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Searcy, D. G. (1975), *Biochim. Biophys. Acta 395,*.535.
von Hippel, P. H., and McGhee, J. D. (1972), *Annu. Rev. Biochem. 41,* 231.
Wilson, D. L., and Geiduschek, E. P. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 514.

Separation of Oligo(adenosine diphosphate ribose) Fractions with Various Chain Lengths and Terminal Structures†

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ABSTRACT: Oligo(adenosine diphosphate ribose) preparations with chain lengths of 3 to 10 adenosine diphosphate ribose units were fractionated according to their chain lengths and their terminal structures by hydroxylapatite column chromatography and then polyacrylamide gel electrophoresis. The peak fractions from the hydroxylapatite column were each separated into two distinct subfractions by gel electrophoresis. The two subfractions were found to differ in chain length and terminal structure. A linear correlation was observed between the mobility and the logarithm of the chain length of oligo(adenosine diphosphate ribose) on gel electrophoresis, irrespective of the terminal structure.

Poly(ADP-Rib)[1] can be synthesized from NAD using isolated nuclei or chromatin of mammalian cells (Chambon et al., 1966; Fujimura et al., 1967; Nishizuka et al., 1967; Sugimura, 1973). ADP-Rib moieties from NAD are linked repeatedly by ribose–ribose (1′-2′) glycosidic bonds to form poly(ADP-Rib). Poly(ADP-Rib) is suggested to bind covalently to histone (Nishizuka et al., 1968; Otake et al., 1969; Smith and Stocken, 1975) and to have a regulatory role in DNA synthesis and mitosis (Burzio and Koide, 1970; Smulson et al., 1971; Miwa et al., 1973).

ADP-ribosylation of protein gives rise to a polymer in which the ADP-Rib units are repeated 1–20 times. In the in vitro reaction, DNA and histone increase both the chain length and the number of poly(ADP-Rib) chains (Yamada and Sugimura, 1973). Addition of DNase I to the system for poly(ADP-Rib) synthesis in vitro increases the number and the chain length of poly(ADP-Rib) formed (Miller, 1975). This paper reports the separation of oligo(ADP-Rib)[2] fractions of various chain lengths and different terminal structures, using hydroxylapatite column chromatography and then polyacrylamide gel electrophoresis. The elution profiles on chromatography and on electrophoresis were reproducible. Hydroxylapatite column chromatography and/or polyacrylamide gel electrophoresis can be used to determine the distribution of oligo(ADP-Rib) fractions of various chain lengths, and to isolate oligo(ADP-Rib) of a desired chain length.

Materials and Methods

Chemicals and Enzymes. NMN, ATP, and *Escherichia coli* alkaline phosphomonoesterase (EC 3.1.3.1) were purchased from Sigma Chemicals Co., St. Louis, Mo. Deoxyribonuclease I (EC 3.1.4.5), pancreatic ribonuclease (EC 3.1.4.22), and snake venom phosphodiesterase (EC 3.1.4.1) were obtained from Worthington Biochemical Corp., Freehold, N.J. Snake venom phosphodiesterase was purified to remove 5′-nucleotidase (EC 3.1.3.5), by the procedure of Sulkowski and Laskowski (1971). Pronase E was purchased from Kaken Chemicals, Tokyo. [*adenine*-8-^{14}C]ATP (47 mCi/mmol) was a product of Schwarz/Mann, Orangeburg, N.Y. Acrylamide and *N,N′*-methylenebisacrylamide were products of Wako Pure Chemical Industries, Ltd., and Seikagaku Kogyo Co., Ltd., Tokyo, respectively. Temed was from Eastman Kodak Co., Rochester, N.Y., and Bio-Gel P-2 and Bio-Gel HTP were from Bio-Rad Laboratories, Richmond, Calif.

Preparation of Oligo(ADP-Rib). Oligo(ADP-Rib) and poly(ADP-Rib) were prepared from [*adenine*-8-^{14}C]ATP and nicotinamide mononucleotide using isolated rat liver nuclei, and purified as described by Sugimura et al. (1971), with the following modification in order to preserve oligo(ADP-Rib). The aqueous layer obtained after the second phenol extraction was subjected to gel filtration on a Bio-Gel P-2 column (2 × 40 cm) equilibrated with 1 mM sodium phosphate buffer (pH 6.8). Oligo(ADP-Rib) was separated from poly(ADP-Rib) by hydroxylapatite column chromatography as described in the following paragraph.

