A STUDY OF THE EFFECTS OF BENZIMIDAZOLE AND KINETIN ON THE METABOLISM OF DETACHED PRIMARY LEAVES OF WHEAT VARIETY KHAPLI

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ABBREVIATIONS

The following abbreviations are used in the text: AA, Ascorbic Acid; AAO, Ascorbic Acid Oxidase; ADP, Adenosine Diphosphate; ATP, Adenosine Triphosphate; BAD, Benzimidazole Adenine Dinucleotide; BMN, Benzimidazole Mononucleotide; BZ, Benzimidazole; C, Centigrade (with numeral); ca., about; D, Density; DBZ, Dark Benzimidazole (floated); DHA, Dehydroascorbic Acid; DNA, Deoxyribonucleic Acid; DW, Dark Water (floated); EDTA, Ethylene-Diamine-Tetraacetate; EMP, Embden-Meyerhof-Parnas; F, Farenheit (with numeral); ft-c, foot candle; FAD, Flavin Adenine Dinucleotide; FMN, Flavin Mononucleotide; g, gram(s); GAO, Glycolic Acid Oxidase; G-6-P, Glucose-6-Phosphate; G-6-PDH, Glucose-6-Phosphate Dehydrogenase; 6-P-G, 6-Phospho-Gluconate; 6-P-GDH, 6-Phospho-Gluconic Dehydrogenase; IAA, Indole Acetic Acid; KN, Kinetin; LW, Light Water (floated); LBZ, Light Benzimidazole (floated); M, molar; m, milli- (Prefix, 10^{-3}); mg, milligram(s); ml, milliliter(s); μ, micro- (Prefix, 10^{-6}); μg, Microgram; NAD and NADH, oxidized and reduced forms of Nicotinamide Adenine Dinucleotide respectively (DPN and DPNH); NADP and NADPH, oxidized and reduced forms of Nicotinamide Adenine Dinucleotide Phosphate respectively (TPN and TPNH); NMN, Nicotinamide Mononucleotide; O.D., Optical Density; PCA, Perchloric Acid; Pi, Po and Pt, Inorganic Phosphorus, Organic Phosphorus and Total Phosphorus respectively; PP, Pentose Phosphate cycle; P-P, Pyrophosphate; ppt, precipitate; ppm, parts per million; RNA, Ribonucleic Acid; %, percentage; TCA, Trichloroacetic Acid; TRIS, Tris (hydroxy methyl) amino methane; wt., weight.

A study of the effects of benzimidazole and kinetin on the metabolism of detached primary leaves of wheat variety Khapli

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ABSTRACT

Primary leaves of wheat variety Khapli were detached and floated on water, benzimidazole (50 ppm) and kinetin (5 ppm). Changes in the concentrations of certain enzymes, coenzymes, nucleic acids and biochemical intermediates were investigated in the leaves and chloroplasts. The chloroplasts were isolated in a nonaqueous medium.

In water floated leaves there was a gradual decline in the levels of insoluble nitrogen, ribonucleic acid, deoxyribonucleic acid, ribonucleic acid to deoxy-ribonucleic acid ratio, ascorbate, α-ketoglutarate, glycolate, pyridine nucleotides, reduced triphosphopyridine nucleotide to triphosphopyridine nucleotide ratio and the activity of alkaline inorganic pyrophosphatase and a rise in the levels of soluble nitrogen, carbohydrates, dehydro-ascorbate, dehydro-ascorbate to ascorbate ratio, glyoxylate, and pyruvate. On the other hand in leaves floated on benzimidazole the gradual decline or rise in the levels of these substances was arrested.

In the chloroplasts from benzimidazole and kinetin floated leaves a higher level of chlorophyll, protein, nucleic acid, pyridine nucleotides (particularly triphosphopyridine nucleotide) and adenosine triphosphate was observed compared to those from water floated leaves.

An improved procedure for the non-aqueous isolation of chloroplasts from wheat leaves and estimation of very low amounts of (10^{-15} moles) pyridine nucleotides in plant extracts is described.

The possible role of benzimidazole and kinetin in retarding senescence of detached wheat leaves is discussed.

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I. INTRODUCTION

Senescence is an expression of events occurring at the cellular and sub-cellular levels. It is an adverse change in the living organism, which is loosely correlated with the passage of time and which leads ultimately to the death of the organism. It is not a single overall process and various factors in varying proportions contribute to it. The endogenous factors responsible for senescence, which control and regulate the processes of metabolism, remain to a great extent obscure and the problem of why tissues eventually die is yet unsolved. MacDonald *et al* (1958) and Muir *et al* (1959) while studying the responses of young and old leaves to metabolic inhibitors have concluded that the balance between the various processes changes with aging and no metabolic system is lost or new system introduced during the life of the organism. According to them any cell component (particulate or soluble enzyme or coenzyme) required in the chain of reactions producing energy is a possible candidate for the role of limiting factor in total energy production.

Well authenticated information on the physiology of aging in plants is scanty and the biochemical changes which occur are poorly understood. The most obvious sign of senescence in plants is loss of leaves. Every autumn witnesses the changing color and death of all leaves on common deciduous trees. Research workers have turned to the problem of leaf-fall in their search for the causes and the nature of cellular death--and a great deal has been learnt by studying leaves isolated from the plant.

To study senescence at the metabolic level, excised plant parts, more particularly leaves, provide an excellent model system. With such a system at least the biochemical and physiological differences that exist between different parts of the same plant can be eliminated. This is a relatively good system, but by no means ideal because it consists of an isolated organ of a whole plant. It does, however, offer many biological and technical advantages. The advantages of detached leaf culture have been reviewed by Yarwood (1946).

In mature leaves, senescence can be accelerated and initiated artificially by excision from the plant and when the leaves are floated on water the symptoms of senescence begin gradually with age. This is to be contrasted with the aging and senescence of attached leaves. Post harvest fruits and vegetables are specific examples of maturing and senescent tissues.

Certain chemicals, i.e. Benzimidazole and Kinetin retard the senescence of detached leaves. Although considerable study has been made regarding the effects of benzimidazole and kinetin, yet the mechanism of action is still unknown. Leaves of Khapli wheat, normally resistant to race 15B-1 of *Puccinia graminis tritici*, become susceptible when detached and floated on water but this breakdown of resistance can be reversed by floating the detached leaves on a solution of benzimidazole or kinetin. Since resistance or susceptibility can be altered with ease, this system is satisfactory for studying (1) the nature of rust resistance as well as (2) the physiology of detached leaves at the biochemical level.

A basic study was undertaken in an attempt to understand more fully the biochemical changes involved in detached leaf senescence and also the effect of benzimidazole or kinetin on these alterations in the hope that the mechanism of action and the biochemical site of activity of these chemicals could be elucidated.

This study is concerned with the effect of benzimidazole on the biochemical composition of detached wheat leaves during senescence, including enzyme systems, nucleic acids and biochemical intermediates. The first part of the thesis deals with the effect of benzimidazole on the biochemical composition of whole detached leaves and the second part deals with the effect of benzimidazole and kinetin on the biochemical composition of chloroplasts. A major emphasis of the work is on the pyridine nucleotide content of whole leaves as well as of chloroplasts and the adaptation of the enzymatic cycling method to the assay of very low amounts of pyridine nucleotides in plant tissues. The results of experiments carried out in establishing the effect of BZ on the drifts in carbohydrate, phosphorus, keto acid, glycolate and ascorbate metabolism, under the experimental conditions employed, are presented in the appendix.

II. LITERATURE REVIEW

This review is an attempt to cover the present knowledge of detached leaf senescence and to summarize reports concerning the effect of benzimidazole or kinetin in delaying senescence of detached leaves.

The metabolism of detached leaves, with particular reference to monocotyledonous plants, is presented in the following pages. At the outset it must be understood that monocotyledons and dicotyledons differ in metabolism and growth processes in a striking and yet incompletely explored manner. The review has been divided into three parts, part (1) deals with the metabolism of detached leaves or plant parts, (2) deals with the effect of benzimidazole and (3) deals with the effect of kinetin in biological systems with special references to senescent metabolism of detached leaves.

(1) Metabolism during senescence in plants or plant parts

Comprehensive reviews on the physiology and biochemistry of detached leaves have been made by Chibnall

(1939), Yarwood (1946) and Varner (1961) and more recently on leaf senescence by Humphries and Wheeler (1963).

It is well established that the detachment of leaves or mutilated parts of leaves from plants quickly predisposes the tissue to a series of degradative changes. Plant physiologists in the 1930's showed that senescence in leaves was accompanied by a fall in the amount of protein in the leaf. This loss of protein is tremendously important for proteins are vital components of the living cell. The chloroplasts responsible for trapping the energy of the sun in the process of photosynthesis contain the green chlorophyll pigments linked to protein in a highly complex way. Any serious disruption of protein production in the cell must result in a loss of function and ultimately death.

Initiation of proteolysis immediately after detachment has been reported (Viets _et al_, 1947; Yemm, 1949; 1950; Richmond and Lang, 1957 and Mothes, 1960). The decrease in protein content is not necessarily due to a lack of carbohydrate, nitrogen or other nutrients (Vickery_et al_, 1946; Mothes and Engelbrecht, 1956) or to inability of the cells themselves to synthesize amino acids (Rogers, 1955), but is due, rather to a failing ability to incorporate amino acids into protein (Racusen and Aranoff, 1954). The decrease in protein content is followed by an accumulation of free amino acids and amides most of which are liberated from proteolysis. Thus normal functions are destroyed because the various metabolic pools are interdependent.

During investigations of the senescence of detached roses Weinstein (1957) found a decrease in dry weight, rapid depletion in free glucose and accumulation of malate, free amino acids and amides. Weinstein and Laurencot (1958) also found a decrease in glucose and fructose but an increase in ribose and xylose during aging. Their data also suggest participation of the TCA cycle in roses and the dark fixation of CO_2 appears to be impaired in aging detached rose petals and stimulated in aging receptacles. Conversion of malate to citrate is also inhibited in aging tissue. They suggested a block in malic dehydrogenase or reduced amounts of pyridine nucleotides or acetyl coenzyme A in aging detached rose tissue.

There is no comprehensive account of the biochemical changes occurring in detached leaves, yet some sporadic reports have appeared.

Aberg (1945, 1949) found a rapid decrease in ascorbic acid content in the darkened excised tomato leaves, maximum decrease being observed in the nearly full grown leaves. That the rate at which ascorbic acid decreases is influenced by temperature suggests some chemical reactions to be involved.

Studies of the keto acids of starving excised wheat leaves by Krupka and Towers (1958) reveal a dramatic increase in glyoxylate levels but no significant change in pyruvate and α-ketoglutarate concentrations.

Using bean pericarp segments, leaf slices of Rhoeo and Mesembryanthemum as test materials Sacher (1959) and Glasziou et al (1960) observed permeability changes, water-logging of intercellular spaces, progressive increase in free space, conspicuous oxidation at the surface of segments, loss of anthocyanin and browning in Rhoeo leaf slices, considerable synthesis of sucrose and drop in glucose, associated with the senescence of water-floated sections.

Weinstein and Porter (1962) demonstrated that leaf sections of tobacco undergo degradative processes during senescence typical of starving whole detached leaves.

A report by Varner _et al_ (1963) on the senescence of cotyledons in germinating peas suggests that excised cotyledons undergoing senescence in the absence of signals from a growing axis (a) lose respiratory control and (b) fail to develop the normal complement of enzymatic activities. Willis _et al_ (1963) suggest that pronounced changes in transpiration rates of leaves may occur when they are severed from the plant. Changes in transpiration rates of excised leaves are mediated by stomatal movement. Release of tension in the conducting system clearly occurs when leaves are severed. The rapid changes in the condition of water supply to the leaf probably leads to a quick and pronounced displacement of the dynamic equilibria of water relations of the cells of the leaf, notably the guard cells and epidermal cells.

A summary of the important biochemical changes known to accompany senescence is given in a tabular form (Table I).

TABLE I

Changes accompanying senescence of detached leaves

Metabolic changes	Plants	Observations	References
Respiration	Barley, Wheat, Cherry laurel	The greater the initial sugar content of the leaves, the greater the initial rate of respiration. Respiratory decline is not constant. Temporary but consistent rise in rate of respiration in the course of general decline. Respiratory rate is limited by available substrates. There is a shift in respiratory substrates from carbohydrates to amino acids.	Yarwood (1946 Varner (1961)
Photosynthesis	Sunflower	Increase in dry weight of leaves in light. Increased photosynthesis due to the greater opening of the stomata of detached leaves. Rate of photosynthesis decreases as the carbohydrates of the leaf are increased. Photosynthesis entirely ceases in leaves with high accumulation of carbohydrates.	Yarwood (1946)
Protein synthesis and degradation	Wheat, Barley, Tobacco Swiss chard, Tomato	Detached leaves supplied with sugar could synthesize proteins in the dark. In leaves starved in the dark protein synthesis ceases and decomposition begins. Protein is gradually decomposed to amides and ammonia. Total nitrogen remains constant for long periods. Protein	Yarwood (1946) Varner (1961)

TABLE I cont'd

Metabolic changes	Plants	Observations	References
		degradation is generally associated with carbohydrate exhaustion.	
Chlorophyll	Wheat, Xanthium	There is a breakdown in chlorophyll content.	Varner (1961) Wang & Waygood (1959) Osborne (1962)
Translocation	Sunflower, Beet	It is reduced or almost eliminated.	Yarwood (1946)
Absorption	Wheat, Brussels Sprout, Tobacco	Detached leaves may absorb water through injured or uninjured surfaces. Greater the water deficiency in leaf tissues, the greater the rate of water uptake.	Yarwood (1946)
Transpiration	Grape, Sycamore	Detachment of turgid leaves causes a sudden but temporary rise in transpiration lasting only a few minutes followed by a slow decline.	Yarwood (1946)
Amino acids, amides and ammonia	Barley, Tobacco	Amides accumulate during first 48 to 96 hrs following excision. Thereafter, the levels of amino acids and amides decline steadily and free ammonium ions accumulate in large quantities.	Yarwood (1946) Varner (1961)

TABLE I cont'd

Metabolic changes	Plants	Observations	References
Ascorbic acid	Tomato	Rapid decrease in ascorbic acid content. Maximum decrease observed in the nearly full grown leaves. Rate of decrease is also influenced by temperature.	Aberg (1945, 1949)
Keto acids	Wheat	Dramatic increase in glyoxylate level but no significant change in pyruvate and α-ketoglutarate levels.	Krupka and Towers (1958)
Nucleic acids	Xanthium	Decrease in the levels of RNA and DNA.	Osborne (1962)

The impairment of protein synthesis in old leaves is the cause, and not the effect of aging (Parthier, 1961). Senescence is closely associated with the declining ability of the cells to make new protein and that protein synthesis is dependent upon special substances which are supplied by the roots appears valid. It is logical to assume that the roots metabolize and supply the blade with certain factors necessary for the continued synthesis of protein. In the absence of root formation in isolated leaves of monocotyledons, any treatment that would retard senescence of leaf blades would appear to substitute either directly or indirectly for the unknown so-called "root-factor".

As benzimidazole or kinetin treatment has delayed the senescence of detached wheat leaves, the following pages include a survey of the literature on the effect of these chemicals on biological systems in general and detached leaves in particular.

(2) Effect of benzimidazole

The effect of benzimidazole on biological systems (Hillman, 1954; Klingensmith, 1959) and on the metabolism of detached leaves (Kapoor, 1963; Humphries and Wheeler, 1963) has been reviewed.

(i) Microorganisms

The effects of benzimidazole on microbial systems include (a) inhibition of the multiplication of influenza virus (Tamm, 1957) (b) inhibition in growth of yeasts and certain bacteria (Wolley, 1944; Klotz and Mellody, 1948) (c) prevention of glycine incorporation into heme (Abbot and Dodson, 1954) (d) blocking of malate synthesis by glucose grown cells of *Saccharomyces cerevisiae* (Creaser, 1955) (e) inhibition of the synthesis in adaptive respiratory enzymes in resting yeast cells (Slominski *et al*, 1954) (f) inhibition in the adaptive formation of enzymes oxidizing benzoate, malonate and L-tyrosine in *Pseudomonas fluorescence* (Kushner and Pascoe, 1955).

(ii) Plants

The effects of benzimidazole on whole plant growth include (a) inhibition of algal growth cultures (Galloway and Krauss, 1959) (b) inhibition of the auxin-induced elongation of etiolated pea epicotyl sections (Galston *et al*, 1953) (c) inhibition of root elongation and increase in frond area in *Lemna* (Hillman, 1955) (d) decreased enzyme activities of polyphenol oxidase, ascorbic acid oxidase, cytochrome oxidase and glycolic acid oxidase in *Lemna minor* (Hillman, 1955) (e) influence on ion uptake of plant roots (Klingensmith and Norman, 1960; Klingensmith, 1961) although the enhancement of ion uptake has now been found to be due to the buffering action of benzimidazole (Parr and Norman, 1962) (f) decline in mitotic index of onion roots (Duncan and Woods, 1953) (g) inhibition in growth rate of flax seedlings (Moore, 1953). Derivatives of benzimidazole have been reported to inhibit the growth of Cranberry bean plants and root formation in cucumber seeds (Rebstock *et al*, 1955, 1957). The present writer also reported the beneficial effects at low concentrations and inhibitory effects at high concentrations on the growth, flowering and yield of rice plants by a derivative of benzimidazole (Misra and Mishra, 1958, 1961).

(iii) Detached leaves

In detached wheat leaves benzimidazole (a) maintains protein levels (Person et al, 1957) (b) stimulates chlorophyll biosynthesis and prevents it from destruction (Wang and Waygood, 1959; Wang et al, 1961) (c) inhibits accumulation of amino acids and water-soluble carbohydrates (Samborski et al, 1958) and (d) maintains the property of resistance to rust in leaves of Khapli variety (Wang et al, 1961). An exhaustive review has been given by Kapoor (1963).

Studies by Wang et al (1961) on the effect of benzimidazole analogues on stem rust and chlorophyll metabolism of detached Khapli wheat leaves suggest that benzimidazole will lose its activity if any member of the elements in its imidazole ring and probably also in its benzene ring is replaced by a different element such as carbon, oxygen, nitrogen or sulphur. Furthermore, compounds in which the methyl, nitro or other group is introduced into the molecule of benzimidazole are either phytotoxic or antagonistically active to their parent compound. They concluded that analogues which were antagonistically active to benzimidazole displayed a similar effect on kinetin. Benzimidazole and kinetin played a similar role in maintaining rust resistance and normal physiology of detached wheat leaves.

Although it has not yet been shown that benzimidazole is a natural factor in plants, yet benzimidazole or 5,6-dimethylbenzimidazole and related compounds occur in animal tissues and bacteria as a part of the molecule of vitamin B_{12}. Kliewer and Evans (1962a, 1962b, 1962c) have isolated, identified and quantitatively assayed the B_{12} coenzyme content of the nodules from Legume, Alder and _Rhizobium meliloti_. They have also furnished evidence that the compound isolated from _R. meliloti_ is a 5,6-dimethylbenzimidozolyl derivative. Fries (1962) established the production of vitamin B_{12} in multicellular green plants by cultivating, aseptically, whole Pea, Wheat and Lupine plants and testing the plant extracts for B_{12} compounds by using various test organisms such as _Escherichia coli_, _Euglena gracilis_ and the red alga _Goniotrichum_.

(3) Effect of kinetin

The effect of kinetin on plant growth and detached leaf senescence has been reviewed by Mothes, 1960; Parthier, 1960 and Miller, 1961.

Kinetin plays an important role in cell division, cell enlargement, shoot initiation, bud elongation, root and leaf growth, breaking of dormancy and increasing dry weight of plants or plant parts.

The main biochemical events after kinetin application include (a) increase in respiration (b) increase in protein and nucleic acid levels (c) acceleration of both RNA-ase and DNA-ase activity and (d) increase in anthocyanin formation.

Publications since 1961 on the kinetin effect in whole plants or plant parts are summarized below: (a) increased tumor formation and growth of tobacco tissue in tissue culture experiments (Schaeffer, 1962) (b) increased fresh as well as dry weight of cell growth in soybean tissue (Miller, 1962) (c) increased elongation and fresh weight of pea internode sections (Katsumi, 1962) (d) made the xylem more normal and altered the epicotyl structure from herbaceous to more-or-less woody, in isolated segments from etiolated pea epicotyl (Sorokin _et al_, 1962) (e) inhibited xanthine oxidase activity (Henderson _et al_, 1962) (f) inhibited powdery mildew development on cucumber leaf disks (Dekker, 1963).

The effects of kinetin on the metabolism of detached leaves include (a) an increased survival of and a delayed protein hydrolysis in detached _Xanthium_ leaves (Richmond and Lang, 1957), (b) stoppage in chlorophyll breakdown (Mothes, 1961), (c) an inhibition not only of the degradation of protein in isolated leaf tissue but also a stimulation in the incorporation of amino acids (Parthier, 1961), (d) an important role in those systems which decisively influence the correlations between various organs (Engelbrecht and Mothes, 1960), (e) an inhibition of starch degradation in excised wheat coleoptiles (Boothley and Wright, 1962), (f) a suppression of protein loss in leaf disks from _Nicotiana tabaccum_ and an increased protein synthesis through accelerated RNA synthesis (Sugiura _et al_, 1962), (g) a temporary arrest in the senescent changes in detached _Xanthium_ leaves by maintaining a relatively high ratio of RNA to DNA. Incorporation of C^{14}-leucine into protein and C^{14}-orotic acid into RNA is increased by kinetin, indicating a stimulation of both RNA and protein synthesis (Osborne, 1962). It is also suggested that the effect of kinetin in retarding senescence of Xanthium leaf cells is mediated through its action in sustaining nucleic acid and protein synthesis (Osborne, 1962).

Gunning and Barkley (1963) report that kinetin postpones senescence in detached oat leaves (7 to 9-days-old). The younger the treated tissue, the more extreme are the effects. In comparatively mature apical regions of leaves, breakdown of chloroplasts is delayed and "insoluble" nitrogen levels are maintained for some time by kinetin application. Respiratory losses of carbon are also reduced. They suggest that if protein (and other) breakdown processes require energy in leaf cells and if kinetin prevents such breakdown, then the energy that would otherwise have been consumed in this way might become available to drive syntheses.

The above review indicates the fragmentary and disjointed knowledge of the chemical and physical factors concerned in leaf senescence and the biochemical changes associated with it and of the role of benzimidazole and kinetin in modifying these factors.

