EFFECT OF KINETIN AND OTHER GROWTH REGULATORS ON CHLOROPHYLL SYNTHESIS IN INTACT, ETIOLATED BEAN SEEDLINGS (PHASEOLUS VULGARIS, VAR. COMMODORE)

by

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INTRODUCTION

Growth in plants depends on the organization of many specialized cells and consists of specific, metabolic pathways. One such pathway is chlorophyll metabolism in plastids which is regulated by several growth factors, both external and internal. Of these factors, growth hormones play an important role in cellular organization and organelle differentiation because of their influence on gene expression.

Cytokinins have been implicated in several aspects of plant growth (65). Recently the requirement of kinetin for chlorophyll production (148, 134) and chloroplast differentiation (134) was interpreted as correlation between a growth regulator and the maturation of an organelle. Therefore, a study was designed using etiolated bean seedlings to investigate the influence of applied kinetin and other growth regulators on chlorophyll synthesis.

LITERATURE REVIEW

Photosynthesis is a photoautotrophic reaction occurring in algae, certain bacteria and higher plants for the formation of organic compounds in chloroplasts from carbon dioxide, water, and sunlight. The combined photolytic and anabolic process results in the evolution of oxygen and water and the synthesis of ATP and $NADPH_2$ which provides the energy for carbon dioxide incorporation into certain carbohydrates and other metabolic compounds (9, 13). The oxidation of these energy-containing compounds provides energy for the growth of most organisms and the maintenance of life itself. Therefore the production of organic matter is dependent on photosynthesis and is hence directly influenced by the physiological makeup of the chloroplasts and the raw materials (carbon dioxide, water, sunlight) available for this process. No attempt will be made to discuss in detail the photosynthetic process because several comprehensive reviews are available (9, 10, 13, 39, 53, 109).

The biochemical evolution of photosynthesis of green algae has been suggested to have emerged from the development of a porphyrin which eventually gave rise to chlorophyll (10, 60, 145) within chloroplasts of unicellular organisms. Considerable work has been published on the basic similarities of plastids in respect to their physiological makeup and biochemical structure in Chlorella, Euglena, and Chlamydomonus and in isolated-intact chloroplasts from higher plants (58, 146). The similarity in plastid (24, 58, 60, 146) and chlorophyll distribution (4) between green algae and higher plants is just one piece of evidence supporting the belief that angiosperms evolved from these unicellular, chlorophyll-containing organisms (89).

Chlorophyll Synthesis and Degradation

In the light reaction, the chlorophyll pigment absorbs and transmits the sun's radiant energy as an excited electron which is passed on to an unknown acceptor. The importance of this energy transduction in photosynthesis has stimulated extensive research in chlorophyll metabolism. This pigment and another tetrapyrrole compound, heme, have a common precursor of protoporphyrin. Heme is found in both plants and animals and functions as a catalyst for respiration while chlorophyll serves as a catalyst to convert radiant energy into chemical energy. Therefore the basic energetics of protoplasm of both plants and animals involves these two pigments which are end products of the same biochemical chain (60).

The biosynthesis of chlorophyll is generally investigated in etiolated plants under illumination. Three stages of greening have been illustrated: photoconversion by light of accumulated protochlorophyll(ide) to chlorophyll(ide), a "lag-phase" in which relatively no pigment is formed, and a net synthesis of chlorophyll that is maintained at a constant rate (47) depending on the species, age, and other factors (126). The formation of protochlorophyll involves several enzymatic reactions. δ-aminolaevulinic acid (δ-AL)[1] is first formed within mitochrondria from the succinate-glycine cycle (118, 119, Fig. 1) although according to Granick (60), it is not known if the synthesis of this metabolite occurs exclusively within mitochondria or if plastids can also produce δ-AL. Both organelles contain their own DNA (51) but whether the coding of the necessary enzymes for δ-AL synthesis comes from nuclear DNA or from the respective organelle DNA is

1. List of Abbreviations

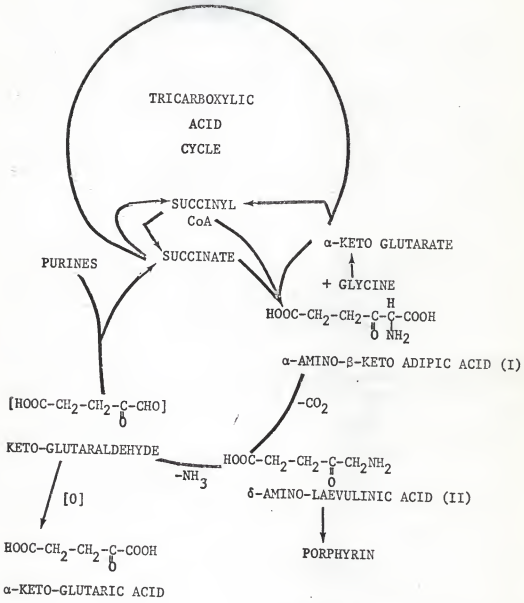


Fig. 1. Succinate-glycine cycle: a metabolic pathway for the synthesis of δ-aminolaevulinic acid and porphyrins [According to Shemin et al. 1955 (119)].

yet to be shown (60).

Studies on the biosynthesis of δ-AL in chicken erythrocytes (56) and Chlorella (26) suggest the requirement of an electron transfer system to form ATP. A portion of the citric acid cycle (α-ketoglutarate and thiamine pyrophosphate, lipoic oxidase, NAD^+, Coenzyme A) is required to form active succinate (succinyl-CoA). Glutamic dehydrogenase is required to oxidize $NADH + H^+$ to NAD^+. A more comprehensive review of the various pathways of succinyl-CoA biosynthesis in erythrocytes and plants has been published by Granick (60). With the addition of glycine, pyridoxal phosphate and δ-AL synthetase, δ-AL is synthesized and then dehydrogenated (139) by δamino-laevulinic acid dehydratase to porphobilinogen, a monopyrrole (139).

$$\text{Succinyl-CoA + Glycine} \xrightarrow[\text{Pyridoxal Phosphate}]{E_1} \text{δ-AL} + CO_2 + \text{CoA}$$

$$2\ \text{δ-AL} \xrightarrow{E_2} \text{PBG} + 2H_2O$$

This enzymatic reaction requiring δ-ALase, involves a condensation of two molecules of δ-AL to form PBG (60). Two bonds are formed; a carbon-carbon bond at (A) (Fig. 2) and a carbon-nitrogen bond at (B) (Fig. 2) with the release of two water molecules. The formation of two bonds for each molecule of PBG formed suggest the action of two enzymes but the presence of one activity peak using zone electrophoresis on starch indicates that only

E_1. δ-AL synthetase: Isolated from chicken erythrocytes and appear to be located on cellular particles presumably mitochondria (115).

E_2. δ-ALase: Isolated from the insoluble portion of ruptured chloroplasts (36, 139). According to Granick, δ-ALase is water soluble and therefore can be extracted from ruptured mitochondria of liver cells (60).

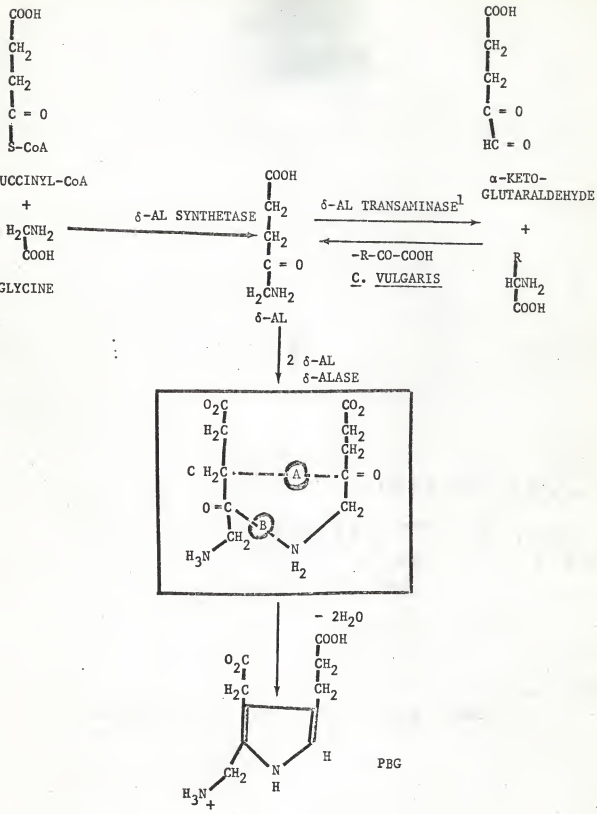


Fig. 2. Biosynthetic pathway of chlorophyll in higher plants and green algae. [According to Bogorad. 1966 (24)]

1. Gassman et al. (50) has detected an enzyme in extracts of Chlorella vulgaris which has the ability to catalyze the formation of δ-AL.

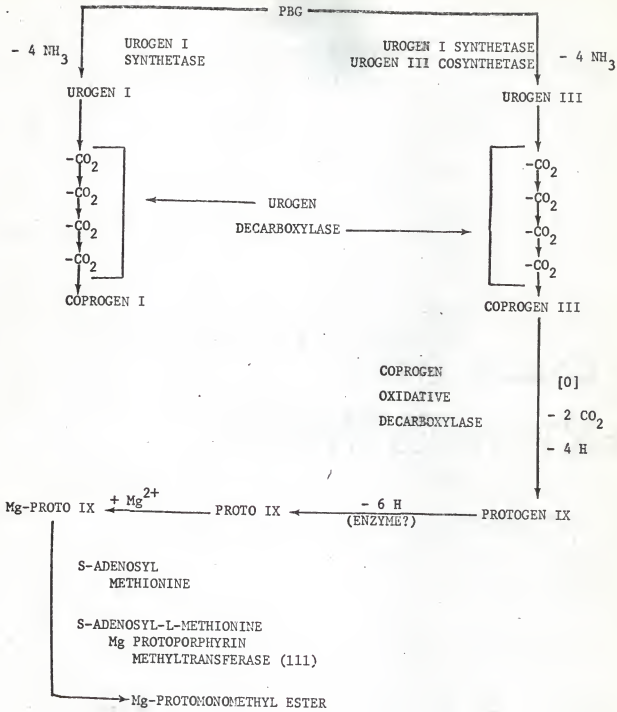
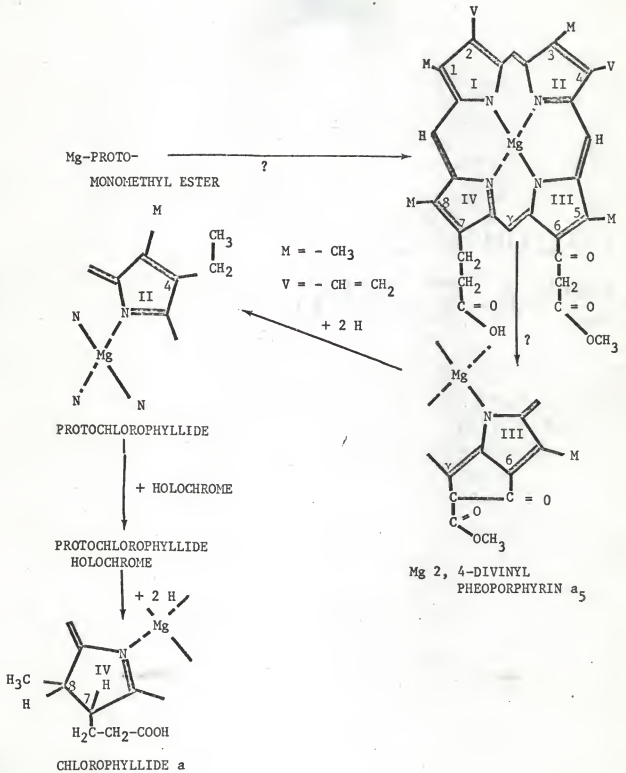


Fig. 2. (Continued)



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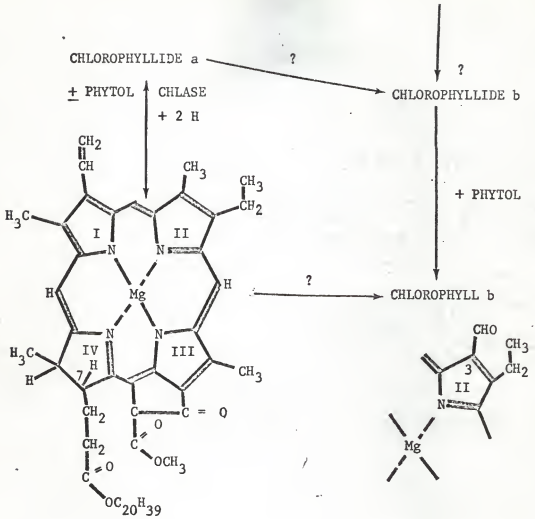

Fig. 2. (Continued)

one enzyme is involved. The enzyme activity (36, 139) increases with greening plastids and is considered to be located in the membranes of the chloroplast and to be related to the development of the grana found in chlorophyll-containing chloroplasts (139). Further comprehensive studies on enzyme activity, stability substrates, inhibitors and action mechanism of δ-ALase have been published (57).