Hydroxylapatite Column Chromatography. Bio-Gel HTP was suspended in 1 mM sodium phosphate buffer (pH 6.8) and packed in a column (1 × 10 cm). The sample (300–400 OD_{260}

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[1] The abbreviations used are: ADP-Rib, adenosine diphosphate ribose; oligo(ADP-Rib), oligo(adenosine diphosphate ribose); poly(ADP-Rib), poly(adenosine diphosphate ribose); Ado(P)-Rib-P, 2′-(5″-phosphoribosyl)-5′-AMP; Ado(P)-Rib, 2′-(ribosyl)-5′-AMP; Ado-Rib-P, 2′-(5″-phosphoribosyl)adenosine; Rib-P, 5′-phosphorylribose; BPB, bromophenol blue; Temed, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] Poly(ADP-Rib) is here defined as polymer containing more than 11 ADP-Rib units and oligo(ADP-Rib) as polymer containing 2 to 10 ADP-Rib units.

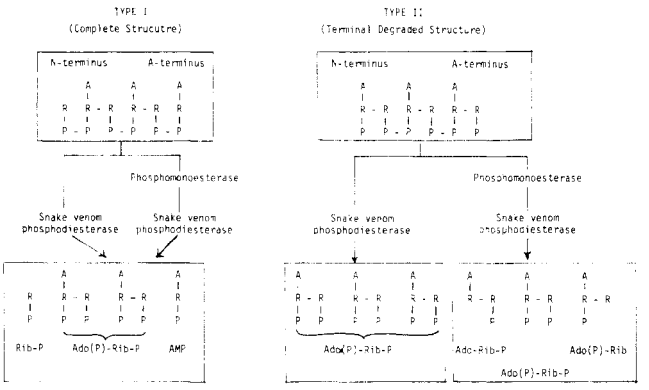


FIGURE 1: Scheme of determination of terminal structure and chain length. The symbols A, R, and P correspond to the adenine, ribose, and phosphate moiety of oligo(ADP-Rib), respectively. Adenine was labeled with ^{14}C at position 8 of the adenine ring.

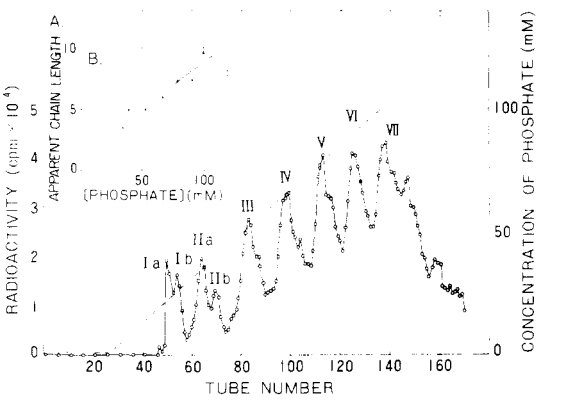


FIGURE 2: (A) Hydroxylapatite column chromatography of oligo(ADP-Rib). Experimental conditions are described under Materials and Methods. The radioactivity and phosphate ion concentrations are indicated as O—O and - - - , respectively. (B) (Inset) Relationship between chain length of oligo(ADP-Rib) and phosphate concentrations at the peak of each fraction.

units) containing oligo(ADP-Rib) and poly(ADP-Rib) was applied to the column. The column was washed with 50 mL of 1 mM sodium phosphate buffer (pH 6.8) and oligo(ADP-Rib) was eluted with 300 mL of a linear concentration gradient of 1 to 100 mM sodium phosphate buffer (pH 6.8), at a flow rate of 15 mL/h. Fractions of 2.5 mL were collected.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out by a slight modification of the method of Peacock and Dingman (1967). The gels used in this study consisted of 25% polyacrylamide (acrylamide–*N,N'*-methylenebisacrylamide, 19:1), 0.09 M Tris–borate buffer (pH 8.3), 25 mM EDTA, 2.2 mM ammonium persulfate, and 13 mM Temed. Gels were poured to a height of 9 cm in glass tubes (0.5 × 10 cm) and pretreated at 90 V for 2 h at room temperature. Samples (15 μL) were mixed with 15 μL of 50% glycerol containing BPB and applied to the gels. Electrophoresis was performed at 90 V, 0.7 mA/tube, for 4 h at room temperature. BPB migrated about 2.5 cm under these conditions. After electrophoresis, gels were cut into 1-mm slices, and the radioactivity was extracted with distilled water containing 0.02% sodium azide on standing overnight at room temperature. The yield of extraction was about 70% of the radioactivity applied to the gel. The mobilities of oligo(ADP-Rib) are expressed as relative mobilities (R_m) to that of BPB.

