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PART II. THE RELATION OF BRANCHED-CHAIN SUGAR ACIDS TO THE CYANIDE INHIBITION OF CO_2 FIXATION

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RESEARCH IN PHOTOSYNTHESIS:

Part I. Biosynthetic Studies on the Chlorophylls and Carotenoids of Algae

Part II. The Relation of Branched-Chain Sugar Acids to the Cyanide Inhibition of CO_2 Fixation

Joan Mary Anderson

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RESEARCH IN PHOTOSYNTHESIS:

PART I. BIOSYNTHETIC STUDIES ON THE CHLOROPHYLLS AND CAROTENOIDS OF ALGAE

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I. INTRODUCTION

"Les vegetaux ne sont que des laboratoires qui..."[1]

The importance of plant pigments in nature cannot be overemphasized. Without these pigments, life as we know it would rapidly vanish, since the ultimate maintenance of life on the earth is dependent on the plant pigments, which are responsible for the conversion of the solar energy into chemical potential energy. Indeed, the advent of aerobic life itself was only possible after the production of oxygen in the atmosphere by green plants.

The plant pigments play a vital role in photosynthesis, which is the process whereby green plants in the light convert water and carbon dioxide to carbohydrates and oxygen. This reaction is separated into two parts: the photolysis of water

$$H_2O \xrightarrow{nh\nu} 2\ [H] + [O]$$

and the reduction of carbon dioxide.

$$CO_2 + 4\ [H] \longrightarrow CH_2O + H_2O$$

In 1782, Senebier, one of the early experimenters in photosynthesis, suggested for the first time the importance of the "green" material in plants.[1] He regarded plants as nothing but laboratories, which were responsible for the maintenance of the atmosphere around us. The wonderful complexity of these "plant laboratories" has fascinated many workers ever since.

Despite the brilliant deductions of Senebier the early investigators of the nineteenth century such as Berthollet, Berzelius and Proust were

concerned only with the chemical composition of the plant pigments. This line of investigation led to the discovery of several selective separatory methods, the most important of which was chromatographic adsorption, a technique which has revolutionized the whole field of chemistry and biochemistry.

Until the advent of labelled atom studies, the concepts of the biosynthesis of the plant pigments lacked corroboration. Much experimental data had been collected on the accumulation of plant pigments under a variety of environmental conditions, but conclusive data could only be furnished by tracer studies. The combination of chromatographic methods and radioactive isotope studies not only led to our present knowledge of the plant pigments but also towards an understanding of the whole vast process of photosynthesis.

HISTORICAL BACKGROUND

Chemistry of chlorophyll

The suggestion of the term "chlorophyll" for the green pigment of plants was made by Pelletier and Caventou in 1818;[2] later in 1832 these authors also realized that chlorophyll was not a single substance, but a mixture.[3] Thirty-two years later the physicist Stokes proved the above hypothesis while investigating fluorescence phenomena.[4] The actual separation of the chlorophyll components was achieved by Tswett, in 1906, by the method of adsorption chromatography.[5]

The elucidation of the chemical structure of chlorophyll was initiated by Willstatter and coworkers.[6] Chlorophyll was shown to contain

magnesium in 1907, to be a phytyl ester in 1907, and the empirical formula was established in 1911.[7] This fundamental work was further developed by Stoll in Switzerland, Conant in America, and most persistently and successfully by Hans Fischer in Germany. The accepted structure of chlorophyll a was published by Fischer in 1939.[7] The only point of doubt was the hydrogen atoms at positions 7 and 8 in ring IV of the chlorophyll molecule (Fig. 1). Lindstead confirmed the presence of the two hydrogens in ring IV at the positions shown, and presented good reasons for assigning a trans-configuration to the chlorophylls and the natural products derived from them.[8]

The formulae and molecular weights of the chlorophylls are as follows:

Chlorophyll a: $C_{55}H_{72}O_5N_4Mg$; mol. wt. 893.48

Chlorophyll b: $C_{55}H_{70}O_6N_4Mg$; mol. wt. 907.46

As can be seen from the formula (Fig. 1), the chlorophylls are dihydroporphin structures, the double bond conjugation of the porphyrin rings being interrupted in ring IV. The chlorophylls may be regarded as methyl phytyl esters of the parent dicarboxylic acids, the chlorophyllins. Phytol ($C_{20}H_{39}OH$) is a diterpene alcohol containing one double bond; it is related to the carotenoids and can be regarded as derivable from vitamin A by hydrogenation.

The chlorophylls are very labile compounds. Treatment with dilute acid will remove the non-ionizable magnesium from the centre of the molecule to produce phaeophytins; stronger acid will remove the phytyl group also to yield phaeophorbides. Removal of the phytol residue without removal of the magnesium atom is accomplished by the enzyme chlorophyllase,

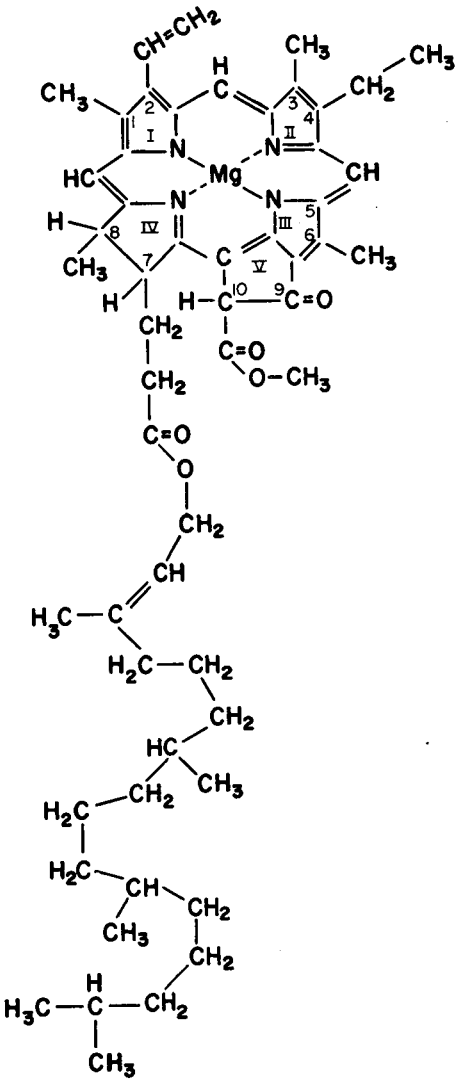

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Fig. 1. Structural formulae for chlorophyll a and b. The methyl group attached to carbon atom 3 in ring II is replaced by a formyl group ($-\overset{H}{C}=O$) in chlorophyll b.

which is present in most plants to a greater or lesser extent. In ethanol or methanol, the phytyl residue is replaced by ethyl or methyl group to give ethyl or methyl chlorophyllide. The action of hot alkali on phaeophytin, phaeophorbide, or the chlorophyllides is to saponify the ester linkage, break the isocyclic ring V and yield chlorin-e, a compound which contains three free carboxyl groups.

Chemistry of the carotenoids

The yellow alcohol-soluble pigments of autumn leaves were called xanthophylls by the Swedish chemist Berzelius, in 1837.[9] At that time it was suggested that the yellow pigments might also occur in green leaves, but this was not established experimentally until 1860 by Fremy[10] and by Stokes,[11] both of whom succeeded in separating the yellow pigments from the chlorophylls in leaf extracts. Later, following the classical work of Tswett[5], the xanthophylls were shown to be a complex mixture of compounds. These compounds may be divided into two classes: the hydrocarbons which are termed "carotenes" and the oxygen-containing derivatives which are termed "xanthophylls";[12] the oxygen can occur in hydroxy-, methoxy-, epoxy-, carboxy- or carbonyl groupings.

The general class is termed the "carotenoids." Karrer's definition states that "carotenoids are yellow to red pigments of aliphatic or alicyclic structure composed of isoprene units (usually eight). It is characteristic of all carotenoids that the arrangement of the isoprene units becomes reversed in the centre of the carotenoid molecule, so that the central methyl groups occupy 1:6 positions instead of 1:5 positions (Fig. 2); this series of conjugated double bonds constitutes the chromophoric system of the carotenoids."[13]

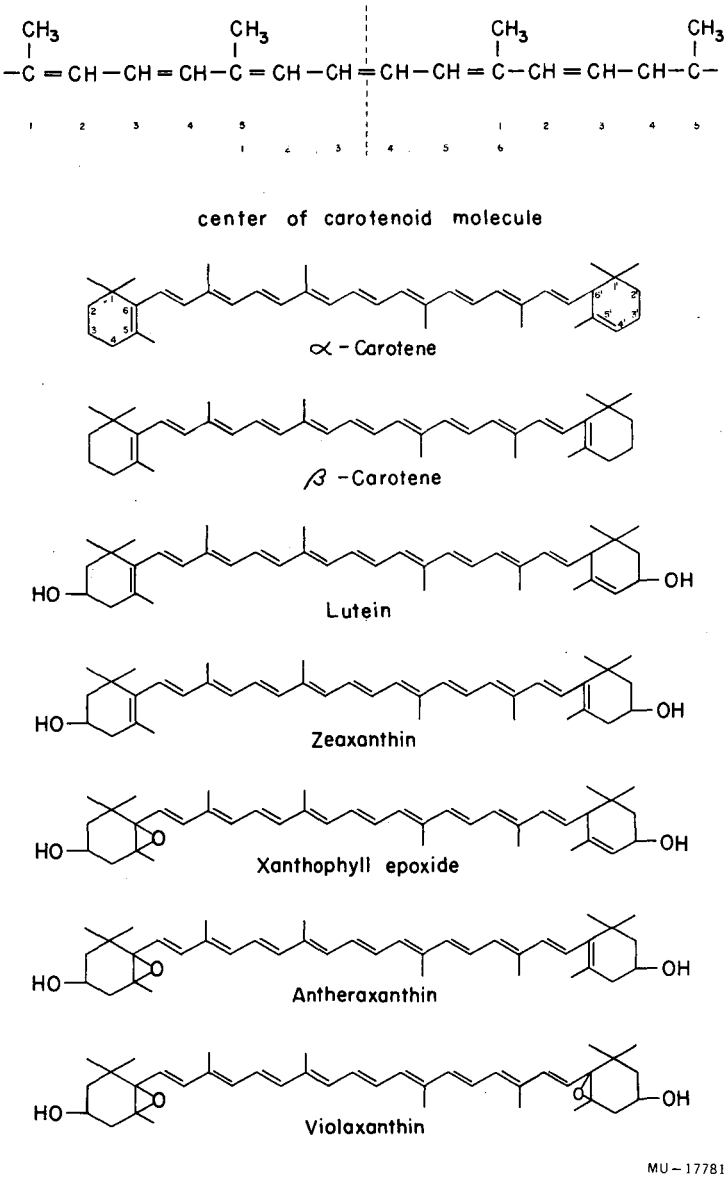

Fig. 2. Structural formulae of the Carotenoids.

The empirical structure of the carotene of carrots and green leaves was established in 1907 as $C_{40}H_{56}$.[14] Zechmeister and Cholnoky in 1928 showed that this material possesses eleven double bonds,[15] and in 1930 Karrer *et al.* proposed the structure of β-carotene which is recognized today[16] (Fig. 2); he synthesized this molecule in 1950.[17] It can be seen that β-carotene possesses a centre of symmetry; oxidative splitting of the molecule at the centre, yields two molecules of vitamin A. The isomeric α-carotene contains eleven double bonds, but only ten of these are conjugated, the last being isolated in the second β-ionone ring (Fig. 2).

Much of the knowledge of the isolation and different varieties of xanthophylls is due to Strain[12], while the chemistry of these molecules is largely due to careful research work of Karrer.[13] The predominating xanthophyll found in nature is lutein, 3,3'-dihydroxy-α-carotene (Fig. 2). The corresponding dihydroxy derivative of β-carotene is zeaxanthin, 3,3'-dihydroxy-β-carotene; the structure of both these substances was elucidated by Karrer.[16] Other carotenoids of interest because of their isolation from algae are the di-epoxides ($C_{40}H_{56}O_4$), violaxanthin whose constitution was established by Karrer to be 5,6,5',6'-di-epoxyzeaxanthin[13], and neoxanthin, which is also a di-epoxide, isolated and characterized by Strain[12]; the structure of neoxanthin, however, is not known. The mono-epoxides ($C_{40}H_{56}O_3$), were also characterized by Karrer.[13]

It is obvious that molecules with structures such as the carotenoids possess, have many spatial possibilities; thus, a polyene with nine conjugated double bonds can theoretically occur in 512 different *cis-trans* isomeric forms. The stereochemistry of the carotenoids has been extensively studied by Zechmeister.[18] The all-*trans* form of a carotenoid, the most

stable form because of its low energy content, can undergo trans-cis isomerization in solution yielding a complex mixture of isomers. Such changes are accelerated by light, heat, and by the addition of acid or iodine. On the basis of x-ray analysis, spectral analysis, and chromatographic analysis, it may be assumed with a very few exceptions that the naturally-occurring carotenoids possess entirely trans-configurations.

Occurrence and distribution of plant pigments

Chlorophyll a occurs in all higher plants, and in all organisms which evolve oxygen in photosynthesis. Chlorophyll b occurs in all higher plants also, and in green algae. In the brown algae, diatoms and dinoflagellates in which chlorophyll b is absent, chlorophyll c occurs in about one-tenth of the total chlorophyll content. Chlorophyll b is replaced by chlorophyll d in red algae. The empirical and structural formulae of both chlorophyll c and d are unknown, although good evidence has been presented by Holt in 1959 to indicate that chlorophyll d is equivalent to 2-desvinyl-2-formyl-chlorophyll a.[19] The purple and brown bacteria contain bacteriochlorophyll, which is closely related to chlorophyll a (Fig. 1); the vinyl group at position 2 in ring I is oxidized to an acetyl group, and ring II is hydrogenated to make bacteriochlorophyll, a tetrahydroporphin derivative. Thus chlorophyll a is the most abundant chlorophyll and its occurrence is universal, except for the purple and brown bacteria.

The ratio of chlorophyll a to b remains remarkably constant, varying only between 2.5 to 4.0; in general, the ratio of chlorophyll a to b is lower in "shade" plants than in "sun" plants.

Carotenoids occur in the grana of the chloroplasts of all higher plants, together with the chlorophylls. The fruits, flowers, roots and

pollens also contain carotenoids which, in general, vary from those present in the chloroplasts. Most of the cryptogams contain carotenoids; however, very large differences occur within the different subgroups, algae, lichens, fungi and so on; indeed, large differences occur within each subgroup. Since over 85 natural carotenoids are known at present, the subject of their distribution in nature is rather complex, but is reviewed by both Karrer and Goodwin.[13,20] The two carotenoids which occur in the highest proportions and in the greatest variety of plants are β-carotene and lutein.

The ratio of the concentration of the chlorophylls to the carotenoids appears not to remain as constant as that of chlorophyll a to b; in general, there is more chlorophyll present than carotenoids. The ratio of the xanthophylls to the carotenes may vary from about 2 to 8.

The chlorophylls and carotenoids are probably present in the chloroplasts, not as the free pigment molecules but complexed to protein, or lipoprotein, macromolecules.

Role of plant pigments in photosynthesis

It is generally conceded that both the chlorophylls and carotenoids have some indispensable function in photosynthesis. The obvious importance of chlorophyll a as the major pigment involved in the absorption of electromagnetic energy and its conversion to chemical energy is indicated by the correspondence of the action spectrum to the absorption spectrum. Little is known about the chemical events in this transformation, or about the changes undergone by chlorophyll in vivo. The type of

approaches being made in this field to study this important mechanism is amply illustrated in the review "The photochemical apparatus -- its structure and function",[21] where reports on photoconductivity, absorption, fluorescence and electron spin resonance experiments are listed.

Although the physical role of the chlorophylls has been shown, there have been attempts to implicate a chemical role for these compounds in photosynthesis. At one time Warburg proposed that carbon dioxide was bound to the chlorophyll a molecule at position 10, ring V (Fig. 1), since the addition of fluoride to plant systems caused the expulsion of CO_2, in stoichiometric amounts equivalent to the chlorophyll present.[22] Franck believes that the hydrogen present in position 10, ring V in the enolic form of chlorophyll a may be involved in the electron transfer which originates from photoactivated chlorophyll.[23] Some evidence for this has been obtained recently by Vishniac and Rose, who found that the C_{10} position mentioned became tritiated in tritiated water studies.[24]

The universal occurrence of the carotenoids as the second major class of autotrophic pigments suggests the importance of these compounds in photosynthesis. This function has been formulated in various ways; as energy transport to and from the chlorophylls,[21,25,26] in terms of energy conversion,[27] or of electron transport.[28] In all probability these various formulations are all manifestations of the same act, which is intimately connected with the absorption of the solar energy and its conversion to chemical energy.

In addition to the above physical functions, several chemical roles have been suggested for the carotenoids. An early attempt to prove a photosynthetic oxygen transport function, using H_2O^{18} was unsuccessful, but this idea may be feasible.[29] A photo-oxidation/protection mechanism was also

suggested and recent work indicates at least the possibility of such a role for carotenoids being tenable.[30] Yet another function has been proposed, that of a non-photosynthetic oxygen transport (i.e., oxygen absorption).[31]

Purpose of study

The purpose of this study was as follows:

(1) The development of rapid and accurate techniques for radioactive tracer studies of the algal pigments.

(2) The determination of the specific radioactivities of the chlorophylls and the carotenoids of algae. It was hoped to gain some insight into the problem of the relationship between chlorophyll a and b, and to look at the possible relationships which might exist between the carotenoids.

II. EXPERIMENTAL PROCEDURES

Standard Abbreviations

The following abbreviations have been employed throughout this text:

ml w p cells	:	volume in ml of wet packed algal cells as measured after standard centrifugation (cf. extraction of algae)
R_f	:	ratio of the distance travelled by the centre of the spot to the distance travelled by the solvent front
O	:	origin of chromatogram
SF	:	solvent front of paper chromatogram
EDTA-Na_4	:	ethylenediaminetetraacetic acid-tetrasodium salt
PPO	:	2,5-diphenyloxazole - primary scintillator "fluor"
POPOP	:	1,4-bis(2-(5-phenyloxazole))benzene -- secondary scintillator "fluor"
MeOH	:	methanol
EtOH	:	ethanol
pet ether	:	petroleum ether

Plant Material

The plants used throughout for the pigment studies were the unicellular green algae, Chlorella pyrenoidosa Chick and Scenedesmus obliquus Turpin (Kuetying); both of these algae are being continually cultured in this laboratory in Myer's medium.[32]

Two methods of culture are employed: variable density and constant density cultures. In the variable density culture method, the liquid culture of algae is inoculated into a large volume of nutrient solution which is contained in a circular, flat-bottomed flask equipped with an inlet and outlet for the addition of nutrient and aeration with 4% CO_2-in-air. The flasks are shaken horizontally in a thermostatically-controlled water bath and illuminated through a transparent panel by fluorescent lights; the intensity of illumination at the bottom of the vessels is about 2,000 foot-candles. Each day a certain volume of algae is removed and more nutrient solution added. Hence the conditions are identical from day to day, although the effective light intensity varies daily, decreasing as the algae increase in density.

The constant density culture apparatus consists of two vertical concentric cylinders, the outer one contains the algae suspension through which 4% CO_2-in-air is bubbled, and water at a controlled temperature is circulated through the inner cylinder. The apparatus is illuminated with a circular bank of fluorescent lights which give a light intensity of about 3,000 foot-candles at the outer surface of the algal suspension. A photocell records the density of the algae, and a solenoid-operated valve allows fresh nutrient to enter the tube, which causes the excess algal suspension to overflow out the top; thus the density is maintained constant.

Extraction of Algae

Several different extraction procedures were used at various times for different purposes; however, in all cases the volume of algal cells

was measured after centrifugation under the same standard conditions. It is important that standard conditions of centrifugation should be employed throughout a study, since all the pigment concentrations will be reported in terms of the volume in ml of wet packed algal cells from which they were extracted.

The conditions of centrifugation are as follows, for the recording of the accurate volume in ml of wet packed algal cells: The algae were spun down in the appropriate sized centrifugation vessels (250, 100 or 40 ml), most of the medium decanted, and the cells stirred up in the remainder of the medium and transferred to tapered centrifuge tubes (B.K.H. sedimentation tubes No. 21068 - 10 ml). The centrifugation was done in an International centrifuge No. 1 (which had been fitted with refrigerating coils so that the centrifugation could be carried out at lower temperature), for 5 min at a speed of 2,850 r.p.m. The cell volume was read, the medium decanted off, and the appropriate extraction method applied immediately to the cells.

Total pigment extract

Algal extracts were needed for the determination of the concentration of the pigments of algae by spectral means, and also for trial chromatography runs. Such total pigment extracts were obtained in the following manner, taking care to work as rapidly as possible and to shield the extracts from the light.

The algal cells were centrifuged under the standard conditions mentioned above, the volume of cells was read before the medium was decanted off, and the cells were then suspended in 98% MeOH (approx. 1.0-1.5 ml MeOH/0.1 ml w p cells which gives an 85-90% MeOH extraction). The methanolic algal

extract was vigorously stirred with a thin glass rod for a few minutes. The tube was then immersed in a steam bath for the minimum possible time, i.e., just until the mixture begins to come to the boil, and then immediately cooled in ice. The tubes were then rapidly centrifuged, using a bench centrifuge, for 1 to 2 minutes at full speed. The methanolic extract was poured off into a tube and cooled in ice in the dark. The cell residue was extracted again in a similar manner. Two such extractions were generally sufficient to give a colourless cell residue; however, if the cells retained any colour, the extraction was repeated. Diethyl ether (ether-MeOH 1:1) was added to the combined cooled, methanolic extract and gently shaken (the formation of emulsions was minimized by the addition of the ether to the alcoholic solution before the addition of the water to form two layers). Sodium chloride solution (5%) was cautiously added to the tubes until two layers were formed. The tubes were centrifuged for one minute, and the ether layer removed and washed two or three times with water to free the ethereal solution from any water-soluble material. (This procedure was carried out in a separatory funnel if more than 0.3 ml w p cells were to be extracted.)

The ethereal layer was transferred to volumetric tubes, dried with a little anhydrous Na_2SO_4 and kept in the dark at 0° until ready for use.

Carotenoid extract

In order to have pure carotenoid extracts, it was possible to remove the chlorophylls by saponification with methanolic KOH. It was of interest to prepare such carotenoid extracts for spectral examination and chromatography and to compare and check the results obtained with the total extract.

The algae were centrifuged using the above conditions, the volume of w p cells noted before decanting off the medium, and a methanolic-KOH solution (aqueous KOH (1 ml of 60% w/v) in 10 ml MeOH), added to the algal cells (approximately 10 ml of KOH-MeOH reagent/0.5 ml w p cells). The cells were vigorously stirred with a thin glass rod and the tubes immersed at 40° for about 5 minutes, in the dark. The cells were centrifuged for 1 minute, the medium decanted off, and the cell residue extracted with MeOH until the methanolic extract was colourless; usually once or twice was sufficient. Finally the cell residues were extracted with ether, and the combined methanolic and ethereal extract, which had been kept in the cold and dark, was shaken gently with ether. Sufficient ether was added to make the solution approximately 1.0 part of MeOH to 0.75 parts of ether. Distilled water was added down the side of the tube or separatory funnel, until two layers were formed. The ethereal layer was washed several times with water, dried over anhydrous Na_2SO_4, and kept in the dark at 0° until ready for use. As long as the ethereal extract was not kept for long, no adsorption of the pigment on the Na_2SO_4 occurred.

The relation of w p cells to the dry weight of algae

The dry weight of the algal cells was obtained in the following manner: Different volumes of the algae, Chlorella and Scenedesmus (2.0, 1.5 and 1.0 ml w p cells) as measured under standard conditions of centrifugation, were subjected to drying to constant weight, either by heating at 40° in an oven, or by lyophilization.

Column Chromatography

Introduction

The method of adsorption chromatography was discovered in 1903 by the Russian botanist Tswett;[33] he described in detail the separation of plant pigments by filtration through columns, followed by development of the pigment bands with pure solvents.[5] He named this method chromatographic adsorption analysis; however, he realized this process was quite applicable to colourless substances also, as has been amply confirmed by this extremely versatile and useful tool. Although Tswett separated plant pigments on a variety of adsorbents such as talc, magnesium and calcium carbonate, the method was left aside for some twenty-five years, and the plant pigments, both chlorophylls and carotenoids, were obtained in the solid form for the first time by partition methods.[6,36]

The use of sucrose as an adsorbent for the separation of the chlorophylls was introduced in 1933,[34] and has remained the most popular adsorbent ever since, possibly because of its cheapness, ready availability, and ease of use.[35,36] Talc, magnesium citrate hexahydrate, and starch have also had limited use as adsorbents for chlorophyll separations.[37] One of the most refined methods is that of Jacobs, et al. who prepared crystalline chlorophyll a and b in 1954.[38] By further improvement of their usual method of adsorption and precipitation of the chlorophylls from an 80% aqueous acetone extract of plant material onto talc, followed by elution and chromatography with benzene-pet ether (1:1) on sugar columns, Stoll and Wiedemann in 1959 also obtained crystalline chlorophyll a and b.[39] This method appears to be faster and easier than that of Jacobs et al.[38]

Column chromatography of the carotenoid mixtures, which contain no chlorophyll, may be carried out on an even greater variety of adsor-

bents, which include alumina, MgO, $Ca(OH)_2$, and mixtures such as MgO-Celite (1:1), in addition to the above-mentioned adsorbents such as starch, sugar, etc.[40] The chlorophylls being more labile than the carotenoids undergo marked decomposition on such "alumina-type" adsorbents (e.g. MgO, Al_2O_3, $CaCO_3$ and Na_2SO_4); hence these are limited to carotenoid separations.

In the experiments for the determination of the specific activities of the plant pigments, a method that was quick, reproducible and allowed separation of both the carotenoids and chlorophylls at one time, was desired. Despite the universal use of sucrose as a suitable adsorbent, some difficulties were experienced with this material; after preliminary investigations, two better adsorbents were found, namely, cellulose powder and polyethylene powder.

Method

For these experiments two different adsorbents were used: cellulose (Whatman ashless powder for chromatography) and polyethylene powder.* The polyethylene powder was characterized by melt indexes which ranged in the samples available from 0.0004 to 0.1. In general, it was found preferable to use polyethylene powder which had a lower melt index; this corresponded to a higher molecular weight. The most satisfactory sample had a mol wt of 360,000.

The adsorbents (cellulose or polyethylene powder) were mixed as a slurry in the appropriate solvent, using a Waring blendor at high speed. The suspension of the adsorbent was then rapidly poured into the

---

* Gift of the Dow Chemical Company, Midland, Michigan

column, which had a plug of glass wool at the bottom to prevent the exit of the adsorbent from the column. Slight pressure was applied to pack the column, either by means of a rubber bulb, or by a gentle stream of air from the air line. Care must be exercised to see that the surface of the column was at all times covered with solvent to prevent air bubbles from entering the adsorbent, as it is being packed down. If the chromatogram should run dry, destruction of the pigments will occur by aerial oxidation; shrinking of the adsorbent and distortion of the zones will also prevent good resolution. When the column was packed, a circle of filter paper, corresponding to the inner diameter of the column, was placed on top of the adsorbent in a horizontal position by means of a glass rod to prevent the top of the column from being disturbed on the addition of the pigment extract.

The columns employed were glass tubes (25 to 35 cm long -- inner diameter 1.5 - 2.5 cm) with capillaries which were bent up into a long "S"-shaped syphon, extending vertically upwards to about 3 in from the top of the column, and then horizontally for about 2 in, and down again with a tapered end.

After the columns had been packed under pressure as described, they were washed with the solvent for several hours, to allow the columns to attain equilibrium and to see if any irregularities had occurred in the packing. The column was wrapped with black paper to exclude all light. The pigment solution was very carefully applied to the top of the column (from which any excess solvent had been removed) by means of a disposable pipette, taking care to run the solution uniformly down the sides of the column so that no one place on the column received extra solution, which would cause "tailing" of the resulting bands.

After the application of the pigment solution, and the washing of the pigment onto the column by the careful addition of a further 10 ml of solvent, the column was then developed under pressure. This was achieved in the following manner: a long glass rod (24 x 0.75 in) with a tapered end was connected to the column by means of a rubber cork; the upper end of the column was attached to a "U"-shaped syphon which led to the solvent reservoir. The solvent was syphoned over into the glass tube by means of a rubber bulb attached to an outlet at the base of the "U" syphon. The solvent then dripped continuously into the column under pressure, the flow rate being about 0.5 ml/min. The fractions from the column were collected in test tubes held on an automatic fraction collector which turned ever 6 min. The tubes were removed, corked, and placed in the dark at 0^{o} and spectral determinations made as soon as possible.

Cellulose columns for total extract: The solvent used was pet ether (b.p. 75^{o}). Fifteen g cellulose powder was needed for every 0.5 ml w p cells extracted. The algae were extracted with MeOH in the usual manner (cf. extraction of algae); the methanolic extract was shaken with pet ether (MeOH-pet ether 2:1) and 4% NaCl solution added until two layers formed. The pet ether layer was washed with water several times, quickly dried over anhydrous Na_2SO_4 and applied to the cellulose column.

After the carotene and lutein bands had emerged, and the chlorophyll a and b band were beginning to travel down the column, the developing power of the solvent was increased by the addition of 1% isopropanol of 0.5% n-butanol. It was important not to do this too soon, or the

chlorophyll b band will begin to catch up with the chlorophyll a band, and poor separation will result.

Cellulose columns for carotenoid extracts: The solvent used was pet ether (b.p. 75°) and 10 g of cellulose powder was required for every 0.4 ml w p cells extracted. The algae were saponified with methanolic KOH (cf. extraction of algae -- carotenoid extract), the ethereal extract was concentrated under reduced pressure, taken up in the minimum volume of pet ether, and placed on the column. The developing power of the solvent was increased by the addition of 2% isopropanol or 1% n-butanol to the pet ether. Toluene could also be used, by gradually reinforcing the pet ether with toluene.

