NICOTINAMIDE DEAMIDASE OF CLAVICEPS

BY

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ABSTRACT

Preliminary studies have indicated that pyridine mono-and di-nucleotides from *Claviceps* may be separated by an acetone precipitation technique.

Nicotinamide deamidase was found to be active in the biosynthesis of pyridine nucleotides in *Claviceps*. The enzyme was purified 67-fold from *Claviceps*, strain SD58 by means of dialysis, sephadex-gel filtration, and acetone precipitation. The Km of the enzyme was found to be 1.087×10^{-7}M. The deamidase demonstrated maximum activity at pH 7.4 and 50°C. The metals, Mn^{++}, Hg^{++}, Fe^{+++}, Cu^{++}, Ca^{++}, and Na^{+}, all inhibited enzyme activity. Magnesium did not affect enzyme activity. The enzyme was specific for nicotinamide and was inhibited by NAD, NADP, NMN, des-NAD and nicotinic acid. The deamidation reaction was found not to be reversible.

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LIST OF ABBREVIATIONS

AMP	-adenosine monophosphate
ATP	-adenosine triphosphate
des-NAD	-des amino nicotinamide adenine dinucleotide
DPM	-disintegrations per minute
g	-gravity
Km	-Michaelis constant
M	-molar
mM	-milli-mole
muM	-milli-micro-mole
NAD	-nicotinamide adenine dinucleotide
NADP	-nicotinamide adenine dinucleotide phosphate
NaMN	-nicotinic acid mononucleotide
NMN	-nicotinamide mononucleotide
P-P	-pyrophosphate
PRPP	-phospho-ribosyl-pyrophosphate
S.A.	-specific activity
ugm	-microgram
Vmax	-maximum velocity of enzyme catalyzed reaction

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I. INTRODUCTION

Since most metabolic processes in living organisms depend upon active enzyme systems, the determination of a complete biosynthetic pathway requires the study of the individual enzymes which catalyze the stepwise reactions of the pathway. The nicotinamide adenine dinucleotide (*NAD) biosynthetic pathway and the enzymes which are involved have been studied in numerous living systems (1-7,14,43).

Recently, Audette (1) determined that cultures of Claviceps purpurea, strain CPM and Claviceps, strain 47A produced NaMN, des-NAD, NAD, NADP, NMN, and N-methylnicotinamide from nicotinic acid or nicotinamide. Subsequently, his investigations suggested the NAD biosynthetic pathway shown in Figure 1. However, deamidation of nicotinamide to form nicotinic acid was not definitely established. Therefore, this study was undertaken in an attempt to determine whether or not nicotinamide deamidase or nicotinamidase (2) was involved in the biosynthesis of NAD in Claviceps.

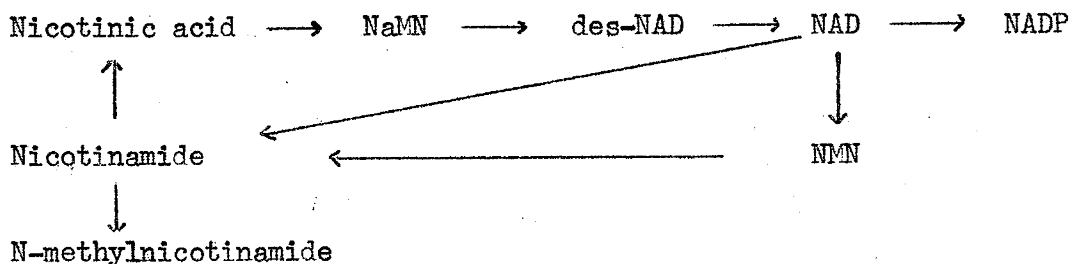

Figure 1. Suggested Pathway of NAD Biosynthesis in Claviceps (1).

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*See list of abbreviations, page iii.

A. <u>Biosynthesis of NAD</u>

In 1951 Leder and Handler (3) reported that incubation of washed human erythrocytes with nicotinamide produced an 8-10 fold increase in pyridine nucleotide synthesis after 20 hours. However, in 1957 in a more extensive study, Preiss and Handler (4) found that human erythrocytes, *in vitro*, utilized low concentrations of nicotinic acid in the synthesis of pyridine nucleotides. Nicotinamide had to be present in high concentrations before it was utilized. Although Preiss and Handler (5) determined that nicotinamide could react with PRPP in the presence of NMN pyrophosphorylase to form NMN, the reaction rate was very slow and the Km of the enzyme was suggested to be too high to be physiologically effective.

Subsequently, in 1958, Preiss and Handler (6) found NaMN and des-NAD to be intermediates in the biosynthesis of NAD from nicotinic acid in acetone powdered erythrocytes. In addition, they determined that yeast autolysates could utilize nicotinic acid but not nicotinamide to produce NAD (7).

As a result of their investigations, Preiss and Handler suggested the general pathway of NAD biosynthesis from nicotinic acid as illustrated in Figure 2.

The enzymes (2) which catalyze the reactions shown in Figure 2 have been identified, viz.:

Reaction 1: Nicotinate phosphoribosyltransferase (Nicotinatenucleotide: pyrophosphate phosphoribosyltransferase E.C. 2.4.2.11);

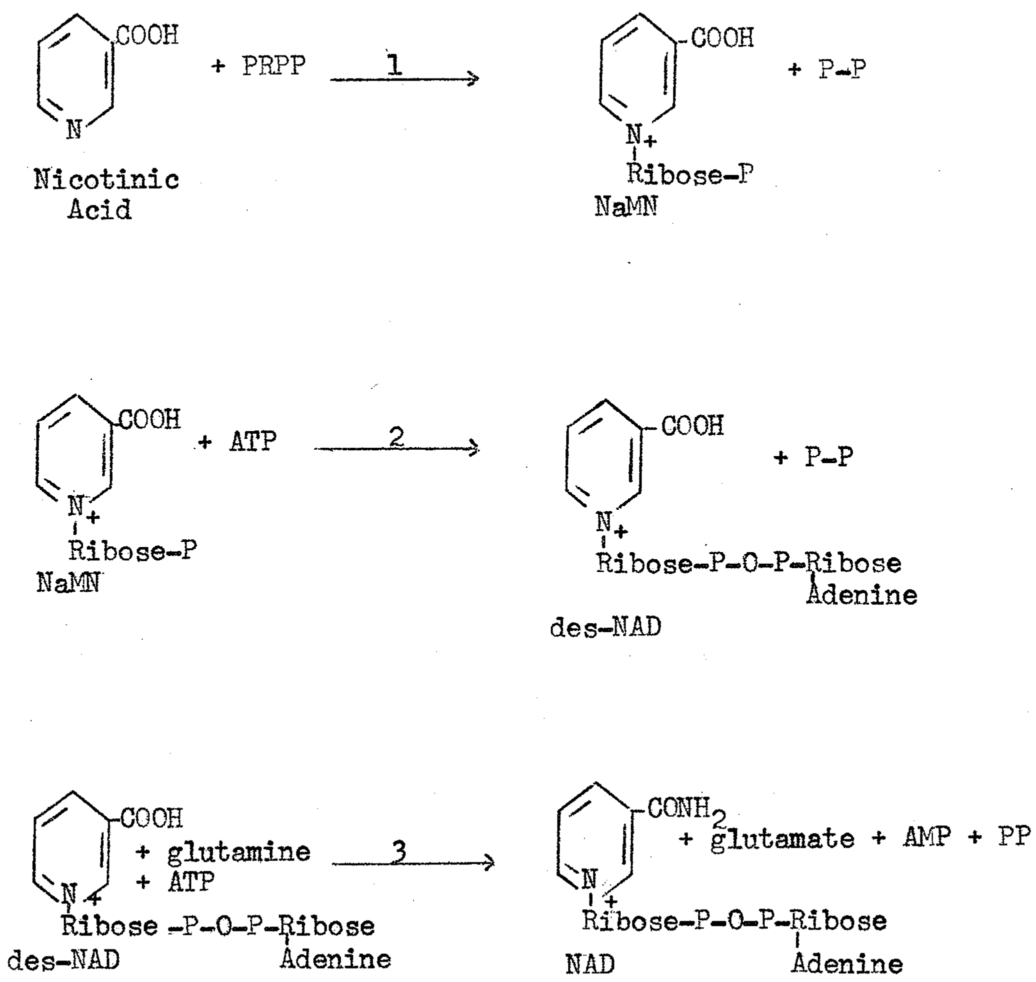

Figure 2. The Preiss and Handler Pathway of NAD Biosynthesis (6,7).

Reaction 2: Nicotinatemononucleotide adenylyltransferase (ATP: nicotinatemononucleotide adenylyltransferase EC 2.77.18) or NAD pyrophosphorylase (ATP: NMN adenylyltransferase EC 2.7.7.1);

Reaction 3: NAD synthetase (deamido-NAD: L-glutamine amidoligase (AMP) EC 6.3.5.1).

The results of some additional significant investigations concerning NAD biosynthesis in various biological systems are mentioned below.

1. Erythrocytes in vitro

Investigations (8-10) indicated that human, pigeon, frog and rabbit erythrocytes could utilize both nicotinic acid and nicotinamide to produce pyridine nucleotides, although the rates of incorporation appeared to vary with the species.

2. Liver and Kidney in vitro

Based upon the observation that mouse liver deamidated the nicotinamide moiety of NMN and that kidney and liver preparations of various birds could deamidate nicotinamide by means of nicotinamide deamidase, Sarma, et al, (11) suggested the biosynthetic pathway shown in Figure 3.

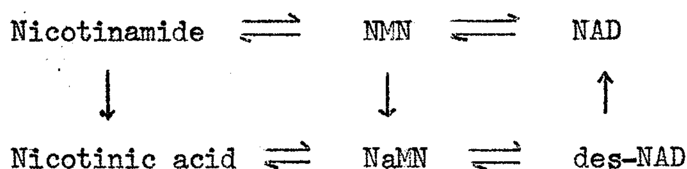

Figure 3. Biosynthetic Pathway of NAD as Suggested by Sarma (11).

3. Mouse liver in vivo

Reports by Langan, et al (10), Narrod, et al, (12), and Hayaishi, et al, (13) have indicated that the "Preiss and Handler Pathway" functions in mice in vivo. However, nicotinamide was found to be a

more efficient precursor of NAD than nicotinic acid at high doses *in vivo* in mouse liver.

4. Tumor cells

Dietrich, *et al*, (14,15) have reported that Ehrlich-Lettre ascites cells could incorporate both nicotinic acid and nicotinamide into NAD.

5. Microorganisms

The NAD biosynthetic pathway in yeast (7) and in *Escherechia coli* (44) has been determined to be the same as the pathway shown in Figure 2, while the possible pathway in the fungus, *Claviceps*, is indicated in Figure 1.

6. Plants

Waller, *et al*, (27) have observed the following reactions in *Ricinus communis* L.:

a) Nicotinamide $\xrightarrow{\text{in vivo}}$ Nicotinic acid + N-methylnicotinamide

b) Nicotinic acid $\xrightarrow{\text{in vitro}}$ NAD + N-methylnicotinic acid

c) NAD $\xrightarrow{\text{in vivo}}$ Nicotinamide + N-methylnicotinamide

B. Nicotinamide Deamidase

1. Investigations of Nicotinamide Deamidase Activity

a) Microorganisms

Hughes and Williamson (16) first reported on deamidase activity in *Lactobacillus arabinosus* 17-5 in 1952. Synthesis of NAD from nicotinamide occurred more rapidly than from nicotinic acid, although, nicotinamide was first deamidated to nicotinic acid.

In 1953 Hughes and Williamson (17) reported that _L. arabinosus_ deamidase rapidly hydrolyzed nicotinamide to nicotinic acid and ammonia, but the enzyme could not catalyze the reverse reaction. Neither nicotinic acid, nicotinamide, nor NAD altered the deamidase activity. _L. arabinosus_ did not degrade NAD. Of 18 other organisms studied only _Staphylococcus albus_ deamidated nicotinamide significantly.

