THE UNIVERSITY OF MANITOBA

NICOTINAMIDE ADENINE DINUCLEOTIDE METABOLISM IN HIGHER PLANTS

A THESIS

SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

by

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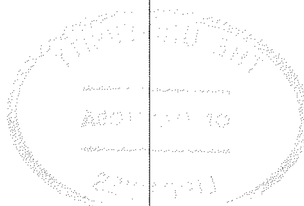
April 1966



LIST OF ABBREVIATIONS

ADP	--	Adenosine diphosphate
ADPR	--	Adenosine diphosphate ribose
AMP	--	Adenosine monophosphate
ATP	--	Adenosine triphosphate
CTP	--	Cytosine triphosphate
D.P.M.	--	Disintegrations per minute
DPN	--	See NAD
GTP	--	Guanosine triphosphate
HCl	--	Hydrochloric acid
ITP	--	Inosine triphosphate
NaAD	--	Nicotinic acid adenine dinucleotide
NAD	--	Nicotinamide adenine dinucleotide
NADP	--	Nicotinamide adenine dinucleotide phosphate
NaMN	--	Nicotinic acid mononucleotide
NaR	--	Nicotinic acid riboside
NMN	--	Nicotinamide mononucleotide
NR	--	Nicotinamide riboside
NRPPRA	--	See NAD
P_i	--	Inorganic phosphate
PP	--	Pyrophosphate
PRPP	--	5-phospho-ribosyl-pyrophosphate
RPPRA	--	See ADPR
TPN	--	See NADP
Tris	--	Tris (hydroxymethyl) aminomethane
TTP	--	Thymidine triphosphate
UTP	--	Uridine triphosphate

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. E. R. Waygood for his advice, guidance and unfailing encouragement during the course of this investigation.

Grateful thanks are also due to Drs. B. D. Sanwal and M. Kapoor for their criticism, suggestions and interest.

The assistance of Miss Maria De Geus, especially in the preparation of specimens, photography and illustrations, is gratefully acknowledged.

Thanks are also due to the many colleagues and members of staff for their help.

Acknowledgements are also made to Dr. D. T. T. Canvin (formerly of the Department of Plant Science) for his assistance and use of equipment for the determination of the radioactivity of the samples.

ABSTRACT

Elodea leaves were floated on water or treated with nicotinic acid nicotinamide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, benzimidazole and kinetin. Nicotinic acid > nicotinamide adenine dinucleotide accelerated the chlorosis of treated leaves. Under similar experimental conditions nicotinamide adenine dinucleotide phosphate caused only a sporadic bleaching of leaves, while nicotinamide was without any effect. Chlorosis appeared only when the treated leaves were illuminated. Detached leaves, maintained in darkness, remained green (non-chlorotic) irrespective of the treatment given or the length of time of treatment. When benzimidazole as well as nicotinic acid or nicotinamide adenine dinucleotide were simultaneously present in the floating medium, benzimidazole overcame the induced senescence and maintained the leaves green. Similar results were obtained with detached wheat leaves (*Triticum aestivum* var. Selkirk).

In another aspect of this study Selkirk wheat leaves were used to investigate the pattern of nicotinamide adenine dinucleotide metabolism in immediately detached leaves and senescent leaves.

Radioactive isotopes of nicotinamide (carbonyl-C^{14}) and nicotinic acid (carboxyl-C^{14}) were allowed to be incorporated by wheat leaves from 5 minutes to 12 hours. The biosynthesis of nicotinamide adenine dinucleotide proceeded from nicotinic acid ---> nicotinic acid riboside ---> nicotinic acid mononucleotide ---> nicotinic acid adenine dinucleotide ---> nicotinamide adenine dinucleotide ---> nicotinamide adenine dinucleotide phosphate, irrespective of the precursor fed. Of the two precursors, nicotinic acid and nicotinamide, nicotinic acid was incorporated more

efficiently. The degradation of nicotinamide adenine dinucleotide demonstrated in vitro was as follows: nicotinamide adenine dinucleotide---> nicotinamide mononucleotide ---> nicotinamide riboside ---> nicotinamide. The nicotinamide would then be deamidated to produce nicotinic acid. Consequently the metabolism of nicotinamide adenine dinucleotide in wheat leaves followed a cyclic pattern with a rapid turnover of the nucleotides. A rigid control of the levels of nicotinamide nucleotides in wheat leaves was indicated by: i) the cyclic pattern of metabolism, ii) rapid labelling of all intermediates, iii) low level of radioactivity of the intermediates, regardless of the length of time allowed for incorporation and, iv) only minor fluctuations in the levels of radioactivity of various intermediates over long periods of isotope incorporation.

Approximately 50% of the nicotinic acid (or nicotinamide) incorporated was detoxified by methylation and accumulated by the leaves as N-methyl nicotinate (trigonelline). The accumulated trigonelline serves as a comparatively innocuous, yet readily available, reserve source of nicotinic acid for the biosynthesis of nicotinamide adenine dinucleotide as required.

The general pattern of nicotinamide adenine dinucleotide metabolism in senescent leaves was similar to that in immediately detached leaves, except that leaves floated on water showed a greater amount of label present in nicotinamide adenine dinucleotide. Leaves treated with benzimidazole accumulated more of the isotope in nicotinamide adenine dinucleotide phosphate.

The biosynthetic and degradative pathways of nicotinamide adenine dinucleotide metabolism in wheat leaves were shown to differ in the following significant details from the pathways described to occur in

animals and microorganisms:

i) a rigid control of intracellular levels of nicotinamide adenine dinucleotide and

ii) although excess of nicotinic acid administered to leaves was detoxified by N^1-methylation, the N-methyl nicotinate thus produced is not excreted but serves as a reserve pool of nicotinic acid for further synthesis of nicotinamide adenine dinucleotide.

iii) Nicotinamide adenine dinucleotide-glycohydrolase activity was not detected in wheat leaves.

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INTRODUCTION

INTRODUCTION

The subterminal stages of the life cycle are distinct as compared to the formative ones in plants and animals alike. The differences between senescence and growth, though morphological, are basically metabolic in nature. The catabolic aspects predominate over the anabolic aspects of metabolism during the senescence. The leaf is probably one of the first organs of a plant to present visible evidence of senescence in the form of chlorosis. In wheat leaves senescence can be studied conveniently _in vitro_ by floating detached leaves on water.

Person _et al_ (1957) discovered that benzimidazole retarded the accumulation of soluble nitrogen and the onset of chlorosis, observed in detached wheat leaves floated on water. Protein synthesis was shown to occur in benzimidazole treated leaves but not in leaves floated on water (Samborski _et al_ 1958). Investigation of the chlorophyll metabolism of detached wheat leaves by Wang and Waygood (1959) and Wang et al (1960, 61) showed that a greater synthesis of chlorophyll occurred and chlorophyll destruction was retarded in benzimidazole treated leaves as compared to leaves floated on water. Kapoor and Waygood (1965 a, b) have reviewed briefly the early investigations of Samborski _et al_ 1958 and the subsequent studies on the 'benzimidazole effect' in this laboratory. From these investigations (Wang and Waygood, 1959; Wang _et al_ 1960, 61; Kapoor and Waygood 1965 a), it is apparent that the 'benzimidazole effect' is

involved in at least two sites of action and one of these being located in the chloroplasts (Waygood, 1965).

The opposite of the protective effect of benzimidazole on chloroplasts was shown to be produced by NAD (Yoshida, 1961). In these experiments NAD was shown to accelerate the senescence of the chloroplasts of detached Elodea leaves.

Kapoor and Waygood (1965, a,b) demonstrated a reaction in wheat embryos in which benzimidazole substituted for the nicotinamide moiety of NAD catalysed by NAD-glycohydrolase. Mishra (1963) see also Waygood (1965) showed that both benzimidazole and kinetin had profound effects on the levels of the oxidized and reduced forms of the nicotinamide nucleotides in whole wheat leaves and chloroplasts from wheat leaves undergoing senescence. For example, the synthesis of NADP in the chloroplasts was markedly enhanced by both benzimidazole and kinetin treatment whereas it (NADP) disappeared from chloroplasts of leaves floated on water.

The purpose of this study was to investigate further the relationships between benzimidazole and NAD in senescence of leaves. Initially studies were made on the effect of benzimidazole in modifying the NAD accelerated senescence of Elodea leaves. Subsequently it was considered essential, if we were to gain any insight into the effect of benzimidazole on control mechanisms, to obtain information on the biosynthetic pathway of the nicotinamide nucleotides by labelled precursor feeding experiments in which wheat leaves were used.

LITERATURE REVIEW

LITERATURE REVIEW

SENESCENCE AND THE EFFECT OF NICOTINAMIDE ADENINE DINUCLEOTIDE

Yoshida (1961) studied the influence of the nucleus on cellular metabolism of Elodea leaf cells plasmolysed with 0.2 M calcium chloride solution. On plasmolysis the protoplasts of some of the leaf cells separated into two parts. One part contained the nucleus whereas the chloroplasts were distributed equally between the two protoplasts. Occassionally, in some cells the two protoplasts were connected with a cytoplasmic bridge. The leaves were maintained in plasmolysing solution for several days. Within four days the chloroplasts in the protoplast containing the nucleus showed a deterioration in size and shape as observed under a light microscope. On the other hand the chloroplasts in the enucleate protoplast increased in size and accumulated starch indicating an increased photosynthetic activity. Yet, if the two protoplasts were connected by a strand of protoplasm the chloroplasts in both deteriorated equally rapidly. Yoshida further demonstrated that addition of NAD but not NADP to the external medium caused the chloroplasts of the enucleate protoplast to deteriorate in a manner similar to those in the protoplast containing the nucleus. On the basis of Brachet's theory (1954) that NAD is synthesized by the nucleus, Yoshida explained the effect of nucleus on the chloroplasts. This suggested that the chloroplasts of the enucleate half were showing an effect similar to the 'benzimidazole effect' even in the absence of benzimidazole.

The work of Yoshida indicates that NAD is deleterious to chloroplasts by accelerating their senescence and suggests perhaps that NAD may, in some manner, be detoxified in the leaf either by conversion to NADP or by pyrophosphatase activity or perhaps by some factor translocated from the roots. In this connection Kapoor and Waygood (1965) have shown that an NAD-glycohydrolase from wheat embryos catalyses the substitution of benzimidazole for the nicotinamide moiety of NAD. One would expect therefore that the NAD levels of leaves may, if this reaction occurs in leaves, decline with benzimidazole treatment. The effect of benzimidazole on the NAD content of leaves was investigated by Mishra (1963) in this laboratory and it was found that neither benzimidazole nor kinetin decreased the NAD content of whole wheat leaves as compared to leaves floated on water. In fact kinetin which elicits responses similar to benzimidazole and benzimidazole and kinetin together maintained the NAD content, above that in leaves floated on water. The comprehensive data reported by Mishra (1963) for whole wheat leaves on the four species of nicotinamide nucleotides is difficult to interpret. However, it did show a tendency for NADP synthesis to be augmented in benzimidazole and kinetin treatment. In further experiments on chloroplasts isolated in non-aqueous media from whole wheat leaves Mishra reported an enhanced synthesis of NADP in chloroplasts from benzimidazole treated leaves and only traces of NADP in the chloroplasts from water floated leaves.

It appears that it is highly improbable that the reaction between NAD and benzimidazole is of any physiological significance in leaves of wheat, especially since the enzyme has not been demonstrated in leaves in this laboratory.

In view of these interactions of benzimidazole and NAD in the senescence of detached leaves and the sparse information available on NAD metabolism, a study of the metabolism of NAD in wheat leaves was undertaken. An historical survey of the literature on NAD metabolism follows.

NICOTINAMIDE ADENINE DINUCLEOTIDE METABOLISM

NOMENCLATURE

Since the discovery of the nicotinamide nucleotide coenzymes and subsequent determination of their structure there has been no unanimity over their nomenclature. Four different systems have been or are in use. These are:

(a) cozymase and phosphocozymase,

(b) codehydrogenase I and codehydrogenase II (codehydrase I and codehyrase II used mainly by the continental workers),

(c) coenzyme I and coenzyme II (abbreviated to Co I and Co II),

(d) diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN).

At the present time, the first system is probably the least, and the fourth the most, in use. The enzyme commission of the

International Union of Biochemistry (Dixon, 1960) has now recommended the names nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) for DPN and TPN respectively and nicotinamide nucleotides instead of pyridine nucleotides.

Since the isolation of the nicotinic acid analogues of nicotinamide mononucleotide and nicotinamide adenine dinucleotide various abbreviations have been used for these analogues in the literature. For instance, nicotinic acid mononucleotide has been abbreviated to as desamido NMN or des NMN or NaMN and similarly several abbreviations have been used for nicotinic acid adenine dinucleotide: desamido DPN, des DPN, desamido-NAD, des NAD and NaAD. To be consistent with the recommendations of the enzyme commission the abbreviations used in this thesis are, NaR for nicotinic acid riboside, NaMN for nicotinic acid mononucleotide and NaAD for nicotinic acid adenine dinucleotide.

DISCOVERY

Harden and Young (1906) demonstrated a thermostable coenzyme required for the activity of "Zymase" during the course of their studies on yeast fermentation. This coenzyme (cozymase: now identified as NAD) was separated from "zymase" by dialysis, but it was not isolated. While studying the "Zwischenferment" from erythrocytes, Warburg and Christian (1931) discovered another coenzyme and named it "The Coenzyme" or "Coenzyme II" (now identified as NADP), as subsequent

studies showed the similarities between the two coenzymes.

STRUCTURE

Warburg and Christian (1935) demonstrated that the coenzyme (NADP) from erythrocytes was composed of one molecule of adenine, two pentose units, three equivalents of phosphoric acid and one molecule of nicotinamide and that nicotinamide was the active group of the coenzyme. Simultaneously von Euler et al (1935) isolated a highly purified "cozymase" (NAD) from yeast and demonstrated that nicotinamide formed a part of the coenzyme. Warburg and Christian (1936) isolated NAD independently and showed that nicotinamide was the active base in both "codehydrases". Von Euler et al (1936) determined the structure and ratio of groups of NAD. Further they demonstrated that the yeast coenzyme contained one phosphate group less than Warburg's coenzyme. It was assumed that the phosphate groups were linked together by a pyrophosphate bond in both the coenzymes. This led Warburg (1936) to name the two coenzymes "Diphospho-Pyridinnucleotid" and "Triphospho Pyridinnucleotid". Kornberg and Pricer (1950) reported the accurate structure of NADP. They demonstrated that the third phosphate of NADP was at 2' position of the ribose portion of adenosine, by specific cleavage of NADP with nucleotide pyrophosphatase to yield NMN and adenosine-2', 5'-diphosphate.

Simultaneous to the delineation of the coenzyme structure, Elvehjem et al (1937) showed that nicotinamide and nicotinic acid were highly effective pellagra preventive factors. Since then a great

deal of work has been done on the vitamin activity and biogenesis of nicotinamide and nicotinic acid on the one hand and the coenzyme properties of NAD and NADP on the other.

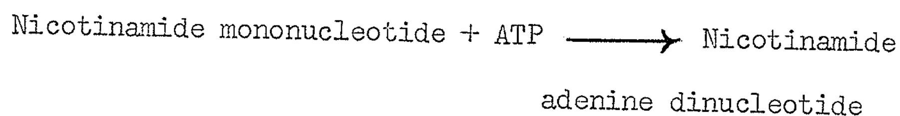
BIOSYNTHESIS OF NICOTINAMIDE ADENINE DINUCLEOTIDE

The elucidation of the biosynthetic pathway of nicotinamide nucleotides was hampered for a long time by two discoveries made some thirty years ago namely, (a) that nicotinamide was the active constituent of the coenzymes I and II (Warburg and Christian, 1935) and (b) Elvehjem's isolation of nicotinamide from liver in 1937 and proving that it was highly effective as a pellagra preventive. Elvehjem's simultaneous observation that nicotinic acid was equally efficient was casually ignored during the following decades. To quote Handler, (1958):

> Since the pyridine nucleotides contain nicotinamide rather than the carboxylic acid, it appeared reasonable to assume that the initial step in further metabolism of nicotinic acid would be the synthesis of nicotinamide. With this thought in mind, I have wasted the time and efforts of a series of graduate students and post doctoral fellows who have sought an enzyme system which would synthesize nicotinamide from nicotinic acid and either ATP or other energy source together with ammonia, glutamine, asparagine, glutamic acid or other nitrogen donor. We have examined human erythrocytes, rat liver and kidney, baker's and brewer's yeast, and a number of microorganisms but with no success.

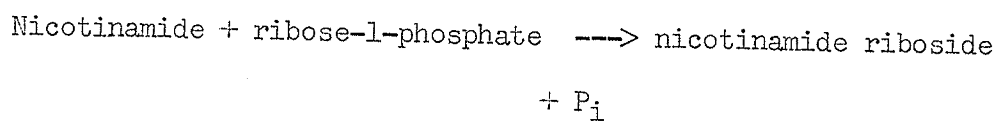
It was generally assumed that nicotinic acid was converted to nicotinamide in the presence of a nitrogen donor and an appropriate energy source.

Kornberg (1950) demonstrated in yeast and liver tissue an enzyme, ATP:NMN adenylyltransferase (E.C. No.2.7.7.1), that catalyses the condensation of NMN with ATP to produce NAD according to the following reaction:



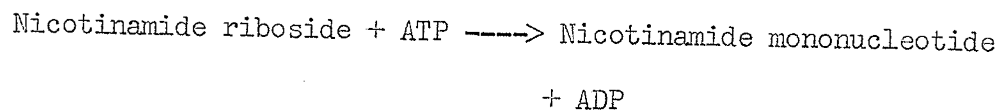
Nicotinamide mononucleotide + ATP $\longrightarrow$ Nicotinamide adenine dinucleotide

Following this Rowen and Kornberg (1951) isolated an enzyme, purine nucleoside:orthophosphate ribosyl transferase (E.C.No.2.4.2.1), which mediates the reaction:



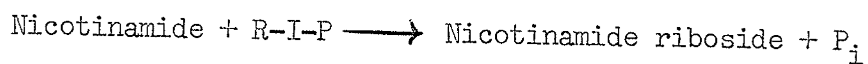
Nicotinamide + ribose-1-phosphate $\longrightarrow$ nicotinamide riboside + P_i

with an equilibrium far to the left at physiological pH values. Also isolated was a weakly active kinase, ATP:N-ribosyl nicotinamide 5'-phosphotransferase (E.C. No.2.7.1.22), converting the nicotinamide riboside to NMN as follows:

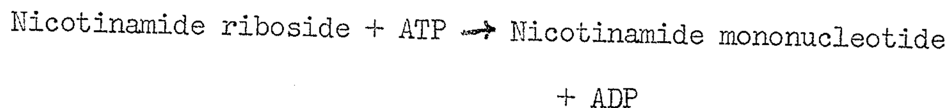


Nicotinamide riboside + ATP $\longrightarrow$ Nicotinamide mononucleotide + ADP

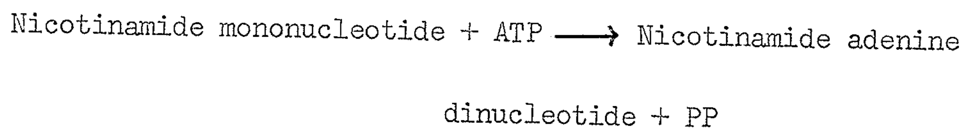
Following the demonstration of these enzymes from yeast and hog liver acetone powder Rowen and Kornberg (1951) proposed the following pathway of NAD biosynthesis.



