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POSTTRANSLATIONAL MODIFICATION OF PROTEINS
BY ADP-RIBOSYLATION

DISSERTATION

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By

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This work presents the development of a highly sensitive and selective chemical assay for mono(ADP-ribose) residues covalently bound to proteins *in vivo*. An extensive review of the literature is presented in the introduction of this work. The physiological functions of mono(ADP-ribosyl)transferase activities associated with certain bacterial toxins (e.g., diphtheria, cholera and pertussis toxins) are well established. However, the roles of endogenous vertebrate transferases are unknown. The elucidation of the roles of these cellular transferases will likely require identification of the physiologically relevant target proteins. Toward this end, it will also be important to identify the types of (ADP-ribose)-protein linkages present *in vivo*. ADP-ribosylation reactions catalyzed by the different bacterial and vertebrate transferases are specific for different amino acid acceptors *in vitro*. However, the vertebrate transferases that have been characterized thus far are NAD:arginine mono(ADP-ribosyl)transferases.

The work presented here describes the development of a chemical assay for the detection of *in vivo* modified, ADP-ribosylated proteins containing N-glycosylic linkages to arginine. Modified histone, elongation factor 2 and

transducin, containing the different known ADP-ribosylated amino acids (arginine, diphthamide and asparagine, respectively) were employed as model conjugates to establish conditions for the quantitative and selective release of ADP-ribose residues bound to proteins via linkage to arginine. Following release from proteins, ADP-ribose residues were isolated by adsorption to a dihydroxyboronyl affinity resin selective for only those nucleotides containing two or more sets of 1,2 cis-diol groups. A highly fluorescent analog, 1,N^6-etheno(ADP-ribose), was then formed by reaction with chloroacetaldehyde. Subsequently, etheno(ADP-ribose) residues were quantified by monitoring fluorescence following anion exchange HPLC.

The assay was applied to the analysis of ADP-ribose residues in adult rat liver. The strategy employed for detection of protein-bound ADP-ribose residues eliminated potential 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 non-covalently bound nucleotides prior to chemical release from proteins. Thus, the studies presented here demonstrate the unambiguous detection and quantification of protein-bound ADP-ribose residues in adult rat liver. "Arginine-linked" mono(ADP-ribose) residues (31.8 pmol/mg protein) were present in vivo at a level almost 400-fold higher than poly(ADP-ribose). 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 previously for carboxlylate ester-linked ADP-ribose. After fractionation of rat liver proteins by gel filtration HPLC, the major peak of "arginine-linked" ADP-ribose residues eluted in the 40-60 kDa region. The later result is consistent with previous suggestions that G-proteins (40-50 kDa) of the adenylate cyclase complex, which are targets for toxin-catalyzed ADP-ribosylation, may also represent target proteins for endogenous transferases.

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CHAPTER I

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is a vitamin-derived, pyridine-containing nucleotide that plays a central role in cellular metabolism. The function of NAD as an electron carrier, coupling biological oxidation-reduction reactions to production of ATP via oxidative phosphorylation, has been understood for quite some time. In its role in cellular energy metabolism, NAD is interconverted between its oxidized and reduced forms (NAD^+ and NADH, respectively) but is not actually consumed or degraded in these reactions. However, in a number of other cellular reactions, NAD^+ is consumed as as a substrate (Table I). Of particular interest are the reactions catalyzed by two general classes of NAD^+:protein ADP-ribosyltransferases, both of which result in the post-translational modification of specific target proteins.

Both prokaryotic and eukaryotic cells, and some bacterial viruses as well, possess mono(ADP-ribosyl)transferase activities which catalyze the release of nicotinamide from NAD^+ and the transfer of the remaining ADP-ribose moiety to specific proteins (Reaction 1).

$$NAD^+ + \text{Protein} \longrightarrow \text{(ADP-ribose)-Protein} + \text{Nicotinamide} + H^+ \quad (1)$$

TABLE I

Enzymes That Utilize NAD^+ As A Substrate

Enzyme	Product(s)[a]
Numerous Dehydrogenases (EC 1.1. - 1.97.)	NADH
NAD^+ Kinase (EC 2.7.1.23)	$NADP^+$
NAD^+ Pyrophosphorylase (EC 2.7.7.1)	NMN^+ + ATP
NAD^+ Pyrophosphatase (EC 3.6.1.9)	NMN^+ + AMP
DNA Ligase (EC 6.5.1.2)	NMN^+ + AMP
NAD^+ Glycohydrolase (EC 3.2.2.5)	ADPR + Nam
ADP-ribosyltransferases (EC 2.4.99.-.)	$X\text{-}(ADPR)_n$ + n(Nam)

[a] Non-standard abbreviations are as follows: ADPR, ADP-ribose; Nam, nicotinamide; $X\text{-}(ADPR)_n$, mono- and poly(ADP-ribosyl)ated acceptor molecules (i.e., polypeptides or individual amino acids).

In addition, eukaryotic cells possess the nuclear enzyme poly(ADP-ribose) polymerase, which catalyzes not only the formation of the (ADP-ribose)-protein conjugate, but also the formation of polymers of ADP-ribose by further addition of ADP-ribose residues (Reaction 2).

$$\text{n(NAD}^+\text{) + Protein} \longrightarrow \text{(ADP-ribose)}_n\text{-Protein + n(Nicotinamide) + n(H}^+\text{)} \qquad (2)$$

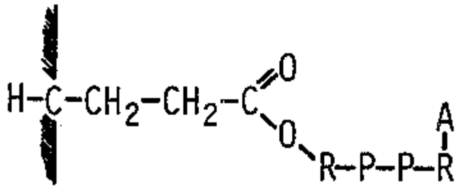
Prokaryotic Mono(ADP-ribosyl)transferases

Certain bacterial toxins represent a group of mono(ADP-ribosyl)transferases whose physiological role is fairly well understood (reviewed in Refs. 1-5). Included in this group are the exotoxins produced by *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli* and *Bordetalla pertussis*. These toxins share a number of common features. First, they are all catalytically inactive as secreted. In the case of diphtheria toxin (actually a phage gene product) and *Pseudomonas* exotoxin A, the molecules are secreted as single polypeptides containing A and B domains, the latter being responsible for binding to cell surface receptors. Cholera toxin, *E. coli* heat-labile enterotoxin and islet-activating protein (IAP), one of the pertussis toxins, are secreted as multimeric proteins composed of at least two types of subunits, designated A and B, with the latter again being required for binding to cell surface receptors. Once associated with their target cells, all of these toxins undergo dissociation of their domains or subunits via limited proteolysis and/or reduction of disulfide bonds, releasing catalytically active mono(ADP-ribosyl)-transferases. The sizes of the active enzymes (fragment- or subunit-A) are very similar for all of the toxins (24-28 kDa). In the absence of suitable proteins or amino acids to serve as ADP-ribose acceptors, all five of these enzymes will function as NAD^+ glycohydrolases, using water as the ADP-ribose "acceptor" and releasing nicotinamide from NAD^+.

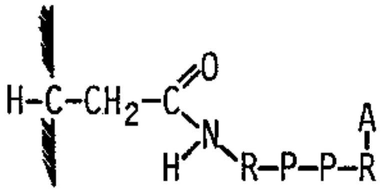
Despite the striking homologies just described, these toxins can actually be divided into three different groups on the basis of the different mechanisms used to affect intoxication of vertebrate cells and the different target proteins and/or amino acids which serve as ADP-ribose acceptors. Specifically, diphtheria toxin (6-8) and Pseudomonas exotoxin A (9) inhibit protein synthesis in vertebrate cells by catalyzing the NAD^+-dependent ADP-ribosylation of elongation factor 2 (EF-2). On the other hand, cholera toxin (10-12) and E. coli heat-labile enterotoxin (13,14) activate adenylate cyclase in animal cells by catalyzing the ADP-ribosylation of the GTP-binding stimulatory subunit (G_s) of the cyclase system. Finally, IAP also activates adenylate cyclase, but does so by a mechanism distinct from that utilized by choleragen and related toxins. IAP catalyzes the ADP-ribosylation of another GTP-binding regulatory component of the cyclase system, namely, the inhibitory subunit (G_i) (15-17).

Although all five toxins catalyze the formation of N-glycosylic linkages between ADP-ribose and amino acids, they differ with respect to the specific amino acids which serve as ADP-ribose acceptors (Fig. 1). Diphtheria (18) and *Pseudomonas* (19) toxins catalyze the ADP-ribosylation of diphthamide, a hyper-modified histidine residue (18). This amino acid is present in EF-2 (one residue per molecule) from all eukaryotic sources examined, including plants (20), but is apparently not found in any other cellular proteins (21). Thus, diphtheria and related toxins exhibit very stringent specificity with respect to both the amino acid acceptor and the target protein which are modified by ADP-ribosylation. Choleragen and related toxins exhibit less stringent specificity and are able to ADP-ribosylate numerous proteins *in vitro*. However, these enzymes are very specific with respect to amino acid requirement, utilizing only arginine and other guanidino-containing compounds as substrates for ADP-ribosylation *in vitro* (13,22). Pertussis toxin (IAP) has recently been shown to specifically modify an asparagine residue in the α-subunit of transducin, the only other *in vitro* substrate besides G_i demonstrated thus far (23).

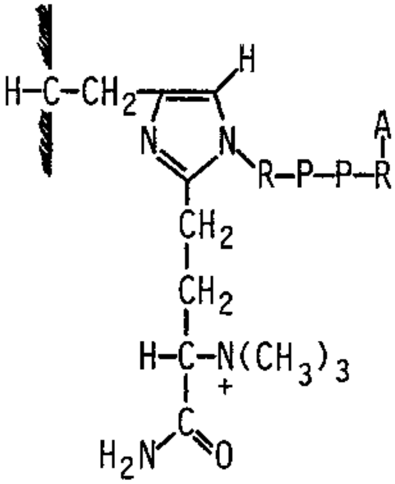
Figure 1. Structures of the known (ADP-ribose)-protein linkages. Diphthamide is the trivial name for 2-[3-carboxyamido-3-(trimethylammonio)propyl]histidine.



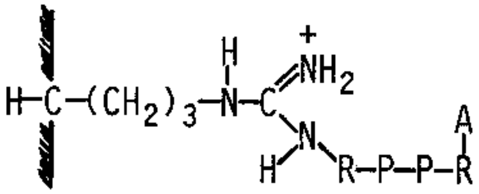
(ADP-ribosyl)glutamate



(ADP-ribosyl)asparagine



(ADP-ribosyl)diphthamide



(ADP-ribosyl)arginine

ADP-ribosylation of Elongation Factor 2 and Inhibition of Protein Synthesis

Eukaryotic elongation factor 2 is a GTP-binding protein (approximate mass = 95 kDa) that catalyzes the translocation of the peptidyl-tRNA and mRNA from the ribosomal A-site to the P-site, with concomitant hydrolysis of GTP. In the absence of ribosomes, however, binding of GTP by EF-2 is not accompanied by hydrolysis (24). Furthermore, hydrolysis of GTP is not required for binding of EF-2 to ribosomes, since other compounds, including GDP and the non-hydrolyzable analog GDP(CH_2)P, can also bind to EF-2 and promote the formation of a ternary complex with ribosomes (25). In studies with the homologous prokaryotic elongation factor (EF-G), it was shown that GDP(CH_2)P could substitute for GTP in the translocation of an aminoacyl-tRNA from the A-site to the P-site of the ribosome. However, in the presence of GDP(CH_2)P, EF-G was required in stoichiometric, rather than catalytic, amounts (26,27). Uncoupling of the GTPase and translocation activities of EF-2 has also been demonstrated (28). These findings, and those from additional studies with specific inhibitors of protein synthesis (e.g., fusidic acid), have led to the conclusion that the GTPase activity of EF-2 (and EF-G) is required for release of the factor from the ribosome (29). Such "recycling" is necessary, since EF-1 and the incoming aminoacyl-tRNA can not interact with ribosomes that contain bound EF-2 (29,30).

Strauss and Hendee (31) demonstrated that cessation of protein synthesis was the first metabolic alteration that could be detected in cells after exposure to diphtheria toxin. In cell-free systems, inhibition of protein synthesis by toxin was shown to be an NAD^+-dependent process (32). Subsequently, two groups of investigators presented evidence for the toxin-catalyzed ADP-ribosylation of EF-2 (7,8,33). The function of ADP-ribosylated EF-2 has been shown to be unaltered with respect to nucleotide binding or the formation of an EF-2•GTP•ribosome complex (24). However, Raeburn et al. (34) and Honjo et al. (35) demostrated that the ribosome-dependent GTPase activity of EF-2 was inhibited by ADP-ribosylation. Thus, it appears likely that diphtheria and related toxins inhibit protein synthesis by maintaining EF-2 in an "activated" state with regard to ribosome binding, thereby preventing GTP hydrolysis, release of EF-2•GDP from the ribosome and, consequently, further addition of amino acids.

The ADP-ribosylation of EF-2 proceeds to completion under physiological conditions, i.e., at neutral pH and saturating $[NAD^+]$ (K_m = 5 μM) (33). However, diphtheria toxin also catalyzes the reverse reaction at acidic pH (e.g., pH 5.2) in the presence of a high concentration of nicotinamide (K_m = 0.5 mM) (33). The ADP-ribosyltransferase activity of diphtheria toxin is inhibited by a number of compounds, including NADH, nicotinamide and GTP (K_i = 36 μM, 0.21 mM and 4.9 mM, respectively) (36,37). As mentioned above, diphtheria toxin exhibits unusual specificity with regard to protein substrates, such that EF-2 from any eukaryotic source will serve as an ADP-ribose acceptor, but the toxin will not modify any other protein (21,33).

A partial explanation for this specificity was provided by analysis of the trypsin-derived ADP-ribosylated peptides of EF-2 from diverse eukaryotic species, including rat, beef, yeast and wheat germ. This study revealed extensive homology in primary structure and the invariable presence of diphthamide, the amino acid acceptor for ADP-ribose, as the penultimate residue in each peptide (20). However, the tryptic peptide (15 amino acids) containing the ADP-ribosylation site was not a substrate for diphtheria toxin, suggesting that recognition of EF-2 by toxin is not solely determined by the presence of diphthamide or the amino acid sequence in this region (38). Nevertheless, the presence of the diphthamide residue is an absolute requirement for the ADP-ribosylation of EF-2 by diphtheria toxin (39-42).

Diphthamide itself is the most complex example of a site-specific posttranslational modification identified to date. Based on structural (18,43,44) and genetic analyses (39-41) and experiments using radiolabelled precursors (21), it has been concluded that at least three distinct enzymatic reactions are required for the biosynthesis of diphthamide from a specific histidine residue in EF-2. Although the sequence of reactions is not known, two of the three reactions require S-adenosylmethionine (SAM). The (3-carboxy-3-aminopropyl) side chain at carbon 2 of the imidazole ring (Fig. 1) is derived from the "backbone" of methionine. The three methyl groups of the trimethylammonio-function are also derived from methionine via SAM. The precursor for amidation of the terminal carboxyl group is unknown at present. It has been further established that the entire cellular content of diphthamide can be accounted for as ADP-ribosylatable residues in EF-2 (21). In view of the complexity of the biosynthesis of diphthamide, its exclusive presence in EF-2 from all eukaryotes, and the inactivation of EF-2 by modification of this residue, it might be predicted that diphthamide must perform an essential role in the function of EF-2. In contradiction to this expectation, however, mutant cells possessing an altered form of EF-2 containing no diphthamide were found to be capable of protein synthesis and growth at the same rates as wild-type cells (39,40,42). Thus, the role of diphthamide in the normal function of EF-2 in protein synthesis is unknown.

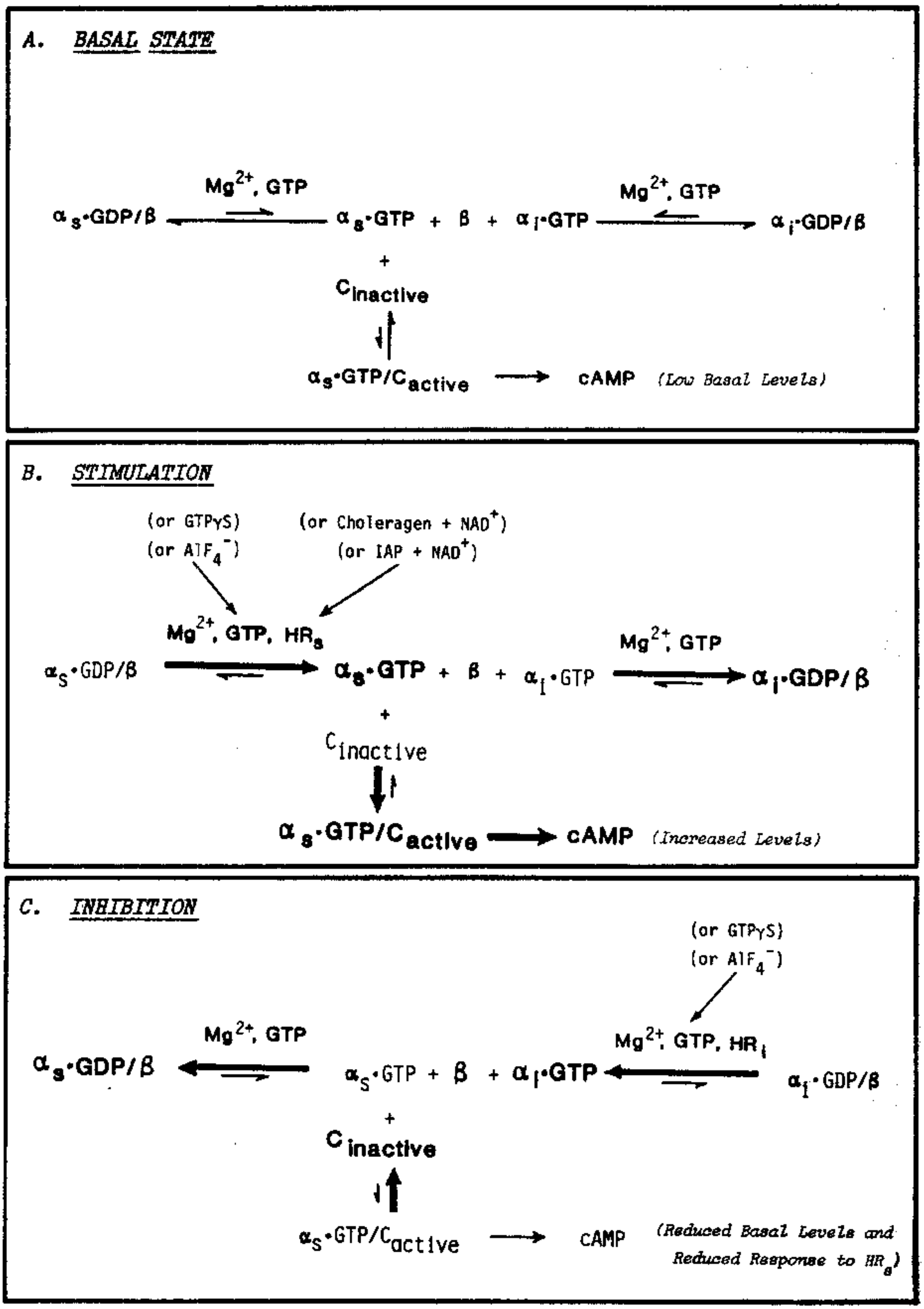
ADP-ribosylation of G-Proteins and Activation Of Adenylate Cyclase

Hormone-sensitive adenylate cyclase is a multiprotein enzyme complex, the components of which are embedded and organized in the matrix of the plasma membrane. The adenylate cyclase system catalyzes the synthesis of cAMP at a relatively low basal rate which can be dramatically altered by catecholamines, polypeptide hormones, acetylcholine, prostaglandins, opioids, numerous drugs and certain bacterial toxins (reviewed in Refs. 45-47). The interaction of these regulatory molecules with specific cell-surface receptors is coupled to either stimulation or inhibition of adenylate cyclase activity, depending upon both the type of ligand and the type of receptor(s) present on the target cell surface. Polypeptide hormones (e.g., ACTH, TSH, glucagon), β-adrenergic agonists (e.g., isoproterenol, epinephrine), prostaglandins (e.g., PGE_1) and toxins (e.g., choleragen, IAP) stimulate adenylate cyclase activity upon binding to their specific receptors. Inhibition of cyclase activity is mediated by interactions between specific receptors and α_2-adrenergic agonists (e.g., epinephrine, norepinephrine), muscarinic (cholinergic) agonists, opioids (e.g., enkephalin) and prostaglandins (e.g., PGE_1). In addition to surface receptors and the cyclase itself, the complex also includes a pair of highly-conserved, homologous regulatory proteins (variously designated G, G/F or N) that bind guanine nucleotides (47). These two G-proteins are responsible for coupling ligand-receptor interactions to cAMP production.

The various stimulatory receptors are linked to the catalytic subunit (designated C) via a common pool of stimulatory G-proteins (G_s) (48,49). Likewise, inhibitory receptors are coupled to C via a common pool of inhibitory G-proteins (G_i). G_s is composed of a 45 kDa α-subunit, a 35 kDa β-subunit and (probably) a 10 kDa γ-subunit (47,50-52). G_i has a homologous subunit arrangement, with a slightly smaller α-subunit (41 kDa) and identical β- and γ-subunits (17,47). The α-subunits of G_s and G_i each contain a single high affinity binding site for guanine nucleotides (17,53) and are the targets for ADP-ribosylation by cholera toxin and IAP, respectively (15-17,50,51). The β-subunit common to both G_s and G_i apparently contains a binding site for Mg^{2+} (54,55).

Modulation of adenylate cyclase activity via G_s and G_i is a complex process that requires "activation" of the G-proteins by dissociation of their respective α_s/β or α_i/β heterodimers (55-60). The basal rate of dissociation is relatively slow, and the reaction requires the presence of Mg^{2+} and either guanine nucleotides or fluoride ions (in the form of an AlF_4^- complex (61)) (Fig. 2). Adenylate cyclase activity is dependent upon the availability of free α_s-polypeptide, which is regulated, in turn, by the relative

Figure 2. Proposed regulation of adenylate cyclase activity by G-proteins. HR_s and HR_i represent the stimulatory and inhibitory hormone-receptor complexes, respectively. See text for details.


A. BASAL STATE
Mg2+, GTP
αs·GDP/β
αs·GTP + β + αi·GTP
Mg2+, GTP
αi·GDP/β
+
Cinactive
αs·GTP/Cactive
cAMP
(Low Basal Levels)
B. STIMULATION
(or GTPγS)
(or AlF4−)
(or Choleragen + NAD+)
(or IAP + NAD+)
Mg2+, GTP, HRs
αs·GDP/β
αs·GTP + β + αi·GTP
Mg2+, GTP
αi·GDP/β
+
Cinactive
αs·GTP/Cactive
cAMP
(Increased Levels)
C. INHIBITION
(or GTPγS)
(or AlF4−)
Mg2+, GTP
αs·GDP/β
αs·GTP + β + αi·GTP
Mg2+, GTP, HRi
αi·GDP/β
+
Cinactive
αs·GTP/Cactive
cAMP
(Reduced Basal Levels and Reduced Response to HRs)

amount of free β-subunit present. More G_i is present in membranes than G_s, and G_i dissociates more readily than G_s. Thus, the basal, steady-state level of β-subunit is in excess of α_s, and the basal adenylate cyclase activity is low (Fig. 2A). In the presence of Mg^{2+} and either non-hydrolyzable analogs of GTP (e.g., GTPγS) or fluoride, G_s and G_i are activated irreversibly, and adenylate cyclase activity is permanently stimulated or inhibited, respectively (Figs. 2B and 2C).

The effect of hormone-receptor interactions is to dramatically increase both the rate and extent of G_s (or G_i) activation in the presence of Mg^{2+} and guanine nucleotides (Figs. 2B and 2C) (54,59,62). Intracellular concentrations of guanine nucleotides are saturating with respect to dissociation of G-proteins, and hormone treatment has no significant effect on the apparent K_m for binding of guanine nucleotides to G_s (54). In striking contrast, the K_m of the system for Mg^{2+} is reduced by a factor of ≈10^4 following hormone receptor interaction (i.e., from ≈6 mM to 0.1-1.0 μM) (54). Thus, the effect of hormone binding is to reduce the apparent K_m for Mg^{2+} from a value well above the intracellular concentration of ≈0.5 mM (63) to a value well below that found in the cell, allowing the system to operate at saturating concentrations of both Mg^{2+} and guanine nucleotides.

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The α_s- and α_i-subunits also contain bound guanine nucleotide in their respective inactive heterodimers. When G_s or G_i, which have been "prelabelled" with bound [^{3}H]GTP, are activated in the presence of guanine nucleotides following hormone-receptor interactions, [^{3}H]GDP is released in a "nucleotide-exchange reaction" (Fig. 2) (49,64). Stimulation of GTPase activity following interactions of receptors with both stimulatory and inhibitory ligands has also been detected (65,66). These results, and those demonstrating irreversible activation by GTP S, have led to the hypothesis that GTP hydrolysis is responsible for the "turn-off" reaction, restoring adenylate cyclase to the basal state (65). Implicit in this hypothesis is the prediction that the G-proteins function as GTPases. Indeed, purified G_s exhibits a GTPase activity that is stimulated in solution by high concentrations of Mg^{2+}, as well as by the hormone-receptor complex when present in reconstituted vesicles (67).

The actual role for the G_s- and G_i-associated GTPase activity is uncertain, however, since GDP itself can fulfill the guanine nucleotide requirements of the hormone-sensitive adenylate cyclase system. For example, GDP binds to both G_s and G_i with an affinity equal to that of GTP (53,57). Furthermore, GDP is equal in effectiveness to GTP at supporting both hormone-induced [^{3}H]GDP release from G_s in membranes (i.e., the nucleotide exchange reaction) (64) and hormone-stimulated adenylate cyclase activity (68). GDP is also equal in effectiveness to GTP at stimulating the guanine nucleotide-dependent increase in rate of dissociation of hormone from the hormone-receptor complex (69). The metabolic significance of the GTPase activity has also been questioned because of the very slow turnover number for the hormone-stimulated GTPase (1.0-1.5 min^{-1}) (65,67). This value is probably accurate, since it is very similar to the rate constant for hormone-stimulated binding of GTP S (1.7-3.4 min^{-1}) (67), which should reflect an upper limit on the turnover number of the GTPase. Nevertheless, a role for the GTPase activity in regulation of G-protein function is strongly supported by the actions of choleragen and IAP (Fig. 2B).

ADP-ribosylation of G_s by cholera toxin does not alter the apparent affinity of α_s for GTP nor the ability of α_s to activate the catalytic subunit of adenylate cyclase (12,70). ADP-ribosylation does, however, result in a 10-fold decrease in the apparent affinity of α_s for the β-subunit (70). Futhermore, ADP-ribosylation of G_s inhibits the hormone-stimulated GTPase activity (71). Finally, ADP-ribosylation of G_s in the presence of GTP results in irreversible activation of adenylate cyclase, even in the absence of a stimulatory hormone (71). This is in contrast to the inability of GTP to support significant adenylate cyclase activity with unmodified G_s (i.e., the basal state, Fig. 2A). Fewer details are available regarding the ADP-ribosylation of G_i, but two prominent effects have been demonstrated. First, ADP-ribosylation of G_i by IAP inhibits both hormone-stimulated GTPase activity (72) and hormone-stimulated release of $[^3H]$GDP from membranes (49). Second, ADP-ribosylation by IAP inhibits the hormone- and guanine nucleotide-induced dissociation of G_i (59). The net consequence is stimulation of adenylate cyclase activity as a result of a decreased concentration of free β-subunit (Fig. 2B). Taken together, these results further support the hypothesis that dissociation-reassociation of G-protein subunits and hydrolysis of GTP are intimately related events in the regulation of adenylate cyclase activity.

The ADP-ribosylation activities of cholera and pertussis toxins are similar to that of diphtheria toxin in several respects, including maximal activity at physiological pH and a high degree of specificity for NAD^+ (K_m = 4 mM and 2.5 μM for choleragen and IAP, respectively) (11,16). Furthermore, cholera toxin also catalyzes the reverse reaction at acidic pH and in the presence of nicotinamide (73). However, there are some additional requirements for ADP-ribosylation unique to the choleragen-catalyzed reaction. In addition to NAD^+, GTP and a protein cofactor are required for ADP-ribosylation of both purified G_s and of G_s in isolated membranes (73-75). Occupation by GTP of the guanine nucleotide binding site on G_s is apparently necessary for choleragen activity. It is of interest that GDP will not support the ADP-ribosylation of purified G_s (74), since GDP can otherwise fulfill the guanine nucleotide requirements of the hormone-sensitive adenylate cyclase system. The required protein cofactor, which has been purified from rabbit liver membranes, is a 21 kDa polypeptide, designated "ADP-ribosylation factor" (ARF) (74). The function of this protein is unknown, but it appears to somehow promote ADP-ribosylation by binding to GTP-liganded G_s (74). A similar protein with the same ability to support toxin-catalyzed ADP-ribosylation of G_s has been identified in the cytosol or membranes from all vertebrate cells examined thus far (76,77). Neither GTP nor ARF are required for the ADP-ribosyltransferase activity of IAP (16,17).

