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RESPONSES OF NICOTINAMIDE NUCLEOTIDES TO CHEMICAL REGULATORS IN BEAN LEAVES

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ABSTRACT

The effects of chemical regulators on the senescence of bean leaves (Phaseolus vulgaris L. var. Brittle Wax) were studied. Discs from first trifoliate leaves were floated on water, benzimidazole, benzyladenine, kinetin, methionine, ethionine, nicotinic acid, NAD or NADP either under continuous illumination or in darkness.

Under both conditions, methionine had no effect on senescence, however, ethionine, an antagonist of methionine, stimulated senescence. Benzyladenine, kinetin, nicotinic acid, NAD or NADP showed chlorotic effect on this tissue under either condition. In darkness, benzimidazole retarded senescence and overcame the chlorotic effect of nicotinic acid and NAD, but not the chlorotic effect of benzyladenine. On the other hand, benzimidazole had no effect under continuous illumination.

The synthesis and breakdown of NAD, using carboxyl-^{14}C labelled nicotinic acid as precursor, in bean leaves followed the Preiss-Handler pathway as in wheat leaves. Trigonelline, a methylated product of nicotinic acid, incorporated 60 to 80% of the radioactivity administered. The effects of chemical regulators on NAD metabolism was also studied. It was found that benzimidazole and kinetin decreased the NAD/NADP ratio, whereas benzyladenine and ethionine increased it. Benzimidazole was also found to decrease trigonelline formation, when fed with labelled nicotinic acid.

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LIST OF ABBREVIATIONS

ADP -- Adenosine diphosphate

ATP -- Adenosine triphosphate

BZA -- Benzyladenine

BZM -- Benzimidazole

ETH -- Ethionine

HCl -- Hydrochloric acid

KIN -- Kinetin

METH -- Methionine

NA -- Nicotinic acid

NaAD -- Nicotinic acid adenine dinucleotide

NAD -- Nicotinamide-adenine-dinucleotide

NADP -- Nicotinamide-adenine-dinucleotide phosphate

NaMN -- Nicotinic acid mononucleotide

NaOH -- Sodium hydroxide

NaR -- Nicotinic acid riboside

NMN -- Nicotinamide mononucleotide

NR -- Nicotinamide riboside

PRPP -- 5-phosphoribosyl-1-pyrophosphate

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INTRODUCTION

Studies in this laboratory, by Godavari (1966) and Waygood *et al.* (1968) using carboxyl-^{14}C labelled nicotinic acid and carbonyl-^{14}C labelled nicotinamide as precursors, have shown that the synthesis and breakdown of NAD in wheat leaves follows a pathway similar to that demonstrated in human erythrocytes by Preiss and Handler (1957). The main difference is that trigonelline (N-methylnicotinic acid), which is known to be a product of nicotinic acid metabolism in plants (Goodwin, 1963), incorporates 60-80% of the radioactivity administered.

Following the studies of Yoshida (1961), it has been shown that NAD accelerates the senescence of detached leaves of *Elodea* floated on aqueous medium. However, nicotinic acid was discovered to be a much more potent bleaching agent of the chloroplasts of *Elodea* than NAD while nicotinamide and trigonelline were non-toxic (Waygood *et al.*, 1968).

The effect of nicotinic acid and NAD could be overcome by kinins, such as benzimidazole and kinetin. These former studies were concerned with monocotyledonous plants only and the purpose of this investigation was to determine whether the synthesis and breakdown of NAD followed a similar pathway in dicotyledonous leaves, for example, bean leaves (*Phaseolus vulgaris* L.). Accordingly, experiments similar to

those of Godavari (1966) and Waygood et al. (1968) were conducted using carboxyl-^{14}C labelled nicotinic acid as a precursor of NAD synthesis. Another precursor of NAD (^{14}C nicotinamide) was not used since the same pattern of intermediates emerged in wheat leaf extracts when either nicotinic acid or nicotinamide was used as a precursor of NAD synthesis.

In other experiments nicotinic acid and NAD were tested on bean leaf discs floating on aqueous media to determine whether they caused chlorosis similar to that in wheat leaves. In addition, several kinins, e.g. benzimidazole, kinetin and benzyladenine, were tested to determine their effect on the senescence of bean leaf discs or on overcoming the possible accelerating effects of certain compounds on senescence.

