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REDUCTION OF CHLOROPHYLL *a*, *a'* AND *b* BY SODIUM BOROHYDRIDE: SEPARATION OF DIASTEREOISOMERIC DESOXO-CHLOROPHYLL ALCOHOLS ON A SUCROSE COLUMN

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SUMMARY

Chlorophyll *a*, *a'* and *b* were reduced with sodium borohydride in pyridine and the desoxo-chlorophyll alcohols (DOCA) produced were separated on a sucrose column. Reduction of chlorophyll *a* (Chl *a*) for 15 min resulted in one principal product, the 9(*R*) 10(*R*) DOCA *a*. Similar treatment of Chl *a'* yielded as the principal reduction product a different diastereomer, presumably the 9(*S*) 10(*S*) DOCA *a*. More unreduced Chl remained also in the latter instance. This was interpreted as an indication of the steric hindrance of the 9-oxo group by the methoxycarbonyl group in Chl *a'*. Reduction of Chl *a* for 30 min resulted in three diastereomeric DOCA as evidenced by the separation on a sucrose column. These were presumably the 9(*S*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) forms of DOCA *a*. The 9(*R*) 10(*R*) form was, however, the principal reduction product also in this instance. When Chl *a'* was reduced for 30 min, the separation on sucrose yielded possibly four diastereomeric DOCA which were thought to possess the 9(*S*) 10(*S*), 9(*R*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) configurations. The 9(*S*) 10(*S*) form was still the principal reduction product. Chl *a'* also yielded appreciable amounts of magnesium-free pigments, which was interpreted as further evidence for the higher pheophytinization tendency of Chl *a'* in comparison with that of Chl *a*. The separation of the products from the reduction of Chl *b* for 30 min yielded five components. Two of them were 10-epimers of Chl *b*-3-methanol and the three others, different diastereomers of DOCA *b*-3-methanol.

INTRODUCTION

In methanol or pyridine–methanol, sodium borohydride ($NaBH_4$) selectively reduces the 9-oxo and 3-formyl groups on chlorophylls (Chl), chlorophyllides (Chld) and their corresponding magnesium-free derivatives (Fig. 1)[1,2]. Applying the $NaBH_4$ reduction to methyl-Chld *a*, Holt[1] reported only one reduction product, the Mg 9-desoxo-9-hydroxymethylpheophorbide *a*. Dilute solutions of $NaBH_4$ in methanol converted methyl-Chld *b* into Mg methylpheophorbide *b*-3-methanol, while larger amounts of this reagent yielded Mg 9-desoxo-9-hydroxymethylpheophorbide

b-3-methanol[1]. No stereochemical specification was given by Holt for these reduction products. Apparently, his chromatographic procedure did not separate the potential stereoisomers of the reduction products. Wolf and Scheer[2] applied $NaBH_4$ reduction to methylpheophorbide *a* and were able to separate three different diastereoisomers as reduction products by preparative thin-layer chromatography (TLC) on silica gel. These were characterized spectroscopically as the 9(*R*) 10(*R*), 9(*S*) 10(*R*) and 9(*S*) 10(*S*) configurations of 9-desoxo-9-hydroxymethylpheophorbide *a* (Fig. 1). The formation of the fourth isomer, possessing the 9(*R*) 10(*S*) configuration, could not be detected.

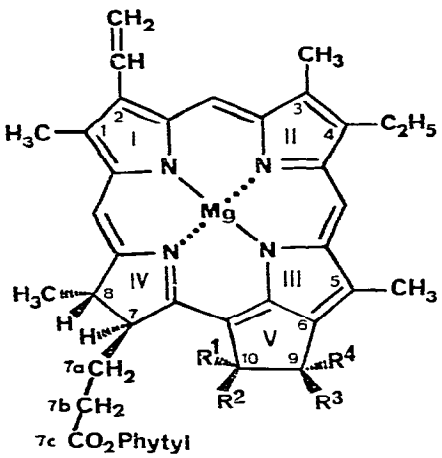

Fig. 1. Structures of chlorophylls and derivatives. In the compounds of the *b*-series, the methyl group at C_3 must be replaced with a formyl group. In methylchlorophyllides, the phytyl group at C_{7c} must be replaced with a methyl group. Pheophytins have 2H in place of Mg. Methylpheophorbides have a methyl group instead of phytyl at C_{7c} and 2H in place of Mg. Chl = chlorophyll; DOCA = desoxochlorophyll alcohol.

No.	R^1	R^2	R^3	R^4	*Designation*
1a	CO_2CH_3	H	= O		Chl *a* = Mg 10(*R*) pheophytin *a*
1b	H	CO_2CH_3	= O		Chl *a'* = 10-epi-Chl *a* = Mg 10(*S*) pheophytin *a*
2a	CO_2CH_3	H	H	OH	9(*R*) 10(*R*) DOCA *a* = 9-desoxo-9(*R*)hydroxy-10(*R*)Chl *a*
2b	CO_2CH_3	H	OH	H	9(*S*) 10(*R*) DOCA *a* = 9-desoxo-9(*S*)hydroxy-10(*R*)Chl *a*
3a	H	CO_2CH_3	H	OH	9(*R*) 10(*S*) DOCA *a* = 9-desoxo-9(*R*)hydroxy-10(*S*)Chl *a*
3b	H	CO_2CH_3	OH	H	9(*S*) 10(*S*) DOCA *a* = 9-desoxo-9(*S*)hydroxy-10(*S*)Chl *a*

In this investigation, the reactivities of chlorophyll *a*, *a'* and *b* with $NaBH_4$ in pyridine were compared by separating the products formed in each instance on a sucrose column. Investigations described elsewhere[3] had definitively established the 10-epimer nature[4,5] of Chl *a'*, formed through the keto-enol tautomerism of the β-keto ester system in ring V. These investigations also suggested that the observed higher solubility of Chl *a'* in non-polar solvents[6], its reduced tendency to form chlorophyll–water adducts[5,6] and greater tendency than Chl *a* to pheophytinize[6] are

best interpreted in terms of the conformational alterations arising from the single stereochemical change at C_{10}. According to this interpretation, the 9-oxo group of Chl *a'* has a reduced coordination tendency owing to steric hindrance by the methoxycarbonyl group which is on the same side of the ring plane as the phytylpropionic acid residue at C_7. The results of this investigation give further information on this effect in Chl *a'*. They also demonstrate that Chl *a* and Chl *a'* yield different diastereoisomers as principal products of reduction with $NaBH_4$ in pyridine. The chromatographic separations of the different diastereoisomeric reduction products of the chlorophylls are described in detail.