Nicotinamide + R-I-P $\longrightarrow$ Nicotinamide riboside + P_i

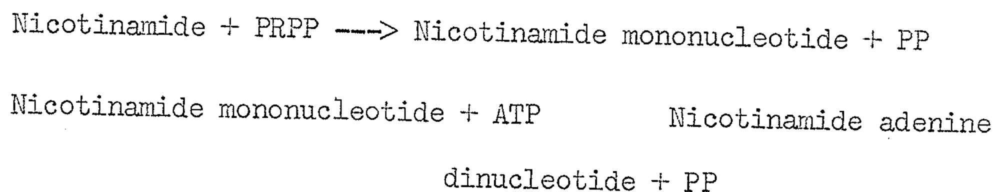


Nicotinamide riboside + ATP $\longrightarrow$ Nicotinamide mononucleotide + ADP



Nicotinamide mononucleotide + ATP $\longrightarrow$ Nicotinamide adenine dinucleotide + PP

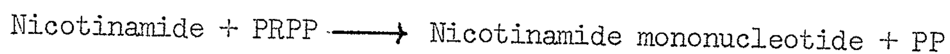
Singer and Kearney (1954) suggested the following modification:



Nicotinamide + PRPP $\longrightarrow$ Nicotinamide mononucleotide + PP

Nicotinamide mononucleotide + ATP Nicotinamide adenine dinucleotide + PP

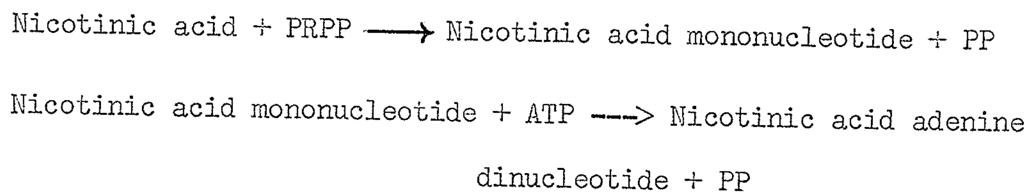
The isolation by Preiss and Handler (1957) of an enzyme, NMN:Pyrophosphate phosphoribosyl transferase (E.C. No.2.4.2.12), from erythrocytes capable of catalysing the reaction:



Nicotinamide + PRPP $\longrightarrow$ Nicotinamide mononucleotide + PP

and the studies of Holzer et al (1961) on tumor cells added further support to the Rowen-Kornberg pathway.

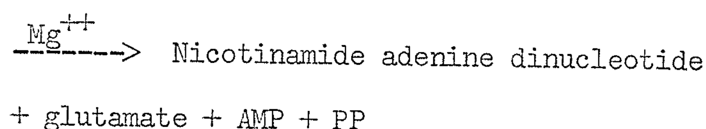
Preiss and Handler (1957) reported the biosynthesis of NAD by human erythrocytes on incubation with nicotinic acid ($-C^{14}$). They isolated the labelled nicotinic acid analogues of NMN and NAD (desamido NMN or NaMN and desamido NAD or NaAD respectively) and demonstrated the conversion of NaAD to NAD in the presence of glutamine, ATP and Mg^{++}. They formulated the following pathway (Preiss and Handler, 1958):



Nicotinic acid + PRPP $\longrightarrow$ Nicotinic acid mononucleotide + PP

Nicotinic acid mononucleotide + ATP $\longrightarrow$ Nicotinic acid adenine dinucleotide + PP

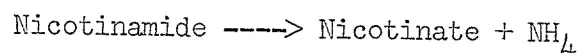
Nicotinic acid adenine dinucleotide + glutamine + ATP



$\xrightarrow{Mg^{++}}$ Nicotinamide adenine dinucleotide

+ glutamate + AMP + PP

The Preiss-Handler pathway as postulated required an enzyme capable of deamidating the nicotinamide, to produce nicotinic acid according to the reaction:



Nicotinamide ----> Nicotinate + NH_4

Such an enzyme, nicotinamide amidohydrolase (E.C. No.3.5.2.*), was reported in bacteria (Hughes and Williamson, 1953), microorganisms (Oka, 1954), fungi (Sarma _et al_ 1964), and mammalian tissue (Petrack _et al_ 1963 and Rajagopalan _et al_ 1958). Nishizuka and Hayaishi (1963) demonstrated that in mammalian tissue 3-hydroxy anthranilic acid ($-C^{14}$) was converted to quinolinic acid ($-C^{14}$). The quinolinic acid in the presence of a decarboxylase** and PRPP gave rise to NaMN. They demonstrated further that NaMN together with ATP and glutamine produced NAD and that NaAD accumulated in the absence of a nitrogen donor.

In plant tissue unlike in animal tissue tryptophan was not converted to nicotinic acid (Dalgleish, 1958). Burkholder (1943) demonstrated the synthesis of nicotinic acid during the germination of oats, wheat, barley and maize. Guha and Das (1957) isolated a bound

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* Enzyme number not available.

** Classification not available.

form of nicotinic acid (niacinogen) and Ghosh (1962) showed an increase in nicotinamide nucleotide levels concomitant with a decrease of niacinogen levels during the germination of cereal grains. Ghosh et al (1963) studied, from extracts of rice seedlings, an enzyme capable of hydrolysing niacinogen with the liberation of nicotinic acid and in the same report it was briefly mentioned that NaAD together with NAD was isolated from the incubation mixture of the extracts of rice seedlings, nicotinic acid-7-C^{14}, PRPP, ATP, glutamine, glutathione and Mg^{++}. No further details were published.

Goodwin (1963) states that Terroine, in 1948, reported that excess nicotinic acid supplied to shoots was converted into trigonelline. Joshi and Handler (1962) showed a disappearance of trigonelline and simultaneous appearance of nicotinamide nucleotides during the germination of pea seeds. Hadwiger et al (1963) isolated labelled trigonelline as well as nicotinic acid from corn embryos after feeding quinolinic acid (-C^{14}) with greater radioactivity distributed in nicotinic acid. Further, homogenates of castor seedlings converted quinolinic acid to NaMN in a PRPP dependent reaction.

The information available on NAD biosynthesis in plants from either nicotinic acid or nicotinamide is both fragmentary and contradictory.

BIOSYNTHESIS OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE

Kornberg (1950) reported a specific kinase, ATP:NAD 2'-phosphotransferase (E.C. No.2.7.1.23), in yeast which converted NAD to NADP

irreversibly, in the presence of ATP. Although several phosphatases were detected which convert NADP to NAD (Kaplan, 1952) no additional information was published on the kinases (except for that of Wang and Kaplan, 1954, from pigeon liver) since Kornberg's work. Pathak and Waygood (1965) unpublished, see also Waygood, 1965) recently demonstrated an NAD kinase in wheat leaves.

DEGRADATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE

Three different degradative pathways for NAD in living systems are:

(a) liberation or substitution of the nicotinamide moiety of NAD succeeded by further conversion of the nicotinamide to other related pyridines or alkaloids and degradation of ADPR produced simultaneously with nicotinamide;

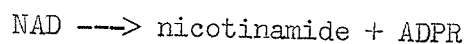
(b) hydrolytic cleavage of the pyrophosphate bond of NAD followed by further degradation of the products - NMN and AMP and,

(c) deamination of the adenine moiety of NAD followed by the cleavage of the pyrophosphate bond.

The first mechanism of the three was extensively studied by Kaplan and coworkers (Kaplan, 1960, see also Zatman _et al_, 1953). This aided considerably in a better understanding of structural relationships of various groups of the coenzyme, the relative importance of each group for coenzyme activity and spatial arrangement within the coenzyme and of the coenzyme with the apoenzyme. Hence the mechanisms of degradation and the enzymes involved are discussed below in a

greater detail in accordance with their relative importance.

(a) A group of enzymes, NAD glycohydrolases (E.C. No.3.2.2.5) investigated mainly in the animal tissue and microorganisms (Handler and Klein, 1942 a, b) are capable of either a hydrolytic scission of the bond between nicotinamide and ribose of NAD,



NAD ---> nicotinamide + ADPR

or a substitution of the nicotinamide moiety of NAD with another base. The mechanism was elegantly elucidated by Zatman et al (1953) and formulated as follows:

$$ARPPN^{+} + En \longrightarrow ARPPR\text{-}En + N$$

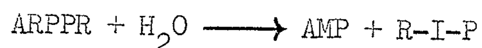
$$ARPPR\text{-}En + X \longrightarrow ARPPRX + En + H^{+}$$

Kaplan and coworkers (Kaplan, 1960, see also Zatman et al, 1953) produced several analogues of NAD using this transglycosidase activity of the animal tissue "DPNases" as they were generally known.

Replacement of nicotinamide from NAD by imidazole in the presence of NAD-glycohydrolases from animal tissues was first reported by Alivisatos and Woolley (1955) and Alivisatos et al (1962 b) demonstrated the substitution of benzimidazole for nicotinamide in NAD mediated by these glycohydrolases. Kapoor and Waygood (1965) have shown a similar exchange reaction between benzimidazole and NAD in whole homogenates of wheat embryos.

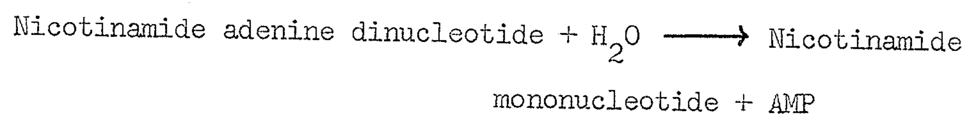
The ADPR (ARPPR) formed by this hydrolysis may then be cleaved

by a pyrophosphatase, dinucleotide nucleotidohydrolase (E.C.No.3.6.1.9) (Jacobson and Kaplan, 1957).



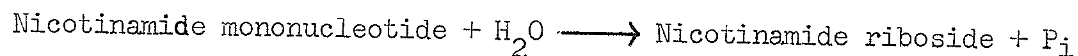
$$\text{ARPPR} + H_2O \longrightarrow \text{AMP} + \text{R-I-P}$$

(b) Kornberg and Pricer (1950) demonstrated a pyrophosphatase (E.C.No.3.6.19) capable of hydrolytically cleaving NAD as follows:



$$\text{Nicotinamide adenine dinucleotide} + H_2O \longrightarrow \text{Nicotinamide mononucleotide} + \text{AMP}$$

Such an enzyme was found in potatoes (Kornberg, 1950) and wheat leaves (Roberts, 1959). The NMN liberated might be further hydrolysed by a 5'-nucleotidase, 5'-ribonucleotide phosphohydrolase (E.C. No.3.1.3.5) (Heppel and Hilmoe, 1951) or a non-specific phosphatase of the phosphoric mono-ester hydrolases (E.C.No.3.1.3) type as described by Roberts (1959):

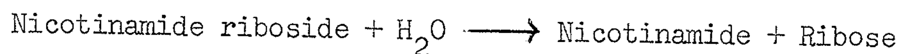


$$\text{Nicotinamide mononucleotide} + H_2O \longrightarrow \text{Nicotinamide riboside} + P_i$$

A phosphorolytic or a hydrolytic ribosyl hydrolase would then liberate nicotinamide from the nucleoside:

$$\text{Nicotinamide riboside} + P_i \longrightarrow \text{Nicotinamide} + \text{R-I-P}$$

or



$$\text{Nicotinamide riboside} + H_2O \longrightarrow \text{Nicotinamide} + \text{Ribose}$$

(c) Finally the adenine moiety of NAD may be deaminated by an aminohydrolase (E.C. No.group 3.5.4 *.; Kalckar, 1947) to produce the

inosinic acid analogue of NAD. Further degradation of the analogue is suspected to follow the pyrophosphorolysis pattern.

The ultimate fate of the nicotinamide liberated is uncertain. In animals it was reported (Perlzweig et al, 1950) to be excreted after methylation. It might be deamidated and participate in a cyclic metabolism of NAD or incorporated into alkaloids.

CONTROL MECHANISMS OF THE BIOSYNTHETIC PATHWAY

The intracellular levels of oxidised nicotinamide nucleotides appear to be rigidly controlled. Transient elevation in the concentration of liver NAD in response to nicotinamide administration was reported in several animals (Kaplan, 1960). In rats a transient depression of the concentration of liver NAD upon administration of ethionine, which traps adenine groups, has been reported (Stekol et al, 1963). Alterations in the physiology of the tissue, such as the onset or degree of senescence were reflected by changes in the concentrations of both the reduced and oxidised forms of NAD and NADP in wheat leaves (Mishra, 1963).

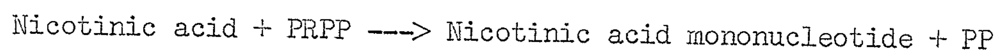
Imsande (1964 a,b, and c) reported a mechanism for the control of NAD biosynthesis from nicotinic acid. The first enzyme in the pathway, nicotinate nucleotide:pyrophosphate phosphoribosyltransferase (E.C. No.2.4.2.11), mediating the reaction,

Nicotinic acid + PRPP ---> Nicotinic acid mononucleotide + PP

was shown to be rate limiting in several bacteria, yeast and rat liver

cell free extracts. A tripolyphosphate was uniformly required, for enzymic activity. With inorganic polyphosphate the enzyme was only one third as active as with ATP. The efficiency of the other nucleotides tested was as follows: ATP = GTP > ITP > CTP > UTP > TTP. ADP and AMP were ineffective. He demonstrated further that, *in vivo*, ATP served to activate the enzyme and that conditions causing an increase in intracellular level of ATP cause an increase in NAD synthesis. Imsande concluded that NAD synthesis was therefore controlled indirectly by regulating the amount of ATP available in the cell.

The ATP dependence of nicotinate phosphoribosyltransferase was further supported by Nakamura *et al* (1964). A thousand-fold purified enzyme preparation, from beef liver, catalysed the reaction:



Nicotinic acid + PRPP ---> Nicotinic acid mononucleotide + PP

only if ATP were present in the incubation mixture. In the absence of ATP the same enzyme preparation mediated the reaction, nicotinic acid mononucleotide ⟶ nicotinic acid riboside + P_i.

The relative ratios of the activities of nicotinate phosphoribosyltransferase and phosphohydrolase remained constant throughout the purification procedure. On sedimentation analysis with sucrose density gradient both activities were obtained in the same fractions in a constant proportion. This led them to postulate that the same protein exhibits either one or the other enzyme activity depending on the presence or absence of ATP. Hence the availability of ATP would control the biosynthesis of NAD.

No information is as yet available on the regulation of NAD biosynthetic pathway in plant tissues.

AGENCIES AFFECTING THE SENESCENCE (CHLOROSIS) OF *ELODEA* AND WHEAT LEAVES

AGENCIES AFFECTING THE SENESCENCE (CHLOROSIS) OF *ELODEA* AND WHEAT LEAVES

As described in the literature review, Yoshida (1961) had shown that chloroplasts of nucleated protoplasts, of plasmolysed *Elodea* leaves, undergo an accelerated senescence in comparison to the chloroplasts of the protoplasts without nucleii. The chloroplasts of the enucleate protoplasts, remained green for a long period of time, increased in size and produced large amounts of starch. This is analogous of the effect of benzimidazole or kinetin which, as Person *et al* (1957) showed, maintained wheat leaves green, for almost a month, when floated on solutions of these compounds. The absence of a nucleus in protoplasts and its effect on the maintenance of chlorophyll could be regarded as similar to benzimidazole effect in the absence of benzimidazole. Since NAD but not NADP caused a breakdown of the chloroplasts of enucleated protoplasts, experiments were conducted to determine if benzimidazole could overcome or antagonise the accelerating effect of NAD.

These experiments were similar to those of Yoshida, except that whole, unplasmolysed *Elodea* leaves were used and treated either with NAD, benzimidazole or both to determine if there were any antagonistic effects.

When these studies were commenced the building, in which our laboratory was located, was undergoing extensive renovation and remodelling, consequently most of the early experiments were not conducted under rigidly controlled environment. Later experiments

were conducted in plant growth chambers, the environmental conditions being stated in the legend of the appropriate figure or the text.

MATERIALS AND METHODS

Elodea plants were obtained from the Hudson's Bay Company Ltd., Winnipeg. In the early experiments (first four experiments described below) plants were grown in aquaria with a layer of soil at the base and tap water (well aerated to remove chlorine). The plants were illuminated with two incandescent lights (25 W). In the later experiments plants were grown in H9agland's complete nutrient solution diluted 10-fold and the lamps were changed to Grolux lamps (25 W, F20T12-GRO, Sylvania, Drummondville, Quebec, Canada). Under these environmental conditions the *Elodea* plants showed a lush growth of the shoot system as well as the root system but this was also accompanied by a rank growth of algae, e.g. *Spirogyra*. However, the *Spirogyra* was easily eradicated by allowing the aquaria to remain in the diffuse light of the laboratory.

Mature leaves from growing apieces of *Elodea* twigs were detached and floated either on water or in solutions of a different combinations, in petri dishes (five centimeters in diameter). Each petri dish contained six leaves selected at random from the growing tips and six ml of the floating medium. The leaves were floated either under continuous illumination or in a 12 to 16 hour light

period. As the kind and intensity of illumination and duration of treatment were different in each experiment the details will be described at the appropriate place in the text. Unless otherwise stated the final concentrations of the following substances dissolved in the floating medium were nicotinic acid, nicotinamide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate at 1×10^{-3} M; benzimidazole 66 mg per litre, kinetin ca 20 mg per litre. The leaves were removed when the treatment showed significant changes in the chlorotic condition (approximately after 2 or 3 days in the early experiments and one day in the later experiments) and then dried between filter papers. The dried leaves were mounted on white cardboard, covered with Scotch tape and subsequently photographed on Kodachrome colourfilm.

The experiments described below were carried out with leaves collected from Elodea twigs grown in de-chlorinated tap water in aquaria and illuminated with two incandescent lights (25 W).

EXPERIMENTAL RESULTS

Experiment I was set up to determine the effects of NAD and benzimidazole when present either separately or together in the floating medium, on the detached leaves. Accordingly detached leaves were treated with NAD (10^{-3} M) or benzimidazole (66 mg per litre) or both NAD and benzimidazole together and illuminated with two incandescent (25 W) lights for three days at room temperature. The results of this experiment are shown in Figure 1. Leaves treated

FIGURE 1. NAD accelerated senescence in *Elodea* leaves and the "benzimidazole effect".

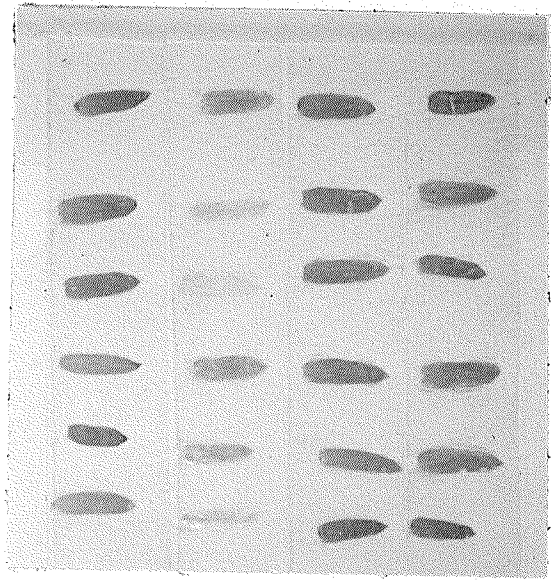
Apical leaves collected from plants grown in tap water and illuminated with two incandescent lights (25 W). Six leaves in 6.0 ml floating medium.

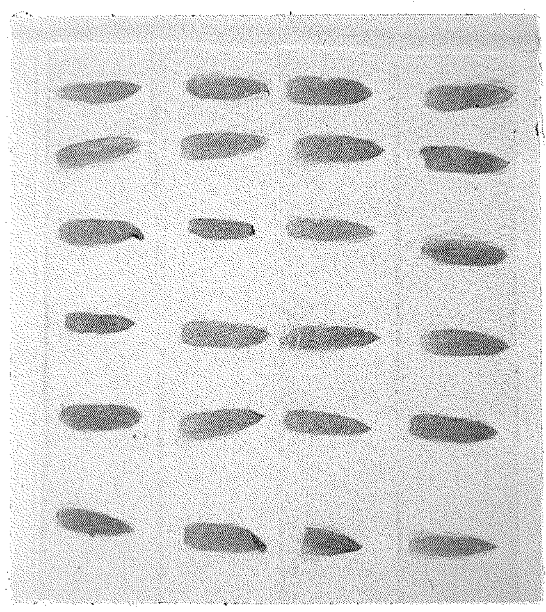
A : leaves floated on water.
B : leaves treated with NAD (10^{-3} M).
C : leaves treated with benzimidazole (66 mg per litre)
D : leaves treated with NAD + benzimidazole

Leaves were illuminated at room temperature for three days with two incandescent lights (25 W) maintained 6" above.

