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SIMULTANEOUS DETERMINATION OF MONO- AND POLY(ADP-RIBOSE) *IN VIVO* BY TRITIUM LABELLING AND DIRECT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION

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SUMMARY

A microanalytical method for the determination of cellular mono-, oligo- and poly(ADP-ribose) has been developed that does not involve enzymatic degradation of oligomers to ribosyladenosine. The method consists of separation of protein-bound mono-, oligo- and poly(ADP-ribose) adducts from soluble nucleotides, followed by hydrolysis and quantitative isolation of AMP [derived from mono-(ADP-ribose)proteins], oligo- and poly(ADP-ribose) by boronate affinity chromatography and subsequent isolation of these nucleotides by HPLC. *cis*-Diols in AMP, oligo- and poly(ADP-ribose) are selectively oxidized by periodate, then reduced by [^{3}H]borohydride. Conditions for the oxidation–reduction steps were optimized, and tritiated AMP, oligo- and poly(ADP-ribose) were quantitatively determined by radiochemical analysis of these components that were isolated by reversed-phase high-performance liquid chromatography (18). A 1-pmol ADP-ribose unit under standard conditions yields $2 \cdot 10^3$–$2.2 \cdot 10^3$ cpm ^{3}H and this sensitivity can be amplified by increasing the specific radioactivity of [^{3}H]borohydride.

INTRODUCTION

Poly(ADP-ribose), which is formed from NAD and is present predominantly in cell nuclei, is a biological macromolecule of nucleic acid-like structure[1,2]. Its cellular function is currently under extensive investigation[3–7]. Determination of polymeric and monomeric ADP-ribose *in vivo* constitutes an important analytical biochemical problem in this area of research. Previously several analytical methods have been reported:

(a) Isotope dilution by endogeneous poly(ADP-ribose) of labelled poly-(ADP-ribose) or mono(ADP-ribose)[8,9].

(b) Antigen–antibody reaction between poly(ADP-ribose) and anti-poly-(ADP-ribose) antiserum[10–12] or conversion of mono(ADP-ribose) to AMP and estimation of AMP by a radioimmunoassay[13].

(c) Fluorometric determination of ribosyladenosine derived from poly(ADP-ribose)[14–16].

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(d) Tritium labelling of ribosyladenosine derived from poly(ADP-ribose)[17].

We have recently developed novel high-performance liquid chromatography (HPLC) methods for the simultaneous determination of *in vitro*-synthesized poly(ADP-ribose) and mono(ADP-ribose) on a reversed-phase column[18] and chain-length analyses on an ion-exchange column[19]. We now report a highly sensitive and specific method for the simultaneous analysis of polymeric and monomeric ADP-ribose *in vivo*, suitable for biochemical experimentation. This method differs from previous procedures[13–17] inasmuch as protein-bound mono- and poly(ADP-ribose) are determined after microisolation of these compounds and, following hydrolysis to AMP and poly(ADP-ribose), analyses are performed directly by borotritiation and HPLC[19] without chemical or enzymatic degradation of polymers into chemical subunits.

EXPERIMENTAL

Materials

Nicotinamide-adenine dinucleotide, oxidized (NAD), Tris–HCl, proteinase K, triethylamine, sodium periodate and potassium borohydride, were purchased from Sigma (St. Louis, MO, U.S.A.); sodium [^{3}H]borohydride (Lot No. 2558124, 1.68 Ci/mmol) from ICN Radiochemicals (Irvine, CA, U.S.A.), [^{14}C]NAD (283 mCi/mmol) from Amersham (Arlington, IL, U.S.A.), boronic acid gel (Affi-gel 601) from Bio-Rad (Richmond, CA, U.S.A.), ammonium carbonate and glacial acetic acid (HPLC grade) from Baker (Phillipsburg, NJ, U.S.A.), potassium phosphate (HPLC grade) from Fisher (Santa Clara, CA, U.S.A.), methanol and acetonitrile (HPLC grade) from Alltech (Deerfield, IL, U.S.A.). All other chemicals used were of reagent grade. Scintillation fluid (Aquasol) was obtained from New England Nuclear (Boston, MA, U.S.A.), and cell culture supplies from the Cell Culture Facility of UCSF (San Francisco, CA, U.S.A.). The 14C rat fibroblasts, containing MMTV promoter, EJ-ras oncogene, and a 4.8 kb Sma-Bgl II DNA sequence construct, isolated by Dr. William M. Lee (Department Microbiology and Immunology), were a gift from Dr. Alex Tseng (Cancer Research Institute).

