

EFFECT OF BENZIMIDAZOLE ON PROTEIN SYNTHESIS, PHOTOPHOSPHORYLATION
AND NICOTINAMIDE ADENINE DINUCLEOTIDE KINASE OF WHEAT LEAVES

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ABSTRACT

The investigations reported in this thesis were a part of an overall research programme in this laboratory to determine the mechanism of action of benzimidazole and kinetin in delaying the onset of visible and metabolic changes occurring during the senescence of detached wheat leaves.

Primary leaves of Khapli (Triticum dicoccum Schübler) and Selkirk (Triticum aestivum L.) wheat senescing under continuous illumination under controlled conditions were used to study the effect of benzimidazole or kinetin on (I) leucine- C^{14} incorporation into cytoplasmic and chloroplastic protein, (II) photophosphorylation by isolated chloroplast fragments and on (III) nicotinamide adenine dinucleotide kinase (EC 2.7.1.23) activity of wheat leaves.

I. Both benzimidazole and kinetin maintained or increased the protein content of leaves as compared to the water controls and increased the specific activity of leucine- C^{14} incorporation into the protein fraction while the specific activity of protein from leaves floated on water showed a less increase or remained fairly constant, indicating that these treatments accelerated protein synthesis rather than inhibiting its breakdown. However, in chloroplasts, the protein content in benzimidazole treated leaves remained at higher levels but there was no increase in the specific activity of protein. These results indicate that of, at least, two different sites involved in the 'benzimidazole effect' (Waygood, E. R. (1965), Plant Physiol. 40: 1242-1246) this particular effect of benzimidazole and kinetin resides outside the chloroplast.

II. Studies on the photophosphorylation of chloroplast fragments were made without the addition of an artificial electron acceptor so that the system should resemble the one occurring naturally. Dicumarol ($6 \times 10^{-5} \text{M}$) was added to inhibit ATPase activity. A 'phosphodoxin' fraction (Black, et al., (1963), Proc. Natl.Acad.Sci. 50: 37-43) isolated from wheat leaves was also tested and shown to accelerate the endogenous rate of photophosphorylation. Chloroplast fragments from leaves floated on water lost their capacity to phosphorylate whereas, those from benzimidazole treated leaves showed an initial substantial increase. Furthermore, benzimidazole was capable of restoring the capacity of chloroplasts to photophosphorylate, especially in the presence of 'phosphodoxin'. A part of these studies has been reviewed briefly by Waygood (vide supra). Studies on the 'photohydrolytic' capacity of chloroplast fragments indicated the rates very similar to those of photophosphorylation.

III. Photophosphorylation and NAD kinase activity are closely inter-related and the latter enzyme was invariably, though not significantly, more active in benzimidazole treated leaves and in leaves grown under relatively high light intensity. NAD kinase was assayed by a spectrophotometric and a fluorimetric method. Doubt was cast upon the validity of the former assay but both methods gave such similar enzyme kinetics that it is highly probable that the 'apparent' NAD kinase activity as measured by the spectrophotometric method, has some enzymatic significance. Both methods showed that the activity of NAD kinase was inhibited 50% by physiological concentrations (10^{-3}M) of orthophosphate

and this was overcome by ATP, the former being a substrate and the latter a product of photophosphorylation. It is speculated and supporting evidence is provided that benzimidazole increases the NADP content of wheat leaves and chloroplasts (Waygood, vide supra) by lowering the orthophosphate content due to its stimulation of photophosphorylation. Benzimidazole per se (10^{-3} M) completely inhibits NAD kinase activity, therefore, it must be a derivative which regulates the 'benzimidazole effect' in wheat leaves.

TABLE OF CONTENTS

	<u>PAGE</u>
LIST OF RADIOACTIVE AND OTHER CHEMICALS USED IN THE EXPERI- MENTS -----	1
LIST OF ABBREVIATIONS -----	2
INTRODUCTION -----	4
REVIEW OF LITERATURE -----	8
I. Role of Benzimidazole and Kinetin in Delaying Senescence -----	8
II. A Senescent Cell and Its Protein Turnover -----	11
III. Energy Levels of Leaves - The Rate of Photophosphory- lation -----	14
IV. NADP Levels and NADP Synthesis in Tissues -----	18
PART I	
EFFECT OF BENZIMIDAZOLE ON PROTEIN SYNTHESIS IN SENESCING LEAVES	
MATERIALS AND METHODS -----	24
RESULTS -----	28
I. Leucine-C ¹⁴ Incorporation into Total Leaf Protein----	28
II. Leucine-C ¹⁴ Incorporation into Chloroplast Protein----	33
PART II	
EFFECT OF BENZIMIDAZOLE ON PHOTOPHOSPHORYLATION OF WHEAT CHLOROPLAST FRAGMENTS	
MATERIALS AND METHODS -----	41
RESULTS -----	46
I. The Endogenous Rate of Photophosphorylation in Wheat Chloroplasts -----	46
II. The effect of Benzimidazole with or without 'Phos- phodoxin' Fraction in Wheat Chloroplast Fragments from Senescing Leaves -----	51

TABLE OF CONTENTS CONTINUED

	<u>PAGE</u>
III. ATPase Activity Associated with Chloroplast Fragments from Senescing Leaves -----	58
IV. The 'photohydrolytic' Activity in the Photophosphory- lation System -----	64
V. Effect of Benzimidazole on Wheat 'phosphodoxin' from Senescent Leaves -----	73
PART III	
STUDIES ON NICOTINAMIDE ADENINE DINUCLEOTIDE KINASE OF WHEAT LEAVES	
MATERIALS AND METHODS -----	77
RESULTS -----	82
I. NAD Kinase Activity-Fluorimetric Method -----	82
II. 'Apparent' NAD Kinase Activity-Spectrophotometric Method -----	97
III. The Effect of Benzimidazole on the Orthophosphate Levels of Wheat Leaves -----	104
IV. NAD and NADP(H) Breakdown by Wheat Leaf Enzyme Preparations -----	110
GENERAL DISCUSSION -----	116
SUMMARY -----	124
BIBLIOGRAPHY -----	127

LIST OF TABLES

<u>TABLE</u>	<u>PAGE</u>
I. Leucine-C ¹⁴ incorporation into total leaf protein of senescing leaves -----	29
II. Leucine-C ¹⁴ into total protein of senescing leaves --	31
III. Leucine-C ¹⁴ into chloroplast protein -----	34
IV. Protein, chlorophyll and DNA content of wheat chloroplasts from senescing leaves -----	38
V. The effect of dicumarol on photophosphorylation and the liberation of orthophosphate in darkness-----	47
VI. The effect of benzimidazole, with or without 'phosphodoxin' fraction, on photophosphorylation in wheat chloroplast fragments from senescing leaves	50
VII. The effect of benzimidazole on photophosphorylation in leaves senescing on water -----	53
VIII. The effect of phenazine methosulfate and adenylate kinase on photophosphorylation in wheat chloroplast fragments from immediately detached leaves-	55
IX. The ATPase activity associated with wheat chloroplast fragments from senescing leaves -----	57
X. The effect of phenazine methosulfate and ADP on ATPase activity -----	62
XI. The effect of light intensity and darkness on the 'hydrolytic' activity of wheat chloroplast fragments -----	65
XII. The rates of orthophosphate liberation in light and darkness in a complete photophosphorylation system with chloroplast fragments from senescing leaves -----	70
XIII. The progress of NAD kinase activity -----	83
XIV. The effect of enzyme concentration on NAD kinase activity -----	83
XV. NAD kinase activity in senescing leaves -----	87
XVI. The effect of benzimidazole on NAD kinase activity---	89
XVII. The effect of orthophosphate on NAD kinase activity--	94

LIST OF TABLES CONTINUED

<u>TABLE</u>		<u>PAGE</u>
XVIII.	The effect of ATP on NAD kinase inhibition by ortho-phosphate -----	96
XIX.	The effect of orthophosphate, ATP and benzimidazole on isocitrate dehydrogenase system -----	98
XXa.	Specificity of ATP as a phosphate donor for 'apparent' NAD kinase activity -----	98
XXb.	The effect of inhibitors on 'apparent' NAD kinase activity -----	99
XXc.	The effect of orthophosphate on NAD breakdown in the presence of enzyme preparations from freshly harvested leaves-Spectrophotometric method -----	100
XXIa.	The effect of 'age' on the orthophosphate content of wheat leaves variety Selkirk or Khapli -----	106
XXIb.	Orthophosphate content of primary and secondary leaves of wheat grown in the growth chamber -----	106
XXII.	The effect of benzimidazole and light on the ortho-phosphate levels of leaves (Selkirk Wheat)-----	107
XXIII.	NAD and NADP breakdown in senescing leaves -----	111
XXIV.	The effect of benzimidazole, kinetin, AMP and ADP on the enzymatic breakdown of NADP -----	111
XXV.	The effect of orthophosphate on the enzymatic breakdown of NAD and NADP-Fluorimetric method -----	112
XXVI.	The enzymatic breakdown of NADP(H) by leaf and chloroplast preparations from freshly harvested leaves -----	112

LIST OF FIGURES

<u>FIGURE</u>		<u>PAGE</u>
I.	Incorporation of leucine-C ¹⁴ into total leaf protein of senescing leaves -----	30
II.	Incorporation of leucine-C ¹⁴ into chloroplast protein of senescing leaves -----	35
III.	Chlorophyll, protein and DNA content of chloroplasts from senescing leaves -----	39
IV.	ATPase activity associated with chloroplast fragments from senescing leaves -----	59
V.	pH curve for light-induced ATPase activity -----	61
VI.	Effect of light intensity on the 'photohydrolytic' activity of chloroplast fragments in a complete photophosphorylation system -----	66
VII.	Effect of 'age' on the 'apparent' orthophosphate utilization by chloroplast fragments in a complete photophosphorylation system -----	71
VIII.	Activation and fluorescence spectra of wheat 'phosphodoxin'.-----	74
IX.	The progress of NAD kinase activity -----	84
X.	The effect of enzyme concentration on NAD kinase activity -----	85
XIa.	pH curve for 'apparent' NAD kinase activity -----	91
XIb.	The effect of ATP concentration on 'apparent' NAD kinase activity -----	92
XIc.	Inhibition of 'apparent' NAD kinase by orthophosphate -----	93
XId.	Reversal of enzyme inhibition by ATP-----	93
XIe.	Inhibition of 'apparent' NAD kinase by kinetin in the reaction system -----	101
XIf.	Inhibition of 'apparent' NAD kinase by benzimidazole in the reaction system -----	101
XIg.	The 'apparent' NAD kinase activity as measured by glutathione reductase assay -----	102

LIST OF RADIOACTIVE AND OTHER CHEMICALS USED

IN THE EXPERIMENTS

L-leucine-U-C¹⁴: (0.16 mg/ml with 0.5 mC/ml) - Radiochemical Centre, Amersham, Buckinghamshire, England.

P³²-orthophosphate (3mC in 1.5 ml 1N HCl): Radiochemical Center.

Albumin Bovine (Fraction V): Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.

Dicumarol: Nutritional Biochemical Corporation.

Benzimidazole: Eastman Kodak, Rochester, New York, U.S.A.

The following chemicals were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

AMP, ADP and ATP-sodium salt

Calf thymus DNA

DL-Isocitrate-trisodium salt

Kinetin

Glutathione (oxidized)

NAD, NADH, NADP and NADPH

PMS

Adenylate kinase-Equine muscle

Alcohol dehydrogenase-Yeast

Isocitric dehydrogenase-Pig heart (NADP specific)(Type IV)

Glutathione reductase (NADP specific)

Hexokinase (Type III)-Yeast

LIST OF ABBREVIATIONS

AMP	-	Adenosine monophosphate
ADP	-	Adenosine diphosphate
ATP	-	Adenosine triphosphate
ADH	-	Alcohol dehydrogenase (EC 1.1.1.1)
AD kinase	-	Adenylate kinase (EC 2.7.4.3)
ATPase	-	ATP diphosphohydrolase (EC 3.6.1.5)
Bz	-	Benzimidazole
c.p.m.	-	Counts per minute
2,4 D	-	2,4-dichlorophenoxyacetic acid
DNA	-	Deoxyribonucleic acid
FMN	-	Flavine mononucleotide
IDH	-	Isocitric dehydrogenase (EC 1.1.1.42)
ITP	-	Inosine triphosphate
Kn	-	Kinetin
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	reduced NAD
NADP	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Reduced NADP
NAD kinase	-	NAD 2'-phosphotransferase (EC 2.7.1.23)
NAD pyrophosphatase	-	Nucleotide pyrophosphatase (EC 3.6.1.9)
PFK	-	Phosphofructokinase (EC 2.7.1.11)
Pi	-	Inorganic phosphate
PMS	-	Phenazine methosulfate
PP	-	Pyrophosphate

LIST OF ABBREVIATIONS CONTINUED

PPNR	-	Photosynthetic pyridine nucleotide reductase
RNA	-	Ribonucleic acid
TCA	-	Trichloroacetic acid
Tris	-	Tris (hydroxymethyl) aminomethane

INTRODUCTION

INTRODUCTION

Studies in this laboratory have been primarily concerned with the elucidation of the mode of action of benzimidazole and kinetin in delaying the visible and metabolic changes occurring during the senescence of detached wheat leaves floated on water under controlled conditions. Some of the physiological changes manifested by leaves senescing on water are as follows; (i) rapid loss of chlorophyll, (ii) decline in protein, nucleic acids and starch, (iii) increase in free amino acids and sugars, (iv) decrease in ATP and NADP contents with a corresponding increase in ADP and NADH levels and finally (v) increase in proteolytic and hydrolytic enzymes (Person, et al., 1957; Samborski, et al., 1958; Wang and Waygood, 1959; Wang, et al., 1960; Mishra, 1963; Godavari, 1966). These degradative changes can be reversed by floating the leaves either on benzimidazole or kinetin solution at a concentration of 50 and 5 mg per liter respectively.

This thesis provides an extension to these investigations which are reviewed in detail later, while the most recent studies including some of the experimental work reported in this thesis, have been summarized by Waygood (1965). The thesis is comprised of three separate sections as described below with a general review of literature, general discussion and summary. Methods and materials, results and discussion of the results are presented separately in each section.

I. THE EFFECT OF BENZIMIDAZOLE ON PROTEIN SYNTHESIS

This investigation was undertaken since it had been shown by

Person, et al., (1957), Samborski, et al., (1958) and repeated many times in this laboratory that benzimidazole treatment increased the protein content of excised wheat leaves. Samborski, et al., (1958) investigated the effect of benzimidazole on the infection of rust in wheat leaves and arrived at a conclusion that benzimidazole treatment stimulated protein synthesis in leaves and caused a drop in free amino acids essential for rust infection. Mishra (1963) reported an increase in protein content in detached leaves which was more profound in light than in darkness. It was, therefore, important to determine whether this increase was due to an acceleration of protein synthesis or an inhibition of its breakdown. A solution to this problem can be obtained by determining the rate of incorporation of leucine-C¹⁴ into cytoplasmic and chloroplast protein since leucine is incorporated solely in the protein fraction. The results on the time study of leucine-C¹⁴ incorporation into total and chloroplast protein fraction of senescing leaves are discussed in the first part of the thesis.

II. THE EFFECT OF BENZIMIDAZOLE ON PHOTOPHOSPHORYLATION

As has been pointed out by Waygood (1965) there are at least two different sites of action in the 'benzimidazole effect' in delaying senescence and one of them resides in the chloroplasts. Benzimidazole treatment showed a selective effect on chlorophyll metabolism of wheat leaves (Wang and Waygood, 1959) and maintained high levels

of ATP in chloroplasts (Mishra, 1963). An investigation was, therefore, undertaken to determine the effect of benzimidazole on the photochemical ATP formation in wheat leaf chloroplasts. Studies on the isolation of wheat 'phosphodoxin' and its effect on the endogenous rate of photophosphorylation, were prompted by the report (Black, et al., 1963a) on its ubiquitous occurrence as a natural cofactor in photophosphorylation.

In order to study the effect of benzimidazole on ATP formation of wheat chloroplasts, it was felt necessary that the reaction system should resemble as closely as possible to the one occurring naturally. For this reason, no artificial electron acceptor was used in the present system. The results on the endogenous rate of photophosphorylation with or without 'phosphodoxin' in the reaction system are given in the second part of the thesis.

A part of these investigations has been reviewed briefly by Waygood (1965).

III. THE EFFECT OF BENZIMIDAZOLE ON NAD KINASE ACTIVITY

Mishra (1963) reported a marked increase in cytoplasmic as well as chloroplast NADP content in wheat leaves senescing under continuous illumination on either benzimidazole or kinetin solution. Studies on the biosynthetic pathway of NAD in wheat leaves provided further evidence that benzimidazole treatment favoured an accumulation of radioactivity in NADP content of wheat leaves fed with radioactive precursors of NAD such as, nicotinic acid (carboxyl- C^{14}) or nicotinamide (carbonyl- C^{14}).

Wheat leaves senescing on water, however, showed a tendency to accumulate most of the label in NAD rather than NADP fraction (Godavari, 1966).

This investigation was concerned with a study on the NAD kinase activity in wheat leaves with a view to explain the increase in NADP levels in leaves treated with benzimidazole. The results on the NAD kinase activity of wheat leaves with a possible regulatory mechanism in NADP synthesis, are summarized in the last part of the thesis.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

I. ROLE OF BENZIMIDAZOLE AND KINETIN IN DELAYING SENESCENCE

The process of cell division, growth, maturity and senescence are sequential phenomena in biological systems. Senescence is the ultimate event of these processes culminating the consequence of metabolic changes that occur at cellular and subcellular levels. Although various theories have been proposed to explain the loss of cellular vitality in senescence, the changes instrumental in cellular 'aging' are not yet fully understood (Varner, 1961). However, recent theories about 'aging' are centered primarily around the impairment of RNA synthesis leading to a gradual decrease in protein content essential for the metabolic activity of a cell (Sacher, 1965). Protein plays a vital role in the structural integrity and the biochemical activity of a cell and any change, either qualitative or quantitative, in protein synthesis is bound to affect the normalcy of a cell (Bonner, 1959; Monod, et al., 1963).

The degradative processes leading to 'aging' of excised plant tissues can be appreciably retarded by the administration of auxins, indole acetic acid, 2,4-D, gibberellic acid and growth regulators such as kinetin and benzimidazole or by the formation of roots on tissues. Gregory and Samantrai (1950) suggested that during root formation, tissues such as leaves receive certain root factors necessary for the synthesis of proteins. Varner, et al., (1963) showed that the course of senescence in pea cotyledons, was controlled by the 'signals' received from the axis of the seedlings. The biochemical nature of these factors controll-

ing senescence in the tissue, although ascribed to hormones and auxins, is unknown (Thimann, 1963). Growth regulators, such as kinetin and benzimidazole appear to substitute for natural factors in delaying senescence in excised plant tissues. They resemble auxins in promoting protein synthesis, cell division and root or callus formation (Person, et al., 1957; Richmond and Lang, 1957; Osborne, 1962; Bachelard and Stowe, 1963).

Action of kinetin. Kinetin (6-furfuryl-aminopurine) characterized as a 'cell division' factor has not been shown to be a natural constituent in plants but kinetin-like substances have been reported from over 40 different species of plants (Helgeson, 1964). Beauchesne (1961) was able to isolate a compound from corn which resembled adenine and which he found was more active than kinetin in stimulating cell division and in promoting bud formation in tobacco tissue culture. 'Zeatin' (6-4, hydroxy-3-methylbut-2-enyl aminopurine), a crystalline substance isolated from corn was also found to be more active than kinetin in its growth promoting action (Letham, 1963). Kinetin-like substances have been isolated from peas, coconut milk and apples (Rogzinska, et al., 1965; Zwar, et al., 1963). Dunn, et al., (1955,1960) reported the occurrence of 6-methyl aminopurine in the DNA of Eschericia coli and in the RNA of wheat germ.

The action of kinetin in various plant tissues and organs, is known to be profound and diversified. In excised leaves and tissues, kinetin not only inhibits protein degradation but also it induces protein synthesis (Mothes and Engelbrecht, 1961; Parthier, 1961).

Application of kinetin to excised Xanthium leaves prevents loss of chlorophyll and stimulates protein as well as RNA synthesis (Osborne, 1962). It induces rooting in the leaves of Acer rubrum (Bachelard and Stowe, 1963) and promotes anthocyanin synthesis in petals of Impatiens balsamina (Klein and Hagen, 1961). Kinetin stimulates synthesis of DNA in excised tobacco pith tissue (Patau, et al., 1957) and barley leaves (Srivastava and Ware, 1965).

Action of benzimidazole. Benzimidazole, another growth factor, has not yet shown to be a natural plant product but benzimidazole or 5,6-dimethyl benzimidazole and closely related compounds have been reported to be an integral part of vitamins and coenzymes (Weissbach, et al., 1959). Coenzyme B₁₂ has been isolated from plant parts such as, nodules of Alder and extracts of germinating peas or wheat and lupine plasts, (Kliwar and Evans, 1962 a,b and c; Fries, 1962).

The action of benzimidazole is similar to that of kinetin in many ways. However, it appears to be more selective in its action for monocotyledons (Wang, et al., 1961). Benzimidazole treatment stimulates protein and chlorophyll synthesis in excised wheat leaves and maintains the property of resistance to rust in detached leaves of Khapli wheat (Wang and Waygood, 1959; Wang, et al., 1960; Samborski, et al., 1958). However, it failed to show any preservative effect on chlorophyll of bean, sunflower or tobacco leaves (Wang, et al., 1961). It also failed to inhibit elongation of etiolated pea stems (Forsyth and Samborski, 1960). On the other hand, kinetin treatment prevents loss of chlorophyll and inhibits cell elongation in the tissues respectively described

above (Osborne and McCalla, 1961).

Mode of action. The exact role played by kinetin and benzimidazole in delaying senescence in excised plant tissues, is not yet fully understood. There is a general agreement that these growth factors regulate protein metabolism in a cell by either exerting their influence at RNA, DNA and ribosomal levels or by controlling enzymatic reactions which participate in protein synthesis at cytoplasmic or organelle levels. Three possible mechanisms have been proposed for such a control; (i) direct participation of the growth factor in the RNA, or DNA molecule (McCalla, et al., 1962; Fox, 1965), (ii) formation of an active metabolite on entry in the cell (Alivisatos, et al., 1962; Kapoor and Waygood, 1965a and b) or (iii) inhibition of hydrolytic enzymes and stimulation of synthetic processes (Osborne, 1962; Ingle, et al., 1965; Srivastava and Ware, 1965, Srivastava, 1965).

II. A SENESCENT CELL AND ITS PROTEIN TURNOVER

There is a gradual decrease in protein levels in an 'aging' or senescent cell. This decline in protein content has been shown to be associated with (i) an increase in respiration rate (Gibbs and Beevers, 1955), (ii) a shift in metabolic pathways (Laties, 1964) and (iii) a change in enzymatic complement (Varner, et al., 1963; Smillie, 1962). Robinson (1956) reported a general increase in proteolytic enzymes in mature root cells. A similar increase in the activity of hydrolytic enzymes has been reported in a variety of 'aging' tissues (Kessler, et al., 1962; Shuster, et al., 1962). Hulme (1958) reported a net increase in

protein nitrogen, during climacteric and active ripening of apple and pear fruits. The climacteric rise in CO_2 production was further shown to be due to the synthesis and increase in activity of malate dehydrogenase (EC.1.1.1.38-40) and pyruvic carboxylase (EC 6.4.1.1) in ripening apples (Hulme, et al., 1963).

The changes in enzyme complement associated with 'aging' need not necessarily be due to de novo synthesis and could be accounted for as either an increase or decrease in the catalytic activity of the existing enzymes or their release from the bound form or their conversion into catalytically active form. The evidence obtained with the use of specific inhibitors or radioactive amino acids, indicates that de novo synthesis of some enzymes, does occur in both the mature and senescing tissues (see below).

Protein synthesis in senescing tissues. (a). Use of specific inhibitors. Matsushita (1959) reported an increase in the levels of RNAase (EC 2.7.7.16) in germinating wheat endosperm and suggested it to be due to de novo synthesis of protein in the tissue. Shannon, et al., (1964) showed that increase in RNAase activity of corn mesocotyl tissue could be prevented by the use of actinomycin D or puromycin.

(b) Use of radioactive amino acids. Much of the evidence on protein turnover in senescent tissue, has been provided by the use of radioactive amino acids in protein synthesis. The first direct evidence for protein turnover in leaves was given by Vickery, et al., (1946). It was found that isotopic nitrogen from N^{15} -ammonia fed to tobacco plants, was incorporated into leaf protein in spite of the net

protein breakdown in this tissue. Christensen and Thimann (1949) reported an increase in protein content with a corresponding decrease in free amino acids during the growth of excised pea internodes cultured in water. Racusen and Foote (1960) fed detached soybean leaves alternately with $C^{14}O_2$ and $C^{12}O_2$ and provided evidence that free amino acids were incorporated into leaf protein at a rate of 2% per hour.

Incorporation of labelled amino acids into protein fraction of excised leaves, roots or isolated chloroplasts, was shown to be inhibited in the presence of chloramphenicol, puromycin or actinomycin (Jacoby and Sutcliffe, 1962; Sissakian, et al., 1965). These findings suggest that mature and senescing cells are capable of protein synthesis, although they show an overall decline in protein levels.

(c) Amino acid complement of senescent tissue. A decline in protein content of mature tissue was reported to be due to the inability of the tissue to incorporate amino acids into protein fraction (Racusen and Aranoff, 1954). One of the possibilities that excised tissues or leaves were lacking in free amino acids, was ruled out by the observations of Rogers (1955) and Racusen, et al., (1954). Rogers (1955) found that leaves treated with sucrose- C^{14} were able to incorporate the radioactivity into every one of the amino acids ordinarily found in protein. Free amino acids do not appear to be a limiting factor for protein synthesis in senescing leaves. Since energy in the form of ATP is required for the synthesis (Webster, 1955; 1957) as well as degradation of proteins (Steinberg and Vaughan, 1956), it is suggested by MacDonald,

et al., (1958) and Muir, et al., (1959) that any factor which affects the energy levels of senescing leaves is a limiting factor in protein turnover.