EXPERIMENTAL

Isolation of chlorophyll a and b

Chlorophyll *a* and *b* were isolated from frozen clover leaves (a mixture of *Trifolium pratense*, *T. hybridum* and *T. repens*) by the improved two-phase extraction method followed by precipitation and separation on a sucrose column[7]. The spectroscopic properties of these chlorophylls matched closely those previously described[7].

Isolation of chlorophyll a'

Chl *a'* was isolated from a Chl *a* preparation that had been standing for *ca.* 6 months in the dark at 4° in light petroleum (b.p. 60–80°) containing 0.5% of 1-propanol. Separation of the chlorophyll mixture on a sucrose column at 4° provided Chl *a'* in a yield of 38% (see Fig. 2). Chl *a'* could also be prepared more rapidly by the alternative methods described previously[3,5,6].

Reduction of chlorophyll by sodium borohydride

$NaBH_4$ (for synthesis, Merck, Darmstadt, G.F.R.) in pyridine (30 mg per 10 ml) was used as reducing agent. The effluent solution of chlorophyll was evaporated to dryness in a rotary evaporator immediately after the chromatographic separation. A 10-ml volume of the $NaBH_4$ solution was added to the residue. After standing for 15 or 30 min, the reaction mixture was evaporated to dryness, and the pigments in the residue were extracted into *ca.* 10 ml of the eluent to be used in the chromatographic separation.

Separation of the reduction products on a sucrose column

A glass column (50 × 3 cm I.D.) was employed in all separations. The column was packed by the slurry method[7]. Light petroleum (b.p. 60–80°) (LP), containing 0.5% of 1-propanol (PrOH), was used as the eluent for separating Chl *a* and *a'* as well as their reduction products, while 2% of PrOH in LP was employed for the separation of the reduction products of the *b* series. The eluent solution of the pigments was introduced into the top of the sucrose column, which was eluted until the components of interest had emerged from the column into the effluent. The absorbances (*A*) of the collected fractions were measured at selected wavelengths by means of a Perkin-Elmer 139 UV–visible spectrophotometer. All chromatographic separations were performed in the dark at 4°. β-Carotene was used as a reference

compound and the migration rate of a component was expressed in terms of the R_c values[7].

Visible absorption spectra

Visible absorption spectra were recorded with a Cary Model 118C 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 diethyl ether by means of a Thunberg tube at reduced pressure.

Solvents

Formamide, required for the isolation of Chl *a* and *b*, was purified by distillation *in vacuo*[8]. The distilled product was used without fractional crystallization. The other solvents employed were of analytical-reagent grade and were used without further purification.

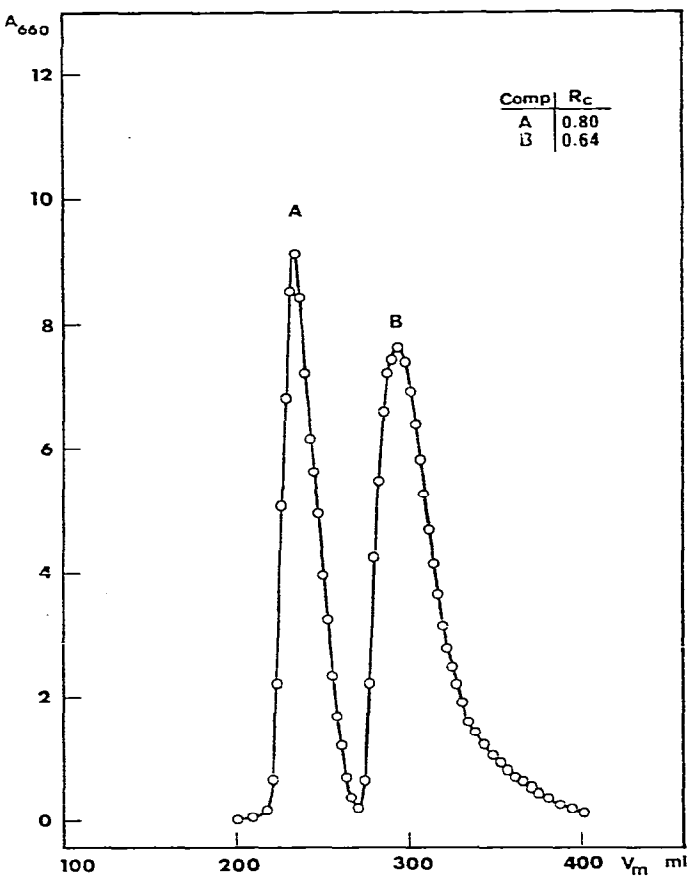

Fig. 2. Separation of chlorophyll *a'* and *a* on a sucrose column. Height of the sucrose layer, h = 44.0 cm. Eluent = 0.5% 1-propanol in light petroleum (b.p. 60–80°). Flow-rate, $\bar{u}$ = 0.9 ml/min. V_m = effluent volume. A = chlorophyll *a'* [= 10(*S*)-chlorophyll *a*], B = chlorophyll *a*. R_c = the elution volume of β-carotene divided by the elution volume of the component.

