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Chlorophylls

I. Separation and Isolation of Chlorophylls *a* and *b* by Multiple Liquid-Liquid Partition

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The fractionation of chloroplast pigments by means of multiple liquid-liquid partition has been studied utilizing three types of solvent system: petroleum ether-ethanol-formamide (PEF), petroleum ether-methanol-formamide (PMF), and petroleum ether-benzene-methanol-formamide (PBMF). Of these, the PBMF-system was found to be most suitable for the separation and isolation of chlorophylls. The PMF-system, although it was most selective, appeared to be unsuitable for this purpose due to the formation of a 10-methoxy-lactone derivative from chlorophyll *a* during fractionation. This allomerization reaction was sensitive to the polarity of the lower phase and did not occur when the PBMF-system was employed, provided the fractionation time did not exceed 24 h. According to the interpretation presented for this observation, the enolization reaction is the first and also the rate-limiting step in the reaction sequence of the allomerization.

In order to extract chloroplast pigments from plant material, a "two-phasic" method was developed. Conventional extraction methods invariably led to the formation of considerable amounts of 10-hydroxy-chlorophylls, these allomerization products being produced in all probability by the enzymatic oxidation of chlorophylls during the extraction. In the "two-phasic" extraction 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 the final method developed, the pigments were extracted from frozen soybean leaves by means of the PMF-system and thereafter separated in the PBMF-system utilizing a partition apparatus of 100 tubes. By this method, chlorophyll *a* and chlorophyll *b*, each of a high degree of purity, were yielded in approximately 24 h, including the time required for the extraction of the pigments from the plant material. The ratio of the absorbance at the Soret band wavelength to the absorbance at the chlorin band wavelength (A_S/A_I) was, in diethyl ether, 1.32 for chlorophyll *a* and 2.88 for chlorophyll *b*. The isolated chlorophyll preparations have been kept frozen in cyclohexane at $-15°C$ for a period of six months and no changes have been observed in their behaviour.

During extraction and fractionation, the chlorophylls are susceptible to a number of chemical transformations which yield various alteration products or "isomers".[1] These transformations include keto-enol tautomerism, epimerization, enzymatic or nonenzymatic oxidation (allomerization), solvolysis, pheophytinization, and photochemical bleaching. Due to these transformations, the isolation of pure chlorophylls from plant material is a difficult task. When chromatographic methods [1–10] are employed for this purpose, two or three successive fractionations are generally required in order to isolate relatively pure chlorophyll *a* and chlorophyll *b*. Even such an extensive purification as this apparently does not guarantee that a chlorophyll preparation of high purity will be obtained.[11,12] It should also be emphasized in this context that *neither* the crystallinity [5,6] of, *nor* a positive Molisch phase test for a chlorophyll preparation is reliable evidence of its purity.

Liquid-liquid partition methods have, in only a few cases, been utilized for the fractionation of chlorophylls. Willstätter and co-workers [13,14] obtained partially purified chlorophyll *a* and chlorophyll *b* by successive distribution of the pigments between petroleum ether and aqueous methanol in a separating funnel. The same solvent system (or a slight modification of it) was later employed for the fractionation of chlorophylls by means of either countercurrent distribution (CCD) [15–17] or Martin-Synge distribution (MSD).[18] However, the results of these fractionations have not been very promising due to the low selectivity of the utilized solvent systems and to the great tendency of the chlorophylls to undergo transformation.

In the present investigation, the authors have undertaken a detailed study on the applicability of the partition principle to the separation and isolation of chlorophylls. As a result of this study, a method yielding both highly-purified chlorophyll *a* and highly-purified chlorophyll *b* in one fractionation can now be described.

EXPERIMENTAL

Equipment and solvents. Multiple liquid-liquid partition was performed utilizing the apparatus developed by Hietala.[19] The properties and operation of this apparatus, as well as methods for the calculation of theoretical distribution curves and partition coefficients, have been described in an earlier publication.[20] The upper phase was employed as the mobile solvent in all fractionations described in this publication. The phase ratio among the different fractionations varied from 0.21 to 0.33. A shaking frequency of 22 cycles/min, an amplitude of $\pm 45°$ and a flow rate of $1-2$ ml/min were utilized in the present study.

Three types of solvent system were employed:

(1) The PEF-system: petroleum ether(3)/ethanol(2)-formamide(1);

(2) The PMF-system: petroleum ether(4)/methanol(3)-formamide (1); and

(3) The PBMF-system: petroleum ether(8)-benzene(1)/methanol(6.75)-formamide (2.25).

The values in parentheses state the initial volume ratios of the solvent components. Occasionally, oxalic acid was added to the lower phase, since it has been proclaimed that allomerization would thereby be prevented.[13] The concentration of the oxalic acid was the same as that employed by Arn *et al.*:[18] 10 mg/l of lower phase.

The solvents required for the distribution apparatus, for the extraction of pigments from the plant material and for spectroscopy were of reagent grade purity. They were utilized as commercially supplied, with the exceptions of diethyl ether and formamide.

