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SEPARATION OF POLY(ADP-RIBOSE) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The homopolymer of ADP-ribose, poly(ADP-ribose), was synthesized *in vitro* by liver nuclei from NAD. The protein–poly(ADP-ribose) adducts were isolated and, after base hydrolysis or proteolysis by proteinase K, the free polymers were separated from NAD, ADP-ribose, AMP and adenosine, and quantitatively determined by reversed-phase chromatography on an Ultrasphere ODS 5-μm column. Oxidation of the polymer by sodium periodate and labeling with ^{3}H by borotritiation maintained the polymeric structure, but its modification was detectable by the chromatographic system employed.

INTRODUCTION

The NAD-derived nuclear polymer of eukaryotic cells, poly(ADP-ribose)[1–3] has been recently identified to exhibit a specific helical conformation[4–6]. Covalent binding of this unique nucleic acid-like macromolecule to prevalently nuclear non-histone proteins[7–9] may represent a supramolecular chromatin network system that appears to influence programmed gene expression as detected by the regulation of cellular phenotype of human fibroblasts[10]. Further study of the physiological function of poly(ADP-ribose) critically depends on specific techniques capable of determining the polymer in various cellular systems. Analytical methods which depend on the assay of the enzymatic degradation products, phosphoribosyl-AMP and AMP[11,12], cannot estimate the chain length of the polymers directly, only as a calculated average value, and it seems desirable to determine poly(ADP-ribose) without its degradation.

We have in the past employed immunochemical[7] and affinity chromatographic[8] methods for the assay of polymers in tissues. The disadvantage of these procedures is that they are relatively laborious.

In the present report we describe the development of a direct chromatographic technique for the determination of *in vitro* generated poly(ADP-ribose) on the basis of a direct separation of the polymers from nucleotides by high-performance liquid chromatography (HPLC). *In vitro* tritiation of the intact polymers provides a sensitive technique for the direct assay of unlabeled poly(ADP-ribose).

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EXPERIMENTAL

Materials

[^{14}C]NAD (269 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.), sodium [^{3}H]borohydride (270 mCi/mmol) from New England Nuclear (Boston, MA, U.S.A.), and NAD, Tris–HCl, proteinase K and sodium periodate from Sigma (St. Louis, MO, U.S.A.). Boronic acid gel (Affi-Gel 601) was obtained from Bio-Rad (Richmond, CA, U.S.A.). Ammonium carbonate and potassium phosphate, both HPLC grade were from Baker and Fisher, respectively. All other chemicals used were reagent grade. Analytical methods were the same as detailed previously[4,5].

Large scale preparation of poly(ADP-ribose) for HPLC standards

The method of the enzymatic synthesis was the same as described[4–6], except the incubation system was scaled up to 300 ml, the NAD concentration (spec. radioactivity 74.9 cpm/mmol) was increased to 5 m*M*, and the temperature of incubation was raised to 37°C for 60 min. The incubation buffer presently used was 100 m*M* Tris–HCl (adjusted to 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* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride and rat liver nuclei, prepared by a published method[13] except Triton X-100 was omitted. The concentration of nuclear protein in the incubate was 3.2 mg/ml. Further modifications were: hydrolysis of protein poly(ADP-ribose) bonds in 14 ml 1 *N* sodium hydroxide for 3 h (instead of overnight), the use of Diaflo ultrafilters with 1 kD cutoff, and the quantitative elution of poly(ADP-ribose) from the boronate resin (15 g, 3 cm × 6 cm column) with 0.5 *M* Tris–HCl (pH 7.0)* a method superior to previous procedures[4,5]. In this system, 14.7% NAD was converted into poly(ADP-ribose), with a recovery of spec. radioactivity within ±7% of the originally added labeled NAD.

Enzymatic synthesis of poly(ADP-ribose) in small incubates, used for experimental HPLC separations

For individual HPLC experiments the preparative incubate was proportionally adjusted to 0.1 ml, the NAD concentration to 2.5 m*M*, and the specific radioactivity to 1700 cpm/nmol. Variations in incubations are given under Results. Reactions were stopped either by (a) 10% perchloric acid (final conc.) at 4°C, perchlorate removed by potassium bicarbonate or (b) by proteinase K, added in two portions (100 μg/mg nuclear protein), and incubation for 2 × 20 min at 37°C. In (a) adducts were hydrolyzed by 1 *M* sodium hydroxide by incubation at 55°C for 2 h and the pH was subsequently adjusted to 6.0.

