A Protein–Protochlorophyll Complex Obtained from Inner Seed Coats of *Cucurbita pepo*

THE RESOLUTION OF ITS TWO PIGMENT GROUPS INTO TRUE PROTOCHLOROPHYLL AND A PIGMENT RELATED TO BACTERIAL PROTOCHLOROPHYLL

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1. The inner seed coats of *Cucurbita pepo* were extracted with aqueous acetone and found to contain pigments with spectra similar to that of protochlorophyll. 2. When the fruits of *C. pepo* were stored the amount of protochlorophyll-like material in the inner seed coats increased and a form of protochlorophyll absorbing at longer wavelength was apparently formed. 3. The pigment was resolved into two forms of protochlorophyll by chromatography on sugar columns. One form with absorption maxima in ether at 432, 535, 571 and 623 mμ was spectroscopically identical with plant protochlorophyll; the other, with absorption maxima at 438, 537, 574 and 624 mμ, was spectroscopically identical with bacterial protochlorophyll isolated from the tan mutant of *Rhodopseudomonas spheroides*. The two phaeoporphyrins obtained from the seed-coat pigments closely resemble the corresponding phaeoporphyrin derivatives of plant protochlorophyll and bacterial protochlorophyll in spectroscopic and partition properties. 4. The pigment in the cells of inner seed coat of *C. pepo* is concentrated in discrete particles of about 1·7 μ diameter. Extracts of the seed coats in a glycerol–glycine buffer were similar in spectroscopic properties to the crude protochlorophyll holochrome, but were not light-transformable. 5. After partial purification of the glycerol–glycine buffer extracts a pigment–protein complex was obtained with absorption maxima at considerably longer wavelengths than in organic solvents. 6. Preparations of the seed-coat protochlorophyll, in the presence of bovine serum albumin, adsorbed on filter paper or in colloidal solution, did not have absorption bands shifted so far to the red region as the natural protein complex isolated from the seed coat. 7. It is suggested that bacterial protochlorophyll (magnesium 2,4-divinylphaeoporphyrin a_5 methyl ester) is involved in the biosynthesis of chlorophyll in both plants and photosynthetic bacteria.

Both green plants and the purple photosynthetic bacteria, the Athiorhodaceae, carry out photosynthesis by reactions that are fundamentally similar and the pigments of chloroplasts and chromatophores are closely related structurally. Bacteriochlorophyll may be considered as derived from chlorophyll *a* by the reduction of a double bond in ring II and the replacement of the vinyl group at position 2 by an acetyl group (Fischer & Stern, 1940). Such similarity of structure suggests that these pigments may have common intermediates in biosynthesis and that chlorophyll *a* may be an intermediate in the formation of the bacterial pigment. In higher plants light is required for the formation of chlorophyll *a*, but pigment formation by the bacteria is not light-dependent (Cohen-Bazire, Sistrom & Stanier, 1957). However, in green algae and germinating seedlings of a number of gymnosperms greening can take place in the dark, showing that there is no absolute requirement for light for chlorophyll *a* formation.

The growth medium of a *Rhodopseudomonas spheroides* mutant (Sistrom, Griffiths & Stanier, 1956) and of *R. spheroides* grown in the presence of 8-hydroxyquinoline (Jones, 1963*b*) contain pigments closely related in spectroscopic properties to phaeophytin *a* and to hydroxyethylphaeophytin *a*, the magnesium-free derivative of a logical intermediate between chlorophyll *a* and bacterial chlorophyll (Jones, 1963*b*, 1964). These facts

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support the view that chlorophyll *a* is an intermediate in bacteriochlorophyll biosynthesis.