The latter was purified immediately before use according to the method of Verhoek,[21] except for the fractional crystallization. The final product resulted in a green to green-yellow colour upon reaction with bromthymolblue. The diethyl ether was treated with a concentrated solution of ferrous sulphate, following which the ether was washed, dried and distilled. The employed boiling point fraction of the petroleum ether was 60 – 80°C. Ethanol (99.5 %) was purchased from Alko Oy (Finland).

A Cary Spectrophotometer Model 15 was used to record the absorption spectra and a Beckman DU Spectrophotometer to measure single absorbances.

Extraction of the pigments from plant material. Frozen soybean leaves were generally utilized as a source of choloroplast pigments. Immediately after harvesting, the leaves were transferred to the dark at a temperature of −15°C and were stored under these conditions until used. For the purpose of comparison, however, fresh or dried leaf material was occasionally employed. The extraction procedures described as follows were performed rapidly and in dimmed light. Methods 1 and 2 describe the extraction of the pigments by slightly modified conventional procedures. Since the conventional procedures consistently led to the formation of considerable amounts of allomerization or other alteration products, regardless of how quickly or carefully the extraction was performed, a "two-phasic" extraction method was developed (Method 3).

Method 1. Sixty grams of frozen soybean leaves were suspended in 400 ml of a petroleum ether(3)-methanol(1) mixture. The suspension was homogenized by means of a Waring Blendor (1 min) and filtered in a Büchner funnel with the aid of suction. The residue in the funnel was re-extracted with 400 ml of the petroleum ether-methanol mixture. The two filtrates were then transferred into a dark bottle, 1 l of concentrated sodium chloride solution was added and the mixture shaken gently. After a few minutes standing, the petroleum ether containing the chloroplast pigments was collected into the upper phase. The phases were then separated by means of a separatory funnel and the lower phase was re-extracted with 100 ml of pure petroleum ether. The related petroleum ether extracts were then washed three times with distilled water (pH 5) and subsequently evaporated to a volume of 38 ml by means of a rotatory evaporator.

Method 2. Two hundred grams of frozen soybean leaves were crushed in the cold and then extracted with 1 l of 80 % (w/w) acetone. The resulting suspension was allowed to stand, with occasional stirring, for 1 h at 4°C, after which it was filtered in a Büchner funnel. The residue remaining in the funnel was re-extracted with 900 ml of 80 % acetone. Following this re-extraction, the leaf material still retained a green colouring. The two filtrates were subsequently treated as in Method 1. The final volume of the pigment-containing petroleum ether solution was 10 ml.

Method 3. One hundred grams of frozen soybean leaves were crushed in the cold and suspended, while in a separatory funnel, in a mixture of 600 ml petroleum ether + 600 ml methanol(3)-formamide(1). Argon gas was continuously bubbled throughout the phase system both before the leaves were suspended therein and also during the suspending procedure. The funnel was then tightly stoppered and the suspension vigorously shaken by hand for 20 min. After phase separation, the lower phase was allowed to drain from the funnel. The upper phase was then permitted to empty directly through a filter into a glass cylinder. The filtrate was washed three times with distilled water and subsequently evaporated to a volume of 15 ml.

Preparation of some chlorophyll derivatives. Magnesium-free chlorophyll derivatives were prepared by treating an ethyl ether solution of the chlorophyll with 13 % hydrochloric acid. The phytyl group was removed by hydrolysis of the ester in 30 % hydrochloric acid. Methyl esters of the derivatives were prepared by means of diazomethane. The ethereal solution of diazomethane was prepared from nitrosomethylurea by saponification, as described by Eistert.[22]

RESULTS

Fractionation employing the PEF-system. The results of the first fractionation experiment are presented in Fig. 1. Several components were resolved by employing the PEF-system with 70 fractionation tubes. However, many of the green components were allomerization products of chlorophylls, since they reacted negatively to phase testing.

