MACROMOLECULAR DERIVATIVES OF NAD^+ IN HEART NUCLEI: POLY ADENOSINE DIPHOSPHORIBOSE AND ADENOSINE DIPHOSPHORIBOSE PROTEINS

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Summary: Rates of poly (ADP-R) formation from NAD^+ were determined in isolated pigeon heart and liver nuclei. In heart nuclei K_m for NAD^+ was 330 μM. On a DNA basis rates were more than twice in heart nuclei than in liver nuclei. The polymer poly (ADP-R) was identified in both nuclear systems by isolation, digestion with snake venom phosphodiesterase and chromatographic separation of phosphoribosyl-AMP and AMP. ADP-R binds to macromolecular nuclear components to form ADP-R derivatives, which upon digestion with snake venom phosphodiesterase yield only AMP, distinguishing these ADP-R compounds from poly (ADP-R).

The nucleic acid-like polymeric product of NAD^+, poly (ADP-R) has been identified by enzymatic degradation with snake venom phosphodiesterase (E.C. 3.1.4.1) to 2'-(5"-phosphoribosyl)-5'-AMP and AMP in chromatin preparations of thymus and liver cell nuclei (1,2,3,cf.4) and nuclei of HeLa cells (5,6). On the basis of the customary enzymatic assay for the poly (ADP-R) synthase, which consists of counting the acid precipitable radioactivity after incubation of labelled NAD^+ with some form of chromatin preparation, it appears that this enzyme is present in a variety of animal cells. Recently an ADP-ribosylated protein was isolated from mitochondria (7); therefore the assumption that poly (ADP-R) is the only macromolecular derivative of NAD^+ in animal cells seems unlikely. Assessment of the biological role of macromolecular derivatives of NAD^+ requires identification of these substances in a variety of animal tissues which have greatly different physiological functions. Pursuing this goal we compared the rates of syntheses and identified poly (ADP-R) in nuclei of heart and liver of 6 week old pigeons. It is known that cardiocytes have a limited capacity for mitosis (8), whereas the wellknown phenomenon of liver regeneration indicates a far greater mitotic potential for hepatocytes. It was assumed that this difference in mitotic potential of the two organs may be reflected in a difference of rates of poly (ADP-R) formation in nuclei isolated from both tissues of the same species. Besides poly (ADP-R) formed from NAD^+, we also find that ADP-R

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Abbreviations: poly (ADP-R) = poly (adenosine diphosphoribose)
ADP-R = adenosine diphosphoribose
NP-40 = nonidet P40, detergent product of Shell Co.
DTT = dithiothreitol

itself can bind covalently to macromolecular components of chromatin. The existence of a family of hitherto unknown macromolecular metabolites of NAD^+ is experimentally demonstrated in the same chromatin preparations where poly (ADP-R) is synthesized.

Materials and Methods. Isolation of purified nuclei from liver and cardiac tissues of 6 week old pigeons (250 g body weight) and assays of DNA and protein were carried out by standard techniques (9). Higher yields of cardiac nuclei were occasionally obtained by substitution of the high density centrifugation step with several centrifugal washings at 700 x g with 0.25 M sucrose-1 mM $MgCl_2$. The source of ^{14}C labelled (uniformly in the adenine portion 253 Ci/mole) NAD^+ was Amersham-Searl Corp. and the radiochemical counting techniques were the same as published previously (7). Labelled ADP-R was prepared from labelled NAD^+ by hydrolysis with calf spleen glycohydrolase (E.C. 3.2.2.5) and isolated by paper chromatography with a yield of 75% (93% purity). Poly (ADP-R) was isolated by a published method (10) except phenol extraction was employed after pronase digestion. At stage 5 (see Table) the isolated poly (ADP-R) was dissolved in 50 mM Tris-acetate (pH 7.2) + 8 mM $MgCl_2$ and digested with purified (14) snake venom phosphodiesterase (Boehringer) at 200 µg/ml concentration for 2 h at 37°. The amount of poly (ADP-R) used for digestion was 4.5 nmoles (32,500 CPM) from liver and 7.8 nmoles (24,000 CPM) from heart nuclei. The entire incubation mixture was applied to Whatman No. 1 chromatographic paper, washed with 80% ethanol and developed in isobutyric acid-30% NH_3-H_2O, 66:1:33 (v/v). Authentic nucleotides were co-chromatographed with samples. Treatment with nucleases prior to snake venom phosphodiesterase was found unnecessary because non-radioactive breakdown products of DNA did not interfere with identification of radioactive nucleotides derived from poly (ADP-R).

