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Scope and Method of Study: A review of the literature was made in regards to the types of chlorophyll present in various organisms and the synthesis of the most common type, chlorophyll a. An attempt was made to emphasize the characteristics of the chlorophylls and to point out the factors affecting their synthesis.

Findings and Conclusions: There are five basic types of chlorophyll, a, b, c, d, and bacteriochlorophyll. Of these chlorophyll a is found in largest amounts in higher plants and is the first to be synthesized. The process of photosynthesis is entirely dependent on the presence of chlorophyll in the living plant cell. The chloroplasts are the units containing chlorophyll in the cells and are the sites of this food-making process.

Factors affecting the synthesis of chlorophyll are as follows: genetic factors, light, oxygen, carbohydrates, nitrogen, magnesium, iron, other mineral elements, temperature, water, etc.

The chemical synthesis of chlorophyll goes through a series of about fifteen steps most of which are fairly well understood and the catalytic enzymes have been partially determined.

Chlorophyll b is believed to be synthesized by the same route as that for chlorophyll a but the final steps are different resulting in a slightly different compound being produced at a later time and in smaller amounts.

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THE SYNTHESIS OF CHLOROPHYLL

By

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CHAPTER I

INTRODUCTION

The purpose of this study is to review the literature pertaining to the types and synthesis of chlorophyll and to present it in a condensed, and, it is hoped, adequate manner.

Chlorophyll is probably the most unique chemical substance known to man. Its characteristic properties enable it to carry on what is undoubtedly the most important chemical reaction of living things, the process of photosynthesis. Without the green plant and its foodmaking properties, life as we know it would be impossible.

By means of chlorophyll, living plant cells can change the energy of the sun into chemical energy. Plant cells take carbon dioxide from the air and water from the soil and change them into simple sugars. The plant can do this only with the aid of chlorophyll and sunlight. Chlorophyll uses the energy of the sunlight to form the sugar molecule which is then transformed into complex carbohydrates, fats, and proteins in the plant.

CHAPTER II

TYPES AND CHARACTERISTICS OF CHLOROPLAST PIGMENTS

Green is the predominant color of the plant kingdom. With few exceptions, leaves are green, as are also many other plant organs such as herbaceous and young woody stems, young fruits, and sepals of flowers. The green coloring of plants is termed chlorophyll, although actually a number of kinds of chlorophyll occur in plants.

Less evident is the fact that the leaves and many other organs of plants also contain yellow pigments. These are seldom apparent except in leaves in which the chlorophyll fails to develop or in which it is destroyed as a result of senescence or other physiological changes. The yellow chloroplast pigments of leaves belong to the group of compounds called carotenoids.

Except in blue-green algae and the photosynthetic bacteria, chlorophylls occur only in the chloroplasts. The carotenoids of leaves are also restricted to the chloroplasts, and this also appears to be true of the carotenoids in the cells of most algae. All of these pigments, it seems, are present only in the grana of the chloroplasts. In certain other plant organs, such as flower petals, the yellow pigments occur in chromoplasts. The chlorophylls and associated

carotenoids are often called the chloroplast pigments. In some kinds of algae, pigments of another group, called the phycobilins, also occur in the chloroplasts. The best known of these are the phycoerythrin of most red and some blue-green algae, and the phycocyanin of most blue-green and some red algae. These two pigments are closely related chemically. Both are colloidal proteinaceous pigments and can be extracted from the cells with hot water.

A number of different kinds of chlorophyll occur in the plant kingdom. Of these, chlorophyll a is most nearly of universal occurrence, being present, as far as is known, in all photosynthetic organisms except the green and purple bacteria. Chlorophyll b is found in all higher plants and in the green algae, but is not present in most other algal phyla. Chlorophyll c is found in the brown algae and diatoms, which do not contain chlorophyll b. Similarly red algae contain chlorophyll d but no chlorophyll b. In the purple bacteria still another kind of chlorophyll called bacteriochlorophyll is present, whereas the green bacteria contain another apparently similar pigment called bacterioviridin. All of these chlorophyll are very similar in chemical composition, and all of them are compounds which contain magnesium. (Meyer, 1960)

Chlorophyll a and b are the characteristic chlorophylls of the higher plants. Neither is water soluble, but both are soluble in a number of organic reagents. Chlorophyll a is readily soluble in absolute ethyl alcohol, ethyl ether, acetone, chloroform, and carbon bisulfide. Chlorophyll b is

soluble in the same reagents, although generally less so. Chlorophyll a is blue-green in solution, and blue-black in the solid state; chlorophyll b is almost pure green in solution, and greenish-black in the solid state.