The determination of chain length by molecular filtration[7] was calibrated by quantitation of phosphoribosyl-AMP/AMP assay (after enzymatic digestion) in each elution peak by quantitative paper chromatography[14].

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* This modification was suggested by Dr. Takeyoshi Minaga.

Oxidation of poly(ADP-ribose) with sodium periodate and reduction with sodium [^{3}H]borohydride

This procedure is an adaptation of a published method[15]. To 0.1 ml of a 3.6 m*M* solution of poly(ADP-ribose) in distilled water was added 0.08 ml of 10 m*M* sodium periodate, and the mixture was incubated in the dark at room temperature for 1 h. Then 0.04 ml of potassium phosphate buffer (0.10 *M*, pH 6.8) and by 0.02 ml of a 0.10 *M* solution of sodium [^{3}H]borohydride in 0.10 *M* potassium hydroxide were added in sequence, and the mixture was incubated in the dark at room temperature for an additional hour. In order to decompose excess borotritiide, 0.10 ml of aqueous acetic acid (2.0 *M*) was added and the mixture was allowed to stand in a fume hood for 1 h. The solution was then evaporated to dryness under a stream of nitrogen at room temperature, and the residue was dissolved in 0.2 ml of distilled water to give an aqueous solution suitable for UV spectroscopy and HPLC analysis. Using sodium [^{3}H]borohydride of a specific radioactivity of 270 mCi/mmol, the specific radioactivity of the resultant tritiated poly(ADP-ribose) was in the range of 63–93 mCi/mmol, corresponding to an average of 58% of non-exchangeable ^{3}H per mol of sodium iodate-reactive residues per ADP-ribose units.

HPLC

Chromatography was performed with the following components: Waters Assoc. (Milford, MA, U.S.A.) Model 600A solvent delivery pumps, a Waters Model 680 Gradient Controller, a Waters Model 730 Data Module, a Beckman-Altex (Berkeley, CA, U.S.A.) Model 155 variabel-wavelength detector, a Radiomatic Intrument and Chemical Co. (Tampa, FL, U.S.A.) Flo-One Model HP radioactivity flow detector, and a Beckman-Altex analytical reversed-phase column (Ultrasphere ODS, 5 μm, 25 cm × 4.6 mm I.D.), with a pre-column packed with the same sorbent as the analytical column. Chromatography was carried out at ambient temperature. Prior to use, the column required pretreatment by washing with buffer C of System I (*ca.* 200 ml) at a rate of 0.5 ml/min. Two solvent gradient programs were utilized to elute poly(ADP-ribose) and/or nucleotides. System I uses three buffers: A, 0.1 *M* potassium phosphate (pH 4.25); B, the same as A but containing 20% methanol; C, 0.1 *M* potassium phosphate (pH 7.0), 1 *M* urea, and 50% acetonitrile. The flow-rate was 2.0 ml/min throughout. Upon sample injection, the gradient was started from 100% A to 100% B in 20 min, using concave gradient curve 9. Elution continued at 100% B for an additional 2 min and then a linear gradient was commenced from 100% B to 100% C during 6 min, and elution was continued at 100% C for *ca.* 10 min. System II uses two buffers: A, 0.1 *M* potassium phosphate (pH 7.0), 1 *M* urea; B, same as A but containing 50% acetonitrile, and the flow-rate was 3.0 ml/min. Upon sample injection, the gradient was started from 100% to 80% A and 0% B to 20% B in 60 min, using a linear gradient (Δ1% per 3 min).

RESULTS AND DISCUSSION

Under the preparative conditions of the polymer synthesis the preponderance of medium chains was apparent, corresponding to an average molecular weight of 5000 to 20,000, an average length of 8–30 ADP-ribose units[7,14].