In 1953, Bogorad and Granick (26) published data which demonstrated the enzymatic similarity of the biosynthesis of heme and chlorophyll. Porphobilinogen, isolated from the urine of patients with acute porphyria, was used as a substrate for Chlorella cells to synthesize porphyrin. The reaction was supported by the algal cells if they were pretreated by being frozen and thawed before the incubation period of 20 hr. at 30°C. These PBG enzymes involved have been suggested to have different degrees of inactivation. This was demonstrated by an experiment in which frozen and thawed Chlorella cell preparations were heated to 55°C before incubation. The heating had no effect on the rate of PBG disappearance but the derivatives and yields of the porphyrin were altered. At the end of the incubation period, all PBG had disappeared. In the control, unheated Chlorella cells, 55% of the new porphyrin (calculated by PBG disappearance) could be accounted for by the initial PBG, while 90% of the final yield in the heat treated extract was accounted for by the metabolized PBG. This suggested that the enzyme which catalize non-porphyrins [purines and other metabolites (26, 112)] in unheated preparations may be resistant to heating at 55°C.

The significant result from heating the preparation (55°C), in comparison to the unheated, is the production of only Uro I. In unheated preparations, the Uro present after a 24-hour period was entirely isomer I, but

after 4-hour induction, isomer III predominated the suspension. Therefore it appears that the enzyme necessary for Uro III production was broken down over the 24-hour incubation period and isomer III, produced before the 4-hour period, was metabolized into other porphyrins. Similar effects of pre-heating were found to occur in whole chick and human red cells (29).

Two hypotheses to explain the critical step which determines the isomer of Urogen formed, were published by Shemin et al. (119) and Bogorad and Granick (26). The former investigators postulated that an intermediate tripyrrylmethane could be formed from a dipyrrole plus PBG. The tripyrrylmethane then splits into a dipyrrylmethane and a monopyrrole. The formation of either of the isomers is dependent on the breakage of the intermediate (Fig. 3). Isomer III formation results in elimination of a 1-carbon compound which appears to be formaldehyde. This hypothesis was substantiated by the formation of this compound from PBG to porphyrin biosynthesis either by heating under acid conditions or by enzymatic conversion in cell-free extracts (119). The second explanation (26) accounted for the fact that only I and III isomers are found in nature by postulating the formation of a branched "T" tetrapyrrylmethane (Fig. 3). If the tetrapyrrylmethane splits at A, only Urogen I could be formed and the splitting at B would result in the production of isomer III. Two possible mechanisms for the synthesis of this "T" intermediate in respect to its stearic-enzymatic relationship with porphyrin synthesis is extensively discussed by Bogorad and Granick (26, Fig. 4) and others (21, 24). Another explanation as to why only isomers I and III are found in nature and not II and IV, is the evolution of the former compounds which are advantageous to the system (60). If PBG is heated in acid solution, all four isomers will be formed. If PBG

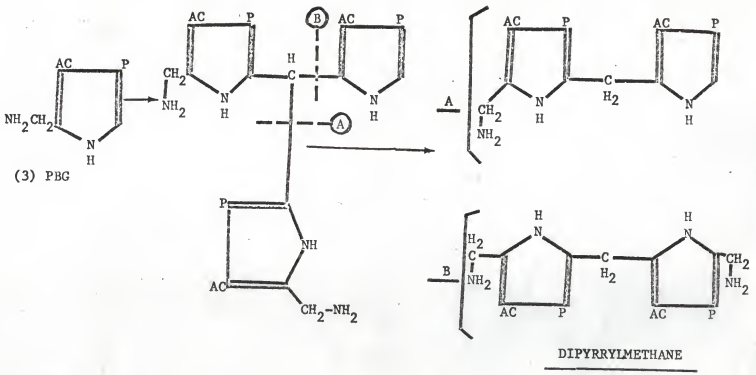

Fig. 3. Shemin et al. (119) hypothesis on the formation of isomers Urogen I and III. The condensation of two molecules of dipyrrylmethane A would give rise to isomer I and the condensation of a mole of A and a mole of B would give rise to isomer III. P = $-CH_2-CH_2-COOH$, AC = $-CH_2-COOH$.

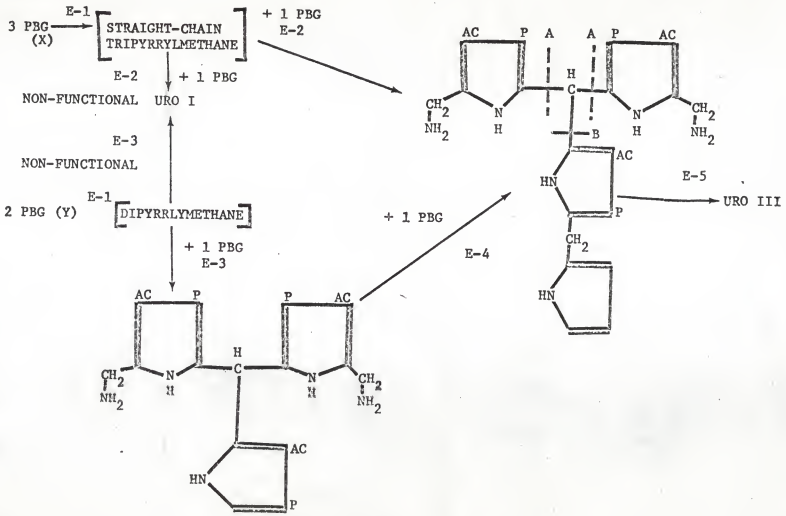

Fig. 4. Bogorad and Granick's (26) hypothesis on the formation of isomers Urogen I and III. Data from Chlorella mutants indicate that Urogen III arises from split at B. E = enzyme, P = $-CH_2-CH_2-COOH$, AC = $-CH_2-COOH$.

is heated in neutral or alkaline solution, only siomer I and III are formed (40). Therefore a slightly basic environment such as sea water would support an enzyme which catalizes these isomers. Granick (60) also stated that Urogen, being an intensely absorbing fluorescent, and photoactive dye, is the first porphyrin which could support biochemical reactions. Uroporphyrin forms a chelating complex with iron thus possessing the capacity to act as a weak redox catalyst. A possible explanation for the additional steps to chlorophyll synthesis is to evolve a more efficient redox and photosynthetic system. This evolution of chlorophyll proceeded from a water-soluble compound to more lipid-soluble compounds which are organized in lipoprotein membranes (60). Several evolved end products have been found in algae and are known to take part in photochemical reactions: protein-bile pigments, phycocyanin and phycoerythrin found in red and blue-green algae (145).

The enzymes associated with the synthesis of isomers I and III are Urogen deaminase (PBG-deaminase) (18) and Urogen isomerase (19). The former enzyme involves the deamination of PBG to form isomer I. The mechanism involves splitting out one molecules of ammonia from the aminomethyl group of PBG and the hydrogen from the unsubstituted α-position of another molecule of PBG. Therefore Urogen I synthesis involves four molecules of PBG to form one molecule of isomer I with four ammonias given off (18). The oxidative product of isomer I (uroporphyrin I) was originally suggested to be formed by an enzyme within a crude preparation of PBG-deaminase from spinach leaves (18). Now it is believed that the oxidized porphyrins are formed because of the lack of deterioration of Urogen isomerase in the porphyrin enzyme system. It was suggested by Rimington (112) and others

(23, 83, 118) that Uro I and Copro I are not reducible in vivo, so cannot become part of the biosynthetic pathway, thus they are end products similar to heme and chlorophyll. It is interesting that these are the compounds which are predominantly found in acute cases of porphyria (26, 112).

Urogen isomerase catalyzes the synthesis of isomer III in the presence of PBG-deaminase. Incubated alone with PBG, this enzyme is inactive (23). Therefore in an enzyme system of both PBG-deaminase and Urogen isomerase, Urogen III is synthesized. When isomerase has deteriorated, possibly because of high temperature, Urogen I is formed (18, 19). Originally it was believed that these isomers were precursors for heme and chlorophyll biosynthesis depending on which enzyme is present (20). Now recent data indicate that Uro I is an end product, Urogen I can serve as a substrate (20) and Urogen III is the biosynthetic intermediate of porphyrin assimilation (55). This was also shown by Neve et al. (101) who reported the conversion of reduced Uro III (uroporphyrinogen III) to Copro and PBG by an enzyme system from lysed duck erythrocytes.

Pure enzymatic preparations used in current studies of Urogen I and III biosynthesis in higher plants have been found: the relatively heat-resistant Urogen I synthetase [formerly called Urogen deaminase (23) and PBG-deaminase (18)] from spinach and the heat susceptible Urogen III cosynthetase from wheat germ (22). Several inhibitors for these synthetases are known (23). Noncompetitive inhibitors such as formaldehyde, amonium hydroxide, NH_4^+, Hg^{++}, p-chloromercurobenzoate seem to reduce the consumption rate of PBG. The competitive inhibitors are PBG analogues such as apsopyrroledicarboxylic acid and isoporphobilinogen. Another inhibitor includes hydroxylamine which strongly interferes with the formation of

Urogen I but has only a slight effect on the rate of PBG consumption. Hydroxylamine also interferes with cosynthetase in Urogen III formation presumably by disruption of the two-enzyme system (23).

Two Coprogen isomers can be formed, through intermediates with 7, 6, and 5 carboxyl groups per each molecule, by decarboxylation of Urogen I and III in vitro (23). Coprogen III is the functional intermediate while Coprogen I and the oxidized isomers are comparable to Uro I and III in that they are end products formed from decarboxylation of Uro (21). The decarboxylation is catalyzed by one enzyme, Urogen decarboxylase. Urogen III with 8 carboxyl groups is decarboxylated successively and at random at the acetic acid side-chains to form Coprogen III which has 4 carboxyl groups. Intermediate compounds with 7, 6, or 5 carboxyl groups have been isolated and used as substrates for Urogen decarboxylase for Coprogen synthesis (135). The enzyme has not been isolated from plant tissue but its existence is indicated by the production of Copro and Proto IX from Urogen III in a cell-free preparation of Chlorella (21). As yet, no cofactors for this enzyme are known. The presence of Hg^{++}, Co^{++}, Mn^{++}, iodoacetate and p-chloromercuribenzoate inhibit Coprogen formation (23).

