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OPTICAL PROPERTIES OF THE PROTOCHLOROPHYLL PIGMENTS

I. ISOLATION, CHARACTERIZATION, AND INFRARED SPECTRA

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SUMMARY

A method for the large-scale preparation of the protochlorophyll pigments from pumpkin seed coats is described. Use is made of polyethylene and sugar chromatographies, currently employed for the preparation of the chlorophyll pigments. The procedure permits the isolation of pure solid samples, in relatively large amounts (20–50 mg from 2 kg of seeds) and with good yields (higher than 40 %).

In confirmation of other reports, two protochlorophyll pigments are obtained. One is the true protochlorophyll *a* and the other one is 4-vinyl protochlorophyll *a*, also called the "bacterial" protochlorophyll or Mg-2,4-divinylpheoporphyrin a_5, esterified with phytol. The pigments were characterized as regards their chromatographic sequence compared to the chlorophylls, their purity, the presence of the phytol, and by their visible and infrared absorption spectra.

Protochlorophyll *a* and 4-vinyl protochlorophyll *a* show clear differences in their visible absorption spectra. The small differences observed in their infrared spectra can be ascribed to the presence of the additional vinyl substituent in 4-vinyl protochlorophyll *a*. The infrared spectra present several distinguishing features. The absence of the skeletal (C=C) vibrations indicates a much higher symmetry of the macrocycle in these molecules compared to the chlorophylls. In carbon tetrachloride, the "aggregated" ketone carbonyl band was observed for protochlorophyll *a* and 4-vinyl protochlorophyll *a* at 1668 cm^{-1}, indicating that this molecule aggregates in a similar way to that of the chlorophyll pigments.

INTRODUCTION

The importance of the protochlorophyll pigments lies in the fact that they are known to be precursors of chlorophyll *a* in the photosynthesis process[1–9]. The properties of the chlorophyll pigments have been studied in greater detail, but our knowledge of both classes of pigments is far from complete. It is clear that an understanding

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of the spectral properties of these porphyrin pigments in the plants, and more precisely in the lipoprotein complexes, requires a knowledge of their aggregation properties[11,12]. Essentially, up to the present time, visible absorption spectroscopy[1–12], chemical reactions, and a few infrared data[6] have been used for their characterization. Unfortunately, a systematic physicochemical study of the protochlorophyll-like pigments has been hampered by the lack of any experimental procedure for a large-scale preparation of these pigments. It is significant to note that for this reason, protochlorophyll has been called an "elusive substance"[13].

A rich source for the preparation of the protochlorophyll pigments is the coats from the Cucurbitaceae (pumpkin) seeds. No transformation of the isolated pigments to chlorophyll *a* in the presence of light has ever been detected. FISCHER AND RÜDIGER[3] have shown that protochlorophyll is present in etiolated leaves in the nonphytolated form (protochlorophyllide), while it is mostly in the phytol ester form (protochlorophyll) in pumpkin seed coats. However, it appears from the work of SIRONVAL, MICHEL-WOLWERTZ AND MADSEN[4] that both forms are in fact present in etiolated leaves, in relative amounts depending upon the age of the seedling. In view of these considerations, we have attempted to isolate, purify, and characterize the green pigments from pumpkin seed coats. Similar attempts have been made by JONES[9], but he was not able to achieve a complete purification of the pigments with good yield. The method described below allows an easy preparation of pure protochlorophyll pigments in relatively large amounts (25–50 mg). It will help in initiating further physicochemical characterization of these pigments.

The presence of a protochlorophyll-like pigment, showing small but clear absorption spectrum differences compared to true protocholorophyll *a*, has been detected in the seed coats of Cucurbitaceae[1,7,8]. It was suggested to be the phytol ester of Mg-2,4-divinylpheoporphyrin a_5, found in bacteria[8]; we will call it here 4-vinyl protochlorophyll *a*. INADA AND SHIBATA[14] have examined the seed coats and ether extracts from various types of pumpkin seeds by visible absorption spectroscopy. They also observed differences in the absorption spectrum of the protochlorophyll present in certain types of seeds compared to the protochlorophyllide from etiolated leaves. They suggested differences between the side chains for these two pigments but did not investigate the question further.

In the choice of the methods of extraction and purification of the pigments, the following published observations were taken into account. Polyethylene chromatography has been used because of its known efficiency in the separation of the plant pigments[5,6], especially the separation of the yellow carotenoid pigments from the green porphyrin pigments[15]. Sugar chromatography[8,13] achieves a successful separation of most of the green pigments by an appropriate choice of the ratio of polar solvent (*n*-propanol) to hydrocarbon (light petroleum, or 2,2,4-trimethylpentane). Mannitol[16] and cellulose[17] have been reported to give very good separation of the plant pigments. Small-scale preparations were tried using these adsorbents; they did not give additional improvement compared to sugar, so that finally commercial powdered sugar was chosen for the large-scale preparation. We have avoided the use of siliceous adsorbants for this purpose because they produce alteration of the chloroplast pigments[18]. Column chromatography is the most useful method for large-scale preparation, while thin-layer chromatography appears to be the most convenient for identification, tests of purity, or small-scale purification.

MATERIALS AND METHODS

General considerations

As often as possible the experiments were carried out in yellow light or in dim light, and the solutions were protected from light by aluminum foil to avoid photodecomposition of the pigments, although no particular instability in this regard has been observed during the manipulations. For example, an ether solution about $2 \cdot 10^{-5}$ M in protochlorophyll did not show any measurable bleaching after 2 h under room light.