III. ENERGY LEVELS OF LEAVES. THE RATE OF PHOTOPHOSPHORYLATION.

A cell requires energy in the form of ATP molecules for biosynthetic, degradative and mechanochemical processes (Jacoby, 1964; Quastel, 1964; Arnon, 1961). But synthesis of ATP also requires energy. Green plants and photosynthetic bacteria are unique in utilizing the electromagnetic radiation energy for this purpose. The conversion of light energy into the chemical bond energy of ATP molecules is termed 'photosynthetic phosphorylation'. This process is distinct from that of 'oxidative phosphorylation' or substrate phosphorylation (Arnon and Whatley, 1954b).

Photophosphorylation by isolated chloroplasts. Arnon, et al., (1954a) discovered that chloroplasts isolated from spinach leaves, when illuminated under suitable conditions, were able to synthesize ATP. Green leaves were shown to contain a paucity of mitochondria (Esau, 1953; James and Das, 1957) and isolated whole chloroplasts, without the aid of mitochondria, were shown to synthesize ATP in the light but not in darkness (Arnon, et al., 1954a). It was, therefore, proposed by Arnon, et al., (1958) that photochemical ATP formation was the main source of energy required for the biosynthetic reactions in leaves.

The primary photochemical reaction in photophosphorylation con-

sisted of the following steps:

- (i) the absorption of photon by the assimilatory chlorophyll pigments,
- (ii) the excitation and expulsion of an electron from the chlorophyll molecules,
- (iii) the transfer of electron through the cofactors and
- (iv) the formation of energy products, such as ATP and NADPH coupled with electron transfer (Arnon, 1964).

The overall process can be summarized as follows:

- (a) $\text{nADP} + \text{nPi} = \text{nATP} \dots\dots\dots$ (Cyclic process)
- (b) $2\text{ADP} + 2\text{Pi} + 2\text{NADP} + \frac{1}{2}\text{H}_2\text{O} = 2\text{ATP} + 2\text{NADPH}_2 + \text{O}_2 + 2\text{H}_2\text{O} \dots\dots$ (non-cyclic).

Cofactors in photophosphorylation. Common 'redox' chemicals such as, methyl phenazonium methosulfate (PMS), ferricyanide and indophenol dyes have been shown to support and stimulate the endogenous rate of photophosphorylation by isolated chloroplasts (Jagendorf and Avron, 1958; Arnon, 1963). These chemicals have not been known to occur naturally in leaves and their role in photophosphorylation under physiological conditions is uncertain. Conversely, substances such as, ascorbate, vitamin K, FMN, cytochrome c, NADP and PPNR have been reported to occur and to catalyze photophosphorylation in chloroplasts isolated from green leaves (Forti and Jagendorf, 1961; Whatley, et al., 1955; Giovanelli and San Pietro, 1959). All these cofactors have been suggested to be involved primarily in electron transfer reactions rather than in the photochemical ATP formation (Black and San Pietro, 1963c).

'Phosphodoxin' as a cofactor in photophosphorylation. Black, et al., (1963) isolated a naturally occurring water soluble compound from spinach leaves and suggested its involvement in photophosphorylation reaction of intact leaves. This substance called 'Phosphodoxin' is a thermostable compound which is able to catalyze photophosphorylation in the absence of exogenous electron acceptors. It shows a response to light intensity similar to that shown by other cofactors and gives a high rate of ATP formation when used with or without PMS in the reaction mixture. Although the mode of action of 'phosphodoxin' in photophosphorylation is unknown, it is found to be of common occurrence in chloroplasts as well as bacterial chromatophores (Black, et al., 1964).

ATPase activity associated with chloroplasts. The ATP/ADP ratio in the leaves is subjected to a change under the catalytic activity of an enzyme ATPase (EC 3.6.1.5)(ATP = ADP + Pi). ATPase activity has been reported to be present in chloroplasts (Hoch, et al., 1963; Avron, 1962) and cell free extracts of blue green alga (Petrack, et al., 1961). The available evidence indicates that there are two light-induced ATPases present in the chloroplasts; one requires sulfhydryl compounds, Mg^{++} ions, PMS and brief exposures to light while, the other needs, PMS, Ca^{++} but not Mg^{++} ions and continuous illumination. The pH optimum for both the activities was the same as for photophosphorylation. The known inhibitors of photophosphorylation such as, sodium fluoride or azide, ammonium chloride and 2,4-dinitrophenol have been shown to inhibit ATPase activity (Avron, 1964).

Avron (1962) suggested a correlation between ATP formation and its light-induced hydrolysis in chloroplasts (referred to as their photohydrolytic capacity, Section II). Molotkorsky and Zheskova (1965) reported a three-fold increase in ATPase activity of chloroplasts under the influence of heat and suggested it to be the measure of mechano-chemical processes undergoing in chloroplasts. Investigations on the light-scattering measurements (Packer, et al., 1963) showed that mitochondria, chloroplasts and photosynthetic bacteria undergoing energy transfer reactions manifested changes in the membrane structure. A protein fraction isolated from spinach chloroplasts showed ATPase activity and manifested structural changes in chloroplasts under the influence of light. The aged chloroplasts, however, developed a requirement for ATP to undergo similar changes. These chloroplasts failed to show light-scattering changes in the presence of ammonium chloride which is a known inhibitor of photophosphorylation. These results led Packer, et al., (1963) to postulate that light-induced ATPase activity was involved in the control of structural changes associated with the electron transfer reactions occurring in photophosphorylation.

Besides the light-induced ATPases, chloroplasts have been reported to possess a small amount of ATPase activity independent of light (Black, et al., 1963b). This enzyme is activated by Mg^{++} ions and high temperatures (Wessels and Baltscheffsky, 1960), or by fragmentation of chloroplasts by sonication (Black, et al., 1963b). Its relationship to the light-induced ATPase activity is not yet clear (Avron, 1964).

IV. NADP LEVELS AND NADP SYNTHESIS IN TISSUES

Formation of NADP has been unanimously shown to be a single step reaction catalyzed by an enzyme NAD kinase (EC 2.7.1.23) with NAD as a substrate. Von Euler and Vestin (1938) reported the synthesis of NADP from NAD and ATP when incubated in the presence of crude yeast extract. Similar results were reported by Mehler, *et al.*, (1948) with the use of crude pigeon liver fractions. The enzyme mediating the phosphorylation of NAD to form NADP, was isolated by Kornberg (1950) from dried brewer's yeast and later by Wang and Kaplan (1954) from pigeon liver. It was subsequently found to be present in rabbit liver and kidney (Katchman, *et al.*, 1951), rat liver (Greenbaum, *et al.*, 1965), alga (Oh-Hama and Miyachi, 1959) and cotyledons and leaves of higher plants (Forti, 1961; Yamamoto, 1961; 1966b; Ogren and Krogmann, 1963, 1965; Waygood, 1965).

The enzyme isolated from different sources, shows a slight variation in substrate specificity. Thus, NAD kinase isolated from either pigeon liver or higher plants, unlike yeast enzyme, is highly specific for its substrate requirements and catalyzes the following reaction:

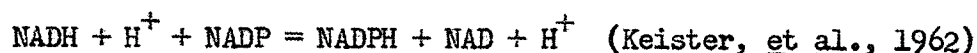


while, the yeast NAD kinase, in addition to reaction 1, catalyzes



Plant NAD kinase is able to use ITP+ADP to phosphorylate NAD to NADP (Yamamoto, 1966b) while, broken cells of Chlorella ellipsodea can use

either ATP or Pi as a phosphate donor (Oh-Hama and Miyachi, 1963). The conversion of NADP to NADPH is catalyzed by an enzyme 'transhydrogenase' (EC 1.6.1.1) according to the following reaction:



Localization of NAD kinase in tissues. Villee and Hagerman (1958) reported the presence of NAD kinase in the soluble fraction of human placenta and suggested the extramitochondrial localization of this enzyme in contrast to the synthesis of NAD which is located in the nuclear and mitochondrial fraction (Morton, 1961; Hogeboom and Schneider, 1952). In leaves, although most of the activity was recovered in the soluble fraction, a small amount was found to be associated with chloroplast fraction as well (Ogren and Krogmann, 1965; Yamamoto, 1966a).

NADP levels in excised tissues. In general, the levels of NAD and NADP were reported to remain fairly constant in the majority of animal and plant tissues with NAD in the oxidised and NADP predominantly in the reduced form (Glock and McLean, 1955; Greengard, et al., 1964; Mishra 1963; Yamamoto, 1966a). Certain hormonal treatments as well as 'aging' were shown to alter the concentration and the existing balance between these two coenzymes. Villee and Hagerman (1958) suggested that the synthesis of NADP was under hormonal control. Clark, et al., (1964) investigated the rate of NADP synthesis in precancerous and cancerous tissue of rat livers. The cancerous tissue treated with carcinogenic dyes showed a significant fall in NADP + NADPH levels over the controls. However, the rate of NADP synthesis as measured by NAD kinase activity was

found to be appreciably higher as compared to the actual levels of this coenzyme in the tissue.

Yamamoto (1963) observed a decrease in NADPH/NADP ratio in older leaves of tobacco while Mishra (1963) showed a decrease with age in NAD/NADH ratio and NADP + NADPH content in wheat leaves senescing on water.

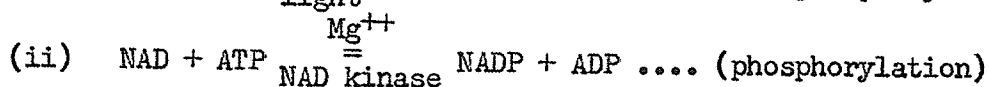
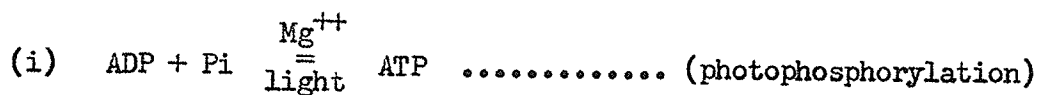
Greenbaum, et al., (1965) measured NAD kinase activity in livers of rats treated with glucogen, thyroxine and pituitary growth hormone and arrived at a conclusion that there was no correlation between NADP levels and NAD kinase activity in the tissue. There was a decline in NADP levels after 3 days of treatment while, NAD kinase isolated from the tissue showed no change in activity. The levels of NAD in the tissue did not appear to limit the rate of NADP synthesis as the injection of nicotinamide gave a 10-fold increase in NAD levels without showing any change in NADP levels. It was concluded from these investigations that NADP synthesis was under other regulatory mechanisms.

Regulation of NADP synthesis. 1. Availability of ATP. Since ATP is one of the substrates for NADP synthesis, it was thought to be implicated in the regulation of NAD kinase activity (Melhuish and Greenbaum, 1961; Greenbaum, et al., 1965; Glock and McLean, 1955; Kaplan and Greenberg, 1944). A correlation between ATP and NADP levels in rats treated with thyroxin, insulin or growth hormone was reported by these workers. Precancerous rat livers showed a decline in ATP (Reid, 1962) and NADP levels but no change in NAD kinase activity (Clerk, et al., 1964). However, Greenbaum, et al., (1965) suggested

that a decrease in ATP levels would affect the NAD kinase activity as the Michaelis constant for NAD kinase for ATP was 18 mM (Wang and Kaplan, 1954) while that for NMN adenylyltransferase (EC 2.7.7.1) an enzyme catalyzing NAD synthesis, was 0.46 mM (Kornberg, 1950).

2. Effect of light. The involvement of light in NADP synthesis was first reported by Oh-Hama and Miyachi (1959) in Chlorella cells. These cells when exposed to light, showed an increase in NADP/NAD ratio, under both the aerobic and anaerobic conditions. Under aerobic conditions, however, the increase in darkness and a decrease in light of NAD content in the cells, was almost equal to the corresponding decrease or increase in the endogenous levels of NADP + NADPH. NADH levels were low in these cells either in light or darkness.

Further investigations with disintegrated algal cells (Oh-Hama and Miyachi, 1963) showed that the photochemical conversion of NAD to NADP was independent of ATP and the algal cells could use Pi as a phosphate donor. Thus, the algal cells exposed to light underwent the following reactions:



with the overall reaction



NAD and NADP undergoing diurnal changes in light and darkness, were suggested to control the 'dark' and 'light' respiratory metabolism in plants (Oh-Hama and Miyachi, 1963).

found to be appreciably higher as compared to the actual levels of this coenzyme in the tissue.

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Ogren and Krogmann (1963,1965) observed similar reversible changes in NAD and NADP + NADPH levels in bean leaves exposed to alternate dark and light periods. Detached wheat leaves also underwent similar changes in light and dark periods (Mishra, 1963).

3. Changes in oxygen levels. Storage organs such as, cotyledons of bean seeds were reported (Yamamoto, 1961) to have low NADP content and a high NADH/NAD ratio. These tissues also failed to show any isocitrate dehydrogenase activity in the absence of exogenous NADP. In contrast, high NADP content was observed in the growing parts of the germinating seeds and in tissues growing under aerobic conditions showing low NADH/NAD ratio. These tissues showed the isocitrate dehydrogenase activity in the absence of added NADP. It was, therefore, suggested by Yamamoto (1961) that isocitrate dehydrogenase activity was controlled by NAD kinase essential for NADP synthesis and that NAD kinase controlled the carbohydrate metabolism in storage organs such as cotyledons.

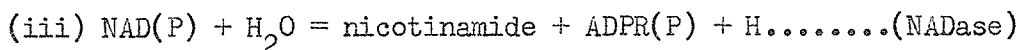
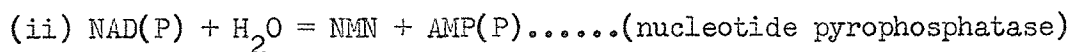
Yamamoto (1966a) later showed that aerobic conditions were essential to maintain NAD in the oxidised state since NADP production was dependent on higher NAD/NADH ratio in the tissues.

4. Redox state of NAD. The reduced NAD was reported to be a potent inhibitor of NAD kinase activity (Wang and Kaplan, 1954; Yamamoto 1966b). High levels of NADH were found under anaerobic (Yamamoto, 1966a) conditions as well as during 'aging' of the tissue (Mishra, 1963). Thus anything such as gaseous condition or enzyme 'transhydrogenase'

catalyzing the reduction of NAD by NADPH, which increased the concentration of NADH was suggested to affect the NAD kinase activity (Yamamoto, 1966a).

5. Ion shift. A high concentration of Mg^{++} ions (9 mM) is required for NAD kinase activity (Kornberg, 1950). This is reported to be 50 times as high as that required for NAD synthesis. It was, therefore, suggested by Greenbaum, et al., (1965), that a shift in ion concentration might influence phosphorylation of NAD to form NADP.

NADP breakdown. A balance between the synthetic and degradative processes, regulates NADP levels in a tissue. The phosphatases cleaving the 2' phosphate of NADP have been shown to be present in green leaves (Forti, et al., 1962). There are three enzymes responsible for the breakdown of NADP in plants; (i) NADP or NADPH-phosphatase (Forti, et al., 1962), (ii) nucleotide pyrophosphatase (EC 3.6.1.9) (Kornberg, 1955) and (iii) NADase (EC 3.2.2.6)(Kaplan, 1955) which catalyze the following reactions:



Among these, enzymes (i) and (iii) are reported to be highly active in decomposing NADP(H) in plant leaves and their activity is shown to be independent of the redox state of NAD (Yamamoto, 1966a).

PART I

EFFECT OF BENZIMIDAZOLE ON PROTEIN SYNTHESIS IN SENESCING LEAVES

MATERIALS AND METHODS

PLANTS

First leaves of Khapli wheat (Triticum dicoccum Schübler) grown either in the growth chamber or in the greenhouse for 7 to 10 days, were used throughout this investigation. The experimental conditions concerning each experiment are described at the appropriate place in the text.

Detached leaf culture. Excised leaves in five gram lots were floated on either 500 ml of distilled water, benzimidazole (50 mg per liter) or kinetin solution (5 mg per liter) in pyrex trays (12" wide x 18" long x 2.5" deep). The trays were covered with Saran Wrap and placed in a growth chamber at 21°, under continuous illumination. The light source was a bank of 12 fluorescent lamps (F72T12), General Electric Company) of white cool light and 6 incandescent lamps of warm daylight. The light intensity (measured by General Electric Light Meter) used in each experiment varied between 750 to 850 ft-c, at the surface of the floated leaves. The leaves used in feeding experiments were harvested at zero time (immediately detached) and at subsequent intervals of 1, 3 and 5 days. Precautions were taken to exclude the leaf samples showing any visible signs of contamination.

Radioactive isotope feeding experiments. The feeding procedure followed by Wang (1959) was adapted in the experiments to feed excised leaves with radioactive isotope. The cut ends of detached leaves (12 to 15 leaves) were placed into 0.3 ml solution of leucine-C¹⁴ (0.016 mg/ml = 5 μ C/ml) in a vial. The vials were then placed in a growth chamber at 21°, under continuous illumination at

750 ft-c, for a period of 5 hours. Sufficient quantities of water were added to the vials to protect the leaves from drying after the feeding solution had been absorbed within first 40 to 50 minutes. The experiments were conducted in triplicate.

Extraction procedure. At the end of each feeding period, leaves were washed, dried on filter paper and weighed. They were then cut into small sections and plunged immediately in 85% boiling ethanol prior to protein extraction, using the method of Wang (1959). The solutions were refluxed successively for 30 minutes, twice in 80% once in 40% and finally in distilled water. The alcohol insoluble fraction was ground in 80% ethanol and centrifuged at 5,000 rpm for 10 minutes. The residue was washed once in each of 80% cold and hot ethanol, ethanol:ethyl ether (1:1, v/v) and ethyl ether. It was then hydrolyzed twice with 1N NaOH at 90° for 15 minutes. The aliquots were combined and made to volume.

ISOLATION OF CHLOROPLASTS

(a) For radioactivity measurements. Chloroplasts were isolated from leaves by the method of Jagendorf and Avron (1958). They were washed once in the grinding medium containing 0.4M sucrose, 0.01M NaCl and 0.05M Tris-HCl buffer, pH 7.5 and the protein was precipitated with 5% TCA (final concentration). The TCA insoluble fraction was washed and hydrolyzed with 1N NaOH at 90° for 15 minutes and made to volume.

(b) For DNA and protein determination. Chloroplasts were isolated by the method of Pollard (1964). Leaves were soaked in 0.01M $MgCl_2$ solution for 2 to 4 hours at 0° and ground in a medium containing 0.3M sucrose, 0.01M $Ca(NO_3)_2$, 0.05M Tris-HCl buffer, pH 7.8 with 6% polyvinyl pyrrolidone. The brei was filtered through 4 layers of Kleenex tissue and the filtrate was centrifuged at 5,000 rpm for 10 minutes. The residue was suspended in a solution containing 0.5M sucrose in 0.05M sodium phosphate buffer, pH 7.5.

The chloroplast fraction was further purified by the sucrose density gradient technique (Messelson, Stahl and Vinograd, 1957). Lusteroid tubes (5.0 ml capacity) were layered successively with 1.5 ml of 70% sucrose solution ($d \approx 1.34$) followed by 2.0 ml of 50% solution ($d \approx 1.22$) and 1.0 ml of chloroplast suspension ($d \approx$ approx. 1.06). The tubes were centrifuged at 39,000 rpm for 30 minutes (running time) using a Spinco swinging bucket preparative ultracentrifuge (Model SW 39L) without using automatic brakes. The two layers separated at the interphase of 50% sucrose solution were collected for DNA and protein determination.

ANALYTICAL PROCEDURES

Determination of DNA. The procedure suggested by Pollard (1964) was used for the extraction of DNA from the chloroplasts. The chloroplast fraction was extracted stepwise with hot methanol, methanol: ethyl ether (1:1, v/v) and ethyl ether. The residue was treated at room temperature with 0.5N perchloric acid for a period of 10 minutes.

This acid washed residue was then used to extract DNA by heating it with 0.5N perchloric acid at 85° for 10 minutes. The supernatant was used for DNA measurements by diphenylamine method (Burton, 1956). Calf Thymus DNA was used as a standard for DNA determination.

The residue left after DNA extraction was washed in methanol, methanol:ethyl ether (1:1, v/v) and ethyl ether solution and hydrolyzed with 1N NaOH for protein determination.

Protein determination. Protein determinations were made by the Folin-Phenol reagent of Lowry, et al., (1951). Crystalline bovine albumin was used as a standard.

Chlorophyll determination. Chlorophyll was determined on 1 gram samples of wheat leaves by the method of Arnon (1949) as modified by MacKinney (1961).

Radioactivity measurements. Aliquots of 20 to 50 μ l were used to measure the radioactivity using Nuclear Chicago (Model No.186) with a D.47 end window gas flow counter with a micromil window.

Colorimetric measurements. All the colorimetric measurements were made by using a 'Spectronic 20' colorimeter. Protein was measured at 500 m μ , DNA at 600 m μ and chlorophyll at 652 m μ . Chlorophyll was also measured in Zeiss Spectrophotometer (Model PMQ II).

RESULTS

I. LEUCINE-C¹⁴ INCORPORATION INTO TOTAL LEAF PROTEIN

The experiments reported in this section were conducted on excised wheat leaves senescing under continuous illumination as benzimidazole was reported to show a pronounced effect on protein synthesis in light as compared to darkness (Person, *et al.*, 1957; Samborski, *et al.*, 1958; Mishra, 1963). Similarly Stephenson, *et al.*, (1956) showed that incorporation of radioactive amino acids into protein fraction of tobacco leaf discs was greater in discs subjected to light than those maintained in darkness. The results from two separate experiments (I and II) on the protein synthesizing capacity of senescing leaves are given in Table I. The mean values of data in Table I together with the percentage increase in the specific activity and radioactivity recovery are summarized in Table II (and Figure 1).

The data in Table I show that there was a considerable loss of protein in detached leaves floated on water for 5 days whereas leaves treated with benzimidazole or kinetin maintained or even increased the protein content. In all treatments leucine-C¹⁴ was incorporated into protein fraction, but the incorporation was considerably greater in leaves floated on benzimidazole or kinetin solution, except that the kinetin treatment showed a decrease on the 5th day of treatment. The fact that these leaves showed a higher level of protein with a corresponding increase in the specific activity, indicates clearly that benzimidazole or kinetin accelerates new protein

TABLE I. Leucine-C¹⁴ incorporation into total leaf protein of senescing leaves.

Treatment	Days floated	mg protein per gram fresh wt		Loss of protein % Control		Total counts x 10 ²		Specific Activity x 10 ²	
		Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
Immediately detached	0	4.55	3.82	(100)	(100)	117.57	150.00	25.84	39.27
Water	1	3.38	3.05	25.72	20.16	94.90	172.50	28.08	56.56
	3	3.35	2.59	26.38	32.20	110.68	222.50	33.04	85.91
	5	2.52	2.10	44.62	45.03	82.73	165.00	32.83	78.57
Benzimidazole	1	4.87	3.10	-	18.85	126.95	275.00	26.07	88.71
	3	3.96	3.60	12.97	5.76	152.74	330.00	38.57	91.67
	5	3.82	3.40	16.05	11.00	220.74	295.80	57.78	87.00
Kinetin	1	4.44	3.59	2.42	6.02	185.94	242.50	41.87	67.55
	3	4.44	3.60	2.42	5.76	232.50	377.50	42.36	104.86
	5	4.18	3.70	8.14	3.15	207.50	205.12	49.64	55.44

Wheat leaves (5 g) detached from 7 to 8 days old Khapli seedlings grown under greenhouse conditions were floated under continuous illumination of 750 ft-c.

Total counts fed: 1.17 x 10⁵ cpm. Feeding period: 5 hours.

FIGURE I. The Incorporation of Leucine-C¹⁴ into Total Leaf Protein of Senescing Leaves.

The lower lines indicate the level of protein while the upper lines indicate specific activity.

