DETECTION, ISOLATION AND CHARACTERIZATION OF CHLOROPHYLLS AND RELATED PIGMENTS DURING RIPENING OF FRUITS AND VEGETABLES

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Denise Yen-ching Lynn Co

1966

This is to certify that the

thesis entitled

"Detection, isolation and characterization of chlorophylls and related pigments during ripening of fruits and vegetables."

presented by

Denise Yen-ching Lynn Co

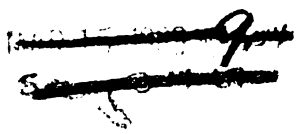
has been accepted towards fulfillment of the requirements for

Ph.D. **degree in** Food Science

Sigmund H. Pilnik
Major professor

Date 11/18/66

O-169



EX A 004

ABSTRACT

DETECTION, ISOLATION AND CHARACTERIZATION OF CHLOROPHYLLS AND RELATED PIGMENTS DURING RIPENING OF FRUITS AND VEGETABLES

by Denise Yen-ching Lynn Co

Thin-layer chromatographic methods using silica gel G adsorbent and several solvent systems were developed. These micromethods permit rapid separation of small amounts of chlorophylls and related pigments. Eight major and eight to ten minor tetrapyrrole pigments can be separated speedily. The results of the thin-layer chromatography were evaluated and compared with column and paper chromatography using known compounds.

Pigment extracts from the peels of progressively ripening bananas, peppers and cucumbers were separated with the thin-layer chromatography developed. The chromatograms of the extracts from green banana peels and cucumber peels appeared very similar to the patterns from extracts of dark green peppers, and underwent similar changes during ripening. Two compounds were the last to disappear and are thought to be degradation products somewhat more stable than chlorophylls a and b. Besides these two compounds, two additional green pigments appeared in acetone extracts of all three experimental fruits. The visible absorption peaks of the latter two were at 418 and 444 nm. No pink fluorescent compounds were found in the fully mature fruits.

The 418 and 444 compounds fluoresce pink under ultraviolet radiation; under daylight the color of the 418 compound resembles that of chlorophyll a, and the 444 compound resembles chlorophyll b. Visible spectra

showed that they are different from chlorophylls *a* and *b*.

Enzymatic de-esterification tests and IR spectra showed that they posess an ester group at the C_7 propionic acid sidechain. Both gave a negative phase test indicating that the isocyclic ring is altered, while the visible spectra showed that they are different from allomerized chlorophylls. The central metal can be removed easily with oxalic acid and the metal-free derivatives can be recomplexed with Cu. They move more slowly than the chlorophylls on a thin-layer chromatogram and their HCl numbers are slightly lower than those of the chlorophylls. This indicates that these two compounds are slightly more basic than chlorophyll and are metal-containing diester chlorophyll-type pigments. They could possibly be derivatives of chlorophylls or of chlorophyll precursors, or even be precursors of chlorophylls.

DETECTION, ISOLATION AND CHARACTERIZATION OF CHLOROPHYLLS AND RELATED PIGMENTS DURING RIPENING OF FRUITS AND VEGETABLES

By

Denise Yen-ching Lynn Co

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science

1966

TO

my parents

ACKNOWLEDGMENTS

The author wishes to express her sincere thanks to Dr. S. H. Schanderl for his guidance and suggestions throughout this research and his patience in correcting the thesis. She expresses appreciation to the members of the guidance committee, Dr. C. L. Bedford, Dr. B. S. Schweigert, Dr. P. Kindel, Dr. D. R. Dilley, and Dr. P. Markakis for reviewing the manuscript.

Special thanks are due to Mr. R. J. Embs and Mrs. Schanderl for reviewing the manuscript and to Mr. P. Coleman for reproducing the photographs.

Last but not the least, she wishes to thank her husband Henry for his continueous encouragement throughout this study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

INTRODUCTION

Chlorophyll is the general name of green pigments in photosynthetic organisms. Besides its biological functions, chlorophyll is important in the field of food science. It reveals the ripeness and quality of many fruits and vegetables (Ramirez and Tomes, 1964; Gortner, 1965). It reflects the keeping quality of some foods (Hall and Mackintosh, 1964), and it indicates various changes during the storage, preparation and preservation of green vegetables (Dietrich et al, 1960; Rogachev et al, 1960; Jones et al, 1962; White et al, 1963; Forsyth, 1964; Schanderl et al, 1965).

Chlorophyll is used as coloring matter in some industrial products, such as in green-colored toothpaste, in soap and in candles (Aries, 1946). It was suggested as a possible paint pigment (Thimann, 1949) and as antibacterial agent (Daly et al, 1939; Burgi 1942; Barnes, 1946; Jorgensen, 1962; Beuchat et al, 1966).

The degradation products of chlorophylls, especially phylloerythrin, were reported to produce skin lesions as a result of photosensitivity in light-colored sheep and cattle (Quin et al,1935; Clare, 1944). Some of the chlorophyll derivatives have been reported to be toxic to man (Hashimoto and Tsutsumi, 1964). However, under normal circumstances, the ingestion of chlorophyll is not harmful (Aronoff, 1953).

Since the degradation of chlorophyll in fruits and vegetables is of considerable importance in the field of food science, research was carried out to study the biodegradation of chlorophyll in some green plant

tissues.

To minimize pigment destruction and alteration during analysis and to detect derivatives which might occur in very low concentrations, a rapid micromethod using the thin-layer chromatographic technique was developed.

Besides the usual chlorophylls, some chlorophyll-type pigments were observed on the thin-layer chromatograms. Studies of these pigments were carried out.

LITERATURE REVIEW

General review

The name chlorophyll was initially given by Pelletier and Caventou in 1818 to describe the pigment responsible for the green color of leaves. Today it is generally extended to all classes of photosynthetic porphyrin pigments.

Fremy (1860) was the first to use a partitioning method to separate plastid pigments between an ether solution containing yellow carotenoids and an acidic aqueous solution of blue-green pheophytins and pheophorbides. He thought "green" chlorophyll was a mixture of two pigments. This hypothesis, later affirmed by Stokes (1864) and Sorby (1873), was not substantiated until 1906, when Tswett separated two chlorophylls using column chromatography and named them chlorophylls α and β, which later became a and b.

The modern era on the study of chlorophyll chemistry was opened by Willstätter and his school in the early 20th century, his research being summarized in "Untersuchungen über Chlorophyll". He first established the correct empirical formulae for the chlorophylls and described their preparation and degradation. He was also responsible for the discovery of chlorophyllase and utilized it in the esterification and de-esterification of the chlorophyllides.

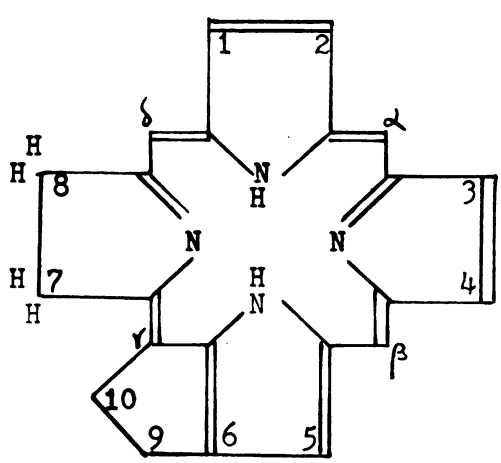
The field of chemistry opened by Willstätter was further developed by his students. By 1940, Fischer, after many years of synthetic, analytical and degradative investigations on both the blood pigments and chlorophylls, established the structures of chlorophylls a and b and

bacteriochlorophyll a.

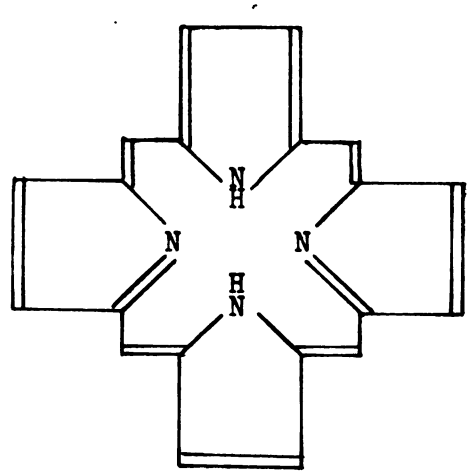
The synthesis studies were initiated by Fischer (1940) but chlorophyll a was not completely synthesized until 1960 (Strell). Woodward et al (1960) were able to synthesize chlorophyll a from simple pyrroles by a series of different methods.

A variety of chlorophylls have been described; chlorophylls a, b, c, and d; bacteriochlorophylls a and b; chlorobium chlorophylls 660 and 650; and their immediate precursors and degradation products, e.g., the pheophytins and pheophorbides of these chlorophylls. Only the structures of chlorophylls a and b and bacteriochlorophyll a are known definitely.

Chlorophylls are magnesium complexes of compounds derived from phorbin (I), which, in turn, are the dihydro derivatives of porphin (II)



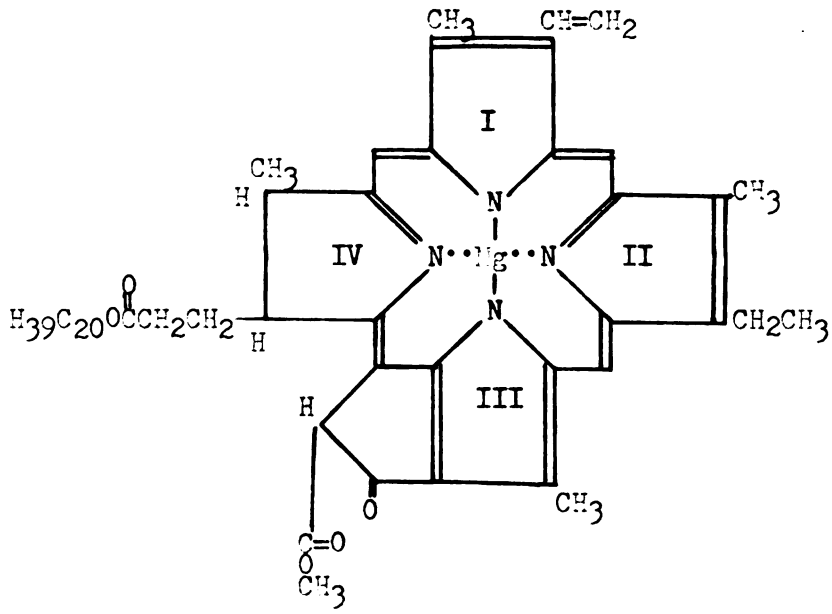
(I)



(II)

with the addition of the isocyclic ring, a cyclopentanone ring connecting C_6 with the γ methene carbon.

The structure of chlorophyll a is shown below (III). Chlorophyll



(III)

b has a formyl in place of the methyl in the 3 position. A chlorophyllide is the derivative resulting from the removal or variation of the alcohol esterified to the C_7 propionic acid group. Pheophorbides and pheophytins are the magnesium-free derivatives of chlorophyllides and chlorophylls, respectively.

Chlorophylls are usually soluble to varying degrees in organic solvents such as ether, acetone, methanol, chloroform and pyridine, and insoluble, when they are pure, in hydrocarbons. Free acid chlorophyllides are less soluble than their esters. Pheophytins and pheophorbides are readily soluble in warm acetic and formic acids and also in aqueous hydrochloric acid, depending on their acid numbers. This constant is lowest for a free acid, increasing with increasing chain length of the esterified alcohol. Ring cleavage to yield chlorins or reduction of

carbonyl groups to alcohols causes a marked drop in the acid number.

All chlorophylls exhibit pronounced absorption bands in the blue-green (Soret) and red regions of the visible spectrum. They are decarbomethoxylated upon prolonged heating of the solutions (Pennington et al, 1964) and are decomposed by acids, alkalies, oxidizing agents, hydrolytic enzymes, oxidative enzymes and intense light (Strain, 1958; Pennington et al, 1964). They are oxidized (allomerized) rapidly when dissolved in relatively inert solvents, such as alcohols, in the presence of air (Strain, 1954). When isolated in the solid state and stored in evacuated and sealed ampules, the chlorophylls may be preserved for long periods without alteration.

Biogenesis and biodegradation of chlorophylls

The biogenesis of the porphyrins, including the chlorophylls, proceeds from the condensation of succinyl coenzyme A with glycine to form δ-aminolevulinic acid which, in turn, is dimerized to porphobilinogen. Four molecules of the latter are condensed to the various porphyrinogens, which are eventually oxidized to protoporphine. Granick (1948) suggested that protoporphine is the bifurcation point for chlorophyll and heme synthesis. A general pathway for subsequent chlorophyll biogenesis has also been proposed (Granick, 1950). Cf. the reviews by Bogorad (1965a, 1965b, 1966).

Observations on the biodegradation of chlorophylls were reported as early as 1845 in connection with morphological changes of the chloroplasts. Subsequently, Tswett (1908) noted the pigment changes during the autumnal discoloration of leaves.

In 1938, Joslyn and Mackinney reported that in a mixture of chlorophylls a and b in 90% acetone, the rate of conversion of chlorophylls to pheophytins was of first order with respect to acid concentration (normality) and possibly second order with respect to chlorophyll concentration. Two years later, Mackinney and Joslyn repeated the same work with pure solutions of chlorophyll a and of chlorophyll b, and confirmed their early statement. In addition, they found that formation of pheophytin a from chlorophyll a was 7-9 times faster than that of pheophytin b from chlorophyll b.

Schanderl et al (1962) reported that in the in vitro conversion of chlorophylls a and b into their respective pheophytins, the rate of conversion of a exceeded that of b by five times, and that the reaction was first order with respect to acid concentration, in agreement with the work of Mackinney and Joslyn.

Cho (1966), using 80% aqueous acetone solvent-acid buffer system, showed that the conversion of chlorophylls a and b to pheophytins a and b follows a general acid catalysis. However, contrary to the above workers' finding, he proposed a new mechanism explaining the rate law and concluded that the rate is second order with respect to hydrogen ion concentration and first order with respect to chlorophyll concentration. He also proposed three resonance forms of chlorophyll b to explain the slower conversion of chlorophyll b to pheophytin b.

Noack (1944) suggested the destruction of chlorophyll in aging leaves could be due to the action of hydrogen peroxide, in heterogeneous catalysis with trivalent metal ions. Chlorophyllides, because of their solubility, were believed to be more easily decomposed by hydrogen peroxide

than chlorophylls. Thus, he supposed that the first step was the hydrolysis of chlorophyll by chlorophyllase. However, Sud'ina and Romanenko (1961) and Sud'ina (1961, 1963) noted that chlorophyllase activity decreased in ripening plant tissues and concluded that it did not participate in the degradation of chlorophyll.

Aronoff and Mackinney (1943) studied the photo-oxidative breakdown of chlorophyll. They obtained pink intermediates when chlorophyll solutions in acetone or benzene were exposed to light in the presence of oxygen, but they did not identify the degradation products of chlorophyll.

Strain (1954) studied the oxidation and isomerization reactions of chlorophyll in killed leaves and noted that oxidation is accelerated by oxidases and that the course of the reactions and the nature of the products depended upon the plant material and its treatment.

Strain (1941) observed that chlorophylls a and b were oxidized to colorless substances in a system consisting of an aqueous extract from soybeans, a fat and oxygen. Holden (1965) found that chlorophyll was bleached by extracts of legume seeds which had lipoxidase activity. The bleaching was inhibited by commercial antioxidants. She concluded that chlorophyll appears to be bleached by co-oxidation during a chain reaction involving peroxidation of fatty acids and the breakdown of hydroperoxide by a heat-labile factor.