FIGURE 2. Effect of darkness on the chlorosis of *Elodea* leaves.

Experimental conditions and symbols used are the same as in Figure 1, except that the leaves were maintained in darkness.


A
B
C
D


A
B
C
D

with NAD were chlorotic while leaves treated with benzimidazole remained green similar to leaves floated on water. When benzimidazole and NAD were both present in the floating medium, benzimidazole overcame the NAD effect and maintained the leaves green (Fig 1).

Experiment II. The influence of light on the opposing effects of NAD and benzimidazole on the senescence of detached leaves was studied in this experiment. Leaves were subjected to the same treatments for three days at room temperature as in Experiment I, except for the leaves being maintained under darkness. The results (Fig 2) showed neither an acceleration of senescence by NAD, nor the retardation of senescence by benzimidazole, nor the mutual antagonism of NAD and benzimidazole when present together in the floating medium. The leaves remained green irrespective of the treatment and the length of time when maintained under darkness. In other experiments (not illustrated here) chlorosis of leaves was not observed even after seven days in the absence of illumination, thereby indicating that light is necessary for the acceleration of senescence by NAD, and therefore for the protective effect of benzimidazole and the mutual antagonism of NAD and benzimidazole to become evident.

Experiment III was designed to clarify the role of light in the expression of the effects of NAD and benzimidazole on the rate of senescence in detached leaves. Accordingly leaves were given the same treatments as in experiments I and II, maintained under darkness for three days at room temperature (c.f. Experiment II) and

then illuminated. Under these conditions NAD treated leaves became chlorotic in 5 hours. Benzimidazole overcame the NAD induced chlorosis and maintained the leaves green (Fig 3), when both NAD and benzimidazole were simultaneously present in the floating medium. This shows again that because the senescence of detached leaves was accelerated by NAD, only when illuminated the protective effect of benzimidazole was evident only in the presence of light.

Experiment IV. In this experiment the effectiveness of NADP as compared to NAD on the acceleration of senescence and its interaction with benzimidazole were studied. Leaves were treated with NAD, NADP, benzimidazole, NAD + benzimidazole and NADP + benzimidazole and illuminated with two incandescent lights for three days at room temperature as in Experiment I. Although leaves floated on NAD solution were bleached in three days, leaves treated with NADP for the same length of time showed an incomplete bleaching (white spots: Fig 4). Under the same experimental conditions benzimidazole maintained the leaves green (Fig 5) even in the presence of either NAD or NADP in the floating medium. In several other experiments detached leaves under illumination on treatment with NAD were consistantly chlorotic while even the partial chlorosis of leaves treated with NADP occurred infrequently. Benzimidazole showed antagonistic effects, consistently overcame the accelerated senescence and maintained the leaves green.

The plant material for the remaining experiments described here,

FIGURE 3. Role of light in senescence and benzimidazole effect.

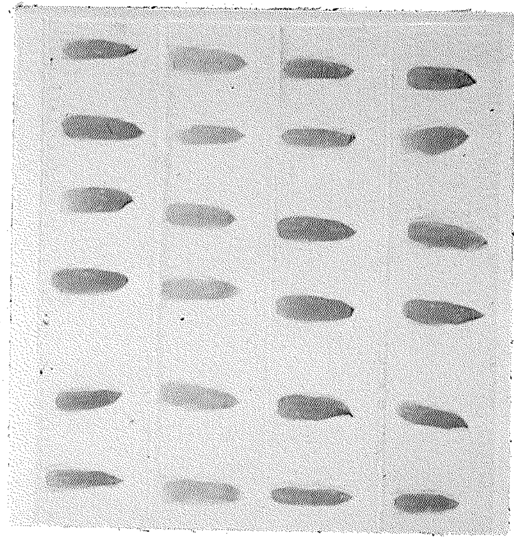
Experimental conditions are the same as in Figure 1, except for illumination. Leaves were maintained under darkness for three days and then illuminated for 5 hr.

A : leaves floated on water
B : leaves treated with NAD
C : leaves treated with benzimidazole
D : leaves treated with NAD + benzimidazole

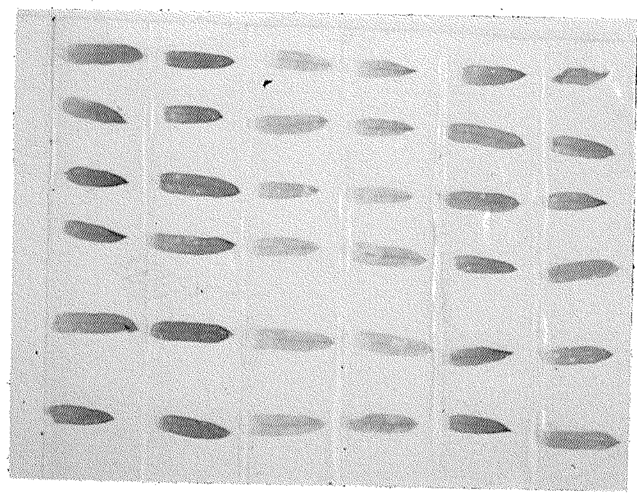
FIGURE 4. Efficacy of nicotinamide nucleotides on acceleration of senescence.

For Experimental conditions see Figure 1.

A : leaves floated on water
B : leaves treated with NAD
C : leaves treated with NADP



A B C D



A A B B C C

was grown in Hoagland's nutrient solution in aquaria equipped with Grolux lamps and the experiments were carried out under controlled environmental conditions in growth chambers.

Experiment V: The difference in the effects of NAD and NADP on accelerating the senescence of detached leaves of Elodea led to a study of the effects of the precursors for the biosynthesis of NAD, i.e. nicotinamide and nicotinic acid on the senescence of detached leaves. Leaves were treated with either nicotinic acid or nicotinamide (5.3×10^{-3} M) or NAD or NADP (1×10^{-3} M), with or without benzimidazole or kinetin in the floating medium, and maintained under continuous illumination (ca 2000 ft-c at 21°) for 20 hours. The results are shown in Figure 6. Leaves treated with nicotinamide remained green while leaves treated with nicotinic acid or NAD were chlorotic. Although Figure 6 does not show the "benzimidazole effect" it was observed nevertheless in several other experiments.

Experiment VI was carried out to study the effect of light on the acceleration of senescence caused by nicotinic acid or NAD (c.f. Experiment II). Leaves were subjected to similar treatments as in Experiment V but maintained in darkness and neither nicotinamide nor nicotinic acid showed any bleaching effect even after three days (Fig 7).

Experiment VII: Since the solutions of nicotinic acid or NAD impart a considerably acidic pH to the floating medium it was necessary

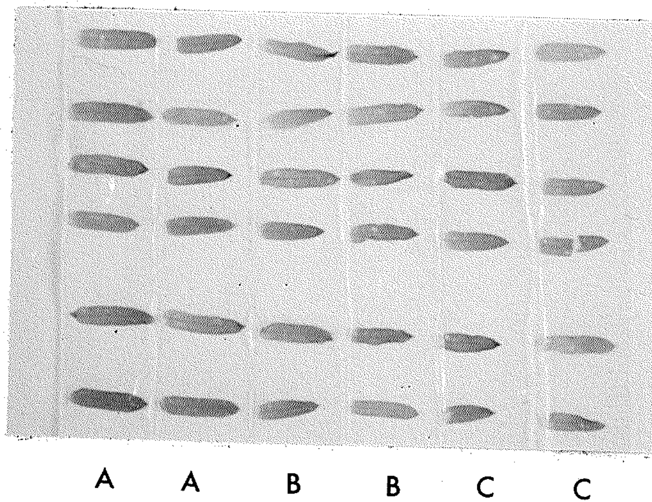
FIGURE 5. Antagonistic effects of benzimidazole x nicotinamide nucleotides.

For experimental conditions see Figure 1.

A : leaves treated with benzimidazole
B : leaves treated with benzimidazole + NAD
C : leaves treated with benzimidazole + NADP

FIGURE 6. Nicotinamide derivatives and their effect on senescence.

Plants were grown in Hoagland's nutrient medium (10-fold diluted) and illuminated with two Grolux lamps (25 W). Six leaves in 6.0 ml medium, maintained in a growth chamber under continuous illumination (2000 ft-c at 21°) for 20 hours. Treated with nicotinamide or nicotinic acid ($5x10^{-3}$M) or NAD or NADP ($1x10^{-3}$M) and with or without benzimidazole (66 mg per litre) or kinetin (20 mg per litre). The solutions were not neutralised.


A
A
B
B
C
C

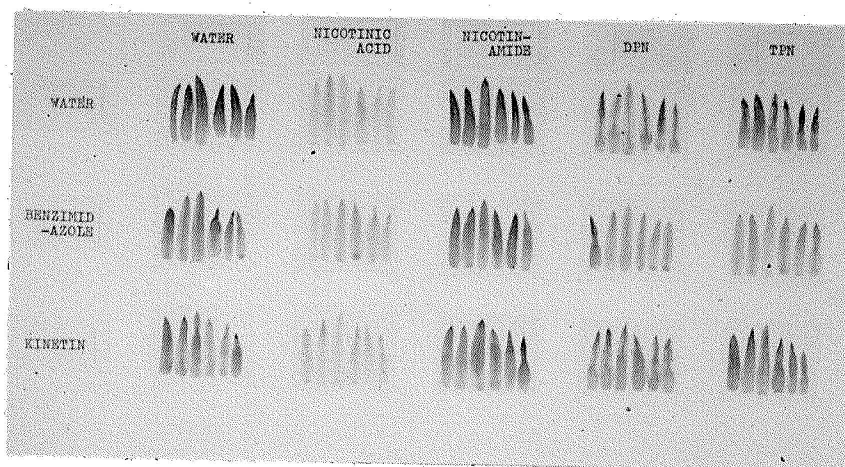
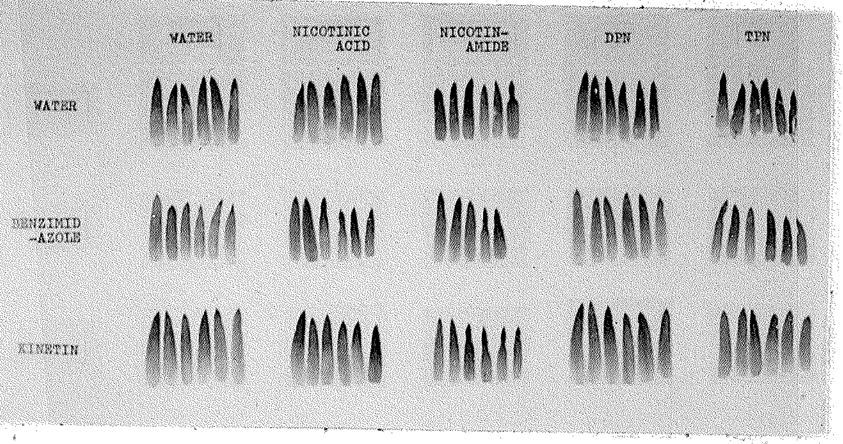

WATER
NICOTINIC ACID
NICOTIN-AMIDE
DPN
TPN
WATER
BENZIMID -AZOLE
KINETIN

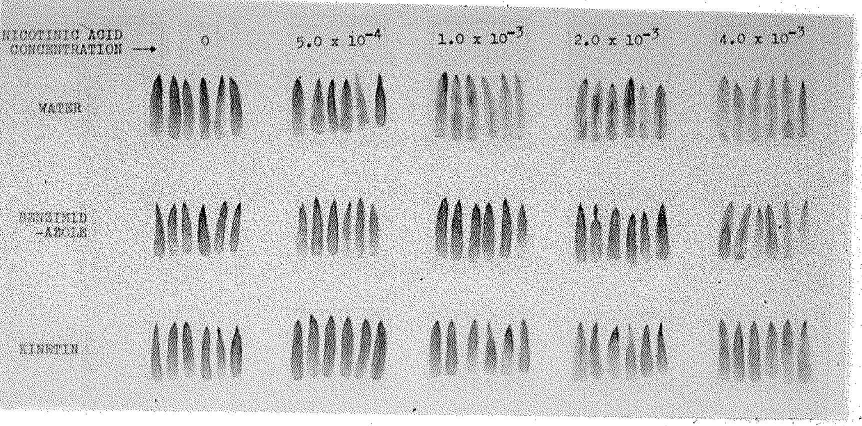
FIGURE 7. The effect of darkness on the acceleration of chlorosis by nicotinamide derivatives.

Experimental conditions similar to those in Figure 6 except for maintaining the leaves in darkness.

FIGURE 8. Acceleration of senescence by increasing concentration of nicotinic acid x benzimidazole or kinetin.

Experimental conditions are similar to those for Figure 6 except that neutralised solutions were used in the treatment of leaves.


WATER
NICOTINIC ACID
NICOTIN-AMIDE
DPN
TPN
WATER
BENZIMID-AZOLE
KINETIN


NICOTINIC ACID CONCENTRATION →
0
5.0 x 10^-4
1.0 x 10^-3
2.0 x 10^-3
4.0 x 10^-3
WATER
BENZIMID-AZOLE
KINETIN

to determine the extent to which the acidity of the medium may be considered a cause for the acceleration of chlorosis of the detached leaves treated with nicotinic acid or NAD. Solutions of nicotinamide, nicotinic acid, NAD and NADP were neutralised to pH 7.0 before use in the floating medium and detached leaves were allowed to senesce under conditions similar to those described for the previous experiment. Nicotinic acid or NAD treated leaves consistently developed chlorosis though more slowly than in un-neutralised solutions. NADP was sporadic in causing this effect while nicotinamide was without any effect. The "benzimidazole effect" was always noticeable and the leaves remained green when benzimidazole was also present in the floating medium together with nicotinic acid or NAD or NADP and if the leaves were maintained under light. There was no visible chlorosis of the leaves in darkness.

Experiment VIII: Since both nicotinic acid and NAD accelerated the senescence of detached, illuminated leaves in the absence of benzimidazole a study was made of the effective range of concentrations of nicotinic acid and NAD (neutralised) that would clearly accelerate the chlorosis of detached leaves illuminated for 40 hours in a growth chamber. Progressively increasing chlorosis was visible in the leaves treated with nicotinic acid at a final concentration of 5.0×10^{-4} M (Fig 8). In a similar study of leaves treated with NAD solution (not illustrated) chlorosis appeared at a higher concentration of NAD (1.0×10^{-3} M). Yet if the leaves were simultaneously treated with benzimidazole, it counteracted the acceleration

of chlorosis of these detached leaves caused by either nicotinic acid or NAD even though the concentration of either one of these substances in the floating medium reached as high a final concentration as 2.0×10^{-3} M.

While nicotinamide and NADP were without any effect on senescence, nicotinic acid and NAD accelerated the senescence in detached, illuminated leaves of *Elodea* as well as Selkirk wheat leaves and benzimidazole overcame the acceleration of senescence in either case. These opposite effects on senescence of detached leaves caused by benzimidazole on one hand and nicotinic acid or NAD on the other led to an investigation of the biosynthetic and degradative pathways of NAD metabolism and the effect of benzimidazole on these pathways in wheat leaves.

THE BIOSYNTHETIC AND DEGRADATIVE PATHWAY OF NICOTINAMIDE

ADENINE DINUCLEOTIDE METABOLISM IN WHEAT LEAVES

THE BIOSYNTHETIC AND DEGRADATIVE PATHWAY OF NICOTINAMIDE ADENINE DINUCLEOTIDE METABOLISM IN WHEAT LEAVES

During the experiments on the senescence of detached leaves, of the two possible precursors of NAD biosynthesis, nicotinic acid and nicotinamide were found to differ considerably in their effect on the senescence of detached leaves floated on water. Hence the metabolic fate of these compounds was investigated taking advantage of the availability of these radioactive compounds. Detached wheat leaves were allowed to incorporate labelled nicotinic acid (carboxyl-C^{14}) and nicotinamide (carbonyl-C^{14}) and the distribution of the isotope in various compounds followed by the techniques as described under Materials and Methods.

MATERIALS AND METHODS

Nicotinic acid (carboxyl-C^{14}) and nicotinamide (carbonyl-C^{14}) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England; NMN, NAD, NADP, Neurospora NAD-glycohydrolase, snake (Crotalus) venom phosphodiesterase, yeast alcohol dehydrogenase, NADP specific isocitric dehydrogenase from beef heart were obtained from Sigma Chemical Company (St.Louis, Mo. U.S.A.); benzimidazole was obtained from Eastman Organic Chemicals (Rochester, New York, U.S.A.); Na A D (desamido NAD) was a generous gift of Dr. N. O. Kaplan.

Selkirk wheat (_Triticum aestivum_ L.) seedlings were grown on vermiculite, either in the greenhouse at various times of the year

or in a growth chamber (Model 16-S, Controlled Environment Ltd., Winnipeg) under continuous illumination. Seven to eight days old primary leaves harvested from these seedlings were used in the experimental studies on the labelled precursor incorporation into nicotinamide adenine dinucleotide and as a source of enzymes as mentioned in the text.

Wherever operations were conducted in cold, they were carried out in a cold laboratory at 4°.

ORTHOPHOSPHORIC MONOESTER PHOSPHOHYDROLASE

This enzyme was used in the identification by enzymatic hydrolysis of the intermediates, (i.e. nicotinamide mononucleotide and nicotinic acid mononucleotide) of the pathways for the biosynthesis and degradation of nicotinamide adenine dinucleotide metabolism isolated from the leaves after incorporation of labelled nicotinic acid or nicotinamide.

The enzyme was prepared according to Schmidt (1955) from bull prostrate glands. Frozen glands were thawed, cut into small pieces with scissors and homogenised in a Waring blendor for two minutes. The suspension was kept overnight in the refrigerator after the addition of a few drops of toluene. The suspension was centrifuged and the turbid supernatant (ca 200 ml) was dialyzed overnight against four litres of distilled water in the cold room. The pH of the opalescent solution was lowered to pH 5.6 by dropwise addition of 1.0 N acetic acid. The precipitate was removed by centrifugation. The supernatant was used without further purification.

NICOTINAMIDE ADENINE DINUCLEOTIDE GLYCOHYDROLASE

This enzyme mediates a substitution of the nicotinamide moiety of nicotinamide adenine dinucleotide molecule with a free base in an exchange reaction. This property of the enzyme was utilized in the synthesis of, 1) radioactive nicotinamide adenine dinucleotide by an exchange between labelled nicotinamide (carbonyl-C^{14}) and the unlabelled nicotinamide moiety of nicotinamide adenine dinucleotide and, 2) nicotinic acid adenine dinucleotide by an exchange between nicotinic acid and nicotinamide adenine dinucleotide.

The glycohydrolase from beef spleen was prepared according to Kaplan (1955). Two hundred grams of fresh beef spleen was homogenized in a Waring blendor with ice and water for four minutes and made to 750 ml with ice water and 15 ml of 1.0 M sodium bicarbonate. The connective tissue was removed by centrifugation at 500 g for ten minutes. One hundred millilitres of the crude material was centrifuged at 20,000 g for 15 minutes and the residue was suspended in 60 to 70 ml of 0.02 N sodium bicarbonate and centrifuged at 500 g for 15 minutes. The supernatant fluid was then centrifuged at 20,000 g for 15 minutes. The residue was suspended in 80 ml of distilled water. The suspension was finally centrifuged at 20,000 g for one minute and the supernatant was used.