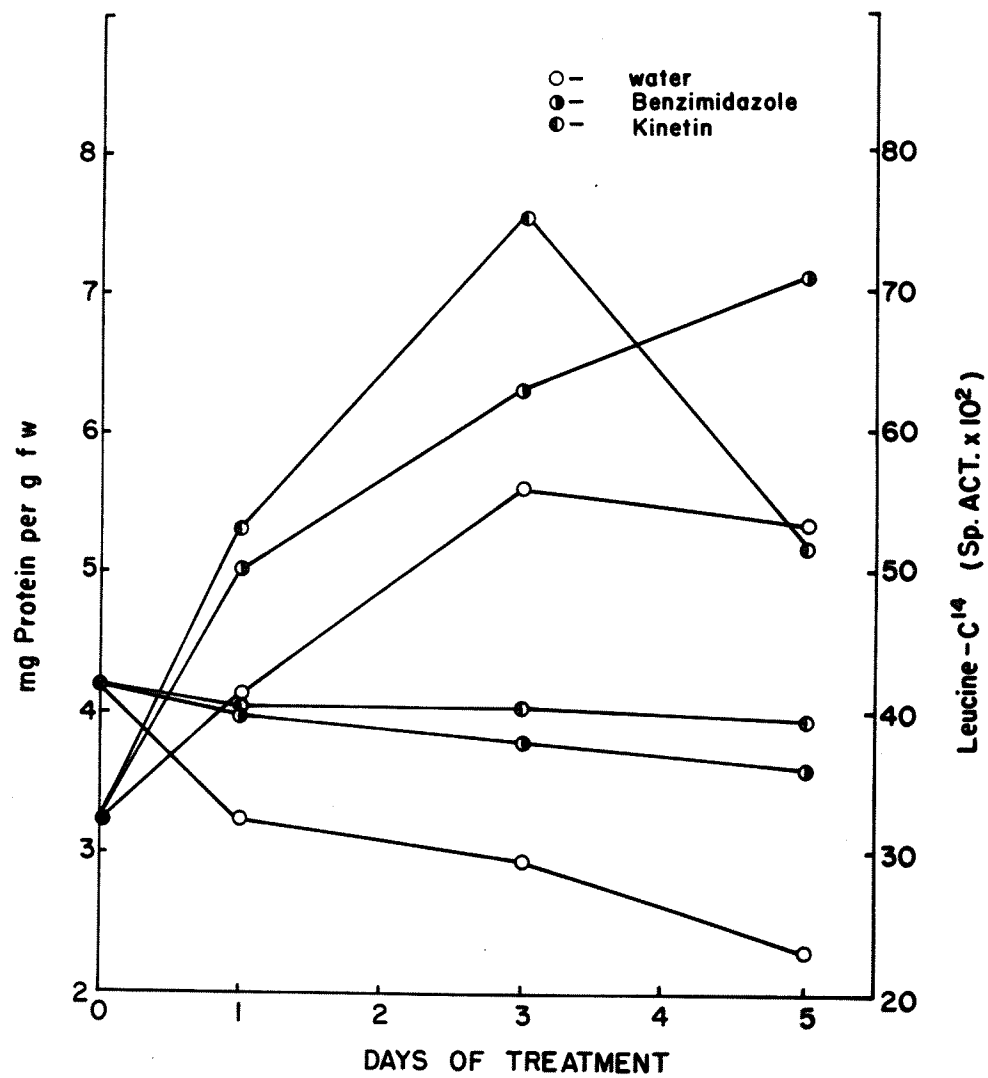


TABLE II. Leucine- C^{14} incorporation into total protein of senescing leaves.*

Treatment	Days floated	mg protein per gram fresh wt	Loss of protein % control	Total counts x 10 ²	Radioactivity recovered % fed **	Specific activity x 10 ²	Increase in specific activity % control
Immediately detached	0	4.18	(100)	133.78	11.43	32.00	(100)
Water	1	3.21	23.30	133.70	11.42	41.65	135.15
	3	2.97	29.00	166.59	14.23	56.09	175.28
	5	2.31	44.80	123.86	10.58	53.61	167.53
Benzimidazole	1	3.98	4.80	200.97	17.17	50.49	157.78
	3	3.78	9.60	240.37	20.54	63.58	198.68
	5	3.61	13.70	258.27	22.07	71.54	223.56
Kinetin	1	4.01	4.10	214.22	18.30	53.42	166.93
	3	4.02	3.90	305.00	26.00	75.80	236.87
	5	3.94	5.80	206.31	17.63	52.36	163.62

* The mean values of data in Table I.

** Calculated on the basis of total Leucine- C^{14} fed per sample: 1.17×10^5 cpm.

synthesis rather than inhibits protein breakdown. If protein breakdown were inhibited by benzimidazole (or kinetin) the specific activity of leucine- C^{14} incorporation would remain constant or the same as that of leaves floated on water (Table II, column 7). A decrease in radioactivity in the alcohol insoluble fraction as indicated by the percent recovery or specific activity of leucine- C^{14} into protein fraction of leaves floated on water for a period of 5 days may be due to the dilution of radioactivity as a result of the increase in the soluble amino acid pools in senescing leaves (Samborski, et al., 1958; Mishra, 1963). However, these leaves also show a decline in total protein levels indicating the shift of balance towards the breakdown rather than protein synthesis in these leaves as compared to those treated with either benzimidazole or kinetin.

The results on the incorporation of radioactivity in the alcohol insoluble fraction indicated the recovery of 12%, 20% and 21% of total leucine- C^{14} (1.17×10^5 cpm) fed to each sample of leaves floated on water, benzimidazole or kinetin solution respectively for 5 days (Table II, column 5).

The loss of protein in detached leaves floated on water is generally the case with senescent leaves. The increase or the maintenance of protein fraction in leaves treated with either benzimidazole or kinetin, has been demonstrated repeatedly in this laboratory and also by other workers (Person, et al., 1957; Richmond and Lang, 1957; Samborski, et al., 1958; Osborne, 1962; Mishra, 1963; Mothes, 1964). Samborski, et al., (1958) reported a 50% loss in insoluble nitrogen content of wheat leaves floated on water for 12 days whereas leaves treated with benzimidazole solution showed no appreciable changes. Mishra (1963) reported a decrease of 23.7% and 19.0% respectively in T.CA insoluble nitrogen fraction of leaves floated for 24 hours on water and benzimidazole solution. This trend was continued further in leaves senescing on water for 4 days while, it was

slowed down with benzimidazole treatment.

A high rate of radioactive- C^{14} incorporation into protein fraction of leaves fed with $C^{14}O_2$ has been shown to be associated with a rapid protein turnover in leaves (Hellebust and Bidwell, 1963a). Xanthium leaf blades treated with kinetin solution for 3 days showed a 4.44% increase (Specific Activity 77.5×10^{-2}) in the rate of leucine- C^{14} incorporation with a corresponding (5.83%) increase in protein content while those senescing on water showed a 46.23% (Specific Activity 39.9×10^{-2}) and 50.76% decrease in the rate of incorporation and protein content respectively, over zero time controls (Specific Activity 74.2×10^{-2})(Osborne, 1962). Similarly, wheat endosperm tissue active in the accumulation of storage protein was shown to incorporate leucine- C^{14} with rates of about 180 cpm per mg nitrogen (Graham, et al., 1964).

II. LEUCINE- C^{14} INCORPORATION INTO CHLOROPLAST PROTEIN

The chloroplasts used for protein and radioactivity determination, were isolated in aqueous medium from leaves fed with leucine- C^{14} under conditions similar to those used for studies on total leaf protein. The data in Table III (and Figure II) show that leaves floated on water for 5 days lost nearly 75% of the chloroplast protein and half of chlorophyll content. Benzimidazole treatment slowed down these losses by 25% and delayed 'chlorosis' in excised leaves. Both the treatments showed a similar increase in the rate of leucine- C^{14} incorporation during the first 24-hour period with a gradual decline in the subsequent treatments which was sharper in benzimidazole treated leaves.

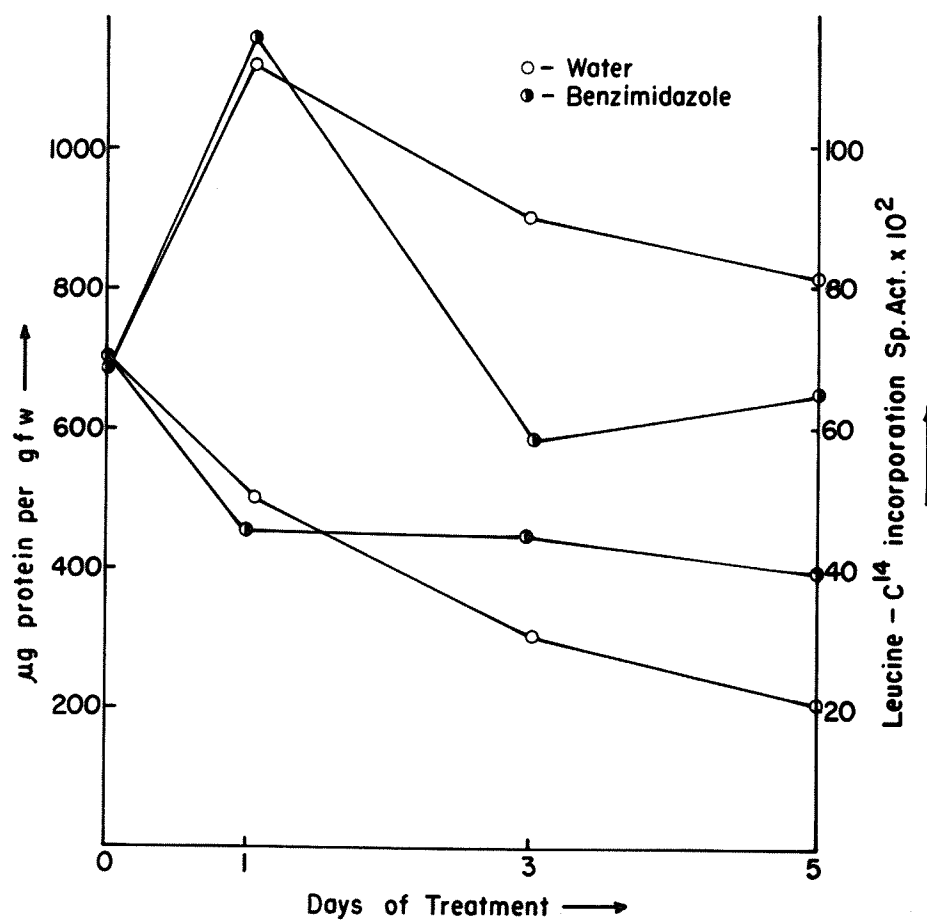
Table III. Incorporation of Leucine- C^{14} into chloroplast protein

Treatment	Days floated	mg chlorophyll per gram fresh wt	Loss of chlorophyll % control	µg protein per gram fresh wt	Loss of protein % control	Total counts $\times 10^2$	Specific activity $\times 10^2$	Increase in specific activity % control
Immediately floated	0	2.0	(100)	709.0	(100)	48.945	69.034	(100)
Water	1	1.1	45.0	500.0	24.5	57.07	114.14	165.338
	3	1.3	35.0	329.4	53.55	30.14	91.49	132.528
	5	1.0	50.0	235.3	66.82	19.41	82.49	119.491
Benzimida-zole	1	1.7	15.0	445.0	37.24	51.73	116.24	168.380
	3	1.6	20.0	450.0	36.57	26.25	59.07	85.566
	5	1.5	25.0	400.0	43.59	26.91	67.275	97.451

Experimental conditions were the same as those given in Table I.

Total counts fed: 1.9×10^5 cpm per sample.

FIGURE II. Incorporation of leucine-C¹⁴ into chloroplast protein of senescing leaves.



In comparison with the data in Table I (or Figure I), it is seen that (i) senescing leaves lost chloroplast protein more rapidly than cytoplasmic protein, (ii) protein turnover in chloroplasts slowed down within 24 hours of detachment and (iii) benzimidazole treatment maintained higher levels of chloroplast protein by retarding the breakdown rather than by stimulation of synthesis suggesting its preferential action at the site of cytoplasmic protein synthesis. Bidwell (1966) reported that the isolation of chloroplasts by techniques similar to those used in the present investigation was found to have resulted in the loss of a large proportion of newly synthesized protein from chloroplasts during the extraction of the tissue. One of the possibilities that the failure to obtain any increase in the specific activity in the chloroplast protein of leaves treated with benzimidazole due to the loss of this newly synthesized protein containing leucine- C^{14} can not therefore, be overruled in the present studies.

Bamji and Jagendorf (1966) have shown that isolated wheat chloroplasts were able to incorporate leucine- C^{14} at the rate of 41.50×10^2 cpm per mg chlorophyll. The rate of incorporation depended on the physiological condition of the chloroplasts and decreased with age of attached leaves. This unusually high rate of leucine- C^{14} incorporation by isolated wheat chloroplasts was suggested to be due to 'the high proportion of labelled protein', which is soluble rather than attached to ribosomes or membranes.

The persistence of the high rates of leucine- C^{14} incorporation into chloroplast protein of leaves senescing on water for 3 or 5 days (Table III, column 8) can possibly be due to the easy accessibility of leucine- C^{14} as a result of permeability changes associated with senescence (Bialeski and Laties, 1963; Sacher, 1966) or loss of

structural components (Waygood, 1965). Fragmentation of chloroplasts has been shown to increase the rate of glycine- C^{14} or leucine- C^{14} into protein fraction (Sissakian, et al., 1965; Stephenson, et al., 1956). Whole chloroplasts of either pea seedlings or tobacco leaves were able to incorporate radioactive amino acid at the rate of 39 cpm/mg protein. This rate was increased to 200 cpm/mg protein in pea seedling chloroplasts fragmented by high speed centrifugation (Sissakian, et al., 1965).

A general decrease in chloroplast protein and chlorophyll content with senescence has been reported by other workers. Wood and Cruickshank (1944) reported a more rapid loss of chloroplast protein than from the remainder of the cell during senescence of Kikuyu grass blades. Wang and Waygood (1959) showed that a decrease in chlorophyll content of wheat leaves floated on water, was prevented by benzimidazole treatment. Mishra (1963), however, reported almost no loss of chloroplast protein and nucleic acids and a slight loss of chlorophyll pigments in wheat chloroplasts isolated in non-aqueous medium from leaves senescing on water for 6 days, and suggested that the breakdown of cytoplasmic protein was more rapid than chloroplast protein in excised leaves.

Zucker, et al., (1962) proved the "validity" of sucrose medium for the isolation of chloroplasts and it also gave consistent results in the present investigation. However, it appeared desirable to check the levels of chloroplast, protein and DNA by using sucrose density gradient techniques to purify the chloroplasts isolated by Pollard's (1964) method which was shown to be more reliable. The results are given in Table IV (and Figure III).

Protein, DNA and chlorophyll content of wheat chloroplasts. The data in Table IV show that protein content of chloroplasts increased

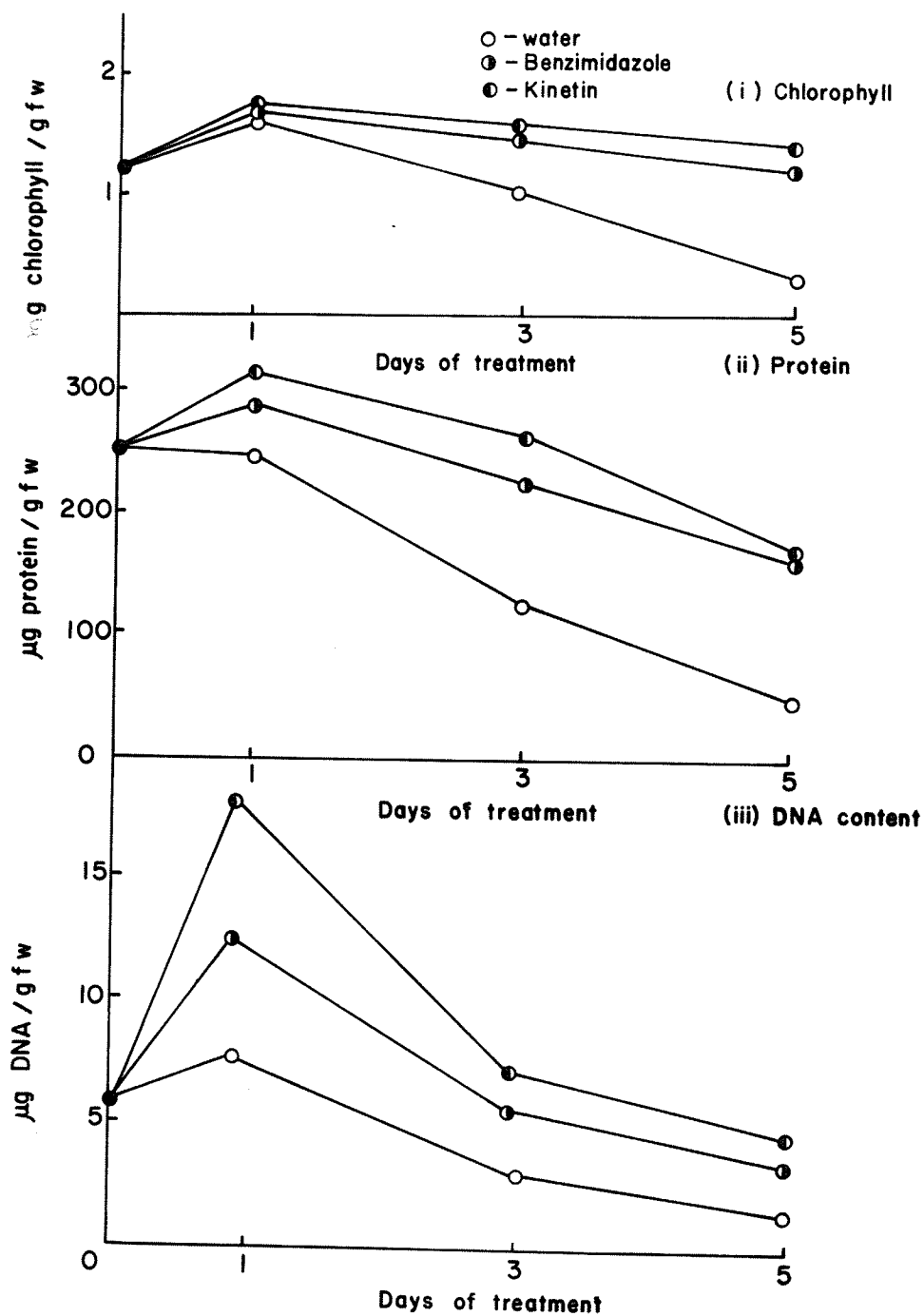
TABLE IV. Protein, chlorophyll and DNA content of wheat chloroplasts from senescent leaves.

Treatment	Days floated	mg chlorophyll per gram fresh wt	Loss of chlorophyll % control	µg protein per gram fresh wt	Loss of protein % control	µg DNA per gram fresh wt	Loss of DNA % control
Immediately detached	0	1.23	Immediately detached	254.4	Immediately detached	5.8	Immediately detached
Water	1	1.60	-	246.0	3.31	7.81	-
	3	1.08	12.20	121.0	52.44	3.12	46.21
	5	0.32	73.99	47.5	81.33	1.50	74.20
Benzimidazole	1	1.70	-	286.0	-	12.60	-
	3	1.49	-	224.0	11.95	5.60	3.50
	5	1.27	-	160.8	36.80	3.37	41.90
Kinetin	1	1.73	-	310.0	-	18.20	-
	3	1.57	-	260.0	-	7.10	-
	5	1.43	-	165.0	35.15	4.68	19.32

The leaves were obtained from wheat seedlings grown for 7 days in the growth chamber at 21° under 2500 ft-c illumination for 16 hours photoperiod and 8 hours darkness. The excised leaves were floated under continuous illumination of 850 ft-c.

FIGURE III. Chlorophyll, protein and DNA content of chloroplasts from senescing leaves.

Chlorophyll (mg), protein (μ g) and DNA (μ g) calculated on the basis of gram fresh weight of treated samples.



during the first 24-hour period of benzimidazole (or kinetin) treated leaves and showed a gradual decline thereafter. All the treatments showed an increase in chlorophyll and DNA content of chloroplasts during this period, indicating the synthesis of chlorophyll. In the subsequent treatments, DNA from all and chlorophyll content from those senescing on water, showed a gradual decline in agreement with the results in Table II.

The leaves used for floating experiments were grown under controlled conditions at 2500 ft-c illumination (16 hours photoperiod and 8 hours darkness) and floated under continuous illumination of 850 ft-c. Thus, the increase in chlorophyll content can be due to the synthesis of chlorophyll in these leaves when transferred from a higher to a lower intensity of light (Rabinowitch, 1956). Formation of chloroplasts with a corresponding increase in protein and DNA content is also shown to be dependent on light (Smillie, 1963; Rhodes and Yemm, 1963; Brawermann, et al., 1962; Sissakian, et al., 1965).

Thus, the slight differences observed in Tables III and IV, were due to the physiological conditions of excised leaves used for the floating experiments.

PART II

EFFECT OF BENZIMIDAZOLE ON PHOTOPHOSPHORYLATION OF WHEAT
CHLOROPLAST FRAGMENTS

MATERIALS AND METHODS

MATERIALS

First leaves of Selkirk wheat (Triticum aestivum L.) grown in the greenhouse for a period of 7 to 10 days were used throughout the investigation.

METHODS

Isolation of chloroplasts. Samples of leaves floated under continuous illumination as described in the previous section, were used for the isolation of chloroplasts. Leaves were harvested at zero time (immediately detached) and at intervals of 1, 3, 5 and 7 days. They were weighed in 1 gram lots and ground in 10 ml of sucrose medium containing 0.4M sucrose, 0.01M NaCl and 0.05M Tris-HCl buffer, pH 7.5 according to the procedure of Jagendorf and Avron (1958). The brei was filtered through 4 layers of cheese cloth and centrifuged at 1000 rpm (200 x g) for 2 minutes. The supernatant was recentrifuged at 3000 rpm (1000 x g) for 10 minutes. The chloroplasts were suspended in 20 to 25 ml of freshly prepared 0.02M sodium ascorbate solution pH 7.5, for 15 to 20 minutes. The excess of ascorbate was removed by centrifugation and the pellet was taken in 5.0 ml of 0.05M Tris-HCl buffer, pH 7.5. The suspension was covered with aluminum foil and stored on ice for immediate use. All manipulations were made at 0 to 4°. Chlorophyll was determined on 0.1 ml suspension, following the method of Arnon (1949).

Preparation of 'phosphodoxin'. 'Phosphodoxin' was isolated from immediately detached leaves using the method of Black, et al., (1963). Ten-day-old primary leaves (100 to 150 grams) were homogenized with 500 ml of chilled acetone in a blender for 2 minutes with the Variac set at 30 to 40 volts. The process was repeated 2 to 3 times in order to remove all chlorophyll. The powder was dried and then extracted with distilled water at 100° for 5 minutes. The water soluble fraction was re-precipitated with acetone. The precipitate was dissolved in water, re-precipitated in cold absolute ethyl alcohol and used for purification by paper chromatography.

'Phosphodoxin' was purified by using one dimensional ascending paper chromatography (Whatman No.3 paper) with n-propanol:water: 1% ammonium hydroxide (3:1:2, v/v/v, pH 10.6) for 20 hours at room temperature. The fraction corresponding to R_f 0.47 was eluted with water, re-extracted with absolute ethanol and the water soluble fraction was used as a source of 'phosphodoxin'.

To investigate the effect of benzimidazole (or kinetin) on the endogenous levels of 'phosphodoxin' wheat leaves (5 gram lots), were floated for 6 days under continuous illumination of 850 ft-c on either water or solutions of benzimidazole and kinetin. 'Phosphodoxin' fractions were prepared from these leaves and were used for spectro-photofluorimetric studies.

Measurement. The stock solution was diluted 10 times with either 0.1N NaOH or 0.1M phosphate buffer (pH 7.0) and used for measuring the fluorescence with Aminco-Bowman Spectrophotofluorometer using

excitation at 350 mμ and emission at 430 mμ.

Determination of orthophosphates. Orthophosphate was determined by the method of Waygood (1948) with KH_2PO_4 solution as a standard. These determinations were made on either 'Spectronic 20' or spectrophotometer (Zeiss, Model PMQ II), at 650 mμ wave length.

Purification of P^{32} solution. Orthophosphate- P^{32} solution was purified according to the method of Avron (1960). The solution was refluxed for 1 hour at 100° , treated with acid washed Norit A and filtered through filter paper. The filtrate was neutralized with 1N NaOH and was diluted with potassium phosphate buffer (pH 7.6) providing a solution of 2 μmoles of orthophosphate containing 1 μC P^{32} per 0.1 ml.

Photophosphorylation by fragmented chloroplasts. The reaction system. The reaction system contained in 1.0 ml final volume the following components in μmoles: Tris-HCl (pH 7.8), 48; ADP (pH 6.8), 2; MgCl_2 , 2; K_2HPO_4 - KH_2PO_4 (pH 7.6), 2 (containing approximately 1 μC P^{32}); glucose, 30; and dicumarol 6 μmoles. The suspension of chloroplasts (0.15 to 0.2 ml = ca 30 μg chlorophyll) and 200 μg (13 units) of yeast hexokinase, were added to make the total volume, in a glass cuvette of 1 cm light path. The reaction mixture contained 0.1 ml 'phosphodoxin' fraction, when present. Both dark and zero time controls were included in each reaction.

Procedure. The cuvettes were illuminated for 30 seconds by a

200W tungsten lamp giving a light intensity of 2500 ft-c, after passing through a water filter maintained at 30°. This was followed by a 30 second dark period. The reaction was terminated by the addition of 0.3 ml of 20% TCA chilled on ice. Each cuvette was then washed with 0.7 ml water and the reaction mixture (2.0 ml) was centrifuged at 5000 rpm for 5 minutes. ATP³² formation was measured by determining the incorporation of radioactivity into organic phosphate fraction following the method of Avron (1960) described below.

Assay of ATP³² formation. Aliquots of the supernatant from TCA denatured reaction mixture, were taken in duplicate in 10 x 150 mm pyrex tubes and were treated with 1.2 ml acetone. The contents were mixed in a Vortex mixer (Scientific Industries, Inc.), and allowed to stand for 10 minutes. The water phase was treated with 0.8 ml of water saturated with a mixture of equal parts of isobutanol and benzene, followed by 7.0 ml of isobutanol-benzene mixture (1:1 v/v) saturated with water. After phase separation, the water phase was treated with 0.8 ml of molybdate reagent containing 5 gram ammonium molybdate and 40.0 ml concentrated sulfuric acid in 100.0 ml solution. The aqueous layer was washed with 0.02 ml of 0.02M KH₂PO₄ solution, followed by 7.0 ml of isobutanol-benzene mixture. The aqueous layer was then used for radioactivity measurements.

The difference between the control and sample reading gave the corrected count while, the count of the supernatant after TCA treatment, gave the total counts introduced. The μ moles of ATP³² formed were calculated from the following equation:

$$\mu\text{moles ATP}^{32} \text{ formed} =$$

corrected cpm $3.3 \times \frac{\text{volume in tube after TCA}}{\text{volume used for assay}} \times \frac{\mu\text{moles Pi in the tube}}{\text{total cpm introduced}}$

Radioactivity measurements for ATP³². Aliquots of samples in 50 μl were dried in aluminum planchets under infra-red light and used for measuring radioactivity with Philips Scaler (Model PW 4035).