Since S-adenosylmethionine is implicated in the methylation of nicotinic acid to trigonelline (Joshi and Handler, 1960), both methionine and its antagonist, ethionine, were tested to determine their effect on (a) the retardation or acceleration of senescence in bean leaf discs and (b) the distribution of ^{14}C in the intermediates of the biosynthesis and breakdown of NAD using carboxyl-^{14}C labelled nicotinic acid.

The experiments on senescence are described in Section I and those on the incorporation of ^{14}C nicotinic acid in Section II of this thesis.

LITERATURE REVIEW

It is well known that aging in leaves is characterized by a decrease in chlorophyll content and an accompanying loss of protein and ribonucleic acid. These symptoms of senescence occur at an accelerated rate in excised mature leaves and in leaf discs floated on water (Osborne, 1962).

During the last decade, several chemical substances (kinins) have been shown to retard the senescence in leaf blade. In 1957, Person et al. demonstrated that both chlorophyll degradation and protein loss, in detached wheat leaves, were retarded by floating them on a solution containing 50 p.p.m. benzimidazole. In the same year, Richmond and Lang (1957) showed that a similar effect could be obtained if excised leaves of Xanthium were maintained with their petioles in 5 p.p.m. of kinetin solution. Mothes and Engelbrecht (1959) sprayed kinetin on some limited areas of leaves of Nicotiana and reported that the retention of chlorophyll was localized in the areas to which kinetin was applied. Recently, Leopold and Kawase (1964) found that the application of benzyladenine to one or more leaves of bean seedlings would retard the senescence of the treated leaves, but induced the senescence of untreated leaves on the same seedlings.

In a study of the influence of the nucleus on the metabolism of plasmolysed Elodea leaf cells, Yoshida (1961) showed that the chloroplasts of nucleated protoplasts under-

went rapid senescence, whereas those of enucleated protoplasts remained green and were photosynthetically active for a long period of time. The addition of NAD to the medium caused a rapid senescence of the chloroplasts of enucleated protoplasts, but NADP had no effect. It has been suggested by Waygood et al. (1968) that the maintenance of the green color in the chloroplasts of enucleated protoplasts is analogous to the effect of benzimidazole, as also may be the effect of kinetin and benzyladenine (Person et al., 1957; Richmond and Lang, 1957; Leopold and Kawas, 1964). Yoshida (1961) implied that the effect of the nucleus on senescence was due to NAD, since NAD is synthesized by the nucleus (Brachet, 1954). Siebert and Humphrey (1965) confirmed that the nucleus is a site of NAD synthesis.

Recently, Godavari (1966) and Waygood et al. (1968) extended these studies in this laboratory and demonstrated that NAD accelerated senescence in unplasmolysed Elodea and wheat leaves, but NADP was not as effective as NAD. In addition, they showed that nicotinic acid, a precursor of NAD synthesis, was considerably more effective as a bleaching agent than NAD. These effects of NAD and nicotinic acid appeared only when the detached leaves were illuminated. However, under the identical conditions, benzimidazole and kinetin were capable of overcoming the accelerating effect of nicotinic acid and NAD on the chlorosis in Elodea and wheat leaves.