In 1954 Oka (18) separated nicotinamide deamidase from the enzyme asparaginase in a partially pure form from beer yeast by autolysis with ethyl acetate and subsequent acetone precipitation. Chaykin, _et al_, (19) in 1967 also found a highly active nicotinamide deamidase in the yeast, _Sacchromyces fragilis_ C 351. The activity was found to be associated with the cell membrane.

Halpern and Grossowicz (20) have demonstrated high deamidase activity in cell free extracts of _Mycobacterium phlei_. Three-acetylpyridine inhibited the activity of the enzyme completely (21).

Nicotinamide deamidase activity has also been demonstrated in the insect larva, _Corcyra cephalomica_ St. (22), _Neurospora crassa_ (23), _Mycobacterium avium_ (24), _Torula cremoris_ (25), and _Aspergillus niger_ (26).

b) Vertebrates

As early as 1945 Johnson, _et al_, (28) reported that some of the nicotinamide given orally to humans was deamidated to nicotinic acid. In 1949 Ellinger, _et al_, (29) reported that the rabbit and guinea pig deamidated nicotinamide. In addition humans, dogs, cats and rats were shown to amidate nicotinic acid to form nicotinamide. Rajagopalan (30) first showed that avian species contained nicotinamide

deamidase in 1958.

In 1961 Greengard, et al, (31,32) demonstrated that hypophysectomy and adrenalectomy in rats both increased liver NAD content on a normal diet. Concurrent administration of either tryptophan, nicotinic acid, or nicotinamide increased the NAD level even more with nicotinamide being most effective. Administration of ACTH or cortisone acetate both reversed the effect of hypophysectomy and adrenalectomy.

In 1963 Petrack, et al, (33) partially purified nicotinamide deamidase from rat and mouse liver. The enzyme was mainly located in the microsomes, with some in the mitochondria, and little in the nuclei and cell sap. This is in contrast to A. niger (26), in which the activity is localized in the soluble cytoplasmic matter of the cell (cell sap).

Subsequently, Greengard, et al, (34) showed nicotinamide deamidase to be four times more active in hypophysectomized rats than in normal rats. This accounted for higher levels of NAD in hypophysectomized animals. Hypophysectomy appeared to remove an inhibitor of the deamidase. These findings contradicted Sarma's report (35) in 1961 that mouse liver did not contain deamidase activity.

Ricci, et al, (36) have reported that rat liver concentration of nicotinic acid increased 70 fold eight hours after the injection of nicotinamide, again demonstrating the deamidase activity.

Chaykin, et al, (37,38) suggested in 1965 that at high levels of nicotinamide in the mouse, the inhibition of nicotinamide deamidase is overcome and nicotinamide may then serve as a precursor of NAD via nicotinic acid.

In 1966 Ijichi, et al, (39) found that, initially, administered nicotinamide-^{14}C was incorporated into NAD without undergoing deamidation, but eventually significant deamidation did occur in the mouse.

Thus nicotinamide deamidase activity has been found in both microorganisms and in vertebrates.

2. Significance of Nicotinamide Deamidase in NAD Biosynthesis

The presence of nicotinamide deamidase in a physiological system provides the system with the "Preiss and Handler Pathway" for the utilization of nicotinamide as a precursor in NAD synthesis. If the enzyme is absent in the system, nicotinamide may be utilized directly via NMN to form NAD or may function as a substrate for NAD-ase. These two latter pathways may also be available even if the deamidase is present.

Nicotinamide deamidase has been suggested to be the rate-limiting step in the synthesis of NAD from nicotinamide via nicotinic acid (33). Greengard, et al, (34) have suggested that this step may be under hormonal control in animals.

Thus the enzyme serves to utilize nicotinamide and control NAD biosynthesis, and hence also the synthesis of NADP, and the reduced forms of these two pyridine nucleotides. These nucleotides are essential for numerous oxidation/reduction reactions in physiological systems.

3. Isolation and Characterization of Nicotinamide Deamidase

Nicotinamide deamidase has been partially purified and characterized from various sources, as is indicated below.

a) Nicotinamide deamidase of *Mycobacterium avium* (24)

The enzyme was relatively sensitive to temperature changes. All activity was destroyed in 15 minutes at 50°C. Freezing caused 13% destruction of activity. Optimum activity of the enzyme was noted at pH 8.3.

The enzyme was specific for nicotinamide and could not attack L-asparagine, L-glutamine or NAD.

Deamidase activity was inhibited by Cu^{++}, Zn^{++}, Fe^{+++}, Cu^{+++}, and Hg^{++}. Metal chelating agents such as dihydrostreptomycin and isonotinic acid hydrazide caused partial inhibition.

b) Nicotinamide Deamidase of *Torula cremoris* (25)

Studies indicated that the deamidase quickly lost its activity above 50°C. However, the presence of substrate provided significant protection between 40 and 50°C. The optimum activity of the enzyme was noted at pH 7.

This enzyme was also specific for nicotinamide. It did not attack L-glutamine, L-asparagine, NAD, NMN, benzamide, glycinamide, N-methylnicotinamide, N-ethylnicotinamide, or N,N-diethylnicotinamide.

From a Lineweaver and Burk (40) plot the Km of the enzyme for nicotinamide was found to be 1.4×10^{-5}M.

Various metals produced varying degrees of inhibition, however, Mg^{++} had no effect. Three-acetyl pyridine inhibited the enzyme competitively while NAD and NADP caused non-competitive inhibition at physiological concentrations. In addition, NAD was found to inhibit the biosynthesis of the nicotinamide deamidase.

c) Nicotinamide Deamidase of Aspergillus niger (26)

High nicotinamidase activity was reported at pH 7.5 at 40°C. The activity was directly proportional to substrate concentration up to 200 mg. of nicotinamide. The enzyme did not attack NAD, NADP, NMN, benzamide, N-methylnicotinamide, L-asparagine, L-glutamine, alpha-naphthaleneamide, or indole-acetamide.

The Km for nicotinamide was determined to be 6.5×10^{-4}M which is slightly higher than the Km for the T. cremoris enzyme.

The nicotinamide deamidase was inhibited by various metal chelating agents. Mg^{++} was the only metal found which could reverse the α,α'-dipyridyl inhibition. Therefore, this enzyme appeared to be dependent on Mg^{++}. Again in contrast to the T. cremoris enzyme, NAD did not inhibit the enzyme.

Nicotinamide deamidases from other sources have been characterized (17,18,33,41). In general the properties were similar to those already mentioned with slight variations. An exception which must be noted is the high Km of 0.181M obtained with the deamidase from mouse liver by Petrack, et al (33). However, Ichiyama (42) has reported the discovery of an enzyme in the gastrointestinal tract of the rat with a much lower Km.

Although the nicotinamide deamidase properties vary, depending upon the source of the enzyme, the overall attributes are similar.

II. EXPERIMENTAL

A. Preliminary Studies On The Biosynthesis Of NAD

1. Materials and Methods

Organism

Claviceps, strain 47A, which was obtained as a slant culture from Dr. R.C.S. Audette, School of Pharmacy, University of Manitoba, Winnipeg, Manitoba was employed in this study.

Stock Cultures

Stock cultures of Claviceps, 47A were maintained on sterile modified McCrea (45) nutrient agar medium at 25°C in the dark. The stock cultures were renewed every 8 to 10 weeks.

Liquid Culture Medium

The NAD biosynthetic study was carried out employing Claviceps 47A grown in a modified Abe's nutrient medium (1). The medium was inoculated aseptically with 15 to 30-day old organisms grown on McCrea slants (45) or with a homogenized suspension of 30-day old organisms grown on Abe's medium (1).

Chemicals

Nicotinamide-7-^{14}C (S.A. 10.7 mc/mM) and nicotinamide-7-^{14}C (S.A. 7.63 mc/mM) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.; Nicotinic acid-7-^{14}C (S.A. 27.9 mc/mM) was purchased from Nuclear Chicago Corp., Des Plaines, Ill., U.S.A.; N-methylnicotinamide iodide was obtained from Calbiochem Corp., Los Angeles, Calif., U.S.A.; nicotinic acid was obtained from The Matheson Co. Incorp., Norwood, Ohio, U.S.A.; and samples of des-NAD-^{14}C and

NaMN-^{14}C were provided by Dr. R.C.S. Audette.

Measurement of Radioactivity

A *Unilux Liquid Scintillation system was used to determine the activity of radioactive samples. Not more than 0.1 ml. of radioactive solutions were dispensed into counting vials of 20 ml. capacity. Where required, radioactive spots from paper chromatograms were diced and placed into counting vials. Radioactive spots corresponding to nicotinic acid-^{14}C were diced and heated in 0.05 ml. hyamine hydroxide (1M solution in methanol) plus 0.2 ml. distilled water at 70°C for thirty minutes. Radioactive spots containing compounds other than nicotinic acid were heated similarly in 0.2 ml. distilled water. (A series of experiments indicated that 90% of the activity on the paper could be detected following this procedure.**) In all cases, after the samples to be counted were suitably prepared in the counting vials, 10 ml. of scintillation fluid (1) of the following composition were added:

POP (2,5-diphenyloxazole)	0.2%
di-Me-POPOP [1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene]	0.05%
OR	
bis-MSB [(bis-(O-Methylstyryl)-benzene]	0.004%
Isopropanol (Fisher Reagent)	40.0%
Toluene (Baker Reagent) to make	100.0%

The counting efficiency for each sample was determined by employing an internal standard of benzoic acid-7-^{14}C (S.A. 6.6×10^3

*Nuclear-Chicago Unilux Model 6850 Bench-top Liquid Scintillation System, Nuclear-Chicago, Des Plaines, Ill.
**All counting data was corrected-based on 90% recovery of activity.

dpm/mg.; New England Nuclear Corp., Waltham, Mass.). All radioactive solutions for scintillation counting were measured with an *Agla micrometer and a 1.0 ml. capacity syringe.

Detection of Radioactivity

Radioactive spots were located on paper chromatograms using a radio chromatogram scanner** as suggested by Audette (1).

Preparation of Nicotinamide-7-^{14}C for Administration

Nicotinamide-7-^{14}C was dissolved in sterile glass distilled water and then sterilized employing a ***Swinny filter prior to use.

Incubation of Cultures with Nicotinamide-7-^{14}C

A prescribed amount of nicotinamide-7-^{14}C was administered aseptically to entire 30-day-old cultures as prepared above. In a variation of the procedure, 30-day-old cultures were homogenized in a stainless steel, chilled, Waring blendor before the nicotinamide-7-^{14}C was administered. The flasks were then shaken at 24°C throughout the incubation period.

Chromatography Procedures Employed (1)

1. Paper

Whatman No. 3MM paper was used in all chromatographic procedures. Chromatograms were developed in glass tanks at room temperature. Three solvent systems were used for separation and purification of the radioactive metabolites by ascending development, namely:

a) Isobutyric acid: ammonia: water (66:1.7:33) (IAW).

b) Pyridine: water (2:1) (PW).

c) Butanol saturated with 3% ammonia (BA).

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*Agla micrometer and syringe obtained from Burroughs Wellcome and Co., Montreal, P.Q., Canada.
**Nuclear-Chicago Actigraph III (Model No. 1002), Nuclear-Chicago, Des Plaines, Ill.
***Swinnex Disposable Filters, Millipore Filter Corp., Bedford, Mass.

The hR_f values obtained in these solvent systems for the pyridine compounds under study are listed in Table 1.

2. Thin Layer Chromatography (TLC)

Compounds which had been isolated on paper chromatograms were identified by thin layer chromatography employing Silica Gel G (Merck) as indicated by Audette (1). Table 2 shows the hRf values of the compounds studied by this procedure.

