

THE UNIVERSITY OF MANITOBA

RESPONSES OF NICOTINAMIDE NUCLEOTIDES TO  
CHEMICAL REGULATORS IN BEAN LEAVES

A THESIS

SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR

THE DEGREE OF MASTER OF SCIENCE

BY

SHIOW-CHYN CHEN

DEPARTMENT OF BOTANY

April 1970



## ABSTRACT

The effects of chemical regulators on the senescence of bean leaves (Phaseolus vulgaris L. var. Brittle Wax) were studied. Discs from first trifoliolate 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-<sup>14</sup>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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## ACKNOWLEDGEMENTS

The author wishes to express her sincere gratitude to Dr. E. R. Waygood for his invaluable advice and guidance during the course of this investigation.

Thanks are due to Dr. H. Lees, Head, Department of Microbiology, for the use of Liquid Scintillation Spectrometer.

Acknowledgements are also given to Mr. M. Bryan for his assistance in photographs.

Finally, the co-operation and help of the colleagues and the staff members of this department are greatly appreciated.

## INTRODUCTION

Studies in this laboratory, by Godavari (1966) and Waygood et al. (1968) using carboxyl-<sup>14</sup>C labelled nicotinic acid and carbonyl-<sup>14</sup>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- $^{14}\text{C}$  labelled nicotinic acid as a precursor of NAD synthesis. Another precursor of NAD ( $^{14}\text{C}$  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  $^{14}\text{C}$  in the intermediates of the biosynthesis and breakdown of NAD using carboxyl- $^{14}\text{C}$  labelled nicotinic acid.

The experiments on senescence are described in Section I and those on the incorporation of  $^{14}\text{C}$  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.

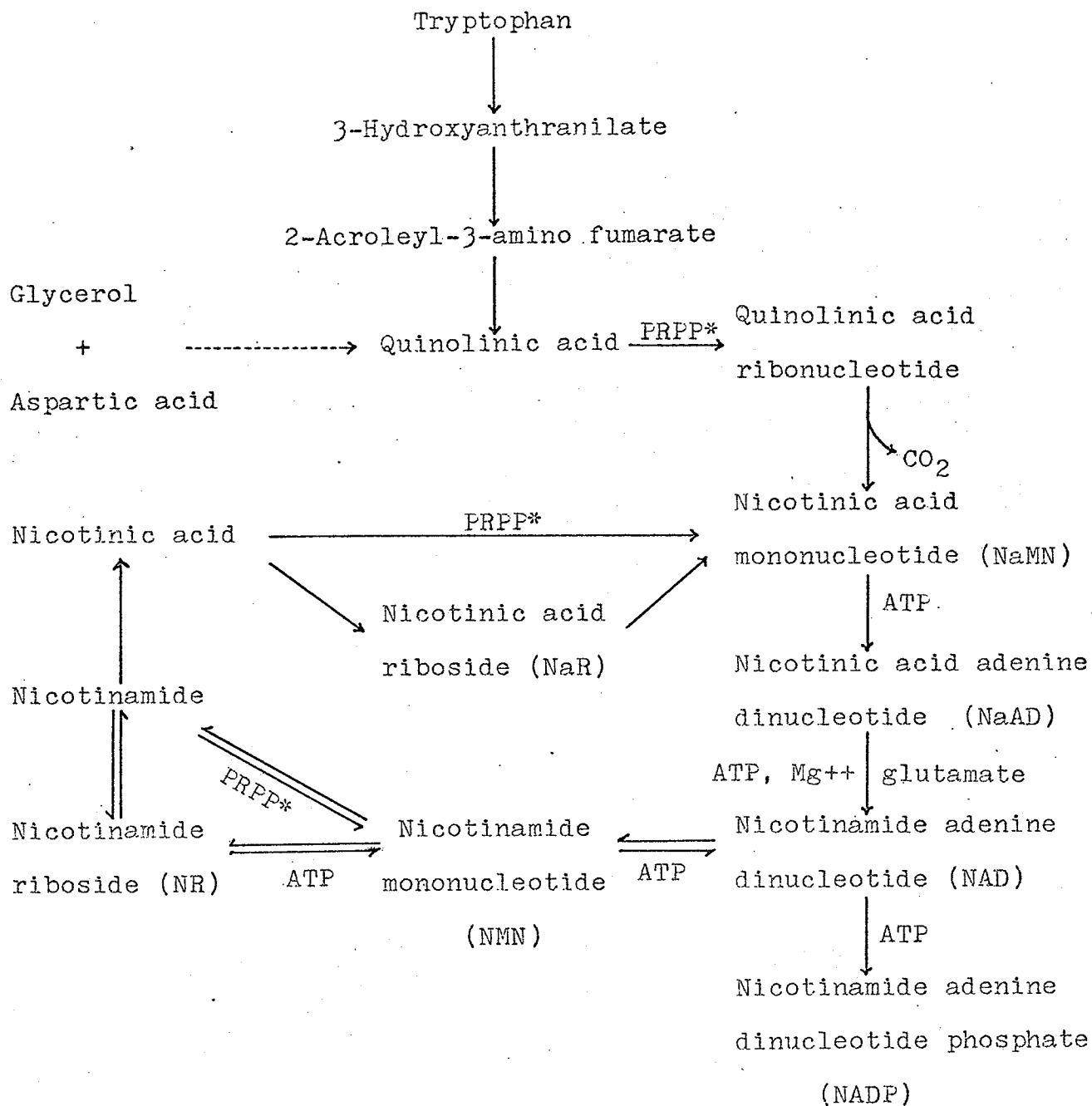
- (1) Preiss-Handler pathway: The exogenous or preformed nicotinic acid is converted into NAD via nicotinic acid mononucleotide and nicotinic acid adenine dinucleotide (Preiss and Handler, 1957).
- (2) Nicotinamide pathway: The exogenous or preformed nicotinamide is converted into nicotinamide mononucleotide (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-<sup>14</sup>C labelled nicotinic acid and carbonyl-<sup>14</sup>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 I.





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  $^{14}\text{C}$  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  $^{14}\text{C}$  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 methylgroup 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 et 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.

SECTION I

EFFECTS OF KININS

ON

THE SENESCENCE OF DETACHED BEAN LEAVES

## MATERIALS AND METHODS

Plant materials: Plants of Phaseolus vulgaris L. var. Brittle Wax were grown from seeds in vermiculite in pots in the greenhouse at various times of the year. Depending on the season, 20 to 40 day old first trifoliolate leaves were excised and washed with deionized distilled water. Generally, the fresh trifoliolate leaves weighed about 0.9g each and the middle leaflet was about 10cm in length.

Chemicals: Kinetin, benzyladenine, NAD and NADP were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.; methionine and ethionine were obtained from Calbiochem, Los Angeles, California, U.S.A.; benzimidazole and nicotinic acid were purchased from Eastman Organic Chemicals, Rochester, New York, U.S.A..

Procedures: For the experiments, bean leaf discs 1.1cm in diameter were punched from the area beside the midrib of the blade with a cork borer. Eight discs were randomized and floated with the abaxial side downwards on 10ml of water or the various solutions in 5 inch diameter petri dishes as described. The petri dishes were then placed in a glass tray, covered with Saran wrap and then placed in a growth chamber at 22°C either under continuous illumination or in darkness. The light source was a bank of 8, cool-white, fluorescent lamps (F48T 12-CW-VHO, SYLVANIA) and 4, warm-daylight, incandescent

lamps, with a total light intensity of 1500ft-c.

The various compounds dissolved in the solutions were as follows: benzimidazole (BZM), kinetin (KIN), benzyladenine (BZA), methionine (METH), ethionine (ETH), nicotinamide-adenine-dinucleotide (NAD), nicotinamide-adenine-dinucleotide-phosphate (NADP) and nicotinic acid (NA). The test solutions were all adjusted to pH 7 with the addition of 0.1N NaOH and the concentrations of the compounds in each solution are stated in the description of each experiment. After 7- or 14-days of incubation the petri dishes containing the leaf discs were examined, their appearance recorded, and then they were photographed.

## EXPERIMENTAL RESULTS

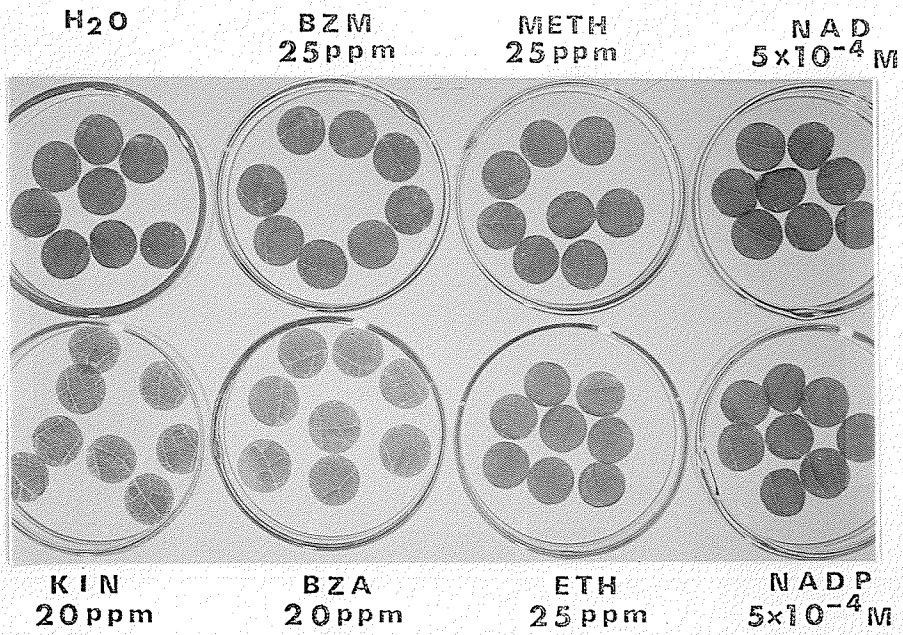
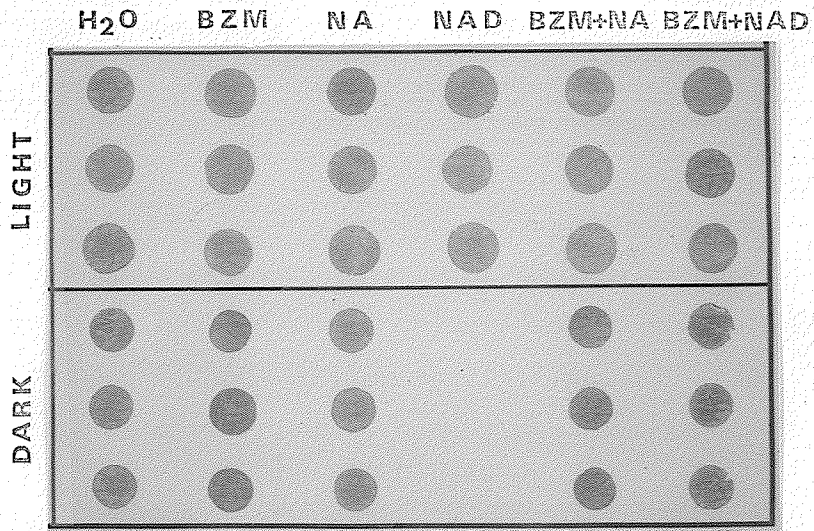
Effect of nicotinic acid, benzimidazole and NAD in light and darkness. It has been shown that benzimidazole overcomes the bleaching effect of either nicotinic acid or NAD on the chloroplasts of either detached Elodea- or wheat-leaves (Godavari, 1966; Waygood et al., 1968). It was therefore of interest to determine whether these compounds had the same effect on bean-leaf discs. Leaf discs from 20- to 40-day-old trifoliolate leaves were floated on either (1) water, (2) a 25 p.p.m. solution of benzimidazole, (3) a solution containing  $5 \times 10^{-4}M$  nicotinic acid, (4) a solution containing  $5 \times 10^{-4}M$  NAD, (5) a mixture of (2) and (3), or (6) a mixture of (2) and (4), all under either continuous illumination, or complete darkness, for 14 days. The results are shown in Fig. 1, although it is difficult to see their effects in this photograph. However, under continuous illumination, leaf discs floated on benzimidazole showed no retardation of senescence when compared with leaf discs floated on water. Furthermore, NAD induced more chlorosis than did nicotinic acid, and the effect of these two compounds was not overcome by benzimidazole as was the case with Elodea leaves (Godavari, 1966; Waygood et al., 1968).

