INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17″ × 23″ black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6″ × 9″ black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Accessing the World's Information since 1938

300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

Order Number 8801778

Studies of nicotinamide adenine dinucleotide metabolism in yeast

Yan, Cong, Ph.D.

City University of New York, 1987

Copyright ©1987 by Yan, Cong. All rights reserved.

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages __✓__
2. Colored illustrations, paper or print ______
3. Photographs with dark background __✓__
4. Illustrations are poor copy ______
5. Pages with black marks, not original copy ______
6. Print shows through as there is text on both sides of page ______
7. Indistinct, broken or small print on several pages __✓__
8. Print exceeds margin requirements ______
9. Tightly bound copy with print lost in spine ______
10. Computer printout pages with indistinct print ______
11. Page(s) __________ lacking when material received, and not available from school or author.
12. Page(s) __________ seem to be missing in numbering only as text follows.
13. Two pages numbered ______. Text follows.
14. Curling and wrinkled pages ______
15. Dissertation contains pages with print at a slant, filmed as received ________
16. Other ____________________

STUDIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE METABOLISM IN YEAST

by

CONG YAN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1987

© 1987

CONG YAN

All Rights Reserved

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5/1/87
Date

Chair of Examing Committee

5-1-1987
Date

Executive Officer

Supervisory Committee

The City University of New York

Abstract

STUDIES OF NICOTINAMIDE ADENINE DINUCLEOTIDE METABOLISM IN YEAST

by

CONG YAN

Adviser: Professor Donald L. Sloan

Nicotinamide phosphoribosyltransferase (N_mPRTase; 2.4.2.12) and quinolinate phosphoribosyltransferase (QPRTase; 2.4.2.19) activities were detected for the first time in yeast and were partially purified and characterized. In addition, the yeast nicotinamide deamidase (YNDase;3.5.1.19) was purified to homogeneity , as determined by the criteria of gel electrophoresis. The enzyme was then characterized in detail. Polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC) gel filtration molecular weight studies showed that YNDase is a monomeric protein with a molecular weight of 34,000. Our kinetic study of this enzyme showed that the K_m of nicotinamide is 34 μM and that there exists an activity-dependant pK equal to 7.8. A pH study indicated that this enzyme has an unusually broad range of pH stability. Chemical modification analysis with N-ethylmaleimide (NEM) revealed that a cysteine residue is located at active center. YNDase was also inactivated by diethylpyrocarbonate (DEP) and the enzyme activity could be protected by substrate. A substrate analogue study showed that nicotinaldehyde is a noncompetitive inhibitor of YNDase. When we incubated YNDase with nicotinaldehyde in the presence of sodium borohydride, a Schiff base was formed, which indicates that a lysine residue is located near to the active center to facilitate the

enzymatic reaction. Amino acid composition analysis was also accomplished and some substrate specificity studies have been performed, revealing a high degree of specificity of YNDase for nicotinamide. In the yeast system, we carried out some studies concerning ADP-ribosylation of proteins. NAD glycohydrolase and ADPR phosphodiesterase activities were discovered. Finally a sensitive HPLC assay method was designed and implemented in our research.

This thesis is dedicated to my wife and parents for their love and encouragement.

ACKNOWLEDGEMENTS

I wish to thank my mentor, Dr. Donald L. Sloan for his support. Whenever I met with difficulties, he always kindly helped, and encouraged me. With incredible dedication, determination and patience, he guided me through a process of thinking, researching and writing. No matter where I am in future, I will always remember those good times when I worked in his laboratory.

It is my pleasure to express my gratitude to the members of my thesis committee, Dr. Glantz, Morton, Dr. Meshnick, Steve, Dr. Schulz, Horst, Dr. Sweeney, William, for their patience in reading my dissertation and listening to my thesis defense, and for useful advice.

I wish to thank my collegures who formed a warm, friendly, helpful and cooperative team. I especially thank Drs. Robert Ashton and Rosalyn Strauss for their contributions and suggestions concerning various aspects of my research. I also wish to thank Dr. Linda Ali for teaching me the use of the HPLC instrument and Florence Wright-Hayward for assisting me in the use of the Macintosch computer.

To anyone else who sustained me, I can say with respect and enthusiasm, that I could not have been able to finish this thesis without your help.

I love my proteins and HPLC.

TABLE OF CONTENTS

	Page
COPYRIGHT PAGE	ii
APPROVAL	iii
ABSTRACT	iv
ACKNOWLEDGEMENT	vi
TABLE OF CONTENTS	viii
LIST OF TABLES	xiii
LIST OF FIGURES	ix
ABBREVIATIONS	xvi
INTRODUCTION	1
MATERIALS	12
METHOD	13
NaPRTase, NmPRTase and QPRTase Investigations	13
A. HPLC Assay	13
B. Partially Purification of NaPRTase, NmPRTase and QPRTase	13
1. Autolysis	
2. Manganese chloride treatment	
3. Ammonium sulfate fractionation	
C. Activity Detection	14

Nicotinamide Deamidase 15

I. Assay Procedure 15

A. HPLC Assay 15

B. NH_3 Detection 15

II. Purification of Nicotinamide Deamidase from Baker's Yeast 16

A. Design of an Optimal Purification Procedure 16

B. Procedures 17

1. Autolysis
2. Ammonium sulfate fractionation
3. Acetone fractionation
4. DEAE cellulose chromatography
5. Phospho-cellulose chromatography
6. Phenyl-Sepharose chromatography
7. HPLC gel-filtration chromatography

III. Protein Determination 22

A. Standard Assay Procedure 27

B. Microassay Procedure 27

IV. Gel electrophoresis 28

A. Protocol for the Preparation of Solution 28

B. Protocol for the Preparation of Glass Columns 29

C. Nondenaturing Gels 30

1. Preparation of separating gel
2. Preparation of stacking gel
3. Application of the sample
4. Staining and destaining

D. Denaturing Gels 32

1. Preparation of separating gel

2. Preparation of stacking gel

3. Treatment and application of sample

4. Staining and destaining

V. Molecular Weight Determination by HPLC 34

VI. pH Stability Study 34

1. Preparation of the buffer solutions

2. Preincubation

3. Activity assay procedure

VII. Kinetic Studies 36

VIII. Specific Chemical Modification of the Nicotinamide Deamidase 36

A. N-ethylmaleimide (NEM) Modification 37

1. Inhibition study

2. Protection study with nicotinamide

3. Protection study with nicotinate

B. Diethylpyrocarbonate (DEP) or Ethoxyformic Anhydride Modification 39

1. Inhibition study

2. Protection study by nicotinamide

3. Enzyme treatment with hydroxylamine

IX. Analogue Studies (Nicotinaldehyde) 41

1. Substrate testing

2. Inhibition and protection

3. Kinetic study of inhibition

4. Flow dialysis (sodium borohydride treatment)

5. Substrate labeling study

X. Amino Acid Composition Study 46

XI. YNDase Substrate Specificity Studies 49

1. Deamidation study of nicotinamide mononucleotide to nicotinate mononucleotide by YNDase

2. N,N-diethylnicotinamide study

ADP-ribosylation 50

I. Assay Procedures 50

A. HPLC Assay 50

B. Radioactive Assay 50

II. Partial Purification 50

A. Purification from Yeast 50

1. Autolysis

2. Ammonium sulfate fractionation

3. Hydroxylapatite chromatography

B. Purification from Calf Thymus 51

III. Kinetic Study 51

A. ADP Ribosylation Study using HPLC 51

B. ADP Ribosylation Study using Radioactivity 52

C. HPLC Gel Filtration Study 52

1. Molecular weight estimation of the NAD hydrolyzing enzyme in yeast

2. HPLC radioactive labeling study of yeast proteins

RESULTS 54

Detection of NaPRTase, NmPRTase and QPRTase Activities by HPLC in Yeast 54

Yeast Nicotinamide Deamidase 61

A. HPLC Assay and NH_3 Detection 61

B. Purification of Nicotinamide Deamidase 61

C. Molecular Weight Determination 65

D. pH Study 65

E. Kinetic Analysis 75

F. Chemcal Modifcation 75

1. NEM Modification

2. DEP Modification

G. Analogue Study 82

H. Amino Acid Composition Study 92

I. Specificity Study 92

ADP Ribosylation 92

A. HPLC Assay Design 92

B. NAD Utilization and ADPR Generation Study 96

C. Relative M. W. Determination of NAD Glycohydrolase in Yeast 105

DISCUSSION 108

REFERENCES 112

CURRICULUM VITAE 115

List of Table

Table	Description	Page
1	Comparisons between Two ADP-ribose Generating Activities	10
2	Summary of the Purification of YNDase	64
3	Amino Acid Composition Analysis of YNDase	93
4	The Comparison of NicotinamideDeamidase from Different Sources	110

List of Figures

Figure	Description	Page
1	*De novo* pathway of NAD synthesis	3
2	Preiss and Handler pathway of NAD synthesis	5
3	Profiles of the elution of YNDase through Phospho-cellulose and DEAE-cellulose chromatography	20
4	Profiles of the elution of YNDase from Phenyl-Sepharose CL-4B chromatography	23
5	Profiles of the elution of YNDase from HPLC gel filtration chromatography	25
6	The separation of the nicotinaldehyde peak from nicotinamide and nicotinate peaks on a Waters μBondapak C_{18} HPLC column	42
7	Amino acid composition of YNDase, as determined by HPLC analysis	47
8	Reverse phase HPLC assay procedure for the pyridine phosphoribosyltransferases	55
9	Elution profiles of the incubation mixture of N_mPRTase and YNDase on μBondapk HPLC column	57
10	Rates of appearance of N_a and NMN and the rate of disappearance of N_m	59
11	HPLC elution profiles of the YNDase assay solution	62
12	Gel electrophoresis of the final YNDase preparation	66
13	Molecular weight determinations of YNDase with	

	HPLC gel filtration	68
14 &15	Molecular weight determinations of YNDase by SDS-gel electrophoresis	70
16	Effects of pH on the YNDase catalyzed reaction	73
17	Double reciprocal plots of the initial velocity of nicotinate formation *versus* nicotinamide	76
18	Dixon plot of log Vmax *versus* pH, as derived from the data shown in Figure 17	78
19	Incubations of YNDase with NEM	80
20	Incubations of YNDase with DEP	83
21	Hydroxylamine treatment of YNDase	85
22	Incubations of YNDase with nicotinaldehyde	88
23	Kinetic analysis of the effect of nicotinaldehyde on the YNDase catalyzed formation of nicotinate	90
24	HPLC elution profile of a solution of nicotinamide, AMP, NAD, nicotinate and ADP-ribose	94
25	NAD degradation by yeast and calf thymus extract	97
26	ADPR incorporation into proteins in yeast and calf thymus, as determined with labeled NAD	99
27	Detection of ADPR degradation in yeast	101
28	Separation of NAD and ADPR degradation activities in yeast by Hydroxylapatite column.	103
29	Radioactive labeling study of yeast crude protein by labeled NAD using HPLC gel filtration column	106

ABBREVIATIONS

ADPR	Adenosine diphosphate ribose
DEP	Diethylpyrocarbonate
HPLC	High performance liquid chromatography
N_a	Nicotinate
NAD	Nicotinamide adenine dinucleotide
N_aMN	Nicotinate mononucleotide
$N_aPRTase$	Nicotinate phosphoribosyltransferase
N_m	Nicotinamide
N_mMN	Nicotinamide mononucleotide
$N_mPRTase$	Nicotinamide phosphoribosyltransferase
QPRTase	Quinolinate phosphoribosyltransferase
YNDase	Yeast Nicotinamide Deamidase

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is the functional form of a very important vitamin molecule, which is involved in almost all forms of life and plays a key role in many cellular events.

In 1867, C. Huber (1) synthesized a drug derived from the oxidation of "nicotines", this was the first time that nicotinic acid was introduced to the scientific world. In 1873, H. Weidel (2) described the elemental analysis and crystalline structure of salts and other derivatives of nicotinic acid in some detail. In 1915, J. Goldberger (3) demonstrated that pellagra was a dietary deficiency disease, which later was identified as a niacin deficiency. In 1935, nicotinamide was found to be an integral part of the coenzymes for "redox" metabolism, coenzyme I (4) and II (5, 6), later named diphosphopyridine and triphosphopyridine nucleotide (DPN and TPN) and now called nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NAD and NADP). Since then, these substances have been studied extensively and many of their functions have been clarified. The structure of NAD was determined in 1936 in the laboratory of Hans Von Euler (7, 8).

NAD provides three major functions in living cells.

1). Through the early work of Harden and Young, Warburg and others, NAD is known to be the most abundant of the respiratory coenzymes. It is an electron carrier in various biological oxidation- reduction systems.

2). It is one of the substrates of DNA ligase in *E. coli*.

3). It is the substrate of poly(ADP-ribose) synthetase and ADP-ribosyl transferase, and in that capacity is involved in cell regulation.

There are three pathways by which living cells can synthesize NAD. The first one is a *de novo* pathway (Figure 1, also termed the Dietrich pathway, 9, 10). In this pathway, tryptophan is utilized in a multistep way to synthesize quinolinic acid, which can be converted by quinolinate phosphoribosyltransferase, QPRTase, (nicotinate nucleotide: pyrophosphate phosphoribosyltransferase, EC. 2.4.2.19) to produce nicotinate mononucleotide (N_aMN). NAD can be synthesized from N_aMN in two steps, the addition of the AMP portion (catalyzed by nicotinate mononucleotide: adenyltransferase) followed by the formation of the amide group (catalyzed by NAD synthetase). The second metabolic pathway (11) is the Preiss-Handler pathway (Figure 2). The nicotinic acid is utilized to synthesize N_aMN through the action of nicotinate phosphoribosyltransferase (EC. 2.4.2.11), where nicotinic acid is obtained either from nutritional sources or from tryptophan metabolism. In the third route, nicotinamide can serve as a precursor for the formation of nicotinamide mononucleotide (N_mMN) through the action of nicotinamide phosphoribosyltransferase (nicotinamidenucleotide: pyrophosphate phosphoribosyl-transferase, EC, 2.4.2.12) . Both the second and third pathways are called salvage pathways.

We have detected all three activities (QPRTase, N_mPRTase and N_aPRTase) in yeast cells in this laboratory , even though the Preiss-Handler pathway is reputed to be predominant (12, 13) in this organism.

Since nicotinamide deamidase plays a very important role in both NAD and ADPR metabolism, it is obvious that the isolation and characterization of this enzyme can help us to understand the nature of the living cells events.

This enzyme has been detected and studied in several mammalian (14, 15) and bacterial (16, 17) sources. Some investigators (18, 19) have proposed that, in mammals, the rate-limiting step in the biosynthesis of NAD from nicotinamide is the deamidation of nicotinamide to nicotinic acid, according to the Preiss-Handler

Figure 1. *De novo* pathway for NAD biosynthesis.

Tryptophan

↓ L-Tryptophan 2,3,-dioxygenase

N'-Formylkynurenine

↓ Kynurenine formamidase

Kynurenine

↓ Kynurenine 3-monoxygenase

3-Hydroxykynurenine

↓ Kynureninase

3-Hydroxyanthranilic Acid

↓ 3-Hydroxyanthranilic acid oxygenase

a-Amino, ß-Carboxy-

Muconic Semialdehyde

↓

CO_2^- CO_2^- N

Quinolinic Acid

PRibPP ↓ QPRTase

CO_2^- N P O OH OH

Nicotinic Acid Mononucleotide

Figure 2. Preiss and Handler pathway for NAD synthesis. A cyclic pathway that involves the nicotinamide deamidase (1) catalyzed formation of nicotinate and the synthesis of NAD, as described by Preiss and Handler, involving nicotinate phosphoribosyltransferase (2), nicotinate mononucleotide adenyltransferase (3) and nicotinamide adenine dinucleotide synthetase (4). Reaction (5) defines a nicotinamide adenine dinucleotide glycohydrolase activity, an adenosine diphosphoribosyltransferase activity or a poly(adenosyl-diphosphoribose) synthetase activity, depending on the molecular structure of (X).

ADP–H_2C O X
HO OH

O
C–NH_2
N+
ADP– H_2C O
HO OH

5

O
C –NH_2
N

1
H_2O
NH_3

O
C –O^-
N

2
PRibPP, ATP
ADP, Pi, PPi

O
C –O^-
N+
PIO– H_2C O
HO OH

3
ATP
PPi

O
C –O^-
N+
ADP– H_2C O
HO OH

4
gln
glu

pathway. This enzyme had been detected previously in yeast (20, 21), but had never been purified. This deamidase catalyzes the following general reaction:

Nicotinamide → Nicotinate (1)

What makes nicotinamide deamidase so important in ADP-ribosylation metabolism is that the enzyme can control the levels of both nicotinamide and NAD, the former is the inhibitor (22) of poly (ADP-ribose) synthetase whereas the later is the substrate. In addition to be a cofactor, NAD has another very important use in metabolism. Since the discovery of poly (ADP-ribose) synthesis in animal tissues in 1966 (23-25), the ADP-ribosylation reaction has been detected in almost all forms of life and almost all compartments of the cell.

Scheme I

ADP-Ribosyl Nicotinamide

The structure of NAD can be considered to be an the adenosine diphosphate-ribosyl moiety (ADP-ribose) attached covalently to nicotinamide, through a β-N-glycosidic linkage.The high energy of this bond [approximately ~8.2 kcal per mole at pH 7 and 25°C (26)] supplies the driving force for the ADP-ribosylation reaction. As described below there are three enzymes that can break this bond.

NAD glycohydrolase hydrolyzes the N-glycosidic linkage of NAD yielding free ADPR and nicotinamde, and a proton (equation 2). Certain NAD glycohydrolases can catalyse a base exchange reaction to yield analogues of NAD, containing various pyridine derivatives in place of nicotinamide. The enzyme acts, therefore, as both a hydrolase and a transglycosidase (equation 3).

$$\text{ADPR-N}_m + H_2O \Longrightarrow \text{ADPR} + \text{N}_m + H^+ \qquad (2)$$

$$\text{ADPR-N}_m + \text{base} \Longrightarrow \text{ADPR-base} + \text{N}_m \qquad (3)$$

ADP-ribosyltransferase catalyzes the transfer of the ADP-ribosyl moiety of NAD to a macromolecular acceptor (X). A single unit of ADP-ribose is transferred with the concomitant release of nicotinamide and a proton. This reaction is termed "mono ADP-ribosylation"

$$\text{ADPR-N}_m + X \Longrightarrow \text{ADPR-X} + \text{N}_m + H^+ \qquad (4)$$

A third type of enzymatic reaction occurs mainly in the nuclei of eukaryotes. In this case, a polymer of ADP-ribose is formed as a moiety attached to a macromolecular acceptor (X) such as a histone. The responsible enzyme is termed poly (ADP-ribose) synthetase.

$$nADPR\text{-}N_m + X \Longrightarrow (ADPR)n\text{-}X + nN_m + nH^+ \quad (5)$$

Thus, ADP-ribosylation reactions can be classified into two major groups: mono (ADP-ribosyl)ation and poly (ADP-ribosyl)ation. The main differences between the two kinds of ADP-ribosylation enents are listed in Table 1. ADPR can be hydrolyzed by ADPR glycohydrolase and/or phosphodiesterase.

ADP ribosylation is involved in so many significant biological functions, such as DNA repair, cell cycle, cellullar differentiation and oncogenesis (27, 28). We are interested in determining if there exists any ADP-ribosylation activity in yeast. These studies have not appeared in the literature with the exception of one preliminary abstract (29).

A sensitive assay method is very important for any enzyme study. Procedures employing spectroscopy and radioactivity are the two most common ways of monitoring an enzyme activity. In the instance of nicotinamide deamidase, both the substrate (nicotinamide) and product (nicotinate) have similiar absorbance properties, thus it is impossible to use normal spectroscopic measurements to detect this enzyme activity. For this reason, most laboratories have used expensive radioactive substrates and time consuming chromatographic separations of substrates from resulting radioactive products (14-17). A few laboratories have used the Koenig reaction (30) with some modifications (21), i.e. the reaction with cyanogen bromide and aromatic amines to give a color. In this laboratory, we designed and developed a totally new HPLC assay method, which is convenient, sensitive and time-efficient (see "Method" section). As a matter of fact, a number of HPLC assay procedures have been developed in this laboratory to study various PRTases in various aspects

TABLE 1: Comparisons Between Two ADP-ribose Generating Activities

	Mono(ADP-ribosyl)ation	Poly(ADP-ribosyl)ation
length of the resultant ADPR chain	single	polymer
chemical nature of the ADP-ribosyl protein bond	N-glycoside	O-glycoside
character of the enzyme	many microbial toxins	eukaryotic enzymes
cellular location of reaction	cytoplasm and cell membrane	nucleus

(31-35). As described in this thesis, we have also applied HPLC successfully to NmPRTase, QPRTase and ADP-ribosylation studies in yeast.

Both the catabolism and anabolism of NAD have important consequences in the cell. In this dissertation we will describe the results of the following research projects. 1). Pyridine nucleotide biosynthesis in yeast has been characterized. 2). The nicotinamide-to-nicotinate interconversion in this organism has been analyzed in detail. 3). Aspects of the breakdown of NAD (ADP-ribose synthesis) in yeast have been examined.

MATERIALS

Baker's yeast (Budweiser brand) was obtained from Valente Yeast Inc. (Flushing, P. O. Box 16, Whitestone, NY, 11357) and calf thymus from Pel-Freez Biologicals (P. O. Box 68, Rogers, Arkansas, 72756). PRibPP (sodium salt), nicotinate (free acid), nicotinamide, quinolinic acid, nicotinamide mononucleotide (N_mMN), adenosine triphosphate (ATP, disodium salt), nicotinate mononucleotide (N_aMN), adenosine diphosphate (ADP, sodium salt) adenosine monophosphate (AMP), ADP-ribose, dithiothreitol, mercaptoethanol, N-ethylmaleimide, diethylpyrocarbonate, acrylamide and the protein standards used in the YNDase structural analysis were obtained from Sigma (St. Louis), whereas sodium dodecyl sulfate, N,N-diethylnicotinamide and nicotinaldehyde were purchased from Aldrich Chemicals (Milwaukee). NAD (Adenine-2,8-H^3) was obtained from ICN Radiochemicals (2727 Campus Drive, Irvine, CA 92715). Sodium borohydride was obtained from Allied-Fisher Scientific (Pittsburgh). The protein purification materials obtained from Bio-rad (Richmond, CA) were Cellex-D and hydroxyapatite (HTP). Phenyl-Sepharose CL-4B was obtained from Pharmacia Inc. (Piscataway, NJ) whereas phospho-cellulose was purchased from the James Rivers Corp. (Berlin, NH). All other commercially available reagents were analytical grade.

METHODS

N_aPRTase, N_mPRTase and QPRTase Investigations

A. HPLC Assay

A Waters HPLC instrument equipped with Model 6000 A and M-45 solvent delivery systems, Model 660 solvent programmer, Model U6K sample injector, Model 440 absorbance detector, and a Houston Omniscribe chart recorder was used in the assay procedure. A single 30 cm x 3.9 mm Waters µBondapak C_{18} column was placed on line with the solvent delivery system at a flow-rate of 1.5 ml/min.. A reversed-phase HPLC elution system was used. The C_{18} column was first equilibrated with 25 mM $NH_4H_2PO_4$ buffer, pH 6.0 which had been clarified by vacuum filtration through a 0.45 µM HA Millipore filter. A linear gradient (from 0% to 100%) of a 10% methanol solution was employed within 20 min. (see Figure 8).

