Journal of Chromatography, 330 (1985) 287–298
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 798

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF *IN VITRO* SYNTHESIZED POLY(ADP-RIBOSE) ON ION-EXCHANGE COLUMNS, SEPARATION OF OLIGOMERS OF VARYING CHAIN LENGTH AND ESTIMATION OF APPARENT BRANCHING

ALAEDDIN HAKAM* and ERNEST KUN*
The Cardiovascular Research Institute and Department of Pharmacology, The University of California, San Francisco, CA 94143 (U.S.A.)
(First received March 8th, 1985; revised manuscript received April 9th, 1985)

SUMMARY

Separated macromolecular fractions of *in vitro* synthesized poly(ADP-ribose) by liver nuclei were subjected to ion-exchange chromatography in a programmed high-performance liquid chromatographic elution system. The effects of ionic strength, pH and temperature on the separation of poly(ADP-ribose) chains were determined. Short chain oligomers (up to $n = 11$) were fractionated into individual components by baseline separation. Each fraction was analyzed for chain length. Trace amounts of Ado(P)Rib(P)Rib(P) found in phosphodiesterase digests were taken as indication of apparent branching. In phosphodiesterase digests of the shorter oligomers, besides traces of the above component, two other digestion products were also observed, presumably representing oligomer termini, one terminal fragment being dominant in short oligomers. Medium and long chain oligomers were partly resolved to individual components, and especially the long oligomers exhibited marked temperature dependent elution patterns. Apparent branching increased with increasing chain length up to about 3% for $n = 44$ and components presumably indicating termini diminished to mere traces. The adenine spectra of all fractions identified individual components.

INTRODUCTION

Structural aspects of poly(adenosine diphosphate ribose), poly(ADP-ribose), the nucleic acid like homopolymer derived from nicotinamide-adenine dinucleotide (NAD) in cell nuclei, that is covalently bound to chromatin proteins, have been reviewed[1,2]. The most conspicuous property of the isolated homopolymer is its secondary structure[3,4] which predicts helix–helix interaction between single strands. A further structural complexity has been identified by the isolation of 2′-[2″-(5‴-phosphoribosyl)-5″-phosphoribosyl]adenosine 5′-monophosphate, Ado(P)Rib(P)Rib(P),

* Recipient of the Research Career Award of the United States Public Health Service.

0021-9673/85/$03.30

as a variable, minor component of phosphodiesterase digest of poly(ADP-ribose), indicating branched structure[5,6]. The Ado(P)Rib(P)Rib(P) product has been identified in 3T3 cells and various animal tissues[7,8] and an ubiquitous occurrence of variable branching proposed. Estimation of poly(ADP-ribose) in these studies has been based on the etheno derivatives of phosphodiesterase and alkaline phosphatase digestion products[9]. Some degree of uncertainty regarding the reported analytical values is based on the apparent 10–20 fold variance between Ado(P)Rib(P)Rib(P) content of liver (on a DNA basis) as published by two different groups (compare ref. 6 with ref. 7). This discrepancy may not necessarily be related to differences in techniques only, and could reflect real biological variations which cannot be estimated at present. We have recently developed a high-performance liquid chromatographic (HPLC) method, capable of direct separation of undegraded poly(ADP-ribose), a procedure based on reversed-phase chromatography[10]. The present work is concerned with the resolution of isolated oligomeric fractions of $(ADP\text{-}ribose)_n$ into components containing discrete chains. The main purpose of the present experiments was to compare the behavior of oligomers of varying chain length at varying temperatures during elutions from an ion-exchange column while monitoring spectra. Enzymatic digestion products of the highly purified polymers, which were obtained by base hydrolysis of protein adducts, have been further analyzed and from the Ado(P)Rib(P)Rib(P) content a correlation between chain length and apparent branching was estimated. As yet structurally unidentified phosphodiesterase degradation products, presumably representing oligomer–protein end-pieces, were quantitatively isolated from short oligomers.

EXPERIMENTAL

Materials

NAD, tris(hydroxymethyl)aminomethane (Tris), proteinase K, alkaline phosphatase (from Bovine intestine Type VII-S) and ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Crotalus adamanteus venom phosphodiesterase was obtained from Pharmacia P-L Biochemicals, Inc. (Piscataway, NJ, U.S.A.), boronic acid gel (Affi-Gel 601), acrylamide and bisacrylamide from Bio-Rad (Richmond, CA, U.S.A.). [^{14}C]NAD from Amersham (Arlington Heights, IL, U.S.A.). Ammonium carbonate and potassium phosphate, both HPLC grade, were purchased from Baker and Fisher, respectively. All other chemicals were reagent grade.