III. MATERIAL AND METHODS

1. Plant Material

The primary leaves of wheat (*Triticum dicoccum* Schübler Var. Khapli) were used throughout the investigations. Seeds of homozygous pure strains of Khapli wheat (obtained from the Canada Department of Agriculture Research Station, Winnipeg) were grown in flats under greenhouse conditions and the primary leaves detached when 7 to 14-days-old. The leaves were cut at 7-10 cm from the tips. The age of the leaves used in each experiment is indicated under the experiment concerned.

Williams (1960) found that primary leaves of wheat seedlings have completed their growth after about eight days and the recent studies of Perkins and Roberts (1960) indicate that 13-day old primary wheat leaves do not incorporate C^{14} into the dihydroporphyrins from which it was assumed that the primary leaves of wheat mature within 7 days after sowing.

2. Detached leaf culture

The detached leaves were weighed into different lots and floated on either water, 50 ppm benzimidazole (Eastman Kodak, Rochester, N.J.) or 5 ppm kinetin (Sigma Chemical Co.) solution in petri dishes (15 cm outside diameter) or glass pyrex trays (12" wide x 18" long x 2.5" deep) depending upon the volume of the sample. For 1 gram lots, usually, 40 ml of the solution were used in petri dishes and for 3 to 10 gram lots, usually, 500 ml of solution were used in trays. The trays were covered with Saran wrap. Care was taken to avoid overlapping of the leaves. The trays were placed in growth chambers either under continuous illumination or complete darkness. The light source was a bank of 16 fluorescent lamps (F72T12, General Electric Co.) eight of which were warm white and 8 day light giving a light intensity of ca. 750 ft-c at the surface of floated leaves (measured by a General Electric light meter). The relative humidity of the growth chamber was ca. 60% and temperature, 70° F.

In some of the experiments instead of continuous illumination the floated leaves were subjected to 16 hr light period followed by an 8 hr dark period in a 24 hr cycle. In these experiments the leaves were harvested at the middle of the light period.

3. Analytical methods

(a) Nitrogen determinations: Nitrogen was determined by a Micro-Kjeldahl technique (MacKenzie and Wallace, 1954). Total nitrogen was determined after complete incineration of the leaf segments (1/2 inch sections) with concentrated sulphuric acid, containing a pinch of $CuSO_4 \cdot 5H_2O:K_2SO_4$ (3:1) and a selenised Hengar granule as catalyst, in a Kjeldahl flask. TCA-insoluble and soluble portions of the leaf were also subjected to the same procedure.

(b) Protein determinations: Proteins were precipitated from solution with 5% TCA (final concentration). Nitrogen was determined in precipitates by a Micro-Kjeldahl technique (MacKenzie and Wallace, 1954) and protein estimated by assuming 16% nitrogen.

In some cases the precipitated protein was solubilized in 1 N NaOH, boiled if necessary and aliquots of the solubilized protein taken for the estimation of protein either using the biuret reagent of Gornall et al (1949) or the Folin-phenol reagent of Lowry et al (1951). In all cases crystalline bovine albumin (Sigma Chemical Co.) was used as the standard.

It was noted that protein values estimated by Micro-Kjeldahl technique were always lower than those estimated either by biuret or phenol reagent.

(c) Carbohydrate determinations: Leaves were cut into 1/2 inch sections, dropped into boiling 80% ethanol and refluxed for 30 minutes. The extraction was repeated twice with 40% ethanol and the extracts combined. The ethanolic extract was concentrated in a flash evaporator and fractionated using the ion-exchange procedure of Wang (1960). Total soluble carbohydrates were determined by use of an anthrone method (Yemm and Willis, 1954).

(d) Phosphorus determination: Phosphate was determined by the procedure of Lowry and Lopez (1946) as adapted by Waygood (1948) for the estimation of inorganic and organic phosphate in plant extracts. Total phosphate was determined as orthophosphate after complete incineration of the sample (equivalent to 0.1-0.2 g fresh wt.) with 2.2 ml of PCA (60%)

in a Micro-Kjeldahl flask.

The method of Allen (1940) and Holden (1952) as modified by Mukherji and Shaw (1962) was used to obtain the various phosphate fractions (inorganic P, organic P, RNA-P and DNA-P).

(e) Keto acid determinations: Keto acids were extracted and quantitatively estimated with a slight modification of the methods already described (Towers and Mortimer, 1956; Krupka and Towers, 1958; Neish, 1957; and Smith, 1960). Leaves (2.5 g fresh wt.) were homogenized in a Waring Blendor containing 100 ml of 95% ethanol and 10 ml of a saturated solution of 2,4-dinitrophenylhydrazine (Eastman Kodak) in 2 N HCl. The homogenate was filtered with suction and the filtrate reduced to volume (approx. 15 ml) in a flash evaporator. The concentrated filtrate was made to about 50 ml by adding water and extracted three times with ethyl acetate. The combined ethyl acetate extracts were washed twice with water and the washed ethyl acetate extract then reextracted with a freshly prepared 1% sodium carbonate solution and the extraction repeated three times until the carbonate extract became colorless. The carbonate solution (pH ca. 10) was cooled in an ice bath and carefully acidified to pH 2.0 by dropwise addition of cold 2 N HCl and the acidified hydrazones washed two times with ethyl acetate. The ethyl acetate extracts were combined and washed three times with distilled water (to remove NaCl) and evaporated to dryness in an air jet at room temperature.

The dried residue was dissolved in 1 ml of ethyl acetate and aliquots (50-100 µl) taken for chromatography. An ascending method using Whatman #1 paper (in an atmosphere of ammonia) and Tert-amyl alcohol:ethanol:water (9:1:4 v/v) as the developing solvent was adopted. The developing solution was allowed to ascend the paper for 16 hours. The chromatograms were dried and sprayed with ethanolic NaOH (NaOH 2% and 90% ethanol). The color with ethanolic NaOH was olive green for α-ketoglutaric acid, reddish brown for glyoxylic acid and brown for pyruvic acids, the latter two acids having two isomeric spots. The spots in the other chromatograms were identified by comparing with the sprayed chromatogram and also by ultra-violet absorbing dark spots. The spots were cut into strips and placed in a test tube with 5 ml of freshly prepared 1% Na_2CO_3 and after 90 minutes the optical density noted in a Spectronic 20 Colorimeter at appropriate wavelengths (α-ketoglutaric - 385 mμ, glyoxylic - 365 mμ and pyruvic - 375 mμ). Elution from the spots adjacent to DNP spots served as the blank in each case.

Standard curves were obtained (Smith, 1960) for each of the three keto acids (pyruvic, α-ketoglutaric and glyoxylic acids were obtained from Nutritional Biochemicals Corp., Ohio) subjected to the same procedure as outlined for the plant extracts. The standard curve was linear between the range 0-20 μg for each of the α-ketoglutaric acid glyoxylic acids and 0-25 μg for the pyruvic acid. The reproducibility of the method was found to be ± 10%.

(f) Glycolic acid: The extraction of glycolic acid was made after Zelitch (1958) and the quantitative estimation as reported by Zelitch (1958) and Sanwal and Waygood (1963). Briefly, the leaves were extracted with hot 0.01 M sodium bisulfite solution, reextracted, centrifuged and the supernatant fluids (approximately 30 ml from 2.5 g of leaves) were used for the separation of glycolic acid on a Dowex 1-x 10-acetate anion exchange resin (200-400 mesh) (obtained from the California Corporation of Biochemical Research, Los Angeles, California). The column was 8 cm in length x 0.7 cm in diameter. Before loading, the column was washed with 10 ml of 6 M acetic acid in order to remove any impurity that may produce color with the glycolic acid reagent. It was further washed with approximately 25 ml of water until the effluent was neutral. The entire extract was slowly added to the column and washed with 30 ml of water which removed neutral and basic compounds. Glycolic acid was eluted with 4 M acetic acid at a flow rate of 1 ml per minute. Recovery of added glycolic acid was about 88%.

Aliquots of the eluate were used for glycolic acid estimation by the colorimetric procedure of Calkins (1943) using 0.01% 2,7-naphthalenediol in concentrated sulfuric acid as the color reagent.

(g) Determination of glycolic acid oxidase activity: The preparation and assay of the enzyme was made as described by Sanwal and Waygood (1963). Five grams of leaves were chilled in the cold room and all subsequent operations were done in the cold (4° C). The leaves were macerated in a chilled mortar with acid-washed sand and 20 ml of 0.1 M phosphate buffer pH 8.3. The homogenate was passed through 4 layers of cheese cloth and centrifuged at 18,000 x g at

0° C for 20 minutes. The supernatant thus obtained was used as the enzyme source.

The standard reaction mixture consisted of 1 ml enzyme (3-6 mg protein); phosphate buffer, pH 8.3, 100 µmoles; FMN 0.3 µmoles; glycolic acid (side arm) 10 µmoles, pH adjusted to 8.0 by KOH; and water to a final volume of 3.0 ml in the main compartment of the Warburg vessel. 0.2 ml of 2 N NaOH with a wick of filter paper was kept in the center well. Final pH of reaction mixture was 8.0. The temperature was 30° C and the gas phase, air.

(h) Flavin nucleotides: The method of Huennekens and Felton (1955) as adapted by Sanwal and Waygood (1963) was used for separating the flavin nucleotides from other cell constituents. Five grams of leaves were ground with 20 ml of 11% TCA in a glass homogenizer for 3 minutes and the homogenate centrifuged at 20,000 x g for 15 minutes and the supernatant filtered to remove any floating debris. All further operations were done in the dark at 3-5° C. The deproteinized acid extract was passed through a column (12 cm long x 0.7 cm diameter) of Florisil 30-60 mesh (a highly selective adsorbent, extremely white, hard powdered magnesiumsilica gel, frequently referred to as a magnesium-silicate, manufactured by Floridin Company, Tallahasse, Florida) previously washed with water. A small plug of glass wool was placed on the top of the column. The flavin nucleotides were absorbed on the top of the column. After the acid extract passed through the column, it was washed with 180 ml of water followed by 90 ml of 0.5% pyridine. In order to avoid inadvertent channeling of the column due to frequent refilling and to increase the flow rate a separatory funnel was attached to the top of the column.

Finally 30 ml of 5% pyridine was used to elute the flavin nucleotides. The flavin eluate was adjusted to pH 8.0 (glass electrode), if necessary, and the pyridine largely removed by four extractions with three volumes of chloroform. The chloroform was removed under vacuum in a flash evaporator.

Estimation of FMN and FAD in the final aqueous extract was done by the fluorimetric method of Burch (1955) using riboflavin, which had been identically treated, as standard. The fluorescence of the extract was measured in a Coleman electronic photofluorometer with B_2 and PC_2 filters before and after hydrolysis. Concentrations of FMN and FAD were calculated from the difference in fluorescence of the hydrolyzed and unhydrolyzed sample (Burch, 1955).

(i) Ascorbic (AA) and dehydroascorbic (DHA) acids: The leaves were homogenized in ice-cold 3% metaphosphoric acid for 3 minutes. The homogenate was centrifuged at 20,000 x g for 15 minutes and the residue reextracted. The combined supernatants were made to volume (2 g fresh wt. leaves/25 ml of solution). The supernatant solution was then used for the quantitative determination of AA and DHA by the method of Hughes (1956). The method involves the estimation of AA in portions of the extract before and after treatment with homocysteine. The reduction of the 2,6-dichlorophenolindophenol dye was measured photoelectrically (at 520 mμ) in a Bausch & Lomb Spectronic 20 Colorimeter.

(j) Determination of ascorbic acid oxidase activity: For assay of ascorbic acid oxidase (AAO), 3 g of leaf tissue were homogenized with 10 ml of ice-cold citrate-phosphate (0.02 M - 0.6 M) buffer pH 5.8 in the cold room (4° C). The homogenate was filtered through 4 layers of cheese cloth and the filtrate made to a volume of 15 ml. Aliquots of the filtrate were assayed for the enzyme activity using standard Warburg respirometer procedures and measuring oxygen uptake over three 10 minute intervals at 30° C after equilibration and tipping.

The assay mixture consisted of 1 ml of leaf homogenate (equivalent to 200 mg fresh wt.), 1 ml of citrate-phosphate buffer pH 5.8, 0.2 ml of L-ascorbic acid containing 1.2 mg and water to make the final volume 3.0 ml. 0.2 ml of 2 N NaOH with a wick of filter paper was kept in the center well.

(k) Estimation of phenolics: The phenol content was determined in 80% methanol (99.8% purity, 'Baker Analyzed' reagent) extracts with the Folin-Ciocalteau (phenol reagent, Fisher Scientific Co.) reagent as described by Spies (1955). Results are expressed in "chlorogenic acid equivalents". The standard curve was linear in the range 0.-240 µg of chlorogenic acid (Nutritional Biochemical Co.) as measured in a Bausch & Lomb Spectronic

20 Colorimeter at 650 mμ.

(1) Nucleic acids: The nucleic acids (RNA and DNA) were isolated and estimated after the modified method of Schmidt and Thannhauser (1945) as recommended by Hutchison and Munro (1961) and adapted by Osborne (1962) to plant tissues. Total ribo- and deoxyribo-nucleic acid content was determined by measurement of the optical density at 260 mμ in a Beckman Model DK-2 ratio recording spectrophotometer scanning between 240 and 340 mμ.

The O.D. at 260 mμ of yeast S-RNA prepared by the phenol method of Gierer and Schramm (1956) was used as the standard. The UV absorptions of the samples were converted to μg RNA.

A standard curve using commercial DNA from Herring sperm (N.B. Co.) was prepared using the absorption of different concentrations of DNA at 260 mμ and the absorptions of the samples at 260 mμ were converted to μg DNA.

The nucleic acids of wheat leaves prepared as above were slightly contaminated with protein, but the absorption peak was always at 260 mμ. No attempt was made to further purify the samples as the comparative effects of water and benzimidazole treatment on nucleic acid contents were studied.

(m) Determination of inorganic pyrophosphatase activity: One gram of leaf material was homogenized in a mortar with 10 ml of ice-cold TRIS buffer (0.05 M TRIS containing 0.08 M cysteine-HCl, final pH 8.0. Cysteine-HCl was added to TRIS just before use). The homogenate was passed through 4 layers of cheese cloth and centrifuged for 10 minutes at 18,000 r.p.m. All operations were maintained at 0-4° C. The supernatant solution, after centrifugation, was used as the source of enzyme. One ml of this supernatant was mixed with 3 ml of TRIS (0.05 M, pH 8.0) buffer, 0.5 ml of 0.02 M $MgCl_2$, 0.1 ml of 2% (w/v) sodium-pyrophosphate (free from inorganic phosphorus) and 0.4 ml of water to make the final volume 5 ml. This mixture was incubated at 30° C in a water bath (Colora ultra thermostat) for 10 minutes. After incubation the reaction was stopped by adding 2 ml of 15% TCA and the mixture centrifuged. Aliquots of the supernatant were taken for the estimation of inorganic phosphorus. As control the inorganic phosphorus contents of extracts treated with TCA at zero time was also determined. The difference between the control and experimental sample was regarded as a measure of alkaline inorganic pyrophosphatase activity. The enzyme activity is expressed as μmoles Pi liberated in 10 minutes at 30° C per gram initial fresh weight of leaves.

4. Enzymatic Cycling Procedure to Estimate Pyridine Nucleotides

The procedure for the determination of pyridine nucleotides adopted by Anderson and Vennesland (1954), Glock and McLean (1955) was followed in the preliminary experiments. Using these methods, the recovery values were not consistent and also a large quantity of leaves was needed. Therefore, a very sensitive enzymatic cycling procedure originally described by Lowry et al (1961) was modified to assay the nucleotides in one gram lots of leaves.

(a) Principle:

NAD or NADH

(1) $$\text{NADH} + \alpha\text{-ketoglutarate} + \text{NH}_4^+ \xrightarrow[\text{(beef heart)}]{\text{glutamic dehydrogenase}} \text{NAD}^+ + \text{glutamate}$$

(2) $$\text{NAD}^+ + \text{lactate} \xrightarrow[\text{(beef heart)}]{\text{lactic dehydrogenase}} \text{NADH} + \text{pyruvate} + \text{H}^+$$

The pyruvate produced after cycling (2500-fold yield) is measured in a second step with added NADH and lactic dehydrogenase.

(3) $$\text{NADH} + \text{pyruvate} + \text{H}^+ \xrightarrow[\text{(Rabbit skeletal muscle)}]{\text{lactic dehydrogenase}} \text{NAD}^+ + \text{lactate}$$

The resulting NAD is measured fluorometrically.

NADP or NADPH

(1) $$\text{NADPH} + \alpha\text{-ketoglutarate} + \text{NH}_4^+ \xrightarrow[\text{(beef heart)}]{\text{glutamic dehydrogenase}} \text{NADP}^+ + \text{glutamate}$$

(2) $$\text{NADP}^+ + \text{glucose-6-P} \xrightarrow[\text{(yeast)}]{\text{G-6-P dehydrogenase}} \text{NADPH} + \text{6-P-gluconate}$$

The 6-P-gluconate produced after cycling (5,000-fold yield) is measured in a second incubation with 6-P-gluconate dehydrogenase and extra NADP.

(3) $$\text{6-P-gluconate} + \text{NADP}^+ \xrightarrow[\text{(rat liver)}]{\text{6-P-GDH}} \text{ribulose-5-P} + \text{NADPH} + \text{H}^+$$

The NADPH produced is measured fluorometrically.

Note:- During cycling the nucleotides were used at concentrations well below their Michaelis constants, consequently reaction rates were proportional to nucleotide concentrations.

(b) Enzyme preparations:

(i) Preparation of 6-P-gluconate dehydrogenase: The isolation and partial purification of the enzyme was done after the method of Lowry et al (1961) with slight modifications. Throughout, the preparation was kept at 0-4° C and all centrifugations were made at about 10,000 x g for 20 minutes in a Servall refrigerated centrifuge at 2° C. The buffer used was 0.025 M phosphate pH 7.5, unless otherwise stated.

Rat livers, 370 g were homogenized in an omnimixer with 9 volumes of buffer. The homogenate was centrifuged and to the supernatant solid ammonium sulfate was added to a concentration of 1 Molar and left overnight in the cold room. The precipitate was discarded and the supernatant made to 2 Molar by adding solid ammonium sulfate. The preparation was left for one hour and the precipitate discarded. To the supernatant solid ammonium sulfate was added to make the concentration 3 Molar and it was left overnight in the cold room. The supernatant was siphoned off and the precipitate dissolved in 3 volumes of buffer and dialyzed for 5 hours at 4° C, against the same buffer containing 0.2 mM EDTA.

The dialyzed preparation was diluted with the buffer to a protein concentration of 1% (measured with Folin-phenol reagent, using Trypsin from Bovine Pancreas 2x crystallized, dialyzed and lyophilized, salt free, obtained from Sigma Chemical Co., as the standard). The nucleic acid was removed as a precipitate with 0.04 volume of 1% protamine sulfate. To the supernatant, solid ammonium sulfate was added to a concentration of 2 Molar with enough 1 N NH_4OH to bring the pH to 7.0. The precipitate was discarded and to the supernatant solid ammonium sulfate was added to a concentration of 2.8 Molar, again adjusting the pH to 7.0 with 1 N NH_4OH.

The supernatant was discarded and the precipitate dissolved in 3 volumes of buffer and dialyzed as before. The sample was brought to a protein concentration of 1% by adding buffer and pH lowered to 6.0 with dilute acetic acid. It was then treated with $Ca_3(PO_4)_2$ gel and the gel was added in 4 steps (1.5 ml of 0.4% gel per ml of sample at each step).

From the last two gel treatments the enzyme was recovered by eluting in two steps using 0.2 M phosphate buffer pH 7.8 at the first step and 0.2 M phosphate buffer at pH 8.2 in the second step (40 ml of buffer per g of gel).

To the combined eluates 3 volumes of 3.5 Molar ammonium sulfate (neutralized) was added and the precipitate used as the source of enzyme. The precipitate was dissolved in 3 Molar ammonium sulfate (neutralized) and kept in the cold room. The enzyme preparation thus prepared contained 33.5 mg protein/ml and the activity was 70 μmoles/mg protein/hr as measured at 340 mμ in a Beckman DK-2 recording Spectrophotometer at 25° C with a final concentration of 0.5 mM 6-P-gluconate; 0.05 mM NADP; 1 mM EDTA, pH 8.0; and 0.05 M TRIS buffer, pH 8.0, in the assay system. (The glucose-6-P-dehydrogenase activity was 0.23 μmoles/mg protein/hr with a final concentration of 2 mM G-6-phosphate, 0.15 mM NADP, 10 mM $MgCl_2$ and 12 mM glyclylglycine buffer, pH 7.5, in the assay mixture).

(ii) <u>Commercial enzymes</u>: Glucose-6-Phosphate Dehydrogenase from Yeast and Crystalline Glutamic Dehydrogenase from Beef Heart were obtained from the Sigma Chemical Co. and

Lactic Dehydrogenase from Beef Heart and Rabbit Skeletal Muscle were obtained from the Worthington Biochemical Corporation. Beef Heart Lactic Dehydrogenase was specially purified as described by Lowry *et al* (1961).

(c) *Extraction procedure*:

The recommended procedure of Lowry *et al* (1961) for extracting the oxidized forms of pyridine nucleotides with 0.01 M H_2SO_4 - 0.1 M Na_2SO_4 was not adopted because such an extract when used with the cycling mixture resulted in 100% inhibition of cycling reactions. The procedure was modified by extracting the leaves with ice-cold 0.02 N HCl.

One gram wheat leaf was homogenized in 10 ml of ice-cold 0.02 N HCl in a mortar and pestle with a little acid-washed sand. The homogenate was quantitatively transferred to a centrifuge tube and after centrifugation the precipitate extracted twice with 5 ml of 0.02 N HCl each time and the combined supernatants made to a volume of 25 ml. 20 µl aliquots were taken either for NAD or NADP assay. The samples were stored at 4° C.

For the reduced nucleotides the procedure of Lowry *et al* (1961) was adopted. One gram of wheat leaves was homogenized in 10 ml of ice-cold 0.02 N NaOH containing 0.5 mM cysteine (cysteine added as cysteine-hydrochloride to the NaOH solution before use) and immediately (within 30 minutes) heated for 20 minutes at 60° C. After centrifugation the precipitate was reextracted twice and the combined supernatants made to a volume of 25 ml. 20 µl aliquots were taken for either NADH or NADPH assay. The samples were stored in the cold room at 4° C.

