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SOME NEW MOLECULAR BIOLOGY OF CHLOROPHYLL-A

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SOME NEW MOLECULAR BIOLOGY OF CHLOROPHYLL-A

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SOME NEW MOLECULAR BIOLOGY OF CHLOROPHYLL *a*

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ABSTRACT

Some observations on the separation of pigments from a plant extract (described in an Appendix) led to new improved procedures for the separation of pure crystalline chlorophyll a and its derivative methyl chlorophyllide a.

The visible absorption spectra of chlorophyll a in solvents of various polarizabilities and dielectric constants, in concentrated solutions, and the crystalline state have been investigated. The investigations on concentrated solutions of chlorophyll a showed deviations from ideality which have been ascribed to dimerisation. The studies on crystals showed characteristic wavelength shifts arising from the molecular crystal forces.

Some general observations on the nature and degree of order in the crystals have been made by X-ray powder diffraction methods.

An investigation of the infrared spectra of chlorophylls showed that previous assignments of the vibrations were essentially correct. An investigation of the vibration frequency shifts caused by different solvents showed that the keto group of the cyclopentanone ring of chlorophyll a was subject to a variety of interactions. From a study of the infrared spectrum of chlorophyll a at varying concentrations and with the use of a magnesium free derivative of chlorophyll a (methyl pheophorbide-a) it was shown that the dimerisation of chlorophyll a was due to a weak bonding between a keto group from one molecule with the magnesium atom of another molecule. This interaction is shown to be the main orienting force which directs the crystallisation of chlorophylls.

Proton nuclear magnetic resonance studies on the chlorophylls have been carried out. From these studies it has been possible to assign chemical shifts with a high degree of certainty to two important protons (delta-methine bridge proton and the proton on the cyclopentanone ring) in the chlorophylls. A Digital Height Analyser has been used to obtain spectra from solutions of chlorophyll a which are of low concentration, and in this way spectra were found which could not be obtained by conventional techniques.

The investigation of the photoinduced paramagnetism of plant extracts and crystalline chlorophyll a have shown that the presence of water on the surface of the extract films and impurities present in chlorophyll a crystals influence the production of free electrons in the samples, when they are exposed to light.

GENERAL INTRODUCTION

Research on photosynthesis, now that the dark reactions of the sugar forming processes in the plant cell have been worked out (1), is being concentrated on the first stages of the photosynthetic process, whereby the light energy from the sun is trapped and transferred into the bonds of highly reactive species. The highly reactive species then go on to carry out the dark reactions. From investigations which have been concerned with the first stages of photosynthesis it is clear that the molecule chlorophyll a plays a major role. There are other plant pigments (chlorophyll b, carotenes, and xanthophylls) which play some part in the primary processes, but whose role would seem to be secondary to that of the chlorophyll a. The molecule chlorophyll a therefore is the controlling molecule in the process whereby the plant cells convert light energy into chemical bond energy. In a similar fashion we talk about the information storage of a cell as being located in desoxyribonucleic acid (DNA). The significance of DNA in cellular processes became clear in 1953 (2) when the sciences of chemistry and physics had reached a much higher stage of development than when the significance of chlorophyll a in cellular processes had been noted fifty years before. Because of the great advances made in the quantum mechanical description of molecules, and experimental techniques for investigating molecular structure, the function of DNA in cells has been actively pursued along lines which have been called Molecular Biology. Surely, molecular biology cannot be restricted to the study of DNA alone, when we have other molecules such as enzymes, hormones, alkaloids, and chlorophylls which play

important roles in the control of cellular processes. The procedures of molecular biology have been so successful that they should be applied to these other molecules. This is the approach that has been used in this study of chlorophyll a chemistry. It would not be true to say that the molecular biology of chlorophyll a has not been investigated until the present time. Indeed, there have been a great many chemical investigations on chlorophyll a which contribute to the molecular biology of chlorophyll a, but these were carried out as specialised investigations, where the techniques were well known to the investigators. The approach taken in this thesis has been to use many techniques which are not necessarily at the limit of their power but can be taken further if they show promise in giving valuable information. By using a range of techniques and trying to reach the rigor of physics, then more coherence is obtained in the investigation of the molecular biology of chlorophyll a. For these reasons the thesis has been entitled "Some New Molecular Biology of Chlorophyll a".

A brief review shall be given in the introduction of what is considered to be the molecular biology of chlorophyll a as it existed before the present investigations were commenced. It will be given in the same sequence as was followed in the investigation, and indications given as to where extensions of the previous knowledge were attempted. Detailed introductions to the chemistry and physics of the various experimental studies and the advances, if any, in the physical chemistry of chlorophyll a will be given in the various subdivisions of the work.

Very broadly, the overall task of the molecular biologist is to

extract the molecule from the cell, reconstruct the physical state and milieu of the molecule in the cell, and then attempt to reproduce the cellular function of the molecule in a test tube (in vitro). He will be guided in the last three objectives by the investigations of the biophysicist who studies the intact cell or portions of the cell.

The first objective, then, is to extract the molecule from various species of cells and purify it to such an extent that the validity of any experiment carried out on the molecule is not called into question over its purity. Questions about the molecule's purity in organic chemical reactions are not as serious as those that can be posed over refined physical chemical measurements of, say, its electronic conductive properties in the solid state. The first separation of chlorophyll a from plants was carried out by Twsett in 1906. Since that time biochemists have isolated chlorophyll a from many plants and continually improved on its state of purity (3,4). New techniques of physical investigation such as electron spin resonance require very pure materials in a crystalline state. From this, it is quite reasonable to expect that this technique would show that previous methods of purification were inadequate. An attempt was therefore made to develop a new procedure for the separation and purification of chlorophyll a which would give satisfactory results when the new physical techniques were applied.

When the molecule can be obtained with reasonable purity, then its structure can be determined by classical organic chemical techniques. If the molecule can be crystallised, then its structure can be determined by physical methods, of which the most general is X-ray

crystallography. Chlorophyll a could not be obtained as crystals until recently, but it could be extracted with sufficient purity so that its structure could be determined by a triumph of classical organic chemistry. The molecular structure which is agreed upon today as being the best representation of chlorophyll a was given by H. Fischer in 1940 (5).(Fig. 1). This structure was confirmed by its partial synthesis in the laboratories of R. B. Woodward (6). There can be little doubt about the structure of chlorophyll a. Since the structure of chlorophyll a was first put forward, the powerful techniques of ultraviolet, visible, infra-red spectroscopy have been refined, and proton nuclear magnetic resonance spectroscopy (NMR) has been discovered. These techniques have been applied to a structural investigation of chlorophyll a only to a small extent. The ultraviolet and visible absorption spectra (of a large molecule) are not very powerful in determining its structural features. However, these techniques have been very valuable in the study of the molecular biology of chlorophyll a because they are the easiest way to characterise the molecule and correlate with spectra obtained from cells, thus ensuring the relevance of the molecule in photosynthesis (7,8). The newer techniques of infra-red and NMR could confirm certain gross features of the molecule's structure and give detailed information about its fine structure. The main value of these techniques would, now, be as tools for following the changes in the chlorophyll a when its function is being investigated. Infra-red spectroscopic studies of chlorophyll a have been carried out since the time of Coblentz (9). Agreement between the results of the most

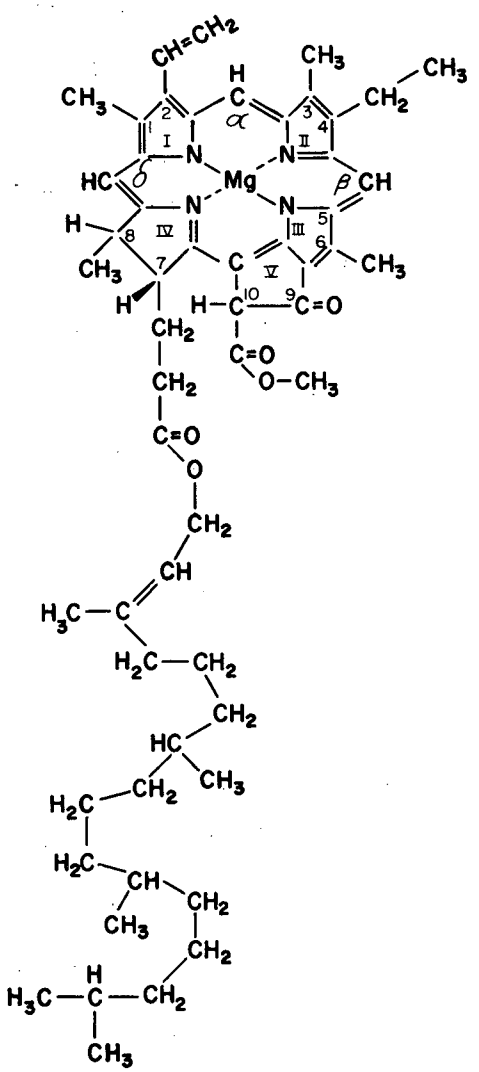

Fig. 1. The molecular structure of chlorophyll a.

recent work has not been satisfactory, especially over assignments of vibrations to the ring V keto group at C_{10} and the C_9 ester group, which have long been thought to take part in a keto-enolisation phenomenon. A reinvestigation of the infra-red spectrum of chlorophyll a and some of its derivatives seemed worthwhile to resolve the conflicting results. NMR studies of chlorophyll a have not been reported, but work done by several laboratories on porphyrins and difficulties with the infra-red studies suggested that the NMR spectrum of chlorophyll a would be extremely valuable. Relating the various types of spectra originating from the isolated molecule to the physical state or reactions of the molecule in the cell is something that has been constantly borne in mind by the various workers.

Reconstructing the physical state of the molecule "in vitro" is a much more difficult task than isolating the molecule from the cell. In the final analysis, an agreement must be obtained between the synthetic model's properties and biophysical measurements on the cell. It is not necessary to wait for the biophysics to become clear and give us good indications of what the model should be before going ahead and making models. The simplest biophysical measurements on the photosynthetic cell which tell us something about the nature of the chlorophyll in the cell are its visible absorption and fluorescence spectra. The red absorption band of the chlorophyll a molecule in the common solvents, such as ether, is shifted by 200 Å further to the red in the cell. Attempts have been made to bring about this shift by changing the dielectric and polarizable properties of the solvent in which the molecule is dissolved. These have not

been successful in bringing about the desired shift. Concentrated solutions (10^{-3} M/liter) of chlorophyll a in ethanol have shown absorption and fluorescence bands which are presumably due to aggregated species. These have shown some correlation with cellular properties (10). The spectroscopy of colloidal forms of chlorophyll a have been studied for some time. It can be seen from the structure of chlorophyll a that it has a large hydrophobic chain attached to a polar head which allows the molecule to form monolayers at a water-air interface. Langmuir (11) noticed this feature of the molecule, and he carried out the construction of monolayers from chlorophyll a. In recent years the optical properties of these monolayers have been studied intensively (12). They show reasonable agreement in their spectral characteristics with those of the intact cell. There is always the possibility that small amounts of crystalline chlorophyll a exist in the cell, and it is of some importance to prepare this physical state of the molecule and study its properties. In the present work, visible light absorption spectral studies have been carried out on chlorophyll a dissolved in various solvents, high concentrations of chlorophyll a in non polar solvents (carbon tetrachloride), and the crystalline state.

The function of chlorophyll a in the first stages of photosynthesis has been studied as a molecular biological problem with two hypotheses. If the chlorophyll a exists in the cell dissolved in some solvent media, then we can hypothesize that its function will obey the laws of photochemistry in solution (13,14). A recent hypothesis (15,16) was that the chlorophyll a could exist in a semicrystalline or crystalline form whose behaviour would follow the physics of organic semiconductors.

Much work has been done on the solution photochemistry of chlorophyll a without any conclusive results being obtained. There are several features of the chlorophyll a structure which could be implicated in reversible reactions with light and water as demanded by the first stages of photosynthesis. For example, much attention has been given to the C_{10} proton which can take part in a reversible reaction involving a keto-enolisation (14). The protons at positions 7 and 8 in the molecule could lead to chlorophyll a undergoing a photochemical oxidation with quinones which are known to be present in plants, conceivably in a close association with the chlorophyll. This aspect has been extensively studied by M. Calvin and his students (13). A very recent development stemming from the NMR studies of porphyrins (17) is the realisation that the delta bridge proton of chlorophyll a could be a unique proton in the molecule. Experiments have shown that the delta proton of chlorophyll a can be exchanged in preference to the alpha and beta protons (18). However, tracer experiments on intact cells suggested that it was the C_{10} proton which had been exchanged (19). We have commenced experiments in this area using NMR to analyse for possible exchanges of the delta proton and the C_{10} proton.

Very little work has been done on the solid state properties of chlorophyll a because it had been very difficult to prepare crystalline chlorophyll a. A large amount of work has been carried out on the physical chemistry of phthalocyanines, which have analogous structures to chlorophyll a (20). The results of these experiments suggest that similar studies on chlorophyll a would be fruitful. We have at first studied the electron spin resonance of extracts from cells which

contained associated plant pigments which are extractable with polar organic solvents. A cursory examination of the ESR properties of the crystalline chlorophyll *a* was also carried out.

No attempt has been made in the introduction, nor will any be made in the conclusions, to give any detailed interpretation of the mechanism of photosynthesis in terms of the molecular biology of chlorophyll *a*. The general conclusions which will be given at the end of the dissertation will follow the general order of topics given in the introduction.

The Extraction, Purification, and Crystallisation of Chlorophylls

Introduction

The first demonstration of the separatory technique of chromatography was carried out by Twsett in 1906 when he showed that an extract of plant pigments could be separated on a column of precipitated chalk. Chlorophyll *a* in a crude form was first separated with this technique. Wilstatter used partition type experiments whereby molecules are distributed between two solvent phases, and as this process is repeated many times because of the differences in the distribution coefficients of the molecules, they can be separated. Through the years chromatography has been found superior to the partition type experiments for the separation of chlorophylls.

The nature of the chromatographic adsorbent has changed from inorganic salts to the more mild organic solids (sugar) which give less breakdown of the delicate chlorophylls. Spectroscopically pure chlorophylls were required by the plant biochemists so that they could set up spectroscopic standards for the analysis of plant materials. Only when the demands of physical chemists became more severe was chlorophyll *a* obtained in a crystalline form. Although several claims were made by various workers that they had obtained crystalline chlorophyll, these did not hold when it was investigated by X-ray diffraction. Jacobs, Vatter, and Holt (21) obtained crystalline chlorophyll in 1954, and shortly afterwards it was again obtained by Swiss workers (22). Both of the methods developed to obtain crystalline chlorophyll *a* were elaborate and required a great deal of manipulative skill. The method of Jacobs *et al.* gave crystals which had poor powder X-ray patterns

but did show characteristic red shifts in the absorption bands of the monomeric species when they formed a molecular crystal. A less involved procedure for the separation of chlorophyll a was desired and one yielding crystals which had more order than those shown in the pictures presented by Jacobs et al. (21). At one stage of our investigations on the ESR of extracts it was not sufficient to separate chlorophyll a, but also necessary to separate the pigments associated with it in the extract. Because of this demand, Jacob's outlined procedure (21), Figure 2, was followed because it, in principle, separated all the other pigments at clearly defined steps of the separation. Great difficulty was found in reproducing their procedure for several reasons. The most troublesome manipulation in their scheme, which is also common to the other separations reported, was the distribution of the chlorophylls and carotenes into the petroleum ether phase, when, quite often, an emulsion was formed which was hard to break, requiring that the experiment be repeated. The other difficult manipulation was the sugar chromatography, which, quite often, stemming from the inefficiency of previous steps, led to the difficult task of separating many pigments and the decomposition products of chlorophylls (pheophytins), the structure of which, along with the other chlorophyll derivatives and associated pigments, is given in Appendix II. Theoretically, the polar molecules, such as xanthophylls, would be distributed into the acetone:water phase when the initial phase separation is carried out between petroleum ether and acetone-water, and the resulting petroleum ether phase is thoroughly washed with acetone-water. This treatment never seemed to be totally effective, and a pale yellow band invariably present on the leading

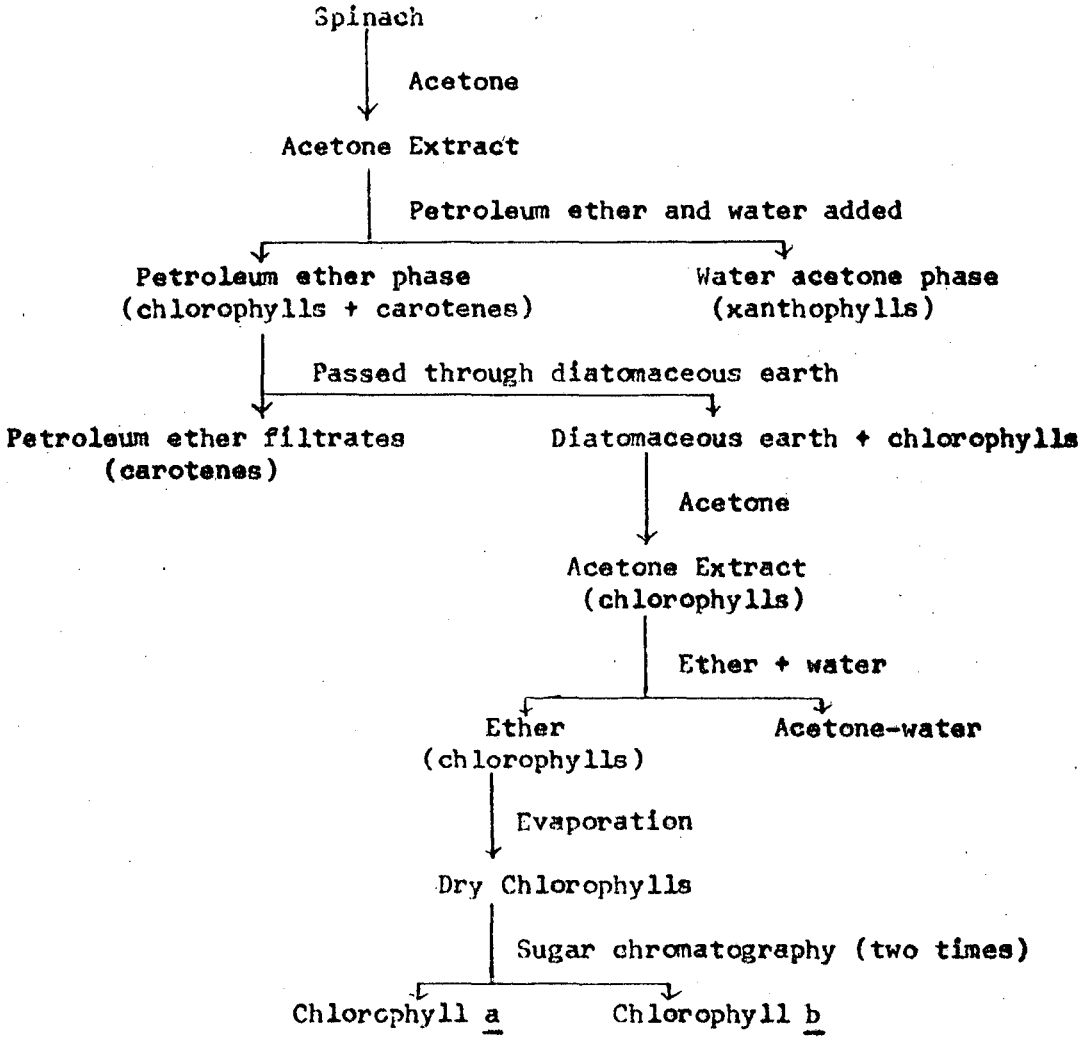

Fig. 2. Outlined Method of Jacobs et al. (21) for Separation of Chlorophylls.

edge of the chlorophyll a on a sugar column (chromatographic separation of chlorophylls a and b) was due to the inefficiency of the phase separation. Although the preferential adsorption of chlorophylls on a diatomaceous earth and the washing through of the carotenes does remove most of the carotenes, it is not highly effective, resulting in varying amounts of carotenes preceding the chlorophylls on the sugar chromatographic column. Furthermore, the chlorophylls were degraded to pheophytins due to the acidic nature of the diatomaceous earth surface. Pheophytins also precede the chlorophylls on a sugar column, and the desired final separation is for chlorophyll a to come through without any pigment preceding it.

A large number of experiments were conducted on plant extracts (along the lines of Holt, et al.). They are not relevant to this thesis but have been fully reported in Appendix I. The most promising result from this work was that polyethylene powder could separate the chlorophylls from the other plant pigments (23), and the separation which we have developed is based on this fact. It was also noted in these experiments that chlorophyll a could be very efficiently separated on sugar from chlorophyll b, and the desired final stage would be the separation of chlorophyll a and chlorophyll b on sugar.

The separation developed in this work is outlined in Figure 3. An acetone extract of the plant material, which is spinach when fairly large quantities (200 mg) of chlorophyll a are required (for smaller quantities, algae are suitable), is prepared. This extract is then passed through a polyethylene powder column which separates the chlorophylls from the other pigments present in the extract.

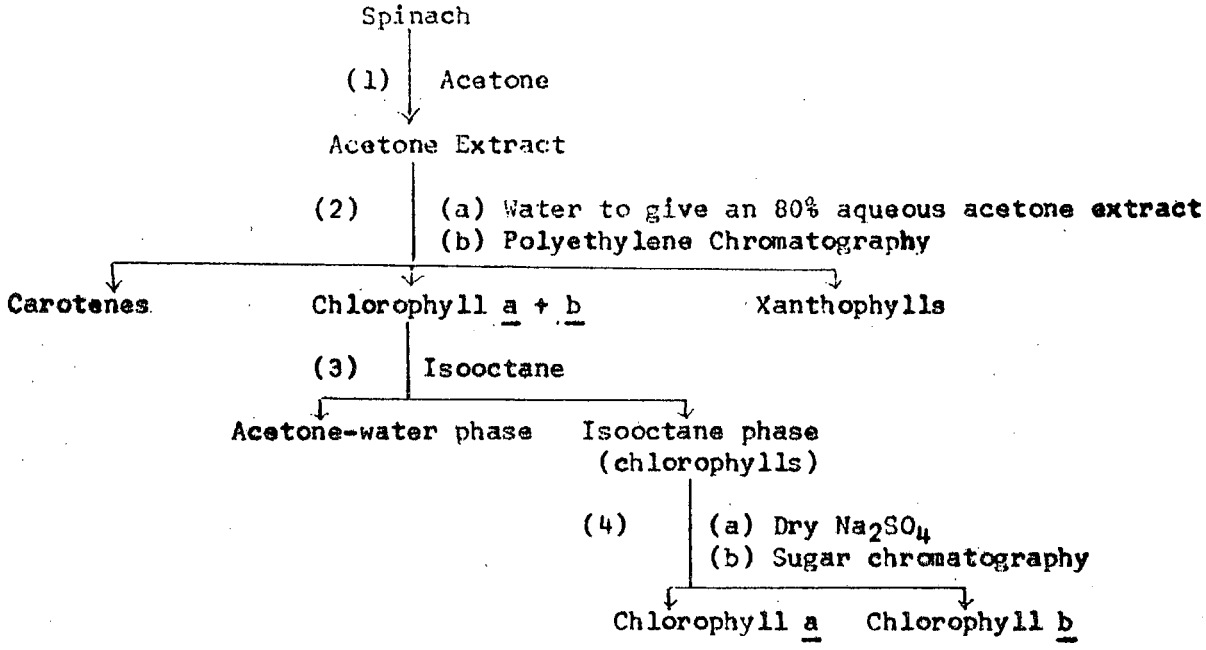

Fig. 3. Chlorophyll a and Chlorophyll b Separation Described in This Work.

Chlorophylls in the acetone-water solution are then transferred into isooctane, which is dried, before carrying out the separation of the chlorophylls on a sugar column. The eluted chlorophylls can be recovered in several solid forms, some of which are crystalline.

A typical procedure for extracting chlorophyll a from Chlorella algae is also described. This procedure differs from that described for the extraction of chlorophyll from spinach only in the collection and extraction of the starting material; from that point on the extraction and purification are carried out in the same manner as was described for the spinach preparation.

A Procedure for the Separation and Purification of Chlorophyll a from Spinach

800 grams of fresh spinach, obtained from a local market, are washed free of contaminants, their main stems are removed, and then they are thoroughly dried with adsorbent paper. It is important to remove as much water as possible, as this can interfere with the water-acetone ratio used in the polyethylene chromatography. The leaf material is ground up for about 2 min in a Waring blender at high speeds with acetone of reagent grade quality, using 500 ml of acetone for every 200 gm of leaves. Filtration of the resulting brei is carried out immediately on a Buechner funnel and the filtrates collected. Dilution of the filtrates with distilled water gives an acetone:water solution (80% v/v) of the pigments, which is then chromatographed on polyethylene powder.

Polyethylene Chromatography of an Acetone-Water Extract of Spinach

Polyethylene chromatography is an example of reversed phase partition chromatography (24) in which the stationary phase is polyethylene

and the mobile phase acetone-water. It has not been possible to find the physical characteristics of the polyethylene powder which control the separating power of this material, but it would seem that powder with a melt-index (M.I.)* of less than 2.8 is satisfactory. At lower M.I. better separations are obtained. The particle size of the powder should be such that a suitable flow rate of solvent (1 ml/min is satisfactory) is obtained.

Polyethylene powder was packed to a height of 50 cm in lots of 600 ml into a glass column, carefully cleaned and dried, of 10 cm diameter and 60 cm in length. This was the ultimate size of column which could be used to give good packing of the powder and could be manipulated with ease. The column had no joints or stopcocks which would lead to contamination because of the lubricants present on the joints. In order to support the powder there is a glass plate on the end of the column with a large number of holes (1 mm in diameter) which are covered with filter paper. After each 600 ml lot of polyethylene was placed in the column it was tapped with a vertical motion on a rubber bung until the powder had reached its minimum volume and then compressed manually with a wooden rammer of a diameter slightly less than that of the glass column. When the column had been packed it was inserted in a 1.5 liter Buechner flask, but no vacuum was applied until 500 ml of 60% (v/v) acetone-water had been added carefully so that the top surface of the powder was not disturbed. Another 500 ml

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*The melt-index is the weight in grams of polyethylene extruded in 10 min at constant temperature through an orifice of specified diameter when a given mass is placed on the driving piston. It has not been possible to relate this empirical test to the molecular structure.

of 60% (v/v) acetone-water was applied to complete the wetting of the polyethylene, and as the last few milliliters of this wash remain on top of the polyethylene surface, application of the pigment extract was commenced. Two liters of the extract were applied to the column, and although the chlorophyll sometimes appears to precipitate out at the top of the column, the procedure should be continued. As the last of the plant extract was about to enter the polyethylene surface development was commenced with 70% (v/v) acetone-water, and this takes the xanthophylls through the column and elutes them, with the chlorophylls becoming evenly distributed on the polyethylene. The experimental apparatus and the order of the pigment bands on the column are shown in Figure 4. Elution with 70% (v/v) acetone-water was continued until the chlorophylls were about to be eluted from the column. The eluates at this stage should be colorless or show only faint traces of yellow xanthophylls. Then the vacuum was broken and a clean receiver was placed under the column and the chromatography recommenced with 80% (v/v) acetone-water, collecting the mixture of chlorophylls a and b. Pheophytins, quinones, and carotenes remain at the top of the column and can be eluted with 100% acetone. The polyethylene can be cleaned with several washes of acetone and ether before being brought back to its original state by a final washing with 70% (v/v) acetone-water. However, this is a laborious process and gives a polyethylene column which is still slightly contaminated and therefore of uncertain value.

Transfer of Chlorophylls from Acetone-Water to Isooctane

To carry out the sugar chromatography the chlorophylls must be in isooctane. The best way to transfer the chlorophylls into isooctane

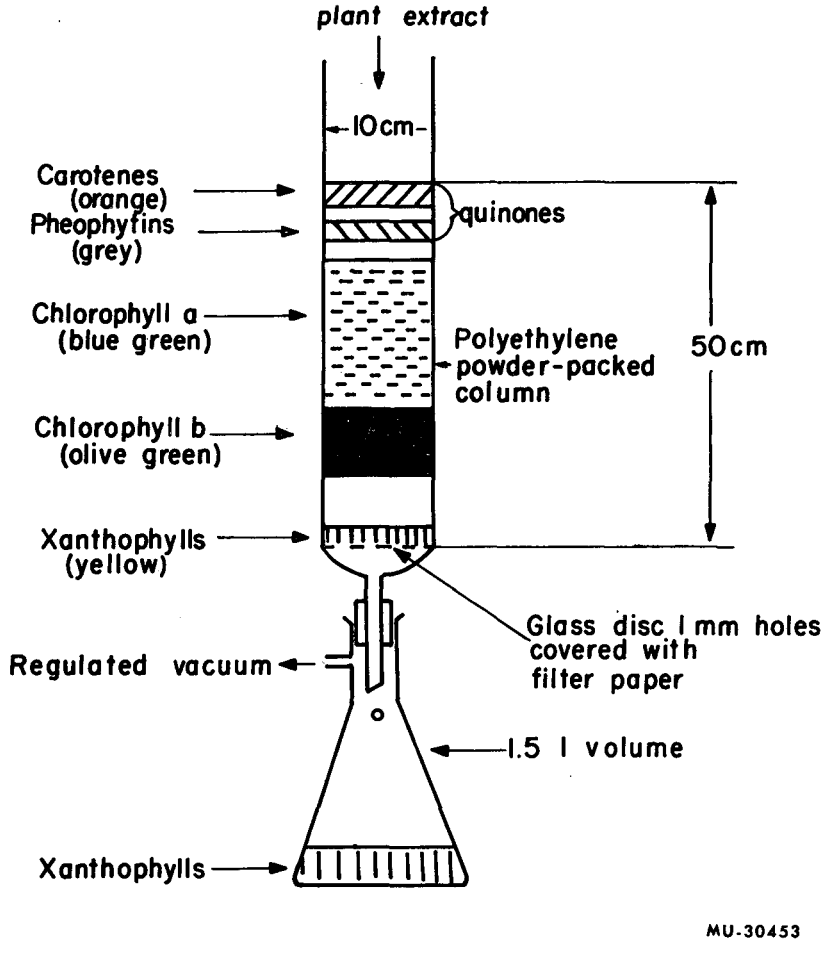

Fig. 4. Polyethylene chromatograph of an acetone-water extract of plant pigments.

was to put a layer of 600 ml isooctane on top of the acetone-water solution of chlorophylls in a 2-liter separatory funnel. The chlorophylls distribute into the isooctane phase with the addition of small amounts of water completing the phase separation. The acetone-water phase was discarded and the acetone washed out of the isooctane with distilled water. This is a critical step, because if too much acetone is washed out the chlorophylls precipitate out, and if too high a concentration of acetone is allowed to remain in the isooctane then the sugar chromatography which follows breaks down. Water washing was continued until the color of the solution went from bluish green to green and then stopped. If the chlorophylls do come out of solution, then the careful addition of drops of pyridine will bring them back in. Finally, the isooctane solution of the chlorophylls was dried with anhydrous sodium sulphate for one hour in the dark, and after filtration the isooctane solution of the chlorophylls was chromatographed on sugar.

Sugar Chromatography of a Mixture of Chlorophyll a and b

Sugar chromatography of the mixture of chlorophylls in isooctane was carried out in the same apparatus which was used for the polyethylene chromatography of the acetone-water spinach extract. Confectioners' sugar (Californian and Hawaiian Sugar Corp.), without any special treatment, was packed to a height of 40 cm in lots of 600 ml, using a similar technique as was used for the polyethylene powder; but joints between the lots must be carefully done--i.e., the excess sugar, after the tamping, was scraped out of the column before the next lot was added. This brand of sugar has been very consistent in its ability

to separate the chlorophylls; however, a few tests on Spreckels brand did not give good results, presumably due to its higher starch content. (Outside of this district it would be better to test the various local brands.) Isooctane, 500 ml, was added to the column and suction applied after the initial addition of the liquid. Just before the last of the wetting solution--great care must be taken at all stages of this step not to allow the sugar to go dry--had entered the sugar surface, the isooctane solution of the chlorophylls was applied. When the last few milliliters of the pigment solution were about to enter the sugar column, a developing solvent of 0.5% by volume of n-propanol in isooctane was added to the column. The distribution of the pigment bands on the column after development is shown in Figure 5 and a photograph of a typical separation of chlorophyll a on sugar shown in Figure 6. Chlorophyll a is eluted from the column, and chlorophyll b can be recovered by continuing elution, by changing the development solvent to 2% n-propanol in isooctane, or by cutting out the chlorophyll b band and then extracting the chlorophyll b with ether or acetone. Chlorophyll a was recovered from the eluate of the column by several procedures, to be described in the next section.

Recovery of Chlorophyll a from Column Eluates

All the chlorophyll a from the sugar column can be recovered by removing the volatile isooctane-n-propanol solvents under vacuum. This procedure cannot effect any further purification and does not lead to crystalline chlorophyll a, but the following two procedures do give crystalline chlorophyll a.

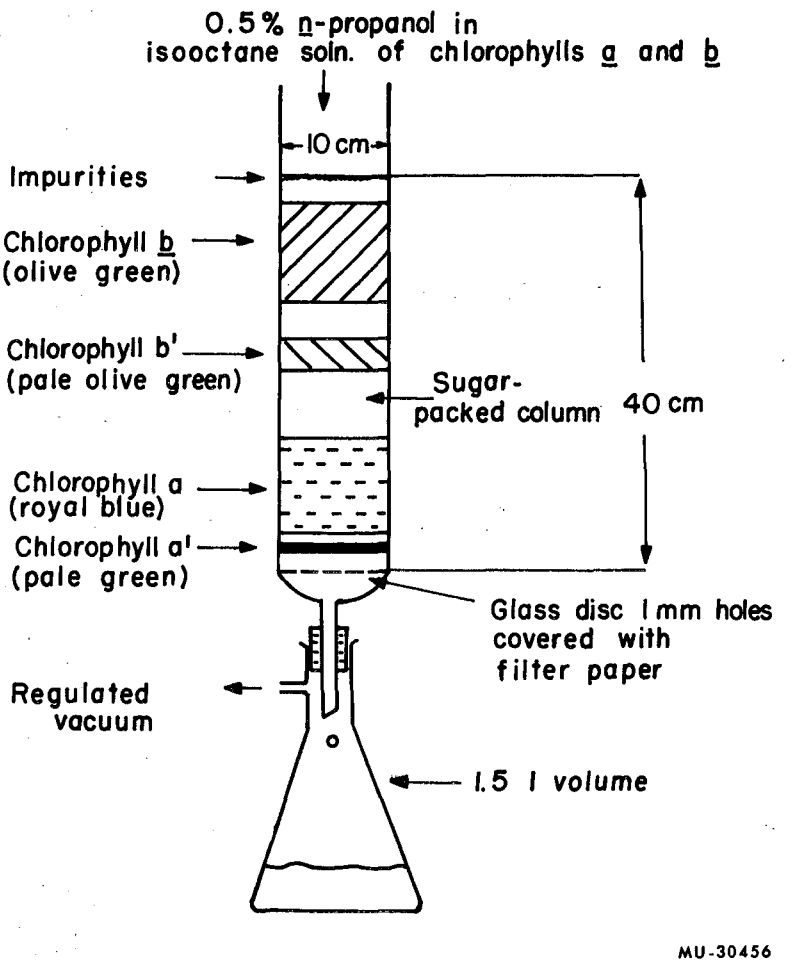

Fig. 5. Sugar chromatograph of chlorophyll a and chlorophyll b mixture in isooctane.

