METABOLISM OF NICOTINAMIDE NUCLEOTIDES IN WHEAT LEAVES INFECTED BY PUCCINIA GRAMINIS TRITICI

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LIST OF ABBREVIATIONS

ATP - Adenosine 5' triphosphate

H-1 and H-6 - Healthy leaves fed radioactive compound for 1 and 6 hours respectively.

10-1 (38-1, 48-1) and 10-6 (38-6, 48-6) - Rust-infected leaves by Race 10 (38, 48-1) after feeding 1 and 6 hours respectively.

NA - Nicotinic acid; NA* - Carboxyl ^{14}C (labelled) nicotinic acid.

NaAD - Nicotinic acid adenine dinucleotide

NAD - Nicotinamide adenine dinucleotide; NADH - reduced form of NAD.

NADP - Nicotinamide adenine dinucleotide phosphate;
NADPH - Reduced form of NADP

NAD(P) - NAD and NADP

NAm - Nicotinamide

NaMN - Nicotinic acid mononucleotide

NaR - Nicotinic acid riboside

NMN - Nicotinamide mononucleotide

NR - Nicotinamide riboside

PEP or P-enolpyruvate - Phosphoenolpyruvate

Pi - Inorganic phosphate

PP - Pyrophosphate

Trig - Trigonelline (N-methyl nicotinic acid).

SAM - S-adenosylmethionine;
SAM* - Methyl ^{14}C (labelled) S-adenosylmethionine

INTRODUCTION

A number of studies have been initiated to investigate the relationship between NAD(P) metabolism and senescence of wheat leaves and to study the effect of kinins, which delay senescence, on this metabolism (Mishra 1963; Godavari 1966; Mishra and Waygood 1968; Waygood *et al* 1968). One effect noted is that benzimidazole and kinetin treatment increases the total NAD(P) content of wheat leaves, particularly that of the chloroplasts. Wheat leaves infected with *Puccinia recondita* have also been shown by Rohringer (1964) to accumulate NAD(P) as compared to uninfected controls and there has been considerable speculation and some evidence (Howard *et al* 1968; Kiraly *et al* 1967) that this obligate parasite induces kinin production in the host-parasite complex.

Shaw and Samborski (1956) have shown that the green islands appearing around uredosori in wheat leaves are 'sinks' for any labelled compounds fed to the leaves (Shaw *et al* 1954). Wang (1961) has shown that there is an increase in the chlorophyll content and a *de novo* synthesis of starch in the green island of rust-infected pinto bean plants. This indicates a very active metabolism in the green island area. Shaw and Samborski (1956) used non-specific compounds such as glucose-1-^{14}C and carbon dioxide ^{14}C which enter into many metabolic pathways, to show their accumulation in the green islands. Nicotinic acid is a precursor only in NAD biosynthesis and if the label from nicotinic acid accumulated in a similar manner in the green islands it would indicate a very active synthesis and breakdown of NAD

in this region. Accordingly, radioautographs were made from rust-infected wheat leaves fed carboxyl ^{14}C labelled nicotinic acid for one and six hours.

There is also the possibility that the photosynthetic pathways operative in the green islands differ from those of healthy leaves and therefore preliminary experiments were undertaken to compare the activity of phosphoenolpyruvate carboxylase (EC 4.1.1.31.) and phosphoenolpyruvate synthetase (Cooper and Kornberg 1965) in healthy and rust-infected wheat leaves. Phosphoenolpyruvate carboxylase has been looked for but never demonstrated in wheat stem rust and bean rust uredospores (Staples and Weinstein 1959). If there was an increase in P-enolpyruvate carboxylase and an appearance of P-enolpyruvate synthetase in the host parasite complex it would indicate that the 4-carbon dicarboxylic acid photosynthetic pathway (Hatch and Slack 1966) was involved in CO_2 fixation. Healthy wheat leaves normally utilize the Calvin cycle (Calvin and Bassham 1957; Calvin and Benson 1948) involving ribulosediphosphate carboxydismutase (Ribulosediphosphate carboxylase EC 4.1.1.f) for CO_2 fixation.