(d) Conditions for optimum cycling:

The cycling reactions were at an optimum when the following modifications to Lowry *et al's* (1961) method were adopted.

(a) The cycling time was doubled, i.e. 60 minutes instead of the 30 minutes for NAD or NADH assay.

(b) The crystalline beef liver glutamic dehydrogenase was used in half the amounts recommended for NADP or NADPH assay.

(c) The sulfate concentration was kept as low as possible by removing most of the sulfate from the enzyme preparation before use.

(e) Fluorometric measurements:

All fluorometric measurements were made in a Turner Fluorometer (G. K. Turner Associates, Palo Alto, California) Model 110 with primary filter no. 110-811 (7-60) at 365 mμ and secondary filter no. 110-821 (75) at 485 mμ using matched round pyrex glass cuvettes (10 x 75 mm).

(f) Actual Procedure

(i) Determination of NAD or NADH:- NAD or NADH (1.25 to 2.5 x 10^{-12} moles) or sample in a volume not exceeding 20 μl were pipetted into pyrex glass tubes (10 x 75 mm). All tubes were brought to the same volume of 20 μl by adding the respective extraction media, if necessary. The tubes were transferred to an ice-bath and volumes of 100 μl of complete cycling mixture (containing TRIS buffer, pH 8.4, 20 μmoles, sodium lactate 10 μmoles, ADP 0.03 μmoles, α-ketoglutarate 0.5 μmoles, ammonium acetate 10 μmoles, Glutamic dehydrogenase 41 μg and Lactic Dehydrogenase from Beef Heart 0.7 μg) kept near 0° C were added to each tube and after quick mixing (time between addition of first and last sample never exceeded 10 minutes) the tubes were transferred to a 25° C water bath for one hour and then to a 100° C water bath for 3 minutes. The tubes were kept in an ice-bath and to each tube 100 µl of a reagent (containing NaH_2PO_4 65 µmoles, K_2HPO_4 15 µmoles, NADH 30 mµmoles and Rabbit Muscle Lactic Dehydrogenase 0.15 µg, the NADH and enzyme being added to ice-cold phosphate just before use) were added and the tubes transferred again to the 25° C water bath for 15 minutes and returned back to the ice bath. 0.2 ml of 1 N HCl was added to each tube with very thorough mixing to destroy the excess of NADH. After 10 minutes 0.5 ml of 9 N NaOH was added to each tube. After heating 10 minutes at 60° C, 2 ml of water were added and the fluorescence read. All assays were done in triplicate.

Blanks and standards were carried out through the entire procedure and were treated as nearly as possible like the sample to be analyzed.

(ii) Determination of NADP or NADPH:- NADP or NADPH (1.25 to 2.5 x 10^{-12} moles) or sample in a volume not exceeding 20 µl were pipetted into pyrex glass tubes (10 x 75 mm). All tubes were brought to the same volume of 20 µl by adding the respective extraction media, if necessary. The tubes were transferred to an ice bath and volumes of 100 µl of complete cycling mixture (containing TRIS buffer pH 8.0, 10 µmoles, Glucose-6-Phosphate 0.1 µmoles, α-ketoglutarate 0.5 µmoles, ADP 10 mµmoles, ammonium acetate 2.5 µmoles, Bovine plasma albumin 20 µg, glutamic dehydrogenase 10 µg and Glucose-6-P Dehydrogenase 0.125 Kornberg Units) kept near 0° C were added to each tube and after mixing the tubes were transferred to a water bath at 38° C for 30 minutes and then to a 100° C water bath for 3 minutes. After cooling 1 ml of a reagent (containing TRIS buffer pH 8.0, 25 µmoles, EDTA 0.25 µmoles, NADP 0.25 µmoles and 1 mg protein of the specially prepared 6-P-Gluconic Dehydrogenase) was added to each tube and after 30 minutes at room temperature 2 ml of water were added and the fluorescence read. All assays were done in triplicate.

Standards which increase in steps of two or three to cover the particular concentration range were provided. Standards and blanks were carried through the entire process, including any procedure before cycling.

5. Isolation of Chloroplasts in a Nonaqueous Medium

As polar substances like cofactors and enzymes may be lost during aqueous isolation owing to leaching, the chloroplasts were isolated in a dry state in a nonpolar organic solvent, petroleum ether-carbon tetrachloride (CCl_4) in an attempt to minimize leaching. The procedures as described by Heber, 1957; Behrens and Thalacker, 1957; Stocking, 1959; Heber and Tyszkiewicz, 1962 and Smillie, 1963, were adopted with some modifications.

Briefly the procedure involves the following steps:

I. Freeze drying of leaves <u>in vacuo</u>.

II. Homogenizing the dry leaves in petroleum ether/CCl_4 (30/70, v/v, d = 1.3) in a precooled omnimixer for 3 minutes.

III. Centrifugation of the broken material at different densities of the organic solvent (d = 1.425 to 1.25) and collection of the chloroplast enriched fraction at the interface of d = 1.350/1.375.

I. Freeze drying of leaves

Fifty grams of leaves were ground in a precooled mortar with solid CO_2 and the frozen leaves were transferred to a glass dish. The dish was placed in a refrigerated (+2° C) desiccator containing P_2O_5. The desiccator was immediately connected to a freeze drying unit operating in a high vacuum system. After CO_2 had escaped, the diffusion pump was switched on and pressure reduced to less than 10^{-4} mm Hg. The freeze drying continued overnight and the freeze-dried leaves stored under vacuum at 4° C until used.

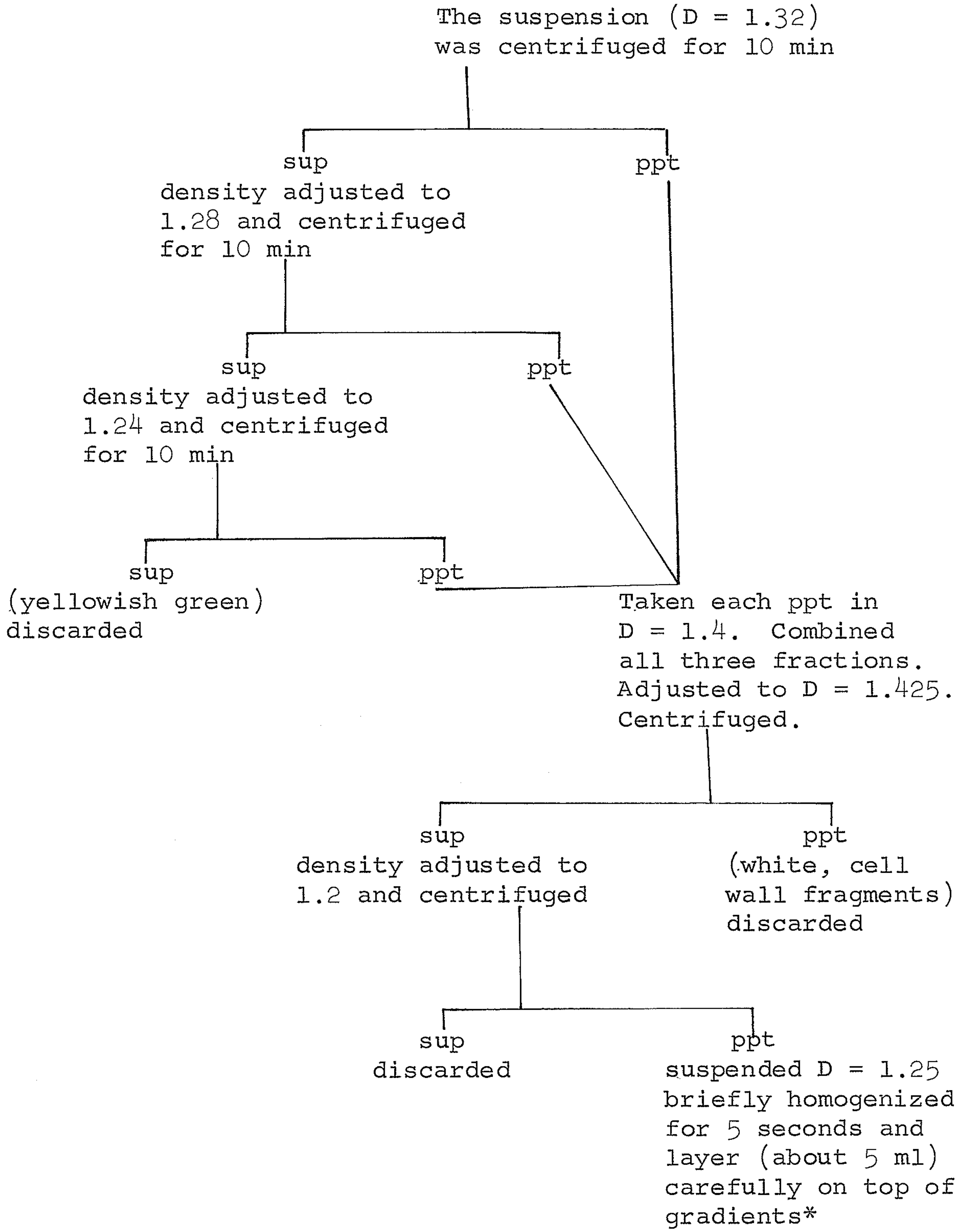
II. Homogenizing in organic solvent

Three grams of freeze-dried leaves were homogenized in a precooled omnimixer with ca. 200 ml of a mixture (density, D = 1.3) of 70 parts CCl_4 and 30 parts petroleum ether (b.p. 30-60° C) for 3 minutes at 120 V. The homogenate was passed through a very thin layer of cotton wool on a Buchner funnel without suction air and the same procedure repeated for the residue recovered from the cotton wool. Passing the homogenate through a thin layer of cotton wool removed most of the cell walls and guard cells, the main contaminant of the chloroplast fraction.

III. Isolation of chloroplasts

A flow sheet of the isolation procedure is given below.

All centrifugations were made in an International clinical centrifuge with a swinging bucket rotor having a maximum speed of 3,000 r.p.m.



*Density gradients: D = 1.425/1.400/1.375/1.350/1.325/1.300.

Five ml of each of D = 1.425, 1.400, 1.325, and 1.300 and 10 ml of each of densities 1.375 and 1.350 were layered in decreasing density on top of each other using cork discs to layer solutions. After centrifuging at top speed for 20 minutes 3 distinct zones were obtained at the interfaces of D = 1.350/1.375 (#1), D = 1.375/1.400 (#2) and D = 1.400/1.425 (#3). #1 was the smallest but the best enriched chloroplast layer followed by #2 and #3 which was the densest and darkest.

The layers were withdrawn and dried under air-jet. After drying, the chloroplasts were stored in a desiccator containing silica gel. The desiccator was kept in the cold room (0-4° C).

Analytical Methods for Estimating Chloroplast Composition

All weighings of dried chloroplast preparations were made on a Cahn Electrobalance Model M-10 (Cahn Instrument Co., Downey, California).

(a) Chlorophyll

Two milligrams of dried chloroplast sample were extracted three times in a glass homogenizer with 85% acetone and the combined extracts made to a volume of 5 ml after centrifugation. The chlorophyll content of the solution was estimated by the method of Arnon (1949) using a Zeiss Spectrophotometer Model PMQ II.

(b) Protein

The residue remaining after chlorophyll extraction was solubilized with 1.0 ml of 1 N NaOH and aliquot of the solubilized sample taken for the estimation of protein by the method of Lowry et al (1951) using crystalline Bovine Albumin (Sigma) as the standard. The colorimetric measurements were made in a Beckman-B Spectrophotometer.

(c) Nucleic Acids

Aliquots of the solubilized protein as obtained from above were taken for the nucleic acid estimation by measuring the O.D. at 260 mμ in a Zeiss Spectrophotometer Model PMQ II.

(d) Pyridine Nucleotides

The extracting media were the same as described before. For the oxidized pyridine nucleotides 2 mg of chloroplasts and for the reduced pyridine nucleotides 3 mg of the chloroplasts were taken. For extracting the pyridine nucleotides the chloroplasts were disrupted in 2 ml aliquots of the extracting media by sonic vibration for 120 seconds at 1.2 amperes in an Ultrasonic disintegrator (Measuring and Scientific Equipment

Ltd., London, England) at +2° C. The alkaline sonic extract was heated for 10 minutes at 60° C. Both the acidic and alkaline sonic extracts were centrifuged in a Servall centrifuge (+2° C) at 20,000 x g for 20 minutes and aliquot (10-20 µl) of the supernatant used for the pyridine nucleotide assay by the enzymatic cycling procedure as described before.

(e) Adenosine Diphosphate (ADP):

(i) Extraction:- The chloroplasts were extracted with hot water and after centrifugation the supernatant chromatographed on Whatman #1 paper and developed in a solvent Ethanol/1.0 M ammonium acetate, pH 3.8, 7/3 (v/v) (System #V, Pabst Circular OR-17) for 19 hours. The ADP spot was detected with reference to the standard (marker) under Ultraviolet light. The chromatogram was eluted with hot water, centrifuged and the supernatant used for ADP assay. The recovery of added ADP was 86%.

(ii) Determination:- The enzymic method as described by Greengard (1956), Glick (1961) and Seraydarian et al (1962) was followed. It is based upon the following reaction sequence.

$$\text{Phosphoenolpyruvate} + \text{ADP} \longrightarrow \text{Pyruvate} + \text{ATP} \quad (1)$$

$$\text{Pyruvate} + \text{NADH} + H^+ \longrightarrow \text{Lactate} + \text{NAD}^+ \quad (2)$$

The enzyme pyruvate kinase (reaction 1) and lactate dehydrogenase from Rabbit Skeletal Muscle (reaction 2) were crystalline preparations and obtained from Sigma Chemical Co. and Worthington Biochemical Corporation respectively. The NADH utilized in reaction 2 was measured fluorimetrically (Greengard, 1956) using a Turner Fluorometer Model 110 with a primary filter at 365 mμ (#7-60) and a secondary filter at 435 mμ (#2A+47B). The decrease in fluorescence was noted until it had approximately reached the endpoint. There was a linear dependence between the amount of ADP in the range 0.3 to 2.4 mμmole and the fluorescence.

The following is an account of a typical assay. Into a glass pyrex tube (10 x 75 mm) was added ADP standard (0.3 to 2.4 mμmoles) or sample to a volume of 0.2 ml. Then the following were added: Triethanolamine buffer pH 7.7, 30 μmoles; EDTA pH 7.4, 1.5 μmoles; $MgSO_4 \cdot 7H_2O$, 60 μmoles, KCl, 240 μmoles; Phosphoenolpyruvate, 10 mμmoles and NADH, 5 mμmoles. The volume was made to 2.7 ml by adding water. This was mixed thoroughly and brought to room temperature and checked for constancy of fluorimetric reading set at 90 to 100 scale units. At zero time 0.3 ml of a mixture containing 36 µg of Rabbit Muscle Lactic Dehydrogenase (Worthington Biochemical Corp.) and 30 µg of Pyruvic Kinase (Sigma Chemical Co.) were added and the decrease in fluorescence noted until it had approximately reached the end point. The change in a blank without ADP or sample was subtracted from the difference.

(f) Adenosine Triphosphate (ATP):

ATP was measured in the hot water extract of chloroplasts by the firefly luminescence assay (Strehler and McElroy, 1957) using a photomultiplier (American Instrument Co. Inc., Silver Springs, Maryland, U. S. A.) attached to a Sargent Recorder Model SR. A "30-second" calibration curve was prepared by plotting meter reading vs. known amounts of ATP (0.06 to 0.18 mµmole), just before use. Recovery of added ATP in the hot water extract was 95%.

Luciferin-luciferase extract was prepared by grinding 50 mg of "live-desiccated" firefly lanterns (Sigma Chemical Co.) in 5 ml of 0.1 M Sodium arsenate buffer pH 7.4 containing

100 mg of $MgSO_4 \cdot 7H_2O$, for about 5 minutes in a pre-cooled mortar in the cold room and centrifuging the extract for 10 minutes in a refrigerated centrifuge.

To a cuvette (1 ml capacity) at room temperature were added 0.1 ml of firefly extract, 0.4 ml of water and at zero time 0.3 ml of ATP or sample were added with thorough mixing. The photomultiplier was adjusted to maximum sensitivity and the photomultiplier reading recorded in a Sargent Recorder. ATP concentration of the sample was calculated from the "30-second" calibration curve.

IV. EXPERIMENTAL RESULTS

1. Effect of benzimidazole on the biochemical composition of whole leaves.

(a) Nitrogen

Numerous authors have reported the changes in protein and soluble nitrogen levels following detachment of leaves. Person *et al* (1957) and Samborski *et al* (1958) found that protein breakdown and accumulation of amino acids occurred on leaves floated on water but not on benzimidazole (BZ).

The experiment reported here was carried out with detached leaves floated either in continuous light or in darkness and the drifts in the level of TCA-insoluble and soluble and total nitrogen are presented in Table II. There was an increase in soluble nitrogen and a decrease in the insoluble nitrogen fraction of the leaves following detachment. Benzimidazole treatment maintained a higher level of insoluble nitrogen and a lower level of soluble nitrogen in detached leaves. The changes were more pronounced in the dark than in the light

TABLE II

Drifts in the levels of total, TCA-insoluble and TCA-soluble (15% TCA) nitrogen of detached wheat leaves.

The leaves were 10-days-old at the time of detachment. All figures are mg nitrogen per g initial fresh weight of leaves. (Mean of duplicate experiments).

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
				Total nitrogen	
0	7.0	-	-	-	-
1	-	5.30	5.12	5.40	4.52
2	-	5.70	4.94	4.80	4.48
3	-	4.13	4.27	4.60	4.40
4	-	4.70	4.35	3.50	4.41
				TCA-insoluble nitrogen	
0	5.35	-	-	-	-
1	-	4.08	4.32	3.66	3.94
2	-	3.76	4.30	3.35	3.40
3	-	3.10	4.00	2.68	3.06
4	-	2.90	3.30	1.80	2.90
				TCA-soluble nitrogen	
0	0.72	-	-	-	-
1	-	0.69	0.54	0.90	0.84
2	-	0.75	0.56	1.43	1.33
3	-	0.98	0.80	1.80	1.39
4	-	1.27	0.93	2.18	1.66

presumably due to a greater protein loss in darkness. There was no significant change in the level of total nitrogen between water and benzimidazole treated leaves.

The time curves given in Fig. 1 show the effect of benzimidazole on the insoluble nitrogen content which is expressed as a percentage of total nitrogen. This fraction includes the proteins and the nucleic acids. The curves indicate that the downward drift in the insoluble nitrogen fraction which normally occurred in water floated leaves was reversed by benzimidazole treatment, where indeed an upward trend was indicated for the first three days following detachment. The trend in the curve indicates that protein and nucleic acid synthesis was promoted in detached leaves by benzimidazole treatment and continuous illumination accelerated the process.

Curves showing changes in the ratio of the soluble and insoluble fractions are given in Fig. 2, which also illustrates the benzimidazole effect in stabilizing protein levels of the detached leaves under continuous illumination. BZ treatment maintained this ratio at a low level.

Samborski <u>et al</u> (1958) concluded that BZ prevents the

Changes in the levels of insoluble nitrogen when expressed as a percentage of total nitrogen (Fig. 1).

Changes in the ratios of soluble-to-insoluble nitrogen (Fig. 2)

The leaves were 10-days-old at the time of detachment.

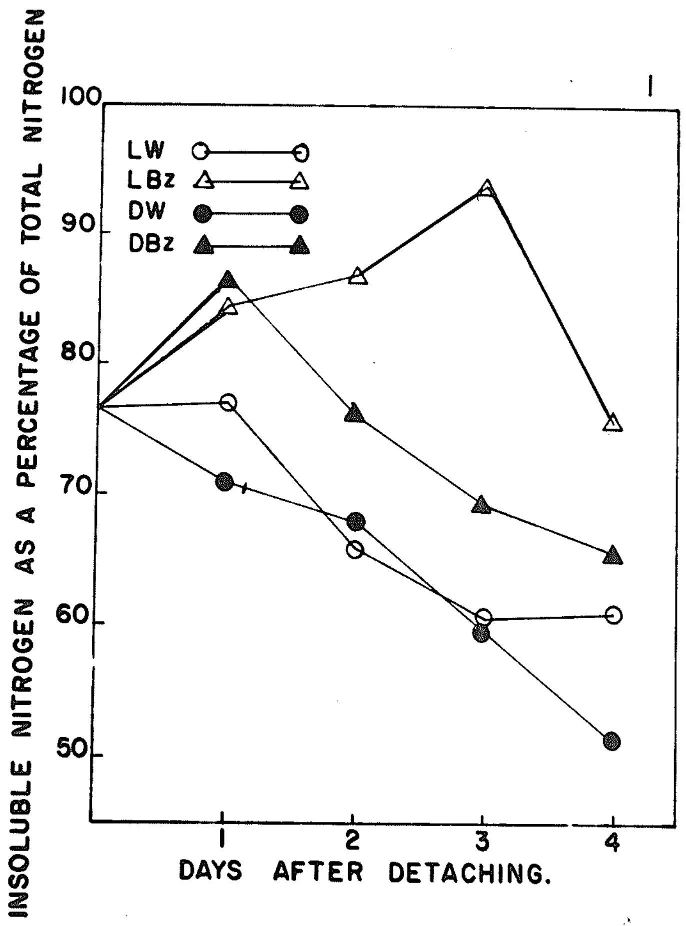
LW - Leaves floated on water under light

LBZ - Leaves floated on benzimidazole under light

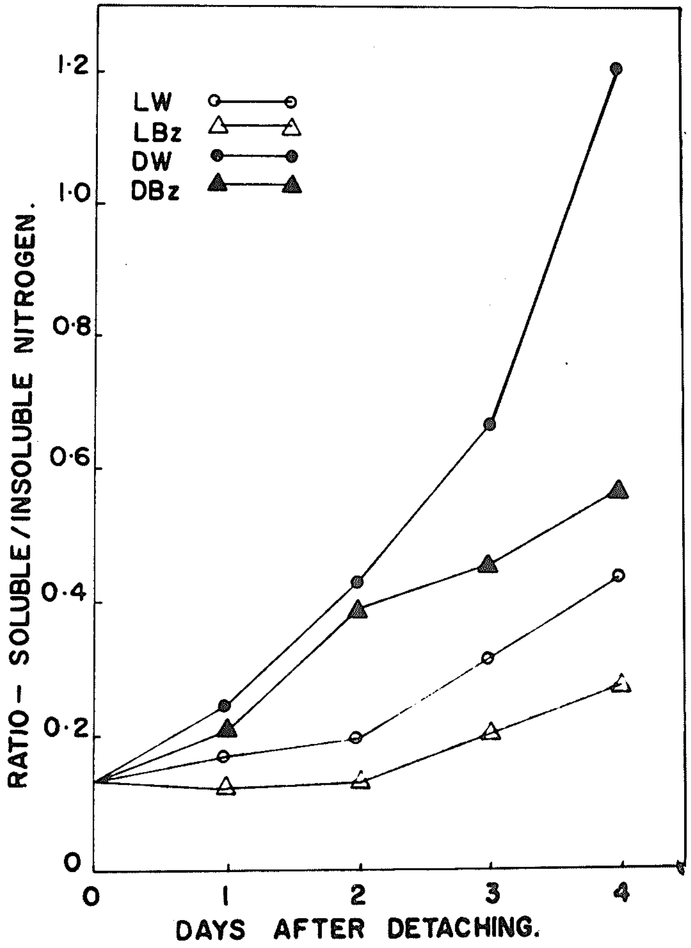
DW - Leaves floated on water under complete darkness

DBZ - Leaves floated on benzimidazole under complete darkness.