Samples (5 µl) from solutions containing substrates and products were injected onto the HPLC with a Hamilton 801 microliter syringe. Nucleotides and bases in the eluent were detected at 254 nm with a different absorbance setting (sensitivity).

B. Partial Purification of N_aPRTase, N_mPRTase and QPRTase Activities

1. Autolysis

One lb of baker's yeast was suspended in a mixture of 1.15 L of 0.3 M potassium phosphate buffer (pH 7.8) and 50 ml of toluene at 37°C with gentle stirring for 4 hrs. The pH was maintained at 7.8 by the periodic addition of 5 N KOH solution. Then the suspension was allowed to stand overnight at 4°C, and was

clarified by centrifugation at 8,000 rpm (10,400 x g) for 20 min.. The supernatant was filtered through glass wool to remove fluffy lipid material and 1150 ml of crude extract were obtained.

2. Manganese chloride treatment

A volume of 55 ml of 1 M $MnCl_2$ (50 mM of final concentration) was added to the autolysate to precipitate nucleic acids after 1 hr and 640 ml of supernatant were collected.

3. Ammonium sulfate fractionation

The following ammonium sulfate "cuts" (protein precipitates) were obtained from the Mn(II)-treated supernatant: 40% (155.5 g ammonium sulfate), 40-50% (46.3 additional g) and 50-60% (49.5 additional g). The precipitates were collected by centrifugation at 10,000 rpm (16,300 x g) and 4 °C for 30 min. and then redissolved in 10 mM phosphate buffer (pH 7.8). These samples were used for determining N_aPRTase, N_mPRTase and QPRTase activities.

C. Activity Detection

Following this purification procedure, volumes of 100 µl of the different ammoniun sulfate fractions were incubated at 37 °C with 1525 µl of 0.2 M Tris-phosphate buffer (pH 8.0), 250 µl of 50 mM $MgCl_2$, 250 µl of 10 mM ATP, 250 µl of 10 mM PRiPP and 125 µl of 20 mM nicotinate (or quinolinate, or nicotinamide). After successive 0, 1, 5, 10, 30 min. incubation times, 0.5 ml of incubation mixture was transferred to another test tube, boiled and filtered for use in the HPLC assay procedure.

Nicotinamide Deamidase

I. Assay Procedure

A. HPLC Assay

The same Waters HPLC instrument and μBondapak C_{18} column, employed in the N_aPRTase, N_mPRTase and QPRTase study, were used here except that a 10% methanol isocratic elution buffer and a flow rate of 1.0 ml/min. were applied (Figure 11). Samples (5 μl) from solutions containing substrates and products were injected onto the HPLC column by using a Hamilton 801 microliter syringe. The concentration of the substrates employed in standard kinetic analysis are listed in figure 11. Nucleotides and bases in the eluent were detected at 254 nm.

B. Ammonia Detection

For this analysis, 400 μl of 20 mM nicotinamide and 10 μl (130 milliunits) of pure enzyme (10 μl of buffer instead in control) were incubated at 37°C for 30 min. (avoid boiling). Thereafter, a small square of red litmus paper,was moistened with deionized water, and pasted into the center of the convex side of a watch glass. One pellet of solid NaOH was then placed in a vial (1.5 cm x 4.5 cm height). With a medicine dropper, one drop of the above incubated solution being tested was allowed to fall onto this pellet. Then the vial was covered with the watch glass, so that the litmus paper was on the under side of the watch glass and completely inside the vial. Care was taken not to let the litmus paper or the moistened part of the watch glass come in contact with the rim of the vial. In less than 1 min., the litmus paper turned blue if ammonium ion was present. In our studies, the sample turned blue immediately, whereas the litmus paper above control solution remained red for at least 5 min.. This indicated the generation of NH_3 during the enzyme incubation.

II. Purification of Nicotinamide Deamidase from Baker's Yeast

A. Design an Optimal Purification Procedure

The design of an optimal chromatographic purification scheme for an unknown protein is an art. A systematic approach, with the objective of optimizing a scheme, can lead to higher recoveries, enhanced yields and high specific activities. Additional benefits include time-saving and cost-effectiveness. Dr. Gail Sofer and V. J. Britton have proposed some basic steps in optimizing a chromatographic purification scheme (36).

In general, information on physicochemical as well as biological properties, such as biospecificity, charge, isoelectric point, surface properties or size of a biomolecule, should be considered when selecting a separation strategy. Other properties of the gels (such as capacity, flow rate, resolving capability and accommodation of sample volume) also are important.

Often the initial step of purification involves salt or/and alcohol (or acetone) precipitation with subsequent desalting to increase the specific activity of the component of interest and remove gross contaminants such as nucleic acids and pigments. Afterward, a high-capacity technique such as an initial chromatography step should be selected. Ion exchange chromatography has an average capacity for a complex mixture as high as 30 mg per ml of gel matrix (37). An advantage to the use of a high-capacity procedure early in the purification scheme is that such techniques are concentrating techniques. Reducing the solution volume usually makes subsequent chromatographic steps easier to perform.

At the end of purification scheme, a high-resolving technique should be used to eliminate closely related contaminants from the component of interest. Some high-

resolving techniques used in the latter part of a scheme include affinity chromatography, chromatofocusing and high performance liquid chromatography.

After each step, a concentration method is necessary. In addition, methods suitable for checking the purity of the sample should be quick, have high resolving capabilities and be suitable for analyzing a wide range of proteins. Electrophoresis, isoelectric focusing and immunoelectrophoresis meet these criteria. Recently high performance liquid chromatography has further reduced the time required for determining purity (38, 39, 40).

For yeast nicotinamide deamidase, after several tries, I observed that the following scheme can be used to obtain pure enzyme: ammonium sulfate precipitation, 50% acetone precipitation, DEAE-cellulose chromatography, phospho-cellulose chromatography, Phenyl-Sepharose chromatograghy and HPLC gel filtration chromatography.

B. Procedures

All steps described were performed in a cold room maintained at 4°C, except where specified.

1. Autolysis

24 lbs of baker's yeast were suspended into a mixture of 8 L of 5mM potassium phosphate buffer (pH 7.0) and 1 L of toluene. This suspension was allowed to stand at 37°C for 4 hrs with gentle stirring in a constant temperature water bath. The pH was maintained at 7.0 by the periodic addition of 5N KOH solution. Thereafter, the autolysis mixture was spun in a Sorvall RC-5 superspeed refrigerated centrifuge for 20 min at 10,400 x g (8,000 rpm) at 4°C. The supernatant was filtered through glass wool to remove fluffy lipid material mixed with toluene. The total volume of 15 L was obtained.

2. Ammonium sulfate fractionation

To this autolysate, 7080 g of ammonium sulfate was added with gentle stirring (70% cut) in the presence of 2~3 ml octanol (an antifoaming agent). The solution was allowed to stand overnight to effect complete precipitation and was spun at 9,000 rpm for 30 min. to collect the protein precipitate. The protein precipitate was redissolved in 5 mM potassium phosphate buffer (pH 7.0) to get a final volume of 4 L. This procedure was then repeated, with the exception that a 40-70% cut of ammonium sulfate was obtained in second time. This protein precipitate was redissolved (4 L) and dialyzed against 20 L of same buffer overnight (the same buffer was changed once).

3. Acetone fractionation

To this 4 L ammonia sulfate fractionated solution, 425 ml (10%, v/v) pre-cooled (-20°C) acetone was added and stirred. This suspension was clarified by centrifugation at -10°C for 30 min. at 9,000 rpm. The denatured precipitate was discard. Another 2.8 L of the same cold acetone was added to 3.5 L supernatant to give 50% acetone fractionation, the suspension was slowly stirred (15 min., 4°C) and then allowed to stand at -20°C for an additional 90 min.. The resulting protein precipitate was collected by centrifugation (-10°C, 30 min., 9,000 rpm), redissolved in 2L in 5 mM phosphate buffer (pH 7.0) and dialyzed overnight against 20 L of the same buffer.

The formula for calculating the amount of acetone to be added is:

$$\text{Volume to add to 1 L to take \% from X to Y} = \frac{1000(Y-X)}{100-Y} \tag{6}$$

At this stage, the YNDase preparation could be stored at -76°C for at least one year without a measurable decrease in activity. A 200 ml portion of the acetone fraction was then purified further through the use of several chromatographic procedures which were tested in several different sequences. The most useful of these sequences will be described.

4. DEAE cellulose chromatography

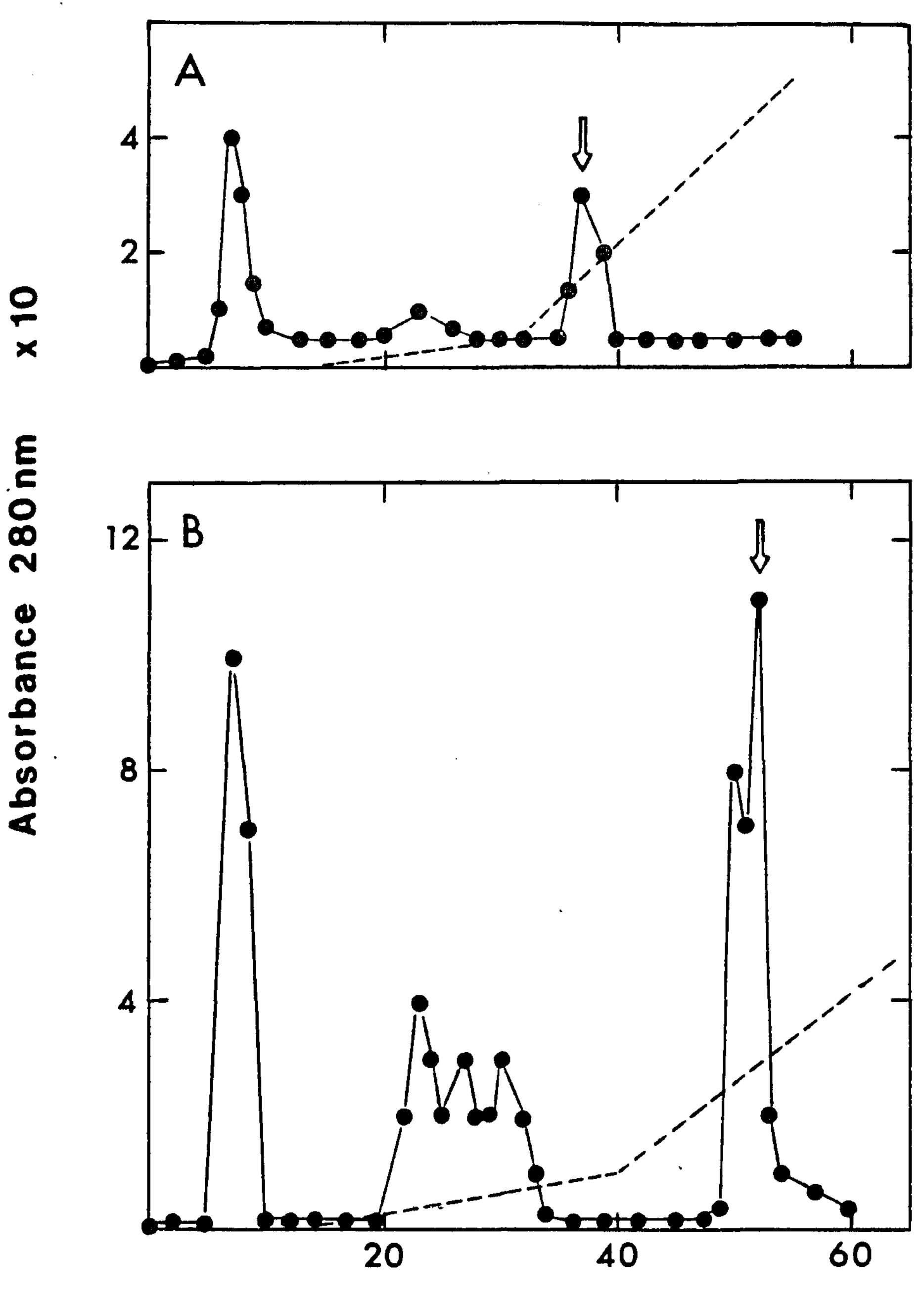
0.2 L of dialyzed solution from the acetone fractionation was layered onto a DEAE cellulose column (7 cm x 20 cm) pre-equilibrated with 5 mM (pH 7.0) potassium phosphate buffer, allowed to be adsorbed for 30 min. and then washed subsequently with 5 mM and 100 mM potassium phosphate buffer (pH 7.0). YNDase activity was not found in this solution. The column was washed continuously with 100-500 mM phosphate gradient whereupon the YNDase activity appeared (Figure 3B). The active fractions were pooled, dialyzed against 5 mM phosphate buffer overnight (pH 7.0, 18 L) and concentrated by selective filtration (Amicon Diaflo Unit, PM-10 filters) to a volume of 15 ml.

5. Phospho-cellulose chromatography

The concentrated YNDase sample was applied to a phospho-cellulose column (2.5 cm x 22 cm) pre-equilibrated with 5 mM phosphate buffer (pH 7.0) and washed with succeeding concentrations of 5 mM and 100 mM phosphate buffer (pH 7.0). The enzyme activity was then removed from the column with a 500 mM elution buffer at pH 7.0 (Figure 3A). The active fractions were again dialyzed and concentrated as described above to a final volume of 17 ml.

6. Phenyl-Sepharose chromatography

Figure 3. Profiles of the elution of YNDase through Phospho-cellulose and DEAE-cellulose. A). As described in "mothods", succeeding 15 ml volumes of the protein eluent from a DEAE-cellulose column, that contained YNDase, were applied to the Phospho-cellulose column equilibrated with 5 mM phosphate buffer (pH 7.0). B). Succeeding 200 ml volumes of the protein extract obtained from the acetone treatment step, were eluted through DEAE cellulose equilibrated with 5 mM phosphate (pH 7.0) through which a phosphate concentration gradient was allowed to develop. The dashed lines in these figures represent our estimation of the phosphate concentration of each fraction. The protein elution profile was developed by measuring the absorbances of these fractions at 280 nm (-●-). The standard HPLC assay procedure, as described in the legend in Figure 11, was employed to monitor the YNDase activity in these fractions.


A
4
2
B
12
8
4
20
40
60
Absorbance 280nm x 10
Fraction Number

A volume of 17 ml of the YNDase preparation was applied to this column (3.5 x 30 cm) pre-equilibrated with 30% ammonium sulfate in 5 mM phosphate (pH 7.0). The protein was washed with this same buffer, and then a gradient from 30% ammonium sulfate to 50% ethylene glycol was applied. I observed that the activity eluted when 50% ethylene glycol was employed to wash the column subsequent to the application of the gradient (Figure 4). The active fractions were again pooled, dialysed and concentrated by Amicon filtration to 4 ml.

7. HPLC gel-filtration chromatography

A Bio-rad TSK-250 column was placed on-line with the Waters 6000 A/U6K system and equilibrated with a mobile phase recommended by the manufacturer (0.05 M sodium sulfate, 0.02 M sodium phosphate, pH 6.8), A series of 10 aliquots (20 μl) of the enzyme solution (from the Phenyl-Sepharose step) were injected onto the column, and 1 ml eluent fractions were collected. Fractions containing YNDase activity were pooled and concentrated to 1 ml final volume as described above (Figure 5). Thereafter this enzyme solution was ready for purity determination (see "Gel Electrophoresis" section).

III. Protein Determination

The concentration of protein in different purification steps were determined with the Bio-Rad Protein Assay Kit. The Bio-Rad Protein Assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (41-43). When compared with other protein assays, especially the widely used Lowry method (44), Bio-rad assay offers several advantages: 1) The Bio-Rad Protein Assay is much easier to use. It requires one reagent and 5 minutes to

Figure 4. Phenyl-Sepharose CL-4B column chromatography of a yeast protein extract containing YNDase activity that was obtained as a 40-60% ammonium sulfate fractionation. The column was equilibrated with 5 mM phosphate buffer (pH 7.0) which contained 30% ammonium sulfate (solution A). The enzyme was eluted with a linear gradient of solution A with solution B (50% ethylene glycol). The YNDase activity (- Δ -) of each fraction was monitored with the HPLC assay procedure described in Figure 11, whereas the protein concentration in these fractions was estimated by measuring their absorbances at 280 nm (-●-). The dashed line represents our estimation of the content (in % of total solvent) of 50% ethylene glycol.

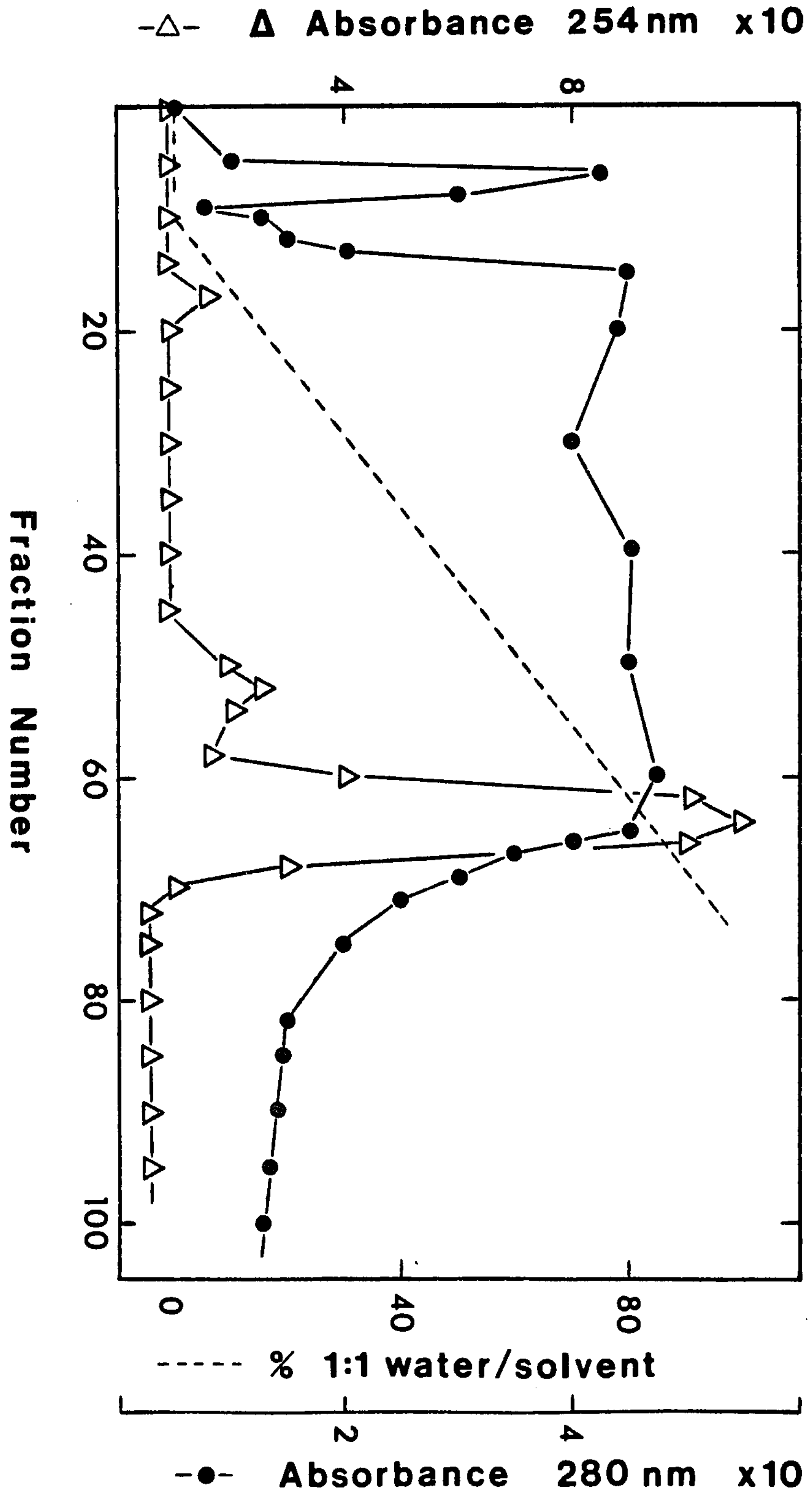

-△- Δ Absorbance 254nm x10
4
8
Fraction Number
20
40
60
80
100
0
40
80
----- % 1:1 water/solvent
2
4
-●- Absorbance 280 nm x10

Figure 5. Profiles of the elution of YNDase through HPLC gel filtration column. Elution conditions were as follows: 0.05 M sodium sulfate and 0.02 M sodium phosphate buffer, pH 6.8; 1 ml/min flow rate; 0.5 cm/min chart speed.

16
12
8
4
0
Elution Volume (ml)

perform whereas three reagents and 30-40 minutes are typical for the Lowry assay. 2) Because the absorbance of the dye-protein complex is relatively stable, the Bio-Rad assay does not require the critical timing necessary for the Lowry assay. 3) The Bio-Rad assay is free from most of the spectral interferences which limit the application of the Lowry assay.

A. Protocol for the Standard Assay Procedure (20-140 μg protein)

Prepare several dilutions of a bovine albumin standard, containing from 0.2 to about 1.4 mg/ml protein (142 μg/ml, 284 μg/ml, 568 μg/ml, 852 μg/ml, 1130 μg/ml, 1420 μg/ml). Prepare a standard curve each time the assay is performed.

1). Place 0.1 ml of standards and appropriately diluted "unknown" samples in clean, dry test tubes. Place 0.1 ml sample buffer in the " blank" test tube.

2). Dilute 1 volume of dye reagent concentrate with 4 volumes of high quality deionized water. Filter through Whatman No. 1 paper or equivalent and store the dilute reagent in a glass container at room temperature (discard the dilute reagent after 2 weeks). Thereafter add 5.0 ml diluted dye reagent to each of above test tubes.

3). Vortex (avoid excess foaming) or mix several times through gentle inversion of the test tube.

4). After a period of from 5 minutes to one hour, measure OD_{595} of the unknown samples through the use of a Coleman Model 101 Hitachi UV-VIS Spectrophotometer.

5). Plot OD_{595} *versus* concentration of standards.

B. Protocol for the Microassay Procedure (1-20 μg protein; ≤25 μg/ml)

Prepare several dilutions of protein standard containing from 1 to 25 μg/ml. Prepare a standard curve each time the assay is performed.

1). Place 0.8 ml of standards and appropriately diluted samples in clean, dry test tubes. Place 0.8 ml sample buffer in "blank" test tube.

2). Add 0.2 ml dye reagent concentrate.

3). Continue as described above.

IV. Gel Electrophoresis (PAGE)

The gel electrophoresis apparatus obtained from Fisher Scientific Company (Buchler Instrument) was used, which consisted of two reservoirs connected electrically by glass tubes containing the polyacrylamide gels. The gel system was prepared in a vertical column which consisted of the stacking gel and separating gel. The LKB D. C. 3371E Power Supply, and an LKB 2209 MultiTemp cooling system were used.

A. Protocol for the Preparation of Solutions:

Solution A (resolving gel buffer): Dissolve 36.30 g of Tris base in 150 ml deionized water. Adjust the pH to 8.8 with 5N HCl. Adjust the final volume to 200 ml with deionized water.

Solution B (stacking gel buffer): Dissolve 12.0 g of Tris base in 150 ml deionized water. Adjust the pH to 6.8 with 2N HCl. Adjust the volume to 200 ml with deionized water and then filter.

Solution C (electrode buffer): Dissolve 14.41 g of glycine, 6.9 g of Tris base (and 0.5 g of SDS for denaturing gel electrophoresis) in 0.8 L of deionized water. Adjust to pH 8.3, and a volume of 1 L with deionized water.