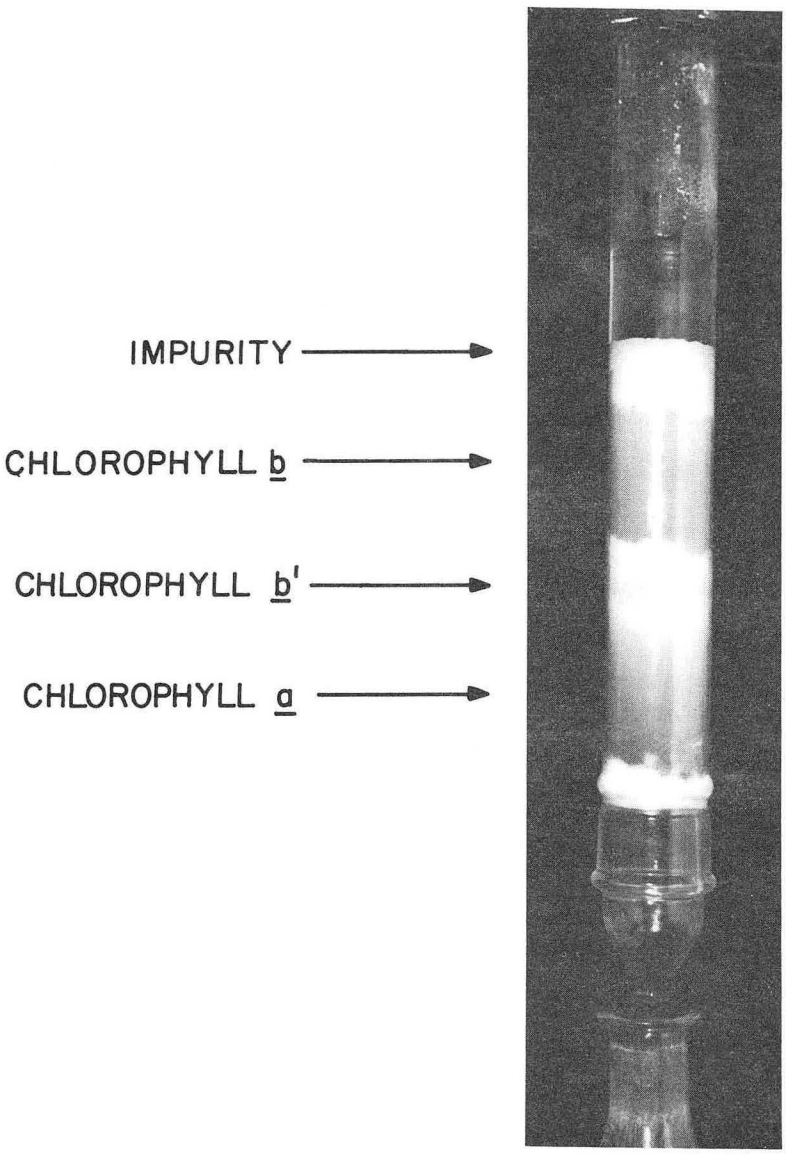

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Fig. 6. Photograph of sugar chromatographic separation of chlorophyll a and chlorophyll b.

The first procedure used was similar to that of Jacobs *et al.* (21). The eluate from the sugar column was transferred to a liter separatory funnel and 500 ml of distilled water added, care being taken not to cause much agitation. Careful agitation was then applied to the separatory funnel, and as the isopropanol diffused from the isooctane into the water the chlorophyll *a* solution went from a deep blue to a dark green. The water was then run off and the washing treatment continued until fine sheets of chlorophyll *a* with a metallic lustre appeared at the isooctane-water interface. After the water was run off, the isooctane slurry of the chlorophyll particles was collected, centrifuged, and the supernatant isooctane siphoned off. The particles can be stored in a refrigerator as a slurry or the isooctane removed in a vacuum dessicator.

The second procedure for obtaining chlorophyll *a* is now the preferred one because the particles are easier to collect and have more order in their structure. The solution of chlorophyll *a* from the column was collected in a flask, which was covered with aluminum foil to protect the chlorophyll *a* from the destructive action of light, and placed in a refrigerator. After about 8 hours, chlorophyll *a* crystals were deposited and collected by centrifugation. Filtration leads to the fine crystals sticking to the filter paper and loss in yield; therefore the supernatant liquid in the centrifuge tube was siphoned off. To preserve the crystalline structure of the crystals, they are stored suspended in the isooctane, but for most uses the isooctane can be removed in a vacuum dessicator.

The Extraction of Chlorophyll a from Algae

The best solvent for breaking down the algae and extracting the pigments is methanol, but it leads to the introduction of allomerised chlorophylls which are readily formed in the presence of methanol and air. However, these allomerised chlorophylls are present in small amounts and are removed on the polyethylene and sugar columns.

A 250 ml sample of Chlorella algae grown under steady state conditions was taken, and this was centrifuged down at 2,000 rpm to remove the culture medium and collect approximately 4 ml of wet packed cells. These were resuspended in 200 ml of distilled water, stirred, centrifuged, and collected again, and the treatment carried out once more. The collected cells were extracted with 80 ml of reagent grade methanol in a flask which was covered with foil to exclude light, and allowed to remain in a refrigerator for one hour. The methanol extract from the cells was recovered by siphoning off the supernatant layer formed on centrifugation. Distilled water was added to the methanol extract from the Chlorella to give a 70% (v/v) solution of the pigments which was suitable for polyethylene chromatography. Separation of the pigments was carried out in an analogous manner to that for the chlorophyll a, using columns of 3 cm diameter and 25 cm in length, with the volumes of solvent scaled accordingly.

Discussion

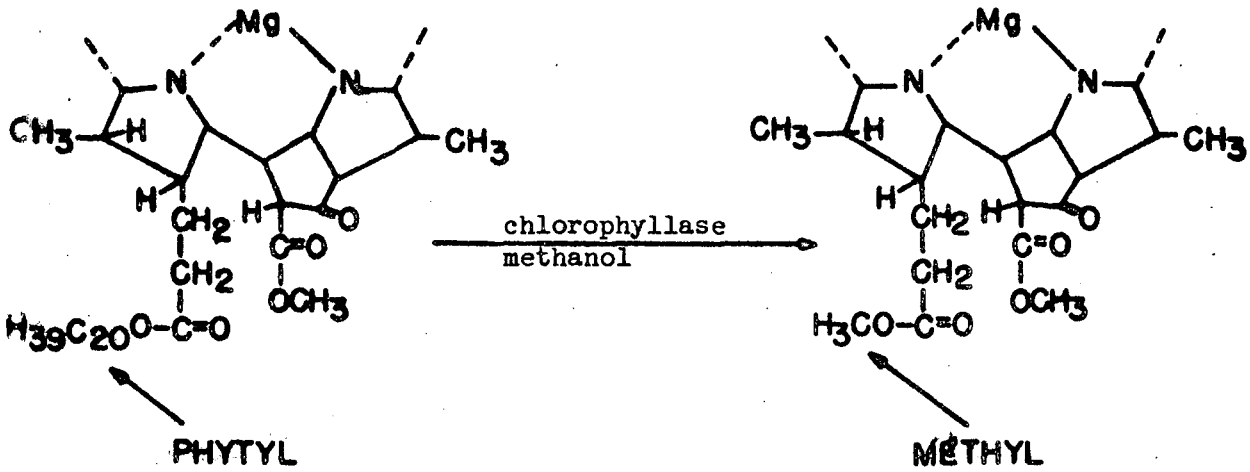
It can be seen from Figures 2 and 3 that the number of steps in the new procedure described in this work is much less than is required in what seems to be the best previously reported procedure. The very irreproducible phase separation between petroleum ether and acetone,

which was included to separate out the xanthophylls from the acetone extract of the plant and is common to all of the previously reported procedures, has been discarded. The xanthophylls were completely removed on the polyethylene column, which did this more efficiently than the phase separation of the older methods. A diatomaceous earth treatment, which is present in some of the older methods, and included to separate the carotenes from the chlorophylls, has become an unnecessary step in the separation because the carotenes remain at the top of the polyethylene column along with pheophytin, which is formed to a varying extent in the extraction of the plant material with acetone. Therefore, the chlorophylls are the only pigments (except for small traces of unknown impurities) which have to be separated by means of sugar chromatography. Because unknown impurities remain at the top of the column, and chlorophyll *b* and *b'* travel after the chlorophyll *a* band, chlorophyll *a* comes through the sugar column without having to pass through a sugar surface on which any other pigment has been previously adsorbed. Chlorophyll *a* crystals (evidence for crystallinity will be presented in the section on the optical properties of chlorophyll *a* in various states) have been prepared without any elaborate procedure. The procedure described is highly reproducible and has been carried out more than twenty times.

After having had good success with the polyethylene column chromatography for the extraction of chlorophyll *a*, an extraction of methyl chlorophyllide, which is the chlorophyll *a* molecule with the photochemically irrelevant phytyl chain substituted by a methyl group, was attempted.

The Extraction and Purification of Methyl Chlorophyllides

It has been known for some time that the phytyl chain of chlorophylls can be exchanged for other smaller alkyl groups such as methyl, by extracting with alcohol certain plants which contain an enzyme called chlorophyllase. The alcohol used for the extraction has the alkyl group which is to be exchanged for the phytyl (25).



Chlorophyll a — Methyl chlorophyllide a

Again, Holt and coworkers have given the most detailed description of the preparation of chlorophyllides (Fig. 7) and have shown that the leaves of Ailanthus altissma were rich in the enzyme chlorophyllase (26). It is not necessary to separate the chlorophyll a and enzyme before carrying out the transfer reaction; all that is required is to grind up the leaves in the presence of methanol, allow the reaction mixture to sit in the dark for three hours at room temperature, and then extract the chlorophyllides from the carotenoids and the phytol alcohol ($C_{20}H_{39}OH$) which has also been formed. Holt and coworkers used a similar method for the extraction of the chlorophyllides from the

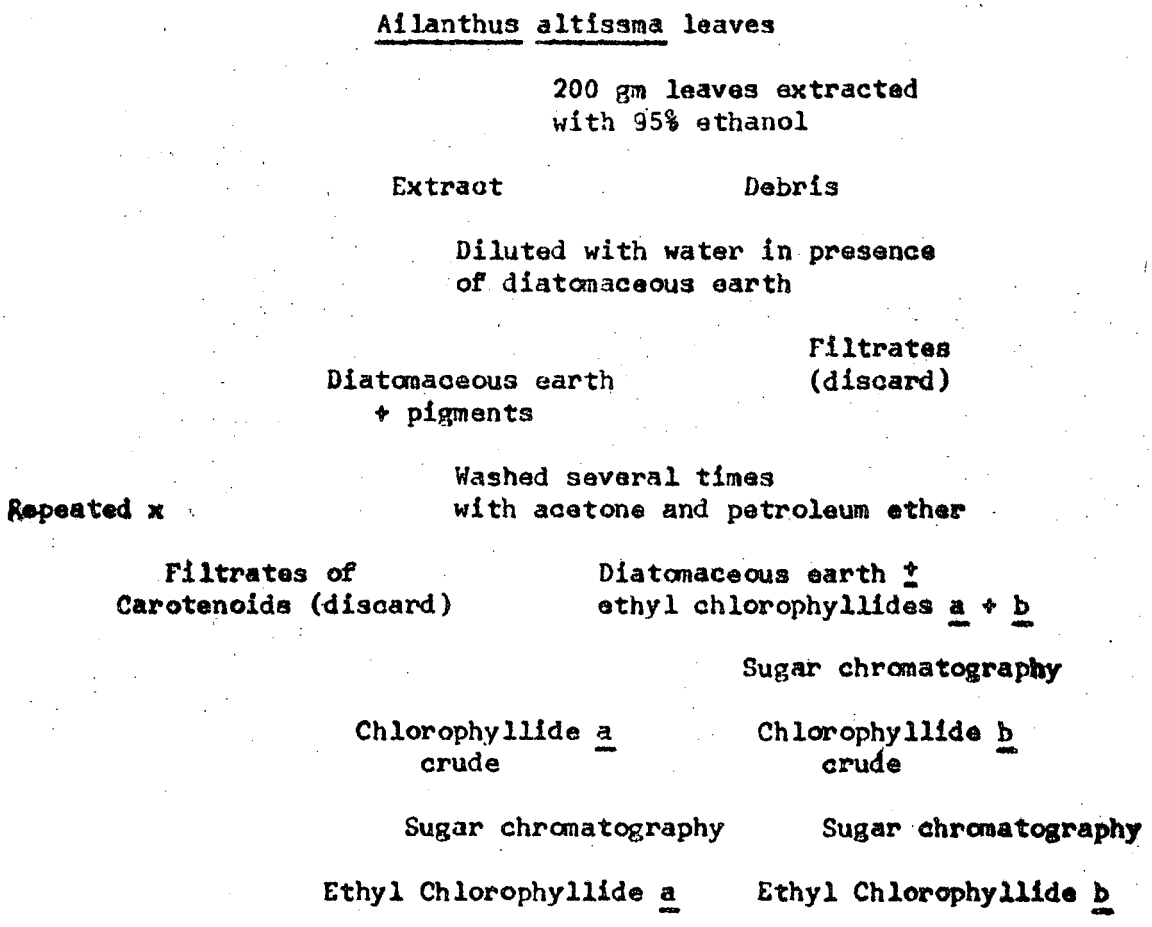


Fig. 7. Procedure of Holt and Jacobs for Extraction and Purification of Ethyl Chlorophyllides.

ethanol extract of the Ailanthus altissma as they had used for the preparation of chlorophylls. Anticipating the difficulties, which have already been described in the introduction to the chlorophyll a preparative procedure, to be also present in their chlorophyllide procedure, and being confident about the general applicability of the new polyethylene procedure to separate chlorophyll type pigments, an attempt was made to separate chlorophyllides by a method analogous to the new chlorophyll a procedure.

Many experiments showed that polyethylene could not separate chlorophyllides, for two reasons: (1) The fundamental breakdown in the polyethylene chromatography of a methanol extract from Ailanthus altissma arises from the fact that for every molecule of chlorophyllide made by the enzyme there was also a molecule of phytol alcohol formed which rendered the surface of the polyethylene ineffective. (2) The phytol alcohol when it was removed from the crude mixture lowered the solubility of the chlorophyllides in aqueous methanol solutions and they precipitated out on the polyethylene column without separating. This gave the clue to a new procedure for the preparation of chlorophyllide a, and again the crux of the problem was to obtain chlorophyllides a and b free of the other plant pigments and especially the surface active phytol alcohol.

The phytol alcohol formed in the enzymic reaction was removed by precipitating the methyl chlorophyllides and carotenoids from the methanol extract of the Ailanthus altissma by adding water under carefully controlled conditions. The precipitate collected by filtration was recrystallized several times from benzene-isooctane mixtures to give a mixture of methyl chlorophyllides a and b almost free of

phytol alcohol and carotenes, which are readily soluble in the benzene-isooctane solvent. Sugar chromatography separated the methyl chlorophyllides and purified the methyl chlorophyllide a, which could be recovered in crystalline form by allowing the eluate from the sugar column (which contained pyridine) to sit on top of water, and as the pyridine diffused into the water, methyl chlorophyllide a crystallized out (Fig. 8).

A Procedure for the Extraction of Methyl Chlorophyllide a from Ailanthus altissma

Ailanthus altissma leaves were gathered from a tree in the University Botanical Garden. When larger supplies were required, they were obtained from a grove about 8 miles from Lafayette, along Upper Happy Valley road. 2,000 gm of leaves were ground up with 5 liters of 100% methanol (reagent grade) in a large Waring Blendor. After the methanolic brei had been allowed to sit for 1 hr in the dark at room temperature it was filtered and the filtrates transferred to a 10-liter flask equipped with an efficient stirrer, and placed in an ice bath (the laboratory sink). Distilled water from a separatory funnel was added at the rate of 2 ml/min until a drop of the flask's contents, when placed on filter paper, showed the presence of a precipitate. The flask with its contents was allowed to sit for 1 hr. Then the supernatant liquid was siphoned off and the precipitate collected on filter paper without vacuum. A wash of isooctane (500 ml) removed much of the carotenes precipitated with the methyl chlorophyllides, and they were completely removed by a series of crystallisations.

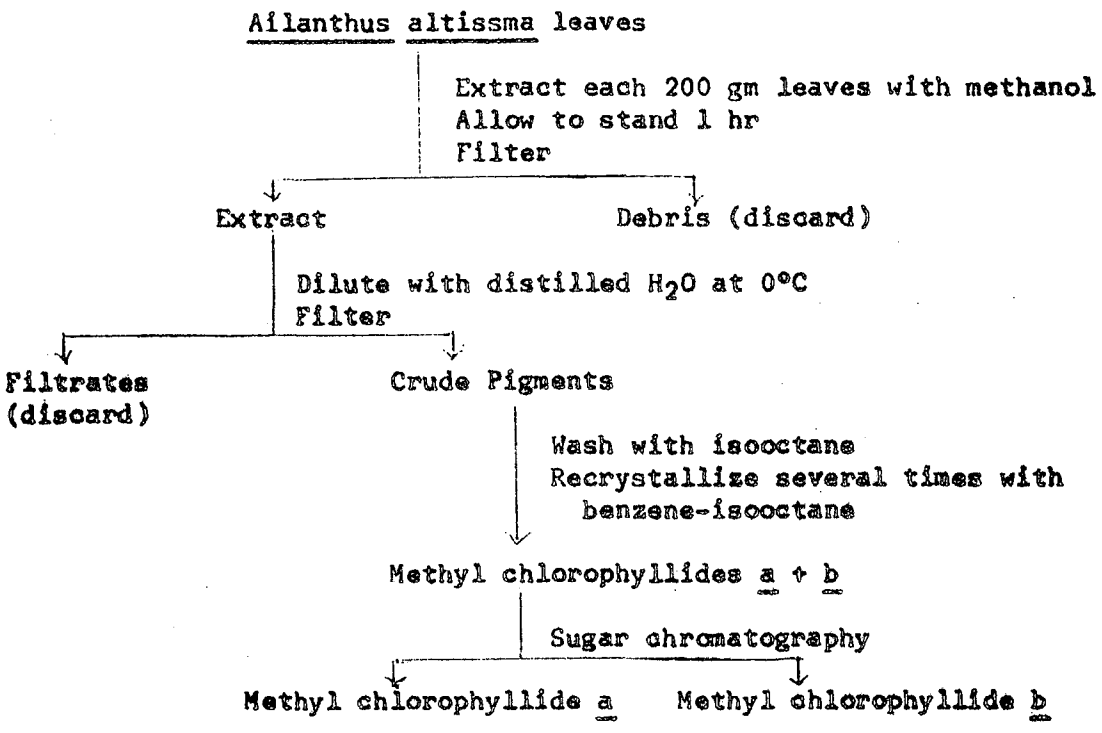


Fig. 8. Methyl chlorophyllide a and Methyl chlorophyllide b Separation, Described in this Work.

The crude mixture of plant pigments was scraped off the filter paper, transferred into a separatory funnel, and dissolved in 100 ml of benzene and 500 ml of acetone. Distilled water was carefully added until two phases were formed, whence the xanthophylls were distributed into the acetone water phase, which was discarded. The benzene solution was then washed with two 500 cc washes of 60% acetone-water to remove the last traces of xanthophyll. The benzene solution of methyl chlorophyllides and carotenes was run off into a beaker placed in an ice bath, and isooctane added until the methyl chlorophyllides crystallized out, which could be detected by spotting drops of the benzene solution on filter paper and observing the crystals formed. The methyl chlorophyllides were collected by filtration, and the carotenes remaining in the isooctane benzene filtrates were discarded. The benzene crystallization procedure was repeated twice more to give the methyl chlorophyllides free from carotenes, and after the last recrystallization they were dired in a vacuum dessicator. The methyl chlorophyllides were then in a suitable state for separation by sugar chromatography into methyl chlorophyllide a and methyl chlorophyllide b. The yield was about 2 gm of mixed methyl chlorophyllides.

Separation of Methyl Chlorophyllides a and b by Sugar Chromatography

Methyl chlorophyllides (100 mg) were dissolved in 200 ml of 20% (v/v) pyridine-isooctane and filtered through a funnel to remove any undissolved material. An apparatus was used similar to that in the sugar chromatography of the chlorophylls, but a column (diameter of 4 cm and length 40 cm) was packed with 1,500 ml of confectioners' sugar in six lots in the same way as was used for the chlorophyll a chromatography. The column was wetted with 250 ml of a solvent mixture

(2.5% pyridine:0.5% n-propanol, 97% isooctane by volume), and as the last of this solution was entering the column, the solution of methyl chlorophyllides was applied. Development of the column after the pigments were adsorbed at the top of the column was done with a solvent mixture of 5% pyridine:0.5% isopropanol:94.5% isooctane, and the methyl chlorophyllide a comes through the column first. The function of the pyridine was to keep the pigments in solution, and the isopropanol resolves the bands of pigments, which the pyridine alone could not accomplish. A trace of methyl pheophorbides came through the column ahead of the methyl chlorophyllide, and therefore the eluate of chlorophyll a which was collected was slightly contaminated by pheophorbide. However, the pheophorbides, because of their greater solubility, were in the mother liquors when the methyl chlorophyllide a was crystallized. This was accomplished quite easily by allowing the eluate from the column to sit on top of a layer of water in a separatory funnel, and as the pyridine diffused into the water, methyl chlorophyllide a crystallized out. The methyl chlorophyllide a was collected by centrifuging the isooctane suspensions from the separatory funnel, and after drying in a vacuum dessicator, a yield of 20 mg was obtained.

Discussion

A simple method for preparing methyl chlorophyllide a, which has the basic chromophoric group of chlorophyll a, in a crystalline form noticeable to the eye, has been devised.

Fewer steps are required in the above procedure than in the previous method of Holt and Jacobs (26). A mixture of chlorophyllides a

and b was obtained by a series of crystallizations without the use of diatomaceous earth, which causes decomposition of the methyl chlorophyllides. A large quantity of the methyl chlorophyllides can be obtained by the above procedure, and these are stored until the components of the mixture are required, when they can be readily obtained by sugar chromatography. The poor solubility of the methyl chlorophyllides in isooctane, even when it contains polar solvents, shows that the phytyl group has been exchanged for a methyl group, and this was confirmed in the infrared studies, where it can be seen that the intensities of the C-H vibrations of the methyl chlorophyllides are less than those present in the infrared spectrum of chlorophyll a. A typical visible absorption spectrum of methyl chlorophyllide a, prepared by the new procedure (Fig. 9), shows that it agrees closely in its general form and transitions with that of chlorophyll a, and it has a ratio of its O.D. blue/O.D. red of 1.29, which indicates that it has good purity. The extinction coefficients of the transitions of methyl chlorophyllide a have yet to be determined. The experiment has been reproduced about five times.

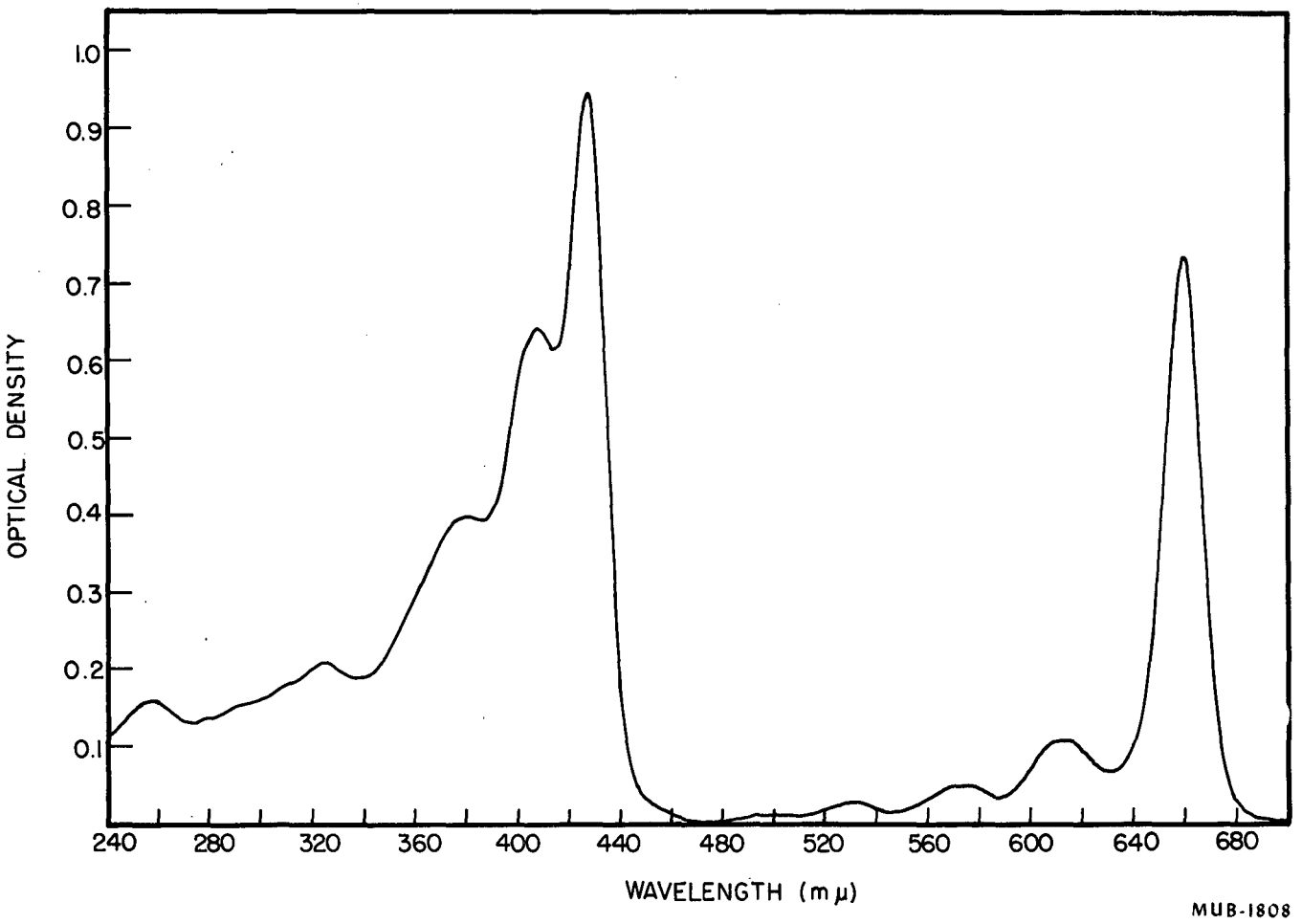


Fig. 9. Visible absorption spectrum of methyl chlorophyllide a.

The Visible and Ultraviolet Spectra of Chlorophylls

Introduction

Investigations of the visible absorption spectra of chlorophylls have been carried out since the first chlorophylls were isolated. As the ease of preparing reproducible samples of chlorophylls was improved, a concurrent improvement in the ease of taking visible and ultraviolet spectra has also taken place. The major uses of the visible absorption spectrum of chlorophyll *a* have been: to estimate the purity of the preparation, to try to relate the spectrum of the isolated chlorophyll *a* with the spectra of plant cells, and for setting up optical standards which are necessary for the determination of chlorophyll content in plants. The latter type of spectroscopic investigation did not concern us, because each worker or laboratory has to set up its own chlorophyll standard, which is dependent on the type of analysis which is used to determine the chlorophyll *a* content of the plant material (4).

Assessing the purity of chlorophyll *a* by means of its absorption spectra and comparing this with other workers' results is not an easy task, because the purity of the solvents used varies from laboratory to laboratory. It is well known that chlorophyll *a* spectra are very sensitive to the nature of the solvent. The absorption spectrum from 2,200 to 7,500 Å of the preparation of chlorophyll *a*, described above, in purified ethyl ether has been carried out without attempting any exhaustive comparative investigation of the spectrum of our sample with others.

The major emphasis in this area of the work was to try to put the chlorophyll *a* in a milieu or physical state which would shift

the absorption bands to values similar to those found for the cell. The chlorophyll a visible absorption spectrum has been studied in solvents of various polarizabilities and dielectric constants. Because the concentration of chlorophyll a is about 10^{-2} M/liter in the cell, a concentration dependence study of chlorophyll a in carbon tetrachloride (non-polar solvent) was made. Finally, the visible absorption spectrum of crystalline chlorophyll a has been taken to find the near-infrared transitions in its spectrum.

The Ultraviolet and Visible Spectra of Chlorophyll a in Ethyl Ether

Procedure

Chlorophyll a crystals, prepared as described in a previous section, were dried for 12 hr under a vacuum of less than 5 microns of Hg. Ethyl ether was purified by washing with sodium carbonate, dried with calcium chloride, distilled from a sodium-mercury amalgam and the middle cut taken. Two weighed quantities of chlorophyll a were dissolved in ethyl ether, in a tightly stoppered volumetric flask (250 ml), to give a concentration of pigment which would give an optical density of about 0.5 to 0.6 in the red and blue absorption bands when measured on the spectrophotometer. Spectra were measured on a Cary Model 14R spectrophotometer, using matched 1 cm cells for the red and blue regions and 5 cm cells for the remaining poorly absorbing regions. Optical densities at wavelengths of maximum absorption were taken point by point. The preparation of solutions was carried out in dim light and at room temperature.

Results

The extinction coefficients were calculated from the concentration of the solutions, the measured optical densities at the various

wavelengths, and a known path length by using the Lambert-Beer relationship:

$$\log \frac{I_0}{I} = O.D. = \varepsilon c l$$

where O.D. is the optical density

c is the concentration in moles/liter

l is the path length of the cell

ε is the molar extinction coefficient

The molecular weight of the chlorophyll a was taken as 893.6.

The average of two determinations of the extinction coefficients at wavelengths between 2,200 and 7,500 Å were taken and plotted against the wavelength to give the spectrum shown in Figure 10, and extinction coefficients at the points of maximum absorption are given in Table I.

Conclusions

The wavelengths of maximum absorption of the chlorophyll a in ethyl ether which were found for chlorophyll a prepared by the new procedure agree in position with those found by previous workers (Fig. 10), but the values of the extinction coefficients are 10% lower than those given by other workers. No explanation of this discrepancy can be given without further extensive experimentation. However, a satisfactory measure of the purity of the chlorophyll a from the spectrum can be obtained by comparing the ratio of the extinction coefficients of the blue band (4,280 Å) to the red band (6,600 Å), which gives a value of 1.29 (27). In other experiments the ratio of the optical densities at the blue and red bands of chlorophyll a in unpurified ether were taken and gave values of 1.25 to 1.16. These results are about 5% less than the values (1.32)

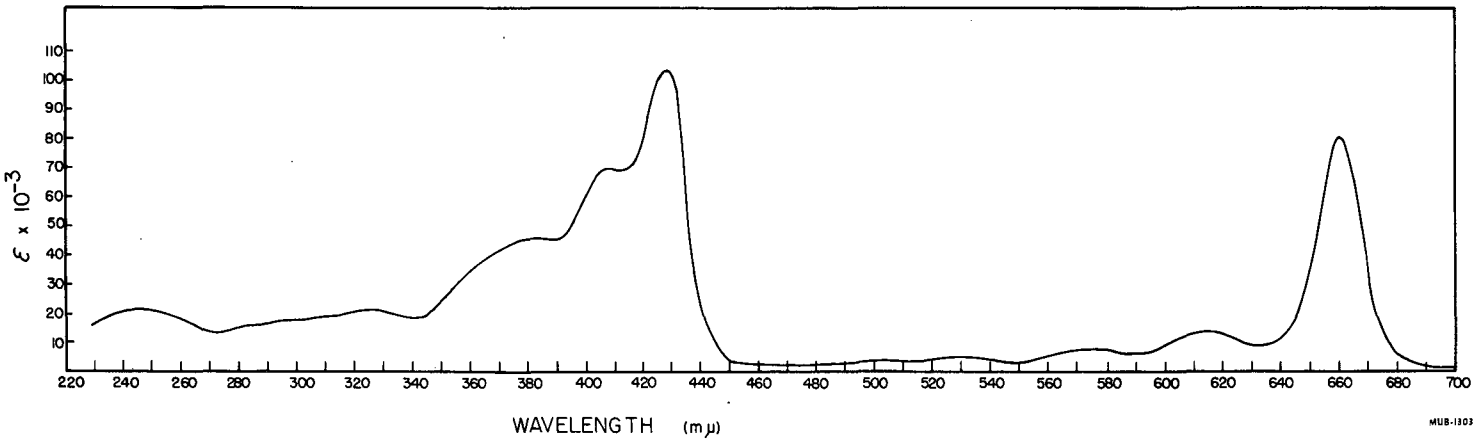


Fig. 10. Visible and ultraviolet absorption spectrum of chlorophyll a in diethyl ether.

Table I

The Extinction Coefficients of Chlorophyll *a* in Ethyl Ether Compared with Those of Other Workers

Anderson

Wavelength (mμ)	660	615	576	532	428	408	380	325
Extinction coefficient ($\varepsilon \times 10^{-3}$)	80.88	13.75	8.03	5.07	104.1	69.4	45.84	21.69

Smith and Benitez (4)

Wavelength (mμ)	662	615	578	533.5	430	410
Extinction coefficient ($\varepsilon \times 10^{-3}$)	90.1	14.6	8.28	3.77	117.5	85.2

Zscheile and Commar (27)

Wavelength (mμ)	660	614	576	532	429	410
Extinction coefficient ($\varepsilon \times 10^{-3}$)	91.2	13.8	7.60	3.70	120.6	85.2

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obtained by other workers [1.304 (8), 1.322 (27)], and this lowering of the blue band intensities indicates that the new procedure for preparing chlorophyll *a* has been effective in removing carotenoids (~ 5%) which absorb strongly in the blue region, thus making the chlorophyll *a* blue band appear more intense than it should. The principles of the chromatography used by other workers suggest that their samples were always liable to contamination by carotenes. Because carotenes are very effective quenchers of fluorescence, an enhanced fluorescence is predicted for our sample of chlorophyll *a* compared with others, but this property of our sample has not been tested. The visible absorption spectrum of chlorophyll *a* has not

been altered to the extent that the theories of the visible absorption spectrum of chlorophyll a are in question (28,29).

Visible and Near Ultraviolet Spectrum of Chlorophyll a in Various Solvents

Chlorophyll a crystals of the same sample were dissolved in various solvents of reagent grade quality to give solutions of approximately 1.5×10^{-5} M/liter concentration. Their visible absorption spectrum was taken on a Cary 14R spectrophotometer in the region of their red and blue bands.

Results

The wavelengths of the main transitions observed and the ratios of the optical densities of the blue to red and violet to red bands are tabulated in Table II along with the refractive index and dielectric constant of the solvent.