The formation of Protogen IX from Coprogen III is an enzymatic decarboxylative and dehydrogenative reaction by Coprogen oxidative decarboxylase which has been prepared from beef liver mitochrondria (115). Broken-cell preparations of Chlorella vulgaris have been shown to catalyze the synthesis of Protogen IX from Urogen III and PBG (26). Ruptured isolated plastids from Euglena gracilis will also support the synthesis of Protogen IX from δ-AL (36). This reaction needs oxygen as an enzymatic coenzyme (115). The possibility of other oxidants supporting this reaction have

been tested but with no success (24). The enzyme mechanism involves the formation of vinyl groups from the propionic acid side-chains in positions 2 and 4 on rings I and II respectively, of Coprogen III by decarboxylation and dehydrogenation (24). The enzyme specificity is two or three times faster for isomer III than isomer I (115).

The identification of biosynthetic steps for chlorophyll metabolism from Proto IX are based on studies of irradiated mutants of Chlorella in which the synthetic chain was blocked at Proto IX, Mg-Proto IX, Mg-protomonomethyl ester, and Mg-vinyl pheoporphyrin-a_5. When placed in sequence, these intermediates indicate a logical series of reactions but these porphyrins are not thought to be true intermediates (60, 135). Granick (60) stated that the verification of these intermediates will be based on substrate-enzymatic reactions. Accumulation of protochlorophyllide-a in etiolated leaves treated with δ-AL suggests that the enzyme system, except for δ-AL synthetase, for porphyrin synthesis exists in the chloroplast (60). Evidence for succinyl-CoA formation in plastids indicates that all enzymes from δ-AL to chlorophyll are present in this organelle (60). Therefore based on the studies of Chlorella mutants (60), plants treated with δ-AL (60), and the purple non-sulphur bacteria (69), the following pathway was proposed; Proto IX → Mg-Proto IX → Mg-protomonomethyl ester → Mg-2,4-divinyl pheoporphyrin-a_5 → protochlorophyllide-a → chlorophyllide-a → chlorophyll-a.

The point of Mg incorporation is unknown. Its insertion after Proto IX formation is based on whether Mg resembles the chelation of iron (39). Mg-Proto IX is esterified fifteen times faster than Proto IX which indicates the following sequence: Proto IX → Mg-Proto IX → Mg-protomonomethyl ester (141). The possibility of this reaction being enzymatic (101) or non-enzymatic (57, 93) is still being investigated.

Mg-protomonomethyl ester has been isolated from a chromatophase fraction - Rhodopseudomonas spherordes - which contained a methylating enzyme, Mg-Proto IX and S-adenosyl methionine (the active methyl) (141). Ethionine and threonine seem to inhibit bacteriochlorophyll synthesis in R. spheroides while methionine reverses the effect of both ethionine and threonine while homoserine will overcome the inhibition of threonine. The action of ethionine inhibits the formation of the methyl ester group by transmethylation from methionine (52).

Mg-protomonomethyl ester has been isolated from a Chlorella mutant and etiolated plants treated with δ-AL. In another mutant, data indicated that this intermediate appeared to be the immediate precursor of protochlorophyllide-a (54). Green et al. (61) has shown formate to be a precursor of the methyl ester group of chlorophyll in Chlorella.

Radmer and Bogorad (111) detected an enzyme in an isolated chloroplast fraction from Zea mays which catalyzes the transfer of the methyl group from (-) S-adenosyl-L-methionine to form Mg-Protomonomethyl ester from Mg-Proto. A similar methyltransferase system has been found in Phaseolus vulgaris which exhibits similar properties as the enzyme system in corn (110).

Relatively nothing is known about the cyclopentanone ring found in Mg-2,4-divinyl pheoporphyrin-a_5: a step between Mg-protomonomethyl ester and protochlorophyllide-a (60). This intermediate has also been isolated from a culture of R. spheroides grown anaerobically in the light in the presence of 8-hydroxyquionotine (25 mm) (69). Smith et al. (135) cited a mechanism which involves the oxidation of the propionic acid side-chain at position 6 (the beta position) to form a ketone group, the cyclization between the alpha carbon of the propionic acid and the gamma carbon of the

porphyrin ring followed by hydrogenation of the vinyl group at position 4 to form protochlorophyllide-a. The enzymes catalyzing this reaction are unknown (14).

In the natural state, protochlorophyll(ide) and chlorophyll are attached to a protein carrier (128). The general term "holochrome" has been given to protochlorophyll or chlorophyll in its natural state. This term was extended to the extracted pigment-protein-complex calling it protochlorophyll holochrome (132). The molecular weight of this complex has been calculated to be 600,000 ± 50,000, from measurements of sedimentation coefficient (18S), diffusion coefficient (2.70×10^{-7} $cm^2/sec.$), and partial specific volume (0.73 ml/g). The ratio of pigment to protein molecule is an average of one to one (14). This complex has been shown to be physiologically active in that upon illumination a large fraction of the protochlorophyll holochrome is converted to its reduced form (132).

According to Granick (60), the protein molecule's role could be enzymatic or structural in nature within a developing chloroplast. The linking of holochrome to pigment takes place after the formation of protochlorophyllide. Whether this is achieved by a specific enzyme or by some other means is unknown (135). Based on Boardman's recent work (14), Trown (144) concluded that protochlorophyll holochrome occurs in a protein moiety with carboxydismutase[1] which collectively is known as fraction I protein. Further information on the properties of this holochrome has been published by Smith and his co-workers (128, 131, 132).

1. Carboxydismutase (ribulose 1,5-diphosphate carboxylase) catalyzes the carboxylation of ribulose 1,5-diphosphate (144).

The formation of chlorophyllide-a in higher plants require a light-mediated reduction at C-7 and C-8 in trans position on ring IV of protochlorophyllide-a (4). Exceptions have been found in some algae and gymnosperms (132), therefore the addition of 2H atoms is probably enzymatic instead of photolytic (151). Smith found that the quantum yield for chlorophyllide formation is about 0.6 which suggests the possibility that one light quantum is necessary for this photoreduction (133). Other insights concerning this conversion are: the rate of transformation is proportional to the light intensity, the action spectrum parallels the absorption spectrum of protochlorophyll, reduction is inhibited by temperature above 55° and decreases as temperature is lowered (135).

Boardman (15) has found that the photo-conversion of an isolated protein pigment complex takes place only when the protochlorophyll is attached to the native protein. The rate of transformation is independent of the initial concentration of the complex. The course of the two hydrogen atoms is unknown. The donor molecule seems to be a part of the moiety and possibly is located in the hydrophobic portion of the complex. Smith (135) has shown that the hydrogen or electron donor most likely is not water.

Smith and Benitez (129) proposed that this conversion is a bimolecular reaction that follows the second order reaction. This has been verified by Virgin (150). From kinetic studies by Boardman (15), the conversion could also fit 2 first order reactions. This is based on the hypothesis that the active protochlorophyll molecules are bound to the protein in two ways (15, 95).

After illumination, chlorophyllide-a undergoes esterification with phytol (3,7,11,15-tetramethyl-2-hexadocen-1-ol) at the propionic acid residue

at position 7 to form chlorophyll-a. Observations have been made by several workers which suggest that phytolation is an enzymatic reaction in vitro (17, 37, 121, 122, 156) by Chlase which also is known to be capable of hydrolytic activity in vitro (85, 94, 103).

$$\text{Chlorophyllide} + \text{Phytol} \underset{\text{Chlase}}{\overset{\rightarrow}{\leftarrow}} \text{Chlorophyll}$$

Smith has stated that this reaction is a thermoreaction and apparently both enzyme and phytol are photo-induced (135). Shimizu et al. (119) suggested that the ratio of chlorophyllide and/or pheophorbide to phytol might regulate the mode of action of Chlase. A low concentration ($0-.5 \times 10^{-4}$M) results in a breakdown of chlorophyll while the levels of .5 to 7.0×10^{-4}M tend to regulate the phytolation of the chlorophyll molecules. In vitro studies on phytolation in tobacco plants have shown ratios of 3.3 and 2.5 of chlorophyllide and pheophorbide, respectively, to phytol. Young plants known for large quantities of chlorophyll such as Picea excelsa (1.82) and Pinus sylvestris (1.52) have ratios which indicate a synthetic activity for Chlase. One possible error was noted by the author in that extremely high acetone concentrations were used to overcome experimental difficulties. Therefore this ratio can be only speculation and remains to be established in nature (122). Granick also indicated that phytolation occurs via a phytol pyrophosphate mechanism instead of the activity of Chlase (60). Thus the questions concerning a possible dual mechanism of synthesis and degradation for Chlase remains unanswered (17).

The characterization of Chlase has been published by Holden (66) and Böger (17). Holden prepared a soluble fraction of Chlase from sugarbeet

leaves. The use of ethanol and ether suggests that in the mature leaf, this enzyme is associated with lipids (66). Ardao and Vennesland extended this concept in saying that Chlase may be present in the lipid-chlorophyll-protein complex (7). In etiolated pea seedlings, the low Chlase activity increases to comparable levels of light grown seedlings within a 48 hr. period (66).

Multiple investigations on the biosynthesis of chlorophyll-b has evolved four biological schemes: (1) chlorophyll-b is formed from chlorophyll-a; (2) chlorophyll-a is formed from chlorophyll-b; (3) chlorophylls (a & b) are independently formed from a precursor (x); and (4) two entirely different pathways exist for each of these chlorophylls (98).

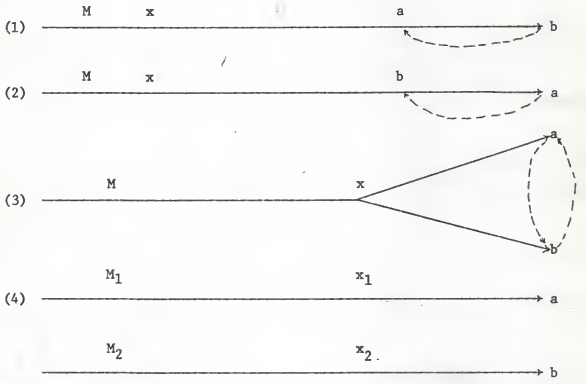

Fig. 5. Four biological schemes for chlorophyll-b synthesis. M represents metabolic pathway [According to Michel-Wolwertz, 1963 (98)].

Only the first and third schemes will be discussed here. The classical concept (1) is sequential in that the methyl group (chlorophyll-a) is oxidized to a formyl group (chlorophyll-b). This concept is supported by several observations: no plants have been found to contain chlorophyll-b without chlorophyll-a (124), protochlorophyllide-b has not been isolated (60), and chlorophyll-a accumulates before chlorophyll-b is detected (24). Recently Shlyk and Nikolayeva (122) illustrated the above sequence by a pulse labelling experiment with barley seedlings which were fed $^{14}CO_2$. Labelled chlorophyll-a activity was higher than labelled chlorophyll-b. Since no chlorophyllide-b was isolated, chlorophyll-b was indicated to be formed from chlorophyll-a. A similar experiment with Chlorella vulgaris using acetate 1-C^{14} illustrated photo-oxidation of chlorophyll-a to chlorophyll-b (98). Total activity with increased light intensity was measured during greening. The increase in chlorophyll production revealed an activity increase parallel to light intensity increase and two stages of each pigment exist which differ in "age". Chlorophyll-b incorporated more radioactive carbon at the stronger light intensities than chlorophyll-a. This was interpreted to indicate that light intensity acts as a regulating mechanism on the synthetic step(s) of chlorophylls: chlorophyll-a biosynthesis is favored by a low light intensity while chlorophyll-b formation is favored by high light intensity. Based on this concept, the author presented the following scheme on chlorophyll-b synthesis.