The HPLC elution pattern of *in vitro* tritiated medium-size polymers by System

I is shown in Fig. 1. Apart from a ^{3}H-containing impurity (elution at 2–3 min) the polymer emerged as a symmetrical peak between 27 and 30 min. Fig. 1 also demonstrates that no contamination with nucleotides was present, since during the first 25 min of elution by System I no nucleotide was detected. The ^{3}H-labeled polymer was collected as HPLC eluate and its apparent molecular size compared to that of the parent poly(ADP-ribose) fractions, which were labeled with ^{14}C only in the adenine moiety and had not been subjected to borotritiation. As shown in Fig. 2, there is good agreement between the macromolecular profile of the tritiated fraction, isolated by HPLC, and the parent sample; therefore, the oxidation and reduction procedure had no influence on the macromolecular nature of the polymer. Borotritiation had no effect on the absorption spectrum of poly(ADP-ribose), either. However, some degree of structural modification due to the opening of the constituent ribose rings could be detected by reversed-phase HPLC of short (3–10 ADP-ribose units) and medium (8–30 ADP-ribose units) oligomers (Figs. 3a and b) in terms of changed retention times.

The specificity of the HPLC method was tested by determining the inhibitory effect of 10 m*M* benzamide (cf. ref. 10) on the *in vitro* synthesis of poly(ADP-ribose). Poly(ADP-ribose) was generated by small-scale enzymatic incubates (see Methods)

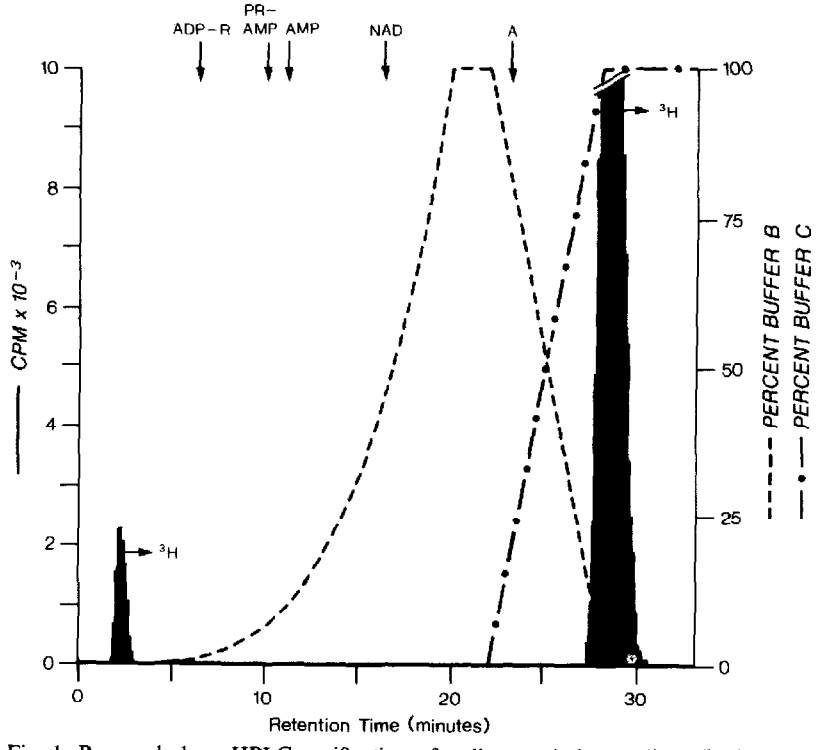

Fig. 1. Reversed-phase HPLC purification of sodium periodate–sodium [^{3}H]borohydride-treated poly(ADP-ribose). The main peak (95% of the total radioactivity) was collected and characterized (see Fig. 3). HPLC elution System I was employed. Curves: ------, % buffer B; -.-.-.-, % buffer C; shaded area = eluted material. ADP-R = adenosine diphosphoribose; PR-AMP = phosphoribosyl-AMP; A = adenosine.