Conclusions

The chlorophyll a spectrum, as has been known for some time, depends markedly on the nature of the solvent in which it is measured. One can see that the spectrum of chlorophyll a in alcohols shows a strange behavior--i.e., the violet and blue bands become of almost equal intensity. From the infrared studies, to be discussed later, it can be seen that alcohols which are hydrogen bonding solvents interact with the molecule in the keto group region and presumably alter the spectrum by influencing the keto group contribution to the electronic transitions. No correlation, using the theoretical description of solvent effects on organic dyes as given by McRae (30), has been attempted because there are too many competing factors present, due to the complicated structure of the chlorophyll a molecule. It is seen

that the highly polarizable solvent carbon disulphide shifts the red band to 672 mμ, which is still 13 mμ less than the red absorption maximum in the visible spectrum of a cell or chloroplast.

Table II

Chlorophyll a Absorption Characteristics in Solvents of Various Polarizabilities and Dielectric Constants

Solvent	Polarizability n^2D	Dielectric const. E.D.	Red (mμ)	Blue (mμ)	Violet (mμ)	O.D. Blue / O.D. Red	O.D. Violet / O.D. Red
Carbon tetrachloride	2.13	2.24	665	431	413	1.29	0.95
α-Chloronaphthalene	2.66	5.04	668	439	415	1.24	0.85
Carbon disulphide	2.65	2.64	672	439	423	1.10	0.912
Acetone	1.85	21.20	662	429	409	1.17	1.02
Ethyl ether	1.82	4.33	661	427	407	1.22	1.08
Dioxane	1.422	2.23	662	433	412	1.28	0.98
Chloroform	2.08	4.72	665	433	415	1.1	0.97
Pyridine	2.28	13.55	668.5	443	419	1.31	0.845
Methanol	1.77	33.64	666	430	415	1.0	1.0
Ethanol	1.85	25.07	664	430	413	0.955	0.955
Propanol	1.91	20.1	666	432	414	0.975	0.93
Butanol	1.95	17.1	667	433	415	0.912	0.965

A Concentration Dependence Study of Chlorophyll *a* in Carbon Tetrachloride

Introduction

A fair number of studies have been made on the spectroscopic properties of aggregated dyes. In all cases the dyes have been ionic--e.g., thionine--and the solvent used in the study has been water (31). It is, perhaps, dangerous to draw conclusions from these results about the aggregation properties of chlorophyll *a*, which is only slightly polar and insoluble in water. Two workers, Lavorel (32) and Weber (33), suggested, on the basis of fluorescence studies, that chlorophyll *a* formed dimers in alcohol.

This suggestion was taken up by Brody (10,34), who studied the phenomenon more thoroughly and concluded, because of the broadening of the red and blue bands in the absorption spectrum of concentrated solutions (10^{-3} M/l) of chlorophyll *a*, that dimers were formed. By subtracting from the spectrum of a concentrated solution of chlorophyll *a* the spectrum of a dilute solution in which the molecule was assumed to be completely monomeric, he obtained a difference spectrum which he claimed was characteristic of a dimer. The assumed splitting of the monomeric peak to give a doublet at 682 mμ and 648 mμ was attributed to dimers present in the concentrated solution. Brody also studied the fluorescence properties of his concentrated solutions. When the fluorescence bands of the solutions were correlated with the fluorescence bands of various algae at low temperatures he found significant agreement (10).

An NMR experiment on chlorophyll *a* in carbon tetrachloride showed anomalous behaviour, which pointed to the aggregation of the chlorophyll

in the very concentrated solutions used (0.05 M/l). In acetone the spectrum could be readily assigned to the structure of chlorophyll a, and so we were dealing in this case with a monomer. In view of these results, the visible absorption in the blue and red regions of the chlorophyll a spectrum was studied, using different concentrations of chlorophyll a in carbon tetrachloride.

Experimental

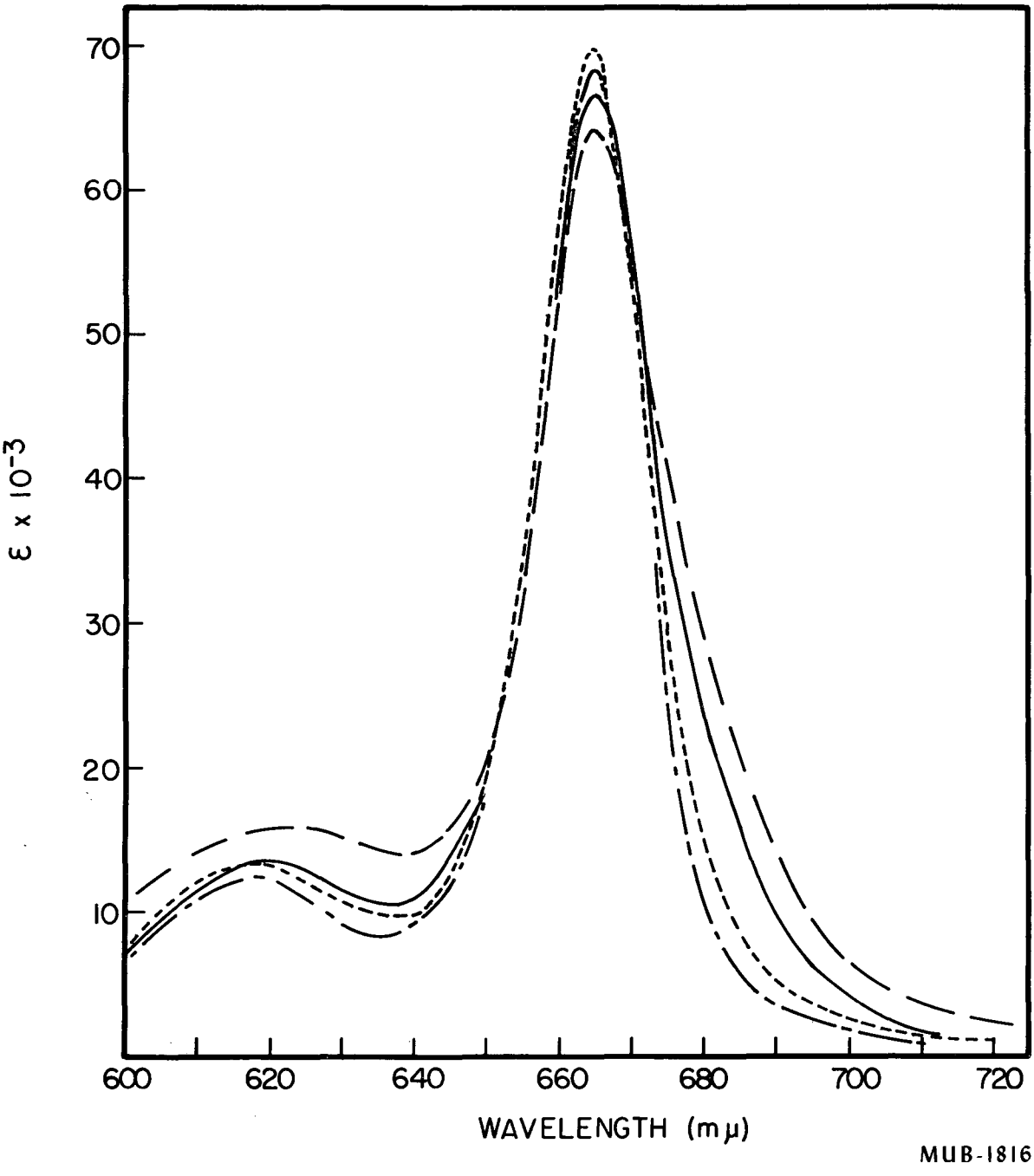
Carbon tetrachloride reagent grade was refluxed over phosphorous pentoxide for 3 hr and then distilled, with the middle fraction taken. Dry crystals of chlorophyll a were weighed out and dissolved in the carbon tetrachloride to give a stock solution of 4.25×10^{-4} M/liter, which was diluted with carbon tetrachloride to give calculated concentrations, giving optical densities of approximately 0.7 when cells of path lengths varying from 2.5×10^{-3} to 1.0 cm were used. The various cell path lengths were obtained by placing spacers in a 1 cm cell, and the spectra were taken on a Cary 14R spectrometer at room temperature.

Results

The extinction coefficients were calculated by the Lambert-Beer relationship, and the extinction coefficients were plotted against the corresponding wavelengths in the regions of the red and blue bands for the different concentrations in Figures 11 and 12 respectively.

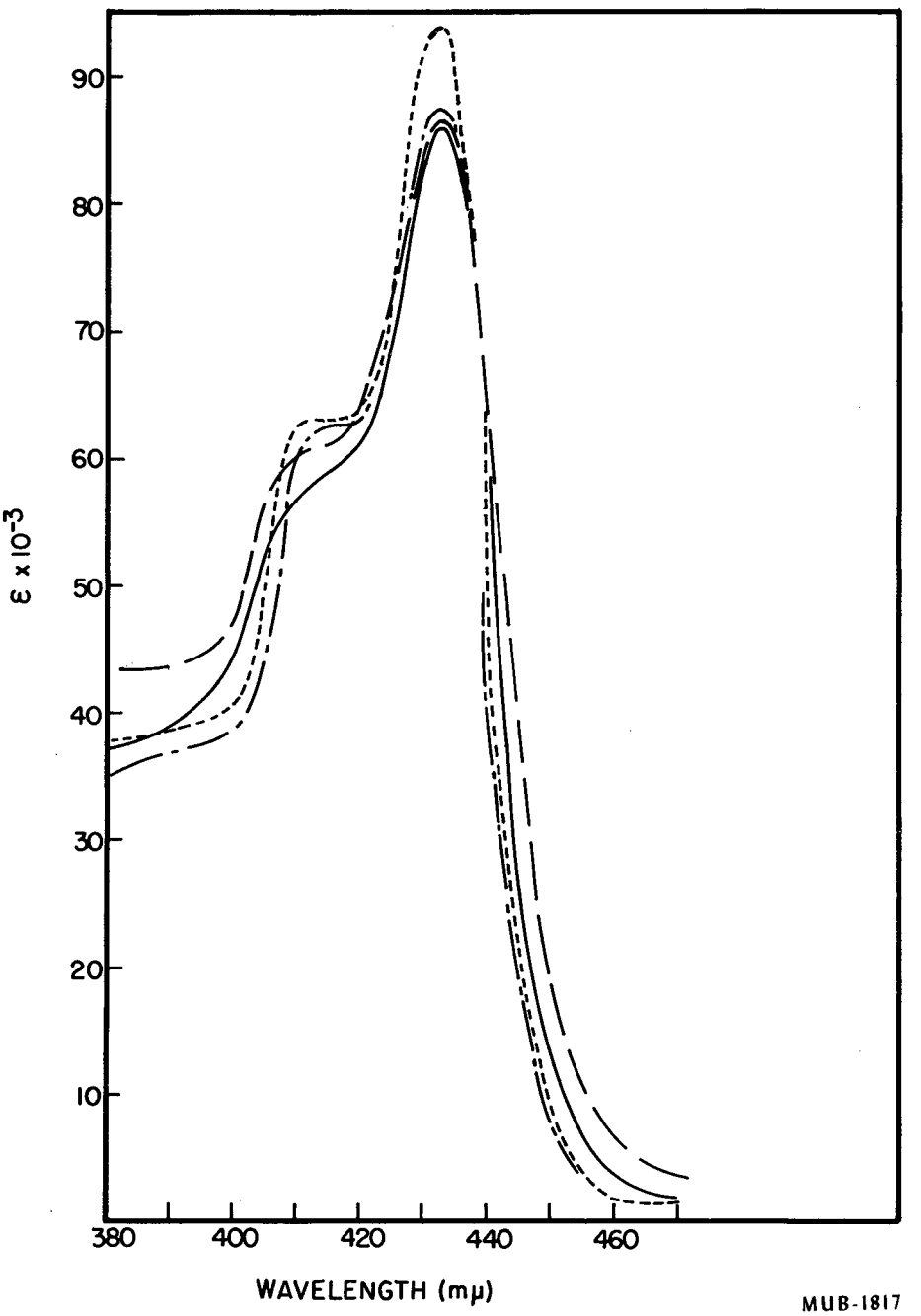
Conclusions

It can be seen from Figures 11 and 12 that the red and blue band shapes are not constant over the range of concentrations of chlorophyll a in carbon tetrachloride studied, and there is an enhanced absorption in the red tails for the bands in the concentrated solutions



— — — — 4.25×10^{-4} M/l of chlorophyll a in carbon tetrachloride
——— 2.15×10^{-4} M/l of chlorophyll a in carbon tetrachloride
- - - - - 4.03×10^{-5} M/l of chlorophyll a in carbon tetrachloride
— - — - 1.01×10^{-5} M/l of chlorophyll a in carbon tetrachloride

Fig. 11. Red absorption band of chlorophyll a in CCl_4 at various concentrations.



– – – – 4.25×10^{-4} M/l of chlorophyll a in carbon tetrachloride
——— 2.15×10^{-4} M/l of chlorophyll a in carbon tetrachloride
- - - - - 4.03×10^{-5} M/l of chlorophyll a in carbon tetrachloride
– – – – 1.01×10^{-5} M/l of chlorophyll a in carbon tetrachloride

Fig. 12. Blue absorption band of chlorophyll a in CCl_4 at various concentrations.

over the bands in the dilute solutions. We are not prepared, on the basis of this experiment or on those of other workers, to ascribe this effect to a rigid dimerisation of the chlorophyll a without further experimentation. Therefore, no attempt was made to derive any thermodynamic constants for the association nor give any geometrical properties of the dimer, as was done by Brody (34) from a theory of McRae and Kasha (35).

We would prefer to ascribe the changes to a type of solvent effect, due to chlorophyll a - chlorophyll a interactions of a non specific nature. In accord with a recent theory of Pople and Longuet-Higgins (36), it is suggested that the excited state of the chlorophyll a molecule experiences London type dispersion interactions due to the close proximity of other chlorophyll a molecules with high polarizability, which bring about a lowering in energy of the excited state, compared with that which the single chlorophyll a molecule would experience when surrounded by carbon tetrachloride molecules. This would result in a lowering of the energy of the electronic transitions or, in other words, the red shifts, which are seen in the spectra. There are, therefore, transitions in the neighborhood of 680 mμ present in the concentrated solutions of chlorophyll a in non polar media.

Because the phenomenon of aggregation has been found in concentrated solutions of chlorophyll a in ethanol (34) and benzene (37), then the occurrence of this property of chlorophyll a is well established. In these systems of concentrated chlorophyll a there are near-infrared absorption characteristics which are similar to those

found in intact cells. For that reason these systems of concentrated chlorophyll *a* should be studied further.

The Optical Properties of Crystalline Chlorophyll *a*

Introduction

The only previous investigation of the optical properties of crystalline chlorophyll *a* was reported by Jacobs *et al.* (21), who showed that their crystals had an optical transition in the near infrared at 735 mμ.

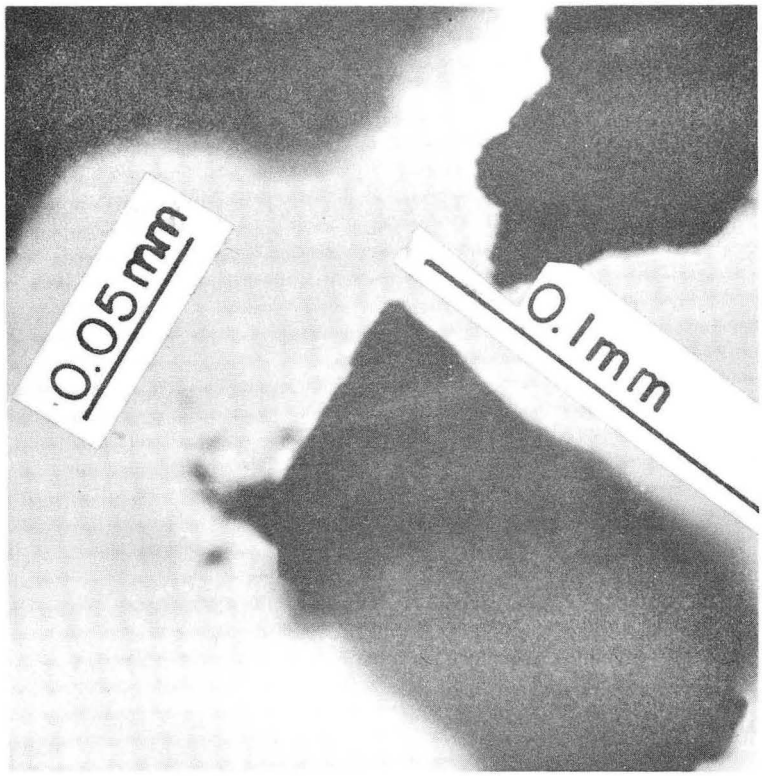
We have now prepared crystals by a method which is in principle the same as that used by these workers, and is the first procedure of preparation which was described in a previous section (38) on the recovery of chlorophyll *a*. This crystal form chlorophyll *a* is designated as chlorophyll *a* (C1). Chlorophyll *a* (C1) is prepared by allowing the n-propanol from the chlorophyll eluate from the sugar column to diffuse into water, and as the n-propanol content of the eluate reaches a low concentration, chlorophyll *a* precipitates out because of its low solubility in isooctane.

We also obtained crystalline chlorophyll *a* from the sugar column eluate without the presence of large quantities of water, and this form of chlorophyll *a* is designated as chlorophyll *a* (C2). Chlorophyll *a* (C2) precipitates spontaneously out of the eluate under conditions described in a previous section. Because chlorophyll *a* (C2) was expected to have more order in its structure, more attention was devoted to this material. Before going on to discuss the optical properties of the crystals, a qualitative discussion of

the crystallinity of chlorophyll a (C1) and chlorophyll a (C2) is given in the following section.

Qualitative Discussion of Chlorophyll a Crystallinity

When an isooctane suspension of chlorophyll a (C2) was observed under a microscope, crystals of chlorophyll a in thin rectangular plates (0.05 mm broad and 0.1 mm long) were seen to be present in a background of smaller crystals (Fig. 13) (39). To convince ourselves thoroughly of the crystallinity of these rather small particles, their X-ray powder diffraction patterns were taken by Dr. W. S. Fyfe (of the Geology Department, University of California), using a Norelco X-ray diffractometer. First of all, the degree of order present in chlorophyll a (C1) was compared with that in chlorophyll a (C2). Suspensions of both these particles in isooctane were smeared on a glass plate and their diffraction pattern taken, giving the spectra in Figures 14 and 15 for chlorophyll a (C1) and chlorophyll a (C2) respectively. From the scattering angle 2θ given by the spectrometer, taking $\lambda = 1.5418$ Å and using the Bragg relationship, $n\lambda = 2d \sin \theta$, the interplanar spacings were obtained and are shown at the various scattering angles on the figures. Chlorophyll a (C1) (Fig. 14) gave poorly defined scatterings from the planes, with spacings of 10.6 - 21 Å and within the planes of the sheets at 4.23 and 6.35 Å. Chlorophyll a (C2), from its spectrum in Figure 15, has a sheetlike structure from the 10.6, 14, and 21 Å spacings, with relatively poor order within the sheets shown by poorly defined spacings at 4.23 and 7.4 Å. Therefore, chlorophyll a (C2) has higher order than chlorophyll a (C1), mainly in the stacking of the sheets, but, as we shall see below, the order in the crystal can be



ZN-3866

Fig. 13. Photograph of typical chlorophyll a crystal.

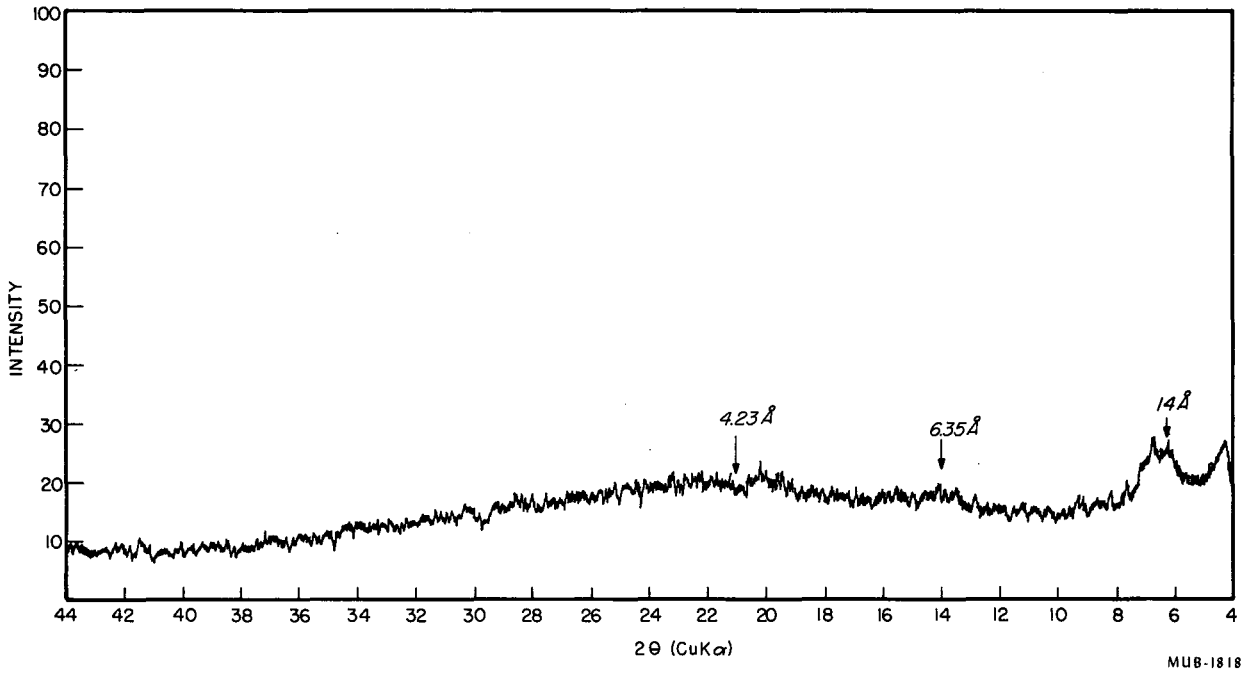


Fig. 14. X-ray powder diffraction spectrum of chlorophyll a (C1).

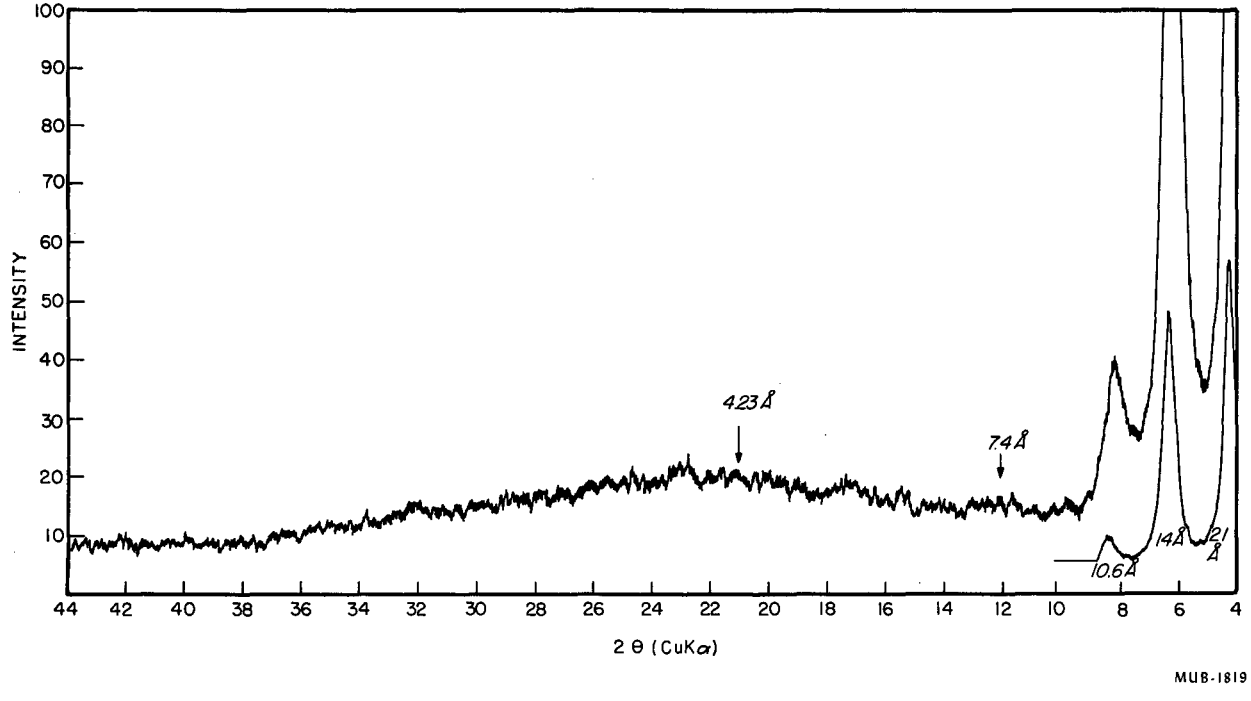


Fig. 15. X-ray powder diffraction spectrum of chlorophyll a (C2).

readily broken down by drying and mechanical treatment.

Drying of the isooctane suspension of chlorophyll a (C2) in a vacuum dessicator gave the spectrum shown in Figure 16, and when the particles, after drying and grinding in an agate mortar, had their diffraction pattern taken, they gave the spectrum in Figure 17. It can be seen from these figures that the order has been partially destroyed, but mainly in three dimensional planes. If it can be assumed that our chlorophyll a (C1) is similar to the preparation of Jacobs et al., then from these simple observations it seems that the chlorophyll a spontaneously crystallized from a column eluate has the highest order yet found. Storing the suspension in isooctane preserves the order. Chlorophyll a (C2) should be the preferred physical state for studying the optical properties and ESR of crystalline chlorophyll a.

Visible and Near Infrared Spectra of Crystalline Chlorophyll a

The visible absorption spectrum of the chlorophyll a (C1) crystals dried in vacuo was first taken by the technique of Shibata, Benson, and Calvin, which uses opal glass close to the cell containing the sample dispersion to correct for scattered light from the particles (40). The spectrum of the dried chlorophyll a (C1) as a Nujol suspension is shown in Figure 18. A distinct red absorption band is observed at 738 mμ, which is close to that obtained by Jacobs et al. (21). From the X-ray observations it is probable that the red peak would be shifted to shorter wavelengths on drying, and therefore the spectrum of the particles suspended in isooctane was taken. A few milligrams of the isooctane suspension of chlorophyll a (C1) were resuspended in

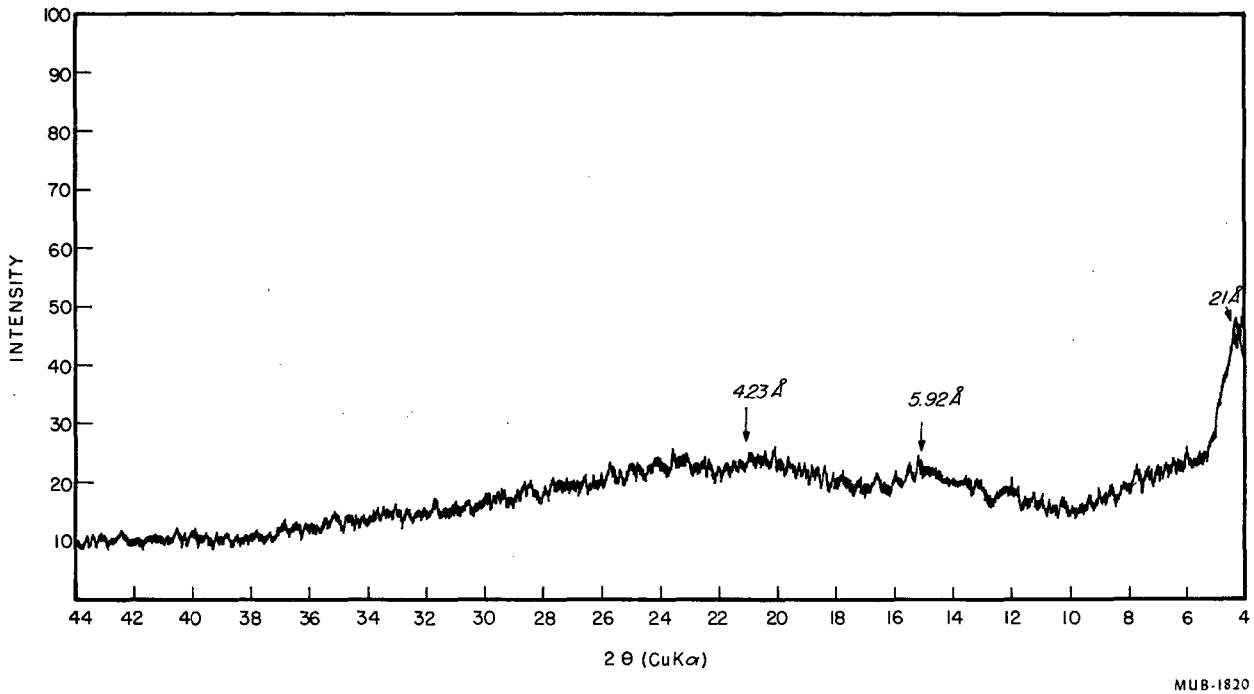


Fig. 16. X-ray powder diffraction spectrum of chlorophyll a (C2) dried under vacuum.

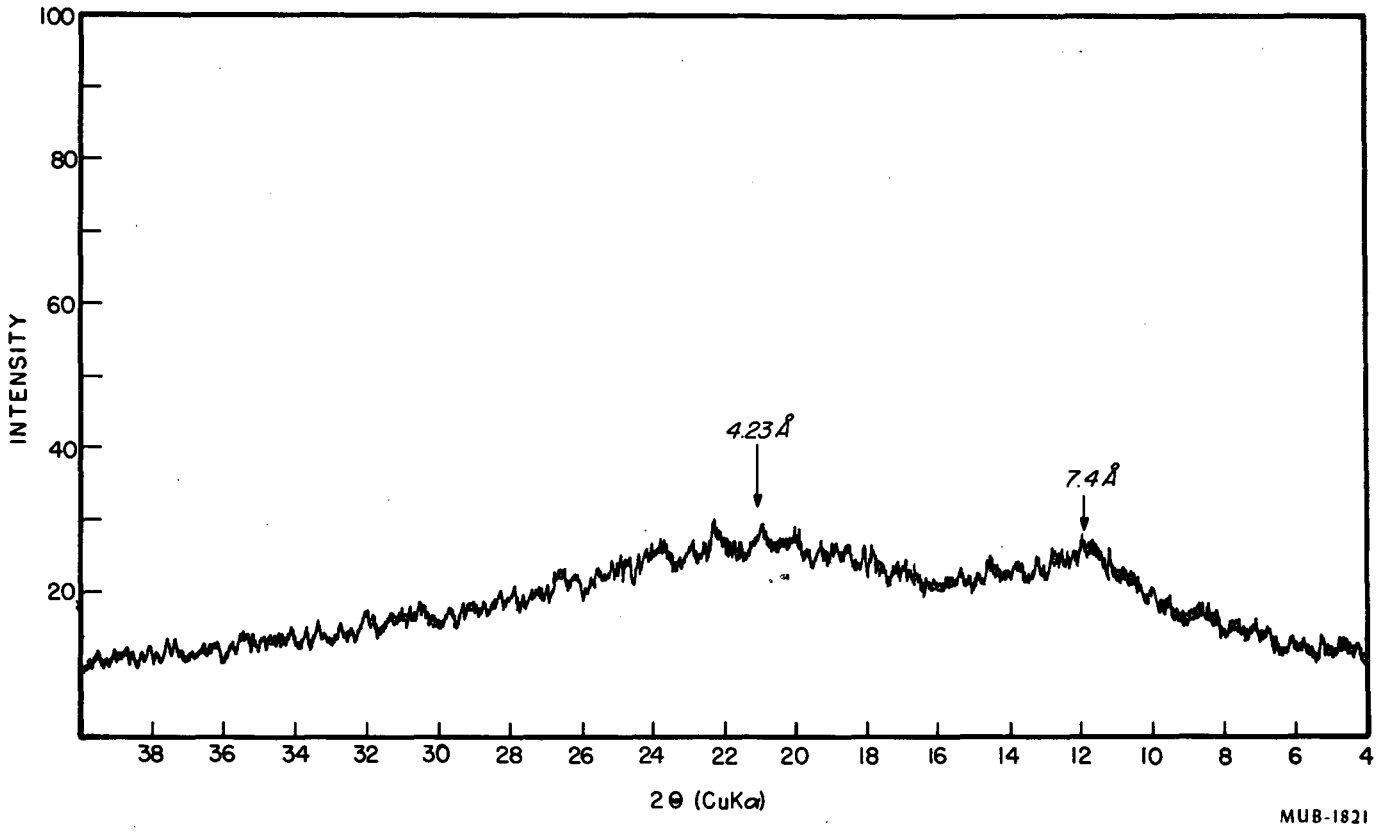


Fig. 17. X-ray powder diffraction spectrum of chlorophyll a (C2) dried in vacuum and ground up.

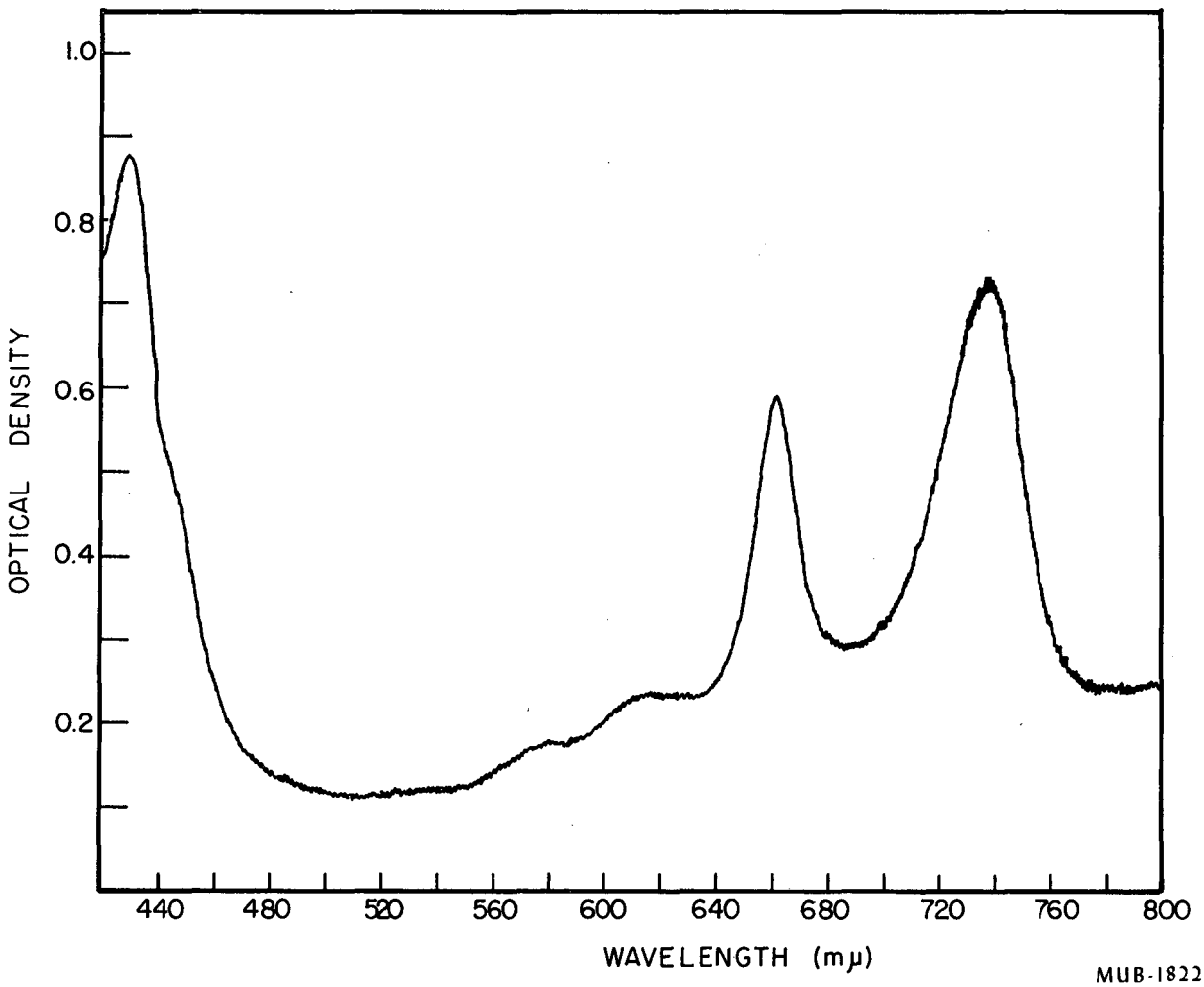


Fig. 18. Visible and near infrared spectrum of chlorophyll a (C1) in Nujol.

isooctane, stirred with a glass rod, and then centrifuged for 20 min at 2,000 rpm. The absorption spectrum of the supernatant isooctane was taken without using any correction for the scattering from the very small crystals of chlorophyll a (C1) present in the isooctane. The red absorption peak of the particles was now found at 744 mμ, which is 6 mμ further to the red than that of the dried particles, indicating that drying also influences, but not to any great extent, the near infrared absorption spectrum of the chlorophyll a crystals.