All of the chlorophylls possess the property of fluorescence--the property manifested by certain substances when illuminated of re-radiating light of wave lengths other than those they absorb. Usually the radiated light is longer in wave length than the incident light. Chlorophyll a in ethyl alcoholic solution exhibits a deep blood-red fluorescence, best seen by viewing the solution in reflected light. Similar solutions of chlorophyll b exhibit a brownish-red fluorescence. Chlorophyll in living cells also exhibits fluorescence.

Willstatter (1928) and his associates isolated chlorophylls a and b in pure form from over two hundred different species of higher plants and found each type to be identical in chemical composition. They also succeeded in determining the molecular formulas of these two chlorophylls. For chlorophyll a this is $C_{55}H_{72}O_5N_4Mg$; for chlorophyll b it is $C_{55}H_{70}O_6N_4Mg$.

Each chlorophyll yields a separate series of degradation products, but one of the products derived from both is an unsaturated primary alcohol which is called phytol ($C_{20}H_{39}OH$). Splitting of the phytol group from the chlorophyll molecule can be accomplished by the enzyme chlorophyllase, which occurs in leaves. Phytol makes up about one-third of the chlorophyll molecule. It has a strong affinity for oxygen

and may be responsible for the reducing action of chlorophyll.

When an ether solution of a chlorophyll is interposed between a source of "white light" and a spectroscope, it can readily be shown that certain wave lengths of light are much more completely absorbed than others. The regions of the spectrum in which complete or nearly complete absorption takes place appear as dark bands. Chlorophylls a and b both show a definite and characteristic absorption spectrum when in solution. The exact appearance of such absorption spectra will vary, however, depending upon the kind of solvent and the concentration and thickness of the layer of solution which is examined. Both chlorophylls exhibit maximum absorption in the blue-violet region and a secondary maximum in the short red.

Chlorophylls are invariably and specifically associated with photosynthesis. All of the chlorophylls appear to participate in photosynthesis in fundamentally the same ways. Their role appears to be twofold: (1) they absorb certain wave lengths of radiant energy and either convert this energy into other wave lengths which are used in photosynthesis, or else transfer the absorbed energy directly to compounds involved in the reaction; (2) they act in the capacity of a catalyst at some stage or stages of the photosynthetic process. The first of these roles is the more obvious of the two, since neither carbon dioxide nor water absorbs radiant energy in the visible range. It is essential, therefore, that the reaction be "sensitized" by a pigment.

The universal presence of the carotenoid pigments in the chloroplasts suggest that they also participate in photosynthesis, but no evidence has yet been found that they actually play any role in the process in the highest plants. There is good evidence, on the other hand, that at least some of the light absorbed by certain carotenoid pigments in diatoms and green and brown algae is used in photosynthesis. Similarly, light absorbed by phycocyanin in some blue-green algae and by phycobilins (phycocyanin and phycoerythrin) in some red algae is used in the process. In some red algae the phycobilins appear to be the primary light absorbers rather than chlorophyll. While other pigments may thus supplement chlorophylls as light-absorbers in the photosynthetic mechanism of at least some species, they cannot substitute for chlorophyll in its catalytic role. No example has ever been found of photosynthesis occurring in a cell which did not contain at least one of the chlorophylls. (Meyer, 1960)

It has never been found possible to accomplish the complete photosynthetic process in vitro by the use of chlorophyll solutions or dispersions. Until very recently the complete process of photosynthesis was not known to occur even in intact chloroplasts isolated from cells. Since it had been known for some time that a part of the photosynthetic process could occur in vitro in the presence of isolated chloroplasts or chloroplast fragments, it was generally considered that other constituents of the living cell system besides the chloroplasts were essential for the occurrence of photosynthesis.

