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The Isolation and Spectral Absorption Properties of Protochlorophyll from Etiolated Barley Seedlings

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Many plants, when grown in continuous darkness, produce leaves which are yellow and are without chlorophyll. However, these etiolated leaves contain a small quantity of a green pigment known as protochlorophyll. Illumination of such leaves causes the protochlorophyll to disappear and chlorophyll to appear. It has been assumed by some[1] that the protochlorophyll is transformed into chlorophyll, but adequate evidence for this transformation has not yet been put forward.

One way to test this assumption is to follow quantitatively the transformation by spectrometric methods. In order to use these methods, it is necessary to have absolute values of the absorption coefficients for both chlorophyll and protochlorophyll. For chlorophyll, absolute values are available,[2] but for protochlorophyll only relative values have been published.[3,4] Inasmuch as we intend to follow quantitatively the transformation of protochlorophyll to chlorophyll by the use of spectrophotometric methods, we have determined the absolute values of the absorption coefficients of protochlorophyll isolated from etiolated barley leaves.

Experimental

In these experiments several preparations of protochlorophyll were made. Since the method of preparation was improved in each successive experiment, only the procedure followed for the last experiment will be described.

Barley Seedlings.—Barley seedlings were grown in a darkroom, in flats containing soil, at a temperature of about 18°. Each flat (14 × 19 in.) was sown with 180 g. of seed. The leaves were harvested in dim green light nine days after sowing the seed. From the fourteen flats of seed sown, a yield of 5.115 kg. of leaves was obtained.

Extraction.—The leaves were divided into 300-g. portions and each portion was immersed in water at 90° for five minutes. This operation was carried out in dim green light so as to avoid transformation of the protochlorophyll to chlorophyll in the living leaves. After the leaves had been killed by the hot-water treatment, this transformation no longer took place in the light so that all subsequent operations could be carried out in the laboratory in ordinary light. The protochlorophyll could have been extracted from the living leaves just as well as from the killed leaves, but the killing of the leaves made it possible to press out more than half the water of the leaves before extracting them with acetone. Thus a considerable saving in acetone could be effected.

The killed leaves were removed from the water and were subjected to a pressure of about 35 kg./sq. cm. in order to free them from as much water as possible. The pressed leaves, 2.398 kg., were cut into short lengths in a chopping bowl. The chopped leaves were extracted in 80-g. lots with 400-ml. portions of acetone in a Waring Blendor. The combined acetone extracts were stored overnight in a cold room.

Isolation of Protochlorophyll.—The acetone extract was filtered and the pigment transferred to ether in the following manner: To each liter of acetone extract was added 750 ml. of ether. In order to wash out the acetone, this solution was run in a stream of drops through two liters of water contained in a large separatory funnel. Attempts to wash the ether more thoroughly resulted in undesirable emulsions. The several lots of ether obtained by the transfers just described were combined and were filtered through a layer of powdered cane sugar, 25.4 cm. in diameter and 7.6 cm. deep. This filtration removed something which caused decomposition of the protochlorophyll when it was subsequently transferred to petroleum ether. The yellow ether filtrate containing the protochlorophyll was concentrated by evaporation of ether at reduced pressure. Petroleum ether was added and the solution again concentrated by evaporation of solvent at reduced pressure. To reduce the ether content still further, petroleum ether was again added to the concentrate and part of the solvent removed by evaporation at reduced pressure. The petroleum ether concentrate, about one liter, was diluted with three liters of petroleum ether and the solution passed over powdered sugar in order to adsorb the protochlorophyll. One column of sugar 21 cm. in diameter and 20 cm. deep, and two columns 3.5 cm. in diameter and 30 cm. deep were used to adsorb the protochlorophyll. In each column, the protochlorophyll was retained by the sugar in a pale, blue-green zone extending two-thirds of the way down the column from the top. The adsorbed protochlorophyll was washed with petroleum ether to remove fats and yellow pigments and then was eluted with about nine liters of ether.