Although G_s is by far the major protein ADP-ribosylated by cholera toxin, several other cellular proteins are also modified (73). As determined by autoradiography after SDS gel electrophoresis, the approximate masses of the minor ADP-ribosylated peptides are 13, 15, 22, 24, 80 and 150 kDa. ADP-ribosylation of all these proteins requires both GTP and ARF activity (3), and modification is inhibited by arginine (78). After modification with $[^{32}P]NAD^+$ and limited proteolysis, G_s and two of the minor proteins (22 and 98 kDa) yielded nearly identical radiolabelled peptide fingerprints (3). These results have led to the suggestion that the toxin-modified proteins may represent a class of GTP-binding proteins that also interact with ARF. One protein not ADP-ribosylated by cholera toxin is G_i, the target for modification by IAP. Conversely, IAP does not ADP-ribosylate G_s (17). However, both toxins catalyze the covalent attachment of an ADP-ribose residue to distinct (and different) sites on the GTP-binding α-subunit of transducin (23,79,80). Transducin is a regulatory protein in retinal rod membranes and exhibits striking structural and functional homology to G_s and G_i (47). ADP-ribosylation by cholera toxin resulted in inhibition of transducin-associated GTPase activity (79), further supporting the suggestion that a closely related family of G-proteins may regulate a diverse set of cellular functions (47).

Bacteriophage Mono(ADP-ribosyl)transferases

Following infection of *E. coli* by bacteriophage T4, host RNA synthesis rapidly stops and transcription of T4 genes proceeds in a well-defined sequential pattern ("immediate early" to "delayed early" to "late" RNA) (81). Within 2-4 minutes after T4 infection, changes can be detected in several of the polypeptides which comprise the *E. coli* RNA polymerase $\alpha_2\beta\beta'\sigma\omega_2$ holoenzyme. Specifically, during the first 2 min after infection, the α-, σ-, β'- and β-subunits are "altered" to varying extents (all <50%) by ADP-ribosylation. During the subsequent 2-3 minutes, the α-subunits are specifically and completely "modified" by ADP-ribosylation (one ADP-ribose residue per molecule of α) (81,82). Two distinct mono(ADP-ribosyl)transferase activities (designated *alt* and *mod*, respectively) have been characterized which catalyze these covalent additions to *E. coli* RNA polymerase, and both of these are encoded in the T4 genome (81,83,84). In fact, the active product of the T4 *alt* gene is actually present within the virion and is injected along with other phage components into the *E. coli* cell (83; reviewed in Refs. 85,86).

The *alt* transferase, responsible for the initial ADP-ribosylation events, is a monomeric protein with a mass of 70 kDa (83). As mentioned above, the alteration reaction is incomplete; i.e., only one of the two α-subunits in the RNA polymerase holoenzyme can be ADP-ribosylated by *alt*, even though the *in vitro* reaction with purified α-subunits is quantitative (83). This enzyme is also rather non-specific and will ADP-ribosylate many different proteins *in vitro*, as well as several *E. coli* proteins in addition to RNA polymerase *in vivo*. In contrast, the *mod* transferase is a protein of 25 kDa that is highly specific both *in vitro* and *in vivo*, catalyzing the ADP-ribosylation of the α-subunits of RNA polymerase and only one additional *E. coli* protein, a polypeptide of 13 kDa (84). Despite the differences between the two enzymes, both utilize arginine as the amino acid acceptor and catalyze the formation of an N-glycosylic linkage between ADP-ribose and the guanidino group of the acceptor.

ADP-ribosylation of E. coli RNA Polymerase And Inhibition of RNA Synthesis

Functional differences were demonstrated between normal and ADP-ribosylated RNA polymerase (formed by the *mod* transferase) when transcription of *E. coli* genes under control of three different promoters was evaluated in an *in vitro* protein synthesis assay (87). ADP-ribosylated RNA polymerase did not support the expression of genes under control of one of the promoters and only weakly supported expression of the other transcriptional units. In contrast, both the modified and unmodified enzymes supported expression of T4 genes equally well, with the exception of certain "middle" phage genes which were only synthesized in the presence of ADP-ribosylated RNA polymerase. Mixed reconstitution experiments confirmed that the observed differences in specificity of the two forms of RNA polymerase were due to ADP-ribosylation of the α-subunits. On the basis of these data from *in vitro* experiments and the coincidence of ADP-ribosylation with changes in transcription specificity *in vivo*, it was concluded that ADP-ribosylation of RNA polymerase was responsible for switching off expression of *E. coli* genes and switching on at least some phage genes.

However, this conclusion was not supported by the work of Horvitz, who identified T4 mutants lacking either the *alt* or *mod* transferase and found them to be viable (88). Goff and Setzer generated double mutants (T4 *alt*⁻*mod*⁻) by genetic recombination (89). Even though ADP-ribosyltransferase activity was completely absent, the double mutants were recovered with the same freguency as wild-type phage, and with no apparent differences in viability or other physiological parameters. Futhermore, double mutants halted *E. coli* RNA synthesis after infection with the same kinetics as wild-type phage. Thus, the physiological role of these two enzymes is unknown. It is possible that ADP-ribosylation of RNA polymerase is only essential under conditions in which other regulatory mechanisms are inoperable. It is also possible that RNA polymerase is only an adventitious substrate and not the physiologically relevant target for these phage-encoded ADP-ribosyltransferases.

The latter possibility was strengthened by the work of Pesce et al., who identified an ADP-ribosyltransferase as a component of another virion, coliphage N4 (90). The N4 transferase was capable of ADP-ribosylating several proteins *in vitro* and about 30 *E. coli* proteins *in vivo*, but the host RNA polymerase was not among those proteins. These workers also found that expression of early phage genes was accomplished by an N4 RNA polymerase which was already present in the virion. However, in a previous report they demonstrated that transcription of late genes was absolutely dependent upon an N4-modified form of *E. coli* RNA polymerase (91). Since the nature of the modification was not determined, it remains possible that ADP-ribosylation of RNA polymerase did occur, but at a later time than that observed for other proteins and, thus, was not detected. It is also possible that the target for the N4 ADP-ribosyltransferase was the N4 RNA polymerase, but this possibility was not explored, and the physiological role of the phage transferases remains unknown.

Eukaryotic Mono(ADP-ribosyl)transferases

In addition to the bacterial toxins that exert their effects on vertebrate cells, animal cells themselves possess mono(ADP-ribosyl)transferase activities. The presence of such enzymes has been demonstrated in avian (92) and human (93) erythrocytes, rat (94,95) and bovine liver (96), rat testis (97), rat and bovine thyroid cells (98,99), rabbit skeletal muscle (100) and polyoma virus-transformed baby hamster kidney cells (96). Further, two different mono(ADP-ribosyl)transferases from turkey erythrocytes (101,102) and a transferase from hen liver nuclei (103) have been purified and extensively characterized.

The first of the two transferases to be purified from turkey erythrocytes (designated transferase A) was a monomeric protein with a molecular mass of 28 kDa (101). At low ionic strength, however, the enzyme formed oligomers of >500 kDa with a concomitant 90% decrease in activity. Dissociation and restoration of enzyme activity could be accomplished by an increase in the concentration of a variety of anions, the effectiveness of which were correlated with their chaotropic activity as defined by the Hofmeister series; i.e., $SCN^{-}>Br^{-}>Cl^{-}>F^{-}>PO_4^{3-}$ (104). Interestingly, in the absence of salts, very low concentrations of histones (20 µg/ml) also maximally activated the transferase by converting oligomers to monomers (93,105). Under the conditions employed, the histones themselves did not serve as ADP-ribose acceptors, and activation was not observed with 11 other proteins tested, nor with polyarginine or polylysine.

The second ADP-ribosyltransferase purified from turkey erythrocytes (transferase B) differed from transferase A in several respects (102). Transferase B was slightly larger, with a mass of 32 kDa. Chaotropic salts, at concentrations required for maximum activation of transferase A, inhibited enzyme B by 40%. Furthermore, concentrations of histones which maximally activated transferase A had no effect on the activity of transferase B, and no change in the size of the B enzyme was detected in the presence of salt or histones.

Like cholera and related toxins (106,107), the ADP-ribosyltransferase A from turkey erythrocytes catalyzes, in vitro, the formation of an α-N-glycosylic linkage between ADP-ribose and the guanidino group of arginine (108), and both avian transferases will also utilize other guanidino-containing compounds as ADP-ribose acceptors. The kinetic properties of the two avian enzymes were similar; the apparent K_m values for the activated transferase A were 15 µM

(105) and 1.3 mM (104) (for NAD^+ and arginine methyl ester, respectively), while the corresponding values for transferase B were 36 µM and 3.0 mM (102). The calculated V_{max} values for transfer of ADP-ribose to arginine methyl ester were 350 µmol/min/mg (101) and 60 µmol/min/mg (102) for the A and B enzymes, respectively.

In addition to the avian erythrocyte enzymes, arginine-dependent mono(ADP-ribosyl)transferases have also been partially purified from human erythrocytes (93) and rat liver (94). The reactions catalyzed by the latter enzymes appear to be similar, if not identical, to those catalyzed by the turkey transferases; the human erythrocyte enzyme was also activated by anions and histones (93). The transferase purified from hen liver nuclei also has properties in common with both of the turkey enzymes (103). The hen liver enzyme has a mass of 27.5 kDa and utilizes histones, polyarginine, arginine methyl ester and other low molecular weight guanidino-containing compounds as acceptors. Like the turkey transferase B, the hen liver enzyme is inhibited by salts and does not aggregate or dissociate in the presence of NaCl. However, the nuclear transferase from hen liver has a higher pH optimum than the other two avian enzymes, and higher apparent K_m values as well (70 µM and 24 mM for NAD^+ and arginine methyl ester, respectively). In the absence of suitable proteins or amino acids to serve as ADP-ribose acceptors, all five of the vertebrate enzymes described will function as NAD^+ glycohydrolases. The properties of the known mono(ADP-ribosyl)transferases are summarized in Table II.

Possible Cellular Functions of Eukaryotic Mono(ADP-ribosyl)transferases

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 proteins via ADP-ribosylation, the role(s) of endogenous eukaryotic mono(ADP-ribosyl)transferases has not been established. Several lines of evidence suggest 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 bacterial toxins (i.e., by ADP-ribosylation of regulatory components of the adenylate cyclase complex) (92,98,99,109-111).

For instance, it has been demonstrated that transferase A from avian erythrocytes catalyzes the NAD^+-dependent activation of adenylate cyclase *in vitro* (92). Blecher and coworkers have also demonstrated both stimulation of adenylate cyclase and ADP-ribosylation of G_s by an endogenous transferase in RL-PR-C hepatocyte membranes in response to the synthetic hormone isoproterenol (109,110). The ADP-ribosylation activity was shown to be inhibited by propranolol, a β-adrenergic antagonist that also blocks hormone stimulation of

TABLE II

Comparison of Mono(ADP-ribosyl)transferases[a]

	Diphtheria Toxin	Cholera Toxin
Molecular Mass (Daltons)[b]	24,000	23,500
K_m (NAD^+)	5 μM	4 mM
NAD^+ Glycohydrolase Activity[c]	Yes	Yes
Amino Acid Acceptor	diphthamide	arginine
Target Protein	EF-2	G_s
Target Protein Activity	GTPase	GTPase
Effect of ADP-ribosylation on Target Protein Activity	Inactivated	Inactivated
Effect of ADP-ribosylation on Cellular Metabolism	Protein Synthesis Inhibited	Adenylate Cyclase Activated

[a] Only one representative for each distinct type of transferase is shown.

[b] Active (or activated) form of the enzyme.

[c] In the absence of an acceptor.

TABLE II (continued)

Pertussis Toxin	T4*mod* Transferase	Avian Erythrocyte Transferase A	Calf Thymus Poly(ADP-ribose) Polymerase
28,000	25,000	28,000	110,000
2.5 µM	140 µM	15 µM	100 µM
Yes	?	Yes	?
asparagine	arginine	arginine	glutamic acid
G_i	α-subunit of RNA polymerase	?	?
GTPase	GTPase[d]	?	?
Inactivated	Inactivated[e]	?	?
Adenylate Cyclase Activated	Host RNA Synthesis Inhibited	?	?

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[d] While technically not a GTPase, RNA polymerase does bind and hydrolyze GTP during RNA synthesis.

[e] ADP-ribosylated RNA polymerase is inactivated with respect to synthesis of *E. coli* RNA only.

adenylate cyclase (109). Further, a large amount of experimental evidence has accumulated which reveals striking structural and functional similarities between bacterial toxins and glycoprotein hormones (reviewed in Ref. 99). Structurally, there are significant homologies in both amino acid sequence and subunit arrangement, as well as homologies in the structures of specific cell surface receptors. Functionally, there are dramatic similarities between the consequences of toxin-receptor and hormone-receptor interactions, including alterations in the physical and electrochemical state of the cell membrane and (perhaps consequently) the activation of adenylate cyclase.

In recent studies, Kohn and coworkers demonstrated that thyrotropin (TSH) stimulated an endogenous (ADP-ribosyl)-transferase activity in rat and bovine thyroid membranes *in vitro* and that NAD^+ added to the membrane preparations enhanced this stimulation (98,111). Further, they showed that the TSH-stimulated ADP-ribosylation of membrane components preceded the hormone stimulation of adenylate cyclase activity, and that the predominant membrane protein ADP-ribosylated following TSH-stimulation was identical to the major ADP-ribosylated product formed after treatment with cholera toxin, i.e., the GTP-binding stimulatory component of the adenylate cyclase complex. In additional studies, DeWolf et al. detected a pyrophosphatase-like enzyme activity associated with thyroid membranes; this activity was capable of hydrolyzing the product of the ADP-ribosyltransferase reaction to 5'-AMP and an acceptor containing ribose phosphate (98). This finding, along with other data, led those investigators to propose that TSH-stimulated ADP-ribosylation of the GTP-binding regulatory protein, followed by further processing to a phospho-ribosylated protein, could lead to the formation of a stable activated state, desensitized to further stimulation by additional TSH. Thus, there is a significant amount of experimental data which supports the hypothesis that ADP-ribosylation in vertebrate cells is part of a mechanism regulating the cellular response to hormonal stimulation.

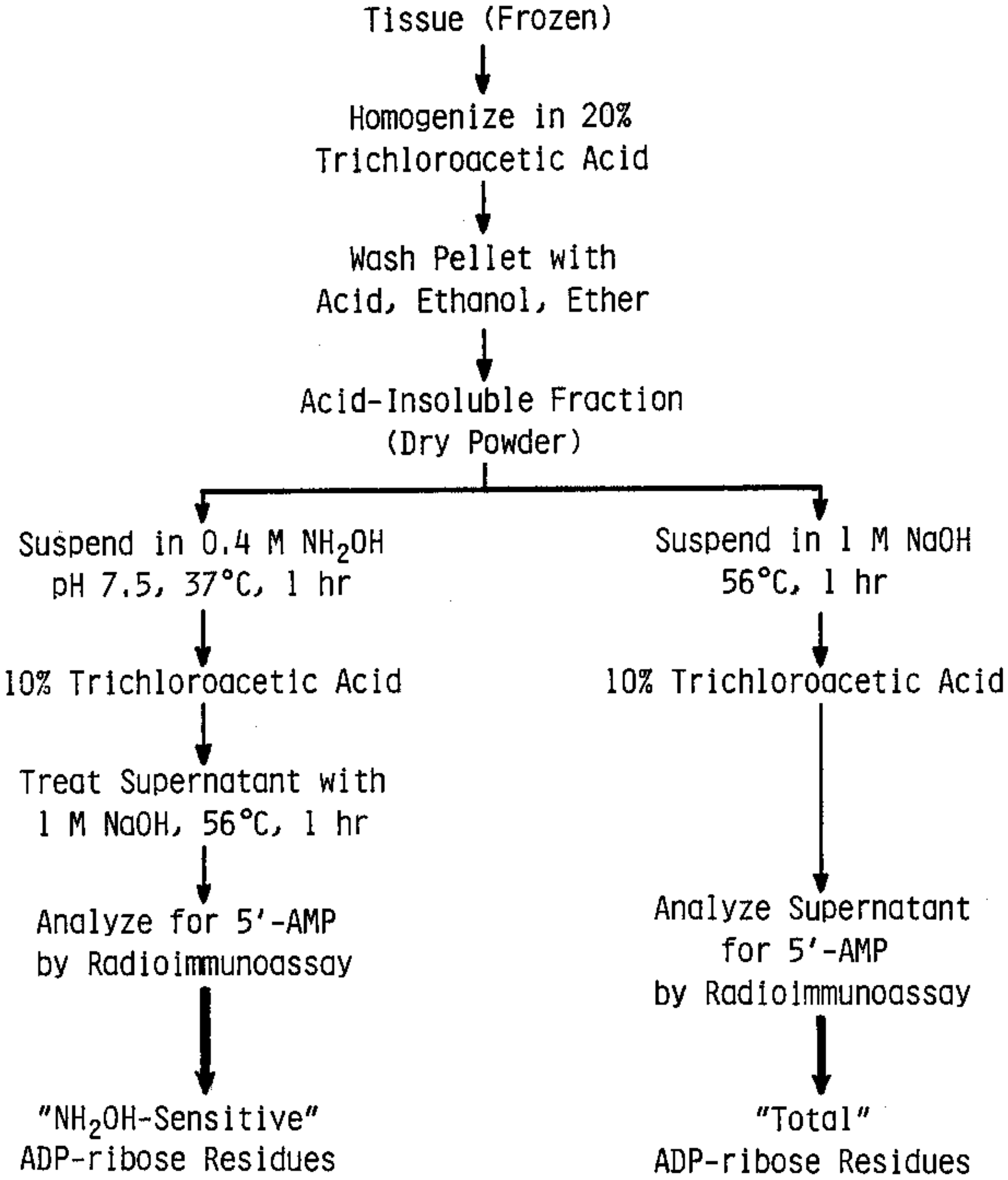
On the other hand, evidence has been presented which does not support this hypothesis (1,92,112-114). First, although the avian erythrocyte transferase A can activate adenylate cyclase in membrane preparations from bovine brain, it does not activate cyclase from turkey erythrocytes or other sources (1,92). Further, the glycopeptide hormones themselves do not appear to possess intrinsic ADP-ribosyltransferse activity (112). Additionally, some of the regions of sequence homology between cholera toxin and the glycopeptide hormones are relatively limited, and their statistical and functional significance has been questioned (113). Finally, in a cultured cell line responsive to the glycopeptide hormone human chorionic gonadotropin (hCG), activation of adenylate cyclase by hCG _in vitro_ did not appear to require NAD$^+$, nor was ADP-ribosylation detected (114). 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. Indeed, Hilz et al. have recently identified a heretofore unrecognized problem associated with studies of ADP-ribosylation reactions *in vitro* (115). Various subcellular fractions possess NAD$^+$ glycohydrolase activities capable of generating significant quantities of radiolabelled ADP-ribose from exogenously added radiolabelled NAD$^+$. Hilz et al. demonstrated that such *in vitro* studies may be particularly susceptible to artifacts resulting from the subsequent non-enzymatic formation of (ADP-ribose)-protein adducts (i.e., Schiff base formation between basic amino acids and the reducing ribose of ADP-ribose).

Detection of ADP-ribosylated Proteins Modified In Vivo

Identification of the target protein(s) modified *in vivo* by mono(ADP-ribosylation) would facilitate elucidation of the physiological role(s) of cellular mono(ADP-ribosyl)transferases. Knowledge of the chemical nature of the linkage(s) that exist(s) *in vivo* is also essential, since the reactions catalyzed *in vitro* are specific for several different amino acid acceptors. As previously described, the transferases identified thus far catalyze the formation of N-glycosylic linkages between ADP-ribose and either diphthamide, arginine or asparagine. In addition, after in vitro incubations with radiolabelled NAD^+, histones were shown to be mono(ADP-ribosylated) via ester linkages to the γ-carboxyl group of glutamic acid residues and to the α-carboxyl group of terminal lysine residues (116-119). (These modifications were presumably catalyzed by the nuclear enzyme poly(ADP-ribose) polymerase.) Thus, the results from in vitro experiments indicate that the (ADP-ribose)-protein conjugates formed in vivo are likely to be heterogeneous, involving several different amino acids.

Indeed, the studies of Hilz and co-workers suggest that such heterogeneity does exist in vivo. Those investigators have presented evidence for the existence of at least two classes of (ADP-ribose)-protein linkages, which were distinguished by their different chemical stabilities (120). This evidence was based on the detection of 5'-AMP following treatment of the acid-insoluble fraction from rat liver with either strong alkali, or neutral hydroxylamine followed by strong alkali (Fig. 3). 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. Hilz and co-workers have described the

Figure 3. Protocol described by Bredehorst et al. (120) for analysis of protein-bound ADP-ribose residues.


Tissue (Frozen)
Homogenize in 20% Trichloroacetic Acid
Wash Pellet with Acid, Ethanol, Ether
Acid-Insoluble Fraction (Dry Powder)
Suspend in 0.4 M NH_2OH pH 7.5, 37°C, 1 hr
10% Trichloroacetic Acid
Treat Supernatant with 1 M NaOH, 56°C, 1 hr
Analyze for 5'-AMP by Radioimmunoassay
"NH_2OH-Sensitive" ADP-ribose Residues
Suspend in 1 M NaOH 56°C, 1 hr
10% Trichloroacetic Acid
Analyze Supernatant for 5'-AMP by Radioimmunoassay
"Total" ADP-ribose Residues

detection and quantification of both classes of residues in Ehrlich ascites tumor cells (121), normal and leukemic lymphocytes (122), rat liver during development (123-125), rat liver and mouse kidney under various nutritional and hormonal conditions (126-128), several hepatoma cell lines (124,129) and, most recently, a variety of mouse tissues (127). The subcellular distribution of these residues in rat liver has also been examined (130). Although absolute levels of "mono(ADP-ribosylated) proteins" (i.e., 5'-AMP residues) were found to vary widely among different cell- and tissue types, some general conclusions have been presented (reviewed in Refs. 124, 131-133).

First, total levels of mono(ADP-ribose)-protein conjugates (i.e., alkali-released 5'-AMP) were correlated with the total cellular levels of NAD (i.e., NAD^+ + NADH) (126,132). Second, the total levels of protein-bound mono(ADP-ribose) residues (detected as 5'-AMP) were much lower than NAD levels (126,132), but were significantly higher, by at least two orders of magnitude, than levels of poly(ADP-ribose) residues in the same cells (120,125,132). Finally, the intracellular levels of hydroxylamine-sensitive (ADP-ribose)-protein conjugates appeared to be correlated with cellular growth rate, while levels of hydroxylamine-resistant conjugates appeared to be correlated with hormonal stimulation and the degree of cellular differentiation (124,131,132).

While the studies described above have established mono(ADP-ribosylation) as a potentially important post-translational modification of proteins, they have so far provided no information concerning either the identity of the proteins modified *in vivo* or the physiological role of ADP-ribosylation reactions. 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 goal of the present study was to detect, quantify, and (ultimately) identify the target proteins modified *in vivo* by mono(ADP-ribosylation). Since the purified cellular transferases studied thus far are specific for arginine (or guanidino-containing analogs) as the ADP-ribose acceptor, we have focused our attention on the detection of proteins modified *in vivo* by N-glycosylic linkages to arginine.

CHAPTER II

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, morpholinopropanesulfonic acid (MOPS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, tris(hydroxymethyl)-aminomethane (Tris base), Coomassie Brilliant Blue G-250, bovine serum albumin (Fraction V), paraldehyde, Sephadex G-25 (medium and superfine), 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), [2,8-^{3}H]adenosine triphosphate (28 Ci/mmol) and [α-^{32}P]adenosine triphosphate (2,000-3,000 Ci/mmol) were purchased from ICN Chemical and Radioisotope Division (Irvine, CA). Bio-Rex 70 (200-400 mesh) and polypropylene Econo-Columns (0.8 cm I.D.) were obtained from Bio-Rad Laboratories (Richmond, CA). Aldrich Chemical Co. (Milwaukee, WI) was the source for cyanogen bromide, 6-aminohexanoic acid and *m*-aminophenylboronic acid hemisulfate. Snake venom phosphodiesterase and calf thymus DNA were from

Worthington Biochemical Corporation (Freehold, NJ). NAD-pyrophosphorylase (pig liver) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and Sepharose 4B was obtained from Pharmacia (Piscataway, NJ). MPS-1 ultrafiltration cells and YMT membranes were provided by Amicon Corporation (Danvers, MA), and Aquasol was obtained from New England Nuclear (Boston, MA). For high performance liquid chromatography, Partisil-10 SAX anion exchange columns (250 mm x 4.6 mm I.D. x 1/4 in O.D.) and Partisil-10 SAX anion exchange resin were purchased from Whatman Chemical Separation (Clifton, NJ), and methanol (HPLC grade) was from Fisher Scientific (Dallas, TX). The Bio-Sil TSK guard column (75 x 7.5 mm, I.D.) and analytical columns (300 x 7.5 mm, I.D.) were from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were reagent grade or the highest purity commercially available.

Diphtheria toxin and eukaryotic elongation factor 2 were kindly provided by Dr. D. Michael Gill (Tufts University). [^{14}C]ADP-ribosylated histone and purified mono(ADP-ribosyl)-transferase A from turkey erythrocytes were the very generous gifts of Dr. Joel Moss (NIH). [^{32}P]ADP-ribosylated transducin was a gift from Dr. Richard Kahn and Dr. Alfred Gilman (UTHSC, Dallas). Purified [adenine-2,8-^{3}H]poly(ADP-ribose) which had been incubated with snake venom phosphodiesterase (phosphatase-free) was provided by Rafael Alvarez-Gonzalez.

CHAPTER III

METHODS

Preparation of Special Solutions

For the studies described here, the following two solutions were prepared fresh as described, and the pH of all solutions used in these investigations were adjusted to the indicated values at 25°C unless specified otherwise. Buffer A contained 6 M guanidinium chloride, 50 mM MOPS and 10 mM EDTA, pH 4.0 (5°C). Buffer B contained 6 M guanidinium chloride, 100 mM NH_4OAc and 10 mM EDTA, pH 9.4 (5°C). Application and elution buffers for dihydroxyboronyl affinity chromatography (especially alkaline solutions containing either NH_4^+ or HCO_3^-) were also prepared immediately before use. Buffers A and B, as well as all other concentrated stock solutions (e.g., 2.5 M NH_4OAc, etc.), were filtered after preparation using Whatman #41 paper. Additionally, all solutions used for high performance liquid chromatography were filtered using 0.45 μm membranes, followed by de-aeration under vacuum.

Synthesis of Dihydroxyboronyl Bio-Rex 70
(DHB Bio-Rex)

DHB Bio-Rex was synthesized by a modification of the procedure described by Wíelckens, et al. (125). Twenty-five g of Bio-Rex 70 (200-400 mesh, sodium form) were suspended in 100 ml of 0.25 M NH_4OAc, pH 5.0, and the pH was adjusted to 5.0 by addition of conc. HOAc. The resin was filtered using a Buchner funnel (coarse frit), washed with 1 l of deionized water and resuspended in 100 ml of deionized water. The pH was adjusted to 5.0 with 6 N HCl, if necessary. Two and one-half g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the suspension and stirred for 15 min. A solution of 2.5 g of m-aminophenylboronic acid in 15 ml of deionized water, adjusted to pH 5.0, was then added to the suspension and stirred for 18 hr in the dark at room temperature. The pH was maintained at 5.0 by periodic adjustment with HCl or NH_4OH as necessary. The resin was washed with 1 l of each of the following solutions (in order): deionized water; 0.1 M NH_4OAc, pH 4.5, containing 1.0 M NH_4Cl; 0.1 M NH_4HCO_3, pH 9.0, containing 1.0 M NH_4Cl; and again with deionized water. Finally, the resin was washed with 100 ml of 0.5 M MOPS buffer, pH 6.0, containing 6 M guanidinium chloride and stored in the dark at 4°C as a 1:1 suspension (packed volume of resin:volume of buffer) in the same buffer (usually about 50 ml each).