When seeds of higher plants are germinated in the dark no chlorophyll is formed but protochlorophyllide (magnesium vinylphaeoporphyrin a_5 monomethyl ester) and its phytyl ester, protochlorophyll, accumulate. Protochlorophyllide in combination with a high-molecular-weight protein forms the protochlorophyll(ide) holochrome that is converted into chlorophyll(ide) *a* holochrome by illumination either in the intact leaves or *in vitro* (cf. Smith, 1960) after extraction into buffer. Pure protein-free protochlorophyllide is not converted into chlorophyll *a* by illumination and differs markedly from the protochlorophyll holochrome in the position and relative intensities of its absorption bands. Chlorophyll synthesis in the photosynthetic bacteria is not light-dependent but a pigment, 'bacterial protochlorophyll', has been found to accumulate in a mutant of *R. spheroides* that cannot synthesize chlorophyll (Stanier & Smith, 1959; Griffiths, 1962). This pigment resembles plant protochlorophyll in spectroscopic properties, but differs slightly from it in the position of the absorption maxima, which are shifted to longer wavelengths. An apparently identical pigment produced by *R. spheroides* grown in the presence of 8-hydroxyquinoline was identified (Jones, 1963*c*) as magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester. It was suggested that this ester may be an intermediate in the biosynthesis of chlorophyll *a* as well as of bacteriochlorophyll since it would fit logically between magnesium protoporphyrin monomethyl ester, which has been found in higher plants (Granick, 1961) and in photosynthetic bacteria (Jones, 1963*a*; Gibson, Neuberger & Tait, 1963), and protochlorophyllide. It appeared possible that 'bacterial protochlorophyll' might be present in the seed coats of Cucurbitaceae, since Stanier & Smith (1959) reported that protochlorophyll from this source absorbed light at slightly longer wavelengths than the usual higher-plant protochlorophyll, although the band maxima did not correspond precisely with those of 'bacterial protochlorophyll'.

The results described in the present paper may be summarized as follows. (i) Two green pigments have been separated from the seed coats of *C. pepo*: one pigment is spectroscopically identical with true protochlorophyll, the other with bacterial protochlorophyll. (ii) Bacterial protochlorophylls from mutant *R. spheroides* (Stanier & Smith, 1959) and 8-hydroxyquinoline-treated *R. spheroides* (Jones, 1963*b*) are spectroscopically and chromatographically identical. This work has been briefly reported (Jones, 1965). (iii) Protochlorophyll pigments in the seed coats of *C. pepo* are present as protein complexes with spectroscopic properties very similar to those of the leaf protochlorophyll(ide) holochrome. These complexes are organized in discrete particles with the seed-coat cells but could be extracted into glycerol buffers and partly purified. (iv) A possible role for these pigments in chlorophyll biosynthesis in *C. pepo* is suggested.

MATERIALS AND METHODS

Biological material. The tan mutant M_5 of *R. spheroides* (Stanier & Smith, 1959) was a gift from Dr June Lascelles (Department of Bacteriology, University of California). It was grown on solid or liquid media (Bull & Lascelles, 1963). Seeds were obtained from commercially grown vegetable marrows (*C. pepo*) that were picked and then stored in a cool room.

Porphyrins. Vinylphaeoporphyrin a_5 monomethyl ester (protochlorophyllide lacking magnesium) was prepared from etiolated bean plants (*Phaseolus vulgaris*) (Jones, 1963*c*). Divinylphaeoporphyrin a_5 monomethyl ester was prepared from the medium of *R. spheroides* grown in the presence of 8-hydroxyquinoline (Jones, 1963*c*).

Hydrochloric acid solutions. Concentrations of aqueous HCl solutions are given as % (w/v). The conc. HCl used was sp.gr. 1·180.

Preparation of ether solutions of seed pigments and bacterial protochlorophyll. Seeds of *C. pepo* were collected from mature fruits. It was observed that in mature seeds nearly all the fluorescent pigment was located in the inner deep-olive-green seed coats, but it proved impractical to detach large quantities of the inner coats from the seeds. The seeds were therefore suspended in about 4vol. of aq. 90% (v/v) acetone and blended in an Ato-Mix blender (Measuring and Scientific Equipment Ltd., London) at top speed for 1min. The resulting suspension was filtered and the particulate material was re-extracted with 90% acetone until little pigmented material was left in the filter cake. The acetone extract was concentrated *in vacuo* under nitrogen, mixed with a large volume of ether and sufficient water was added to transfer all the pigments into the ether phase, which was collected, concentrated and used in subsequent purification steps. It contained much oily material that interfered with the usual adsorption-chromatographic procedures and these oils were largely removed by partitioning between heptane and methanol in an apparatus for continuous multi-stage extraction (see below).

Bacterial protochlorophyll was prepared from the growth medium of the tan mutant *R. spheroides* (Stanier & Smith, 1959) grown in 10l. batches with continuous aeration. The cells were removed by centrifuging and the medium was extracted with ether. The pigments, largely a mixture of bacterial protochlorophyll and its magnesium-free derivative, were purified by chromatography on polyethylene columns (Jones, 1963*b*).