It has now been shown, however, that under the proper conditions the complete process of photosynthesis can occur in isolated chloroplasts. The important principle emerging from this significant discovery is that the chloroplast is the complete photosynthetic unit in plant cells. The chloroplasts, therefore, appear to be the unit of protoplasm in which the entire process of photosynthesis occurs either inside the cell or, under the right conditions, outside the cell. The chloroplast is regarded as a complete photosynthetic unit containing multienzyme systems divided into three main groups, each controlling an increasingly complex phase of photosynthesis; photolysis of water, photosynthetic phosphorylation, and CO₂ fixation.

CHAPTER III

FACTORS INFLUENCING CHLOROPHYLL SYNTHESIS

A number of conditions are known to be necessary for or at least to influence greatly the synthesis of chlorophylls in plants. Absence of any one of these factors will inhibit chlorophyll synthesis, resulting in the condition often called chlorosis. This term is most frequently applied when the failure of chlorophylls to develop is the result of a deficiency of one of the essential mineral elements. Different types of chlorosis may develop in the leaves of any species depending upon the factor limiting chlorophyll formation. The following discussion of the factors influencing chlorophyll synthesis refers primarily to the formation of chlorophylls a and b in the higher plants. (Meyer, 1960)

Certain genetic factors are necessary for the development of chlorophylls as shown by the behavior of some varieties of maize in which a certain proportion of the seedlings produced cannot synthesize chlorophylls, even if all environmental conditions are favorable for their formation. As soon as the food stored in the grain is exhausted such albino seedlings die. This trait is inherited in such strains of maize as a Mendelian recessive and hence is apparent only in

plants homozygous for this factor. More or less similar genetic effects on chlorophyll synthesis have been demonstrated in a number of other species.

Light is usually necessary for the development of chlorophyll in the angiosperms. In the algae, mosses, ferns, and conifers, however, chlorophyll synthesis can occur in the dark as well as in the light, although the quantity produced is often less in the absence of light than in its presence. In a few angiosperms such as seedlings of water lotus (*Nelumbo*) and in the cotyledons of citrus embryos, chlorophylls can also develop in the absence of light.

Relatively low intensities of light are generally effective in inducing chlorophyll synthesis. All wave lengths of the visible spectrum will, if their energy value is adequate, cause chlorophyll development in etiolated seedlings except those longer than 680 m μ .

Like other complex compounds synthesized in plants, chlorophyll represents the terminal product of a long chain of reactions. Most of the steps in this sequence of reactions are known and will be discussed in Chapter IV. Dark-grown seedlings of higher plants are usually yellow in color, but actually contain traces of a green pigment, called protochlorophyll. Chemically this compound is closely related to the other chlorophylls, differing from chlorophyll a only in having two less hydrogen atoms in the molecule. There is good evidence that protochlorophyll is the immediate precursor of chlorophyll a. Reduction of protochlorophyll

to chlorophyll a appears to be the last step in the synthesis of this latter compound. In the higher plants, at least, this reaction occurs only in the light, and the protochlorophyll is the light-absorbing agent for its own transformation to chlorophyll a.

If a solution of chlorophyll in an organic solvent is exposed to bright light, its color soon fades because of a destructive effect of light upon the chlorophyll. This is probably a photo-oxidation process, since it is accompanied by the absorption of oxygen. In the absence of oxygen and reactive compounds, chlorophyll is remarkably stable against illumination. (Linschitz, 1958)

Strong light also brings about the disintegration of the chlorophylls in leaves, although at a less rapid rate than in chlorophyll solutions. In leaves exposed to intense light, therefore, synthesis and decomposition of chlorophylls are probably going on simultaneously. In accord with this concept are the results of one investigator who found in a number of species of plants that the chlorophyll content per unit leaf weight or per unit leaf area increased with decreasing light intensity until a relatively low intensity was reached. Further decrease in light intensity below this value caused a decrease in chlorophyll content.

In the absence of oxygen, etiolated seedlings fail to develop chlorophylls even when illuminated under conditions otherwise favorable for chlorophyll formation. Oxygen must therefore be required in some of the steps in the sequence of reactions whereby chlorophyll is synthesized.

Etiolated leaves which have been depleted of soluble carbohydrates fail to turn green even when all of the other conditions to which they are exposed favor chlorophyll synthesis. When such leaves are floated on a sugar solution, sugar is absorbed and chlorophyll formation occurs rapidly. A supply of carbohydrate foods is therefore essential for the formation of chlorophyll.

