Alteration of Chloroplast Pigments by Chromatography with Siliceous Adsorbents

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Siliceous adsorbents isomerize neoxanthin and violaxanthin, the principal epoxy carotenoid pigments of leaves, by converting the epoxy groups into furanoid groups. Neoxanthin, with one epoxy group, yields neochrome with one furanoid group. Violaxanthin, with two epoxy groups, yields luteoxanthin with one epoxy group and one furanoid group; and luteoxanthin yields auroxanthin with two furanoid groups. In chromatographic separations on siliceous adsorbents, this isomerization of epoxy carotenoids is accelerated by nonpolar organic wash liquids that permit very strong adsorption of the pigments. The isomerization is greatly accelerated when the adsorbent plus the pigment and wash liquid are exposed to air so that the organic liquid evaporates, as in the isolation of the pigments after separation by thin-layer or column chromatography. This isomerization occurs very rapidly when the organic wash liquid is evaporated from the adsorbent plus the pigment in a vacuum or in a stream of dry nitrogen. It occurs even faster in a stream of moist nitrogen. Ammonia vapors retard the isomerization. This isomerization is essentially the same as that produced when the epoxides are treated with acid. The chlorophylls are also altered by many siliceous adsorbents. These results demonstrate important limitations in the use of siliceous adsorbents for the chromatography of the chloroplast pigments.

FOR SOME 60 YEARS, the isolation and identification of the leaf pigments has depended, to a large degree, upon the use of chromatographic methods (*1–4*). For the chromatographic isolation of the chlorophylls, exceptionally mild adsorbents such as the polysaccharides have been required (*1–7*); for the separation of many of the carotenoid pigments from one another, more adsorptive powders such as activated magnesia (*8*), alumina (*9*), and lime (*10*) have been necessary. With these chromatographic systems, the leaf pigments were usually found to be chlorophylls-*a* and -*b*, neoxanthin, violaxanthin, lutein, traces of zeaxanthin, traces of other xanthophylls, and β-carotene plus or minus α-carotene (*1–17*).

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Recently, various siliceous adsorbents such as silica gel (*6, 15, 16, 18–26*) and calcium silicates (*16*) have been utilized for the column and thin-layer chromatography of the chloroplast pigments. Some of the separations with these siliceous adsorbents have provided an increased number of green, gray, and yellow zones, which have been attributed to reactions with the adsorbent and to additional pigments in the leaves themselves.

Chromatography of the individual leaf pigments with silica gel has indicated that the chlorophylls (*19, 20*), and even carotene (*6*), are altered by this adsorbent. Moreover, carotene epoxides, which are related to the epoxy xanthophylls of leaves, are altered by the mild adsorptive alumina (*27–29*).

Because of the contradictory interpretations of all these observations, we have restudied the chromatography of the leaf pigments on various siliceous adsorbents. To this end, the untreated leaf extracts and the saponified extracts, without the chlorophylls, were examined by column chromatography and by thin-layer chromatography using various preparations of the siliceous adsorbents with various organic wash liquids.

EXPERIMENTAL

Materials and Procedures. VARIABLE CONDITIONS. Numerous variable conditions have been examined in the use of siliceous adsorbents. These variables include the adsorbent preparations themselves, their treatment (including conditions of storage, hydration, and activation), their use in columns and thin layers, and the use of various wash liquids ranging from nonpolar to weakly polar substances. Other variable conditions include the treatment of the adsorbent plus the pigment—e.g., by exposure to air, to inert gases, to vacuum, and to various solvents.

Special care was taken in the handling of the plant material, the extracts, and the solutions of the pigments. The composition of solvent mixtures for the extraction and for the chromatography of the pigments is often critical. It is specified as per cent or proportion by volume.

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LEAF EXTRACTS. Procedures for the preparation of the leaf extracts were minor modifications of those employed in our studies of the paper chromatography of the chloroplast pigments (*6*). The leaves were from cocklebur (*Xanthium*) grown in a greenhouse. A 6- or 12-gram portion of the fresh leaves freed of midribs was extracted with 120 or 240 ml of cold acetone in a chilled blender. The mixture was centrifuged, and the pigments were then transferred to cold petroleum ether (bp 65–110° C) by the addition of this solvent, equal to the volume of the acetone, and excess salt solution.

The green petroleum ether solution was washed twice with water and evaporated to dryness at reduced pressure with a rotary evaporator maintained below 35° C. The residue was dissolved in 3 or 6 ml of petroleum ether. Small portions of this green solution were employed for the chromatographic experiments.

For the removal of the chlorophylls by saponification, the acetone extract of the leaf material was treated with a solution of 3 or 6 grams of KOH in 30 or 60 ml of methanol. After 20 minutes the carotenoid pigments were transferred to ether plus petroleum ether, 1:1, by the addition of this mixture, equal to the volume of acetone, and excess salt solution. The yellow layer was washed with water and evaporated at reduced pressure with a rotary evaporator. The residue was dissolved in 3 or 6 ml of ether plus petroleum ether 1:1.

COLUMNS AND LOADING. Columns were prepared in tubes of 1-cm i.d. by 25 cm. The adsorbent was packed dry and in small portions to a height of 20 cm. From 50 to 400 μl of the pigment solutions were used. The sample was washed into the dry column with 0.5 to 1.0 ml of the wash liquid. Additional wash liquid was then added until the liquid front reached the bottom of the column. Vacuum was seldom employed to accelerate the percolation which was roughly equal to that in thin layers. The individual pigment zones were removed with a special, thin, semicylindrical stainless-steel spatula.