Detection of Pyridine Compounds (1)

The following procedures were employed to detect pyridine-type compounds on paper chromatograms:

1. Ultraviolet light (253 mu) was employed to locate ultraviolet absorbing compounds.

2. Paper chromatograms were exposed to a mixture of methyl ethyl ketone and concentrated ammonia (1:1 v/v), which was stirred constantly, for one hour in a closed glass tank. After exposure to this mixture NMN, NAD and NADP produced a blue-white fluorescence when viewed under long wave ultraviolet light (366 mu). However, NaMN and des-NAD did not fluoresce (46).

3. Paper chromatograms were also exposed to cyanogen bromide vapours and then sprayed with p-aminobenzoic acid (47). Nicotinic acid produced a yellow colored spot while nicotinamide produced a deep yellow-orange color following this treatment.

Isolation and Purification of Radioactive Pyridine Compounds (1)

The isolation and purification procedure of radioactive pyridine compounds from *Claviceps* 47A after incubation with nicotinamide-7-^{14}C

TABLE 1

*hR_f VALUES OF PYRIDINE COMPOUNDS ON PAPER CHROMATOGRAMS

Solvent**	IAW	PW	BA
Compound:			
NMN	45	29	0
NaMN	20	44	0
des-NAD	27	62	0
NAD	46	63	0
NADP	29	26	0
Nicotinamide	84	81	61
Nicotinic Acid	74	76	21
N-Methyl-nicotinamide	79	69	10

0 - Remained at origin

*hR_f (R_f x 100)

**IAW - Isobutyric acid: ammonia: water (66:1.7:33)

PW - Pyridine: water (2:1)

BA - Butanol saturated with 3% ammonia

TABLE 2

*hR_f VALUES OF PYRIDINE COMPOUNDS ON SILICA GEL G

THIN LAYER PLATES DEVELOPED IN WATER

Compound	*hR_f
Nicotinic acid	80
Nicotinamide	41
N-Methyl-nicotinamide	7
NAD	0
NMN	0

0 - Remained at origin

*$hR_f = (R_f \times 100)$

is summarized in Figure 4. Nicotinamide-7-^{14}C (S.A.10.7 mc/mM; 46.7 muM; 1.11 x 10^6 dpm) was incubated with shaking for six hours at 24°C with each of seven, 30-day-old, Claviceps 47A cultures. The mycelium was separated from the medium by filtering and washed with distilled water. The filtrate, fraction A, was diluted volumetrically to 800 ml. with water and suitable aliquots were counted and chromatographed. The mycelium was homogenized in a chilled Waring stainless steel microblendor with five volumes of cold 5% trichloroacetic acid (TCA) for three minutes. The resulting suspension was centrifuged at 15,000 x g. for thirty minutes at 0°C. The pellet obtained was resuspended in 5% TCA and recentrifuged. The two supernatants were combined and diluted volumetrically to 200 ml. with 5% TCA to produce fraction B, and aliquots were counted. Five volumes of acetone at -20°C. were added to the solution and the mixture was stored overnight at -20°C. The suspension was centrifuged at 0°C at 15,000 x g. for twenty minutes. The resulting pellet was successively washed with acetone and peroxide-free ether at 0°C and the washings were added to the supernatant. The washed pellet was dried in vacuo and was dissolved in distilled water to make 25 ml. of fraction C prior to chromatographic analysis and scintillation counting.

The acetone and ether were removed from the supernatant in vacuo at 20°C. The resulting aqueous solution was extracted with peroxide-free ether. The final aqueous solution, fraction D, and ether extract, fraction E, were concentrated to 25 ml. and 10 ml. fractions, respectively. Suitable aliquots of the two solutions were counted, and the radioactive metabolites were separated by paper chromatography. The distribution of radioactivity in Fractions A-E is listed in Table 3.

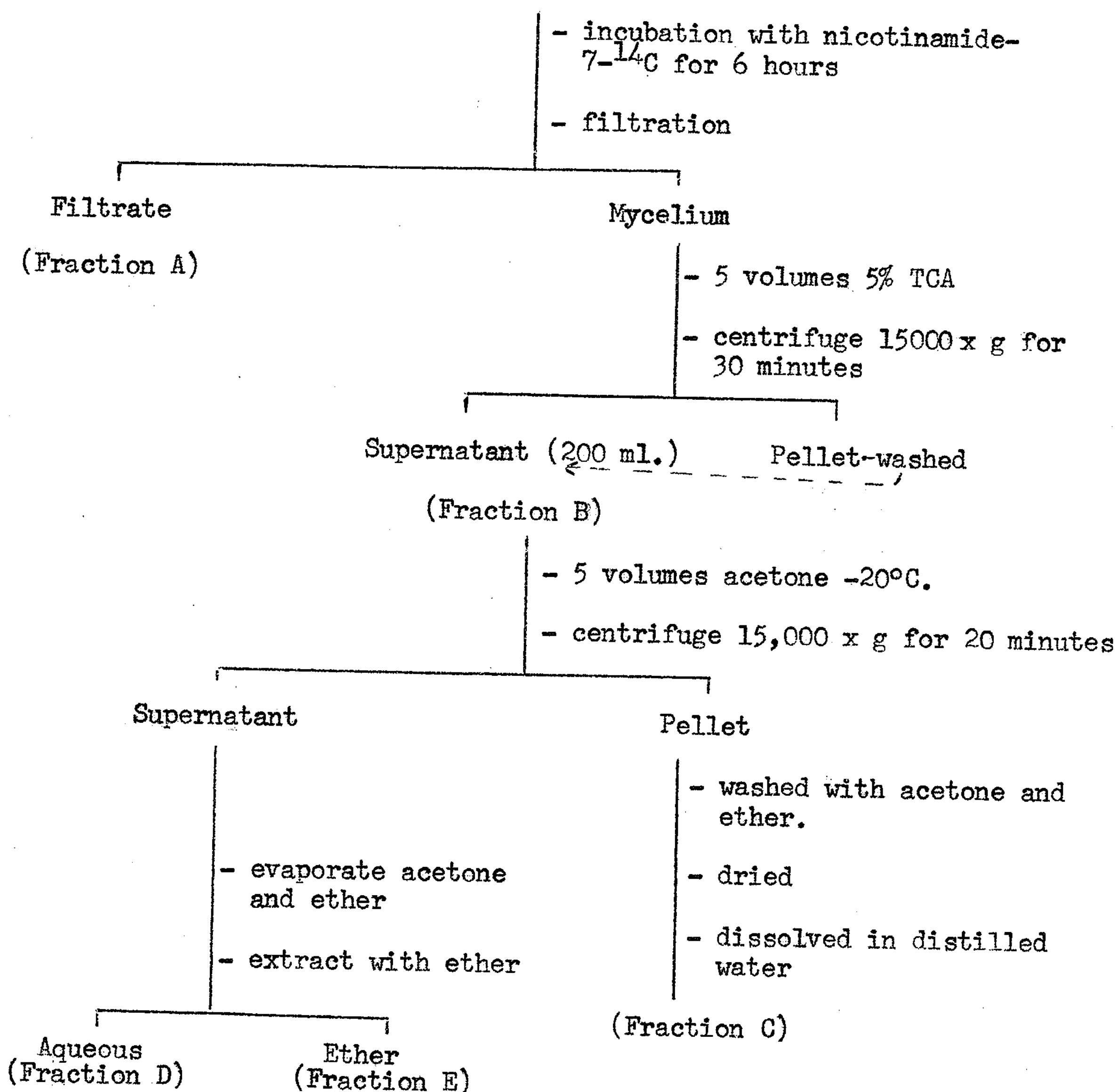

Figure 4. Summary of the Isolation and Purification Procedure of Radioactive Pyridine Compounds From Claviceps 47A After Incubation With Nicotinamide-7-^{14}C.

Portions of fractions A,C,D, and E were chromatographed on Whatman 3 MM paper in the IAW solvent system. Figures 5 and 6 show radiochromatographic scans of fractions C and D, respectively, developed in IAW. Separated radioactive bands from all fractions were eluted with distilled water, freeze-dried and the residues were dissolved in minimum quantities of distilled water. The relative distribution of radioactivity in the bands was determined. The eluted bands of fractions A and E were rechromatographed on silica gel plates in water and on Whatman 3 MM paper in butanol-ammonia. The eluted bands of fractions C and D were rechromatographed on Whatman 3 MM paper in pyridine-water and butanol-ammonia. The relative distribution of radioactivity in the final, separated bands was determined.

2. Experimental Results

a) Study With Intact Cultures of Claviceps 47A

Following the incubation of Claviceps 47A cultures with nicotinamide-7-^{14}C, as previously described, the incubation medium, fraction A, and fraction B, which was produced from the mycelium, were analyzed for radioactivity. Fractions C,D, and E were derived from fraction B by appropriate extraction procedures. The distribution of radioactivity in the various fractions is shown in Table 3.

Table 4 indicates the percentage distribution of radioactivity in the respective bands obtained from fractions A,C,D, and E in the I.A.W. system.

Rechromatography of bands 3 and 4, obtained from fractions A and E, on TLC and paper in the butanol-ammonia system indicated that nicotinic acid and nicotinamide were the only compounds present in these fractions.

The results of rechromatographing IAW bands 1 and 2 of fraction C and bands 1-3 of fraction D on paper in the pyridine-water system are listed in Table 5. The identity of specific compounds with specific hR_f values is included. According to Shuster and Goldin (48) fractions C and D should contain primarily dinucleotides and mononucleotides, respectively.

The results of rechromatographing IAW bands 4 and 5 of fraction D on paper in butanol-ammonia are listed in Table 6. The possible identity of the compounds with specific hR_f values is included.

The distribution of radioactivity in the various pyridine compounds which were isolated from the mycelium by means of paper chromatography

TABLE 3

THE DISTRIBUTION OF RADIOACTIVITY IN VARIOUS FRACTIONS OBTAINED FROM 30-DAY-OLD CULTURES OF CLAVICEPS 47A INCUBATED WITH *NICOTINAMIDE-7-^{14}C.

Fraction	DPMX10^6	% of Total Activity Fed
A	1.112	14.31
B	5.946	76.53
**C	0.867	11.16
**D	4.380	56.37
**E	0.085	1.09

*Each culture was incubated with Nicotinamide-7-^{14}C (S.A. 10.7 mc/mM; 46.7 muM; 1.11 x 10^6 dpm).

Legend:

Fraction A - total, diluted incubation medium (Filtrate).

Fraction B - supernatant remaining after TCA precipitation.

**Fractions C,D,E obtained from Fraction B.

Fraction C - acetone-precipitated dinucleotides.

Fraction D - acetone-soluble mononucleotides.

Fraction E - ether extract of mononucleotide fraction.

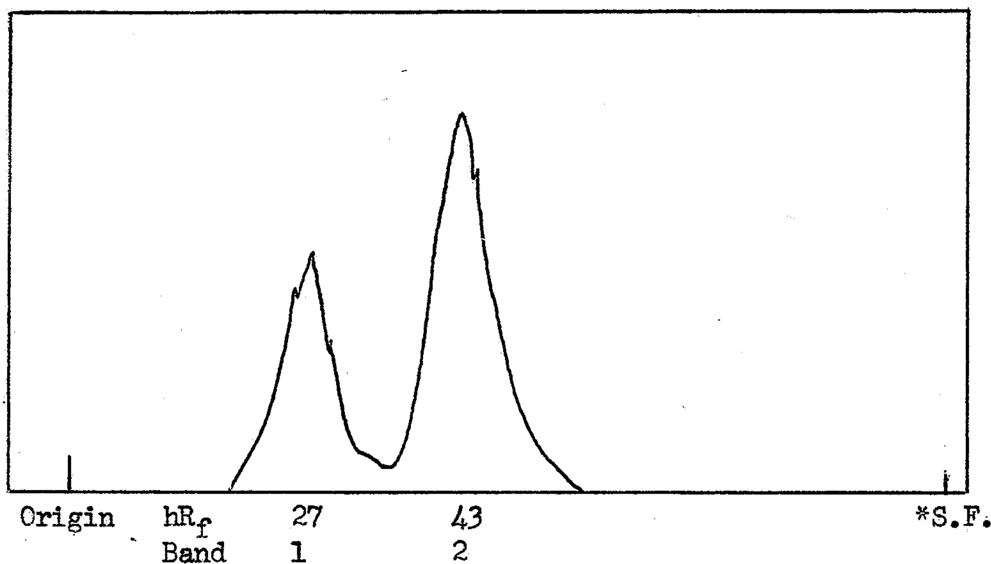

Figure 5. Radiochromatographic scan of fraction C, consisting of dinucleotides, developed in isobutyric acid-ammonia-water. 0.5 ml. of a total 25 ml. fraction was streaked over 1 inch of Whatman 3MM paper. The Actigraph III settings were: 300 cpm scale; 10 second interval; 60 cm./hour; 1050 volts.