Cellulose columns for separation of chlorophyll a and b: The same conditions were employed for the separation of pure chlorophyll a and b as mentioned above, under cellulose columns for the total extract. However, it is not necessary to elute the chlorophyll a and b bands from the column; indeed, it was preferable to remove these bands mechanically in order to save time, and hence limit the decomposition of the pigments. After the clear separation of these bands from each other, and from the xanthophyll epoxides which were situated above the chlorophyll zones, the column was freed from excess solvent by gentle suction. The adsorbent was forced out of the column by applying an air stream to the bottom of the column, and the middle of the chlorophyll a and b zones cut out, transferred to beakers and extracted with ether. The ethereal solution of the chlorophylls was filtered and placed in the dark at 0°C.

The various fractions obtained from the above cellulose columns were checked for purity by spectroscopic examination, and the pure fractions concentrated under reduced pressure and applied to paper chromatograms as a further test for homogeneity.

Polyethylene columns for total extract: The solvent used here was 80% or 85% aqueous MeOH. Fifteen g polyethylene powder was required for every 0.5 ml w p algal cells extracted. The methanolic extract (cf. extraction of algae) was placed on top of the column and sufficient distilled water to make the solution up to 80% aqueous MeOH was added. An alternative procedure was to add about 2 g of polyethylene powder to the methanolic algal extract obtained after the algae had been boiled in MeOH. This slurry was diluted to 80% aqueous MeOH and poured onto the column. It was somewhat easier to obtain a uniform initial zone of pigments on the column by this latter method. The addition of the water to the methanolic algal extract caused some slight precipitation of the chlorophylls and the carotene; the precipitate dissolved during the course of development of the chromatogram and did not hinder the separation obtained.

After the separation and elution of the xanthophyll di- and mono-epoxides from the column, the developing power of the solvent was increased, by reinforcing the aqueous MeOH concentration up to 95%.

Polyethylene columns for carotenoid extracts: The solvent used here was 85% MeOH and 10 g of polyethylene powder (reused, low density product of mol wt 360,000) was required for every 0.4 ml w p cells. Improved separations were obtained if the column had been prewashed with EDTA-Na_4. After packing the column in the usual manner, a solution of 1 g EDTA-Na_4 (tetrasodium salt) in 500 ml of 50% aqueous MeOH was added to the column, followed by extensive washing with 80% MeOH to re-requilibrate the column and to wash out the excess EDTA, before applying the carotenoids to it. The methanolic KOH extract was thoroughly extracted with ether,

washed with water to remove the alkali, and concentrated under reduced pressure to dryness, then dissolved in MeOH, placed on the column and diluted to 85% aqueous MeOH.

Polyethylene columns for separation of chlorophyll a and b: Aqueous MeOH (80%) was used as the solvent here, and 15 g of polyethylene powder was used for every 0.5 ml w p cells extracted. It was feasible to extrude the zones of chlorophylls mechanically as described in the corresponding cellulose columns; as the separation on polyethylene columns was faster than on cellulose columns, in most cases the bands were eluted from the polyethylene columns.

The various fractions eluted were checked spectroscopically, concentrated under reduced pressure and transferred to ether by the addition of 5% NaCl solution to the ether-MeOH mixture(1:2). The ethereal fractions were chromatographed on paper to check their purity.

Paper Chromatography

Introduction

Interestingly enough, paper chromatography of a sort was first demonstrated with the partial separation of plant pigments by the method of capillary analysis. Goppelsroeder in the period 1850 to 1910 studied this procedure, whereby the components of a solution were partially resolved by capillary action when a strip of filter paper was dipped into the solution of pigments to be resolved. In 1906, M. Tswett[5], the founder of modern chromatography, and Goppelsroeder[41] independently partially separated plant pigments on paper for the first time by this method of capillary analysis.

Paper chromatography is a separation process based primarily on liquid-liquid partition on a paper support which retains the nonmobile phase in contact with the mobile phase which flows over it. The method was introduced by Martin in 1941, and the complete details of the process were presented, in a now classical paper, in 1944.[42] The theoretical ideas and experimental techniques used today are essentially the same as those developed by Martin.

Despite its early introduction, the methods of paper chromatography of the plastid pigments have been of a rather preliminary nature and have found only limited application in radioactive tracer work. The most recent review by Šestak covers the work from 1952 to 1957;[43] prior to 1952 the method was virtually unused.

One- and two-dimensional techniques in ascending, descending, horizontal and circular arrangements have been described, utilizing a variety of papers, sometimes after treatments such as drying, washing or impregnation of the paper. The chromatograms have been developed with a variety of polar or nonpolar organic solvents, or their mixtures.

Methods

Whatman No. 4 and No. 3MM filter papers were used for the separation of the plant pigments. Since more material could be placed on the origin of the thicker (No. 3MM) paper, this was, in general, used. It was especially important to use the thicker filter paper when it was necessary to elute the spots for spectral or radioactive determinations. The total pigment extract which could be placed on the origin of a Whatman No. 3MM sheet (46 by 57 cm) was roughly equivalent to 0.6 to 0.8 mg

pigment, or to 0.1 ml w p cells. In the case of the carotenoid extract, it was possible to place on the origin the extract from 0.15 ml w p cells, which corresponded to about 0.2 mg pigment.

Various vessels and chromatographic chambers were used for paper chromatography. In all cases it was absolutely essential to have the vessel completely light-tight; this was achieved by covering the outside with black paper. It was also essential to allow the solvent mixture to equilibrate in the vessel for at least eight hours, before the first chromatogram was run.

Trial chromatograms were made in graduate cylinders (5,000 ml) which were stoppered by rubber corks. Paper clips were attached by rubber bands to two drawing pins fastened to the inside of the cork; the paper strips were suspended from these clips and ascending chromatograms made. It was possible to run these chromatograms in the cold room at 0°, also to run under a nitrogen atmosphere. Descending chromatograms could be made in these graduate cylinders by means of a little glass trough being suspended from the cork; a separatory funnel was placed through the cork, by means of which the solvent could be admitted. Chromatograms either by the ascending or descending method took 1.5 to 2 hours to run.

Fast trial chromatograms of more than one spot could be made in glass tanks (12 in. x 8 in. by 8 in.) which were fitted with a ground glass top. A glass support, consisting of a horizontal bar with two wide "V"-shaped legs at either end, stood in the tank and the papers could be clipped to this upright, and ascending chromatograms made in about one hour's time.

The large sheets of filter paper were run in the usual chromatographic boxes and took about three hours to run about three-quarters of

the length of the paper.[32] These chromatograms could not be made at 0° since the boxes are permanent fixtures in a 25° thermostatically-controlled chromatography room.

The pigment spots were spotted on an origin which was about three inches from the edge of the paper, in ether or pet ether solution. It was preferable to use ethereal solutions, since the extract dried very quickly and no time was lost in getting the chromatogram into the chromatographic chamber. Pigment extracts that have been spotted on the filter paper and left for a short time in the light, show a marked decomposition, after subsequent development. In order to obtain uniform and "compact" origins, it was preferable to have fairly concentrated, ethereal solutions to spot on chromtograms.

A variety of solvents were tested. Satisfactory solvent systems found were as follows:

(1) Toluene

(2) Pet ether-isopropanol (100:2.5)

(3) Pet ether-Isopropanol-toluene (100:2.5:2.5) in a toluene-saturated chamber.

(4) Pet ether-n-butanol (100:2.0).

Successful two-dimensional chromatograms were made by using solvent (1) for the first dimension and solvent (4) for the second dimension.

At the beginning of this work, a large number of solvent systems were tested in order to find satisfactory resolution of the pigments. Solvent mixtures were found almost on a trial and error basis, e.g. start with a nonpolar solvent such as pet ether and gradually reinforce it, with a variety of polar solvents. In the same manner, each solvent was taken in turn and mixed with various other solvents. Rather than tabulate

large lists of the various combinations of solvents tried for this part of the work, a summary of the findings from these trial chromatograms will be included in Section IV, in the results and discussions of paper chromatography of the pigments.

In the case of paper chromatography of carotenoid pigments, either of single compounds or of carotenoid extracts, a sharper resolution could be obtained using Whatman No. 3MM sheets which had been soaked for half an hour in EDTA-Na_4 (tetrasodium salt) and then thoroughly washed for 8 hours with distilled water in a rectangular trough with a perforated bottom for applying suction. After the papers were dried over-night in an oven at 40°, they were ready for use.

The purity of the various chromatographic bands was checked by elution of the spots and spectroscopic examination of the pigments. If the extracts that had been chromatographed were fresh, the elution was possible in diethyl ether. The bands were cut out, dissected into small strips, placed in small beakers, and covered with the minimum amount of ether sufficient to extract the pigment from the paper. Actually it was just as quick and satisfactory to cut a large zone (i.e., a 12 in. zone from a one-dimensional chromatogram on regular Whatman sheets), into four pieces, and place these in a minimum volume of ether in a small beaker, and let the pigment elute upwards to the top of the strips, then cut off the resulting smaller pigment area. The pigments were eluted in the absence of light, as rapidly as possible.

If the chromatograms were made from older pigment extracts, sometimes ether was not sufficient to elute the spots. If quantitative elution was desired, MeOH-benzene (4:1) was used for carotenoid spots, and

isopropanol-isopropyl ether (1:9) for the chlorophylls.[44] However, it was, in general, better to elute with ether even if all the pigment did not come off from the paper, since the measurements to be made were of the specific activity of the pigment. Such a measurement does not involve knowledge of the total concentration of any substance, but only of the quantity whose radioactivity was subsequently measured.

Centrifugally-Accelerated Paper Chromatography

Introduction

The technique of centrifugally-accelerated chromatography was developed by H. J. McDonald in 1957.[45] The combination of two vectors, chromatography and centrifugal acceleration, make it possible to separate substances in very short times, for example, 15 minutes instead of 8 hours.

The apparatus consists of a motor-driven, disc-shaped head rotating in a horizontal plane; the paper is held horizontally inside this unit and the solvent is fed in a continuous jet stream under pressure, through a very narrow aperture in the head.

The literature contains separations of water-soluble compounds, such as dyes and amino acids,[46] but as yet there is no mention of separation of any other compounds. Therefore, a study was made of the fundamental factors involved in obtaining good separations and reproducible chromatograms of the algal pigments.

Apparatus and Methods

The chromatograms were made on the commerically-available instrument called a chromatofuge, which was assembled and operated in the

prescribed manner.*

The chromatofuge was placed in the 25° thermostatically-controlled chromatography room. An additional solvent arm and feed device was made, and both of the solvent bottles were set up above the machine, their support arms being clamped together up on a higher rod to be out of the way of opening and closing the machine. Tygon tubing was used throughout; the spacer ring on the chromatofuge head was made of rubber and the non-polar solvents used caused considerable stretching of this ring, to the point where the ring refused to stay around the outside of the head. A section was removed and the ring refitted on. It was found, however, that the ring shrank again to its original shape after the machine had not been used for several days, and the cutout piece had to be reinserted.

The delivery jets of the glass tips, through which the solvent is forced out under pressure in a thin jet stream, are very fine; indeed, it is essential that no dust be present in the solvent or tubes or these tips will be continually being blocked or partially blocked up. In the latter case, the solvent will emerge in droplets which has a disastrous effect on the resulting chromatogram. In order to fill the pressure solvent bottle without removing the cork and sealing ring, a special graduated cylinder was devised with two side-arm stopcocks, one at the top and the other at the bottom of the cylinder. The solvent was filtered through a sintered glass funnel into the special cylinder by applying vacuum to the upper stopcock. The solvent was then transferred to the pressure solvent bottle by means of a piece of tubing attached from the lower end of the cylinder to the longer tube of the pressure solvent bottle and by applying vacuum to the shorter tube of the solvent pressure bottle.

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* Labline No. 5060 Chromatofuge. Labline, Inc., Chicago, Illinois (1958).

Whatman No. 1 and 3 MM circles (18 in.) and circles cut from No. 17 MM sheets were used for centrifugal chromatograms as well as Schleicher and Schuell circles (50 cm.). Since most of the spots were required for spectroscopic examination or respotting, it was desirable to work on a larger scale and most of the work was done on the Whatman No. 3 MM papers. Actually, it was feasible to cut out the circles from the rectangular sheets of chromatographic paper and this was done whenever the circles were not available.

The material was placed on a circular origin which must be at least 8 cm in diameter; if the diameter was any smaller some of the pigments would run inwards to the centre of the circle, instead of migrating outwards. It was most satisfactory to apply the pigments in ethereal solutions as the ether evaporated so quickly; however, other solvents were also feasible. Since it was difficult to make a uniform circle of material on the origin by hand, a thin glass capillary was held in a compass and the circle traced. This method failed entirely, as no capillary device could be found which permitted the uniform addition of the pigment from an ethereal extract, although such a capillary device was quite satisfactory with aqueous solutions. With practice it was possible to make very thin uniform circles of pigment on the origin by holding the paper vertically with one hand and applying the extract with the other. Papers were spotted in the cold room with as little light as possible.

The total extract which could be placed on such a circular origin on Whatman No. 3 MM sheet was about 0.3 - 0.4 mg pigment, i.e., 0.05 ml w p cells. In the case of the carotenoid extract it was possible to place an

extract from 0.05 ml w.p cells which corresponded to about 0.1 - 0.2 mg pigment. About three times as much material could be placed on the circular origin of a Whatman No. 17 MM paper; since the paper was so thick it was necessary to spot the material on both sides or else the pigment zones on the underside of the paper, after development, would have lower R_f's.

It was also desirable to use a liner for the chromatofuge head. A sheet of Whatman No. 3 MM paper is placed in close contact with the bottom of the head, by cutting holes in the sheet so that the stainless steel points protruded through the liner paper. Some of the solvent was placed on this liner and the machine was rotated for 5 minutes in order to saturate the chamber head with the solvent fumes, before placing the paper to be developed in the chromatofuge. In subsequent runs, about 10 to 20 ml of solvent was poured onto the liner, the paper placed in position and developed. It was not necessary, in the subsequent "runs" to rotate the machine for a 5 minute period, before development of the paper.

The solvent was fed into the machine under nitrogen pressure; the pressure range employed could vary between 4 to 15 p.s.i. depending on the capacity of the glass delivery jet and the type of solvent used. The length of time of running the chromatogram, and the speed at which the head was rotated (between 340 to 960 rpm) also depended on the solvent and paper used. In order to avoid repetition, the precise conditions used (speed, nitrogen pressure, time of development, etc.) will be tabulated in Section IV, where the R_f values of the plant pigments under a variety of conditions will be presented.

In the case of pet ether-isopropanol solvents, it was necessary to mix the solvent several days before it was required for use. Fresh

solvent mixtures used under the same running conditions gave anomalous results. (The solvent did not travel nearly as far and the separations obtained were very poor.)

Reversed paper chromatography: Methanol was shaken mechanically for 10 minutes with paraffin oil (Squibb's mineral oil) and left standing until the two phases had completely separated (8 - 10 hours). The upper methanolic layer, after filtration, was used as the solvent. Whatman No. 1 or 3 MM circles were drawn slowly through a 2.5 to 7% solution of the equilibrated oil in ether and left for a few minutes to dry in the hood; no liner was used.

Spectroscopy

All visible spectra were measured by using a Cary Model 14M recording spectrophotometer. The wave length scan was 10 or 25 A per second, the chart drive was 120 in/hr, and the slit width < 0.2 mm. As the resolution of absorption was not changed by the use of scanning speeds up to 25 A per second, this speed was used for ordinary determinations. The absorption cells were exactly matched for transmissivity. Small absorption cells (0.8 ml) were used when the amount of solution available was small.

All solvents used were of Baker's C.P. analyzed reagent grade. The petroleum ether (B.R. 65-110°) was purified by shaking with concentrated H_2SO_4 (10% w/v) until the acid layer was colourless. The pet ether was then shaken with water to remove most of the acid, once with 10% Na_2CO_3 solution, and again with water. If the aqueous layer was

neutral to litmus, the pet ether was then dried over anhydrous $CaCl_2$ for 24 hours. After filtration, the pet ether is distilled through an efficient column and the fraction (b.p. 70-75°) collected. The diethyl ether was tested for absence of peroxides, stood for 24 hours over NaOH, and distilled.

Radioactive measurements

Aluminium planchets were cleaned by rinsing in alcohol, and a circle was drawn with a grease pencil just inside the circumference. A few drops of 2 M acetic acid and diluted detergent (Aquet-H_2O, 1:500 w/v) were placed in the centre of the planchet, and the radioactive sample added (by hand) to the plate as it rotated on a turntable. Drying was accomplished in a stream of air, or by placing the planchets in the hood under an infrared lamp.

The plates were counted in an automatic counter,* equipped with a G.M. tube with a "micromil" window, for the maximum time, i.e. for 25,600 counts, and allowed to recycle through a sufficient number of times to insure that the desired statistical accuracy was obtained.

In order to count individual planchets or spots on radioautographs, a Scott tube, covered with a Mylar end-window** was employed; Q gas/Wood used in the counters. The radioactive areas on paper chromatograms were detected by exposure of the paper to du Pont x-ray film, type 507.

Liquid scintillation counting: The counter used was the liquid scintillation spectrometer, "Tri-Carb"*** operated in the prescribed manner.

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* Automatic counter, Model C-110A, Nuclear-Chicago Corp., Chicago.

** Mylar is duPont's registered trademark for its polyester film.

*** "Tri-Carb" liquid scintillation spectrometer, Model 214, Packard Instrument Co., Inc., La Grange, Illinois.

Scintillation solution No. 2 used for aqueous samples: Toluene (1.0 l), p-dioxane (2.0 l), abs. EtOH (1.2 l), naphthalene (260 g), PPO (26 g), POPOP (0.5 g).

Scintillation solution No. 6 used for nonpolar samples: Toluene (3.0 l), PPO (15.89 g), POPOP (0.3 g).

The aliquots of radioactive material to be counted (must not exceed 500 λ) were pipetted accurately with a lambda pipette into glass vials with aluminium-lined screw tops, which contained 10 ml of the appropriate phosphor solution, No. 2 or No. 6, and counted in the machine a sufficient number of times to obtain the desired statistical accuracy. The first count, minus the background of the blank sample, is termed C_1.

The variability of the composition of the sample can be controlled by the use of an internal standard. The C^{14} internal standard used was toluene-1-C^{14}, which had been distilled and diluted to an appropriate concentration. One hundred λ of toluene-C^{14} (1.4×10^5 dpm/ml) was pipetted into the counted sample and the vial recounted; this count minus the background is termed C_2.

$$\text{Efficiency of counting} = \frac{C_2 - C_1}{\text{std.activity added}} = \frac{C_2 - C_1}{1.4 \times 10^4}$$

$$\text{d/m of sample} = \frac{C_1}{\text{efficiency}}$$

In order to prevent contamination of the glass vials, these were only used once. The internal standard was added to every sample that was counted, to compensate for the variability of the composition of the solvent. Since some of the samples to be counted were intensely green or yellow in colour, another factor, "colour quenching", needed

to be considered. Such strongly coloured samples will tend to absorb some of the light emitted by the scintillator fluor, and, consequently, lower the sensitivity of the radioactive measurements. The variation in efficiency is illustrated below and shows the necessity of using the internal standard for every sample to be counted.

Ethereal samples in scintillation solution No. 6

Sample No.	C_1 c/10 min	C_2 c/10 min	$C_2 - C_1$	Efficiency	d/10 min
1	560	104,571	104,011	0.7429	754
2	4,202	110,507	106,305	0.7593	5,534
3	868	104,588	103,720	0.7409	1,171
4	2,130	107,522	105,392	0.7528	2,829
5	1,487	105,565	101,078	0.7430	2,001
	Aqueous samples in scintillation solution No. 2				
1	7,486	84,713	77,227	0.5516	13,570
2	1,118	88,612	87,494	0.6249	17,890
3	4,944	78,367	73,423	0.5244	9,428
4	6,442	81,353	74,911	0.6351	10,144
5	469,360	541,453	72,093	0.5278	889,276

Specific Radioactivity of Pigments

Series I - preliminary experiments

These preliminary experiments were designed to determine the specific radioactivity of chlorophyll a and b using one-dimensional chromatography for the separation of the pigments.

After harvesting, the algae Scenedesmus or Chlorella were centrifuged and resuspended in the nutrient solution used for culturing the algae to give a 1% solution (ml w p cells/suspension vol). Fifty ml of this 1% algal suspension was placed in the "lollipop" of the "steady state apparatus." Illumination was provided by reflector spotlights with illuminated both sides of the vessel with a light intensity of approximately 7,000 foot-candles on either side of the "lollipop", which was jacketed with a water cooler. Excessive heat production was avoided by the use of water-cooled infrared filters placed between the reflector spotlights and the "lollipop."

The "steady state apparatus" was designed by Wilson in 1953[47] and has been considerably improved over the years by Bassham.[48*] The apparatus consists of a closed gas circulating system, as schematically represented in Fig. 3. An ionization chamber attached to a vibrating reed electrometer records the quantity of C^{14} in the system. The levels of CO_2 and O_2 in the system are detected by analysers. A pH-statt is included in the appartus so that the pH may be maintained constant throughout the course of an experiment. The CO_2 and $C^{14}O_2$ uptake, oxygen evdution and the pH are recorded on a multipoint recorder during

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* I wish to thank Dr. James A. Bassham for permission to use the "steady state apparatus" and Mrs. Martha Kirk for helping me to operate it.

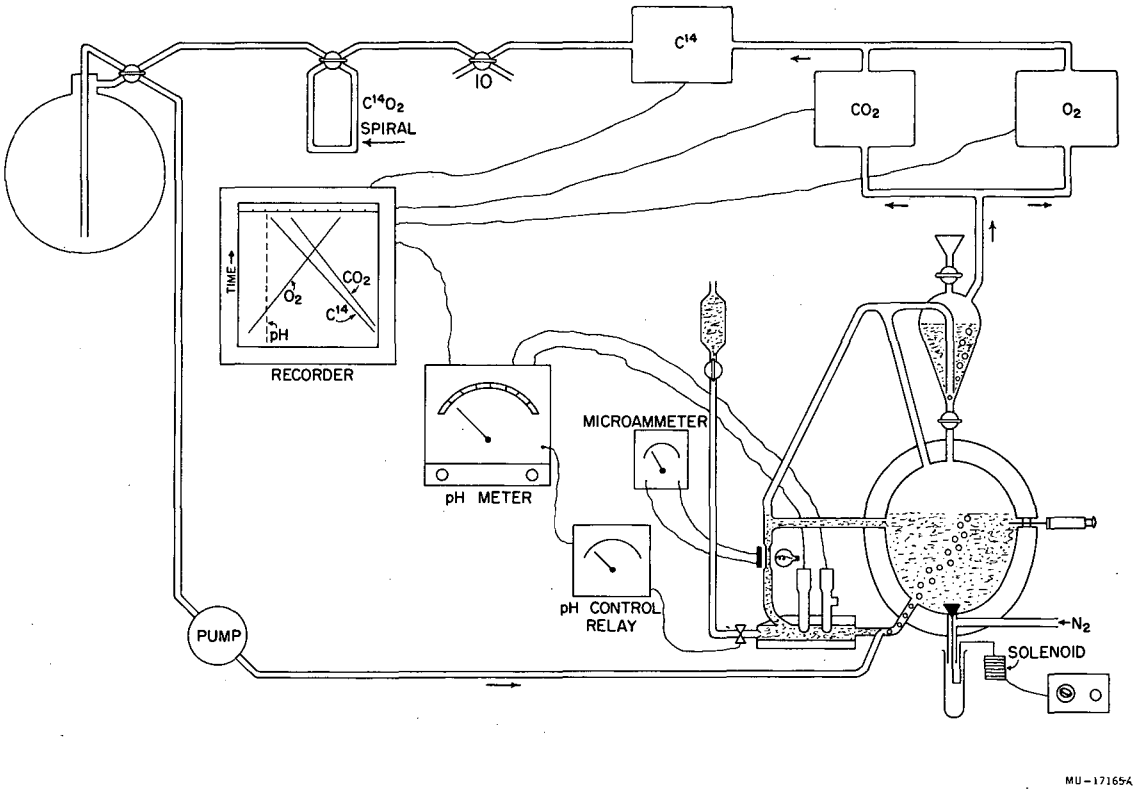

Fig. 3. Schematic representation of the "steady state" apparatus. Algae suspension in "lollipop" at bottom right hand side, and the C^{14}, CO_2 and O_2 analysers at the top right hand side of the diagram.

the experiment. Since the total volume of the system is known, the quantitative rate of CO_2 uptake and oxygen evolution can be determined.

For long-time experiments it was necessary to fill the large reservoir (5,375 ml) shown at the left hand side of Fig. 3 with 4% CO_2-in-air before beginning the experiment, so that the algae would have a sufficient amount of CO_2 for adequate growth during the course of the experiment. In the experiments of Series I and II, the pH-statt was not incorporated in the "steady state apparatus," nor was the machine calibrated for the quantitative uptake of CO_2 or oxygen evolution.

As soon as it was ascertained that the algae were photosynthesizing adequately (i.e., satisfactory rate of CO_2 uptake and O_2 evolution) the radioactivity was injected through a stopper at the side of the "lollipop" (Fig. 3). One ml of 0.036 M $NaHC^{14}O_3$ was added, followed by 0.5 ml of 0.01 M HCl to attempt to buffer the system. Samples were removed at various time intervals and more nutrient solution was added as the algae grew, to maintain a somewhat constant density.

After centrifugation of the radioactive algae, the supernatant was discarded and the algal cells were rinsed with MeOH (1 ml) and extracted with pet ether-MeOH (3:7) (4 ml of the pet ether-MeOH solution/0.1 ml w p cells). After centrifugation and separation of the cell residue, the extract was washed with 4% NaCl solution, and the pet ether layer removed and extracted several times with 90% MeOH to remove most of the xanthophylls. The pet ether layer was dried over anhydrous Na_2SO_4.

The pigments were separated by one-dimensional chromatography and their specific activity determined by planchet counting.

Series II

These experiments were designed to measure the specific radioactivity of chlorophyll a and b. Since the separation of the pigments by one-dimensional chromatography proved inadequate, the chlorophylls were first separated by column chromatography, followed by paper chromtography.

The experiments were carried out in the "steady state apparatus" as previously described under Series I, except that the 50 ml of 1% algal suspension was allowed to photosynthesize for the total time; no samples were withdrawn at time intervals in order to have sufficient material to work with.

The algae were extracted with boiling methanol (cf. extraction of algae), and the methanolic extract divided in half. One-half of the solution was applied to the top of a polyethylene column, and the other half was extracted with pet ether (pet ether-MeOH 1:2 v/v). It was necessary to add 4% NaCl to transfer the chlorophylls to the organic layer. The pet ether layer was then washed several times with 90% MeOH, removed and dried over anhydrous Na_2SO_4 before application to the cellulose column. After separation of the pigments by column chromatography, the individual pigments were re-chromatographed on paper.

Series III

The specific radioactivities of the chlorophylls and carotenoids were determined, after the separation of the pigments, by centrifugally-accelerated chromatography.

The "steady state apparatus" was used with the pH-statt; the pH was maintained constant at 6.5 throughout the course of the experiment, by the addition of 0.5 M NH_4OH by the pH-statt. For "longer time" experiments with C^{14} of a higher specific activity it was advantageous to generate the $C^{14}O_2$ from $BaC^{14}O_3$ rather than use $NaHC^{14}O_3$ as was done in the earlier experiments. The $C^{14}O_2$ was generated in the conventional manner on a vacuum line by the addition of 25 ml of concentrated H_2SO_4 to 40 mg of $BaC^{14}O_3$ (50 μc/mg) and frozen out in a spiral which was subsequently attached to the "steady state machine." (Fig. 3)

The algae were suspended in a diluted medium instead of the nutrient solution:, $(NH_4)_2HPO_4$ (40 mg/l), $MgSO_4 \cdot 7H_2O$ (20 mg/l), NH_4Cl (20 mg/l), KNO_3 (25 mg/l), 0.0005 M NH_4NO_3, trace elements, Arnon's sA-4* (1 ml/l), Fe-versenol** (1 ml/l).

Samples were taken out at different time intervals and extracted in the usual manner; one-half of each sample was saponified with methanolic KOH (cf. p 16). The pigments were transferred to ether, the ethereal extract washed thoroughly, and the ethereal extracts placed on the origin of circular chromatograms. After development of the chromatograms, the pigment zones were cut out and eluted with ether in small beakers in the dark. The volume of the ether solution/pigment was recorded, and aliquot placed in the glass vial for scintillation counting, and the remainder of the solution placed in a small absorption cell, and the concentration determined by the Cary Model 14 spectrophotometer.

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* D. I. Arnon; Am. J. Bot. 25, 352 (1938).

** 37.2 g Versenol in 500 ml H_2O plus $FeSO_4 \cdot 7H_2O$ (24.9 g) diluted to a litre and aerated overnight.

III. SPECTROSCOPIC EQUATIONS FOR PIGMENT CONCENTRATIONS

Introduction

The estimation of the pigments in plant materials depends on two operations: the complete extraction of the pigments and the reliable measurement of the concentration of the pigments present in the extract. The methods available for the determination of the pigment concentrations are based on four procedures: colorimetry, spectrophotometry, fluorimetry and the estimation of the magnesium content of chlorophyll, if the chlorophylls are the only-magnesium containing substances present. The most convenient and accurate method for the determinations of the chlorophyll and carotenoids, both as mixtures or as the pure compounds, is the spectrophotometric method. The microdetermination of the chlorophylls by colorimetry, however, provides an accurate quantitative method.[49]

The quantitative relation between the concentration and the light absorption of any pigment is defined by the Beer-Lambert law, which may be expressed mathematically as:

$$\alpha = 1/cd \cdot \log_{10}(I_o/I) \qquad (1)$$

where α = specific absorption coefficient in l/g cm*

d = length in cm of the light path through the solution

I = intensity of the light transmitted by the solution

I_o = intensity of the light transmitted by the solvent

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* Since it has been customary in the literature of plant pigments to use the specific absorption coefficient, rather than the more usual molar extinction coefficient ε ($\alpha \cdot$mol wt = ε), this convention has been observed throughout.