HPLC

HPLC was performed with the following components: Model 6001 solvent-delivery pumps (Waters Assoc., Milford, MA, U.S.A.), Waters Model 680 gradient controller, Waters Model 730 data module and Model 1040A high-speed spectrophotometric detector (Hewlett-Packard, Santa Clara, CA, U.S.A.). Chromatographic data were stored in a Hewlett-Packard Model 9121D disc memory system and plotted by a Hewlett-Packard HP 7470A graphic plotter. The columns employed were AX100 and AX300 (Synchrom Inc., Linden, IN, U.S.A.), depending on the nature of the analyses. Column temperature was maintained by a Waters temperature control accessory.

Solvent gradients involved at least two of the following buffer systems: A, 0.05 *M* potassium phosphate (pH 4.25); B, 0.05 *M* potassium phosphate (pH 4.25), 1 *M* ammonium sulfate and 30% (v/v) methanol; C, same as B but containing 0.2% (v/v) trifluoroacetic acid (pH 2). Flow-rate was 0.6 ml/min in all cases. Chain length analyses of poly(ADP-ribose) was based on spectral analyses of emerging fractions from the AX300 column with the following solvent gradient: 100% A to 100% B in 90 min (linear) and from 100% B to 100% C (linear) in 45 min. For average chain length determination degradation products of the oligomers by phosphodiesterase were analyzed on the AX100 column with the linear gradient from 100% A to 50% B, 50% A in 45 min.

Gel electrophoresis was performed by the method reported in ref. 11.

Alkaline phosphatase dephosphorylations were performed by the method reported in ref. 12.

Phosphodiesterase digestion of poly(ADP-ribose)

In 100 μl of incubation mixture containing 50 m*M* Tris–hydrochloric acid (pH 7.8) and 5 m*M* magnesium chloride, two A_{260} units of poly(ADP-ribose) were incubated with one unit of enzyme at 37°C for 1 h. The mixture then was treated with 20 μg of proteinase K at 37°C for an additional hour. Samples were directly injected into the HPLC without further deproteinization.

The chain length of polymers, *n*, was calculated by a modification of a published formula[13] as follows

$$n = \frac{[\text{AMP}] + [\text{PR-AMP}] + [\text{Ado(P)Rib(P)Rib(P)}] + [\text{end fraction}]}{[\text{AMP}] - [\text{Ado(P)Rib(P)Rib(P)}]}$$

where PR-AMP denotes 2′-(5″-phosphoribosyl)adenosine 5′-monophosphate, and end fraction = probable attachment site to protein acceptors of as yet unknown structure (see Results and discussion and Fig. 5).

Preparation of poly(ADP-ribose)

The incubation system (300 ml total volume) for large scale polymer synthesis was composed of 100 m*M* Tris–hydrochloric acid (pH 8.5 at 25°C), 67 m*M* sucrose, 10 m*M* calcium chloride, 0.8 m*M* magnesium chloride, 0.5 m*M* dithiothreitol, 0.2 m*M* ethylenediaminetetraacetate (EDTA), 0.1 m*M* phenylmethanesulfonyl fluoride and 5 m*M* NAD containing ^{14}C-labeled NAD with a specific radioactivity of 74.9 cpm/nmol. The quantity of rat liver nuclei isolated from male Wistar rats was 3.2 mg per ml of incubation mixture. Nuclei were isolated as reported[10]. Following incubation for 60 min at 37°C in a shaking flask the reaction was terminated by addition of 32 ml of cold (4°C) 50% (w/v) trichloroacetic acid, centrifuged at 3100 *g* and the pellet washed sequentially with two 170-ml, one 75-ml and one 37.5-ml portion of cold (4°C) 5% trichloroacetic acid. The radioactivity in the final wash (supernatant) was less than 8.4% of that in the sediment. The sediment was washed three times with 37.5-ml portions of diethyl ether, and, after removal of traces of ether by vacuum, it was dissolved in 14 ml of 1 *M* sodium hydroxide, stirred for 3 h, centrifuged at 3100 *g* and the supernatant adjusted to pH 9 with 3 *M* ammonium carbonate. This treatment yields free poly(ADP-ribose) which, after subsequent purification, contains

