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Covalent modification of proteins by metabolites of NAD$^+$

(ADP-ribose/ribose-5-phosphate/bovine serum albumin/histone/polyamine Schiff bases)

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ABSTRACT **Covalently bound adducts of poly(L-lysine), bovine serum albumin, lysine rich histone (f_1) and deoxyribonucleotidase I (DNase, EC 3.1.4.5) with adenosine diphosphoribose and ribose-5-phosphate were prepared at pH 7.4 and 9.5. Macromolecular adducts of bovine serum albumin and histone (f_1) were isolated by gel filtration and electrophoresis. Reduction of products by $NaBH_4$ did not dissociate the ribose-5-phosphate moiety from macromolecules. Specific introduction of ^3H into the adducts also indicated Schiff base formation. The reaction of ribose-5-phosphate with ϵ-amino groups of histone (f_1) approached 70–90% saturation. Spermine and spermidine also react with adenosine diphosphoribose and ribose-5-phosphate to form 1:1 Schiff bases. It is proposed that high turnover of cellular NAD$^+$ is the source of aldehydic metabolites which may regulate macromolecular metabolism by covalent modification of nuclear proteins, whereas polyamines serve as modulators of this control cycle.**

Enzymatic transfer of ADP-ribose from NAD$^+$ to a mitochondrial protein (1), and direct association of ADP-ribose with macromolecular constituents of nuclei (2), indicate that poly(ADP-ribose) of chromatin is not the only macromolecular product of NAD$^+$ in animal cells. The operation of NAD$^+$ glycohydrolase (NAD$^+$ nucleosidase, EC 3.2.2.5) in mitochondria (3) and in nuclei (4), and of the specific glycohydrolase which degrades poly(ADP-ribose) to ADP-ribose in nuclei (5–8), provides known enzymatic pathways for ADP-ribose formation in these DNA containing organelles. Because it is estimated that the turnover of NAD$^+$ in HeLa cells is about 20 times higher than required to maintain the NAD$^+$ pool size during growth (9), it is unclear whether the catabolic products of NAD$^+$ which should be available in abundance have an as yet unrecognized biological function. Association of ADP-ribose with regulatory macromolecules generating protein products that are covalently altered is one of the possible consequences of high cellular turnover of NAD$^+$.

The first question requiring clarification is the identification of the nature of the chemical bond formed between ADP-ribose and macromolecules (2). We are describing the interaction of ADP-ribose and of ribose-5-phosphate with purified model substances: poly(L-lysine), lysine rich (f_1) histone (10), crystalline bovine serum albumin (BSA) (18), crystalline deoxyribonuclease I (DNase, EC 3.1.4.5) (11), spermine, spermidine, and L-lysine. Some conditions chosen for these studies are physiological (pH 7.4); therefore, it is predictable that the reactions described can occur *in vivo*. The model substances chosen are known cellular constituents, and thus conclusions drawn are likely to be of physiological relevance. Identification of the adducts of ADP-ribose and of ribose-5-phosphate with macromolecules and polyamines as Schiff bases is the experimental basis of further, more biologically oriented investigations.

Abbreviations: BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; ADP-R, adenosine-diphosphoribose.

METHODS

ADP-ribose (uniformly ^{14}C-labeled in the adenine portion) was prepared from labeled NAD$^+$ (271 Ci/mol, obtained from Amersham Searle Corp.). Aliquots of labeled NAD$^+$ (50 μCi) were incubated with 13 mg of particulate spleen NAD$^+$ nucleosidase (obtained from Sigma) in 50 mM Tris-Cl (pH 7.3) at 37° for 15 min in a reaction volume of 4 ml. The reaction was stopped with 2 ml of 30% trichloroacetic acid. The sediment was discarded and trichloroacetic acid removed from the supernatant by repeated extractions with ethyl ether until the aqueous phase had a neutral pH. After freeze-drying, the material was dissolved in a minimum volume of H_2O, spotted on Whatman no. 1 chromatographic paper, washed free of Tris with 80% ethanol, and developed with the isobutyric acid system (12). The radioactive ADP-ribose was eluted with H_2O; its purity was 93%, recovery 70–75% (2), and its radioactivity was 51,000–93,000 cpm per μl, depending on the volume of eluent. Radioactive ribose-5-phosphate (35 Ci/mol) was purchased from Schwarz/Mann Corp. Spermine and spermidine hydrochloride, L-lysine, and picrylsulfonic acid were of analytical grade. Poly(L-lysine) (average molecular weight = 186,000) and desoxyribonucleotidase I were obtained from Sigma, and crystalline bovine serum albumin from Miles Laboratories. A sample of f_1 histone (10) was a gift of David R. Cole (University of California, Department of Biochemistry, Berkeley, Calif.). Gel electrophoresis of bovine serum albumin was performed in the presence of 5% sodium dodecyl sulfate (13), and of histone in acid-urea gel (14). Analytical and radiochemical techniques were the same as reported previously (1–3). High voltage electrophoresis was carried out in a Savant instrument at pH 6.4 (0.5 M pyridine-acetate buffer) at 4000 V for 30 min on Whatman no. 1 filter paper. Amino acids and amines were determined by a quantitative ninhydrin test (15).