In darkness, bean leaf discs floated on water showed less chlorosis than their counterparts in light. For those floated on benzimidazole, it appeared that senescence was retarded to a certain extent. However, with NAD treatment, leaf discs

Fig. 1. Effect of nicotinic acid, benzimidazole and NAD on bean-leaf discs under continuous illumination and darkness for 14 days.

Fig. 2. Effect of kietin, benzimidazole, methionine, ethionine, etc. on bean-leaf discs under continuous illumination for 7 days.





became flaccid and translucent and were difficult to remove from the petri dishes. This accounts for their absence from the photograph. Although nicotinic acid showed chlorosis under this condition, it was a less potent bleaching agent of bean-leaf discs than was NAD. It also appeared that benzimidazole overcame the chlorotic effect produced by NAD or nicotinic acid.

Effect of kinetin, benzimidazole, methionine, ethionine, etc.

in light and darkness. Both kinetin and benzyladenine have been shown to retard the senescence of various dicotyledonous leaves, including bean leaves (Richmond and Lang, 1957; Mothes and Engelbrecht, 1959; Leopold and Kawase, 1964). Accordingly, these compounds were tested to determine their effect on bean-leaf discs under conditions of continuous illumination. Discs were floated for 7 days on solution containing either 20 p.p.m. kinetin (KIN) or benzyladenine (BZA), 25 p.p.m. methionine (METH), ethionine (ETH) or benzimidazole (BZM), or  $5 \times 10^{-4}M$  NAD or NADP. All solutions were adjusted to pH 7.0 with 0.1N NaOH. The results are shown in Fig. 2, which indicates that discs treated with either benzimidazole, methionine, NAD, or NADP were not visibly different from those floated on water. It was also noted that NAD did not accelerate senescence as it does in Elodea leaves (Waygood et al., 1968). On the other hand, discs floated on kinetin, benzyladenine and ethionine showed some interveinal chlorosis, and the former two treatments significantly increased the area of the discs. The experiment shown in Fig. 3 was a repetition of the experiment described above

and illustrated in Fig. 2 except that the leaf discs were maintained in continuous darkness. Benzimidazole, methionine, NAD and NADP showed little or no effect. Kinetin induced a slight chlorosis, but treatment with benzyladenine caused a severe chlorotic condition while ethionine caused the leaves became translucent.

Effect of various concentrations of chemical regulators on floated bean-leaf discs maintained under continuous illumination for 7 days. The chemical 'regulators' used in the previous experiments were tested at various concentrations for 7 days under continuous illumination. A control experiment of bean-leaf discs floating on water was not used since such leaf discs showed no difference from treatment with 25 p.p.m. benzimidazole, 25 p.p.m. methionine, or either  $5 \times 10^{-4}M$  NAD or NADP.

(a) Benzimidazole and methionine. Fig. 4 clearly indicates that varying the concentration of either of these two substances from 10 to 50 p.p.m. did not induce any visible differences in either the acceleration or the retardation of senescence of bean leaf discs.

(b) Benzyladenine and kinetin. In comparison with bean leaf discs floated on water (Figs. 1 and 2), interveinal chlorosis was evident at a concentration of 5 p.p.m. with both test chemicals and chlorosis increased with increasing concentration of the chemicals, up to 25 p.p.m. (Fig. 5). As in the previous experiments the leaf discs increased in area.

Fig. 3. Effect of kinetin, benzimidazole, methionine, ethionine, etc. on bean-leaf discs in darkness for 7 days.

Fig. 4. Effect of various concentrations of benzimidazole and methionine on bean-leaf discs under continuous illumination for 7 days.

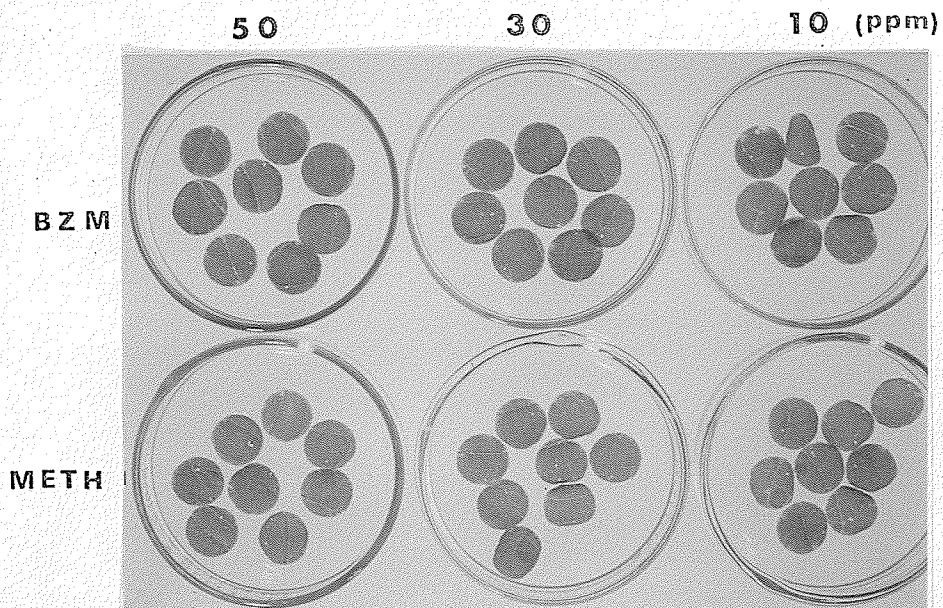
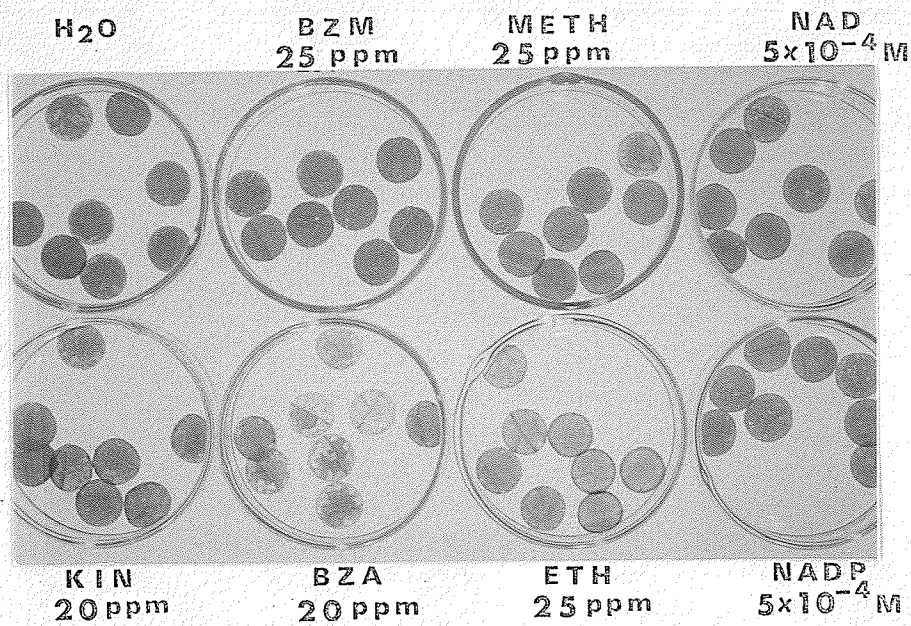
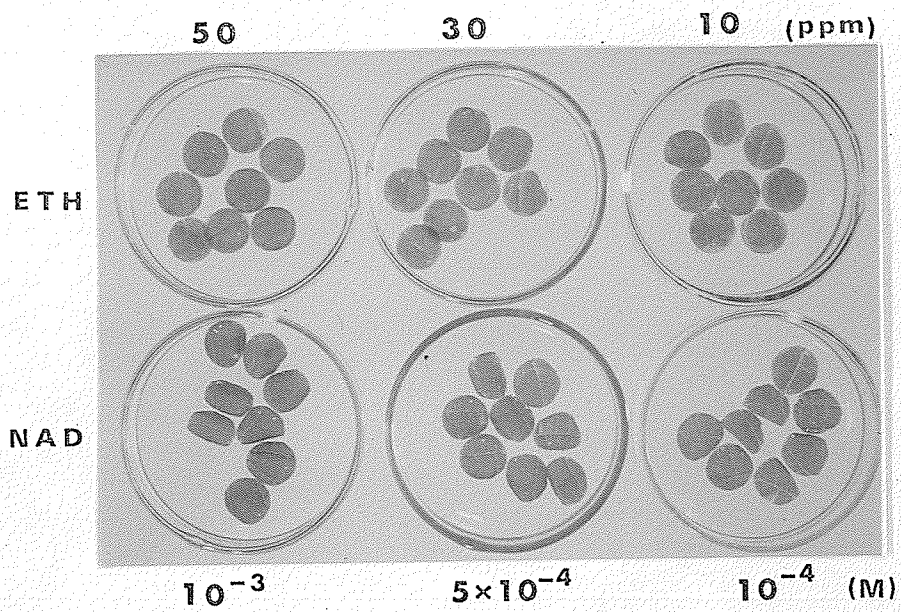
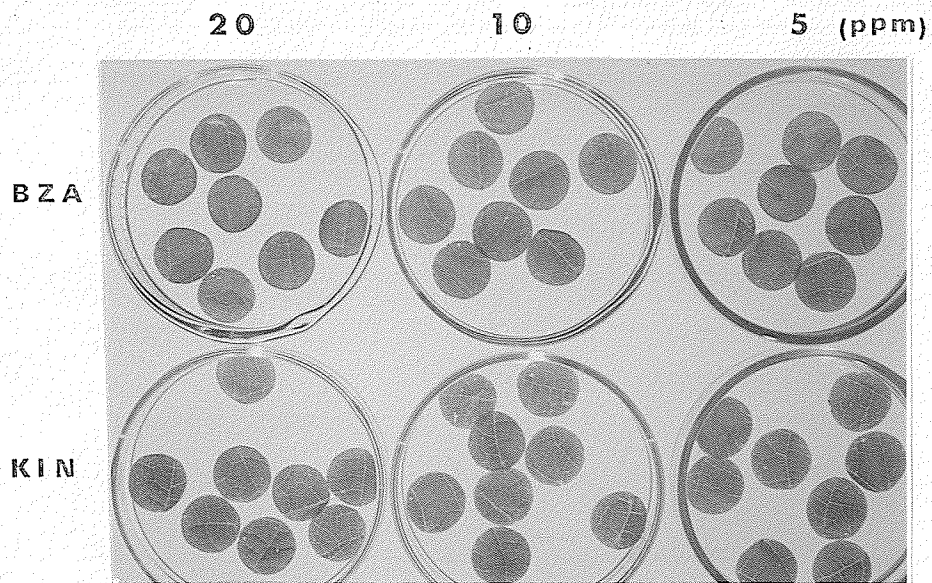