Godavari (1966) using carboxyl ^{14}C labelled nicotinic acid and carbonyl ^{14}C nicotinamide has shown that the pathway of biosynthesis and breakdown of NAD in wheat leaves follows that previously shown by Preiss and Handler (1957) for human erythrocytes. Accordingly, following the procedures of Godavari (1966) using carboxyl ^{14}C labelled nicotinic acid, studies were undertaken to determine if the same pattern of intermediates of NAD synthesis and breakdown occurred in

LITERATURE REVIEW

The effect of benzimidazole in delaying the senescence of wheat leaves was first reported by Person et al (1957).

Wang and Waygood (1959) have shown that the level of chlorophyll in Khapli wheat leaves floating on 50 ppm aqueous benzimidazole in continuous light becomes more or less stabilized at the 6-day level for another 5 days, whereas the level in control leaves floating on water drops rapidly within 6 days. Wang et al (1960) have also shown that the rates of incorporation of glycine-2-^{14}C and succinate 2,3-^{14}C into the chlorophyll are maintained at the same rate as in immediately detached leaves by benzimidazole treatment, whereas the rates of incorporation are drastically reduced in leaves floated on water. This indicates that benzimidazole exerts its effect on the chlorophyll metabolism of detached wheat leaves either by protecting the chlorophyll from rapid destruction or stimulating chlorophyll synthesis.

In studies on the effect of benzimidazole and metal ions on the development of stem rust in detached leaves of wheat, both benzimidazole and the nickel ion have been shown to have a preservative effect on the green pigment of leaves (Wang 1959). Wang also showed that benzimidazole could reverse the breakdown of resistance which occured in detached Khapli wheat leaves, floated on water, to Puccinia graminis tritici, Race 15B-1. Since benzimidazole has no effect on rust development in susceptible leaves, its action in maintaining resistance must result from its effects on the metabolism of the host

(Person and Forsyth 1958). Also, there is strong evidence that kinetin may play a similar role to that of benzimidazole in maintaining rust resistance and normal physiology of detached wheat leaves (Wang et al 1961). Investigations indicate that there were at least two different sites of action involved in the benzimidazole effect and one of these must reside in the chloroplast (Wang and Waygood 1959).

Yoshida (1961) has shown that chloroplast in enucleated protoplasts of plasmolyzed detached cells in Elodea leaves remained green for a long period, became enlarged, and photosynthesized while in comparison chloroplasts in nucleated protoplasts senesced rapidly. This phenomenon is analogous to the effect of benzimidazole in maintaining chlorophyll levels in wheat leaves (Wang et al 1960; 1961).

Yoshida also showed that NAD in the medium accelerated the senescence of chloroplasts in enucleated protoplasts whereas, NADP was ineffective. Other experiments have demonstrated that NAD accelerates the senescence of detached, unplasmolysed Elodea leaves as well as detached leaves of Selkirk wheat but this effect of NAD can be overcome by benzimidazole as well as kinetin (Godavari 1966). The toxicity of NAD and the relative inocuousness of NADP suggest that the level of pyridine nucleotides may play physiological roles in the leaf. Mishra (1963) has determined the pyridine nucleotide content in whole detached wheat leaves or chloroplasts isolated in nonaqueous media from immediately detached leaves and from those leaves floated on water or benzimidazole by using the enzymatic cycling method of Lowry et al (1961).