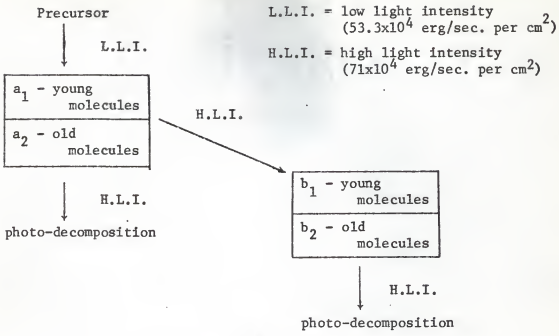

Fig. 6. A possible scheme showing the effect of light intensity on the biosynthesis of chlorophylls (a & b). [According to Michel-Wolwertz, 1963, (98)].

According to this scheme, the young chlorophyll-a molecules could be called "protochlorophyll-b" (98).

The third scheme, proposed by Smith (134), is a parallel production of both chlorophylls (a & b). This alternate is based on the proportional rate of greening found in etiolated leaves under illumination (16). Smith and French (135) theorized that if chlorophyll-b is formed from chlorophyll-a then upon termination of synthesis, the conversion would continue and the ratio in older plants would differ from young chlorophyllous plants. This has not been found to occur. Two possible explanations were cited by the authors. The first explanation was that the ratio of rates of the chlorophylls from a common precursor in young seedlings and the final ratio of concentrations of older plants are the same. Secondly, a feedback controlling

mechanism exists which regulates the ratio of chlorophylls (a & b). Virgin (151) also stated that chlorophyll-b formation is independent of chlorophyll-a accumulation. The linear relationship between the chlorophylls seems to be more of an accidental phenomena than of any significance in pigment biosynthesis. Protochlorophyll synthesized under etiolated conditions exists in two forms: the unphytolated form, protochlorophyll (650 nm), which makes up the main portion of protochlorophyll. A possible link between protochlorophyll (636 nm) which is inactive in photo-conversion and slowly disappears in the light and chlorophyll-b which appears one hour later was cited by Virgin. The synthesis of chlorophyll-a from protochlorophyll (650 nm) and chlorophyll-b from protochlorophyll (636 nm) is support for parallel pathways for the synthesis of chlorophylls (a & b). Overwhelming evidence for this hypothesis would be the isolation of chlorophyllide-b; none was found by Virgin (153). The extracts of these two pigments (636 and 650 nm) are identical in absorption and fluorescence data which indicates that the difference is in the holochrome rather than in the pigment (153). In conclusion, the mechanism for the terminal biosynthesis of chlorophyll remains unknown (16, 151).

Environmental Influences on Chlorophyll Accumulation

As previously stated, chlorophyll formation in etiolated higher plants can be demonstrated to be divided into three separate stages: protochlorophyll formation, the lag-phase, and chlorophyll accumulation (47). A similar mechanism has been suggested by Virgin to occur in vivo (153). The second and third stages are commonly called greening in which the etioplast

matures into a chloroplast (84, 157) and additional chlorophyll is formed. During the first 2 to 3 hr. of illumination, chlorophyll formation is relatively slow followed by an acceleration of chlorophyll accumulation (151). Treatment of various intermittent light periods (red and white irradiations followed by darkness) on dark-grown wheat leaves revealed that all chlorophyll-a synthesized during greening is formed via protochlorophyll. Therefore the rate of chlorophyll-a formation is regulated by the rate of protochlorophyll formation (152).

Using the basic technique described above, Virgin (151) demonstrated that etiolated barley leaves would regenerate protochlorophyll within a range of 22° to 30°C. Rapid chlorophyll-a accumulation occurred between 2 to 3 hr. at 22° to 30°C. and chlorophyll-b was synthesized about an hour later at a corresponding rate. Friend (44) found a similar range (20° to 30°C.) to exist in etiolated wheat seedlings and demonstrated an increasing parallel with light intensity.

For photo-conversion of protochlorophyll, temperature dependency is low (129). At -195°C. no reduction occurs, but at -70°C. a fairly rapid transformation takes place and increases with temperature to 40°C. At higher temperatures, the reaction does not take place. This indicates that the reaction is not entirely photochemical but a possible enzymatic process plays an important role. Whether this involves the holochrome complex (129) or the esterification of chlorophyllide-a (153, 159) remains an open question.

The effect of light on chlorophyll synthesis seems to be at least two-fold (153). First, light converts protochlorophyllide into chlorophyllide-a with the addition of 2 hydrogen atoms (153) and possibly responsible for the oxidative formation of chlorophyll-b (98). Secondly, light seems to regulate

the formation of protochlorophyll-precursors possibly through the red, far-red system (152, 154, 157, 160). Several interesting experiments by Virgin (152) on chlorophyll formation under continuous and intermittent light have revealed certain variations in greening. Red (610-690 nm) and blue light (410-550 nm) support similar lag-phase curves for pigment formation under continuous irradiation. Pretreatment with a short impulse of red or blue light results in the elimination of the lag-phase under red light while blue light supports a lag-phase during greening. The difference was cited to be a time factor required to reach the saturation point: for red light, 10 to 30 sec. and for blue light 5 to 10 min. This means that the two light qualities differ in sensitivity (22:1 ratio) which suggests a possible explanation why blue light has little to no effect on the elimination of the lag-phase (152).

If greening is linked to the phytochrome mechanism then a far-red light should nullify chlorophyll formation. Price et al. (108) verified this reversal phenomena thus establishing chlorophyll synthesis as another of the physiological responses to the phytochrome system. The absorbed light is not associated with the photoconversion (159) but with certain metabolic and growth responses mediated by this photoreceptor (84).

The quantity as well as the quality of light regulates protochlorophyll synthesis (151). Low light intensities (8 to 200 ft.c.) stimulate comparable greening curves. Higher intensity (600 ft.c.) results in a noticeable increase in pigment formation. Friend (44) has shown that the higher the light intensity, the higher the rate of chlorophyll production and the maximal chlorophyll content in leaves. The high level of chlorophyll formation obtained at 2,500 ft.c. took two weeks for accumulation. It is also evident

that the newly formed chlorophyll seems to be destroyed under high light intensity (87, 126). Madsen (87) stated that accumulation under this condition could take place if the duration of irradiation is sufficiently short (1/2000 sec.).

The age of seedlings has been shown to have an effect on the lag-phase of chlorophyll synthesis (126). Younger bean plants (2-, 3- and 4-day old) synthesize chlorophyll linearly during the first 6 hr. of illumination. A definite lag-phase is noted in 6-day old seedlings. Only a small amount of chlorophyll is formed in a 11-day old seedling during a 6 hr. irradiation period. If the leaves are incubated with exogenous substrates such as 0.25 M sucrose (161), then the capacity to accumulate chlorophyll reappears (126). Under these conditions, the lag-phase is shortened to 2 hr. followed by a rapid accumulation of chlorophyll. This suggests a sucrose or cotyledon factor necessary for greening is present in younger seedlings, then is depleted in 6-11 day old seedlings resulting in a lag-phase. The nature of this factor is not known (126). Plants from 11 to 21 days showed no major difference among the time courses of chlorophyll synthesis except the amount of chlorophyll produced is less (126).

Nutritional Studies on Chlorophyll Synthesis

It has long been known that iron is essential for the growth of higher plants and is involved in chlorophyll synthesis. Porphyrin enzymes, catalase and peroxidase, have been reported to be deficient in chlorotic leaves in comparison to normal leaves (41). Iron deficiency results in a depression of catalase while an iron/manganese ratio seems to affect the level of peroxidase in barley leaves (2). Other studies have shown iron to be involved with δ-AL synthesis (32, 90, 91) and the conversion of Coprogen III to Proto

(67). Hsu and Miller (67) found similar incorporation into chlorophyll-a of δ-amino [$^{14}C_4$] laevulinic acid and [$^{14}C_2$] succinyl CoA + glycine in normal and iron-deficient tobacco leaves when iron was added. Hsu and Miller showed a Copro accumulation to occur in culture media containing leaf discs and low iron concentrations. With the addition of iron in both iron-deficient and and normal leaves, this porphyrin decreased markedly. These data indicate that the formation of Proto from Coprogen requires iron. Marsh (91) measured the porphyrins which accumulated in the cultured leaves. The predominant porphyrin under iron-deficiency conditions were Proto and a small amount of Copro. Hsu and Miller found that most of the Copro had been secreted from the leaves into the incubation medium. Indirect evidence in support of iron's role as a cofactor to Coprogen III oxidase in mitochondria is the enzymatic inhibition of o-phenanthroline which is a chelating agent with a high affinity for iron (115). More data is yet to be obtained before the role of iron is to be fully understood (67).

In algal cells, it was found that the balance of nitrogen and glucose sources (N/G) determines the cellular pigmentation (120, 142). Bleached cells were induced by a low N/G ratio and green cells resulted from higher N/G ratios. This suggests that chlorophyll synthesis is affected by the relative amounts of the nitrogen source (urea) and the carbon source (glucose) instead of the concentration of the nitrogen or carbon sources alone.

Magnesium, a constituent of the chlorophylls, has an effect on the pigment level as well as on chloroplast formation. Magnesium deficient leaves possess chloroplasts with few thalykoids and no grana (143). Kirk (73) stated that algal cells vary between species in respect to magnesium deficiency and chlorophyll content. An internal pool of Mg^{2+} might explain this

difference.

Trace elements have been shown to have the following effect in barley leaves: (1) boron, copper, cobalt and molybdenum aid in the accumulation of chlorophyll, (2) cobalt, molybdenum and especially copper retard the breakdown of chlorophyll in the dark, (3) cobalt, molybdenum increase the stability of the chlorophyll-protein complex (136).

Differentiation of Plastids

Most of the studies on differentiation of plastids used etiolated seedlings under illumination. Morphogenesis of plastids in green plants will not be discussed here. A recent publication by Kirk covers this area adequately (73).

Electron micrographs of higher plants under etiolation have revealed cytoplasmic spheres approximately 0.2μ to 1μ in diameter which have been identified as plastid primordia. The inner membrane seems to invaginate to form small vesicles which extend toward the center of the spheres. If the plant is maintained in the dark, the entire structure enlarges with the development of more vesicles (25, 73). Eventually the terminal, dark etioplasts containing one or two prolamellar bodies in each plastid is formed (63). These structures are made up of adjoining membranous masses of interconnecting tubules sometimes called the crystalline centers. Occasionally a thalykoid extends from the crystalline centers to the inner layer of the plastid envelope. The appearance of starch grains, if any, are located near the thalykoid (75). The formation of a prolamellar body (deposition of membranous material around ribosomes) seems to be regulated by a dark mechanism which controls the ratio of polyribosomes to single ribosomes (63). The

location of the protochlorophyll(ide) holochrome is unknown (63) but it is thought to be within the crystalline centers or in the thalykoids which extend into the stroma (75, 77).

