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Modification of Proteins by Mono(ADP-ribosylation) in Vivo†

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ABSTRACT: We have pursued the detection of in vivo modified, ADP-ribosylated proteins containing N-glycosylic linkages to arginine. ADP-ribosylated histone, elongation factor 2, and transducin, containing the different known ADP-ribosylated amino acids (arginine, diphthamide, and cysteine, respectively), were employed as model conjugates to establish conditions for the selective detection of adenosine(5′)diphosphoribose (ADP-ribose) residues bound to arginine. We report here the detection and quantification of protein-bound ADP-ribose residues in adult rat liver with linkages characteristic of arginine. These mono(ADP-ribose) residues were present in vivo at a level of 31.8 pmol/mg of protein which is 400-fold higher than polymeric ADP-ribose residues. A minor fraction (23%) of the ADP-ribose residues detected were bound via a second, more labile linkage with chemical properties very similar to those described for carboxylate ester linked ADP-ribose.

Both prokaryotic and eukaryotic cells, and some bacterial viruses as well, possess enzyme activities which catalyze the release of nicotinamide from NAD^+ and transfer of the remaining adenosine(5′)diphosphoribose (ADP-ribose)[1] moiety to specific proteins. Certain bacterial toxins represent a group of such mono(ADP-ribosyl)transferases whose physiological role is fairly well understood [reviewed by Vaughan & Moss (1981)]. For example, diphtheria toxin (Collier, 1967; Honjo et al., 1968; Gill et al., 1969) and *Pseudomonas* exotoxin A (Iglewski & Kabat, 1975) inhibit protein synthesis in vertebrate cells by catalyzing the ADP-ribosylation of elongation factor 2 (EF-2). Cholera toxin (Gill, 1975; Moss et al., 1976; Cassel & Pfeuffer, 1978), *Escherichia coli* heat-labile enterotoxin (Moss & Richardson, 1978; Gill & Richardson, 1980), and islet-activating protein (one of the pertussis toxins) (Katada & Ui, 1982a; Bokoch et al., 1983) irreversibly activate adenylate cyclase in animal cells by catalyzing the ADP-ribosylation of GTP-binding regulatory proteins of the cyclase system. Diphtheria (Van Ness et al., 1980), cholera (Moss & Vaughan, 1977), and pertussis (West et al., 1985) toxins modify proteins in vivo by catalyzing the ADP-ribosylation of either diphthamide (a hypermodified histidine), arginine, or cysteine residues, respectively.

In addition to bacterial toxins, animal cells themselves possess mono(ADP-ribosyl)transferase activities. The presence of such enzymes has been demonstrated in avian (Moss & Vaughan, 1978) and human (Moss & Stanley, 1981a) erythrocytes, rat (Moss & Stanley, 1981b; Richter et al., 1983) and bovine (Iglewski et al., 1984) liver, rat testis (Momii & Koide, 1982), rat and bovine thyroid cells (DeWolf et al., 1981; Kohn, 1978), rabbit skeletal muscle (Soman et al., 1983), and polyoma virus transformed baby hamster kidney cells (Lee &

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[1] Abbreviations: ADP-ribose, adenosine(5′)diphosphoribose; MOPS, 4-morpholinepropanesulfonic acid; etheno(ADP-ribose), 1,N^6-etheno-adenosine(5′)diphosphoribose; HPLC, high-performance liquid chromatography; EF-2, eukaryotic elongation factor 2; buffer A, 50 mM MOPS, 6 M guanidinium chloride, and 10 mM EDTA, pH 4.0 (5 °C); buffer B, 100 mM ammonium acetate, 6 M guanidinium chloride, and 10 mM EDTA, pH 9.4 (5 °C); EDTA, ethylenediaminetetraacetic acid; DHB, dihydroxyboronyl; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; kDa, kilodaltons.

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Iglewski, 1984). Two different mono(ADP-ribosyl)transferases from turkey erythrocytes (Moss et al., 1980; Yost & Moss, 1983) and a transferase from hen liver nuclei (Tanigawa et al., 1984) have been purified and extensively characterized. Like cholera toxin, the purified vertebrate transferases catalyze, in vitro, the formation of N-glycosylic linkages between ADP-ribose and the guanidino group of arginine (Oppenheimer, 1978; Moss et al., 1979). Although the physiological effects of the bacterial toxins on animal cells are explainable (at least in part) in terms of the activation or inhibition of specific enzymes via ADP-ribosylation, the role(s) of endogenous mono(ADP-ribosyl)transferases has (have) not been established. Several lines of evidence are consistent with the hypothesis that one physiological role of cellular transferases might be the activation of adenylate cyclase, in response to hormone–receptor interactions, by a mechanism analogous to that utilized by the bacterial toxins (Moss & Vaughan, 1978; DeWolf et al., 1981; Kohn, 1978; Vitti et al., 1982; Beckner & Blecher, 1981; Reilly et al., 1981). Other evidence has been presented which does not support this hypothesis (Vaughan & Moss, 1981; Kurosky et al., 1977; Moss et al., 1978; Rebois et al., 1983). However, interpretations of both the positive and negative results must be made cautiously, since they are based on data obtained almost exclusively from in vitro experiments.

Knowledge of both the chemical nature of ADP-ribose–protein linkage(s) present in vivo and also the identity of the target protein(s) themselves is essential for defining the role(s) of cellular ADP-ribosyltransferases. The different mono(ADP-ribosyl)transferases identified thus far catalyze the covalent modification of three different amino acids. In addition, after in vitro incubation of nuclei with radiolabeled NAD^+, histones were mono(ADP-ribosylated) [presumably by poly(ADP-ribose)polymerase] via ester linkages of the γ-carboxyl group of glutamic acid residues and to the α-carboxyl group of terminal lysine residues (Riquelme et al., 1979; Burzio et al., 1979; Ogata et al., 1980a,b). Thus, it is likely that the (ADP-ribose)–protein conjugates formed in vivo also involve different amino acids. Nevertheless, since the purified vertebrate transferases studied thus far are specific for arginine (or guanidino-containing analogues) as the ADP-ribose acceptor in vitro, we have focused our efforts on the detection of proteins modified in vivo by N-glycosylic linkages to arginine.

Experimental Procedures

Materials. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): nicotinamide, NMN^+, ADP-ribose, guanidine hydrochloride, histone (type II-A, calf thymus), hydroxylamine hydrochloride, 4-morpholinepropanesulfonic acid (MOPS), Sephadex G-25 (superfine), bovine serum albumin, adenine, adenosine, AMP, ADP, ATP, NAD^+, and the etheno derivatives of the last six compounds. Chloroacetaldehyde (45% in water) was purchased from ICN Pharmaceuticals, Inc. (Plainview, NY). [8-^{14}C]Adenosine triphosphate (58 mCi/mmol) and adenosine [α-^{32}P]triphosphate (2000 Ci/mmol) were purchased from ICN Chemical and Radioisotope Division (Irvine, CA). Calf thymus DNA was from Worthington Biochemical Corp. (Freehold, NJ), and Aquasol was obtained from New England Nuclear (Boston, MA). Diphtheria toxin and eukaryotic elongation factor 2 were kindly provided by Dr. D. Michael Gill (Tufts University). [^{32}P]ADP-ribosylated transducin was a gift from Dr. Richard Kahn and Dr. Alfred Gilman (UTHSC, Dallas). All other chemicals used were reagent grade or the highest purity commercially available.