Determination of ATPase activity. The reaction system. The reaction system contained in μmoles : Tris-HCl (pH 7.8), 48; MgCl_2 , 5; ATP (pH 6.8), 2; and the suspension of chloroplasts (ca $\equiv$ 30 μg chlorophyll) in a glass cuvette of 1 cm light path. Water was added to make the final volume of 1.0 ml.

Assay for light-induced ATPase. The cuvettes were illuminated for 10 minutes under conditions similar to those used in photophosphorylation experiments. The reaction was terminated by the addition of cold TCA and the supernatant was used for the determination of orthophosphate.

'Photohydrolytic' activity (ATPase, ADPase or non-specific phosphatase) in photophosphorylation system. The complete reaction system as that used for photophosphorylation studies, was used to determine the 'photohydrolytic' activity of chloroplast fragments except that the reaction system contained no radioactive phosphate.

The cuvettes were illuminated for a total of 30 seconds with two successive periods of 15 seconds light and 15 seconds darkness (totalling 1 minute reaction), under the conditions similar to those described for photophosphorylation studies. The excess of orthophosphate liberated at the end of 1 minute was taken as the measure of 'photohydrolytic' activity associated with chloroplast fragments.

RESULTS

As pointed out in the Introduction, the object of this investigation was to study the effect of benzimidazole on the endogenous rate of photophosphorylation in wheat chloroplasts, accordingly no exogenous electron acceptors were used in the system. Preliminary investigations indicated that the various methods tried to isolate chloroplasts active in photophosphorylation, were unsuitable with the exception of Avron's (1960) method using ascorbate and with dicumarol present in the reaction system (Jagendorf, et al., 1964). The results of the effect of dicumarol on photophosphorylation in wheat chloroplasts are given in Table V.

I. THE ENDOGENOUS RATE OF PHOTOPHOSPHORYLATION IN WHEAT CHLOROPLASTS

The effect of dicumarol. Table V shows a typical experiment on the effect of dicumarol on photophosphorylation in wheat chloroplasts. The data show that P_i (orthophosphate) was phosphorylated in the light reaction with dicumarol in the reaction system whereas, it was liberated in the absence of dicumarol. Since, dicumarol also inhibited P_i liberation in darkness, it is possible that it inhibited ADP breakdown by ADPase or by a non-specific enzyme which also acted as ATPase in light. Another possibility of dicumarol acting as a catalyst in the electron transfer reaction, cannot be

TABLE V. The effect of dicumarol on photophosphorylation and the liberation of orthophosphate in darkness.

System*	<u>μmoles Pi per hour per mg chlorophyll</u>	
	Light**	Darkness
Without dicumarol	+ 7.0	+ 7.0
With dicumarol	- 3.75	+ 0.25

* The reaction system contained in μmoles: Tris-HCl, pH 8.2, 48; MgCl₂, 2; ADP(pH 6.8), 1; K₂HPO₄, 0.5; glucose, 30 and hexokinase (13 units) and chloroplast suspension (capacity 50 μg chlorophyll) in a final volume of 1 ml. Chloroplasts were not treated with ascorbate.

** Illumination at 2500 ft-c for 5 minutes followed by darkness for 5 minutes. Dark controls were kept in darkness for 10 minutes. The other conditions were the same as given in 'Methods'.

+ = indicates liberation of orthophosphate

- = indicates utilization of orthophosphate

overruled in the present system. Jagendorf, et al., (1964), however, suggested that dicumarol might be effective in inhibiting ATPase activity without inhibiting photophosphorylation.

The addition of dicumarol, above or below $6 \times 10^{-6} \text{M}$ was found to be ineffective in obtaining consistent results (not shown in Table V). The endogenous ATP formation generally varied between 4 to 10 $\mu\text{moles per hour per mg chlorophyll}$ (Table V) and it was further increased with chloroplasts treated with sodium ascorbate (Table VI). In all subsequent studies, therefore, ascorbate treated chloroplasts (referred to as chloroplast fragments) were used for photophosphorylation with dicumarol in $6 \times 10^{-6} \text{M}$ concentration in the reaction system, but with no other exogenous electron acceptor.

Cereal leaves are notoriously unsuitable for photophosphorylation studies (Heber, 1960; Mifflin and Hageman, 1963). Mifflin and Hageman (1963) reported a failure to obtain any endogenous ATP formation in maize chloroplasts with no artificial cofactors. Shen and Shen (1962) however, reported 20 to 22 $\mu\text{moles of ATP formation (per hour per mg chlorophyll)}$ with wheat chloroplasts in the absence of added cofactors. These chloroplasts were reported to possess no ATPase activity which might account for these appreciably high endogenous rates.

Chloroplasts isolated from dicotyledonous leaves have been shown to give comparatively higher endogenous rates and have been used extensively for photophosphorylation studies (Arnon, et al., 1954a,b; Jagendorf, and Avron, 1958; Smillie and Krotkov, 1959; Avron, 1960;

Forti and Jagendorf, 1961; Whatley, et al., 1963). The endogenous rates for spinach or swiss chard chloroplasts vary from 2.2 to 10 μ moles ATP per hour per mg chlorophyll (Avron, 1960; Jagendorf and Avron, 1958).

The role of ascorbate. The data in Table VI are from wheat chloroplasts that have been treated with sodium ascorbate to obtain partially fragmented chloroplasts as suggested by Avron (1960). The ascorbate treatment showed a 4 to 8 fold increase in the rate of ATP formation in chloroplasts from immediately detached leaves. This rate of photophosphorylation in chloroplast fragments varied from 15 to 96 μ moles ATP per hour per mg chlorophyll (Table VI).

As mentioned in Methods, these chloroplasts were prepared by suspending them in 20 to 25 ml of 0.02M sodium ascorbate for 15 to 20 minutes in the cold, centrifuging and resuspending them in 0.05M Tris-HCl buffer, pH 7.5. If it is assumed that 10% ascorbate remained with chloroplasts, it can be calculated that approximately 0.4 μ moles ascorbate was present in the reaction mixture if it had not undergone any degradation. Therefore, the acceleration of photophosphorylation by ascorbate, observed in wheat chloroplasts may be due to its catalytic activity as a cofactor. Whether any residual dehydroascorbate was operating as an electron acceptor remains to be established. The possibility of such a labile factor participating in the system is suggested by the rapid deterioration of activity in chloroplasts stored for more than two hours.

TABLE VI. The effect of benzimidazole, with or without 'phosphodoxin' fraction, on photophosphorylation in wheat chloroplast fragments from senescing leaves.

μ moles ATP³² formed per hour per mg chlorophyll (at pH 7.8)

Treatment	Experiment No	Days Floated					
		0		1		3	
		-PD *	+PD	-PD	+PD	-PD	+PD
Immediately detached	I	20.0	168.0	..	..	..	..
	II	15.0	28.0	..	..	..	..
	III**	96.0	348.0	..	..	..	..
Water	I	..	..	96.0	69.0	0.0	64.0
	II	..	..	24.0	22.0	34.0	38.0
	III	..	..	60.0	0.0	54.2	108.4
Benzimidazole	I	..	..	125.0	0.0	0.0	217.0
	II	..	..	50.0	67.0	20.0	67.5
	III	..	..	219.0	428.0	280.0	408.0

* PD = 'Phosphodoxin'

** The average of two experiments carried out on the same chloroplast preparation. Primary leaves of Selkirk wheat grown under greenhouse conditions for 7 to 8 days were floated under continuous illumination of 850 ft-c.

The reaction system and conditions were the same as given in 'Methods'. The values given in the table are corrected for 'dark' controls.

Addition of ascorbate to either homogenizing medium or reaction system, has been shown to retain the activity and to stimulate ATP formation in swiss chard chloroplasts (Avron, 1960). These chloroplasts showed the rate of 2000 to 2500 μ moles ATP³² formation (per hour per mg chlorophyll) as against 1000 μ moles ATP³² when PMS (0.09 μ moles) was supplemented with sodium ascorbate (10 μ moles) in the reaction system. Since no other reducing agent such as cystein or glutathione was found to replace ascorbate in its requirement for photophosphorylation, it was suggested that it influenced some enzyme active in ATP formation (Avron, 1960). Marre' et al., (1958) reported the presence of 'monodehydro ascorbate reductase' in washed chloroplasts and proposed a catalytic role for ascorbate-dehydroascorbate in photophosphorylation. Ascorbate has been shown to be a cofactor in photophosphorylation (Whatley, et al., 1955) and addition of ascorbate in concentrations as low as 5×10^{-4} M has been reported to stimulate ATP formation in spinach chloroplasts (Forti and Jagendorf, 1961).

Similarly, the use of cystein for the isolation of maize chloroplasts, has been shown to increase the initial rate of ATP formation with PMS (0.3 μ moles) from 3 to 145 μ moles ATP per hour per mg chlorophyll (Mifflin and Hageman, 1963).

II. THE EFFECT OF BENZIMIDAZOLE WITH OR WITHOUT 'PHOSPHODOXIN' FRACTION ON PHOTOPHOSPHORYLATION IN WHEAT CHLOROPLAST FRAGMENTS FROM SENESCING LEAVES

The results of three separate experiments conducted to study the effect of benzimidazole on the endogenous rate of photophosphoryla-

tion in wheat chloroplast fragments, in the presence or absence of wheat 'phosphodoxin', are given in Table VI.

The data in Table VI show that wheat leaves senescing on water showed a general decrease in the rate of ATP³² formation, with or without 'phosphodoxin' in the reaction system although addition of 'phosphodoxin' increased the initial rate in the majority of treatments. Benzimidazole treatment showed a stimulation in the initial rate of ATP³² formation which invariably showed a further 2-fold increase with 'phosphodoxin' in the reaction system.

The results presented in Table VII show that benzimidazole treatment also restored, at least in the presence of 'phosphodoxin', the rate of photophosphorylation in chloroplast fragments isolated from leaves senescing on water for 3 days. The results given in Table VII are the continuation of Experiment III in Table VI and are presented in the form given by Waygood (1965).

The chromatographically pure 'phosphodoxin' fraction showed the presence of a few weak 'ninhydrin' positive spots and enhanced Pi liberation in photophosphorylation studies with orthophosphate. It is not known whether any of the catalytic activity of wheat 'phosphodoxin' in ATP³² formation (Tables VI and VII) was due to the 'ninhydrin' positive fraction.

The stimulatory effect of benzimidazole on photophosphorylation appears to be an indirect one since, reaction systems containing benzimidazole ($6 \times 10^{-3}M$) generally showed liberation of Pi (orthophosphate) which indicated that it might be inhibitory for photophosphorylation. Further, Büchel, *et al.*, (1966) reported the inhibition of 'Hill' reaction and 'cyclic' photophosphorylation in grasses with benzimidazole

TABLE VII. The effect of benzimidazole on photophosphorylation in leaves senescing on water.

μ moles ATP³² per hour per mg chlorophyll (at pH 7.8)

Treatment	Days Floated									
	0		1		3		5		7	
	-PD	+PD	-PD	+PD	-PD	+PD	-PD	+PD	-PD	+PD
Immediately detached	228*	360	..	..	..	..	..	..	..	..
	(96)	(348)								
Water	..	..	60	180	54	108	0	0	..	..
			(60)	(0)	(54)	(108)				
Benzimidazole	..	..	430	633	280	440	0	75	0	0
			(219)	(428)	(280)	(408)		(0)		
Water (0-3 days)										
Benzimidazole (3-7 days)	..	..	..	..	..	..	65	168	108	324
							(0)	(93)	(0)	(194)

* The figures in parentheses denote the rate of photophosphorylation corrected for 'dark' controls.

The reaction system and conditions were the same as given in Table VI. (Continuation of Experiment No.III).

and its substitution compounds in concentrations as low as 10^{-6} M. The uncoupling of ATP formation was shown to be related to the structure of benzimidazole substitution compounds.

A decrease in ATP formation with a corresponding decrease in ATP levels of senescent leaves appears to be a common phenomenon. Smillie and Krotkov (1959) reported a decrease in the rate of photophosphorylation from 27 μ moles ATP in 9-days old to 9.9 μ moles ATP (per hour per mg chlorophyll), in 28-days old leaves of pea seedlings. Mishra (1963) reported a decrease in ATP with a corresponding increase in ADP content, in chloroplasts of wheat leaves senescing on water for 6 days while, benzimidazole treatment retained high ATP and low ADP levels in chloroplasts.

Spinach 'phosphodoxin' has been reported to give a 200-fold increase in the endogenous rate of photophosphorylation from 1.5 μ moles ATP to 196 μ moles ATP per hour per mg chlorophyll (Black, *et al.*, 1963a).

The effect of phenazine methosulfate and adenylate kinase on photophosphorylation in chloroplast fragments from immediately detached leaves. Other experiments were conducted to investigate the effect of phenazine methosulfate as a cofactor for photophosphorylation in wheat chloroplast fragments. Adenylate kinase was also included in these studies since it was shown to stimulate the rate of ATP^{32} formation in swiss chard chloroplast fragments when supplemented with PMS (0.09 μ moles) (Avron, 1961). The results of these investigations are given in Table VIII.

TABLE VIII. The effect of phenazine methosulfate and adenylate kinase**on photophosphorylation in wheat chloroplast fragments from freshly harvested leaves.

$\mu\text{moles ATP}^{32}$ formed per hour per mg chlorophyll
(at pH 7.8)

System	ATP^{32} formed	Increase in the rate of photophosphorylation % control
Complete*	16.6	(100)
+ phenazine methosulfate	0.0	0.0
+ 'phosphodoxin'	44.0	265.06
+ 'phosphodoxin' + phenazine methosulfate	167.0	1006.02
+ adenylate kinase + phenazine methosulfate	30.0	180.72

* The reaction system and conditions were the same as given in Table VI. The cofactors when present, were used in the following concentration: phenazine methosulfate, 0.1 μmole ; adenylate kinase, 0.5 μg and 'phosphodoxin', 0.1 ml.

** Adenylate kinase was dialysed overnight against distilled water to remove ammonium sulfate from the commercial preparation used.

Primary leaves of Selkirk wheat grown in the greenhouse for 8 days were used for the preparation of chloroplast fragments.

The data in Table VIII show that PMS alone failed to stimulate any photophosphorylation in wheat chloroplast fragments. The addition of 'phosphodoxin' to the reaction system either alone or with PMS increased the endogenous rate by approximately 3 and 10-folds respectively. The system was also active in photophosphorylation, when PMS was supplemented with adenylate kinase although, the increase in the rate was not as great as that observed with 'phosphodoxin' fraction.

Spinach or swiss chard chloroplasts have been shown to give maximum rates (2000 to 2500 $\mu\text{moles ATP}^{32}$ per hour per mg chlorophyll) of 'cyclic' or 'non-cyclic' photophosphorylation with PMS as a cofactor (Avron, 1960; Avron and Jagendorf, 1958). Maize chloroplasts, however were reported to show no great response to PMS alone as a cofactor (Mifflin and Hageman, 1963). In accord with the results given in Table VIII, PMS ($5 \times 10^{-5}\text{M}$) in combination with 'phosphodoxin' was shown to double the rate of photophosphorylation in spinach chloroplasts (Black, *et al.*, 1964). Similarly, adenylate kinase was reported to accelerate the endogenous rate from 10 $\mu\text{moles ATP}^{32}$ to 640 $\mu\text{moles ATP}^{32}$ per hour per mg chlorophyll, when used in combination with PMS (0.09 μmoles) in swiss chard chloroplasts which contained neither ATPase nor adenylate kinase activity (Avron, 1961).

The failure to obtain ATP^{32} formation in wheat chloroplast fragments with PMS as a cofactor, may be due to an increase in the light-induced ATPase activity (Hoch and Martin, 1963; Avron, 1962) (Table IX). Plant ATPase (EC 3.6.1.5) has been shown to act also on ADP

TABLE IX. The ATPase activity associated with wheat chloroplast fragments from senescing leaves.

μ moles Pi liberated per hour per mg chlorophyll
(at pH 7.8)

Treatment	Days floated	Light*	Darkness
Immediately detached	0	87.7	61.9
Water	1	33.0	16.0
	3	13.2	15.4
	5	23.5	16.1
Benzimidazole	1	38.0	27.0
	3	18.7	26.6
	5	8.0	8.0

* Continuous illumination at 2500 ft-c for 10 minutes.

Primary leaves of Selkirk wheat seedlings grown in the greenhouse for 9 to 10 days were used for the experiments.

The reaction system and conditions were the same as given in 'Method' under ATPase assay.

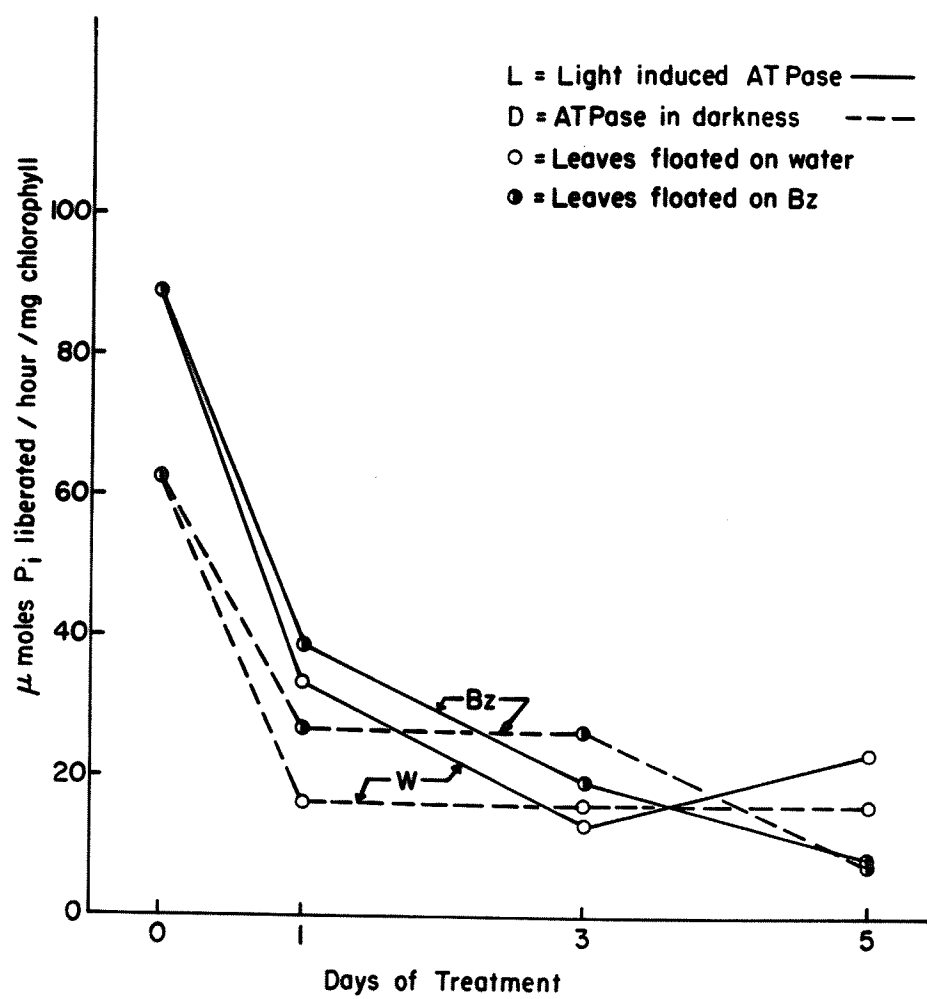
(Kalckar, 1944). The levels of ADP thus, appear to limit photophosphorylation with PMS in the reaction system (Table VIII), as it is supported by the fact that the addition of adenylate kinase to the system, supported photophosphorylation by catalyzing the formation of ADP from ATP and AMP ($ATP + AMP = 2 ADP$). The effect of 'phosphodoxin' in stimulating PMS mediated photophosphorylation is, however, difficult to comprehend.

III. ATPASE ACTIVITY ASSOCIATED WITH CHLOROPLAST FRAGMENTS FROM SENESCING LEAVES

Preliminary investigations to determine the rates of ATP formation in wheat chloroplasts with the use of orthophosphate (non-radioactive) suggested the presence of very active ATPase (or ADPase) associated with these preparations. It was, therefore, decided to examine the presence of this activity in senescing leaves. The results of the ATPase activity associated with leaves senescing under continuous illumination on either water or benzimidazole solution are given in Table IX (Fig. IV).

The data in Table IX (Fig. IV) indicate that there was a loss of light-induced ATPase activity with senescence, suggesting a correlation between the structural integrity of chloroplasts and ATPase activity associated with them. The ATPase activity in darkness (Black, et al., 1963b; Wessels and Baltscheffsky, 1960) showed an initial decrease in leaves senescing for 24 hours but remained steady thereafter. Leaves treated with benzimidazole maintained both the light-induced and 'dark' ATPase activity at slightly higher levels (Table IX).

FIGURE IV. ATPase activity associated with chloroplast fragments from senescing leaves.



As reported in the Review of Literature, there are two light-induced and one 'dark' ATPase activity associated with chloroplasts (Avron, 1964). Of the two light-induced ATPases, one requires PMS or sulfhydryl compounds, Mg^{++} ions and a brief exposure to light while the other requires PMS, Ca^{++} ions (but not Mg^{++} ions) and continuous light. Both the activities have been implicated with the structural changes in chloroplast membrane associated with electron transfer and photophosphorylation reactions (Packer, et al., 1963; Dilley and Vernon, 1964). The ATPase activity present in darkness does not change upon 'aging' and its role in photophosphorylation is obscure (Avron, 1964).

Effect of pH on the light-induced ATPase activity. The experiments reported in Table IX were conducted at pH 7.8. Investigations on the effect of pH on the light-induced ATPase activity shown in Figure V, indicated that the greatest activity was at pH 6.0. The pH values below this range were not tested.

Effect of phenazine methosulfate and ADP on ATPase activity.

Phenazine methosulfate has been reported to activate the light-induced ATPase activity (Hoch and Martin, 1963; Avron, 1962, 1963), whereas, ADP has been shown to be a competitive inhibitor for PMS catalyzed light-induced ATPase activity (Avron, 1962). Investigations were, therefore, conducted to study the effect of both PMS and ADP on the ATPase activity of wheat chloroplast fragments. The results given in Table X show that PMS as well as ADP activated ATPase in light but had no effect in darkness. The results also indicated that light-

FIGURE V. The reaction system and conditions were the same as given in Table IX except that chloroplasts from immediately detached leaves were used throughout the experiment.

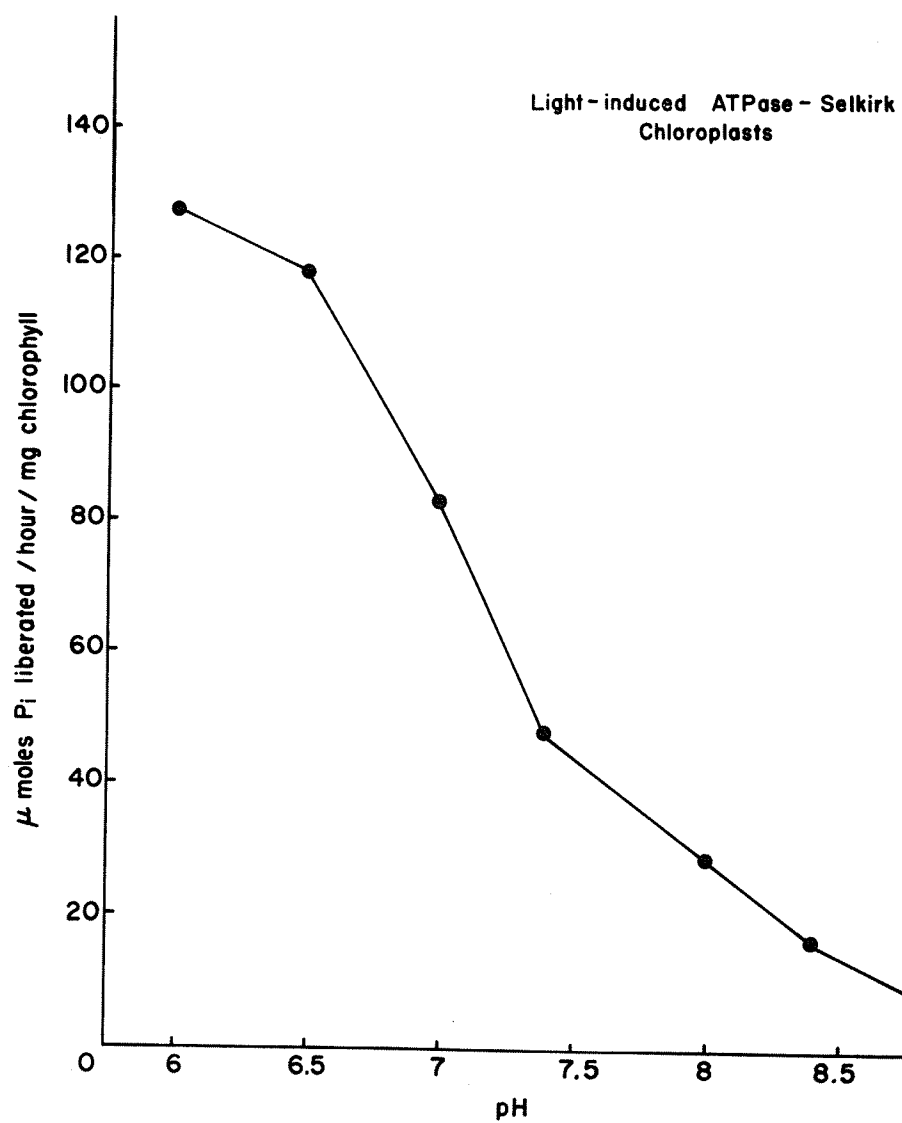


TABLE X. The effect of phenazine methosulfate and ADP on ATPase activity.

μ moles orthophosphate liberated per hour per mg chlorophyll
(at pH 6.0)

System	Light	Darkness
Complete	145.16	87.0
+ phenazine methosulfate (0.1 μ mole)	190.0	79.0
+ ADP (4.0 μ moles)	170.0	97.0

The reaction system and conditions were the same as given in Table IX.

