THE UNIVERSITY OF MANITOBA

STUDIES ON THE SITE OF BENZIMIDAZOLE ACTION AND ITS METABOLISM IN DETACHED WHEAT LEAVES

A Thesis

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ABSTRACT

The investigations comprising this thesis were a continuation of the overall research programme being pursued in this laboratory to determine specifically the site and mechanism of action of benzimidazole in delaying the senescence of detached wheat leaves. In order to determine the intracellular localization of benzimidazole or its derivative, primary leaves of wheat (Triticum aestivum L. var. Selkirk) were allowed to incorporate tritiated benzimidazole under illumination for 6 hours in a growth chamber. Radioautographic studies showed that benzimidazole or its derivative was localized in the chloroplasts. Electron microscope autoradiography gave unequivocal evidence that it was associated with the lamellar structure of the grana. Studies on the fatty acid content revealed that linolenic acid was the principal fatty acid in wheat leaves which is predominantly localized in the chloroplasts (Benson, A.A., Natl. Acad. Sci., National Research Publ. 1145. Washington: pp. 571-574 (1963); and Wolf, F.T. and R.B. Bridgs Biochemistry of Chloroplasts. 1:188-194, 1966). Leaves senescing on benzimidazole and kinetin with a photoperiod of 16 hours and 8 hours darkness showed that treatment with benzimidazole augmented the linolenic acid content after a period of 86 hours; a decline in the level was observed after 114 hours and 136 hours; but the linolenic acid content was still considerably higher than that in leaves floated on water. Kinetin treatment did not show as great a stimulating effect

as that observed with benzimidazole, but it still exhibited a preservatory effect in the linolenic acid content. These studies further support the findings (Waygood, E.R., Plant Physiol., 10:1242-1246, 1965) that one of the sites of action of benzimidazole is in the chloroplasts and it could be closely associated with the synthesis of lamellar lipids.

Benzimidazole is metabolized by wheat leaves in light as well as in darkness and incorporated into a greenish coloured pigment isolated from the chloroform soluble fraction of leaves. Only free benzimidazole was found in the water soluble fraction. Rigorous analyses indicated that it was a single compound which did not move in an electric field and exhibited properties characteristic of phaeophytin. It is suggested that benzimidazole may chelate with a porphyrin and that the so-called benzimidazole-phaeophytin complex is an artefact of isolation. A few experiments were undertaken to ascertain its biological role but these were not sufficiently extensive to come to any conclusions. Further studies are required to elucidate its mechanism of action and its possible role in delaying senescence of detached wheat leaves.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
CoA	Coenzyme A
DNA	Deoxyribonucleic acid
2,4 D	2,4- dichlorophenoxy acetic acid
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
PPO	2,5-Diphenyl oxazole
POPOP	1,4-bis-[2-(5-phenyl oxazolyl)]-benzene
PRPP	5-phosphoribosyl-1-pyrophosphate
RNA	Ribonucleic acid
Tris	Tris(hydroxymethyl) aminomethane

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INTRODUCTION

INTRODUCTION

In the life of an organism at least three phases can be recognized which are (i) a period of growth (ii) a period of maturation and finally (iii) senescence. The sequential progression of these phases is based on an interplay between the synthetic and degradative processes. Involved in this interplay are various physiological, enzymatic and chemical changes. During the early stages of development, the synthetic processes involved in the cell division and cell enlargement predominate over the degradative processes. A dynamic equilibrium exists between the synthetic and degradative processes during maturation which is followed by senescence wherein the degradative processes become predominant by a shift in the equilibrium favouring them. This subtle balance leads to events which result in the ultimate death of an organism. Senescence may therefore, be taken to represent the final stage of development and differentiation.

Although the problem of development and differentiation of organisms has provoked considerable attention, the problem of senescence especially at the biochemical level has not received sufficient attention until recent years. The biochemistry of ageing especially with regard to higher plants has been reviewed by Varner (1961).

Early in the autumn the leaves of deciduous trees undergo colour changes as a result of the preferential destruction of the green chlorophylls.

These changes represent a natural sequence of events termed 'senescence'. Detached leaf cultures have been employed extensively in studies of this phenomenon because of the ease of artificially inducing senescence by detaching leaves from the plant. The first visible symptom of senescence is yellowing of the leaves which is the resultant of a host of other metabolic derangements. Since the green colour of the leaves is due to an abundance of chlorophyll within the chloroplasts, the yellowing of leaves is a direct reflection on the state of activity of the chloroplasts in the leaves. Similarly, the protein levels in detached leaves undergo a rapid decline. As a result of these and other metabolic changes the life span of the leaf cell is considerably reduced (Michael, 1935; Chibnall and Willshire, 1954).

Certain substances, e.g., benzimidazole, 'cytokinins', gibberellins indole acetic acid and other growth regulating substances tend to alter or regulate the onset of senescence although they do not reverse this process. This suggests that the cells destined for breakdown or senescence are still capable of being induced into a state of spontaneous reversion. These substances regulate the synthetic activity of the cells in some fashion resulting in events which delay their imminent death.

Kinetin retards the protein degradation and thus delays the yellowing of detached Xanthium leaves (Richmond and Lang, 1957). Tobacco leaves on treatment with kinetin not only retain their green colour but also their capacity for photosynthesis, RNA and protein synthesis (Wollgiehn and Parthier, 1964). Treatment of barley leaves with kinetin results in

a decreased rate of respiration (Udvardy et al., 1964).

Some of the biological changes associated with wheat leaves treated with benzimidazole may be summarized as follows:

(a) Increased content of (i) chlorophyll, (ii) proteins, (iii) nucleic acids and (iv) starch (Pearson et al., 1957; Samborski et al., 1958; Wang, 1959; Wang and Waygood, 1959).

(b) An increase in ATP and $NADP^+$ contents with a corresponding decrease in ADP and NADH levels (Mishra and Waygood, 1968).

(c) Maintenance of the fine structure of the chloroplast with a more closely integrated structure of the intra- and inter-grana (Waygood, 1965; Yoshida et al., in preparation).

(d) A marked increase in the rate of photophosphorylation (Pathak, 1966).

The work embodied in this thesis is a continuation of the overall research programme being continued in this laboratory on visual and metabolic changes encountered in detached wheat leaves during senescence. The objective of these studies was to elucidate the site and mechanism of the action of benzimidazole. Since kinetin produces similar metabolic changes some of the studies were also undertaken to determine its effect.

The thesis is divided into two sections, the objective being as follows:

I. (a) Autoradiographic studies on the site of benzimidazole action:

Waygood (1965) provided experimental evidence that there are at least two sites involved in the benzimidazole effect in delaying

senescence, and one of them is intimately associated with the chloroplasts. Yoshida et al., (in preparation) demonstrated a profound effect of benzimidazole on the fine structure of chloroplasts as evidenced with a more closely integrated intra- and inter-grana lamellae. Benzimidazole treatment resulted in enhanced chlorophyll synthesis (Wang and Waygood, 1959) as well as 'phosphodoxin' content which augmented photophosphorylation by isolated chloroplast fragments (Pathak, 1966). The effect of benzimidazole in exhibiting this diverse spectrum of biological activity, all associated with the chloroplast, led to an autoradiographic evaluation of the incorporation of tritiated benzimidazole into the chloroplast. Electron microscopic investigations were also carried out to resolve exactly where this activity was associated within the fine structure of the chloroplast.

(b) Effect of benzimidazole and kinetin on the fatty acid content:

Marked increases in ATP and $NADP^+$ levels have been reported by Mishra and Waygood (1968) both in the cytoplasmic and chloroplast fractions in wheat leaves senescing under continuous illumination when treated with benzimidazole or kinetin. Studies (Godavari, 1966) on the incorporation of nicotinic acid (carboxyl-C^{14}) or nicotinamide (carbonyl-C^{14}) showed that benzimidazole treatment increased the accumulation of the label into $NADP^+$ in wheat leaves. The overall reaction as described by Mishra and Waygood (1968) is as follows:

$$NAD^+ + NADH \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} NADP^+ + NADPH$$

The partial reactions suggested (Mishra and Waygood, 1968) are due to the operation of NAD^+ Kinase, NADPH transhydrogenase, photosynthetic electron transfer and biosynthetic reactions:

$$2\ NAD^+ + 2\ ATP \longrightarrow NADP^+ + ADP \quad \text{(kinase)}$$

$$NADP^+ + NADH \longrightarrow NADPH + NAD^+ \quad \text{(transhydrogenase)}$$

$$NADP^+ + H^+ + e \longrightarrow NADPH \quad \text{(Electron transfer)}$$

$$NADPH + X \longrightarrow NADP^+ + XH \quad \text{(Biosynthetic)}$$

and the overall reaction occurring as a consequence of the activity of these reactions will be as:

$$NAD^+ + NADH + 2ATP + X + H^+ + e \longrightarrow NADP^+ + NADPH + 2ADP + XH$$

Earlier findings (Waygood, 1965) showed that benzimidazole had a pronounced effect on the lamellar structure of the chloroplasts. Studies carried out in the present investigations have revealed that the action of benzimidazole is associated with the lamellae of the chloroplast. Light and dark reactions and electron transport leading to oxygen evolution take place in the lamellar structures of the chloroplast.

Lipids constitute approximately 50% of dry weight of the lamellae of the chloroplasts (Park and Pon, 1961; Lichtenthaler and Parks, 1963). Linoleic and linolenic acids are synthesized from oleic acid by two aerobic dehydrogenations. McMahon and Stumpf (1964) isolated an enzyme system from safflower seeds catalyzing the reaction oleyl CoA $\xrightarrow[O_2]{NADPH}$ linoleic acid. The formation of linolenic acid from linoleic probably involves a similar type of reaction. Two main

functions intimately associated with the chloroplasts are (1) formation of ATP and $NADPH_2$ generated in the light by photophosphorylation and (2) utilization of these two substrates for endergonic biocehmical reactions. On the basis of evidences accumulated, a study of the fatty acid content of wheat leaves undergoing senescence was undertaken since lipid synthesis has been shown to be tightly coupled to photophosphorylation (Stumpf et al., 1963).

II. Metabolism of Benzimidazole:

A survey of the literature reveals that although considerable investigations have been carried out on the physiological effects of "cytokinins," benzimidazole and their analogues on leaves undergoing senescence, few studies have been carried out as to how these compounds are metabolized by plants despite the potential significance of these substances.

Kapoor (1963) demonstrated the formation of benzimidazole mononucleotide and benzimidazole adenine dinucleotide in wheat embryos. These studies were, therefore, initiated in an attempt to determine the metabolic products of benzimidazole in wheat leaves and, if possible, to determine the locus of its action.

LITERATURE REVIEW

LITERATURE REVIEW

The phenomenon of senescence represents the final phase of events in the life cycle of an organism resulting in visual and metabolic changes at the cellular and sub-cellular level. Very little insight has been gained about the changes which play their role associated with these processes (Varner, 1961). The onset of degradative processes leading to senescence of leaves on detachment, can be altered considerably by the administration of auxins, indole acetic acid, 2,4-D,gibberellic acid; growth substances, cytokinins such as kinetin and benzimidazole or by the formation of roots on tissues (Gregory and Samantrai, 1950). Treatment of detached bean leaves with N^6-benzyl adenine resulted in decreased rates of respiration and retention of chlorophyll (Maclean and Dedolph, 1964). Cytokinin like activity was also exhibited by certain 6-substituted purines (Strong, 1956); 1-substituted adenine (Hamzi and Skoog, 1964), and substituted urea (Bruce et al., 1965).

Naturally occurring cytokinins:

A large number of compounds exhibiting cytokinin like biological activity have been isolated from over forty different species of plants (Helgeson, 1964). Some of the natural sources of substances exhibiting 'phytokinin' activity are: sunflower root-exudate (Kende, 1964); immature maize seeds (Miller and Witham, 1964; Beauchesne et al., 1964; Shantz and Steward, 1964), germinating peas, apple fruits (Zwar et al., 1963); tomato

fruits (Maia, 1964); yeast (Miller and Witham, 1964); coconut milk (Shantz and Steward, 1964); coconut endosperm (Miller, 1956; Strong, 1956; aesculus fruits (Shantz, 1964); corn-steep water (Fox and Miller, 1959); bleeding sap of vine (Loeffler and Overbeck, 1964); crown gall tumor tissue (Wood 1964). Another compound, 6-N(cis-4-hydroxy-3-methyl but 2-enylamiao)-9-B-D-ribofuranosyl purine having cytokinin activity have been isolated from the S-RNA of immature corn kernels, garden peas and spinach (Hall 1967a and b). A number of purine compounds besides kinetin were tested and it was concluded that the activity of kinetin depends on both adenine and the furfuryl side chain. The purine ring alone failed to show any kinetin effect (Wang et al., 1961).

The biochemical sequence of events during senescence although attributed to hormones and auxins is still unknown (Thimann, 1963). Srivastava and Ware (1965) suggested that the primary action of kinetin was in the suppression of the destruction of the activity of ribonuclease (E.C. 2.7.7.16) and deoxyribonuclease (E.C. 3.1.4.5.); however, Potapezyk (1959) reported an increase in the activity of ribonuclease on treatment with kinetin. Most of the studies have explained the effect of 'cytokinins' on the impairment of RNA and protein synthesis, as a consequence of which the life span of the cell is considerably prolonged (Sugerira et al., 1962; Salunkhe et al., 1962; Sacher, 1965; Srivastava, 1965). However, Anderson and Rowan (1966) have attributed that the first observed action of kinetin was involved in the inhibition of the increase in the concentration of α-amino nitrogen occurring in the untreated tobacco discs which in turn

maintains the mechanism of protein synthesis, a process involving several RNA fractions.

Senescence and chlorophyll destruction:

The investigations on the fate of chlorophylls during senescence showed that chlorophylls tend to be destroyed at a faster rate (Seybold, 1943; Jeffrey and Griffith, 1947; Wolf, 1956). These results were further substantiated by the data of Wolf (1956) who in a study of the pigments in the leaves of twenty-five species of green leaves has shown that chlorophyll decreases from 60.4% to 56.2% during yellowing of the leaves. The chain of events that takes place in the chlorophyll molecule during senescence remains unresolved. It seems cleavage of the chlorophyll molecule into small fragments occurs (Seybold, 1943). Goodwin (1958) reported that the chlorophylls from senescent leaves, though similar in their chromatographic behaviour, differ in their spectroscopic properties in light petroleum with red maxima appearing at 625 mμ and 635 mμ instead of 662 mμ and 644 mμ, respectively.

Action of kinetin:

Kinetin, a cytokinin delayed the loss in chlorophyll, protein and RNA content of detached leaves (Richmond and Lang, 1957); and tobacco leaf discs (Osborne, 1962; Sugerira et al., 1962). Miller (1961) has reviewed the effects of cytokins on the biochemical and physiological processes in higher plants. The diverse spectrum of biological activity exhibited by kinetin may be summarized as follows:

(i) evokes mitotic activity in callus tissues from tobacco and soya bean (Miller and Witham, 1964).

(ii) induction of secondary shoot elongation (Wickson and Thimann, 1958 and Sachs and Thimann, 1964).

(iii) inhibition of the pea stem segment elongation (Miller 1956).

(iv) enhancement in the anthocyanin formation in detached petals of Impatiens balsamina spp. (Klein and Hagen 1961).

(v) increased germination of lettuce seeds in the dark (Miller, 1956, 1958).

Mothes and Engelbrecht (1961) showed that numerous substances in the untreated parts of excised mature leaf, when sprayed with kinetin migrated to the 'kinetin-locus'. The treated tissue behaves physiologically like young tissue as evidenced by the accumulation of certain substances in treated areas.

Senescence and green island formation:

Bushnell (1966) by application of single drops of Ni^{++}, Co^{++}, or kinetin demonstrated the induction of similar patterns in the delay of senescence in detached wheat leaves. Treatment of the tissue by a single drop method resulted in the formation of green island around the point of application which remained green for a longer period compared to the distant parts of the leaves that became chlorotic. Yeast extract coconut milk and benzimidazole also prevented the green pigment from destruction.

Infection and green island formation:

Harding et al., (1968) investigated the pattern of pigment retention during the infection of Albugo candida induced 'green island' development on Brassica juncea. The studies demonstrated that the photosynthetic apparatus in the green island does not get damaged as evidenced (i), in the retention of chlorophyll (ii), ability to fix $^{14}CO_2$ (iii), electron micrographs confirmed that delayed chlorophyll breakdown and maintenance of photosynthesis were accompanied by a less rapid breakdown of chloroplast grana, indicating in essence that the photosynthetic apparatus in the green island remains undamaged during infection.

Metabolism of N^6-benzyladenine:

Carpenter and Cherry (1966) suggested that N^6-benzyladenine effects the synthesis of RNA at the nucleotide precursor level. McCalla et al., (1962) reported that N^6-benzyladenine was converted to benzyladenosine in senescing leaves of Xanthium. Amongst the other products recovered were benzyladenyllic acid, adenylic, guanyllic and inosinic acids; besides, a substantial amount of the label was also detected in urea and ureides. The studies of Hampton et al., (1956) and Von Saltza (1959) have shown that both riboside as well as ribotide of kinetin were as effective as kinetin in their activity in promoting growth of callus tissue in vitro. Fox (1966) presented evidence that both intact benzyladenine and its metabolites were incorporated into soluble RNA.

Effect of benzimidazole on senescence:

Pearson et al., (1957) first showed a dramatic effect of benzimidazole on detached wheat leaves. Retainment of green colour was observed in leaves

treated with benzimidazole compared to leaves floated on water which became chlorotic. Samborski et al., (1958) demonstrated loss of proteins with a corresponding increase in amino acid content of detached wheat leaves undergoing senescence on water. These metabolic dearrangements were arrested considerably on treatment with benzimidazole. Extensive studies carried out in this laboratory by Wang and Waygood (1959) have provided evidence that chlorophyll is involved intimately in these processes. The earlier work on the subject has been briefly reviewed by Kapoor (1963); Mishra (1963) and Pathak (1966).

Effect of benzimidazole and kinetin on chlorophyll metabolism:

Wang et al., (1960) working on the chlorophyll metabolism in detached wheat leaves reported that leaves treated with benzimidazole showed an enhanced rate of incorporation of glycine-2-C^{14} and succinate-2-3-C^{14} into chlorophyll (chloroform fraction), compared to leaves floated on water. The rate of incorporation of the isotopes were approximately two- to four-fold increments higher on treatment with benzimidazole. The incorporation of glycine-2-C^{14} by leaves was maintained by benzimidazole treatment even after four days of detachment while the rates of incorporation of leaves undergoing senescence on water were able to incorporate only 50% of glycine-2-C^{14} as by immediately detached leaves. The rate of glycine incorporation in leaves floated on water further decreased to 25% to that of the immediately detached leaves, whereas prior treatment with benzimidazole maintained the normal rate of incorporation.