no detectable traces of protein[4]. Proteins were removed by extraction with 800 ml of water-saturated phenol followed by centrifugation at 3100 *g*. The phenol phase was washed with 30 ml of aqueous 1 *M* ammonium carbonate, and the aqueous phases were combined, which contained 92% of the total radioactive material. Traces of phenol in the aqueous phase were removed by extraction with 100 ml of diethyl ether, and residual ether was removed by a stream of nitrogen. The resultant aqueous solution was passed through a 6 × 3 cm column (15 g) of boronate resin[10] previously washed and equilibrated with 1 *M* ammonium carbonate. At a flow-rate of 0.4 ml/min, fractions of 8 ml each were collected and monitored at 260 nm. When absorbance in the effluent reached baseline, elution with 0.5 *M* Tris–hydrochloric acid (pH 7.0) was initiated. In contrast to elution with water[3], Tris–hydrochloric acid recovered about 90% of the applied radioactivity from the boronate column. Fractions containing radioactivity were combined and passed through a Millipore membrane filter (0.1 μm pore size) to remove particles of the boronate resin, then concentrated on a Diaflo Ultrafilter (UM-2) having a molecular cut off of 1000 daltons. About 7% of the radioactivity passed through the filter representing nucleotides. The supernatant was freeze-dried and applied to a Sephadex G-50 column. Fig. 1 shows the macromolecular distribution of poly(ADP-ribose). Fractions 10–13 contain long chains, fractions 14–22 medium chains and 23–36 short chains of poly(ADP-ribose). These pooled fractions were desalted by a Diaflo Ultrafilter (UM-2), where salt removal was monitored by conductivity measurements. The purity of the polymer at this stage has been established[3,4]. The amounts of recovered polymers were: 20.3 mg of long chain, 48.2 mg of medium chain and 13.0 mg of short chain poly(ADP-ribose), as determined by spectrophotometry (A_{260nm}), using $\varepsilon = 15 \cdot 10^3$ l mol^{-1} cm^{-1} per monomeric unit. The specific radioactivity of the poly(ADP-ribose) was within 7% of that of the starting NAD, indicating no appreciable dilution by trace amounts of non-labeled endogenous NAD or polymer, or by trace amounts of other UV absorbing macromolecules. In this system 14.7% of NAD was converted to poly(ADP-ribose) as determined by acid precipitable radioactive material.

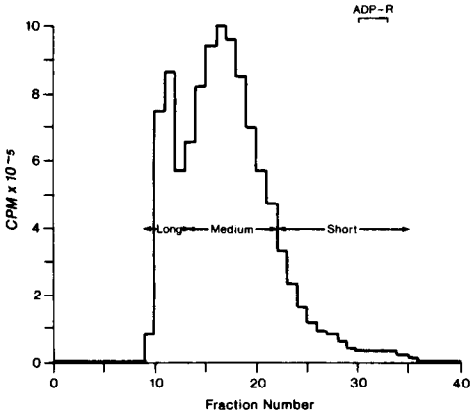

Fig. 1. Elution profile of poly(ADP-ribose) on Sephadex G-50 column (93 × 1.2 cm). The elution and equilibration buffer was 100 m*M* Tris–hydrochloric acid (pH 7.0) containing 1 *M* sodium chloride, and fraction volumes were 4 ml, flow-rate was 12 ml/h. Bracket at upper right denotes where mono ADP-ribose elutes.

For highly radioactive poly(ADP-ribose) markers (26 000 cpm per nmol) the concentration of NAD was reduced to 2.5 m*M*, without alteration of other components, as described above.

RESULTS AND DISCUSSION

When unfractionated poly(ADP-ribose), synthesized to contain high radioactivity as described in the Experimental section, was subjected to HPLC on an anion-exchange column with a pore size of 300 Å using a ternary solvent gradient, elution patterns as shown in Fig. 2 were observed. During the first 90 min of the chromatogram, in which elution is effected by increasing ionic strength (linear gradient from zero to 1.0 *M* ammonium sulfate), the early peaks show baseline separation from each other, but with longer retention times resolution decreases and is eventually lost at about 80 min. Then at 90 min, with ionic strength held constant, a linear pH gradient (0 to 0.2% trifluoroacetic acid in buffer B) with a duration of 35 min is carried out. With elution being effected by decreasing pH, a single sharp peak at 118 min is observed. This peak is most probably a family of long chain polymers which are protonated, thus made uncharged at lower pH (see buffer B), and are thus desorbed from the column at higher acidity (buffer C). Following that peak no further radioactive material was eluted.

The exact reason for the loss of resolution with increasing chain lengths is not known but it can be assumed that the ion-exchange separation is incomplete because with longer chain lengths the percentage charge difference between consecutive polymers diminishes. It is also apparent that with longer chain lengths there is a proportionately greater amount of branching in the polymer (see below), which adds to the complexity of the polyanion.

A correlation between pore size of the packing matrix and the resolution of oligomers was sought. It was found that pore size of 300 Å was optimal because at 100 Å smaller oligomers separated but resolution was lost above $n = 10$, and on the other hand between 300 and 1000 Å no improvements of resolution were detectable.

