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APPLICATION OF ELUTION ANALYSIS TO THE STUDY OF CHLOROPHYLL TRANSFORMATIONS BY COLUMN CHROMATOGRAPHY ON SUCROSE

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SUMMARY

The transformations of chlorophyll (Chl) *a* and *b* were studied by applying elution analysis to chromatographic separations on a sucrose column. When the Chl *a*–water adduct was heated in 1-propanol at 50° for 15 min, subsequent separation on sucrose revealed three principal products which were identical with Chl *a* according to their visible absorption spectra and yielded positive reactions to the Molisch phase test. Only minor amounts of oxidation products were observed in this instance. When water-free Chl *a* was heated under similar conditions, separation on sucrose yielded, in addition to Chl *a*, considerable amounts of Chl *a'* and oxidation products. On standing in 1-propanol at 25° for 4 days, Chl *a* was completely allomerized. Separation on sucrose revealed three pairs of oxidation products, each pair consisting of the 10(*S*) and 10(*R*) isomers. When a light petroleum solution of chloroplast pigments was extracted with 70% aqueous methanol, the separation of the precipitated chlorophylls on sucrose yielded three chlorophyll components in both series but virtually no oxidation products. As the most plausible interpretation, it is suggested that the most slowly migrating chlorophylls arose from the presence of considerable amounts of the enol of chlorophyll in the samples. When these samples were introduced on to the sucrose column, the hydrogen-bonded water molecules were removed from the enol. This rendered the enol susceptible to transformation during the separation on sucrose. As very little oxygen was present, principally Chl *a* and *a'* were produced. Although no enol could be detected in the effluent, the lifetime of the enol was considered to be long enough to cause the appearance of shoulders corresponding to the slowly migrating chlorophylls. These results confirm the enol forms of chlorophyll play a key role in chlorophyll oxidation and epimerization. They also show that the use of methanol–water mixtures for the purification of chlorophyll preparations requires special precautions in order to prevent the enolization and epimerization reactions.

INTRODUCTION

Owing to their exceptional lability, the chlorophylls (Chl) are susceptible to a number of chemical transformations (Fig. 1) which yield various products and iso-

mers[1–4]. This lability derives from the high reactivity of the moderately strained isocyclic ring V in the Chl molecule (1a). Because of the activation of the C_{10} atom by the methoxycarbonyl group, the C_{10} hydrogen is enolizable (1a→2)[1,5]. The enol forms (2,3,4) probably occur as intermediates in the epimerization (1a→1b)[6–8], pyrochlorophyll formation (1a→1e)[9,10], solvolysis (1a→9)[11,12] and allomerization (1a→7a–d)[6,10,13–16] reactions.

In 1973, Hynninen and Assandri[16] advanced evidence for the effect that the allomerization resulting in the formation of purpurin 7-lactone derivatives (7a–d) is preceded by the enolization reaction. They proposed a mechanism for the allomer-

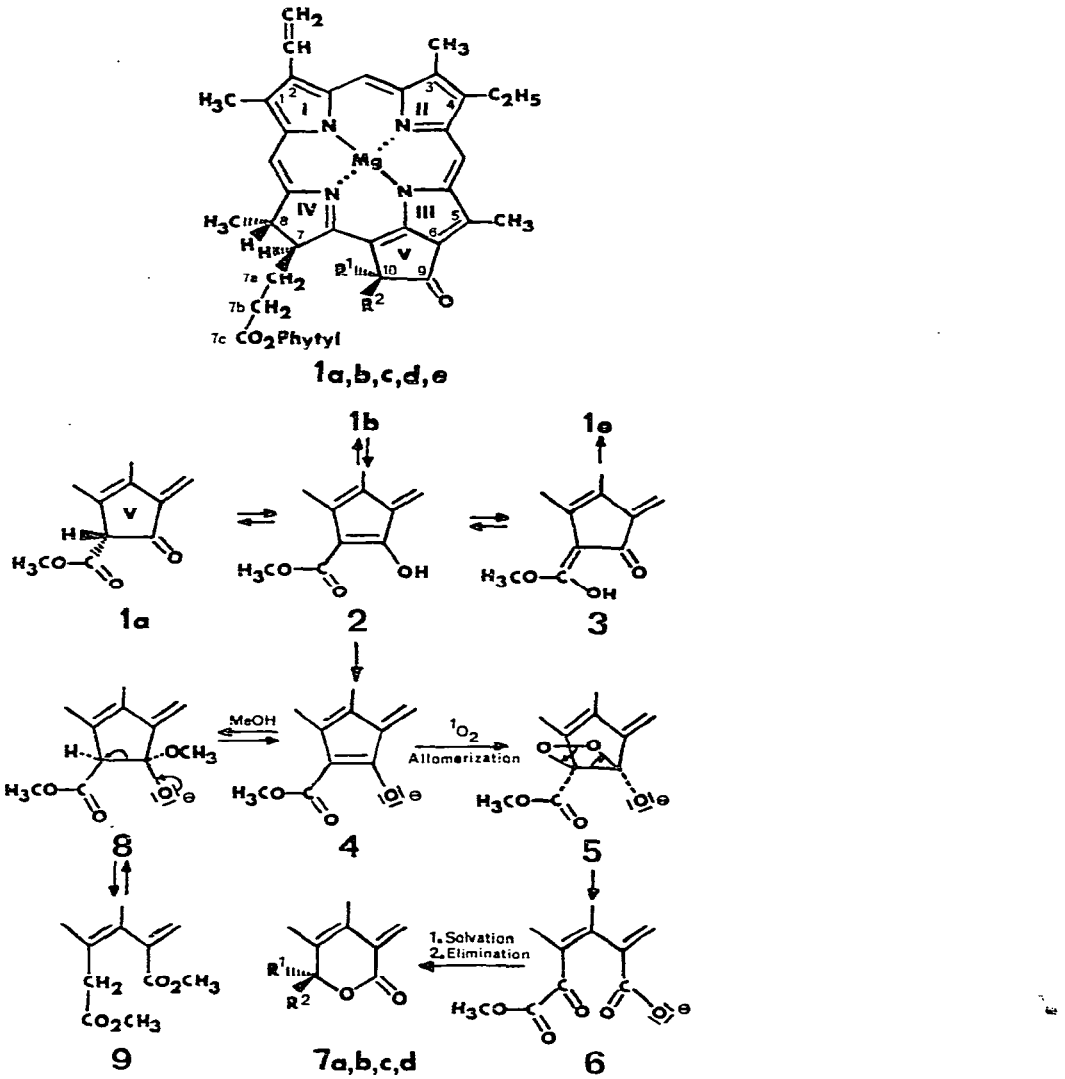

Fig. 1. Structure and reactions of chlorophyll. In the corresponding derivatives of the *b* series, the methyl group at C_3 must be replaced with a formyl group. Chlorophyllides have 1H in place of phytyl at C_{7c} and pheophytins have 2H in place of Mg. In pheophorbides the phytyl must be replaced with 1H and the Mg with 2H.