A few milligrams of chlorophyll a (C2) suspension were resuspended in isooctane, stirred with a glass rod, and then centrifuged for 20 min at 2,000 rpm. The absorption spectrum of the supernatant isooctane which contained the small chlorophyll a particles was then taken at different temperatures, and without making any corrections for the scattering of the small particles present. The resultant spectra are: at room temperature, Figure 19a; after heating to 50° C for 10 min, Figure 19b; and then after allowing the heated solution to sit overnight in a refrigerator, Figure 19c.

The isooctane suspension at room temperature had a red absorption maximum at 748 mμ, which is 4 mμ to longer wavelengths than chlorophyll a (C1), and absorption in the region characteristic of monomeric chlorophyll a at 660 mμ. There was also a shoulder on the 660 mμ peak at 675 mμ which is characteristic of perturbed-monomer transitions of the type discussed for concentrated solutions of chlorophyll a in carbon tetrachloride. On heating the suspension to 50° C, the peak at 748 mμ in the absorption spectrum had decreased in intensity, but still with a maximum in the region of 748 mμ and indicating the

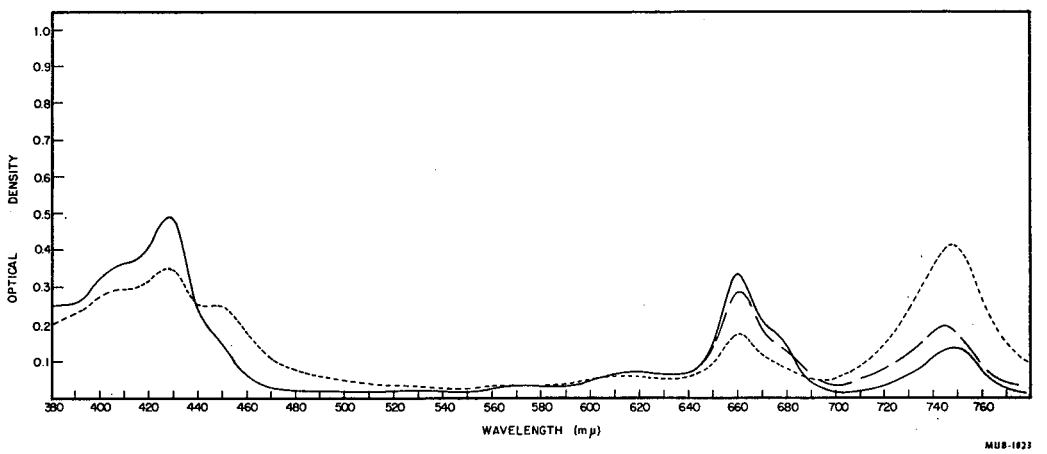


Fig. 19. a, b, and c. Visible and near infrared absorption spectra of chlorophyll a (C2) in isooctane.

a. ----- Absorption spectrum of chlorophyll a (C2) microcrystals in isooctane.
b. ——— Absorption spectrum of chlorophyll a (C2) microcrystals in isooctane after heating.
c. — — — Absorption spectrum of chlorophyll a (C2) microcrystals in isooctane after cooling heated material.

presence of crystals. In the heated isooctane suspension there was now an increased intensity of the 660-675 mμ absorption bands relative to the absorptions arising from the crystallites, indicating that the crystallites had broken down to give monomers, and dimers. On cooling the heated isooctane suspension, a partial recovery of the original spectrum obtained from the starting suspension was obtained. The near infrared absorption peak was now at 745 mμ, and there was a corresponding decrease in the intensities of the absorption bands arising from the monomer and dimers of the chlorophyll a present in the isooctane.

Conclusions

A crystalline form of chlorophyll a (C2) has been obtained which has a red absorption peak at 4 mμ to longer wavelengths than the crystalline chlorophyll a (C1) which is obtained by a water precipitation technique. The effect of heating on chlorophyll a crystals in isooctane suggests that chlorophyll a can exist in a crystalline form, a monomeric form, and a perturbed-monomeric form. Because cooling of the heated suspension caused a small reversibility of the aggregation, care should be taken when interpreting the spectral results from investigations of cells at low temperatures, in which the chlorophylls could be aggregating. This aggregation of chlorophylls present in the cells could lead to artifact absorption bands rather than an intensification of transitions already present in the cellular material.

Chlorophyll a can exist in cells mainly in non-crystalline form, or in very small amounts as the crystalline form; otherwise

absorption of light by cells in the region of 750 mμ would have been observed, and this, indeed, has not unambiguously been found.

The Infrared Spectra of Chlorophyll a and Its Derivatives

Introduction

The infrared spectra of chlorophyll a and its derivatives have been intensively investigated for some time (9,41). Most of the vibrations in the infrared spectrum of chlorophyll a have, therefore, been assigned to all the prominent structural features of the molecule, such as the ring V keto group, the ester groups at C_7 and C_{10}, and the C-H vibrations of the phytol group. There seemed to be little that could be added to the infrared spectroscopy of chlorophyll a. However, in 1955, Holt et al. (42,43), with the reactions of chlorophyll a and its molecular biology in mind, found in the most exhaustive investigation up to that point that the infrared spectrum of chlorophyll a in non-polar solvents (carbon tetrachloride) was different in the region characteristic of the ring V keto group from that of the molecule in polar solvents (ether). The most striking difference between the infrared spectrum in the two classes of solvents was the presence of a vibration (1,650 cm^{-1}) in the carbon tetrachloride solutions, which was attributed by these workers to an enolic form of chlorophyll a. Chlorophyll a was therefore in a tautomeric equilibrium when dissolved in non-polar solvents:

Further, chlorophyll a, it was claimed, could undergo this phenomenon, but its derivatives, chlorophyll b and pheophytin a, could not. The presence of the formyl group in chlorophyll b and the removal of the magnesium from chlorophyll a, with the replacement of this ion with two protons to give pheophytin a, were the factors in the molecular structure which made the effect specific to chlorophyll a. If this anomaly in the infrared spectrum of chlorophyll a could be attributed to the keto-enolisation of the molecule, and was specific to it, then this tautomerism has important consequences in the chemical reactions of the molecule, and perhaps its molecular biology.

The infrared spectroscopy of chlorophyll a can be used as a possible analytical means of following the exchange of the O^{16} isotope on the C_9 keto group with the O^{18} isotope from heavy water, which should give a calculated shift of 40 cm^{-1} to lower frequencies for the C=O vibration. Now, having a new sample of chlorophyll a and some of its derivatives available, a reexamination of the assignments of the vibrations in the infrared spectra of chlorophyll a and its derivatives (Appendix I) was in order. This work, although not novel, was required as a sound basis for a study of the anomaly found in the infrared spectrum of chlorophyll a, and a laboratory catalogue of the infrared spectra of chlorophylls would be built up which would be of value for future studies of chlorophyll a chemistry. Interpretation of molecular spectra at frequencies below 1,600 cm^{-1} is not possible because of the complexity of the molecular structures involved; nevertheless, the region below 1,600 cm^{-1}, called "the fingerprint region", is valuable for identification purposes.

An emphasis was placed on the study of the infrared spectrum of chlorophyll a in various solvents, in different solid forms, and at various concentrations in a non-polar solvent (carbon tetrachloride), because Holt's claim that chlorophyll a existed in a keto-enol equilibrium in non-polar solvents (carbon tetrachloride) is certainly probable. However, the infrared measurements require concentrations of chlorophylls (1×10^{-2} M/l) in carbon tetrachloride which are greater by a factor of 400 in concentration over the concentrations (4×10^{-4} M/l) which we used in the visible spectroscopy. As one spectroscopic investigation of the visible absorption region of carbon tetrachloride chlorophyll solutions showed deviations from ideality which were thought to arise from intermolecular perturbations between chlorophylls, it would be reasonable to suppose that the anomalous features of the infrared spectra of chlorophyll a in non-polar solvents were due to the same interaction and give a more detailed insight into the association.

Experimental

Chlorophyll a and chlorophyll b were obtained by previously described techniques and were dried for 24 hr in a vacuum dessicator before the solutions were made up. The mixture of pheophytins a and b were prepared by adding a few drops of concentrated hydrochloric acid to a solution of chlorophyll a and b in acetone water. The chlorophylls had been separated from the other pigments on a polyethylene column before treatment with acid. The pheophytins formed in the acetone water solvent were then transferred to a small quantity of isooctane, which was removed under vacuum to give the

solid pheophytins. Methyl-chlorophyllide a was obtained by the previously described procedure, and methyl pheophorbide a was given to us by Professor R. B. Woodward and Dr. A. Stoll. Solutions were made up with reagent grade solvents except the carbon tetrachloride, which was dried and distilled from phosphorous pentoxide. The infrared spectra were taken on a Beckman IR7 spectrometer.

Assignments of Vibrations in the 4,000 cm^{-1} to 1,600 cm^{-1} Region of the Infrared Spectrum of Chlorophyll a and Its Derivatives

The infrared bands in chlorophyll a and its derivatives were assigned from the correlations between molecular structure and vibration frequencies to be found in the monographs of Bellamy (44) and Brugel (45) and guided by the results of Holt (42), who studied derivatives which were not available to us. In this section the possible hydroxyl stretching frequencies (in the region of 3,400 cm^{-1}) will not be discussed and the anomaly which exists in the carbonyl stretching region (1,700-1,640 cm^{-1}) noted, leaving these details for a fuller discussion in the following section, which treats the anomaly in more detail. The representative reproductions of the spectra from chlorophyll a are as follows: chlorophyll a in carbon tetrachloride, Figure 20; chlorophyll a crystals (C2) (smeared on a silver chloride plate as a thick isooctane suspension and then allowing the isooctane to evaporate off), Figure 21; chlorophyll a crystals (C1) in potassium bromide, Figure 22; and chlorophyll a in 1% pyridine:carbon tetrachloride, Figure 23. The most striking high frequency vibrations, common to all of the spectra, are those at 2,960, 2,930, and 2,870 cm^{-1}; these were best resolved in carbon tetrachloride and are clearly C-H stretching vibrations originating from the large number of C-H bonds present in the phytol chain. This assignment was confirmed by the

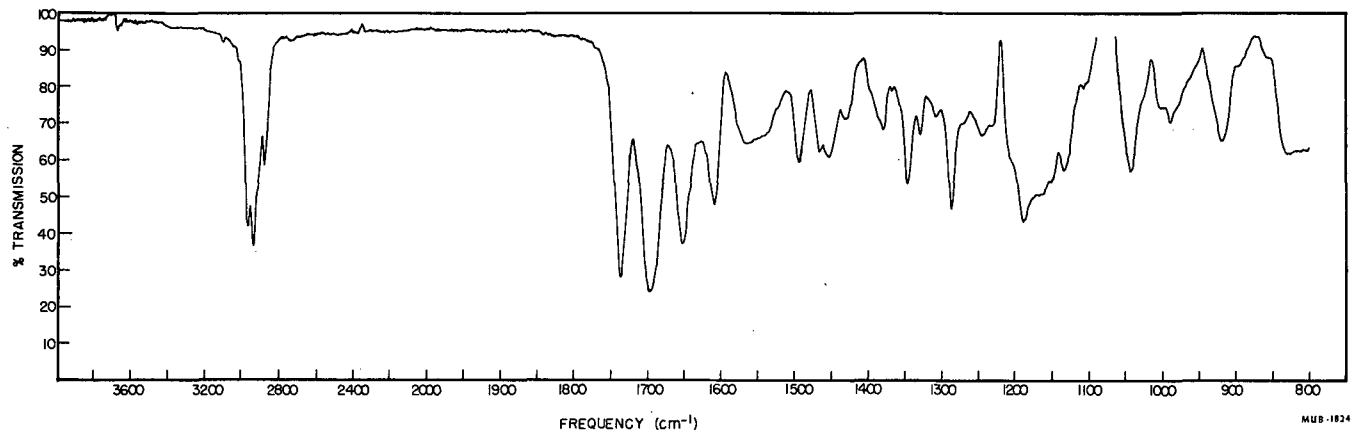


Fig. 20. Infrared spectrum of chlorophyll a in carbon tetrachloride.

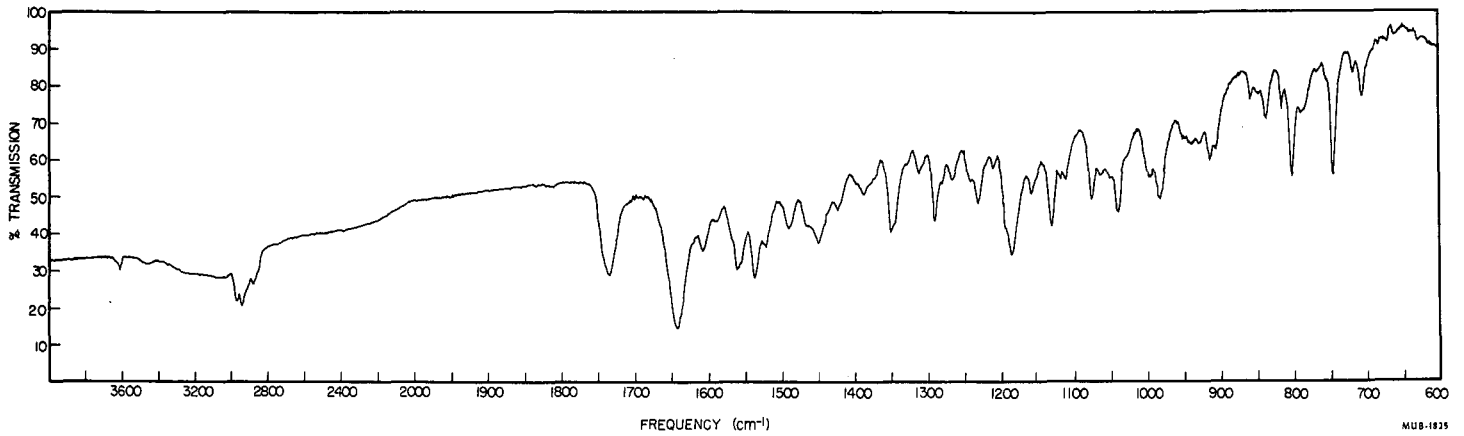


Fig. 21. Infrared spectrum of chlorophyll a crystals (C2) smeared on a silver chloride plate.

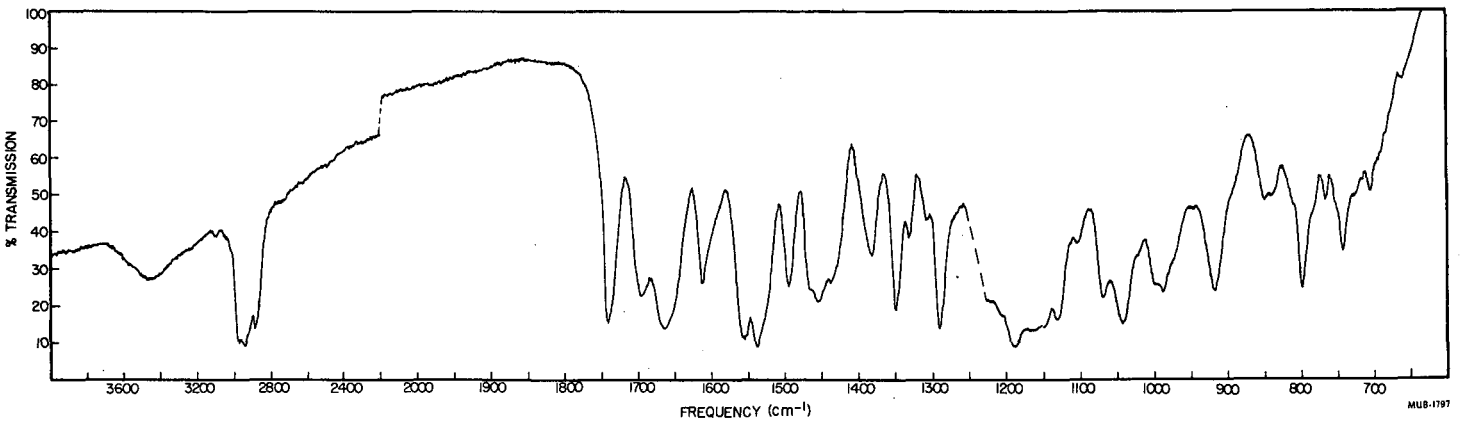


Fig. 22. Infrared spectrum of chlorophyll a crystals (C1) in potassium bromide disk.

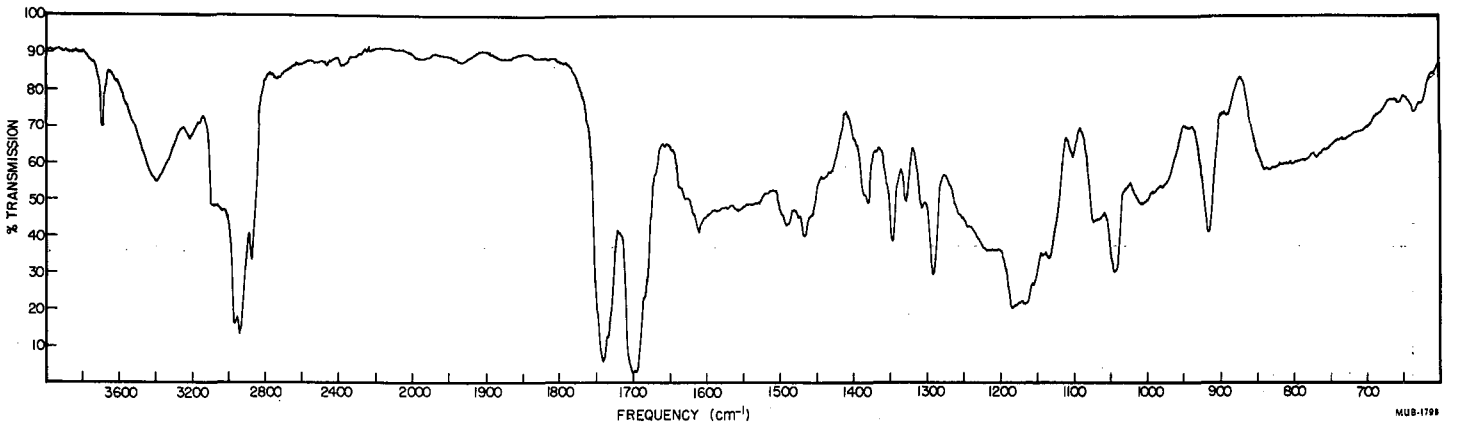


Fig. 23. Infrared spectrum of chlorophyll a in 1% pyridine carbon tetrachloride.

lower intensities observed in the methyl-pheophorbide a spectrum (Fig. 26) and methyl chlorophyllide a (Fig. 28) spectra. It is not worthwhile to try to assign the particular C-H vibrations originating from the phytol chain, as it is not a relevant part of the molecule--i.e., as far as its photochemistry is concerned.

The next vibration present in all of the spectra is in the region of 1,740 cm^{-1} and, according to Bellamy (44), normal saturated esters, of which there are two representatives in the chlorophyll a molecule at C_7 and C_{10}, have C=O stretching vibrations from 1,735 to 1,750 cm^{-1}. It appears that in chlorophyll a the ester groups vibrate at fairly similar frequencies and in general cannot be resolved, but it is pointed out that in weakly acidic solvents which can hydrogen bond to these ester groups the band is split--in methanol (Fig. 29F) two vibrations at 1,735 and 1,715 cm^{-1} are distinct, and in chloroform the vibration at 1,730 cm^{-1} has a shoulder at 1,715 cm^{-1} (Fig. 29E). Therefore, the vibration which occurs between 1,735 and 1,740 cm^{-1} has been assigned to the C_1 and C_{10} ester groups, with the exact position of the band showing a small solvent shift.

The next vibration is in the region of 1,700 cm^{-1}, and its intensity depends on the physical state of the chlorophyll a, when the infrared spectrum is taken on the crystals, and on the nature of the solvent. Chlorophyll a in carbon tetrachloride and potassium bromide show only one band at 1,642 cm^{-1} with the disappearance of the 1,700 cm^{-1} vibration, whereas chlorophyll a in pyridine (Fig. 23) shows one vibration at 1,700 cm^{-1} and the vibration at 1,655 cm^{-1} has disappeared. These are the only striking and unequivocal changes in

the chlorophyll *a* spectrum when its environment is changed, and it is clear that the 1,700 cm^{-1} and 1,655 cm^{-1} vibrations are interdependent, with the 1,700 cm^{-1} vibration being the basic vibration and subject to shifts to lower frequencies when the molecule's environment is changed. Keto-groups have vibrations in the range 1,725-1,690 cm^{-1}, with shifts to lower frequencies when they are conjugated with C=C bonds or if they are present in ring systems. The vibration at 1,700 cm^{-1} is assigned to the C_9 keto group, and the 1,655 cm^{-1} vibration associated with it discussed in the next section.

The remaining vibration in the chlorophyll *a* spectrum which can be assigned is at 1,610 cm^{-1}, which does not undergo solvent shifts and, unfortunately, is on the edge of some vibrations due to the solvent, and therefore changes in the intensity of this vibration with changes in solvent are not easily discerned. Having assigned all the keto groups present and noting that the only other type of bonds present in the molecule which could give rise to the vibration at 1,660 cm^{-1} would be a vinyl group and a >C=C< present in an aromatic system, Holt (43) showed that bacteriochlorophyll which does not have a vinyl group in its structure still has the 1,610 cm^{-1} vibration in its spectrum, and therefore it must arise from the vibration of an aromatic >C=C< bond. Falk *et al.* (46) have shown that in molecules where the cyclopentanone ring is broken--e.g., chloroporphyrin-e_6--the band at 1,610 cm^{-1} is not noticeable in the infrared spectrum. This suggests that it is close to the ring V of the molecule, and therefore the 1,610 cm^{-1} vibration is assigned to the carbon-carbon double bond in ring IV, adjacent and partially conjugated with the ring V keto-group. If this vibration is due to the group assigned to it,

some variation in its intensity is expected when its milieu is changed because of its partial interaction with the C_9 keto group. This solvent effect seems to occur, but this was hard to confirm because many of the solvents have absorptions close to the 1,610 cm^{-1} vibration.

The assignments for the spectrum of chlorophyll b in carbon tetrachloride, shown in Figure 24, must essentially be the same as those given for chlorophyll a, but the vibrations arising from a $C\overset{H}{\underset{O}{}}$ group must now be present. The assigrments are, therefore: the group of vibrations from 2,960-2,870 cm^{-1} to the C-H's of the phytyl group and the alkyl groups on the chlorin; the vibration at 1,740 cm^{-1} to the ester groups at C_7 and C_{10}; the vibration at 1,705 cm^{-1} to the C_9 keto group; and the vibration at 1,610 cm^{-1} to the carbon-carbon double bond in ring IV and adjacent to the C_9 keto group.

In the chlorophyll b spectrum there is a new, weak but distinct vibration at 2,740 cm^{-1}, which is just outside the range 2,900-2,700 cm^{-1} quoted by Bellamy (44) for the C-H vibration of a formyl group. The vibration present at 2,740 cm^{-1} must, however, be due to the C-H in the formyl group at C_3 in the chlorin nucleus. There is also a new vibration at 1,670 cm^{-1} which only changes its shape when the chlorophyll b spectrum is taken in various solvents, and again, according to Bellamy, the formyl group in saturated aldehydes have C=O vibrations in the region 1,680-1,660 cm^{-1}. Therefore, the vibration at 1,688 cm^{-1} is assigned to the C=O vibration of the formyl group at C_3 of the chlorophyll b molecule. The presence of both these vibrations at 1,670 cm^{-1} and 2,740 cm^{-1} help to confirm the assignments of each one taken alone.

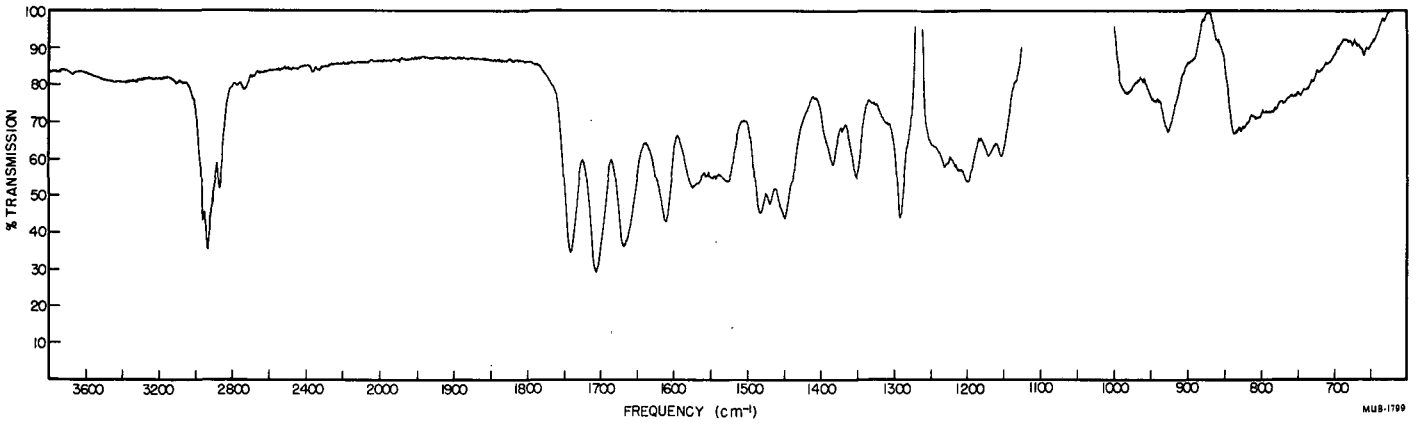


Fig. 24. Infrared spectrum of chlorophyll b in carbon tetrachloride.

In the spectrum of a mixture of pheophytins a and b, shown in Figure 25, all the vibrations found for chlorophyll a and b should be present, but now a vibration characteristic of two N-H groups which are present in the pheophytins should also be present. There is a vibration at 3,400 cm^{-1} which is assigned to the N-H vibrations. It should be noted that another difference in the infrared spectrum of pheophytins when compared with that of the chlorophylls dissolved in carbon tetrachloride is the presence of a sharp vibration at 1,650 cm^{-1} arising from pheophytin b. The removal of the solvent dependent band in magnesium-free derivatives is shown conclusively by studies on the methyl pheophorbides.

In the spectrum of methyl pheophorbide a, shown in Figures 26 and 27, the N-H vibrations occur at 3,400 cm^{-1}, and a decreased intensity of the C-H vibrations (2,960-2,860 cm^{-1}) is noticeable, which is expected when the phytyl group is replaced by a methyl group. It is also clear that the spectrum of methyl pheophorbide a in carbon tetrachloride and chloroform are almost identical in the 1,800-1,550 cm^{-1} region, indicating that this molecule was not behaving in the same way as chlorophyll a.

Methyl chlorophyllide a, whose spectrum (Fig. 28) could not be measured readily in a non polar solvent, did show the same spectral characteristics as chlorophyll a except for the decreased intensities of the C-H vibrations. We remark that this is all that is required to show that our preparation of methyl chlorophyllide a was sound.

A tabulation of the assignments given to the vibrations of chlorophyll and its derivatives are given in Table III.

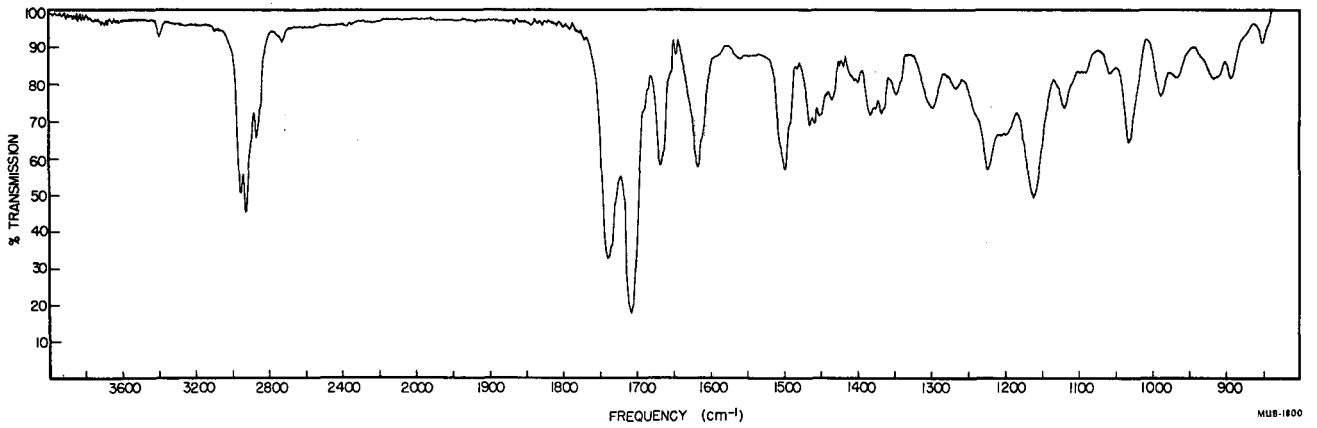


Fig. 25. Infrared spectrum of a mixture of pheophytins a and b in carbon tetrachloride.

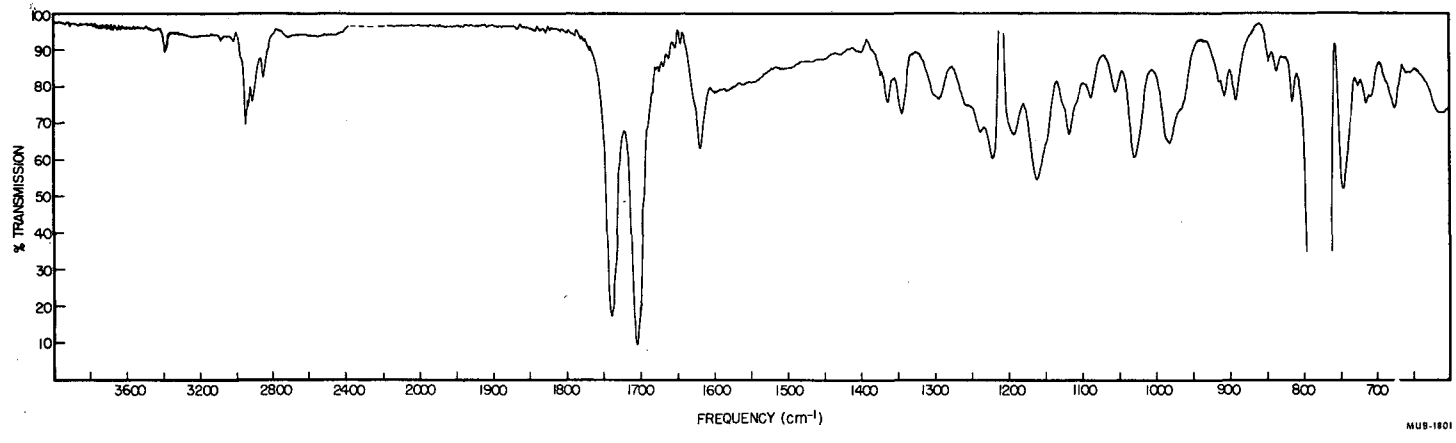


Fig. 26. Infrared spectrum of methyl pheophorbide a in carbon disulfide.

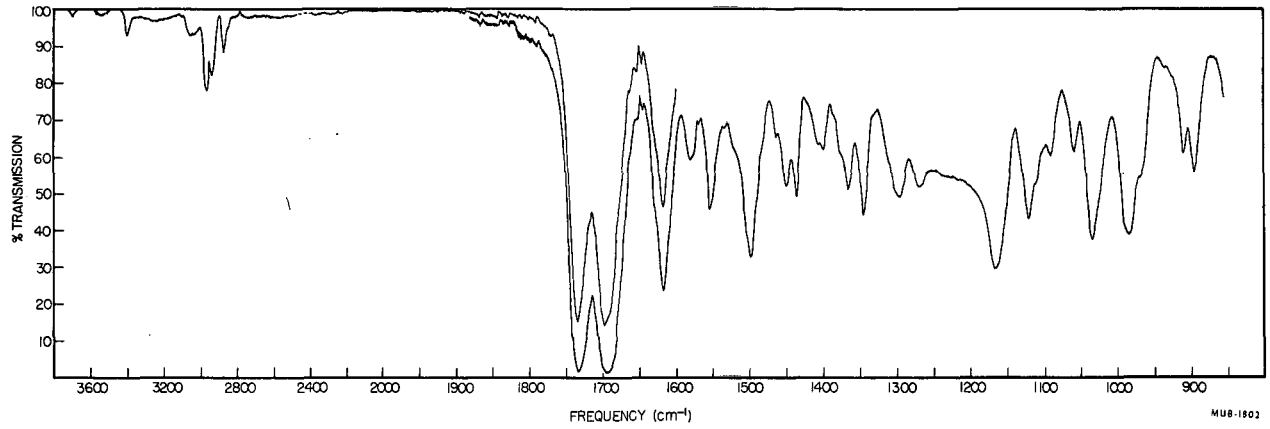


Fig. 27. Infrared spectrum of methyl pheophorbide a in chloroform.