Chloroplast fragments prepared from immediately detached leaves were used in the reaction system.

induced ATPase activity associated with wheat chloroplasts, unlike the activity associated with spinach or swiss chard chloroplasts, showed no requirement for PMS as a cofactor and showed high endogenous rates even in its absence.

The ratio of photochemical ATP³² formation to light-induced ATP hydrolysis at pH 7.8 appears to be approximately 1 for wheat chloroplast fragments (96 μ moles ATP³²/87.7 μ moles ATP, data from Tables VI and IX respectively), in the absence of PMS as a cofactor. This ratio was found to be 50 (2000 μ moles ATP³² synthesized/40 μ moles ATP³² hydrolyzed) for swiss chard chloroplasts which showed a complete dependence on PMS (0.1 μ mole) for ATP hydrolysis (Avron, 1962). Petrack and Lipmann (1961) reported the rates of 200 μ moles ATP hydrolysis per hour per mg chlorophyll, in spinach chloroplasts activated by light in the presence of PMS (0.4 μ moles). The rate of PMS catalyzed photophosphorylation in these chloroplasts, was 250 to 750 μ moles ATP per hour per mg chlorophyll.

In comparison with spinach or swiss chard chloroplasts, wheat chloroplast fragments showed high rates of light-induced ATPase activity which might account for the failure to obtain photophosphorylation in the absence of dicumarol and also for low endogenous rates of ATP formation. These preparations also showed very high 'dark' ATPase activity (Table X) as compared to that present in spinach or swiss chard chloroplasts (Avron, 1962; Black, *et al.*, 1963b). Spinach chloroplasts were reported to hydrolyze 3 to 6 μ moles ATP per hour per mg chlorophyll in darkness and this rate was increased to 15 μ moles ATP (hydrolyzed per hour per mg

chlorophyll) with chloroplast fragments prepared by sonication (Black, et al., 1963b).

IV. THE 'PHOTOHYDROLYTIC' ACTIVITY IN THE PHOTOPHOSPHORYLATION SYSTEM

The results of the previous experiments (Tables IX and X) indicated high rates of ATPase activity (light-induced as well as that present in darkness) associated with wheat chloroplast fragments in the absence of either PMS or sulfhydryl compounds in the reaction system designed solely to determine ATP breakdown and not photophosphorylation. Accordingly, it was of interest to compare the rate of ATP (or ADP) breakdown in light and in darkness, in the complete system used for photophosphorylation. Chloroplast fragments prepared from immediately detached leaves by the ascorbate treatment procedure, were used to study the rate of Pi (orthophosphate) liberation in the complete system illuminated under different light intensities. Since ADP was present in the reaction system, it is not possible to distinguish the contribution of ATPase, ADPase or a non-specific phosphatase to the breakdown of adenine nucleotides. It is for this reason, the liberation of Pi (orthophosphate) in the complete system at the end of light or 'dark' reaction, is discussed under 'photohydrolytic' activity rather than ATPase or ADPase activity. The results of experiments are presented in Table XI (Fig. VI).

The data in Table XI (Fig. VI) show that Pi liberation in the light reaction (two successive periods of 15 seconds light and 15 seconds darkness, totalling 1 minute), increased with increasing light

TABLE XI. The effect of light intensity and darkness on the 'hydrolytic' activity of wheat chloroplast fragments.

μ moles orthophosphate per hour per mg chlorophyll
(at pH 7.8)

Light intensity for light reaction ft-c.	Light (L) (1 minute)*	Darkness (D) (1 minute)	L-D	L-D/2	μ moles Pi utilized/ hour/mg chlorophyll
500	+319.0	+638.7	-319.7	- 0.3	..
1000	..	..	..	..	(96.0)
1200	+451.6	+637.0	-185.4	+133.1	..
1500	+638.7	+638.7	0.0	+319.4	(65.0)
2500	+806.4	+638.7	+167.7	+487.1	..
3500	+774.0	+638.7	+135.3	+454.7	..
4500	+967.0	+638.0	+329.0	+648.0	..

* Two successive periods of 15 seconds light and 15 seconds darkness, totalling 1 minute reaction.

The reaction system was the same as that used for photophosphorylation studies.

L = denotes reaction system illuminated for 1 minute* (Two successive light periods with two dark periods).

D = Darkness for 1 minute.

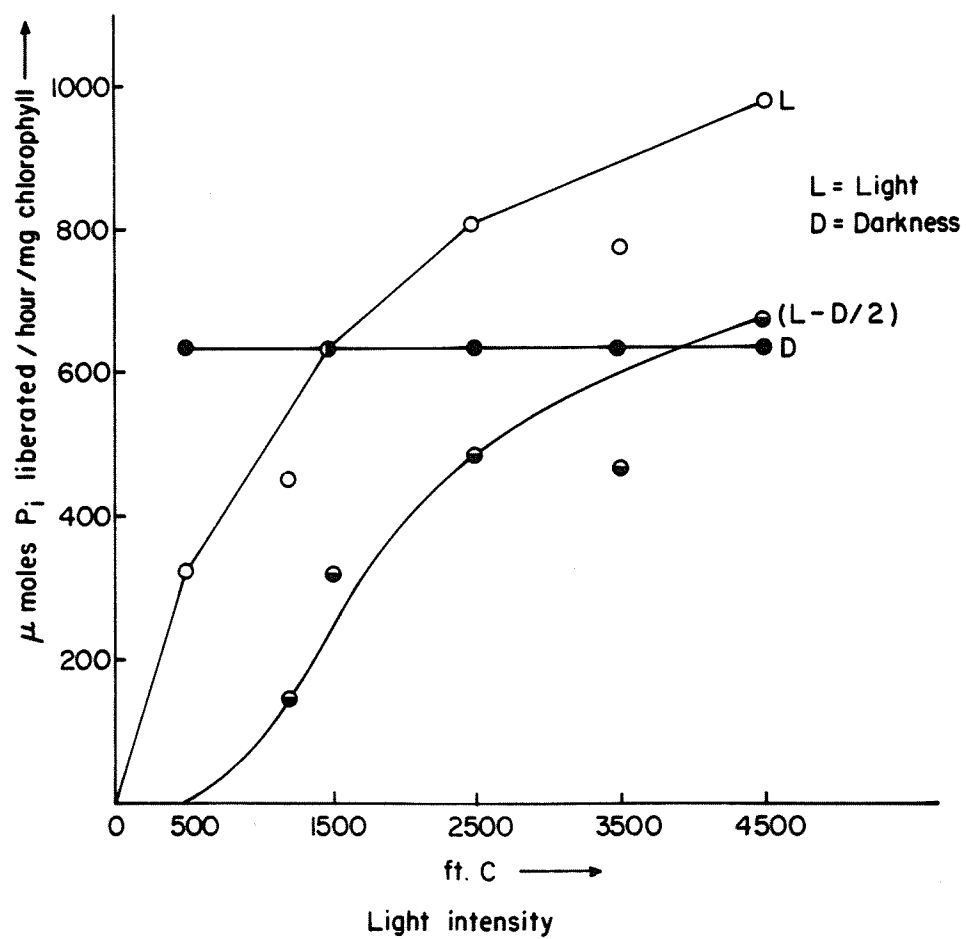
L-D = Pi liberation in 1 minute total period.

L-D/2 = Pi liberated in 30 seconds of light period after subtracting one-half of dark period (30 seconds).

- = orthophosphate utilized

+ = orthophosphate liberated

FIGURE VI. Effect of light intensity on the 'photohydrolytic' activity of chloroplast fragments in a complete photophosphorylation system.



intensity whereas, the rate was constant in the 'dark' controls (1 minute darkness). The rates of Pi liberation were higher than those observed for ATPase activity in ATPase system (Table IX). It may be coincidental that the rate of Pi liberation at the lowest light intensity of 500 ft-c is almost identical to one-half of that in darkness, suggesting but not proving, that the Pi liberation at 500 ft-c is due primarily to the breakdown occurring in darkness and that light inhibits dark ATPase (or ADPase) or fails to stimulate it. However, as the light intensity is increased, it induces an ATPase (or ADPase) and this component together with that in darkness ($D/2$) contributes to the total Pi liberation. On this basis, the light-induced Pi liberation is equal to the rate in 'light' minus half of the rate in darkness. If the light-induced ATPase (or ADPase) is related to photophosphorylation, then the value ($L-D/2$) is a measure of photophosphorylation (Fig.VI).

On the other hand, if the assumption that Pi liberation in light or darkness is catalyzed by one enzyme is incorrect and light does not inhibit 'dark' ATPase (or ADPase) activity then perhaps the value $L-D$ is a more valid measure of photophosphorylation. The data for $L-D$ at 500 and 1200 ft-c are negative indicating phosphate uptake which would suggest that photophosphorylation occurs only at low light intensity. This is supported by the fact that photophosphorylation could be detected occasionally at these low light intensities (Table XI, column 6).

Figure VI shows the effect of light intensity on Pi liberation in the total light period of 1 minute (L) (30 seconds light and 30 seconds

darkness split) and in the light period of 30 seconds (L-D/2). The dark controls have also been plotted but of course, show no relationship to light intensity. Both the light-induced ATPase (or ADPase) values (L and L-D/2) show that light saturation for the maximum Pi liberation is 4500 ft-c or above. It is difficult to assess which of these data is more significant but the effect of light intensity on ATPase (or ADPase) activity indicates some causal relationship between it and photophosphorylation as suggested by Avron (1962).

Jagendorf and Avron (1958) showed that light saturation required for photophosphorylation in the complete reaction system, depended on the cofactors used. With PMS (0.05 μ moles) as a cofactor in the system, spinach chloroplasts showed light saturation at 4400 ft-c for rapid rates of photophosphorylation and 'Hill' reaction, whereas, light saturation at 1500 ft-c was achieved with FMN (0.05 μ moles) as a cofactor. In the present system no exogenous cofactor was present although, dicumarol was added to inhibit ATPase activity and chloroplasts were treated with ascorbate and the data obtained in the experiments (Table XI) may be typical of such a system.

Jagendorf and Hind (1963) reported a transient light-induced ATPase activity in chloroplasts which showed no requirement for either Ca^{++} ions or sulfhydryl compounds for activation. However, the light-induced ATPase activity was accelerated by a trace of ADP, Pi and pyocyanine in the reaction system and showed the time course for the activation identical to that for photophosphorylation. It is not known whether the activation of Pi liberation by wheat chloroplast

fragments (Table XI) was due to ATPase similar to that reported by Jagendorf and Hind (1963) or it was due to ADPase which was inhibited by dicumarol at low light but activated at high light intensities.

The rates of orthophosphate liberation in the complete system with chloroplast fragments from senescing leaves. It appeared from the results given in Table XI that (i) liberation of Pi in the light reaction (L-D) could serve as a measure of the 'apparent' rate of photophosphorylation in wheat chloroplasts and (ii) light saturation at low light intensity could be used to measure such a rate. The data in Table XII (Fig. VII) are the outcome of such experiments designed to measure the rates of Pi liberation in light and darkness in a complete photophosphorylation system used with chloroplast fragments from senescing leaves. Leaves senescing under continuous illumination of 850 ft-c on either water or solutions of benzimidazole and kinetin, were used to evaluate these rates (Pi liberation in L-D) with those of ATP³² formation in senescing leaves (Tables VI and VII). Similarly, it was interesting to compare these rates with the ATPase activity of senescing leaves measured under continuous illumination in ATPase system (Table IX).

The data in Table XII show that the liberation of Pi in a system with chloroplast fragments from leaves senescing on water, showed a gradual decrease either in light or darkness. Benzimidazole (or kinetin) treatment enhanced the initial rate (zero time control) of Pi liberation both in light and darkness and maintained it at a higher

TABLE XII. The rates of orthophosphate liberation in light and darkness in a complete photophosphorylation system with chloroplast fragments from senescing leaves.

μmoles Pi liberated per hour per mg chlorophyll
(at pH 7.8)

Treatment	Days floated	Light*(L) (1 minute)	Darkness (D) (1 minute)	(L-D) (1 minute)
Immediately detached	0	+125.0	+250.0	-125.0
	2**	+ 78.12	+156.25	- 78.13
Water	4	+ 62.50	+125.00	- 62.50
	7	..	..	..
	2	+156.20	+468.70	-312.50
Benzimidazole	4	+234.30	+421.80	-187.50
	7	+ 98.68	+148.20	- 49.52
	2	+156.0	+625.0	-469.0
Kinetin	4	+535.6	+758.9	-223.3
	7	+187.6	+281.2	- 93.6

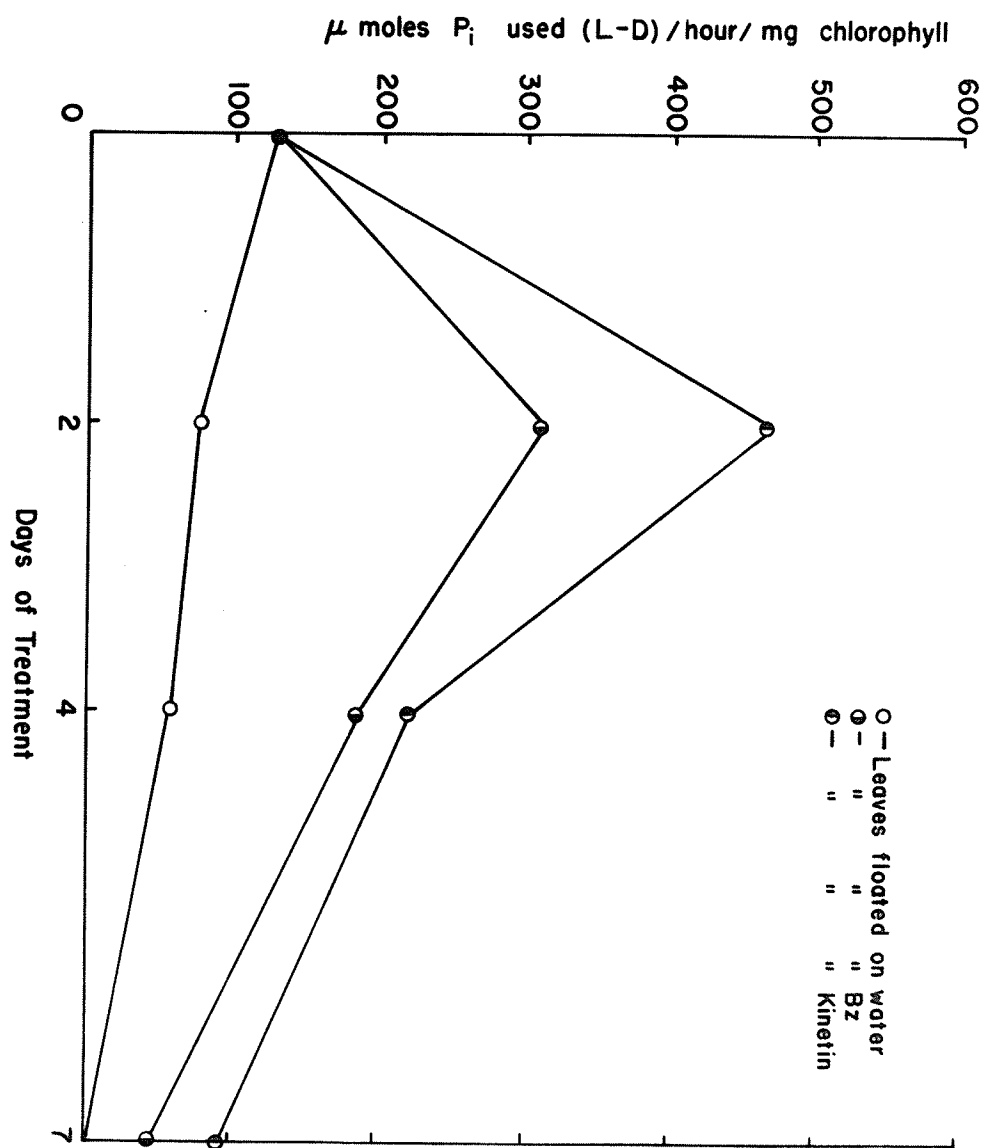
The reaction mixture and conditions were the same as given for Table XI.

* Two successive periods of 15 seconds light and 15 seconds darkness totalling 1 minute reaction.

** Light intensity of 1200 ft-c was used for illuminating chloroplast fragments from leaves senescing for 2 days and it was increased to 2500 ft-c for those prepared from leaves senescing for 4 to 7 days.

Primary leaves of Selkirk wheat grown in the greenhouse for 9 to 10 days were used for the experiment.

FIGURE VII. Effect of 'age' on the 'apparent' ortho-phosphate utilization by chloroplast fragments in a complete photophosphorylation system.



level for a period of 4 days. In comparison with the rates of photophosphorylation (Table VI), these rates show a close similarity, suggesting that the method may serve as a measure of the 'apparent' rate of ATP formation in light.

The chloroplast preparations from leaves senescing for 4 days or more, showed the requirement of higher light intensity for the liberation of Pi in light. For this reason, the light intensity of 2500 ft-c was used to illuminate the reaction system containing these preparations. The decrease in the rate of Pi liberation as well as its dependence on high light intensity with 'age', indicate that Pi liberation was dependent on the structure of chloroplasts and it was mediating the synthetic rather than degradative processes.

Figure VII shows the difference in Pi liberation (L-D) in the light and dark reaction. There was an acceleration in the rate of Pi liberation with benzimidazole (or kinetin) treatment and it resembled the trend in photophosphorylation rather than ATPase activity shown in Figure V. Although, the present evidence does not permit to state whether Pi liberation in light and darkness is catalyzed by one enzyme or there are two entities responsible for the light and dark reaction respectively, it suggests that Pi liberation in the complete system is dependent on the physiological condition of chloroplasts and it may be related to the intermediate(s) of photophosphorylation as indicated by Avron (1962).

V. EFFECT OF BENZIMIDAZOLE ON WHEAT 'PHOSPHODOXIN' FROM SENESCENT LEAVES

'Phosphodoxin' has been reported to be a natural cofactor for photophosphorylation in intact leaves (Black, et al., 1963a). Benzimidazole treatment increased the endogenous rate of photophosphorylation in leaves. It was, therefore, decided to study the effect of benzimidazole on 'phosphodoxin' levels of leaves senescing under continuous illumination. Accordingly, 'phosphodoxin' fraction was isolated from leaves floated for 6 days under continuous illumination of 850 ft-c on either water or solutions of benzimidazole and kinetin. The results of these experiments are given in Figure VIII.

It is evident from Figure VIII, that 'phosphodoxin' fraction isolated from wheat leaves gave a fluorescent peak at 430 m μ with the activation maximum at 350 m μ . Benzimidazole (or kinetin) treatment showed a slight increase in the initial (zero time control) levels of 'phosphodoxin' as measured by relative fluorescence. Leaves floated on water, however, showed a slight loss in this fraction.

'Phosphodoxin' from spinach leaves, was reported to give a slightly different peak which was reported to be at 440 m μ with activation maximum at 358 m μ (Black, et al., 1963c). It thus appears, that 'phosphodoxin' preparations from different plant materials, show different fluorescent peaks characteristic of the species.

FIGURE VIII. Activation and fluorescence spectra of wheat 'phosphodoxin'.

Solvent: 0.1M phosphate buffer, pH 7.0.

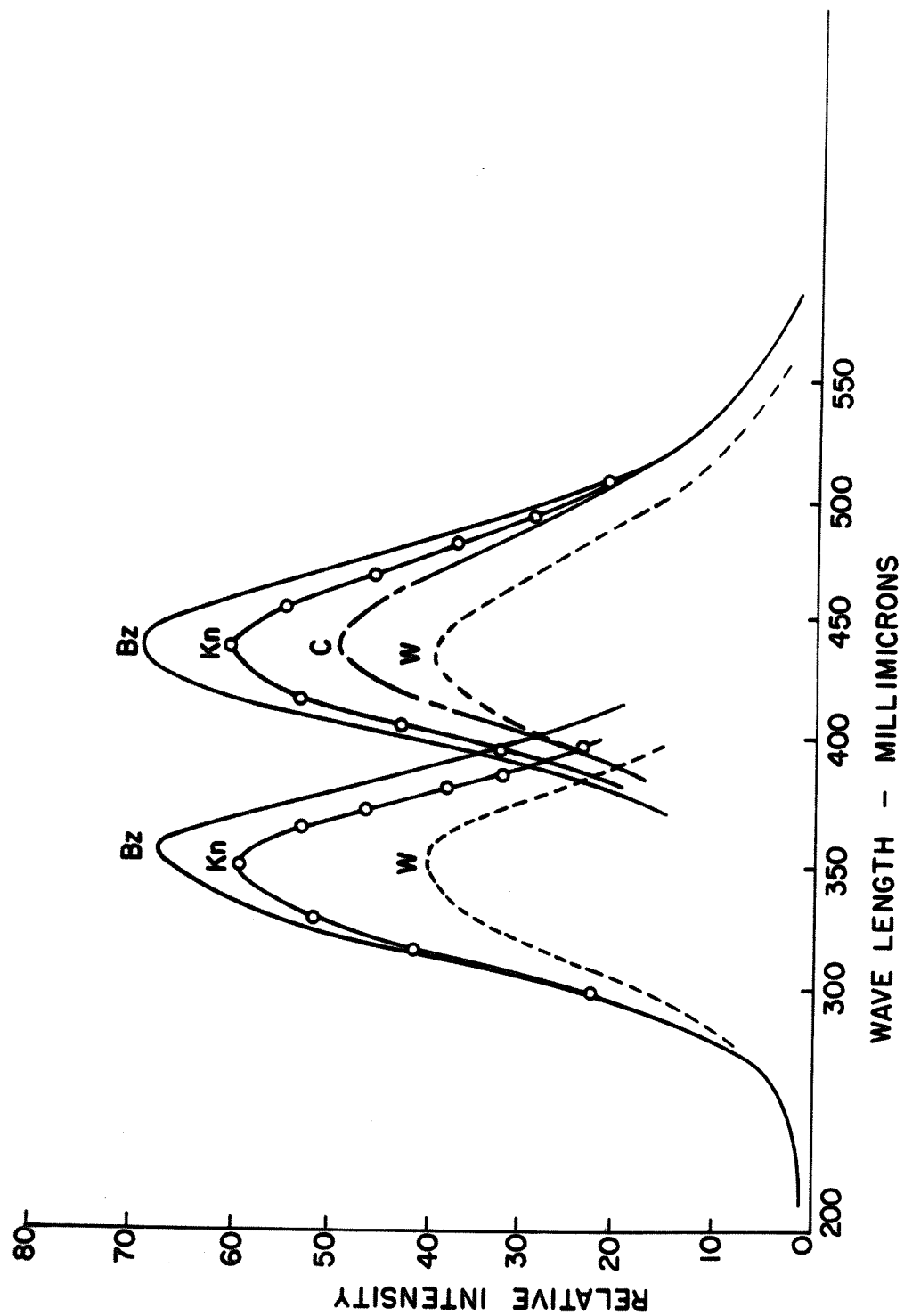
Concentration: 10%

C: control-immediately detached

W: floated on water

Bz: floated on benzimidazole

Kn: floated on kinetin



PART III

STUDIES ON NICOTINAMIDE ADENINE DINUCLEOTIDE KINASE
OF WHEAT LEAVES

Preliminary experiments on NAD kinase involved a one-step system in which NADP formed by the reaction $\text{NAD} + \text{ATP} = \text{NADP} + \text{ADP}$, was reduced by the specific NADP-isocitrate dehydrogenase system and measured at 340 m μ in a spectrophotometric procedure similar to that of Wang and Kaplan (1954). It was noted that the active preparations were obtained from wheat leaves grown under high light intensity or from leaves floated on benzimidazole solution under continuous illumination (kinetin was not tested).

The complete system always showed an increase in absorbancy at 340 m μ whereas the controls did not. However, it was impossible to show NADP formation using a two-step system (Kornberg, 1950; Wang, 1955). The activity was inhibited 50% by orthophosphate at a concentration of 10^{-3}M and this inhibition was overcome by ATP. ADP and NADH also inhibited the activity. The activity was later found to be due to increased turbidity in the system which registered as absorbancy at 340 m μ . The formation of either NADP or NADPH has never been demonstrated directly in these investigations nor in any other studies reported so far (Kornberg, 1950; Ogren and Krogmann, 1965; Yamamoto, 1966b).

It appeared necessary to use a more sensitive method for measuring NAD kinase activity. The fluorimetric procedure of Jacobson and Astrachan (1957) was finally adopted and the results obtained by this method were so similar to those arising from the spectrophotometric method that it appeared possible that the turbidity resulting from the presence of all components of NAD kinase system might have some

enzymatic significance. Accordingly, some of the experiments by spectrophotometric method are included in the latter part of this section and the absorbancy at 340 mμ is referred to as 'apparent' NAD kinase activity.

MATERIALS AND METHODS

MATERIALS

Primary leaves of Selkirk wheat (Triticum aestivum L.) grown in the growth chamber at 21° for a period of 10 to 12 days under 1200 to 1500 ft-c light intensity (16 hours photoperiod with 8 hours darkness), were floated on benzimidazole solution for 24 hours under continuous illumination of 850 ft-c. The leaves were harvested and lyophilized after being killed by liquid nitrogen. The crude enzyme prepared from the leaf powder was used for NAD kinase measurements by the fluorimetric method.

METHODS

I. THE FLUORIMETRIC METHOD FOR NAD KINASE MEASUREMENTS

The extraction of enzyme. The extraction procedure as described by Ogren and Krogmann (1965) was used to prepare the crude enzyme. Lyophilized leaf powder (500 mg) was ground in a chilled mortar and pestle with 20 ml 0.05M Tris-HCl buffer, pH 7.5. The brei was stirred for 20 minutes in the cold and centrifuged at 18,000 rpm for 20 minutes. The pellet was re-extracted with 5 ml of the buffer and centrifuged as before. The combined supernatant was frozen for 2 days, thawed and centrifuged to remove the greenish precipitate. The clear supernatant was used as the enzyme preparation.