RESULTS

Incubation of ADP-ribose or ribose-5-phosphate with macromolecules listed in Table 1 resulted in covalent binding as determined by acid precipitation of products and radiochemical assay (1–3). Covalent binding was determined after incubation at 37° for 20 min as a relative measure of initial velocities, and after 6 hr, as a measure of reaction equilibrium. It is evident that both ADP-ribose and ribose-5-phosphate were bound to macromolecules. The extent of binding at 20 min was larger for ribose-5-phosphate than for ADP-ribose. Alkaline pH promoted binding, but significant reactions occurred also at pH 7.4. Binding approached different plateaus at both pH values. With physiological cellular conditions, in the presence of excess acceptor macromolecules, the quantity of macromolecularly bound ADP-ribose and ribose-5-phosphate should be dependent only on rates of formation of the aldehydic metabolites of NAD$^+$. For this reason the results obtained after 6 hr incubation

Table 1. Covalent binding of adenosine-diphosphoribose (ADP-R) and ribose-5-phosphate (R-5-P) to macromolecules

Experimental conditions	pH	Binding in nmol of ADP-R or R-5-P/μmol of macromolecule: 20 min incubation	6 hr incubation	Molar saturation of ϵ-NH_2 groups (%)
Poly(L-lysine) (0.5 mg) + ADP-R	7.4	144	514	0.05
	9.5	235	409	0.04
Poly(L-lysine) (0.5 mg) + R-5-P	7.4	212	1759	0.14
	9.5	302	1350	0.10
Lysine-rich histone (0.34 mg) + R-5-P	7.4	33	180	67
	9.5	133	327	87
Lysine-rich histone (0.34 mg) + ADP-R	7.4	25	97	33
	9.5	84	125	39
Lysine-rich histone (0.6 mg) + ADP-R	7.4	40		
+ 10 mM spermidine		28 (−30%)		
+ 10 mM spermine		28 (−30%)		
BSA (1 mg) + ADP-R	7.4	29		
+ 10 mM spermidine		19 (−34%)		
+ 10 mM spermine		18 (−37%)		
BSA (1 mg) + ADP-R	7.4	25	158	21
	9.5	74	191	25
BSA (1 mg) + R-5-P	7.4	33	140	23
	9.5	83	317	48

Appropriate amounts of macromolecules were incubated with 2 mM aldehydic metabolite of NAD^+ in a final volume of 20–30 μl with either 100 mM phosphate (pH 7.4) or carbonate-bicarbonate (pH 9.5) buffers. Acid precipitable counts were determined. Poly(L-lysine) derivatives were precipitated either as the trinitrophenylamine derivatives (in 8% $NaHCO_3$) or with 25% trichloroacetic acid in the presence of 6 M urea. Incubation for 6 hr was carried out: 1 hr at 37°, and 5 hr at room temperature. Values in parentheses refers to the decrease in binding due to the presence of spermine or spermidine.