It was found that the pyridine nucleotide content decreased in the leaves floated on water and increased in the leaves floated on benzimidazole with the concomitant changes in chlorophyll (Waygood 1965; Mishra and Waygood 1968). With isolated chloroplasts, however, a general loss in the content of NAD was observed during both treatments. Chloroplasts from leaves floated on water had lost all their NADP after 6 days, whereas those treated with benzimidazole or kinetin increased or maintained their NADP level (Mishra and Waygood 1968; Waygood et al 1968). These studies indicated that benzimidazole and kinetin treatment maintained, at least partially, the level of total pyridine nucleotides and enhanced the conversion of NAD to NADP, the latter disappearing rapidly from chloroplasts of leaves floated on water. Similar techniques were used by Rohringer (1964) to measure pyridine nucleotide contents of Puccinia recondita uredospores germinated for 2.5 and 6 hours and wheat leaves (Triticum sativum L. var. Lee) infected with Puccinia recondita. This study of pyridine nucleotides in uredospores revealed high levels of NAD(P) which were halved within 6 hours of germination. Initially, the NAD concentration was also high in both resistant and susceptible infected leaf tissue, i.e. 2 days after inoculation, but at 5 days it continued to increase in the latter but decline to normal levels in the former. Rohringer also found that infected areas of susceptible tissue accumulated large amounts of NAD, NADP and NADPH. By electron microscopic studies, Howard et al (1968) have observed that the chloroplasts from the center of Albugo candida induced green islands on Brassica juncea cotyledons still contained well formed grana and abundant starch grains 96 hours after

detachment, whereas the chloroplasts of detached non-infected Brassica juncea cotyledons lost all grana structure within 84 hours of detachment. Infection induced green islands contain chloroplasts which have the same well formed structure as those found in benzimidazole and kinetin treated wheat leaves (Waygood 1965).

Some substances, extracted from Erysiphe graminis DC spores and this mildew infected barley leaves have been implicated in the induction of starch accumulation and green island formation in healthy tissues (Bushnell and Allen 1962). Also, a high cytokinin activity was recorded in rust-infected pinto bean and broad bean leaves by bioassay techniques which were based on the chlorophyll-retention test (Kiraly et al 1967). Accordingly, although cytokinins have not been isolated from green islands, there is strong circumstantial evidence for their involvement on the chlorophyll retention.

As to the mechanism by which kinins overcome the effect of NAD in accelerating senescence of a leaf, or chloroplast, Kapoor and Waygood (1965) have suggested 3 possible pathways by which the exogenous NAD may be 'detoxified'. (A) By the action of NAD pyrophosphatase, which is known to be present in wheat (Roberts 1959), (B) Benzimidazole promotes the phosphorylation of NAD to NADP by NAD kinase, or (C) Benzimidazole substitutes the nicotinamide moiety of NAD and results in benzimidazole nucleoside by NAD nucleosidase (Kapoor and Waygood 1965).

A fairly comprehensive review of the literatures on the biosynthetic pathway of nicotinamide nucleotides was presented by

Godavari (1966). Godavari investigated the pattern of NAD metabolism in immediately detached wheat leaves var. Selkirk and senescent leaves of this variety by using carbonyl ^{14}C nicotinamide and carboxyl ^{14}C nicotinic acid. His results indicated that the biosynthesis of NAD in both types of leaf studied followed the Preiss and Handler pathway (1957) which proceeded from nicotinic acid ———> nicotinic acid riboside ———> nicotinic acid mononucleotide ———> nicotinic acid adenine dinucleotide ———> nicotinamide adenine dinucleotide ———> nicotinamide adenine dinucleotide phosphate. The degradation of NAD, as demonstrated in vitro was as follows: Nicotinamide adenine dinucleotide ———> nicotinamide mononucleotide ———> nicotinamide riboside ———> nicotinamide. However, approximately 50% of the nicotinic acid incorporated was detoxified by methylation and accumulated in the leaves as N-methyl nicotinic acid (trigonelline). Godavari also noted that the effect of nicotinic acid in accelerating the bleaching of detached leaves is greater than that of NAD. Prasad and Waygood (unpublished results) have demonstrated that NAD greatly inhibited the formation of trigonelline from nicotinic acid. This suggested a possible explanation for the bleaching effect of nicotinic acid on the basis of the transmethylation of nicotinic acid to trigonelline, for a successful kinetin-like activity of trigonelline in yellowing test was reported by Karsten (1966). The activity of 500 mg of trigonelline per litre was equivalent to that of 0.1 mg of kinetin per litre.