Synthesis of Dihydroxyboronyl Sepharose 4B (DHB Sepharose)

DHB Sepharose was synthesized by a modification of the procedure described by Sims, et al. (134). One hundred ml of packed Sepharose 4B were washed five times with 400 ml portions of 0.1 M NaCl in a Buchner funnel (coarse frit), with drying between washes. The gel was washed with 1 l of deionized water, resuspended in 100 ml of deionized water and chilled in an ice bath. Twenty-five g of finely divided cyanogen bromide suspended in 50 ml of deionized water were added to the gently stirred suspension, and the pH was immediately raised to (and maintained at) 11 ± 0.2 by the dropwise addition of 2 M NaOH. After 30 min, the suspension was removed from the ice bath and the temperature was allowed to slowly increase to 15-20°C, at which it was maintained by addition of ice chips. When the pH was stable for 5 min (usually about 1 hr after removal from the ice bath), 50 g of crushed ice were added and the gel was rapidly filtered and washed with 800 ml of cold 0.1 M $NaHCO_3$, pH 9.0. The gel was then resuspended in 100 ml of cold 0.1 M $NaHCO_3$, pH 9.0, containing 10 g of 6-aminohexanoic acid and stirred at 4°C for 18 hr. The pH was maintained at 9.0 by periodic addition of HCl or NaOH as necessary. The gel was filtered, washed with 5 l of deionized water and resuspended in 400 ml of deionized water. The pH was adjusted to 5.0 with NaOH, and 1 g of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride was added to the suspension and stirred for 15 min. A solution containing 0.8 g of m-aminophenylboronic acid hemisulfate in 15 ml of deionized water, adjusted to pH 5.0, was then added to the suspension and stirred for 18 hr in the dark at room temperature. The pH was maintained at 5.0 by periodic adjustment with HCl or NaOH as necessary. The gel was filtered and washed with 1 l of 0.5 M NaCl followed by 10 l of deionized water, then stored in the dark at 4°C as a 1:1 suspension (packed volume of resin:volume of water) in deionized water.

Equilibration of Dihydroxyboronyl Bio-Rex For Affinity Chromatography

DHB Bio-Rex was washed and equilibrated immediately before use. For column chromatography, 1-ml aliquots of the stock suspension of DHB Bio-Rex (equivalent to 0.5 ml packed bed volume) were added to Econo-columns and equilibrated by washing sequentially with 10 ml of each of the following solutions: (i) application buffer (unless specified otherwise, 0.25 M NH_4OAc, pH 9.0, 25°C); (ii) deionized water; and (iii) application buffer. Alternatively, for batch adsorption chromatography, the required amount of resin (1 ml of stock suspension per sample) was sedimented (1,500 x g, 5 min) in a conical polypropylene centrifuge tube and the liquid was decanted. The resin was resuspended in 2 volumes of cold Buffer B, and the pH was adjusted to 9.4 (5°C) with concentrated NH_4OH. The resin was sedimented, the liquid was decanted, and the resin was washed 3 times with 10 volumes of cold deionized water and then resuspended in 8 volumes of cold Buffer B.

Equilibration of Dihydroxyboronyl Sepharose For Affinity Chromatography

DHB Sepharose was washed and equilibrated immediately before use. For column chromatograpy, 1-ml aliquots of the stock suspension of DHB Sepharose (equivalent to 0.5 ml packed bed volume) were added to Econo-columns and equilibrated by washing sequentially with 5 ml of each of the following solutions: (i) application buffer (unless specified otherwise, 0.25 M NH_4Cl, pH 9.0, 25°C); (ii) elution buffer (10 mM H_3PO_4, 25 mM KCl, pH 2.3, 25°C; and (iii) application buffer.

Determination of Binding Capacity of Dihydroxyboronyl Bio-Rex for NAD^+

Five DHB Bio-Rex columns were prepared as described above. Immediately prior to application, a 10 mM solution of NAD^+ in application buffer was prepared and diluted with the same buffer to each of the following concentrations (6 ml each): 0.2, 0.5, 1, 2 and 5 mM. Identical aliquots (1-5 μl) of radiolabelled NAD^+ (approx. 0.02 μCi of either [^3H], [^{14}C]

or [^{32}P]) were added, and 5 ml of each solution (containing 1, 2.5, 5, 10 and 25 µmol of NAD$^+$, respectively) were applied to columns of DHB Bio-Rex. After washing with 10 ml of application buffer, the bound NAD$^+$ was eluted with 5 ml of deionized water and quantified by liquid scintillation counting. The binding capacity was defined as the maximum amount of NAD$^+$ which could be applied while maintaining a binding efficiency $\geq$90%.

Determination of Binding Capacity of Dihydroxyboronyl Sepharose for NAD$^+$

Five DHB Sepharose columns were prepared as described above. Immediately prior to application, a 1 mM solution of NAD$^+$ in application buffer was prepared and diluted with the same buffer to each of the following concentrations (6 ml each): 0.02, 0.05, 0.1, 0.2 and 0.5 mM. Identical aliquots (1-5 µl) of radiolabelled NAD$^+$ (approx. 0.02 µCi of either [^{3}H], [^{14}C] or [^{32}P]) were added, and 5 ml of each solution (containing 0.1, 0.25, 0.5, 1.0 and 2.5 µmol of NAD$^+$, respectively) were applied to columns of DHB Sepharose. After washing with 10 ml of application buffer, the bound NAD$^+$ was eluted with 5 ml of elution buffer and quantified by liquid scintillation counting. The binding capacity was defined as the maximum amount of NAD$^+$ which could be applied while maintaining a binding efficiency $\geq$90%.

Strong Anion Exchange High Performance Liquid Chromatography (SAX HPLC)

The chromatographic apparatus used for separation of nucleosides and nucleotides included a Beckman Model 330 liquid chromatograph, a Whatman Partisil-10 SAX column (250 mm x 4.6 mm I.D.) preceded by a guard column (50 mm x 1.5 mm I.D.) containing the same material, a Varian Fluorichrom fluorometer and an Instrumentation Specialities Company 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 cut-off 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 using one of two different solvent systems. For analysis of samples containing ATP, a "high salt" buffer system, containing 1 M KCl and 0.25 M KH_2PO_4, pH 4.7, was employed. For other samples, separations were effected using a "low salt" buffer system, containing 0.1 M KH_2PO_4, pH 4.7.

Gel Filtration High Performance Liquid Chromatography

The chromatographic apparatus used for separation of proteins was essentially the same as that described above for SAX HPLC, with the following exceptions. First, for some experiments, the injector was equipped with a 250-μl sample loop. Second, two different column configurations were employed: (i) two Bio-Sil TSK-125 columns (300 x 7.5 mm I.D.) connected in series; or, (ii) a Bio-Sil TSK-250 column connected in series to a Bio-Sil TSK-400 column (300 x 7.5 mm I.D., each). In both configurations, the analytical columns were preceded by a Bio-Sil TSK-125 guard column (75 x 7.5 mm, I.D.). Column equilibration and protein separations were accomplished at a flow rate of 1.0 ml/min using Buffer A.

Preparation of [adenine-8-^{14}C]Nicotinamide Adenine Dinucleotide ([^{14}C]NAD$^+$)

Radiolabelled NAD$^+$ was synthesized from [8-^{14}C]ATP using NAD$^+$ pyrophosphorylase (ATP:NMN adenylyltransferase, EC 2.7.7.1) and purified by affinity chromatography on DHB Sepharose. The procedure used for synthesis was similar to others described previously (135,136). The incubation volume was 2.79 ml containing 125 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$, 20 mM NMN$^+$, 0.2 mM [^{14}C]ATP (50 μCi) and 0.01 units/ml of NAD$^+$ pyrophosphorylase (0.2 mg lyophilized powder/ml, equivalent to 0.06 mg enzyme/ml). The mixture was incubated at 37°C for 15 min, then ethanol was added to a final concentration of 28% (v/v) and the incubation was continued for 2 hr. The mixture was placed on ice and diluted to 30 ml with 0.25 M NH_4OAc, pH 8.8. This solution was then applied to a 3-ml DHB Sepharose column (0.7 x 8 cm) in three portions of 10 ml; the column was washed with 10 ml of 0.25 M NH_4OAc, pH 8.8, after each of the first two applications and with 30 ml of the same buffer after the final application. $[^{14}C]NAD^+$ was eluted with 5 ml of 10 mM H_3PO_4, neutralized with NaOH, and concentrated to dryness. The sample was dissolved in 3 ml of a 1:1 mixture of ethanol:sodium phosphate (20 mM, pH 7.0) and stored at -20°C. The overall recovery was 60% (30 μCi) and the purity was >95% as judged by HPLC analysis.

Preparation of [adenine-2,8-^{3}H]Nicotinamide Adenine Dinucleotide ($[^3H]NAD^+$)

Radiolabelled NAD^+ was prepared from [2,8-^{3}H]ATP by a procedure similar to that described above. The incubation volume was 0.72 ml containing 125 mM Tris-HCl, pH 7.6, 15 mM $MgCl_2$, 4 mM NMN^+, 40 μM $[^3H]ATP$ (1 mCi, added in 50% ethanol; final ethanol conc. = 28% v/v), and 0.01 units/ml of NAD^+ pyrophosphorylase (0.2 mg lyophilized powder/ml, equivalent to 0.06 mg enzyme/ml). The mixture was incubated at 37°C for 1 hr, then diluted to 5 ml with 0.25 M NH_4OAc, pH 8.8 (application buffer) and applied to a 0.5-ml DHB Sepharose column (equilibrated as described above). The column was washed with 10 ml of application buffer and 0.5 ml of 10 mM H_3PO_4. $[^3H]NAD^+$ was eluted with 2 ml of H_3PO_4, neutralized with NaOH, and concentrated to 1 ml. The sample was then diluted to 2 ml with ethanol and stored at -20°C. The overall recovery was 87% (0.87 mCi) and the purity was >95% as judged by HPLC analysis.

Preparation of [adenylate-^{32}P]Nicotinamide Adenine Dinucleotide ($[^{32}P]NAD^+$)

Radiolabelled NAD^+ was prepared from [α-^{32}P]ATP by a procedure similar to that described above. Radiolabelled ATP 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 of pyruvate kinase, 0.3 units/ml of NAD^+ pyrophosphorylase (6 mg lyophilized powder/ml, equivalent to 2 mg enzyme/ml) and, when added to the Corex tube (above), 5-7 μM [^{32}P]ATP (1 mCi). 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 0.5-ml DHB Bio-Rex column (equilibrated as described above). The column was washed with 5 ml of 1 M NH_4HCO_3, pH 9.0, followed by 10 ml of 0.25 M NH_4HCO_3 and 0.5 ml of deionized water. $[^{32}P]NAD^+$ was 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.

Preparation of Radiolabelled ADP-ribose

[8-^{14}C]ADP-ribose and [α-^{32}P]ADP-ribose were prepared from the corresponding radiolabelled 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 (equilibrated as described above). The column was washed with 10 ml of application buffer and 0.5 ml of 10 mM H_3PO_4, 25 mM KCl, pH 2.3 (elution buffer). Radiolabelled ADP-ribose was 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 1,N^6-etheno(ADP-ribose)

Authentic etheno(ADP-ribose) was prepared from etheno-(NAD$^+$) by alkaline incubation and affinity chromatography as described for preparation of radiolabelled ADP-ribose. This preparation was used to establish the exact elution position of etheno(ADP-ribose) following anion exchange HPLC.

Preparation of Radiolabelled $1,N^6$-etheno(ADP-ribose)

Etheno([8-^{14}C]ADP-ribose) and etheno([α-^{32}P]ADP-ribose) were synthesized from the corresponding radiolabelled ADP-ribose by reaction with chloroacetaldehyde and purified by affinity chromatography on DHB Sepharose. The procedure used for synthesis was similar to that reported previously for other adenine-containing compounds (134). The incubation volume was 5.0 ml containing 0.25 M NH_4OAc, pH 4.5, 0.1 M chloroacetaldehyde and 1-50 µM ADP-ribose (>0.05 µCi/ml [^{14}C]ADP-ribose alone, or 5 µCi/ml [^{32}P]ADP-ribose plus unlabelled ADP-ribose). The tube was capped tightly and incubated at 60°C for 4 hr, then cooled to room temperature; the pH of the solution was adjusted to 9.0 (25°C) with concentrated NH_4OH. Radiolabelled etheno(ADP-ribose) was isolated by affinity chromatography as described for preparation of radiolabelled ADP-ribose and stored at -20°C. The overall recovery was 85-88%.

Preparation of [-^{32}P]ADP-Ribosylated Elongation Factor 2 ([^{32}P]EF-2)

The procedure used for synthesis of [^{32}P]EF-2 was similar to that described by Gill (137). 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 EF-2 (provided by D. M. Gill and estimated to contain 1 pmol/µl) and 2 µl diphtheria toxin (also from D. M. Gill). The mixture was incubated at 37°C for 30 min, placed on ice, and 10 µl of bovine serum albumin (10 mg/ml) were added. Next, 25 µl of ice cold trichloroacetic acid (100% w/v) were 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 (12,000 x g for 5 min). The supernatant was carefully removed using a micro-aspirator. The pellet was washed twice with 500 µl of trichloroacetic acid (20%), three times with 500 µl of anhydrous diethylether, and dried under vacuum. The dried pellet was redissolved 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 EF-2).

Preparation of Sephadex G-25 (superfine) Desalting Columns

Thirty g of Sephadex G-25 (superfine) (enough for 20 columns) were hydrated in excess distilled water overnight and then filtered. For each column, 6.1 g of moist Sephadex were resuspended in 5 ml of Buffer A, and 10 ml of this suspension (equivalent to approx. 5.5 ml packed bed volume) were transferred to a polypropylene Econo-Column. Columns were centrifuged at 600 x g for 5 min, using 15-ml polypropylene centrifuge tubes to collect the effluents. To completely equilibrate the Sephadex, 2.5 ml of Buffer A were added, the columns were centrifuged as above, then another 2.5 ml of Buffer A were added and the columns were capped and stored at 5°C. Prior to sample application, the columns were centrifuged at 600 x g for 15 min.

Preparation of the Acid-Insoluble Fraction From Rat Liver

Adult rats (Sprague-Dawley, 6 mo old, male) were sacrificed, and the livers were quickly removed and frozen in liquid nitrogen. Samples which were not processed immediately were stored at -80°C until used. Frozen livers were quickly weighed and then placed in a large mortar containing liquid nitrogen. The frozen tissue was powdered using the mortar and pestle and then transferred to a 50-ml conical polypropylene centrifuge tube. Five volumes of cold 20% trichloroacetic acid were added and the tube was immersed in an ice bath. The tissue was homogenized using a Brinkman Polytron homogenizer at a setting of 6 for two 30-sec periods, interrupted by a 30-sec period to insure cooling. After centrifugation (1,500 x g, 10 min), the supernatant was decanted, the pellet was returned to the mortar containing liquid nitrogen and the trichloroacetic acid extraction and centrifugation were repeated. The pellet following the second trichloroacetic acid extraction was again powdered in liquid nitrogen, transferred to the centrifuge tube and extracted in 5 volumes of cold ethanol (100% absolute) using the Polytron homogenizer. Following centrifugation, the supernatant was decanted, and the pellet was resuspended in 5 volumes of cold ethanol by vortexing. After centrifugation (1,500 x g, 5 min), the supernatant was decanted and two extractions with anhydrous ether were performed as described for the final ethanol extraction. The residual ether was removed by vacuum, and the acid-insoluble fraction (a fine powder) was stored at -20°C until analyzed. The yield of dry powder for several different preparations was 158 ± 9 mg per g (wet wt.) of tissue (±S.D., n=4).

Determination of DNA Content in the Acid-Insoluble Fraction from Rat Liver

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 $HClO_4$ with 3 M $HClO_4$. After 15 min, samples were centrifuged (1,500 x 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 agitation as above at 70°C for 30 min. The samples were then incubated on ice for 15 min and centrifuged as above. The supernatants were carefully removed and adjusted to 1.5 M $HClO_4$ (with 3 M $HClO_4$) for analysis. DNA content was estimated by the method of Burton (138) as modified by Richards (139), except that the reagent contained 3% (w/v) diphenylamine and 0.01% (v/v) paraldehyde in glacial acetic acid, and the sample and reagent were combined in a ratio of 1:1. For use as the standard, calf thymus DNA (5 mg) was dissolved in KOH, precipitated and washed as described above for samples, then dissolved in 500 volumes of 0.5 M $HClO_4$ at 70°C and incubated on ice (as above). After adjusting to 1.5 M $HClO_4$, an aliquot of the supernatant was diluted with 1.5 M $HClO_4$ and the absorbance at 260 nm was determined. The "DNA" concentration of the standard was determined using the value $\varepsilon_{260}^{mg/ml} = 26.97$, which was calculated from the base content of calf thymus DNA. (This value is close to the value of 30 used by Levy et al. without documentation (140)). The acid-insoluble fraction from rat liver contained 4.01 ± 0.14 µg DNA per mg of dry powder (± S.D., n=4).

Determination of Protein Content in the Acid-Insoluble Fraction from Rat Liver

For protein analysis, samples of the acid-insoluble fraction (typically 25 mg) were dissolved in Buffer A as described below. Protein content was estimated by the method of Bradford (141), using bovine serum albumin (dissolved in Buffer A) as the standard. The $\varepsilon_{280}^{mg/ml}$ for bovine serum albumin in Buffer A was 0.642, which was very similar to the corresponding value of 0.660 in 0.15 M NaCl (142). The acid-insoluble fraction from rat liver contained 693 ± 79 µg protein per mg of dry powder (±S.D., n=12).

Dissolution of the Acid-Insoluble Fraction From Rat Liver

The desired amount of "tissue powder" (up to 375 mg) was weighed in a 15-ml polypropylene centrifuge tube, and 40 volumes of cold Buffer A were then added. Samples were dissolved using a Buchler Evapo-mix gyrorotary shaker at 0-5°C. Effective dissolution was also achieved by vigorously stirring the sample in the polypropylene tube, using a triangular stir bar designed for use in conical tubes. A Dounce homogenizer and the "B" (loose-fitting) pestle were also used to facilitate dissolution of some samples, but adherence to glass surfaces resulted in some sample loss with this technique. Following dissolution, the pH of the resulting extract (usually about 3.5) was adjusted to 4.0 with 5-10 µl of 1 N NH_4OH.

Separation of Proteins from Free Nucleotides in the Acid-Insoluble Fraction from Rat Liver

Gel filtration HPLC. After dissolving samples of the acid-insoluble fraction in Buffer A (above), aliquots (250 µl) were subjected to gel filtration HPLC as described earlier, using two Bio-Sil TSK-125 columns connected in series.

Sephadex G-25 Column Chromatography. After dissolving samples of the acid-insoluble fraction in Buffer A (above), aliquots (500 µl) were applied to a column of Sephadex G-25 (medium; 45 x 0.9 cm I.D.) equilibrated in Buffer A at room temperature. Fractions eluting between 9 and 14 ml were pooled as the "protein fraction." Fractions eluting between 14 and 23 ml were pooled as the "nucleotide fraction."

Dialysis. After dissolving samples of the acid-insoluble fraction in Buffer A (above), aliquots (1 ml) were dialyzed (tubing = 12-14,000 MW cut-off) at 5°C for 24 hr against three changes of Buffer A (20 volumes each).

Precipitation from concentrated formic acid. The dissolution of trichloroacetic acid-insoluble material in formic acid followed by re-precipitation has been described previously for removal of nucleotides trapped in trichloroacetic acid pellets after protein kinase assays

(143). The acid-insoluble fraction from rat liver was dissolved in 20 volumes of cold formic acid (90-98%), then diluted 10-fold with cold deionized water. Cold trichloroacetic acid (100% w/v) was immediately added to a final concentration of 20%. The sample was placed on ice for 15 min, then centrifuged (1,500 x g, 10 min). The pellet was washed twice with 100 volumes of anhydrous diethylether, and residual ether was removed by vacuum. The resulting powder was then dissolved in Buffer A as described above.

Sephadex G-25 (superfine) column centrifugation. For routine analyses, a modification of the column centrifugation method (144) for removing low molecular weight compounds from solutions of macromolecules was adopted. The acid-insoluble fraction from rat liver was dissolved in Buffer A as described above. For each sample to be analyzed, a 500-µl aliquot of the dissolved sample was applied to a 5.5-ml column of Sephadex G-25 (superfine) and centrifuged at 600 x g for 15 min, collecting the effluent in a 15-ml polypropylene centrifuge tube. To achieve quantitative recovery of proteins, an additional 250-µl aliquot of Buffer A was applied to each column and the columns were centrifuged as above, collecting the effluents in the same tubes (designated "Protein Fraction"). Low molecular weight compounds were eluted by application of 2.5 ml of Buffer A to each column and centrifugation as above (designated "Nucleotide Fraction").

Assay for Mono(ADP-ribose) Residues in the Acid-Insoluble Fracton from Rat Liver

Chemical release of protein-bound ADP-ribose. Neutral hydroxylamine (6 M NH_2OH, 6 M NH_4Cl, 50 mM MOPS, 10 mM EDTA, pH 7.0) was prepared fresh by dissolving hydroxylamine hydrochloride in an equimolar amount of concentrated NH_4OH and adding 0.1 volume of concentrated MOPS/EDTA buffer (0.5 M MOPS, 0.1 M EDTA, pH 7.0). The samples were adjusted to pH 7.0 by addition of NH_4OH, and 0.2 volume of neutral hydroxylamine was added to aliquots (typically 500 µl) of each sample. For samples incubated in the absence of hydroxylamine, 0.2 volume of a solution containing 6 M NH_4Cl, 50 mM MOPS, 10 mM EDTA, pH 7.0 (neutral buffer) was added. Samples were capped tightly and incubated for 12 hr at 37°C.

Isolation of ADP-ribose by batch adsorption affinity chromatography. After incubation, samples were placed on ice and the pH was adjusted to 9.4 (5°C) by addition of concentrated NH_4OH. Next, 4.5 ml of the suspension containing freshly washed DHB Bio-Rex (equilibrated as described above, and equivalent to 0.5 ml of packed resin) were added to each sample. Samples were vigorously agitated in a Buchler Evapo-mix shaker for 30 min at 0-5°C. The resin was sedimented (1,500 x g, 5 min) and washed once with 5 ml (i.e., 10 volumes) of cold Buffer B and twice with 5 ml of a cold solution of 1 M ammonium acetate pH 9.4 (5°C). Following the last wash, 1 ml of 1 M HCl was added to the resin and the suspension was warmed to room temperature. The suspension was then transferred to an Econo-Column, and the eluate was collected in a 15-ml polypropylene centrifuge tube. An additional 4 ml of deionized water were applied to each column and the eluates collected. To each eluate (5 ml), ammonium acetate (2.5 M, pH 4.5) was added to a final concentration of 0.25 M.

Formation of 1,N^6-etheno(ADP-ribose). Chloroacetaldehyde (7 M) was added to each sample to a final concentration of 0.1 M, and the samples were capped tightly and incubated for 4 hr at 60°C. Samples were cooled to room temperature and prepared for HPLC analysis by affinity chromatography on DHB Sepharose columns, as follows. Concentrated NH_4OH was used to adjust the pH of the samples to 9.0 (25°C), and samples were applied to freshly prepared DHB Sepharose columns (equilibrated as described above). Columns were then washed with 10 ml of 0.25 M NH_4Cl, pH 9.0, and 0.5 ml of elution buffer (10 mM H_3PO_4, 25 mM KCl, pH 2.3). ADP-ribose was eluted with 2 ml of elution buffer (the pH of the eluate was 4.5-5.0) and stored at -20°C until analyzed.

Analysis by anion exchange HPLC. The chromatographic apparatus used for analytical separation and quantification of etheno(ADP-ribose) was the same as that described above for SAX HPLC. Analyses were accomplished isocratically at a flow rate of 1.0 ml/min using 0.1 M KH_2PO_4 buffer, pH 4.7.

CHAPTER IV

RESULTS

Affinity Chromatography Using Dihydroxyboronyl Resins

The use of the modified protocols described for synthesis of dihydroxyboronyl affinity resins resulted in a significant increase in the nucleotide binding capacity of the resins, while maintaining the previously described selectivity (134,145). The binding and elution characteristics were also more consistent from one preparation of resin to another when the modified protocols were employed for synthesis. The NAD^+ binding capacity of each preparation of affinity resin was routinely determined to verify that there were no significant differences between preparations, thereby eliminating such differences as a source of experimental variability. Typical results from the determinations of binding capacities are shown in Figs. 4 and 5, and the values obtained for several preparations were 6.05 ± 0.58 μmol NAD^+ per 0.5 ml of DHB Bio-Rex ($\bar{x}$ ± s.d., n=3) and 0.77 ± 0.18 μmol NAD^+ per 0.5 ml of DHB Sepharose ($\bar{x}$ ± s.d., n=3).

Conditions for the efficient binding and elution of ADP-ribose have been described previously for the boronate matrices prepared according to the originally published

Figure 4. Determination of binding capacity of dihydroxyboronyl Bio-Rex for NAD^+. Different amounts of radiolabelled NAD^+ (1-25 μmol) were applied to columns of DHB Bio-Rex and the amount bound to (and eluted from) each column (▲) was determined as described in "METHODS." The percent of applied NAD^+ that bound to (and eluted from) each column (●) was then calculated.