The solvents used for the extractions of the plant pigments were reagent grade without further purification.

The chromatographic procedure of ANDERSON AND CALVIN[15] was used throughout this work for the separation of the pigments from the plant extracts, *i.e.*, polyethylene chromatography (Dow Chemical Co., Midland, Mich.; melt index 3.5) removing the yellow pigments and sugar chromatography (C and H powdered sugar) for the separation of the green pigments.

The purity test of the samples can be made very conveniently and quickly on thin layers of sugar[19], mannitol[16], or cellulose[17] (Eastman Chromagram cellulose plate, No. 6064). 2,2,4-Trimethylpentane ("isooctane") containing various amounts of added *n*-propanol (0.25–2 %, v/v) was used as developing solvent; light petroleum *plus* *n*-propanol gave very similar results. Developers containing methanol or ethanol[17] were avoided because they could produce allomerization of the pigments. The chromatographic adsorption sequence of the protochlorophyll pigments compared to the chlorophyll pigments was determined by performing co-chromatographies of two or three pigments at a time.

The presence of the phytol in the different pigments obtained was checked by silica-gel thin-layer chromatography[20] (Eastman Chromagram silica gel plate, No. K301R) after hydrolysis with methanol–KOH (ref. 21).

The absorption spectra in the ultraviolet and visible regions were recorded using a Cary 14R spectrophotometer; the solvent was anhydrous ether (Baker analyzed reagent, peroxide content 0.00001 %).

The infrared spectra were measured with a Beckman IR 7 spectrophotometer under experimental conditions similar to those used by KATZ *et al.*[22] for the chlorophyll pigments. The instrument was equipped with a condensor accessory for the use of a microcell (0.1-mm pathlength). The samples consisted of about 0.5–1 mg of the pigments dissolved in 20 μl of carbon tetrachloride (Matheson, Coleman, and Bell, spectroquality reagent) or tetrahydrofuran (Mallinckrodt, stabilized, analytical reagent; peroxide content max. 0.015 %). The solvent and the sample spectra were recorded separately, without compensation in the reference beam.

Extraction and purification of protochlorophyll pigments from pumpkin seeds

We have essentially reproduced the observations of JONES[9] in the preparation process, but a number of interesting facts and important improvements have been made which we would like to describe here because we think that they will be very helpful for future work in this field.

In agreement with the observations of INADA AND SHIBATA[14], the content of the various protochlorophyll pigments depended on the variety of pumpkin seeds used.

The "Calabaza type" (available commercially from Ferry–Morse Seed Co., Mountain View, Calif.) had a very high protopheophytin content. The "Styriac type" (kindly provided as a small sample by Dr. J. Harrington and Mr. P. Koostra of the Department of Vegetable Crops, University of California, Davis, Calif.), on the contrary, showed both a high total green pigment content and high protochlorophyll content. Finally, a variety available from Belgium (T. Mercenier, Herbalist, Flobecq, Belgium) was found particularly valuable for a large-scale preparation for two reasons: the amount of protochlorophyll with respect to protopheophytin was high and the seeds were sold "hulled". This last variety has then been used in the preparation described below.

Extraction. Two kg of naked pumpkin seeds, in lots of 200–300 g, were immersed in about 1 l of water, washed twice with this volume of water, and then left in the water for a few minutes. Small portions of seeds were then removed from the water, slightly dried with adsorbent paper, and extracted with portions of acetone. The decoloration of the seeds was almost instantaneous. Each acetone fraction was reused several times for the extraction of further seed fractions until the effectiveness of the coat pigment extraction became low. The removal of the coat mentioned by FISCHER AND RÜDIGER[3] and rightly considered by JONES[9] as impractical for large quantities of seeds does not appear necessary for efficient extraction, provided one starts with naked seeds. Direct extraction with acetone–water (100:80, v/v) from the dry seeds was unsuccessful. Immersion in water probably causes a dilation of the coat membrane, allowing the acetone to penetrate and extract the pigments. JONES[9] made the extraction successfully with acetone–water (90:100, v/v) directly on the seeds. This apparent discrepancy with our observations could be due to the fact that the seeds used by JONES were not completely dry, since he was collecting them from mature fruits. Otherwise, it is likely that a very great volume of solvent was needed to achieve the extraction. Moreover, JONES broke the seeds in a Waring blender for their extraction, which resulted in an unfortunate introduction of oils and fats in the extract. This very undesirable consequence is greatly reduced with our procedure.

The extraction process was designed to obtain an acetone extract as concentrated as possible, which greatly facilitates the chromatographic procedure. The combined acetone extracts (2–3 l for 2 kg of seeds) were clarified by filtration. A spectrum was recorded for the determination of the total protochlorophyll content (using an extinction coefficient of 36.9 $l \cdot g^{-1} \cdot cm^{-1}$ at 622 nm (ref. 23)) and relative amounts of protochlorophyll and protopheophytin (from the absorbance at 622 and 565 nm). The acetone extract, containing about 100 mg of protochlorophyll, was stored overnight in a refrigerator.