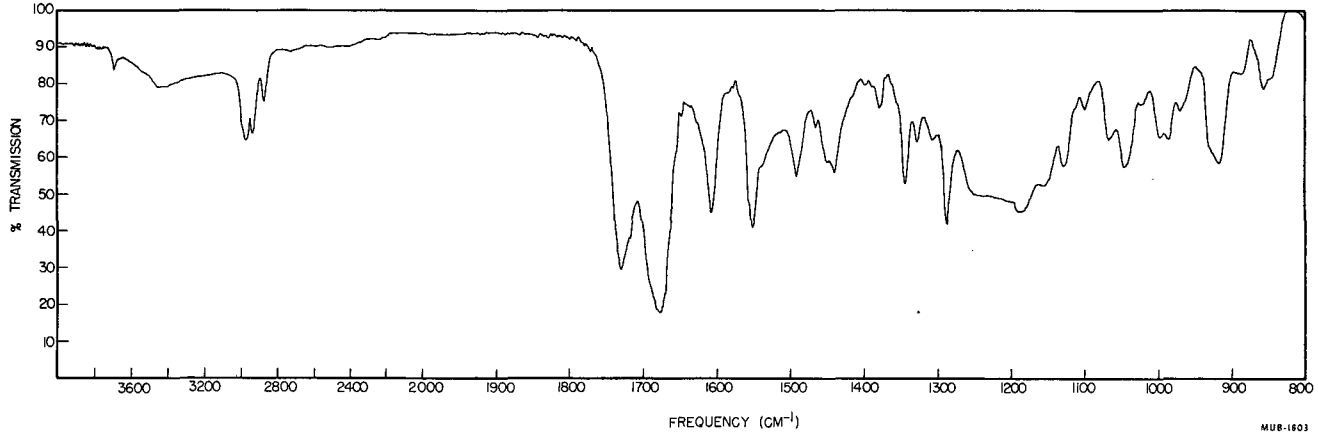


Fig. 28. Infrared spectrum of methyl chlorophyllide a in chloroform.

A. Chlorophyll a (C2) on silver chloride
B. Chlorophyll a in KBr
C. Carbon disulfide
D. Benzene
E. Chloroform
F. Methanol
G. Ether
H. Pyridine

TABLE III

Assignments of the Vibrations in the Infrared Spectra of Chlorophyll and Its Derivatives

(Frequencies in cm^{-1})

Molecule	Solvent	NH	C-H	Formyl C-H	Ester C_7 and C_9 >C=O	Keto C_9 C=O	Aggreg. Band	Formyl C=O	>C=C<
Chlorophyll a (C2)	Crystals on AgCl	-	2870 2970	-	1735	-	1642	-	1608
Chlorophyll a (C1)	Crystals in KBr	-	2880 2980	-	1740	1695	1665	-	1610
Chlorophyll a	Carbon Tetrachloride	-	2870 2960	-	1735	1695	1650	-	1610
Chlorophyll a	Carbon Disulphide	-	2860 2970	-	1736	1695	1650	-	-
Chlorophyll a	Benzene	-	2860 2970	-	1735	1694	1650	-	1610
Chlorophyll a	Chloroform	-	2860 2960	-	1730 1715	1678	Shoulder on 1678 (?)	-	1610
Chlorophyll a	1% Pyridine in Carbon Tetrach.	-	2975 -2970	-	1740	1700	-	-	1610
Chlorophyll a	Ethyl Ether	-	-	-	1738	1708	-	-	1608
Chlorophyll a	Methanol	-	-	-	1735 1715	1665	(?)	-	-
Chlorophyll a	0.5% MeOH in CCl_4	-	-	-	1735	1695	1650	-	1610

TABLE III (Cont'd.)

Molecule	Solvent	NH	C-H	Formyl C-H	Ester C_7 and C_9 >C=O	Keto C_9 C=O	Aggreg. Band	Formyl C=O	>C=C<
Chlorophyll b	Crystals in KBr	-	2870 2980	2740	1740	1705	Shoulder on 1670	1670	1610
Chlorophyll b	Carbon Tetrachloride	-	2870 2960	2740	1740	1705	Shoulder on 1668	1668	1610
Chlorophyll b	Ethyl Ether	-	-	-	1743	1710	-	1670	1610
Chlorophyll b	Chloroform	-	-	-	1725 1732	1695	Shoulder on 1655	1662	1610
Pheophytins a & b	Carbon Tetrachloride	3400	2860 -2960	2720	1740	1705	-	1668	1615
Methyl Pheophorbide a	Carbon Tetrachloride	3400	2860 -2960	-	1740	1705	-	-	1620
Methyl Pheophorbide a	Chloroform	3400	2870 -2960	-	1735	1696	-	-	1610

The Infrared Spectrum of Chlorophyll a in Various Solvents, in Different Solid Forms, and at Various Concentrations in a Non-polar Solvent

The purpose of this study was to investigate the nature of the phenomenon leading to the presence of a vibration at 1,650 cm^{-1} which appeared in the chlorophyll a spectrum when the molecule was dissolved in non-polar solvents but was absent in the spectrum when taken in polar solvents.

In Figure 29 is shown the infrared spectrum of chlorophyll a in various solvents in the region 1,800-1,550 cm^{-1}, and it can be seen immediately that the spectrum of chlorophyll a in this region varies markedly with the state of aggregation of the chlorophyll a and the nature of the solvents. In the spectrum of crystalline chlorophyll a (C2) on a silver chloride plate the vibration at 1,700 cm^{-1} is completely absent and has presumably shifted to 1,642 cm^{-1}, whereas the poorer crystalline form of chlorophyll a (C1) in potassium bromide, which in itself could lead to a breakdown of the order of the crystal, does not have a vibration at 1,642 cm^{-1} but has been shifted to 1,665 cm^{-1} with a vibration at 1,695 cm^{-1} plainly present. It has already been shown that the spectrum of chlorophyll a in carbon tetrachloride (Fig. 20), and also in the two other non-polar solvents, carbon disulfide and benzene, that there are vibrations at 1,695 cm^{-1} and 1,650 cm^{-1}. The intensity of the 1,650 cm^{-1} vibration in the benzene solution of chlorophyll has been reduced. When the chlorophyll a is dissolved in weakly acid solvents, hydrogen bonding to the keto groups in the chlorophyll a molecule results in pronounced solvent shifts of the keto group

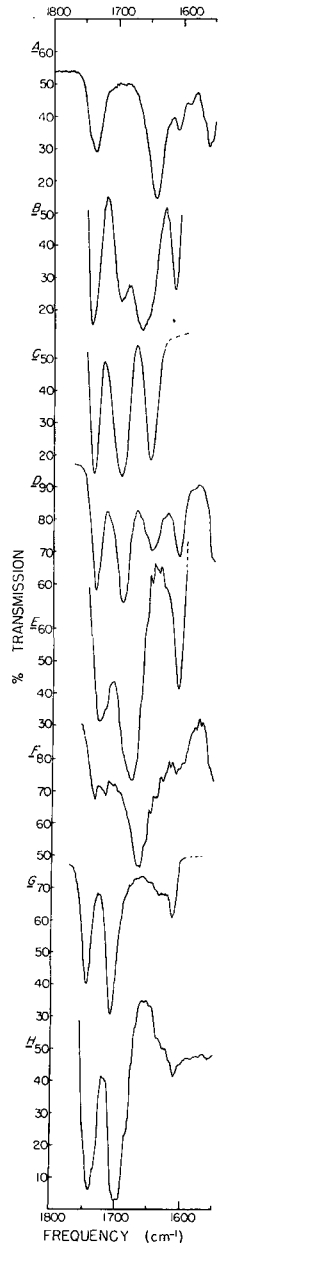


Fig. 29. The 1,650-1,800 cm^{-1} region of the infrared spectrum of chlorophyll a dissolved in various solvents.

vibrations. The chloroform hydrogen bonding interaction partially splits the vibration in the region of 1,740 cm^{-1}, which has been ascribed to the two ester groups at C_7 and C_{10}, to give a band at 1,730 cm^{-1} with a shoulder at 1,715 cm^{-1}, and in methanol the effect is more pronounced, resulting in two distinct vibrations at 1,735 cm^{-1} and 1,715 cm^{-1}. Between these vibrations and the vibration at 1,610 cm^{-1} there is a broad intense vibration at 1,678 cm^{-1} in chloroform and 1,665 cm^{-1} in methanol, which is ascribed to the ring V keto group undergoing a variety of hydrogen bonding effects with the solvent, and perhaps the 1,650 cm^{-1} vibration lying underneath. In weakly basic solvents and non-hydrogen bonding molecules (as far as the chlorophyll *a* molecule is concerned) there is only one sharp vibration, at 1,708 cm^{-1} in the ethyl ether and 1,700 cm^{-1} in the pyridine, between the 1,740 cm^{-1} and the 1,610 cm^{-1} vibrations also present in the spectrum. In these solvents, therefore, the interaction which leads to the presence of a vibration in the region of 1,650 cm^{-1} has been removed. The strange behaviour of chlorophyll *a* in various states of aggregation and in various solvents has now been demonstrated. Consideration shall now be given to what happens to a chlorophyll *a* derivative's infrared spectra where a formyl group has replaced a methyl group at C_3 (chlorophyll *b*), or there has been removal of the magnesium atom from the center of the ring and introduction of two protons (methyl pheophorbide *a*). Both of these replacements could alter the electronic properties of the molecules and prevent keto-enolisation, as was suggested by Holt (43).

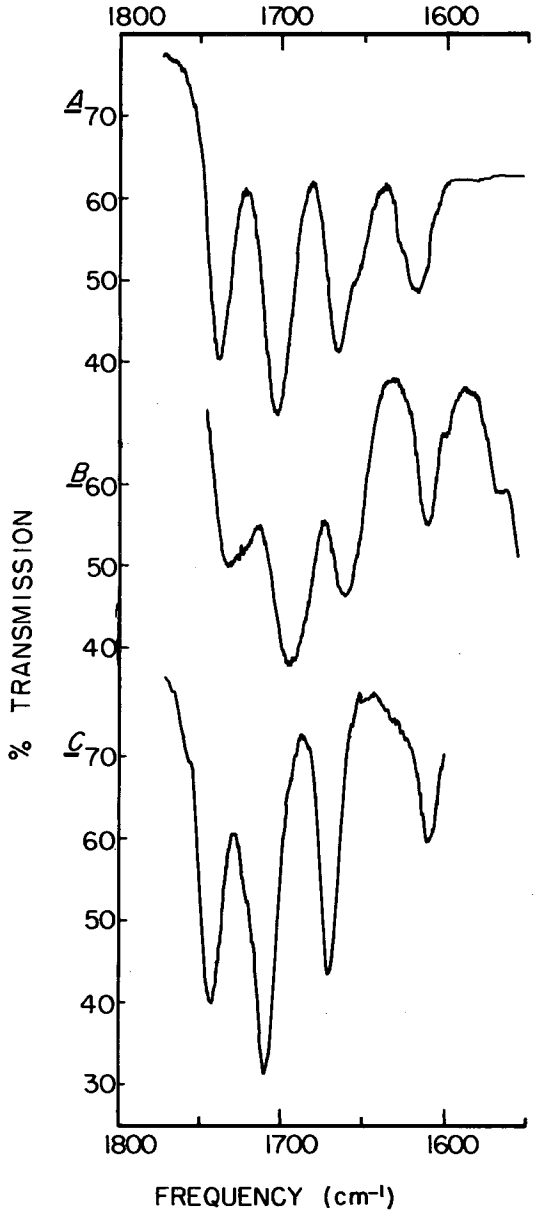
In chlorophyll *b* the C=O vibration of the aldehyde group obscures

the region of interest, but it can be seen in Figure 30, where the spectrum of chlorophyll b in the three classes of solvents has been given, that the chlorophyll b spectrum is similar to chlorophyll a in carbon disulfide. However, the C=O formyl vibration at 1,655 cm^{-1}, which is mainly due to the C=O vibration of the formyl group, also has a pronounced shoulder in carbon disulfide which is not present in the chlorophyll b spectrum in ethyl ether. This assymetry of the 1,655 cm^{-1} vibration of chlorophyll b in carbon disulfide is ascribed to an underlying vibration similar to that found in chlorophyll a solutions in non-polar solvents and indicates therefore that chlorophyll b shows the same solvent interaction behaviour as chlorophyll a. This is in contradiction to the work of Holt (43).

The methyl-pheophorbide a's spectrum (Figs. 26 and 27), as has already been pointed out, is identical in a polar and a non-polar solvent, and therefore the presence of the magnesium atom is in fact a factor in the production of the vibration at 1,650 cm^{-1} in solutions of chlorophyll derivatives.

If keto-enolisation was present in the non-polar solvents, then it could conceivably be broken down in the polar solvents, and we must therefore not only look at the keto region for evidence of the effect, but also in the hydroxyl stretching region, where the polar solvents presumably would show some effect.

Unfortunately, even in carefully dried chlorophyll a water is likely to be present due to bonding with chlorophyll a. The majority of the chlorophyll a spectra show vibrations of small intensity, presumably due to the presence of hydroxyl bonds, but they cannot be

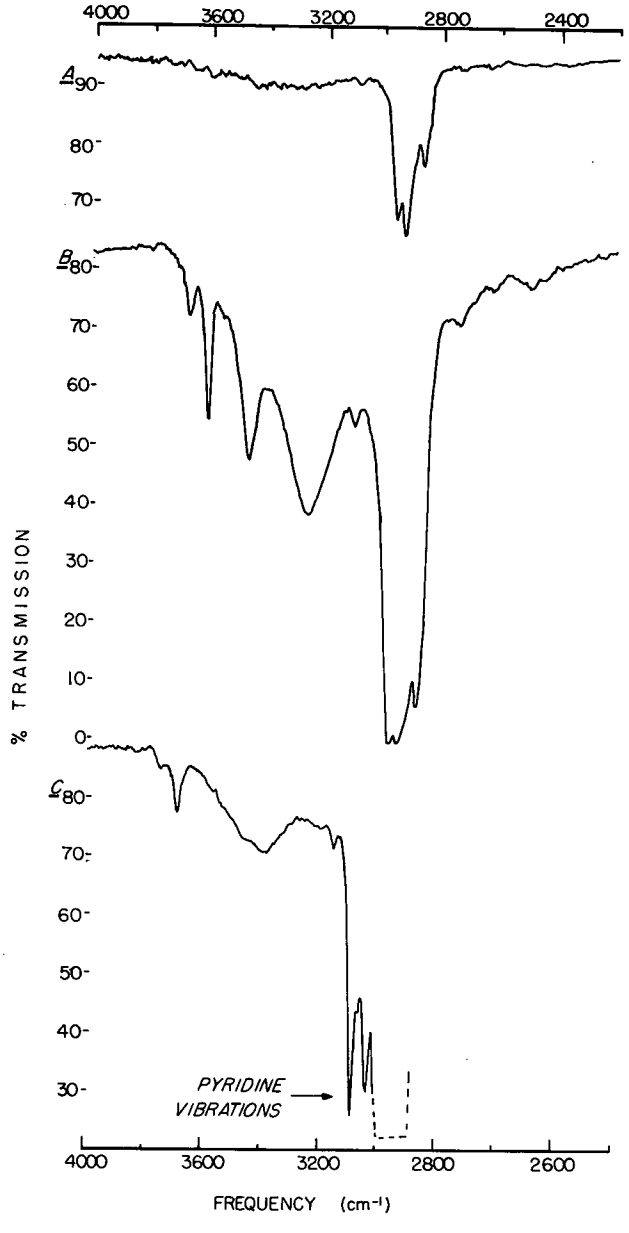


A. Carbon disulfide
B. Chloroform
C. Diethyl ether

Fig. 30. The 1,650-1,800 cm^{-1} region of the infrared spectrum of chlorophyll b dissolved in various solvents.

assigned readily and certainly not to an intramolecular hydrogen bond. The infrared spectrum of chlorophyll a (Fig. 31), dried for 24 hr under a vacuum of less than 5 microns, was obtained in carbon tetrachloride dried over phosphorus pentoxide using a long path-length to accentuate the vibrations in this region. This spectrum shows several vibrations which are tentatively ascribed to free water or water loosely bound to chlorophyll a (47). When the spectrum of the same sample is taken in 0.2% pyridine-carbon tetrachloride (Fig. 31), these vibrations are removed and a broad band at 3,400 cm^{-1} is obtained, which is due to water bound to pyridine (47). One further fact is that the vibration at 1,740 cm^{-1}, due to the ester groups, splits on strong association with an hydroxyl group, as shown by the spectrum, when taken in methanol. It is seen from the spectra in non-polar solvents that this vibration is unsplit, which it should be if an enol form was present. There would seem to be a very broad and weak band which underlies the C-H regions, but, because of the limited range of solution strengths which are accessible, this region could not be investigated more thoroughly. It would seem, therefore, that traces of water are causing the vibrations found in this region, and the investigation was concentrated on the carbonyl region, to study the anomalous effect.

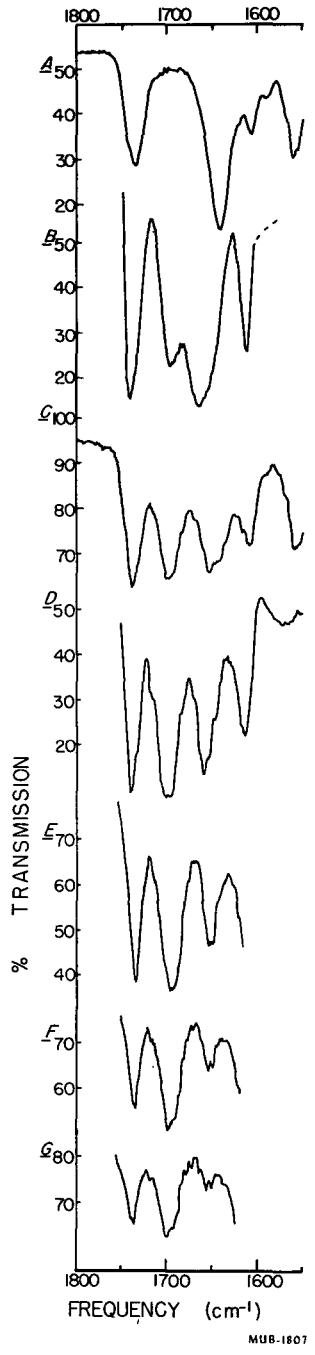
If chlorophyll a was undergoing a keto-enol tautomerisation in non-polar solvents, and the presence of the vibrations at 1,700 cm^{-1} and 1,600 cm^{-1} were evidence for the presence of both forms, then it is expected that relative intensities of these vibrations would change as the concentration of the chlorophyll a was varied in a non-polar



A. Chlorophyll a 2.24×10^{-2} M/l in CCl_4, path length 0.1 mm
B. As in "A", but with path length 1 mm
C. 2.24×10^{-2} M/l in 0.2% pyridine-CCl_4, path length 1 mm.

Fig. 31. The '-OH' region of chlorophyll a in various solvents.

solvent. The effect of changing the chlorophyll a concentration on the intensities of the vibrations between 1,800-1,550 cm^{-1} is shown in Figure 32, and it can be seen that on going from high to low concentrations the intensity of the 1,650 cm^{-1} band decreases, whereas the intensity of the 1,700 cm^{-1} band increases with respect to the 1,740 cm^{-1} vibration. This demonstrates that there is some intermolecular association between the chlorophyll a molecules which leads to a shift of 50 cm^{-1} to lower frequencies for the C=O vibration of the ring V keto group, while the ester group vibrations remain unaltered. The shape of the 1,650 cm^{-1} vibration also undergoes changes on dilution, pointing to several steric interactions with the keto group, with no specific interaction being present. It has already been shown by the study of the methyl pheophorbides that the presence of magnesium in the center of the molecule is necessary for this association in non-polar solvents. What there must be, then, in non-polar solutions of chlorophylls a and b is a weak contact interaction between the ring V keto group of one chlorophyll molecule with the central magnesium of another chlorophyll molecule, as shown in Figure 33. It is well known that ethers, ketones, and pyridine form 1:1 complexes with chlorophyll a molecules (48) at the central magnesium atom, and so the sterically hindered ring V keto group of a chlorophyll a molecule, even in concentrated solutions of these solvents, could not displace the ligands donated from the solvent; because of this, the chlorophyll is prevented from associating. The infrared spectrum in the region 1,650-1,800 cm^{-1} of chlorophyll a (C2) crystals (which, from the X-ray diffraction studies are known to be



A. Chlorophyll a on silver chloride
B. Chlorophyll a in KBr
C. 2.24×10^{-2} M/l
D. 1.01×10^{-2} M/l
E. 1.72×10^{-3} M/l
F. 4.36×10^{-3} M/l
G. 8.72×10^{-4} M/l

Fig. 32. Concentration dependence study of chlorophyll a in carbon tetrachloride.

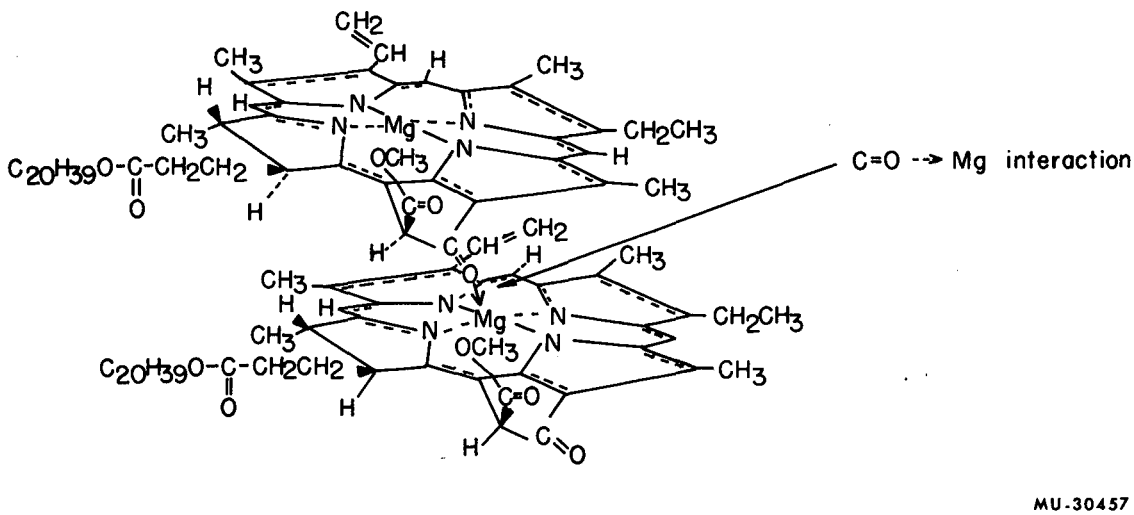


Fig. 33. Chlorophyll - chlorophyll dimerisation.

highly ordered) and chlorophyll a (C1) (which has poorer order than C2 and probably has several defects in its structure) have been given in Figure 32, to show that on going to crystalline form the aggregation becomes nearly complete in chlorophyll a (C1) and complete in chlorophyll a (C2), thus leading to shifts of 50 cm^{-1} for the 1,700 cm^{-1} vibration. The main crystal force present is a weak interaction between magnesium of one molecule and the keto group of an adjacent molecule, giving a stacking of the molecules similar to a distorted pack of cards in one dimension.

Conclusions

The infrared spectra of chlorophyll a and some of its more important derivatives have been obtained and assignments of the vibrations arrived at without any conflict with previous workers. The assignments of the vibrations are given in Table III.

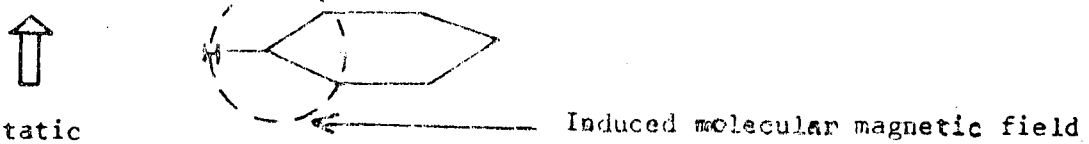
It has been shown that chlorophyll a and most likely that chlorophyll b both associate in non-polar solvents through a weak carbonyl-magnesium interaction, leading to a vibration in the region of 1,650 cm^{-1}, which was formerly ascribed to an enolic form of the chlorophyll a present when the molecule was undergoing a keto-enol tautomerism. A major interaction in crystalline chlorophyll is a weak carbonyl-magnesium bond, and this could be proved if a crystal structure determination was made on the crystals.

A more detailed understanding of the infrared of chlorophyll a in various states of aggregation and solvent milieu has been obtained and could perhaps be used for investigating the physical state of the molecule in the cell and give a surer guide to the reactions of the molecule in solution, especially its exchange reactions with H_2O^{18}.

Proton Nuclear Magnetic Resonance of Chlorophyll a

Introduction

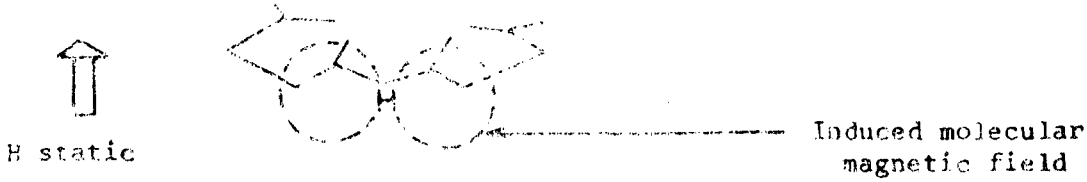
The technique of proton nuclear magnetic resonance (NMR) has been widely used for investigating the structures of large organic molecules (49), and recently the chemical shifts of proton resonances from porphyrins have been assigned to the already known groups in these molecules (50-54). A striking feature of the NMR spectra of porphyrins is the resonances in the region of 600 cps on the low field side of those originating from the internal standard tetramethyl silane (TMS) and which were readily assigned to the methine-H protons. The protons of benzene also occur at low field, and this result was ascribed to the circulation of the aromatic pi electrons around the ring, thereby inducing magnetic lines of force which are in the same sense as the static magnetic field at the peripheral protons on the ring (49-55).



Benzene in a magnetic field

A similar explanation to that given for the low field benzene proton resonances was given for the low field proton resonances of porphyrins. The assumption was made that the porphyrins are aromatic molecules, and therefore planar in structure, which is a necessary condition for the circulation of the electrons. However, a recent crystal structure determination of nickel etioporphyrin (56) showed that it was non-planar, and if this non-planarity of porphyrins is in general true,

then the above interpretation of their NMR will not be correct. It is still, however, possible for the circulating pi electrons in each of the pyrrole rings which make up the porphyrins to have induced magnetic fields which give fields in the region of the methine-H bridge protons. Each methine proton is then in



Methine proton in a magnetic field

the field of the resultant of two molecular magnetic fields, arising from the two pyrrole rings on either side of it. There is a second order effect from the pi electrons of the double bond present in the methine bridge, which is smaller. Chlorins, of which chlorophylls are an example, have one of the pyrrole rings reduced (in our nomenclature, ring IV), and therefore the proton which is situated between a pyrrole ring I and a reduced ring IV only has a magnetic field contribution from one ring, whereas the alpha and beta methine-H protons see contributions from two pyrrole rings. This should make the resonance of the delta methine proton come at higher field than the alpha and beta protons.

Because the chlorins, according to the reasoning given above, should show similar NMR behaviour to the porphyrins, it should be possible from the reported correlations on the porphyrins to give assignments to the chemical shifts of protons on the chlorins. In particular, the delta methine-H proton, for reasons given above, and the C_{10} proton, which is uncoupled to any other protons in the system, should both therefore appear as a single line with an intensity

equivalent to one proton.

Our major interest in the NMR of chlorophylls was as a tool for following exchange reactions of chlorophyll a or chlorophyll b *in vivo* or *in vitro*. If all of a particular proton in a sample of chlorophyll was substituted by deuterium, the proton resonance for that proton would disappear, showing that one proton out of seventy-two had been exchanged--a result hard to obtain by other techniques of analysis. With a correct assignment of a particular proton's chemical shift, it should also be possible to locate the site of the exchange in the molecule.

Experimental

The chlorophylls were prepared as described previously, and the methyl pheophorbide a was obtained from Professor R. B. Woodward. The pigments were dissolved in deuterated solvents (acetone-d_6 and chloroform-d) or in carbon disulfide, to give a saturated solution of approximately 0.04 M/l, and sealed in tubes designed to sit snugly in the spectrometer probes.

Tetramethylsilane (TMS) was added to the solution of the chlorophylls as an internal reference. In certain cases, due to the poor stability of the apparatus, which made the calibration unreliable at low fields, the proton of the undeuterated chloroform of the solvent was used as a reference for low field chemical shifts. Resonances are reported in terms of the chemical shift (δ), where

$$\delta = \frac{\nu_{proton} - \nu_{TMS}}{60} \quad \text{p.p.m.}$$

and a positive sign given to peaks which occur on the low field side of TMS.

The spectra were taken on a Varian A-60 NMR spectrometer operating at 60 Mc/sec at the probe temperature (30°). The spectrum of chlorophyll a in carbon disulfide was obtained by computer averaging, using a C.A.T. computer (Model 400-2-Pulse Height Analyzer, Technical Measurement Corp., North Haven, Conn.), over many spectra run on a Varian A-60 spectrometer at Varian Associates laboratory. The scanning rates were 1 cps/sec or 0.5 cps/sec in the low field region of the protons and the error in the chemical shifts (δ) $\pm$ 0.05.

Results and Discussion

The spectra of the chlorophylls are given as recorded on the spectrometer chart, because, although the majority of the assignments to be given will probably not be altered with further investigations, there are a few--especially those in the high field region close to the large number of resonances originating from the phytyl protons--which must be at this time considered tentative. Unfortunately, it is not possible at the present time to give the spectra of the methyl chlorophyllides because of their extremely poor solubility in the solvents which can be used for NMR, but the spectra can no doubt be found in the future by using the C.A.T. technique.

A starting point for the assignments of the chemical shifts is to make use of the correlations between structure and chemical shifts obtained by previous workers for the porphyrins given in Table IV.

The simplest molecule structurally in the series of molecules studied was methyl pheophorbide a, which does not have a phytyl chain. The proton resonances from the phytyl chain are in fact irrelevant, and obscure resonances, originating from hydrogens belonging to groups on the chlorin ring. Methyl pheophorbide a is insoluble in acetone-d_6

TABLE IV

The Chemical Shifts of the Protons in Chlorophylls

δ in p.p.m. from Tetra methyl silane

Group Proton	Molecule: Solvent:	Methyl Pheophorbide a Chloroform-d	Chlorophyll a Acetone-d_6	Chlorophyll b Chloroform-d	Chlorophyll a (Predicted from spectra of previous work)
Chlorin ring protons					
Methine H		9.25 9.05 δ 8.5	9.85 9.30 δ 8.09	9.75 9.30 δ 8.09	9.73 9.57 δ 8.90
Formyl-3 H		-	-	9.30	-
Vinyl 2 $CH=CH_2$		8.0 7.8 7.7 7.5	8.19 8.00 7.90 7.68	7.68 (poorly resolved)	8.3
Vinyl 2 $CH=CH_2$		6.28 6.12	6.30 6.00 6.14	6.12 5.70 (poorly resolved)	6.40 6.10
C_{10}-H		5.95	5.84	5.75	5.35
Ethyl CH_2CH_3		4.35 (quartet)	4.25 (quartet)	3.98	3.94
C_{10} $-OCH_3$		3.6	3.8	3.7	3.8
C_8 OCH_3		3.6	-	-	-

(Cont'd.)

TABLE IV (Cont'd.)

Molecule: / Solvent:	Methyl Pheophorbide a Chloroform-d	Chlorophyll a Acetone-d_6	Chlorophyll b Chloroform-d	Chlorophyll a (Predicted from spectra of previous work
Chlorin ring protons (Cont'd.)				
Methyl	3.95	3.55	3.25	3.6
1, 3 and 5	3.3	3.3	3.2	3.2
	2.59	3.2		3.2
8-CH_3	2.5	2.58 (?)	2.67	2.28
Ethyl $CH_2\underline{CH_3}$	1.9	2.38	2.2	1.69
8-CH_2CH_2-C(=O)	1.9	1.8	1.9	-
	2.5	2.38		
Phytol Protons				
CH_2O Me Me C=C C=C CH H Me	-	5.17	5.0	-
		1.72	1.80	
		1.18	1.20	

but soluble in chloroform, and its spectrum in this solvent is shown in Figure 34. There are three distinct proton resonances at low field δ = 9.25-8.5, whose intensities are equivalent to single protons and according to the introductory argument and previous work are clearly due to the methine-H protons. The δ-proton differs from the alpha and beta protons because it is in close proximity to one pyrrole ring, unlike the alpha and beta protons, which are between two pyrrole rings. The resonance at δ = 8.5, which is at the highest field of the three single downfield resonances, is therefore assigned to the δ-proton. The resonances at δ = 9.25 and 9.05 are assigned to the alpha and beta protons, without being able to distinguish between the specific protons. Just on the low field side of the δ-proton resonance (8.5) there is a quartet (δ = 8.19, 8.00, 7.90 and 7.68) which has a total intensity equivalent to one proton, and from previous studies (53) and the argument that it is downfield, it suggests π-electron conjugation with a complex splitting due to coupling with other protons. This quartet can only arise from the single proton nearest the ring of the 2-vinyl group. There is a complex group of resonances in the region of δ = 6.00, and having an intensity equivalent to three protons. If the resonance at δ = 5.95 is not taken into account (with an intensity approximately equivalent to one proton) and we then consider the remaining resonances in conjunction with the quartet previously assigned, the structure of the lines is characteristic of a vinyl group. The protons in this region had also been previously assigned by other workers to the two protons remaining on the vinyl group, and therefore the resonances at δ 6.28 and 6.12 were assigned to the two protons on the 2-vinyl group, but

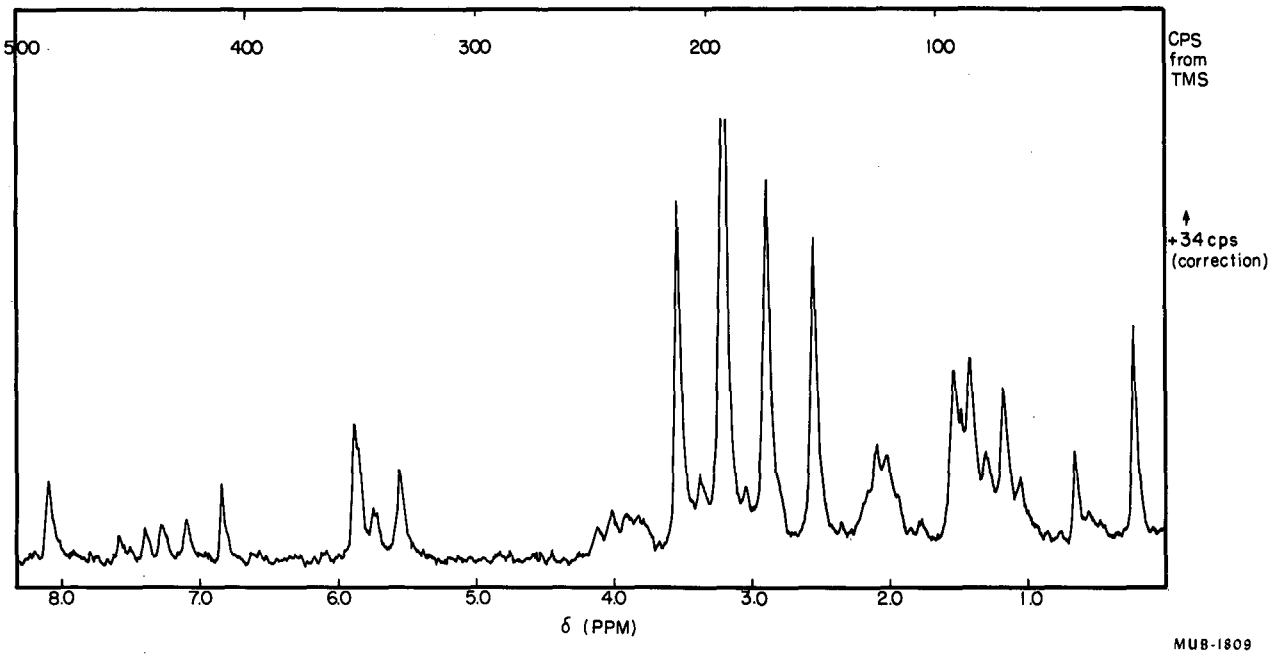


Fig. 34. NMR spectrum of methyl pheophorbide a in chloroform-d. (See also page 95.1).