Fig. 5. Effect of various concentrations of benzyladenine and kinetin on bean-leaf discs under continuous illumination for 7 days.

Fig. 6 Effect of various concentrations of ethionine and NAD on bean-leaf discs under continuous illumination for 7 days.



(c) Ethionine and NAD. Treatment of leaf discs with ethionine concentrations of from 10 to 50 p.p.m. (Fig. 6), all resulted in interveinal chlorosis similar to that obtained with 25 p.p.m. ethionine, as illustrated in Fig. 2. Leaf discs floated on NAD did not show any difference at concentrations from  $10^{-4}$  to  $10^{-3}M$ .

Effect of benzimidazole, methionine, NAD and NADP under both continuous illumination and darkness for 14 days. In the earlier experiments, in which bean-leaf discs were floated for 7 days under continuous illumination or in darkness, it was found that benzimidazole, methionine, NAD and NADP showed little or no effect (Figs. 2 and 3). Accordingly, the experiment was repeated, with two kinds of concentration of each regulator and the time of floating being extended to 14 days. There appears to be no significant difference between this and the previous experiment, except that the leaf discs treated with NAD or NADP were greener than those of the control water treatment. However, bacterial infection was found on one or two leaf discs of those treated with NAD. On the other hand, when leaf discs were floated for 14 days in darkness (Fig. 8), methionine showed no effect, but with benzimidazole treatment the leaf discs had maintained their original green colour. With NAD or NADP treatment the discs were both chlorotic and translucent.

Effect of benzimidazole on NAD treatment in darkness. Benzimidazole has been shown to overcome the accelerating effect of NAD on the senescence of Elodea leaves under continuous illumination.



Fig. 7. Effect of floating period on bean leaf discs under continuous illumination for 14 days.

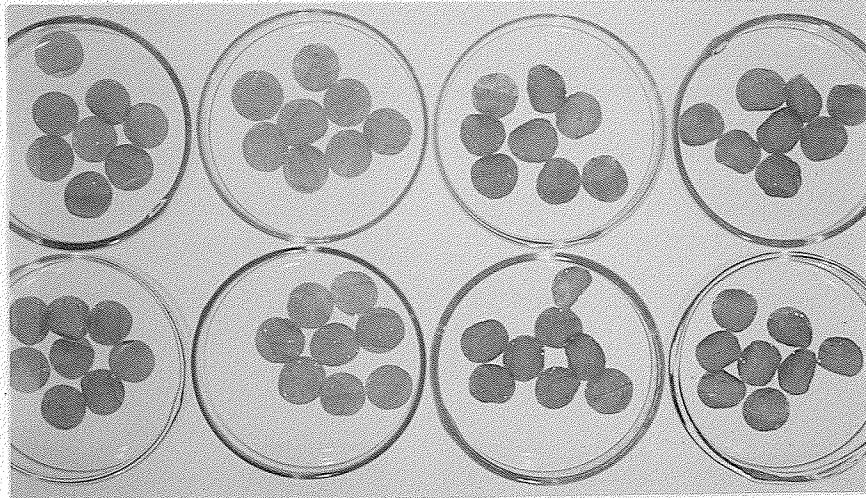
Fig. 8. Effect of floating period on bean leaf discs in darkness for 14 days.

METH  
25 ppm

BZM  
50 ppm

NAD  
 $10^{-3} M$

NADP  
 $10^{-3} M$



H<sub>2</sub>O

25 ppm

$5 \times 10^{-4} M$

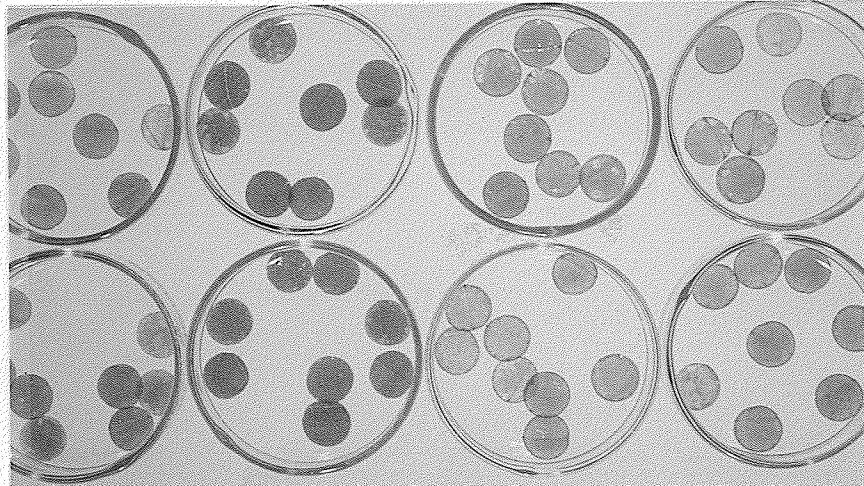
$5 \times 10^{-4} M$

METH  
25 ppm

BZM  
50 ppm

NAD  
 $10^{-3} M$

NADP  
 $10^{-3} M$



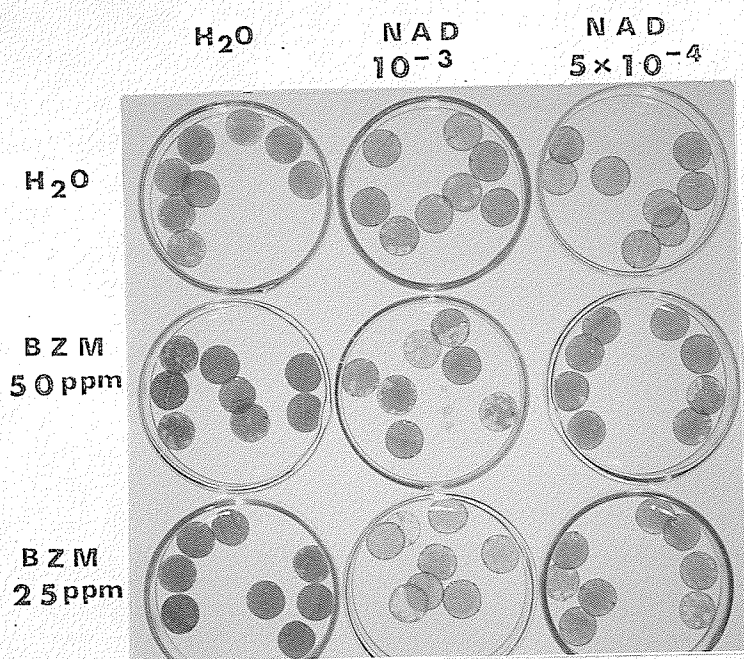
H<sub>2</sub>O

25 ppm

$5 \times 10^{-4} M$

$5 \times 10^{-4} M$

Fig. 9. Effect of benzimidazole on NAD treatment on bean leaf discs in darkness for 14 days.