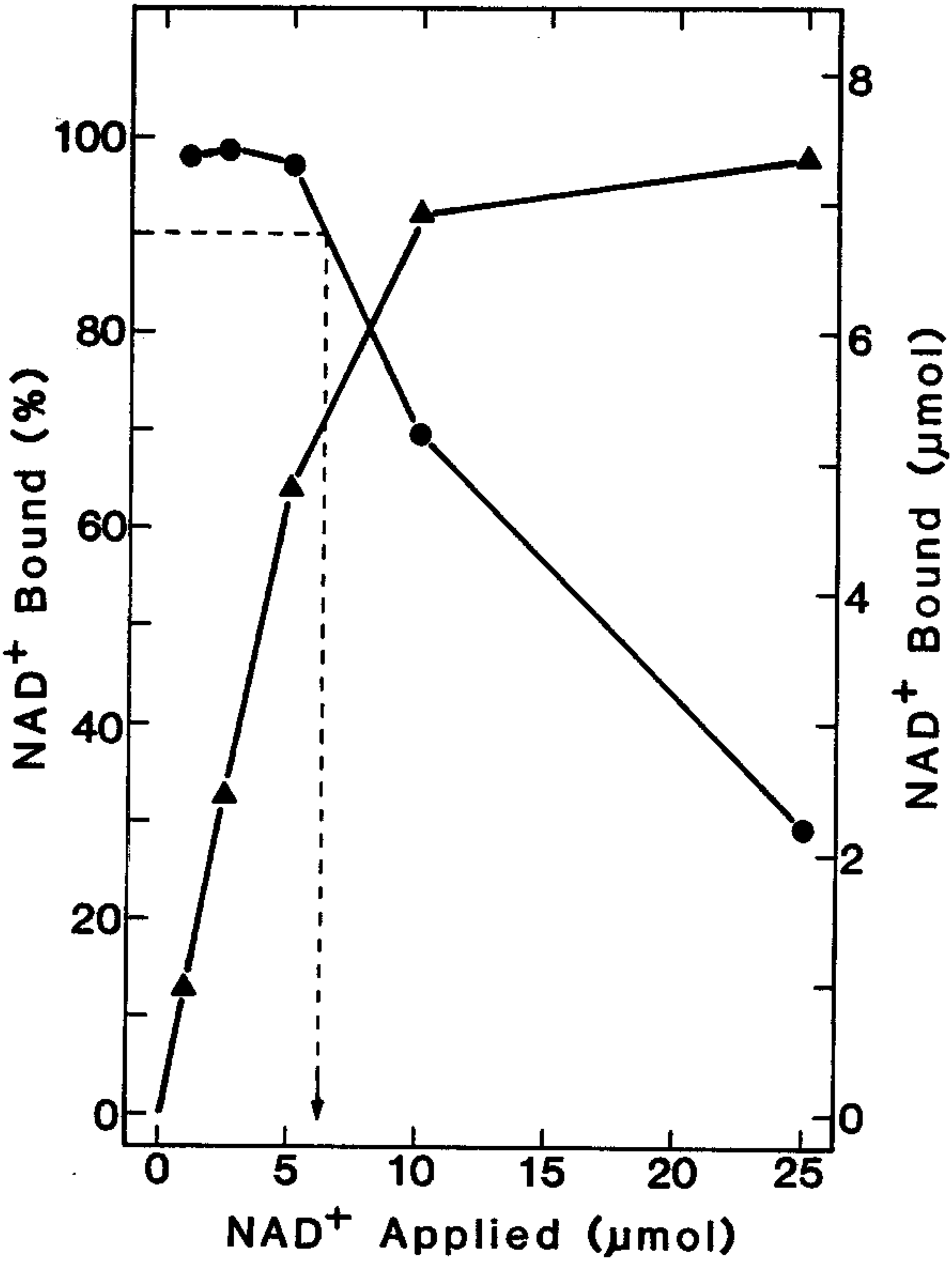
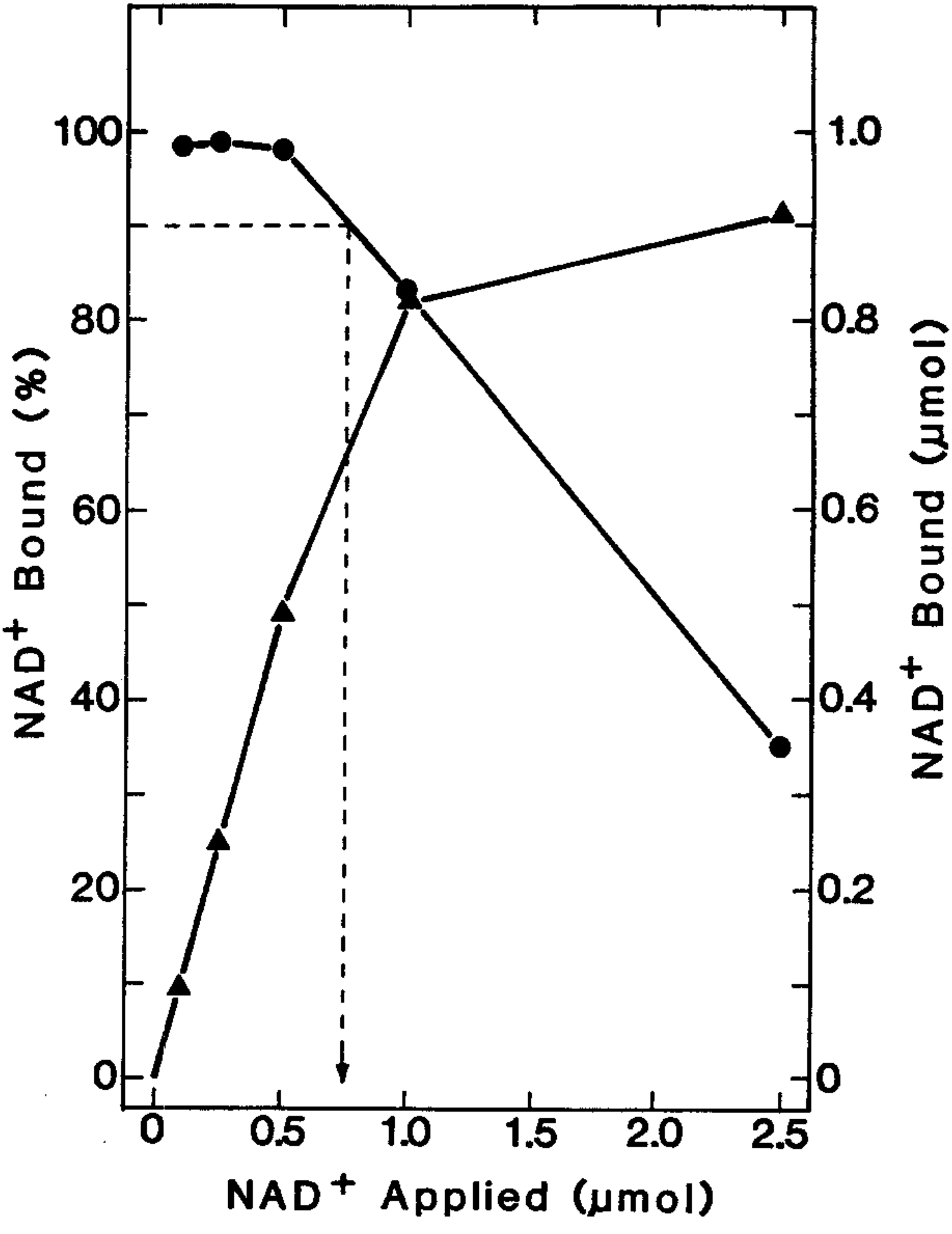

NAD$^+$ Bound (%)
100
80
60
40
20
0
NAD$^+$ Bound (μmol)
8
6
4
2
0
0
5
10
15
20
25
NAD$^+$ Applied (μmol)

Figure 5. Determination of binding capacity of dihydroxyboronyl Sepharose for NAD$^+$. Different amounts of radiolabelled NAD$^+$ (0.1-2.5 μmol) were applied to columns of DHB Sepharose and the amount bound to (and eluted from) each column (▲) was determined as described in "METHODS." The percent of applied NAD$^+$ that bound to (and eluted from) each column (●) was then calculated.


NAD⁺ Bound (%)
NAD⁺ Bound (µmol)
NAD⁺ Applied (µmol)
0
20
40
60
80
100
0
0.2
0.4
0.6
0.8
1.0
0
0.5
1.0
1.5
2.0
2.5

protocols (145). However, neither quantitative binding nor complete elution of ADP-ribose were obtained when those conditions were applied to the resins used in this study. Quantitative binding of ADP-ribose was effected by increasing the pH of the application buffer to 9.0 (25°C) (or 9.4, 5°C). Replacement of Na^+ with NH_4^+ in the application buffers and, for DHB Sepharose, addition of 25 mM KCl to the elution buffer permitted complete elution of ADP-ribose from the boronate resins.

An additional difficulty was encountered with the resins used in this study. 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 for analysis of tissue samples. DHB Bio-Rex was chosen for this procedure on the basis of its higher binding capacity and greater mechanical stability.

Separation of ADP-ribose and Related Compounds by SAX HPLC

A variety of HPLC systems have been described for the separation of nucleotides and nucleosides. These systems have been based on either strong anion exchange (146-148) or paired-ion reversed-phase chromatography (149,150). Using these previous studies (149-151) as a guide, two different paired-ion reversed-phase HPLC systems were developed for the isocratic separation of adenine-containing nucleotides. One solvent system permitted the analysis of samples containing ATP, while the second system was optimized for separation of ADP-ribose from related compounds (Fig. 6). However, the tetrabutylammonium hydroxide employed as the ion-pairing reagent produced an irreversible and continuous (almost daily) decrease in column resolution. Concomitant with the changes in resolution, pure nucleotides exhibited anomolous chromatographic behavior, eluting as asymetrical or even "doublet" peaks (Fig. 7). Thus, while initial portions of this study were conducted using paired-ion chromatography, strong anion exchange HPLC was employed for all subsequent investigations, including those described here.

Two isocratic solvent systems were developed for SAX HPLC with the same basic resolution characteristics as those developed for paired-ion chromatography. That is, a "high salt" buffer system permitted the elution of ATP within a reasonable amount of time, while a "low salt" buffer system was optimized for resolution of ADP-ribose and etheno(ADP-ribose) from related compounds (Figs. 8-11). The "high salt" solvent system was routinely used to verify the purity of commercial preparations of radiolabelled ATP, as well as to establish that no radiolabelled ATP was present after synthesis and purification of radiolabelled NAD^+. With the "low salt" buffer system, etheno(ADP-ribose) was separated from

Figure 6. Separation of ADP-ribose and related compounds by paired-ion reversed phase HPLC. The chromatographic apparatus was the same as that described for SAX HPLC in "METHODS." The column was an Altex Ultrasphere-I.P. reversed-phase column (250 x 4.6 mm I.D.), the solvent was 10 mM NaH_2PO_4, pH 7.0, containing 3 mM tetrabutylammonium hydroxide and 15 % (v/v) methanol. Separations were accomplished isocratically at 1.0 ml/min and 2,000 psig. Numbered peaks in panel A are (1) NMN^+, (2) NAD^+, (3) adenosine, (4) AMP and (5) ADP-ribose. In panel B, numbered peaks are (1) etheno(NAD^+), (2) etheno(AMP) and (3) etheno(ADP-ribose).

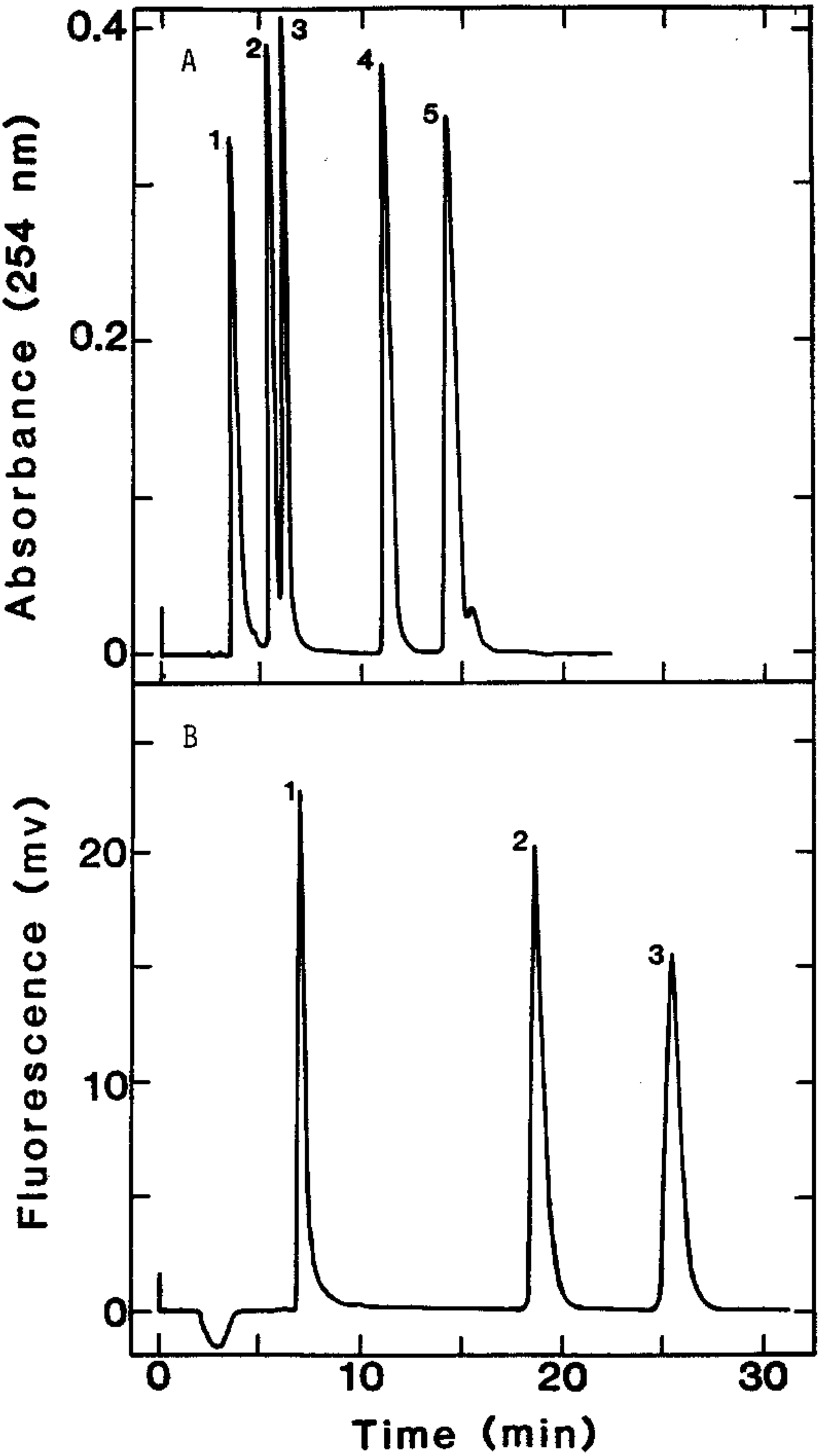

A
B
1
2
3
4
5
1
2
3
Absorbance (254 nm)
0.4
0.2
0
Fluorescence (mv)
20
10
0
0
10
20
30
Time (min)

Figure 7. Loss of resolution due to deterioration of the reversed-phase column caused by the ion-pairing reagent. Nucleotides were analyzed using (A) the system described in the legend to Fig. 6 or (B) the same system containing 25% (v/v) methanol. Numbered peaks are (1) NMN^+, (2) NAD^+, (3) AMP, (4) ADP-ribose, (5) ADP and (6) ATP.

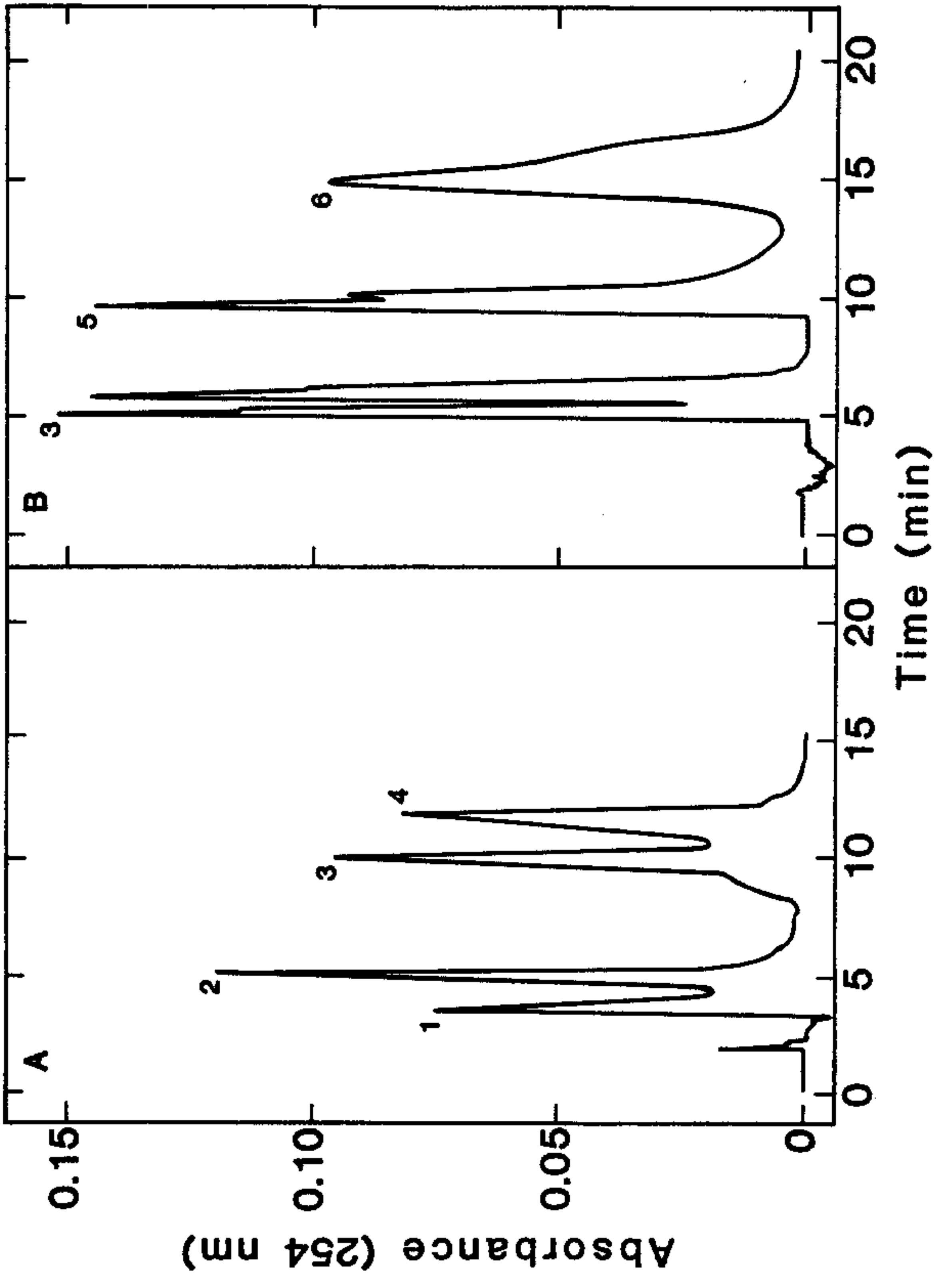

A
B
1
2
3
4
3
5
6
Absorbance (254 nm)
0
0.05
0.10
0.15
Time (min)
0
5
10
15
20

Figure 8. Separation of adenine nucleotides by anion exchange HPLC using the "high salt" buffer system. Compounds were separated as described in "METHODS" at a pressure of 200 psig. Numbered peaks are (1) NAD^+, (2) AMP, (3) ADP-ribose, (4) ADP, (5) ATP, (6) [2,8-^{3}H](phosphoribosyl)AMP and (7) [2,8-^{3}H](diphosphoribosyl)AMP. (The identity of peak 7 was later confirmed by injecting a larger amount of radioactivity). Prior to injection of the phosphodiesterase-treated [^{3}H]poly(ADP-ribose), the enzyme was separated from the nucleotides by centrifugation according to operating instructions provided with the Amicon MPS-1 ultrafiltration cell and YMT membranes.

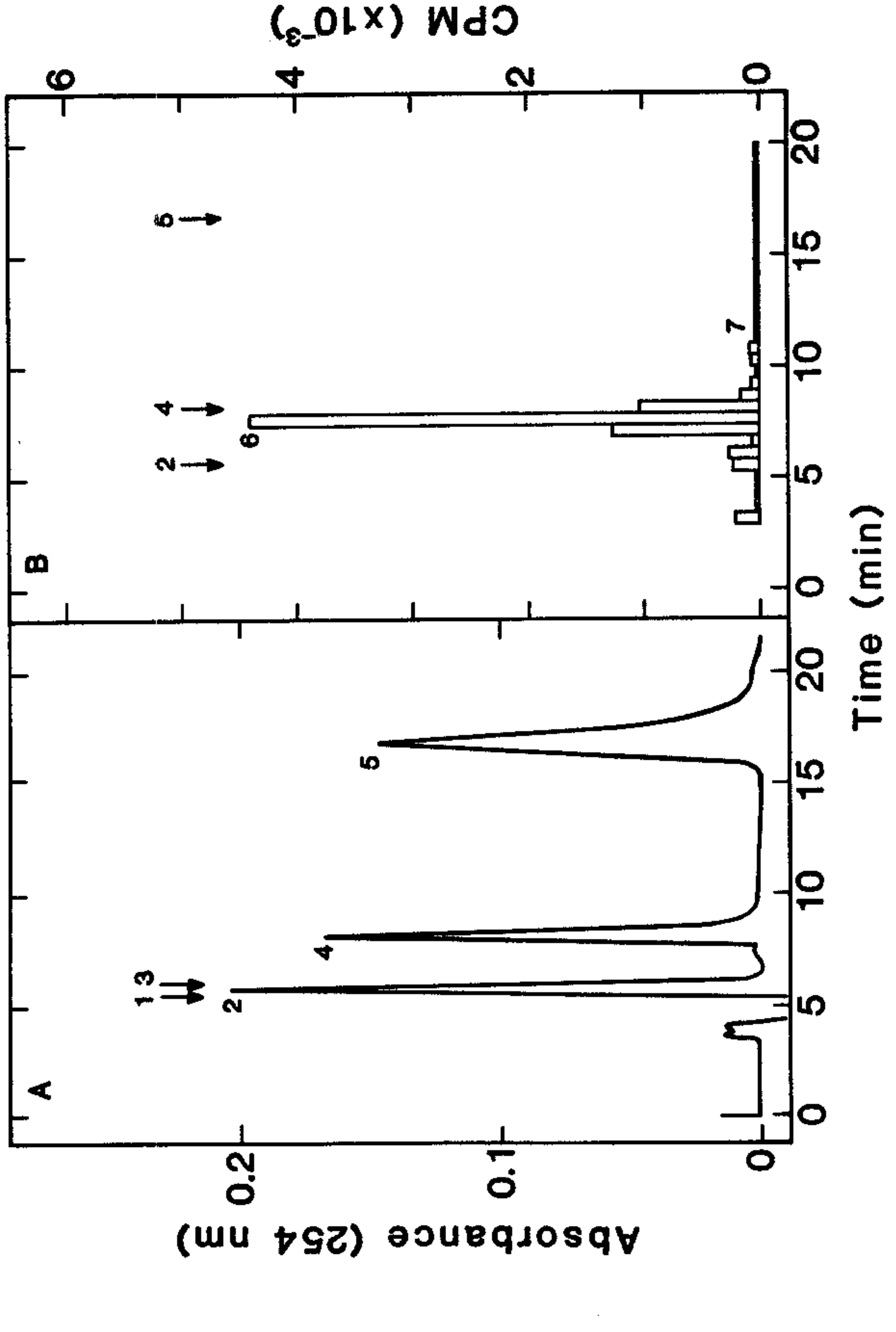

A
B
Absorbance (254 nm)
0.2
0.1
0
CPM (x10^{-3})
6
4
2
0
Time (min)
0
5
10
15
20
1 3
2
4
5
6
7

Figure 9. Separation of adenine nucleotides by anion exchange HPLC using the "low salt" buffer system. Compounds were separated as described in "METHODS" at a pressure of 200 psig. Numbered peaks are (1) NAD^+, (2) AMP, (3) ADP-ribose, (4) NADH, (5) $[2,8-^3H]$(phosphoribosyl)AMP and (6) ADP. The arrow at 5.0 min indicates a 10-fold increase in sensitivity of the absorbance monitor, and numbers on the left ordinate represent absorbance after the change in sensitivity. See legend to Fig. 8 for details of the preparation of the $[^3H]$(phosphoribosyl)AMP prior to injection.

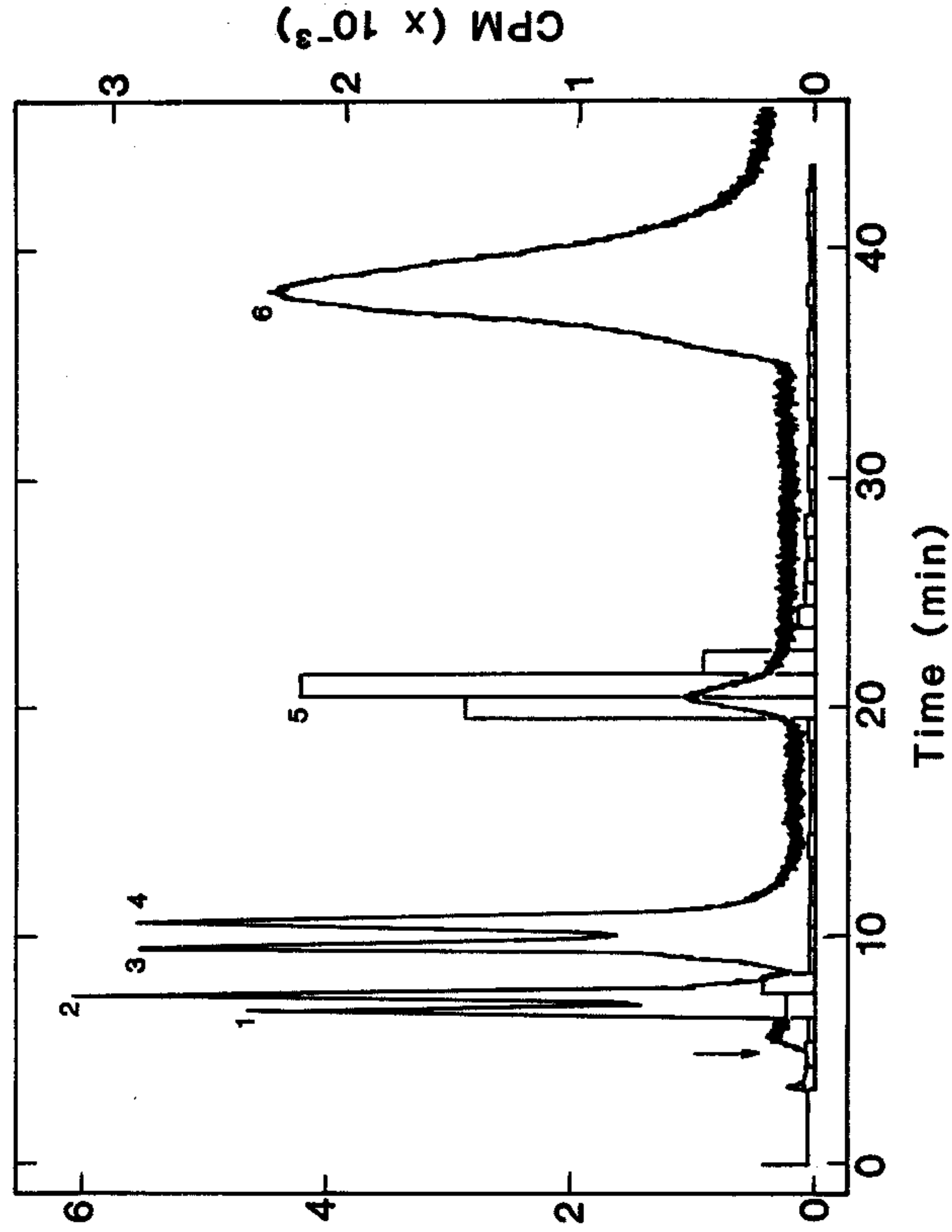

Absorbance (x 10³, 254 nm)
CPM (x 10⁻³)
Time (min)
0
2
4
6
0
1
2
3
0
10
20
30
40
1
2
3
4
5
6

Figure 10. Separation of adenine nucleotides and inorganic phosphate by anion exchange HPLC using the "low salt" buffer system. Compounds were separated as described in "METHODS." Numbered peaks are (1) NAD^+, (2) AMP, (3) ADP-ribose and (4) [^{32}P] inorganic phosphate. The arrow at 5.5 min indicates a 10-fold increase in sensitivity of the absorbance monitor as described in the legend to Fig. 9.