Protein and chlorophyll determinations. The protein content of the enzyme preparation was measured by the spectrophotometric procedure

of Warburg and Christian (1941). Chlorophyll determinations were made on separate fractions of leaf powder following the method of Arnon (1949). Protein was measured at 260-280 m μ and chlorophyll at 652 m μ on Zeiss spectrophotometer (Model PMQ II) (Warburg and Christian, 1941; Arnon, 1949).

NAD kinase assay. The conversion of NAD to NADP by NAD kinase activity was measured by the modification of the fluorimetric method (Jacobson and Astrachan, 1957). The method involves the fluorimetric measurements of NAD remaining in the reaction system following NAD kinase activity. The NADP formed is reduced by isocitrate dehydrogenase and any NADPH or NADH formed is destroyed by acid treatment. The fluorescence of NAD was developed by alkali treatment (Kaplan, *et al.*, 1951) and the loss of NAD (corrected for any NADH formation in the system) was taken as the measure of NADP formed by NAD kinase.

The reaction system. The reaction system for the assay contained in μ moles the following components in a final volume of 1.0 ml: Tris-HCl, pH 7.5, 48; MgCl₂, 5; NAD (prepared in Tris-HCl buffer, pH 7.5), 0.2; nicotinamide, 20; isocitrate, 10; ATP (neutralized to pH 6.0), 2; 0.2 mg isocitrate dehydrogenase and enzyme preparation (containing 0.4 to 0.8 mg protein). All assays were run in triplicate. The mean of two experiments was taken for each enzyme preparation.

Procedure. The reaction system was incubated for 30 minutes at room temperature. The reaction was stopped with the addition of 0.2 ml

50% TCA, heated for 2 minutes at 95° to destroy NADPH (or any NADH) and centrifuged to remove the denatured protein. The supernatant was used to measure the remaining NAD in the system. Aliquots of 0.1 ml supernatant, were treated in duplicate with 3.9 ml of 5N NaOH, boiled for 5 minutes at 100° and the fluorescence determined.

Fluorimetric measurements were made on Turner Fluorometer (Model 110) with primary filter No.110-811 (7-60) at 365 m μ and the secondary filter No.110-821 (75) at 485 m μ , using pyrex glass cuvettes No.110 x 801.

II. SPECTROPHOTOMETRIC ASSAY FOR 'APPARENT' NAD KINASE ACTIVITY

The enzyme preparation. Primary leaves of Selkirk wheat were ground in a mortar and pestle with 5 times (w/v) of grinding medium containing 0.4M sucrose, 0.01M NaCl and 0.05M Tris-HCl buffer, pH 7.5. The brei was filtered through 4 layers of cheese cloth, centrifuged at 18,000 rpm for 20 minutes and the supernatant dialyzed for 12 to 16 hours against 200 volumes of 0.02M KHCO_3 and 0.003M KF. The dialysate was used for NAD kinase assay.

In some experiments, the dialysate was further purified by solid ammonium sulfate fractionation. The fraction separating between 0 to 45% saturation was dissolved in Tris-HCl buffer, pH 7.5, dialyzed overnight against 0.02M KHCO_3 and centrifuged. The supernatant was used for the enzyme assay.

Assay. The following components in μ moles were used in the final volume of 2.0 ml: Tris-HCl, pH 7.3, 50; $MgCl_2$, 5; nicotinamide, 4.5; sodium fluoride, 1; isocitrate, 10; NAD (pH 5.0), 0.4; isocitrate dehydrogenase, 1 mg and the enzyme (0.1 to 0.2 ml). The reaction was started by the addition of ATP (pH 5.8, 2.0 μ moles) and the increase in absorbancy at 340 m μ was recorded every minute for 10 minutes using silica cuvettes of 1 cm light path. NAD left in the reaction system after 10 minutes was measured enzymatically with alcohol dehydrogenase system (Racker, 1955a). In some experiments, NAD kinase activity (NADPH formed in one-step reaction) was also checked with glutathione-NADP specific glutathione reductase system (Racker, 1955b).

III. NAD AND NADP(H) BREAKDOWN IN LEAF EXTRACTS

Ten grams of freshly harvested leaves were ground with 20 ml 0.15M KCl-0.02 M $KHCO_3$ solution. The brei was filtered through 4 layers of cheesecloth and centrifuged first at 5000 rpm for 5 minutes and then at 18,000 rpm for 20 minutes. The supernatant was dialyzed overnight against 200 volumes of 0.02M $KHCO_3$, centrifuged and used as the crude enzyme.

The chloroplasts separated after the first centrifugation were washed twice with 0.05M Tris-HCl buffer, pH 7.5, suspended in 10 ml of the buffer and used for the assay.

The reaction system contained the following components in μ moles in the final volume of 2.0 ml: Tris-HCl, pH 7.5, 50; $MgCl_2$, 5; nicotinamide,

20; sodium fluoride, 1; NADP(H) or NAD (in Tris-HCl buffer, pH 7.5), 0.2 or 0.25; and 0.2 to 0.5 ml of the enzyme preparation. After the incubation at room temperature, the reaction was stopped by heating at 95° for 1 minute, the denatured protein was removed and NADP remaining in the supernatant was determined by isocitrate dehydrogenase system (Ochoa, 1955). Alcohol dehydrogenase was used to determine NAD content (Racker, 1955a) while, either isocitrate dehydrogenase or glutathione reductase system (Racker, 1955b) was used to determine NADPH.

Both nicotinamide and sodium fluoride are components of the NAD kinase system and accordingly were present in this reaction system.

IV. ORTHOPHOSPHATE DETERMINATIONS IN LEAVES.

Wheat leaves either freshly harvested or those floated on water or benzimidazole solution, were weighed in one gram lots and ground in 5% TCA chilled on ice. The brei was centrifuged at 5000 rpm for 2 minutes and the supernatant was used to determine orthophosphate content by the method of Waygood (1948).

RESULTS

I. NAD KINASE ACTIVITY-FLUORIMETRIC METHOD

Preliminary experiments indicated that the most active enzyme preparations were obtained from leaves floated on benzimidazole for 24 hours under continuous illumination. Therefore, in several experiments these have been designated as controls. The progress of activity for the enzyme from such leaves is shown in Table XIII and Figure IX. The slowing down of activity suggests the presence, in the crude enzyme of NAD(P) nucleosidase (EC 3.2.2.6) or NADP(H) phosphatase (Forti, *et al.*, 1962).

Table XIV and Figure X show that there is a linear relationship between protein concentration and enzyme activity. It is calculated from the data (Table XIV) that approximately 90 μ moles NADP was formed per hour per mg protein. On the chlorophyll basis, this rate is approximately 1148 μ moles NADP per hour per μ mole chlorophyll. Rates of NADP formation close to 65 μ moles per hour per mg protein were reported in tobacco and spinach leaves (Yamamoto, 1966b). Ogren and Krogmann (1965) reported 0.24 μ moles NADP formation per minute per μ mole chlorophyll (14.4 μ moles NADP/hour/ μ mole chlorophyll), in spinach leaves. This rate is almost 12 times greater than that observed in wheat leaves.

NAD kinase activity in senescing leaves. Wheat leaves floated on benzimidazole or kinetin solution under a photoperiod of 16 hours light and 8 hours darkness, were reported to show an increase in NADP and NADPH levels as compared to those floated on water which showed an

TABLE XIII. The progress of NAD kinase activity

Incubation period minutes	mmoles NADP per 0.8 mg protein	mmoles NADP/ μmoles chlorophyll
10	17	283.3
20	22	366.6
30	27	449.94
45	30	499.94

The reaction system and experimental conditions were the same as given in 'Methods' for the fluorimetric assay.

TABLE XIV. The effect of enzyme concentration on NAD kinase activity.

Enzyme mg protein	mmoles NADP/ 30 minutes	mmoles NADP/ 30 minutes/ μmoles chlorophyll
0.4	18	600.0
0.8	30	500.0
1.2	56	622.0

The reaction system and experimental conditions were the same as given for Table XIII.

FIGURE IX. The progress of NAD kinase activity.

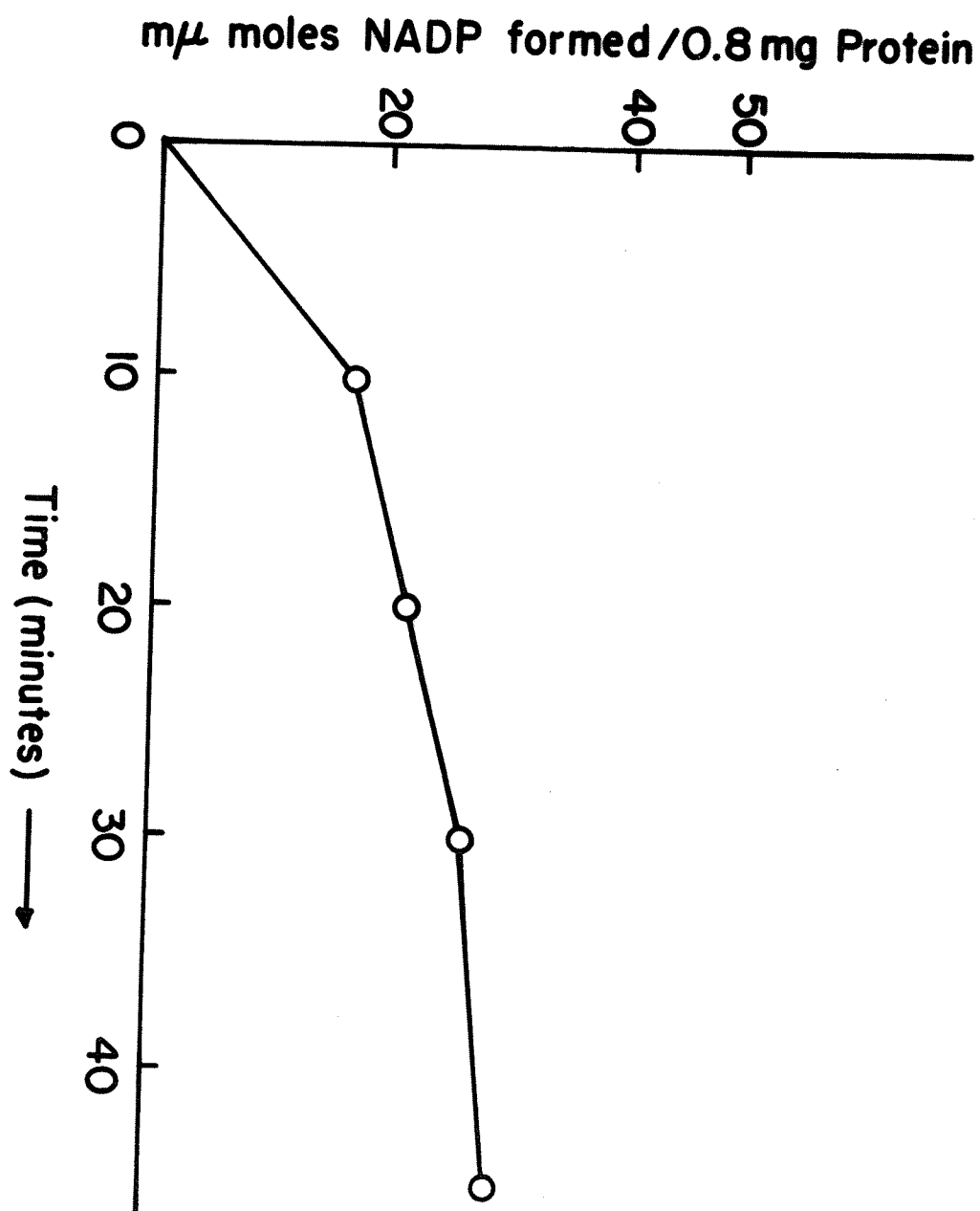
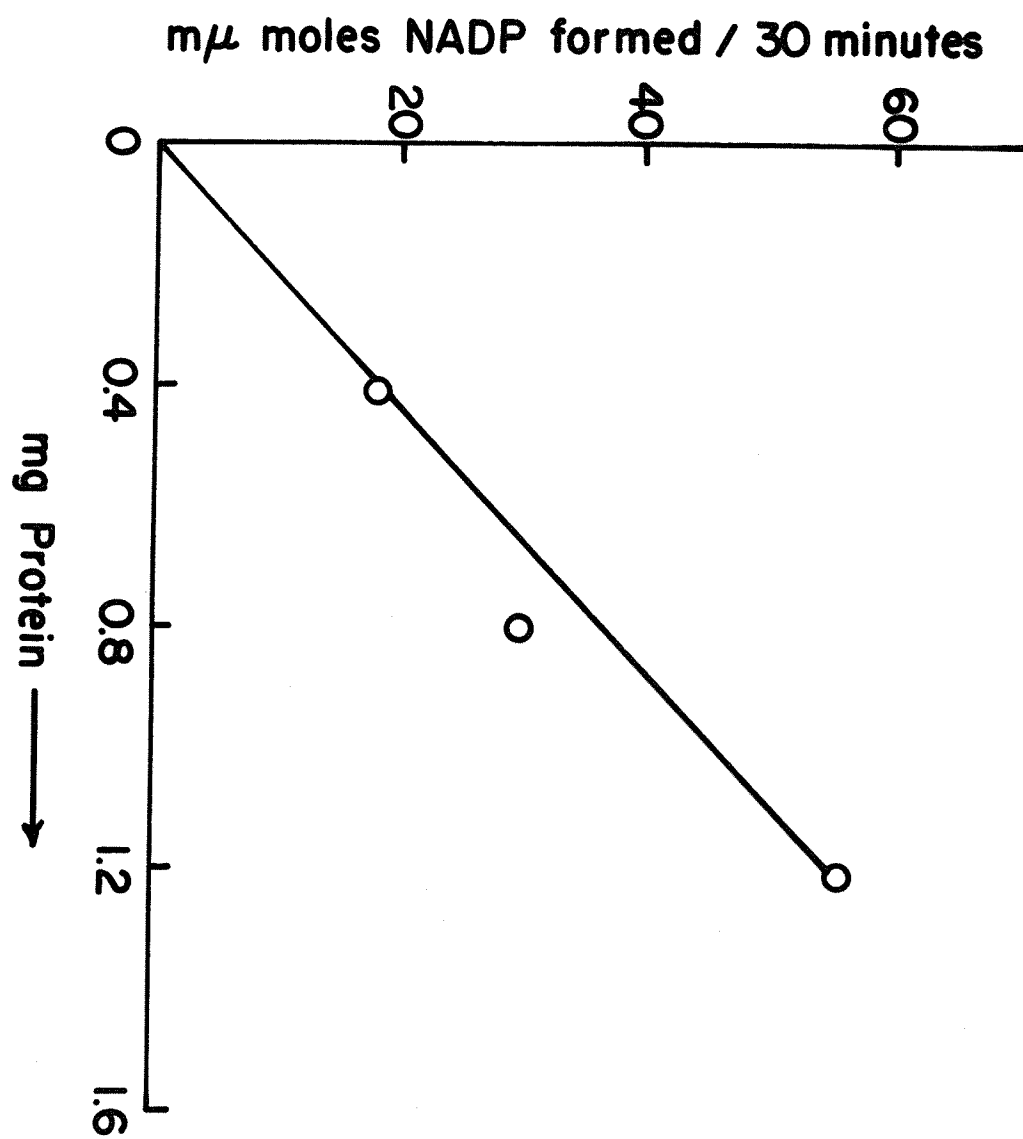


FIGURE X. The effect of enzyme concentration on NAD
kinase activity.



increase in NADP levels (Mishra, 1963). Furthermore, non-aqueous chloroplast preparations from leaves floated on these solutions under continuous illumination were reported to show an increase in NADP levels. Investigations were, therefore, undertaken to study the NAD kinase activity in leaves floated for 48 hours under continuous illumination of 850 ft-c, on either water, benzimidazole or kinetin solution. The rates of NADP formation in these leaves were compared to those observed in leaves floated on benzimidazole solution for 24 hours under similar conditions. The results of these investigations are given in Table XV.

It is evident from Table XV, that senescing leaves do not show any significant loss in NAD kinase activity. Benzimidazole treatment did not show any increase in the enzyme activity whereas, kinetin treatment showed a slightly less activity as judged by the levels of NADP synthesized. It appears, therefore, that the loss of NADP levels associated with senescence (Mishra, 1963; Yamamoto, 1963) and the increase in NADP(H) levels in leaves treated with benzimidazole (or kinetin) solution (Mishra, 1963) may not necessarily be due to the respective loss or increase in NAD kinase activity of leaves. In accord with these findings, no correlation between NADP levels observed in senescent or hormone treated tissue and NAD kinase activity associated with these tissues, was reported in rat livers (Greenbaum, *et al.*, 1965; Clark, *et al.*, 1964).

The effect of benzimidazole on NAD kinase activity. Since benzimidazole and kinetin have been shown to stimulate the synthesis

TABLE XV. NAD kinase activity in senescing leaves.

Enzyme source	mmoles NADP formed per hour per mg protein		mmoles NADP formed per hour per μ mole chlorophyll	
	Experiment No.		Experiment No.	
	I	II	I	II
Control (benzimidazole (24)*)	75.9	..	500.0	..
Water (48)**	66.0	58.5	436.0	390.0
Benzimidazole(48)	70.0	65.5	466.0	436.0
Kinetin (48)	48.0	50.7	458.0	422.0

* The enzyme prepared from leaves floated on benzimidazole for 24 hours under continuous illumination of 850 ft-c.

** The enzyme preparations from leaves floated on the respective solution for 48 hours under similar conditions.

The plants were grown in the growth chamber for 9 days at 21° under 1500 ft-c (16 hours photoperiod and 8 hours darkness).

of NADP(H) in wheat leaves and their chloroplasts, it was of importance to determine their effect directly in the NAD kinase reaction system. Both benzimidazole and kinetin had previously been shown to inhibit 'apparent' NAD kinase activity by the spectrophotometric method (Figs. XIe and f, discussed later). The data in Table XVI also show that benzimidazole solution at a concentration of $6.0 \times 10^{-3}M$ completely inhibits NAD kinase activity as measured by the more sensitive and valid fluorimetric method. However, this is a relatively high concentration of benzimidazole and the inhibition may have no physiological significance. Indeed, this effect cannot be invoked to explain the marked increase in the NADP content of wheat leaf chloroplasts as found by Mishra (1963) (see Waygood, 1965). The inhibition of NAD kinase activity by benzimidazole is similar to that reported for phenylalanine deaminase (EC 4.3.1.a) by Zucker (1965). This enzyme was inhibited 80 to 90% by benzimidazole at a concentration of $5.0 \times 10^{-3}M$. Since, the author has previously shown (Table XV), that benzimidazole treatment did not necessarily show a significant increase in NAD kinase content of leaves, the effect of benzimidazole must be indirect either by a derivative, e.g., benzimidazole nucleotide (Kapoor and Waygood, 1965a) activating the enzyme or as considered later or by its effect on the orthophosphate or ATP content of leaves.

Regulation of NAD kinase activity. As pointed out in the introduction to this section, the only active preparations showing 'apparent' NAD kinase activity (spectrophotometric method), were obtained from leaves grown under high light intensity or floated on benzimidazole

solution. Such leaves have lower orthophosphate levels than those grown under low light intensity or in leaves floated on water. It was, therefore, speculated that orthophosphate might have some regulatory effect on NAD kinase activity. Studies were, therefore, made on the effect of orthophosphate on 'apparent' NAD kinase activity and later by the fluorimetric assay. As will be discussed later, orthophosphate inhibits 'apparent' activity (Fig.XIc) and the data in Table XVII show that orthophosphate at a concentration of 10^{-3} M inhibited by 50% the enzyme activity determined by the fluorimetric method. There is no report available (as far as the author is aware of) on NAD kinase inhibition by orthophosphate present in physiological concentration, in the reaction system.

Orthophosphate at the concentrations more than 2.2 mM, has been shown to inhibit sugar nucleotide phosphorylase catalyzing the reaction $\text{NDP-hexose} + \text{P}_i \longrightarrow \text{NDP} + \text{hexose-1-P}$, where X is a purine or pyrimidine nucleoside (Cabib, *et al.*, 1965). Similarly, purified NADP(H) phosphatase from pea leaves, has been reported to be inhibited by orthophosphate when present at the concentration of 5.0×10^{-5} M (Forti, *et al.*, 1962).

The effect of ATP on NAD kinase inhibition by orthophosphate.

Since orthophosphate was inhibitory to the enzyme activity, it was decided to study the effect of ATP on NAD kinase activity in overcoming this inhibition for two reasons; (i) orthophosphate is one of the substrates of photophosphorylation and (ii) ATP has been reported to

FIGURE XIa. pH curve for 'apparent' NAD kinase activity.

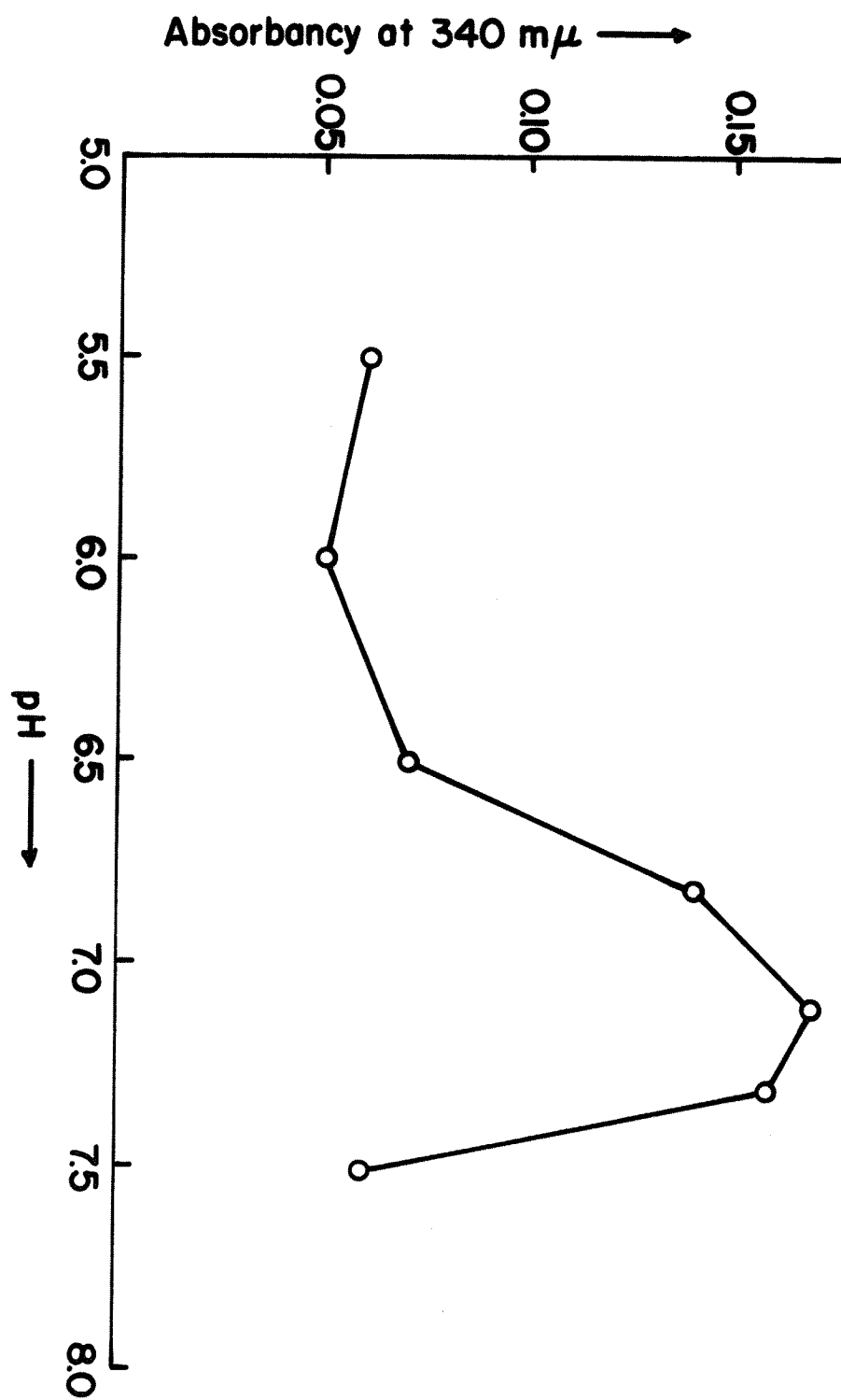


FIGURE XIb. The effect of ATP concentration on
'apparent' NAD kinase activity.

