Proc. Nat. Acad. Sci. USA
Vol. 72, No. 1, pp. 205–209, January 1975

Natural Occurrence of Poly(ADP-Ribosyl) Histones in Rat Liver

([^{14}C]ribose/HCl-extraction/CM-cellulose/histone f1/2′-[5″-phosphoribosyl]-5′-AMP)

KUNIHIRO UEDA, AKIRA OMACHI*, MASASHI KAWAICHI, AND OSAMU HAYAISHI

Department of Medical Chemistry, Kyoto University Faculty of Medicine, and Laboratory of Molecular Biology, Institute for Chemical Research, Kyoto University, Kyoto 606, Japan

Contributed by Osamu Hayaishi, October 25, 1974

ABSTRACT **Poly(ADP-ribose) bound to histones has been isolated from rat liver. When [^{14}C]ribose was administered intraperitoneally to rats at a dosage of 300–750 μg (100–250 μCi)/100 g, approximately 1% of the radioactivity was recovered in the acid (5% Cl_3CCOOH)-insoluble material of the liver nuclei 2 hr after injection. Of the acid-insoluble radioactivity, 4.5–9% was extractable with 0.25 N HCl. Carboxymethyl-cellulose column chromatography of the HCl-extracted material revealed that the radioactivity cochromatographed with histone subfractions f1 and, to a lesser extent, f2 and f3. Part of the protein-bound radioactivity was rendered acid-soluble by treatment with either snake venom phosphodiesterase or neutral NH_2OH. From the enzyme digest, 5′-AMP and ψADP-ribose [2′-(5″-phosphoribosyl)-5′-AMP] were recovered, while the NH_2OH treatment yielded ADP-ribose monomer and, presumably, oligomer. These observations indicate that ADP-ribose is attached to histones *in vivo* and is present both as a monomer and a polymer.**

Poly(ADP-ribose), a macromolecule synthesized from NAD in chromatin (1–5), has been assumed to be covalently bound to nuclear proteins, principally histones (6–8). Although this poly(ADP-ribosyl)ation of proteins has been well established *in vitro* using various nuclear preparations (for reviews, see refs. 9 and 10), there is no report that has demonstrated the existence of the protein-bound polymer *in vivo*. Doly and Mandel (11) offered the first evidence that the polymer exists in hen liver by isolating 2′-(5″-phosphoribosyl)-5′-AMP (abbreviated ψADP-ribose) from phosphodiesterase digests of phenol-extracted material after injection of [^{32}P]orthophosphate. More recently, Colyer *et al.* have reported the isolation of poly(ADP-ribose) from cultured mouse L-cells labeled with [^{3}H]adenosine, with no reference made of any binding to proteins (12). Smith and Stocken (13) and Dietrich *et al.* (14), on the other hand, briefly described the occurrence of protein-bound ADP-ribose in rat liver nuclei. Neither of these reports, however, presented any evidence for the existence of polymerized† ADP-ribose attached to nuclear proteins.

Recently, we carried out a series of experiments to determine the conditions for labeling NAD *in vivo* and, thereby, poly(ADP-ribose). The results, which have been presented elsewhere (15), suggested that the injection of [^{14}C]ribose serves best for this purpose. Coinjection of [^{3}H]adenine also proved to be useful for following adenine-derived compounds. In this communication, new data obtained by employing these precursors are presented which enable us to report the first unequivocal evidence for the natural existence of poly(ADP-ribosyl) histones in mammalian tissues.