1


INSOLUBLE NITROGEN AS A PERCENTAGE OF TOTAL NITROGEN
100
90
80
70
60
50
LW
LBz
DW
DBz
1
2
3
4
DAYS AFTER DETACHING.

2


RATIO – SOLUBLE/INSOLUBLE NITROGEN.
1·2
1·0
0·8
0·6
0·4
0·2
0
LW
LBz
DW
DBz
0
1
2
3
4
DAYS AFTER DETACHING.

downward drift in alcohol-insoluble nitrogen content which normally occurs in detached leaves. The present investigation supports their results in that BZ maintained a higher level of TCA-insoluble nitrogen and a lower level of TCA-soluble nitrogen compared to water floated leaves.

The existence of protein turnover in leaf tissue has long been suggested or implied (Webster, 1959) and in excised leaves which are typically in a state of protein loss, Racusen _et al_ (1960) found a protein synthetic rate of roughly 2% per hour for freshly excised leaves and a lowered protein synthesis (or turnover) with increased time of excision. It is reasonable to assume that BZ increased protein synthesis which balanced senescence so as to produce an overall high level in TCA-insoluble nitrogen content.

(b) _Nucleic acids_

An impairment of nucleic acid synthesis may be a possible feature of cellular senescence and an attempt has therefore been made in this experiment to compare the levels of RNA and DNA in the detached leaves floated on water or benzimidazole. It was also hoped that the effect of BZ in retarding senescence in leaves may be mediated through its action in sustaining nucleic acid synthesis.

The leaves used in this experiment were 9-days-old at the time of detachment and were floated either in a 16 hr. light period followed by 8 hr. darkness in a 24 hr. day cycle or in complete darkness.

The absolute values reported for RNA and DNA in wheat leaves may be taken into consideration with caution. As emphasized earlier these nucleic acid preparations were not very pure because of some protein contaminations. The values can only be taken into account when it concerns only comparative figures between treatments. The nucleic acid assays were done in triplicate.

The absolute values for RNA and DNA in plant parts as reported recently, have been summarized below for comparing the values for wheat leaves as reported in Tables III and IV.

		µg/g fresh wt		
Plant	Part	RNA	DNA	Author
Citrus sinensus	leaf (6 wk old)	1,249	265	Monselise *et al* (1962)
Xanthium pennsylvanicum	leaf	1,052	340	Osborne (1962)
Xanthium pennsylvanicum	veg, bud	4,900	-	Ross (1962)
Peanut	cotyledon	880	150	Cherry (1962)
Olea europea	leaf	1,600*	600*	Kessler *et al* (1962)
Ligustrum sinensis	leaf	5,200*	400*	Kessler *et al* (1962)
Pisum sativum	leaf (9 day-old)	3,420*	150*	Smillie & Krotkov (1961)
Phaseolus vulgaris	leaf	1,260*	545*	Nieman *et al* (1963)

*Assuming phosphorus as 10% of nucleic acid. The values reported by these authors were as RNA-P or DNA-P.

(i) Ribonucleic acid (RNA)

The changes in the levels of RNA during a period of senescence extending over 7 days are presented in Table III. The data clearly show the continuous fall in the level of RNA more rapidly in the dark than in the light and at a faster rate in water than in the BZ floated leaves. This fall is also associated with the yellowing and loss of

TABLE III

Drifts in RNA content of detached wheat leaves.

Leaves were 9-days-old at the time of detachment.
(μg/g initial fresh wt of leaves)

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
0	1221	-	-	-	-
3	-	741	873	700	829
5	-	617	801	567	764
7	-	596	700	487	753

TABLE IV

Drifts in DNA content of detached wheat leaves.

Leaves were 9-days-old at the time of detachment.
(μg/g initial fresh wt of leaves)

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
0	473	-	-	-	-
3	-	494	400	407	436
5	-	422	465	349	465
7	-	414	422	262	451

chlorophyll and protein, symptomatic of senescence. BZ treatment maintained higher levels of RNA both in light and darkness. The fall in the levels of RNA was initially slower in the BZ floated leaves. The higher levels of RNA in BZ floated leaves may be correlated with the increased amount of TCA-insoluble nitrogen as reported earlier (Table II) for the BZ floated leaves.

(ii) Deoxyribonucleic acid (DNA)

Table IV summarizes the drifts in the DNA levels during senescence. There was no significant drop in the levels of DNA either in light or in darkness by BZ treatment. Only the darkened leaves on water showed progressively decreased DNA contents with time. Compared to water floated leaves the levels of DNA were slightly higher in BZ floated leaves in light, but were significantly higher in the dark. It appears there was little or no synthesis of DNA during the senescing period and this would accord with the normal absence of cell division in mature excised leaves.

(iii) RNA/DNA ratio

A fall in the ratio (Table V) of total RNA to total

TABLE V

RNA/DNA ratio of detached wheat leaves.

Leaves were 9-days-old at the time of detachment.

		Light		Dark	
Days after detachment	I.D.	water	benzimidazole	water	benzimidazole
0	2.58	-	-	-	-
3	-	1.50	2.18	1.72	1.90
5	-	1.46	1.73	1.63	1.64
7	-	1.43	1.66	1.85	1.67

DNA was observed in the water floated leaves, which were undergoing rapid senescence. In the BZ floated leaves, where senescence was retarded or delayed, the ratio was higher than in the water floated leaves. Perhaps, in the BZ floated leaves, there was a net increase in the levels of RNA per unit DNA, which in other words would indicate a stimulation in the synthesis of RNA in BZ floated leaves. BZ maintained this ratio high and the effect was only significant in light presumably due to a light-dependent synthesis of RNA.

Osborne (1962) found a decline in levels of RNA, DNA and RNA/DNA ratio with the progress of senescence in detached xanthium leaves and the addition of kinetin temporarily maintained a relatively high ratio of RNA to DNA and higher levels in RNA and DNA.

(c) Drifts in the level of pyridine nucleotides

Pyridine nucleotides are important cofactors in the regulation of cellular metabolism and this experiment was designed to test to what extent these nucleotide concentrations are altered in the detached leaves floated on water, BZ, KN, or BZ + KN.

The purposes of this experiment were also to verify

(1) Whether or not the PN pattern in leaves alters with senescence, because Jedeikin and Weinhouse (1955) have reported the post-mortem stability of NAD and NADH in rat liver.

(2) Whether there is any interrelationship between the changes in the levels of certain PNS and the metabolic pattern of leaves during senescence.

(3) Whether the effect of BZ or KN in delaying the onset of senescence in leaves could be correlated with the relative levels of pyridine nucleotides (Yoshida, 1961).

(4) Whether there is any change in the oxidation-reduction state of leaves accompanying senescence because the preliminary investigation on ascorbate metabolism suggested that the oxidation-reduction state of NADP was influenced by BZ treatment.

(5) Whether the reaction, between NAD and BZ leading to the formation of BAD, reported by Kapoor (1963) from her *in vitro* experiments occurs also *in vivo*. If such a reaction does occur *in vivo* then the levels of NAD in BZ floated leaves would be altered.

The leaves used in this experiment were 14-days-old at the time of detachment and were floated in 16 hr. light followed by an 8 hr. dark period in a 24 hr. day cycle and the leaves harvested (1) just before the end of the 16 hr. light period or (2) just before the end of the 8 hr. dark period.

(i) Assay for NAD-ase or NADP-ase

To assure that no NAD-ase or NADP-ase activity was present in the acid extract (because of the extraordinary stability of mammalian NAD-ase towards acid), a 0.9 ml aliquot of the acid-extract was incubated with 0.1 ml of either NAD or NADP solution containing 16 mg/ml and incubated for 24 hours either at 4° C or for 1 and 24 hours at 38° C. The NAD and NADP contents were measured before and after incubation using a glutamic acid-glutamic dehydrogenase system. The observations are presented in Table VI. It is obvious that there was no loss of either NAD or NADP if kept at 4° C, however, there was only a slight loss in NAD at 38° C after 24 hours.

(ii) Recovery experiments

Leaf samples were extracted as described before. The

TABLE VI

Δ O.D./30 seconds assay system at 340 mμ

Sample	'0'	38° C		+4° C
	time	1 hr	24 hrs	24 hrs
NAD #1	0.05	0.05	0.04	0.05
#2	0.05	0.05	0.04	0.05
NADP #1	0.07	0.07	0.07	0.07
#2	0.08	0.08	0.08	0.08

<u>Assay system</u>

Phosphate buffer 0.12 M, pH 7.6 - 0.56 ml

L-glutamic acid 0.5 M, pH 7.0 - 0.06 ml

Incubated mixture - 0.15 ml

L-glutamic dehydrogenase* - 100 units (approx.)

final vol. 0.8 ml

*From beef liver; ammonium sulfate suspension (20 mg protein/ml). Lot #G52B - 200 (Sigma Chemical Co.)
1 mg protein = 11,000 units

pyridine nucleotide solution was added at the time of grinding in amounts of 3.12 mμmoles to each sample except sample #3 where 1.56 mμmoles were added. Standards consisted of NAD, NADP, NADH or NADPH and were prepared in respective acid and alkaline solution followed by the same temperature treatment as the samples.

Assays were conducted in triplicate. All values recorded in Table VII are expressed as mμmoles per g fresh weight of leaves. The standard deviation was ± 5% for NAD and NADH assay and ± 13% for NADP and NADPH assay. Recovery of added nucleotides was satisfactory in all cases, there being 96-100% recovery of NAD, 96-98% recovery of NADH, 99% recovery of NADP and 92-97% recovery of NADPH.

(iii) Light and dark effect on PN levels

No matter what the treatments were the results show (Table VIII and X) the level of NADPH was higher at the end of the 16 hr. light periods, the reverse being the case for NADH. No significant difference was observed in the level of NADP but NAD level was low at the end of the 16 hr. light periods and high after the dark periods.

The levels of total NADP + NADPH were high and NAD +

TABLE VII

Recovery of pyridine nucleotides added to wheat leaves at the time of extraction

All values are recorded as mμmoles (10^{-9} moles) per g fresh weight of leaves.

Sample	NAD^+			NADH			$NADP^+$			NADPH		
	Calculated	Found	% Recovery	Calculated	Found	% Recovery	Calculated	Found	% Recovery	Calculated	Found	% Recovery
#1		6.25										
#1 + NAD^+	9.37	9.37	100									
#2		10.10										
#2 + NAD^+	13.22	12.70	96.5									
#3					8.95							
#3 + NADH				10.51	10.10	96						
#4					7.80							
#4 + NADH				10.92	10.70	98						
#5								4.15				
#5 + NADP							7.27	7.18	99			
#6											6.46	
#6 + NADPH										9.58	9.35	97
#7											6.65	
#7 + NADPH										9.77	9.20	94
#8											6.25	
#8 + NADPH										9.37	8.60	92

Mean standard deviation: NAD and NADH - 5%; NADP and NADPH - 13%.

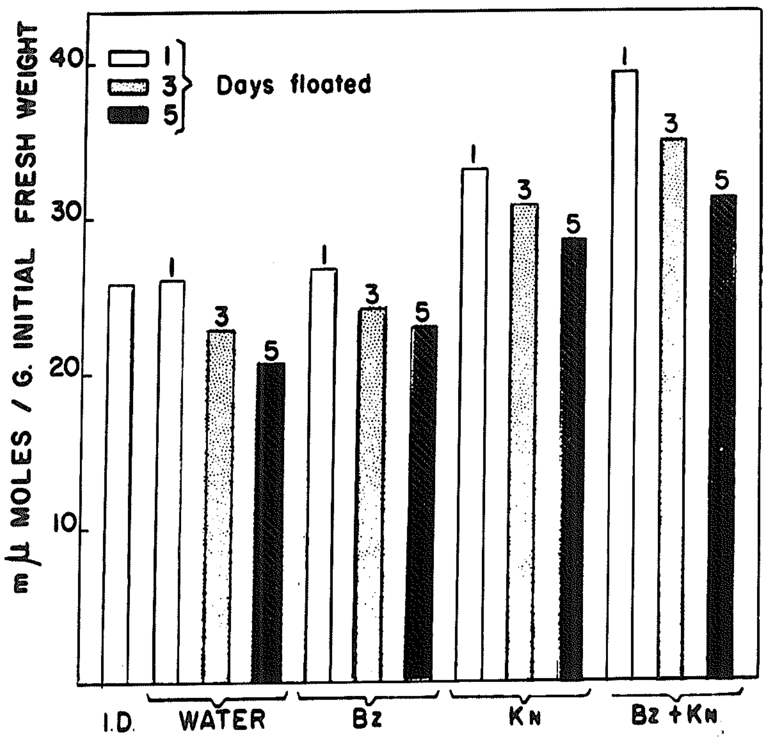
NADH low at the end of 16-hr. light periods, the reverse being true at the end of 8-hr, dark periods. The ratios of NADPH/NADP were high and NADH/NAD low at the end of light periods than the same at the end of dark periods. It is known (Walker, 1962) that as a consequence of cyclic and non-cyclic photophosphorylation the ratio of NADPH/NADP will be at a higher level in the light and will tend to diminish in the dark.

(iv) Effect of senescence on PN levels

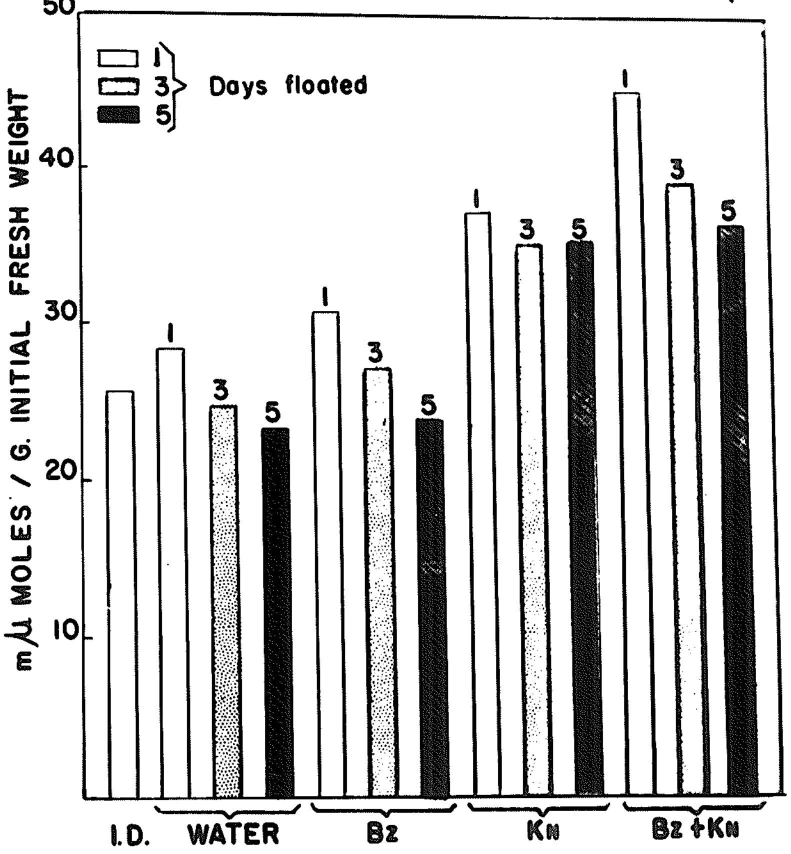
The changes in the levels of total PN at the end of the light (Fig. 3) and dark (Fig. 4) periods indicate a very little loss (ca. 20%) after a 5-day period of detachment. The levels of individual PN at the end of light (Table VIII) and dark (Table X) periods show a decrease in the levels of NAD, NADP and NADPH and an increase in the levels of NADH at the end of light periods only. There was practically no loss in the NADP + NADPH levels. The NADPH/NADP ratio decreased whereas NADH/NAD ratio increased with the progress of senescence.

At the end of dark periods the levels of NAD increased but the levels of NADH, NADP and NADPH gradually decreased with time. There was no loss in the NAD + NADH levels.

3


mµ MOLES / G. INITIAL FRESH WEIGHT
10
20
30
40
1
3
5
Days floated
I.D.
WATER
Bz
Kn
Bz + Kn
1
3
5
1
3
5
1
3
5
1
3
5

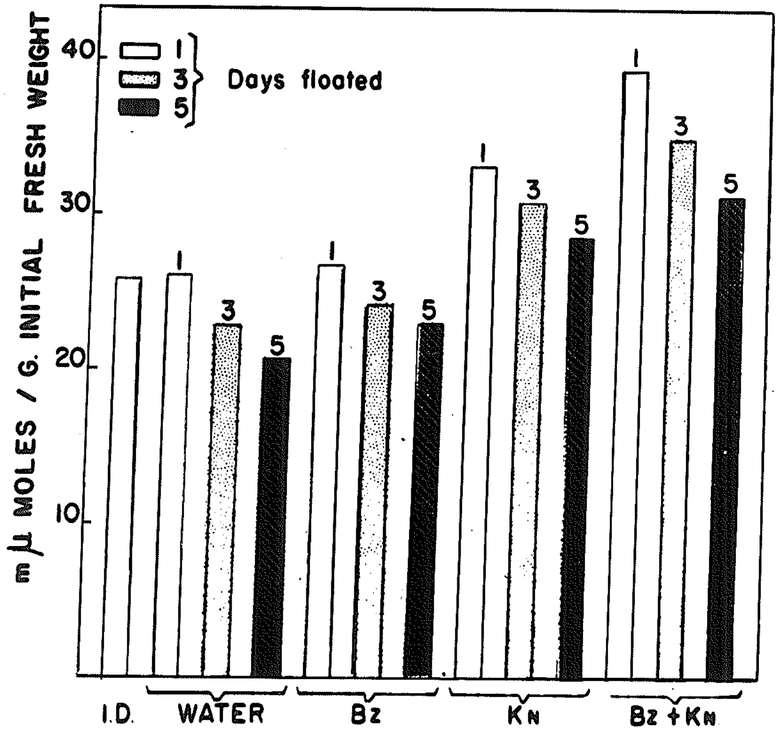
4


mµ MOLES / G. INITIAL FRESH WEIGHT
10
20
30
40
50
1
3
5
Days floated
I.D.
WATER
Bz
Kn
Bz + Kn
1
3
5
1
3
5
1
3
5
1
3
5

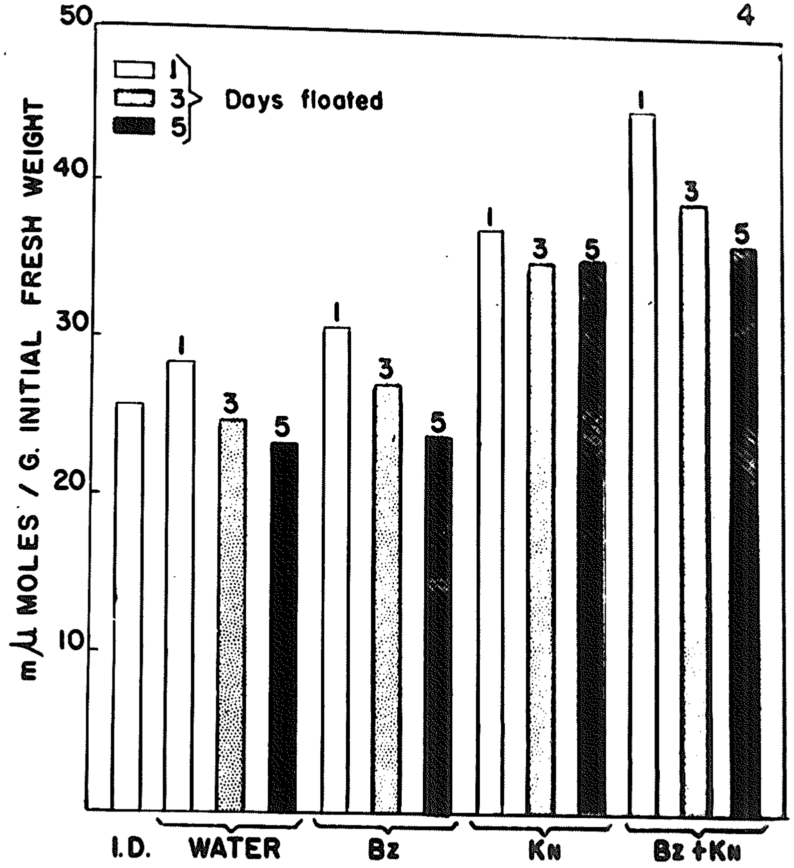
Drifts in the total pyridine nucleotide levels of leaves at the end of light periods (Fig. 3), and dark periods (Fig. 4).

Leaves were 14-days-old at the time of detachment.

3


mμ MOLES / G. INITIAL FRESH WEIGHT
40
30
20
10
1
3
5
Days floated
I.D.
WATER
Bz
Kn
Bz + Kn

4


mμ MOLES / G. INITIAL FRESH WEIGHT
50
40
30
20
10
1
3
5
Days floated
I.D.
WATER
Bz
Kn
Bz + Kn

The NADH/NAD ratio gradually decreased with time.

(v) Effect of BZ or KN on PN levels

Tables VIII and X summarize the drifts in the levels of PN by various treatments and tables IX and XI indicate the pattern of changes in their ratios. Figs. 5 and 6 show the fluctuation in the ratios of NADP/NADPH and NAD/NADH respectively at different periods during senescent metabolism in the various treatments. Evidently the differences between the curves are probably not significant.