NICOTINAMIDE RIBOSIDE

Selkirk wheat (7 to 8 day old primary) leaves were harvested, wrapped in wet paper towels and chilled. The leaves were ground in a

mortar with sand and 20 ml of phosphate buffer, pH 7.0 (10^{-3} M). The brei was filtered through four layers of cheesecloth and centrifuged at 500 g for five minutes. The supernatant was dialyzed overnight against four litres of cold distilled water with continuous stirring in the cold.

Approximately 750 mg of NAD was dissolved in 20 ml of distilled water and neutralized to pH 7.0. To this 20 ml of the dialyzed whole homogenate was added and incubated at 37° with a few drops of toluene. The reaction was terminated by immersion in boiling water for three minutes. The mixture was centrifuged at 20,000 g for 20 minutes. The supernatant was loaded onto a Dowex-1-10 (formate) column and the column was washed with ca 200 ml of distilled water. The effluents were pooled and concentrated. Most of the adenine, adenosine and nicotinamide crystallised out. The mother-liquor contained nicotinamide riboside relatively free of these contaminants.

NICOTINIC ACID RIBOSIDE AND NICOTINIC ACID MONONUCLEOTIDE

The riboside and ribotide of nicotinic acid were prepared from nicotinamide riboside and its mononucleotide respectively by chemical deamidation of the nicotinamide moiety as described by Atkinson and Morton (1960). Nicotinamide riboside (or NMN) was suspended in acetic anhydride (10 ml per mmole) and kept at -10° while an equal volume of nitrous anhydride prepared by gentle heating of a mixture of concentrated nitric acid and granular arsenic trioxide, was condensed in the reaction vessel. The suspension was maintained at 10° to 15° for one hour, and the volatile material was then removed by a stream of dry air.

The dried reaction products were dissolved in a small volume of distilled water and neutralised with 1.0 N sodium hydroxide. Further purification of the product by passing through Dowex-1-10 (formate) resin column according to the procedure of Atkinson and Morton (1960) was not carried out.

NICOTINIC ACID ADENINE DINUCLEOTIDE

This was prepared according to the proceedure described by Honzo *et al* (1964). Approximately 700 micromoles of nicotinamide adenine dinucleotide, 60 mmoles of nicotinate and 4.0 mmoles of Tris-HCl buffer (pH 7.5) in a final volume of 70 ml were incubated with 1.6 g of beef spleen NAD-glycohydrolase. The incubation was carried out in a 200 ml Erlenmeyer flask at 37° with occasional shaking. Progress of the reaction was followed by determination of nicotinic acid adenine dinucleotide in aliquots from the incubation mixture by, (a) measurement of CN-addition product of the total N-ribosyl bond present and, (b) alcohol dehydrogenase assay for the residual nicotinamide adenine dinucleotide. When the dehydrogenase assay had reached a minimum and the cyanide reaction began to decrease, the reaction was stopped by the addition of 10 ml of 20% perchloric acid. The denatured protein was centrifuged off and washed twice with 30 ml of 2% perchloric acid. The supernatant was adjusted to pH 7.0 with 10.0 N KOH and the precipitated perchlorate discarded. The clear supernatant was decanted onto a Dowex-1x10 formate, 200-400 mesh (1.2 x 32 cm) column and the column was washed with 0.2 N formic acid until no more UV-absorbing material

was eluted from the column. Nicotinic acid adenine dinucleotide was then eluted by application of a formic acid concentration gradient (mixing chamber, 450 ml of 0.2 N formic acid; reservoir 2.0 N formic acid). The elution of nicotinic acid adenine dinucleotide was followed by measuring the absorbancy at 260 mμ of each of the 5.0 ml fractions collected. The dinucleotide was located in the 150 ml to 250 ml effluent volume and the combined fractions were lyophilised.*

TRIGONELLINE

This was synthesized essentially according to the proceedure described by Sarett et al (1940). Nicotinic acid (1.0 g), methyl iodide (0.6 ml, sp.gr. 2.12), and excess of moist silver oxide** were suspended in 10.0 ml of methanol and heated at 100° for one hour with stirring. The mixture was cooled, filtered and the filtrate dried overnight under air jets. The residue was dissolved in 30 ml of hot ethanol and trigonelline was allowed to crystalize overnight from the clear solution in the cold.

INDUCTION OF SENESCENCE IN LEAVES

Primary leaves of Selkirk wheat (7 to 8 days old) were excised

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* The spectra of the CN-addition products of nicotinamide riboside, nicotinic acid riboside and nicotinic acid mononucleotide are given in Appendix 1.

** Silver oxide was prepared by dropwise addition of 5.0 N KOH to a concentrated solution of silver nitrate until a dark brown precipitate begins to form instead of a dark grey precipitate. The precipitate was collected by filtration.

and floated on either water or 50 mg per litre benzimidazole solution in glass trays (ca leaves 5 g fr wt per 500 ml per tray). The trays were covered with saran wrap and placed in a growth chamber (21°) under continuous illumination (4200 ft-c) and allowed to senesce for two days.

FEEDING OF RADIOACTIVE COMPOUNDS TO LEAVES

Primary leaves of Selkirk wheat were cut to four inches in length. The cut ends were dried quickly with Kleenex and inserted into glass vials (1.0 x 3.5 cm), at the rate of 18 leaves per vial. The vials contained 0.1 ml of the isotope (nicotinic acid or nicotinamide-C^{14}, one µc). The leaves were allowed to absorb the isotope solution under continuous illumination (4200 ft-c at 21°). As soon as the leaves in each vial have taken up the isotope solution an equal volume (0.1 ml) of distilled water was added to the vial and gently swirled so that any traces of isotope adsorbed on the wall of the vial would be dissolved and absorbed by the leaves. This step was repeated twice. Subsequently the vials were filled with distilled water. Four vials (from each isotope treatment) were removed at selected time intervals (one hour intervals up to six hours and thereafter at two hour intervals up to twelve hours). Leaves from two vials were mixed, the cut ends dried, rapidly weighed and cut into 2-5 mm sections and plunged into 30 ml of boiling 95% ethanol for 15 to 20 minutes.

EXTRACTION OF LABELLED INTERMEDIATES

The killed leaves were successively extracted twice with 80% ethanol, twice with 40% and twice with water for 15 to 20 min using less than 30 ml of extractant. These extracts were pooled (final vol 200-220 ml) and dried overnight under a jet of air at room temperature.

The dried material was separated into two fractions, (1) a chloroform fraction containing the chlorophyll pigments and lipid materials, (2) a water fraction containing all the water soluble substances and the bulk of the radioactivity. This was achieved by phase transfer between two immiscible solvents, water as the polar solvent and chloroform as the non-polar solvent and the following was the procedure. The dried material was repeatedly extracted by washing with small aliquots of chloroform to remove the pigments and lipid fractions. The chloroform extract was transferred to a separatory funnel and washed with water several times to remove any traces of contaminating water soluble material in the chloroform extract. The water washings were saved and pooled together in a second separatory funnel. The chloroform insoluble, residual dry matter was then extracted repeatedly with small aliquots of water. The water extract was then transferred to the second separatory funnel containing the water washings of the chloroform extract. The water extract was washed several times with chloroform to remove contaminating pigments and lipid material. These chloroform washings were added to the chloroform extract contained in the first separatory funnel.

The pooled washings with and extracts of a given solvent, chloroform or water as the case may be, under 30 ml in total volume were dried overnight under air jets at room temperature. The dry matter was quantitatively taken up in five millilitres of the water or chloroform.

PAPER CHROMATOGRAPHY

Aliquots of 0.2 ml of water soluble material were streaked on Whatman No.1 filter paper. Three solvent systems were used:

I Isobutyric acid: ammonia: water = 66:1:33 at pH 3.8

II One molar ammonium acetate: 95% ethanol = 3:7 at pH 5.0

III n-Butanol saturated with water in an atmosphere of ammonia

Development was carried out overnight using the ascending technique at 20°.

PAPER ELECTROPHORESIS

Acid washed Whatman 3 mm paper (12" x 11.5") was used. The combined extract was applied and the spots allowed to dry. The paper was then placed in a Spinco, Durram type, cell and equillibriated for 15 to 20 minutes, with the solvent, sodium acetate 0.05 M at pH 5.0 (Siliprandi _et al_, 1954). Electrophoresis was carried out in the cold room with a Shandon power supply unit at 500 v, 32 mA for five hours.

RADIOAUTOGRAPHY

Kodak no screen medical X-ray film was used for contact exposure to radioactive isotopes on developed chromatograms. The films were developed with Elon-hydroquinon (Kodak D-19b) X-ray film developer (Hodgeman, 1954) for five minutes at 18° and fixed with Quick-Fix (Edwal Scientific Products Corporation, Chicago, U.S.A.)

DETERMINATION OF RADIOACTIVITY

Radioactivity determinations were carried out with the aid of Nuclear Chicago (Model 725) liquid scintillation system. The radioactive areas from the paper chromatograms were cut into small squares and placed in counting vials containing one millilitre of 50% ethanol. After an interval of one to two hours, ten millilitres of dioxane scintillation fluid* was added. The samples were counted for ten minutes. When pigments were present counting was carried out, with and without internal standard.

DETECTION OF PYRIDINIUM COMPOUNDS

Pyridinium compounds were detected by the following methods:

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*Scintillation fluid:

Dioxane	833 ml
Ethylene glycol monethyl ether	167 ml
Naphthalene	50 g
2,5 Diphenyloxazole (PPO)	4 g
[2-(5-phenylaxazolyl)] benzene (POPOP)	0.1 g
Final volume	1 litre

(1) Radioautography on X-ray films and the corresponding areas were marked on the chromatograms.

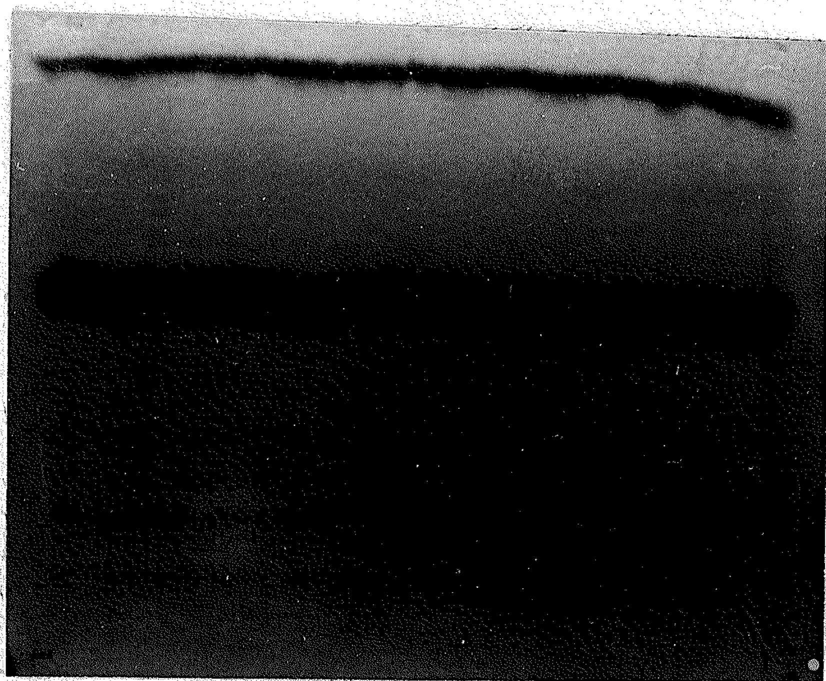
(2) Exposure of chromatograms for one hour to the vapours of methyl ethyl ketone: Ammonia (1:1) in a closed chamber. Quarternary pyridinium compounds (nicotinamide derivatives) fluoresce under ultraviolet light whereas tertiary pyridinium compounds (nicotinic acid derivatives) do not fluoresce (Kodicek and Reddi, 1951).

(3) Spraying the chromatogram with 1% picryl chloride in 95% ethanol, dried at room temperature, followed by exposure to ammonia fumes. Nicotinamide and nicotinic acid give characteristic red spots (Dawson et al, 1959).

EXPERIMENTAL RESULTS

Immediately detached wheat leaves were fed radioactive nicotinic acid or nicotinamide ($-C^{14}$) under continuous illumination. The water soluble compounds were extracted as described under Materials and Methods. Aliquots of the extract were streaked on Whatman No.1 filter paper. The chromatograms were developed overnight in Solvent I and radioautographed on X-ray film. A typical radioautograph (Fig 9), nicotinamide fed for 12 hr showed nine radioactive areas on each chromatogram, corresponding to nine possible intermediates of NAD biosynthetic pathway including NADP. The radioactive areas were numbered from I to IX in order of their occurrence successively from the solvent front to the starting line

FIGURE 9. Typical radioautograph.
For explanation see text.



and for ease of reference a tentative identification list of these compounds is given in Table 1.

TABLE 1. Identification List of Compounds I to IX.

Compound No.	Identification	Abbreviation
I	Nicotinamide	-
II	Nicotinic acid	-
III	Nicotinamide riboside	NR
IV	Trigonelline	-
V	Nicotinic acid riboside	NaR
VI	Nicotinamide mononucleotide	NMN
VII	Nicotinamide adenine dinucleotide	NAD
VIII	Nicotinic acid mononucleotide	NaMN
IX	A mixture of nicotinic acid adenine dinucleotide and nicotinamide adenine dinucleotide phosphate	NaAD and NADP respectively

IDENTIFICATION

In order to identify the various intermediates of the NAD biosynthetic pathway, water extracts were separated with solvent I by paper chromatography and radioautographed. Radioactive areas corresponding to the spots on the radioautograph were cut out of the chromatogram and eluted with water in the cold. The eluants, from several

strips, of the same compound were pooled and lyophilized. Since the original extract contained all other water soluble substances of the leaves there were considerable amounts of contaminants in a given radioactive compound. With whole leaf extracts the technique of paper chromatography was used in preference to chromatographic separation on resin columns for the following two reasons:

(1) The quantities of the labelled precursors were small, e.g. 10 µg per g fr.wt.

(2) To avoid the inevitable loss of some of the intermediates due to non-adsorption on columns.

Due to the extreme lability of the pyridinium ring and the lengthy time involved in radioautography and occasional exposure to ultraviolet light during visual inspection, considerable losses of the intermediates were encountered during their isolation.

The identification of the intermediates was based mainly on cochromatography, electrophoresis and alkaline hydrolysis with 0.1 N sodium hydroxide at 100° for 10 minutes which breaks the N-ribosyl bond of the pyridinium ring.

Chromatography and electrophoresis were carried out as described in Methods. Compounds I and II (Fig 9) were identified as nicotinamide and nicotinic acid respectively, compounds III, IV and V migrated to positions between nicotinic acid and nicotinamide mononucleotide and were assumed to be nicotinamide riboside, an unknown derivative of the pyridinium ring and nicotinic acid riboside. Compounds VI and VII

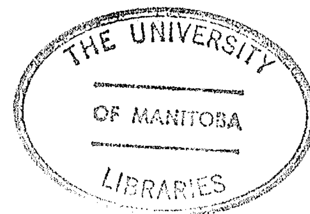
had the same excursion values as nicotinamide mononucleotide and nicotinamide adenine dinucleotide. Compound IX had an R_f value the same as that of nicotinic acid adenine dinucleotide and nicotinamide adenine dinucleotide phosphate in Solvent I and was, therefore considered to be a mixture of nicotinic acid adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. Compound VIII was assumed to be nicotinic acid mononucleotide based on the relative migratory properties reported in the literature (Nishizuka and Hayaishi, 1963).

Compounds III to IX were hydrolysed with 0.1 N sodium hydroxide at 100° for 10 minutes and the products were identified by paper chromatography (Solvents I and III) and by electrophoresis. Compounds III, VI and VII tentatively identified by cochromatography as nicotinamide riboside, nicotinamide mononucleotide and nicotinamide adenine dinucleotide produced nicotinamide after alkaline hydrolysis. Compound V underwent complete hydrolysis to nicotinic acid under the experimental conditions described above and this,together with the known relative migratory properties of nicotinic acid riboside (Nishizuka and Hayaishi, 1963), give further support to its identification as nicotinic acid riboside. On the other hand the complete hydrolysis of compound IV to nicotinic acid was only achieved by treatment with 2.0 N sodium hydroxide at 100° for one hour indicating that this compound (IV)* is possibly either Trigonelline (Joshi and Handler, 1962) or Niacinogen (Ghosh et al, 1963) both of which have been reported to be present in the grasses. The hydrolysis of compound IX resulted in the libera-

* See Appendix 4 for further proof of the identity of compound IV.

tion of approximately equal amounts of nicotinic acid and nicotinamide supporting the tentative identification that compound IX is a mixture of nicotinic acid adenine dinucleotide and nicotinamide adenine dinucleotide phosphate.

Further proof of the identity of these intermediates was obtained by enzymatic hydrolysis and cochromatography and electrophoresis of the products. Compound VI (nicotinamide mononucleotide) on incubation with orthophosphoric monoester phosphohydrolase and snake venum phosphodiesterase (contaminated by a monoesterase) produced nicotinamide riboside. Compound VII (nicotinamide adenine dinucleotide) treated with Neurospora NAD-glycohydrolase produced nicotinamide and when treated with phosphodiesterase (contaminated with monoesterase) liberated nicotinamide mononucleotide and nicotinamide riboside. The mononucleotides and ribosides of both nicotinic acid and nicotinamide were identified as the products of hydrolysis by snake venom phosphodiesterase of compound IX, a mixture of nicotinic acid adenine dinucleotide and nicotinamide adenine dinucleotide phosphate.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LIBRARIES

INCORPORATION OF PRECURSORS

The biosynthetic pathway of nicotinamide adenine dinucleotide in wheat leaves was followed with the aid of the incorporation of carbon-14 isotopes of nicotinic acid or nicotinamide by leaves for varying intervals of time (1 to 12 hr). In a time course study of the incorporation of labelled nicotinamide or nicotinic acid two microcuries of the isotope (0.2 ml of 10^{-3} M solution = 0.2 micromole or 0.02 mg of

FIGURE 10. Incorporation of nicotinamide-C^{14} and the distribution pattern of radioactivity in a time course study.