MATERIALS AND METHODS

Injection of Radioactive Precursors and Preparation of HCl-Extract. Male Wistar rats, weighing 90–110 g, were employed throughout this study. The radioactive precursors, D-[*1*-^{14}C]-ribose (49.9 Ci/mole) (New England Nuclear Corp.) and [*8*-^{3}H]-adenine (27 Ci/mmol) (the Radiochemical Center, Amersham) were freed of their solvent vehicles by evaporation *in vacuo* and dissolved in saline (0.15 M NaCl) at concentrations of 10 or 4 mM (0.5 or 0.2 mCi/ml) and 37 μM (1 mCi/ml), respectively. In the main experiments reported herein, 0.5 ml of the ribose solution and 0.25 ml of the adenine solution were mixed and injected intraperitoneally into each rat. The animal was put in a metabolic chamber and maintained for 2 hr under ventilation with CO_2-free air. The animal was then etherized and the liver was quickly removed. The liver was homogenized in either 3 volumes of ice-cold 20% Cl_3CCOOH (or 5% $HClO_4$) or 5 volumes of 0.25 M sucrose containing 3.3 mM $CaCl_2$ with the aid of a Potter-Elvehjem type tissue grinder. The acid homogenate was centrifuged for 10 min at 10,000 × *g*. The precipitate was treated with 20% Cl_3CCOOH and centrifuged as before. This washing procedure was repeated three more times so that the final material was contaminated by less than 0.5% of the total acid-soluble radioactivity. The sucrose homogenate was centrifuged for 5 min at 800 × *g*, and the precipitate obtained (*crude nuclei*) was subjected to acid washing as was applied to the acid homogenate. The acid-insoluble material prepared by either of these procedures was extracted with 5 volumes (per original liver weight) of 0.25 N HCl by stirring for 30 min at 4°. After the mixture was centrifuged for 15 min at 15,000 × *g*, the supernatant fraction was collected and the precipitate was reextracted as above. Five extracts obtained in this way were combined, concentrated approximately 50-fold with the aid of a Diaflo (Amicon) ultrafiltration device equipped with a UM-2 membrane, and centrifuged for 60 min at 105,000 × *g*. The final, slightly yellow supernatant fraction is referred to as the "HCl-extract."

Carboxymethyl (CM)-Cellulose Column Chromatography. Analysis of the HCl-extract with a CM-cellulose column was carried out by the method of Johns *et al.* (16). The sample, which contained 5.5 mg of protein in 1 ml, was dialyzed over-

Abbreviation: ψADP-ribose, 2′-(5″-phosphoribosyl)-5′-AMP.

* On leave from Department of Physiology, College of Medicine, University of Illinois at the Medical Center, Chicago, Ill.

† The term "polymerized (or poly) ADP-ribose" denotes not only a large polymer but an oligomer in which ADP-ribose is linked by a ribose (1′ → 2′) ribose linkage.

TABLE 1. *Incorporation of [^{14}C]ribose and [^{3}H]adenine into the acid-soluble, acid-insoluble, and acid-insoluble, HCl-extractable material of liver*

Exp.	Starting preparation and precursor (dose)	Incorporation into material*		
		Acid-soluble (thousand cpm)	Acid-insoluble (thousand cpm)	Acid-insoluble, HCl-extractable (thousand cpm)
I.	Crude nuclei (1)†			
	[^{14}C]Ribose (5 μmol, 250 μCi)	2,076	4,640	207
	[^{3}H]Adenine (9.3 nmol, 250 μCi)	ND	ND	72
II.	Whole Cl_3CCOOH-homogenate (2)†			
	[^{14}C]Ribose (5 μmol, 250 μCi)	11,900	5,330	295
	[^{3}H]Adenine (9.3 nmol, 250 μCi)	ND	ND	49
III.	Whole $HClO_4$-homogenate (5)†			
	[^{14}C]Ribose (2 μmol, 100 μCi)	4,504	2,163	189

ND = not determined.
* Per animal.
† Number of rats employed.

night against 500 ml of 0.1 M sodium acetate buffer (pH 4.2) and applied on a CM-cellulose column (0.8 × 35-cm) equilibrated with the same buffer. The column was washed with the equilibrating buffer until no more radioactivity appeared and then eluted stepwise with approximately 100-ml portions of 0.17 M sodium acetate buffer (pH 4.2) containing 0.42 M NaCl, 0.01 N HCl, and 0.02 N HCl, respectively. The fractions obtained (2.5 ml each) were assayed for total or acid-insoluble radioactivity by the use of a Millipore filter (6) and for protein by measuring the absorbance at 220 nm.

Digestion with Snake Venom Phosphodiesterase and Analysis of Products. A sample of the HCl-extract (11 mg of protein) was neutralized with NaOH, and was incubated for 48 hr with snake venom phosphodiesterase (200 μg) in a mixture (2.5 ml) containing 250 μmol of Tris–acetate buffer (pH 8.8) and 25 μmol of $MgCl_2$. The phosphodiesterase preparation was obtained from Sigma Chemical Co. or Boehringer Mannheim GmbH and was further purified by passage through a Bio-Rad AG 50 column to remove a contaminating 5′-nucleotidase (17). The enzyme digestion was terminated by addition of 5% (final) $HClO_4$ and the mixture was centrifuged at 15,000 × *g* for 10 min. The supernatant fraction was neutralized with KOH, and was applied, together with nonradioactive markers (5′-AMP and ADP-ribose), on a Dowex 1-formate column (×2, 200–400 mesh; 0.8 × 35-cm). The column was eluted sequentially with linear gradients of 0–0.3 N HCOOH, 0.3–4 N HCOOH, and 4–6 N HCOOH containing 0–0.6 N NH_4HCOO. The fractions were examined for ^{3}H and ^{14}C radioactivity by placing aliquots in a PCS scintillator (Amersham/Searle) in a Beckman DPM-100 or Packard Tri-Carb 3385 liquid scintillation spectrometer.