The protochlorophyll was purified by repeated adsorptions on sugar and by precipitation as follows[5]: The protochlorophyll was transferred from the ether eluate to petroleum ether by the evaporation procedure already described and was adsorbed from petroleum ether in two successive adsorptions. Each time the protochlorophyll zone was washed thoroughly with petroleum ether in order to remove fats and yellow pigments. The protochlorophyll was then adsorbed from benzene and was washed with benzene. This removed most of the protopheophytin and yellow pigment. A readsorption from benzene and a washing with benzene containing 10% ether removed yellow pigment and the remainder of the protopheophytin which accompanied the protochlorophyll. Again the protochlorophyll was adsorbed from benzene and was washed with benzene containing 20% ether. This treatment moved the protochlorophyll band down the column and freed it from the more strongly adsorbed contaminants. Finally, by adsorption from benzene, the protochlorophyll was collected on a sugar column in a small zone, 4 cm. in diameter and 1.5 cm. deep. The protochlorophyll band was washed with petroleum ether and then was eluted with ether.

The washing of the column with petroleum ether was for the purpose of removing benzene so that the final ether eluate could be used for making reliable absorption measurements in the ultraviolet. Unfortunately, all of the benzene was not removed and the absorption measurements in

(1) For discussion see Inman, Rothemund and Kettering, "Biological Effects of Radiation," B. M. Duggar, Editor, McGraw-Hill Book Co., Inc., New York, N. Y., 1936, p. 1093.

(2) Zscheile and Comar, *Bot. Gaz.*, **102**, 463 (1941).

(3) H. Rudolph, *Planta*, **21**, 104 (1933–1934).

(4) Seybold and Egle, *ibid.*, **29**, 119 (1938–1939).

(5) After each adsorption the protochlorophyll zone was separated mechanically, the pigment was eluted from the sugar with ether and was transferred to the solvent required for the next adsorption by the evaporation procedure already described.

the ultraviolet portion of the spectrum could not be made on this ether eluate.

Of the final ether eluate, 15 ml. was taken for spectral absorption measurements and magnesium determination, and the remaining 67 ml. for precipitation and further purification of the protochlorophyll. The pigment contained in the 67 ml. was transferred to 25 ml. of benzene by the evaporation procedure and was precipitated by the addition of 50 ml. of petroleum ether, b.p. <50°. The mixture was set in an ice-bath and the pigment allowed to settle. The precipitate was collected by centrifugation, was washed with low-boiling petroleum ether, and was dried *in vacuo*. The yield was 10.2 mg. Two more precipitations were carried out in analogous fashion, the yields being 7.5 and 7.2 mg., respectively. The protochlorophyll obtained by the final precipitation contained 2.73% magnesium which agrees with the theoretical percentage calculated from the formula proposed by Fischer and Oestreicher.[6]

Seven days were required for the operations carried out between the harvesting of the leaves and the final adsorption of the protochlorophyll. All manipulations after killing the leaves were performed under the ordinary conditions of light intensity and temperature of the laboratory (about 20 to 22°). Whenever it was necessary to store solutions of the pigment for more than a few hours, the solutions were placed in a refrigerator in the dark. Protochlorophyll appears to be moderately stable in ether solution, relatively less so in acetone and methanol solutions. It is relatively unstable in petroleum ether and should not be left in contact with this solvent longer than is required for whatever manipulations are necessary.

Absorption Spectrum Measurements.—The absorption measurements were made by means of the Beckman quartz spectrophotometer Model DU. The proper functioning of the instrument was assured by checking the correctness of the wave length scale by means of the spectral lines from the mercury arc and the optical density scale by measuring the absorption of a standard solution (Weigert's solution).[7,8]

The approximate widths of the spectral bands used for measurement in different regions of the spectrum were

Wave length in mμ	700	600	500	400	300
Band width in mμ	1.8	1.4	0.7	0.6	0.9

Specific absorption coefficients were calculated by the usual formula

$$\alpha = D/lc$$

where α is the specific absorption coefficient, l is the length of light path through the solution in centimeters, c is the concentration of the pigment in grams per liter, and D is the optical density of the solution compared to pure solvent.

The range of optical density values (D) measured on ether solutions of protochlorophyll in different portions of the spectrum were

Wave length range, mμ	Density range, D
700–635	0–0.2
635–605	0.2–0.73
605–525	0.1–0.2
525–460	0.025–0.1
450–320	0.2–0.7

The concentration of the protochlorophyll in solution was determined by either one of two methods: either from the weight of precipitated protochlorophyll dissolved in a known volume of solvent, or from the weight of protochlorophyll derived from the magnesium content of the solution on the assumption that protochlorophyll contains 2.73% magnesium. The magnesium content of the solution was estimated colorimetrically by the Titan-yellow method as described by Smith.[9]

Specific absorption coefficients of protochlorophyll dissolved in ether, acetone and methanol were determined.

