PREPARATION AND CHARACTERIZATION OF MODEL CONJUGATES FOR THE STUDY OF PROTEINS MODIFIED BY ADP-RIBOSE.

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PREPARATION AND CHARACTERIZATION OF MODEL CONJUGATES FOR THE STUDY OF PROTEINS MODIFIED BY ADP-RIBOSE.

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Modification of proteins by ADP-ribose has been shown to be a versatile modification with respect to the amino acid side chain used as acceptor.

The first part of this dissertation describes the preparation of model cojugates for potential amino acid acceptors for ADP-ribose. The chemistry of the linkages between ADP-ribose and the model conjugates was studied and used to develop selective chemical release conditions to differentiate different types of ADP-ribose linkages. Aplication of these conditions led to the identification in endogenous proteins of two new linkages characteristic of ADP-ribosyl acetals such as serine, threonine or tyrosine. The synthesis of a fluorescent derivative of ADP-ribosyl histidine led to the identification of histidine as endogenous acceptor of ADP-ribose.

The second part describes the preparation of model conjugates to study protein glycation by ADP-ribose. The chemistry of protein glycation by hexoses has been extensively studied; however the chemistry of glycation with

ADP-ribose is poorly understood. Model conjugates were prepared using *n*-butylamine and ADP-ribose and two stable products formed were shown to be ketoamines. The adducts could be distinguished from known enzyme catalyzed linkages by their stability in neutral hydroxylamine and their rapid release at pH 9.0. Unique release products were found following pH 9.0 hydrolysis and this information was used as a parameter to determine ADP-ribose glycation *in vitro*. Histone H1 with a high lysine content residues and its presence on chromatin where the turnover of ADP-ribose polymer after DNA damage occurs makes it a likely biological relevant protein. The studies reported here with glycated histone H1 by ADP-ribose showed that ketoamine adducts were formed.

The results described here will allow the study of the biological importance of ADP-ribose glycation and also allow differentiation on crude extracts between enzymatic modifications from protein ADP-ribose glycation that can occur due to the presence of NAD glycohydrolases.

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LIST OF ABBREVIATIONS

Abbreviations	Full Name
ADPR	Adenosine diphosphoribose
AGE	advanced glycosylated end products
CHES	2[cyclohexylamino] ethane sulfonic acid
Con A	concanavalin A
C_{18}	carbon 18
DHB	dyhydroxyboronyl
DNA	deoxyribonucleic acid
D_2O	deuterium oxide (deuterated water)
E. coli	*Escherichia coli*
MHz	megahertz
HPLC	high performance liquid chromatography
ε-ADPR	1, N6-etheno adenosine diphosphoribose
MOPS	3-[N-morpholino] propane sulfoni acid
NAD	nicotiamide adenosine dinucleotide
NADase	NAD glycohydrolase
NMR	nuclear magnetic resonance
PBA	phenylboronyl affinity
RNA	ribonucleic acid
TFA	trifluoracetic acid

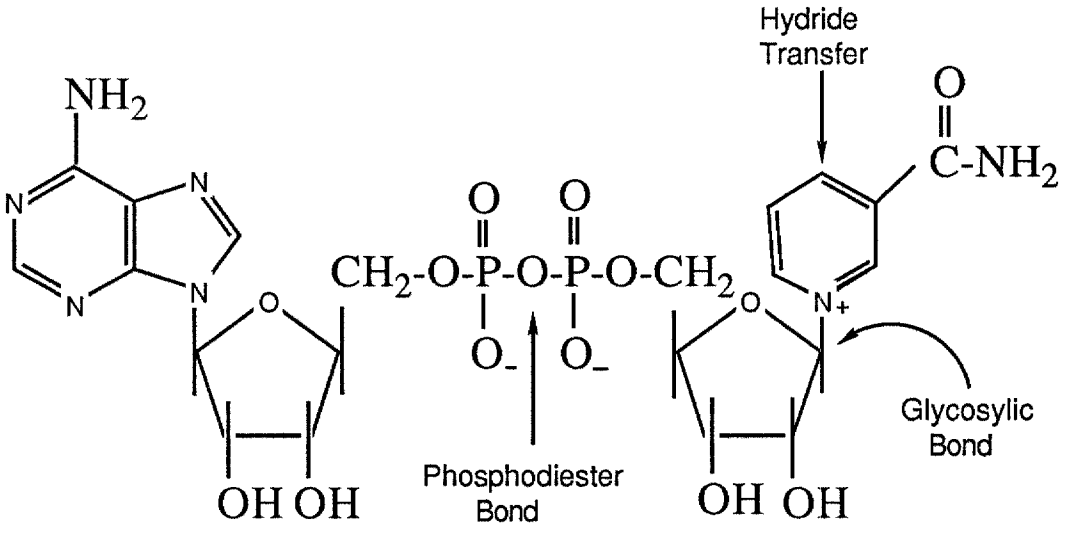
CHAPTER I

INTRODUCTION

NAD[1] is a very versatile molecule due in large part to the presence of multiple bonds with a high group transfer potential (Fig. 1). These linkages make NAD well suited to a number of group transfer reactions frequently encountered in biological chemistry.

The first class of reactions discovered to NAD were oxidation-reduction reactions. Here NADH uses its reduced pyridine ring to transfer a hydride ion to several different acceptors resulting in the formation of NAD. The enzymes that catalyze these reactions are called dehydrogenases. They are a very diverse group of enzymes that play a central role in the energy metabolism of the cell. Another type of reaction that uses a group transfer potential of NAD is catalyzed by DNA ligase from *E. coli* (65, 107). Here, the group transfer potential of the pyrophosphate bond is used to transfer the AMP moiety of NAD to the phosphate of the 5' terminal end of
a nicked strand of DNA. The pyrophosphate bond that is formed subsequently reacts with the hydroxyl group on the 3' terminal end to seal the DNA. Finally, NAD also uses the

Figure 1. Structure of NAD. The arrows indicate the bonds with high group transfer potential.


Hydride
Transfer
NH2
O
C-NH2
N
N
N
N
O
CH2-O-P-O-P-O-CH2
O
O
O-
O-
O
N+
Glycosylic
Bond
OH OH
Phosphodiester
Bond
OH OH

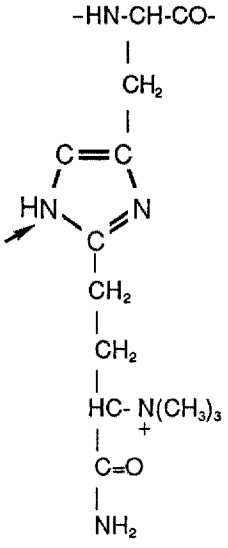
high group transfer potential of the glycosylic linkage between nicotinamide and ribose. The free energy of hydrolysis of this bond is used to make possible the transfer of ADP ribose to a variety of nucleophilic acceptors (Fig. 2). Transfer reactions involving ADP-ribose can be grouped into two general classes: those involving the formation of ADP-ribose polymers and those involving the transfer of a single ADP-ribose.

Poly(ADP-ribosyl)ation occurs in the nucleus of eukaryotic cells (44) and is catalyzed by poly (ADP-ribose) polymerase. Three distinct reactions are involved in the formation of the polymer. First there is a transfer of ADP ribose to the γ carboxylate from internal glutamate, the β carboxylate from internal aspartate or a carboxylate on the C terminal lysine of a histone protein. The next reaction is the sequential addition of ADP-ribose units using the anomeric carbon of the incoming ribose to form (1"-2') ribosyl-ribose bonds. This reaction generates linear chains of ADP-ribose. The third reaction consists of the formation of a branch on the linear polymer of ADP-ribose by using the anomeric carbon of an additional ribose to form (1'''-2'') ribosyl-ribose bonds (75,79).

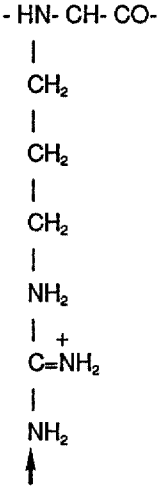
Three classes of monomeric ADP-ribosylation have been described: i) monomeric ADP-ribose protein transfer reactions, ii) NAD hydrolysis reactions, and iii) cyclic ADP-

Figure 2. Amino acids modified by mono and poly (ADP-ribosyl)ation. The arrows indicate the sites of modification.

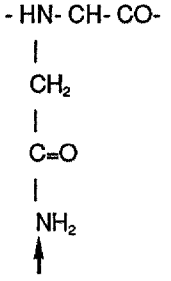
Diphtamide



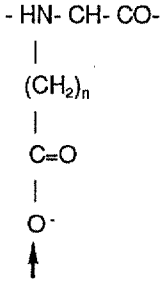
Arginine



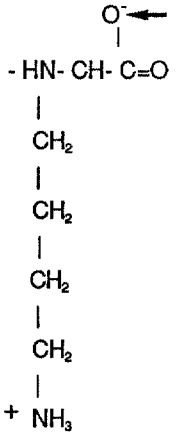
Asparagine



Glutamic acid (n=2)
Aspartic acid (n=1)



COOH-terminus (lysine)



ribose formation.

The best understood of the monomeric ADP-ribose transfer reactions are catalyzed by bacterial toxins that modify the activity of key regulatory proteins such as heterotrimeric G proteins and elongation factor 2 of protein synthesis (38). This monomeric ADP ribosylation occurs on a variety of acceptor nucleophiles in proteins. The amino acids modified are cysteine, arginine, asparagine, and diphthamide (Fig. 2). Animal cells also contain endogenous enzymes that catalyze monomeric ADP-ribose transfer to proteins (93,138, 145).

NAD can also be used by NAD glycohydrolases (NADases) as substrates that hydrolyze NAD to form free ADP-ribose and nicotinamide. This free ADP-ribose is sufficiently reactive to result in subsequent protein glycation. The specific function of NADases is not clear.

Finally, it has been found recently that the N^6-amino group of adenine can serve as a nucleophile to form a cyclic ADP-ribose molecule. The enzyme that catalyzes this reaction is ADP-ribosyl cyclase (62,63).

ADP-ribose polymer metabolism

The formation of ADP-ribose polymers involves three different reactions catalyzed by a single enzyme, poly (ADP-ribose) polymerase. These reactions include initiation of

the ADP-ribose chains on protein, linear elongation of the polymer and branching as described above. This enzyme also can catalyze NAD hydrolysis. The polymer size ranges from a few to over 250 residues. The protein acceptors of this polymer are nuclear proteins such as histones (97, 106, 131), lamins (29) and poly ADP-ribose polymerase itself (49, 101, 102). When poly (ADP-ribose) polymerase modifies itself, long polymers are produced. When histones and lamins are the acceptor proteins shorter polymers are produced (72). The high sensitivity to neutral hydroxylamine of the glycosidic bond formed between the first unit of ADP-ribose and protein has suggested that most of the poly (ADP-ribosyl) protein bonds are carboxyl esters (97). However, it has been reported that a minor portion of proteins modified by ADP-ribose polymers contains hydroxylamine resistant bonds (1).

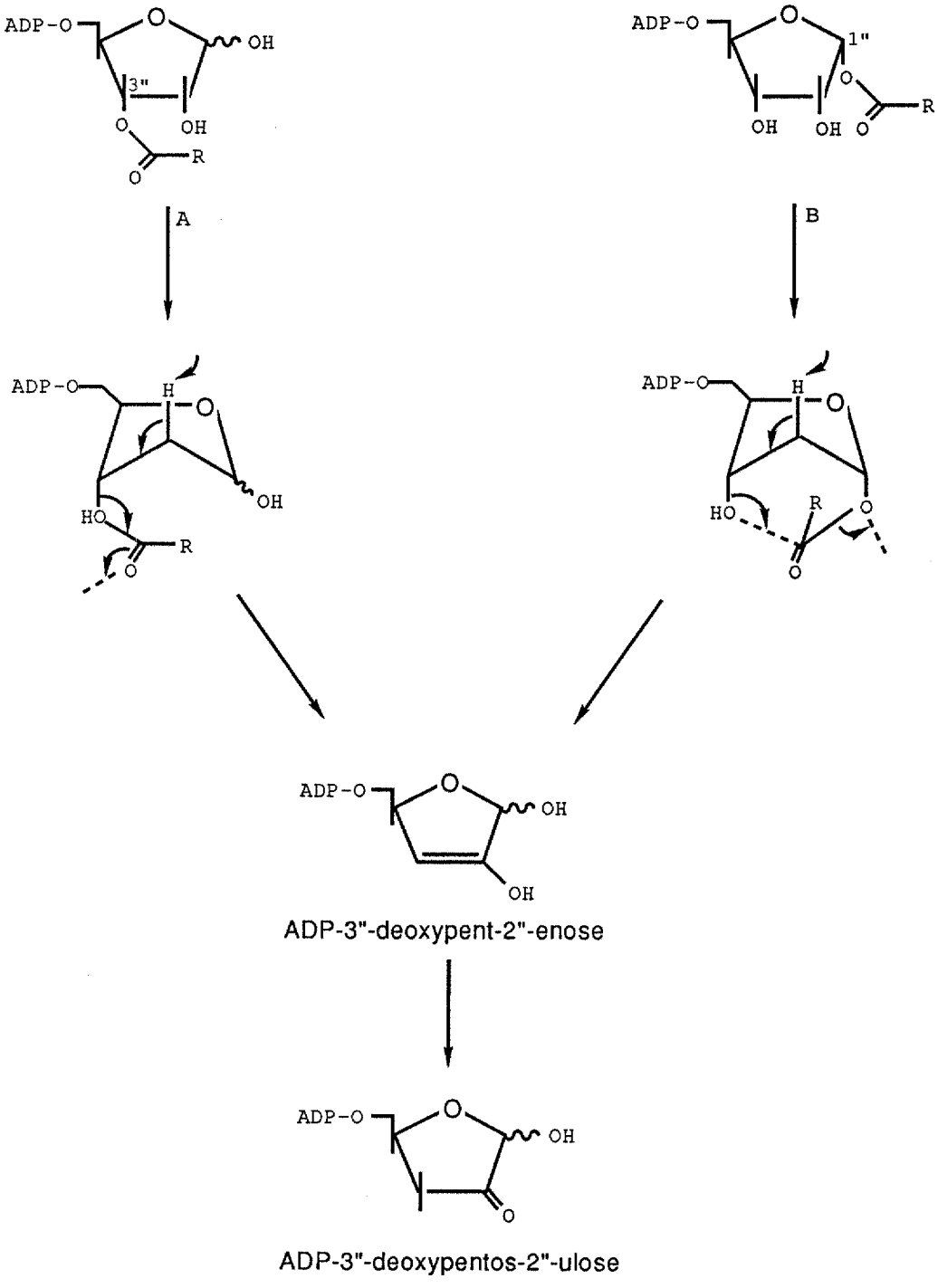
Poly(ADP-ribosyl)ation is a reversible event that is activated by DNA strand breaks (103, 143). There are two enzymes involved in the catabolism of the polymer. Poly ADP-ribose glycohydrolase (78, 80, 138), which splits the ribose-ribose glycosidic bond and ADP-ribosyl protein lyase (55, 105), which non hydrolytically cleaves the glycosidic bond between the last unit of ADP-ribose and the acceptor protein. The product of the glycohydrolase degradation of the polymer is free ADP-ribose. Mechanistically, this enzyme rapidly hydrolyzes long polymers in a processive manner, and short

polymers more slowly in a distributive manner (32). The implication is that the long polymers bound to poly (ADP-ribose) polymerase are shorter lived, and the short polymers of other acceptor proteins are longer lived. This suggests different functions for the different sizes of the polymer. It has been postulated that the rate limiting step in the turnover of the polymer is the removal of the protein proximal ADP-ribosyl groups from acceptor proteins (9, 140). This step is catalyzed by ADP-ribosyl protein lyase. The product of the reaction is a dehydrated form of ADP-ribose, ADP-3"-deoxypent-2"-enose, whose structure is shown in Fig. 3 (55).

Monomeric Protein ADP-ribose transfer reactions

Heterotrimeric guanyl nucleotide-binding proteins usually termed G-proteins are key factors for the relay of transmembrane signals received outside the cell to its interior (26, 119). For example, they connect the receptor for specific agonists such as hormones or neurotransmitters to the enzyme producing amplification of the signal through generation of a second messenger. The second messengers then lead to a cellular response. Enzymes modulated by G-proteins include adenylate cyclase, which produces cAMP from ATP, cGMP phosphodiesterase, which catalyzes the hydrolysis of cGMP to

Figure 3. Proposed mechanisms for the product formed by ADP-ribosyl protein lyase.


ADP-O
OH
3"
OH
O
R
O
A
ADP-O
1"
O
R
OH
OH
O
B
ADP-O
H
O
OH
HO
R
O
O
ADP-O
H
O
HO
R
O
O
ADP-O
OH
OH
ADP-3"-deoxypent-2"-enose
ADP-O
OH
O
ADP-3"-deoxypentos-2"-ulose

5'GMP, and phospholipase C, which forms diacyl-glycerol and inositol phosphate, and phospholipase A_2, involved in the formation of arachidonic acid.

G-proteins are composed of three different subunits: α, β, and γ (12, 26, 100, 119). Receptors that can either stimulate or inhibit adenyl cyclase upon binding of an agonist are connected to specific G-proteins, G_s and G_i, respectively. For visual excitation a rhodopsin receptor is connected to a G protein called transducin (Gt), which activates cGMP phosphodiesterase activity (24). G_o is yet another class of G-proteins that may be involved in regulation of ion channels (40, 66, 118, 129). The α-subunits of G-proteins bind, and hydrolyze GTP and interact with effector enzymes. The β subunits appear to be very similar in all G proteins (69) and the γ subunits seem involved in G-protein binding to the membrane via an isoprenyl group (142,94). The mechanistic basis for the receptor mediated activation of G-proteins has been extensively studied. For example, it is known that G-proteins are inactive when $G\alpha$ with bound GDP is associated with $G\beta\gamma$. Recently, it has been reported that the α subunit is myristoylated and this modification is required for interaction with the $\beta\gamma$ subunit (64). When this complex interacts with the agonist activated receptor the exchange of GDP for GTP occurs. This GTP binding exchange promotes the dissociation of the complex from the receptor and the release

of the α subunit from the trimeric complex. Then Gα·GTP interacts with effector while Gβγ is recycled. The turn-off mechanism of Gα involves hydrolysis of GTP to GDP by its intrinsic GTP hydrolase (GTPase) activity.

Bacterial toxins can alter the activity of α subunits by monomeric ADP-ribosylation. Apparently ADP-ribosylation affects the interaction of Gα subunit with the receptor. Different toxins use different G proteins as substrate for ADP-ribosylation. Cholera toxin and *Escherichia coli* heat labile enterotoxin (89) catalyze the transfer of ADP-ribose to Gsα and Gtα. This modification results in inhibition of Gα GTPase activity with stabilization of active [Gα.GTP] dissociated from Gβγ and persistent activation of adenylate cyclase(20). Modification by cholera toxin also promotes release of GDP from Gα, thereby opening the site for GTP binding and formation of the active Gα·GTP complex (16, 17). The amino acid modified is arginine through its guanidino group (113, 133). Cholera toxin also catalyzes ADP-ribosylation of free arginine, agmatine (87), automodification (91, 127) and other proteins not related to Gsα.

Pertussis toxin catalyzes the transfer of ADP-ribose to Giα, Goα, and Gtα (21, 52, 53, 96, 117, 134, 137). The modification occurs on the thiol group of a cysteine residue four amino acids from the carboxyterminus of Gtα(137). In the other two substrates of pertussis toxin there is also a

cysteine in the same position (135). This suggests that they may be ADP-ribosylated in the same location. ADP-ribosylation by Pertussis toxin stabilizes the inactive [Gα·GDP]Gβγ complex, which results in the interruption of signal propagation from agonist-receptor complexes (41, 60, 134) and prevention of formation of receptor G protein complexes with high affinity for agonist.

Monomeric ADP-ribosylation catalyzed by toxins is also involved in altering the activity of other key regulatory proteins in the cell. For example, diphteria toxin and *Pseudomonas aeruginosa* exotoxin catalyze the transfer of ADP-ribose to the amino acid diphtamide (108), which is a hypermodified histidine residue present in elongation factor 2 of protein synthesis. The elongation factor, which is required for growth of nascent polypeptide, is inactivated and protein synthesis is inhibited. This can result in cell death. Finally, *Clostridium botulinum* C2 toxin (4, 104) catalyzes ADP-ribose transfer to an arginine of non-muscle actin and *C. botulinum* C3 toxin modifies a group of 20 to 25 kilodalton membrane proteins on an asparagine residue (115).

The observation that key regulatory proteins are the target of the ADP-ribosylating bacterial toxins has suggested the possibility of the existence of endogenous enzymes that catalyze this transfer reaction. Indeed, enzymes in animal cells have been found that catalyze similar reactions to

cholera toxin and *E. coli* heat-labile enterotoxin. Moss has described a family of enzymes isolated from turkey erythrocytes (93, 138, 145) that catalyze the ADP-ribosylation of arginine, agmatine and histones with the same stereochemistry as the toxins, an α-ADP-ribosyl arginine linkage from β-NAD.

Two arginine mono ADP-ribosyltransferases, termed A and B, have been purified from cytosol. A similar transferase has been isolated from turkey erythrocyte membranes, termed C, and another from the nucleus, termed A'. It is interesting that some of these enzymes are stimulated by salt and histones, as is true for transferases A and A'. In fact the latter has been proposed to play an interrelated role with poly(ADP-ribose) polymerase in modulating chromatin structure.

Endogenous enzymes have also been found for the ADP-ribosylation of elongation factor 2 (61). This enzyme ADP-ribosylates a diphtamide residue of elongation factor 2 (EF2). Iglewski recently reported that mutant cells in EF2 can still be ADP-ribosylated by the endogenous enzyme but not by the bacterial toxins suggesting a difference in the active site of these enzymes. ADP-ribosylation by the cellular enzyme has been proposed to be involved in regulation of protein synthesis under an as yet unknown set of circumstances. Tanuma et al. (126) have reported a human

erythrocyte mono ADP-ribosyltransferase that modifies Giα on a cysteine residue. Because pretreatment with Pertussis toxin blocks the activity of this enzyme on Giα, it has been suggested that the same site is ADP-ribosylated with both enzymes.

Cyclic ADP-ribose

Cyclic ADP-ribose is a recent metabolite discovered by Lee and coworkers (62). The proposed structure corresponds to an ADP-ribose in which the 6-amino group of the adenine ring forms a glycosylic bond with the anomeric carbon of ribose. It is an ubiquitous molecule in mammalian and invertebrate tissues (62). The enzyme catalyzing this reaction has been termed ADP-ribosyl cyclase. Evidence is accumulating for the role of cyclic ADP-ribose as a second messenger involved in the mobilization of intracellular calcium by a pathway different from that regulated by inositol trisphosphate. The mammalian ADP-ribosyl cyclase is membrane bound. It is important to mention that a previously described NADase from *Aplysia californica* was found to be an ADP-ribosyl cyclase although it is a cytosolic enzyme (34, 63). This suggests the possibility that at least some membrane bound NADases found in mammalian cells could represent cyclase activity. If cyclic ADP-ribose is a second messenger, degradative

enzymes for cyclic ADP-ribose must be postulated. Evidence for the existence of this putative hydrolase of cyclic ADP-ribose has recently been obtained (Unpublished results).

NAD glycohydrolases

NADases catalyze the hydrolysis of NAD to free ADP-ribose and nicotinamide. These enzymes are found in both procaryotes (8, 123) and eukaryotes (11, 30, 45, 51). The specific properties of the enzymes depend on the source. When microorganisms are the source they are soluble, resist inhibition by nicotinamide and lack a transglycosylation reaction (146). This reaction refers to the exchange of the nicotinamide portion of NAD with a variety of other nucleophiles. In general, NADases from mammalian sources are membrane bound and have been reported in endoplasmic reticulum, plasma membrane, and nuclear membrane fractions (11, 30, 132, 147). They also function as transglycosidases. The biological function of NAD glycohydrolases is not known. The rationale for an enzyme that cleaves NAD to free ADP-ribose and nicotinamide is not apparent. It could be that NADases are ADP-ribosyltransferases whose physiological substrates have not been identified yet. The glycohydrolase activity of these enzymes could be due to the absence of a true substrate. It should be mentioned that protein, mono

ADP-ribosyltransferases can behave as NADases. Also, it could be that NADases, by producing high local concentrations of ADP-ribose, could modify specific proteins through glycation. In support of this idea, mitochondrial proteins have been found to be specifically ADP-ribosylated by glycation (35, 125). The other possibility is that the combined action of ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activity mimic NADase activity in membrane preparations.

Although the physiological function of NADases is not known, they have been extensively studied mechanistically. The use of different nucleophiles has helped to elucidate the substrate requirements for the exchange reaction of the different mammalian NADases. For example, alcohols with a linear chain from 1 to 7 carbons have been studied as substrates (144). Facilitation of the alcoholysis reaction with an increase in chain length was observed. This suggests the presence of a hydrophobic pocket in the active site of the enzyme. It was also found that the formation of pyridine nucleotide analogs relative to the hydrolytic product, free ADP-ribose, correlated well with the nucleophilicity of the pyridine bases studied (144). Imidazole derivatives were not as effective substrates as the pyridine bases but were more effective than alcohols as ADP-ribose acceptors in the transglycosylation reaction (5). It is also interesting that

among different imidazoles the rate of formation of products varied dramatically. For example, it was found that histamine is a better substrate than imidazole or histidine. In fact histidine was a very poor substrate. When imidazole acetic acid was used, no product was detected. By comparing these results, there is an indication that a negative charge provided by the carboxylate group decreases the formation of product, and a positive effect is seen by the presence of a positive charge. This correlates well with the postulation that a carboxylate group is present in the active site of mammalian NADases.

Many potentially useful nucleophiles, such as guanidine and alkylamine compounds have been tested in the transglycosidation reaction without success (144). In alkylamines, the small proportion of deprotonated amino groups at pH 7.6 and the lability of the aminoriboside formed can explain the lack of success in detecting a product.

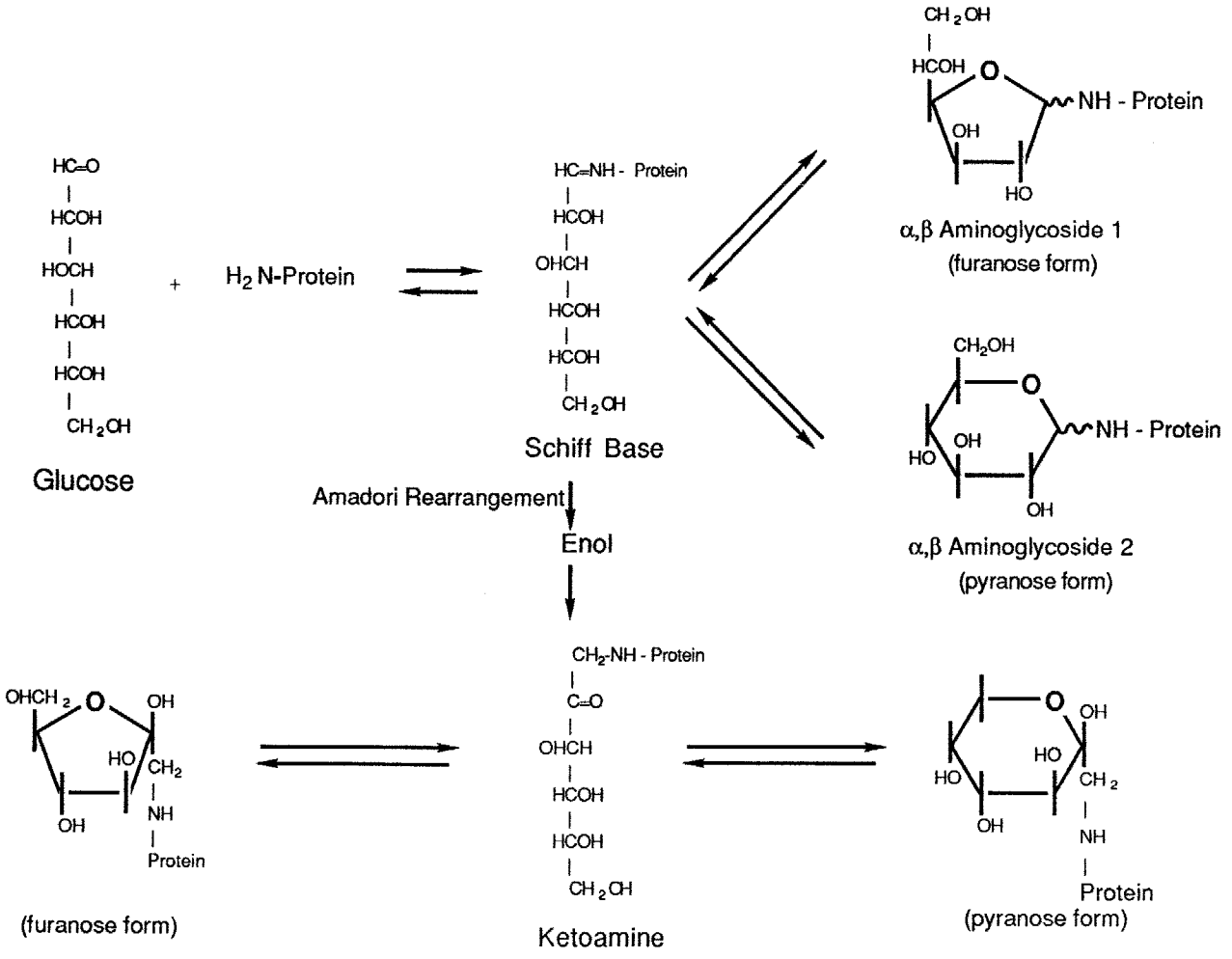
The poor stability of mammalian NADases is a disadvantage in their use as biosynthetic tools. However Anderson and coworkers (144) characterized a NADase from *Bungarus fasciatus* that is stable at 37 °C and pH 7.6 and performs the transglycosylation reaction. Also, this enzyme can be immobilized on Con A-Sepharose without any loss in stability or catalytic activity. This provided us with an attractive reagent to prepare ADP-ribosylated conjugates to study the

chemistry of linkages between ADP-ribose and proteins, which is one of the topics of this dissertation.

Glycation of proteins

It is well established that a reducing sugar can react through its aldehydic group with amino groups of proteins. Investigations in food research for many years have targeted glycation of proteins involved in the browning of food (37). This reaction can generate several possible products. These are shown in Fig. 4 using glucose as the source of reducing sugar. It is known that an amino group of lysine nucleophilically attacks the electrophilic carbonyl group provided by the aldehydic function of the reducing sugar. The first product formed following dehydration is a Schiff base. This can be stabilized by the formation of both a furanose ring and a pyranose ring, and these products are termed aminoglycosides. The Schiff base can also rearrange by an intramolecular oxidation reduction reaction to form an enol. The latter can tautomerize to a ketone. This reaction, known as an Amadori rearrangement, produces a ketoamine that also can be stabilized by the formation of either pyranose or furanose rings. The aminoglycoside can, through its Schiff base, give rise to the original lysine and

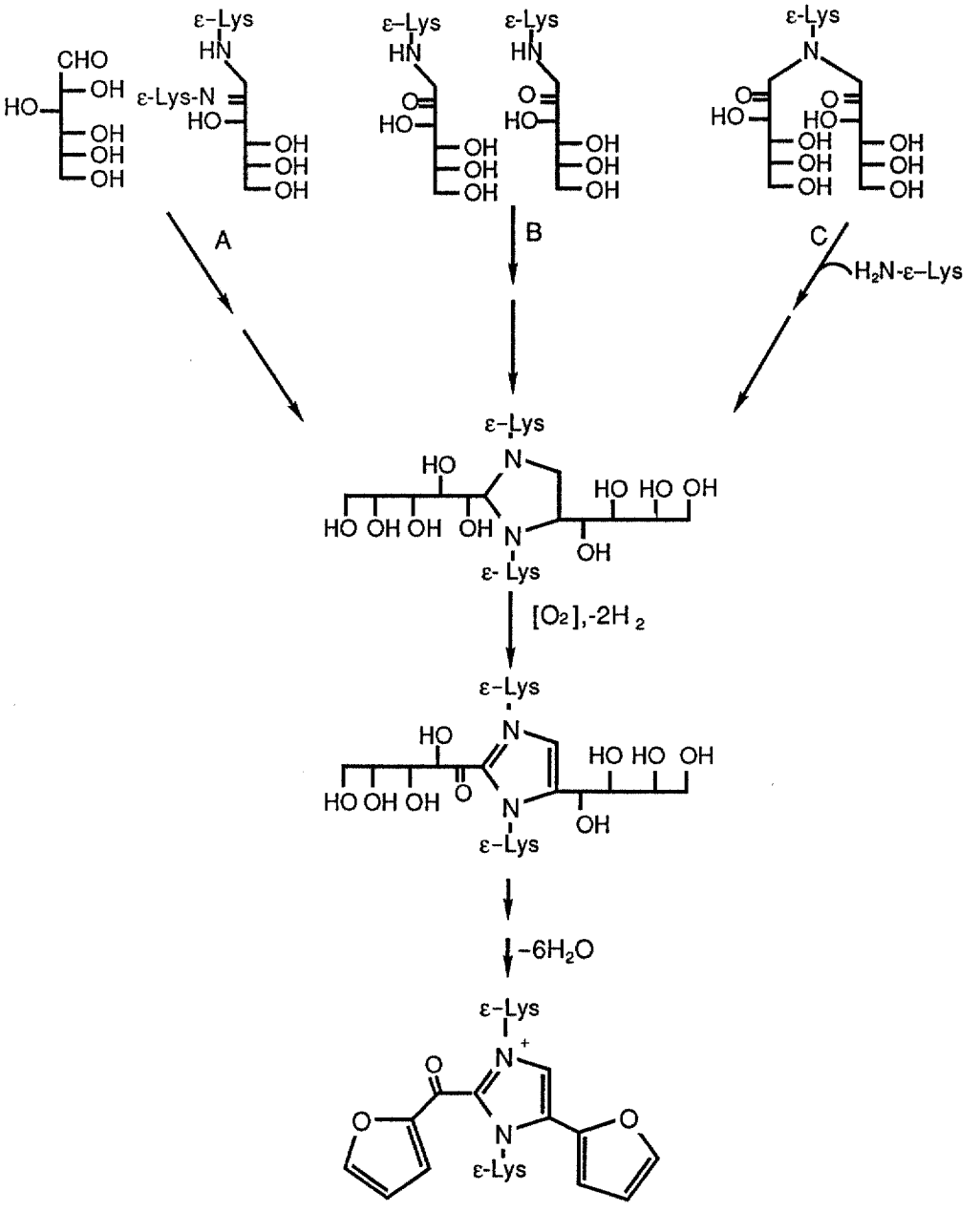
Figure 4. Possible products of the reaction of glucose with amino groups from lysine residues of proteins.


HC=O
HCOH
HOCH
HCOH
HCOH
CH2OH
Glucose
+
H2N-Protein
HC=NH - Protein
HCOH
OHCH
HCOH
HCOH
CH2OH
Schiff Base
Amadori Rearrangement
Enol
CH2OH
HCOH
O
NH - Protein
OH
HO
α,β Aminoglycoside 1
(furanose form)
CH2OH
O
NH - Protein
HO
OH
OH
α,β Aminoglycoside 2
(pyranose form)
OHCH2
O
OH
HO
CH2
NH
Protein
(furanose form)
CH2-NH - Protein
C=O
OHCH
HCOH
HCOH
CH2OH
Ketoamine
O
OH
HO
CH2
NH
Protein
(pyranose form)

sugar from which it was formed. The ketoamine has been reported to be more stable than the aminoglycoside but can slowly decompose to give a variety of fluorescent chromophoric products that give a brown color to solutions containing them. It should be mentioned that glycation also can occur by an oxidative pathway (2, 3).

Considerable interest has been generated in the study of the effects of glycation on the structure and function of proteins *in vivo* (31, 42). *In vivo*, the most important factors for glycation are the reducing sugar concentration and incubation time. Diabetes mellitus results in higher than normal amounts of blood glucose for prolonged periods of time. This leads to the potential glycation of proteins, which may alter their structure and thus their function. It is known that once the ketoamine is formed it can slowly react intra or intermolecularly with other amino groups. Further rearrangements create a very reactive fluorescent adduct, referred as advanced glycosylation end product (AGE). These can crosslink proteins (18, 19) and modify their structure and function (Fig.5). It has been proposed, for example, by Cerami and others that highly reactive fluorescent adducts formed from long lived Amadori glycosylated proteins contribute to the pathophysiological condition of Diabetes mellitus (54). This mechanism has been proposed to be at least partially responsible for the

Figure 5. Proposed mechanism for advanced glycosylation end product (AGE) formation and its involvement in the protein-protein cross-linking.