HPLC

HPLC was performed with the following instrumental components: Waters Assoc. (Milford, MA, U.S.A.) Model 6001 solvent delivery pumps, Waters Model 680 gradient controller, Waters Model 730 data module, and Hewlett-Packard (Santa Clara, CA, U.S.A.) Model 1040A high speed spectrophotometric detector. Chromatographic data were stored in a Hewlett-Packard Model 9121D disc memory system and plotted by a Hewlett-Packard HP 747A graphic plotter. HPLC eluate fractions were collected with an Isco (Lincoln, NE, U.S.A.) Model (Retriever No. III) fraction collector and counted on a Beckman (Palo Alto, CA, U.S.A.) LS 3800 scintillation counter. The column employed was a Beckman-Altex (Berkeley, CA, U.S.A.) analytical reversed-phase column (Ultrasphere ODS, 5 μm, 25 cm $\times$ 4.6 mm I.D.), with a precolumn packed with the same sorbent as the analytical column. Chromatography was carried out at ambient temperature. The buffer system employed was: buffer A, 0.10 *M* potassium phosphate (pH 4.25); buffer B, same as A but containing 20% methanol; buffer C, 0.10 *M* potassium phosphate (pH 7.0), 1.0 *M*

urea and 50% acetonitrile. The flow-rate was 1.5 ml/min throughout. Upon sample injection the gradient started from 100% A to 100% B in 20 min, a concave gradient, curve 9, was used. 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.

Isolation of poly and mono(ADP-ribose) present in vivo from cells. Cultures of 14C rat fibroblasts were maintained in Dulbecco's Modified Eagle Medium + 10% fetal calf serum + penicillin/streptomycin at 33°C in 5% carbon dioxide and air atmosphere. Doubling time of the cells was 24 h and, at the time of confluency, cells were removed by trypsin treatment, sedimented at 500 *g* (room temperature), washed twice by centrifugation in phosphate buffered saline and counted in a hemocytometer. For a detailed experimental description of isolation of poly(ADP-ribose) see ref. 19. Briefly, the cell pellet was extracted with 20% (w/v) trichloroacetic acid to remove soluble nucleotides. For each extraction, the pellet was dispersed by sonication [power setting 2, four sets of twenty pulses on a Bronson Sonifier (Bronson Sonic Power Co., Danbury, CT, U.S.A.)], with cooling in an ice-water bath, followed by centrifugation at 4°C (3000 *g* for 20 min). Extraction was repeated six times to assure complete removal of nucleotides which otherwise may be trapped in the acid-insoluble material. The protein-bound polymers and monomers were isolated by successive base hydrolysis, proteolysis by proteinase K, followed by phenol extraction and phenyl boronate chromatography. Elution of poly(ADP-ribose) and of AMP (which is derived from mono(ADP-ribose) from the boronate column (7 cm × 0.7 cm) was performed with 1 *M* triethylammonium acetate* (pH 6.3) instead of Tris–HCl. After freeze-drying, the material was dissolved in distilled water and subjected to periodate oxidation and sodium [^{3}H]borohydride reduction (see below).