Preparation of Dihydroxyboronyl Affinity Resins. Dihydroxyboronyl-Bio-Rex 70 (DHB-Bio-Rex) was prepared as described by Wielckens et al. (1981, 1984). The preparation of dihydroxyboronyl-Sepharose 4B (DHB-Sepharose) has also been described previously (Jacobson et al., 1984). The NAD^+ binding capacity (defined as the maximum amount of NAD^+ which could be applied while maintaining a binding efficiency $\geq$90%) was routinely determined for each preparation of affinity resin (Alvarez-Gonzalez et al., 1983). The values obtained for the different preparations used in this study were 6.05 $\pm$ 0.58 μmol of NAD^+/0.5 mL of DHB-Bio-Rex ($\bar{x} \pm$ SD, n = 3) and 0.77 $\pm$ 0.18 μmol of NAD^+/0.5 mL of DHB-Sepharose ($\bar{x} \pm$ SD, n = 3). The two affinity resins were equilibrated immediately before use as described previously (Jacobson et al., 1984).

Preparation of Radiolabeled NAD^+ and ADP-ribose. [*adenine*-8-^{14}C]Nicotinamide adenine dinucleotide ([^{14}C]-NAD^+) was synthesized from [8-^{14}C]ATP by using NAD^+ pyrophosphorylase (ATP:nicotinamide-nucleotide adenylyltransferase, EC 2.7.7.1) and subsequently purified by affinity chromatography on DHB-Sepharose as described previously (Alvarez-Gonzalez et al., 1983). The procedure used for synthesis was also similar to others described by Ueda & Yamamura (1971) and Poirier et al. (1978). The purity was >95% as judged by HPLC analysis. [*adenylate*-^{32}P]-Nicotinamide adenine dinucleotide ([^{32}P]NAD^+) was synthesized from [α-^{32}P]ATP according to a protocol developed by Dr. Robert C. Benjamin (unpublished results). [α-^{32}P]ATP (1 mCi) was transferred to a siliconized Corex tube (15 mL) and lyophilized. The incubation volume was 50 μL containing 50 mM Tris base, 30 mM phosphoenolpyruvate, 2 mM $MgCl_2$, and 1.6 mM NMN^+, and was adjusted to pH 7.5 with NaOH. The reaction mixture also contained 30 units/mL pyruvate kinase, 0.3 unit/mL NAD^+ pyrophosphorylase (6 mg of lyophilized powder/mL, equivalent to 2 mg of enzyme/mL), and, when added to the Corex tube (above), 5–7 μM [^{32}P]ATP. The mixture was incubated at 37 °C for 30 min, then diluted to 10 mL with 1 M NH_4HCO_3, pH 9.0 (application buffer), and applied to a DHB-Bio-Rex column (0.5-mL packed volume, previously equilibrated by consecutive 10-mL washes with application buffer, deionized water, and again with application buffer). The column was washed with 5 mL of application buffer, followed by 10 mL of 0.25 M NH_4HCO_3, pH 9.0, and 0.5 mL of deionized water. [^{32}P]NAD^+ was then eluted with 4 mL of deionized water. The eluate was collected in a siliconized Corex tube (15 mL) and lyophilized. The sample was dissolved in 3 mL of 10 mM sodium phosphate, pH 6.0, and stored at −20 °C. The purity was >95% as judged by HPLC analysis.

[8-^{14}C]ADP-ribose and [α-^{32}P]ADP-ribose were prepared from the corresponding radiolabeled NAD^+ by alkaline incubation and purified by affinity chromatography. An equal volume of 0.2 M NaOH was added to an aliquot containing either [^{14}C]- or [^{32}P]NAD^+, and the mixture (typically 50–500 μL) was incubated at 37 °C for 30 min. The incubation mixture was adjusted to pH 9.0 by addition of an equal volume of 0.2 M NH_4Cl, pH 4.5, and diluted to 5 mL with 0.25 M NH_4Cl, pH 9.0 (25 °C) (application buffer). The sample was applied to a 0.5-mL DHB-Sepharose column previously equilibrated by consecutive 5-mL washes with application buffer, elution buffer, and again with application buffer. The column was washed with 10 mL of application buffer and 0.5 mL of 10 mM H_3PO_4 and 25 mM KCl, pH 2.3 (elution buffer). Radiolabeled ADP-ribose was then eluted with 2 mL of elution buffer (the pH of the eluate was 4.5–5.0) and stored

at −20 °C. The overall recovery was 55–60%. The purity was >90% as judged by HPLC analysis.

Preparation of Radiolabeled ADP-ribosylated Proteins. [*adenine*-^{14}C]ADP-ribosylated histone (^{14}C-histone) was prepared by using purified NAD–arginine ADP-ribosyltransferase from turkey erythrocytes as described previously (Moss et al., 1983). The reaction was terminated by addition of cold trichloroacetic acid to a final concentration of 20% w/v. Following centrifugation, the pellet was washed twice with anhydrous diethyl ether, dried under gentle vacuum, dissolved in a cold solution (5 °C) containing 50 mM MOPS, pH 4.0, 6 M guanidinium chloride, and 10 mM EDTA (buffer A), and stored at −20 °C. The procedure used for synthesis of [^{32}P]ADP-ribosylated elongation factor 2 (^{32}P-EF-2) was similar to that described by Gill (1972). The incubation volume was 100 μL containing 20 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 43 nM [^{32}P]NAD^+ (5.5 μCi), 5 μL of EF-2 (provided by D. M. Gill and estimated to contain 1 pmol/μL), and 2 μL of diphtheria toxin (also from D. M. Gill). The mixture was incubated at 37 °C for 30 min and placed on ice, and 20 μL of bovine serum albumin (10 mg/mL) was added. Next, 25 μL of ice-cold trichloroacetic acid (100% w/v) was added, and the sample was incubated on ice for 10 min. The sample was diluted to 500 μL with ice-cold trichloroacetic acid (20% w/v) and centrifuged (12000*g* for 5 min). The supernatant was carefully removed with a microaspirator. The pellet was washed twice with 500 μL of trichloroacetic acid (20%) and 3 times with 500 μL of anhydrous diethyl ether and dried under vacuum. The dried pellet was dissolved in buffer A and stored at −20 °C. The recovery of radiolabel as ^{32}P-EF-2 was 61% (3.35 μCi; 2.65 pmol of EF-2).

Preparation of the Acid-Insoluble Fraction from Rat Liver. The procedure for tissue preparation has been described previously (Jacobson et al., 1984). Briefly, the tissue was frozen in liquid nitrogen, powdered with a pestle in a large mortar containing liquid nitrogen, and then homogenized in 20% trichloroacetic acid by using a Brinkmann Polytron homogenizer. After centrifugation, the pellet was twice pulverized and homogenized as described previously, first in 20% trichloroacetic acid and then in 100% ethanol. The acid-insoluble material was then washed once more (by vortexing and centrifugation) with 100% ethanol and twice with anhydrous diethyl ether, and dried by gentle vacuum. The yield of acid-insoluble material (a fine, dry powder) for several different preparations of rat liver was 158 ± 9 mg/g (wet weight) of tissue ($\bar{x}$ ± SD, n = 4). The acid-insoluble fraction obtained from rat liver contained 4.01 ± 0.14 μg of DNA/mg of dry powder ($\bar{x}$ ± SD, n = 4) and 693 ± 79 μg of protein/mg of dry powder ($\bar{x}$ ± SD, n = 12).

