IN VIVO OCCURENCE OF BOUND ADP-RIBOSE ‡

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1. Introduction

The polymerization of the ADP-ribose moiety of NAD in eukaryotic cells is catalyzed by a nuclear enzyme [1]. Purification of the polymerized material [2, 3] demonstrates that the chain length produced in vitro varies from approx. 4–33 ADP-ribose units. The polymeric material is in part bound to nuclear protein, much of the material being bound to nuclear histones [4]. The ability to label nuclei after incubation with labeled NAD varies through the cell cycle [5–7]. More recent studies [8] indicate that at least in the case of rate, most (70–80%) of the labeled material bound to nuclear material after incubation with [^{14}C] NAD is not poly (ADP-ribose) but the monomer ADP-ribose, most of which can be made acid soluble by incubation with cold neutral NH_2OH. Until very recently only one report [9] has been forthcoming indicating the in vivo formation of poly (ADP-ribose. This communication documents our efforts to demonstrate the presence of ADP-ribose derivatives in vivo and presents data indicating that ADP-ribose is formed in vivo in rat liver tissue and that this material is made acid soluble by extraction with ice-cold neutral hydroxylamine. Employing this procedure, we were unable to detect the presence of poly (ADP-ribose).

2. Materials and methods

2.1. *Chemicals and enzymes*

$^{32}P_i$, acid-free, was obtained from New England Nuclear, Inc. RNAase and DNAase and thin layer cellulose plates were obtained from E. Merck, Darmstadt, GFR. Snake venom phosphodiesterase was purchased from Boehringer u. Söhne GmbH. Pronase, grade B, was obtained from Calbiochem. Hydroxylapatite spheroids (75 μm particles) were the generous gift of Dr. A.R. Thomas, of the Atomic Energy Research Establishment, Harwell, England.

2.2. *Isolation of ADP-ribose derivatives*

Ten unstarved female albino SIV-50 rats (250 g) were each injected with 10 mCi $^{32}P_i$. Two hours later the animals were sacrificed by decapitation after ether anesthesia, the livers removed and nuclei isolated employing the procedure of Blobel and Potter [10]. The nuclei, containing 106 mg protein, were washed twice with 10 ml of 0.25 M TKM buffer (0.05 M Tris–Cl, pH 7.5, containing 0.025 M KCl and 0.05 M $MgCl_2$). Two-thirds of the nuclear material was suspended in 10 ml of 3% cold (0°C) PCA, extracted for 5 min and then centrifuged. The acid-insoluble material was then washed 4–5 times with cold 3% PCA followed by two washings with Tris-Cl buffer, pH 7.8. The neutral acid-insoluble nuclear material was then suspended in 8 ml of ice-cold 0.4 M NH_2OH, pH 7.0, and incubated at 0°C for 1 hr. The material was centrifuged and the resulting pellet washed once with Tris–Cl buffer, pH 8.2. The NH_2OH-soluble material was combined with the wash and an equal volume of 6% ice-cold PCA was added. The supernatant material obtained after centrifugation was neutralized with KOH. After removal of the insoluble potassium perchlorate, non-radioactive 5′-AMP and ADP-ribose were added as carriers and the sample, after appropriate dilution,

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† *Abbreviation*: ADP-ribose = adenosine diphosphoribose.

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was placed on a Dowex-1-2X formate column and eluted stepwise with 1.0 M, 6.0 M formic acid and 6.0 M formic acid containing 0.4 M ammonium formate. The fractions from each elution step containing radioactivity were combined, lyophilized and applied to thin layer cellulose plates and developed in the following solvents: isobutyrate–concentrated NH_4OH–H_2O (66:1:33, v/v/v), ETOH–1.0 M ammonium acetate, pH 7.5 (7:3, v/v), and 0.1 M PO_4, pH 6.8–$(NH_4)_2SO_4$–*N*-propanol (100:60:2, v/w/v). The location of the radioactivity on the thin layer plates was determined using a chromatographic scanner and the results quantitated employing liquid scintillation spectrometry. All counts are corrected to the day of injection.

The NH_2OH-insoluble material was then digested with pronase, DNAase and RNAase, extracted with phenol (cold RNA was added at this point as a marker) and placed on a hydroxyapatite column in 0.1 M PO_4, pH 6.8, and eluted with a PO_4 gradient according to the procedure of Sugimura [3]. This procedure separates ADP-ribose polymer according to mol. wt. Fractions containing radioactivity but no detectable nucleic acids as measured by A_{260}, but which would theoretically contain poly (ADP-ribose) of 15–20 ADP-ribose units, were subjected to phosphodiesterase digestion followed by chromatography on thin layer cellulose plates employing the solvents listed above in an attempt to identify the basic units of poly (ADP-ribose), 2′-phosphoribosyl AMP and 5′-AMP.