Determination of the Terminal Structures of Oligo(ADP-Rib). The terminus having the AMP portion of the ADP-Rib unit of complete oligo(ADP-Rib) (type I) is designated as the A terminus and the other terminus as the N terminus.

As shown later, two different structures of each terminus were obtained. Two examples of several possible structures of oligo(ADP-Rib) with a chain length of three are shown in Figure 1. If complete oligo(ADP-Rib) (type I) is digested with venom phosphodiesterase, regardless of previous digestion with alkaline phosphomonoesterase, the products will be 1 molecule of Rib-P from the N terminus, 2 molecules of Ado(P)-Rib-P, and 1 molecule of AMP from the A terminus. If terminal degraded oligo(ADP-Rib) (type II) is digested with venom phosphodiesterase, the products will be 3 molecules of Ado(P)-Rib-P with no AMP. Previous digestion with alkaline phosphomonoesterase, however, will produce 1 molecule of Ado-Rib-P from the N terminus, 1 molecule of Ado(P)-Rib-P, and 1 molecule of Ado(P)-Rib from the A terminus.

Thus, using combinations of these two enzymes, the A-terminal and N-terminal structures of mixtures of type I, type II, and other possible structures can be determined. The number of A termini can be estimated as the total number of AMP and Ado(P)-Rib. As the number of N termini should be equal to the number of A termini, the number of Rib-P from N termini (Figure 1) was calculated as the number of A termini minus the number of Ado-Rib-P liberated from the N terminus of terminal degraded oligo(ADP-Rib) (type II).

Terminal dephosphorylation with alkaline phosphomonoesterase (15 μg/mL) was done by the method of Matsubara et al. (1970a) and then the preparation was subjected to complete digestion with snake venom phosphodiesterase (12.5 μg/mL) (Figure 1). The digestion products were analyzed by paper chromatography with a mixture of 0.1 M sodium phosphate (pH 6.8), ammonium sulfate, and 1-propanol (100:60:2, v/w/v) as solvent. After development, the paper (No. 51A of Toyo Roshi Co., Ltd., Tokyo) was cut into 2-cm strips and the radioactivity of each strip was counted. Ado-Rib-P, AMP, Ado(P)-Rib, and Ado(P)-Rib-P, which have different mobilities (Shima et al., 1969), were identified by their mobilities relative to that of adenosine, AMP, or ATP. Though Ado(P)-Rib and AMP have different mobilities, they were not clearly separated from each other. Therefore, the content of Ado(P)-Rib was calculated by subtracting the AMP content, from the sum of the contents of Ado(P)-Rib and AMP. The content of AMP was measured exactly in samples digested with snake venom phosphodiesterase alone (see Results), and the sum of the contents of Ado(P)-Rib and AMP was determined in samples treated with alkaline phosphomonoesterase.

Calculation of Chain Length. The chain length is expressed as the number of adenine residues per chain. It was calculated by the following equation.

$$\text{chain length} = \frac{[\text{Ado(P)-Rib-P}] + [\text{Ado-Rib-P}]}{[\text{AMP}] + [\text{Ado(P)-Rib}]} + 1$$

Estimation of Phosphate. Phosphate was estimated by the method of Josse (1966).

Results

Hydroxylapatite Column Chromatography. Figure 2A shows the typical pattern of oligo(ADP-Rib) on hydroxylapatite column chromatography. All the oligo(ADP-Rib) and poly(ADP-Rib) were adsorbed to the column and oligo(ADP-Rib) was eluted in seven radioactive fractions with a linear concentration gradient of 1–100 mM sodium phos-

TABLE I: Properties of Oligo(ADP-Rib) Fractionated by Hydroxylapatite Column Chromatography.