Analysis by Anion-Exchange HPLC. The chromatographic apparatus used in this study for separation and analysis of nucleotides included a Beckman Model 330 liquid chromatograph, a Whatman Partisil-10 SAX column (250 mm × 4.6 mm i.d.) preceded by a guard column (50 mm × 1.5 mm i.d.) containing the same material, a Varian Fluorichrom fluorometer, and an Instrumentation Specialties Co. Model UA-5 ultraviolet absorbance monitor (attached in series after the fluorometer). The injector was equipped with a 1.0-mL sample loop. The fluorometer was equipped with a deuterium lamp, a 220I excitation filter, and a 370-nm cutoff emission filter (Corning no. 3-75). The absorbance monitor was equipped with a type 6 optical unit, 19-μL flow cells, and a 254-nm band-pass filter. Separations were accomplished isocratically at a flow rate of 1.0 mL/min by using 100 mM potassium phosphate buffer, pH 4.7.

Table I: Separation of Protein-Bound ADP-ribose from Free Nucleotides by G-25 Column Centrifugation[a]

treatment	radiolabeled ADP-ribose (dpm)	
	protein-bound [^{14}C]ADP-ribose	free [^{32}P]ADP-ribose
column centrifugation		
protein fraction	20010 (97%)	990 (2%)
nucleotide fraction	493 (2%)	44964 (96%)

[a] After the acid-insoluble fraction from rat liver was dissolved, either [^{14}C]ADP-ribosylated histone (20 530 dpm) or free [^{32}P]ADP-ribose (53 130 dpm) was added, and identical aliquots (500 μL) were subjected to fractionation by G-25 (superfine) column centrifugation as described under Experimental Procedures.

Determination of in Vivo Levels of Mono(ADP-ribose) Residues. The assay for mono(ADP-ribose) residues in acid-insoluble fractions derived from cells or tissues has been described in detail elsewhere (Jacobson et al., 1984). Briefly, the acid-insoluble material obtained from rat liver (as described previously in this paper) was dissolved in 40 volumes of cold buffer A which contains 6 M guanidinium chloride (approximately 17 mg of protein/mL), and the pH of the resulting solution (usually about 3.5) was adjusted to 4.0 with NH_4OH. The dissolved sample was centrifuged briefly (1500*g*, 5 min) to remove a small amount of insoluble material. To ensure that the sample was free of noncovalently bound nucleotides, a modification of the column centrifugation method (Helmerhorst & Stokes, 1980) for removing low molecular weight compounds from solutions of macromolecules were employed. Aliquots of the dissolved sample were applied to columns of Sephadex G-25 (superfine) and centrifuged, and the effluents (denoted "protein fraction") were collected for analysis as described previously (Jacobson et al., 1984). In some experiments, low molecular weight compounds were subsequently eluted for analysis by application of 2.5 mL of buffer A to each column, followed by centrifugation and collection as described earlier (denoted "nucleotide fraction"). The efficiency of the column centrifugation method for separating protein-bound ADP-ribose from free ADP-ribose is shown in Table I. Chemical release of protein-bound ADP-ribose residues was accomplished by incubating samples at neutral pH in the presence or absence of hydroxylamine. After incubation, free ADP-ribose was isolated by affinity chromatography using DHB-Bio-Rex.[2] ADP-ribose was then reacted with chloroacetaldehyde to form the highly fluorescent analogue 1,N^6-etheno(ADP-ribose), which was then prepared for HPLC analysis by affinity chromatography using DHB-Sepharose. Etheno(ADP-ribose) was separated from residual interfering substances by anion-exchange HPLC and quantified by monitoring the fluorescence. Some additional details regarding quantitative detection of etheno(ADP-ribose) are provided below.

Quantitative Formation and Detection of Etheno(ADP-ribose). Authentic etheno(ADP-ribose) was prepared from etheno-NAD^+ by alkaline incubation and affinity chromatography as described for preparation of radiolabeled ADP-

[2] Conditions for the quantitative binding and elution of ADP-ribose have been described previously for the boronate matrices used in this study (Alvarez-Gonzalez et al., 1983). However, when tissue-derived samples, with high concentrations of protein and nucleic acid, were applied to columns of either DHB-Bio-Rex or DHB-Sepharose, the flow rates became excessively slow. Therefore, a batch adsorption procedure was adopted as an alternative to column chromatography, and DHB-Bio-Rex was chosen on the basis of its higher binding capacity and greater mechanical stability.

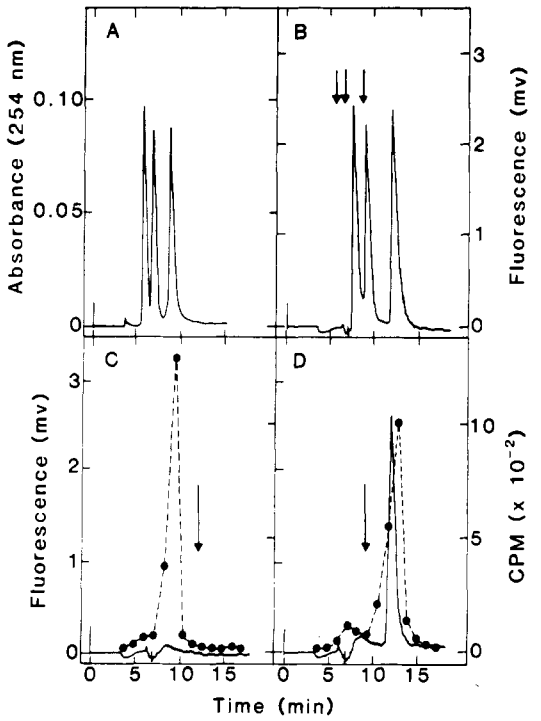

FIGURE 1: Separation of NAD^+, AMP, and ADP-ribose and their etheno derivatives by HPLC and quantitative conversion of ADP-ribose to etheno(ADP-ribose). (A) Five to ten nanomoles each of NAD^+, AMP, and ADP-ribose (in the order or elution) was separated isocratically as described under Experimental Procedures. (B) Etheno-NAD^+ (approximately 100 pmol) and 5–10 pmol each of etheno-AMP and etheno(ADP-ribose) (in the order of elution) were also separated as described. The arrows in panel B are for reference, indicating the elution positions of the non-etheno compounds. Also shown are chromatograms of [^{14}C]ADP-ribose (17 pmol injected) after incubation in the absence (C) or presence (D) of chloroacetaldehyde as described under Experimental Procedures. The arrows in panels C and D are for reference, indicating the elution postions of etheno(ADP-ribose) and ADP-ribose, respectively. The inflection at 6.5 min in panels B, C, and D reflects a 20-fold increase in sensitivity of the fluorometer. Numbers on the ordinate represent fluorescence after the change in sensitivity.