Polyethylene chromatography. Water was then added to the acetone extract to the limit of solubility of the pigments, the amount of water necessary being determined by a test on a small portion of the extract. Under these conditions the blue-green solution with red fluorescence becomes dark green and slightly opalescent. This step produces good adsorption in a relatively concentrated band during the introduction on the polyethylene column (10 cm × 40 cm; previously washed with 1–2 l of acetone–water (60:100, v/v) under slightly reduced pressure). At the end of the introduction of the acetone extract, the green pigment zone extended one-half of the column length from the top, and the yellow pigment elution had started. The column was washed with acetone–water (70:100, v/v) to eliminate fats, oils, and yellow pigments until the

eluate became almost colorless. The acetone concentration was then increased to 80 %, resulting in a very slow movement of the principal green band toward the bottom of the column. A further increase of the acetone concentration to 90 % eluted the protochlorophyll pigments as a blue-green solution with red fluorescence with partial overlap of the following olive-green zone of the protopheophytin. The complete elution of protopheophytin can be achieved with pure acetone, giving an intensely "red" solution. Attempts to reach a better separation of the protochlorophyll and protopheophytin pigments by various adjustments of the acetone concentration between 80 and 90 % were unsuccessful. The acetone eluates were stored overnight in a refrigerator.

Transfer to a hydrocarbon solvent

It was observed that the protochlorophyll fraction (5 l) was only partially transferred to isooctane, leaving a green aqueous phase. Consequently, the transfer was first made to ether from the acetone–water solution, as done by other authors[3,9,23], the ether phase being washed with water, concentrated, and diluted with isooctane to reach an ether concentration of about 10 %. It is known that for the chlorophyll pigments, the presence of a minimum or 10–20 % of a polar solvent is necessary to avoid precipitation from a hydrocarbon solution[24]. The isooctane fraction was dried over anhydrous Na_2SO_4. The yield of the protochlorophyll recovered at that point of the preparation was about 50 %, the remaining quantities being lost on the polyethylene column and in the water phase during the transfer.

Sugar chromatography of the isooctane fraction

The isooctane solution was adsorbed on a sugar column (10 cm × 40 cm; 2 kg of powdered sugar) previously washed with 1–1.5 l of pure isooctane, and vacuum was applied to increase the flow rate. When all the solution had been added to the column, the protochlorophylls formed a zone 15–20 cm deep. The remaining protopheophytin and yellow pigments were completely eluted by washing with pure isooctane.

Under these conditions, the protochlorophyll band appeared clearly divided into two bands of equal importance (3–4 cm deep each), a blue-green zone, less adsorbed, and a green zone, 3–4 cm higher on the column. It is to be noted that the same two bands were observed in the preparation from each of the varieties of pumpkin seeds mentioned above. Nevertheless, it is known that the content of the two protochlorophyll pigments depends on the period of storage of the pumpkin[9]. During the washing with pure isooctane, these bands practically do not move. The washing was continued until the eluate became colorless and the protopheophytin band was no longer visible on the column: protopheophytin forms a pale grey-green zone difficult to detect on the sugar column, except under normal room light intensity.

The wash liquid was then changed to 0.25 % *n*-propanol–isooctane, which caused the pigments to move slowly, the separation between the two protochlorophyll bands increasing on the way down the column. The same solvent was used until the blue-green protochlorophyll *a* band was completely eluted. An increase of *n*-propanol concentration to 0.5 % then eluted the green 4-vinyl protochlorophyll *a* band. It is important to mention that a wash liquid constituted of 0.5 % propanol–isooctane, used immediately after the elimination of the protopheophytin and yellow pigments, was shown to produce a partial overlapping of the two protochlorophyll bands. JONES[9]

did not succeed in making a good separation of these bands (called F_1 and F_2 by him) with a satisfactory yield. We find that a careful adjustment of the *n*-propanol concentration to 0.25 % in volume allows a complete separation, giving the pure protochlorophyll *a* and 4-vinyl protochlorophyll *a* fractions. Identification of these two fractions will be considered in the RESULTS.

The two protochlorophyll eluates were separately concentrated by evaporation under reduced pressure at 40–50°, to about 100 ml, washed several times with water to remove the *n*-propanol and contaminants inevitably present in the sugar. If the pigments started to precipitate at the water interface, they were redissolved by the addition of a small amount of ether, a solvent easily eliminated in the next evaporation process. The two solutions were then further concentrated to 10–20 ml and allowed to stand overnight in a freezer (—15 to —20°). Protochlorophyll *a* did not appear to precipitate in appreciable amount at this temperature, while 4-vinyl protochlorophyll *a* was almost completely separated, leaving a clear green supernatant after centrifugation in a clinical centrifuge for 1–2 min. To precipitate protochlorophyll *a*, the solution was set in solid CO_2 for 2–4 h. Separation was achieved by centrifugation, leaving a green supernatant further concentrated to 2–4 ml for the recovering of the remaining pigment. The precipitates were then dried under vacuum at room temperature for 2–3 days. Protochlorophyll *a* was obtained as a waxy solid weighing about 20 mg. It is not completely clear to us whether this waxy state is due to some residual fats or oils, or just to a lack of crystallization during the precipitation process as is also observed for other chlorophyll pigments. (See RESULTS, *Infrared spectra*, for a detailed discussion of this point.) 4-Vinyl protochlorophyll *a*, about 20 mg also, was a crystalline solid, easy to manipulate and obtained in a nice powdered state. The total yield in the protochlorophylls from the extraction to the attainment of the dry solid samples was about 40–50 %.