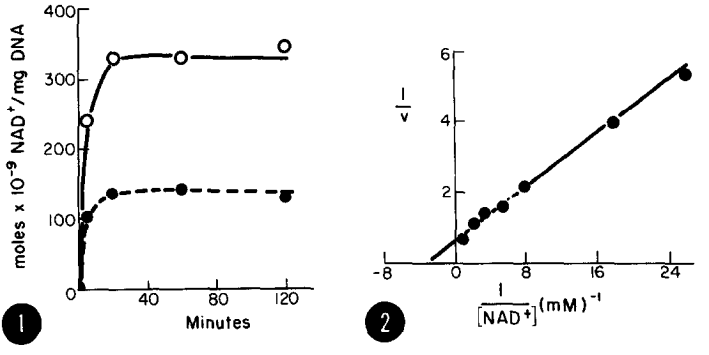


Fig. 1: Time course of poly (ADP-R) formation from NAD^+ (3.0 mM). The incubation system (100 µl) contained 30 mM $MgCl_2$, 4 mM KF, 60 mM KCl, 100 mM Tris-Cl (pH 8.0), 0.5 mM EDTA $(Na)_2$, 2 mM DTT and ^{14}C-NAD^+ 8 x 10^5 CPM. In the liver system 2.0 mg protein (Lowry) and 0.4 mg DNA (Burton), and in the heart system 0.4 mg protein and 0.1 mg DNA were present. t = 37°. Acid precipitable radioactivity was determined by the glass fiber filter technique using 6% $HClO_4$ as protein precipitating agent.

Fig. 2: Saturation of poly (ADP-R) synthase of heart nuclei by NAD^+. v = nmoles NAD^+ converted to poly (ADP-R) in 3 minutes at 37°, pH 8.0, K_m = 330 µM. Conditions for the assay are described in legend of Fig. 1.

Results and Discussion. Rates of poly (ADP-R) formation from NAD^+ (3 mM) as expressed as moles x 10^{-9} NAD^+ converted to poly (ADP-R) per mg DNA were more than twice in heart nuclei than in liver nuclei (Fig. 1). Whether the increased capacity of heart nuclei as compared to liver nuclei is due to higher enzyme content or to differences in enzyme regulation is at present unknown. As shown in Fig. 2, the K_m of NAD^+ for heart nuclei is 330 μM, similar to K_m values reported for rat liver (250 μM, cf. 12) and other poly (ADP-R) synthase systems (11).

Extremely low poly (ADP-R) synthase activities of rat heart nuclei were reported recently (13). The concentration of NAD^+ used by this author (13) was 1.2 μM, which is 1/210th of the K_m found for this enzyme in rat tissues (12); consequently the reported results have no quantitative meaning.

That acid precipitable radioactive material (Figs. 1, 2) was predominantly poly (ADP-R) was proved by its isolation (Table) and identification (Fig. 3).

TABLE

Stages of purification (see Ref. 10)	Liver	Heart
1. First TCA precipitate	108	250
2. After NaOH treatment	92	210
3. After pronase	92	210
4. After phenol extraction	83	210
5. After ethanol acetate precipitation	75	167

The incubation system (2.3 ml) contained the same molar concentrations of ingredients as shown in legend to Fig. 1. The amount of nuclei per system was 61 mg protein, 12 mg DNA for liver and 43 mg protein, 2.4 mg DNA for heart. Results are expressed as nmoles of ADP-R residues per mg DNA.