In spite of the fact that large numbers of chlorophyll derivatives of oxidative and hydrolytic nature have been prepared in vitro, none of these have been observed as intermediates in the biodegradation in plant tissues, probably because of the extreme speed of the reactions (Egle, 1944). It appears that a rapid cleavage into small fragments must occur, because

no large, obviously derived compounds are observed (Seybold, 1943). Egle (1960), reviewing the literature on chlorophyll breakdown, indicated that there is still very little known about the biochemical processes involved in chlorophyll breakdown.

Chromatographic separation of chlorophylls

Chlorophylls may be separated from one another and from many other plant constituents by partition between immiscible solvents. Separations may involve only one or a few partitions using selected solvent pairs, or they may be based upon multiple partitions. The most effective procedure for the separation of chlorophylls from one another, their various alteration products, and the carotenoid pigments is chromatography with adsorbents, such as cellulose, starch or powdered sugar.

Chromatographic separations may be qualitative or quantitative, on an ultramicroscale or on a preparative scale. Quantitative methods provide inidividual pigments that may be estimated fluorometrically (French, 1960) colorimetrically or spectrophotometrically (French, 1960; Smith and Benitez, 1955).

Chromatographic methods (Heftmann, 1961) may be employed in many different modifications, namely, columnar chromatography, one- or two-dimensional paper chromatography, radial paper chromatography with or without acceleration by centrifugal force, and thin-layer chromatography.

The first columnar chromatographic separation was performed by Tswett (1906), who separated the pigments of plant extract on columns of powdered calcium carbonate or sugar, and obtained a separation of different colored zones. He separated the green pigments well enough to indicate

the presence of two.

Zscheile (1934a) was able to prepare chlorophylls a and b by using a talcum adsorption column for the final purification step of chlorophyll a and fractional precipitation from petroleum ether for the b component. In 1941, Zscheile and Comar reported the successful separation of chlorophylls using sucrose columns alone. They paid particular attention to the changes chlorophylls can undergo on drying and redissolving, stating that drying must be avoided and that the purified chlorophyll solution must be used immediately after purification.

The chromatography of plant extracts on powdered sugar columns was reviewed by Smith and Benitez (1955) and by Strain (1958).

The first separation of a petroleum ether extract of dried leaves on a sheet of filter paper was carried out by Brown in 1939. He was able to obtain 2 green chlorophylls, two or three xanthophylls and a series of carotenes.

Since Brown's experiment, the paper chromatography of leaf pigments has been modified in many ways and applied to the separation of chloroplast pigments (Linskens, 1955). Up to 1958, there were about 50 reports dealing with paper chromatography of plastid pigments (Šesták, 1958). Most solvent systems used were combinations of different proportions of petroleum ether, benzene, acetone, alcohol, toluene, or chloroform. Various methods were also applied, such as normal phase, reverse phase, development on filter paper or glass paper, in one or two dimensions, etc. The most widely used solvent systems for two-dimensional separations were first developed by Bauer in 1952, using "spezial benzin"-petroleum ether-acetone (10:2.5:2, by volume) for the first development and "spezial benzin"-petroleum ether-

acetone-methanol (10:2.5:1:0.25, by volume) for the second development. They were later modified by Sironval (1954), who substituted benzene for "benzin" in Bauer's solvent I and had a successful one-dimensional separation of chlorophylls on paper.

Holden (1962) used several solvent systems for the separation of chlorophylls a and b and some of their breakdown products, e.g., pheophytins, pheophorbides and chlorophyllides, by paper chromatography. Although the eight pigments could not be separated with one solvent mixture, any one could be separated from the others by varying the proportions of petroleum ether, benzene, and acetone in the solvent and making small alterations in the developing conditions.

Recently, Michel-Wolwertz and Sironval (1965) reported that by using paper chromatography, several chlorophylls a and b from chlorella extracts had been separated. The spectra of the isolated pigments were reported and discussed.

The classical method of chromatography of chlorophylls on an adsorbent column is somewhat time-consuming and is more suitable for separating larger quantities of pigments. To obtain good separations of chlorophylls on paper chromatograms, it is necessary to use solvents of relatively high boiling point (Green, 1958). Thin-layer chromatography has been developed as a method for the rapid separation of many types of compounds (Stahl, 1963). The rapidity of this technique makes it especially useful for labile compounds, such as chloroplast pigments.

Colman and Vishniac (1964) reported a separation of chlorophylls a and b on a powdered sugar thin-layer plate two-dimensionally. Nutting et al (1965), using the same material, separated pheophytins a and b one-

dimensionally with 5% acetone in Skellysolve B within 3½ hours.

Bacon (1965) separated chlorophylls a and b, chlorophyllides and pheophytins by thin-layer chromatography on cellulose one-dimensionally with the solvent system of petroleum ether-acetone-n-propanol (9:1:0.045, by volume). R_f values for chlorophylls a and b and some of their derivatives were given, but he noted these were approximate values since they vary with, for example, the brand of cellulose powder and the total quantity of pigments applied, or whether the pigments are applied as a mixture rather than as separate spots.

Kieselguhr with admixtures, fat-treated kieselguhr, and fat-treated cellulose also have been used as adsorbents (Hager and Bertenrath, 1962; Egger, 1962). Schneider (1966) evaluated these methods. In addition, he developed a method, also using cellulose plates, which separated chlorophylls a and b when successively developed by two solvent systems in the same direction. He reported that the yield of pigment cannot be improved if sugar, ascorbate or cysteine is added to the plates to prevent oxidation of the pigments, or if chromatography is accomplished in an atmosphere of nitrogen.

Schaltegger (1965), using silica gel for one-dimensional separation of chloroplast pigments in cherry leaves, was able to separate four carotenoids, three chlorophylls a and two chlorophylls b, pheophytin and porphyrin.

Kim (1966) used Gas-chrom P for column and thin-layer chromatography, and kieselguhr G coated with triolein for the complete fractionation of bacteriochlorophyll and its degradation products by thin-layer chromatography.

Chlorophyllase

Chlorophyllase (chlorophyll chlorophyllido-hydrolase) was discovered over fifty years ago by Willstätter and Stoll (1910). It is widely distributed in plants and may be of considerable importance in the metabolism of the plant (Holden, 1963).

The breakdown of chlorophyll by chlorophyllase *in vivo* was suggested by Noack (1944). The hypothesis that chlorophyllase takes part in the biosynthesis of chlorophyll but does not take part in its breakdown *in vivo* was proposed by Sud'ina and Romanenko (1961) and reemphasized by Sud'ina (1961, 1963).

Whatever its function *in vivo*, chlorophyllase nevertheless catalyses *in vitro* the removal of the phytyl group from chlorophylls *a* and *b* and pheophytins *a* and *b*; bacteriochlorophyll is also a substrate (Fischer and Lambrecht, 1938). Klein and Vishniac (1961) reported that chlorobium chlorophyll-650 was hydrolyzed by rye chlorophyllase. Thus, it has become an important tool for testing the presence of phytyl or any other alcohol esterified in the C_7 propionic position of the chlorophyll.

Krossing (1940) found that the enzyme was located in the chloroplast fraction of spinach leaves. By digitonin treatment, Ardao and Vennesland (1960) isolated chloroplastin, a chlorophyll-lipoprotein complex, from spinach chloroplasts and found that it had chlorophyllase activity.

Holden (1961) succeeded in preparing soluble chlorophyllase from the leaves of sugar beet (*Beta vulgaris* var. saccharifera) and reported that the optimum conditions for activity of the partly purified enzyme are pH 7.7 at 25° and an acetone concentration of 40%.

Shimizu and Tamaki (1962) succeeded in obtaining water soluble

chlorophyllase from tobacco (Nicotiana Tabacum var. bright yellow) leaves. They used n-butanol and aqueous solution of sodium chloride as solvents in isolating the enzyme. In a subsequent study (1963), they reported the phytylation by chlorophyllase on chlorophyllide and pheophorbide in vitro.

Hydrochloric acid number (basicity test)

The distribution of porphyrin and phorbin compounds between organic solvents and aqueous solutions of various concentrations of HCl has been very useful for separating mixtures of these compounds and for identifying individual members of the groups. The two-phase system most widely used is ether and hydrochloric acid. This method of separation was first introduced by Willstätter and Mieg in 1906 for the separation of a number of different porphyrin-like substances.

Willstätter (1913) assigned to each compound a characteristic number, the hydrochloric acid number, which is the weight percent of hydrochloric acid in a solution that extracts two thirds of the chlorophyll derivative (Mg-free compound) when thoroughly shaken with an equal volume of the ether solution.

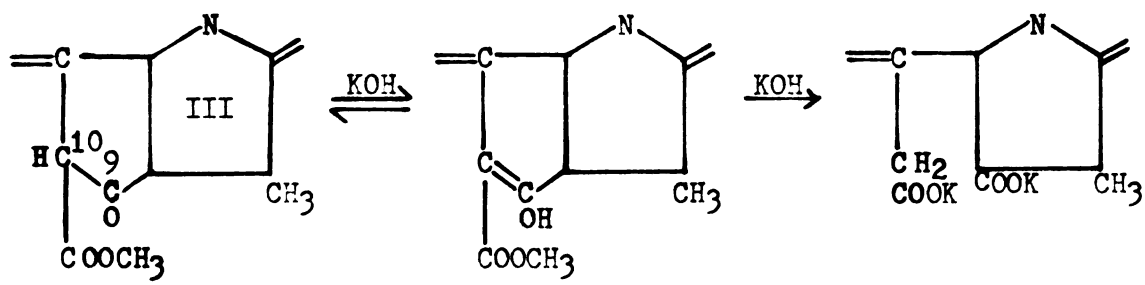
A qualitative test of this kind is very useful in determining whether the chlorophyll molecule still contain the phytyl group.

Molisch phase test

This test, developed by Molisch (1896), consists of underlayering an ether solution of a chlorophyll with an approximately equal volume of a 28 to 30% (w/v) methanolic potassium hydroxide solution.

The mechanism of the phase test was suggested by Stoll and

Wiedemann (1952) as an enolization of the hydrogen atom on C_{10} to the carbonyl group at C_9. This forms a new double bond between C_9 and C_{10} which enters the conjugation of the phorbin system and causes the color change, yellow in the case of chlorophyll a and reddish in the case of chlorophyll b. The characteristic color only lasts from a few seconds to a few minutes. Further action of the alkali hydrolyzes the linkage between C_9 and C_{10} forming the green compounds; thus, the alcoholic layer finally becomes about the same color as the original ether solutions. These reactions are represented for pheophorbide a by the following structures (Smith and Benitez, 1955):



green pheophorbide a yellow enolate green chlorin e_6

The reaction is at first reversible, but further action at the linkage towards the final stage is irreversible.

A positive phase test indicates that the cyclopentanone ring (the isocyclic ring) has not been oxidized, or the carbomethoxy group at C_{10} has not been removed. However, a considerable fraction of the chlorophyll has to be allomerized to show a negative phase test. Also, the positive phase test is no guarantee of the absence of allomerization. If the chlorophyll is oxidized by air in the presence of alcohol to form a hydroperoxide on C_{10} (Fischer and Stern, 1940), there is no longer an

enolizable hydrogen on C_{10}, and a negative phase test results.

Visible absorption spectra

Prior to the study of infrared and proton magnetic resonance spectra, the visible absorption spectra of chlorophylls and related compounds were the most used physical properties for determining the nature of substituents.

Zscheile, Hogness and Young in 1934 developed a photoelectric method and Zscheile (1934b) used it to measure quantitatively the absorption spectra of the chlorophylls a and b in ether solution. He provided the data for quantitative determinations of chlorophyll concentrations by spectrophotometric measurements between 3950 and 7800 Å as well as a method for the determination of percent composition of mixtures of a and b, with an accuracy of 1 percent or better.

In 1941, Mackinney studied the effects of solvents on the absorptivities of chlorophylls a and b. Values obtained in anhydrous ether and anhydrous acetone may be compared with similar values in methanol aqueous acetone. From the values at 645 and 663 nm, he derived two equations for the calculation of the concentrations of the two chlorophylls in 80% acetone extracts from plant material.

In the same year, Zscheile and Comar (1941) reported the successful separation of chlorophylls on sucrose columns, and they noted that the absorption spectrum in ether solution is very sensitive to previous treatment of the solution.

In 1943, Harris and Zscheile studied the effects of solvents upon the visible absorption spectra of chlorophylls a and b. They prepared

solutions of chlorophyll a in thirteen solvents and of chlorophyll b in five solvents by direct elution from sucrose adsorption columns, and found that the type of solvent greatly changed the absorption spectra.

Holt and Jacobs (1954) studied the spectroscopy of chlorophylls and ethylchlorophyllides a and b and their pheophorbides, and described procedures for the preparation of the sample and separation by column chromatography. With spectral analysis, they found that the replacement of phytol by a short-chain alcohol group or a hydrogen had little or no effect on the molar absorptivities in ether, acetone, or dioxane.

Smith and Benitez (1955) reviewed the literature on the spectral determination of chlorophyll. Chlorophyll solutions were prepared according to the procedure of Zscheile and Comar (1941) and the absorption spectra determined. A comparison of the absorption maxima and absorptivities of chlorophylls and pheophytins reported by Zscheile and Comar (1941) and Mackinney (1940) with their values showed very good agreement in most regions of the spectrum.

Vernon (1960) undertook a critical evaluation of the methodology in the spectral determination of chlorophylls a and b, pheophytins a and b, total chlorophylls, total pheophytins, and percent retention of chlorophylls. He derived a series of equations and checked their reliability against the magnesium titration method of Robinson and Rathbun (1959). Good agreement was found between the chlorophyll values calculated by the two methods. However, the magnesium method cannot be used to differentiate between chlorophylls a and b or to determine several constituents in one solution.

A comment on the spectrophotometric determination of chlorophyll was made by Bruinsma (1961). He proposed the equation for the determination

of total chlorophyll. In 1963, the same author gave a brief review of the quantitative analysis of chlorophylls a and b in plant extracts and gave a different equation for calculating the ratio of chlorophyll a to b.

Infrared spectra

Infrared spectra of various chlorophylls and their derivatives have not been used for quantitative analysis; however, they appear to offer possibilities for comparative and identification purposes.

The first examination of the infrared spectrum of chlorophyll and the important related compounds was made by Stair and Coblentz in 1933. Weigl and Livingston (1953), using improved instrumentation, analyzed the infrared spectra of chlorophylls a and b and bacteriochlorophyll.

Holt and Jacobs (1955) further demonstrated the applicability of infrared spectroscopy to structural problems in chlorophyll chemistry. Sidorov and Terenin (1961) examined the spectra of the divalent metal derivatives of pheophytin a and pyropheophytin a.

Katz et al (1963) reported that the infrared spectra of chlorophylls a and b in carbon tetrachloride, chloroform, and benzene in the 1600-1750 cm^{-1} region are best explained on the basis of intermolecular aggregation involving co-ordination of ketone and aldehyde carbonyl oxygen atoms of one molecule with the central magnesium atom of another.

Katz et al (1966) reviewed the work of infrared in the book "The Chlorophylls" and presented IR absorption spectra for chlorophylls a and b, pheophytins a and b, pyrochlorophyll a and other chlorophylls.

MATERIALS AND METHODS

I. Materials

A. Spinach: Market purchased fresh spinach was used for the preparation of known pigments.

B. Banana: Bananas (Gros Michelle), without ethylene pretreatment, were obtained from the American Fruit Company in Detroit. The bananas were received in the stages #1 to #2 of ripeness according to the standard color index number of the Fruit Dispatch Company (Fig. 1). The bananas were stored at 15-16°C or ripened at room temperature to stages #4 and #6.

