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Chlorophylls. V.* Isolation of Chlorophylls *a* and *b* Using an Improved Two-phase Extraction Method Followed by a Precipitation and a Separation on a Sucrose Column

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Chlorophylls *a* and *b*, each of a high degree of purity, were isolated by means of a method consisting of an improved two-phase extraction method followed by a precipitation of the chlorophylls as aggregates and separation of the precipitated chlorophylls on a sucrose column. The spectroscopic properties of the chlorophylls purified by this means closely matched those previously described for pure chlorophylls. On using the method, 50 g of frozen clover leaves yielded approximately 20 mg of chlorophyll *a* and 3 mg of chlorophyll *b*. The advantage of the method is that the transformation of the chlorophylls into various alteration products can be minimized. The two-phase extraction method followed by precipitation also permits the preparation of large amounts of pure chlorophyll mixture, from which mixtures of pheophytins or pheophorbides *a* and *b* are easily obtained by conventional methods.

The chromatographic techniques employed for the separation and isolation of chloroplast pigments have been extensively reviewed by Holden,[2] Sestak,[3] Strain and Svec,[4,5] and Strain and Sherma.[6,7] Since 1971 several additional reports of investigations on this subject have appeared.[8–14] The inorganic adsorbents (silica gel, Kieselguhr), frequently used in these investigations, are not suitable for the separation of chlorophylls, since the adsorbents cause alteration of these pigments.[5,8,16,17] The reversed-phase partition thinlayer chromatography (RP-TLC), a method originally developed by Egger,[15] appears to be suitable for analytical purposes, but is unsuitable for preparative isolation, since the impregnating triglycerides are eluted from the chromatograms and interfere seriously with subsequent re-chromatography.[11] Hynninen and Ellfolk [14] isolated chlorophylls *a* and *b* by the Martin-Synge distribution (MSD).[18] In the final method developed, chloroplast pigments were extracted from frozen soybean leaves by means of a two-phase extraction method, and thereafter separated in the PBMF-system [light petroleum (8) – benzene (1)/methanol (6.75) – formamide (2.25)] employing a partition apparatus of 100 tubes.

The original two-phase extraction method [14] as performed in a separatory funnel and under an argon atmosphere has since proved to be somewhat cumbersome. In addition, the method developed suffers from the restriction that it requires the availability of a partition apparatus which is rather difficult to prepare. In the present publication, the author reports the combination of an improved two-phase extraction method with a precipitation and a separation of the precipitated chlorophylls on a sucrose column.

EXPERIMENTAL

Extraction of the pigments from plant material. Frozen clover leaves (a mixture of *Trifolium pratense*, *T. hybridum* and *T. repens*) were used as a source of chloroplast pigments. Immediately after harvesting, the leaves were transferred to the dark at a temperature of −30 °C and were stored under these conditions until

* Part IV. See Ref. 1.

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used. The improved two-phase extraction method described as follows was performed rapidly and in dimmed light.

Fifty grams of frozen clover leaves were suspended in a mixture of 400 ml of light petroleum (b.p. 60 – 80 °C) + 300 ml of methanol + 100 ml of formamide in a 2 l decanter. Before suspending the leaves, the solvent mixture was cooled to a temperature below 0 °C in a freezer. The suspension was immediately homogenized by means of an Ultra-Turrax homogenizer (1 min, full speed). The phases were allowed to separate while gently stirring the mixture occasionally. After phase separation, which took about 5 min, the upper phase (*ca.* 300 ml) was decanted off, transferred into a separatory funnel and washed several times with cold (0 °C) CO_2-free distilled water until the chlorophylls aggregated.

Separation of the chlorophylls from carotenoid pigments by precipitation. The suspension containing the aggregated chlorophylls, carotenes and xanthophylls was allowed to stand for 2 h or overnight at – 30 °C in a freezer to make the precipitation of the chlorophylls complete. After this, the precipitated chlorophylls were separated from the suspension by centrifugation (+4 °C, 10 300 *g*, 10 min.) The green-yellow supernatant containing the carotenoids and the possible small amount of pheophytin *a* and chlorophyll *a'* was carefully decanted off and discarded. The precipitate was washed with cold (– 30 °C) light petroleum (b.p. 60 – 80 °C, *ca.* 160 ml). After centrifugation, the wash-liquid was decanted off and the chlorophyll precipitate was dissolved in 160 ml of cold peroxide-free diethyl ether. The solution was evaporated to a volume of *ca.* 10 ml by means of a rotatory evaporator.