Fraction	Concn of Phosphate at Fraction Peak (mM)	Previous Treatment with Alkaline Phosphomonoesterase	Radioact. of Products with Snake Venom Phosphodiesterase[a]				Apparent Chain Length
			From N Terminus		From A Terminus		
			Ado-Rib-P	Ado(P)-Rib-P	Ado(P)-Rib	AMP	
IIa	34	−	1.4	88.4	0.0	10.2	
		+	19.8	51.0	19.0	10.2	3.4
IIb	38	−	4.9	76.2	0.0	18.9	
		+	14.9	64.4	1.7	18.9	4.9
III	52	−	0.6	90.7	0.0	8.7	
		+	16.4	63.8	11.1	8.7	5.1
IV	66	−	0.4	93.5	0.0	6.1	
		+	12.5	70.9	10.5	6.1	6.0
V	78	−	0.0	94.9	0.0	5.1	
		+	10.4	75.9	8.6	5.1	7.3
VI	90	−	0.2	94.5	0.0	5.3	
		+	9.5	77.0	8.2	5.3	7.4
VII	100	−	0.2	95.4	0.0	4.4	
		+	8.7	80.8	6.1	4.4	9.6

[a] Percentages of the total radioactivity are shown.

phate buffer (pH 6.8). Poly(ADP-Rib) was eluted with a much higher phosphate buffer concentration. The seven radioactive peaks in the chromatogram, designated as fractions I to VII in Figure 2A, were not symmetrical and each had a shoulder behind the peak. In peaks I and II this shoulder appeared as a small peak (Ia, Ib, IIa, and IIb in Figure 2A).

The reproducibility of this hydroxylapatite column chromatography was good: for example, fraction III, eluted with 52 mM phosphate buffer in the first column chromatography, was eluted, as a single peak with 54 mM phosphate buffer on rechromatography. On rechromatography, each of the other fractions was also eluted as a single peak, at nearly the same phosphate buffer concentration as in the first chromatography. The specific activities of fractions II–VII, i.e., the ratio of radioactivity to A_{260}, were 30–50% of the values calculated from the specific activity of the [^{14}C]ATP used. Therefore, these fractions were still contaminated with oligonucleotides, even though the purification involved treatments with DNase and RNase. Fraction I was heavily contaminated, judging from its specific activity, so that it was not characterized further.

The oligo(ADP-Rib) fractions were eluted from the hydroxylapatite column in order of increasing chain length, as shown in the last column of Table I. A linear relationship was observed between the apparent chain lengths of oligo(ADP-Rib) and the concentrations of phosphate buffer at the peaks of the fractions, as shown in Figure 2B.

The 6th and 7th columns of Table I show that all the fractions except fraction IIb had two types of A terminus. The heterogeneity in each oligo(ADP-Rib) fraction was also suspected from the fact that each peak had a shoulder on hydroxylapatite column chromatography. Therefore these fractions were subjected to polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. All the fractions from the hydroxylapatite column except fractions IIb and VII were separated into two peaks on electrophoresis, as shown in Figure 3. The faster running peak was designated as subfraction A, and the slower one as subfraction B. The R_m value of fraction IIaB corresponded to that of fraction IIb. Therefore, fraction

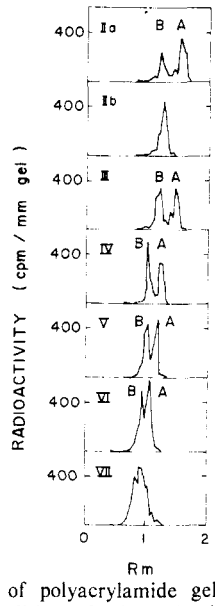


FIGURE 3: Patterns of polyacrylamide gel electrophoresis of oligo(ADP-Rib). The conditions of polyacrylamide gel electrophoresis are described under Materials and Methods. Each column represents the pattern of one fraction obtained by hydroxylapatite column chromatography.

IIaB probably represents cross-contaminating fraction IIb, and this is supported by results on its chain length and terminal structure (Table II). Fraction VII was not well separated in this condition.