Estimation of chlorophyll a or b concentrations

The chlorophylls are not stable crystalline substances which are readily available for standardization purposes. The pure chlorophylls obtained in different laboratories are reported to have different spectroscopic standards. These differences may arise from impurities in the solvents or from impure pigments, from differences in spectroscopic equipment, or from the nonuniformity of the isomeric composition of the chlorophyll. If the estimation of the pigment concentration is important, it is essential that the wave length maxima and the specific absorption coefficients employed are checked, under the conditions of the analysis employed. This subject has been comprehensively reviewed by Smith and Benitez in 1955 and the wave length maxima and absorption coefficients of various workers are summarized.[35]

In order to determine the wave length maxima and absorption coefficients of pure chlorophyll a and chlorophyll b, these compounds were obtained from alcoholic algal extracts and separated by column chromatography (on polyethylene and on cellulose columns). Fig. 4 shows the quantitative absorption curves obtained for pure chlorophyll a and chlorophyll b in ether. The specific absorption coefficients obtained agreed closely with those of Smith and Benitez,[35] and differed from Zscheile and Comars' earlier findings.[50] It is of interest to note that the specific absorption coefficients determined independently, from the colorimetric estimation of magnesium content by Falk[49], are in agreement with the spectroscopic data of Smith and Benitez.[35]

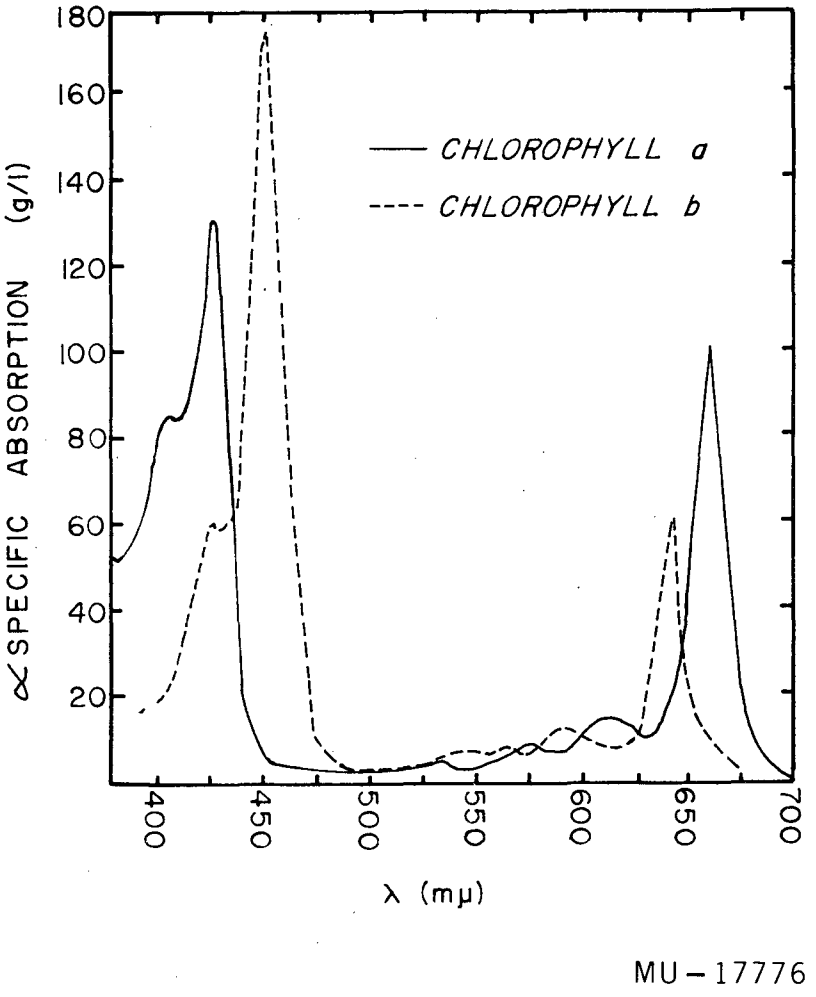


Fig. 4. The absorption spectra of chlorophyll a and chlorophyll b in ether.

The concentration of chlorophyll a or b may be calculated from Eq.(1), by substitution of the experimentally found optical density at a particular wave length and the appropriate specific absorption coefficient.

Spectroscopic criteria for the purity of chlorophyll a and b

Isolated chlorophylls are extremely labile; the magnesium is relatively easily lost from the chlorophylls to give the corresponding phaeophytin derivatives. Hence, it is important to have some criteria available, to judge whether such contamination by pheophytin has occurred. Normally with the precautions taken of storing the ethereal extracts in the dark at $0^{o}C$ under nitrogen, such decomposition is minimized; also, all spectra are determined with as little time delay as possible.

After comparison of the chlorophyll and pheophytin spectra, Zscheile and Comar defined two quantities, R_a and R_b, which permitted rapid detection of any pheophytin contamination.[50] R_a and R_b are the ratios of the long wave length absorption of chlorophylls a and b to their corresponding absorption coefficients at 505 and 520 mμ respectively, where the pheophytins have conspicuous absorption maxima. The average values obtained for R_a was 52.4 and for R_b, 18.9. The similar equations derived from my data are as follows:

$$R_a = \alpha_{662}/\alpha_{505} = 100.9/2.4 = 42.04 = \log_{10}(I_o/I)_{662}/\log_{10}(I_o/I)_{505}$$

$$R_b = \alpha_{644}/\alpha_{520} = 62.0/3.5 = 17.7 = \log_{10}(I_o/I)_{644}/\log_{10}(I_o/I)_{520}$$

Any contamination by pheophytin is indicated by a decrease in R_a or R_b.

Detection of carotenoids in chlorophyll samples was particularly important in the case of paper chromatography, where there was frequent overlap of pigment spots due to the limited range available for separation.

The carotenoids absorb light in the blue region of the visible spectrum, from 520 mμ downwards. Thus a comparison of the ratio of the red absorption of the chlorophyll spectrum to that of the blue absorption would reveal the presence of any carotenoids. These ratios are defined as follows:

$$R_{xa} = \alpha_{662}/\alpha_{430} = 135/131 = 1.03 = \log_{10}(I_o/I)_{662}/\log_{10}(I_o/I)_{430}$$

$$R_{xb} = \alpha_{644}/\alpha_{455} = 62.0/174.8 = 0.355 = \log_{10}(I_o/I)_{644}/\log_{10}(I_o/I)_{455}$$

A decrease in the values obtained for R_{xa} or R_{xb} would thus indicate any carotenoid contamination.

Estimation of chlorophyll a and b in a plant extract

In any solution in which more than one component contributes to the absorption at the particular wave lengths employed, the values of the optical densities of the individual components are assumed to be additive (this relationship is quite satisfactory, unless some sort of energy interaction occurs between the molecules involved). It is necessary in such a solution to measure the optical densities at more than one wave length; in order to obtain maximal accuracy, wave lengths are chosen where the absorption of the components differs as much as possible. The concentration of the pigments may then be calculated by means of the following simultaneous equations:

$$\log_{10}(I_o/I)' = \alpha'_a C_a d + \alpha'_b C_b d \qquad (2)$$

$$\log_{10}(I_o/I) = \alpha''_a C_a d + \alpha''_a C_b d \qquad (3)$$

where the prime (') and double prime (") refer to the wave lengths employed, and the notation used is the same as in Eq.(1). Subscript a refers to chlorophyll a, b to chlorophyll b, and c to total carotenoid content of algae.

The equations used throughout for the calculation of chlorophyll a and b concentrations in ethereal solutions, containing both chlorophylls and carotenoids, are obtained by substitution of the appropriate absorption coefficients in the above Eqs.(2) and (3). These equations are then solved by determinants to give:

$$C_a(\text{mg/l}) = 10.1 \log_{10}(I_o/I)_{662} - 1.01 \log_{10}(I_o/I)_{644} \qquad (4)$$

$$C_b(\text{mg/l}) = 16.4 \log_{10}(I_o/I)_{644} - 2.57 \log_{10}(I_o/I)_{662} \qquad (5)$$

The above equations are identical with those of Smith and Benitez[35] (since the α's are identical). Fig. 5 shows a typical absorption spectra of a Chlorella and a Scenedesmus total ethereal extract; Eqs.(4) and (5) were determined from such curves as these.

The total concentration of the chlorophylls may be obtained by the determination of the optical density at coincident points of the chlorophyll a and b spectra. In this case the general Eq.(2) is reduced to:

$$\log_{10}(I_o/I)' = \alpha'(C_a + C_b)$$

Since the carotenoids absorb light below 520 mμ, this region is not applicable for the choice of coincident points. Comar and

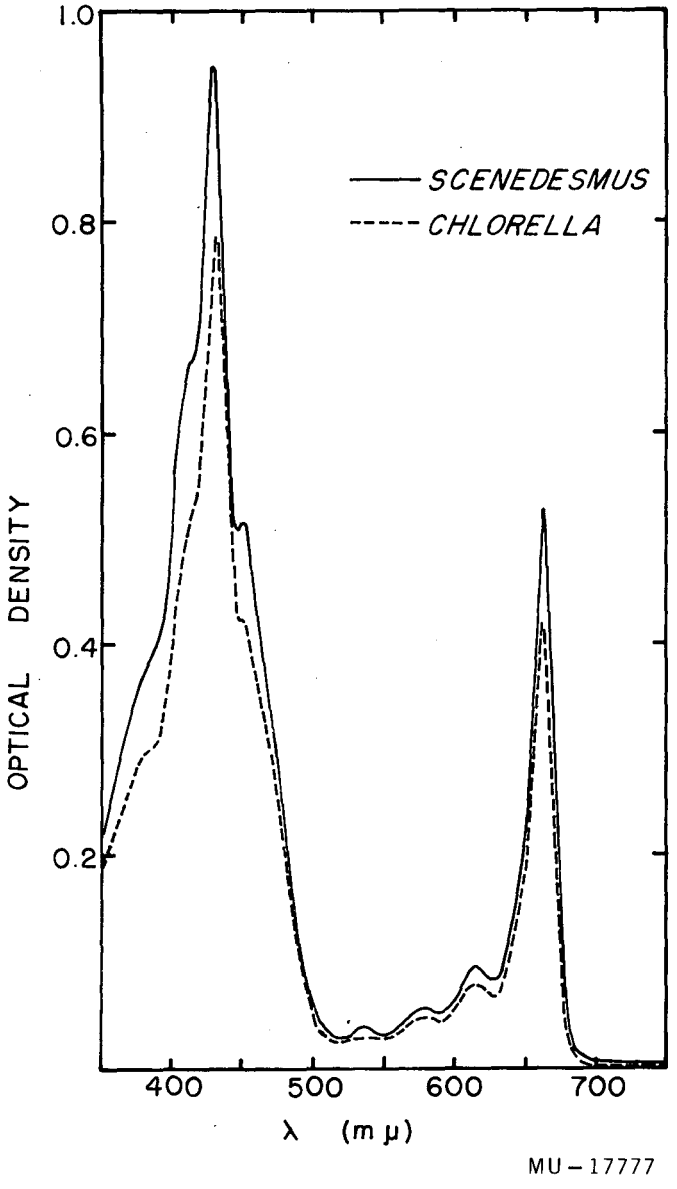


Fig. 5. The absorption spectra of the total ethereal extracts from Chlorella and Scenedesmus.

Zscheile found that the addition of carotene to pure mixtures of chlorophyll *a* and *b* showed an effect above 520 mμ.[51] Calculations of the chlorophyll content at 546 and 589 mμ showed an apparent decrease in the amount of chlorophyll present (as calculated from wave lengths greater than 600 mμ); hence, it was inferred that the carotene must be having some effect on the light absorption in the region between 520 and 590 mμ. It would be of considerable interest to confirm these findings (the amount of absorption in this region is low, thus the experimental error incurred is greater). If such a phenomenum was found to be true, it would be of importance in the physical concepts of chlorophyll-carotenoid interactions and might hint at the possibility of charge transfer of energy between these molecules in the chloroplasts.

The coincident points about 590 mμ occur at 604 and 638 mμ, where α = 11.1 and 14.0, respectively. Thus the equations for the total concentration of chlorophyll present in any extract are as follows:

$$C_{(a+b)}(mg/l) = \log_{10}(I_o/I)_{638}/0.014 \qquad (6)$$

$$C_{(a+b)}(mg/l) = \log_{10}(I_o/I)_{604}/0.011 \qquad (7)$$

Spectroscopic determination of the carotenoids

In order to obtain the carotenoids as the principal light-absorbing substances of the methanolic algal extract, the chlorophylls may be removed by saponification. By partition of the products between ether and aqueous MeOH, the carotenoids could be transferred to the ether layer, while the saponified chlorophylls remained in the aqueous layer.

As no pure carotenoids were available for standardization purposes, the various absorption coefficients reported in the literature were consulted.[40] The average molecular weight of a carotenoid mixture from algae is equal to 568. (The average distribution of the carotenoids in algae was found to be approximately as follows: 10% carotenes, 70% lutein, 10% mono-epoxides and 10% di-epoxides.) The average value of the molecular absorption coefficient of the various carotenoids involved, at the wave length of maximal absorption, has been calculated as follows:

$$\mathcal{E} = (1.35 \pm 0.06) \times 10^{+5} \text{ g mols/l}$$

therefore $\alpha = 240$ l/g.cm.

Carotenoid mixtures obtained from both Scenedesmus and Chlorella showed the maximum to occur at 443 mμ as illustrated in Fig. 6. The derived equation for the carotenoid concentration of algal extracts is therefore:

$$C_c(\text{mg/l}) = \log_{10}(I_o/I)_{443}/0.24 \qquad (8)$$

Spectroscopic determination of carotenoids in the presence of chlorophylls

An independent method of checking the carotenoid concentration is possible, thus eliminating the necessity for the saponification of the chlorophylls. An extension of the general Eq.(2) to include three light-absorbing components gives the following equation:

$$\log_{10}(I_o/I)' = \alpha'_a C_a d + \alpha'_b C_b d + \alpha'_c C_c d \qquad (9)$$

The maximum wave length of the carotenoid ethereal extract obtained from algae, 443 mμ, was chosen as the reference wave length since the

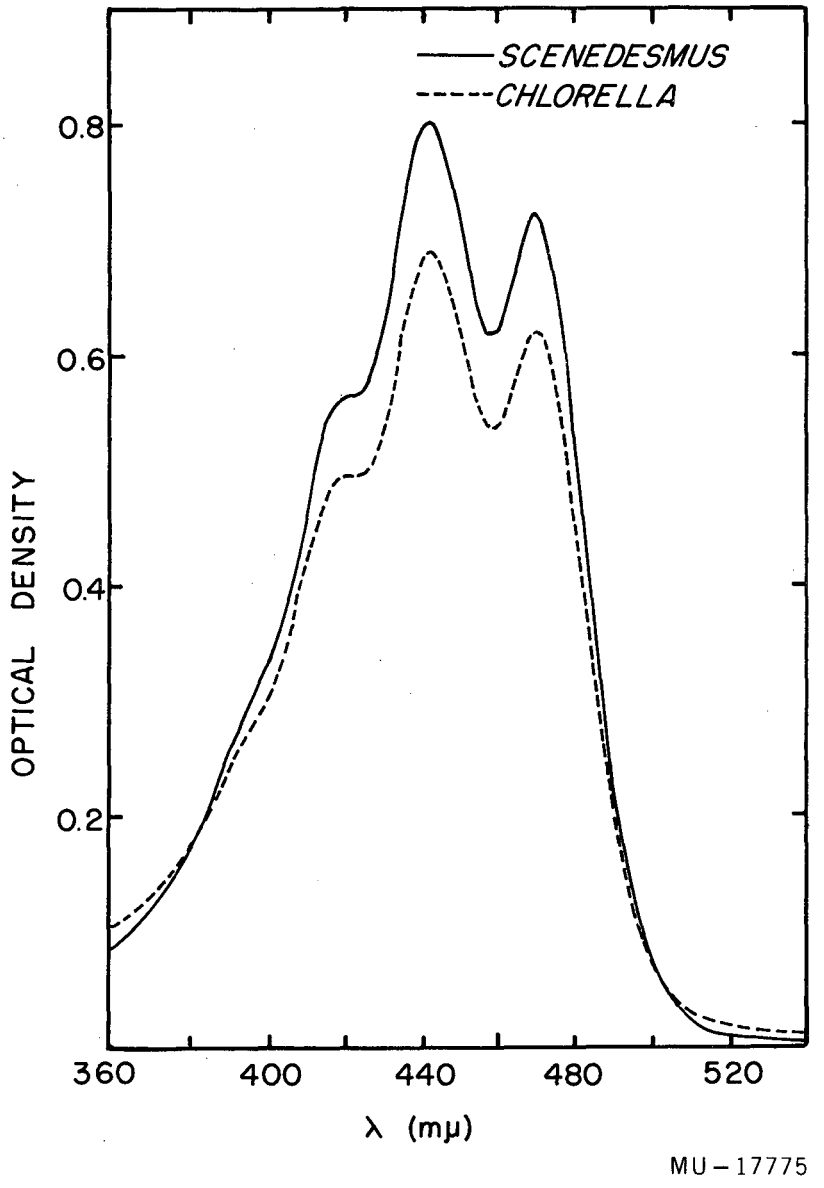


Fig. 6. The absorption spectra of the carotenoid extracts from Chlorella and Scenedesmus in ether.

absorption coefficient had been calculated here, and found to be equal to 240. Substitution of the known α_a and α_b into Eq.(9) gives:

$$\log_{10} (I_o/I)_{443} = 35\ C_a + 88\ C_b + 240\ C_c \qquad (10)$$

The values of C_a and C_b as obtained from Eqs.(4) and (5) are substituted into Eq.(10) to give the following equation for carotenoid concentration:

$$C_c(mg/l) = \frac{\log_{10}(I_o/I)_{443} - 0.035\ C_a - 0.088\ C_b}{0.24} \qquad (11)$$

Total concentration in terms of wet packed cells of algae

All results are recorded in terms of ml of wet packed algae cells, packed under standard conditions of centrifugation as described in the Experimental Section in order to have constancy. Since the ethereal solution obtained, in general, was too concentrated to measure spectroscopically, an aliquot was diluted with ether to a measured concentration. After having obtained the concentration of the pigment in mg/l by means of the appropriate equation, the concentration in terms of mg/ml w p cells was determined by the following equation:

$$C \text{ pigment (mg/ml w p cells)} = C(mg/l) \times \frac{X}{1000} \times \frac{Y}{Z \cdot V} \qquad (12)$$

where X = volume in ml of the ethereal solution obtained from extraction of the methanolic algae extract

Z = volume in ml of the above solution X which was diluted for measurement on the Cary Model 14 spectrophotometer

Y = volume in ml to which aliquot Z was diluted

V = volume in ml w p cells which were extracted to give solution X

IV. RESULTS AND DISCUSSION

The chlorophylls and carotenoids

The green algae, Chlorella pyrenoidosa and Scenedesmus obliquus, were used for the plant pigment studies. There are several reasons for using algae rather than higher plants in such a study. Unicellular algae may be grown in a nonvarying steady state of growth indefinitely; the control of conditions such as nutrient, light intensity, density and so on is relatively easy to maintain. This enables experiments to be carried out with plant material of a constant physiological state. The pigments of higher plants are subject to both daily and seasonal changes in pigment concentration.

Secondly, in working with unicellular algae there is a statistically large number of organisms so that the effects of individual variations are eliminated. It is virtually impossible to culture higher plants under the same conditions. Even one set of plants in a greenhouse is subjected to many variations. The "doubling" time of the algae grown in this laboratory is about 12 hours (i.e. the time necessary for the concentration of fats, proteins, and so on to be doubled). This time stays remarkably constant, indicating a fast and uniform growth rate.

A third reason for the choice of algal cells was that Chlorella cells have been reported to contain an unusually high amount of chlorophyll (up to 5 % of the dry weight, in contrast to higher plants which, in general, contain 0.5 - 1.5% of the dry weight).

Finally, these organisms have been used extensively in photosynthetic studies and their culture is adequately maintained in

this laboratory, both by variable density and constant density culture methods (cf. p. 18). The conditions of culturing algae vary from laboratory to laboratory; the physiological conditions such as light, medium, temperature, aeration, and so on, exert a significant effect of the pigment concentration of any plant material. This should be kept in mind when considering the results.

It is now possible to grow algae by the "synchronous" culture, where the cells are all at the same stage of development in the life cycle.[52] In Chlorella, two distinct forms of cells are found, "light cells" and "dark cells." The latter are smaller in size, richer in chlorophyll, stronger in photosynthetic activity and weaker in respiration than the former. When illuminated, these "dark cells" increase in mass and finally divide into 2-, 4- or 8-autospores and the cycle is repeated. Obviously pigment studies on algal cells which had been grown in "synchronous" culture would represent a more sophisticated approach than has been possible in any plant experiments up to this time. It would be of interest to observe the increase and decrease in the chlorophyll a and b concentrations in the "dark" and "light" cells.

A variety of extraction procedures are reported in the literature for the quantitative removal of the plant pigments.[35] The most usual solvents being employed for the initial extraction are acetone, methanol or pet ether. Extractions are done at liquid nitrogen, room, or higher temperatures. Perhaps the most widely used method for routine analysis of the chlorophyll content is that of Mackinney, whereby the plant material is extracted in 80% aqueous acetone and the concentration estimated directly in the aqueous

acetone by spectrophotometric means.[53] It is difficult to obtain rapid and complete extraction of the algal pigments by this method, however, and the initial extraction was carried out with MeOH. Despite the nondesirability of heating the solvent due to the lability of the chlorophylls, this method was employed, as extraction with MeOH in the cold took a longer time and some of the chlorophyll was lost because it decomposed to methyl chlorophyllides a and b. In particular, the chlorophyll b seemed to decompose to a greater extent, giving an apparently lower ratio of chlorophyll a to b than was obtained by the hot extraction. As long as the hot methanolic solution of algae was rapidly cooled in ice before centrifugation, no loss of the chlorophylls occurred.

In the case of extraction of the carotenoids, it is routine practice to saponify the chlorophylls with methanolic KOH.[40] This procedure not only removes the chlorophylls, but also any neutral fats which might interfere with subsequent chromatography of the pigments. Only one carotenoid, astaxanthin, is unstable in alkali, and it is fortunate that this carotenoid has never been found in any of the algae examined.[40]

The spectrophotometric method employed has been discussed (Section III); after methanol extraction, the pigments are transferred to ether, and the concentration measured directly on the Cary Model 14 spectrophotometer. The variation of the chlorophyll and carotenoid concentration of Chlorella is illustrated in Table 1. The first thing to notice is the remarkable constancy of the chlorophyll a to b ratio which was found to be 3.18 in Chlorella (average of over 100 determinations), and in Scenedesmus to have a slightly

Table 1. Variation in chlorophyll and carotenoid content of *Chlorella pyrenoidosa**.

Column no.	1	2	3	4	5	6	7
Compound conc. calculated from	Chlorophyll a (mg/l).Eq.(4)	Chlorophyll b (mg/l).Eq.(5)	a/b	Total chlorophyll mg/ml w p cells	Carotenoids** mg/ml w p cells Eq.(11)	Carotenoids/ mg/ml w p cells	(a+b)/c
1	3.36	1.06	3.16	7.28	1.00	0.96	7.3
2	2.44	0.78	3.13	7.40	1.04	1.19	7.1
3	7.07	2.20	3.21	7.69	1.10	1.12	6.9
4	3.37	1.04	3.24	7.10	0.96	0.98	7.3
5	6.53	2.05	3.18	7.06	1.10	1.04	7.0
6	7.75	2.37	3.27	7.34	1.08	1.21	6.8
7	3.70	1.10	3.36	6.91	1.09	0.90	6.9
8	6.24	1.96	3.18	7.22	1.01	1.06	7.0
9	9.73	3.33	2.92	7.66	1.02	1.20	6.9
10	2.99	0.93	3.21	7.13	1.06	1.05	6.7
11	3.20	1.01	3.16	7.21	1.01	0.98	7.1

* The algae used were cultured in the constant density culture tube; the measurements were made on random days during May, 1959.

** Column 5 represents the conc. of carotenoids obtained in the total extract.

/ Column 6 represents the conc. of carotenoids obtained from a separate aliquot of the cells after saponification to remove the chlorophylls.

lower value of 3.10. Actually, in all of the plants ever examined this ratio of chlorophyll a to b only varies between about 2.5 to 4.0. This ratio is usually lower in "shade plants" than in "sun plants." Most of the green algae behave as "shade plants" under natural conditions; however, in culturing in the laboratory where they receive adequate illumination, this ratio is raised to about 3.0.

In general, it was found that less variation of the pigment concentrations was obtained from the algae grown under constant density, rather than variable density culture methods; hence, the former algae were used for all the specific radioactivity experiments.

Columns 5 and 6, Table 1, show the amount of carotenoids obtained from the total extract, and the carotenoid extract, respectively. The average variation between these two methods is about ± 4%, which is within the experimental error inherent in the various measurements of the w p cell volume, the total extractability of the pigment from the cells, and so on. Hence, it was not necessary in the radioactive experiments each time to take a separate aliquot of algae and saponify it in order to have the initial concentration of carotenoid, as it could be accurately obtained from the total extract.

The chlorophyll to carotenoid ratio remains fairly constant also, the average value from the determinations made being 7.0. In general, in plants this ratio is more variable than the chlorophyll a to b ratio; variation is shown between different classes of plants, and in higher plants it depends to some extent on the state of etiolation of the leaves. The ratio may be anywhere between 4.0 to 8.0. On looking at the results in the literature, it is safe to make a generalisation that the concentration of chlorophyll to carotenoids is higher in green algae than in higher plants.

Finally, it may be inferred that the constancy of the pigment ratios obtained in these studies is a reflection of the steady state of growth obtained under the culturing conditions employed, since no variations greater than those shown in Table 1 were found over a period of three years investigation.

Under the conditions of centrifugation employed to measure the volume of w p algal cells, experiments of lyophilization and drying of the cells at 40° show that 1 ml w p cells of either Scenedesmus or Chlorella are equivalent to 200 ± 4.0 mg of dry cells (8 determinations). Therefore, the total concentration of the chlorophyll pigments is about 7.2 mg/ml w p cells, or 3.6% of the dry weight, while the carotenoid content is only 0.55% of the dry weight.

Identification of the carotenoids

The absorption bands of the carotenoids in the visible are characterised by 2, or more usually 3, intense bands near the "blue" end of the spectrum. Depending on the extension of the bands into the blue or green, the colour of the pigments may be yellow, orange, or red. With increasing polarity or polarisability of the solvent, the absorption bands are displaced towards longer wave lengths. Changing from ether to carbon disulphide, for example, results in a bathochromic shift of 43 mμ for β-carotene. On the other hand, transition from a nonpolar solvent to a polar solvent of the same polarisability (e.g. from ether to EtOH) makes very little change.

Although the carotenoids possess relatively complex structure, the absorption spectra of these compounds are comparatively simple in character. Karrer has compiled a list of empirical relations that

exist between the constitution and absorption spectra of the carotenoids, which proves very useful in the identification of these compounds.[13] The absorption of light results in the electronic oscillation along the axis of the polyene chain; thus, the λ_{max} will occur at longer wave lengths, and the intensity of absorption will increase, as the number of conjugated ethylenic double bonds increases, Also, the length of the chain will be changed by the cis- or trans-character of the molecule, which will result in differences in intensity and wave length maxima.

Since no reliable crystalline samples of the carotenoids could be obtained for the standardisation of the algal carotenoids, the identification was made by comparison of the absorption spectra with those reported in the literature. It should be mentioned that the only positive means of identification of the carotenoids is by mixed-melting point determinations.

Some of the reliable values for the absorption maxima of the carotenoids are listed in Table 2 (the formulae of these compounds are shown in Fig. 2). The following structures were assigned to the carotenoids obtained from algae (purity of the compounds obtained by column chromatography on cellulose and polyethylene was checked by rechromatography on paper and columns), on the basis of the correspondence of the absorption maxima listed below, to those shown in Table 2.

Table 2. The absorption maxima of plant carotenoids in various solvents.

Carotenoid	No. of conjugated double bands	λ_{max} (mμ)			Solvent	References
α-carotene	10	420	445	475	hexane	40,13
β-carotene	11	425	451	482	hexane	40
		425	450	477	hexane	13
α- and β-carotene mixtures		428	452	480	ether	55
		425	446	480		54
lutein	10	421	446	476	EtOH	12
		422	446	476	ether	55
		420	447	477	hexane	40
zeaxanthin	11	423	451	483	hexane	40
		--	453	481	EtOH	12
xanthophyll epoxide	9	--	445	473	EtOH	13
		--	442	471	pet ether	13
violaxanthin	10	419	442	472	EtOH	12
		420	441	470	ether	55
		416	438	468	ether	54
neoxanthin	?	413	437	466	EtOH	12
		414	436	464	ether	55

α- and β-carotene

λ_{max} (mμ)				
425	446	475	in ether	(Fig.7)
426	448	476	in pet ether	
425	445	473	in 80% MeOH	

Since the absorption maxima of β-carotene are about 5 mμ greater than those of α-carotene, due to the addition of an extra conjugated double bond, it is possible to tell immediately whether or not one or more carotenes are present; the spectral data shown above indicate a mixture. Subsequent chromatography of the carotenes obtained from polyethylene columns on calcium hydroxide, showed that the carotene mixture contained both α- and β-carotene.[44]

lutein

λ_{max} (mμ)				
422	445	472	in ether	(Fig.7)
422	446	472	in pet ether	
422	445	472	in 80% MeOH	

The absorption maxima correspond to those shown in Table 2 for lutein. The corresponding β-dihydroxy xanthophyll is zeaxanthin: the absorption maxima of zeaxanthin are about 6 mμ greater than those of lutein. All attempts at rechromatography, both on columns and paper with a great variety of solvents, showed no separation of the dihydroxyxanthophyll into two bands; hence it may be inferred that only lutein is present in Chlorella and Scenedesmus and, if any zeaxanthin is there, it must be less than 1% of the total dihydroxy xanthophyll content. A mixed-melting point determination would be the only method to see if traces of zeaxanthin were present.

