# INVESTIGATIONS OF THE CHLOROPLAST PIGMENTS OF HIGHER PLANTS, GREEN ALGAE AND BROWN ALGAE AND THEIR INFLUENCE UPON THE INVENTION, MODIFICATIONS, AND APPLICATIONS OF TSWETT'S CHROMATOGRAPHIC METHOD*,**

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## I. SIGNIFICANCE AND MYSTERIES OF THE GREEN AND YELLOW PLANT PIGMENTS

For many centuries, observant men have recognized that virtually all life depends upon the intrinsic activities of green plants[1]. This primordial nutritional role of plants is indicated by the classification of animals as herbivores—those that eat herbage—and carnivores—those that prey upon the consumers of herbage.

Long before the evolution of modern laboratories and preceding the extensive use of scientific instruments, green pigments were recognized as indispensable parts of the plant's self-nourishing or autotrophic apparatus. Plants that lacked the green

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withered and died. Plants with the green throve and accumulated the organic matter essential to the nutrition of animals.

Both sunlight and the green pigments were necessary for plant growth. In the dark, all plants failed to grow and to accumulate their organic substance. In the light, only those plants with their unique green pigments, called chlorophylls[1-3], grew normally, and only the green parts of the plants contributed to the production of organic matter and the liberation of oxygen.

There have long been indications that yellow carotenoid pigments always occur in the green parts of plants[1,3-8]. Plants deficient in green pigments, because of genetic or nutritional deficiencies, were usually yellow. Seedlings from seeds germinated in the dark did not turn green but became yellow instead. When these yellow, etiolated seedlings were exposed to light, the characteristic chlorophylls formed rapidly and obscured the yellow pigments. In the autumn, the leaves of many plants lost their green pigments but retained much yellow, which, presumably, had been masked by the green before it disappeared[4]. In a few plants, this loss of the chlorophyll was accompanied by the formation of conspicuous, red, water-soluble pigments that were unrelated to the yellow carotenoid pigments[4].

About forty years ago, these old observations led STRAIN to pen the following verses. Even now, after many intensive pigment studies, these stanzas pose numerous unanswered questions concerning the number, the identity, the formation, and the reactions of the essential green and yellow pigments.

*Nature's Mystic Greens and Yellows*

The interface of earth and sky
Is interlaced with green.
This is a happy circumstance
In Nature's plan supreme.

In plants, from algal cells to trees,
The basic greens abound.
But greens themselves are not alone,
Bright yellows, too, are found.

These greens and yellows play one role
On which all lives depend.
They utilize the force of light
To make the stuffs that mend.

The yellows, greens—how manifold?
What are their chemistries?
Their syntheses? Their evolutions?
Their bio-mythologies?

## 2. PLURALITY OF THE CHLOROPLAST PIGMENTS

Due to the inherent limitations of chemical and biological techniques, answers to the first of the questions in these stanzas must often precede examination of the others. How varied are the green and yellow pigments? Yet, to answer this question, the specialist must contemplate another. How reliable are the methods for the extraction, isolation, description and identification of the components of the pigment mixtures found in the green parts of plants?

Over the years, many techniques have been used to isolate and describe the individual pigments of green plants. With the microscope, the indispensable green[2,3] and yellow[4-6] pigments were observed together in specialized microscopic organs, the chloroplasts, that occur within the plant cells. With special extraction procedures and with improved separatory methods, such as partition between two immiscible liquids, these green and yellow pigments were isolated from the chloroplasts of various kinds of plants[2-8]. With the improvement of isolation procedures based upon precipitation and crystallization, and with the perfection of descriptive techniques, such as absorption spectroscopy, numerous chloroplast pigments were recognized. Frequently, however, the separatory procedures and the descriptive methods were inadequate so that many natural mixtures were incompletely separated; hence, the identification of the components was sometimes inaccurate.

There were other ramifications of these analytic studies of the chloroplast pigments. Numerous yellow pigments were isolated from mixtures found in diverse, yellow, plant parts. Some of these pigments were identical with those found in the green chloroplasts. An example is the carrot-root carotene, which was ultimately found to be a mixture of two closely-related, isomeric pigments, $\alpha$- and $\beta$-carotene, plus traces of other similar pigments, $\gamma$-, $\delta$-, and $\zeta$-carotene[5]. Many additional yellow pigments obtained from yellow plant organs differed but slightly from those of the chloroplasts. Examples are lycopene of tomatoes and eschscholtzxanthin of the petals of the California poppy[5].

In time, about half a dozen green chlorophylls[2,3,6-8] and several dozen yellow carotenoid[5,6] pigments were isolated from the green parts of plants. Nearly two hundred additional carotenoid pigments have now been isolated from yellow plant organs, from various animal products, and by chemical synthesis[5]. The structural formulas of some of these chloroplast pigments are presented in APPENDICES I and II. For reasons cited below, the pigments in these appendices are limited primarily to those of higher plants, green algae and brown algae.

## 3. DIVERSE INTERESTS IN CHLOROPLAST PIGMENTS

As new scientific concepts were developed, special interest was focused on the chloroplast pigments by way of many disciplines. Energeticists found Nature's chain of energy linked to the production of food and fuel through the absorption and utilization of solar energy by the chloroplast pigments[1,7], as implied in Fig. 1. Agriculturists related crop production to the greenness of their plants, which varied with the inorganic nutrition or fertilization. Plant physiologists and photochemists established a
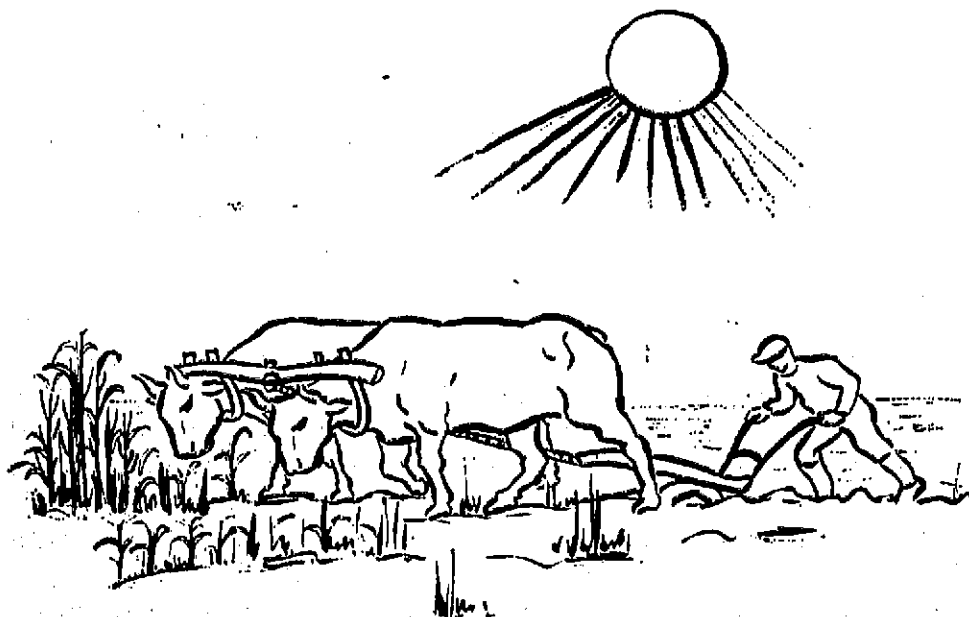
Fig. 1. Nature's chains of energy. Radiant energy absorbed by the chloroplast pigments of green plants serves for the production of oxygen and organic matter, the primal basis for food, fodder, and organic fuels.

relationship between the spectral absorption properties of the principal pigments and their role in the production of oxygen and organic matter[7]. Biochemists studied the complex biosynthesis and the natural degradation of the green and yellow pigments, and they established that some of these yellow pigments are the natural precursors of vitamin A, which is required by many animals[5]. Organic chemists determined the chemical composition and the molecular structure of many of the green and yellow pigments[2]. Biologists, particularly ecologists, recognized the unique role of plants not only for the organic nourishment of animals but also for maintenance of the chemical balance of the biosphere[1-6].

Special systems or combinations of green and yellow pigments were found to be characteristic of particular kinds of plants. These pigment systems indicated taxonomic or evolutionary relationships among organisms of the major plant groups. The close chemical relationships among the components of these pigment systems and the presence of the same green chlorophyll, chlorophyll a, in all autotrophic plants indicated that the photosynthetic pigment systems originated from a common source many eons ago[6].

