NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE OF CLAVICEPS

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ABSTRACT

THE PRESENT STUDIES HAVE INDICATED THAT CULTURES OF CLAVICEPS SD-58 COULD CONVERT NICOTINAMIDE-7-^{14}C INTO THE PYRIDINE MONO - AND DINUCLEOTIDES WHICH WERE SEPARATED BY AN ACETONE PRECIPITATION METHOD.

CULTURES OF CLAVICEPS SD-58 INCUBATED SIMULTANEOUSLY WITH NICOTINAMIDE 7-^{14}C AND ^{32}P RESULTED IN AN INCREASE IN THE SPECIFIC ACTIVITY OF THE NMN-^{14}C PORTION (NICOTINAMIDE-^{14}C) OF NAD FOR THREE CONCENTRATIONS OF NICOTINAMIDE (10^{-6}M, 10^{-5}M AND 10^{-4}M). THE SPECIFIC ACTIVITY OF THE NMN-^{32}P PORTION (RIBOSE-5-PHOSPHATE-^{32}P) FROM THE SAME NAD REMAINED CONSTANT OVER THE RANGE OF CONCENTRATIONS OF NICOTINAMIDE. SIMILAR STUDIES EMPLOYING NICOTINIC ACID-7-^{14}C AND ^{32}P PRODUCED SIMILAR CHANGES IN SPECIFIC ACTIVITY OF THE NMN-^{14}C (NICOTINAMIDE-^{14}C) AND NMN-^{32}P (RIBOSE-5-PHOSPHATE-^{32}P) FOR THE 10^{-6}M AND 10^{-5}M CONCENTRATIONS OF NICOTINIC ACID. HOWEVER, THE 10^{-4}M CONCENTRATION OF NICOTINIC ACID PRODUCED AN INCREASED SPECIFIC ACTIVITY IN THE NMN-^{14}C PORTION AND A SLIGHT DECREASE IN THE NMN-^{32}P SPECIFIC ACTIVITY. THESE SIMULTANEOUS ^{32}P: NICOTINAMIDE-7-^{14}C AND ^{32}P: NICOTINIC ACID-7-^{14}C INCORPORATION STUDIES INDICATED THAT THE NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE ENZYME SYSTEM WAS ACTIVE IN THE BIOSYNTHETIC PATHWAY OF PYRIDINE NUCLEOTIDE SYNTHESIS IN CLAVICEPS SD-58.

THE NAD GLYCOHYDROLASE WAS FOUND TO BE ASSOCIATED WITH THE NUCLEAR AND MICROSOMAL FRACTIONS OF CULTURES OF CLAVICEPS SD-58.

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LIST OF ABBREVIATIONS

AMP	-ADENOSINE MONOPHOSPHATE
ATP	-ADENOSINE TRIPHOSPHATE
CPM	-COUNTS PER MINUTE
DES-NAD	-DES AMINO NICOTINAMIDE ADENINE DINUCLEOTIDE
DPM	-DISINTEGRATIONS PER MINUTE
G	-GRAVITY
M	-MOLAR
MM	-MILLI-MOLE
MUM	-MILLI-MICRO-MOLE
NAD	-NICOTINAMIDE ADENINE DINUCLEOTIDE
NADᵗASE	-NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE
NADP	-NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE
NAMN	-NICOTINIC ACID MONONUCLEOTIDE
NMN	-NICOTINAMIDE MONONUCLEOTIDE
P	-INORGANIC PHOSPHATE
P-P	-PYROPHOSPHATE
PRPP	-PHOSPHO-RIBOSYL-PYROPHOSPHATE
P.S.I.	-POUNDS PER SQUARE INCH
TRIS	-TRIS (HYDROXYMETHYL) AMINO ETHANE
S.A.	-SPECIFIC ACTIVITY
UGM	-MICROGRAM

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I. INTRODUCTION

METABOLIC PROCESSES IN LIVING ORGANISMS DEPEND UPON A NUMBER OF FACTORS, ONE OF WHICH IS ACTIVE ENZYME SYSTEMS. THE MOST STRIKING BIOCHEMICAL ROLE OF THESE ENZYMES IS THEIR ABILITY TO AFFECT, IN A SPECIFIC AND EFFICIENT MANNER, THE WIDE SPECTRUM OF RATES OF REACTIONS THAT CONSTITUTE THE LIFE PROCESS. COINCIDENTALLY, HOWEVER, THESE ENZYMES MAY BE SUSCEPTIBLE TO A WIDE VARIETY OF CONTROLS. THEREFORE, IN ORDER TO DETERMINE THE COMPLETE BIOSYNTHETIC PATHWAY OF A COMPOUND OR A GROUP OF COMPOUNDS, IT IS NECESSARY TO STUDY THE INDIVIDUAL ENZYMES WHICH CATALYZE THE INDIVIDUAL REACTIONS THROUGHOUT THIS PATHWAY.

SUCH A GROUP OF COMPOUNDS ARE THOSE INVOLVED IN THE NICOTINAMIDE ADENINE DINUCLEOTIDE (*NAD) BIOSYNTHETIC PATHWAY. THE COMPOUNDS AND THE ASSOCIATED ENZYMES WHICH ARE INVOLVED IN THE NAD BIOSYNTHETIC PATHWAY HAVE BEEN STUDIED IN NUMEROUS LIVING SYSTEMS (1-9).

AUDETTE (1) HAS SHOWN THAT SAPHROPHYTIC CULTURES OF CLAVICEPS, STRAIN 47A, A CLAVINE ALKALOID PRODUCING FUNGUS, AND CLAVICEPS PURPUREA, STRAIN CPM, A NON-ALKALOID PRODUCING FUNGUS, COULD UTILIZE NICOTINIC ACID AND NICOTINAMIDE TO PRODUCE MONO-AND DINUCLEOTIDES OF NICOTINIC ACID AND NICOTINAMIDE.

AFTER INCUBATION OF THE FUNGAL CULTURES WITH NICOTINIC ACID OR NICOTINAMIDE, THE ISOLATED COMPOUNDS IDENTIFIED INCLUDED NICOTINIC ACID MONONUCLEOTIDE (NAMN), NICOTINIC ACID ADENINE DINUCLEOTIDE (DES-NAD), NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD), NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADP), NICOTINAMIDE MONONUCLEOTIDE (NMN), AND

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*SEE LIST OF ABBREVIATIONS, PAGE III.

N-METHYL NICOTINAMIDE.

THE FOLLOWING NAD BIOSYNTHETIC PATHWAY WAS PROPOSED TO OCCUR IN THE CLAVICEPS SPECIES STUDIED (FIGURE 1).

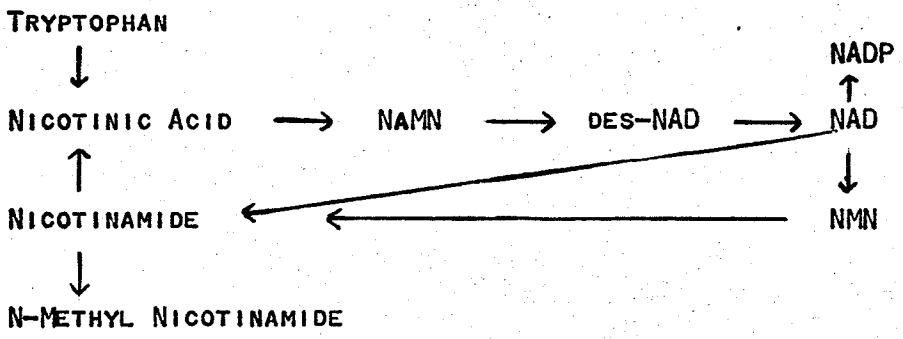

FIGURE 1. SUGGESTED PATHWAY OF NAD BIOSYNTHESIS IN CLAVICEPS (1).

THE CONVERSION OF NICOTINAMIDE TO NICOTINIC ACID THROUGH A DEAMIDATION PROCESS IN CLAVICEPS WAS CONFIRMED BY SIEMENS (10). THE ENZYME, NICOTINAMIDE DEAMIDASE, REQUIRED FOR THIS CONVERSION, WAS PURIFIED BY MEANS OF DIALYSIS, SEPHADEX-GEL FILTRATION AND ACETONE PRECIPITATION. THE Km OF THE ENZYME WAS FOUND TO BE 1.087×10^{-7}M AND IT DEMONSTRATED MAXIMUM ACTIVITY AT pH 7.4 AND 50°C. THE DEAMIDATION PROCESS WAS FOUND NOT TO BE REVERSIBLE. AUDETTE (1) SUGGESTED THE ABSENCE OF THE NMN PYROPHOSPHORYLASE SYSTEM BUT THE ABSENCE OR PRESENCE OF THE NAD-GLYCOHYDROLASE SYSTEM (NAD'ASE (2)) WAS NOT ESTABLISHED. THE PRESENT STUDY WAS UNDERTAKEN IN AN ATTEMPT TO DETERMINE WHETHER NAD-GLYCOHYDROLASE (2) WAS INVOLVED IN THE BIOSYNTHETIC PATHWAY OF NAD IN CLAVICEPS.

A. BIOSYNTHESIS OF NAD

SEVERAL SYNTHETIC PATHWAYS REGARDING THE BIOSYNTHETIC FORMATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD) IN BIOLOGICAL SYSTEMS HAVE BEEN PROPOSED. INITIALLY, ROWEN AND KORNBERG (11) OBSERVED THAT AN ENZYME

FROM HOG LIVER COULD PROMOTE THE FOLLOWING REACTION:

$$\text{Nicotinamide} + \text{Ribose-1-phosphate} + H^+ \rightleftharpoons \text{Nicotinamide Riboside} + P$$

(WHERE P REPRESENTS INORGANIC PHOSPHATE). KORNBERG (12, 13) ALSO DESCRIBED AN ENZYME WHICH CATALYZED THE FOLLOWING REACTION:

$$ATP + \text{Nicotinamide riboside} \longrightarrow NMN + ADP.$$

HOWEVER, IT WAS DOUBTFUL AS TO WHETHER THIS ENZYME PLAYED A PART IN NAD SYNTHESIS (12). IN 1950, KORNBERG (13) DISCOVERED AND PURIFIED NAD PYROPHOSPHORYLASE FROM HOG LIVER AND BREWER'S YEAST. THIS ENZYME PROMOTES THE FOLLOWING REVERSIBLE REACTION:

$$ATP + NMN \rightleftharpoons NAD + PP$$

(WHERE PP REPRESENTS PYROPHOSPHATE). SINCE THE EQUILIBRIUM CONSTANT FOR THIS ENZYME REACTION WAS FOUND TO BE 0.45, IT CONTRIBUTED MORE TO THE BREAKDOWN OF NAD THAN TO ITS SYNTHESIS.

IN 1951, LEDER AND HANDLER (3) REPORTED THAT THE INCUBATION OF WASHED HUMAN ERYTHROCYTES WITH NICOTINAMIDE LED TO A SIGNIFICANT ACCUMULATION OF NMN. PREISS AND HANDLER (4) FOUND THAT HUMAN ERYTHROCYTES, IN VITRO, UTILIZED LOW CONCENTRATIONS OF NICOTINIC ACID FOR THE SYNTHESIS OF THE NUCLEOTIDES; AND ALSO INDICATED THAT NICOTINAMIDE COULD REACT WITH PRPP (PHOSPHORIBOSYL PYROPHOSPHATE) IN THE PRESENCE OF NMN PYROPHOSPHORYLASE TO FORM NMN (5). THE NMN PYROPHOSPHORYLASE ISOLATED FROM THE HUMAN ERYTHROCYTES, HOWEVER, WAS RESTRICTED IN DISTRIBUTION AND ALSO EXHIBITED A HIGH Km VALUE WHICH THE AUTHORS (5) CONCLUDED WOULD PRECLUDE EFFECTIVE SYNTHESIS UNDER THE PHYSIOLOGICAL CONDITIONS DESCRIBED.

PREISS AND HANDLER IN 1958 (6) OBSERVED THAT AFTER INCUBATION OF NICOTINIC ACID -7-^{14}C WITH HUMAN ERYTHROCYTES AND WITH AN ACETONE POWDER

PREPARED FROM HUMAN ERYTHROCYTES TWO RADIOACTIVE NUCLEOTIDES, NAMN AND DES-NAD COULD BE IDENTIFIED. SUBSEQUENTLY, THEY DETERMINED THAT YEAST AUTOLYSATES COULD UTILIZE NICOTINIC ACID BUT NOT NICOTINAMIDE TO PRODUCE NAD (7).

ON THE BASIS OF THEIR STUDIES, PREISS AND HANDLER SUGGESTED THE FOLLOWING GENERAL PATHWAY FOR NAD BIOSYNTHESIS FROM NICOTINIC ACID (FIGURE 2).

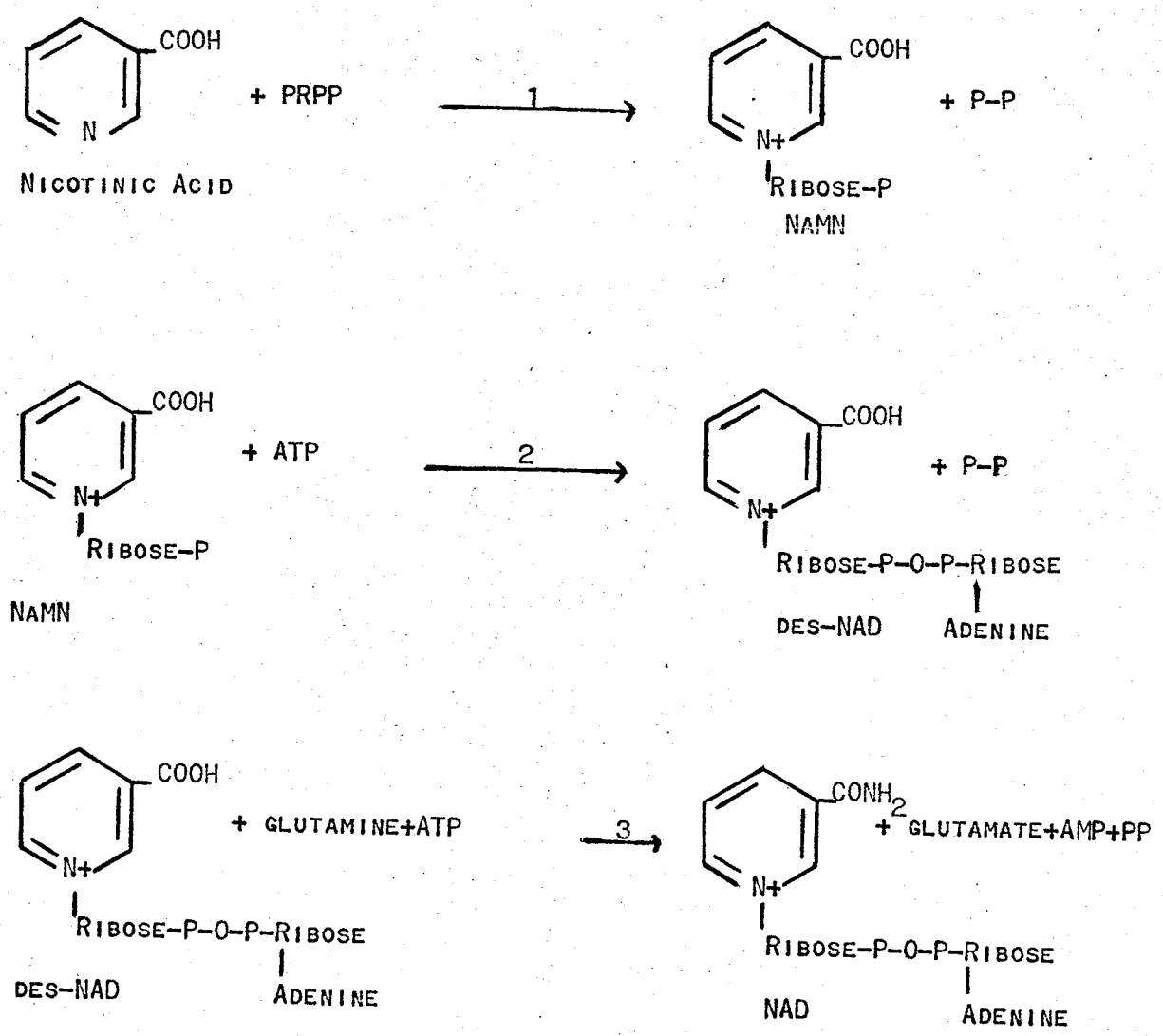

FIGURE 2. THE PREISS AND HANDLER PATHWAY OF NAD BIOSYNTHESIS (6,7).

THE ENZYMES (2) WHICH CATALYZE THE REACTIONS AS SHOWN IN FIGURE 2 HAVE BEEN IDENTIFIED, VIZ.:

REACTION 1: NICOTINATE PHOSPHORIBOSYLTRANSFERASE (NICOTINATE-NUCLEOTIDE PYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE, E.C. 2.4.2.11);

REACTION 2: NICOTINATEMONONUCLEOTIDE ADENYLYLTRANSFERASE (ATP: NICOTINATEMONOMUCLEOTIDE ADENYLTRANSFERASE, E.C. 2.7.7.18) OR NAD PYROPHOSPHORYLASE (ATP: NMN ADENYLTRANSFERASE, E.C. 2.7.7.1);

REACTION 3: NAD SYNTHETASE (DEAMIDO-NAD: L-GLUTAMINE AMIDO-LIGASE (AMP), E.C. 6.3.5.1.

A SUMMARY OF NAD BIOSYNTHESIS STUDIES IN VARIOUS BIOLOGICAL SYSTEMS FOLLOWS.

1. ERYTHROCYTES IN VITRO

IN VITRO STUDIES (14,15) HAVE SHOWN THAT HUMAN, MONKEY, GUINEA-PIG, FROG AND RABBIT ERYTHROCYTES PRODUCE THE NICOTINAMIDE CONTAINING NUCLEOTIDES (PYRIDINE NUCLEOTIDES) FROM BOTH NICOTINIC ACID AND NICOTINAMIDE. HOWEVER, THE RATE OF INCORPORATION OF EITHER NICOTINIC ACID OR NICOTINAMIDE WAS FOUND TO VARY WITH THE SPECIES.

2. LIVER AND KIDNEY IN VITRO

INVESTIGATIONS BY SARMA ET AL (16) SUGGEST THAT MOUSE LIVER COULD DEAMIDATE THE NICOTINAMIDE MOIETY OF NMN AND THAT LIVER PREPARATIONS OF VARIOUS AVIAN SPECIES (DOVE, PIGEON, PARROT, AND DUCK) COULD DEAMIDATE NICOTINAMIDE BY MEANS OF THE NICOTINAMIDE DEAMIDASE ENZYME SYSTEM. SARMA ET AL (16) SUGGESTED THE FOLLOWING NAD BIOSYNTHETIC PATHWAY (FIGURE 3).

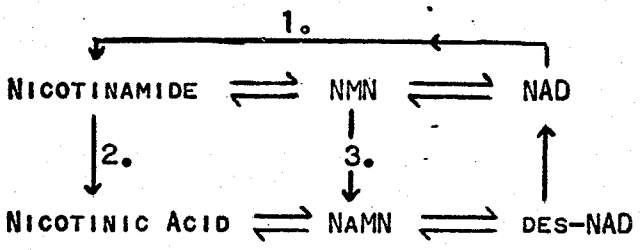

FIGURE 3. BIOSYNTHETIC PATHWAY OF NAD AS SUGGESTED BY SARMA ET AL (16).

SARMA ET AL (16) SUGGESTED THAT REACTION 1, FIGURE 3, WAS MEDIATED BY THE ENZYME NAD GLYCOHYDROLASE (E.C.3.2.2.5.); REACTION 2 WAS MEDIATED BY THE ENZYME NICOTINAMIDE DEAMIDASE AND REACTION 3 WAS MEDIATED BY NMN DEAMIDASE.

3. MOUSE LIVER IN VIVO

REPORTS BY LANGAN ET AL (15), NARROD ET AL (17) AND HAYAISHI ET AL (18) 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.

4. TUMOR CELLS

DIETRICH ET AL (8, 19) OBSERVED THAT EHRLICH-LETTRE ASCITES CELLS INCORPORATED BOTH NICOTINIC ACID AND NICOTINAMIDE INTO NAD.

5. MICROORGANISMS

THE "PREISS AND HANDLER PATHWAY" OF NAD BIOSYNTHESIS HAS BEEN SHOWN TO EXIST IN YEAST (7) AND IN ESCHERICHIA COLI (20). IMSANDE (20) FOUND THAT THE PROPERTIES OF THE ENZYMES REQUIRED FOR SYNTHESIS OF NAD IN E. COLI WERE ESSENTIALLY THE SAME AS THOSE FOUND IN MAMMALIAN TISSUE AND YEAST. HOWEVER, AMMONIA WAS FOUND TO BE A BETTER AMIDE DONOR THAN GLUTAMINE FOR THE FORMATION OF NAD.

IN THE FUNGUS, CLAVICEPS, NAD BIOSYNTHESIS IS PROPOSED TO TAKE

PLACE AS SHOWN IN FIGURE 1.

6. PLANTS

WALLER AND CO-WORKERS (21) HAVE OBSERVED THE FOLLOWING REACTIONS IN *RICINUS COMMUNIS* L.:

A) NICOTINAMIDE $\xrightarrow{\text{IN VIVO}}$ NICOTINIC ACID + N-METHYL NICOTINAMIDE

B) NICOTINIC ACID $\xrightarrow{\text{IN VITRO}}$ NAD + N-METHYL NICOTINAMIDE

C) NAD $\xrightarrow{\text{IN VIVO}}$ NICOTINAMIDE + N-METHYL NICOTINAMIDE

ALSO, NICOTINIC ACID HAS BEEN REPORTED TO BE A PRECURSOR OF NAD IN RICE SEEDLINGS. (22)

FROST *ET AL* (23) OBSERVED THAT NICOTINIC ACID, QUINOLINIC ACID AND NAD SERVED AS PRECURSORS OF NICOTINE IN *NICOTIANA RUSTICA*.

WAYGOOD *ET AL* (24) ALSO HAVE SHOWN THAT NICOTINIC ACID AND NICOTINAMIDE WERE INCORPORATED INTO THE PYRIDINE NUCLEOTIDES; NICOTINIC ACID RIBOSIDE, NMN, NAD, DES-NAD AND NADP, AND TRIGONELLINE IN WHEAT LEAVES.

B. NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE

1. DEFINITION

NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE (E.C.3.2.2.5) (NADⁱ ASE, NAD NUCLEOSIDASE) INDUCES THE HYDROLYSIS OF NAD (2) ACCORDING TO THE SCHEMATIC REACTION INDICATED: WHERE N REPRESENTS NICOTINAMIDE, R REPRESENTS RIBOSE, P REPRESENTS PHOSPHATE AND A REPRESENTS ADENINE.

$$\overset{+}{\text{NRPPRA}} + H_2O \longrightarrow \text{ARPPR} + \text{N} + H^+$$

IN REALITY, THE ENZYME NADⁱASE BESIDES POSSESSING HYDROLYTIC ACTIVITY, POSSESSES TRANSGLYCOSIDASE ACTIVITY; THAT IS, IT CAN PROMOTE THE TRANSFER

OF THE ARPPR GROUP.

THE ABILITY OF THE NADᵀASES TO CATALYZE THE EXCHANGE REACTION BETWEEN NAD AND FREE NICOTINAMIDE, OR STRUCTURALLY RELATED COMPOUNDS, CREATES THE POSSIBILITY OF FORMING STRUCTURAL ANALOGS OF NAD (25).

2. INVESTIGATIONS OF NAD GLYCOHYDROLASE ACTIVITY

A) MICROORGANISMS

I) NEUROSPORA CRASSA

IN 1951, NASON ET AL (26) FOUND THAT NADᵀASE LEVELS INCREASED 10-20 FOLD WHEN NEUROSPORA CRASSA WAS GROWN ON A ZINC-DEFICIENT MEDIUM, AS COMPARED TO N. CRASSA GROWN ON A ZINC-CONTAINING MEDIUM. BIOTIN DEFICIENCY WAS ALSO FOUND TO PROMOTE A SIMILAR INCREASE IN NADᵀASE LEVELS. THE NAD GLYCOHYDROLASE ENZYME FROM N. CRASSA, PURIFIED BY KAPLAN ET AL (27), WAS FOUND TO HYDROLYZE THE NICOTINAMIDE RIBOSIDE LINKAGE OF NAD. THE ENZYME WAS FOUND TO BE ACTIVE OVER A pH RANGE, 3 TO 9; WAS NOT INFLUENCED BY METAL BINDING AGENTS OR METAL IONS AND WAS NOT INHIBITED BY THE PRESENCE OF NICOTINAMIDE.

ALTHOUGH THERE IS NO DIRECT EVIDENCE TO SUGGEST THAT THE GLYCOHYDROLASE ENZYME FROM N. CRASSA MAY POSSESS TRANSGLYCOSIDASE ACTIVITY, GROSSMAN AND KAPLAN (36) HAVE SHOWN THAT ERGOTHIONINE COULD INDUCE NICOTINAMIDE SENSITIVITY TO THE NADᵀASE FROM N. CRASSA.

II) MYCOBACTERIUM

KERN AND NATALE (28) ISOLATED A NAD GLYCOHYDROLASE ENZYME FROM MYCOBACTERIUM BUTYRICUM WHICH HAD SIMILAR CHARACTERISTICS TO THE NADᵀASE ENZYME ISOLATED FROM NEUROSPORA. THE ISOLATED ENZYME REQUIRED HEAT ($100^{o}C$ FOR 1 MINUTE) FOR ACTIVATION AND IT PROMOTED THE CLEAVAGE OF NAD AT THE NICOTINAMIDE-RIBOSIDE LINKAGE. THE ENZYME, HOWEVER, DID NOT

CATALYZE THE "NICOTINAMIDE EXCHANGE REACTION".

M. BUTYRICUM NAD'ASE PROMOTES THE CLEAVAGE OF NAD AND NADP; EXHIBITED A BROAD pH OPTIMUM (pH5 TO pH9) AND WAS NOT INHIBITED BY THE PRESENCE OF NICOTINAMIDE. THE Km VALUE FOR NICOTINAMIDE AS DETERMINED BY SUZUKI (29) WAS 1.6×10^{-3}M.