2-furoyl-4(5)-(2-furanyl)-1H-imidazole (FFI)

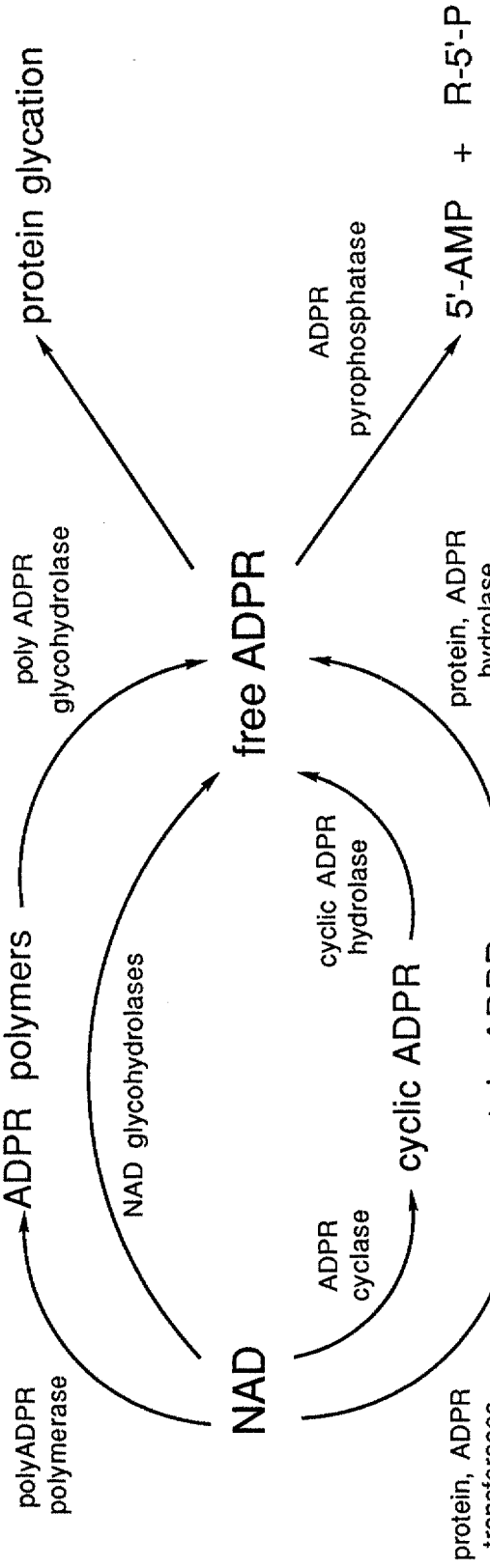
accelerated cataract formation associated with this disease. Also this phenomena has been implicated in the aging of long lived proteins such as lens crystallins, collagen, elastin and myelin proteins (81). It has been suggested that glycosylated collagen or elastin could promote lipid accumulation in the arterial wall by covalently trapping LDL, via the highly reactive post Amadori degradation products. There is evidence that fluorescent adducts can either trap soluble plasma proteins, act as signals for macrophage recognition and uptake (14,15), or induce mutations in double stranded plasmid DNA (141).

Recently, 2 furoyl-4(5)-(2-furanyl)-1H-imidazole (FFI) has been identified as one of these fluorescent adducts (110). Its structure is shown in Fig.5. It is noteworthy that nucleic acids are long lived molecules in the resting cell and their glycation might be implicated in the process of age dependent changes in the genetic material (141).

Protein glycation can occur with several different sugars found in the cell such as glucose, glucose 6-phosphate (10), glyceraldehyde 3-phosphate (39), and in the focus of this glycation of proteins by hexoses other sugars may behave differently.

Studies of ADP-ribose transfer reactions have shown that free ADP-Ribose is a product of many metabolic pathways. These are summarized in Fig. 6. Thus, this complex pentose nucleotide is a likely participant in the glycation of

Figure 6. Metabolic pathways for the formation and utilization of free ADP-ribose.


protein glycation
poly ADPR glycohydrolase
ADPR pyrophosphatase
5'-AMP + R-5'-P
ADPR polymers
free ADPR
protein, ADPR hydrolase
NAD glycohydrolases
cyclic ADPR hydrolase
cyclic ADPR
protein-ADPR
polyADPR polymerase
ADPR cyclase
NAD
protein, ADPR transferases

proteins. There are at least two fates of free ADP-ribose. One involves the possible modification of proteins through glycation and the other involves its catabolism by a highly specific ADP-ribose pyrophosphatase (73). The existence of the latter suggests the importance for the cell to have a mechanism to minimize protein glycation by free ADP-ribose. The possible effects of ADP-ribose glycation related to ADP-ribose polymer metabolism are particularly interesting due to its intimate interaction with chromatin proteins and DNA. The turnover of the polymer is very fast in response to DNA damage with a half-life of approximately 1 min (47). This generates high local concentration of ADP-ribose in the microenvironment of the chromatin. Taking this into account it is likely that this sugar may contribute to the slow glycation of proteins, especially histones, which have a high lysine content.

It is known that there is a sharp age-related decline of the amount of transcription *in vivo* and in the RNA template activity of chromatin in vitro. There have been indications that DNA in active regions of chromatin is more vulnerable to damage such as single stranded breaks, dimers, and other modifications (70). Such damage, selectively accumulated in active chromatin, could reduce and alter its transcriptional potential without serious effect on chromatin higher order structure. The age-related increase in the denaturation

temperature of DNA in chromatin depends on several different biochemical factors. These include: crosslinks between DNA strands, increase of the proportion of chromatin proteins tightly bound to DNA, and possible increase of different crosslinks between chromatin proteins, which render chromatin structures more compact. Any of the events described above could involve glycation. This suggests that when poly ADP-ribose formation is involved in DNA repair, the result of this metabolism may significantly contribute to the glycation of the chromatin proteins over time. In addition the possible glycation of DNA and chromatin proteins could contribute to the aging of the cell. It is important to design strategies to study possible changes in chromatin proteins due to glycation by ADP-ribose and its accumulation with both the age and the frequency of DNA damage.

In contrast to the well studied glycation of proteins using hexoses such as glucose, the ADP-ribosylation adducts formed with proteins have not been well characterized. Kun *et al.* (59) have reported that stable adducts are formed when ADP-ribose and ribose react with peptides such as polylysine and proteins although the adducts formed were not characterized. Hilz *et al.*(35) more recently have reported specific proteins that are non enzymatically ADP-ribosylated in liver mithochondria. To elucidate the nature of the amino acid modified in the mitochondrial proteins, three likely ADP-

ribosylated polypeptides were prepared using polylysine, polyhistidine and polyarginine. By comparing the conditions required for release of ADP-ribose from the peptides with the mitochondrial proteins, Hilz and coworkers (35) concluded that the amino acid modified in the latter must be an amino acid other than arginine, histidine or lysine. The lack of knowledge about the specific adducts formed with either the ADP-ribosylated peptides reported by Kun or the ADP-ribosylated proteins found by Hilz makes it very difficult to assess the nature of the products formed.

Chemical Stability of the linkages between ADP-ribose and proteins

ADP-ribose modification of proteins can occur through both enzymic and glycation mechanisms. In the enzymic reactions catalyzed by ADP-ribose protein transferases, a number of amino acids can serve as acceptors for this modification. The glycation reaction using free ADP-ribose involves glycation of proteins at lysine residues or amino terminal groups. Thus, our current knowledge clearly shows the versatility of ADP-ribose as a biological group transfer agent. To understand the biological relevance of the different classes of ADP-ribose transfer reactions, it is important to distinguish between an enzymic vs glycation

reactions and, if enzymic, which class of transferase may be involved. Thus, another major aim of this dissertation was to study the chemistry of the linkages between ADP-ribose and the different acceptors found in proteins.

Two overall approaches have been used to study the different types of ADP-ribose linkage chemistry, namely the study of protein models and the study of low molecular weight model conjugates. The use of protein models involves proteins modified by a bacterial toxin or a cellular mono-ADP-ribosyltransferase for monomeric transfer reactions and the combined action of poly(ADP-ribose) polymerase and poly (ADP-ribose) glycohydrolase for polymeric transfer reactions. Finally the incubation of free ADP-ribose with histones or polyamino acids has been used for glycation reactions (59). The range of linkages involved in monomeric transfer has been expanded in recent years from the glycosylic linkages involving diphtamide and arginine to include asparagine and the thioglycosidic bond formed with cysteine.

The relative stability to chemical treatment with neutral hydroxylamine has been important in the development of our understanding of ADP-ribose linkage chemistry. The properties of hydroxylamine that makes it suitable for the chemical characterization of the glycosylic and glycosidic linkages are due to its nucleophilic character. To be reactive, a nucleophilic reagent requires the presence of an

electrophilic center. For poly(ADP-ribose) linkages involving a glycosidic bond between a carboxylate from internal glutamates and aspartates or C terminal lysines, the electrophilic center is provided by a carbonyl group in the ADP-ribosylated adduct formed. This linkage when treated with the highly nucleophilic reagent hydroxylamine is very reactive. Alkaline conditions such as 1 M NaOH also break this glycosidic bond from the protein. In contrast, diphtamide, arginine, and cysteine have important differences in their sensitivity to treatment with hydroxylamine. In diphtamide (Fig.2), the chemical stability of the glycosylic linkage formed with ADP-ribose is due to the specific chemical properties of the modified imidazole ring. First this ring lacks a target for a nucleophilic reagent, such as hydroxylamine or NaOH. In addition the nitrogen of the imidazole ring involved in the formation of the glycosylic linkage is a weak base due to the participation of its pair of electrons in the resonance of the ring. This makes this linkage very resistant to hydrolysis with neutral hydroxylamine and with either strong alkali or minerals acids.

The first report of an ADP-ribosyl arginine linkage was shown in the α polypeptide of RNA polymerase from *E. coli* after infection with the bacteriophage T_4 (27). In this report the protease digestion of the modified protein

produced two peptides, Thr-Val-Arg and Val-Arg. This data suggested that arginine was involved in the glycosylic linkage with ADP-ribose due to the presence of its nucleophilic group. The next question was which nucleophilic group of the C-terminal arginine was involved in the glycosidic linkage: the C-terminal carboxylate or the guanidino group. The $t_{1/2}$ obtained with different chemical conditions such as hydroxylamine (4 M, pH 4.8, 37°C; $t_{1/2}$ 105 min) and alkali (0.25 M NaOH, 37 °C; $t_{1/2}$ 75 min) suggested that a guanidino group from arginine was involved in the formation of the glycosylic bond. The possibility of a carboxylate linkage was discarded due to the relative stability in alkali conditions. Fig. 2 shows the structure of the adduct formed with arginine. The selective release conditions for this linkage were developed in this laboratory by Payne *et al.* (109). A histone was used as a subtrate for a specific arginine ADP-ribosyl transferase. This modified histone was used as model for selecting specific chemical release conditions for this linkage. It was found that 1 M neutral hydroxylamine at 37 °C released 95 % of the ADP-ribose bound to histone in 6 h. This chemical treatment made it possible to distinguish the arginine linkage from diphtamide (EF-2) and cysteine (transducin), which were shown to be stable under these conditions (109). Although ADP-ribose was reported to be the product of this reaction, the most likely

initial product formed is the oxime of ADP-ribose. By using this selective chemical release, Payne, *et al.* were able to detect endogenous rat liver proteins with linkages indistinguishable from ADP-ribosyl arginine.

Fig. 2 shows that the carbon from the guanidino group constitutes an electrophilic center. The center formed by the presence of three nitrogens bound to a carbon is less reactive than the one formed using a carbonyl group. This is due at least in part to the higher electrophilic character of oxygen as compared to nitrogen. This is reflected in the kinetics of hydrolysis catalyzed by hydroxylamine. Arginine ADP-ribose requires at least 6 h at 37 °C with hydroxylamine for release of the glycosylic bond while O-carboxylate linkages from poly ADP-ribosylated proteins require minutes.

Recently, selective chemical release conditions have been developed for distinguishing cysteine (46) from arginine, dipthtamide and carboxylate ADP-ribose linkages. For the development of this selective release condition, transducin ADP-ribosylated at cysteine, was used as a model. Mercuric salts are known to break down thioglycosidic bonds (56) from sugars. Meyer *et al.*(71) used mercuric salts with pertussis toxin modified transducin to show that ADP-ribose was released from the thioglycosidic linkage as expected. Jacobson et al. showed that these conditions did not alter the other linkages and thus mercuric salts have allowed the

detection of endogenous rat liver proteins modified at this amino acid (46). Although the exact mechanism for the attack of this linkage by mercuric ion is not known, the formation of a transition complex of mercury with sulfur may be involved in the lability of the linkage.

The asparagine linkage similarly to the diphtamide linkage, is resistant to all known chemical release conditions tested despite having a nucleophilic center, the carbonyl group in the amide function. Apparently the delocalization of electrons between the carbonyl group and the nitrogen partially neutralizes the positive density charge on the carbon, similar to the peptide bond of proteins. This makes the nucleophilic attack by reagents such as hydroxylamine, and alkali ineffective.

Research Prospectus for this dissertation

ADP-ribosylation protein transfer reactions involve the transfer of ADP-ribose to a variety of nucleophilic groups on the side chain of amino acids of proteins as discussed above. This is a very versatile posttranslational modification with respect to the protein substrate and to the amino acid to which ADP-ribose is bound since cysteine, arginine, asparagine, diphtamide (a hypermodified histidine), aspartate and C-terminal lysine are known to be modified. This

versatility makes likely the existence of other potential nucleophiles from the side chain of amino acids that might be modified by ADP-ribosylation.

One of the aims of this dissertation is to test this possibility. Further, this research aimed to study the basic chemistry of the known and other potential linkages between protein nucleophiles and ADP-ribose. It is important to understand the basic chemistry of the different glycosidic linkages formed between ADP-ribose and the amino acid in order to elucidate the biological function(s) of ADP-ribose transfer reactions. Fig. 7 shows the experimental approach that has been previously developed in this laboratory to search for endogenously ADP-ribosylated proteins. This protocol is dependent on the selective chemical release of ADP-ribose from the modified amino acid. One approach to this problem has involved the use of models proteins modified at known amino acids by bacterial toxins. There are several limitations regarding this approach. First modified protein models are required in large amounts for structural characterization. Second, the complexity of the data obtained increases with the size of the molecule. Third, the number of known toxins that are ADP-ribosyltransferases limit the range of amino acids that may be modified. To overcome these difficulties we have chosen an alternative approach, which involves the synthesis of low molecular weight model

Figure 7. Procedure for detection of endogenous proteins covalently modified by ADP-ribose monomers

Detection of Endogenous Proteins Covalently Modified by ADP-ribose Monomers

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Protein

↓

Removal of non covalently bound ADP-ribose

↓

Selective chemical release of intact ADP-ribose

↓

Isolation of ADP-ribose using boronate resin

↓

Formation of fluorescent derivative of ADP-ribose

↓

Quantification by HPLC-fluorescence monitoring

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conjugates for this study. These can be prepared in large amounts, facilitate the interpretation of the data obtained with characterization techniques, and increase the spectrum of model conjugates that can be studied.

Another major aim of this dissertation was to achieve the chemical and structural characterization of conjugates formed with ADP-ribose and butylamine to study the chemistry of the linkage between ADP-ribose and lysine from proteins. These characterizations will allow the search for proteins modified at lysine residues or N-terminal an amino groups *in vivo* and also to distinguish protein, mono-ADP-ribose transferase activities from non enzymic reactions in *in vitro* experiments.

CHAPTER II

MATERIALS

ADP-ribose, *n*-butylamine, MOPS, NAD, L-arginine hydrochloride, histidine hydrochloride, snake venom (*Bungarus fasciatus*), imidazole, pyrophosphate (tetrasodium salt), L-serine ethyl ester (hydrochloride), L-threonine methyl ester (hydrochloride), Concanavalin A Sepharose 4B, and CHES were purchased from Sigma Chemical Co. An analytical HPLC column (C_{18} mBondapak 10 mm 3.9 mm x 300 mm) was purchased from Waters. A preparative HPLC column (C_{18} Dynamax-300A, 5mm x 300 mm) was from Rainin. Hydroxylamine hydrochloride, Acetonitrile (HPLC grade) and ammonium formate were from Fisher Scientific. D_2O, 1,2 o-dinitrobenzene, chloroacetaldehyde, ethanol (99.8 and 99.9 %), premium NMR tubes, 5mm, were from Aldrich Chemical Co. Potassium phosphate, water (omnisolv) and propanol was from EM Science. PBA-30 was from Amicon. Methanol (HPLC grade), and isobutyl alcohol was from Mallinckrodt. Acrylamide was from United States Biochemical Corp. N, N'-Methylene-bis-acrylamide was from Bio-Rad. Trifluoroacetic acid (HPLC grade) was from Applied Biosystems. Ecolume was from ICN Biomedicals.

CHAPTER III

METHODS

Immobilization of Crude Snake Venom NADase

Sixty six mg of crude *B. fasciatus* snake venom was dissolved in 10 ml of 10 mM potassium phosphate, pH 7.0. This solution was incubated at 37 °C with 1 mM EDTA to irreversibly inactivate phosphodiesterase. The enzyme preparation was incubated overnight at 4 °C. Then after filtration the NADase solution (10 ml), containing 42 units of NADase activity, was incubated with 10 ml of packed Con A Sepharose gel in a total of 50 ml of 50 mM potassium phosphate, pH 7.5. After 30 min stirring at 37 °C all the NADase was bound to the gel and only a trace of phosphodiesterase activity remained. The Con A-Sepharose bound NADase gel was washed three times with potassium phosphate, 50 mM, pH 7.5 and assayed for NADase and phosphodiesterase activities. The enzyme was stored to a final volume of 50 ml of 50 mM sodium pyrophosphate and potassium phosphate, pH 7.5 at 4 °C.

Reaction of n-butylamine and ADP-ribose

The reaction mixture contained 100 mM potassium phosphate buffer, pH 8.0, 25 mM ADP-ribose, 3 M *n*-butylamine. Incubation was at 37 °C. Aliquots were taken at 0, 4, 8, and 12 h and diluted to 1 ml with 50 mM potassium phosphate buffer, pH 8.0, and subjected to analytical reversed-phase HPLC. The elution was perfomed isocratically with 100 mM potassium phosphate, pH 6.0, 5% methanol, at a flow rate of 1 ml/min at room temperature. Detection was at 254 nm using an ISCO model 228 detector.

^{1}H and ^{13}C NMR analysis of products 1 and 2

Fifty ml of the reaction of *n*-butylamine and ADP-ribose were prepared. The products were purified using a preparative C_{18} Dynamax-300A 5 mm column using 5 mM potassium phosphate and 1% methanol at pH 6.0 for isocratic elution. Fractions containing the eluted peaks were pooled, and lyophilized. This sample was adjusted to pH 6.0 and washed twice with 99.8% D_2O and once with 99.9% D_2O. The final sample was dissolved in 99.9% D_2O containing 50 mM potassium phosphate, pH 6.0 to a final concentration of 7 mM.

A Varian XL-300 NMR Spectrometer operating at 299.9 MHz for ^{1}H and 75.4 MHz for ^{13}C was used to acquire spectral data

^{1}H NMR spectral parameters were as follows: sweep width,

4400 Hz; data points, 32K; acquisition time, 3.6 s; acquisition delay, 1 s; 32 acquisitions in double precision mode. ^{13}C NMR spectral parameters were as follows: sweep width, 18000 Hz; data points, 32 K; acquisition time, 1 s; acquisition delay, 2 s; 20000-25000 acquisitions in double precision mode. ^{1}H spectra were referenced to HOD at 4.68 ppm and ^{13}C spectra were referenced using the referencing software of the instrument.

Colorimetric reaction of products 1 and 2 with o-dinitrobenzene

To a reaction mixture containing 5 mM potassium buffer, pH 6.0 and 60 µmoles of products 1 or 2, was added 50 ml of (20 %, w/v) sodium hydroxide. This reaction mixture was stirred for one minute. Subsequently, 100 µl of an aqueous solution of 0.2 % o-dinitrobenzene were added. The complete reaction mixture was kept on ice and stirred. After one minute the appearance of a purple color indicated the presence of the ketoamine.

Reaction of products 1 and 2 with hydroxylamine

The reaction mixture contained 100 mM MOPS buffer, pH 7.0, 100 mM purified products 1 or 2, and 1 M hydroxylamine. At 0, 20, 40, 60, and 80 min aliquots were diluted to 1 ml with 50 mM potassium phosphate buffer, pH 6.0, and subjected to

analytical reversed-phase HPLC as described above.

^{1}H NMR analysis of oximes from products 1 and 2

The purification procedure and preparation of the oximes for ^{1}H NMR analysis were the same as described for products 1 and 2.

pH 9.0 hydrolysis of ^{14}C products 1 and 2

The reaction mixture contained 100 mM CHES buffer, pH 9.0, 100 µM purified ^{14}C products 1 and 2. Incubation was at 37 °C. At 0, 20, 40, and 60 min aliquots were diluted to 1 ml with 100 mM potassium phosphate buffer, pH 6.0 and subjected to analytical reversed-phase HPLC. An isocratic elution was used with 50 mM potassium phosphate, pH 5.0 and room temperature. Fractions were collected and counted in a scintillation counter.

Borohydride reduction of ketoamines 1 and 2

To a reaction mixture containing 100 µM ketoamines 1 and 2, 50 mM sodium phosphate, pH 6.0, 100 mg of sodium borohydride was slowly added at 0 °C and the pH was adjusted to 7.0. After the addition of borohydride was completed the reaction mixture was incubated at 25 °C for 1 h at pH 5.0.

Preparation and purification of etheno derivatives of pH 9.0 hydrolysis products of ketoamines 1 and 2

One hundred µl of a 20 µM solution of pH 9.0 hydrolysis products from ketoamines 1 and 2 in 200 mM CHES buffer were diluted in 5 ml of 1 M ammonium formate buffer, and the pH was adjusted to 4.6. Eighty µl of cloroacetaldehyde were added. This reaction mixture was incubated for 4 h at 60 °C in the dark. After completion, 5 ml of 1 M ammonium formate was added and the pH was adjusted to 8.6. This solution was added to a previously equilibrated PBA-30 column. The column was washed with 10 ml of 1 M ammonium formate buffer at pH 8.6. Then 3 ml of water were added followed by 3 ml of 1 M ammonium formate pH 4.6.

Preparation of ADP-ribosyl arginine

A 100 µl solution containing 2.5 mM NAD, 100 mM Arginine and 50 mM potassium phosphate pH 7.5 was added 5 µl of mono ADP-ribosyl transferase A. A control without adding transferase A was run parallelly. 5 ml aliquots were diluted to 1 ml potassium phosphate 100 mM and injected to a reversed-phase HPLC. The elution was performed isocratically in 100 mM potassium phosphate, 5 % methanol, pH 6.0 with a flow rate of 1 ml/min. Nucleotides were detected using uv at 254 nm.

Preparation of ethyl, methyl, and propyl ADP-ribose

The reaction mixture contained 2.5 mM NAD, 15 ml of immobilized NADase, 50 mM potasium phosphate and 250 mM methanol, 250 mM ethanol or 250 mM propanol and the final pH was 7.5. The incubation was at 37 °C for 24 h. The appropriate controls were run parallelly. Aliquots were diluted to 1 ml potassium phosphate 100 mM, pH 6.0. A similar HPLC system was used as described above for arginine ADP-ribose preparation

Preparation of propionyl ADP-ribose

A 100 µl reaction mixture containing 2.5 mM NAD, 20 µl of immobilized NADase, 50 mM potassium phosphate, and 1 M propionic acid, at pH 6.0 was incubated at 37 °C for 24 h. 10 µl aliquots were diluted to 1 ml potassium phosphate pH 6.0. The HPLC analysis and the detection system were similar to the ones used for arginine ADP-ribose.

Preparation of imidazoyl ADP-ribose

A 100 µl reaction mixture contained 2.5 mM NAD, 20 ml immobilized NADase, 50 mM potassium phosphate, and 1 M imidazole and the pH was 7.0. Incubation was at 37 oC for 24 h. 10 µl aliquots were taken and diluted to 1 ml 50 mM potassium phosphate buffer, pH 6.0, and subjected to analytical reversed-phase HPLC. The elution was performed isocratically with 100 mM potassium phosphate, pH 6.0, 5 %

methanol, at a flow rate of 1 ml/min at room temperature. Detection was at 254 nm using an ISCO model 228 detector.

Preparation of ADP-ribosyl histidine

The reaction mixture contained 50 mM potassium phosphate buffer, pH 6.0, 2.5 mM NAD, 10 µl of immobilized NADase and 500 mM histidine. Incubation was at 37 °C for 24 h. Appropriate controls were run without NADase or histidine. Aliquots were taken and diluted to 1 ml with 50 mM potassium phosphate, pH 6.0, and subjected to analytical reversed-phase HPLC as described above for arginine ADP-ribose.

Etheno ADP-ribosyl histidine preparation

Five µmol of purified histidine ADP-ribose were diluted in 5 ml of 250 mM ammonium formate buffer, pH 4.6. To this solution 80 µl of cloroacetaldehyde were added. Incubation was 60 °C for 4 h. This reaction mixture was diluted to a final volume 10 ml and the pH adjusted to 8.6 in 250 mM ammonium formate buffer. This solution was applied to a previously equilibrated DHB-Sepharose column. 10 ml of 250 mM of ammonium formate were used to wash the column. Then 2 ml of water were added to the column and finally eluted two times with 2 ml of ammonium formate.

Formic acid hydrolysis of methyl, ethyl, propyl, serine ethyl ester or threonine methyl ester ADP-ribose

A solution containing 100 μM of methyl, ethyl, propyl, serine ethyl ester or threonine methyl ester ADP-ribose, 44 % formic acid was incubated at 37 °C. Aliquots were taken every ten minutes. The same HPLC analysis system as described for the preparation of arginine ADP-ribose was used.

NaOH treatment of imidazoyl ADP-ribose

A 100 μl reaction mixture contained 100 μM purified imidazoyl ADP-ribose, and 1 M NaOH. Aliquots were taken every 30 min for 3 h. The HPLC analysis is the same as described for the preparation of arginine ADP-ribose.

Preparation of serine ethyl ester and threonine methyl ester ADP-ribose

The 100 μl reaction mixture contained 2.5 mM NAD, 20 μl immobilized NADase, 50 mM potassium phosphate buffer, pH 6.0, 1 M serine ethyl ester or 750 mM threonine methyl ester. Incubation was at 37 °C for 24 h. The HPLC analysis was the same as described for the preparation of arginine ADP-ribose.

NMR of methyl, ethyl, propyl, serine methyl ester, and threonine methyl ester ADP-ribose

The purification procedure for methyl, ethyl, and propyl

ADP-ribose and the sample preparation for analysis by NMR were the same as described for ketoamines 1 and 2. Serine ethyl ester and threonine methyl ester were purified in DHB-Sepharose as described for the purification of etheno histidine ADP-ribose. The NMR sample preparation was the same as the ketoamines 1 and 2.

NMR of imidazoyl and ADP-ribosyl histidine

The purification procedure and the sample preparation for NMR analysis were the same as described for ketoamines 1 and 2.

Preparation and purification of glycated histone H1 by ^{32}P ADP-ribose

Histone H1 (500 μM), 100 μCi of ^{32}P ADP-ribose (50 μM) was incubated in 100 μl of 10 mM potassium phosphate pH 8.0 for 4 h. The pH was adjusted to 5.0 and the glycated histone purifed by HPLC. A gradient of 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile (buffer B) was used. The initial concentration of buffer A was 80% and buffer B 20%. The run lasted 8 min and the final concentrations were buffer A 55 % and buffer B 45 %. The collected fractions were pooled and lyophilized and the final pH adjusted to 5.0 with ammonium formate 10 mM.

SDS-PAGE electrophoresis of glycated histone H1

Purified glycated histone H1 was applied to SDS polyacrylamide gel. The separating gel preparation contained 0.375 M Tris-HCl, pH 8.8, 1% (w/v) SDS, 12% Acrylamide/Bis (degas for 15 minutes at room temperature), 0.5 % ammonium persulfate and TEMED 50 µl. The Stacking gel preparation contained 0.125 M Tris, pH 6.8, 1.0 % (w/v) SDS, 4.0 % Acrylamide/Bis, 0.5 % ammonium persulfate and 0.1 % TEMED. Histone H1 was used as a control and run parallely with glycated histone H1.

HPLC analysis of the pH 9.0 degradation products of glycated histone H1

A similar HPLC system used for separation of the pH 9.0 degradation products for ketoamines 1 and 2 was used to analyzed the pH 9.0 degradation products of ^{32}P glycated histone H1.

CHAPTER IV

RESULTS-PART I: MODEL CONJUGATES FOR ENZYMIC MODIFICATION OF PROTEINS BY ADP-RIBOSE

Preparation of methyl, ethyl, and propyl ADP-ribose using snake venom NADase

Two approaches have been used to prepare model conjugates as described in this dissertation, namely an enzymatic approach for enzymic modifications and a direct chemical synthesis approach for glycation by ADP-ribose. For the enzymatic approach snake venom NADase from *Bungarus fasciatus* was used. As described in the introduction, this enzyme catalyzes a transglycosylation reaction. This property of the enzyme makes it useful to prepare model conjugates of ADP-ribose. The *Bungarus fasciatus* enzyme was used for most applications because, in contrast to other mammalian NADases, this enzyme is stable at 37 °C, pH 7.5 and can be immobilized on Con A-Sepharose without significant loss of activity as was shown by Yost and Anderson (118). A crude extract of snake venom can be used to prepare the immobilized enzyme without any further purification once EDTA is added to inactivate the contaminating pyrophosphatase activity.

Initial studies began with the preparation of conjugates that mimic the linkage between ADP-ribose and serine, threonine, tyrosine or hydroxyproline. When the hydroxyl group of the side chain of these amino acids is used to form a bond with the anomeric carbon of ADP-ribose, an acetal functional group is formed. To prepare acetal conjugates of ADP-ribose, methanol, ethanol, and propanol were selected as model nucleophiles. NADase was incubated with NAD and the respective alcohol at 37 °C, pH 7.5 for 24 h. Analysis of the products was monitored using reversed-phase HPLC with uv detection at 254 nm as shown in Figs. 8, 9, and 10. These figures show the HPLC profiles of the acetal conjugate preparation using methanol, ethanol, and propanol, respectively. The control shown in panel A was done to test the possibility of a nonenzymic reaction between the alcohol and NAD. Panel B shows the incubation of the enzyme with NAD in the absence of the alcohol. This was a positive control to check the activity of the enzyme and to determine that the enzyme preparation did not contain any uv absorbing material that would interfere with the detection of the product. Panel C shows the incubation of NAD, enzyme, and the respective alcohol. In Fig. 2C a new peak appeared that eluted at 9 minutes when methanol was used as the alcohol. In Figs. 3C, and 4C, when ethanol and propanol were used, respectively, new peaks eluted following nicotinamide.

Figure 8. HPLC profile of the reaction of snake venom NADase, NAD and methanol. Panel A shows the incubation of NAD and methanol. Panel B shows the incubation of NADase and NAD. Panel C shows the complete reaction mixture. The arrow indicates the putative model conjugate formed in the reaction. The detection system was uv at 254 nm.

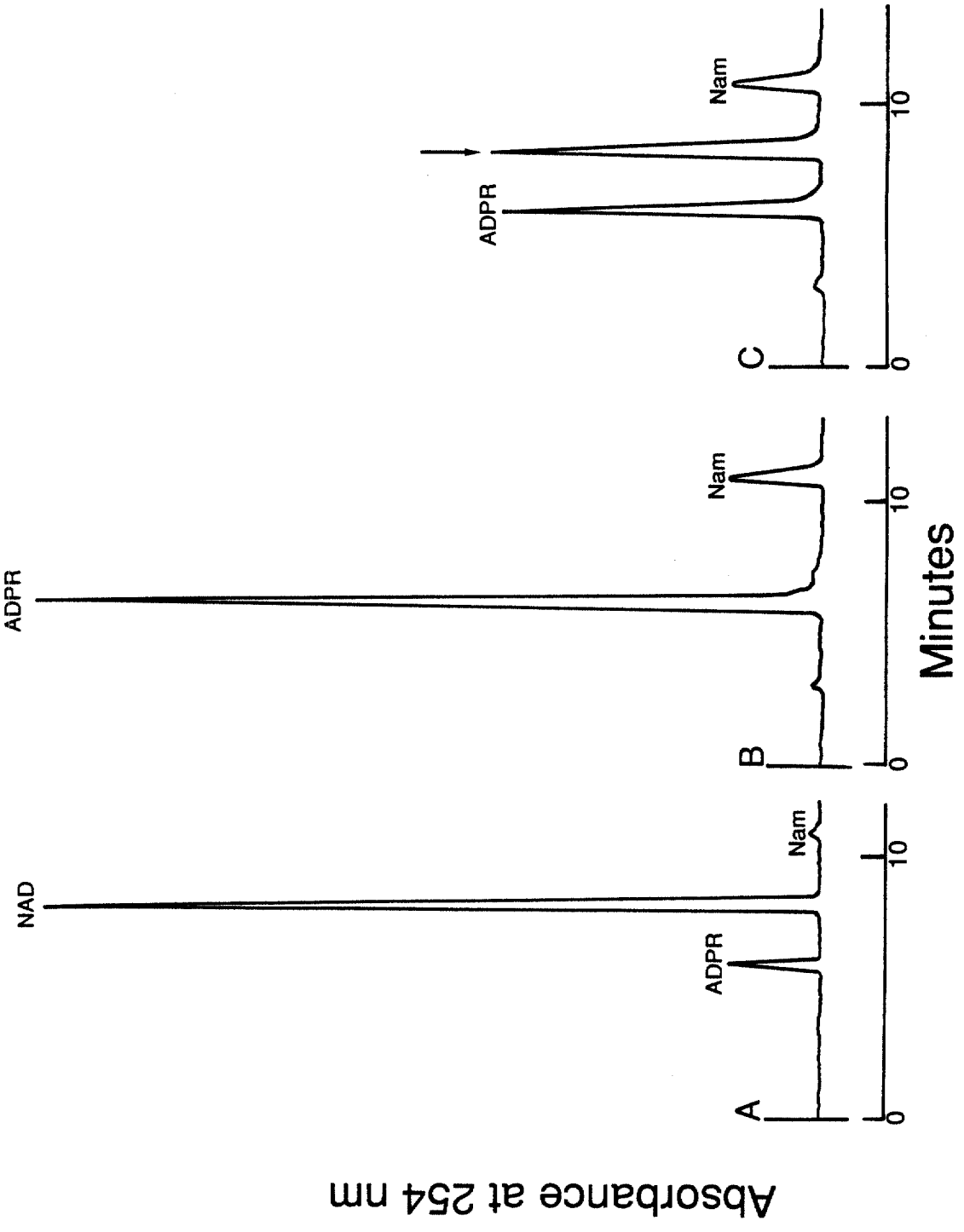

Absorbance at 254 nm
A
B
C
NAD
ADPR
Nam
ADPR
Nam
ADPR
Nam
0
10
0
10
0
10
Minutes

Figure 9. HPLC profile of the reaction of snake venom NADase, NAD and ethanol. Panel A shows the incubation of NAD and ethanol. Panel B shows the incubation of NADase and NAD. Panel C was the complete reaction mixture. The arrow indicates the product obtained using NADase. The detection was uv at 254 nm.