A test of the purity of the samples by thin-layer chromatography was made as described above. None of the two protochlorophyll pigments showed any trace of colored impurity.

The drastic drying conditions (100° under vacuum) used for the chlorophyll *a* pigments[13] have been tried in the case of the protochlorophyll pigments and have yielded degraded samples, showing identical absorption spectra compared to the native samples, but complete disappearance of the optical activity. Pyrolysis affecting the cyclopentenone ring presumably occurred during the drying at 100°.

Preparation of the protopheophytin pigments

The removal of the Mg^{2+} from the protochlorophyll pigments can be achieved by treatment of an ether solution of these pigments with 25 % HCl as for the chlorophyll pigments[9].

In our case, protopheophytin *a* was also obtained by a chromatographic procedure identical to the one used for the protochlorophylls, starting from the Calabaza type of pumpkin seeds in view of its high content in this pigment (see above). During the sugar chromatography, protopheophytin *a* appeared as a single band on the column, and was eluted by pure isooctane giving a "red" solution. This solution was concentrated to 10–20 ml by evaporation, and the pigment started to precipitate at room temperature. The precipitation was almost complete after a few hours at 4–5°, and the solid protopheophytin in crystalline form was easily collected by centrifugation

during 2–3 min in a clinical centrifuge, leaving a very pale green supernatant. The precipitate was washed several times with isooctane and collected each time by centrifugation after cooling for a few hours at 4–5°. Finally, the protopheophytin sample was dried under vacuum at room temperature. Drying at 100° resulted, as for protochlorophyll, in a degradation of the product (presumably pyrolysis) with no change in the absorption spectra but with complete disappearance of the optical activity. From 800 g of seeds (Calabaza type) 10 mg of protopheophytin was obtained.

RESULTS

Chromatographic behavior

The chromatographic sequences of the protochlorophyll pigments compared to the chlorophyll pigments are given in Table I, as determined by thin-layer chromatography on sugar, mannitol, and cellulose. The mobilities of protochlorophyll *a* and 4-vinyl protochlorophyll *a* indicate that they are fully esterified. In fact, the phytol was identified in protochlorophyll *a*, 4-vinyl protochlorophyll *a* and protopheophytin *a* by the chromatographic procedure mentioned in the experimental section. The

TABLE I

CHROMATOGRAPHIC SEQUENCES OF THE PROTOCHLOROPHYLL PIGMENTS COMPARED TO THE CHLOROPHYLLS

On mannitol and cellulose plates			*On sugar plate***
Pigments	*Color of zones*	R_F*	
Protopheophytin *a*	Grey-green	0.1	Protopheophytin *a*
Pyrochlorophyll *a*	Blue green	0.52	4-Vinyl proto-chlorophyll *a*
4-Vinyl protochlorophyll *a*	Yellow green	0.54	Pyrochlorophyll *a*
Protochlorophyll *a*	Green	0.6	Chlorophyll *a*
Chlorophyll *a* (and protochlorophyll *a* + pyrochlorophyll *a*)	Blue green	0.63	Chlorophyll *a'*
Chlorophyll *a'* (protochlorophyll *a* + chlorophyll *a*)	Pale blue green	0.65	Protochlorophyll *a*
Pheophytin *a*	Olive green	0.85	Pheophytin *a*
Pyropheophytin *a*	Olive green	0.9	Pyropheophytin *a*

* On mannitol plate. Developer: 0.5 % propanol–isooctane.

** Color of zones in the 2nd column, for the corresponding pigments; developer: 0.5 % propanol–isooctane.

chromatographic sequences are the same on mannitol and cellulose but slightly different on sugar. On the cellulose and sugar plates the spots have long tails, while on mannitol they are much better defined, as also observed by SMITH, BRIEDENBACH AND RUBENSTEIN[16], and R_F values can be determined. A very peculiar behavior was observed on the cellulose and mannitol plates for the spots corresponding to the co-chromatographed protochlorophyll *a plus* chlorophyll *a*, and also for protochlorophyll *a plus* pyrochlorophyll *a*. Both mixtures moved faster than the components and did not show any separation into two bands. Other co-chromatographies showed the normal separation in bands; for example, 4-vinyl protochlorophyll *a plus* chlorophyll *a* as well as 4-vinyl protochlorophyll *a plus* pyrochlorophyll *a* were separated readily.

TABLE II

SPECTRAL PROPERTIES OF THE PROTOCHLOROPHYLL PIGMENTS IN ETHER

Relative absorbances of the bands are given in parentheses; blue band taken as 100. sh stands for shoulder.

Compounds	*Absorption (nm)*							*Ref. No.*
Protochlorophyll *a*	623 (12.3)		571 (4.6)		535 (2.2)		432 (100)	23,33
	623		571		535		432	9
	622 (12)	602 (2.93)	570 (4.4)	556 (sh 2.75)	533 (2.05)	438 (sh 76.5)	432 (100)	This study
4-Vinyl protochlorophyll *a*	624		574		537		438	8,9
	622 (10)		571 (6.5)		532.5 (2.73)		438 (100)	7
	622 (10.75)	605 (sh 2.6)	572 (5.9)	558 (sh 3.5)	536 (2.15)	444 (sh 75.5)	437 (100)	This study
Bacterial protochlorophyll *a*	624 (12.2)		574 (6.8)		537 (2.8)		438 (100)	5
Protopheophytin *a*	638 (0.96)		585 (6.75)	565 (9.85)	524 (4.6)		417 (100)	33
	639		586	565	524		417	9
	638 (0.9)		588 (7)	565 (10)	524 (5.3)	432 (sh 57)	418 (100)	This study
4-Vinyl protopheophytin *a*	644		591	567	527		421.5	9
Bacterial protopheophytin *a*	642 (0.93)		590 (6.1)	568 (9.1)	527 (5.4)		421.5 (100)	5