may be extrapolated to cellular conditions. When polyamines were present simultaneously, the binding of ADP-ribose or of ribose-5-phosphate (not shown) was significantly inhibited. This inhibition was due to a reaction between the aldehydic metabolites of NAD^+ and one basic amino group of the polyamines to form Schiff bases. As illustrated in Fig. 1, high voltage electrophoresis readily separated unreacted ADP-ribose, unreacted polyamines, and the products. These were eluted and identified by paper chromatography as the 1 to 1 adducts of ADP-ribose and polyamines, or of L-lysine (not shown), as determined by radioactivity and ninhydrin analyses (15). The stoichiometry was established by elution of products (Fig. 1) and subsequent analyses: 138 nmol of spermidine reacted with 131 nmol of ADP-ribose; 60 nmol of L-lysine reacted with 52 nmol of ADP-ribose. The same 1:1 stoichiometry was obtained when ribose-5-phosphate was substituted for ADP-ribose. An aqueous solution of the isolated spermine or spermidine ADP-ribose adducts completely decomposed to ADP-ribose and to the polyamines upon standing at room temperature for 24 hr. In 1 M HCl (at 37° for 30 min) the main product (80%) was AMP, derived from ADP-ribose by acid hydrolysis. Both the primary decomposition product, ADP-ribose, and its secondary product, AMP, which was produced by acid hydrolysis of ADP-ribose, were isolated and identified by paper chromatography (1–3). Both aldehydic products of NAD^+ formed 1:1 Schiff base adducts with polyamines which strongly indicated that the same type of reaction occurred between ϵ-amino groups of lysine residues of macromolecules and the carbonyl group of NAD^+ metabolites. This was tested further by the isolation of macromolecular derivatives and subsequent assays which are characteristic for macromolecular Schiff bases (16). The ADP-ribose-BSA compound was readily isolated by molecular filtration on a Sephadex G-50 column (50 cm × 1 cm; void volume = 22.5 ml; flow rate of 12 ml/hr) with 50 mM Tris·Cl (pH 8.5) as shown in Fig. 2. Unreacted ADP-ribose quantitatively separated (second peak) from bound ADP-ribose (first peak). The total radioactivity determined by direct assay for radioactivity and by acid precipitation was the same in the ADP-ribose-BSA compound. Gel electrophoresis of BSA and of the ADP-ribose-BSA adduct further demonstrated that ADP-ribose (determined by radioactivity) coincided with the protein band. It should be noted that BSA and ADP-ribose-BSA were not preincubated with $NaDodSO_4$ in the customary manner (13), as a precaution to prevent the hydrolysis of the pyrophosphate bond of ADP-ribose. Because of insufficient exposure to $NaDodSO_4$, both the monomer and dimer were visible as incompletely separated bands. The ADP-ribose-BSA adduct had less tendency to separate than native BSA (Fig. 3). Electrophoretic behavior of histone (f_1) in acid-urea gel (14), and of the same histone after covalent modification with ADP-ribose, is illustrated in Fig. 4. Binding of ADP-ribose markedly changed the contour of the histone band which indicates that covalent modification by ADP-ribose altered electrophoretic mobility. The time-course of covalent binding of ribose-5-phosphate to lysine rich histone and to crystalline DNase I is shown in Fig. 5. When the ribose-5-phosphate-histone or the DNase I compounds were reduced with $NaBH_4$ according to Recsei and Snell (16), the expected acid stable products were obtained. As seen from Fig. 5, the amount of the radioactive ribose-5-phosphate covalently bound to histone or to DNase I and acid precipitable was nearly the same before and after reduction with $NaBH_4$ which indicates that the Schiff base and not the carbinolamine was predominantly formed under our experimental conditions. The macromolecular adducts of ADP-ribose and ribose-5-phosphate were resistant to hydrolysis with hydroxylamine at pH 4.6; thus, the possibility of phosphoramide linkage (17) was excluded. This possibility was also eliminated by the results which showed that ribose-5-phosphate forms the same type of macromolecular adducts as ADP-ribose. This leaves the carbonyl group as the only reactive group shared by