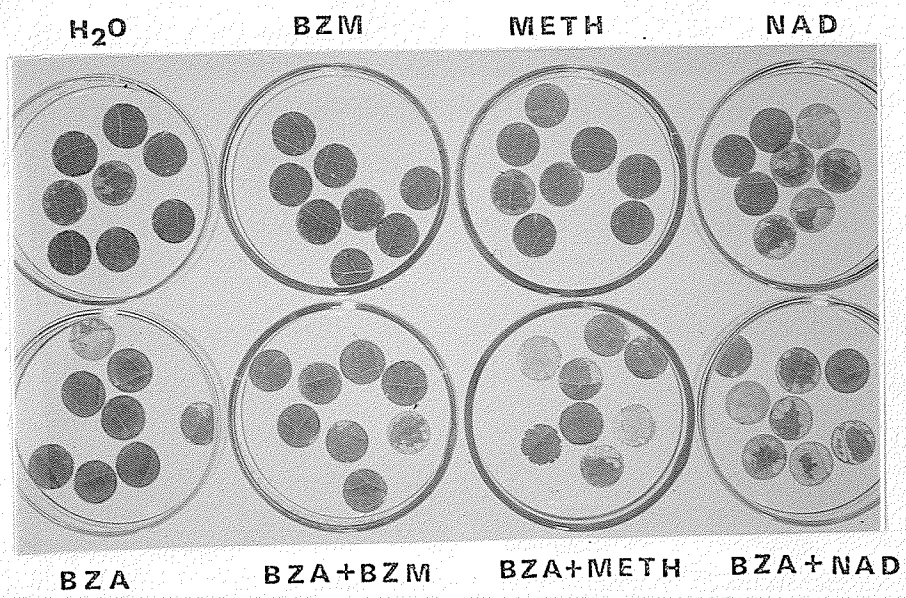
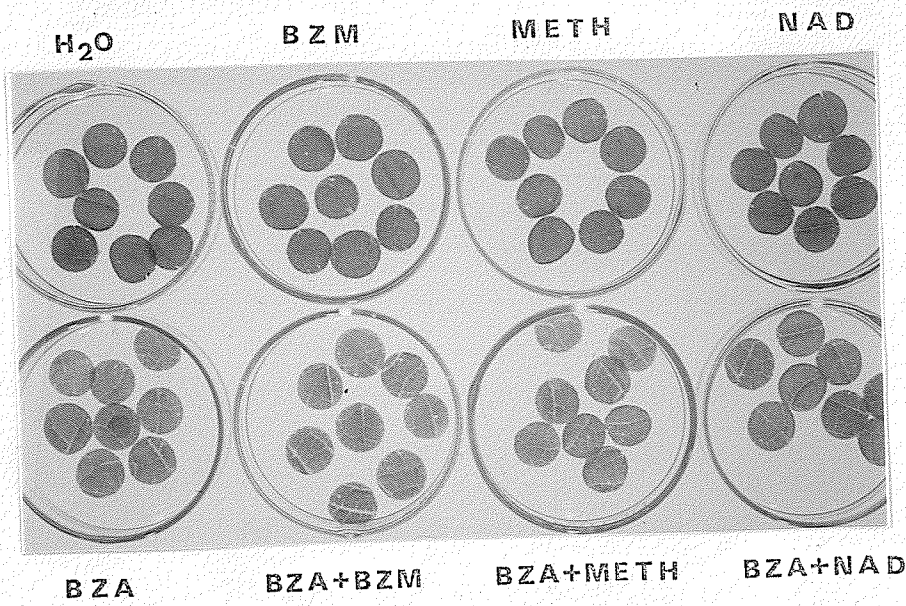


With bean-leaf discs NAD had little or no effect in light (Fig. 7), but in darkness as shown in Fig. 8, NAD produced a chlorotic and translucent condition. The results of an experiment using discs floated for 14 days in darkness (Fig. 9) showed that 50 p.p.m. of benzimidazole overcame to a certain extent the effect of  $5 \times 10^{-4}M$  of NAD. At higher concentrations of NAD or lower concentrations of benzimidazole, chlorotic and translucent condition was general.

Effect of benzyladenine on benzimidazole, methionine and NAD treatments. Since benzyladenine treatment induced chlorosis in bean-leaf discs when they were under continuous illumination or in darkness, an experiment was undertaken to determine whether benzimidazole, methionine or NAD could overcome the accelerating effect of benzyladenine or chlorosis. Leaf discs were floated for 7 days on either  $H_2O$ , solution of 25 p.p.m. benzimidazole or methionine, or  $5 \times 10^{-4}M$  NAD, all test solutions with and without 20 p.p.m. benzyladenine. Fig. 10 shows that none of these regulators overcame the effect of benzyladenine on bean-leaf discs which were under continuous illumination. Although benzimidazole maintained the green colour of the discs in darkness (Fig. 8), it was unable to overcome the chlorotic condition produced by benzyladenine in darkness (Fig. 11). Both methionine and NAD accelerated the chlorosis produced by benzyladenine in darkness.

Fig. 10. Effect of benzyladenine on benzimidazole, methionine and NAD treatments on bean-leaf discs under continuous illumination for 7 days.

Fig. 11. Effect of benzyladenine on benzimidazole, methionine and NAD treatments on bean-leaf discs in darkness for 7 days.



SECTION II

NICOTINAMIDE-ADENINE-DINUCLEOTIDE METABOLISM

IN

BEAN LEAVES



## MATERIALS AND METHODS

Plant materials: Bean plants (Phaseolus vulgaris L. var. Brittle Wax) were grown in vermiculite in the greenhouse. Trifoliolate leaves, including their petioles, were removed for test purposes.

Chemicals: Carboxyl- $^{14}\text{C}$  labelled nicotinic acid (specific activity, 27.9mc per mmole) was purchased from the Radiochemical Center, Amersham, Buckinghamshire, England.

Feeding of  $^{14}\text{C}$  nicotinic acid to leaves: The procedure followed by Wang (1959) was used in these experiments to feed leaves with radioactive nicotinic acid. The cutends of detached petioles (3cm in length) were inserted into small vials (0.4 x 2cm) containing 0.018  $\mu\text{mole}$  of the radioactive compound. These experiments were conducted in a growth chamber under continuous illumination of 400ft-c and 22° for various periods of time. Sufficient quantities of water were added to the vials to prevent desiccation of leaves.

Induction of senescence in leaves: The two methods used to induce senescence were as follows:

(a) The trifoliolate leaves were first fed with the solutions as described in Section I in a growth chamber at 22° under continuous illumination (400ft-c) for 40 hours. This was followed by feeding with radioactive nicotinic acid for 6 hours.

(b) The trifoliolate leaves after feeding with radioactive compound (6 hours) were cut into small pieces. These were then floated on 20 ml of the solutions in petri dishes (10cm in dia-

meter) as described in Section I. The petri dishes were then placed in a growth chamber either under continuous illumination or in darkness for 40 hours. The concentrations of various solutions are stated in the section of Experimental Results.

Extraction and fractionation: Leaves were cut into small sections and plunged into boiling 95% ethanol and extracted twice in 85% ethanol for 30 minutes, followed by 40% ethanol and then water. The alcoholic and water extracts were combined and evaporated to dryness. The residue was extracted with chloroform to remove chlorophylls, carotenoids and lipids. The residue was then dissolved in water and made to a volume of 5ml. Fractions were stored in the deep freeze.

Paper chromatography and radioautography: Aliquots of the 0.3ml water soluble fraction were streaked on Whatman No. 1 paper. The chromatograms were developed in the solvent (isobutyric acid :  $H_2O$  :  $NH_4OH$  = 66:33:1, v/v/v, pH 3.8) for 28 hours by ascending technique at 20 . Radioautographs were obtained by exposing the chromatograms on Kodak 'no screen' medical X-ray film for two weeks. The films were developed with Elonhydrogenon (Kodak D-19b) X-ray film developer (Hodgeman et al., 1954) for five minutes at 18° and fixed with Quick-Fix (Edwal Scientific Products Corporation, Chicago, U.S.A.).

Detection of pyridinium compounds: Pyridinium compounds were detected by the following methods:

- A. Authentic compounds were used as markers to identify the compounds.
- B. The chromatograms were exposed to the vapors of methyl ethyl ketone : ammonium (1:1 in v/v ) in a closed glass tank for about one hour. Nicotinamide derivatives fluoresce under ultraviolet light, but nicotinic acid derivatives do not fluoresce (Kodicek and Reddi, 1951).
- C. Chromatograms were sprayed with 1% picryl chloride in 95% ethanol, dried at room temperature, and exposed to ammonia fumes. After one hour, nicotinic acid and nicotinamide turned into reddish coloured spots (Dawson et al., 1962).

Measurement of radioactivity: Radioactivity was measured with Liquid Scintillation Spectrometer, Packard Instrument Company, U.S.A.. Compounds corresponding to each radioactive area or spot on the radioautograph were cut out from the developed chromatogram. The compounds were eluted from the paper with 0.2ml of 50% ethanol for 4 hours at room temperature. Ten milliliters of dioxane scintillation fluid\* was added and the samples were counted for 5 minutes. All counts were corrected for background. The radioactivity of 0.3ml aliquots of the water soluble fractions was also determined at the same time.

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\*Scintillation liquid contained 100g of naphthanene, 7g of 2,5 diphenyloxazole (PFO) and 0.3g of 1,4 Bis (2,5) phenyloxazolyl benzene (POPOP) in 1000ml of P-dioxane.

## EXPERIMENTAL RESULTS

### Feeding $^{14}\text{C}$ nicotinic acid to immediately detached bean leaves.

Labelled nicotinic acid ( $0.018\mu\text{mole}$ ) was fed immediately to freshly detached trifoliolate-bean leaves for periods of from 2 to 48 hours. An 0.3ml aliquot of the water soluble fraction of the extracted leaves was counted and another 0.3ml aliquot was chromatographed and radioautographs were prepared as described in Methods and the results are shown in Fig. 12. The compounds were identified by markers and also by comparison with the radioautographs of Godavari (1966) and Waygood *et al.* (1968). It is clear that the label in nicotinic acid is incorporated into nicotinamide, trigonelline, NAD, and the intermediates of NAD synthesis and breakdown within 2 hours. After 6 hours the pool of radioactive nicotinic acid is virtually depleted. Quantitative measurements of the radioactivity in each compound are shown in table I and some are graphically illustrated in Fig. 13. As with wheat leaves the radioactivity in trigonelline increases from 0 to almost 80% of the total incorporated within 6 hours, the same time period within which the nicotinic acid pool is virtually depleted.

A short time course study (Fig. 14) shows that nicotinic acid is rapidly metabolized by bean leaves with its radioactivity appearing in most of the intermediates of NAD synthesis and breakdown within 10 minutes of feeding. Trigonelline is labelled within 5 minutes of feeding. It appears therefore that the metabolic pathways of NAD biosynthesis and breakdown in bean

Fig. 12. Radioautographs of the intermediates of NAD metabolism after incorporation of  $^{14}\text{C}$  nicotinic acid in a time course study from 2 to 48 hours.

NAm : Nicotinamide  
NA : Nicotinic acid  
NR : Nicotinamide riboside  
TRIG : Trigonelline  
NMN : Nicotinamide mononucleotide  
NAD : Nicotinamide adenine dinucleotide  
NaAD : Nicotinic acid adenine dinucleotide  
NADP : Nicotinamide adenine dinucleotide phosphate



TABLE I

The percentage of various intermediates of NAD metabolism  
in a time course study of  $^{14}\text{C}$  nicotinic acid incorporation.