Both benzimidazole and kinetin exhibited similar effects although

benzimidazole appeared to be more selective for monocotyledons than kinetin. Sunflower, tobacco and bean tissue treated with benzimidazole did not show any preservative effect on the retention of chlorophyll pigments (Wang et al., 1961). Osborne and McCalla (1961) reported that kinetin retained the chlorophyll pigments in leaf discs of senescing Xanthium leaves while benzimidazole treatment showed only a slight effect.

Structural specificity of benzimidazole:

Wang et al., (1960, 1961) arrived at the conclusion that both the benzene ring as well as the imidazole moiety were essential for its action in (i) delaying senescence and (ii) rust resistance of detached leaves. Any substituents in the ring were either phytotoxic or they induced chlorosis symptoms. Substitution of methyl, amino, or other substituents in the benzimidazole molecule will change in its action on both rust development as well as on the chlorophyll metabolism. Hao (1960) adduced further information that benzimidazole protects chlorophyll from destruction both in light as well as in darkness. A recent report (Kuraishi and Toshio, 1967) has, however, shown that 4-benzyl amino-benzimidazole was also active in its effect on the retention of the green pigment. Wang et al., (1961) showed that the action of benzimidazole in delaying the destruction of the green pigment was selective for monocotyledonous plants.

Pyridine nucleotide levels in senescing leaves:

Mishra (1963) showed that leaves (as well as chloroplasts isolated from them) treated with benzimidazole and kinetin maintained a higher level of $NADP^+$ and lower level of NAD^+. Godavari (1966) substantiated that prior

treatment of leaves with benzimidazole enhances the incorporation of nicotinic acid (carboxyl-C^{14}) and nicotinamide (carbonyl-C^{14})(the precursors of pyridine nucleotides) into $NADP^+$. Yamamoto and Ohyama (1962) showed that treatment of tobacco and spinach leaves with kinetin also stimulated the synthesis of pyridine nucleotides.

Pyridine nucleotide levels in leaf rust:

Studies carried out by Rohringer (1964) on the drifts in the pyridine nucleotide content in both resistant and susceptible tissues showed that NAD^+ concentration was initially high in the inoculated areas of both susceptible as well as resistant tissues, the concentration of which increased in the susceptible but decreased to normal levels in the resistant tissue. He further demonstrated that inoculated areas of susceptible reacting tissues accumulated larger amounts of NADH, $NADP^+$ and NADPH.

Maintenance of the fine structure of the chloroplast:

Electron microscope studies carried out by Yoshida et al., (in preparation) revealed that chloroplasts isolated from wheat leaves floated on water showed deterioration of the lamellae and a loss of electron dense particles as compared to chloroplasts isolated from immediatley detached leaves. In contrast to this, the chloroplasts from benzimidazole treated leaves had more closely spaced grana and inter-grana lamellae and more electron dense particles. In another aspect of these studies Pathak (1966) found that the rates of photophosphorylation follow closely with the fine structure of the chloroplasts on treatment with benzimidazole. In the

chloroplasts isolated from leaves floated on water the rate of photochemical ATP production was decreased to zero after five days.

Effect of benzimidazole on etiolated leaves:

Wang et al., (1960) demonstrated that treatment of etiolated leaves with benzimidazole stimulated the production of chlorophyll pigments.

Vitamin B_{12}-like compounds containing benzimidazole:

Benzimidazole has not been shown to be a natural factor even though 5,6-dimethyl benzimidazole and other related compounds have been shown to be an integral part of structure of vitamin B_{12}-like factors (Weisbach et al., 1959). Coenzyme B_{12} has been isolated from nodules of alder and extracts of germinating peas, wheat and lupine plants (Kliewar and Evans 1962a and b; Fries, 1962). Vitamin B_{12} has been shown to stimulate the synthesis of chlorophyll in etiolated leaf sections (Godnev and Shabelskaia 1966).

Metabolism of benzimidazole:

Wang (1959) reported that benzimidazole in wheat leaves was readily metabolized and incorporated as such into a complex array of compounds. Three compounds were present in the alcohol-soluble fraction as free compounds and one in a bound form in the nucleic acid fraction. Hydrolysis of the latter with hot trichloroacetic acid liberated a compound which migrated in acidic solvents. The compound on drastic hydrolysis yielded benzimidazole. To elucidate further the nature of the compound, leaves were fed with ribose-C^{14}; both ribose as well as benzimidazole were recovered from the compound in the nucleic acid fraction. The compound was

tentatively identified to be either a nucleoside or nucleotide of benzimidazole.

Formation of benzimidazole mono-nucleotide:

Subsequently, Kapoor and Waygood (1965) demonstrated and partially purified an enzyme system from wheat embryos which catalyzed the conversion of benzimidazole to its nucleotide in a PRPP-dependent reaction mediated by benzimidazole mononucleotide pyrophosphorylase. A similar reaction with kinetin as the substrate was also demonstrated by these authors.

Formation of benzimidazole adenine dinucleotide:

Kapoor and Waygood (1965) showed that benzimidazole could substitute for the nicotinamide moiety of NAD^+ with the formation of a benzimidazole analogue of NAD^+, benzimidazole adenine dinucleotide. The enzyme system catalysing the splitting of the pyridinium-N-ribosyl bond of NAD^+ and its substitution by benzimidazole has been demonstrated in wheat embryos.

Fatty acids in leaves:

Menke (1938) showed that all the lipids of the leaf were localized in the chloroplasts of spinach (chloroplasts 30.9%; cytoplasm 0.5% of the dry matter). Chibnall and Channon (1927) found that the leaves of Brasica oleracea contained 10.7% of saturated fatty acids while the rest were of the unsaturated series and of these, linoleic and linolenic acids (9, 12, 15 octadecatrienoic acid) were the main constituents. Smith and Chibnall (1932) reported that in the forage grasses Dactylis glomerata and Lolium perenne the saturated fatty acids constituted only 11 and 12%, respectively.

The figures for Dactylis and Lolium were 16 and 22% for oleic; 31 and 26% for linoleic and 42 and 40% for linolenic, respectively.

Sisakyan and Smirnov (1956) found that fatty acids constituted 57.8% of the dry weight of the chloroplasts of sugar beet; 48.8% in sunflower and 60% in red clover. Debuch (1961) analyzed the fatty acid composition of the chloroplasts of Allium porrum and reported that linolenic acid constituted 71.3% of the total fatty acids. Hawke (1963) reported the linolenic acid content of rye-grass to be 75%.

Plants are rich in glycolipids which are associated mainly with the chloroplasts. Crombie (1958) first demonstrated a high concentration of glycolipids in the chloroplasts of photosynthetic organisms. This finding was subsequently confirmed in different laboratories (Weeink, 1961; 1963; Sastry and Kates, 1963; Debuch, 1961 and Zill and Harmon, 1962). Linolenic acid was the main constituent of galactolipids viz., monogalactosyl-dilinolenin and digalactosyl dilinolenin which are concentrated in the chloroplasts. In both of these lipids linolenic acid was present to the extent of 94 - 96% (Benson, 1963; Sastry and Kates, 1963; Wolf et al., 1966). Similar results have been reported from different sources. Wolf et al., (1962) reported linolenic acid to be the major fatty acid of spinach chloroplasts. Newman (1962) showed that the young leaves of bush bean contained more C18 unsaturated fatty acids while the older ones contained more of saturated acids. Further studies by this author (1966) revealed that the chloroplasts isolated from senescent squash leaves contained a reduced amount of C18 unsaturated fatty acids especially linolenic acid.

Crombie (1958) earlier recognized the presence of α-linolenate in the chloroplasts of the chlorophyllous tissue, since then, the lipids of leaves, chloroplasts, and photosynthetic organisms have been examined by various workers (Allen et al., 1964; Bloch and Chang, 1964; O'Brien and Benson, 1964). The findings have been confirmed that α-linolenate to be primarily concentrated in the lipids of the chloroplasts or chromatophores of the photosynthetic organisms (Erwin and Bloch, 1963). The stimulation of unsaturated fatty acid synthesis in chloroplast lipoprotein by light was first observed by Osipova (1960). Newman (1962) found an increased concentration of unsaturated acids in bush bean leaves after exposure to illumination.

Newman, (1962) in an attempt to correlate the influence of darkness on the photosynthetic rate and plastid lipids showed that the galactolipids and associated fatty acids (linolenic acid constituted the major proportion) were present in relatively low concentration in dark-treated plastids. The increased exposure of the plastids to light caused an augmentation in galactosyl glyceride content thereby increasing in turn their linolenic acid content. A marked increased in the linolenic acid content of watermelon cotyledons during the process of greening was reported by Crombie (1958).

Erwin and Bloch (1962) demonstrated a correlation between the physiological activity and fatty acid in Euglena gracillis. When this organism was grown in light, α-linolenate was the major fatty acid which virtually disappeared when grown in the dark. Mutant strains which lost the ability

to photosynthesize, lack ∝-linolenate as well. A somewhat similar situation exists in the blue-green alga Anabena variabilis by having a large percentage of ∝-linolenate and the absence of the same in the related but colourless Beggiatoa. Wintermans (1960) reported that the green leaves of alder, Sambucus nigra contain higher galactolipid content than the yellow leaves from the same species. All these studies revealed that decreased rates of photosynthesis lead to decreases in the concentration of galactolipids and linolenic acid in the leaf (Wallace and Newman, 1965). Studies with lower plant forms revealed that galactolipids and linolenic acid were stimulated or depressed with light or dark treatments (Rosenberg, 1964). Contradictory to these reports James and Nichols (1966) were of the view that trans-3-hexadecenoic acid was synthesized during the course of greening; the authors came to this conclusion because of its apparent absence under etiolated conditions. Chang and Ludin (1965) in agreement with Erwin and Bloch (1962, 1963) were of the opinion that ∝-linolenic acid, a major fatty acid of galactolipids, may have a specific function in photosynthetic electron transport, rather than being required simply as a part of the lipoprotein in the chloroplast structure as suggested by O'Brien and Benson (1964).

An investigation into the dietry factors which influence the composition of bovine milk lipids demonstrated that the young leaf tissue of rye grass contained higher level of lipids than the older tissue (Hawke, 1963). Wallace and Newman (1965) reported that the young leaves of squash have a lower ratio of saturated to unsaturated fatty acid than the older ones.

Newman (1962) showed that the chloroplasts isolated from Bush bean grown in the light contained a higher ratio of unsaturated to saturated fatty acids compared to the protoplastids of dark grown material. Gibbel and Kurtz (1956) showed that both saturated and unsaturated fatty acids were synthesized from acetate. Sisakyn and Smirnov (1956) first showed higher rates of C^{14}-acetate incorporation into long chain fatty acids in isolated chloroplasts in light than in the dark. These results have been confirmed subsequently (Smirnov, 1960). Stumpf and James (1963) have shown that incorporation of C^{14}-acetate into fatty acids of isolated lettuce chloroplasts in the presence of CoA, Mn^{2+}, CO_2, and $NADP^+$. A twofold stimulation in the presence of light was reported by these authors owing to the provision of a continuous supply of ATP by photophosphorylation; they suggested that lipid synthesis was tightly coupled to this process. The effect of light in stimulating the synthesis of fatty acids are explainable as a consequence of the photosynthetic reduction of $NADP^+$ (Davenport, 1960; Keister et al., 1961). Mudd and McManus (1965) reported that stimulation of the fatty acid synthesis in light could be equalled in the dark provided adequate amounts of NADPH were available in the reaction system. The co-factors which could supply NADPH for the reductive steps of fatty acids synthesis such as $NADP^+$ and glucose-6-phosphate favour the incorporation of acetate into lipids.

SECTION I.

PART I.

Studies on the Site of Benzimidazole Action in Wheat Leaves

AUTORADIOGRAPHIC STUDIES ON THE SITE OF BENZIMIDAZOLE ACTION

The principle involved in this technique is that a radiographic emulsion produces a latent image by a charged particle radiation. The radiation energy, absorbed in individual silver halide crystals, produces development sites which can be developed chemically to yield silver grains, and the location of the isotopes can then be studied by recording the position of the radioactive material incorporated into the specimen. Thus, a photographic emulsion placed close to a radioactive sample for a sufficient period of time produces latent images in the emulsion and after photographic processing, the developed silver grains can then be located with microscope image. Accordingly, a study of the distribution of tritiated benzimidazole in the chloroplast was undertaken to evaluate the incorporation of the isotope into it. The electron microscopic studies further resolve in high optical resolution making it feasible to locate the incorporation of the radioactivity into the fine structure of the chloroplast.

MATERIALS AND METHODS

MATERIALS

Tritiated benzimidazole uniformly labelled was purchased from Radiochemical Centre, England (1.5 mc/ml). Its purity was verified by chromatography. Glutaraldehyde was an aqueous 25% solution (Fisher Scientific Co.). Polyvinyl toluene latex was obtained from Dow Chemical Co., Midland, Michigan, the average particle size being 268 mu. Autographic stripping emulsion, Edwal Quick Fix, NTE emulsion and Ektol developer were purchased from Kodak Co., London, England. All other reagents were of analytical grade.

METHODS

Feeding of tritiated benzimidazole:

Eight-day-old primary wheat leaves (Triticum aestivum var. Selkirk) grown in the greenhouse were used in the experiments. Six leaves were fed in glass vials with 0.10 μl of 1.5 uc of tritiated benzimidazole having a specific activity of 0.018 uc/mM for six hours in the growth chamber (21^{o}) at a light intensity of 1200 ft.-candles. As the solution was absorbed by the leaves, water was added to prevent dessication of leaves due to transpiration losses.

Isolation of chloroplasts:

After the feeding period, the leaves were wrapped in a wet paper towel, chilled for 10 minutes in order to facilitate grinding and homogenized in a mortar in the cold room with buffered sucrose glutaraldehyde

solution, according to the procedure of Barrnett (1962) and Sabatini et al., (1963). The solution contained 10.5 ml of 0.1 M phosphate buffer, pH 7.4; 10.5 ml of 0.4 M sucrose solution and 6.5 ml of 25% commercial aqueous glutaraldehyde solution freshly prepared. Addition of glutaraldehyde lowered the pH to 6.8. The leaf debris was removed by filtering through four layers of cheese cloth and the resulting filtrate was centrifuged at 200 x g at 1^{o} for 2 min in order to remove cell debris. The supernatant was centrifuged for 10 min at 700 x g at 0^{o}. The resulting pellet was suspended in 5 ml of the above buffer and again recentrifuged at 700 x g for 10 min. This treatment was necessary in order to remove the radioactivity which might be absorbed on the surface of the chloroplasts. The resulting pellet was resuspended in the buffer and mixed with a drop of polyvinyl latex. The suspension was used for autoradiographic studies.

Mounting of the chloroplasts:

Acid-cleaned microscopic slides were completely immersed in a solution of gelatin and chrome alum of the following composition: gelatin 5.0 g; chrome alum 0.5 g and water to make a volume of 1 litre. The slides were dried in a rack. A drop of chloroplast suspension was placed on a slide and spread with another slide.

Autoradiography:

Exposure of the sample:

The mounted chloroplasts were exposed to autoradiographic Kodak stripping emulsion AR-10. The thickness of the emulsion was 5 μ coated on a gelatin layer of 10 μ. The procedure for applying the stripping emulsion

and processing was essentially that in the Kodak instruction sheet as follows. The emulsion was cut with a razor sufficient to cover the whole of the specimen on the slide with a margin of approximately 1/2-in. wide all around. By means of the tip of a forceps the section of the emulsion layer from the glass plate was stripped and placed on the surface of water with the emulsion side downward. The emulsion layer was then lifted from the water by raising the slide underneath it. The slides were kept in a light-proof box at 4^{o}. In initial experiments an exposure period of 2 - 3 days was found adequate.

Processing:

The strips were developed employing the standard procedure as outlined in Kodak data sheet: Kodak D-19b - 4 min., rinsed with water, quick fix for 6 - 8 min., followed by a water rinse. The specimen and the superimposed emulsion was dried in a stream of cold air and the slides were then mounted in glycerine jelly (Boyd, 1955).

Evaluation of Grain Counts:

Quantitative determination of counts were made by recording the number of silver grains visible under the microscope on the emulsion above a chloroplast. A circle of approximately 3 μ in diameter was inscribed on a cover slip which was positioned in the eyepiece. The number of grains on the emulsion above the chloroplasts were recorded by focussing into the circle and counting their number. The grains in the background were recorded by encircling the latex droplet into the circle in an adjoining area near to the position of the chloroplast. In both these cases the

positions were selected at random on the slide. Counts were made at different positions on the slide.

Micro-photographs:

Micro-photographs of the chloroplasts exposed to autoradiography were taken at a magnification of 400 x and 900 x using a monochromatic mercury blue filter that transmits at 460 mμ and an exposure time of 2 sec.

Electron microscope autoradiography:

Chloroplast suspension:

The centrifuged pellets of chloroplasts isolated from Selkirk wheat leaves used in experiments with the light microscope were followed in the electron microscopic studies.

Methods:

The preparation procedure followed was essentially after Hess (1966).

Fixing and staining procedures:

(1) The chloroplast pellet was fixed in 3% acrolein and 3% glutaraldehyde cacodylate buffer for 1.5 hours at room temperature. The solution consisted of 0.3 ml acrolein; 1.2 ml 25% glutaraldehyde; 3.5 ml distilled water; 5.0 ml 0.2 M sodium cacodylate buffer, pH 7.4.

(2) Washed several times in the above buffer; diluted 1:1 with distilled water for one hour at room temperature and post-fixed in 1% osmium tetroxide for 2 hours in an ice bath.

(3) Rinsed several times in diluted buffer and post-stained in a stoppered container with aqueous uranyl acetate for 1 day.

(4) Washed with distilled water.

(5) Dehydrated through alcohol series: 50, 70, and 90% 15 min. each, and finally in absolute alcohol twice for 30 min.

(6) Induced into cross linked methacrylate resin 4 times for 30 min.

Ultra thin sections were prepared with an LKB 4800 ultramicrotome; sections were picked up on ordinary copper grids and stained with Reynold's lead citrate for 8 minutes. Lead citrate was prepared as follows: 1.33 g of lead nitrate and 1.76 g sodium citrate were mixed with 30 ml of distilled water and shaken for one minute. The solution was allowed to stand for 30 minutes with intermittent shaking in order to ensure complete conversion of lead nitrate to citrate. Then, 8.0 ml of N NaOH was added and the suspension diluted to 50 ml with distilled water. The pH of the solution at this stage was 12.0. After staining the sections were washed with 0.02 N NaOH and then with distilled water. The sections were allowed to dry.