Visible absorption spectra

A comparison of the visible spectra of the protochlorophyll pigments in ether with published data appears in Table II, in the form of the wavelengths of absorption maxima and the relative absorbance of the peaks. The most important feature of these data is the difference between the spectra of the two protochlorophyll pigments. Three main spectral characteristics differentiate protochlorophyll *a* and 4-vinyl protochlorophyll *a*. The blue band (Soret band) of 4-vinyl protochlorophyll *a* is shifted by about 5–6 nm to longer wavelengths compared to that of protochlorophyll *a*, and the absorbance ratio of the 620-nm band to the 570-nm band is clearly higher for protochlorophyll *a* than for 4-vinyl protochlorophyll *a*. These two facts account for the differences in color of the two pigments on the sugar column. Finally, the small band at 602 nm in protochlorophyll *a* appears only as a shoulder in the spectrum of 4-vinyl protochlorophyll *a* (see also Part II, ref. 25).

The conclusions from our observations, together with the chemical reactions and spectral properties[6], are those given by JONES[9]. The protochlorophyll *a* fraction is true protochlorophyll *a*. The 4-vinyl protochlorophyll *a* is the "bacterial" protochlorophyll, esterified in the present case by the phytol. From the work of JONES[5,6,8,9], it appears that 4-vinyl protochlorophyll *a* differs from protochlorophyl *a* by the presence of a second vinyl group, in place of the ethyl group in Ring II.

Infrared spectra

The infrared spectra of protochlorophyll *a* and 4-vinyl protochlorophyll *a* in carbon tetrachloride are presented in Fig. 1. Relatively little difference appears in the

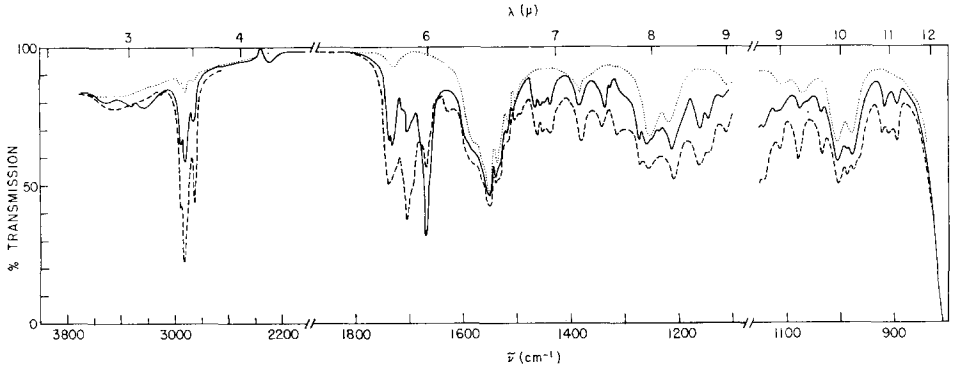


Fig. 1. Infrared spectra of protochlorophyll *a* (————) and of 4-vinyl protochlorophyll *a* (– – – – – –) in CCl_4 solution (0.1-mm path; about 25–50 mg·ml^{-1}). ·····, solvent spectrum.

infrared spectrum of 4-vinyl protochlorophyll *a* with respect to that of protochlorophyll *a*. Additional peaks or peaks of different shape occur in the 1600–1700, 1325–1340, 1060–1075, and 910-cm^{-1} regions; their significance will be examined in the DISCUSSION in connection with the presence of the second vinyl substituent. The positions of the bands for protochlorophyll *a* and 4-vinyl protochlorophyll *a* in the 1600–800-cm^{-1} region are tabulated in Table III.

The intense C–H band showing three peaks at 2855, 2930 cm^{-1} and 2955 cm^{-1} (Fig. 1) is a further indication of the presence of the phytol[26,27].

A comparison of the infrared spectra of protochlorophyll *a* and 4-vinyl protochlorophyll *a* in the carbonyl region (1600–1750 cm^{-1}) appears in Fig. 2 for different

TABLE III

INFRARED ABSORPTION BANDS OF THE PROTOCHLOROPHYLL PIGMENTS IN THE 1600–800-cm^{-1} REGION (IN CARBON TETRACHLORIDE)

Protochlorophyll a	*4-Vinyl protochlorophyll a*	*Assignments*
1465	1463	C–H bending (phytol)
1455	1455	
1436	1436	
1384	1380	
1335	1342	
1324	—	
—	1313	
1305 (shoulder)	1300 (shoulder)	
1272	1270	Bending of ring
1210	1210	
1160	1163	C=O stretching
1144	1145	
1113	1113	
1074	1078	
1060 (shoulder)	—	
1035	1035	
985	987	Vinyl C–H bending
917	923	
908 (shoulder)	910	
893	895	