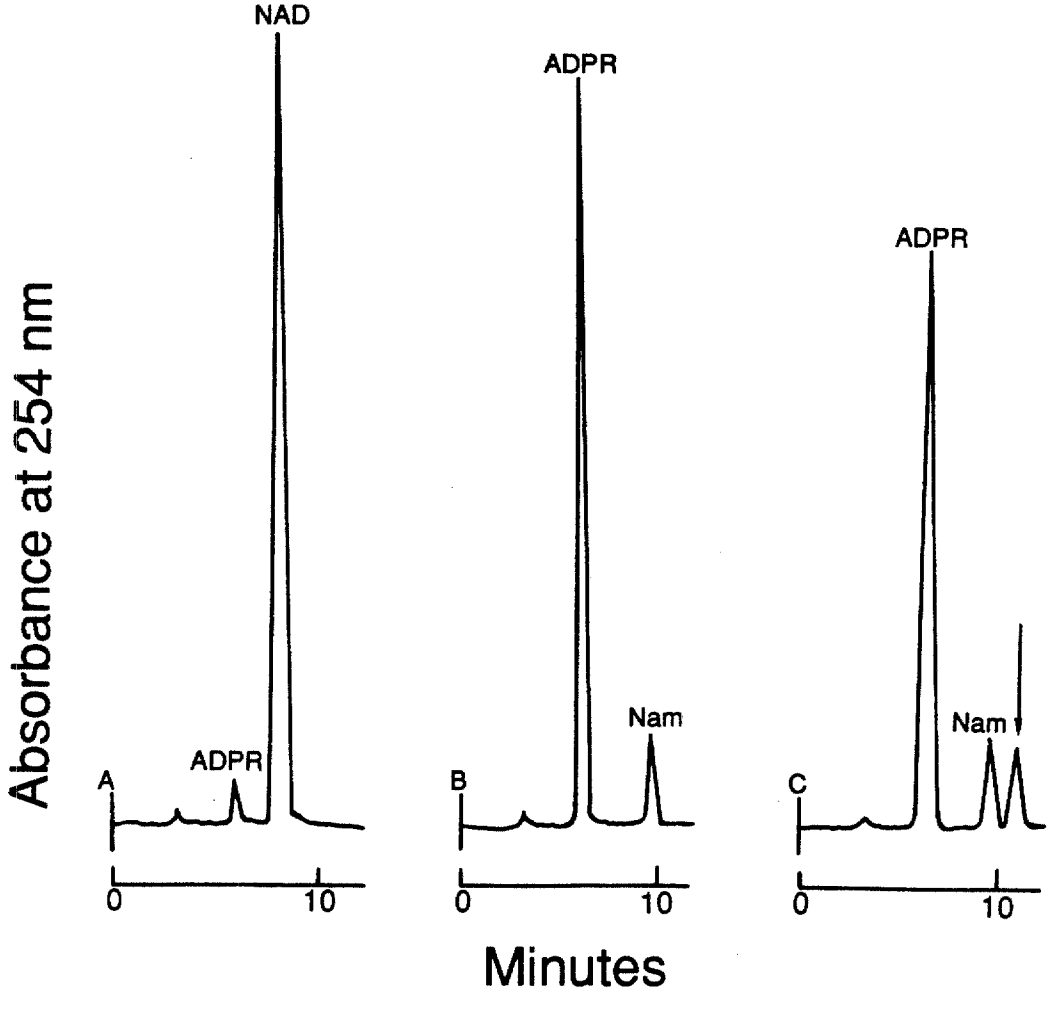
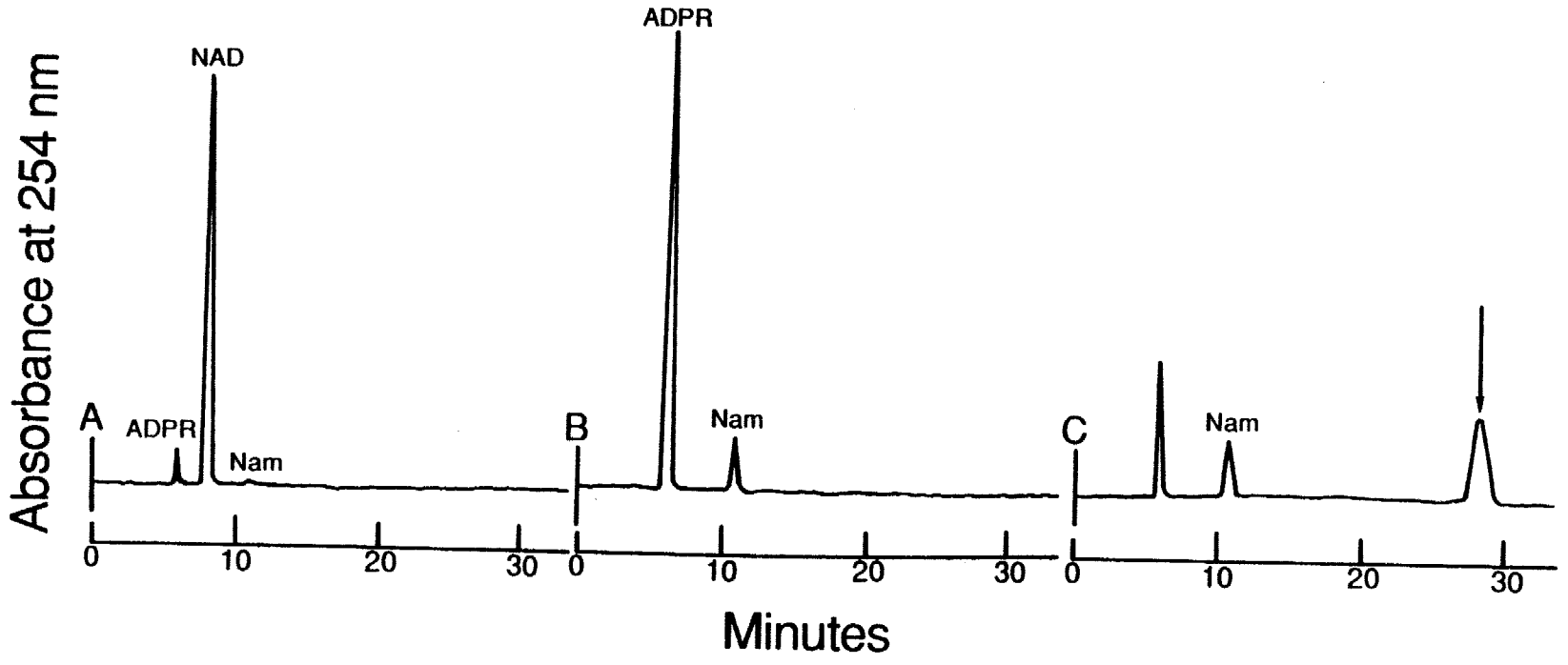

NAD
ADPR
ADPR
Nam
Nam
A
B
C
ADPR
0
10
0
10
0
10
Absorbance at 254 nm
Minutes

Figure 10. HPLC profile of the reaction of snake venom NADase, NAD and propanol. The reaction mixture was incubated at 37 °C in 100 mM potassium phosphate and sodium pyrophosphate at pH 7.0. Panel A shows the incubation of NAD and propanol. Panel B shows the incubation of NADase and NAD. Panel C was the complete reaction mixture. The arrow indicates the product formed in the NADase reaction mixture. The detection was uv at 254 nm.

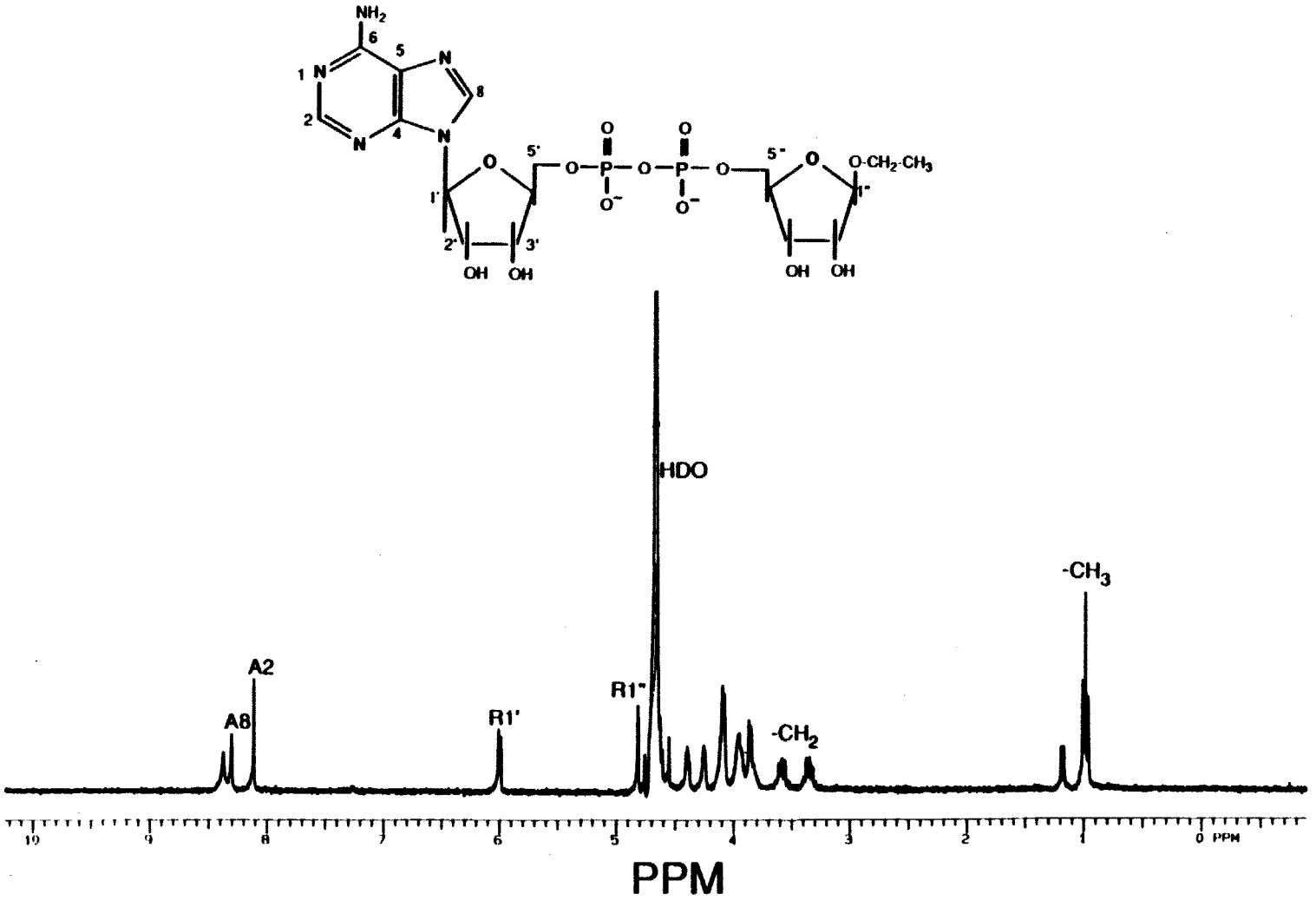

Absorbance at 254 nm
A
ADPR
NAD
Nam
B
ADPR
Nam
C
Nam
0
10
20
30
0
10
20
30
0
10
20
30
Minutes

We hypothesized that these new peaks were the putative methyl, ethyl, and propyl ADP-ribose.

^1H NMR of the putative ethyl ADP-ribose

Prior to study the chemistry of the acetal linkages, it was necessary to obtain a definitive characterization of the structure of the compounds obtained. NMR was used as the primary characterization method due to the detailed structural information that it provides. For proton NMR (^1H NMR) the data obtained reflects the environment of non exchangeable protons in the molecule. A sample for ^1H-NMR was obtained using preparative reversed-phase HPLC. The product was washed several times in 99% D_2O. The pH was adjusted to 6.0 and the final concentration was adjusted to 2 to 5 mM. Fig. 11 shows the ^1H-NMR for the compound obtained from ethanol and NAD. There were three diagnostic signals for the presence of the adenosine portion of the molecule. The first two were provided by the chemical shifts shown at 8.05 and 8.35 ppm that correspond to the H2 and H8 of the adenine ring. The third appeared at 6.0 ppm and corresponds to the the anomeric proton (R1') for the linkage between adenine and ribose. The region of 4-5 ppm correponds to the remaining chemical shifts for the protons of the two riboses. At 3.35 and 3.60 ppm appeared the chemical shifts for the two

Figure 11. ^{1}H-NMR of the putative ethyl ADP-ribose product. The 12 min product observed in the HPLC profile (Fig. 9) was isolated using preparative reversed-phase HPLC. After lyophilization a sample for NMR was prepared in D_2O.


NH2
6
5
N
1 N
8
2
N
4
N
O
5'
O
P
O
O
P
O
5"
O
O-CH2-CH3
1'
1"
2'
3'
OH
OH
OH
OH
HDO
-CH3
A2
A8
R1'
R1"
-CH2
10
9
8
7
6
5
4
3
2
1
0 PPM
PPM

diasteromeric hydrogens of the methylene portion in the ethyl alcoxide part of the adduct. The methyl group of the ethyl alcoxide was observed at 0.95 ppm. At 4.8 ppm the anomeric hydrogen of the ethyl bound to ribose is shown. In total, this information provided evidence that the structure proposed for this compound, 1" ethyl ADP-ribose, was correct.

Although the putative products for methyl and propyl ADP-ribose were not analyzed by NMR the chemical properties were similar to ethyl ADP-ribose. Therefore, methyl and propyl ADP-ribose were assumed to have an analogous structure to that proposed for ethyl ADP-ribose.

Synthesis of ADP-ribosyl conjugates of serine ethyl ester and threonine methyl ester

In order to develop acetal model conjugates with closer resemblance to the product formed between ADP-ribose and serine or threonine, serine ethyl ester and threonine methyl ester were used as the nucleophiles for the preparation of the ADP-ribosylated adducts. Shown in Figs. 12 and 13 are the reversed-phase HPLC profiles of incubations using threonine methyl ester, and serine ethyl ester respectively. Panel A in each figure shows the control using ADP-ribose and the amino acid ester. This control was used because the amino acid ester contains a free amino group that can react nonenzymatically with ADP-ribose to form, through a Schiff

Figure 12. HPLC profile of the incubation of snake venom NADase, NAD and threonine methyl ester. Samples were subjected to HPLC with uv detection at 254 nm. Panel A shows the incubation of ADP-ribose and threonine methyl ester. Panel B shows the incubation of NADase and NAD. Panel C shows the incubation of the complete reaction mixture using uv as the detection system.

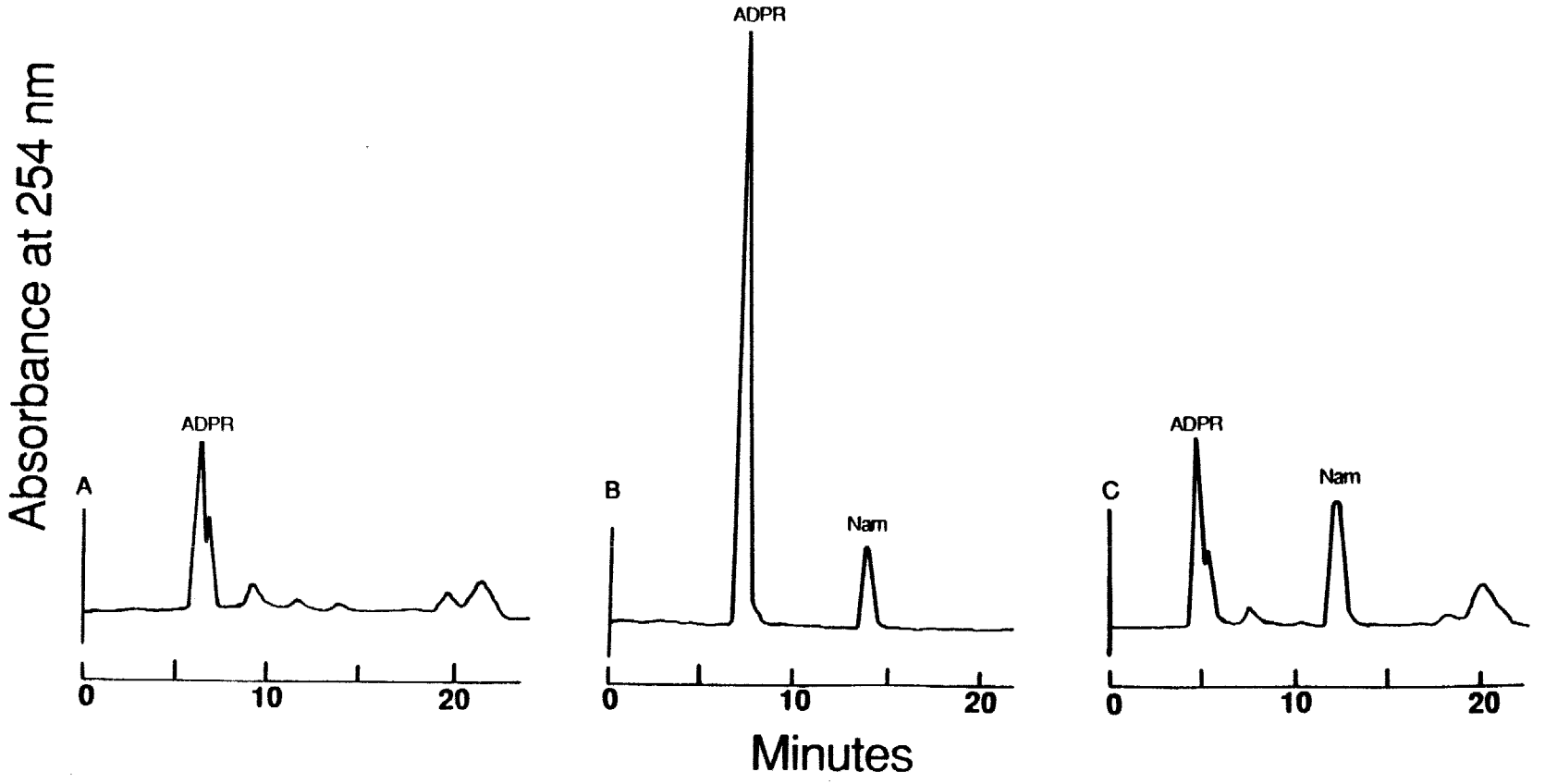
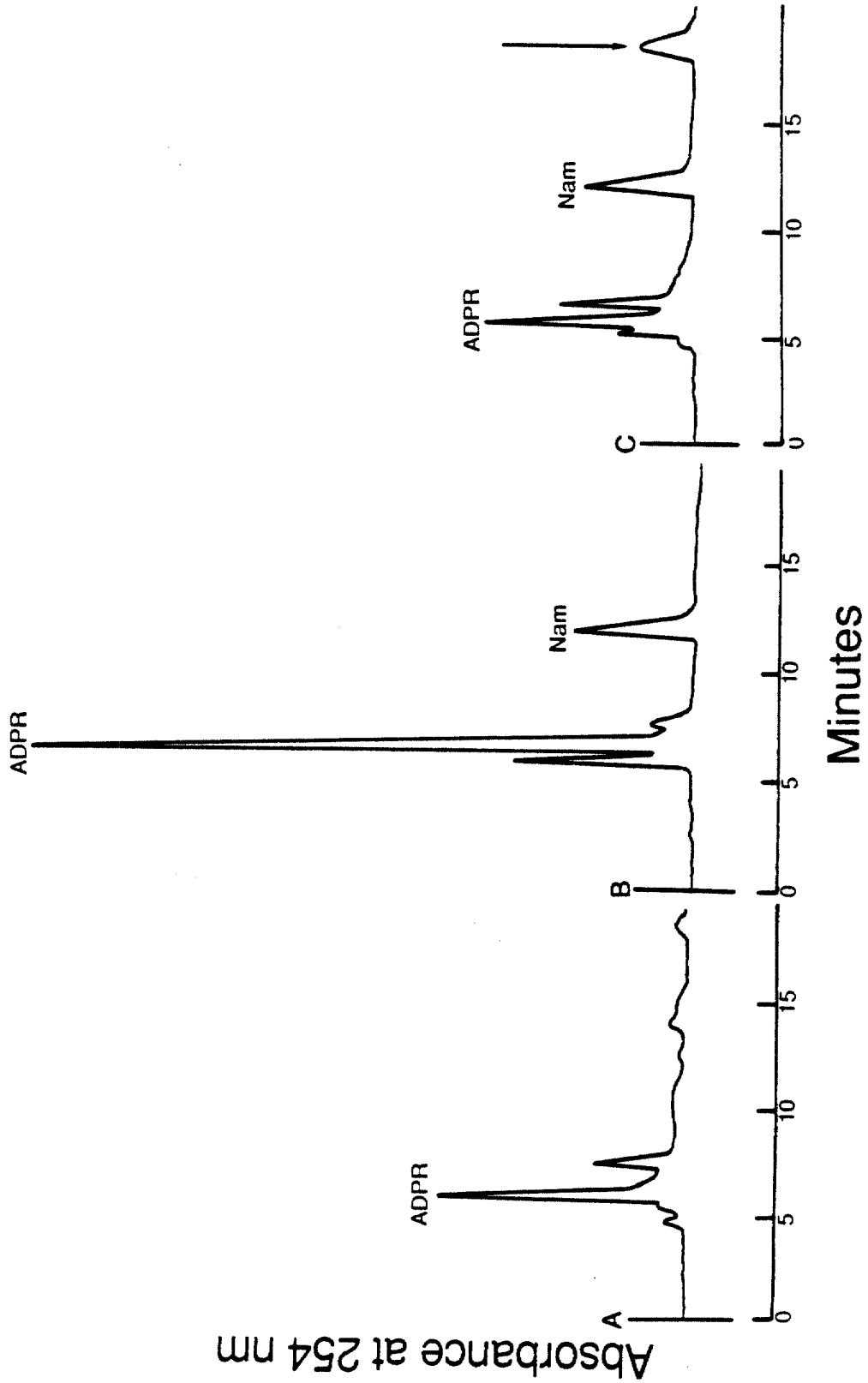

Absorbance at 254 nm
A
ADPR
0
10
20
B
ADPR
Nam
0
10
20
C
ADPR
Nam
0
10
20
Minutes

Figure 13. HPLC profile of the incubation of snake venom NADase, NAD and serine ethyl ester. Samples were subjected to reversed-phase HPLC with uv detection at 254 nm. Panel A shows the incubation of ADP-ribose and serine ethyl ester. Panel B shows the incubation of NADase and NAD. Panel C shows the incubation of the complete reaction mixture. The arrow indicates the product obtained in the NADase reaction.


Absorbance at 254 nm
A
ADPR
B
ADPR
Nam
C
ADPR
Nam
0
5
10
15
Minutes

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Either the original page was missing or the original pagination was incorrect.

ethyl ester ADP-ribose. The presence of the adenosine portion of the molecule was shown by the peaks at 8.05 and 8.35 ppm that correspond to the H2 and H8 of adenine ring. The signal at 6.0 ppm showed the presence of the anomeric hydrogen for adenine bound to ribose. The peaks at 1.0, 3.35 and 3.6 ppm suggested the presence of the methyl and the two diasteromeric hydrogens of the methylene group, respectively, of the ester portion of the amino acid. The signal at 4.87 ppm indicated the presence of the anomeric hydrogen for serine ethyl ester bound to ribose. The signals between 3.5 and 4.7 ppm corresponded to the remaining hydrogens of the two riboses and to the α and β hydrogens of the amino acid. To confirm unequivocally the latter signals ^{13}C-NMR was used. Fig. 15 shows the ^{13}C-NMR analysis of this adduct. The carbonyl of the ester portion of the amino acid appeared at 176 ppm. At 60.1 and 62 ppm the signals for the α and β carbons of the amino acid ester were observed. At 69 and 71 ppm the signals for both carbons #5 from the two ribose moieties were seen. At 74 and 75 ppm the carbons at position 3 were observed. At 78.0 and 78.1 ppm the signals corresponding to position 2 carbons from the two riboses appeared. At 85 and 87 ppm the carbon #4 signals from the two riboses were shown. At 110 and 91 ppm, the signals corresponding to the anomeric carbons for the amino acid ester and the anomeric carbon of ribose bound to adenine,

Figure 14. ^{1}H-NMR of the putative serine ethyl ester ADP-ribose. The new product shown in HPLC analysis was isolated as described in methods. The assignments are based in reported adenine containing compounds (76).

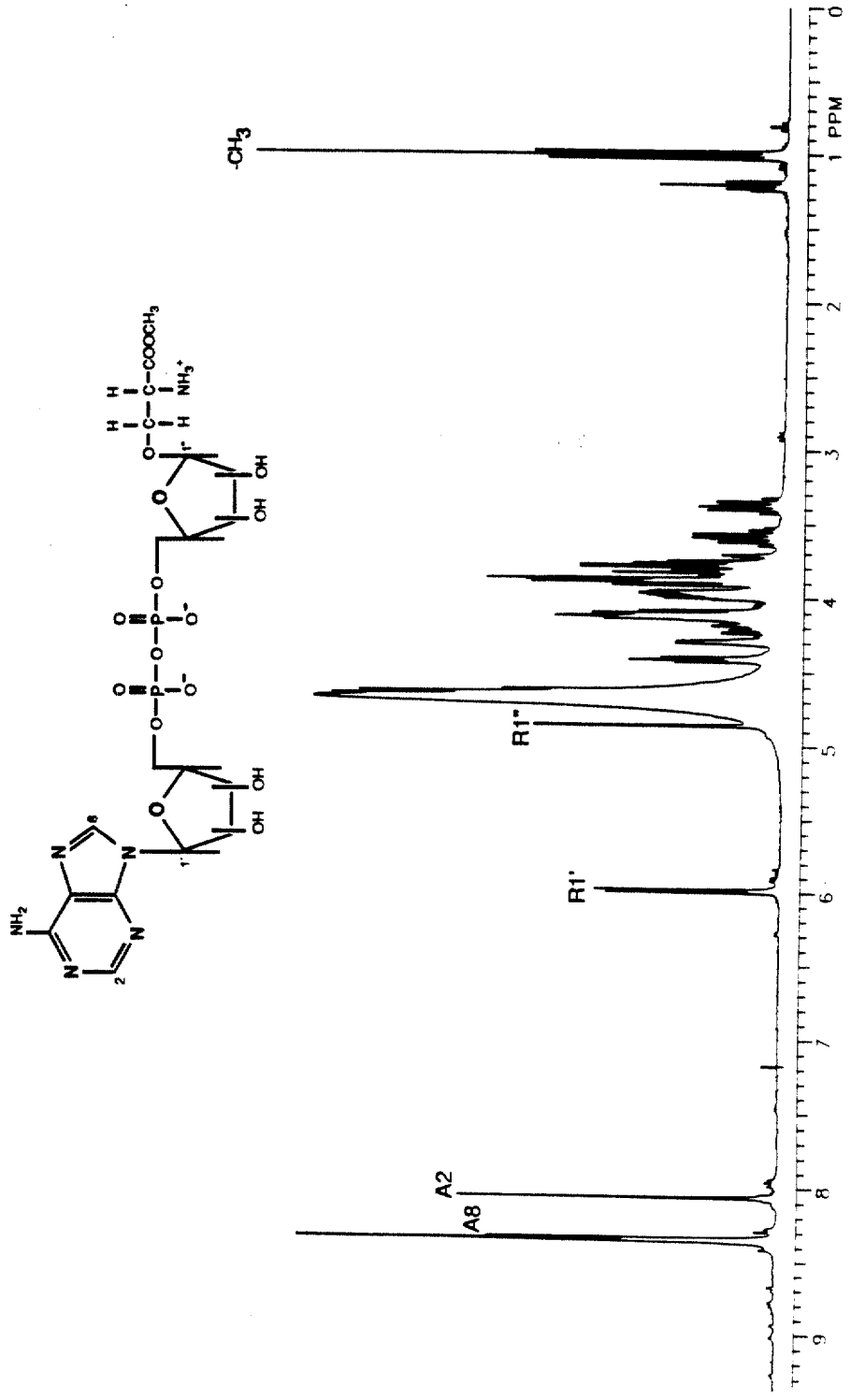
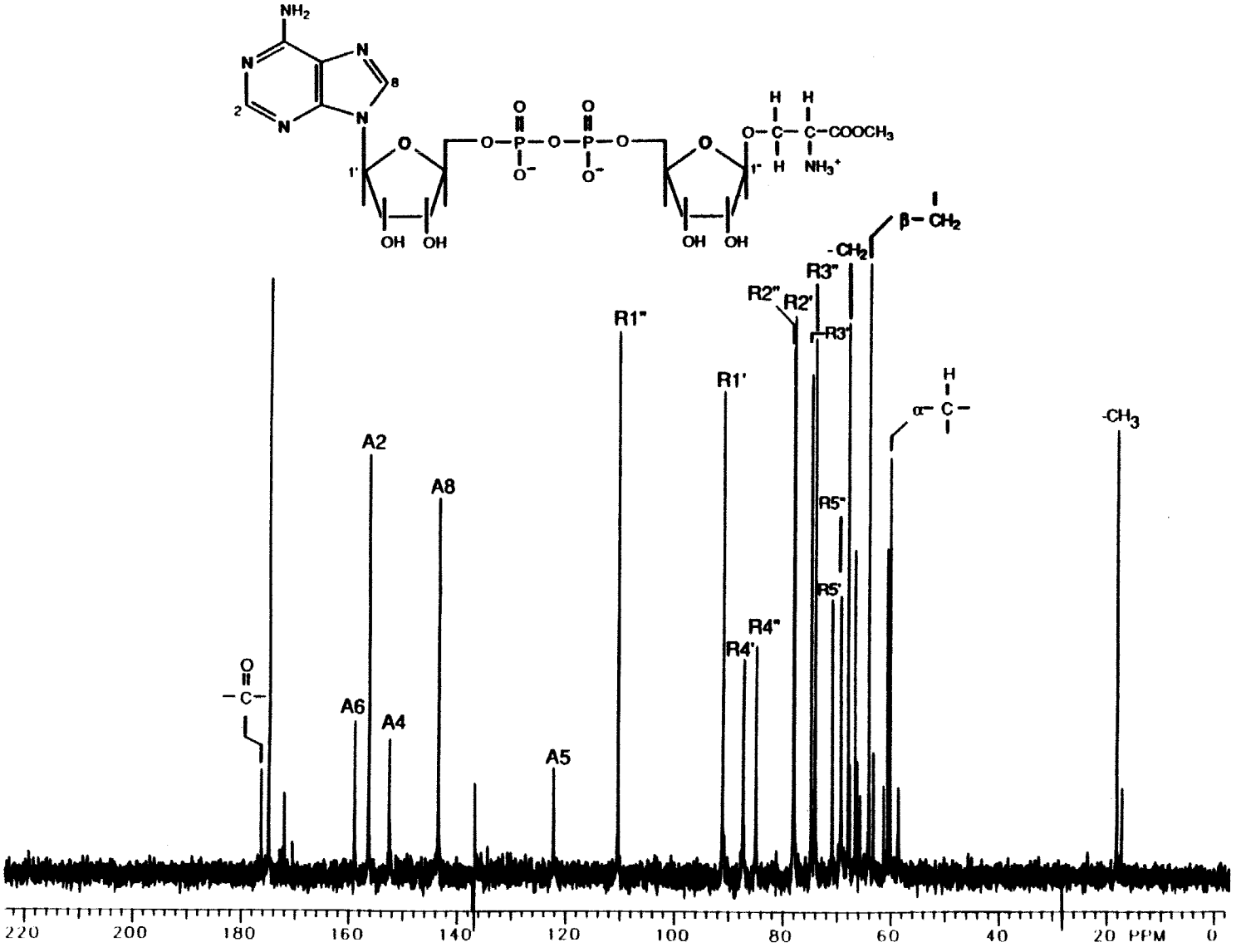

A8
A2
R1'
R1''
CH3
1 PPM

Figure 15. ^{13}C-NMR of the putative serine ethyl ester ADP-ribose.


NH2
N
N
8
2
N
N
1'
O
O
P
O⁻
O
P
O⁻
O
O
1"
H
H
O
C
C
COOCH3
H
NH3+
OH
OH
OH
OH
β- CH2
-CH2
R3"
R2"
R2'
R3'
R1"
R1'
α- C
H
-CH3
A2
A8
R5"
R5'
R4"
R4'
O
C
A6
A4
A5
220
200
180
160
140
120
100
80
60
40
20
PPM
0

were observed. These data provided evidence that the structure of this adduct is serine ethyl ester ADP-ribose.

^{1}H and ^{13}C NMR of the putative threonine methyl ester ADP-ribose

The putative threonine methyl ester was also analyzed using NMR. The sample for ^{1}H-NMR was prepared by using DHB-S chromatography and HPLC, similar to the preparation of the serine ethyl ester conjugate. The sample was washed several times in D_2O. Fig. 16 shows the ^{1}H-NMR spectra of this adduct. At 8.15 and 8.40 ppm appeared the signals corresponding to H2 and H8 from the adenine ring. The anomeric hydrogen for adenine bound to ribose was seen at 6.0 ppm. At 1.2 ppm was shown a doublet corresponding to the methyl group bound to the β carbon in the threonine methyl ester portion of the molecule. A singlet at 3.22 ppm corresponded to the methyl ester of the amino acid. The α hydrogen was seen as a doublet at 3.54 ppm. To clearly identify the remaining signals of this complex molecule, ^{13}C NMR was used. Fig. 17 shows the ^{13}C NMR spectrum. At 178 ppm is observed a signal corresponding to the carbonyl from the ester portion of the amino acid. A peak at 111 ppm corresponded to the anomeric carbon of the amino acid bound to ribose. A signal at 64 ppm indicated the presence of the

Figure 16. ^{1}H-NMR of the putative threonine methyl ester ADP-ribose. The product was isolated using reversed-phase HPLC as described in Methods.

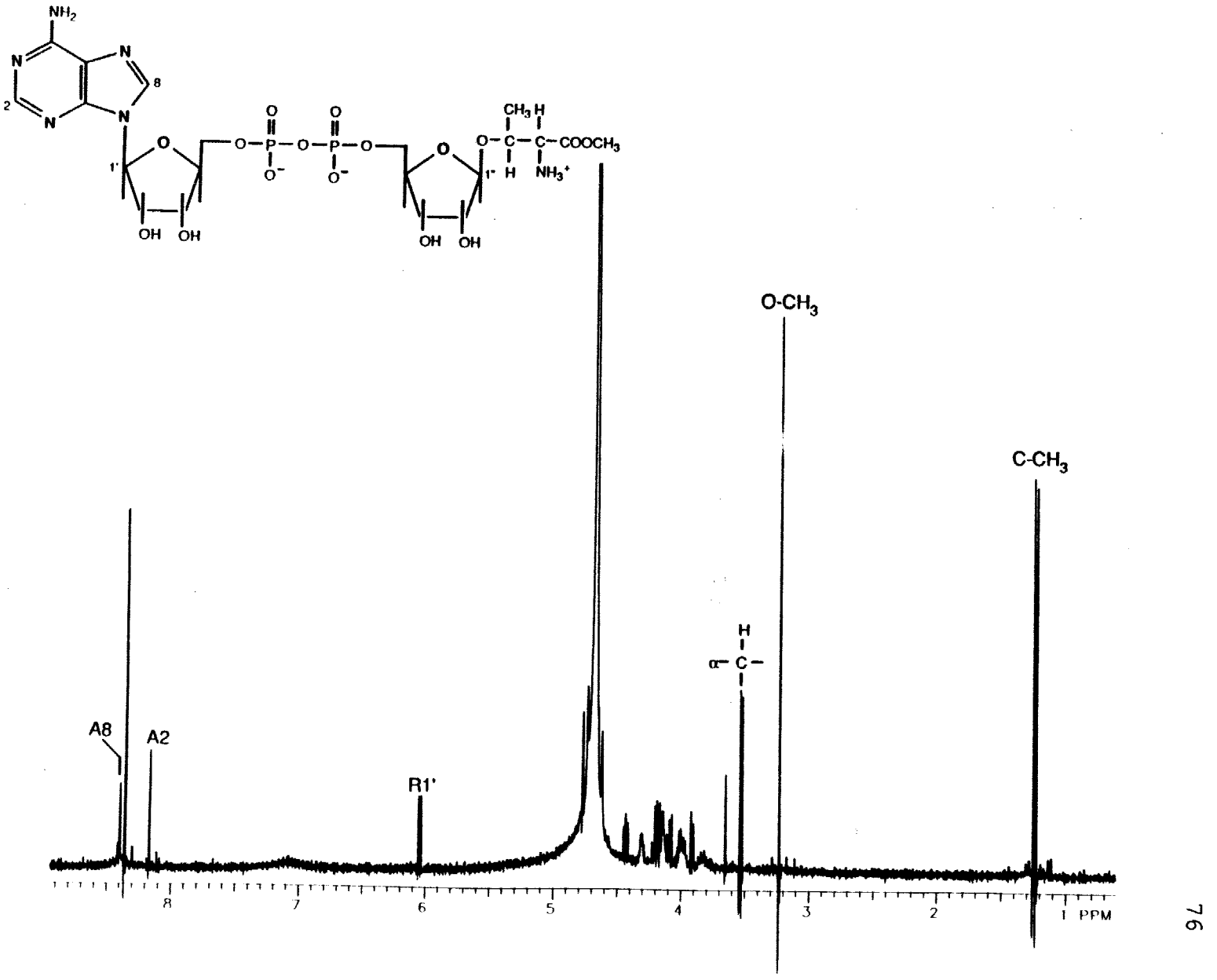
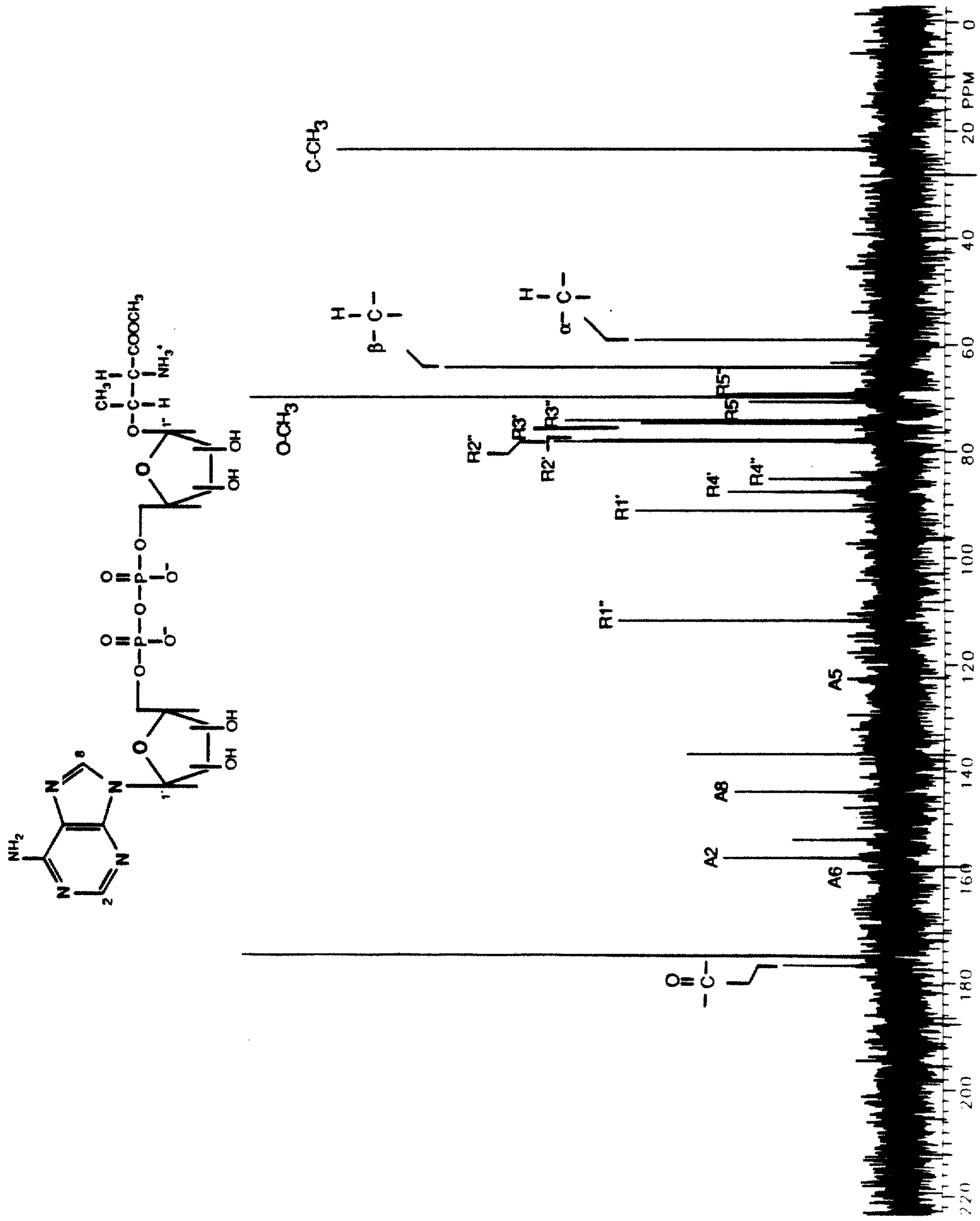

NH2
N
N
8
2
N
N
1'
O
O
O
P
O⁻
O
P
O⁻
O
O
CH3 H
C
C
COOCH3
H
NH3+
1'
OH OH
OH OH
O-CH3
C-CH3
H
α C
A8
A2
R1'
8
7
6
5
4
3
2
1 PPM

Figure 17. ^{13}C-NMR of the putative threonine methyl ester ADP-ribose.