Post-stained grids were evaporated with approximately 60A^{o} carbon layer over the specimen. Then they were coated with Kodak NTE monolayer emulsion (drop and drain method) in a dark room. The emulsion was prepared according to the procedure of Salpeter and Bachmann (1964).

One gram of NTE emulsion gel was dissolved in 10 ml of distilled water at 60-70^{o} in water bath in a light-proof container using safety light. The diluted emulsion was stirred for 10 minutes and the resulting

contents were centrifuged at 11,000 rpm. After cooling the clear supernatant was discarded carefully. The concentrated emulsion was again dissolved in warm water at 60-70^{o} in a water bath (1 ml distilled water per gram primary emulsion weight) for 10 minutes and the emulsion was next diluted with 4 ml of water per gram of the original emulsion. A few drops of the re-diluted centrifuged emulsion (kept at 60^{o}) were placed with a syringe onto the slide. The emulsion was then drained and air-dried in a vertical position. In reflected white light the emulsion layer on glass slides showed interference colours which depended on their thickness. Silver interference colour demonstrated the emulsion to be monolayer.

Emulsion-coated specimens were exposed at 4^{o} for over two months. The specimens were then developed after the exposure period using the procedure outlined below.

1% acetic acid rinse	10 sec
Developed with Kodak Dektol developer	2 min at 24^{o}
Kodak rapid fixer	5 min
Distilled water rinse	5 min

Developed specimens were finally examined in a Phillips EM-75 electron microscope and photographed.

EXPERIMENTAL RESULTS

Localization of sites:

Experiments were carried out to determine whether or not sites for the uptake of tritiated benzimidazole were localized on/in the chloroplasts after feeding wheat leaves.

Chloroplasts isolated from wheat leaves incubated with tritiated benzimidazole for 6 hours and developed after an exposure period of 2 - 3 days showed a relatively heavy distribution of silver grains in the emulsion above the chloroplast preparation. In background areas selected at random very few silver grains were detected.

Grain counts in the chloroplast and in adjoining areas in the background selected at random are presented in Table I. Very few, an average of 2 grains were noticed in the background whereas a high incorporation, an average of 15, was observed in the chloroplast.

Microphotograph Figures 1 - 4 of the chloroplast and silver grains indicate that most of the activity is either absorbed on or within the chloroplasts.

Control experiments in which leaves were fed with cold benzimidazole and following the same procedure as that with tritiated benzimidazole, showed no silver grains.

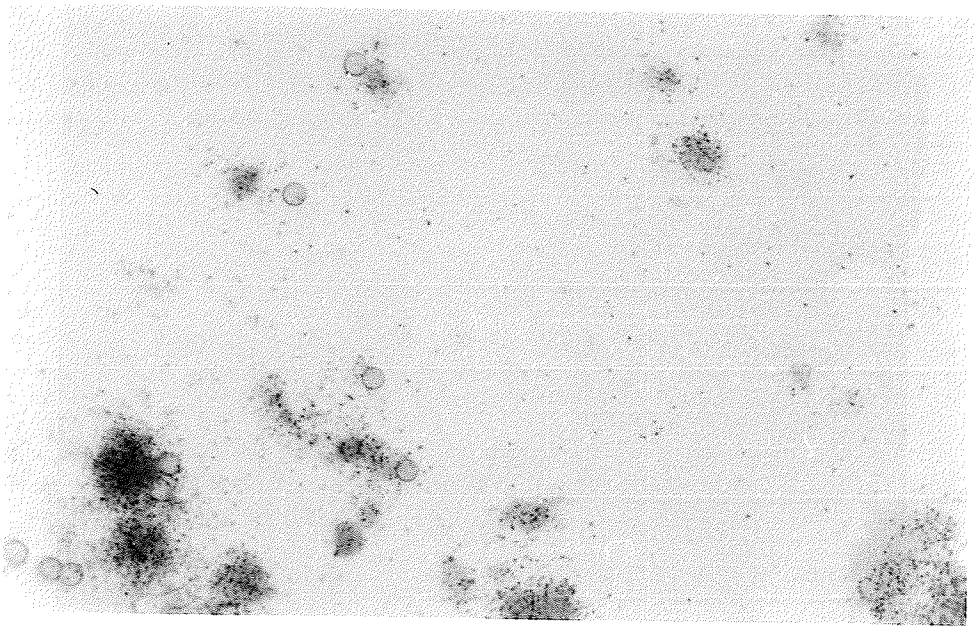
TABLE I QUANTITATIVE ESTIMATION OF SILVER GRAINS IN WHEAT LEAF CHLOROPLASTS

Sample No.	Number of silver* grains in chloroplast background		Net grains incorporated into the chloroplast
1	16	4	12
2	20	2	18
3	11	0	11
4	29	4	25
5	22	1	21
6	27	1	26
7	14	1	13
8	18	1	17
9	7	1	6
10	15	6	9
11	10	2	8
12	14	1	13
13	9	1	8
14	11	2	9
15	12	1	11
16	19	0	19
17	11	3	8
18	8	0	8
19	7	2	5
20	19	3	16
Average	15	2	13

* Grains were counted using a magnification of 100X with oil emulsion and mercury blue filter.

FIGURE 1. Micro-autoradiograph of isolated chloroplasts after feeding tritiated benzimidazole to Selkirk wheat leaves showing heavy incorporation of radioactivity, focussed through chloroplasts and silver grains 400X.

FIGURE 2. Same as above focussed through silver grains 400X.



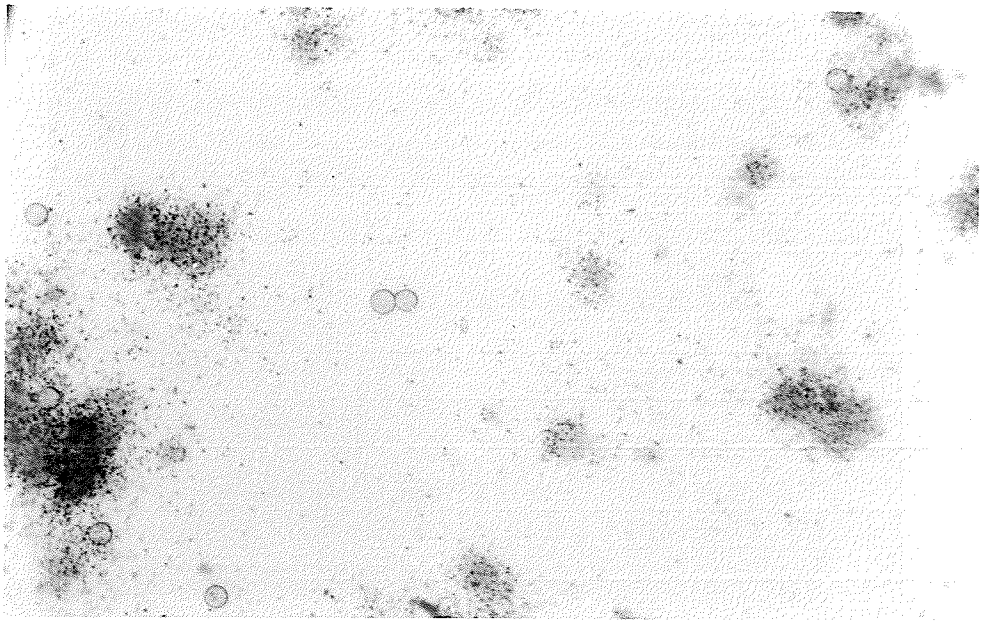
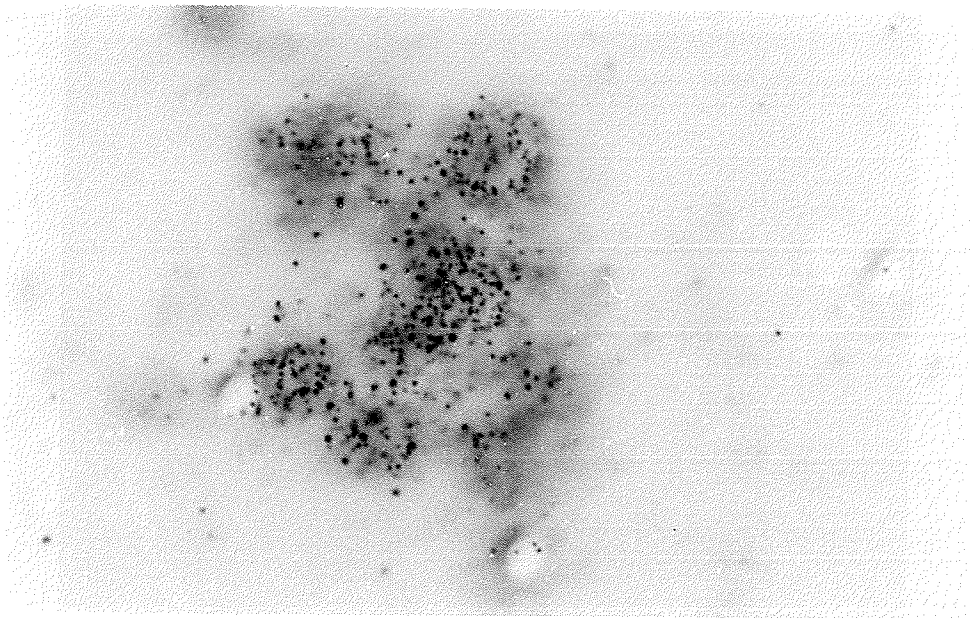
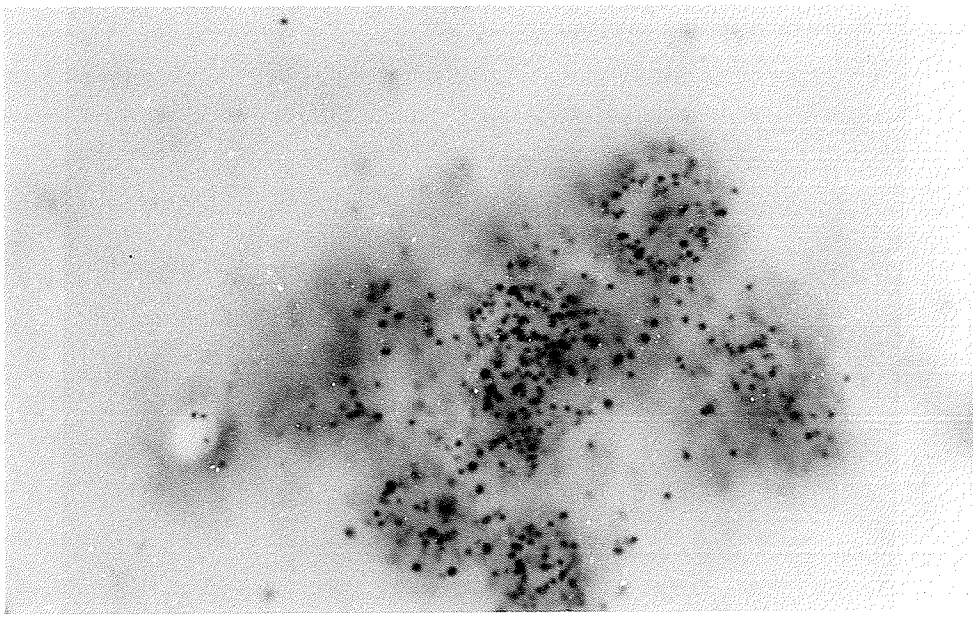


FIGURE 3. Micro-autoradiograph of isolated chloroplasts after feeding tritiated benzimidazole to Selkirk wheat leaves showing heavy incorporation of radioactivity in the chloroplasts, focussed through chloroplasts and silver grains 900X.

FIGURE 4. Same as above focussed through silver grains 900X.





Electron Microscopic Autoradiography:

In order to determine whether the tritiated benzimidazole was associated with the fine structure of the chloroplast, and if so, to specifically locate its precise site, autoradiographic studies were extended to the use of electron microscopy using Phillips EM-75 B electron microscope.

Electron micrographs (Figure 5 A.) of wheat chloroplasts developed after an exposure period of two months revealed the chloroplast to have a well defined skeletal system of the lamellar system, although the double membranes of the chloroplasts were mostly ruptured. Silver grains were associated with the lamellar system, and not with the stroma matrix. The electron micrographs further revealed that most of these silver grains were concentrated especially on the grana structure in the chloroplast. Some very minute background radiations were also noticeable which might have been caused by leaching of the radioactive particles because of the ruptured double membranes.

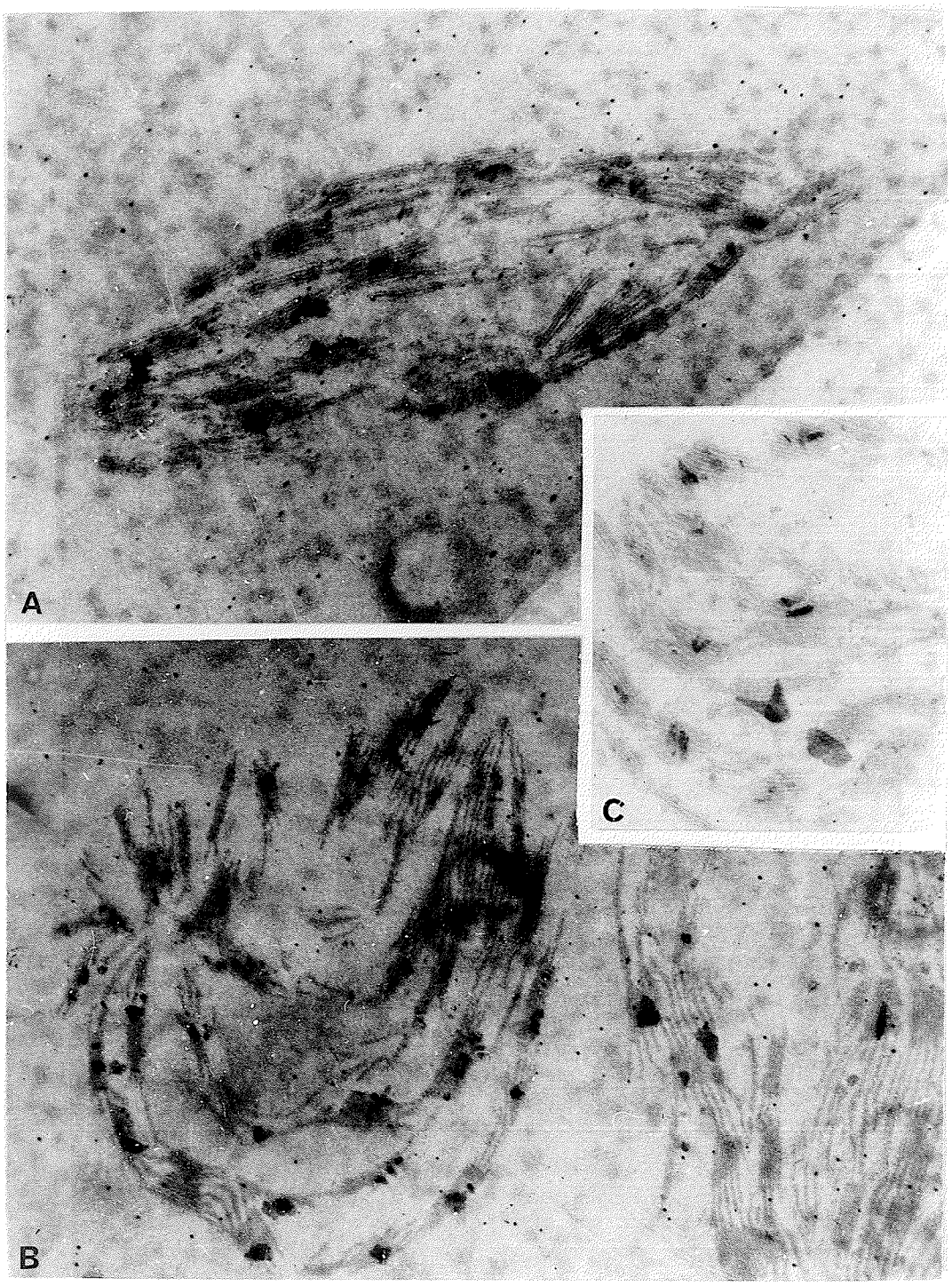
The chloroplasts in the mesophyll cells lie close to the periphery of the cell. In a typical longitudinal section through the plastid axis, grana and stroma lamellae are aggregated in linear layers extending throughout the entire chloroplast in parallel fashion. A striking feature was that in many chloroplasts the lamellae did not extend in a straight line across the plastid but instead bend back to form a boat-shaped structure as can be seen in Figure 5 B., which represents a view of slanting tangential side profile cut along the axis of the chloroplast. It is

FIGURE 5. Radioautograph of a section through a chloroplast isolated from wheat leaves after feeding tritiated benzimidazole. Sections were coated with a monolayer of centrifuged Kodak NTE emulsion and developed after an exposure period of two months.

A. Grana and stroma lamellae structure is clearly retained, silver grains can be seen on the grana structure.

B. Boat-shaped transformation of the chloroplast structure. The orientation of grana fretwork skeleton are curved to form a boat shape in a cross-section view of the chloroplast.

C. A part of the chloroplast with pointed silver grains showing the direction of radiation.


A
B
C

reasonable to interpret that this transformation from ordinary discoid shape into boat shape is a characteristic phenomenon of the chloroplasts. The bending tendency of the lamellar structure on treatment with benzimidazole into a boat-shaped structure is consistent with the results earlier reported by Yoshida et al., (in preparation). Silver grains were found located on the grana structure. Figure 5 C. shows that some of these grains are pointed showing the direction of radiation.

These studies clearly pinpoint that benzimidazole is incorporated in the grana associated with the lamellar structure of the chloroplasts.

PART II

Fatty Acid Content of Wheat Leaves Undergoing Senescence

FATTY ACID CONTENT OF WHEAT LEAVES UNDERGOING SENESCENCE

In the light of recent work, it has been shown that leaves contain large amounts of galactolipids (Weenik, 1963). Their presence as the main lipid fraction involved in photosynthesis suggests a relationship between the occurrence of chlorophyll and galactolipids (Benson, 1964 1967) in leaves and their apparent absence in seeds. Because of the possible role of monogalactosyl dilinolein and digalactosyl dilinolein in photosynthesis, the concentrations of linolenic acid in the leaves is of considerable importance. Changes in the level of linolenic acid in the leaves during senescence may lead to the later enzyme activities of photophosphorylation and fatty acid synthesis -- the two processes which are tightly coupled with each other. The metabolic changes would shift the equilibrium of the cell in such a manner so that the cell may cope as it approaches senescence. Accordingly, a study of the effect of benzimidazole and kinetin in the changes of fatty acids on wheat leaves was undertaken.