THIN LAYERS AND LOADING. Thin layers of the adsorbents were spread on five glass plates, 20 by 20 cm, using a slurry of the powder with water and a Desaga spreader set for a layer thickness of 0.25 mm. The amount of water in the slurries and the presence or absence of binders is described below for each adsorbent. All plates were dried overnight at room temperature. Some were activated at 110° C for 0.5 hour and cooled in air for 5 minutes before use. The pigment solutions, 0.25 to 20 μl, were placed at a starting line 2.5 cm from one edge of the plate. The plates were stood in 500 ml of the wash liquid in a large, covered cylindrical glass jar, 28 cm in diameter by 29 cm, lined with thick filter paper (*6*). The development, which was not started until the jar was saturated with the vapors of the wash liquid, was continued until the solvent front had moved 15 cm beyond the starting line.

WASH LIQUIDS. The purest organic liquids were employed for formation of the chromatograms. The petroleum ether employed as a wash liquid, either alone or in mixtures with other substances, was the low boiling fraction, bp 20–40° C.

LOCATION OF PIGMENT ZONES. The zones of the individual pigments, both in columns and in thin layers, were located visually. At low loading (0.25 μl) in thin layers, the separation of the zones was much greater than at higher concentrations, but the minor zones were very difficult to locate. At very high loading (more than 20 μl), the zones were incompletely separated. Obviously, the conditions that favored maximum separation led to diminished detectability, particularly of the minor constituents. Because of the intense color of the carotenoids, the zones were as readily detectable by visual observation as by treatment with the vapors of concentrated HCl.

COLOR REACTIONS WITH HCL. The blue color produced by the action of concentrated HCl upon carotenoid pigments dissolved in diethyl ether has long been employed as a basis of description and identification. With this reagent violaxanthin yields a deep-blue color in the acid; the other leaf carotenoids remain yellow (*3*, *11*). Separated in paper and exposed to the vapors of concentrated HCl, the zones of violaxanthin yielded a stable blue color; the zones of neoxanthin were green–blue; and the zones of the other carotenoids were unaffected (*6*). Separated in most siliceous adsorbents, particularly in those of marked sorption capacity, the zones of all the carotenoids were turned blue-green, blue, or blue-purple by the HCl vapors. In the thin layers, the zones of lutein and of carotene were of the deepest blue near the periphery and were yellow-blue in the more concentrated central regions. Upon removal from the acid vapors, the lutein and carotene zones returned to their yellow color in a few minutes. The neoxanthin, the violaxanthin, and their alteration products remained blue. In layers of the mildest siliceous adsorbents, such as talc, lutein and carotene remained yellow upon exposure to the vapors of concentrated HCl.

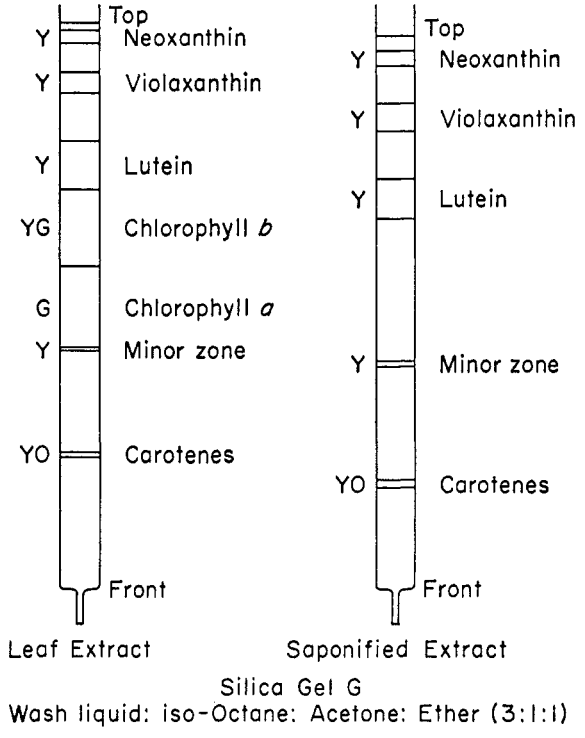

Figure 1. Leaf pigments (left) and leaf carotenoids (right) separated in columns of Silica Gel G

SPECTRAL ABSORPTION PROPERTIES. The spectral absorption curves of the carotenoid pigments were determined in ethanol. There were two principal reasons for this choice of solvent. The separated pigments were eluted with ethanol; hence, it was not necessary to transfer them to another solvent. Also, spectral absorption curves for the leaf carotenoids and for the acid isomerization products had already been determined in this solvent (*3*, *13*, *15*, *30*, *31*).

Chromatography with Silica Gels. SILICA GEL G, ACCORDING TO STAHL. Columns of Silica Gel G (E. Merck AG, Brinkmann Instruments, Inc.) provided effective separation of the leaf pigments, which were adsorbed from petroleum ether and washed with isooctane:acetone:ether, 3:1:1. Under these conditions, the two chlorophylls formed contiguous zones below the lutein zone as shown in Figure 1. With ethanol, each of the pigments was eluted completely.