C. Pepper: Bell peppers, Capsicum frutescens, were grown in a green house. Selected fruits in their progressive stages of ripening were deseeded and extracted.

D. Cucumber: Cucumbers, Cucumis sativus (white spine variety), were obtained from the Unversity farm. Green peels of approximately one mm thickness were extracted.

E. Chlorophyllase: Leaves of Ailanthus altissima, growing on campus, were used for enzyme preparation.

II. Methods

A. Preparation of known pigment solutions:

1. Chlorophylls: Market-purchased fresh spinach leaves were extracted with cold acetone in a mortar with glass sand. A small amount of $MgCO_3$ was added before extraction to neutralize the acids (Mackinney, 1940). The crude extract was dried under vacuum and redissolved in petroleum ether (P.E.,

b.p. 30-60°), then chromatographed on a powdered sugar column following the procedure of Strain (1958). The zones corresponding to chlorophylls a and b were collected separately.

2. Pheophytins: A portion of chlorophyll a or b solution obtained as above was dried in vacuum and redissolved in 5 ml acetone, and 0.1-0.2g of oxalic acid was added. The mixture was allowed to stand at room temperature for one hour for complete conversion to the metal-free compounds. The pigments were transferred to diethyl ether and washed with water to remove the oxalic acid.

3. Chlorophyllides: Chlorophyllides were prepared according to Holt and Jacobs (1954). Ailanthus altissima leaves were ground with acetone in the proportion of 3:7 (w/v). The ground mixture was allowed to stand in the dark at room temperature for 12 hours. After centrifugation at 1114 x G for 10 minutes, the green supernatant solution was transferred to ether, dried further, and redissolved with P.E. The pigment solution was chromatographed on a powdered sugar column, and the zone containing chlorophyllides a and b was collected. This was repeated once.

4. Pheophorbides: The pheophorbides were obtained from the chlorophyllides by treatment with oxalic acid, as described above for the preparation of pheophytins.

B. Extraction of pigments from plant materials:

1. Pepper: Five-gram samples of the carpel tissue of pepper at four selected stages of ripeness (Fig. 2) were ground with

Fig. 1. Standard color index numbers of banana ripeness, ranging from dark green (#1) to fully mature (#6) and beyond. (Courtesy of Fruit Dispatch Company.)

Fig. 2. Increasing stages of maturity for three varieties of bell peppers (Capsicum frutescens), ranging from very immature (left) to full maturity (right). Variety 035 which has been used in this experiment shows (from left to right) an increase in chlorophyll content, two intermediate stages and full maturity.

acetone in a mortar at 2°C under minimum illumination. Pure glass sand was added to aid grinding, and a pinch of magnesium carbonate was used to neutralize the acids liberated from the tissue. Five to six extractions of 5 ml acetone each were carried out speedily and the extracts combined. Ten to fifteen ml of ether was mixed with the acetone extract. Saturated aqueous NaCl solution was added until two layers were formed, the pigmented material being in the upper (ether) phase. The lower layer was discarded. The mixture was washed repeatedly with water to remove acetone and then dried over anhydrous Na_2SO_4.

2. Banana: Bananas at three different ripening stages, #1-2, #4 and #6 according to the standard color index (Fig.1), were used. Only the peels were extracted for pigments. Ten discs of banana peels were obtained with #12 cork borer, total surface 34.6 cm^2, and the pigments extracted as the peppers.
3. Cucumber: Peels of 1 mm thickness were obtained from green and over-mature yellow cucumbers. The pigments were extracted as with peppers, but not quantitatively.

C. Preparation of chlorophyllase:

1. Chloroplast extraction: Forty g amounts of washed, deribbed Ailanthus leaves were cut and blended in a Waring Blendor with 3 volumes of cold 0.35M NaCl solution for 1 min. The homogenate was filtered through four layers of cheese cloth. The filtrate was centrifuged at 278 x G for 3 min. The supernatant was recentrifuged at 1114 x G for 20 min. The green

chloroplasts, collected in the bottom of the tube, were resuspended in 0.35M NaCl and centrifuged at 1114 x G for 20 min. The washing was repeated 4-5 times until the supernatant was free of green color. All preparations were carried out in a cold room at 2°C.

2. Extraction of chlorophyllase from chloroplasts: The prepared chloroplasts were resuspended in 20 ml of 1% NaCl solution. Forty ml of n-butanol were added to the chloroplast suspension slowly with vigorous stirring at room temperature; the stirring was continued for 5 min. after the addition of butanol. The mixture, after centrifugation at 1114 x G for 20 min., was separated into three distinct layers: a clear yellow aqueous lower layer, a layer of yellowish light particles on the interface, and an upper butanolic layer containing the dissolved green pigments. The lower aqueous layer was removed by suction through a long pipette and filtered. The filtrate was dialyzed against four changes of deionized water for 12-15 hours at 2°C to remove the n-butanol. The dialyzate was briefly centrifuged (232 x G, 20 seconds) in a clinical centrifuge to bring down the insoluble particles. Prechilled acetone was added to the supernatant to make a 70% acetone solution. After standing in the cold for 12 hours, the enzymes were centrifuged (232 x G, 5 min.) in a clinical centrifuge. The precipitate was washed twice with 70% acetone. The washed precipitate was taken up with 10 ml of water and centrifuged to remove the insoluble material. The soluble

enzyme solution was ready for use. The activity was not lost when stored at 0°C for 2 months.

3. Determination of chlorophyllase activity: One part of enzyme solution was mixed with 2 parts of 0.02M sodium citrate solution and incubated with 3 parts of an acetone solution of the pigment in the dark at room temperature. A control was prepared with water substituted for the enzyme solution. After 3 to 12 hours, the mixture was separated on silica gel thin-layer plates with modified Bauer solvent I. Two spots appeared on the plate. The appearance of the pigment with lower R_f value indicates that the phytyl group or C_7 esterified moioty of the chlorophyll was de-esterified by chlorophyllase.

D. Chromatography:

1. Thin-layer chromatography

a. Preparation of thin-layer plates: The adsorbents used for the thin-layer plates were silica gel with binder (silica gel G, E. Merck, Darmstadt, Germany), or without binder (Joseph Crossfield and Sons, Ltd., Warrington, England), as well as fluorescent silica gel G_f (E. Merck, Darmstadt, Germany). Five grams of silica gel powder were mixed with approximately 15 ml of deionized water in a screw-cap vial. After vigorous shaking for 20 seconds, the slurry, which had a pH of 6.2-6.5, was poured on a carefully cleaned grease-free plate (20cm x 20cm). The plate was tilted in various directions to obtain a uni-

form coat, and dried overnight at room temperature on a level surface. For comparisons, some coated plates were partially dried at room temperature for 20 minutes, then in an oven at 85°C for 30 minutes and cooled in a desiccator. By this method, a layer of evenly distributed silica gel with a known quantity per unit area was obtained, and the thickness of the layer can be adjusted readily by varying the amount of silica gel used. The average thickness of the silica gel coating was 0.24-0.25 mm. Only freshly prepared plates were used (Co and Schanderl, 1966b).

b. One-dimensional chromatography: The pigment samples were applied immediately after preparation approximately 1.5 cm from the bottom edge of the plates with capillary tubes under dim light. A graduate micro-pipette was used for quantitative chromatography of the extracts of peppers and bananas. The plates were developed in the dark at 16°C with the first modified Bauer solvent, described below, in a glass chamber saturated with P.E. After 40 minutes, the solvent front had moved about 19 mm. The plates were viewed under ultra-violet (UV, 366nm) radiation and photographed immediately or traced on onion skin paper. For permanent preservation, the developed chromatograms were, after brief air-drying, sprayed with Neatan solution and thoroughly dried in a ventilated oven at 50°C for about 5 minutes. A special adhesive tape (19cm wide roll) was carefully applied to the surface of each chromatogram

and peeled off again, taking with it the layer of silica gel and pigment spots. The preserved chromatograms were placed in a cellophane folder and kept in the dark without pronounced color change even after one year.

c. Two-dimensional chromatography: The pigment preparations were successively applied in one spot at one corner of the plate about 2 cm from the edges, under dim light. Replicate plates were developed in the dark in a glass chamber saturated with P.E. at 16°C using the solvent systems described below. Immediately following development, the chromatograms were photographed under daylight or UV, or traced on onion skin papers or preserved with Neatan solution as described above.

d. Solvent systems:

Solvent system I (modified Bauer solvents)

1st dimension: Benzene-P.E.-acetone (10:2.5:2, by vol.) 40 minutes.

2nd dimension: Benzene-P.E.-acetone-methanol (10:2.5:1:0.25, by vol.) 40 minutes.

Solvent system II

1st dimension: Benzene-P.E.-acetone-methanol (10:2.5:1:0.25, by vol.) 40 minutes.

2nd dimension: P.E.-acetone-n-propanol (8:2:0.05, by vol.) 40 minutes.

Solvent system III

1st dimension: Benzene-P.E.-acetone (10:2.5:2, by vol.)

40 minutes.

2nd dimension: P.E.-acetone-n-propanol (9:1:0.45, by vol.) 2 hours.

2. Paper chromatography: The same pigment samples used on thin-layer were spotted on Whatman No. 1 paper and chromatographed one-dimensionally, descendingly, with Holden's (1962) solvent mixture of P.E.-benzene-acetone (4:1:0.5, by vol.) in a P.E. saturated chamber at room temperature. The time required for a satisfactory separation was 12 hours. Photographs were taken immediately after development.

E. Identification of the pigments:

1. Phase test: The Molisch phase test was carried out by under-layering an ether solution of a pigment with an approximately equal volume of 28-30% (w/v) methanolic potassium hydroxide solution. In a positive test a colored ring is formed at the interface of the two phases, the color being yellow in the case of chlorophyll a and reddish in the case of chlorophyll b.

2. HCl number: The ether solution of the pigment was thoroughly shaken with equal volume of various concentrations (w/v) of HCl solutions. The weight percent of HCl in a solution that will extract 2/3 of a chlorophyll derivative from a equal volume of ether is the HCl number of that pigment.

3. C_7 esterification test: The procedure for the test was the same for determination of the chlorophyllase activity.

4. Copper complexing of pigments:

a. Pheophytinization of pigments: The pigment in acetone was mixed with a small amount of oxalic acid for 1 hour in the dark. Approximately 15-20 ml of ether was added, followed by several successive washings with deionized water to remove the acid and acetone.

b. Copper complexing of pigments: The metal-free pigment can complex with Cu^{++} rather easily during refluxing of the ether pigment solution with a few crystals of $CuCl_2$ under neutral or slightly acidic conditions. The metal-free pigment in ether solution was transferred to a 50 ml round bottom flask, several crystals of $CuCl_2$ were added, and the solution refluxed for approximately 1 hour. The color of the ether solution changed from grey to green within 20 minutes, but the refluxing was continued for one hour until there was no fluorescence under UV radiation.

5. Visible spectra: All pigments which fluoresced pink under UV on silica gel G plates were each eluted with ether. The more polar ones were eluted with either acetone or methanol. The eluates were evaporated under vacuum in a microevaporator, Rotary Evapo-Mix (Buchler Instruments, Fort Lee, N. J.) or by blowing a stream of nitrogen into the solutions. The pigments were redissolved in diethyl ether for spectral evaluation. A Bausch and Lomb spectronic 505 recording spectrophotometer was used to obtain the spectra. A Beckman DU spectrophotometer with a Gilford attachment was used for the

detailed study of some spectral regions. For the pigments which were present in very low concentrations, a special procedure was used: Two appropriately cut strips of black plastic each with a 10 mm horizontal slit, 1 mm high, were inserted in front of the sample and reference cuvettes. This did not effect the width of the light path but reduced the height so that a complete qualitative spectrum of the pigment solution could be obtained with only 0.1 ml in the microcuvette (Scientific Cell Co., 10x3.6 mm).

6. Infrared spectra:

   a. Sample preparation: Thin-layer chromatography on silica gel was carried out to isolate the compounds. The isolated pigments were purified alternately with thin-layer and paper chromatography at least 4 times each, using the systems as described in previous sections. The purified pigments were carefully dried in a vacuum desiccator over $CaCl_2$ for at least 12 hours in the dark before the IR measurement.

   b. IR spectra: The IR spectra were obtained with a Perkin-Elmer 337 grating IR spectrophotometer fitted with beam condensor and an attenuator inserted in the reference beam. The normal slit width and slow scan were used in all the measurements. The sample was dissolved in a small amount of ether and transferred on micro KBr plates. The IR spectra from 4000-400 cm^{-1} were recorded.

RESULTS AND DISCUSSION

I. Thin-layer and paper chromatographic separation of the pigments prepared from the sugar column.

The separation of the pigments prepared by the sugar column and their Mg-free derivatives on paper and thin-layer plates coated with silica gel G is illustrated in Fig. 3.

Both chromatographic methods demonstrate that more than one compound can be separated from the single zone obtained from a sugar column. On the thin-layer chromatogram, the chlorophyllides remained at the origin, and the pheophorbides began to move only after 40 minutes of development. Pheophytin a moved most rapidly and separated well from b, which followed it closely. The chlorophylls had R_f values of about 0.4, chlorophyll a being slightly ahead of chlorophyll b. A number of pigments moved ahead of chlorophyll a, and some partially separated spots could be seen behind chlorophyll b. Paper chromatography with Holden's solvent showed very good separation; the chlorophyll a band from the sugar column separated into at least four compounds and the chlorophyll b into three. However, it needed at least 12 hours to achieve satisfactory results. This is a disadvantage, compared to the fast thin-layer chromatography, because it may allow the formation of the pheophytin. However, the separation of pheophorbides and chlorophyllides was superior to that obtained with thin-layer chromatography.

Fig. 4 shows a one-dimensional thin-layer chromatogram photographed

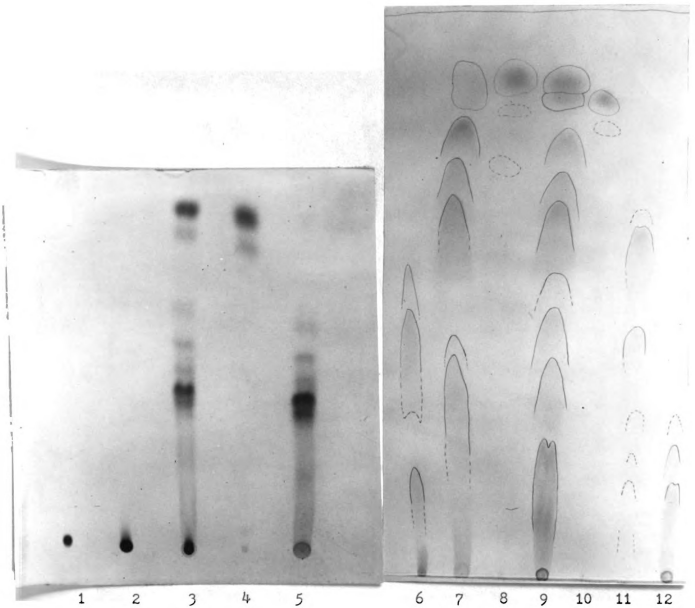


Fig. 3. Separation by one-dimensional thin-layer chromatography using modified Bauer solvent I (left) and descending paper chromatography with Holden's solvent (right). Pigments applied were: (1) pheophorbides, (2) chlorophyllides, (3) mixture of all pigments, (4) pheophytins a and b, (5) chlorophylls a and b, (6) chlorophyll b, (7) chlorophyll a, (8) pheophytin a, (9) mixture of all pigments, (10) pheophytin b, (11) pheophorbides, and (12) chlorophyllides. Spots visible on the paper chromatogram under daylight are outlined with a solid line and under UV with broken lines.