Preparation of phaeophytins. Ether solutions of the protochlorophylls prepared from seed-coat extracts were shaken with 25% HCl, which was rapidly neutralized with sodium acetate, the ether layer being washed with water until free from acid, washed with 15% HCl to remove free phaeoporphyrins and then again washed free of acid.

Removal of oils from seed extracts by countercurrent separation. The mixture of ether-soluble compounds

obtained from the seeds of *C. pepo* was transferred to methanol-saturated heptane to give as concentrated a solution as possible. This was continuously partitioned against heptane-saturated methanol in an apparatus developed at these Laboratories (Hartley, Howes & McLauchlan, 1965) that has ten stirring vessels. Each vessel contained as lower phase heptane-saturated methanol (about 20ml.) and as upper phase methanol-saturated heptane (about 20ml.). The heptane solution of seed pigments was added to the first vessel and heptane-saturated methanol passed through the apparatus. Most of the oily components remained in the stationary heptane layer and were retained in the first five or six vessels, and the protochlorophyll-like pigments were eluted in the moving methanol phase. Some resolution of the protochlorophyll-like pigments occurred, pigments with absorption bands at longer wavelengths than true protochlorophyll being found at higher concentrations in the early eluate and pigments with true protochlorophyll spectra being richer in the latter eluate, but neither pigment was free from the other. These operations were carried out in the dark or in subdued light.

Thin-layer chromatography. Separation of pigments was carried out on thin layers of Kieselgel HF254 (E. Merck A.-G., Darmstadt, Germany) developed with light petroleum (b.p. 100–120°)–propan-2-ol–water (400:40:1, by vol.) (Hager & Bertenrath, 1962) or with heptane–acetone (7:3, v/v). Thin layers of sucrose (Colman & Vishniac, 1964) and of mannitol (Smith, Breidenbach & Rubenstein, 1965) were also used and developed with 2% (v/v) methanol in light petroleum (b.p. 40–60°), 0·5% (v/v) propan-1-ol in light petroleum (b.p. 40–60°) or 5% (v/v) acetone in light petroleum (b.p. 40–60°). The pigments were detected by their intense red fluorescence in u.v. light.

Preparative partition chromatography. For the resolution of mixtures of very similar protochlorophyll-like pigments the method of Hughes & Holt (1962) was used. Ester groups were first hydrolysed by extracting the pigments from the crude mixture into 12N-HCl and allowing the HCl solution to stand for 30min., after which ether and water were added and the HCl was neutralized with sodium acetate. The pigments in the ether phase were washed free from acetic acid with distilled water. The resulting phaeoporphyrins were then fractionated from ether with HCl, and the fraction obtained between 10% and 15% HCl was collected, neutralized with sodium acetate, transferred to ether and washed with water until free from acid. It would be expected that any porphyrins derived from protochlorophylls of interest in this work would be collected in this fraction (Jones 1963c). This mixture was then applied to a column of Celite (Johns Manville, 545) that had been dry-packed under suction, washed with 14% HCl saturated with ether and then equilibrated with acid-saturated ether. The column was developed with acid-saturated ether. Separation is based on the differing partition of porphyrins between HCl and ether: compounds of low HCl number (cf. Falk, 1964) would be retained in the acid phase whereas compounds of high HCl number would tend to move with the ether phase (Hughes & Holt, 1962).

Preparative adsorption chromatography. Mixtures of pigments in light petroleum (b.p. 40–60°) were applied to columns of icing sugar equilibrated with light petroleum, the columns developed with light petroleum and then allowed to run dry. Fractions of interest were cut out, extracted in ether, transferred to light petroleum (b.p. 40–60°)–benzene (1:1, v/v) and rechromatographed on a sugar column equilibrated with light petroleum–benzene mixture, which was also used to develop the column. After development the column was allowed to drain and the bands were cut out and eluted.