RESULTS AND DISCUSSION

Separation of chlorophyll a and a'

An example of a complete separation of Chl *a'* (A) and Chl *a* (B) on a sucrose column is shown in Fig. 2. In this instance the mixture of the epimeric chlorophylls was obtained as a result of prolonged standing of Chl *a* (7.0 mg) in LP containing 0.5% of PrOH in the dark at 4°. The mixture contained 2.2 mg (38%) of Chl *a'* and 3.6 mg (62%) of Chl *a*. A molar absorptivity of $8.63 \cdot 10^4$ l · mole^{-1} · cm^{-1} was used for the determination of the amounts of Chl *a* and *a'*[9,10]. Small amounts of oxidation (allomerization) products, not further characterized, remained on the sucrose column in this separation.

Separation of the products from the reduction of chlorophyll a and a' for 15 min

When Chl *a* (3.6 mg, B in Fig. 2) was reduced with $NaBH_4$ in pyridine for 15 min at room temperature, the separation of the products on sucrose yielded the results shown in Fig. 3. The green components A and C were spectroscopically

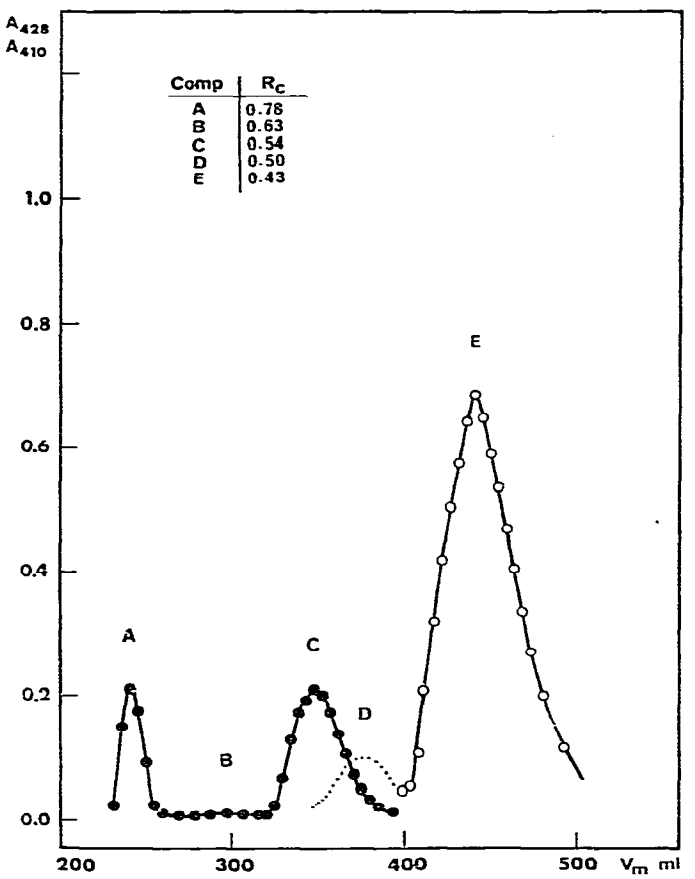

Fig. 3. Separation of the products from the $NaBH_4$ reduction of chlorophyll *a* for 15 min. h = 43.0 cm. Eluent as in Fig. 2. $\bar{u}$ = 1.0 ml/min. ○, A_{410}; ●, A_{428}. A = Chlorophyll *a'*; B = 10-propoxy-lactone derivative; C = chlorophyll *a*; D and E = 9(*S*) 10(*S*) and 9(*R*) 10(*R*) configurations of POCA *a*, respectively.