MATERIALS AND METHODS

MATERIALS

Eight-day-old primary wheat leaves (Triticum aestivum var. Selkirk) grown in the growth chamber with a photo period of 16 hours and an 8-hour dark period were used in these experiments. All reagents used were of analytical grade.

METHODS

Treatment and manipulation of leaves:

Approximately 15-gram lots of 9-day-old primary leaves of wheat 4 inch in length were floated in pyrex glass trays (13.5" X 8.5") on a 500 ml of solution of benzimidazole 50 ppm; or kinetin 5 ppm or on distilled water. The trays were covered with "saran wrap" which was punctured in order to permit aeration. The trays were transferred to a growth chamber and illuminated with a light intensity of 1600 ft.-candles in a photo period of 16 hours and an 8-hour dark period. After the expiry of the period indicated, leaves were collected, surface blotted with absorbent tissue to remove excess water and 10 g fresh weight of leaves were used for the estimation of fatty acids.

Extraction of lipids:

The leaves were cut into 5 mm segments and plunged into boiling 95% ethanol for 1 min. Leaves were extracted successively for 30 min. at room temperature with the following solvents: (a) acetone, (b) twice with chloroform-methanol 2:1 (v/v).

The extracts from each of the above steps were combined. Water-soluble impurities were removed by layering the extract under a large volume of water in a separatory funnel and left overnight in the cold room at 0-4°. During this rather long period a fluffy material appeared at the interface of water and chloroform. The water layer was removed and the white material was re-dissolved by the addition of more methanol. The extract was dried under an atmosphere of nitrogen and finally taken up in hexane.

Methanolysis of lipids:

Preparation of methyl esters was carried out essentially by the procedure of Kates (1964). The crude lipid extract obtained above was refluxed with 5 ml of methanolic 2NHCl (Methanol - 12N HCl 5:1 (v/v) for approximately 5 hours on a water bath at 60°. After refluxing the flask was cooled to room temperature and the volume of the methanol phase was adjusted with 0.5 ml of water in order to enhance phase separation. The methyl esters of the fatty acids were extracted 5 times using 5 ml portions of redistilled petroleum ether (B.P. 30 - 60°) for each extraction. The combined petroleum ether extracts were dried over anhydrous sodium sulphate to remove traces of water and evaporated to a suitable volume (approximately 0.5 ml) in an ester tube at a temperature of 58°.

Gas-liquid chromatography:

Fatty acid composition of the methyl esters was determined by gas-liquid chromatography (James, 1960) in a Beckman GC-2 gas chromatograph

fitted with a thermal conductivity detector. A 6' x 3/16" O.D steel column packed with Butane-diol-succinate (BDS) on 70 - 80 mesh Chromosorb W was employed. Analysis were carried out at 220^{o} and 250 ma current with Helium as the carrier gas at a pressure of 40 P.S.I. and a flow-rate of 62.5 ml per minute. An aliquot of 10 μl was injected into the gas chromatograph for analysis, however, whenever necessary this was adjusted to give a suitable recorder response depending on the concentration of the fatty acid content. The mount of constituent fatty acid present in the chromatographed sample was determined by integration of the area under the peak using a disc chart integrator. Identification of the major components was done by comparing the retention times with those obtained with pure standards. The peak having a retention time corresponding to that with methyl palmitate has been identified as 14:0 (myristiate), 16:0 (palmitate), 16:1 (palmitoleate), 18:0 (stearate), 18:1 (oleate), 18:2 (linoleate), 18:3 (linolenate).

EXPERIMENTAL RESULTS

The fatty acid composition of immediately detached wheat leaves and of those senescing on 50 ppm benzimidazole; 5 ppm kinetin and water as determined by gas chromatography is given in Table II. Of the fatty acids which were identified, saturated fatty acids made up only 13.93% of the total fatty acids. Palmitic, oleic, linoleic and linolenic acids were the principle fatty acids present in wheat leaves.

The presence of arachiodinic acid having a retention time of 20:4 reported in three species of mosses and four species of ferns (Wolf, 1966) was not detected by gas chromatography in wheat leaves. A higher ratio of saturated to unsaturated fatty acids was observed in these studies compared to those reported from other angiosperms (Wolf, 1966). The fatty acid present in largest quantity (66.5% of the total fatty acids) was an 18-carbon fatty acid with a retention time similar to that of linolenic acid. The results from wheat leaves are in general agreement with those reported from wheat-leaf chloroplasts, (Wolf, 1966) but differ markedly in their composition of the fatty acids. These Authors reported palmitic acid content of 19.3%; while the present investigations showed palmitic acid to represent only 10.24% of the total fatty acids in wheat leaves. These differences could possibly be attributed to different photo period and light intensity.

Treatment of leaves with benzimidazole for 86 hours showed a marked increase of 14.87% in the linolenic acid content, while those floated on

TABLE II FATTY ACID COMPOSITION OF SELKIRK WHEAT LEAVES UNDERGOING SENESCENCE

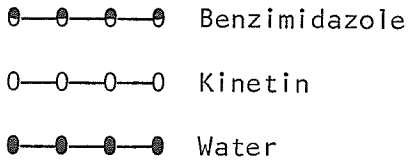
Treatment	Hours	Percentage* Fatty Acid per 10 Gram Initial Fresh Weight of Leaves							Linolenic Acid % of Control
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	
Immediately detached	-	0.50	10.24	3.08	3.19	9.23	7.25	66.51	100.00
Water	38	0.63	14.37	3.75	2.50	6.25	11.25	61.25	92.09
	86	0.41	13.65	3.29	2.59	8.47	10.58	61.00	91.72
	114	0.53	14.24	4.51	6.07	8.57	8.93	57.14	85.91
	135	0.67	19.78	7.67	2.77	8.13	6.77	54.20	81.49
Benzimidazole 50 ppm	38	0.43	9.48	5.41	4.30	9.01	8.11	63.26	95.11
	86	0.20	6.34	0.52	5.06	6.22** 0.57**	4.87	76.40	114.87
	114	0.34	13.64	4.66	3.64	7.27	9.10	61.35	92.24
	135	0.28	13.87	6.07	7.51	6.94	9.83	55.49	83.43
Kinetin 5 ppm	38	0.39	12.94	5.17	6.20	6.11	8.47	60.71	91.28
	86	0.30	11.84	3.51	2.20	8.02	8.33	65.80	98.93
	114	0.41	10.79** 1.31**	0.87	6.71	6.99	6.54	66.38	99.81
	135	0.13	14.58** 1.21**	4.86	3.47	8.08	7.99	59.69	89.75

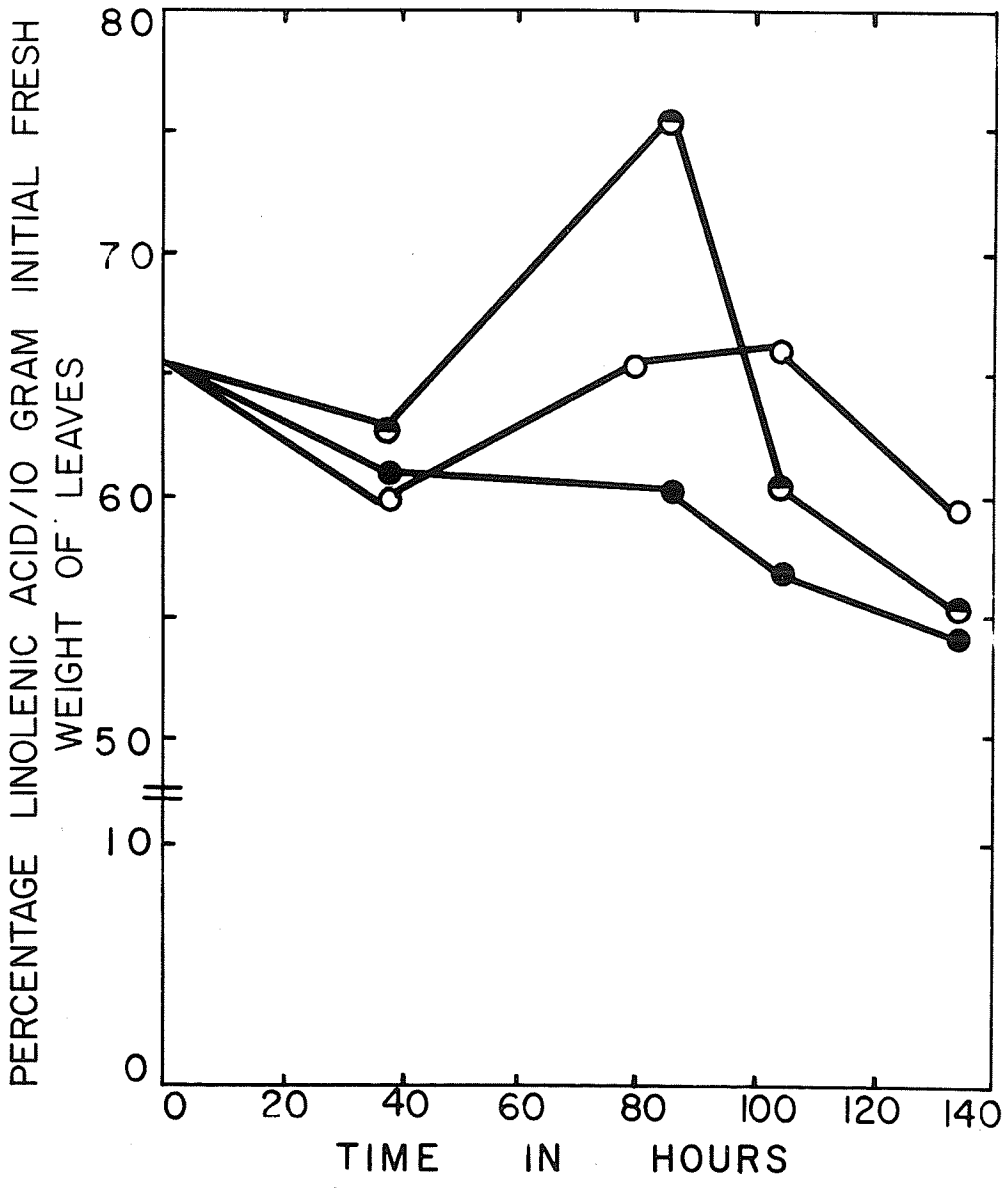
* Percent of total fatty acids as methyl esters assuming a total recovery of 100 percent. The values are average of three experiments. Primary leaves grown in the greenhouse for eight days were used. The details of experimental conditions were as in Methods.

** An unidentified shoulder appeared in these cases.

14:0 myristiate, 16:0 palmitate, 16:1 palmitoleate, 18:0 stearate, 18:1 oleate, 18:2 linoleate, 18:3 linolenate.

Figure 6. Changes in the level of linolenic acid in detached Selkirk wheat leaves undergoing senescence.




PERCENTAGE LINOLENIC ACID/10 GRAM INITIAL FRESH WEIGHT OF LEAVES
80
70
60
50
10
0
0
20
40
60
80
100
120
140
TIME IN HOURS

water lost as much as 8.3% of linolenic acid. During further treatment of wheat leaves for a period of 114 hours and 135 hours the linolenic acid content of the leaves decreased by 22.63% and 31.44% respectively, but these values were still higher by 6.33% and 2% compared to those undergoing senescence on water. Kinetin treatment of wheat leaves showed no increase in their linolenic acid content as found in the case of benzimidazole; nevertheless, the results indicate that kinetin maintains the linolenic acid content as compared to those floated on water.

The increased linolenic acid content of wheat leaves (Figure 6) treated with benzimidazole is presumably due to the increased content of galactosyl glyceride content in the chloroplasts; the synthesis of which is presumably stimulated on treatment with benzimidazole by its incorporation into the lamellar structure of the chloroplasts. A small shoulder next to oleic appeared on sampling leaves after a period of 86 hours in the case of leaves treated with benzimidazole and a similar shoulder was observed with kinetin treatment having a retention time next to palmitic acid. Since the quantities were so small, no attempt was made to identify these minor components.

SECTION II.

Metabolism of Benzimidazole in Detached Wheat Leaves — Formation of Benzimidazole-phaeophytin Complex and its Biological Role

METABOLISM OF BENZIMIDAZOLE IN DETACHED LEAVES

It is evident from the literature review that although much attention has been focussed on the visual and metabolic changes occurring in wheat leaves undergoing senescence on benzimidazole or kinetin, studies on the mechanism of its action, however, have been meagre. In spite of some experimentation the mechanism of delaying senescence by these substances is still a subject of speculation.

It is also evident from the experimental results in this Section that benzimidazole was exclusively associated with a green coloured pigment that was isolated from the chloroform fraction of wheat leaves. Accordingly, experimental methods were designed to identify the green coloured pigment and to determine its biological role, if any. Six types of feeding experiments were undertaken to study the compounds derived from benzimidazole after its entry into detached leaves as follows:

I. Wheat leaves

II. Wheat leaf sections

III. Wheat leaf sections vacuum infiltrated

IV. Etiolated leaves

V. Partially etiolated leaves

VI. Partially green leaves

The last one can be considered as midway between completely etiolated and fully green.

MATERIALS AND METHODS

CHEMICALS

Radioactive benzimidazole labelled in 2-C^{14} position (specific activity 0.975 mc/mM) and unlabelled product were purchased from Calbiochem Los Angeles 63; and Eastman Organic Chemicals, Rochester 3, New York respectively. These were used without any further purification as no evidence of impurities was found during the course of experimentation. D-ribose-C^{14} and P^{32} orthophosphate were products of Calbiochem and Radiochemical Centre, Amersham, England, respectively. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Upsala, Sweden. Instant thin layer plates were procured from Gelman Instruments, Michigan. Cellulose ion exchange papers marketed by the Whatman Co., were used.

PLANT MATERIAL

1. Wheat leaves:

First leaves of wheat (Triticum aestivum L. var. Selkirk) grown in the greenhouse for a period of about eight days were used throughout the course of these investigations. The procedure used by Wang (1959) was essentially followed in these experiments. The leaves were excised with a razor blade and the cut ends placed in distilled water; the leaves were cut to 4-inch lengths at their cut ends. Twenty leaves were placed with their cut ends in each of two small vials containing 10 µl of radioactive benzimidazole solution (0.01 µc) containing 19400 cpm and transferred to a growth chamber (21°). The vials were kept

under cool white light provided from a fluorescent and incandescent arrangement at a light intensity of 1600 ft.-candles for 3, 5, and 9 hours during which time care was taken to replenish water in the vials periodically to prevent desiccation of the leaves. The dark controls were maintained by placing the leaves covered under a small bell jar the surface of which was coated with black paint. After the treatment the leaves were removed from the vials and weighed. The leaves were cut into 1/4 inch lengths and killed immediately in 95% boiling ethanol. The leaf sections were then further extracted twice by refluxing in 80% ethanol, once with 50% ethanol and finally with 40% ethanol, each extraction taking 30 minutes. The extracts were evaporated to dryness under a stream of filtered air at room temperature, the dried residue was then partitioned between chloroform and water soluble fractions. This was achieved by washing the chloroform soluble fraction with water and the water soluble fraction with chloroform; care was taken that all the chloroform soluble pigments were completely removed from the water soluble fraction. The fractions were dried under a stream of filtered air overnight.

II. Wheat leaf sections:

These experiments were conducted in order to determine if the greater cut surface contributed to any differences in the incorporation of the label. Leaves from 9 day old Selkirk wheat were excised and cut into 1 cm sections with a sharp razor blade. One gram fresh weight of leaf sections were placed in a flask containing 10 μl of benzimidazole-2-C^{14} containing 19400 cpm solution and the flasks were covered with

Saran wrap. The flasks were transferred to a growth chamber at 21° and a light intensity of 1600 ft.-candles. Water was added as and when required. Dark controls were maintained as previously described for wheat leaves. After the total feeding period of 3 and 5 hours the leaf sections were killed with 95% boiling ethanol. Further extractions and fractionations were carried out as described for the whole leaves.

III. Wheat leaf sections vacuum infiltrated:

Selkirk wheat leaves, 9 days old, grown in the greenhouse were cut to 1 cm length with a razor blade, 1 g fresh weight of these were placed in a 50 ml pyrex vacuum flask containing 10 µl of a solution of benzimidazole-2-C^{14} containing 19400 cpm. Flasks were evacuated for 2 minutes after every half hour for a period of 3 to 5 hours. Immediately after evacuation the flasks were returned to the growth chamber at a light intensity of 1600 ft.-candles and at 21°. Dark controls were maintained as previously described. The leaf sections were then killed with boiling 95% ethanol and extracted as described previously.

IV. Etiolated leaves:

The leaves were obtained by growing in complete darkness at room temperature for 9 days at which time the leaves had attained a length of approximately 15 cm. Leaves were excised under a weak green light and cut into 4 inch lengths. Twenty leaves were placed with their cut ends in a glass vial containing 10 µl of radioactive benzimidazole, containing 19400 cpm; and placed in a growth chamber at a light intensity of 1600 ft.-candles. Dark controls were maintained as previously outlined. The

etiolated leaves on exposure to light turned yellowish-green during the course of the experiment. Water was added to the vials as and when required. The total feeding period in these experiments were 3 and 5 hours. After the expiry of the feeding period the leaves were killed in 95% boiling ethanol. Further extractions and fractionation were carried out as outlined previously.

V. Parially etiolated leaves:

Leaves were grown in complete darkness and prior to incubating with benzimidazole were exposed to a light intensity of 100 ft.-candles at room temperature for 1 hour. Nine day old wheat leaves were cut into 4-inch lengths and 20 leaves were incubated with 10 μl of benzimidazole solution, containing 19400 cpm in glass vials at a light intensity of 1600 ft.-candles for 3 and 5 hours. Simultaneously leaves were fed in darkness and further procedures were the same as described previously.

VI. Partially green leaves (midway to fully green):

These leaves are referred to as partially green as they have been exposed to light for a greater period of time compared to partially etiolated leaves. In these experiments leaves were grown in complete darkness and exposed to a light intensity of 400 ft.-candles in the growth chamber for 2 hours prior to incubation. After this treatment leaves were excised and cut to a length of 4 inches. Twenty leaves were fed with 10 ul of benzimidazole-2-C^{14} containing 19400 cpm, in light as well as in darkness in the growth chamber following the same procedure as described. Feeding was continued for a period of 3 and 5

hours. After the feeding period the leaves were killed, extracted and fractionated as described.