RESULTS

Spectroscopic examination of crude ether-soluble pigments from the pale-yellow seeds of young freshly harvested marrows showed that almost no protochlorophyll-like pigments were present. The extract was yellow, absorbing strongly in the region 400–500mμ but with an absorption band in the red region around 664mμ (see Fig. 1), suggesting the presence of a mixture of chlorophylls *a* and *b*. After storage of intact marrows for 4 weeks there was a marked change in the appearance of seeds and of acetone extracts of seeds. The seeds had become olive green, and the extracts were deep purple and exhibited intense red fluorescence in u.v. light. The extract of mature seeds had an absorption spectrum with maxima in ether at about 432, 573 and 624mμ, although some absorption at 664mμ was still present (see Fig. 1). The bands at 432, 573 and 624mμ correspond well with those of protochlorophyll and they indicated a protochlorophyll content of about 1% of the dry weight of seed coat on the basis of the specific extinction coefficient given by Smith & Benitez (1955). A comparison with the absorption spectrum in ether of etiolated-leaf protochlorophyll showed that the spectrum of seed-extract pigment had a shoulder at about 438mμ that was absent from the leaf pigment. Further experiments showed that the amount of '438mμ material' was variable and tended to increase with the period of storage of the

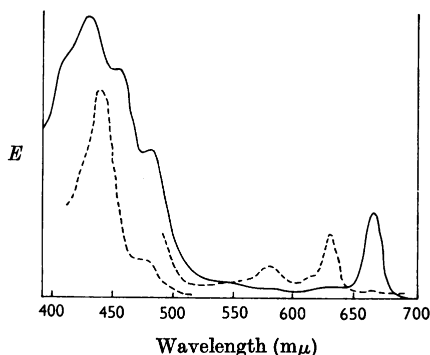


Fig. 1. Spectra in ether of pigments obtained by acetone extraction of seed coats of *C. pepo*: ——, extract of young seeds; ----, extract of old seeds. The latter extract was diluted by the addition of 2 parts of ether for measurements in the region below 500mμ.

marrow. In one extract the main absorption band was at 438 mμ and the shoulder at 432 mμ but this was exceptional. Since the main absorption band of the 'bacterial protochlorophyll' in mutants and in inhibited cultures of *R. spheroides* (Stanier & Smith, 1959; Griffiths, 1962; Jones, 1963*c*) is at 437–438 mμ it appeared possible that the seeds contained a mixture of true protochlorophyll and 'bacterial protochlorophyll'.

Thin-layer chromatography in a variety of systems yielded three red fluorescent spots, none of which remained at the origin, and the fastest moving of which predominated. In all cases, when the major spots were eluted into ether the absorption spectra were very similar. Each spot appeared to contain a mixture of 432 mμ- and 438 mμ-absorbing substances, with the 438 mμ-absorbing material richer in the tail of the spot. When this major component was run through a sucrose column, a green component (F_1) was incompletely separated from a slower olive-green component (F_2). Repeated chromatography of these fractions was necessary before good resolution was achieved and the yields were very low. The spectra of these pigments in ether are given in Fig. 2. Component F_1 ($\lambda_{max.}$ at 432, 535, 571 and 623 mμ) is spectroscopically identical with true protochlorophyll; component F_2 ($\lambda_{max.}$ at 438, 537, 574 and 624 mμ) is identical with (i) the bacterial protochlorophyll (magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester) described by Jones (1963*b*,*c*) and (ii) with the protochlorophyll that was obtained (see the Materials and Methods section) by ether extraction and chromatography of the medium of the tan mutant of *R. spheroides* that cannot synthesize bacteriochlorophyll. When components F_1 and F_2 were treated with acid to remove magnesium the absorption spectra of the resulting phaeophytins differed markedly but closely resembled the corresponding derivatives of protochlorophyll and bacterial protochlorophyll respectively.

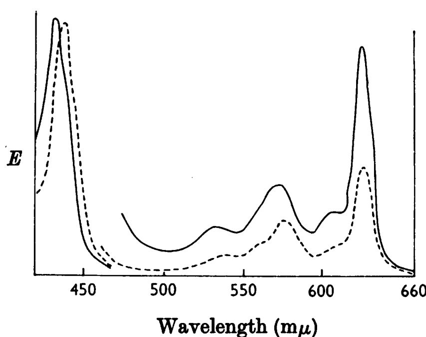


Fig. 2. Absorption maxima in ether of the protochlorophylls obtained from seed coats of *C. pepo* after extraction into acetone and light petroleum (40–60°) and repeated chromatography on sucrose columns. ——, Spectrum of F_1, the faster-moving fraction, diluted to one-eighth concentration for measurements in u.v. region; - - - -, spectrum of F_2, diluted to one-quarter concentration for measurements in u.v. region.