Since nitrogen is a part of chlorophyll molecules it is not surprising to find that a deficiency of this element in the plant retards chlorophyll formation. Failure of chlorophylls to develop is one of the commonly recognized symptoms of nitrogen deficiency in plants.

Magnesium is also a part of chlorophyll molecules. Deficiency of magnesium in plants results in the development of a characteristic mottled chlorosis of the older leaves.

In the absence of iron in an available form, green plants are unable to synthesize chlorophylls and the leaves soon become blanched or yellow in color. While not a constituent of chlorophyll molecules, iron is essential for its synthesis. The exact function is not known. (Lascelles, 1956).

In the absence of manganese, copper or zinc, more or less characteristic chloroses develop in plants. These, along with other substances, apparently play at least indirect roles in the synthesis of chlorophyll.

In general, chlorophyll synthesis can occur over a wide range of temperatures. In etiolated wheat plants,

synthesis of chlorophylls occurs at any temperature within the range 3 to 48° C., but is most rapid between 26° and 30° C. Somewhat similar results have been obtained on chlorophyll synthesis in potato tubers except that synthesis appears to be the most rapid in the approximate range 11 to 19° C. The range of temperatures over which chlorophyll synthesis occurs, as well as the temperature of most rapid synthesis, undoubtedly varies considerably from one species to another.

Water is very essential to the synthesis and normal functioning of chlorophyll. One fact which may have a significance for the problem of photosynthesis is a peculiar affinity of chlorophyll for water. Dissolved in absolutely dry benzene, chlorophyll shows no fluorescence. Traces of water--it is sufficient to open the flask containing the dry chlorophyll solution to the air--restore the fluorescent state of chlorophyll. Desiccation of leaf tissues not only inhibits synthesis of chlorophylls but seems to accelerate disintegration of the chlorophylls already present. A familiar example of this effect is the browning of grass during droughts. (Livingston, 1949)

The mechanism of chlorophyll synthesis is very sensitive to any type of physiological disturbance within the plant. Many other conditions besides those already discussed, such as lack of certain other mineral elements, deficient aeration of the roots, infestations of insects, or infection with bacterial, fungus, or viral diseases may induce directly or indirectly partial or complete chlorosis of the leaves. The failure of chlorophyll synthesis to take place normally is

often one of the first observable symptoms of almost any upset of the metabolic conditions within a plant.

CHAPTER IV

THE CHEMICAL SYNTHESIS OF CHLOROPHYLL a

It has been known for some time that chlorophyll a is formed by the photoconversion of protochlorophyll present in dark grown leaves. Light is not required for the synthesis of protochlorophyll but it is necessary for the final steps in the production of chlorophyll a.

In the formation of chlorophyll a the biosynthetic chain starts with acetate and glycine as has been demonstrated by experiments with carbon-14. (Della Rosa, 1953). The acetate is converted to succinic acid through the tricarboxylic acid cycle. Beyond this step the pathway shown in Figure 1 seems most reasonable.

Most, if not all, of these steps are enzymatically catalyzed and a review of the enzymes involved that are found in photosynthetic organisms will follow. All the enzymes have not been isolated and none of these plant enzymes have been completely purified. By one method or another, however, their existence seems assured.

An enzyme catalyzing step I has been isolated from spinach by Kaufman and Alivisatos (1955). A name was not given to this enzyme.