Chromatography on sucrose column. The conventional chromatographic procedure on a sucrose column,[4–6] was employed in the separations. Icing sugar (The Finnish Sugar Co., Helsinki, Finland), containing 0.69 % tricalcium phosphate, was used in the separations as provided, with the exception of one separation where the sugar was dried at +80 °C for 1 h before use. The sugar was mixed with the eluent (light petroleum, b.p. 60 – 80 °C, containing 0.5 % 1-propanol) to form a slurry,

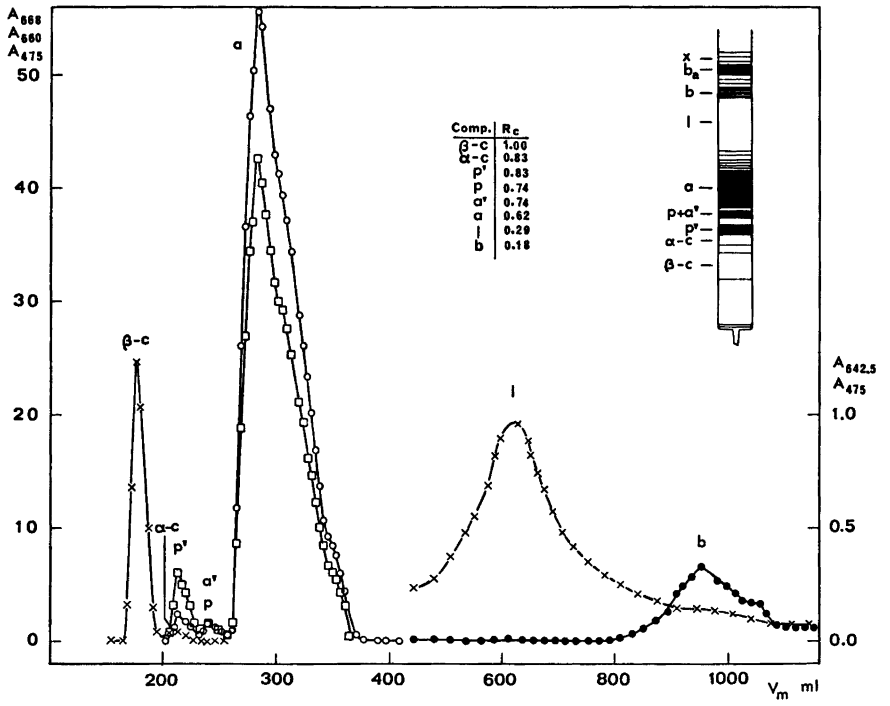

Fig. 1. Separation of chloroplast pigments on a sucrose column. Height of the sucrose layer = $h = 40.0$ cm. Flow rate = $u = 0.7$ ml/min. V_m = effluent volume. × = A_{475}, □ = A_{668}, ○ = A_{660}, and ● = $A_{642.5}$. Components from β-c to a: left-hand absorbance scale; components l and b: right-hand absorbance, scale. β-c = β-carotene, α-c = α-carotene, p' = pheophytin *a'*, p = pheophytin *a*, a' = chlorophyll *a'*. a = chlorophyll *a*, l = lutein, b = chlorophyll *b*, b_a = aggregated chlorophyll *b*, and x = xanthophyll The upper right-hand part of the figure shows the situation before β-c had emerged from the column.