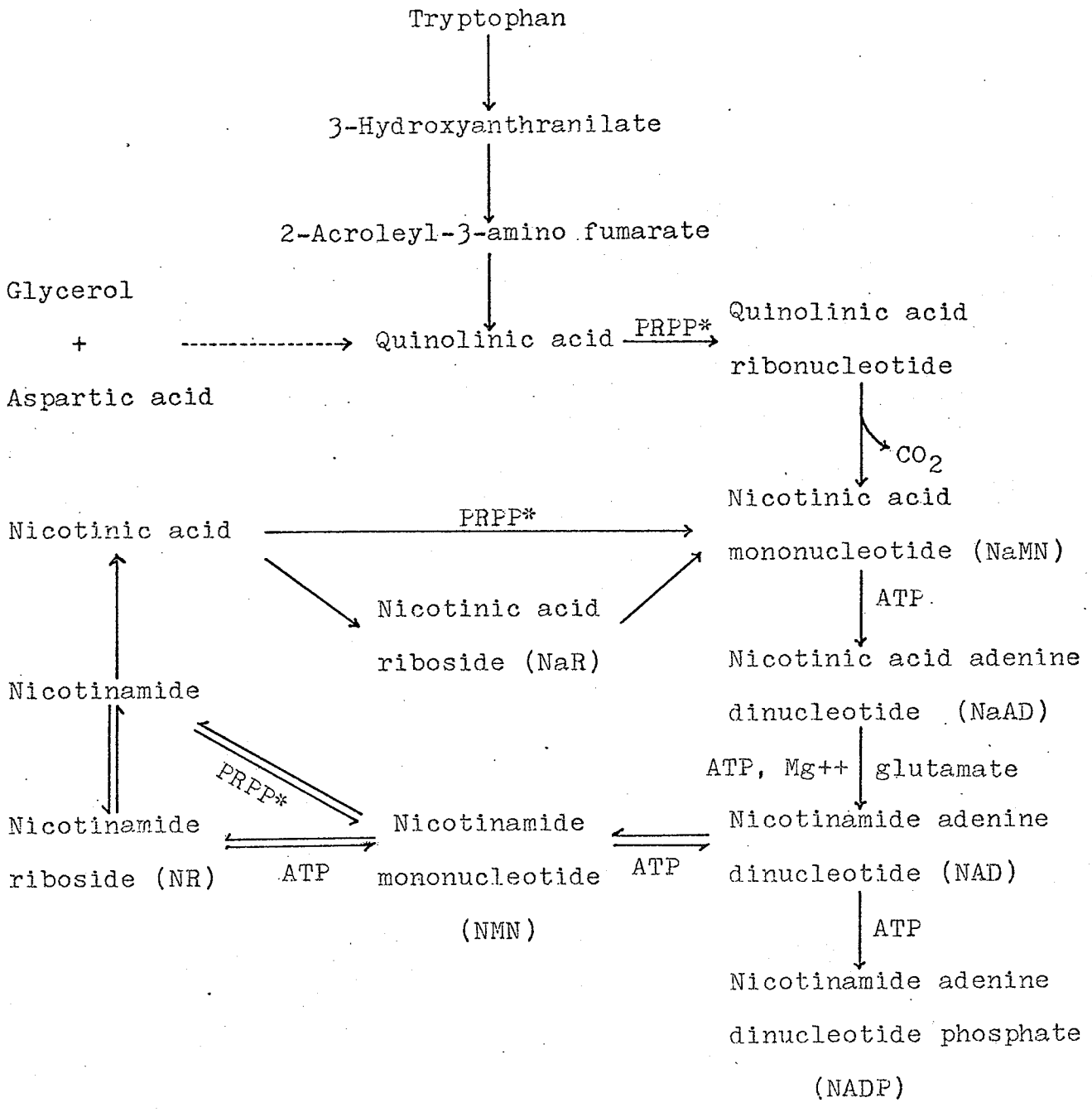
In an investigation of the relationship between nicotinamide nucleotide content and kinins in wheat in this laboratory, Mishra (1963) and Mishra and Waygood (1968) found that the total nicotinamide nucleotide content was increased following treatment with kinins. There was a diurnal rhythm in all treatments with an increase of NADP(H) and a decrease of NAD(H) during the photoperiod and the opposite case in darkness. However, when isolated chloroplasts were floated on water, they lost all their NADP within 6 days. On the other hand, those treated with benzimidazole or kinetin increased or at least maintained their level of NADP.

In order to investigate NAD metabolism, a brief study is described here. It is established that there are four fundamentally different pathways of NAD biosynthesis in organisms. (1) Preiss-Handler pathway: The exogenous or preformed nicotinic acid is converted into NAD via nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide (Preiss and Handler, 1957). (2) Nicotinamide pathway: The exogenous or preformed nicotinamide is converted into nicotinamide mononucleotide (Preiss, and Handler, 1957) which then becomes NAD (Kornberg, 1950a). (3) Tryptophan pathway: Quinolinic acid is synthesized from tryptophan and the quinolinic is converted to quinolinic acid ribonucleotide which enters the Preiss-Handler pathway via nicotinic acid mononucleotide and NAD is produced (Nishizuka and Hayaishi, 1963). (4) Aspartate-glycerol pathway: Quinolinic acid is synthesized from small molecules, such as aspartate and glycerol or related

substances, and it is then converted into NAD by way of nicotinic acid mononucleotide as above (Isquith and Moat, 1966). another nicotinamide nucleotide, NADP, is formed from NAD. This reaction is catalyzed by NAD kinase, ATP:NAD 2'-phosphotransferase (E.C.No. 2.7.1.23) (Kornberg, 1950b).