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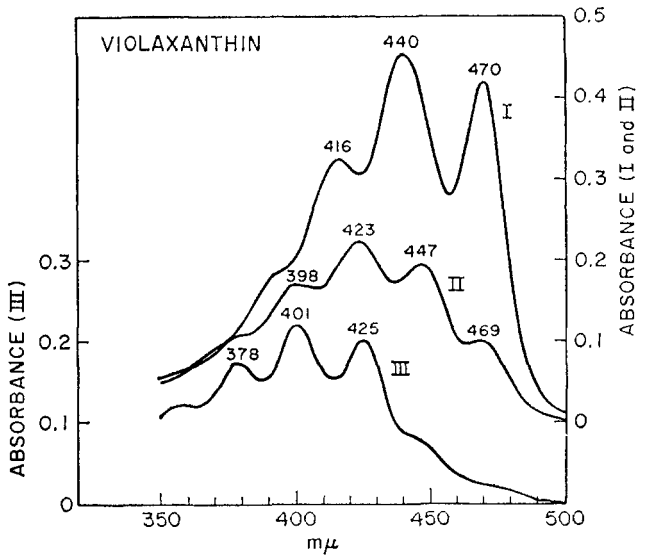

Figure 2. Spectral absorption curves of pigments eluted after adsorption of violaxanthin in columns and layers of Silica Gel G

Wash liquid: isooctane, acetone, ether, 3:1:1. Curve I, zone from column without drying (unchanged violaxanthin). Curve II, zone from thin layer with partial drying (unchanged violaxanthin, plus luteoxanthin, plus auroxanthin). Curve III, zone from column dried in moist nitrogen (auroxanthin)

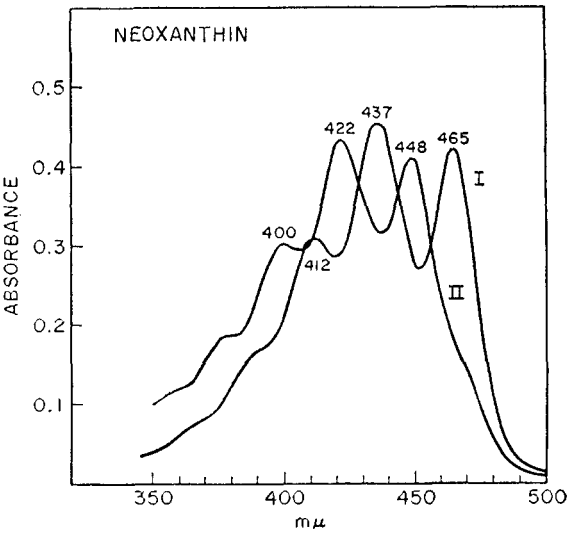

Figure 3. Spectral absorption curves of pigments eluted after adsorption of neoxanthin in columns of Silica Gel G

Wash liquid: isooctane, acetone, ether, 3:1:1. Curve I, zone without drying (unchanged neoxanthin). Curve II, zone dried in moist nitrogen (neochrome)

The spectral absorption curves of the carotenoids were like those of the natural pigments. The curves of the chlorophylls were slightly altered. With many other wash liquids, the chlorophylls formed diffuse zones overlapping the xanthophyll zones.

Columns of Silica Gel G provided excellent separations of the principal carotenoids in the saponified leaf extracts as indicated in Figure 1. The separations were obtained with petroleum ether:acetone, 7:3, isooctane:acetone:ether, 3:1:1, and also with these solvent mixtures plus 1% *N,N*-dimethylaniline. From the top of the column, the principal zones were neoxanthin, violaxanthin, lutein, and carotene. Minor zones often appeared just above and below the lutein zone. Each of the major pigments was eluted cleanly and without alteration by ethanol. Spectral absorption curves for the eluted, unchanged violaxanthin and neoxanthin are shown in Figures 2 and 3, respectively.

When the zones of the individual carotenoid pigments were removed from the columns and placed in a rapid stream of moist nitrogen for 0.5 hour, the neoxanthin and the violaxanthin faded rapidly. As shown by the spectra in Figures 2 and 3, the pigments eluted from these "dried" adsorbents corresponded to those of furanoid pigments formed by isomerization of epoxy carotenoid pigments. Thus neoxanthin, with one epoxy group, yielded neochrome with one furanoid group (*32*). Absorption maxima in ethanol were: neoxanthin, 437 and 465 mμ; neochrome, 422 and 448 mμ. Violaxanthin, with two epoxy groups, yielded luteoxanthin with one furanoid group and one epoxy group. This pigment then yielded auroxanthin with two furanoid groups (*11*, *30*). Absorption maxima in ethanol were: violaxanthin, 441 and 470 mμ; luteoxanthin, 424 and 446 mμ; auroxanthin, 401 and 427 mμ. The products formed by the action of the siliceous adsorbents were chromatographically and spectrometrically identical with the rearrangement products formed by the action of acetic acid upon the neoxanthin and violaxanthin dissolved in ethanol (*30*).

When zones of the adsorbed pigments were placed in a stream of dry nitrogen for 0.5 hour, the violaxanthin and the neoxanthin were only partially isomerized. Dimethylaniline in the wash liquid had very little effect in retarding the isomerization of the neoxanthin and the violaxanthin. The lutein and the carotene were unaltered. When the carotenoids adsorbed on the silica Gel G were exposed to a stream of nitrogen saturated with the vapors of concentrated ammonium hydroxide, there was no alteration of the pigments.