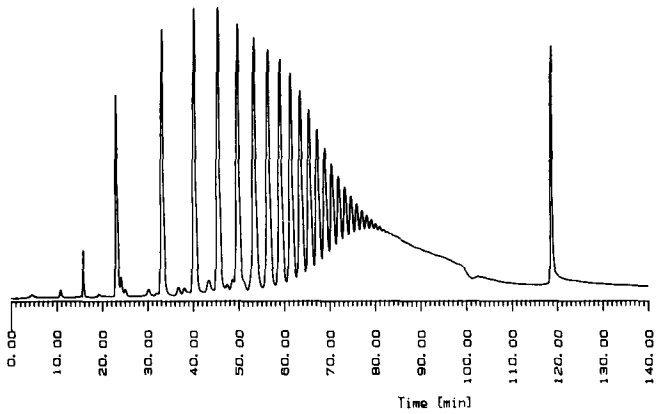

Fig. 2. Chain length analysis of unfractionated poly(ADP-ribose) (100-μl injection containing 2.50 A_{260} units) performed at 75°C monitored by UV detector, 0.184 a.u.f.s. at 260 nm on AX300 column (250 × 4.1 mm).

Therefore, as a compromise, the pore size of 300 Å was chosen for the separation of all oligomers of ADP-ribose, whereas nucleotide digestion products (by phosphodiesterase) were chromatographed on column of a pore size of 100 Å.

Further studies were undertaken on poly(ADP-ribose) that had been fractionated on a Sephadex G-50 column (see Fig. 1) into short, medium and long chains. Since the effects of temperature on the resolution and recovery of nucleic acids on ion-exchange columns are well known, the effects of varying elution temperatures on the resolution of poly(ADP-ribose) fractions were determined. Fig. 3A shows the effect of increasing temperature on the resolution of the medium chain length polymer. By increasing from 29°C (a) to 50°C (b) and to 75°C (c), better resolution is achieved, the peaks become progressively sharper yet there is no apparent change in retention times. The same effects were observed with short chain poly(ADP-ribose) (not shown). There was no evidence of degradation of poly(ADP-ribose) at the elevated temperatures.

The effect of temperature on the behavior of long chain poly(ADP-ribose), however, is different (Fig. 3B). At 29°C (a), the long chain polymer, eluting in the pattern of a broad peak (long I) and a sharp peak (long II), is obtained with relatively low recovery. On going to 50°C (b) the sharp peak shows considerable increase in intensity. Further increase in temperature to 75°C (c) partly "melts" the sharp peak into the broad peak indicating a temperature dependence of both peaks. This type of behavior is partly predictable from the structural melting curves seen with isolated polymers[3,4].

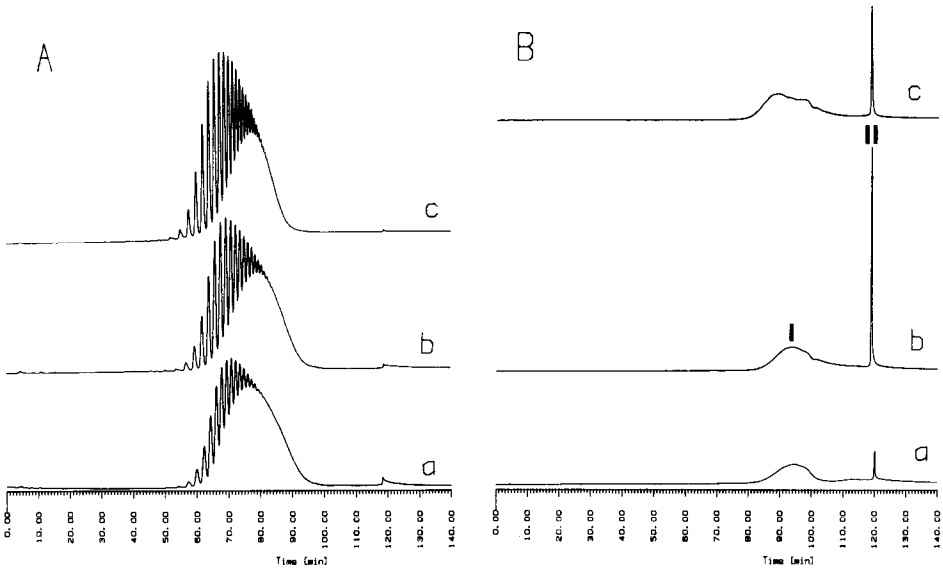

Fig. 3. Effect of column temperature on the HPLC elution pattern of (A) medium chain length and (B) long chain length poly(ADP-ribose) from AX300 column (250 × 4.1 mm) at (a) 29, (b) 50 and (c) 75°C, monitored at 260 nm. In (A) at each temperature 2.40 A_{260} units in 90 μl were injected and an ordinate of 0.500 a.u.f.s. uniformly applies to each chromatogram. In (B) at each temperature 2.24 A_{260} units in 40 μl were injected and an ordinate of 1.60 u.a.f.s. uniformly applies to each chromatogram. Note: the broad peak eluted between 80 and 105 min is designated long chain population I and the sharp peak eluting at about 120 min is designated long chain population II.