C-CH3
O-CH3
β-C
α-C
R5'
R5"
R3'
R3"
R2'
R2"
R4'
R4"
R1'
R1"
A5
A8
A2
A6
PPM
0
20
40
60
80
100
120
140
160
180
200
220

β carbon of the ethyl group in the amino acid ester. The remaining signals of the two riboses appeared in the region of 60 to 90 ppm. This information provided evidence of the structure proposed for this adduct, 1" threonine methyl ester ADP-ribose.

Chemical cleavage of acetal ADP-ribose adducts

An ultimate goal of these studies is to search for endogenous proteins modified *in vivo* with acetal linkages to ADP-ribose. Thus experiments to investigate the specific conditions that allow the selective cleavage of these conjugates were conducted.

Fig. 18 shows reversed-phase HPLC analysis of the formic acid 44% (w/v) treatment of ethyl ADP-ribose. Panel A shows ethyl ADP-ribose at zero time and panel B shows the reaction mixture after 40 min. In the latter, significant hydrolysis of this adduct was demonstrated by the production of ADP-ribose. Fig. 19 shows the kinetics of the chemical release of the ethyl adduct with acid (44% formic acid, 37 ^{o}C), hydroxylamine (1 M, pH 7.0, 37 ^{o}C), and alkali treatment (1.0 M, 37 ^{o}C). From these data, it was shown that this conjugate is stable to hydroxylamine and alkali treatment while acid hydrolysis occurs with a $t_{1/2}$ of 22 min. Similar analyses were carried out with the other acetal conjugates (data not

Figure 18. Analysis by HPLC of the hydrolysis of ethyl ADP-ribose in formic acid. Ethyl ADP-ribose was incubated in 44% formic acid at 37 °C. The released product appears to be ADP-ribose.

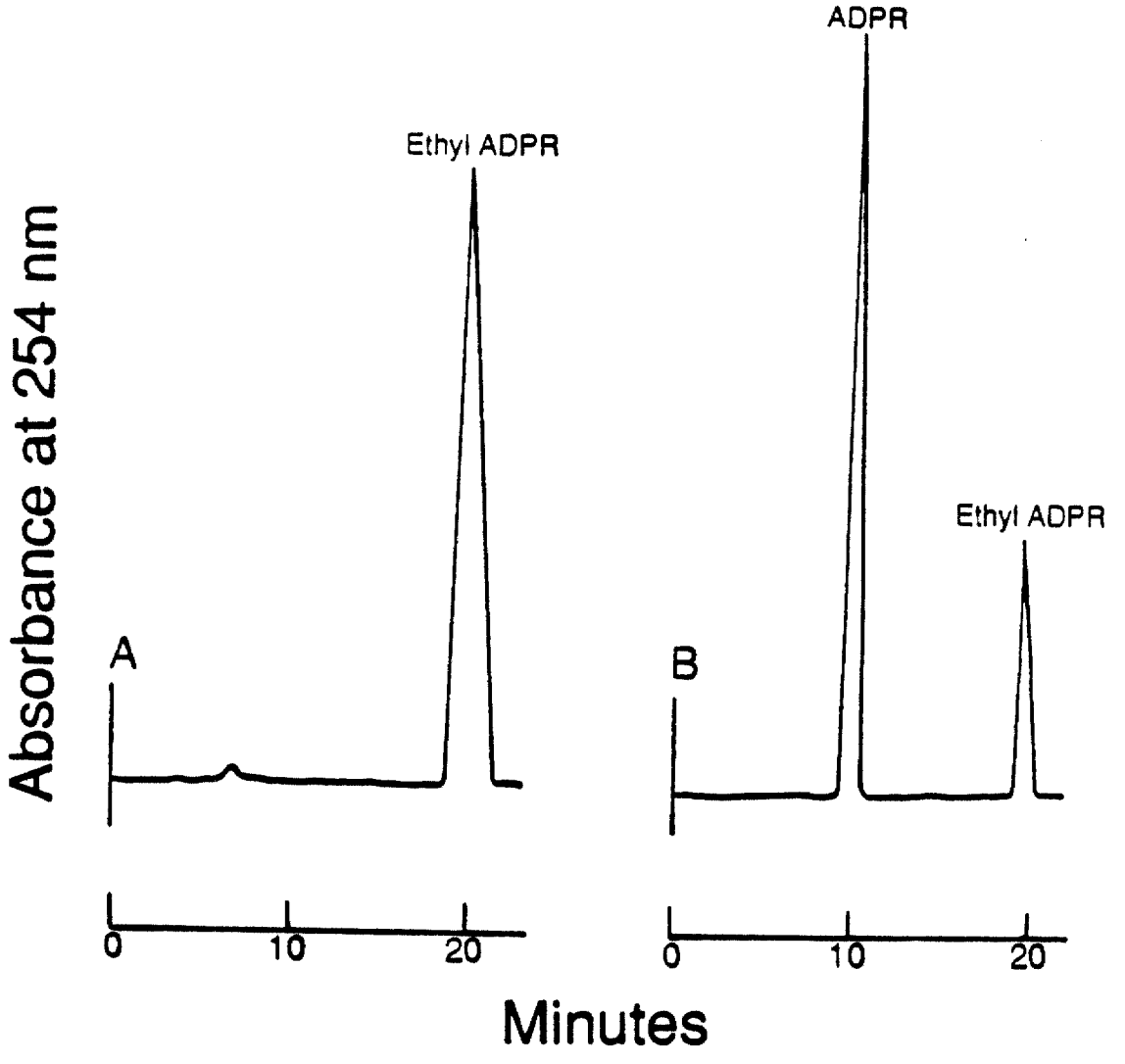
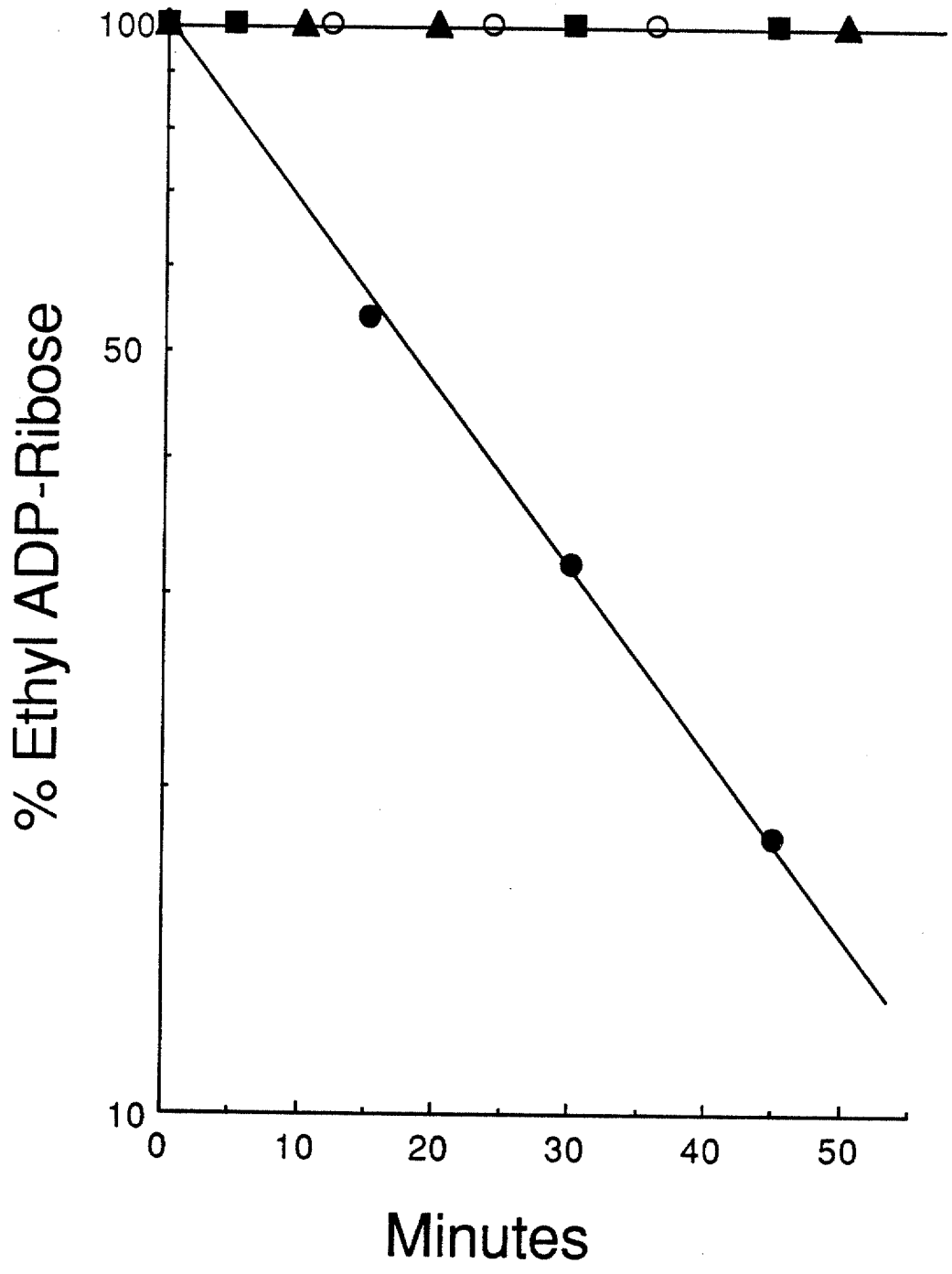

Absorbance at 254 nm
A
Ethyl ADPR
0
10
20
B
ADPR
Ethyl ADPR
0
10
20
Minutes

Figure 19. Kinetics of acid hydrolysis for ethyl ADP-ribose. Purified ethyl ADP-ribose was incubated in 44 % formic acid, hydroxylamine (1 M, MOPS, pH 7.0), and NaOH (1 M), at 37 °C. $HgCl_2$ (10 mM) was used at 25 °C Aliquots were taken and diluted to 1 ml with potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm. (○) $HgCl_2$; (▲) NaOH; (■) hydroxylamine; (●) formic acid 44%.


100
50
10
% Ethyl ADP-Ribose
0
10
20
30
40
50
Minutes

shown).

Fig. 20 shows the kinetics of the acid hydrolysis of the various acetals prepared. The $t_{1/2}$ for propyl ADP-ribose was 15 min, 19 min for ethyl ADP-ribose, 22 min for serine ethyl 42 min for methyl ADP-ribose, and 50 min for threonine methyl ester ADP-ribose. The table below summarizes the chemical release conditions for the acetal linkages. For these purposes a conjugate was considered stable when its $t_{1/2}$ was at least 10 h.

ADP-ribosyl linkage	Formic Acid(44%)	NH_2 OH (1M, pH 7.0)	Hg^{2+} (10 mM)	CHES pH9.0	NaOH (1 M)
Acetal	Released	Stable	Stable	Stable	Stable

Preparation of imidazoyl ADP-ribose

Imidazoyl ADP-ribose was prepared as an analogue of the glycosylic linkage between histidine and ADP-ribose. Fig. 21 shows an HPLC profile for the preparation of this conjugate.

Panel A shows a control in which NAD and imidazole were incubated to check the possibility of a nonenzymic reaction leading to the formation of a product. Panel B represents a positive control for snake venom NADase activity. Panel C

Figure 20. Kinetics of hydrolysis in formic acid for acetal conjugates of ADP-ribose. The purified acetals were incubated in 44 % formic acid at 37 °C. Aliquots were taken and diluted to 1 ml with potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm. (■) Propyl ADP-ribose; (●) Ethyl ADP-ribose; (□) ADP-ribosyl serine ethyl ester; (▲) ADP-ribosyl threonine methyl ester; (○) methyl ADP-ribose.

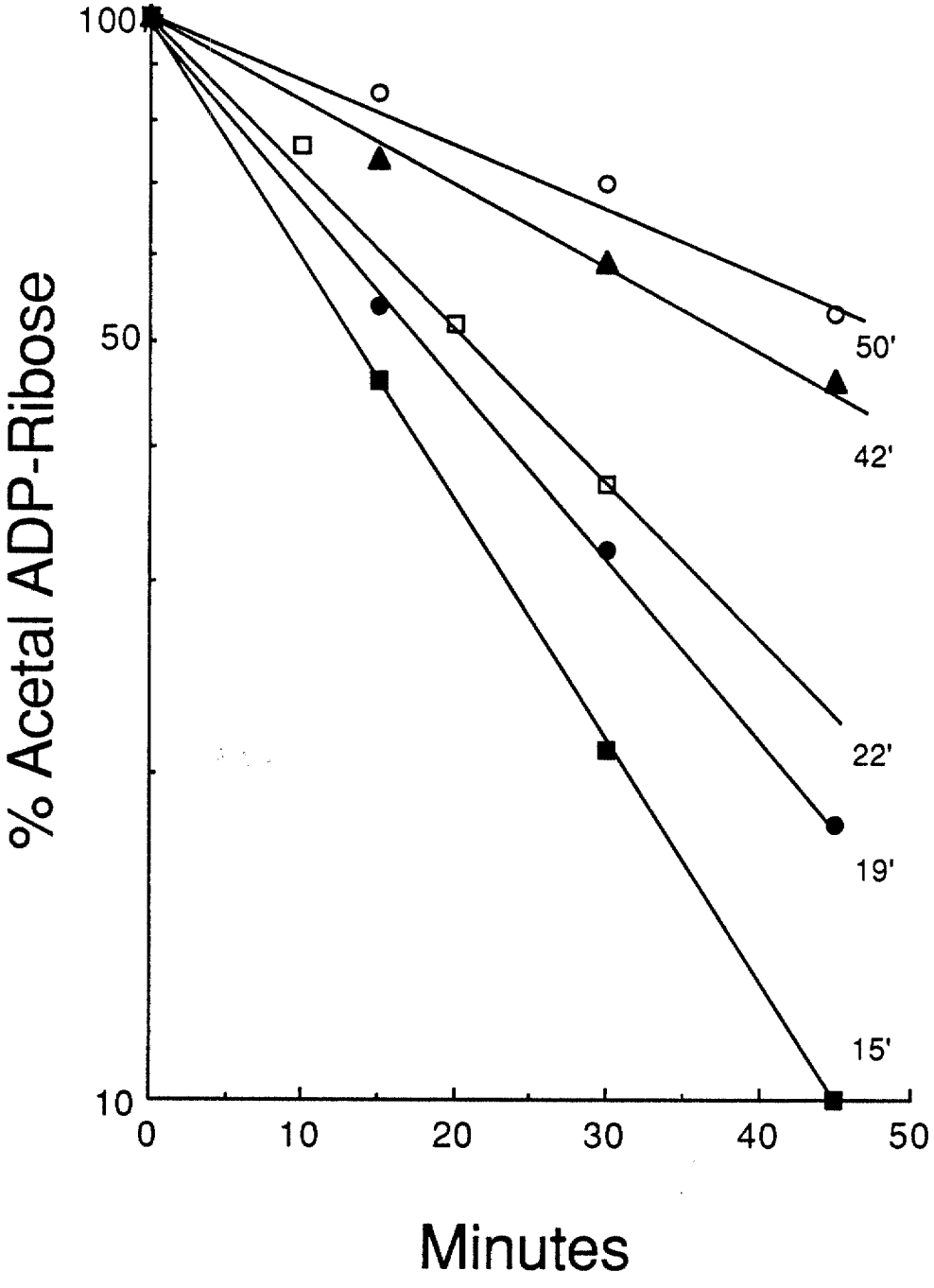
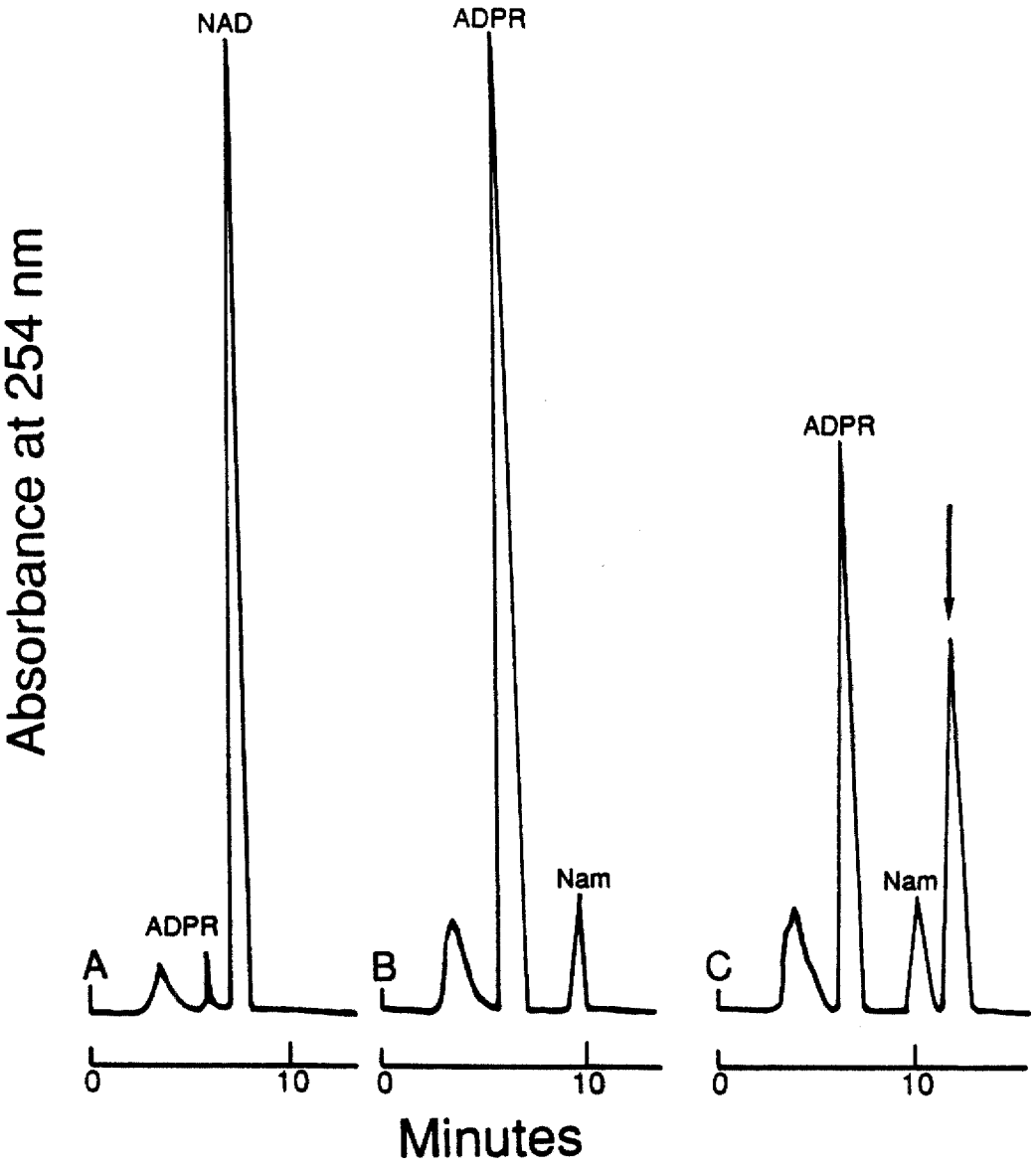

100
50
10
% Acetal ADP-Ribose
0
10
20
30
40
50
Minutes
50'
42'
22'
19'
15'

Figure 21. HPLC analysis of the reaction of NADase, NAD and imidazole. Panel A shows the incubation of NAD and imidazole. Panel B shows the incubation of NAD and NADase. Panel C shows the incubation of NADase, NAD and imidazole. The arrow shows the product formed in the reaction.


NAD
ADPR
ADPR
Nam
ADPR
Nam
A
B
C
Absorbance at 254 nm
0
10
0
10
0
10
Minutes

shows the complete reaction mixture. A new peak appeared at 11 min as indicated by the arrow.

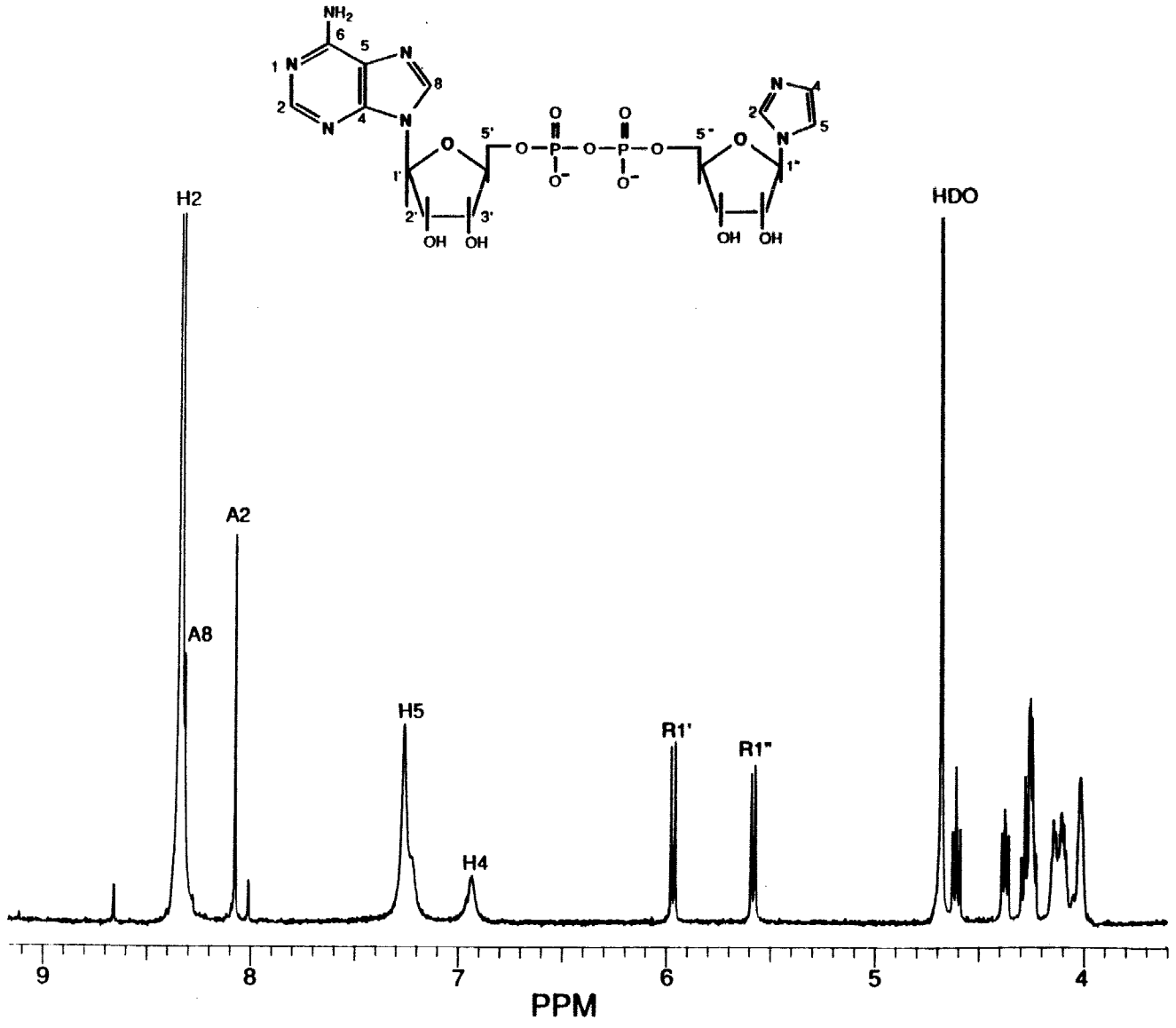
^{1}H-NMR of the putative imidazoyl ADP-ribose

The product was characterized using NMR. The sample was prepared using preparative reversed-phase HPLC. The sample was washed three times with D_2O. Fig. 22 shows the ^{1}H-NMR of the putative imidazoyl ADP-ribose sample. The signals at 6.9, 7.29, and 8.36 ppm corresponded to H4, H5, and H2 respectively of the imidazole ring. This assignment was based on published spectra of imidazole and imidazole containing molecules such as histidine (132). At 8.1 and 8.32 ppm the signals for H2 and H8 respectively of the adenine ring were observed. The anomeric hydrogens appeared at 5.9 and 5.6 ppm and represent the adenine bound to ribose and the imidazole bound to ribose. The hydrogens of the two riboses of the molecule appeared in the region 4.0-4.6 ppm. This information provided evidence that the new peak corresponds to imidazoyl ADP-ribose.

Preparation of ADP-ribosyl histidine

To search for a histidine-like conjugate to ADP-ribose in endogenous tissue proteins, it was necessary to prepare

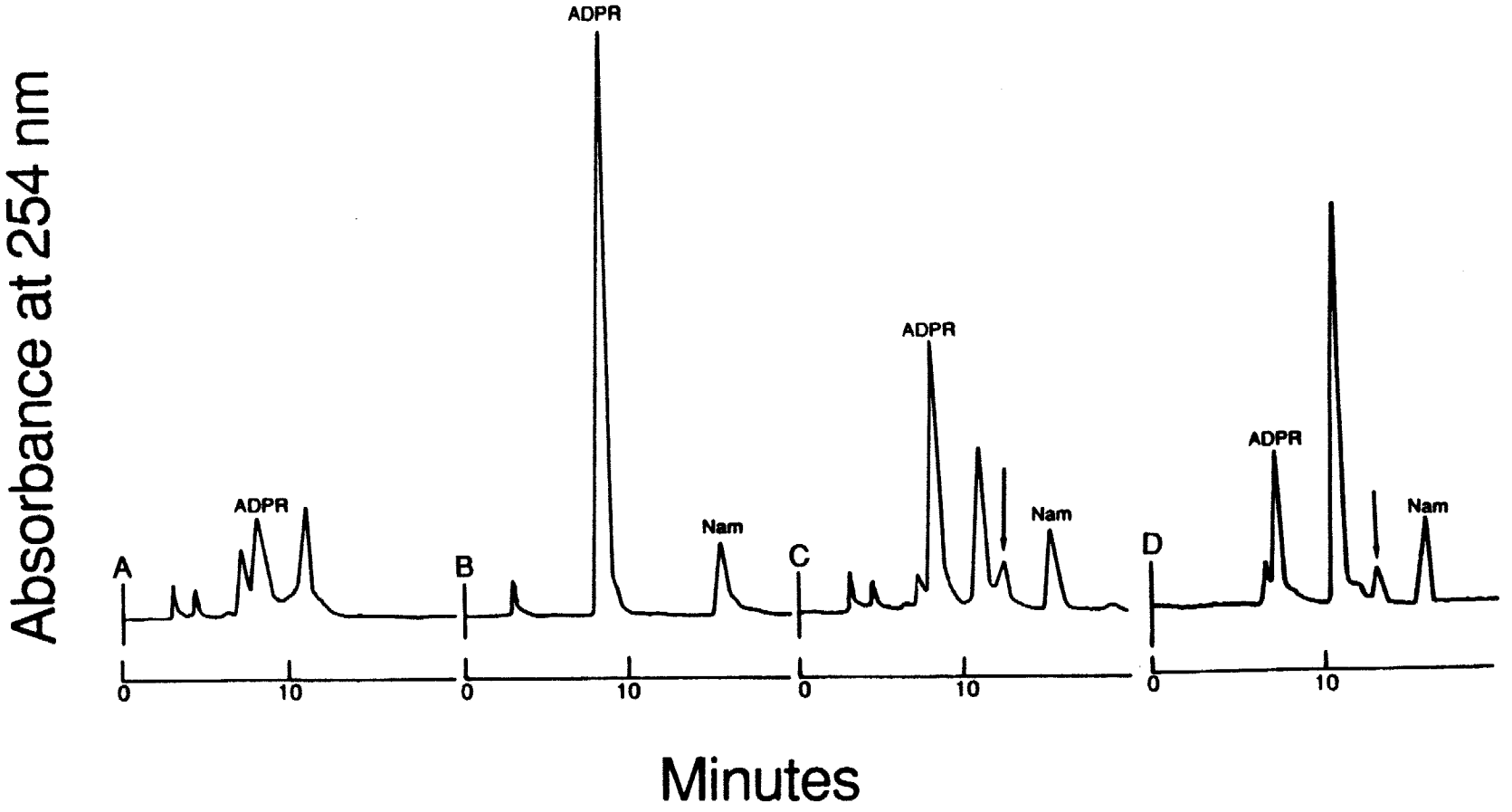
Figure 22. ^{1}H-NMR of imidazole ADP-ribose. A reversed-phased HPLC purified product was subjected to NMR analysis as described in Methods. The assignments were based on data of adenine and imidazole containing molecules.


H2
A8
A2
H5
H4
R1'
R1"
HDO
9
8
7
6
5
4
PPM

synthetically histidine ADP-ribose which would be used as a standard. Again, snake venom NADase was used to prepare this conjugate. Fig. 23 shows the reversed-phase HPLC analyses of the products of this reaction. Panel A corresponds to the incubation of ADP-ribose and histidine. This control was used to determine whether a product was formed through a Schiff base reaction between the α amino group of the amino acid and ADP-ribose. Since ADP-ribose is produced by the hydrolysis of NAD to ADP-ribose and nicotinamide by NADase, a control for this reaction was needed. Panel B shows a positive control to check the activity of NADase. Panel C corresponds to the complete reaction mixture. The arrow denotes the appearance a new peak that did not appear in the controls. Since the glycosylic bond between ADP-ribose and imidazoyl is resistant to NaOH treatment, the putative histidine ADP-ribose peak was assumed to behave similarly. The analysis of sample on panel C, treated with alkali prior to HPLC, is shown on panel D. The product shown by the arrow is stable to these conditions. This new product was retained on a DHB-Sepharose column, which provided evidence that this new peak contained ADP-ribose.

The standard prepared above was converted to the etheno derivative in order to be detected with very high sensitivity, which are needed for in *in vivo* studies. Histidine ADP-ribose was treated with cloroacetaldehyde for 4

Figure 23. HPLC profile of the preparation of histidine ADP-ribose. The upper panel shows in panel A the incubation of ADP-ribose with histidine. Panel B shows the incubation of NADase with NAD, and panel C shows the complete reaction mixture of NADase, NAD and histidine. The Panel D shows the same reaction mixture as an panel C, treated with NaOH (1 M). The arrow shows the position of the putative histidine ADP-ribose.


Absorbance at 254 nm
ADPR
ADPR
Nam
ADPR
Nam
ADPR
Nam
A
B
C
D
0
10
0
10
0
10
0
10
Minutes

h at 60 °C. After purification by DHB-S the etheno derivative appears as a single peak in anion exchange chromatography (data not shown)

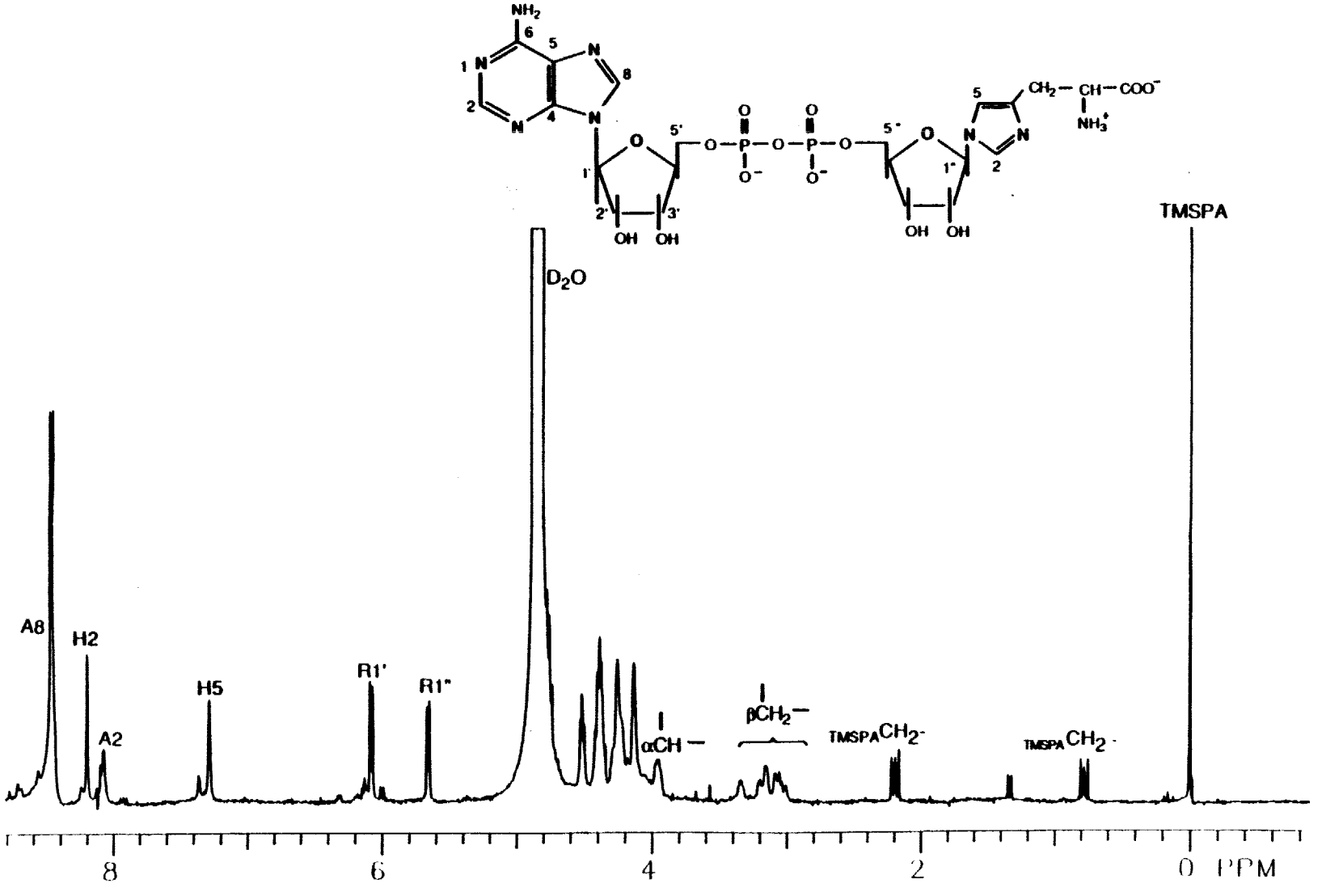
^{1}H-NMR of the putative ADP-ribosyl histidine

To elucidate the structure of the conjugate, NMR was used. Fig. 24 shows the ^{1}H-NMR spectrum. The presence of the adenine ring was shown by the signals at 8.05 and 8.23 ppm corresponding to H2 and H8 of the purine ring, respectively. At 6.05 ppm was shown a peak corresponding to the hydrogen of the anomeric carbon when adenine is bound to ribose. Two peaks at 7.23 and 8.20 ppm were the signals for the imidazole ring of histidine corresponding to H5 and H2, respectively. At 5.8 ppm a doublet was shown indicating the presence of an anomeric hydrogen when imidazole is bound to ADP-ribose. At 3.50 and 3.95 ppm were shown multiplets corresponding to the α and β hydrogens respectively of the histidine moiety of the molecule. This data provided evidence that the product obtained is histidine ADP-ribose.