Separation of these two pigments was more readily carried out after conversion into phaeoporphyrins. A problem with this method of separation was the low solubility that made large-scale purification very difficult. On a small scale, however, very good separation of two phaeoporphyrins was achieved. The pale-green faster-moving band (P_1) had a spectrum in ether (Fig. 3) identical with that of divinylphaeoporphyrin a_5, and the dark-green slower-moving band (P_2) had a spectrum identical with that of monovinylphaeoporphyrin a_5 (the phaeoporphyrin derived from protochlorophyll). The spectroscopic properties are compared in Table 1.

The bacterial phaeoporphyrins derived from mutant and inhibited *R. spheroides*, when partitioned between ether and different concentrations of hydrochloric acid, had almost identical distributions (Table 2), which suggests that they have the same alkyl substituents as well as the same chromophores.

During thin-layer chromatography the bacterial protochlorophyll from mutant *R. spheroides*, or from chelator-inhibited cultures of *R. spheroides*, did not move from the origin in any of the solvents used (see the Materials and Methods section), whereas the protochlorophyll fraction F_2 moved well up the plates. This indicates that the F_2 protochlorophyll, unlike the bacterial pigment, is fully esterified. This was confirmed by its very high hydrochloric acid number (about 25) and by its failure to pass from ether into aqueous alkali. The F_1 pigment of seeds was also fully esterified.

Properties of seed-coat protochlorophylls in vivo. A microscopic examination of inner seed coats

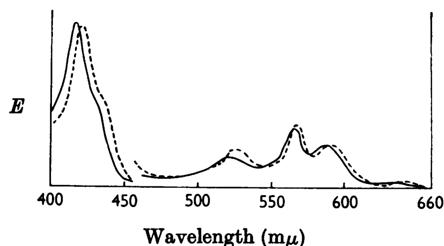


Fig. 3. Absorption spectra in ether of the phaeoporphyrins obtained from the protochlorophylls of seed coats of *C. pepo*, as described in the Materials and Methods section. - - - -, Spectrum of the faster-moving band P_1; ——, spectrum of the slower-moving band P_2.

Table 1. *Spectroscopic properties of ether solutions of the phaeoporphyrins derived from protochlorophylls of various origins*

Source of phaeoporphyrin	Absorption maxima (mμ)					Band III/ band IV extinction ratio
	Band I	Band II	Band III	Band IV	Soret	
Phaeoporphyrin from etiolated-leaf protochlorophyll	638	586	565	524	417	2·1
Seed-coat phaeoporphyrin P_1	639	586	565	524	417	2·1
Phaeoporphyrin from bacterial protochlorophyll of tan mutant of *R. spheroides*	644	591	567	527	421.5	1·6
Phaeoporphyrin from bacterial protochlorophyll of 8-hydroxy-quinoline-treated *R. spheroides*	644	591	567	527	421·5	1·6
Seed-coat phaeoporphyrin P_2	644	591	567	527	421·5	1·6

Table 2. *Comparison of distributions between ether and hydrochloric acid of phaeoporphyrins from seed-coat protochlorophyll, protochlorophyll from tan mutant of* R. spheroides *and protochlorophyll from 8-hydroxyquinoline-treated* R. spheroides

The phaeoporphyrins in ether solution were shaken with an equal volume of HCl of appropriate strength and the concentration of phaeoporphyrin remaining in the upper phase was determined spectrophotometrically by its extinction at 421·5 mμ.

Concn. of HCl (%, w/v)	Phaeoporphyrin remaining in ether (%)		
	Phaeoporphyrin P_2 from seed coat	Phaeoporphyrin from tan mutant of *R. spheroides*	Phaeoporphyrin from 8-hydroxy-quinoline-treated *R. spheroides*
11	73	69	70
12	58	55	57
13	42	41·5	41
14	28	26·5	25
15	20	18·5	18

isolated from mature seeds of *C. pepo* showed that the green protochlorophyll was located in discrete particles of an average diameter of 1·7 μ. In some preparations these particles appeared to be gathered within a membrane inside the cell, but such preparations could not always be obtained. This organization of protochlorophyll into discrete structures resembles the situation in etiolated leaves, where the protochlorophyll(ide)–protein complex (the holochrome) is located in bodies of diameter 0·7–1·3 μ (Boardman & Wildman, 1962). The spectrum of the pigment within the intact seed coats had a maximum at about 648 mμ, not at 624 mμ as would be expected for protochlorophyll in organic solvents. Attempts were therefore made to extract a protochlorophyll–protein complex from seed coats by using methods successful in the isolation of protochlorophyll holochrome.