Time (hours)	2	4	6	8	10	12	24	48
NAm	5.40	13.24	7.38	11.41	11.70	15.46	11.42	3.18
NA	42.70	12.25	2.76	3.95	3.24	1.84	2.33	1.29
NR	4.00	3.81	5.13	4.27	2.99	4.64	2.64	1.02
TRIG	40.53	61.11	77.09	68.59	73.83	70.38	75.80	84.86
NaR	0.80	1.02	1.32	1.10	1.00	1.14	0.91	0.60
NMN	0.72	0.64	1.01	0.81	0.80	0.81	0.44	1.50
NAD	2.61	4.11	2.91	2.79	3.09	2.46	3.62	1.00
NaMN	0.40	0.36	0.42	0.50	0.44	0.57	0.38	0.44
NaAD	0.57	0.56	0.58	0.39	0.40	0.50	0.38	0.37
NADP	0.41	0.43	0.42	0.45	0.56	0.56	0.38	0.39
Original Streak	0.31	0.34	0.26	0.29	0.28	0.31	0.28	0.17
Total Percentage	98.45	97.87	99.28	94.55	96.43	98.67	98.58	94.82
Total Counts*	346,319	389,243	345,519	277,886	312,975	255,340	402,373	357,650

\*These values were obtained from an aliquot of 0.3ml of water soluble fraction, which was counted for 5 minutes.

Fig. 13. Fluctuations in the levels of radioactivity of some intermediates in NAD metabolism up to 48 hours after incorporation of  $^{14}\text{C}$  nicotinic acid.

NA : Nicotinic acid

NAm : Nicotinamide

NR : Nicotinamide riboside

TRIG : Trigonelline

NAD : Nicotinamide-adenine-dinucleotide



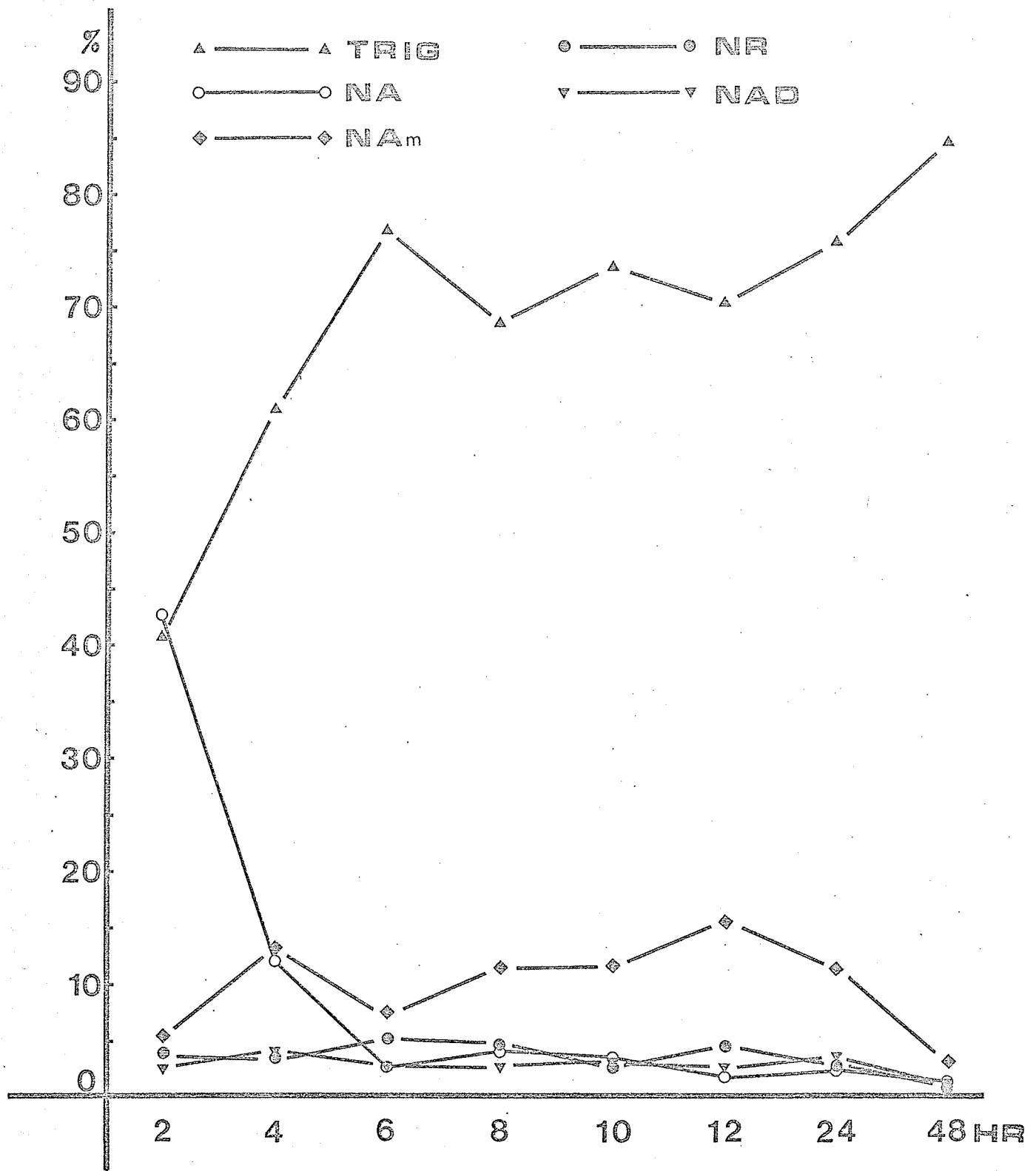


Fig. 14. Radioautographs of the intermediates of NAD metabolism after the incorporation of  $^{14}\text{C}$  nicotinic acid in a short time course study.

The abbreviations shown are the same as those in Fig. 12.



NAm

NA

NR

TRIG

NAD

NaAD

NADP

5

5

10

10

15

20MIN

leaves is identical with that formed in wheat leaves (Godavari, 1966).

Feeding  $^{14}\text{C}$  nicotinic acid prior to senescence on regulators. Godavari (1966), Mishra and Waygood (1968) and Waygood et al. (1968) have shown that benzimidazole treatment of wheat leaves increases their pyridine nucleotide content especially NADP. Prasad and Waygood (unpublished) have shown that NAD inhibits the conversion of nicotinic acid to trigonelline. Greenbaum et al. (1964) have also shown that ethionine caused a substantial fall in NADP and NADPH in rat liver. Accordingly a series of experiments were undertaken to determine the effect of these and other compounds on the labelling pattern after feeding  $^{14}\text{C}$  nicotinic acid to leaves which had been floated on various solutions either under continuous illumination or in darkness.

In the first experiment the trifoliate leaves were detached from the plant and fed  $^{14}\text{C}$  nicotinic acid for 6 hours. The leaves were then weighed, cut into small sections and floated on 20ml of: (1) 25 p.p.m. benzimidazole, methionine, or ethionine, (2) 10 p.p.m. benzyladenine or kinetin, (3)  $5 \times 10^{-4}\text{M}$  NAD. After 40 hours, the leaf sections were killed, extracted, and radioautographs were prepared as described.

The labelling pattern under continuous illumination is shown in Fig. 15 and the quantitative data are given in Table II. There appears to be no significant difference between the treatments except that the total radioactivity incorporated into each

Fig. 15. Radioautographs of the intermediates of NAD metabolism after feeding  $^{14}\text{C}$  nicotinic acid to bean leaves for 6 hours prior to allowing them to senesce on solutions of various regulators under continuous illumination. The abbreviations are the same as those in Fig. 12.

Fig. 16. Radioautographs of the intermediates of NAD metabolism after feeding  $^{14}\text{C}$  nicotinic acid to bean leaves for 6 hours prior to allowing them to senesce on solutions of various regulators in darkness. The abbreviations are the same as those in Fig. 12.

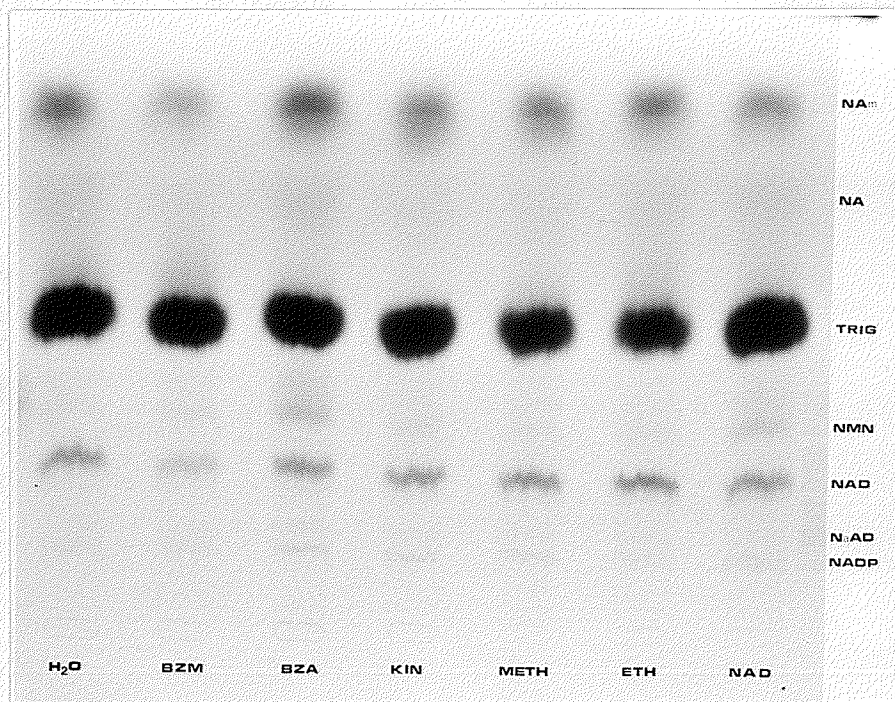
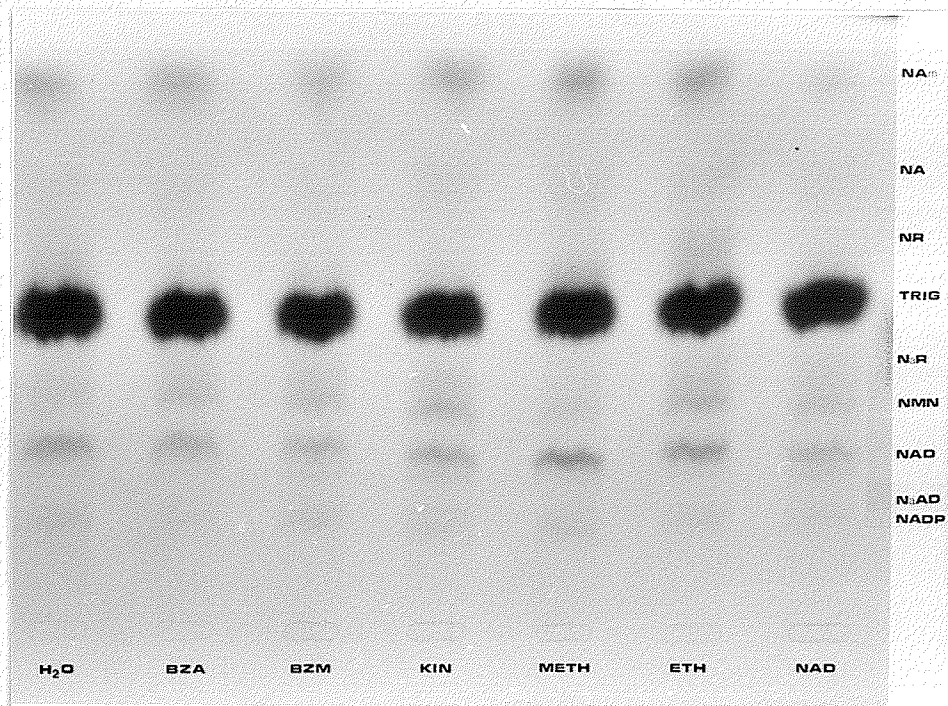


TABLE II

The percentage of various intermediates of NAD metabolism in a study of  $^{14}\text{C}$  nicotinic acid incorporation prior to senescence on regulators under continuous illumination.