TABLE I

VISIBLE ABSORPTION SPECTRA OF CHLOROPHYLL DERIVATIVES

No.	*Derivative**	*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	10(*R*) Chl *a* (2B)§§	274–362	0.5% PLP	661.0(1.27), 613(7.97), 575(15.0), 530(29.9), 496 (59.8), 428.5(1.00), 409(1.54), 381(2.52), $w_{1/2}$ 16.4, $w_{S1/2}$ 38.0
2	10(*R*) Chl *a* (4E)	305	0.5% PLP	661.0(1.29), 614(7.84), 575(15.5), 530(30.5),496 (48.4), 429.0(1.00), 409(1.50), 382(2.37), $w_{1/2}$ 17.2, $w_{S1/2}$ 40.3
3	10(*R*) Chl *a* (6F)	339	0.5% PLP	660.5(1.21), 615(7.66), 576(15.7), 529(29.2), 494 (51.1), 428.0(1.00), 409(1.23), 382(2.27), $w_{1/2}$ 16.1, $w_{S1/2}$ 41.5
4	10(*R*) Chl *a* + impurity (3C+3D)	316–380	EE	660.5(1.44), 636(6.14), 616(7.73), 577(14.4), 527 (25.8), 496(46.8), 410(1.0), 384(2.14), $w_{1/2}$ 18.2, $w_{S1/2}$ 43.6
5	10(*R*) Chl *a* (7F) + impurity (7E)	372	0.5% PLP	660.0(1.40), 635(7.17), 614(8.6), 574(28.7), 531 (25.3), 599(19.5), 428.0 (1.23), 408.0(1.00), $w_{1/2}$ 17.8, $w_{S1/2}$ 61.7
6	10(*S*) Chl *a* (2A)	218–264	0.5% PLP	661.0(1.25), 613(7.44), 574(14.3), 531(26.0), 496 (45.1), 428.5(1.00), 409(1.39), 383(2.26), $w_{1/2}$ 17.3, $w_{S1/2}$ 40.7
7	10(*S*) Chl *a* (3A)	227–264	EE	660.5(1.43), 614(8.02), 575(15.9), 529(29.0), 494 (52.6), 429.0(1.00), 409(1.36), 383(2.29), $w_{1/2}$ 18.6, $w_{S1/2}$ 40.7
8	10(*S*) Chl *a* (4C)	237	0.5% PLP	661.0(1.26), 614(7.73), 574(20.2),531(47.2), 496 (85.0), 428.5(1.00), 409(1.46), 383(2.37), $w_{1/2}$ 17.6, $w_{S1/2}$ 39.9
9	10(*S*) Chl *a* (6B)	250	0.5% PLP	661.0(1.23), 613(7.44), 573(14.9), 530(19.8), 499 (22.3), 429.0(1.00), 409(1.24), 382(1.98), $w_{1/2}$ 16.7, $w_{S1/2}$ 55.6
10	10(*S*) Chl *a* (7B)	248	0.5% PLP	661.0(1.21), 613(8.22), 569(19.5), 528 (22.4),498 (19.5), 428.5(1.00), 408(1.04), 382(1.89), $w_{1/2}$ 17.2, $w_{S1/2}$ 68.9
11	10(*S*) Chl *a* (7B) + impurity (7C)	263	0.5% PLP	660.5(1.36), 614(8.89), 568(22.4), 527(22.4), 499 (16.0), 428(1.19), 404.0(1.00), $w_{1/2}$ 18.4, $w_{S1/2}$ 66.9
12	9(*R*) 10(*R*) DOCA *a* (3E)	403–492	EE	633.0(3.97), 589(30.1), 548(68.4), 515(33.4), 410.0(1.00), $w_{1/2}$ 18.6, $w_{S1/2}$ 17.8
13	9(*R*) 10(*R*) DOCA *a* (4G)	447	0.5% PLP	634.0(3.62), 590(42.7), 549(94.0), 516(47.0), 410.0(1.00), $w_{1/2}$ 17.8, $w_{S1/2}$ 17.2
14	9(*R*) 10(*R*) DOCA *a* (6H)	439	0.5% PLP	634.5(3.51), 591(28.7), 550(31.6), 516(31.6), 410.5(1.00), $w_{1/2}$ 16.7, $w_{S1/2}$ 17.7
15	9(*R*) 10(*R*) DOCA *a* (6H)	439	EE	633.5(3.99), 591(26.6), 550(42.8), 516(26.6), 410.5(1.00), $w_{1/2}$ 18.8, $w_{S1/2}$ 18.6
16	9(*R*) 10(*R*) DOCA *a* (7I)	489	0.5% PLP	635.0(4.52), 602(31.1), 551(52.9), 518(48.1), 410.0(1.00), $w_{1/2}$ 25.1, $w_{S1/2}$ 25.8
17	9(*R*) 10(*S*) DOCA *a* (7H)	440	0.5% PLP	635.0(3.30), 593(47.0), 549(61.1), 514(33.9) 408.5(1.00), $w_{1/2}$ 15.3, $w_{S1/2}$ 19.6
18	9(*S*) 10(*R*) DOCA *a* (6I)	487	0.5% PLP	634.5(3.50), 592(32.0), 550(54.9), 516(32.0), 410.5(1.00), $w_{1/2}$ 15.7, $w_{S1/2}$ 17.8
19	9(*S*) 10(*R*) DOCA *a* (6I)	487	EE	633.5(4.12), 590(36.5), 550(43.1), 516(31.6) 410.5(1.00), $w_{1/2}$ 17.8, $w_{S1/2}$ 18.0

TABLE I *(continued)*

No.	Derivative*	V_m** (ml)	Solvent***	Peak positions (nm), peak ratios (R)§, and half-widths of the red absorption band and the Soret band, $w_{I1/2}$ and $w_{S1/2}$ (nm)
20	9(*S*) 10(*R*) DOCA *a* (7J)	533	0.5% PLP	635.0(4.43), 602(23.0), 552(48.1), 515(34.7), 500(32.9), 410.5(1.00), $w_{I1/2}$ 30.5, $w_{S1/2}$ 28.2
21	9(*S*) 10(*S*) DOCA *a* (4F)	369	0.5% PLP	635.5(3.51), 596(33.1), 548(99.3), 514(38.6), 408.5(1.00), $w_{I1/2}$ 17.8, $w_{S1/2}$ 20.1
22	9(*S*) 10(*S*) DOCA *a* (6G)	376	0.5% PLP	635.5(3.16), 590(29.3), 449(61.6), 514(30.8), 409.0(1.00), $w_{I1/2}$ 15.9, $w_{S1/2}$ 17.6
23	9(*S*) 10(*S*) DOCA *a* (6G)	376	EE	633.5(3.81), 591(33.7), 550(78.7), 515(35.0), 409.5(1.00), $w_{I1,2}$ 17.4, $w_{S1/2}$ 17.9
24	9(*S*) 10(*S*) DOCA *a* (7G)	410	0.5% PLP	635.5(3.26), 595(33.9), 550(96.2), 515(36.1), 408.5(1.00), $w_{I1/2}$ 15.3, $w_{S1/2}$ 19.6
25	10(*R,S*) Pheophytin *a* (4B)	214	0.5% PLP	668.0(2.10), 611(14.2), 559(55.5), 532(13.2), 504 (10.5), 408.5(1.00), $w_{I1/2}$ 17.8, $w_{S1/2}$ 50.1
26	10(*S*) Pheophytin *a* (7A)	227	0.5% PLP	668.0(1.82), 610(14.3), 560(44.5), 532(11.1), 504(9.27), 465(27.8), 408.5(1.00), $w_{I1/2}$ 15.7, $w_{S1/2}$ 52.1
27	10(*R,S*) Pheophytin *a* + impurity (6A)	230	0.5% PLP	669.5(2.60), 635(10.9), 613(15.0), 563(34.1), 530 (13.8), 496(10.4), 399.0(1.00), $w_{I1,2}$ 21.7, $w_{S1/2}$ 48.5
28	DOPA *a* (4D)	274	0.5% PLP	653.5(2.94), 605(29.4), 549(106), 498(10.6), 395.0 (1.00), $w_{I1/2}$ 16.1, $w_{S1/2}$ 35.6
29	DOPA *a* (6D)	287	0.5% PLP	654.0(2.39), 603(20.3), 550(37.8), 520(33.4), 498(10.3), 397.0(1.00), $w_{I1/2}$ 16.1, $w_{S1/2}$ 41.3
30	DOPA *a* (7D)	299	0.5% PLP	653.0(2.57), 624(47.4), 597(38.5), 549(103), 498 (9.81), 395.0(1.00), $w_{I1/2}$ 12.5, $w_{S1/2}$ 34.1
31	10-Propoxylactone derivative (3B)	278–316	EE	654.0(2.68), 609(10.9), 560(24.4), 520(21.8), 500 (25.4), 413.5(1.00), $w_{I1/2}$ 37.6, $w_{S1/2}$ 28.2
32	10-Propoxylactone derivative (6E)	316	0.5% PLP	653.5(1.81), 609(10.8), 564(23.8), 522(27.8), 486 (55.6), 415.5(1.00), $w_{I1/2}$ 21.0, $w_{S1/2}$ 36.4
33	Mg unstable chlorin 7-methylphytyl ester		EE	650.0(2.52), 610(12.3), 566(23.2), 522(31.4), 417.0 (1.00), $w_{I1/2}$ 24.3, $w_{S1/2}$ 34.2
34	10(*R*) Chl *b*-3-methanol (8D)	418–428	EE	654.5(1.75), 614(9.12), 576(22.6), 530(31.2), 498 (51.5), 429.0(1.00), 411(1.51), 382(3.08), $w_{I1/2}$ 18.8, $w_{S1/2}$ 37.2
35	10(*S*) Chl *b*-3-methanol (8A)	263–296	2.0% PLP	654.5(1.87), 617(7.03), 575(19.7), 533(28.1), 501 (40.3), 430.0(1.00), 413(1.17), 373(2.99), $w_{I1/2}$ 20.7, $w_{S1/2}$ 42.7
36	9(*R*) 10(*R*) DOCA *b*-3-methanol (8F)	548–558	EE	629.0(5.01), 582(55.1), 517(48.4), 413.5(1.00), $w_{I1/2}$ 19.4, $w_{S1/2}$ 14.9
37	9(*S*) 10(*R*) DOCA *b*-3-methanol (8G)	642–651	EE	629.0(4.86), 580(50.0), 517(38.6), 413.5(1.00), $w_{I1/2}$ 20.9, $w_{S1/2}$ 15.3
38	9(*S*) 10(*S*) DOCA *b*-3-methanol (8E)	460–469	EE	628.5(5.01), 580(67.8), 516(52.4), 413.0(1.00), $w_{I1/2}$ 20.6, $w_{S1/2}$ 16.1