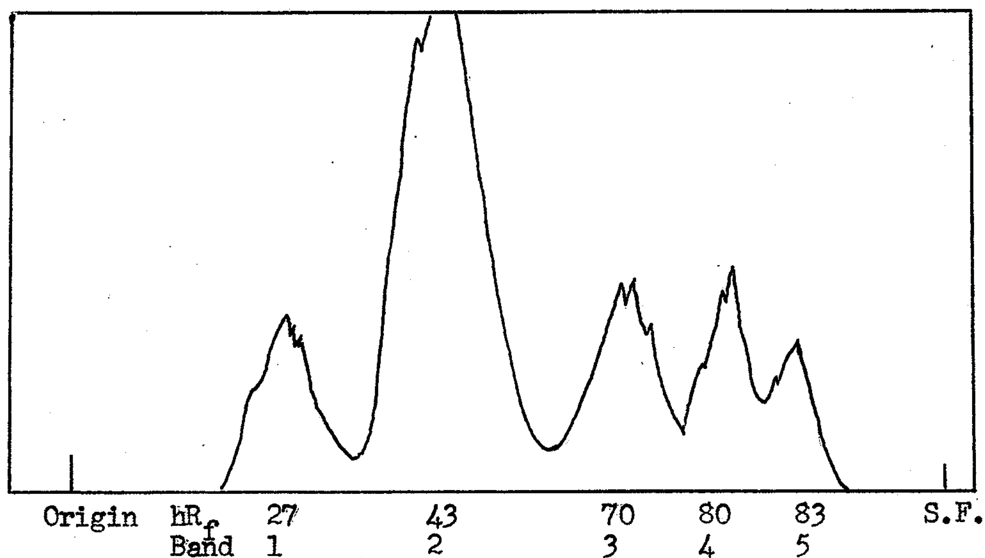

Figure 6. Radiochromatographic scan of fraction D, consisting of mononucleotides, developed in isobutyric acid-ammonia-water. 0.5 ml. of a total 25 ml. fraction was streaked over 1 inch of Whatman 3MM paper. The Actigraph III settings were: 300 cpm scale; 10 second interval; 60 cm./hour; 1050 volts.

*S.F.-Solvent front.

TABLE 4

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN THE BANDS

FROM FRACTIONS A,C,D, AND E AS SEPARATED IN IAW.

**Fraction	Band (IAW)	hR_f	Distribution of Radioactivity in Band Within Each Fraction (%)
A	3*	70	21.43
	4	81	78.57
C	1	27	47.49
	2	43	52.51
D	1	27	8.96
	2	43	42.18
	3	70	18.68
	4	80	17.07
	5	83	13.11
E	3	72	60.48
	4	81	39.52

*The band numbers refer to specific hR_f values in all fractions.

**See legend Table 3.

TABLE 5

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN THE BANDS FROM FRACTIONS C AND D AS SEPARATED IN PW.

Fraction	Band (IAW)	hR_f (PW)	Metabolite	Distribution of Radioactivity Within Band (%)
C	1	0	*ND	21.11
		28	NADP	31.85
		**37	ND	31.93
		44	ND	2.61
		69	des-NAD	4.54
		83	Nicotinamide	7.96
C	2	64	NAD	100
D	1	8	ND	39.58
		15	ND	24.39
		28	NaMN (NADP)	23.25
		59	des-NAD	7.45
		77	Nicotinic acid	5.33
D	2	26	NMN	15.09
		36	ND	13.00
		61	NAD	63.36
		77	Nicotinamide	8.55
D	3	***79	Nicotinamide	100

*ND - Not identified

**C_1-hR_f 37 - may have been NaMN resulting from the hydrolysis of des-NAD

***D_3-hR_f 79 - was identified as nicotinamide by paper chromatography in BA and IAW solvent systems and silica gel TLC.

However, nicotinamide was also concentrated in D_5-hR_f 83 as shown in Table 6 .

Therefore, the former compound may have been a nicotinamide-containing compound resulting from nucleotide hydrolysis.

This distribution pattern, not previously noted,(1) may have been due to the acetone-precipitation procedure.

TABLE 6

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN BANDS 4 AND 5 FROM FRACTION D AS SEPARATED IN BUTANOL-AMMONIA

Fraction	Band (IAW)	hR_f (in butanol-ammonia)	Metabolite	Distribution of Radioactivity Within Band (%)
D	*4	16	Nicotinic acid	94.44
		58	Nicotinamide	5.56
D	*5	17	Nicotinic acid	16.49
		58	Nicotinamide	83.51

*Bands 4 and 5 contained predominantly nicotinic acid and nicotinamide, respectively. Cross contamination occurred because bands were not entirely separated in IAW.

is indicated in Table 7. These compounds accounted for 1.63×10^6 dpm of the 7.77×10^6 dpm of nicotinamide-7-^{14}C administered.

TABLE 7

THE PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN PYRIDINE-CONTAINING COMPOUNDS AFTER INCUBATION OF *CLAVICEPS* 47A WITH NICOTINAMIDE-7-^{14}C.

Compound	Distribution of Recovered Radioactivity (%)
Nicotinamide	36.37
Nicotinic acid	25.56
NaMN	2.03
NMN	3.55
des-NAD	0.87
NAD	18.43
NADP	1.50
Not-identified	11.69

b) Study With Homogenized Cultures of Claviceps 47A

i) Homogenization of the Mycelium With Growth Medium

Instead of incubating an intact culture of Claviceps 47A with nicotinamide-7-^{14}C, the mycelium was first homogenized in a chilled, sterile, stainless steel Waring micro-blendor in the growth medium and was then incubated with shaking for six hours at 24°C. with nicotinamide-7-^{14}C (S.A. 10.7 mc/mM; 46.7 muM; 1.11 x 10^6 dpm). Analysis indicated that 85.5% of the initial activity was located in the supernatant, fraction A. Nicotinic acid accounted for 9% of the recovered radioactivity and nicotinamide accounted for 81% of the recovered activity. Trace amounts of NMN and NAD were detected in fraction A, indicating that NMN pyrophosphorylase may function in NAD biosynthesis in Claviceps.

ii) Homogenization of the Mycelium With Phosphate Buffer

In a variation of the above procedure, the mycelium of a culture of Claviceps 47A was first homogenized in 25 ml. 0.05M phosphate (KH_2PO_4-Na_2HPO_4), pH 7.4, buffer (54), hereafter, referred to as "phosphate buffer". The homogenate was then incubated with shaking for six hours at 24°C. with nicotinamide-7-^{14}C (S.A. 10.7 mc/mM; 46.7 muM; 1.11 x 10^6 dpm). The following cofactors were added to the incubation medium (5,7):

ATP	(1.4 uM/ml.)	0.1 ml.
PRPP	(2 uM/ml.)	0.1 ml.
$MgCl_2$	(20.5 uM/ml.)	0.1 ml.
Glucose	(0.56 M/ml.)	0.2 ml.
Glutamine	(20 uM/ml.)	0.1 ml.

Analysis showed that 75.08% of the initial activity was recovered in the supernatant, fraction A. Traces of NMN and NAD were detected by paper chromatography, but 67.31% of the activity was recovered as nicotinic acid and 31.65% as nicotinamide. The high concentration of nicotinic acid in the phosphate medium suggested that deamidation of nicotinamide occurred during the incubation. This finding prompted an endeavour to isolate and purify nicotinamide deamidase from Claviceps.

B. Partial Purification And Characterization Of Nicotinamide Deamidase From *Claviceps*

1. Materials and Methods

Organisms

The organisms used in this study were:

a) *Claviceps*, strain 47A, provided by Dr. R.C.S. Audette, University of Manitoba.

b) *Claviceps*, strain SD58, obtained from Dr. H.G. Floss, University of Purdue, Lafayette, Indiana, U.S.A.

Liquid Culture Medium

Claviceps, strain 47A, was grown on a modified Abe's (1) nutrient medium while *Claviceps*, strain SD58, was grown on a medium of the following composition:

Mannitol	50 Gm.
Sucrose	50 Gm.
KH_2PO_4	0.1 Gm.
$MgSO_4.7H_2O$	0.3 Gm.
$FeSO_4.7H_2O$	10 mg.
$ZnSO_4.7H_2O$	4.4 mg.
Yeast Extract	3 Gm.
Succinic Acid	5.4 Gm.
Distilled Water to make	1 litre

The medium was adjusted to pH 5.4 with ammonium hydroxide (52).

The media were sterilized and the organisms were propagated as previously described.

Studies to Determine Experimental Conditions Required to Demonstrate High Nicotinamide Deamidase Activity

The following fractionation procedure is summarized in Figure 7. All operations were carried out at about 5°C. unless otherwise specified. Single 30-day-old cultures of Claviceps 47A were homogenized in either 0.25 M. sucrose, 0.1 M. phosphate buffer, pH 7.4, or 0.05M. phosphate buffer, pH 7.4, by either of the following methods:

a) In a Potter homogenizer for 20 minutes.

b) In a Lourdes grinder for 3 minutes.

c) In a Waring micro-blender for 2 minutes.

d) With a wedgewood mortar and pestle plus sand for 5 minutes (mycelium: sand-3:1 w/w).