NH_2OH Treatment. Hydroxylaminolysis of the HCl-extract was performed at neutral pH, as described previously (7), according to the method of Lipmann and Tuttle (18). The reaction mixture contained 100 μmol of potassium phosphate buffer (pH 7.0), 1 mmol of NH_2OH, 875 μmol of NaOH and a sample of the HCl-extract in a total volume of 1 ml (final pH around 6.5). The mixture was incubated for 20 min at 37°. Before and after the treatment, the acid-insoluble radioactivity was monitored in aliquots of the reaction mixture that were acidified and washed with 5% Cl_3CCOOH on a Millipore filter (6). After the NH_2OH treatment, the whole mixture was put on a Dowex 1-formate column, eluted, and analyzed as described above for the phosphodiesterase digests.

Paper Chromatography. Two solvent systems were employed: 1, isobutyric acid–1 M NH_4OH–0.1 M Na_2EDTA (100:60:1.6) and 2, 0.1 M potassium phosphate (pH 6.8)–$(NH_4)_2SO_4$–*n*-propanol (100:60:2). In both systems, descending chromatography was performed. Authentic nonradioactive markers were cochromatographed and located under an ultraviolet lamp. The radioactivity was determined in 1-cm strips in a liquid scintillation spectrometer.

RESULTS

Incorporation of [^{14}C]Ribose and [^{3}H]Adenine into Liver Tissue. [^{14}C]Ribose and [^{3}H]adenine administered intraperitoneally were actively incorporated into the liver. The distribution profile of the radioactivity among acid-soluble, acid-insoluble, and acid-insoluble, HCl-extractable material 2 hr after injection is shown in Table 1. Irrespective of the acid used for initial homogenization (Cl_3CCOOH or $HClO_4$) or the dose administered, approximately two-thirds of the labeled ribose that was recovered in the whole tissue was found in the acid-soluble fraction and one-third was found in the acid-insoluble material (Exp. II and III). The latter represented about 1% of the administered dose. When crude nuclei were isolated following tissue homogenization with sucrose (Exp. I), much less acid-soluble radioactivity was found therein, while the amount of acid-insoluble radioactivity recovered in nuclei was almost identical to that found in the acid-insoluble material from the whole tissue homogenate (Exp. II). This suggests that the ribose that was converted to an acid-insoluble form under the present conditions was located entirely in the nucleus.

Part of the acid-insoluble radioactivity was found to be extractable with 0.25 N HCl. When the nuclear material, doubly labeled with [^{14}C]ribose and [^{3}H]adenine, was treated with HCl, 4.5% of the acid-insoluble ^{14}C was extracted together with almost comparable amounts of acid-insoluble ^{3}H (corrected for the difference in counting efficiency).

Association of Ribose and Adenine with Histones. The HCl-extract prepared as above is known to contain a majority of the histone proteins. When the nuclear HCl-extract was