For most of these diverse studies, knowledge of the properties of the individual chloroplast pigments has been prerequisite. The development and application of methods for the separation and isolation of the individual chlorophylls and carotenoids opened the doors to the determination of their physical and chemical properties, their molecular structures, and their biological functions and relationships[2-8]. One of these special preparative methods proved to be exceptionally selective, and it was destined to be useful with various kinds of chemical substances.

## 4. EARLY ATTEMPTS TO PREPARE THE INDIVIDUAL CHLOROPLAST PIGMENTS

Many investigations, about a century ago, revealed the chloroplast pigments to be so labile that they could not be recovered unchanged after attempts to separate them from one another by various chemical procedures[4]. With milder, sorptive methods, however, as by the addition of adsorptive alumina, magnesia, or charcoal to an extract of green leaves, the green pigments were sorbed, and the unaltered yellow pigments remained in the solution. These procedures demonstrated the presence of the yellow pigments, but the natural mixtures of these yellow substances could not be resolved further by this single adsorptive step[4].

In 1864 and 1873, by distribution of the chloroplast pigments of leaves between two immiscible solvents (such as carbon disulfide and aqueous alcohol), STOKES[9] and SORBY[10] found two green and two yellow pigments. These two green pigments differed with respect to their color and spectral absorption properties. The greener or "blue-green" pigment was the less polar of the two. It was called chlorophyll $a$. The yellow-green pigment was the more polar and dissolved more readily in alcohol than the chlorophyll $a$. It was called chlorophyll $b$[9,10]. The yellow pigments were more readily soluble in the alcohol than the chlorophyll $b$. They were similar to the leaf yellow that had been extracted from yellow autumn leaves and named xanthophyll by BERZELIUS in 1837[11,12].

Many additional partition experiments with various immiscible solvents (such as benzene or petroleum ether and aqueous alcohol) confirmed and extended the earlier results. Some of these improved partition experiments indicated that several xanthophyll-like substances were present in leaves. Unfortunately, these partition procedures did not effect a complete resolution of the xanthophyll mixtures. Consequently, there were numerous conflicting conclusions regarding the properties of the leaf xanthophylls, their nomenclature, and their identity[4].

The partition procedures also revealed that carotene (then called carotin) was present in the leaf extracts. This yellow pigment, known as a crystallizable constituent of yellow carrot roots since 1826, remained in solution with the xanthophylls when the chlorophylls were removed from leaf extracts with adsorptive charcoal or alkalies. When all the pigments in the leaf extracts were examined by partition between immiscible solvents (such as petroleum ether or benzene and aqueous alcohol), the carotene separated with the chlorophyll $a$ and was obscured by the latter's deep green color. Removal of the chlorophyll $a$ by saponification provided yellow solutions of the carotene. Most of these partition methods for the separation of the chloroplast pigments for the period of 1860 to 1911 were carefully reviewed by PALMER in his American Chemical Society Monograph on the carotenoid pigments[4], which was published in 1922 (pp. 29–43).

In 1904, a dynamic, analytical procedure, based upon differential migration and known as capillary analysis, was applied to the separation of the xanthophylls contained in the extracts of yellow flowers[13]. For separation of these xanthophylls by capillary analysis, an alcoholic solution of the pigments was allowed to rise into strips of sorptive filter paper. Under these conditions, overlapping zones of the yellow pigments were formed. The roles of sorbability and solubility in these separations were not established.

By 1904 capillary analysis was already widely known as a unique, mild, separatory method. This separatory procedure had been described forty-one years before by SCHÖNBEIN[14], and it had been widely applied by his student, GOPPELSROEDER, who published a very extensive summary article in 1901[15]. This article was republished as a book in 1906[16]. An analogous separatory procedure had been employed earlier by RUNGE, a German dye chemist, who had also published a book about this method in 1855[17,18]. He allowed dye solutions to flow continuously into sheets of filter paper and observed a series of concentric rings containing the several colored substances. Under these conditions, only a portion of the least sorbed pigment advanced beyond the more sorbed components and separated completely from them. If the overlapping or cross-contaminated pigment zones were separated mechanically, each pigment could be eluted from its zone in the paper and submitted to further purification by repetition of the separatory procedure. In this way, partial resolution of the mixture was achieved, and some of the major components of the mixture could be recovered[14-18].

Separatory procedures similar to capillary analysis, but with columns of porous sorptive powders in place of paper, had long been utilized for the partial resolution of mixtures of various kinds of substances. These applications included the selective sorption of ions by kaolin[19], by soils[20,21] and by rolls of paper pressed into glass tubes[22]. They also included the partial resolution of petroleum in columns of sorptive earth[23-25]. In most of these applications, as in capillary analysis, the solution of the mixture was allowed to flow continuously into the sorptive medium thus providing overlapping zones of the components of the mixture. These flow-of-solution methods were unsuitable for resolution of the complex natural mixtures of the similar labile, yellow and green chloroplast pigments.

## 5. IMPORTANCE OF CHLOROPLAST PIGMENTS TO THE INVENTION AND ADOPTION OF CHROMATOGRAPHIC ANALYSIS

At the beginning of the twentieth century, while the capillary analytical studies of flower xanthophylls were under way[13], a similar but much more effective differential migration method of analysis was devised by the Russian botanist and plant physiologist, MICHAEL TSWETT. His method was first employed for the separation of the chloroplast pigments of leaves, and it was demonstrated most convincingly with these colored substances. It was mentioned in several preliminary reports and then described in great detail in 1906[26]. It was called chromatographic adsorption analysis, because of its use with pigments and because the separations depend upon the selective or preferential adsorption of the components of the mixture. In this chromatographic procedure, by contrast with the continuous flow-of-solution procedures, only a little of the solution of the pigments was placed in a porous column of sorptive powder. This small zone of sorbed pigments was then washed with fresh solvent. This washing formed the chromatogram, because the components of the mixture migrated at different rates and separated as discrete zones with little overlapping or cross-contamination. Of all the separatory methods that have been tested for the separation and isolation of the chloroplast pigments, TSWETT's unique chromatographic or sorptographic procedure has contributed much more than any of the others. It has provided a very flexible and adaptable procedure.
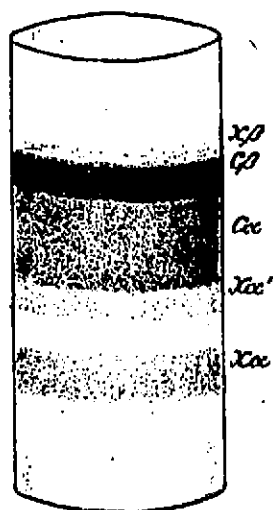
Fig. 2. Chromatogram of leaf pigments separated in a column of precipitated chalk washed with carbon disulfide (Tswett[26]).

Tswett's illustration of his separation of the chloroplast pigments in the chromatographic column[26] is reproduced as Fig. 2. The adsorbent was dried precipitated chalk, and the solvent or wash liquid was carbon disulfide.

Tswett not only described a unique analytical procedure, but he also understood the physical basis of the separations that he obtained. He made extensive tests of the sorbents and the solvents[27]. He provided the nomenclature that is common today after sixty-six years of usage[26]. These terms are: chromatographic adsorption analysis, the chromatographic column, the chromatogram, formation of the chromatogram, chromatographic zones, chromatographic sequences. None of this nomenclature is applicable to the other differential migration methods of analysis described above. This emphasizes the fundamental procedural differences between chromatography and the similar flow-of-solution procedures.

Progress in studies of the chloroplast pigments and the modifications and applications of chromatography have often been intimately interrelated. These relationships are revealed by a consideration of the contributions of chromatography to the separation of the pigments. Moreover, improvements and refinements of chromatography for use with chloroplast pigments paved the way for numerous applications of this technique to the separation and isolation of various kinds of substances, colorless as well as colored, inorganic as well as organic, gaseous and vaporous as well as dissolved.

Studies of the chloroplast pigments had unanticipated effects upon the adaptation and application of Tswett's chromatographic adsorption method. Most of the workers who soon adopted the technique were also experienced investigators of the green and yellow pigments, as indicated by several of the papers cited here in chronological order[28-34]. The unique usefulness of the Tswett method in their hands soon led many of them to prepare books and reviews concerning the chromatographic method as is indicated by a corresponding series of references, also in chronological order[35-43]. This early realization of the importance of chromatography as an analytical or preparative tool led to a continuing stream of reports and books concerning this novel technique.