As shown in Fig. 3, digestion of isolated poly (ADP-R) at stage 5 with purified snake venom phosphodiesterase (14) yields phosphoribosyl-AMP and small amounts of AMP, identified by the ADP-R and AMP markers. It is known that the snake venom enzyme does not act on phosphoribosyl-AMP, but hydrolyzes ADP-R. These results identify poly (ADP-R) synthesized by both nuclear preparations. Inhibition by nicotinamide (4.3 mM inhibits 77%) and by thymidine (4.3 mM inhibits 78%) as also reported by others (1,2,3,15) identifies poly (ADP-R) synthase.

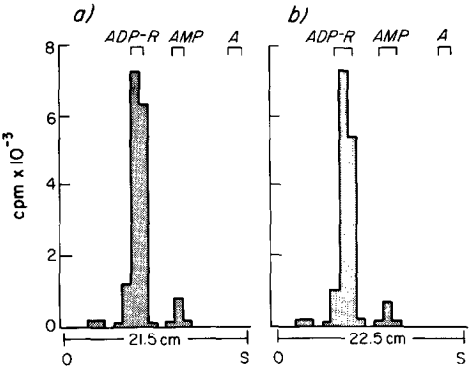
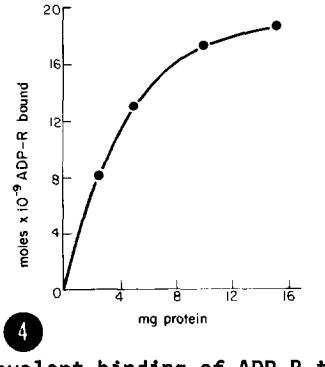


Fig. 3: Identification of poly (ADP-R).
A = adenosine,
O = origin of chromatogram,
S = solvent front,
a) liver nuclei,
b) heart nuclei.
The average chain length of poly (ADP-R) was 14 for the liver and 16 for the heart.

Incubation of a nuclear preparation of heart with ADP-R (6.6 mM) under similar conditions as used for the biosynthesis of poly (ADP-R), except in the absence of added NAD^+, yields covalently bound ADP-ribosylated macromolecules. When the precipitable radioactive material was digested with snake venom phosphodiesterase (see Methods) the only radioactive nucleotide released was AMP, which identifies covalently bound ADP-ribose. Binding of ADP-R to heart nuclear preparations is shown in Figs. 4 and 5.



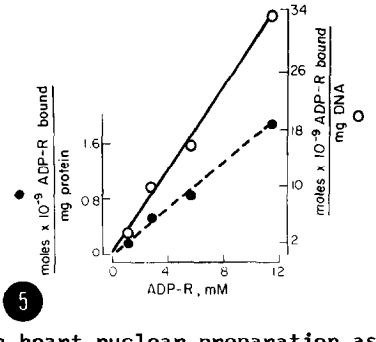


Fig. 4: Covalent binding of ADP-R to pigeon heart nuclear preparation as a function of protein concentration. The reaction system calculated for 1 ml volume contained 16 mM Na phosphate (pH 8.0) 6.6 mM ADP-R (8.3×10^6 CPM) and increasing amounts of heart nuclei (31 mg protein and 1.7 mg DNA per ml) in 50 mM Tris-Cl, 1 mM DTT, 1% NP-40. Time of incubation 20 min. at 37°.

Fig. 5: Covalent binding of ADP-R as a function of ADP-R concentration. Conditions are the same as described in legend of Fig. 4. The concentration of protein was 13 mg/ml, DNA = 0.7 mg/ml.

When the amounts of macromolecular products formed from ADP-R are calculated on a DNA basis (fig. 5) as was done for poly (ADP-R) the values found are of the same order of magnitude (nmoles/mg DNA) as poly (ADP-R). The macromolecular ADP-R products are not specific for heart nuclei; they represent a mixture of proteins containing covalently bound ADP-R. The molecular structure of these substances will be described elsewhere.

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