* Chl = chlorophyll; DOCA = desoxochlorophyll alcohol; DOPA = desoxopheophytin alcohol.

** V_m = effluent volume.

*** 0.5% (2%) PLP = 0.5% (2%) 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.

§§ The number and letter in parentheses refer, respectively, to the figure number and to the component in that figure.

closely similar to Chl *a* (Table I; 1, 6). As both components also reacted positively to the Molisch phase test, they probably represent Chl *a'* and Chl *a*. A trace amount of another component (B) appeared between A and C. The R_c value, the negative phase test and the visible absorption spectrum of B suggest that it may be a 10-propoxylactone derivative. The principal component E (blue) showed a visible absorption spectrum (Table I; 12) closely similar to that described for 9-desoxo-9-hydroxymethyl-Chld *a*[1]. At first, component E appeared to be the only reduction product. Re-examination of the absorption spectrum of C, however, revealed that it was contaminated by a component that had a red band at 636 nm. In addition, the spectrum measured at $V_m = 380$ ml showed a stronger red band at 636 nm than that expected on the basis of the elution profile of E. It seemed likely, therefore, that a small amount of another reduction product (stereoisomer), in addition to E, was located between C and E. The approximate amount and position of this component (D) was estimated from the measured spectra. Small amounts of oxidation products remained in the upper part of the column. The main oxidation product was a bright-green component that was almost immobile. The slow migration rate and the nature of the absorption spectrum (Table I; 33) of this component suggest that it is presumably a 10-hydroxylactone derivative (Mg-unstable chlorin 7-methyl-phytyl ester)[11,12].

Fig. 4 shows the separation of the $NaBH_4$ reduction products of Chl *a'* (2.2 mg, A in Fig. 2). The reduction and separation conditions employed were similar to those used in the separation shown in Fig. 3. A trace amount of an unidentified pigment (A) appeared first in the effluent. Components B and C were characterized as pheophytin *a* and Chl *a'* on the basis of their R_c values, positive reactions to the phase test and visible absorption spectra (Table I; 8, 25). The spectrum measured at $V_m = 274$ ml (Table I; 28) provided evidence that D was a small amount of 9-desoxo-9-hydroxypheophytin *a* (DOPA *a*)[1]. The principal green component E in the effluent series was identified as Chl *a* [$R_c = 0.62$, positive reaction to the phase test, visible absorption spectrum (Table I; 2) closely similar to that of Chl *a*]. The blue pigments F and G exhibited visible absorption spectra (Table I; 13, 21) very similar to that of 9-desoxo-9-hydroxymethyl-Chld *a*[1]. F and G were, however, slightly different from each other in the positions of the Soret bands. The Soret band of F shows a blue shift of 1.0–1.5 nm. Comparison of the R_c values indicates that F and G in Fig. 4 correspond to D and E in Fig. 3. The relative amounts of the two reduction products are, however, different in Figs. 3 and 4. In Fig. 3, the slower migrating blue component (E, $R_c = 0.43$) is the principal reduction product, whereas in Fig. 4, the faster moving blue component (F, $R_c = 0.51$) is the principal reduction product. These results are best interpreted on the basis of stereoisomerism. The two blue components presumably differ from each other in the configurations at C_9 and C_{10}. As Chl *a* possesses an *R* configuration at C_{10}, it would yield the 9(*R*) 10(*R*) DOCA *a* (2a) as the principal reduction product. In this case, the *R* configuration at C_9 is more likely than the *S* owing to the greater possibility for hydrogen bond stabilization in 2a than in 2b (Fig. 5). Being epimeric with Chl *a* at C_{10}, Chl *a'* would yield the 9(*S*) 10(*S*) DOCA *a* (3b) as the principal reduction product, as now the 9(*S*) configuration is more favourable than the 9(*R*) on the basis of hydrogen bond stabilization considerations (see 3a and 3b in Fig. 5). The fact that there is a smaller amount of another stereoisomer, in addition to the principal re-