Fig. 1 *(continued)*

No.	R^1	R^2	*Designation*
1a	CO_2CH_3	H	Chl *a* = Mg 10(*R*)pheophytin *a*
1b	H	CO_2CH_3	Chl *a'* = 10-epi-Chl *a* = Mg 10(*S*)pheophytin *a*
1c	CO_2CH_3	OH	10(*R*)hydroxy-Chl *a*
1d	OH	CO_2CH_3	10(*S*)hydroxy-Chl *a*
1e	H	H	Pyro-Chl *a*
2,3			Enol forms of Chl *a*
4			Chl enolate anion
5			Cyclic peroxide or dioxetane derivative of Chl *a*
6			Mg purpurin 7-methylphytyl ester
7a	CO_2CH_3	$OCH_2CH_2CH_3$	Mg 10(*R*)propoxypurpurin 7-lactone methylphytyl ester = Mg 10(*R*)purpurin 7-lactone propyl ether methylphytyl ester
7b	$OCH_2CH_2CH_3$	CO_2CH_3	Mg 10(*S*)propoxy-purpurin 7-lactone methylphytyl ester = Mg 10(*S*)purpurin 7-lactone propyl ether methylphytyl ester
7c	CO_2CH_3	OH	Mg 10(*R*)hydroxypurpurin 7-lactone methylphytyl ester = Mg 10(*R*) unstable chlorin 7-methylphytyl ester
7d	OH	CO_2CH_3	Mg 10(*S*)hydroxypurpurin 7-lactone methylphytyl ester = Mg 10(*S*) unstable chlorin 7-methylphytyl ester
8			Intermediate in the solvolysis of ring V
9			Mg chlorin e_6 dimethylphytyl ester

ization, according to which molecular oxygen adds to the double bond between C_9 and C_{10} of the enolate anion (4). In further detail, this mechanism signifies the addition of singlet oxygen ($^1O_2$) to the activated double bond of the enolate ion to yield a labile cycloperoxide or dioxetane intermediate (5), which undergoes cleavage, thus resulting in a purpurin 7-derivative (6) with a free carboxyl group at C_6. This compound thereafter is converted through solvation and elimination reactions[16] to the 10-hydroxy- or 10-alkoxypurpurin 7-lactone derivatives (7a–d). Experimental evidence for the participation of singlet oxygen in the allomerization has been obtained recently[17].

In this work, the relationship among the enolization, epimerization and allomerization reactions of Chl was investigated in further detail. Conventional chromatography on sucrose[3,4] was used as a separation method. Elution analysis was applied extensively to this technique, in order to improve the determination of potential zone spreading effects, resolution and number of components. 1-Propanol (PrOH) was selected as the allomerizing solvent, since allomerization is much slower in this alcohol than in the more polar methanol (MeOH). This fact might render it possible to observe some of the relatively labile intermediates in the reaction sequence. The potential transformations occurring in aqueous MeOH were also of interest, as this solvent mixture is used in the standard isolation procedure[3,18] for extracting impurities from Chl preparations and as it is well known that allomerization does not take place (or proceeds only very slowly) in aqueous MeOH whereas it occurs rapidly in absolute MeOH. Evidence can now be forwarded for the effect that the water molecules in aqueous alcohol mixtures prevent allomerization, presumably by forming hydrogen bonds with the enol (3). This paper also reports that the allomerization of Chl *a* in PrOH results in three pairs of oxidation products, each pair being separable on the sucrose column into 10(*S*) and 10(*R*) isomers.

EXPERIMENTAL

Extraction of chloroplast pigments

Chloroplast pigments were extracted from plant material by employing the improved two-phase extraction method[19]. Frozen clover leaves (a mixture of *Trifolium pratense*, *T. hybridum* and *T. repens*) were also used as a source of the pigments.

Treatment of the chloroplast extract with 70% aqueous methanol

A light petroleum (b.p. 60–80°) (LP) solution of the chloroplast pigments (300 ml) was extracted with 70% aqueous MeOH (*ca.* 12 × 300 ml). The leaf xanthophylls (lutein, violaxanthin and neoxanthin) are effectively removed by this procedure[19] (different concentrations of water in MeOH were used by Strain and co-workers[3,18] to extract impurities from LP solutions of Chl). After the aqueous MeOH extraction, the LP solution containing the carotenes, the Chls and their potential transformation products, was washed several times with cold distilled water until the Chls precipitated. The mixture was permitted to stand at −30° for 2 h to make the precipitation of the Chls complete. The precipitated Chls were collected from the suspension by centrifugation[19]. The washed precipitate was then dissolved in diethyl ether (10 ml) and the solution was evaporated nearly to dryness at reduced pressure. The residue was dissolved in 3 ml of the eluent to be used in the chromatographic separation.

Chromatography on a sucrose column

The chromatographic separations were performed in the dark at 4°. A glass column (50 × 3 cm I.D.) was packed with icing sugar (Finnish Sugar Co., Helsinki, Finland) by the slurry method[19]. Before use, the sugar was passed through a 100-mesh sieve. In order to prevent evaporation of the solvent and to make working in a cold room safe, the upper end of the column, having a ground-glass joint, was connected with Teflon tubing to the solvent bottle. The lower end of the column was similarly connected with Teflon tubing to an automatic fraction collector. These arrangements made it possible to apply elution analysis to the chromatographic separations. After the introduction of the eluent solution of the pigments into the top of the sucrose layer, the column was eluted with 0.5% PrOH in LP (b.p. 60–80°) until the components of interest had emerged from the column. The collected fractions (the tubes were closed with cork stoppers) were measured at selected wavelengths by means of a Perkin-Elmer UV–visible spectrophotometer. The components which remained on the column after the elution were characterized on the basis of their spectroscopic properties after first removing the sugar layers containing the absorbed pigments through the upper end of the column and then eluting the pigments from the layers with diethyl ether. β-Carotene was used as a reference compound and the migration rate of a component was expressed in terms of the R_c values[19].

Heating of chlorophyll in propanol

Approximately 10 mg of purified[19] Chl *a*, containing variable amounts of water, were dissolved in 10 ml of PrOH. The solution was heated at 50° for 15 min. In one instance, the solution was permitted to stand in the dark at 25° for 4 days. At the end of this period, the Chl solution was rapidly evaporated nearly to dryness at reduced pressure. The residue was dissolved in 2 ml of the eluent.