Contrary to the loss in total PNS in water floated leaves some synthesis occurred in the KN or BZ + KN floated leaves. The synthesis is reflected in the reduced form of the nucleotides.

(1) Effect of Benzimidazole

At the end of the light periods there was no significant difference between the water and BZ floated leaves except that the NADPH/NADP ratios were low in the water floated leaves and high in BZ floated leaves.

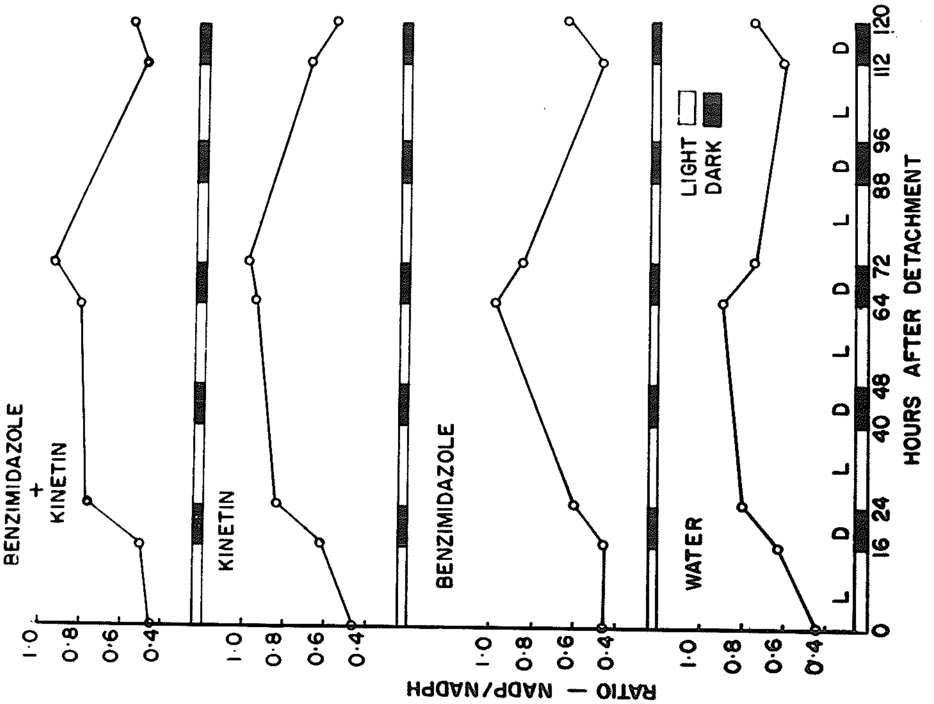
The difference in PN levels at the end of dark periods is of some interest, the NAD levels being high and

Drifts in the ratios of NADP-to-NADPH (Fig. 5).

Drifts in the ratios of NAD-to-NADH (Fig. 6).

Leaves were 14-days-old at the time of detachment.

5


RATIO — NADP/NADPH
BENZIMIDAZOLE + KINETIN
1·0
0·8
0·6
0·4
KINETIN
1·0
0·8
0·6
0·4
BENZIMIDAZOLE
1·0
0·8
0·6
0·4
WATER
1·0
0·8
0·6
0·4
LIGHT
DARK
L D L D L D L D L D
0 16 24 40 48 64 72 88 96 112 120
HOURS AFTER DETACHMENT

6

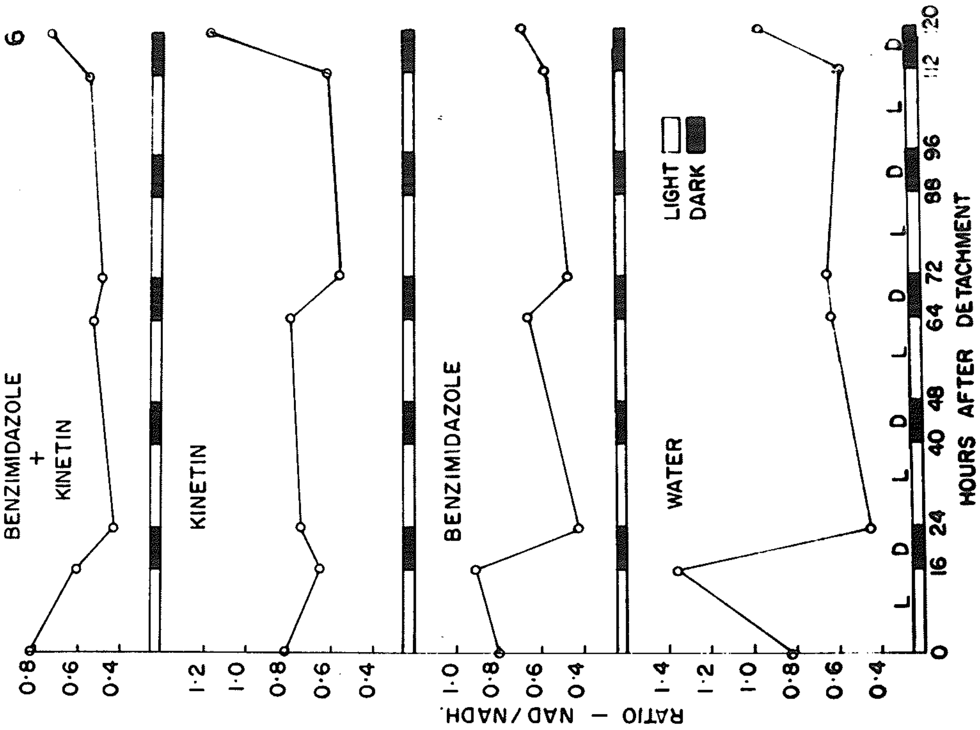

RATIO — NAD/NADH.
BENZIMIDAZOLE + KINETIN
0·8
0·6
0·4
KINETIN
1·2
1·0
0·8
0·6
0·4
BENZIMIDAZOLE
1·0
0·8
0·6
0·4
WATER
1·4
1·2
1·0
0·8
0·6
0·4
LIGHT
DARK
L D L D L D L D L D
0 16 24 40 48 64 72 88 96 112 120
HOURS AFTER DETACHMENT

TABLE VIII

Drifts in the levels of pyridine nucleotides of leaves at the end of the 16 hour light periods

(values as mμmoles per g initial fresh wt of leaves)

Treatment	Days	NAD	NADH	NAD + NADH	NADP	NADPH	NADP + NADPH	Total PN
I.D.	0	6.49	7.85	14.34	3.64	7.83	11.47	25.81
Water	1	5.13	3.78	8.91	6.61	10.53	17.14	26.05
	3	3.47	5.59	9.06	6.61	7.29	13.90	22.96
	5	3.47	6.19	9.66	4.18	6.75	10.93	20.59
BZ	1	3.47	3.92	7.40	6.07	13.23	19.30	26.70
	3	3.32	4.83	8.15	7.96	8.10	16.06	24.21
	5	3.93	6.49	10.42	2.97	6.21	9.18	19.60
KN	1	3.62	5.28	8.90	9.45	14.98	24.43	33.33
	3	5.13	6.34	11.47	9.45	9.85	19.30	30.77
	5	5.74	9.06	14.80	5.80	8.10	13.90	28.70
BZ + KN	1	3.92	6.34	10.27	9.86	19.57	29.43	39.70
	3	3.78	7.09	10.87	10.80	13.23	24.03	34.90
	5	5.74	10.11	15.85	5.13	10.26	15.39	31.24

Standard Deviation: NAD and NADH ± 5%; NADP and NADPH ± 13%.

TABLE IX

Drifts in the ratios of pyridine nucleotides of leaves at the end of the 16 hour light periods

Treatment	Days	$\frac{NADPH}{NADP}$	$\frac{NADH}{NAD}$	$\frac{NAD + NADP}{NADH + NADPH}$	$\frac{NADH + NADPH}{NAD + NADP}$	$\frac{NAD + NADH}{NADP + NADPH}$	$\frac{NADP + NADPH}{NAD + NADH}$
I.D.	0	2.15	1.21	0.65	1.55	1.25	0.80
Water	1	1.59	0.74	0.82	1.22	0.52	1.92
	3	1.10	1.61	0.78	1.28	0.65	1.54
	5	1.61	1.78	0.59	1.69	0.88	1.13
BZ	1	2.18	1.13	0.56	1.80	0.38	2.61
	3	1.02	1.46	0.87	1.15	0.51	1.97
	5	2.09	1.65	0.54	1.84	1.13	0.88
KN	1	1.59	1.46	0.65	1.55	0.36	2.74
	3	1.04	1.24	0.90	1.11	0.59	1.68
	5	1.40	1.58	0.67	1.49	1.06	0.94
BZ + KN	1	1.99	1.62	0.53	1.88	0.35	2.87
	3	1.23	1.88	0.72	1.40	0.45	2.21
	5	2.00	1.76	0.53	1.88	1.03	0.97

TABLE X

Drifts in the levels of pyridine nucleotides of leaves at the end of the 8 hour dark periods.

(value as mµmoles per g initial fresh wt of leaves)

Treatment	Days	NAD	NADH	NAD + NADH	NADP	NADPH	NADP + NADPH	Total PN
I.D	0	6.49	7.85	14.34	3.64	7.83	11.47	25.81
Water	1	4.38	9.81	14.19	6.48	7.83	14.31	28.50
	3	6.49	9.81	16.30	3.64	4.72	8.37	24.67
	5	7.09	7.25	14.34	3.91	5.13	9.04	23.38
BZ	1	4.38	9.96	14.34	6.21	10.39	16.60	30.94
	3	5.73	12.08	17.81	4.32	4.99	9.31	27.12
	5	5.43	7.70	13.13	4.18	6.48	10.66	23.79
KN	1	6.49	8.76	15.25	10.12	11.88	22.00	37.25
	3	8.61	14.94	23.55	5.94	5.67	11.61	35.16
	5	12.38	9.36	21.74	5.13	8.91	14.04	35.78
BZ + KN	1	6.34	14.80	21.14	10.53	13.63	24.16	45.30
	3	8.15	16.61	24.76	7.02	7.29	14.31	39.07
	5	9.06	11.92	20.98	5.67	9.72	15.39	36.37

Standard Deviation: NAD and NADH ± 5%; NADP and NADPH ± 13%.

TABLE XI

Drifts in the ratios of pyridine nucleotides of leaves at the end of 8 hour dark periods.

Treatment	Days	$\frac{NADPH}{NADP}$	$\frac{NADH}{NAD}$	$\frac{NAD + NADP}{NADH + NADPH}$	$\frac{NADH + NADPH}{NAD + NADP}$	$\frac{NAD + NADH}{NADP + NADPH}$	$\frac{NADP + NADPH}{NAD + NADH}$
I.D.	0	2.15	1.21	0.65	1.55	1.25	0.80
Water	1	1.21	2.24	0.62	1.62	1.00	1.01
	3	1.30	1.51	0.70	1.43	1.95	0.51
	5	1.31	1.02	0.89	1.12	1.59	0.63
BZ	1	1.67	2.28	0.52	1.92	0.86	1.16
	3	1.16	2.11	0.59	1.70	1.91	0.52
	5	1.55	1.42	0.68	1.47	1.23	0.81
KN	1	1.17	1.35	0.81	1.24	0.69	1.44
	3	0.95	1.74	0.71	1.42	2.03	0.49
	5	1.74	0.76	0.96	1.04	1.55	0.65
BZ + KN	1	1.29	2.33	0.59	1.69	0.87	1.14
	3	1.04	2.04	0.63	1.58	1.73	0.58
	5	1.71	1.32	0.68	1.47	1.36	0.73

NADH levels low in water floated leaves compared to the BZ floated leaves. Both NADP and NADPH levels were low in water floated leaves and high in BZ floated leaves. The trends for NADPH/NADP ratios were the same as found at the end of the light periods. The levels of total oxidized nucleotides were high and reduced nucleotides low in water-floated leaves compared to the BZ floated leaves.

(2) Effect of Kinetin

Kinetin had a marked effect in enhancing the PN levels at the end of both light and dark periods. At the end of the light periods NAD and NADH levels increased but NADP and NADPH levels decreased. The same trend was observed at the end of dark periods.

(3) Effect of BZ + KN

Mainly the kinetin effect was reflected in this treatment and in some cases the effect was additive.

The salient observations of this experiment were

(a) There was very little loss of PNS during senescence.

(b) Some synthesis of PN was indicated by KN or BZ + KN treatments.

(c) NADPH/NADP ratios were higher at the end of light periods and these ratios gradually decreased accompanying senescence.

(d) The effect of BZ or KN in delaying senescence was due to their ability to maintain high values for the ratios of NADPH/NADP.

(e) The oxidation-reduction status of leaves as measured by by the NAD + NADP/NADH + NADPH ratios gradually increased during senescence at the end of dark periods and BZ or BZ + KN treatments maintained these ratios relatively low.

However, the difficulties in interpreting the data on the pyridine nucleotide pattern of the senescing whole leaves demanded further investigations at the chloroplast level and the next section deals with the biochemical composition of chloroplasts with particular reference to pyridine and adenine nucleotides.

2. Effect of benzimidazole or kinetin on the biochemical composition of chloroplasts

The first symptom of senescence is that chlorosis occurs in the chloroplasts. Moreover, there is a compartmentalization of cellular substances in the leaves. It was thought that any change in the composition of chloroplasts by BZ or KN treatments may account for their effects in delaying the senescence and preserving the green pigment and protein of the detached leaves.

As the formation of chloroplasts is light dependent (Smillie, 1963), the leaves were floated under continuous illumination, assuming that BZ or KN may enhance chloroplast development in light. Moreover, some of the investigations presented in the appendix suggested the pronounced effect of BZ under continuous light. The leaves used in this experiment were 10-days-old at the time of detachment and were floated for 6 days under continuous light and the chloroplasts isolated in a non-aqueous medium.

The yield of chloroplast fractions from 3 g of freeze dried leaves are tabulated below for the different treatments.

(value in mg)

Treatment	days floated	chloroplast fraction #1	#2	#3	Total
I.D.	0	35	113	173	323
water	6	21	112	160	293
BZ	6	105	90	185	380
KN	6	45	95	167	307

In subsequent experiments, for the estimation of the chloroplast composition, only fraction #1 was used.

(a) Chlorophyll

Fig. 7 shows the effect of BZ or KN on the levels of chlorophyll in the chloroplasts of senescing leaves. The chlorophyll content in the chloroplasts of immediately detached leaves is 3.2% on a dry weight basis. Although no accurate data are available yet Stocking (1959) has reported a value of 2% for his pure chloroplasts (density = 1.32) isolated from tobacco leaves in a non-aqueous medium. The values reported by various authors using an aqueous isolation technique for spinach leaves are 16% (Granick, 1938), 7.6-8.3% (Menke, 1940), 8% (Smith, 1941) and 4-6% (Bot, 1942), chlorophyll, on a chloroplast dry matter basis. Jagendorf and Wildman (1954) using an aqueous isolation technique have also reported 8-10% chlorophyll on a chloroplast dry weight basis for tobacco leaves. In the absence of any data for wheat or any monocot leaves the value reported here seems reasonable and approximates those of the other reports for spinach or tobacco leaves.

There was a very slight loss in the chlorophyll content of the chloroplasts of water-floated leaves, but an increase has been noted after BZ or KN treatments. The increase in the chlorophyll level would indicate some synthesis and would support the earlier contemplation of Wang et al (1960) that BZ either functions as or can take the place of a natural factor which is necessary in the biosynthesis of chlorophyll in wheat leaves. Perhaps KN affects the same way as BZ, but is less effective than BZ in chlorophyll biosynthesis of wheat leaves.

(b) Protein

There was practically no change (Fig. 8) in the protein levels of chloroplasts in water floated leaves but

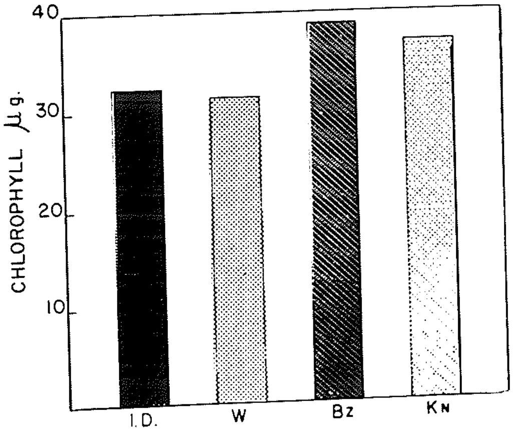
Drifts in the chlorophyll (Fig. 7), protein (Fig. 8) and nucleic acids (Fig. 9) levels in the chloroplasts.

Nucleic acids value in arbitrary units.

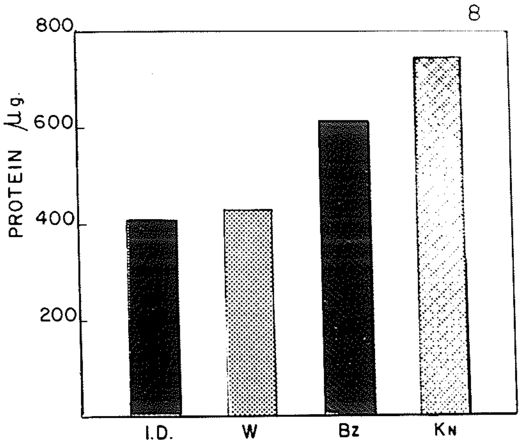
I.D. - Immediately detached leaves (10-days-old)

W - Water)
)
BZ - Benzimidazole) floated for 6 days under continuous
) light.
KN - Kinetin)

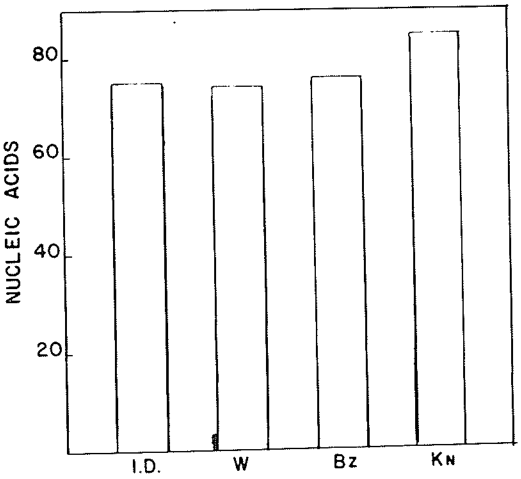
7


CHLOROPHYLL μg.
40
30
20
10
I.D.
W
Bz
Kn

8


PROTEIN μg.
800
600
400
200
I.D.
W
Bz
Kn

9


NUCLEIC ACIDS
80
60
40
20
I.D.
W
Bz
Kn

like chlorophyll there was an increase in chloroplast proteins in the BZ or KN treated leaves. Perhaps these two processes are related and the synthesis of chloroplast protein in leaves is to some extent interdependent with chlorophyll synthesis.

The value reported for immediately detached leaves as 40.6% protein on a chloroplast dry weight basis agrees closely with other values reported on the same basis (50%, Rabinowitch, 1945; 44.8%, Menke, 1940; 35-55%, Stocking, 1956). The total nitrogen content has been reported as 8% for non-aqueous chloroplasts (Stocking, 1959) and 8-10% for aqueous chloroplasts (Jagendorf et al, 1954) of tobacco leaves.

The chloroplast composition after BZ or KN treatment results in a net increase in protein levels. The maintenance of chloroplast protein in water floated leaves would indicate that cytoplasmic protein breaks down more rapidly than chloroplast protein which ultimately leads to a net protein loss from the whole leaves undergoing senescence. Increase in amounts of chlorophyll by BZ or KN treatment is associated with increase in chloroplast protein.

(c) Nucleic acids

The same trend as protein is observed for the nucleic acid levels (Fig. 9) expressed in arbitrary units. It would not be unreasonable to use chlorophyll content as an index of chloroplast development because the synthesis of chloroplast proteins is similarly affected. If nucleic acid is required for the light-dependent development of chloroplasts then any increase in the nucleic acid levels would reflect in the protein and chlorophyll content of chloroplasts.

The results of the present investigation suggest that leaves floated for 6 days in light are never damaged with respect to their chloroplast composition but when the leaves are floated on BZ or KN the chlorophyll, protein and nucleic acid levels in the chloroplasts usually surpass those of water floated leaves.

The role of BZ or KN in retarding senescence would appear to be closely linked to their ability to increase protein, nucleic acid, and chlorophyll contents of chloroplasts.

(d) Pyridine nucleotides

Green leaf chloroplasts have been recognized as possessing several biochemical abilities Because of the central importance of pyridine nucleotides in biological oxidation-reduction reactions, the concentrations of these coenzymes in the chloroplasts are of considerable interest. Changes in their levels or in the relative ratios of the oxidized and reduced forms, during senescence, would ultimately lead to altered enzyme activities in the chloroplasts. These altered enzymic patterns would modify the efforts of the cell and it would not be unreasonable to assume that such changes are clearly desirable so that a leaf cell may cope with a different spectrum of metabolic events as it approaches senescence.

Any effect of BZ or KN in modifying the levels or in the ratios of pyridine nucleotides would account for delaying senescence. The aim of this experiment was to study if any such changes actually occur in the chloroplasts and whether BZ or KN treatments regulate such changes (Yoshida, 1961).

(i) Pyridine nucleotide levels in chloroplasts

The levels of pyridine nucleotides (Table XII) in the

TABLE XII

Drifts in the pyridine nucleotide levels of chloroplasts

(Values as 10^{-12} moles per mg dry wt of chloroplasts)

Treatment	NAD	NADH	NAD + NADH	NADP	NADPH	NADP + NADPH	Total PN	As % of I.D.
I.D.	399	22.7	421.7	113	17.6	130.6	552.3	100
Water	54	40.8	94.8	trace	14.9	14.9	109.7	19
BZ	177	30.2	207.2	224	13.5	237.5	444.7	80
KN	104	28.7	132.7	79.6	20.1	99.7	232.4	42

Standard Deviation:

NAD and NADH ± 5%
NADP and NADPH ± 13%

chloroplasts of immediately detached leaves indicate higher values compared to those reported by Krogman (1958) and Das and Crane (1961) for spinach chloroplasts isolated in an aqueous medium. There is every possibility of some leaching during the isolation of chloroplasts in an aqueous medium and in the words of Krogman (1958) "In view of the many instances of loss of water-soluble material during the isolation of chloroplasts, this seemingly low value for pyridine nucleotide content need not reflect on the condition in the intact cell".