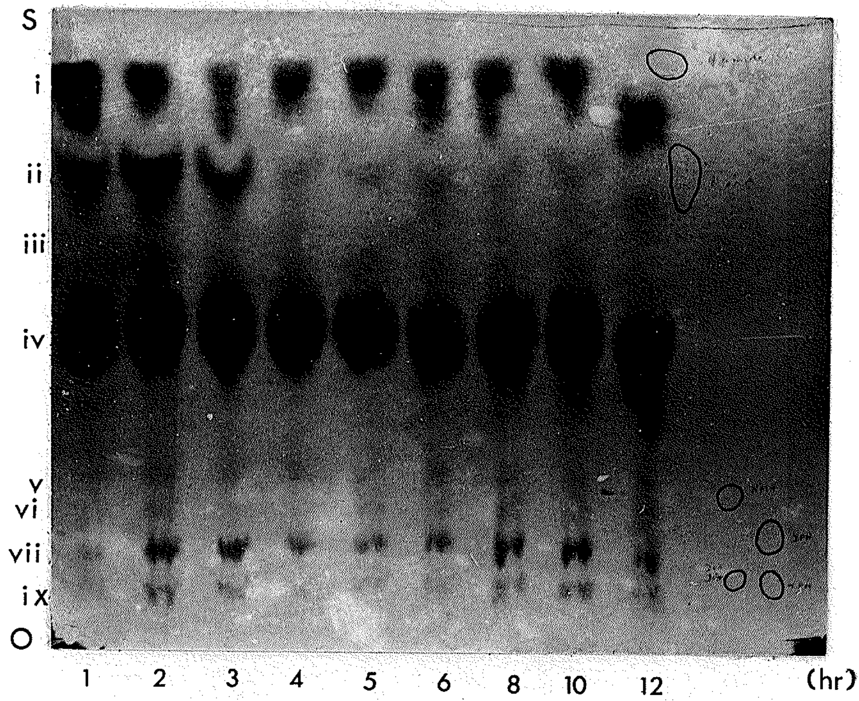
s = solvent front
i = nicotinamide
ii = nicotinic acid
iii = NR
iv = Trigonelline
v = NaR
vi = NMN
vii = NAD
ix = NaAD + NADP
o = origin

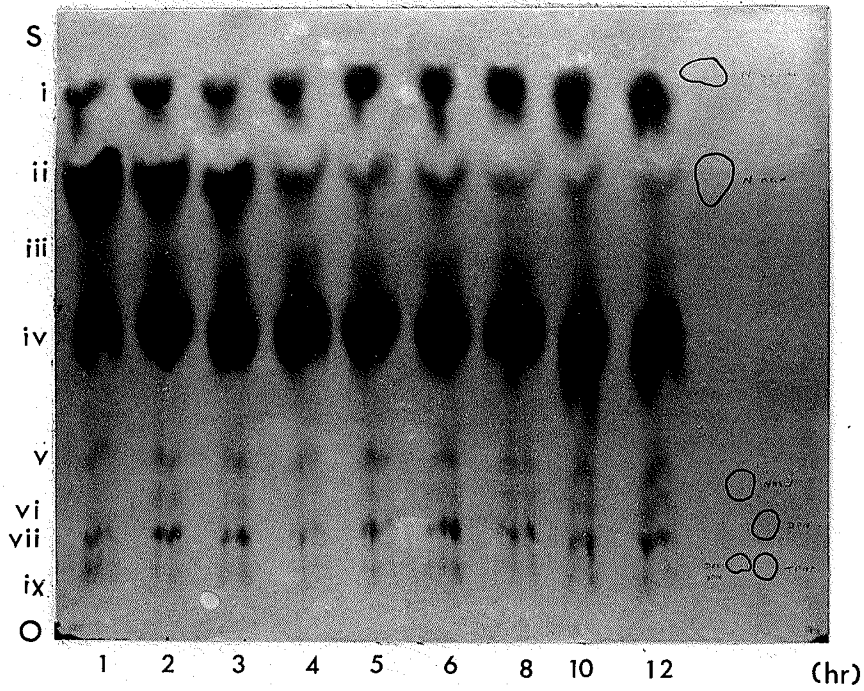
Chromatograms developed in Solvent I overnight. Radioautographs developed after two weeks.

FIGURE 11. Incorporation of nicotinic acid-C^{14} and the distribution pattern as radioactivity in a time course study.

s = solvent front
i = nicotinamide
ii = nicotinic acid
iii = NR
iv = Trigonelline
v = NaR
vi = NMN
vii = NAD
ix = NaAD + NADP
o = origin

Chromatograms developed in Solvent I overnight. Radioautographs developed after two weeks.


S
i
ii
iii
iv
v
vi
vii
ix
O
1
2
3
4
5
6
8
10
12
(hr)


S
i
ii
iii
iv
v
vi
vii
ix
O
1
2
3
4
5
6
8
10
12
(hr)

the material in 0.2 ml of water) were fed to approximately two grams fresh weight of leaves. The intermediates of NAD biosynthesis were separated by paper chromatography and located by radioautography as explained in Methods. Figures 10 and 11 illustrate the radioautographs of a time course experiment in which immediately detached Selkirk wheat leaves were fed radioactive nicotinamide (Fig 10) or nicotinic acid (Fig 11) from 1 to 12 hours. The pattern of incorporation of the labelled precursors by leaves for the periods of time in hours, 1, 2, 3, 4, 5, 6, 8, 10 and 12 are shown, reading from left to right, by the vertical columns in both figures. Total radioactivity found in each of the compounds is given in Appendix 2 and 2a and the fluctuations in the levels of radioactivity of these compounds are graphically illustrated in Figures 12 and 13.

Irrespective of the precursor fed and the period of time (1 to 12 hr) allowed for it to be metabolised by the leaves, the general pattern of labelling remained the same as shown in these figures (10-13). Nicotinamide or nicotinic acid appears in the extracts following the absorption of nicotinic acid or nicotinamide by the leaves. The level of the precursor and the product (nicotinamide or nicotinic acid as the case may be) showed a steady decrease. Nicotinamide riboside (Compound III) after an initial accumulation begins to decrease and nearly disappears after about six hours. Most of the label appears to be channeled into Trigonelline (Compound IV). Regardless of whether the precursor fed was the amide or the acid, Trigonelline (Compound IV) appears to accumulate the maximum amount

FIGURE 12. NAD metabolism: fluctuations in the levels of radioactivity of various intermediates after nicotinamide-C^{14} incorporation up to 12 hours.

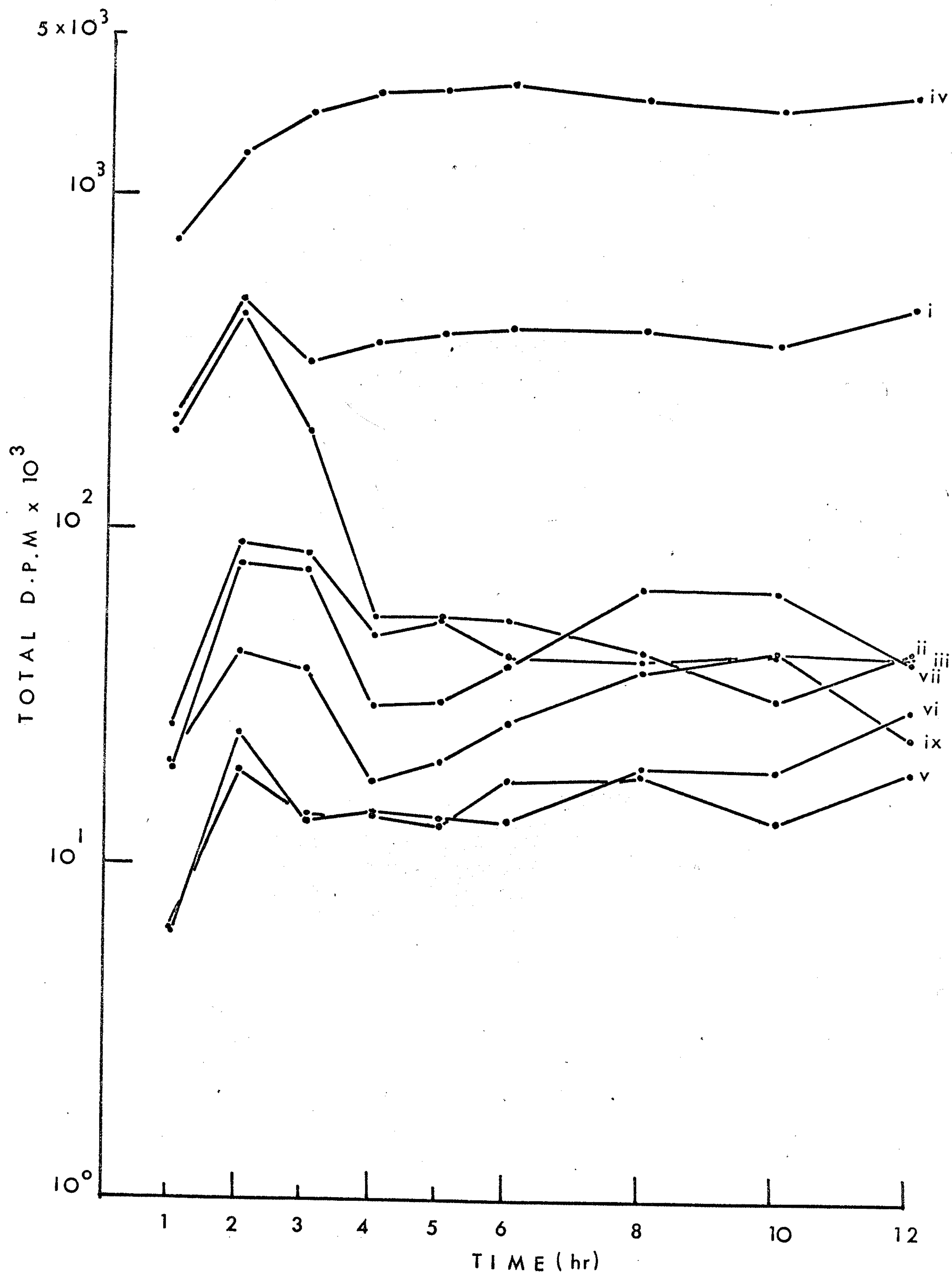
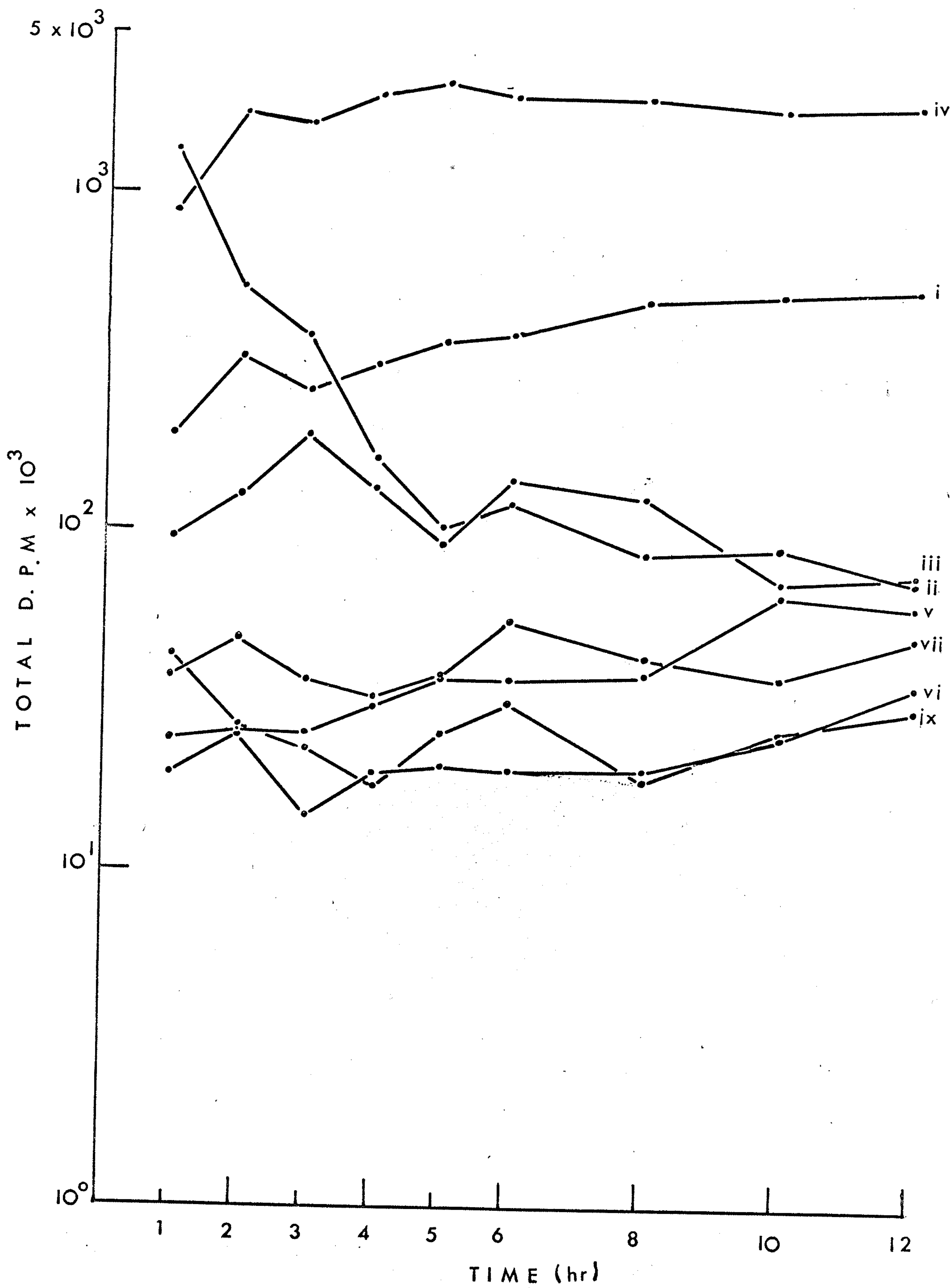

NICOTINAMIDE (—C14)
5 x 10^3
10^3
10^2
10^1
10^0
TOTAL D.P.M x 10^3
TIME (hr)
1
2
3
4
5
6
8
10
12
iv
i
ii
iii
vii
vi
ix
v

FIGURE 13. NAD metabolism: fluctuations in the levels of radioactivity of various intermediates after nicotinic acid-C^{14} incorporation up to 12 hours.


NICOTINIC ACID $-C^{14}$
5×10^3
10^3
10^2
10^1
10^0
TOTAL D. P. M $\times 10^3$
1
2
3
4
5
6
8
10
12
TIME (hr)
iv
i
iii
ii
v
vii
vi
ix

of the label (almost 40-50% of the total fed) very early and remains highly radioactive indicating an accumulation of this product (Figs 10-13). Unlike nicotinamide riboside (Compound III), nicotinic acid riboside (Compound V) remains at a low level of radioactivity, particularly if nicotinamide were the precursor incorporated. However, the ribosides of both nicotinic acid and nicotinamide showed at least a two-fold increase in radioactivity if nicotinic acid was fed to the leaves (Figs 12, 13).

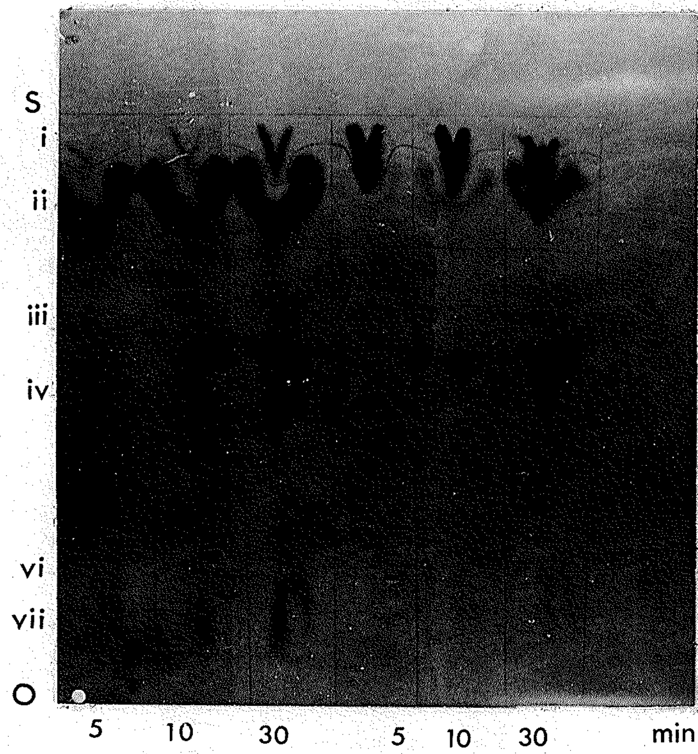
The incorporation of the label at the levels of the bases and nucleosides sharply contrasts with the levels of radioactivity present in the nucleotides. Barely detectable amounts of nicotinic acid mononucleotide (Compound VIII) accumulate, following the assimilation of the isotope and then only after 12 hours (Fig 9). The radioactivity at the level of nicotinamide mononucleotide (Compound VI), nicotinamide adenine dinucleotide (Compound VII) and nicotinic acid adenine dinucleotide together with nicotinamide adenine dinucleotide phosphate remained consistently low while exhibiting fluctuations in the label incorporated (Figs 12, 13).

Since the general pattern of distribution of the radioactivity was the same in both amide and acid over long periods of incorporation, a short term experiment was carried out to determine the order in which the compounds became radioactive. Figure 14 illustrates the results of a short term experiment in which leaves were fed with either nicotinic acid or nicotinamide, each for 5, 10 and 30 minutes

FIGURE 14. Distribution pattern of radioactivity in a short term, time course study of incorporation of nicotinic acid-C^{14} and nicotinamide-C^{14}.

s = solvent front
i = nicotinamide
ii = nicotinic acid
iii = NR
iv = Trigonelline
vi = NMN
vii = NAD
o = origin

Chromatograms developed in Solvent I overnight.
Radioautographs developed after two weeks.


S
i
ii
iii
iv
vi
vii
O
5
10
30
5
10
30
min

respectively from left to right.

After five minutes of incorporation nicotinamide appears in leaves fed with nicotinic acid and nicotinic acid in those leaves fed with nicotinamide. Nicotinamide riboside (Compound III), nicotinic acid riboside (Compound V), nicotinamide mononucleotide (Compound VI), nicotinic acid adenine dinucleotide (Compound IX) and nicotinamide adenine dinucleotide phosphate (Compound IX) were not detectable in both cases in the short time period of this experiment.

However, traces of trigonelline (Compound IV) and nicotinamide adenine dinucleotide (Compound VII) appear in leaves fed with nicotinic acid but not in those fed with nicotinamide.

After a ten minute interval trigonelline (Compound IV) and nicotinamide adenine dinucleotide (Compound VII) became distinct on the radioautograph (Fig 14) and traces of radioactivity are visible at the level of nicotinic acid riboside (Compound V) and nicotinamide mononucleotide (Compound VI) in the extracts from leaves allowed to incorporate nicotinic acid, whereas trigonelline (Compound IV) and nicotinamide adenine dinucleotide (Compound VII) could barely be detected in the extracts of leaves fed with nicotinamide.

On metabolising nicotinic acid for thirty minutes considerably more radioactive material was detectable in both trigonelline (Compound IV) and nicotinamide adenine dinucleotide (Compound VII) and at the same time traces of nicotinamide riboside (Compound III),

nicotinic acid riboside (Compound V) and nicotinamide mononucleotide (Compound VI) appeared. On the other hand in leaves fed with nicotinamide all the radioactivity was confined to trigonelline (Compound IV) and nicotinamide adenine dinucleotide (Compound VII).

Having determined, from these experiments, the sequence of incorporation, the efficacy of precursors and the biosynthetic pathway of nicotinamide adenine dinucleotide metabolism it was decided to study the effects, if any, of senescence on the biosynthesis of nicotinamide adenine dinucleotide in wheat leaves.

BIOSYNTHESIS OF NICOTINAMIDE ADENINE DINUCLEOTIDE IN WHEAT LEAVES

Selkirk wheat leaves, 7-8 day old, were detached and floated on water or treated with benzimidazole solution for two days under continuous illumination. The leaves were then allowed to absorb either nicotinic acid (carboxyl-C^{14}) or nicotinamide (carbonyl-C^{14}) for two hours. The water soluble compounds were extracted and separated by paper chromatography. From the radioautographs of these chromatograms no differences were evident in the pattern of labelling between, (a) the senescent leaves, (b) and immediately detached leaves. The pattern of isotope distribution appeared to be similar in both cases and Fig 15 illustrates the pattern obtained after incorporation of the precursors by senescent leaves. The results of various treatments (in duplicate) as seen in this figure are, from left to right, extracts of leaves floated on water or treated with benzimidazole and allowed to incorporate nicotinic acid followed by

FIGURE 15. Patterns of isotope distribution in senescent leaves after incorporation of nicotinic acid-C^{14} and nicotinamide-C^{14}.