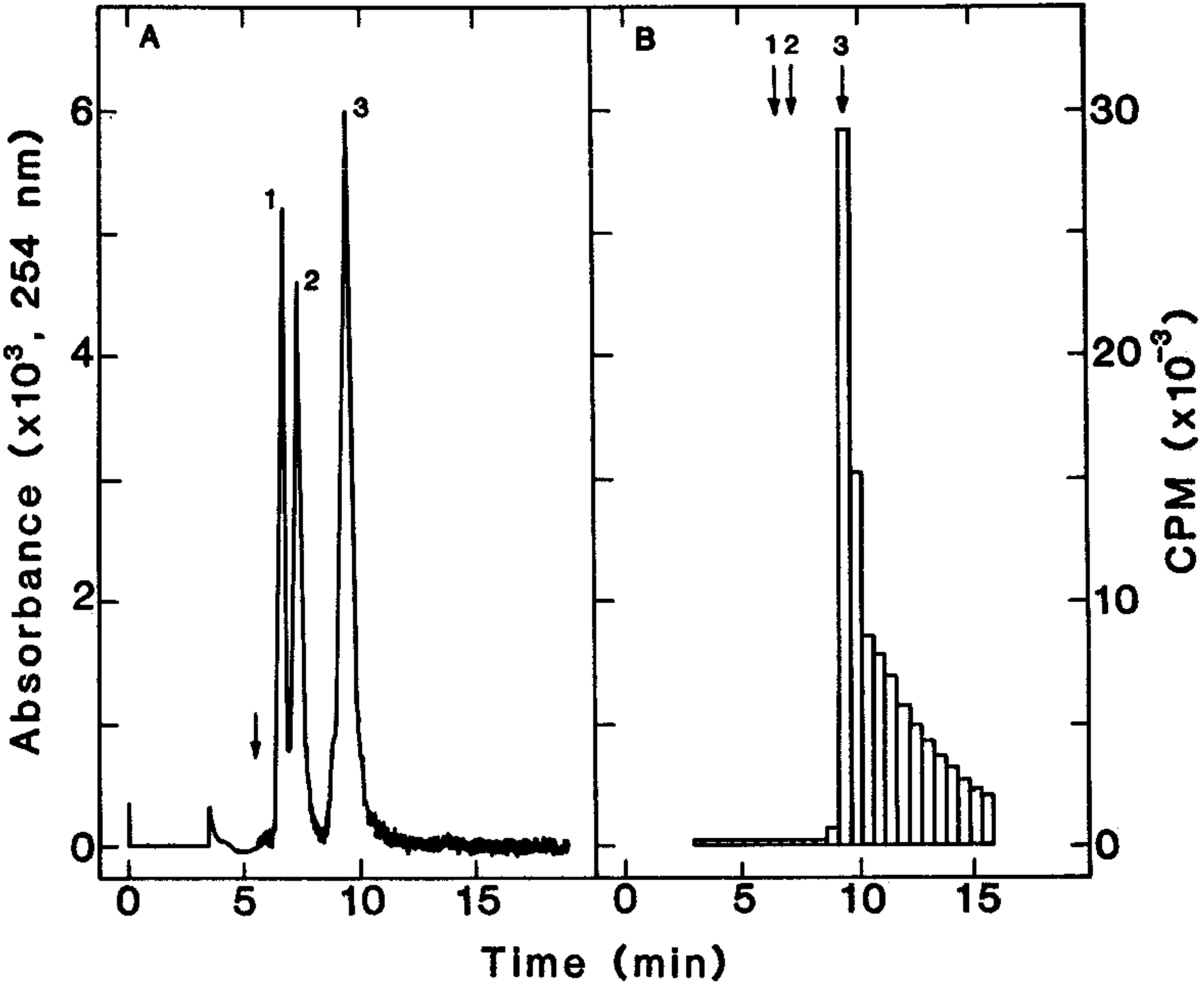
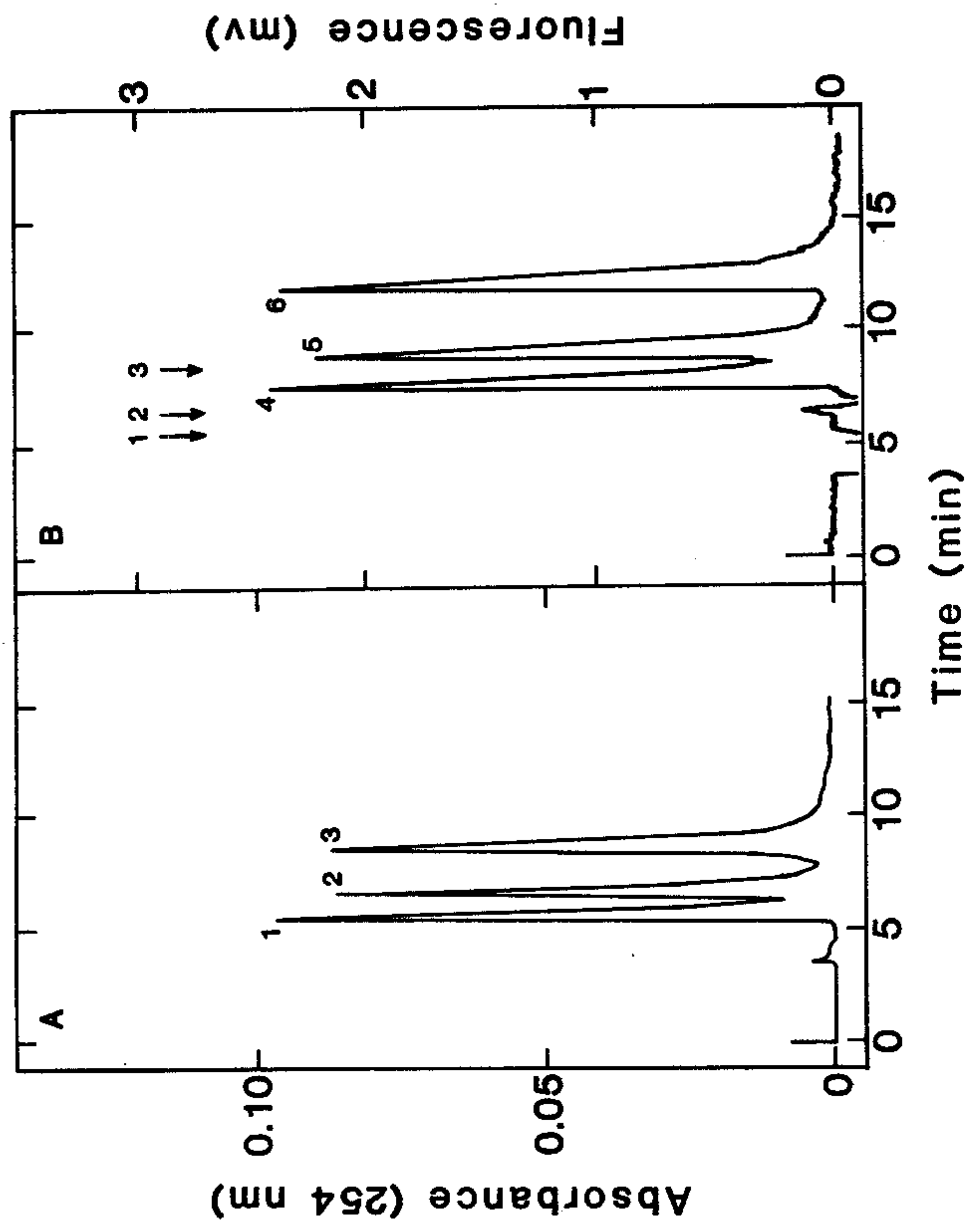

A
B
1
2
3
1 2
3
Absorbance (x10³, 254 nm)
0
2
4
6
CPM (x10⁻³)
0
10
20
30
0
5
10
15
0
5
10
15
Time (min)

Figure 11. Separation of adenine and ethenoadenine nucleotides by anion exchange HPLC. Compounds were separated as described in "METHODS," using the "low salt" buffer system. Numbered peaks are (1) NAD^+, (2) AMP, (3) ADP-ribose, (4) etheno(NAD^+), (5) etheno(AMP) and (6) etheno(ADP-ribose.


A
B
Absorbance (254 nm)
0
0.05
0.10
Fluorescence (mv)
0
1
2
3
Time (min)
0
5
10
15
1
2
3
4
5
6
1 2 3

other nucleotides in less than 15 min. Retention times for etheno(ADP-ribose) and all the related compounds examined are summarized in Table III. In addition to providing optimum resolution and a relatively short analysis time (<20min) for etheno(ADP-ribose), the "low salt" buffer also exhibited a relatively low degree of fluorescence quenching (19%) (Fig. 12). Fluorescence of ethenoadenine-containing compounds was quenched dramatically (49%) in the "high salt" buffer.

Quantitative Formation and Detection Of Etheno(ADP-ribose)

Optimal conditions for the formation of a fluorescent derivative from ADP-ribose were identical to those for formation of ethenoadenosine and similar to conditions reported previously for the formation of etheno derivatives of other adenine-containing compounds (134). Maximum fluorescence was observed after incubation of either adenosine or ADP-ribose with 0.1 M chloroacetaldehyde for 4 hr at 60°C and pH 4.5 (Fig. 13). Analysis by UV spectroscopy verified that an ethenoadenine derivative was present following incubation of [^{32}P]ADP-ribose with chloroacetaldehyde (Fig. 14). Anion exchange HPLC analysis established that etheno(ADP-ribose) was the fluorescent product of the reaction between [^{14}C]ADP-ribose (or [^{32}P]ADP-ribose) and chloroacetaldehyde. The results depicted in Fig. 15 confirmed that ADP-ribose was stable during derivatization and that it was quantitatively

TABLE III

Retention Times For Adenine-Containing Nucleotides And Inorganic Phosphate

Compound	Retention Time (min)	
	"Low Salt" Solvent System[a]	"High Salt" Solvent System[b]
NAD^+	6.74	5.60
AMP	7.40	5.87
etheno(NAD^+)	8.44	n.d.[c]
ADP-ribose	9.57	6.30
P_i	9.66	n.d.
etheno(AMP)	10.13	n.d.
NADH	10.60	n.d.
etheno(ADP-ribose)	13.95	n.d.
(phosphoribosyl)AMP	20.45	7.77
ADP	38.05	8.23
(diphosphoribosyl)AMP	n.d.	10.64
ATP	∞[d]	17.07

[a] "Low Salt" Solvent System = 0.1 M KH_2PO_4, pH 4.7.

[b] "High Salt" Solvent System = 0.25 M KH_2PO_4, 1 M KCl, pH 4.7.

[c] n.d. = not determined.

[d] ATP was not eluted from the column in 133 min.

Figure 12. Quenching of ethenoadenosine fluorescence by phosphate buffers of different ionic strengths. The fluorescence of ethenoadenosine (27 nM) was determined in water and in different concentrations of KH_2PO_4, pH 4.7 (or KH_2PO_4 plus 1 M KCl).

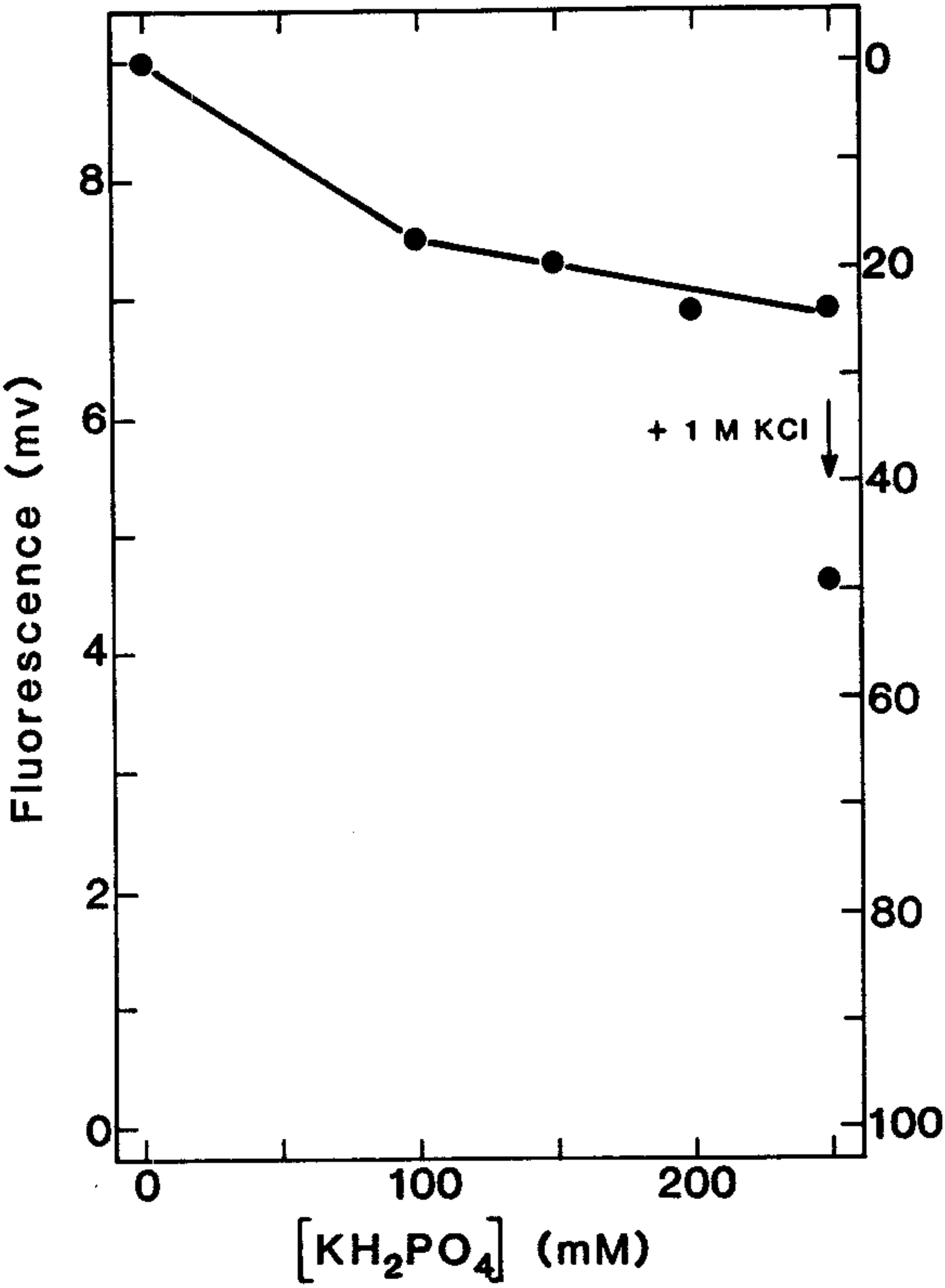

Fluorescence (mv)
0
2
4
6
8
Quench (%)
0
20
40
60
80
100
+ 1 M KCl
0
100
200
$[KH_2PO_4]$ (mM)

Figure 13. Formation of fluorescent derivatives of adenosine and ADP-ribose following reaction with chloroacetaldehyde. Fluorescence was determined after incubation at 60°C in 1.0 ml of 0.2 M sodium citrate, pH 4.5, containing: 100 µM adenosine plus 20 mM (■) or 100 mM (●) chloroacetaldehyde; 100 µM ADP-ribose plus 100 mM chloroacetaldehyde (▲); or 100 mM choloracetaldehyde alone (○).

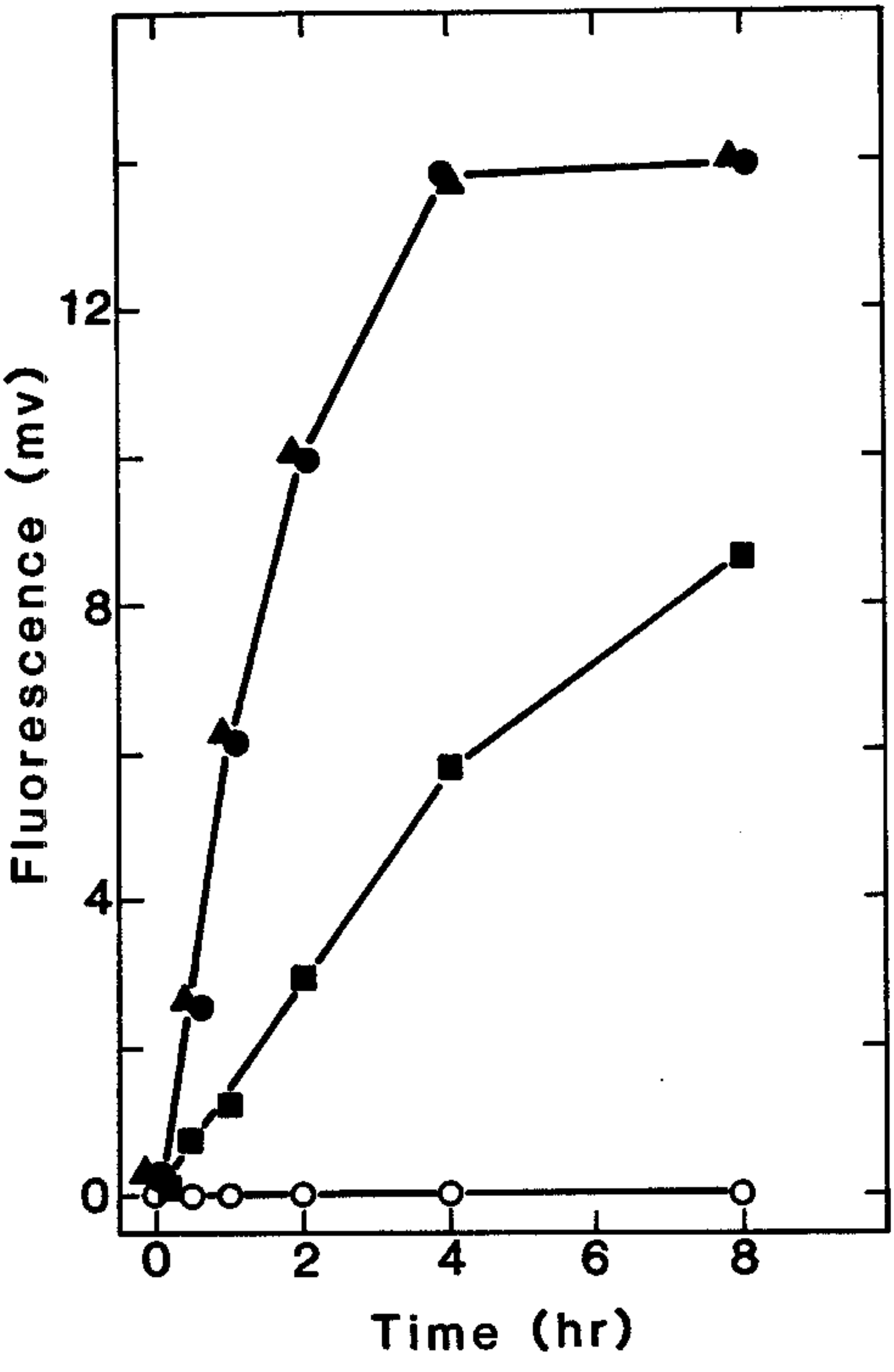

Fluorescence (mv)
0
4
8
12
Time (hr)
0
2
4
6
8

Figure 14. UV spectra of ADP-ribose before and after reaction with chloroacetaldehyde. Curve A is the UV spectrum obtained for ADP-ribose (43 μM) before reaction with chloroacetaldehyde. Curve B is the spectrum obtained for the fluorescent derivative of ADP-ribose (45 μM) after addition of purified [^{32}P]ADP-ribose, reaction with chloroacetaldehyde and purification by affinity chromatography as described in "METHODS."

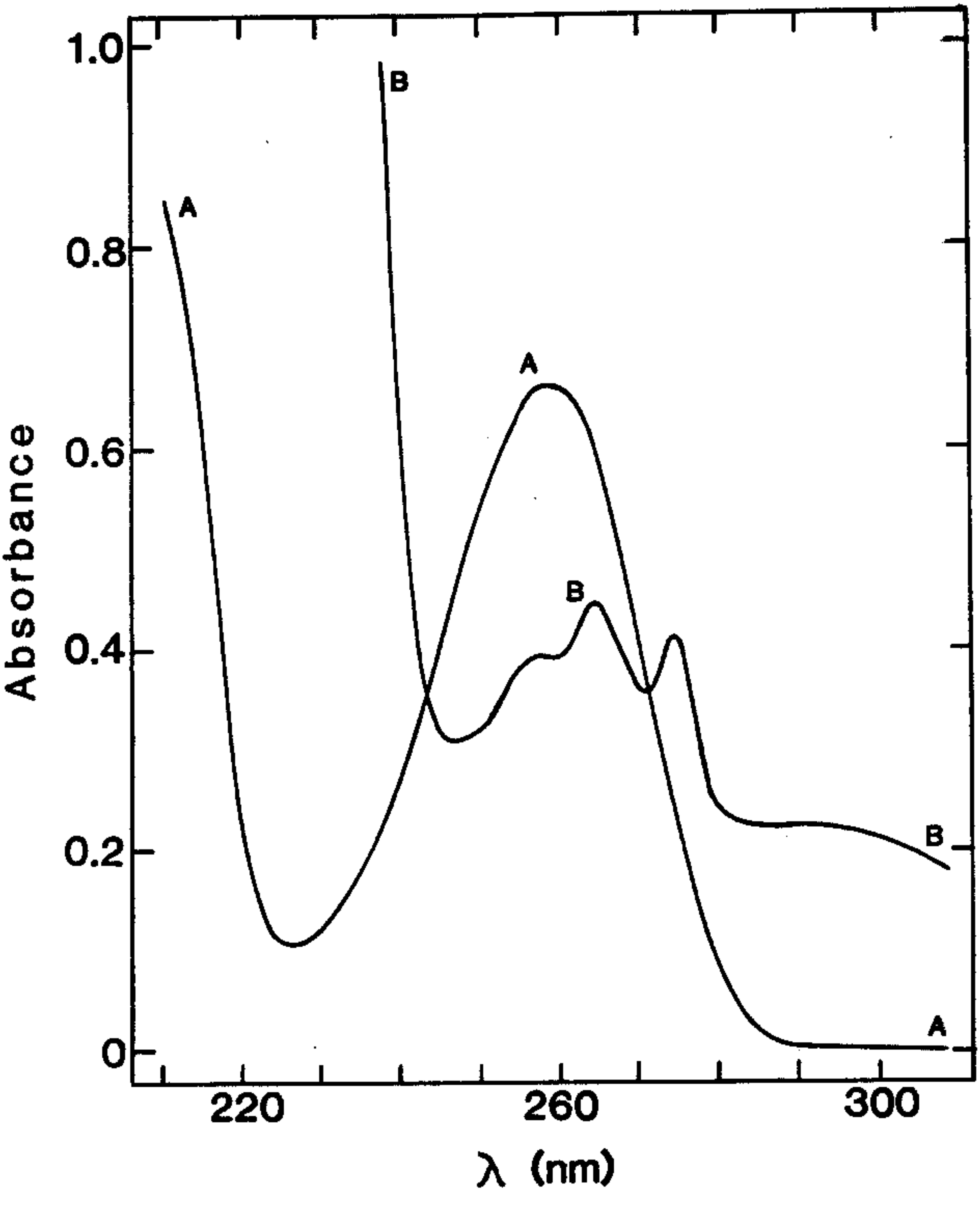
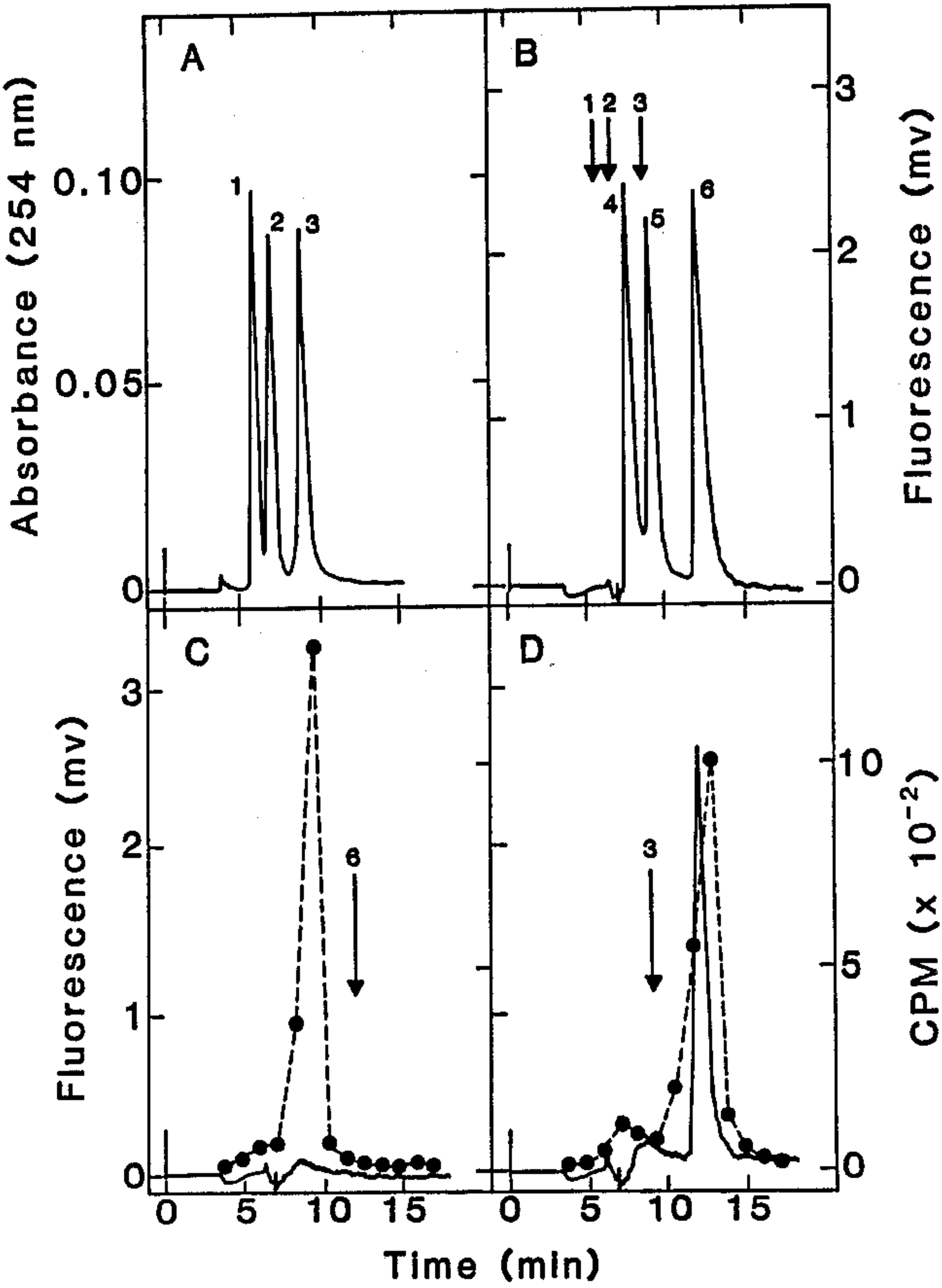

Absorbance
1.0
0.8
0.6
0.4
0.2
0
220
260
300
λ (nm)
A
B
A
B
B
A

Figure 15. Separation of adenine nucleotides and their etheno derivatives by HPLC, and quantitative conversion of ADP-ribose to etheno(ADP-ribose). Numbered peaks are (1) NAD^+, (2) AMP, (3) ADP-ribose, (4) etheno(NAD^+), (5) etheno-(AMP) and (6) etheno(ADP-ribose). (A) Five to ten nmol each of NAD^+, AMP and ADP-ribose were separated isocratically as described in "METHODS." (B) Etheno(NAD^+) (approx. 100 pmol) and 5-10 pmol each of etheno(AMP) and etheno(ADP-ribose) 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 in "METHODS." The arrows in panels C and D are for reference, indicating the elution positions 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.

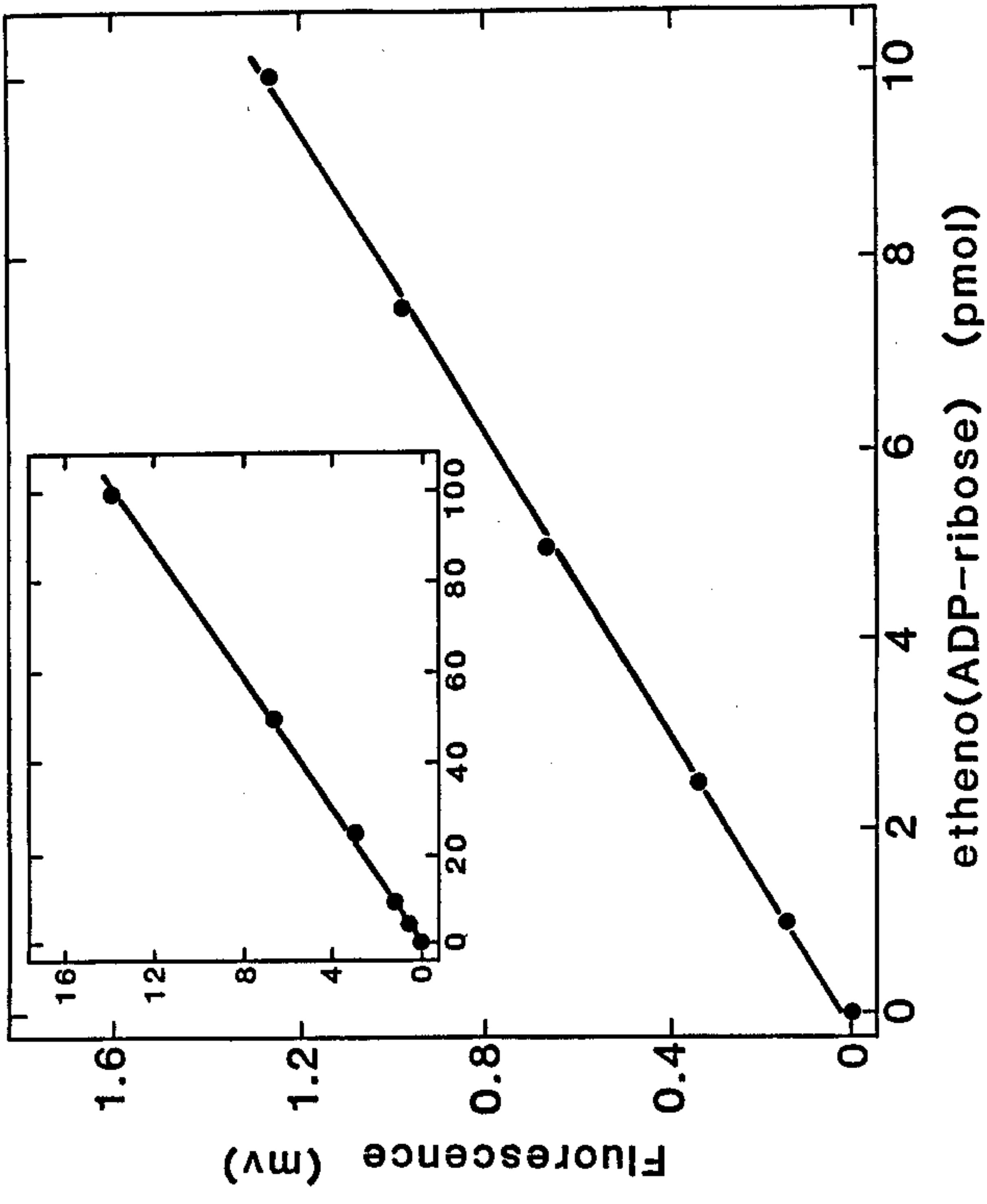

A
B
C
D
Absorbance (254 nm)
0.10
0.05
0
1
2
3
4
5
6
Fluorescence (mv)
3
2
1
0
CPM (x 10^{-2})
10
5
0
Time (min)
0
5
10
15

converted to etheno(ADP-ribose). Approximately 6 nmol of purified [^{14}C]ADP-ribose, which had been standardized both spectrally and by specific radioactivity, were derivatized as described above. The etheno([^{14}C]ADP-ribose) was then used to construct a standard curve for quantification of fluorescence following SAX HPLC. The relationship between fluorescence and the quantity of etheno([^{14}C]ADP-ribose) injected was linear over the entire range examined (1 to 100 pmol) (Fig. 16).