Under illumination, protochlorophyllide is converted into chlorophyllide and the crystalline centers dissociate into numerous vesicles extend in rows through the stroma (73, 77). The rate of extension of the vesicles appears to increase with light intensity (73). This stage persists during the lag-phase of chlorophyll biosynthesis (25). The fusing of these primary thalykoids from grana during the stage of rapid accumulation of chlorophyll (77).

Because the dissociation of the prolamellar body occurs at the same time and responds to the same light quality as the photo-conversion of protochlorophyllide, it has been suggested that the two processes might be functionally related (77). Chlorophyll synthesis is known to be controlled by the phytochrome system (108) but the transformation of tubes to vesicles in plastids can be stimulated by both red and far-red light (77). Therefore some unknown pigment must exist or the protochlorophyllide molecule might absorb the light energy required for the dissociation of the prolamellar body. Klein (77) stated that the activation of δ-AL synthetase system, the photo-conversion of protochlorophyllide, etc. are controlled by light of low energy, but the dispersal of vesicles is a high energy requiring process in which more than an induction illumination is required (77). In etiolated bean leaves, Klein (78) demonstrated that plastids which contain little to no chlorophyll can form closed-end lamellae structures in the same region of prolamellar bodies. Therefore these structural changes and the variation of chlorophyll content occur independently (77). Further investigation is

required in understanding the relationship between the photoreduction of protochlorophyllide and the structural changes in plastids (73, 77).

The final stage of chloroplast development is the formation of the photosynthetic apparatus. The soluble enzymes and cytochrome f linked to the photosynthetic process change slightly during greening. This indicates that the photosynthetic activity (O_2 evolution, Hill reaction, $NADP^+$ photoreduction, and cytochrome f photo-oxidation) parallels the synthesis of chlorophyll and lamellae formation (105). Electron micrographs illustrate growth and association of lamellae into grana with an increase of chlorophyll formation. The lipophilic tail is associated with the lipid portion of the lamellae while the hydrophilic head resides within the protein layer of the double layer membrane (lamellae). This complex is referred to as the lipid-chlorophyll-protein complex and could possibly be the force that bonds the protein and lipid layers together. Once the phytol portion is associated with the lipophilic carotenoid pigments, resonance transfer of energy (electrons) is initiated between the carotenoid and chlorophyll pigments. The shift of absorption maximum by chlorophyll complex from 673 nm to 676 nm and the aggregation of chlorophyll molecules results in a resistance against photo-oxidation of chlorophyll. It is believed that the resistance to bleaching, development of energy transfer and oxygen evolution follows similar time courses. Thus, the development of function follows the development of structure (35).

Dependency on DNA, RNA and Protein Synthesis.

Cell growth is based on cell division and cell enlargement. Considerable evidence supports the concept that cell growth is regulated by DNA, RNA,

and protein synthesis (43, 102, 104) and water uptake (30). Recent studies have indicated that plastids have the ability to make some of their DNA and most of their RNA, to undergo organelle division, and to form some amino acids. They also seem to synthesize most, if not all, of their proteins. This genetic system of chlorophyll and protein biosynthesis is responsive to light. Thus, these biochemical studies support the concept that plastids are autonomous, self-duplicating organelles (51, 73).

Kirk et al. (74) and others (48, 49, 88, 107) have found that the inhibition of protein synthesis also inhibits chlorophyll formation. Two explanations by Kirk (74) and Gassman and Bogorad (48, 49) have been published. First, Kirk believes chlorophyll synthesis requires the formation of some structural protein within the lamellae in stoichiometric relation or possibly, of the formation of the protein moiety of protochlorophyllide holochrome (72, 74). Kupke (80) stated that there is a functional relationship between protochlorophyllide holochrome and an "18-S" protein which seems to be identical to fraction I protein (80, 144). This "18-S" protein appears and its concentration increases parallel with protochlorophyllide in the etiolated leaf in the dark and with chlorophyll during greening. The significance of this correlation is that the concentration of total soluble protein increased with the "18-S" protein which suggests that plastid differentiation is related to this protein thus regulation of chlorophyll accumulation (80). It also seems that this protein is involved with several activities such as the soluble enzymes in the Calvin cycle (33).

Secondly, Gassman and Bogorad (48, 49) interpreted the prevention of chlorophyll formation by inhibitors of protein synthesis by stating that an

essential enzyme(s) for δ-AL formation is labile thus requiring continuous synthesis; when terminated by inhibitors, chlorophyll formation stops. This contention was supported by the overcoming of the inhibition of chloramphenicol on both chlorophyll synthesis and the regeneration of protochlorophyllide by the addition of δ-AL. Actinomycin D prevented greening after 4 hr. of illumination which indicated a necessity for continuous production of a specific DNA-dependent RNA species during chlorophyll accumulation. Thus, chlorophyll synthesis is controlled by a catalytic enzyme, most likely, δ-AL synthetase (48). Granick (59) found that etiolated leaves supplied with a certain level of δ-AL produced a corresponding amount of chlorophyll. Also in Rhodopseudomonas spheroides cells, the enzyme, δ-AL synthetase, has been shown to be extremely labile (34).

Kirk (74) stated that chloramphenicol at high concentrations (2 mg/ml) inhibits oxygen uptake in corn mitochondria and eliminates phosphorylation. Thus it seems possible that this antibiotic may effect chlorophyll synthesis in other ways than just through the inhibition of protein synthesis. Using another inhibitor (actidione-10 mg/ml), which acts at the ribosomal level, Kirk (72, 74) showed that δ-AL did not relieve the inhibition of chlorophyll synthesis.

The Molecular Biological Pathway

A tremendous amount of research stimulated by the work of Watson and Crick (155) has resulted in a genetic concept that each gene codes the synthesis of an appropriate protein and then a specific enzyme which regulates the different phases of plant growth (1, 27, 28). The environmental factors

such as light, temperature, oxygen, water and minerals influence directly and indirectly the intermediary metabolism (photosynthesis, carbohydrates, amino acids, hormones, respiration, etc.) of the plant. These components of plant metabolism in turn influence every sequence of the molecular biological pathway (1).

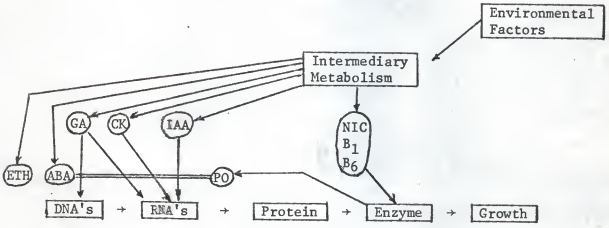

Fig. 7. Scheme representing the sequential influence of environmental factors, and intermediary metabolism, specifically growth hormones, on the molecular biological pathway. Abbreviations: abscisic acid, ABA; auxin, IAA; cytokinin, CK; ethylene, ETH; gibberellin, GA; nicotinic acid, NIC; peroxidases, PO; pyridoxine, B_6; and thiamin, B_1. (Thiamin, nicotinic acid, and pyridoxine, function in prosthetic groups of specific respiratory enzymes.) [Modified from Addicott, 1968 (1)].

Plant hormones have been considered as having a critical influence on this pathway illustrated above (1). Using specific antibiotics, gibberellins, cytokinins and auxins are believed to stimulate directly or indirectly the expression of genes, that is, the coding of specific RNA's (43, 45, 79, 92, 101, 103). Interaction of these "promoting" hormones with one another, with specific "inhibitory" hormones such as abscisic acid and ethylene results in a controlling mechanism on plant growth (11, 38, 46, 81, 116, 147). Further

information on hormonal control of plant and cell growth are available (65, 117).

The chlorophyll molecule as already discussed is an essential component of photosynthesis and plant growth. Several recent publications (12, 138, 148, 158) have discussed the influence of certain growth regulators on chlorophyll synthesis. Gibberellic acid tends to dilute pigment content in wheat seedlings in that the rate of cell growth exceeds the rate of chlorophyll synthesis (158). In an albino tobacco mutant (Nicotiana tabacum) cultured on solid agar medium, chlorophyll production was influenced by IAA and kinetin, whereas an inhibition was noticed when IAA and 2,4-D were added as hormonal supplements (149). Stetler and Laetsch (138) also found an enhancement of chlorophyll synthesis by kinetin in another N. tabacum variety. Proplastids in callus tissue were found to require the presence of this hormone for grana formation. This correlation between kinetin and plastid differentiation was not duplicated with other growth regulators such as auxin or gibberellin (138). Therefore, the present investigation was carried out on etiolated bean seedlings in an attempt to show a kinetin stimulation in chlorophyll synthesis and to demonstrate that indoleacetic-3-acid and gibberellic acid have no direct effect on chlorophyll accumulation.

MATERIALS AND METHODS

Seedlings of Phaseolus vulgaris, var. Commodore were used in this study. Seeds were surface sterilized in 3% NaOCl for 8 to 10 min. followed by 3 washings in tap water. Then the seeds were allowed to take up tap water for 2 to 3 hr. and germinated in presoaked vermiculite held in polyethylene trays. Unless mentioned, constant temperature of 26°C ± 1° and constant illumination at an intensity of 510 ft.c. ($10x10^4$ erg/sec. per cm^2) within a growth chamber were maintained. Irradiation was provided by General Electric cool white Power Groove fluorescent tubes and was filtered through 4 layers of cheesecloth.

All manipulations of seedlings before illumination were performed under a safe green light. An aerosol spray kit was used to apply each treatment (100 cc) to the etiolated seedlings. Nine hours before illumination, the following treatments were applied: distilled water (control), kinetin (1.5 mg/l), GA_3 (2 mg/l), IAA (2 mg/l), puromycin (1 mg/l) and kinetin (1.5 mg/l) plus puromycin (1 mg/l).

Chlorophyll Determination of Seedlings Treated with Growth Regulators

Primary leaves from 9-day old seedlings were excised, weighed, immersed in 12 ml of 80% acetone plus a pinch of $CaCO_3$, and ground with sand in a mortar. The acetone extract was filtered through a suction filter. Two additional washings with acetone of 7 ml each were made in a similar manner and added to the original extract. Then 20 ml of ether was added to the combined extract. The acetone-ether mixture was poured through a long-stem funnel into a separatory funnel (180 ml capacity) which contained 100 ml of distilled water. The movement of the organic solvent through the water

removed most of the acetone resulting in a two layer solution. The pigment-ether layer was separated from the water-acetone layer and washed twice in a similar manner. Care was taken to avoid emulsification. The chlorophyll-ether solution was adjusted to 15 ml by addition or evaporation of ether then stored in a refrigerator (7°C.) for a period not more than 10 hr. The O.D.'s of protochlorophyll and chlorophylls a and b were determined at wavelengths 624 nm, 663 nm and 644 nm, respectively, on a Beckman DU Spectrophotometer. Protochlorophyllide was determined using the equations of Anderson and Boardman (6) and chlorophylls (a & b) were determined using the equations of Smith and Benitez (130).

Chlorophyll Determination of Seedlings Treated with Puromycin

The initial procedure as described above was followed with the exception that the 80% acetone extract was not transferred to ether. After the filter suction process, the pigment-acetone extract was centrifuged (1,000g) to sediment any remaining debris. The chlorophyll-acetone solution was adjusted to 15 ml by addition or evaporation of acetone then stored in a refrigerator (7°C.). The O.D.'s of the chlorophylls (a & b) were determined at 663 and 645 nm, respectively, on a Beckman DU Spectrophotometer. Chlorophylls (a & b) were determined by a nomogram (74).