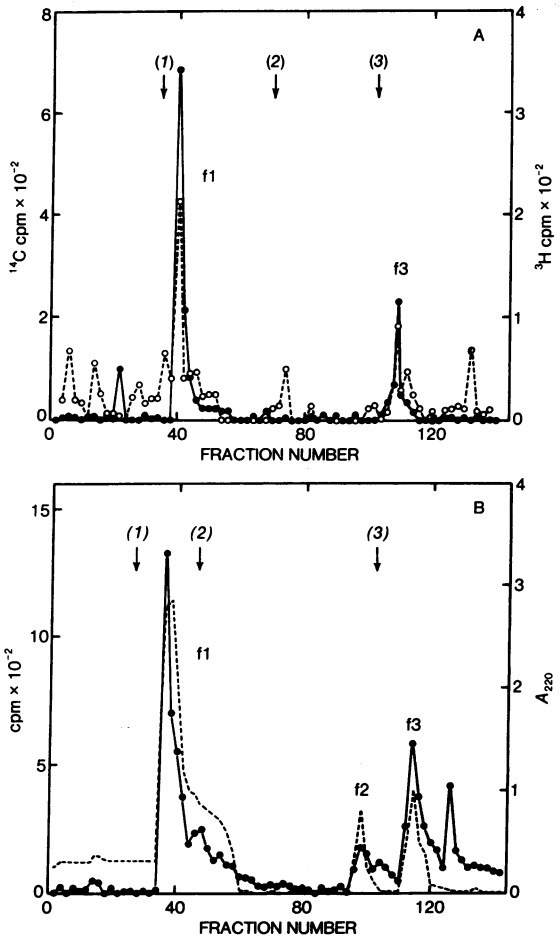

FIG. 1. Analysis of an HCl-extract on a CM-cellulose column. The column charged with the HCl-extract was eluted stepwise with (*1*) 0.17 M sodium acetate (pH 4.2) and 0.42 M NaCl, (*2*) 0.01 N HCl, and (*3*) 0.02 N HCl. (A) HCl-extract of crude nuclei, ●——● ^{14}C; O- - -O ^{3}H. (B) HCl-extract of whole $HClO_4$-homogenate, ●——● ^{14}C (acid-insoluble); - - - A_{220}.

passed through a CM-cellulose column according to the procedure of Johns *et al.* (16), the ^{14}C and ^{3}H radioactivity cochromatographed with histone subfractions f1 and f3 (Fig. 1A). When the material from the whole acid-homogenate was analyzed under the same conditions (Fig. 1B), a major radioactive peak was again found with f1 and a minor one with f3, but, in most cases, an additional small peak was observed with the f2 subfraction eluted with 0.01 N HCl.

Association of [^{14}C]ribose with histones was further substantiated by chromatography of the HCl-extract on an Amberlite CG-50 column by the method of Luck *et al.* (19). From this column, the radioactivity coeluted with proteins, both at 9% and from 20 to 35% guanidine·HCl in 0.1 M sodium phosphate buffer (pH 6.8).

Identification of Poly(ADP-Ribose). The radioactive components bound to the histone proteins were analyzed by treatment with venom phosphodiesterase and NH_2OH. Fig. 2 is the elution profile of a phosphodiesterase digest through a Dowex 1-formate column. By this enzyme treatment, approximately 20–40% of the bound ^{14}C and ^{3}H was rendered acid-soluble. Among a number of radioactive products, the material that eluted with the 5′-AMP marker and the material that appeared just before the ADP-ribose marker (fractions I and II, respectively) appeared particularly significant. Each fraction had both ^{14}C and ^{3}H, and their elution positions corresponded to those of the digestion products of poly (ADP-ribose), i.e., 5′-AMP and ψADP-ribose. Further identification of these substances as such was performed by paper chromatography. In Fig. 3 are shown the paper chromatograms of fractions I and II in solvent system 1. The former substance

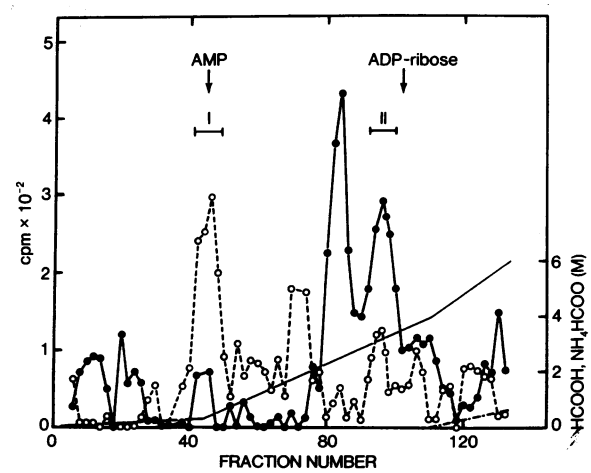

FIG. 2. Analysis of phosphodiesterase digests of HCl-extract on a Dowex 1-formate column. The products resulting from digestion with snake venom phosphodiesterase were analyzed as described under *Materials and Methods.* ●——● ^{14}C; O- - -O ^{3}H; —— HCOOH; -·-·- NH_4HCOO. Arrows indicate the elution positions of authentic 5′-AMP and ADP-ribose. Bars (fractions I and II) represent those fractions that were combined for subsequent analyses.