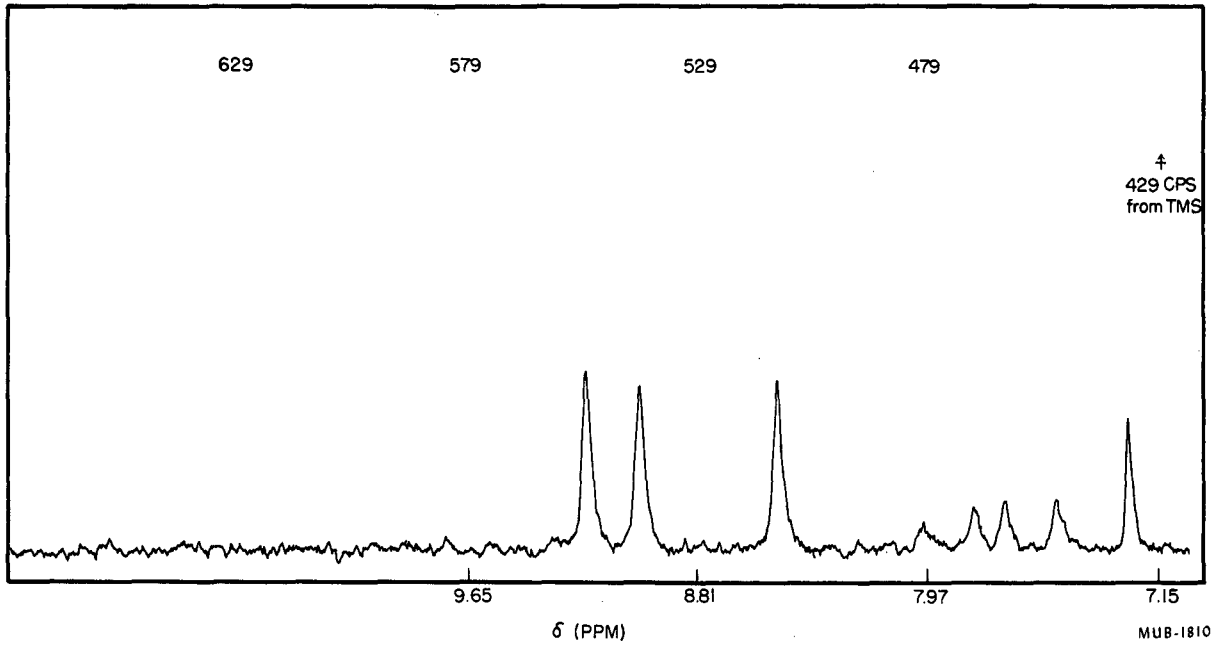


Fig. 34. NMR spectrum of methyl pheophorbide a in chloroform-d.
(See also page 95).

the coupling constants between the protons have not been evaluated because of the poor resolution possible from the spectrometer. There is only one remaining single proton in the molecule which is presumably uncoupled to other protons, and therefore the resonance at δ 5.95 was assigned to the C_{10}-H proton on ring V. At δ 4.35 there is a quartet which could only arise from the coupling of a CH_2 group with a CH_3 group, and it is at low field, indicating that it is close to the ring current. The resonance at δ 4.35 was therefore assigned to the CH_2 of the 4-ethyl group on ring II. The methoxy group protons, of which there are six in the methyl pheophorbide a, have been shown in many molecules to have chemical shifts in the region of δ 3.60. The most intense resonance line in the methyl pheophorbide a spectrum is at δ 3.60 and is clearly due to the methoxy group's protons. There are three methyl groups in the methyl pheophorbide a molecule, and these were easily assigned to the resonance lines at δ 3.95, 3.3 and 2.59 without being able to assign them to specific methyl groups.

The assignments from this stage on must be considered more tentative than those given above. The remaining protons are not under the influence of the ring currents and for this reason are not distinctly separated. There are two groups of resonances at δ 2.5 and δ 1.9 which belong to the CH_3 of the ethyl group at C_4 and the groups on ring IV which are subject to spin-spin coupling, causing a complexity that is hard to unravel without the use of molecular analogues (which were not available) or the use of spin-spin decoupling experiments (57). Because many of the porphyrins showed the CH_3 of the ethyl group to be within the range of δ = 1.73-1.97, then the CH_3 group of the 4-ethyl group in methyl pheophorbide a can be

assigned to part of the region around δ = 1.9. Caughey (54) assigned the 8-CH_3 group in chlorin-e_6 trimethyl ester to a resonance at δ = 2.28, and therefore part of the resonance at δ = 2.5 was assigned to this group. However, the intensity of this resonance is greater than that required for three protons and is about equivalent to four and is probably due to contributions from other groups in this region. The resonances of the remaining protons present in the $-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-O$ and C-C groups are assigned to the remainder of the resonances present at δ = 2.5 and 1.9.

The assignments of the resonances of chlorophyll a in acetone-d_6 (Fig. 35 are essentially the same as those given for methyl pheophorbide a, and the phytol group resonances are readily picked out from the spectrum of phytol alcohol (No. 346) given in the Varian NMR catalogue (58). Here again, the resonances of the groups on the reduced ring IV are hard to unravel because they are obscured by the intense resonances of the phytol proton resonances. However, these resonances are not particularly relevant to our problem.

Chlorophyll b differs from chlorophyll a in structure only in the replacement of a 3-methyl group for a formyl group. This change in structure is evident in the NMR spectrum of the chlorophyll b (Fig. 36), where there were only two resonances (δ = 3.25 and 3.2) which were characteristic of two methyl groups instead of three, as in the chlorophyll a. The aldehyde proton resonance is a unique resonance because of its proximity to a keto group which has a large magnetic anisotropy, resulting in proton resonances in the region of δ = 9-10. Because the resonance at δ = 9.30 in the chlorophyll b

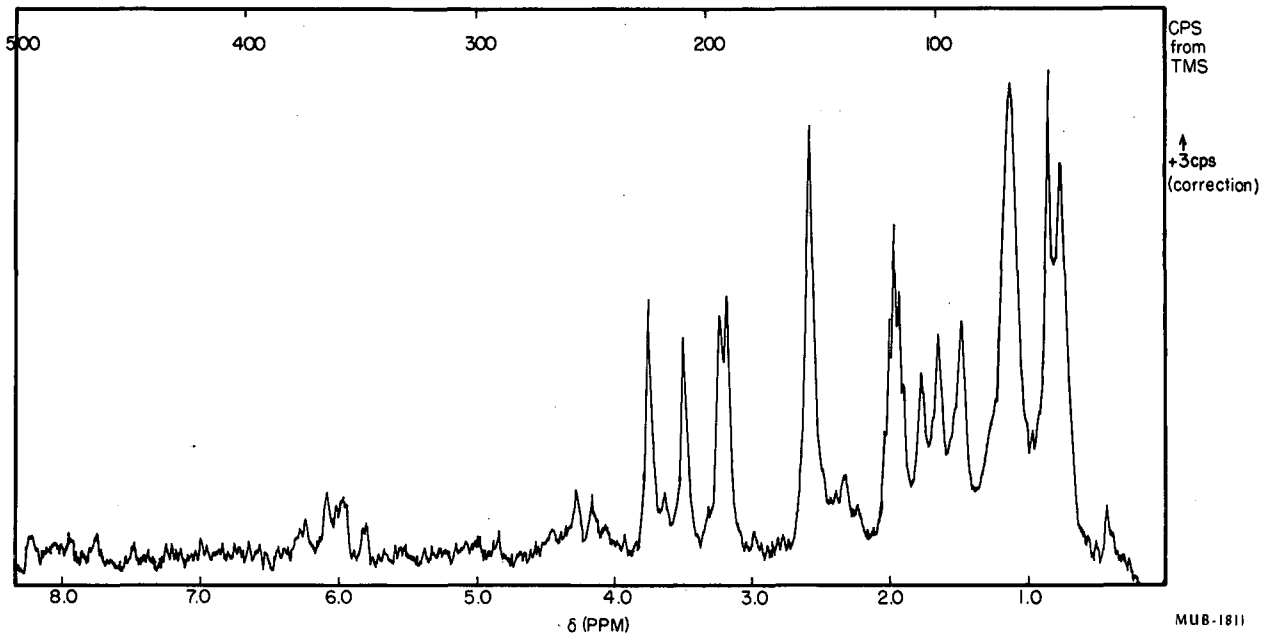


Fig. 35. NMR spectrum of chlorophyll a in acetone-d_6.
(See also page 98.1).

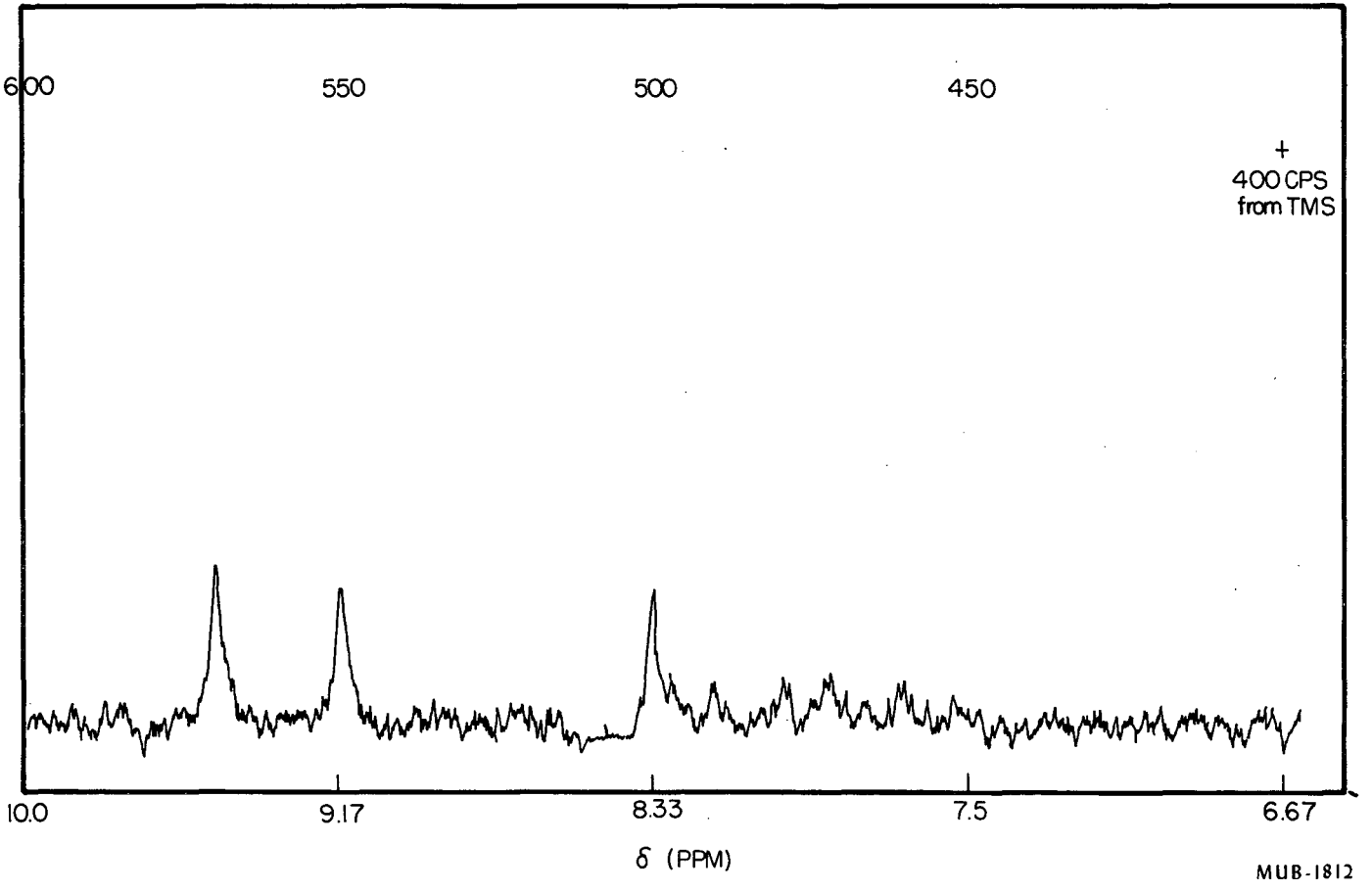


Fig. 35. NMR spectrum of chlorophyll a in acetone-d_6.
(See also page 98).

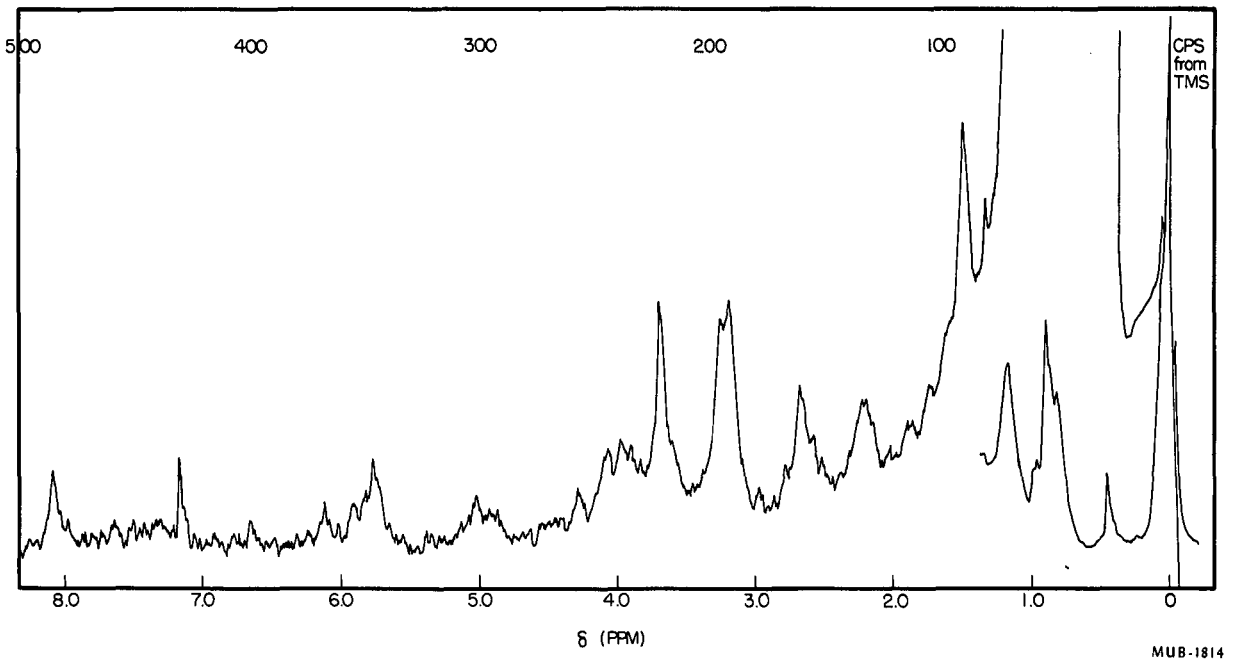


Fig. 36. NMR spectrum of chlorophyll b in chloroform-d.
(See also page 99.1).

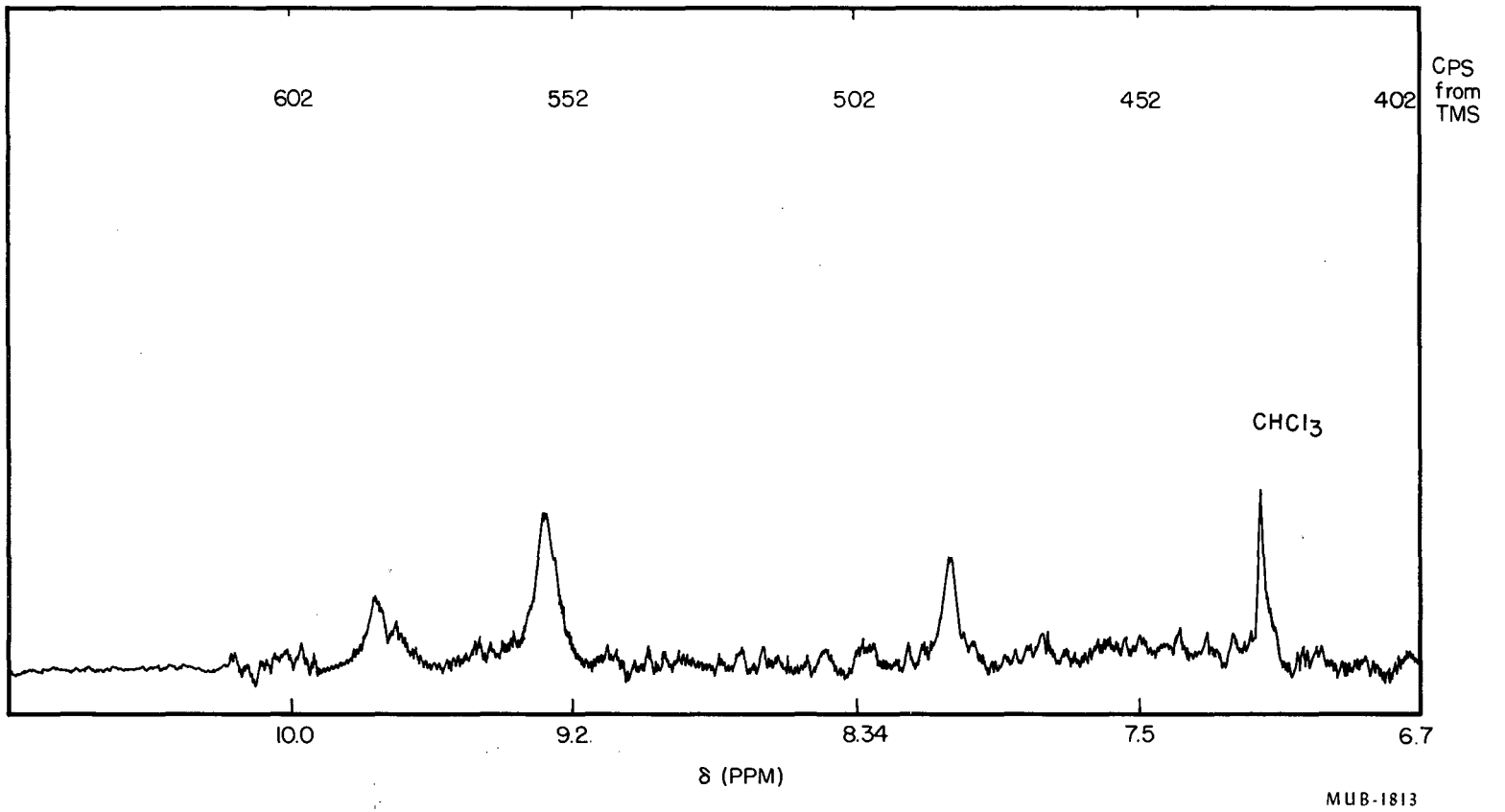
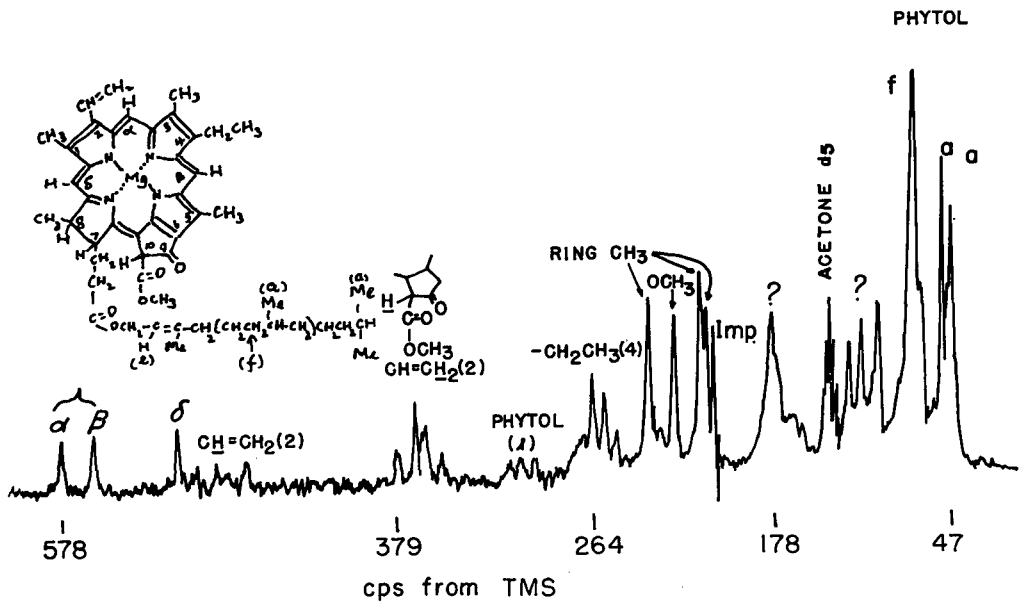


Fig. 36. NMR spectrum of chlorophyll b in chloroform-d.
(See also page 99).

spectrum has an intensity equivalent to two protons, it is concluded that part of it is due to the proton on the 1-formyl group.

In some of the first experiments an attempt was made to obtain the NMR spectrum of chlorophyll a in carbon tetrachloride or carbon disulfide, in order to investigate the solvent effects on chlorophyll a which had been detected in the infrared spectroscopic experiments. At the time that the NMR experiments were attempted on solutions of chlorophyll a in carbon tetrachloride the anomaly in the infrared studies on the same system were ascribed to keto-enol tautomerism. However, a reasonable spectrum could not be obtained from solutions of chlorophyll a in non-polar solvents from the Varian A-60 spectrometer due to poor sensitivity, but through the use of the continuous averaging of many spectra (59) with a C.A.T. computer at Varian Associates laboratory, clearly defined NMR spectra of chlorophyll a in carbon disulfide (Fig. 37) and acetone-d_6 (Fig. 38) were obtained.

It can be seen from the chlorophyll a spectrum in acetone that no new knowledge had been gained from this more elaborate experiment, but a proton NMR spectrum of chlorophyll a at high concentrations ($\sim$ 0.05 M/l) in a non-polar solvent (carbon disulfide) was obtained, which could not be obtained by conventional means. All that can be said at the present time about the NMR of chlorophyll a in non-polar solvents is that the spectrum is not characteristic of the monomeric chlorophyll a and is no doubt due to the same type of intermolecular interactions which were detected in the visible and infrared spectroscopy. The collected assignments of the chemical shifts are given in Table IV.



MU-30536

Fig. 37. Chlorophyll a saturated solution in acetone-d_6 signal/noise improvement using C. A. T.

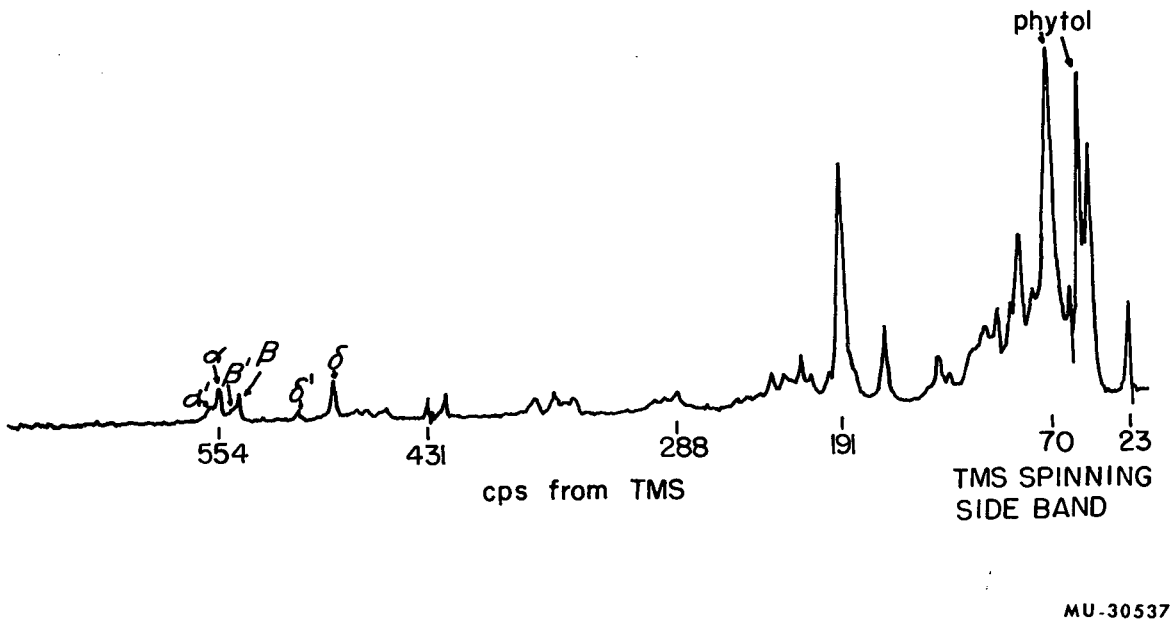


Fig. 38. Chlorophyll a saturated solution in carbon disulfide signal/noise improvement using C. A. T.

Conclusions

The NMR of chlorophyll *a*, chlorophyll *b*, and methyl pheophorbide *a* have been obtained, and their most relevant protons (as far as exchange studies are concerned) at C_{10} and the delta methine bridge have been found to have chemical shifts in the region of δ = 7.5-5.95 and δ = 8.5-8.1 respectively. The structure of the proton-proton splitting arising from the vinyl group changes in the different molecules studied and reflects the alteration of the pi electron conjugation throughout the molecule, due to the presence of different substituents. However, the introduction of a magnesium atom into methyl pheophorbide *a* to give chlorophyll *a* does not essentially alter the positions of the peripheral proton chemical shifts, and therefore the magnesium does not, by this test, seem to alter the electronic environment of the peripheral groups on the ring to any great extent.

The NMR spectra of chlorophyll *a* in non-polar solvents at high concentrations are difficult to obtain without the use of a computer for averaging runs of many spectra. In the future it should be possible by the use of a computer to obtain the NMR spectrum of chlorophyll *a* in non-polar solvents at low concentrations and also perhaps from a concentration dependence study, learn more about the chlorophyll-chlorophyll interactions.

The Electron Spin Resonance (ESR) of Plant Extracts and Chlorophyll a

Introduction

A logical step in the investigation of unpaired electrons induced by light in photosynthetic systems is the study of ESR signals from the organic solvent extracts of the original systems. It is a reasonable supposition that chlorophyll a, which makes up a large fraction of the extracted mixture of pigments, is an important factor in the production of light-induced ESR signals, in these extracts. However, one must remember that in ESR experiments the physical form of the molecules in the cell and the other pigments with which it is associated can influence the production and nature of the unpaired electrons. Trying to reproduce the construction of the cell's structure, in which the chlorophyll a's physical form is probably that of a disordered monolayer (12,13), is not easy and could not be attempted until a sample of pure chlorophyll a had been obtained.

Sogo, Jost, and Calvin (60) made preliminary experiments on methanol extracts of Chlorella and Rhodospirillum rubrum, relatively pure photosynthetic pigments, and mixtures of these pigments. The ESR signals obtained in their tentative experiments were irreproducible and differed in their signal characteristics (line width, rise and decay time) from the biological material. The extract of plant pigments when made into films gave signals that were dependent on the gases absorbed on their surface, and their line width was much narrower than that of the biological materials which they had previously investigated.

S. S. Brody, et al. (61) have investigated the ESR of solutions and microcrystals of a sample of chlorophyll a obtained by the pro-

cedure of Jacobs, Valter, and Holt (21), which, presumably, was the purest sample of chlorophyll a obtained up to that time. The crystals had a dark signal, and a photo-induced signal with a quantum yield of 7%. The solutions of chlorophyll a (10^{-3} M/l in pyridine) had no dark signal, but a photo-induced paramagnetism of 3% quantum yield was obtained when the samples were tested in an ESR spectrometer.

The present work reported here was in the nature of "sighting experiments", designed to get some feeling for the manipulation of the extracts of plant pigments and, from the crude measurements, some suggestions of paths to be taken in a more detailed study of ESR signals in extracts of biological materials. This work, in fact, prompted the search for a means of separating the plant extract into its components (Appendix II) and obtaining a sample of extremely pure chlorophyll a, which could then be combined (under controlled conditions) with other plant pigments to give a system with properties similar to the original plant extract or even the cell.

The ESR signal which resulted from the crystalline chlorophyll a obtained by the procedure already described is compared with the signals obtained by Brody from crystalline chlorophyll a prepared by the method of Jacobs, Vatter, and Holt (21).

Preparation of Films for the ESR Spectrometer

The plant material from which the extracts were prepared was Chlorella grown under steady state conditions. A 250 ml sample of Chlorella culture was centrifuged at 2,000 rpm for 10 min to remove the culture medium. The wet-packed algae (about 5 cc) were washed twice with distilled water, extracted with 100 ml of 100% methanol

(reagent grade) at room temperature for one hour, and the insoluble residue centrifuged down. The methanol extract was made into a sample suitable for testing in the ESR spectrometer (described in reference 60) in three ways:

(1) The extract obtained by the above procedure had the solvent removed under vacuum in a 200 ml flask. The sticky material in the flask was scraped out and applied to a silvered copper rod.

(2) The flask was rinsed with 2 ml of petroleum ether, and this petroleum ether solution of pigments was placed in a quartz sample tube with dimensions 8 cm by 8 mm O.D. The solvent was carefully evaporated off with the application of gentle vacuum, leaving a film of pigments (carotenes and chlorophylls) about 0.5 mm thick on the lower 1.5 cm of the tube.

(3) 100 ml of the methanol extract was treated with 50 ml of petroleum ether in a separatory funnel, with the addition of distilled water, drop by drop, until two phases were obtained. This phase separation removed the majority of the xanthophylls and left the chlorophylls and carotenes in the supernatant phase, which was collected and evaporated onto the lower part of the quartz tube in the same manner as in procedure (2).

The preparation of the films is summarized in Figure 39.

Electron Spin Resonance of Plant Pigment Extracts

The film prepared by method (1), when tested in the spectrometer for ESR signals, gave no signals when it was dry, but when it was "doped" with small (unknown) quantities of water it gave white light-induced signals. Several samples gave signals of similar form (line

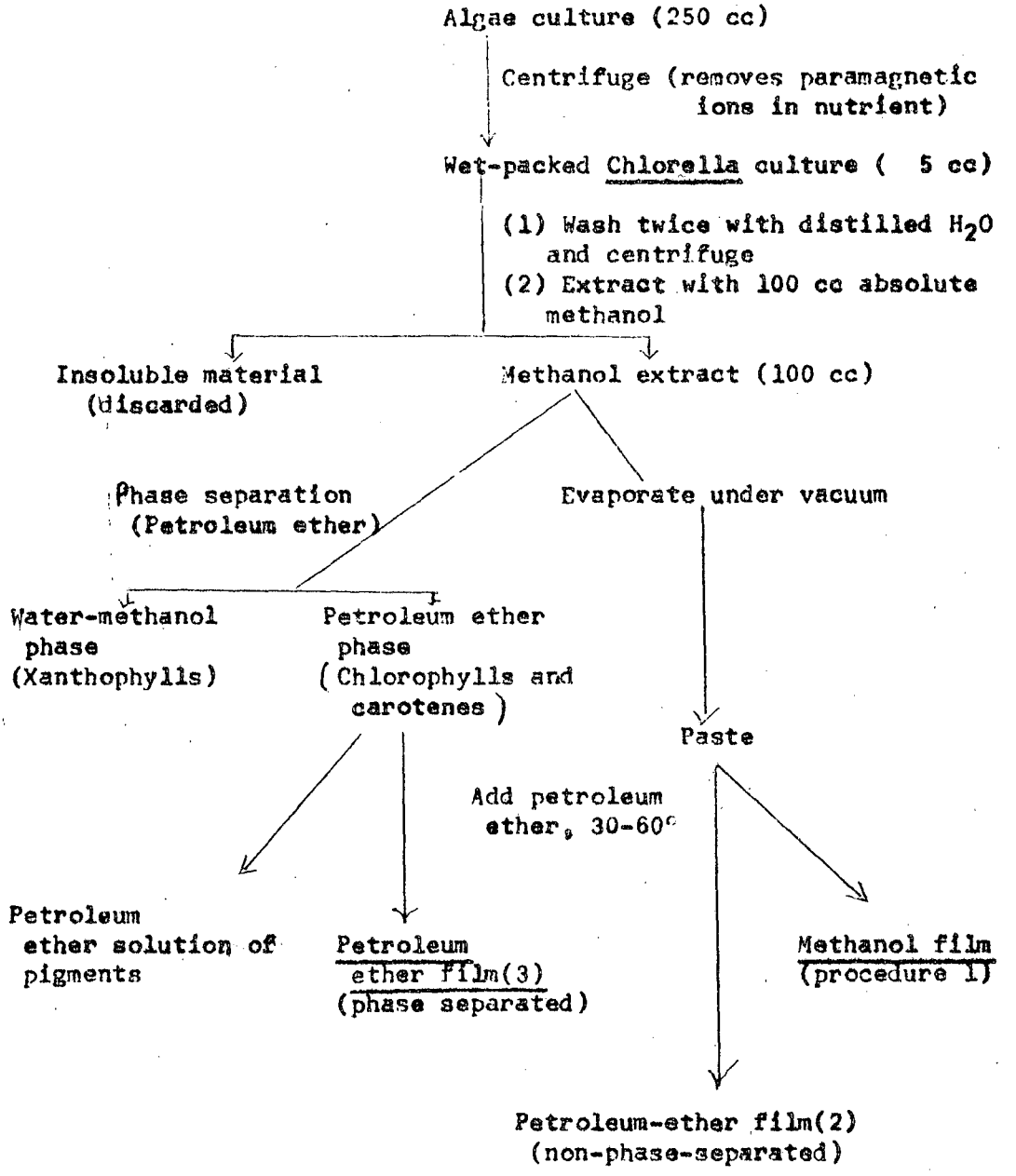


Fig. 39. Chlorella Extraction and Film Preparation

width about 6 gauss) but were not reproducible in intensity and rates of decay as had been found by Sogo, et al.

Films prepared by method (2) (using petroleum-ether washes of the extract), deposited on the quartz tube, gave much higher yields of free spins and were more reproducible from sample to sample. Again the addition of water was necessary for the production of spins, and now this variable was controlled more carefully, without ascertaining at this stage the reasons for the higher signal intensities and with better reproducibility of the signals obtained.