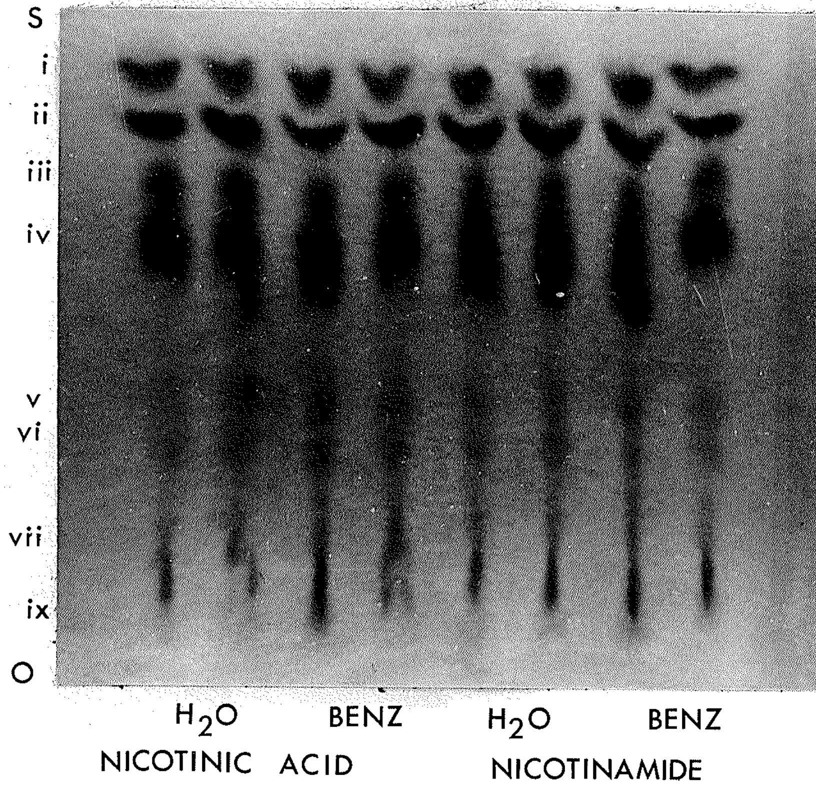
s = solvent front
i = nicotinamide
ii = nicotinic acid
iii = NR
iv = Trigonelline
v = NaR
vi = NMN
vii = NAD
ix = NaAD + NADP

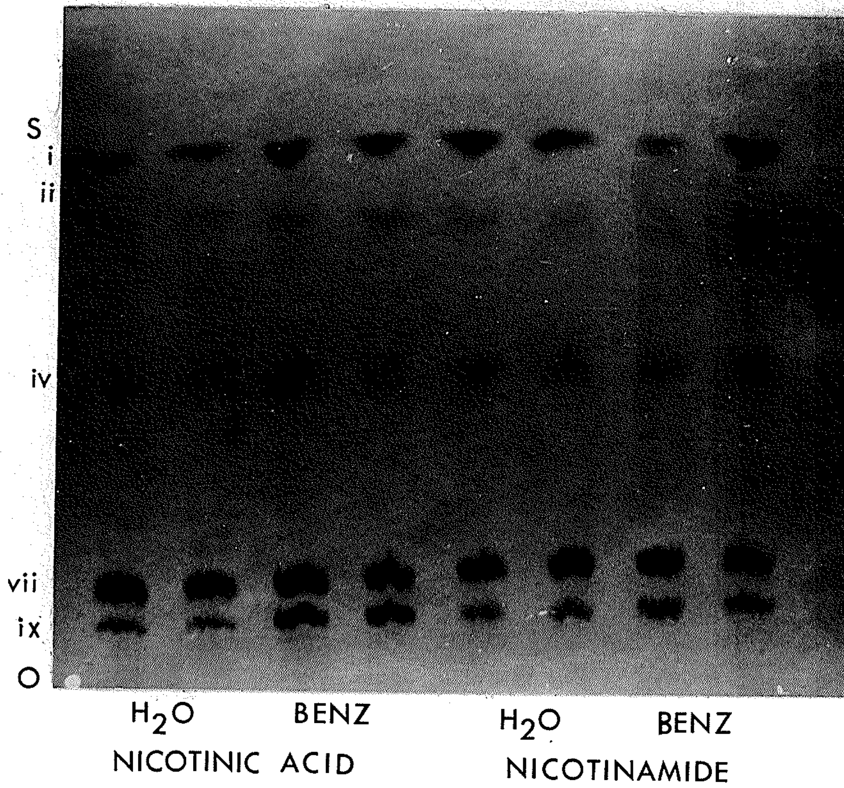
Chromatograms developed in Solvent I overnight. Radioautographs developed after two weeks.

FIGURE 16. Patterns of isotope accumulation in the nucleotides in senescent leaves.

s = solvent front
i = nicotinamide
ii = nicotinic acid
iii = NR
iv = Trigonelline
v = NaR
vi = NMN
vii = NAD
ix = NaAD + NADP

Chromatograms developed in Solvent I overnight. Radioautographs developed after two weeks.


S
i
ii
iii
iv
v
vi
vii
ix
O
H_2O
BENZ
H_2O
BENZ
NICOTINIC ACID
NICOTINAMIDE


S
i
ii
iv
vii
ix
O
H_2O
BENZ
H_2O
BENZ
NICOTINIC ACID
NICOTINAMIDE

extracts of leaves floated on water or treated with benzimidazole and fed with nicotinamide. Since there were large amounts of interfering water soluble substances in the aqueous extracts, chromatographic separation was not satisfactory. Advantage was taken of the fact that acetone precipitated NAD from water solutions in acidic pH. Accordingly one millilitre aliquots of whole water extracts were acidified with HCl until, on application to congo red paper a royal blue colour developed. Six volumes of cold acetone (-10°) was added and the solution stored overnight in a freezer (-15°). The white precipitate was collected by centrifugation, washed twice with cold acetone and once with cold ether. It was dried, overnight, in a dessicator under vacuum. The dry powder was dissolved in one ml of distilled water and chromatographed. Radioautography of the developed chromatograms (Fig 16) clearly showed the presence of larger amounts of nucleotides in benzimidazole floated leaves compared to those floated in water. Furthermore in both the cases nicotinic acid was incorporated into the nucleotides to a greater extent than nicotinamide.

In subsequent experiments three sets (in duplicate) of detached leaves were allowed to incorporate nicotinamide (C^{14}) for two hours under continuous illumination, one set of leaves was killed and extracted immediately, the second set of leaves was floated on water and the third set treated with benzimidazole. Both the groups of leaves were maintained under continuous illumination for two days. The radioautograph (Fig 17), from left to right, shows the distri-

FIGURE 17. Patterns of isotope distribution in leaves allowed to senesce after incorporation of nicotinamide-C^{14}.

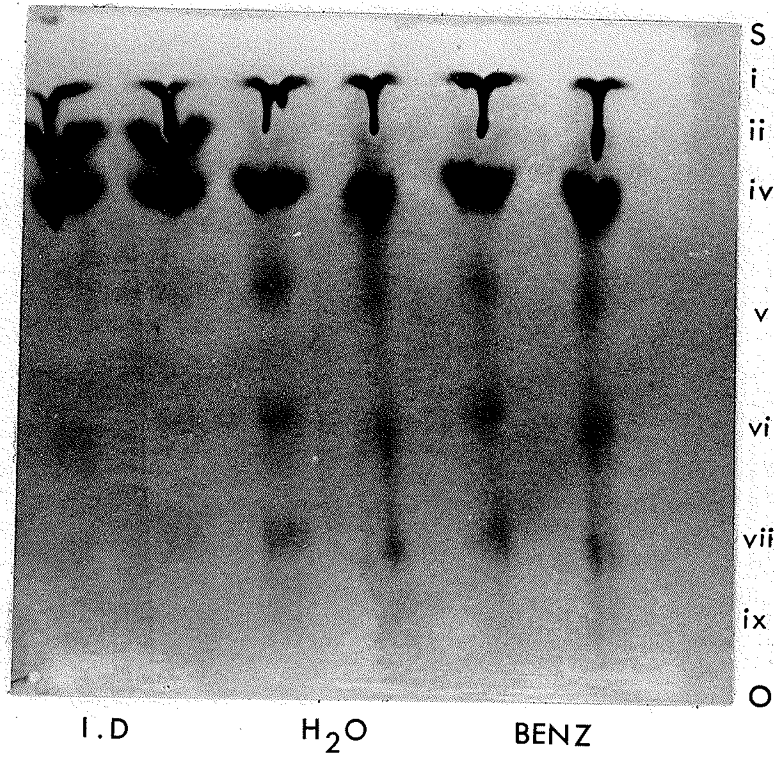
s = solvent front
i = nicotinamide
ii = nicotinic acid
iv = Trigonelline
v = NaR
vi = NMN
vii = NAD
ix = NaAD + NADP
o = origin

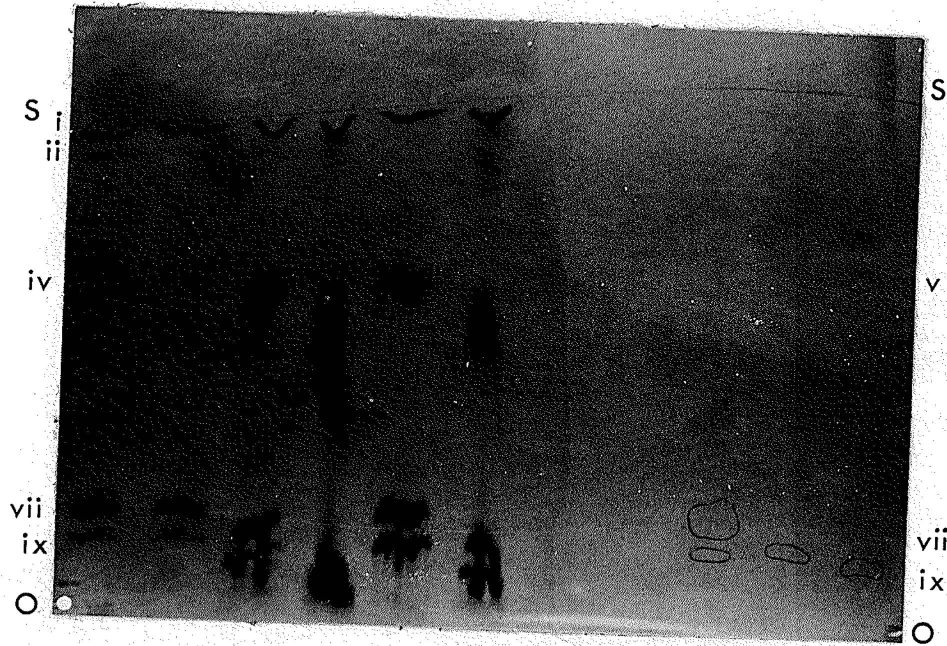
Chromatograms developed in Solvent I overnight. Radioautographs developed after two weeks.

FIGURE 18. Patterns of isotope accumulation in the nucleotides in leaves fed with nicotinamide-C^{14} and allowed to senesce.

s = solvent front
i = nicotinamide
ii = nicotinic acid
iv = Trigonelline
v = NaR
vi = NMN
vii = NAD
ix = NaAD + NADP
o = origin

Chromatograms developed in Solvent I overnight. Radioautographs developed after two weeks.


S
i
ii
iv
v
vi
vii
ix
O
I.D
H_2O
BENZ


S
i
ii
iv
vii
ix
O
S
v
vii
ix
O

bution of label in various intermediates from the extracts of immediately detached leaves, leaves floated on water and leaves treated with benzimidazole. Evidently, the treated leaves conformed in general to the pattern exhibited by the leaves immediately detached and fed with nicotinamide. The only striking difference observed, was that nicotinic acid and nicotinamide riboside, Compounds II and III respectively, were not detectable in the extracts of leaves either floated on water or treated with benzimidazole. Although extracts of immediately detached leaves fed for two hours showed only traces of nicotinic acid riboside and nicotinamide mononucleotide (Compounds V and VI) no significant differences were noticeable in NAD and NaAD + NADP (Compounds VII and IX). Therefore, the nicotinamide nucleotides were precipitated from whole extracts as in the previous experiment. The radioautographs of the precipitated nucleotides showed a greater accumulation of the label in the extracts of leaves floated on water and benzimidazole treated leaves than in the immediately detached leaves (Fig 18). Benzimidazole treated leaves showed a greater labelling of NADP while leaves floated on water accumulated more label in NAD.

DEGRADATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE

The degradative aspects of NAD metabolism were studied *in vitro*. Five grams of leaves (seven to eight days old) were ground in a mortar with sand and 20 ml Tris-HCl buffer pH 7.0 (5×10^{-2} M). The brei was passed through cheesecloth and centrifuged at 500 g for three

minutes. The precipitate (sand, fibres and cell walls) was discarded. The supernatant was dialyzed overnight against four litres of cold distilled water in a cold room. The dialyzed whole homogenate was incubated with NAD at 37° for 30 minutes at pH 5, 6, 7 and 8. The reaction was stopped by immersion in boiling water for three minutes. The precipitated protein was removed by centrifugation. Aliquots of the supernatant were streaked on Whatman No.1 filter paper and developed with solvent II using an ascending technique. From this it was observed that at pH values of seven and above, the products of hydrolysis are mainly nicotinamide mononucleotide and adenosine monophosphate whereas at pH values below seven especially at pH 6.0 nicotinamide riboside and adenosine accumulated maximally accompanied by small quantities of nicotinamide and adenine.

DISCUSSION

DISCUSSION

During the senescence of an organism, the degradative aspects of metabolism become dominant over the synthetic aspects. The alteration of the equilibria of a few key processes would shift appropriately the overall metabolic pattern of the organism. A wide variety of changes are accompanied by an alteration of one such process namely, the metabolism of NAD. Benzimidazole and kinetin, it appears, are moderators of at least this process. The evidence for the importance of NAD in the senescence of detached leaves came from two different sources.

As described in detail in the Literature Review, Yoshida (1961) showed that chloroplasts in the protoplasts of plasmolysed *Elodea* leaves lacking a nucleus remained green and synthesized starch for a considerable time, in comparison to nucleated protoplasts which showed a rapid degeneration of their chloroplasts. When NAD was added to the medium in which the leaves were floated, the green chloroplasts in the enucleated protoplasts underwent a rapid degeneration similar to those in the nucleated protoplasts. Yoshida, (1961) argued that since NAD is presumed to be synthesized by the nucleus (Brachet, 1954) its production under these abnormal conditions accelerates oxidative senescence. The presumed lack of NAD production in enucleated protoplasts prevents the occurrence of oxidative senescence.

Since benzimidazole maintains both *Elodea* and wheat leaves green for a considerable time it was argued that the absence of the nucleus

in Yoshida's experiments was showing something similar to the "benzimidazole effect".

Mishra, (1963) in a study of the metabolic changes of detached wheat leaves under various conditions, presented comprehensive data on the levels of oxidised and reduced forms of NAD and NADP. The data reported by Mishra on the drifts in the levels, of all four species of nicotinamide nucleotides of the wheat leaves under different treatments, determined following a light or dark period is difficult to interpret. The NAD level of leaves floated on water declined as compared to that of immediately detached leaves. Benzimidazole and benzimidizole and kinetin together maintained the level of NAD above that in leaves floated on water and a tendency for increased NADP synthesis was shown by leaves treated with benzimidazole or kinetin. In further experiments on chloroplasts isolated in non-aqueous media from whole wheat leaves, Mishra reported an enhanced synthesis of NADP in chloroplasts from benzimidazole treated leaves and only traces of NADP in the chloroplasts isolated from leaves floated on water. The data reported by Mishra (1963) suggest that the changes in the ratios of reduced and oxidised forms of the nicotinamide nucleotides are significant and would cause wide metabolic changes (see also Waygood, 1965).

Hence the independent observations by Yoshida (1961) and Mishra (1963) on the different aspects of senescence in detached leaves which led to the present investigation are:

(i) in the absence of a nucleus, the chloroplasts in the protoplasts of plasmolysed cells of Elodea leaves remain

green, i.e. show the "benzimidazole effect" and the presence of a nucleus or exogenously added NAD accelerate the oxidative senescence of such chloroplasts, as reported by Yoshida, and

(ii) benzimidazole influences the levels of nicotinamide nucleotides in detached wheat leaves, reported by Mishra.

In the present investigation into the effects of NAD and benzimidazole on senescence, whole unplasmolysed Elodea leaves were used. Detached leaves floated on NAD solutions were bleached consistently (Fig 1) while in those treated with NADP only occasional partial chlorosis occurred (Fig 4). This suggests the possibility that of the two nucleotides of nicotinamide, NAD alone accelerates chlorosis directly and that NADP functions more indirectly by serving as a source of NAD, through the action of the ubiquitous plant phosphomonoesterases. The activity of the phosphomonoesterases could then account for the sporadic effect of NADP on chlorosis of leaves floated on water. Since NAD and NADP differed considerably in their effectiveness on the acceleration of chlorosis, the two possible precursors of NAD biosynthesis, i.e. nicotinamide and nicotinic acid were tested in the floating medium of Elodea leaves. Nicotinic acid alone was found to be effective and even more so than NAD in accelerating the chlorosis of leaves (Fig 6). Both nicotinic acid and NAD cause the chlorosis by a property other than the acidity of their solutions, was shown by floating the leaves on (a) neutralised solutions of these compounds (Fig 8) and (b) on dilute solutions of HCl.

Elodea leaves treated with either NAD or nicotinic acid failed

to develop chlorosis when maintained under darkness (Fig 2, 7), unlike leaves treated similarly but illuminated. Since the leaves became chlorotic under illumination but not if maintained under darkness, it was argued that light must be a factor in the acceleration of senescence of leaves by NAD. Further evidence for the role of light was obtained by the appearance of chlorosis in the "dark controls" only after they were exposed to light for a sufficient length of time (Fig 3).

As described in greater detail in the Introduction and Literature Review, Person _et al_ in 1957, showed that benzimidazole retarded the onset of chlorosis in detached wheat leaves floated on water. Wang and Waygood (1959), (Wang _et al_, 1960,1961), reported that wheat leaves treated with benzimidazole, as against leaves floated on water, showed a greater synthesis of chlorophyll and a retardation of chlorophyll degradation. Mishra (1963), demonstrated the influence of benzimidazole on the levels of nicotinamide nucleotides in wheat leaves and chloroplasts isolated from wheat leaves. Furthermore Alivisatos (1962) in rat liver and Kapoor and Waygood in 1965b in wheat embryos have demonstrated _in vitro_, the formation of benzimidazole analogues of NAD.

In the present investigation on the accelerated senescence of _Elodea_ leaves, the effects of benzimidazole were studied. The chlorosis of _Elodea_ leaves treated with nicotinic acid or NAD or NADP was consistently prevented and the leaves were maintained green when benzimidazole was also included in the floating medium (Figs, 1, 3 and 5). Since the acceleration of chlorosis by nicotinic acid or NAD was evident

only when the leaves under treatment were illuminated, the "benzimidazole effect" also becomes evident, in the case of leaves treated and illuminated. Analogue formation of benzimidazole with NAD does not appear to play a significant role in the retardation of leaf chlorosis by benzimidazole, particularly since the enzyme mediating the exchange reaction (NAD-glycohydrolase) was not found in the leaves, in this laboratory.

However, benzimidazole appears to alter the metabolism of NAD in some unknown manner and thus retard the breakdown of chlorophyll, in the presence of either nicotinic acid or NAD. Under normal (healthy) conditions NAD apparently exists in a dynamic equilibrium with NADP on one hand and the precursors on the other as illustrated in Figures 10 to 13. Following the incorporation of radioactive isotopes of precursors, the amount of label in the nucleotides does not show a steady increase but oscillates between a high and low levels of incorporation. Significant changes occurred only if the leaves were allowed to senesce under controlled (by treating with benzimidazole) or uncontrolled conditions (by floating on water) as seen in Figures 15 to 18. More radioactivity was present in the nucleotides isolated from the treated leaves than in immediately detached leaves. Furthermore a greater incorporation of the label was shown into NADP than into NAD in leaves treated with benzimidazole as compared to leaves floated on water (c.f. Mishra, 1963; see also Waygood, 1965). From this it is evident that the metabolic pattern of NAD is altered, however slightly, in senescence and benzimidazole affects both senescence and NAD metabolism in a significant manner.