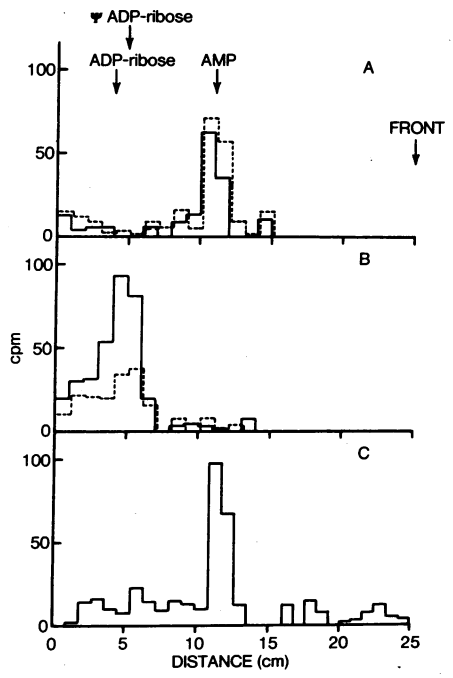

FIG. 3. Paper chromatography of fractions I and II obtained from phosphodiesterase digests of HCl-extract. Fractions I (A) and II (B) prepared as depicted in Fig. 2 and fraction II treated with *Escherichia coli* alkaline phosphatase (C) were chromatographed on paper in solvent system 1 (see *Materials and Methods*). —— ^{14}C; - - - ^{3}H. Authentic ψADP-ribose was prepared by enzymatic hydrolysis of poly(ADP-ribose) synthesized *in vitro* with chromatin (6).

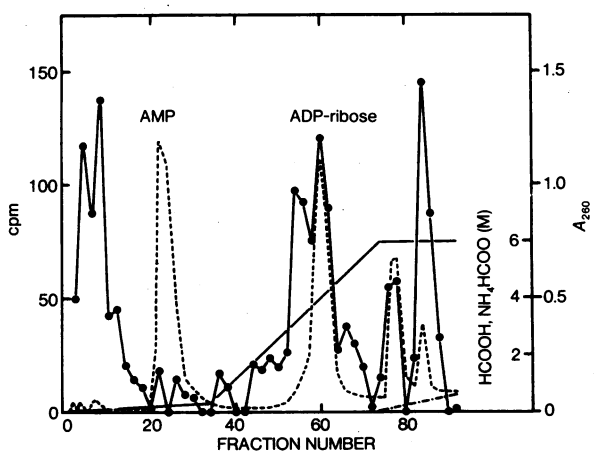

FIG. 4. Analysis of NH_2OH-treated HCl-extract on a Dowex-1 column. The HCl-extract was prepared from the acid-insoluble fraction of the whole $HClO_4$-homogenate. ●——● ^{14}C; - - - A_{260}; —— HCOOH; -·-·- NH_4HCOO.

cochromatographed with authentic 5'-AMP and the latter with ψADP-ribose with respect to both ^{14}C and ^{3}H. In solvent 2, fractions I and II also comigrated with these authentic markers.

To substantiate the existence of ψADP-ribose in the phosphodiesterase digests, the following two procedures were performed on the putative material. The first treatment was digestion with alkaline phosphatase (Fig. 3C). After digestion, the main peak of ^{14}C migrated in the position of the partially dephosphorylated product of ψADP-ribose, close to 5'-AMP (20). The other technique employed for identifying ψADP-ribose was acid hydrolysis. After treatment of fraction II with 1 N HCl for 10 min at 100°, the ^{14}C and ^{3}H chromatographed on paper with ribose-5-phosphate and adenine, respectively, a result that was previously observed with authentic ψADP-ribose (21). Since there is no known compound that yields ψADP-ribose under the present conditions, except poly(ADP-ribose), the existence of ψADP-ribose in the phosphodiesterase digests is unequivocal proof of the presence of a polymer of ADP-ribose and indicates that the material bound to histones is not just an ADP-ribose monomer.

The existence of an ADP-ribose monomer was explored by treating the HCl-extract with neutral NH_2OH. This treatment has been shown to effect the release of mono- and oligo(ADP-ribose) from histones that are ADP-ribosylated *in vitro* (7, 22). In the case of the *in vivo* material, 5–40% (fluctuating with the sample) of the acid-insoluble ^{14}C was converted to an acid-soluble form by this treatment. Upon chromatography of the released material on Dowex 1 (Fig. 4), ADP-ribose appeared to be present, since the radioactive material that eluted from the Dowex 1 column with marker ADP-ribose also cochromatographed with authentic ADP-ribose on paper in solvent 1. The radioactivity eluted by the NH_4HCOO gradient in 6 N HCOOH was partially acid-insoluble and may represent oligomers of ADP-ribose.