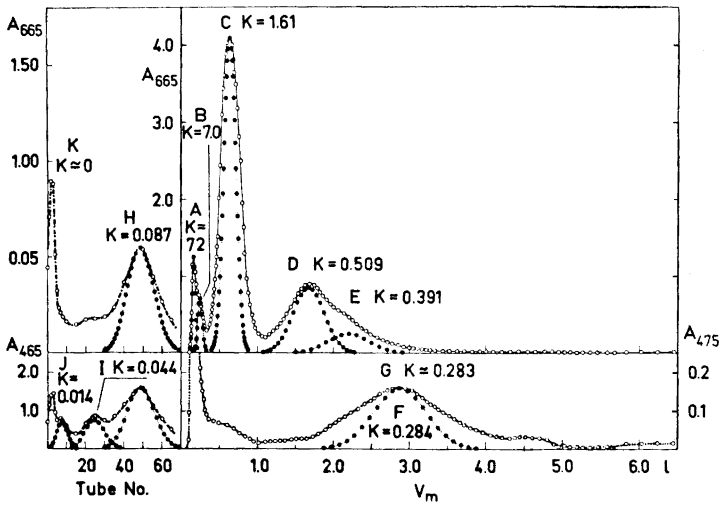

Fig. 1. Separation of chloroplast pigments employing the PEF-system. 10 ml of the extract prepared according to Method 1 were sampled into tubes r = 0,..,3. Number of fractionation tubes utilized $= N = 70$. Average volume of the mobile phase in a partition unit $= v_m = 2.31$ ml; average volume of the stationary phase in a partition unit $= v_s = 11.19$ ml. Total volume of effluent eluted from the apparatus $= V_m = 6443$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring the absorbances of the effluent fractions or of the lower phases in the tubes: — $= A_{665}$, ··· $= A_{475}$, -·- $= A_{652}$, and -- $= A_{465}$. A = β-carotene, B = pheophytin *a*, C = chlorophyll *a*, D = 10-hydroxy-chlorophyll *a*, E = chlorophyll *b*, F = lutein, G = Mg-*b*-purpurin 7-lactone-alkyl ether-methylphytyl ester, H = 10-hydroxy-chlorophyll *b*, I = Mg-unstable rhodin-methylphytyl ester, J = neoxanthin, and K = chlorophyllide.

In the effluent series, the first two components, A and B, eluted from the apparatus, were spectroscopically identified as β-carotene and pheophytin *a*, respectively. Components A and B were only partially resolved, due to their large partition coefficients ($K_A = 72.3$, $K_B = 7.0$). The blue-green component C yielded both a positive Molisch phase test and an absorption spectrum closely resembling that of pure chlorophyll *a* (Table 1; a). The green component D was identified as 10-hydroxy-chlorophyll *a* upon the basis of the following characteristics: (1) the visible absorption spectrum of D (Table 1; d) resembled that of chlorophyll *a*;[23,24] (2) the phase test was negative; and (3) the extraction of D from an ethyl ether solution into 10 % aqueous potassium hydroxide by shaking for 2 h converted the pigment into Mg-unstable chlorin-monomethyl ester and a small amount of Mg-purpurin 18.[25] These products were identified both spectroscopically and by esterification with diazomethane, which yielded a mixture of Mg-purpurin 7-trimethyl ester and Mg-purpurin 18-monomethyl ester.[25] Component D has since been found to be identical with the compound isolated by means of multiple liquid-liquid partition from an allomerization mixture of chlorophyll *a* in methanol.[26] Furthermore, it appears evident that component D is identical to the chlorophyll derivative found by Arn *et al.*[18]

Table 1. Spectroscopic properties of the chlorophylls and their derivatives.

Compound [a]	Solvent	Peak positions (nm)								Peak ratios	
		I	II	III	IV	V	VI	S	Ss1	A_S/A_I	A_S/A_{Ss1}
a. Chlorophyll *a* (1C)	Pe	661.5	615	577	532	498		429.0	412	1.31	1.33
b. Chlorophyll *a* (2C)	Ee	661.0	615	577	532	498		430.0	410	1.32	1.59
c. Chlorophyll *a* (4C)	Ee	660.0	614	576	530	497		429.0	409	1.32	1.56
d. 10-Hydroxy-chlorophyll *a* (1D)	Pe	661.5	614	575	530	499		428.0	413	1.27	1.20
e. Mg-purpurin 7-lactone-alkyl ether-methylphytyl ester (3D)	Pe	652.5	608	566	523			417.0		1.82	
f. Mg-unstable chlorin-methylphytyl ester (4I)	Pe	654.0	608	566	523			418.0		1.88	
g. Unstable chlorin(-monomethyl ester)	Ee	668.0	611	560	528	498	468	399.0		2.50	
h. Pheophytin *a*	Ee	667.0	609	560	532	503	467	408.0		2.14	
i. Pheophorbide *a*	Ee	667.0	609	560	533	504	467	408.0		2.07	
j. Chlorophyll *b* (3F)	Ee	642.0	593					453.0	430	2.89	2.64
k. Chlorophyll *b* (4D)	Ee	642.5	595					452.5	430	2.88	2.55
l. 10-Hydroxy-chlorophyll *b* (1H)	Pe	645.5	600					452.0	430	2.97	1.80
m. Mg-*b*-purpurin 7-lactone-alkyl ether-methylphytyl ester (1G)	Pe	631.5	585	536				443.0		4.42	
n. Mg-unstable rhodin-methylphytyl ester (1I)	Ee	630.0	584	535				443.0		4.58	
o. Unstable rhodin(-monomethyl ester)	Ee	653.0	598	522				427.0		6.53	
p. Pheophytin *b*	Ee	654.0	599	555	525			433.0	412	4.81	2.28
q. Pheophorbide *b*	Ee	655.0	599	555	525			433.0	412	4.83	2.04

[a] The number and letter in parentheses refer, respectively, to the figure number and to the component in that figure.
S = Soret band, Ss1 = 1st satellite of the Soret band; Ee = ethyl ether, Pe = petroleum ether.

and assumed by them to be an "artifact" produced by the action of oxalic acid.