Films were deposited as described above, on the quartz ampule, and then evacuated with a vacuum pump at 10 microns pressure for 6 hr to remove traces of methanol, petroleum ether, water, and adsorbed oxygen. The sample was protected from light by placing a jacket around the quartz tube, all the previous steps being carried out in dim light. The sample was sealed under vacuum, placed in the ESR spectrometer, and the visible light from a projection lamp was then shone on the sample. At this stage no dark signal, and only a very small light-induced signal, was obtained. The sample kept at room temperature was then exposed in an apparatus, shown in Figure 40, to a definite vapor pressure of water which had been boiled and then degassed several times under vacuum to remove oxygen. The whole apparatus was evacuated for 3 hr to remove any traces of oxygen and then the oxygen-free water vapor from "A" at 0° C, obtained by an ice water bath around the tube containing the water, was allowed to come into contact with the film of pigments. Stopcocks C and D were then closed and the sample tested for dark and light-induced signals. The experiment was

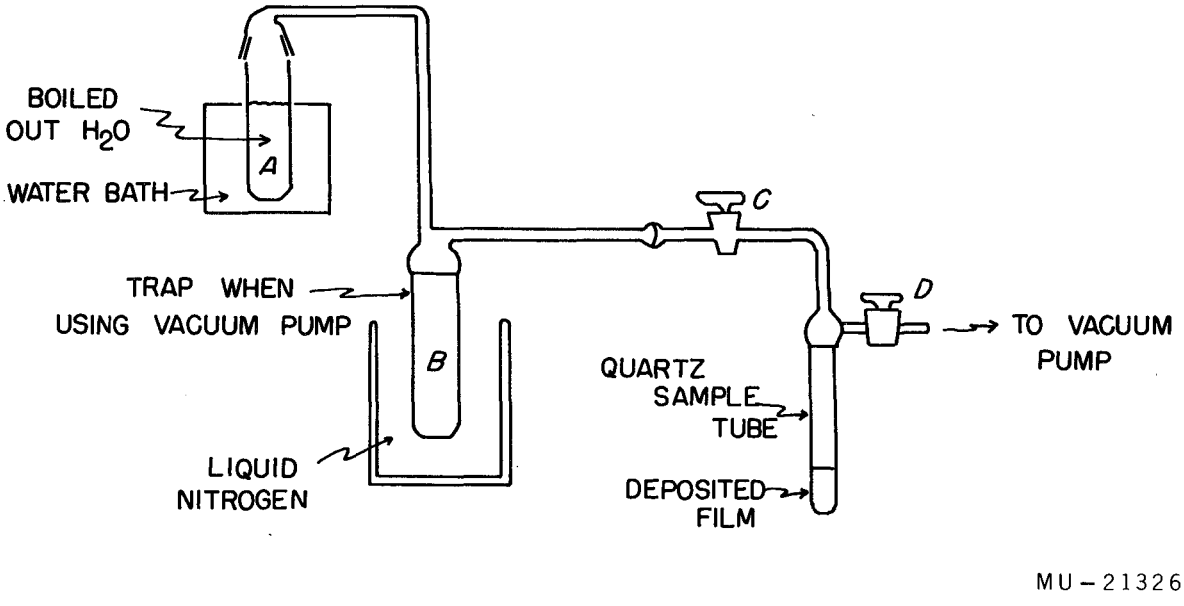


Fig. 40. Apparatus for applying water vapor to sample.

repeated on the same film after desorbing the water from a previous experiment, then increasing the vapor pressure of water, obtained by altering the temperature of the water bath around A. In each case a small dark signal was obtained, and a white light-induced signal (Fig. 41) of intensity varying with the amount of water "doping" and of narrow line width (3 gauss) was found.

Two observations can be made on the dynamics of the photo-induced signal: 1) The steady state concentration of photo-induced spins, as a function of water vapor pressure, and 2) the rate of decay of the spins after the light is turned off. Since the line width of the signals remained constant as they decayed, the concentration of spins was taken to be proportional to the amplitude difference between the peaks of the differentiated absorption curve.

Figures 42 and 43 represent the data from the above experiments. It is seen in Figure 42 that the steady state population of unpaired spins goes through a maximum at approximately 4.7 mm ambient water vapor pressure. For a given method (2) of producing the sample films, the decay rates, as shown by the slope of curves in Figure 43 at various vapor pressures, with the order of increasing vapor pressure I to IV, are relatively independent of water vapor pressure. However, a sample prepared by method (3) (involving a phase separation which removed the xanthophylls), and with ambient water vapor pressure equal to 4.7 mm Hg, showed both the equilibrium amplitude (value at $t = 0$) and the rate of decay to be markedly different from samples produced by the other techniques, as shown by dotted curve V in Figure 43.

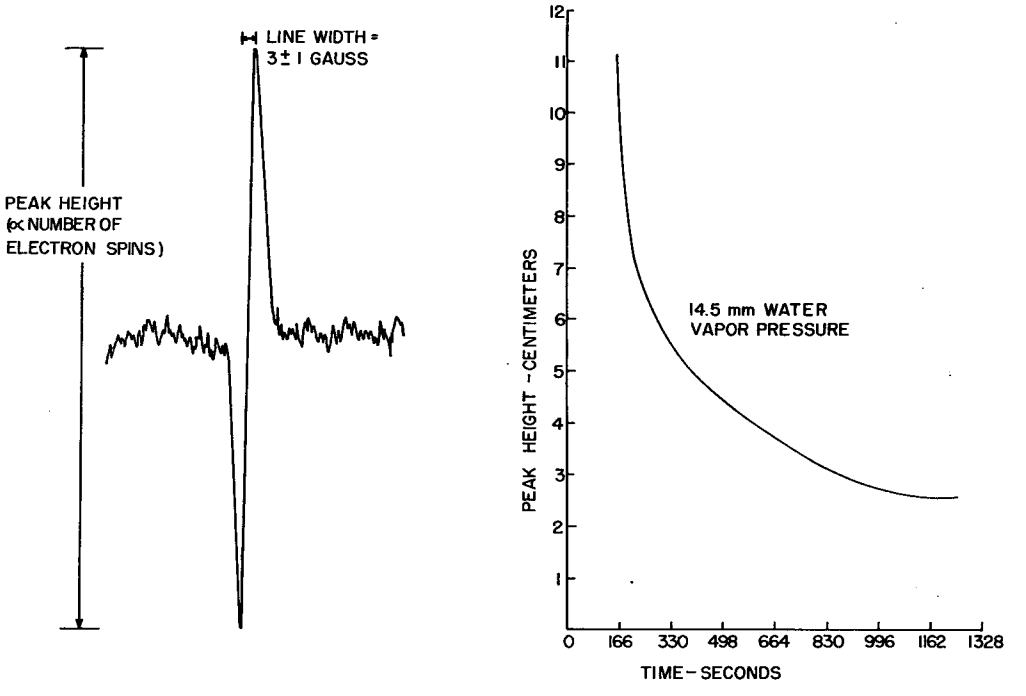


Fig. 41. Light-induced signal in plant extract film.

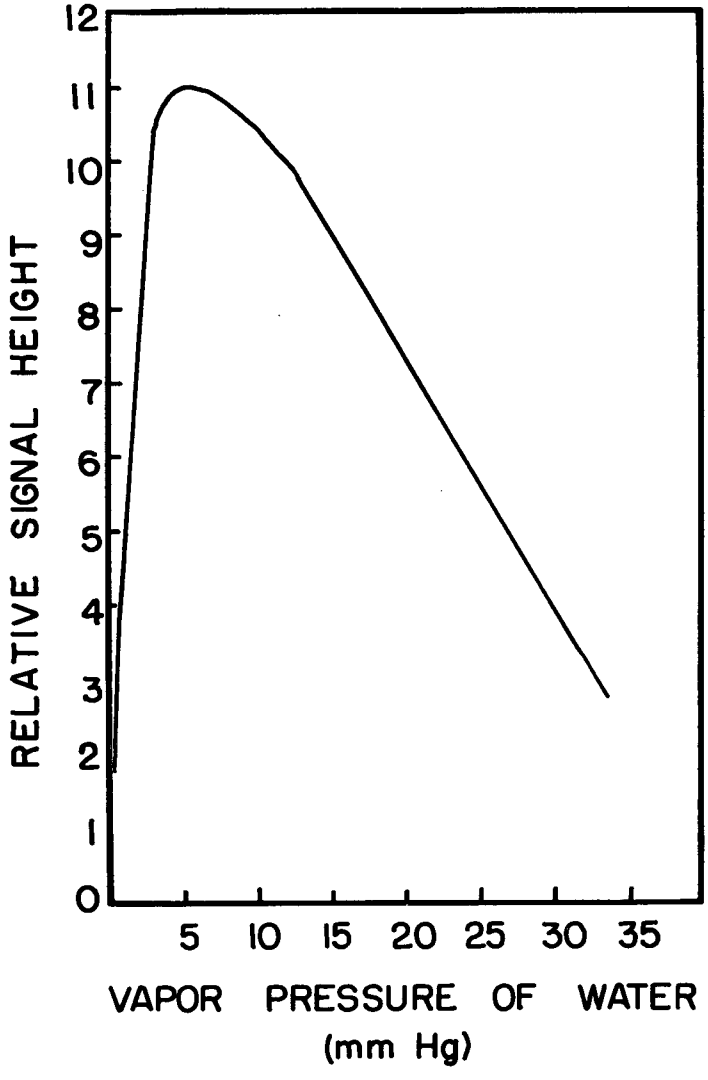


Fig. 42. Concentration of spins in an extract as a function of water vapor pressure.

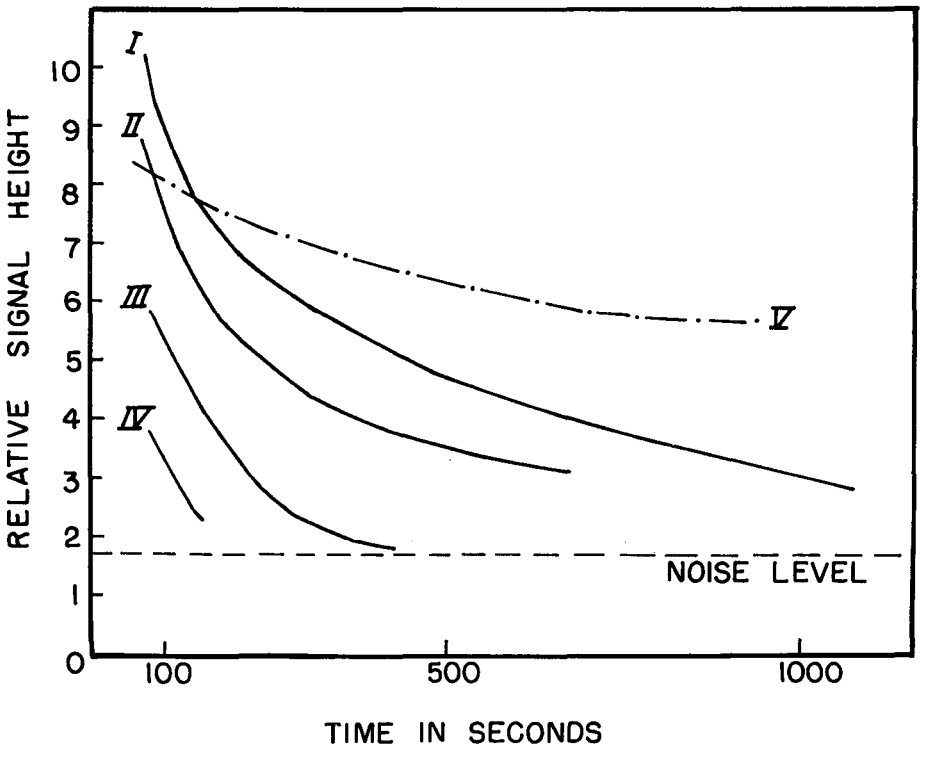


Fig. 43. Rates of decay of spins in an extract at a given water vapor pressure. I. 4.67 mm HgH_2O; II. 14.5 mm HgH_2O; III. 19.8 mm HgH_2O; IV. 31.8 mm HgH_2O; V. 4.67 mm HgH_2O.

Production of Spins in Plant Extract Solutions

It is of interest to find out whether or not solutions of the extracted pigments produce signals under the influence of light, because if a free radical were formed it could exhibit fine structure which could lead to its identification. Methanol solutions of the pigments could not be tested with sufficient concentration of pigments present to give a signal because the dielectric constant of methanol is such that microwaves from the spectrometer were strongly absorbed. However, the methanol-extracted material can be transferred into petroleum ether and investigated in the ESR spectrometer.

A petroleum ether solution was placed in the quartz tube and the light-induced signals observed as the petroleum ether was evaporated off with weak vacuum. Only when solid material appeared as the solvent was evaporated off did small signals appear.

ESR of Crystalline Chlorophyll a (C2)

About 0.25 ml of a heavy suspension of chlorophyll a (C2) in isooctane was smeared on a small quartz plate of area 1 cm^2, which was inserted in a quartz tube between two Teflon spacers such that the film of chlorophyll a crystals was exposed to a maximum intensity of light when placed in the spectrometer. The red light used was isolated from the white light of a 1,000 W projection lamp by passing the white light first through an 8 cm cell of water to remove the infrared radiation and through a Corning 2030 filter which transmits red light from 648-750 mμ.

Two samples from different preparation of chlorophyll a (C2) crystals gave similar results in the preceding experiment.

The chlorophyll *a* crystals showed a small symmetrical dark signal in the region of $g \approx 2$, and of line width 15 gauss, when placed in the ESR spectrometer. Shining red light on the sample induced a symmetrical signal in the region of $g \approx 2$, which took about 15 min to reach a steady state intensity of about four times that of the dark signal. When the light was turned off there was a slow decay of the signal, which after 10 hr had not returned to the intensity of the dark signal. The shapes of the ESR signals in the dark and light are compared in Figure 44, and the kinetics of the production and decay of the signal are shown in Figure 45.

The dependence of the ESR signals when water vapor was placed on the surface of the crystals was not studied.

Discussion of the ESR Signals from Extracts of Plant Pigments and Crystalline Chlorophyll *a*

It would be presumptuous to make any strong conclusions from the very tentative experiments described above, but they have demonstrated the importance of two variables in the ESR experiments--namely, the necessity for water in the production of signals from plant extracts and the effect of purity on the signal characteristics from chlorophyll *a* preparations. Terennin, *et al.* (62) have confirmed our water effect on the ESR signals in preparations of chlorophylls which to us are of unknown purity. The character of the signals we obtained from our preparation of chlorophyll *a* differs from those obtained by Brody (61) using crystals prepared by a different procedure. Brody's dark signal is similar to ours, but his light-induced signal is assymetrical, arising from the superposition of a narrow signal on a broad signal, while ours is symmetrical and similar to our dark signal,

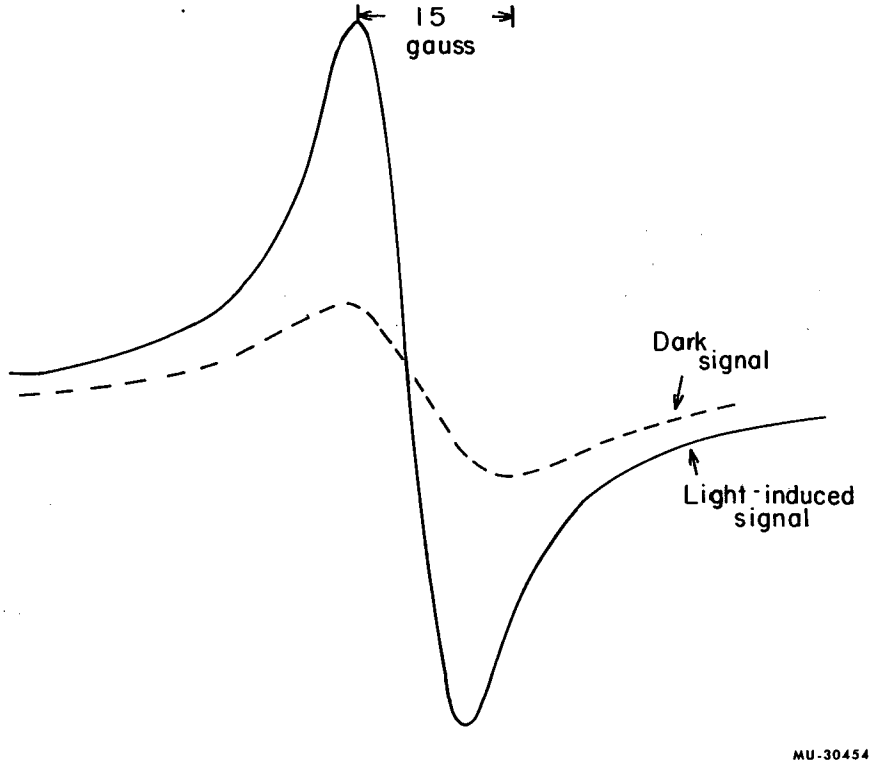


Fig. 44. ESR signals in chlorophyll a crystals.

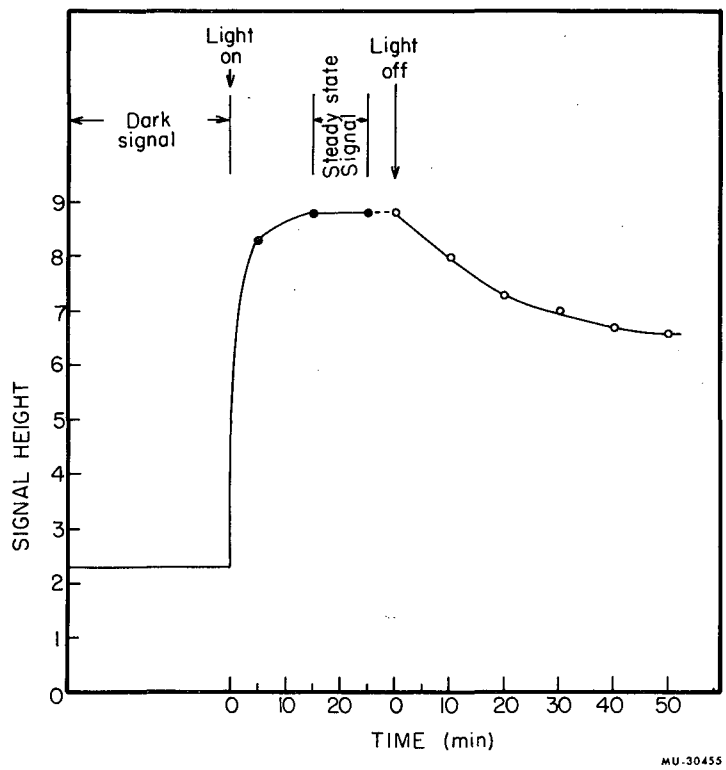


Fig. 45. Time dependence of ESR signals in chlorophyll a crystals.

both signals presumably being due to the same type of electrons. Brody's asymmetrical signal could be due to the poorer order of the crystal, but more likely due to traces of a xanthophyll impurity which we feel are present in the crystals he used.

The origin and nature of the signals which have been obtained in the above experiments are not known. It is felt that experiments on crystalline chlorophyll a (C2) should be carried out on either the crystalline material or on monolayers, both of which could be "doped" with the pigments (xanthophylls, carotenes, and quinones) which are present in the plant extracts. These experiments should give valuable information about the nature of unpaired electrons in chlorophyll a and organic dyestuffs in general.

APPENDIX I

Separation of Plant Pigments

A sound technique for separating the pigments present in plants into the broad classes of xanthophylls, carotenes, and chlorophylls is a necessity for studies on the pigments' reactions *in vivo* and as a means of obtaining samples for studying their *in vitro* properties. The extraction of the pigments from cellular material is carried out with a polar solvent (acetone or methanol). In the methods developed over the years this extract was subjected to various treatments designed to arrive at the isolation of one of the molecules in a group--e.g., chlorophyll *a*--without attempting to recover all the molecules from the original extract. The most elaborate schemes for separating the extract were devised for chlorophyll *a*, and, indeed, if some of the steps in the procedure had been highly efficient then the separation of the molecules into their classes would have been achieved. The principles of a typical chlorophyll *a* separation are along the following lines: The polar xanthophylls can be transferred into a water-acetone phase when an acetone extract of the plant material is treated with a non-polar solvent such as petroleum ether, which takes up the chlorophylls and carotenes. Carotenes can be separated from the chlorophylls by passing the petroleum ether solution, containing both types of pigments, over a diatomaceous earth, on which the chlorophylls are adsorbed but the carotenes pass on. In principle then, the three classes of pigments have been separated with an efficiency depending on the power of the phase separation to isolate the xanthophylls and the effectiveness of the diatomaceous earth to separate the carotenes from the chlorophylls. Chromatography can

then be used to separate the various classes of pigments into their individual members. The presence of quinones in the plant extracts had been demonstrated by Bishop (63), and this was a type of molecule which could contribute to the ESR properties of our plant extracts.

After the preliminary investigation of the ESR properties of plant extracts it was obvious that the extract would have to be broken up into its constituents and an account made of all the pigments present in the extract before a proper study of the ESR properties of the extract could be made. The above type of separation was investigated following the procedure of Jacobs et al. (21), as a means of separating the plant pigments, and each fraction was tested for the presence of plastoquinone by means of the sodium borohydride test (63). The ultraviolet absorption spectra of chlorophyll a and b were measured to see if their spectrum had a band at 255 mμ and what happened to it when the chlorophyll was reduced with sodium borohydride.

Isolation of Plant Pigments from Spinach (I)

Spinach was used rather than Chlorella, as it could be obtained in large quantities and the principles worked out for spinach could be used for an analysis of methanol extracts of Chlorella. The outlined procedure for separating the pigments is shown in Figure 46.

Preparation of an Acetone Extract from Spinach

After the large veins were cut out of the spinach, it was washed and dried on blotting paper. 180 gm of the spinach was ground with 500 ml of reagent grade acetone in a Waring Blendor. The mixture was filtered through a 5-in Buechner funnel and the cake washed with 50 ml of acetone to remove any traces of the remaining pigment, giving

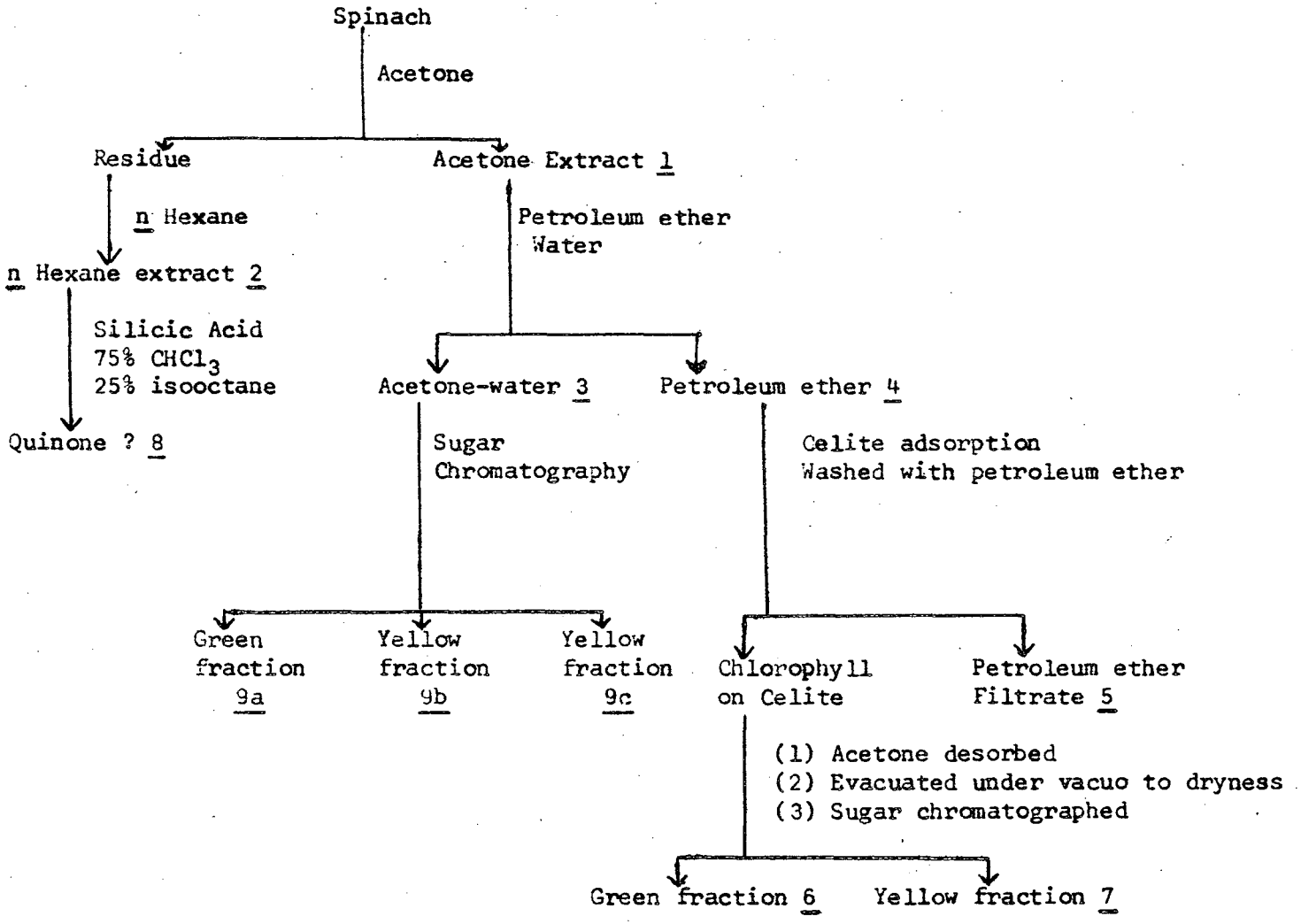


Fig. 46. Isolation of Pigments from an Acetone Extract of Spinach

the acetone extract (marked "1" in Fig. 46). After the cake was sucked dry, it was washed with five lots of 40 ml of n-hexane to give the n-hexane extract ("2" in Fig. 46).

Partitioning the Xanthophylls into the Polar Phase

100 ml of the acetone extract were placed in a separatory funnel and 30 ml of petroleum ether added; 50 ml of 0.5% sodium chloride solution were carefully added down a filter funnel, whose outlet was under the surface of the petroleum ether, until two phases were obtained. There were then two phases, one an acetone-water phase ("3" in Fig. 46) containing the xanthophylls and the other a petroleum ether phase ("4" in Fig. 46) containing the chlorophylls and carotenes.

Adsorption of the Chlorophylls on Celite and the Elution of Carotenes

The petroleum ether phase after several washings with 0.5% sodium chloride solution was poured into a 3-in diameter Buechner funnel packed to 1/2 in from the top with Celite (diatomaceous earth) and washed with 200 ml of petroleum ether (b.p. 30-60°) until the petroleum ether filtrates ("5" in Fig. 46) were colorless. This method was unsatisfactory because channeling of the Celite occurred, which allowed some of the chlorophylls to pass through, thus contaminating the carotenoids. Another approach was to pack a column 20 cm long and 5.5 cm in diameter with Celite, either by dry packing or as a slurry with petroleum ether. However, this procedure is also quite difficult to reproduce and brings about some alteration of the chlorophylls to pheophytins, especially in the presence of light.

Chromatography with Silicic Acid

Chromatographic columns (0.5 cm in diameter), inserted in a Buechner flask so that gentle suction could be applied, were packed

to a depth of 18 cm with silicic acid (Mallinckrodt 100 mesh) with slight tapping of the added portions to make them homogeneous. The column was washed with isooctane (Philips Spectroscopic Grade) and the sample dissolved in 10 ml of isooctane added to the column. The column was developed with 25% chloroform:75% isooctane to elute the carotenoids and then with 75% chloroform:25% isooctane to carry the possible quinone away from the material at the top of the column. The band that travels with the 75% chloroform:25% isooctane ("8" in Fig. 46) is cut out of the column to prevent decomposition and tested for quinone.

Chromatography with Sugar

Columns (2 cm diameter) inserted in a Buechner funnel so that gentle suction could be applied were dry packed with sugar (Californian and Hawaiian [C&H] powdered cane sugar) to a depth of 20 cm. The chlorophyll mixture, desorbed with acetone and evaporated down, was applied to the sugar column in a solution of 1 ml pyridine and 10 ml petroleum ether, and the column was developed with a mixture of benzene (4 parts), hexane (95 parts), and pyridine (1 part) to give one green zone ("6" in Fig. 46) and one yellow zone ("7" in Fig. 46). When the technique was applied to the evaporated acetone-water phase, it gave two yellow zones ("9b" and "9c" in Fig. 46) and one green zone ("9a" in Fig. 46). The bands were cut out and eluted with ether and their spectra taken.

Preparation of Solutions for Spectral Analysis

In searching for the quinone or characterising the compound eluted from a column, it is necessary to measure the spectrum in a particular solvent. In testing for the quinone the best solvent for the ultraviolet spectral analysis is 95% ethanol, in which the borohydride

reduction is best carried out. In most cases it is best to evaporate down the extract or solution of the substance eluted from a column under vacuum with rotary evaporator to remove the benzene or acetone remaining from previous steps. The sample is divided into two parts, and one part is treated for 15 min with several milligrams of sodium borohydride, which is then removed by centrifugation. The spectra taken on a Cary 14R Spectrometer of the various fractions (before and after reduction with sodium borohydride) are shown in Figure 47.

Discussion of the Separation of Pigments from Spinach

The separation of the pigments by the above procedure was too irreproducible for ESR work, and because the changes in the ultraviolet spectra of the extract solutions on sodium borohydride reduction could not be satisfactorily interpreted, there was still uncertainty about the location of the quinone in various parts of the extraction procedure.

Chlorophylls were seen to change from a bluish to an olive green color on the Celite column. Whether this was a photochemical reaction or due to the acidity of the surface of the Celite has not been ascertained, but on separating the resultant material the main product separated on a sugar column was pheophytin a, identified by means of its spectrum (Fig. 48).

The changes in the ultraviolet spectrum of the chlorophylls due to sodium borohydride reduction were unknown, and investigation of their spectra in the region between 220 mμ and 280 mμ showed nothing of interest either before or after reduction with sodium borohydride. The changes in their visible spectrum on sodium borohydride reduction do show remarkable changes (Figs. 49 and 50), but the substances formed cannot be readily identified from their spectra.

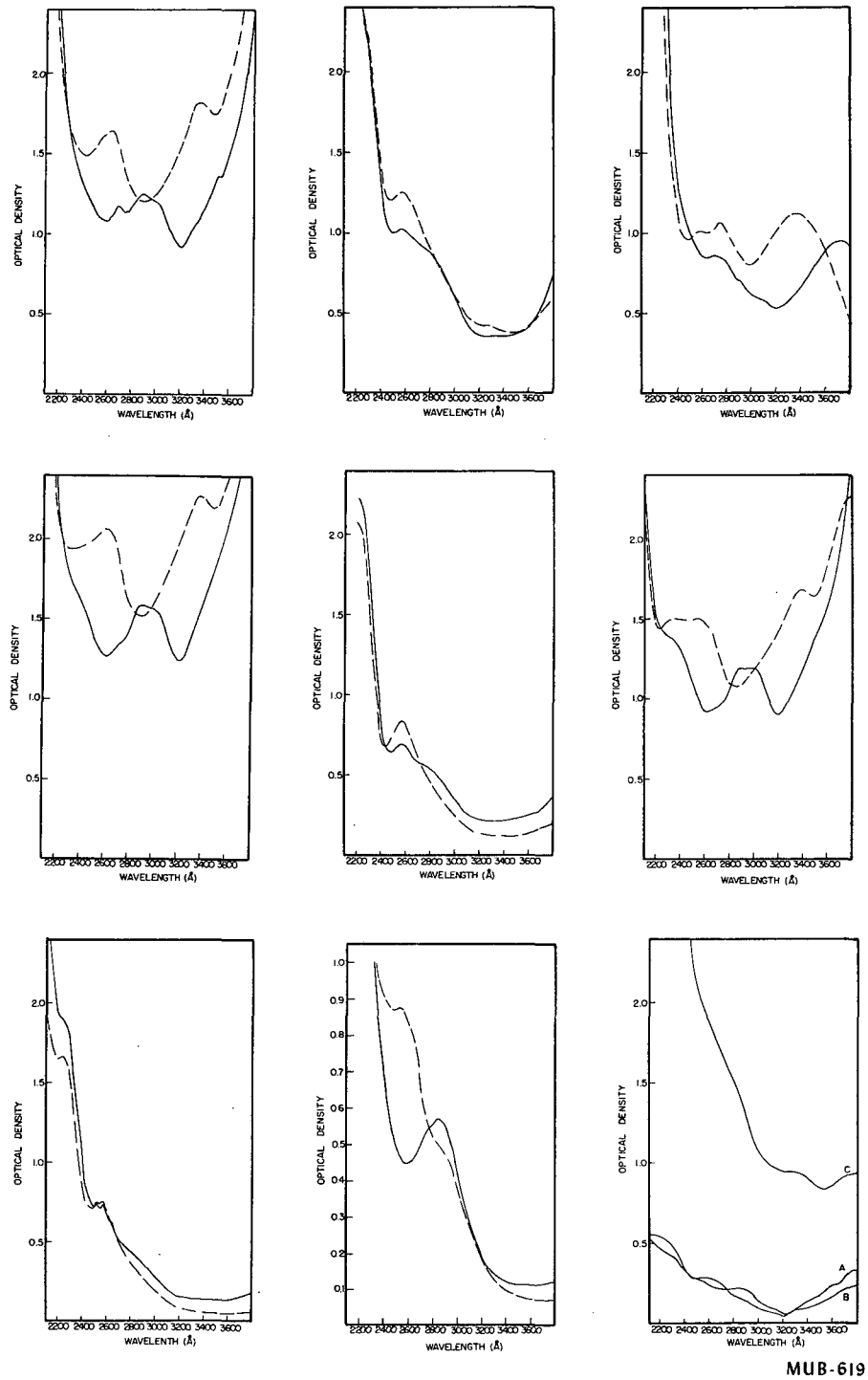


Fig. 47. Spectra of various fractions from extraction scheme shown in Fig. 46.