NAD Metabolism Intermediate	Regulator						
	Control**	BZM	BZA	KIN	METH	ETH	NAD
NAM	3.97	3.47	4.38	4.45	4.95	4.13	3.34
NA	1.10	0.94	1.21	1.28	1.80	1.78	1.20
NR	1.88	1.14	1.34	1.25	1.97	2.70	1.00
TRIG	57.90	51.72	52.91	53.51	54.55	54.61	52.07
NaR	1.72	1.26	1.14	1.51	1.07	1.30	1.14
NMN	0.91	1.50	1.25	1.80	1.04	2.27	1.08
NAD	1.95	1.54	2.13	1.88	2.42	2.07	1.35
NaMN	0.41	0.56	0.35	0.51	0.51	0.56	0.54
NaAD	0.29	0.30	0.31	0.39	0.36	0.30	0.36
NADP	0.43	0.57	0.32	0.79	0.57	0.44	0.46
Original Streak	0.14	0.23	0.14	0.20	0.18	0.12	0.29
Total percentage	70.70	63.23	66.48	67.57	69.42	70.28	62.83
Total Counts*	423,680	370,836	383,084	401,251	402,036	421,450	360,320

\*These values were obtained from an aliquot of 0.3ml of water soluble fraction, which was counted for 5 minutes.

\*\*Control solution consists of  $\text{H}_2\text{O}$  only

TABLE III

The percentage of various intermediates of NAD metabolism in a study of  $^{14}\text{C}$  nicotinic acid incorporation prior to senescence on regulators in darkness.

NAD Metabolism Intermediate	Regulator						
	Control**	BZM	BZA	KIN	METH	ETH	NAD
NAm	8.26	7.25	12.17	9.58	13.45	15.35	6.00
NA	2.15	1.41	2.08	1.15	1.74	2.26	1.26
NR	2.52	4.00	1.13	0.77	2.47	4.62	1.15
TRIG	72.99	78.12	73.50	80.75	69.90	59.92	82.27
NaR	0.63	0.93	1.14	0.65	1.04	0.96	1.07
NMN	0.65	0.94	1.73	0.43	0.99	1.17	2.03
NAD	2.62	1.76	2.51	2.50	3.93	5.43	1.12
NaMN	0.32	0.39	0.44	0.31	0.79	0.65	0.40
NaAD	0.26	0.36	0.35	0.31	0.41	0.54	0.21
NADP	0.34	0.42	0.41	0.46	0.67	0.66	0.38
Original Streak	0.19	0.21	0.22	0.19	0.31	0.27	0.12
Total Percentage	90.93	95.79	96.02	97.10	95.70	91.83	96.01
Total Counts*	442,726	320,397	473,618	411,511	263,675	232,853	470,476

\*These values were obtained from an aliquot of 0.3ml of water soluble fraction, which was counted for 5 minutes.

\*\*Control solution consists of  $\text{H}_2\text{O}$  only



of the intermediates of NAD metabolism and most particularly into trigonelline, is considerably lower than that found in immediately detached leaves (cf. Fig. 12 and Table I).

A similar experiment in continuous darkness (Fig. 16 and Table III) shows a considerably greater labelling of trigonelline at almost the same level as in immediately detached leaves without inducing senescence except treatment with ethionine (Fig. 12 and Table I). In addition, there was a greater percentage of radioactivity in NAD and less in NADP. However, there appeared to be no significant difference between treatments.

Feeding  $^{14}\text{C}$  nicotinic acid together with regulators. In this experiment,  $^{14}\text{C}$  nicotinic acid was fed to leaves and, immediately after it had been absorbed, the leaves were then fed with various regulators. The leaves were killed after 6 hours of feeding. The results are shown in Fig. 17 and Table IV. The control in water is not shown in Fig. 17, since the leaves accidentally wilted and absorbed very little  $^{14}\text{C}$  nicotinic acid. Hence the data for the water control in Table IV is taken from the time course study given in Table I.

The radioautographs and the quantitative data indicate that with the exception of methionine treatment, all other treatments slowed down the rate of disappearance of  $^{14}\text{C}$  nicotinic acid (cf. previous figures and tables). Much more trigonelline is formed in the presence of methionine, whereas less is formed in the presence of benzimidazole, ethionine or NAD. Although water

Fig. 17. Radioautographs of the intermediates of NAD metabolism after feeding  $^{14}\text{C}$  nicotinic acid together with regulators to bean leaves for 6 hours. The concentrations of the regulators are as follows:

Benzimidazole : 50 p.p.m.  
Benzyladenine : 20 p.p.m.  
Kinetin : 20 p.p.m.  
Methionine : 50 p.p.m.  
Ethionine : 50 p.p.m.  
NAD :  $10^{-3}\text{M}$



TABLE IV

The percentage of various intermediates of NAD metabolism in a study of  $^{14}\text{C}$  nicotinic acid incorporation together with various regulators.

NAD Metabolism Intermediate	Regulator						
	Control**	BZM	BZA	KIN	METH	ETH	NAD
NA <sub>m</sub>	7.38	32.14	14.95	21.73	14.94	15.03	15.61
NA	2.67	6.17	7.54	4.30	3.13	6.04	12.29
NR	5.13	9.36	6.48	8.23	10.44	19.76	16.18
TRIG	77.09	42.06	60.92	55.15	64.01	50.82	43.77
NaR	1.32	0.95	0.87	0.80	1.16	1.37	1.42
NMN	1.01	0.90	1.04	1.16	0.74	0.81	1.30
NAD	2.91	4.95	4.11	4.37	3.40	4.24	4.35
NaMN	0.42	0.48	0.43	0.47	0.38	0.58	1.12
NaAD	0.58	0.80	0.70	0.81	0.55	0.62	2.33
NADP	0.42	0.95	0.55	0.81	0.37	0.32	0.62
Original Streak	0.26	0.49	0.33	0.39	0.27	0.25	0.44
Total Percentage	99.28	99.25	97.92	98.22	99.39	99.84	99.43
Total Counts*	345,519	480,950	472,603	403,040	505,743	468,106	421,720

\*These values were obtained from an aliquot of 0.3ml of water soluble fraction, which was counted for 5 minutes.

\*\*Control solution consists of H<sub>2</sub>O only

control treatment has a high label in trigonelline, it should be noted that the data is taken from a different set of experiments. The lower amount of label in trigonelline with NAD treatment may be due to the dilution by cold trigonelline, which is formed from regulator NAD. The concentrations of the regulators are given in the legend to Fig. 17.

Feeding  $^{14}\text{C}$  nicotinic acid to leaves after senescence. The trifoliolate leaves were fed with various regulators at the concentrations given in Fig. 17. After 40 hours they were fed with  $^{14}\text{C}$  nicotinic acid for 6 hours. As with wheat leaves (Godavari, 1966; Waygood *et al.*, 1968) chromatography of the water soluble fraction of senescent leaves was unsatisfactory owing to the interference presumably of other senescence products (Fig. 18). Accordingly, the nucleotides were precipitated by acetone. Two millilitre aliquots of the soluble fraction were acidified with 0.1N HCl and 6 volumes of cold ( $-10^\circ$ ) acetone was added and the mixture was stored overnight at  $-15^\circ$ . The white precipitate was collected by centrifugation, washed twice with cold acetone and once with ether. It was dried in a desiccator under vacuum and dissolved in 1.0ml  $\text{H}_2\text{O}$ . Radioautographs (Fig. 19) were prepared as described in Methods, and the quantitative data are given in Table V. The data show that all treatments increased the labelling of NAD and NADP when these are compared with the water control. They were most heavily labelled in the benzyladenine treatment but there was also a significant labelling of NaAD.

Fig. 18. Radioautographs of the intermediates of NAD metabolism after feeding  $^{14}\text{C}$  nicotinic acid to bean leaves after senescence on various regulators.

The abbreviations are the same as those in Fig. 12.

Fig. 19. Radioautographs of acetone precipitated nicotinamide nucleotides from the same water extracts as those in Fig. 18.