ribose. The separation of key adenine- and ethenoadenine-containing nucleotides by anion-exchange HPLC is illustrated in Figure 1A,B. With the isocratic system employed, etheno(ADP-ribose) was separated from other nucleotides in less than 15 min. Retention times for additional related compounds, including adenosine, NADH, phosphoribosyladenosine monophosphate (*iso*-ADP-ribose), and ADP, were 5, 11, 20, and 38 min, respectively (ATP was not eluted from the column in 133 min). Optimal conditions for the formation of a fluorescent derivative from ADP-ribose were identical with those for formation of ethenoadenosine and similar to conditions reported previously for the formation of etheno derivatives of other adenine-containing compounds (Sims et al., 1980). Maximum fluorescence was observed after incubation of either adenosine or ADP-ribose with 0.1 M chloroacetaldehyde for 4 h at 60 °C and pH 4.5 (not shown). Anion-exchange HPLC analysis established that etheno(ADP-ribose) was the fluorescent product of the reaction between ADP-ribose and chloroacetaldehyde. The results depicted in Figure 1C,D confirmed that ADP-ribose was stable during derivatization and that it was quantitatively converted to etheno-(ADP-ribose). Approximately 6 nmol of purified [^{14}C]-ADP-ribose, which had been standardized both by spectral methods and by specific radioactivity, was derivatized as described earlier. The etheno[^{14}C]ADP-ribose was then used to construct a standard curve for quantification of fluorescence

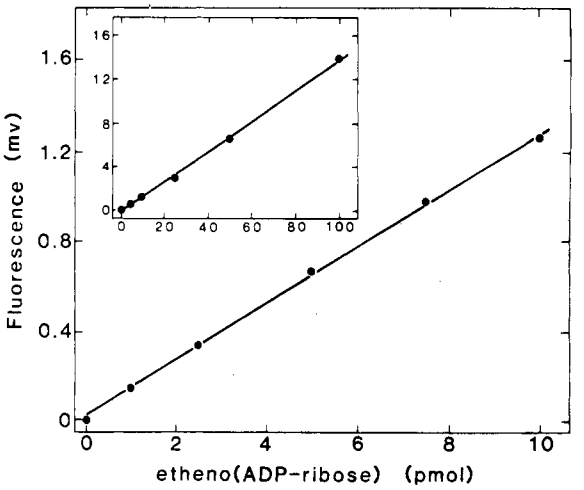

FIGURE 2: Standard curve for the quantification of etheno(ADP-ribose) following anion-exchange chromatography. Etheno[^{14}C]-ADP-ribose (58 mCi/mmol; 2.6 μM) was diluted to appropriate concentrations, and 1-mL aliquots were analyzed by anion-exchange HPLC with fluorescence detection as described under Experimental Procedures.

following anion-exchange chromatography. The relationship between fluorescence and the quantity of etheno[^{14}C]ADP-ribose injected was linear over the entire range examined (1–100 pmol) (Figure 2).

Miscellaneous. For DNA analysis, samples of the acid-insoluble fraction (typically 50 mg) were suspended in 100 volumes of 0.1 M KOH and dissolved by agitation in a Buchler Evapo-mix shaker at 45 °C for several hours. Samples were then placed on ice and adjusted to 0.5 M $HClO_4$. After 15 min, samples were centrifuged (1500*g*, 15 min), and the pellets were washed with 100 volumes of cold (5 °C) 0.5 M $HClO_4$. After centrifugation, the pellets were dissolved in 40 volumes of 0.5 M $HClO_4$ by incubation at 70 °C for 30 min. The samples were then incubated on ice for 15 min and centrifuged as described previously. The supernatants were carefully removed and adjusted to 1.5 M $HClO_4$ for analysis. DNA content was estimated by the method of Burton (1956) as modified by Richards (1974), using calf thymus DNA (prepared as described previously for samples) as the standard. For protein analysis, samples of the acid-insoluble fraction were dissolved in buffer A as described earlier. Protein content was estimated by the method of Bradford (1976), using bovine serum albumin (dissolved in buffer A) as the standard.

RESULTS

Selective Release of Arginine-Linked ADP-ribose Residues from Proteins. Mono(ADP-ribosylated) histone was synthesized with purified NAD–arginine ADP-ribosyltransferase and used as a model conjugate to establish reaction conditions necessary for the selective release from proteins of intact ADP-ribose residues bound to arginine. The acid-insoluble fraction obtained from rat liver was dissolved in buffer A which contains 6 M guanidinium chloride, and [^{14}C]ADP-ribosylated histone was added. Aliquots of this sample were subjected to various treatments; then free [^{14}C]ADP-ribose and protein-bound [^{14}C]ADP-ribose were separated by column centrifugation (see Table I) in order to evaluate the stability of the ADP-ribose–arginine linkage. The linkage was completely stable when the ADP-ribosylated histone was dissolved in 6 M guanidine at pH 4.0 (i.e., buffer A) and stored at −20 °C for as long as 1 year (data not shown) or when incubated in the same solution at neutral pH and 37 °C for 12 h (Figure 3A). The ADP-ribose–histone linkage was cleaved in the

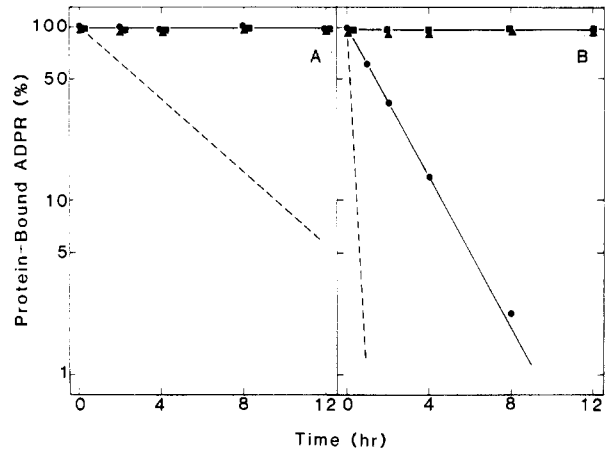

FIGURE 3: Stability of different ADP-ribose–protein linkages. Samples of [^{14}C]ADP-ribosylated histone (●), [^{32}P]ADP-ribosylated EF-2 (■), or [^{32}P]ADP-ribosylated transducin (▲) were added to liver extract (4000–10 000 dpm/0.5 mL) and incubated at pH 7.0, 37 °C, in the absence (panel A) or presence (panel B) of 1 M NH_2OH. The amount of ADP-ribose remaining bound to protein was determined after removal of free ADP-ribose by column centrifugation as described under Experimental Procedures. The dashed line in each panel represents the stability of the ADP-ribose–glutamate linkage as reported previously (see text).

presence of hydroxylamine at neutral pH, with a half-life of 1.5 h in the presence of 1 M neutral hydroxylamine (Figure 3B). Approximately 95% of the ADP-ribose residues were released from histone by incubation in the presence of 1 M neutral hydroxylamine for 6 h. When the more dilute hydroxylamine concentration previously employed by Bredehorst et al. (1978) (0.4 M, 37 °C, pH 7.5) was used, the half-life of the ADP-ribose–histone linkage was recently reported to be about 2 h (Moss et al., 1983). Under those conditions, we also obtained a value of 2 h (not shown), even though our reaction mixture contained additional, potentially interfering components (e.g., 6 M guanidinium chloride and the acid-insolbule material derived from rat liver). The stability of [^{14}C]ADP-ribose in the presence of neutral hydroxylamine was examined by anion-exchange HPLC analysis. After incubation under conditions employed for quantitative release of covalently bound ADP-ribose from protein, greater than 98% of the free [^{14}C]ADP-ribose remained intact (data not shown). Confirmation that ADP-ribose was released intact from protein was obtained in subsequent experiments described later (see Figure 5).