In vitro preparation of poly(ADP-ribose) standard. The method employed was described previously[18], except that for elution of poly(ADP-ribose) from the boronate column 1 *M* triethylammonium acetate (pH 6.3) was used instead of Tris–HCl.

Preparation of 2'-(5'-phosphoribosyl) adenosine 5'-monophosphate (PR-AMP). The preparation PR-AMP standard was carried out as described previously[19].

Tritium "labelling" of poly(ADP-ribose) and AMP

(a) *Oxidation of poly(ADP-ribose) and AMP with sodium periodate.* Samples containing up to 50 nmol of *cis*-diol groups in 0.100 ml of distilled water were combined with 0.010 ml of 10 m*M* sodium periodate, mixed in a vortex mixer, and allowed to react in the dark at room temperature for 30 min. This reaction time assures complete oxidation of the 2',3'-*cis*-diols of the ribose moieties of the polynucleotide to the corresponding dialdehydes without breakage of the polymer backbone[18]. The oxidized sample was immediately reduced *in situ* (see below).

(b) *Reduction of oxidized poly(ADP-ribose) and AMP with sodium [^{3}H]borohydride.* The periodate-oxidized sample of the polynucleotide or AMP was chilled, buffered by addition of 0.005 ml of 0.10 *M* potassium phosphate (pH 6.6) followed by 0.005 ml of a 0.10 *M* solution of sodium [^{3}H]borohydride in 0.10 *M*

* In contrast to Tris–HCl, triethylammonium acetate quantitatively elutes poly and mono(ADP-ribose) from the boronate gel and, due to its volatility, can be removed by freeze-drying, thus eliminating the need for desalting.

potassium hydroxide. The resultant solution, which was mildly basic (pH 8–9), was allowed to react in the dark at room temperature for 30 min. Excess reducing agent was then decomposed by addition of 0.025 ml of aqueous acetic acid (2.0 *M*) and the sample was dried in the fume hood under a stream of nitrogen. In order to assure complete removal of exchangeable tritium, the dried sample was redissolved in 0.100 ml of aqueous acetic acid (0.50 M) and then redried, and this step was repeated once more. The dried sample was then dissolved in distilled water and an aliquot corresponding to $2 \cdot 10^6$–$10 \cdot 10^6$ cells was injected into the HPLC system.

RESULTS AND DISCUSSION

The chemical reactions on which this method is based are: (a) the oxidation by sodium periodate of the 2′,3′-*cis*-diol groups of the ribose moieties in poly(ADP-ribose)* and related nucleotides, wherein the ribose rings are opened by a splitting of the carbon–carbon bond of the *cis*-diol and two aldehyde groups are formed, and (b) the reduction by [^{3}H]borohydride of these aldehydes to primary alcohol groups. In the reduction of each aldehyde group, a C–^{3}H bond is formed which, being non-exchangeable with water hydrogens, constitutes a stable isotopic labelling.

The procedure for periodate oxidation–[^{3}H]borohydride reduction which we employ represents a modification of a method previously reported for oxidation–reduction of monomeric ribonucleosides[20] and ribosyladenosine[17,21]. Because our approach, unlike that of previous workers, is to oxidize poly(ADP-ribose) in its polymeric form without degradation, it was desirable to determine conditions for the reactivity of the polymer with sodium periodate. In a previous study[18] we showed that poly(ADP-ribose) indeed undergoes periodate oxidation–[^{3}H]borohydride reduction without disruption of the polymeric backbone, but we did not determine the time requirements for optimal conditions. At a concentration of 0.5 m*M* poly(ADP-ribose) units and 1.0 m*M* sodium periodate in distilled water at 25°C (reactions followed by decrease in UV absorbance at 310 nm as described[22]) the initial halflife of oxidation of the polymer (4.0 min) (measured by sodium periodate uptake) is four times longer than that of adenosine (1.0 min) and ten times that of AMP (0.4 min). However, the rate of reaction is still sufficiently fast so that, at room temperature with 2 equivalents of periodate present, oxidation is complete within 30 min.