Many observations support the hypothesis that a substance diffusing from the site of infection alters the normal metabolism of the host tissues. Fungi seem to affect the photosynthetic process by affecting

the chloroplast. Rusted wheat leaves retain chlorophyll in regions termed green islands at the periphery of the uredosori. Wang (1961) has shown that Uromyces phaseoli induced an increase in the chlorophyll content of pinto bean leaves at the periphery of uredosorus as it developed.

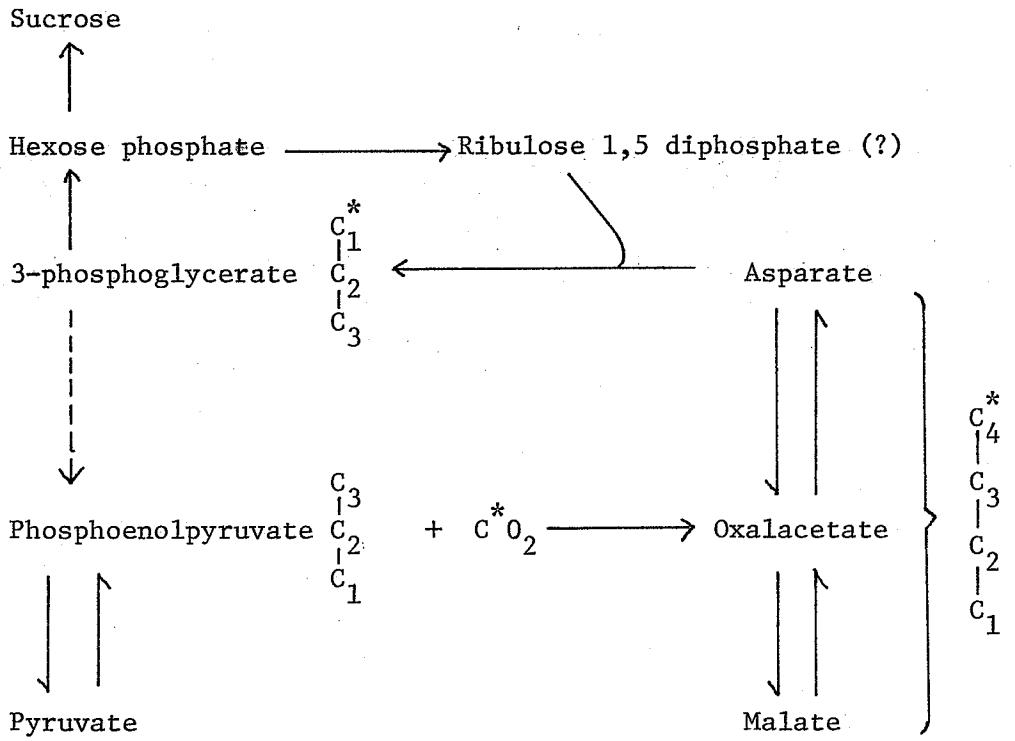
Shaw and Samborski (1956) determined quantitatively the accumulation of glucose-1-^{14}C, or its products, in 10-day old rust-infected tissue. They reported that rusted tissue accumulated 2.4 times as much radioactivity as did healthy tissues cut from comparable uninfected leaves. Similar results for the accumulation at the uresosori of fed compound has also been reported in 19 host parasite combinations by Yarwood and Jacobson (1955) using $N_2{}^{35}S$, $H_3{}^{32}PO_4$ and ^{14}C sucrose. It was also found that the mesophyll cells in the green islands of rust-infected wheat leaves incorporated more ^{32}P into ribonucleic acid than did corresponding cells of uninoculated leaves (Rohringer and Heitefuss 1961). All of this evidence suggests that the area around uredosori are active metabolically and can be considered as a 'sink' for all metabolites.

Scott and Smillie (1963) have stated that the overall rate of photosynthesis in Erysiphe graminis-infected barley leaf segments decreased when compared with non-infected leaves. However, if one recaculates their data on the basis of chlorophyll content, it is found that the photosynthetic rate is actually increased. Allen (1942), in an earlier study employing Erysiphe graminis-infected wheat leaves, showed that there is no decline in photosynthetic activity per unit of chlorophyll. Since the chlorophyll content of the infected leaves is

reduced by infection, it is only the overall photosynthetic activity that decreases (Shaw and Samborski 1956; Sempio 1950; Allen 1942). Therefore it is entirely possible that the chlorophyll retained (Wang 1961), or reformed (Allen 1942), in the green islands may be more efficient in photosynthesis.