Ironically, adverse criticism of TSWETT's novel chromatographic technique soon came from accomplished investigators of the pigments[3,44,45]. Most of these scientists relied upon the partition methods and never reported any attempts to repeat or apply TSWETT's procedure. See section 8.

In 1967, sixty-one years after the publication of TSWETT's classic report in the *Berichte der deutschen botanischen Gesellschaft*, an English translation of his material was presented in the *Journal of Chemical Education*[46]. It included a reproduction of TSWETT's drawing of his equipment and his chromatogram, the latter reproduced here as Fig. 2, as noted already. The translation was accompanied by a critique concerning the novelty, pertinence, and influence of TSWETT's contribution[47].

As indicated above, TSWETT's method stands in sharp contrast to the earlier differential migration techniques based upon flow-of-solution into the sorptive medium[4,13-23]. It is unjustifiable, therefore, for recent reviewers[24,25,48-50] to regard these flow-of-solution methods as equivalent to the developmental procedure described by TSWETT. It is also not true, as stated by some reviewers[50,51], that TSWETT's work was forgotten until the 1930's. In fact, there was extensive use of TSWETT's method for the examination of the yellow and green chloroplast pigments before the 30's, as shown by the citations in this section[28-30,35], in PALMER's book[4] and also in the sections 7 and 8.

There seems to be no justification for the statement that TSWETT "... was convinced that chlorophyll, as isolated by other researchers, was not a single substance"[51]. Before TSWETT's studies, experiments with the physical partition methods had already established the presence of chlorophylls *a* and *b* in leaves and had served for their separation and isolation[9,10].

## 6. TSWETT's EXPERIENCE WITH THE CHLOROPLAST PIGMENTS OF LEAVES AND OF BROWN ALGAE

TSWETT had produced a technique destined to influence the course of progress in studies of the chloroplast pigments of all kinds of plants[6,8]. In his hands, however, this adaptable method was applied primarily to the pigments of the higher plants. Although interested in the coloring matters of brown algae, he examined these special pigments principally by the use of partition methods. Brown seaweeds exhibit an unusual color change from olive-brown to green when the plants are dipped into hot water, allowed to stand in fresh water, or treated with anesthetics such as ether. This color change had been attributed to removal of a water-soluble pigment that TSWETT was seeking along with the chloroplast pigments[4,52,53].

With extracts of leaves, the sequence and the color of the zones in TSWETT's chalk column and the pigments contained therein are listed herewith. The pigment names are those employed by TSWETT[26].

Uppermost—Yellow, xanthophyll $\beta$ (most sorbed)

Olive-green, chlorophyllin $\beta$

Blue-green, chlorophyllin $\alpha$

Yellow, xanthophyll $\alpha'$ + xanthophyll $\alpha''$

Yellow, xanthophyll $\alpha$

Lowest —Orange-red, carotin (non-sorbed)

TSWETT's chromatographic experiment revealed two green pigments identical with the chlorophylls previously isolated by the partition methods, namely, the weakly-polar, blue-green or green chlorophyll *a* and the more-polar, yellow-green chlorophyll *b*[9,10]. These chromatographic observations indicated that the leaf green was composed primarily of these two chlorophylls, because no other green pigments could be detected.

In the chromatographic column with carbon disulfide as the wash liquid, the leaf xanthophyll separated into three zones. Two of these, the $\beta$ and the $\alpha$, were well separated. Two additional xanthophylls, xanthophyll $\alpha'$ and xanthophyll $\alpha''$, formed contiguous or overlapping zones that separated from each other only after the column was washed with benzene. This result indicated that the leaf xanthophylls were limited to a maximum of four, separable pigments. Unfortunately, the individual xanthophylls were not described well enough so that they could be recognized with certainty should they be isolated from other natural sources.

In TSWETT's experiments, the carotene was not adsorbed and moved through the column with the solvent front. Had the leaf carotene been a mixture, it would not have been separated under these conditions.

TSWETT's chromatographic results demonstrated that the pigments separated in a characteristic sequence. This sequence could serve as a basis for the description of the individual components of the mixture, and it could also serve for the comparison of different preparations of each pigment.

As surveyed by PALMER[4] (pp. 93–100), green and special yellow pigments had often been separated from the brown algae (phaeophytes) by the early partition methods. The principal green pigment was identical with the predominant chlorophyll *a* of leaves. A second, very polar green pigment, called chlorofucine, was described by SORBY[10]. Fucoxanthin, an unusually polar xanthophyll, was found in the brown algae and diatoms. Another xanthophyll and carotene were also isolated[10].

With partition methods, TSWETT isolated chlorophyll *a* and the chlorofucine of SORBY[10], which he called chlorophyllin $\gamma$[52,53]. He confirmed the presence of carotene, fucoxanthin, and another xanthophyll. There is no evidence that he attempted to resolve the brown algal pigments with his chromatographic method.

With partition methods and the fluorescence microscope, WILSCHKE confirmed the presence of chlorophyll *a* and chlorophyllin $\gamma$ in brown algae[54]. He preferred to call chlorophyllin $\gamma$ or chlorofucine chlorophyll *c*, a terminology now widely adopted.

TSWETT's primary interest in the chloroplast pigments of leaves and of brown algae has prompted us to consider here primarily the chromatography of the similar combinations of pigments found in flowering plants (spermatophytes), ferns (pteridophytes), mosses and liverworts (bryophytes) and most green algae (chlorophytes)[6,8]. We have also included the chromatography of the unique chloroplast pigments of the brown algae[6,8].

## 7. EARLY STUDIES OF THE PIGMENTS OF LEAVES AND OF BROWN ALGAE BY TSWETT'S METHOD

After TSWETT's report on the chromatography of the pigments of leaves, plant physiologists and biochemists soon adopted his technique for further investigation of

the leaf pigments. As early as 1912, ROGOWSKI, in DHÉRÉ's laboratory, employed TSWETT's technique for examination of the pigments of leaves and of snail livers[55]. Similar studies were reported by DHÉRÉ AND VEGEZZI[56] in 1916. In a series of papers in 1914, PALMER AND ECKLES[29] investigated the pigments of the green fodder supplied to dairy cows. They confirmed that leaves contain carotene, not sorbed in the chalk columns, several xanthophylls, and the two chlorophylls, *a* and *b*. These workers also examined the carotenoid pigments of milk fat from cows fed green plant material. The principal milk pigment proved to be carotene. Obviously the cow transferred the carotene to the milk fat in preference to the more abundant xanthophylls and chlorophylls of the fodder. Much of this work was summarized in PALMER's classical monograph[4].

In the middle 1920's there were further investigations of the pigments of leaves, flowers and fruits by the use of TSWETT's method. Quantitative aspects of the extractions and separations were studied, and the separation of two hydrocarbon carotenoids, carotene and lycopene, was reported[57]. The TSWETT method was employed to separate rhodoxanthin, a ketonic xanthophyll, from the leaf xanthophylls and from carotene[58]. It was also used to examine the leaf xanthophyll isolated by partition and crystallization[59].

From all these citations and those in section 5, TSWETT's method was not forgotten in the second and third decades of this century, as claimed by several reviewers[49-51]. Moreover, in this period, scientists with an interest in pigments and in chromatography were productive in various geographic regions, as shown by publications in England, France, Germany, Russia and the United States.

Most of the early studies of the pigments of brown algae had been based upon the use of partition methods, as noted above. In 1911, however, CZAPEK[28] examined an extract of brown algae with the TSWETT technique. He reported only chlorophyll *a*, fucoxanthin and "xanthophyll", overlooking the chlorophyllin γ (chlorophyll *c*) and carotene observed by SORBY[10] and TSWETT[52,53] with partition methods.

In most of these early applications of TSWETT's method, very small quantities of the individual pigments were isolated. Consequently, their description and identification were based largely upon the comparative chromatographic sequences, the absorption spectra, and the color reactions with acids. Larger quantities of the chloroplast pigments were needed for more extensive investigations of their properties. Experiments to this end led to the use of the TSWETT method on a large scale with activated adsorbents and on a much larger scale with mild adsorbents. They also led to conflicting results and conclusions concerning the number and nature of the leaf carotenoids isolated by partition and crystallization methods.