Mono(ADP-ribosylated) EF-2 and transducin were also used as model conjugates to evaluate the stabilities of the other two reported linkages, ADP-ribose–diphthamide and ADP-ribose–cysteine, respectively. In contrast to the susceptibility of the ADP-ribose–arginine linkage to cleavage by hydroxylamine, the ADP-ribose–diphthamide and ADP-ribose–cysteine linkages were stable for 12 h in the presence of 1 M neutral hydroxylamine (Figure 3). In fact, no release was detected following incubation at a higher concentration (3 M) or for a longer time (21 h). These results are in agreement with and extend the findings of previous reports in which these two linkages were found to be stable in the presence of neutral hydroxylamine for at least 2 h (Nishizuka et al., 1969; Hsia et al., 1984). The carboxylate ester linkage between ADP-ribose and histone has been well characterized with respect to stability at neutral pH in the absence and presence of hydroxylamine, with reported $t_{1/2}$ values of 3–4 h and 5–10 min, respectively (Nishizuka et al., 1969; Riquelme et al., 1979; Burzio et al., 1979; Ogata et al., 1980a,b) (Figure 3). The stabilities of the arginine–, cysteine–, and diphthamide–

Table II: Stability of Different ADP-ribose–Protein Linkages in the Presence of Neutral Hydroxylamine or Strong Alkali[a]

	ADP-ribose released (%) in		
	NH_2OH (37 °C)		
ADP-ribose acceptor	0.4 M, pH 7.5, 1 h	1 M, pH 7.0, 12 h	NaOH (56 °C) 1 M, 1 h
arginine (histone)	27	98	88
cysteine (transducin)	ND[b]	<1	60
diphthamide (EF-2)	<1	<1	<1

[a] Samples of liver extract containing 4000–10 000 dpm/0.5 mL either [^{14}C]ADP-ribosylated histone, [^{32}P]ADP-ribosylated transducin, or [^{32}P]ADP-ribosylated EF-2 were incubated in the presence of neutral hydroxylamine or strong alkali as described by Bredehorst et al. (1978). Aliquots (500 μL) were then fractionated by G-25 (superfine) column centrifugation as described under Experimental Procedures, and the amounts of radiolabeled ADP-ribose in the protein and nucleotide fractions were quantified. For comparison, identical samples were also incubated in the presence of neutral hydroxylamine as described under Experimental Procedures. [b] ND = not determined.

ADP-ribose conjugates were also evaluated by employing the "differential release" conditions utilized previously by Bredehorst et al. (1978) (i.e., brief treatment with dilute hydroxylamine vs. treatment with strong alkali). As shown in Table II, treatment with 1 M NaOH resulted in almost complete release of arginine-linked ADP-ribose. A significant fraction of the ADP-ribose–arginine linkages was also cleaved during the brief treatment with 0.4 M neutral hydroxylamine. In contrast, the cysteine- and diphthamide-linked ADP-ribose residues were completely resistant to hydroxylamine treatment, and release during treatment with alkali was either incomplete (cysteine) or negligible (diphthamide).

Protein-Bound Mono(ADP-ribose) Residues in Adult Rat Liver. The acid-insoluble fraction from adult rat liver was prepared and dissolved in buffer A as described under Experimental Procedures. Trapped nucleotides were separated from proteins by column centrifugation, and aliquots of the protein fraction were then incubated in the presence of 1 M neutral hydroxylamine at 37 °C for 12 h. Quantitative detection of ADP-ribose following release from protein involved (i) isolation of ADP-ribose by affinity chromatography, (ii) formation of a highly fluorescent analogue, etheno(ADP-ribose), and (iii) quantification by monitoring the fluorescence following anion-exhcange HPLC. As shown in Figure 4A, a peak of fluorescent material eluted at the position of etheno-(ADP-ribose). A series of control analyses were also performed which established the identity of the fluorescent material as etheno(ADP-ribose). First, the observed fluorescence was not due to contaminants originating in the reagents or to endogenous fluorescent compounds in the extract (panels B and C, respectively, of Figure 4). Second, treatment of the sample with purified snake venom phosphodiesterase resulted in elimination of the original fluorescent peak and generation of a peak eluting at the position of etheno-AMP (Figure 4D). Finally, when [^{14}C]ADP-ribosylated histone was added to an aliquot of liver extract and analyzed as described, the radioactivity was coincident with the fluorescent peak in the elution position of etheno(ADP-ribose) (Figure 5). It has been reported previously that a hydroxylamine derivative of ADP-ribose was actually the product released upon cleavage of the ADP-ribose–arginine linkage (Skorko et al., 1977; Moss et al., 1983); ADP-ribose and its hydroxylamine derivative were indistinguishable by high-voltage paper electrophoresis (Skorko et al., 1977) and anion-exchange HPLC (D. A. Yost and J. Moss, unpublished results). Thus, it is probable that the residues detected as etheno(ADP-ribose) in the present study

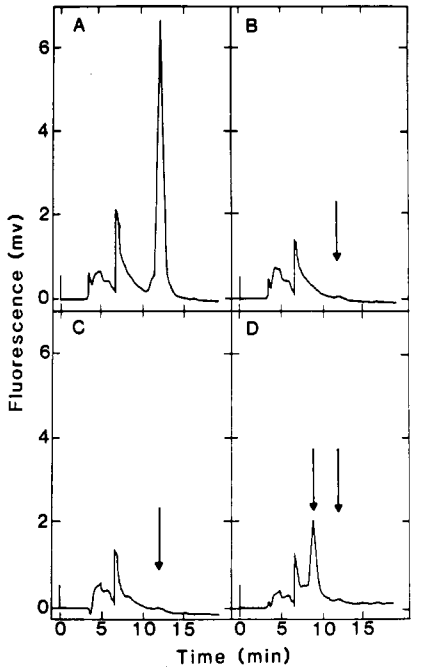

FIGURE 4: Measurement of protein-bound mono(ADP-ribose) residues in adult rat liver. Rat liver was subjected to analysis as described under Experimental Procedures (A). Control analyses included omission of extract (B), omission of chloroacetaldehyde (C), and treatment with purified snake venom phosphodiesterase (1 unit/mL, 37 °C, 3 h) prior to derivatization and affinity chromatography (D). Arrows in panels B–D are for reference, indicating the elution positions of etheno(ADP-ribose) (B and C) and both etheno-AMP and etheno(ADP-ribose) (D). The inflection at 6.5 min in each chromatogram reflects a 10-fold increase in the sensitivity of the fluorometer. Numbers on the ordinate represent fluorescence after the change in sensitivity.