Dissolution of the Acid-Insoluble Fraction From Adult Rat Liver

A variety of aqueous solvents were tested for their ability to dissolve the acid-insoluble fraction from rat liver. Solutions containing 1% SDS, 8 M urea or a combination of these compounds were ineffective, even with incubation at 37°C for 12 hr or 100°C for 10 min. Concentrated formic acid (90-98%) effectively dissolved the dry, acid-insoluble material at 0-5°C, but some precipitation occurred when attempts were made either to neutralize the solution or to dilute it with deionized water. The acid-insoluble material derived from rat liver was also dissolved effectively by a solution containing 6 M guanidinium chloride and remained in solution over a wide range of pH values (3-9) and when stored at -20°C. A small amount of the acid-insoluble fraction that remained insoluble in 6 M guanidinium

Figure 16. 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 in "METHODS."


Fluorescence (mv)
0
0.4
0.8
1.2
1.6
0
2
4
6
8
10
etheno(ADP-ribose) (pmol)
0
4
8
12
16
0
20
40
60
80
100

chloride could be removed by brief centrifugation; this material (a translucent pellet) apparently did not contain protein, since the protein concentration was identical before and after centrifugation.

Separation of Protein-Bound ADP-ribose From Free Nucleotides

The ability to separate protein-bound ADP-ribose from free nucleotides was crucial to this study for two reasons. First, it was important to demonstrate that any ADP-ribose residues detected in tissue samples were protein-bound, and not artifacts derived from nucleotides trapped in the acid-insoluble material during preparation. Second, the separation of free and protein-bound ADP-ribose was necessary in order to monitor the release of ADP-ribose residues from proteins, and thereby establish the conditions required for quantitative cleavage of (ADP-ribose)-protein linkages. Free and protein-bound ADP-ribose in the acid-insoluble fraction from rat liver were effectively separated by each of five different techniques, including (i) gel filtration HPLC, (ii) Sephadex G-25 gel filtration column chromatography, (iii) dialysis, (iv) precipitation from concentrated formic acid prior to dissolution in Buffer A and (v) Sephadex G-25 (superfine) column centrifugation. The resolution achieved by all five techniques was comparable; 92-98% of the [^{14}C]ADP-ribosylated histone was recovered following each treatment, while 96-98% of the free ADP-ribose was eliminated (Figs. 17 and 18, and Tables IV and V). However, the column centrifugation technique was ultimately adopted as the standard procedure, since it was faster and more convenient for routine analysis of multiple samples.

Quantitative Release of Arginine-Linked ADP-ribose Residues from Proteins

Mono(ADP-ribosylated) histone, which had been synthesized using purified NAD:arginine ADP-ribosyltransferase, was used as a model conjugate to establish reaction conditions necessary for the quantitative release from proteins of intact ADP-ribose residues bound to arginine. As described in the preceding section, the results from gel filtration HPLC and Sephadex G-25 column chromatography established that >95% of the radiolabel in the histone sample was, indeed, bound to protein (Figs. 17 and 18). In preliminary experiments, the stability of the ([^{14}C]ADP-ribose)-histone linkage was evaluated using gel filtration HPLC. As seen in Fig. 19A, the linkage was relatively stable at pH 8.8, with only about 10% of the residues released after 1 hr at 37°C. However, a significant fraction of the ADP-ribose residues (32%) were released in the presence of neutral hydroxylamine under the conditions employed by Hilz and coworkers (120) (Fig. 19B). Continued incubation with 0.4 M neutral

Figure 17. Separation of protein-bound ADP-ribose from free nucleotides by gel filtration HPLC. Samples of blue dextran, ADP-ribose and [^{14}C]ADP-ribosylated histone in Buffer A were subjected to gel filtration HPLC analysis using two TSK-125 analytical columns connected in series as described in "METHODS."

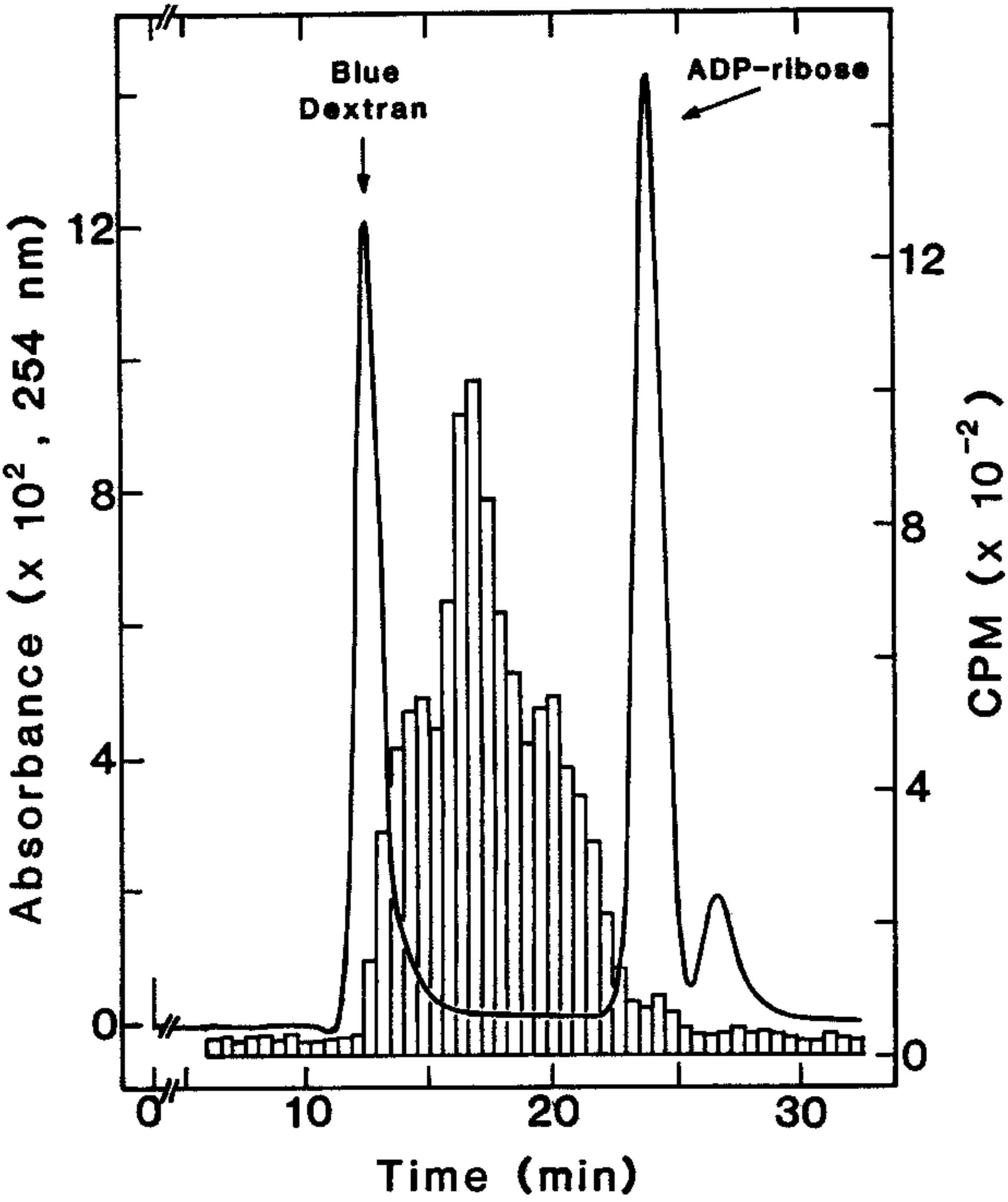

Blue
Dextran
ADP-ribose
Absorbance (x 10^2, 254 nm)
0
4
8
12
CPM (x 10^{-2})
0
4
8
12
Time (min)
0
10
20
30

Figure 18. Separation of [^{14}C]ADP-ribosylated histone and rat liver proteins from free ADP-ribose by Sephadex G-25 column chromatography. [^{14}C]ADP-ribosylated histone (▲) or [^{14}C]ADP-ribose (■) were added to samples of the acid-insoluble fraction from rat liver (dissolved in Buffer A). Aliquots (0.5 ml) were applied to a Sephadex G-25 column as described in "METHODS," and the effluent was analyzed for radioactivity and UV absorbance (●).

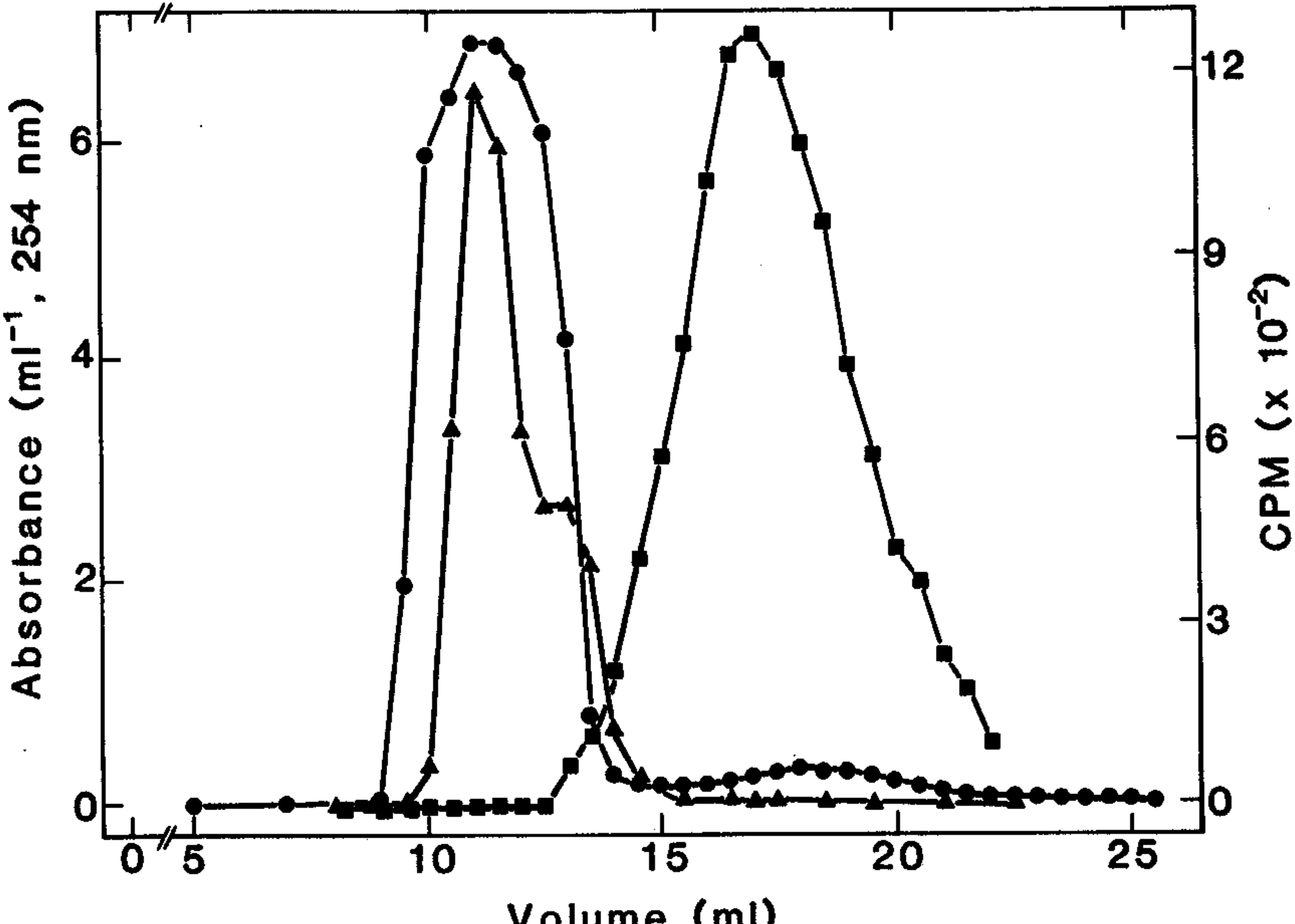

Absorbance (ml^{-1}, 254 nm)
0
2
4
6
Volume (ml)
0
5
10
15
20
25
CPM (x 10^{-2})
0
3
6
9
12

TABLE IV

Separation of Protein-Bound ADP-ribose From Free Nucleotides By Dialysis Or Acid Precipitation

Treatment	Radiolabelled ADP-ribose (dpm)	
	Protein-bound $[^{14}C]$ADP-ribose	Free $[^{14}C]$ADP-ribose
None (Sample in Buffer A)	7,113	19,520
Dialysis	6,650 (98%)[a]	380 (2%)
None (Sample in Formic Acid)	10,820	30,910
Trichloroacetic Acid	9,500 (92%)[a]	510 (2%)

[a] Values for % recovery have been adjusted to account for the presence of a small amount ($\leq$5%) of radiolabel not bound to protein.

Samples of the acid-insoluble fraction from rat liver were dissolved in either Buffer A or formic acid as described in "METHODS." Either $[^{14}C]$ADP-ribosylated histone or free $[^{14}C]$ADP-ribose was added to identical aliquots, which were then subjected to dialysis or precipitation with trichloroacetic acid as described in "METHODS."

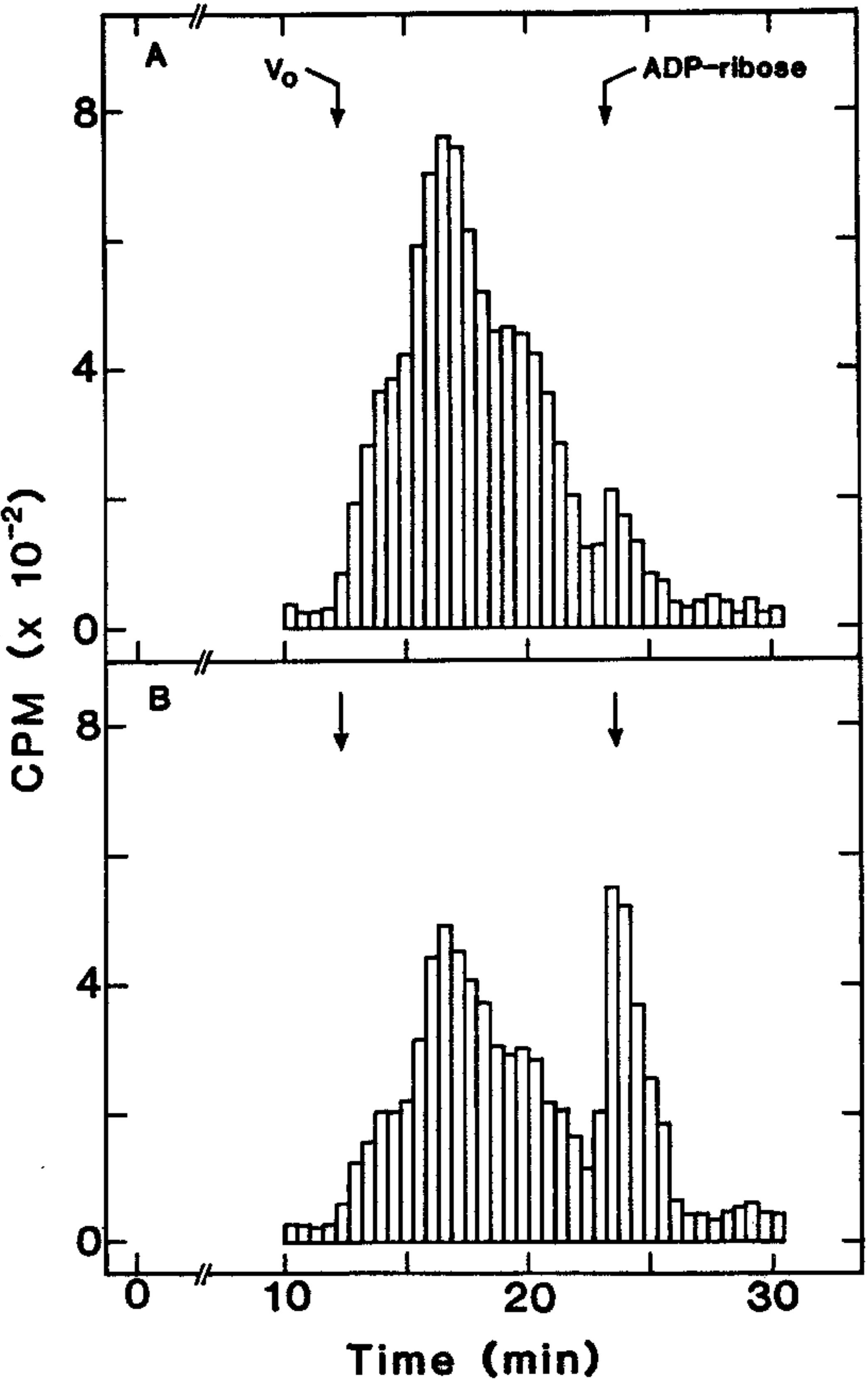
TABLE V

Separation of Protein-Bound ADP-ribose From Free Nucleotides by G-25 Column Centrifugation

Treatment	Radiolabelled ADP-ribose (dpm)	
	Protein-bound $[^{14}C]$ADP-ribose	Free $[^{32}P]$ADP-ribose
None	20,530	53,130
Column Centrifugation		
"Protein Fraction"	20,010 (97%)	990 (2%)
"Nucleotide Fraction"	493 (2%)	44,964 (96%)

After dissolving the acid-insoluble fraction from rat liver, either $[^{14}C]$ADP-ribosylated histone or free $[^{32}P]$ADP-ribose was added, and identical aliquots (500 µl) were subjected to fractionation by G-25 (superfine) column centrifugation as described in "METHODS."

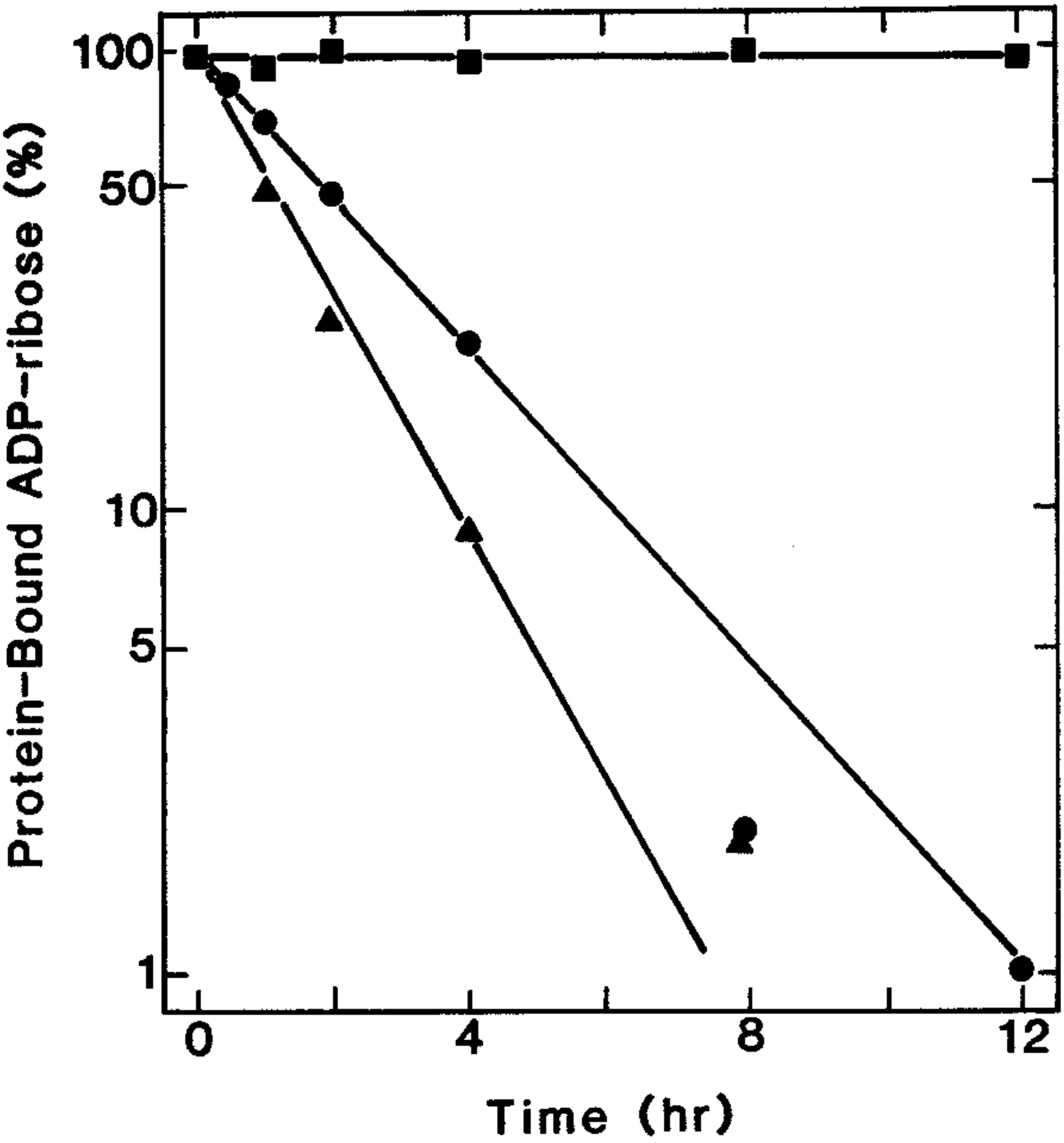
Figure 19. Preliminary evaluation of the stability of the (ADP-ribose)-histone linkage by gel filtration HPLC. [^{14}C]ADP-ribosylated histone in Buffer A was incubated at 37°C for 1 hr (A) at pH 8.8 or (B) in the presence of 0.4 M NH_2OH at pH 7.5. Free and protein-bound [^{14}C]ADP-ribose were separated by gel filtration HPLC as described in "METHODS" and the legend to Fig. 17.


A
B
V_o
ADP-ribose
CPM (x 10^{-2})
0
4
8
0
4
8
0
10
20
30
Time (min)

hydroxylamine for 12 hr at 37°C resulted in the release of 98% of the protein-bound ADP-ribose residues.

The stability of the (ADP-ribose)-histone linkage was characterized in more detail using Sephadex G-25 column chromatography. 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 yr. In the presence of the acid-insoluble fraction from rat liver (dissolved in Buffer A), the linkage was also completely stable at neutral pH and 37°C for at least 12 hr (Fig. 20). The t values for the (ADP-ribose)-histone linkage in the presence of 0.4 M or 3 M neutral hydroxylamine were 2 hr and 1 hr, respectively. The half-life value in the presence of the more dilute hydroxylamine concentration is in agreement with the value recently reported by Moss et al. (152), even though our reaction mixture contained additional, potentially interfering components (e.g., 6 M guanidinium chloride and the acid-insoluble 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 from protein, greater than 98% of [^{14}C]ADP-ribose remained intact (data not shown). Confirmation that ADP-ribose was released intact from protein was obtained in subsequent experiments described below (e.g., see Fig. 23).

Figure 20. Stability of the (ADP-ribose)-histone linkage in the presence of the acid-insoluble fraction from rat liver. [^{14}C]ADP-ribosylated histone was added to samples of the acid-insoluble fraction from rat liver (dissolved in Buffer A). The samples were incubated at pH 7.4 in the absence (■) or presence (●) of 0.4 M NH_2OH, or at pH 7.0 in the presence of 3.0 M NH_2OH (▲) at 37°C. Aliquots (0.5 ml) were removed at the indicated times, and free and protein-bound [^{14}C]ADP-ribose were separated by Sephadex G-25 column chromatography and quantified as described in "METHODS."


Protein-Bound ADP-ribose (%)
100
50
10
5
1
0
4
8
12
Time (hr)

Selective Release of Arginine-Linked ADP-ribose Residues from Proteins

Mono(ADP-ribosylated) EF-2 and transducin were also used as model conjugates to evaluate the stabilities of the other two known N-glycosylic linkages, (ADP-ribose)-diphthamide and (ADP-ribose)-asparagine, respectively. In contrast to the susceptibility of the (ADP-ribose)-arginine linkage to cleavage by hydroxylamine, the (ADP-ribose)-diphthamide and (ADP-ribose)-asparagine linkages were stable for 12 hr in the presence of 1 M neutral hydroxylamine (Fig. 21). In fact, no release was detected following incubation at a higher concentration (3 M) or for a longer time (21 hr). 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 hr (23,153,154). 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 hr and 5-10 min, respectively (116-119,153) (Fig. 21). The stabilities of the arginine-, asparagine- and diphthamide-(ADP-ribose) conjugates were also evaluated employing the differential release conditions utilized previously by Hilz and coworkers (i.e., brief treatment with dilute hydroxylamine vs treatment with strong alkali) (120). As shown in Table VI (and earlier

Figure 21. 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 (4,000-10,000 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 in "METHODS." The dashed line in each panel represents the stability of the (ADP-ribose)-glutamate linkage as reported previously (116-119).