THE NAD'ASE ENZYME HAS ALSO BEEN CHARACTERIZED FROM MYCOBACTERIUM TUBERCULOSIS $H_{37}Rv$ (30-32). IN VITRO STUDIES WITH CRUDE CELL-FREE EXTRACTS OF M. TUBERCULOSIS $H_{37}Rv$, HAVE SHOWN THAT THE ENZYME IS FIRMLY BOUND TO, OR IN A COMPLEX WITH, AN INHIBITOR. LIKE M. BUTYRICUM, NAD'ASE FROM M. TUBERCULOSIS H_{37} Rv MUST BE HEAT ACTIVATED. HOWEVER, IT DIFFERS IN THAT IT HAS VERY WEAK ACTIVITY (Km FOR NAD WAS 0.33 mM AND FOR NADP - 0.31 mM); IT EXHIBITS MAXIMUM ACTIVITY AT pH 6.5 AND REACTS EQUALLY WELL WITH NAD OR NADP. THE NAD GLYCOHYDROLASE FROM M. TUBERCULOSIS $H_{37}Rv$ EXHIBITED NO "EXCHANGE REACTION" CAPABILITIES.

THE IN VIVO STUDIES WITH M. TUBERCULOSIS $H_{37}Rv$ (32) INDICATED THAT THE NAD GLYCOHYDROLASE ENZYME WAS IN AN ACTIVE STATE COMPARED WITH AN INHIBITED STATE IN CELL-FREE EXTRACTS IN VITRO.

III) STREPTOCOCCI

LAZARIDES AND BERNHEIMER (33) OBSERVED THAT NAD GLYCOHYDROLASE WAS AN EXTRACELLULAR PRODUCT OF GROWTH OF CERTAIN STRAINS OF STREPTOCOCCI BELONGING TO GROUPS A, C AND G BUT NOT OF OTHER STREPTOCOCCI. AMONG THE STREPTOCOCCI BELONGING TO GROUP A, C OR G EXAMINED, SOME BUT NOT ALL STRAINS PRODUCED AN EXTRACELLULAR ENZYME WHICH WAS CAPABLE OF CATALYZING THE CLEAVAGE OF NAD AT THE NICOTINAMIDE-RIBOSE LINKAGE.

TABLE 1 SHOWS THAT NAD'ASE ACTIVITY IS ASSOCIATED ONLY WITH PARTICULAR SEROLOGICAL TYPES.

Type	Frequency of Production of NADᵗase by selected types of Group A Streptococci		
	Previously Tested	Newly Tested	Total
1	0/8	0/25	0/33
3	5/5	18/18	23/23
4	13/14		13/14
5	1/3	0/19	1/22
6	3/3	17/17	20/20
12	52/56	0/2	52/58
14	0/1	0/12	0/13
19	0/3	0/35	0/38
24		0/14	0/14

Table 1. Frequency of Production of NADᵗase by Selected Types of Group A Streptoccus (33).

Streptococci of types 3, 4, 6 and 12 produced NADᵗase with regularity while types 1, 5, 14, 19 and 24, did not produce the enzyme. NAD glycohydrolase isolated from Streptococci pyogenes, Group A, strain 4 (34) was found to be heat labile, exhibited maximum activity in the pH range 6.5 - 8.5 and had a Km value of 4.8×10^{-4}M.

iv) Bacillus subtillus

Investigations with Bacillus subtillus (35) have shown that this organism contained an active NADᵗase capable of splitting the pyridine coenzyme at the nicotinamide riboside linkage. Like M. butyricum, the NADᵗase from B. subtillus required heat for activation and was associated with a heat-labile protein inhibitor.

v) Aspergillus niger

Sarma et al (37) have shown that Aspergillus niger contained the NAD glycohydrolase enzyme system. The enzyme isolated was found to be in the 100,000 x g supernatant; exhibited highest activity over a pH range of 7.5 - 9.0; was active only towards NAD and NADP and did not attack NMN, N-methylnicotinamide or NADH. NADᵗase from A. niger had a Km value for NAD of 7.963×10^{-6}M and did not require a metal ion for activation.

Fehrenbach (38) has also suggested that Aspergillus contains an active NAD glycohydrolase enzyme system.

Evidence for the presence of the NAD glycohydrolase enzyme system has also been reported for Chromabacterium violaceum (28, Proteus vulgaris X-19 (strain #6380) (35), and yeast (39).

B) Vertebrates

Enzyme inactivation of NAD in animal tissues has been known from the early studies of Von Euler et al (40) in 1937 and Lennerstrand (41) in 1936. Since these initial investigations, there has been extensive work done on the characterization, purification and determination of the role of the NAD glycohydrolase enzyme system in vertebrates. Unlike the majority of the NADᵗase isolated from microbial sources which hydrolyze the nicotinamide-riboside linkage of NAD, the animal NAD glycohydrolases have the capacity to catalyze two different reactions: the hydrolysis of NAD at the nicotinamide riboside linkage to form adenosine diphosphate ribose and nicotinamide or the transfer of the adenosine diphosphate ribose (ADPR) moiety of NAD to pyridine derivatives such as nicotinamide, acetyl-pyridine, thionicotinamide. (Exchange Reaction) (42).

INITIAL OBSERVATIONS BY MANN AND QUASTEL (43) AND HANDLER AND KLEIN (44, 45) HAVE SHOWN THAT NICOTINAMIDE INHIBITED THE CLEAVAGE OF NAD AT THE NICOTINAMIDE RIBOSIDE LINKAGE. ZATMAN ET AL (46) HAVE FURTHER SHOWN THAT THE NICOTINAMIDE INHIBITION WAS DUE TO AN "EXCHANGE" BETWEEN FREE NICOTINAMIDE AND THE BOUND NICOTINAMIDE OF NAD. THIS "EXCHANGE" IS ILLUSTRATED BELOW WHERE N* REPRESENTS NICOTINAMIDE LABELLED WITH ^{14}C IN THE CARBOXAMIDE CARBON.

NRPPRA (NAD) + N* ⟶ N* RPPRA + N

NAD GLYCOHYDROLASE HAS BEEN FOUND WIDELY DISTRIBUTED IN ANIMAL TISSUES WITH THE LUNG AND SPLEEN POSSESSING THE HIGHEST CONCENTRATIONS (49). THE NADᵀASE ACTIVITY HAS BEEN REPORTED TO BE ASSOCIATED CHIEFLY WITH THE MICROSOMAL FRACTION, ALTHOUGH ACTIVITY HAS ALSO BEEN FOUND IN THE NUCLEAR FRACTION (47-50). THE ASSOCIATION OF THE NAD GLYCOHYDROLASE SYSTEM IN THE MICROSOMAL FRACTION OF MAMMALIAN TISSUES HAS LED TO PROBLEMS IN SOLUBILIZATION. DEOXYCHOLATE, DIGITONIN AND SONIC TREATMENT HAVE PROVED RELATIVELY UNSUCCESSFUL FOR SOLUBILIZATION PROCEDURES. HOWEVER, USE OF LIPOLYTIC AND PROTEOLYTIC ENZYMES PRODUCES A SOLUBLE PREPARATION WITH MAXIMUM ACTIVITY (51-55).

SWISLOCKI AND KAPLAN (51) HAVE SHOWN THAT NAD GLYCOHYDROLASE FROM PIG BRAIN IS ASSOCIATED WITH THE MICROSOMAL PARTICLES. THE NADᵀASE OBTAINED HAD AN APPROXIMATE MOLECULAR WEIGHT OF 26,800, EXHIBITED CONSTANT ACTIVITY OVER A pH RANGE OF 5.5 TO 8.0 AND SHOWED SPECIFICITY ONLY FOR OXIDIZED NAD AND NADP (ACTION ON NADP WAS ONLY 45% THAT OF NAD).

SWISLOCKI ET AL (51) ALSO FOUND THAT THE NAD GLYCOHYDROLASES ISOLATED FROM VARIOUS ORGANS (I.E. BRAIN, LIVER, SPLEEN, LUNG) OF A PARTICULAR MAMMALIAN SPECIES HAD SIMILAR MOLECULAR WEIGHTS (SEE TABLE 2).

ORGAN	BRAIN	LIVER	SPLEEN	LUNG
SPECIES	MOLECULAR WEIGHT	MOLECULAR WEIGHT	MOLECULAR WEIGHT	MOLECULAR WEIGHT
PIG	26,000	25,000	25,000	23,000
BEEF	24,000	-	23,000	-
SHEEP	25,000	-	-	-
DOG	50,000	55,000	-	-
MOUSE	80,000	80,000	-	-
RAT	70,000	85,000	-	-
HORSE	85,000	90,000	80,000	-
RABBIT	85,000	85,000	-	-
HUMAN	-	90,000	-	-

- UNDETERMINED

TABLE 2. THE APPROXIMATE MOLECULAR WEIGHTS OF NAD GLYCOHYDROLASES ISOLATED FROM VARIOUS ORGANS OF DIFFERENT MAMMALS (51,52).

HOWEVER, STUDIES USING NAD GLYCOHYDROLASE, ISOLATED FROM THE BRAIN OF VARIOUS MAMMALS, SHOWED THAT THE ELECTROPHORETIC ABILITY OF LOW MOLECULAR WEIGHT NADᵀASES WAS MARKEDLY DIFFERENT FROM THE HIGH MOLECULAR WEIGHT NADᵀASES (52). LOW MOLECULAR NAD GLYCOHYDROLASES (PORCINE, BOVINE) OF APPROXIMATELY 25,000 MIGRATED TOWARD THE CATHODE AT pH 7.0; WHEREAS HIGH MOLECULAR WEIGHT NAD GLYCOHYDROLASES (HORSE, MOUSE, RABBIT, HUMAN AND MOUSE EHRLICH ASCITES TUMOR CELLS) OF APPROXIMATELY 70,000 TO 90,000 MIGRATED TOWARD THE ANODE. NOTWITHSTANDING THE VARIABILITY IN MOLECULAR WEIGHT AND ELECTROPHORETIC MOBILITY, THE SOLUBLE MAMMALIAN NAD GLYCOHYDROLASES EXHIBITED SIMILAR CATALYTIC PROPERTIES IN THAT THEY WERE SENSITIVE TO NICOTINAMIDE INHIBITION AND THEY POSSESSED TRANSGLYCOSIDASE ACTIVITY.

IN ADDITION TO THE ABOVE OBSERVATIONS, SWISLOCKI ET AL (52) FOUND THAT THE NADᵀASES OF HIGH MOLECULAR WEIGHT EXHIBITED MULTIPLE FORMS AFTER ELECTROPHORESIS. EACH FORM POSSESSED NADᵀASE ENZYME ACTIVITY. NAKAZAWA ET AL (42) FOUND THAT THERE WERE AT LEAST TWO DIFFERENT NAD GLYCOHYDROLASES PRESENT IN RAT LIVER; ONE LOCALIZED IN THE MICROSOMAL FRACTION AND THE OTHER LOCALIZED IN THE NUCLEAR FRACTION. THE NADᵀASE LOCALIZED IN THE MICROSOMES WAS ASSOCIATED WITH THE ENDOPLASMIC RETICULUM AND POSSESSED HYDROLYTIC AS WELL AS TRANSGLYCOSIDIC ACTIVITY. THE MICROSOMAL NADᵀASE REPRESENTED MORE THAN 90% OF THE TOTAL CELLULAR NADᵀASE ENZYME ACTIVITY. THE NUCLEAR NADᵀASE REPRESENTED APPROXIMATELY 5% OF THE TOTAL NADᵀASE ACTIVITY AND WAS FOUND TO BE ASSOCIATED WITH CHROMATIN. NUCLEAR NADᵀASE, SIMILAR TO THE MICROSOMAL NADᵀASE, EXHIBITED BOTH HYDROLYTIC AND TRANSGLYCOSIDIC PROPERTIES. THE PROPERTIES OF BOTH THE MICROSOMAL AND NUCLEAR NAD GLYCOHYDROLASES OF RAT LIVER ARE OUTLINED IN TABLE 3 (42).

Properties	Microsomal NADᵗase	Nuclear NADᵗase
Inhibitor	Nicotinamide	Nicotinamide
Exchange Reaction	Positive	Positive
Km for NAD	1.7×10^{-4}M	2.5×10^{-4}M
Optimum pH	6.4	7.6 - 8.0
Heat Stability	Stable	Labile
Specificity	NAD, NADP	NAD
*Dᵗase Treatment	Resistant	Sensitive

Table 3. Properties of Microsomal and Nuclear NAD Glycohydrolases from Rat Liver (42).

* Dᵗase represents Deoxyribonuclease

Data presented by Banay-Schwartz et al (56) suggested that at least two NADᵗases were present in rat liver; one firmly bound to the microsomal fraction and the other more loosely bound to the nuclear fraction and partially extractable. This confirmed Nakazawaᵗs results (42).

Zatman et al (57) found that there was some species difference with respect to inhibition of mammalian NADᵗases by certain pyridine compounds. NADᵗases from ruminants such as beef, lamb, goat and deer were strongly inhibited by isonicotinic acid hydrazide (INH-sensitive); and NADᵗases from rodent, horse, pig, human and frog tissues were not inhibited by isonicotinic acid hydrazide (INH-insensitive). The INH-insensitive enzymes formed analogues of INH and acetylpyridine more readily than did the sensitive enzymes. Other differences between the two groups were seen with pyridine, which inhibited the beef spleen enzyme (sensitive) but stimulated the pig brain enzyme (insensitive); and 3-acetylpyridine,

WHICH STIMULATED BOTH THE PIG BRAIN ENZYME (INSENSITIVE) AND THE BEEF SPLEEN ENZYME (SENSITIVE), BUT HAD A MUCH GREATER EFFECT ON THE FORMER.

INVESTIGATIONS FOR NAD GLYCOHYDROLASE ACTIVITY HAVE ALSO BEEN CARRIED OUT WITH ASCITES TUMOR CELLS. DIETRICH AND FULLER (8) FOUND THAT IN INTACT ASCITES CELLS, THE SIMULTANEOUS UPTAKE OF ^{32}P AND NICOTINAMIDE $-^{14}C$ INTO THE NMN PORTION OF NAD, IN THE ABSENCE OF L-GLUTAMINE, RESULTED IN LITTLE CHANGE IN THE SPECIFIC ACTIVITY OF ^{32}P INCORPORATION AS A RESULT OF NICOTINAMIDE ADDITION. HOWEVER, THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE $-^{14}C$ IN THE NMN MOIETY OF NAD WAS MARKEDLY INCREASED. THEY CONCLUDED THAT THE NICOTINAMIDE INCORPORATION APPARENTLY TOOK PLACE IN THE ABSENCE OF DE NOVO BIOSYNTHESIS, WHICH SUGGESTED THAT NICOTINAMIDE $-^{14}C$ WAS INCORPORATED INTO NAD VIA THE NAD GLYCOHYDROLASE ENZYME. GRUNICHE ET AL (58) REPORTED A SIMILAR OBSERVATION BUT INDICATED THAT ONLY A SMALL PART OF THE TOTAL UPTAKE OF NICOTINAMIDE $-^{14}C$ INTO NAD, IN ASCITES CELLS, WAS CATALYZED BY THE NAD GLYCOHYDROLASE ENZYME SYSTEM.

BOCK ET AL (59) FOUND NAD GLYCOHYDROLASE ACTIVITIES IN THE NUCLEAR AND MICROSOMAL FRACTIONS OF EHRLICH ASCITES CELLS. THE NUCLEAR NADᵀASE WAS ASSOCIATED WITH CHROMATIN WHEREAS THE MICROSOMAL NADᵀASE WAS ASSOCIATED WITH THE PLASMA MEMBRANE. TABLE 4 OUTLINES THE PARTICULAR PROPERTIES OF EACH ENZYME FROM ASCITES CELLS AS DETERMINED BY BOCK ET AL (59).

Properties	Ascites cell Microsomal NADᵗase	Ascites cell Nuclear NADᵗase
Km for NAD	3.0×10^{-4}M	6×10^{-4}M
Optimum pH	6.5	7.5
Specificity	NAD, NADP, APAD*, NMN	NAD, NADP, APAD*
DNase Treatment	Resistant	Sensitive

* APAD - 3-acetyl pyridine analogue of nicotinamide

Table 4. Properties of Microsomal and Nuclear NADᵗases from Ascites Cells. (59).

c) Plants

Higher plants have also been found to contain the NAD glycohydrolase enzyme system. Hasse and Schleyer (60) in 1961 found that NAD was degraded by extracts from Lupinus luteus. The degradation of NAD was catalyzed by NAD nucleosidase and NAD pyrophosphatase enzyme systems. In 1966 Maini et al (61) found that tumor tissue of Rumex acetosa and Althea rosea (hollyhock) in vitro possessed NADᵗase activity whereas normal tissue of the same plants did not. The NAD glycohydrolase enzyme as characterized from the tumor tissue of Rumex and Althea had an optimum pH of 3.5; it acted upon NAD, NADP and des-amino-NAD but not on NMN; it was inhibited by nicotinamide; it was inactivated by heat at 80°C and it was contained in the particulate fraction of the cell debris. Recent studies carried out on tumor tissue of Rumex acetosa (62) have shown that these tissues appear to possess two NAD glycohydrolases. Enzyme 1 was localized in the 480 x g fraction whereas Enzyme 2 was localized in the 5090 and 20,000 x g fractions. Enzymes 1 and 2 showed an optimum

pH of 4.0 and 4.5 respectively; both acted upon NAD, NADP, deamino-NAD and thionicotinamide adenine dinucleotide (action on the latter two compounds was at a reduced rate) and both were inhibited by nicotinamide. Enzyme 1 exhibited a Km value for NAD of 4×10^{-4}M whereas Enzyme 2 had a Km value for NAD of 1.7×10^{-4}M. The presence of the NAD glycohydrolase system in tumor tissues has been implicated as the reason why low levels of pyridine nucleotides are found in malignant tissues (63 - 65).

Therefore, there is wide distribution of NAD glycohydrolase enzyme activity in animal, microbial and plant tissue.

3. Significance of NAD Glycohydrolase in NAD Biosynthesis

The physiological functions of NAD glycohydrolases remain to be determined. The existence of more than one NADᵗase within a single cell or tissue makes the role of determining the function of this enzyme much more complex.

However, a few observations can be made regarding the effect of this enzyme in NAD biosynthesis. Joshi and Handler (66) have suggested that in living cells at least part of the nicotinamide produced *in vivo* by the action of NAD glycohydrolase on NAD could be reused by deamidation to nicotinic acid and conversion to NAD via the Preiss and Handler Pathway. The two enzymes, NAD glycohydrolase and nicotinamidase, would thus play a key role in the metabolism of NAD and would constitute a "salvage loop" in organisms where the *de novo* pathway was genetically blocked or absent, such as in the neoplastic condition (67). Organisms operating under these conditions exhibit a nutritional requirement for nicotinic acid or nicotinamide. Where the *de novo* pathway was operable, the NADᵗase pathway was present as a spare pathway. Alternately, the following suggestion

MAY BE FUNCTIONAL. SINCE THE LOSS OF THE NICOTINYL STRUCTURE FROM THE CYCLE MAY OCCUR AT THE LEVEL OF NICOTINAMIDE OR NICOTINIC ACID, THE NAD'ASE ENZYME COULD CONVERT NAD TO NICOTINAMIDE WHICH COULD BE DEAMIDATED TO NICOTINIC ACID. THESE COMPOUNDS COULD BE EXCRETED AS DERIVATIVES OR AS THE FREE COMPOUND. THUS, NAD'ASE MAY SERVE AS A REGULATOR OF NAD LEVELS (67).

GOPINATHAN ET AL (68) HAVE SUGGESTED THAT NAD GLYCOHYDROLASE IN MYCOBACTERIUM BUTYRICUM H37Rv MAY SERVE AS THE MECHANISM FOR CELLULAR CONTROL OF THE ACTION OF NAD. THEY SUGGESTED THAT THE NAD'ASE IN AN INHIBITED STATE WAS SUBJECT TO NUTRITIONAL CONTROL AND, THEREFORE, MAY REGULATE SIGNIFICANTLY THE OXIDATIVE METABOLISM OF THE ORGANISM.

EVIDENCE BY GREEN AND BODANSKY (64) HAS SUGGESTED THAT NAD GLYCOHYDROLASE PLAYED A ROLE IN THE MAINTENANCE OF INTRACELLULAR NAD CONCENTRATIONS. THEIR RESULTS INDICATED THAT A DECREASED RATE OF GLYCOLYSIS WAS ASSOCIATED WITH AN INCREASE IN NAD'ASE LEVELS. THIS, THEREFORE, EMPHASIZED THE ROLE OF THE NAD GLYCOHYDROLASE IN MAINTAINING CELLULAR NAD LEVELS ADEQUATE FOR GLYCOLYSIS OR FOR METABOLIC SEQUENCES INVOLVING NAD.

THE ROLE OF NAD GLYCOHYDROLASES AS TRANSGLYCOSIDASES REMAINS YET TO BE DETERMINED AS DOES THE COMPLETE ROLE OF NAD GLYCOHYDROLASES AS HYDROLASES. THE FACT THAT MORE THAN ONE NAD'ASE MAY EXIST WITHIN A SINGLE CELL OR TISSUE MAKES THE PROBLEM EVEN MORE COMPLEX. HOWEVER, THE KNOWLEDGE THAT A GIVEN ENZYME IS ASSOCIATED WITH A SPECIFIC ORGANELLE MAY HELP TO DETERMINE THE PHYSIOLOGICAL ROLE OF THE NAD'ASE ENZYMES AS THE FUNCTIONS OF THE ORGANELLE, IN WHICH THEY ARE LOCATED, ARE FURTHER CLARIFIED.

II. EXPERIMENTAL

A. Materials and Methods

Organism

Claviceps, strain SD-58, which was obtained as a slant culture from Dr. H. G. Floss, Purdue University, Lafayette, Indiana, U.S.A. in September 1968, was employed in this study.

Liquid Culture Medium

The NAD biosynthetic study was carried out employing *Claviceps*, strain SD-58 grown in a nutrient medium, designated SD Media, of the following composition:

Mannitol	50.0 Gm
Sucrose	50.0 Gm
KH_2PO_4	0.1 Gm
$MgSO_4 \cdot 7H_2O$	0.3 Gm
$FeSO_4 \cdot 7H_2O$	0.10 mg
$ZnSO_4 \cdot 7H_2O$	4.4 mg
Yeast Extract	3.0 Gm
Succinic Acid	5.4 Gm
Distilled Water to make	1 litre

The medium was adjusted to pH 5.4 with concentrated ammonium hydroxide (69).

The fungal cultures were grown in 50 ml. of SD nutrient media (69) which was dispensed in 250 ml. Erlenmeyer flasks, stoppered with non-absorbent cotton and sterilized by autoclaving at 121°C for twenty minutes. The medium was inoculated *aseptically with a homogenized suspension (1.0 ml.) of a 14-day-old organism grown on SD media. The inoculated culture flasks were maintained in the dark in an incubator at 26°C $\pm$1 throughout the growth period of 12 days.

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*A Cleanline Laminar Flow Hood was employed, Model CHC 24-4, Controlled Environment Equipment Corporation, 344 South Avenue, Whitman, Massachusetts, U.S.A.

CHEMICALS

NICOTINAMIDE -7-^{14}C (SPECIFIC ACTIVITY 10.7 MC/MM), NICOTINAMIDE -7-^{14}C (S.A. 5.09 MC/MM) PHOSPHORIC ACID -^{32}P(0.02N HCL),CARRIER FREE, AND NICOTINIC ACID -7-^{14}C (S.A. 10.2 MC/MM WERE PURCHASED FROM NEW ENGLAND NUCLEAR, MONTREAL, QUEBEC, CANADA; NICOTINIC ACID -7-^{14}C (S.A. 27.9 MC/MM) WAS PURCHASED FROM NUCLEAR CHICAGO CORP., DES PLAINES, ILL., U.S.A.; NICOTINAMIDE WAS PURCHASED FROM SIGMA CHEMICAL COMPANY, ST. LOUIS, MISSOURI, U.S.A.; AND REFERENCE STANDARDS OF NICOTINAMIDE MONONUCLEOTIDE, β-DIPHOSPHOPYRIDINE NUCLEOTIDE (β-NAD), RIBOSE -5- PHOSPHATE (DISODIUM SALT), ADENOSINE MONOPHOSPHATE (FREE ACID) WERE PURCHASED FROM THE SIGMA CHEMICAL COMPANY, ST. LOUIS, MISSOURI, U.S.A.

ENZYME PREPARATIONS

PHOSPHODIESTERASE (TYPE II: LYOPHILIZED POWDER) FROM CROTALUS ADAMANTEUS VENOM WAS PURCHASED FROM SIGMA CHEMICAL COMPANY, ST. LOUIS, MISSOURI, U.S.A.

MEASUREMENT OF RADIOACTIVITY

THE *UNILUX LIQUID SCINTILLATION SYSTEM WAS USED TO DETERMINE THE ACTIVITY OF RADIOACTIVE SAMPLES. SAMPLES OF NOT MORE THAN 0.1 ML. OF RADIOACTIVE SOLUTIONS WERE DISPENSED INTO COUNTING VIALS OF 20 ML. CAPACITY AND TEN ML. OF SCINTILLATION FLUID WERE ADDED TO THE VIAL BEFORE COUNTING. WHERE REQUIRED, RADIOACTIVE SPOTS FROM THE CHROMATOGRAMS WERE ELUTED FROM THE CHROMATOGRAM WITH DISTILLED WATER. THE ELUATE WAS FROZEN,

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*NUCLEAR-CHICAGO UNILUX MODEL 6850 BENCH TOP LIQUID SCINTILLATION SYSTEM, NUCLEAR CHICAGO, DES PLAINES,ILL., U.S.A.

*LYOPHILIZED AND THE RESIDUE RECONSTITUED VOLUMETRICALLY IN DISTILLED WATER. ALIQUOTS OF THE RECONSTITUTED PORTION WERE EMPLOYED FOR COUNTING. A SERIES OF EXPERIMENTS EMPLOYING NICOTINAMIDE -7-^{14}C AND PHOSPHORIC ACID -^{32}P INDICATED THAT THE RECOVERY OF THE ACTIVITY ELUTED FROM THE PAPER WAS 90% OF THE AMOUNT CHROMATOGRAPHED.