Isolated inner seed coat (1 g.) was ground in a chilled mortar with 10–15 ml. of a glycine buffer containing 30% (v/v) of glycerol and 70% (v/v) of 0·04 M-glycine (sodium salt)–sodium hydroxide buffer, pH 9·6. The extract was filtered through muslin and the pulp re-extracted with the glycerol–glycine buffer. The filtrates were combined and centrifuged at 10 000 *g* for 15 min. and the resulting supernatant was centrifuged at high speed for 1 hr. (50 000 rev./min. in the MSE Super Speed 50 centrifuge). The absorption spectrum of the supernatant fluid (I) is shown in Fig. 4, together with the spectrum in ether of the pigment after extraction into acetone and transfer to ether. The marked shift of absorption bands between the spectrum *in vivo* and the spectrum *in vitro* closely resembles the situation found in the protochlorophyll holochrome, where the absorption maximum in the red region *in vivo* is at about 640 mμ, compared with the maximum in ether at 623 mμ, and the intensity of the band in the blue region is low relative to the red band (cf. Smith, 1960). An

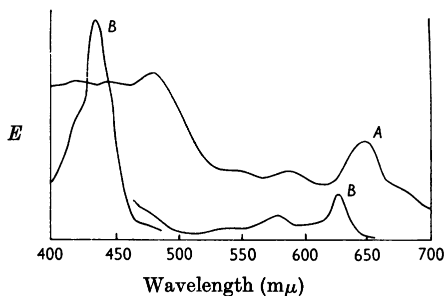


Fig. 4. Absorption spectrum of seed-coat extract in 30% glycerol–70% 0·04M-glycine buffer, pH 9·6, compared with the spectrum of an extract made in acetone, followed by transfer to ether. *A*, Spectrum of glycerol–glycine extract; *B*, spectrum in ether.

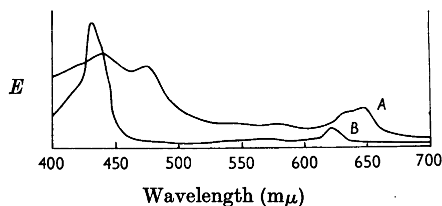


Fig. 5. Spectrum of the partly purified protochlorophyll–protein complex from seed coats of *C. pepo*. *A*, Spectrum in 0·05M-glycine buffer, pH 9·6; *B*, spectrum after extraction of pigments with acetone and transfer into ether.

attempt was made to purify this pigment complex by methods previously used for the purification of the protochlorophyll holochrome (Smith, 1960; Boardman, 1962). The fluid I was treated with ammonium sulphate at pH 9·6 and the fraction precipitated between 20% and 50% saturation was collected. The precipitate was dissolved in 0·05M-glycine buffer, pH 9·6, a procedure that required several hours' stirring, and the solution was applied to a column of Sephadex G-100. The coloured material was not retained on the column and was collected. The spectrum of the effluent (II) (Fig. 5) shows that there is a tendency for the relative absorption at 442mμ to increase and for the band at about 640mμ to split, possibly because there are two different chromophores in the preparation. This fraction has strong protein absorption at 260mμ.

Properties of the protochlorophyll–protein complex. This partly purified complex (II) sedimented on high-speed centrifugation (30min. at 140000*g*), a phenomenon that has been noted with the protochlorophyllide holochrome. When the complex was treated with acetone the resulting mixture of protochlorophylls resembled that present in the crude extract (Fig. 5). The complex was remarkably stable to heat: 10min. at 100° caused only very slightly increased absorption at about 445mμ accompanied by a very slight fall in the absorption of the shoulder at 636mμ. The increased light-scattering from the suspension caused by precipitation of protein necessitated the use of wider slits and may have obscured slight differences in absorption maxima. Since heat denaturation of the protein had little effect on the visible absorption spectrum it seemed possible that the characteristic high ratio of absorption in the red region to absorption in the blue region was due to the presence of polymeric forms of the pigment, perhaps adsorbed non-specifically to proteins. Since it is known that aggregates of chlorophyll *a* have spectroscopic properties resembling those of chlorophyll *a in vivo* (e.g. Krasnovsky & Kosobutskya, 1953; Brody & Brody, 1965), various colloidal and aggregated preparations of the seed-coat pigment were made in attempts to achieve properties resembling those found in the protein complex.