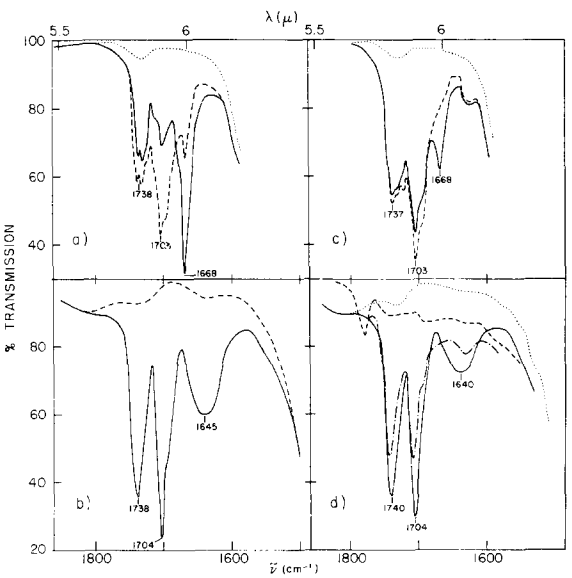


Fig. 2. Infrared spectra of the protochlorophyll pigments in the carbonyl region: a. Protochlorophyll *a* in CCl_4 (————) and in CCl_4 + 0.5% ethanol (– – – –); solvent CCl_4 (·····). b. Protochlorophyll *a* in tetrahydrofuran (————); solvent tetrahydrofuran (– – – –). c. 4-Vinyl protochlorophyll *a* in CCl_4 (————) and in CCl_4 + 0.5% ethanol (– – – –); solvent CCl_4 (·····). d. 4-Vinyl protochlorophyll *a* in tetrahydrofuran (————) and in ether (—·—·—); solvent tetrahydrofuran (·····), solvent ether (– – – –).

solvents: carbon tetrachloride in which protochlorophyll *a* and 4-vinyl protochlorophyll *a* aggregate and carbon tetrachloride *plus* 0.5 % ethanol, ether and tetrahydrofuran in which they are present in the monomer form, as indicated by absorption and circular dichroism spectral data (to be discussed elsewhere)[28].

An important experimental observation was made with the protochlorophyll *a* pigment and needs to be discussed in detail here. In the infrared spectra of protochlorophyll *a* in carbon tetrachloride, recorded with the waxy solid obtained in the preparation procedure (see MATERIAL AND METHODS), no aggregated ketone carbonyl band was observed at 1668 cm^{-1}, while 4-vinyl protochlorophyll *a* showed that band clearly. As we pointed out in the description of the purification procedure, we did not know at that time if this waxy state was the normal state of this pigment in the precipitation conditions used or if some impurity (oil or fat) was still present. We were then inclined to think that the absence of the additional band at 1668 cm^{-1} for protochlorophyll *a* in carbon tetrachloride was due to some impurity and we decided to make a further purification of this pigment. For that purpose, protochlorophyll *a* was applied to a sugar column as a solution in 10 % ether–isooctane. The pigment band was then washed on the column for several hours with pure isooctane, and eluted afterwards with 0.25 % propanol–isooctane. The eluate was washed with water and concentrated to 1–2 ml. This time, the pigment precipitated almost completely at −15° overnight, leaving a clear green supernatant, while the isooctane solution of the initial pigment had required the solid CO_2 temperature to induce appreciable precipitation. Furthermore, after drying, the solid protochlorophyll *a* was in a crystalline form, in contrast with the waxy state obtained initially. This was a clear indication that we had removed the impurity causing these effects. The measurement of the infrared spectrum of the repurified protochlorophyll *a* sample in carbon tetrachloride was difficult for solubility reasons; the spectrum is the one represented in Fig. 1, where the aggregated band at 1668 cm^{-1} is now very intense. Only minor changes appeared in the rest of the infrared spectrum for the repurified protochlorophyll *a* compared to the waxy solid protochlorophyll *a*. No modification of the visible absorption spectrum in ether nor of the infrared spectrum in tetrahydrofuran was observed after this further purification.

It is to be emphasized that the waxy solid protochlorophyll *a* did show the typical aggregation behavior in carbon tetrachloride in the electronic absorption spectrum and the circular dichroism[28]. The impurity was playing the role of disaggregating agent in the infrared measurement apparently because of the high concentration used.

The intensity of the aggregated band appeared to depend upon the concentration and the conditions of drying of the samples. The spectra presented in Fig. 1 were obtained with samples dried for at least 5 days under vacuum at room temperature. If, after dissolution in a polar solvent, the solvent was evaporated quickly under vacuum and the pigment redissolved immediately in carbon tetrachloride the intensity of the aggregated band was then very low.

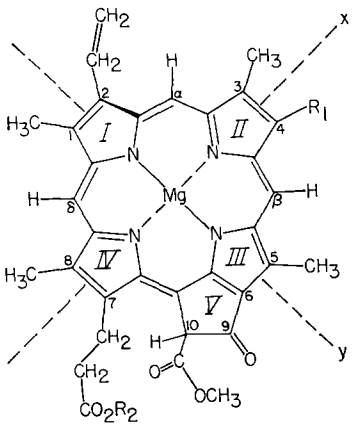
As shown by the dashed curve in Fig. 2a, protochlorophyll *a* was not completely disaggregated by the addition of 0.5 % ethanol to the carbon tetrachloride in this concentration range, while 4-vinyl protochlorophyll *a* was. However, the peak at 1668 cm^{-1} disappeared for both pigments in tetrahydrofuran. In this last solvent a broad band appeared around 1645 cm^{-1}. This effect was reversible; after evaporation

of the tetrahydrofuran and redissolution in carbon tetrachloride, the 1645-cm^{-1} band was no longer present.