## 8. OBSERVATIONS DIFFERING FROM THOSE OF TSWETT

While TSWETT was engaged with his chromatographic studies of the chloroplast pigments, WILLSTÄTTER AND MIEG[44] carried out a large-scale isolation of the crystalline xanthophyll from 100 kg of the dried leaves of the nettle (Urtica). For the separation of this xanthophyll from the other pigments, partition and crystallization methods were employed. The chemical composition of the crystalline product, determined by combustion, corresponded to the formula $C_{40}H_{56}O_2$. As a consequence, the preparation

could not have contained significant quantities of xanthophyll oxidation products or of other xanthophylls with greater proportions of oxygen. It was concluded, therefore, contrary to Tswett's observations, that only one xanthophyll was present in leaves. Tswett's several xanthophylls were regarded as alteration products of the principal xanthophyll. This conclusion, which was re-emphasized in 1913 by the Willstätter and Stoll book on chlorophyll[3], undoubtedly retarded the adoption of Tswett's method. Furthermore, World War I, which engaged the attention of many research laboratories, hindered the adoption of the new technique, and it put an end to the publications by Tswett.

In 1925, Schertz[59] prepared leaf xanthophyll by the partition and crystallization methods. Examined by Tswett's chromatographic method, this preparation provided a single zone. This result was believed to support the contention that Tswett's several xanthophylls were alteration products of a single leaf xanthophyll.

With partition methods, as noted above, both Sorby[10] and Tswett[52,53] had found chlorofucine or chlorophyllin γ among the pigments of brown algae[2]. With similar partition methods, however, Willstätter and Stoll[3] and Willstätter and Page[45] had not found this green pigment, nor had Czapek[28] with his application of Tswett's technique. Most of these observations, which had failed to reveal the presence of chlorophyllin γ, were found, in time, to result from various unanticipated conditions.

Some of the early extraction, partition and crystallization procedures employed with the pigments of leaves and of brown algae permitted the loss of certain components of the natural pigment mixtures. Such losses sometimes occurred before the chromatographic separations were attempted. The correct interpretation of the number and the nature of the individual pigments resulted from improvements of the preparatory techniques and from effective modifications of Tswett's chromatographic method. Owing to the lability of some of the pigments, no single chromatographic system sufficed for separation and isolation of all the components of the natural mixtures. Investigations of these chromatographic procedures were stimulated tremendously by the discovery that certain carotenes serve as provitamins A[60]. Consequently, effective methods for examination of the carotenes were required.

## 9. CHROMATOGRAPHIC METHODS FOR SEPARATION OF THE CHLOROPLAST CAROTENES

By the 1930's there were indications that the carotene isolated from carrot roots was different from the corresponding pigment isolated from leaves (lower melting point and absorption maxima at shorter wavelengths than those of the leaf carotene). For a more critical examination of the carotene from these two sources by chromatography, however, it was necessary to find active adsorbents that would sorb this pigment reversibly and selectively without altering it. Surprisingly, three different adsorbents were developed independently and in a short period in three different laboratories. These sorptive materials were activated alumina (introduced by Kuhn and Lederer[61]), lime (employed by Karrer and Walker[62] and Karrer et al.[63]) and activated magnesia (described by Strain[64]). The specially-activated magnesia, prepared from the bitterns after solar concentration of sea water, was first marketed as Micron Brand Magnesium Oxide No. 2641, but it is now available as SeaSorb Magnesia,

No. 43. When employed in chromatographic columns with petroleum ether as the wash liquid, these sorbents separated carrot root carotene into two principal components, namely, α- and β-carotene, and three minor components, which separated in the sequence[65], γ (most sorbed), δ-, ζ-, β-, and α- (least sorbed). A special carotene, ε-carotene, from diatoms was less sorbed than the α-carotene[66].

The activated magnesium oxide was so finely divided that columns of it filtered very slowly. Consequently, to facilitate rapid filtration, one part of the magnesia was mixed with one to two or more parts of non-sorptive, heat-treated, diatomaceous earth (Celite 545). When pressed into glass tubes, this dry mixture provided columns that permitted rapid filtration and yielded unusually even zones of the sorbed pigments even when the tubes were 8 to 10 cm in diameter[8].

All the activated adsorbents bound the polar chlorophylls very strongly and altered them so that the natural green pigments could not be recovered. The xanthophylls were also bound very strongly, but these could be separated and recovered by washing the columns with solvents that are more polar than petroleum ether[67]. It was possible, therefore, to separate the chloroplast carotenes by chromatography of the untreated extracts of plant material[68]. It was also practicable to separate the carotenes in extracts from which the chlorophylls and natural fats had been removed by saponification with alcoholic potassium hydroxide followed by partition of the pigments between ether or petroleum ether and aqueous alkali[64-70].

With activated magnesia as the chromatographic adsorbent, the leaf carotene of most autotrophic plants was found to be β-carotene. Some plants contained small proportions of α-carotene in addition to the β-carotene[70,71]. Only a few plants, such as the siphonalean green algae, contained more α-carotene than β-carotene[6,8,71]. Brown algae from various geographic locations yielded primarily β-carotene[72].

## 10. CHROMATOGRAPHIC METHODS FOR SEPARATION OF THE CHLOROPLAST XANTHOPHYLLS

The chromatography of the carotenes opened the way to the examination of the xanthophylls in plant extracts treated in various ways. As noted above, SCHERTZ had shown that leaf xanthophyll isolated by partition methods and crystallized from alcohol yielded primarily a single pigment when examined by TSWETT's procedure[59]. A partial separation of leaf xanthophyll by fractional crystallization was reported by KUHN et al.[73]. A chromatographic separation of one of these fractions provided a major component like that observed by SCHERTZ[59] and a minor component identical with a xanthophyll, violaxanthin, obtained from yellow pansies[74]. The additional xanthophylls observed by TSWETT (Fig. 2) were not found. It was possible that they were artifacts, or they had been lost in the partition and crystallization procedures.

The principal leaf xanthophyll isolated by SCHERTZ and by KUHN et al. was identical with a pigment that had been isolated from various animal sources and that had been called lutein[4]. These results led to a modification of the nomenclature for the leaf xanthophylls. The principal leaf xanthophyll was to be called lutein. The term "xanthophyll", previously adopted for the crystalline xanthophyll from leaves[8] was proposed as a generic name for all these alcohol-soluble carotenoids[73]. In many instances, however, lutein and xanthophyll were used synonymously, and "xanthophylls" became the generic term.

The possibility for the loss of some of the xanthophylls in the preliminary preparative procedure was reduced by a special crystallization method. To this end, the chlorophylls were removed from the leaf extracts by saponification and partition, and the xanthophylls were crystallized from the concentrated ether solutions by the addition of petroleum ether. Under these conditions, the xanthophyll mixture separated from the solution with a minimum loss of the pigments[68].

Chromatography of the leaf-xanthophyll mixture obtained by crystallization from petroleum ether demonstrated that several of these carotenoid pigments occur together[68]. With activated magnesia as the sorbent and with 1,2-dichloroethane as the wash liquid, a number of clearly-defined yellow zones was obtained. A strongly sorbed pigment was a new xanthophyll called neoxanthin. Violaxanthin separated well below the neoxanthin and just above a small zone of zeaxanthin, an isomer of lutein obtained from corn (Zea). See Appendix II. Then there appeared a wide zone of lutein and below this a very small zone of cryptoxanthin. Three additional zones appeared in the magnesia columns. Two of these, with pigments having absorption spectra like that of flavoxanthin, remained near the top of the column; a third, designated as "isolutein", formed a zone in the lutein zone itself. All three of these pigments may have been formed from other xanthophylls by the action of traces of HCl in the 1,2-dichloroethane. This solvent, especially recovered preparations, slowly liberated HCl. Moreover, this acid rapidly converted violaxanthin into strongly-adsorbed, flavoxanthin-like xanthophylls. When the use of the 1,2-dichloroethane was eliminated, as by the use of petroleum ether plus acetone or plus n-propanol, the three additional xanthophylls were not observed[6,8].

The separation of the leaf xanthophylls in columns of magnesia was found to vary a great deal with respect to variation of the wash liquid. For example, the sequence and the separability of these pigments with petroleum ether plus acetone or n-propanol as the wash liquid was very different from that obtained with 1,2-dichloroethane, as shown in Table 1.