RADIOACTIVE SAMPLES WERE PLACED IN COUNTING VIALS AND SCINTILLATION FLUID OF THE FOLLOWING COMPOSITION WAS ADDED:

POP (2,5 - DIPHENYLOXAZOLE)	0.2%
BIS-MSB [(BIS-(O-METHYLSTYRYL)-BENZENE]	0.004%
ISOPROPANOL (FISHER REAGENT)	40.0%
TOLUENE (BAKER REAGENT) TO MAKE	100.0%

THE COUNTING EFFICIENCY FOR CARBON -^{14}C CONTAINING SAMPLES WAS DETERMINED BY EMPLOYING AN INTERNAL STANDARD OF BENZOIC ACID -7-^{14}C (S.A. 6.6 x 10^3 DPM/MG.; NEW ENGLAND NUCLEAR CORP., BOSTON, MASS.). ALL RADIOACTIVE SOLUTIONS FOR SCINTILLATION COUNTING WERE MEASURED WITH AN **AGLA MICROMETER AND A 0.5 ML. CAPACITY SYRINGE.

DETECTION OF RADIOACTIVITY

RADIOACTIVE COMPOUNDS WERE LOCATED ON PAPER CHROMATOGRAMS USING A RADIOCHROMATOGRAM SCANNER***. THE SETTINGS OF THE VARIOUS CONTROLS WERE

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*EMPLOYING THE VIRTIS UNITRAP, MODEL 10-100, THE VIRTIS COMPANY INC., GARDINER, NEW YORK.

**AGLA MICROMETER AND SYRINGE MADE BY BURROUGHS WELLECOME AND CO., LONDON, ENGLAND AND DISTRIBUTED IN CANADA BY WARNER-CHILCOTT LAB. CO. LIMITED, 2200 EGLINTON AVENUE, EAST, SCARBOROUGH, ONTARIO, CANADA.

***NUCLEAR-CHICAGO ACTIGRAPH III (MODEL NO. 1002), NUCLEAR CHICAGO, DES PLAINES, ILL.

AS FOLLOWS:

HIGH VOLTAGE	1050 VOLTS
SECONDS	10
SCAN SPEED	VARIABLE
COUNTS PER MINUTE	VARIABLE
GAS (98.7% HELIUM; 1.3% BUTANE)	7 LBS. P.S.I.

RADIOACTIVE COMPOUNDS WERE LOCATED ON THIN LAYER PLATES USING THE *THIN LAYER CONVEYOR ATTACHMENT," IN CONJUNCTION WITH THE **RADIOCHROMATOGRAM SCANNER.

PREPARATION OF NICOTINAMIDE -7-^{14}C, NICOTINIC ACID -7-^{14}C AND PHOSPHORIC ACID -^{32}P FOR ADMINISTRATION

SAMPLES CONTAINING NICOTINAMIDE -7-^{14}C AND NICOTINIC ACID -7-^{14}C WERE DISSOLVED IN STERILE GLASS DISTILLED WATER AND STERILIZED EMPLOYING A ***SWINNEY FILTER PRIOR TO USE. UNDILUTED SAMPLES OF PHOSPHORIC ACID -^{32}P WERE ADDED DIRECTLY USING ASEPTIC TECHNIQUE WHEREAS SAMPLES OF PHOSPHORIC ACID -^{32}P WHICH WERE DILUTED WITH DISTILLED WATER WERE STERILIZED EMPLOYING A ***SWINNEY FILTER.

INCUBATION OF CULTURES WITH RADIOACTIVE COMPOUNDS

THE REQUIRED AMOUNT OF THE COMPOUND UNDER STUDY (NICOTINAMIDE -7-^{14}C, NICOTINIC ACID -7-^{14}C OR PHOSPHORIC ACID -^{32}P) WAS ADMINISTERED ASEPTICALLY TO THE CULTURE FLASKS OF 12-DAY-OLD CULTURES OF CLAVICEPS. THE FLASKS

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*NUCLEAR-CHICAGO THIN-LAYER-PLATE CONVEYOR SYSTEM,(MODEL NO. 1006), NUCLEAR CHICAGO, DES PLAINES, ILL.

**NUCLEAR-CHICAGO ACTIGRAPH III (MODEL NO. 1002), NUCLEAR CHICAGO, DES PLAINES,ILL.

***SWINNEX DISPOSABLE FILTERS, MILLIPORE FILTER CORP., BEDFORD, MASS., CATALOGUE #SXHA0130S.

WERE SHAKEN ON A WATER-BATH SHAKER* AT 37°C THROUGHOUT THE INCUBATION PERIOD.

CHROMATOGRAPHY PROCEDURES EMPLOYED (1)

1. PAPER

WHATMAN No. 3MM PAPER WAS USED IN ALL CHROMATOGRAPHIC PROCEDURES AND THE CHROMATOGRAMS WERE DEVELOPED IN GLASS TANKS AT ROOM TEMPERATURE. THE FOLLOWING FOUR SOLVENT SYSTEMS WERE USED FOR SEPARATION AND PURIFICATION OF THE ISOLATED RADIOACTIVE COMPOUNDS BY ASCENDING DEVELOPMENT:

A) ISOBUTYRIC ACID: AMMONIA: WATER (66:1.7:33) pH 3.8 (IAW) (70)

B) PYRIDINE: WATER (2:1) (PW) (70)

C) BUTANOL SATURATED WITH 3% AMMONIA (BA) (1)

D) ETHANOL: 1M AMMONIUM ACETATE (7:3) (EAA) ADJUSTED TO pH5 WITH CONCENTRATED HYDROCHLORIC ACID. (70)

THE hR_F (R_F x 100) VALUES OBTAINED IN THESE SOLVENT SYSTEMS FOR THE PYRIDINE COMPOUNDS UNDER STUDY ARE LISTED IN TABLE 5.

*WATER - BATH SHAKER, EBERBACH CORPORATION, ANN ARBOR, MICHIGAN.

TABLE 5

*HR_F VALUES OF PYRIDINE COMPOUNDS ON PAPER CHROMATOGRAMS

SOLVENT**	IAW	PW	BA	EAA
COMPOUND				
NMN	45	29	0	45
NAD	46	62	0	23
NADP	29	26	0	21
NICOTINAMIDE	84	81	61	81
NICOTINIC ACID	76	75	21	78
N-METHYL NICOTINAMIDE	79	69	10	70

0 - REMAINED AT ORIGIN

*HR_F = (R_F x 100); WHATMAN #3 MM PAPER

**IAW ISOBUTYRIC ACID: AMMONIA: WATER (66:1.7:33) pH 3.8

PW - PYRIDINE: WATER (2:1)

BA - BUTANOL SATURATED WITH 3% AMMONIA

EAA - ETHANOL: 1M AMMONIUM ACETATE (7:3) pH 5.0.

2. Thin Layer Chromatography (TLC)

Non-nucleotide pyridine containing compounds, which had been isolated from paper chromatograms, were identified by thin layer chromatography employing Silica Gel G (Merck) as indicated by Audette (1). Detection of radioactivity on thin layer plates was done according to procedures previously described. Table 6 shows the HR_F values of the compounds studied by this procedure.

TABLE 6

*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

*$HR_F = (R_F \times 100)$

Detection of Pyridine Compounds (1)

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

1. Short wavelength ultraviolet light * (253 mu) was employed to locate ultraviolet absorbing compounds. (e.g. nicotinamide)

2. Developed chromatograms containing pyridine compounds 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, the nicotinamide nucleotides 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 (71).

3. Paper chromatograms were also exposed to cyanogen bromide vapours for a period of 1 hour and then sprayed with p-aminobenzoic acid (72). Nicotinic acid produced a yellow colored spot, while nicotinamide produced a deep yellow-orange color following this treatment.

Detection and Assay of Ribose-5-Phosphate

The following procedures were used to detect and assay ribose-5-phosphate which was obtained from the basic hydrolysis of nicotinamide mononucleotide.

1. Detection of Ribose-5-Phosphate

Paper chromatograms were sprayed with aniline phthalate reagent (73):

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*Chromato-Vue Model C5, Ultra-Violet Products, Inc., San Gabriel, California, U.S.A.

0.93 GRAMS OF ANILINE

1.66 GRAMS OF O-PHTHALIC ACID DISSOLVED IN 100 ML. OF WATER-SATURATED BUTANOL.

AFTER SPRAYING THE CHROMATOGRAM WITH THE ANILINE PHTHALATE REAGENT, IT WAS HEATED TO 105°C FOR 10 MINUTES AND THE RIBOSE-5-PHOSPHATE APPEARED AS A DARK BROWN SPOT. THE HR_F VALUES OBTAINED IN THE SOLVENT SYSTEMS EMPLOYED FOR RIBOSE-5-PHOSPHATE ARE LISTED IN TABLE 7.

TABLE 7

*HR_F VALUES OF RIBOSE-5-PHOSPHATE ON PAPER CHROMATOGRAMS

SOLVENT**	IAW	BA
COMPOUND		
RIBOSE-5-PHOSPHATE	20	0

0 - REMAINED AT ORIGIN

*$HR_F = (R_F \times 100)$

**IAW ISOBUTYRIC ACID: AMMONIA: WATER (66:1.7:33) PH 3.8

BA BUTANOL SATURATED WITH 3% AMMONIA

2. ASSAY FOR RIBOSE-5-PHOSPHATE

RIBOSE-5-PHOSPHATE WAS ASSAYED ACCORDING TO THE METHOD OF HORECKER ET AL AS DESCRIBED BY ASHWELL (74).

Isolation and Purification of Radioactive Pyridine Compounds

The isolation and purification procedures of the radioactive compounds from *Claviceps* after incubation with nicotinamide -7-^{14}C, nicotinic acid -7-^{14}C or phosphorus -^{32}P are summarized in Figure 4 and Figure 5.

Figure 4 outlines the summary of the isolation and purification procedures for the radioactive pyridine compounds from *Claviceps* after incubation with nicotinamide -7-^{14}C.

Figure 5 is an outline for the subsequent isolation and purification procedures employed for ^{32}P containing compounds.

The radioactive labelled compound or compounds under study were incubated with shaking for six hours at 37°C with individual 12-day-old *Claviceps* cultures. The mycelium was separated from the medium by filtration and washed with distilled water. The filtrate, fraction A, was diluted volumetrically to 200 ml. with water; and suitable aliquots were counted and chromatographed when required. The mycelium was homogenized in a Virtis "45" Hi Speed Homogenizer* at high speed with five volumes of cold 5% trichloroacetic acid (TCA) for three minutes. The resulting suspension was centrifuged** at 15,000 x g.(12,000 R.P.M.) 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 150 ml. with 5% TCA to produce fraction B. Aliquots of fraction B were counted

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*Virtis "45" Hi Speed Homogenizer, Virtis Co. Inc., Gardiner, New York.

**IEC Refrigerated Centrifuge, Model B-20, with "870" Head for 50 ml. capacity tubes; International Equipment Company, 300 Second Avenue, Needham Heights, Massachusetts.

WHERE NECESSARY. FIVE VOLUMES OF ACETONE AT -20°C WERE ADDED TO THE SOLUTION AND THE MIXTURE WAS STORED FOR 24 HOURS 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 A DESICCATOR UNDER VACUUM AND WAS DISSOLVED IN DISTILLED WATER TO MAKE 25 ML. OF FRACTION C PRIOR TO CHROMATOGRAPHIC ANALYSIS AND SCINTILLATION COUNTING.

WHEN REQUIRED, THE ACETONE AND ETHER WERE REMOVED FROM THE SUPERNATENT IN VACUO AT 20°C. THE RESULTING AQUEOUS SOLUTION WAS EXTRACTED WITH PEROXIDE-FREE ETHER. THE RESULTING AQUEOUS SOLUTION, FRACTION D, WAS SEPARATED FROM THE ETHER EXTRACTION, FRACTION E. THE SOLUTIONS, FRACTION D AND FRACTION E, WERE CONCENTRATED TO 25 ML. AND 10 ML. FRACTIONS, RESPECTIVELY. FRACTION D AND FRACTION E CONTAINED THE AQUEOUS AND ETHER SOLUBLE MONONUCLEOTIDES, RESPECTIVELY.

FRACTION C, THE DINUCLEOTIDE FRACTION, WAS LYOPHILIZED AND DISSOLVED IN DISTILLED WATER TO MAKE 5 ML. (FIG. 5). THE ENTIRE AQUEOUS EXTRACT WAS CHROMATOGRAPHED ON WHATMAN 3 MM PAPER AND DEVELOPED ASCENDINGLY IN THE IAW SOLVENT SYSTEM. THE SEPARATED BAND CORRESPONDING TO NAD WAS ELUTED WITH DISTILLED WATER, LYOPHILIZED AND THE RESIDUE DISSOLVED IN A MINIMUM QUANTITY OF DISTILLED WATER. THIS PARTIALLY PURIFIED NAD PREPARATION WAS THEN RECHROMATOGRAPHED IN THE EAA SOLVENT SYSTEM. THE PURIFIED NAD WAS ELUTED WITH DISTILLED WATER, LYOPHILIZED AND THE RESIDUE DISSOLVED IN A MINIMUM AMOUNT OF DISTILLED WATER.

THE ISOLATED NAD WAS HYDROLYZED EMPLOYING THE PHOSPHODIESTERASE SNAKE VENOM INTO NMN AND AMP USING THE FOLLOWING MODIFIED PROCEDURE (75,76).

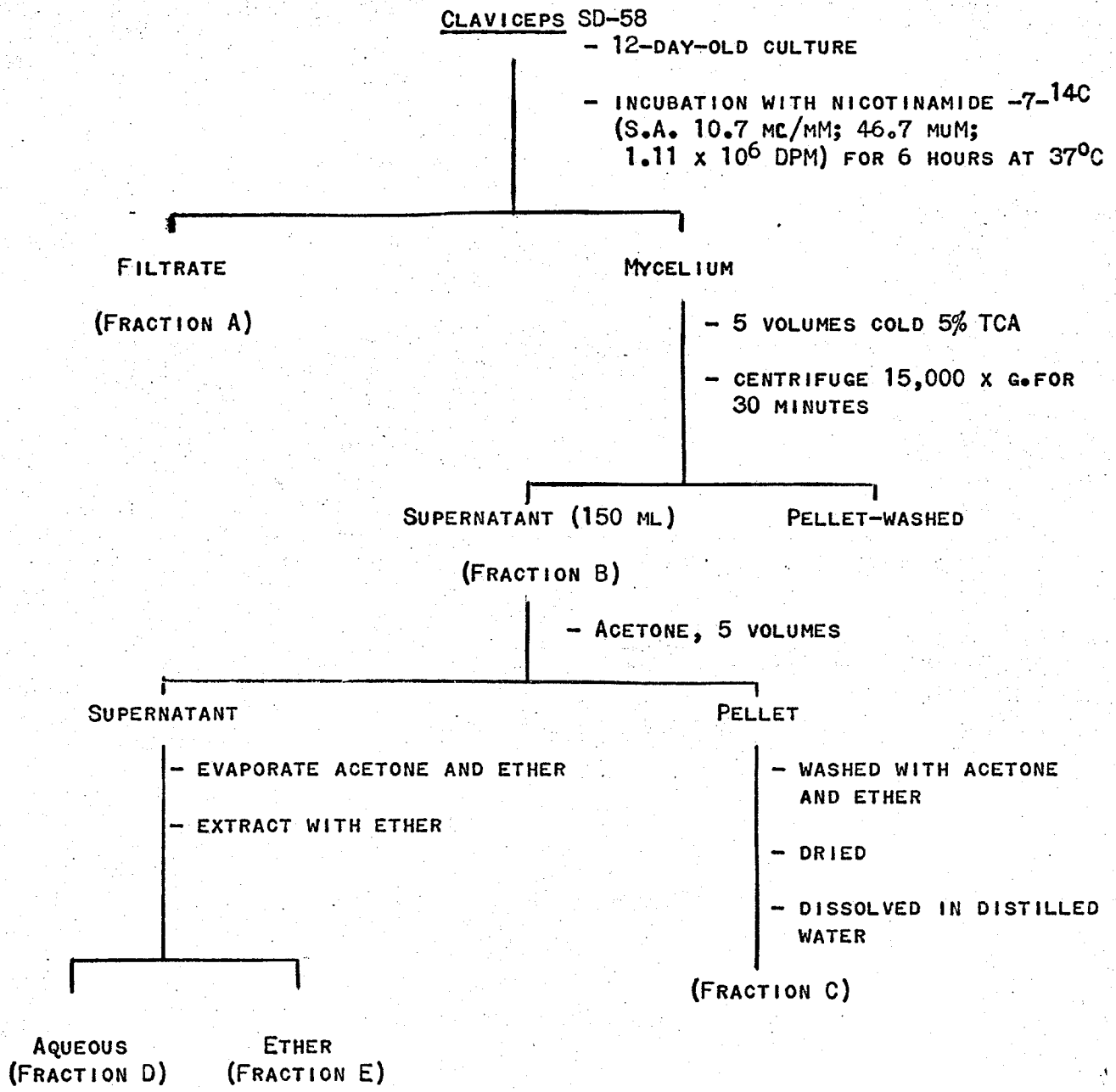

FIGURE 4. SUMMARY OF THE ISOLATION AND PURIFICATION PROCEDURES OF RADIOACTIVE PYRIDINE COMPOUNDS FROM CLAVICEPS SD-58 AFTER INCUBATION WITH NICOTINAMIDE -7-^{14}C.

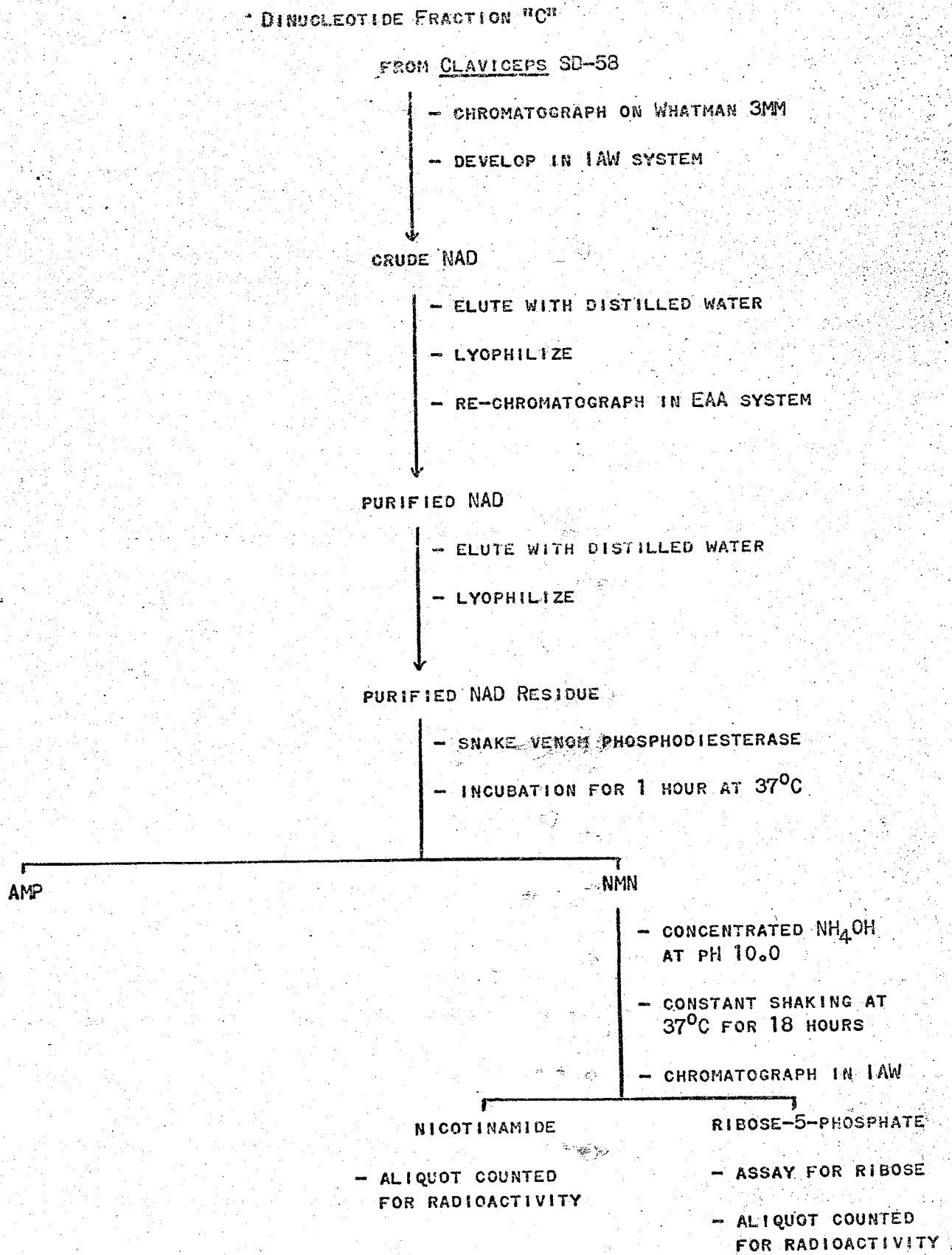

FIGURE 5. SUMMARY OF THE ISOLATION AND PURIFICATION FOR THE HYDROLYTIC PRODUCTS OF NAD AND NMN

THE ISOLATED NAD WAS INCUBATED AT 37°C FOR 1 HOUR IN A MIXTURE OF 1.0 ML. TRIS-ACETATE BUFFER, pH 8.8; 0.3 ML. OF 0.3 M MAGNESIUM ACETATE AND 1.0 ML. OF PHOSPHODIESTERASE ENZYME PREPARATION CONTAINING 0.8 MG PROTEIN/ML. FOLLOWING THE INCUBATION, THE RESULTANT MIXTURE WAS LYOPHILIZED, RECONSTITUTED IN DISTILLED WATER AND CHROMATOGRAPHED IN THE EAA SYSTEM. THE PURIFIED NMN WAS ELUTED FROM THE CHROMATOGRAM WITH DISTILLED WATER, LYOPHILIZED AND RECONSTITUTED IN 5.0 ML. OF DISTILLED WATER. THE NMN SAMPLE WAS ADJUSTED TO pH 10 WITH CONCENTRATED AMMONIUM HYDROXIDE AND SHAKEN FOR 18 HOURS AT 37°C. AFTER THE HYDROLYSIS PROCEDURE, THE SAMPLE WAS NEUTRALIZED WITH DILUTE ACETIC ACID, FROZEN AND LYOPHILIZED. THE RESIDUE, DISSOLVED IN A MINIMUM AMOUNT OF WATER, WAS THEN CHROMATOGRAPHED IN THE IAW SOLVENT SYSTEM. A RADIOCHROMATOGRAPHIC SCAN OF THE CHROMATOGRAPHED MATERIAL REVEALED TWO RADIOACTIVE COMPONENTS, ONE WITH AN R_F SIMILAR TO NICOTINAMIDE AND THE OTHER WHICH MIGRATED WITH RIBOSE-5-PHOSPHATE. THE TWO RADIOACTIVE COMPONENTS WERE IDENTIFIED AS NICOTINAMIDE AND RIBOSE-5-PHOSPHATE BY EXPOSURE OF THE CHROMATOGRAM TO CYANOGEN BROMIDE (72) AND ANILINE PHTHALATE (73) RESPECTIVELY. NICOTINAMIDE AND RIBOSE-5-PHOSPHATE WERE FURTHER IDENTIFIED BY CHROMATOGRAPHY IN THE BA SOLVENT SYSTEM. PREISS AND HANDLER (6) REPORTED THAT ADENOSINE MONOPHOSPHATE (AMP) MIGRATED WITH NMN IN THE ETHANOL-AMMONIUM ACETATE SYSTEM. THEREFORE, AFTER THE HYDROLYSIS OF NAD BY THE SNAKE VENOM PHOSPHODIESTERASE PREPARATION TO FORM NMN AND AMP, THE ELUATE ELUTED FROM THE CHROMATOGRAM WOULD CONTAIN NMN AND AMP. IT WAS NECESSARY, THEREFORE, TO DETERMINE WHETHER THE BASIC HYDROLYSIS FOR NMN AFFECTED THE AMP IN THE PREPARATION IN SUCH A WAY THAT IT MAY HAVE CONTRIBUTED TO THE AMOUNT OF RIBOSE-5-PHOSPHATE PRODUCED.

COLD ADENOSINE MONOPHOSPHATE (NON-RADIOACTIVE) WAS DISSOLVED IN A MINIMUM VOLUME OF DISTILLED WATER AND ADJUSTED TO pH 10 WITH CONCENTRATED AMMONIUM HYDROXIDE. THE PREPARATION WAS SHAKEN FOR 18 HOURS AT 37°C. AFTER THIS TREATMENT, THE SAMPLE WAS NEUTRALIZED, FROZEN AND LYOPHILIZED. THE RESIDUE, DISSOLVED IN A MINIMUM AMOUNT OF DISTILLED WATER, WAS CO-CHROMATOGRAPHED WITH UNTREATED AMP AND DEVELOPED IN THE IAW SOLVENT SYSTEM. EMPLOYING THE short WAVELENGTH ULTRAVIOLET LIGHT (253 MU), ONLY ONE ULTRAVIOLET ABSORBING SPOT COULD BE LOCATED WITH AN HR_F SIMILAR TO AMP. EXPOSURE OF THE CRHOMATOGRAM TO ANILINE PHTHALATE REAGENT PRODUCED NO EVIDENCE FOR THE PRESENCE OF RIBOSE-5-PHOSPHATE.

TABLE 8 OUTLINES THE HR_F VALUES FOR AMP AND RIBOSE-5-PHOSPHATE IN THE TWO SOLVENT SYSTEMS EMPLOYED.

TABLE 8

*HR_F VALUES OF ADENOSINE MONOPHOSPHATE

ON PAPER CHROMATOGRAMS

SOLVENT **	IAW	EAA
COMPOUND		
ADENOSINE MONOPHOSPHATE	54	44
RIBOSE-5-PHOSPHATE	20	NOT DETERMINED

*HR_F - (R_F x 100)

**IAW ISOBUTYRIC ACID - AMMONIA - WATER 66:1.7:33, pH 3.8

EAA ETHANOL - 1M AMMONIUM ACETATE 7:3 pH 5.0.

THE RADIOACTIVE AREAS CORRESPONDING TO NICOTINAMIDE -^{14}C AND RIBOSE -5-PHOSPHATE -^{32}P WERE ELUTED WITH DISTILLED WATER, LYOPHILIZED AND RE-CONSTITUTED IN 5.0 ML. OF DISTILLED WATER. ALIQUOTS OF THE RESPECTIVE

LABELLED SOLUTIONS WERE COUNTED BY A LIQUID SCINTILLATION COUNTER.