Solution D (acrylamide solution): Dissolve 30 g of acrylamide and 0.8 g of bisacrylamide in 85 ml of deionized water. Adjust the volume to 100 ml with deionized water and filter. Store in the dark at 4°C.

Solution E (sample buffer 0.0625 M Tris): The following materials are added together: 25 of ml Tris-SDS stock (pH 6.8), 2 g of SDS, 10 ml of glycerol, 5 ml of 2-mercaptoethanol, 0.1 ml of 1% bromophenol blue. Add deionized water to 100 ml, and store in a tightly sealed bottle.

Tris-SDS stock solution (0.25 M Tris): Dissolve 39.4 g of Trizma-HCl and 2 g of SDS and adjust the pH to 6.8. Add deionized water to a final volume of 1000 ml.

Staining solution: Dissolve 0.625 g of Coomassie brilliant blue in a mixture of 113.5 ml of deionized water, 113.5 ml of methanol and 23 ml of glacial acetic acid (5:5:1). When the dye is fully dissolved, filter the solution through Whatman No. 1 filter paper or its equivalent.

Destaining solution: Mix 875 ml of deionized water with 50 ml of methanol and 75 ml of glacial acetic acid.

Tracking dye (0.05%): Dissolve 5.0 mg of bromophenol blue (anion) in 10.0 ml of deionized water.

B. Protocol for the Preparation of Glass Columns:

The glass tubes (15 cm x 5 cm) were cleaned by immersing them in a cleaning solution and rinsed extensively with deionized water (cleaning solution: hot nitric acid or 10 g chromic acid in 200 ml hot sulfuric acid).

C. Nondenaturing Gels (PAGE)

To check the purity of nicotinamide deamidase, it is necessary to separate intact proteins. Gels may be prepared under condition where proteins are not denatured. In these cases, protein separation depends on a combination of differences in protein molecular size and shape as well as charge.

1. Preparation of separating gel

The glass gel columns were closed at one end with a rubber stopper and placed in a vertical position. All the solutions necessary for polymerization were warmed to room temperature. It was better to evacuate the solutions with an aspirator or vacuum pump because it is important to reduce the solutions to a low partial pressure of air, since oxygen acts as chain terminator in the polymerization process. Gloves were recommended to wear because of toxic acrylamide.

To make 7.5% polyacrylamide separating gel, 4.88 ml of solution D, 5.00 ml of solution A, 9.90 ml of deionized water, 0.07 ml of 10% (w/v) fresh ammonium persulfate and 0.01 ml of TEMED (N,N,N',N'-tetramethylethylenediamine, a free radical scavenger) were added to a beaker, mixed well (try to avoid bubbles, if necessary, aspirate immediately to remove air bubble) and transferred to the glass columns by a syringe as quickly as possible. Once ammonium persulfate had been added, the gels started to polymerize. Immediately after this step, a 3-4 mm layer of water was gently placed on top of the gel solution. After an overlayering of the gel solution with water, the interphase was visible and soon disappeared. It became visible again after completion of polymerization. This process took about 30 min. to

1 hr, and depended on the freshness of the solution. When polymerization was complete, each tube was inverted and drained of excess water, which was carefully removed with Kimwipes.

2. Preparation of stacking gel

To make 3% polyacrylamide stacking gel, the following solutions were added to a beaker and mixed well: 2.00 ml of solution D, 5 ml of solution B, 12.80 ml of deionized water, 0.20 ml of fresh ammonium persulfate (10%, w/v), 0.03 ml of TEMED. The appropriate amount of stacking gel solution was placed on top of the separating gel. Immediately thereafter, a layer of 3-4 ml of deionized water was carefully placed on top of the polymerizing solution, by observing the precautions mentioned above. After polymerization, excess water was removed and the upper part of the column was rinsed carefully with the electrode buffer (solution C).

3. Application of the sample

Place solution C into the lower buffer reservoir. The gel columns were positioned in the electrophoresis apparatus. Gas bubbles trapped in the columns must be (and were) removed. 80 µl (12.2 µg) of purified nicotinamide deamidase, with 10% glycerol or sucrose (which allowed the samples to sink to bottom of the sample well in the stacking gel) and one drop of 0.05% tracking dye, were loaded on top of the column gel. The sample should be low in inorganic salts and preferably dialyzed against the same buffer used in the upper reservoir. Electrophoresis was carried out in the cold room at 0-4 °C. 3 mA/tube current was used and maintained. A change of voltage should be observed during the course of electrophoresis. A drastic change in the potential gradient is an indication of overheating. When the band of the tracking dye reached the lower end of the separating gel, the power supply was disconnected and the column samples were removed from the apparatus.

4. Staining and destaining

The gels were removed from the column by gently injecting deionized water between the wall of the glass tube and the gel using a syringe. The syringe was moved in a circular motion around the gel as it was inserted and water was injected. After removing each gel from the column, they were placed gently in test tubes and covered with the staining solution. They were allowed to stand for 3 hrs at room temperature and then removed from the stain and rinsed with deionized water. The gels were transferred to capped plastic test tubes containing holes . These tubes were placed in a 1 liter flask full of destaining solution, and stirred gently using a magnetic stirrer at room temperature. It was better to place a piece of wool in the flask to absorb the staining dye.

D. Denaturing Gels (SDS-PAGE)

SDS gel electrophoresis is a variation of PAGE. The electrophoretic mobility of protein is determined in the presence of sodium dodecyl sulfate (SDS, $CH_3(CH_2)_{11}OS_3^-Na^+$) and 2-mercaptoethanol. 2-mercaptoethanol breaks disulfide linkages and keeps the sulfhydryl groups reduced. The anionic detergent SDS binds to the hydrophobic regions of protein (about 1.4 mg of SDS per mg protein) and denatures them into rodlike polypeptides. The diameter of these partical appears to be roughly constant and the length is proportional to the length of the polypeptide chain. Consequently the charge/size ratio is virtually identical for all proteins, therefore separation by SDS-PAGE depends only on molecular weight of the protein. Most proteins in crude mixture are soluble in SDS and bind it avidly. Even the most basic proteins are converted to their acidic SDS derivatives and thereby migrate toward the anode at pH 7-9. SDS is usually sufficient to halt the effects of proteases,

protein kinases and protein phosphotases. Thus, SDS-PAGE is a fine method to determine the purity, M.W., and subunit composition of protein and polypeptide.

1. Preparation of separating gel

See "Nondenaturing Gels" section.

2. Preparation of stacking gel

See "Nondenaturing Gels" section, except that 0.10 ml of 10% (w/v) SDS was added to the 3% polyacrylamide solution.

3. Treatment and application of sample

1). Treatment of Sample. 80 µl of deamidase protein (7 µg/50 µl) with 80 µl solution E were mixed and incubated them at room temperature for 30 min.. They then were placed in boiling water for 2 min. and cooled down to room temperature.

2). Treatment of standard protein. 2 mg of bovine albumin, egg albumin, trypsinogen, beta-lactoglobulin, lysozyme and "dalton mark" were weighed out, and dissolved in 1 ml of solution E. These samples were then incubated and boiled as before.

3). Application of sample. See "Nondenaturing Gels" section with a couple of exceptions. a). 120 µl treated sample and 50 µl standard proteins were loaded on to the columns. b). SDS electrophoresis was carried out at 10°C which was controlled by LKB 2209 MultiTemp cooling system. The temperature control system is necessary because of SDS precipitation under 10°C.

4. Staining and destaining

See "Nondenaturing Gels" section.

V. Molecular Weight Determination by HPLC

The Bio-rad TSK-250 column was used under the same conditions that were used to purify the enzyme. To establish the molecular weight of this enzyme, five protein standards (bovine serum albumin, beta lactoglobulin, egg albumin, trypsinogen and lysozyme) were eluted through the column sequentially to obtain a molecular weight standard curve by plotting elution volume *versus* log molecular weight. The position of deamidase in the elution profile with 1 ml/min. flow rate was monitored at absorbance of 254 nm.

VI. pH Stability Study

A key requirement in most enzyme kinetic analyses is to control the pH at all times. The pH can influence the velocity of an enzyme-catalyzed reaction because the active sites on enzymes are composed of ionizable groups that must be in the proper ionic form in order to maintain the conformation of active site, facilitate the binding of the substrates or catalyze the reaction. On the other hand, substrates themselves may contain ionizable groups and only one ionic form of that substrate may bind to the enzyme or undergo catalysis. The pH effect on the K_m and V_{max} will disscused in the "Kinetic Study" *(vide infra).*

The effects of pH on the stability of an enzyme must be taken into account in any study of the effect of pH on substrate binding and catalysis because changes in enzyme activity could result from the existence of the improper ionic form of the enzyme (or substrate or both), from inactivation of the enzyme, or from a combination of these effects.

1. Preparation of the buffer solutions

Following buffer solutions were chosen to use in pH study:

Buffer	pKa	pH Study Range
50 mM Acetic Acid	4.75	4.0, 4.5, 5.0, 5.5, 6.0
50 mM Potassium Phosphate	7.21	6.0, 6.5, 7.0, 7.5
50 mM Tris buffer	8.30	8.0, 8.5, 9.0
50 mM CHES	9.50	9.0, 9.5, 10.0, 10.5

A glycylglycine buffer (pK_b 8.4) was also tested, but these solutions absorbed at 254 nm, thus interfering with the HPLC detection.

2. Preincubation

A volume of 5 µl of purified enzyme (65 milliunits) was incubated with 45 µl of each buffer (pH 4-10.5) at room temperature for 10 min..

3. Activity assay

The above preincubated enzyme solution was transferred to an assay solution (100 µl of 10 mM nicotinamide, 1850 µl of 50 mM potassium phosphate buffer, pH 7.0), and the incubation was continued for 0, 3, 6 and 9 min.. At each time point, 0.5 ml of incubated enzyme assay solution was removed and transferred to a test tube, boiled for 2 min. and assayed by HPLC as described in the "Assay Procedure" section.

VII. Kinetic Study

To examine the effect of pH on the K_m and V_{max} of nicotinamide deamidase, the HPLC assay procedure as described in "Assay Procedure" section was employed. The kinetic analysis was performed in the following manner.

1). To the incubation mixture (2.5 ml) was added 25 µl of the 1:100 dilution enzyme (0.975 milliunits), 0.125 ml of the appropriate concentration of nicotinamide (0.4, 1.0, 2.0, 4.0 mM) to give a final concentration of 0.02, 0.05, 0.10, 0.20 mM, and in addition 2.35 ml of 50 mM acetate (pH 5, 5.5), or phosphate (6.0, 6.5, 7.0, 7.5), or Tris-phosphate (pH 8.0, 8.5), or CHES (pH 9.0) buffer was included.

2). The above described mixtures were incubated for 0, 2, 4, 6, 8 min.. Thereafter aliquots of 0.5 ml of solution were transferred to a test tube and boiled for 2 min. to stop the reaction.

3). 5 µl of sample was then injected onto the HPLC column.

VIII. Specific Chemical Modification of the Nicotinamide Deamidase

At the active site of every enzyme, some essential amino acid groups play key roles in binding, or catalyzing substrates to products. To identify such groups is obviously important for an understanding of the mechanisms by which enzymes catalyze reactions. Chemical modification is a major powerful tool for identifying these amino acids. Protection by substrates or related compounds has been used to selectively prevent the modification of active-center residues. If an enzyme activity is retained following modification in the presence of substrate but is lost in its absence, it is usually assumed that a group in the active site has been protected by substrate. Since our pH kinetic study suggested that at least one essential protonated amino acid

residue is present at the YNDase active site, chemical modification analyses were initiated to see if this amino acid could be identified.

A. N-ethylmaleimide (NEM) Modification

$$\text{(P)–SH} + \text{maleimide-}NC_2H_5 \xrightarrow{pH > 5} \text{(P)–S–succinimide-}NC_2H_5 \qquad (7)$$

$$\text{(P)–}NH_3 + \text{maleimide-}NC_2H_5 \xrightarrow{pH > 7} \text{(P)–NH–succinimide-}NC_2H_5$$

Since the pK_b of YNDase (7.8) is close to that of a cysteine side chain (8.33), the chemical modification of sulfhydryl groups was performed first. NEM is a highly specific reagent for protein sulfhydryl groups below pH 7. At pH 7, its reaction rate with simple thiols is approximately 1000-fold greater than that with corresponding simple amino compounds. At higher pH values, reactions with amino groups becomes increasingly more significant (45).

1. Inhibition study

1). Preincubation: Volumes of 200 µl of 50 mM potassium phosphate buffer (pH 6.0), 2 µl of stock enzyme (26 milliunits) and 2 µl of various concentration of NEM were added to a test tube to give a final concentrations of 0.1 µM, 0.5 µM, 1.0

μM, 10.0 μM of NEM respectively. The mixtures were incubated at room temperature.

2). Activity Assay: The above incubated mixtures were transferred at 0, 2, 5, 10, 15 min. to a 0.49 ml of assay solution (485 μl of 50 mM potassium phosphate buffer, pH 6.0, 5 μl of 20 mM nicotinamide, 0.2 mM final concentration). Then these solutions were incubated at 37 °C for an additional 20 min.. The reaction was stopped by boiling and treated as described in the "Assay Procedure" section.

3). Detection of activity by HPLC: These method was described in the "Assay Procedure" section.

2. Substrate protection by nicotinamide

Volumes of 200 μl of 50 mM potassium phosphate buffer (pH 6.0), 2 μl of stock enzyme (26 milliunits), 2 μl of various concentration of nicotinamide were mixed to a final given concentration of 10 μM, 20 μM and 40 μM of nicotinamide. Then 2 μl of 100 μM NEM (final concentration equal to 1 μM) was added to these test tubes, and were incubated at room temperature. The following steps were the same as the inhibition study.

3. Product protection by nicotinate

These experiments were carried out in the same manner as the substrate protection study, except that 20 μM, 40 μM, 80 μM, and 120 μM of protection concentration of nicotinate were used.

B. Diethylpyrocarbonate (DEP) Modification

DEP reacts with histidyl residues (carbethoxylation) as shown in following equation (46, 47):

P–imidazole + DEP ($O=C(OCH_2CH_3)–O–C(OCH_2CH_3)=O$) $\xrightarrow{pH\ 4\text{-}7}$ (8)

P–imidazole–N–C(=O)–OCH_2CH_3 + CH_3CH_2OH + CO_2

NH_2OH

P–imidazole–N–C(=O)–O–CH_2CH_3 $\xrightarrow{pH\ 7}$ (9)

P–imidazole–NH + HO(H)N–C(=O)–O–CH_2CH_3

DEP can also react with a variety of nucleophilic residues which occur in proteins, including sulfhydryl, arginyl and tyrosyl residues. Hydroxylamine can remove the carbethoxy group from modified histidyl and tyrosyl residues and recover the enzyme activity (equation 9), but not from modified lysyl or sulfhydryl residues. Thus, if it can be shown that the activity of DEP-treated enzyme can be recovered by

incubation with hydroxylamine, histidyl or tyrosyl group may be present at active site.

1. Inhibition study

1). Preincubation: A total of 2 μl of stock enzyme (26 milliunits) and 5 μl, 10 μl or 30 μl of 20 mM DEP (final concentration equal to 0.5 mM, 1.0 mM, and 3.0 mM respectively) were placed into 200 μl of 50 mM potassium phosphate buffer (pH 7.0), and incubated at room temperature.

2). Activity assay: A volume of 5 μl of DEP treated enzyme were transferred at 0, 5, 10, 15, 20, 30 min. into a 0.495 ml of assay solution (470 μl of 50 mM potassium phosphate buffer, pH 7.0, 25 μl of 2 mM nicotinamide, final concentration of 0.1 mM), and the incubation was continued for 15 min. at 37°C. Thereafter the enzyme activity was detected by HPLC, as described in the "Assay Procedure" section.

2. Protection study by nicotinamide

The study was carried out under the same conditions as those employed in the inhibition study, except that 0.5 mM, 1 mM of nicotinamide were involved in the preincubation with 3 mM DEP.

3. Enzyme treatment by hydroxylamine

1). Studies of hydroxylamine's effect on nicotinamide deamidase activity. 5 μl of stock enzyme (65 milliunits) was placed into 470 μl of 50 mM potassium phosphate buffer solutions, pH 7.0, each of which contained 1 mM, 5 mM, 10 mM of hydroxylamine respectively. These were incubated at 4°C for 1 hr. Then 25 μl of 2 mM nicotinamide (final concentration equal to 0.1 mM) was added to the

preincubated mixture to initiate the enzyme reaction. The incubation was allowed to continue for an additional 15 min., boiled and detected by HPLC.

2). Dialysis experiment: 10 μl of stock enzyme (130 milliunits) was added to 1 ml of 10 mM H_2NOH (dissolved in 50 mM potassium phosphate buffer, pH 7.0). Control experiment made use of 1 ml of buffer instead. These solutions were incubated at 4°C for 2 hrs. Thereafter 5 μl of the hydroxylamine-treated enzyme was incubated with an assay solution (495 μl of 50 mM potassium phosphate buffer with 0.1 mM nicotinamide) for another 15 min., and treated as usual. The remainder of treated enzyme was placed in dialysis tubing, and dialysed against 5 mM potassium phosphate buffer at 4°C for 6 hr. A volume of 5 μl of this dialyzed enzyme was added to assay solution, incubated in the same way.

Since hydroxylamine inactivated the deamidase (see "Results" section), I could not continue the decarbethoxylation study.

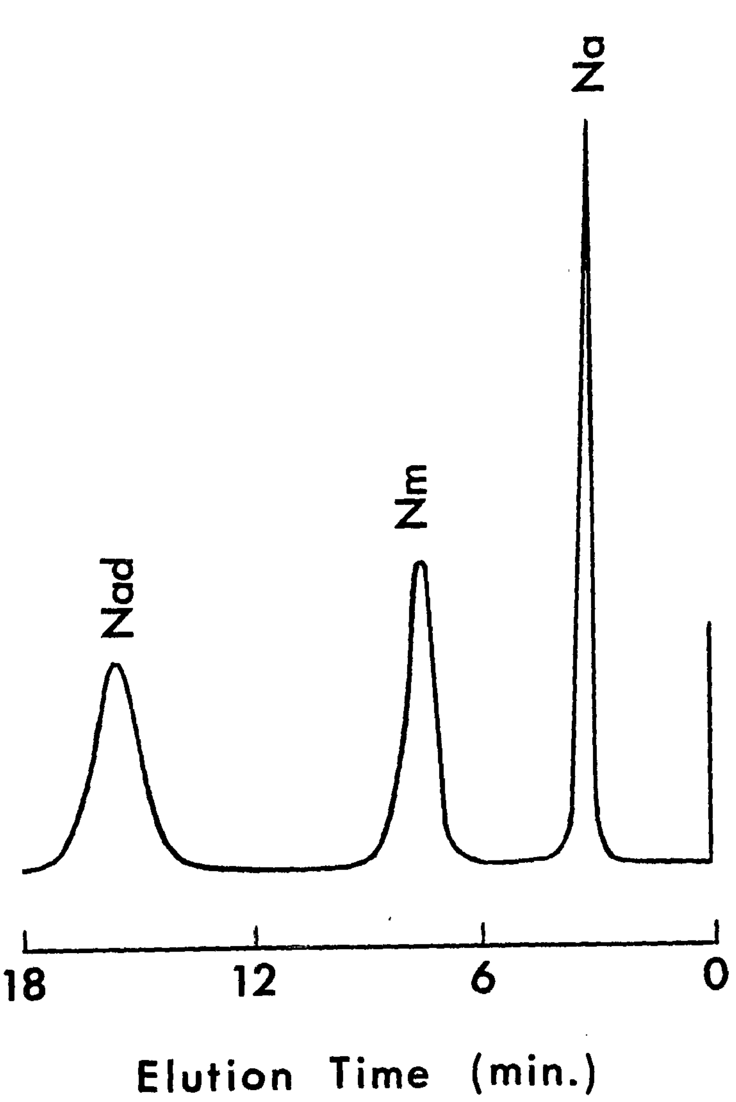
IX. Analogue Study (Nicotinaldehyde)

Nicotinaldehyde is a structural analogue of nicotinamide and has an absorbance at 254 nm wavelength. However, I can separate it from nicotinate and nicotinamide by the HPLC with a 10% methanol eluent when the assay solution is eluted through the μBondapak C_{18} column, as shown in Figure 6.

1. Substrate testing

A volume of 2.0 ml of 50 mM potassium phosphate buffer (pH 7.0) which contained 1mM nicotinaldehyde and 2 μl of stock enzyme was incubated at 37°C for 0, 10, 20, 30 min.. At each time point, 0.5 ml of the incubated solution was removed, boiled and treated as in the nicotinamide study.

Figure 6. The separation of the nicotinaldehyde peak from nicotinamide and nicotinate peaks on a Waters Associates reversed-phase μBondapak C_{18} HPLC column. Elution condition: 10% methanol elution buffer; 1 ml/min flow rate; 0.5 cm/min chart speed.



Na
Nm
Nad
18
12
6
0
Elution Time (min.)

2. Inhibition and protection

Volumes of 2 μl of stock enzyme (26 milliunits) and 4 μl, 20 μl of 100 mM nicotinaldehyde (final concentrations of 2 mM and 10 mM respectively) were added to a 200 μl of 50 mM potassium phosphate buffer. The mixtures were incubated at room temperature. Then,10 μl of preincubated enzyme solution was transferred to the assay solution (475 μl of potassium phosphate, pH 7.0, 25 μl of 20 mM nicotinamide) at 0, 5, 10, 20, 30 min.. Detection of the enzyme activity was accomplished with the HPLC, as described in the "Assay Procedure" section.

The protection experiments were carried out under the same conditions employed in the inhibition studies, except that 20 mM nicotinamide and 2 mM nicotinaldehyde were preincubated with the enzyme.

3. Kinetic inhibition studies

Concentrations of 0 μM, 16.5 μM, 33.0 μM and 49.5 μM of nicotinaldehyde (inhibitor) were mixed with 20 μM, 50 μM, 100 μM and 200 μM of nicotinamide (substrate) respectively, in 50 mM potassium phosphate buffer pH 6.5 and 3.25 milliunits/0.5 ml of enzyme (25 μl of 1:100 diluted stock enzyme). Incubations were carried out at 37°C. At time points of 1, 2, 4, 6 min., aliquots (0.5 ml) of the solution were taken, boiled and detected by HPLC, as descibed in "Assay Procedure" section.

4. Flow dialysis (sodium borohydride treatment)

1). Detection of the $NaBH_4$ effect on YNDase: First, 4 μl of 1 M of $NaBH_4$ and 2 μl of stock enzyme (26 milliunits) were added to a 200 μl of 50 mM potassium phosphate buffer (pH 7.0). These solutions were incubated at room temperature and then 10 μl of incubated solution was transferred to the assay solution at 0, 10, 20, 30

min.. Then these solutions were incubated for an additional 15 min., and the enzyme activity was detected as usual.

2). Flow dialysis study:

Step 1. 2 ul of stock enzyme (26 milliunits) was added to a 200 μl of 50 mM potassium phosphate buffer (pH 7.0). Thereafter 10 μl of diluted enzyme solution was transferred to the assay solution to detect the activity (counted as 100% initial activity).

Step 2. A volume of 2 μl of 1 M nicotinaldehyde was added to the rest of enzyme solution, incubated at room temperature for 10 min.. Then 10 μl of treated enzyme solution was transferred to the assay solution to detect the activity.

Step 3. 4 μl of 1 M $NaBH_4$ was added to the nicotinaldehyde-treated enzyme solution for an additional 20 min. incubation at room temperature. A volume of 10 μl of this enzyme solution was then transferred to the assay solution to detect the activity.