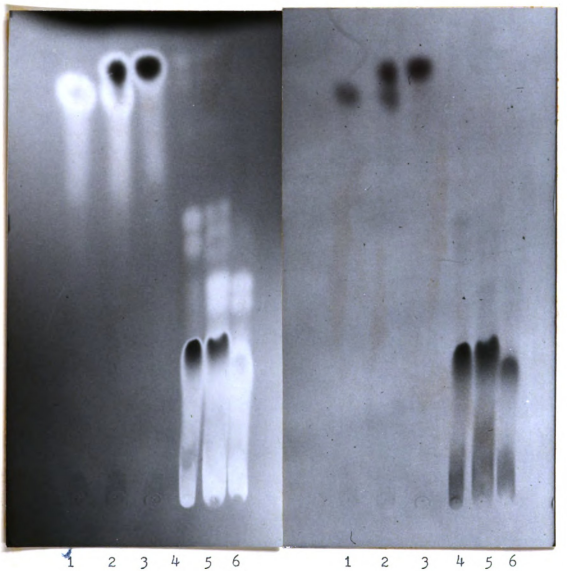


Fig. 4. One-dimensional thin-layer chromatography of prepared pigments developed with modified Bauer solvent I photographed under daylight (left) and UV (right). The pigments applied were: (1) pheophytin b, (2) pheophytins a and b, (3) pheophytin a, (4) chlorophyll a, (5) chlorophylls a and b, and (6) chlorophyll b.

under daylight and under UV. A number of compounds are barely visible under daylight, but under UV two prominent spots are ahead of chlorophyll a and two are ahead chlorophyll b. The spots marked by circling with a pin can easily be scraped off and eluted with suitable solvents. Acetone or methanol was used to elute those components more polar than the chlorophylls (lower R_f), and was then evaporated under vacuum with a micro-evaporator apparatus or under a stream of nitrogen, and the pigments redissolved in diethyl ether for spectral analysis. Diethyl ether was used directly for eluting the chlorophylls and the less polar compounds.

Two-dimensional separation by thin-layer chromatography of the pigments prepared on sugar columns and developed by the modified Bauer solvent systems is demonstrated in Fig. 5. The chromatogram was photographed under daylight (left) and UV (right). As in the UV photograph of the single-dimensional chromatogram, the pheophytin and chlorophyll spots appear dark, although they fluoresce pink (light spots) in lower concentrations. The UV photograph shows clearly that the spots which had appeared in one-dimensional chromatography were separated into several more spots. The chlorophylls separate into three spots; spectral analysis showed that the very left one was chlorophyll a, very right one was chlorophyll b, and a mixture of chlorophyll a and b, possibly isomers, was in the center. Numerous solvent systems were tried with silica gel thin-layer plates throughout this experiment, but only the two most effective ones are reported here with the modified Bauer solvent system.

The modified solvents used did not change the order but accomplished

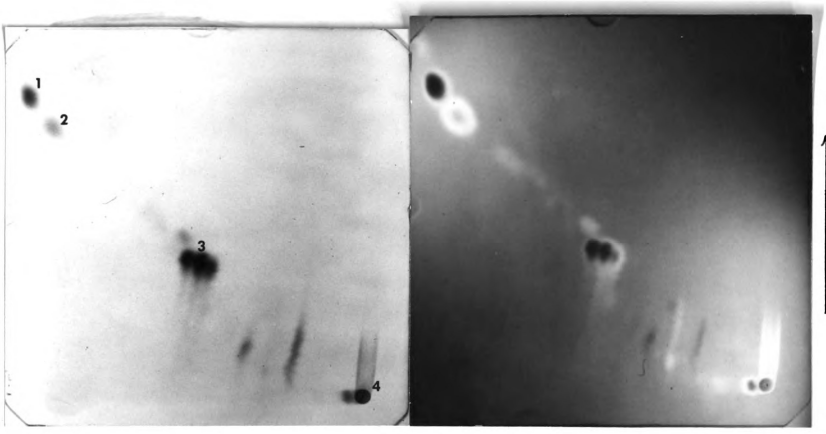


Fig. 5. Two-dimensional thin-layer chromatograms of a pigment mixture developed with solvent system I photographed under daylight (left) and UV (right). Pheophytins a and b appear in the upper left corner (1 and 2). The chlorophylls are grouped together near the center (3), and the pheophorbides are unresolved to the left of the origin (4), where the chlorophyllides remained. The separation of smaller spots between these known compounds is enhanced.

better separation especially in the chlorophyll region. Good separation of chlorophylls into four spots, two of the chlorophyll a and two of the chlorophyll b spectrum, is shown in Fig. 6a. The spots appear even more compact than with the modified Bauer solvent system, and the time required is the same. In Fig. 6b, it can be seen that the solvent system III gave the best separation of the chlorophylls into four distinct spots, again two of chlorophyll a and two of the chlorophyll b spectrum. The time required for the first dimension was also only 40 minutes, but the second dimension required approximately two hours for development.

Chlorophyllides and pheophorbides could not be separated in the first dimension by any of these three solvent systems, and pheophorbides were separated only slightly in the second dimension.

With the modified Bauer solvent system, the compounds having less polarity than chlorophylls separated quite well. Five are readily visible in daylight, and seven can be detected under UV. Those moving more slowly, i.e., compounds more polar than chlorophylls, also separated reasonably well into four or more spots.

Fig. 6a shows the chromatogram which was developed with Benzene-P.E.-acetone-methanol (10:2.5:1:0.25, by vol.) followed by P.E.-acetone-n-propanol (8:2:0.05, by vol.) on silica gel G. This method appears most appropriate for work on biodegradation, where the separation of unknown compounds with mobilities similar to the chlorophylls is of importance.

The solvent system III used in Fig. 6B showed promise for chlorophylls themselves, but the length of time of development might permit

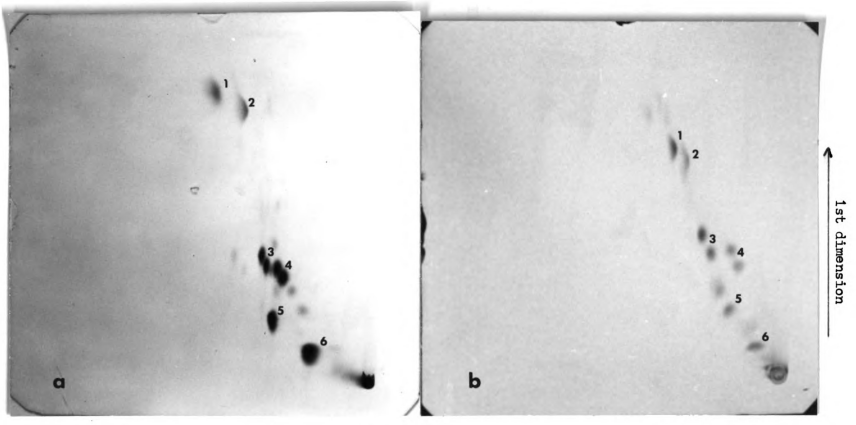


Fig. 6. Two-dimensional thin-layer chromatograms of a pigment mixture developed with solvent systems II (6a) and III (6b). Besides a large number of smaller spots, there appear well resolved spots, including pheophytin a (1), pheophytin b (2), two chlorophylls a (3), two chlorophylls b (4), and compounds "418" (5) and "444" (6). The advantage of the slower solvent III is seen in the separation of the chlorophylls.

formation of artifacts.

Besides the room-dried silica gel plates described above, the oven-dried silica gel plates and the fluorescent silica gel (silica gel G_f) coated plates were also used. The comparison of several replicate developments showed no difference when using either room-dried or oven-dried plates or silica gel with or without binder. The fluorescent silica gel powder showed no particular advantage in the detection of the pink fluorescent chlorophyll derivatives studied here. For these reasons, room-dried silica gel G (with binder) was used to prepare the thin-layer plates throughout all succeedings experiments.

II. Thin-layer chromatography of pigments extracted from the fruits at progressively ripening stages.

Work in the study of changes in chlorophyll during the ripening of fruit was first conducted on bananas because of their availability throughout the year, and because the change from deep green to full yellow occurs within 4-8 days at room temperature. Later, the cultured bell peppers were used for three main reasons: (1) the significant color change, which some varieties of green peppers undergo, leads ultimately to a fruit free of chlorophyll or drivatives; (2) the concentration of easily extractable pigments in the carpel tissue is high, and there are no undesired interfering substances, such as the polyphenols in banana peel; and (3) they are easier to cultivate in a greenhouse under controlled conditions. Since the ripening of peppers takes a considerably longer time than that of bananas, a large number of peppers were planted at various time intervals, so that there was

a continuous supply of the fruit at different ripening stages for study throughout the whole year.

The variety of Capsicum frutescens used in this work undergoes a change from light green to dark green. The dark green color remains unchanged for a long period, during which it could be assumed that the enzymes are formed which carry on the complete destruction of chlorophyll. During a period which can be as short as 24 hours, a change in color occurs from dark green through olive green and bright orange, to the dark red color of the fully ripened fruit.

Cucumbers were not used until later in this experiment. Since the pigments extracted from ripening peppers and bananas showed the same patterns on thin-layer chromatograms during every stage of ripening, the question arose whether the pigments from cucumbers would show the same patterns; therefore, pigments in the extracts of green and yellow cucumber peels were determined qualitatively on thin-layer chromatography.

Typical two-dimensional thin-layer chromatograms of pepper extracts at four stages of ripeness are shown in Fig. 7. The color of the peppers during stage one of ripeness is whitish yellow; 2, dark green; 3, olive orange; and 4, bright orange, as is shown in Fig. 2. The subsequent stage of dark red did not yield any fluorescent chlorophyll derivatives. The spots were named A, B, C, D, E, F, G, and H, according to their appearance in the first dimension of the chromatogram in stage 3, when the maximum number of compounds was present. Some separated further in the second dimension. The first stage shows chlorophyll a and chlorophyll b and, between them, the two isomers,

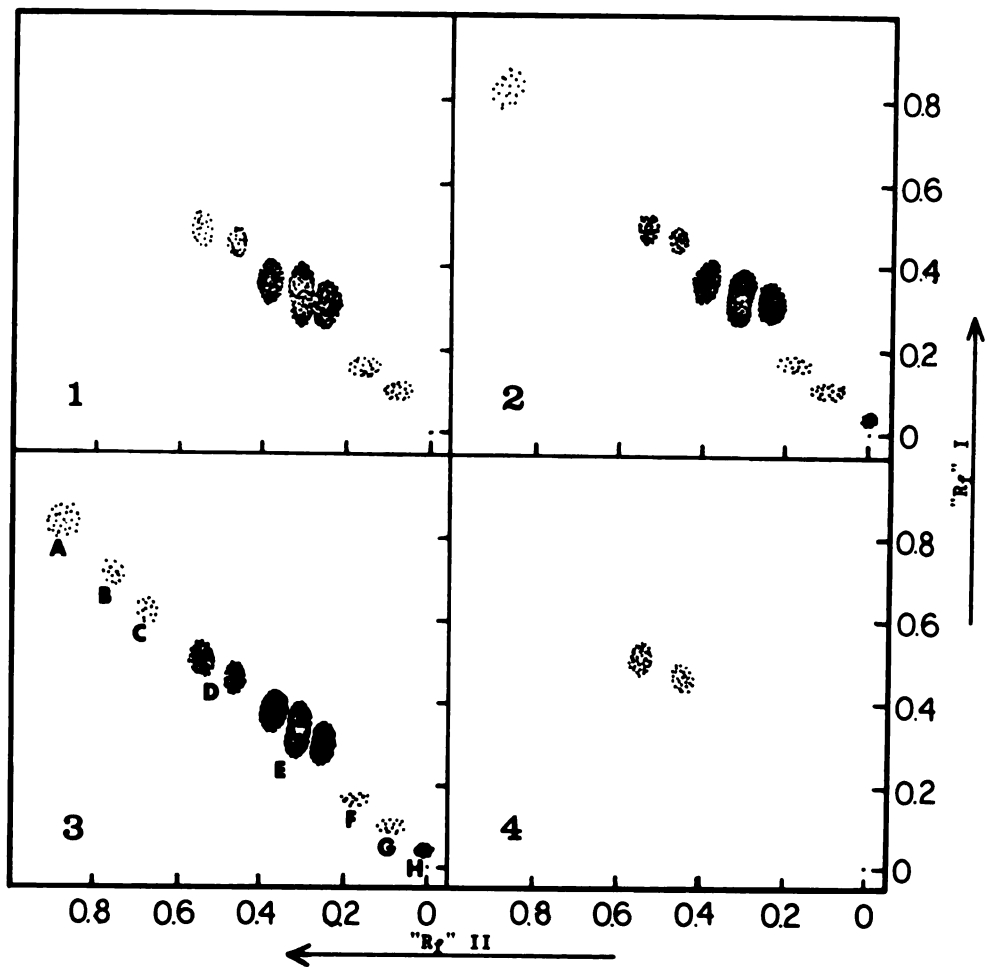


Fig. 7. A schematic drawing of two-dimensional chromatograms showing the changes in pigments during four progressive stages of ripening of Capsicum frutescens. Stages 1 and 2 represent those of increasing greenness, stage 3 the turning (breaking) point, and 4 the one just prior to complete ripeness.

which are difficult to separate from each other and have the same spectral characteristics as chlorophylls a and b. Ahead of chlorophyll a are two spots. These belong to a compound here called D, which later separated into two more compounds. Behind the chlorophyll group appear spots F and G. Notably absent at this stage are spot A (pheophytin) and spot H. The fact that a chromatogram can be made without any formation of pheophytin indicates that the procedure of extraction and chromatography minimizes artifacts.

In stage 2, the same pigments were found as in stage 1 but in higher concentrations. Therefore, in stages 1 and 2 there was a buildup of chlorophylls; however, there was probably some biodegradation, since pheophytin and spot H appeared in stage 2. The next stage, 3, showed the appearance of two substances, B and C, of higher R_f values than either the chlorophylls or compound D. They were present in very small amounts and, at this stage, did not permit good spectral identification. Stage 4 depicted the only two spots remaining after all derivatives had disappeared. They were D_b and D_a.

The spots on the silica gel plate were scraped off and eluted with ether, and their spectra are shown in Figs. 8 and 9. The elution of the spot called H was difficult, since it was very tightly adsorbed on the silica gel. The spectrum of this compound was therefore obtained by spotting the original extract on a sugar plate prepared according to Colman and Vishniac (1964) and developed with the solvent of Grob et al (1960), neglecting all compounds but the most polar one. With this method, H was easily eluted with diethyl ether for spectroscopy, and its identity was checked by rechromatography on silica gel.

Fig. 8. Absorption spectra of pheophytin *a* (spot A) and three typical compounds obtained from *Capsicum* extracts (solid lines) and from spinach extract (broken line). Spot C is a compound less polar than the chlorophylls and F, G and H are more polar derivatives. The spectrum of H shows some characteristics of chlorophyll *a* with trace amounts of *b*.