ALIQUOTS OF THE RIBOSE-5-PHOSPHATE CONTAINING SAMPLE WERE ASSAYED EMPLOYING THE ORCINOL-PENTOSE REACTION ACCORDING TO THE METHOD OF HORECKER ET AL (74). DIETRICH AND FULLER (8) REPORTED THAT THE HYDROLYSIS OF THE RIBOSYL BOND OF NMN BY AMMONIUM HYDROXIDE WAS QUANTITATIVE UNDER THE CONDITIONS USED, YIELDING EQUIMOLAR AMOUNTS OF RIBOSE-5-PHOSPHATE AND NICOTINAMIDE. ON THE BASIS THAT THIS YIELDS AN EQUIMOLAR AMOUNT OF THESE TWO COMPOUNDS, THE VALUE OBTAINED FROM THE ORCINOL-PENTOSE REACTION FOR RIBOSE-5-PHOSPHATE WAS EMPLOYED FOR CALCULATING THE EQUIMOLAR AMOUNTS OF NICOTINAMIDE WHICH WERE OBTAINED FROM THE SAME HYDROLYSIS.

EMPLOYING THE RESULTS OF THE COUNTING AND OF THE ORCINOL-PENTOSE ASSAY AND SUBSEQUENT CALCULATIONS, FOR THE QUANTITIES OF NICOTINAMIDE, THE SPECIFIC ACTIVITY (CPM/MU MOLE)OF THE COMPOUNDS WERE CALCULATED.

EXPERIMENTAL CONDITIONS REQUIRED TO DEMONSTRATE NAD GLYCOHYDROLASE ACTIVITY IN CLAVICEPS IN VITRO

THE FRACTIONATION PROCEDURES EMPLOYED FOR OBTAINING NUCLEAR AND MICROSOMAL FRACTIONS FROM CLAVICEPS MYCELIUM ARE OUTLINED IN FIGURE 6. SINGLE 12-DAY-OLD CULTURES OF CLAVICEPS WERE HOMOGENIZED IN 0.1M PHOSPHATE BUFFER [98.2 ML. 0.5M KH_2PO_4 AND 34.0 ML. 0.5M Na_2HPO_4, DILUTED TO 1 LITRE (77)] PH 6.4 BY EITHER OF THE FOLLOWING METHODS:

A) USE OF THE VIRTIS HOMOGENIZER AT HIGH SPEED FOR 3 MINUTES OR

B) WITH A WEDGEWOOD MORTAR AND PESTLE PLUS SAND (MYCELIUM:SAND-3:1 W/W) WITH GRINDING FOR 5 MINUTES.

THE CRUDE HOMOGENATE WAS FILTERED THROUGH NYLON STOCKING OF MEDIUM POROSITY AND THE FILTRATE WAS CENTRIFUGED AT 900 X G.(3000 R.P.M.) FOR

6 MINUTES AT 0°C. THE PELLET WAS WASHED WITH 0.1M PHOSPHATE BUFFER AND RECENTRIFUGED. THE PELLET, CONSISTING OF CELLULAR DEBRIS, INTACT CELLS AND NUCLEI WAS RETAINED AS FRACTION I. THE SUPERNATANTS WERE COMBINED AND RECENTRIFUGED AT 1000 X G.(3200 R.P.M.) 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. (14,500 R.P.M.) FOR 20 MINUTES TO SEDIMENT THE MITOCHONDRIA. THE PELLET OBTAINED, FRACTION III WAS DISCARDED. THE COMBINED SUPERNATANTS WERE RETAINED AS FRACTION IV AND ANALYZED FOR NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE ACTIVITY.

FOR THE DETERMINATION OF NAD GLYCOHYDROLASE ACTIVITY IN FRACTION II, THE PELLET WAS SUSPENDED IN 10 ML. OF THE HOMOGENIZATION MEDIUM (PHOSPHATE BUFFER). TO AN ALIQUOT OF 2.5 ML. OF FRACTION II, NICOTINAMIDE-7-^{14}C (1940 DPM) AND COLD NAD (20.0 MG.) WERE ADDED AND TO ANOTHER ALIQUOT OF 2.5 ML. NAD-7-^{14}C (1254 DPM) WAS ADDED. THE PREPARATIONS WERE INCUBATED FOR 2 HOURS AT 37°C ON A SHAKING WATER BATH. THE MICROSOMAL SUPERNATANT, FRACTION IV, WAS TREATED SIMILARLY USING 5.0 ML. OF THE TOTAL SUPERNATANT (14.0 ML.) FOR EACH INCUBATION. NO ADDITIONAL HOMOGENIZATION MEDIUM WAS ADDED BEFORE THE INCUBATION. IMMEDIATELY FOLLOWING THE 2 HOUR INCUBATION, ALL PREPARATIONS WERE CENTRIFUGED AT 25,000 X G. FOR 20 MINUTES. THE RESULTING SUPERNATANTS WERE FROZEN, LYOPHILIZED AND RECONSTITUTED IN A MINIMUM AMOUNT OF DISTILLED WATER PRIOR TO CHROMATOGRAPHY OF THE TOTAL RESIDUE IN THE IAW SOLVENT SYSTEM. THE PEAK AREAS OF THE RESULTANT RADIOACTIVE COMPOUNDS (NICOTINAMIDE, NICOTINIC ACID AND NAD) AS DETERMINED BY A *PLANIMETER, WERE USED AS A MEASURE OF THE HYDROLYTIC AND TRANSGLYCOSIDIC ACTIVITY OF NAD GLYCOHYDROLASE OF THE NUCLEAR FRACTION

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*ARISTO PLANIMETER DISTRIBUTED BY HUGHES-OWENS CO. LTD., 464 HARGRAVE ST., WINNIPEG, MAN.

(FRACTION II) AND MICROSOMAL FRACTION (FRACTION IV).

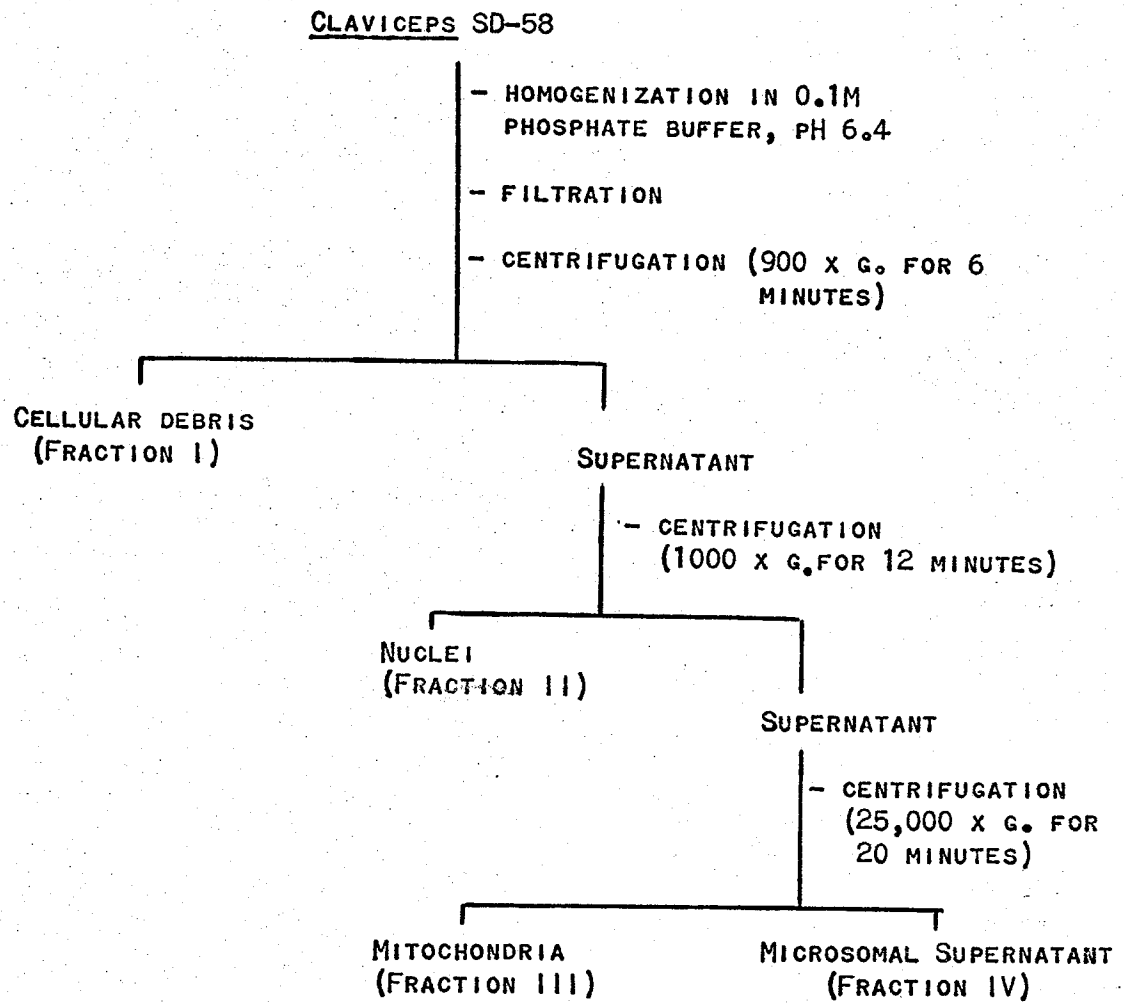

FIGURE 6. SUMMARY OF THE FRACTIONATION PROCEDURES OF CLAVICEPS SD-58 FOR IN VITRO STUDIES

B. Experimental Results

1. Incorporation of Nicotinamide into Pyridine Nucleotides in the Intact Culture of Claviceps SD-58.

Twelve day old cultures of Claviceps SD-58 were incubated with nicotinamide -7-^{14}C (S.A.10.7 mc/mM; 1.11 x 10^6 DPM per culture) employing the procedures previously described in Materials and Methods. Following the 6 hour incubation period, the incubation medium, fraction A, (Figure 4) and fraction B (Figure 4), which was obtained from the mycelium, were analyzed for radioactivity. Fractions C, D and E were obtained from fraction B according to the extraction procedure outlined in Figure 4. The distribution of radioactivity which was obtained in the various fractions is outlined in Table 9. The values given are those for the total activity obtained from four cultures which were subsequently fractionated and characterized.

Table 10 indicates the percentage distribution of radioactivity in the respective bands obtained from fractions A, C, D and E when chromatographed in the IAW solvent system.

Rechromatography of the compounds eluted from bands 3 and 4, obtained from fractions A and E, on paper in the butanol-ammonia system (Table 5) and on TLC using water as the developing solvent (Table 6) indicated that nicotinic acid and nicotinamide were the only compounds present in the fractions.

According to Shuster and Goldin (78), Fractions C and D should contain primarily dinucleotides and mononucleotides respectively. Rechromatography of the eluted IAW developed bands 1 and 2 of fractions C and D on paper in the pyridine-water system produced the results as shown

TABLE 9

THE DISTRIBUTION OF RADIOACTIVITY OBTAINED IN VARIOUS FRACTIONS FROM FOUR 12-DAY-OLD CULTURES OF CLAVICEPS SD-58 INCUBATED WITH *NICOTINAMIDE 7-^{14}C.

FRACTION	**DPMX10^6	% OF TOTAL ACTIVITY FED
A	0.284	6.4
B	3.503	78.9
***C	0.236	5.32
***D	2.950	66.44
***E	0.084	1.88

*EACH CULTURE WAS INCUBATED WITH NICOTINAMIDE -7-^{14}C (S.A. 10.7 MC/MM; 46.7 MUM, 1.11 x 10^6 DPM)

**RESULTS ARE FOR THE TOTAL ACTIVITY RECOVERED FROM FOUR CULTURES.

FRACTION A - TOTAL, DILUTED INCUBATION MEDIUM (FILTRATE)

FRACTION B - SUPERNATANT REMAINING AFTER TCA PRECIPITATION OF THE MYCELIUM

***FRACTIONS C, D AND E WERE OBTAINED FROM FRACTION B

FRACTION C - ACETONE-PRECIPITATED DINUCLEOTIDES

FRACTION D - ACETONE-SOLUBLE MONONUCLEOTIDES

FRACTION E - ETHER EXTRACT OF MONONUCLEOTIDE FRACTION

TABLE 10

THE DISTRIBUTION OF RADIOACTIVITY IN THE BANDS OBTAINED FROM FRACTIONS A, C, D AND E AS SEPARATED BY CHROMATOGRAPHY IN THE IAW SYSTEM

Fraction	Band	HR_F	Distribution of Radioactivity in Band Within Each Fraction (%)
A	3*	70	74.68
	4	81	25.32
C	1	27	33.46
	2	45	42.85
	4	84	23.69
D	1	25	8.53
	2	45	69.76
	3	70	18.61
	4	77	3.10
E	3	72	58.36
	4	81	41.64

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

Fraction A - total, diluted incubation medium where Band 3 corresponds to nicotinic acid and Band 4 corresponds to nicotinamide.

Fraction C - acetone-precipitated dinucleotides where bands 1, 2 and 4 correspond to des-NAD (NADP), NAD and nicotinamide respectively.

Fraction D - acetone-soluble mononucleotides where bands 1, 2, 3 and 4 correspond to NaMN (NADP), NMN, nicotinic acid and nicotinamide, respectively.

Fraction E - ether extract of mononucleotide fraction where Band 3 corresponds to nicotinic acid Band 4 corresponds to nicotinamide.

IN TABLE 11 WHICH INDICATE THE IDENTITY OF SPECIFIC COMPOUNDS WITH THEIR RESPECTIVE HR_F VALUES.

THE RESULTS OF RECHROMATOGRAPHING THE ELUTED IAW BANDS 3 AND 4 OF FRACTION D AND BAND 4 OF FRACTION C (TABLE 10) ON PAPER IN THE BUTANOL-AMMONIA SYSTEM ARE LISTED IN TABLE 12. THE IDENTITY OF SPECIFIC COMPOUNDS WITH SPECIFIC HR_F VALUES IS INCLUDED.

FIGURE 7 REPRESENTS A RADIOCHROMATOGRAPHIC SCAN OF THE SUPERNATANT OBTAINED AFTER TCA PRECIPITATION OF THE MYCELIUM, FRACTION B, WHICH WAS OBTAINED FOLLOWING NICOTINAMIDE -7-^{14}C INCUBATION. THE CHROMATOGRAM, DEVELOPED IN THE IAW SOLVENT SYSTEM, SHOWED FOUR BANDS WHERE BAND 1 CORRESPONDS TO DES-NAD AND NADP; BAND 2 CORRESPONDS TO NMN AND NAD; BAND 3 CORRESPONDS TO NICOTINIC ACID AND BAND 4 CORRESPONDS TO NICOTINAMIDE.

TABLE 11

IDENTIFIED COMPOUNDS IN THE BANDS FROM FRACTIONS C AND D AS SEPARATED IN THE PYRIDINE-WATER SYSTEM

FRACTION	BAND IAW	HR_F (PW)	COMPOUND
C	1	28	NADP
		67	DES-NAD
		83	*NICOTINAMIDE
C	2	64	NAD
C	4	81	NICOTINAMIDE
D	1	26	NADP
D	2	30	NMN
		62	NAD
		77	*NICOTINAMIDE

FRACTION C - ACETONE-PRECIPITATED DINUCLEOTIDES

FRACTION D - ACETONE-SOLUBLE MONONUCLEOTIDES

*NICOTINAMIDE OCCURS AS A RESULT OF THE HYDROLYSIS OF THE RESPECTIVE NUCLEOTIDES.

D - BAND 1 - UNPRECIPITATED NADP

D - BAND 2 - HR_F 62-UNPRECIPITATED NAD.

TABLE 12

COMPOUNDS IDENTIFIED IN THE BANDS FROM FRACTIONS C AND D AS SEPARATED IN BUTANOL-AMMONIA

Fraction	Band (1AW)	HR_F BA	Compound
C	4	60	*Nicotinamide
D	3	20	Nicotinic Acid
	4	60	Nicotinamide

Fraction C - acetone-precipitated dinucleotides.

Fraction D - acetone-soluble mononucleotides

*Nicotinamide occurs as a result of hydrolysis of the corresponding nucleotides.

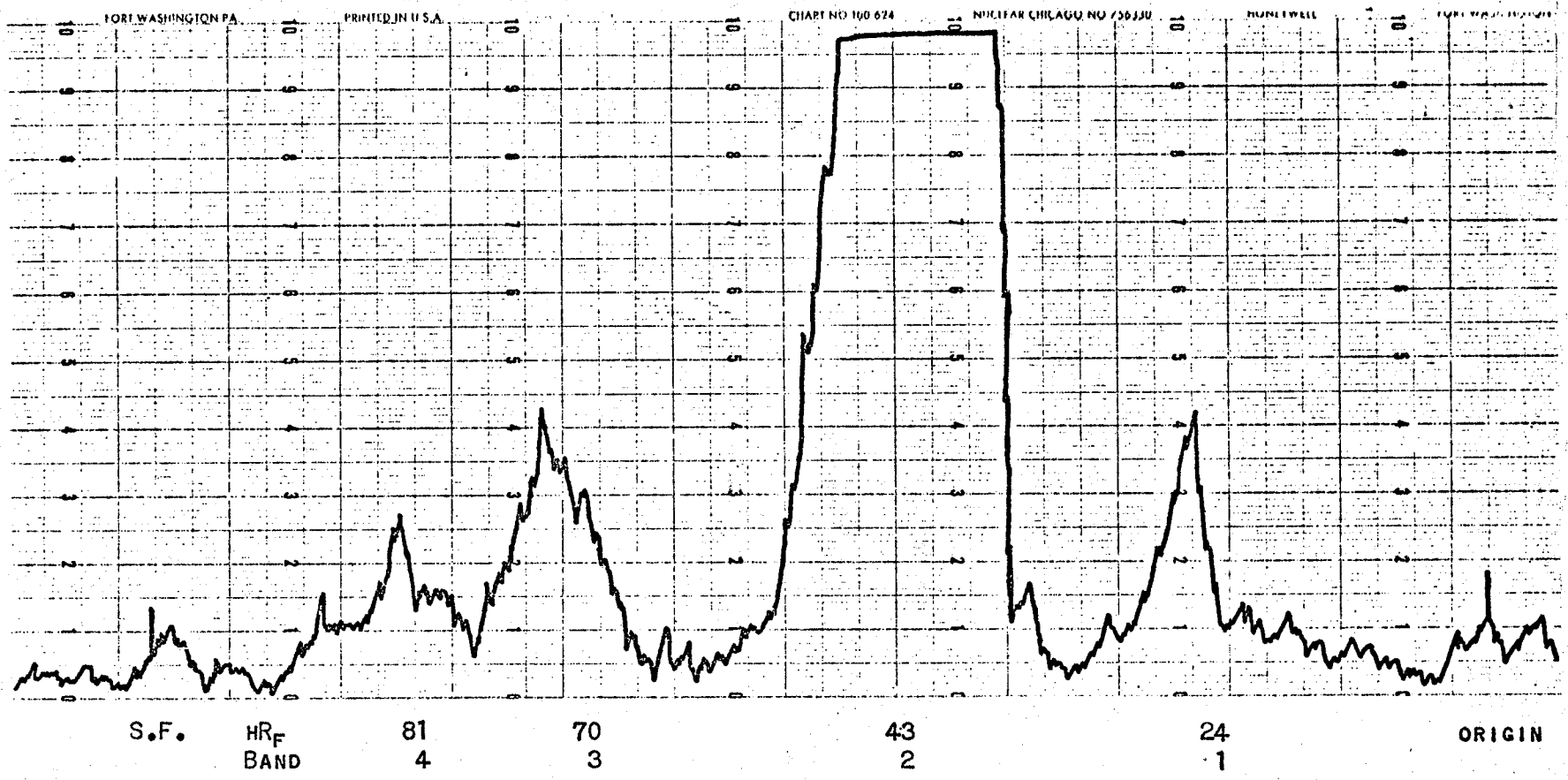

FIGURE 7. RADIOCHROMATOGRAPHIC SCAN OF *FRACTION B, OBTAINED FROM CULTURES INCUBATED WITH NICOTINAMIDE -7-^{14}C AND DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. A 0.1 ML ALIQUOT OF A TOTAL OF 200 ML. WAS STREAKED OVER 1 INCH OF WHATMAN 3 MM PAPER. THE ACTIGRAPH III SETTINGS WERE: 100 CPM SCALE; 10 SECOND INTERVAL; 60 CM./HOUR; 1050 VOLTS.

*FRACTION B - SUPERNATANT OBTAINED AFTER TCA PRECIPITATION OF THE MYCELIUM.

BAND 1 CORRESPONDS TO DES-NAD AND NADP.

BAND 2 CORRESPONDS TO NMN AND NAD.

BAND 3 CORRESPONDS TO NICOTINIC ACID.

BAND 4 CORRESPONDS TO NICOTINAMIDE.

A RADIOCHROMATOGRAPHIC SCAN OF FRACTION C, THE DINUCLEOTIDE FRACTION, IS SHOWN IN FIGURE 8. BAND 1 OF FRACTION C CORRESPONDS TO THE FOLLOWING COMPOUNDS; NADP AND DES-NAD AND WAS FOUND TO CONTAIN NICOTINAMIDE WHICH PRESUMABLY OCCURRED DUE TO HYDROLYSIS OF THE NICOTINAMIDE NUCLEOTIDES, BAND 2 CORRESPONDS TO NAD AND BAND 3 CORRESPONDS TO NICOTINAMIDE WHICH OCCURRED AS A RESULT OF THE HYDROLYSIS OF NICOTINAMIDE CONTAINING NUCLEOTIDES. FIGURE 9 IS A RADIOCHROMATOGRAPHIC SCAN OF FRACTION D, THE MONONUCLEOTIDE FRACTION. BAND 1 OF FRACTION D CONSISTS OF NAMN AND NADP AND DES-NAD (UNPRECIPITATED DINUCLEOTIDES), BAND 2 CORRESPONDS TO NMN AND NAD AND CONTAINED NICOTINAMIDE AS A HYDROLYSIS PRODUCT. BAND 3 CORRESPONDS TO NICOTINIC ACID. NICOTINAMIDE ACTIVITY, ALTHOUGH NOT OBVIOUS IN FIGURE 9, WAS DETECTED AT HR_F 77 ON ALL SCANS.

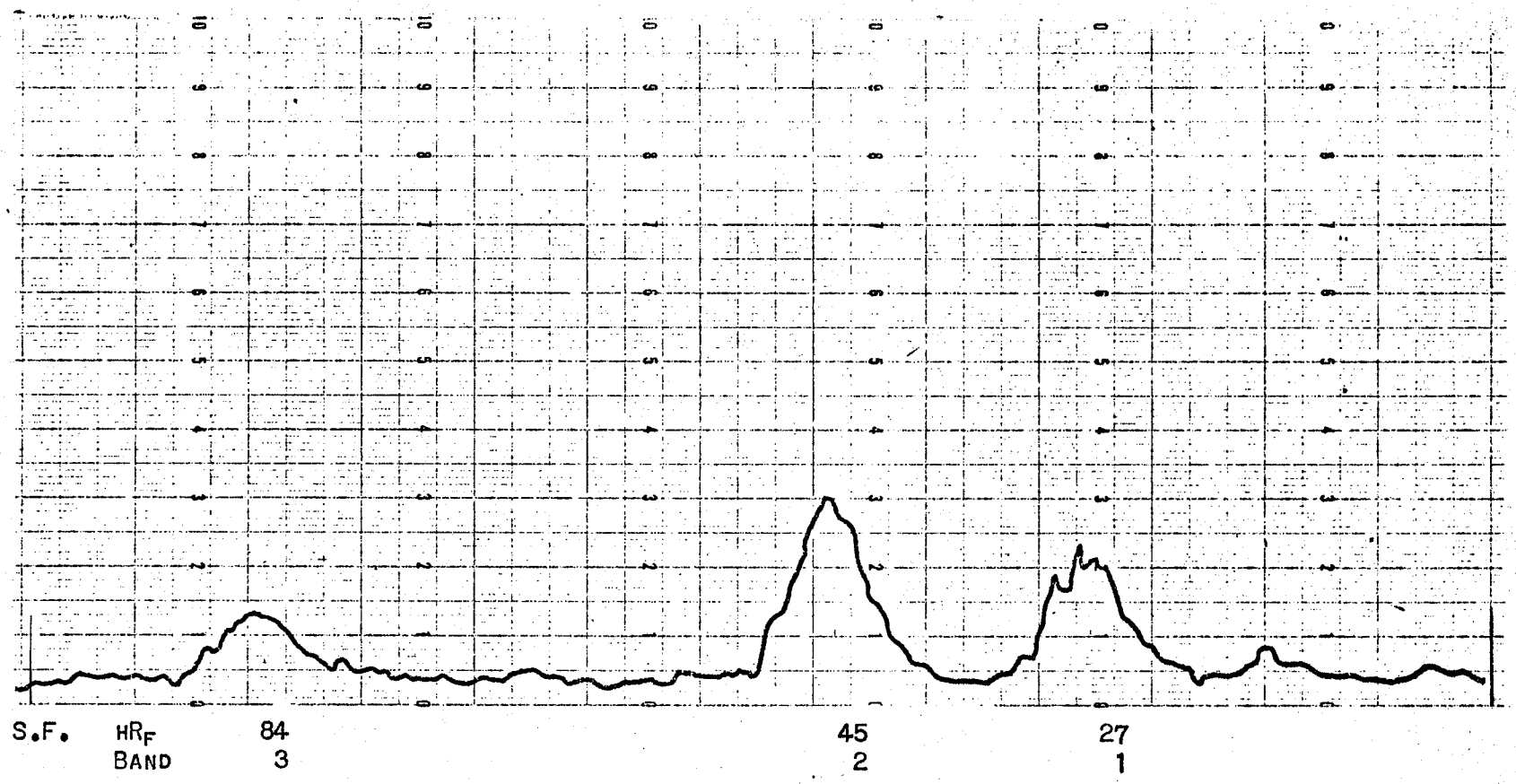

FIGURE 8. RADIOCHROMATOGRAPHIC SCAN OF THE DINUCLEOTIDES, FRACTION C, OF A NICOTINAMIDE -7-^{14}C INCUBATION, DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER- A 0.5 ML. ALIQUOT OF 25 ML WAS STREAKED OVER 1 INCH OF WHATMAN 3MM PAPER. THE ACTIGRAPH III SETTINGS WERE: 500 CPM SCALE; 10 SECOND INTERVAL; 60 CM/HOUR; 1050 VOLTS.