The low recovery observed at 29°C was investigated further as follows. The sharp peak (long II) in Fig. 3B was isolated and re-injected at 29°C yielding poor recovery as expected. Then, after the column had been washed and re-equilibrated with starting buffer at 29°C, the column temperature was elevated to 50°C and the elution gradient was repeated (without any further sample injection), resulting in the elution of long peak II that had been tightly absorbed at 29°C. Subsequent repetition

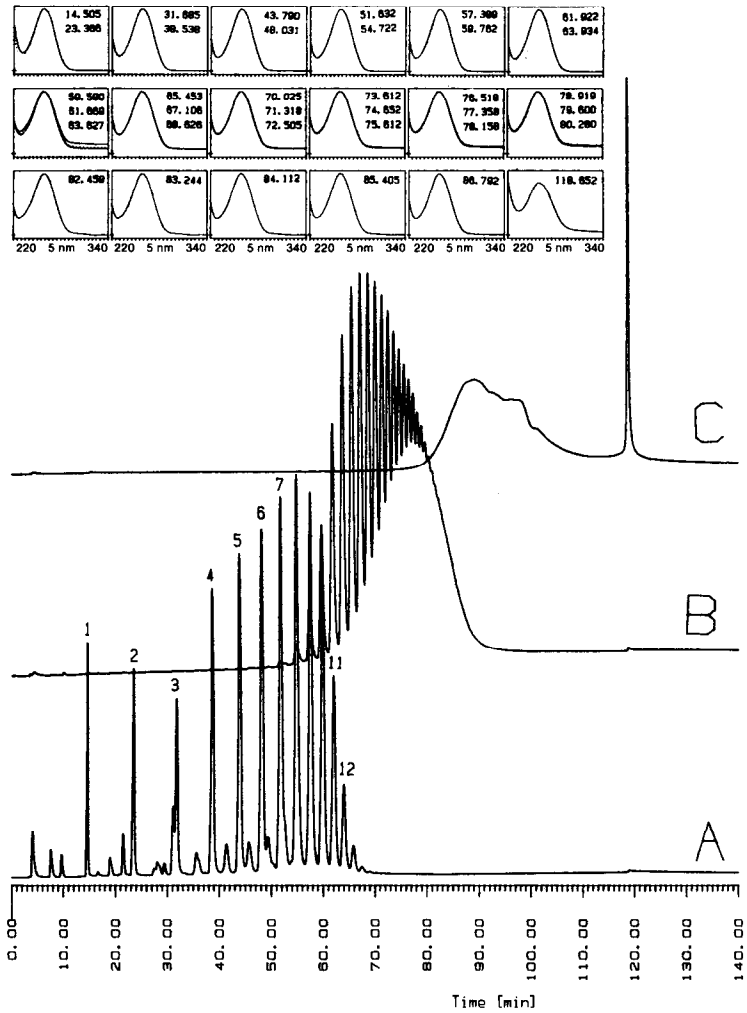

Fig. 4. HPLC analysis of (A) short, (B) medium and (C) long chain poly(ADP-ribose). Chromatography on AX300 column (250 × 4.1 mm) was performed at 75°C and monitored by UV detector at 260 nm. For (A), injection was 100 μl containing 2.70 A_{260} units, 0.290 a.u.f.s.; (B), 90 μl containing 2.40 A_{260} units, 0.195 a.u.f.s.; (c) 40 μl containing 2.24 A_{260} units, 0.374 a.u.f.s. In each chromatogram the intensity of highest peak is adjusted to full scale. Selected peaks were UV-scanned at their apexes from 220 to 340 nm, and the spectral plots are shown in the rows of insets. The top row of insets are from (A) (two superimposed in each plot), the middle row from (B) (three superimposed in each plot) and the lower row from (C) (one spectrum in each plot). In each plot the retention times are given in the upper right hand corner.

of the gradient at higher temperature (75°C) did not result in the appearance of any further UV-absorbing material. It is evident that the composite of long chain polymers can be quantitatively recovered at an appropriate temperature (50–75°C) but resolution beyond two composite peaks is not possible in this system, with the exception of long peaks I and II which can be obtained in individual fractions by the manipulation described above. It should be noted that, in contrast, recoveries for short and medium chain lengths are essentially quantitative at low temperature (29°C) as well as at higher temperatures (50–75°C).