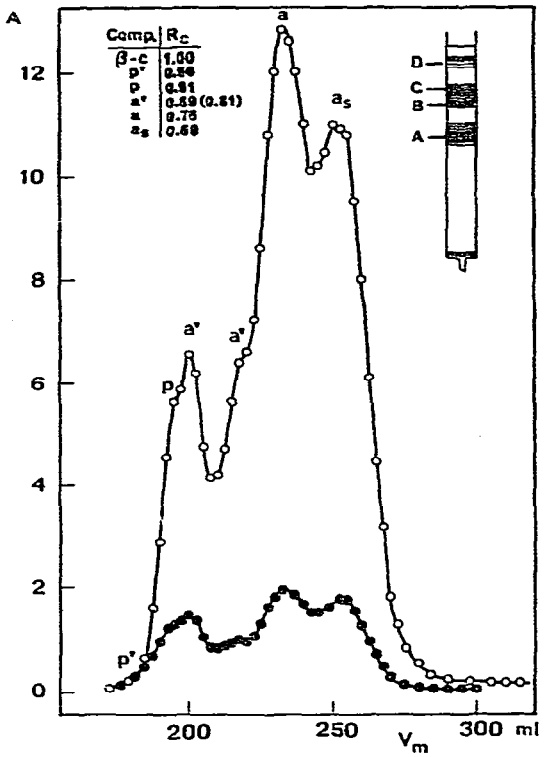

Fig. 2. Separation of the products formed from crystalline chlorophyll *a* in 1-propanol employing chromatography on a sucrose column. Height of the sucrose layer, $h = 40.0$ cm. Eluent = 0.5% 1-propanol in light petroleum (b.p. 60–80°). Flow-rate = 1.0 ml/min. Effluent volume = V_m. ○, A_{660}; ●, A_{675}. The upper right-hand part of the figure shows the components which remained on the column after a volume of 325 ml had passed through. p′ and p = pheophytins p′ and p; a′, a and a_s = chlorophylls *a*′, *a* and a_s. A = Mg 10-propoxypurpurin 7-lactone MePhy ester; B = 10-hydroxychlorophyll *a*; C = Mg 10-hydroxypurpurin 7-lactone MePhy ester; D = chlorophyllide *a* + impurities. R_c = the elution volume of β-carotene divided by the elution volume of the component.

Absorption spectra

Visible absorption spectra were recorded with a Cary Model 118C spectrophotometer. Measurements were performed directly upon the effluent as well as after transferring the pigments into diethyl ether by means of a Thunberg tube at reduced pressure. The spectroscopic properties of the separated components are given in Table I.

Solvents

Formamide was purified by distillation *in vacuo*[19]. The other solvents used were of analytical-reagent grade and were employed without further purification.

RESULTS AND DISCUSSION

Separation of the alteration products formed from chlorophyll a in propanol

When crystalline Chl *a*, *i.e.*, the Chl *a*–water adduct, was heated in PrOH at 50° for 15 min, the chromatographic separation revealed the partial separation of

TABLE I

ELECTRONIC ABSORPTION SPECTRA OF CHLOROPHYLL DERIVATIVES

No.	*Derivative*	*Symbol**	*V_m** (ml)*	*Solvent****	*Peak positions (nm), peak ratios (R)[§] and half-widths of the red absorption band and the Soret band, $w_{1/2}$ and $w_{S1/2}$ (nm)*
1	Chl *a'* (1b)	2a'	220	PLP	661.0(1.19), 613(7.50), 574(14.6), 530(26.0), 496(47.0), 428.0(1.00), 412(1.26), 382(2.20), $w_{1/2}$ 17.8, $w_{S1/2}$ 42.9
2	Chl *a'* (1b)	3a'	245	PLP	661.0(1.15), 613(7.68), 574(15.9), 530(31.9), 497(70.5), 428.5(1.00), 409(1.44), 382(2.33), $w_{1/2}$ 16.6, $w_{S1/2}$ 39.9
3	Chl *a'* (1b)	3a'	245	EE	660.0(1.28) , 612(8.87), 573(16.9), 530(29.8), 496(52.7), 428.0(1.00), 408(1.50), 381(2.44), $w_{1/2}$ 17.1, $w_{S1/2}$ 38.9
4	Chl *a'* (1b)	4a'	232	PLP	661.5(1.15), 612(7.05), 574(14.0), 532(19.2), 503(24.8), 427.5(1.00), 409(1.15), 380(1.90), $w_{1/2}$ 17.8, $w_{S1/2}$ 64.8
5	Chl *a'* (1b)	6a'	177	PLP	661.5(1.21), 614(7.70), 575(14.1), 531(23.1), 500(35.1), 429.0(1.00), 410(1.42), 383(2.31), $w_{1/2}$ 17.4, $w_{S1/2}$ 40.7
6	Chl *a'* (1b) + pheophytin *a*	2a',p	198	PLP	661.0(1.22), 612(6.33), 574(10.6), 532(13.3), 504(17.7), 428.0(1.00), 409(1.19), 386(1.94), $w_{1/2}$ 17.1, $w_{S1/2}$ 67.5
7	Chl *a* (1a)	2a	235	PLP	661.0(1.18), 614(6.82), 576(11.0), 531(17.4), 496(26.8), 429.0(1.00), 410(1.39), 382(2.42), $w_{1/2}$ 16.4, $w_{S1/2}$ 39.1
8	Chl *a* (1a)	2a	235	EE	660.0(1.29), 612(7.33), 573(11.5), 529(17.5), 496(25.9), 428.0(1.00), 408(1.56), 379(2.61), $w_{1/2}$ 17.0, $w_{S1/2}$ 37.9
9	Chl *a* (1a)	3a	286	PLP	661.0(1.21), 614(7.75), 575(15.1), 529(30.7), 496(66.2), 429.0(1.00), 410(1.45), 382(2.44), $w_{1/2}$ 16.6, $w_{S1/2}$ 39.0
10	Chl *a* (1a)	3a	286	EE	660.0(1.31), 613(8.42), 575(16.3), 529(31.2), 496(69.7), 428.5(1.00), 409(1.54), 381(2.56), $w_{1/2}$ 17.2, $w_{S1/2}$ 38.3
11	Chl *a* (1a)	4a	274	PLP	660.0(1.24), 613(7.58), 574(15.4), 527(26.8), 494(56.7), 426.0(1.00), 412(1.11), 380(2.23), $w_{1/2}$ 18.8, $w_{S1/2}$ 45.0
12	Chl *a* (1a)	6a	193	PLP	661.0(1.22), 613(7.65), 575(13.7), 531(20.4), 500(27.8), 428.5(1.00), 410(1.32), 382(2.15), $w_{1/2}$ 17.1, $w_{S1/2}$ 43.2
13	Chl a_s	$2a_s$	255	PLP	661.0(1.22), 614(8.71), 576(15.6), 530(32.0), 496(77.4), 428.5(1.00), 409(1.52), 381(2.49), $w_{1/2}$ 17.0, $w_{S1/2}$ 38.7
14	Chl a_s	$3a_s$	433–539	PLP	660.0(1.23), 614(7.32), 570(19.1), 527(16.4), 494(51.2), 426.0(1.00), 410(1.21), 380(2.19), $w_{1/2}$ 19.0, $w_{S1/2}$ 44.8
15	Chl a_s	$4a_s$	385–550	PLP	661.0(1.20), 613(7.28), 570(24.6), 527(24.6), 494(53.0), 427.5(1.00), 410(1.16), 380(2.26), $w_{1/2}$ 19.2, $w_{S1/2}$ 44.5
16	Chl a_s	$6a_s$	224	PLP	660.5(1.21), 613(7.98), 574(15.1), 530(28.2), 494(52.7), 428.5(1.00), 409(1.53), 381(2.52), $w_{1/2}$ 17.4, $w_{S1/2}$ 38.2