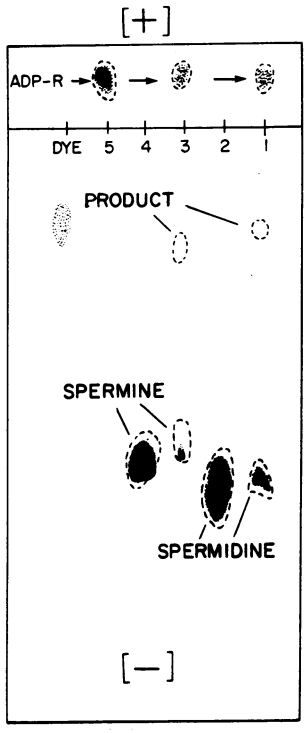

FIG. 1. Paper electrophoresis of spermine and spermidine adducts with ADP-ribose (ADP-R). The reaction mixtures consisted of spermine (2 μmol) and spermidine (2 μmol), each incubated with ADP-ribose (2 μmol) in a volume of 200 μl at 37° for 30 min at pH 9.5 (0.25 M carbonate-bicarbonate buffer), and components were separated by paper electrophoresis (see *Methods*). The spots were visualized both by ninhydrin and chlorox spray on the same paper. (*1*) Spermidine:ADP-R; (*2*) spermidine; (*3*) spermine:ADP-R; (*4*) spermine; (*5*) ADP-R; dye = Coomassie brilliant blue.

both ADP-ribose and ribose-5-phosphate. When the reaction between ribose-5-phosphate, histone (f_1), and DNase I reached a plateau, the molar saturation of ε-amino residues of lysine in histone (19) and DNase I (11) approximated 70–90%. The reactivity of ADP-ribose was about ½ of that of ribose-5-phosphate, presumably because of steric reasons. Only about ⅓ of ε-amino groups of BSA (18) bound ribose-5-phosphate, and a small molar fraction of amino groups of poly(L-lysine) was re-

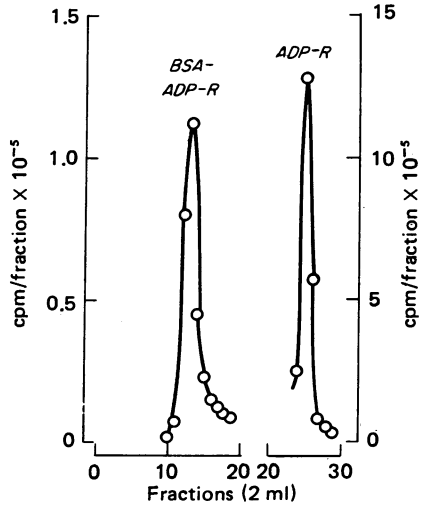

FIG. 2. Elution profile of BSA-ADP-R mixture on a Sephadex G-50 column, calibrated with dextran blue and AMP. The adduct, prepared by incubating BSA (72.5 nmol), ADP-R (200 nmol), and [^{14}C]ADP-R (1 μCi) in a volume of 100 μl at pH 9.5 (0.5 M CO_3-HCO_3) for 30 min at 37°, was eluted from the Sephadex column with Tris buffer (50 mM, pH 8.5). Left ordinate, cpm of ADP-ribose-BSA compound; right ordinate, cpm of unreacted [^{14}C]ADP-ribose.

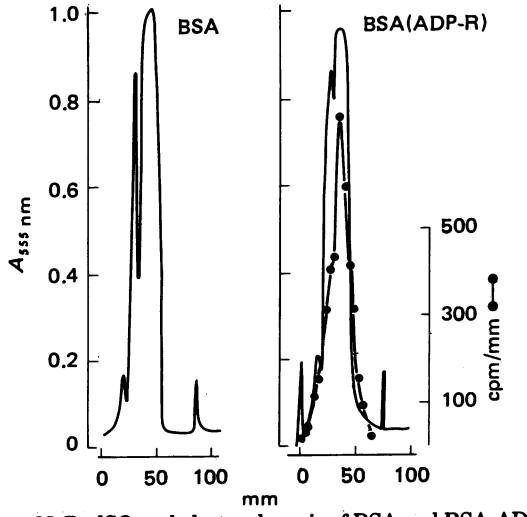

FIG. 3. NaDodSO$_4$-gel electrophoresis of BSA and BSA-ADP-R adduct. The control and the adduct contained 50 μg of protein. Preparation of adduct is described in legend of Fig. 2. The gel electrophoresis was carried out in 10% acrylamide gel at a current density of 8 mA per gel for 2 hr. The gels stained with Coomassie brilliant blue were scanned at 555 nm (solid lines) in a Gilford spectrophotometer. The duplicate (unstained) gels were cut in 1 mm sections and dissolved by incubating in H_2O_2 (30%, 0.5 ml) at 50° for 16 hr. Unreacted hydrogen peroxide was destroyed with ascorbic acid (0.2 ml, 20% wt/vol). Radioactivity was measured (–●–●–●–) after adding soluene (0.3 ml) and aquasol (5 ml).