The chain lengths of individual peaks were determined according to established methods (see Experimental and ref. 13). A composite of individual chromatograms obtained for short, medium and long polymers is shown in Fig. 4. HPLC resolution of short chains ($n_{average}$ = 6.96), that were eluted from Sephadex between fractions 23 to 36 (Fig. 1), is demonstrated in Fig. 4A, where each emerging peak was analyzed by its UV spectrum (A_{260} to A_{340}). Twelve major peaks separated (ending at retention time 63 min) and each fraction exhibited characteristic adenine absorbance shown in the inset spectra. For the short chain length group (top row of insets) two superimposed spectra were recorded in each plot and retention times are indicated in the upper right corner of the plot. Each major peak was collected and analyzed further. Peak 1 is AMP and peaks 2–4 are short oligomers (n = 2–4). Peaks 5–10 were sufficient for chain length analyses by phosphodiesterase digestion and the results of these analyses are given in Table I. Increasing chain length coincides with an increase in apparent branching from 0.34% (n = 7.1) to 0.5% (n = 11.0). Separation and spectral identification of peak fractions were carried out for medium ($n_{average}$ = 18.6, eluate fraction 14–22, see Fig. 1) and long ($n_{average}$ = 44.0, eluate fraction 10–13, see Fig. 1) size oligomers as illustrated in Fig. 4B and C, respectively. UV absorbance spectra for medium chain length peaks are shown in the middle row of insets (with three superimposed spectra in each plot) and those for long chain length

TABLE I

COMPOSITION OF PHOSPHODIESTERASE DIGESTS OF ADP-RIBOSE OLIGOMERS

a = Major terminal fragment (see Fig. 5); peak x in Fig. 5 is not included because it has no adenosine absorbance.

Sample	*% a*	*% AMP*	*% PR-AMP*	*% Ado(P)Rib(P)Rib(P)*	*Chain length*
Short chains					
Peak 5	8.15	17.38	74.46	–	5.8
Peak 6	6.22	14.35	79.09	0.34	7.1
Peak 7	5.31	12.51	81.81	0.36	8.2
Peak 8	4.85	11.08	83.65	0.41	9.3
Peak 9	3.98	10.20	85.30	0.46	10.3
Peak 10	3.40	9.59	86.49	0.50	11.0
*Medium**	1.26	6.5	91.10	1.14	18.6
*Long***	1.79	5.29	89.80	3.02	44.0
I	3.07	4.60	89.95	2.37	44.8
II	1.57	5.21	90.1	3.10	47.4

* Incompletely separated medium length fractions were analyzed in one pool.

** Long chains were pooled for analyses.

peaks shown in the lower row of insets (one spectrum per plot). In all cases the retention times are indicated in the upper right corners of the plots.

Although partial resolution into individual peaks of medium chain length poly(ADP-ribose), $n_{average} = 18.6$, is apparent and spectral identification of each peak as adenine nucleotide clearly defines the oligomers, baseline separation as obtained for short chains does not take place (Fig. 4B). However, more than eighteen individual peaks are discernable but their individual chain lengths cannot be determined because of incomplete separation (see footnotes in Table I).

As given in Table I, the average chain length of polymers eluting in long peaks I and II differ somewhat and apparent branching is increased from 2.37 to 3.1%. The most probable explanation for the behavior of long chains in this HPLC system is a simultaneous contribution of both inter-helical forces[3,4] and increased branching, producing a network of polymers that fails to be resolved on the basis of charge separation.

The evidence for branching was examined by separation of the products of phosphodiesterase digestion of poly(ADP-ribose) (Fig. 5). The lower part (5A) and upper part (5B) of the figure illustrate the resolution of digests of short and long chain polymers respectively. In both cases AMP and PR-AMP were identified, which are the result of cleavage of the terminal and internal pyrophosphate linkages respectively. The nucleotide Ado(P)Rib(P)Rib(P)* is derived from branching points in the polymer (refs. 5, 6, 8) in the long chain (5A), indicating the occurrence of multiple long chains at branching points.

In Fig. 5 peaks labeled a and x are observed in addition to the peaks identified as AMP, PR-AMP and Ado(P)Rib(P)Rib(P). Peak x, seen only in the long chain digest, does not have an adenine UV spectrum and no attempt has been made to identify this minor component. Peak a, observed in both short and long chain digests, does display an adenine UV spectrum and contains radioactivity and, therefore, was clearly derived from the polymer. It is not a degradation product formed during polymer preparation since phosphodiesterase treatment of poly(ADP-ribose) from differing biological preparations gave the same peak a components. Furthermore, it is not due to any contaminating enzymes in phosphodiesterase since treatment of PR-AMP with phosphodiesterase did not produce traces of peak a. Also, if phosphodiesterase had a phosphatase contamination the amount of peak a should be independent of chain length. In view of the arguments that internal branching, and terminal polymer fragments have been accounted for by PR-AMP, Ado(P)-Rib(P)Rib(P) and AMP respectively, it is reasonable to postulate that peak a is the fragment deriving from the protein attachment site. The existence of such termini has been suggested but no exact chemical structures have been established. We are currently preparing a quantity of peak a material for elucidation of its chemical structure by NMR spectroscopy, which will be reported elsewhere. It should be noted that we do have some additional chemical evidence bearing on its structure. If PR-AMP is subjected to limited digestion by alkaline phosphatase, a quantity of material is generated which in the HPLC elutes with the same retention time as peak a. Thus

---

* Ado(P)Rib(P)Rib(P) was identified by nuclear magnetic resonance (NMR) spectrometry, in agreement with published results[6].