17	Chl *b'*	6b'	280	PLP	642.0(2.51), 597(11.5), 568(17.6), 540(21.4), 450.5(1.00), 427(1.49), $w_{1/2}$ 33.5, $w_{s1/2}$ 41.7
18	Chl *b'*	6b'	495	PLP	642.0(2.74), 594(14.4), 564(22.8), 545(23.2), 451.0(1.00), 427(2.64), $w_{1/2}$ 17.4, $w_{s1/2}$ 22.4
19	Chl *b*	6b	537	PLP	642.0(2.74), 594(14.5), 564(23.5), 544(24.2), 450.5(1.00), 426(2.58), $w_{1/2}$ 17.2, $w_{s1/2}$ 22.1
20	Chl b_s	$6b_s$	604	PLP	642.0(2.84), 594(14.8), 564(22.8), 544(23.2), 450.5(1.00), 426(2.80), $w_{1/2}$ 16.6, $w_{s1/2}$ 21.1
21	Chlorophyllide *a* + impurity	2D		EE	659.0(1.61), 613(8.08), 568(17.9), 530(14.5), 494(13.2), 425.5(1.00), 410(1.08), 380(1.78), $w_{1/2}$ 23.3, $w_{s1/2}$ 68.0
22	Chlorophyllide *a* + impurity	3D		EE	656.0(2.24), 614(8.28), 419.0(1.00), $w_{1/2}$ 27.3, $w_{s1/2}$ 86.1
23	Pheophytin *a'*	2p'	177	PLP	668.0(1.90), 610(13.1), 561(40.6), 504(9.32), 471(15.0), 408.5(1.00), $w_{1/2}$ 16.8, $w_{s1/2}$ 53.3
24	Pheophytin *a'* + pheophytin *a*	4p',p	213	PLP	668.5(1.87), 610(14.3), 561(42.1), 532(11.5), 504(9.53), 468(26.0), 408.5(1.00), $w_{1/2}$ 16.2, $w_{s1/2}$ 52.5
25	P_{ox} (7a,b)	2A		EE	652.0(1.94), 604(11.9), 561(24.6), 482(32.8), 417.0(1.00), $w_{1/2}$ 18.0, $w_{s1/2}$ 33.0
26	P_{ox} (7a,b)	3 lact	320-331	PLP	655.0(1.67), 609(9.40), 567(20.4), 522(27.2), 490(60.6), 417.0(1.00), $w_{1/2}$ 21.7, $w_{s1/2}$ 38.6
27	P_{ox} (7a,b) + Chl a_s	3 lact, a_s	358	PLP	659.0(1.29), 613(7.63), 573(16.0), 527(24.2), 497(41.3), 420.0(1.00), 413(1.04), 384(2.21), $w_{1/2}$ 20.5, $w_{s1/2}$ 49.2
28	P_{ox} (7a,b)	3A		EE	657.0(1.54), 611(8.60), 566(19.8), 524(24.6), 488(41.0), 419.0(1.00), $w_{1/2}$ 21.3, $w_{s1/2}$ 43.7
29	P_{ox} (7a)	5B	222-276	PLP	652.5(1.93), 606(12.1), 562(25.6), 520(28.6), 487(57.2), 417.0(1.00), $w_{1/2}$ 19.0, $w_{s1/2}$ 31.6
30	P_{ox} (7b)	5A	185	PLP	652.0(1.97), 606(12.4), 563(26.3), 520(31.0), 486(70.2), 417.0(1.00), $w_{1/2}$ 18.8, $w_{s1/2}$ 32.0
31	P_{ox} (7a,b) + Chl *a* (1a)	4 lact, a	274-291	PLP	655.0(1.68), 609(9.63), 567(21.1), 522(28.1), 490(67.5), 417.0(1.00), $w_{1/2}$ 22.4, $w_{s1/2}$ 49.3
32	P_{ox} (7c)	5F	2608	EE	651.0(1.96), 605(12.5), 561(26.8), 519(28.1), 484(52.4), 416.5(1.00), $w_{1/2}$ 19.8, $w_{s1/2}$ 31.6
33	P_{ox} (7d)	5E	2071-2608	EE	651.5(2.11), 605(13.5), 562(27.6), 519(31.2), 483(54.6), 417.0(1.00), $w_{1/2}$ 18.6, $w_{s1/2}$ 29.5
34	P_{ox} (7c,d)	3C		EE	653.0(1.77), 609(11.3), 565(42.9), 525(65.5), 490(200), 418.5(1.00), $w_{1/2}$ 20.2, $w_{s1/2}$ 37.3
35	P_{ox} (7c,d) + P_{ox} (1c,d)	2C,B		EE	659.5(1.38), 611(8.42), 567(20.9), 526(25.0), 494(40.0), 423.5(1.00), 410(1.15), 380(1.78), $w_{1/2}$ 20.1, $w_{s1/2}$ 45.6
36	P_{ox} (1c,d)	3B		EE	662.0(1.18), 615(6.07), 574(17.5), 533(26.0), 490(39.0), 428.0(1.00), 410(1.24), 380(1.94), $w_{1/2}$ 19.7, $w_{s1/2}$ 47.4
37	P_{ox} (1c)	5D	1400-1705	EE	660.0(1.27), 613(8.9), 570(15.9), 527(20.2), 488(27.8), 426.5(1.00), 410(1.43), 380(2.47), $w_{1,2}$ 18.5, $w_{s1/2}$ 44.7
38	P_{ox} (1d)	5C	820-1198	EE	661.5(1.25), 614(7.94), 571(15.3), 527(14.5), 494(13.8), 427.5(1.00), 402(0.963), $w_{1/2}$ 21.3, $w_{s1/2}$ 73.8

* The number and letter(s) refer, respectively, to the figure number and to the component in that figure.