As indicated above, weakly polar solvents that permitted migration and separation of the pigments did not promote isomerization of the xanthophylls. By contrast, a nonpolar solvent, petroleum ether, that allowed the pigments to be so strongly sorbed that they did not migrate, promoted partial isomerization of the neoxanthin and violaxanthin even though the solvent was not permitted to evaporate. The two latter pigments also underwent partial isomerization when adsorbed from weakly polar solvents and then washed with petroleum ether.

Thin layers of the Silica Gel G were prepared from a slurry of 25 grams of the adsorbent with 50 ml of water. The sorption capacity and the selectivity of the air-dried and the heated or activated layers were very similar. When the solvents were the same as those employed with columns, comparable separations were obtained, as may be seen by comparison of Figures 1 and 4. Somewhat better separations of the strongly sorbed xanthophylls were obtained with petroleum ether:acetone, 7:3, as solvent.

If the plates were removed from the migration tanks and the zones of the pigments removed quickly and eluted with ethanol, the violaxanthin was partially isomerized, as indicated in Figure 2. The neoxanthin was only slightly altered, and the other carotenoids were unaltered. But if the plates were allowed to dry while the adsorbent was scraped loose, both the neoxanthin and the violaxanthin were completely isomerized, the lutein and carotene remaining unaltered.

Silica Gel G made into thin layers, dried in air, scraped loose, and packed into columns separated the carotenoid pigments in the same way as the untreated silica gel. With this treated adsorbent, there was no isomerization of the neoxanthin and the violaxanthin unless the zones of the adsorbed pigments were dried in air or in moist nitrogen.

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For the chromatographic comparison of the isomerized xanthophylls with the parent pigments, neoxanthin and violaxanthin were treated with acetic acid in ethanol (*30*). The reaction products were then compared with the saponified leaf pigments in thin layers of Silica Gel G with three different solvent mixtures, namely, petroleum ether:acetone, 7:3, and isooctane:acetone:ether, 3:1:1 and 2:2:1. The auroxanthin from the violaxanthin formed somewhat elongated zones moving a little faster than the violaxanthin zones and following closely the trailing regions of the lutein zones. Neochrome, from neoxanthin, provided somewhat elongated zones with the leading boundaries moving but slightly faster than the neoxanthin zones.

SILICAR. Columns of 200–325-mesh Silicar (Mallinckrodt Chemical Works) provided a poor separation of the major leaf carotenoids; the violaxanthin and the lutein formed overlapping zones when the solvent was benzene:acetone, 7:3. With this chromatographic system, the violaxanthin zone, eluted with ethanol, yielded a mixture of violaxanthin, luteoxanthin, and auroxanthin. The other carotenoids were unaltered. Owing to the poor resolution of the xanthophylls, the effect of drying the adsorbent plus the pigments separated from the natural mixtures was not examined further.

To test the stability of the individual xanthophylls on Silicar, the neoxanthin and violaxanthin were prepared from the saponified extracts of cocklebur leaves in columns of powdered sugar (*3*). Each pigment was then adsorbed in a separate column of Silicar from benzene:acetone, 7:3. The zones were removed and divided into two parts. One part was eluted immediately with ethanol. The other part was dried in a stream of moist nitrogen for 0.5 hour, and the pigments were eluted with ethanol. The freshly eluted neoxanthin was unaltered. The dried and eluted portion was completely isomerized. The freshly eluted violaxanthin was a mixture of the original pigment plus the two isomerization products, luteoxanthin and auroxanthin. The dried and eluted violaxanthin zone yielded only the completely isomerized product, auroxanthin.

Thin layers of Silicar were prepared from a slurry of 30 grams of the adsorbent plus 4.7 grams of $CaSO_4$ and 70 ml of water. The sorption capacity of the air-dried and of the activated Silicar plates with benzene:acetone, 7:3, as solvent exceeded that of the Silica Gel G plates, but the resolution of the pigments was incomplete. Both the chlorophylls and the carotenoids formed trailing zones, due presumably to alteration of the individual pigments. Spectra of the neoxanthin and the violaxanthin, which were taken immediately after these compounds had been eluted, indicated that the neoxanthin had not been isomerized but that the violaxanthin had been extensively rearranged, as had been observed in columns.

SILICA GEL LAYERS ON FILM. Chromagram Sheets of silica gel without fluorescent indicator (Type K301R2, Eastman Kodak Co.) were used under the same conditions employed with thin layers on glass plates. Activated, unactivated, and moistened and dried sheets gave similar results. With the untreated leaf extracts, the separations of the pigments, which varied with the wash liquid and with the loading of the adsorptive layer, were usually complete. The sequence was neoxanthin (most adsorbed), violaxanthin, chlorophyll-*b*, lutein, chlorophyll-*a*, and carotenes. Usually the separation of the lutein and the chlorophyll-*b* was most difficult, especially at the higher loading, 10 to 20 μl. The wash liquids were: benzene:acetone, 7:1; benzene:acetone, 7:3; isooctane:acetone:ether, 3:1:1; isooctane:acetone:carbon tetrachloride, 3:1:1; ethylene dichloride:ethyl acetate, 4:1; and petroleum ether:acetone, 7:3. The last four of these wash liquids also provided excellent separations of the carotenoids in the saponified leaf extracts, yielding zones of neoxanthin, violaxanthin, lutein, and carotenes, from which the unaltered pigments were eluted with ethanol.