Step 4. The rest of the treated enzyme solution was added to a flow dialysis chamber, dialysed against 50 mM potassium phosphate buffer, pH 7.0. Afterward 10 μl of dialysed enzyme solution was transferred to the assay solution to detect the activities after 30, 60, 120, 170. min. time points.

Step 5. The enzyme activity was detected by HPLC as usual.

Control experiments carried out in parallel, with the exception that step 3 was omitted.

5. Substrate labeling study

A volume of 4 μl of 1 M sodium borohydride (20 mM final concentration), 4 μl of 20 mM nicotinamide (0.4 mM final concentration, in a control experiment only buffer was added) and 2 μl of stock enzyme (26 milliunits) were added to 190 μl of 50 mM potassium phosphate buffer (pH 6.0). This mixture was incubated at room

temperature for 0, 5, 10, 20, 30 min.. At each time point, 20 μl of preincubated enzyme was transferred to an assay solution (475 μl of 50 mM potassium phosphate buffer, pH 6.0, 5 μl of 20 mM nicotinamide), and the incubation was continued for an additional 10 min. at 37°C. Activity was detected by HPLC as described above.

X. Amino Acid Composition Analysis

I prepared the following samples for amino acid composition studies of YNDase.

Sample 1: YNDase

56 μg of pure enzyme, from under -70°C, was lyophilized to dryness.

Sample 2: YNDase + NEM

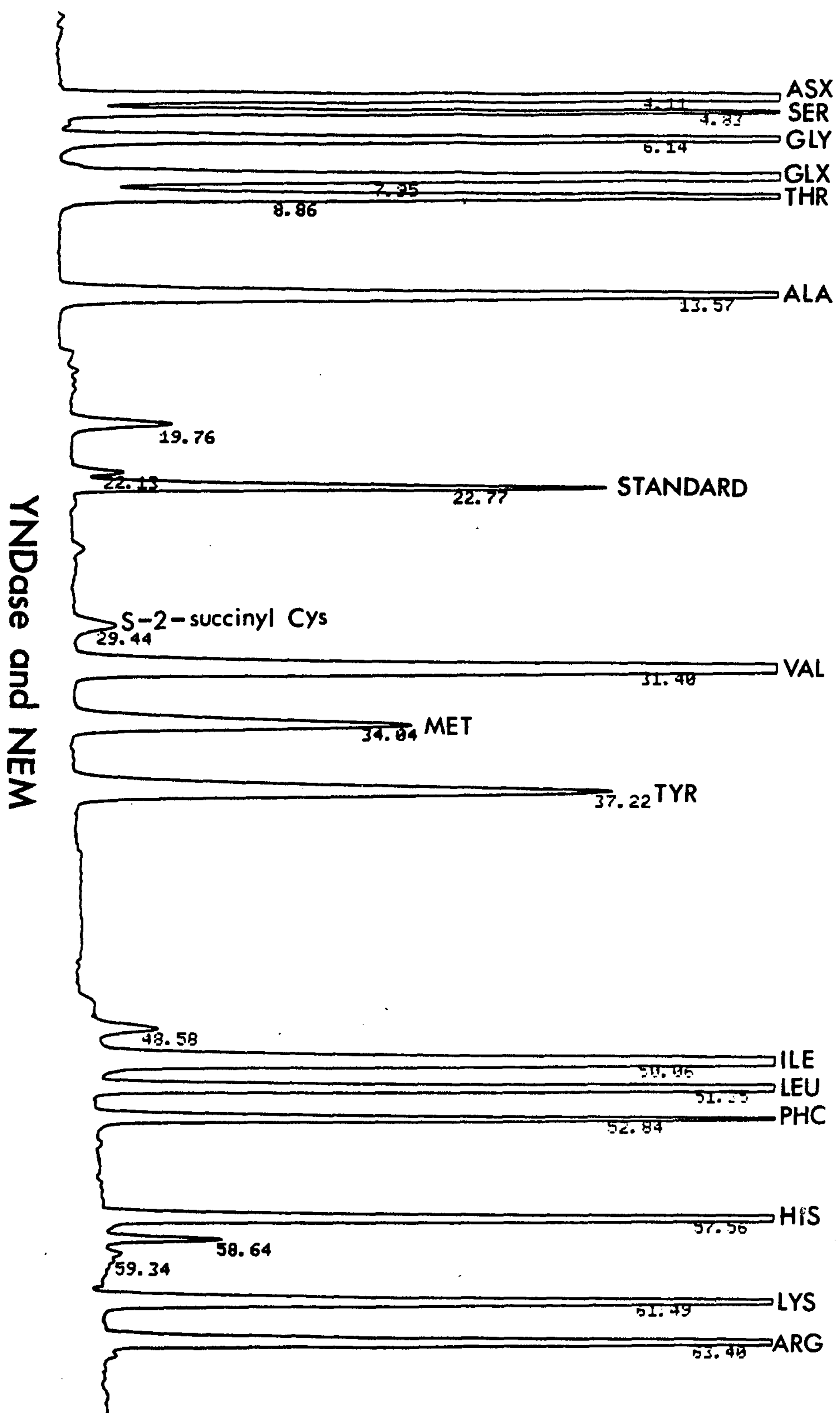
56 μg of pure enzyme was incubated with 80 μl of 0.1 M NEM (final concentration was 80 μM) at room temperature for 30 min., and then frozen and lyophilized to dryness.

Sample 3: Cysteine + NEM

10 mg of cysteine (M. W. equal to 121) and 11 mg of NEM (M. W. equal to 125) were incubated in 300 ml of 50 mM potassium phosphate buffer (pH 6.0) at room temperature for 30 min.. This sample also was frozen and lyophilized.

The prepared samples were sent to Dr. Stanley Stein, NJ. CABM, Waksman Institute of Microbiology, Piscataway, NJ. The amino acid composition of YNDase was determined in presence and absence of NEM by Clement Woghiren in this laboratory according to a published procedure (48). Figure 7 shows the amino acid elution profile of sample 2 via HPLC.

Figure 7. Amino acid composition of YNDase as determined by HPLC analysis



YNDase and NEM

XI. Specificity Study of Nicotinamide Deamidase

1. Deamidation study of nicotinamide mononucleotide to nicotinate mononucleotide by YNDase.

I wished to examine if nicotinamide deamidase in yeast catalyzes the following reaction.

$$N_mMN \implies N_aMN + NH_3 \qquad (10)$$

Thus, 2 ml of 1mM N_mMN (prepared with 50 mM potassium phosphate buffer, pH 7.0) was incubated with 2 µl of stock enzyme (26 milliunits) at 37°C. At 1, 5, 10, 20 min., 0.5 ml volumes of the incubation solution were transferred to a test tube, boiled and filtered for an HPLC assay.

2. N,N-diethylnicotinamide study

I examined N,N-diethylnicotinamide as a YNDase substrate. For this experiment, 2.5 ml of 1 mM of N,N-diethylnicotinamide (prepared with 50 mM potassium phospate buffer, pH 7.0) was incubated with 2 µl of stock enzyme (26 milliunits) at 37°C. At 0, 5, 10, 20, 30 min., 0.5 ml of incubation solution was transferred to a test tube, boiled and filtered for this HPLC assay.

ADP-ribosylation

I. Assay Procedures

A. HPLC Assay Procedure

The following HPLC assay conditions were used during our examination of the ADP ribosylation activity.

- elution buffer: 10% methanol with 30 mM potassium phosphate-buffer at pH 5.5,
- flow rate: 1.0 ml/min.,
- chart speed: 0.5 cm/min.,
- detection wavelength: 254 nm.

The same Waters HPLC instrument and μBondapak C_{18} column employed in the nicotinamide deamidase study were used for these experiments.

B. Radioactivity Assay Procedure

The radioactivity assays of ADP-ribose synthetase or transferase activities for both yeast and calf thymus were carried out under the same conditions as described in Reference 49, except that a Tracor Analytic scintillation counter was utilized.

II. Partial Purification of Enzymes

A. Purifications from Yeast

1. Autolysis

The procedures were the same as described in the PRTase purification study, except that 50 mM Tris-Cl (pH 8.0 at room temperature) containing 0.3 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol and 50 mM sodium bisulfite (buffer A) was used.

2. Ammonium sulfate fractionation

283.2 g ammonium sulfate was added to 0.6 L of yeast crude extract to make 70% protein cut. After 2 to 3 hrs of gentle stirring in the cold room (4°C), the solution was spun at 9,000 rpm in a GSA rotor for 30 min.. The supernatant was discarded and the precipitated protein was redissolved to a volume of 300 ml with buffer A and dialysed against 4 L of buffer A overnight.

3. Hydroxylapatite chromatography

30 ml of 70% ammonium sulfate fractionated solution was applied to a hydroxylapatite column pre-equilibrated by buffer A. The column was eluted by 250 ml of buffer A (pH 8.0), 80 ml buffer A containing 30 mM potassium phosphate (pH 8.0) and 300 ml of buffer A also containing 1 M potassium phosphate. The resulting protein profile is shown in Figure 28.

B. Purifications From Calf Thymus

The crude extract of calf thymus was obtained by the method of Ito et al (49).

III. Kinetic Study

A. ADP Ribosylation Study with HPLC

For each time point, 50 µl of 1 M Tris-Cl buffer (pH 8.0), 50 µl of 100 mM $MgCl_2$, 50 µl of 10 mM dithiothreitol, 25 µl of 20 mM NAD (1mM NAD of final

concentration), 50 μl of 1 mg/ml DNA, 50 μl of 1 mg/ml histone and either 225 μl of calf thymus extract or 70% ammonium sulfate fractionated yeast protein (or crude extract) were added to a test tube. These mixtures were incubated at 37 °C for 0, 5, 10, 20, 30 min. respectively. Thereafter, the samples were filtered and injected onto the HPLC.

B. ADP Ribosylation Study with Radioactivity

The standard reaction mixture, in a total volume of 0.1 ml contained 10 μl of 1 M Tris-Cl buffer (pH 8.0), 10 μl of 100 mM $MgCl_2$, 10 μl of 10 mM dithiothreitol, 5 μl of 20 mM NAD* (Adenine-2,8-H^3, 1.39 x 10^4 dpm/nM), 10 μl of 1 mg/ ml histone, 10 μl of 1 mg/ml DNA and 45 μl of protein solution. The reaction was carried out at room temperature for 0, 5, 10, 20, 50 min. respectively and terminated by the addition of 5 ml of 10% trichloroacetic acid (TCA). The solutions were then poured onto 25 mm #31 glass filters (Schleicher & Schuell), and washed with 5 ml of 5% TCA 3 times. The filters then were placed into vials and the radioactive contents were evaluated with the scintillation counter. This radioactive assay was used to detect poly(ADP-ribose) synthetase or ADP-ribose transferase.

C. HPLC Molecular Sieve Filtration Study

1. An approximate molecular weight determination of the NAD hydrolysis enzyme in yeast

A volume of 5 μl of yeast protein sample from hydroxylapatite purification step was injected onto a Bio-rad TSK 250 gel filtration column and then eluted with a buffer containing 0.1 M Na_2SO_4 and 0.02 M NaH_2PO_4 (pH 6.8). The fractions solution were collected at about 1 ml/tube. From each sample tube, 100 μl of solution was transferred to an assay solution (325 μl of 100 mM Tris-Cl buffer, 50

μl of 0.1 M $MgCl_2$, 25 μl of 20 mM NAD). The activity was detected with HPLC as described in "Assay Procedures" section. Five protein standards (bovine serum albumin, beta lactoglobulin, egg albumin, trypsinogen and lysozyme) were eluted under the same conditions.

2. Radioactivity labeling study of yeast protein by HPLC

A volume of 200 μl of the yeast protein solution derived from the hydroxylapatite purification step (or 70% ammonium sulfate fractionation) was incubated with 50 μl of 20 mM labeled NAD (1.39 x 10^4 dpm/nM), 100 μl of 0.1 M Tris-Cl buffer, 50 μl of 0.1 M $MgCl_2$, 50 μl of DNA (1 mg/ml) and 75 μl histone (1 mg/ml) at room temperature. At 0, 1, 3, 5 hrs, 5 μl of incubation solution was injected onto a Bio-rad TSK 250 gel filtration column, and eluted with a buffer containing 0.1 M Na_2SO_4 and 0.02 M NaH_2PO_4. The fractions were collected at 1 ml/vial. To each vial was added 4 ml of hydroflour scintillation liquid per vials and these vials were then counted.

RESULTS

Detection of N_aPRTase, N_mPRTase and QPRTase Activities by HPLC in Yeast

As shown in Figure 8, N_aPRTase as well as N_mPRTase and QPRTase activities were all observed in crude yeast extract by using reversed-phase HPLC. These three enzymes were monitored by including ATP in each of the assay solutions. As can be seen in Figure 8A, very little QPRTase activity was observed under these conditions, whereas considerable concentration of N_mMN and N_aMN were synthesized (Figure 8B, 8C; N_mMN's position was slightly behind the N_aMN peak) which indicated the existence of relatively large amounts of N_mPRTase and N_aPRTase in yeast. To our knowledge, this marks the first time that N_mPRTase and QPRTase have been detected in yeast. It was during this survey of the pyridine nucleotide productions, that I detected a PRibPP- and ATP-independent nicotinamide-to-nicotinate transition (Figure 9).

The protein extract that was observed to contain the three pyridine phosphoribosyltransferases was fractionated through the use of ammonium sulfate salting-out procedures. A redissolved 40-60% fraction was observed to contain both N_mPRTase and N_aPRTase activities but not to contain any detectable QPRTase. Moreover, I have observed that N_mPRTase is essentially independent of added ATP for activity (Figure 10), in contrast to the yeast N_aPRTase-catalyzed reaction which requires ATP as a substrate (50) and in contrast to the N_mPRTase activities from mammaliam sources, which require ATP as an allosteric effector (51).

Figure 8. Reverse phase HPLC assay procedure for monitoring pyridine phosphoribosyltransferases (PRTase) activities. The concentrations of the assay components in the incubation mixture were: 0.05 M Tris-phosphate buffer (pH 8.0), 1 mM $MgCl_2$, 1 mM ATP (when appropriate), 1 mM PRibPP, 1 mM nicotinamide or nicotinate or quinolinate and 100 μl of the protein extract. The final solution volume was 0.5 ml. (A) HPLC elution profile after incubation of the extract with quinolinate, (B) this profile after incubation with nicotinamide, (C) this profile after incubation with nicotinate. Elution conditions are as described in the "Methods" section. Elution times are in units of min.

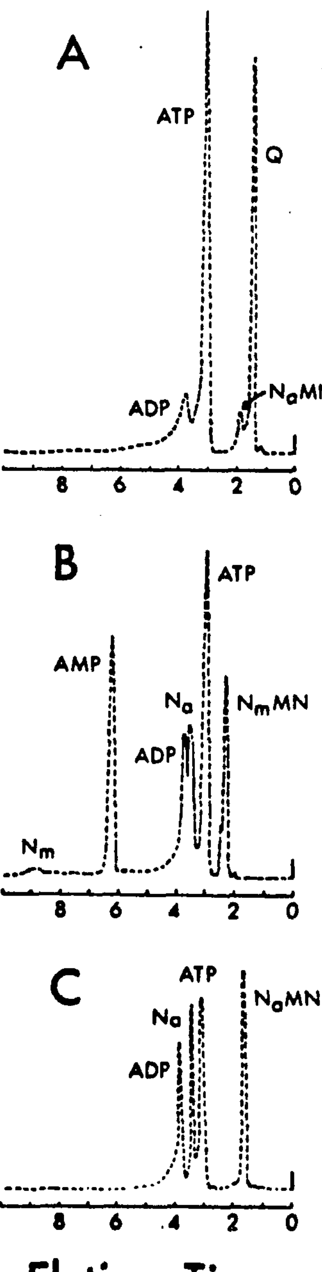
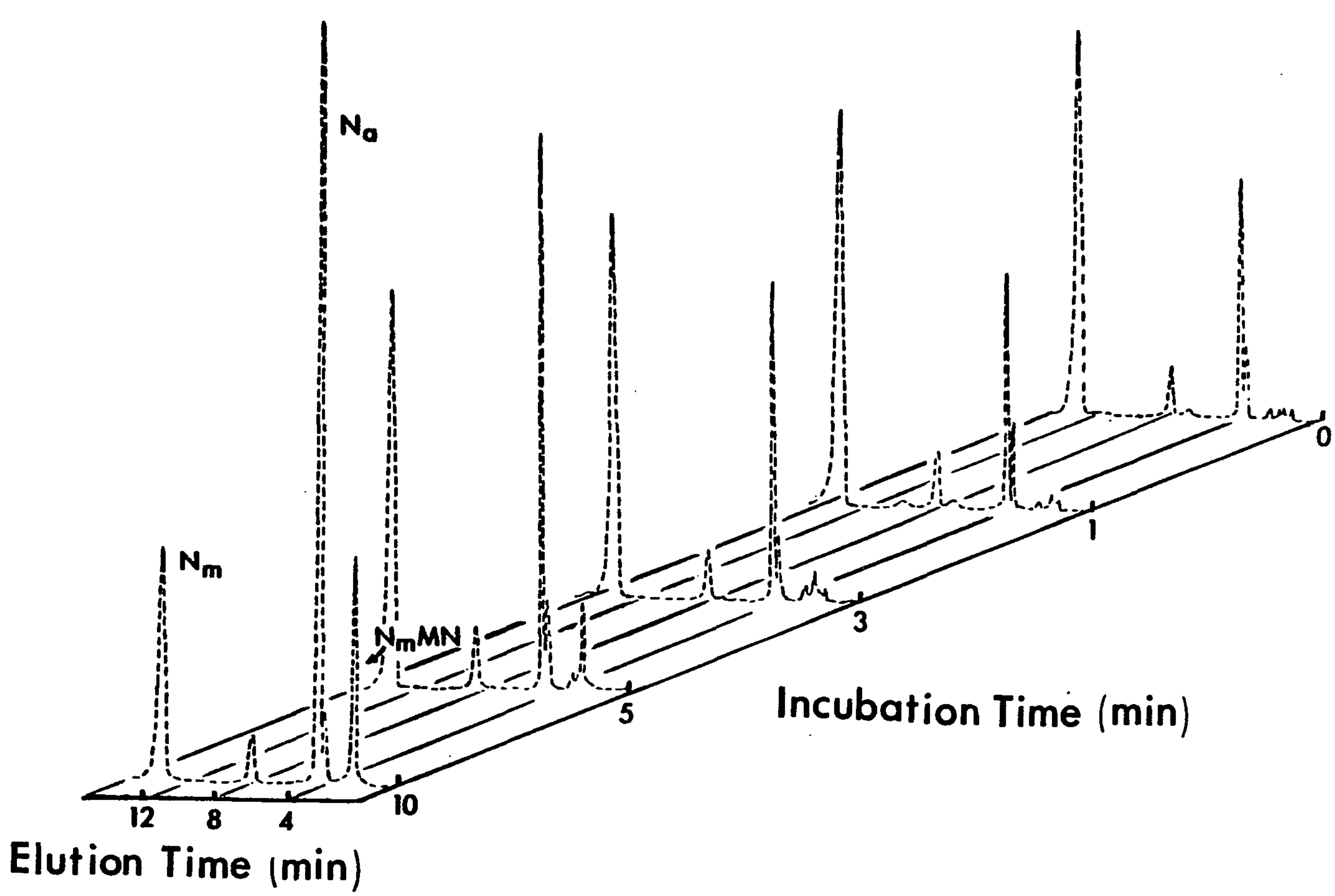
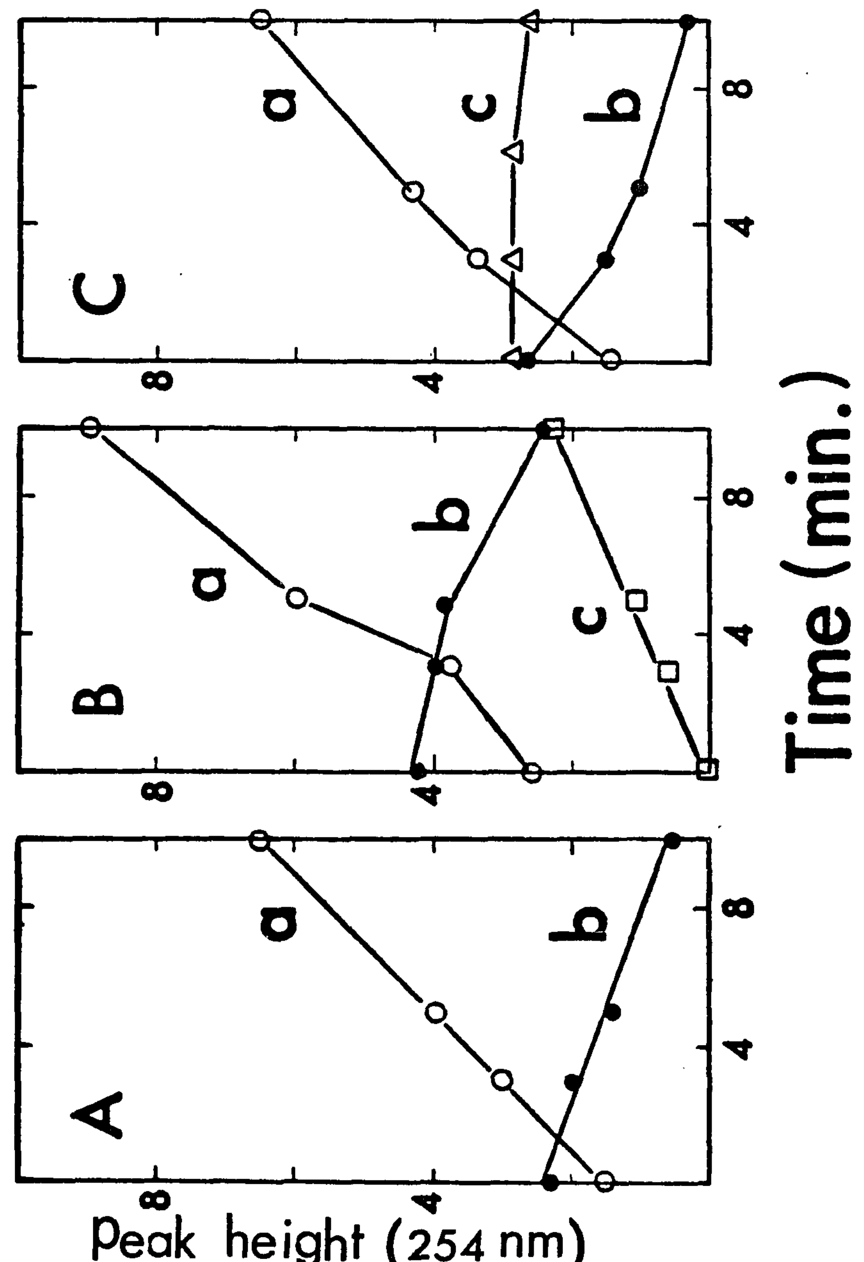

A
ATP
Q
ADP
N_aMN
8
6
4
2
0
B
ATP
AMP
N_a
N_mMN
ADP
N_m
8
6
4
2
0
C
ATP
N_aMN
N_a
ADP
8
6
4
2
0
Elution Time

Figure 9. Elution profiles over a 10-min time period of the incubation mixture defined in Figure 8, which included nicotinamide but not ATP. Elution conditions with a methanol-phosphate gradient are as described in the "Methods" section.