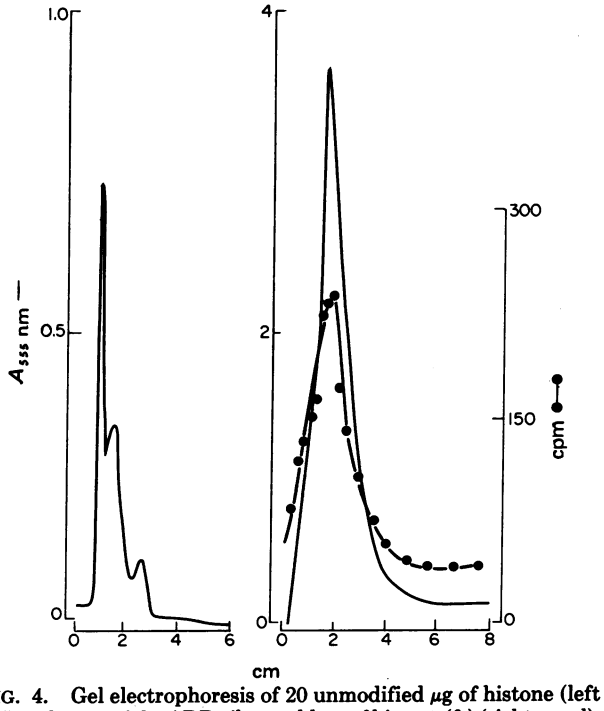

FIG. 4. Gel electrophoresis of 20 unmodified μg of histone (left panel) and 90 μg of the ADP-ribose adduct of histone (f_1) (right panel). Gel electrophoresis was done in acid–urea gel (14), 2 mA per gel for 7 hr at 4°. Gels were stained with amido black, destained, and scanned (see legend of Fig. 3). The amount of modified histone was increased to 90 μg to increase the sensitivity of detection in changes of electrophoretic behavior and to correlate scan with radioactivity (right ordinate). Methods for gel slicing and detection of radioactivity are described in the legend of Fig. 3.

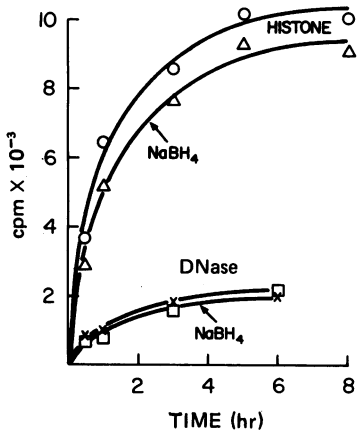

FIG. 5. Time course of the association of ribose-5-phosphate with histone (f_1) and desoxyribonucleotidase I. Histone (17 nmol) and DNase I (22 nmol) were incubated at 37° with 2 mM ribose-5-phosphate (66,000 cpm) in carbonate-bicarbonate buffer (100 mM, pH 9.5) and acid precipitable radioactivity was determined before and after reduction with $NaBH_4$ (cf. 16). The molar reactivity of ribose-5-phosphate with lysine residues of DNase I was 70%. ○ = histone without $NABH_4$. △ = histone after treatment with $NABH_4$. × = DNase I without $NaBH_4$. □ = DNase I after reduction with $NaBH_4$.

active. No correlation to arginine residues could be found. The stability of the Schiff bases of macromolecules was much greater than that of polyamines because neighboring electron donor groups on proteins predictably stabilize this bond (cf. 20).

The most direct proof that the binding of ribose-5-phosphate to poly(L-lysine) and histone was due to the formation of a Schiff base was obtained by the specific introduction of ^3H into adducts by NaB^3H_4 according to Recsei and Snell (16). Acid precipitable ^3H was determined with the poly(L-lysine)/ribose-5-phosphate compound, whereas unreacted ^3H was removed from the tritiated histone/ribose-5-phosphate compound by dialysis. Unlabeled adducts of macromolecules and ribose-5-phosphate were prepared by incubation at pH 9.5 for 24 hr at room temperature. Subsequent treatment with tritiated $NaBH_4$ was carried out at 0° for 1 hr (cf. 16) with both native macromolecules (control) and phosphoribosylated macromolecules. The difference in ^3H between native macromolecules and macromolecules incubated with ribose-5-phosphate was the measure of Schiff base formation. As seen from Table 2, the large incorporation of ^3H clearly indicated the formation of Schiff bases. Specific tritiation of the Schiff bases of BSA was complicated by the large incorporation of ^3H into this protein which presumably indicates the presence of exchangeable H atoms (see also control in Table 2).