A small amount of material yielding a positive phase test was eluted at 2250 ml (component E), although it was poorly resolved from components D and F and could not be spectroscopically identified. The similarity of its partition coefficient ($K_E = 0.391$) to that of component E in Fig. 2 ($K_E =$

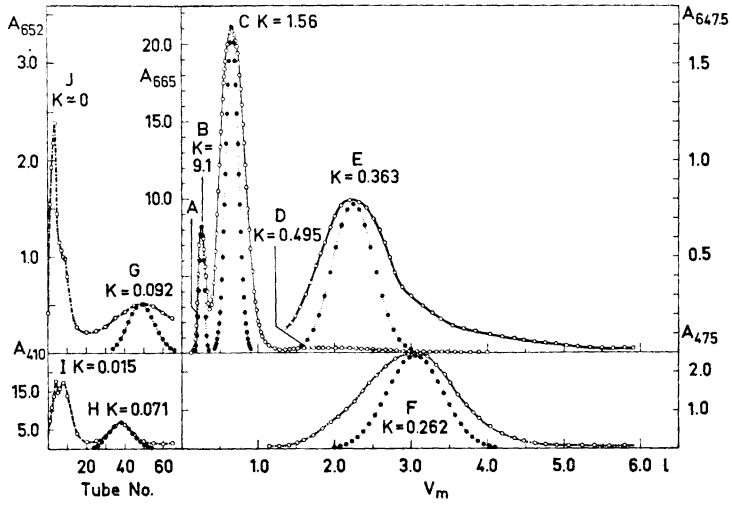

Fig. 2. Separation of chloroplast pigments employing the PEF-system. 10 ml of the extract prepared according to Method 2 were sampled into tubes r = 0,..,4. $N = 70$. $v_m =$ 2.60 ml; $v_s = 10.90$ ml. $V_m = 5910$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring the absorbances of the effluent fractions or of the lower phases in the tubes: – $= A_{665}$, — $= A_{647.5}$, ··· $= A_{475}$, -·- $= A_{652}$, and —— $= A_{410}$. A = β-carotene, B = pheophytin *a*, C = chlorophyll *a*, D = 10-hydroxy-chlorophyll *a*, E = chlorophyll *b*, F = lutein, G = 10-hydroxy-chlorophyll *b*, H = violaxanthin, I = neoxanthin, and J = chlorophyllide.

0.363) indicates, however, that component E of Fig. 1 is probably a small amount of intact chlorophyll *b*.

Component F was spectroscopically identified as xanthophyll (lutein). A slight absorption peak (632 nm) within the lutein fraction ($V_m = 3000 - 3825$ ml) revealed that this fraction was overlapped by some chlorophyll derivative. Indeed, the fractions eluted within the range $V_m = 3925 - 5000$ ml contained principally this derivative, which was purified by extracting the lutein from the petroleum ether solution by means of methanol-water. The derivative yielded a negative phase test and was spectroscopically very similar to the pigment characterized as a 10-methoxy-lactone derivative (Mg-*b*-purpurin 7-lactone-methyl ether-methylphytyl ester) by Pennington *et al.*[24] (Table 1; m). Further evidence regarding the 10-alkoxy-lactone nature of component G was given by the following properties: (1) the pigment was not extracted from an ether solution by 10 % aqueous potassium hydroxide;[25] (2) the visible absorp-

tion spectrum of the magnesium-free derivative resembled that of unstable rhodin monomethyl ester; and (3) treatment with diazomethane did not convert the pigment into Mg-*b*-purpurin 7-triester.[25] The pigment has since been found to closely resemble the primary allomerization product of chlorophyll *b* in methanol.[26]

At this stage, several pigments were still remaining in the apparatus, as shown in Fig. 1. Component H is undoubtedly 10-hydroxy-chlorophyll *b*, since it possessed properties analogous to those of 10-hydroxy-chlorophyll *a*, *viz.* (1) the visible absorption spectrum (Table 1; l) resembled that of chlorophyll *b*; (2) the phase test was negative; and (3) the agitation of an ethyl ether solution of H with 10 % aqueous potassium hydroxide for 2 h resulted in derivatives (Mg-unstable rhodin monomethyl ester and Mg-*b*-purpurin 18) which were soluble in the aqueous alkali phase. Component I was characterized as Mg-unstable rhodin methylphytyl ester. Its visible absorption spectrum (Table 1; n) closely resembled that of component G. The other components remaining in the apparatus could not be characterized with certainty, since they were only partially resolved. Fraction J appeared to contain a carotenoid (neoxanthin) as its principal component. The chlorophyllides, *etc.*, remained at the sampling end of the apparatus.

It was concluded from the results presented in Fig. 1 that oxidation of the chlorophylls had occurred primarily during extraction of the pigments from the plant material, since deviations from the theoretical values were rather small in the case of the chlorophyll *a* fraction (component C). Therefore, it was considered necessary to employ a different method of extracting the pigments from the plant material.

Fig. 2 presents the results obtained from the second fractionation. In this case, the pigments were extracted according to Method 2 and again separated by means of the partition apparatus utilizing PEF as the solvent system. The relative amount of 10-hydroxy-chlorophyll *a* (component D) separated in this instance is much smaller than that distinguishable as a result of the first fractionation (Fig. 1). More intact chlorophyll *b* (component E) was probably also obtained in this case. However, the concentration profile of chlorophyll *b* reveals a lengthy tail, thus indicating that a slow transformation of chlorophyll *b* to a 10-ethoxy-lactone derivative had probably occurred during the fractionation.