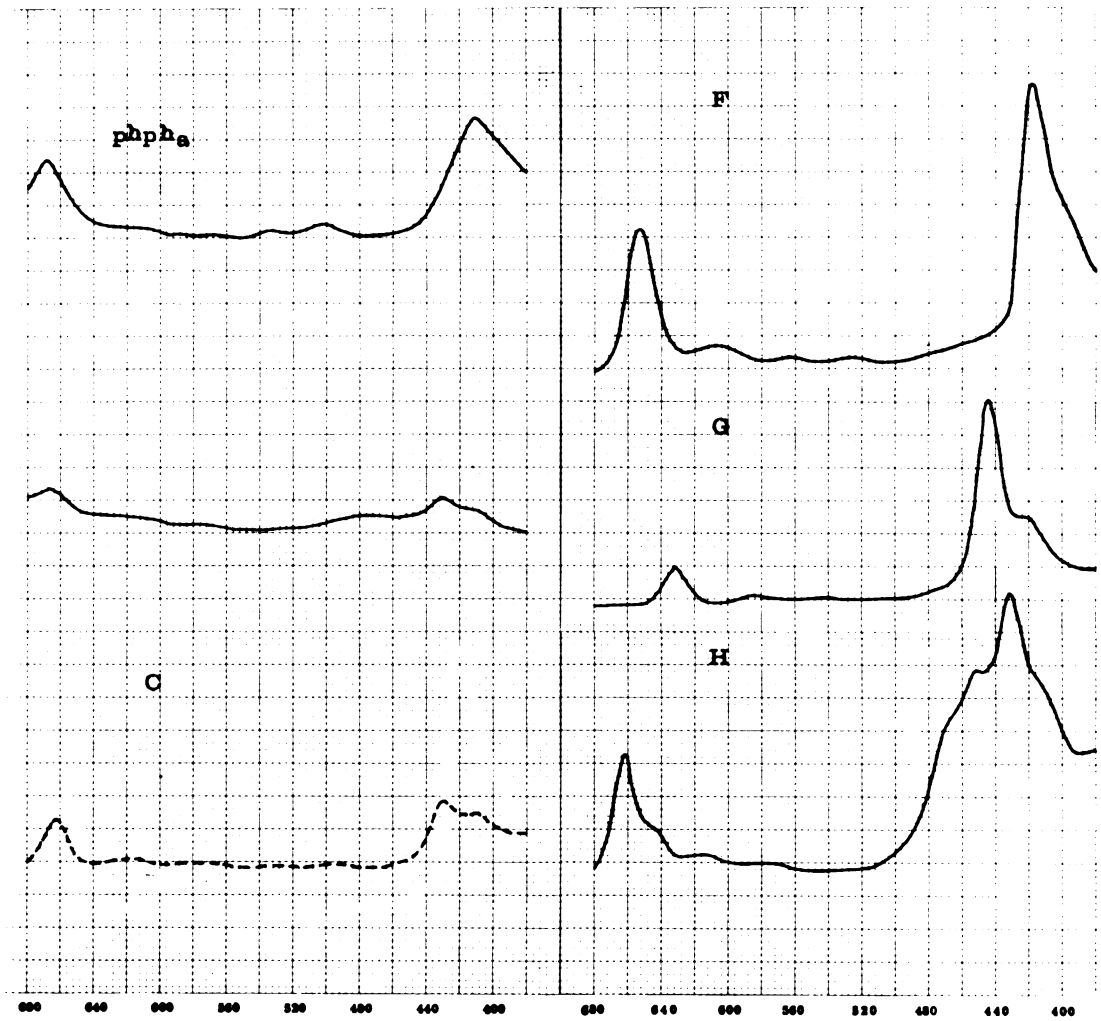

phph$_a$
F
G
H
C

Fig. 9. Absorption spectra of four chlorophylls (right column) and two related compounds (left column) obtained from Capsicum extracts (solid lines) and from spinach extracts (broken lines). The spectra of the chlorophylls named here a_1 and b_2 are quite pure, but the middle spots show overlapping and double peaks both in the red and blue regions of the spectrum.

chphs
a_1
$a_2(b_1)$
$b_1(a_2)$
b_2
D_b
D_a
680
640
600
560
520
480
440
400

Fig. 8 shows the spectrum of pheophytin a (Zscheile and Comar, 1941), found in the spot called A. No spectrum is available for B, since it was never present in large enough amounts to be eluted. A rather weak spectrum of compound C is shown, matched by the best spectrum obtained of C from spinach extracts, shown as a dotted line. A clear spectrum of F and G could be obtained, but H appeared to contain more than one compound.

The upper left of Fig. 9 shows a spectrum of the faster moving component of spot D. Since it showed characteristics of chlorophyll b, it was called D_b. The other component, shown below in the solid line, was called D_a. To aid identification, both spectra are shown, matched by the spectra of the corresponding spots (dotted line) obtained in larger quantities from spinach extracts.

On the right side of Fig. 9 are the chlorophyll spectra. The first one is pure chlorophyll a. The second one appears to be the isomer of a with traces of b. The third one appears to be the isomer of b with traces of a, and the last one is pure chlorophyll b.

The D compounds are of interest during ripening, since they disappear last. They are present in small amounts throughout the other stages but appear to increase during stage 3. Their degradation appears to be slower, and they are, therefore, retained longer than any other fluorescent compound. The compounds F and G, which exhibited characteristics of chlorophyll absorption spectra, have not been reported in the literature. The color of spots F and G is very similar to that of chlorophylls a and b, respectively. As with the other, it is not clear at present whether or not these compounds are

degradation products of chlorophyll precursors remaining from the biosynthesis (Schanderl and Lynn, 1966).

The thin-layer chromatogram of the extract from green banana peels in stage #1-2 appeared very similar to the pattern from extracts of peppers in stage 2. The same was true for the banana peels in stage 3-4 and peppers of stage 3, and for the banana peels in stage 5 and peppers of stage 4.

For the cucumbers the pigment extracted from the green peel displayed similar chromatographic patterns as peppers in stage 2 and those from the light yellow-green peel as peppers in stage 4.

The dark red pepper, very ripe yellow banana peel, and light yellow cucumber peel did not yield any fluorescent chlorophyll derivatives.

III. Some studies of two chlorophyll-type green pigments.

Besides the usual green and yellow pigments, two green pigments, i.e., spots F and G were observed in low concentrations on thin-layer chromatograms of extracts of all three plant materials used. From their spot sizes and color intensities they were estimated to represent about 5% or less of total green pigments. Since they exhibited more stability than chlorophyll and were similar in color, it seemed desirable to determine some of their characteristics.

A. Chromatography of the pigments

These two pigments moved more slowly than chlorophylls under the conditions described and were well separated between chlorophylls and chlorophyllides when the latter were added to a pigment

extract. The two pigments were eluted from the silica gel with acetone or methanol more readily than with ether, which is commonly used for the chlorophylls.

Under UV light, both pigments displayed pink fluorescence. Under daylight, the color of the higher R_f compound was bluish-green. Its spectrum in diethyl ether showed maxima at 418 nm and 655 nm. The color of the slower-moving (low R_f) compound was green with a slightly yellowish tinge, and it had absorption maxima at 444 nm and 630-632 nm. For brevity, they are referred to as the 418 and 444 compounds (Co and Schanderl, 1966a).

B. Artifacts.

Several steps were taken to check the possible formation of artifacts. The plant extract was chromatographed one-dimensionally on a thin-layer plate coated with cellulose powder (Whatman CC41), according to the precedure described by Bacon (1965). Chlorophylls a and b occurred as two wide but well separated zones moving ahead of a weakly pink fluorescent zone, which was the 444 compounds. Compound 418 could not be detected. From this it can be concluded that at least the 444 compound is not an artifact formed on the surface of the silica gel plates but existed in the plant extracts. To locate the 418 compound on the cellulose plate, a known purified 418 compound was co-chromatographed with a plant extract, as well as being spotted as a single spot at the side of the extract. On this chromatogram, the plant extract separated as before into three zones (two wider ones followed by the 444 zone). The added 418 compound did not separate from the chlorophylls. The 418

compound spotted on the side moved about as far as the chlorophyll b zone. However, the chlorophyll a zone, when eluted and rechromatographed on a silica gel plate, yielded back the added 418 compound. This variation of R_f value with the amount and even the number of compounds in the mixture was observed on cellulose by Bacon (1965). These experiments showed that cellulose is not satisfactory as adsorbent using Bacon's solvent for the separation of these minor pigment constituents in plant extracts.

To determine whether the 418 compound was formed on silica gel, the plant extract was spotted on a silica gel plate and developed as before. After the spot of 418 had been spectrally identified, all other compounds that had separated besides the 418 compound were recombined and rechromatographed on a new silica gel plate. Even after several replications, no trace of the 418 compound could be found. From this it can be concluded that the 418 compound found in this experiment was not an artifact under the conditions of the chromatography. The plant extracts were also chromatographed on powdered sugar columns, as described by Strain (1958), but these minor constituents of the plant extract were not detected. Similar difficulties with sugar columns were reported by Michel-Wolwertz and Sironval (1965). Also, sugar plates were used following the method of Colman and Vishniac (1964), but the separation was not satisfactory.

C. Removal of the metal and formation of a copper-complex.

A variety of metals, such as Mg, Zn, and Cu, may be complexed with the porphyrin ring. Usually, the metal is chelated in the

center of the ring, and it is also possible to prepare doubly complexed porphyrins, e.g., Mg-Cu porphins *in vitro* (Aronoff).

So far, all the naturally occurring plant chlorophylls reported are Mg-porphyrins; the central Mg can be removed from the chlorophyll molecule with carboxylic acids (oxalic or acetic) but is difficult to replace *in vitro*. The Mg-free chlorophylls are usually grayish in color, and their formation leads to discoloration in food.

Zinc and copper are readily introduced into the ring but can be removed only with strong acid. The Mg- and Zn- complexes fluoresce pink under UV radiation, while copper complexes do not (Schanderl *et al*, 1965).

1. Pheophytinization (removal of the metal) of the 418 and 444 compounds:

   When these two pigments were treated with oxalic acid in acetone, a color change occurred. The maximum absorption peaks in diethyl ether underwent a hypsochromic shift (towards shorter wavelength) in the blue region and a bathochromic shift (towards longer wavelength) in the red region. The similar shifts of absorption maxima also occur when chlorophylls become pheophytins.

   On a thin-layer chromatogram each metal-free derivative of 418 and 444 compounds had higher R_f values than its corresponding pigment. This relation is the same as that between pheophytins to chlorophylls. The migration of the metal-free compounds and their parent pigments on a two-dimensional

silica gel thin-layer plate developed with solvent system III is shown in Fig. 10.

2. Cu-complexes of the compouds:

Besides Mg, other divalent metals, e.g., Zn or Cu can be introduced into phorbin by refluxing the ether pigment solution with either crystalline $CuCl_2$ or zinc acetate under neutral or slightly acidic conditions. In this study the formation of Cu-complexes of the pigments was achieved by refluxing the ether solutions of the pigment with $CuCl_2$ crystals in presence of trace amounts of water. The color of the ether solution changed from grey to green within 20 minutes, but the refluxing was continued at least an hour until there was no fluorescence under UV radiation.

After the refluxing was completed, the pigment solution was cooled, then transferred to a separatory funnel, and washed with deionized water to remove the excess copper. The solution was dried on anhydrous sodium sulfate. Spectra were obtained with a Bausch and Lomb spectronic 505, and a detailed study was carried out in a Beckman DU with a Gilford readout unit, as described before. The absorption spectra of the Cu-complexes of the pigments are shown in Figs. 13 and 14.

D. De-esterification of the pigments:

In this study it was found that chlorophyllase exists in the chloroplasts of Ailanthus leaves. Thus, extracting the enzyme from carefully prepared chloroplasts is an important purification procedure. For this reason, chloroplasts were prepared, the

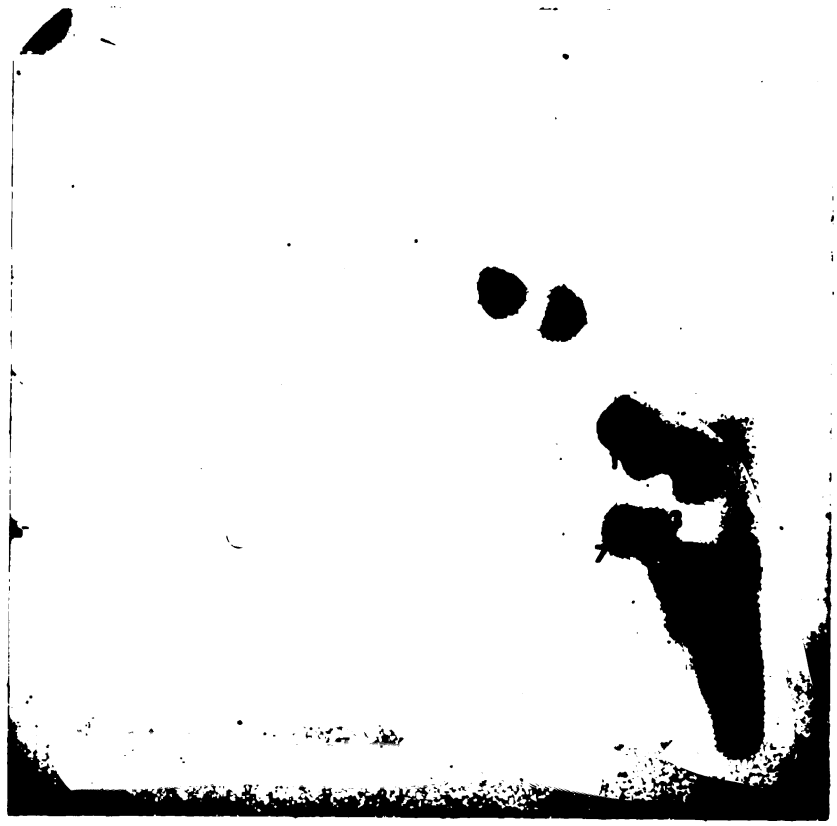


Fig. 10. Two-dimensional thin-layer chromatography of the 418 and 444 compounds and their metal-free derivatives developed with solvent system III. Spots are:
(1) chlorophylls a, (2) chlorophylls b, (3) metal-free derivative of 418, (4) 418 compound, (5) metal-free derivative of 444 compound, (6) 444 compound, (7) a carotenoid.

pigments removed with butanol, and the enzyme solution dialyzed against prechilled deionized water in the cold for 12 hours. The water was changed four times until there was no trace of butanol odor. Holden (1961) reported that up to 60% of the enzyme activity was lost in her preparations when the enzyme was dialyzed against distilled water in the cold for 48 hours; the reason was not given. Since the purpose of using chlorophyllase in this study was to test the existence of an esterified group at the 7-propionic group, partial loss of enzyme activity was not important. Also the butanol which dissolved in the aqueous layer during the pigment extraction might interfere with the later experiments.

Besides 70% acetone precipitation, the ammonium sulfate precipitation procedure was also tried, but the enzyme recovery was very poor. The same result had also been mentioned by Holden (1961).

Chlorophyllase action *in vitro* usually required the presence of a solvent such as acetone (40-70%) or methanol (70-80%). To avoid the use of methanol, the de-esterification study was carried out in a system containing 3 parts of acetone, which dissolved the substrate, 2 parts of 0.02M sodium citrate solution, and 1 part of enzyme solution. The de-esterification was carried out in the dark at room temperature.

The 418 and 444 compounds were not fully converted after 12 hours incubation; but besides the pigments themselves, each gave a spot which moved more slowly than its parent compound on thin-

layer chromatograms. This indicates that these two pigments had been hydrolyzed by chlorophyllase at a somewhat slower rate than chlorophylls or pheophytins.

E. Molisch phase test.

A positive Molisch phase test is considered to be proof of the presence of the 9-keto, the 10-hydrogen, and the 10-carboxymethyl groups. Both pigments gave a negative phase test, indicating that the isocyclic ring has been affected; therefore, they are possibly not phorbins, but chlorins.

F. Hydrochloric acid number (basicity test).

The hydrochloric acid numbers of the pheophytins of the 418 and 444 compounds are presented in Table 1:

Table 1. Hydrochloric acid numbers of chlorophyll derivatives and of the metal-free derivatives (pheophytins) of the 418 and 444 compounds.