** V_m = effluent volume.

*** PLP = 0.5% 1-propanol in light petroleum (b.p. 60-80°); EE = diethyl ether.

§ R = quotient of absorbance at Soret band divided by absorbance at wavelength indicated.

three principal components (Fig. 2). These components are designated Chl *a'*, *a* and a_s (a_s = slowly migrating Chl *a*). In spite of their different migration rates on the sucrose column, the three Chls exhibited virtually identical visible absorption spectra (Table I: 6, 7, 8, 13). As all three reacted positively to the Molisch phase test[1], none of them can be an allomerization product. Chl *a'* had previously been shown[8] in a definite manner to be the 10-epimer of Chl *a* [Chl *a'* and *a* have 10(*S*) and 10(*R*) configurations, respectively]. The appearance of the third Chl "isomer" (a_s) in the concentration profile is a new feature not observed before in the separations of Chl *a'* and *a*. In the front part of the concentration profile, two slight shoulders (p' and p) were detected and the absorption spectra (Table I: 6,23) indicated the presence of pheophytin *a*-like pigments at these points.

Another shoulder appeared at V_m = 218 ml. As the absorption spectrum (Table I: 1) at this point matched closely that of Chl *a*, it seems likely that a new amount of Chl *a'* was formed during the separation. Minor amounts of allomerization products remained on the column in this fractionation. The lowest zone (A) was presumably (Table I: 25) a 10-propoxypurpurin 7-lactone derivative (7a,b). The middle zone (B,C) seemed to consist of two allomerization products (Table I: 35), the 10-hydroxy-Chl *a* (1c,d) and 10-hydroxypurpurin 7-lactone derivative (7c,d). The narrow yellow-green band (D), which remained at the top of the sugar layer, may be principally chlorophyllide *a* (Table I: 21). Support for the above identification comes from the separations described below. It should be noted, in addition, that no stereochemical specification at C_{10} of the allomerization products was possible in this instance owing to their incomplete resolution.

Fig. 3 presents the results from the re-fractionation of the effluent components (V_m = 164–300 ml) in Fig. 2. The elution analysis revealed only two principal components (a' and a) which had the spectroscopic properties of Chl *a* (Table I: 2,3,9,10). In this instance, however, the concentration profile of Chl *a* showed an unusually long tail. The effluent fractions in the range V_m = 433–539 ml contained principally Chl *a*-like pigment (Table I: 14). The presence of a lactone derivative was spectroscopically (Table I: 26, 27) detected in the fractions corresponding to V_m = 320–358 ml. Larger amounts of allomerization products remained on the sucrose column in this separation in comparison with those in Fig. 2. The components in bands A–D were characterized on the basis of their visible absorption spectra (Table I: 22, 28, 34, 36) and chemical properties[16,20]. On the basis of the evidence given below, it seems likely that bands C and B represent racemic mixtures of the 10-hydroxypurpurin 7-lactone derivative (7c,d) and 10-hydroxy-Chl *a* (1c,d), respectively. Band A and the lactone derivative in the effluent fractions (V_m = 220–358 ml) presumably represent the 10(*R*) and 10(*S*) configurations, respectively, of the 10-propoxypurpurin 7-lactone derivative (7a,b).

In order to establish the significance of the presence of water in the Chl *a*–PrOH solution, the following experiment was performed. Chl *a* was first purified on a sucrose column. The effluent solution of Chl *a* was then evaporated nearly to dryness in a rotating evaporator. The residue was dissolved in 10 ml of PrOH and the solution was heated at 50° for 15 min. After this period, the solution was evaporated to dryness and the residue dissolved in 2 ml of the eluent. The separation of this water-free sample on a sucrose column yielded the results shown in Fig. 4, which reveals two essential features. Firstly, the amount of Chl *a'* is unusually high. Secondly, the

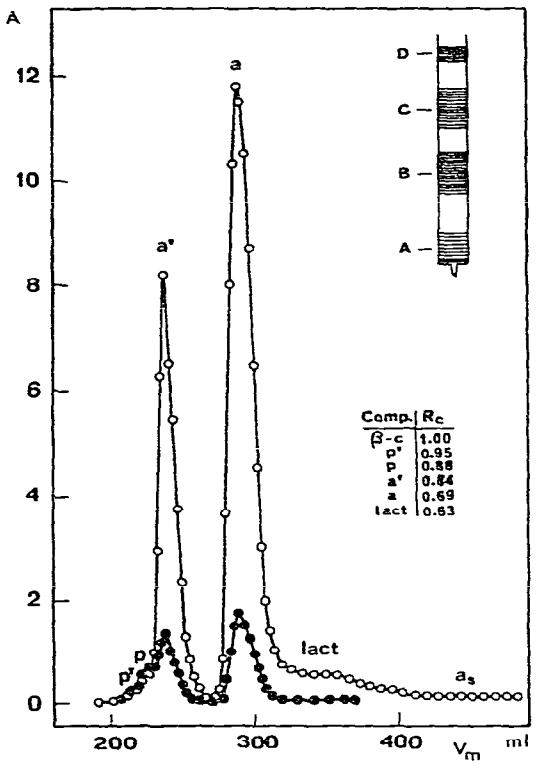

Fig. 3. Re-fractionation of the effluent components in Fig. 2. The effluent solution of the pigments in Fig. 2 was evaporated to dryness in a rotary evaporator and the residue was dissolved in 2 ml of the eluent. This sample was separated on a newly prepared sucrose column. h = 45.0 cm. Eluent as in Fig. 2. Flow-rate = 0.7 ml/min. ○, A_{660}; ●, A_{675}. The upper right-hand part of the figure shows the components that remained on the column after a volume of 500 ml had passed through. Lact = Mg 10-propoxypurpurin 7-lactone MePhy ester; other abbreviations as in Fig. 2.

concentration profile of Chl *a* exhibits even more extensive broadening than that in Fig. 3. This implies that allomerization had been taking place during fractionation. The presence of a lactone derivative could be spectroscopically (Table I: 31) detected in the fractions corresponding to V_m = 274–350 ml. The long tail (V_m = 385–550 ml), however, seemed to contain principally Chl *a*-like pigments (Table I; 15). Considerable amounts of allomerization products, not characterized in detail, remained on the column in this fractionation.