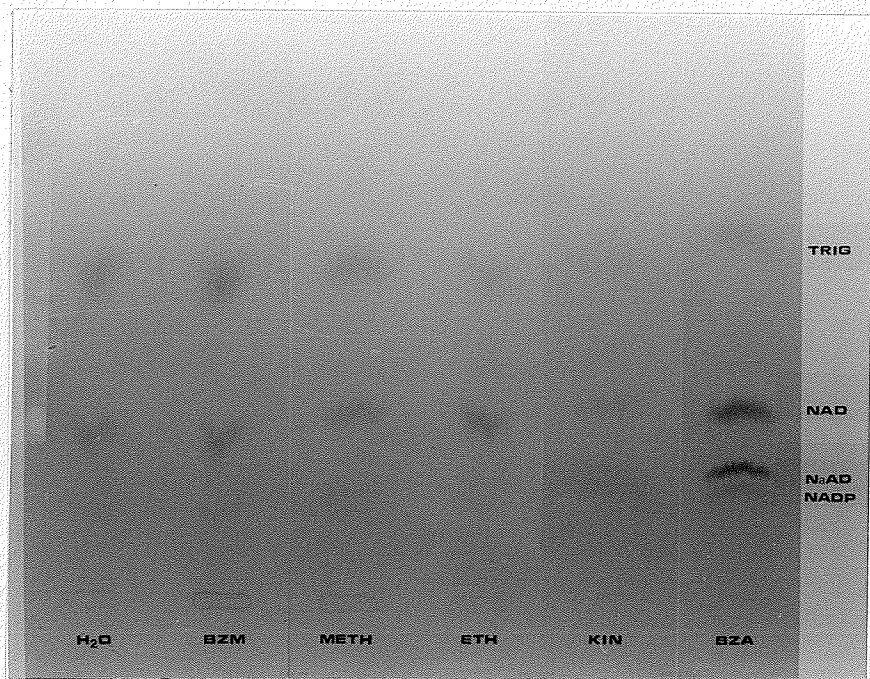
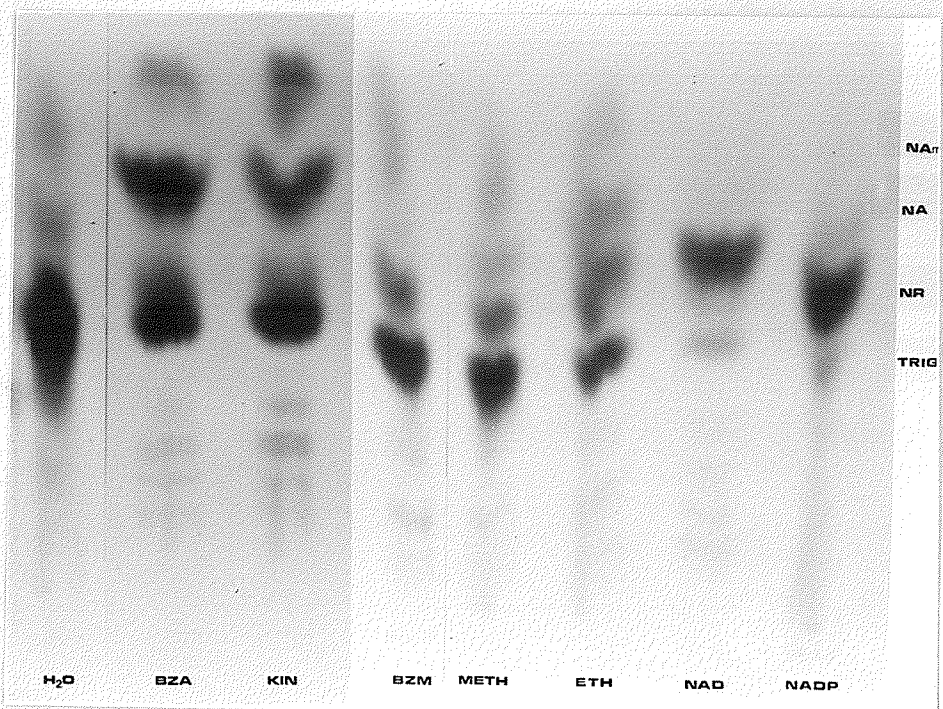


TABLE V

The counts of radioactive pyridine nucleotides in a study of  $^{14}\text{C}$  nicotinic acid incorporation after induction of senescence treated with the following regulators.

NAD Metabolism Intermediate	Regulator					
	Control*	BZM	BZA	KIN	METH	ETH
NAD	1,920	3,040	8,610	2,780	2,760	3,502
NaAD			7,307	1,190		610
NADP	690	1,470	2,670	1,340	1,210	1,210
Total Counts	387,630	356,760	324,363	326,460	343,680	286,815

(Fractions used were 0.3ml, which was counted for 5 minutes.)

\*Control solution consists of  $\text{H}_2\text{O}$  only



## DISCUSSION

In studying the effect of benzimidazole on chlorophyll metabolism of detached etiolated and green leaves of Khapli wheat, Wang and Waygood (1959) showed that benzimidazole increased the rate of chlorophyll formation and decreased the rate of chlorophyll destruction in both light and darkness. However, in dicotyledonous plants, such as bean, sunflower and tobacco, Wang et al. (1961) found that benzimidazole had no effect on the maintenance of chlorophyll in leaf tissue. They suggested that this kinin was selective for monocotyledons.

Further work has been done in this laboratory. The growing apices of Elodea were floated on water or solutions of NAD, NADP, nicotinic acid and nicotinamide in the presence and absence of benzimidazole or kinetin under continuous illumination or in darkness (Waygood et al., 1968). Under continuous illumination, both NAD and nicotinic acid accelerated chlorosis in this tissue, and nicotinic acid was a more potent bleaching agent than NAD. However, neither NAD nor nicotinic acid had any effect in darkness. NADP and nicotinamide had little or no effect on senescence of this tissue. It was also shown that benzimidazole and kinetin overcame the accelerating effect of NAD and nicotinic acid on the induced senescence under continuous illumination.

In the present studies, leaf discs from trifoliolate leaves were floated on either water, or solutions of benzimi-

dazole, nicotinic acid, NAD or mixtures of these either under continuous illumination or in darkness for 14 days. It was shown that both NAD and nicotinic acid stimulated senescence of bean leaf discs (Fig. 1). At the same concentration ( $10^{-3}M$ ), NAD appeared to be a more effective bleaching agent than nicotinic acid. Under continuous illumination, benzimidazole had no effect on senescence retardation, nor did it overcome the chlorotic effect of NAD and nicotinic acid.

In darkness, benzimidazole appeared to retard senescence of bean leaf discs and both NAD and nicotinic acid were found to stimulate senescence. Both bleaching effects were overcome to a certain extent by benzimidazole. The results of this experiment are quite different from those observed with Elodea leaves in which the effect of NAD and nicotinic acid could be overcome only under continuous illumination (Godavari, 1966; Waygood et al., 1968).

The floating period of this experiment, which was 14 days, raised the problem of the artificial contribution of bacterial infection to the senescence process. Accordingly, the senescence process was followed for a shorter floating period, 7 days, in light and darkness. The results (Figs. 2 and 3) indicated that benzimidazole, methionine, NAD and NADP had little or no effect on senescence under both conditions. However, it was shown that NAD did accelerate senescence in Elodea leaves under continuous illumination within 3 days (Godavari,

1966; Waygood et al., 1968). On close examination, it was found that a pH factor was probably implicated in the acceleration of senescence by NAD in Elodea leaves. Since the pH of  $10^{-3}$ M NAD is 3.8, and the solution was not neutralized in the experiment on Elodea leaves, the acidic pH might have exerted or stimulated the chlorotic effect under continuous illumination. The pH of a 50 p.p.m. solution of benzimidazole is 6.5. The mixture of  $10^{-3}$ M NAD and 66 p.p.m. benzimidazole (Godavari, 1966) is presumed to have a pH value between 3.8 to 6.5. Since benzimidazole did not show any effect on Elodea leaves but overcome the effect of NAD, the reversing effect of benzimidazole on NAD in that experiment could have been due to an increase in pH value. In the present studies with bean-leaf discs, the solutions were all neutralized to pH 7 by NaOH. In a 7 day floating period, there was no observable difference between the effects of benzimidazole, methionine, NAD or NADP and those of water (Figs. 2 and 3), nor was there any relationship between concentration and senescence under continuous illumination (Figs. 4 and 6).

Kinetin and benzyladenine have been reported to stimulate growth and delay senescence in various leaves (Kuraish and Okumura, 1956; Mothes and Engelbrecht, 1959; Leopold and Kawase, 1964). In Figs. 2 and 3, the leaf discs treated with kinetin and benzyladenine showed some interveinal chlorosis under both conditions, but there was an increase in the surface

area of leaf discs under light conditions. These data imply that 20 p.p.m. of kinetin or benzyladenine induced growth and chlorosis together. Srivastava (1967) reported that both kinetin and benzyladenine promoted RNA synthesis at low concentration but inhibit it selectively at higher concentrations. He stressed that the concentration was important for their effects. In this experiment, the concentration of 20 p.p.m. of kinetin and benzyladenine may have been so high as to bring about the chlorotic effect on bean leaves, but still induced growth. The results in Fig. 5 show that chlorosis was stimulated and areas of the discs were increased when the concentration of kinetin and benzyladenine was increased to 20 p.p.m.. This observation supports the above explanation.

S-adenosylmethionine is involved in the methylation of both the C-10 carbon of chlorophyll (Radmer and Bogorad, 1967) and the pyridinium N of nicotinic acid (Joshi and Handler, 1960). To examine this further, leaf discs were floated for 7 days on methionine and its antagonist, ethionine, to determine whether these amino acids regulate senescence. Figs. 2 and 3 show that methionine had no effect, but ethionine induced chlorosis in bean leaf discs under both conditions. Furthermore, the chlorosis was shown to be related closely to concentration of ethionine (Fig. 6). Discs exposed to methionine for 14 days were still not affected (Figs. 7 and 8). This could be accounted for if the amino acid is the source of the C-10 methyl group of

chlorophyll, and thus there may be sufficient methyl-group donor for chlorophyll synthesis in leaf tissues. Farber et al. (1964) reported that the reaction of ethionine with ATP to produce S-adenosylethionine was more rapid than the formation of S-adenosylmethionine from methionine and ATP in rat liver, thus the observed bleaching effect of ethionine is assumed to be due to ATP depletion.

The results in Fig. 7 show that NAD and NADP maintained the green color of leaf discs under continuous illumination. This result is quite different from that shown in Fig. 1, in which NAD induced chlorosis of bean-leaf discs. Thus, it is difficult to define the effect of NAD or NADP on bean leaves. Moreover, the bacterial infection which was found on the leaf discs treated with NAD (Fig. 7) makes the issue more confused.

In darkness, NAD and NADP did stimulate the appearance of chlorosis (Fig. 8); the leaf discs became translucent and chlorotic. This chlorotic effect was overcome by benzimidazole (Figs. 1 and 9), a compound which retarded senescence only in darkness (Figs. 8 and 9).

Since benzyladenine was found to accelerate senescence under both conditions, leaf discs were treated with benzimidazole, methionine, and NAD, all with or without benzyladenine. It was shown that methionine and NAD accelerated the chlorotic inducing effect of benzyladenine in darkness (Fig. 11). Benzimidazole was unable to reverse the chlorotic effect of benzyladenine even

in darkness (Figs. 10 and 11). Further discussion about benzyladenine will be given in later section.