The time requirement for reduction by borohydride of the oxidized groups (dialdehydes) was determined by HPLC analyses of dialdehyde reduction. The reversed-phase HPLC system[18] readily separates poly(ADP-ribose) from nucleotides (Fig. 1). Fig. 1 also shows the elution peak identifying AMP which has been periodate-oxidized and borohydride-reduced, denoted by an asterisk**, which we employ as a primary standard. Fig. 2 presents our kinetic study of the reduction of periodate-oxidized AMP (AMP-dialdehyde) by borohydride to [^{3}H]AMP. At an initial concentration of 0.48 m*M* AMP-dialdehyde and 4.8 m*M* borohydride at room temperature (pH 9), reduction to [^{3}H]AMP is rapid and complete within 10 min. As seen

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* In poly(ADP-ribose) each monomeric unit contains two ribose moieties, but only one of these has a *cis*-diol available for oxidation.

** In the figures an asterisk denotes [^{3}H]-labelling through periodate oxidation–[^{3}H]borohydride reduction.

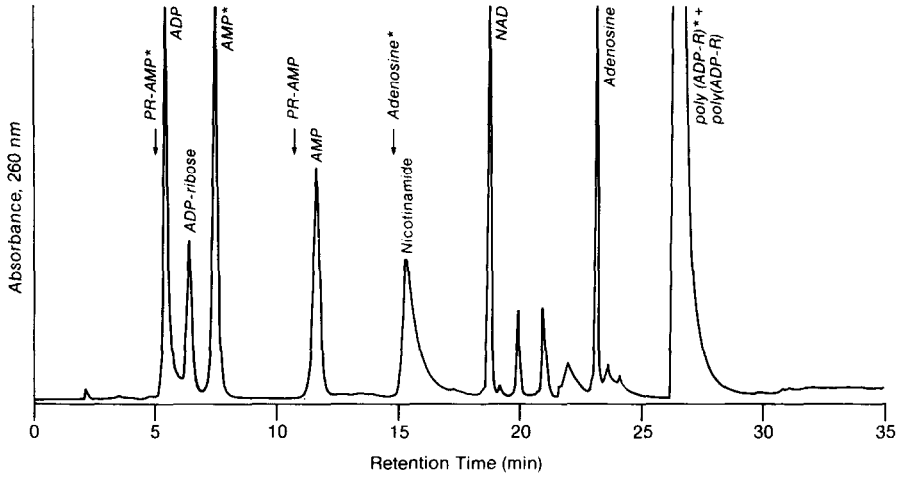

Fig. 1. Reversed-phase HPLC analysis of poly(ADP-ribose) and monomeric nucleotides. Aliquots (50 μl) were injected, containing standards. Chromatography was performed at room temperature and monitored by UV at 260 nm; with a recorder scale of 0.15 a.u.f.s. Arrows indicate the positions of compounds not included in the standard mixture, but their elution times were determined separately. An asterisk denotes [^{3}H]-labelling.

in Fig. 2, at zero reaction time AMP-dialdehyde displays itself as a broad elution peak, centered at *ca.* 4 min. At a reaction time of 0.25 min this broad peak is largely transformed to two new peaks, at retention time 4.90 min, and at 7.45 min. The first of these new peaks is presumably the partially reduced species (*i.e.*, with one aldehyde group still unreacted), while the second is the fully reduced product, [^{3}H]AMP. This becomes evident as the reaction progresses, and at reaction time of 10 min essentially all of the material is consolidated into the [^{3}H]AMP peak.