The crude homogenate was filtered through nylon and the filtrate was centrifuged at 900 x g. for 6 minutes at 0°C. The pellet was washed with the respective homogenization medium, described above, and recentrifuged. The pellet, consisting of cellular debri, intact cells and nuclei was retained as fraction I. The supernatants were combined and recentrifuged at 1000 x g. for 12 minutes. The pellet, consisting of nuclei, was washed, recentrifuged, and retained as fraction II. The combined supernatants were centrifuged at 25,000 x g. for 20 minutes to sediment the mitochondria. The pellet was washed, recentrifuged, and was retained as fraction III. Initially, the supernatant was retained as fraction IV and analyzed for nicotinamide deamidase activity, but subsequently, it was centrifuged at 130,000 x g. for 60 minutes in order to sediment the microsomal fraction. The microsomal fraction was washed, recentrifuged, and retained as fraction V while the

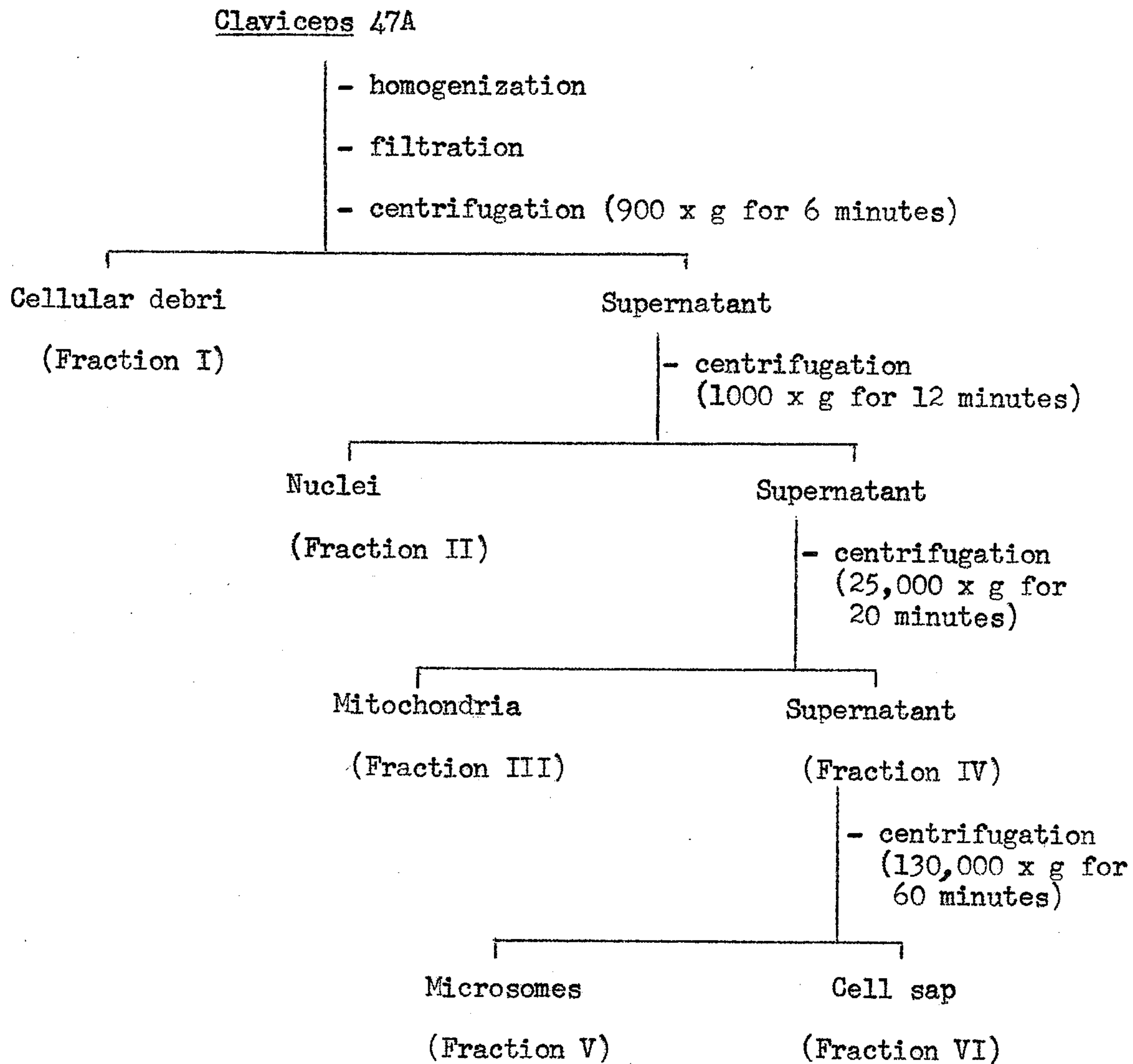

Figure 7. Summary of Fractionation Procedure of Claviceps 47A.

superantant was kept as fraction VI.

For the determination of nicotinamide deamidase activity in the fractions, aliquot portions of the pellets were suspended in 10 ml. volumes of homogenization medium containing the specified cofactors and nicotinamide-7-^{14}C, and the preparations were incubated for specific periods of time ranging from 15 minutes to 6 hours at 37°C. The fractions IV and VI were treated similarly, except no additional homogenization medium was added before the incubation. After the incubation period, the preparations were immersed in boiling water for 3 minutes. The resulting precipitates were then removed by centrifugation at 25,000 x g. for 20 minutes. Aliquot portions of the supernatants were counted and chromatographed on Whatman 3 MM paper in the BA and IAW solvent systems. Radioactive nicotinamide and nicotinic acid were located, identified, and determined quantitatively by the procedures previously described. The amount of nicotinamide-7-^{14}C which was converted to nicotinic acid-7-^{14}C after incubation was taken as a measure of nicotinamide deamidase activity in a fraction.

Nicotinamide Deamidase Purification Procedure

a) Assay of Enzyme Activity

The enzyme preparations obtained at each step of the purification procedure were assayed for deamidase activity.

The standard enzyme assay mixtures contained 0.25 ml. of the enzyme preparation in 0.05 M. phosphate buffer, pH 7.4, and nicotinamide-7-^{14}C in varying concentrations, viz.: 0.8 ugm; 1.6 ugm; 2.4 ugm; 3.2 ugm; 4.0 ugm; 4.8 ugm; 5.6 ugm. A blank control consisted of the buffer and nicotinamide-7-^{14}C (2.4 ugm.). The preparations were

incubated with slow shaking at 37°C for 30 minutes. The reaction was terminated by immersing the incubation tubes in boiling water for 3 minutes. The precipitated protein was removed by centrifugation and a 0.1 ml. aliquot of the supernatants was chromatographed on paper in butanol:ammonia. The amount of nicotinamide-7-^{14}C deamidated to nicotinic acid-7-^{14}C, as determined by counting the respective radioactive spots on the developed chromatograms, was taken to be a measure of the deamidase activity.

The amount of deamidation occurring in each assay mixture was determined. The enzyme deamidated nicotinamide at varying rates, depending upon the concentration of nicotinamide-7-^{14}C (i.e. 0.8 ugm; 1.6 ugm; 2.4 ugm; 3.2 ugm; 4.0 ugm; 4.8 ugm; 5.6 ugm). The deamidase activity of the enzyme was calculated on the basis of the assay mixture which demonstrated the highest rate of deamidation. The unit of activity was defined as the amount of enzyme (protein) required to convert 0.01 ugm (0.082 muM) of nicotinamide-7-^{14}C to nicotinic acid-7-^{14}C in 30 minutes under the given conditions. Specific activity was expressed as units per mg. of protein.

b) <u>Preliminary Purification Procedures</u>

Initially attempts were made to purify nicotinamide deamidase from <u>Claviceps</u>, strain 47A. The 130,000 x g. supernatant, as prepared above using phosphate buffer, was employed as the crude enzyme extract. The extract was subjected to ammonium sulphate precipitation and calcium phosphate gel treatment as employed by Joshi and Handler (25). However, these procedures completely destroyed the enzyme activity.

Treatment of nicotinamide deamidase preparations of <u>Claviceps</u>,

strain SD58, with ammonium sulphate also denatured the enzyme. Attempts to employ charcoal, as employed by Petrack, et al, (33), to purify the deamidase did not produce an active enzyme. However, the enzyme was partially purified by the procedure indicated below.

c) Nicotinamide Deamidase Purification

Claviceps SD58, which was obtained during the course of the study, was employed instead of Claviceps 47A as a source of nicotinamide deamidase because the former organism grew more rapidly than the latter. (Claviceps SD58 demonstrated maximum alkaloid production in 15 days while Claviceps 47A required 37 days to attain maximum alkaloid production.)

All procedures were carried out at about 5°C unless otherwise indicated. The mycelium of 15-day-old cultures of Claviceps, strain SD58, was ground with sand (3:1 w/w) and phosphate buffer in a wedgewood mortar. The homogenate was appropriately filtered and centrifuged, by the procedure previously outlined, to yield a final 130,000 x g. supernatant which was employed as the crude enzyme extract. An aliquot portion was retained for protein analysis, by the method of Lowry (49), and for assay of nicotinamide deamidase activity as previously described (page 32).

The crude extract was dialyzed employing cellophane tubing (Cenco) for six hours at 5°C. against 12 l. of distilled water. The dialysate was frozen and lyophilyzed and the residue was dissolved in a minimum volume of phosphate buffer. The resulting preparation was subjected to Sephadex G-75-120 (Sigma) gel filtration. (A 35 x 2 cm. column was previously washed with 300 ml. phosphate buffer.) The column was eluted with phosphate buffer, to produce forty 5 ml. fractions.

Each fraction was assayed for protein content and enzyme activity. Figure 8 demonstrates the protein content and the relative amount of deamidase activity in each fraction. The fractions with significant enzyme activity were combined. Suitable aliquots of the solution were retained for protein and enzyme activity analysis while the remaining volume was subjected to acetone precipitation at -15°C. (25). Nine ml. of acetone at -15°C. were added per 13 ml. of solution containing 1 mg. protein/ml. The mixture was allowed to stand for 60 minutes with frequent stirring, and the resulting precipitate was removed by centrifugation at 23,000 x g. for 20 minutes. The precipitate was found to be inactive. An additional 14 ml. portion of acetone was added to the supernatant, the mixture was allowed to stand for 120 minutes with frequent stirring, and the resulting precipitate was removed by centrifugation at 23,000 x g. for 30 minutes. The nicotinamide deamidase-containing precipitate was dried in vacuo and was dissolved in phosphate buffer. The protein concentration and deamidase activity of this fraction were determined. After the preparation had been frozen for 24 hours, an inactive precipitate formed which was removed by centrifugation at 23,000 x g. for 30 minutes.

A portion of the supernatant was subjected to paper electrophoresis, employing Whatman 3MM paper in phosphate buffer, at 210 volts for 140 minutes. Protein bands were located by spraying strips of the electrophoresis papers with 0.3% ninhydrin in 95% ethanol. The bands were individually eluted from the paper with minimum volumes of phosphate buffer. The resulting solutions were assayed for nicotinamide deamidase activity.

Since only minute quantities of active nicotinamide deamidase

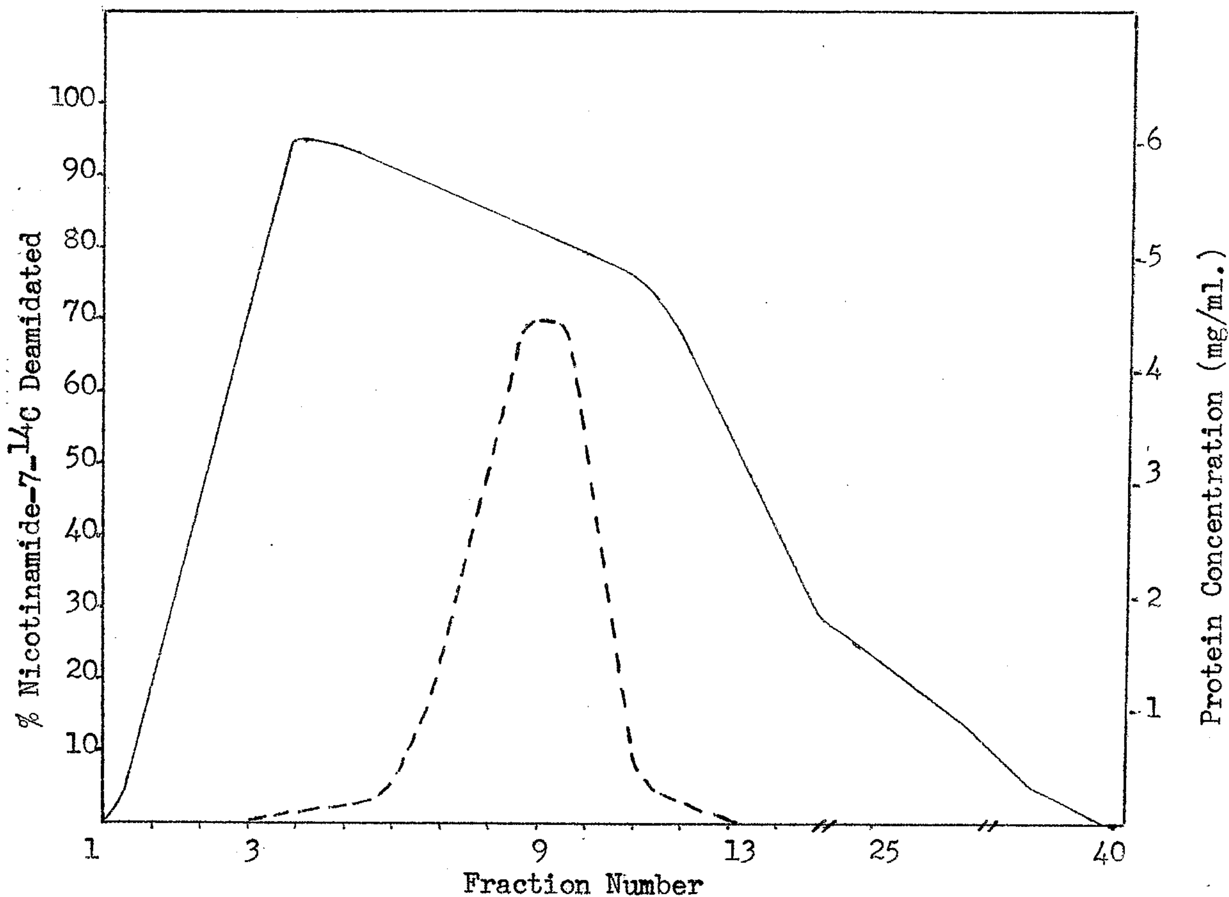

Figure 8. Concentration of Protein and Location of Nicotinamide Deamidase Activity in 5 ml. Fractions Collected From a Sephadex G-75-120 Gel Column (35 x 2 cm.).