Na
Nm
NmMN
0
1
3
5
10
Incubation Time (min)
12
8
4
Elution Time (min)

Figure 10. Rate of appearances of nicotinate (a) and N_mMN (c) and rate of disappearance of nicotinamide (b). The assay conditions and incubation mixture were as described in Figure 8 except that: (A) PRibPP and ATP were not present, (B) 1 mM PRibPP was present but ATP was not present and (C) 1 mM ATP was present but PRibPP was not present.


A
B
C
a
b
c
8
4
Peak height (254 nm)
Time (min.)

Yeast Nicotinamide Deamidase

A. HPLC Assay and NH_3 Detection

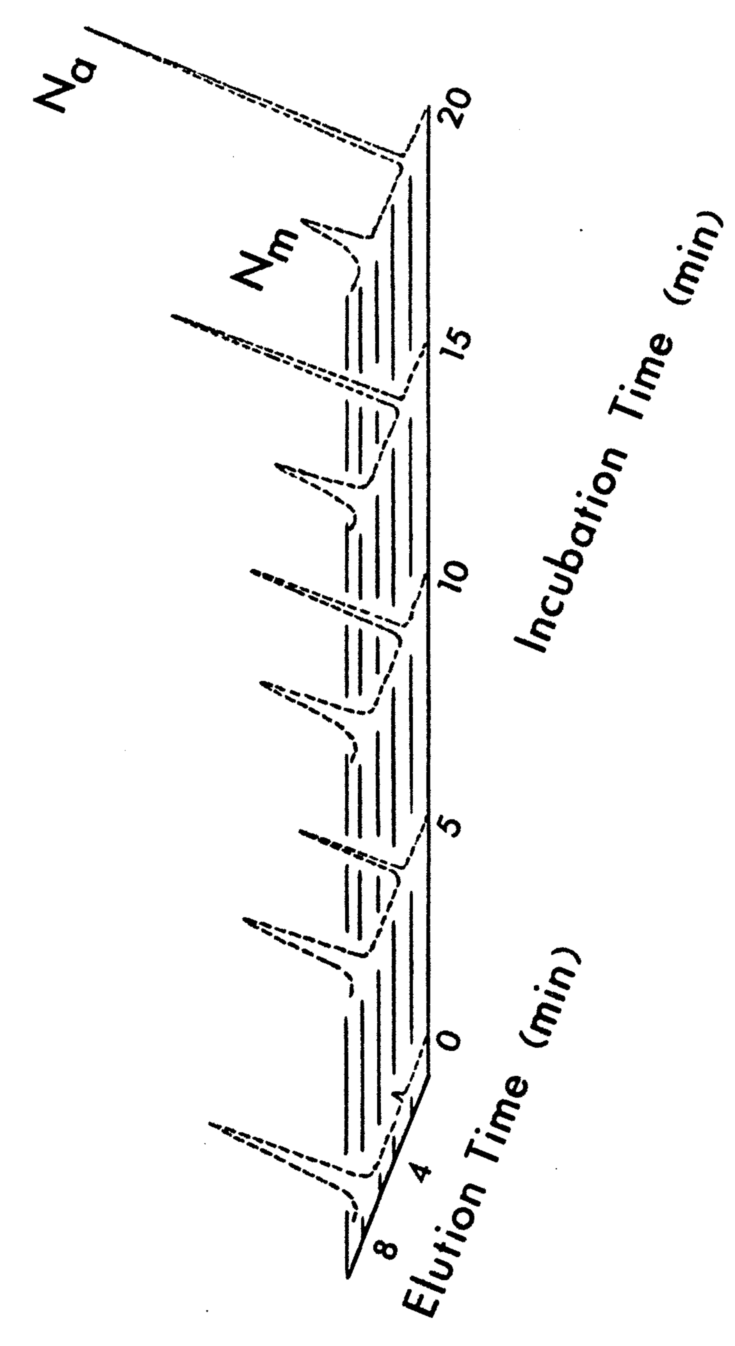
A standard profile of a HPLC elution of a mixture of nicotinate and nicotinamide by 10% methanol is shown in Figure 11. Two peaks are clearly separated. During the time course of incubation with YNDase, I monitored product (nicotinate) concentration increases and substrate (nicotinamide) concentration decreases. In our kinetic study, I converted the peak height to concentration by construction of a standard curve of peak height *versus* concentration. I also monitored the NH_3 generation during the YNDase incubation with nicotinamide.

B. Purification of Nicotinamide Deamidase

A summary of the six-step purification procedure of this enzyme from a baker's yeast extract is given in Table 2. This table lists the total protein, protein concentration, total activity, specific activity, yield and purification fold. At least, a 5000-fold purification was obtained. The purified YNDase from this procedure was homogeneous by the criteria of poly-acrylamide gel electrophoresis (Figure 12A) and SDS gel electrophoresis (Figure 12B).

From Table 2, we note the following. a). Without removing significant amounts of protein by HPLC gel filtration, the purification factor increased dramatically (about 35 fold). There are several possibilities that would explain this phenomenon. 1) The diluted form of YNDase is much more active than the concentrated aggregated form of YNDase; 2) An small molecular weight inhibitor had been removed. b). Even though the inclusion of Phenyl-Sepharose chromatography in the procedure, subsequent to the Phospho-cellulose step, did not improve the specific activity of the YNDase preparation, this step has been included in our recommended procedure,

Figure 11. HPLC profiles of the YNDase assay solution. The incubations were carried out under conditions described in the "Method" section. The assay solutions contained 4.7 ml of 50 mM phosphate (pH 7.0), 0.25 ml of 20 mM nicotinamide and 5 μl of YNDase (67 milliunits). At the appropriate time interval, an aliquot of solution was removed, filtered and injected onto the HPLC column under the following elution conditions: 0.1 sensitivity, 1 ml/min flow rate, 0.5 cm/min chart speed, 10% methanol elution buffer.



N_a
N_m
20
15
10
5
0
Incubation Time (min)
4
8
Elution Time (min)

TABLE 2: Summary of the Purification of Nicotinamide Deamidase from Yeast.

Procedure	Volume (ml)	Conc. (mg/ml)	Total mg X 10^3	Units* /ml	Sp. Act. (units/mg)	Total units	Yield (%)	Purification (factor)
40-70% ammonium sulfate	206	73	15	2.4	0.03	500	100	1
50% acetone	60	63	7	6.3	0.1	360	72	3
DEAE cellulose	800	0.5	0.4	0.3	0.6	240	49	20
Phospho-cellulose	17	1.1	0.2	4.6	4.2	78	16	140
Phenyl-Sepharose	4	2.8	0.1	13	4.8	53	11	160
HPLC gel filtration**	1	0.14	--	24	175	--	--	5800

* One unit of YNDase is the amount that catalyzes the formation of 1 μmole of nicotinate per minute under 37°C at pH 7.

** To date 10 samples (20 ul each) have been injected onto the Bio-rad TSK-250 column . The eluent that contained YNDase activity was concentrated to 1 ml.

because two protein contaminants remained with the preparation after HPLC gel filtration when an elution through Phenyl-Sepharose was not performed.

C. Molecular Weight Determination

Even though the gel electrophoresis, illustrated in Figure 12A in the "Purification" section, shows that the native form of YNDase is defined by a single band, this technique cannot tell us the precise molecular weight of this enzyme, because under nondenaturing conditions, the mobility of a protein molecule in PAGE depends on its molecular weight, shape and charge. But the molecular weight of a native protein can be determined with molecular sieve column chromatography. As shown in Figure 13, a YNDase elution profile, obtained by HPLC gel filtration, indicates that the molecular weight of YNDase is about 34,000, a relatively small protein similar to those found in prokaryotic organisms (17), but quiet different from those in eukaryotic organism, which lie within the range of 150,000-300,000 (14, 15, 52).

The SDS gel electrophoresis performed in the presence of mercaptoethanol shows a single protein band with a molecular weight of approximately 34,000, as determined through comparisons with proteins of known molecular weights (Figure 14, 15).

By comparing the molecular weight of YNDase under nondenaturing conditions with that of the denatured YNDase subunit, I conclude that YNDase is a monomeric protein with a molecular weight of 34,000.

D. pH Studies

As shown in Figure 16, YNDase is active over an unusually wide pH range and exibits optimum activity between pH 6.0-8.5. A significant loss of enzymatic activity was observed when YNDase was incubated for 10 min. at pH values outside the 4.5 and 9.0 range.

Figure 12. Gel electrophoresis of the homogeneous nicotinamide deamidase preparation. (A) Under non-denaturing conditions: 7.5% acrylamide resolving gel, 2.5 hr run at 4°C. To this gel was added 80 μl of final enzyme solution (9 μg) along with 1% bromophenol blue. (B) SDS-PAGE: 10% acrylamide resolving gel, 4.5 hr run at 15°C. Prior to the application of the sample, the enzyme was dissolved in a Tris-SDS buffer (pH 6.8) that contained glycerol, mercaptoethanol and 1% bromophenol blue.

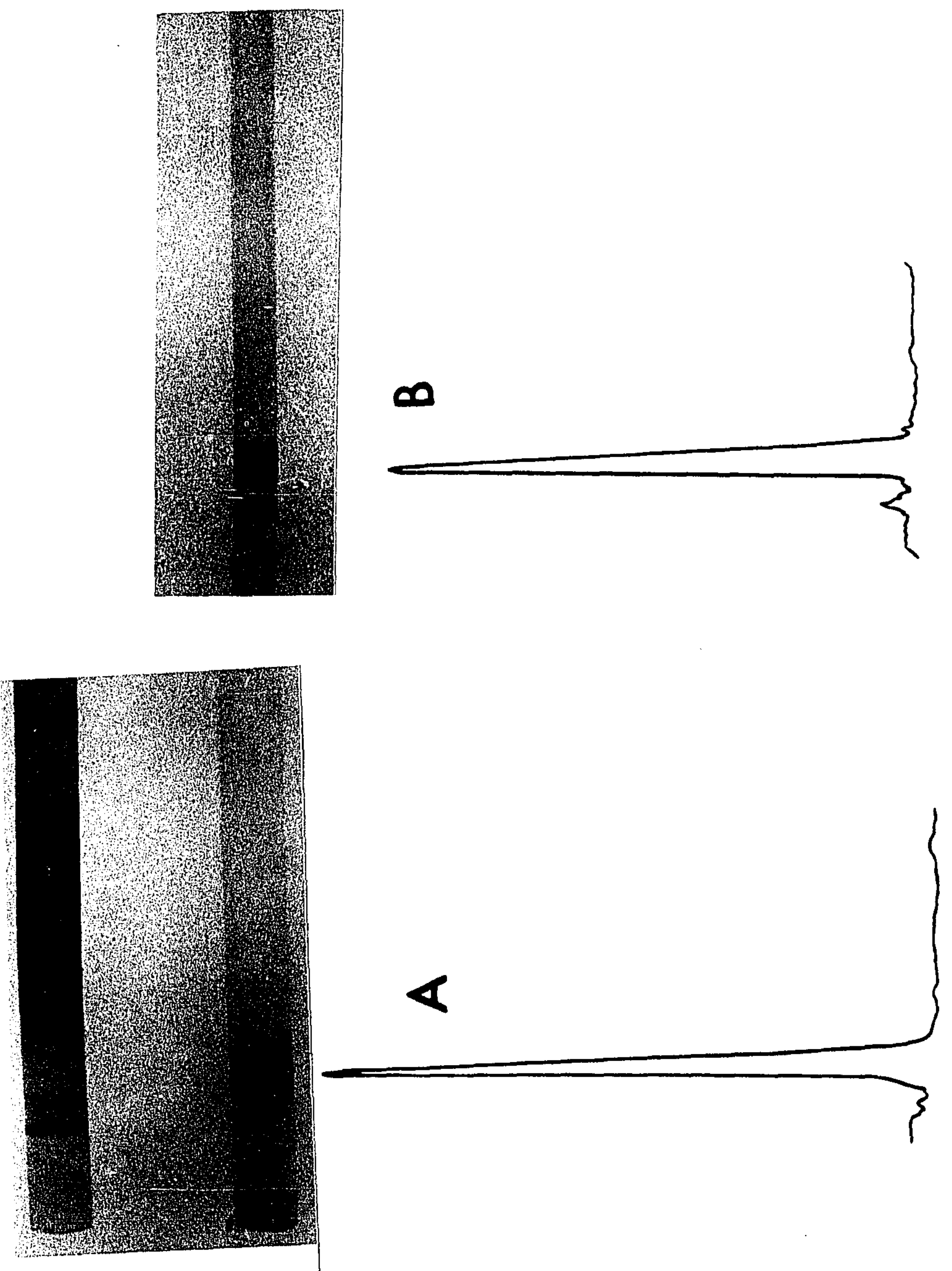
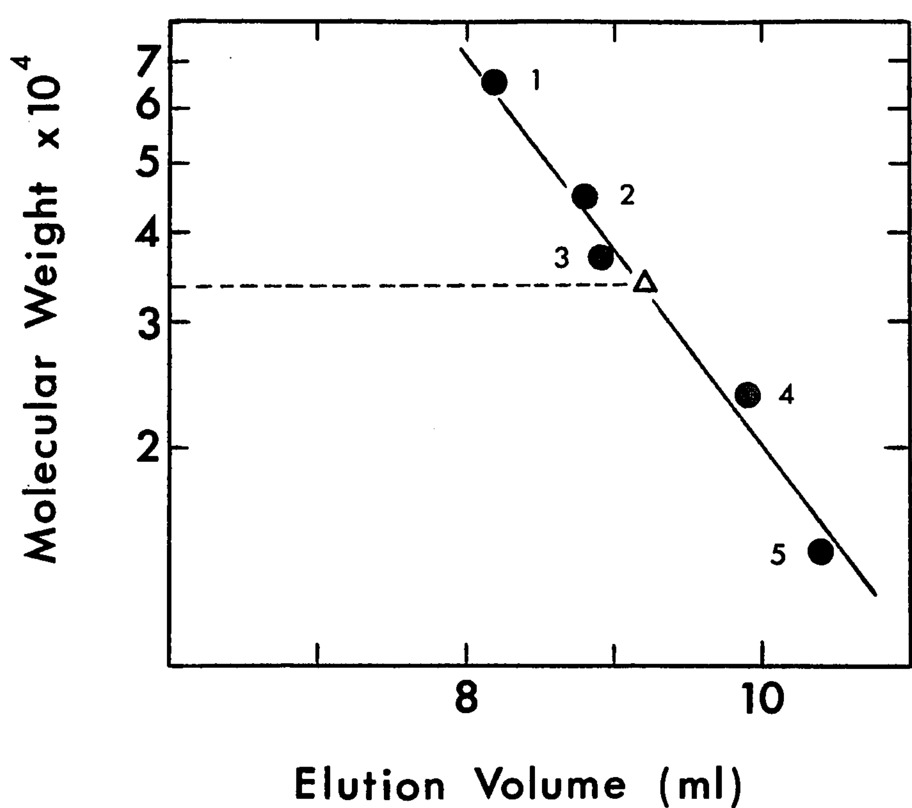

B
A

Figure 13. Molecular weight determinations of YNDase by HPLC. A plot of the molecular weights of standard proteins [1). bovine serum albumin (66,000), 2). egg albumin (45,000), 3). beta lactogobulin (36,800), 4). trypsinogen (24,000), 5). lysozyme (14,300)] and purified YNDase *versus* the volume required to elute them through a Bio-rad TSK-250 molecular sieve HPLC column. The conditions of the electrophoresis and HPLC experiments are as described in "Method" section.


Molecular Weight x 10^4
7
6
5
4
3
2
1
2
3
4
5
8
10
Elution Volume (ml)

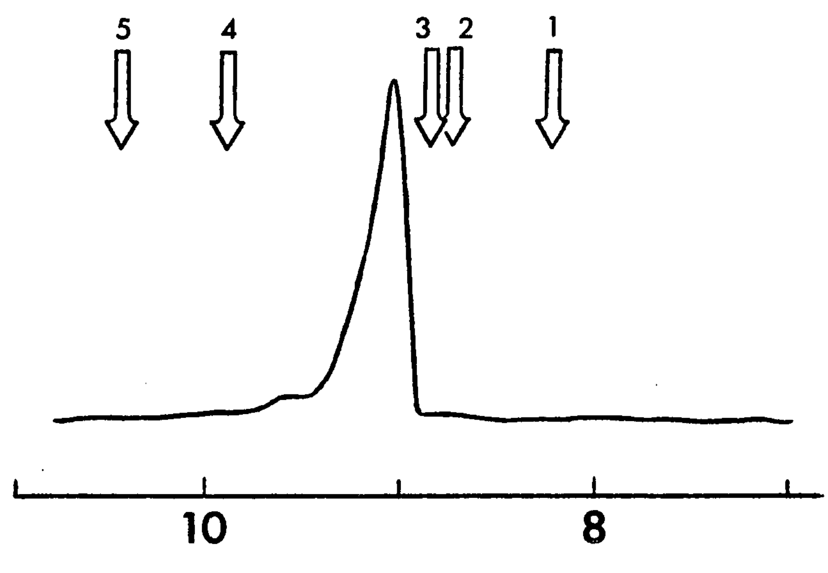
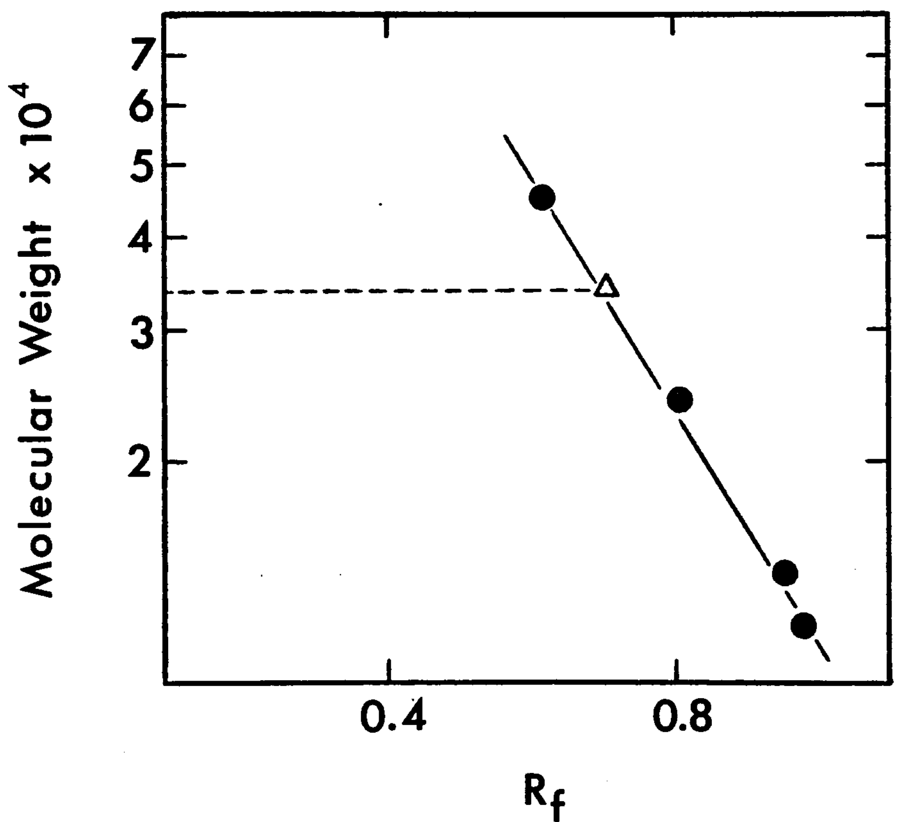

5
4
3
2
1
10
8
Elution Volume (ml)

Figure 14 and 15. Molecular weight determinations of YNDase by SDS-gel electrophoresis. A plot of the molecular weights of standard proteins [egg albumin (45,000), trypsinogen (24,000), beta lactoglobulin's subunit (18,400), lysozyme (14,300)] and purified YNDase *versus* their R_f values from gel electrophoresis runs of these proteins.


Molecular Weight x 10⁴
7
6
5
4
3
2
0.4
0.8
Rf

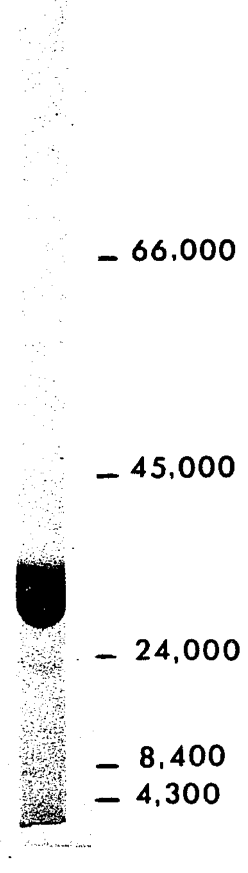
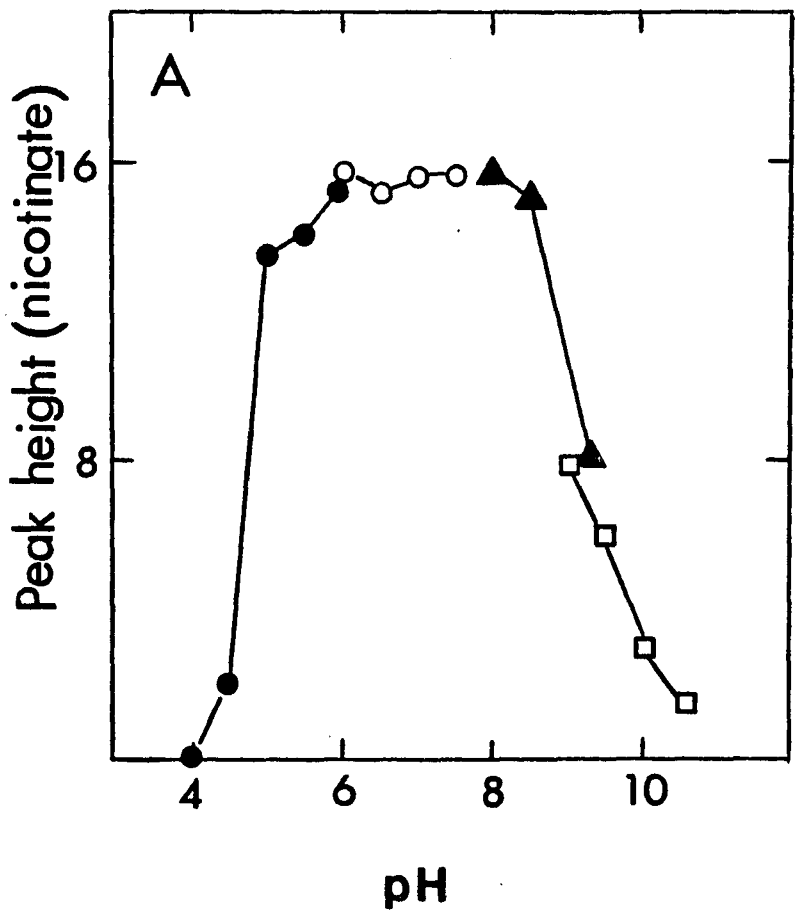

66,000
45,000
24,000
8,400
4,300

Figure 16. Effects of pH on the stability of YNDase catalyzed reaction. Enzyme activity *versus* pH of an incubation (10 min) mixture of YNDase and the appropriate buffer. These buffers were: 50 mM acetate (-●-), phosphate (-○-), Tris-Cl (-▲-), CHES (-□-). The enzymatic activity was measured at pH 7.0 with the standard procedure described in the text and Figure 11.