Protein Determination

Primary leaves of 9-day old etiolated seedlings were treated 9 hr. before illumination with kinetin (1.5 mg/l), puromycin (1 mg/l) and the combination of kinetin plus puromycin at the same concentrations as when applied

separately. Leaf samples of 1 gm were taken at 0, 2 and 4 hr. of illumination. Whole isolated etioplasts[1] were obtained by a modified method of Klein and Poljakoff-Mayber (76). The leaves were ground in a cold mortar with 5 ml of a phosphate-sucrose buffer (0.2M monopotassium and disodium phosphate plus 0.7M sucrose) adjusted at pH 7.5. Then the brei was squeezed through two layers of cheesecloth followed by a centrifugation at 350g for 7 min. at 0°C. The supernatant was decanted and centrifuged at 1000g for 20 min. which sediments the whole etioplasts.

The chloroplasts were obtained by a modified method of Arnon et al. (8). The leaf brei was obtained by the procedure described above (76) then centrifuged for 1 min. at 0°C. and 200g. The leaf homogenate was decanted and centrifuged for 7 min. at 1000g which sediments the whole chloroplasts.

The protein within the plastids were precipitated by 5 ml of 10% trichloroacetic acid. The white precipitate was collected by centrifugation and stored in 5 ml of 1% trichloroacetic acid at 7°C. for protein determination by Lowry's method (86).

1. This term has been suggested by Kirk because it is more descriptive of plastids isolated from etiolated seedlings (73).

RESULTS

Preliminary Experiments

Preliminary experiments using etiolated seedlings of Phaseolus vulgaris, var. Kentucky Wonderbean were designed to find the optimal range of light intensity and the concentration of kinetin for enhancing chlorophyll synthesis. During the initial phase of chlorophyll-a accumulation, a slight inhibition was found under the illumination of 1800 ft.c. and treatment with 10 mg/l of kinetin (Table 1). Little to no effect was noted with the radiation of 1800 ft.c. and the treatment of 5 mg/l of kinetin. These conditions also supported a top level of chlorophyll-a accumulation of 565 ug/g fr. wt. after 40 hr. in the light (Table 2). The next experiment showed a slight inhibition of chlorophyll-a accumulation with 510 ft.c. and 5 mg/l of kinetin (Table 3). These data indicated that the following were required for illustrating kinetin stimulation of chlorophyll production: a lower concentration of kinetin with a light intensity of 510 ft.c. plus a bean variety containing a higher level of chlorophyll.

A contamination problem by an unknown species of Penicillium was noted. Surface sterilizing agents 0.1% $HgCl_2$ and 3% NaOCl were employed and the latter seemed to be the most effective.

Response of Chlorophyll Synthesis to the Application of Kinetin

The application of kinetin to etiolated bean seedlings 9 hr. before illumination enhances chlorophyll synthesis (Fig. 8). The pigment level during the first 4 hr. was higher in the leaves treated with kinetin than in

the untreated, which indicates an increase of protochlorophyll production before the light period (152). The lag-phase in the treated leaves was completed after 4 hr. and the leaves appeared visibly green after 6.5 hr. of irradiation. Untreated leaves were delayed approximately 2 hr. behind the treated leaves in initiating Stage III. During the accumulation of chlorophyll-a, one distinct shoulder was found in both treated and untreated leaves between hr. 12 and 16 of the light period. The possibility that this shoulder actually corresponds to the initial phase of chlorophyll-a accumulation was considered. The amount of chlorophyll-a produced in etiolated, 9-day old bean leaves irradiated for 5 min. of continuous light was approximately 35 ug/g fr. wt. (3). If the above interpretation was correct, little to no net synthesis of chlorophyll-a would occur for 12 hr. This is not the case (Table 4).

Table 4. Chlorophyll-a accumulation (ug/g fr. wt.) in etiolated bean seedlings of P. vulgaris, var. Commodore which were treated with kinetin (1.5 mg/l) and deionized water.

Treatment	4 hr.	8 hr.	12 hr.
Kinetin	75	177	243
Control	59	129	181

Therefore the initiation of chlorophyll-a accumulation occurred after 4 hr. in the light and 8 hr. before the shoulder was detected. Two possible interpretations for the presence of this shoulder could be related to the synthesis of chlorophyll-b and a lag in lamellae formation during chlorophyll-a accumulation. Supporting evidence for these interpretations will be discussed later.

Traces of chlorophyll-b appeared approximately 8 hr. after illumination and 1.5 hr. following the initiation of chlorophyll-a accumulation. A linear increase of chlorophyll-b synthesis was noted for the first 24.5 hr. followed by a slight accumulation. With the appearance of chlorophyll-b, the ratio of chlorophyll-a to b approaches 3.1 found in mature primary leaves. The presence of applied kinetin decreases the time necessary for this process (Table 5).

The regeneration of protochlorophyllide begins after the photo-conversion of the relatively small amounts of protochlorophyllide synthesized in etiolated seedlings (150). After 6½ hr. of greening, protochlorophyll(ide) was detected and found to increase slightly in an irregular manner for the next 28 hr. (Table 6).

Table 6. The regeneration of protochlorophyllide (ug/g fr. wt.) in etiolated bean seedlings of P. vulgaris, var. Commodore which had been treated 9 hr. before illumination with kinetin (1.5 mg/l) and deionized water.

Treatment	4 hr.	6½ hr.	8 hr.	10½ hr.	12 hr.	16 hr.
Control	5	23	14	51	44	34
Kinetin	10	33	23	61	46	80

24½ hr.	28½ hr.	34 hr.
79	74	112
92	78	142

The reliability of these data is in doubt because it is a well established fact that protochlorophyllide detection is negligible after a few minutes of greening. During continuous illumination, the regenerated protochlorophyllide has been shown to be either photo-decomposed or photo-converted to

chlorophyllide-a (140).

The Effect of Other Growth Regulators

The time course pattern of greening in etiolated bean seedlings treated with IAA and GA_3 were basically similar to the pattern stimulated by kinetin with the exception that IAA and GA_3 did not appear to enhance chlorophyll synthesis. At the concentration used, IAA (1 mg/l) showed a slight inhibition of chlorophyll metabolism (Fig. 9) and GA_3 (2 mg/l) had relatively no effect (Fig. 10) for the first 24 hr. The measurement taken at 30 hr. showed a slight increase in the accumulation of both chlorophylls (a & b) in leaves treated with GA_3. The substantiation of this finding in the next measurement (37 hr.) was not possible because of partial pigment loss during acetone to ether transference. From the ratios of chlorophyll a/b, no indication of a control rate of pigment formation was noted in the primary leaves treated with IAA or GA_3.

Similar to kinetin treated leaves, a shoulder was evident between hr. 13 and 16. In the experiment with GA_3, both chlorophylls (a & b) and their respective controls exhibited little to no net chlorophyll accumulation during this 3 hr. period. When the seedlings were treated with IAA, this shoulder was not as evident in chlorophyll accumulation and was absent from the synthesis of chlorophyll-b. Whether this lag in chlorophyll production in both GA_3 and IAA treated seedlings was significant to the terminal stages of chlorophyll metabolism will be discussed later.

Dependency of Chlorophyll Synthesis on Protein Metabolism

Chlorophyll synthesis has been shown to be dependent on protein synthesis (48, 49, 72, 74). These reports were verified when puromycin (1 mg/l) was applied to etiolated been seedlings. The presence of this protein inhibitor lessened the rate of chlorophyll accumulation (Table 6). After 9 hr. of greening, the accumulation of chlorophyll-a was 1.7 times more in the etiolated leaves treated with kinetin than the leaves treated with puromycin. This indicated that puromycin probably interferred with chlorophyll formation through the synthesis of a catalytic enzyme(s) (48, 49) or a structural protein necessary for lamellae formation (72, 74).

When puromycin was applied with kinetin, the rate of accumulation was comparable to kinetin (1.5 mg/l) when applied by itself. The mode of action of puromycin involves the detachment of nascent polypeptides from ribosomal particles (99). Yarmolinsky and DeLa Haba (162) proposed that puromycin acts as a group-specific analogue of aminoacyl t-RNA's. Recent evidence has been cited that besides having a coding function, these aminoacyl t-RNA's are responsible for the attachment of the nascent peptides to the ribosomes (68). Kinetin has been shown to be incorporated into soluble RNA (43) so the elimination of the inhibition of puromycin on chlorophyll synthesis by the presence of exogenous kinetin seems to be a dilution process. The reason for this interpretation is based on the following: the concentration used in this experiment was lower (0.01×) than the levels used by Gassman and Bogorad (48). A characteristic feature of the action of puromycin has been reported that the reaction lessened after the detachment of 30-35% of the polypeptide chains from the ribosomes (68). Also puromycin (1 mg/l) only slightly inhibited

protein synthesis in chloroplasts of etiolated bean leaves during the preillumination period. After 2 hr. in the light, no effect of puromycin on protein synthesis was found (Table 7). Relatively no increase in chloroplast protein was noted during the first 4 hr. of greening in leaves treated with kinetin and kinetin plus puromycin (Table 7).

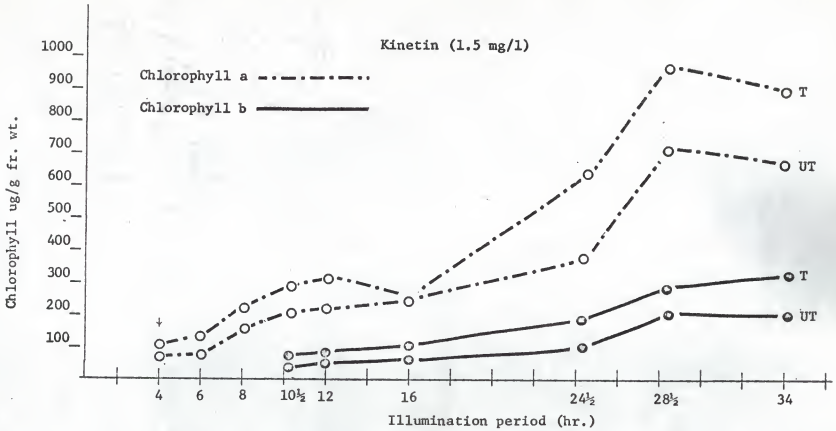

Fig. 8. The effect of kinetin on chlorophyll formation by dark grown seedlings of P. vulgaris, var. Commodore. Etiolated leaves were treated 9 hr. before illumination. The course of greening represents the combination of two experiments measured at different time intervals. ↓ represents the initiation of chlorophyll accumulation, T represents the treated seedlings, UT represents the control.

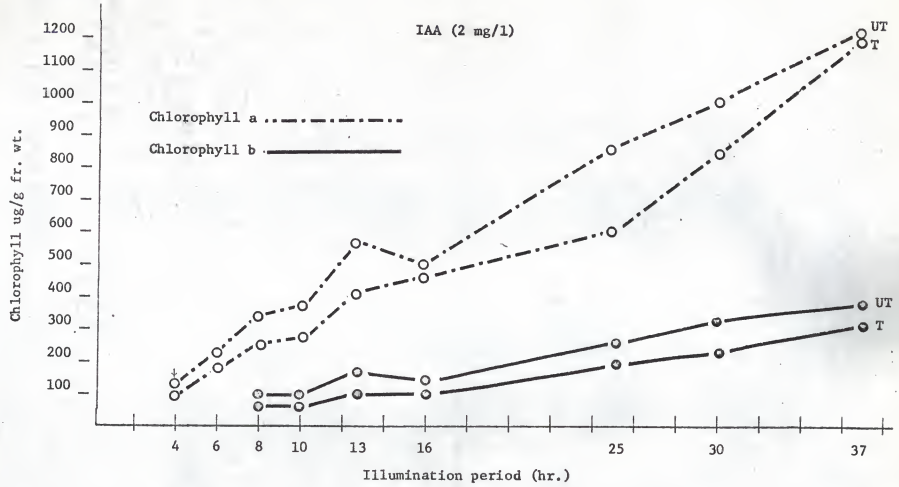

Fig. 9. The effect of IAA on chlorophyll formation by dark grown seedlings of P. vulgaris, var. Commodore. Etiolated leaves were treated 9 hr. before illumination. Each datum represents one measurement. ↓ represents the initiation of chlorophyll accumulation, T represents the treated seedlings, UT represents the control.