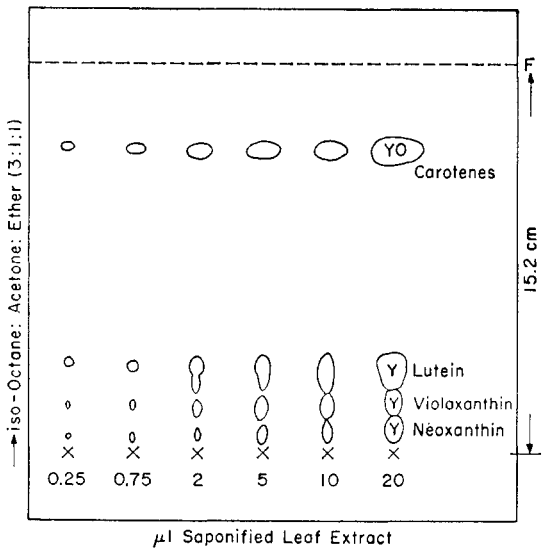

Figure 4. Leaf carotenoids separated by adsorption in a thin layer of Silica Gel G, the degree of separation varying with the loading of the adsorbent

Wash liquid: isooctane, acetone, ether, 3:1:1

When the zones of the individual carotenoids were cut from the chromatograms and dried in a current of moist nitrogen for 0.5 hour, the violaxanthin yielded a mixture of the unaltered pigment with roughly equal proportions of luteoxanthin and auroxanthin. The neoxanthin yielded a little neochrome along with unaltered neoxanthin. The lutein and the carotene were unaltered.

STABILIZED SILICA GEL LAYERS. The "instant thin layer chromatography" sheets (Chromatography Media, ITLC Type S, Gelman Instrument Co.) prepared from silica gel and glass fibers provided rather inefficient separations of the leaf pigments. The chlorophyll zones were very strongly adsorbed and overlapped the zones of the strongly sorbed carotenoids. The wash liquids included petroleum ether: ethyl acetate, 10:1; 13:1; 16:1 and 20:1 and benzene: acetone, 7:3.

In the ITLC sheets, the carotenoid pigments in the saponified extracts were clearly separated into four principal zones, especially in the loading range of 1 to 10 μl, with petroleum ether:ethyl acetate, 13:1, as wash liquid. The neoxanthin and violaxanthin eluted from the moist sheets or from the sheets exposed to moist nitrogen were unaltered.

Chromatography with Siliceous Earths. HEAT-TREATED DIATOMACEOUS EARTH. Columns of untreated Celite 545 (Johns-Manville) were weakly sorptive and not very selective. With leaf extracts and with petroleum ether plus 5% acetone as wash liquid, chlorophylls-*a* and -*b* were well separated above carotene and below neoxanthin, but the zones of violaxanthin and of lutein were not clearly differentiated. With the saponified extracts, the carotenoid pigments were also poorly separated. They formed only three principal zones instead of the usual four, the chromatogram being incompletely developed by the time the wash liquid had reached the bottom of the columns.

Thin layers of Celite 545 were prepared from 25 grams of the Celite, plus 3.9 grams of $CaSO_4$, plus 75 ml of water, which were mixed in a blender. Separations were made in layers that had been air-dried and in those that had been activated.

Separation of the untreated leaf extracts in the thin layers often led to trailing of the green zones, particularly at the higher loading, 10 to 20 μl. Petroleum ether plus approximately 0.38% propanol or 10% acetone gave better separations than petroleum ether with larger or smaller proportions of these solvents.

In the thin layers of Celite, the saponified pigments yielded four principal zones corresponding to neoxanthin, violaxanthin, lutein, and carotenes when the wash liquid was petroleum ether:acetone, 9:1.

Preparations of neoxanthin and violaxanthin eluted from sugar columns were readsorbed on Celite 545 in columns and washed with petroleum ether plus 10% acetone. The pigments eluted from the undried portions of the Celite with ethanol were unaltered. Those eluted from portions dried in moist nitrogen for 0.5 hour were also unaltered.

ACID-WASHED, HEAT-TREATED, DIATOMACEOUS EARTH. The separations of the leaf pigments on the commercial acid-washed Celite 545, also obtained from Johns-Manville, were similar to those on the untreated Celite 545. Neoxanthin and violaxanthin were isolated with sugar columns, adsorbed on the acid-washed Celite, and washed with petroleum ether plus 10% acetone. The pigments eluted from the Celite with ethanol were slightly altered, but when the Celite plus the pigments were exposed to a stream of moist nitrogen, the eluted pigments were partly isomerized.

ACID-WASHED, CELITE ANALYTICAL FILTER-AID. The separations of the chloroplast pigments on this commercial product of Johns-Manville were similar to those on the untreated Celite 545. Neoxanthin and violaxanthin, isolated in a sugar column and adsorbed in a column of this filter-aid, were partially changed when eluted with ethanol. When this filter-aid plus the pigments were exposed to a stream of moist nitrogen before elution of the pigments with ethanol, each xanthophyll was isomerized completely.

