by the fact that poly(ADP-Rib)s with up to 65 residues gave discrete bands on electrophoresis. Moreover, poly(ADP-Rib) had the same mobility on re-electrophoresis as on the first electrophoresis and also gave almost the same electrophoretic pattern in the presence of 7 M urea or 0.1% SDS. Thus the only explanation for this discrepancy is that poly(ADP-Rib)s larger than $(ADP\text{-}Rib)_{30}$ have a branched structure with several *A termini*.

DISCUSSION

An electrophoretic method was established that can resolve poly(ADP-Rib) by a size difference of one residue on polyacrylamide gel. Using this method the number of residues in a polymer can be measured easily and accurately. Moreover, the size distribution of molecules in a polymer composed of a population of various sized molecules can be determined. This system is useful for analysis of poly(ADP-Rib). Hydroxylapatite column chromatography has been used in previous studies to fractionate poly(ADP-Rib)s with different chain lengths. However, as shown in Fig. 2, although fractionation by hydroxylapatite column chromatography was satisfactory for poly(ADP-Rib)s with less than 20 residues, it was not effective for much larger poly(ADP-Rib)s. In contrast, polyacrylamide gel electrophoresis, which can be used to separate poly(ADP-Rib)s with different numbers of residues, gave discrete bands with poly(ADP-Rib)s of up to at least 65 residues.

The existence of such large molecules was surprising, since the largest polymer ever reported previously was $(ADP\text{-}Rib)_{29}$ (13). We also detected the existence of poly(ADP-Rib)s which seemed to have an even larger intrinsical high molecular weight than $(ADP\text{-}Rib)_{65}$, because this material consistently showed little mobility on polyacrylamide gel electrophoresis under various denaturing conditions. Comparison of the mobility of poly(ADP-Rib) with that of Hap digests of fd DNA, indicated that the maximum size of poly(ADP-Rib) is over 4.5×10^5 daltons. Previously, broad size distribution of poly(ADP-Rib) with an S value of 2S to 10S was

Table 1. Chain length of poly(ADP-Rib) fractionated by polyacrylamide gel electrophoresis

Band number*	Rm	Radioactivity of products with snake venom phosphodiesterase, cpm		Chain length‡
		total	*A terminus*+	
1	1.43	—§	—	—
2	1.31	1,523	358	4.25 (4)
3	1.20	1,195	226	5.29 (5)
4	1.12	1,943	335	5.80 (6)
5	1.05	2,334	335	6.97 (7)
6	0.98	1,614	194	8.32 (8)
7	0.92	1,751	181	9.67(10)
8	0.87	—	—	—
9	0.84	—	—	—
10	0.79	2,449	194	12.6 (13)
11	0.75	—	—	—
12	0.71	2,791	195	14.3 (14)
13	0.68	2,037	133	15.3 (15)
14	0.65	2,338	149	15.7 (16)
15	0.62	2,125	122	17.4 (17)
30	0.40	2,622	90	29.1 (29)
39-41	0.29-0.31	2,380	83	28.7 (29)
43-47	0.26-0.28	3,059	128	23.9 (24)
60-70	0.18-0.21	3,077	138	22.3 (22)
(origin)	0-0.01	10,378	452	23.0 (23)

*Poly(ADP-Rib) of bands number 1-8, 9-15 and 30-(origin) was extracted from gels after electrophoresis of fraction I, fraction II and fraction III, respectively.

+Radioactivity in the *A terminus* is the sum of that in AMP and 2'-ribosyl-AMP in the products with snake venom phosphodiesterase.

‡The nearest integer is shown in parenthesis.

§Not determined.

demonstrated on sucrose density gradient centrifugation (16,17), and the high molecular weight poly(ADP-Rib)(S value of 12S) was isolated (18). However, the molecular features of these high molecular weight poly(ADP-Rib) have not been clarified (16-18).

Poly(ADP-Rib)s with 30, 40, 45 and 65 residues were demonstrated to have chain lengths of about 30 by the conventional

method. The possibility that the molecules aggregated was excluded by the fact that they gave clear discrete bands on electrophoresis. The chain length was calculated as the ratio of total radioactivity to that derived from the *A terminus*, and thus the chain length should be smaller when a polymer had several *A termini*. The apparent discrepancy between the chain length and the size of the polymer can, therefore, be explained by supposing that poly(ADP-Rib)s have several *A termini* in a single molecule. This supposition was in agreement with the electron microscopic observations showing that poly(ADP-Rib) has a branched structure with about 30 residues per branch (manuscript in preparation). And the material indicating the branched structure, containing one mole of adenine, three moles of ribose and three moles of phosphorus, was obtained among the digested products of high molecular weight poly(ADP-Rib) with snake venom phosphodiesterase (manuscript in preparation).

In preparation of this paper, Adamietz et al. (19) reported the method for determination of chain length in poly(ADP-Rib) samples by polyacrylamide gel electrophoresis in the size range of 8-33 ADP-Rib residues. According to our system, good separation was obtained even for $(ADP\text{-}Rib)_n$ from monomer to $(ADP\text{-}Rib)_8$. Moreover, poly(ADP-Rib)s larger than $(ADP\text{-}Rib)_{30}$ were separated and detected at least up to $(ADP\text{-}Rib)_{65}$.

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ABBREVIATIONS

ADP-Rib, adenosine diphosphate ribose; poly(ADP-Rib), polymer of ADP-Rib; $(ADP\text{-}Rib)_n$, poly(ADP-Rib) with n residues; BPB, bromophenol blue; XC, xylene cyanol FF.

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