DISCUSSION

Since the discovery of poly(ADP-ribose) in 1966, the demonstration of its natural occurrence has been pursued by many investigators with a variety of methods. Until very recently, however, all attempts to demonstrate it *in vivo* were unsuccessful except that of Doly and Mandel (11). In 1968, we reported a covalent linkage of the polymer to nuclear proteins, presumably to histones (6), suggesting that the polymer *in vivo* may also be found in a protein-bound form. The present study was undertaken to isolate poly(ADP-ribosyl)-histones from mammalian tissues, on the basis of this line of reasoning.

Our method consisted, in principle, of two critical steps, i.e., injection of [^{14}C]ribose into the whole rat and extraction of acid-insoluble liver material with dilute HCl. The use of labeled ribose as a precursor appears to have two advantages; firstly, it is more selectively incorporated into NAD than into other nucleotides and, secondly, the radioactivity of the ribose in NAD is enriched in the NMN half, as formerly noted by Shuster and Goldin (23). In a typical experiment, specific radioactivities of NAD and 5'-AMP recovered from the acid-soluble fraction were 239 and 100 cpm/nmol, respectively, and the ratio of radioactivity of the NMN moiety to that of the 5'-AMP moiety in NAD was 3.1.

Extraction of histone proteins with dilute mineral acid seems to be essential for the detection of poly(ADP-ribose), since [^{14}C]ribose injected under the present conditions was actively incorporated into various biological macromolecules. A preliminary analysis has revealed that [^{14}C]ribose-derived, acid-insoluble radioactivity is distributed among RNA, DNA, and lipid, at least. Therefore, direct treatment of Cl_3CCOOH-washed material with venom phosphodiesterase yields a variety of compounds which can mask the presence of ψADP-ribose in subsequent analyses.

HCl-extraction has to be applied on the crude nuclear preparation or on the Cl_3CCOOH-washed liver material; the identical procedure applied to nuclei which were further purified by high-speed centrifugation (75,000 × *g*, 60 min) through 2.2 M sucrose failed to give a significant amount of "labeled" histone protein. In this case, degradation of the labeled material probably ensues during the purification procedure.

The presence of adenine in a nuclear basic protein fraction was reported in rat thymus by Ord and Stocken (24). As a consequence of analyzing protease digests, the same group reported recently that ADP-ribose is present in association with histone F1 and proposed that the attachment is via a serine-phosphate residue (13). Dietrich *et al.* also reported the *in vivo* binding of ADP-ribose to proteins, employing [^{32}P]-orthophosphate as an *in vivo* probe (14). It seems to us, however, that their identification of ADP-ribose may be preliminary and equivocal, since we have occasionally encountered ambiguous material which behaves like ADP-ribose during various chromatographic analyses but which cannot be degraded chemically or enzymatically to the expected products. In this connection, the possibility of noncovalent attachment of ADP-ribose to protein needs also to be ruled out. At present, the formation of ψADP-ribose by phosphodiesterase degradation appears to provide the best proof for the natural occurrence of bound ADP-ribose, albeit of a polymeric form.

Poly(ADP-ribose) obtained *in vivo* is partially degraded by snake venom phosphodiesterase and is also released from proteins by treatment with NH_2OH. However, the *in vivo* material is less susceptible to both treatments compared to poly(ADP-ribose) produced *in vitro* (7). Alkaline pH also barely solubilized the acid-insoluble material produced *in vivo* (7). Whether these discrepancies are due to some fundamental difference in structure between the *in vivo* and *in vitro* mate-

rials or simply reflect a difference in chain length of the polymer must be determined by further investigation. In this connection, it may be noteworthy that the average chain length of natural poly(ADP-ribose) bound to histones is about 2 to 5, as estimated from the ratio of [^{14}C]ψADP-ribose to [^{14}C]5'-AMP in the phosphodiesterase digests (Fig. 2), if one assumes equal specific radioactivity and purity of these products. This chain length appears to be greater than that of *in vitro* material, which is mostly monomeric (6).

We thank Drs. S. Narumiya and J. Oka for their excellent technical assistance in the earlier part of this work and Dr. C. Edson for valuable advice on the preparation of this manuscript. This work was supported in part by the National Science Foundation (GF-32308 to A.O.) and by research grants from the Waksman Foundation of Japan, Inc., Princess Takamatsu Cancer Research Fund, and the Scientific Research Fund of the Ministry of Education of Japan.

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