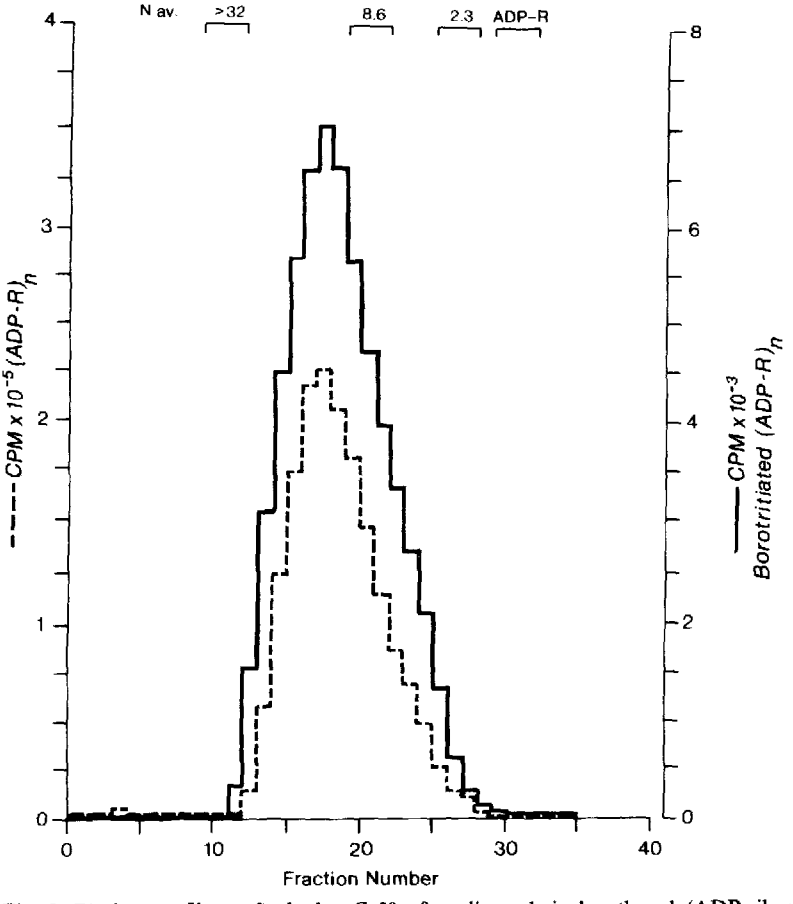

Fig. 2. Elution profile on Sephadex G-50 of medium chain length poly(ADP-ribose) before and after treatment with sodium periodate–sodium [^{3}H]borohydride. Curves: -----, before; ——, after. N_{av} = number average of ADP-ribose units.

and chromatographed using System I. The results of these experiments are shown in Fig. 4. The difference between (a) and (b) consists of the inhibition of the polymerase by benzamide exemplifying the role of the enzyme in (b), corresponding to an almost complete absence of poly(ADP-ribose). A comparison of methods capable of quantitative release to the free polymer from covalent binding to proteins is illustrated in Fig. 5. In Fig. 5a polymers were released from covalent bonds to proteins by base hydrolysis and in Figure 5b by treatment with proteinase K, and analyzed by System I. Based on the areas determined under poly(ADP-ribose) in Figs. 5a and 5b, the difference between the two methods was found to be negligible (see Table I); thus, from the viewpoint of the analytical method they are equivalent. It is of interest that the phosphoribosyl-AMP could not be detected in nuclear incubates, thus, no significant pyrophosphorolytic degradation of the polymer is apparent. The AMP and adenosine are formed from ADP-ribose, as determined in separate experiments (not shown). The elution position of authentic phosphoribosyl AMP is indicated in Fig. 1.