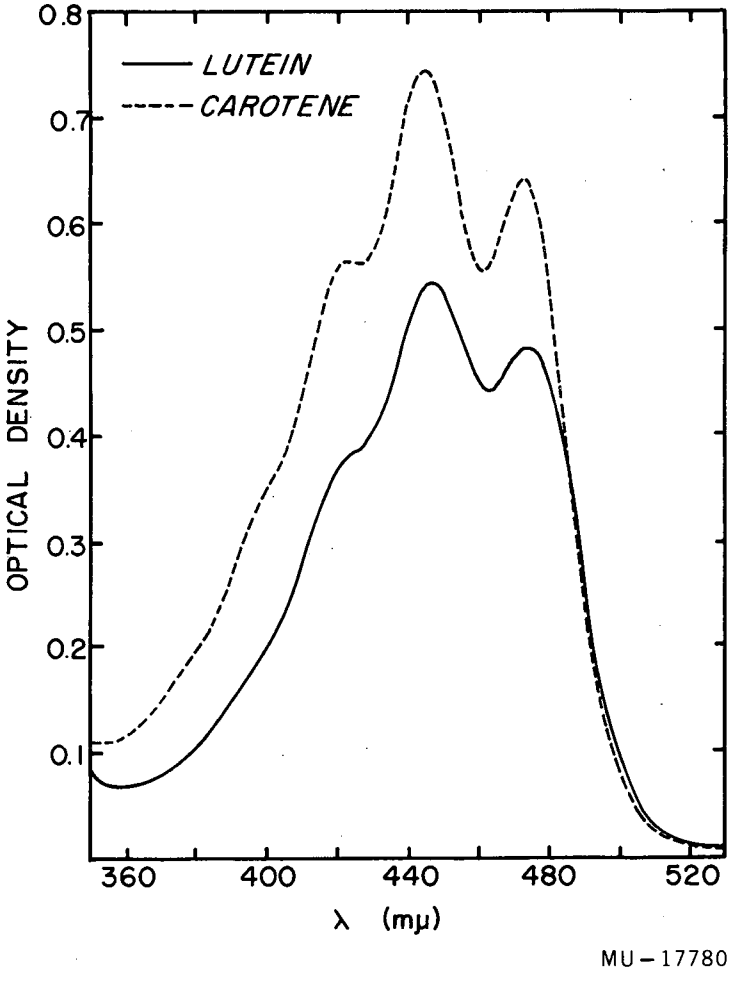


Fig. 7. Absorption spectra of the carotene mixture and lutein from green algae, in ether.

xanthophyll mono-epoxide

No mono-epoxides were found to be present in the carotenoids obtained in these studies. Blass reported that a xanothophyll mono-epoxide was present,[54] but for reasons stated below under pigment X, this "mono-epoxide" has been termed neoxanthin.

violaxanthin

λ_{max} (mμ)	421	440	470	in ether	(Fig.8)
	421	439	469	in 80% MeOH	

The di-epoxide, violaxanthin, undergoes a hypsochromic displacement in its absorption spectrum on the addition of acid, due to isomerisation of the di-epoxide to a di-furanoid isomer. This shift is approx. 20 mμ per epoxide group. The hypsochromic displacement is illustrated in Fig. 8 where the 470 mμ band is shifted to 426 mμ and the 440 mμ band to 401 mμ.

pigment X (neoxanthin)

λ_{max} (mμ)	414	436	466	in ether	(Fig.9)
	413	435	465	in 80% MeOH	

Neoxanthin is a cis-di-epoxide of unknown constitution ($C_{40}H_{56}O_4$). The assignment of a cis-configuration was given, because one cis-ethylenic double bond causes a hypsochromic shift of 3-4 mμ as compared to the isomer with the all trans-configuration (i.e. violaxanthin). The carotenoid termed pigment X would appear to be neoxanthin for the following reasons:

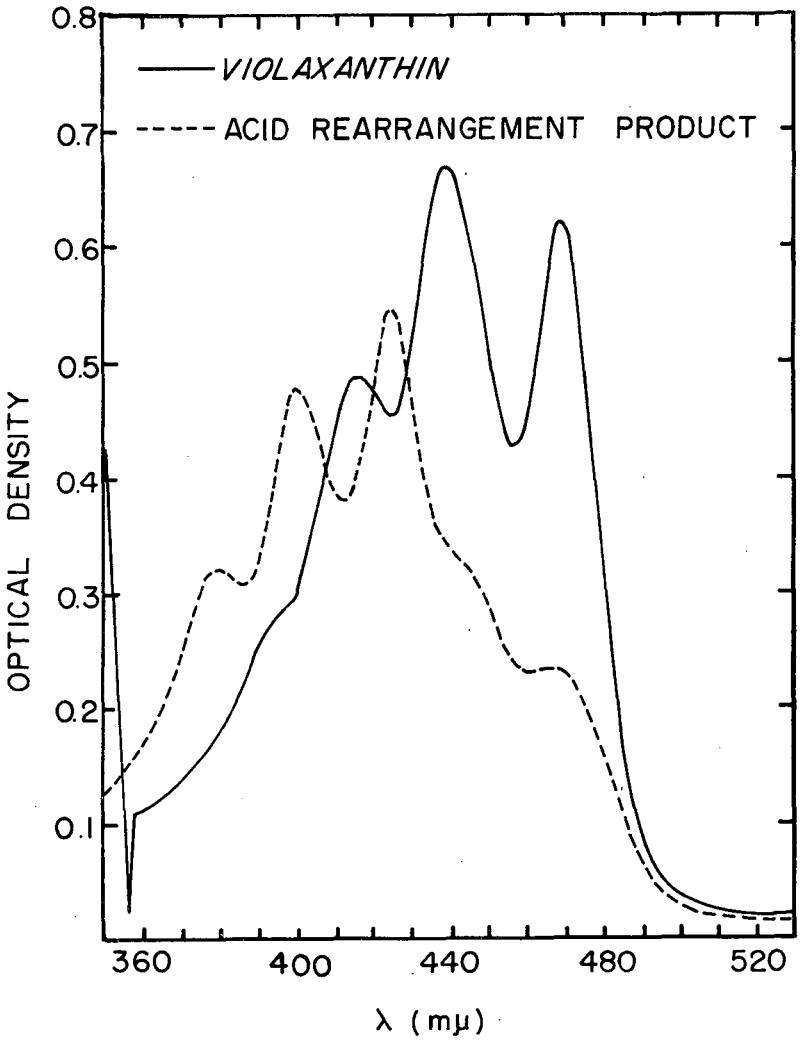


Fig. 8. The absorption spectrum of violaxanthin from green algae, in ether, and the acid rearrangement curve (1% HCl).

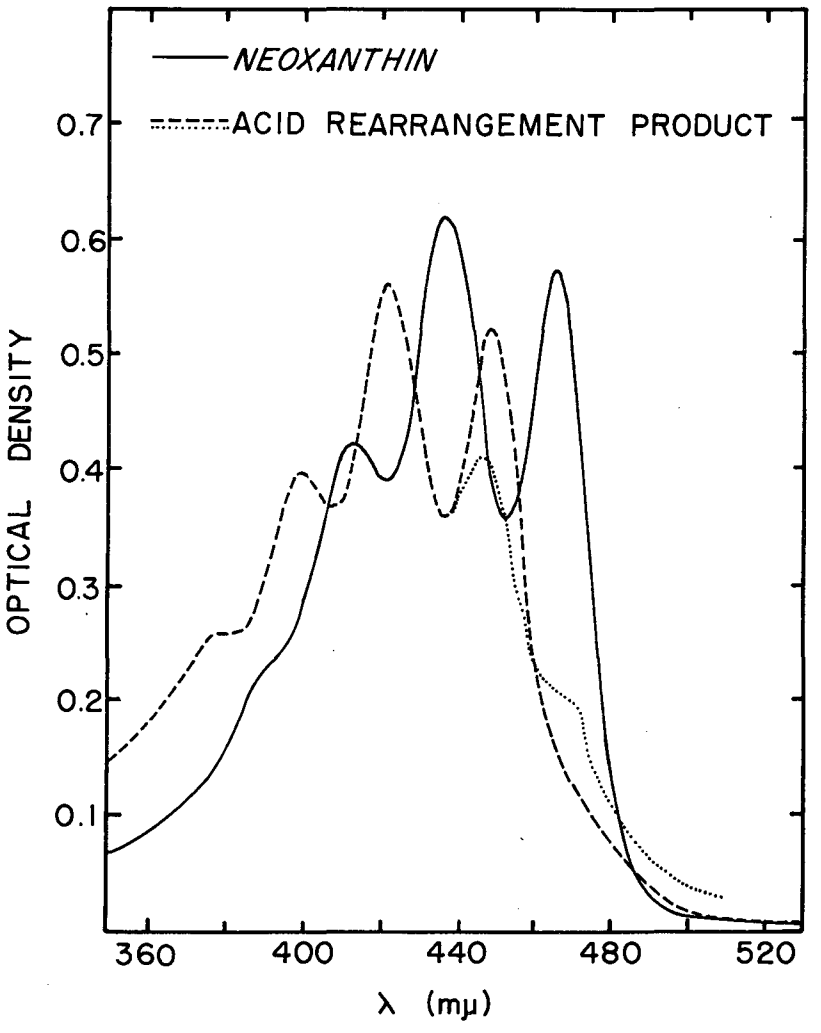


Fig. 9. The absorption spectrum of neoxanthin from green algae, in ether, and the acid rearrangement curve(6% HCl). The lower peak at 449 mμ on the acid rearrangement curve was obtained with weaker acid (1% HCl).

1) The agreement of the λ_{max} of pigment X with those reported for neoxanthin (Table 2).

2) The appearance of a "cis"-peak 114 mμ lower than the longest wave length maxima, which is a characteristic of a cis-configuration. Applying all the empirical rules of Karrer's, it would be inconceivable that a cis-mono-epoxide would have such high absorption maxima as those found for pigment X.[13]

3) It is an empirical rule that the R_f values of the carotenoid molecules depend on the number of oxygens present in the molecules.[43] Pigment X scarcely moves from the origin of paper chromatograms and is adsorbed very strongly on cellulose columns. Violaxanthin, which is a di-epoxide, travels further on paper chromatograms and is less strongly adsorbed on cellulose columns. It seems unlikely that a mono-epoxide would show greater adsorptive properties than violaxanthin does.

4) Both Strain and Goodwin reported that neoxanthin is present in Chlorella.[56,57]

5) The acid rearrangement curve is shown in Fig. 9. Unfortunately, no such spectrum for neoxanthin has been reported in the literature, beyond the fact that this compound gives a characteristic blue colouration with HCl, as does violaxanthin. It will be noted that the curve obtained with 1% HCl corresponds to that of violaxanthin, although the intensity of absorption is somewhat different. Stronger acid caused an increase in the intensity of the 449 mμ band (Fig. 9). The action of acid on di-epoxides causes the isomerisation of the epoxide groups. Acid will cause cis-trans isomerisation also. Because pigment X is already a cis-isomer it is not surprising that

the acid rearrangement curve should be a little different from that of violaxanthin which has an all trans-configuration.

On the basis of the above evidence, this pigment has been called neoxanthin.

Concentrations of the carotenoids in algae

Strain sates that Chlorella pyrenoidosa contains α-, β-, and ε-carotenes, lutein, violaxanthin and neoxanthin.[56] In a determination of the carotenoid content of Chlorella vulgaris, Goodwin found roughly the same distribution of carotenoids in the light and in the dark, which he reported to be β-carotene, 10%; lutein and a trace of violaxanthin, 75-80%, and neoxanthin, 10%.[57] From this study it is evident that considerably more than a "trace" of violaxanthin is present. In numerous and very careful experiments on the carotenoid concentration obtained from polyethylene columns, Blass found the following carotenoid concentrations in the light.[58]

	conc. in 10^{-8} mole/ml w p cells
α-carotene	30
β-carotene	30
lutein	155
"xanthophyll-epoxide" (neoxanthin)	50
violaxanthin	47

These values are the average of eight column separations and the figures show a ± 4% variation. It should be noted that these figures give a total carotenoid concentration of 1.8 mg/ml w p cells, which is higher than that

obtained in my results by spectrophotometric determination of the total carotenoid extract (column 5, Table 1). This is partly due to the different specific absorption coefficients used by Blass.[58]

The average percentage composition of the carotenoids as determined both from polyethylene and cellulose columns, was the following:

	% composition
carotenes	14
lutein	52
neoxanthin	16
violaxanthin	16

Therefore, the average xanthophyll to carotene ratio is 6.0. In general, in both higher plants and green algae this ratio varies from 4.0 to 6.0. It is also of interest that Haskin in 1942 reports almost identical ratios for the pigments of Chlorella pyrenoidosa: a xanthophyll to carotene ratio of 6.1, and a chlorophyll a to b ratio of 3.2.[59] (The ratios obtained in these studies were 6.0 and 3.18, respectively.)

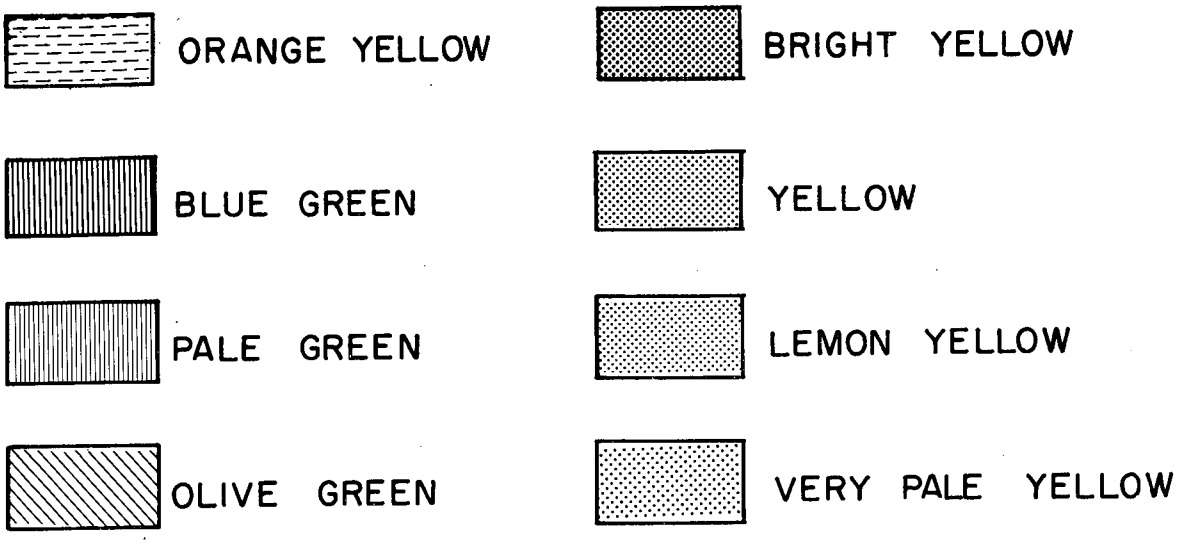
The carotenoids of the green algae resemble those of higher plants more closely than do any other class of algae, although the xanthophylls are less complicated than those found in the higher plants. It is an interesting fact that, whenever the dominant carotene is β-carotene as in green algae and higher plants, then the dihydroxy xanthophyll will be the α-isomer, that is, lutein rather than zeaxanthin (β-dihydroxy xanthophyll).

Column chromatography

Despite the universal use of sucrose columns for the separations of plant pigments, this adsorbent has several disadvantages. It is difficult to obtain constant adsorbent qualities in commercial sucrose and, in some cases, the polar solvents used for dissolving the pigments may also dissolve the sucrose; hence, extra time is involved in the preparation of the extract.

Strain and Sato demonstrated that the sorptive qualities of chromatography paper are similar to those of cellulose used in a column, for the separation of plant pigments. They concluded that "sucrose columns give sharper resolution than cellulose." The eluent used was pet ether-n-propanol (99.4:0.6 v/v).[60] Preliminary investigations proved that cellulose was an excellent adsorbent, however; the column was uniformly packed under slight pressure, using a slurry of cellulose in pet ether. The cellulose had no chemical action on the chlorophylls and a very good separation of the a band from the b band was obtained. The rate of percolation of the eluant was faster than in sucrose columns, yet no loss of resolution occurred during development.

The order of elution of the pigments roughly parallels that obtained on sucrose columns: The carotenes are most easily eluted, followed by the dihydroxy xanthophylls; chlorophyll a precedes chlorophyll b, and it was necessary to add n-butanol (3%) to elute the chlorophyll b and the succeeding xanthophyll mono- and di-epoxides. The separation is shown in Fig. 10. This method was investigated concurrently by a group of Australian workers, who also found cellulose to be a most satisfactory adsorbent.[61] They used isopropanol or MeOH (1%) to in-



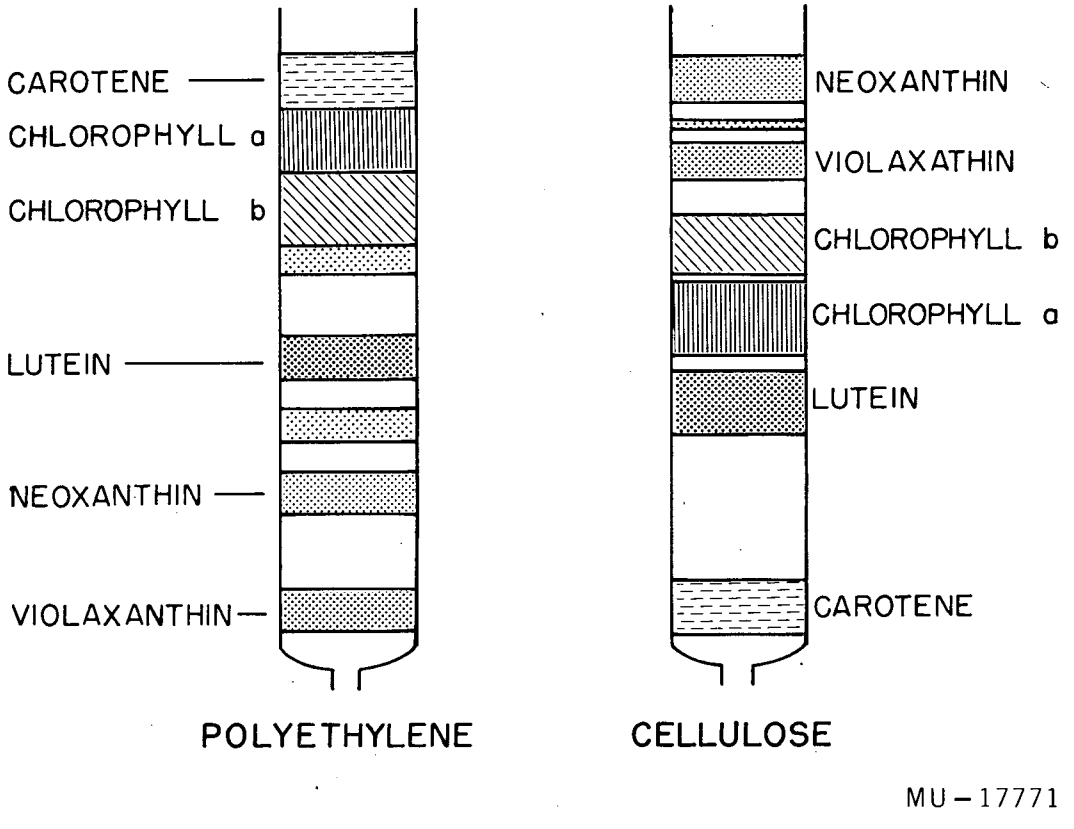


Fig. 10. Separation of the pigments of Chlorella on a polyethylene column (left with 80% aqueous MeOH and on a cellulose column (right) with pet ether. The colour chart for the pigment zones is shown above.

crease the eluting power of the solvent. In investigations for suitable adsorbents for the separation of carotenoids, Blass found that polyethylene powder was a useful adsorbent.[54] In the course of this investigation, it was found that polyethylene powder was also a satisfactory adsorbent for chlorophyll and carotenoid separations, as no decomposition of the chlorophylls took place on the column. Excellent resolution was obtained, the column was relatively easy to pack uniformly, and the rate of percolation was satisfactory.

The order of elution of the pigments is completely the reverse of that obtained on cellulose or sucrose adsorbents. The xanthophyll di- and mono-epoxides were eluted first, followed by lutein, chlorophyll b, then a, and lastly the carotenes, as is shown in Fig. 10.

This reversal of separation is analogous to that obtained in reversed phase paper chromatography, where impregnation of the paper with oil or vaseline causes a reversal of the pigment bands. It is possible on sucrose columns by the use of different solvent combinations to obtain reversal of contiguous bands,[61] but this is the first time an adsorbent has been found which completely reverses the elution pattern, as happens on polyethylene powder.

Since each adsorbed component may leave behind it a trail of adsorbed material as it passes down the column, the most adsorbed components are contaminated by the traces of pigments that preceded them. This is especially true of the chlorophylls which generally travel close to one another. Thus, to be able to obtain such a complete reversal, as on polyethylene columns, is very advantageous. The chlorophyll b band is likely to be obtained in a purer state from polyethylene than from cellulose or sucrose columns.

Undoubtedly the process of adsorption is important in the development of cellulose columns with a nonpolar solvent, but on the polyethylene columns, with 80% aqueous MeOH where the pigment zones are the reverse of those found on cellulose or sucrose columns, it would appear that partition is playing a dominant role. Not only are the chlorophyll a and b bands reversed, but the whole order of the carotenoids is also reversed.

Although the conventional methods for chromatography of the carotenoids extract are on "alumina-type" adsorbents,[40] both cellulose and polyethylene were found to be satisfactory. The polyethylene columns which had been washed by EDTA-Na_4 before development gave excellent separations, the order of elution being violaxanthin, neoxanthin, lutein and the carotenes.

Paper chromatography

Despite the different solvents mixtures used in one-dimensional chromatography, the pigments are resolved in the following general order: O -dihydroxy xanthophylls, chlorophyll b, chlorophyll a, monohydroxy xanthophylls, phaeophytins, carotenes -- S F. The R_f values of the carotenoids depend on the number of oxygen atoms in the molecule; the carotenes with no oxygen run at the solvent front in all cases, except with pure polar solvents, while the di-epoxides with four oxygen atoms have much lower R_f values; neoxanthin seems always to have a lower R_f value than the isomeric di-epoxide violaxanthin (Table 3).

Table 3. R_f values of algal pigments from ascending chromatograms.

[Conditions: 0 to SF: 40 cm; time of development, 1.5 to 2 hours. Whatman No. 3 MM. Average R_f values for 6 runs - deviation ± 0.02]

Solvent: Toluene			pet ether-isopropanol-toluene (100:2.5:2.5)	
SF.	Total extract	Carotenoids	Total extract	Carotenoids
carotene	0.98	0.98	0.96	0.97
lutein	0.88	0.86	0.90	0.86
violaxanthin	0.70	0.66	0.77	0.72
chlorophyll a	0.29		0.62	
chlorophyll b	0.19		0.28	
neoxanthin	0.14	0.13	0.15	0.15
Solvent: pet ether-isopropanol (100:2.5)			pet ether-isopropanol(100:2.5) in toluene-sat. chamber	
SF.	Total extract	Carotenoids	Total extract	Carotenoids
carotene	0.98	0.98	0.98	0.98
lutein	0.56	0.50	0.88	0.84
chlorophyll a	0.23		0.79	
violaxanthin	in tail of chlor. a	0.31	in tail of chlor.a	0.72
chlorophyll b	0.12		0.45	
neoxanthin	0.09	0.04	0.30	0.17

Trial chromatograms for the separation of chlorophyll a and b were obtained using a variety of mobile phases (the extract had the xanthophylls removed, by partition of the ether solution with 90% MeOH).

Mobile phases and description of separation:

(a) acetone-isopropanol-water (100:25:25)

Good separation of chlorophyll a from b, but marked tailing of the spots.

(b) toluene-methanol (100:1-4)

Good separation and compact spots. Increase of R_f's with increasing concentration of MeOH.

(c) ethyl acetate and ethyl acetate-water mixtures

No separation until the water concentration was 25% and then marked tailing of the spots.

(d) MeOH - water (85:15)

Fair separation of the chlorophylls.

(e) Pet ether-n-butanol (100:1.5)

Excellent separation of the chlorophylls; slight tailing of chlorophyll a.

(f) toluene-acetone-isopropanol (100:4; 2 v/v)

Excellent separation and compact spots.

As far as the chlorophylls are concerned, good resolution may be obtained with nonpolar solvents which have small quantities of polar solvents mixed with them. The addition of water to the mobile phase results in marked spreading out of the chlorophyll spots and should, therefore, be avoided.

Table 3 illustrates that the addition of polar to nonpolar solvents seems to be of great importance for the satisfactory resolution of both the carotenoids and chlorophylls. With pet ether alone, the R_f's of chlorophyll *a* and *b*, and the di-epoxides are all less than 0.1, which is obviously unsatisfactory. On the other hand, mixtures containing large amounts of polar solvents, or of pure polar solvents, result in all the pigments moving with the solvent front which, again, gives poor resolution. The R_f value of lutein is increased in solvents which contain some toluene, as compared to the pet ether-alcohol mixtures. This is an advantage, because in the latter solvents the lutein shows a tendency to "stick" with chlorophyll *a*, despite increasing the concentrations of alcohol in the pet ether.

Figs. 11, 12 and 13 illustrate the pigment zones obtained with three solvent systems, in order to give some idea of the resolution and the size of the pigment bands. The actual width of the zones seems to depend on the solvent employed and to bear little relation to the actual concentration of the pigment; thus, although lutein is 52% of the carotenoid extract, the lutein band is rarely wider than those of the di-epoxides, violaxanthin and neoxanthin which are present in only one-third of the lutein concentration. In general, the chlorophyll *a* band is wider than the *b* band, as would be expected.

Two-dimensional chromatography gives greater resolution of the pigments. Because of the slowness of this method, it is not so favorable for the unstable plant pigments. Satisfactory solvents for separation are:

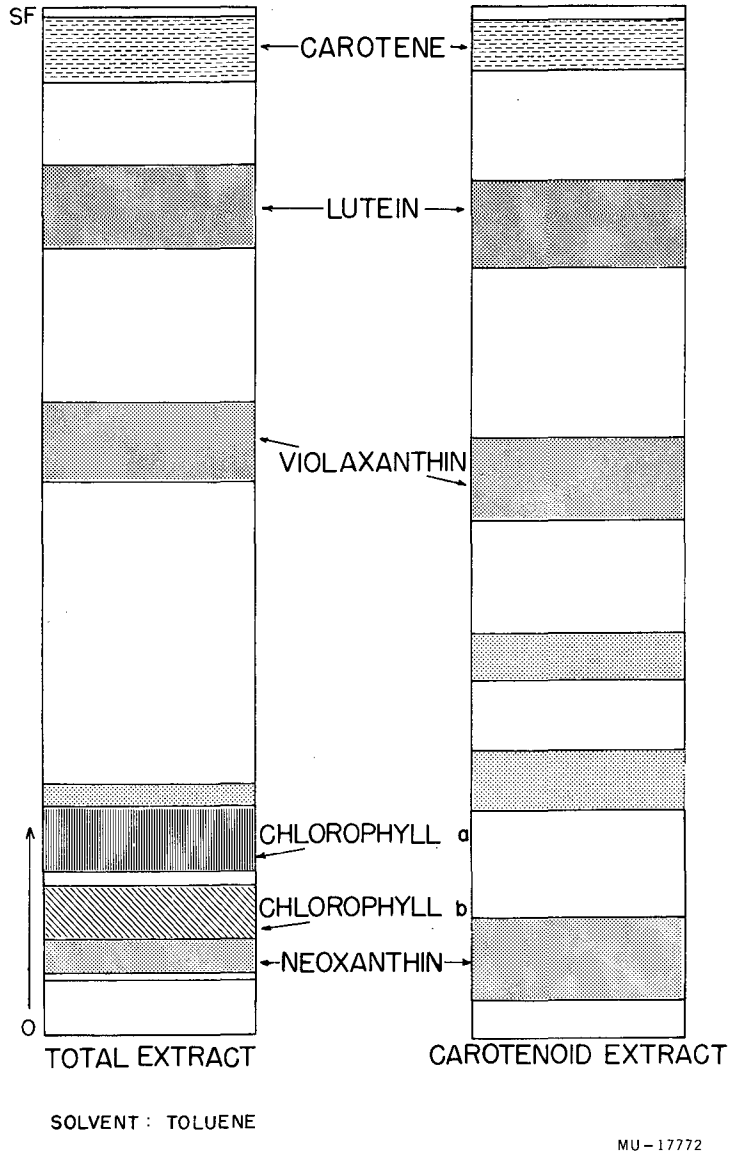


Fig. 11. Descending paper chromatograms of the total pigments and carotenoid extracts of Chlorella, using toluene as the mobile phase. The colour chart for the pigment zones is shown in Fig. 10.

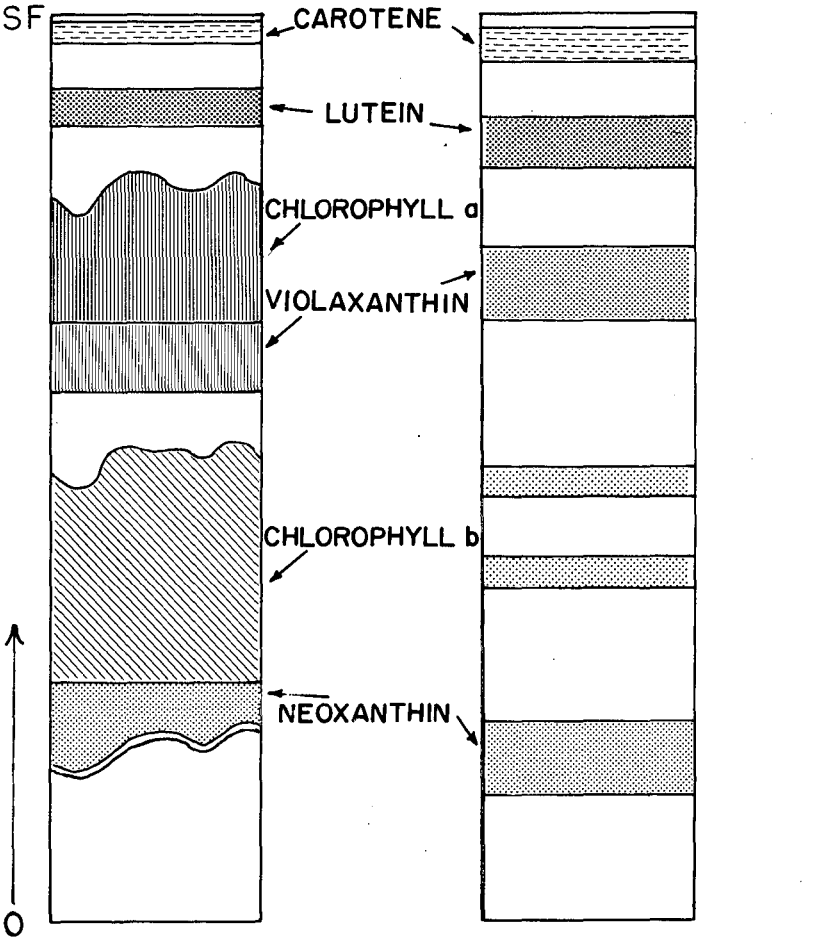


Fig. 12. Descending paper chromatograms of the total pigment and carotenoid extracts of Chlorella, using pet ether-isopropanol (100:2.5) as the mobile phase, in a toluene-saturated chamber. The colour chart for the pigment zones is shown in Fig. 10.