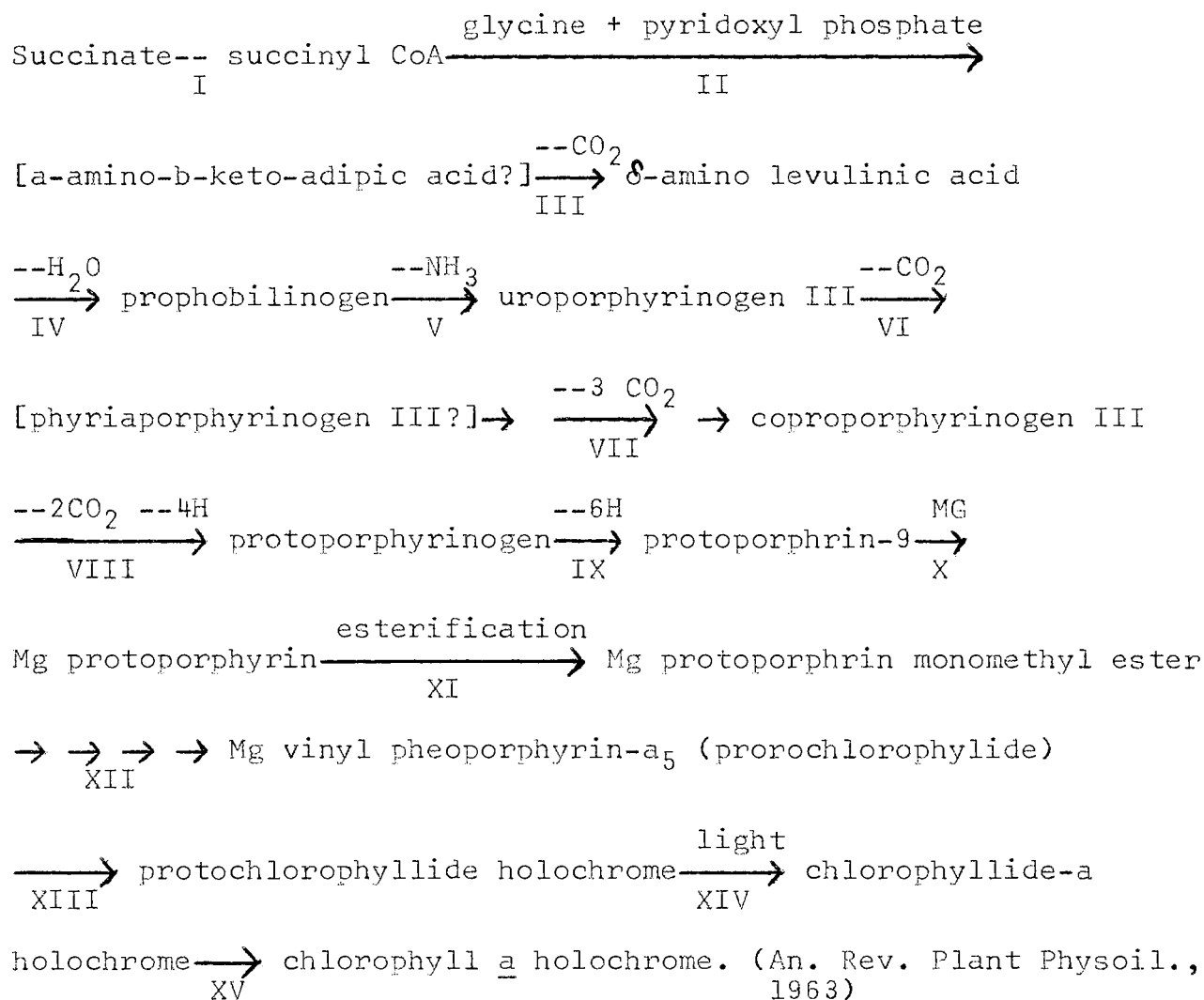


Figure 1

Steps II and III should be considered together because it is known that succinyl-CoA plus glycine and pyridoxyl phosphate form δ -aminolevulinic acid, whether or not the reaction passes through the questionable α -amino- β -keto adipic acid. The catalyzing enzyme of this reaction is called δ -aminolevulinic acid synthetase. This enzyme has been prepared in soluble form from Rhodopseudomonas spheroides by precipitation with 30 per cent ammonium sulfate. (Kikuchi, 1958).

The δ -aminolevulinic dehydrase is the enzyme facilitating step IV, the condensation of δ -aminolevulinic acid to porphobilinogen. Schmid and Shemin (1955) have prepared highly purified protein from duck erythrocytes which carries out this reaction but this specific enzyme has not been isolated from plants.

Step V, porphobilinogen to uroporphyrin III is the result of two enzymes: (a) porphobilinogen deaminase, and (b) uroporphyrinogen isomerase.