Testing compound	Traces extracted by HCl, %	HCl number	Almost completely extracted by HCl, %
Pheophytin-418	22	26-27	32
pheophytin-444	29	33	35
*pheophytin a	25	29	32
*pheophytin b	30	35	--
*pheophorbide a	12	15	17
*pheophorbide b	16	19.5	22
*methyl pheophorbide a	13	16	18
*methyl pheophorbide b	17	21	23

*from Willstätter (1913).

The above table shows that the HCl number of the metal-free

418 compound is lower than that of pheophytin a but much higher than that of either pheophorbide a or methyl pheophorbide a. This is also true for the metal-free 444 compound, in comparison with the chlorophyll b series. It indicates that the new pigments, the 418 and 444 compounds, are slightly more basic than chlorophylls a and b; therefore, either a shorter sidechain is esterified at the 7-propionic group or some other functional group or groups in the pigment molecules are different from those of the chlorophylls, thus contributing the increase in basicity.

G. Visible spectra of the 418 and 444 compounds and their derivatives.

The visible spectra of the 418 and 444 compounds in 1 ml of ether were determined. Then the ether solution was evaporated to dryness, and the pigments were redissolved in 1 ml of methanol. Their spectra in methanol were determined, and the results are shown in Figs. 11 and 12.

Under daylight, the color of the 418 compound resembles that of chlorophyll a, and the 444 compound resembles that of chlorophyll b, but the visible spectra showed that they are different from chlorophylls a and b. When ether was used as a solvent, the absorption maxima of the 418 compound are located 11 nm lower in the blue region and 6 nm lower in the red region than those of chlorophyll a, while those of the 444 compound are located 9 nm lower in the blue region and 11 nm lower in the red region than those of chlorophyll b.

Their absorption maxima also distinguished them from allomerized chlorophylls in both ether and methanol solvents. Com-

Fig. 11. Visible absorption spectra of the 418 compound in ether (solid line) and methanol (broken line) solutions. The spectrum in 1 ml of ether solution was recorded first; then the ether was evaporated to dryness, and the pigment was redissolved in 1 ml of methanol for spectral analysis.

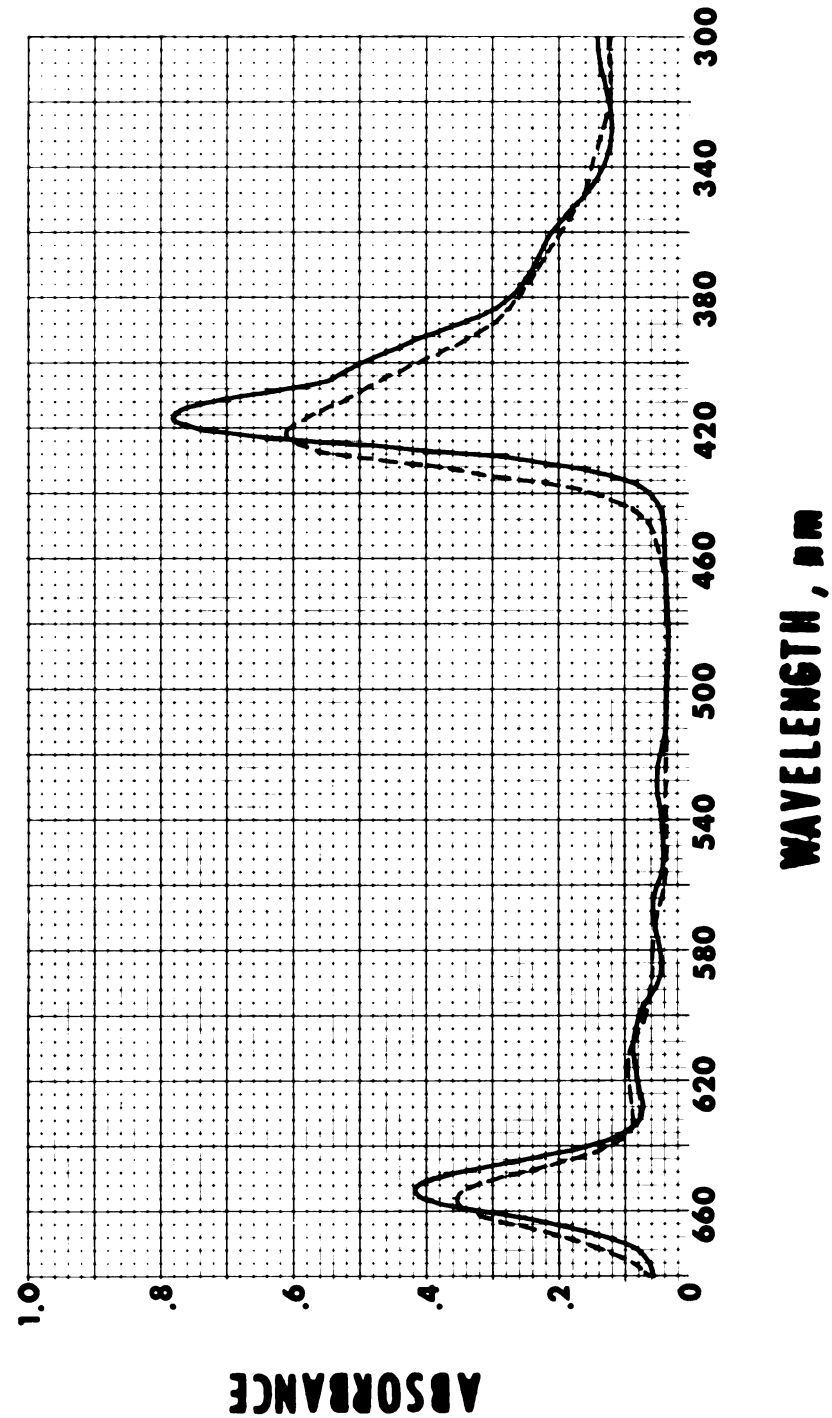
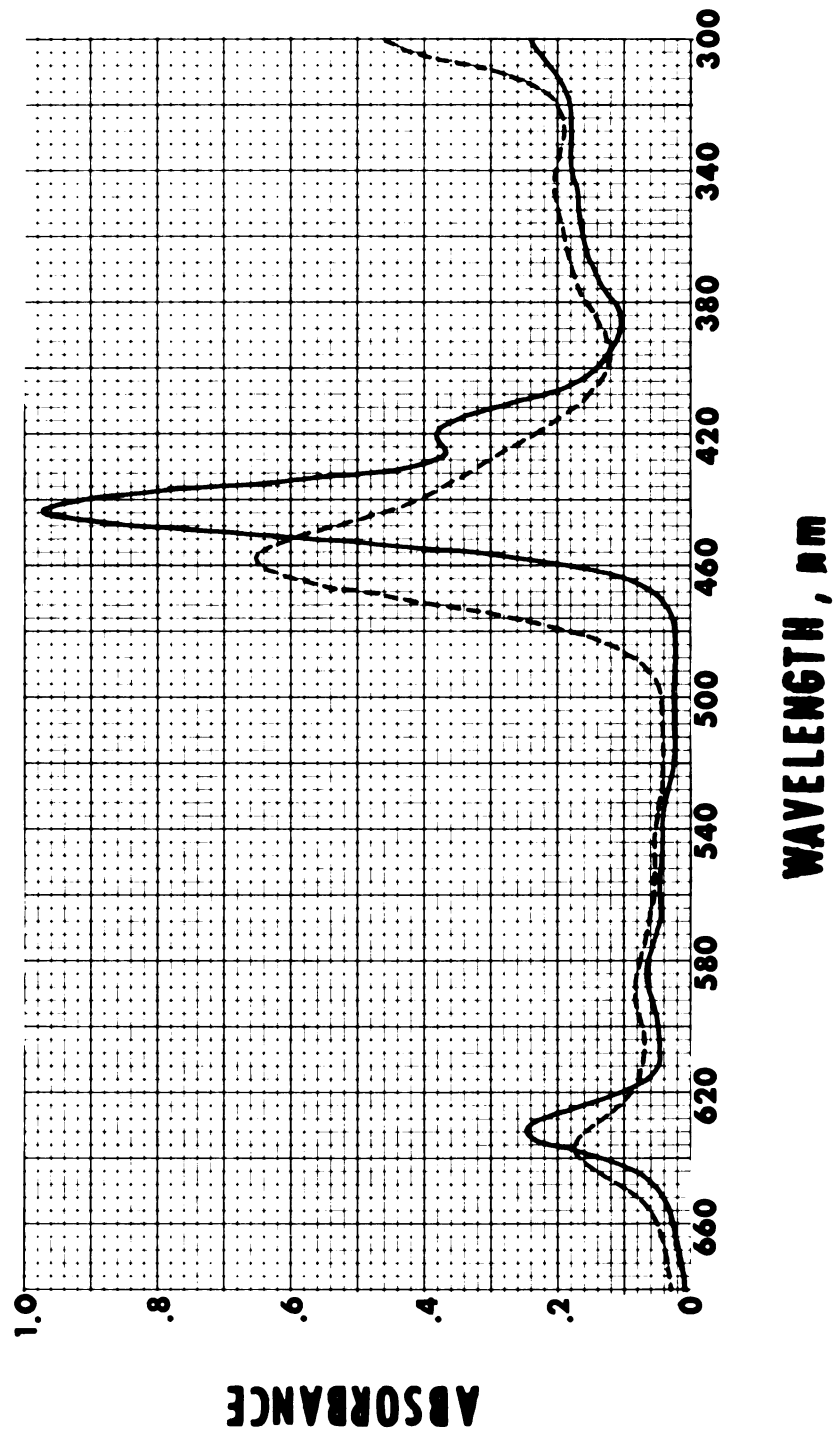

ABSORBANCE
1.0
.8
.6
.4
.2
0
660
620
580
540
500
460
420
380
340
300
WAVELENGTH, nm

Fig. 12. Visible absorption spectra of the 444 compound in ether (solid line) and methanol (broken line) solutions. The spectrum in 1 ml of ether solution was recorded first; then the ether was evaporated to dryness, and the pigment was redissolved in 1 ml of methanol for spectral analysis.


ABSORBANCE
1.0
.8
.6
.4
.2
0
660
620
580
540
500
460
420
380
340
300
WAVELENGTH, nm

pound 418 exhibits absorption peaks at 417.5-418 nm and 653-655 nm in ether, and 421 nm and 655-657 nm in methanol, while those of allomerized chlorophyll a are located at 427.5 nm and 660 nm in ether, and 432.5 and 665 nm in methanol (Pennington et al, 1964). Compound 444 exhibits absorption peaks at 442.5-444 nm and 631 nm in ether and 456 nm and 635 nm in methanol, while those of allomerized chlorophyll b are at 450 nm and 641 nm in ether, and 467 nm and 652.5 nm in methanol (Pennington et al, 1964).

The absorption spectra of the metal-free compounds of these two pigments also were examined in ether and methanol. The absorption spectra from 300 nm to 680 nm are shown in figs. 13 and 14. Both compounds exhibited a hypsochromic shift in the blue region and a bathochromic shift in the red region, which is typical when the Mg is removed from a chlorophyll molecule.

The Cu-complex of the 418 compound underwent a bathochromic shift both in the blue and red regions in ether. The Cu-complex of the 444 compound underwent a bathochromic shift in the blue region but a hypsochromic shift in the red region in ether. It was not possible to obtain the absorption spectra of the Cu-complexes in methanol because of their instability in this solvent. The absorption spectra of these metal-free compounds and their Cu-complexes in ether solution are shown in Figs. 13 and 14.

H. Infrared spectra of the 418 and 444 compounds.

Infrared spectra of the 418 and 444 compounds are shown in

Fig. 13. Visible absorption spectra of the metal-free derivative of the 413 compound (solid line) and its Cu-complex compound (broken line) in ether.

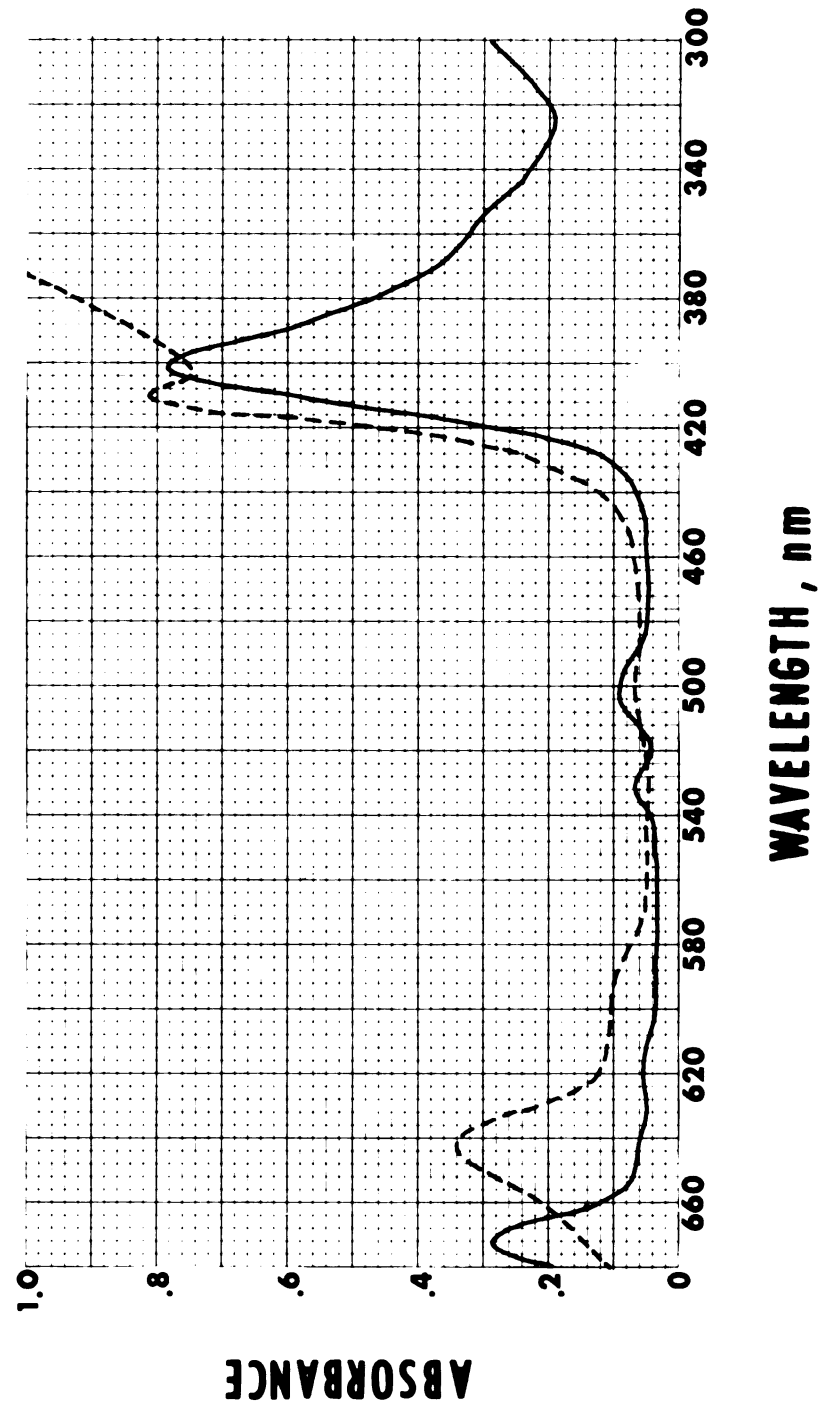

ABSORBANCE
1.0
.8
.6
.4
.2
0
300
340
380
420
460
500
540
580
620
660
WAVELENGTH, nm

Fig. 14. Visible absorption spectra of the metal-free derivative of the 444 compound (solid line) and its Cu-complex compound (broken line) in ether.