Chemical stability of imidazoyl ADP-ribose

The ultimate goal of these studies was to identify proteins modified by ADP-ribose at histidine residues *in*

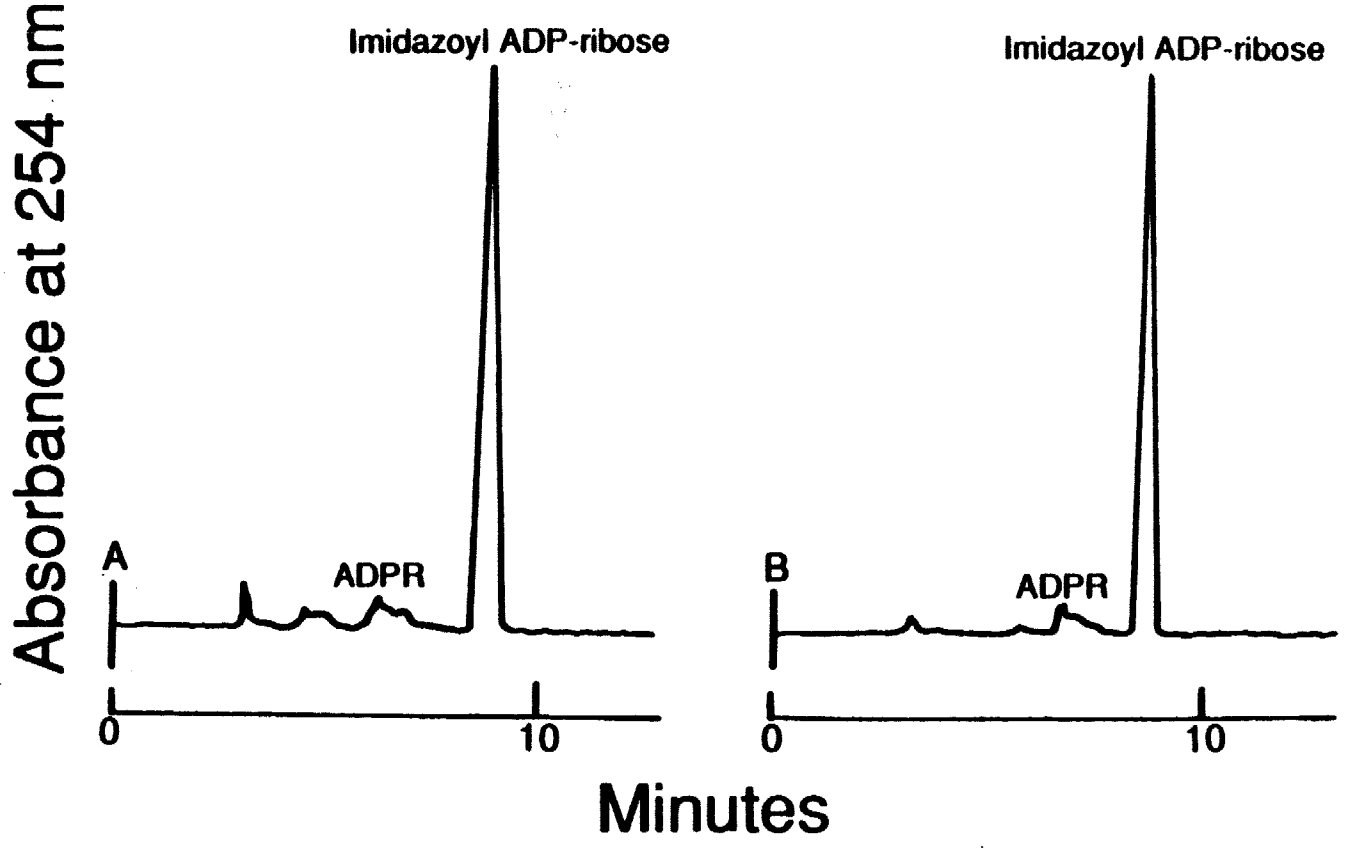
Figure 24. ^{1}H-NMR of the putative histidine ADP-ribose. The NMR sample was prepared as described in Methods.


A8
H2
A2
H5
R1'
R1"
D2O
αCH
βCH2
TMSPACH2
TMSPACH2
TMSPA
8
6
4
2
0 PPM

vivo. Thus experiments were designed to investigate the selective chemical release conditions that could differentiate imidazoyl containing conjugates from all the other known linkages to ADP-ribose. Fig. 25 shows a HPLC profile of imidazoyl ADP-ribose treated with alkali (1 M NaOH, and 37 ^{o}C). This conjugate was stable for at least 3 h. Similar results were obtained with all release conditions tested such as hydroxylamine (1 M, pH 7.0 and 37 ^{o}C), acid (44% formic acid, 37 ^{o}C) and mercury treatment (mercuric acetate 10 mM, room temperature). Histidine-like linkages behave as asparagine and diphtamide counterparts in being stable to all the chemical release conditions used. The table below shows a summary of the chemical release conditions for imidazoyl ADP-ribose and imidazole containing amino acids such as histidine and diphtamide.

ADP-Ribosyl linkage to:	Formic Acid(44%)	Hydroxylamine (1M, pH 7.0)	Hg^{2+} (10 mM)	CHES, pH 9.0	NaOH (1 M)
Histidine	Stable	Stable	Stable	Stable	Stable

Figure 25. HPLC profile of alkali treatment of imidazoyl ADP-ribose. Imidazoyl ADP-ribose was incubated in NaOH (1 M) at 37 °C. The right panel shows imidazoyl ADP-ribose at 0 min and left panel shows it at 2 h.


Absorbance at 254 nm
Imidazoyl ADP-ribose
Imidazoyl ADP-ribose
A
ADPR
B
ADPR
0
10
0
10
Minutes

Preparation of arginine ADP-ribose

It was important to have a standard of arginine ADP-ribose for comparing the chemical properties of this glycosylic bond with the known ADP-ribosyl amino acid linkages. Transferase A from turkey erythrocytes (70) was used. Fig. 26 shows the HPLC profile of this reaction. Panel A shows the incubation of arginine with NAD. Panel B shows the incubation of ADP-ribose with arginine to account for products resulting from the Schiff base reaction of the α amino group of the amino acid with the reducing sugar. Panel C shows the complete reaction mixture. The arrow indicates the products corresponding to arginine ADP-ribose (136).

Preparation of propionyl ADP-ribose

It was of considerable interest to prepare a conjugate that is an analogue of ADP-ribose glutamate. This conjugate is desired in the study of enzymes of ADP-ribose polymer metabolism and in differentiating between products formed by different classes of ADP-ribose transfer enzymes.

NADase was used to prepare a conjugate that contains an alkyl carboxylate bound to ADP-ribose to form an anomeric carboxylate linkage. Fig. 27 shows the reversed-phase HPLC analyses of the preparation of such a conjugate using propionate. Panel A shows the product of NAD incubated with

Figure 26 HPLC profile for the preparation of ADP-ribosyl arginine. Panel A shows the incubation of 2.5 mM NAD with 50 mM arginine in 50 mM potassim phosphate buffer pH 7.0. Panel B represents the incubation of 2.5 mM ADP-ribose with 50 mM arginine in 50 mM potassium phosphate pH 7.0 to account for Schiff base products. Panel C shows the complete reaction mixture that includes arginine, NAD, with the same concentration as mentioned above, 1 unit of transferase A, in 50 mM potassium phosphate pH 7.0.

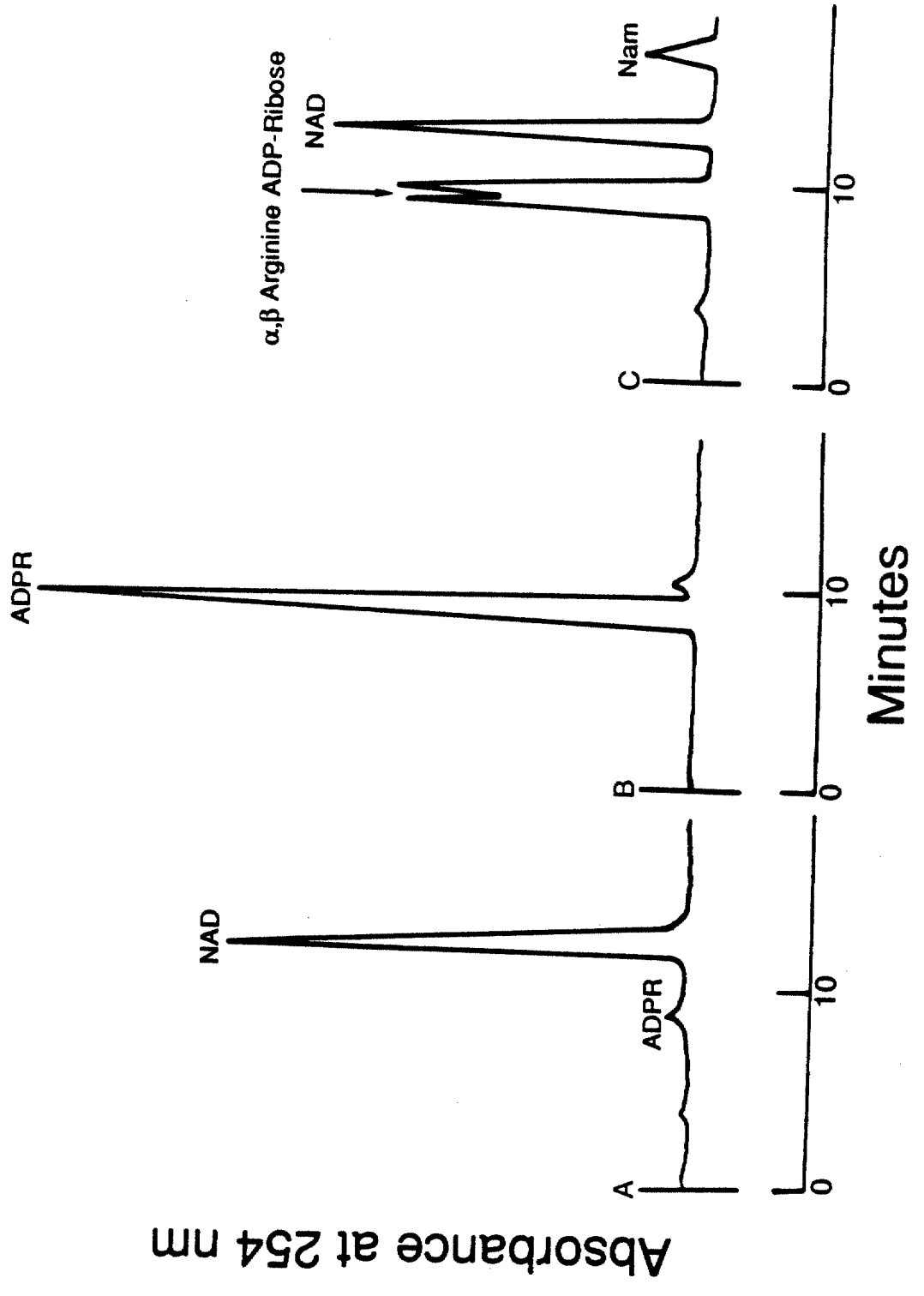
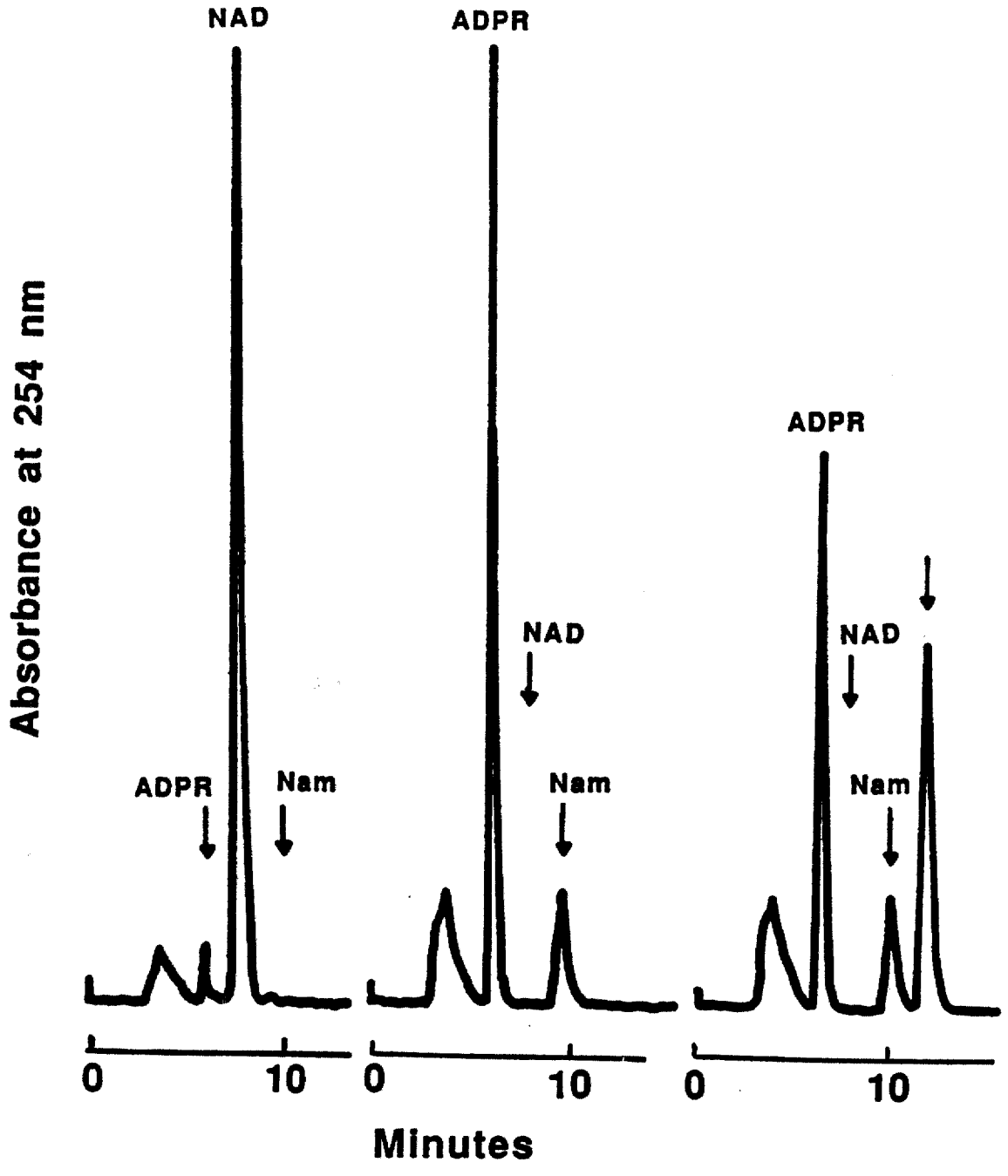

Absorbance at 254 nm
A
ADPR
NAD
B
ADPR
C
α,β Arginine ADP-Ribose
NAD
Nam
0
10
0
10
0
10
Minutes

Figure 27. HPLC profile of the preparation of propionyl ADP-ribose. Panel A is a control in which NAD was incubated with propionic acid. Panel B represents the incubation of NAD with NADase to check for the viability of the enzyme. Panel C shows the complete reaction mixture containing NAD, propionic acid and NADase. The arrow shows the product formed in the reaction. The arrow indicates the product obtained.


NAD
ADPR
ADPR
NAD
Nam
ADPR
NAD
Nam
NAD
Nam
Absorbance at 254 nm
0
10
0
10
0
10
Minutes

propionic acid, to check the possibility of a nonenzymic reaction between these reagents. NADase was incubated with NAD to check the activity of the enzyme as shown in panel B. The complete reaction mixture is seen in panel C, in which a new peak appeared eluting after nicotinamide as indicated by the arrow.

NMR of the putative propionyl ADP-ribose

Similarly to the approach above with other conjugates, NMR was used to assess the identity of the putative conjugate. A sample was prepared using a similar procedure as with all the other conjugates. Fig. 28 shows an ^{1}H-NMR spectrum of this sample. The presence of an adenine ring is suggested by the signals at 8.22 and 8.38 ppm corresponding to H2 and H8, respectively, of the purine ring. A triplet at 1.09 ppm corresponded to the methyl group of the propionate moiety of the molecule. At 2.4 ppm appeared a quartet corresponding to a methylene group vicinal to a methyl group. A doublet 6.07 ppm corresponded to the anomeric hydrogen of adenine bound to ribose. At 5.9 ppm is shown a doublet that corresponded to the anomeric hydrogen of propionate bound to ribose. The remaining hydrogens for the two riboses are shown in the region of 4.0 to 4.5 ppm. To distinguish clearly this region, ^{13}C-NMR was used. Fig. 29 shows the ^{13}C-NMR spectrum

Figure 28. ^{1}H NMR of the putative propionyl ADP-ribose. The product obtained in the complete reaction mixture shown in Fig. 27 was purified by reversed-phase HPLC as described in Methods and a sample for NMR was prepared.

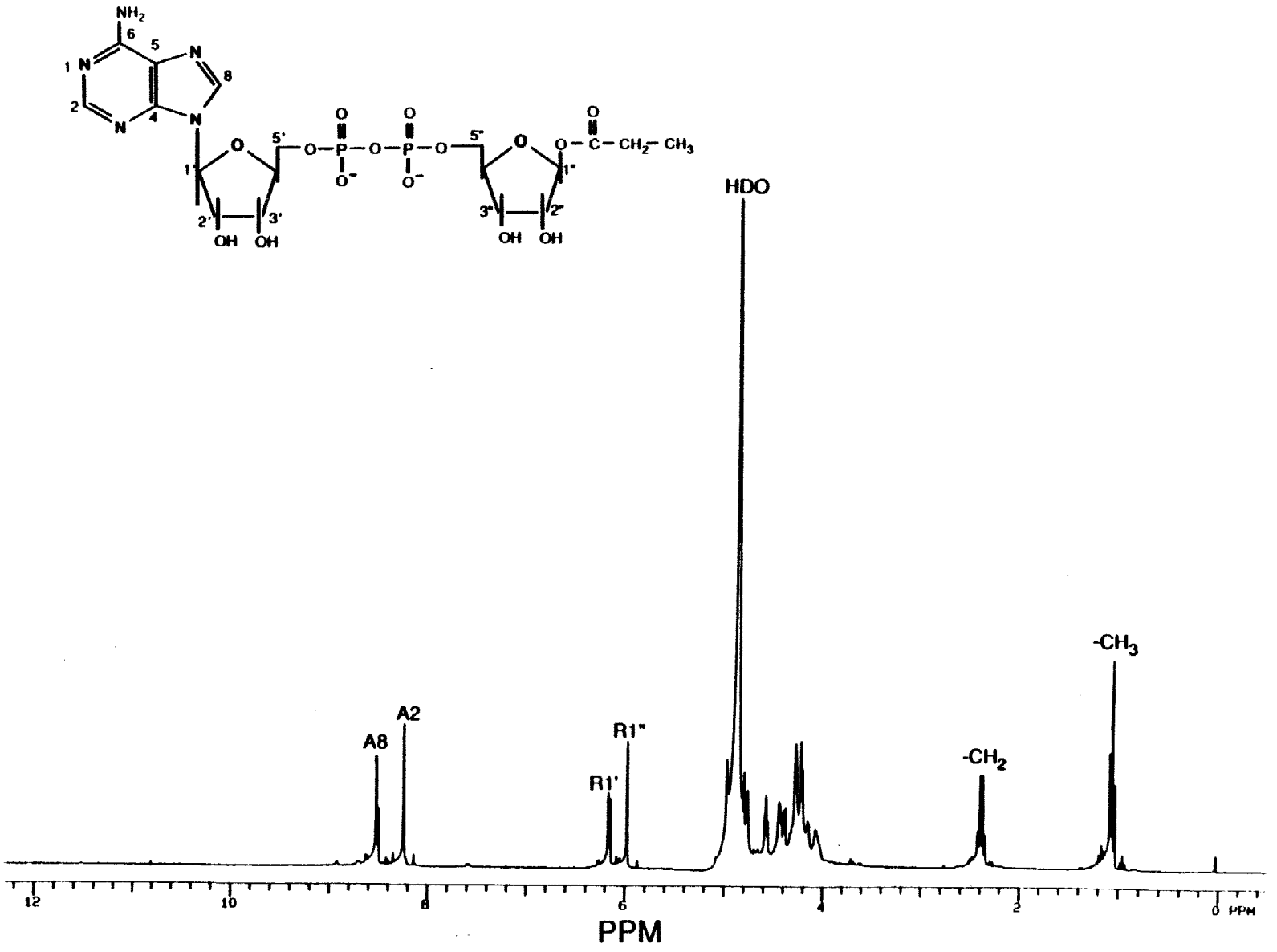
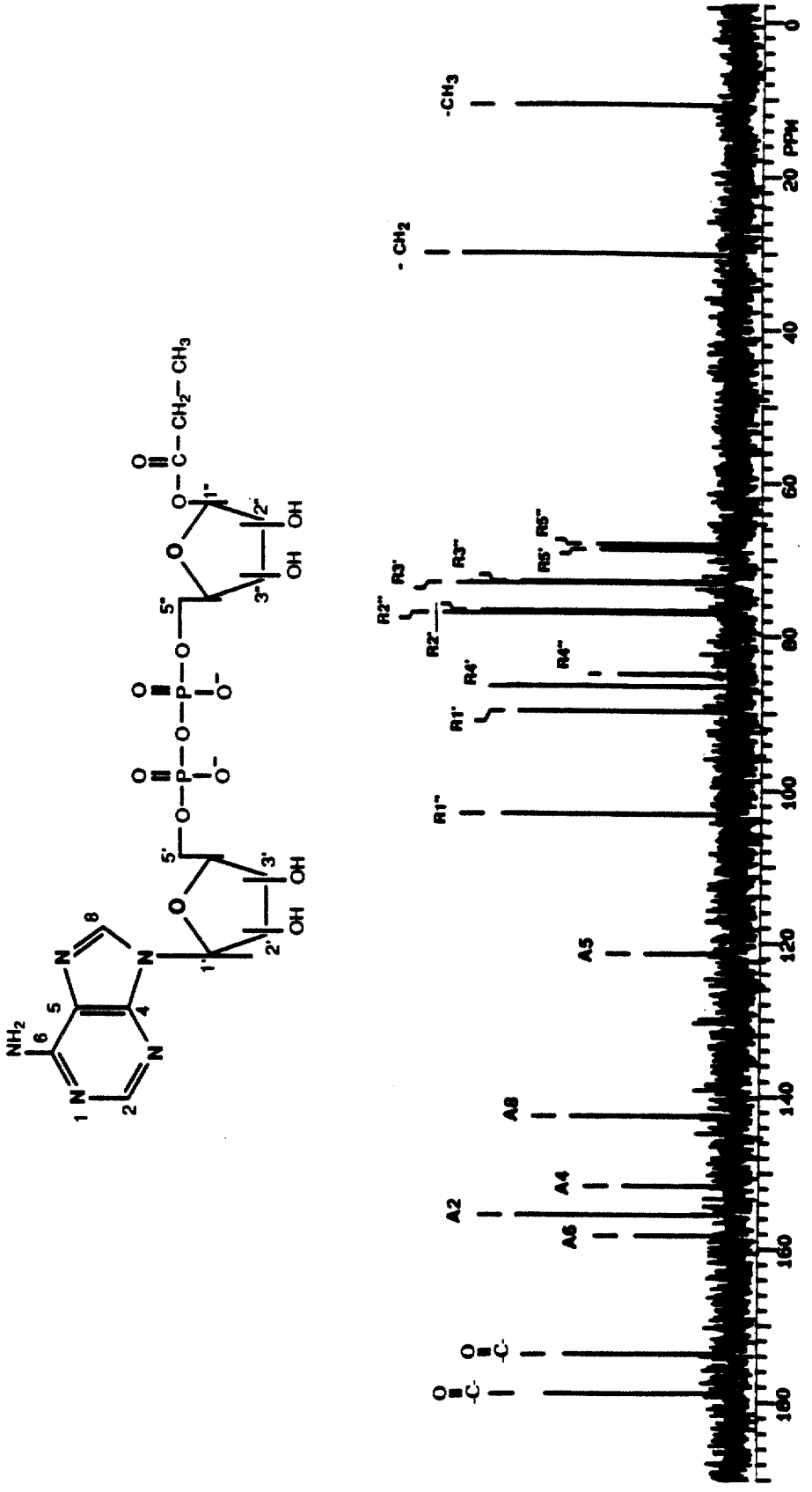

HDO
A8
A2
R1'
R1"
-CH2
-CH3
12
10
8
6
4
2
0 PPM
PPM

Figure 29. ^{13}C NMR of the putative propionyl ADP-ribose. A similar procedure for the preparation of ^{1}H NMR sample was followed.


NH2
6
5
4
8
N
N
N
N
1
2
1'
2'
3'
4'
5'
OH
OH
O
P
O⁻
O
C
CH2
CH3
1"
2"
3"
4"
5"
-CH3
-CH2
R3'
R3"
R5' R5"
R2"
R2'
R4'
R4"
R1'
R1"
A5
A8
A4
A2
A6
0
20 PPM
40
60
80
100
120
140
160
180

for this sample. At 174 ppm is shown the carbonyl group for the ester conjugate. At 82.5 and 83.5 ppm are shown the signals for the C4 of the two riboses. Two peaks at 77.0 and 77.3 ppm corresponded to C2 of both riboses. At 72.2 and 72.5 ppm appeared C3 for the riboses and 67.8 and 68.5 ppm the peaks for C5 were observed. This data provided definitive evidence that the structure of this product is propionyl ADP-ribose.

Chemical stability of propionyl ADP-ribose

The stability of this conjugate was tested using with the goal of differentiating it from the other known linkages to ADP-ribose and to compare it with the analogous linkage in histones. Fig. 30 shows the kinetics of release of propionyl ADP-ribose in hydroxylamine (1 M, 37 ^{o}C, pH 7.0). In Fig. 31 is shown the HPLC analysis of this reaction. The released product co-migrated with ADP-ribose. The half life of the conjugate was 8.0 min. The table below shows a summary of the chemical release conditions used.

ADP-Ribosyl linkage to:	Formic Acid(44%)	Hydroxylamine (1M, pH 7.0)	Hg^{2+} (10 mM)	CHES, pH 9.0	NaOH (1 M)
Propionate	Stable	Rapidly	Stable	Released	Released

Figure 30. Kinetics of hydrolysis in hydroxylamine of propionyl ADP-ribose. Purified propionyl ADP-ribose was incubated in 1 M hydroxylamine, pH 7.0, 100 mM MOPS, at 37 °C. Aliquots were taken and diluted to 1 ml with potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm.

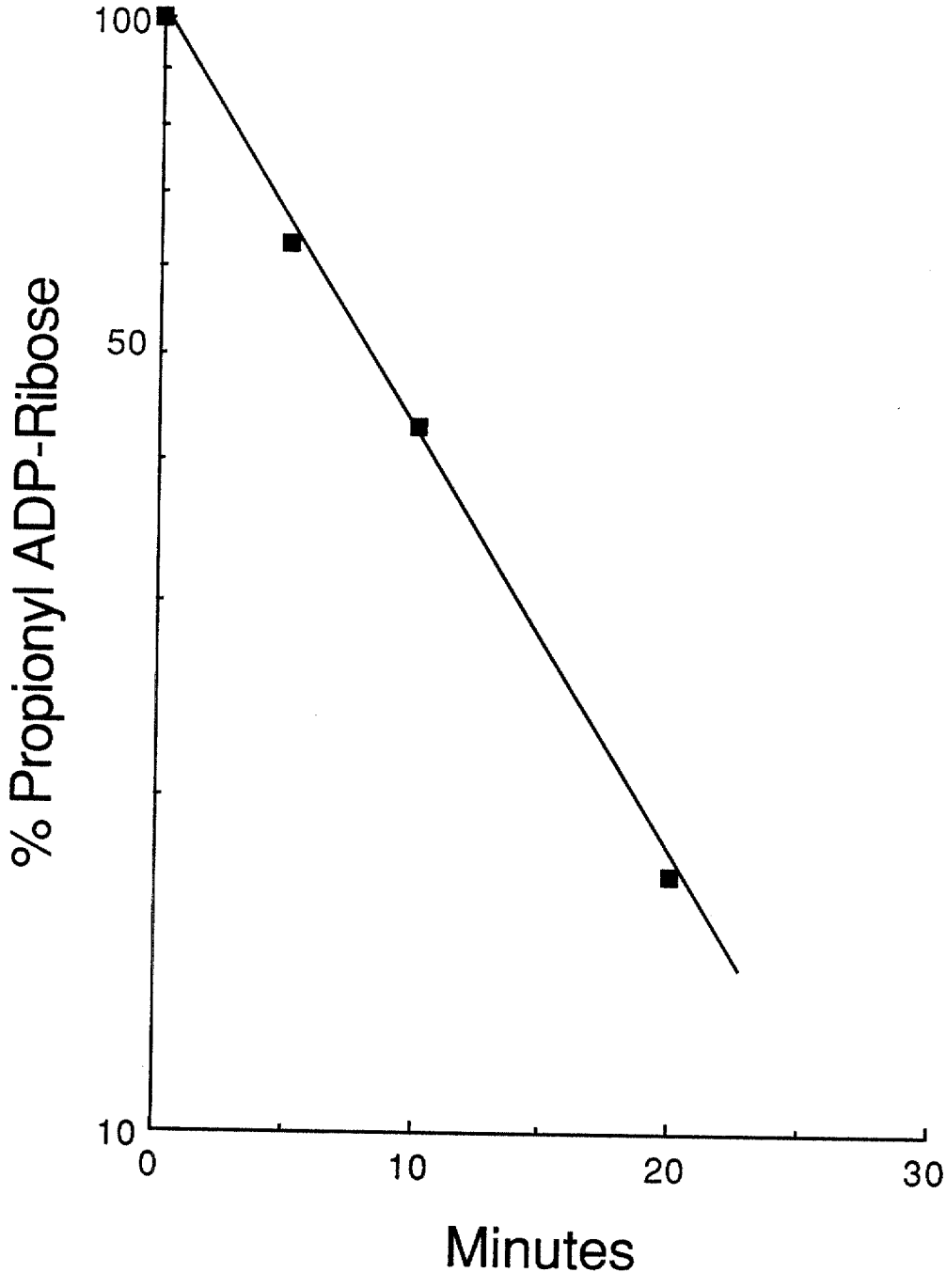
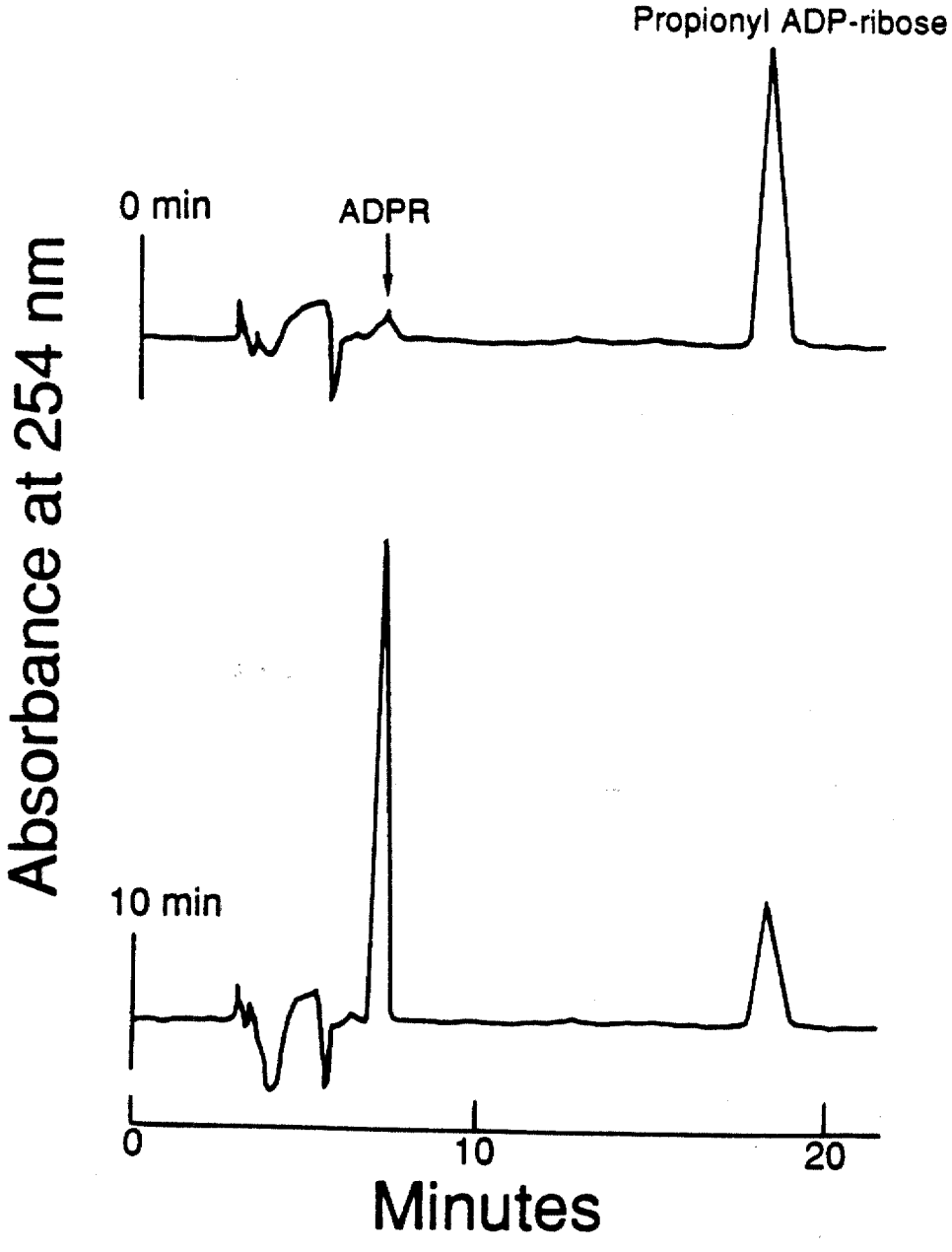

100
50
10
% Propionyl ADP-Ribose
0
10
20
30
Minutes

Figure 31. HPLC analysis of the hydroxylamine release product of propionyl ADP-ribose. Purified propionyl ADP-ribose was treated with 1M hydroxylamine. Reversed-phase HPLC was used to monitor the reaction with uv detection at 254 nm. Panel A shows the reaction at 0 minutes and Panel B shows the reaction at 10 minutes incubation at 37 ^{o}C.


Propionyl ADP-ribose
0 min
ADPR
Absorbance at 254 nm
10 min
0
10
20
Minutes

PART II: MODEL CONJUGATES FOR PROTEIN GLYCATION BY ADP-RIBOSE

Reaction of n-butylamine with ADP-ribose

The reaction of hexoses such as glucose with amines has been studied previously in detail (129, 127, 124, 128, 131, 47, 36, 69). Fig. 32 shows analogous products that may be formed between ADP-ribose and an alkyl amine group such as that found in lysine. This series of reactions shows the formation of a Schiff base adduct that can undergo either a ring closure to form α and ß aminoribosides or an Amadori rearrangement to form a ketoamine. In order to characterize the adducts formed between *n*-butylamine and ADP-ribose, the reactants were incubated at 37 °C, pH 8.0 and the products were monitored by reversed-phase HPLC. Fig. 33 shows analysis by HPLC over a 6 h period. At 2 h, primarily a single product was formed while at longer times a second product was observed. These compounds were termed products 1 and 2, respectively. When the pH of the reaction was in the range of 4.0 to 6.0, only product 1 was formed.

Figure 32. Possible products of the reaction of amines with ADP-ribose.