It is further worth noting that (a) a minor peak is observed at retention time 23.5 min (Fig. 2). We have observed that if the reaction mixture is unbuffered, this peak, which possibly represents a dimeric aldehyde species, increases in size although still remaining a minor component. Therefore, in order to minimize this minor product we include potassium phosphate (pH 6.6) in an amount equivalent to the potassium hydroxide in the carrier solvent of the borohydride reagent. Thus, when borohydride is added to the periodate-oxidized nucleotide, the resultant solution has a pH of about 9, which is sufficiently basic to prevent decomposition of borohydride by aqueous protons, but not so strongly basic as to catalyze aldehydic adducts. (b) Excess sodium periodate, which is carried over from the oxidation step, does not interfere with the reduction reaction. Even when the amount of excess periodate was intentionally tripled, no inhibition of the borohydride reduction of the dialdehyde was observed. (c) If the borohydride reaction mixture is allowed to stand at room temperature for periods up to an hour, no decomposition of [^{3}H]AMP is detected during HPLC.

Based on the rapidity of the borohydride reduction of AMP-dialdehyde, we estimate that a 30-min reaction time would be ample for complete reduction of periodate-oxidized poly(ADP-ribose) to [^{3}H]poly(ADP-ribose). This was confirmed by reacting oxidized poly(ADP-ribose) with [^{3}H]borohydride (in quantities analogous