So far the information available on NAD metabolism and especially

Rowen-Kornberg pathway:

Nicotinamide + R-1-P ----> NR + P_i (1)

NR + ATP ----> NMN + ADP (2)

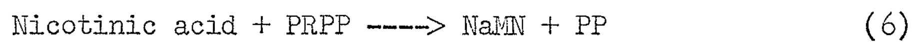
NMN + ATP ⟶ NAD + PP (3)

Singer and Kearney modification:

Nicotinamide + PRPP ----> NMN + PP (4)

NMN + ATP ----> NAD + PP (5)

Preiss-Handler pathway:


Nicotinic acid + PRPP ----> NaMN + PP (6)

NaMN + ATP ----> NaAD + PP (7)

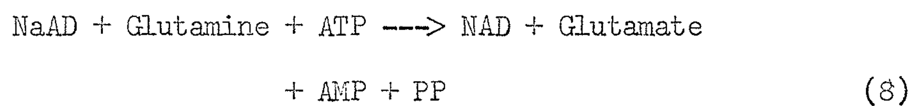
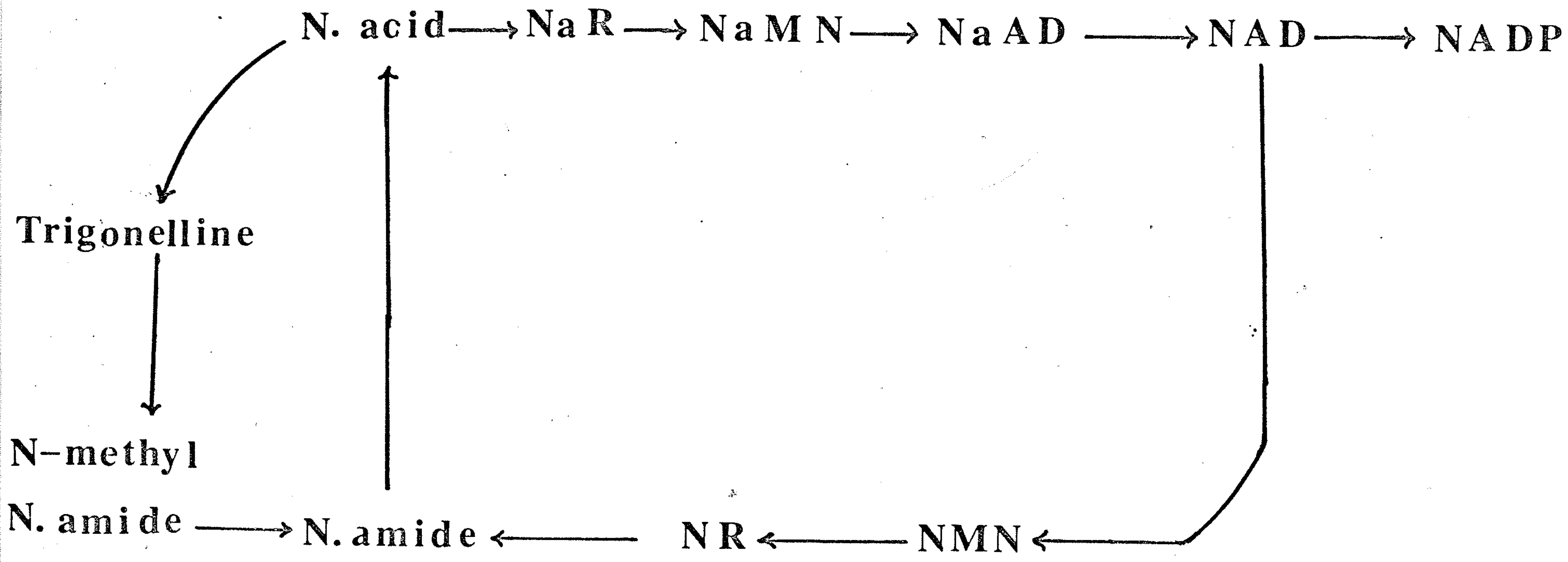

NaAD + Glutamine + ATP ---> NAD + Glutamate + AMP + PP (8)

FIGURE 19.

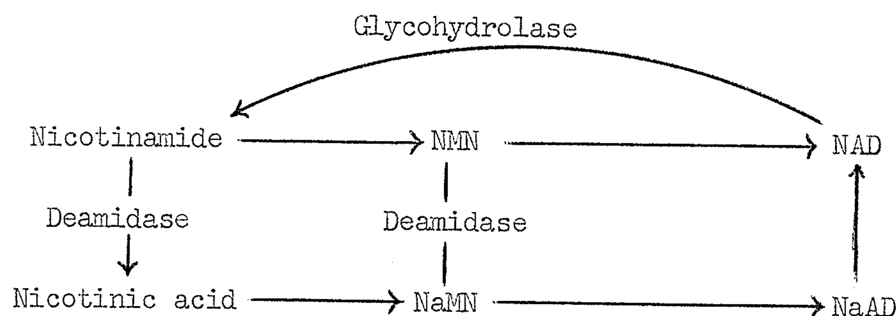
Pathways of NAD Biosynthesis

FIGURE 20. Proposed scheme of cyclic metabolism of NAD.



N. acid
NaR
NaMN
NaAD
NAD
NADP
Trigonelline
N-methyl
N. amide
N. amide
NR
NMN

on biosynthesis of NAD has come mainly from investigations on animal tissues and microorganisms. As described in the Literature Review Rowen and Kornberg (1951) suggested that nicotinamide was the precursor in the biosynthesis of NAD and degradation was by a reversal of the synthetic process (the reactions of this pathway of NAD metabolism and the modifications later on suggested are given in Figure 19 and for ease of reference the reactions are mentioned in the text by their number). The evidence was based on the study of individual enzymes and not the complete pathway. Reaction (1) can occur in animals but requires a high concentration of nicotinamide, low P_i concentration and at the physiological pH values the equilibrium is well over to the left, all of which are unlikely to be realised *in vivo*. Further, only a weakly active kinase (E.C. No.2.7.1.22) was shown by Rowen and Kornberg (1951) to mediate reaction (2). Kornberg (1950) purified enzymes from hog liver and brewers yeast, which catalyse reaction (3) but with an equilibrium constant to the left. Singer and Kearny (1954) suggested that reactions (1) and (2) of the Rowen-Kornberg pathway be replaced by (4). Preiss and Handler (1957) reported the presence of an enzyme mediating reaction (4) from erythrocytes. Preiss and Handler (1958) proved that in yeast and mammalian tissue nicotinic acid was incorporated into NAD through reactions (6) to (8). Handler (1958) further suggested that nicotinamide is deamidated to produce nicotinic acid and that synthesis and degradation of NAD might proceed through different pathways. Sarma *et al* (1961) confirmed the operation of Preiss-Handler pathway of NAD synthesis in avian tissues and postulated a cyclic pathway for the metabolism of NAD as follows:



Further evidence for the operation of this cyclic pathway was furnished by Gholson and Kori (1964) in Bacillus subtilis.

From the experimental results described in the present study, several significant features of the NAD metabolism in wheat leaves are brought to light.

During the time course study of the incorporation of labelled precursors by wheat leaves lasting from 1 to 12 hours, irrespective of the precursor incorporated, whole aqueous extracts of these leaves contained : nicotinamide (Compound I), nicotinic acid (Compound II), nicotinamide riboside (Compound III, NR), trigonelline (Compound IV), nicotinic acid riboside (Compound V, NaR), nicotinamide mononucleotide (Compound VI, NMN), nicotinic acid mononucleotide (Compound VIII, NaMN), nicotinamide adenine dinucleotide (Compound VII, NAD), nicotinic acid adenine dinucleotide (Compound IX, NaAD) and nicotinamide adenine dinucleotide phosphate (Compound IX, NADP) (Fig 9). The presence of nicotinamide as well as nicotinic acid together with their derivatives (ribosides, mono- and di-nucleotides) in the extracts of leaves fed with nicotinamide is inconsistent with the Singer-Kearney modification (1954) of NAD biosynthesis as postulated by Rowen and Kornberg (1951), for such

a scheme would require deamidation at every level beginning with nicotinamide up to the dinucleotide (NAD) to account for the presence of nicotinic acid and its derivatives (NaR, NaMN and NaAD). Although Sarma et al (1961) reported an amidohydrolase, from avian tissue, capable of deamidation of nicotinamide and NMN to nicotinic acid and NaMN respectively, deamidation of NR and NAD were not shown. Furthermore the conversion of NaAD to NAD was reported from several sources (Kaplan, 1960, 1961) the reverse reaction has not been reported. The presence of the nicotinic acid derivatives in the extracts of leaves fed with nicotinamide, is required by the Preiss-Handler pathway of NAD biosynthesis.

As described under Experimental Results, in a short term experiment on the incorporation of precursors (nicotinic acid-7-C^{14} or nicotinamide-7-C^{14}) by wheat leaves,on feeding the leaves with nicotinic acid for 5 minutes nicotinamide, trigonelline and NAD become labelled. After a ten minute interval radioactivity was detectable in NaR and NMN, while after metabolising nicotinic acid for 30 minutes considerably more of the label was present in trigonelline and NAD and traces of radioactivity appeared not only in NaR and NMN but in NR as well. On the other hand, in the extracts of leaves fed with labelled nicotinamide for 5 minutes the only other substance to acquire the isotope was nicotinic acid and after ten minutes traces of radioactivity appear in nicotinic acid, trigonelline and NAD and no other intermediates of NAD metabolism become radioactive even after 30 minutes. From this it can be seen that the labelled C^{14} from nicotinic acid appears in other intermediates more rapidly than the labelled C^{14} from nicotinamide. Such

a rapid distribution of the isotope would occur if only the labelled parent compound (i.e. nicotinic acid) is more directly in the path of biosynthesis and not a breakdown product of NAD such as nicotinamide.

When immediately detached leaves were allowed to absorb isotopes of nicotinic acid or nicotinamide, the levels of radioactivity distribution (Figs 10 to 13) show that more of the label from nicotinic acid than from nicotinamide appears in the various intermediates. Similarly leaves floated on water or treated with benzimidazole accumulated more of the radioactivity in the nucleotides, after incorporation of nicotinic acid rather than after the incorporation of nicotinamide (Figs 15 and 16). Furthermore leaves fed with nicotinamide and then floated (on water or benzimidazole solution) show no traces of free nicotinic acid (Fig 17) unlike the immediately detached leaves (Figs 10 to 13) thereby indicating the rapid utilization of nicotinic acid under these conditions.

Since the degradation of NAD, as described under experimental results, is brought about by a three-step hydrolysis (the second mechanism, p.15) the presence of NMN, NR and nicotinamide in the extracts of leaves can be accounted for, irrespective of the precursor incorporated.

From these findings it is apparent that the Preiss-Handler pathway of NAD biosynthesis is operative in plants rather than the Rowen-Kornberg pathway. The point that the Preiss-Handler pathway is operative in plants generally rather than as a peculiarity of wheat (a monocotyledon) is also supported by the isolation of NaMN, on incubation of Quinolinic acid ($-C^{14}$) and PRPP with whole homogenates of castor seedlings (Hadwiger *et al*, 1963).

The minor fluctuations in intermediates instead of a steady incorporation of the isotopes into the nucleotides during the time course study (Figs 10 to 13) is indicative of a rapid turnover of the nucleotides during normal metabolism. The label accumulates to a greater extent in the nucleotides when the leaves are senescent (Figs 16 to 18). Similarly when the leaves were fed nicotinamide, its radioactivity level continued to decrease for about six hours as it is slowly incorporated(via nicotinic acid) into the nucleotides. Following this the radioactivity of nicotinamide showed an upward trend indicating a liberation of the amide from the nucleotides (Figs 10 to 13). The rapid appearance of the labelled intermediates of both the biosynthetic and degradative pathways suggests that NAD metabolism follows a cyclic pattern similar to that reported by Sarma et al (1961) in avian tissues. By such a cyclic mechanism the nicotinamide is recovered and used repeatedly without a significant loss. NAD and NADP could be rapidly synthesized as required from a pool of nicotinamide or some derivative which is perhaps less toxic to the plant than either nicotinic acid or NAD.

From the studies carried out on animals administered high doses of nicotinamide by Kaplan et al, (1956) and low doses of nicotinic acid by Langan et al (1959), a several-fold increase in the liver concentration of NAD was reported. NAD metabolism of the wheat plant appears to differ significantly in this respect from that of animal tissues and microorganisms.

In the present study with wheat leaves the incorporation of pre-

cursors does not seem to enhance the NAD level (Figs 10-13). Such a change in the nucleotide level as for example found in animal tissues, was noticeable only when the leaves were senescent (Figs 16 and 18). Hence the intracellular concentrations of the nicotinamide nucleotides must be controlled rigidly through a cyclic metabolic pathway. The proposed cyclic pathway of NAD metabolism is shown in Figure 20.

From the proposed pathway of NAD metabolism in wheat leaves (Fig 20) and the experimental results presented here, several significant features of NAD metabolism in the wheat plant are evident. They are; (a) formation and accumulation of trigonelline, (b) apparent interconversion of nicotinamide and nicotinic acid, (c) and a rigid control of the intracellular levels of the nicotinamide nucleotides.

On feeding the wheat leaves with nicotinamide or nicotinic acid one of the first products formed is trigonelline (Compound IV, N-methyl nicotinate)(Fig 14). Trigonelline appears after 5 and 10 minutes successively after the incorporation of the precursors nicotinic acid and nicotinamide respectively. Trigonelline continues to accumulate (almost 50% of the label) and remains at a high level of radioactivity (Figs 10 to 13) with minor fluctuations. It arises possibly through the mediation of a methyl donor such as methionine. Since nicotinic acid is highly toxic to leaves (Figs 6 to 8), it is presumably detoxified by methylation in the leaves to produce the betaine-trigonelline, in an analogous reaction to the excretion of methyl nicotinamide by animals after ingestion of nicotinamide in excess of the body requirements as reported by Ellinger (1948) according to Goodwin (1963).

Joshi and Handler (1960) demonstrated <u>in vivo</u> methylation of nicotinic acid by a methyl pherase (S-adenosyl methionine: nicotinate N-methyl transferase, E.C. No.2.1.1.b) from pea seedling extracts in the presence of methionine and ATP. Although this interconversion of nicotinic acid and trigonelline have been suggested and the simultaneous disappearance of trigonelline and an increase in the nicotinamide nucleotide content shown earlier (Burkholder,1943; Blake, 1954; Handler, 1958) the enzyme was isolated and shown to be substrate specific for nicotinic acid, in 1960, by Joshi and Handler.

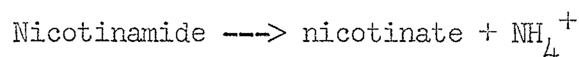
The source of free nicotinamide in animals, is by the degradation of NAD either through NMN (---> NR ---> nicotinamide) as described by Kornberg (1950) and Rowen and Kornberg (1951) or through the direct liberation of nicotinamide by NAD-glycohydrolase type reaction demonstrated by Handler (1954).

In the present investigation degradation of NAD was shown to proceed <u>in vitro</u> through NMN ---> NR ---> nicotinamide and NAD-glycohydrolase activity was not detected in wheat leaves. Yet radioactive nicotinamide appears in the aqueous extracts of leaves fed with nicotinic acid-7-C^{14} for 5 minutes (Fig 14), simultaneously with trigonelline and NAD but no other intermediates of the synthetic or degradative pathway (NaR, NaMN, NaAD or NMN and NR) show any labelling at this stage (Fig 14). The three possible explanations that exist for the origin of nicotinamide are discussed below.

(i) Nicotinamide could have arisen through the NAD-glycohydrolase activity on NAD but all attempts at demonstration of this

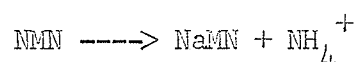
activity in the leaves have been unsuccessful.

(ii) Amido-hydrolases capable of deamidating nicotinamide and NMN to yield nicotinic acid and NaMN respectively according to the following reaction:



Nicotinamide ---> nicotinate + NH_4^+

or



NMN ----> NaMN + NH_4^+

are known to occur in animal tissues and micororganisms (Sarma et al, 1961; Potrack et al, 1963) although the reverse reaction was not reported so far. It is possible that the same enzyme responsible for the deamidating of nicotinamide also mediates the amidation of nicotinic acid, in a reverse reaction, or

(iii) nicotinamide might arise through the second mechanism of NAD degradation (p.15) coupled with rapid recycling as, for example, in animal tissues the nicotinamide nucleotides turn over at least 3 or 4 times a day (Kaplan 1960,1961). Since the simultaneous appearance of the label (Fig 14) in nicotinamide, trigonelline and NAD together with the known mechanism of degradation of NAD by wheat leaf extracts *in vitro*, a rapid cyclic metabolism appears to be the most suitable explanation. Furthermore, as suggested by Kaplan (1960) (see also 1961), the half life of nicotinamide nucleotides is, possibly, much shorter than hitherto suspected.

As explained under Experimental Results, irrespective of the labelled precursor incorporated, the radioactivity appears rapidly in all the possible intermediates of NAD metabolism including NADP (Figs 10 to 13). Even after 12 hours of incorporation of the precursor, none of these compounds (except trigonelline) show a steady accumulation but minor fluctuations in the level of radioactivity (c.f. the reports for animal tissues, Kaplan et al (1956) and Langan et al (1959)). Considerable changes in the levels of radioactivity of NAD and NADP appear only in the senescent leaves (Figs 15 to 18). A cyclic metabolic pathway alone, with trigonelline serving as a reserve pool, would best explain these results.

In conclusion, NAD biosynthesis in the wheat plant is shown to proceed via the Preiss-Handler pathway. The efficacy of nicotinic acid in accelerating senescence (similar to NAD) in detached Elodea leaves is suggestive of the same pathway being operative in Elodea plant as well. Perhaps this pathway of NAD biosynthesis is of wider occurrence than it is known to be. The intracellular levels of the nicotinamide nucleotides are rigidly controlled in the wheat plant through a cyclic pathway of NAD metabolism. Under senescent conditions the pathway being somehow impaired, NAD tends to accumulate. Benzimidazole brings a shift of equilibria to favour the accumulation of NADP instead of NAD. How this is brought about is not clear at this time.

SUMMARY

SUMMARY

1. The acceleration of senescence (chlorosis) by NAD in detached *Elodea* leaves floated on water was investigated in the present study together with the influence of benzimidazole on this accelerated senescence and also on NAD metabolism in detached leaves.

2. It was shown that NAD consistently accelerated chlorosis and that chlorosis of leaves was sporadic when treated with NADP.

3. The two precursors of NAD biosynthesis, namely nicotinamide and nicotinic acid, were tested in the floating medium. Of the two precursors, nicotinic acid alone accelerated the senescence while nicotinamide was without effect.

4. Nicotinic acid was more efficient than NAD in the acceleration of chlorosis of the detached leaves.

5. Acidity of the solutions of nicotinic acid or NAD was eliminated as the cause for the acceleration of chlorosis by:

   i) the appearance of chlorosis in leaves treated with neutralized solutions,

   ii) the absence of accelerated chlorosis in leaves treated with dilute acid solution and,

   iii) the absence of chlorosis in leaves treated with non-neutralized solutions and maintained for long periods in darkness.

6. Evidence was presented to show that the rapid degradation of chlorophyll in detached leaves treated with nicotinic acid or NAD takes place only if the leaves were illuminated. Chlorosis was not evident in leaves maintained in darkness.

7. Benzimidazole was shown to exhibit antagonistic properties towards NAD and overcome the accelerated chlorosis of detached leaves. This "benzimidazole effect" was also demonstrated in leaves treated with nicotinic acid and benzimidazole simultaneously.

8. The mutually antagonistic effects of benzimidazole on one hand and nicotinamide derivatives on the other, in accelerating the senescence of detached leaves led to a study of the pathway of NAD metabolism in leaves and the effect of benzimidazole on the metabolism of NAD. Accordingly wheat leaves were fed with radioactive isotopes of nicotinamide (carbonyl-C^{14}) and nicotinic acid (carboxyl-C^{14}), for 5 minutes to 12 hours.

9. Biosynthesis of NAD was shown to proceed from nicotinic acid ---> NaR ---> NaMN ---> NaAD ---> NAD ---> NADP, i.e., by the Priess-Handler pathway of NAD biosynthesis.