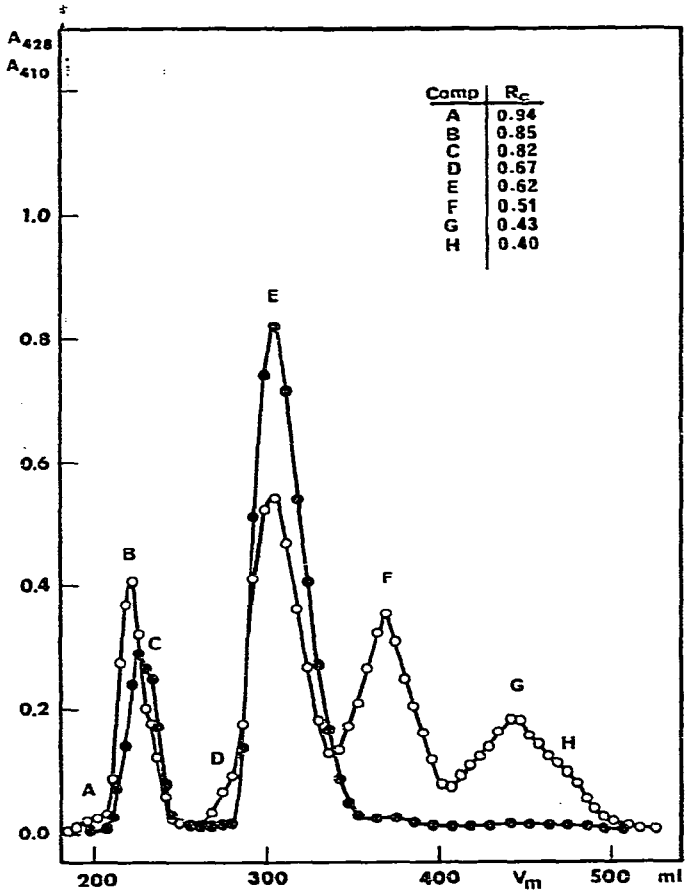

Fig. 4. Separation of the products from the $NaBH_4$ reduction of chlorophyll *a'* for 15 min. h = 42.3 cm. Eluent as in Fig. 2. $\bar{u}$ = 1.2 ml/min. ○, A_{410}; ●, A_{428}. A = Pheophytin *a'*(?); B = pheophytin *a*; C = chlorophyll *a'*; D = DOPA *a* (configurations unknown); E = chlorophyll *a*; F and G = 9(*S*) 10(*S*) and 9(*R*) 10(*R*) configurations of DOCA *a*, respectively.

duction product, in Figs. 3 and 4 can be explained by the enolization and epimerization reactions occurring in pyridine during the reduction period. The small amount of base probably present in the $NaBH_4$ preparation may be expected to accelerate these reactions.

On comparing the results in Figs. 3 and 4, it is also evident that there is more unreduced chlorophyll in Fig. 4 and that the difference between the amounts of the two stereoisomeric reduction products is smaller in Fig. 4. This is interpreted to imply that Chl *a'* is more resistant to reduction by $NaBH_4$ owing to steric hindrance of the 9-oxo group by the methoxycarbonyl group. The fact that there is more Chl *a* than Chl *a'* in Fig. 4 is presumably due to the easy interchangeability of these epimers. Chl *a'* was probably converted into Chl *a*, partly during the reduction and partly during the preparation of the sample for chromatography.

There is a third difference between the results in Figs. 3 and 4. In Fig. 4, the presence of the magnesium-free derivatives (pheophytin *a* and DOPA *a*, configurations unknown) was clearly demonstrated, whereas neither of these pigments