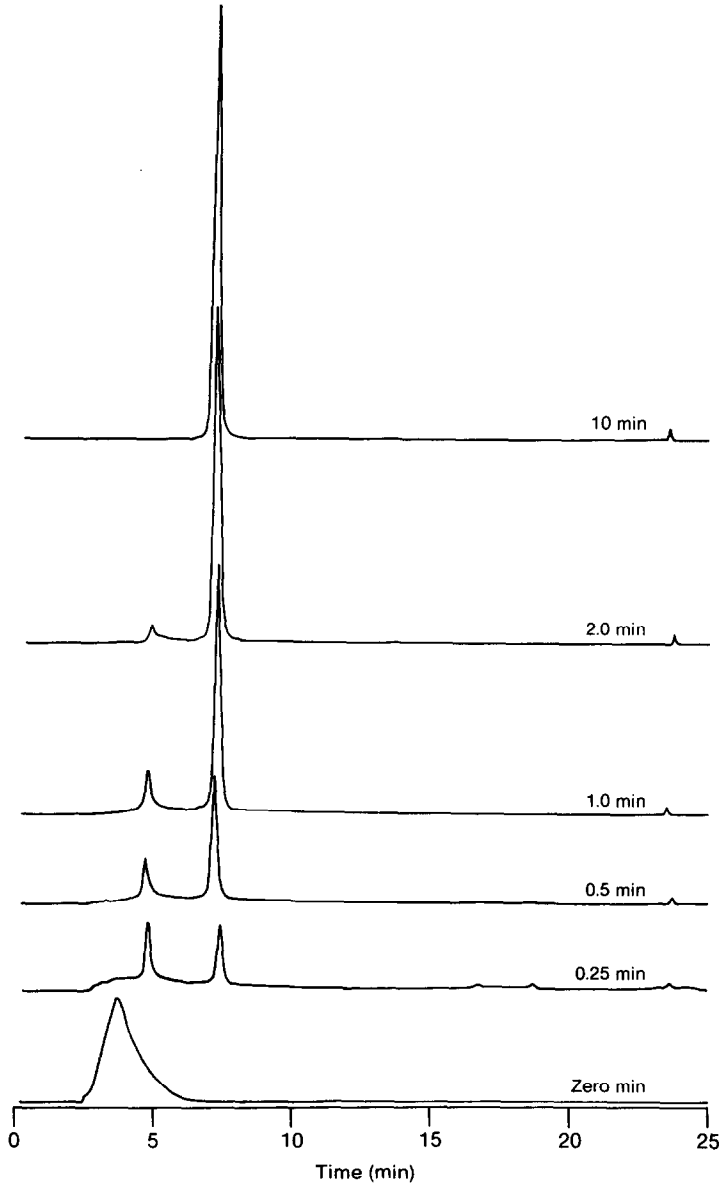

Fig. 2. Reversed-phase HPLC monitoring of the borohydride reduction of periodate-oxidized AMP. Oxidized AMP (500 nmol) was mixed with 5000 nmol of borohydride in 4.5 m*M* potassium phosphate (pH 9) in a final volume of 1100 μl. Aliquots (110 μl) were removed at specific times, immediately quenched by addition of 50 μl of 2.0 *M* acetic acid, dried, dissolved in 100 μl of water and a 30-μl portion (corresponding to 15 nmol of oxidized AMP) was injected for HPLC. Chromatography was performed at room temperature with UV detector at 260 nm. The ordinate was 0.300 a.u.f.s. in each chromatogram. The reaction times are indicated on each plot.

to those detailed above for AMP) for 30 min, isolating the polymer by HPLC (see below), determining its specific radioactivity, and then re-subjecting the material to a new cycle of reduction with a large excess of [^{3}H]borohydride (100 ×). No further incorporation of radioactivity (no change in specific radioactivity) was detected, proving that during the initial 30 min complete reduction was effected. In addition, the specific radioactivity of the [^{3}H]poly(ADP-ribose) was consistently the same as that of [^{3}H]AMP.

Concerning the HPLC behavior of the tritiated derivatives of the structural constituents of poly(ADP-ribose) (*i.e.*, [^{3}H]AMP, [^{3}H]PR-AMP, and [^{3}H]adenosine) on the ODS reversed-phase column, it is predictable that these molecules have shorter retention times than their unreacted parent compounds. Table I gives the retention times for adenosine, AMP and PR-AMP and their tritiated derivatives. The shorter retention times are expected because oxidation–reduction of the secondary hydroxyl groups of the ribose ring (2′,3′-*cis*-diol) results in breakage of the ring into open chain primary alcohols. However, as we have previously shown[18], oxidation–reduction of poly(ADP-ribose) does not alter significantly its retention time in this elution system (which is system I, cf. ref. 18).

For quantitation of poly(ADP-ribose) by conversion to [^{3}H]poly(ADP-ribose), we simultaneously carry out generation of [^{3}H]AMP from AMP as primary external standard. Fig. 3A shows the elution profile of 5 nmol [^{3}H]AMP, as followed by radioactivity counts of the HPLC eluate fractions. Repeated preparations of [^{3}H]AMP routinely gave us a total of about $2.1 \cdot 10^6$ cpm/nmol [^{3}H]AMP. At this specific radioactivity it is possible to detect subpicomol amounts of tritiated product. If additional sensitivity is required, sodium [^{3}H]borohydride of higher specific radioactivity can be employed.

When analyzing poly(ADP-ribose) content of biological samples, we routinely ran parallel samples of AMP, subjecting them to the same reactions and monitoring the specific radioactivity of [^{3}H]AMP as an indication of complete labelling efficiency.

Fig. 3B shows the elution profile of radioactivity of a doubly-labelled sample of [^{3}H]poly(ADP-ribose) (4 nmol), derived from [^{14}C]poly (ADP-ribose) (generated by *in vitro* incubation of [^{14}C]NAD with rat liver nuclei[18]). From this chromatogram, it is clear that [^{3}H]-labelling has occurred only where [^{14}C]-label is present, except for the first elution peak at about 2.5 min (denoted as peak 1) which has virtually no

TABLE I

COMPARISON OF RETENTION TIMES OF PARENT COMPOUNDS AND TRITIATED DERIVATIVES OF STRUCTURAL CONSTITUENTS OF POLY(ADP-RIBOSE)

Tritiated derivatives obtained by periodate oxidation–[^{3}H]borohydride reduction.