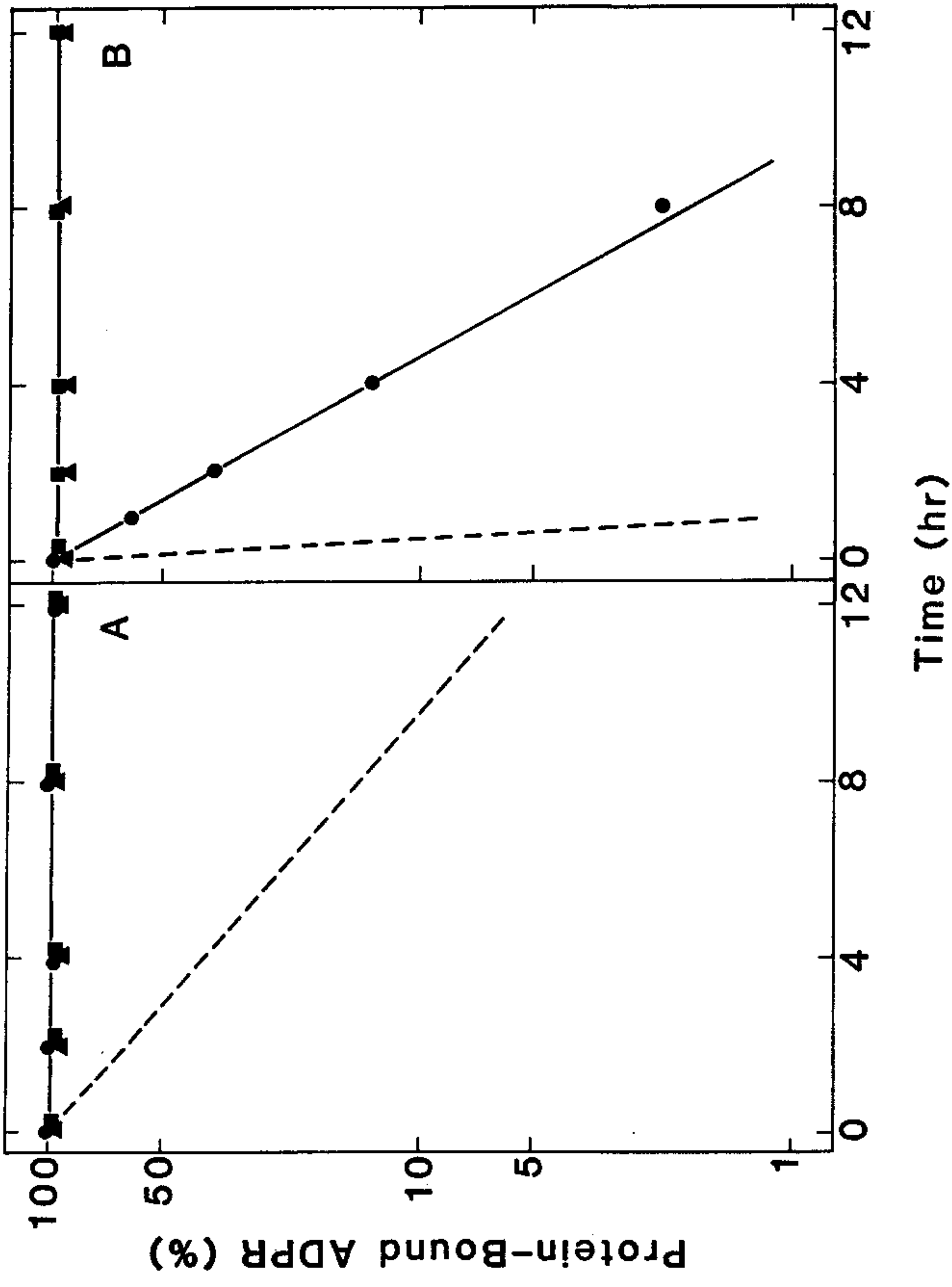

A
B
Protein-Bound ADPR (%)
100
50
10
5
1
Time (hr)
0
4
8
12
0
4
8
12

TABLE VI

Stability of Different (ADP-ribose)-Protein Linkages In the Presence of Neutral Hydroxylamine or Strong Alkali

ADP-ribose Acceptor	ADP-ribose Released (%)		
	NH_2OH (37°C)		NaOH (56°C)
	0.4 M, 1 hr pH 7.5	1 M, 12 hr pH 7.0	1 M 1 hr
Arginine (histone)	27	98	88
Asparagine (transducin)	n.d.[a]	<1	60
Diphthamide (EF-2)	<1	<1	<1

[a] n.d. = not determined

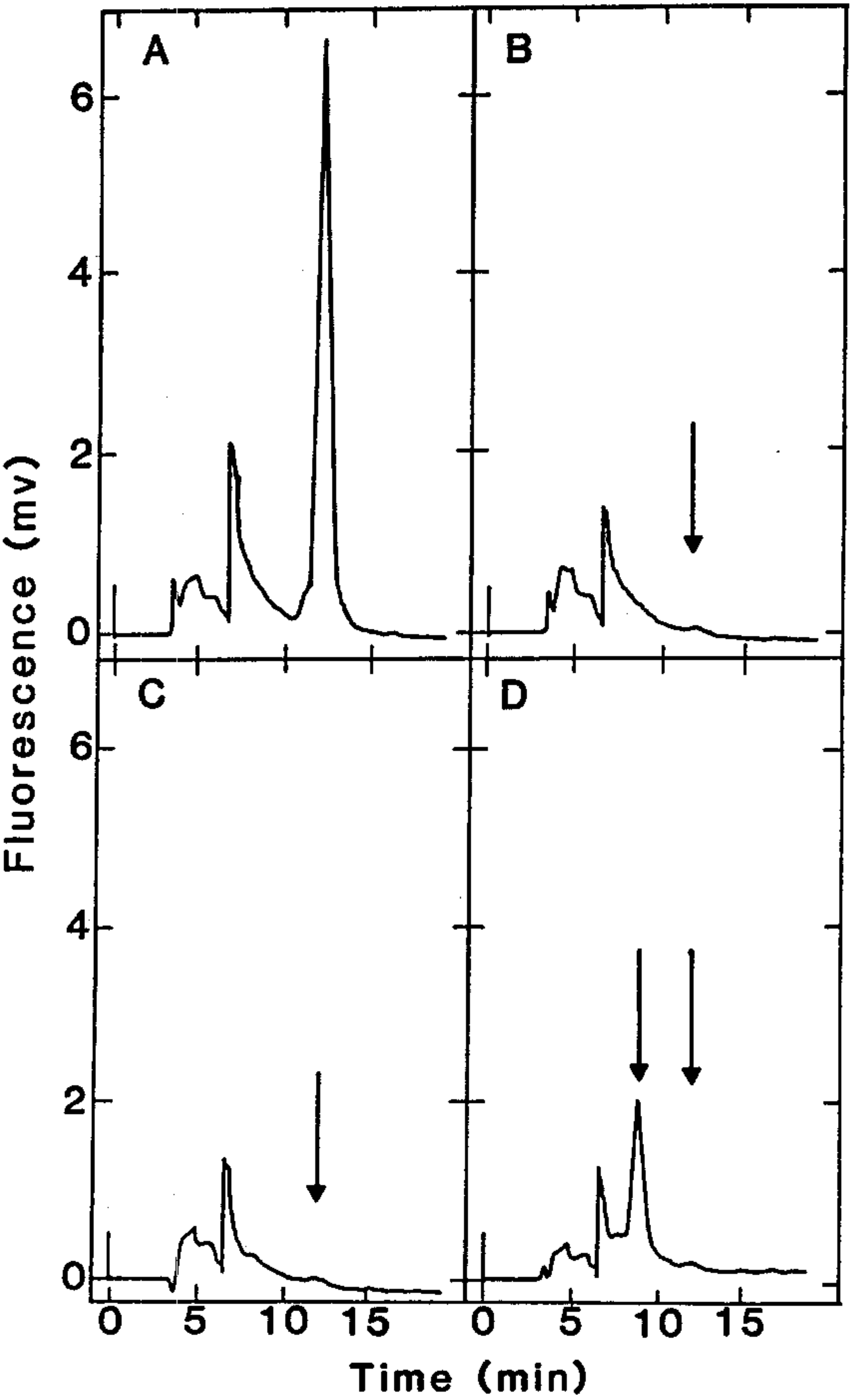
Samples of liver extract containing 4,000-10,000 dpm/0.5 ml of either [^{14}C]ADP-ribosylated histone, [^{32}P]ADP-ribosylated transducin or [^{32}P]ADP-ribosylated EF-2 were incubated in the presence of neutral hydroxylamine or strong alkali as described by Bredehorst et al. (120). Aliquots (500 µl) were then fractionated by G-25 (superfine) column centrifugation as described in "METHODS," and the amounts of radiolabelled ADP-ribose in the "protein" and "nucleotide" fractions were quantified. For comparison, identical samples were also incubated in the presence of neutral hydroxylamine under the conditions described in "METHODS" for the assay of protein-bound mono(ADP-ribose) residues.

in Fig. 19), a significant fraction of the (ADP-ribose)-arginine linkages was cleaved during the brief treatment with 0.4 M neutral hydroxylamine. Treatment with 1 M NaOH resulted in almost complete release of arginine-linked ADP-ribose. In contrast, the asparagine- and diphthamide-linked ADP-ribose residues were completely resistant to hydroxylamine treatment, and release during treatment with alkali was either incomplete (asparagine) or negligible (diphthamide).

Detection of 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 in "Methods." 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 hr. Quantitative detection of ADP-ribose following release from protein involved (i) isolation of ADP-ribose by affinity chromatography, (ii) formation of a highly fluorescent analog, 1,N^6-etheno(ADP-ribose), and (iii) quantification by monitoring fluorescence following anion exchange HPLC. As shown in Fig. 22A, 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

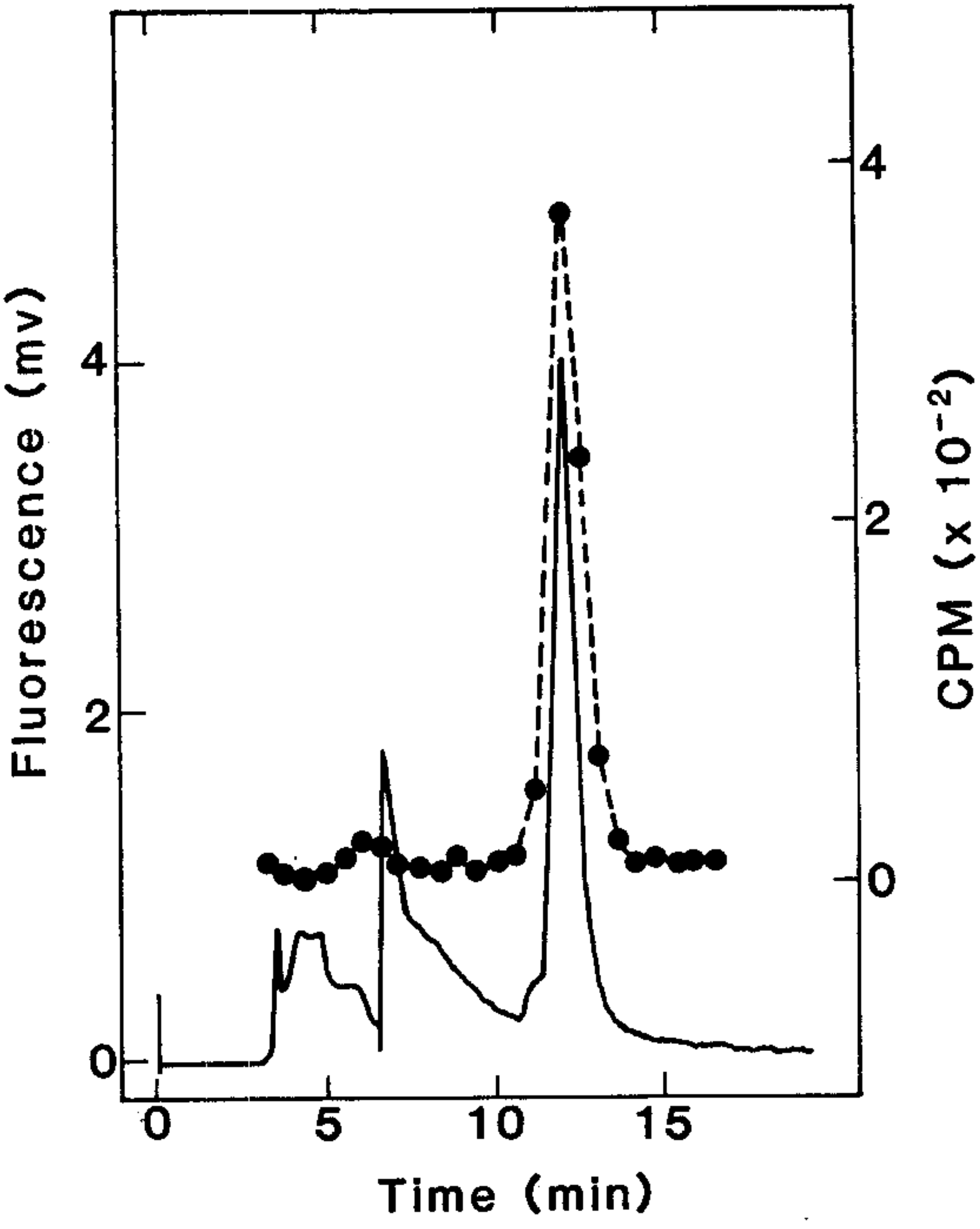
Figure 22. Measurement of protein-bound mono(ADP-ribose) residues in adult rat liver. Rat liver was subjected to analysis as described in "METHODS." (A) Control analyses included omission of extract (B), omission of chloroacetaldehyde (C), and treatment with purified snake venom phosphodiesterase (1 unit/ ml, 37°C, 3 hr) 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.


A
B
C
D
6
4
2
0
Fluorescence (mv)
0
5
10
15
Time (min)

to contaminants originating in the reagents or to endogenous fluorescent compounds in the extract (Figs. 22B and 22C, respectively). 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) (Fig. 22D). 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) (Fig. 23). 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 (84,152); ADP-ribose and its hydroxylamine derivative were indistinguishable by high voltage paper electrophoresis (84) and anion exchange HPLC (Yost, D. A. and Moss, J., personal communication). Thus, it is probable that the residues detected as etheno(ADP-ribose) in the present study 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

Figure 23. 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 in "METHODS," 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.


Fluorescence (mv)
0
2
4
CPM (x 10^{-2})
0
2
4
0
5
10
15
Time (min)

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, recovery of etheno[^{14}C]ADP-ribose from [^{14}C]ADP-ribosylated histone in the absence of hydroxylamine was only 4%. 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%.

The values obtained from replicate analyses of rat liver in the absence or presence of hydroxylamine are shown in Table VII ("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 VII, column 3).

TABLE VII

ADP-Ribose Residues in the Acid-Insoluble Fraction From Adult Rat Liver

Treatment	ADP-Ribose (pmol/mg protein)		
	A	B	[B - A]
	$-NH_2OH$	$+NH_2OH$	NH_2OH-Requiring Residues
None[a]	80.0	181.0	101.0
Column Centrifugation			
Protein Fraction[b]	9.7 ± 1.5	41.5 ± 1.5	31.8 ±0.8
Nucleotide Fraction[a]	72.5	146.9	74.4
	82.2	188.4	106.2

[a] Average of duplicate determinations (range ≤ ±8% for all values)
[b] Mean ± S.D. (n=6)

After dissolving the acid-insoluble fraction from rat liver, samples (containing 2.41 mg protein) were incubated in the absence or presence of hydroxylamine and analyzed for total ADP-ribose residues as described in "METHODS." 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 protein).

Not surprisingly, when the acid-insoluble fraction from rat liver was dissolved and analyzed directly with no pretreatment to eliminate non-covalently bound nucleotides, higher values for ADP-ribose were obtained in both the absence and presence of hydroxylamine (8.2-fold and 4.4-fold higher values, respectively), and 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 large amount of ADP-ribose residues not associated with the "protein fraction" (Table VIII). 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 (155).

However, an unexpected result was the observation that ADP-ribose residues requiring hydroxylamine for detection (Table VII, column 3) were significantly lower in the "protein fraction" after column centrifugation; almost 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. Qualitatively similar results were observed when other techniques were employed in place of column

TABLE VIII

Relative Amounts of ADP-ribose in Acid-Insoluble Fractions from Adult Rat Liver

Number of Acid Extractions	ADP-ribose Residues Detected[a]		
	A Unfractionated Sample	B "Protein Fraction"	[A/B]
1	9.8	1.00	9.8
2	4.4	0.97	4.6
3	3.0	0.88	3.4
4	2.6	0.82	3.1

[a] Relative Values.

Different samples of the acid-insoluble fraction from rat liver were prepared as described in "METHODS," except that the number of extractions with trichloroacetic acid was varied. Samples of each preparation were dissolved, incubated in the presence of hydroxylamine and analyzed for ADP-ribose residues as described in "METHODS." The "protein fraction" from each sample, obtained by Sephadex G-25 column chromatography as described in "METHODS," was also analyzed as described for the unfractionated samples.

centrifugation to remove non-covalently bound nucleotides; 40-70% of the hydroxylamine-requiring ADP-ribose residues were eliminated from the protein fraction by these treatments (Table IX). One explanation for this result could be that a significant quantity of low molecular weight ADP-ribosylated peptides are 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 (Fig. 24), NADH was completely degraded; approximately 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 (Fig. 25). Subsequently, the trichloroacetic acid-treated samples were incubated in the absence or presence of hydroxylamine and analyzed for ADP-ribose. As seen in Table X, 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.

TABLE IX

Relative Amounts of ADP-ribose After Treatments To Remove Free Nucleotides From Preparations Of Rat Liver Proteins

Treatment	ADP-ribose Residues Detected[a]		
	A $-NH_2OH$	B $+NH_2OH$	[B - A][b] NH_2OH-Requiring Residues
Experiment I[c]			
None	0.44	1.00	0.56
Column Centrifugation	0.05	0.23	0.18 (-68%)
Experiment II[d]			
None	0.47	1.00	0.53
Dialysis	0.07	0.38	0.30 (-43%)
Trichloroacetic Acid[e]	0.09	0.31	0.22 (-58%)

[a] Relative values.

[b] Numbers in parentheses are the decreases in NH_2OH-requiring residues detected after each treatment.

[c] Data from Table VII.

[d] Data obtained with a different preparation of acid-insoluble material than that used in Experiment I.

[e] Sample dissolved in formic acid and precipitated with trichloroacetic acid.

Samples of acid-insoluble fractions from rat liver were dissolved in either Buffer A or formic acid, subjected to the indicated treatment and analyzed for ADP-ribose residues as described in "METHODS."

Figure 24. Analysis of trichloroacetic acid-treated NAD^+ by anion exchange HPLC. NAD^+ (≈200 pmol) was analyzed by anion exchange HPLC ("METHODS") (A) before and (B) after treatment with trichloroacetic acid at 0°C as described in the text. Numbered arrows represent elution positions of (1) NAD^+ and (2) ADP-ribose. The arrow at 5.5 min indicates a 10-fold increase in sensitivity as described in the legend to Fig. 9.

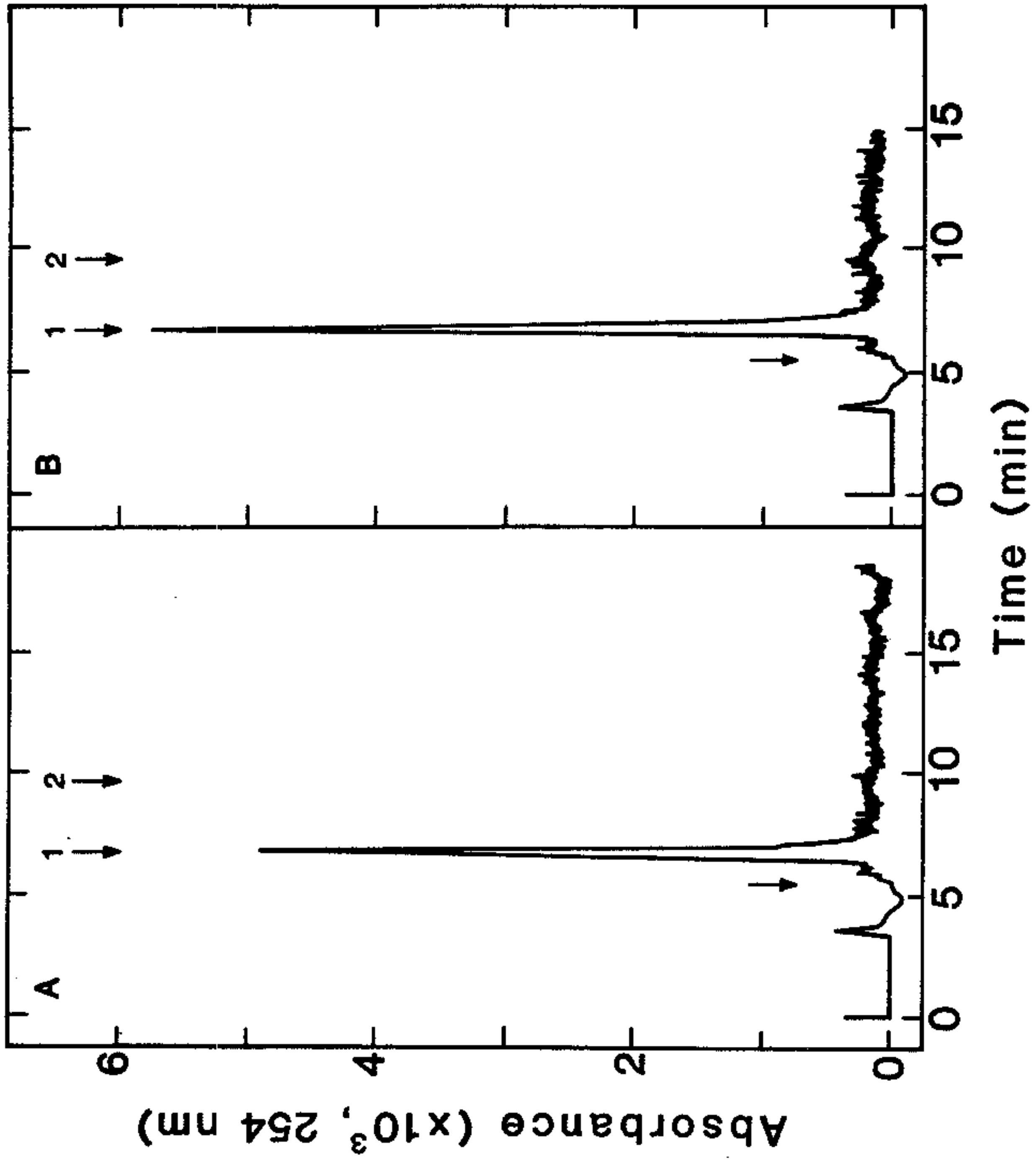

A
B
1
2
Absorbance (x10^3, 254 nm)
0
2
4
6
Time (min)
0
5
10
15

Figure 25. Analysis of trichloroacetic acid-treated NADH by anion exchange HPLC. NADH (≃650 pmol) was analyzed by anion exchange HPLC ("METHODS") (A) before and (B) after treatment with trichloroacetic acid at 0°C as described in the text. Numbered arrows represent elution positions of (1) ADP-ribose and (2) NADH. The arrow at 5.5 min indicates a 10-fold increase in sensitivity as described in the legend to Fig. 9.

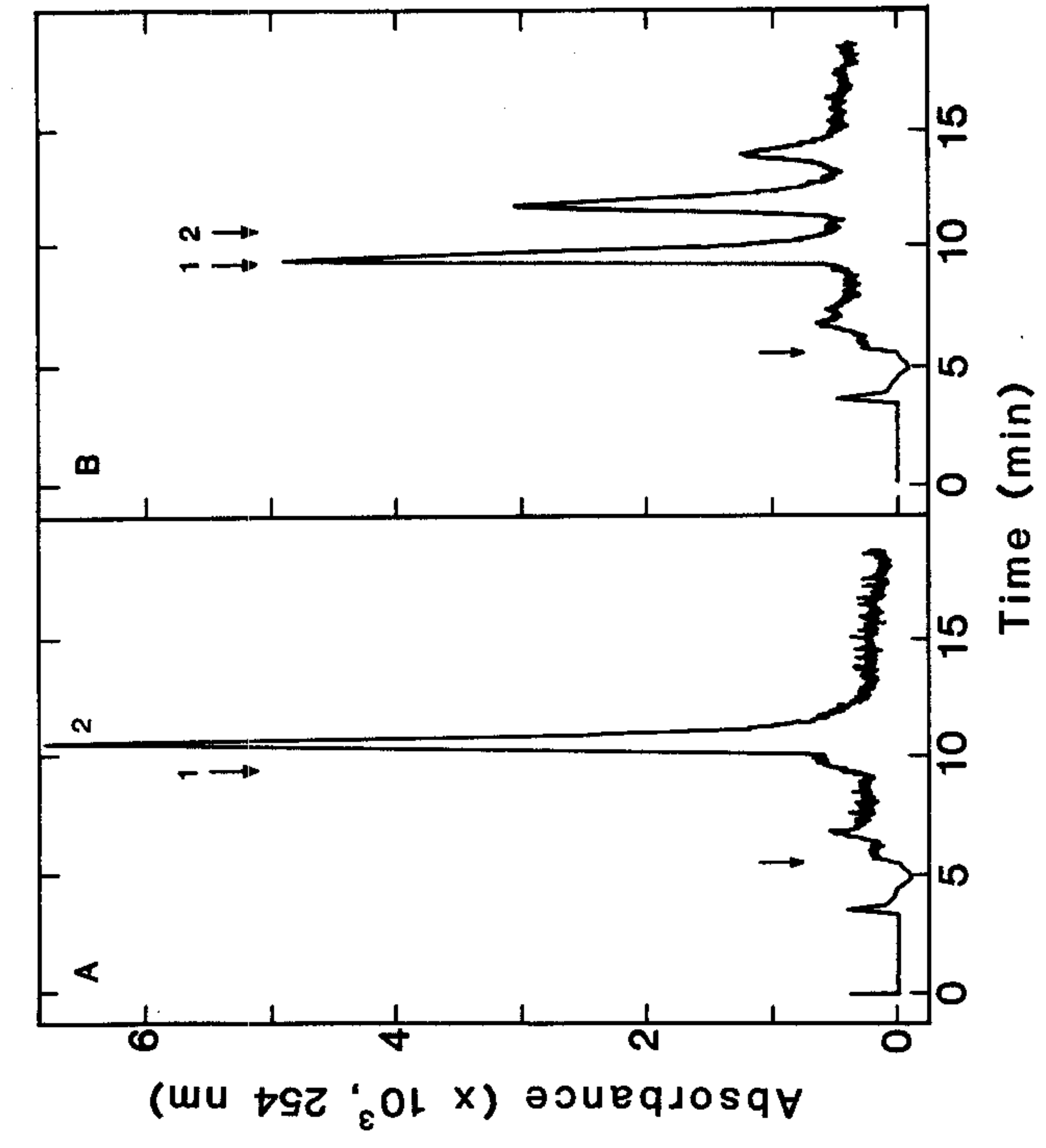

A
B
1
2
1 2
0
2
4
6
Absorbance (x 10³, 254 nm)
0
5
10
15
Time (min)

TABLE X

ADP-ribose Residues From NAD^+ and NADH Following Acid Extraction

Nucleotide Treated	ADP-ribose (pmol)		
	A $-NH_2OH$	B $+NH_2OH$	[B - A] NH_2OH-Requiring Residues
NAD^+	95 (19%)	106 (21%)	11 (2%)
NADH	297 (59%)	436 (87%)	139 (28%)

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

Release of ADP-ribose Residues from Rat Liver Proteins

The time course for release of ADP-ribose residues from rat liver proteins modified *in vivo* was determined in the presence or absence of hydroxylamine. As shown in Fig. 26, release of ADP-ribose by hydroxylamine was essentially complete after 8-12 hr 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 non-covalently bound ADP-ribose, representing about 3% of the total free "nucleotide fraction" prior to G-25 column centrifugation (Table VII). This value is in agreement with the previous estimate of the "desalting" efficiency of the column centrifugation technique (Table V).

Kinetic analysis of the release of protein-bound ADP-ribose residues in the presence of hydroxylamine revealed two first-order components (Fig. 27), suggesting that 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)

Figure 26. 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 non-covalently bound nucleotides ("METHODS"). Aliquots of the "protein fraction" were then incubated at pH 4.0, 0°C (●) or pH 7.0, 37°C in the absence (▲) or presence (■) of 1 M NH_2OH. At the indicated times, samples (containing 2.32 mg protein) were subjected to column centrifugation, and free ADP-ribose in the "nucleotide fraction" was quantified by HPLC analysis following affinity chromatography and derivatization ("METHODS").