Results

The specific absorption coefficients of protochlorophyll in ether solution are shown in Fig. 1. The absorption values for the curve (open circles) were obtained from measurements on a solution prepared from a known weight of the precipitated and dried protochlorophyll dissolved in a known volume of ether. The values plotted by solid circles were derived from measurements on a solution of protochlorophyll obtained by the final elution of the pigment from the adsorption column with ether. In each case, the concentration of the pigment was calculated from the magnesium content of the solution.

In Fig. 2 the absorption coefficients of protochlorophyll dissolved in ether, acetone and methanol are given in graphical form. The concentrations of the solutions were based on weighed quantities of the precipitated and dried pigment. It should be pointed out that for the data presented in Figs. 1 and 2, the concentrations of the solutions of the precipitated and dried pigment would be the same whether calculated from the weight of pigment added or from the magnesium content of the solutions determined analytically. This arises from the fact that the dried pigment used in the preparation of these solutions contained 2.73% magnesium, the value used for the conversion of the magnesium content of solutions to weight of protochlorophyll.

For convenience, the wave lengths and corresponding absorption coefficients at the maxima of the principal absorption bands of protochlorophyll in the visible portion of the spectrum are summarized in Table I. The values in the table for unprecipitated protochlorophyll dissolved in ether are the ones plotted in Fig. 1 with solid circles; the values for precipitated and dried protochlorophyll dissolved in ether, acetone and methanol are the ones plotted in Fig. 2.

TABLE I

WAVE LENGTHS AND SPECIFIC ABSORPTION COEFFICIENTS FOR THE ABSORPTION MAXIMA OF PROTOCHLOROPHYLL IN THE VISIBLE PORTION OF THE SPECTRUM[a]

Unprecipitated Ether		Precipitated and dried Ether		Acetone		Methanol	
λmax., mμ	α	λmax., mμ	α	λmax., mμ	α	λmax., mμ	α
623	39.9	623	36.9	623	34.9	629	27.2
571	14.9	571	14.0	571	13.5	578	10.5
535	7.2	535	6.8	535	6.9	...	...
432	325.5	432	305.9	432	270.5	434	177.3

[a] The values presented in this table were obtained from measurements on individual solutions and are not average values. Portions of the same batch of precipitated and dried protochlorophyll were used for preparation of the ether, acetone and methanol solutions on which these measurements were made.

(6) Fischer and Oestreicher, *Z. physiol. Chem.*, **262**, 243 (1939–1940).

(7) Smith, THIS JOURNAL, **58**, 247 (1936).

(8) Zscheile, Comar and Mackinney, *Plant Physiol.*, **17**, 666 (1942)

(9) Smith, THIS JOURNAL, **69**, 1942 (1947).