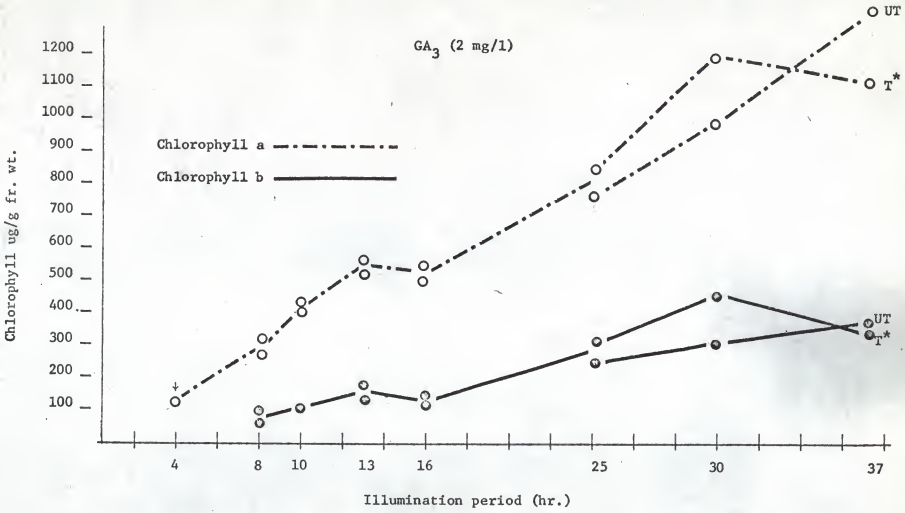

Fig. 10. The effect of GA_3 on chlorophyll formation by dark grown seedlings of P. vulgaris, var. Commodore. Etiolated leaves were treated 9 hr. before illumination. Each datum represents an average of two measurements. ↓ represents the initiation of chlorophyll accumulation, T represents the treated seedlings, UT represents the control. *Partial pigment loss in one of the two measurements during pigment-acetone transferrance to pigment-ether solution.

Table 1. Pigment contents (ug/g fr. wt.) during the initial portion of chlorophyll-a accumulation in P. vulgaris, var. Kentucky Wonderbean sprayed with kinetin (10 mg/l) and deionized water. Light intensity of 1800 ft.c. was maintained. Each datum represents one experiment.

	Control	Kinetin
12 hr.	157	136
14 hr.	338	292

Table 2. Pigment contents (ug/g fr. wt.) during the initial portion of chlorophyll-a accumulation in P. vulgaris, var. Kentucky Wonderbean sprayed with kinetin (5 mg/l) and deionized water. Light intensity of 1800 ft.c. was maintained. Each datum represents the mean of three experiments.

	Control	Kinetin
12 hr.	37	49
14 hr.	81	94
40 hr.	565	---

Table 3. Pigment contents (ug/g fr. wt.) during the initial portion of chlorophyll-a accumulation in P. vulgaris, var. Kentucky Wonderbean sprayed with kinetin (5 mg/l) and deionized water. Light intensity of 510 ft.c. was maintained. Each datum represents one experiment.

	Control	Kinetin
4 hr.	70	52
9 hr.	204	141

Table 7. Protein determination (mg/ml) of isolated plastids from etiolated seedlings of P. vulgaris, var. Commodore which were treated with deionized water, kinetin (1.5 mg/l), kinetin (1.5 mg/l) plus puromycin (1 mg/l) and puromycin (1 mg/l). Primary leaves were treated 9 hr. before illumination. Leaf samples (1 g) were taken at 0, 2, and 4 hr. during greening. [1]Etiolated seedlings treated with deionized water were subject to a flash of white light during the time of spraying. The primary leaves were pale green at 0 hr.

	Control	Kinetin	Kinetin & Puromycin	Puromycin
0 hr.	13.8	11.8	12.1	8.8
2 hr.	10.8	10.9	11.8	11.8
4 hr.	10.9	10.9	12.5	13.8

DISCUSSION

Terminal Biosynthetic Pathway of Chlorophyll Production

Chlorophyll-b biosynthesis is one of many areas of research in which technical refinement involving labeling of porphyrin is necessary before this in vivo pathway can be revealed (5). Until this has been accomplished, only speculations can be made concerning this problem. The two predominant schemes to date are concerned with a sequential formation of chlorophyll-a to b (98, 123, 125) and a parallel formation from a common precursor (134, 151, 153). Which of these alternatives is correct remains to be shown.

The data reported in this paper seems to fit the sequential formation of chlorophyll-a to b. The basis for this interpretation rests on the following: the pattern of chlorophyll-a formation (Fig. 8) is comparable to Michel-Wolwertz proposed scheme (Fig. 6), in that the shoulder found during chlorophyll-a accumulation occurs approximately at the time of chlorophyll-b detection. Michel-Wolwertz found that in photo-oxidating light ($71x10^4$ erg/sec. per cm^2-H.L.I.), the total activity of chlorophyll-a strongly increases during the first 2 hr. followed by a slow increase until the 6th hr. followed by a decrease in total activity. Under these same irradiated conditions, the total activity of chlorophyll-b increased steadily for 4 hr. followed by a steep rise until the 6th hr. Between the 6th hr. and the 8th hr., a decrease was noted. Under non-photo-oxidating conditions ($53.3x10^4$ erg/sec. per cm^2-L.L.I.), the total activity of both chlorophylls (a & b) respond differently (98). Michel-Wolwertz's interpretation of these experiments was that a distinct relationship occurred between the photo-oxidation of chlorophyll-a and the abrupt increase in

chlorophyll-b.

The scheme as proposed by Michel-Wolwertz (98) indicates that young molecules of chlorophyll-a will enter either one of two reservoirs: old molecules of chlorophyll-a or young molecules of chlorophyll-b. The rate of chlorophyll-b formation seems to be dependent on the rate of saturation of the reservoir of young chlorophyll-a molecules. This saturated rate seems to occur after 4 hr. in the light.

This scheme of chlorophyll-b formation appears comparable to the data reported in this paper. This shoulder in chlorophyll-a accumulation found between hr. 12 to 16 could possibly represent an unsaturated rate of chlorophyll-a production. After 12 hr. of greening, a certain portion of the accumulated chlorophyll-a molecules could be photo-oxidized to chlorophyll-b molecules. This would result in a decrease in net accumulation of chlorophyll-a which could not be altered without an increased rate of synthesis of protochlorophyll formation.

One difficulty in comparing the results reported in this paper and the data cited by Michel-Wolwertz (98) is the variation in the technique used, especially the difference in light intensity. Michel-Wolwertz based his interpretation on the difference in photo-oxidation potential between 53.2×10^4 and 71×10^4 erg/sec. per cm^2. Photo-oxidation of chlorophyll-a was found to occur at lower light intensity but at a slower rate (98). This could possibly explain why little to no net increase of chlorophyll-a occurred for 4 hr. during Stage III of chlorophyll formation and could also explain why approximately 34 hr. was necessary for the completion of Stage III (Fig. 8).

The second possible interpretation of this shoulder could be an association between chlorophyll formation and morphogenosis of plastids (73).

Table 5. The ratios of chlorophyll a/b in etiolated bean seedlings of P. vulgaris, var. Commodore which were treated with Kinetin (1.5 mg/1), IAA (2 mg/1) and GA_3 (2 mg/1). Primary leaves were treated 9 hr. before illumination.

	Control	Kinetin		Control	IAA	GA_3
24.5 hr.	4.04	3.80	25 hr.	3.38	3.06	2.52
28.5 hr.	3.43	3.49	30 hr.	3.23	3.65	2.58
34 hr.	3.29	2.83	37 hr.	3.80	3.96	2.59

Table 6. The effect of puromycin on chlorophyll synthesis by dark grown seedlings of P. vulgaris, var. Commodore. Puromycin (1 mg/1) and kinetin (1.5 mg/1) and the combination of these chemicals were applied 9 hr. before illumination. The values in the table represent the amount of chlorophylls (a & b) synthesized after 5, 7 and 9 hr. in the light.

	Chlorophyll b ug/ml			Chlorophyll a ug/ml		
	Kinetin	Kinetin & Puromycin	Puromycin	Kinetin	Kinetin & Puromycin	Puromycin
5 hr.	1.9	1.9	1.2	5.9	7.5	4.5
7 hr.	2.9	3.6	1.9	8.9	10.2	5.5
9 hr.	3.5	2.4	2.8	10.6	6.9	6.4

A lag in grana formation during chlorophyll accumulation could account for the appearance of a shoulder in Stage III. This will be discussed at a later time.

Stimulation of Chlorophyll Production by Kinetin

The enhancement of chlorophyll formation by exogenous kinetin was first proposed by Stetler and Laetsch (138) and Venketeswaran (148, 149) in 1965. The former investigators found that the functional relationship between kinetin and chlorophyll formation was also involved with plastid differentiation. In the presence of kinetin (1 mg/l), tobacco tissue originally isolated from Maryland Mammoth supported chloroplasts with extensive grana development after 45 days in the light. Plastids from tissue callus cultured in the light for 28 days were similar in size to the normal chloroplasts but exhibited aggregations of vesicles. The light-grown chloroplasts cultured for 45 days in the presence of kinetin synthesized 1.5×10^{-3} mg of chlorophyll/mg dry wt., which is close to the level found in mature tobacco leaves. This value is approximately 1/8th[1] of the total chlorophyll produced (1,300 ug/g fr.wt.-Fig. 8) in etiolated bean seedlings which were treated with kinetin and maintained in the light for 34 hr.

Stetler and Laetsch (138) attempted to demonstrate the same kinetin effect with an auxin and a gibberellin but to no success. This corresponds to the evidence presented in this report and others (158). Stetler and Laetsch (138) stated that GA_3 inhibits chloroplast maturation despite its stimulation of growth. It seems that the induction of chloroplast matruation and growth rate are inversely related (137).

The stimulation of chlorophyll formation by kinetin (10 mg/l) in isolated pumpkin cotyledons (Cucurbita pepo, var. King of Mammoths) was reported by Banerji and Laloraya (12). These data are in conflict with

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1. In tobacco leaves, fresh weight is approximately 10% dry plus 90% water.

the evidence reported in this paper. Kinetin, at the high physiological concentration of 10 mg/l, induced an inhibitory effect on chlorophyll formation (Table 1). Several observations could account for the difference in optimal levels for kinetin stimulation of chlorophyll production: different plant organs of different genera tested and the use of excised cotyledons instead of treating the intact seedlings.