DIATOMACEOUS EARTH, KIESELGUHR G, ACCORDING TO STAHL. Columns of Kieselguhr (Brinkmann Instruments, Inc.) or of Kieselguhr plus Celite, 1:1 by weight, failed to produce satisfactory separation of the pigments in the untreated leaf extracts, largely because of the trailing and spreading of the chlorophyll zones. The solvents employed were petroleum ether plus acetone, 5 to 10%, and petroleum ether plus propanol, 0.25%.

With the saponified leaf extracts, the Kieselguhr and the Kieselguhr plus Celite columns provided an effective separation of the carotenoids into the usual four principal zones. Better separations were obtained with 5% acetone in petroleum ether than with 10% acetone for a wide loading range, 75 to 650 μl. The pigments eluted from a Kieselguhr column with ethanol were unaltered. Drying the individual zones in a stream of moist nitrogen partially isomerized the violaxanthin but not the neoxanthin, lutein, or carotene.

With thin layers formed from 25 grams of Kieselguhr and 50 ml of water, there was also a poor separation of the pigments in the leaf extracts due to spreading of the chlorophyll zones. The best separations were obtained at the lowest loading, 0.25 μl, when the solvent was petroleum ether plus 10% acetone.

In the thin layers, the carotenoids in the saponified extracts were well separated. With petroleum ether plus 10% acetone as solvent and with a loading range of 1 to 20 μl, these carotenoids yielded neoxanthin, violaxanthin, lutein, and carotene with a trace of pigment between neoxanthin and violaxanthin. The violaxanthin eluted from the freshly prepared chromatograms was but slightly isomerized. The other carotenoids were unaltered.

TALC. Columns of talc (USP talc or talcum powder, Mallinckrodt Chemical Works) filtered so slowly that this adsorbent was mixed with two parts of Celite by weight before it was packed into the chromatographic tubes. With the leaf extracts and with petroleum ether plus 10% acetone, the chlorophylls streaked through the adsorbent, especially in the region above the lutein zone. In this respect, the separations were unsatisfactory.

In columns of talc plus Celite and with petroleum ether plus 10% acetone, the saponified extracts yielded well separated zones of neoxanthin, violaxanthin, lutein, and carotene. For these separations a loading range of 35 to approximately 600 μl was satisfactory. Spectra of the pigments eluted from the neoxanthin and violaxanthin zones indicated little or no isomerization.

Thin layers were prepared from 30 grams of talc, 3.9 grams of $CaSO_4$, and 60 ml of water. In these layers, talc proved to be a very weak adsorbent. With the untreated leaf extracts, adsorption was very weak from petroleum ether:acetone, 4:1, providing the sequence: neoxanthin, violaxanthin, chlorophyll-*b*, lutein, chlorophyll-*a*, and carotene. With petroleum ether plus 5% acetone the pigments were too firmly bound, but with 10% acetone there was an excellent separation at the lower loadings, 0.25 and 0.75 μl. The sequence was then neoxanthin, chlorophyll-*b*, violaxanthin, chlorophyll-*a*, lutein, and carotenes.

With the saponified leaf extracts washed with petroleum ether plus 10% acetone, there was an excellent separation of the four principal zones, neoxanthin, violaxanthin, lutein, and carotene. All zones were readily visible at all loadings, 0.25 to 20 μl. The spectra of the freshly eluted neoxanthin and violaxanthin indicated little or no isomerization. Poor separations were obtained with benzene-acetone mixtures, and there was some isomerization of the epoxy carotenoids.

Chromatography with Synthetic Silicates. MAGNESIA–SILICA GEL. Columns were prepared from 100–200-mesh Florisil (Floridin Co.) which was activated at 1200° F. The Florisil bound the chlorophylls so firmly that a satisfactory separation of the green and yellow pigments could not be obtained. With the saponified extracts, however, four principal zones were separated, and the individual pigments were eluted unchanged. When the moist zones of the adsorbent plus pigment were dried in moist nitrogen for 0.5 hour, the neoxanthin was completely isomerized to neochrome; the violaxanthin was almost completely isomerized to auroxanthin; and the lutein and carotene were unaltered.

CALCIUM SILICATE, MICRO-CEL C. In columns of Micro-Cel C (particle size <0.1 micron, Johns-Manville), the pigments in the untreated leaf extracts were not readily separated, primarily because of the strong, irreversible adsorption of the chlorophylls. By contrast, the carotenoid pigments were readily separated into four major zones, namely, neoxanthin, violaxanthin, lutein, and carotene with a minor zone between lutein and carotene and another between lutein and violaxanthin. When the solvent was petroleum ether plus 15% acetone, the pigments from the four major zones were eluted without alteration by ethanol. When the zones were dried in a stream of moist nitrogen, the neoxanthin was almost completely isomerized to neochrome, and the violaxanthin yielded mostly auroxanthin plus some luteoxanthin plus a little residual violaxanthin.

Thin layers of Micro-Cel C were prepared from 15 grams of this adsorbent, 3.0 grams of $CaSO_4$, and 98 ml of water. In these layers, petroleum ether plus 15% acetone provided the usual four principal carotenoid zones plus a minor zone located between lutein and violaxanthin and detected only at the higher loading, 5 to 20 μl. After activation of the thin layers on the glass plates, only the four major zones were obtained. Benzene plus 15% acetone provided equivalent separations.