BAND 1 CONSISTS OF NADP, DES-NAD.

BAND 2 CORRESPONDS TO NAD.

BAND 3 CORRESPONDS TO NICOTINAMIDE WHICH OCCURRED AS A RESULT OF THE HYDROLYSIS OF THE CORRESPONDING NUCLEOTIDES.

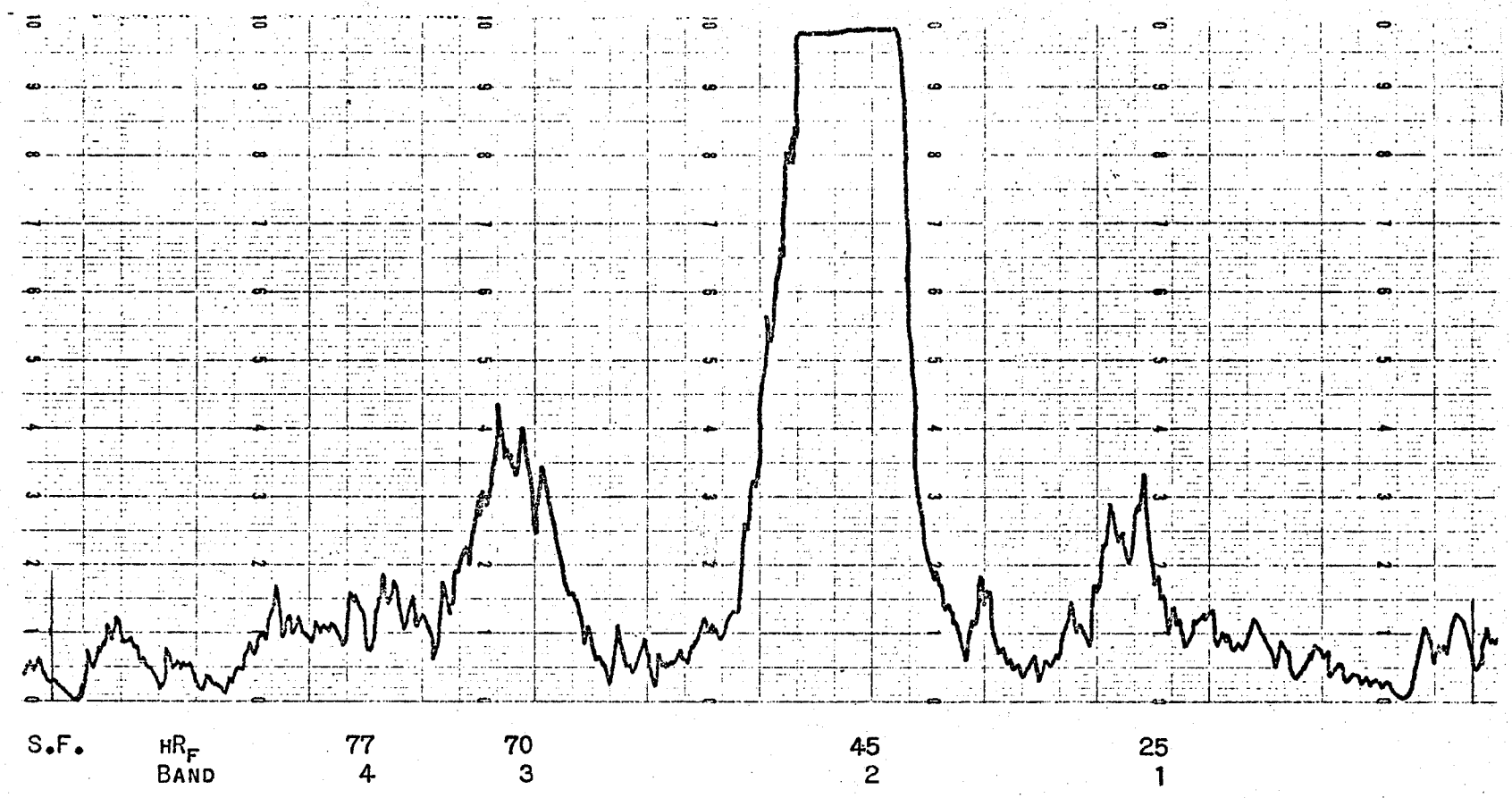

FIGURE 9. RADIOCHROMATOGRAPHIC SCAN OF THE MONONUCLEOTIDES FRACTION D, OF A NICOTINAMIDE -7-^{14}C INCUBATION, DEVELOPED IN ISOBUTYRIC ACID - AMMONIA-WATER. A 0.5 ML. ALIQUOT OF A TOTAL 200 ML. FRACTION WAS STREAKED OVER 1 INCH OF WHATMAN 3 MM PAPER. THE ACTIGRAPH SETTINGS WERE: 150 CPM SCALE; 10 SECOND INTERVAL; 60 CM./HR; 1050 VOLTS.

BAND 1 - CONSISTS OF NaMN, DES-NAD, NADP.
BAND 2 - CONSISTS OF NMN, NAD AND NICOTINAMIDE.
BAND 3 - CORRESPONDS TO NICOTINIC ACID.

NICOTINAMIDE ACTIVITY, ALTHOUGH NOT OBVIOUS, WAS DETECTED AT HR_F 77 ON ALL SCANS. THIS OCCURRED AS A RESULT OF HYDROLYSIS OF CORRESPONDING NUCLEOTIDES.

2. ^{32}P Incorporation into Pyridine Nucleotides in Intact Cultures of Claviceps SD-58.

Individual flasks of twelve-day-old cultures of Claviceps SD-58 were incubated with 0.05 ml. of undiluted radioactive ^{32}P labelled phosphoric acid (S.A. 1 mc/19.0 x 10^{-3}M per 1.0 ml.). Two culture flasks were withdrawn from the shaker after two, four and six hour incubation periods. The two culture flasks from each incubation period were combined and processed as outlined in Figure 4 to yield purified NAD.

An aliquot of the crude NAD isolated from the duplicate extracts was chromatographed and developed in the IAW solvent system. Figure 10 indicates the distribution of activity in the dinucleotide fraction C, from the six hour incubation period. The radiochromatographic scans for the similar fractions from the two and four hour incubation periods produced results similar to those shown for the six hour period. Rechromatography of the eluted band at hR_F 45 in IAW from the six hour incubation period on paper in ethanol-ammonium acetate system produced the radiochromatogram shown in Figure 11. A similar radiochromatographic scan was obtained for the preparations from the two and four hour incubation periods. Using the planimeter, it was found that the ratio of the NAD peak area from equivalent amounts of the 2,4 and 6 hour incubation extracts was 1:1.4:2.1, respectively. Therefore, the six hour incubation period was found to be suitable to give adequate ^{32}P labelled - NAD levels for subsequent studies.

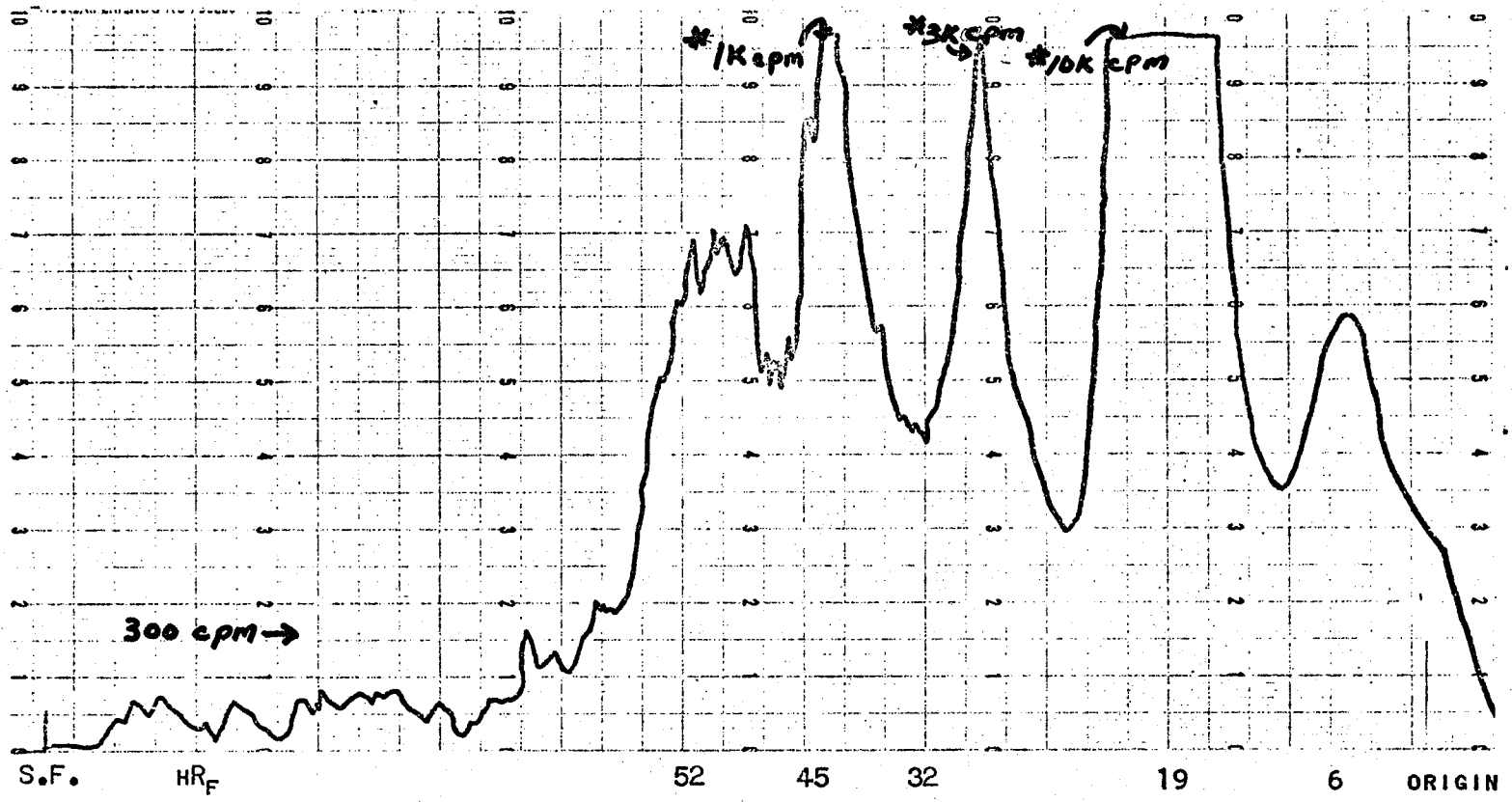

FIGURE 10. RADIOCHROMATOGRAPHIC SCAN OF THE DINUCLEOTIDE FRACTION, FRACTION C, AFTER SIX HOUR INCUBATION WITH ^{32}P, DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. THE ACTIGRAPH SETTINGS WERE: VARIABLE CPM; 10 SECOND INTERVAL; 60 CM./HOUR; 1050 VOLTS.

*CPM SCALE INDICATOR CHANGED TO THE SETTING INDICATED AT THIS POINT.

FRACTION C - ACETONE-PRECIPITATED DINUCLEOTIDES

BAND HR$_F$6 - NOT IDENTIFIED.
BAND HR$_F$19- NOT IDENTIFIED.
BAND HR$_F$32- CORRESPONDS TO NADP REFERENCE STANDARD.
BAND HR$_F$45- CORRESPONDS TO NAD REFERENCE STANDARD.
BAND HR$_F$52- CORRESPONDS TO AMP REFERENCE STANDARD.

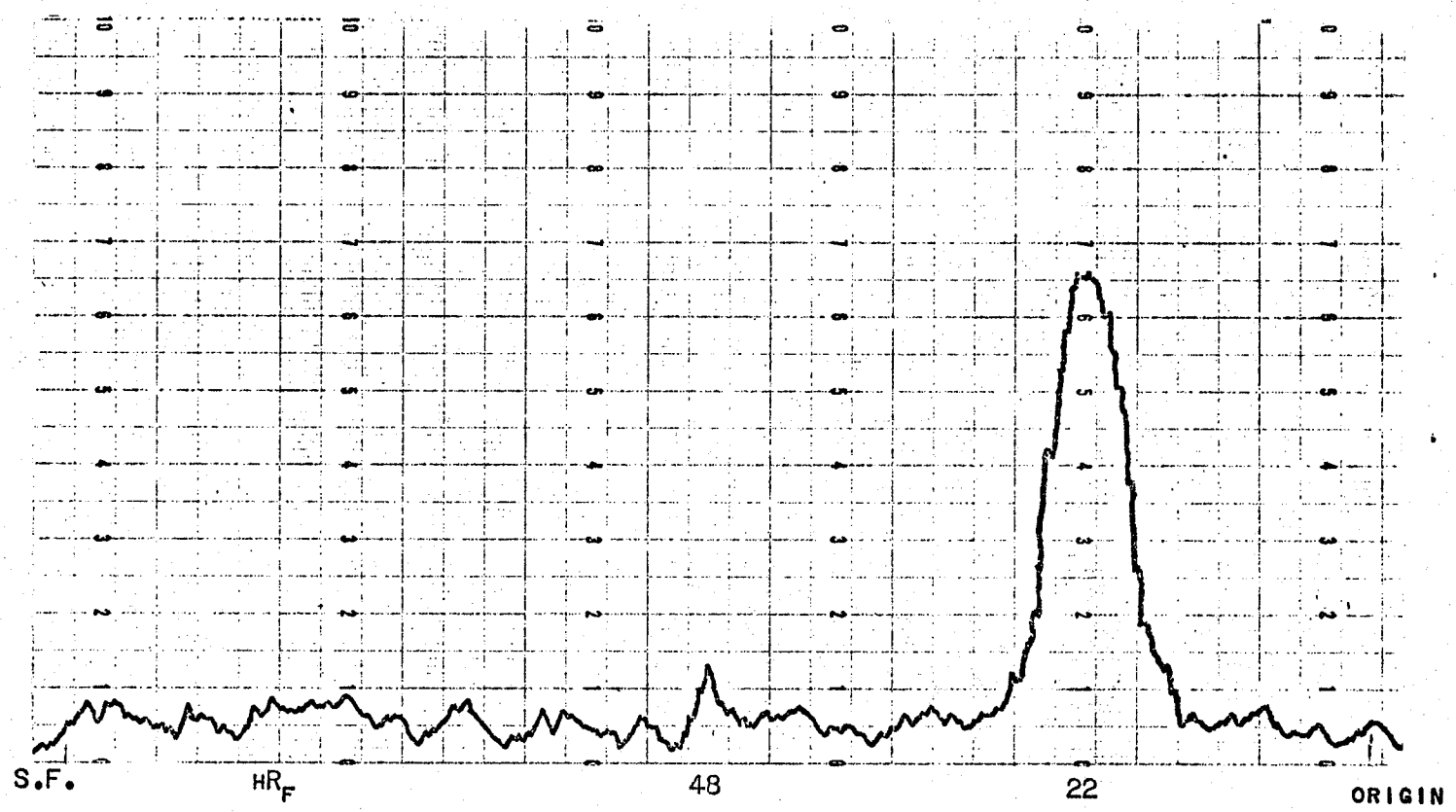

FIGURE 11. RADIOCHROMATOGRAPHIC SCAN OF THE ELUTED BAND FROM FRACTION C, HR$_F$ 45 IN IAW, FROM A 6 HOUR INCUBATION WITH ^{32}P, DEVELOPED IN ETHANOL--AMMONIUM ACETATE. THE ACTIGRAPH SETTINGS WERE: 300 CPM SCALE; 10 SECOND INTERVAL; 60 CM./HOUR; 1050 VOLTS.

FRACTION C - ACETONE-PRECIPITATED DINUCLEOTIDES.

BAND HR$_F$22 - CORRESPONDS TO NAD REFERENCE STANDARD.

BAND HR$_F$48 - UNIDENTIFIED AND MAY HAVE OCCURRED AS A HYDROLYSIS PRODUCT OF NAD.

3. Simultaneous ^{32}P and Nicotinamide -7-^{14}C Incubation with Intact Cultures of Claviceps SD-58.

Duplicate flasks of twelve-day-old cultures of Claviceps SD-58 were incubated with a constant concentration (0.05 ml. of phosphoric acid - ^{32}P (S.A. 1 mc/19.0 x 10^{-3}M per 1.0 ml.) and the following concentrations of nicotinamide -7-^{14}C; 10^{-6}M, 10^{-5}M and 10^{-4}M. The specific activity of the added nicotinamide was 5.09 mc/mM for the 10^{-6}M and 10^{-5}M concentrations of nicotinamide and 0.169 mc/mM for the 10^{-4}M level of nicotinamide. Molar concentrations were calculated on the basis that the total incubation volume per flask of Claviceps was 50 ml.

Duplicate sets of culture flasks containing each molar concentration of nicotinamide-7-^{14}C and ^{32}P were processed according to the procedure outlined in Figures 4 and 5 to produce purified NAD. Figure 12 is a radiochromatographic scan of Fraction C, the dinucleotide fraction, after a six-hour incubation with ^{32}P and nicotinamide-7-^{14}C.

Figures 13 and 14 indicate the distribution of activity for the phosphodiesterase cleavage of the isolated NAD (^{32}P and nicotinamide-7-^{14}C containing) to NMN (Figure 13) and the ammonia hydrolysis of NMN (nicotinamide-7-^{14}C and ribose-5-phosphate -^{32}P containing) to nicotinamide and ribose-5-phosphate (Figure 14) for the 10^{-6}M concentration of nicotinamide, respectively. The radioactive peak at hR_F 40 (Figure 14) represents unhydrolyzed NMN. Similar results were obtained for the 10^{-5} and 10^{-4} molar concentrations of nicotinamide. The assays for ribose-5-phosphate and nicotinamide were carried out according to the procedures previously described. Examination of the chromatogram upon which the NMN hydrolyzed extracts were developed indicated the presence of AMP (very

LOW ACTIVITY) AT AN HR_F OF 48 IN THE IAW SOLVENT SYSTEM (FIGURE 14). THE ACTIGRAPH III SETTINGS WERE SUCH THAT THE AMP COULD ONLY BE DETECTED AT HIGH SENSITIVITY; 100 CPM VERSUS 3 K CPM FOR NICOTINAMIDE -7-^{14}C AND RIBOSE -5-PHOSPHATE -^{32}P AS WAS DONE FOR THE SCAN REPRESENTED IN FIGURE 14.

AS PREVIOUSLY MENTIONED, THE HYDROLYTIC PRODUCTS OF THE SNAKE VENOM PHOSPHODIESTERASE CLEAVAGE, AMP AND NMN, RAN TOGETHER IN THE SOLVENT SYSTEM (ETHANOL-AMMONIUM ACETATE) USED IN THIS STUDY. AS OUTLINED UNDER THE SECTION ON MATERIALS AND METHODS, TESTS WERE CARRIED OUT TO DETERMINE WHETHER AMP WAS AFFECTED BY THE ALKALINE HYDROLYSIS. THE RESULTS INDICATED THAT AMP WAS UNAFFECTED BY THIS PROCEDURE WITH NO BREAKDOWN TO FORM DETECTABLE RIBOSE -5- PHOSPHATE.

THE RATE OF INCORPORATION OF ^{32}P AND VARYING CONCENTRATIONS OF NICOTINAMIDE-7-^{14}C INTO THE NMN PORTION OF NAD IN INTACT CULTURES OF CLAVICEPS SD-58 ARE PRESENTED IN TABLE 13. IN THE ABSENCE OF ADDED NICOTINAMIDE, SOME ^{32}P WAS INCORPORATED INTO THE NMN PORTION OF NAD. INCREASING CONCENTRATIONS OF NICOTINAMIDE -7-^{14}C ($10^{-6}M$, $10^{-5}M$, AND $10^{-4}M$) HAD LITTLE EFFECT UPON THE SPECIFIC ACTIVITY OF THE ^{32}P FOUND IN THE NMN PORTION OF NAD (FIGURE 15). HOWEVER, THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C ISOLATED FROM THE SAME PREPARATION OF NMN INCREASED WITH INCREASING CONCENTRATIONS OF NICOTINAMIDE-7-^{14}C (FIGURE 15). CORRECTING FOR THE DILUTION OF THE RADIOACTIVITY OF THE NICOTINAMIDE AT THE $10^{-4}M$ LEVEL (S.A. 0.169 MC/MM VERSUS S.A. 5.09 MC/MM) FOR THE OTHER LEVELS OF NICOTINAMIDE THE INCREASED NICOTINAMIDE INCORPORATION WAS VERY MARKED.

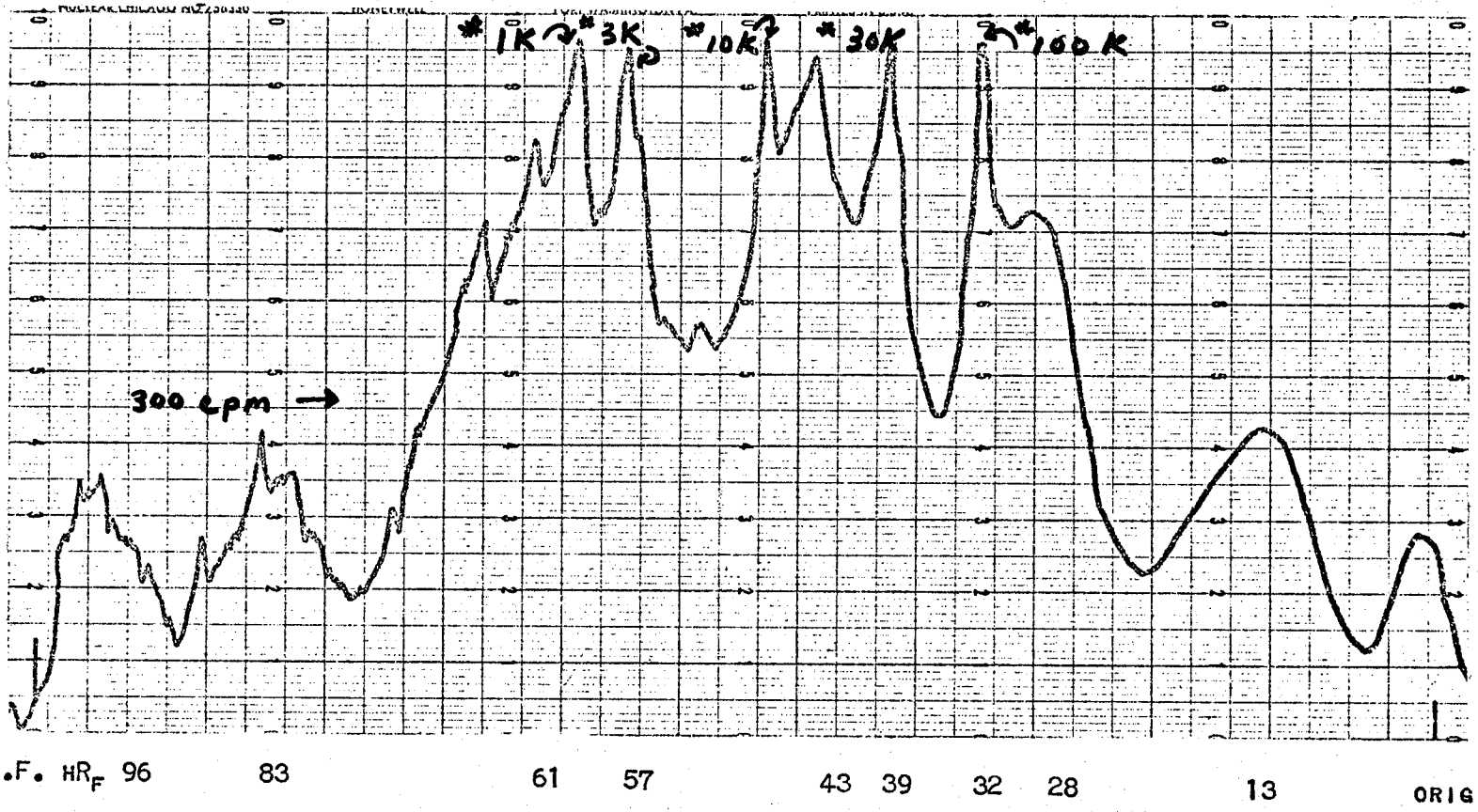

FIGURE 12. RADIOCHROMATOGRAPHIC SCAN OF THE DINUCLEOTIDES, FRACTION C, OF A NICOTINAMIDE -7-^{14}C (10^{-6}M) AND ^{32}P INCUBATION, DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. THE ACTIGRAPH SETTINGS WERE: VARIABLE CPM; 10 SECOND INTERVAL, 60 CM./HOUR; 1050 VOLTS.

*CPM SCALE INDICATOR CHANGED TO THE SETTING INDICATED AT THIS POINT.

FRACTION C - ACETONE-PRECIPITATED DINUCLEOTIDES.

BANDS AT HR$_F$ 13, 39, 57, 61 AND 96 NOT IDENTIFIED.
BANDS AT HR$_F$ 28 AND 32 CORRESPOND TO DES-NAD AND NADP.
BAND AT HR$_F$ 43 CORRESPONDS TO NAD
BAND AT HR$_F$ 83 CORRESPONDS TO NICOTINAMIDE WHICH OCCURRED DUE TO HYDROLYSIS OF NICOTINAMIDE CONTAINING NUCLEOTIDES.