TABLE 1

XANTHOPHYLLS OF LEAVES AND OF MOST GREEN ALGAE AND THEIR SEQUENCES IN MAGNESIA COLUMNS WASHED WITH 1,2-DICHLOROETHANE (I), WITH PETROLEUM ETHER PLUS 25% ACETONE (II), AND WITH PETROLEUM ETHER PLUS 3 TO 5% n-PROPANOL (III)

| I | II | III |
|---|---|---|
| Neoxanthin (most sorbed) | Zeaxanthin + Neoxanthin | Zeaxanthin |
| Violaxanthin | | Neoxanthin |
| Zeaxanthin | Lutein | Violaxanthin |
| Lutein | Violaxanthin | Lutein |
| Cryptoxanthin (least sorbed) | Cryptoxanthin | Cryptoxanthin |

Because of their pronounced affinity for adsorbents, the xanthophyll mixtures could be separated on the mildest sorbents, such as cellulose and powdered sugar, provided the least polar liquids, as petroleum ether plus 0.5 to 1.0% n-propanol, were employed for formation of the chromatograms. The xanthophylls separated with these sorptive systems were identical with those isolated with the sorptive magnesia[6,8].

TABLE 2

XANTHOPHYLLS OF LEAVES AND OF MOST GREEN ALGAE AND THEIR SEQUENCES IN COLUMNS OF
MAGNESIA (WASHED WITH PETROLEUM ETHER PLUS 3 TO 5% $n$-PROPANOL) AND IN COLUMNS OF
POWDERED SUGAR (WASHED WITH PETROLEUM ETHER PLUS 0.5 TO 1% $n$-PROPANOL)

| Magnesia | Sugar |
|----------|-------|
| Zeaxanthin | Neoxanthin |
| Neoxanthin | Violaxanthin |
| Violaxanthin | ⎰ Zeaxanthin |
|  | + |
| Lutein | ⎱ Lutein |
| Cryptoxanthin | Cryptoxanthin |

The separation and sequences of the xanthophylls of leaves and of green algae in columns of powdered sugar were very different from those observed in columns of magnesia. These differences, observed with similar wash liquids, are illustrated by Table 2. It is noteworthy that zeaxanthin and lutein are readily separable in the magnesia columns, but they are inseparable in the sugar columns.

Activated magnesia has much greater adsorption capacity than powdered sugar. For this reason, much greater quantities of the xanthophylls may be separated in the magnesia columns than in sugar columns of the same dimensions.

After all these chromatographic results were available, there were still contradictory reports concerning the identity of the xanthophylls from leaves and from green algae. Lutein epoxide was reported to be an important xanthophyll of leaves[75] and of some green algae[76], but these results have never been verified[6,8,77,78], even with a variety of sorptive systems.

There has also been some confusion concerning the identity of neoxanthin. The same or a similar leaf xanthophyll was called foliaxanthin[79]. Eventually, an extensive collaborative study[80] confirmed that foliaxanthin was, in fact, identical with the previously-described neoxanthin[68].

All these and many additional chromatographic studies[2,5,6,8], some of which are described in the next section, indicate that the xanthophylls of seed plants, ferns, mosses, liverworts, and most green algae are those listed in Tables 1 and 2.

Some species of green algae and some isolates (but not all) of a given species contain an additional xanthophyll[81-84] called loroxanthin[81]. In chromatographic columns of powdered sugar developed with petroleum ether plus propanol, loroxanthin forms a yellow zone between neoxanthin and violaxanthin. In magnesia columns with the same wash liquid, loroxanthin is sorbed above the neoxanthin. Structurally, loroxanthin is unique in that it contains a hydroxyl substituent on an "in chain" methyl group[8,81,82]. See Appendix II.

Saponification followed by partition and chromatography was not effective with the xanthophylls of siphonalean green algae or of brown algae. The siphonalean green algae contain two xanthophylls in addition to those found in the other green algae. One of these, siphonein, is an ester, and it is converted into the other, siphonaxanthin, by saponification. Three groups of investigators have established the structure of siphonaxanthin as a ketonic derivative or loroxanthin[81-84]. See Appendix II. Saponification also altered the principal xanthophyll, fucoxanthin, of brown algae.

## II. CHROMATOGRAPHIC SEPARATIONS OF THE CHLOROPHYLLS AND XANTHOPHYLLS OF HIGHER PLANTS AND GREEN ALGAE

For isolation of the labile chlorophylls and xanthophylls, it was necessary to avoid the use of alkalies and the alkaline adsorbents. Precipitated chalk, employed by TSWETT[26] and by ZSCHEILE[85] for the separation of chlorophylls *a* and *b*, was not very selective[86]. Consequently, much attention has been devoted to the examination of mild, selective, sorption systems that will separate the chlorophylls and the xanthophylls from one another.

From TSWETT's early reports, sugar (which he powdered himself) was a mild adsorbent exhibiting many desirable sorptive properties. Because powdered sugar (with 3% starch to prevent caking) is now a cheap commercial product, it was tested extensively as a sorbent for the separation of the green and the yellow pigments in leaf extracts[6,8,36]. Experience showed that a very weakly-polar wash liquid, such as petroleum ether, was essential to permit adequate sorption of the pigments. Moreover a little alcohol added to the petroleum ether improved the selectivity of the system. Alcohols of low molecular weight, such as methanol or ethanol, were less effective than those of higher molecular weight and often separated from the petroleum ether. *n*-Propanol was a bit more effective than the isopropyl compound. Higher alcohols, which were also effective, were difficult to remove from the petroleum ether by extraction with water. Accordingly, *n*-propanol has been used most extensively with petroleum ether for the separation of the chloroplast pigments in columns of powdered sugar[6,8]. This system separated chlorophylls *a* and *b* and their respective, interconvertible isomers *a'* and *b'*, which are formed spontaneously and reversibly in heated plant material or in the extracts[87-89]. See Appendix I. It also separated most of the xanthophylls from one another as illustrated by Table 3 and Fig. 3. It confirmed the identity of several of the xanthophylls separated with magnesia columns and with sugar

TABLE 3

TOTAL PIGMENTS AND THE CAROTENOID PIGMENTS OF LEAVES AND OF MOST GREEN ALGAE SEPARATED IN COLUMNS OF POWDERED SUGAR WITH PETROLEUM ETHER PLUS 0.5% *n*-PROPANOL AS THE WASH LIQUID

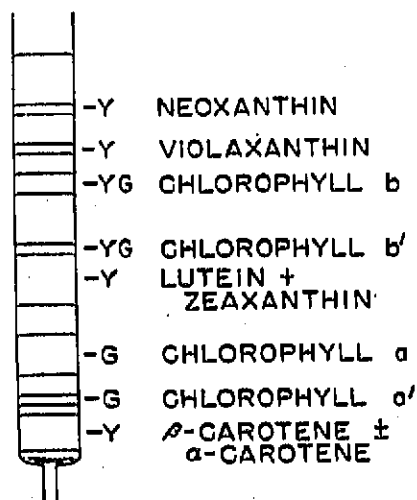| Total pigments | Carotenoid pigments |
|---|---|
| Neoxanthin (most sorbed) | Neoxanthin |
| Violaxanthin | Violaxanthin |
| Chlorophyll *b* | |
| (Chlorophyll *b'*) | |
| + | |
| Zeaxanthin | Zeaxanthin |
| + | + |
| Lutein | Lutein |
| Chlorophyll *a* | |
| (Chlorophyll *a'*) | |
| Cryptoxanthin | Cryptoxanthin |
| β-Carotene | β-Carotene |
| + | + |
| α-Carotene (least sorbed) | α-Carotene |

Fig. 3. Chromatogram of leaf pigments in a column of powdered sugar washed with petroleum ether plus 0.5% *n*-propanol (STRAIN[6]).

columns after saponification of the chlorophylls. In spite of its efficacy, however, powdered sugar did not separate all the pigments, notably the non-sorbed carotenes and the isomeric lutein and zeaxanthin. For the separation of each of these pigment mixtures, columns of activated magnesia were especially selective, as already noted in section 10.

Columns of powdered sugar about 0.5 cm in diameter have served for separations on a very small scale[43]. Columns some 8 to 10 cm in diameter and 36 to 38 cm long, with about 1.5 kg of powdered sugar, have been employed for separations on a preparative scale. These large columns are readily prepared with dry powdered sugar freshly rubbed through a wire sieve with a pestle[8]. About ten of these large columns

TABLE 4

TOTAL PIGMENTS AND THE CAROTENOID PIGMENTS OF MOST SIPHONALEAN GREEN ALGAE (AFTER SAPONIFICATION OF THE CHLOROPHYLLS) SEPARATED IN COLUMNS OF POWDERED SUGAR WITH PETROLEUM ETHER PLUS 0.5% *n*-PROPANOL AS THE WASH LIQUID

| *Total pigments* | *Carotenoid pigments* |
| --- | --- |
| Siphonaxanthin | Siphonaxanthin |
| Neoxanthin | Neoxanthin |
| Violaxanthin | Violaxanthin |
| Siphonein | |
| Chlorophyll *b* | |
| ⌠(Chlorophyll *b'*) | |
| ∣    + | |
| ⟨Zeaxanthin | ⌠Zeaxanthin |
| ∣    + | ∣    + |
| ⌊Lutein | ⌊Lutein |
| Chlorophyll *a* | |
| (Chlorophyll *a'*) | |
| Cryptoxanthin | Cryptoxanthin |
| ⌠β-Carotene | ⌠β-Carotene |
| ⟨    + | ⟨    + |
| ⌊α-Carotene | ⌊α-Carotene |

are required for the preliminary separation of the pigments from 1 kg of very green spinach leaves (freed of the midribs).