Extraction of leaves at room temperature:

In some experiments leaves were extracted with 85% acetone or 80% ethanol at room temperature in order to prevent the destruction of chlorophyll pigments.

Extraction with 85% acetone:

Feeding of leaves was carried out essentially in the same manner as detailed earlier. One g of 4 inch long leaves were fed with 10 μl solution of benzimidazole, containing 19.4×10^3 cpm, for 4 hours at a light intensity of 1600 ft.-candles. After the feeding period leaves were cut into 1/2 inch sections and homogenized in a mortar with 10 ml of 80% ethanol, this step was repeated twice and the residue was then extracted twice with 10 ml of 80% cold acetone. Care was taken that all the extractable green pigments were recovered. The residual mass was extracted with hot boiling ethanol in graded series. The extract from each step was evaporated to dryness and the distribution of radioactivity in different fractions was evaluated. Samples were submitted to electrophoresis using the buffer systems described later in this Section.

Extraction of chloroplasts after feeding leaves:

Nine day old wheat leaves were fed with benzimidazole-2-C^{14} both in darkness as well as under light following essentially the same procedure as detailed earlier. A solution of radioactive benzimidazole, containing 7.2×10^5 cpm was fed to leaves for a period of 4 hours.

After the feeding period the chloroplasts were isolated following the procedure of Jagendorf and Avron (1958). Leaves were cut with a razor blade and homogenized in a small teflon homogenizer containing a solution of 0.05M phosphate buffer; 0.4M sucrose and 0.01M KCl first for 15 seconds at 35% and then for 10 seconds at 58% of line voltage. The resulting brei was filtered through 4 layers of cheese cloth centrifuged at 300 X g for 3 minutes in order to remove cell debris and then at 1000 X g for 10 minutes. The pellet was washed again with the same buffer. The debris left after extraction of the chloroplasts was extracted with boiling ethanol and further extracted similar to the procedure described for whole leaves. The extracts were combined with the supernatant. The supernatant after sedimentation of chloroplasts was dried and made to a volume of 10 ml.

Aliquots were taken for determination of radioactivity. Chloroplasts were extracted with boiling ethanol, the extract was dried and further fractionated into chloroform and ethanol fractions. The radioactivity incorporated into each fraction was determined.

Counting of fractions:

The dried materials were taken up in the appropriate solvent either chloroform or water and made to a final volume of 5 ml. An aliquot of 10 μl from each fraction was examined for its radioactive content in a scintillation counting spectrophotometer as described below.

Scintillation counting:

Counting of chloroform and ethanol extracts was carried out with a

Packard Tricarb liquid scintillation spectrophotometer Model 314 EX-2 which detected the photo emission with photomultiplier tubes and recorded the data automatically. By measuring the amount of emission at different energies on three separate channels, a check on the activity of each analysis was obtained. Counting of each sample was achieved with 10 µl aliquots in glass vials using 15 ml of scintillation liquid and counting at a gain setting of 7.75% for C^{14}; and 55.7% for P^{32}. The samples were counted for 10 minutes. In order to correct for quenching, the chloroform soluble fractions containing the pigments were recounted using an external standard and the corrections applied thereto. The efficiency of these fractions were only 13 per cent. The scintillation liquid consisted of:

PPO - 7 g, POPOP - 3 g, and Naphatalene - 100 g, made to 1 litre (Loewus, 1961).

Electrophoresis:

Following the fractionation of the wheat leaf extracts, 100 µl of the extract was applied to Whatman No. 1 paper strips 4 X 30 cm and subjected to electrophoresis for different lengths of time ranging from 1 to 3 hours and at 500 volts in a Spinco Durum type electrophoresis cell attached to a Shandon power supply unit in the cold room. Three buffer systems were mainly used as the solvents during the course of the experimentation.

(i) 0.5N Acetic acid, pH 2.0

(ii) Formate formic acid, pH 3.6

(iii) Tris-borate buffer, pH 8.6 made by dissolving

2.0 M Tris - 25 ml 0.25 M boric acid 125 ml.

However, an evaluation of different buffer systems at different concentrations and varying either the length of time or voltage was also done.

During preliminary experiments the sample was applied in the middle of the strip but during the course of the experimentation the sample was applied near the cathodic or anodic end of the paper depending upon the experimental conditions in order to allow more distance to be traversed by the compound(s) of interest.

Visual localization of the spots was achieved by examination directly under U.V. illumination (filter transmitting light with a wave length of 253.7 mμ). No corrections were made for differences in viscosity of the buffers or for shrinkage of the paper on drying. The buffers, voltage gradients and time used for electrophoresis are given in Table VI. The positions of the benzimidazole marker and its product after electrophoresis were determined by means of a Geiger-Muller tube; the estimated central position of the spot was used for distance measurements.

Radioactivity was qualitatively detected using a Philips electronic counter, Model PW 4035, fitted with an amperex 200 NB end window counter tube. The electrophoretic strips were scanned for the radioactivity with a Nuclear Chicago D-47 automatic gas flow counter, without end window connected to an Actigraph II Model C-100 (Nuclear Chicago) rate-meter.

LOCATION OF RADIOACTIVE AREAS

Autoradiography:

Radioactive bands were located by exposing the strips to X-ray films (Agfa) 14 X 17 inches for 15 - 20 days depending upon the radioactivity contained in the strips. The films were developed with Kodak D-19b-Elon-hydroquinone for 5 minutes (Hodgeman, 1954), followed by Edwal Quickfix for 3 minutes.

The films were intensified as and when required using solutions of the following composition:

Solution A: Water - 750 ml, diglycollic acid - 60 ml and sodium hydroxide - 30 g

Solution B: Water - 100 ml, $Fe_2(NO_3)_3$ $9H_2O$ - 14 g, $KF.2H_2O$ - 1 g

Solution C: Water - 100 ml, pot. ferricyanide - 5 g, sodium nitrite - 1 g

Solution A, B, and C, were prepared by adding the constituents in the following order: Solution B was added to Solution A in 2 minutes with stirring. Solution C was added to A plus B in 2 minutes with stirring. Further agitation was continued for 1 minute. The films were rinsed for 3 - 8 minutes in this solution.

CHROMATOGRAPHY

Paper:

Aliquots (100 - 200 μl) of the chloroform as well as water soluble extract were used for uni-dimensional chromatographic separation on Whatman No. 1 filter paper. The extracts were applied in the form of spots or streaks. The chromatograms were developed for about 2 hours in an ascending manner. The following systems were used for developing the chromatograms:

(1) n-butyl alcohol-0.05N acetic acid, 2:1 (v/v)

(2) Propyl alcohol - 1% ammonium hydroxide, 2:1 (v/v)

(3) Isoamyl alcohol saturated with 0.1M citrate buffer (pH 9.0)

(4) 3% Ammonium chloride (w/v)

(5) Ligroin-benzene-chloroform-acetone-isopropyl alcohol 50:35:10:0.5:0.17 (v/v)

(6) n-hexane with 0.5% n-propyl alcohol (v/v)

(7) Ligroin-acetone-methanol 400:1 (v/v)

(8) Toluene-methanol 400:1 (v/v)

(9) Ligroin-petroleum ether-acetone 20:5:4 (v/v)

(10) Acetonitrile - 0.4M phosphate buffer, pH 6.8 80:20 (v/v)

(11) Acetone - 30% glacial acetic acid

(i)	1	: 1	(v/v)
(ii)	2	: 1	(v/v)
(iii)	3	: 1	(v/v)
(iv)	4	: 1	(v/v)

THIN LAYER CHROMATOGRAPHY

The possibility of effecting separation on thin layers of silica gel, cellulose powder MN-300 and micro crystalline avicel were also tried.

Silica gel G:

Glass plates 20 x 20 cm were used to support the absorbent layer. A slurry was prepared by mixing 20 g of the absorbent 200 mesh (E. Merck and Co.) and 600 mg of calcium sulphate as a binder and the plates coated in the usual manner (Stahl, 1961). The following solvent systems were used to develop the plates:

(1) Tertiary butanol - n-propanol-ethanol - 25% ammonium hydroxide-water

40:40:10:45:15 (v/v)

(2) Methanol-isopropanol - 25% ammonium hydroxide-water

45:30:15:10 (v/v)

(3) t-butyl alcohol-glacial acetic acid-water

3:1:1 (v/v)

(4) Isopropanol-ammonium hydroxide-water

16:1:3 (v/v)

(5) Isopropanol - 0.2NHCl:water

670:176:154 (v/v)

(6) Ethanol:ammonium acetate

8:2 (v/v)

"Avicel" microcrystalline cellulose:

Thirty grams of superfine grade avicel micro crystalline cellulose (American Viscose Corporation Marcus, Hook, Philadelphia) was suspended in 500 ml of 5 mM Versene and the suspension was allowed to stand for 30 minutes, filtered through a Buchner funnel and washed with distilled water. The slurry was poured into a Desaga-Brinkmann applicator with a slit width of 250 μ and the cleaned glass plates were coated by moving the applicator to the other end. In order to avoid edge effects the plates were separated from each other immediately after coating and allowed to dry at room temperature overnight.

Cellulose MN-300:

Twenty-five ml of a 10% (w/v) polyethyleneimine hydrochloride solution (pH 5.5-6.0) was dialysed against 4 litres distilled water overnight and made up to a 1% solution with water (Randerath, 1964); a suspension of MN cellulose powder consisting of 20 g of the powder with 125 ml of the dialysed 1% polyethyleneimine solution was homogenized in an electric mixer for 5 minutes and the plates coated as described previously.

The "Avicel" microcrystalline as well as Cellulose MN-300 plates were developed using the following solvent system:

(i) Isobutanol-isopropanol-water

1:1:1 (v/v)

(ii) Chloroform-methanol-ammonia

80:25:0.1 (v/v)

(iii) Water-acetone

4:1 (v/v)

(iv) Saturated ammonium sulphate-1M-sodium acetate-isopropanol

80:18:2 (v/v)

INSTANT THIN LAYER CHROMATOGRAPHY

Instant thin layer (Type SG Gelman Instrument Co., Ann Arbor, Michigan) chromatographic sheets which combines the merits of silica gel with glass fibre were used. The sheets were activated by heating at 100^{o} for 1 hour before using and developed for 20 minutes. The

solvent systems used for effecting the separation were:

(i) n-propanol-ethyl acetate-water

65:25:10 (v/v)

(ii) Acetone-methanol-water

8:2:1 (v/v)

(iii) Petroleum ether-ethyl acetate

10:1 (v/v)

CELLULOSE ION EXCHANGE PAPER CHROMATOGRAPHY

The following anion and cation cellulose exchange papers were used:

Anion type: (i) Diethyl amino ethyl cellulose DE-20

(ii) Amino ethyl cellulose AE-20

Cation type: (i) Cellulose phosphate PO-20

(ii) Cellulose citrate CT-30

(iii) Carboxy methyl cellulose CM-50

The solvent systems employed to effect the separation were:

(i) Ligroin-benzene-chloroform-acetone-isopropyl-alcohol

50:35:10:0 5:0 17 (v/v)

(ii) Ligroin-acetone-methanol

30:1:0 03 (v/v)

(iii) Acetone - 3% glacial-acetic acid

1:3 (v/v)

Autoradiography and preservation of thin layers:

Autoradiographs were prepared in the conventional manner by super imposing X-ray film upon each chromatogram and exposing for a period of one to two weeks and developing in the conventional manner. The radio-active areas on the chromatograms were located by comparison with the autoradiographs. The layers were removed from the glass plates by spray-ing with 'netan' (Merck and Co.,) onto the layers until the plates were moistened and dried in a current of air. The colourless adhesive film that formed on drying was spread over the chromatogram and the glass plates immersed in water. The layer was next peeled off gently and stored.

Fractionation on Sephadex LH-20:

Sephadex is a cross linked dextran which on swelling in an organic solvent gives a three dimensional network thereby acting as a molecular sieve. Sephadex LH-20 has the advantage as it can be used with organic solvents.

Ten grams of Sephadex LH-20 were placed in a beaker and suspended in three times its volume of chloroform. The suspension was poured into the column. Sephadex LH-20 because of its low specific gravity floats in chloroform. Dextran was therefore secured in position in the column by means of a teflon membrane and glass beads placed over it. One ml of the chloroform extract, containing 57,200 cpm were loaded onto the column and elution was achieved with 500 times its volume of chloroform. The flow rate was regulated to 0.5 ml/min and fractions of 5 ml each were collected. The fractions were evaporated to dryness and the residue

taken up in 15 ml of scintillation liquid and counted in the scintillation spectrophotometer as described earlier.

Ribose 1-C^{14} and P^{32} experiments:

Eight-day-old primary wheat leaves 4 inch in length were floated on a solution of 200 ppm benzimidazole in pyrex glass trays under continuous illumination for two days. After this period 2 g lots were placed in glass vials containing 0.1 ml of D-ribose 1-C^{14} or P^{32} orthophosphate for 4 hours in the growth chamber at a light intensity of 1,600 ft.-candles. All subsequent operations for handling the leaves were the same as described earlier.

Ethanol and chloroform extracts were examined for their radioactivity and subjected to electrophoresis in different buffer system gradients. The electrophoretic strips were exposed to X-ray film and the developed spots examined for their radioactive content.

EXPERIMENTAL RESULTS

The experimental results presented concern the incorporation of benzimidazole-2-C^{14} in wheat leaves, leaf sections, leaf section vacuum infiltrated and wheat leaves under varying degrees of etiolation.

METABOLISM OF BENZIMIDAZOLE-2-C^{14}

1. Detached leaves:

Exogenously supplied benzimidazole-2-C^{14} was readily incorporated into detached Selkirk wheat leaves. The results in Table III indicate that a substantial amount (84.82%) of the isotope fed was recovered in the chloroform soluble fraction within a feeding period of three hours. Feeding leaves further for a period of 5 and 9 hours resulted in a higher percentage of incorporation from 90.68% to 91.85%, respectively into the chloroform soluble fraction, but these figures were not significantly different. It appears that benzimidazole is incorporated in wheat leaves readily within a period of 3 hours into the chloroform soluble fraction of the leaves. The water soluble fraction contains only 15.18% of the total radioactivity incorporated in 3 hours. (Table III). During the feeding period from 3 - 9 hours, a gradual decrease in the incorporation of benzimidazole-2-C^{14} into the water soluble fraction was observed from 15.18% in 3 hours to 9.32% in 5 hours which further declined to 8.15% in 9 hours. A similar pattern in the distribution of radioactivity into the chloroform soluble fraction was obtained when the leaves were fed in the dark. The figures were 74.61% during a

period of 3 hours; 80.60% in 5 hours and 86.60% in 9 hours. These rates of incorporation were comparatively lower compared to those fed under light. This slightly low rate of incorporation may be due to the effect of light in exerting the higher rates of incorporation into the chloroform soluble fraction. Despite the comparatively low values of the chloroform/water soluble observed in leaves fed under darkness the ratio remained more or less the same.

II. Leaf sections:

The experiments with wheat leaf segments were undertaken to determine whether benzimidazole is incorporated more readily by this technique in wheat leaves. One cm leaf sections, fed with benzimidazole-2-C^{14} for a period of 3 and 5 hours revealed that 86.45% and 93.35% of the radioactivity was recovered in the chloroform soluble fraction under light; the corresponding figures in darkness were 77.30% and 87.26% (Table III). The slightly higher rates of benzimidazole incorporation into the chloroform soluble fraction observed in leaf segments compared to those of whole leaves may be attributed to the greatly cut surface of the leaf segments. A similar pattern in the incorporation of the radioactivity was observed in leaf segments fed under darkness. Little difference was found in the water soluble fractions.

III. Leaf sections vacuum infiltrated:

These studies were undertaken in order to determine if vacuum infiltration promoted the incorporation of benzimidazole into leaf sections. One cm leaf sections were fed for 3 and 5 hours as described in Methods.

TABLE III INCORPORATION OF BENZIMIDAZOLE-2-C^{14} INTO WHEAT LEAVES

Experimental Group		Hours Feeding	Experiment No.	Fresh Weight (g)	Radioactivity cpm/fraction		Percentage* radioactivity incorporated into	
					water soluble	chloroform soluble	water soluble	chloroform soluble
Leaves	Light	3	1	1.16	2750	16150	14.55	85.45
			2	1.18	1130	16300	15.81	84.19
		5	1	1.84	1675	16610	9.16	90.84
			2	1.19	1682	16060	9.48	90.52
		9	1	1.05	1270	16850	7.01	92.99
			2	1.17	1710	16700	9.29	90.71
	Dark	3	1	1.08	4800	12400	27.91	72.09
			2	1.20	4000	13500	22.86	77.14
		5	1	1.21	4120	14600	22.10	77.90
			2	1.27	3050	15200	16.71	83.29
		9	1	1.25	3080	16400	15.81	84.19
			2	1.03	2080	16800	11.01	88.99

Continued

TABLE III. Continued

Experimental Group		Hours Feeding	Experiment No.	Fresh Weight (g)	Radioactivity cpm/fraction		Percentage* radioactivity incorporated into	
					water soluble	chloroform soluble	water soluble	chloroform soluble
Leaf Sections	Light	3	1	1.26	2640	17400	13.17	86.83
			2	1.16	2650	16400	13.92	86.08
		5	1	1.18	1262	18340	6.44	93.56
			2	1.25	1270	17260	6.85	93.15
	Dark	3	1	1.08	4520	14780	23.42	76.58
			2	1.13	3890	13850	21.93	78.07
		5	1	1.24	2280	16500	12.15	87.85
			2	1.02	2430	15800	13.33	86.67
Leaf Sections Vacuum Infiltrated	Light	3	1	1.29	4962	12350	28.66	71.34
			2	1.30	5060	13470	27.30	72.70
		5	1	1.18	1938	15325	11.22	88.78
			2	1.20	1988	15775	11.19	88.81
	Dark	3	1	1.06	4150	14225	22.58	77.42
			2	1.12	4400	14338	23.48	76.52
		5	1	1.24	3912	14686	21.03	78.97
			2	1.05	4200	14050	23.01	76.99

* The values are the mean of two experiments based on total counts recovered.

For these time periods (Table III) 72.02% and 88.97% of the radioactivity was incorporated under light into the chloroform soluble fraction; the corresponding figures under darkness were 76.97% and 77.98%. The results indicate that vacuum infiltration has little effect on the incorporation in light, but it may have assisted incorporation in darkness. The data show that the bulk of the radioactivity is incorporated into the chloroform soluble fraction irrespective of the treatment.