An ether solution of the seed-coat pigments was dried on to filter paper or transferred to 10% (v/v) dioxan in 0·05M-glycine buffer, pH 9·6, or to 1% (w/v) bovine serum albumin. An extract of seed-coat pigments in 0·5% Triton X-100 was also prepared. The spectroscopic properties of these preparations and of some naturally occurring protochlorophyll complexes are given in Table 3.

DISCUSSION

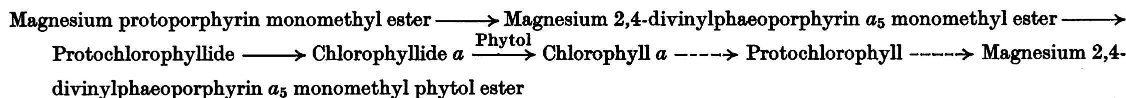
The examination of extracts of seeds of *C. pepo* confirmed previous reports that the seed coats contain a protochlorophyll that is different in spectroscopic properties from the protochlorophyllide of etiolated leaves. Separation of the pigments by chromatography into material with a maximum in the blue region at 438mμ and material with a maximum at 432mμ (Fig. 2) may explain reports in the literature (e.g. Stanier & Smith, 1959; Inada & Shibata, 1960) of protochlorophylls with anomalous absorption maxima, since these workers may well have been examining mixtures of pigments. It may also explain the claim by Seybold (1937) for the existence of a protochlorophyll *b*, although direct comparison with his data, which were obtained by the use of a simple spectroscope, is not possible. Later work has failed to support any claim for the existence of a protochlorophyll *b* in etiolated leaf tissue, where chlorophyll *b* is not detected for several hours after the formation of chlorophyll *a* has been induced by illumination (Smith, 1960), and it appears that another explanation for the presence of this second protochlorophyll must be sought.

Table 3. *Spectroscopic properties of complexes of protochlorophylls*

B indicates a spectrum, as obtained for protochlorophyll *in vivo* (cf. Fig. 2), where the ratio of absorption in the blue region to absorption in the red is high; R indicates a spectrum (cf. Fig. 6) where the ratio is low, as in protochlorophyll in organic solvents.

	Band max. (mμ)		
Mode of formation of complex	Blue region	Red region	Spectrum type
1. 10% dioxan colloid in 0·05 M-glycine, pH 9·6*	439	626	R
2. 5% acetone colloid in 0·05 M-glycine, pH 9·6	442	628	R
3. 5% acetone colloid in 1% bovine serum albumin*	443	628	R
4. Extract of seed coats, adsorbed on filter paper*	442	628	R
5. 0·5% Triton X-100 extract of seed coats*	442	628	B
6. *R. spheroides* tan mutant *in vivo*	approx. 440 (shoulder)	632	R
7. Etiolated-leaf protochlorophyll holochrome†	442	640	R
8. Non-light-convertible protochlorophyll of *Chlorella* mutant, *in vivo*‡	?	631	?

* Mixed protochlorophyll pigment obtained by acetone extraction of seed coats.
† Smith (1960).
‡ Granick (1961).



Scheme 1.

In Table 1 a comparison of the properties of the phaeoporphyrins derived from the longer-wavelength seed-coat protochlorophyll and from bacterial protochlorophyll shows the virtual identity of these pigments; both have the characteristic low band III/band IV ratio. This emphasizes their difference from the same derivative of 'true' protochlorophyll (Jones, 1963*c*) (cf. also Fig. 3). Tables 1 and 2 also show that the pigment obtained from mutant bacteria appears to be the same as that derived from bacteria grown in the presence of 8-hydroxyquinoline and identified as 2,4-divinylphaeoporphyrin a_5 monomethyl ester (Jones, 1963*c*). Since the positions and ratios of the absorption bands of porphyrins are characteristically affected by the nature of the substituents it may be assumed that the long-wavelength form of protochlorophyll found in seed coats is also an ester of magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester, presumably the phytol ester. This identification is supported by the close similarity of the absorption bands of the magnesium-containing complexes of these pigments (both have $\lambda_{max.}$ in ether at 438, 537, 574 and 624 mμ; Fig. 2 and Jones, 1963*b*). Such a pigment is a logical biosynthetic precursor of protochlorophyll.