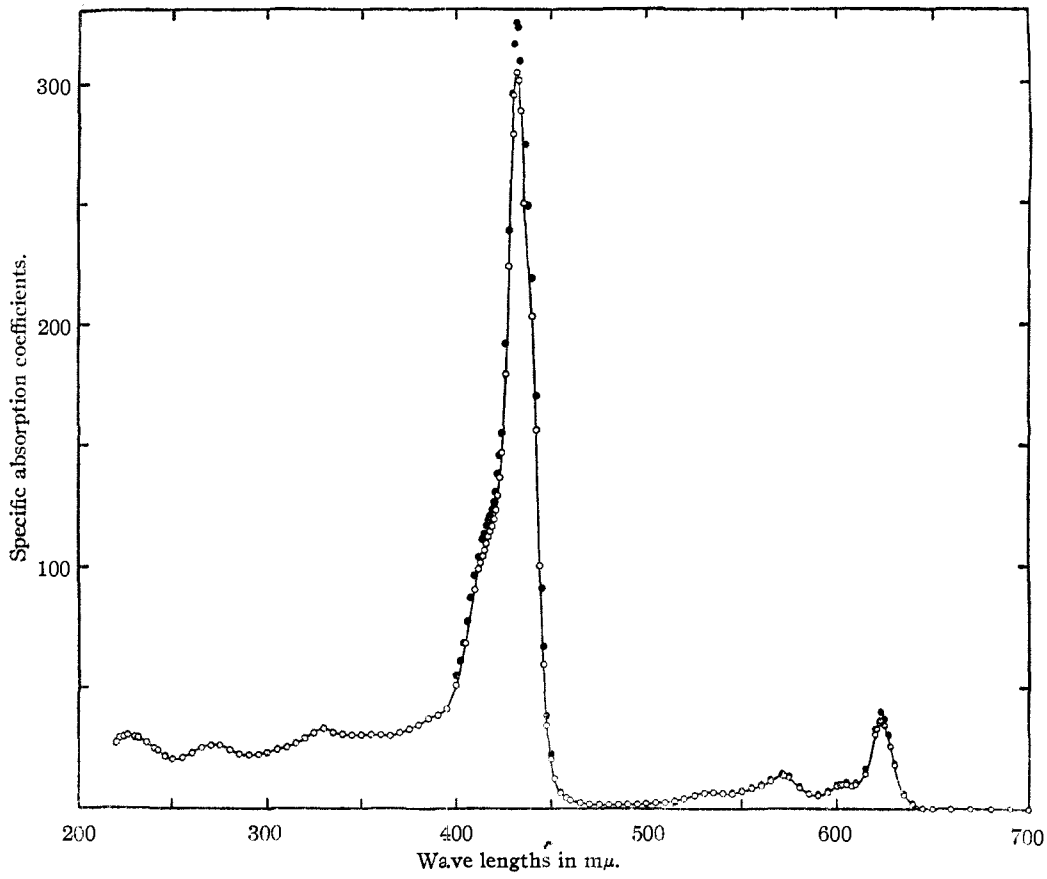

Fig. 1.—The absorption spectrum of protochlorophyll in ether solution: comparison of the absorption spectrum of unprecipitated protochlorophyll (●) with the precipitated and dried protochlorophyll (○).

Individual measurements on three different ether solutions of precipitated and dried protochlorophyll from the best lot of pigment obtained thus far, the preparation of which has already been described, are given in Table II, preparation number IV. The average values for these determinations are: 623 mμ, 36.9 ± 0.2, and 432 mμ, 300.8 ± 5.0. This comparison gives an estimate of the reproducibility of the spectroscopic measurements.

TABLE II

COMPARISON OF THE SPECIFIC ABSORPTION COEFFICIENTS OF THE TWO PRINCIPAL ABSORPTION MAXIMA OF PROTOCHLOROPHYLL FROM DIFFERENT LARGE-SCALE PREPARATIONS IN ETHER SOLUTION

Prepn. number	Treatment	Specific absorption coefficients at mμ 623	432
I	Unprecipitated	36.2	...
		38.8	306.4
II	Precipitated and dried	34.3	274.1
		34.0	274.8
III	Unprecipitated	34.2	270.4
	Precipitated and dried	34.9	276
IV	Unprecipitated	39.9	325.5
	Precipitated and dried	36.9	305.9
		37.5	...
		36.5	295.8
	Average	36.3 ± 0.4	291.1 ± 4.9

Three other preparations of protochlorophyll were made on the same scale previous to the one described in this paper. In these experiments as complete purification of the pigment was not attained as in this last preparation. However, many spectrometric measurements were made on ether solutions of the chromatographically separated pigment and specific absorption coefficients calculated from the magnesium content of these solutions. Also measurements were made with ether solutions of the precipitated pigment and absorption coefficients calculated on the basis of magnesium content. All the reliable values obtained for the maxima at 623 mμ and 432 mμ for all four preparations are given in Table II. The average value for ten determinations at wave length 623 mμ is 36.3 ± 0.4 and from seven determinations at wave length 432 mμ is 291.1 ± 4.9.