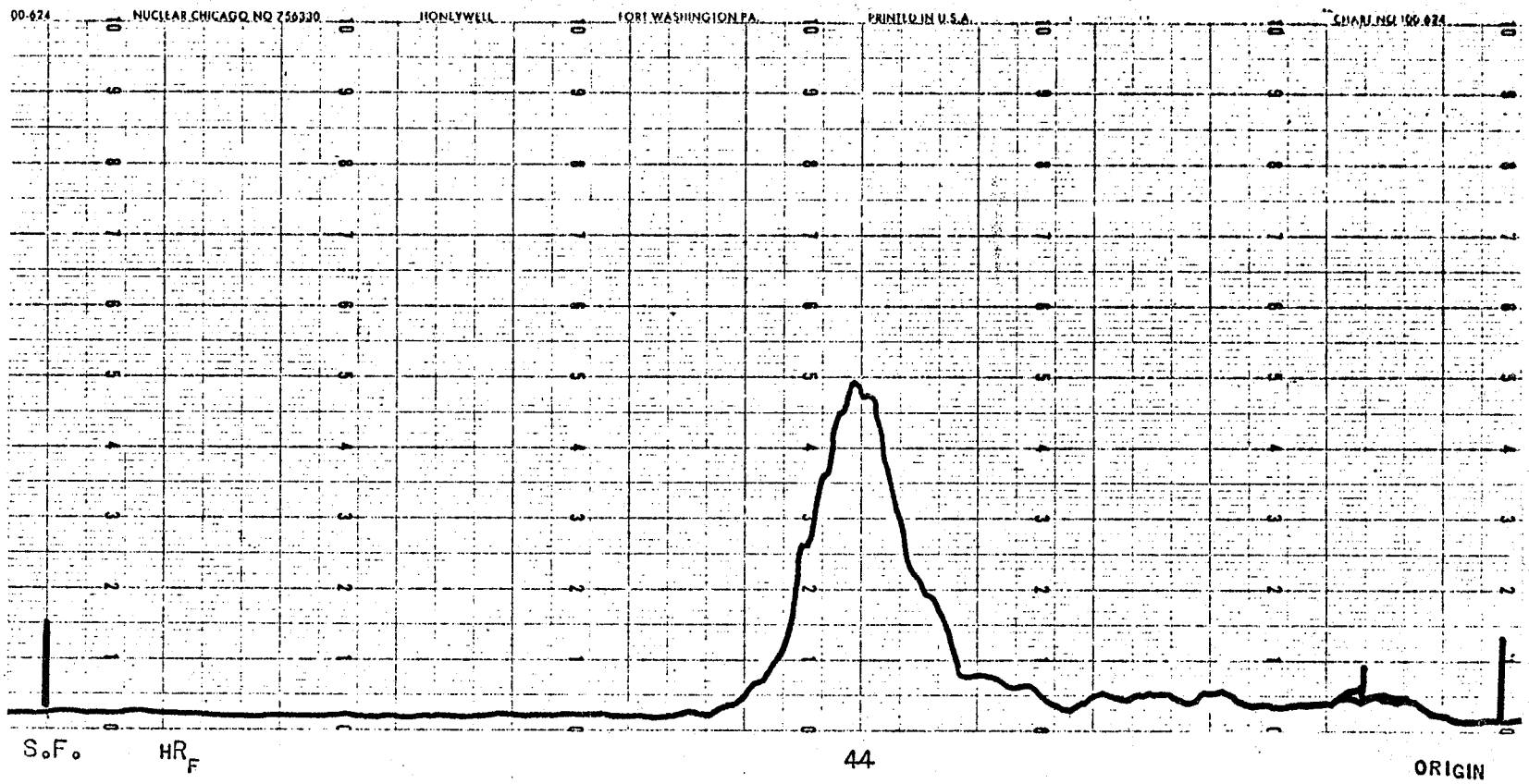

FIGURE 13. RADIOCHROMATOGRAPHIC SCAN OF THE RESIDUE FROM THE CLEAVAGE OF NAD (^{32}P AND NICOTINAMIDE-7-^{14}C LABELLED) TO NMN BY (SNAKE VENOM) PHOSPHODIESTERASE FOR THE 10^{-6}M CONCENTRATION OF NICOTINAMIDE-7-^{14}C; DEVELOPED IN ETHANOL-AMMONIUM ACETATE. THE ACTIGRAPH III SETTINGS WERE: 1 K CPM SCALE; 10 SECOND INTERVAL; 60 CM/HOUR; 1050 VOLTS.

BAND AT 44 CORRESPONDS TO NMN AND AMP.

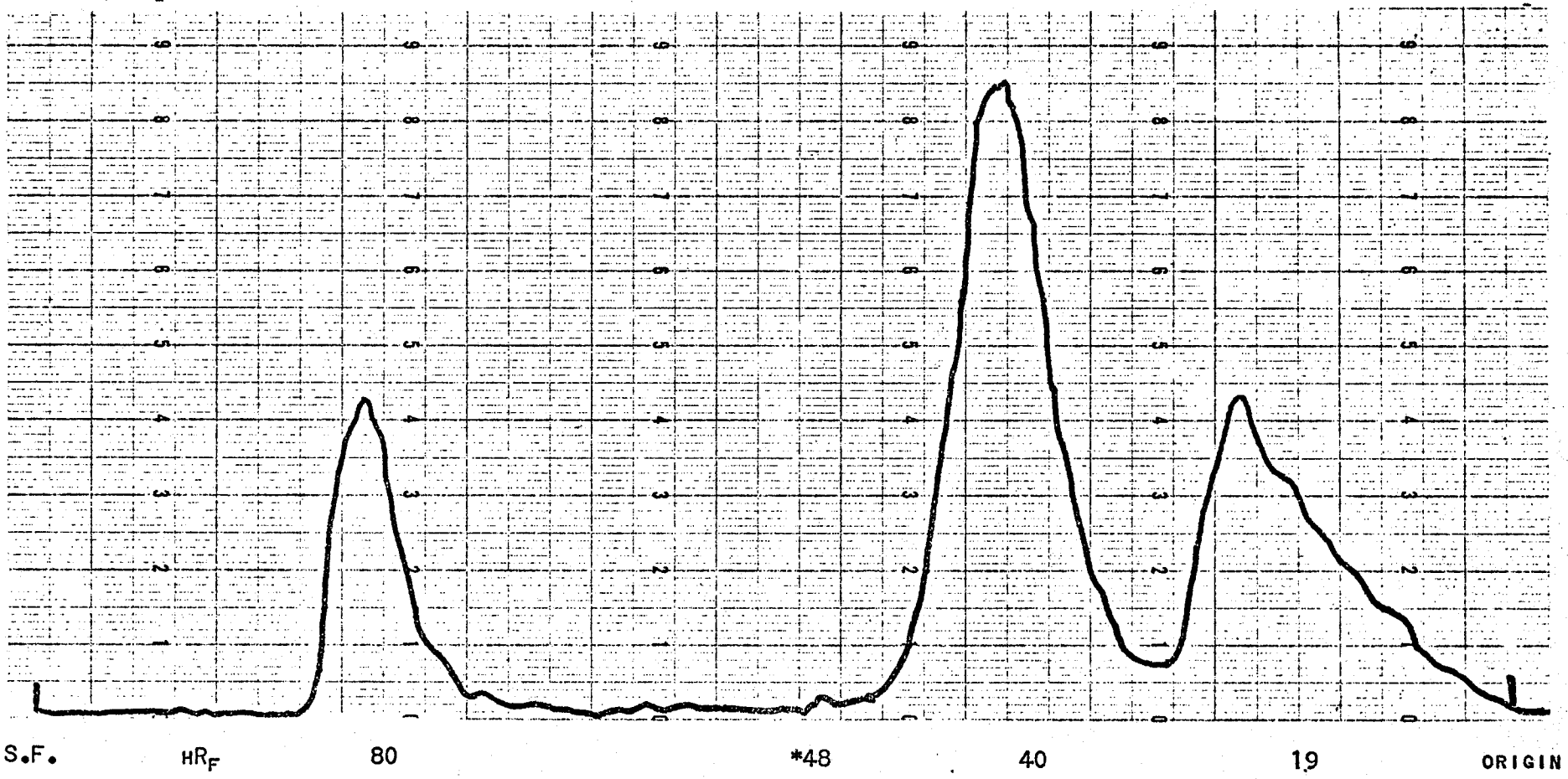

FIGURE 14. RADIOCHROMATOGRAPHIC SCAN OF THE RESIDUE FROM THE AMMONIA HYDROLYSIS OF NMN (^{32}P AND NICOTINAMIDE -7-^{14}C CONTAINING) TO NICOTINAMIDE AND RIBOSE -5- PHOSPHATE, FOR THE 10^{-6}M CONCENTRATION OF NICOTINAMIDE -7-^{14}C; DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. ACTIGRAPH III SETTINGS WERE 3 K CPM SCALE; 1050 VOLTS; 60 CM./HOUR; 10 SECOND INTERVAL.

BAND AT HR$_F$ 19 CORRESPONDS TO RIBOSE-5-PHOSPHATE.
BAND AT HR$_F$ 40 CORRESPONDS TO UNHYDROLYZED NMN.
BAND AT HR$_F$ 80 CORRESPONDS TO NICOTINAMIDE.

*THE PRESENCE OF AMP IS INDICATED AT HR$_F$ 48.

TABLE 13

EFFECT OF NICOTINAMIDE LEVELS ON INCORPORATION OF NICOTINAMIDE -7-^{14}C AND ^{32}P

INTO THE NMN PORTION OF NAD IN DUPLICATE CULTURES OF CLAVICEPS

Nicotinamide Concentration	Nicotinamide-7-^{14}C from NMN Portion of NAD		Ribose-5 Phosphate -^{32}P from NMN Portion of NAD		S.A. of Ribose -5-Phosphate cpm/muM	S.A. of Nicotinamide cpm/muM
	cpm	muM	cpm	muM		
-			8,643	273	32	
*10-6M	□ 16,840	330	15,458	330	47	51
**10-5M	32,800	339	15,957	339	47	97
***10-4M	****112,800	383	19,032	383	50	318

**** Corrected for dilution factor

* Each of 2 culture flasks contained nicotinamide-7-^{14}C (0.695 x 10^6DPM, 5.09 mc/mM) and 0.05 ml. of ^{32}P - phosphoric acid (S.A. 1 mc/19.0 x 10^{-3}M per 1 ml.).

** Each of 2 culture flasks contained nicotinamide -7-^{14}C (6.95 x 10^6DPM, 5.09 mc/mM) and 0.05 ml. of ^{32}P - phosphoric acid (S.A. 1 mc/19.0 x 10^{-3}M per 1 ml.).

*** Each of 2 culture flasks contained nicotinamide -7-^{14}C (2.78 x 10^6DPM, 0.169 mc/mM) and 0.05 ml. of ^{32}P - phosphoric acid (S.A. 1 mc/19.0 x 10^{-3}M per 1.0 ml.)

□ Values given are from duplicate cultures.

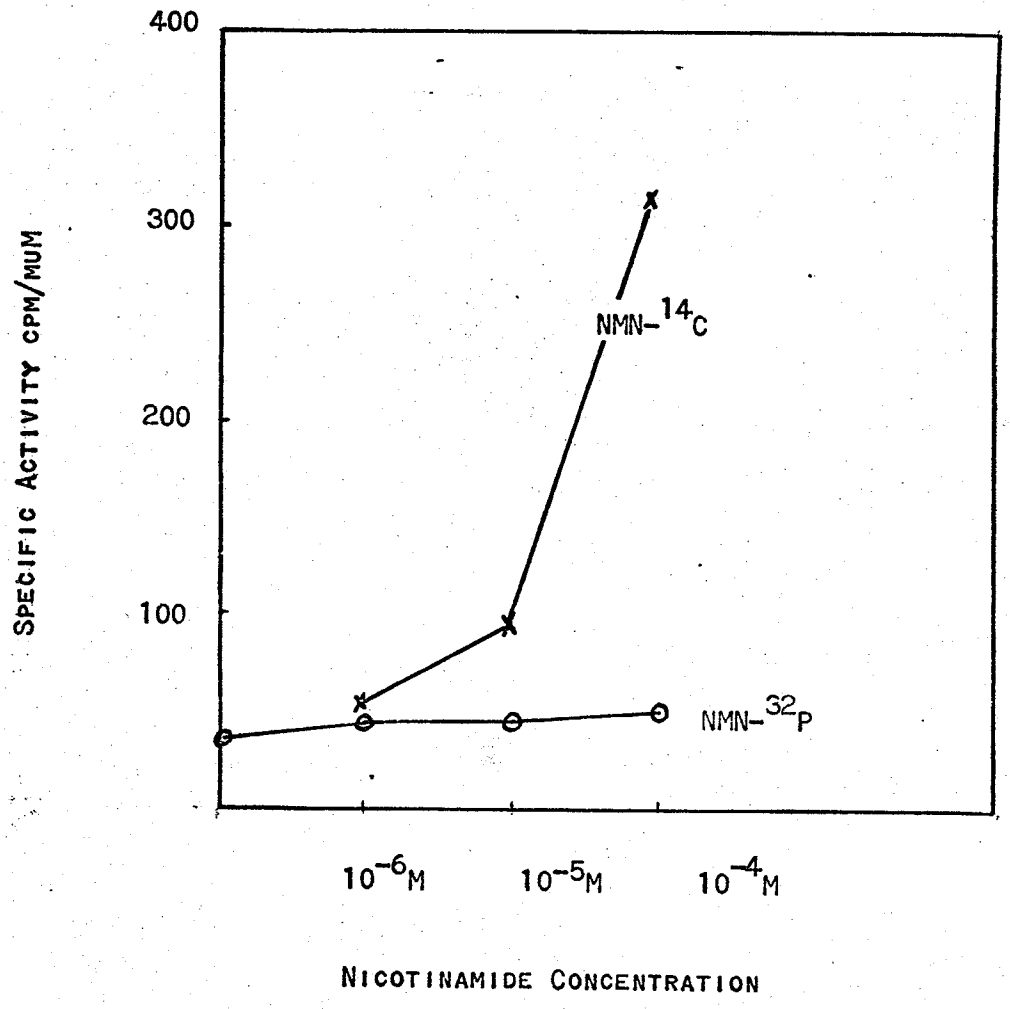

FIGURE 15. THE EFFECT OF NICOTINAMIDE LEVELS ON THE INCORPORATION OF NICOTINAMIDE -7-^{14}C AND ^{32}P INTO THE NMN MOIETY OF NAD IN INTACT CULTURES OF CLAVICEPS SD-58. THE SPECIFIC ACTIVITY OF THE ^{32}P WAS 1 MC/19.0 X 10^{-3}M AND THE SPECIFIC ACTIVITY OF THE ADDED NICOTINAMIDE WAS 5.09 MC/MM FOR ALL LEVELS EXCEPT THE 10^{-4}M LEVEL, WHERE THE ACTIVITY WAS 0.169 MC/MM. THE DATA FOR THE ^{14}C SPECIFIC ACTIVITY AT THE 10^{-4}M CONCENTRATION OF NICOTINAMIDE HAVE BEEN CORRECTED TO MAKE IT EQUIVALENT TO THE OTHER LEVELS EXAMINED.

• NMN -^{32}P
X NMN -^{14}C

4. Simultaneous ^{32}P and Nicotinic Acid -7-^{14}C Incorporation into NAD in Intact Cultures of Claviceps SD-58.

Duplicate sets of flasks of twelve-day-old cultures of Claviceps were incubated with a constant concentration (0.5 ml.*) of phosphoric acid -^{32}P (S.A. 1 mc/19.0 x 10^{-3}M) and varying concentrations (10^{-6}M and 10^{-4}M) of nicotinic acid -7-^{14}C. The specific activity of the added nicotinic acid was 27.9 mc/mM for the 10^{-6}M concentration, 9.3 mc/mM for the 10^{-5}M concentration and 0.93 mc/mM for the 10^{-4}M concentration. Molar levels were calculated on the basis that the total incubation volume per flask of Claviceps was 50 ml.

Duplicate sets of culture flasks containing each molar concentration of nicotinic acid -7-^{14}C and ^{32}P were treated as outlined in Figures 4 and 5. Representative scans for the cleavage of NAD to NMN and the hydrolysis of NMN to ribose-5-phosphate and nicotinamide are shown in Figures 16 and 17, respectively.

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*1 ml. of original phosphoric acid was diluted to 10 ml. volumetrically with distilled water from which 0.5 ml. was pipetted using the Agla syringe.

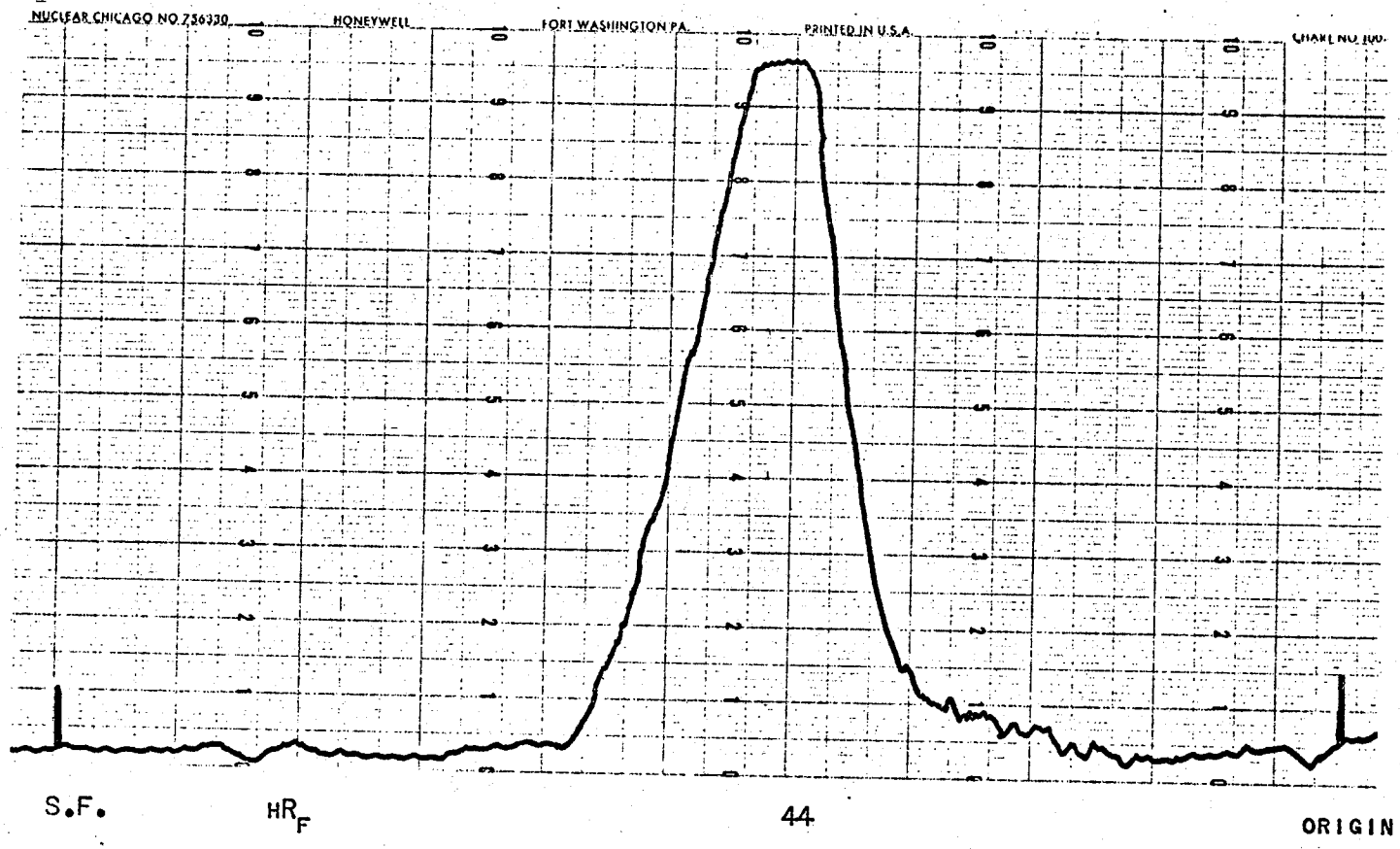

FIGURE 16. RADIOCHROMATOGRAPHIC SCAN OF THE RESIDUE FROM THE CLEAVAGE OF NAD (^{32}P AND NICOTINAMIDE -7-^{14}C CONTAINING) TO NMN BY SNAKE VENOM PHOSPHODIESTERASE FOR THE 10^{-6}M CONCENTRATION OF NICOTINIC ACID-7-^{14}C, DEVELOPED IN ETHANOL-AMMONIUM ACETATE. ACTIGRAPH III SETTINGS WERE: 15 K CPM SCALE; 10 SECOND INTERVAL; 60 CM./HOUR; 1050 VOLTS.

BAND AT HR$_F$ 44 CORRESPONDS TO NMN AND AMP.

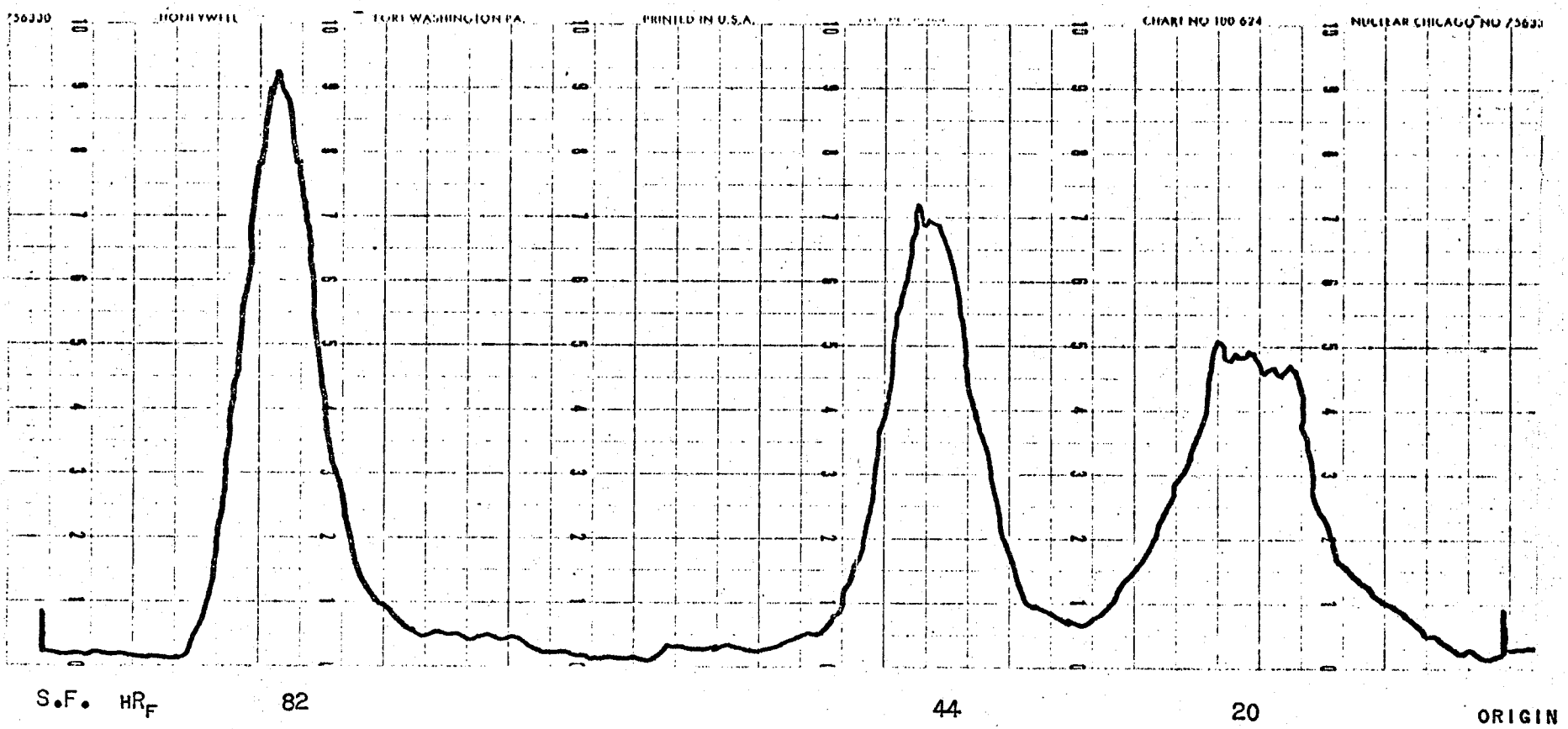

FIGURE 17. RADIOCHROMATOGRAPHIC SCAN OF THE RESIDUE FROM THE AMMONIA HYDROLYSIS OF NMN (^{32}P AND NICOTINAMIDE -7-^{14}C CONTAINING) TO NICOTINAMIDE AND RIBOSE-5-PHOSPHATE FOR THE 10^{-6}M CONCENTRATION OF NICOTINIC ACID-7-^{14}C, DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. ACTIGRAPH III SETTINGS WERE: 1 K CPM SCALE; 10 SECOND INTERVAL; 60 CM/HOUR; 1050 VOLTS.

BAND AT HR$_F$ 20 CORRESPONDS TO RIBOSE-5-PHOSPHATE.

BAND AT HR$_F$ 44 CORRESPONDS TO NMN.

BAND AT HR$_F$ 82 CORRESPONDS TO NICOTINAMIDE.

THE ASSAY FOR RIBOSE-5-PHOSPHATE AND NICOTINAMIDE WAS CARRIED OUT ACCORDING TO THE PROCEDURES PREVIOUSLY DISCUSSED.

THE RATE OF INCORPORATION OF ^{32}P AND NICOTINIC ACID-7-^{14}C INTO THE NMN PORTION OF NAD IN INTACT CULTURES OF CLAVICEPS IS PRESENTED IN TABLE 14. ^{32}P IN THE ABSENCE OF NICOTINIC ACID WAS INCORPORATED INTO THE NMN PORTION OF NAD. INCREASING CONCENTRATIONS OF NICOTINIC ACID-7-^{14}C ($10^{-6}M$, $10^{-5}M$ AND $10^{-4}M$) RESULTED IN PARALLEL CHANGES IN THE SPECIFIC ACTIVITY OF THE ISOLATED RIBOSE-5-PHOSPHATE -^{32}P AND NICOTINAMIDE-^{14}C ISOLATED FROM THE NMN MOIETY OF NAD (FIGURE 18). ADDITIONS OF NICOTINIC ACID-7-^{14}C TO GIVE CONCENTRATIONS OF $10^{-6}M$ AND $10^{-5}M$ RESULTED IN A SUBSTANTIAL INCREASE IN THE SPECIFIC ACTIVITIES OF BOTH THE NICOTINAMIDE (I.E. 149 TO 2,239) AND RIBOSE-5-PHOSPHATE (I.E. 52 TO 153) IN THE NMN PORTION OF NAD. INCREASING THE CONCENTRATION OF NICOTINIC ACID-7-^{14}C TO $10^{-4}M$ HOWEVER DID NOT PRODUCE A CHANGE IN THE SPECIFIC ACTIVITY OF THE ^{32}P IN THE NMN MOIETY OF NAD FROM THE $10^{-5}M$ CONCENTRATION. HOWEVER, AT THE SAME CONCENTRATION OF NICOTINIC ACID-7-^{14}C, $10^{-4}M$, THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C IN THE NMN MOIETY OF NAD INCREASED 2.5 FOLD. CORRECTING FOR THE DILUTION OF THE RADIOACTIVITY OF THE NICOTINIC ACID AT THE 10^{-5} AND $10^{-4}M$ CONCENTRATIONS, THE INCREASED NICOTINIC ACID INCORPORATION INTO THE NMN MOIETY OF NAD APPEARED SUBSTANTIAL.