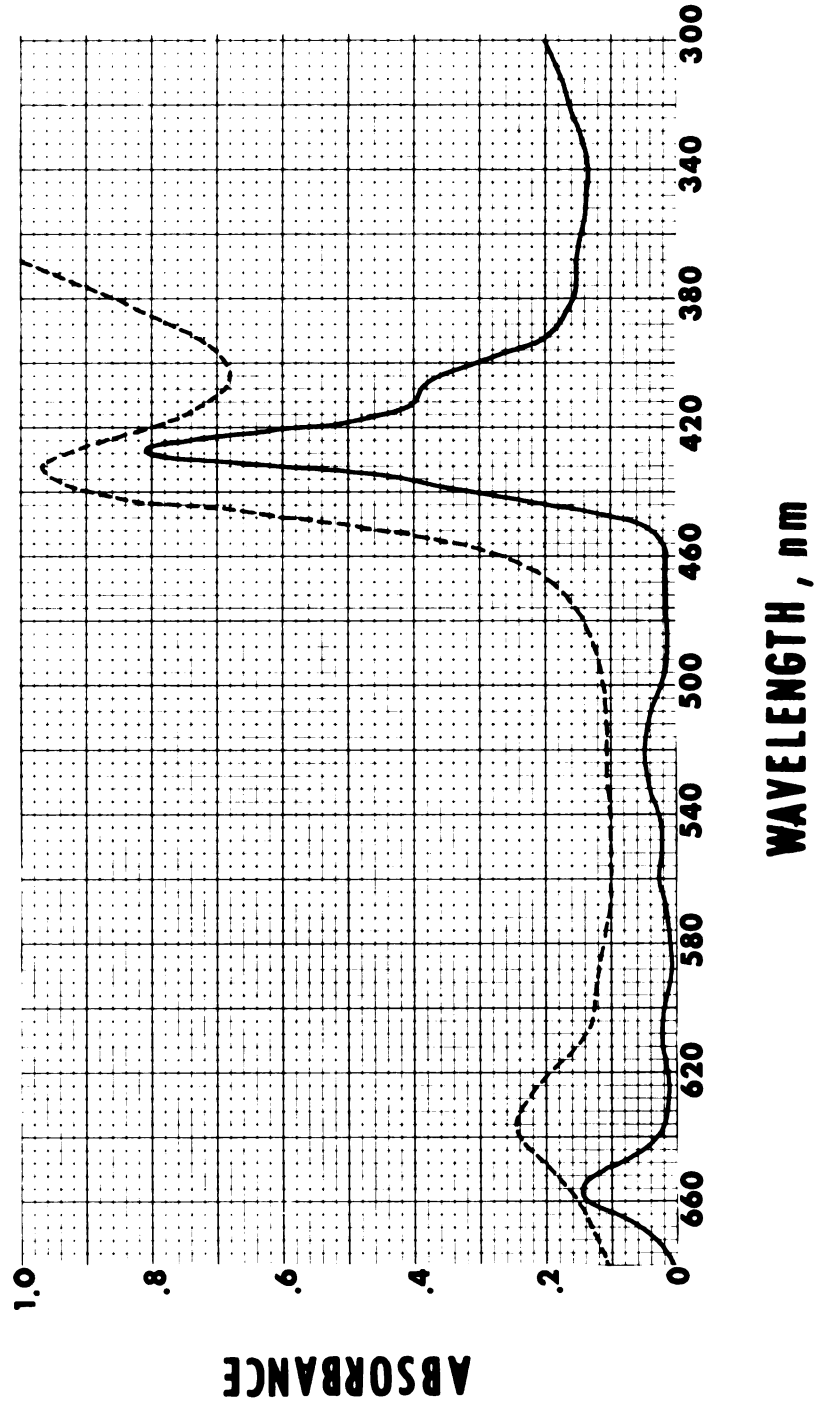

ABSORBANCE
1.0
.8
.6
.4
.2
0
300
340
380
420
460
500
540
580
620
660
WAVELENGTH, nm

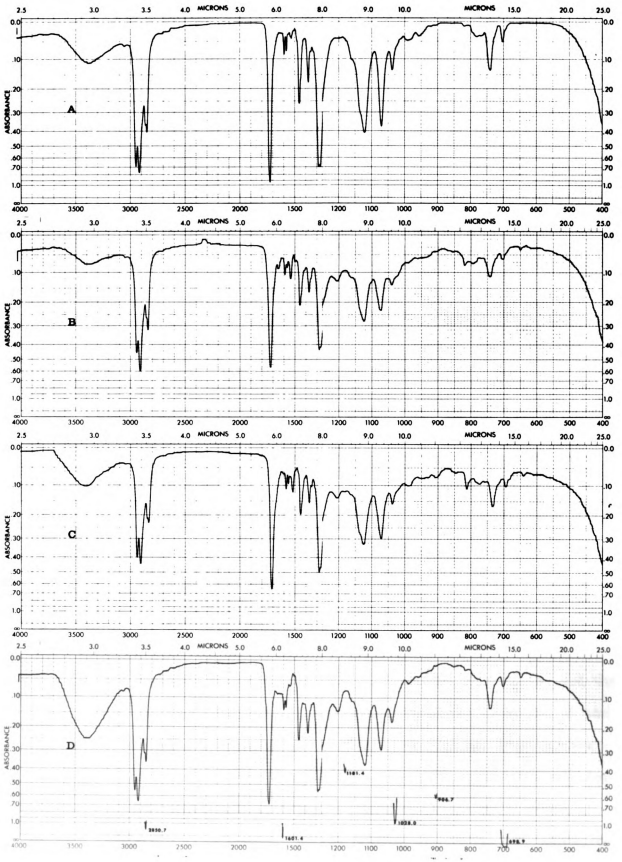
Fig. 15A, and B, respectively. Fig. 15C and D are the IR spectra of two compounds formed during the preparation of the 418 and 444 compounds. They moved more slowly than the above compounds but exhibited the same absorption maxima, i.e., at 418 and 444 nm, respectively, in the visible region. They are referred to as the second 418 and 444 compounds. For discussion these spectra are divided into four regions according to Katz (1966): 4000-2700 cm^{-1}, 1750-1600 cm^{-1}, 1600-1300 cm^{-1} and 1300-400 cm^{-1}.

1. The 4000-2700 cm^{-1} region: All spectra had a broad band centered at approximately 3400 cm^{-1}. This band can be attributed to N-H stretching vibration which appears at 3370-3400 cm^{-1} and is more pronounced in the case of metal-free derivatives. It also can be logically assigned to an O-H stretching vibration of water, since water is extremely difficult to remove from chlorophylls and their derivatives, even when they are in crystalline forms.

   The absorption in the region of 2965-2955 cm^{-1} can be assigned to C-H stretching of $-CH_3$; that at 2940-2925 cm^{-1} to antisymmetric C-H stretching of $-CH_2-$; and that at 2865-2880 cm^{-1} to overlapping of symmetric $-CH_3$ and $-CH_2-$ stretching. The methene and vinyl CH stretching vibrations are too weak to be observed, while the C_8, C_9 and C_{10} hydrogen absorption peaks are most likely masked by the much more numerous methyl and methylene bands.

   The strong absorption bands at 2965-2880 cm^{-1} show that the phytyl (or farnesyl in the case of chlorobium chlorophyll)

Fig. 15. Infrared spectra of the solid 418 (curve A) and 444 (curve B) compounds on KBr plates. Curves C and D are the IR spectra of the solid second 418 and 444 compounds on KBr plates.


MICRONS
ABSORBANCE
A
B
C
D
3930.7
1601.4
1161.4
1028.0
966.7
876.7

group contribute a large part of the intensity of these bands. However, the ratio of the absorption at 2925 cm^{-1} over that at 3400 cm^{-1} is 7 for curve A; 4 for curve C; 7.5 for curve B; and 2.64 for curve D. This difference in ratios may be useful to interpret the mobilities, where those having the ratios of 7 and 7.5 moved faster on a thin-layer chromatogram than those having the ratios of 4 and 2.64.

2. The 1750-1600 cm^{-1} region: The strong and sharp absorption band at 1715-1735 cm^{-1}, appearing in all spectra, can be assigned to C_7 and C_{10} ester C=O groups. But the absorption band at 1708-1701 cm^{-1} for the stretching mode of the ketone carbonyl group does not appear in any of the four curves. It can be assumed that the absorption band of the C_9 keto group overlaps with that of the ester keto groups of C_7 and C_{10}. Other differences are: curve D shows higher absorption backgrounds between 1700-1600 cm^{-1}, and there is a small but distinct band at 1650 cm^{-1} in curve C.
3. The 1600-1300 cm^{-1} region: The medium intensity absorption peaks that appear in this region mostly arise from the skeletal vibrations of the tetrapyrrolic macrocycle, and the carbon-hydrogen bending modes of the phytyl group and of the alkyl substituents of the chlorin ring. The spectra show bands at 1560-1550 cm^{-1}, 1540-1520 cm^{-1}, 1500-1490 cm^{-1} and 1355-1345 cm^{-1} that may be assigned to the C=C and C=N skeletal vibrations of the chlorin ring. The CH_3 antisymmetric

bending is absorbed at 1456-1448 cm^{-1} and gem-dimethyl in phytyl or farnesyl gives a doublet at 1385-1375 cm^{-1}.

There is a peak at 1520 cm^{-1} in curves A and C, but it shifts to 1540 cm^{-1} in curves B and D, and the small peak at 1500 cm^{-1} appears in curves B and D but not in curves A and C.

4. The 1300-400 cm^{-1} region ; finger print region: Spectra in this region are very complex and generally associated with molecular motions in- and out-of-plane, involving many atoms, and bending and breathing vibrations of ring structures.

The absorption peak at 1200-1160 cm^{-1} can be assigned to an ester antisymmetric carbon-oxygen stretching vibration, and peaks at 1170-1035 cm^{-1} can be assigned to symmetric ester carbon-oxygen stretching modes. The 1135-1115 cm^{-1} peak is contributed by pyrrole ring-breathing mode. The N-H out-of-plane bending mode absorbs at 720-710 cm^{-1}, and vinyl C-H out of plane bending mode gives rise to an absorption at 990-980 cm^{-1} and at 923-910 cm^{-1}.

There is a broad band at 1210 cm^{-1} in curves B, C, D, but not in curve A.

The 418 and 444 compounds fluoresce pink under ultraviolet radiation; under daylight the color of the 418 compound resembles that of chlorophyll a, and the 444 compound resembles chlorophyll b. Visible spectra showed that they are different from chlorophylls a and b. Enzymatic de-esterification tests and IR spectra showed that they posess an ester group at the

C_7 propionic acid sidechain. Both gave a negative phase test, indicating that the isocyclic ring is altered, while the visible spectra showed that they are different from allomerized chlorophylls. The central metal can be removed easily with oxalic acid, and the metal-free derivatives can be recomplexed with Cu. They move more slowly than the chlorophylls on a thin-layer chromatogram, and their HCl numbers are slightly lower than chlorophylls. These indicate that the two compounds are probably slightly more basic than chlorophyll and are metal-containing diester chlorophyll-type pigments. They could possibly be derivatives of chlorophylls or of chlorophyll precursors, or even be precursors of chlorophylls.

SUMMARY AND CONCLUSIONS

Thin-layer chromatographic methods using silica gel G adsorbent and several solvent systems were developed. These micromethods permit rapid separation of small amounts of chlorophylls and related pigments. Eight major and eight to ten minor tetrapyrrole pigments can be separated speedily. The results of the thin-layer chromatography were evaluated and compared with column and paper chromatography using known compounds.

Pigments were extracted from fruit tissues with acetone instead of methanol to avoid allomerization. Magnesium carbonate was added to neutralize the cytoplasmic acids to minimize pheophytin formation. Pigmeent extracts from the peels of progressively ripening bananas, peppers and cucumbers were separated with the thin-layer chromatography developed. The chromatograms of the extracts from green banana peels and cucumber peels appeared very similar to the patterns from extracts of dark green peppers, and underwent similar changes during ripening. Two compounds were the last to disappear and are thought to be degradation products somewhat more stable than chlorophylls *a* and *b*. Besides these two compounds, two additional green pigments appeared in acetone extracts of all three experimental fruits. The visible absorption peaks of the latter two were at 418 and 444 nm. No pink fluorescent compounds were found in the fully mature fruits.

The 418 and 444 compounds fluoresce pink under ultraviolet radiation; under daylight the color of the 418 compound resembles that of chlorophyll *a*, and the 444 compound resembles chlorophyll *b*. Visible spectra showed that they are different from chlorophylls *a* and *b*.

Enzymatic de-esterification tests and IR spectra showed that they posess an ester group at the C_7 propionic acid sidechain. Both gave a negative phase test, indicating that the isocyclic ring is altered, while the visible spectra showed that they are different from allomerized chlorophylls. The central metal can be removed easily with oxalic acid, and the metal-free derivatives can be recomplexed with Cu. They move more slowly than the chlorophylls on a thin-layer chromatogram, and their HCl numbers are slightly lower than those of the chlorophylls. This indicates that these two compounds are slightly more basic than chlorophyll and are metal-containing diester chlorophyll-type pigments. They could possibly be derivatives of chlorophylls or of chlorophyll precursors, or even be precursors of chlorophylls.

BIBLIOGRAPHY

Ardao, C. and Vennesland, B. 1960. Chlorophyllase activity of spinach chloroplastin. Plant physiol. 35, 368.

Aries, R. S. 1946. Chlorophyll. Chemurgic Digest 5, 100.

Aronoff, S. 1953. The chemistry of chlorophyll. Advances in Food Research 4, 133.

Aronoff, S. and Mackinney, G. 1943. The photo-oxidation of chlorophyll. J. Am. Chem. Soc. 65, 956.

Bacon, M. F. 1965. Separation of chlorophylls a and b and related compounds by thin-layer chromatography on cellulose. J. Chromatog. 17, 322.

Barnes, T. C. 1946. Electrical measurements of the healing rate of human skin. Hahnemannian Monthly 81, 8.

Bauer, L. 1952. Trennung der Karotinoide und Chlorophylle mit Hilfe der Papierchromatographie. Naturwissenschaften 39, 88.

Beuchat, L. R., Lechowich, R. V., Schanderl, S. H., Co, Denise Y. C. and Mcfeeters, R. F. 1966. Inhibition of bacterial growth by chlorophyllide a. Quarterly Bulletin of the Mich. Agri. Expt. Station 48, 411.

Bogorad, L. 1965a. Chlorophyll Biosynthesis. In "Chemistry and Biochemistry of Plant Pigment", Goodwin, T. W., ed., Academic Press, N. Y.

Bogorad, L. 1965b. Porphyrins and bile pigments. In "Plant Biochemistry", Bonner, J. and Varner, J. E., eds., Academic Press, N. Y.

Bogorad, L. 1966. The biosynthesis of chlorophylls. In "The Chlorophylls", Vernon, L. P. and Seely, G. R., eds., Academic Press, N. Y.

Brown, W. G. 1939. Micro separations by chromatographic adsorption on blotting paper. Nature 143, 377.

Bruinsma, J. 1961. A comment on the spectrophotometric determination of chlorophyll. Biochim. Biophys. Acta 52, 576.

Bruinsma, J. 1963. The quantitative analysis of chlorophylls a and b in plant extracts. Photochem. and Photobiol. 2, 241.

Burgi, S. 1942. The wound-healing action of the chlorophyll and blood pigments. Z. ges. exptl. Med. 110, 259.