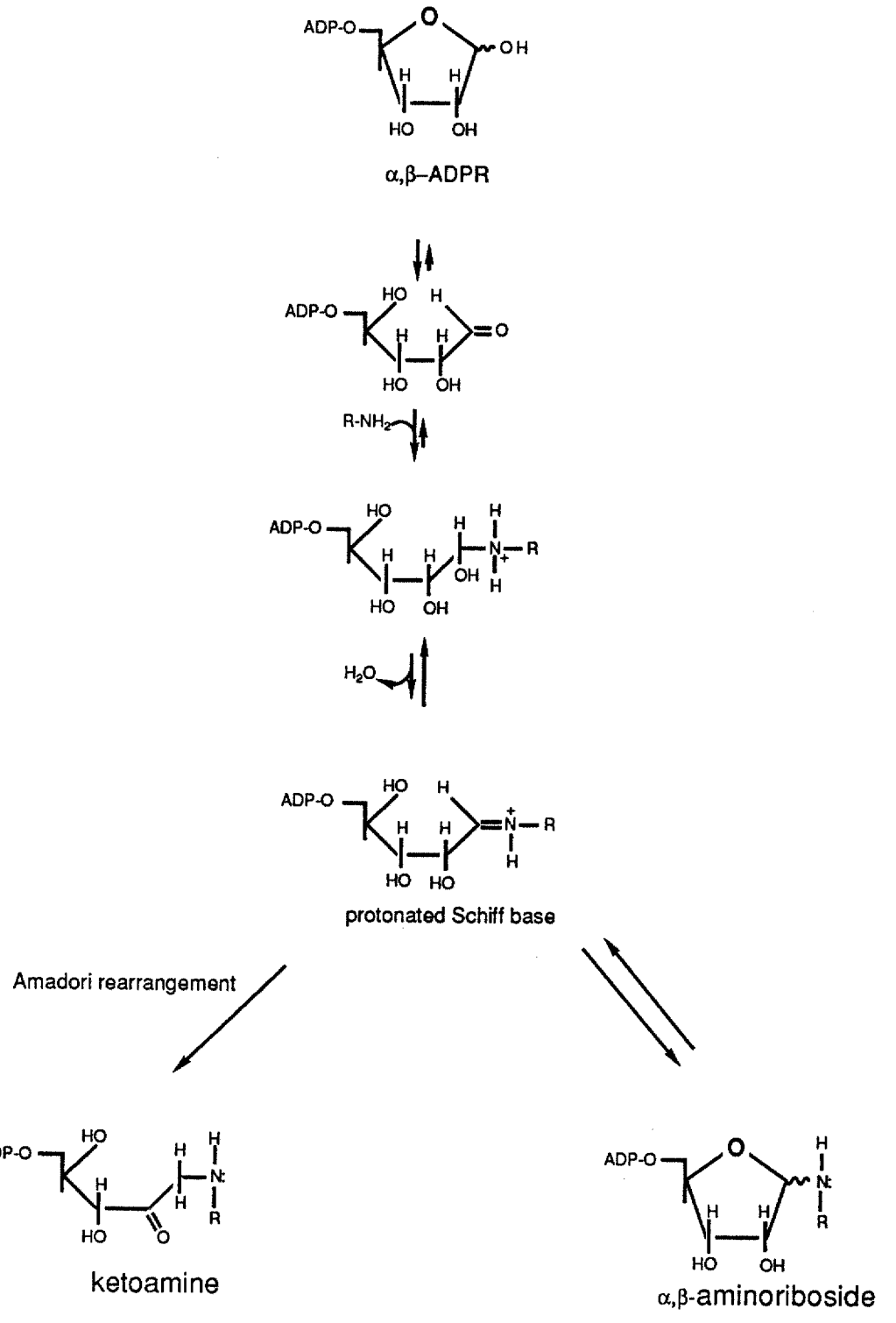
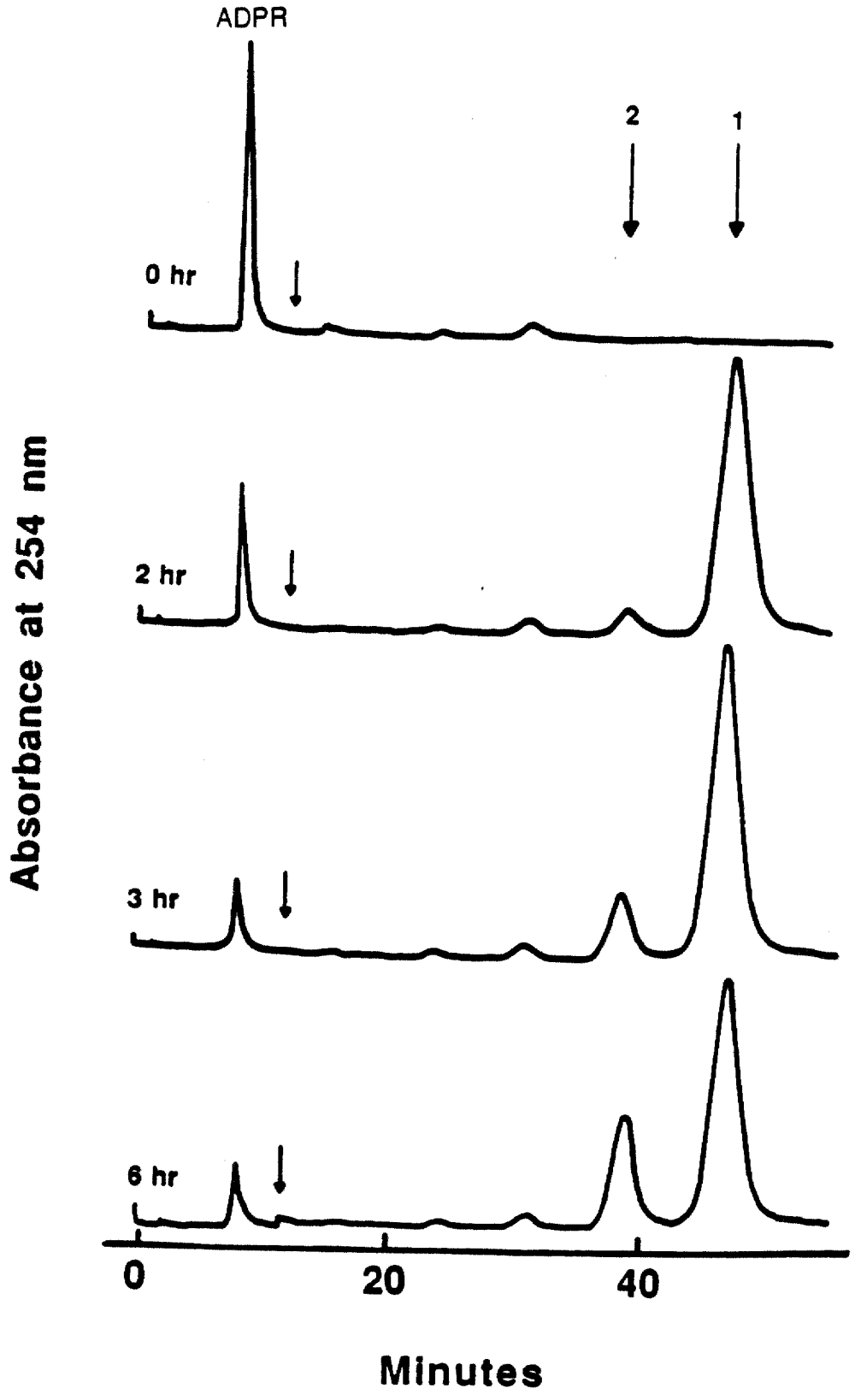

ADP-O
O
OH
H
H
HO
OH
α,β–ADPR
ADP-O
HO
H
H
H
O
HO
OH
R-NH2
ADP-O
HO
H
H
H
OH
N+
R
H
HO
OH
H
H2O
ADP-O
HO
H
H
H
N+
R
H
HO
HO
protonated Schiff base
Amadori rearrangement
ADP-O
HO
H
H
H
N
R
HO
O
ketoamine
ADP-O
O
H
N
R
H
H
HO
OH
α,β-aminoriboside

Figure 33. Time course of incubation of *n*-butylamine with ADP-ribose. The reaction mixture contained 100 mM potassium phosphate buffer, pH 8.0, 25 mM ADP-ribose, 3 M *n*-butylamine. Incubation was at 37 °C. At the times indicated aliquots were diluted to 1 ml with 50 mM potassium phosphate buffer, pH 6.0, and subjected to reversed-phase HPLC with uv detection at 254 nm. At 10 min of each run, the sensitivity was increased by a factor of 10 as indicated by the arrow. The position of ADP-ribose is shown. The positions of products 1 and 2 are shown by the numbered arrows.


ADPR
2
1
0 hr
2 hr
3 hr
6 hr
Absorbance at 254 nm
0
20
40
Minutes

^{13}C NMR analysis of products 1 and 2

The formation of 2 products suggested two likely possibilities: (i) the two peaks could correspond to the formation of α and ß anomers of an aminoriboside, or (ii) the peaks could represent formation of an aminoriboside and a ketoamine. In order to test these possibilities products 1 and 2 were isolated by preparative reversed-phase HPLC and analyzed by ^{13}C NMR as shown in Figs. 34 and 35. Product 1 was prepared at pH 5.0 since it was quite unstable at higher pH. For both products, absorptions appeared in four well defined regions of the spectrum. Peaks corresponding to the four *n*-butyl group carbons occurred between 10 and 55 ppm as expected, and the signals at 207 and 208 ppm were clearly from a ketone carbonyl carbon. The adenine ring carbons appeared between 120 and 160 ppm in accord with previously reported data for ADP-ribose and related adenine containing compounds (125). The spectrum of product 1 showed additional peaks at 174 and 118 ppm and additional signals in the butylamine region that very likely correspond to the enol form of a ketoamine (see discussion). Thus, the spectral analysis of products 1 and 2, by virtue of the signals at 207 and 208 ppm, respectively, indicated that they were both ketoamines. In the case of product 2 this conclusion was further supported by the observation that the region between

Figure 34. ^{13}C NMR analysis of product 1. Product 1 was prepared as described in Methods. NMR assignments for the ribose and adenine regions are based on published data of related adenosine containing compounds (125).

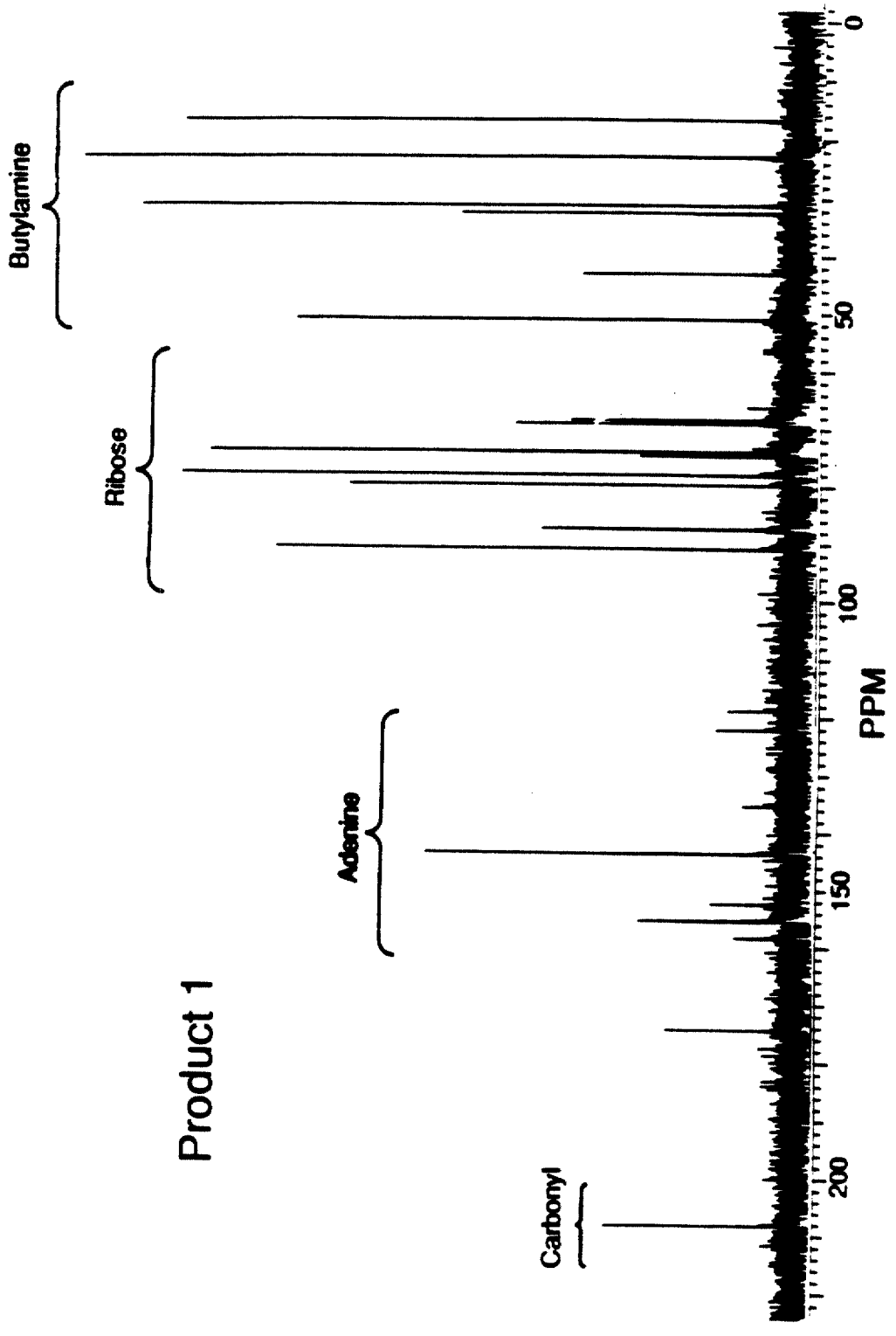
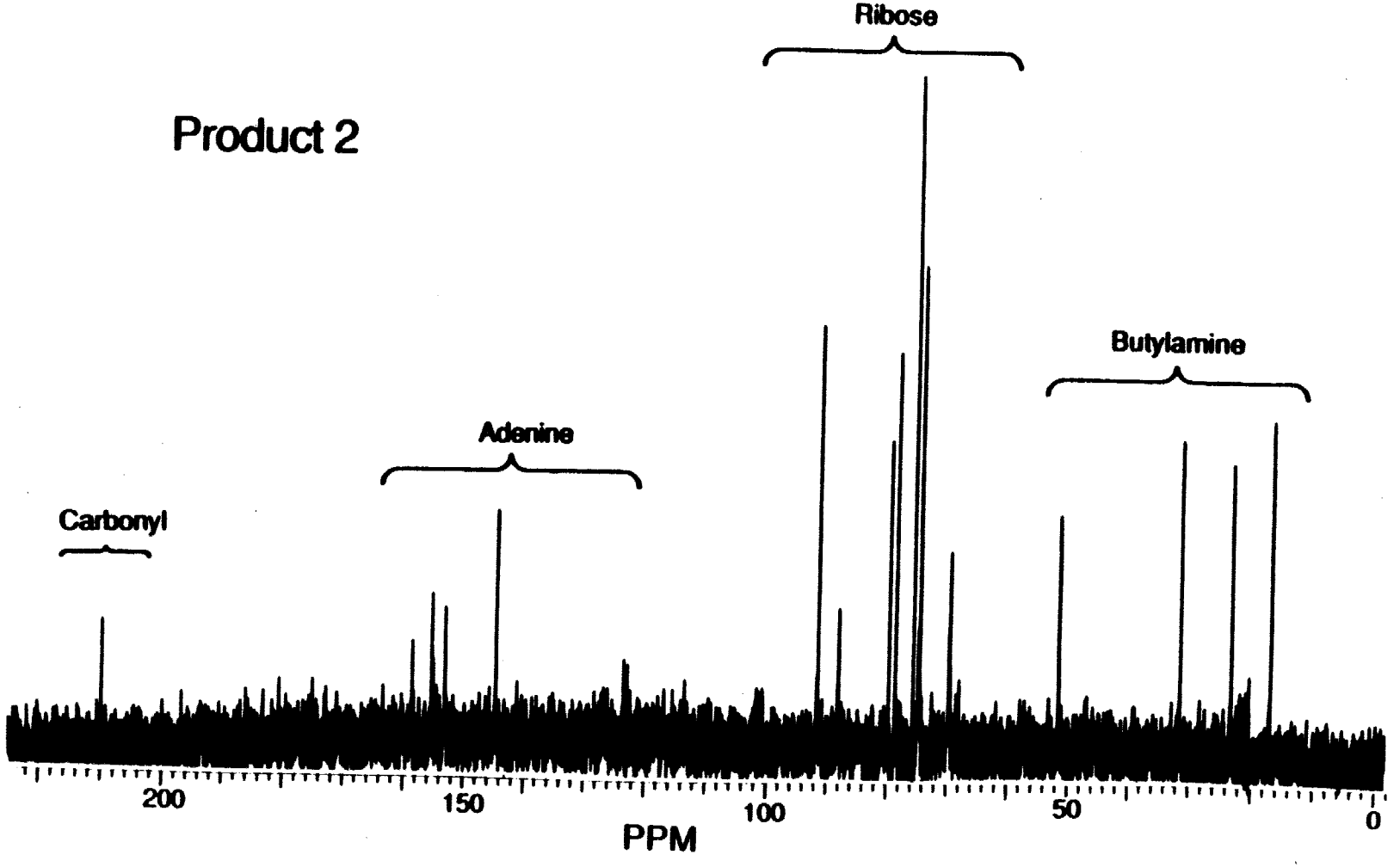

Product 1
Butylamine
Ribose
Adenine
Carbonyl
200
150
100
50
0
PPM

Figure 35. ^{13}C NMR analysis of product 2. Product 2 was prepared as described in Methods. NMR assignments for the ribose and adenine regions are based on published data of related adenosine containing compounds (125).


Product 2
Ribose
Butylamine
Adenine
Carbonyl
200
150
100
50
0
PPM

65 and 90 ppm contained only 7 rather than the 9 absorptions expected for one ribofuranose and four carbons from an opened ring. Proton exchange with D_2O at both carbons α to a carbonyl would lead to coupling, increased relaxation times, and line broadening, which attenuated these signals into the base line noise.

In order to further test for the presence of a carbonyl group in these compounds, the color test described by Fearon *et al.* (123), using o-dinitrobenzene was used. The mechanism proposed for the reaction with this reagent in shown in Fig. 36. Ketoamines give a purple color in this test while aminoribosides do not. When products 1 and 2 were incubated with this reagent, an intense purple color was obtained for both, further indicating that they were ketoamine products produced by an Amadori rearrangement. Taken together, these data provided evidence that both products 1 and 2 are ketoamines.

Borohydride treatment of products 1 and 2

In order to further characterize ketoamines 1 and 2, they were reacted with the reducing agent borohydride. This reagent reduces carbonyl groups to alcohols. Figs. 37 and 38 shows the HPLC analysis of this reaction. Panel A in both Figures shows the respective ketoamines at 0 min. Panel B in

Figure 36. Proposed Mechanism for the reaction of ketoamines and o-dinitrobenzene.

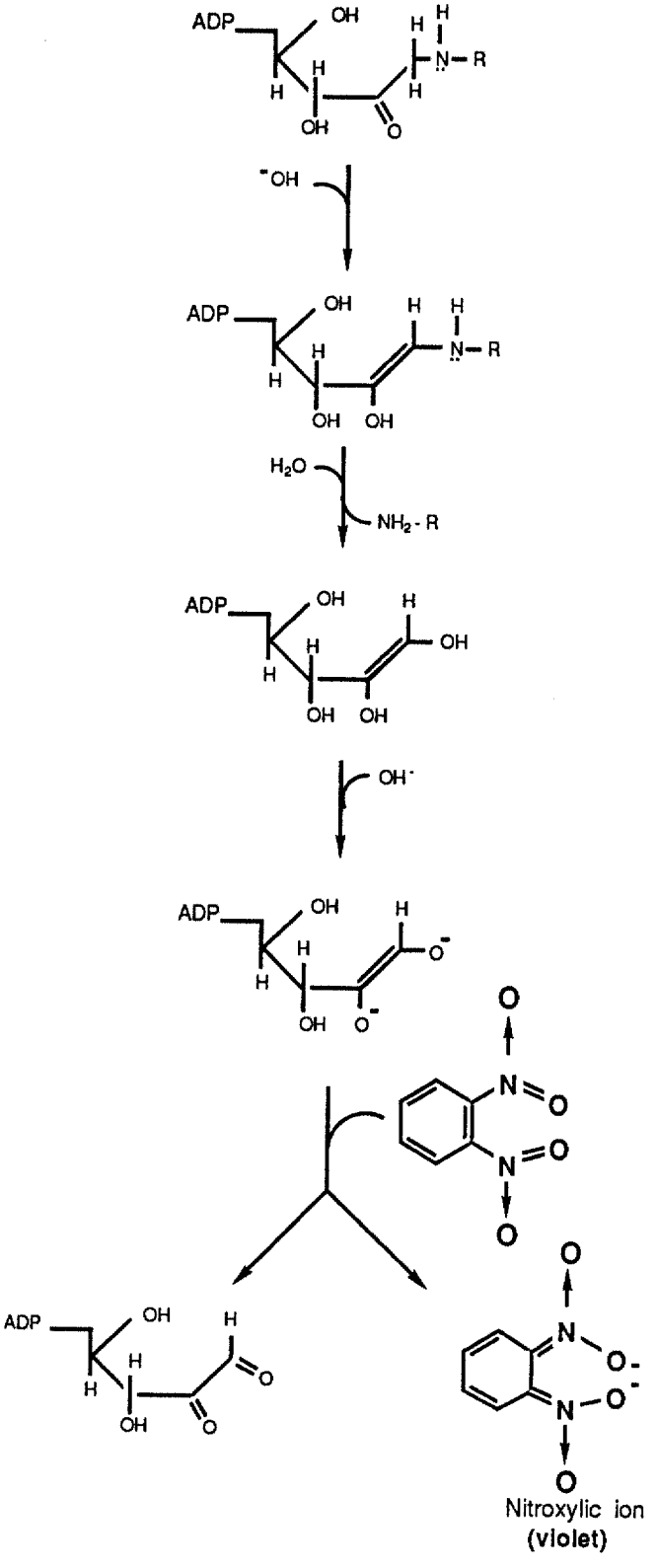

ADP
OH
H
N
R
OH
O
⁻OH
ADP
OH
H
N
R
H
OH
OH
H₂O
NH₂ - R
ADP
OH
H
OH
H
OH
OH
OH⁻
ADP
OH
H
O⁻
H
OH
O⁻
O
N
O
N
O
O
ADP
OH
H
H
H
O
OH
O
O
N
O⁻
N
O⁻
O
Nitroxylic ion
(violet)

Figure 37. HPLC analysis of the sodium borohydride treatment of ketoamine 1. Purified ketoamine 1 was incubated with sodium borohydride as described in Methods. Panel A shows the HPLC profile of this reaction at 0 min. Panel B represents this reaction after 1 h.

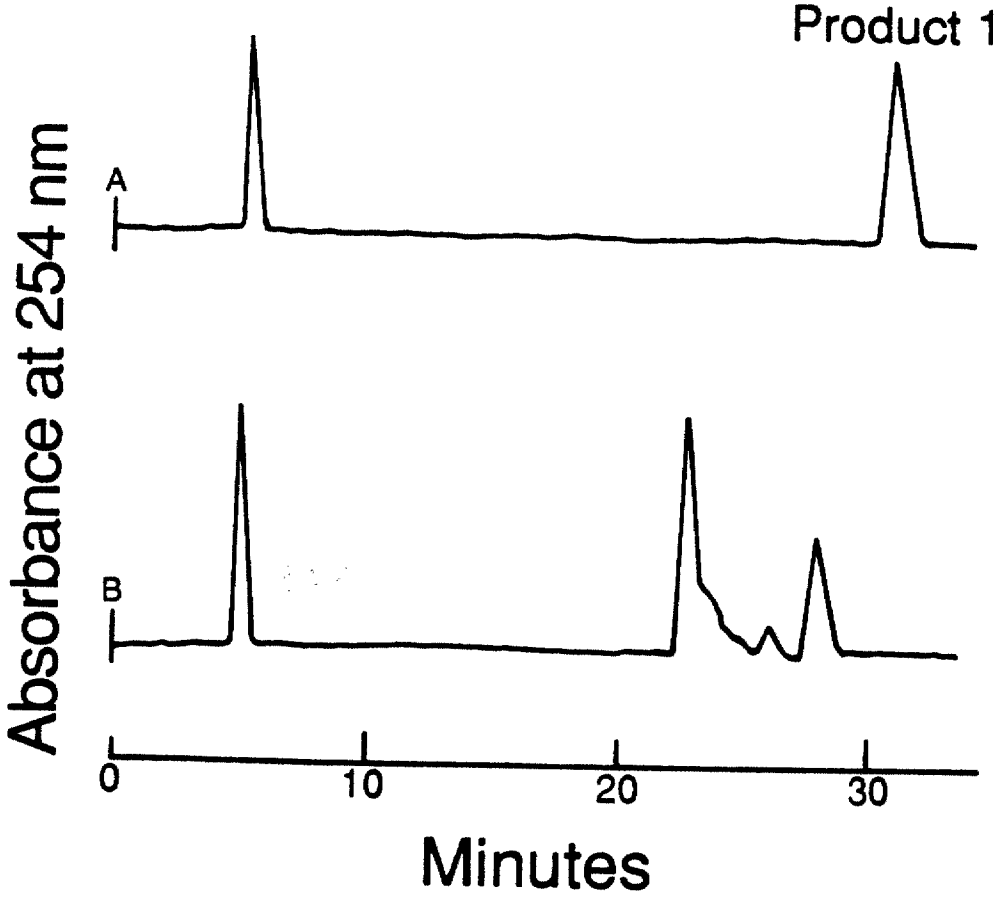

Product 1
A
B
Absorbance at 254 nm
0
10
20
30
Minutes

Figure 38. HPLC analysis of the treatment of ketoamine 2 with sodium borohydride. Purified ketoamine 2 was reduced with sodium borohydride as described in Methods. Panel A shows the HPLC profile of this reaction at 0 min while panel B at 1 h.

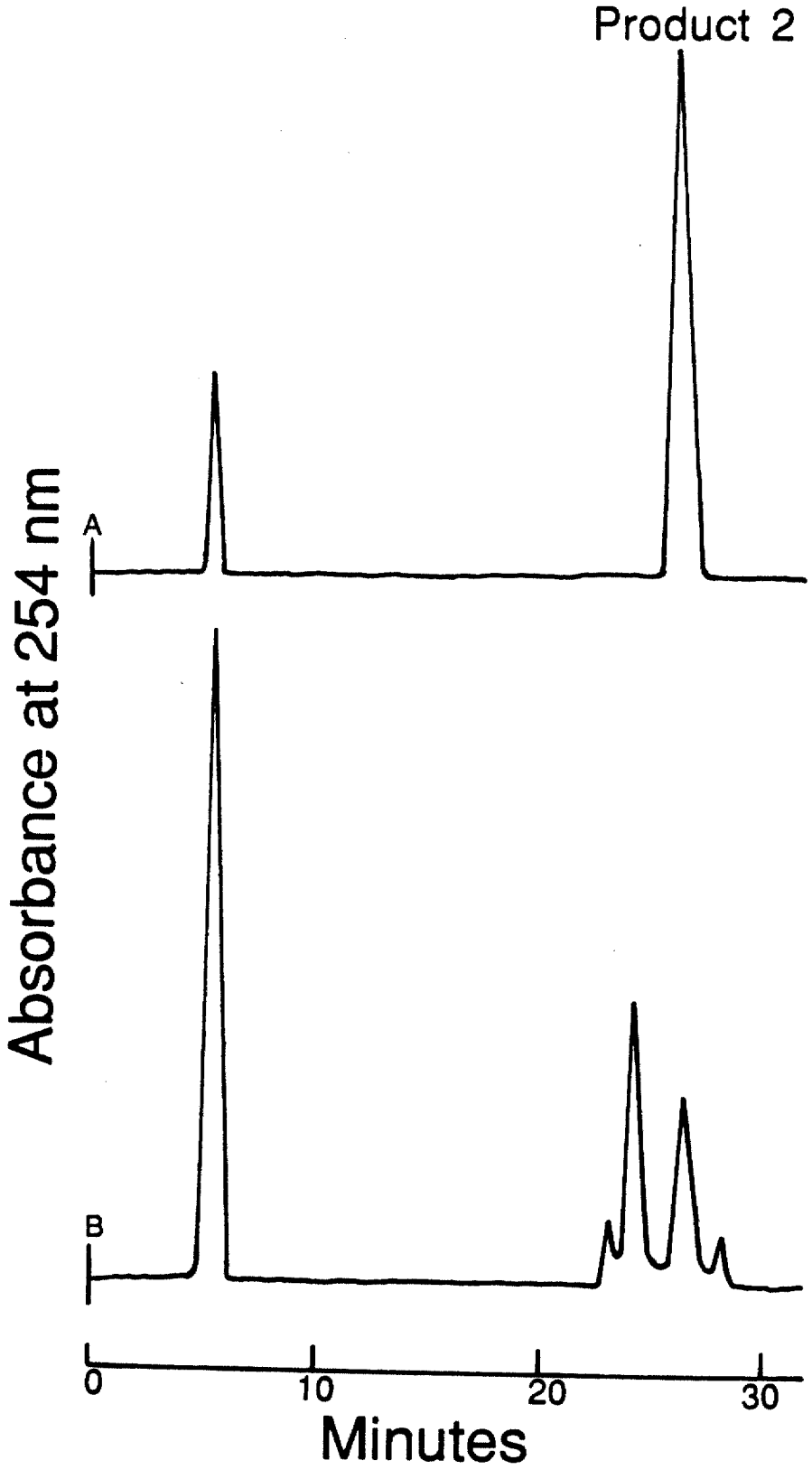

Product 2
Absorbance at 254 nm
A
B
0
10
20
30
Minutes

Fig. 37 shows that ketoamine 1 four products were formed, two of them in significantly larger amounts. Panel B in Fig. 38 shows that ketoamine 2 also formed four products although analogous to ketoamine 1, two of them are in higher amounts.

Reaction of ketoamines 1 and 2 with hydroxylamine

In order to further characterize the putative ketoamines, they were incubated with 1 M hydroxylamine at pH 7.0 and analyzed by reversed-phase HPLC, as shown in Figs. 39 and 40 for ketoamines 1 and 2, respectively. After 2 h of incubation at 37 ^{o}C, as shown in Fig. 39, ketoamine 1 was quantitatively converted to a single product. Analysis of the product by ^{13}C NMR showed the disappearance of the peak at 207 ppm and the appearance of a peak at 160 ppm, which is characteristic of an oxime. This compound has been termed oxime 1. When ketoamine 2 was incubated with hydroxylamine (Fig. 40) and analyzed by reversed-phase HPLC, two products (termed oximes 2 and 3) were formed.

^{1}H NMR characterization of the oximes obtained from the putative ketoamines

In order to identify the positions of the carbonyl groups in ketoamines 1 and 2, the three oximes obtained were

Figure 39. Time course of the reaction of ketoamine 1 with hydroxylamine. Purified ketoamine 1 was incubated in 100 mM MOPS, pH 7.0, 1 M hydroxylamine at 37 °C. At the times indicated, aliquots were diluted to 1 ml with 100 mM potassium phosphate buffer, pH 6.0.

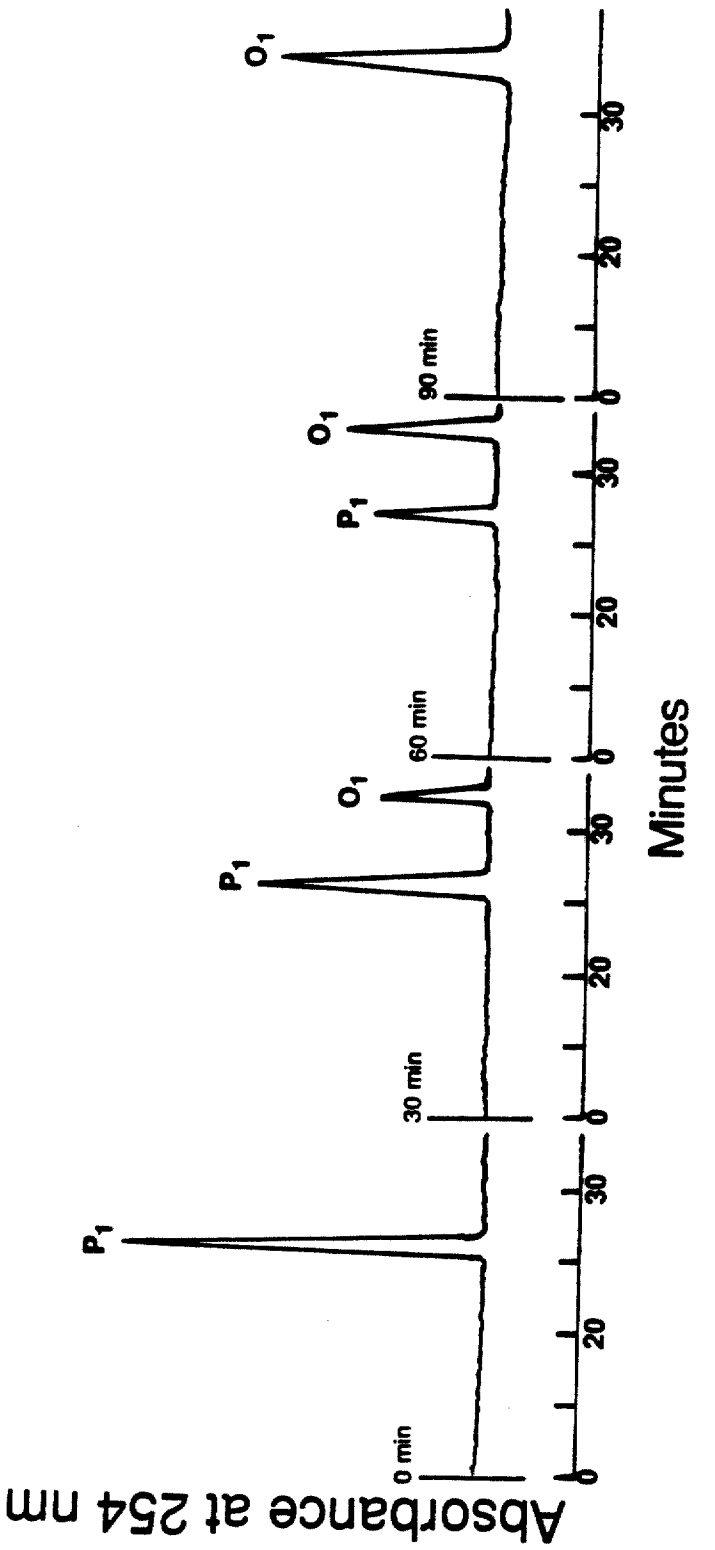
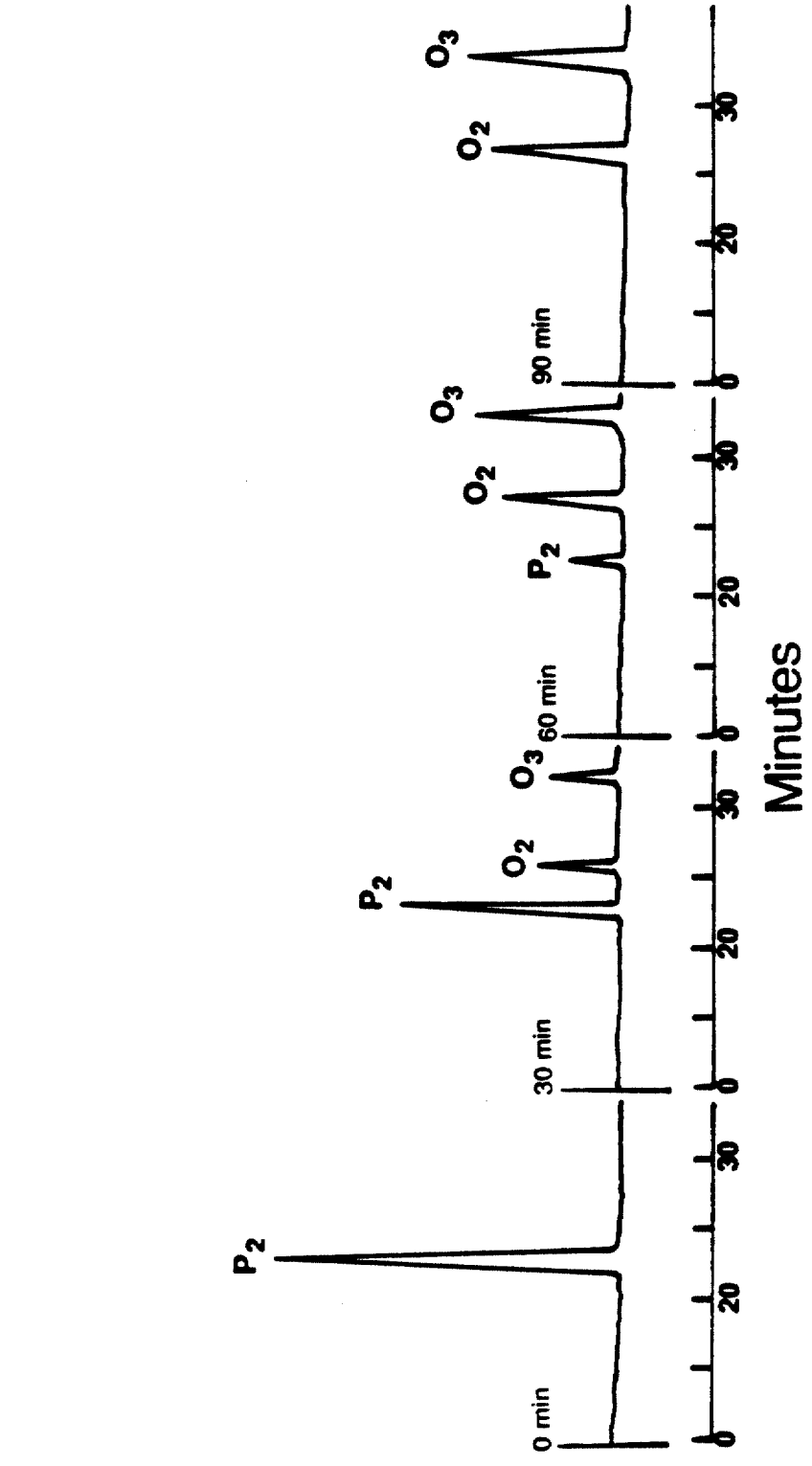

Absorbance at 254 nm
Minutes
0 min
30 min
60 min
90 min
P1
O1
0
20
30

Figure 40. Time course of the reaction of ketoamine 2 with hydroxylamine. Purified ketoamine 2 was incubated in 100 mm MOPS, pH 7.0, 1 M hydroxylamine at 37 °C. At the times indicated, aliquots were diluted to 1 ml with 100 mM potassium phosphate buffer, pH 6.0.