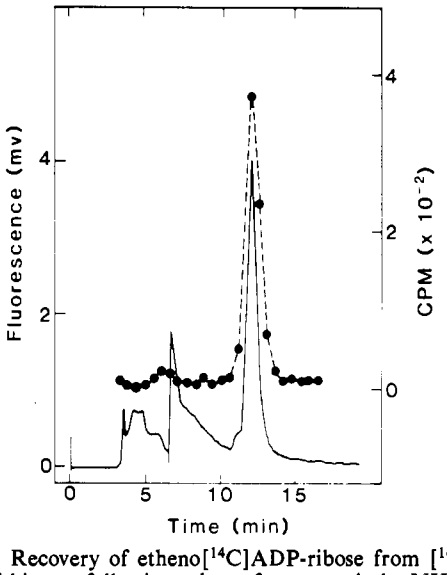

FIGURE 5: Recovery of etheno[^{14}C]ADP-ribose from [^{14}C]ADP-ribosylated histone following release from protein by NH_2OH and derivatization with chloroacetaldehyde. A sample of liver extract containing [^{14}C]ADP-ribosylated histone was subjected to analysis as described under Experimental Procedures, and both fluorescence and radioactivity were quantified. The inflection at 6.5 min in the fluorescence chromatogram reflects a 10-fold increase in the sensitivity of the fluorometer. Numbers on the left-hand ordinate represent fluorescence after the change in sensitivity. The closed circles represent radioactivity.

were, in fact, etheno(ADP-ribosyl)oxime or a mixture of etheno(ADP-ribose) and etheno(ADP-ribosyl)oxime.

The addition of [^{14}C]ADP-ribosylated histone to a sample prior to analysis also provided a control for assessment of recovery through the entire procedure. Overall recovery of etheno[^{14}C]ADP-ribose from [^{14}C]ADP-ribosylated histone added at the beginning of the analysis was routinely 55–60%. The losses were not selective, since they were incurred following quantitative release of ADP-ribose from protein. This conclusion is further supported by the observation that when free [^{14}C]ADP-ribose (or [^{32}P]ADP-ribose) was added to an aliquot of liver extract following G-25 column centrifugation, the overall recovery was the same as that obtained with the [^{14}C]ADP-ribosylated histone. When rat liver was subjected to analysis in the absence of hydroxylamine, a peak of etheno(ADP-ribose) was also observed. As expected, since the ADP-ribose–arginine linkage is stable at neutral pH, only a small amount (≤4%) of etheno[^{14}C]ADP-ribose was recovered from [^{14}C]ADP-ribosylated histone incubated in the absence of hydroxylamine. In the absence of hydroxylamine, the overall recovery of free [^{14}C]ADP-ribose (or [^{32}P]ADP-ribose), added to an aliquot of liver extract following G-25 column centrifugation, was routinely 65–70%.

Table III: ADP-ribose Residues in the Acid-Insoluble Fraction from Adult Rat Liver[a]

treatment	ADP-ribose (pmol/mg of protein) −NH_2OH	+NH_2OH	NH_2OH-requiring residues
none[b]	80.0	181.0	101.0
G-25 column centrifugation			
protein fraction[c]	9.7 ± 1.5	41.5 ± 1.5	31.8 ± 0.8
nucleotide fraction[b]	72.5	146.9	74.4
total	82.2	188.4	106.2

[a] After the acid-insoluble fraction from rat liver was dissolved, samples (containing 2.41 mg of protein) were incubated in the absence or presence of hydroxylamine and analyzed for total ADP-ribose residues as described under Experimental Procedures. Aliquots (500 μL) of the liver extract were also subjected to fractionation by G-25 (superfine) column centrifugation prior to incubation and analysis of equivalent samples (i.e., equivalent to the untreated samples containing 2.41 mg of protein). [b] Average of duplicate determinations (range ≤±8% for all values). [c] Mean ± SD (n = 6).

The values obtained from replicate analyses of rat liver in the absence or presence of hydroxylamine are shown in Table III ("protein fraction"). The amount of ADP-ribose observed following incubation at neutral pH alone represented 23% of that obtained by neutral hydroxylamine treatment. The difference between the amounts of ADP-ribose observed in the presence and absence of hydroxylamine should provide an estimate of the amount of ADP-ribose bound to proteins via linkages that require hydroxylamine for cleavage; i.e., ADP-ribose bound to proteins via an N-glycosylic linkage to arginine (Table III, column 4).

Not surprisingly, when the acid-insoluble fraction from rat liver was dissolved and analyzed directly with no pretreatment to eliminate noncovalently bound nucleotides, higher values for ADP-ribose were obtained in both the absence and presence of hydroxylamine (8.9-fold and 4.5-fold higher values, respectively) (Table III). These additional residues were accounted for after column centrifugation when the nucleotide fraction was analyzed. Additional trichloroacetic acid extractions during preparation of the acid-insoluble fraction failed to significantly decrease the amount of ADP-ribose detected in the nucleotide fraction (data not shown). These data are consistent with our original concern that trapping of even a small fraction of the total cellular NAD^+ or NADH in the trichloroacetic acid pellet could lead to a large overestimation of levels of protein-bound mono(ADP-ribose), especially since NADH is degraded to ADP-ribose under acidic conditions (Bredehorst et al., 1979).

Table IV: ADP-ribose Residues from NAD^+ and NADH following Acid Extraction[a]

nucleotide treated	ADP-ribose (pmol)		
	$-NH_2OH$	$+NH_2OH$	NH_2OH-requiring residues
NAD^+	95 (19)	106 (21)	11 (2)
NADH	297 (59)	436 (87)	139 (28)

[a] NAD^+ and NADH (750 nmol of each) were treated with 20% Cl_3CCOOH at 0 °C in sham tissue extractions, and the Cl_3CCOOH was subsequently removed by ether extractions and lyophilization. After being dissolved in buffer A, 500 pmol of each sample was incubated in the absence or presence of hydroxylamine and analyzed for ADP-ribose residues as described for tissue samples under Experimental Procedures. Numbers in parentheses refer to the percent of input nucleotide converted to ADP-ribose.

However, an unexpected result was the observation that ADP-ribose residues requiring hydroxylamine for detection (Table III, column 4) were significantly lower in the protein fraction after column centrifugation; more than 70% of those residues were found in the nucleotide fraction. This was surprising, since ADP-ribose residues derived from trapped nucleotides would not be expected to contribute to those values. Similar results were observed when other techniques (including dialysis) were employed in place of column centrifugation to remove noncovalently bound nucleotides. One explanation for this result could be that a significant quantity of low molecular weight ADP-ribosylated peptides is present in rat liver. Alternatively, there could be acid modification products of NAD^+ or NADH present which require hydroxylamine for conversion to ADP-ribose. To examine the second possibility, NAD^+ and NADH were treated with trichloroacetic acid in a "sham" tissue preparation. Following trichloroacetic acid treatment, spectrophotometric measurements and analysis by anion-exchange HPLC confirmed that, while NAD^+ was stable, NADH was completely degraded; approximately 40–50% of the NADH was converted to ADP-ribose, and the remainder was degraded to two additional compounds with retention times distinct from all other adenine-containing nucleotides examined (not shown). Subsequently, the trichloroacetic acid treated samples were incubated in the absence or presence of hydroxylamine and analyzed for ADP-ribose. As seen in Table IV, treatment of acid-extracted NADH with hydroxylamine significantly increased the amount of ADP-ribose produced. Thus, conversion of acid modification product(s) of NADH to ADP-ribose by hydroxylamine could account for most, if not all, of the additional ADP-ribose residues detected in the nucleotide fraction after hydroxylamine treatment.