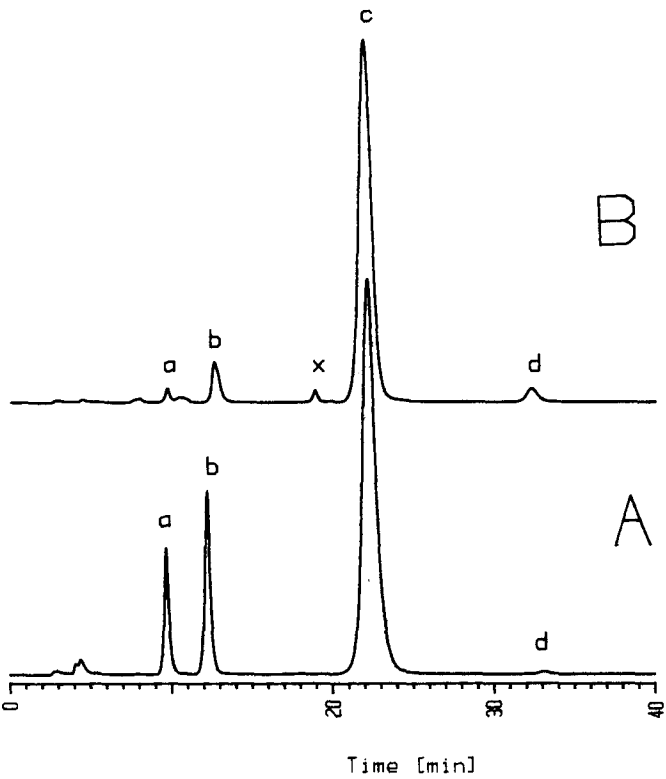

Fig. 5. HPLC analysis of phosphodiesterase digestion products of (A) short chain and (B) long chain length poly(ADP-ribose) (*cf.*, Fig. 1) on AX100 column (250 × 4.1 mm) performed at 50°C and monitored at 260 nm. In (A) 0.60 A_{260} units in 100 μl were injected with recorder at 0.907 a.u.f.s., and in (B) 1.08 A_{260} units in 100 μl, 1.509 a.u.f.s. Peaks a and x denote unknown components suspected to be terminal groups; b = AMP; c = PR-AMP; d = Ado(P)Rib(P)Rib(P).

it seems that peak a has a structure related to PR-AMP except with a phosphate group absent.

In connection with peak a and separation of poly(ADP-ribose) using anion-exchange HPLC, it is appropriate to include here an account of additional related studies. If samples of isolated short chain polymer peaks (see Fig. 4A and Table I) are subjected to gel electrophoresis, each peak resolves into two unequal bands in the gel. Thus it is evident that each polymer peak observed in the HPLC is actually composed of two sub-populations. This finding is consistent with earlier results using hydroxylapatite chromatography[11]. We have found that if a single isolated HPLC peak, *viz.*, peak 8, having the two sub-populations is treated with alkaline phosphatase and then re-injected into the HPLC, then two sub-populations are resolved (see Fig. 6A and B). Therefore, alkaline phosphatase reacts differently with one sub-population than with the other. It is reasonable to assume that the difference is explained by a terminal phosphate group present in only one of the components that is cleavable by the phosphatase. This conclusion presupposes that, before treatment with alkaline phosphatase, the two sub-populations (differing with respect to presence or absence