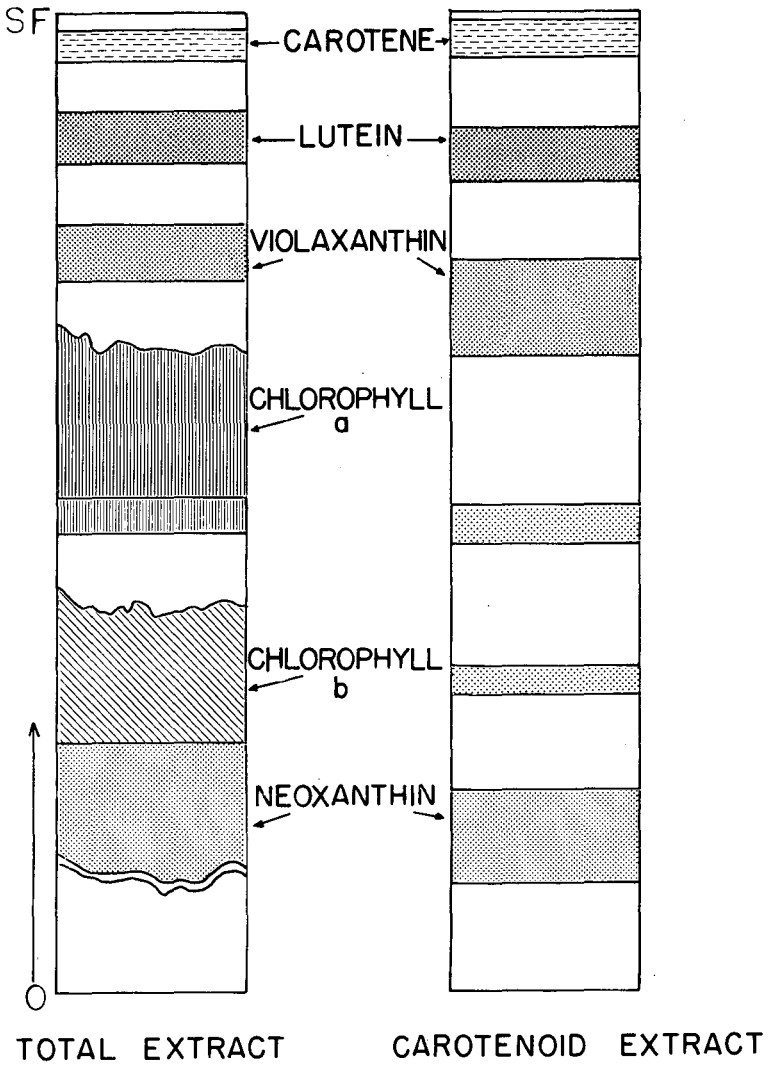


Fig. 13. Descending paper chromatograms of the total pigment and carotenoid extracts of Chlorella, using pet ether-isopropanol-toluene (100:2.5:2.5) as the mobile phase, in a toluene-saturated chamber. The colour chart for the pigment zones is shown in Fig. 10.

(a) 1. pet ether

2. pet ether-n-propanol (100:0.6 v/v)[60]

(b) 1. toluene

2. pet ether-n-butanol (100:2 v/v)

Methods based on reverse phase chromatography also have been employed. In this technique, the paper is impregnated with vaseline, olive or paraffin oil, and a strongly polar solvent used as the developer. The sequence of development is the reverse of that obtained with the usual chromatographic methods (where small amounts of water are retained on the cellulose). Circular chromatograms impregnated with paraffin oil were used,[61] but this method was not found to offer any advantages over the conventional methods.

Descending and ascending chromatograms were employed; in general, the method of ascending chromatography was used for strip chromatograms and the descending method for the larger sheets of paper. Ascending chromatograms under nitrogen atmosphere gave less decomposition of the pigment spots, but due to the disturbance of the solvent atmosphere, poorer resolution was obtained.

The results of the decomposition of the algal pigments on a typical paper chromatogram are as follows:

An ethereal extract (0.58 mg) of algal pigments was placed on the origin (15 cm) of a Whatman No. 3 MM sheet and developed for 2.5 hours with pet ether-isopropanol-toluene (100:2.5:2.5) by descending chromatography. The bands were eluted with ether and the concentration determined spectrophotometrically.

	conc. of pigment (mg x 10^{-2})
carotene	1.28
lutein	3.01
violaxanthin	1.01
chlorophyll *a*	24.10
chlorophyll *b*	11.08
neoxanthin	0.99
Total	41.47

This represents a 72% recovery of the pigments from a typical chromatogram. Part of the loss is due to the incomplete elution of the pigment by the ether, and part is due to decomposition of the pigments. However, as paper chromatograms were used for specific activity measurements only, and not for a quantitative measure of the pigment concentration, this does not matter. With pet ether-isopropanol as the mobile phase, the average recovery was 75%, and with strip chromatograms which ran for a shorter time (1 hour), it was 80%. Strip chromatogram runs in the cold room under nitrogen gave a 85% recovery, but the resolution of the bands was inferior.

Centrifugally-accelerated paper chromatography

First of all, it should be pointed out that the chromatograms obtained from the chromatofuge, regardless of the velocity of rotation, are not circular in shape but elliptical. This results from an inherent property of the fibre structure of the paper; the major axis of the ellipse is parallel to the long side of the rectangular sheet, and the Whatman circles commercially available show this elliptical pattern; therefore, they must be cut from the same paper as the rectangular sheets. This is not a disadvantage, however; regard-

less of whether the measurements for the R_f values are made on the long axis, the short axis, or intermediate positions of the ellipse, these are the same within the experimental error (± 0.02 R_f units).

It was found that the resolution of the carotenoids and chlorophylls was the same as that obtained in conventional ascending or descending chromatography. That is, any solvent which gave satisfactory resolution in the conventional methods could be used for the centrifugally-accelerated chromatograms, although it was necessary to determine suitable running conditions for the chromatofuge.

In order to obtain reproducible R_f's, it is necessary to have a flow rate of solvent such that the perimeter of the wetted area does not increase after the rotor and solvent flow are stopped; with such a flow rate the paper is said to be "minimally wet." Also, it is important not to have such a high flow rate that flooding of the paper will occur during development, as this causes distortion of the zones.

The placement of the circular origin is important; it must not be less than 7.5 cm in diameter or else some of the pigments will flow inwards. It was found that an increase of 1.0 cm for the diameter of the circular origin resulted in an increase of about 0.04 for intermediate R_f values. It was not advantageous to increase the diameter beyond 12 cm for the separation of total pigment extracts, since under those conditions only spots with R_f values less than 0.3 would be separated.

In general, under the same conditions of nitrogen pressure, velocity of rotation, and with the same delivery jet, different

solvents will travel different distances. For example, at 500 rpm, 7.5 p.s.i. and a flow rate of 2.0 ml/min, toluene travelled at 15 cm; pet ether (bp 75°) 13 cm; pet ether (bp 30-60°) 6 cm; and pet-ether-isopropanol (100:2.5) 14 cm in 10 minutes.

Also, with different grades of paper, the distance travelled by the solvent will vary. With a flow rate of 2.5 ml./min of toluene at 500 rpm, the following results were obtained:

	time of development (min)	0 →SF. (cm)
Whatman No. 1	10	16
	12.5	17.5
	15	19
Whatman No. 3 MM	10	14
	12.5	15
Whatman No. 17 MM	10	6
	20	14
	25	16

It can be seen that increasing the time results in a non-linear extension of the solvent front. The area wetted by the solvent increases rapidly up to a certain point and then tapers off. Obviously when trying out a new grade of paper or a different solvent, it is necessary to make some trial runs with no pigments on the paper to find a satisfactory time.

Yet another variable needs to be considered: the use of the liner paper. This is essential with most solvents, in particular with pet ether. Even with the heavier Whatman No. 3 MM or 17 MM paper it is necessary to have a liner.

Table 4 illustrates the R_f's obtained, using the same solvent and conditions, at different velocities. In general, it is seen that above 450 rpm the R_f values decrease as the velocity of rotation increases, although for a change of 50 rpm the decrease in R_f's is within the experimental error, but considering the changes obtained over the complete range of velocities available (i.e., 350-950 rpm), the changes are significant. It will be noticed that between 350 and 450 rpm the R_f's increase with increasing velocity, instead of decreasing as they do in the range 450-950 rpm. Hence the "optimum speed" for this solvent is 450 rpm; the speed at which the R_f values are a maximum will be termed the "optimum speed."

For several other solvents a similar set of runs were made at varying velocities and the same type of pattern was found as that of Table 4. Instead of listing the whole gamut of R_f values obtained, Table 5 shows the R_f's obtained at the "optimum speed." It is seen that this "optimum" speed is not constant for different solvents, but it seems to lie nearer the lower velocities of rotation.

Comparison of the R_f's listed in Tables 4 and 5 to those of Table 3 (descending chromatograms) shows the similarity obtained with the same solvents. In all cases carotene runs right at the solvent front. Solvents with a higher proportion of toluene are useful for separation of the carotenoids; on the other hand, the chlorophylls a and b travel further in pet ether-isopropanol solvents. Figs. 14 and 15 show the width of the pigment zones obtained; it may be seen that the bands are very narrow and well-defined. They are less than one-half as wide as those obtained on descending or ascending chromatograms, that have been run the same distance as the chromatofuge chromatograms.

Table 4. Chromatofuge R_f's.

Conditions: Whatman No. 3 MM paper (unwashed). Solvent: pet-ether isopropanol (100:2.5); flow rate of 2.5 ml/min; 7.5 p.s.i. nitrogen pressure, temp. 25°; liner saturated with 20 ml of solvent (unnecessary for toluene); time of development, 10 min; circular origin, 10 cm. The R_f values reported are the average of 3 separate runs at each velocity quoted.

Speed in rpm	350	400	450	500	550	600	650	750	850	950
carotenes	0.91	0.92	0.94	0.94	0.96	0.95	0.97	0.96	0.97	0.98
lutein	0.46	0.47	0.56	0.47	0.44	0.42	0.36	0.35	0.32	0.30
chlorophyll *a*	0.44	0.45	0.47	0.38	0.37	0.30	0.26	0.27	0.23	0.22
chlorophyll *b*	0.27	0.26	0.26	0.23	0.23	0.16	0.14	0.14	0.11	0.08
neoxanthin	0.06	0.08	0.10	0.13	0.11	0.11	0.08	0.05	0.05	0.04

Note: As in ascending or descending chromatograms, with petether-isopropanol (100:2.5), violaxanthin is masked in the bottom of the chlorophyll *a* spot.

Table 5. Chromatofuge R_f's of algal pigments at "optimum speed".*

Conditions: Whatman No. 3 MM paper (unwashed); flow rate of 2.5 ml/min; 7.5 p.s.i. nitrogen pressure; liner saturated with 20 ml of solvent (except unnecessary for toluene); time of development 10 min; 10 cm circular origin. R_f values reported are the average of 3 separate runs at the velocity quoted.

Solvent: Toluene; "optimum speed" 600 rpm			Solvent: Toluene-pet ether (4:1), "optimum speed" 500 rpm	
Compound	Total extract	Carotenoid extract	Total extract	Carotenoid extract
carotene	0.96	0.97	0.98	0.97
lutein	0.89	0.90	0.90	0.90
violaxanthin	0.82	0.80	0.78	0.74
chlorophyll *a*	0.34		0.37	
chlorophyll *b*	0.19		0.27	
neoxanthin	0.04	0.06	0.07	0.07
Solvent: Toluene-pet ether (1:1); "optimum speed" 750 rpm			Solvent: Toluene-pet ether (1:3); "optimum speed" 500 rpm	
carotene	0.98	0.94	0.98	0.95
lutein	0.83	0.85	0.75	0.73
violaxanthin	0.70	0.50	0.48	0.35
chlorophyll *a*	0.33		0.30	
chlorophyll *b*	0.22		0.19	
neoxanthin	0.10	0.07	0.12	0.17

* "Optimum speed" is defined as that velocity of rotation of the chromatofuge where the R_f's of the pigments are at a maximum.

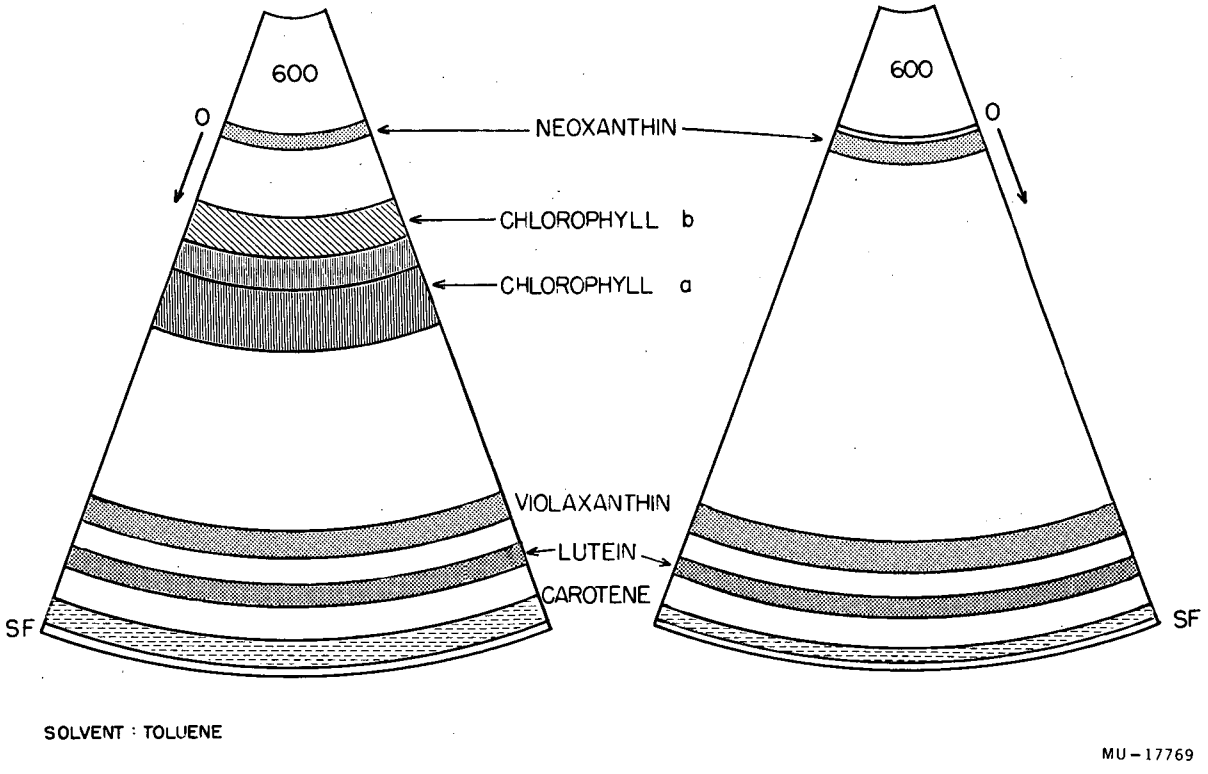


Fig. 14. Sectors of centrifugally-accelerated paper chromatograms of total pigment and carotenoid extracts from Chlorella, using toluene as the solvent, at 600 rpm; time of development, 12.5 min; flow rate of 2.5 ml/min; 0→SF. = 17 cm, Whatman No. 3 MM paper. The colour chart for the pigment zones is shown in Fig. 10.

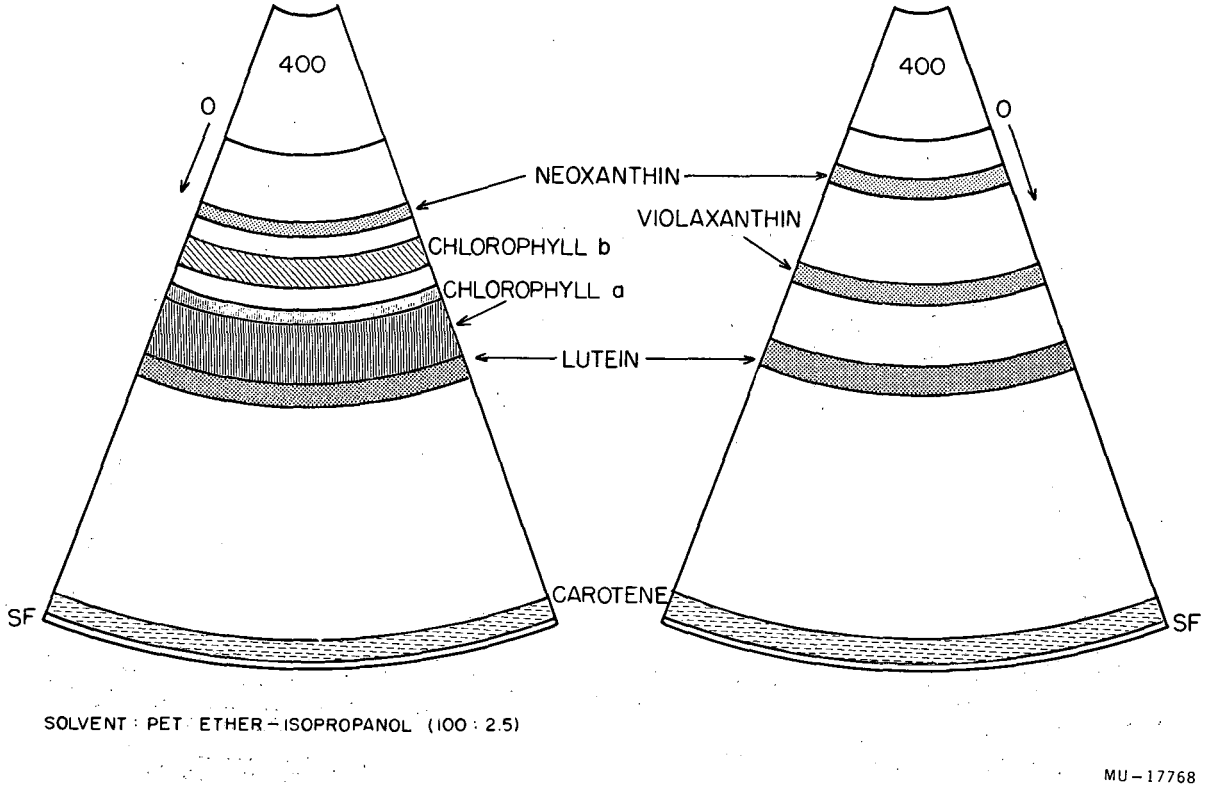


Fig. 15. Sectors of centrifugally-accelerated paper chromatograms of total pigment and carotenoid extracts from Chlorella, using pet ether-isopropanol (100:2.5) as the solvent at 400 rpm. Time of development, 10 min; flow rate of 2.5 ml/min. 0→SF. = 17 cm; Whatman No. 3 MM paper. The colour chart for pigment zones is shown in Fig. 10.

In the hopes of obtaining better separation of chlorophyll a and b reverse phase chromatography was tried. The MeOH used as the solvent for the paraffin oil-impregnated paper showed a great tendency to flood the papers. A flow rate of 1.25 ml with a 4 p.s.i. of nitrogen pressure was necessary, which meant at least 15 minutes development time. The results obtained were disappointing; the bands were diffused and streaking into one another. All the pigments other than the carotene, which stayed right at the origin had R_f's greater than 0.6, which meant that they were all "jammed together" despite the variety of conditions tried.

The quantitative recovery of pigments from a typical chromatogram was checked. Algal pigments (0.21 mg) were placed on the origin of a Whatman No. 3 MM paper and developed for 10 minutes with a flow rate of 2.3 ml/min at 500 rpm, using toluene. The spots were eluted immediately with ether and the concentration measured spectrophotometrically.

	conc. of pigment ($mg \times 10^{-3}$)
carotene	3.12
lutein	18.24
violaxanthin	2.98
chlorophyll a	119.10
chlorophyll b	39.61
neoxanthin	3.11
Total	186.16

Thus 88% of the pigments placed on the origin were recovered, which is much better than ascending chromatography where the average is about 70%. Since the chromatograms take 10 to 15 minutes instead of

2 to 3 hours to be developed, there is less time for decomposition of the pigments to occur. The pigment zones are easily eluted with ether and less time is needed to obtain colourless paper; hence there is a smaller loss in this operation than on the ascending or descending chromatograms. It would probably be advantageous to operate the chromatofuge at lower temperature than 25°; it was not possible to do this with our machine.

In summary, it can be said that centrifugally-accelerated chromatography appears to be very promising for the plant pigments. The advantages of this method are:

(1) the rapid development time (10-15 minutes)

(2) the narrowness and compactness of the pigment zones

(3) the ease of elution of the pigment zones and the greater recovery of the pigments after elution.

(4) the many variable factors available, velocity of rotation, solvent flow and so on which permit more control over the resolution than in conventional methods. (These many variables might also be classed as a disadvantage as some time is needed to find the optimum conditions for resolution).

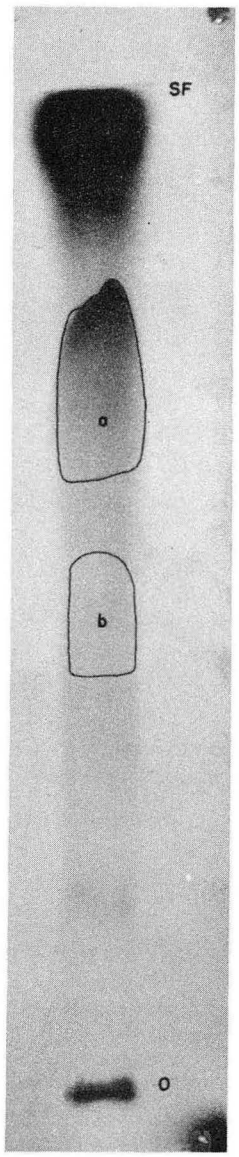
Specific Radioactivity of Pigments

Series I results

At the beginning of this study, it had been hoped that the specific radioactivities of the plant pigments might be determined after the resolution of the plant pigments by paper chromatography. This technique of paper chromatography has been used extensively since its introduction in 1944,[42] and together with radioactive tracers has been used for the separation of minute quantities of many biological materials.

Most of the experimental reports on paper chromatography of the plant pigments were qualitative;[43] the question of the application of the method for radioactive studies had not been considered. The initial work showed that the pigments could be fairly adequately resolved on one-dimensional chromatograms. Since there are at least five carotenoids and two chlorophylls present in the algal extract, it seemed advantageous to achieve partial separation of the pigments, before the application of paper chromatography. This was achieved by the saponification of the chlorophylls to obtain a carotenoid extract, and by partition of the 90% methanolic algal extract with pet ether to remove the xanthophylls in the pet ether layer, and given an extract containing only the chlorophylls and carotenes.

Despite the good resolution of the chlorophylls on paper, the radioautographs were disappointing; Fig. 16 illustrates a typical radioautograph. The complete streaking of the radioactivity from the chlorophyll *a* to *b* spot shows the danger of measuring the radioactivity of these pigments by merely counting the spots on the paper. A similar



SOLVENT : TOLUENE – ACETONE–
ISOPROPANOL (100 : 2.5 : 2.5)

ZN-2218

Fig. 16. Radioautograph of the chlorophylls from Scenedesmus after 2 hours photosynthesis with $C^{14}O_2$. The lines around a and b represent the green areas of chlorophyll a and b of the corresponding paper chromatogram.

streaking of radioactivity was obtained on carotenoid radioautography. Obviously it was desirable to measure the concentration of the pigment spectrophotometrically, rather than assume no decomposition of the pigment had occurred on the paper. Therefore, the spots were eluted and the concentration determined. It was found that only about 70% recovery of the pigments was obtained. Subsequent rechromatography of the spectroscopically pure pigments showed a diminution in the streaking, but the concentration of some of the pigments was too small for accurate spectral work. Two-dimensional chromatography resulted in a better separation of the pigments, and the spots on the chromatograms coincided with darkened areas on the radioautographs; however, the concentration of the pigments after elution was vanishingly small.

The extreme lability of both the chlorophylls and the carotenoids is obvious from a glance at their chemistry. The time involved in better chromatographic separation, for example, with two-dimensional chromatography, is sufficient for the decomposition of the pigments to occur to a marked extent, and yet the whole success of the method depends on adequate chromatographic separations; thus an impasse has been reached. No validity can be attached to the results obtained by this method; hence, they are not quoted. It should be emphasized that caution is needed, when dealing with the paper chromatographic separation of any labile substances.

Series II results

Since only very small quantities of pigments can be resolved on paper, this is not a good method for the initial separation of the pigments. Even with prior partial separation by partition, the amounts of pigments in an extract that can be placed on the origin of a paper chromatogram are small, and part of the failure of the experiments of Series I can be ascribed to this fact. The next attempt undertaken utilised the method of column chromatography, which has been used extensively for the preparation of plant pigments, for both crystalline compounds and spectroscopic standards.[35,40]

In 1957, Blass determined the radioactivity of the successive fractions eluted from a polyethylene column with 80% MeOH. Very little correspondence between the chromatographic distribution of the radioactivity and the light-absorbing pigments was found.[58] Similarly, Shlyk in Russia divided a sucrose column after partial development with pet ether into zones and compared the radioactivity of the zones with the pigment concentration. He found high radioactivity at the top of the column extending into the chlorophyll b zone.[62] A similar result is obtained with cellulose; the carotenes which are eluted first are contaminated with colourless substances which run both before and after the carotenes. It is obvious that even though the pigments may be obtained in a spectroscopically pure state from column separations, no validity can be attached to radioactive measurements at this point.

It is therefore necessary to purify these pigments further; this was achieved by paper chromatography. Since the pigments after

column separation are present as single substances (except for colourless contaminants) it was possible to apply more material to the origin of a chromatogram, than was the case in Series I experiments. Subsequent elution of the spots from the chromatogams gave adequate material for the accurate measurements of concentration. Moreover, a correspondence was obtained between the pigment areas on the chromatogram and the darkened areas of the radioautograph. The presence of colourless contaminants was implicated by darkened areas on the radioautographs, which did not coincide with the pigment areas.

The difference in radioactivities of the chlorophylls, after separation by cellulose and polyethylene columns, is shown in Table 6. It is an advantage to use both cellulose and polyethylene columns, because of the reversal of the development of the pigment zones. Chlorophyll a precedes chlorophyll b on cellulose columns; therefore, the tailing decomposition products of chlorophyll a may cause contamination of chlorophyll b. The values for the specific radioactivity of chlorophyll b are lower from the cellulose than from the polyethylene columns. Table 6 also illustrates the lower specific activities obtained after the second separation on paper; these values are considerably lower than those determined for the pigments that had been separated on cellulose or polyethylene columns and are therefore likely to be much more accurate.

The molar radioactivities of the Scenedesmus pigments after one hour photosynthesis with $C^{14}O_2$ are listed in Table 7. These values are obtained after the separation of the pigments on a polyethylene

Table 6. Specific radioactivity of the pigments from *Scenedesmus* after photosynthesis with $NaHC^{14}O_3$ (11.1 μc/μM).

Chromatographic separation	Chlorophyll a (μc/μM)	Chlorophyll b (μc/μM)
8.5 hours photosynthesis		
polyethylene	2.12	1.71
	1.79	(0.29)
	2.38	1.35
paper	1.48	0.41
(pet ether-n-butanol) 100:3	0.92	0.67
	1.47	0.59
7.5 hours photosynthesis		
cellulose	1.98	1.49
	1.67	1.71
	1.83	1.89
polyethylene	1.81	1.28
	1.72	1.42
paper	1.31	0.89
(pet ether-n-butanol) 100:3	1.22	0.62
	1.17	0.44

Table 7. Molar radioactivity of *Scenedesmus* pigments after one hour photosynthesis with $NaHC^{14}O_3$ (11.1 μc/μM).

Pigment	μc/μM
violaxanthin	0.074
neoxanthin	0.006
lutein	0.078
α-carotene	0.814
β-carotene	0.700
chlorophyll *a*	0.147
chlorophyll *b*	0.056

column and further rechromatography on paper, using toluene as the mobile phase. Since the carotenes cannot be separated into the α- and β-isomers on paper, it is necessary to use calcium hydroxide columns.[44] There is a disadvantage to such a strong adsorbent as calcium hydroxide; although it allows a better chromatographic separation of the isomers, marked decomposition of the pigments occurs. Also, the carotenes obtained from both cellulose and polyethylene columns are strongly contaminated with colourless radioactive substances. In order to obtain accurate data for the carotenes, it would be advantageous to remove both the chlorophylls and the xanthophylls from the algal extract (saponification of the chlorophylls and partition with pet ether to remove the xanthophylls). The figures shown in Table 7 are not the average values obtained from many experiments done. When the main sources of error are considered, i.e., the presence of radioactive colourless contaminants and the decomposition on the paper, it is obvious that both of these errors will tend to give high values for the specific activities of the pigments. The experimental errors involved in the radioactive and concentration measurements are of a much lower magnitude (2%). Thus, the lowest specific activities of the pigments have been quoted in Table 7, since they are believed to be the most reliable data obtained.