Absorbance at 254 nm
0 min
30 min
60 min
90 min
P_2
O_2
O_3
Minutes
0
20
30

analyzed by ^{1}H NMR. Figs. 41, 42, and 43 show a portion of the ^{1}H NMR spectrum of oximes 1, 2, and 3, respectively. The ^{1}H NMR of oxime 1 in Fig. 41 shows a pair of triplets at 2.83 and 2.95 ppm represented methylene protons on the nitrogen proximal carbon of the butylamino group. The presence of two absorptions is consistent with the expectation of a mixture of syn and anti isomers of an oxime. The diastereotopic methylene protons on the carbon α to both the oxime and nitrogen appeared as a pair of doublets (J=13.0 Hz) at 3.75 and 3.87 ppm. Finally, the doublet (J=7.0 Hz) at 4.26 ppm must be the proton on carbon 3" since this hydrogen can be coupled to a single vicinal proton only if the imino carbon is located at position 2". Thus, these data are consistent only with the oxime having an imino carbon at position 2".

Fig. 42 shows the ^{1}H NMR spectrum of oxime 2. Again, the mixture of *syn* and *anti* oxime isomers has led to two overlapping absorptions centered at 2.83 ppm for methylene protons on the nitrogen proximal carbon of the butylamino group. However, they are more nearly equivalent than in oxime 1 indicating that the site of isomerism is farther removed from the butylamino group. The key diagnostic information in this spectrum is the pair of doubled doublets (dd, J=11.8, 4.3 Hz and dd, J=11.8, 6.4 Hz) centered at 3.44 ppm. These are the methylene protons on the carbon α to nitrogen and represent the AB portion of an ABC spin system.

Figure 41. ^{1}H NMR spectra of oxime 1. The oxime was prepared as described in Methods. The region corresponding to ribose and the nitrogen proximal methylene group from butylamine is shown.

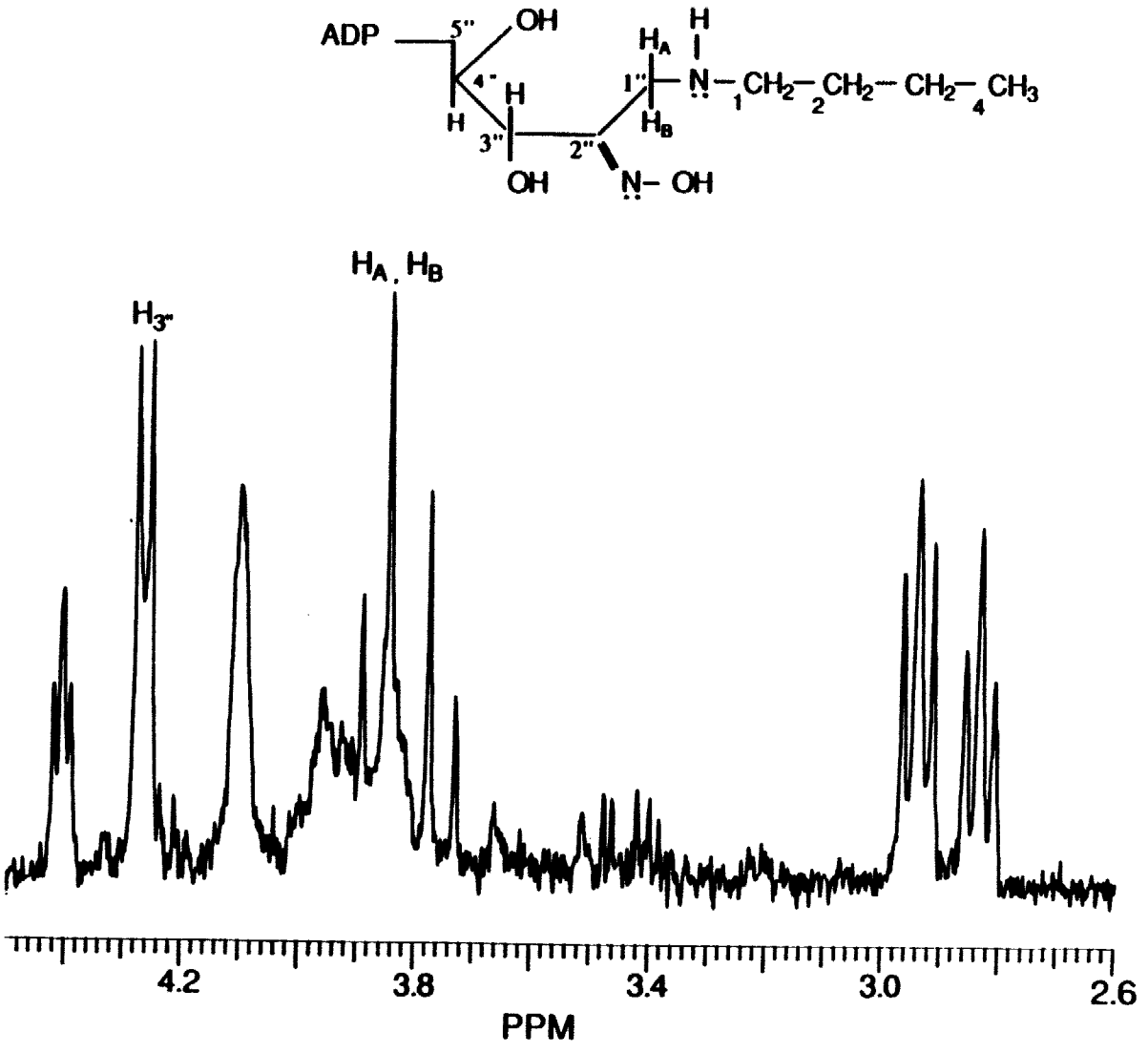

ADP
5''
OH
4''
H
H
3''
OH
2''
N– OH
H
H_A
1''
H_B
N
1
CH2
2
CH2
CH2
4
CH3
H_A, H_B
H_3''
4.2
3.8
3.4
3.0
2.6
PPM

Figure 42. ^{1}H NMR spectrum of oxime 2. The oxime was prepared as described in Methods. The region corresponding to ribose and the nitrogen proximal methylene group from butylamine is shown.

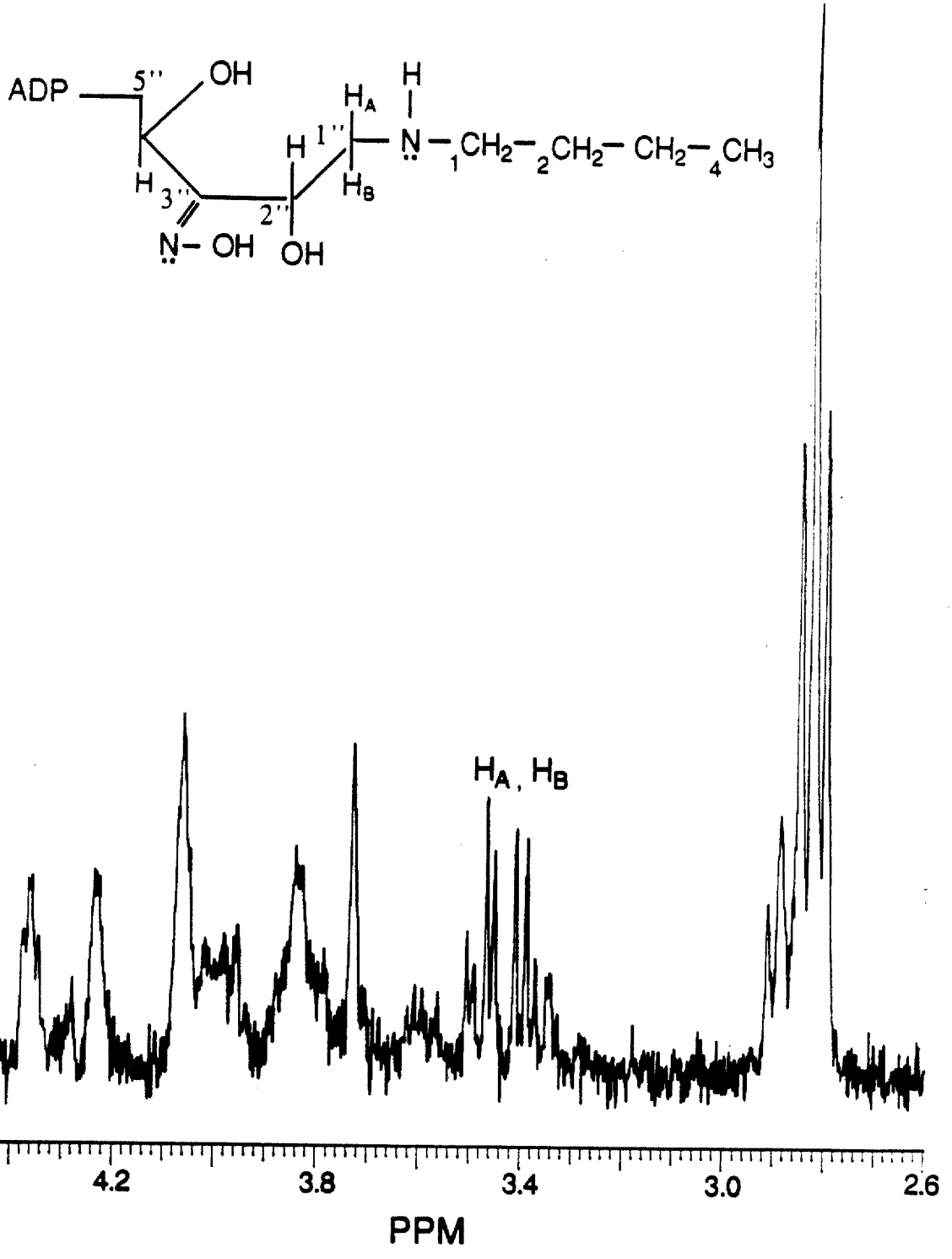
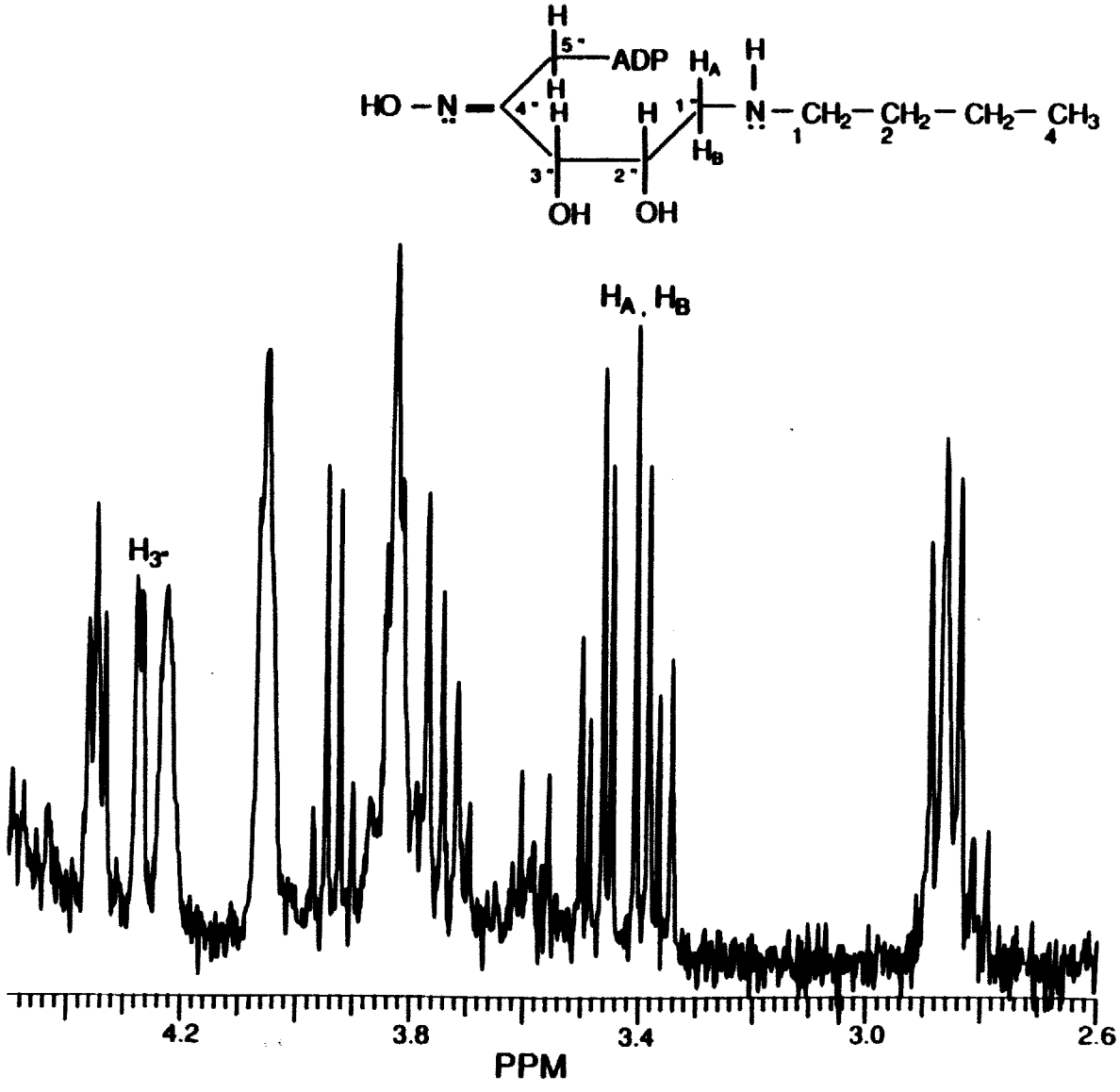

ADP
5''
OH
H
3''
N– OH
2''
OH
H
1''
H_A
H_B
H
N
CH2–1 CH2–2 CH2– CH2–4 CH3
H_A , H_B
4.2
3.8
3.4
3.0
2.6
PPM

Figure 43. ^{1}H NMR spectrum of oxime 4. The oxime was prepared as described in Methods. The region shown is the same as for oxime 2 and 3 (Figs. 42, and 43).


H
5'
ADP
H_A
H
HO – N
4'
H
H
1'
N – CH_2 – CH_2 – CH_2 – CH_3
H_B
3'
2'
OH
OH
H_3'
H_A, H_B
4.2
3.8
3.4
3.0
2.6
PPM

These data are consistent with the proposal that oxime 2 is derived from a ketoamine with the carbonyl at position 3".

Fig. 43 shows the ^{1}H NMR spectrum of oxime 3. If this oxime is indeed a mixture of syn and anti isomers, the siteof isomerism is well removed from the carbon a to nitrogen since there is a single triplet at 2.85 ppm for the methylene protons on the nitrogen proximal carbon of the butylamino group. The ABC spin system for protons on carbons 1 and 2 is a prominent spectral feature. A pair of doubled doublets (dd, J=11.8, 4.3 Hz and dd, J=11.8, 6.4 Hz) centered at 3.42 ppm represent the geminal protons of the methylene group. The vicinal methine proton was easily located at 3.6 ppm by examining the COSY spectrum of the molecule (data not shown). This analysis indicates that oxime 3 was derived from a ketoamine with a carbonyl at position 4.

Stability studies of products 1 and 2

The chemical stability of ketoamines 1 and 2 has been studied with the aim of identifying conditions that would allow the differentiation of ADP-ribose lysine glycation products from the other ADP-ribosyl amino acids that can occur in proteins by the action of protein, mono-ADP-ribosyltransferases.

Previous studies from this laboratory (43,88, 122) along

with studies described in this dissertation have characterized the chemical stability of linkages between ADP-ribose and amino acid side chains known to be modified by protein, mono-ADP-ribosyltransferases (43, 88, 122). Table I summarizes chemical stability properties for ketoamines 1 and 2 and compares them to linkages between other amino acid side chains and ADP-ribose. An ADP-ribose conjugate was considered stable when its $t_{1/2}$ was at least 10 h. Both ketoamines 1 and 2 were relatively stable in 44% formic acid with $t_{1/2}$ values of 13 and 16 h, respectively (data not shown). Also they were stable to Hg+2 treatment (data not shown) and stable in hydroxylamine as described above. Both ketoamines were unstable in NaOH (1 M). The key difference was the lability of both ketoamines at pH 9.0. Fig. 44 shows that the ketoamines 1 and 2 were cleaved with $t_{1/2}$ of 16 and 32 min, respectively at pH 9.0. For comparison, ADP-ribosylarginine is also shown.

Figs. 45 and 46 shows the result of incubation of ketoamine 1 and 2 at pH 9.0, respectively. Figures 47 and 48 shows the HPLC profile of the pH 9.0 hydrolysis of ^{14}C radiolabel ketoamines 1 and 2 respectively. For reference purposes the main hydrolysis products from ketoamines 1, have been labeled k1a and k1 b and, from ketoamine 2, k2a and k2b. While the nature of the main products has not yet been established, thet did not co-migrate with ADP-ribose.

Table I: Stability of the linkage between amino acid side chains and ADP-Ribose

ADP-Ribosyl linkage to:	Formic Acid(44%)	Hydroxylamine (1M, pH 7.0)	Hg^{2+} (10 mM)	CHES, pH 9.0	NaOH (1 M)
Carboxylate	Stable	Rapidly Released	Stable	Released	Released
Arginine	Stable	Slowly Released	Stable	Stable	Released
Cysteine	Stable	Stable	Released	Stable	Released
Histidine (Diphtamide)	Stable	Stable	Stable	Stable	Stable
Asparagine	Stable	Stable	Stable	Stable	Stable
Serine, Threonine	Released	Stable	Stable	Stable	Stable
Lysine (ketoamines)	Stable	Stable	Stable	Released	Released

Figure 44. Kinetics of the hydrolysis at pH 9.0 for ketoamines 1 and 2, and ADP-ribosyl arginine. Purified ketoamines 1 and 2, were incubated in 100 mM CHES buffer, pH 9.0, at 37 °C. Aliquots were taken and diluted to 1 ml with potassium phosphate buffer, pH 6.0. Samples were subjected to reversed-phase HPLC with detection at 254 nm. (■) ketoamine 1; (●) ketoamine 2; (▲) ADP-ribosyl arginine.

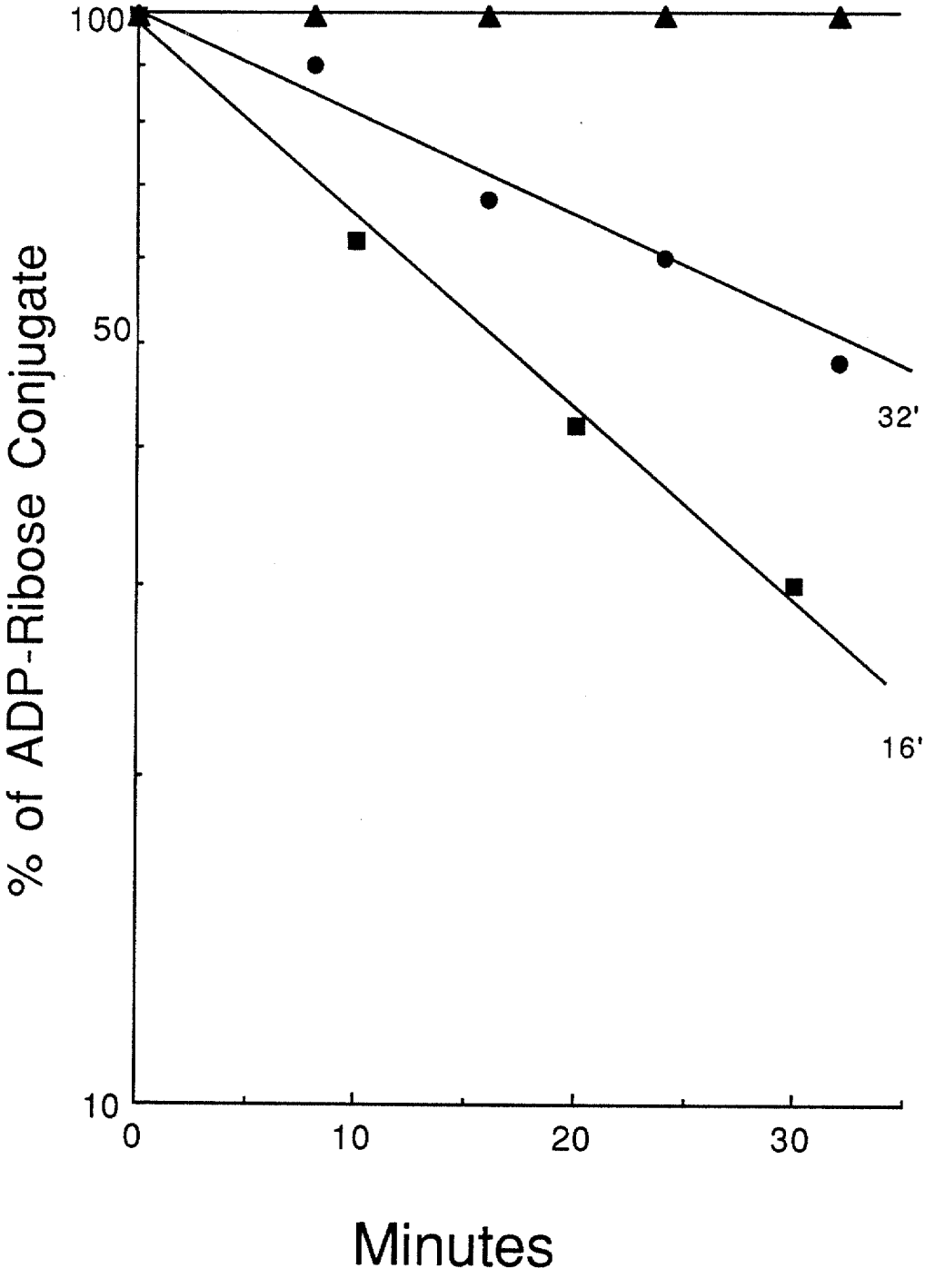

100
50
10
0
10
20
30
% of ADP-Ribose Conjugate
Minutes
32'
16'

Figure 45. Analysis by HPLC of the hydrolysis of ketoamine 1 at pH 9.0. Purified product 1 was incubated at pH 9.0 as described in Fig. 45. The positions of ADP-ribose and AMP are shown. Panel A shows the pH 9.0 incubation at 0 minutes. Panel B shows the products obtained after 90 minutes. Panel C shows the same sample as panl B with added ADP-ribose and AMP.

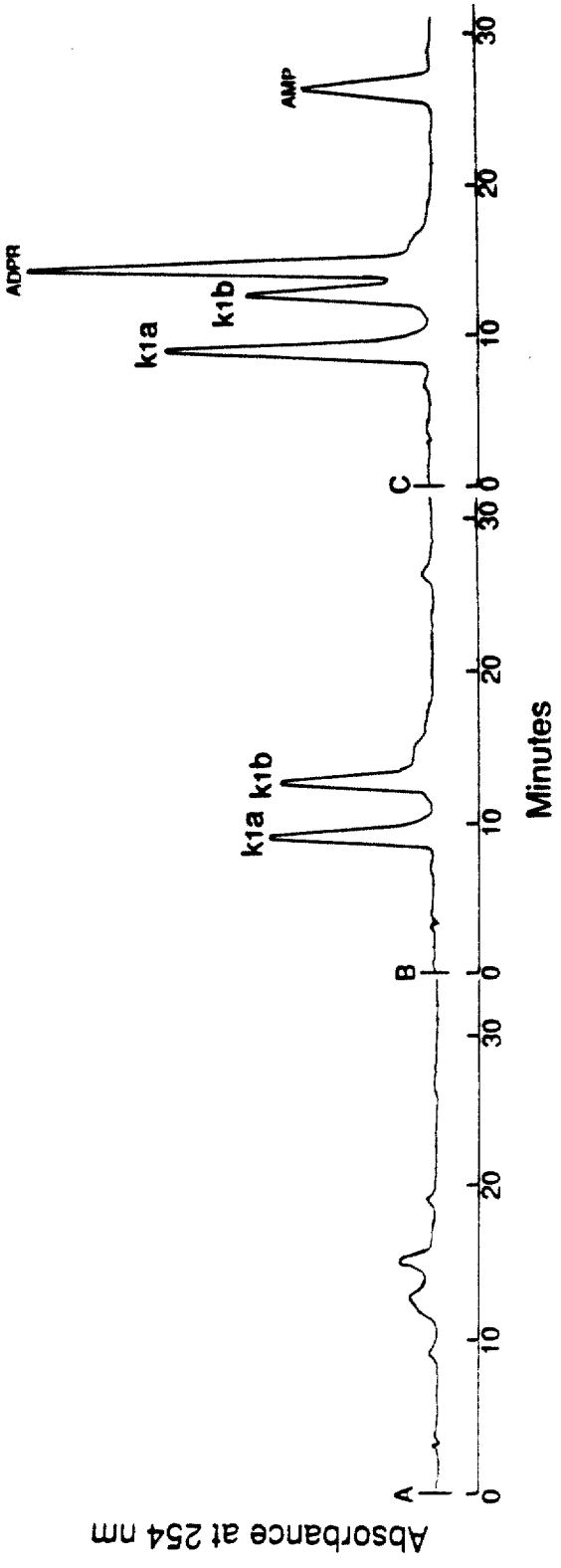

Absorbance at 254 nm
A
B
C
k1a
k1b
k1a k1b
ADPR
AMP
0
10
20
30
Minutes

Figure 46 Analysis by HPLC of the hydrolysis of ketoamine 2 at pH 9.0. Purified ketoamine 2 was incubated at pH 9.0 as described in Fig. 45. The positions of ADP-ribose and AMP are shown. Panel A shows the reaction at 0 min. Panel B shows the reaction at 90 minutes. Panel C is the same reaction shown in panel B and added ADP-ribose and AMP.

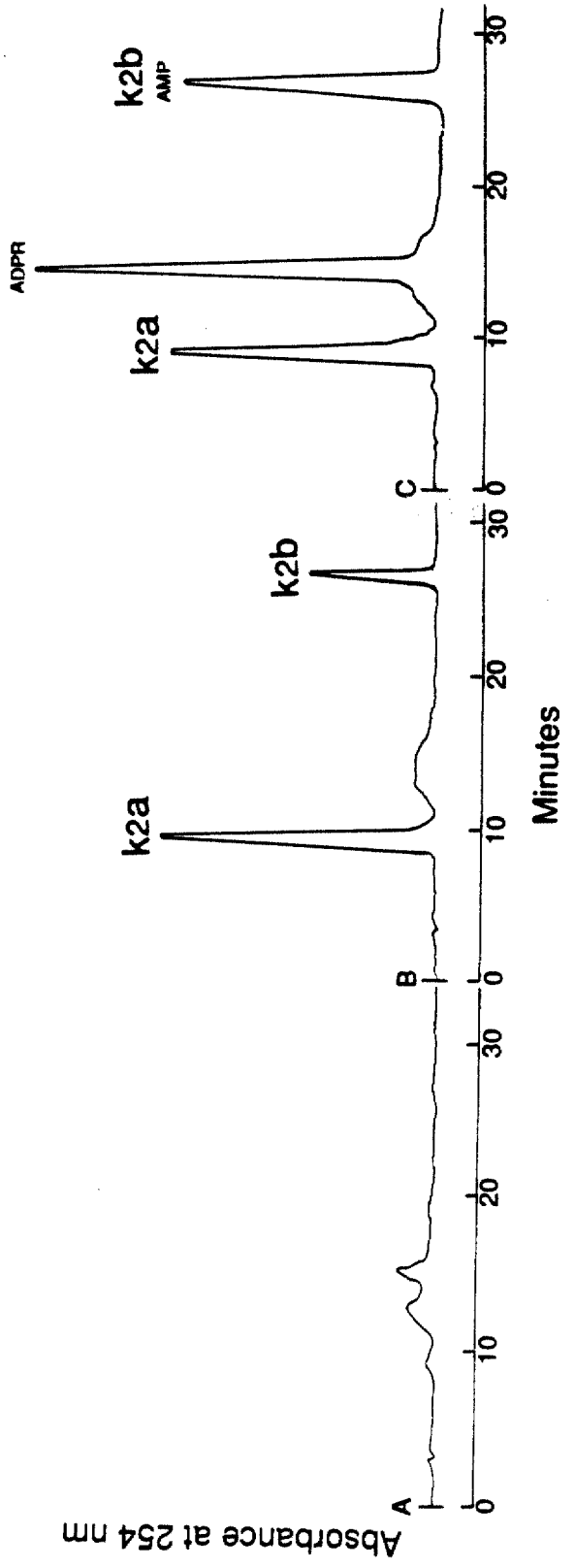

Absorbance at 254 nm
A
B
C
k2a
k2b
k2a
k2b
ADPR
AMP
0
10
20
30
Minutes

Figure 47. HPLC of pH 9.0 hydrolysis of ^{14}C radiolabel ketoamine 1. Purified ^{14}C label ketoamine 1 was incubated in CHES buffer, 200 mM, pH 9.0 at 37 °C. An aliquot was taken after 90 minutes and analyzed by isocratic reversed-phase HPLC as described in Methods. The chromatogram shows the HPLC profile of the degradation products obtained after 90 minutes at 37 °C.

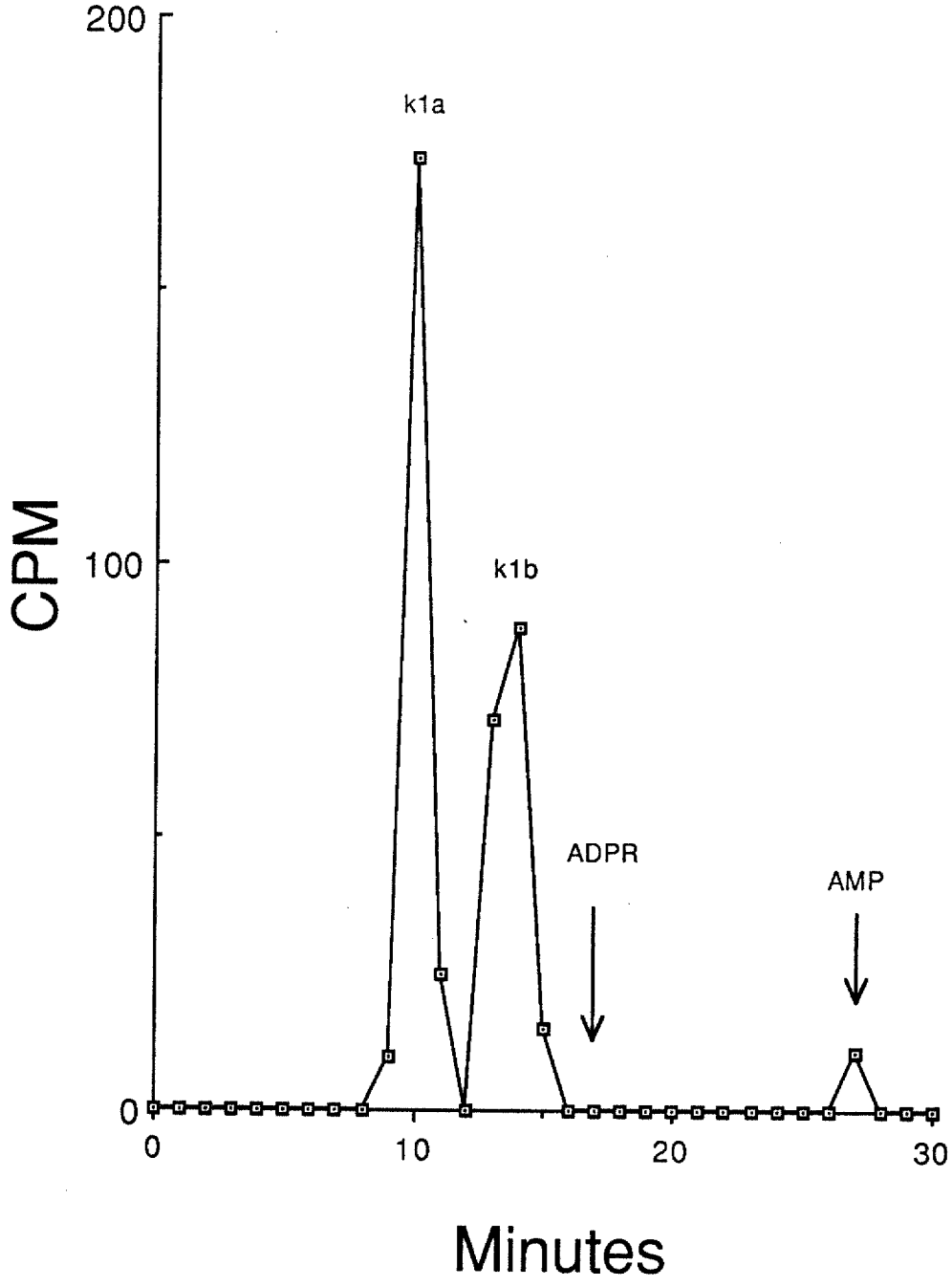
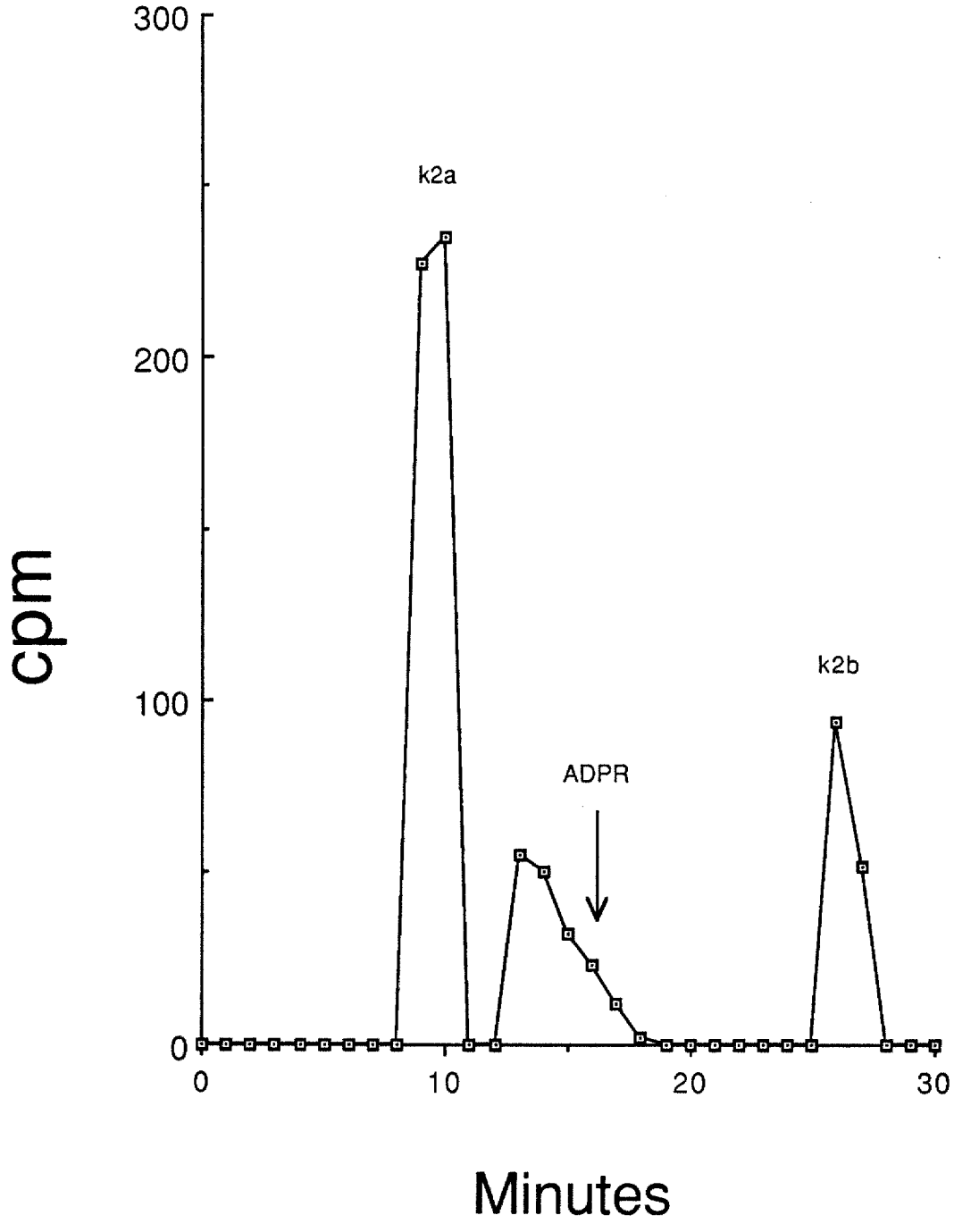

200
100
0
CPM
k1a
k1b
ADPR
AMP
0
10
20
30
Minutes

Figure 48. HPLC of pH 9.0 hydrolysis of ^{14}C radiolabel ketoamine 2. Purified ^{14}C label ketoamine 2 was incubated in CHES buffer, 200 mM, pH 9.0 at 37 °C. An aliquot was taken after 90 minutes and analyzed by isocratic reversed-phase HPLC as described in Methods. The chromatogram shows the HPLC profile of the degradation products obtained after 90 minutes at 37 °C.