Setting aside this disagreement on the optimal kinetin levels, the evidence cited in this paper indicates that this growth regulator enhances chlorophyll production. But the mechanism for cytokinin action remains unknown (82). The ability of kinetin to delay senescence by maintaining nucleic acid and protein synthesis plus a partial retention of total chlorophyll in Xanthium leaves (106) implicates the chloroplast as a possible site for cytokinin action (82). Mothes and Engelbrecht (100) found that labelled amino acids accumulated in treated (kinetin) areas of tobacco leaves. The author's interpretation was that kinetin delays senescence by establishing a gradient of metabolites towards the treated area resulting in accumulation. Another interpretation of senescence is the loss of ribosomes (113) and that the presence of kinetin retards the rapid loss of polyribosomes and ribosomes in excised leaves which would otherwise occur when floated on water. In the presence of kinetin, only a slight decline of protein content was noted. Therefore it was suggested that kinetin stimulated RNA synthesis which in turn suppressed the activities of ribonuclease and peptidases: enzymes which have been found to increase during senescence (114).

Recently three laboratories have found that cytokinins may have a distinct relationship between certain t-RNA's. Skoog et al. (127) found

that t-RNA from yeast, liver, and E. coli exhibits cytokinin activity in a tobacco callus bioassay. Ribosomal RNA from yeast was found to be inactive (127) which indicates that cytokinins were not generally present (65). Hall et al. (64) isolated a nucleoside, N^6-(Δ^2-isopentenyl) adenosine (a cytokinin) from a soluble RNA fraction of spinach and garden peas. Fox (43) reported the incorporation of C^{14} into several RNA components in kinin-requiring tobacco and soybean tissue cultured on a medium containing N,6-benzyladenine-8-C^{14}. The majority of the label was found in t-RNA, especially adenylic and guanylic acids (RNA nucleotides). Therefore it appears that cytokinins have been found in at least two t-RNA's (serine and tyrosine) and absence from five others (arginine, glycine, phenylalanine, valine, and alanine) (65). In the serine t-RNA, the function of the cytokinin, which is adjacent to the anticodon, has been suggested to bring about an orientation of the polynucleotide chain which facilitates base pairing with the m-RNA. Estimates have indicated that the number of t-RNA molecules containing cytokinins is rather small (65). Thus it is tempting to conclude that the action of cytokinins is through t-RNA metabolism and in some way, modifies the biological activity of t-RNA (65). Whether the localization of cytokinins in specific t-RNA molecules indicates a functional relationship between growth regulators and growth-morphogenesis in tissues remains to be established (127).

Photo-induction of Grana Formation During Greening

When etioplasts are exposed to light, plastid components increase from a low level to a high, maximum level after many hours of greening. This induction has been shown to be triggered by the phytochrome system (108) with white, red and blue light being the most effective and far-red light the least effective (73, 96). These data are compatable with the photoconversion of protochlorophyllide except that the holochrome pigment itself is the photoreceptor (73). The initial light treatment results in an immediate increase in RNA synthesis (31), especially the 28S (r-RNA) fraction as indicated by the incorporation of $^{32}PO_4^{-3}$ after 4 hr. in irradiated chloroplasts of Euglena cells (163). The 4S (t-RNA) fraction was shown to have low activity at the same time. After 16 hr., the 4S region became evident and after 72 hr., it was apparent that this area was one of three active regions (4S, 10S-14S, 19S) (163). In contrast to the RNA fraction, chloroplast protein increased only slightly for the first 24 hr. (31) which is in agreement with the data reported in this paper. Further evidence for this slight increase is indicated by the increased activity of photosynthetic enzymes (42, 73) and others involved with chlorophyll biosynthesis: δ-AL synthetase and Chlase (73).

When etioplasts are initially exposed to light, protochlorophyllide is converted to chlorophyllide and the prolamellar bodies appear as clusters of vesicles which disperse and then fuse forming long tubular membranes extending through the stroma (70). Kahn has presented evidence which indicates that the protochlorophyll holochrome is a component of these tubular membranes (70). Recently this tube transformation has been interpreted as a structural change in that the order of membranes is lost but the continuity

of these membranes remains intact (63). Substantiating the data presented by Klein et al. (77, 78), Kahn demonstrated an association between tube transformation and the photo-conversion of protochlorophyllide (71). Based on the data found by Kahn and other investigators, Kahn hypothesized that low light energy absorbed by protochlorophyllide initiated the photo-conversion of protochlorophyllide holochrome, which makes up the membraneous tubes of the prolamellar bodies. The donation of hydrogen atoms by macromolecular protein within the membranes coincide with rearrangement of bonds within the protein macromolecules. After a period of time, this changed bonding allows for a new order of configuration (71).

The second stage of greening corresponds with the extension of the tubular membranes and the lag-phase of chlorophyll biosynthesis (25, 73). With sufficient high light intensity, this phase can occur without chlorophyll formation which indicates that this structural change is not the cause of the lag-phase in greening (73).

Gunning and Jagoe (62) estimated that, after the end of the lag-phase of greening in oat seedlings, no de novo synthesis of membranes had occurred. This further indicates that the dispersal of the prolamellar bodies (tube transformation) and vesicle extension were the results of rearrangement of membranes (73). The primary lamellae along certain regions were observed to have doubled forming two thylakoids and from this time on, it appears that chlorophyll production paralleled thylakoid formation (73). Therefore a lag in lamellae formation could possibly result in photo-destruction of newly formed chlorophyll molecules which would account for the shoulder found during Stage III. This "stacking" was considered to be the first stage of grana formation which required 4 hr. of illumination (62). At

this time, Zeldin and Schiff (163) found that the RNA fraction with the highest activity was the 28S fraction and relatively low activity occurred in the 4S region.

After 10 hr. of illumination, plastids in dark-grown oat leaves exhibited stacks of 3 or 4 thylakoids: the prolamellar body still remained and several osmiophilic globules were located in the same region (62). After 20 hr. of illumination, the stacking process had continued and 3 to 8 thylakoid-grana were visible. Several interpretations have been cited concerning the ways in which grana formation occurs. For further discussion of this subject, these reports are recommended (73, 97).

In approximately 45 hr., the formation of the ultrastructure of chloroplasts in etiolated leaves of P. vulgaris is completed. The number of plastids per leaf remains constant ($2x10^8$) which indicates that organelle division does not take place during grana formation (73). During this same 45 hr. period, the 4S fraction becomes evident (163), and protein synthesis increases 2 fold over the last 24 hr. (31) and the photosynthetic apparatus has been functional for 35 hr. (73). The significance of the time course pattern of RNA fractions in relation to the fine structural changes in chloroplast differentiation and chlorophyll synthesis is unknown.

The use of specific antibiotics in greening experiments has revealed the requirement of nucleic acid and protein synthesis for chlorophyll production. Two hypothesis have been cited: the dependency of chlorophyll formation seems to be in catalytic amounts (48, 49) or stoichiometric in nature (74). Based on the belief that no de novo synthesis of membranes occurred during the lag phase (62) and chlorophyll formation and vesicle dispersal occurred independently (73), the application of a protein

inhibitor immediately before the lag-phase most likely would have the greatest effect on the level of various enzymes necessary for chlorophyll formation. When applied during chlorophyll accumulation, the inhibitor probably would interfere with both enzyme levels and the synthesis of lamellar macroproteins since chlorophyll and grana formation goes hand-in-hand at this time (73). The verification of one or possibly, to various degrees, both of these hypothesis remains to be shown.

The use of puromycin and other t-RNA inhibitors not only demonstrates the requirement of nucleic acid and protein synthesis for chlorophyll development (48, 49) but also indicates the involvement of the 4S fraction and cytokinins in chlorophyll production. The use of actinomycin D and actidione indicates the requirement of a functioning DNA-like RNA (48) and r-RNA (74) fraction, respectively. Therefore future studies on chlorophyll biosynthesis and plastid morphogenesis under the influence of various growth regulators, especially cytokinins, and specific protein inhibitors, are inviting.

SUMMARY

Etiolated bean seedlings (Phaseolus vulgaris, var. Commodore) were germinated in presoaked vermiculite. Intact seedlings (9-day old) were treated with deionized water (control), kinetin (1.5 mg/l), GA_3 (2 mg/l), IAA (2 mg/l), puromycin (1 mg/l) and puromycin-kinetin (1 mg/l: 1.5 mg/l, respectively). Primary leaf samples were obtained periodically and chlorophyll determinations were made. The presence of kinetin enhanced chlorophyll production during stage II and III whereas GA_3 and IAA did not. The presence of puromycin interfered with chlorophyll accumulation while in combination with kinetin, this inhibition was not observed. Chlorophyll content at 12 to 16 hr. revealed a shoulder during Stage III. A correlation between this observation and the terminal biosynthetic pathway of chlorophyll a → b was suggested.

Protein determinations of dark-grown seedlings treated with kinetin, puromycin and kinetin-puromycin were made of isolated chloroplasts at 0, 2 and 4 hr. The application of puromycin had a varied effect on the level of chloroplast protein. At 0 hr. a slight inhibition was noted whereas the lack of an effect of puromycin on chloroplast protein after 2 hr. of illumination was attributed to the low level of application and its mode of activity. Similar to the data on chlorophyll production, kinetin, when applied jointly with puromycin, overcame the effect of the protein inhibitor. Relatively no increase of chloroplast protein synthesis in kinetin, and kinetin-puromycin treated leaves was found to occur during the first 4 hr. of greening. Based on the similarity of the mode of activity of both puromycin and kinetin, t-RNA synthesis is required for nucleic acid and protein metabolism and a specific 4S species is implicated for chlorophyll

production. The interference of chlorophyll production because of the presence of puromycin illustrates the dependency of chlorophyll synthesis on protein synthesis. Whether this dependency is enzymatic in amount or a structural relationship remains to be established.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
δ-AL	δ-aminolaevulinic acid
δ-ALase	δ-aminolaevulinic acid dehydrase
δ-AL synthetase	δ-aminolaevulinic acid synthetase
Chlase	Chlorophyllase
Copro	Coproporphyrin
Coprogen	Coproporphyrinogen
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
Mg-proto IX	Magnesium protoporphyrin
Mg-protomonomethyl ester	Magnesium-protoporphyrin IX monomethyl ester
m-RNA	Messenger-ribonucleic acid
nm	Nanometer
NAD^+, NADH	Nicotinamide adenine dinucleotide and its reduced form
$NADP^+$, NADPH	Nicotinamide adenine dinucleotide phosphate and its reduced form
O.D.	Optical density
Protochlorophyllide-a	Magnesium vinyl pheoporphyrin a_5
PBG	Porphobilinogen
Proto IX	Protoporphyrin IX
Protogen IX	Protoporphyrinogen IX
RNA	Ribonucleic acid
t-RNA	Transfer-ribonucleic acid
Uro	Uroporphyrin
Urogen	Uroporphyrinogen

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EFFECT OF KINETIN AND OTHER GROWTH RECULATORS ON CHLOROPHYLL SYNTHESIS IN INTACT, ETIOLATED BEAN SEEDLINGS (PHASEOLUS VULGARIS, VAR. COMMODORE)

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ABSTRACT

Primary leaves were excised from dark-grown bean seedlings (Phaseolus vulgaris, var. Commodore) which were treated with kinetin, gibberellic acid, indolacetic-3-acid, and puromycin. The application of kinetin enhanced chlorophyll production while other growth regulators, gibberellic acid, indolacetic-3-acid, were ineffective. The time course pattern of chlorophyll formation indicated a shoulder during Stage III which suggests a sequential pathway for chlorophyll-b biosynthesis. The application of puromycin demonstrated the requirement of protein synthesis for chlorophyll metabolism.

Protein levels of isolated chloroplasts were determined. Little to no increase of total protein was noted in experiments using kinetin and puromycin-kinetin. The presence of puromycin inhibited protein synthesis before illumination of seedlings and no inhibition was noted thereafter.

Morphogenesis of plastids is discussed in respect to chlorophyll and grana formation.