Etiolated, partially etiolated and partially green leaves:

The results obtained from etiolated, partially etiolated and partially green leaves fed with benzimidazole-2-C^{14} are shown in Table IV. The data showed that etiolated leaves incorporated 68.40% and 74.73% of the radioactivity in light during a period of 3 and 5 hours; the corresponding figures in darkness were 56.42% and 65.90%; etiolated leaves incorporated as much as 43.62% and 34.10% of the radioactive benzimidazole in darkness into the water soluble fraction during a period of 3 and 5 hours, respectively, compared to those fed under light which incorporated only 31.60% and 25.27%. Partially etiolated leaves incorporated 79.33% and 82.25% in light during a period of 3 and 5 hours into the chloroform soluble fraction; the corresponding figures in darkness were 62.48% and 73.41%. Leaves on half way to greening incorporated 82.08% and 87.82% in light into the chloroform soluble fraction during a period of 3 and 5 hours, while the figures in darkness were 76.72% and 87.80% respectively. It is evident from these results that benzimidazole is incorporated in etiolated, partially etiolated and leaves halfway to greening exhibiting a similar pattern in their incorporation.

TABLE IV. INCORPORATION OF BENZIMIDAZOLE-2-C^{14} INTO ETIOLATED, PARTIALLY ETIOLATED AND PARTIALLY GREEN WHEAT LEAVES

Experimental Group		Hours Feeding	Experiment No.	Fresh Weight (g)	Radioactivity cpm/fraction		Percentage* radioactivity incorporated into	
					water soluble	chloroform soluble	water soluble	chloroform soluble
Etiolated Leaves	Light	3	1	1.07	6900	11620	37.25	62.75
			2	0.95	4750	13550	25.95	74.05
		5	1	1.02	5620	13300	29.70	70.30
			2	1.05	3750	14250	20.83	79.17
	Dark	3	1	0.94	8200	10450	43.96	56.04
			2	1.04	8400	11050	43.29	56.81
		5	1	0.96	7150	12500	36.38	63.62
			2	0.98	6650	14250	31.81	68.19
Partially Etiolated Leaves	Light	3	1	1.14	3900	14200	21.54	78.46
			2	1.02	3800	15400	19.79	80.21
		5	1	0.92	3900	14200	21.54	78.46
			2	0.94	2700	16650	13.95	86.05
	Dark	3	1	1.12	7700	11800	39.48	60.52
			2	1.06	6900	12500	35.56	64.44
		5	1	1.08	4800	14000	25.53	74.47
			2	1.06	5300	13200	28.64	71.36

Continued

TABLE IV. Continued

Experimental Group		Hours Feeding	Experiment No.	Fresh Weight (g)	Radioactivity cpm/fraction		Percentage* radioactivity incorporated into	
					water soluble	chloroform soluble	water soluble	chloroform soluble
Partially Green Leaves	Light	3	1	1.02	3200	15650	16.97	83.03
			2	1.09	3500	15050	18.86	81.14
		5	1	1.18	2650	16600	13.76	86.24
			2	1.12	1950	16450	10.59	89.41
	Dark	3	1	0.94	4800	14350	25.06	74.94
			2	1.12	4200	15400	21.47	78.53
		5	1	1.14	3000	15950	15.83	84.17
			2	1.08	1580	16850	8.57	91.43

* The values are the mean of two experiments based on total counts recovered.

ANALYSIS OF THE CHLOROFORM AND WATER SOLUBLE FRACTION

Electrophoresis:

The data in Tables III and IV show that the bulk of the radioactivity fed to leaves was associated with the chloroform soluble fraction. In a preliminary electrophoresis experiment using acetic acid and Tris-borate buffer, the radioactivity associated with the water soluble fraction had the same electrophoretic mobility as the benzimidazole marker; whereas there was no movement of the unknown compound from the chloroform soluble fraction in either of these two solvents. Neither was there any free benzimidazole in the chloroform fraction (Table V).

The chloroform soluble fraction from several different feeding experiments was then submitted to electrophoresis in ten different solvents (Table VI). In no case did the radioactive compound from the chloroform soluble fraction move from its origin in either anionic or cationic solvents and in no case was benzimidazole shown to be present. The radioactive chloroform soluble compound was a greenish coloured pigment. In etiolated leaves it was greenish yellow, pale yellow in leaves fed in darkness and became progressively green in partially etiolated and partially green leaves.

Figures 7 and 8 show the autoradiographs of the chloroform and water soluble fraction of leaves fed in light and darkness after electrophoresis in 0.5 N acetic acid. The sole radioactive water soluble compound moved to the same position as the benzimidazole marker

TABLE V. RELATIVE ELECTROPHORETIC MOBILITY OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT ISOLATED FROM WHEAT LEAVES AFTER FEEDING BENZIMIDAZOLE-2-C^{14}

Compound	Mobility in	
	Tris-borate buffer (migration to anode)	0.5 N Acetic acid (migration to cathode)
Benzimidazole	1.0	1.0
Chloroform soluble extract	0.0*	0.0*
Water soluble extract	1.0	1.0

* Compound did not move from the point of application; all the radioactivity was associated with the green pigment.

Electrophoresis was performed in (i) Tris-borate buffer, pH 8.6 at a voltage of 500 v for 200 minutes applying a gradient of 16.7 v/cm, (ii) 0.5 N acetic acid, pH 2.0 employing a voltage of 400 v for 180 minutes and a gradient of 13.3 v/cm.

TABLE VI. ELECTROPHORETIC MOBILITIES OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT ISOLATED FROM WHEAT LEAVES AFTER FEEDING BENZIMIDAZOLE-2-C^{14} UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Buffer	Concentration (M)	pH	Voltage	Time (min)	Mobility† cm^2/volt x min	
					Benzimidazole	Compound derived from chloroform extract
Oxalate	0.10	1.85	600	90	0.434	0*
Acetic acid	0.50	2.00	400	180	1.300	0
Glutamate	0.05	2.55	700	150	1.482	0
Formate	0.05	3.60	500	180	18.662	0
Citrate	0.10	4.00	900	100	6.250	0
Acetate	0.20	5.30	800	120	7.400	0
Phosphate	0.10	6.90	800	60	0.208	0
Tris	0.10	8.25	700	120	-2.160	0
Tris-borate	**	8.60	500	180	-1.440	0
Glycyl-glycine	0.10	9.60	800	120	-2.400	0

Effective length of paper 30 cm; experimental conditions as described under Methods.

Anionic buffers were sodium salts excepting potassium phosphate; cationic buffers were chlorides.

* Compound failed to move from the point of application.

** Made by dissolving 165 ml of 2.0 M Tris and 835 ml of 0.25 M boric acid.

-ve value indicates movement towards the anode.

† Arbitrarily expressed as mobility cm^2/volt x minutes, distance measured from the origin to its movement towards the pole under consideration.

FIGURE 7. Autoradiograph of the electrophoretic separation of the chloroform and water soluble extract in 0.5 N acetic acid after feeding benzimidazole-2-C^{14} to detached wheat leaves fed under light.

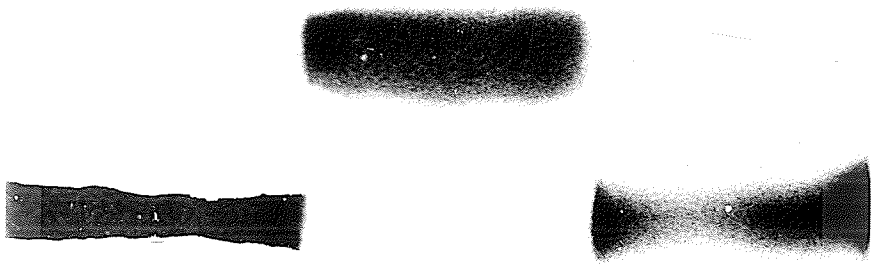
1. Benzimidazole reference which moved 1.7 cm from the point of origin.

2. Chloroform soluble extract of wheat leaves which did not migrate from the point of origin.

3. Water soluble extract of wheat leaves migrating to the same position as benzimidazole reference.

O - Origin

Electrophoresis was performed in 0.5 N acetic acid pH 2.0 employing a voltage of 400 volts for 180 minutes.



1 2 3

FIGURE 8. Autoradiograph of the electrophoretic separation of the chloroform and water soluble extract in 0.5 N acetic acid after feeding benzimidazole-2-C^{14} to detached wheat leaves fed under darkness.

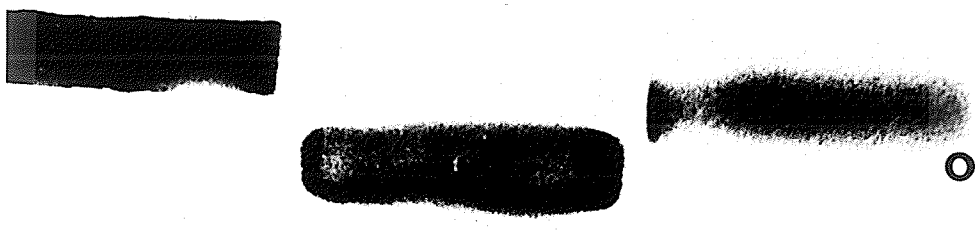
1. Benzimidazole reference which moved 1.7 cm from the point of origin.

2. Chloroform soluble extract of wheat leaves which did not migrate from the point of origin.

3. Water soluble extract of wheat leaves migrating to the same position as benzimidazole reference.

0 - Origin

Electrophoresis was performed in 0.5 N acetic acid, pH 2.0 employing a voltage of 400 volts for 180 minutes.



1 2 3

whereas the green radioactive pigment from the chloroform soluble fraction remained at the origin.

Figure 9 shows an autoradiograph of the two fractions from partially green leaves after electrophoresis in Tris-borate buffer. Scanning the strips showed that all the radioactivity was associated with the green pigment whether it was from leaves fed benzimidazole-2-C^{14} in light or darkness.

Paper chromatography:

Eleven different solvent systems normally used for the separation of chlorophyll pigments were tested with the chloroform soluble fraction. In nine solvents the green pigment as well as the benzimidazole marker failed to move from the point of origin. However, in acetonitrile-phosphate buffer and in solvents containing varying concentrations of acetone with 30% acetic acid, both the benzimidazole marker and the green pigment moved to different positions. The Rf values are given in Table VII. Again, the chloroform soluble fraction revealed only a single radioactive spot which was green in colour.

The radioactivity associated with the water soluble fraction had the same Rf as the benzimidazole marker and indicates that its radioactivity is due to unmetabolized benzimidazole in the leaves.

Thin layer chromatography:

Silica gel, cellulose powder and Gelman instant thin layer plates Type SG, were used with a variety of solvents to determine whether this green pigment in the chloroform soluble extract was a single compound.

FIGURE 9. Autoradiograph of the electrophoretic separation of the chloroform and water soluble extract in Tris-borate buffer, pH 8.6 after feeding benzimidazole-2-C^{14} to detached partially green wheat leaves.

1. Benzimidazole reference which moved 2.0 cm from the point of origin.

2. Water soluble extract of wheat leaves fed under light migrating to the same position as benzimidazole reference.

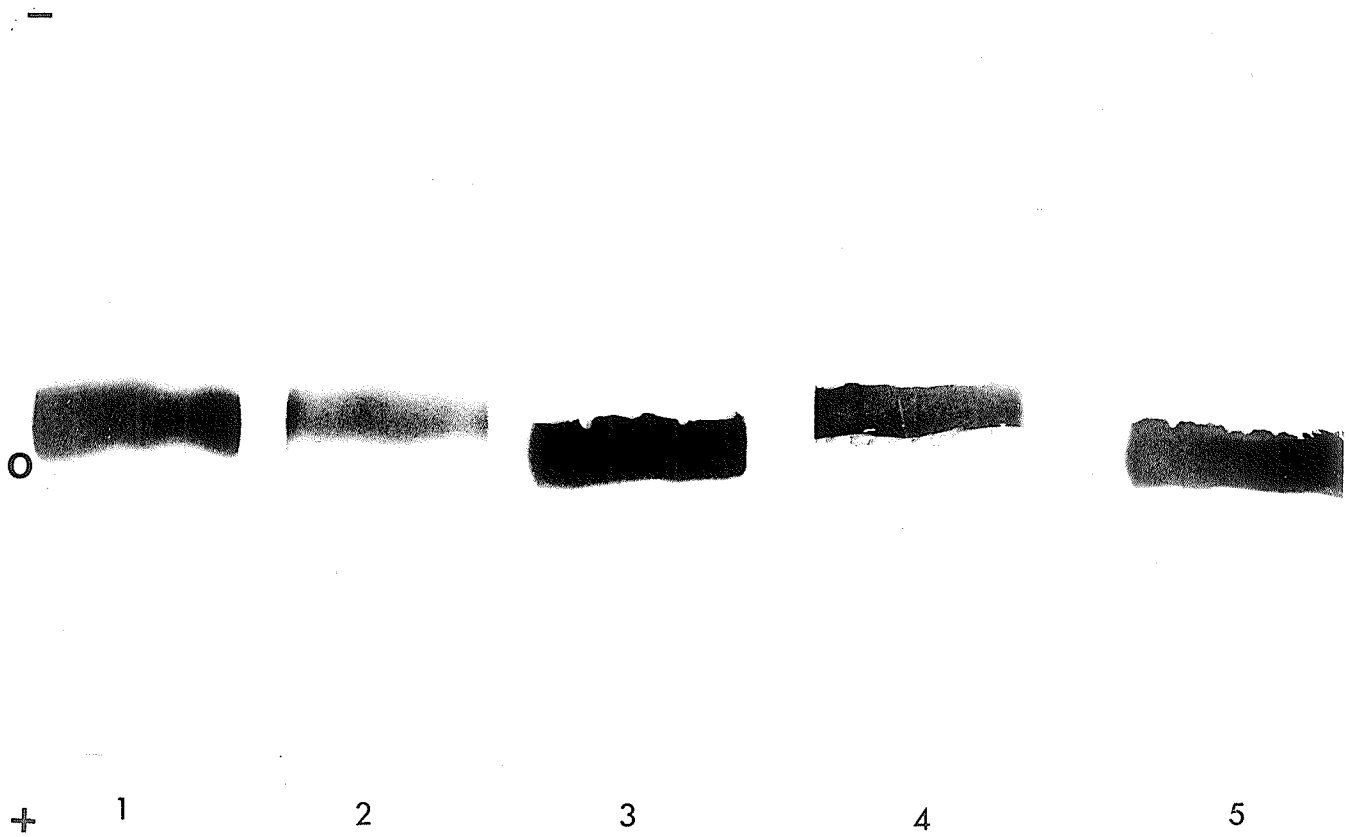
3. Chloroform soluble extract of wheat leaves fed under light which did not migrate from the point of origin.

4. Water soluble extract of wheat leaves fed under light migrating to the same position as benzimidazole reference.

5. Chloroform soluble extract of wheat leaves fed under light which did not migrate from the point of origin.

O - Origin

Electrophoresis was performed in Tris-borate buffer pH 8.6 employing a voltage of 500 volts for 180 minutes.


-
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+
1
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3
4
5

TABLE VII. Rf VALUE OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT CHROMATOGRAPHED ON WHATMAN NO. I FILTER PAPER

Solvent No.	Solvent system	Rf Value of		
		Benzimidazole	Chloroform soluble extract	Water soluble extract
1.	n-butyl alcohol - 0.5 N acetic acid - 2:1 (v/v)	0*	0	0
2.	Propyl alcohol - 1% ammonium hydroxide 2:1 (v/v)	0	0	0
3.	iso-amyl alcohol saturated** with 0.1 M citrate buffer pH 9.0	0	0	0
4.	3% Ammonium chloride (w/v)	0	0	0
5.	Ligroin-benzene-chloroform-acetone-isopropyl alcohol 50:35:10:0.5:0.17	0	0	0
6.	n-hexane with 0.5% n-propyl alcohol	0	0	0
7.	Ligroin-acetone-methanol 30:1:0.03 (v/v)	0	0	0
8.	Toluene-methanol 400:1	0	0	0
9.	Ligroin-petroleum ether-acetone 20:5:4 (v/v)	0	0	0
10.	Acetonitrile:0.4 M phosphate buffer pH 6.80 80:20 (v/v)***	0.590	0.841	0.590
11.	Acetone:glacial acetic acid†			
	(i) 1:1 (v/v)	0.640	0.830	0.630
	(ii) 2:1 (v/v)	0.857	0.795	0.850
	(iii) 3:1 (v/v)	0.800	0.932	0.800
	(iv) 4:1 (v/v)	0.775	0.966	0.775

Continued

TABLE VII. Continued

* Indicates that the compound failed to move from the point of application in the particular solvent.

** Five per cent solution of citric acid, pH adjusted with ammonium hydroxide to 9 and agitated with an equal volume of Iso-amyl alcohol.

*** Eight volumes of acetonitrile were mixed with 2 volumes of 0.4 M sodium phosphate buffer, pH 6.86. Paper was sprayed with sodium phosphate buffer, pH 6.86 and dried before spotting the compound.

† Thirty ml of glacial acetic acid made up to 100 ml with distilled water.

The results are tabulated in Tables VIII, IX and X. Autoradiography and scanning showed that there was only one radioactive compound in either the chloroform or water soluble fraction. Radioactivity was associated with the green pigment in the former and with a compound that moved to the same position as benzimidazole-2-C^{14} in the water soluble fraction.

Cellulose ion exchange chromatography:

Whatman anion and cation exchange papers were also used to determine whether the green pigment could be separated into two or more components. When Solvent Systems (1) or (2) were used neither benzimidazole nor the green pigment moved from the point of origin. However, in Solvent (3) acetone 30% acetic acid (1:3 (v/v), the green pigment separated into two radioactive areas at Rf 0.975 and 0.740 on aminoethyl cellulose AE-20 ion exchange paper. The former is close to the Rf of benzimidazole (Table XI) and suggests that benzimidazole is part of the green pigment complex which can be displaced under special conditions. If it were a loose moiety this would have occurred more frequently, but this was the only case heretofore in which the green pigment separated into two compounds.

Column chromatography:

One milliliter of the chloroform soluble extract obtained from leaves fed with benzimidazole-2-C^{14} was loaded onto a column of Sephadex LH-20 and eluted with 500 ml of chloroform. Five milliliter fractions were collected and dried in a stream of filtered air. Figure 10 shows that the bulk of the radioactivity was associated with a green pigment of relatively high molecular weight in fractions 3 to 18.