Comparative tests with neochrome and auroxanthin, prepared by acetic acid isomerization of the respective xanthophylls, showed that the former was adsorbed between the neoxanthin and the violaxanthin zones while the latter was adsorbed between the violaxanthin and the lutein zones.

CALCIUM SILICATE, MICRO-CEL E. Separations of the saponified and the unsaponified pigments in columns of Micro-Cel E (particle size <0.1 micron, Johns-Manville) were similar to those obtained in columns of Micro-Cel C. Moreover, the eluted pigments were unaltered. When the zones of the individual pigments were dried in moist nitrogen, the neoxanthin and the violaxanthin were partially isomerized, and the lutein and carotene were also altered. These changes were indicated by some increase of the absorption in the 400-

to 440-mμ region and by a small decrease in the 470-mμ region.

Thin layers of Micro-Cel E were prepared from 15 grams of this adsorbent, 3.0 grams of $CaSO_4$, and 108 ml of water. With petroleum ether plus 15% acetone, the saponified leaf extracts yielded zones of the four major pigments but with seven small additional zones. With benzene plus 15% acetone, there were four principal zones and three minor zones.

ARC SILICA 800. Because of its fineness, the arc silica (Pittsburgh Plate Glass Co.) was mixed with twice its weight of Celite 545 before use in columns. In spite of its fineness, this silica was a very weak adsorbent. Even in a mixture with Celite, the percolation of the solvent was so slow that few experiments were carried out.

COMPARATIVE RESULTS AND CONCLUSIONS

Selectivity of Siliceous Adsorbents. The selective properties of all the siliceous adsorbents were similar to those of powdered sugar and cellulose (paper) (*1–3, 6*). This was indicated by the chromatographic sequences and by the inseparability of lutein and zeaxanthin. Employed with suitable precautions, the milder siliceous adsorbents permitted the separation of most of the leaf pigments already isolated by chromatography on sugar (*3, 17*) and on paper (*1, 2, 6*). The sequence of the pigments on the mild adsorbents was usually neoxanthin (most adsorbed), violaxanthin, chlorophyll-*b*, lutein plus zeaxanthin, chlorophyll-*a*, and carotenes (β- plus or minus α-carotene), although variations of the position of the chlorophylls relative to lutein and violaxanthin were also observed. (See Figure 1.)

These observations point not only to important precautions necessary in the use of siliceous adsorbents but also to limitations of the selectivity of these adsorbents. As on cellulose and sugar (*3, 33*), the separations on siliceous adsorbents are most effective with substances that differ in polarity, such as neoxanthin, $C_{40}H_{56}O_4$ (3 —OH plus 1 epoxide); violaxanthin, $C_{40}H_{56}O_4$ (2 —OH plus 2 epoxides); and lutein, $C_{40}H_{56}O_2$ (2 —OH). Siliceous adsorbents are not effective with substances that differ little in polarity but which do differ in the arrangement of the double bonds, as do lutein $C_{40}H_{56}O_2$ (2 —OH, 11 double bonds, 10 conjugated) and zeaxanthin $C_{40}H_{56}O_2$ (2 —OH, 11 double bonds, 11 conjugated). Adsorbents, such as magnesia, lime, and alumina, which distinguish among the unsaturation of the carotenoids (*10–13, 33*), are known to alter the chlorophylls (*3, 6*).

Alteration of the Pigments. The separation of the pigments, the number of the pigment zones, and the properties of the eluted pigments often varied enormously. The most striking alteration of the carotenoid pigments occurred when the wash liquid was permitted to evaporate from the siliceous adsorbents before elution of the adsorbed materials. This effect varied greatly with the adsorbent as summarized in Table I.

The isomerization of neoxanthin and violaxanthin on siliceous adsorbents is analogous to the isomerization of these pigments with acid. As this acid-induced isomerization is known to take place with very weak acids, such as acetic acid in ethanol (*30*), one might suspect that the isomerization on the siliceous adsorbents is stimulated by acidity of the silicates themselves. There are, however, several facts contrary to this view. Virtually all the siliceous adsorbents tested in this study provided neutral liquid phases when made into a slurry with distilled water. Adsorption of the pigments on most of the silicates without subsequent evaporation of the solvent did not produce the isomerization; hence acid, if present, was not effective in the presence of the neutral and inert organic liquids. The weakly basic dimethylaniline, added to the wash liquids before and during the chromatography, did not prevent isomerization of the epoxy xanthophylls when the more volatile solvents were evaporated. By contrast, evaporation of the wash liquid in the presence of ammonia vapor did prevent the isomerization. Even though the epoxide isomerizations with acids and with silicates yielded the same isomerization products, it is possible that the acids and the silicates do not react with the epoxides in exactly the same way.

Table I. Isomerization of Neoxanthin and Violaxanthin Adsorbed on Various Siliceous Adsorbents, "Dried" in Moist Nitrogen, and Eluted with Ethanol

Adsorbent	Neoxanthin	Violaxanthin
Silica Gel G	Isomerized	Isomerized
Silicar	Isomerized	Isomerized
Chromagram sheet	Slightly isomerized	Isomerized
Chromatography media, Type S	Unaltered	Unaltered
Celite 545	Unaltered	Unaltered
Celite 545 acid-washed	Partly isomerized	Partly isomerized
Celite analytical filter-aid	Isomerized	Isomerized
Kieselguhr G	Unaltered	Partly isomerized
Talc	Unaltered	Unaltered
Florisil	Isomerized	Isomerized
Micro-Cel C	Mostly isomerized	Mostly isomerized
Micro-Cel E	Partly isomerized	Partly isomerized

No attempt has been made to discover how the siliceous adsorbents alter the chlorophylls. These pigments are so labile and are altered in so many ways that one might expect a variety of products (*7, 34*) depending upon the adsorption conditions (*19, 20, 35*).