One of the main objectives of the present investigation was to determine whether the biosynthesis and breakdown of NAD in bean leaves followed the Preiss-Handler pathway (1958) as in wheat leaves (Godavari, 1966). When labelled nicotinic acid was incorporated into NAD, the following intermediates were identified : nicotinamide, nicotinic acid, nicotinamide riboside, trigonelline, nicotinic acid riboside, nicotinamide mononucleotide, nicotinamide adenine dinucleotide, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (Fig. 13). These intermediates are the same as those which occurred in wheat leaves (Godavari, 1966). The findings that labelled trigonelline appeared 5 minutes after feeding and its radioactivity increased from 0 to 80% of the total, and that the labelled nicotinic acid pool was depleted in 6 hours, were similar to those of Godavari (1966) with wheat leaves. These observations indicate that the metabolic pathways of NAD biosynthesis and degradation in bean leaves may be identical to those in wheat leaves.

In order to investigate the effect of regulators on NAD metabolism,  $^{14}\text{C}$  nicotinic acid was fed to leaves (i) before senescence was induced by regulators, (ii) together with the regulators, and (iii) after senescence was induced by regulators. When labelled nicotinic acid was fed prior to senescence by

regulators under light conditions for 40 hours, it was found that the total radioactivity, particularly in trigonelline, was much lower than that in leaves which were extracted immediately after feeding (Fig. 15). However, there was no significant difference between the various treatments under light conditions. With ethionine treatment in darkness, there was chlorosis and less label in trigonelline but more label in NAD, relative to the water control (Fig. 12 and Table I). There was no significant difference between the other treatments in darkness. It appeared that light was involved in the degradation of trigonelline, but the regulators were not. Thus, it seems likely that in darkness, the chlorosis which ethionine produced was due to the reduction of trigonelline or promotion of NAD synthesis or both.

When labelled nicotinic acid and regulators were incorporated together, it was found that methionine increased trigonelline formation, but benzimidazole, ethionine and NAD decreased it. The effect of NAD on trigonelline reduction may be due to the dilution effect of cold trigonelline, which is formed from regulator NAD.

When labelled nicotinic acid was fed to leaves after regulator-induced senescence, the results (Fig. 18) were not as clear as the previous ones. After precipitation with acetone, the nucleotide residue was radioautographed. The results are shown in Fig. 19. It was not possible to detect trigonelline

formation from these chromatograms. In each treatment, NAD and NADP increased to a certain extent compared with the water control. The data showed that bean leaves produced larger amounts of NAD and NADP after the induction of senescence by regulators than on water.

Mishra (1963) and Waygood et al. (1968) showed that NADP and NADPH were increased in wheat leaves during a 16 hour photoperiod, that NAD and NADH were increased after 8 hours in darkness, and that they underwent reciprocal changes in light and darkness during senescence. They also showed that benzimidazole or kinetin increased or maintained the NADP level in chloroplasts isolated from leaves which had been floated for 6 days. From the feeding of labelled nicotinic acid or nicotinamide to detached leaves of wheat floated on either water or benzimidazole, it was found that the incorporation of label into NAD was about the same in both treatments, whereas its incorporation into the NADP and nicotinic acid dinucleotide pool was greater in benzimidazole treated leaves. Since NADP induced only a sporadic bleaching of Elodea leaves, it was therefore suggested that NAD was detoxified to NADP, and the effect of benzimidazole on the maintenance of chlorophyll was due to the suppression of NAD synthesis so that NAD did not accumulate above toxic threshold levels (Godavari, 1966; Waygood et al., 1968). The NAD/NADP ratio in bean leaves was investigated. The NAD/NADP ratio after each treatment (Table VI) was calculated.



TABLE VI\*

NAD/NADP ratio in various treatments.

	Regulator						
	Control**	BZM	BZA	KIN	METH	ETH	NAD
Feeding $^{14}\text{C}$ nicotinic acid prior to senescence on regulators under continuous illumination.	4.53	2.70	6.66	2.38	4.25	4.70	2.93
Feeding $^{14}\text{C}$ nicotinic acid prior to senescence on regulators in darkness.	7.70	4.20	6.12	5.43	5.89	8.23	2.95
Feeding $^{14}\text{C}$ nicotinic acid together with regulators.	6.93	5.21	7.47	5.80	9.19	13.25	7.01
Feeding $^{14}\text{C}$ nicotinic acid after induction of senescence treated with regulators.	2.78	2.07	3.23	2.08	2.28	2.89	

\*This table is calculated from Tables II, III, IV and V.

\*\*Control solution consists of  $\text{H}_2\text{O}$  only

from the data in Tables II thru V. Lower NAD/NADP ratios were obtained after treatment with benzimidazole and kinetin in each experiment. These results indicate that benzimidazole and kinetin increased NADP formation, and confirm the results of Mishra and Waygood (1968) and Waygood et al. (1968). On the other hand, higher NAD/NADP ratios were obtained with benzyladenine and ethionine treatment, which indicates that benzyladenine and ethionine decreased NADP formation. It should be noted that the lower NAD/NADP ratios obtained when leaves were floated on NAD solutions were probably due to the dilution of the radioactive NAD by cold NAD.

Waygood et al. (1968) showed that, in wheat leaves, the effect of benzimidazole on NAD was due to its ability to augment the conversion of NAD into NADP. This may not be true for bean leaves, because benzimidazole did not overcome the chlorotic effect of benzyladenine (Fig. 11), and NAD and NADP had almost the same effect on senescence of bean leaf discs (Figs. 2, 3, 7, 8, and 9). Under the conditions used, NAD did not appear to be detoxified to NADP.

When leaves were floated on various solutions under continuous illumination, the ratios of NAD/NADP were generally lower than when they were floated in darkness (Table IV). This confirms the results of Waygood et al. (1968) that light stimulates NADP formation. Goldthwaite and Leetsch (1967) have reported that the chemical growth regulators indoleacetic acid,

2,4-dichlorophenoxyacetic acid, gibberellic acid, kinetin and benzyladenine are relatively ineffective in retarding senescence in bean primary leaves under light conditions. The senescence retardation is dependent on white light. They suggested that light inducing photosynthesis caused the ineffectiveness of growth regulators on senescence retardation in bean leaves. The present data show that benzimidazole retards senescence in darkness, but not under continuous illumination, and thus, it is doubtful that light is a factor involved in the process of senescence. Light stimulated trigonelline degradation and NADP formation (Tables II and VI), however, we cannot find a relationship between the absence of a benzimidazole effect on senescence retardation under light condition, and the increase in trigonelline degradation or in NADP formation caused by light.

The major questions arising from these investigations are how benzimidazole retards senescence and how it overcomes the effects of nicotinic acid, NAD and NADP. In the present work, it was found that benzimidazole did not retard senescence, nor did it overcome the chlorotic effect of nicotinic acid, NAD or NADP under continuous illumination. In darkness, it did maintain chlorophyll content and overcome the chlorotic effect of nicotinic acid, NAD or NADP. It is presumed that the mechanism of action of benzimidazole in any case should be the same. Waygood (1965) found that benzimidazole treatment of leaves produced a more closely integrated structure of the

inter- and intra-grana lamellae of wheat leaf chloroplasts, and increased the rate of photophosphorylation of wheat leaf chloroplast fragments. He suggested that one of the action sites of benzimidazole was within the chloroplast. Since the effect of benzimidazole on senescence retardation of bean leaves is different from that of Elodea or wheat leaves, a question arise as to whether the mode of action of benzimidazole in bean leaves is similar to that in wheat leaves.

According to present data, there are three possible mechanisms of action of benzimidazole. Firstly, on the basis of long floating period, it is possible that NAD or NADP will promote bacterial action which stimulates the senescence of leaf discs. Nicotinic acid, a precursor of NAD or NADP synthesis, was converted into NAD or NADP and, therefore, has the same effect as that of NAD or NADP. Benzimidazole has bactericidal action, and can reverse the effect of nicotinic acid, NAD or NADP. If this suggestion is correct, the study here may explain the results of Wang et al. (1961) and their suggestion that benzimidazole is selective for monocotyledons.

The second possibility is that the chlorotic effect of nicotinic acid, NAD or NADP was caused by their conversion into trigonelline. Since the synthesis of trigonelline will lower the concentration of S-adenosylmethionine (Joshi & Handler, 1966), which is a methyl group donor for chlorophyll formation (Radmer & Bogorad, 1967), benzimidazole may reduce trigonelline formation

(Fig. 17), and therefore, maintain the S-adenosylmethionine for chlorophyll synthesis. Thus, the chlorotic effect of nicotinic acid, NAD or NADP would be overcome by benzimidazole. However, it is difficult to explain the effect of benzimidazole alone. Since a methyl group donor, methionine, had no effect on senescence retardation, it seems there is no requirement to maintain the S-adenosylmethionine content in leaf tissues.

The third possibility is that benzimidazole can substitute for the nicotinamide moiety of NAD to produce benzimidazole adenine dinucleotide in a reaction catalyzed by NAD nucleosidase (Kapoor and Waygood, 1965). The chlorotic effect of NAD is then reduced to some extent by benzimidazole.

So far, it has been well shown that exogenous cytokinins in senescing leaves and leaf discs induce synthesis of protein, RNA, lipid and starch (Anderson and Rowan, 1966; Mothes and Engelbrecht, 1959; Osborne, 1962; Sugiura, Umemura and Oota, 1962), suppress ribonuclease and deoxyribonuclease activity (Srivastava, 1967), and prevent the respiratory increase which normally follows detachment (Person et al., 1957). However, it is difficult to explain the reversing effect of benzimidazole on nicotinic acid, NAD, or NADP. To understand the mode of action of benzimidazole, further work must be done.

## SUMMARY

1. The effects of benzimidazole, benzyladenine, kinetin, methionine, ethionine, nicotinic acid, NAD or NADP on the senescence and on the NAD metabolism of bean trifoliolate leaves have been studied.
2. NAD, NADP and nicotinic acid accelerated senescence in darkness, and NAD was a more effective bleaching agent on the senescence of bean leaf discs than nicotinic acid.
3. Methionine had no effect; nevertheless, ethionine, an antagonist of methionine, caused chlorosis on bean leaves both under continuous illumination and in darkness.
4. Benzyladenine and kinetin, at the concentration of 20 p.p.m., accelerated the senescence and increased the surface area of bean leaf discs either under continuous illumination or in darkness.
5. In darkness, benzimidazole retarded senescence of bean leaf discs and overcame the chlorotic effect of nicotinic acid or NAD.
6. The metabolic pathway of NAD in bean leaves was similar to that in wheat leaves, following the Preiss-Handler pathway.
7. Light stimulated the degradation of trigonelline and decreased NAD/NADP ratio.
8. Benzyladenine and ethionine increased NAD/NADP ratio, however, benzimidazole and kinetin decreased it.
9. Benzimidazole reduced trigonelline production, but methionine increased it.

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