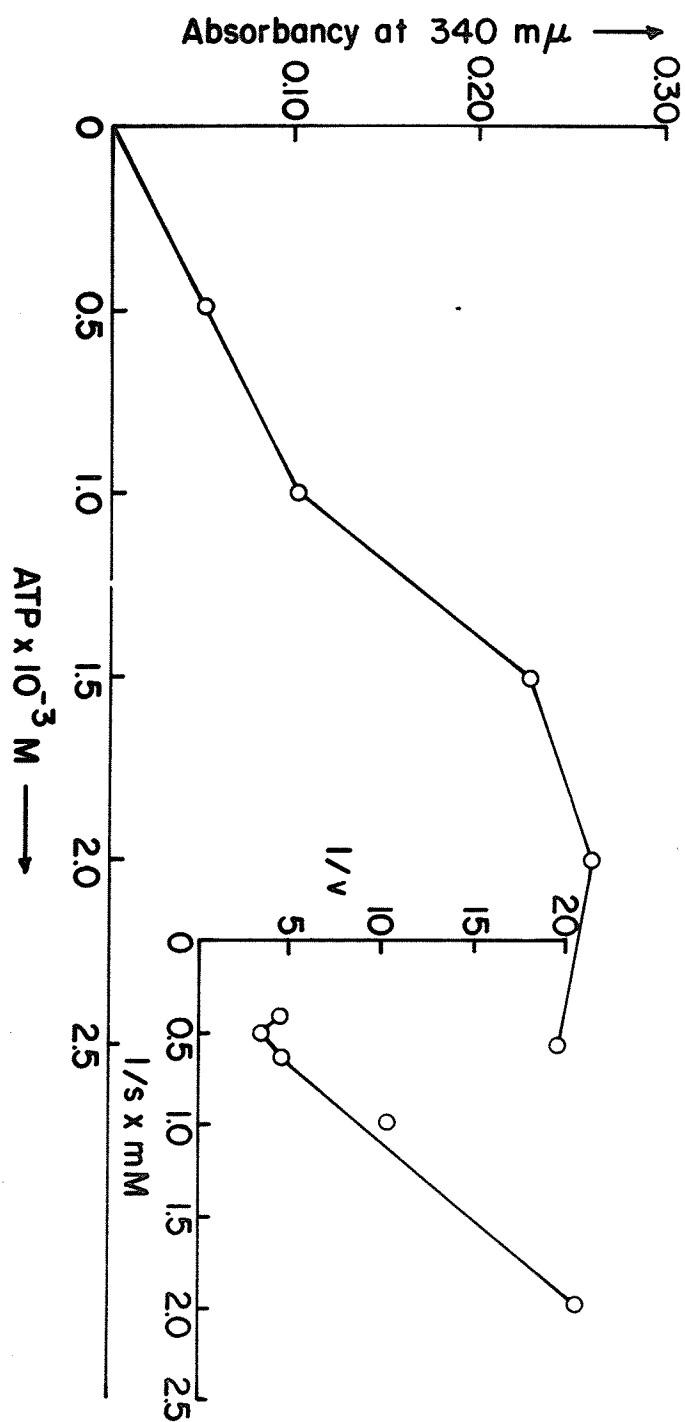


FIGURE XIc. Inhibition of 'apparent' NAD kinase by orthophosphate.

FIGURE XIId. Reversal of enzyme inhibition by ATP.

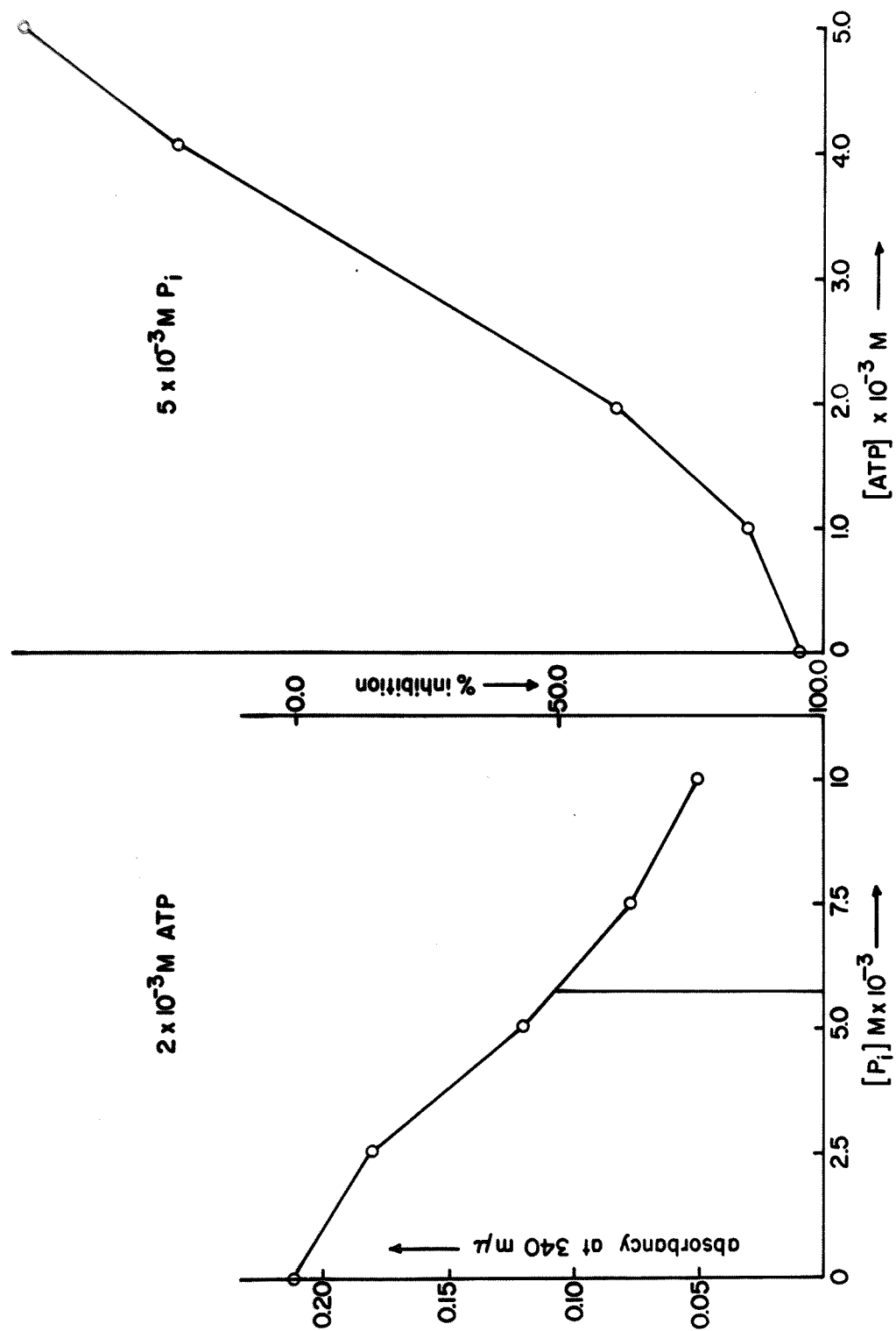


TABLE XVII. The effect of orthophosphate on NAD kinase activity.

Expt. No.	Assay system	mmoles NADP per hour per mg protein	mmoles NADP per hour per μ mole chlorophyll	Inhibition of NADP synthesis % control
I	Control	194.8	695.6	(0.0)
	+Pi 10^{-2} M	0.0	0.0	100.0
	+Pi 10^{-3} M	80.8	288.52	59
	+Pi 10^{-4} M	137.6	491.34	29
II	Control	194.4	602.6	(0.0)
	+Pi 10^{-2} M	3.2	9.91	98
	+ Pi 10^{-3} M	105.2	326.09	46
	+Pi 10^{-4} M	122.8	380.65	37

The reaction system and experimental conditions were the same as given for Table XIII.

Potassium phosphate buffer (0.1M, pH 7.2) was used as the source of orthophosphate (Pi).

regulate NAD kinase activity in rat liver tissue (Greenbaum, et al., 1965). The results of these experiments are given in Table XVIII.

It is evident from Table XVIII, that ATP could overcome, to a certain extent, the inhibition of NAD kinase by orthophosphate. ATP in concentrations higher than 5 μ moles was found to be slightly inhibitory which might possibly be due to the substrate inhibition (Expt.No.I). ATP, however, appears to be a controlling factor for NADP synthesis in senescent leaves (Expt. No.II). The enzyme preparation from leaves floated on water for 49 hours showed a higher potential for NADP synthesis with an increase in ATP levels in the reaction system. It may be noted that the samples of leaves used in these two experiments (Expt. No.I and II), were not from the same lot and the control rates of NADP synthesis in these experiments can not, therefore, be compared. These experiments were chosen to show that the effect of ATP in overcoming the enzyme inhibition by orthophosphate was the same in leaves floated on either benzimidazole solution for 24 hours (Expt. No.I) or water for 48 hours under continuous illumination (Expt. No.II).

It was also important to determine that the ATP-orthophosphate effect was on NAD kinase activity and not on isocitrate dehydrogenase system used to reduce NADP formed by NAD kinase activity ($\text{NAD} + \text{ATP} = \text{NADP} + \text{ADP}$) in the reaction system. The effect of ATP, Pi (orthophosphate), and other cofactors (used in the complete reaction system for NAD kinase assay), on the isocitrate dehydrogenase assay, was

TABLE XVIII. The effect of ATP on NAD kinase inhibition by orthophosphate.

nmol NADP formed per hour per mg protein

Expt. No.	Assay system	μ moles ATP used						
		1	2	3	4	5	6	8
I*	Control	..	158.8	..	..	..	..	..
	+Pi 10^{-3} M	0.0	..	51.2	120.0	116.0	92.0	76.0
II**	Control	..	58.4	..	..	..	..	..
	+Pi 10^{-3} M	..	19.0	..	47.0	..	63.0	..

* The enzyme prepared from leaves floated on benzimidazole solution for 24 hours at 850 ft-c.

** The enzyme prepared from another lot of leaves floated on water for 48 hours at 850 ft-c.

studied by the spectrophotometric method (Ochoa, 1955).

The effect of orthophosphate, ATP and benzimidazole on isocitrate dehydrogenase system. The results of the effect of Pi, ATP and benzimidazole on the isocitrate dehydrogenase system, are given in Table XIX, which show that neither Pi, ATP nor benzimidazole had any inhibitory effect on the assay system in question.

Another possibility, that Pi was reacting with NAD and thereby limiting NAD kinase activity might be ruled out on the basis of the reports of Colowick, et al., (1951) and Griffiths (1964). NAD has been reported to react with orthophosphate in biological systems but the existence of such a compound was not detected by any spectral changes of NAD in the presence of 1M primary, secondary or tertiary phosphate (Colowick, et al., 1951). Griffiths (1964) reported no changes in the absorption maxima in the region of 290 to 300 mμ with either phosphorylated or non-phosphorylated NAD derivatives.

In the present investigation, ATP-Pi effect on NAD kinase was observed by both the fluorimetric and spectrophotometric methods (Tables XVII and XVIII, and Figs. XI c and d, respectively), and confirm the findings that NAD kinase activity in wheat leaves is regulated by ATP-orthophosphate levels.

II. 'APPARENT' NAD KINASE ACTIVITY-SPECTROPHOTOMETRIC METHOD

Some of the results obtained by this method are given in Tables XX a, b and c and Figures XI a,b,c,d,e,f and g and are discussed briefly.

TABLE XIX. The effect of orthophosphate, ATP and benzimidazole on isocitrate dehydrogenase system.

Reaction system	Absorbancy at 340 mμ after 10 minutes
Control* (0.2 μmoles NADP)	0.58
+ Nicotinamide (20 μmoles)	0.58
+ ATP (10 μmoles)	0.58
+ Pi (10 μmoles)	0.59
+ benzimidazole (1.6×10^{-2} M)	0.58
with NAD (0.2 μmoles)	0.01

* The reaction system contained the following components in a final volume of 2 ml: Tris-HCl, pH 7.5, 96 μmoles; $MgCl_2$, 5 μmoles; isocitrate, 10 μmoles; and isocitrate dehydrogenase 1 mg protein. The reaction was started with 0.2 μmoles of NADP solution and the increase in absorbancy at 340 mμ was recorded for 10 minutes.

TABLE XXa. Specificity of ATP as a phosphate donor for 'apparent' NAD kinase activity.

Phosphate donor	Absorbancy at 340 mμ after 10 minutes
ATP (4 μmoles)	0.275
ADP (4 μmoles)	0.0
AMP (4 μmoles)	0.01
GTP (5 μmoles)	0.028
UTP (5 μmoles)	0.048

The reaction system was same as given in 'Methods' for 'apparent' NAD kinase assay. Epzyme preparation from freshly harvested leaves, was used.

GTP = guanosine triphosphate
UTP = uridine triphosphate

TABLE XXb. The effect of inhibitors on 'apparent'
NAD kinase activity.

Inhibitor	Concentration (final)	Inhibition % control
Potassium cyanide	$5.0 \times 10^{-3} \text{M}$	80.00
Na-pyrophosphate	$5.0 \times 10^{-3} \text{M}$	16.00
	$5.0 \times 10^{-4} \text{M}$	7.00
Meta phosphate	$5.0 \times 10^{-2} \text{M}$	100.00
NADH	$6.0 \times 10^{-4} \text{M}$	100.00
Dicumarol	$1.0 \times 10^{-6} \text{M}$	0.00
Sodium fluoride	$2.0 \times 10^{-4} \text{M}$	0.00
Orthophosphate	$5.0 \times 10^{-3} \text{M}$	48.00
ADP	$2.0 \times 10^{-2} \text{M}$	68.00

The reaction system was the same as given for Table XXa.

TABLE XXc. The effect of orthophosphate on NAD breakdown in the presence of enzyme preparation from freshly harvested leaves.

Enzyme assay	Absorbancy at 340 mμ			
	Expt.No.I		Expt. No. II	
	control	+Pi	control	+Pi
NAD kinase	0.13	0.08	0.149	0.065
NAD left (alcohol dehydrogenase assay)	0.25	0.24	0.55	0.57
Total NAD used	0.38	0.32	0.69	0.635
NAD lost by enzymatic breakdown	0.48	0.54	0.13	0.20
Alcohol dehydrogenase control (0.4 μmoles NAD)	..	.. 0.86	..	.. 0.83

* Pi used = 5.0×10^{-3} M. The reaction was observed for NAD kinase activity for 10 minutes, NAD left in the system was then reduced by alcohol dehydrogenase system.

FIGURE XIe. Inhibition of 'apparent' NAD kinase by
kinetin in the reaction system.

FIGURE XIIe. Inhibition of 'apparent' NAD kinase by
benzimidazole in the reaction system.

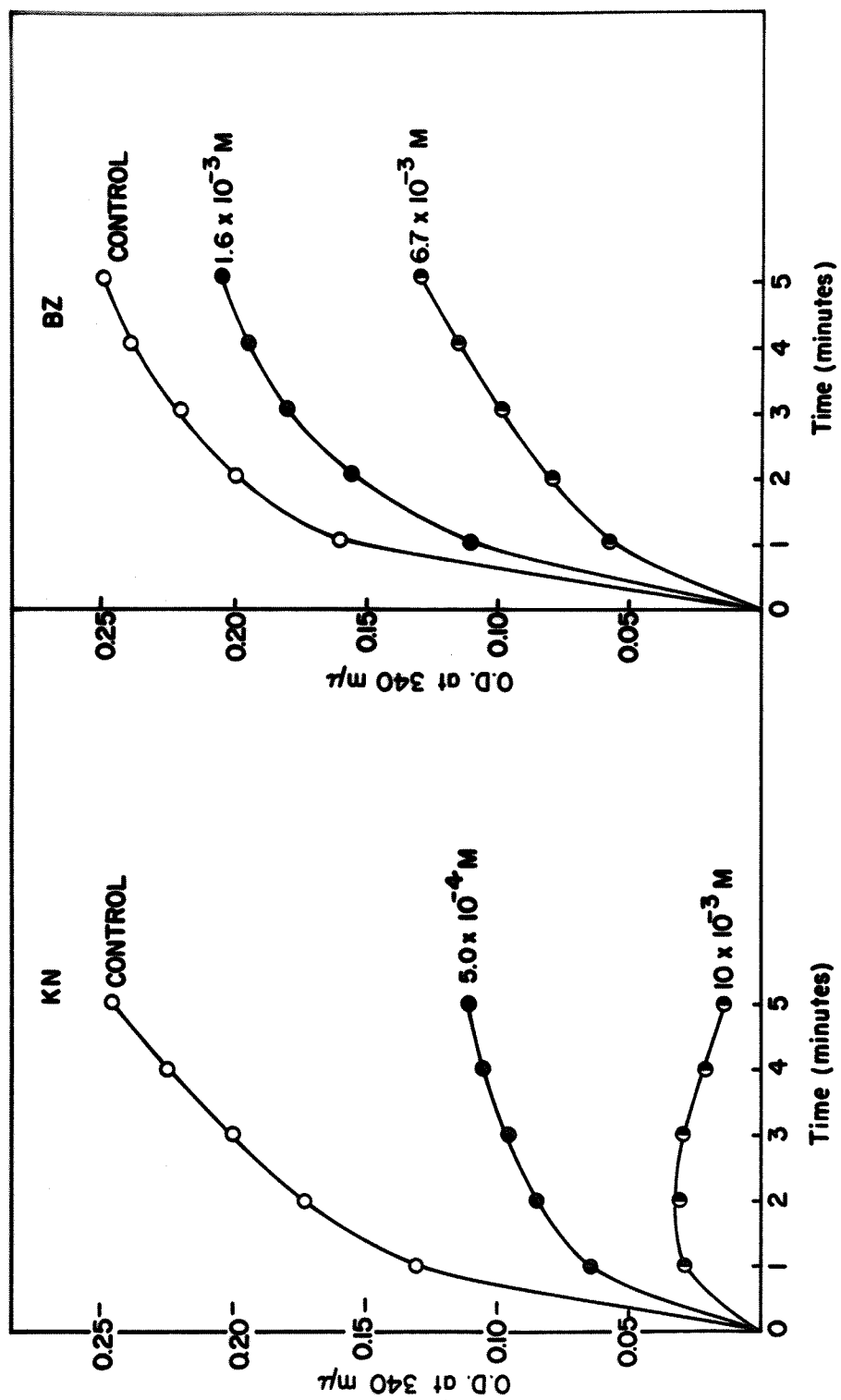
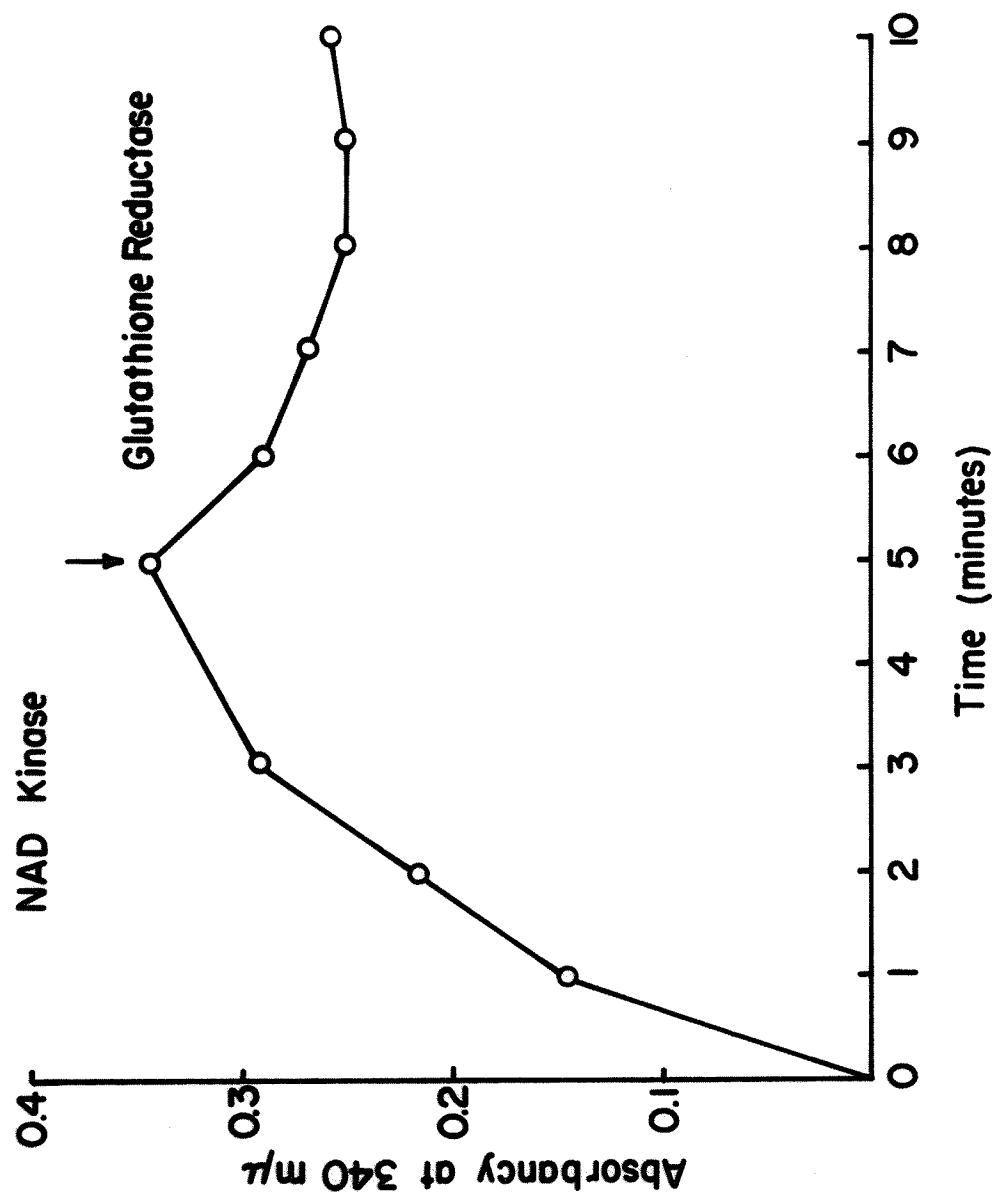


FIGURE XIg. The 'apparent' NAD kinase activity as
measured by glutathione reductase assay.



As shown in Table XXa, ATP was found to be the only nucleotide which showed the activity as registered by turbidity. The 'apparent' activity was inhibited by potassium cyanide, sodium pyrophosphate, metaphosphate, orthophosphate, ADP and NADH in the reaction system (Table XXb). NADH has been reported to be a specific competitive inhibitor of NAD kinase (Wang and Kaplan, 1954; Wang, 1955; Yamamoto, 1966b), which lends support to the argument that 'apparent' NAD kinase activity may have some enzymatic significance. Figure XIa shows the optimum pH for the 'apparent' activity. The effect of ATP concentration on the 'apparent' activity is shown in Figure b, the kinetics of which suggest that ATP may be a regulatory factor for the activity. As mentioned previously, the inhibition of the activity by orthophosphate and its reversal by ATP, are shown in Figures XI c and d respectively. These results are similar to those obtained by the fluorimetric method using enzyme preparations from lyophilized leaves floated on benzimidazole solution for 24 hours under continuous illumination of 850 ft-c. Figures XI e and f show the inhibition of the activity by kinetin and benzimidazole solution respectively. The data in Figure XI g provide evidence that, at least part of the increase in absorbancy at 340 m μ , was due to NADP reduction and not completely due to the turbidity developed in the reaction system, as shown by the use of glutathione reductase system. Glutathione reductase system is not specific for NADPH, but does react with NADH very slowly. In this investigation, the absorbancy produced in the NAD kinase system in the absence of either isocitrate

or isocitrate dehydrogenase or that possibly produced by the reduction of NAD to NADH in the presence of the crude enzyme preparations used for NAD kinase assay, was not decreased by the use of glutathione reductase assay (not shown in the Figure). It is suggested from the data (Fig. XI g) that the partially purified enzyme (0 to 45% ammonium sulfate fraction used for the assay), was able to give the 'apparent' rate of 0.043 μ moles (NADP) per 10 minutes per 0.5 mg protein (516 μ moles/hour/mg protein). These values are almost 5.7 times greater than those obtained by the fluorimetric method (90 μ mmole s/hour/mg protein)(Table XIV).

Spinach or tobacco leaf NAD kinase activity was reported to be optimum at pH 6.8 (Yamamoto, 1966b). ADP has been shown to inhibit pigeon liver NAD kinase (Wang and Kaplan, 1954; Wang, 1955). Wang and Kaplan (1954) reported the specificity of ATP as a phosphate donor for pigeon liver NAD kinase. Similarly, yeast NAD kinase has been shown to be inhibited 41% by sodium pyrophosphate at a concentration of $2.0 \times 10^{-2}M$ (Kornberg, 1950). All these results strongly suggest that the 'apparent' activity has some enzymatic significance.

III. THE EFFECT OF BENZIMIDAZOLE ON THE ORTHOPHOSPHATE LEVELS OF WHEAT LEAVES

Since orthophosphate inhibited NAD kinase activity, it was speculated that benzimidazole might be operating by influencing orthophosphate levels in leaves which in turn influenced the NAD kinase system. Accordingly, experiments were undertaken to determine the effect

of benzimidazole on the endogenous levels of orthophosphate in wheat leaves. In the preliminary experiments (by the spectrophotometric method) it was noted that there was considerably less activity in older than younger leaves. Since it would be expected that orthophosphate levels would increase with 'age', primary and secondary leaves were also tested for orthophosphate levels. The results of these experiments are given in Tables XXIa and b and XXII.

The effect of 'age'.The effect of 'age' on orthophosphate levels of primary leaves of Selkirk and Khapli wheat grown under greenhouse conditions, is shown in Table XXIa. The data indicate that there was an increase with 'age' in the orthophosphate content of leaves and Khapli wheat showed a higher Pi (orthophosphate) content than Selkirk wheat. Wheat seedlings grown under greenhouse conditions showed fluctuations in Pi levels in response to changes in daily light intensities.

The data in Table XXb show orthophosphate levels of primary and secondary leaves grown under controlled conditions at 21° under the light intensity of 1500 ft-c (16 hours photoperiod and 8 hours darkness). It is evident from the data that these leaves showed lower levels of Pi which were further found to be lower in primary than in secondary leaves.

The reaction in photophosphorylation is $ADP + Pi = ATP$. Thus, the levels of orthophosphate in leaves subjected to light are expected to change with changes in the rate of 'cyclic' or 'non-cyclic'

TABLE XXIa. The effect of 'age' on the orthophosphate content of wheat leaves variety Selkirk or Khapli.

μ moles Pi per gram fresh weight

Age in days	Selkirk Wheat		Khapli Wheat	
	Expt.No.		Expt.No.	
	I	II	I	II
9	27.67	-	22.26	-
12	18.46	30.59	24.74	32.59
13	31.05	29.65	30.59	32.75
14	31.57	30.59	33.47	28.50
15	30.59	33.43	40.73	29.58

The seedlings were grown under greenhouse conditions.
(natural day-light illumination).