Compound	*Retention time (min)*	
	Parent compound	*Tritiated derivative*
PR-AMP	10.40	5.12
AMP	11.62	7.45
Adenosine	22.84	14.80

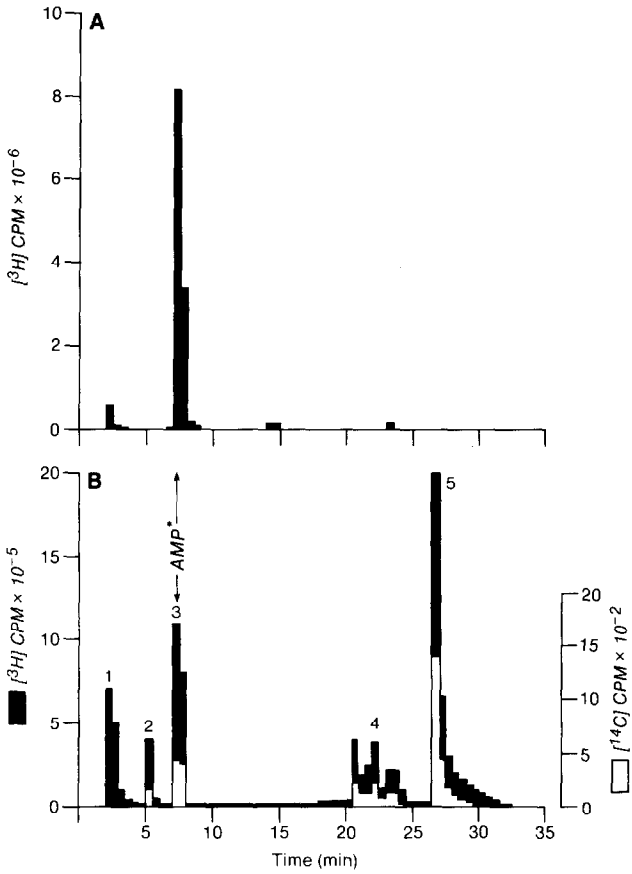

Fig. 3. Reversed-phase HPLC analysis of (A) [^{3}H]AMP and (B) [^{14}C, ^{3}H]poly(ADP-ribose), derived from *in vitro*-generated poly(ADP-ribose). (A) [^{3}H]AMP (5 nmol) in 20 μl of water was injected and fractions of the eluate were collected and counted every 0.5 min. (B) A 20-μl sample, containing 4 nmol of [^{14}C, ^{3}H]poly(ADP-ribose), were injected and 0.5-min fractions of the eluate were collected and counted. The left ordinate represents [^{3}H] and the right ordinate [^{14}C]. 1 = background peak, 2 = [^{14}C,^{3}H]PR-AMP, 3 = [^{14}C,^{3}H]AMP, 4 = short chain [^{14}C,^{3}H]poly(ADP-ribose) (n = 2–6), and 5 = [^{14}C,^{3}H]poly(ADP-ribose) (n = 7–50).

retention time in the column. Even in blank samples containing no nucleotide such a tritium-containing peak is observed.

We have found that the elution profile and specific radioactivity of [^{3}H]poly(ADP-ribose) is not affected when the amount of poly(ADP-ribose) is decreased significantly relative to the excess sodium periodate and [^{3}H]borohydride reagents. Thus, when we subjected 50 pmol of poly(ADP-ribose) to the same exact conditions as 5 nmol, the [^{3}H]poly(ADP-ribose) gave the same elution profile and specific radioactivity. However, peak 1, which is not derived from the polymer, is observed to be larger and constitutes the most intense peak in the chromatogram (not shown). Since in biological samples the amount of poly(ADP-ribose) is not known prior to analysis, it is important that the amounts of excess reagents do not interfere with complete conversion to [^{3}H]poly(ADP-ribose).

In Fig. 3B, the doubly-labelled [^{14}C, ^{3}H] peaks are identified as follows: peaks 2 and 4 are [^{14}C, ^{3}H]PR-AMP and very short oligomers respectively, while peaks 3 and 5 are [^{14}C, ^{3}H]AMP and [^{14}C, ^{3}H]poly(ADP-ribose), respectively.