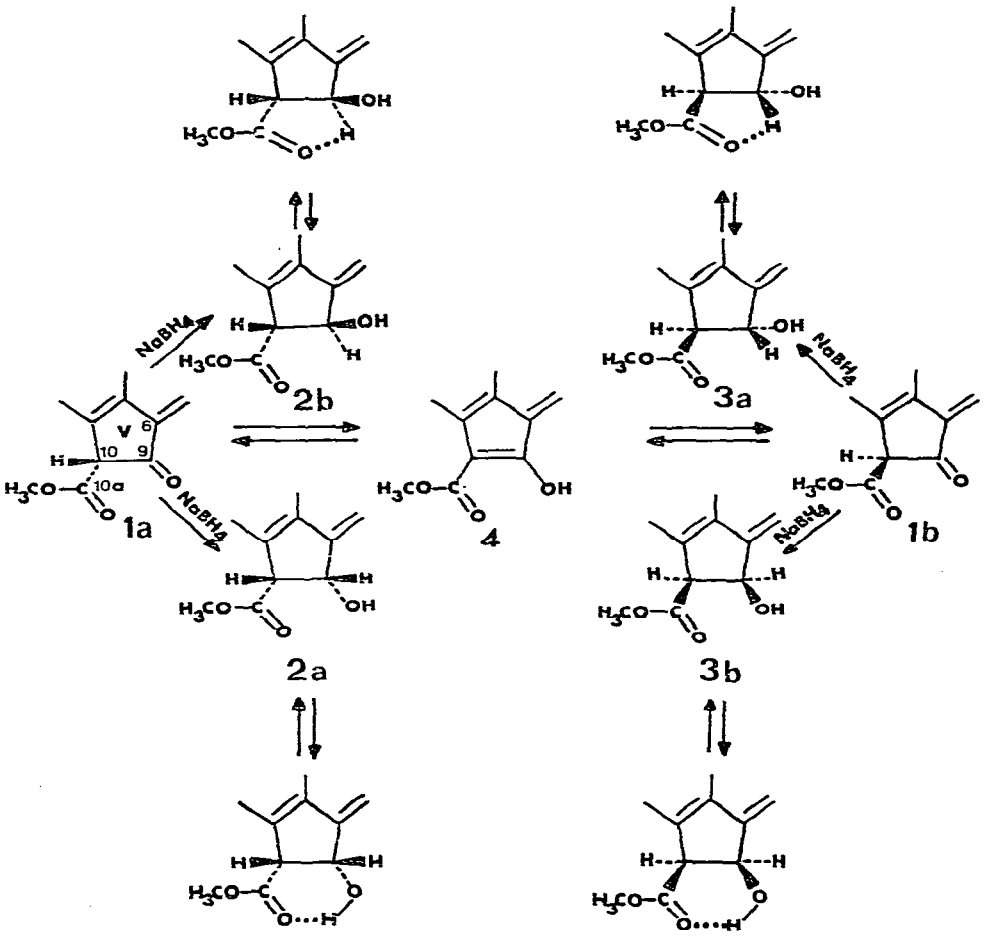

Fig. 5. Possibilities for intramolecular hydrogen bonding in the four diastereoisomers (2a, 2b, 3a, 3b) of DOCA *a*. 1a = chlorophyll *a*; 1b = chlorophyll *a'*; 4 = enol form of chlorophyll *a*.

could be detected in Fig. 3. This is further evidence for the greater tendency of Chl *a'* to pheophytinize in comparison with that of Chl *a*[6]. A small amount of oxidation products remained on the sucrose column also in the separation shown in Fig. 4. The slight shoulder marked H in Fig. 4 possibly signifies the presence of a small amount of a third diastereoisomeric reduction product of chlorophyll.

Separation of the products from the reductions of chlorophyll a and a' for 30 min

Reduction of Chl *a* (6.8 mg) by $NaBH_4$ in pyridine for 30 min at room temperature resulted in the formation of three different blue reduction products as shown by the separation on a sucrose column (Fig. 6, components G, H and I). The absorption spectra (Table I; 14, 15, 22, 23) and R_c values of G and H in Fig. 6 indicate that these components correspond to D and E in Fig. 3 and F and G in Fig. 4. Component I in Fig. 6 is new reduction product not detected in the previous separations [except perhaps as a slight shoulder (H) in the separation shown in Fig. 4]. Components G and H in Fig. 6 possessed slightly different absorption spectra. Con-

sistent with the above results, the difference between the positions of the Soret bands of G and H was 1.0–1.5 nm. Components H and I, on the other hand, were spectroscopically identical (Table I; 15, 19). The R_c values, visible absorption spectra and hydrogen bond stabilization considerations suggest that components G, H and I represent the 9(*S*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) configurations of DOCA *a* (3b, 2a and 2b). The fact that the formation of the third diastereoisomeric DOCA (I) could be clearly detected in this instance was presumably due to the longer reduction time used in the experiment. TLC on cellulose[13] using pyridine–light petroleum (1:9) as the solvent system separated a mixture of components G, H and I into three spots which exhibited red fluorescence under UV light. The probable character of the minor components, A–F in Fig. 6, is indicated in the legend to this figure. The visible absorption spectra of these components appear in Table I (3, 9, 27, 29, 32).

When Chl *a'* (4.1 mg) was subjected to reduction with $NaBH_4$ in pyridine for 30 min, the separation of the reduction products on sucrose yielded the results shown

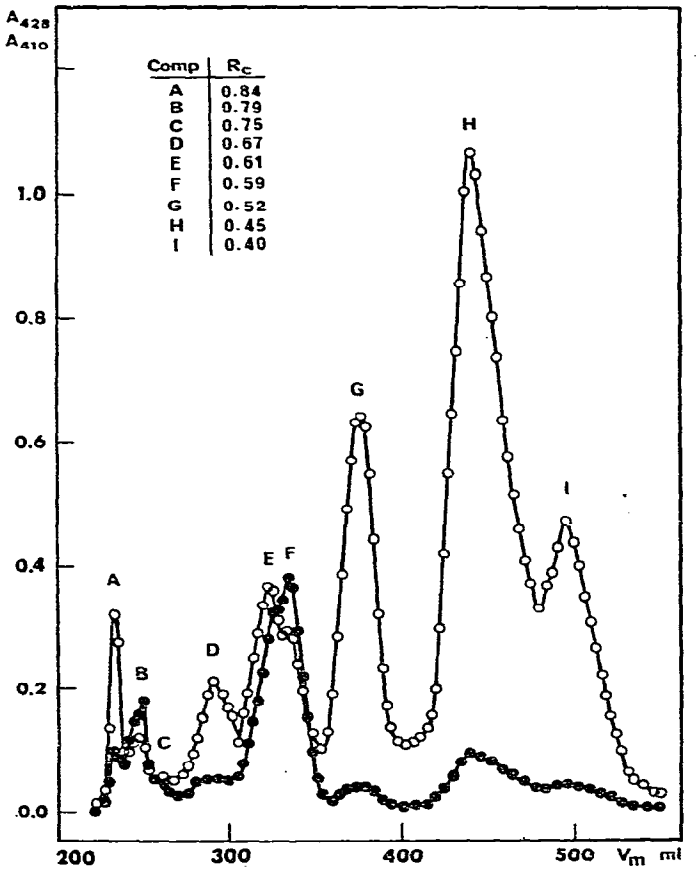

Fig. 6. Separation of the products from the $NaBH_4$ reduction of chlorophyll *a* for 30 min. h = 43.1 cm. Eluent as in Fig. 2. $\bar{u}$ = 1.0 ml/min. ○, A_{410}; ●, A_{428}. A = Pheophytin *a'*; B = chlorophyll *a'*; C = unidentified impurity; D = DOPA *a* (configurations unknown); E = 10-propoxylactone derivative; F = chlorophyll *a*; G, H and I = 9(*S*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) configurations of DOCA *a*, respectively.