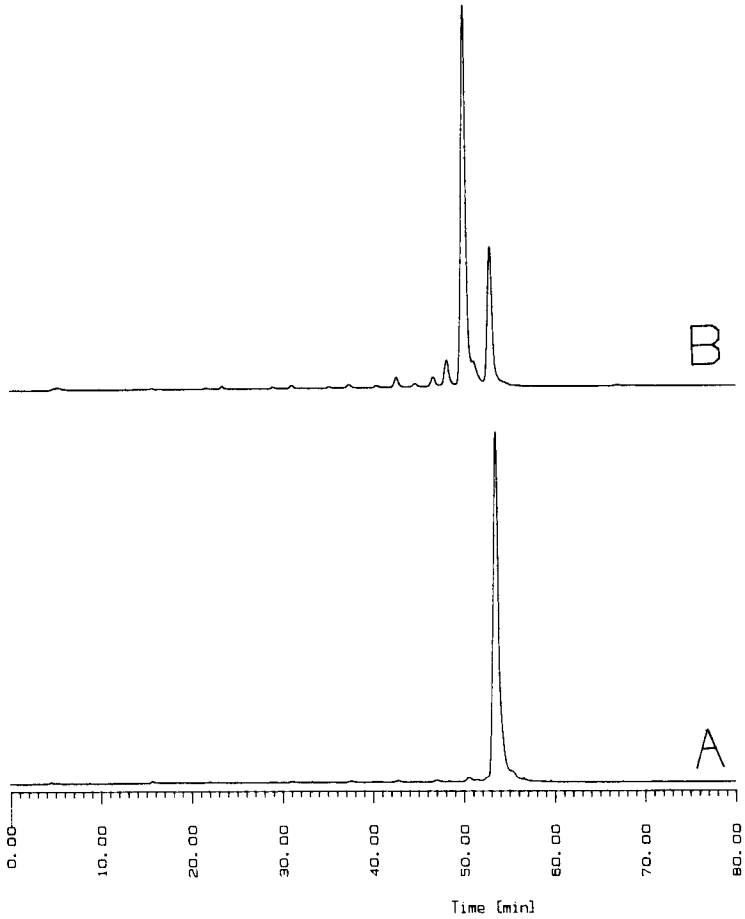

Fig. 6. HPLC analysis of isolated peak 8 from Fig. 4, (A) before and (B) after treatment with alkaline phosphatase. Chromatography was performed on AX300 column (250 × 4.1 mm) at 75°C and monitored by UV detector at 260 nm. In (A), 0.74 A_{260} units in 30 μl was injected with recorder scale at 0.653 a.u.f.s., and in (B) 0.55 A_{260} units in 100 μl, 0.358 a.u.f.s.

of a terminal phosphate group) must also differ in some other, compensatory way such as having one less or greater chain length and this results in co-migration on the anion-exchange resin and co-elution as a single peak.

ACKNOWLEDGEMENTS

This research was supported by F-49620-81-C-0007 (Air Force Office of Scientific Research) and HL-27317 (National Institute of Health). We thank Dr. Jerome McLick for helpful discussions and assistance in preparing the manuscript. The use of Tris–hydrochloric acid as an eluent for the boronate column was suggested by T. Minaga. We are indebted to Dr. N. J. Oppenheimer, UCSF School of Pharmacy, for NMR analyses.

REFERENCES

1 T. Sugimura, M. Miwa, H. Saito, Y. Kanai, I. Ikejima, M. Terado, M. Yamada and T. Utakoji, *Adv. Enzyme Regul.*, 18 (1980) 195.
2 E. Kun, T. Minaga, E. Kirsten, G. Jackowski, J. McLick, L. Peller, S. M. Oredsson, L. Marton, N. Pattabiraman and G. E. Milo, *Adv. Enzyme Regul.*, 21 (1983) 177.
3 T. Minaga and E. Kun, *J. Biol. Chem.*, 258 (1983) 725.
4 T. Minaga and E. Kun, *J. Biol. Chem.*, 258 (1983) 5726.
5 M. Miwa, N. Saikawa, Z. Yamajzumi, S. Nishimura and T. Sugimura, *Proc. Natl. Acad. Sci. U.S.A.*, 67 (1979) 595.
6 M. Kanai, M. Miwa, Y. Kuchino and T. Sugimura, *J. Biol. Chem.*, 257 (1982) 6217.
7 H. Juarez-Salinas, V. Levi, E. L. Jacobson and M. K. Jacobson, *J. Biol. Chem.*, 257 (1982) 607.
8 H. Juarez-Salinas, H. Mendoza-Alvarez, V. Levi, M. K. Jacobson and E. L. Jacobson, *Anal. Biochem.*, 131 (1983) 410.
9 J. L. Sims, H. Juarez-Salinas and M. K. Jacobson, *Anal. Biochem.*, 106 (1980) 296.
10 A. Hakam, J. McLick and E. Kun, *J. Chromatogr.*, 296 (1984) 369.
11 M. Tanaka, M. Miwa, K. Hayashi, K. Kubota, T. Matsushima and T. Sugimura, *Biochemistry*, 16 (1977) 1485.
12 J. B. Crowther, J. P. Caronia and R. A. Hartwick, *Anal. Biochem.*, 124 (1982) 65.
13 M. Kawaichi, K. Ueda and O. Hayaishi, *J. Biol. Chem.*, 256 (1981) 9483.
14 H. Kawamitsu, H. Hoshino, H. Okada, M. Miwa, H. Momoi and T. Sugimura, *Biochemistry*, 23 (1984) 3771.