___Protein concentration (mg/ml.).

-Determined for each of the forty 5 ml. fractions collected.

---Location of nicotinamide deamidase

-Based on the % nicotinamide-7-^{14}C (4 ugm.) deamidated by 0.25 ml. of each fraction in 30 minutes at 37°C.

could be recovered by means of electrophoresis, the enzyme solution remaining after the "freezing precipitation" became the object of enzyme characterization.

The Km of the deamidase preparation was determined by incubating a specific amount of protein with increasing concentrations of nicotinamide-7-^{14}C for 30 minutes at 37°C and determining the amount of nicotinamide-7-^{14}C converted to nicotinic acid-7-^{14}C.

The effect of pH on nicotinamide deamidase activity was determined by incubating a specific amount of protein with a specific amount of nicotinamide-7-^{14}C at varying pH. The samples were acidified appropriately with 1% HCl and basified with 1% NaOH.

The nicotinamide deamidase activity in the incubation mixture at different temperatures was determined.

The effect of the presence of metals, EDTA, and compounds, such as, nicotinic acid, NAD, NADP, NMN, des-NAD, and N-methylnicotinamide on nicotinamide deamidase activity was determined. These substances were incubated with the enzyme preparation in the presence of nicotinamide-7-^{14}C and deamidation was observed.

Finally the specificity of the deamidase was studied by incubating the enzyme with NAD-^{14}C, NADP, and NMN-^{14}C. Deamidation products of these compounds were determined by the paper chromatographic procedures which were previously recorded for pyridine-containing compounds.

2. Experimental Results

a) Conditions for Nicotinamide Deamidase Activity

Preliminary studies indicated that most satisfactory fractions were obtained by grinding the cultures in a mortar with sand. In the initial experiments, before the necessary cofactors for the enzyme

reaction were known, the compounds previously mentioned (page 27) were added to each incubation mixture. Nicotinamide-7-^{14}C (S.A.7.63 mc/mM; 32.7 muM; 5.55 x 10^5 dpm) was incubated with 10 ml. of the prepared fractions in all cases. Under all experimental conditions investigated, employing cellular fractions, the only radioactive pyridine-containing compounds which were detected were nicotinamide-7-^{14}C and nicotinic acid-7-^{14}C. That is, none of the fractions studied produced any intermediates of the pyridine nucleotide biosynthetic cycle even after 6 hours of incubation.

In all preliminary experiments the nicotinamide deamidase activity was found to be concentrated primarily in fraction IV (i.e. 25,000 x g. supernatant) after 105 minutes of incubation at 37°C. Later experiments demonstrated some deamidase activity in fraction II (i.e. nuclei) while most of it was found in fraction VI (i.e. 130,000 x g. supernatant), the soluble portion of the cell.

The effect of different homogenization and incubation media on the extent of conversion of nicotinamide-7-^{14}C to nicotinic acid-7-^{14}C is shown in Table 8. The presence of sucrose appeared to inhibit the deamidase activity while the presence of phosphate was found to be essential for significant deamidation to occur. However, excess phosphate (0.1M) appeared to cause inhibition of enzyme activity, as was demonstrated by fractions II and IV.

The effect of possible cofactors in the 10 ml. incubation medium on deamidase activity in the various fractions is indicated in Table 9. The results suggested that none of the cofactors employed were required for the enzyme reaction to occur.

TABLE 8

THE PERCENTAGE CONVERSION OF *NICOTINAMIDE-7-^{14}C TO NICOTINIC ACID-7-^{14}C

IN VARIOUS HOMOGENIZATION MEDIA

**Homogenization Medium / Fraction	10 ml. 0.25 M Sucrose	7ml. 0.25 M Sucrose + 3 ml. Phosphate Buffer	10 ml. 0.25 M Sucrose + 0.001 M $MgCl_2$	10 ml. 0.1 M Phosphate Buffer pH7.4	10 ml. 0.05 M Phosphate Buffer	10 ml. 0.05 M Phosphate Buffer + 0.001 M $MgCl_2$
I -cells, cellular debri	***2.4	3.4	3.3	4.3	7.9	4.2
II -nuclei	2.1	2.1	5.7	4.9	5.6	15.2
III-mitochondria	0	1.2	0	--	--	1.3
IV -cell sap + microsomes	3.6	--	12.0	16.0	91.3	95.1
V -microsomes	--	4.2	1.3	--	--	0
VI -cell sap	--	30.9	34.8	--	--	95.8

*Nicotinamide-7-^{14}C (S.A. 7.63 mc/mM; 32.7 muM; 5.55×10^5 DPM)
**The fractions were incubated in the homogenization media indicated.
***The numbers included refer to the percentage of nicotinamide-7-^{14}C converted to nicotinic acid-7-^{14}C in 105 minutes at 37°C
--Fractions not studied

TABLE 9

THE PERCENTAGE CONVERSION OF NICOTINAMIDE-7-^{14}C TO NICOTINIC ACID-7-^{14}C IN THE PRESENCE AND ABSENCE OF COFACTORS

Incubation Medium / Fraction	0.25 M Sucrose + 0.001 M $MgCl_2$ (10 ml)		Phosphate Buffer + 0.001 M $MgCl_2$ (10 ml)	
	Presence of Cofactors**	Absence of Cofactors	Presence of Cofactors	Absence of Cofactors
A-cells, cellular debri	*3.3	4.9	4.2	4.9
B-nuclei	5.7	---	15.2	---
C-mitochondria	0	0	1.3	5.1
D-cell sap + microsomes	12.0	16	95.1	93.0
E-microsomes	1.3	0.5	0	-----
F-cell sap	34.8	33.6	95.8	94.8

*The numbers included refer to the percentage of nicotinamide-7-^{14}C (S.A. 7.63 mc/mM; 32.7 muM; 5.55 x 10^5 dpm) converted to nicotinic acid-7-^{14}C.

**The cofactors employed/10 ml. incubation mixture are listed on page 27.

---Fractions not studied under the given conditions.

b) Nicotinamide Deamidase Purification

In a typical experiment nicotinamide deamidase was partially purified from 10 cultures of 15-day- old *Claviceps*, strain SD58. The cultures were initially homogenized employing sand with 65 ml. of phosphate buffer, and appropriately centrifuged and washed to produce 110 ml. of crude enzyme extract (i.e. 130,000 x g. supernatant). The dialyzed extract was frozen and lyophilized, the residue was dissolved in 3 ml. phosphate buffer, and the solution was subjected to sephadex filtration. Fractions seven to eleven inclusive contained most of the enzyme activity and were combined to produce 29 ml. of solution. Twenty-six ml. of the enzyme preparation (1 mg. protein/ml.) were treated with 18 ml. of acetone at -15°C. The resulting precipitate was inactive and the supernatant was further treated with 28 ml. of acetone to produce an active precipitate. The precipitate was dried and then dissolved in 10 ml. of phosphate buffer. Freezing of this preparation for 24 hours resulted in the formation of an inactive precipitate which was removed by centrifugation. One and a half ml. of the final active solution were subjected to paper electrophoresis. One protein band migrated 16 mm. from the origin toward the anode. Three bands migrated toward the cathode at the following distances from the origin: 9 mm., 20 mm., 39 mm.

The electrophoretic migration pattern is illustrated in Figure 9 . Incubation of the eluted protein from the bands with nicotinamide-7-^{14}C (1.11×10^5 dpm.) for one hour indicated that all deamidase activity was associated with the band which moved 9 mm. from the origin. Accurate protein assays could not be carried out due to a lack of enzyme preparation. The purification procedure is summarized in Table 10.

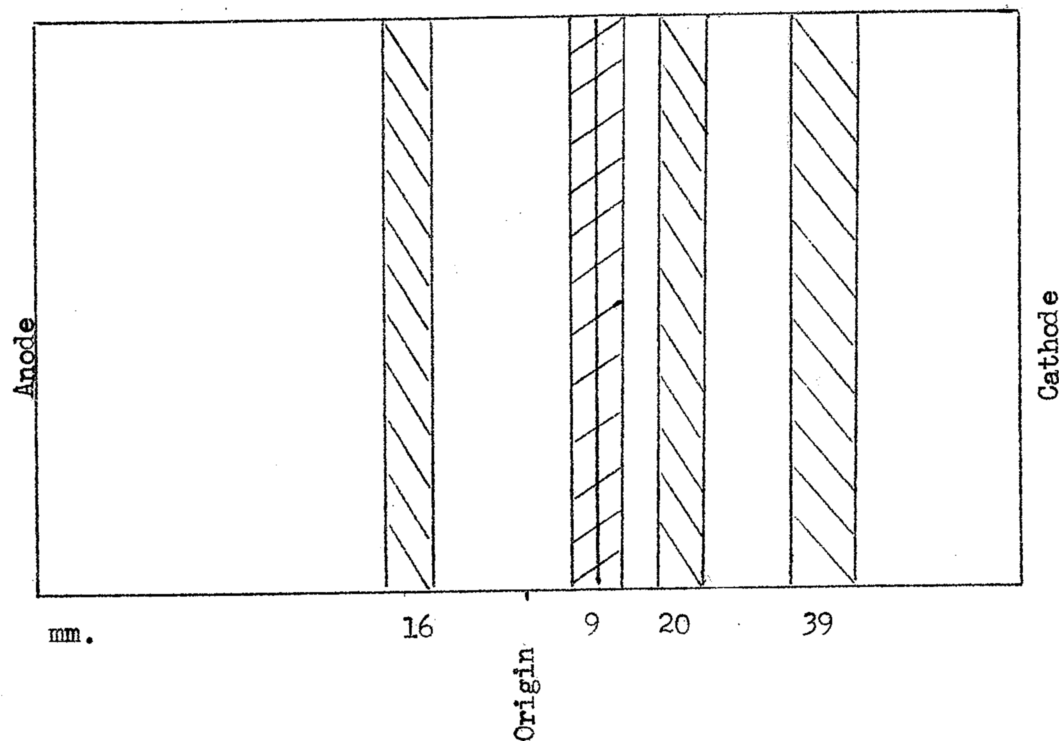

Figure 9. Separation of Protein Constituents of the 67-Fold Enzyme Preparation by Paper Electrophoresis.

-Protein band containing nicotinamide deamidase.

Whatman 3 MM paper was employed in 0.05M phosphate buffer pH 7.4; 210 volts; 140 minutes. The bands were located by spraying half inch strips of the paper with 0.3% ninhydrin in 95% ethanol.