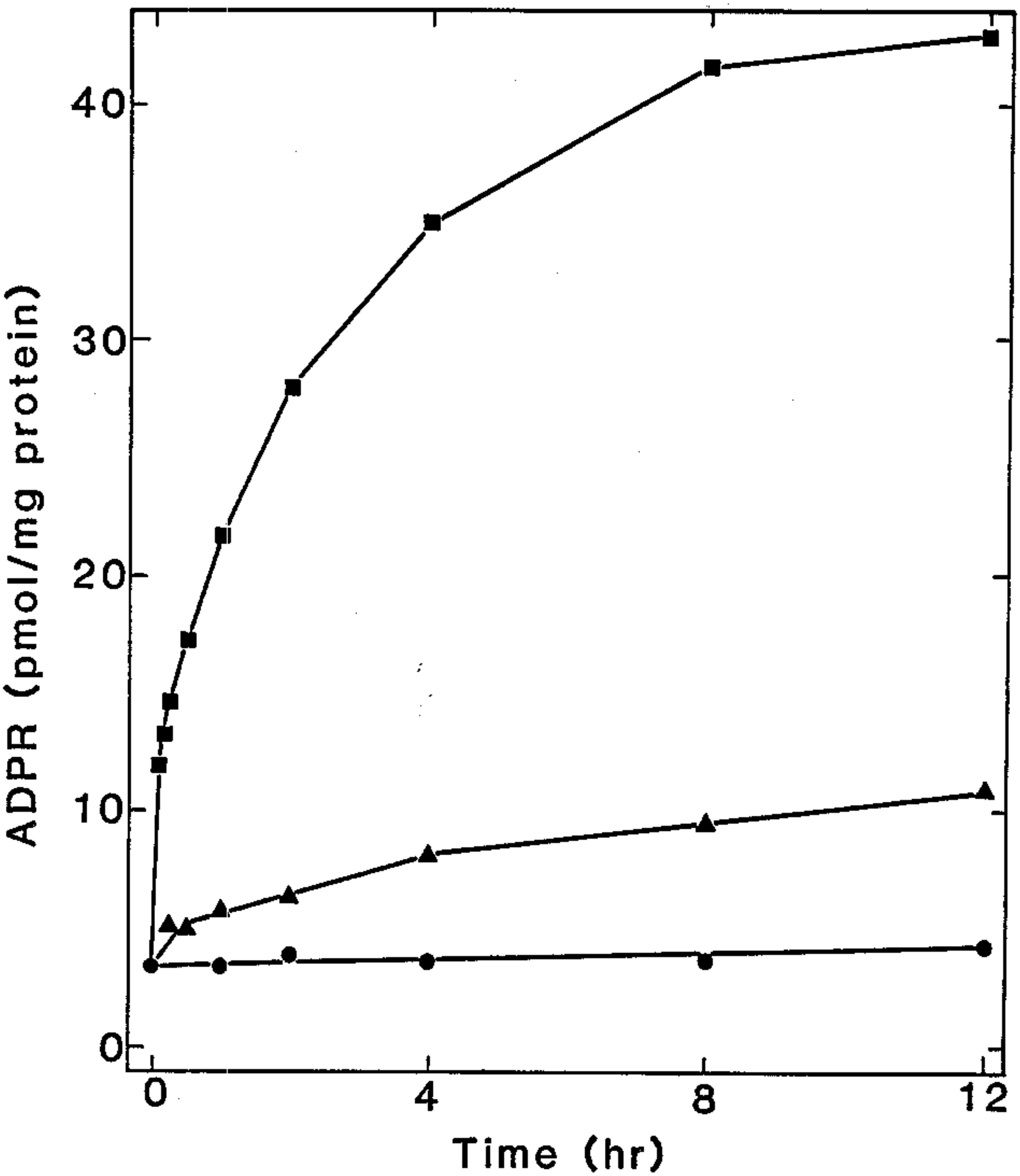
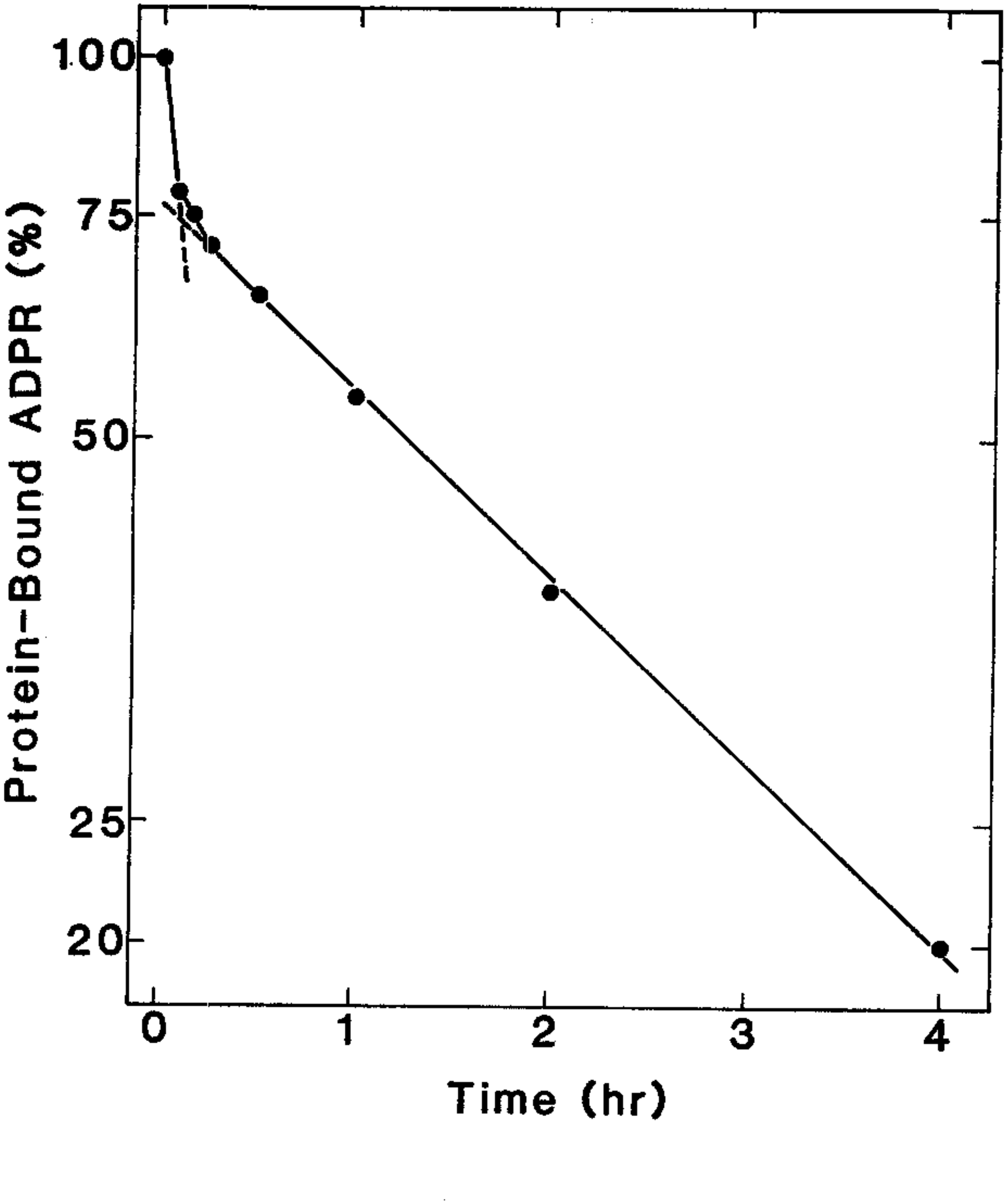

ADPR (pmol/mg protein)
0
10
20
30
40
Time (hr)
0
4
8
12

Figure 27. Release of ADP-ribose from rat liver proteins by neutral hydroxylamine. Experimental details are the same as described in 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).


Protein-Bound ADPR (%)
100
75
50
25
20
0
1
2
3
4
Time (hr)

had a half-life of approximately 2 hr. 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 hr (not shown).

Size Distribution of Rat Liver Proteins ADP-ribosylated at Arginine In Vivo

The presence of 6 M guanidinium chloride has a dramatic effect on the behavior of proteins during gel filtration chromatography; even relatively small proteins can elute at or near the column void volume. As determined by the supplier (see Bio-Rad catalog), the normal fractionation range of TSK-125 columns (5-80 kDa) is reduced to 1-25 kDa in the presence of guanidinium chloride. This was also shown in Fig. 17, in which histones in Buffer A eluted with retention times characteristic of much larger proteins. Thus, columns with the largest available exclusion limit were required to achieve even modest resolution of total rat liver proteins dissolved in 6 M guanidinium chloride. Two TSK columns (TSK-250 and -400; see "METHODS") were connected in series, equilibrated in Buffer A, and calibrated with proteins of known molecular masses (Figs. 28 and 29). Samples of the acid-insoluble fraction from rat liver (dissolved in Buffer A) were incubated at pH 7.0 and 37°C for 4 hr, then subjected to G-25 column centrifugation. This

Figure 28. Calibration of TSK columns and analysis of [^{32}P]ADP-ribosylated EF-2 by gel filtration HPLC. Mixtures (in Buffer A) of (A) blue dextran and ADP-ribose, or (B) protein standards (Bio-Rad) and [^{32}P]ADP-ribosylated EF-2 were analyzed by gel filtration HPLC using TSK-250 and -400 analytical columns connected in series ("METHODS"). Numbered peaks in panel B are (1) thyroglobulin, (2) IgG, (3) [^{32}P]ADP-ribosylated EF-2, (4) ovalbumin, (5) myoglobin and (6) vitamin B_{12}.

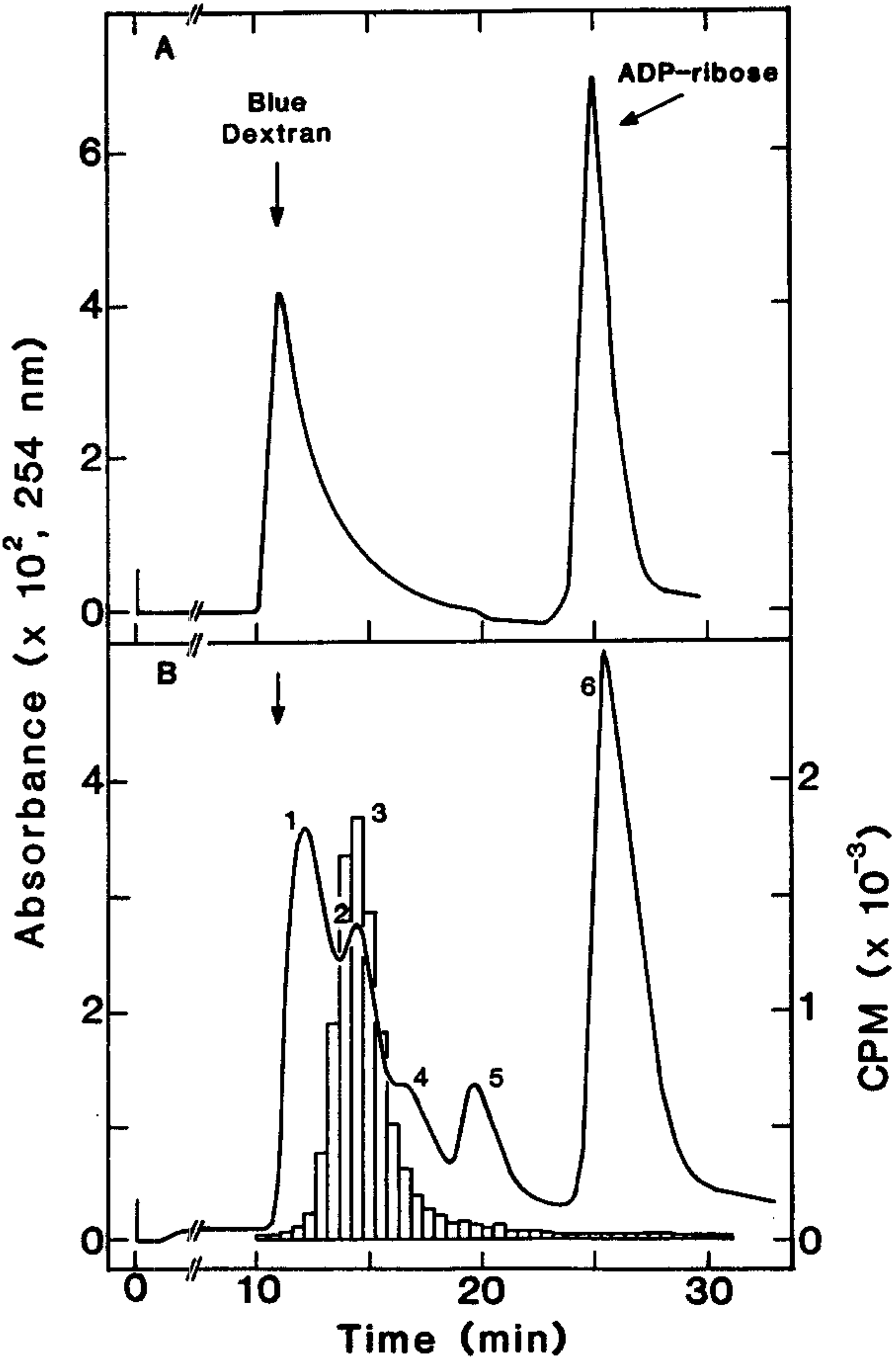
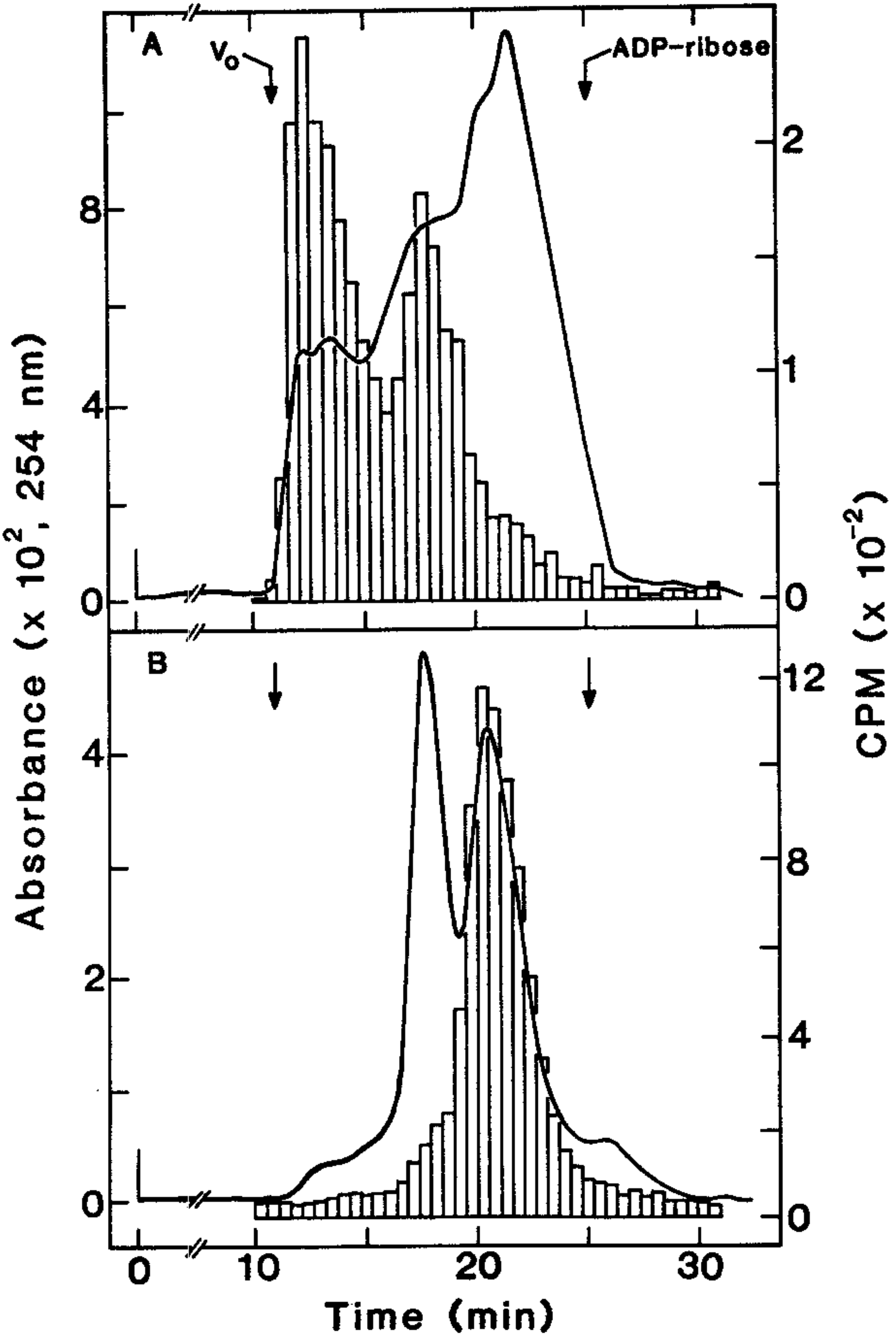
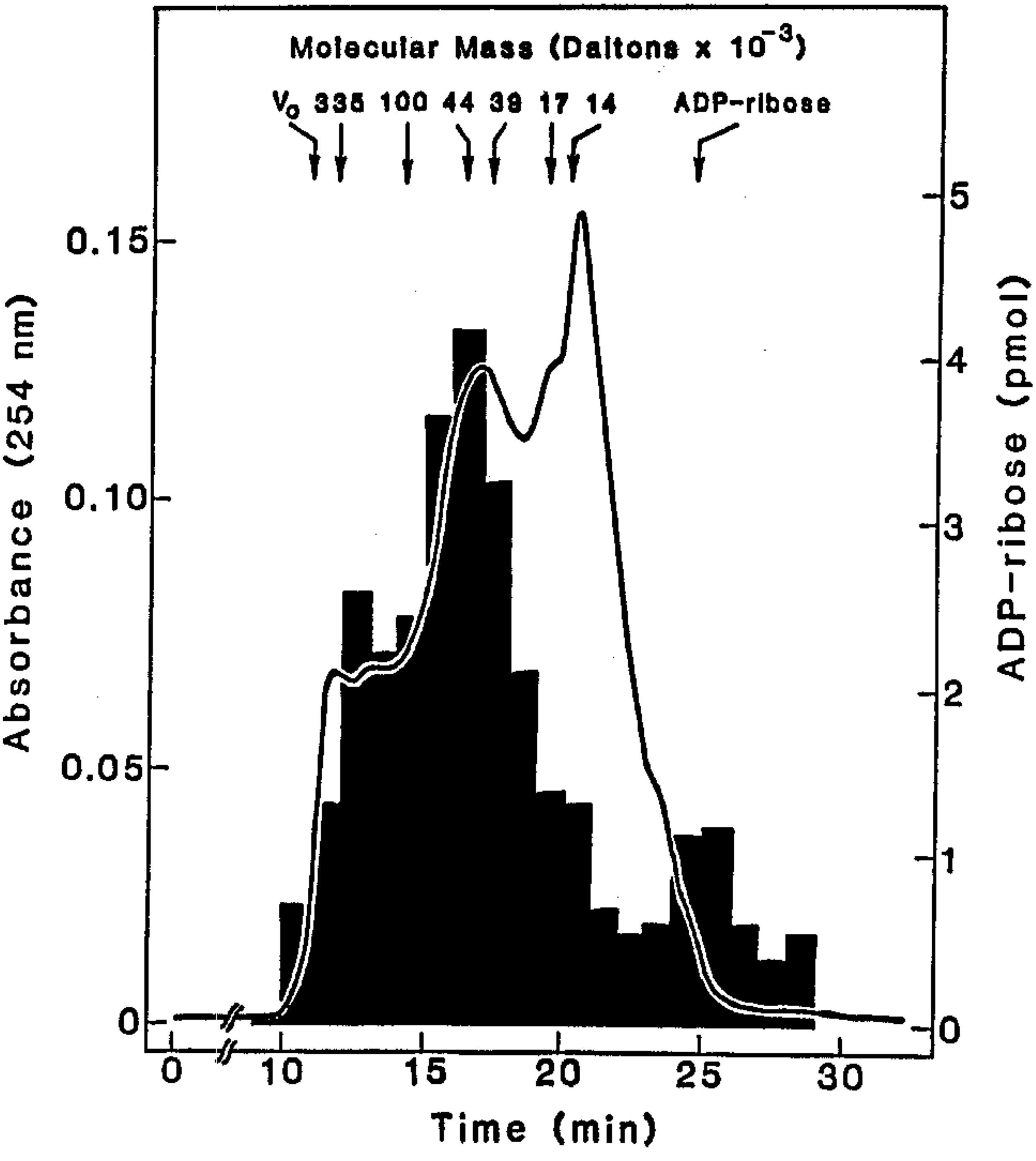

A
Blue Dextran
ADP-ribose
B
1
2
3
4
5
6
Absorbance (x 10^2, 254 nm)
CPM (x 10^{-3})
Time (min)
0
2
4
6
0
10
20
30
1
2

Figure 29. Analysis of [^{32}P]ADP-ribosylated transducin and [^{14}C]ADP-ribosylated histone H2B by gel filtration HPLC. Mixtures (in Buffer A) of either (A) rat liver proteins and [^{32}P]ADP-ribosylated transducin, or (B) histones (Sigma II-A) and [^{14}C]ADP-ribosylated histone H2B were analyzed by gel filtration HPLC as described in the legend to Fig. 28 and "METHODS."


A
Vo
ADP-ribose
B
Absorbance (x 10^2, 254 nm)
CPM (x 10^{-2})
0
4
8
0
2
4
0
1
2
0
4
8
12
0
10
20
30
Time (min)

procedure (i.e., preincubation followed by column centrifugation) eliminated most of the trapped nucleotides, as well as most of the ADP-ribose residues bound to proteins via linkages that did not require hydroxylamine for release. Rat liver proteins were then separated by gel filtration HPLC and, fractions were treated with hydroxylamine and analyzed for ADP-ribose residues. As shown in Fig. 30, ADP-ribose residues were associated with every fraction. However, a major peak of protein-bound ADP-ribose eluted in the 40-60 kDa region. The amount of ADP-ribose residues decreased sharply in the region containing smaller proteins (i.e., smaller than 40 kDa).

Figure 30. Fractionation of rat liver proteins modified *in vivo* by mono(ADP-ribosyl)ation at arginine. The acid-insoluble fraction from rat liver (dissolved in Buffer A) was incubated at pH 7.0 and 37°C for 4 hr. After removal of free ADP-ribose by Sephadex G-25 column centrifugation, aliquots of the protein fraction were subjected to gel filtration HPLC. Fractions of 1 ml were collected and either analyzed for protein or incubated with neutral hydroxylamine and analyzed for ADP-ribose ("METHODS").


Molecular Mass (Daltons x 10^{-3})
V_o
335
100
44
39
17
14
ADP-ribose
Absorbance (254 nm)
0.15
0.10
0.05
0
ADP-ribose (pmol)
5
4
3
2
1
0
0
10
15
20
25
30
Time (min)

CHAPTER V

DISCUSSION

The identification of target proteins modified *in vivo* by cellular mono(ADP-ribosyl)transferases has been complicated by serious technical difficulties associated with detection and quantification of this posttranslational modification. Radiolabelled NAD^+ is not transported into cells and, thus, can not be used. The use of radiolabelled precursors of NAD^+, to which cells are permeable, is complicated by dilution into intracellular pools, as well as by intracellular metabolism resulting in incorporation of the precursors into a variety of macromolecular species. Hilz and coworkers have previously described methodology designed to quantify proteins mono(ADP-ribosylated) *in vivo* (120). This 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 radio-immunoassay. Although Hilz and coworkers have applied their methodology extensively in the analysis of protein-bound ADP-ribose residues from a variety of cell types, reports of such measurements by other investigators have not appeared. One potential difficulty for other laboratories in adopting this protocol is the requirement for production and rigorous characterization of highly specific antibodies against 5'-AMP.

There are several additional inherent limitations in this methodology as well. First, protein-bound mono(ADP-ribose) is present in tissues in very small amounts relative to potentially interfering substances, including AMP and the oxidized and reduced forms of NAD. Of particular concern are NAD^+ and NADH, which are converted to ADP-ribose under alkaline (156) or acidic (155) conditions, respectively. The trapping of only a small fraction of the total cellular content of these nucleotides in acid-insoluble fractions and their subsequent conversion to 5'-AMP could potentially lead to a large overestimation of ADP-ribose covalently bound to proteins. Furthermore, for analysis of samples as described by Hilz and coworkers, the acid-insoluble fraction (a dry powder) was only "suspended" in control or hydroxylamine-containing solutions. Since the sample was not actually dissolved, the possibility exists that some residues remained inaccessible during treatment, and would only be observed after incubation in hot alkali, a condition more likely to actually dissolve the acid-insoluble material.

Thus, experiments _in vivo_ have been hindered by the lack of a reliable and generally applicable method for the detection of mono(ADP-ribosylated) proteins. To circumvent this limitation, we have developed an assay designed to detect the products formed _in vivo_ by the known cellular mono(ADP-ribosyl)transferases, i.e., (ADP-ribose)-protein conjugates containing N-glycosylic linkages to arginine. The development of an assay for the quantitative detection of ADP-ribose residues bound to proteins via linkage to arginine was guided by several criteria. First, conditions for cleavage of ADP-ribose residues from proteins should permit the discrimination of (ADP-ribose)-arginine linkages from the other known linkages. Second, since none of the degradation products of ADP-ribose (i.e., ADP, AMP, adenosine and adenine) are unique, the structural integrity of the ADP-ribose molecule should be preserved following release from proteins. Third, the detection of ADP-ribose released from proteins should permit quantification of very small amounts of this nucleotide.

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 non-covalently bound nucleotides prior to chemical release of ADP-ribose from proteins. In addition, both of the affinity resins used in this study have been extensively characterized and have been shown to retain only those nucleotides containing two or more sets of 1,2 *cis*-diol groups, facilitating the separation of ADP-ribose from most other nucleotides and from the bulk of cellular nucleic acids and proteins (145). Such selectivity was essential for detection of small quantities of protein-bound ADP-ribose against the high "background" levels of adenine-containing compounds initially present. Additional selectivity was provided by the conditions employed for both the formation and detection of the fluorescent etheno-derivative of ADP-ribose (157,158). Finally, the resolution of etheno(ADP-ribose) from other nucleotides and nucleosides by anion exchange HPLC provided a third source of selectivity and, in combination with fluorescence detection, also provided the necessary sensitivity.

Mono(ADP-ribosylated) histone, containing an (ADP-ribose)-protein linkage of defined composition, was used to determine the conditions necessary for the quantitative cleavage of this type of linkage. Although it has yet to be unequivocally established, the bond between ADP-ribose and histone is almost certainly an N-glycosylic linkage between the 1-carbon of the terminal ribose and a guanidino nitrogen of an arginine residue. The preceeding conclusion is supported by several lines of evidence. First, the turkey erythrocyte enzyme used to synthesize the (ADP-ribose)-histone conjugate is specific for guanidino-containing acceptor molecules (92,108,152). Further, the chemical stability of the linkage was indistinguishable from that of ADP-ribose bound to agmatine (an arginine analog), which was also synthesized using the turkey erythrocyte transferase (152). The chemical stability of the (ADP-ribose)-histone linkage in the presence of hydroxylamine was also very similar to that reported for the linkage between ADP-ribose and the α-subunit of E. coli RNA polymerase, in which the covalent modification was shown to be an ADP-ribosylated arginine (81). Thus, using ADP-ribosylated histone as a model conjugate, two sets of incubation conditions were established, one in which the linkage was completely stable, and a second in which the bond was quantitatively cleaved. When tissue samples were then analyzed using the same two sets of incubation conditions, the majority of the ADP-ribose residues detected were bound to proteins via linkages with the same properties as those of the arginine linkage in the modified histone.

It should be pointed out that, while 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, non-enzymatically formed (ADP-ribose)-protein conjugates could be present *in vivo* (115), and cleavage of such linkages by hydroxylamine could complicate interpretation of the data presented here. However, recent studies have shown that the Schiff base adducts between ADP-ribose and poly(arginine), poly(lysine) and poly(histidine) were all considerably more stable than enzymatically formed (ADP-ribose)-arginine (Koch, R., Jacobson, M. K. and Hilz, H., manuscript in preparation).. In the presence of hydroxylamine (3 M, pH 7.0, 37°C), $t_{1/2}$ values for the non-enzymatic adducts were approximately 4 hr, as compared to the corresponding value of about 1 hr for the enzymatically formed conjugate (Fig. 20). These results, together with those showing the kinetics of release of ADP-ribose residues from proteins modified in vivo (Figs. 26 and 27), suggest that non-enzymatic adducts, if present in vivo, are not quantitatively significant.

However, it should also be pointed out that (ADP-ribose)-diphthamide, (ADP-ribose)-asparagine and similarly stable linkages may exist in vivo, in which case, these would not be detected at all with the incubation conditions employed. The latter possibility is strengthened by several recent reports. First, a cellular enzyme capable of ADP-ribosylation of EF-2 was identified in virus-transformed BHK cells (96) and in beef liver (96,159). 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^{+} (154).

Hilz and coworkers have presented evidence for the existence *in vivo* of two classes of (ADP-ribose)-protein linkages, which were distinguished on the basis of different chemical stabilities (120). One class, designated "hydroxylamine-sensitive," was released from acid-insoluble fractions during brief treatment with 0.4 M hydroxylamine at 37°C and neutral pH. The second class of linkages, designated "hydroxylamine-resistant," was released with strong alkali (1 M NaOH, 56°C, 1 hr). The N-glycosylic linkage to arginine was clearly "hydroxylamine-resistant," since more than 70% of the [^{14}C]ADP-ribose remained bound to histone after brief treatment with dilute hydroxylamine. However, as demonstrated here and in a recent report by Moss et al. (152), 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 (120). The overall values we obtained for mono(ADP-ribose) residues bound to rat liver proteins were 2- to 3-fold lower than those reported by Hilz and coworkers. However, it is interesting to note that our value of 5.50 nmol/mg DNA (31.8 pmol/mg protein) for "arginine-linked" ADP-ribose residues is reasonably close to their value of 7.12 nmol/mg DNA for ADP-ribose bound via "hydroxylamine-resistant" linkages (120). The levels of "arginine-linked" ADP-ribose residues we detected were about 400-fold higher than poly(ADP-ribose) levels (14.3 pmol/mg DNA) (160), but still represented less than 1% of the total NAD content in rat liver (126,132) (Table XI). 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 and coworkers (120,125,126,132).

The results described here are also at least 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) (98,99). 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 might be assumed to be subverting a normal

TABLE XI

Estimated Levels of NAD and ADP-ribose Residues In Adult Rat Liver

ADP-ribose Moieties Present As...	Amount Detected	
	pmol per mg protein	Relative Values
NAD^+ + NADH[a]	3,110	1.0
"Arginine-Linked" Mono(ADP-ribose)	31.8	0.01
"Glutamate-Linked" Mono(ADP-ribose)	9.7	0.003
Poly(ADP-ribose)[b]	0.083	0.00003

[a] Calculated from the data of Bredehorst, et al. (126).

[b] Calculated from the data of Jacobson, et al. (160).

regulatory process, are G_s and G_i, with masses in the same range as the *in vivo* modified proteins (39-45 kDa).

We have demonstrated the complete release from protein of one class of ADP-ribose residues under conditions in which the second class remained bound to protein (i.e., neutral pH in the absence of hydroxylamine). The half-life of the more labile class of linkages at neutral pH in both the presence and absence of hydroxylamine was very similar to that reported previously for ADP-ribose bound to histones via carboxylate ester linkages (116-119,153). It is also clear that the second, major class of (ADP-ribose)-protein linkages *in vivo* has properties very similar to those of N-glycosylic linkages to arginine residues (81,152). However, since the chemical identities of the linkages between proteins and (ADP-ribose) *in vivo* have yet to be established, we refer to these two classes as "arginine-like" and "carboxylate ester-like" only for the sake of simplicity. The work described here should provide the capability for isolating and identifying the individual acceptor proteins modified by ADP-ribosylation *in vivo*, as well as for rigorously identifying the amino acids which serve as acceptors in the modified proteins.

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