The time course for relase of ADP-ribose residues from rat liver proteins modified in vivo was determined in the presence or absence of hydroxylamine. As shown in Figure 6, release of ADP-ribose by hydroxylamine was essentially complete after 8–12 h of incubation. In the absence of hydroxylamine, there was also a time-dependent release of ADP-ribose residues, amounting to about 20–25% of the total residues released by hydroxylamine. Only a slight release of protein-bound ADP-ribose residues was detected when the incubation was carried out at pH 4 and 0 °C. The latter (control) incubation also revealed the presence of a small amount of noncovalently bound ADP-ribose, representing about 3% of the total free nucleotide fraction prior to G-25 column centrifugation (Table III). This value is in agreement with the estimate of the efficiency of the column centrifugation technique for the removal of trapped nucleotides (Table I). Kinetic analysis of the release of protein-bound ADP-ribose residues in the presence of hydroxylamine revealed two pseudo-first-order

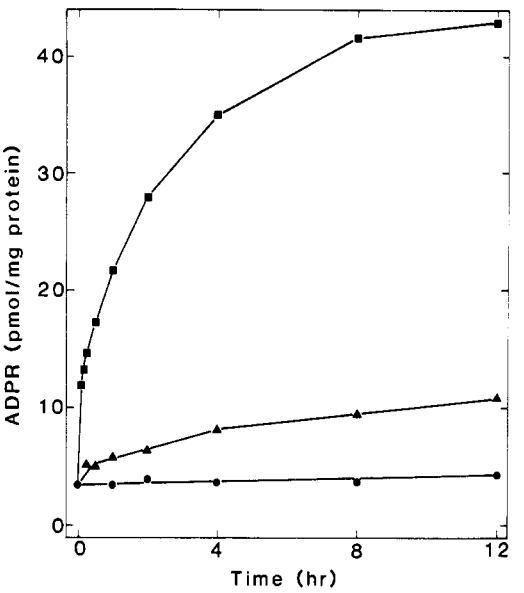

FIGURE 6: Stability of ADP-ribose–protein linkages formed in vivo. The acid-insoluble fraction from rat liver was dissolved (pH 4.0, 0 °C) and immediately subjected to column centrifugation to eliminate noncovalently bound nucleotides (Experimental Procedures). Aliquots of the protein fraction were then incubated at pH 4.0, 0 °C (●), or at pH 7.0, 37 °C, in the absence (▲) or presence (■) of 1 M NH_2OH. At the indicated times, samples (containing 2.32 mg of protein) were subjected to column centrifugation, and free ADP-ribose in the nucleotide fraction was quantified by HPLC analysis following affinity chromatography and derivatization (Experimental Procedures).

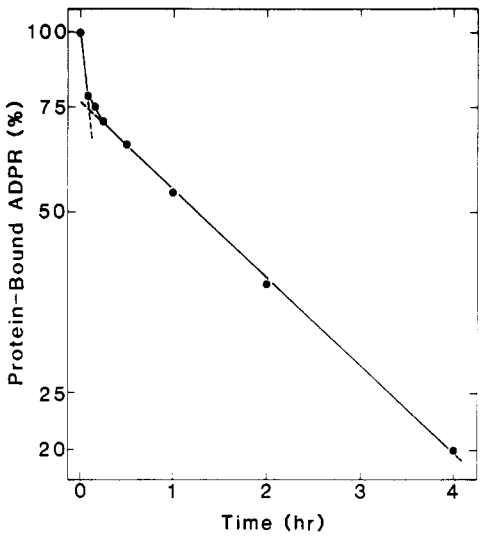

FIGURE 7: Release of ADP-ribose from rat liver proteins by neutral hydroxylamine. Experimental details are the same as those described in the legend to Figure 5. Values for protein-bound ADP-ribose were corrected for residual (trapped) free nucleotides by subtracting the value obtained at t = 0, pH 4.0, 0 °C (see text).

components (Figure 7), suggesting that at least two distinct classes of mono(ADP-ribosylated) proteins are present in adult rat liver. In the presence of 1 M neutral hydroxylamine, one class had a half-life of approximately 5 min, while the other class (representing 75–80% of the total) had a half-life of approximately 2 h. Kinetic analysis of the release of ADP-ribose residues from proteins in the absence of hydroxylamine yielded essentially a single first-order component and an estimate for the half-life of 3–3.5 h (not shown).

Discussion

The studies presented here demonstrate the unambiguous detection of ADP-ribose residues bound to proteins in vivo.

Our strategy for detection of protein-bound ADP-ribose residues eliminated artifacts arising from trapped nucleotides (or their degradation products), since the acid-insoluble material was completely dissolved in a strongly denaturing solution and freed of noncovalently bound nucleotides prior to chemical release of ADP-ribose from proteins. In addition, mono(ADP-ribosylated) histone, EF-2, and transducin, containing ADP-ribose–protein linkages of defined composition, were used to establish conditions necessary for the selective and quantitative release of ADP-ribose residues bound to arginine. When tissue samples were then analyzed under the same incubation conditions, the majority of the ADP-ribose residues detected were bound to proteins via linkages with the same properties as described for arginine linkages. A minor fraction (23%) of the ADP-ribose residues detected was bound via a second, more labile linkage with chemical properties very similar to those described for carboxylate ester linked ADP-ribose. Since the chemical identities of the linkages between proteins and ADP-ribose in vivo have yet to be rigorously established, we refer to these two classes as "arginine linked" and "carboxylate ester linked" only for the sake of simplicity. Although the incubation conditions employed here were selective for distinguishing arginine-linked ADP-ribose residues from those involved in the other known linkages, it is possible that additional, as yet unknown, linkages may exist in vivo. Such linkages could have properties similar to those of ADP-ribosylated arginine and would, therefore, contribute to the levels of ADP-ribose residues we have detected as arginine-linked. For instance, nonenzymatically formed ADP-ribose–protein conjugates could be present in vivo (Hilz et al., 1984), and cleavage of such linkages by hydroxylamine would complicate interpretation of the data presented here. However, recent studies have shown that the Schiff base adducts between ADP-ribose and polyarginine, polylysine, and polyhistidine were all considerably more stable than enzymatically formed ADP-ribose–arginine (R. Koch, M. K. Jacobson, and H. Hilz, unpublished results). In the presence of hydroxylamine (3 M, pH 7.0, 37 °C), $t_{1/2}$ values for the nonenzymatic adducts were all approximately 4 h, as compared to the corresponding value of about 1.5 h for the enzymatically formed arginine conjugate. These results, together with those showing the kinetics of release of ADP-ribose residues from proteins modified in vivo (Figures 6 and 7), suggest that nonenzymatic adducts, if present in vivo, are not quantitatively significant.

It should also be pointed out that ADP-ribose–diphthamide, ADP-ribose–cysteine, and similarly stable linkages may exist in vivo, in which case these would not have been detected with the incubation conditions employed in this study. The possibility of other linkages is strengthened by several recent reports. First, a cellular enzyme capable of ADP-ribosylation of EF-2 was identified in virus-transformed BHK cells (Lee & Iglewski, 1984) and in beef liver (Iglewski et al., 1984). The reaction was reversed by diphtheria toxin at low pH in the presence of nicotinamide, suggesting that the cellular transferase modifies diphthamide. Second, formation of ADP-ribosylated proteins with linkages not susceptible to cleavage by hydroxylamine was observed following incubation of membranes from mammalian cells with [^{32}P]NAD$^+$ (Hsia et al., 1985).