The assignments of the bands will be considered in the DISCUSSION. Our results are in general agreement with the data of INHOFFEN AND BIERE[29] except that the position of the peaks are 10–20 cm^{-1} higher in our case. We must note that our infrared spectra of chlorophyll *a* determined under the same conditions, showed all its bands at positions in perfect agreement with the published values[22].

DISCUSSION

In confirmation of the work of JONES[9], two protochlorophyll pigments have been isolated from pumpkin seed coats. By the extraction and purification procedures described in the present study, they can be obtained as pure solid samples, in relatively large amounts. One of the two pigments is true protochlorophyll *a* (esterified by phytol), the other is the phytol ester of the so-called "bacterial" protochlorophyll, characterized by the presence of a second vinyl group in place of the ethyl on Ring II. These observations justify the previous results of STANIER AND SMITH[7]. They do not explain the reports of SEYBOLD[30] on the presence of a protochlorophyll *a* and a protochlorophyll *b* pigment, because neither of the two pigments displays an absorption spectrum similar to those published by this author. Neither 4-vinyl protochlorophyll *a* nor its phytol-free analogue has been reported to be present in etiolated leaves, despite the suggestion of JONES that the pathways for chlorophyll biosynthesis are the same in higher plants as they are in bacteria[9]. This last point has been previously discussed by JONES[8,9] and ELLSWORTH AND ARONOFF[10], and will not be considered here.



Protochlorophyll *a*: $R_1 = C_2H_5$; R_2 = phytyl. 4-Vinyl protochlorophyll *a*: $R_1 = CH{=}CH_2$; R_2 = phytyl.

Protochlorophyll *a*, 4-vinyl protochlorophyll *a*, and protopheophytin *a* have been further characterized by their chromatographic sequence, and visible and infrared spectra in the present study.

A detailed analysis of the electronic absorption properties will appear in Part II, ref. 25. The differences between the absorption spectra of protochlorophyll *a* and 4-vinyl protochlorophyll *a* are in agreement with the observations of JONES[9].

Our discussion of the infrared spectra of the protochlorophyll pigments will be guided by the excellent analysis made on the chlorophyll pigments by KATZ *et al.*[22,26] and by ANDERSON AND CALVIN[27], and by the data available for the porphyrins[31].

In the 4000–2700-cm^{-1} region, the protochlorophyll pigments show a broad band of low intensity around 3450 cm^{-1}, probably due to traces of water in the pigments. No exhaustive drying of the pigments was attempted in view of the sensitivity of these pigments to an increase of the temperature (see MATERIALS AND METHODS). In the C–H stretching region, the assignment of the three bands is made by comparison with the data of FOX AND MARTIN[32] on the infrared spectra of olefins. The asymmetric C–H vibration of the $-CH_3$ group appears at 2955 cm^{-1}, the very intense asymmetric C–H stretching mode of the $-CH_2$ group at 2930 cm^{-1}, and the slightly weaker overlapping band of the symmetric $-CH_3$ and $-CH_2$ stretching vibrations at 2855 cm^{-1}. The comparison between protochlorophyll *a* and 4-vinyl protochlorophyll *a* enables us to conclude that the vinyl C–H stretching vibrations are masked by the absorption due to the numerous $-CH_2$ and $-CH_3$ bands (especially those coming from the phytol chain), as pointed out by KATZ, DOUGHERTY AND BOUCHER[26].

In the carbonyl region, the characteristic ester and ketone carbonyl bands are observed in all the solvents investigated (see Table IV and Fig. 2). In carbon tetrachloride, the characteristic additional band around 1650 cm^{-1} was observed for protochlorophyll *a* and 4-vinyl protochlorophyll *a* and can be directly assigned to the aggregated ketone carbonyl band, as found for the chlorophyll pigments[26,27]. The aggregation scheme proposed by ANDERSON AND CALVIN[27] is then valid for the protochlorophyll pigments. The relative intensity of the aggregation peak in various experimental conditions has been discussed in the results. Other evidence for the aggregation of protochlorophyll *a* and 4-vinyl protochlorophyll *a* in carbon tetrachloride was obtained from circular dichroism and absorption spectra[28]. It was also observed,

TABLE IV

INFRARED BANDS OF THE PROTOCHLOROPHYLL PIGMENTS IN THE CARBONYL REGION (1600–1750 cm^{-1})

Absorbances for 0.1-mm path are given in parentheses.

Compound	*Solvent*	*ν (cm⁻¹)*				
		Ester carbonyl	*Ketone carbonyl*	*Aggregated ketone*	*?*	*Vinyl C–H*
Protochlorophyll *a*	CCl_4	1738 (0.165)	1703 (0.155)	1668 (0.51)	—	—
	CCl_4 + 0.5% ethanol	1738 (0.215)	1705 (0.35)	1668 (0.17)	—	—
	Tetrahydrofuran	1738 (0.415)	1704 (0.62)	—	1645 (0.20)	—
	Ether	1742 (0.27)	1707 (0.28)	—	1630 (0.05)	—
	$CHCl_3$*	1720	1680	1660	—	—
4-Vinyl protochlorophyll *a*	CCl_4	1737 (0.24)	1703 (0.38)	1668 (0.14)	—	1625
	CCl_4 + 0.5% ethanol	1737 (0.25)	1703 (0.43)	—	—	1625
	Tetrahydrofuran	1740 (0.43)	1705 (0.55)	—	1640 (0.14)	—
Protopheophytin *a*	CCl_4	1738 (0.14)	1710 (0.175)	—	—	—

* From ref. 29.

similarly, that protochlorophyll *a* and vinyl protochlorophyll *a* are in the monomer form in the 10^{-4}–10^{-5} M concentration range in carbon tetrachloride *plus* 0.5 % ethanol, in pyridine, in ether, and in tetrahydrofuran.