TABLE 10

SUMMARY OF PURIFICATION OF NICOTINAMIDE DEAMIDASE FROM

CLAVICEPS, STRAIN SD58

Procedure	Volume (ml.)	Protein (mg.)	Specific Activity (units/mg.)	Units	Purification (-fold)
Crude extract	110	330.0	35.3	11,649	1
Sephadex filtration	29	28.42	653.6	18,575	18.52
Acetone precipitation	10	6.04	1777.8	10,738	50.36
Freezing precipitation	10	3.13	2364.1	7,400	66.97
Electrophoresis - deamidase activity detected in a protein band which migrated toward the anode (Figure 9)					

Specific Activity - expressed as units/mg. protein.

Unit - defined as the amount of enzyme required to convert 0.01 ugm. (0.082 muM) of nicotinamide-7-^{14}C to nicotinic acid-7-^{14}C in 30 minutes under the given conditions.

Total units= mg. (protein) x specific activity

Purification= specific activity of fraction ÷ specific activity of crude extract.

c) <u>Enzyme Characterization</u>

The 67-fold purified enzyme preparation was characterized as indicated below.

i) <u>Km</u>

The results of incubating 0.1 ml. or 31.3 ugm. of protein preparation with increasing concentrations of nicotinamide-7-^{14}C are summarized in Table 11.

The Km of the enzyme preparation was determined to be 1.087 x 10^{-7}M from a Lineweaver and Burk (40) plot as is illustrated in Figure 10. The plot is an average of four trials. The deamidase activity of the enzyme at each step of purification appeared to be inhibited by high concentrations of nicotinamide.

ii) <u>pH</u>

Figure 11 indicates the effect of incubating 0.1 ml. or 31.3 ugm. of the enzyme with 2.0 ugm. of nicotinamide-7-^{14}C for 30 minutes at 37°C. at varying pH. The optimum pH was found to be about 7.4. The deamidase activity was significantly inhibited in acid media while it maintained relatively high activity over a pH range of 7.4-11.

iii) <u>Temperature</u>

The effect of temperature on nicotinamide deamidase activity is illustrated in Figure 12. Samples of 0.1 ml. or 31.3 ugm. of enzyme were incubated with 2.0 ugm. of nicotinamide-7-^{14}C for 30 minutes at varying temperatures. The optimum deamidase activity was noted at about 50°C. When the enzyme preparation was heated at 100°C. for 2 minutes before incubation with nicotinamide-7-^{14}C no deamidation occurred. Freezing the enzyme preparation for one month did not cause

TABLE 11

THE AMOUNT OF NICOTINAMIDE-7-^{14}C DEAMIDATED BY 31.3 UGM.

OF ENZYME PREPARATION IN 30 MINUTES

Sample Number	*(S) Amount of Nicotinamide-7-^{14}C Added (ugm.)	**(V) Amount of Nicotinamide-7-^{14}C Deamidated (ugm.)
1	0.80	0.490
2	1.60	0.590
3	2.00	0.650
4	2.40	0.580
5	3.20	0.720
6	4.00	0.500
7	4.80	0.460

*S = substrate concentration in terms of ugm. nicotinamide-7-^{14}C (S.A. 7.63 mc/mM) added.

**V = velocity of the reaction in terms of the number of ugm. nicotinamide-7-^{14}C deamidated in 30 minutes under the given conditions (based on radioactivity).

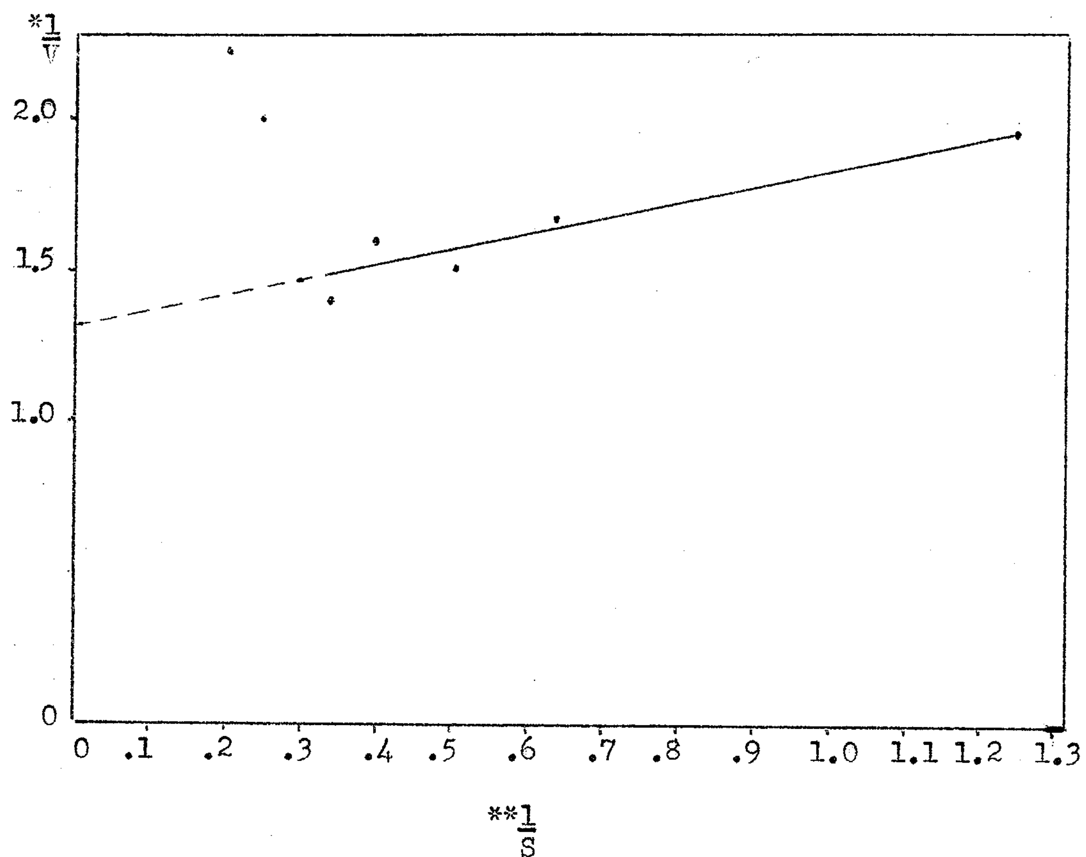

Figure 10. Lineweaver and Burk (40) Plot Employed to Calculate Km of Nicotinamide Deamidase.

$*\frac{1}{V}$ = the positive reciprocal of the substrate concentration

$**\frac{1}{S}$ = the positive reciprocal of the substrate concentration

Km Calculation:

Slope of extrapolated curve $= \frac{Km}{Vmax}$ (40) = 0.548

$\frac{1}{Vmax}$ = 1.32 (distance from (0,0) to the point of intersection of the line and the vertical axis .)

Km = 0.4154 ugm-based on 31.3 ugm protein
Km = 13.272 ugm or 1.087×10^{-7} M.-based on 1 mg. protein

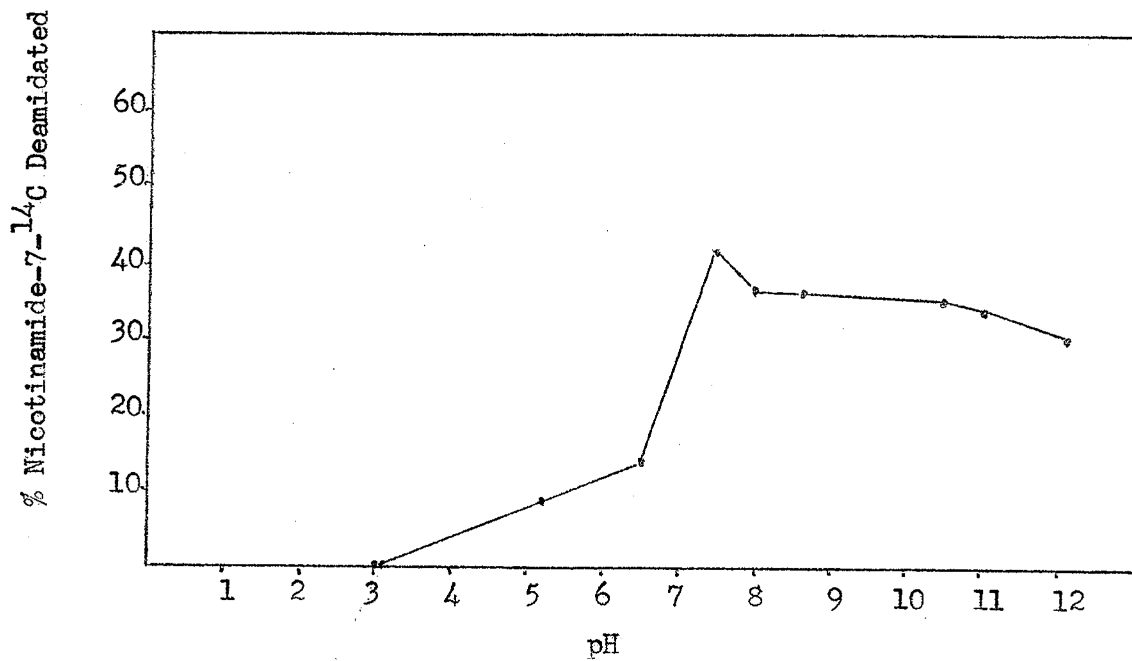

Figure 11. The Effect of pH on Nicotinamide Deamidase Activity

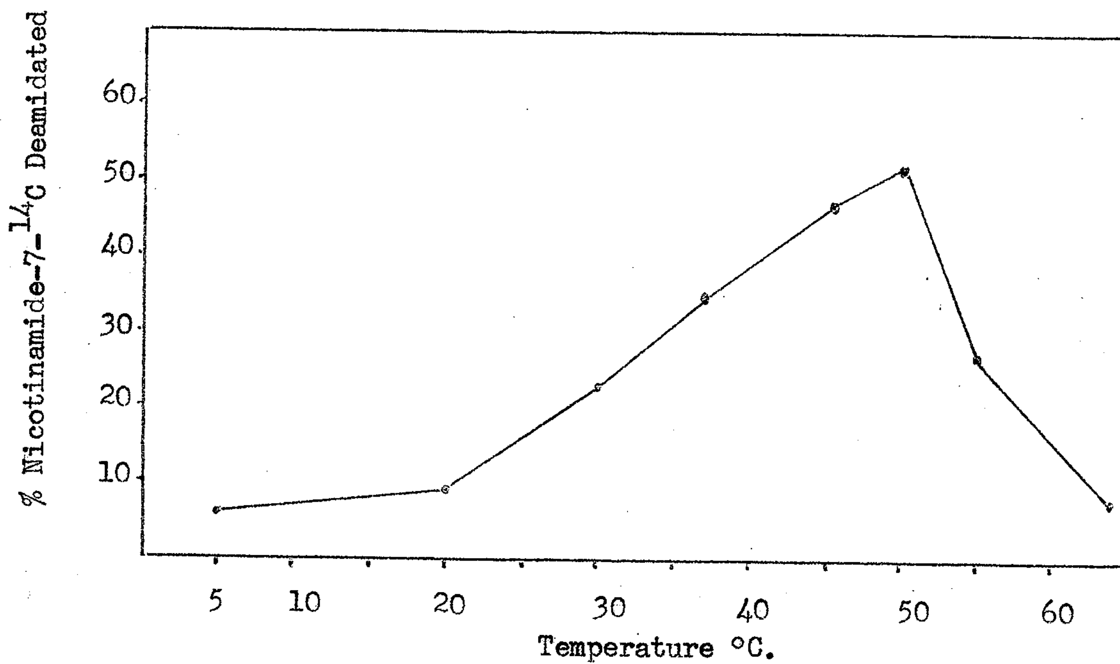

Figure 12. The Effect of Temperature on Nicotinamide Deamidase Activity

denaturation.