Each pigment zone in the sugar column is usually contaminated with traces of the pigments that precede it. For careful preparative work, therefore, the pigment from each zone should be purified further by recovery and re-adsorption in fresh columns of sugar[8]. Carotenoid pigments that are not altered by magnesia may be purified by re-adsorption in columns of this adsorbent. Chlorophylls and carotenoid pigments that are not separated in the sugar columns must be re-adsorbed with more selective chromatographic systems, as described in section 12.

As noted in section 10, some green algae contain loroxanthin in addition to the pigments shown in Table 3. In the sugar columns, loroxanthin is sorbed above chlorophyll $b$ between neoxanthin and violaxanthin.

All but one species of siphonalean green algae yielded siphonein and siphonaxanthin, in addition to the chlorophylls and carotenoids[72] shown in Table 3. The sequence of these pigments in the sugar columns is indicated by Table 4.

## 12. CHROMATOGRAPHY OF THE CHLOROPHYLLS AND CAROTENOIDS OF BROWN ALGAE

TSWETT's interest in the pigments of the brown algae and the divergent results quoted in section 8 prompted additional investigations of the green and yellow pigments of these organisms. As a practical matter, chlorophyll $c$ was extractable with difficulty from freshly dried algae. Moreover, chlorophyll $c$ extracted with methanol or ethanol was not readily transferred to petroleum ether. With suitable precautions to prevent loss of the $c$ and with columns of powdered sugar washed with petroleum ether plus propanol, the principal pigments were chlorophylls $c$, neofucoxanthins A and B, fucoxanthin, violaxanthin, chlorophyll $a$ and carotene, as shown in Fig. 4[8]. With columns of activated magnesia, the carotene of various species proved to be $\beta$-carotene, as noted in section 9.



-LG  CHLOROPHYLLS $c_1 + c_2$

-OY  NEOFUCOXANTHIN  A
-OY  NEOFUCOXANTHIN  B

-O  FUCOXANTHIN
-Y  VIOLAXANTHIN

-G  CHLOROPHYLL  a
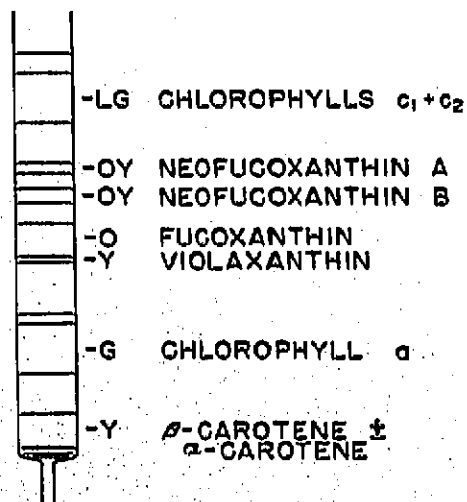
-Y  $\beta$-CAROTENE $\pm$
    $\alpha$-CAROTENE

Fig. 4. Chromatogram of the pigments of brown algae in a column of powdered sugar washed with petroleum ether plus 0.5 to 2% $n$-propanol (STRAIN AND SVEC[8]).

Treatment of the brown algae in ways that usually retard enzymatic changes (extraction with high proportions of alcohol or preliminary immersion in boiling water) always provided the same combination of pigments. This indicated that the several green and yellow pigments were natural constituents of the chloroplasts. The possibility that some of them may have been alteration products was virtually excluded, excepting the partial cis-neofucoxanthins that are formed spontaneously in solutions of fucoxanthin[90-92]. From these results, the striking change in the color of many brown algae from yellow-brown to green when the thalli are heated (or treated with the vapors of organic solvents) cannot be attributed to gross chemical changes of the pigments. It is probably due to alteration of their physical arrangement or distribution in the highly-specialized structure of the normal chloroplasts.

Several different lines of investigation indicated that the natural chlorophyll $c$ might be a mixture of two compounds. In early chromatographic experiments, adsorption of the extracts of brown algae in columns of powdered sugar often provided two contiguous yellow-green zones of chlorophyll $c$. Although this result indicated the presence of at least two similar components of the chlorophyll $c$, elution of the total chlorophyll $c$ followed by re-adsorption in fresh sugar columns failed to effect a satisfactory separation[93]. The mass spectrum and the nuclear magnetic resonance (NMR) of crystalline chlorophyll $c$, obtained after chromatography of the extracts of brown algae, also indicated that two pigments were present[94].

The two components of chlorophyll $c$ were separated by chromatography in columns of a specially-prepared polyethylene with acetone as the wash liquid. The less sorbed constituent was called $c_1$; the more sorbed component was $c_2$[95]. The $c_1$ proved to be tetradehydropheoporphyrin $a_5$ monomethyl ester; the $c_2$ was the corresponding hexadehydro compound[96,97]. In contrast to the other chlorophylls, which are esterified, the $c_1$ and $c_2$ are unesterified at the propionyl group. See Appendix I.

From these results, the polyethylene–acetone combination was remarkably selective for two substances of large molecular weight that differ only by two protons. Although powdered sugar was not very selective for the separation of the $c_1$ and $c_2$, it was much more effective for separation of the methyl esters of $c_2$ and $c_1$[98]. The reasons for these variations of the chromatographic selectivity are not clear.

## 13. CHROMATOGRAPHY OF ISOTOPICALLY-MODIFIED CHLOROPLAST PIGMENTS

Thus far chromatographic methods have not served for the separation of various isotopically-modified chloroplast pigments from one another. Neither the $^{13}C$ modifications, which are formed naturally, nor the fully-deuterated modifications, which are formed by growing plants in heavy water[99], are separable from each other or from the usual forms in the chromatographic systems studied thus far.

The isotopic modifications of the pigments are determined by mass spectroscopy. For these determinations, the isotopically-modified forms of each pigment must first be isolated together, free of other substances. For the resolution of the several pigments, each with its isotopically-modified forms, there is no substitute for the chromatographic methods described in the preceding sections.

## 14. PAPER CHROMATOGRAPHY OF THE CHLOROPLAST PIGMENTS

In 1939, the chromatographic separation of substances by differential migration from a narrow initial zone of the mixture in sorptive paper was demonstrated by BROWN[100] with the pigments of leaves. This development of the chromatogram in paper represented a great advance over the flow-of-solution into paper as employed by RUNGE[17], GOPPELSROEDER[15,16] and others[13,14,18] many years before. It was a high point in the beginning of paper chromatography.

For this chromatographic separation in paper, a spot of the pigment mixture, dissolved in carbon disulfide, was placed in a sheet of filter paper. Then fresh carbon disulfide was added slowly to the center of this spot. The radial flow of the wash liquid caused the pigments to migrate differentially so that they formed a series of concentric, circular, ring-like zones as shown in Fig. 5. The pigments in these zones and their sequence correspond to those separated by one-way flow of the wash liquid in columns of precipitated chalk as reported by TSWETT and illustrated in Fig. 2.
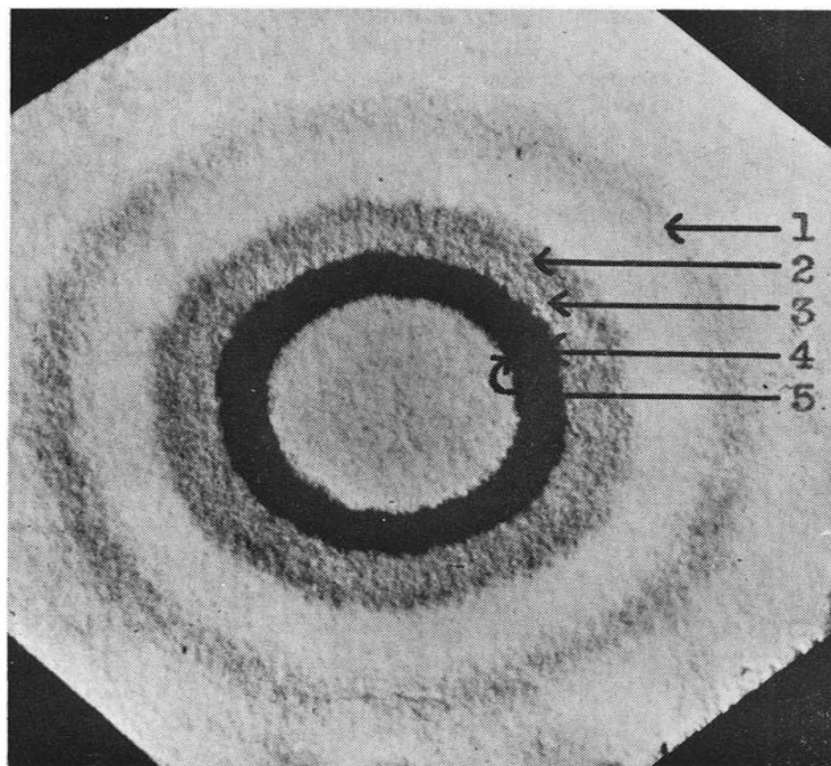