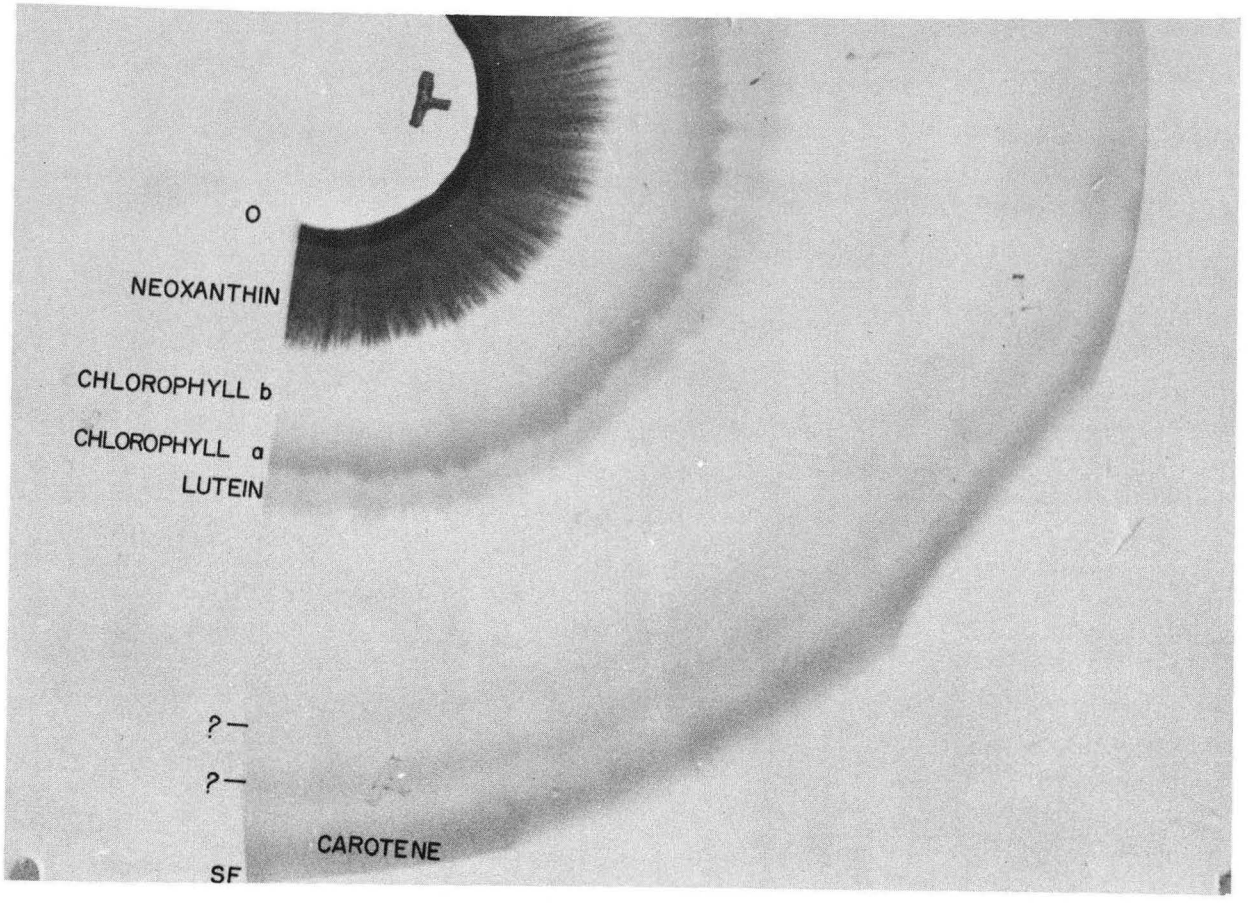
Although the results here are much more valid, it can only be said that they represent relative values for the specific activities of the pigments. The main source of trouble inherent in this method is the time lag in the paper chromtographic separation. Al-

though the pigments may be obtained in a spectroscopically pure state after elution from the paper chromatograms, at least 30% of the pigment is lost either on the development of the paper or in the elution, thus the measurements may yet be too high. Crystallisation of the pigments after the column separation would be the only way to check this.

Series III results

In an attempt to eliminate the time lag mentioned above, the method of centrifugally-accelerated chromatography was investigated. The resolution of the pigments was found to be very satisfactory by this means. Perhaps the greatest advantage was the shorter time involved in the development of the chromatograms (10 minutes instead of 2 to 3 hours). Elution of the pigment spots was much easier from these chromatograms than from the conventional paper chromatograms, and only about 10% of the pigment was lost during the development and elution, which is some 20% better than with the paper chromatograms.

Fig. 17 shows a radioautograph of an algal extract; it can be seen that two colourless substances with marked radioactivity are present near the solvent front, just above the carotenes. Also there is considerable activity at the origin. This could be decreased by adequate washing of the ethereal extracts before their application to the paper. On the other hand, complete streaking of the radioactivity was obtained with the reverse phase chromatograms.



ZN-2219

Fig. 17. Radioautograph of the total pigment extract from Chlorella after 2 hours photosynthesis with $C^{14}O_2$. The centrifugally accelerated chromatogram was developed with pet ether-isopropanol (100:2.5) at 400 rpm. Time of development, 10 min; flow rate of 2.5 ml/min; 0 → SF, 16.5 cm; Whatman No. 3 MM paper. The coloured zones of the chromatogram corresponded to the darkened areas of the radioautograph.

A typical distribution of the radioactivity in the various pigments is listed in Table 8. It can be seen that only about 10% of the radioactivity is present in the ethereal extracts. Subsequent washing of this extract results in a loss of about 35% of that radioactivity. Thus the total activity fixed in the algal pigments and various fats which accompany them is about 3% of the total.

As might be expected, the separation of the pigments on centrifugally accelerated chromatograms, without some initial separation, is not satisfactory. The values obtained are too high due to possible contamination of the pigments by colourless lipids. Since carotene was always running at the solvent front, and, therefore, highly contaminated, no measurements were made of its specific activity. It can be seen that the variations are too great in Table 9, to attach any validity to the results listed, except in a relative manner. Chlorophyll a has, in general, a higher activity than chlorophyll b; lutein and violaxanthin have similar activities, while neoxanthin has a lower activity than the other carotenoids (except at the longer times of photosynthesis). It should be noted that the relative distributionof the specific activities of Table 9 are similar to those shown in Tables 6 and 7.

It will be remembered that the main source of error in the experiments of Series II was the time lag involved in the rechromatography of the spectroscopically pure pigments on paper. Not only did marked decomposition of the pigments occur on development of the paper chromatograms, but it was difficult to elute the spots.

Table 8. The distribution of radioactivity in the various fractions obtained from *Chlorella* after photosynthesis with $C^{14}O_2$ (5.0 μc/μM).

Photosynthesis time in hr	Total extract d/m/ml w p cells	Percentage of total fixation			
		Supernatant	Methanolic extract	Ethereal extract	Cell residue
1.0	98.4×10^7	8	35	9	58
2.0	129×10^7	14	27	8	59
2.5	141×10^7	13	42	10	35
3.0	188×10^7	15	41	16	28

Table 9. Molar radioactivity in μc/μM of the pigments from *Chlorella* after photosynthesis with $C^{14}O_2$ (11.1 μc/μM) and separation by centrifugally-accelerated chromatography.

Carotenoid extract:	Toluene at 700 rpm			Total extract: pet ether-isopropanol (100:2.5) at 400 rpm	
Photosynthesis time	Molar radioactivity in μc/μM				
in hrs	neoxanthin	violaxanthin	lutein	chlorophyll *a*	chlorophyll *b*
1.0	0.11	0.44	0.27		
	0.19	0.87	0.59		
2.0		1.12	0.94		
3.0	0.63	1.96	1.7		
		1.24			
		0.73			
5.0				1.69	0.76
6.0	1.71	1.36	0.55	0.79	0.36
	1.34	0.69	1.06	0.84	0.21
		1.65	1.62		

Total extract: Pet ether-isopropanol (100:2.5) at 400 rpm

	chlorophyll *a*	chlorophyll *b*	lutein
1.25	0.76	0.36	0.77
2.25	1.16	0.15	0.98
3.25	0.96	--	0.98
4.25	1.43	0.71	2.12
	1.62		2.61
	1.45		
5.25	1.85	0.46	1.78
	2.03		1.50

This is not the case with centrifugally-accelerated chromatograms. Although it is not feasible to separate the pigments from the colourless contaminants by centrifugally-accelerated chromatography alone, it is believed that this technique could be used to great advantage, after the prior separation of the pigments by column chromatography.

Discussion

The earlier stages of the biosynthesis of the chlorophylls have been well established; it has been shown both by *in vivo* and *in vitro* reactions that glycine and acetate combine to form the pyrrole nucleus, porphobilinogen, which, in turn, is converted to protoporphyrin. The terminal steps in the biosynthesis are not understood as yet.

The *de novo* synthesis of chlorophylls *a* and *b* in *Chlorella vulgaris* was studied in 1953.[62] It was shown that after seven days of exposure of the algae to radiocarbon in the light, the specific activities of chlorophyll *a* and *b* were equal. A similar result was obtained by Shlyk after the exposure of the etiolated leaves of several higher plants to C^{14} for 60 hours in the light.[63]

Shlyk also studied the incorporation of radiocarbon into the green leaves of higher plants and found a large difference in the specific activities of chlorophyll *a* and *b*.[64] The specific activity of chlorophyll *a* was two to three times greater than that of *b*, even after 24 hours exposure. This result was confirmed by experiments herein described with *Chlorella* and *Scenedesmus*, where the

specific radioactivity of chlorophyll a was always greater than that of b, the ratio being about 2 to 3.

It is of interest to look at the data in regard to the problem of the relationship between chlorophyll a and b. Firstly, it may be said that the possibility of conversion of chlorophyll b to a is definitely excluded, since a substance with a lower specific activity cannot be a precursor to one with a higher specific activity. Also, it may be unequivocally stated that chlorophyll a and b are not rapidly interconverted in plants; otherwise the specific activity of the chlorophylls would be the same in green plants, which is not the case.

All the studies so far undertaken seem to indicate a common pathway for the formation of chlorophyll a and b, at least up to a certain point. Shlyk found that the percentage distribution of the radioactivity in three fragments of the chlorophyll a and b molecules, the methyl ester, the phytyl chain and the chlorin ring was the same, although the specific activity of the chlorophyll a was greater than that of b.[64] Degradations as such were not performed in this study, but it was noted that the specific activities of methyl chlorophyllide a and b had the same ratio as the original chlorophylls from which they were derived; hence, the distribution was the same in the phytyl chains also.

Two possibilities remain: Either chlorophyll a is a precursor to chlorophyll b, or the chlorophylls are derived from a common precursor (either the porphobilinogen or porphin ring stage) and are formed independently of one another in the latter stages of the biosynthetic chain.

Studies dealing with the photochemistry and chemistry of the protochlorophyll halochrome indicate that this protein-porphyrin complex is the initial chlorophyll precursor.[65] Earlier experiments with etiolated seedlings indicated the prior formation of chlorophyll a, followed by chlorophyll b in a manner which seemed to indicate that chlorophyll b was being derived from a. More refined studies show that this is not necessarily so; indeed, Smith believes that chlorophyll a is not a precursor to b since their rate of formation is parallel, and if chlorophyll b was being derived from a, the rate of formation of chlorophyll b should be proportional to the concentration of chlorophyll a, which is not the case.

The only way to explain the facts of the higher specific activities of chlorophyll a is to suggest that it is a precursor to b, or that its turnover rate is greater, or both. This can only be resolved when reliable data can be obtained over a range of time intervals; all the data so far are at isolated times. Since the de novo synthesis was studied after such a long time, it is scarcely surprising that the chlorophylls have attained equilibrium. Had the data obtained in these experiments been of a more reliable nature, it would have been possible to follow the increase in the specific activity of chlorophyll a as compared to that of b. This would furnish an answer to the question. It is believed that such measurements could be obtained using the methods of column chromatography together with centrifugally-accelerated paper chromatography.

It is also interesting to speculate on the possible relations between the carotenoids. During the course of this work, Sapozhnikov

reported the differences in the carotenoid concentrations of higher plants, in the light and in the dark. He found a reciprocal relation between lutein and violaxanthin which was influenced by the light; in the dark, the concentration of lutein was decreased and that of the di-epoxide violaxanthin increased, while the reverse reaction took place in the light. The depression of the violaxanthin concentration by light was confirmed by Blass in these algae experiments, although the correspondingly smaller percentage increase of lutein was not observed.[44] The proposed relation between these substances is supported by the fact that their specific activities are very nearly equal (Tables 6, 7 and 9). Such a light-dark interconversion between lutein (xanthophyll) and violaxanthin is indicated in Fig. 18. This scheme would require that the hydration of lutein, and its dehydrogenation to violaxanthin, be a dark, enzymatic reaction, while the expulsion of molecular oxygen from violaxanthin giving back lutein, be a light-dependent reaction. A similar result would, however, be attained if a dark, direct oxidation by molecular oxygen of lutein, and a photoreduction of violaxanthin, took place. An unequivocal distinction between these two alternatives would be provided by the demonstration of isotopic oxygen content in violaxanthin, isolated from plants which had photosynthesized in H_2O^{18}.

In contrast to this suggested photosynthetic oxygen transport scheme, Cholnoky et al. envisage a nonphotosynthetic transport system (i.e. oxygen absorption) as shown in Fig. 18, whereby zeaxanthin is oxygenated to the mono-epoxide, antheraxanthin (Fig. 2) which may then give up its oxygen to another substrate and return to lutein.[31,67]

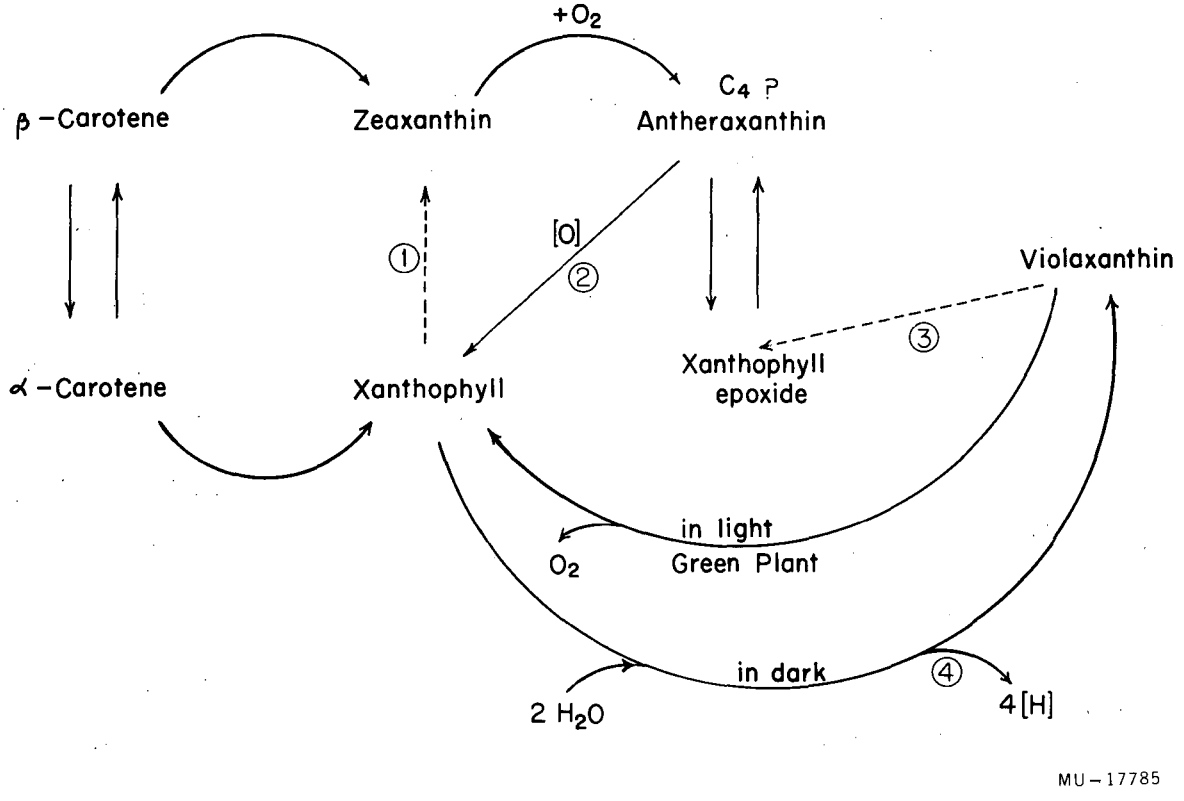


Fig. 18. Possible dynamic relations between the carotenoids

(1) rate-limiting in green plants
(2) [O] available as oxidant for other systems
(3) possible slow step
(4) [H] ultimately for CO_2 reduction

Perhaps this system may be coupled to the lutein-violaxanthin system in plants which contains both mono- and di-epoxides, or a slow leak from the violaxanthin directly into the mono-epoxide may occur, as shown in Fig. 18 (step 3).

Finally, the specific activities of the carotenes are higher than those of the other carotenoids, which might implicate these compounds as precursors to the more oxygenated forms just discussed. In general, it has been found that the more reduced carotenoids are precursors to the more oxidized forms.[30,31,67]

V. CONCLUSION

The determination of the specific radioactivities of the chlorophylls and carotenoids of algae after photosynthesis with $C^{14}O_2$ is a formidable task, due to the extreme lability of these compounds. The whole success of the method depends on adequate chromatographic separations of the pigments from the colourless contaminants which are closely associated with them, and yet the time involved for such separations is sufficient for marked decomposition of the pigments to occur. It is suggested that the techniques of column chromatography, followed by centrifugally-accelerated paper chromatography of the spectroscopically pure pigments, may resolve this problem.

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RESEARCH IN PHOTOSYNTHESIS:

PART II. THE RELATION OF BRANCHED-CHAIN SUGAR ACIDS TO THE CYANIDE INHIBITION OF CO_2 FIXATION

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PART II

THE RELATION OF BRANCHED-CHAIN SUGAR ACIDS TO THE CYANIDE INHIBITION OF CO_2 FIXATION

I. INTRODUCTION

The use of inhibitors in the study of biological reactions has often been crucial. Not only do specific inhibitors help clarify the sequence of certain metabolic pathways, but they also frequently permit the accumulation of intermediate products which might otherwise have remained undetected. They are an invaluable source of information concerning the nature and mechanism of action of the enzyme, upon which they act.

Cyanide has been one of the most widely used enzyme inhibitors, especially in the study of respiratory enzymes. It is clear that this ion is a very unspecific enzyme inhibitor; more than forty-five cyanide-sensitive enzymes, which include haemoproteins and iron or other metal-containing enzymes, have been reported.[1] Firstly, it may combine with, or actually remove the metal of an enzyme. It may act as a reducing agent to break the essential disulphide linkages of the enzyme, or it may react chemically with carbonyl groups of the enzyme or of some co-factor associated with it, or even attack the substrate.

Many inhibitors have been used in the study of photosynthesis including cyanide, fluoride, hydroxylamine and so on. The sensitivity of photosynthesis to cyanide was discovered in 1919 by Warburg.[2] Since that time, a host of experiments have been reported which deal with the effects of low and high concentrations of cyanide(under

different intensities of light on photosynthesis.[3] Due to the enormous variability of the experiments performed, it is almost impossible to interpret the data. The majority of studies indicated that cyanide exerts a profound influence on photosynthesis.

In 1957, Kandler examined the effect of cyanide on the pattern of carbon dioxide fixation on the alcohol-water soluble fractions of Chlorella[4]. The algae were photosynthesised for ten seconds in the light in $C^{14}O_2$, followed by the addition of KCN. After a further ten seconds, the algae were killed by the addition of boiling alcohol, to stop the enzyme reactions. The most striking features of these experiments, were the decrease in the C^{14} activity of phosphoglyceric acid and the increase in that of the diphosphates, as compared to normal photosynthesis fixations. Treatment of the eluted diphosphate area yielded two new spots on radioautographs, besides the other well-known sugars. These spots were found to exhibit an acid-lactone behavior.

It is the purpose of this study to establish in more detail the role of cyanide in the formation of this new compound in Chlorella.

II. EXPERIMENTAL

Abbreviations

The following abbreviations will be used throughout Part II of this thesis:

CN^-	cyanide
HmDP	hamamelonic acid diphosphate
PGA	phosphoglyceric acid
RuDP	ribulose diphosphate

General Methods

All the solvents were evaporated under reduced pressure using a rotary evaporator * in conjunction with dry ice and liquid nitrogen traps, at 10-100 mμ; it was not advisable to allow the bath temperature to exceed 40°.

Ultraviolet and visible spectra were determined by the Cary 14 recording spectrophotometer, Model 14M. The radioactive measurements were made in the usual manner (cf. Part I p.33).

Anions and cations were removed by the weakly basic and strongly acidic Amberlite IR-4B and IR-120 resins, respectively. The ion exchanger was stirred in a beaker several times with distilled water, and washed into the column, which was then backwashed to eliminate air bubbles and channels. It was not desirable to use strongly basic anion exchange resins, because the alcohol groups of the sugars are very weak acids.

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* Rinco Instrument Company, Greenville, Illinois.

Paper chromatography

Whatman No. 4 sheets were used throughout, after washing the paper with oxalic acid according to Benson.[5] Ascending chromatograms were made in the boxes routinely used in this laboratory;[6] the material was spotted on an origin three inches from the bottom and side of the paper. Phenol-water (72:28 w/v) was used for the development of the longer dimension of the paper, and the solvent was run for eight to ten hours, until it had reached the edge of the paper. After drying at room temperature for at least eight hours, the papers were developed with butanol-propionic acid-water for six to eight hours, until the solvent had reached the edge of the chromatograms. Equal volumes of n-butanol-water (1,246:84 v/v) and propionic acid-water (620: 790 v/v) were mixed just before use, for the second-dimension solvent.

Ferric chloride spray for gallic acid, hamameli-tannin and hamamelose: 0.1% of aqueous $FeCl_3$ solution was sprayed on the chromatograms; blue-gray spots indicated a positive reaction with the above compounds.

Tollen's silver spray for sugars:[7] 2.3 ml of a saturated $AgNO_3$ solution was placed in a graduated cylinder and concentrated NH_4OH added cautiously, until the precipitate initially formed had just dissolved, and the volume made up to 100 ml with methanol. The papers were sprayed with this solution, dried in an oven at 90-100° for several minutes, and then washed with a 4% $Na_2S_2O_3$ solution; this solution was found to reduce the background colour of the chromatogram more successfully than the 5% NH_3 solution normally used.

Sensitive silver spray for sugars: in all cases where a more sensitive silver spray was needed, the silver dip method was employed.

The dried chromatogram (in the case of the "phenol" papers, the excess of phenol was removed by steaming the paper and redrying it) was quickly dipped in a bath containing 0.5 ml of a saturated $AgNO_3$ solution in 100 ml of acetone (to which water had been added drop by drop, to dissolve the precipitate initially formed). The paper was then pinned on a wooden rack, and left for a few minutes to dry, and sprayed with ethanolic sodium hydroxide (0.5 M NaOH in ethanol). The excess background was removed by washing the paper in a 4% $Na_2S_2O_3$ solution, because this solution gave a paler and more uniform background than NH_3.

Column chromatography of sugars

Cellulose columns have been employed for the separation of sugars and found to give satisfactory results for small quantities of materials[8]. These columns are rather difficult to handle, if it is desired to separate over 500 mg of any mixture, or if the R_f's of the substances do not differ appreciably. This is the case with the aldo- and keto-pentoses, as the R_f's differ by less than 0.1 R_f units. Separations of 500 mg mixtures of xylose and xylulose on cellulose columns, using either butanol-propionic acid-water or butanol-ethanol-water (5: 1:4) were disappointing, and only about half of the xylulose was recovered uncontaminated with xylose.

A useful tool was developed in 1954 by Hagdahl and Danielson, who found that it was possible to roll sheets of filter paper very tightly and use this roll as the column adsorbent[9]. The ChroMax pressurized paper chromatography column, Model LKB-3502 * was assembled

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* Ivan Sorvall, Inc. Norwalk, Connecticut, Distributor for L K B-Produktor, Stockholm, Sweden.

in the manner prescribed using the polyethylene plugs at both ends in order to obtain maximum sensitivity from the column. The solvent used was the upper layer of a n-butanol-ethanol-water (5:1:4) mixture. The homogeneity of the column was tested, by applying a solution of methyl red (6 mg/10 ml of the above solvent) to the top of the column, and observing the emergence of the dye zone from the bottom of the column by means of a mirror. This lower surface was instantly coloured with the dye solution when the mantle pressure was 0.5 kg/cm^2, hence the column was developed with this over-pressure supplied from the ballast flask. The solvent was fed into the column from a large reservoir (3 l); since the polyethylene tubing (2mm) through which the solvent entered the column was so fine, the tubing became brittle and the solvent leaked out around the glass capillary to which it was attached. This difficulty was eliminated by using a piece of tapered glass tubing with a narrow bore at one end, and placing the polyethylene tubing inside the glass tubing, rather than over it. Under the conditions stated, the flow rate of the column was 45 ml/hour. Since the saturation volume of the column was 400 ml, the minimal flow time for a substance with an R_f of 1.0 would be 400/45 or 8.88 hours.

The sugar solution to be separated was applied to a test strip of the same paper as used in the column, and ascending chromatograms made in the solvent, with which the column was to be developed. For a xylose-xylulose mixture the R_f's obtained were 0.3 and 0.4, respectively, with butanol-ethanol-water (5:1:4).

The time in hours for the elution of any substance from the column is obtained by dividing the minimal flow time, by the R_f of the substance in question. Thus xylose should be eluted in 22.2 hours and xylulose in 29.6 hours, after the application of the mixture to the column. The eluate was collected in test tubes in an automatic fraction collector (4.5 ml/tube). An aliquot from each tube was applied to the origin of Whatman No. 4 sheets and chromatographed in n-butanol-propionic acid-water. Spraying the paper with Tollen's reagent showed the sugar content of each tube. Typical separation for a xylose-xylulose mixture (0.8 g), which was analysed 21.5 hours after application to the column, was as follows:

Fraction No.	Sugar content
1 to 8	zero
8 to 34	pure xylulose
34 to 79	zero
80 to 88	pure xylose

The solvent was evaporated under reduced pressure and a 95% recovery of the estimated xylulose in the mixture was obtained.

In a similar manner arabinose (R_f = 0.32) and ribulose (R_f = 0.38) could be satisfactorily separated. This method also gave separation of hamamelose from gallic acid with which it was contaminated after hydrolysis of the hamameli-tannin, since the R_f of gallic acid was 0.66 and that of hamamelose, 0.28 under the conditions employed above.

Electrophoresis

Unwashed paper was used for electrophoretic studies, which were carried out on an apparatus similar to that employed by Foster[10];

glycine buffer (0. 1 M) pH 9.75 was used and the experiment was run at 600 v. (10-20 ma.) for 3 hours at room temperature.

Bial-Orcinol test for pentoses

The concentration of the pentoses (less than 50 μg) were determined by the Bial reaction with orcinol-HCl[11]. As there was considerable variation in the methods used from laboratory to laboratory, some standardisation was needed. The orcinol used was freshly recrystallised in benzene and made up just prior to use; orcinol in ethanol (0.2 ml of 10% w/v) and 2 ml of $FeCl_3$ solution in concentrated H Cl (0.1% $FeCl_3$ in conc. HCl w/v) were added to the pentose solution in water to be tested (2 ml). The mixture was thoroughly stirred, and a glass marble placed on top of the test tube, which was heated for 40 min. in a boiling water bath. The test tube was then rapidly cooled, the volume made up to 5.0 ml with distilled water and the optical density at 670 mμ measured. It was necessary to measure the optical density within 20 minutes of cooling the solution, as a change in optical density occurred after 30 minutes standing.

A standard curve was calibrated for arabinose and xylose (pentose dried at 80°/10 mm/24 hrs.); 10, 20, 30 and 40 μg of the pentose were sunjected to this reaction, and the optical density at 670 mμ plotted against the concentration of pentose. A straight line was obtained for both xylose and arabinose.

By using the following data, a mixture of the aldo- and keto-pentose could be analysed. The extinction coefficient given by ribulose is 50% of that given by any aldopentoses[12]. The ratio of the optical

density at 540 mμ compared with that at 670 mμ for both arabinose and xylose, was constant at 0.22. The data for the ketoses were those reported by Cohen.[13]

$$\varepsilon 540/\varepsilon 670 = 1.05$$

Thus the equations for determining the ratio of the aldopentose (x) to the ketopentose (y) in a solution to be analysed, were as follows:

$$\log_{10} I_o/I^{670} = 0.022\,x + 0.011\,y$$
$$\log_{10} I_o/I^{540} = 0.0048\,x + 0.1155\,y$$

The Effect of Cyanide on Carbon Dioxide Fixation in Chlorella

Photosynthesis experiments

The cultures of Chlorella pyrenoidosa used in these experiments were grown in the continuous, constant density culture apparatus under the standard conditions employed in this laboratory[6]. After harvesting, the algae were centrifuged and resuspended in distilled water to give a 1% solution (ml w p cells/vol. of suspension). Aliquots of 10 ml were placed in the vessels illustrated in Fig. 1. These modified "lollipops" were designed to allow sufficient light intensity for a small volume of algae; the extra volume at the top of the vessel was included to permit rapid killing of the algae by injection of boiling alcohol, rather than running the algae out into a flask containing the ethanol. Illumination was provided by reflector spotlights, which gave a light intensity of approximately 7,000 f.c. on either side of the vessels. Excessive heat was avoided by the use of infrared, water-cooled filters placed between the reflector spotlights and the photosynthesis vessels.

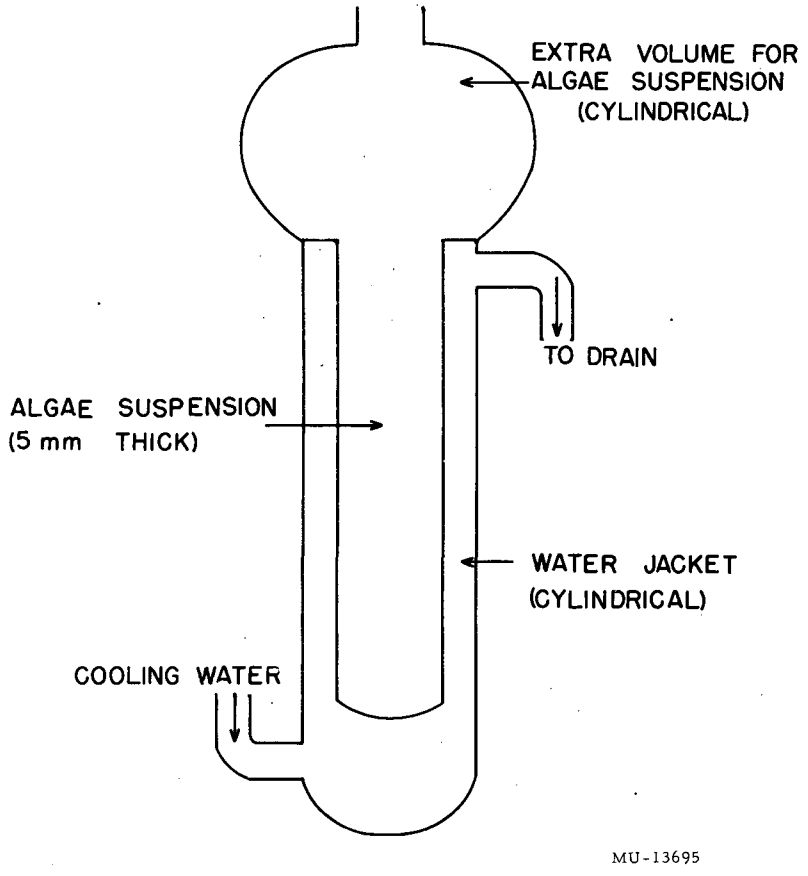

Fig. 1. Modified "lollipop" for photosynthesis experiments with cyanide.