Fractionation employing the PMF-system. Since the selectivity of the PEF-system was unsatisfactory, especially in regard to chlorophyll *b* and lutein, a second solvent system was utilized. Preliminary determination of the partition coefficients for chlorophyll *a*, chlorophyll *b* and lutein indicated that the PMF-system would be considerably more selective than the PEF-system. Therefore, the former solvent system was employed in the fractionation to be described in this section. In order to avoid the occurrence of allomerization during extraction of the pigments from the plant material, a "two-phasic" extraction method (Method 3) was simultaneously introduced.

The results subsequent to these alterations in the conditions of extraction and fractionation are presented in Fig. 3, where it can be seen that the PMF-system is indeed considerably more selective than the PEF-system. Chlorophyll *b* and lutein (components F and G) are now almost completely separated

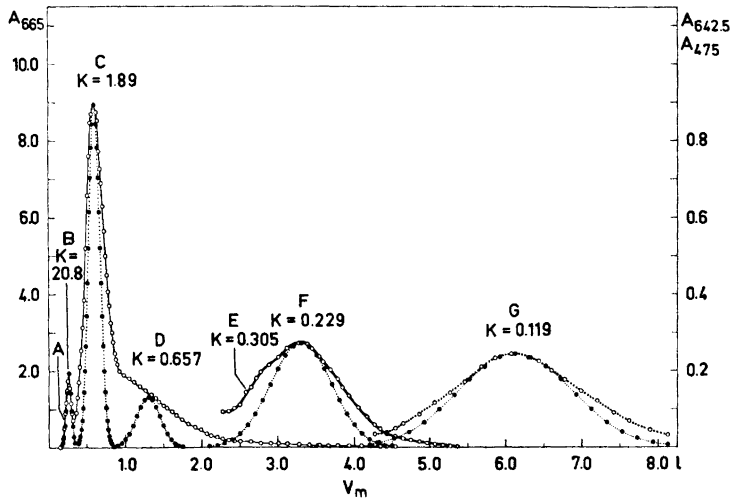

Fig. 3. Separation of chloroplast pigments employing the PMF-system. 15 ml of the extract prepared according to Method 3 were sampled into tubes r = 0,..,4. $N = 70$. $v_m = 3.27$ ml; $v_s = 10.23$ ml. $V_m = 8125$ ml; flow rate = 2 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring the absorbances of the effluent fractions: — $= A_{665}$, — — $= A_{642.5}$, and ··· $= A_{475}$. A = β-carotene, B = pheophytin *a*, C = chlorophyll *a*, D = Mg-purpurin 7-lactone-methyl ether-methylphytyl ester, E = 10-hydroxy-chlorophyll *a*, F = chlorophyll *b*, and G = lutein.

from each other. However, a change has occurred in the concentration profile of chlorophyll *a*, the character of which clearly indicates that chlorophyll *a* (C) has undergone a partial transformation to some derivative (D) during the fractionation. The material eluted at $V_m = 600$ ml yielded a clearly positive phase test as well as an absorption spectrum closely resembling that of pure chlorophyll *a*. The material eluted at $V_m = 1300$ ml gave, on the contrary, a negative phase test and was spectroscopically quite different from chlorophyll *a*. These facts indicated that the allomerization of chlorophyll *a* had now occurred in the apparatus. The absorption spectrum of pigment D (Table 1; e) was the same as that described by Holt [25] for Mg-purpurin 7-lactone-methyl ether-dimethyl ester and by Pennington *et al.*[24] for the 10-methoxy-lactone derivative of chlorophyll *a* (Mg-purpurin 7-lactone-methyl ether-methylphytyl ester). In a manner analogous to that of the 10-alkoxy-lactone derivative of chlorophyll *b*, component D had the following properties: (1) it was not extracted from an ethyl ether solution by 10 % aqueous potassium hydroxide; (2) the visible absorption spectrum of the magnesium-free derivative closely matched that of the unstable chlorin monomethyl ester; and (3) treatment with diazomethane did not convert the pigment into Mg-purpurin 7-triester. Component D has since been found to be identical with the primary allomerization product of chlorophyll *a* in methanol.[26]

Although chlorophyll *a* appeared to be very unstable in the PMF-system, chlorophyll *b* (component F), on the contrary, revealed practically no tailing

at all in this system, thus suggesting some difference in the allomerization behaviour of the two chlorophylls. The fractions eluted within the range $V_m = 3250 - 4000$ ml contained spectroscopically pure chlorophyll *b* (Table 1; j). The initial portion of the concentration zone, however, contained as impurity a small amount of 10-hydroxy-chlorophyll *a*, which was indicated by the measurement of the red absorption peak of the fractions eluted from 2300 to 3250 ml. This small amount of impurity was probably formed during the extraction of the pigments from the plant material, as in the previous experiments.