Fig. 5. Chromatogram of leaf pigments in a sheet of filter paper washed with carbon disulfide (BROWN[100]).

The photograph reproduced as Fig. 5 was submitted by BROWN with his manu-script on the separation of chloroplast pigments by chromatography in paper[100], but it was not published with the article. Later it was loaned to STRAIN and reproduced in a popular article[101]. Unfortunately, the proper acknowledgment was deleted in the course of publication. At this late time, the photograph, its description and its source are finally presented together. It is also significant that BROWN suggested the use of

various powdered sorbents with the paper, thus providing, independently, the basis for thin-layer chromatography at an early stage in the development of this subject.

This utilization of paper for chromatographic separations became known as radial or circular paper chromatography. It opened the way for several other geometric modifications of paper chromatography that were utilized with a great variety of substances. These include one-way or lineal paper chromatography based upon the flow of the wash liquid through spots of the mixture in strips or sheets of paper. They also include two-way paper chromatography based upon flow of one wash liquid through a spot in a sheet of paper followed by transverse flow of a different wash liquid across the first chromatogram. As is well known, all these procedures employed with paper were later modified for use with thin layers of various sorbents thus improving the applicability of the chromatographic technique. In fact, paper chromatography may now be regarded as a special modification of thin-layer chromatography.

Since that early demonstration of radial paper chromatography, virtually all the geometric modifications of chromatography have been utilized with the chloroplast pigments. Except for some spurious double zoning effects and alteration of the pigments by certain sorbents, the same pigments have always been obtained by all these modifications of the chromatographic procedures. Conversely, the chloroplast pigments have provided readily observable substances for use in the demonstration of all these chromatographic techniques[102-104].

## 15. CHARACTERISTICS OF CHROMATOGRAPHY FROM STUDIES OF THE CHLOROPLAST PIGMENTS

Chromatographic investigations of the chloroplast pigments have revealed many of the distinctive properties of TSWETT's sorptographic method. The separations were observed to depend upon the differential migration of the individual substances from the small initial zone of the mixture in the porous sorptive medium. This migration of the sorbed substance was caused by flow of fresh solvent, which exerts the same force upon all the components remaining in solution. The differential migration results, therefore, from a selective resistive force, namely, the selective or preferential sorption of the components of the mixture. As illustrated by the results in Tables 1 to 4, this selective sorption or selectivity is a function of the sorbed substances, the properties of the sorptive medium, and the properties of the solvent employed as the wash liquid. With a particular mixture, the separability may vary with the sorbent, the solvent, or both sorbent and solvent. As a consequence, the separation of substances must be described in relation to the chromatographic system: the mixture, the sorbent and the solvent.

The migration of the sorbed pigments depends upon their dynamic sorption. They must be reversibly sorbed and desorbed without concomitant alteration. Achievement of this condition with labile substances, such as the chloroplast pigments, has been based upon numerous empirical tests. Even now, sixty-six years after publication of TSWETT's illuminating report, the preparation, selectivity and choice of sorbents are based largely upon empirical experimentation. Chromatography of the pigments is an art.

For a particular sorbent and solvent combination, the sequence of the separated

pigments may be made the basis for their description and comparison. This sequence may also be made the basis for the comparison of preparations isolated from different sources. A more direct comparison may be effected by chromatography of a mixture of the two preparations. For this "mixed chromatography" each preparation should be chromatographically homogeneous.

## 16. SORBABILITY AND MOLECULAR STRUCTURE FROM CHROMATOGRAPHY OF THE CHLORO-PLAST PIGMENTS

Basic and useful relationships among sorbability, selectivity, and molecular structure have resulted from the chromatographic comparison of the green and yellow pigments. For example, the hydrocarbon polyene carotenes are not sorbed on powdered sugar from the least polar wash liquids, such as petroleum ether. Consequently, there is little if any affinity between the pigments and the active surface or interface of the sugar itself. With magnesia as the sorbent, however, there is a significant affinity between the hydrocarbon pigments and the adsorptive surface. This affinity, as reflected by the chromatographic sequence, is selective. It increases with the unsaturation of the pigments, as indicated by the number of the double bonds. For similar structures, the sorbability also increases with the conjugation of the double bonds, as with $\beta$- and $\alpha$-carotene. See Appendix II.

By contrast with the hydrocarbon carotenes, the hydroxycarotenes or xanthophylls are readily sorbed on powdered sugar. Because there is little or no affinity between the polyene system and the sugar, the sorption must be due to the hydroxyl groups. Consequently, one would not expect sugar to serve for the separation of the dihydroxycarotenoids lutein and zeaxanthin, which differ primarily in the conjugation of their eleven double bonds. See Appendix II. This expectation is in accord with the experimental observation that zeaxanthin, dihydroxy-$\beta$-carotene, and lutein, dihydroxy-$\alpha$-carotene, yield a single zone in the sugar columns. In the magnesia columns, wherein the polyene system as well as the hydroxyl groups are attracted to the sorptive surface, the zeaxanthin and the lutein are readily separated, as indicated in Tables 1 and 2.

In columns of powdered sugar, carotenoids that differ primarily in the number of hydroxyl groups, such as cryptoxanthin, zeaxanthin and neoxanthin with 1, 2 and 3 hydroxyl groups, respectively, yield a sequence with the adsorbability proportional to the number of the hydroxyl groups. The sorbability of neoxanthin may be influenced by the presence of the epoxy group, but this epoxide effect must be small, as shown by chromatographic studies of the carotene epoxides, which are not sorbed in columns of powdered sugar.

With the hydroxycarotenoids and with activated magnesia as the sorbent, the sorbability increases with the number and conjugation of the double bonds and with the number of the hydroxyl groups. For example, zeaxanthin is much more sorbed than lutein, and both these pigments are more sorbed than cryptoxanthin. It is remarkable that zeaxanthin is about as strongly sorbed as neoxanthin. Zeaxanthin is more sorbed than violaxanthin, its diepoxide. In the diepoxide, there is an epoxy group in each terminal ring in place of the two double bonds; hence, the sorbability due to these two epoxide groups is slightly less than that due to the two additional double bonds in zeaxanthin itself. See Appendix II.

Chromatography often provides clues to changes of the pigments brought about by chemical reactions. The conversion of siphonein to the more sorbed siphonaxanthin by alkali, summarized in section 11, indicates that the natural pigment is an ester. The location of the siphonein above the lutein and zeaxanthin, as shown in Table 4, indicates that the natural siphonein is only partially esterified. When treated with acid chlorides in pyridine, the hydroxyxanthophylls are completely esterified, yielding esters that are much less sorbed than the unesterified pigments.

A combination of chemical changes with chromatographic examination of the products provided a clue to the isomerization of chlorophyll *a* to *a'* and *b* to *b'*. The pheophytins, obtained by removal of the magnesium from the *a* and the *b*, also formed labile, interconvertible isomers. Moreover, substitution of phytyl by methyl provided the methyl chlorophyllides *a* and *b*, each of which formed a labile isomer that was reconvertible to the parent chlorophyllide. From these results, neither the magnesium atom nor the phytyl ester could be involved in the chlorophyll isomerization. The location for the isomerization was, therefore, in the tetrapyrrolic nucleus, and the most likely structural explanation was a *cis–trans* transfer of the carbomethoxy group and the adjacent proton through enolization of the keto group[88]. This conclusion was confirmed by NMR observations on the labile *a'* and *b'* isolated by chromatography[89].