The experiments were carried out in a closed hood provided with four holes, fitted with rubber gloves. Two people were needed for the actual experiments.

The algae were pre-illuminated for 20 minutes in the light, while a continuous stream of 1% CO_2 in air was bubbled through the algae suspension. At the commencement of the experiment the air stream was shut off, and $NaHCO_3$ was added. After two minutes of photosynthesis, with occasional stirring by means of a glass rod with a horizontal handle which was placed in each vessel, KCN was added. After a further 15 seconds photosynthesis, the algae were killed by the rapid injection of 40 ml of boiling ethanol from a large syringe.

The photosynthesis experiments were of three types:

(1) control experiment with $NaHC^{14}O_3$, designated as $C^{14}O_2$.

(2) experiment with $NaHC^{14}O_3$ and KCN, designated as $C^{14}O_2$, CN^-.

(3) experiment with $NaHCO_3$, and $KC^{14}N$, designated as CO_2, $C^{14}N^-$.

The concentration of the radioactive material when used was the same as that of the nonradioactive material; the $NaHCO_3$ was 0.0013 M and the KCN was 0.15 to 0.24 M.

The radioactive algae were then centrifuged, the supernatant retained, and the residue successively extracted with 10 ml of boiling 20% ethanol, and 10 ml of boiling water. The activities of the various extracts were determined by drying suitable aliquots on aluminum planchets and counting in the conventional manner (cf. Part I p.33). The extracts were combined, evaporated to a small volume (about 3 ml) and chromatographed in phenol-water, first dimension and butanol-propionic acid-water, second dimension (cf. chromatography p.120).

The diphosphate area was eluted from the chromatograms into tapered centrifuge tubes (3.0 ml) and either used as such, or hydrolysed. Either acid or enzymic hydrolyses of the diphosphate eluate were accomplished in the following manner:

(1) HCl (sufficient to give a final concentration of 1.0 M) was added to diphosphate eluate, the tube sealed, and heated at 120° for 16 hours.

(2) After concentrating the diphosphate eluate to a volume of 50 λ in a vacuum desiccator (P_2O_5/10 mm/12 hrs.), 50 λ of purified Polidase-S [13] (Schwarz Laboratories, Inc.) in 50 λ of acetate buffer at pH 5.0 (0.2 M in acetate and 0.01 M in Mg^{++}) was added and the tube heated at 37° for 10 hours. An alternative procedure was to use acid prostatic phosphatase* [14] (5 λ) in 50 λ of the above acetate buffer.

C^{14}-labelled cyanide

A modification of the method reported by Sixma, et al., [15] was used to prepare the dry samples of $KC^{14}N$**[16]. A mixture of $BaC^{14}O_3$, ammonium chloride and potassium was ignited in an evacuated tube at 640° for 70 min. The $HC^{14}N$ was generated by the addition of perchloric acid, and trapped in the theoretical amount of potassium methoxide at -78°; the methanol was subsequently evaporated at room temperature to leave the dry sample of $KC^{14}N$.

Concentration and specific activity of cyanide

In order to determine the concentration and specific activity of $KC^{14}N$ prepared in this laboratory, or from commercial samples, it was

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* Gift of Prof. H. A. Barker to this Laboratory, for which we are very grateful.

** I wish to thank Dr. R. M. Lemmon and Miss B. Fingerman for the preparation of $KC^{14}N$.

necessary to develop satisfactory microanalytical techniques. The methods available were unsuitable for the determinations of micro-quantities of this very expensive radioactive cyanide. Since this work was completed, a thesis has been written by Moyer dealing with this problem, and the methods he has developed appear to be very satisfactory.[17]

The concentration of the cyanide was determined by a modification of the cyanogen bromide technique,[18] using the spectrophotometer rather than the Klett-Summerson Photoelectric Colorimeter. KCN (1 to 10 μg) was diluted in water to 1 ml, and saturated bromine water (0.5 ml) was added. The excess bromine was destroyed by the addition of $NaAsO_2$ (0.5 ml of 0.1 M). 5 ml of a pyridine solution (25% freshly redistilled pyridine in water containing 2 ml of conc. HCl) was then added, followed by benzidine hydrochloride (0.2 ml of a 2% solution in water). A red colouration was produced which intensified on standing. After one and a half hours no changes in the optical density or λ_{max} were observed. The absorption spectrum obtained showed two peaks of equal intensity, at 513 and 413 mμ. Samples of varying known concentrations of KCN were prepared, and the optical density at 413 mμ determined at the same time interval after the solution was prepared, i.e. 90 minutes. A linear curve was obtained, relating the optical density to concentration and from this curve the unknown KCN concentration could be determined. It was necessary to recalibrate this curve for every run made, because it appeared to change slightly (up to $\pm$ 5% each time). A variety of factors could be causing this, such as temperature, pH, the amount of light the samples received, and so on. Consistent results were

obtained, however, if the standard linear curve was calibrated at the same time as the concentrations of the unknown were determined.

In order to measure the radioactivity of the cyanide, it was not possible to count the cyanide solutions directly; even if the solution was highly alkaline, slight hydrolysis of the salt always occurred, releasing minute quantities of $HC^{14}N$. To bring the C^{14} in the cyanide to a form suitable for counting, it was necessary to convert the cyanide quantitatively to a nonvolatile solid; satisfactory results were obtained with prussian blue ($Fe_4(Fe(CN)_6)_3$). $KC^{14}N$ (5 λ) was diluted to 1 ml in distilled water, and 250 λ of this solution added to KCN (250 λ of 1.0 M); the nonradioactive cyanide was used in order to obtain a sizeable amount of precipitate. $FeSO_4$ (40 λ of 1.0 M) was added to the cyanide solution to form potassium ferrocyanide, followed by the addition of $FeCl_3$ (50 λ of 1.0 M) to give a deep blue precipitate of prussian blue. The mixture was warmed and two drops of HCl (5 M) added. The precipitate was washed four times with water (2 ml portions) by centrifugation, finely ground up with a glass rod and made up to 2 ml in ethanol. Aliquots (containing less than 25 λ) were plated onto planchets, which had been weighed on the Mettler microbalance. The c/m/mg of prussian blue were converted to c/m/mg KCN plus $KC^{14}N$, from which the specific activity of the $KC^{14}N$ could be obtained.

Preparation of Hamamelonic Acid

Extraction of hamameli-tannin

Powdered witch hazel bark (Hamamelis virginica)* was extracted by three different methods shown in the flow sheet diagrams of

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* Penick and Co., c/o H. R. Laist, San Francisco, California.

Tables 1 and 2. Method I was the method of choice, which was used in all subsequent extractions. Pure hamameli-tannin crystallises in fluffy white clusters from water, m. pt. 154°. (Found: C, 49.6; H, 4.12: $C_{20}H_{20}O_{14}$ requires C, 49.15; H, 4.15%).

Hydrolysis of hamameli-tannin

Hamameli-tannin (5 g) was hydrolysed with 2 M HCl (50 ml) for 50 hours at 60°. The reaction mixture was partially concentrated under reduced pressure at left at 0° for some hours; the precipitated gallic acid was removed by centrifugation. The filtrate was concentrated under reduced pressure to dryness, several times to remove the hydrochloric acid and then dissolved in water (40 ml). The solution was then extracted with ether in a continuous liquid extractor for 48 hours using an infrared lamp to heat the ether. The aqueous layer was evaporated to give a colourless syrup of hamamelose (1.9 g) which had traces of gallic acid still present as shown by paper chromatography. A better method was continuous liquid liquid extraction with n-butanol for 48 hours under reduced pressure, when all traces of gallic acid were removed from the hamamelose syrup.

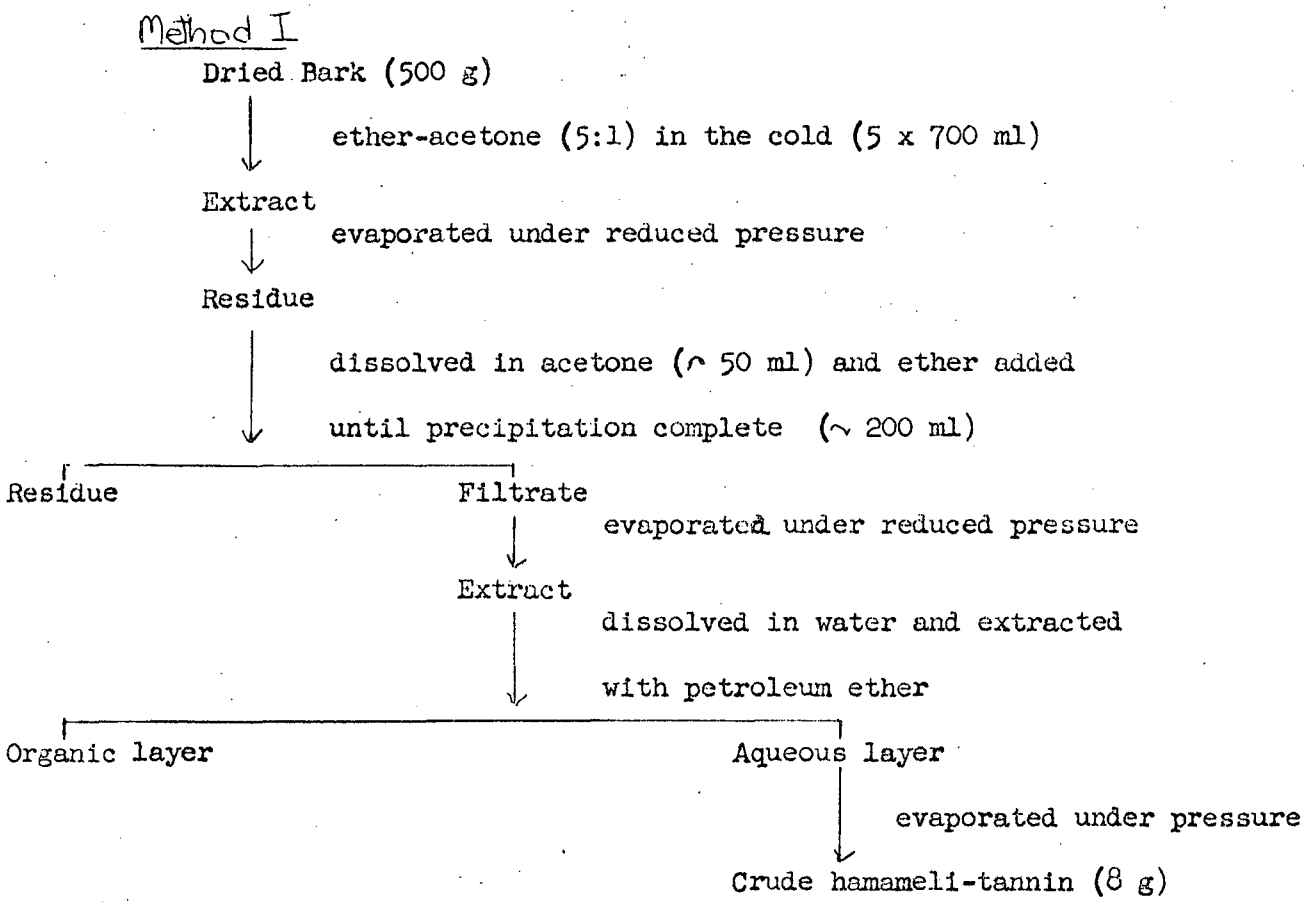
It was also possible to obtain complete separation of the hamamelose from the other substances by chromatography with n-butanol-ethanol-water (5:1:4) on a ChroMax column (cf. p.121).

The following R_f values were obtained in descending chromatograms:

		hamamelose	hamameli-tannin	gallic acid
(1)	phenol-water	0,56	0.35	0.27
(2)	n-butanol-propionic acid water	0.34	0.42	0.59
(3)	n-butanol-ethanol-water	0.26	0.34	0.64

TABLE I

Extraction of Hamameli-tannin



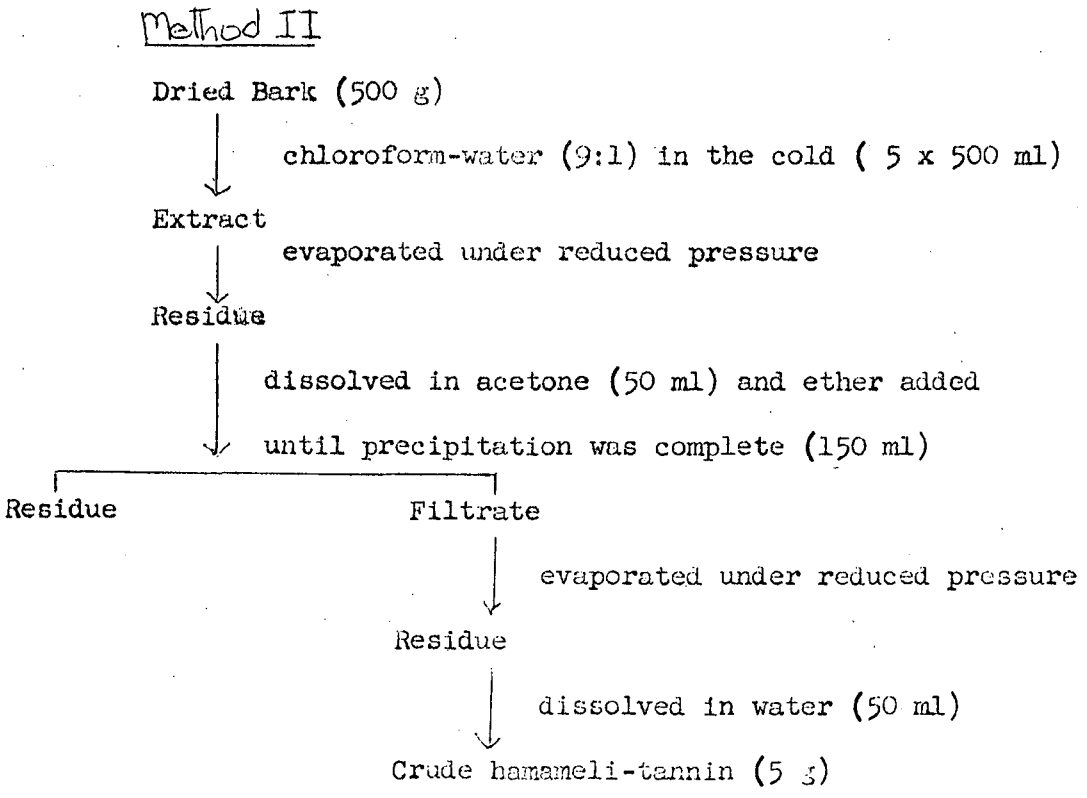
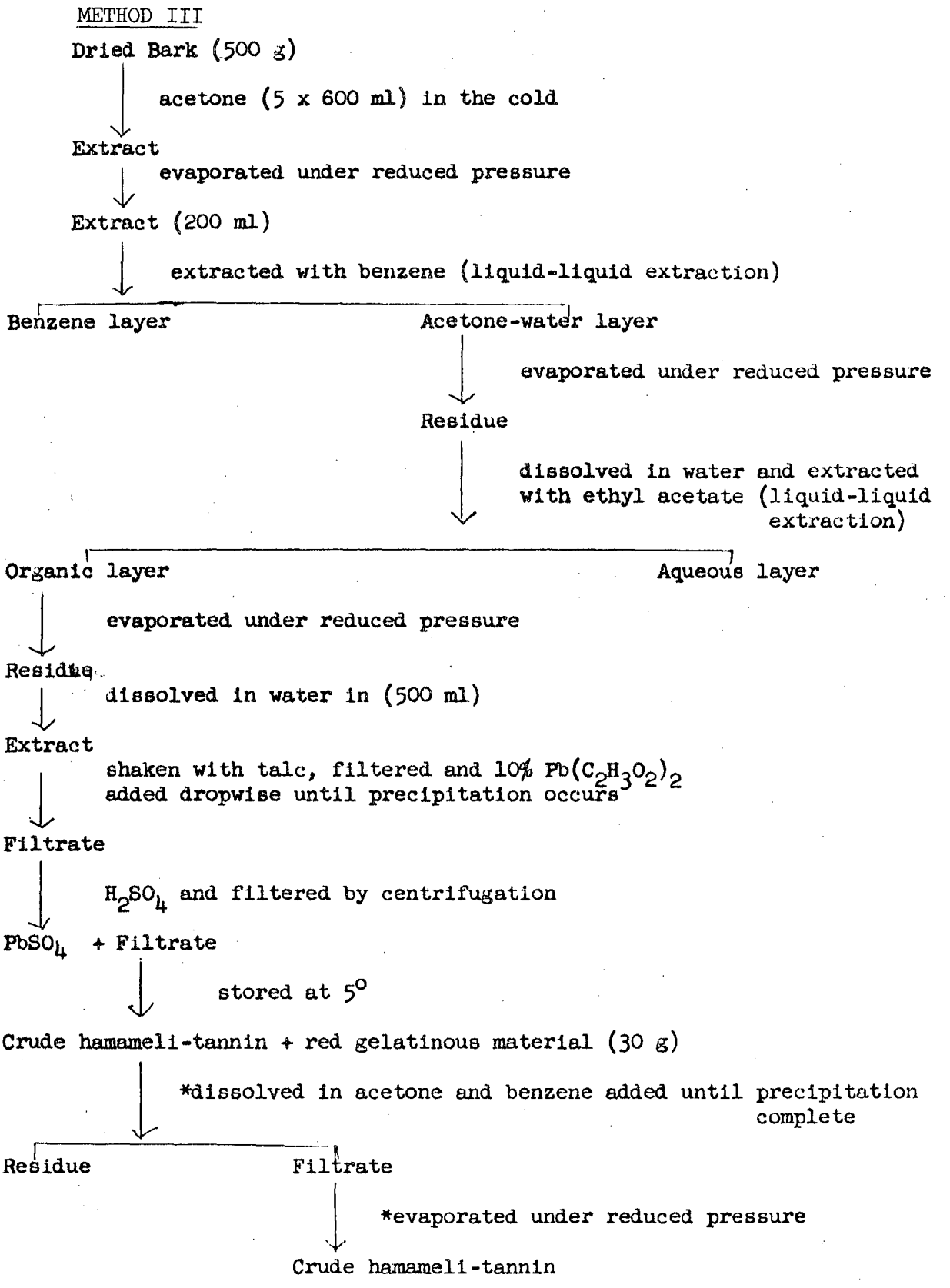


TABLE 2



* Two steps repeated until the red gelatinous material is removed to give pure hamameli-tannin (6.7 g).

Oxidation of hamamelose

Hamamelose (1.8 g) was dissolved in water (20 ml), containing suspended $CaCO_3$ (12 g), the solution cooled to 5°, and the bromine (1 ml) slowly added. After the mixture had been shaken for 24 hours at 25°, air was bubbled through the solution to liberate any gaseous bromine, the mixture was filtered, and the filtrate concentrated under reduced pressure. The syrup was filtered through a column of amberlite IR-120, H^+ form (100 ml resin), and again concentrated under reduced pressure several times to remove the hydrogen bromide. A solution of brucine (12.5 g) in EtOH (40 ml) was added to the syrup and the solution kept at 5° for several days. The crude salt (7.5 g) was dissolved in a minimum amount of water and the excess brucine removed by extraction with chloroform. After the addition of ethanol, white needles of the brucine salt were obtained (4.0 g) m.p. 174°. (Found: C, 61.51; +H, 6.39; N, 4.98. $C_{29}H_{36}N_2O_{10}$ requires C, 60.83; H, 6.34; N, 4.89%).

The hamamelonic acid was generated from a solution of the brucine salt in water (10 ml), by the addition of NaOH (6 ml of 1.0 M). The precipitated brucine was removed by centrifugation, and the supernatant was passed through a column of amberlite IR-120, H^+ form (100 ml of resin). The effluent was concentrated under reduced pressure to give a colourless syrup of hamamelonic acid (1.0 g). The acid syrup (0.8 g) was heated in a steam bath for two hours, triturated with acetone, and left to crystallise for days at room temperature. All attempts to prepare the crystalline lactone failed.

Chromatography of hamamelonic acid resulted in the formation of both an acid and a lactone spot on the paper in the usual solvent

systems. If an aliquot of the acid was sealed in a tube and heated at 100° for two hours with 0.1 M HCl, chromatography gave only the lactone spot, while similar treatment of hamamelonic acid with NaOH (0.1 M) furnished the acid on chromatography. The following R_f values were obtained:

		hamamelonic acid	hamamelonic acid lactone
(1)	phenol-water	0.20	0.60
(2)	n-butanol-propionic acid-water	0.24	0.42

Preliminary Attempts at the Syntheses of Branched-Chain Sugar Acids

Syntheses of the ketopentoses

Preparation of D-ribulose: D-arabinose (20 g) was refluxed gently for four hours in freshly distilled pyridine (200 ml.) with the exclusion of all moisture. The reaction mixture was evaporated under reduced pressure, distilled water added and the mixture re-evaporated under reduced pressure; this was repeated several times until all traces of pyridine were removed. The resulting syrup was dissolved in absolute ethanol (40 ml), allowed to cool and some crystals of arabinose added. The mixture was left for several days, during which time some of the unreacted arabinose crystallised out. After filtration, the syrup was evaporated under reduced pressure, dissolved in absolute ethanol (30 ml) and left for three more days. Yield of recovered arabinose: 14 to 16 g. The concentrated mother liquors yielded a brown syrup which consisted of about 70 to 80% ketose as determined by optical rotation or by the Bial-Orcinol test (cf. p.124). The unreacted arabinose was recrystallised from 95% ethanol (150-200 ml) and dried in a vacuum desiccator (P_2O_5/10 mm/24 hrs).

Preparation of D-xylulose: this ketopentose was prepared from D-xylose, using the same method as that for D-ribulose.

Isolation of the ketopentose

(1) Ribulose: o-nitrophenylhydrazine was prepared according to the method of Vogel given for p-nitrophenylhydrazine, by the diazotisation of o-nitroaniline with alkaline sodium sulphite.[19]

The o-nitrophenylhydrazones of ribulose and arabinose (5 g. of an 80% ketose mixture) were prepared by the method of Glatthaar and Reichstein[20]. The crude solid isolated had a m.p. of 160°, and subsequent crystallisation of this material with absolute ethanol yielded a red crystalline solid (7 g), m.p. 163°. (Found: N, 20.1. $C_{11}H_{15}N_3O_6$ requires N, 14.7%). This solid was dissolved in 80% ethanol and refluxed with benzaldehyde (8 ml) for one hour. After cooling, the mixture was filtered as an oil; the aqueous layer was extracted several times with ether to remove any remaining benzaldehyde, and evaporated under reduced pressure to give a syrup, which was dissolved in 95% ethanol. Crystals of arabinose (3.9 g) were obtained. The red solid isolated above was arabinose-o-nitrophenylosazone (Found: N, 20.1. $C_{17}H_{18}N_6O_7$ requires N, 20.0%). This experiment was repeated but the majority of the attempts to prepare the ribulose-o-nitrophenylhydrazone (m.p. 162°) failed.

(2) xylulose: The formation of the p-bromophenylhydrazones of xylulose and xylose were prepared according to the method of Schmidt.[21] Although these derivatives have the same melting point (128°), they could be successfully separated by fractional crystallisation and the xylulose regenerated by warming the xylulose-p-bromophenylhydrazone with an aqueous solution of benzaldehyde.

(3) xylulose or ribulose: N-benzyl-N-phenylhydrazine hydrochloride (6.3 g) was dissolved in water (15 ml), treated with sodium acetate (2.5 g) and added to the syrup (5 g) obtained above. After heating gently on a water bath for three hours, the mixture was left over night, partially concentrated under reduced pressure and left until the yellow crystals of D-arabinose-benzylphenyl-hydrazone (m.p. 174°) separated out (8.3 g). The filtrate was evaporated under reduced pressure, dissolved in water (150 ml) to which benzaldehyde (3.2 ml) was added, and refluxed at 70° for 30 minutes. After cooling, the mixture was filtered as an oil; the aqueous layer was extracted several times with ether to remove any remaining benzaldehyde and evaporated under reduced pressure to give a yellow syrup which was dissolved in 80% EtOH, decolourized with charcoal and evaporated again. This syrup corresponded to 96% ribulose (1.5 g).

(4) Ribulose or xylulose: a mixture of the aldo- and keto-pentose which contained 80% of the ketose, was dissolved in 30 ml of water, and bromine (2 ml), benzoic acid (3.3 g) and barium benzoate (20 g) added to the reaction mixture, which was then shaken automatically for 48 hours at room temperature. A stream of nitrogen was bubbled through the reaction mixture, to drive out any excess bromine for about 30 minutes, and the solid material was separated by centrifugation. The filtrate was extracted several times with chloroform to remove the excess benzoic acid. The aqueous layer was treated with H_2SO_4 (6 ml of 3 M) and the barium sulphate removed by centrifugation. The supernatant was concentrated under reduced pressure to about 10 ml, and passed through Amberlite-IR-4B, OH^- form (150 ml resin), the effluent concentrated under reduced pressure and passed through Amberlite-IR-120, H^+ form (150 ml resin).

Analysis of the mixture by chromatography showed that some aldonic acid and aldonic lactone remained. It was necessary to recycle the solution through ion exchange resin, until the pure ketose (2.1 g) was obtained.

The following R_f values were obtained in the usual solvent system:

		arabinose	ribulose	xylose	xylulose
(1)	phenol-water	0.59	0.74	0.52	0.67
(2)	butanol-propionic acid-water	0.49	0.61	0.45	0.57

Attempted syntheses of 2-C-hydroxymethyl-D-pentonic acids

The ketoses D-ribulose or D-xylulose were reacted with a little more than equimolar proportions of KCN.

(i) Ketose (2 g) in 5 ml of water together with $NaHCO_3$ (5 ml of 2 M) was added to a frozen solution of 5 ml of KCN (0.8 g) and NaOH (5 ml of 2 M), and left at 0° for one day and at room temperature for a further two days.

(ii) Ketose (2 g) in 5 ml of water was added to a solution of KCN (0.8 g in 5 ml) and HCl (5 ml of 2 M) and left at 0° for one day, and a further two days at room temperature. Reaction mixture (i) was hydrolysed by heating at 60° in a stream of nitrogen for 6 to 8 hours. Reaction mixture (ii) was hydrolysed with $Ba(OH)_2$ (4 g) for 4 to 8 hours, until no more ammonia was evolved, and dilute H_2SO_4 added to precipitate the barium sulphate, which was removed by centrifugation. After concentrating the reaction mixtures (i) or (ii), they were passed through Amberlite IR-120, H^+ form (200 ml resin) and the effluents concentrated under reduced pressure. Attempts to resolve the acids were carried out in the same manner as described previously for hamamelonic acid (cf. p.136).

III RESULTS AND DISCUSSION

The Effect of Cyanide on the Carbon Dioxide Fixation in *Chlorella*

The following photosynthesis experiments were performed:

(1) 2 minutes $NaHC^{14}O_3$; designated as $C^{14}O_2$

(2) 2 minutes $NaHC^{14}O_3$ followed by 15 seconds KCN; designated as $C^{14}O_2,CN^-$

(3) 2 minutes $NaHCO_3$ followed by 15 seconds $KC^{14}N$; designated as $CO_2,C^{14}N^-$

Table 3 illustrates the fixation of radiocarbon in *Chlorella*; the two sets of figures listed, refer to separate experiments performed at different times. It can be seen that the addition of KCN (final concentration M/80) causes a 50% inhibition of the total fixation. About 50% of the total activity was extractable in the initial alcohol extraction and a further 5%, in the 20 % alcohol and water extractions. In the case of the CO_2, $C^{14}N^-$ experiment, about 85% of the total activity is extractable in the initial alcoholic extract. This is a large figure, but it is scarcely surprising, because the $C^{14}N^-$ would be mainly reacting chemically, with the sugars and alcoholic-extractable materials, in the cells.

The total fixation of the radiocarbon in *Chlorella*, for five different experiments is listed in Table 4. With a final concentration of 0.0125 M KCN there is about 50% inhibition of the carbon dioxide fixation, while with a lower concentration of KCN (0.0016 M), the fixation was about 75% of the normal value. Thus the addition of cyanide, under the conditions of these experiments was inhibiting.

Table 3. The total fixation of radiocarbon in the various extracts of Chlorella in c/m/ml w p cells x 10^7.

Photosynthesis Experiment*		Total Fixation	80% alcohol extract	20% alcohol extract	water extract	cell residue
$C^{14}O_2$	(1)	39.2	21.6	1.6	0.4	0.2
	(2)	39.0	16.2	0.2	0.3	0.4
$C^{14}O_2CN^-$	(1)	18.3	13.0	2.4	0.2	0.9
	(2)	19.5	(28.6)	0.4	0.2	0.4
CO_2, $C^{14}N^-$	(1)	7.9	6.8	0.9	0.07	0.8
	(2)	9.4	7.9	0.1	0.06	0.3

* Conditions of photosynthesis experiments: 1% suspension in distilled water. 10 ml suspension per experiment. Light intensity, 7,000 foot candles. 20 min pre-illumination with 1% CO_2 in air. 2 min photosynthesis with $NaHCO_3$ or $NaHC^{14}O_3$ (0.013 M, 23.9 μc/μM), followed by 15 sec photosynthesis with KCN or $KC^{14}N$ (0.15 M, 23.9 μc/μM). Killing with boiling ethanol (final conc. 80%). The molarities of the radioactive and nonradioactive substances were the same. Experiments (1) and (2) were performed on different days.