Krogman (1958) reported a recovery of 98% for added NAD, while Das and Crane (1961) reported only 70% recovery for added NADP in their respective assay methods. For spinach chloroplasts Das and Crane (1961) found 0.078 µg NADP/mg chlorophyll whereas the present observation of 2.6 µg NADP/mg chlorophyll in wheat leaf chloroplasts, is 30 times higher. Krogman's report (1958) for the combined value of NAD + NADP was 3.02 µg/mg chlorophyll for the same spinach chloroplasts, whereas the present report is 10.7 µg/mg chlorophyll which is about 3 times higher.

As no other published data are available on the pyridine nucleotide content in chloroplasts, this experiment suggests that the concentrations of these nucleotides are much higher than those reported by the previous workers. The higher levels reported here are primarily due to the isolation procedure in a non-aqueous medium and sensitive enzymatic cycling method for estimating very low amounts of these nucleotides.

The results suggest that in the chloroplasts there is a very high level of NAD compared to NADP but the level of NADH is slightly higher than NADPH. The NAD + NADH contents are 3 times higher than the NADP + NADPH contents. There is more of oxidized nucleotides than the reduced forms. The total pyridine nucleotides are 1.36 mμmoles/mg protein in the chloroplast and 17 mμmoles/mg chlorophyll. There is one molecule of each of NAD, NADH, NADP and NADPH per 90, 1666, 323, and 2000 molecules of chlorophyll respectively. Krogman (1958) found a maximal value of one molecule of oxidized pyridine nucleotide per 2500 molecules of chlorophyll in spinach. That NADPH/NADP ratios are different (3 times higher) from the NADH/NAD ratio indicates separate physiological roles played by the NADP and NAD enzyme systems in the chloroplasts.

(ii) Effect of BZ or KN on the pyridine nucleotide levels of chloroplasts

Table XII shows that the levels of NAD decreased in the chloroplasts from water floated leaves whereas the levels of NADH increased. By BZ or KN treatment, NAD decreased to a lesser extent but there was virtually no change in NADH. In the case of NADP there was an almost complete loss in the chloroplasts from water floated leaves and a two fold increase by BZ treatment. The total levels of pyridine nucleotides exhibited a drastic fall in the chloroplasts from water floated leaves whereas BZ or KN treatment maintained the levels of these nucleotides with a loss of only 20% or 58% respectively.

Levels of individual PNS are expressed on a protein basis (Table XIII) and levels of the total PNS on a chlorophyll and protein basis (Fig. 10) after treatment with BZ and KN. The same trend is observed when the values are expressed on a dry weight basis. Assuming most of the proteins as enzymes, perhaps senescent metabolism brings about a derangement in the basic or normal enzyme-coenzyme patterns of the chloroplasts and BZ treatment prevents these changes to a considerable extent.

TABLE XIII

Pyridine nucleotide levels in chloroplasts

(Values as 10^{-12} moles/mg protein)

Treatment	NAD	NADH	NAD + NADH	NADP	NADPH	NADP + NADPH	Total PNS
I.D.	982.75	55.91	1038.66	278.32	43.34	321.67	1360.34
water	126.76	95.77	222.53	trace	34.97	34.97	307.50
BZ	289.68	49.42	339.11	366.61	22.09	388.70	727.82
KN	138.85	38.32	177.16	106.27	26.83	133.11	310.28

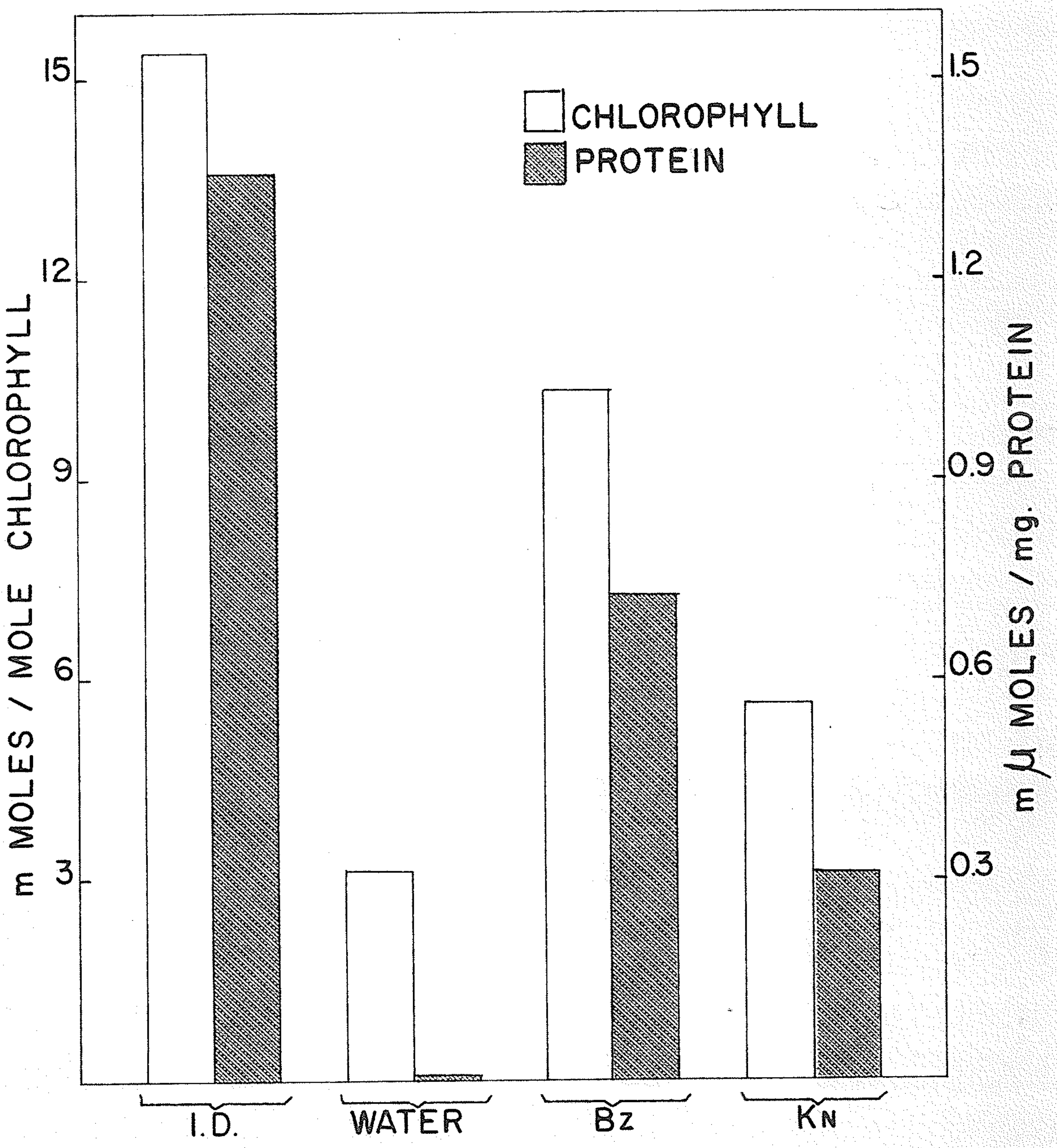


Fig. 10. Drifts in the total pyridine nucleotide levels in chloroplasts on a chlorophyll and protein basis.

The figures on the relative levels of oxidized and reduced nucleotides, as shown in Figs. 11, 12, 13 and 14 as ratios are very interesting. The NAD/NADH ratios decrease in the chloroplasts from water floated leaves but are maintained at a value of 1/3 or 1/5 of the original ratio by BZ or KN treatment respectively. Similarly the NADP/NADPH ratios are doubled by BZ treatment and halved by KN treatment. The ratios of NADP + NADPH/NAD + NADH which became half in the chloroplasts of water floated leaves, increased 3 times by BZ treatment and 2 times by KN treatment. The NAD + NADP/NADH + NADPH ratios were also lowered in the chloroplasts of water floated leaves but were maintained partially by BZ or KN treatment.

These results suggest that the photosynthetic ability will be higher in the chloroplasts of BZ or KN treated leaves, as deduced from high values of NADP content and NADP + NADPH/NAD + NADH ratio (Fig. 13).

To sum up, these results suggest that BZ or KN has a profound effect in maintaining the PN content of chloroplasts. If these values are taken into consideration and compared with the results reported previously for the whole

Drifts in the ratios of

NADP-to-NADPH (Fig. 11)

NAD-to-NADH (Fig. 12)

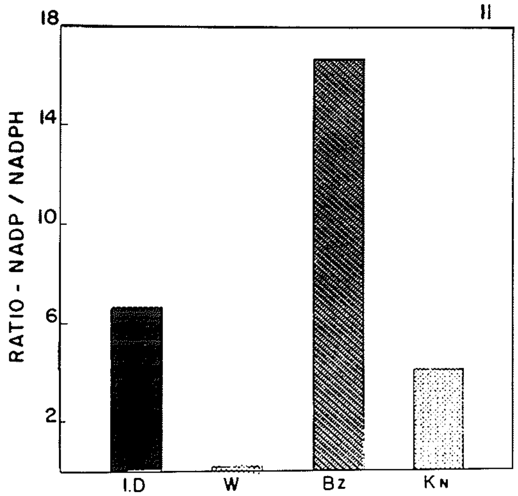
NADP + NADPH-to-NAD + NADH (Fig. 13)

and

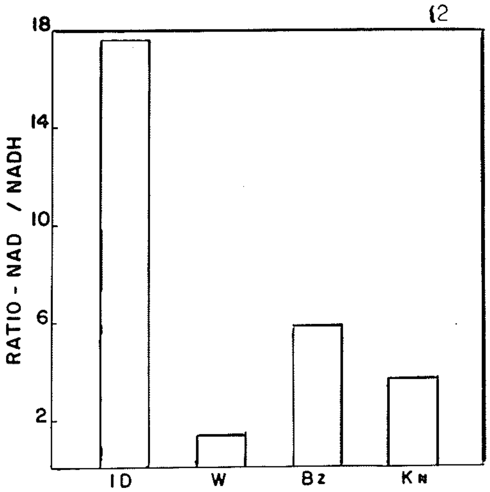
NAD + NADP-to-NADH + NADPH (Fig. 14)

in the chloroplasts.

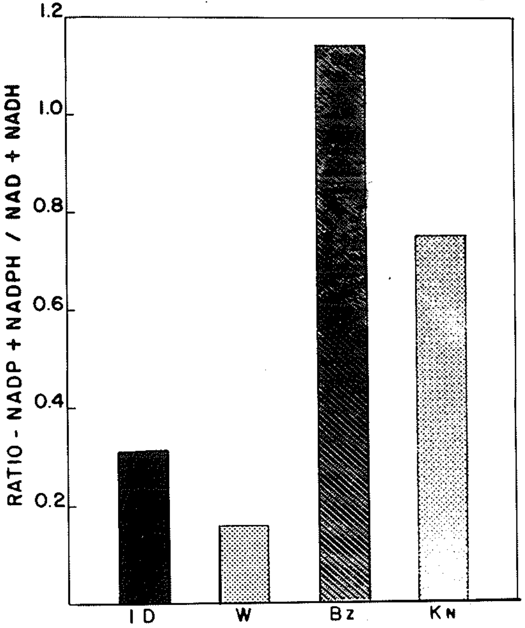
11


18
14
10
6
2
RATIO - NADP / NADPH
I.D
W
Bz
Kn

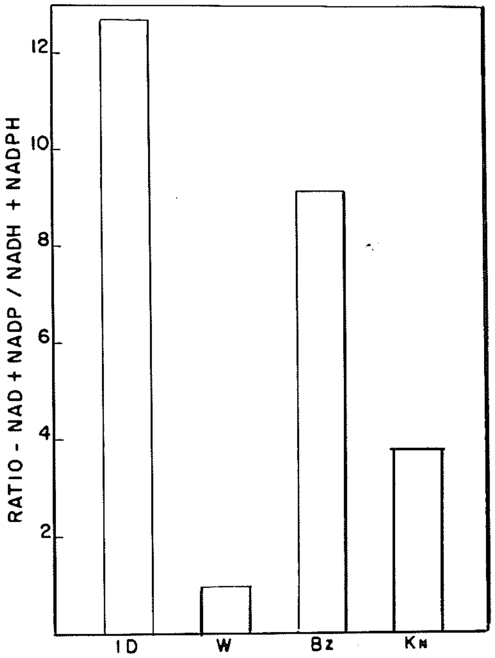
12


18
14
10
6
2
RATIO - NAD / NADH
I D
W
Bz
Kn

13


1.2
1.0
0.8
0.6
0.4
0.2
RATIO - NADP + NADPH / NAD + NADH
I D
W
Bz
Kn

14


12
10
8
6
4
2
RATIO - NAD + NADP / NADH + NADPH
I D
W
Bz
Kn

leaves, then one would assume that by BZ treatment perhaps the chloroplast integrity is maintained and by floating on water this integrity is lost with a leaching of the PNS from the chloroplast to the cytoplasm. However, the variability of the data indicate that the overall levels of pyridine nucleotides in tissue may be of no value and it is of significance only when the levels are determined in the various compartments of the cell.

(e) Adenine nucleotides

Adenine nucleotides are important cofactors in the regulation of cellular metabolism. The obvious importance of the problem whether senescence is dependent upon a continual energy supply, made it imperative to determine the changes of ATP level (or rather, of the ATP/ADP ratio) in the chloroplasts. Moreover, the changes in the pyridine nucleotide levels of chloroplasts suggested further changes in the adenine nucleotide levels could be related with it.

The changes in the level of ADP and ATP are presented in Fig. 15. In the chloroplasts of water floated leaves there was a reduction in the magnitude of the increase in ATP encountered in the chloroplasts of BZ or KN floated

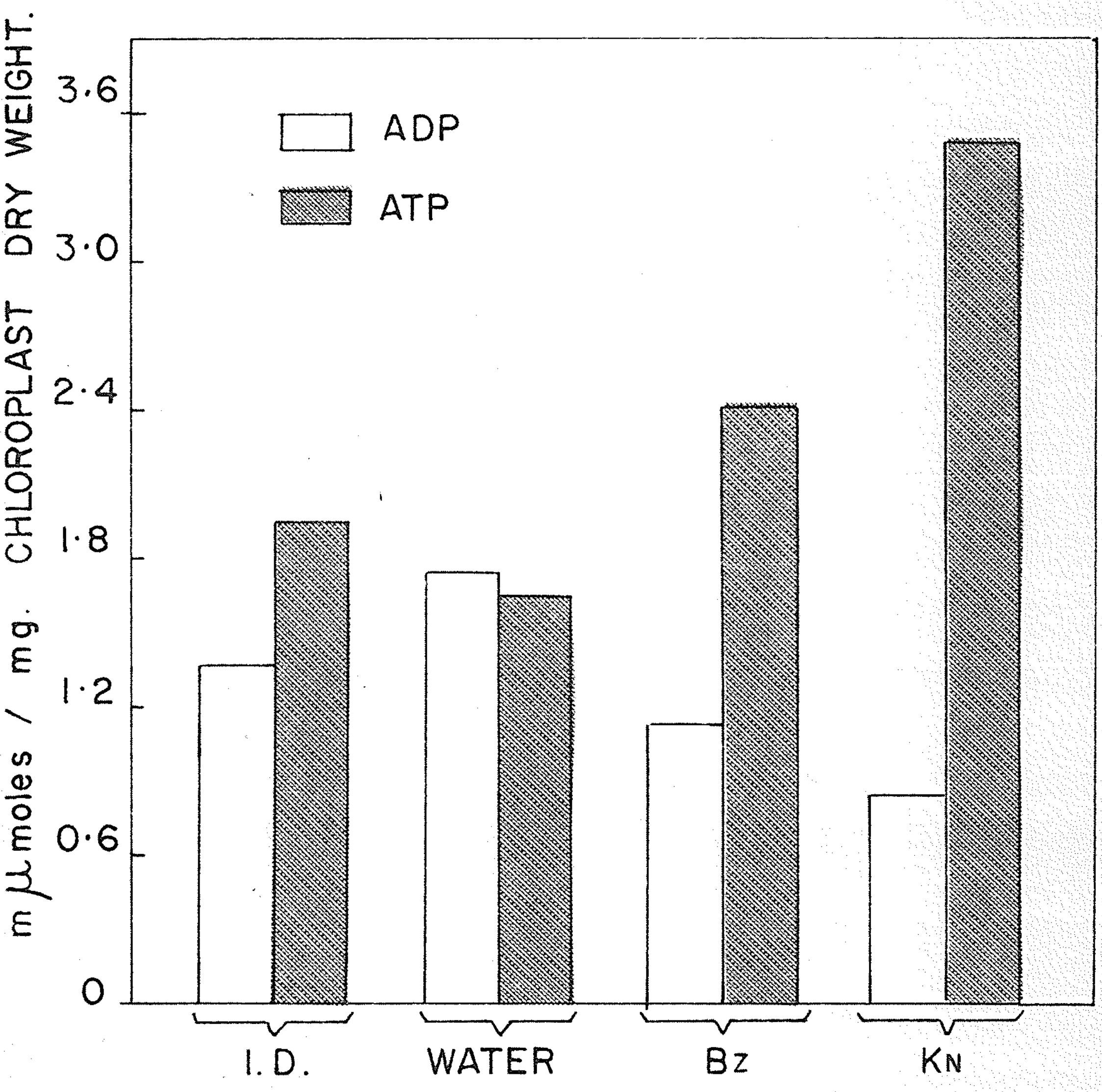


Fig. 15. Changes in the ADP and ATP levels in chloroplasts.

leaves. On the other hand, the ADP levels increased in the chloroplasts of water floated leaves but decreased in the chloroplasts from BZ or KN floated leaves. This may suggest that the assimilating power generated by the chloroplasts in the presence of light is maintained by either BZ or KN treatment.

Arnon et al (1958) have shown that the Hill reaction is accompanied by phosphorylation. In the absence of CO_2, illuminated chloroplast fragments, supplemented with an aqueous extract of chloroplasts, ADP, Pi and NADP carry out the reaction

$$2\ ADP + 2\ Pi + 2\ NADP + 4\ H_2O \longrightarrow 2\ ATP + O_2 + 2\ NADPH + 2\ H^+$$

Thus the excitation energy of chlorophyll can be used for the simultaneous reduction of NADP and phosphorylation of ADP. It is reasonable to speculate that this cyclic photophosphorylation is intimately concerned with senescent metabolism. In the chloroplasts from water floated leaves where NADP is lost ADP would accumulate and ATP level would decrease due to the failing ability of the chloroplasts to phosphorylate. In the chloroplasts from BZ or KN floated leaves, where NADP is present at a sufficient level, phosphorylation will continue and ATP will increase.

The ATP/ADP ratios decreased in the chloroplasts of water floated leaves but increased in the chloroplasts from BZ or KN floated leaves.

The action of BZ or KN on energy metabolism could be interpreted to involve increased synthesis of high energy phosphate bonds by maintaining a greater efficiency of the phosphorylating process.

V. DISCUSSION

In this laboratory two approaches have been made to elucidate the mechanism of action of BZ and KN in delaying the senescence of detached Khapli wheat leaves. In the first, Kapoor (1963) investigated the enzymic systems in wheat embryos which catalyze the synthesis of BMN and benzimidazole riboside or ribotide, which may be the active compound responsible for the benzimidazole effect, but this has not as yet been demonstrated.

The second approach made in the present study is concerned with the changes occurring during senescence in certain metabolites, enzymes and coenzymes, in particular the oxidized and reduced forms of the pyridine nucleotides. The first section deals with the changes occurring in nitrogen, nucleic acids and pyridine nucleotide levels of leaves floated on water and BZ; the second section with the changes occurring in the chloroplasts isolated from leaves floated on water, BZ and KN and the appendix deals with the changes involving carbohydrate, phosphorus, keto acids, glycolate and ascorbate metabolism of leaves floated on water and BZ.

It was noted that in leaves floated on water protein levels dropped with a concommitant increase in non-protein nitrogen but this drop was negligible in the leaves floated on BZ. These changes in protein levels were accompanied by changes in RNA levels. The stimulation of protein synthesis without a compensatory rise in the rate of protein degradation occurs in leaves floated on BZ.

A direct control of BZ on the oxidation-reduction state of the ascorbate system could trigger events leading to the final physiological effects observed in this investigation. The effect of BZ appears to be dependent upon, or associated with, shifts in the ascorbic acid system towards a reducing potential. Firstly, senescence may be correlated with a decrease in ascorbic acid concentration or in other words with a more oxidized potential of the ascorbate system. Secondly, the delay of senescence by BZ treatment results in a higher level in ascorbic acid concentration, or a more reduced potential of this system. There is little evidence as to how BZ causes alterations in the oxidation-reduction state of the cell, but BZ induced inhibition of AAO (Hillman, 1955) may account, at least in part, for the accumulation of ascorbic acid.

In detached leaves floated on water there was a progressive increase in DHA/AA ratio. A rise in this ratio would be expected to occur in a dying tissue (Sahai and Shaw, 1961). It seems that in the leaves floated on BZ, a shift occurs in the oxidation-reduction state through the NADP redox system. The DHA/AA and NADPH/NADP ratios in the BZ floated leaves suggest that the whole NADP-glutathione-ascorbate sequence is shifted towards a reduced potential. This should involve three possible mechanisms:

(a) an increased flux of electrons from respiratory substrates towards NADP

(b) a decrease in the activity of some NADPH-oxidizing systems

(c) a decrease in the activity of an ascorbate oxidizing system.

Yoshida (1961) demonstrated a relationship between NAD and senescence in the leaves of _Elodea_ _densa_. He came to the conclusion that NAD was the factor responsible for the initiation of chlorosis. Besides the effect of NAD on accelerating the senescence of Elodea leaves, Yamamoto (1963) has reported that the ratio of NADPH/NADP was higher in young tobacco leaves than in the older ones, and that this ratio decreased in every organ during senescence.

If NAD is responsible for chlorosis, BZ and KN are expected to counteract this effect. Unpublished data from this laboratory (Waygood and associates) indicate that BZ overcomes the accelerating effect of NAD on chlorosis of Elodea leaves. It was, therefore, considered of interest to investigate the levels of oxidized and reduced PNS in leaves floated on water, BZ and KN. A similar investigation for the isolated chloroplasts from these leaves was also undertaken.