TABLE VIII. Rf VALUE OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT USING THIN LAYER CHROMATOGRAPHY ON SILICA GEL G

Solvent No.	Solvent system	Rf Value of		
		Benzi-midazole	Chloroform soluble extract	Water soluble extract
1.	Tertiary-butanol-n-propanol-ethanol-25% ammonium hydroxide-water 40:40:10:45:15 (v/v)	0.760	0.340	0.740
2.	Methanol-isopropanol-25% ammonium hydroxide-water 45:30:15:10 (v/v)	0.810	0.331	0.810
3.	t-butyl alcohol-glacial acetic acid-water 3:1:1 (v/v)	0.470	0.541	0.470
4.	Isopropanol-ammonium hydroxide-water 16:1:3 (v/v)	0.590	0.500	0.590
5.	Isopropanol-0.2N Hcl:water 670 176 154 (v/v)	0.640	0.680	0.640
6.	Ethanol-ammonium-acetate 8:2 (v/w)	0.750	0.700	0.750

TABLE IX. Rf VALUE OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT USING THIN LAYER CHROMATOGRAPHY ON MN-300 CELLULOSE POWDER AND AVICEL MICROCRYSTALLINE CELLULOSE

Solvent No.	Solvent System	Rf Value On					
		MN-300 Cellulose Powder			Avicel Microcrystalline Cellulose		
		Benzi-midazole	Chloroform soluble fraction	Water soluble fraction	Benzi-midazole	Chloroform soluble fraction	Water soluble fraction
1.	Isobutanol-isopropanol-water 1:1:1 (v/v)	0.760	0.700	0.760	0.830	0.240	0.830
2.	Chloroform-methanol-ammonia 80:25:0.1 (v/v)	0.295	0.140	0.295	0.640	0.130	0.640
3.	Water:acetone 4:1 (v/v)	0.650	0.520	0.650	0.430	0*	0.430
4.	Saturated ammonium sulphate IM sodium acetate:isopropanol 80:18:2 (v/v)	0.300	0.071	0.300	0.640	0*	0.640

* No movement of the green coloured compound was observed in the solvent system.

TABLE X. Rf VALUE OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT USING GELMAN INSTANT THIN LAYER PLATES TYPE SG

Solvent No.	Solvent system	Rf Value of		
		Benzimidazole	Chloroform soluble extract	Water soluble extract
1.	n-propanol-ethyl acetate-water 65:25:10 (v/v)	0.910	0.950	0.900
2.	Acetone-methanol-water 8:2:1 (v/v)	0.890	0.590	0.890
3.	Petroleum ether-ethyl acetate 10:1 (v/v)	0*	0	0

* No movement of the compound was observed in the particular solvent system.

TABLE XI. Rf VALUE OF BENZIMIDAZOLE AND ITS METABOLIC PRODUCT ON WHATMAN ION EXCHANGE PAPERS

	Type of Paper	Rf Value in Solvent System (3)		
		Benzi-midazole	Chloroform soluble	Water extract
	Anion exchanger			
(i)	Diethylamino ethyl cellulose DE-20	0.875	0.936	0.875
(ii)	Aminoethyl cellulose AE-20	0.980	0.975 0.740	0.980
	Cation exchanger			
(i)	Cellulose phosphate P-20	0.362	0.974	0.362
(ii)	Cellulose citrate CT-30	0.740	0.923	0.740
(iii)	Carboxy methyl cellulose CM-50	0.770	0.976	0.770

* Thirty ml of glacial acetic acid made to 100 ml with distilled water.

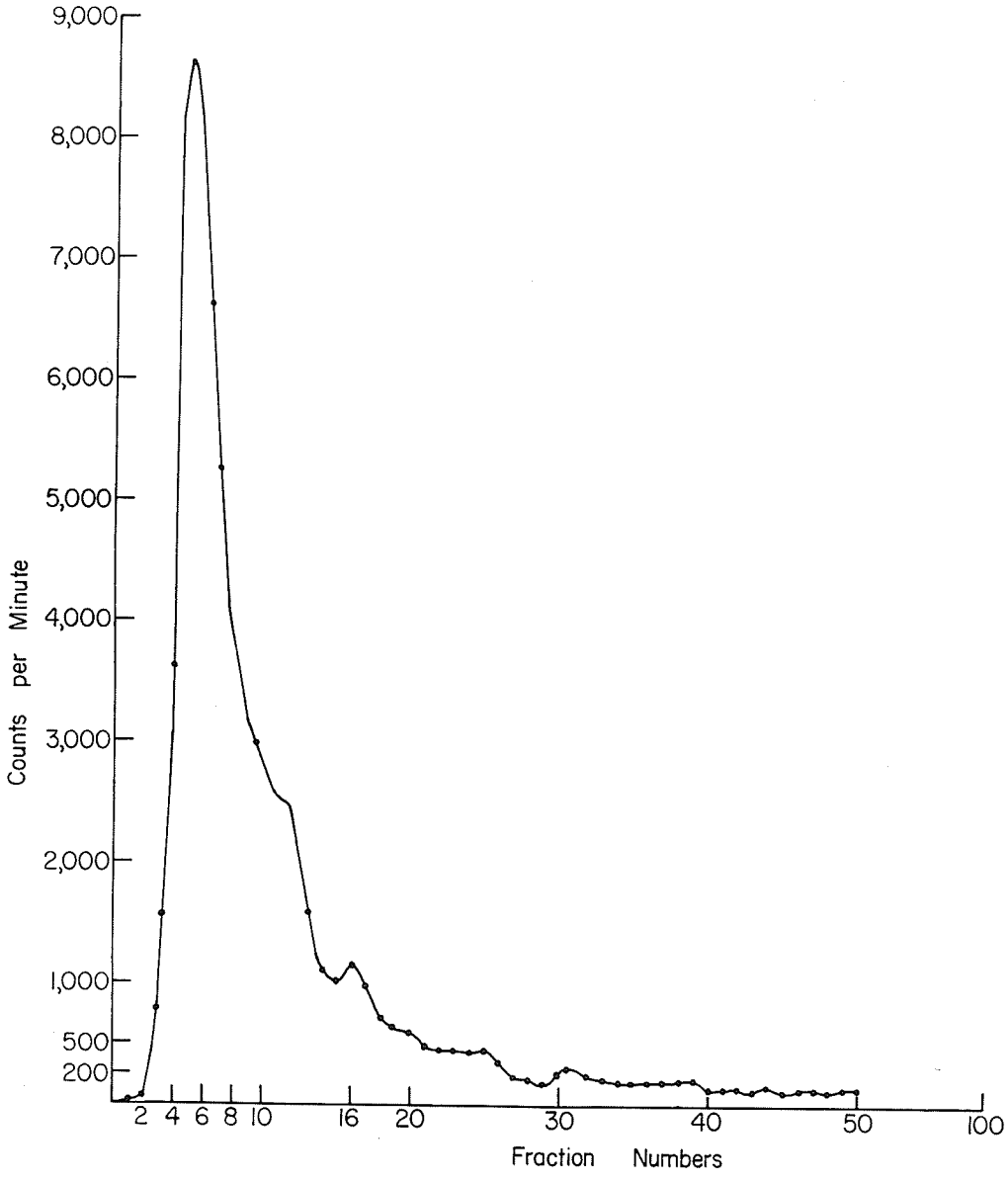
N.B. Neither benzimidazole nor the compound derived from chloroform soluble extract moved from the point of application on these types of paper in (1) and (2) solvent systems.

(1) Ligroin-benzene-chloroform-acetone-isopropyl-alcohol 50:35:10:0.5:0.17 (v/v)
(2) Ligroin-acetone-methanol 30:1:0.03 (v/v)
(3) Acetone - 30% acetic acid 1:3 (v/v)

FIGURE 10. Fractionation of chloroform soluble extract on a column of Sephadex-LH 20.

The peak represents an average of three fractionations.

Fractions from 4 - 18 with which the bulk of the radioactivity was associated were green in colour.


9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
500
200
Counts per Minute
2 4 6 8 10 16 20 30 40 50 100
Fraction Numbers

ANALYSIS OF THE GREEN PIGMENT

(a) Visible absorption spectrum:

The spectrum of the green pigment (0.02 mg) as recorded by a Unicam SP 800 Spectrophotometer in 85% acetone in glass cuvettes of 1 cm light path is shown in Figure 11. There was a strong absorption band at 412 mμ with a shoulder at 443 mμ and other small bands at 508 546, 610 and 672 mμ.

After reduction with potassium borohydride the absorption band at 412 mμ was displaced to 402 mμ, the shoulder at 443 mμ disappeared and the band at 672 shifted to 670 mμ.

(b) Molisch phase test:

If this radioactive green pigment is related to chlorophyll or phaeophytin containing a cyclopentanone ring with a C-10 hydrogen atom and a carbomethoxy group, it should generate the Molisch reddish brown coloured intermediate after the addition of alkaline potassium borohydride (Holt, 1965). The green pigment gave a positive Molisch phase test. The absorption band at 666 mμ shifted to 672 mμ.

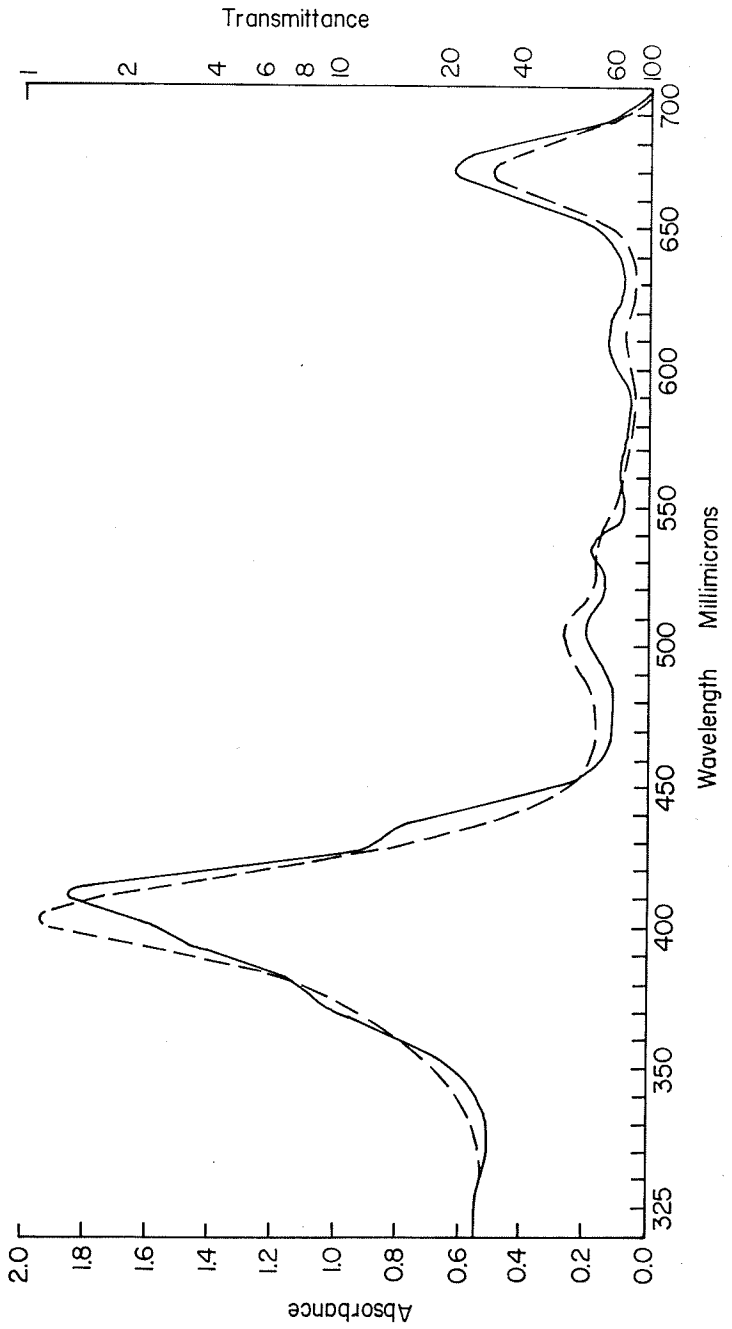
(c) Hydrolytic products:

Samples of the benzimidazole green pigment after separation on a column of Sephadex LH-20 were hydrolysed in 100 ml flasks with (a) 3 ml of 6 N HCl for 6 hours at 100^{o} and (b) 3ml of 6 N NaoH at 100^{o} for 6 hours. The hydrolysed product and washings were collected and dried in a stream of filtered air. The hydrolysed extracts were spotted on Whatman No. 1 filter paper strips and submitted to electrophoretic

FIGURE 11. Visible absorption spectra of the benzimidazole-green pigment isolated from wheat leaves.

———————— represents the spectra of the benzimidazole green coloured compound in 85% acetone.

- - - - - - - represents the spectra of the benzimidazole green coloured compound on treatment with potassium borohydride.


Transmittance
1
2
4
6
8
10
20
40
60
100
Absorbance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
325
350
400
450
500
550
600
650
700
Wavelength Millimicrons

separation in 0.5 N acetic acid, pH 2.0 for 3 hours at a voltage of 400 volts. Under the experimental conditions the benzimidazole marker moved 1.7 cm from the point of application. The hydrolytic product derived from the green coloured compound moved to the same distance as the benzimidazole marker. Figure 12 shows the diagrammatic representation of these results.

(d) Other moieties:

Since Wang (1959) has shown that benzimidazole forms a riboside in wheat leaves and Kapoor and Waygood (1968a, b) demonstrated the formation of a nucleotide or a dinucleotide by enzymatic reactions in wheat embryos it was of interest to determine whether ribose or orthophosphate were present in the benzimidazole green pigment.

Leaves treated with benzimidazole were fed ribose-1-C^{14} and $KH_2P^{32}O_4$ as described in Methods. Ribose-1-C^{14} was metabolized into at least four compounds in the water soluble fraction one of which moved to the same position as free benzimidazole (Table XII (a)). No attempts were made to identify these compounds. There was no radioactive ribose at the point of origin in the chloroform soluble extract which is the position of the green radioactive pigment (Figure 13). Experiments carried out with P^{32} orthophosphate showed that although substantial amounts of the radioactivity were incorporated into the chloroform soluble fraction (Table XII (b)), presumably with the lipid fraction, no phosphate was found to be associated with the green pigment.

(e) Detection of Copper:

In order to characterize the presence of copper associated with the

FIGURE 12. Plan of the electropherograms of the chloroform and water soluble extract of wheat leaves after feeding benzimidazole-2-C^{14} and its hydrolytic product.

A. Benzimidazole marker
Chloroform extract
Water soluble extract

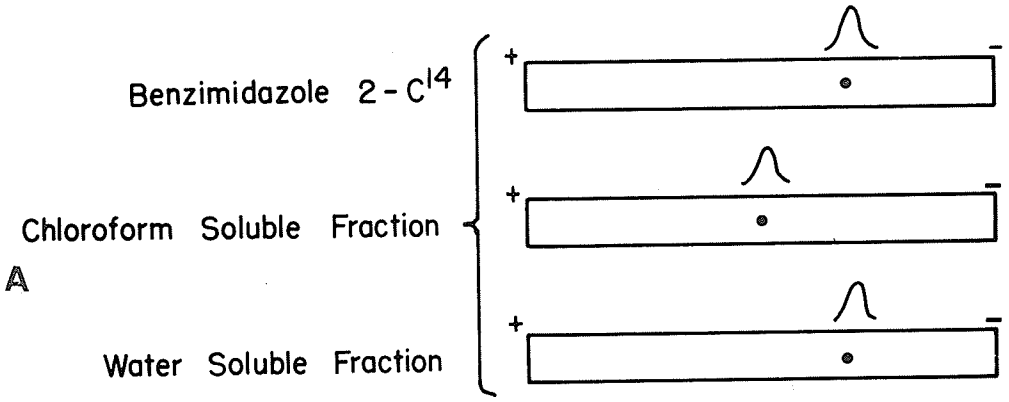
The rectangles represent electropherograms with the substance indicated at the left margin.

0 - Indicates the position on the electropherograms to which the compound migrated under the experimental conditions.

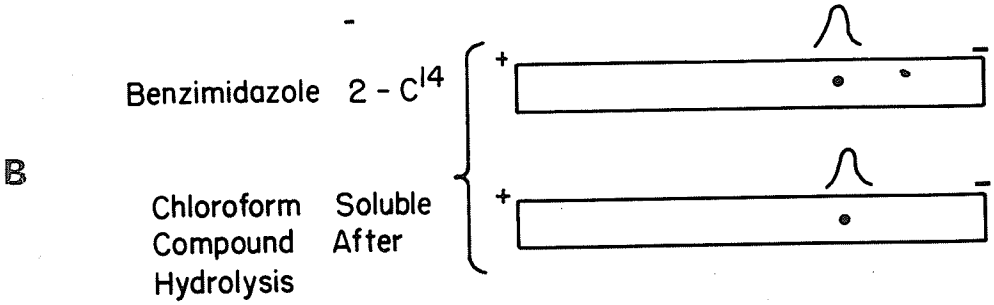
The bell-shaped curves above the rectangles symbolize the strip counter records.

B. The chloroform extract hydrolysed in 6N HCl or 6N NaOH; and the compounds observed on electrophoresis.

Electrophoresis was carried out in 0.5N acetic acid, pH 2.0 at a voltage of 400 v for 3 hours.


Benzimidazole 2 - C14
Chloroform Soluble Fraction
Water Soluble Fraction
+
-

A


Benzimidazole 2 - C14
Chloroform Soluble Compound After Hydrolysis
+
-

B

TABLE XII. (a) INCORPORATION OF D-RIBOSE-1-C^{14} INTO LEAVES FLOATED ON BENZIMIDAZOLE

Experiment No.	Radioactivity cpm/fraction	
	Chloroform soluble	Water soluble
1	0.8×10^3	28.2×10^3
2	1.2×10^3	28.6×10^3

The values are the means of two experiments.
Details of the experimental conditions are given in Methods.

