DISTRIBUTION OF BOUND ADP-RIBOSE DERIVATIVES IN NUCLEAR PROTEIN OF RAT LIVER NUCLEI

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SUMMARY--Most of the acid-insoluble radioactivity produced by incubation of rat liver nuclei with [^{14}C]NAD is rendered soluble by treatment with cold neutral hydroxylamine. The substances released by hydroxylamine have been determined to be (adenosine diphosphoribose)oligomer, adenosine diphosphoribose, 5'-AMP and adenosine, the greatest activity being found in the adenosine diphosphoribose fraction. The distribution of hydroxylamine-sensitive radioactive material in the nuclear proteins varies with the fractionation method employed. Regardless of the method employed, the "histones" contained only small amounts of hydroxylamine-insensitive radioactive material [poly(adenosine diphosphoribose)]

INTRODUCTION--In a previous communication, it was shown that only a small fraction of acid-insoluble radioactivity formed after incubation of rat-liver nuclei with [^{14}C]NAD, represents poly(ADP-ribose)[2] (1). In continued efforts to understand better the polymerization and transfer reactions of NAD with cell nuclei, we provide in this paper some further data on the chemical nature of products formed from NAD by nuclear enzymes *in vitro*. For this purpose, the separation of acid-insoluble radioactivity into hydroxylamine soluble and insoluble material, the fractionation of nuclear macromolecules into several fractions, as well as the chromatographic identification of the different products formed from NAD, were employed.

MATERIALS AND METHODS--Radiochemicals. ([U-^{14}C]adenine)NAD (167 Ci/mole) was obtained from the Radiochemical Centre, Amersham. ([8-^{14}C]adenine)ATP (6 Ci/mole) was from New England Nuclear, Boston, Massachusetts. Chemicals. Dowex-1-2X and thin layer cellulose plates were obtained from E. Merck, Darmstadt. NAD and snake venom phosphodiesterase were obtained from Boehringer u. Soehne, GmbH, Mannheim. Protosol was obtained from New England Nuclear, Boston, Massachusetts.

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[2]ADP-ribose = adenosine diphosphoribose

Isolation of ADP-ribose derivatives. Albino SIV-50 rats (250 grams) of mixed sex were sacrificed by decapitation after ether anesthesia, the livers removed and nuclei isolated employing the procedure of Blobel and Potter (2).

Rat liver nuceli (1-10 mg protein) were incubated with 10^{-3} M [^{14}C]NAD (0.2-2 x 10^6 cpm) in 3 ml of a Tris-Cl buffer, pH 8.2, containing 0.025 M KCl and 0.005 M $MgCl_2$ (TKM buffer) for 10 min. at 25°. The mixture was immediately cooled to 0° in an ice bath. The labeled nuclei were then washed with 5 ml portions of the TKM buffer until the radioactivity of the wash approached background (6-8 times). Nuclear proteins were fractionated employing the procedure of Kostraba and Wang (3). Nuclei were also fractionated into histones and nonhistone fractions using the procedure of Grunicke, Potter and Morris (4). Labeled nuclei or labeled nuclear proteins were incubated in 8 ml of ice-cold 0.4 M NH_2OH, pH 7.0, at 0° for 1 hour. 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, was placed on a Dowex-1-2X formate column and eluted stepwise with 1.0 M, 6.0 M formic acid and 6.0 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 NH_4 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. The NH_2OH-insoluble material was then dissolved in Protosol and quantitated for radioactivity as previously reported (1).

Characterization of the Radioactive Material made Acid Soluble by Treatment

with NH_2OH. In all the thin layer systems utilized as well as the ion exchange column, radioactivity always migrated with carrier adenosine, 5'-AMP and ADP-ribose. In addition phosphodiesterase digestion of the ADP-ribose demonstrated labeled 5'-AMP as a reaction product. Similar treatment of the material suspected of being ADP-ribose oligomer demonstrated 2'-phosphoribosyl ADP-ribose and 5'-AMP as products of the reaction.

Analysis of chain length of ADP-ribose polymer was carried out employing the procedure of Shima et al. (5). Protein was determined employing the procedure of Lowry et al. (6) with bovine serum albumin as a standard. Radioactivity was assayed employing standard liquid scintillation spectrometric techniques and, where applicable, cpm were converted to dpm employing [^{14}C] toluene as an internal standard.

RESULTS AND DISCUSSION--

Composition of Radioactive Material made Acid Soluble by Treatment with NH_2OH.

The distribution of the nuclear-bound radioactivity which is solubilized by cold neutral NH_2OH in rat liver nuclei is shown in Table 1. The label can be accounted for by 4 compounds: (ADP-ribose)oligomer, ADP-ribose, 5'-AMP and adenosine, most of the activity being found in the ADP-ribose fraction. These compounds are probably bound to nuclear material covalently since they were acid insoluble until treated with cold neutral NH_2OH. The observation that ca. 25% of the [^{14}C]NAD consumed can be accounted for in the form of bound 5'-AMP and adenosine is most interesting. [^{14}C]NAD appears to be the true precursor since incubations under identical conditions with [^{14}C]ATP resulted in no incorporation of radioactivity into rat liver nuclei. Whether the nuclear-bound adenosine and 5'-AMP are catabolic products of ADP-ribose metabolism or represent a pathway which is independent of ADP-ribose metabolism is not known and cannot be determined by the data presented here. If the formation of nuclear-bound adenosine and 5'-AMP from NAD is an independent pathway, this would present a new and intriguing observation and might indicate multiple functions in nuclear metabolism of ADP-ribose. If on the other hand, these bound compounds are products