300
200
100
0
cpm
k2a
ADPR
k2b
0
10
20
30
Minutes

Affinity purification chromatography purification of the hydrolysis products at pH 9.0 from ketoamines 1 and 2

With the aim of purifying the hydrolysis products of ketoamines 1 and 2 chromatography on Amicon PBA-30 was used. This resin has the property of retaining polyhydroxy molecules such as sugars through the formation of the borate complex. Purified ^{14}C labelled ketoamines 1 and 2 were incubated in CHES buffer at pH 9.0, 37 °C for 2.5 h at which point they were completely hydrolyzed. The resulting solution was diluted in ammonium formate buffer, pH 8.6 to a final concentration of 2 μM and applied to a previously protein model to initiate studies of glycation of proteins equilibrated PBA-30 column at 4 °C. Tables II, and III shows the result of this fractionation with the hydrolysis products of ketoamines 1 and 2 respectively. The radiolabel was

PBA-30 fractionation of the etheno derivatives of pH 9.0 hydrolysis products of ketoamines 1 and 2

The etheno derivatives of the pH 9.0 hydrolysis products of ketoamines 1 and 2 were prepared as described in Methods. Due to the fluorescence properties of the etheno derivatives the detection of these will allow much higher sensitivity of detection required for *in vivo* studies. Tables IV, and V

Table II: PBA-30 fractionation of the pH 9.0 hydrolysis product of ketoamine 1

Fraction	cpm	%
Flow through at pH 8.6	110	1.2
Wash at pH 8.6	15	0.2
Water	7745	82
Wash at pH 4.6	1625	17

9720 cpm were applied with a total recovery of 98%

Table III: PBA-30 fractionation of the pH 9.0 hydrolysis product of ketoamine 2

Fraction	cpm	%
Flow through at pH 8.6	770	1.4
Wash at pH 8.6	400	0.7
Water	44210	77
Wash at pH 4.6	11610	21

57730 cpm were applied with a total recovery of 99 %

show the results of the fractionation of the etheno derivatives on PBA-30 at 4 °C derived from ketoamine 1 and 2, respectively. These adducts behaved as expected, similarly to the underivatized adducts as shown in Table II and III.

Glycation of histone H1 by ADP-ribose

It was important to investigate the glycation of proteins by ADP-ribose once the basic chemistry with low molecular models had been established. Histone H1 was used as the since it has a high lysine content it has been reported that this protein is mono ADP-ribosylated during DNA damage in which ADP-ribose polymer turnover is induced making glycation of the protein *in vivo* a likely possibility. ^{32}P-ADP-ribose was incubated with histone H1 for 4 h at 37 °C , pH 8.0. C_8-Reversed-phase HPLC was used to purify the glycated histone. Fig. 49 shows the HPLC profile of the purified ^{32}P radiolabel glycated histone. The top panel shows the crude reaction mixture of histone and ^{32}P ADP-ribose. The bottom panel shows the HPLC purified radiolabel histone H1. It is noteworthy to mention that purified histone H1 is stable at pH 5.0, 0 °C.

Gel Electrophoresis of ^{32}P-glycated Histone H1

Gel electrophoresis was used to confirm that histone H1

Table IV: PBA-30 fractionation of etheno derivatives of the pH 9.0 hydrolysis product of ketoamine 1

Fraction	cpm	%
Flow through at pH 8.6	995	9.0
Wash at pH 8.6	260	2.4
Water	6835	63
Wash at pH 4.6	2735	25

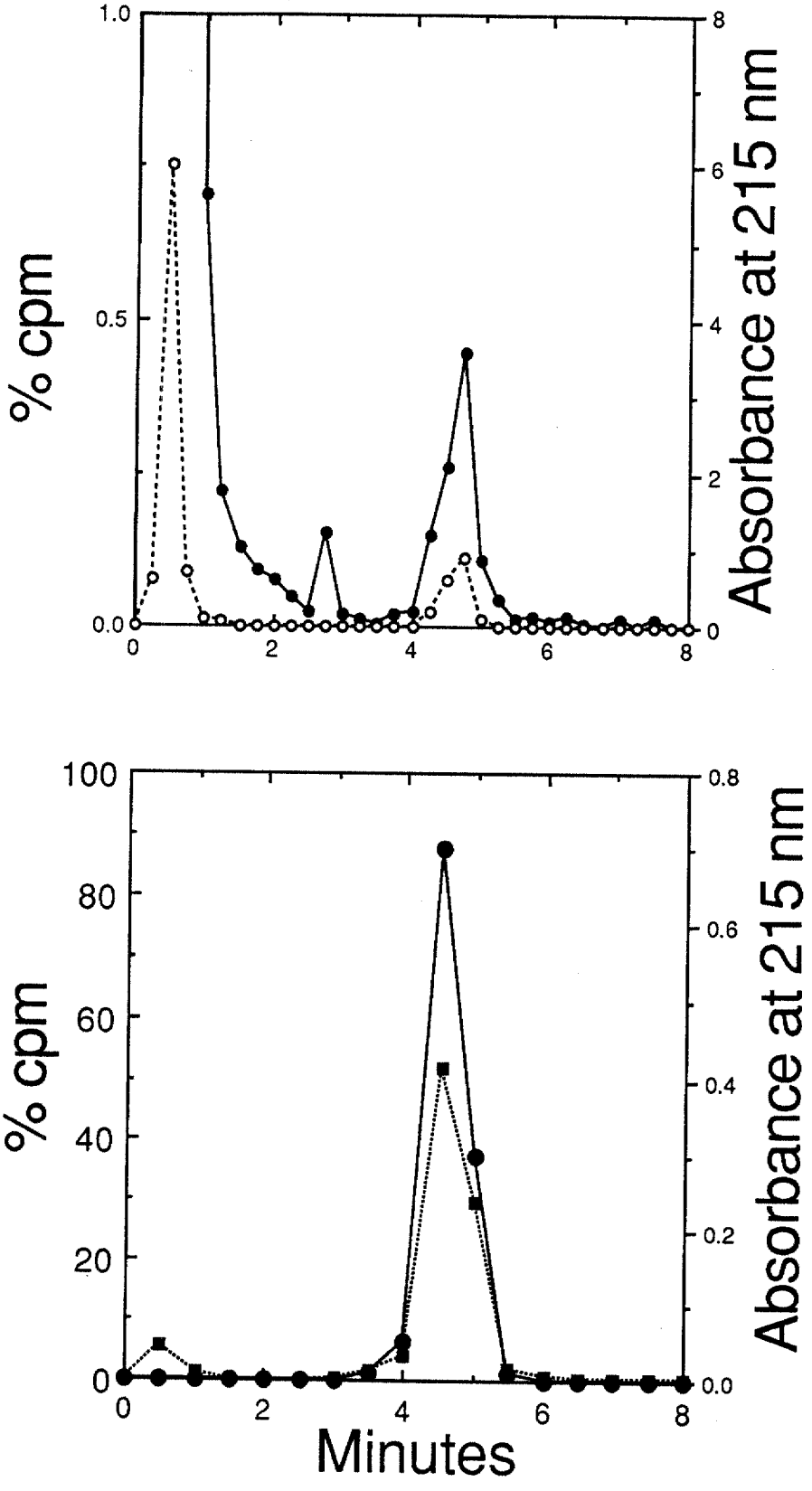
12546 cpm were applied with a total recovery of 86%

Table V: PBA-30 fractionation of etheno derivatives of the pH 9.0 hydrolysis product of ketoamine 2

Fraction	cpm	%
Flow through at pH 8.6	100	0.2
Wash at pH 8.6	80	0.1
Water	29140	50
Wash at pH 4.6	28780	49

61360 cpm were applied with a total recovery of 95 %

Figure 49. HPLC profile of the purified ^{32}P glycated histone H1. ^{32}P ADP-ribose excess was removed from the reaction mixture by an HPLC gradient of 0.1% TFA in H_2O and 0.1% TFA in acetonitrile. Panel A shows the original reaction mixture, % cpm (●) and absorbance at 215 nm, (○) Panel B shows the HPLC purification of ^{32}P histone H1 conjugate, % cpm (●) and absorbance at 215 nm (○).


% cpm
Absorbance at 215 nm
Minutes
1.0
0.5
0.0
8
6
4
2
0
100
80
60
40
20
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8

had been glycated by ^{32}P ADP-ribose. SDS-PAGE was used for the separation. Fig. 50 Panel A shows the stained gel that had been incubated with ^{32}P ADP-ribose. Panel B shows the autoradiogram. This result clearly show that histone H1 was modified

Chemical stability of glycated histone H1

Histone H1 radiolabel with ^{32}P ADP-ribose was treated under similar chemical conditions as the ketoamines obtained with ADP-ribose and *n*-butylamine. In 1 M neutral hydroxylamine at 37 ^{o}C the histone H1 conjugate was stable. In 44 % formic acid, at 37 ^{o}C the conjugate was also stable. When histone H1 conjugate was treated at pH 9.0, 37 ^{o}C the ^{32}P was rapidly released with a half life of 20 min (Fig. 51). Reversed-phase HPLC was used with detection at 215 nm. Table VI summarizes the chemical release conditions used.

HPLC analysis of pH hydrolysis of degradation products from histone H1

It was of interest to compare the pH 9.0 degradation products obtained with the histone conjugate with the ketoamines 1 and 2. Fig. 52 shows the HPLC analysis of ^{32}P radiolabel histone H1 conjugate. The histone conjugate

Figure 50. SDS-PAGE electrophoresis of purified glycated histone H1. Panel A shows the SDS-PAGE electrophoresis anlysis of purified ^{32}P glycated histone H1(lane 1). Panel B shows the autoradiogram of the respective sample.

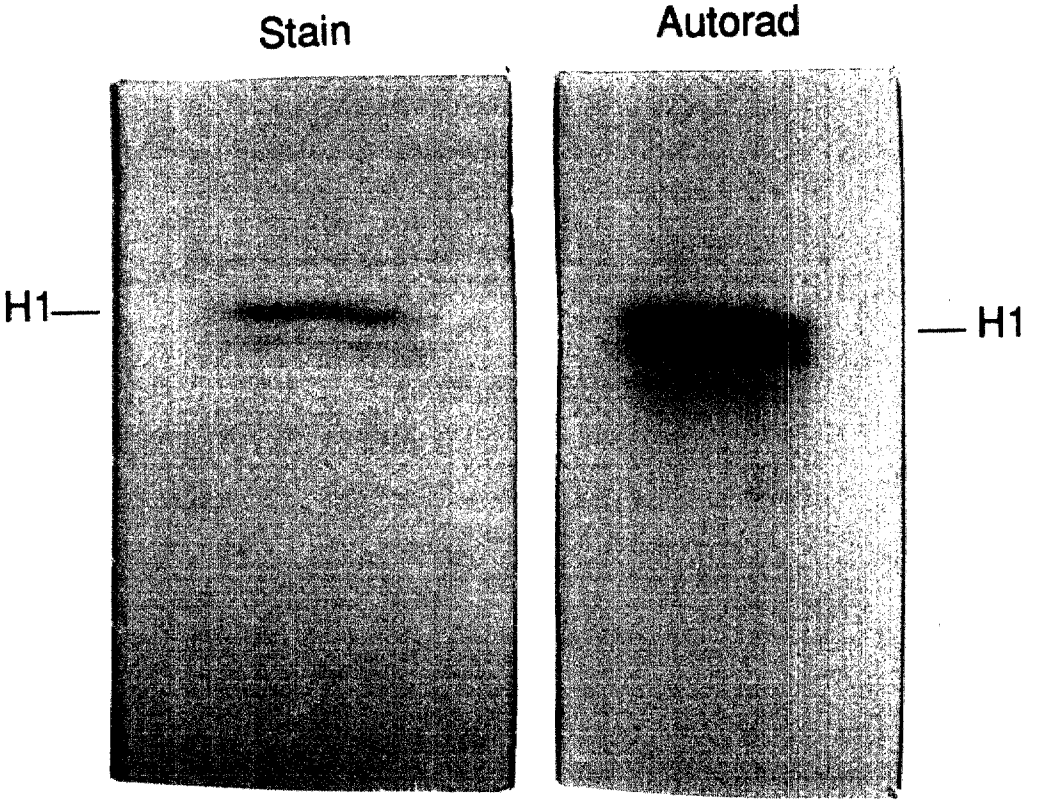

Stain
Autorad
H1
H1

Figure 51. Kinetics of the pH 9.0 hydrolysis of glycated histone H1. Purified histone H1 was incubated in 200 mM CHES buffer pH 9.0 (■), and, 1 M hydroxylamine pH 7.0 (▲). Aliquots were taken at 0, 20, 40, and 60 minutes. In the HPLC analysis the running buffer used was the same as the one described in Fig. 48.

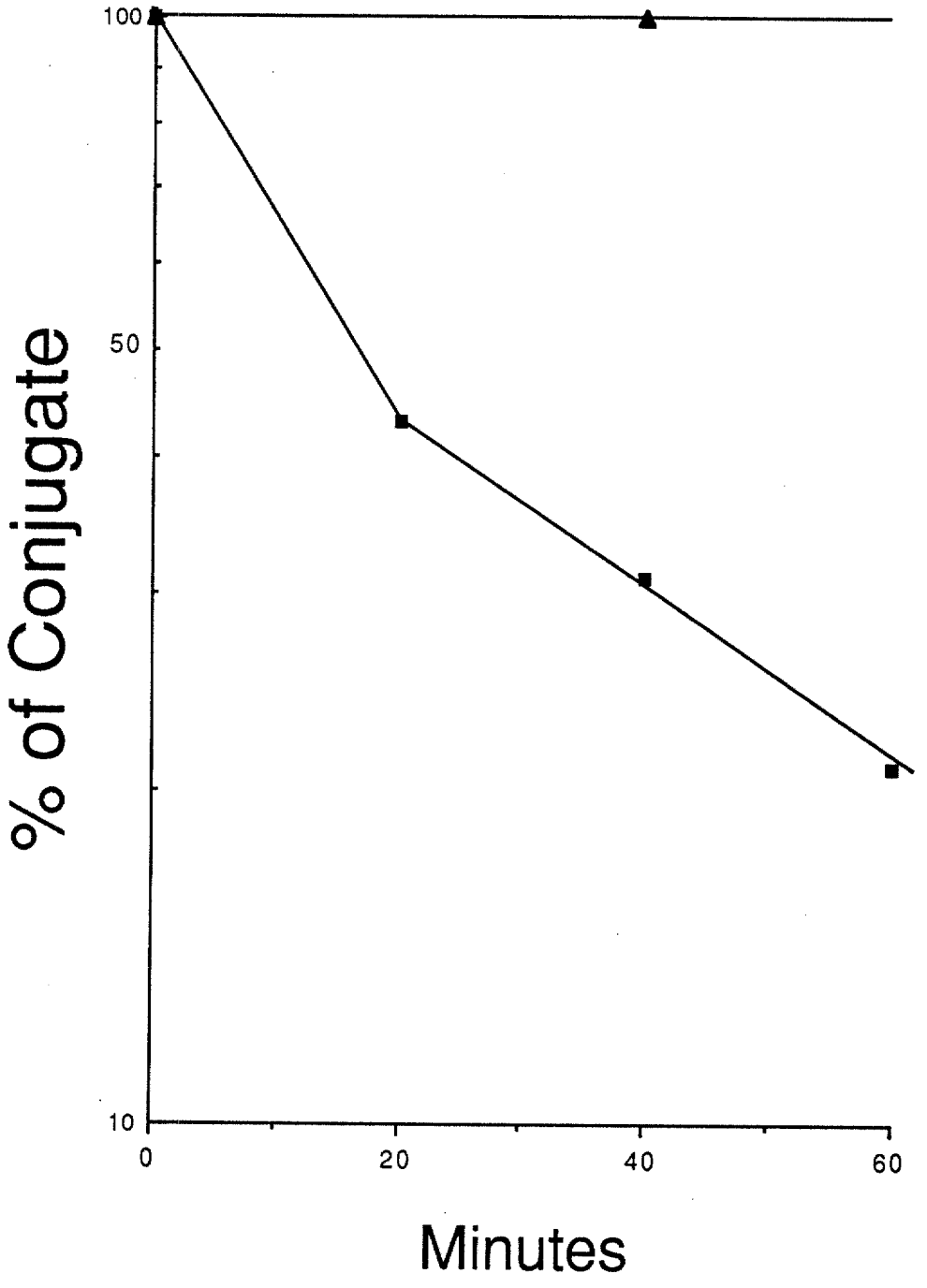
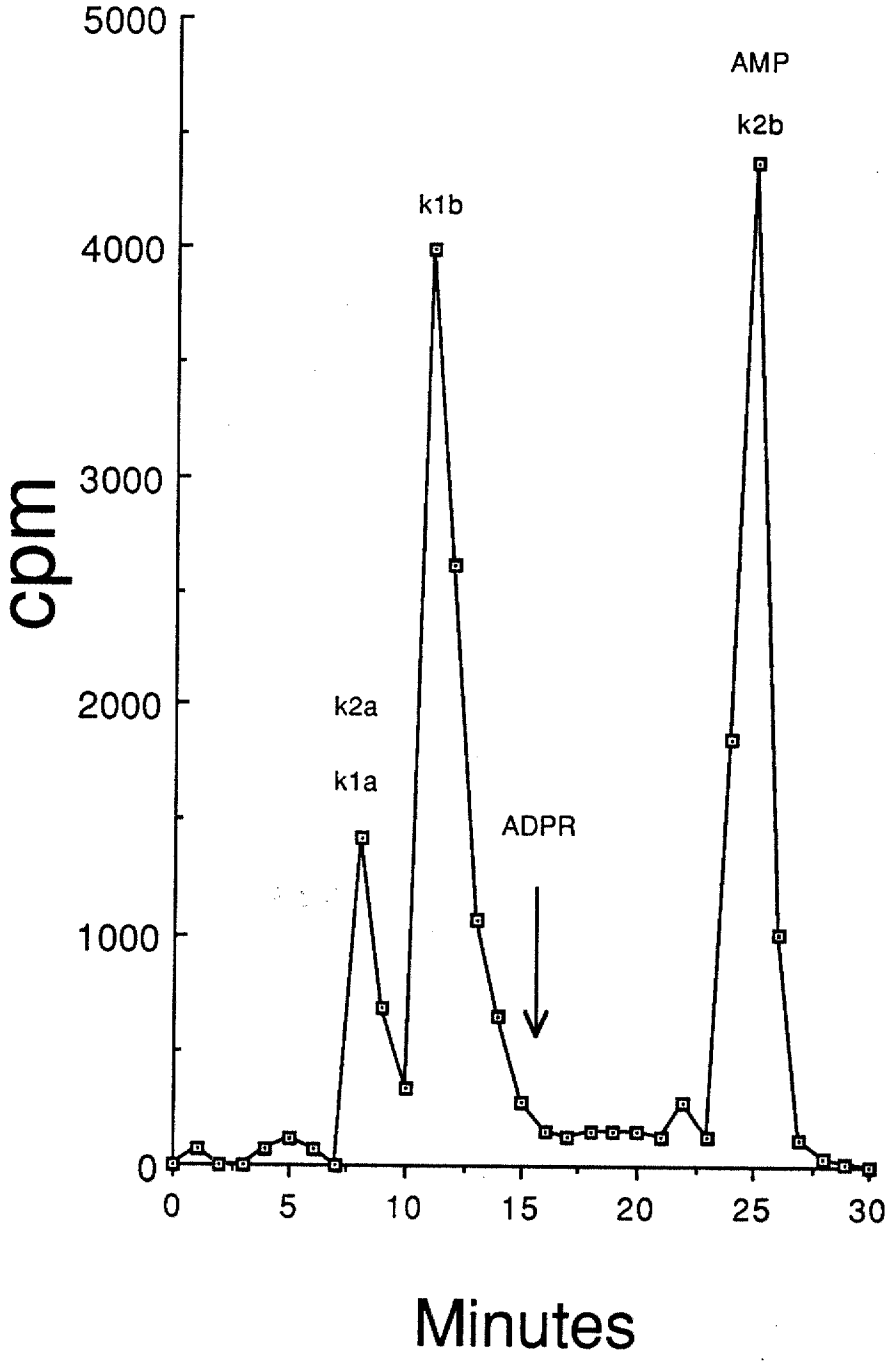

100
50
10
% of Conjugate
0
20
40
60
Minutes

Table VI: Chemical Stability of Glycated Histone H1 and *n*-Butylamine Model Conjugates

ADP-Ribosyl conjugate of:	Formic Acid(44%)	Hydroxylamine (1M, pH 7.0)	CHES, pH 9.0 ($t_{1/2}$)
Histone H1	Stable	Stable	20 minutes
Ketoamine 1	Stable	Stable	16 minutes
Ketoamine 2	Stable	Stable	32 minutes

produced in larger amounts two products that co-migrated with k1b and k2b, AMP. It is important to determine the nature of these products to establish wether or not the low molecular weight conjugates and the histone conjugate give the same hydrolysis products.

Figure 52. HPLC analysis of pH 9.0 degradation products of ^{32}P glycated histone H1. An isocratic reversed-phase HPLC column was used and the running buffer was 50 mM potassium phosphate pH 5.0. For comparison the ^{32}P degradation products from ketoamines 1 and 2 are indicated.


5000
4000
3000
2000
1000
0
cpm
AMP
k2b
k1b
k2a
k1a
ADPR
0
5
10
15
20
25
30
Minutes

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CHAPTER V

DISCUSSION

The ultimate goal for the preparation of these model conjugates is to search for proteins modified *in vivo* by ADP-ribose that could lead to the resolution of important biological questions.

The acetal linkages prepared here such as ADP-ribosyl serine ethyl ester and threonine methyl ester were found to be labile in acid (formic acid 44%). This provided a method to search for proteins modified at serine, threonine, tyrosine or other kind of hydroxyl containing amino acid since all the other classes of linkages to ADP-ribose are stable in acid. Indeed studies with crude extracts from rat liver protein showed evidence for the presence of this class of modification in proteins *in vivo* (47). In these studies two classes of acid labile proteins were shown, one is alkali stable with a similar $t_{1/2}$ to the acetal conjugates and a second class that is alkali labile.

There is similarity in the chemistry of the acetal O-glycosidic bond formed with hydroxyl groups from proteins, and the anomeric carbon of carbohydrates, such as N-acetyl

galactosamine in glycoproteins, and the acetal linkage formed between protein and the complex pentose nucleotide, ADP-ribose. It is reported in the glycoprotein field that the glycosidic linkage formed with serine or threonine is labile to either acid and alkali treatment (144). The mechanism proposed for the alkali hydrolysis is the β elimination of the alcoxide moiety formed between the hydroxyl group of the amino acid and the anomeric carbon of the carbohydrate, to form a double bond. A prerequisite for β elimination is the presence of α hydrogens in the amino acid molecule. The ADP-ribosyl amino acid ester acetal conjugates described here, ADP-ribosyl serine ethyl ester and threonine methyl ester, contain α hydrogens to the amino acid and therefore should be hydrolyzed by alkali. The data obtained shows here is that the ADP-ribosyl amino acid esters are resistant to alkali. This difference in stability between the O-glycosylated proteins and the ADP-ribosyl amino acid ester acetals suggests the requirement of the peptide backbone for the cleavage of the acetal bond by alkali. It is likely that the peptide bond increases the acidity of the α hydrogens to the amino acid.

Propionyl ADP-ribose was prepared to study the chemistry of the glycosidic linkage at glutamate or aspartate when chromatin proteins are modified by ADP-ribose. The stability of the model conjugate was compared with ADP-ribose modified

histones and some differences were found. The $t_{1/2}$ at pH 7.0 for the conjugate was 2 days and for histones was 5-6 h. One possible explanation is the stereochemistry of the glycosidic linkage formed. The model conjugate prepared with NADase has been reported to be β while the ADP-ribose modified histone has shown to be α. Also the effect of surrounding groups in the protein could affect the stability of the glycosidic linkage. However it should be noted that there is hetereogenity in the linkages of the ADP-ribosylated histones since also there are hydroxylamine resistant bonds. This suggests histones are modified on more than different classes of amino acids. In fact, it has been proposed that the ester glycosidic linkage migrates from position 1 to 3 as shown in Fig.3 and other possibilities for this heterogeneity will be described later in the discussion.

The occurrence of phosphorylation on the hydroxyl group of tyrosine by tyrosine kinase activity was discovered originally as the result of cellular transformation by Rous sarcoma virus, but now is known to be a normal event associated with growth factors (58). Glycoproteins have been shown to be glycosylated at asparagine, serine (7), threonine and more recently by tyrosine residues (114), it should be pointed out that only one paper has supported this finding and the results should be taken with caution. The observation of acetal linkages in rat liver proteins, which

are alkali resistant argues for the existence of ADP-ribosylation on tyrosine. This is due to the observation that this amino acid does not contain a hydroxyl group in the β carbon of the amino acid, which would make it alkali labile. Although the ultimate proof of the existence of this linkage is yet to be demonstrated, it has opened interesting possibilities for the biological role of mono ADP-ribosylation of proteins.

Imidazoyl ADP-ribose was resistant to all the release chemical conditions of the glycosylic bond tested. This is not surprising if it is considered that the ADP-ribose moiety contained a glycosylic bond between adenine and ribose that is very stable and structurally similar to the one formed between imidazole and ADP-ribose.

It was important to know the chemistry of the imidazoyl ADP-ribose conjugate since this would allow the identification and purification of ADP-ribosyl histidine. This is due to the low yield of the product and the complexity of the HPLC profile of the reaction caused by the additional peaks when the α amino group of the amino acid reacts with ADP-ribose as shown in panel A and C of Fig. 23. By knowing that imidazoyl ADP-ribose was resistant to alkali the HPLC profile was simplified by treatment with alkali, which breaks down Schiff bases products, as shown in panel D of Fig. 23.

ADP-ribosyl histidine after conversion to the etheno

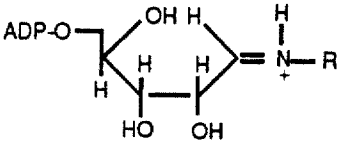
derivative was used as a standard to search for the modification of rat liver proteins at this amino acid. Studies in this laboratory showed that after extensive protease digestion of rat liver proteins and conversion to etheno form, the ADP-ribosylated amino acid co-migrated with the histidine standard. After collecting this product from HPLC, amino acid analysis was carried out and shown to contain histidine. This provided strong evidence for the existence of ADP-ribose modification at histidine residues.

In protein glycation by hexoses, the Schiff base formed between the hexose and amine can be stabilized by ring closure to form either a furanose or pyranose ring (6). Similarly, the ketoamine product of the Amadori rearrangement also can be stabilized by ring closure to either a furanose or pyranose ring. Under the conditions used here, a stable aminoriboside adduct of ADP-ribose and *n*-butylamine derived from a Schiff base was not detected. Although the ADP-ribose adduct could conceivably form an aminoriboside, it may be less stable than the hexose counterpart. It is also likely that the ketoamine adduct derived from ADP-ribose is less stable than the corresponding hexose ketoamine since with ADP-ribose ring formation is not possible. This may have implications with respect to the rate of formation of post Amadori products that are known to occur with ketoamines. These products are formed through further reaction of the

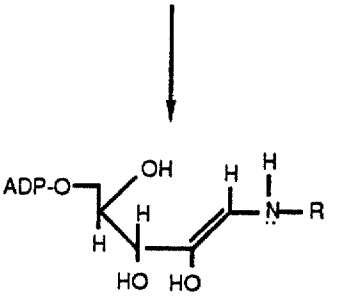
carbonyl group with other amino groups. It would be expected that for ketoamines derived from ADP-ribose, the formation of post Amadori products would be more rapid than with hexoses due to the higher relative concentration of carbonyl groups. The inability of ADP-ribose derived ketoamines to undergo ring closure also may explain the formation of multiple ketoamines (Fig. 33). A possible mechanism for the formation of ketoamines 1 and 2 is shown in Fig. 47. This involves migration, through an enol intermediate, of the carbonyl from position 2 to position 3. By an analogous mechanism the carbonyl could migrate to position 4. This mechanism has been suggested in the reaction of reducing monosaccharides with phenylhydrazine and also in the oxidation of aldoses and ketoses with Tollen's reagent, Fehling's, and Benedict's solutions (83).

The treatment of ketoamines 1 and 2 with hydroxylamine resulted in the formation of stable oximes (Figs. 39, 40). NMR analysis of the oximes provided information that was not obtainable from the ketoamines. In the ketoamines the acidic hydrogens α to the carbonyl group are exchangable with D_2O and thus were eliminated from the ^1H NMR spectrum. The presence of these hydrogens in the oximes aided in the interpretation of the data. The ^1H-NMR of the oxime derived from ketoamine 1 showed that the carbonyl is at position 2. This conclusion was also supported by the observation that

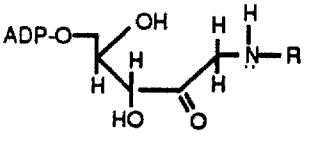
Figure 53. Proposed mechanism for the formation of ketoamines 1 and 2.



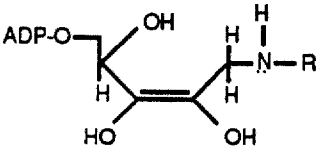
Protonated Schiff base



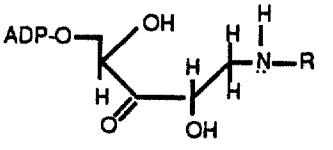
Enol



Ketoamine 1



Enol



Ketoamine 2

ketoamine 1 was the first stable product formed and that under slightly acidic conditions it was the only product formed.

The formation of two oximes from ketoamine 2 (Fig. 40) suggested that it was either a mixture of two compounds or that a further rearrangement occurred in the presence of hydroxylamine. The analysis of ketoamine 2 by ^{13}C NMR (Fig. 35) clearly showed that it was a single compound. Thus, we postulate that hydroxylamine is serving as a base that promotes migration of the carbonyl group from carbon 3 to carbon 4. The carbonyl group in ketoamine 2 cannot be definitively assigned to position 3 or position 4 from the ^{1}H NMR of the oximes (Figs. 42, 43). However, we would predict that ketoamine 2 has the carbonyl group in position 3 based on the stability of the ketoamines. Under mild alkaline conditions ketoamine 2 was much more stable than ketoamine 1. This may be the result of hydrogen bonding between carbonyl and adjacent hydroxyl groups, which results in stabilization. For a ketoamine with the carbonyl in position 3, two hydroxyl groups can potentially form stabilizing hydrogen bonds with the carbonyl group while ketoamine 1 has a single adjacent hydroxyl group capable of hydrogen bonding. Similarly, a ketoamine with the carbonyl group in position 4 would have only a single adjacent hydroxyl group. Since no differences in the stabilities would be expected between ketoamines with

carbonyl groups in either positions 2 or 4, ketoamine 2 most probably has the carbonyl group in position 3.

In earlier studies, Hilz and coworkers have reported glycation by ADP-ribose of polyhistidine and polyarginine in addition to polylysine (35, 36). In this study, when ADP-ribose was incubated with imidazoyl or guanidino compounds under a wide variety of conditions similar to those used by Hilz and coworkers, no products were detected (data not shown). It is possible that this difference in reactivity is due to some chemistry unique to the polymers of histidine or arginine used as acceptors. Alternatively it is possible that the glycation observed with polyhistidine and polyarginine was occurring only at the terminal α amino groups.

The stability of the ketoamines reported here is very similar to the stability of the mitochondrial protein conjugates reported by Hilz and coworkers (35) and the cytosolic protein conjugates reported by Tanaka and coworkers (125). This suggests that the amino acid modified in these proteins was lysine and that the adduct(s) represented one or more ketoamine(s) resulting from an Amadori rearrangement. Recent evidence has shown that glycation by glucose can occur at specific lysine residues in albumin *in vivo* (42). The preferential sites observed for glycation such as Lys-Lys and Lys-His-Lys led these investigators to propose that a basic

amino acid adjacent to the site of glycation promotes the Amadori rearrangement via combined acid-base catalysis. This proposal is also supported by the observation that the site of glycation of hemoglobin A_{IC} contains an adjacent histidine residue (54). Thus, it is possible that a similar mechanism leads to the specific glycation of mitochondrial and cytosolic proteins by ADP-ribose (35, 125).

Free ADP-ribose is a product of metabolism generated at many locations within the cells (Fig. 6). At present, its metabolic fates are poorly understood. The definitive detection of free ADP-ribose levels *in vivo* presents difficult technical challenges. Extractions normally used to obtain nucleotide pools are not suitable since acid extraction converts cellular NADH to free ADP-ribose and alkaline extraction converts NAD to free ADP-ribose. Extractions at neutral pH do not inactivate NADase activity, which generates free ADP-ribose. In view of these technical obstacles to the definitive detection of free ADP-ribose *in vivo*, its levels may have to be monitored indirectly via protein glycation. The potential for protein glycation by free ADP-ribose at a particular cellular location will be related to the relative rates of formation and catabolism. At our current state of knowledge, the nucleus is a likely site of protein glycation by ADP-ribose. Cellular recovery from DNA damage involves the activation by DNA strand breaks

of poly(ADP-ribose) polymerase. This results in high rates of formation of ADP-ribose polymers in chromatin and their rapid turnover to free ADP-ribose (47). The generation of high local concentrations of free ADP-ribose in close proximity to histones rich in lysine residues raises the likelihood of histone glycation. Indeed, Kreimeyer *et al.* (57) have reported the occurrence of monomeric ADP-ribose conjugates of histone H1 in large excess over poly(ADP-ribose) conjugates following DNA damage in hepatoma cells. While proteins carrying monomeric ADP-ribose residues will occur as a consequence of ADP-ribose polymer turnover, these residues are linked to the acceptor proteins by carboxylate ester linkages that are very sensitive to hydroxylamine (97). In contrast, the histone H1 conjugates observed by Kreimeyer, *et al.* were stable in hydroxylamine. Taken together with the stability of the glycation model conjugates reported here, these histone conjugates are very likely to represent *in vivo* glycation by ADP-ribose.

The chemical stability studies of the ketoamines (Fig. 44, Table I) show that they can be differentiated from ADP-ribosyl amino acids that occur in proteins by the action of protein, mono ADP-ribosyltransferases (Table I). The selective release of glycation products from proteins at pH 9.0 should be possible. Further, the results described here (Figs. 45, 46, 52) suggest these products are very likely

unique to ADP-ribose glycation. Thus, they should prove very useful to monitor glycation of proteins by ADP-ribose both *in vitro* and *in vivo*. The structure of these hydrolysis products is currently being investigated.

It is likely that cells possess mechanisms to minimize protein glycation by ADP-ribose. Most likely a metabolic system that degrades ADP-ribose exists. This is supported by the finding of a highly specific ADP-ribose pyrophosphatase, which has a low K_m (0.5 μM) for ADP-ribose (73). Also, polyamines may be protective against glycation by serving as acceptors of ADP-ribose (139). Nevertheless, a variety of nonenzymic modifications of proteins occurs as a function of age and glycation has been implicated in this process (15). The results reported here should be useful in examining the possible role of protein glycation by ADP-ribose in biological systems.

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