Albugo candida-infected Brassica juncea cotyledons incorporated 5-6 times more $^{14}CO_2$ four days after detachment than did similar non-infected cotyledons (Howard et al 1968). A similar stimulation of photosynthetic CO_2 uptake has been detected by Livine (1964) in infected organs of diseased bean and safflower plants prior to fungal sporulation. In addition to the increased efficiency of the chlorophyll in green islands, Livine speculated that infection might also influence the carbon cycle of photosynthesis, i.e. it is possible that green island photosynthesis follows a different pathway than the normal Calvin cycle which is operative in most healthy plants. Hatch and Slack (1966) proposed a scheme (Scheme 1) for photosynthetic fixation of CO_2 in sugar-cane leaves by measuring the rates of labelling of 3-phosphoglycerate and hexose monophosphates and studying the distribution of radioactivity in their individual carbon atoms of these compounds after feeding $^{14}CO_2$ to the plant. It has been a viewpoint in this investigation that the Hatch and Slack cycle may be the pathway for photosynthetic CO_2 fixation in rust-infected wheat leaves.

Scheme 1



Scheme 1. Proposed pathway for photosynthetic fixation of CO_2 in sugarcane leaves. The broken arrow indicates a minor pathway. (Hatch and Slack 1966).

MATERIALS AND METHODS

Plant materials: Two species of wheat, Triticum compactum Host var. Little Club and Triticum aestivum L. var. Marquis were grown in 6 inch plastic pots under the greenhouse condition at ca. 21°C. Primary leaves of the plants were infected with Puccinia graminis Pers. f. sp. tritici Eriks. and Henn. Race 10, 38 and 48-1 respectively 8 to 10 days after sowing.

The infection type produced by these races on the two varieties of wheat are shown in Table 1 and follows the designation used by the Canada Department of Agriculture, Winnipeg, Manitoba. Variety Little Club with a type 4 infection is susceptible to all stem-rust races employed whereas Marquis is moderately resistant to Race 10 and 38 and very resistant to Race 48-1. The minus sign denotes a slightly greater resistance than the integral number. Both varieties of wheat and the three rust races were obtained from Dr. G.J. Green, Canada Department of Agriculture, Winnipeg to whom the author is grateful.

Table 1

Infection types by the rust races on the two varieties

	Rust Race		
Variety	10	38	48-1
Little Club	4	4	4
Marquis	2-	2--	1

Inoculation: Primary leaves of wheat were inoculated with the three races of uredospores about 10 days after sowing when the primary leaves were 4-5 inches in length and just as the secondary leaves were emerging. The leaves were finely sprayed with water and the spores rubbed evenly on to the epidermis manually between thumb and forefinger. After inoculation the pots containing the plants were covered with plastic bags for 24 hours to maintain 100% relative humidity. Under these conditions, it took an additional 7 to 10 days before symptoms of the infection appeared and another 3 to 5 days before uredial pustules and surrounding green islands, if any, appeared. In summer when light intensity was higher, the leaves were inoculated about 7 days after sowing and the onset of the infection and sporulation stages was accelerated.

Feeding of radioactive compounds to leaves: Rust-infected leaves were detached and immersed in water when sporulation and green islands formation were evident. Primary leaves of the same width and having the same degree of infection (on the basis of numbers of pustules per area) were selected and cut into apical 4 inch lengths. About 20 such leaves (ca. 1.5 grams) were placed with their cut ends in vials containing 0.1 ml of the test radioactive compound. These were either carboxyl ^{14}C nicotinic acid or methyl ^{14}C S-adenosylmethionine with specific activities of 27.9 µc/mM and 43.9 µc/mM respectively. About 1 to 3 µc of the radioactive isotopes were fed to the detached leaves. Healthy uninfected leaves were used as controls. These experiments