In tetrahydrofuran and in ether a broad band appears near 1640 cm^{-1}. This band could be attributed to keto–enol tautomerism. In the event of enolisation, however, the pigments would not retain optical activity because during the exchange reaction both positions of the carbomethoxy group on C-10 would be equally probable and a diastereoisomeric mixture would be formed. No evidence of epimerization at room temperature is observed, but for technical reasons we have been unable to investigate the circular dichroism in the high pigment concentration range (0.1 M) used for the recording of the infrared spectra. We have also not analyzed the influence of the concentration on the infrared spectra. Enolisation should not be strongly concentration dependent, however, so that the presence of the normal circular dichroism spectra in tetrahydrofuran at a concentration of about 10^{-5} M makes this explanation implausible. We thus fail to give an interpretation of the infrared band at 1640 cm^{-1}.

A very striking difference between the infrared spectra of the chlorophyll and protochlorophyll pigments is the absence of the skeletal vibrations for the latter. These vibrations appear around 1600, 1550, 1500, and 1350 cm^{-1} in the chlorophyll spectra; none is present in the spectra of the protochlorophylls. This difference reflects the high symmetry of the tetrapyrrolic macrocycle in these compounds. In fact, as pointed out by KATZ, DOUGHERTY AND BOUCHER[26], the 1550-cm^{-1} band is characteristic of the chlorins and does not appear in the infrared spectra of the porphyrins[31] nor in the tetrahydroporphyrins[16]. Finally, KATZ, DOUGHERTY AND BOUCHER[26] have ruled out the possibility that the 1610-cm^{-1} band arises from the vinyl substituent on C-2, by the fact that it is present in the spectra of chlorins that do not have a vinyl group, and absent in porphyrins that do. In fact, 4-vinyl protochlorophyll *a* presents a weak but clearly defined band around 1625 cm^{-1} in carbon tetrachloride and in carbon tetrachloride *plus* 0.5 % ethanol, while protochlorophyll *a* does not. We think that this band results from the presence of the second vinyl substituent at C-4, in 4-vinyl protochlorophyll *a*. In tetrahydrofuran this band is probably masked by the broad band at 1640 cm^{-1}. Our data thus give a further explanation of the fact that the vinyl vibration is not observed in the infrared spectra of the chlorins, because of the presence of the much more intense skeletal vibrations, and is not clearly defined for the porphyrins containing only one vinyl substituent. These observations are in agreement with the results of WETHERELL, HENDRICKSON AND MCINTYRE[31] who found a band at 1610 cm^{-1} for protoporphyrin (vinyl substituents on C-2 and C-4) and no band for mesoporphyrin (vinyl substituents replaced by ethyl).

The bands around 1450, 1380, 1340, and 1315 cm^{-1} are assigned (by comparison with the chlorophylls[26]) to C–H bending modes coming essentially from the phytol chain. A measurement of the infrared spectra of protochlorophyllide *a* would clarify this question.

In the fingerprint region (1300–650 cm^{-1}) KATZ, DOUGHERTY AND BOUCHER[26] have assigned the bands at 990 and 920 cm^{-1} for the chlorophyll pigments to the vinyl C–H out-of-plane bending mode. We differ with this assignment, at least for the 920-cm^{-1} band, because in that case the band would be as intense in the protochlorophylls as in the chlorophylls, and even more intense for 4-vinyl protochlorophylls *a*.

Since it is not so, we are inclined to follow the suggestion of WETHERELL, HENDRICKSON AND MCINTYRE[31] according to which those bands would be masked in the chlorins, probably by deformation vibrations of the ring structure. In the protochlorophylls they are uncovered and appear at 985–990 cm^{1-} and 910–920 cm^{-1} (4-vinyl protochlorophyll *a* shows clearly an additional band at 910 cm^{-1} compared to protochlorophyll *a*). By comparison with the infrared spectra of the chlorophylls, the bands in the 1200–1000-cm^{-1} region are assigned to C–O stretching modes[26], while the 1273-cm^{-1} band would correspond to bending of the tetrapyrrol macrocycle.

Finally, we wish to emphasize that our infrared spectra data give additional support to the reported structure of 4-vinyl protochlorophyll *a* (ref. 9).

With the ready availability of the purified protochlorophylls, the problem of the aggregation properties of these pigments in various solvents is now open to further investigation. A detailed analysis by visible and infrared absorption spectroscopy, fluorescence, and circular dichroism appears in Part II, ref. 25. By comparison of the circular dichroism and magnetic circular dichroism spectra of the protochlorophylls with those of the chlorophylls and related pigments, we are able to determine important relationships between these properties and aspects of molecular structure. The optical activity of these pigments will be discussed along with the problem of aggregation in nonpolar solvents in a following publication[28].

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