TABLE 14

EFFECT OF NICOTINIC ACID LEVELS ON THE INCORPORATION OF NICOTINIC ACID-7-^{14}C AND ^{32}P INTO THE NMN PORTION OF NAD IN DUPLICATE CULTURES OF CLAVICEPS

Nicotinic Acid Concentration	Nicotinamide-7-^{14}C from NMN Portion of NAD		Ribose-5-Phosphate-^{32}P from NMN Portion of NAD		S.A. for Ribose-5-PO_4 cpm/mum	S.A. for Nicotinamide cpm/mum
	cpm	mum	cpm	mum		
-			8,750	250	35	
**10^{-6}M	◘ 19,980	135	7,043	134	52	149
***10^{-5}M	*469,710	211	32,163	211	153	2,239
****10^{-4}M	*944,100	165	25,182	165	152	5,701

* CORRECTED FOR DILUTION FACTOR

** EACH OF TWO CULTURE FLASKS CONTAINED NICOTINIC ACID-7-^{14}C (3.33×10^6DPM, S.A. 27.9 mc/mM) AND 0.5 ML. OF ^{32}P-PHOSPHORIC ACID (S.A. 1 mc/19.0×10^{-3}M IN 10 ML.).

*** EACH OF TWO CULTURE FLASKS CONTAINED NICOTINIC ACID-7-^{14}C (1.11×10^7DPM, S.A. 9.3 mc/mM) AND 0.5 ML. OF ^{32}P-PHOSPHORIC ACID (S.A. 1 mc/1.9×10^{-3}M IN 10 ML.).

**** EACH OF TWO CULTURE FLASKS CONTAINED NICOTINIC ACID-7-^{14}C (1.11×10^7DPM, S.A. 0.93 mc/mM) AND 0.5 ML. OF ^{32}P-PHOSPHORIC ACID (S.A. 1 mc/19.0×10^{-3}M IN 10 ML.).

◘ VALUES GIVEN ARE FROM DUPLICATE CULTURES.

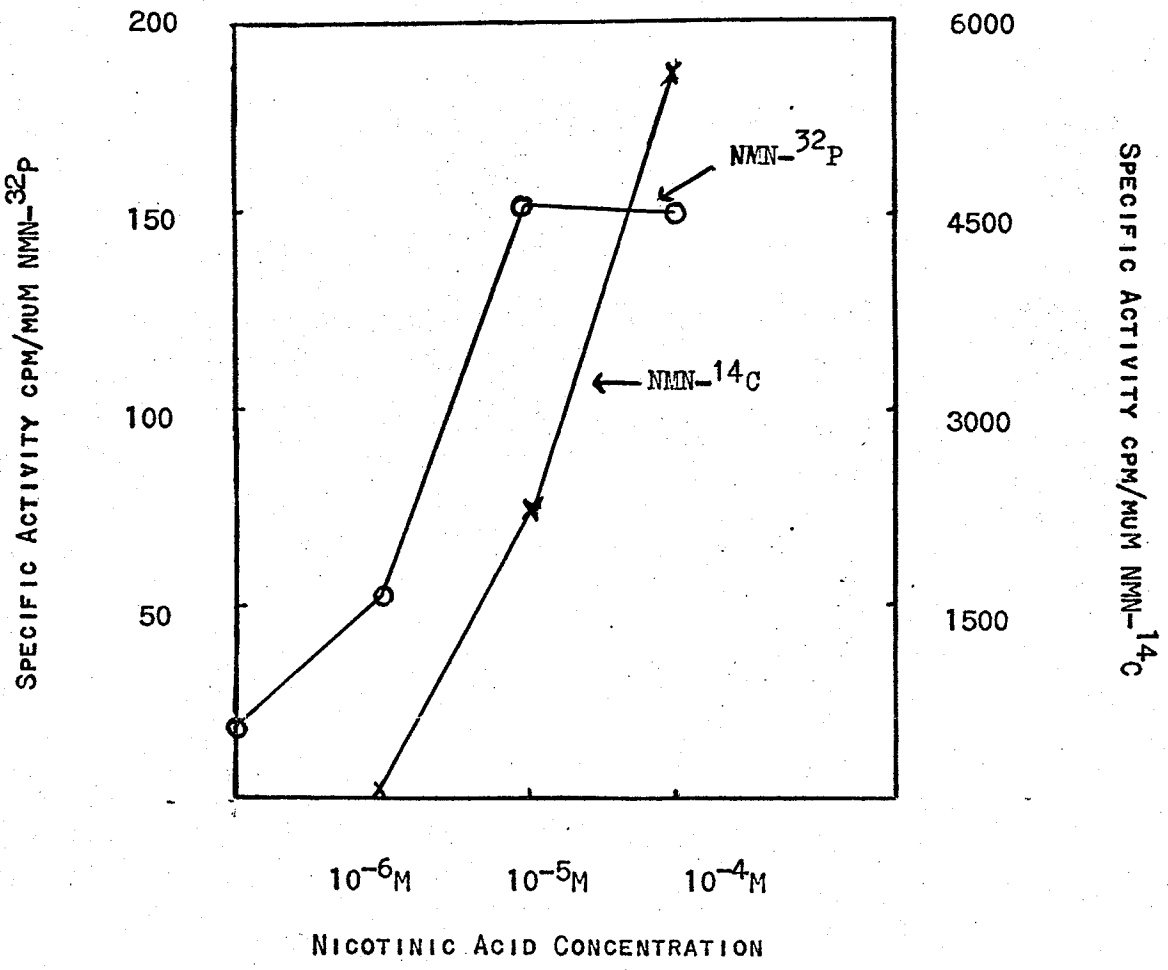

FIGURE 18. EFFECT OF NICOTINIC ACID LEVELS ON THE INCORPORATION OF NICOTINIC ACID-7-^{14}C AND ^{32}P INTO THE NMN MOIETY OF NAD IN INTACT CULTURES OF CLAVICEPS SD-58. THE SPECIFIC ACTIVITY OF THE ADDED ^{32}P WAS 1 MC/19.0 X 10^{-3}M AND THE SPECIFIC ACTIVITY OF THE ADDED NICOTINIC ACID WAS 27.9 MC/MM FOR THE 10^{-6}M CONCENTRATION; 9.3 MC/MM FOR THE 10^{-5}M CONCENTRATION AND 0.93 MC/MM FOR THE 10^{-4}M CONCENTRATION. THE DATA FOR THE ^{14}C SPECIFIC ACTIVITY AT THE 10^{-5} AND 10^{-4}M LEVELS OF NICOTINIC ACID HAVE BEEN CORRECTED TO MAKE IT EQUIVALENT TO THE OTHER LEVEL TESTED.

o NMN-^{32}P
X NMN-^{14}C

5. Demonstration of NAD Glycohydrolase In Vitro Activity

Preliminary studies indicated that the most satisfactory fractions were obtained by grinding the cultures in a Wedgewood mortar with sand as compared to those obtained by Virtis homogenization.

Employing the fractionation procedure outlined in Figure 6, a bi-modal distribution of NAD glycohydrolase activity was observed. Enzyme activity was observed in the nuclear fraction, *fraction II (i.e.1000 x g. pellet - Figure 6) and in the microsomal supernatant, fraction IV (i.e. 25,000 x g. supernatant - Figure 6).

The nuclear fraction, fraction II (Figure 6) was found to possess NADᵀase enzyme activity capable of hydrolytic and transglycosidic functions as shown in Figures 19 and 20. Incubation of an aliquot (2.5 ml.) of the nuclear pellet, fraction II, suspended in 8.0 ml. of 0.1 M phosphate buffer, pH 6.4, for 2 hours at 37°C in the presence of radioactive NAD-^{14}C showed that the NAD-^{14}C had been cleaved at the nicotinamide ribose linkage to form radioactive nicotinamide (Figure 19). The nicotinamide-^{14}C was then deamidated to nicotinic acid-^{14}C due to the presence of the nicotinamidase in the nuclear fraction. The presence of the nicotinamidase in the nuclear fraction confirms the observations reported by Siemens (10)

In addition, an aliquot (2.5 ml.) of the suspended nuclear pellet (total volume of 8 ml.), fraction II, in 0.1M phosphate buffer (pH 6.4) was incubated for 2 hours at 37°C in the presence of radioactive nicotinamide -7-^{14}C and cold NAD (non-radioactive) 20 mg (30mM). Figure 20 illustrates the deamidation of approximately 27% of nicotinamide-7-^{14}C to form

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*Fraction II (nuclear) and fraction IV (microsomal) were the only fractions studied for NAD glycohydrolase activity.

NICOTINIC ACID-^{14}C WHICH RESULTED FROM THE NICOTINAMIDASE ENZYME SYSTEM. CONCURRENTLY, APPROXIMATELY 23% OF THE NICOTINAMIDE-7-^{14}C UNDERWENT AN EXCHANGE REACTION WITH THE BOUND NICOTINAMIDE PRESENT IN THE NAD MOLECULE FORMING RADIOACTIVELY LABELLED NAD-^{14}C (FIGURE 20).

THEREFORE, AS SHOWN IN FIGURES 19 AND 20, THE NUCLEAR FRACTION CONTAINS AN ACTIVE NAD GLYCOHYDROLASE SYSTEM CAPABLE OF HYDROLYTIC AND TRANSGLYCOSIDIC ACTIVITIES ALONG WITH AN ACTIVE NICOTINAMIDASE SYSTEM.

INCUBATION OF AN ALIQUOT (5.0 ML.) OF THE MICROSOMAL SUPERNATANT (TOTAL VOLUME 14.0 ML.), FRACTION IV WITH NAD-^{14}C (1254 DPM) FOR 2 HOURS AT 37°C SHOWED THAT THE NAD-^{14}C WAS HYDROLYZED AT THE NICOTINAMIDE-RIBOSIDE LINKAGE TO FORM NICOTINAMIDE-^{14}C WHICH IN TURN WAS DEAMIDATED BY NICOTINAMIDASE TO FORM NICOTINIC ACID (FIGURE 21). ANOTHER ALIQUOT (5.0 ML.) OF THE MICROSOMAL SUPERNATANT (TOTAL VOLUME 14.0 ML.) WAS INCUBATED UNDER THE SAME CONDITIONS WITH NICOTINAMIDE-7-^{14}C (1254 DPM AND COLD NAD (20.0 MG.). AS SHOWN IN FIGURE 22, THE NICOTINAMIDE-7-^{14}C WAS DEAMIDATED TO FORM NICOTINIC ACID (56%). THERE WAS NO EVIDENCE OF THE FORMATION OF NAD-^{14}C WHICH INDICATED THAT THERE WAS AN ABSENCE OF THE EXCHANGE REACTION BETWEEN THE BOUND NICOTINAMIDE OF NAD AND NICOTINAMIDE-7-^{14}C. FIGURE 21 ILLUSTRATES THAT THE MICROSOMAL SUPERNATANT POSSESSES A NADᵀASE ENZYME SYSTEM CAPABLE OF HYDROLYZING NAD AT THE NICOTINAMIDE-RIBOSIDE LINKAGE TO FORM NICOTINAMIDE. HOWEVER, THE MICROSOMAL SUPERNATANT WAS FOUND TO LACK AN NADᵀASE CAPABLE OF TRANSGLYCOSIDASE ACTIVITY (41) AS SHOWN IN FIGURE 22. TABLE 15 INDICATES THE DISTRIBUTION OF ACTIVITY IN THE COMPOUNDS FORMED WHEN ALIQUOTS OF THE NUCLEAR FRACTION (FRACTION II) AND THE MICROSOMAL FRACTION (FRACTION IV) WERE INCUBATED WITH NAD-^{14}C OR NICOTINAMIDE-7-^{14}C AND COLD NAD.

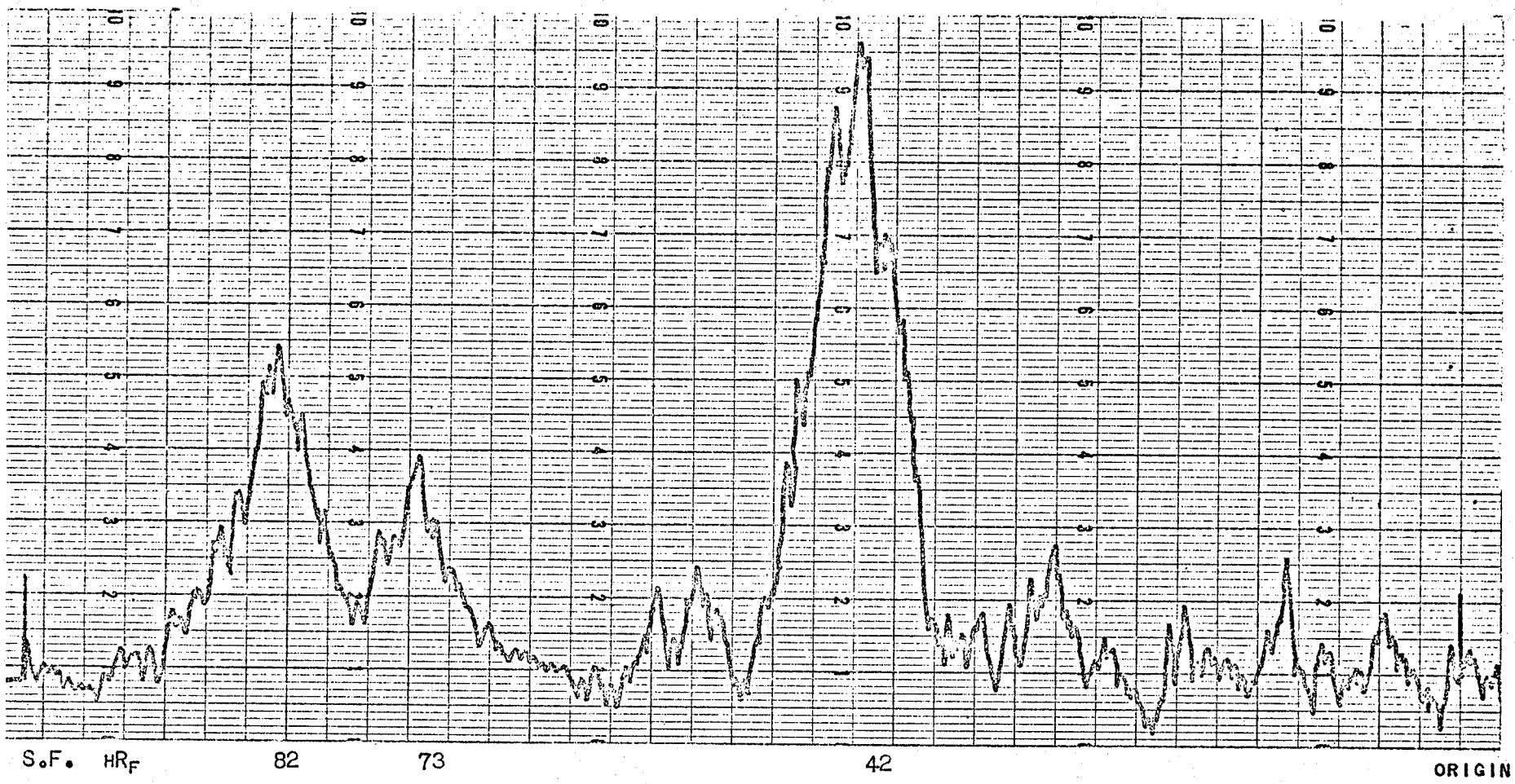

FIGURE 19. RADIOCHROMATOGRAPHIC SCAN OF THE NUCLEAR FRACTION, FRACTION II, INCUBATED AT 37°C FOR 2 HOURS WITH NAD-^{14}C; DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. THE ACTIGRAPH III SETTINGS WERE: 100 CPM SCALE; 10 SECOND INTERVAL; 60 CM./HOUR; 1050 VOLTS.

BAND AT HR_F 82 CORRESPONDS TO NICOTINAMIDE.

BAND AT HR_F 73 CORRESPONDS TO NICOTINIC ACID.

BAND AT HR_F 42 CORRESPONDS TO NAD

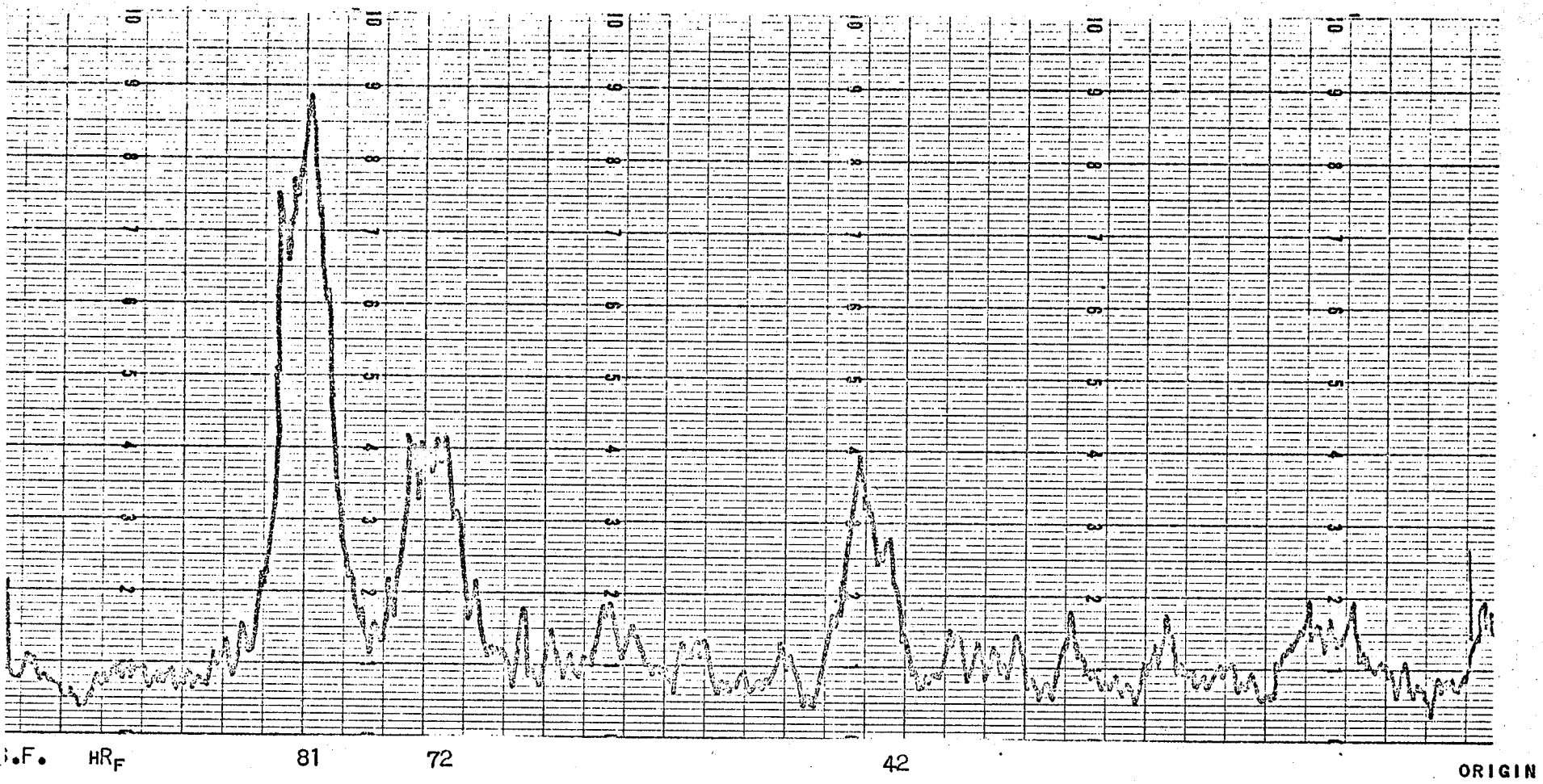

FIGURE 20. RADIOCHROMATOGRAPHIC SCAN OF THE NUCLEAR FRACTION, FRACTION II, INCUBATED AT 37°C FOR 2 HOURS WITH NICOTINAMIDE-7-^{14}C AND COLD NAD; DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. THE ACTIGRAPH III SETTINGS WERE: 150 CPM SCALE 10 SECOND INTERVAL; 60 CM/HOUR; 1050 VOLTS.

BAND AT HR_F 81 CORRESPONDS TO NICOTINAMIDE.

BAND AT HR_F 72 CORRESPONDS TO NICOTINIC ACID.

BAND AT HR_F 42 CORRESPONDS TO NAD.

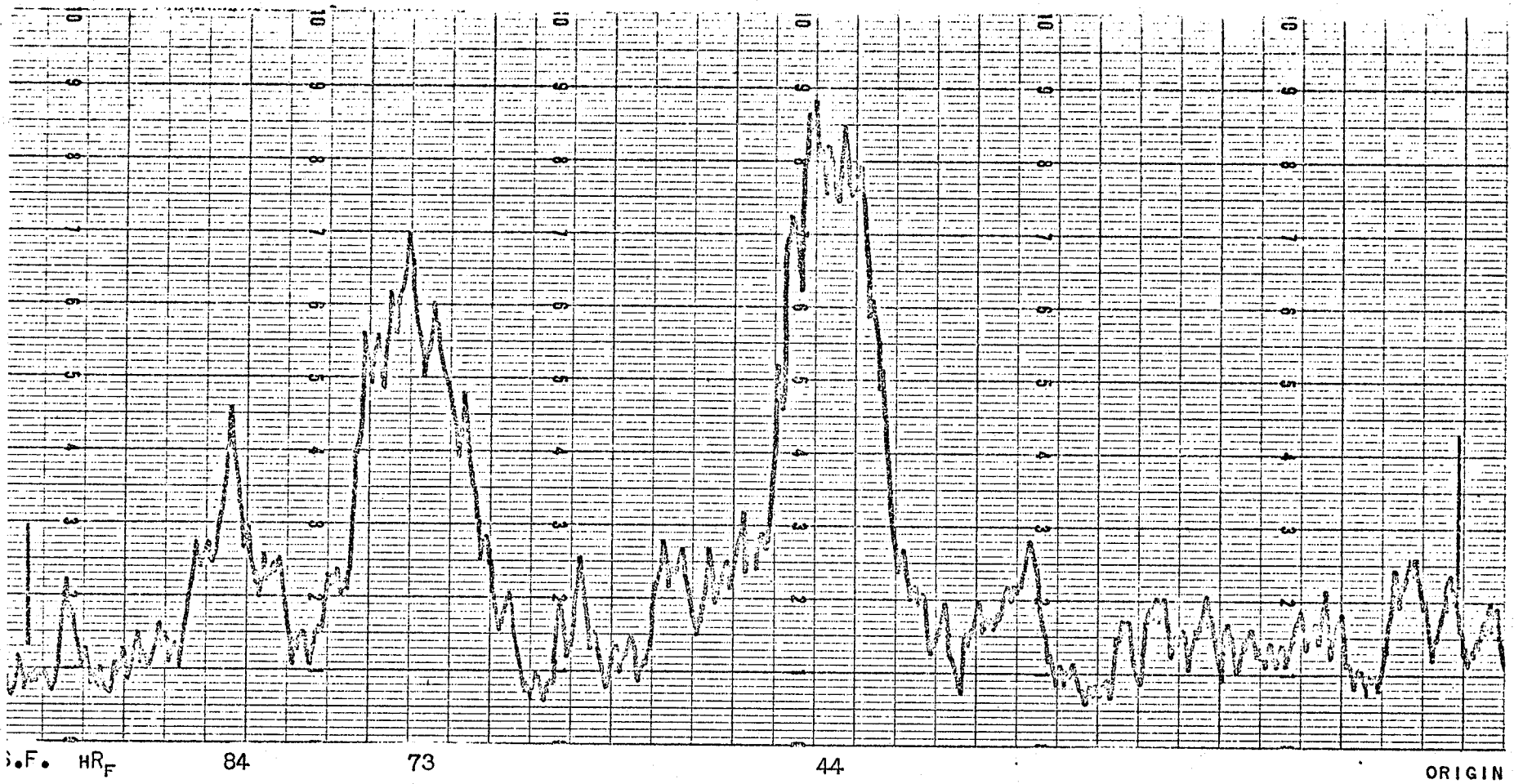

FIGURE 21. RADIOCHROMATOGRAPHIC SCAN OF THE MICROSOMAL SUPERNATANT, FRACTION IV, INCUBATED AT 37°C FOR 2 HOURS WITH NAD-^{14}C; DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. THE ACTIGRAPH III SETTINGS WERE: 100 CPM SCALE; 10 SECOND INTERVAL; 60 CM/HOUR; 1050 VOLTS.

PEAK AT HR$_F$ 84 CORRESPONDS TO NICOTINAMIDE.

PEAK AT HR$_F$ 73 CORRESPONDS TO NICOTINIC ACID.

PEAK AT HR$_F$ 44 CORRESPONDS TO NAD.