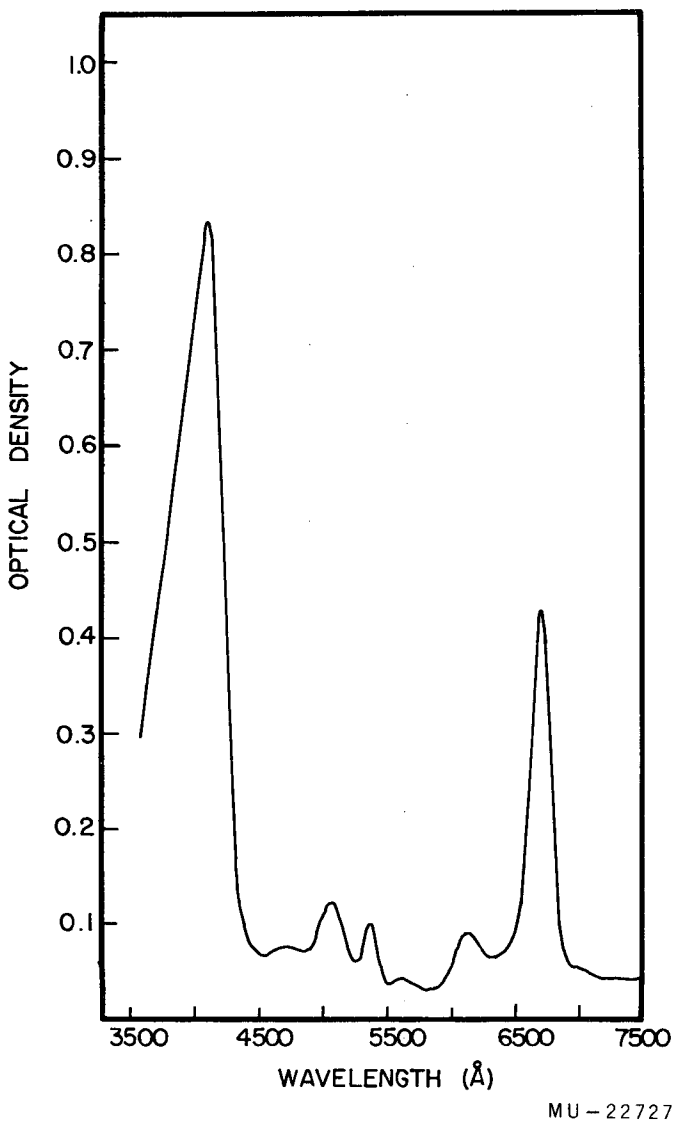


Fig. 48. Absorption spectrum of pheophytin a in ether.

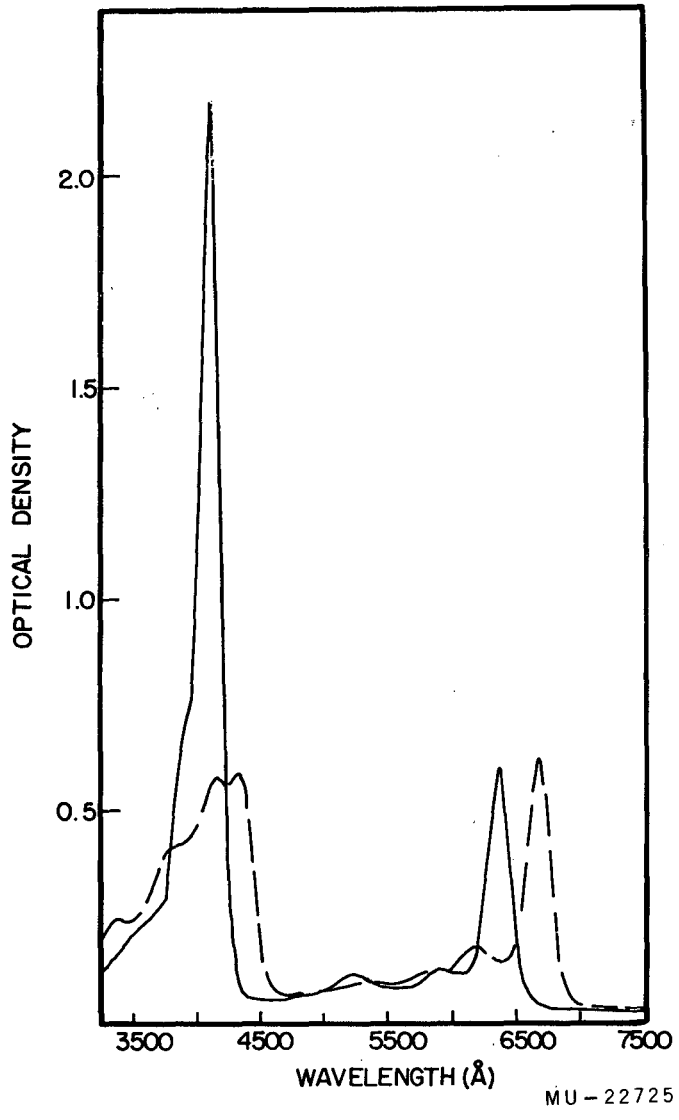


Fig. 49. Reduction of chlorophyll a with sodium borohydride ($NaBH_4$).
– – – – Visible spectrum of chlorophyll a in ethanol
——— Visible spectrum of chlorophyll a reduced with $NaBH_4$.

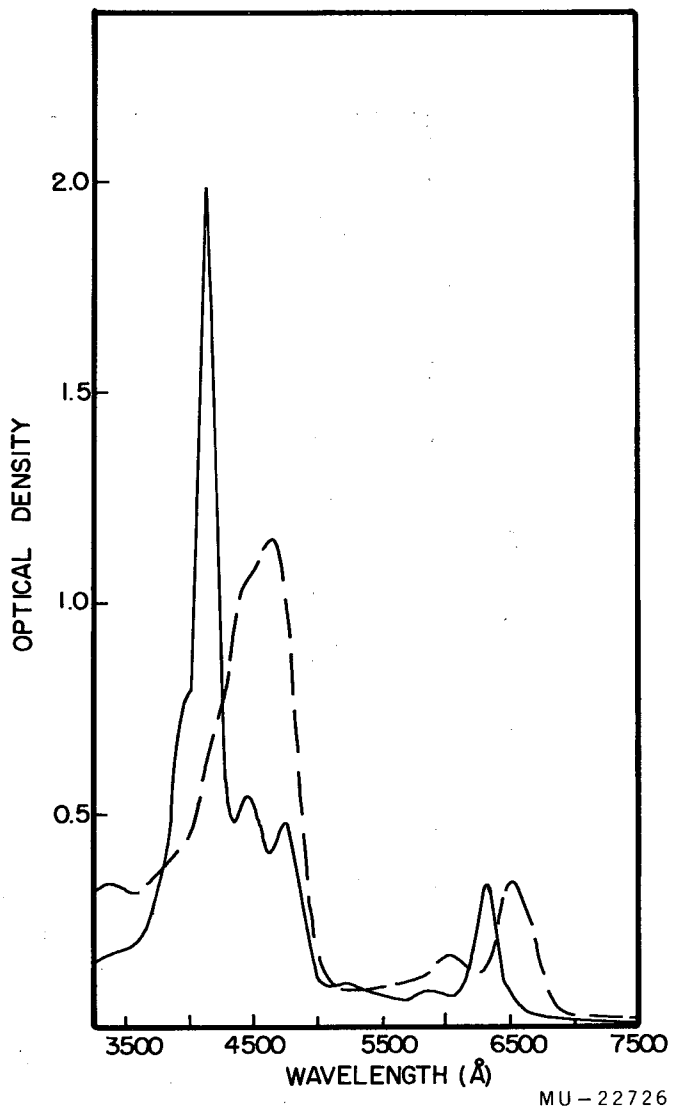


Fig. 50. Reduction of chlorophyll b with sodium borohydride ($NaBH_4$).
– – – – Visible spectrum of chlorophyll b in ethanol
——— Visible spectrum of chlorophyll b reduced with $NaBH_4$.

To remove essentially the carotenes and quinones from the plant material, Bishop's method (63) of extracting freeze-dried chloroplasts and Chlorella with petroleum ether was tried as a means of isolating the small quantities of quinone present, but the method was not successful.

Because of the inconsistency of the spinach (winter caused the formation of large amounts of carotenoids in the plants) and the questionable value of the Celite adsorption technique, it was decided to tackle again the separation of methanol extracts of Chlorella. In the "acetone-spinach separation" the acetone-water phase on evaporation under vacuum produced a sticky mass which was hard to chromatograph; we anticipated a similar difficulty would present itself with the methanol-water phase separation. Joan Anderson (23) separated the photosynthetic pigments from methanol-water solutions on polyethylene columns, and this could be tried on the methanol-water phase which would result when we treated the methanol extract of the algae with isooctane.

The partitioning of the xanthophylls into the methanol-water phase and the chlorophylls into the petroleum ether phase would still be practiced, as the ESR properties of the films prepared from these two phases were different; however, the Celite adsorption of the chlorophylls would be dropped.

Isolation of Pigments from Methanol Extracts from Chlorella (II)

The general outline of the separation procedure is as shown in Figure 51. Samples were taken as indicated and their pigments transferred to ether that had been washed with sodium carbonate and then redistilled from sodium.

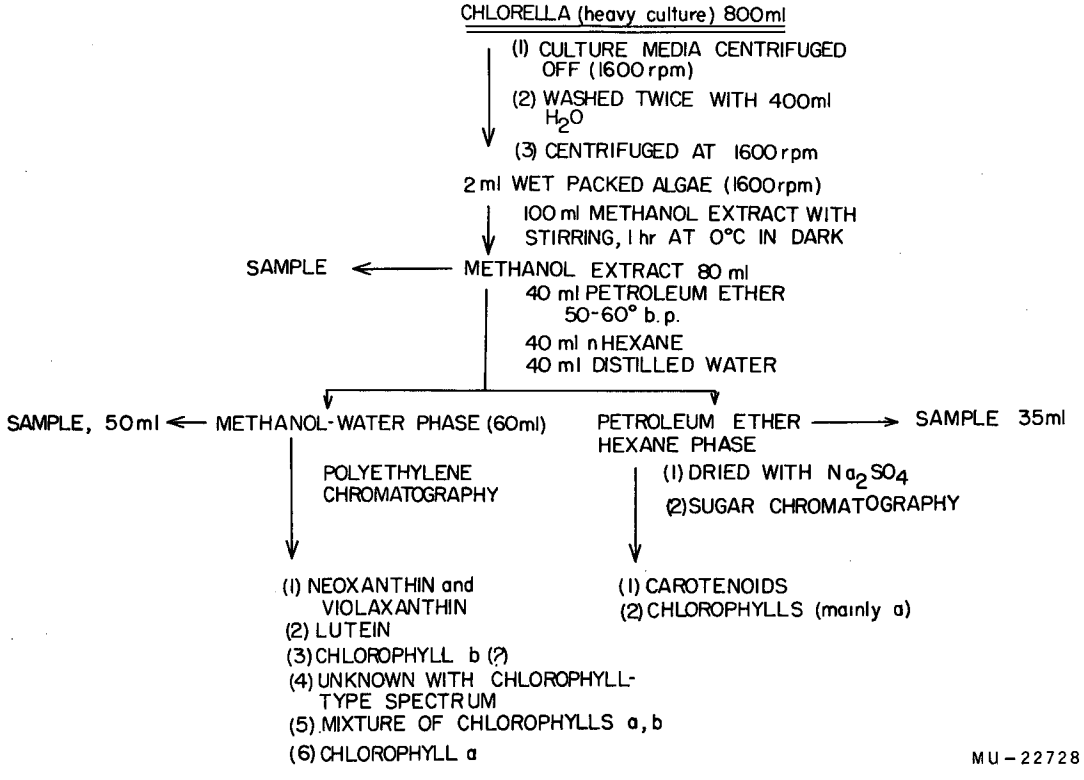


Fig. 51. Isolation of plant pigments from Chlorella.

Polyethylene Chromatography

Columns (2 cm diameter) in a Buechner flask, so that gentle suction could be applied, were packed in small lots (30 ml beakers-full) with polyethylene powder (MI = 0.044) to a depth of 20 cm, with a piece of filter paper placed on top. The methanol-water phase was applied directly to the column, which was then developed with 80% aqueous methanol. The various zones on the column were eluted from the column with this solvent; after the first zone had been eluted the column was developed with 95% methanol to give a complete elution of all material on the column. The eluted pigments were transferred to purified ether by the technique described in Part I without the addition of sodium chloride, and their visible spectra measured on a Cary Model 14R Spectrometer.

Sugar Chromatography

A column (2 cm diameter) was packed to a depth of 18 cm with C&H confectioners' sugar and inserted in a Buechner funnel, so that slight suction could be applied to effect a suitable flow rate of solvent (1 ml/min) through the sugar column. The petroleum ether fraction, from the phase separation of the original methanol extract, was applied to the sugar column and then developed with 1.5% isopropanol in isooctane. The yellow fraction of the carotenoids was washed through and then the chlorophylls. The eluted fractions were evaporated under vacuum to remove the isooctane;isopropanol solvent and the solids redissolved in ether to determine their spectrum.

Discussion of Part II

The use of powdered polyethylene as a chromatographic adsorbent for the separation of the pigments in the methanol-water phase was

successful. The pigments separated are listed in Figure 51 in order of their elution from the polyethylene column, and their spectra are shown in Figures 52, 53, and 54.

The appearance of pigments (Fig. 53), one with absorption bands at 638 mμ and 448 mμ and the other at 645 mμ and 455 mμ, is of interest, and although they have visible spectra which are characteristic of chlorophylls, we have not been able to identify them; however, they are probably allomerised chlorophylls introduced by the extracting agent (methanol). The pigments separated on sugar are shown in Figure 55, and the larger than reported intensity of the ultraviolet region of the carotenoids' ultraviolet spectra should be noted. In one experiment we managed to elute a pale yellow band, coming just after the carotenes, which had strong ultraviolet absorption and was perhaps due to the presence of quinones.

The procedure given in Part II allows an excellent mapping of the pigments extracted from Chlorella with methanol and opens up the extract for detailed testing of the constituent pigments for photo-induced paramagnetism. We also note that polyethylene chromatography has separated the three classes of pigments but did not separate the chlorophylls satisfactorily under the conditions we used. Further, the separation of the chlorophylls from a phase separated isooctane solution of a mixture of chlorophylls and carotenoids always led to carotenoids passing through the column before the chlorophylls. From these two observations we concluded that if we could completely separate the carotenoids from the chlorophylls on a polyethylene column and then be faced with separating the chlorophylls on a sugar column, we could probably have an excellent procedure for separating chlorophyll a.

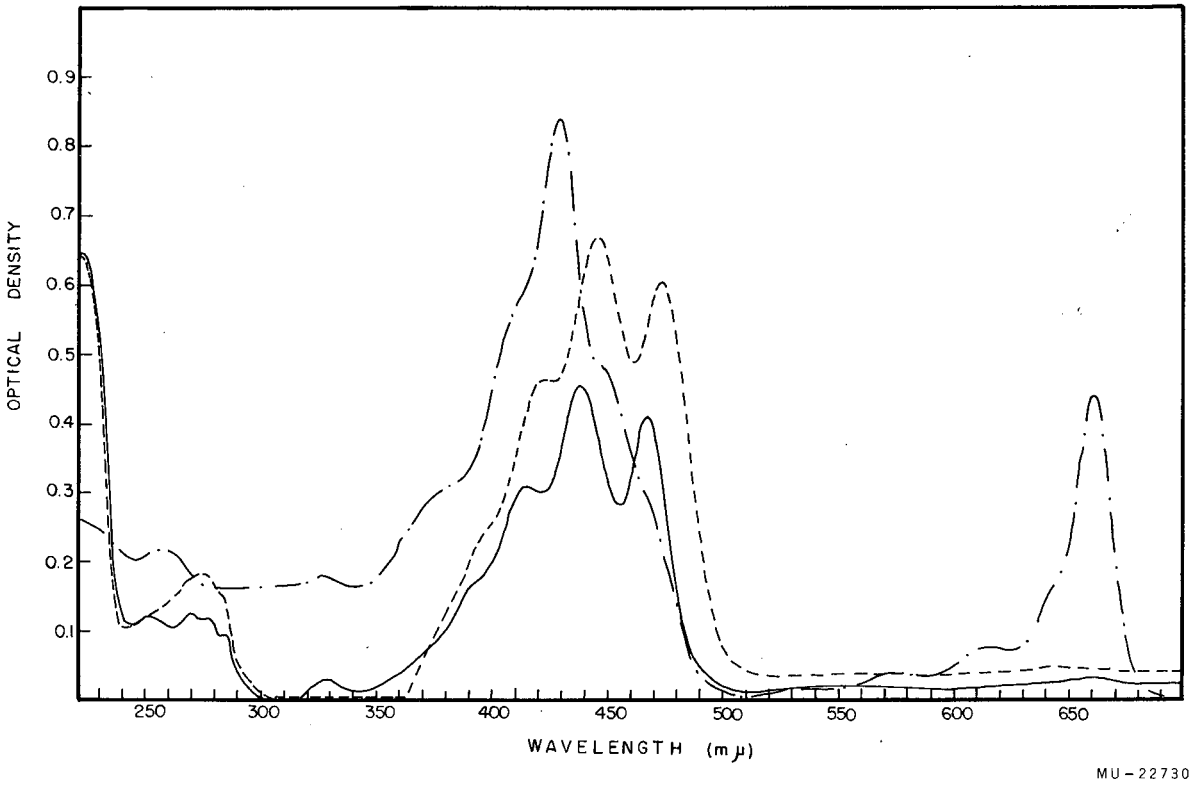


Fig. 52. Polyethylene chromatography. Fractions 1 and 2.

—·— · Methanol-water phase
----- Fraction 1. Neoxanthin.
——— Fraction 2. Lutein.

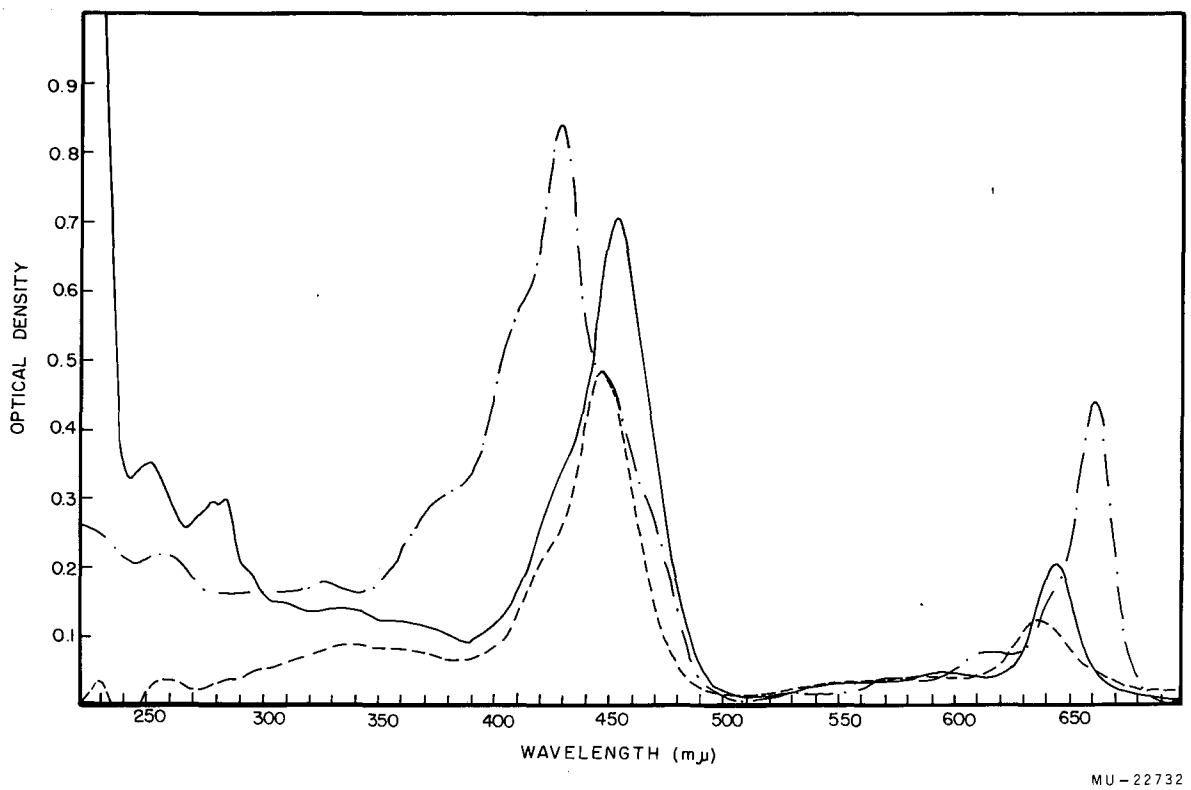


Fig. 53. Polyethylene chromatography. Fractions 3 and 4.

— • — • — MeOH-H_2O phase, containing all the pigments.
— — — — Fraction 3. Protochlorophyll.
———— Fraction 4. (Chlorophyll b).
Or 3 and 4 could be allomerised chlorophylls.

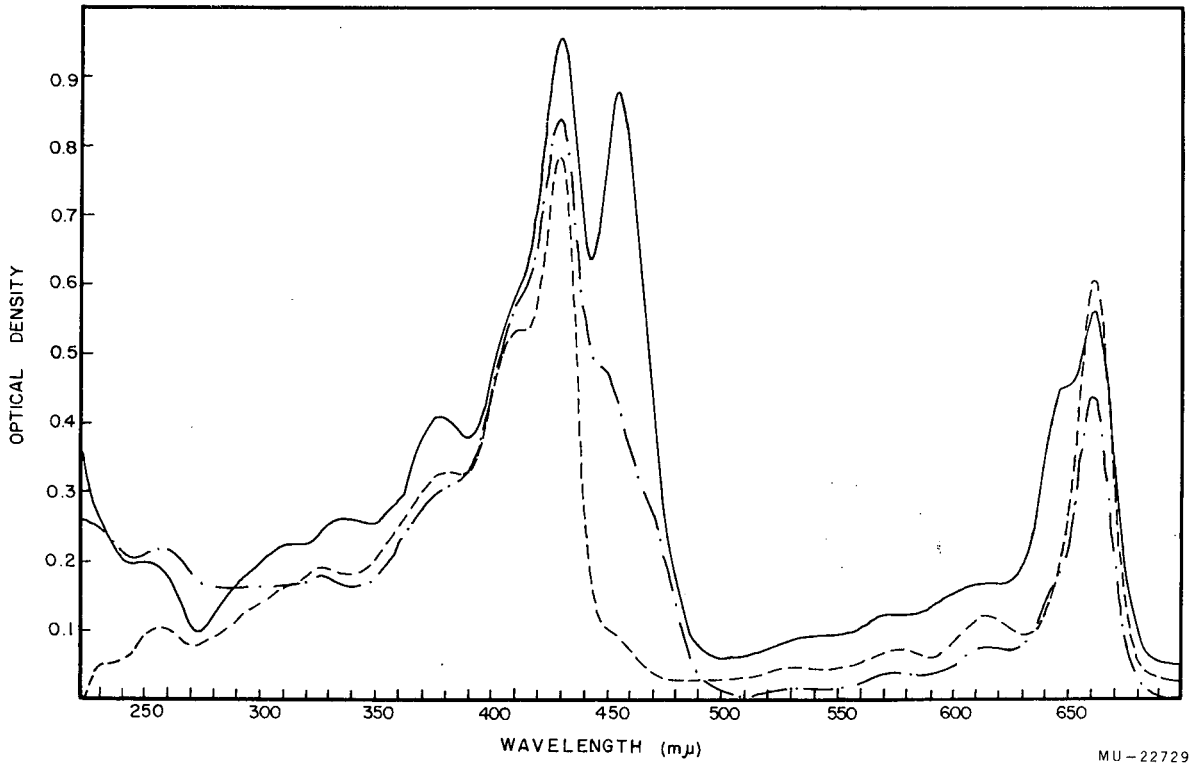


Fig. 54. Polyethylene chromatography. Fractions 5 and 6.

—•—•— MeOH-H_2O phase, containing all the pigments
——— Chlorophyll a and b. Fraction 5.
– – – – Chlorophyll $\bar{a}$. Fraction 6.
Solvent: ether.

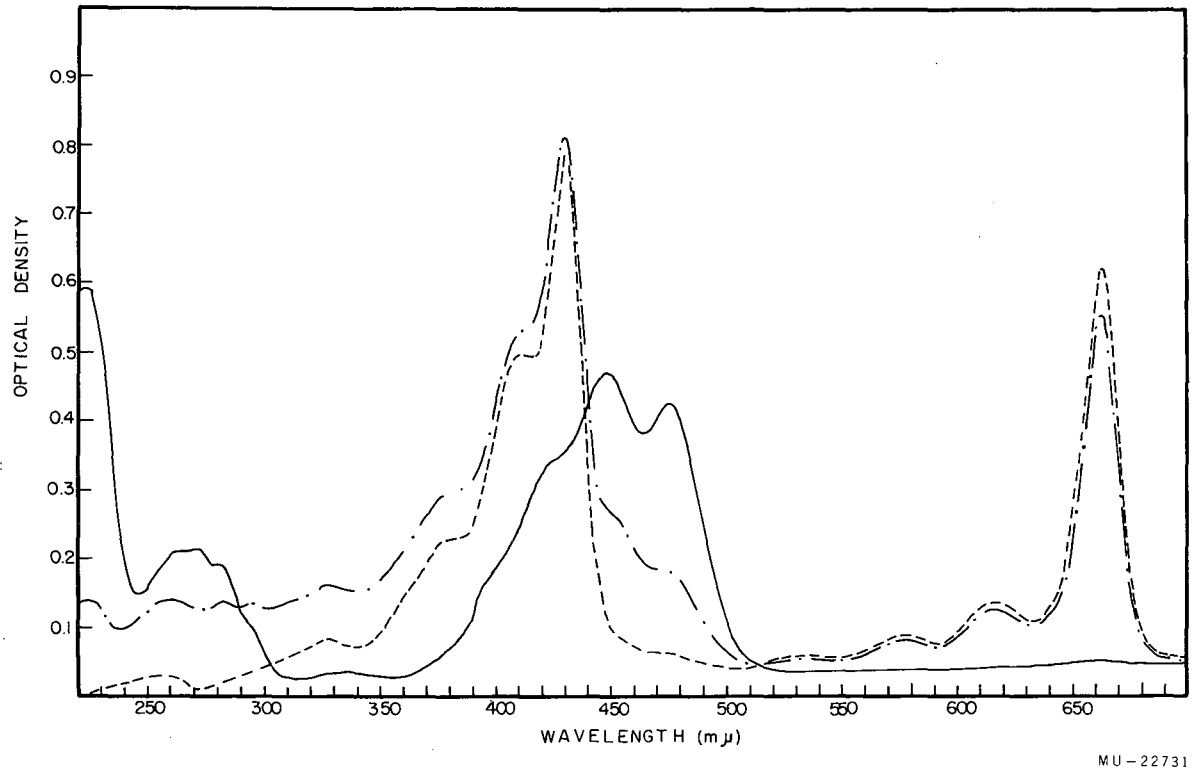


Fig. 55. Sugar chromatography of petroleum ether fraction.
- - • - - • - - Petroleum ether phase absorption spectrum
- - - - - - - - - Carotenes
- - - - - - - - Chlorophyll a absorption spectrum in ether.

Separation of Chlorophylls from a Methanol Extract of Chlorella with a Combination of Polyethylene-Sugar Chromatography (III)

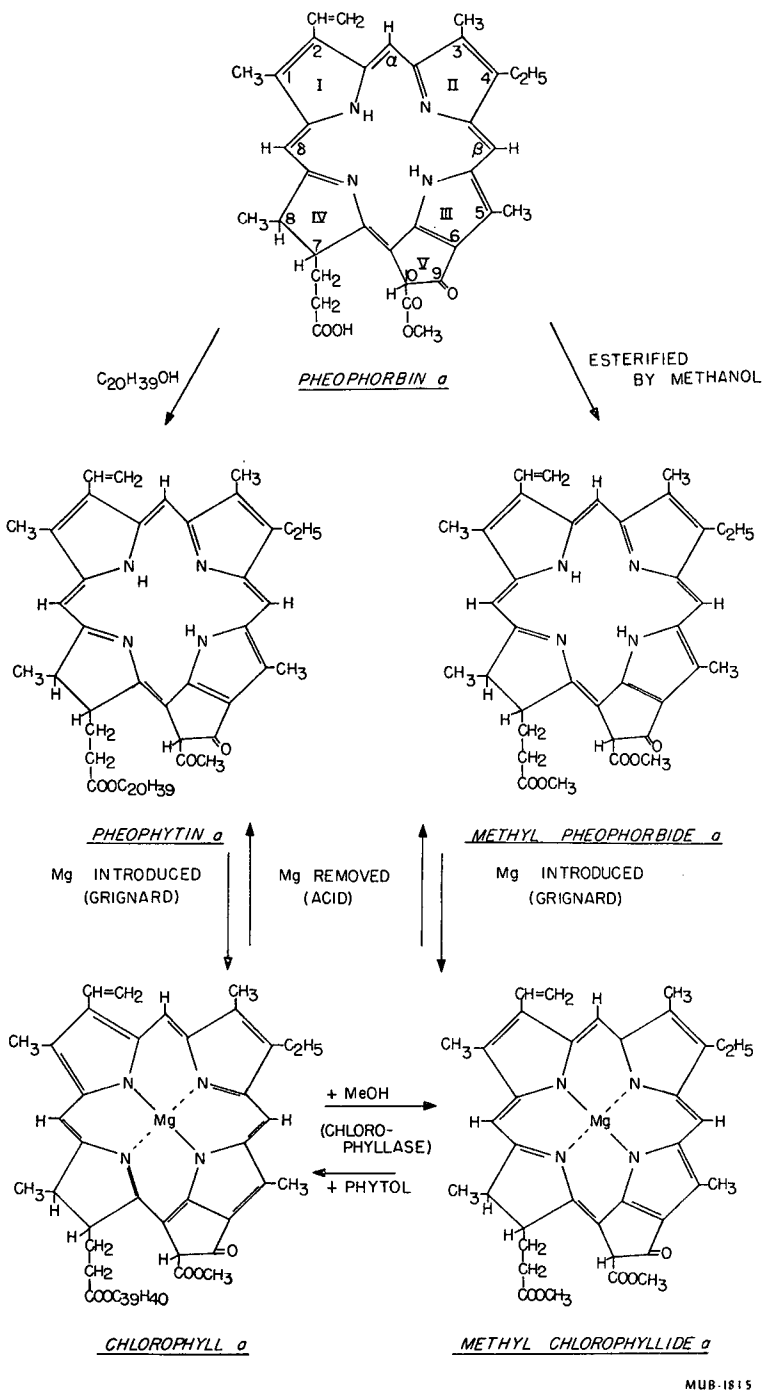
The methanol extract of the Chlorella algae was prepared as follows: 250 ml of Chlorella culture was entrifuged at 2,000 rpm for 10 min to remove the culture medium. The wet packed algae (about 5 cc) were washed twice with distilled water, extracted with 100 ml of 100% methanol (acetone could be used as an extracting solvent, but it was not as effective as methanol in extracting all the pigments) at room temperature for one hour, and the insoluble residue centrifuged down. The supernatant methanol extract of the pigments was removed and diluted to make a 70% methanol-water solution of the pigments, which was then chromatographed on polyethylene as described in Part II. The chlorophylls were eluted from the polyethylene column with 90% methanol-water and collected free from xanthophylls. The chlorophylls were then transferred into 20 ml of isooctane and chromatographed on sugar by the procedure described in Part II, but now each lot of sugar was tamped down with a wooden rammer to give a more solid column of sugar. Elution of chlorophyll a was carried out with 1% isopropanol in isooctane, and the only predominant pigments on the column were chlorophyll a and b with small traces of green pigments, which were probably allomerised chlorophylls formed because of the use of methanol as an extracting solvent. The eluted chlorophyll a in this experiment was a deep blue, whereas the chlorophyll a obtained by the phase separated procedure was more greenish blue, presumably because of the presence of carotenoids. When the eluted chlorophyll a was recovered by evaporating

off the isooctane under vacuum and its spectrum taken in purified ether, the ratio of the optical densities of the blue to red bands was 1.19.

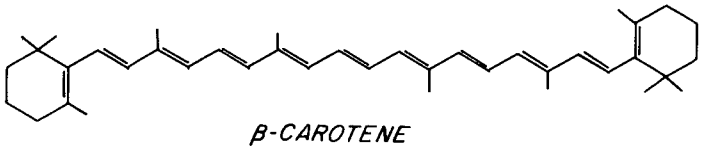
Discussion

A very simple method which had originally been found by Anderson et al. (23) for separating the three classes of pigments (chlorophylls, carotenes, and xanthophylls) found in plants had been confirmed. Chlorophyll a could be separated from the chlorophyll fraction, from the methanol water solution, in excellent purity by means of sugar chromatography. Two awkward steps, the distribution of chlorophylls into isooctane from the original extract and the separation of the carotenes from chlorophylls on diatomaceous earth, had been dispensed with in the extraction of chlorophyll a.

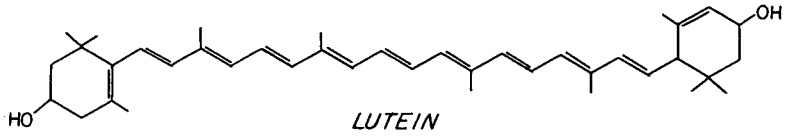
By scaling up the procedure described above and using acetone rather than methanol, which reacts with the chlorophylls, changing to an 0.5 n-propanol-isooctane solvent rather than a 1% isopropanol-isooctane solvent, which gives better resolution of the pigments on the sugar column, an elegant method for preparing hundreds of milligram quantities of chlorophyll a and chlorophyll b could be obtained, leading to samples of chlorophyll a suitable for a study of the molecular biology of chlorophyll a.



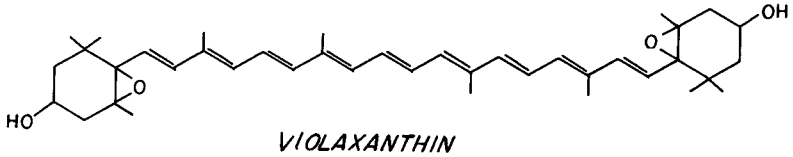
Chlorophyll a and its derivatives.



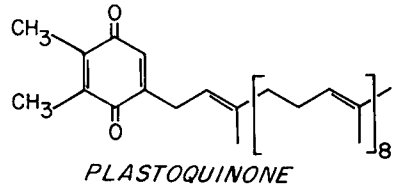
β-CAROTENE



LUTEIN
(XANTHOPHYLL



VIOLAXANTHIN
(XANTHOPHYLL)



PLASTOQUINONE

MU-30452

Pigments associated with chlorophyll a.

GENERAL CONCLUSIONS

A simple procedure for preparing extremely pure crystalline chlorophyll *a* has been developed, as well as an improved method for preparing crystalline methyl-chlorophyllide *a*, which is the photochemically active site of the chlorophyll *a* molecule.

These procedures, which are much simpler to carry out than those devised heretofore, will allow the molecular biology of chlorophyll *a* to be more conveniently investigated in general.

The chlorophyll *a* prepared by this procedure has been used to reevaluate many of the molecule's spectroscopic properties, which had in certain cases been uncertain.

We have shown conclusively from infrared and visible absorption spectroscopy that chlorophyll *a* aggregated to form dimers under certain conditions, and the specific site of the bonding between molecules has been found. The dimers have, we think, physical properties similar to the physical state of the chlorophyll *a* in the cell.

Some new knowledge, from nuclear magnetic resonance, has been found concerning the fine structure of chlorophylls, which can be used to study the possible chemical functions of the molecule in photosynthesis.

We have demonstrated that our purified crystalline chlorophyll *a* has ESR properties which are different from previous preparations. These properties of our sample show that we are now dealing with a sample of chlorophyll *a* whose electronic properties will be more reproducible because of the removal of unknown impurities. This puts us in a good position for learning more about the energy transfer among chlorophyll *a* molecules--a topic of great importance in its molecular biology.

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