3. Results and discussion

The nuclei from the rats administered $^{32}P_i$ (106 mg protein) contained 6.4×10^7 cpm. 3.2×10^6 and 5.0×10^5 cpm were extracted by PCA and NH_2OH, respectively. The material made acid-soluble by cold neutral NH_2OH extraction contained 4.4×10^4 cpm. This material was placed on a Dowex column followed by thin layer chromatography as discussed under Materials and methods. The results are shown in table 1. Considering the vast number of phosphate-containing intermediates in intact cells, the material rendered acid soluble by treatment with neutral NH_2OH is remarkably uncomplicated, only 6 compounds being observed. This would seem to indicate

Table 1
Distribution of radioactivity in acid-soluble material extracted by cold neutral hydroxylamine.

Compound	cpm	% Fraction
P_i	5 800	13
5′-AMP	11 000	25
ADP-ribose	4 700	11
Unknown *	1 300	3
Unknown **	21 000	48

* Elutes from Dowex 1-formate column in same position as ADP-ribose oligomer.

** Uncharacterized material not retained by Dowex formate column. Thin layer chromatography indicates two compounds.

that a select group of compounds are attached to nuclear material in bond types sensitive to cold neutral NH_2OH. These compounds are P_i, 5′-AMP, ADP-ribose, and an unknown compound which elutes from a Dowex column where ADP-ribose oligomer would appear. This compound, although sensitive to phosphodiesterase, was present in amounts too low to permit a valid characterization. In addition, two compounds determined by thin layer chromatography were observed in the material not retained by the Dowex column. No attempts have been made to characterize these latter two compounds.

Characterization of 5′-AMP and ADP ribose: in all the thin layer solvent systems utilized as well as the ion exchange column, radioactivity was always coincidental with carrier 5′-AMP and ADP-ribose. In addition, phosphodiesterase digestion of the ADP-ribose demonstrated that the products of the reaction were labeled 5′-AMP and ribose 5-PO_4.

These studies indicate that ADP-ribose is bound to rat liver nuclear protein in vivo. Since the number of counts recovered, 4700 (11% of all the material made acid soluble by treatment with NH_2OH) is low (see table 1), one must be concerned as to whether this radioactivity actually results from NH_2OH extraction or is merely residual acid-soluble material not fully extracted by the PCA. Since the labeled compound administered was $^{32}P_i$, all the acid-soluble nucleotides would be labeled. Since 5′-AMP, ADP-ribose and 'ADP-ribose oligomer' are the only nucleotides found to be acid soluble after NH_2OH treatment, one can conclude that these compounds are

a result of the NH_2OH extraction. The presence of a large amount of 5′-AMP is of interest. Whether this material is derived from ADP-ribose by enzymatic action during the preparation of the nuclei is unknown. Such a reaction is known to occur in vitro (L.S. Dietrich, unpublished data).

The acid-insoluble, NH_2OH-insensitive nuclear material treated with pronase, DNAase and RNAase yielded 3.9×10^6 cpm that were insoluble in acidic ethanol. 7.0×10^5 cpm were eluted from the hydroxyapatite column in the region where NH_2OH-insensitive poly (ADP-ribose) synthesized in vitro would be found. This material was digested by phosphodiesterase and the products subjected to thin layer chromatography. Only 5′-mononucleotides were found, indicating that most, if not all, of the radioactivity eluting from the hydroxyapatite column came from nucleic acids not completely digested by DNAase or RNAase, rather than from poly (ADP-ribose). No evidence of the presence of 2′-phosphoribosyl AMP was obtained. Taking into consideration the sensitivity of the analyses, if poly (ADP-ribose) were present, less than 7000 cpm would be contained in this compound.

The inability to determine the presence of poly (ADP-ribose) utilizing the procedure employed is not surprising. If the same ratio of ADP-ribose: poly (ADP-ribose) exists in vivo as in vitro, then the poly (ADP-ribose) present in this preparation would be approx. 1500 cpm. Other investigators, in particular Ueda (private communication), have unsuccessfully attempted to find polymer in the same manner. Very recently, Kidwell‡, employing another approach, established the probable presence of poly (ADP-ribose) in lymphocytes, and Ueda‡ has isolated 2′-phosphoribosyl AMP from histones extracted from rat liver nuclei that had been labeled with $^{32}P_i$ in vivo. Our observations demonstrating the presence of acid-insoluble ADP-ribose which is solubilized by NH_2OH complement the findings of these investigators. Thus, there should remain little doubt that ADP-ribose and its derivatives occur in vivo.

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