The radioactive fractions obtained by electrophoresis were eluted from the gel, and the chain length of each oligo(ADP-Rib) was determined. Their R_m values, products with snake venom phosphodiesterase, and chain lengths are summarized in Table II. Each subfraction had a chain length very close to an integer. Moreover, the chain length of subfraction A was always 2 residues shorter than that of subfraction B of the same fraction. The chain lengths of the subfractions in A increased in integer order from fraction IIa to VI. Those of the sub-

TABLE II: Properties of Oligo(ADP-Rib) Fractionated by Polyacrylamide Gel Electrophoresis.

Fraction	R_m	Previous Treatment with Alkaline Phosphomonoesterase	Radioact. of Products with Snake Venom Phosphodiesterase				Chain[a] Length
			From N Terminus		From A Terminus		
			Ado-Rib-P	Ado(P)-Rib-P	Ado(P)-Rib	AMP	
IIaA	1.56	−	2.3	93.2	4.5	0.0	
		+	28.5	36.0	35.5	0.0	2.8 (3)
IIaB	1.22	−	0.4	81.5	0.0	18.1	
		+	4.2	76.5	1.3	18.1	5.2 (5)
IIb[b]	1.27	−	4.9	81.1	0.0	18.9	
		+	17.2	62.1	1.8	18.9	4.8 (5)
IIIA	1.33	−	0.0	100.0	0.0	0.0	
		+	24.2	50.7	25.1	0.0	4.0 (4)
IIIB	1.07	−	6.3	77.1	0.0	16.6	
		+	10.3	73.0	0.1	16.6	6.0 (6)
IVA	1.16	−	1.4	97.0	1.6	0.0	
		+	20.1	60.7	19.2	0.0	5.2 (5)
IVB	0.96	−	0.3	88.1	0.0	11.6	
		+	7.8	77.3	3.3	11.6	6.7 (7)
VA	1.10	−	3.6	91.5	4.9	0.0	
		+	15.0	68.6	16.4	0.0	6.1 (6)
VB	0.94	−	2.0	89.6	0.0	8.4	
		+	6.6	80.9	4.1	8.4	8.0 (8)
VIA	0.96	−	2.1	95.1	2.8	0.0	
		+	12.8	73.3	13.9	0.0	7.2 (7)
VIB	0.85	−	1.4	91.5	0.0	7.1	
		+	5.3	83.2	4.4	7.1	8.7 (9)
VII	0.75	−	0.2	95.4	0.0	4.4	
		+	8.7	80.8	6.1	4.4	9.6 (10)

[a] The nearest integer is shown in parentheses. [b] Fractions IIb and VII consisted of single peaks.

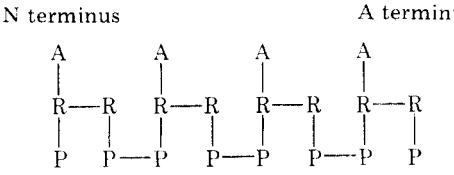
fractions in B also increased in this way. The terminal structures of subfraction A and subfraction B differed (Table II), but a linear relationship was observed between the logarithm of the chain length (L) and the R_m value, irrespective of the terminal structure. The relationship could be expressed as follows:

$$R_m = 2.24 - 1.50 \log L \qquad (3 \leq L \leq 10)$$

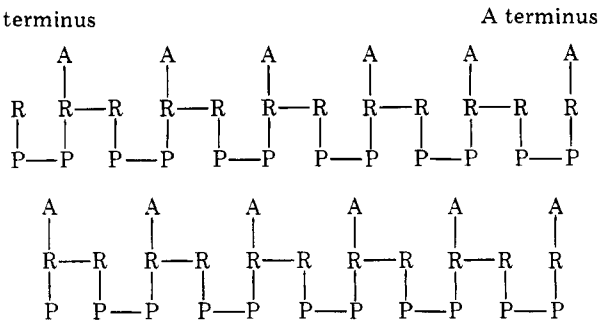
Subfraction A and subfraction B of fraction III were subjected to reelectrophoresis. Subfraction A gave a single peak with a R_m value of 1.35, which was very close to the value of 1.33 obtained in the first electrophoresis. Subfraction B also gave a single peak with a R_m value of 1.42, which was identical with that in the first electrophoresis. Very good reproducibility was also observed on reelectrophoresis of subfractions A and B of other fractions.