Fig. 4 represents a typical chromatogram obtained from a biological sample containing poly(ADP-ribose) from 14C rat fibroblasts. Poly(ADP-ribose) from $1.08 \cdot 10^8$ cells was isolated and dissolved in 300 μl of water. A 100-μl portion was subjected to the periodate oxidation–[^{3}H]borohydride reduction procedure (see Experimental section). An aliquot of the [^{3}H]-labelled product corresponding to $4.5 \cdot 10^6$ cells was chromatographed. The ^{3}H counts under the [^{3}H]poly(ADP-ribose) peak totalled $1.8 \cdot 10^6$ cpm, corresponding to 175 pmol poly(ADP-ribose) per 10^6 cells. The AMP, measured as [^{3}H]AMP, which represents the mono(ADP-ribose) content of the cells, was 14.3 pmol per 10^6 cells. This means that a 1-pmol ADP-ribose unit is equivalent with $2.2 \cdot 10^3$ cpm [^{3}H]. Since the method is very sensitive and reproducible, a much smaller number of cells than specified above is sufficient to yield quantitated amounts of [^{3}H]AMP and [^{3}H]poly(ADP-ribose) radioactivity.

The intracellular poly(ADP-ribose) concentrations reported by others[14,23–25] and obtained by different techniques in our laboratory (cf. ref. 26) are in the same order of magnitude as results obtained by the present technique. However, comparison with the reported method based on ethenoadenine fluorescence[16] is difficult for several reasons. Cellular concentrations of ribosyladenosine are shown as relative fluorescence values and the range of detection (1–60 pmol) was calculated from the fluorescence of standards without analytical data from biological material[16].

Furthermore, the internal standard, as stated (cf. ref. 16) was oligo-ADP-ribose (average number of monomeric units, $n_{aver} = 2.5$), which has been eluted by hydrochloric acid from the affinity column. As we have shown[19] the chromatographic

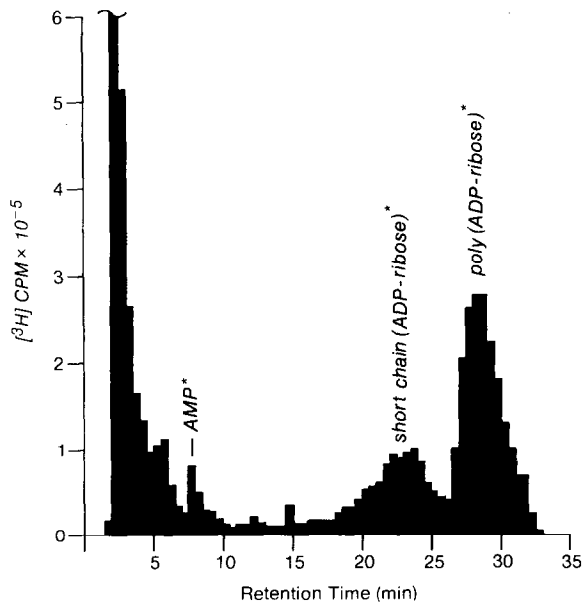

Fig. 4. Reversed-phase HPLC quantitation of poly(ADP-ribose) present in 14C cells.

behavior of oligo- and poly(ADP-ribose) are significantly different and we also find (unpublished results) that polymeric ADP-ribose, with n_{aver} = 30–50 is not eluted from boronate affinity columns by acidic eluants. Therefore, the possibility cannot be excluded that by acid elution of oligo(ADP-ribose) that has been isolated from biological material, some or all of long chain polymers remain on the affinity column and may escape detection.

These discrepancies, besides being attributable to the technical problem of failure to elute long polymers from affinity columns by acidic eluants[16], could reflect also cell-specific variations[27]. This problem concerned with comparison of varying cell types is the subject of a separate report.

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