iv) Effect of Metals

Various metals were added to the incubation mixtures of 31.3 ugm. protein and 2.0 ugm. of nicotinamide-7-^{14}C in a concentration of 0.005 M (25). The effects of the metals on deamidation are depicted in Table 12. The results are an average of two trials. All the metals studied caused some inhibition of enzyme activity, with Hg^{++} and Cu^{++} causing 100% inhibition. Magnesium, when employed as a cofactor, did not alter deamidase activity.

v) Effect of Metal Chelating Agent

The addition of 0.002 M (25) EDTA to the incubation mixture of 31.3 ugm. of protein and 2.0 ugm. of nicotinamide-7-^{14}C stimulated the enzyme activity. In control samples 22.10% of the nicotinamide was deamidated while the presence of EDTA caused 25.22% deamidation. These values are averages of three trials.

vi) Effect of NAD-Cycle Intermediates

Mixtures of 31.3 ugm. of enzyme preparation, 2.0 ugm. of nicotinamide-7-^{14}C, and 0.6 ugm or 1.2 ugm. quantities of specified compounds were incubated for 30 minutes at 37°C. The amount of deamidation of nicotinamide-7-^{14}C occurring in the presence of these compounds is indicated in Table 13. All the pyridine nucleotides studied inhibited the enzyme activity to some extent, with NAD and NADP being most effective. N-methylnicotinamide and nicotinic acid did not cause significant inhibition.

vii) Enzyme Specificity

Nicotinamide deamidase did not deamidate NAD-^{14}C, NADP, and

TABLE 12

EFFECT OF METALS ON NICOTINAMIDE DEAMIDASE ACTIVITY

Metal	% Nicotinamide-7-^{14}C Deamidated	% Inhibition
Hg^{++}	0	100
Cu^{++}	0.91	97.33
Fe^{+++}	22.70	33.37
Mn^{++}	29.82	12.47
Ca^{++}	31.57	7.38
Na^{+}	33.27	2.35
Control*	34.07	0

*Incubation mixture consisted of the following:

Protein (31.3 ugm. in 0.1 ml. Phosphate Buffer)

Nicotinamide-7-^{14}C (S.A. 7.63 mc/mM; 2.0 ugm; 2.78×10^5 dpm).

The metals were added in 0.1 ml. phosphate buffer to produce a final 0.005 M. concentration.

TABLE 13

EFFECT OF NAD-CYCLE INTERMEDIATES ON NICOTINAMIDE DEAMIDASE ACTIVITY

Compound Added	% Nicotinamide-7-^{14}C Deamidated	
	0.6 ugm of added compound	1.2 ugm of added compound
NAD	20.71	20.95
NADP	20.29	19.59
des-NAD	21.39	---
NMN	21.42	---
Nicotinic Acid	21.72	24.03
N-methylnicotinamide	22.09	---
Control*	22.10	24.35

---concentration not studied

*Incubation mixture consisted of the following:

Protein (31.3 ugm. in 0.1 ml. Phosphate Buffer)

Nicotinamide-7-^{14}C (S.A. 7.63 mc/mM; 2 ugm; 2.78 x 10^5 dpm).

The compounds were added in 0.1 ml. phosphate buffer.

NMN-^{14}C. The enzyme preparation was also incubated with asparagine and glutamine but conclusive results could not be obtained. The enzyme did not catalyze the amidation of nicotinic acid to form nicotinamide.

III. DISCUSSION

The preliminary studies on the biosynthesis of NAD in Claviceps, strain 47A, were undertaken (i) to produce radioactive pyridine-containing compounds which could be used in subsequent studies with nicotinamide deamidase; (ii) to determine whether pyridine mono-and dinucleotides of Claviceps could be effectively separated by acetone precipitation (48); (iii) to determine whether nicotinic acid-7-^{14}C could be produced by incubating intact and homogenized cultures of Calviceps with nicotinamide-7-^{14}C.

After incubating 30-day-old Claviceps 47A with nicotinamide-7-^{14}C for six hours, 14% of the administered activity was located in the incubation medium while 76% was in the mycelium. The radioactivity in the medium consisted of nicotinic acid-7-^{14}C (21%) and nicotinamide-7-^{14}C (79%). The pyridine nucleotides were confined to the mycelium. In a similar study Audette (1) employing 28-day-old Claviceps 47A, reported that 50% of the activity remained in the incubation medium and 44% in the mycelium. Nicotinamide-7-^{14}C was the only compound detected in the medium. The 2-day age difference between the cultures in the two studies may have accounted for these variations. Audette (1) reported that alkaloid and NAD content of Claviceps 47A varied significantly with the age of the cultures.

Pyridine-containing compounds reported by Audette (1) to be involved in NAD biosynthesis in Claviceps were identified and isolated chromatographically as indicated in Tables 4-6. The isolated NAD-^{14}C, and NMN-^{14}C were employed subsequently, in the determination of

nicotinamide deamidase specificity. N-methylnicotinamide was not detected.

The acetone precipitation procedure of Shuster and Goldin (48) concentrated NADP-^{14}C and NAD-^{14}C into the precipitated fraction and NMN-^{14}C into the acetone-soluble fraction. The acetone-soluble fraction was contaminated with relatively high concentrations of NADP-^{14}C, NAD-^{14}C, and des-NAD-^{14}C. Therefore, the acetone precipitation of dinucleotides does not appear to have been complete. No evidence of nicotinamide mononucleotide contamination, however, could be found in the dinucleotide fraction.

Studies indicated that the entire, intact culture of _Claviceps_ was required in order for the NAD biosynthesis cycle to proceed. Phosphate appeared to be essential for deamidation of nicotinamide-7-^{14}C to occur in homogenized cultures. Since no significant amounts of nucleotides could be identified after incubation of homogenized cultures with nicotinamide-7-^{14}C and since a high conversion to nicotinic acid-7-^{14}C was detected, it is suggested that nicotinamide deamidase functions in _Claviceps_. (No evidence was obtained to indicate that NAD glycohydrolase was actively present in _Claviceps_ to catalyze an exchange reaction between nicotinamide and NAD).

Preliminary studies indicated that nicotinamide deamidase of _Claviceps_ was recovered in the soluble portion of the cell when either 0.05 M phosphate buffer or 0.25 M sucrose was used to homogenize the cultures. Although the former medium may have disrupted some of the subcellular organelles and thus released any enclosed nicotinamide deamidase into the cytoplasm, the latter medium should not readily

damage these structures. A portion of the activity did appear to be associated with the nuclei. Sarma, et al, (26) reported that the nicotinamide deamidase of Aspergillus niger was also confined to the soluble portion of the cell.

Nicotinamide deamidase of Claviceps appeared to require the presence of phosphate for optimum activity. Other compounds which were examined as cofactors were not essential. Techniques of sephadex filtration and acetone precipitation resulted in a 67-fold purified enzyme preparation from Claviceps SD58.

Sephadex filtration of the crude dialyzed enzyme preparation caused a significant increase in the total units of enzyme activity. This could have been due to the removal of enzyme inhibitors as was also reported by Kimura (24).

The acetone-precipitated product was separated into four protein bands by electrophoresis, and the deamidase activity was found to be concentrated in one protein band which migrated toward the cathode. However, the specific activity of this preparation could not be determined due to the minute quantity recovered.

Although a linear curve could not be definitely determined on a Lineweaver and Burk (40) plot, the Km of the Claviceps nicotinamide deamidase was calculated to be 1.087×10^{-7}M. This value is significantly lower than 1.4×10^{-5}M, determined for the enzyme of Torula cremoris, (25) and 6.5×10^{-4}M determined for the enzyme of A. niger (26). Therefore, it is suggested that the nicotinamide deamidase of Claviceps could function efficiently at very low concentrations of nicotinamide. The Lineweaver and Burk (40) plot indicated that the enzyme was

inhibited by relatively high concentrations of nicotinamide.

Thus nicotinamide deamidase could be a rate-limiting enzyme in NAD biosynthesis from nicotinamide in Claviceps. Upon excessive hydrolysis of NAD to form relatively large amounts of nicotinamide, the deamidase may be inhibited. Therefore, the Preiss and Handler (6.7) NAD biosynthetic cycle would be inhibited. During periods when the organism would require large amounts of NAD, hydrolysis of NAD would be retarded, the nicotinamide concentration would decline, the rate of deamidation would increase, and, accordingly, the Preiss and Handler pathway could function. Petrack, et al, (33) suggested that nicotinamide deamidase was a rate-limiting enzyme in pyridine nucleotide biosynthesis in rat liver.

Highest deamidase activity was noted at pH 7.4. This is similar to the optimum pH of the enzyme from other sources (24-26). Acid conditions inhibited the enzyme and, therefore, it is suggested that the enzyme could not be purified by means of ammonium sulphate precipitation because of the resulting low pH. The enzyme retained relatively high activity from pH 7.4 to 11.

The deamidase activity was inhibited significantly at low temperatures and demonstrated maximum activity at about 50°C. Denaturation occurred very rapidly at temperatures above 50°C. The deamidases from other sources (24,26) underwent denaturation at temperatures above 40°C. The deamidase of T. cremoris (25) also showed optimal activity at 50°C.

Various metals at 0.005 M concentration caused significant enzyme inhibition, similar to that reported for deamidases of other sources

(24,25). The Hg^{++} and Cu^{++} ions, which produced complete inhibition, precipitated the protein of the enzyme during incubation. Since Na^{+} produced 2.35% inhibition, it is likely that the enzyme was also inhibited by the Na^{+} which was present in the 0.05M phosphate buffer which was used throughout the study. This is supported by the fact that 0.002 M EDTA stimulated enzyme activity in the 0.05 M phosphate buffer incubation mixture.

The EDTA-stimulation of deamidase activity is in contrast to the inhibitory effect of metal chelating agents on nicotinamide deamidase of Mycobacterium avium (24) and A. niger (26). Joshi and Handler (25) reported that EDTA had no effect on nicotinamide deamidase activity of T. cremoris.

As indicated in Table 13, NAD, NADP, des-NAD, NMN, and nicotinic acid all caused inhibition of nicotinamide deamidase activity. NAD and NADP were the most effective inhibitors, indicating that concentrations of these dinucleotides in Claviceps may to some extent control the amount of nicotinamide utilized in NAD biosynthesis via nicotinic acid. Nicotinic acid, the immediate end-product of the deamidation did not appear to inhibit the reaction significantly in the concentrations studied.

Nicotinamide deamidase of Claviceps did not deamidate NAD-^{14}C, NADP, or NMN-^{14}C, and, therefore, appeared to be specific for nicotinamide under the experimental conditions. In addition, the deamidation reaction was found not to be reversible.

The following procedures could possibly be employed to obtain a more highly purified enzyme preparation:

1) Sephadex G-150-40 (Sigma) gel filtration.
   - A higher grade of sephadex may yield a more efficient protein separation than Sephadex G-75-120 which was used in this study.
2) Isoelectric focusing (53).
   - Could be employed after acetone precipitation to yield protein separation based on isoelectric values.
3) Large scale, high voltage electrophoresis.
4) Recrystallization.

IV. SUMMARY

Studies have indiacted that nicotinamide deamidase is active in the biosynthesis of pyridine nucleotides in Claviceps. The enzyme was partially purified and characterized. The enzyme appeared to be associated primarily with the soluble portion of the cell and was dependent upon the presence of phosphate. The Km of the enzyme was determined to be 1.087×10^{-7}M. The enzyme demonstrated maximum activity at pH 7.4 and 50°C. Various metal ions inhibited enzyme activity while EDTA stimulated deamidation. Pyridine-containing compounds, other than nicotinamide, associated with the pyridine nucleotide biosynthetic cycle, inhibited enzyme activity. The enzyme did not deamidate NAD-^{14}C, NADP, or NMN-^{14}C and did not amidate nicotinic acid to form nicotinamide.

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