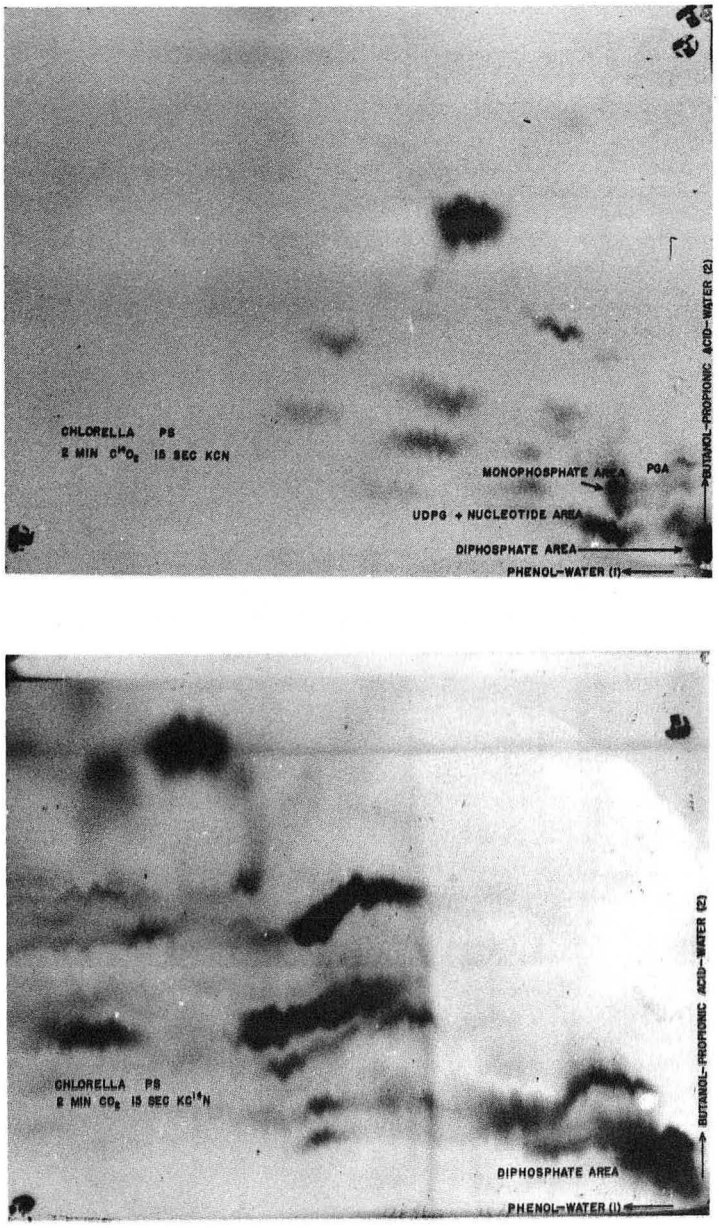
Table 4. The total fixation of radiocarbon in Chlorella in c/m/ml w p cells x 10^7

Photosynthesis Experiment No. *	1	2	3	4	5
$NaHC^{14}O_3$	0.0013 M 11.1 μc/μM	0.013 M 23.9 μc/μM	0.013 M 23.9 μc/μM	0.0013 M 11.1 μc/μM	0.0013 M 11.1 μc/μM
$KC^{14}N$	0.24 M 15.4 μc/μM	0.15 M 23.9 μc/μM	0.15 M 0.52 μc/μM	0.02 M 0.52 μc/μM	0.02 M 0.52 μc/μM
$C^{14}O_2$	--	39.2	39.0	15.0	19.8
$C^{14}O_2$, CN^-	4.8	18.3	19.5	13.8	13.7
CO_2, $C^{14}N^-$	15.8	7.9	9.8	0.68	0.32

* Same conditions of photosynthesis as listed in Table 3.

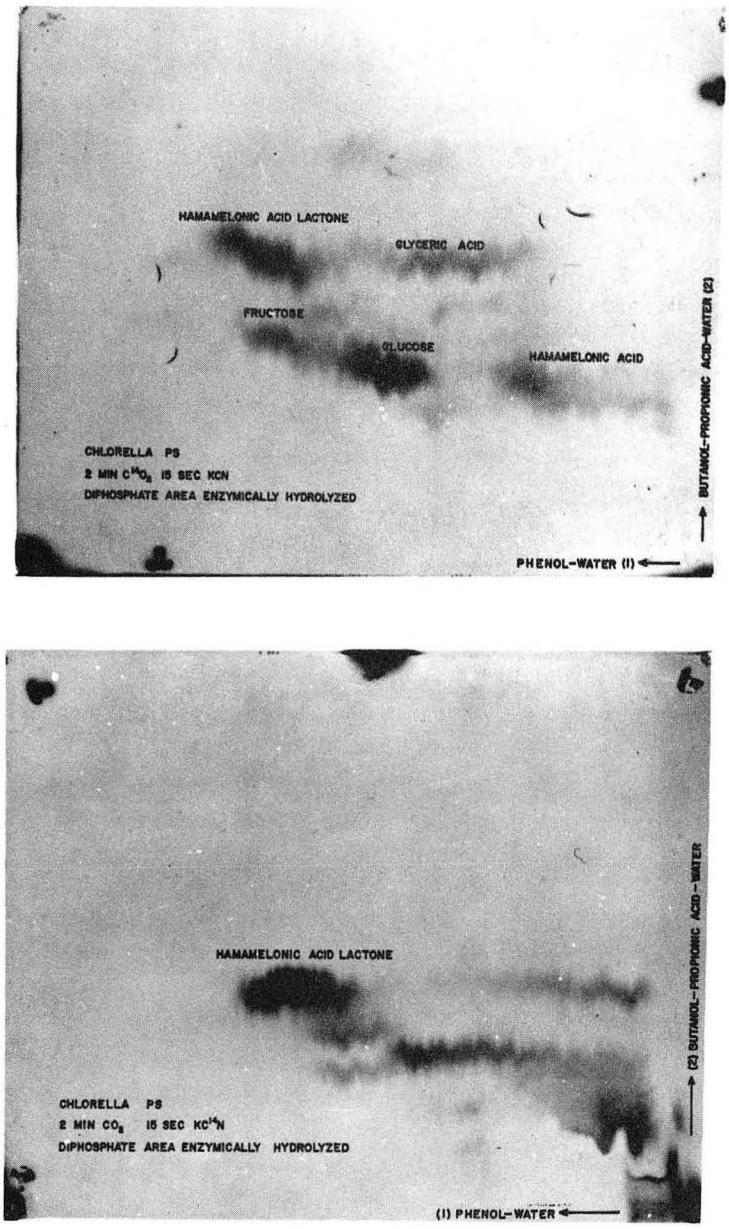
A radioautograph of a typical $C^{14}O_2, CN^-$ experiment is shown in Figure 2. The general picture is rather similar to that of a normal photosynthesis experiment, in the absence of cyanide. From a cursory examination of this radioautograph, it might appear that cyanide was acting only as an enzyme poison, by killing the enzyme and thus permitting an accumulation of the intermediates on the photosynthesis pathway. That this is far from the truth, may be seen by examination of the CO_2, $C^{14}N^-$ radioautograph (Fig. 2). The sole source of radioactivity here is the cyanide; hence all the spots are products of chemical reactions of the cyanide, with the materials present in the cells. The final concentration of the KCN used is fairly high (0.02 M), but even with lower final concentrations of KCN (0.0125 M and 0.0016 M) a similar pattern is obtained. These results indicate the importance of the chemical role of cyanide in studies of inhibition; it is suggested that this factor has been overlooked in many of the studies made. It should be emphasized that the reaction of cyanide with the carbonyl groups of the sugars, is a particularly facile and almost quantitative reaction.

The radioautographs of the enzymatically hydrolysed diphosphate areas of the above experiments are illustrated in Fig. 3. These radioautographs show a large amount of a new compound, which has not been detected before in normal photosynthesis experiments. This same material was found on the radioautographs of the acid hydrolysed diphosphate areas of the $C^{14}O_2$, CN^- and $CO_2, C^{14}N^-$ experiments (Fig. 4). The conditions of the acid hydrolysis were rather drastic and under these conditions, the glucose and fructose obtained with enzymic hydrolysis, will be degraded to volatile compounds.



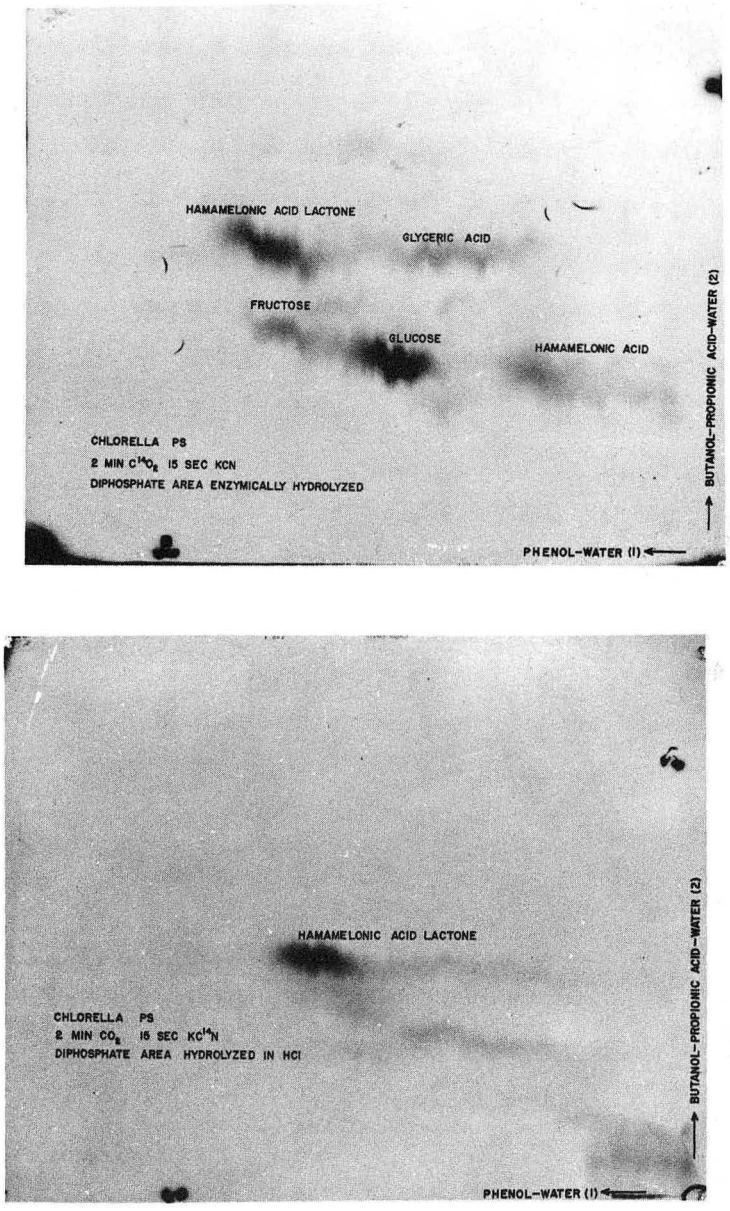
ZN-2217

Fig. 2. Radioautographs of chromatograms of the hot alcohol-water extract of Chlorella. The following conditions of photosynthesis were employed: 2 min. with CO_2 followed by the addition of KCN (final conc. 0.2M) for a further 15 sec. and injection of boiling alcohol (end conc. 80%) to kill the algae. Upper radioautograph of a $C^{14}O_2$, CN^- experiment; lower radioautograph of CO_2, $C^{14}N^-$ experiment.



ZN-2215

Fig. 3. Radioauthographs of the chromatograms of the enzymatically hydrolysed diphosphate areas of the $C^{14}O_2$, CN^- (upper) and the CO_2, $C^{14}N^-$ (lower) experiments of Figure 2.



ZN-2216

Fig. 4. Radioautographs of the HCl hydrolysed diphosphate areas of the $C^{14}O_2$, CN^- (upper) and the CO_2, $C^{14}N^-$ (lower) experiments of Figure 2.

As it has been demonstrated that cyanide must be involved in the formation of this new compound, the possibility was investigated that it had been produced by the action of cyanide on ribulose diphosphate[22]. The reaction of KCN with RuDP yielded an acidic diphosphate material. A comparison of both the electrophoretic and chromatographic behavior of this synthetic diphosphate to the diphosphate area from the $C^{14}O_2$, CN^- experiment showed that the two substances were indistinguishable under these conditions. Hydrolysis of the synthetic diphosphate ($KC^{14}N$ + RuDP) yielded a material which exhibited acid-lactone properties. Cochromatography of this material with authentic hamamelonic acid* (2-C-(hydroxymethyl)-D-ribonic acid) showed the similarlity of these substances. It was found that the radioactivity and the silver spray of the chromatogram did not exactly coincide[22]. This was almost certainly due to the fact that the radioactive material consists of both epimeric acids, as the action of $KC^{14}N$ on RuDP would be an asymmetric synthesis; the name hamamelonic acid diphosphate (HmDP) has been given to this material.

Cochromatography of the acid hydrolysis products of the $C^{14}O_2$,CN^- and the CO_2, $C^{14}N^-$ experiments, with hamamelonic acid (prepared from HmDP) showed the identity of these materials. This result was confirmed by Kandler, who found coincidence of the acid hydrolysate of the diphosphate area of a cyanide-inhibited photosynthesis experiment with hamamelonic acid by cochromatography[23]. The <u>in vivo</u> hamamelonic acid would actually consist of the epimeric acids, but no separation into two acid spots was ever found on paper chromatograms; hence the proportion present of these epimeric acids is not known.

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* We are grateful to Prof. O. Th. Schmidt for a sample of hamamelonic acid.

Thus is has been established that the new material present in the photosynthesis experiments with cyanide is hamamelonic acid diphosphate, produced by the action of cyanide on RuDP, either inside the algae, or during the extraction procedure. It might be wondered why no other cyanohydrin reaction products were detected in the $C^{14}O_2$, CN^- experiments. Under the conditions of photosynthesis employed, it is known that the pool of RuDP is large, accounting for 80 to 90% of the sugar diphosphates;[24] thus it is not surprising that the bulk of the cyanohydrin addition is with RuDP. This in vitro reaction has been shown to be extremely facile[22].

Preparation of hamamelonic acid

In view of the establishment of the branched-chain sugar acids which were obtained in the in vivo experiments described in the preceding section, it was desired to prepare an authentic sample of one of these acids in order to study its chemical properties. Several naturally-occurring branched-chain sugar acids have been isolated from plants and microorganisms;[25] hamamelonic acid may be obtained from natural sources in the following manner.

The bark of the North American shrub, Hamamelis virginica (commonly referred to as witch hazel) contains, in addition to another tannin, about 1 to 1.5% of hamameli-tannin[26]. This tannin was shown to have the composition of a di-O-galloylhexose (I)[27,28]. Acid or enzymic hydrolysis of hamameli-tannin yields two moles of gallic acid, and one mole of a strongly reducing hexose, hamamelose (II), whose structure has been established as 2-C-(hydroxymethyl)-D-ribose (II) (Fig.5).[(29)30]

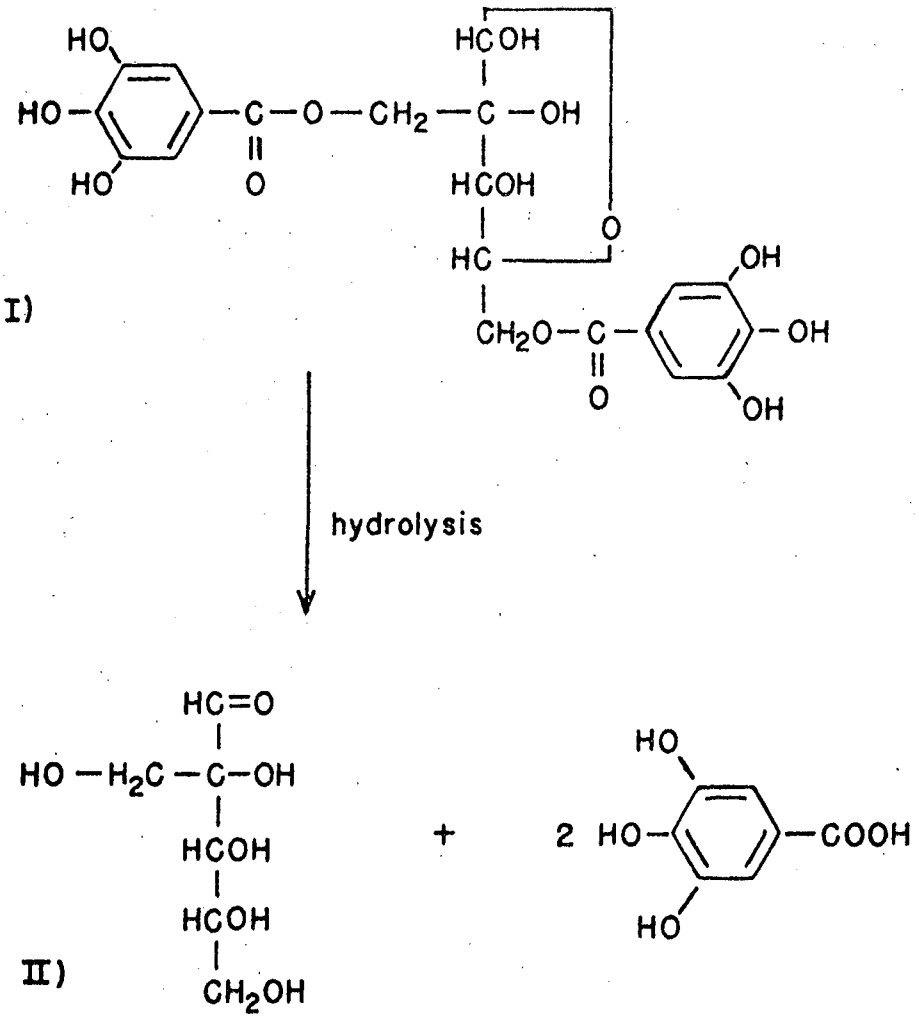


Fig. 5

Several procedures for the extraction of the tannin from the dried bark have been described; these are summarized in flow sheet diagrams (Tables 1 and 2). It was found that Method III gave the best yield of hamameli-tannin corresponding to 1.4% of the weight of the dried bark; however, this is a long and tedious extraction procedure. The complete removal of a red gelatinous material upon which the white crystals of the tannin are formed, is extremely difficult. This material is present in large amounts, only if acetone has been used in the initial extraction of the bark. Method II gave the lowest yield, 0.75%, and had no advantageous features. Although Method I gave a slightly lower yield than Method III (1.25%), it is definitely the method of choice. It is a relatively quick and easy to perform and the crystallised tannin is relatively free from the red gelatinous material of Method III.

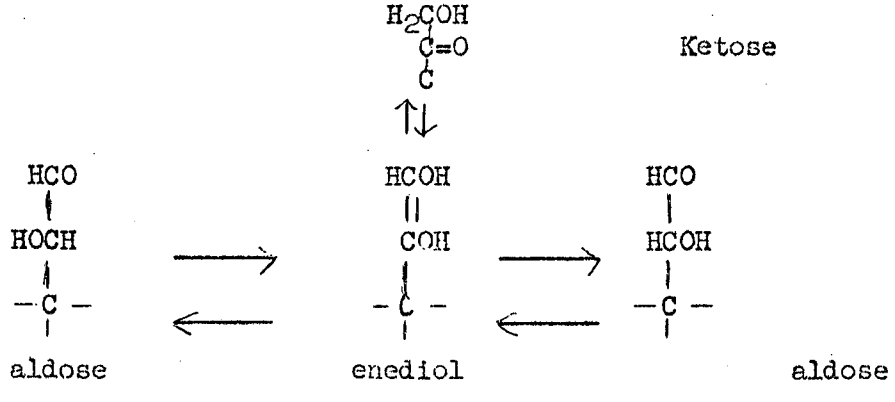
The crystalline hamameli-tannin may be hydrolysed, enzymatically by tanase[28], or by acid[31]. Freudenberg used 1 M H_2SO_4 for the hydrolysis, but a slightly improved yield of hamamelose and an easier work-up procedure, was obtained using 2 M HCl. The separation of gallic acid from the sugar, hamamelose is difficult. The original method used involves precipitation of the gallic acid by the alternate addition of small quantities of Pb $(CO_3)_2$ and Pb $(C_2H_3O_2)_2 \cdot 3\ H_2O$ and subsequent removal of the excess cation[31]. It was hoped that the use of ion exchange resins might facilitate the removal of gallic acid, but this proved unsatisfactory. A better separation was obtained using the pressurized paper chromatography column, Chromax. Liquid liquid extraction of the reaction mixture with ether was only moderately successful; a cleaner product was obtained by continuous liquid extraction with n-butanol under reduced pressure.

The hamamelose was oxidized with mercuric oxide in the presence of calcium carbonate to give a non-crystalline calcium salt which from the free acid was generated. An improved yield of hamamelonic acid was obtained, using bromine as the oxidizing agent.

Preliminary Attempts at the Synthesis of Branched-Chain Sugar Acids

Syntheses of the ketopentoses

Although sugars exhibit moderate stability to acids, they are readily affected by alkalies, even under mild conditions. The simplest isomerization reaction of the reducing sugars is the Lobry de Bruyn and Alberda van Ekenstein transformation[32]. This reaction may be utilised for the preparation of ketoses from aldoses by isomerization with pyridine. This reaction is postulated to proceed through an intermediate enediol; with the loss of asymmetry of carbon atom 2, the two epimeric aldoses will be in equilibrium with the corresponding ketose as shown below.



Thus D-erythro-pentulose (D-ribulose) may be synthesized from D-arabinose or D-ribose; the former pentose was chosen because it is cheaper to buy, and D-threo-pentulose (D-xylulose) may be synthesized from D-xylose.

Although some of the unreacted pentose may be isolated from the reaction mixture by crystallisation, it is most important to remove the last traces of it from the ketose syrup. The classical method for the isolation of the ketose from the aldose is by the formation of substituted phenylhydrazones[32]. These reactions are time consuming, and the substituted phenylhydrazones are often difficult to isolate in a crystalline form; also, the yield is rather poor. Ribulose may be isolated by the formation of the aldo- and keto-o-nitrophenylhydrazones. This reaction appeared to be rather unreliable, because in some cases the arabinose-o-nitrophenyl-osazone was preferentially formed.

Both xylulose and ribulose do not react with N-benzyl-N-phenyl-hydrazine; therefore, the aldoses will form the substituted hydrazones, and the ketoses may be isolated from the reaction mixture. It is difficult, however, to obtain the ketose in a pure state by this reaction.

Another method employed utilises the formation of the mono- and di-isopropylidene derivatives of the sugar mixture[33]. These substances may be separated by fractional distillation. The difficulty inherent in this technique is the crystallisation of the isopropylidene derivatives, although the yields are quite good.

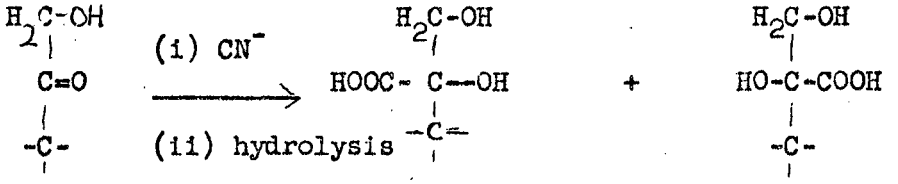
The aldoses may be oxidized to the corresponding aldonic acids, using bromine as the oxidant in a slightly acid, buffered solutions; [34] the ketose do not react under such conditions. The aldonic acid may be removed by the use of ion exchange resins. This method gives pure ketose, although the ion exchange chromatography

necessary is rather tedious. A simpler method is that of pressurized paper roll chromatography, using a CroMax column, where the pure ketose may be isolated easily.

Attempted synthesis of 2-C-hydroxymethyl-pentonic acids.

The cyanohydrin synthesis as devised by Kiliani is one of the oldest reactions in carbohydrate chemistry; the sugars were treated with an aqueous solution of hydrogen cyanide, in the presence of a little ammonia. This method has been modified by the addition of the sugar, to a solution of sodium cyanide which has been buffered at pH 9.0. The cyanohydrins which are normally not isolated are hydrolysed with alkali, and the resulting epimeric acids are separated by fractional crystallisation of the lactones, metallic or alkaloidal salts, the phenylhydrazides and so on.[32]

In the case of the ketoses, the cyanohydrin reaction will result in the formation of branched-chain epimeric acids.



In 1934, Schmidt et. al. prepared the four 2-C-hydroxymethyl-pentonic acids, by the action of an aqueous solution of hydrogen cyanide on D-ribulose and D-xylulose.[30] After hydrolysis of the cyanohydrins with barium hydroxide, the barium salts of the acid mixture were isolated and treated with dilute sulphuric acid. The free acid-lactone mixture was then precipitated with $Ca(OH)_2$; the calcium salts

were reacted with an equivalent amount of oxalic acid and the epimeric acids separated by fractional crystallisation of their phenylhydrazides. This method was repeated in this study and found to have several disadvantages; a considerable amount of material is lost in the purification of the various salts, since the acids are in equilibrium with their γ-lactones at the pH's used. Furthermore, the separation of the phenylhydrazides seems somewhat tenuous as the melting points differ by only a few degrees.

In an attempt to overcome these difficulties, the reaction mixture, after the hydrolysis of the cyanohydrins, was passed through a cation exchange resin column, and the effluent concentrated under reduced pressure. The syrup was dissolved in water and concentrated under reduced pressure several times to remove any traces of cyanide. The brucine salts of the mixture were then made. Attempted fractional crystallisation of the brucine salts was not a success. Unfortunately, no time was left to pursue this problem of the resolution of the 2-C-hydroxymethyl-D-pentonic acids any further.

It would be expected that one of the lactones of an epimeric pair of acids might be more easily hydrolysed than the other. This could be ascertained by studying the rate of hydrolysis of the four epimeric acids, at a fixed pH using a pH-statt. If the lactone mixture was placed on a weakly basic ion exchange column, the more easily hydrolysed lactone should be preferentially adsorbed on the column, while the less easily hydrolysed lactone should be eluted from the column. Gluconic and mannonic acids have been separated by this means.[34]

The cyanohydrin synthesis has been used frequently for the extension of the carbon chain of aldoses, and more recently for the preparation of carbon 14-labelled carbohydrates from the aldoses[35]. The application of the method to ketoses has been limited. Apart from the work of Schmidt's on the pentoses[30], the only other ketose used has been fructose, from which the α- and β-fructoheptonic acids have been isolated[29].

Preliminary micro-experiments on a wide variety of ketoses with $KC^{14}N$ indicate that a large number of compounds had been formed, in addition to the acids. This was shown by the radioautographs of the above reaction mixtures, after hydrolysis. Differences in the pH of the initial ketose-cyanide reaction product made a difference in the distribution of the compounds on the radioautographs. Since these reactions are asymmetric syntheses, it would be of considerable interest to make a conformational study of the ketose cyanohydrin reaction. In the aldose series, it has been found that the predominant epimeric acid formed, will be the one in which the hydroxyl groups on carbon 2 and 4 have a trans-configuration[36]. The pH has also been found to influence the reaction products[32].

It is difficult to determine the configuration of the asymmetric tertiary carbon in branched-chain carbohydrates, as there are no compounds of established configurations to which they may be compared. Schmidt related α- and β-fructoheptonic acids to gluconic and mannonic acids, respectively, on the basis of rules of rotation;[29] similarly, hamamelonic acid was assigned the configuration of 2-C-hydroxymethyl-D-ribonic acid[30]. Woods and Neish in some experiments

with α-fructoheptonic acid prepared 2-C-hydroxymethyl-D-xylonic acid, whose structure followed from the method of synthesis.[37] A recent paper by Bose and Chatterjee contains a generalisation which covers all the optical rotation rules so far proposed.[38] It is of interest that all the data given for the branched-chain sugar acids fit in with this correlation.

It would be advantageous to prepare the hydroxymethyl-pentonic acids for another reason; in the carboxylation of ribulose-1, 5-diphosphate to form 3-phosphoglyceric acid, Calvin has postulated that the reaction proceeds via a six-carbon sugar keto diphosphate intermediate, 2-carboxy-3-ketopentitol-1,5-diphosphate.[39] Moses has tentatively identified this compound in normal photosynthesis experiments; hydrolysis of the diphosphate groups, followed by boro-hydride reduction of this compound yielded a polyhydroxy acid related to hamamelonic acid or its isomer.[40] Thus, the stereochemistry of these in vivo compounds could be ascertained by the preparation of model compounds.

DISCUSSION

It has been demonstrated that the new material which appears in photosynthesis experiments, by using high concentrations of cyanide, is hamamelonic acid diphosphate together with some epimer. This material has not been detected in normal photosynthesis experiments, without the addition of cyanide. The source of hamamelonic acid diphosphate is clearly a cyanohydrin reaction on RuDP. Kandler

found that it was necessary to have a light period, between the addition of cyanide and the injection of boiling alcohol to kill the algae, in order to form appreciable amounts of HmDP.[4] Under these conditions the net fixation of radiocarbon in PGA diminishes. Furthermore, the radiocarbon fixed as hamamelonic acid diphosphate is consistent with the amount disappearing from the pentose phosphate and PGA pools. It has been well established that the initial carboxylation reaction of photosynthesis is the combination of CO_2 with RuDP, followed by the hydrolysis of the product to form two molecules of PGA[6,41]. Thus, in the light period after the addition of cyanide, it would appear that the algae still have the ability to reduce PGA for a short time; then it may be assumed that the carboxydismutase system associated with the initial carboxylation is in some way blocked. This will result in the accumulation of the RuDP from the other products of the photosynthetic cycle, and the cells will no longer fix CO_2 as efficiently as before.

IV. CONCLUSION

In an investigation of the action of cyanide on photosynthesis, green algae have been treated with radioactive cyanide. A multitude of products have been found to be formed in very short exposure times. One of these was identified with a material formed when algae are given radioactive CO_2 and nonradioactive KCN. This material has been identified as the cyanide addition product of ribulose-1,5-diphosphate. Upon hydrolysis it gives a branched-chain sugar acid (or mixture of isomers) closely related to hamamelonic acid. Perhaps the most important aspect of this work is the demonstration of the chemical role of cyanide.

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