Fractionation employing the PMF-system was repeated with a fresh pigment extract prepared according to Method 3. The results were similar to those presented in Fig. 3. The addition of oxalic acid to the lower phases of the solvent systems utilized in the extraction and fractionation procedures was of no help in preventing the formation of the 10-methoxy-lactone derivative of chlorophyll *a*.

Since the 10-alkoxy-lactone derivative did not form from chlorophyll *a* during the first two fractionations in which the PEF-system was employed, it was concluded that the transformation was correlated with the polarity of the lower phase in the fractionation solvent system. The lower phase of the PMF-system is evidently more polar than the lower phase of the PEF-system,

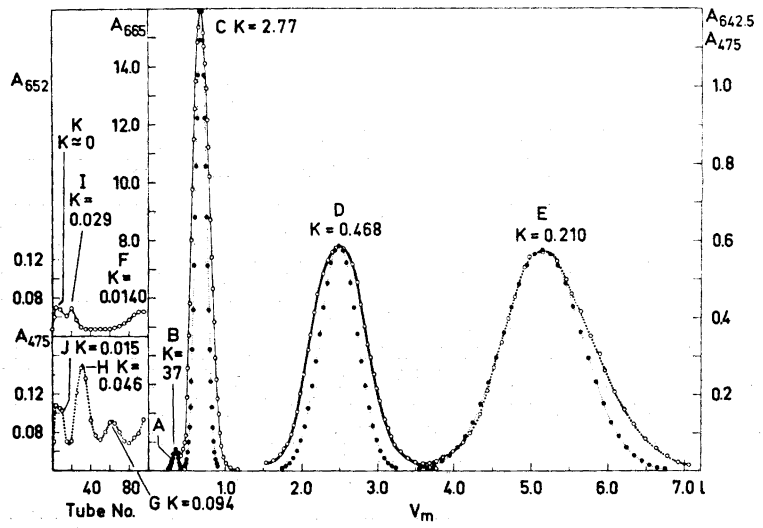

Fig. 4. Separation of chloroplast pigments employing the PBMF-system. 15 ml of the extract prepared according to Method 3 were sampled into tubes r = 0,..,4. N = 100. $v_m = 3.25$ ml; $v_s = 10.25$ ml. $V_m = 7050$ ml; flow rate = 2 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring the absorbances of the effluent fractions or of the lower phases in the tubes: — = A_{665}, — = $A_{642.5}$, ··· = A_{475}, and -·- = $A_{652\ nm}$. A = β-carotene, B = pheophytin *a*, C = chlorophyll *a*, D = chlorophyll *b*, E = lutein, F = 10-hydroxy-chlorophyll *b*, G = one form of violaxanthin, H = a second form of violaxanthin, I = Mg-unstable chlorin-methylphytyl ester, J = neoxanthin, and K = chlorophyllide.

since methanol is only partially miscible with petroleum ether (60 – 80°C), whereas ethanol is completely miscible with it. In order to test this hypothesis, an additional solvent component was sought which would induce greater non-polarity within the lower phase of the PMF-system without appreciably impairing the selectivity of the system as a whole. Such an additional solvent component appeared to be benzene.

Fractionation employing the PBMF-system. The results of a fractionation utilizing the PBMF-system are presented in Fig. 4. The pigments were extracted from the plant material in exactly the same manner as for the experiment presented in Fig. 3. In spite of this fact, the results of Fig. 4 differ considerably from those of Fig. 3. Firstly, the amount of pheophytin *a* fractionated was negligible with the PBMF-system, and, secondly, utilizing this solvent system, chlorophyll *a* emerged from the apparatus as a symmetrical peak with only slight deviations from theoretical values. The eluted interval between 1300 and 1500 ml was almost colourless, thus demonstrating that the resolution of chlorophyll *a* and chlorophyll *b* was practically absolute. Traces of 10-hydroxy-chlorophyll *a* could be spectroscopically detected in the initial portion of the concentration zone of chlorophyll *b*.

The resolution of chlorophyll *b* and lutein is superior in this case when compared with the result presented in Fig. 3. This is obviously due to the fact that the initial 3500 ml of the effluent was re-utilized, after evaporation of the fractions, in order to drive lutein out of the apparatus. The composition of the upper phase probably altered somewhat during the evaporation. This also explains the slight asymmetry of the concentration profile of lutein. No alkoxy-lactone derivative of chlorophyll *b* could spectroscopically be detected between chlorophyll *b* and lutein. Only traces of pigments remained in the apparatus following the complete elution of lutein. The probable character of these pigments is mentioned in the legend to Fig. 4.