There was only 20% loss in the level of total PN after a 5 day period in water floated leaves. The levels of NAD, NADP, and NADPH decreased but NADH increased with the progress of senescence under conditions of photosynthesis. BZ floated leaves showed no significant difference from the water floated leaves except that the NADPH/NADP ratios were lower in the water floated leaves than in the BZ floated ones.

In plants, NADP serves as the coenzyme for G-6-P dehydrogenase and isocitric acid dehydrogenase. G-6-P and isocitrate are considered the key substrates between the EMP and PP pathways. In attached leaves the EMP pathway will be functional because of the low values of NADP + NADPH/NAD + NADH. The increase in this ratio will be conducive to an increased intensity of the PP cycle. Such an increase has indeed been observed for detached leaves. However, the relative activities of the EMP and PP cycle will be influenced by the NADPH/NADP ratio. This ratio is rather low in the water floated leaves when compared to that of BZ floated leaves and thus the proportion of the PP cycle will be higher in the water floated leaves. The observed PN pattern indicates that the EMP sequence is of major importance in attached leaves and that the PP sequence plays an important role in detached leaves. Gibbs and Beevers (1951) demonstrated that in embryonic root tissue the EMP pathway is the dominant glucose degradatory mechanism. However, in progressively older tissue the importance of the EMP pathway declines and is gradually replaced by the PP pathway. Perhaps the same situation is encountered in the attached and detached leaves.

In wheat leaves, which possess enzymes of both the EMP and PP pathways (Waygood and Rohringer, 1963; Lunterstadt *et al*, 1962; Lunterstadt, 1963) the unresolved question concerns the internal influence(s) which apparently determines the fate of G-6-phosphate. In attached wheat leaves, which appear to respire hexose mainly *via* the EMP sequence, an internal influence may be an overall high NADPH/NADP ratio, despite the possibility of spatial separation and storage pools. Clearly, the possibility exists that as leaves are detached, a low ratio of NADPH/NADP combined with a high NADH/NAD ratio could shunt G-6-P into the PP pathway. BZ treatment prevents the increased activity of the PP pathway in the detached leaves by maintaining a high NADPH/NADP ratio.

The results presented also provide some evidence for the mechanism of PP shunt during senescence. Of the two alternative possibilities, (1) increased availability of cofactors (NADP) and (2) increased activation of the enzymes involved, the results would seem to favour the first possibility in the absence of any direct evidence for the second one.

Sacher (1959) has reported the effect of IAA in delaying senescence and Marre and Bianchetti (1961) have reported an increased ratio in NADPH/NADP by IAA treatment. The findings in the present investigation indicate that BZ like IAA delays senescence by maintaining a high NADPH/NADP ratio.

In order to elucidate further the mechanism of BZ and KN in delaying senescence, the quantitative changes occurring in the biochemical composition of chloroplasts were investigated. The chloroplasts were chosen since it is in these structures that the first symptoms of senescence occur, e.g., chlorosis.

Heber (1962) has ascertained that protein synthesis actually takes place within the chloroplasts and that the photosynthetic products are directly incorporated into protein. In the chloroplasts from BZ or KN floated leaves an increase in the levels of chlorophyll and protein has been observed. The increase in the chloroplast protein is presumably due to an accelerated synthesis and incorporation of photosynthetic products. Increase in the protein level of chloroplasts occurs directly by (a) maintaining the chloroplast structure and its capacity to synthesize protein, (b) maintaining high levels of ATP which is required in the first step of protein synthesis (Davie _et al_, 1956; Hoagland _et al_, 1956) (c) enhancing certain processes involved in protein synthesis or indirectly, by stimulating the synthesis of ribonucleic acid (Wollgiehn, 1961; Osborne, 1962; Sugiura _et al_, 1962).

Analysis of PN levels of chloroplasts indicated that the levels of NAD were decreased and the NADH levels were increased in the chloroplasts from leaves floated on water. NAD decrease was comparatively less in the chloroplasts from BZ and KN floated leaves. In the chloroplasts of the latter there was no significant change in the levels of NADH. There was a loss of NADP from the chloroplasts of water floated leaves but a two fold increase was observed in the NADP levels in the chloroplasts from BZ floated leaves. The total levels of both oxidized and reduced PNS exhibited a drastic fall in the chloroplasts from water floated leaves but in the chloroplasts from BZ or KN floated leaves the observed loss was only 20% and 58% respectively.

However, due to the lack of information concerning the activities of NAD- and NADP-linked dehydrogenases and reductases in the steady state the interpretation of metabolic equilibrium in the chloroplasts is unexplained at present. Nevertheless, the extremely low NADP levels in the chloroplasts from water floated leaves may be correlated with the low ATP content.

The levels of ATP were higher and ADP were lower in the chloroplasts from BZ or KN floated leaves than in those from the water floated ones. In the chloroplasts from water floated leaves ADP accumulation was due to loss of NADP while ATP decrease may be due to the failure of the chloroplasts to phosphorylate. In the chloroplasts of BZ and KN floated leaves where NADP is present at a sufficient level, phosphorylation will continue so that the level of ATP will obviously increase. It is assumed that in chlorophyll deficient or starving leaves, the ATP regenerating system is not sufficiently active. This assumption is strengthened by the actual demonstration of low levels of ATP in the chloroplasts of water floated leaves. Perhaps these light dependent responses are inhibited in the chloroplasts of water floated leaves and stimulated in the chloroplasts of BZ or KN floated leaves. Moreover, these responses may be intimately concerned with the structural integrity of the chloroplasts and protein synthesizing ability.

The mechanism of action of BZ could be explained if it could be shown by analogy with bacterial systems (Friedman and Harris, 1962) that it could be incorporated into factors resembling vitamins or coenzymes of group B_{12}. The existence of vitamin B_{12}-like factors has been reported for wheat plants (Fries, 1962). Besides its incorporation into a cofactor, BZ may also be considered as a weak auxin because of its structural resemblance to that of the indole ring. One of the compounds listed by Went (1949) as a "hemi-auxin" (a compound which increases auxin sensitivity of the split pea-epicotyl curvature test when used as a pretreatment) was benzimidazole-acetic acid, though benzimidazole was not tested. Perhaps BZ interferes with some metabolic system in which indole or an indole derivative plays some essential part and thus regulates the auxin economy.

An important finding in the present investigation is the demonstration that pyridine nucleotide and ATP levels are maintained in the chloroplasts by BZ or KN treatment. The two important enzymic reactions that are known to occur in the chloroplasts are

$$(1)\ \text{NAD} + \text{ATP} \xrightarrow{\text{NAD-kinase}} \text{NADP} + \text{ADP}$$

$$(2)\ \text{NADP} + \text{NADH} \xrightleftharpoons{\text{transhydrogenase}} \text{NAD} + \text{NADPH}$$

The presence of NAD-kinase has been noted in wheat leaf chloroplasts (Mishra and Waygood, unpublished). Keister _et al_ (1962) and Smillie (1963) have reported the presence of a transhydrogenase in chloroplasts. It appears that these reactions are intimately involved with the BZ or KN effect. Since reaction (1) is irreversible and NAD is a pool for NADP, it can be visualized that by transhydrogenation the reduced NAD reacts with NADP giving NAD again. The product, NADP, of the first reaction could then be utilized in photosynthesis and also act as a pool for further synthesis of NADP. The maintenance of NAD and ATP in the chloroplasts of BZ and KN floated leaves would favour the first reaction but the decrease in the level of both NAD and ATP from the chloroplasts of water floated leaves would obviously curtail NADP formation. The alterations in the level of NADP would result in a change in the phosphorylative capacity of the chloroplast, at present considered the key mechanism for energy conservation.

Although this study has not pinpointed the actual site(s) of action of BZ or KN, their significant effects on the levels of reduced and oxidized forms of pyridine nucleotides, especially in the chloroplasts, suggest that these are the structures in which both BZ and KN exert their primary effect. This may be a direct effect on membrane structure, which would indirectly affect the synthesis of ATP, NADP and chlorophyll. In this regard it is interesting to note that recently Devi and Sarkar (1963) have shown that cobalt _per se_ can activate, the incorporation of leucine-C^{14} into s-RNA and proteins, in rat livers, in the absence of ATP. They have concluded that this cobalt activated amino acid-incorporating system is an enzyme catalyzed reaction and cobalt has some stimulatory effect on protein biosynthesis in mammalian tissues.

BZ is known to chelate cobalt (Skraup, 1919; Hofmann,

1953) and if the cobalt activation phenomenon is real and occurs in plants then BZ may assist in the transport of cobalt across biological membranes. Studies are underway in this laboratory to ascertain this point.

VI. SUMMARY

A biochemical investigation involving the changes in the composition of whole leaves and chloroplasts senescing on water, benzimidazole or kinetin has shown that

(1) The increase in soluble nitrogen and decrease in insoluble nitrogen fractions of the leaves floated on water was prevented by benzimidazole treatment. Benzimidazole also had a stabilizing effect on the protein levels of detached leaves.

(2) The levels of RNA, DNA, RNA-to-DNA ratio were lower in water floated leaves but were higher in the benzimidazole floated ones.

(3) There was very little loss of pyridine nucleotides during senescence and kinetin or benzimidazole + kinetin treatment increased the pyridine nucleotide levels of leaves. The NADPH/NADP ratio was higher at the end of light periods by benzimidazole treatment.

(4) The NAD + NADP/NADH + NADH ratio gradually increased during senescence, when measured at the end of dark periods but was relatively low on the benzimidazole floated leaves.

(5) Chloroplasts from benzimidazole or kinetin floated leaves exhibited an increase in the levels of chlorophyll, protein and nucleic acid.

(6) In the chloroplasts from water floated leaves, the levels of NAD decreased but NADH increased. With benzimidazole or kinetin treatment NAD decreased to a lesser extent but virtually there was no change in NADH.

(7) The NADP levels were immeasurably low in the chloroplasts from water floated leaves but there was a two fold increase with benzimidazole treatment.

(8) The total levels of pyridine nucleotides exhibited a drastic fall in the chloroplasts from water floated leaves, whereas benzimidazole and kinetin treatment maintained the levels of these nucleotides with a loss of only 20% and 58% respectively.

(9) The ratios of NADP + NADPH/NAD + NADH decreased to half their value in the chloroplasts of water floated leaves after a 6-day period of detachment but increased 3 fold with benzimidazole and 2 fold with kinetin treatment.

(10) In the chloroplasts of water floated leaves the levels of ADP were higher and ATP were lower. On the

other hand, the ADP level was lower and ATP levels higher in the chloroplasts from benzimidazole or kinetin floated leaves.

(11) A procedure for the non-aqueous isolation of chloroplasts from wheat leaves is described.

(12) The enzymatic cycling method of Lowry *et al* (1961) for estimating very low amounts (10^{-15} moles) of pyridine nucleotides is modified and adapted to their measurement in plant extracts.

(13) The results of experiments in establishing the effects of benzimidazole on carbohydrate, phosphorus,keto acids, glycolate and ascorbate metabolism of detached leaves, undergoing senescence are presented in an appendix.

(14) The possible significance of these results has been discussed.

VII. APPENDIX

Experimental Results and Discussion

(a) Carbohydrates

That carbohydrates occupy a key role in metabolism as a source of energy is well recognized. Alcohol soluble carbohydrates are increased considerably by treatments which induce susceptibility in wheat leaves (Forsyth *et al*, 1958). The experiment reported here was conducted in an attempt to elucidate the changes in the carbohydrates in leaves undergoing senescence and the effect of BZ on senescent metabolism and on the maintenance of rust reaction in detached leaves.

Alcohol-soluble carbohydrates only have been determined and individual carbohydrates were not determined separately. The carbohydrate levels are expressed as glucose equivalents. The leaves were floated either in continuous light or darkness following detachment.

The curves in figure 16 show the levels of soluble carbohydrates in water and BZ floated leaves in light and dark. There was always a higher level of soluble carbo-

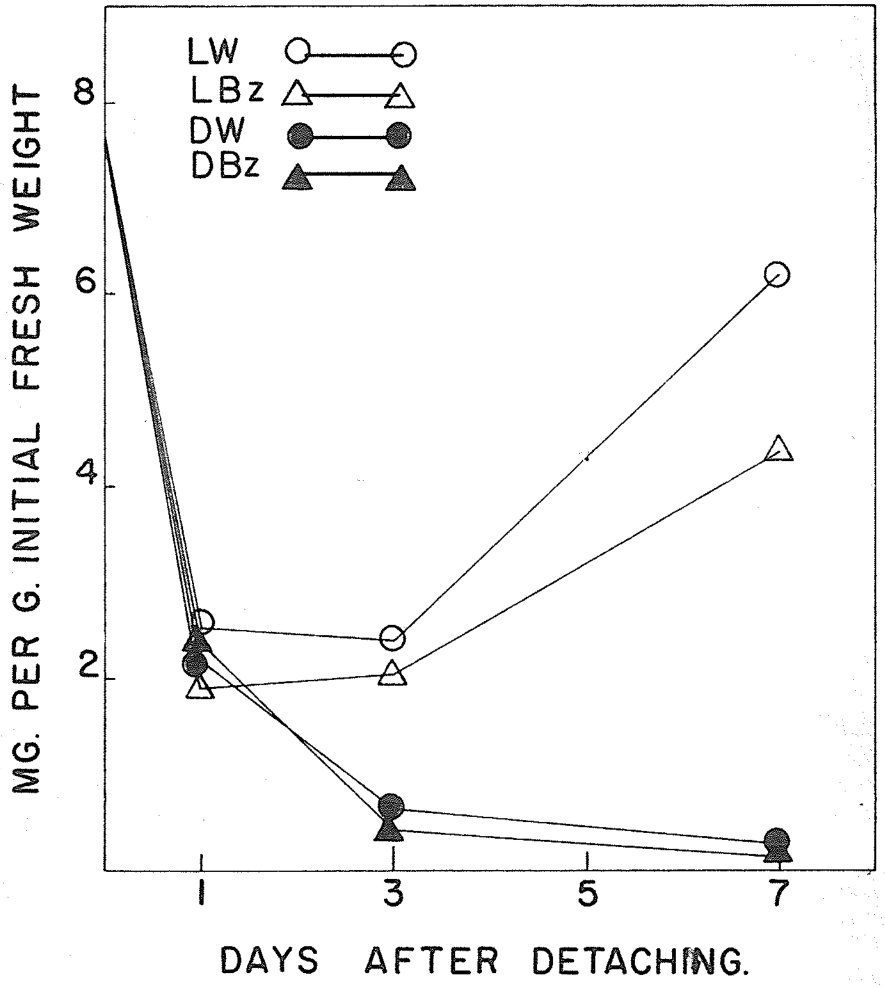


Fig. 16. Drifts in the levels of total alcohol-soluble carbohydrates.
The leaves were 7-days-old at the time of detachment.

hydrates in water floated leaves. In general, the curves in light exhibited two well-marked phases. (1) an initial relatively rapid decrease with time and (2) a phase during which there was some increase with time. The low level of soluble carbohydrates by BZ treatment indicates increased utilization of carbon (assuming sugar as a source of energy and structural material in the elaboration of protoplasm) presumably in synthetic reactions in the BZ floated leaves.

The present results though qualitatively corroborating the findings of Samborski et al (1958) are yet quantitatively different. Samborski et al reported a value of 36 mg in water floated and 20 mg in BZ floated wheat leaves (per g fresh wt) var. Khapli after a 7-day period of detachment, whereas the present report indicates 62 mg for water floated and 43 mg for BZ floated leaves after the same period of detachment. However, this value is in close agreement with the reports of the same author (1961) for Little Club wheat.

(b) Phosphorus

As phosphorus plays an important role in living tissue and the effect of detachment on phosphate metabolism has not been reported, the present investigation was carried out in order to see if there was any change at all between BZ and water floated leaves. The leaves used in this experiment were 7-days-old at the time of detachment and were cultured in 16 hr. light followed by an 8 hr. dark period in a 24 hr. day cycle, for the light cultured leaves and in total darkness for the dark cultured leaves.

Table XIV reports the changes in phosphate levels after 1 to 7 days following excision. Inorganic (Pi), organic (Po) and total (Pt) phosphorus decreased steadily with the progress of senescence BZ treatment lowered the levels of all these phosphate fractions. Comparing light and dark treatments, the latter enhanced the level of Pi. The Pi/Po ratio was higher in BZ treated leaves. Excision resulted in a decrease in the Pt content. The levels of Pi, Po and Pi/Po ratios decrease with the progress of senescence. BZ treatment maintained a low level of Pt, Pi and Po but a high level in the Pi/Po ratio compared to water floated leaves. BZ treatment also caused a small but consistent increase in RNA-P and DNA-P. These results should be taken into consideration bearing in mind the limitations of the

TABLE XIV

Changes in the levels of phosphate fractions (inorganic, organic, total, RNA-P and DNA-P) in detached wheat leaves.

Leaves were 7-days-old at the time of detachment.
(mg per g initial fresh weight of leaves)

Days after detachment	I.D.	Light water	Light benzimidazole	Dark water	Dark benzimidazole
		inorganic P (Pi)			
0	5.42	-	-	-	-
1	-	5.42	4.67	7.17	6.43
3	-	5.23	4.08	5.83	5.40
7	-	2.80	2.33	2.92	2.50
		organic P (Po)			
0	2.75	-	-	-	-
1	-	2.92	2.50	1.00	1.15
3	-	2.35	1.62	2.00	1.60
7	-	2.03	1.17	2.78	1.46
		total P (Pt)			
0	8.17	-	-	-	-
1	-	8.34	7.17	8.17	7.58
3	-	7.58	5.70	7.83	7.00
7	-	4.83	3.50	5.70	3.96

Table XIV cont'd

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
			RNA-P		
0	0.875	-	-	-	-
1	-	0.510	0.643	0.350	0.650
3	-	0.321	0.321	0.175	0.247
7	-	0.117	0.192	0.058	0.117
			DNA-P		
0	1.040	-	-	-	-
1	-	0.500	0.847	0.510	0.643
3	-	0.495	0.903	0.453	0.523
7	-	0.292	0.378	0.221	0.308

methods used. But as observed in previous experiments (Table III) BZ treatment maintained the level of RNA compared to water floated leaves.

The data presented in Table XIV indicate a leakage of phosphorus compounds to the floating medium. This would account for such a large decrease in the Pi and Pt content after a 7-day period of detachment. However, no attempt was made to estimate the phosphorus level in the floating medium. The study shows no major differences in the phosphate metabolism by BZ treatment.

(c) Keto acids

Since the keto acids, particularly glyoxylic, pyruvic and α-ketoglutaric acids are well known intermediates in carbohydrate, organic acid and amino acid metabolism in higher plants and these acids are also involved in both anabolic and catabolic pathways, a study of the effect of BZ on the levels of these acids was undertaken.

The excised leaves used in this experiment were 8-days old at the time of detachment and were kept either in a 16 hr. light period followed by an 8 hr. dark period for the light cultured leaves or in complete darkness.

The levels of glyoxylate (Fig. 17), pyruvate (Fig. 18) and α-ketoglutarate (Fig. 19) are the mean of duplicate experiments.

(i) Glyoxylic acid

Glyoxylate reached a very high level in both light and dark during the senescent metabolism of the detached leaves. Accumulation was greater in light than in dark treated leaves. BZ treatment maintained the level of glyoxylate low and further in the presence of light the glyoxylate content was even lower than in immediately detached leaves. BZ appears to have no significant effect in the dark. The level of glyoxylate rose more or less steadily to a very high value 3 to 7 days after detachment and this behavior can be contrasted with that in aging wheat leaves, where a descending level of glyoxylate with time has been reported by Krupka and Towers (1958). The present experiment supports the finding of Krupka and Towers (1958) on the behavior of glyoxylate levels in excised Thatcher wheat leaves, during starvation in the dark, where the same trend as observed here was reported.

(ii) Pyruvic acid

The changes in the levels of pyruvate, the only substrate common to the EMP pathway and the TCA cycle, presented in Fig. 18, exhibit the same trend as observed for glyoxylate. BZ also maintained the level of pyruvate low only in light. Light had a significant effect on the accumulation of pyruvate in the excised leaves.

The increased level of pyruvic acid in the water floated leaves in light as compared with BZ floated leaves indicates the operation of some pathway for the dissimilation of glucose to pyruvate other than the EMP pathway of glycolysis. As shown and discussed in previous experiments on the pyridine nucleotide levels of whole leaves, there is evidence for the participation of the Pentose-Phosphate pathway in the water floated leaves and BZ treatment preventing the increased operation of the PP pathway in detached leaves by maintaining a high NADPH/NADP ratio.

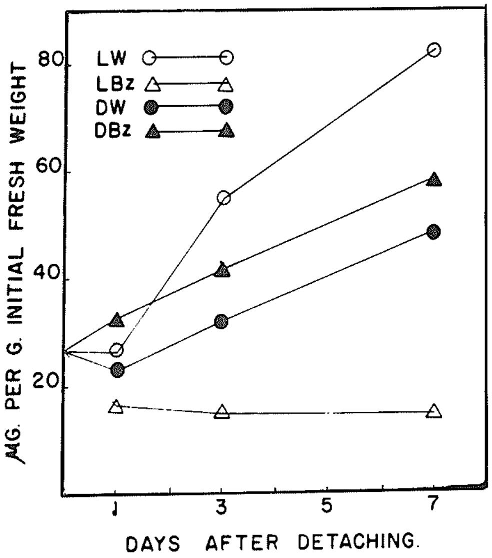
(iii) α-Ketoglutaric acid

The curves in Fig. 19 indicate the effect of BZ in maintaining the level of α-ketoglutarate low. The behavior of this acid was opposite to that of pyruvate and glyoxylate.

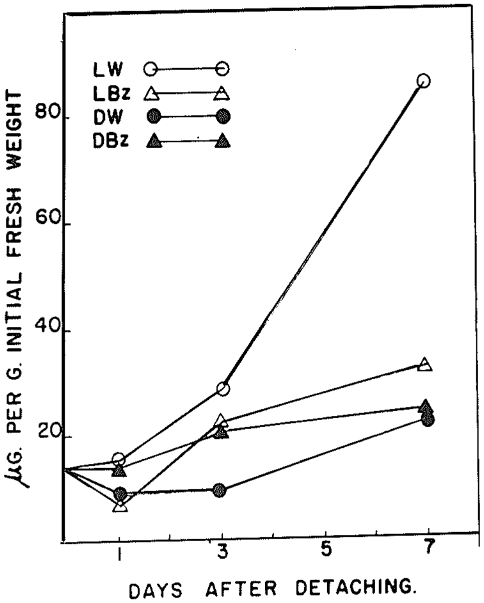
Drifts in the levels of glyoxylic (Fig. 17), pyruvic (Fig. 18) and α-ketoglutaric acid (Fig. 19).