Uroporphyrinogen decarboxylase (Bogorad, 1960; Mauzerall, 1958) is the enzyme active in steps VI and VII. In these steps uroporphyrinogen, the hexahydroporphyrin with eight carboxyl groups, is decarboxylated stepwise and at random at the acetic acid side-chains to form the hexahydro-prophyrinogen, copropoeophyrinogen, with four carboxyl groups. The enzyme has been isolated from hemolyzed rabbit red cells by zone electrophoresis on starch.

Step VIII is catalyzed by coproporphyrinogen oxidase. This enzyme has been prepared from beef liver mitochondria by solubilization with thioglycollate and fractionation with 40 to 70 per cent saturated ammonium sulfate. The enzyme activity has been concentrated about twenty times. The enzyme is highly specific for coproporphyrinogen and acts two or three times faster on series III than series I.

The enzymatic action of steps IX through XIII was not definitely determined at the time of this study.

Step XIV is the photochemical conversion of protochlorophyllide to chlorophyllide a. The final step (XV) in the formation of chlorophyll a is the phytylation. This takes place immediately after chlorophyllide a is formed. It is a thermochemical reaction. Apparently both chlorophyllase and phytol are formed by the initial illumination.

Immediately following the photoproduction of chlorophyllide a spectral changes occur at room temperature. The long wave length absorption peak shifts from about 684 to 670 mu. Experiments on glycerine extracts of holochrome indicated that a spectral shift could take place at the "protochlorophyll" stage which would carry over to the "chlorophyll" stage.

CHAPTER V

THE PATHWAY TO CHLOROPHYLL b

In the biosynthesis of chlorophyll a build up of a precedes the accumulation of b. In most all plants, the ratio between the two runs fairly constant, about three a to one b, but a always appears first. This might indicate that b was synthesized through a, and when working with carbon 14 the maximum radioactivity is lower in b than a and is reached at a later time. However the curves of specificity activity of a and b as a function of time do not intersect as they would if one was converted into another.

The question is what is the precursor of chlorophyll b. Much work has been done in this area, but no definite answer has been agreed upon. Following are four possibilities as to the relationship between a and b and some of the related ideas.

Chlorophyll a comes from chlorophyll b. This has been almost entirely rejected because a is formed first and only after large amounts of a are formed does b appear. Also many plants have only chlorophyll a.

Chlorophyll b comes from chlorophyll a. This is the classical concept of the synthesis of chlorophyll b and has many observations to support it. Genetic sequence indicates a going to b through work with mutant genes.

Chlorophyll a and b arise by separate paths from a common intermediate. From data at present time, this seems most probable. Etiolated leaves placed in the light formed chlorophyll a first but when chlorophyll b began to form, its rate of increase was proportional to the rate of increase of a. These data strongly suggested the side-by-side production of chlorophylls a and b and not sequential formation. Young plants containing labeled protochlorophyll, when illuminated four to six hours, produced chlorophylls a and b of equal specific activity. This interpretation states that chlorophylls a and b come independently from the same preformed protochlorophyll.

Chlorophylls a and b are formed independently from different building blocks. This idea seems impossible due to the close structural resemblance of the two pigments. Also the experiment where chlorophylls a and b of equal specific activity were obtained from the same labeled intermediate would indicate this process to be impossible.

In concluding I believe that it is evident that chlorophyll b is synthesized by the same route as that for chlorophyll a but the final steps are different resulting in a slightly different compound being produced at a later time and in smaller amounts.

CHAPTER VI

SUMMARY

There are five basic types of chlorophyll, a, b, c, d, and bacteriochlorophyll. Of these chlorophyll a is found in largest amounts in higher plants and is the first to be synthesized. The process of photosynthesis is entirely dependent on the presence of chlorophyll in the living plant cell. The chloroplasts are the units containing chlorophyll in the cells and are the sites of this foodmaking process.

Factors affecting the synthesis of chlorophyll are genetic factors, light, oxygen, carbohydrates, nitrogen, magnesium, iron, other mineral elements, temperature, water, etc.

The chemical synthesis of chlorophyll goes through a series of about fifteen steps most of which are fairly well understood and the catalytic enzymes have been partially determined.

Chlorophyll b is believed to be synthesized by the same route as that for chlorophyll a but the final steps are different resulting in a slightly different compound being produced at a later time and in smaller amounts.

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