A
Peak height (nicotinate)
16
8
4
6
8
10
pH

E. Kinetic Analysis

Values for V_{max} and K_m for the YNDase catalyzed reaction, as determined from a double reciprocal plot of the velocity of nicotinate formation *versus* nicotinamide concentration, were determined over a pH range of 5-9 (Figure 17). A K_m value of 34 μM was calculated for nicotinamide over a 5.5-7.5 pH range , whereas an optimal V_{max} value equal to 1.58 $\pm$ 0.2 μmoles nicotinate formed per min. per liter was calculated. An enzyme pK value of 7.8 was obtained from Dixon plots (Figure 18A, 18B) of log V_{max} and log V_{max}/K_m *versus* pH based on the following equation (53), where the molecular constants K_a and K_b represent two proton dissociations defining a bell-shaped activity curve:

$$v = \frac{Vmax}{(1+[H^+]/K_a + K_b/[H^+]) + (K_m/[S])(1 + [H^+]/K_a + K_b/[H^+])} \qquad (11)$$

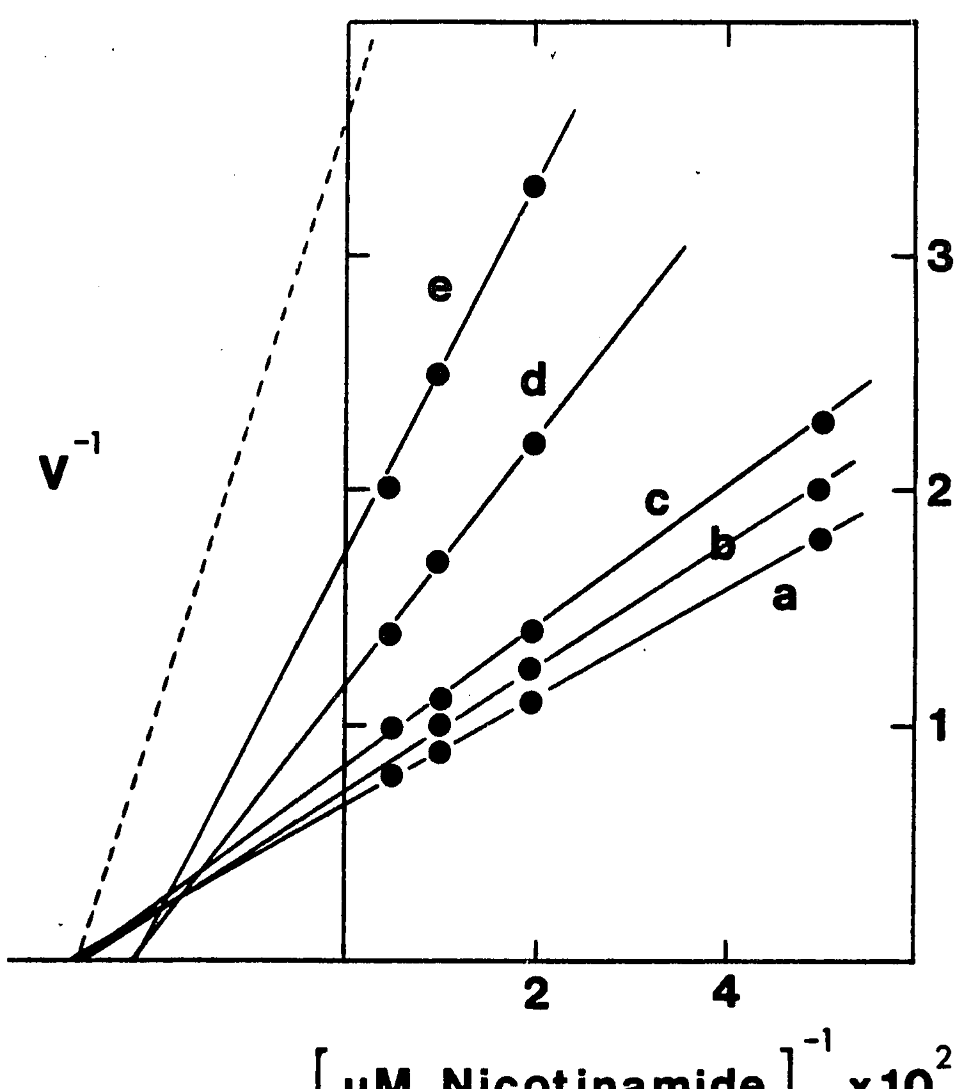
This result suggested that at least one essential protonated amino acid residue must be present at the YNDase active site. Since cysteine side chains are known to have pK_a values of 8.33, which is close to that of YNDase, I chose cysteine residues as our first candidate for a chemical modification study.

F. Chemical Modification

1. N-ethylmaleimide modification

Figure 19 shows the results of an incubation of NEM with YNDase. As shown in Figure 19A, a time-dependent and reagent concentration-dependent loss of enzyme activity takes place during these incubations at pH 6.0. Very small concentrations (as low as 1 μM) of NEM inactivate YNDase completely after 15 min.. Figure 19B and 19C show the protection of the enzyme activity against this inactivation by different

Figure 17. Double reciprocal plots of the initial velocity of nicotinate formation from the addition of 0.5 units (μmoles nicotinate formed/min) of YNDase activity *versus* nicotinamide concentration over a pH range: a) this line represents the data collected in phosphate buffer at pH 6.0, 6.5 and 7.0 and in acetate buffer at pH 5.5; b) data collected in acetate buffer at pH 5.0; c) data collected in phosphate buffer at pH 7.5; d) data collected in Tris buffer at pH 8.0. e) data collected in Tris buffer at pH 8.5. The data collected in CHES buffer at pH 9 is not shown but is defined by a dashed line with a y-intercept at 1/v = 3.7 and an x-intercept at 1/[S] = 3.3. The assay conditions were as described in Figure 11.



e
d
c
b
a
V⁻¹
3
2
1
2
4
[µM Nicotinamide]⁻¹ x10²

Figure 18. Dixon plot of log V_{max}/K_m and log V_{max} *versus* pH, as derived from the data shown in Figure 17.

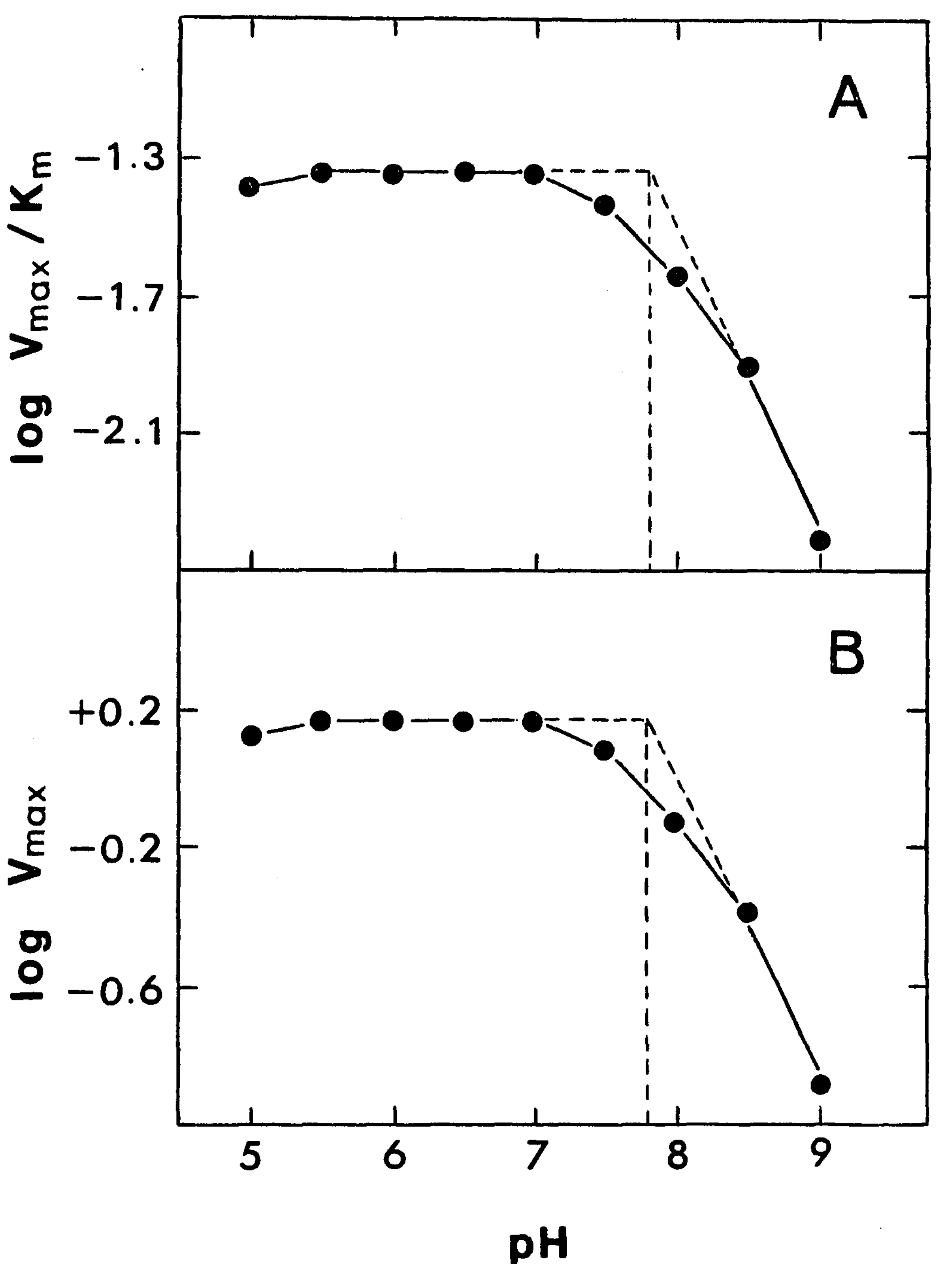
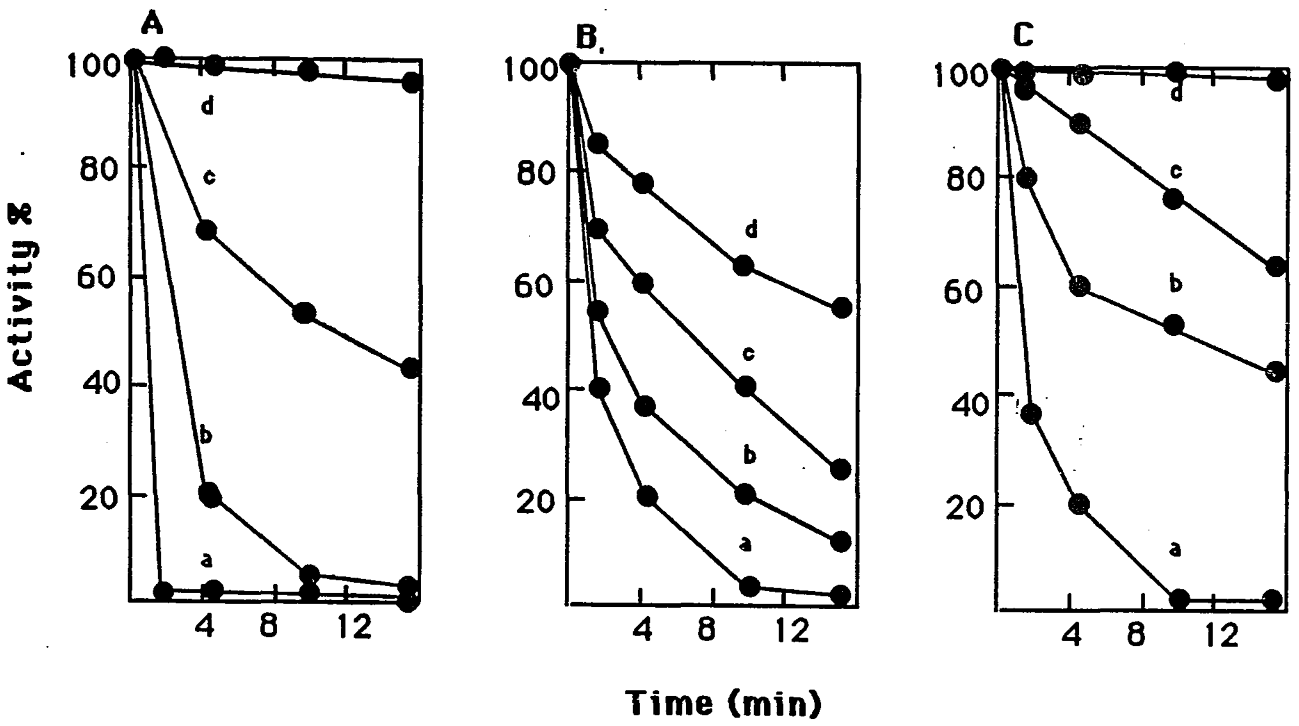

A
B
log Vmax / Km
-1.3
-1.7
-2.1
log Vmax
+0.2
-0.2
-0.6
5
6
7
8
9
pH

Figure 19. Incubations of nicotinamide deamidase with N-ethyl maleimide (NEM). (A) Incubations in the presence of the following concentrations of NEM: a) 10 μM, b) 1μM, c) 0.5 μM and d) 0.1 μM. (B) Incubations in the presence of a) 1 μM NEM and no substrate, b) 1 μM NEM and 10 μM nicotinamide, c) 1 μM NEM and 20 μM nicotinamide, d) 1 μM NEM and 40 μM nicotinamide. (C) Incubations in the presence of a) 1 μM NEM and no product, b) 1 μM NEM and 40 μM nicotinate, c) 1 μM NEM and 80 μM nicotinate, d) 1 μM NEM and 120 μM nicotinate.


A
B
C
Activity %
100
80
60
40
20
4
8
12
a
b
c
d
Time (min)

concentrations of nicotinamide and nicotinate. Interestingly only relatively low concentrations are needed since a covalent bond is formed between these reagents and the enzyme. Since NEM is known to react with cysteine residues preferentially over amino-containing residues by a factor of at least 1000 fold at pH 7.0 or lower (45), these results suggest that cysteine may be present at YNDase active site.

2. Diethylpyrocarbonate modification

The results of incubation of diethylpyrocarbonate (DEP) with YNDase are illustrated in Figure 20. As shown in Figure 20A, there occurs a time-dependent and DEP concentration-dependent loss of enzyme activity during these incubations. Semi-log plots of these results were nonlinear since DEP was decomposed rapidly during the time-course of these incubations. Relatively low concentrations (0.5 mM and 1mM) of nicotinamide serve to protect YNDase from inactivation by 3 mM DEP (Figure 20 B). Since DEP can react with several amino acid residues, these results suggest the presence of histidine, lysine, cysteine, arginine, or tyrosine residues at the active site of YNDase. Histidine and tyrosine modifications can usually be distinguished by the ability of hydroxylamine to reverse the inactivation (47), but in the case of YNDase, an incubation of the enzyme with hydroxylamine alone led to a complete loss of activity (Figure 21) and even after dialysis, the enzyme activity could not be recovered. So, we cannot state which specific amino acid residue at active site is reacting with DEP.

G. Analogue Study

Wolfenden and others (54-57) have demonstrated that aldehydic substrate analogues can bind tightly and reversibly at enzyme active sites where cysteine or serine residues are known to form covalent intermediates with amide-containing substrates. I therefore elected to examine the kinetic effects of nicotinaldehyde on the YNDase-catalyzed nicotinate formation. First of all, nicotinaldehyde does not serve as

Figure 20. Incubations of nicotinamide deamidase with diethyl pyrocarbonate (DEP). (A) Incubations in the presence of a) 0.5 mM DEP, b) 1 mM DEP and c) 3 mM DEP. (B) Incubations in the presence of a) 3 mM DEP plus 1 mM nicotinamide, b) 3 mM DEP plus 0.5 mM nicotinamide and c) 3 mM DEP. Assay conditions were as described in Figure 11.

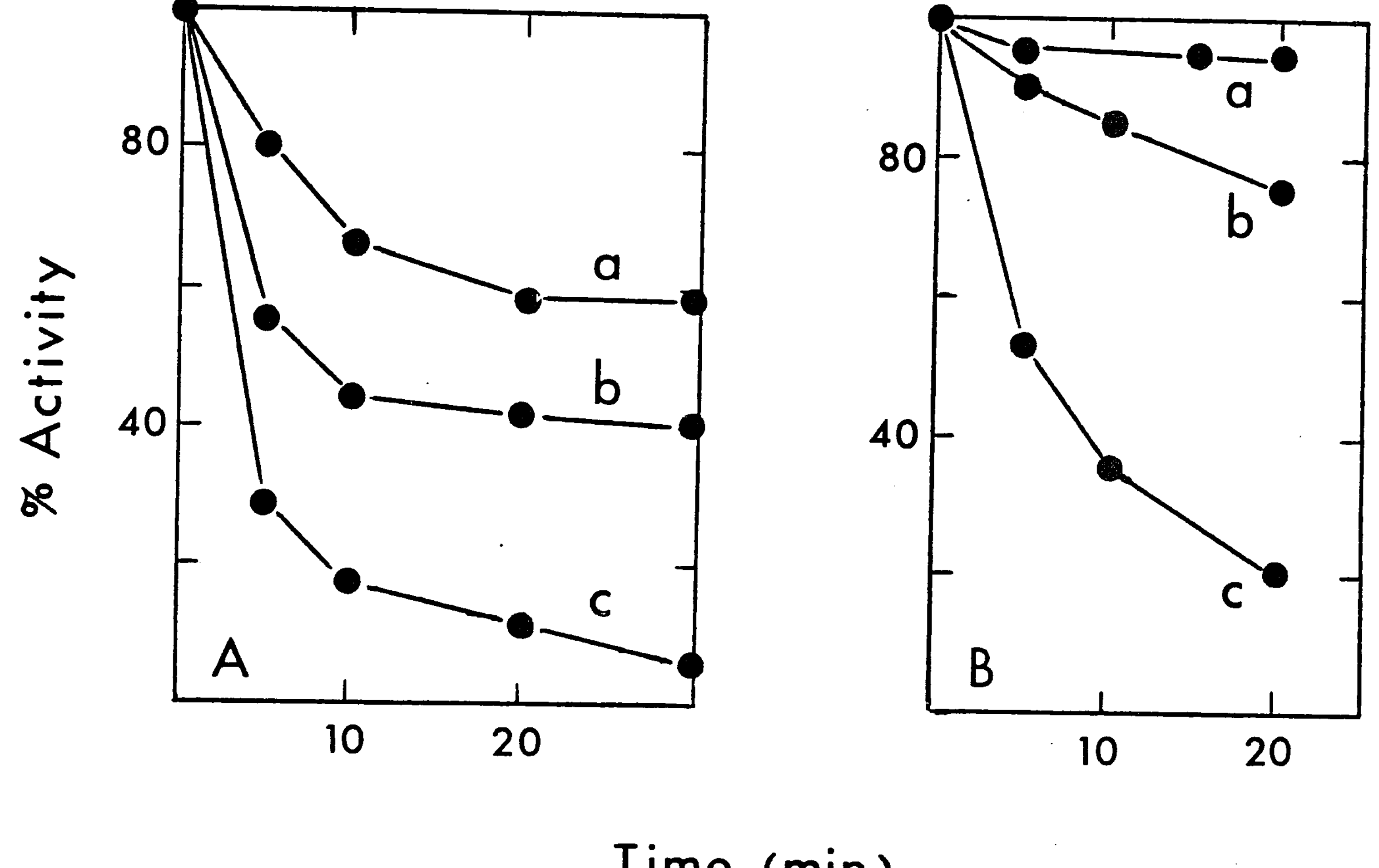
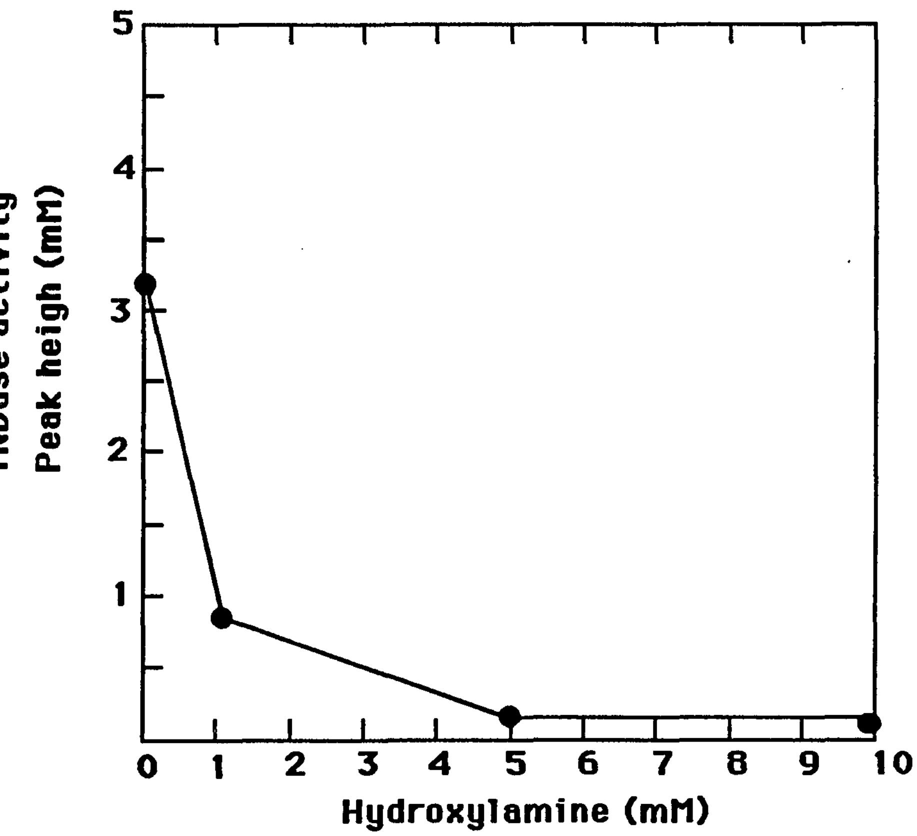

% Activity
80
40
a
b
c
A
10
20
80
40
a
b
c
B
10
20
Time (min)

Figure 21. Effects of the presence of different concentrations of hydroxylamine of YNDase of activity, monitored as defined in Figure 11.