Recent work suggests that the precursor of chlorophyll *a* is non-phytolated (Wolf & Price, 1957). Further, the enzyme chlorophyllase that carries out the phytolation of chlorophylls (Shimizu & Tamaki, 1963) appears to be specific for pigments, such as chlorophylls *a* and *b* and bacteriochlorophyll, that are hydrogenated in ring IV (cf. Holden, 1963), and protochlorophyll is not a substrate (Sudyina, 1963). It may be that these phytolated protochlorophylls are not formed en route to chlorophyll *a* but are the products of a reverse reaction, i.e. there is an equilibrium in the later stages of a light-independent chlorophyll *a* biosynthesis. Thus, when the utilization of chlorophyll *a* is blocked, some is dehydrogenated by a reversed action of the enzymes concerned in the synthesis of the non-phytolated intermediates to yield these phytolated 'precursors'. A scheme for such a sequence is given in Scheme 1. Alternatively, the seed coats may contain a 'phytolase', differing from the usual chlorophyllase, that can esterify pigments that are unsaturated in ring IV. The observation that the porphyrin pigments of immature seeds are largely chlorophylls (Fig. 1), and that subsequently protochlorophyll and finally the long-wavelength form of seed-coat protochlorophyll accumulate, supports either suggestion. Similar experimental observations were reported by Godnev, Rotfarb & Akulovich (1963), who observed that there was a change with age in the absorption spectrum of

pumpkin seed-coat protochlorophyll. In very young seeds they found chlorophylls *a* and *b* and at a later stage protochlorophyll. Finally, in mature seeds, the absorption band maxima in the blue region shifted 4–6 mμ to longer wavelengths.

The action spectrum for conversion of protochlorophyllide into chlorophyllide *a* has a maximum at about 650 mμ (Koski, French & Smith, 1951), which corresponds well with the absorption maximum of the protochlorophyllide holochrome; a minor protochlorophyll component absorbing *in vivo* at 635 mμ is unconverted. Even in *Chlorella*, which normally synthesizes chlorophyll in the dark, a light-dependent mutant requires light of about 650 mμ for chlorophyll synthesis (Bryan & Bogorad, 1963). This suggests that specific binding to a protein is essential for conversion and makes the observation that the protochlorophylls of seed coats are bound to protein and absorb light around 640–650 mμ (Figs. 4 and 5) of great interest. Attempts to produce protochlorophyll complexes absorbing light at longer wavelengths (Table 3) have been unsuccessful and suggest that the natural complexes may have some specific binding sites. These natural complexes may permit interconversions where the pigments of the mutant *R. spheroides* are not convertible into protochlorophyllide.

The organization of the pigments in the seed coats into discrete bodies is a further resemblance to the situation found in etiolated leaves, where the protochlorophyllide holochrome is found in organized structures. In some preparations of the seed-coat protochlorophyll complex two bands were visible in the red region at about 634 and 648 mμ (Fig. 5). It is possible that this 634 mμ material may correspond to the non-convertible protochlorophyll, found in etiolated leaves, with an absorption maximum at about 635 mμ.

The pigment pattern of the inner seed coats of Cucurbitaceae has many features in common with those of the developing plastid. The biosynthetic sequence in the seed coat appears, however, to be working in the reverse direction. The first detectable pigment is chlorophyll and this is replaced successively by protochlorophyll–protein complexes and a 'bacterial protochlorophyll'–protein complex. Unfortunately difficulties in the purification of the seed-coat protein complexes have prevented a close comparison of the proteins of holochromes from leaf and seed coat, but the detection of a pigment closely similar to 'bacterial protochlorophyll' in plant tissue supports the suggestion that magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester is involved in chlorophyll synthesis in both plants and photosynthetic bacteria.

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