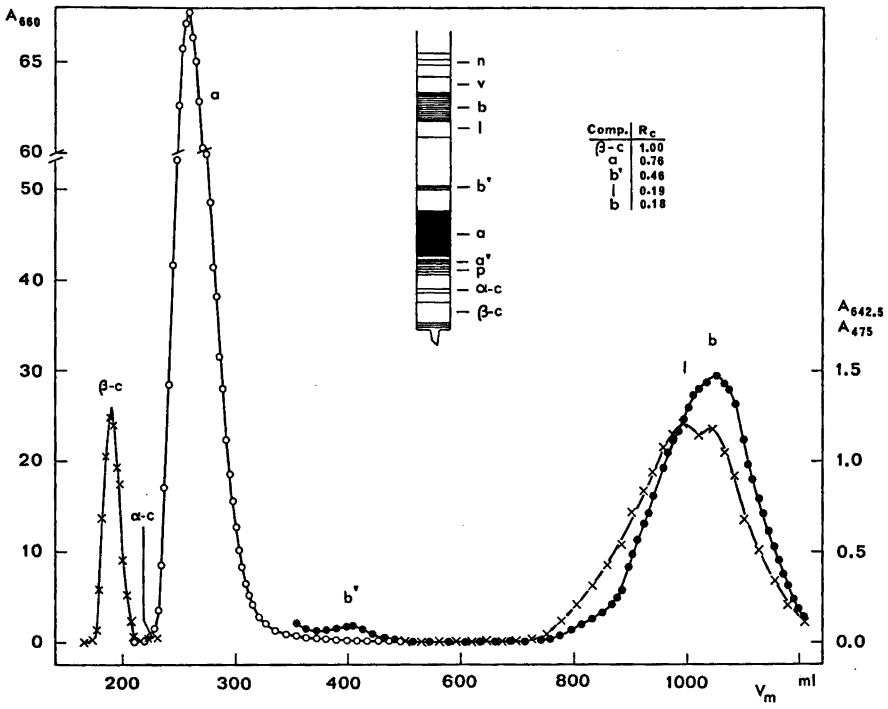

Fig. 2. Separation of chloroplast pigments on a sucrose column. $h = 42.8$ cm. $u = 0.6$ ml/min. × = A_{475}, ○ = A_{660}, and ● = $A_{642.5}$. Components β-c, α-c, and a: left-hand absorbance scale; components b′, l, and b: right-hand absorbance scale. β-c = β-carotene, α-c = α-carotene, a = chlorophyll *a*, b′ = chlorophyll *b*′, l = lutein, b = chlorophyll *b*, v = violaxanthin, and n = neoxanthin. The upper middle part of the figure shows the situation before β-c had emerged from the column.

which was then poured into a glass column having an inside diameter of 3.0 cm and a height of 50 cm. The sugar particles were allowed to settle until no descending movement of the layer was observed.

The pigments in the sample to be fractionated were first made soluble by the following procedure. The light petroleum or diethyl ether dispersion of the mixture of the pigments was evaporated under reduced pressure to near dryness and the residue was dissolved in 4 ml of the eluent. The solution was evaporated under vacuum to near dryness (evaporating was stopped when there were a few drops of the eluent left). The residue was dissolved in 2 to 4 ml of the eluent and the solution was introduced into the top of the column with a pipette. The pigments were allowed to absorb into the sugar layer to form a narrow initial zone and the column was then eluted until chlorophyll *b* had emerged completely (when the flow-rate was 0.6 ml/min, this took about 17 h). β-Carotene was used as a reference compound to facilitate the comparison of different separation results and the identification of the components.

The migration rate of a component was expressed in terms of R_c-values: R_c = the elution volume of β-carotene/the elution volume of the component. If β-carotene was not included in the mixture to be separated, the sugar column was first "calibrated" with a small amount of pure β-carotene before the actual separation was started. The absorbances (A) of the collected fractions were measured at selected wavelengths by means of a Perkin-Elmer 139 UV-VIS spectrophotometer. All chromatographic separations were performed in the dark at a temperature of about +4 °C.

Absorption spectra. The visible absorption spectra of the components were recorded utilizing a Cary Model 118 C spectrophotometer. Measurements were performed directly upon the effluent after the components had emerged from the column as well as after they had been transferred into ethyl ether by means of a Thunberg tube at reduced pressure.

Solvents. Formamide was purified by distillation *in vacuo*.[19] Depending on the purity of the commercial product, 2 to 4 distillations were required to obtain a formamide which reacted

green-yellow to bromothymol blue. This product was used without fractional crystallization. The other solvents used were of reagent grade purity and were employed without further purification.

RESULTS

Separation of chloroplast pigments. Fig. 1 presents the results from the fractionation of the chloroplast pigments obtained by the improved two-phase extraction method. Two yellow pigments, presumably β- and α-carotene, were first eluted from the column. The α-carotene was not completely separated from pheophytin *a'* which appeared as a grey lustrous zone. A small amount of green chlorophyll *a'* + pheophytin *a* was spectroscopically detected between pheophytin *a'* and chlorophyll *a*. Chlorophyll *a* was eluted as a broad zone and shows two small shoulders on the right hand side of its elution curve. These may have resulted from the keto-enol tautomerization [14–20] of the chlorophyll. Another probable reason for these shoulders is the small amount of diethyl ether used to dissolve the pigments in the sample. It was noted that the ether causes broadening and doubling of the zones at the beginning of the fractionation. The doubling effect may result from aggregation caused by the use of diethyl ether. The ether washes the small amount of 1-propanol from the top of the sugar layer whereafter the aggregation of chlorophyll occurs rapidly. This effect was observed clearly in the case of chlorophyll *b* which was divided into two zones. The yellow-green zone (soluble chlorophyll *b*) emerged from the column after the yellow lutein. The other zone (aggregated chlorophyll *b*) remained at the top of the sugar layer and appeared as a dark grey-green zone which was practically immobile. The aggregated chlorophyll *b* probably overlapped the other leaf xanthophylls (violaxanthin and neoxanthin). No chlorophyll *b'* could be spectroscopically detected between chlorophyll *a* and lutein. *Ca.* 30 mg of spectroscopically pure chlorophyll *a* (Table 1; a,b) was obtained from this fractionation ($V_m = 260 - 365$ ml). Chlorophyll *b* (less than 1 mg; $V_m = 850 - 1100$ ml), however, was contaminated by xanthophyll. The molar absorptivities used for the estimation of the amounts were 8.63×10^4 l