As shown in Table I, there is a small amount of perchloric acid-soluble

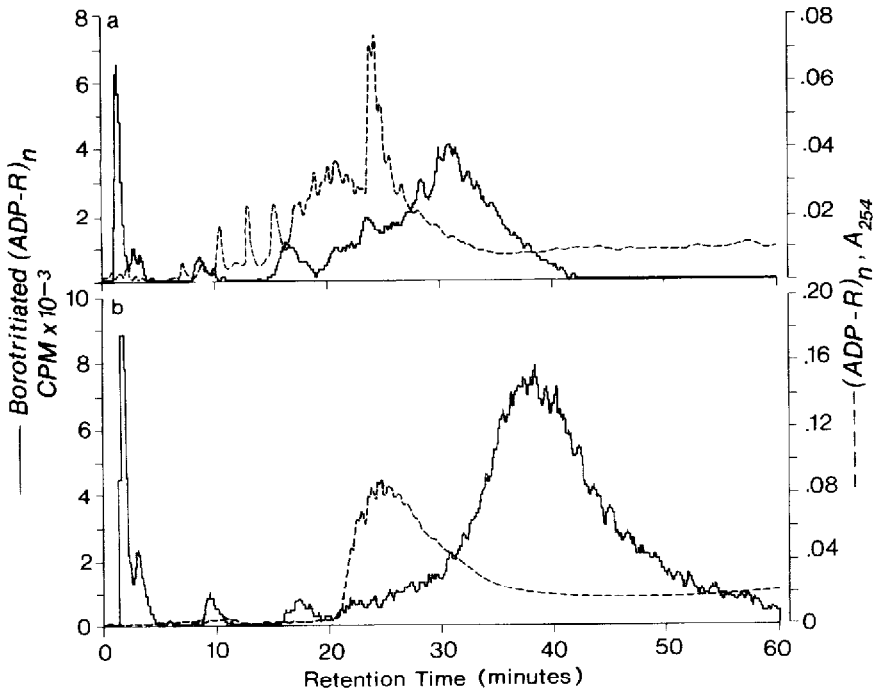

Fig. 3. Reversed-phase HPLC analysis of (a) short chain length and (b) medium chain length poly(ADP-ribose). The same samples were analyzed before (UV detection; ------ = right ordinate) and after (cpm —— = left ordinate), borotritiation, indicating that treatment with sodium periodate and sodium [^{3}H]borohydride increases retention time. In (a) $4.6 \cdot 10^5$ cpm and in (b) $1.1 \cdot 10^6$ cpm were injected, and elution System II was employed.

TABLE I

QUANTITATIVE DISTRIBUTION OF NAD, ITS ENZYMATIC PRODUCTS AND POLY(ADP-RIBOSE) DETERMINED UNDER VARYING CONDITIONS

Results are expressed as percentage conversion of NAD to products.

No.	*Experimental conditions*	*Nucleotides*				*Poly(ADP-ribose)*	
		ADP-R	*5'-AMP*	*NAD*	*Adenosine*	*Determined by HPLC*	*Determined by radioactivity of acid ppt.*
1	Perchloric acid-soluble	11.6	1.5	80.7	0.9	2.9	–
2	20-Min incubation* with benzamide	15.4	3.3	75.3	3.1	0.8	0.1
3	20-Min incubation*	23.1	2.5	53.2	2.6	14.7	12.7
4	40-Min incubation*	26.4	3.1	48.2	4.0	12.7	13.5
5	40-Min incubation followed by sodium hydroxide treatment	1.4	71.0	0.4	2.9	12.4	13.5

* Hydrolyzed by proteinase K.

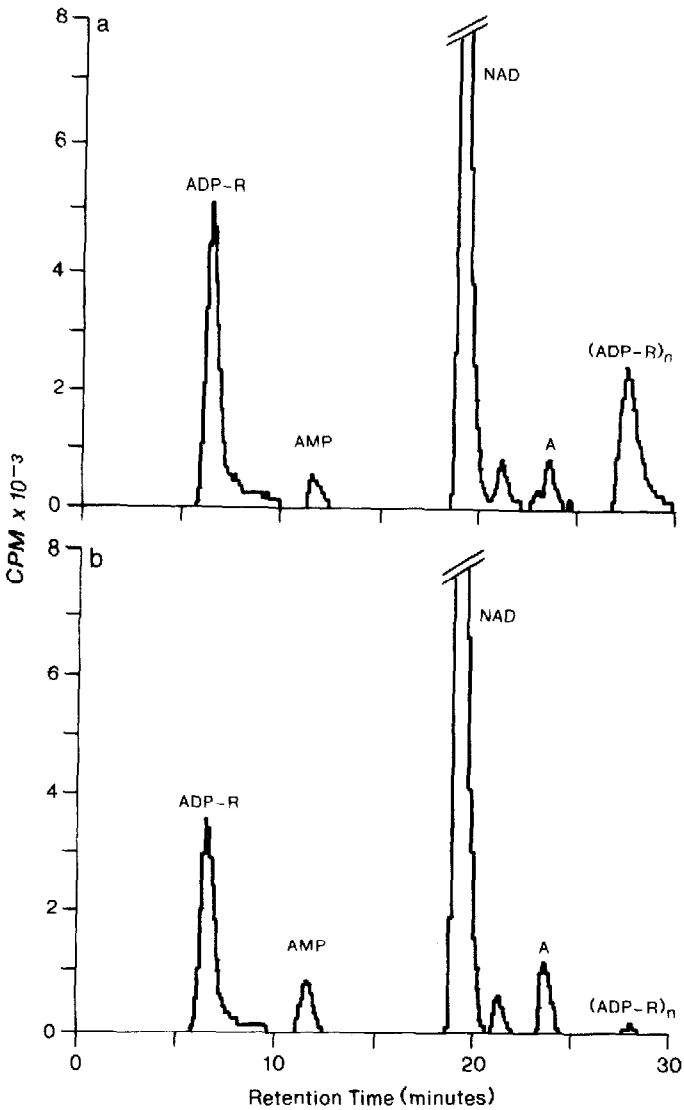

Fig. 4. Reversed-phase HPLC analysis of ^{14}C-labeled products from incubation of rat liver nuclei with [^{14}C]NAD in the absence (a) and the presence (b) of 10 m*M* benzamide. In (a) $1.74 \cdot 10^5$ cpm and in (b) $1.82 \cdot 10^5$ cpm were injected and elution System I was used. A = adenosine. There is a trace of unknown component following NAD.