TABLE 1

Distribution of Radioactivity in Acid-Soluble

Material Extracted by Cold Neutral Hydroxylamine

Compound	dpm	% Fraction
(ADP-ribose)oligomer	18,000	19
ADP-ribose	50,500	55
5'-AMP	5,900	6
Adenosine	18,200	20
	92,600	

Nuclei incubated for 10 minutes with 10^{-3} M [^{14}C]NAD at 25°. 91,000 dpm placed on column.

of ADP-ribose or poly(ADP-ribose) degradation, then one must conclude that there is yet another system that transfers the AMP moiety to protein during degradation of ADP-ribose derivatives. If this linkage were in the 2' position then the 5'-PO_4 would be free for hydrolysis leaving nuclear bound adenosine as a product. The premise that these observations are not artifacts of an in vitro system is strengthened by the observation that 5'-AMP is observed in vivo in the acid soluble fraction obtained from the NH_2OH treatment of acid-extracted liver nuclei made from rats that had been injected with ^{32}Pi (7).

Distribution of Radioactive Material made Acid Soluble by NH_2OH Treatment in Nuclear Proteins as a Function of the Procedure Employed. When one extracts rat liver nuclei that have been incubated in [^{14}C]NAD with dilute mineral acid, most of the radioactivity is bound to proteins ("histones") extracted by acid (8,9). These observations are confirmed in Table 2 and extended to show that most but not all of the radioactivity ("histone" or "nonhistone" bound) is sensitive to release by NH_2OH. If, however, one employs the procedure of Kostraba and Wang (9)[3]

[3]Employing this procedure the chromatin is first solubilized in 1 M salt and then the DNA-histone complex is precipitated by lowering the salt concentration followed by diluted mineral acid treatment of the DNA-histone complex to extract the histones.

TABLE 2

Distribution of ^{14}C Solubilized by Cold Neutral NH_2OH in Histone and non-histone Components of Rat Liver Nuclei

	NH_2OH Soluble Material	NH_2OH Insoluble Material
	dpm	dpm
Histone	92,000	2,703
Non-histone	57,350	7,550
	149,350	9,253

Nuclei incubated for 10 minutes with 10^{-3} M [^{14}C]NAD at 25°. Histones extracted with acid employing procedure of Grunicke et al. (4). Values are the average of 2 experiments.

where the acid soluble proteins are not extracted until removal of the "acidic proteins", only 34% of the radioactivity is found in the "histone" proteins (Table 3) compared with a 60% found when all the acid soluble proteins are extracted (Table 2).

Employing the Kostraba and Wang procedure, approximately 53% of the radioactivity was found in the "acidic protein" fractions. Over 80% of the poly(ADP-ribose) (NH_2OH insensitive material) is found in the material 1) not soluble in 1 M salt ("residual protein") and 2) material not extracted from the DNA-histone complex ("DNA"). These data demonstrate that the nuclear bound radioactivity derived from [^{14}C]NAD is not associated primarily with the "histones", most of the activity being found in the "acidic proteins" and not the "histones." This leads one to suggest that some of the ADP-ribose derivatives are bound to acid soluble proteins which are not histones. It is possible that during the precipitation of the DNA-histone complex the products are partitioned between the "DNA" and the "acidic proteins." The overwhelming presence of the NH_2OH insoluble material [poly(ADP-ribose)] in the 1 M salt insoluble material ("residual proteins") and the residue after histone extraction from the DNA-histone complex is highly significant. It appears clear from the data that neither "histones" nor "acidic

TABLE 3

The Distribution of ^{14}C Solubilized by Cold Neutral NH_2OH in Components of Rat Liver Nuclei

	NH_2OH Soluble Material	NH_2OH Insoluble Material
	dpm	dpm
Histones	34,100	540
Acidic Proteins	53,500	855
Residual Proteins	9,910	3,825
"DNA"	4,150	1,960
	101,660	7,180

Nuclei incubated for 10 minutes with 10^{-3} M [^{14}C]NAD at 25°. Nuclear protein fractionated employing procedure of Kostraba and Wang (3). Results are the average of 2 experiments.

TABLE 4

Distribution of ^{14}C Extracted from Rat Liver Nuclear Components by Cold Neutral NH_2OH

	Adenosine	AMP	ADP-ribose	(ADP-ribose)Oligomer
	cpm	cpm	cpm	cpm
Histone	<100	410	2422	165
Acidic Proteins	1710	1080	3508	355
Residual Proteins	758	594	730	405
"DNA"	<100	<100	642	362
Total	2468	2084	7302	1287

Nuclei incubated for 10 minutes with 10^{-3} M [^{14}C]NAD at 25°. Nuclear protein fractionated employing procedure of Kostraba and Wang (3). Results are the average of 2 experiments.

proteins" contain more than minor amounts of poly(ADP-ribose).

Some interesting observations on the distribution of the compounds present in the NH_2OH solubilized material from these protein fractions are shown in Table 4. Under these conditions it was found that ADP-ribose was located primarily in the "histones" and "acidic proteins". On the other hand, most of the ADP-ribose oligomer was found in the "residual proteins" and "DNA", little (13%) being found in the histones. Virtually all the 5'-AMP and adenosine were shown to be in the acidic proteins and "residual proteins" (ca 90%), no adenosine being detected in the histones and DNA fractions.

These data demonstrate the complexity of products bound to nuclear protein after incubation with [^{14}C]NAD and point out that at our present level of knowledge of the problem, one must be cautious in correlating "ADPR polymerase" with biological activity and/or nuclear location.

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