Terminal Structure of Oligo(ADP-Rib). The terminal structures of the subfractions of oligo(ADP-Rib) obtained by polyacrylamide gel electrophoresis were determined as described in the Materials and Methods. The products of oligo(ADP-Rib) obtained by digestion with snake venom phosphodiesterase were AMP and Ado(P)-Rib-P, with small amounts of Ado(P)-Rib and Ado-Rib-P (Table II). Therefore, the A- or N-terminal structure of oligo(ADP-Rib) must be one of the structures represented as type I or type II in Figure 1. Oligo(ADP-Rib), in which terminal phosphomonoesters of type II had been removed, should not be present in these preparations. The A- and N-terminal structures of oligo(ADP-Rib) in fraction IIIA and fraction IIIB were determined as follows.

Fraction IIIA yielded only Ado(P)-Rib-P on digestion with snake venom phosphodiesterase without previous treatment with alkaline phosphomonoesterase, as shown in Table II. On treatment with alkaline phosphomonoesterase and then snake venom phosphodiesterase, it yielded Ado-Rib-P and Ado(P)-Rib in addition to Ado(P)-Rib-P. The ratio of Ado-Rib-P to Ado(P)-Rib-P to Ado(P)-Rib was 1:2:1, so that the only possible structure of subfraction A was an oligomer of Ado(P)-Rib-P; the terminal structure of fraction IIIA was that of type II in Figure 1. The complete structure of fraction IIIA was thus concluded to be:



The product from the A terminus of fraction IIIB after snake venom phosphodiesterase digestion either with or without previous treatment with alkaline phosphomonoesterase was AMP (Table II). The A-terminal structure of fraction IIIB was that of type I in Figure 1. The amount of Rib-P in the N terminus (the complete structure of oligo(ADP-Rib)) was calculated as described in Materials and Methods and it was found that the ratio of Rib-P to Ado-Rib-P was 6.4:10.3. The N-terminal structure of fraction IIIB was a mixture of those of types I and II in Figure 1. The complete structure of fraction IIIB was thus concluded to be:



The terminal structures of the other fractions were estimated in the same manner as those of fractions IIIA and B (Table II).

Discussion

In this work a good procedure was developed for separation of oligo(ADP-Rib) according to chain length and terminal structure. The procedure involved hydroxylapatite column chromatography and then polyacrylamide gel electrophoresis: the fractions obtained by hydroxylapatite column chromatography were separated into two subfractions by polyacrylamide gel electrophoresis. Differences were found in the chain lengths and terminal structures of these two subfractions. Using a combination of these two methods, oligo(ADP-Rib) with a certain chain length and terminal structure can be isolated or analyzed.

From calculations of molecular charge it was concluded that the separation of oligo(ADP-Rib) on hydroxylapatite column chromatography was mainly due to differences in molecular charge. Each fraction from the hydroxylapatite column contained about equal amount of two components (subfraction A and subfraction B) of oligo(ADP-Rib) (Figure 3). This will be the reason why a linear correlation was observed between the apparent chain length of oligo(ADP-Rib) and the concentration of phosphate buffer required for its elution from the column. If it is possible to prepare oligo(ADP-Rib) with a homogeneous terminal structure, i.e., either type I or type II only, by inhibiting the activity of the degrading enzyme, it will be possible to prepare oligo(ADP-Rib) of a certain chain length by hydroxylapatite column chromatography alone.

Polyacrylamide gel electrophoresis is known to be a good method for separation of oligodeoxyribonucleotides and poly(A) with widely different chain lengths (Maniatis et al., 1975; Pinder and Gratzer, 1974). Oligo $d(pTpA)_{3-30}$ are separated in integer order using 20% polyacrylamide (Jovin, 1971). In this work we found that polyacrylamide gel electrophoresis was also a good method for separation of oligo(ADP-Rib) for analytical and preparative purposes. Electrophoretic mobility is known to be affected by molecular charge, size, and shape (Jovin, 1971). In our system, molecular size seemed to be the main factor in the separation, for the mobility did not depend upon the terminal structure.