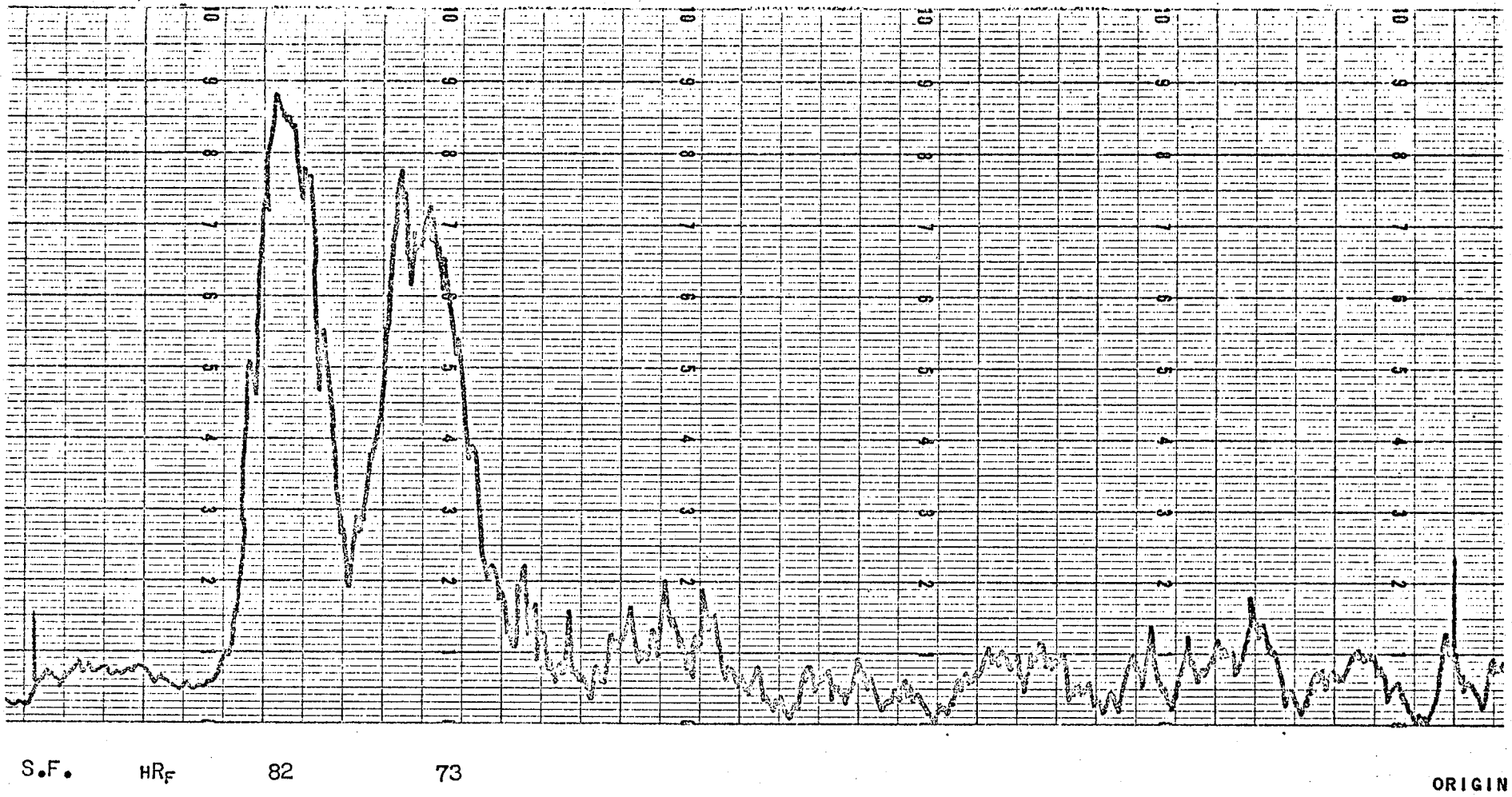

FIGURE 22. RADIOCHROMATOGRAPHIC SCAN OF THE MICROSOMAL SUPERNATANT, FRACTION IV, INCUBATED AT 37°C FOR 2 HOURS WITH NICOTINAMIDE-7-^{14}C AND COLD NAD; DEVELOPED IN ISOBUTYRIC ACID-AMMONIA-WATER. THE ACTIGRAPH III SETTINGS WERE: 100 CPM SCALE; 10 SECOND INTERVAL; 60 CM/HOUR; 1050 VOLTS.

PEAK AT HR_F 82 CORRESPONDS TO NICOTINAMIDE.

PEAK AT HR_F 73 CORRESPONDS TO NICOTINIC ACID.

TABLE 15

DEMONSTRATION OF NAD GLYCOHYDROLASE ACTIVITY (HYDROLYTIC AND TRANSGLYCOSIDIC) IN THE NUCLEAR AND MICROSOMAL FRACTIONS OF CLAVICEPS SD-58

FRACTION*		% FORMATION OF PYRIDINE COMPOUNDS		
	SUBSTRATE	NICOTINAMIDE	NICOTINIC ACID	NAD
NUCLEAR A	NAD-^{14}C	**51.47	16.14	48.53
NUCLEAR B	NICOTINAMIDE -^{14}C+NAD COLD	49.74	26.86	23.40
MICROSOMAL A	NAD-^{14}C	**60.10	42.52	39.90
MICROSOMAL B	NICOTINAMIDE -^{14}C+COLD NAD	44.34	55.66	0

*NUCLEAR A - 2.5 ML. OF THE NUCLEAR FRACTION (8.0 ML.) INCUBATED WITH NAD-^{14}C (FIG. 19).

NUCLEAR B - 2.5 ML. OF THE NUCLEAR FRACTION (8.0 ML.) INCUBATED WITH NICOTINAMIDE-7-^{14}C AND COLD NAD (FIG. 20).

MICROSOMAL A - 5.0 ML. OF THE MICROSOMAL SUPERNATANT (14 ML.) INCUBATED WITH NAD-^{14}C (FIG. 21).

MICROSOMAL B - 5.0 ML. OF THE MICROSOMAL SUPERNATANT (14.0 ML.) INCUBATED WITH NICOTINAMIDE-7-^{14}C AND COLD NAD (FIG. 22).

**THE CUMULATIVE PERCENTAGE INDICATED REPRESENTS THE AMOUNT OF NICOTINAMIDE FORMED PLUS THE AMOUNT WHICH WAS DEAMIDATED.

III. DISCUSSION

THE PRESENT STUDIES ON THE BIOSYNTHESIS OF NAD IN *CLAVICEPS* STRAIN SD-58 WERE UNDERTAKEN (I) TO PRODUCE RADIOACTIVE PYRIDINE CONTAINING COMPOUNDS WHICH COULD BE USED IN SUBSEQUENT STUDIES; (II) TO DETERMINE THE DISTRIBUTION PATTERN OF PYRIDINE CONTAINING MONO - AND DINUCLEOTIDES IN CLAVICEPS SD-58; AND (III) TO DETERMINE BY *IN VIVO* AND *IN VITRO* STUDIES WHETHER THE NAD GLYCOHYDROLASE ENZYME SYSTEM IS FUNCTIONAL IN *CLAVICEPS* SD-58.

NICOTINIC ACID-7-^{14}C (75%) AND NICOTINAMIDE-7-^{14}C (25%) CONSTITUTED 6.4% OF THE ADMINISTERED ACTIVITY WHICH WAS LOCATED IN THE INCUBATION MEDIUM OF 12-DAY-OLD CULTURES OF *CLAVICEPS* SD-58 INCUBATED WITH NICOTINAMIDE-7-^{14}C FOR 6 HOURS. APPROXIMATELY 79% OF THE ADMINISTERED ACTIVITY WAS LOCATED IN THE MYCELIUM WHICH CONTAINED THE PYRIDINE MONO - AND DINUCLEOTIDES. SIEMENS (10), IN A SIMILAR STUDY EMPLOYING 30-DAY-OLD CULTURES OF *CLAVICEPS*, STRAIN 47A, REPORTED THAT 14% OF THE ACTIVITY REMAINED IN THE INCUBATION MEDIUM AND 77% IN THE MYCELIUM. USING 28-DAY-OLD CULTURES OF *CLAVICEPS* 47A, BUT A DIFFERENT EXTRACTION PROCEDURE, AUDETTE (1) FOUND THAT 50% OF THE ACTIVITY REMAINED IN THE INCUBATION MEDIUM AND 44% IN THE MYCELIUM. THESE DIFFERENCES INDICATE THAT THE STRAIN, AGE AND METHOD OF PROCESSING THE FUNGUS HAVE AN INFLUENCE ON THE DISTRIBUTION OF RADIOACTIVITY.

AFTER THE TCA EXTRACTION OF THE MYCELIUM, THE ACETONE PRECIPITATION METHOD OF SHUSTER AND GOLDIN (78) WAS EMPLOYED TO CONCENTRATE THE DINUCLEOTIDES (I.E. NAD-^{14}C) INTO THE PRECIPITATED FRACTION AND THE MONONUCLEOTIDES (I.E. NMN-^{14}C) INTO THE ACETONE SOLUBLE FRACTION. HOWEVER, IN THE PRESENT STUDIES, THE ACETONE-SOLUBLE FRACTION WAS CONTAMINATED WITH

RELATIVELY HIGH AMOUNTS OF DINUCLEOTIDES INDICATING THAT THE ACETONE PRECIPITATION OF THESE COMPOUNDS WAS NOT COMPLETE. THERE WAS, ON THE OTHER HAND, NO EVIDENCE OF MONONUCLEOTIDE CONTAMINATION IN THE DINUCLEOTIDE FRACTION. THE PYRIDINE CONTAINING COMPOUNDS WERE IDENTIFIED CHROMATOGRAPHICALLY (TABLES 10 AND 11) AND WERE THE SAME AS THOSE REPORTED TO BE INVOLVED IN NAD BIOSYNTHESIS IN CLAVICEPS (1). THE NAD-^{14}C ISOLATED BY THE ACETONE PRECIPITATION METHOD (78) WAS SUBSEQUENTLY EMPLOYED FOR NAD GLYCOHYDROLASE EXPERIMENTS.

THE PRESENT STUDIES, EMPLOYING CLAVICEPS SD-58, INDICATED THAT 5.32% OF THE ADMINISTERED ACTIVITY WAS CONCENTRATED IN THE DINUCLEOTIDE FRACTION AS COMPARED TO 66.44% IN THE MONONUCLEOTIDE FRACTION. IN A SIMILAR STUDY, SIEMENS (10) EMPLOYING CLAVICEPS, 47A, OBSERVED THAT 11.16% OF THE ADMINISTERED ACTIVITY WAS CONCENTRATED IN THE DINUCLEOTIDE FRACTION AS COMPARED TO 56.37% IN THE MONONUCLEOTIDE FRACTION. THESE RESULTS INDICATE THAT THE CULTURES OF CLAVICEPS SD-58 PRODUCE A GREATER AMOUNT OF PYRIDINE MONONUCLEOTIDES, I.E. (NMN) [66.44% VS 56.37%] THAN CLAVICEPS 47A. CONVERSELY, THE CLAVICEPS 47A CULTURES PRODUCE MORE PYRIDINE DINUCLEOTIDES, NAMELY NAD, THAN DOES CLAVICEPS SD-58. [11.16% VS 5.32%].

NICOTINAMIDE INCORPORATION INTO NAD HAS BEEN POSTULATED TO OCCUR BY THREE POSSIBLE WAYS:

1. VIA NMN FORMATION AND INCORPORATION INTO NAD (5, 11, 13), THE NMN PYROPHOSPHORYLASE PATHWAY.

2. DIRECT INCORPORATION OF FREE NICOTINAMIDE INTO NAD THROUGH THE ACTION OF THE NAD GLYCOHYDROLASE SYSTEM (46), AND

3. BY DEAMIDATION TO NICOTINIC ACID AND ITS CONVERSION TO NAD BY THE PREISS-HANDLER PATHWAY (6).

AUDETTE (1) CONCLUDED THAT THE NMN PYROPHOSPHORYLASE PATHWAY WAS

ABSENT IN *CLAVICEPS* SPECIES BASED UPON THE OCCURRENCE OF NMN AS A HYDROLYSIS COMPOUND AND THE OBSERVATION THAT NICOTINAMIDE RIBOSIDE COULD NOT BE DETECTED IN EITHER OF TWO STRAINS OF THE FUNGUS EXAMINED, *CLAVICEPS* 47A AND *C. PURPUREA* CPM.

SIEMENS (10) HAS SHOWN IN *CLAVICEPS* 47A AND SD-58 THAT THERE IS AN ACTIVE DEAMIDASE ENZYME SYSTEM CAPABLE OF DEAMIDATING NICOTINAMIDE TO FORM NICOTINIC ACID WHICH CAN ENTER THE PREISS-HANDLER PATHWAY FOR NAD BIOSYNTHESIS.

STUDIES BY AUDETTE (1) SUGGESTED THAT NICOTINAMIDE INCORPORATION INTO NAD IN *CLAVICEPS* SPECIES MAY OCCUR FROM A DIRECT INCORPORATION OF FREE NICOTINAMIDE INTO NAD THROUGH THE ACTION OF THE NAD GLYCOHYDROLASE ENZYME SYSTEM.

EXPERIMENTS IN THIS STUDY HAVE BEEN DESIGNED TO STUDY SIMULTANEOUSLY THE UPTAKE OF NICOTINAMIDE -7-^{14}C AND ^{32}P INTO THE NMN PORTION OF THE NAD MOLECULE. THE UPTAKE OF ^{32}P INTO THE NMN PORTION OF NAD IS CONSIDERED TO BE A MEASURE OF THE SYNTHESIS, *DE NOVO*, OF NAD (8). IMSANDE AND HANDLER (79) HAVE FOUND THAT THE ^{32}P ENTERS THE PYRIDINE NUCLEOTIDE THROUGH LABELLED 5-PHOSPHORIBOSYLPYROPHOSPHATE (PRPP) IN THE CASE OF THE NICOTINIC ACID PRECURSOR (FIGURE 2-REACTION 1). NMN SYNTHESIS WITH NICOTINAMIDE AS A PRECURSOR WAS FOUND TO REQUIRE PRPP (5) OR IN THE CASE OF THE SYNTHESIS OF NMN VIA THE NUCLEOSIDE, RIBOSE-5-PHOSPHATE AND ATP WERE REQUIRED (11). THE SIMULTANEOUS UPTAKE OF NICOTINAMIDE-7-^{14}C AND ^{32}P INTO THE NMN PORTION OF NAD IN INTACT CULTURES OF *CLAVICEPS* SD-58 SHOULD GIVE AN EVALUATION REGARDING THE ACTIVITY OF THE ENZYME, NAD GLYCOHYDROLASE.

CULTURES OF *CLAVICEPS* SD-58 INCUBATED WITH ^{32}P AND NICOTINAMIDE -7-^{14}C SIMULTANEOUSLY PRODUCED THE RESULTS AS SHOWN IN FIGURE 15. LITTLE

CHANGE WAS NOTED IN THE SPECIFIC ACTIVITY OF ^{32}P INCORPORATION INTO THE NMN PORTION OF NAD WHEREAS THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C IN THE NMN PORTION OF NAD INCREASED MARKEDLY (A 5-6 FOLD INCREASE WAS NOTED IN SPECIFIC ACTIVITY OF THE NMN-^{14}C PORTION OF NAD BETWEEN THE 10^{-6} M AND 10^{-4} M CONCENTRATIONS OF NICOTINAMIDE). DIETRICH AND FULLER (8) OBSERVED, IN ASCITES CELLS IN VITRO, THAT THE SPECIFIC ACTIVITY OF THE NMN -^{32}P PORTION OF NAD REMAINED UNCHANGED WHEREAS THE SPECIFIC ACTIVITY OF NICOTINAMIDE-^{14}C IN THE NMN PORTION OF NAD INCREASED ABOUT 9 FOLD FROM 10^{-6} M TO 10^{-3}M CONCENTRATIONS OF NICOTINAMIDE. THIS INCORPORATION OF NICOTINAMIDE (IN THE FORMER AND LATTER STUDIES) APPARENTLY TOOK PLACE IN ABSENCE OF BIOSYNTHESIS. THIS SUGGESTS THAT UNDER THE PRESENT EXPERIMENTAL CONDITIONS, THE NICOTINAMIDE WAS INCORPORATED INTO NAD VIA THE NAD GLYCOHYDROLASE ENZYME PATHWAY. THE INCREASE IN SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C IN THE NMN MOIETY OF NAD (5-6 FOLD) WOULD RESULT FROM A DIRECT EXCHANGE REACTION BETWEEN THE NICOTINAMIDE-7-^{14}C IN THE INCUBATION MEDIA AND THE BOUND NICOTINAMIDE IN THE NMN PORTION OF NAD PRESENT IN THE INTACT CULTURE OF CLAVICEPS.

SIMULTANEOUS INCORPORATION OF NICOTINIC ACID-7-^{14}C AND ^{32}P INTO THE NMN PORTION OF NAD OF CLAVICEPS PRODUCED THE RESULTS AS SHOWN IN FIGURE 18. THE RESULTS INDICATE THAT THE UPTAKE OF NICOTINIC ACID-7-^{14}C WAS CORRELATED WITH A MARKED UPTAKE OF ^{32}P INTO THE NMN MOIETY OF NAD. A 15-FOLD INCREASE IN THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C FROM THE NMN PORTION OF NAD WAS NOTED BETWEEN THE 10^{-6} AND 10^{-5}M CONCENTRATIONS OF NICOTINIC ACID WHEREAS A 2.5-FOLD INCREASE WAS NOTED BETWEEN NICOTINIC ACID CONCENTRATIONS OF 10^{-5}M AND 10^{-4}M. A 3-FOLD INCREASE IN THE SPECIFIC ACTIVITY OF THE NMN-^{32}P PORTION OF NAD WAS NOTED BETWEEN THE 10^{-6}M AND

10^{-5}M CONCENTRATIONS OF NICOTINIC ACID. THEREFORE, UNDER THE EXPERIMENTAL CONDITIONS EMPLOYED, THE INTACT CULTURES OF CLAVICEPS SD-58 HAVE UNDERGONE A RAPID SYNTHESIS DE NOVO OF NAD FROM NICOTINIC AND ^{32}P. HOWEVER, IT MUST BE NOTED THAT THE 10^{-4}M LEVEL OF NICOTINIC ACID SUPPRESSED ^{32}P INCORPORATION INTO THE NMN MOIETY OF NAD WHILE AN INCREASE IN THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C IN THE NMN MOIETY OF NAD OCCURRED. DIETRICH AND FULLER (8), USING ASCITES CELLS IN VITRO, SHOWED THAT THERE WAS ABOUT A 4-FOLD INCREASE IN THE SPECIFIC ACTIVITY OF THE NMN-^{14}C PORTION OF NAD BETWEEN 10^{-6}M AND 10^{-5}M CONCENTRATIONS OF NICOTINIC ACID. CONCURRENTLY, THEY SHOWED A 3-FOLD INCREASE IN THE SPECIFIC ACTIVITY IN THE NMN-^{32}P PORTION OF NAD BETWEEN NICOTINIC ACID CONCENTRATIONS OF 10^{-6}M AND 10^{-5}M. INCREASING THE MOLAR CONCENTRATION OF NICOTINIC ACID TO 10^{-3} RESULTED IN A DECREASE IN NMN-^{32}P SPECIFIC ACTIVITY. USING RESULTS FROM A DIFFERENT EXPERIMENT, NOT INVOLVING ^{32}P, DIETRICH AND FULLER (8) STATED THAT AT THE 10^{-3}MOLAR CONCENTRATION OF NICOTINIC ACID THE SPECIFIC ACTIVITY OF THE NMN-^{14}C PORTION DECREASED ALSO. IN THE PRESENT STUDIES, HOWEVER, THE NMN-^{14}C SPECIFIC ACTIVITY OF NAD CONTINUED TO INCREASE WHILE THE NMN-^{32}P SPECIFIC ACTIVITY OF NAD DECREASED AT THE 10^{-4} CONCENTRATION OF NICOTINIC ACID. LACKING A SATISFACTORY EXPLANATION, IT MAY BE SUGGESTED THAT THE 10^{-4}M CONCENTRATION OF NICOTINIC ACID MAY EXHIBIT AN INHIBITORY ACTION ON THE VELOCITY OF THE KINASE ENZYME WHICH CONVERTS RIBOSE-5-PHOSPHATE AND ATP, IN THE PRESENCE OF Mg^{++} IONS, TO PRPP.

THE ^{32}P: NICOTINAMIDE-7-^{14}C AND ^{32}P: NICOTINIC ACID-7-^{14}C INCORPORATION STUDIES INTO THE NMN MOIETY OF NAD PRODUCED BY INTACT CULTURES OF CLAVICEPS SD-58 HAVE INDICATED THAT THE NAD GLYCOHYDROLASE ENZYME

SYSTEM IS FUNCTIONAL.

AS OUTLINED IN FIGURE 6, FRACTIONATION STUDIES WERE CARRIED OUT TO DETERMINE WHETHER PARTICULAR CELLULAR COMPONENTS EXHIBITED NAD GLYCOHYDROLASE ACTIVITY.

THE RESULTS INDICATED THAT THE NUCLEAR FRACTION (FIGURE 6) POSSESSED A NAD GLYCOHYDROLASE ENZYME SYSTEM CAPABLE OF HYDROLYZING NAD-^{14}C AT THE NICOTINAMIDE-RIBOSE LINKAGE TO FORM NICOTINAMIDE (FIGURE 19) AND CAPABLE OF PROMOTING AN EXCHANGE BETWEEN FREE NICOTINAMIDE-^{14}C AND THE BOUND NICOTINAMIDE OF NAD (FIGURE 20). IN ADDITION, THE MICROSOMAL FRACTION (FIGURE 6) WAS FOUND TO CONTAIN A NAD'ASE SYSTEM CAPABLE OF HYDROLYTIC ACTIVITY BUT LACKING THE TRANSGLYCOSIDIC CAPACITY (FIGURE 21 AND 22).

THE PRESENCE OF AN ACTIVE NICOTINAMIDASE ENZYME SYSTEM IN THE NUCLEAR AND MICROSOMAL FRACTIONS OF CLAVICEPS SD-58 CONFIRMED THE RESULTS OF SIEMENS (10).

VARIOUS AUTHORS (48, 50, 56, 61-63) HAVE OBSERVED THAT NAD GLYCOHYDROLASE ACTIVITY WAS ASSOCIATED WITH THE NUCLEAR AND MICROSOMAL FRACTIONS OF THE PREPARATIONS USED. THE PRESENT STUDY INDICATES THAT THE NUCLEAR AND MICROSOMAL FRACTIONS CONTAIN NAD GLYCOHYDROLASE ENZYME SYSTEMS. THE QUESTION ARISES AS TO WHETHER THE ENZYMES FOUND IN THE NUCLEAR AND MICROSOMAL ARE THE SAME OR DIFFERENT.

IF ONLY ONE ENZYME IS PRESENT, IS IT CAPABLE OF BOTH HYDROLYTIC AND TRANSGLYCOSIDIC ACTIVITY OR IF MORE THAN ONE ENZYME IS PRESENT DOES A PARTICULAR NAD'ASE PERFORM A SPECIFIC FUNCTION? THESE QUESTIONS WILL BE ANSWERED ONLY WHEN THE ENZYMES FROM THE INDIVIDUAL FRACTIONS ARE ISOLATED AND CHARACTERIZED.

ALTHOUGH THE PHYSIOLOGICAL FUNCTIONS OF NAD'ASES ARE STILL UNKNOWN, THE PRESENCE OF NAD GLYCOHYDROLASE IN CLAVICEPS, SD-58 COULD SERVE AS A

"SALVAGE LOOP" (67) WHEN THE DE NOVO PATHWAY OF NAD BIOSYNTHESIS IS GENETICALLY BLOCKED OR ABSENT. THIS ENZYME MAY SERVE AS A REGULATOR OF NAD LEVELS SINCE LOSS OF THE NICOTINYL STRUCTURE FROM THE NAD CYCLE COULD OCCUR AT THE LEVEL OF NICOTINIC ACID OR NICOTINAMIDE. THE ROLE OF NAD GLYCOHYDROLASE AS A TRANSGLYCOSIDASE IN CLAVICEPS REMAINS TO BE DETERMINED.

IV. SUMMARY

STUDIES HAVE INDICATED THAT NICOTINAMIDE-7-^{14}C WAS CONVERTED INTO THE PYRIDINE MONO - AND DINUCLEOTIDES BY SAPHROPHYTIC CULTURES OF CLAVICEPS SD-58.

THE FOLLOWING COMPOUNDS WERE IDENTIFIED AFTER INCUBATION WITH NICOTINAMIDE-7-^{14}C:

NICOTINIC ACID

NICOTINIC ACID ADENINE DINUCLEOTIDE (DES-NAD)

NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD)

NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADP), AND

NICOTINAMIDE MONONUCLEOTIDE (NMN).

INCUBATION OF CLAVICEPS SD-58 CULTURES WITH INCREASING CONCENTRATIONS OF NICOTINAMIDE-7-^{14}C AND A CONSTANT LEVEL OF ^{32}P RESULTED IN AN INCREASED INCORPORATION (6-FOLD) OF NICOTINAMIDE-7-^{14}C INTO THE NICOTINAMIDE MONONUCLEOTIDE MOIETY OF NICOTINAMIDE ADENINE DINUCLEOTIDE BETWEEN THE 10^{-6} M AND 10^{-4} M CONCENTRATIONS OF NICOTINAMIDE. THE SPECIFIC ACTIVITY OF THE ^{32}P INCORPORATED INTO THE SAME NICOTINAMIDE MONONUCLEOTIDE WAS NOT AFFECTED BY NICOTINAMIDE CONCENTRATION.

CLAVICEPS SD-58 CULTURES INCUBATED WITH INCREASING CONCENTRATIONS OF NICOTINIC ACID-7-^{14}C AND A CONSTANT LEVEL OF ^{32}P (PHOSPHORIC ACID) PRODUCED CORRESPONDING CHANGES IN THE SPECIFIC ACTIVITY OF THE NICOTINAMIDE-^{14}C AND ^{32}P IN THE NICOTINAMIDE MONONUCLEOTIDE MOIETY OF NICOTINAMIDE ADENINE DINUCLEOTIDE EXCEPT AT THE 10^{-4} M CONCENTRATION OF NICOTINIC ACID WHERE ^{32}P INCORPORATION DECREASED AND THE NICOTINIC ACID-7-^{14}C INCORPORATION CONTINUED TO RISE.

THE PRESENT STUDIES HAVE INDICATED THAT NAD GLYCOHYDROLASE IS ACTIVE

IN THE BIOSYNTHETIC PATHWAY OF PYRIDINE NUCLEOTIDE SYNTHESIS IN CLAVICEPS SD-58.

THE NAD GLYCOHYDROLASE WAS FOUND TO BE ASSOCIATED WITH THE NUCLEAR (HYDROLYTIC AND TRANSGLYCOSIDIC ACTIVITY WERE 51% AND 23% RESPECTIVELY) AND MICROSOMAL (HYDROLYTIC AND TRANSGLYCOSIDIC ACTIVITY WERE 60% AND 0%, RESPECTIVELY) FRACTIONS OF CULTURES OF CLAVICEPS SD-58.

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