TABLE XII. (b) INCORPORATION OF ORTHOPHOSPHATE-P^{32} INTO LEAVES FLOATED ON BENZIMIDAZOLE

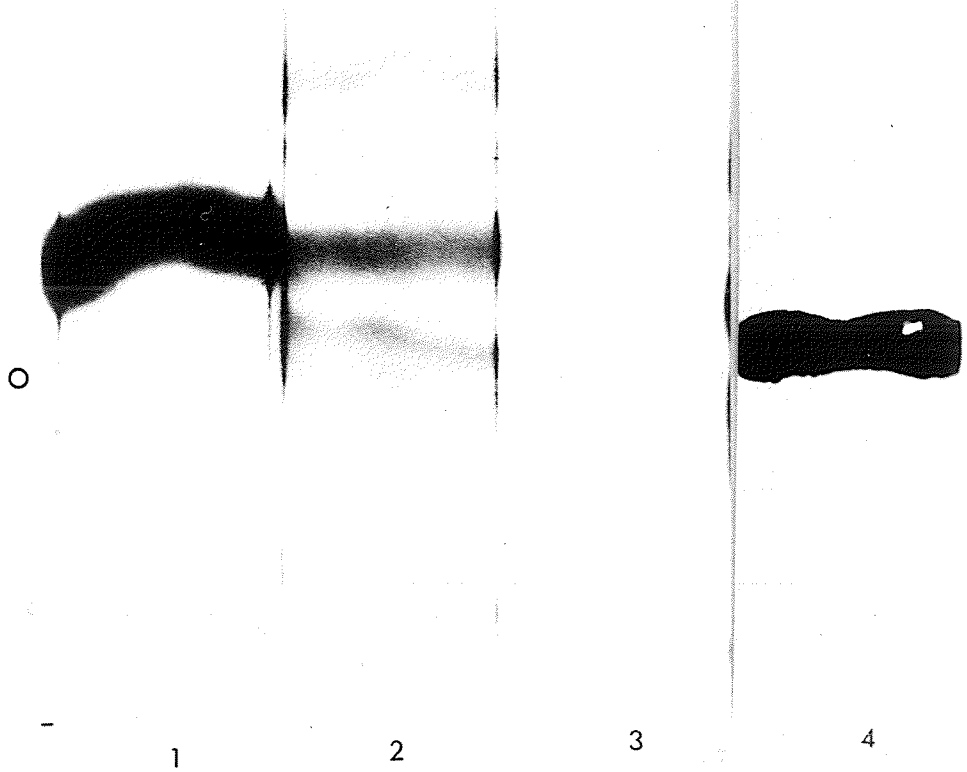
Experiment No.	Radioactivity cpm/fraction	
	Chloroform soluble	Water soluble
1	12.2×10^3	15.6×10^3
2	9.2×10^3	16.8×10^3

The values are the means of two experiments.
Details of the experimental conditions are given in Methods.

FIGURE 13. Autoradiograph of the electrophoretic separation of the chloroform and water soluble extract in 0.5 N acetic acid, pH 2.0 after feeding leaves with ribose-1-C^{14} to detached wheat leaves previously floated on a solution of 200 p.p.m benzimidazole.

1. Benzimidazole reference which moved 1.7 cm from the point of origin.
2. Water soluble extract of wheat leaves fed ribose-1-C^{14}.
3. Chloroform soluble extract of wheat leaves fed ribose-1-C^{14}.
4. Chloroform soluble extract from wheat leaves fed benzimidazole-2-C^{14}.

0 - Origin


O
-
1
2
3
4

green pigment benzimidazole derivative, these fractions obtained on a column of Sephadex LH-20 were dissolved in 1 ml of acetone. The absorption of the resulting solution was measured at 3247 A^{o} in a Perkin-Elmer Model 303 atomic absorption spectrophotometer. Copper was found to be associated with these fractions from 10 to 15 p.p.m.

EXTRACTION OF LEAVES AT ROOM TEMPERATURE

Acetone extraction procedure:

In order to resolve the nature of the green pigment derivative conditions were required in which the pigment moiety of this derivative would be prevented from being converted into phaeophytin. Both heat treatment as well as the action of organic solvents are known to effect this process (Bacon and Holden, 1967). Consequently, the leaves were extracted with 80% acetone after being allowed to metabolise benzimidazole-2-C^{14}. However, as is evident in Table XIII(a), the results revealed that even after 5 extractions with 80% acetone at room temperature only 82.55% of the radioactivity could be recovered, extraction of the green coloured pigment was also not complete and that treatment with hot ethanol had to be restored in order to recover both the residual green coloured pigment as well as the radioactivity quantitatively which accounted for 17.45% of the radioactivity.

Ethanol extraction procedure:

In separate experiments attempts were made to isolate the above compound from the leaves with 80% ethanol at room temperature. As is shown in Table XIII (b) only 76.50% of the radioactivity could be recovered

TABLE XIII. (a) INCORPORATION OF BENZIMIDAZOLE-2-C^{14} INTO WHEAT LEAVES EXTRACTED WITH 80% ACETONE

Experiment No.	Extractable Radioactivity cpm/fraction	
	80% acetone extraction	Hot ethanol extraction
1	15500	3030
2	15010	3438

The figures are mean values of two experiments.
Leaves were extracted five times with 80% acetone.
Details of the experimental conditions are as given in Methods.

TABLE XIII. (b) INCORPORATION OF BENZIMIDAZOLE-2-C^{14} INTO WHEAT LEAVES EXTRACTED WITH 80% ETHANOL

Experiment No.	Radioactivity cpm/fraction		
	80% ethanol	80% acetone	Hot ethanol
1	15075	1625	2165
2	15870	1350	2130

The figures are mean values of two experiments.
Leaves were extracted five times with 80% ethanol.
Details of the experimental conditions are as given in Methods.

after five successive extractions with ethanol at room temperature. A further treatment with hot ethanol was required to effect the complete recovery of the radioactivity as well as the green coloured pigment. These extracts on electrophoresis in different buffer systems as given in Table VI, revealed a single green coloured compound from leaves with which all the radioactivity was associated.

These results clearly show that chlorophyll cannot be quantitatively extracted from leaves floated on benzimidazole and that some residual chlorophyll still remains in the leaves with which radioactivity is associated.

The residual radioactivity as well as the green coloured compound in the leaves lend further support that benzimidazole undergoes chelation with the chlorophyll molecule which is firmly bound in the chloroplasts.

Radioactive green pigment complex in isolated chloroplasts:

The experiments reported in Section I show that benzimidazole was readily incorporated into the chloroplast. Accordingly, experiments were undertaken to evaluate the distribution of radioactivity into the chloroplasts and the supernatant fluid of leaves fed with benzimidazole-2-C^{14}.

The results from two separate experiments are given in Table XIV. The data shows that even though a considerable proportion of the radioactivity fed to leaves was recovered in the chloroplasts, a much higher proportion was recovered in the supernatant fluid. The chloroform soluble fraction of the chloroplast constituted 82.7% of the radioactivity. A

TABLE XIV. DISTRIBUTION OF BENZIMIDAZOLE GREEN PIGMENT COMPLEX IN THE ISOLATED CHLOROPLAST AND SUPERNATANT FRACTIONS

Experiment No.	Treatment	Fraction	Radioactivity* cpm/fraction	
			Chloroform soluble	Water soluble
1.	Light	Chloroplast	5.4×10^4 6.6×10^4	1.0×10^4 1.2×10^4
		Supernatant	6.0×10^5 5.3×10^5	5.9×10^4 7.2×10^4
2.	Dark	Chloroplast	5.8×10^4 5.5×10^4	2.0×10^4 2.6×10^4
		Supernatant	5.3×10^5 5.2×10^5	10.9×10^4 11.3×10^4

Primary leaves of Selkirk wheat grown in the green house for 9 to 10 days were used in these experiments.

Chloroplasts were isolated following the procedure of Jagendorf and Arnon (1958).

Fractionation of chloroform soluble and water soluble fractions were achieved as given in Methods.

* The values are the mean of two experiments based on total counts recovered.

major proportion of the radioactivity in the supernatant fraction (89.72%) still remained associated with the chloroform soluble fraction which, on further resolution, showed a single green coloured compound in electrophoretic as well as chromatographic behaviour. The pattern of the distribution of radioactivity in dark fed leaves showed a similar trend.

The higher proportion of the radioactivity in the supernatant fluid compared to that in the chloroplasts could presumably be attributed to leaching through the chloroplast membranes which are very fragile; alternatively, it could be that there are at least two sites involved in the benzimidazole action in wheat leaves as previously speculated (Waygood 1965).

BIOLOGICAL ROLE OF THE BENZIMIDAZOLE GREEN PIGMENT DERIVATIVE

The photosynthetic apparatus has been shown to reside in the chloroplasts. The photolysis of water is carried out in the lamellar system; the enzymes for carrying out the Hill reaction are known to be associated within the lamellar system (Park and Pon, 1961). The absorption of light energy by chlorophyll and the accessory pigments is confined to the aggregated discs of the grana. The present studies revealed that the action of benzimidazole was associated with the grana in the lamellae of the chloroplast; furthermore, the compound recovered was intimately associated with the chlorophyll molecule. These observations let to suggest that the action of this derivative must have some direct effect on the activities of the chloroplast in the in vitro system. It was considered that by studying the Hill reaction of isolated chloroplasts it might be possible

to determine whether or not the derivative had a stimulatory effect on the photolysis of water (Hill, 1937). Experiments were therefore carried out to measure the effect of the derivative isolated from wheat leaves on the activity of the Hill reaction.

In an attempt to determine the biological role of the green pigment a few experiments were carried out to determine its effect on the activity of the Hill reaction (Vishniac, 1957).

Twenty milligrammes of the green pigment derivative from wheat leaves were eluted on a column of Sephadex LH-20 then suspended in 2 ml of water. Varying amounts of the suspension (from 0.05 - 0.5 ml) were included in the reaction vessel to determine the effect of this complex on the activity of the Hill reaction.

Increasing concentrations of the suspension from 0.05 - 0.5 of the benzimidazole green pigment neither stimulated nor inhibited the rate of the Hill reaction activity of the chloroplasts.

The enzymatic events taking place in the process of oxygen evolution are still obscure. Catalase was implicated as a possible candidate in the reactions leading to the evolution of oxygen as hydrogen peroxide accumulates to a certain extent in the living tissue. It was argued that O_2 evolution could take place by splitting of H_2O_2 mediated by catalase.

$$H_2O_2 \longrightarrow H_2O + 1/2\ O_2$$

A few experiments were therefore conducted to determine if the benzimidazole green coloured complex had any effect on the activity of the catalase.

Varying concentrations of the derivative suspension from 0.05 - 0.4 ml (10 mg/ml) had neither an inhibitory nor stimulatory effect on the activity of the catalase reaction.

The experiments conducted were not sufficiently extensive to establish a specific biochemical role for the benzimidazole green coloured derivative and further studies on this are being continued.

DISCUSSION

DISCUSSION

The phenomenon of senescence represents the terminal phases of growth and differentiation in an organism and a number of factors have been catalogued as the possible causes for ageing. The events governing these processes are not very well understood (Varner, 1961). Perhaps it is convenient to visualize ageing as a process which renders an organism less able to withstand the stresses and strains of life. A number of changes in the metabolic events take place during the process of senescence and substances like benzimidazole exert their influence on the metabolism of the senescent tissue such as detached leaves. (Mishra 1963). It appears logical to assume that either the synthetic or degrative processes in the cell are regulated by benzimidazole which alters the metabolic activities of the cell; as a consequence of these changes the life span of the detached leaf is considerably extended.

The first approach to elucidate the mechanism of action of benzimidazole was to determine the intercellular localization of it or its derivative by feeding tritiated benzimidazole and making autoradiographs of the cellular components. The autoradiographic studies presented in Section I (Figures 1 - 4) revealed, with the 'light field' microscope that its action was localized either in or on the chloroplasts. Electron microscope autoradiography (Figure 5) confirmed that the action of benzimidazole was localized within the lamellar structure of the grana.

The lamellar structure is composed of a lipid-protein-pigment structure (Weier et al., 1966) and whereas the protein and pigment content of leaves have been shown to be increased by benzimidazole treatment (Mishra 1963; Wang et al., 1960) there have been no determinations of the lipid content under the same treatment. When fatty acid determinations were made it was shown that linolenic acid, which is the major component of monogalactosyl diglyceride and digalactosyl diglyceride in the chloroplasts (Benson, 1963), was considerably increased in leaves floated on benzimidazole solution as compared to those floated on water (Figure 6). Treatment of leaves with kinetin resulted in the maintenance of the linolenic acid at a level comparable to that in immediately detached leaves (Table II).

It has been proposed (Waygood, 1965) that benzimidazole treatment of wheat leaves results in a more highly integrated structure of the lamellae. There are more lamellae and they are more closely spaced (Yoshida, unpublished). Since glycolipids are the major lipid constituents and linolenic acid is the chief fatty acid associated with the diglyceride moiety it is possible that the effect of benzimidazole and kinetin treatment is closely related to the synthesis of unsaturated fatty acids such as linolenic acid in the chloroplasts of wheat leaves. Unsaturated fatty acids are formed by the oxidative desaturation of long chain acyl CoA derivatives by oxygen and NADP (Bloch, 1963). For example stearyl CoA is desaturated to oleyl CoA:

$$\text{Stearyl CoA} \xrightarrow[O_2]{\text{NADP}} \text{oleyl CoA}$$

McMahon and Stumpf (1964) have demonstrated a particulate system from developing safflower seeds that catalyses the conversion of oleyl CoA to linoleic acid by NADP and oxygen. The formation of linolenic acid from linoleic presumably involves a similar type of oxidative desaturation although this has not been demonstrated. The observed increase in the linolenic acid content of wheat leaves treated with benzimidazole in the present studies (Figure 6) may either stem from or follow a metabolic precursor in the synthesis of unsaturated fatty acids, which is presumably stimulated by benzimidazole. Since it is known that benzimidazole and kinetin treatment of wheat leaves augment or preserve the NADP content of chloroplasts in wheat leaves (Mishra and Waygood, 1968) it is difficult to distinguish whether the effect of kinins on lipid preservations is a direct or indirect effect on the photosynthetic capabilities of the chloroplasts.

Studies involving the metabolism of benzimidazole-2-C^{14} in wheat leaves have shown that it is incorporated into a green coloured derivative which appears to be a benzimidazole-phaeophytin complex in the chloroform soluble fraction after extraction with boiling ethanol. By rigorous and extensive electrophoretic (Table VI) and chromatographic procedure (Tables VII - XI) it was shown to be a single compound. The interpretation is that this was the tritiated compound shown to be associated with the lamellar structure of the grana. The question arises as to whether it is this complex derivative which is responsible directly or indirectly for the increased content of NADP in chloroplasts of leaves treated with

benzimidazole (Mishra and Waygood, 1968) or on the other hand, is this compound directly or indirectly concerned with lipid synthesis in the chloroplast lamellae which produces a more integrated structure capable of producing NADP more efficiently. These questions cannot be answered by the experimental observations in this thesis. The project was designed to isolate and characterize the nature of the benzimidazole derivative in wheat leaves. Kapoor and Waygood (1968) had shown the presence of enzymes catalysing the formation of benzimidazole riboside and benzimidazole adenine dinucleotide in wheat embryos. These differences may be explainable as arising out of chlorophyllous and non-chlorophyllous tissue.

A single compound was recovered from leaves fed with benzimidazole-2-C^{14} under varying degrees of etiolation from completely etiolated to those on halfway to greening as characterized by their electrophoretic and chromatographic behaviour (Figure 8). These results are explainable that benzimidazole undergoes chelation with the protechlorophyll molecule when the compound recovered is yellow in completely etiolated leaves greenish yellow in partially etiolated, which becomes progressively green in leaves on halfway to greening as chlorophyll is being synthesised on exposure to light from protochlorophyll — the immediate precursor of the former. It is imperative to conclude that benzimidazole undegoes chelation with the chlorophyll molecule which leads to the stabilization of the pigment-protein-lipid complex in the lamellae of the chloroplast — the site of chlorophyll localization. As a consequence of this act the

destruction of the green coloured pigment is considerably prevented which in turn leads to the reactions of the cyclic photophosphorylation to be continued for an extended period in the lamellar system *in vivo*. It is reasonable to interpret that the stimulatory effect exerted by benzimidazole on chlorophyll destruction as well as on its formation as proposed by Wang and Waygood (1959), is probably as a consequence of chelation which is the primary event in delaying the senescence of detached leaves as a result of which, other metabolic activities of the chloroplast are able to be continued. Intact benzimidazole was recovered on hydrolysis of the green coloured compound (Figure 12); the results suggest that a specific structural integrity of benzimidazole is involved in the maintenance of its action of chlorophyll destruction in detached wheat leaves and is consistent with the evidence marshalled by Wang *et al.*, (1959) that both benzene ring as well as imidazole moiety are essential for the manifestation of its action in delaying the senescence of detached leaves.

There was insufficient time to study in depth the biological role of the benzimidazole-phaeophytin complex. In any case it was considered an artefact of extraction and unless some special means of extraction are devised without altering its *in vivo* biological activity (if any) it will be difficult to assess its biological importance. The questions remain for further investigations.

SUMMARY

SUMMARY

The investigations with tritiated benzimidazole were undertaken to locate specifically the intracellular localization of its action in wheat leaves. Studies were made on the changes in the fatty acid content of wheat leaves undergoing senescence on benzimidazole and because of the parallel metabolic changes exhibited by kinetin, the studies on its effect on fatty acids were also undertaken. Of prime importance in these investigations was the metabolism of benzimidazole in wheat leaves to determine, if possible, the locus of its action with a view to explaining the mechanism of this action. The salient results of these investigations are:

1. The autoradiographic studies with the light field microscope revealed that the action of benzimidazole was localized either in or on the chloroplasts.

2. Electron microscope autoradiography gave unequivocal proof that its action was localized within the lamellar structure of the grana.

3. Linolenic acid was found to be the principal unsaturated fatty acid associated with wheat leaves.

4. Treatment of wheat leaves with benzimidazole enhanced their linolenic acid content which presumably represents that of the chloroplasts in the leaves.

5. Wheat leaves, on treatment with kinetin, although not acting in the same manner as with benzimidazole, still maintained their linolenic acid content as compared to water floated leaves.

6. The studies on the metabolism of benzimidazole-2-C^{14} in wheat leaves showed that the major proportion (88.95%) of the radioactivity administered to wheat leaves was recovered associated with the green coloured compound which appears to be a benzimidazole-phaeophytin complex isolated from the chloroform soluble fraction of leaves.

7. Leaf sections, with and without vacuum infiltration showed a similar pattern in their incorporation of benzimidazole.

8. Leaves fed in darkness exhibited a similar trend in the distribution of radioactivity.

9. The visible absorption spectra exhibited by the compound showed that benzimidazole chelates with the chlorophyll molecule; the fractionation behaviour of the compound on Sephadex LH-20 indicated it to be a high molecular weight compound.

10. No ribose-1-C^{14} or P^{32}, fed to leaves previously floated on benzimidazole, was found associated with this green coloured compound.

11. Acid and alkaline hydrolysis resulted in the liberation of free benzimidazole.

12. Leaves under varying degrees of etiolation from completely etiolated to midway to greening, showed higher incorporation of radioactivity into the water soluble fraction than compared with green leaves. The compound recovered from completely etiolated leaves was yellow

coloured which became progressively green from completely etiolated to those midway to greening.

13. As at the present time, with few studies, no biological role could be attributed to the compound per se. The significance of these investigations has been discussed in relation to the mechanism of benzimidazole action in wheat blades.

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