Table 1. Spectroscopic properties of the chlorophylls

Compound			Solvent	Peak positions (nm) and peak ratios (R^c) I		II		III		IV		V		S		Ss1		Ss2	
				nm	*R*	nm	*R*	nm	*R*	nm	*R*	nm	*R*	nm	*R*	nm	*R*	nm	*R*
a.	Chl[d] *a*	(1)[a]	Effl[d]	661.0	1.21	615.5	8.02	578.0	15.8	531.0	31.7	(495)[b]	(62.0)	429.0	1.00	411.0	1.54	381.0	2.50
b.	Chl *a*	(1)	Ee	659.0	1.29	613.0	7.97	575.0	12.7	528.5	18.2	(495)	(23.7)	428.0	1.00	411.5	1.47	380.0	2.36
c.	Chl *b*	(1)	Effl	642.5	2.83	596.0	17.0							451.0	1.00	427.0	2.53		
d.	Chl *a*	(2)	Effl	660.5	1.18	614.0	7.67	575.0	14.5	531.0	27.0	(494)	(48.0)	428.5	1.00	411.0	1.48	381.0	2.41
e.	Chl *a*	(2)	Ee	660.0	1.28	613.5	8.33	575.0	15.7	530.0	28.4	(496)	(52.5)	428.0	1.00	410.5	1.53	380.0	2.53
f.	Chl *a*	(3)	Effl	660.0	1.23	614.0	8.03	577.0	15.4	530.5	30.8	(496)	(61.7)	428.5	1.00	410.5	1.53	381.0	2.50
g.	Chl *a*	(3)	Ee	659.0	1.31	613.0	7.80	575.0	16.7	529.0	30.0	(495)	(60.0)	428.0	1.00	409.5	1.58	379.0	2.62
h.	Chl *b*	(3)	Effl	642.0	2.77	594.0	15.3							451.0	1.00	427.0	2.88		
i.	Chl *b*	(3)	Ee	642.5	3.06	592.0	18.1							451.0	1.00	427.0	2.90		

[a] Number in parentheses refers to figure number. [b] Peak positions and ratios stated in parentheses are approximate. [c] R = quotient of absorbance at Soret band divided by absorbance at wavelength indicated; S = Soret band, Ss1 = 1st satellite of Soret band, Ss2 = 2nd satellite of Soret band; [d] Chl = chlorophyll, Ee = diethyl ether, Effl = 0.5 % 1-propanol in light pertoleum, 60 – 80 °C.

mol^{-1} cm^{-1} for chlorophyll *a* and 5.61×10^4 l mol^{-1} cm^{-1} for chlorophyll *b*.[4,21]

The above fractionation was repeated on a fresh pigment extract with the difference that no diethyl ether was used in the sample. The pigments were made soluble in this case using the procedure described in EXPERIMENTAL. The elution curves of chlorophylls *a* and *b* had no shoulders in this case (Fig. 2). Neither could any aggregated chlorophyll *b* be detected at the upper part of the column. Chlorophyll *b* was, however, almost completely overlapped by lutein, and the pheophytins as well as chlorophyll *a'* cannot be distinguished in the effluent curve (they were observed as partly resolved on the column). A small amount of chlorophyll *b'* could be detected in this case between chlorophyll *a* and lutein.

On drying the sugar at +80 °C for 1 h before packing the column, chlorophyll *b* was eluted from the column before lutein and violaxanthin. The resolution of chlorophyll *b* from lutein was, however, poor also in this case.