Kaplan (1960) has shown that the degradation of the pyridine nucleotide is the only biological source of nicotinamide, which is formed by the hydrolysis of the ribose-nicotinamide bond by NAD glycohydrolase (E.C.No. 3.2.2.5), or by cleavage of NAD and NADP by nucleotide pyrophosphatases to produce nicotinamide mononucleotide (NMN) followed by glycohydrolase activity. Sarma et al. (1961) and Joshi and Handler (1962) have also indicated that a portion of nicotinamide produced in vivo by the action of NAD glycohydrolase may be reutilized by deamidation to nicotinic acid and conversion to NAD via the Preiss-Handler pathway. Therefore, they presented a cyclic scheme for the degradation and resynthesis of NAD which is illustrated in scheme I.

In the study of NAD metabolism in wheat plant fed with carboxyl-^{14}C labelled nicotinic acid and carbonyl-^{14}C labelled nicotinamide, Godavari (1966) has shown that the synthesis of NAD in wheat leaves follows a pathway similar to that found in human erythrocytes (Preiss and Handler, 1957) and the cyclic metabolism of NAD is similar to that shown in Scheme 1.



Scheme 1: Cyclic metabolism of NAD.

*PRPP: 5-Phosphoribosyl-1-pyrophosphate

When wheat leaves (Triticum aestivum L. var. Selkirk) were floated on water or benzimidazole solution for 2 days and then fed with labelled ^{14}C nicotinic acid and nicotinamide, the pattern of incorporation of radioactivity was similar to that in immediately detached leaves except that the incorporation of the label into NADP and nicotinic acid adenine dinucleotide (NaAD) pool was visibly greater in the extracts from benzimidazole treated leaves (Godavari, 1966; Waygood et al., 1968). In subsequent experiments, three sets of detached leaves were allowed to incorporate ^{14}C nicotinamide for two hours under continuous illumination and then one set of leaves was killed and extracted immediately and the other two sets were floated on water or benzimidazole solution, respectively, under continuous illumination for two days. They showed that no significant difference was noticeable in NAD, NaAD and NADP but there was a greater accumulation of the label of the nicotinamide nucleotide in the extract of leaves floated on water and benzimidazole than in the immediately detached leaves, and that the benzimidazole treated leaves showed a greater label in NADP while leaves floated on water accumulated more label in NAD.

In the biosynthetic pathway of NAD from nicotinic acid, Joshi and Handler (1960) showed that S-adenosylmethionine was the main methyl group donor for the formation of trigonelline. Goodwin (1963) also showed that when exogenous nicotinic acid was fed into pea shoots, the endogenous levels remained constant,

but the excess exogenous nicotinic acid was converted into trigonelline. In this laboratory, Godavari (1966) and Waygood at al. (1968), using wheat plants, confirmed these results and they showed that the radioactivity incorporated into the trigonelline reached 75 to 80% of the radioactivity administered.

However, Radmer and Bogorad (1967) suggested that the methyl group occurring at the C-10 carbomethoxy group of chlorophyll was donated by S-adenosylmethionine which was formed from ATP and methionine in Zea mays. Recently, Chan et al. (1968) supported this hypothesis with respect to wheat leaves. It appears that in the formation of trigonelline there is competition with chlorophyll for the methyl group from S-adenosylmethionine.

On the other hand, Farber et al. (1964) found that ethionine was an antagonist of methionine, since ethionine became S-adenosylethionine at the expense of ATP more rapidly than S-adenosylmethionine formed from methionine in rat liver. These compounds, methionine and ethionine, were also used in this investigation, in order to determine whether or not the effect of nicotinic acid on senescence is due to its methylation into trigonelline.