All these observations and correlations show that the chromatographic behavior of the chloroplast pigments and many other organic substances is related to their molecular structure, to the degree and conjugation of the unsaturation, and to the nature and number of various substituent groups as hydroxyl, epoxy, and ester. In the light of these correlations, chromatography has become a useful adjunct to other methods for the determination of composition, molecular structure, natural condition, and function of many naturally-occurring substances.

## 17. RAMIFICATIONS OF CHROMATOGRAPHY

Tswett's basic experiment paved the way for numerous chromatographic investigations as applied to the pigments from various kinds of plants and many other products of biological origin. Progress in this area indicated numerous modifications of this technique, and it paved the way for the use of the columnar and paper techniques with all kinds of chemical substances.

Experience with the chromatography of the chloroplast pigments led not only to the concepts of driving and resistive forces in the differential migration from an initially narrow zone, but also to the technique of electrochromatography. In this procedure, the ions in a narrow zone of mixture placed in a stabilized electrolytic solution are caused to migrate differentially under the driving force of an electrical potential[105]. This technique was also combined with transverse flow of background solution so that the separations could proceed batchwise or continuously, thus combining the selective features of chromatography with those of electrical migration[106]. These methods and concepts, developed directly from chromatographic experience with the chloroplast pigments, led to the demonstration of another continuous procedure for the separation of gases by thermal gravitational diffusion[107].

Apart from Tswett's interest in the chloroplast pigments and beyond his discovery of chromatography, the chromatographic technique was utilized with all kinds of substances. It was applied to ionized substances and to vapors and gases by

ion-exchange chromatography, and by gas chromatography, respectively. The latter modification, as well as high-speed liquid chromatography, has been adapted in recent years to use with sensitive automatic detection methods for substances as they are washed through the columns. In this way, the method has been employed quantitatively with very small amounts of mixtures.

TSWETT's invention of chromatography illustrates an old principle in science: one experiment often stimulates many more. Put another way: the answer to one question often raises many more.

In 1941 an early book on chromatography by STRAIN contained the following introductory quotation[43]: *A good technique sometimes renders more service to science than the elaboration of highly theoretical speculations*. The pertinence of this citation is now supported by sixty-six years of productive work with the chromatographic method. But, in view of the contribution of chromatography in many fields, the following statement seems more apt: *A good technique is often indispensable to progress in science*.

APPENDIX I

*Formulas, structures and some properties of the chlorophylls from higher plants, green algae and brown algae*

Chlorophyll *a*

   Formula: $C_{55}H_{72}O_5N_4Mg$. Mol. wt.: 893.48. % Mg: 2.722
   Structure: see below
   Abs. max. ether: 428.5 nm; $e$, 125,000. 660 nm; $e$, 96,600. Ratio: 1.294



(PHYTYL)

Chlorophyll $a'$
>    Formula: $C_{55}H_{72}O_5N_4Mg$. Mol. wt.: 893.48. % Mg: 2.722
>    Structure: H– and –C(O)OCH$_3$ at C-10 exchanged to form spatial isomer of $a$

Deuteriochlorophyll $a$
>    Formula: $C_{55}D_{72}O_5N_4Mg$. Mol. wt.: 965.48. % Mg: 2.519
>    Structure: H– replaced by D– in structural formula of $a$
>    Abs. max. ether: 428.0 nm; $e$, 116,100. 659.0 nm; $e$, 88,600. Ratio: 1.310

Deuteriochlorophyll $a'$
>    Formula: $C_{55}D_{72}O_5N_4Mg$. Mol. wt.: 965.48. % Mg: 2.519
>    Structure: D– and –C(O)OCD$_3$ at C-10 of deuteriochlorophyll $a$ exchanged to
>    form the spatial isomer

Chlorophyll $b$
>    Formula: $C_{55}H_{70}O_6N_4Mg$. Mol. wt.: 907.46. % Mg: 2.680
>    Structure: –CHO in place of –CH$_3$ at C-3 in structural formula of $a$
>    Abs. max. ether: 452.5 nm; $e$, 175,300. 642.0 nm; $e$, 61,800. Ratio: 2.837

Chlorophyll $b'$
>    Formula: $C_{55}H_{70}O_6N_4Mg$. Mol. wt.: 907.46. % Mg: 2.680
>    Structure: H– and –C(O)OCD$_3$ at C-10 of $b$ exchanged to form the spatial isomer

Deuteriochlorophyll $b$
>    Formula: $C_{55}D_{70}O_6N_4Mg$. Mol. wt.: 977.46. % Mg: 2.488
>    Structure: H– replaced by D– in chlorophyll $b$
>    Abs. max. ether: 451.0 nm; $e$, 165,700. 640.5 nm; $e$, 57,900. Ratio: 2.862

Deuteriochlorophyll $b'$
>    Formula: $C_{55}D_{70}O_6N_4Mg$. Mol. wt.: 977.46. % Mg: 2.488
>    Structure: D– and –C(O)OCD$_3$ at C-10 of deuteriochlorophyll $b$ exchanged to
>    form the spatial isomer

Chlorophyll $c_1$
>    Formula: $C_{35}H_{30}O_5N_4Mg$. Mol. wt.: 610.54. % Mg: 3.980
>    Structure: –CH=CHCOOH in place of the propionyl phytyl ester at C-7, double
>    bond at C-7 to C-8 in structural formula of $a$
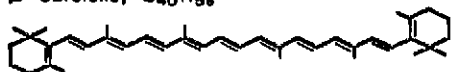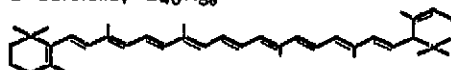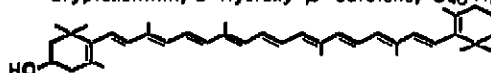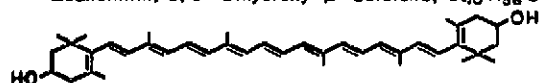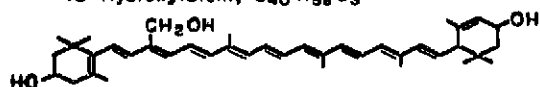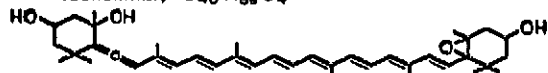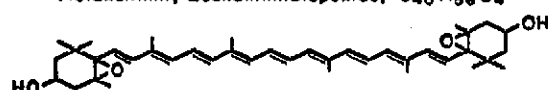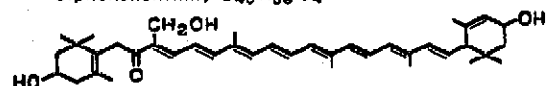>    Abs. max. ether: av. 444.4 nm, 628.2 nm. Ratio: 10.0

Chlorophyll $c_2$
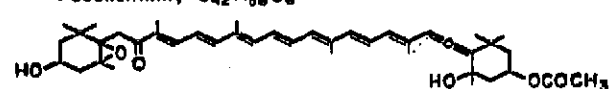>    Formula: $C_{35}H_{28}O_5N_4Mg$. Mol. wt.: 608.53. % Mg: 3.997
>    Structure: as $c_1$ but with –CH=CH$_2$ in place of ethyl at C-4
>    Abs. max. ether: av. 448.4 nm, 628.8 nm. Ratio: 14.5

## APPENDIX II
### Formulas and Structures
### of Some Chloroplast Carotenoid Pigments

$\beta$-Carotene; $C_{40}H_{56}$

$\alpha$-Carotene; $C_{40}H_{56}$

Cryptoxanthin, 3-Hydroxy-$\beta$-carotene; $C_{40}H_{56}O$

Zeaxanthin, 3, 3'-Dihydroxy-$\beta$-carotene; $C_{40}H_{56}O_2$

Lutein, 3,3'-Dihydroxy-$\alpha$-carotene; $C_{40}H_{56}O_2$

Loroxanthin, 3, 3', 19-Trihydroxy-$\alpha$-carotene,
19-Hydroxylutein; $C_{40}H_{56}O_3$

Neoxanthin; $C_{40}H_{56}O_4$

Violaxanthin, Zeaxanthindiepoxide; $C_{40}H_{56}O_4$

Siphonaxanthin; $C_{40}H_{56}O_4$

Siphonein; Monoester of Siphonaxanthin

Fucoxanthin; $C_{42}H_{58}O_6$

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