TABLE XXIb. Orthophosphate content of primary and secondary leaves of wheat grown in the growth chamber.

Age in days	Selkirk Wheat		Khapli Wheat	
	Primary leaves	Secondary leaves	Primary leaves	Secondary leaves
8	8.79	-	11.08	-
9	8.63	-	11.53	-
10	12.68	-	12.58	-
13	10.26	-	13.47	-
14	12.01	22.48	13.73	18.71
15	16.69	21.90	19.74	18.60

Wheat seedlings were grown in the growth chamber at 21° under 1500 ft-c light intensity (16 hours photoperiod and 8 hours darkness).

TABLE XXII. The effect of benzimidazole and light on the orthophosphate levels of leaves (Selkirk wheat).

			Lot No.			
Wheat seedlings grown at ft-c (16 hours photoperiod and 8 hours darkness)			I	II		
			850		3000	
Age in days			8		9	
μ moles Pi per gram fresh weight of immed- iately detached leaves.			27.5		22.0	
Leaves floated on*			μ moles Pi per gram fresh weight			
Days floated			Light		Darkness	
(850 ft-c)			Lot No.		Lot No.	
			I	II	I	II
		Leaves	25.26	23.4	25.26	23.4
Water	1	Medium	1.4	0.9	1.2	0.9
		Total	26.66	24.3	26.46	24.3
		Leaves	23.4	17.5	25.26	23.4
Benzimidazole	1	Medium	0.9	0.6	0.49	0.6
		Total	24.3	18.1	25.75	24.0

* One gram leaves from each lot were floated on 150 ml of either water or benzimidazole solution for 24 hours under continuous illumination of 850 ft-c or in darkness.

photophosphorylation. An increase in Pi content with 'age' may possibly be due to a decrease in photophosphorylation as a result of physical or physiological changes such as, the crowding of leaves or impairment of the chloroplast structure. The rate of photophosphorylation has been shown to decrease with 'age' (Smillie and Krotkov, 1959) and 'aging' tissues have been reported to accumulate Pi^{32} more into inorganic than in the organic form (Bieleski and Laties, 1963).

The effect of benzimidazole and light. In order to study the effect of benzimidazole on the endogenous levels of orthophosphate, primary leaves of Selkirk wheat grown under controlled conditions at 21° under two different light intensities of 850 ft-c and 3000 ft-c (16 hours photoperiod and 8 hours darkness) respectively, were floated on either water or benzimidazole solution for 24 hours under continuous illumination of 850 ft-c. The control sets were floated for 24 hours in darkness. The results of these investigations are given in Table XXII.

The data in Table XXII show that leaves initially grown at 850 ft-c (lot I) showed a slight decrease whereas, those grown at 3000 ft-c (lot II) showed an increase in orthophosphate content when floated on water for 24 hours. However, there was no further change observed in leaves floated in darkness. On the other hand, benzimidazole treated leaves from both lot I and II showed a decrease in orthophosphate levels when exposed to light but not in darkness. This 'benzimidazole

effect¹ was more pronounced in leaves (lot II) initially grown at higher light intensity (3000 ft-c, 16 hours photoperiod with 8 hours darkness). Leaves floated on water in either light or darkness, showed a greater loss of orthophosphate in the floating medium than those floated on benzimidazole solution. This loss may be due to permeability changes in leaves senescing on water (Sacher, 1966).

Thus, a decrease in orthophosphate levels of leaves floated on benzimidazole solution under continuous illumination, may be due to the increase in photophosphorylation as shown in Section II (Tables VI and VII). The increase in photophosphorylation in these leaves may account for a low Pi/ATP ratio which could influence NADP synthesis with a resultant increase in NADP(H) content observed by other workers (Mishra, 1963; Waygood, 1965; Godavari, 1966).

Primary leaves of Selkirk wheat showed 1.6×10^{-2} M orthophosphate per ml fresh juice, which appears to be sufficiently high to inhibit NAD kinase completely. It is, however, possible that the activation or inhibition of NAD kinase may depend on Pi/ATP ratio at the site of NADP synthesis (e.g. in chloroplasts) and benzimidazole treatment may influence these levels with a resultant stimulation in NADP synthesis in leaves exposed to light. As reported earlier in the Review of Literature, the NADP/NAD ratio undergoes diurnal changes in green leaves and alga, when exposed to alternating light and dark periods (Oh-Hama and Miyachi, 1963; Ogren and Krogmann, 1963; 1965; Mishra, 1963) and this ratio changes during senescence (Mishra, 1963; Yamamoto, 1963). It is thus, possible that changes in Pi-ATP levels in

light and darkness or during senescence control NADP synthesis in leaves.

IV. NAD AND NADP(H) BREAKDOWN BY WHEAT LEAF ENZYME PREPARATIONS

Investigations on NAD and NADP(H) breakdown in the reaction system used for NAD kinase activity, were prompted by the failure to obtain NAD kinase activity by a two-step reaction of Kornberg (1950). There was a 15 to 40% breakdown of NAD or NADP(H) when used with the enzyme preparations from lyophilized leaf powder. The results of NAD and NADP breakdown in the complete reaction system (containing nicotinamide and sodium fluoride used to inhibit the breakdown of NAD and ATPase activity, respectively, (Wang and Kaplan, 1954; Greenbaum, *et al.*, 1965)) by the enzyme preparations from lyophilized leaf powder are given in Tables XXIII, XXIV and XXV whereas, those from freshly harvested leaves are given in Table XXVI.

Breakdown activity in senescing leaves. The rate of NAD or NADP breakdown in leaves floated on either water, benzimidazole or kinetin solution for 48 hours under continuous illumination of 850 ft-c is given in Table XXIII. The enzyme preparation from leaves floated on benzimidazole solution for 24 hours under similar conditions was used as a control.

The data in Table XXIII show that there was an increase which was more pronounced for NADP than NAD breakdown, in leaves senescing on water or benzimidazole solution for 48 hours. Kinetin treatment prevented

TABLE XXIII. NAD and NADP breakdown in senescing leaves.

Enzyme preparation	% loss per mg protein per 15 minutes		Increase in breakdown % control	
	NAD	NADP	NAD	NADP
Control (benzimidazole (24))*	46.0	31.0	(0.0)	(0.0)
Water (48)	62.0	58.0	34.00	86.00
Benzimidazole (48)	56.0	45.0	21.00	45.00
Kinetin (48)	41.0	39.0	..	26.00

* The enzyme preparations used for NAD kinase assay in Table XV, were used to study the rate of NAD or NADP breakdown by spectrophotometric method given in 'Methods'.

TABLE XXIV. The effect of benzimidazole, kinetin, AMP and ADP on the enzymatic breakdown of NADP.

Incubation time in minutes	% loss of NADP	
	0	15
Complete system*	22	28
+ Bz ($8.0 \times 10^{-4}M$)	22	28
+ Kn ($5.0 \times 10^{-4}M$)	22	28
+ AMP ($2.5 \times 10^{-3}M$)	19	28
+ ADP ($2.5 \times 10^{-3}M$)	35	30

* The reaction system was the same as that given in 'Methods'. 0.9 mg protein in the enzyme preparation was used. The incubation was at room temperature.

TABLE XXV. The effect of orthophosphate on the enzymatic breakdown of NAD and NADP-fluorimetric assay.

Substrate	Relative fluorescence			<u>μmoles broken</u>	
	Control	+Enzyme *	+Pi ** with enzyme	Enzyme	Pi
NAD (0.2 μmoles)	39	39	36	0.0	0.015
NADP (0.2 μmoles)	48	48	44	0.0	0.017

*Enzyme preparation showing the least amount of breakdown activity was chosen to study the effect of inorganic phosphate on the enzymatic breakdown of NAD and NADP. The enzyme contained 0.9 mg protein in the reaction system used for fluorimetric assay. Incubation time 30 minutes at room temperature.

**Pi used in 10^{-2} M final concentration.

TABLE XXVI. The enzymatic breakdown of NADP(H) by leaf and chloroplast preparations from freshly harvested leaves.

Source of Enzyme	Incubation time (minutes)	<u>μmoles recovered</u>				Breakdown	
		<u>NADP</u>		<u>NADPH</u>		% control	
		Control	Enzyme	Control	Enzyme	NADP	NADPH
Wheat leaf	0	0.22	0.03	0.25	0.12	86.00	52.0
Supernatant	15	0.22	0.0	..	..	100.0	..
Chloroplast suspension	15	0.22	0.15	..	..	31.00	..

Reaction system and conditions were the same as given in 'Methods'. Enzyme preparations from freshly harvested leaves were used (0.1 to 0.2 ml).

NAD breakdown and retarded NADP breakdown as compared to the controls. The increase in the rate of NADP breakdown in senescing leaves floated on water, may be responsible to a certain extent, for the low NADP content observed in these leaves by other workers (Mishra, 1963; Waygood, 1965).

The effect of benzimidazole, kinetin, AMP and ADP on the enzymatic breakdown of NADP. Further experiments were conducted to investigate the effect of benzimidazole or kinetin solution on the rate of the enzymatic breakdown of NADP by wheat leaf enzyme preparations. ADP or AMP were included in the studies since both these nucleotides were shown to inhibit NAD kinase activity (Wang and Kaplan, 1954; Wang, 1955). The data in Table XIV show that the NADP breakdown was neither inhibited nor activated by the presence of either benzimidazole, kinetin or AMP in the complete reaction system. ADP, however, showed a slight activation in the rate.

The effect of orthophosphate on the enzymatic breakdown of NAD and NADP-Fluorimetric method. Preliminary experiments on the 'apparent' NAD kinase activity by the spectrophotometric method indicated that there was a slight activation of NAD breakdown by orthophosphate in the reaction system. The results of these experiments are given in Table XXc. It was, therefore, decided to check these results by the fluorimetric method. The results of these investigations given in Table XXV show that the enzymatic breakdown of NAD(P) was slightly activated by orthophosphate ($10^{-2}M$). However, this

tions also showed the NADP breakdown activity, which was less than that of leaf preparations (supernatant fraction). The results explain the failure to obtain any NADP synthesis by a two-step reaction method (Kornberg, 1950) used for NAD kinase assay in the preliminary experiments.

GENERAL DISCUSSION

GENERAL DISCUSSION

It is revealed from the foregoing results that detached wheat leaves retained the capacity to incorporate leucine- C^{14} into cytoplasmic protein for a period of 5 days. Benzimidazole and kinetin treatment enhanced leucine- C^{14} incorporation into total leaf protein by 93% and 89% respectively (Table II, column 8), as compared to the increase of 59% in leaves floated on water for the entire period under observation. Thus, benzimidazole and kinetin treatment showed an over-all increase of 34% and 30% respectively, in leucine- C^{14} incorporation and retained high protein content as compared to leaves floated on water. These results provide a direct evidence that benzimidazole (or kinetin) treatment maintained high levels of protein by acceleration of protein synthesis and not by inhibiting its break down. The lower specific activity or the per cent recovery of radioactivity into cytoplasmic protein of leaves floated on water under similar conditions may be due to the dilution of labelled leucine in the soluble amino acid pools which have been shown to increase in these leaves (Samborski, et al., 1958; Mishra, 1963). It is also possible that most of the leucine- C^{14} may be associated with the soluble amino acids localized in the metabolically inactive pools such as vacuoles or other cytoplasmic inclusions (Oaks, 1965a) and is unable to participate in protein synthesis. Benzimidazole or kinetin treatment, on the other hand, may control the size of metabolically active pool or transport the accumulated amino acids from the inactive to active pool essential for protein synthesis.

The observed increase in leucine- C^{14} incorporation into the protein content of leaves floated on benzimidazole (or kinetin) solution may result from two possible mechanisms; (i) activation of protein synthesizing

capacity and (ii) inhibition of aminopeptidase enzymes which catalyze the breakdown of peptide linkages. For want of adequate evidence, it is not possible to postulate which of these two mechanisms accounted for the increase in specific activity of leucine- C^{14} which appears to be real on the basis of high protein levels maintained in these leaves (Table I). Although a fraction of radioactivity recovered in the protein fraction may be due to exchange reactions (Webster, 1955) or transaminase reactions between leucine and related amino acids such as valine, isoleucine, nor-leucine or phenylalanine (Fincham and Boulter, 1956), the increase in specific activity appears to be substantially due to new protein synthesis in this tissue in a similar way as that reported by Oaks (1965a) in Maize root tips. Any increase in specific activity of leucine- C^{14} due to bacterial contamination in benzimidazole treated leaves is completely excluded on the basis of the bacteriostatic nature of benzimidazole solution (Wobley, 1944).

Maize root tips were shown to incorporate exogenous leucine- C^{14} rapidly into the precursor pool of amino acids active in protein synthesis (Oaks, 1965a). After a lag of 6 minutes, approximately 73% of the radioactivity was recovered in the alcohol insoluble fraction with a corresponding decrease in the alcohol soluble fraction. The preferential uptake of exogenous leucine- C^{14} by the excised tissue was reported to be due to the inhibition of endogenous leucine synthesis (Oaks, 1965b), as the synthesis of leucine, threonine, lysine and arginine from acetate- $2-C^{14}$ fed to maize root tips, was shown to be inhibited by the supply of the respective amino acids to the reaction system.

In excised wheat leaves floated under continuous illumination, there are two possible sources of amino acid available for protein synthesis; amino acids derived from protein breakdown and those synthesized under the influence of light. Hellebust and Bidwell (1963b) reported that in attached wheat leaves the amino acids used for protein synthesis in chloroplasts during photosynthesis, were from small pools of amino acids that received carbon directly from the newly photoassimilated carbon. It is possible that benzimidazole or kinetin treatment, through its protective effect on chloroplasts (Waygood, 1965; Shaw and Manocha, 1965) and chlorophyll pigments (Tables III and IV) retains the capacity of leaves to synthesize amino acids and proteins (Andreyeva, *et al.*, 1954; Hellebust and Bidwell, 1963b) and to synthesize ATP during photosynthesis. The accumulation of amino acids in kinetin treated areas of tobacco leaves was shown to be the 'result' and not the 'cause' of protein synthesis (Mothes and Engelbrecht, 1961).

The failure to obtain any increase in leucine- C^{14} incorporation into chloroplastic protein of leaves floated on benzimidazole solution may possibly be due to, at least three reasons; (i) benzimidazole treatment did not accelerate protein synthesis in chloroplasts but only protected its breakdown to a certain extent, (ii) the exogenous leucine- C^{14} fed to excised leaves was not available for protein synthesis in these organelles due to permeability barriers or (iii) the newly synthesized protein containing radioactive leucine was lost from chloroplasts during the isolation and extraction procedure (Bidwell, 1966). Based on the first two assumptions it is indicated that of, at least, two different sites involved in the 'benzimidazole effect' in delaying senescence in wheat leaves (Waygood, 1965) this particular effect of benzimidazole (on protein synthesis) resides outside the chloroplasts.

Investigations on photophosphorylation of wheat chloroplasts showed that benzimidazole treatment increased the endogenous rate in leaves floated under continuous illumination for a period of 3 days. This rate was further increased by the addition of 'phosphodoxin' fraction, to the reaction system (Table VI). Furthermore, benzimidazole treatment also restored the capacity to photophosphorylate, at least in the presence of 'phosphodoxin', in leaves senescing on water for 3 days (Table VII). The increase in photophosphorylation by chloroplast fragments from leaves treated with benzimidazole, may be due to its preservative effect on the fine structure of chloroplasts (Waygood, 1965) and 'phosphodoxin' (Figure VIII) as well as NADP content (Mishra, 1963; Godavari, 1966), both of which act as natural cofactors in photophosphorylation (Black et al., 1963; Arnon, et al., 1958).

The tendency of chloroplasts, isolated from leaves floated on water, to show a decrease in photophosphorylation may be due to either the loss of chlorophyll pigments, fine structure, 'phosphodoxin' and NADP content or an increase in hydrolytic enzymes such as, phosphatases. Roberts (1956, 1963) reported the presence of a group of phosphatases in wheat leaves with a narrow substrate specificity. Investigations conducted in this laboratory (Pathak and Waygood) suggested a 92% increase (over zero time controls) in glucose-6-phosphatase (EC 3.1.3.9) activity associated with wheat chloroplasts from leaves senescing on water for 6 to 7 days. Benzimidazole and kinetin treatment, however, prevented this increase by 77%. It may be noted that in the present investigation, ATP^{32} formed in photophosphorylation was trapped as glucose-6-P³² which may account for the failure to recover any radioactivity in the organic phosphate fraction in chloroplast preparations from leaves senescing on water for 3 days or more.

The radioactivity recovered in the organic phosphate fraction may

result from the exchange between endogenous ATP and Pi^{32} supplied and need not necessarily be due to photophosphorylation. However, the experimental evidence reported for swiss chard chloroplasts, suggests the absence of such exchange reactions in chloroplasts (Jagendorf and Avron, 1958; Avron, 1964). Swiss chard chloroplasts also did not show any exchange reactions between ADP and ATP^{32} in the absence of ATPase activity (Avron, 1961). Wheat chloroplasts, however, possess active ATPase (Table IX), ADPase or non-specific phosphatase(s) (Table XI) and it is possible that some of the label of ATP^{32} may have been lost due to these activities even though dicumarol was shown to arrest ATPase activity to an appreciable extent (Table V).

Benzimidazole (and kinetin) treatment resulted in increasing the 'photohydrolytic' activity as well as the activity present in darkness in the complete reaction system used for photophosphorylation (Table XII). A decrease in this activity in chloroplasts from leaves senescing on water and its dependence on higher light intensities with 'age' suggest its role in synthetic rather than degradative processes. The light-induced ATP-ase activity has been shown to be associated with the conformational changes of chloroplasts undergoing electron transfer and photochemical reactions (Packer, et al., 1963; Dilley and Vernon, 1964), suggesting its dependence on the integrity of chloroplast structure. The 'photohydrolytic' activity as measured by the difference in the rate of orthophosphate liberation in light and darkness, in a complete reaction system exposed to low light intensity (i.e. under conditions of negligible light-induced ATPase activity), may serve as a tentative measure of photophosphorylation in systems such as wheat

chloroplasts which are not very suitable for photophosphorylation studies (Table XII, Fig.VII). These rates resembled those of photophosphorylation, in wheat chloroplast fragments from senescing leaves (Table VII).

Photophosphorylation and NAD kinase activity are closely interrelated. High levels of NAD have been shown to induce senescence in wheat (Godavari, 1966) and Elodea leaves (Yoshida, 1961) and benzimidazole treatment has been reported to reverse 'NAD effect' in wheat or Elodea leaves (Godavari, 1966). With the maintenance of ATP generating system benzimidazole treatment controls the levels of orthophosphate (Table XXII) and ADP (Mishra, 1963) in leaves exposed to light and provides energy for NADP synthesis with a corresponding decrease in NAD levels. NAD kinase activity was invariably more in benzimidazole treated leaves and in leaves grown under relatively high light intensity. Ogren and Krogmann (1965) studied the steady state levels of nicotinamide nucleotides in bean leaves exposed to varying light intensities and showed that with an increase in light intensity from zero to 2000 ft-c, there was a decrease in NAD (from 37 mpmoles to 18 mpmoles/ μ moles chlorophyll) and an increase in NADP (from 9 mpmoles to 17 mpmoles/ μ mole chlorophyll) as well as light-induced NADPH accumulation (from 8 mpmoles to 16 mpmoles/ μ mole chlorophyll). The conversion of NAD to NADP was shown to be further increased with the corresponding increase in the light intensity (Ogren and Krogmann, 1965).

Wheat NAD kinase was inhibited 50% by physiological concentrations (10^{-3} M) of orthophosphate (Table XVII) and this inhibition was overcome

by ATP (Table XVIII) the former being the substrate and the latter a product of photophosphorylation. It is speculated and supporting evidence is provided that benzimidazole increases the NADP content of wheat leaves by lowering the orthophosphate content (Table XXII) due to its stimulation of photophosphorylation (Tables VI and VII). The regulation of NAD kinase by orthophosphate/ATP ratio, is in conformity with the observations of other workers, on diurnal changes in NADP/NAD ratio in leaves exposed to alternating light and dark periods (Ogren and Krogmann, 1963; 1965; Oh-Hama and Miyachi, 1963; Mishra, 1963; Waygood, 1965).

NAD(P) breakdown by enzyme preparations from wheat leaves, was slightly activated by orthophosphate (10^{-2} M) (Tables XXV and XXc) but it was not of a magnitude high enough to account for the inhibition of NADP synthesis observed in the presence of orthophosphate. These results, however, suggest the possibility that the synthesis and breakdown of NADP may be catalyzed by one and the same enzyme. Forti, et al., (1962) reported that purified NADP(H) phosphatase from pea leaves, was inhibited competitively by orthophosphate (5×10^{-3} M), which may suggest, but does not prove, that both NADP(H) phosphatase and NAD kinase activity may be mediated by a single protein moiety.

The potential for NADP synthesis in leaves did not appear to change appreciably with 'age' or benzimidazole treatment (Table XV) and benzimidazole per se (10^{-3} M) inhibited NAD kinase (Table XVI), 'cyclic' photophosphorylation and 'Hill reaction' (Buchel, et al., 1966). It is still uncertain whether the intact molecule or some derivative of

benzimidazole is responsible for its effect. Preliminary investigations on the incorporation of benzimidazole-2-C¹⁴ into RNA fraction of wheat leaves fed under continuous illumination (for 5 hours at 850 ft-c), gave negative results although, some of the radioactivity was recovered in the protein fraction (Pathak and Waygood). Wang (1959) reported similar association of radioactivity with the protein fraction of leaves fed with benzimidazole-2-C¹⁴ and suggested it to be due to the formation of vitamin B₁₂-like factor. Wang, et al., (1961) further reported that the intact molecule of benzimidazole was required for the 'benzimidazole effect' observed in detached leaves and any substitution in either benzene or imidazole ring was found to be phytotoxic. Although no active metabolite of benzimidazole has been recovered from wheat leaves, it has been shown to compete with orotate for 5-phosphate-alpha-D-ribosylpyrophosphate to form benzimidazole nucleotide in the presence of enzymes isolated from wheat embryos (Kapoor and Waygood, 1965a). It is thus possible that some derivative(s) of benzimidazole is responsible for its effect on the processes studied in the present investigation.

SUMMARY

SUMMARY

This investigation was undertaken to study the effect of benzimidazole on the synthesis of cytoplasmic and chloroplast protein, on the rate of photophosphorylation and NAD kinase activity of excised wheat leaves senescing under continuous illumination. Because of its similarity in action with benzimidazole, the effect of kinetin on the above-mentioned processes was also studied in the present investigation.

The salient results in each section are as follows:

1. Benzimidazole (or kinetin) treatment accelerates protein synthesis in detached leaves rather than inhibiting its breakdown.
2. The incorporation of leucine- C^{14} into cytoplasmic protein is enhanced by 34.02% and 29.82% respectively, in leaves treated with benzimidazole or kinetin solution as compared to that in leaves senescing on water for 5 days.
3. Senescing leaves lose chloroplast protein more rapidly than cytoplasmic protein and protein turnover in chloroplasts is slowed down within 3 days of detachment.
4. Benzimidazole treatment protects the loss of chlorophyll pigments and chloroplast protein to an appreciable extent but fails to stimulate any protein synthesis in chloroplasts of leaves senescing for more than 1 day under continuous illumination.
5. Wheat chloroplasts are unsuitable for photophosphorylation studies but give better results when treated with ascorbate solution and used with dicumarol in the reaction system.
6. Benzimidazole treatment enhances photophosphorylation in wheat chloroplast fragments. The enhancement is more pronounced when 'phosphodoxin' is present in the reaction system.

7. Benzimidazole treatment restores the rate of photophosphorylation of wheat chloroplast fragments, at least in the presence of 'phosphodoxin', even after the system has deteriorated during senescence on water.
8. Benzimidazole (or kinetin) treatment maintains the endogenous 'phosphodoxin' content at higher levels in leaves senescing for 6 days under continuous illumination.
9. The light-induced ATPase activity associated with wheat chloroplast fragments is stimulated in the presence of phenazine methosulfate or ADP in the reaction system and is decreased with 'aging'.
10. An increase in 'photohydrolytic' activity in response to increasing light intensity and its decrease with 'aging' of leaves senescing on water suggest a correlation between photochemical synthesis and hydrolysis of ATP in chloroplasts. Benzimidazole (or kinetin) treatment increases the 'photohydrolytic' as well as the activity present in darkness in a complete photophosphorylation system of chloroplast fragments from senescing leaves.
11. The difference in the levels of orthophosphate liberation in light and dark period in the complete reaction system exposed to low light intensity, may serve as a qualitative measure of the rate of photophosphorylation in systems such as wheat chloroplasts in which 'phosphatases' activities predominate over photophosphorylation reactions under high light intensities.
12. NAD kinase activity is demonstrated in extracts of wheat leaves. The enzyme activity is inhibited by physiological concentrations

(10^{-3}M) of orthophosphate or by benzimidazole ($6 \times 10^{-3}\text{M}$) solution in the reaction system.

13. The enzyme inhibition by orthophosphate is overcome by ATP suggesting a regulatory mechanism.
14. Benzimidazole (or kinetin) treatment does not alter the rate of NADP synthesis appreciably although, it prevents to a certain extent, the breakdown of NAD or NADP in senescing leaves.
15. The enzymatic breakdown of NAD or NADP by wheat leaf enzyme(s) is slightly activated by orthophosphate (10^{-2}M).
16. The breakdown of NADP is more rapid than NADPH breakdown by wheat leaf enzyme(s).
17. Benzimidazole treatment lowers the orthophosphate levels in excised leaves senescing under continuous illumination but it has no effect on orthophosphate levels of leaves kept in darkness.
18. The 'benzimidazole effect' on protein or NADP synthesis of excised leaves appears to be through its protective effect on chloroplasts and acceleration of photophosphorylation in leaves exposed to light with a resultant decrease in P_i/ATP ratio.

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