Separation of the chlorophylls after precipitation from the extract. In the final method developed for the isolation of chlorophylls *a* and *b*, the pigments were first precipitated from the chloroplast extract obtained by the two-phase extraction method (see EXPERIMENTAL) and thereafter separated on a sucrose column. As can be observed from Fig. 3, the separation problem is remarkably simplified on precipitating the chlorophylls. This procedure effectively separates chlorophylls *a* and *b* from the carotenoids, pheophytins and chlorophyll *a'*. None of these pigments could be detected either in the effluent or on the column. Chlorophyll *a* was eluted from the column as a narrow peak. Traces of chlorophyll *b'* were spectroscopically detected at $V_m = 310$ ml (note that the absorbance scale for chlorophyll *b'* and *b* is different from that for chlorophyll *a*). The front part of

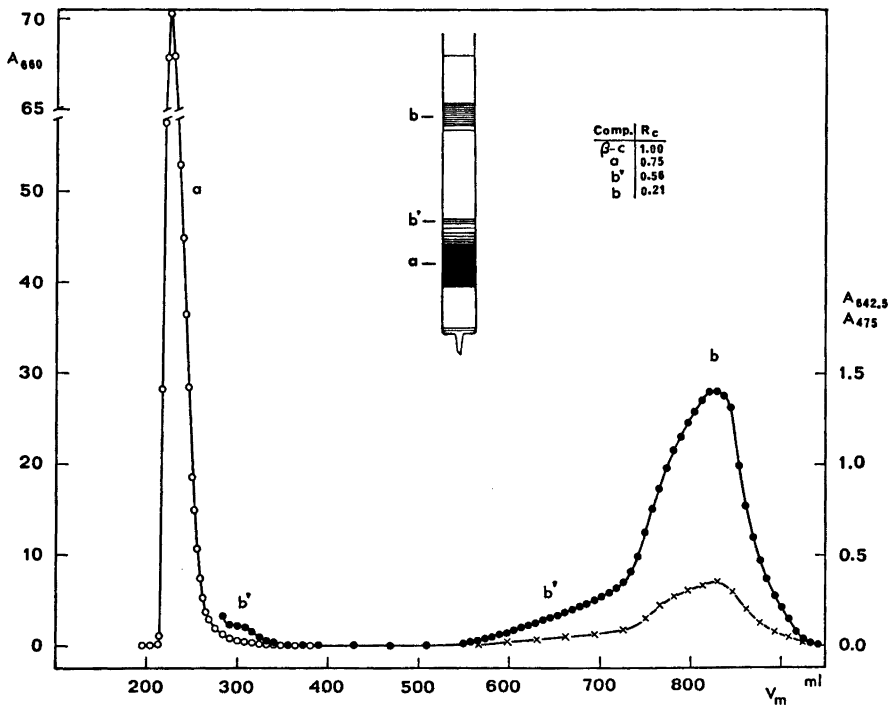

Fig. 3. Separation of chlorophylls on a sucrose column. $h = 40.0$ cm. $u = 0.6$ ml/min. ○ = A_{660}, ● = $A_{642.5}$, and × = A_{475}. Component a: left-hand absorbance scale; components b' and b: right-hand absorbance scale. a = chlorophyll *a*, b' = chlorophyll *b'*, and b = chlorophyll *b*. The upper middle part of the figure shows the situation before a had emerged from the column.

the chlorophyll *b* zone was, however, broadened. This phenomenon indicates that a slow transformation of chlorophyll *b* to chlorophyll *b'* had occurred during the separation. This effect can be minimized by using a shorter column or by removing the sugar layer with chlorophyll *b* adsorbed on it through the upper end of the column and then eluting chlorophyll *b* from the sugar with diethyl ether. The spectroscopic properties of chlorophylls *a* and *b* from the fractionation of Fig. 3 are presented in Table 1; f–i. These properties closely matched those previously [14] reported for chlorophylls *a* and *b* obtained by multiple liquid-liquid partition. Approximately 20 mg of chlorophyll *a* (V_m = 214 – 256 ml) and 3 mg of chlorophyll *b* (V_m = 669 – 901 ml), each of a high degree of purity, were yielded by the fractionation in Fig. 3. Both chlorophyll preparations reacted positively to the phase test.

DISCUSSION

Owing to the susceptibility of the chlorophylls to a number of chemical transformations which yield various alteration products, the isolation of pure chlorophylls is known to be a difficult task.[4] These transformation reactions take place during extraction as well as during fractionation. In 1966 Strain and Svec [4] wrote: "The preparation and isolation of relatively pure chlorophylls..., require care and have been accomplished by only a few investigators". They gave nine references to the investigators capable of doing the isolation.