Natural Pigments of Leaves. The observations reported here confirm the earlier conclusions that leaves contain chlorophylls-*a* and -*b*, neoxanthin, violaxanthin, lutein, and carotenes as the principal pigments. Because the siliceous adsorbents alter both the chlorophylls (*19, 20*) and the carotenoids, isolation of the alteration products cannot be accepted as support for the view that these modified pigments are normal constituents of leaves (*35*). Such conclusions should be based upon the isolation of the pigments under conditions and with adsorbents that are unlikely to contribute to the alteration.

Owing to their availability, the chloroplast pigments have frequently been employed to demonstrate separations by various chromatographic methods (*18, 25*). Numerous observations have revealed, however, that the pigments may be converted to similar colored substances by various reactions in the plant, during their extraction, during their storage and handling, upon heating the solutions, and upon evaporation of the solvent (*3, 14, 34*). Now this lability of the pigments upon adsorption demonstrates, afresh, the care that must be

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exercised in isolating the natural pigments free of their alteration products.

Hazards in the Use of Siliceous Adsorbents. The experiments reported herein show that several of the chloroplast pigments are altered by siliceous adsorbents, the degree of alteration varying with the adsorbent, the wash liquid, and the treatment of the pigment plus the adsorbent. In the light of this experience, siliceous adsorbents should not be relied upon as inert chromatographic media.

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Coupled Ligand Chromatography, Applications to Trace Element Collection and Characterization

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A new general approach to the preparation of chelating resins is demonstrated whereby ligands (e.g., dithizone, oxine) are diazo-coupled to a modified carboxymethylcellulose matrix. Dithizone-coupled powdered cellulose (DC) has been used to effect the quantitative recovery of trace elements from known mixtures. An HCl gradient-elution DC column technique is described for the preconcentration, partial separation, and recovery of trace elements from sea water. The use of sheet-form DC is demonstrated as a medium for spot tests and for precipitation chromatography.

THE MEANS DESCRIBED for collecting trace elements from natural waters include ion exchange chromatography (*1*), partition ion exchange chromatography (*2*), and chelating resin chromatography (*3*, *4*) to name a few. Where selective isolation of ions is required, the application of chelating resins as reviewed by Schmuckler (*5*) appears to be the method of choice. However, there is no simple general method reported to date for the preparation of such resins. We have diazo-coupled several ligands such as dithizone and oxine to benzidine–carboxymethylcullulose which was prepared by the carbodiimide reaction of Sheehan and Hess (*6*). The products formed are thus simply prepared from readily available reactants and the generality of the diazo coupling reaction is such that one may in principle attach any chelating function to the matrix in this way. To date we have prepared ligand celluloses in powder form from dithizone, oxine, cupferron, quinalizarin, 2-thenoyltrifluoroacetone, phenylarsonic acid and *p*-dimethylamino-benzilidene rhodanine. We have used the dithizone cellulose powder to recover trace elements from known mixtures and from sea water because its fine particle size and insolubility make it particularly suitable for this purpose. We chose to demonstrate the use of the same material in sheet form as a medium for spot tests and precipitation chromatography (*7*).

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EXPERIMENTAL

Reagents and Materials. All reagents used were of reagent grade except benzidine "suitable for detection of blood" supplied by Matheson, Coleman and Bell. The matrix used was Cellex CM carboxymethylcellulose (CMC) supplied as the sodium form in 100–200 mesh particle size at 0.72 meq/gram carboxymethyl (CM) capacity by BIO-RAD Laboratories, Richmond, Calif. *N,N'*-dicyclohexylcarbodiimide (NDCC) was supplied by Calbiochem, Los Angeles, Calif.

Diazonium Cellulose. The CMC was converted to the hydrogen form with 4*N* HCl, washed until the washes were neutral and stirred for 1–2 days in a 10% (w/v) aqueous methanol solution which was excess in NDCC and benzidine. It was then washed free of excess reagents with successive washes of methanol, toluene, methanol, and water and diazotized by the direct method. The bright yellow product was washed free of residual nitrite with 10% (w/v) aqueous sulfamic acid, then water. It may be stored damp in a dark freezer for at least a week without loss of activity.

Coupling. A 2–5% (w/v) solution excess in potassium dithizonate at pH (11–12) was stirred with fresh diazonium cellulose overnight at 10°–12° C. The maroon product was washed free of base and treated with iron-free 2*M* HCl to yield the green active dithizonic acid form. This was washed to neutrality with water distilled twice from glass and stored dry away from light and air which slowly deactivated it.

The other ligands noted above coupled readily in 10% aqueous methanol to which a few drops of pyridine were added. The red or purple products are not apparently affected by light or air.

Material Properties. Dithizone cellulose (DC) in contact with heavy metal solutions assumes a characteristic and diagnostically useful color. This suits it for identifying spot tests. Sheet DC made from Whatman CM-81 CMC paper

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