YNDase activity
Peak heigh (mM)
0
1
2
3
4
5
6
7
8
9
10
Hydroxylamine (mM)

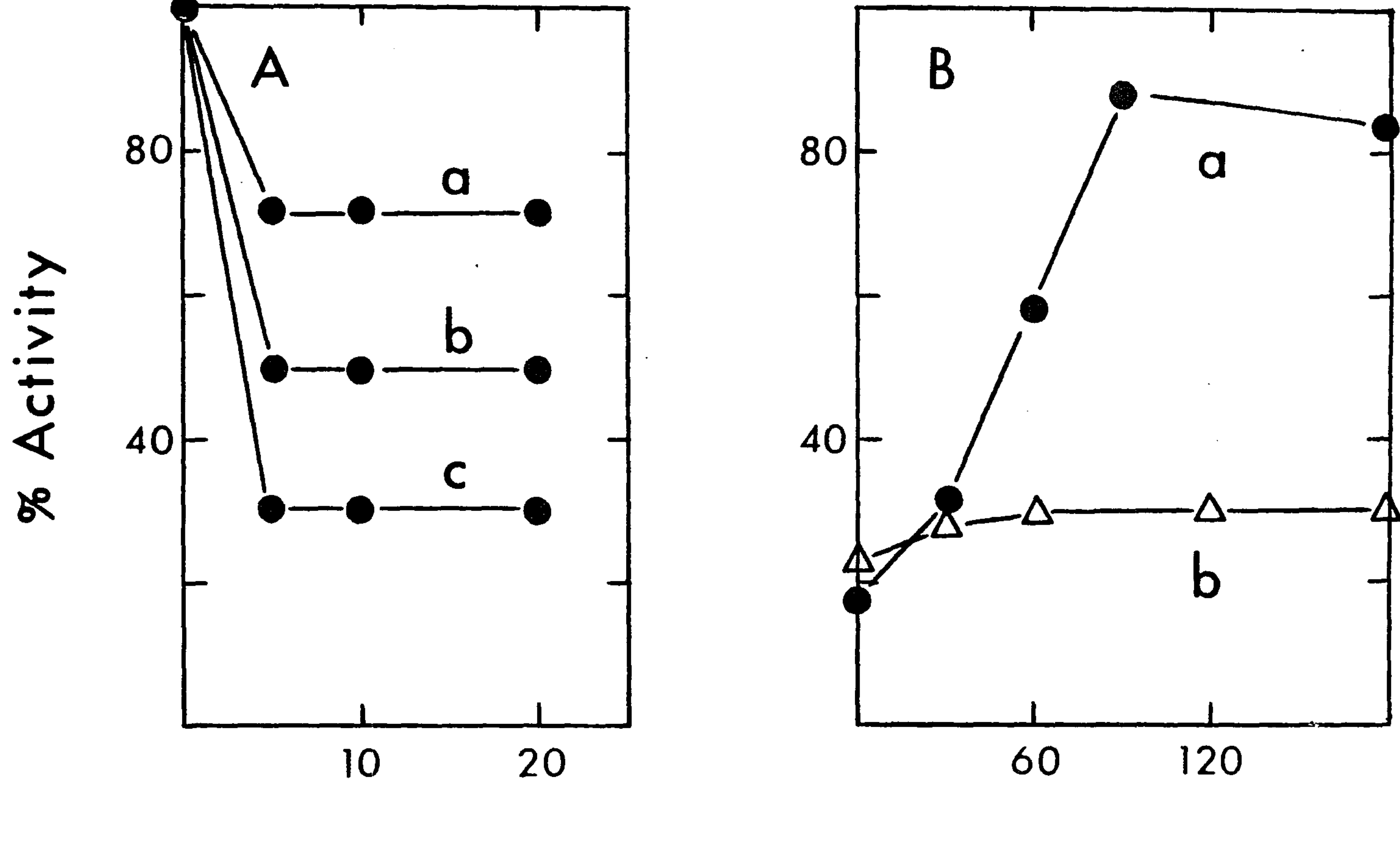
a substrate for YNDase, since during an incubation of nicotinaldehyde with YNDase, nicotinate formation was not detected.

Our inhibition and protection studies (Figure 22A) show that the loss of YNDase activity is not time-dependant in the presence of different nicotinaldehyde concentration, and that fairly high concentrations (20 mM) of nicotinamide are needed to protect against the loss of the enzyme activity by 2 mM nicotinaldehyde. These results suggest that the nicotinaldehyde is a reversible inhibitor possible acting through the formation of a Schiff base intermediate (55) between the enzyme and the aldehyde group of this substrate analogue. This suggestion was confirmed by the flow dialysis experiment (Figure 22B), during which the YNDase activity was nearly all recovered unless the incubation had been performed in the presence of a reducing agent, sodium borohydride. In a control experiment, it was shown that this irreversible loss of enzymatic activity was not due to the addition of the borohydride since a 30 min. incubation with this reagent led to no detectable inactivation of YNDase.

As shown in Figure 23A and 23B, nicotinaldehyde is an apparent noncompetitive inhibitor of nicotinate formation (aldehyde is a competitive inhibitor in the L-Asparaginase-catalyzed and pepain-catalyzed reaction, 56, 57), and is defined by an inhibition constant (68 μM) that is slightly larger than the K_m (34 μM).

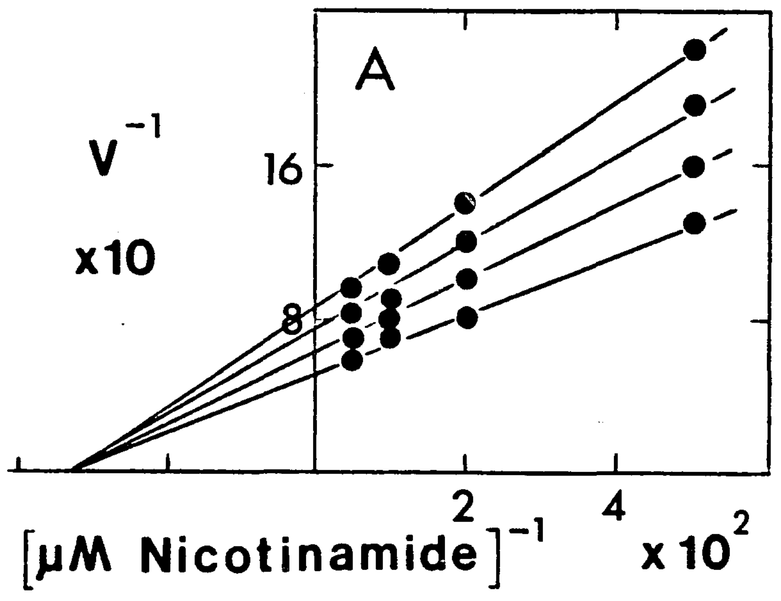
To distinguish further whether the Schiff base intermediate is formed at the active site or a secondary site, a so-called substrate labeling experiment was carried out, similar to those performed with acetoacetate decarboxylase and aldolase (58). I incubated the enzyme with nicotinamide and sodium borohydride under the same conditions as those used in the nicotinaldehyde incubations. I did not monitor any activity loss in this case. There are two possibilities here. First, no Schiff base was formed at the active site. Second, there is a Schiff base formation, but sodium borohydride could not enter the active site. If this is true, the situation should be the same for both nicotinamide and nicotinaldehyde. So, Schiff base formation between

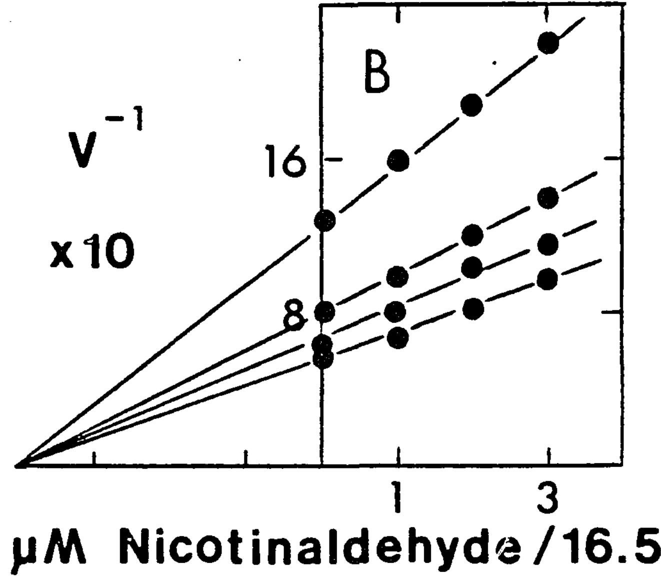
Figure 22. Incubations of nicotinamide deamidase with nicotinaldehyde. (A) Incubations in the presence of a) 2 mM nicotinaldehyde and 20 mM nicotinamide, b) 2 mM nicotinaldehyde and c) 10 mM nicotinaldehyde. (B) Reactivation of the enzyme through flow dialysis, subsequent to incubations with a) 10 mM nicotinaldehyde and b) 10 mM nicotinaldehyde plus 20 mM sodium borohydride. Assay conditions were as described in Figure 11.



Time (min)

Figure 23. Kinetic analysis of the effect of nicotinaldehyde on the YNDase catalyzed formation of nicotinate. (A) Double reciprocal plot of the initial velocity (µmoles nicotinate formed per min) *versus* nicotinamide concentration, and the following series of nicotinaldehyde concentrations: no addition (bottom line), 16.5 µM, 33µM and 49.6 µM (top line). (B) Plot of the reciprocal velocity *versus* nicotinaldehyde concentration. The concentrations of nicotinamide employed were: 200 µM (bottom line), 100 µM, 50 µM and 20 µM (top line). The assay conditions were as described in Figure 11.


A
V⁻¹
x10
16
8
2
4
[μM Nicotinamide]⁻¹ x10²


B
V⁻¹
x10
16
8
1
3
μM Nicotinaldehyde/16.5

the enzyme and nicotinaldehyde is not likely to occur at the active site but is not excluded at a secondary site. This is consistent with the noncompetitive pattern in the kinetic study, that is, nicotinaldehyde alters the YNDase activity by reacting at a secondary site that contains a reactive lysine residue (or an N-terminal amino group) on it. This site may be located near to the active site.

H. A Composition Study

As shown in Table 3, all of the YNDase cysteine residues are apparently modified by NEM and appear in the HPLC elution profile as S-2-succinyl cysteine, just prior to the elution of valine.

I. Specificity Study

Our study showed that YNDase in yeast cannot catalyse the conversion of N_mMN to N_aMN nor hydrolyse the N, N-diethylnicotinamide. It is therefore a relatively specific enzyme.

ADP Ribosylation

A. HPLC Assay Design

In order to monitor the NAD utilization by poly(ADP-ribose) synthetase, or ADP-ribose transferase or NAD glycohydrolase, I designed a HPLC assay procedure to supplement our radioactive assay. The advantage of this HPLC procedure is that we can monitor different peaks simultaneously. As shown in Figure 24, I obtained a very good separation of NAD, ADPR, nicotinate, nicotinamide and AMP peaks by using μBondapak C_{18} column. The experimental conditions are as described in "Methods".

Table 3: Amino Acid Composition of Nicotinamide Deamidase from Yeast Before and After Chemical Modification by N-Ethyl Maleimide.

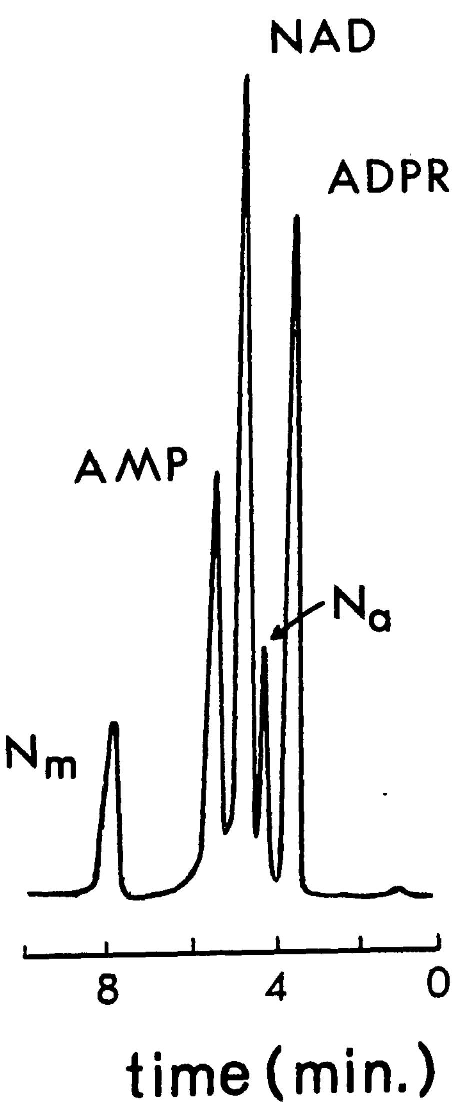
Amino Acid	Number of Residues (enzyme)	Number of Residues (NEM + enzyme)
Asx	44 ± 4	43 ± 3
Ser	12 ± 2	9 ± 1
Gly	17 ± 2	15 ± 2
Glx	27 ± 3	25 ± 3
Thr	17 ± 2	14 ± 1
Ala	17 ± 1	16 ± 1
Val	25 ± 3	23 ± 2
Met	6 ± 1	6 ± 1
Tyr	14 ± 2	13 ± 2
Ile	20 ± 1	21 ± 1
Leu	23 ± 3	22 ± 2
Phe	8 ± 1	8 ± 1
His	16 ± 1	17 ± 1
Trp	1	1
Lys	24 ± 3	28 ± 3
Arg	14 ± 2	12 ± 1
(Pro)	--	--
Cys	6 ± 1[a]	--
Succ-Cys	--	7 ± 3[b]
Calc. MW[c]	34,227	33,187

a). Total cysteine was determined separately by performic acid oxidation to cysteic acid.

b). Total NEM-modified cysteine was estimated from the area of an HPLC elution peak of S-2-succinyl cysteine.

c). These values were calculated by assuming that 10 proline residues are present in the YNDase structure.

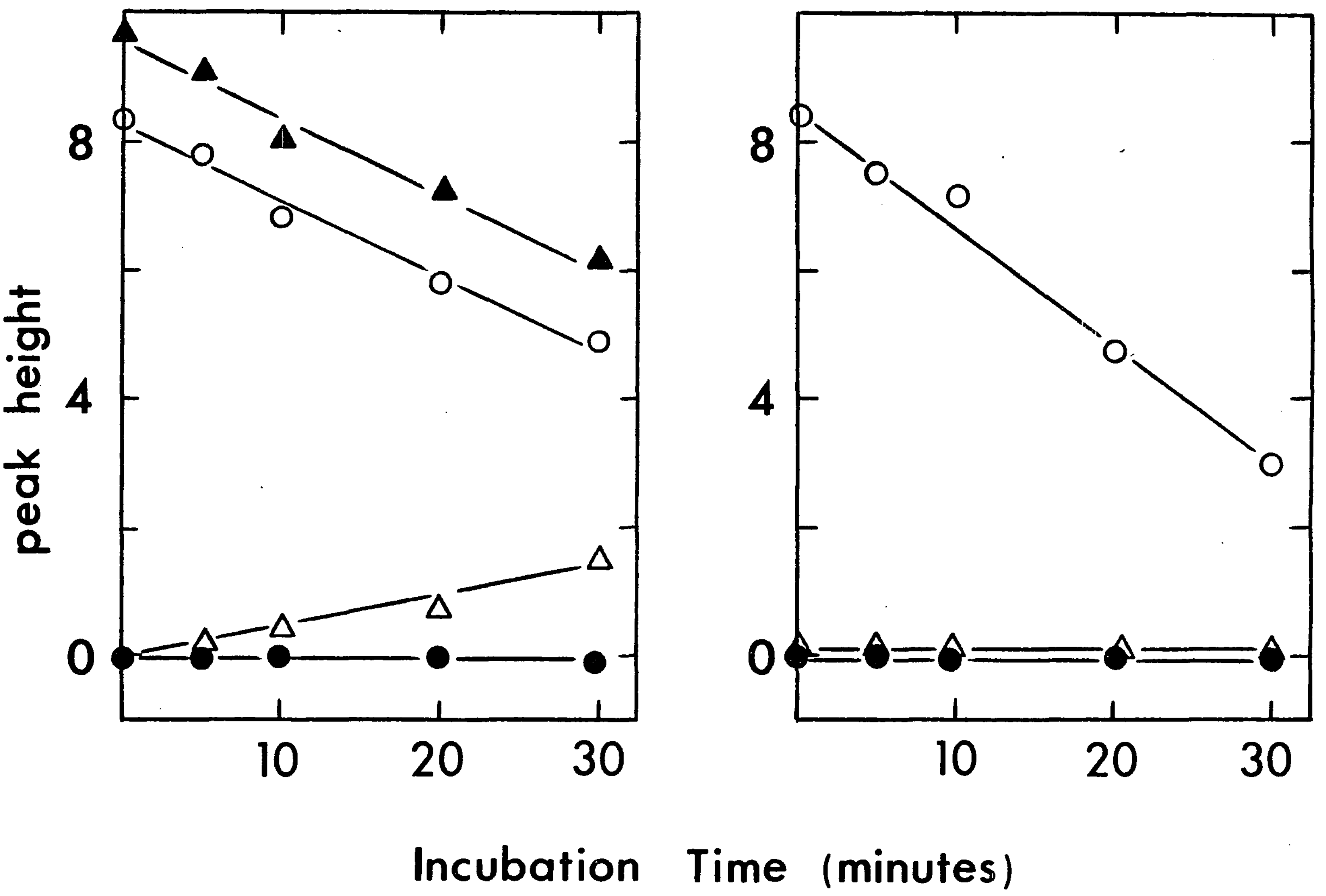
Figure 24. HPLC elution profile of a solution of nicotinamide, AMP, NAD, nicotinate and ADP-ribose.


NAD
ADPR
AMP
Na
Nm
8
4
0
time (min.)

B. NAD Utilization and ADPR Generation Study

As I mentioned in the "Introduction", there are three major enzymes which can hydrolyze NAD to form ADPR and nicotinamide, namely NAD glycohydrolase which generates free ADPR and nicotinamide, ADP-ribosyl transferase which can hydrolyze the NAD and transfer the ADP-ribose moiety to an acceptor, and poly(ADP-ribose) synthetase which can hydrolyze the NAD, transfer the ADP-ribose moiety to an acceptor and synthesize a poly(ADP-ribose). As shown in Figure 25A, NAD peaks decreased at the same velocity in the absence or presence of DNA and histones. When I incubated NAD with the yeast protein extract, ADPR remained at the same level but the AMP peak increased. Figure 25B shows that, in calf thymus tissue, only NAD was utilized in the presence of DNA and histone and that no free ADPR nor AMP was formed in this case. This is because in calf thymus, poly(ADP-ribose) synthetase used almost all the ADPR to modify target proteins. Our radioactive assay showed that, in the case of calf thymus (Figure 26B), labeled ADPR was indeed incorporated into target proteins, whereas no ADPR incorporation occured in yeast (Figure 26A). These results suggest that the increase of AMP concentration in Figure 25A may be due to the degradation of ADPR by other enzymes (ADPR phosphodiesterase). This suggestion is proven by results shown in Figure 27A and 27B. When I incubated ADPR with the yeast enzyme preparation, ADPR was utilized and AMP was formed. The hydrolysis of NAD and ADPR could be performed by one enzyme complex or by two separate enzymes in yeast. Figure 28 shows that through the hydroxylapatite column chromatography, two activities could be separated, indicating that two separate enzymes perform these different functions. Thus, it seems that, in yeast, abundant NAD glycohydrolase activity exists which hydrolyses NAD to nicotinamide, and ADPR that is further hydrolysed by a phosphodiesterase to generate AMP.

Figure 25. Changes in the concentration of NAD [in the presence (-○-) and absence (-▲-) of calf thymus DNA and calf thymus histones], AMP (-△-), and ADPR (-●-). The data on the left was determined for a yeast extract, whereas the data on the right was generated for an extract of calf thymus.



peak height
8
4
0
10
20
30
8
4
0
10
20
30
Incubation Time (minutes)

Figure 26. Rate of appearance of labeled proteins after incubation of either a yeast extract (A) or a calf thymus extract (B) with tritiated-NAD, 1 M Tris-Cl (pH 8.0), 100 mM Mg(II), 10 mM dithiothreitol, and an aliquot of calf thymus DNA and histones.

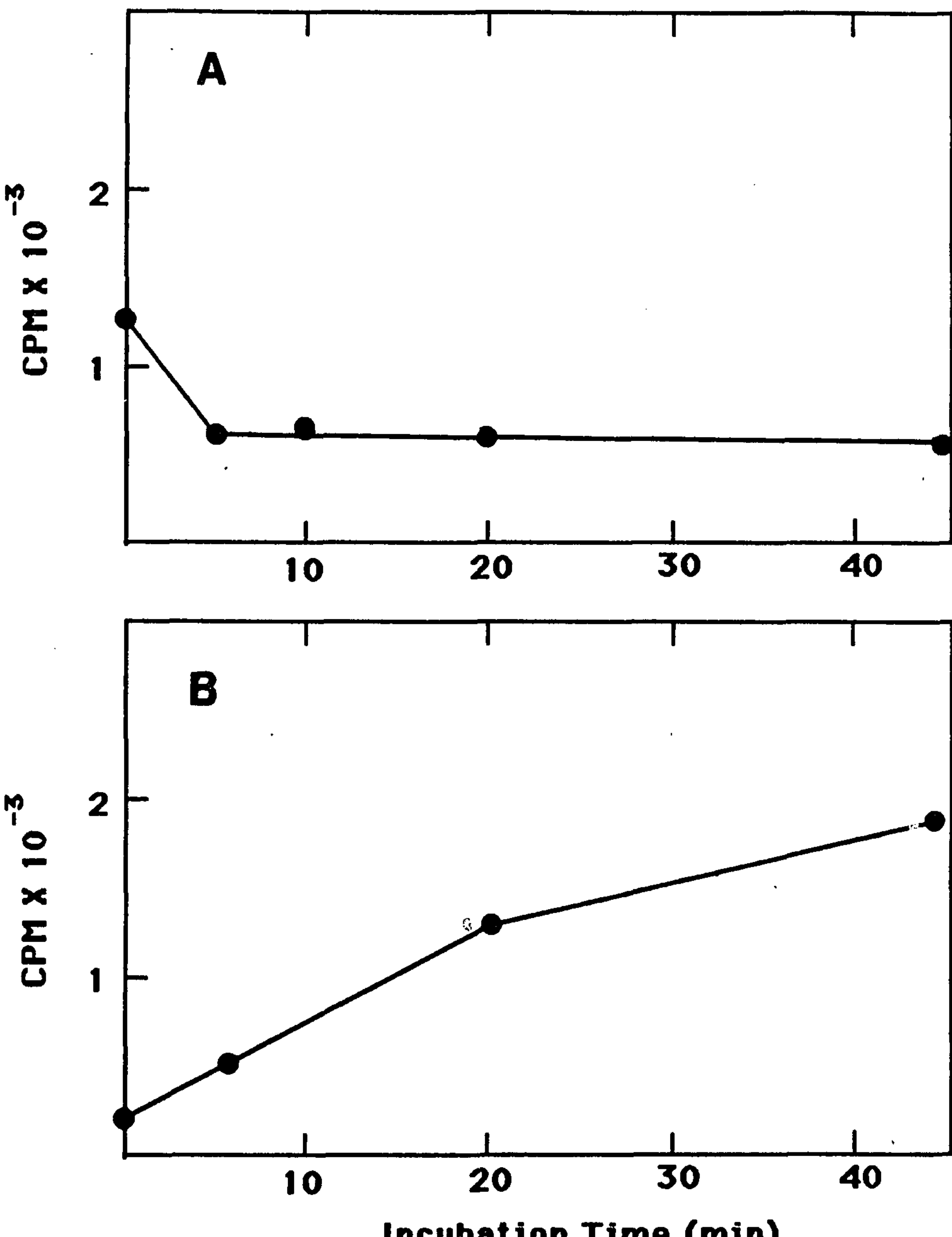

A
B
CPM x 10^{-3}
2
1
10
20
30
40
Incubation Time (min)

Figure 27. Assay procedures that define the presence of ADPR phosphodiesterase in incubation mixtures with (-●-) and without (-■-) the addition of an aliquot of yeast extract.