10. The degradation was shown to begin with a hydrolysis of the pyrophosphate bond of NAD to produce NMN and AMP and the NMN degraded further, by successive hydrolytic scission of the phosphate (to produce NR) followed by the cleavage of ribose to liberate nicotinamide. The nicotinamide would then undergo deamidation to produce nicotinic acid.

11. The metabolism of NAD was shown to follow a cyclic pattern very rapidly. The fast turnover of the nucleotides resulted in only minor fluctuations in the levels of various intermediates with only

a slight accumulation of radioactivity in the intermediates of NAD metabolic pathway.

12. Nicotinic acid was found to be detoxified by methylation to N-methyl nicotinate (trigonelline) and accumulated. From this reserve pool the precursor (nicotinic acid) can be liberated as required by a removal of the methyl group.

13. Senescent leaves conformed with immediately detached leaves in the general pattern of NAD metabolism, although leaves floated on water showed a tendency to accumulate more of the radioactivity from the precursor in NAD. Benzimidazole treatment of leaves, on the other hand, shifted the equilibrium in favour of NADP accumulation.

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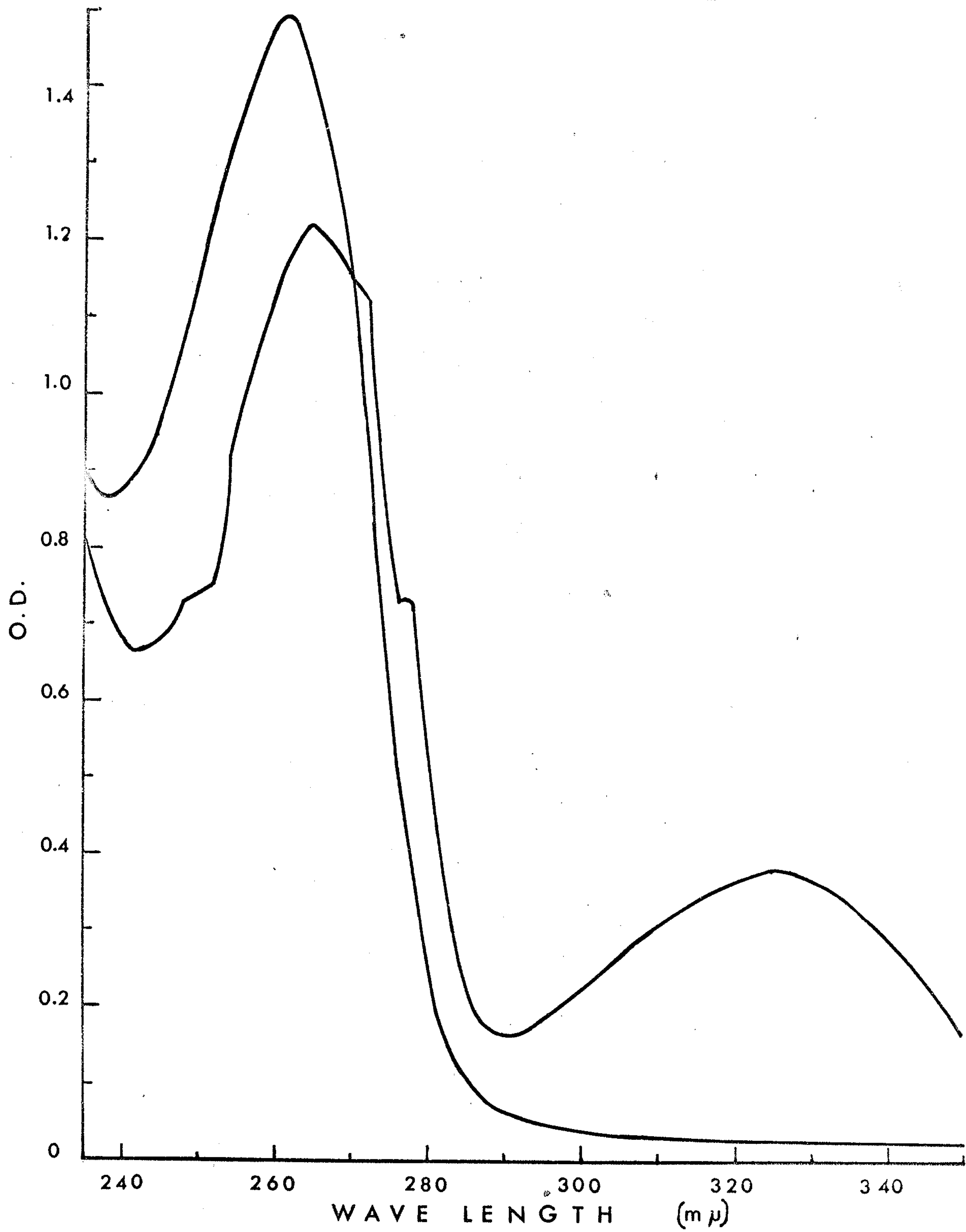
APPENDICES

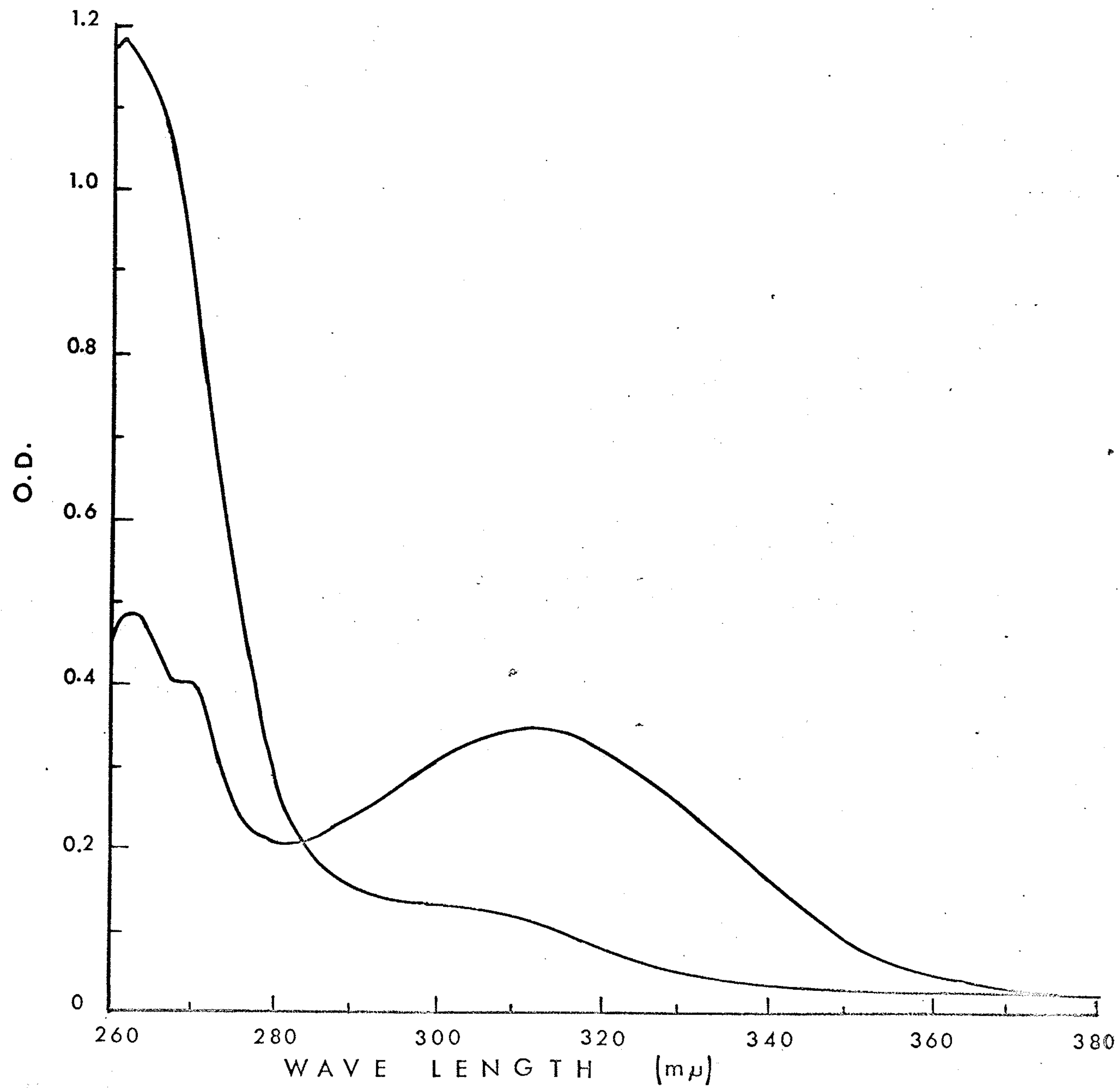
APPENDIX 1a

SPECTRUM OF NICOTINAMIDE RIBOSIDE



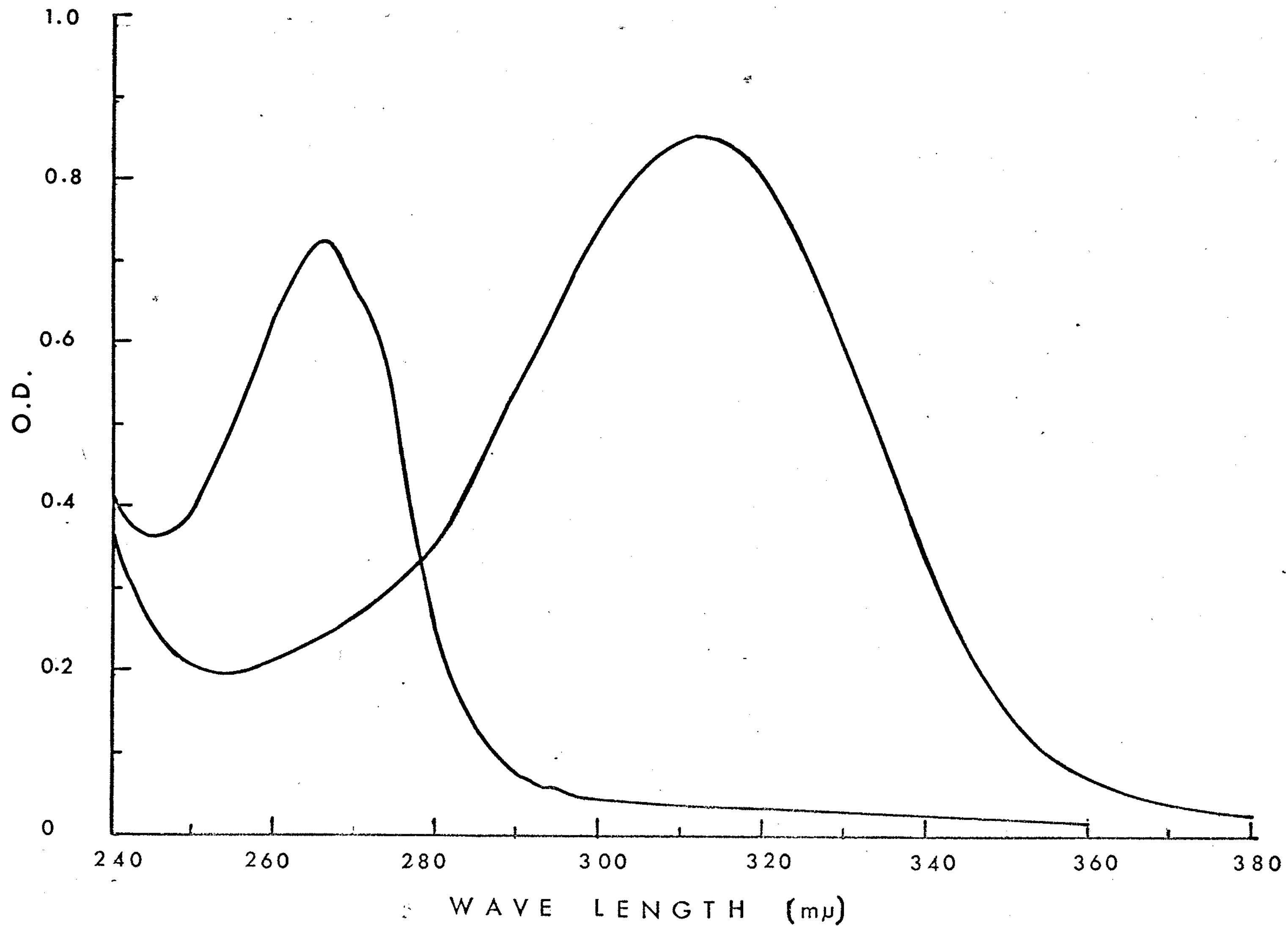
APPENDIX 1b

SPECTRUM OF NICOTINIC ACID RIBOSIDE



APPENDIX 1c

SPECTRUM OF NICOTINIC ACID MONONUCLEOTIDE



APPENDIX 2

NICOTINAMIDE-C^{14} INCORPORATION AND FLUCTUATIONS IN THE TOTAL RADIOACTIVITY OF VARIOUS INTERMEDIATES OF NAD METABOLISM IN A TIME COURSE STUDY

Time (hr)	1	2	3	4	5	6	8	10	12
Fr.wt.Leaves(g)	1.772	1.70	1.88	1.795	1.80	1.79	1.80	1.772	1.805
Nicotinamide*	214.5	497.6	313.6	360.5	388.1	405.0	404.8	36.8	473.7
Nicotinic acid	195.3	446.9	195.2	54.4	54.9	54.0	43.8	31.2	43.0
NR	26.0	91.9	84.8	48.4	54.2	42.6	41.5	43.6	43.2
Trigonelline	721.6	1,320.9	1,784.8	2,038.4	2,079.4	2,162.8	1,989.8	1,816.9	2,032.7
NaR	6.4	19.2	14.2	14.0	13.0	17.9	18.5	13.5	19.2
NMN	6.3	25.0	13.7	14.2	13.8	13.5	19.8	19.4	29.8
NAD	19.6	79.4	76.0	29.9	31.7	38.3	67.6	66.3	43.5
NaAD+NADP	20.4	43.7	38.6	17.7	20.2	26.7	38.1	43.5	24.8

* For this and the following, the total radioactivity is given as disintegrations per minute x 10^3.

APPENDIX 2a

NICOTINIC ACID-C^{14} INCORPORATION AND FLUCTUATIONS IN THE TOTAL RADIOACTIVITY OF VARIOUS INTERMEDIATES OF NAD METABOLISM IN A TIME COURSE STUDY

Time (hr)	1	2	3	4	5	6	8	10	12
Fr.wt.Leaves (g)	1.765	1.650	1.71	1.870	1.71	1.80	1.782	1.685	1.995
Nicotinamide*	192.5	328.7	256.2	304.4	380.9	382.9	482.1	501.5	521.5
Nicotinic acid	1,330.9	521.0	380.3	161.2	102.9	120.8	86.0	90.7	72.6
NR	95.1	127.1	191.5	132.4	91.7	142.7	125.1	71.8	77.7
Trigonelline	887.9	1,735.0	1,616.7	1,987.9	2,166.1	1,979.8	1,963.7	1,829.9	1,891.5
NaR	24.2	25.8	25.2	30.1	37.0	36.6	38.0	68.6	60.7
NMN	19.2	25.2	14.1	19.3	20.1	19.9	19.9	25.0	36.0
NAD	37.5	48.6	36.6	32.4	37.5	54.5	43.1	37.5	49.5
NaAD+NADP	43.1	26.2	22.8	17.9	25.1	31.4	18.4	25.7	30.7

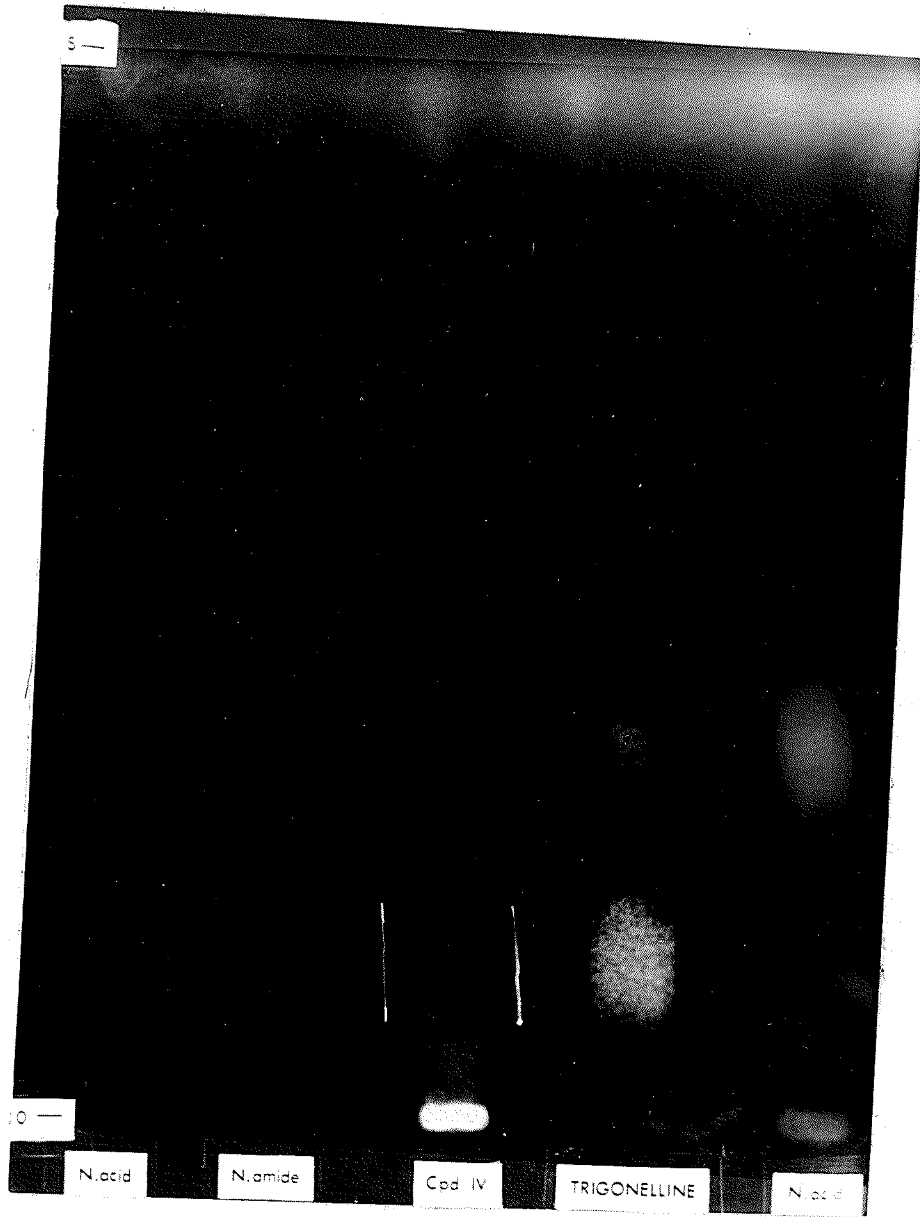
* For this and the following, the total radioactivity is given as disintegrations per minute x 10^3

APPENDIX 3.

Cochromatography of compound IV and trigonelline was carried out by ascending technique on Whatman No.1. filter paper with the following solvent systems:

1. The upper phase of glacial acetic acid:water: n-butanol (250:60:250).
2. 70% Ethanol + 30% ammonium acetate, adjusted to pH 5.0 with HCl.
3. n-Butanol saturated with 15% ammonium hydroxide.
4. Isopropanol 80% made 2N with respect to HCl.
5. 95% Ethanol + concentrated ammonium hydroxide (95:5).
6. Upper phase of n-butanol:acetone:water (45:5:50).

Direct prints of the chromatograms were prepared by a brief exposure to UV light (figure facing). Identical Rf values were obtained in six solvent systems with both compound IV and trigonelline, the former isolated from extracts of leaves fed with labelled precursors and the later synthesized from nicotinic acid by chemical means.



5 —
0 —
N.acid
N.amide
Cpd IV
TRIGONELLINE