The fractionation presented in Fig. 4 resulted in about 80 mg of pure chlorophyll *a* ($V_m = 513 - 918$ ml) and 8 mg of pure chlorophyll *b* ($V_m = 2327 - 3091$ ml), as estimated upon the basis of spectrophotometric concentration determinations. When the effluent solutions of chlorophyll *a* and chlorophyll *b* were cooled to 0°C and washed several times with distilled water, aggregated or "crystalline" chlorophylls were obtained. In agreement with the investigations of Jacobs *et al.*,[5,27] Anderson and Calvin,[28] and Sherman and Wang,[29] the solid chlorophyll *a* exhibited red and blue absorption maxima at 748 and at 451 nm, respectively. When a small amount of ethanol was added to an aggregated solution of chlorophyll *a* in petroleum ether, the normal spectrum of chlorophyll *a* was restored.[30]

The spectroscopic properties of the purest chlorophyll *a* and chlorophyll *b* preparations, isolated by the final method developed, appear in Table 1; c and k. Treatment of ethyl ether solutions of these chlorophyll preparations with 13 % and 30 % hydrochloric acid produced spectroscopically pure pheophytins and pheophorbides, respectively (Table 1; h, i, p and q). The isolated chlorophylls in the aggregated state have been stored in cyclohexane at −15°C for six months and no changes have been observed in their characteristics.

Selectivities of the solvent systems. The selectivities of the three solvent systems employed can easily be compared upon the basis of the partition coefficient, as presented in Table 2. For example, with regard to chlorophylls

Table 2. Partition coefficients of the chloroplast pigments and their alteration products.

Compound	PEF	PMF	PBMF
β-Carotene	72.3	156.7	∞
Pheophytin *a*	8.02[a]	20.8	42.1[a]
Chlorophyll *a*	1.59[a]	1.89	2.83[a]
Mg-purpurin 7-lactone-alkyl ether-methylphytyl ester	0.947	0.657	0.750
10-Hydroxy-chlorophyll *a*	0.502[a]	0.305	0.697[a]
Chlorophyll *b*	0.377[a]	0.229	0.482[a]
Mg-*b*-purpurin 7-lactone-alkyl ether-methylphytyl ester	0.283		0.250
Lutein	0.273[a]	0.119	0.238[a]
10-Hydroxy-chlorophyll *b*	0.091		0.140
Violaxanthin	0.071		0.094
Mg-unstable rhodin-methylphytyl ester	0.044		
Mg-unstable chlorin-methylphytyl ester			0.029
Neoxanthin	0.014		0.015
Chlorophyllides	0.000	0.000	0.000

[a] Average value of two fractionations.

a and *b*, the separation factor, $\beta = K_1/K_2$, has the values 8.2, 5.9, and 4.2, when the PMF-, PBMF-, and PEF-solvent systems, respectively, are utilized. Thus the selectivity of the PBMF-system lies approximately midway between the selectivities of the other two solvent systems.

DISCUSSION

The results presented demonstrate the possibilities of liquid-liquid partition methods regarding the fractionation of such labile compounds as the chlorophylls. They also serve as a good example of the decisive importance of selecting the proper solvent system, when applying these methods to difficult fractionation problems.

Success in the development of the final isolation method must be ascribed to the formulation of an appropriate solvent system for the fractionation, as well as to the development of an improved method for the extraction of the pigments from the plant material with minimal production of allomerization products. In the "two-phasic" extraction method, the pigments are immediately transferred into the nonpolar phase after having been detached from the chloroplasts by the polar phase. Thus, little or no transformation can occur during the extraction procedure.

The formation of alkoxy-lactone derivatives from the chlorophylls was observed to depend sensitively upon the polarity of the fractionation solvent system. This observation can be satisfactorily explained by the reaction scheme presented in Fig. 5. According to this scheme, the first step in the reaction sequence of allomerization is keto-enol tautomerism (reaction 1). Solvents such as aliphatic hydrocarbons, carbon tetrachloride, and water, which cause the chlorophylls to occur in an aggregated state,[5,27–32] preclude the possibility of enolization. In electron donor solvents (ethyl ether, acetone, pyridine, dioxane, tetrahydrofuran, alcohols, *etc.*), however, the chlorophylls occur as monomers and will therefore undergo keto-enol tautomerization. The extent to which enolization occurs in these solvents is, however, sensitive to the particular properties of the solvent concerned. Thus, in some solvents (*viz.* the ethers), the free enol form (II) is not further transformed. In this case, the chlorophylls in all probability exist predominantly as the all-keto form.[33] In solvents possessing relatively weak hydrogen bonding capabilities (pyridine, tetrahydrofuran, petroleum ether containing a small amount of propanol), the free enol can be transformed into the chelated enol (III),[34] which may then be stabilized by additional resonance (IV). In such a case, the total concentration of the enol form may be appreciable. In polar solvents