Among the chemical transformations of the chlorophylls, the "isomerization" to chlorophylls *a'* and *b'*[20,22–24] and the allomerization,[25,26] which is essentially oxidation at C-10 of the isocyclic ring V of chlorophyll, occur very easily in solvents containing methanol, ethanol or 1-propanol. The formation of chlorophylls *a'* and *b'* is known to occur also in pyridine.[20,22] Since these solvents are frequently included in solvent mixtures used for extraction and separation, special care is required to avoid the transformations.

The conventional procedures, in which 80 % acetone or light petroleum – methanol is employed for the extraction of chloroplast pigments, were consistently found to lead to considerable amounts of allomerization or other alteration products.[14] This was explained by the reaction scheme,[14] according to which the allomerization as well as the "isomerization" to chlorophylls *a'* and *b'* are preceded by the enolization reaction. The enolization was supposed to occur in electron donor solvents such as ethyl ether, acetone, pyridine, dioxane, tetrahydrofuran and alcohols in which the chlorophylls occur as monomers. The free enol formed was subsequently presumed to undergo further transformation depending on the polarity of the solvent. In Lewis bases the free enol is probably ionized to the labile enolate anion which is rapidly attacked by oxygen to yield a cyclic peroxide derivative as an intermediate (assuming an addition mechanism [26] for this reaction, one should take into account the possibility that also the free enol may react with $^1O_2$). The peroxide derivative thereafter converts into various oxidation products whose nature depends on the solvent.[26] In solvents possessing relatively weak hydrogen bonding capabilities such as pyridine, tetrahydrofuran and light petroleum containing a small amount of 1-propanol the free enol was assumed to convert to the hydrogen chelate. This was considered to be identical with the chlorophyll' isomer.[20]

On the basis of the foregoing, it is understandable why we so easily get allomerization products or chlorophyll prime isomers on trying to isolate chlorophylls. We have to use polar solvents in the extraction, since on employing nonpolar solvents for this purpose, very little chlorophyll is released from the chloroplasts.[4] Then, however, more or less of the free enol is produced during the extraction. Since water is, as a rule, included in the extracting solvent, the further transformation of the free enol is prevented by the formation of hydrogen bonds between water (or formamide) and the free enol. In subsequent phases of the isolation, the hydrogen bonded water molecule may be stripped off and the free enol may then convert to the enolate anion or the prime isomer.

The reported fact that salts accelerate the allomerization [27] also becomes now comprehesible. On using concentrated salt solutions in the extraction, the salt ions compete with the free enol for water molecules. Consequently, water is removed and the free enol becomes susceptible to transformations. Therefore, con-

centrated salt solutions should not be used in the extraction of the pigments (it has been a common practice in the conventional extraction procedures to add saturated sodium chloride solution in order to transfer the pigments from the polar phase into the light petroleum phase!).

To minimize the enolization of chlorophyll, the extraction procedure should be rapid. This requirement is fullfilled by the two-phase extraction method developed by the present author. In this method, the pigments are loosened from the chloroplasts by the polar lower phase and transferred immediately into the nonpolar upper phase, where the chlorophylls do not undergo alteration. In its improved form, the two-phase extraction method has proved to be very easy and rapid to perform. No argon or nitrogen atmosphere is necessary in this modification owing to its rapidity and the low temperature used.

The precipitation of the chlorophylls as aggregates is an efficient procedure for the separation of the chlorophylls from carotenoids. This separation is required before the chromatography on a sucrose column, since a weak point of the chromatographic procedure is that chlorophyll *b* is overlapped by lutein. This difficulty can be eliminated by extracting the lutein from the light petroleum solution of the pigments with aqueous methanol.[4] However, this method is not recommended by the present author, since, although the allomerization does not occur in aqueous methanol, the enolization does, and when the water molecules are removed, the free enol becomes susceptible to transformation.

The time requirement of the new isolation method is comparable to that of the liquid-liquid partition method.[14] Starting from the packing of the sucrose column, approximately 24 h were needed to obtain pure chlorophylls *a* and *b*.

The scale of the isolation method described can be easily increased. It is possible to prepare large amounts of chlorophyll mixture utilizing the two-phase extraction method followed by precipitation. Mixtures of pheophytins *a* and *b* as well as pheophorbides *a* and *b* are easily obtained from a pure chlorophyll mixture by conventional methods.[14]

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