Leaves were 8-days-old at the time of detachment.

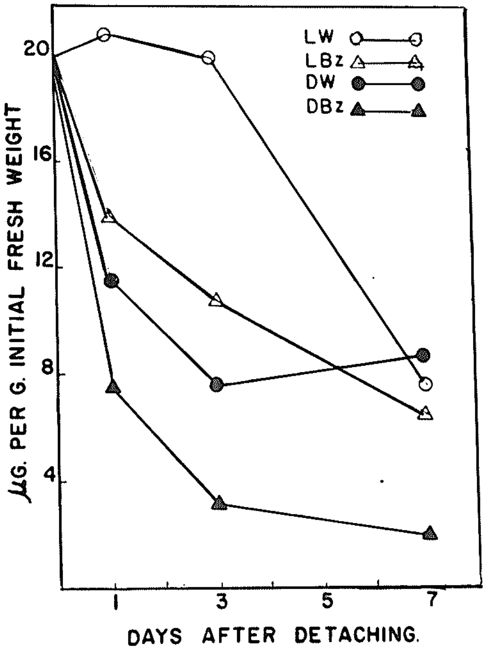
17


LW
LBz
DW
DBz
80
60
40
20
µG. PER G. INITIAL FRESH WEIGHT
1
3
5
7
DAYS AFTER DETACHING.

18


LW
LBz
DW
DBz
80
60
40
20
µG. PER G. INITIAL FRESH WEIGHT
1
3
5
7
DAYS AFTER DETACHING.

19


LW
LBz
DW
DBz
20
16
12
8
4
µG. PER G. INITIAL FRESH WEIGHT
1
3
5
7
DAYS AFTER DETACHING.

The level of α-ketoglutarate decreased with time after excision. In dark the level of this acid was lower than in light. Krupka and Towers (1958) found that the α-ketoglutarate content of aging wheat leaves declined steadily, but there was no significant change in the excised leaves during starvation in the dark.

(d) Glycolate metabolism

It is suggested that glycolic acid oxidase (GAO) can function in terminal respiration in green plants and in oxygen uptake of leaf tissue (Zelitch, 1958). Recently Wang and Waygood (1962) suggested a metabolic pathway of glyoxylate-serine metabolism in wheat leaves. Since the synthesis of glycine during photosynthesis is thought to involve the oxidation of glycolate to glyoxylate followed by amination or transamination an investigation of the role of the key enzyme GAO in detached leaves was undertaken. The glycolate-glyoxylate cycle in wheat leaves following detachment and the effect of BZ on this metabolic pathway has been particularly studied.

Throughout these experiments the leaves were floated either in continuous light or darkness.

Glycolate oxidation *in vivo*

Preliminary experiments were carried out by measuring the capacity of leaf segments of floated leaves to oxidize glycolate *in vivo*. This capacity gradually decreased with the progress of detachment period. Leaves floated in light had a higher rate of oxidizing glycolate than the corresponding leaves in the dark. BZ floated leaves showed a decreased rate of glycolate oxidation *in vivo*.

(i) Glycolic acid oxidase (GAO) activity

The *in vivo* experiment of glycolate oxidation warranted a further investigation of the enzyme system in cell free extracts. The data are presented in Table XV. The enzyme assays were done in duplicate. The activity of GAO increased after a 4-day period of detachment. There was a decrease in enzyme activity after detachment, but it gradually increased with the time of detachment. However, there was no significant change in the total enzyme content (calculated as total units per gram initial fresh weight of leaves) by the various treatments. However, the specific activity of GAO was lower in BZ floated leaves. Hillman (1955) also found a decrease in GAO activity in BZ treated

TABLE XV

Effect of BZ on GAO activity measured in homogenates of wheat leaves undergoing senescence.

Leaves were 11-days-old at the time of detachment.

Days after detachment	water			benzimidazole		
	units activity*/ g fr wt	mg protein/ g fr wt	units activity/ mg protein	units activity/ g fr wt	mg protein/ g fr wt	units activity/ mg protein
			LIGHT			
0	297	24.3	12.4	297	24.3	12.4
2	249	23.0	10.8	216	26.2	8.3
3	259	20.5	12.6	258	27.0	9.5
4	276	21.9	12.6	293	27.2	10.8
			DARK			
0	297	24.3	12.4	297	24.3	12.4
2	182	13.8	13.2	219	15.1	13.9
3	204	12.5	16.3	230	15.9	14.5
4	239	10.0	23.9	245	13.4	18.3

*One unit of activity is given as that amount of enzyme which was required to uptake 1 μlo$_2$ per 10 minutes. Values are mean of duplicate enzyme assays.

fronds of Lemna minor.

The decrease in specific activity of GAO in BZ floated leaves may be related to the enzyme-coenzyme relationships. A deficiency of FMN, the coenzyme of GAO, may account for the decreased enzymic activity. It has been demonstrated by the work of Alivisatos et al (1962) and Kapoor (1963) that BZ reacts with NAD replacing the nicotinamide moiety of the pyridine nucleotide molecule. By analogy it was thought that BZ would also react with FMN and FAD and replace the isoalloxazine ring of the molecule yielding BMN and BAD respectively. If such a reaction is possible in vivo then a decrease in the GAO activity is possible in BZ floated leaves presumably due to loss of FMN.

(ii) Flavin nucleotides

In an attempt to confirm the idea mentioned above the levels of FMN and FAD were determined in the floated leaves. The results given in Tables XVI,XVII and XVIII are mean of triplicate assays.

The quantity of total flavins (FMN and FAD) began to decrease after detachment. In the BZ floated leaves

TABLE XVI

Concentration of FMN in detached wheat leaves.

Leaves were 9-days-old at the time of detachment.
(µg per g initial fresh wt of leaves)

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
0	0.65	-	-	-	-
1	-	0.90	0.84	1.02	0.93
3	-	0.71	0.69	0.72	0.53
5	-	0.65	0.58	0.38	0.35

TABLE XVII

Concentration of FAD in detached wheat leaves.

Leaves were 9-days-old at the time of detachment.
(µg per g initial fresh wt of leaves)

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
0	0.49	-	-	-	-
1	-	0.35	0.42	0.08	0.08
3	-	0.34	0.28	0.13	0.20
5	-	0.32	0.23	0.36	0.21

TABLE XVIII

Concentration of total flavins (FMN and FAD) in detached wheat leaves.

Leaves were 9-days-old at the time of detachment.
(μg per g initial fresh wt of leaves)

Days after detachment	I.D.	Light		Dark	
		water	benzimidazole	water	benzimidazole
0	1.14	-	-	-	-
1	-	1.25	1.26	1.10	1.01
3	-	1.05	0.97	0.85	0.73
5	-	0.97	0.81	0.74	0.56

there were lower levels of FMN and FAD compared to water floated leaves. Though the values may not be of any significant difference yet the trend is important. The levels of FMN were low in 3-5 days after detachment in the dark. The interesting phenomenon was the sharp drop in the FAD level immediately following detachment in the dark which, however, gradually increased with time. The flavin content of wheat leaves presented here closely approximates to that reported by Mer (1955).

If the results obtained _in vitro_ actually correspond to the conditions _in vivo_ one reasonable expectation would be the accumulation of the substrate glycolic acid in the BZ floated leaves, provided the glycolate is not forced into other metabolic pathways. To verify this assumption a quantitative analysis of glycolic acid was undertaken.

(iii) Glycolic acid

Table XIX summarizes the drifts in glycolate levels in detached leaves in continuous light and darkness. The leaves used were 7-days-old at the time of detachment and concentrations of glycolic acid are not calculated back to 100% recovery. Actually glycolate accumulated in the BZ

TABLE XIX

Concentration of glycolic acid in detached wheat leaves.

Leaves were 7-days-old at the time of detachment.
(µmoles per g initial fresh weight of leaves*)

		Light		Dark	
Days after detachment	I.D.	water	benzimidazole	water	benzimidazole
Experiment #1					
0	2.53	-	-	-	-
1	-	2.00	2.00	1.00	2.00
2	-	1.26	2.00	0.53	1.47
5	-	1.05	2.53	0.53	-
Experiment #2					
0	2.63	-	-	-	-
1	-	2.13	2.26	1.32	2.42
2	-	1.63	2.52	0.63	1.63
5	-	1.53	3.26	0.53	0.76

*Recovery of added glycolate was ca. 88%. Values reported are not calculated back to 100% recovery values.

floated leaves under continuous illumination. The increased content of glycolate in light would indicate some synthesis, rather than the inhibition of glycolate oxidation by BZ treatment. On the other hand, glycolate disappeared rapidly in the darkened leaves. The effect of BZ in maintaining a high level of glycolate is significant under continuous illumination. This result would be consistent with the observation for glyoxylate levels (Fig. 17) reported earlier.

(e) Ascorbate metabolism

In an attempt to explain the action of BZ with certain oxidation-reduction systems of the cell, the present investigation was carried out to estimate the concentration of ascorbic acid (AA) in detached leaves. It seems quite possible that shifts in the oxidation-reduction state of the cell, may play an important role in the metabolism of detached leaves. The role of ascorbic acid in (a) an electron transferring system during photosynthesis and (b) respiration, (Waygood, 1950) has been recently reviewed (Mapson, 1962) and its role in the physiology of host-parasite relations has been reported by Sahai and Shaw (1961).

The leaves used in this experiment were cultured either in 16 hr. light followed by 8 hr. darkness in a 24 hr. day cycle or in complete darkness.

(i) Ascorbic acid (AA)

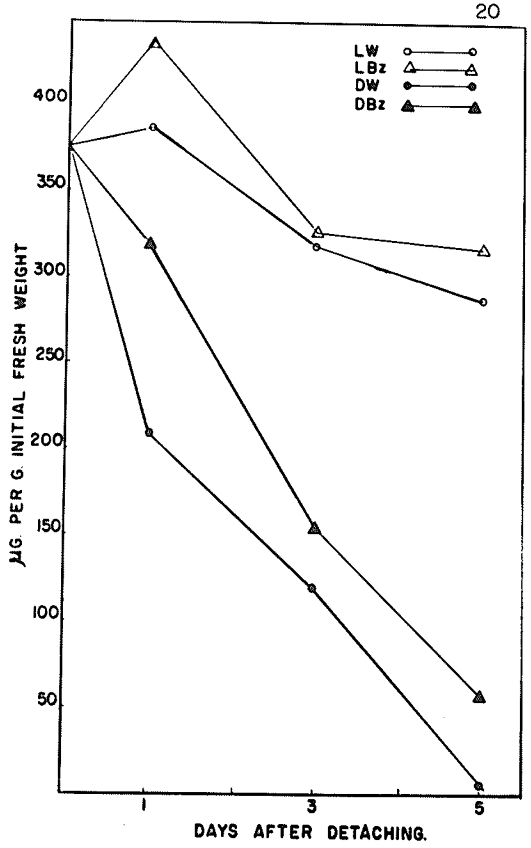
The changes in the levels of ascorbic acid are shown in Fig. 20. The values reported for Khapli wheat leaves are in close agreement with the values reported by Sahai and Shaw (1961), but differ markedly from those reported by Kiraly and Farkas (1962) for the same variety of wheat leaves.

The concentration of ascorbic acid was always higher in the BZ floated leaves. The disappearance of ascorbic acid in darkened leaves after a 5-day period of detachment is most probably due to carbohydrate depletion. Aberg (1949) also found a decrease in ascorbate levels in the detached darkened leaves of parsley, spinach and lettuce. The slight change in ascorbate levels under illumination is most probably due to some light-conditioned synthesis, which is probably connected with the assimilation of carbon dioxide (Aberg, 1945). A recent report (Constable, 1963) suggests the location of ascorbate in the chloroplasts and its

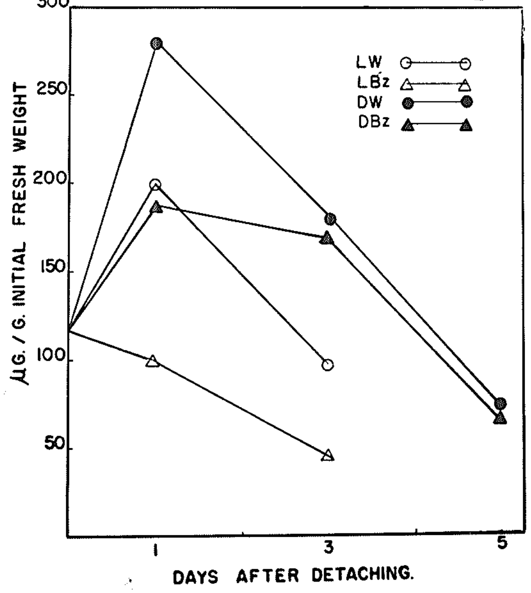
Drifts in the levels of ascorbic acid (Fig. 20), dehydroascorbic acid (Fig. 21) and ratios of dehydroascorbic-to-ascorbic acid (Fig. 22).

Leaves were 10-days-old at the time of detachment.

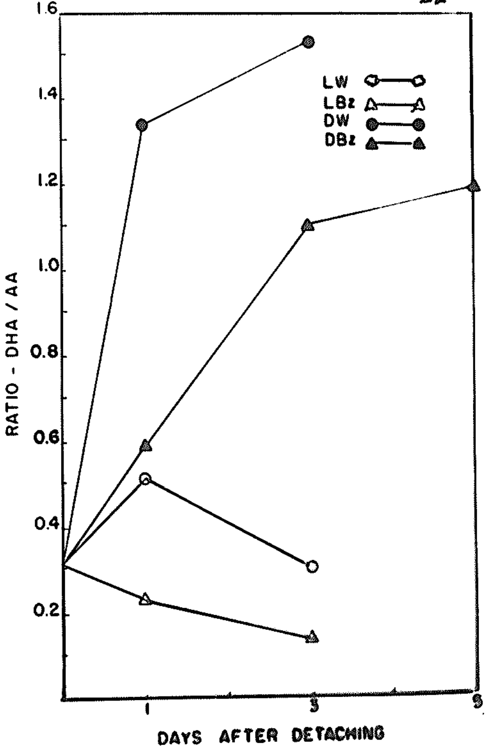
20


LW
LBz
DW
DBz
μG. PER G. INITIAL FRESH WEIGHT
400
350
300
250
200
150
100
50
1
3
5
DAYS AFTER DETACHING.

21


LW
LBz
DW
DBz
μG. / G. INITIAL FRESH WEIGHT
300
250
200
150
100
50
1
3
5
DAYS AFTER DETACHING.

22


LW
LBz
DW
DBz
RATIO - DHA / AA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
3
5
DAYS AFTER DETACHING

preservation may be a factor in the maintenance of chlorophyll in the BZ floated leaves.

(ii) Dehydroascorbic acid (DHA)

Since the ratio of reduced to oxidized AA is a critical factor in metabolism, analysis was made for the DHA content of the detached leaves.

Fig. 21 shows the drifts in the level of DHA. With the technique used it was not possible to estimate the very low amounts of DHA in 5-day floated leaves in light. Where BZ induced an increased level in AA the concentration of DHA was consistently lower. On the other hand, the level of DHA was consistently higher in water floated leaves where the level of AA was lower. There was no significant change in the level of DHA by BZ treatment in the dark.

(iii) Ascorbic acid oxidase (AAO)

Preliminary experiments were done to check on possible changes in AAO activity in the homogenates during this period. The changes in the AAO activity are presented in Table XX but the autooxidation rate with boiled homogenate has not been corrected for the values reported as an attempt has been made to compare the enzyme activity in the water

TABLE XX

Effect of BZ on the AAO activity measured in homogenates of wheat leaves undergoing senescence

Leaves were 10-days-old at the time of detachment.

Days after detachment	water			benzimidazole		
	units activity*/ g fr wt	mg protein/ g fr wt	specific activity units activity/ mg protein	units activity/ g fr wt	mg protein g fr wt	specific activity units/mg protein
			LIGHT			
0	311	25	12.5	311	25	12.5
1	385	26	14.8	367	26	14.1
3	364	26	14.0	370	29	12.8
5	306	20	15.3	294	26	11.3
			DARK			
0	311	25	12.5	311	25	12.5
1	400	22	18.2	338	23	14.7
3	368	20	18.4	316	23	13.8
5	295	15	19.6	228	20	11.4

*One unit of activity is that amount of enzyme which was required to take 1 $\mu l O_2$ per 30 minutes under the conditions specified. Values are the mean of duplicate assays.

and benzimidazole floated leaves. Treatment with BZ resulted in a reduction in the specific activity of AAO in the leaf homogenate. The reduction in the activity was correlated with the high level of AA in BZ floated leaves. Detachment induced an increase in the specific activity of AAO. Little or no significant change was observed in the total enzyme units present in leaves, calculated on a gram fresh weight basis. The increased specific activity in AAO during senescence may be correlated with the disappearance of AA from the leaves with time.

(iv) DHA/AA ratio

The ratio (DHA/AA) presented as curves in Fig. 22 increased rapidly in darkened leaves. The ratio was invariably high in water floated leaves. It is, of course, evident that a rise in oxidation-reduction potential would be expected in a senescing tissue and a rise in this potential would ultimately accompany death of the leaves. That BZ maintains the ratio low is an important point worth consideration in elucidating the effect of BZ in maintaining the normal physiology of detached leaves.

(v) Phenolics

Phenolics occupy an important place in the aromatic metabolism and have also been implicated in rust resistance (Kargopolova, 1937; Newton and Anderson, 1929; Kiraly and Farkas, 1962). The changes in the levels of phenolics (expressed as chlorogenic acid equivalents) summarized in Table XXI are the mean of triplicate assays. There seems to be very little change in the levels of free phenolics in detached leaves. BZ maintained the levels of phenolics high only in light. There was no effect of BZ in the dark. The decrease in the level of phenols in water floated leaves corroborates well the idea of glucoside formation (Hutchinson *et al*, 1958), due to increase in the levels of soluble carbohydrates (Fig. 16) in the water floated leaves.

(f) Changes in alkaline inorganic pyrophosphatase activity

The significant increase in the NAD and NADP levels in the BZ, kinetin (KN) and BZ + KN floated leaves, as reported earlier, warranted a further investigation of the enzymic aspects of such changes. Kornberg (1950) reported the enzymatic mechanism by which NAD could be synthesized from NMN and ATP by the reaction

$$\text{NMN} + \text{ATP} \longrightarrow \text{NAD} + \text{P-P}$$

TABLE XXI

Concentration of total phenolic substances in detached wheat leaves.

Leaves were 8-days-old at the time of detachment. (mg chlorogenic acid equivalent per g initial fresh weight of leaves)

Days after detachment	I.D.	Light water	Light benzimidazole	Dark water	Dark benzimidazole
0	7.04	-	-	-	-
3	-	6.79	8.05	10.00	7.14
5	-	6.65	7.58	8.32	7.25
7	-	5.74	7.47	6.35	6.64

With the formation of NAD, an equivalent amount of pyrophosphate ions are also formed. Removal of pyrophosphate ions enzymatically leads to a shift in the equilibrium towards the formation of more NAD. The actual enzyme responsible for the removal of pyrophosphate ions must be the alkaline pyrophosphatase since it acts at an alkaline pH and it has an immeasurably high affinity for the substrate (Nagana, 1952, 1954). Therefore any increase in the activity of alkaline pyrophosphatase would be manifested in an increase of NAD.

This experiment was designed primarily for studying the comparative distribution of the alkaline inorganic pyrophosphatase by different treatments with the progress of senescence. The enzyme activity is expressed as µmoles-Pi liberated in 10 minutes by the enzyme present in 1 g initial fresh weight of leaves. The leaves used in this experiment were kept under continuous light and floated on water, BZ (50 ppm), KN (5 ppm) and BZ + KN (final concentration 50 and 5 ppm respectively).

Preliminary experiments in assaying the enzyme activity indicated augmentation by Mg^{++} ions, and cysteine dependency. The pH optimum was 8.0.

The pattern of the enzyme activity in the detached leaves is presented in Table XXII. The values are the mean of triplicate enzyme assays. There was a sharp increase following 3 days of detachment in the various treatments. The effects of BZ or KN on the enzyme activity was in descending order

BZ + KN > KN > BZ > water

The increased activity of the treated leaves gradually decreased with time. The declining activity in water floated leaves was restored or even increased by the various treatments. Kinetin was more effective than benzimidazole in restoring the activity of this enzyme and the effect of BZ and KN together was similar. The increased activity of this enzyme by BZ or KN treatment would obviously reflect on the higher levels of NAD by these treatments.

TABLE XXII

Activity of alkaline inorganic pyrophosphatase. Leaves floated in continuous light and were 7-days-old at the time of detachment.

(value* expressed as μmoles Pi liberated/10 min/g initial fresh wt at 30° C)

Days after detachment	I.D.	water	BZ	KN	BZ + KN
0	38.7	-	-	-	-
3	-	38.7	45.2	45.2	54.8
5	-	25.8	35.5	48.4	40.3
7	-	19.3	25.8	30.7	32.3

*Mean of triplicate enzyme assays.

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ATP LEVELS IN CHLOROPLASTS

values in mμmoles/mg

Treatment	Chloroplast dry wt.	Chlorophyll	Protein
I.D.	1.94	60	4.78
Water	1.65	52	3.88
BZ	2.41	62	3.95
KN	3.48	94	4.66

ADP LEVELS IN CHLOROPLASTS

values in mμmoles/mg

Treatment	Chloroplast dry wt.	Chlorophyll	Protein
I.D.	1.36	42	3.36
Water	1.74	55	4.09
BZ	1.15	29.6	1.88
KN	0.84	22.7	1.12

CHANGES IN CHLOROPLAST COMPOSITION

values in µg per mg chloroplast dry wt

Treatment	Chlorophyll	Protein	N.A.	NAD	NADH	NADP	NADPH	Total	ADP	ATP	Total
I.D.	32.4	406	19.3	0.26	0.02	0.08	0.013	0.373	0.58	0.98	1.56
Water	31.5	426	20.0	0.04	0.03	Trace	0.011	0.081	0.74	0.84	1.58
BZ	38.8	611	20.4	0.12	0.02	0.17	0.009	0.319	0.49	1.22	1.71
KN	37.0	749	23.5	0.07	0.02	0.06	0.015	0.165	0.36	1.76	2.12