When Chl *a* was dissolved in PrOH and the solution was permitted to stand in the dark at 25° for 4 days, the separation of the products on a sucrose column yielded the results shown in Fig. 5. Two deep blue components, A and B, first appeared in the effluent. The R_c values of these components are not comparable to those in the above fractionations, as the PrOH concentration of the eluent was probably higher than 0.5% at the beginning of the separation. The visible absorption spectra (Table I; 29,30) and chemical properties of A and B were consistent with the properties of the 10-alkoxypurpurin 7-lactone derivative described previously[16,20]. Consequently, it seems likely that components A and B represent the 10(*S*) and 10(*R*) configurations of this derivative (7a,b). The extended elution analysis provided evidence for the fact

that there exist two stereoisomers of the other allomerization products as well. Components C and D were both found spectroscopically (Table I; 37,38) to be similar to Chl *a*. Since, in addition, the chemical properties of C and D were similar to those described for 10-hydroxy-Chl *a*[16,20], they presumably represent the 10(*S*) and 10(*R*) configurations of this compound (1c,d). Components E and F exhibited spectroscopic and chemical properties consistent with those of the 10-hydroxypurpurin 7-lactone derivative[16,20]. Therefore, these components probably represent the 10(*S*) and 10(*R*) configurations of the last-mentioned derivative (7c,d).

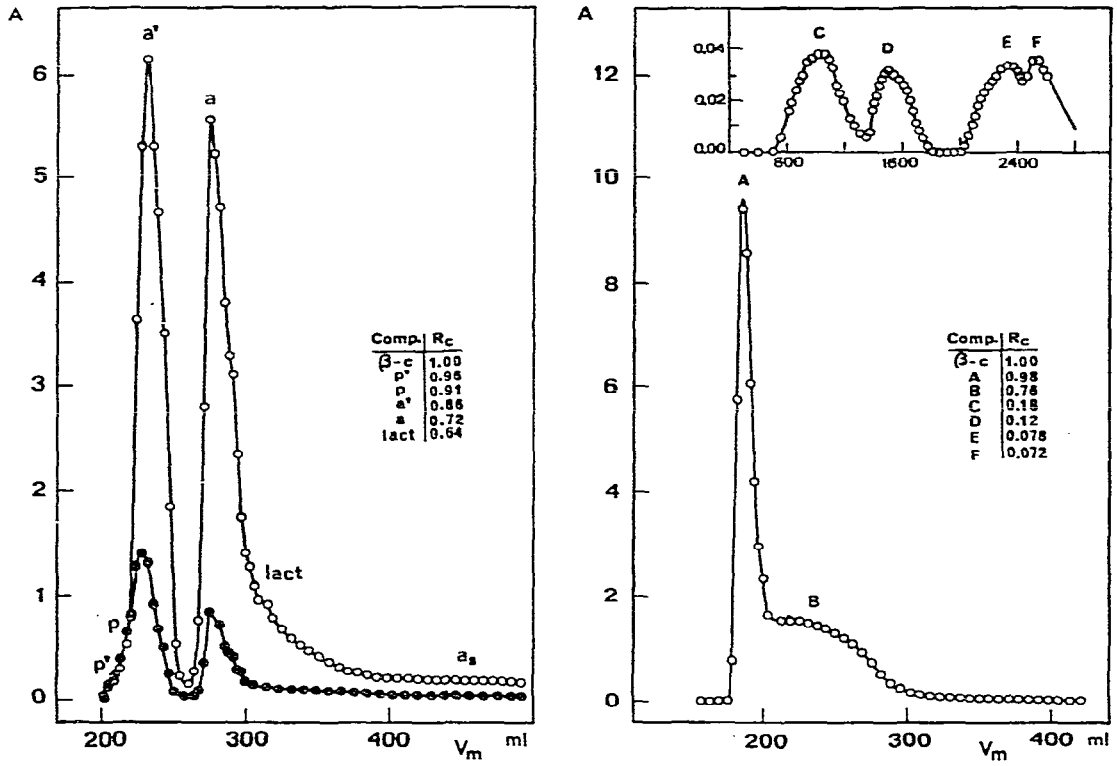

Fig. 4. Separation of the products formed from water-free chlorophyll *a* in 1-propanol employing chromatography on a sucrose column. *h* = 45.0 cm. Eluent as in Fig. 2. Flow-rate = 0.65 ml/min. ○, A_{660}; ●, A_{675}. Abbreviations as in Figs. 2 and 3.

Fig. 5. Separation of the products formed from chlorophyll *a* in 1-propanol after standing at 25° for 4 days. *h* = 42.8 cm. Eluent as in Fig. 2. Flow-rate = 0.8 ml/min. ○, A_{660}. A and B = 10(*S*) and 10(*R*) configurations, respectively, of Mg 10-propoxypurpurin 7-lactone MePhy ester; C and D = 10(*S*) and 10(*R*) configurations, respectively, of 10-hydroxychlorophyll *a*; E and F = 10(*S*) and 10(*R*) configurations, respectively, of Mg 10-hydroxypurpurin 7-lactone MePhy ester.

Separation of the products formed from chlorophylls in 70% aqueous methanol

The results obtained on washing the LP solution of chloroplast pigments with 70% aqueous methanol prior to precipitation and chromatography on sucrose are shown in Fig. 6. No distinct Chl *a'* zone separated in this instance (in the precipitation, Chl *a'* remains principally in solution owing to its high solubility[19]). The concentration profile of Chl *a*, however, exhibits unusual curvatures on both sides. This

suggests that a slow transformation of Chl *a* to Chl *a'* had occurred during the separation. The result obtained in the *b*-series is comparable to that presented in Fig. 2 for the *a*-series. The concentration profile of Chl *b* shows a distinct shoulder which consists of slower migrating Chl b_s. At the same time, there appears a slight shoulder of a more rapidly migrating component which presumably is a new amount of Chl *b'* formed during the separation. As in the *a*-series, all *b*-components possessed closely similar absorption spectra (Table I; 17–20) and reacted positively to the phase test. No characterizable amounts of allomerization products remained on the column in this instance.