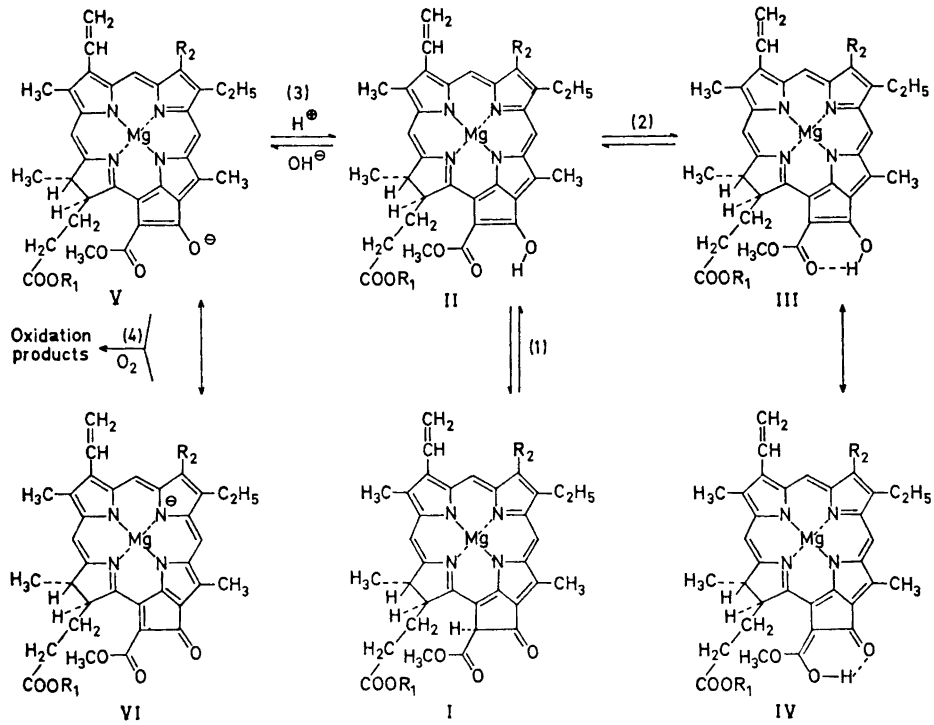

Fig. 5. Probable reactions of the chlorophylls. R_1 = phytyl; in chlorophyll *a*, R_2 = methyl, and in chlorophyll *b*, R_2 = formyl.

(Lewis bases: OH^-, OCH_3^-, *etc.*), the free enol is probably ionized to the enolate anion (V). This anion has been assumed to be the intermediate in the Molisch phase test and in the allomerization of the chlorophylls.[25,35–38] The enolate anion is unstable, being rapidly attacked by oxygen to yield various oxidation products whose nature depends upon the solvent.

The drastic colour change that occurs upon formation of the enolate anion during the phase test has been tentatively explained by assuming [39] that the negative charge becomes principally centred upon the pyrrolenine nitrogen atom, as is illustrated in Fig. 5 (VI). This assumption is based upon the research of Woodward and Scaric,[40] who observed that the pyrrolenine nitrogen atom tends to attract electrons. A second possible explanation to the change of colour upon formation of the enolate is obtained on assuming that the anion forms a diradical.[38] This alternative appears to explain satisfactorily the instability of the intermediate in the presence of oxygen. It also agrees with the fact that measured spectra of the phase test intermediate exhibit strong absorption bands at 500 – 550 nm which disappear in the presence of oxygen.[25,38]

The slow formation of the alkoxy-lactone derivative during fractionation suggests that reactions 1 and 3 are the rate-limiting steps in the reaction sequence of allomerization. This proposition is also in accord with the fact that no drastic colour change can be observed when allomerization occurs. Under conditions promoting allomerization, either reaction 1 or reaction 3 (or both) is slower than reaction 4. Thus, the unstable enolate anion is oxidized as soon as it is formed. Under the conditions prevalent during a positive phase test, however, the enolate is in all probability produced more rapidly than consumed.

Strain [23] has presented evidence to the effect that hydroxy-chlorophylls are produced enzymatically during the extraction of the pigments from leaves. The results obtained in this laboratory are in agreement with this viewpoint. The mechanism of the enzymatic allomerization may be quite different from that of the nonenzymatic.

The spectroscopic properties of the pure chlorophylls have long been a matter of much debate and controversy. An important criterion regarding the purity of a chlorophyll preparation has been the ratio of the absorbance at the Soret band wavelength to that at the chlorin band wavelength (A_S/A_I). Anderson and Calvin [10] (refer also to Aronoff [41]) obtained a ratio of 1.19 for chlorophyll *a* and claimed that the purity of this compound when isolated by chromatography on columns of powdered polyethylene was superior to that when prepared by other means. The purest chlorophyll *a* preparations obtained in this laboratory had a ratio of 1.32, which agrees very well with the generally accepted value of 1.31 – 1.32.[3,4,8,42,43] Moreover, the A_S/A_I ratio of 2.88 obtained for the purest chlorophyll *b* preparation agrees quite well with values (2.82 – 3.01) reported in the literature.[3,4,8]

The final isolation method resulted in a good yield of chlorophyll *a* (80 mg/100 g frozen soybean leaves).[1,10] The yield with regard to chlorophyll *b* (8 mg/100 g frozen soybean leaves) is low, but can easily be increased by adding water to the phase system in Method 3, before the phases are separated. A second possible way of obtaining higher yields of chlorophyll *b* is to utilize a greater number of fractionation tubes, thereby achieving better resolution of

10-hydroxy-chlorophyll *a* and chlorophyll *b*. In such a case, however, it is apparently important to increase the flow rate so that the chlorophyll *b* will be completely eluted from the apparatus within about 24 h. If this time limit is exceeded appreciably, then some lactonization of the chlorophyll *b* may occur during fractionation.

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