Some of the protochlorophyll isolated from the etiolated barley seedlings was converted to protopheophytin. The protopheophytin was dissolved in ether and its absorption coefficients measured. The absorption coefficients determined are not sufficiently accurate for publication at the present time. However, the wave lengths and relative in-

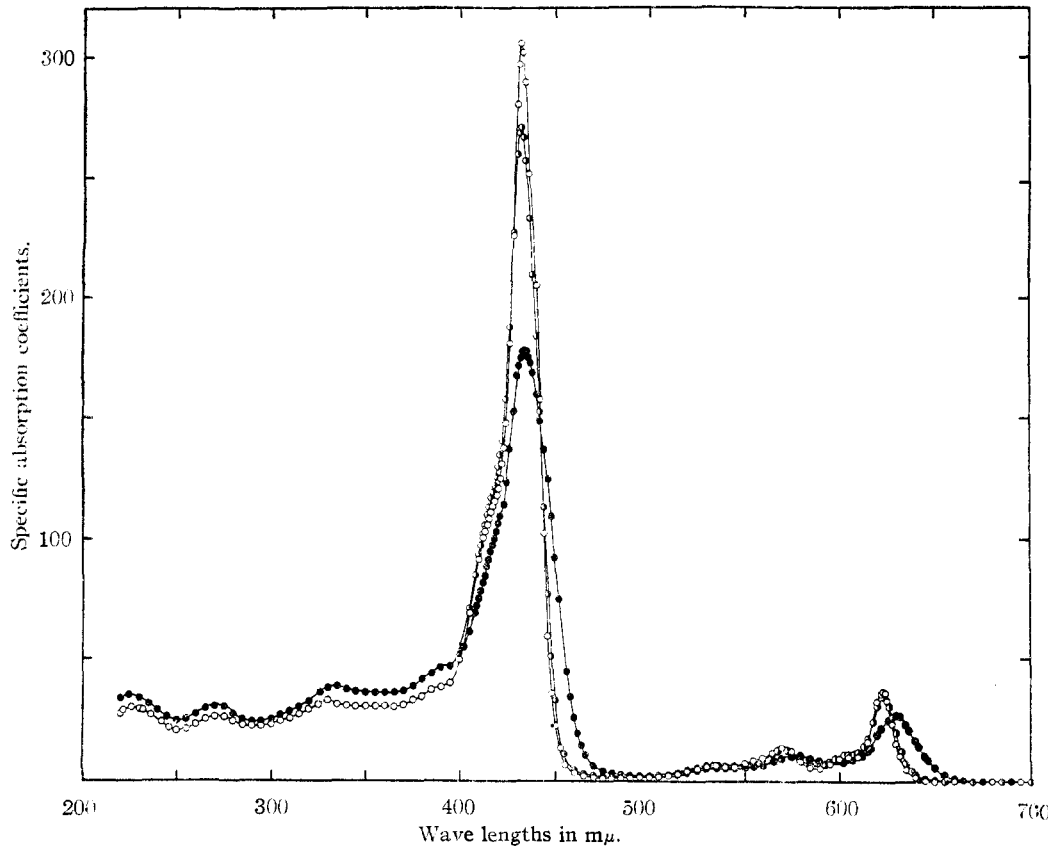

Fig. 2.—The absorption spectrum of precipitated and dried protochlorophyll dissolved in ether (○), acetone (◑), and methanol (●).

tensities of the absorption bands which we obtained will be compared with the values obtained by others. The methods used by the others gave only end-absorption beyond 460 mμ and did not detect the intense narrow band at wave length 417 mμ. (*cf.* Table III).

rophyll from pumpkin seed coats, dissolved in ether, determined spectrophotometrically by Rudolph,[3] *viz.*, 621 and 571 mμ, and the corresponding values obtained by us for protochlorophyll from etiolated barley seedlings, 623 and 571 mμ, agree very well. There is a marked similarity also

TABLE III

THE WAVE LENGTH AND RELATIVE INTENSITIES OF THE ABSORPTION BANDS OF PROTOPHEOPHYTIN DISSOLVED IN ETHER

	Wave lengths in mμ					Relative intensity				
Band number →	I	II	III	IV	V	V	III	II	IV	I
Etiolated barley leaves	638	585	565	524	417					
Pumpkin seed coats[4]	640–638	607–585	574–553	538–525	460–		III	II	IV	I
Synthetic[6]	–640	592.3	567.0	526.6	461–		III	II	IV	I

Discussion

The name protochlorophyll has been used indiscriminately to designate the green pigment contained either in etiolated leaves or in the seed coats of pumpkin seeds.[10] Usually it has been assumed that the protochlorophyll in all etiolated seedlings and in pumpkin seed coats is identical,[11] but sufficiently careful comparison has not been made to establish this. However, the positions of the two long wave-length absorption maxima of protochlorophyll in the positions and the relative intensities of the absorption bands of protopheophytin obtained from three different sources, as the comparison in Table III shows.