Hilz and co-workers have also described a method designed to quantify proteins mono(ADP-ribosylated) in vivo (Bredehorst et al., 1978). Their methodology involved the treatment of acid-insoluble fractions with either strong alkali or neutral hydroxylamine followed by strong alkali to release ADP-ribose and convert it to 5′-AMP, which was then quantified by radioimmunoassay. The 5′-AMP residues detected after hydroxylamine treatment were attributed to ADP-ribose bound to proteins via "hydroxylamine-sensitive" linkages. Additional 5′-AMP residues detected following incubation of the acid-insoluble fraction with strong alkali were ascribed to "ADP-ribose–protein conjugates having "hydroxylamine-resistant" linkages. Both classes of 5′-AMP residues have been detected in a wide range of cells and tissues under various nutritional and hormonal influences [reviewed by Hilz et al., (1982a,b)]. While those studies have established mono(ADP-ribosylation) as a potentially important posttranslational modification of proteins, neither the identity(ies) of the proteins modified in vivo nor the physiological role(s) of ADP-ribosylation reactions has (have) yet been determined. Furthermore, even the identities of the ADP-ribose–protein linkages present in vivo remain unknown, since stability of these bonds in the presence of either neutral hydroxylamine or hot alkali has no known physical correlate. The N-glycosylic linkage to arginine was clearly hydroxylamine resistant, since more than 70% of the [^{14}C]ADP-ribose reamined bound to histone after brief treatment with dilute hydroxylamine (Table II). However, as demonstrated here and in a recent report by Moss et al. (1983), this hydroxylamine-resistant linkage was completely cleaved by incubation for longer times and/or with higher concentrations of hydroxylamine. Thus, stability in the presence of hydroxylamine is only a relative parameter for some linkages, and it is probable that those ADP-ribose residues previously detected as having hydroxylamine-sensitive linkages were also derived, at least in part, from ADP-ribose–protein conjugates containing hydroxylamine-resistant linkages.

Since different conditions were used to effect release of protein-bound ADP-ribose residues, it is difficult to directly compare the results presented here with those previously reported (Bredehorst et al., 1978). The overall values we obtained for mono(ADP-ribose) residues bound to rat liver proteins were 2–3-fold lower than those reported by Hilz and co-workers. An explanation for this discrepancy is suggested by results presented here (Tables III and IV), which show that trapping of free nucleotides during preparation of the acid-insoluble fraction from tissue can lead to significant overestimation of protein-bound ADP-ribose residues. Since Hilz and co-workers ultimately detected 5′-AMP following alkaline treatment, the contribution by trapped nucleotides was probably constant with or without prior treatment with hydroxylamine. Thus, values for ADP-ribose residues bound via hydroxylamine-resistant linkages should be at least a rough approximation of "arginine-linked" residues. It is encouraging, therefore, that our value of 5.50 nmol/mg of DNA (31.8 pmol/mg of protein) for arginine-linked ADP-ribose residues is reasonably close to their value of 7.12 nmol/mg of DNA for ADP-ribose bound via hydroxylamine-resistant linkages. The levels of arginine-linked ADP-ribose residues we detected were about 400-fold higher than ADP-ribose units in poly(ADP-ribose) (14.3 pmol/mg of DNA) (Jacobson et al., 1983) but still represented less than 1% of the total NAD content in rat liver (Bredehorst et al., 1980). These quantitative relationships, as well as the results suggesting that at least two distinct types of ADP-ribose–protein linkages exist in vivo, are also in general agreement with the findings of Hilz et al. (1982a,b).

The results described here and in another recent report (Jacobson et al., 1985) are also consistent with the hypothesis that ADP-ribosylation of G proteins may be a normal cellular regulatory mechanism (e.g., in response to hormone–receptor

interactions). Following preliminary size fractionation experiments, most of the arginine-linked ADP-ribose residues were associated with rat liver proteins in the mass range of 40–60 kDa. The target proteins for ADP-ribosylation by cholera and pertussis toxins, which may be subverting normal regulatory processes, are G_s and G_i, with masses in the same range as the in vivo modified proteins (39–45 kDa). Furthermore, enzyme activities have also been identified which are capable of "processing" ADP-ribosylated proteins (Jacobson et al., 1985; Smith et al., 1985). These results suggest that mono(ADP-ribosylation) is a reversible modification, again consistent with the hypothesis that ADP-ribosylation reactions represent regulatory posttranslational modifications of proteins. We are currently pursuing the isolation and identification of the individual acceptor proteins modified by ADP-ribosylation in vivo, as well as the rigorous identification of the amino acids which serve as acceptors in the modified proteins.

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A DNA Template Recognition Protein: Partial Purification from Mouse Liver and Stimulation of DNA Polymerase α[†]

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ABSTRACT: A protein that specifically enhances up to 13-fold the rate of copying of poly(dT) template by DNA polymerase α was partially purified from chromatin of regenerating mouse liver cells. This stimulatory protein, designated herein factor D, also increases 2–3-fold the activity of polymerase α with heat-denatured DNA and with primed, circular single-stranded ϕX174 DNA. However, factor D has no detectable effect on the copying by polymerase α of poly(dG), poly(dA), and poly(dC) templates. Activity of mouse DNA polymerase β is not affected by factor D with all the tested templates. In contrast to polymerase α, factor D is resistant to inactivation by *N*-ethylmaleimide and calcium ions, but it is readily heat-inactivated at 46 °C and is inactivated by trypsin digestion. Partially purified factor D is not associated with detectable activities of DNA polymerase, DNA primase, deoxyribonucleotidyl terminal transferase, and endo- or exodeoxyribonuclease.

The replication of genomic DNA in eucaryotes is mediated by a number of enzymes and proteins that are thought to form a multiprotein replicative complex (Noguchi et al., 1983; Reddy & Pardee, 1983; Ottiger & Hübscher, 1984). Within that putative complex, DNA polymerase α is deemed to play a central role in the nucleotide selection and polymerization processes (Reddy & Pardee, 1983; Hübscher, 1983a,b). Although the precise molecular structure of polymerase α is still under debate, there is a general agreement that this high molecular weight enzyme consists of a number of subunits and that it copurifies and interacts with primase (Hübscher et al., 1982; Tseng & Ahlem, 1983; Hübscher, 1983b; Yagura et al., 1983; König et al., 1983; Wang et al., 1984), P^1,P^4-bis(5′-adenosyl) tetraphosphate (Ap_4A) binding protein (Rapaport et al., 1981; Baril et al., 1983; Rapaport & Feldman, 1984), and primer recognition proteins (Lamothe et al., 1981; Pritchard & DePamphilis, 1983; Pritchard et al., 1983). The identification and characterization of polypeptides, which together with the polymerase α catalytic core form an active replicative complex, is essential for the elucidation of the details of DNA replication in animal cells. In addition to the extensively investigated catalytic subunit of polymerase α, DNA primase was recently shown to be either a dissociable component of polymerase α or an inseparable part of the polymerase molecule (Tseng & Ahlem, 1983; Hübscher, 1983b; Yagura et al., 1983; König et al., 1983; Wang et al., 1984). Several investigators have identified additional protein factors that associate with polymerase α to increase its affinity to various primer templates. Protein cofactors that enhance the activity of polymerase α with sparsely primed DNA templates such as denatured DNA have been described (Otto et al., 1977; Richter et al., 1978; Novak & Baril, 1978). Two such stimulatory cofactors, proteins C_1C_2 from human and monkey cells, were found to act as primer recognition proteins (Lamothe et

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