Cho, D. H. 1966. Kinetics of the Acid Catalyzed Pheophytinization of Chlorophylls. Doctoral thesis, Univ. of Calif.

Clare, N. T. 1944. Photosensitivity disease in New Zealand III. The photosensitizing agent in facial eczema. New Zealand J. Sci. Technol. 25A, 202.

Co, D. Y. C. Lynn and Schanderl, S. H. 1966a. The occurrence of 418- and 444-nm chlorophyll-type compounds in some green plant tissues. Phytochem. in press.

Co, D. Y. C. Lynn and Schanderl, S. H. 1966b. Separation of chlorophylls and related plant pigments by two-dimensional thin-layer chromatography. J. Chromatog. in press.

Colman, B. and Vishniac, W. 1964. Separation of chloroplast pigments on thin layers of sucrose. Biochim. Biophys. Acta 82, 616.

Daly, S., Heller, G. and Schneider, E. 1939. Effect of chlorophyll derivatives and related compounds on the growth of mycobacterium tuberculosis. Proc. Soc. Exptl. Biol. Med. 42, 74.

Dietrich, W. C., Boggs, M. M., Nutting, M. D. and Weinstein, N. E. 1960. Time-temperature tolerance of frozen foods XXIII. Quality changes in frozen spinach. Food Technol. 14, 522.

Egger, K. 1962. Dünnschichtchromatographie der Chloroplastenpigmente. Planta 58, 664.

Egle, K. 1944. Untersuchungen über die Resistenz der Plastidenfarbstoffe. Bot. Archiv. 45, 93.

Egle, K. 1960. Biologischer Chlorophyllabbau. In "Encyclopedia of Plant Physiology", V/1, 354, Ruhland, W., ed., Springer Verlag.

Fischer, H. and Lambrecht, R. 1938. Verhalten von Chlorophyll-Derivaten gegen Chlorophyllase. Hoppe-Seyl. Z. 253, 253.

Fischer, H. and Orth, H. 1937. "Die Chemie des Pyrrols", vol. 2, part I, Akad. Verlagsges., Leipzig.

Fischer, H. and Stern, A. 1940. "Die Chemie des Pyrrols", vol. 2, part II, Akad. Verlagsges., Leipzig.

Forsyth, W. G. C. 1964. Physiological aspects of curing plant products. Ann. Rev. Plant Physiol. 15, 443.

Fremy, E. 1860. Chimie appliquée a la végétation. Compt. Rend. 50, 405.

French, C. S. 1960. The chlorophylls *in vivo* and *in vitro*. In "Handbuch der Pflanzenphysiologie", Ruhland, W., ed., V/1, Springer, Berlin.

Goodwin, T. W. 1958. Studies in carotenogenesis 24. The changes in carotenoid and chlorophyll pigments in the leaves of deciduous trees during autumn necrosis. Biochem. J. 68, 503.

Gortner, W. A. 1965. Chemical and physical development of the pineapple fruit IV. Plant pigments. J. Food Sci. 30, 30.

Granick, S. 1948. Protoporphyrin 9 as a precursor of chlorophyll. J. Biol. Chem. 172, 717.

Granick, S. 1950. Magnesium vinyl pheoporphyrin a_5, another intermediate in the biological synthesis of chlorophyll. J. Biol. Chem. 183, 713.

Green, M. 1958. Chromatography of chlorophylls. Federation Proc. 17, 439.

Grob, E. C., Eichenberger, W. and Pflugshaupt, R. P. 1961. Zur Bildung der Carotinoide in Blättern, Blüten and Früchten. Chim. Aarau 15, 565.

Hager, A. and Bertenrath, T. 1962. Verteilungschromatographische Trennung von Chlorophyllen und Carotinoiden grüner Pflanzen an Dünnschichten. Planta, 58, 564

Hall, J. L. and Mackintosh, D. L. 1964. Chlorophyll catalysis of fat peroxidation. J. Food Sci. 29, 420.

Harris, D. G. and Zscheile, F. P., Jr. 1943. Effects of solvent upon absorption spectra of chlorophylls a and b; their ultraviolet absorption spectra in ether solution. Bot. Gaz. 104, 515.

Hashimoto, Y. and Tsutsumi, J. 1964. Isolation of pyropheophorbide a as a photo-dynamic pigment from the liver of abalone, Haliotis discus Hanai. Agri. and Biol. Chem. 28, 467.

Heftmann, E., ed. 1961. "Chromatography". Reinhold, N. Y.

Holden, M. 1961. The breakdown of chlorophyll by chlorophyllase. Biochem. J. 78, 359.

Holden, M. 1962. Separation by paper chromatography of chlorophylls a and b and some of their breakdown products. Biochim. Biophys. Acta 56, 378.

Holden, M. 1963. The purification and properties of chlorophyllase. Photochem. and Photobiol. 2, 175.

Holden, M. 1965. Chlorophyll bleaching by legume seeds. J. Sci. Fd. Agric. 16, 312.

Holt, A. S. and Jacobs, E. E. 1954. Spectroscopy of plant pigments. I. Ethyl chlorophyllides a and b and their pheophorbides. Am. J. Bot. 41, 710.

Holt, A. S. and Jacobs, E. E. 1955. Infra-red absorption spectra of chlorophylls and derivatives. Plant Physiol. 30, 553.

Jones, I. D., White, R. C. and Gibbs, E. 1962. Some pigment changes in cucumbers during brining and brine storage. Food Technol. 16, 96.

Jorgensen, E. G. 1962. Antibiotic substances from cells and culture solutions of unicellular algae with special reference to some chlorophyll derivatives. Physiologia Plantarum 15, 530.

Joslyn, M. A. and Mackinney, G. 1938. The rate of conversion of chlorophyll to pheophytin. J. Am. Chem. Soc. 60, 1132.

Katz, J. J., Closs, G. L., Pennington, F. C., Thomas, M. R. and Strain, H. H. 1963. Infrared spectra, molecular weights, and molecular association of chlorophylls a and b, methyl chlorophyllides, and pheophytins in various solvents. J. Am. Chem. Soc. 85, 3801.

Katz, J. J., Dougherty, R. C. and Boucher, L. J. 1966. Infrared and NMR spectroscopy of chlorophyll. In "The Chlorophylls", Vernon, L. P. and Seely, G. R., eds., Academic Press, N. Y.

Kim, W. S. 1966. Complete fractionation of bacteriochlorophyll and its degradation products. Biochim. Biophys. Acta 112, 392.

Klein, A. O. and Vishniac, W. 1961. Activity and partial purification of chlorophyllase in aqueous systems. J. Biol. Chem. 236, 2544.

Krossing, G. 1940. Versuche zur Lokalisation einiger Fermente in den verschiedenen Zellbestandteilen der Spinatblätter. Biochem. Z. 305, 359.

Linskens, H. F., ed. 1955. "Papierchromatographie in der Botanik". Springer, Berlin, Göttingen, Heidelberg.

Mackinney, G. 1940. Criteria for purity of chlorophyll preparations. J. Biol. Chem. 132, 91.

Mackinney, G. and Joslyn, M. A. 1940. The conversion of chlorophyll to pheophytin. J. Am. Chem. Soc. 62, 231.

Mackinney, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem., 140, 315.

Michel-Wolwertz, M. R. and Sironval, C. 1965. On the chlorophylls separated by paper chromatography from chlorella extracts. Biochim. Biophys. Acta 94, 330.

Molisch, H. 1896. Eine neue mikrochemische Reaction auf Chlorophyll. Ber. deut. botan. Ges. 14, 16.

Noack, K. 1944. Uber der biologischen Abbau des Chlorophylls. Biochem. Z. 316, 166.

Nutting, M. D. Voet, M. and Becker, R. 1965. Powdered sugar adsorbent for detection of chlorophyll components in thin-layer chromatography. Anal. Chem. 37, 445.

Pelletier, F. and Caventou, J. B. 1818. Sur la matière verte des feuilles. Ann. Chim. et Phys. 9, 194.

Pennington, F. C., Strain, H. H., Svec, W. A. and Katz, J. J. 1964. Preparation and properties of pyrochlorophyll a, methyl pyrochlorophyllide a, pyropheophytin a, and methyl pyropheophorbide a derived from chlorophyll by decarbomethoxylation. J. Am. Chem. Soc. 86, 1418.

Quin, J. F., Remington, C. and Roeto, G. C. S. 1935. Studies on the photo-sensitization of animals in South Africa, VIII. The biological formation of phylloerythrin in the digestive tracts of various domesticated animals. Onterstepoort J. Vet. Sci. Animal Ind. 4, 463.

Ramirez, D. A. and Tomes, M. L. 1964. Relationship between chlorophyll and carotenoid biosynthesis in Dirty-red (green-flesh) mutant in tomato. Botan. Gaz. 125, 221.

Robinson, H. M. C. and Rathbun, J. C. 1959. The determination of calcium and magnesium in blood using one indicator. Can. J. Biochem. and Physiol. 37, 225.

Rogachev, V. I., Frumkin, M. L., Koval'skaya, L. P. and Egorova, K. V. 1960. Changes in pigments of green peas during sterilization with heat and with γ-rays. Konserv. i Ovoshchesushil. Prom. 15, 19.

Schaltegger, K. H. 1965. Dünnschichtchromatographische Bestimmung von Chlorophyllen und Carotinoiden aus Kirschbaumblättern. J. Chromatog. 19, 75.

Schanderl, S. H., Chichester, C. O. and Marsh, G. L. 1962. Degradation of chlorophyll and several derivatives in acid solution. J. Org. Chem. 27, 3865.

Schanderl, S. H. and Lynn, D. Y. C. 1966. Changes in chlorophylls and spectrally related pigments during ripening of Capsicum frutescens. J. Food Sci. 31, 141.

Schanderl, S. H., Marsh, G. L. and Chichester, C. O. 1965. Color reversion in processed vegetables I. Studies on regreened pea purees. J. Food Sci. 30, 312.

Schanderl, S. H., Marsh, G. L. and Chichester, C. O. 1965. Color reversion in processed vegetables II. Model system studies. J. Food Sci. 30, 317.

Schneider, H. S. W. 1966. Eine Einfache Methode zur Dünnschichtchromatographischen Trennung von Plastidenpigmenten. J. Chromatog. 21, 448.

Šesták, Z. 1958. Paper chromatography of chloroplast pigments. J. Chromatog. 1, 293.

Seybold, A. 1943. Autumn leaf coloring. Botan. Arch. 44, 551.

Shimizu, S. and Tamaki, E. 1962. Chlorophyllase of tobacco plants I. Preparation and properties of water soluble enzyme. Botan. Mag. (Tokyo) 75, 462.

Shimizu, S. and Tamaki, E. 1963. Chlorophyllase of tobacco plants II. Enzymic phytylation of chlorophyllide and pheophorbide in vitro. Archives Biochem. Biophys. 102, 152.

Sidorov, A. N. and Terenin, A. N. 1960. Infrared spectra of chlorophyll and its analogues. Opt. Spectry. (USSR) (English Transl.) 8, 254.

Sironval, C. 1954. Mesure de l'activité de la chlorophyllase par chromatographie sur papier. Physiol. Plantarum 7, 523.

Smith, J. H. C. and Benitez, A. 1955. Chlorophylls: Analysis in plant materials. In "Moderne Methoden der Pflanzenanalyse", Paech, K. and Tracey, M. V., eds., IV, Springer, Berlin.

Sorby, H. C. 1873. On comparative vegetable chromatology. Proc. Roy. Soc. 21, 442.

Stahl, E. 1962. "Dünnschicht Chromatographie", Springer, Berlin.

Stair, R. and Coblentz, W. W. 1933. Infrared absorption spectra of some plant pigments. J. Res. Natl. Bur. Std. 11, 703.

Stokes, G. G. 1864. On the supposed identity of biliverdin with chlorophyll, with remarks on the constitution of chlorophyll. Proc. Roy. Soc. 13, 144.

Stoll, A. and Wiedemann, E. 1952. Chlorophyll. Fortschr. Chem. Forsch. 2, 538.

Strain, H. H. 1941. Unsaturated fat oxidase: specificity, occurrence and induced oxidations. J. Am. Chem. Soc. 63, 3542.

Strain, H. H. 1954. Oxidation and isomerization reactions of the chlorophylls in killed leaves. Agr. Food Chem. 2, 1222.

Strain, H. H. 1958. Chloroplast pigments and chromatographic analysis. Ann. Priestley Lectures, 32nd.

Strell, M., Kalojanoff, A. and Koller, H. 1960. Teilsynthese des Grundkörpers von Chlorophyll a, des Phäophorbids a. Angew, Chem. 72, 169.

Sud'ina, E. G. 1961. Place and role of chlorophyllase reaction in the biosynthesis of chlorophyll. Proc. 5th Intern. Congr. Biochem., Moscow, 6, 512. Pergamon press, Oxford.

Sud'ina, E. G. 1963. Chlorophyllase reaction in the last stage of biosynthesis of chlorophyll. Photochem. and Photobiol. 2, 181.

Sud'ina, E. G. and Romanenko, E. 1961. Changes in pigment content and chlorophyllase activity in fruits. Ukrain, Botan. Zhur. 18, 3.

Thimann, K. V. 1949. U.S. patent 2,481,366, Sept 6.

Tswett, M. 1906. Adsorptionsanalyse und Chromatographische Methode. Anwendung auf die Chemie des Chlorophylls. Ber. Deut. Botan. Ges. 24, 384.

Tswett, M. 1908. Über die Verfärbung und die Entleerung des absterbenden Laubes. Ber. dtsch. bot. Ges. 26a, 88.

Vernon, L. P. 1960. Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal. Chem. 32, 1144.

Weigl, J. W. and Livingston, R. 1953. Infrared spectra of chlorophyll and related compounds. J. Am. Chem. Soc. 75, 2173.

White, R. C., Jones, I. D. and Gibbs. E. 1963. Determination of chlorophylls, chlorophyllides, pheophytins, and pheophorbides in plant material. J. Food Sci. 28, 431.

Willstätter, R. and Mieg, W. 1906. Ueber eine Methode der Trennung und Bestimmung von Chlorophyllderivaten. Ann. 350, 1.

Willstätter, R. and Stoll, A. 1913. "Untersuchungen über Chlorophyll", Springer, Berlin. "Investigations on Chlorophyll", translated by Schertz, F. M. and Merz, A. R. 1928. The science press printing company, Lancaster, Pa.

Woodward, R. B., Ayer, W. A., Beaton, J. M., Bickelhaupt, F., Bonnett, R., Buchschacher, P., Closs, G. L., Dutler, H., Hannah, J., Hauck, F. P., Ito, S., Langemann, A., LeGoff, E., Leimgruber, W., Lwowski, W., Sauer, J., Valenta, Z. and Volz, H. 1960. The total synthesis of chlorophyll. J. Am. Chem. Soc. 82, 3800.

Zscheile, F. P., Jr. 1934a. An improved method for the purification of chlorophylls a and b; quantitative measurement of their absorption spectra; evidence for the existence of a third component of chlorophyll. Bot. Gaz. 95, 529.

Zscheile. F. P., Jr. 1934b. A quantitative spectro-photoelectric analytical method applied to solutions of chlorophylls a and b. J. Phys. Chem. 38, 95.

Zscheile, F. P., Jr. and Comar, C. L. 1941. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectrum. Bot. Gaz. 102, 463.

Zscheile, F. P., Jr., Hogness, T. R. and Young, T. F. 1934. The precision and accuracy of a photoelectric method for comparison of the low light intensities involved in measurement of absorption and fluorescence spectra. J. Phys. Chem. 38, 1.