In this work the N terminus was assumed not to be bound to nuclear protein components, because the linkage between oligo(ADP-Rib) and nuclear protein was reported to be labile at pH 8.0 (Nishizuka et al., 1969). In this work oligo(ADP-Rib) was prepared at pH 8.0 (Sugimura et al., 1971). This procedure should be useful for analyzing the binding of oligo(ADP-Rib) and nuclear proteins, if oligo(ADP-Rib) is prepared at a lower pH, e.g., pH 5 (Sugimura, 1973).

Half the oligo(ADP-Rib) prepared here was the type II oligomer (terminal degraded structure) shown in Figure 1. The type I oligomer (complete structure) may be produced either by the process of chain elongation or by degradation of preformed poly(ADP-Rib) by ribose-ribose bond splitting enzyme (Miwa et al., 1974). The type II oligomer seemed to be produced from intact poly(ADP-Rib) by endonucleolytic hydrolysis of the pyrophosphate bond with phosphodiesterase. Phosphodiesterase from rat liver hydrolyzing poly(ADP-Rib) has been reported, but it hydrolyzes poly(ADP-Rib) exonucleolytically (Futai et al., 1968), from the A terminus (Matsubara et al., 1970b). The presence of the type II oligomer suggests the existence of another new phosphodiesterase hydrolyzing poly(ADP-Rib) endonucleolytically, like snake venom phosphodiesterase (Matsubara et al., 1970a) or exonucleolytically from both termini. Further investigations on this are necessary.

References

Burzio, L., and Koide, S. S. (1970), *Biochem. Biophys. Res. Commun. 40,* 1013.

Chambon, P., Weill, J. D., Doly, J., Strosser, M. T., and Mandel, P. (1966), *Biochem. Biophys. Res. Commun. 25,* 638.

Fujimura, S., Hasegawa, S., and Sugimura, T. (1967), *Biochim. Biophys. Acta 134,* 496.

Futai, M., Mizuno, D., and Sugimura, T. (1968), *J. Biol. Chem. 243,* 6325.

Josse, J. (1966), *J. Biol. Chem. 241,* 1938.

Jovin, T. M. (1971), *Methods Enzymol. 21,* 179.

Maniatis, T., Jeffrey, A., and deSande, H. (1975), *Biochemistry 14,* 3787.

Matsubara, H., Hasegawa, S., Fujimura, S., Shima, T., Sugimura, T., and Futai, M. (1970a), *J. Biol. Chem. 245,* 3306.

Matsubara, H., Hasegawa, S., Fujimura, S., Shima, T., Sugimura, T., and Futai, M. (1970b), *J. Biol. Chem. 245,* 4317.

Miller, E. G. (1975), *Biochem. Biophys. Res. Commun. 66,* 280.

Miwa, M., Sugimura, T., Inui, N., and Takayama, S. (1973), *Cancer Res. 33,* 1306.

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

Nishizuka, Y., Ueda, K., Honjo, T., and Hayaishi, O. (1968), *J. Biol. Chem. 243,* 3765.

Nishizuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O. (1967), *J. Biol. Chem. 242,* 3164.

Nishizuka, Y., Ueda, K., Yoshihara, K., Yamamura, H., Takeda, M., and Hayaishi, O. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 781.

Otake, H., Miwa, M., Fujimura, S., and Sugimura, T. (1969), *J. Biochem. (Tokyo) 65,* 145.

Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6,* 1818.

Pinder, J. C., and Gratzer, W. B. (1974), *Biochim. Biophys. Acta 349,* 47.

Shima, T., Hasegawa, S., Fujimura, S., Matsubara, H., and Sugimura, T. (1969), *J. Biol. Chem. 244,* 6632.

Smith, J. A., and Stocken, L. A. (1975), *Biochem. J. 147,* 523.

Smulson, M., Henriksen, O., and Rideau, C. (1971), *Biochem. Biophys. Res. Commun. 43,* 1266.

Sugimura, T. (1973), *Prog. Nucleic Acid Res. Mol. Biol. 13,* 127.

Sugimura, T., Yoshimura, N., Miwa, M., Nagai, H., and Nagao, M. (1971), *Arch. Biochem. Biophys. 147,* 660.

Sulkowski, E., and Laskowski, M. (1971), *Biochim. Biophys. Acta 240,* 443.

Yamada, M., and Sugimura, T. (1973), *Biochemistry 12,* 3303.