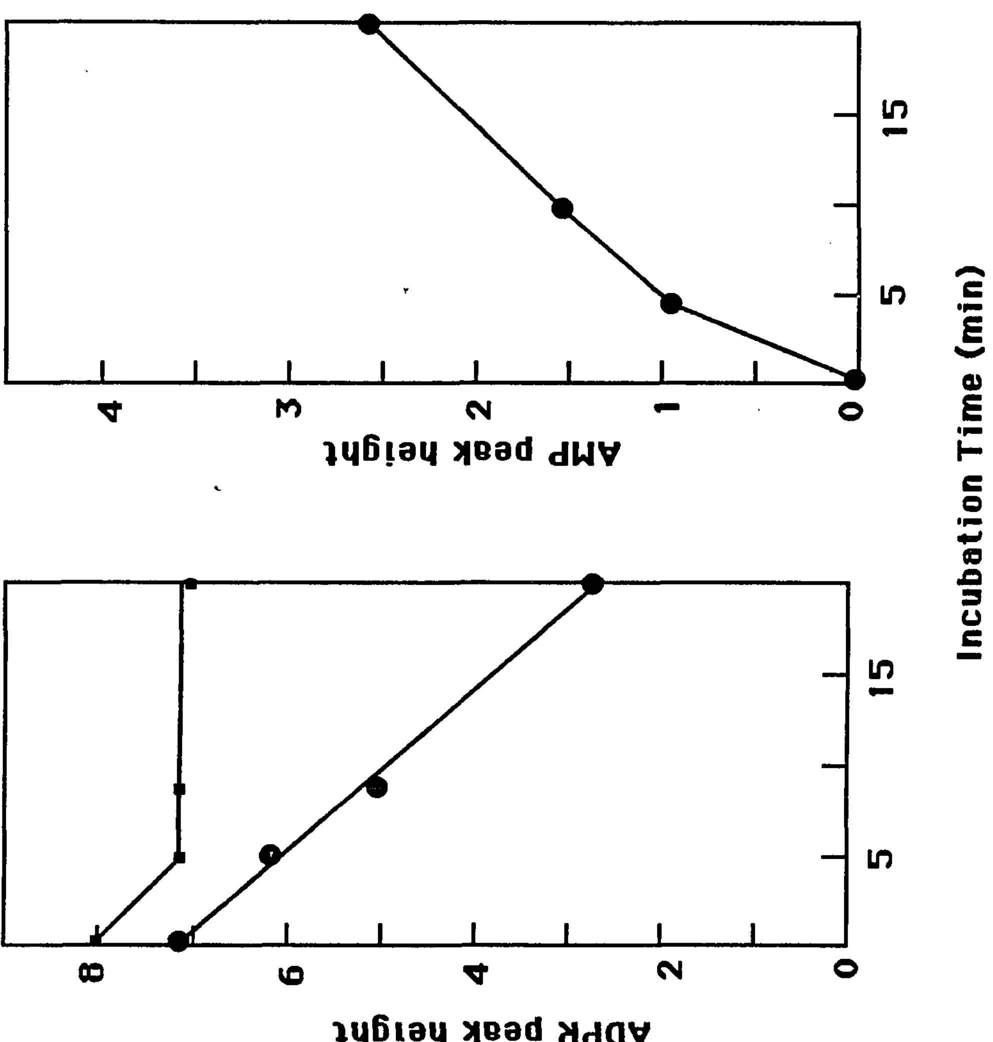

ADPR peak height
0
2
4
6
8
5
15
AMP peak height
0
1
2
3
4
5
15
Incubation Time (min)

Figure 28. Profiles of the elution of enzymatic activities of NAD and ADP-ribose degradation in yeast through hydroxylapatite chromatography. The column was eluted by 250 ml of buffer A (pH 8.0), 80 ml buffer A with 30 mM potassium phosphate and 300 ml of buffer A with 1 M potassium phosphate.

Buffer A: 0.3 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol and 50 mM sodium bisulfite.

Protein

NAD activity

ADPR activity

NAD and ADPR hydrolysis activities

0 1 2 3 4 5 6

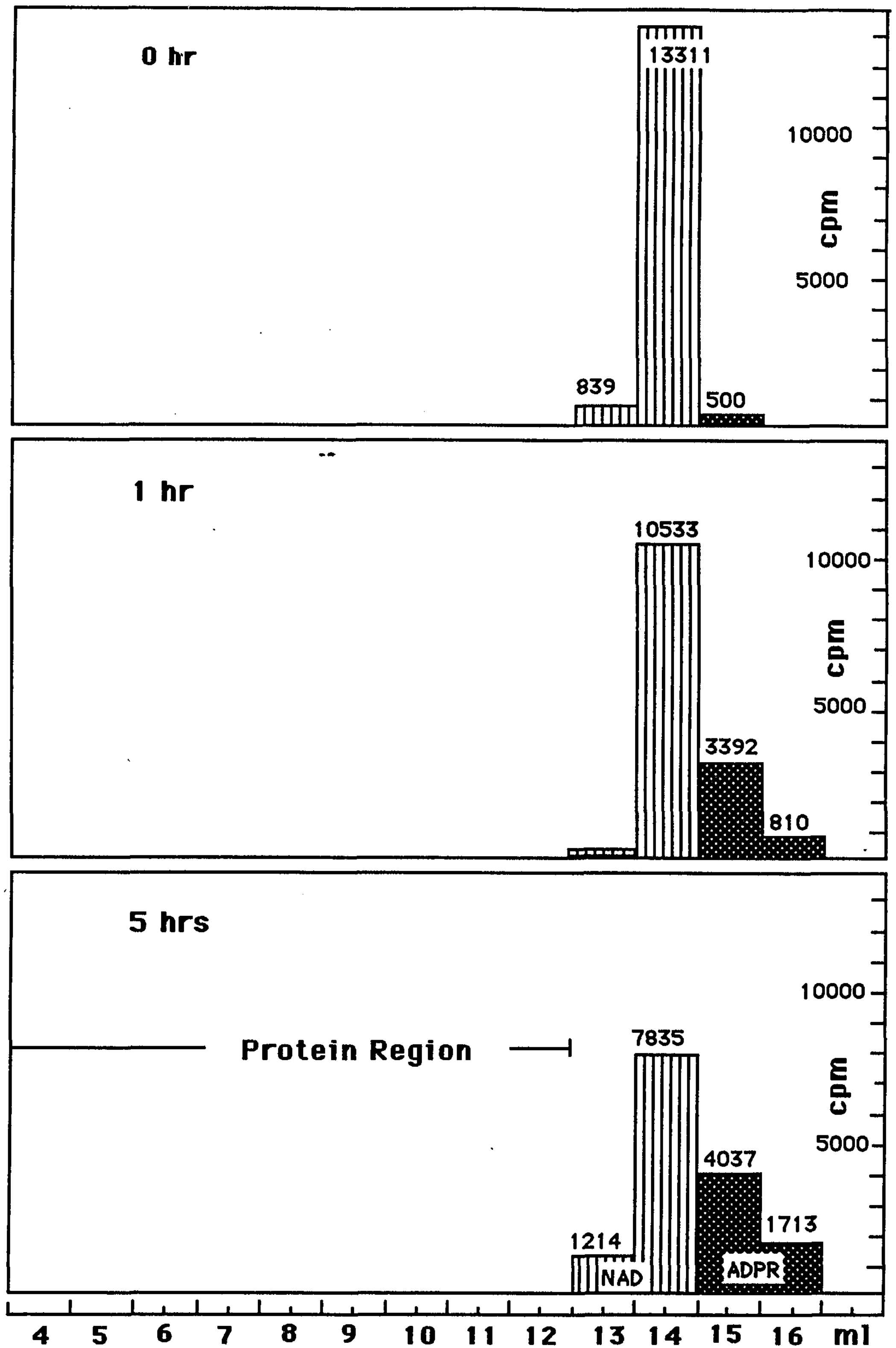
0 10 20 30 40 50 60 70

Fraction

C. Relative M. W. Determination of NAD Glycohydrolase in Yeast

The protein elution peak of HPLC gel filtration where NAD is hydrolyzed during the incubation, is located at around molecular weight of 300,000 (the separation range of TSK 250 column is 1,000-300,000, so the molecular weight of NAD glycohydrolase may be larger than 300,000). Figure 29 is another HPLC gel filtration experiment which shows again that NAD glycohydrolase may exist in yeast, because free labeled ADPR is formed, but no protein incorporation has occured. This result agrees with that defined in Figure 26A.

Figure 29. Radioactive labeling study of yeast crude protein by tritiated NAD using a Bio-rad TSK 250 HPLC gel filtration column. The condition is described in "Method" section.


0 hr
13311
839
500
10000
5000
cpm
1 hr
10533
3392
810
10000
5000
cpm
5 hrs
Protein Region
7835
4037
1713
1214
NAD
ADPR
10000
5000
cpm
4 5 6 7 8 9 10 11 12 13 14 15 16 ml

DISCUSSION

As described in the "Introduction", there are at least three pathways through which living organisms can synthesize NAD. In yeast, both *de novo* and salvage pathways exist and I was the first to detect the QPRTase and N_mPRTase activities in yeast. It seems that yeast cells depend on salvage pathways much more than the *de novo* pathway, because only very little QPRTase activity was detected in comparison with the tremendous N_aPRTase and N_mPRTase activities that were found. This could be interpreted as follows. In the evolutionary course of events, yeast is the simplest eukaryotic single cell and it cannot do everything. In order to save limited energy and material, this organism tries to use the materials which already exist rather than synthesize them. In highly developed eukaryotic organisms (mammalian cells), different organs are highly differentiated and interrelated so that the system can work more efficiently. They try to synthesize their own materials in order to be independant from the outside environment. Thus, significant QPRTase activity exists in these tissue (kidney and liver), and mammals can therefore utilize tryptophan resulting from the turnover of proteins (conditions of starvation) to produce the important pyridine-containing nucleotides. However this ability is limited, and so niacin is still a dietary requirement for most animals. In contrast to this metabolism in mammals, tryptophan degradation in bacteria is directed primarily towards indole formation. Further experimentation is required, to determine if NAD metabolism in yeast most resembles mammalian or bacterial tissues.

As part of this investigation of pyridine nucleotide metabolism, an adequate purification procedure was designed and implemented to isolate YNDase from yeast. This marks the first time that yeast nicotinamide deamidase has been purified completely. Moreover, the molecular weight, enzyme subunit composition, pH

activity profile, kinetic analysis, amino acid composition, chemical modification and substrate analogue studies of YNDase have been accomplished. Again, when I compare YNDase with bacterial and mammalian nicotinamide deamidases, I see that the properties of YNDase are much closer to those of bacteria. This enzyme has a relatively small molecular weight, a single subunit and wide pH activity range (Table 4). In addition, the K_m values for nicotinamide determined for the YNDase and bacterial NDases are consistent with the formation of a relatively tight enzyme-substrate complex. In contrast, all of these results differ from those obtained for the mammalian enzymes that have been examined to date.

Wolfenden and his colleagues (59, 60 and references therein) have demonstrated that active-site directed aldehydic reagents can serve as potent reversible inhibitors and transition state analogues for a variety of enzymatic reactions that involve the breaking of amide or peptide linkage, including the papain (61), L-asparaginase (56) and elastase (62) catalyzed reactions. Since there are similarities between the papain-catalyzed breaking of a peptide bond (apparently involving the formation of an acyl enzyme intermediate with a cysteine residue, 61) and the asparaginase-catalyzed deamidation of asparagine (perhaps involving a cyclic tetrahedral intermediate,56), I elected to test whether nicotinaldehyde, a substrate analogue, was a potent inhibitor of YNDase activity.

Our studies show that nicotinaldehyde is a noncompetitive inhibitor with a relatively weak affinity, and this interaction may not reflect the tightly-held transition state that appears during nicotinate formation. However our chemical modification studies suggest that a cysteine residue is indeed present at the active-site (a covalent thioester bond may be formed between an acyl group of the substrate and the sulfhydryl group of a specific cysteine residue of the enzyme). Nicotinaldehyde may still react at or near to the active site with a lysine residue (secondary-site), which can bind nicotinaldehyde more efficiently than can cysteine at this location. This essential

Table 4: The Comparison of Nicotinamide Deamidases from Different Sources

Source	Km (M)	M.W.	Number of subunits	Optimum pH	Substrate specificity	Inhibitors	Ref.
Baker's yeast	3.3×10^{-5}	34,000*	1	6.0-8.5	high	---	63
Flavobacterium peregrinum	2×10^{-7}	48,000	---	6.5-7.5	high	heavy metal ions P-chloromercuri-benzoate EDTA	17
Tornla cremoris	1.4×10^{-5}	100,000	---	6.0-7.0	high	heavy metal ions NAD, NADP 3-Acetylpyridine	64
Lb arabinosus	1.5×10^{-3}	---	---	4.5-8.0	high	heavy metal ions	65
Rabbit Liver	4×10^{-2}	196,000-218,000 (glycoprotein)	2 or 4	7.4	low**	surface active agents, thyroxine, ammonium sulfate, diisopropylfluoro-phosphate, 8-hydroxyquinoline, o-phenanthroline	15, 52
Mouse C1300 neuroblastoma	6×10^{-6}	230,000 (glycoprotein)	4	7.2	---	sulfhydryl heavy metal ions EDTA cysteine dithiothreitol NAD (comp. inhibition)	14

* YNDase has not been examined as if it is a glycoprotein.

** This enzyme can hydrolyse a variety of amides and esters, specific for the pyridine ring.

lysine residue may be useful in neutralizing negatively charged groups that result from the deamidation event.

Since baker's yeast contains an efficient YNDase, this organism is able to make use of both nicotinamide and nicotinate as nutrients to bring about the synthesis of NAD through the use of the Preiss-Handler pathway. In addition, this enzyme may be present to salvage the nicotinamide that results from the formation of ADP-ribose from NAD. It is now well known that many organisms can use NAD as a substrate to generate ADPR which then serves as a protein regulator. In yeast, I detected abundant NAD glycohydrolase activity (with a relative molecular weight 300,000) and ADPR phosphodiesterase activities, but failed to detect poly(ADP-ribose) synthetase or ADP-ribose transferase. Further investigation has to be performed in this area, concerning these activities in yeast. The question of when, during evolution, ADP-ribose became an important metabolic regulator may be answered through such studies.

Through the entire NAD metabolism study, I designed several HPLC assay systems and succesfully applied them in our research. This unique and powerful assay method can easily be used in analyzing complicated and difficult systems (the simultaneous monitoring several substrates and products which have the same wavelength absorbance). I and my colleagues also have applied HPLC in various aspects of enzyme analyses (kinetic studies, molecular weight determinations, amino acid composition studies), which cannot be achieved by other methods. It will always be my pleasure to use HPLC methods in my future research.

REFERENCES

1. Huber, C. (1867) *Ann. der Chemic und Pharmacie.* **65,** 271

2. Weidel, H. (1873) *Ann. der Chemic und Pharmacie.* **165,** 328

3. Goldberger, J., Waring, C. H., and Willits, D. G. (1915) *Public Health Report* **30,** 3117

4. Von Euler, H., Ahlers, H., and F. Schlenk, Z. (1936) *Physiol. Chem.* **124,**113

5. Warburg, O., and Christian, W. (1934) *Biochem. Zeitschr.* **274,** 112

6. Warburg, O., and Christian, W. (1935) *Biochem. Zeitschr.* **275,** 464

7. Von Euler, H., Albers, H., and Schlenk, Z. F. (1935) *Physiol. Chem.* **237,** 180

8. Von Euler, H., Albers, H., and Schlenk, Z. F. (1936) *Physiol. Chem.* **240,** 113

9. Dietrich, L. S., Fuller, L., Martinez, L., and Yero, I. L. (1966) *Fed. Proc.* **24,** 480

10. Dietrich, L. S., Fuller, L., Martinez, L., and Yero, I. L. (1966) *J. Biol. Chem.* **241,** 188-191

11. Preiss, J., and Handler, P. (1958) *J. Biol. Chem.* **233,** 488-492

12. Sloan, D. L., Ali, L. Z., Aybar-Batista, D., Yan, C., and Hess, S. L. (1984) *J. Chromatog.* **316,** 43-52

13. Yan, C., and Sloan, D.L. (1986) *Fed. Proc.* **45,** 1503

14. Wintzerith, M., Dierich, A., and Mandel, P. (1980) *Biochem. Biophys. Acta* **613,** 191-202

15. Kirchner, J., Watson, J. G., and Chaykin, S. (1966) *J. Biol. Chem.* **241,** 953-960

16. Joshi, J. G., Calbreath, D. F., and Handler, P. (1972) *Meth., in Enzymol.* **18,** 180-185

17. Tanigama, Y., Shimoyama, M., and Ueda, I. (1980) *Meth. Enzymol.* **66,** 132-136

18. Petrack, B., Greengard, P., Craston, A., and Kalinsky, H. J. (1963) *Biochem. Biophys. Res.* Commun., **13,** 472

19. Petrack, B., Greengard, P., Craston, A., and Sheppy, F.(1965) *J. Biol. Chem.,* **240,** 1725

20. Calbreath, D. F., Joshi, J. G., and Handler, P. (1969) *ACS, Div. Biochem., 158th National Meeting, New York, (Abstract.)* 267

21. Fuller, L., and Dietrich, L. S. (1971) *Anal. Biochem.* **39,** 538

22. Purnell, M. R., Whish, W. J. D. (1980) *Biochem. J.* **185,** 775-77

23. Chambon, P., Weill, J. D., Doly, J., Strosser, M. T., and Mandel, P. (1966) *Biochem. Biophys. Res. Commun.* **25,** 638-43

24. Sugimura, T., Fujimura, S., Hasegawa, S., and Kawamura, Y. (1967) *Biochem. Biophys. Acta* **138,** 438-441

25. Nishizuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O. (1967) *J. Biol. Chem.* **242,** 3164-71

26. Zatman, L. J., Kaplan, N. O., and Colowich, S. P. (1953) *J. Biol. Chem.* **200,** 197-212

27. Hayaishi, O., and Ueda, K. (1977) *Ann. Rev. Biochem.* **46,** 95-116

28. Ueda, K., and Hayaishi, O. (1985) *Ann. Rev. Biochem.* **54,** 73-100

29. Miwa, M., Iijima, H., Kondo, T., Kata, M., Kawamitsu, H., et al. (1983) *Seikagaku* **55,** 547 *(Abstract)* (In Japanese)

30. Koenig, W. J., and Prakt., J. (1904) *Chem.* **69,** 105; **70,** 19

31. Ashton, R. W., and Sloan, D. L. (1984) *J. Chromatog.* **317,** 245-250

32. Ali, L. Z., and Sloan, D. (1983) *J. Biochem.* **22,** 3419- 24

33. Ali, L. Z., and Sloan, D. L. (1982) *J. Biol. Chem.* **257,** 1149-1155

34. Hanna, L.S., and Sloan, D. L. (1980) *Anal. Biochem.* **103,** 230-234

35. Sloan, D, L. (1984) *Adv. in Chromatog.* **Vol. 23,** 97-125

36. Sofer, G., and Britton, V. J. (1983) *BioTechniques,* **Nov/Dec,** 198-203

37. Scopes, R. (1982) *"Protein Purification Principles and Practice", Springer-Verlag, New York*

38. Britton, V. J., Beadling, L.C., and Barry., A. (1983) *Liquid chromatography,* **1,** 176-179

39. Kallman, I., G. Lindgren and L. Fagerstam. (1982) *Second International Symposium on HPLC of Proteins, Peptide and Polynucleotides, Baltimore, Maryland, (abstract)* No. 217

40. Mizigaki, J., Kimura, C., Nishimaki, T., Yamamato, H., Sugi, M., Nishimura, S., and Yamanaka., H. (1982) *J. Biochem.* **92,**1671-1674

41. Reisner, A. H., Nemes, P., and Bucholtz, C. (1975) *Anal. Biochem.,* **64,** 509

42. Faqakes de St. Groth, S., et al. (1963) *Biochim. Biophys. Acta,* **71,** 377

43. Sedmak, J. J., and Grossberg, S. E. (1977) *Anal. Biochem.*, **79,** 544

44. Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951) *J. Biol. Chem.*, **193,** 265

45. Means, G. E., and Feeney, R. E. (1971), *"Chemical Modification of Proteins", Holden-Day, Inc,* 110-114 and 219-220

46. Means, G. E., and Feeney, R. E. (1971), *"Chemical Modification of Proteins", Holden-Day, Inc,* 81-83

47. Miles, E. W., *Meth, in Enzymol.* **41,** 431-442

48. Stein, S., and Brink, L. (1981) *Meth, in Enzymol.* **79**

49. Ito, S., Shizuta, Y., and Hayaishi, O. (1979) *J. Biol. Chem.*, **254,** 3647-3651

50. Kosaka, A., Spivey, H. O., and R. I. Gholson (1977) *Arch. Biochem. Biophys.*, **179,** 334

51. Powanda, M. C., Muniz, O., and Dietrich, L. S. (1969) *Biochemistry,* **8,** 1869

52. Su, S., Albizati, L., and Chaykin, S. (1969) *J. Biol. Chem.* **244,** 2956-2965

53. Tipton K. F., and Dixon B. F., *"Contemporary Enzyme Kinetics and Mechanism [5]" (Effects of pH on Enzymes),* 97-148

54. Melchior, W. B., Jr., and Fahrney, D. (1970) *Biochemistry,* **9,** 251-258

55. Walsh, C. (1979) *"Enzymatic Reaction Mechanisms", W. H. Freeman and Co., San Francisco,* 748

56. Westerik, J. O., and Wolfenden, R. (1974) *J. Biol. Chem.* **249,** 6351-6353

57. Westerik, J. O., and Wolfenden, R. (1972) *J. Biol. Chem.* **247,** 8195-8197

58. Means, G.E., and Feeney, R.E. (1971) *"Chemical Modification of Proteins", Holden-Day, San Francisco,* 25-26

59. Wolfenden, R. (1976) *Ann. Rev. Biophys.* **5,** 271-306

60. Wolfenden, R. (1977) *Meth., in Enzymol.* **46,** 15-28

61. Bender, M., and Brubacher, L. (1966) *J. Amer. Chem. Soc.* **88,** 5880-5889

62. Thompson, R. C. (1973) *Biochemistry* **12,** 47-51

63. Yan, C., and Sloan, D. L. (1987) *J. Biol. Chem.* (accepted)

64. Joshi, J. G., and Handler, P. (1962) *J. Biol. Chem.* **237,** 929-935

65. Hughes, D. E., and Williamson, D. H. (1953) *Biochem. J.* **55,** 851-856.

CURRICULUM VITAE

Name: Cong Yan

Working Address: Chemistry Department of City College, Convent Ave. at 138th ST.
New York, N.Y. 10031

Home Address: 30-10 Hobart ST., Woodside, New York 11377

Telephone No.: (718)-956-7811 (home). (212)-690-8302 (lab.)

Birthdate: July 19, 1957

Citizenship: China

Education:

B. S., Virology, Wuhan University (China), 1982

Master of Philosophy, Biochemistry, City University of New York, 1985

Ph.D., Biochemistry, City University of New York, 1987

Research experience: Research Assistant (1983 -present).

Professional Societies: American Chemical Society, 1985 - present.

Honor: 1987 Graduate Student Award, Chemistry Department, CCNY

Publications:

1). Sloan, D. L., Ali, L. Z., Aybar-Batista, D., Yan., C., and Hess, S.L. (1984) "Enzymatic Assay Procedures that Employ High- Performance Liquid Chromatography: Competition Between Phosphoribosyltransferases for A Common Substrate". *J. Chromatog.* **316,** 43-52.

2). Yan, C., and Sloan, D. L. (1986) *Fed. Proc.* **45,** 1503 (abstract).

3). Yan, C., and Sloan, D. L. (1987) "Studies of Nicotinamide Production and Utilization in Yeast". *Fed. Proc.* **46** (abstract in press)

4). Yan, C., and Sloan, D. L. (1987) "Purification and characterization of Nicotinamide Deamidase From Yeast". *J. Biol. Chem.* **261,** in press.

5) Yan, C., Martin, C., and Sloan, D. L. (1987) "Procedures for the Detection of ADP-Ribosylation Events in Yeast". *Analyt. Biochem.* (in preparation).