DISCUSSION

The chemical interaction of aldehydic carbohydrates, e.g., of glucose with peptides to form an *N*-glucoside which passes through the Amadori rearrangement to the more stable 1-amino-1-deoxy-D-fructose compound is well known (21). It was also shown that glucosyl-valyl-histidine is the most probable structure of the glucose-peptide isolated from hemoglobin A_{Ic} (22). The terminal peptide of hemoglobin after reduction with BH_4^- indicated Schiff base formation between glucose and the α-amino group of the terminal valine (23) and suggested an interconversion of the glycosylamine with the Schiff base (cf. 22). The interaction of sugars with lysine residues of proteins has been extensively studied from a nutritional point of view (24, 25), but the reaction of ADP-ribose and ribose-5-phosphate with polyamines and ε-NH_2 groups of lysine residues of macromolecules, especially of histone f_1, has not been investigated. A nonenzymatic formation of an adduct between ADP-ribose and histamine was reported (26), but the chemical structure of this substance was not clarified. It was believed previously that the ADP-ribose/histamine adduct was the result of NAD^+ glycohydrolase catalyzed exchange between nicotinamide and ADP-ribose of NAD^+ (cf. 26). Another type of reaction of the aldehydic glycolytic metabolite, pyruvaldehyde, with succinic dehydrogenase which involved protein bound thiol groups has also been described (27).

Table 2. Tritium uptake into histone (f_1) and poly(L-lysine) adducts of ribose-5-phosphate as calculated per 10 nmol of macromolecules

Macromolecules	^3H uptake (cpm)	Difference due to the adduct (cpm)
Histone (control)	38,000	—
Histone-ribose-5-phosphate adduct	64,000	26,000
Poly(L-lysine) (control)	52,600	—
Poly(L-lysine)-ribose-5-phosphate adduct	214,000	162,600

Histone (68 nmol) and poly(L-lysine) (2.4 nmol) were incubated with 2 mM ribose-5-phosphate at 37° in 100 mM carbonate-bicarbonate buffer (pH 9.5) for 24 hr. The reaction mixture was treated with $NaBH_4$ (1 mg containing 5.5×10^8 cpm of ^3H) at 0° (cf. 16), and macromolecular radioactivity was determined in the histone adduct after removal of unreacted ^3H by dialysis (until radioactivity of dialysate reached background levels). In the poly(L-lysine) adduct, macromolecular radioactivity was determined by acid precipitation in 6 M urea.

As shown, the Schiff bases of ADP-ribose and ribose-5-phosphate formed with macromolecules are stable at pH 7.5; therefore, this covalent modification is most likely irreversible under physiological conditions. It is predictable that the quantity of irreversibly modified macromolecules depends only on the rates of formation of aldehydic metabolites from NAD^+. Therefore, availability of NAD^+ and the activities of NAD^+ glycohydrolase to form ADP-ribose and of phosphodiesterase to degrade ADP-ribose to ribose-5-phosphate are rate-limiting enzymatic reactions of covalent protein modification. The hitherto unexplained role of chromatin-bound poly(ADP-ribose) may well be related to *in situ* liberation of ADP-ribose by nuclear poly(ADP-ribose) glycohydrolase. Histones, structurally associated with chromatin (cf. 28) or nonhistone proteins can be directly modified by Schiff base formation between enzymatic products of poly(ADP-ribose) and ε-amino groups of lysine residues of macromolecules. This storage function of poly(ADP-ribose) for inhibitory aldehydic metabolites is meaningful in terms of localized release of relatively high concentrations of aldehydes in the chromatin at sites where macromolecular interaction can critically regulate DNA transcription. The resulting alteration of chromatin is predictably complex because different responses would be expected if, e.g., histone or nonhistone proteins were modified first. A further complication is introduced by the presence of polyamines which—as shown here—can trap aldehydes and protect macromolecular acceptors. The as yet unexplained biological role of polyamines (29) may be rationalized by this mechanism. Present results in conjunction with the known high cellular turnover of NAD^+ (9) suggest that NAD^+ metabolism could serve as a signal mechanism between metabolic and epigenetic systems.

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