Seybold and Egle[4] have reported the presence of protochlorophylls *a* and *b* from the seed coats of pumpkin seeds. The presence of the two forms has been called in question by Fischer and Oestreicher,[6] who could not establish the presence of a *b* component. Our experiments have also given no indication of the presence of two protochlorophyll components in etiolated barley leaves. If a distinction should be made between two components, the positions of the absorption bands of the

(10) Stoll and Wiedemann, "Fortschritte der Chemie organische Naturstoffe," Vol. I, Julius Springer, Wien, 1938, pp. 159–254.

(11) Rothemund, "Medical Physics," Year Book Publishers, Chicago, Ill., 1944, pp. 154–180.

protochlorophyll described in this paper agree more nearly with those of the protochlorophyll *a* than with those of the protochlorophyll *b* reported by Seybold and Egle.

In the action spectrum for chlorophyll formation, Frank[12] observed a narrow, intense band in the blue region of the spectrum. This band had no counterpart in the published absorption spectra of protochlorophyll, the supposed precursor of chlorophyll. The absorption spectrum reported in this paper shows that protochlorophyll possesses such a band. This observation strengthens the assumption that protochlorophyll is the light-absorbing agent active in the photochemical formation of chlorophyll.

Some doubt exists on the advisability of determining the absorption coefficients of chlorophylls *a* and *b* after drying.[2] On account of this, the coefficients for protochlorophyll were measured both before and after precipitation and drying. The values obtained for the unprecipitated pigment are on the average somewhat higher than for the precipitated and dried pigment—about 5% higher at the absorption maxima—but the difference is not great (*cf.* Table II).

The use of the precipitated and dried pigment for the measurement of absorption spectra has the advantage that spectra can be obtained in different solvents on the same batch of material without contaminating the solutions with foreign solvents. In plant physiological work, however, in which extracts of plant material will be used without isolation and purification of protochlorophyll, the use of the absorption values for the unprecipitated pigment may yield slightly more reliable analytical results than the use of the values for the precipitated and dried pigment.

Absorption values have been obtained for the precipitated and dried protochlorophyll in ether, acetone, and methanol. Because of the larger number of determinations made for ether solutions, the values obtained for this solvent have a higher degree of certainty than those obtained for solutions in acetone and methanol. In the red region of the spectrum the absorption maximum of protochlorophyll when dissolved in ether lies 6 mμ to shorter wave lengths than it does when protochlorophyll is dissolved in methanol. This difference is about midway of the differences obtained for solutions of chlorophylls *a* and *b* in these solvents, *viz.*, 4 and 8.5 mμ, respectively.[13]

Probably the most useful portion of the absorption curve of protochlorophyll for plant physiological work is the absorption band in the red region of the spectrum. This band can be observed in crude extracts of etiolated leaves and its intensity can be measured uninfluenced by the absorption of the yellow pigments. Because of the disparity between the absorption coefficients of protochlorophyll and of chlorophylls *a* and *b* at the absorption maxima of the two chlorophylls in the red region of the spectrum, small quantities of these chlorophylls could be detected if present in extracts of etiolated leaves containing protochlorophyll. The specific absorption coefficients of the three pigments in ether solution are as follows: protochlorophyll at 660 mμ, 0.2, and at 642.5 mμ, 1.4; for chlorophyll *a* at 660 mμ, 102.1[2]; and for chlorophyll *b* at 642.5 mμ, 56.8.[2]

Summary

Protochlorophyll has been isolated in a high degree of purity from etiolated barley seedlings. Its absorption in the visible portion of the spectrum has been measured for ether, acetone and methanol solutions. The wave lengths and specific absorption coefficients of the two principal absorption maxima for solutions of the precipitated and dried pigment in each of the three solvents are as follows: ether, 623 mμ, 36.9 sq. cm./mg. and 432 mμ, 300.8; acetone, 623 mμ, 34.9 and 432 mμ, 270.5; and methanol, 629 mμ, 27.2 and 434 mμ, 177.3. The corresponding values for the unprecipitated and undried pigment dissolved in ether are 623 mμ, 39.9 and 432 mμ, 325.5.

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(12) Frank, *J. Gen. Physiol.*, **29**, 157–179 (1946).

(13) Harris and Zscheile, *Botan. Gaz.*, **104**, 515 (1943).