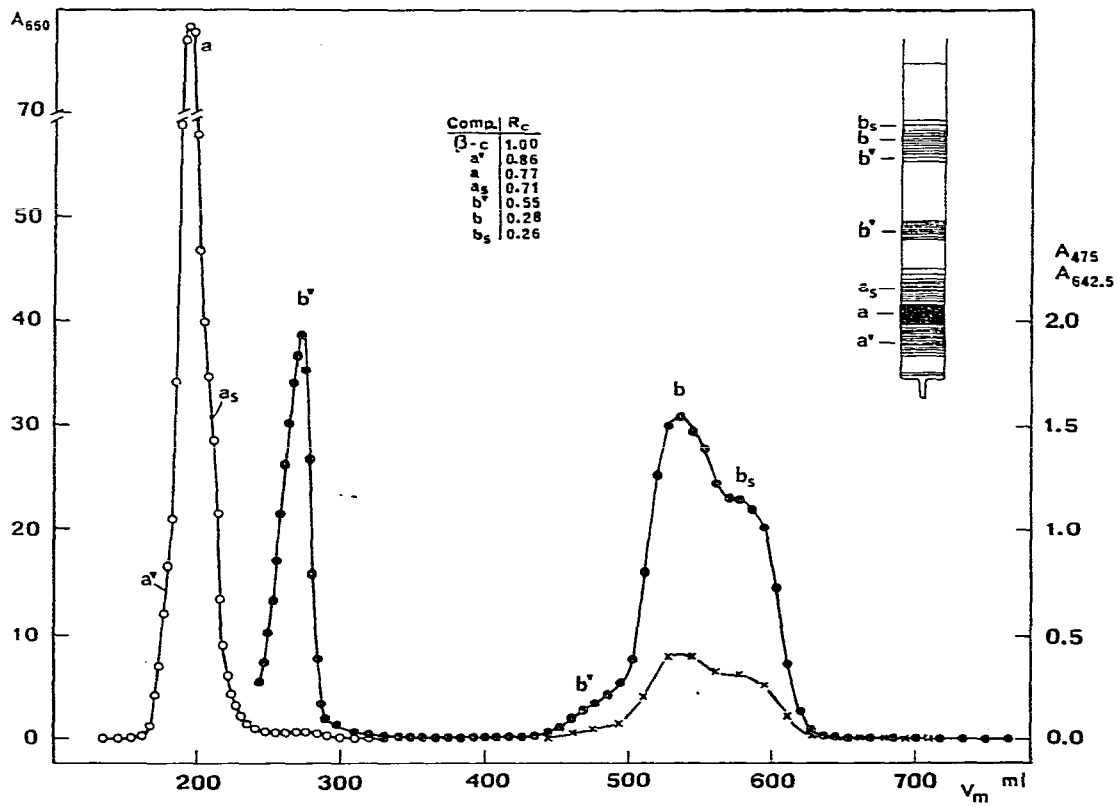

Fig. 6. Separation of the products formed on first washing the light petroleum solution of chloroplast pigments with 70% aqueous methanol and then precipitating the chlorophylls. h = 37.0 cm. Eluent as in Fig. 2. Flow-rate = 0.9 ml/min. ○, A_{660} (left-hand scale); ●, $A_{642.5}$; ×, A_{475} (right-hand scale). a', a and a_s = chlorophyll *a'*, *a* and a_s; b', b and b_s = chlorophyll *b'*, *b* and b_s, respectively. The upper right-hand part of the figure shows the situation before the most rapidly moving component (a') had emerged from the column.

Re-fractionation of the components in the fractions corresponding to V_m = 150–230 ml and V_m = 450–630 ml (Fig. 6) yielded the results presented in Fig. 7. In the *a*-series, a distinct Chl *a'* zone separated from Chl *a* and a new amount of Chl *b'* appeared after Chl *a*. The concentration profile of Chl *b* shows only a slight shoulder corresponding to the slowly migrating Chl b_s. The front part of the Chl *b* profile,

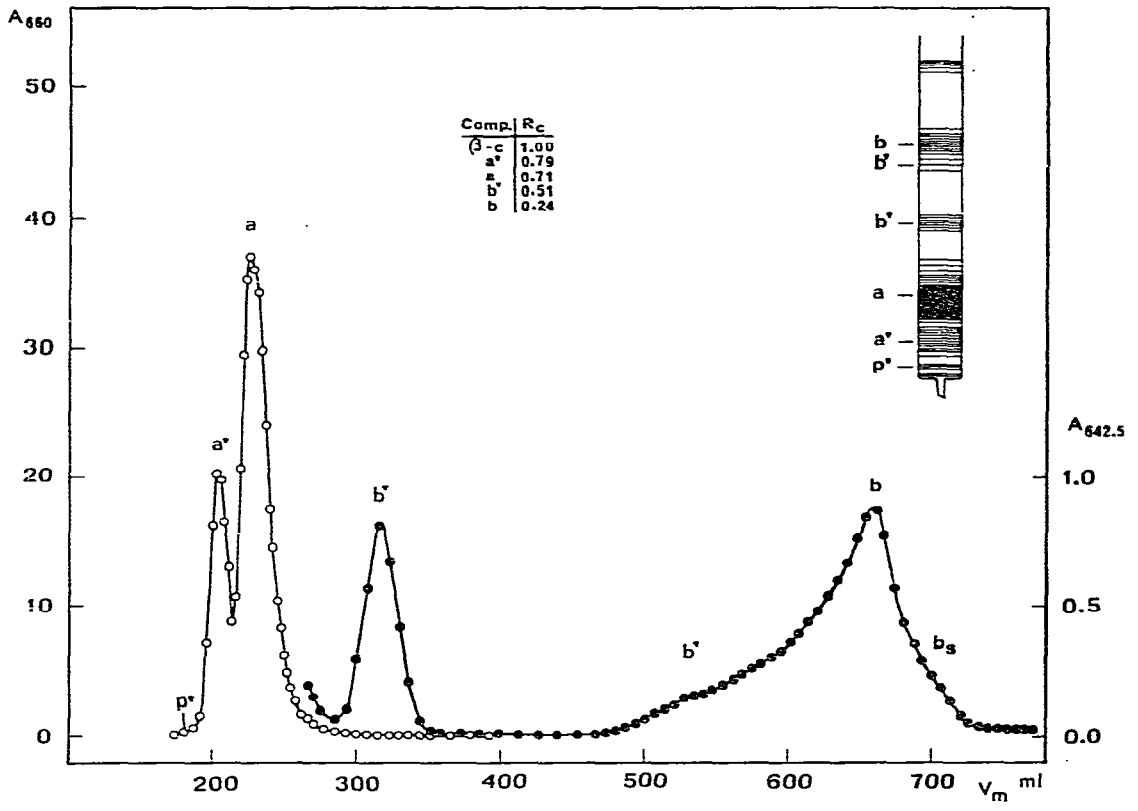

Fig. 7. Re-fractionation of the components in the fractions corresponding to V_m = 150–230 ml and V_m = 450–630 ml in Fig. 6. h = 37.3 cm. Eluent as in Fig. 2. Flow-rate = 0.7 ml/min. ○, A_{660} (left-hand scale); ●, $A_{642.5}$ (right-hand scale). Abbreviations as in Fig. 6. The upper right-hand part of the figure shows the situation before the most rapidly migrating component (a') had emerged from the column.

however, is extensively broadened, which signifies the formation of Chl b' during the separation.