poly(ADP-ribose), as determined by HPLC (No. 1). This fraction may contain oligopeptides covalently attached to the polymer, which could be the result of trace proteolysis of polymer–protein adducts. This question is under investigation. The presence of benzamide (No. 2) results in profound inhibition of polymer synthesis, determined either by HPLC or by acid precipitation. The ADP-ribose increments found under these conditions (No. 2) and after varying incubation (Nos. 3 and 4) indicate NAD-glycohydrolase activities that are probably unrelated to the polymerase reaction. When polymer–protein adducts are hydrolyzed by sodium hydroxide

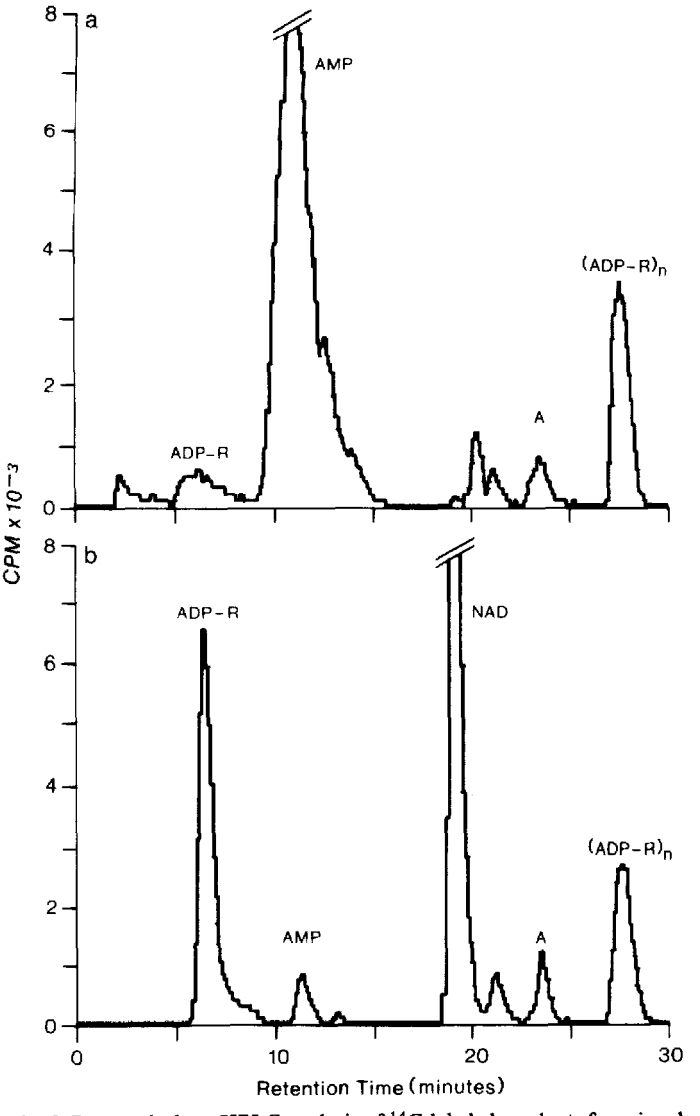

Fig. 5. Reversed-phase HPLC analysis of ^{14}C-labeled products from incubation (40 min) of rat liver nuclei with [^{14}C]NAD followed by treatment with (a) 1 *M* sodium hydroxide and (b) proteinase K. In (a) 2.59 · 10^5 cpm and in (b) 2.07 · 10^5 cpm were injected and elution System I was used.

(No. 5) both NAD and ADP-ribose are degraded to AMP, as would be expected, but the yield of poly(ADP-ribose) is the same after hydrolysis of polymer-protein adducts by proteinase K (compare refs. 4 and 5), determined either by HPLC or by isotope analysis of acid-precipitable products before hydrolysis. This method of HPLC analysis of poly(ADP-ribose) is being presently applied for the assay of endogenous polymers in animal tissues under varying experimental conditions.

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