On trying to interpret the appearance of the slowest migrating Chls (a_s and b_s), the following possibility should be considered. As Chl–water adducts were used in the samples for the separations in Figs. 2 and 6, it is possible that water molecules remained coordinated to the magnesium atoms of a part of the Chl molecules even on the sucrose column. These Chl molecules would be expected to migrate slower than those without coordinated water. The observed simultaneous formation of the prime Chls would then, however, be difficult to account for. As enolization is necessarily involved in the epimerization at C_{10}[8], it would require the assumption that Chl · H_2O molecules are enolized more easily than Chl molecules, which does not seem very likely.

The shoulders corresponding to the slow-migrating Chls could be alternatively explained by the aggregation occurring at the beginning of the separation[19]. No aggregation effects, however, were observed in the separations shown in Figs. 2 and 6. Moreover, the simultaneous formation of the prime Chls has not been observed in instances where aggregation has caused retardation or broadening of Chl zones[19].

The most plausible interpretation of the results described above is based on the keto–enol tautomerism of Chl. When Chl *a* was heated in PrOH in the presence of some water, the enolization of the β-keto ester system presumably occurred. The further transformation of the enol to Chl *a'* or allomerization products was, however, prevented by water, which can be hydrogen bonded to the enol. In this context, it should be noted that water can also hydrogen bond to the keto form of Chl. The bonds formed in this instance are evidently not strong enough to prevent the enolization completely. Owing to the stabilization effected by water, the aqueous alcohol solutions of Chl may contain the enol form of Chl in higher concentrations than is normally observed. As a rule, the concentration of the enol form(s) of Chl in organic solvents is very low and its lifetime is possibly only a few seconds[8]. The stabilization effected by water through hydrogen bonding, however, presumably increases both.

On the above basis, it seems likely that the slowly migrating Chls (a_s and b_s) arose from the presence of the enol form of Chl in the samples used in the fractionations. When these samples were introduced into the sucrose column, the hydrogen-bonded water molecules were removed from the enol. This rendered the enol susceptible to transformation during the separation on sucrose. As very little oxygen was present in the sucrose column (the solubility of oxygen in light petroleum is low), the free enol was principally converted into Chl *a* and *a'*. These reactions were, however, relatively slow at the low temperature used in the separations and, consequently, the lifetime of the enol was long enough to cause the appearance of the shoulder corresponding to the slowly migrating Chl. The enol form of Chl may be expected to move more slowly than the keto form, as the 9-desoxo-9-hydroxy-Chls move more slowly than their parent compounds on sucrose[21]. The visible absorption spectra measured on the effluent did not reveal the presence of the enol form of Chl, since at the moment the fractions were collected most of the enol originally present in the sample had already been transformed into Chl *a* and *a'*. In addition, small amounts of the enol would be difficult to detect by visible absorption spectroscopy, as the chlorin band of the enol is virtually at the same position as that of the keto form of Chl[8].

The separations shown in Figs. 3 and 4 demonstrate that the epimerization and allomerization reactions of Chl occur readily in PrOH solutions containing very little or no water. In this instance, the lifetime of the enol is short and it is converted into Chl *a'* or the allomerization products as rapidly as it is produced.

The results described offer additional evidence for the reaction scheme presented in Fig. 1. It is also important that they should be taken into consideration in the isolation of Chls[19]. The above results do not support the use of MeOH–water mixtures for the purification of Chl preparations, as appreciable amounts of the prime Chls may be produced as a consequence of these treatments. If they are used for that purpose, it is advisable to cool the mixtures before the extractions and to work as rapidly as possible to minimize the extent of the enolization of Chl.

ACKNOWLEDGEMENT

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REFERENCES

1 G. R. Seely, in L. P. Vernon and G. R. Seely (Editors), *The Chlorophylls*, Academic Press, New York, London, 1966, p. 67.
2 A. H. Jackson, in T. W. Goodwin (Editor), *Chemistry and Biochemistry of Plant Pigments*, Vol. 1, Academic Press, London, New York, San Francisco, 1976, p. 1.
3 H. H. Strain and W. A. Svec, in L. P. Vernon and G. R. Seely (Editors), *The Chlorophylls*, Academic Press, New York, London, 1966, p. 21.
4 H. H. Strain and W. A. Svec, *Advan. Chromatogr.*, 8 (1968) 119.
5 R. C. Dougherty, H. H. Strain and J. J. Katz, *J. Amer. Chem. Soc.*, 87 (1965) 104.
6 H. H. Strain, *J. Agr. Food Chem.*, 2 (1954) 1222.
7 J. J. Katz, G. D. Norman, W. A. Svec and H. H. Strain, *J. Amer. Chem. Soc.*, 90 (1968) 6841.
8 P. H. Hynninen, M. R. Wasielewski and J. J. Katz, *Acta Chem. Scand.*, in press.
9 F. C. Pennington, H. H. Strain, W. A. Svec and J. J. Katz, *J. Amer. Chem. Soc.*, 86 (1964) 1418.
10 G. W. Kenner, S. W. McCombie and K. M. Smith, *J. Chem. Soc. Perkin Trans. I*, (1973) 2517.
11 F. C. Pennington, S. D. Boyd, H. Horton, S. W. Taylor, G. D. Wulf and J. J. Katz, *J. Amer. Chem. Soc.*, 89 (1967) 3871.
12 P. H. Hynninen, *Acta Chem. Scand.*, 27 (1973) 1771.
13 L. G. Johnston and W. F. Watson, *J. Chem. Soc.*, (1956) 1203.
14 A. S. Holt, *Can. J. Biochem. Physiol.*, 36 (1958) 439.
15 F. C. Pennington, H. H. Strain, W. A. Svec and J. J. Katz, *J. Amer. Chem. Soc.*, 89 (1967) 3875.
16 P. H. Hynninen and S. Assandri, *Acta Chem. Scand.*, 27 (1973) 1478.
17 P. H. Hynninen, *Tetrahedron Lett.*, submitted for publication.
18 H. H. Strain, M. R. Thomas and J. J. Katz, *Biochem. Biophys. Acta*, 75 (1963) 306.
19 P. H. Hynninen, *Acta Chem. Scand.*, B, 31 (1977) 829.
20 P. H. Hynninen and N. Ellfolk, *Acta Chem. Scand.*, 27 (1973) 1463.
21 P. H. Hynninen, *J. Chromatogr.*, 175 (1979) 89.