in Fig. 7. The concentration profile seems to reveal the presence of four diastereoisomeric DOCA (G, H, I and J). The R_c values, visible absorption spectra (Table I; 16, 17, 20, 24) and hydrogen bond stabilization considerations suggest that G, H, I and J represent the 9(*S*) 10(*S*), 9(*R*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) configurations of DOCA *a* (3b, 3a, 2a and 2b). Consistent with the results in Fig. 4, the component possessing an R_c value of *ca.* 0.5 was the principal DOCA also after reduction for 30 min. The ratio of the amounts of components I and J in Fig. 7 is probably not correct, as the fractions from 500 to 550 ml contained some impurity with a red absorption band at 650 nm. This impurity is presumably an allomerization product (a lactone derivative?) which also absorbs at 410 nm. Consequently, the actual amount of DOCA in fractions from 500 to 550 ml is probably considerably lower than that revealed by the concentration profile in Fig. 7.

Another essential feature revealed by Fig. 7 is the formation of large amounts of 10(*S*) pheophytin *a* (A) and DOPA *a* (D). Component A presumably has prin-

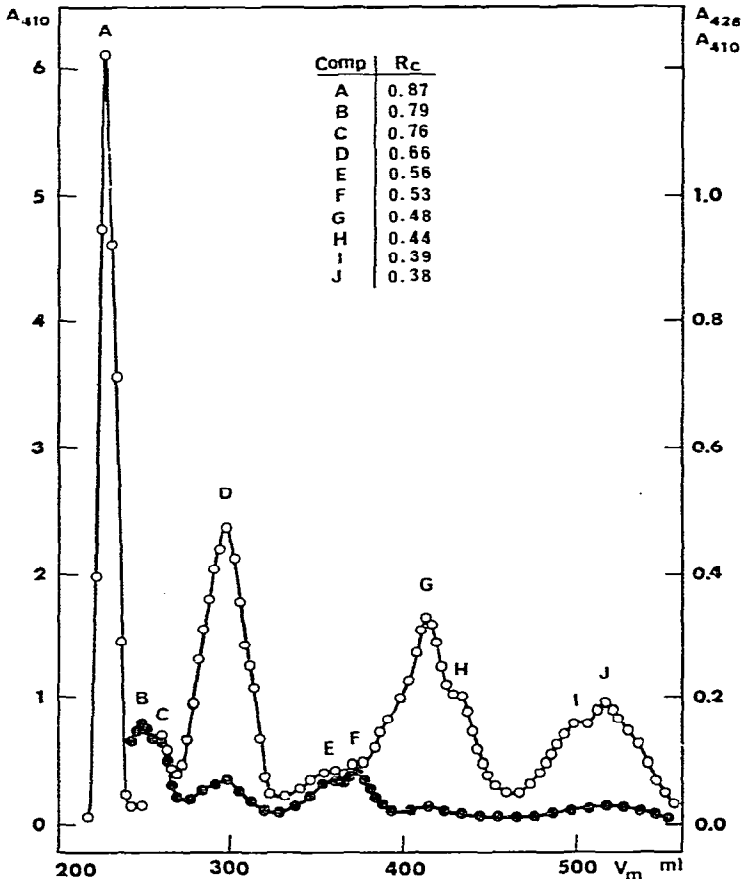

Fig. 7. Separation of the products from the $NaBH_4$ reduction of chlorophyll *a'* for 30 min. h = 44.0 cm. Eluent as in Fig. 2. $\bar{u}$ = 1.0 ml/min. ○, A_{410}; ●, A_{428}. A = Pheophytin *a'*; B = chlorophyll *a'*; C = unknown impurity; D = DOPA *a* (configurations unknown); E = 10-propoxylactone derivative (?); F = chlorophyll *a*; G, H, I and J = 9(*S*) 10(*S*), 9(*R*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) configurations of DOCA *a*, respectively.

cipally the 10(*S*) configuration, as it is derived from Chl *a'* through the loss of the magnesium atom. D possibly contains more than one stereoisomer which do not separate owing to the high R_c values. Two reasons can account for the large amounts of the magnesium-free pigments produced in the reduction shown in Fig. 7. Firstly, magnesium may be released from Chl *a'* as a result of the formation of a transient complex between $NaBH_4$ and Chl *a'*. Secondly, pyridine, which was used as a solvent, is capable of coordinating to the fifth and sixth positions of the central magnesium atom. This may be sufficient to abstract the magnesium from Chl *a'*. Comparison of the results in Figs. 6 and 7 appears to indicate more clearly than the results in Figs. 3 and 4 that the bonds between the magnesium atom and the nitrogen atoms are weaker in Chl *a'* than in Chl *a*. The observed greater tendency of Chl *a'* to pheophytinize was previously[3] interpreted to arise from the conformational alterations caused by the single stereochemical change at C_{10}. These alterations presumably also affect the bonds between the magnesium atom and the nitrogen atoms. The X-ray measurements by Chow *et al.*[14] show that in ethylchlorophyllide *a* dihydrate the five-coordinate magnesium is displaced by 0.39 Å from the plane of three nitrogen atoms. In this compound, a water molecule occupies the fifth coordination site of the magnesium. The conformational alterations occurring with Chl *a'* may be expected to increase this perturbation, which results in weakening of the bonds between the magnesium atom and the nitrogen atoms. On this basis, it seems likely that ligands such as pyridine or water, which can form relatively strong bonds with the fifth coordination site of the magnesium atom, are liable to cause the abstraction of the magnesium from Chl *a'*.

Separation of the products from the reduction of chlorophyll b for 30 min

Reduction of Chl *b* (1.3 mg; the preparation may have contained some Chl *b'*) with $NaBH_4$ in pyridine for 30 min at room temperature yielded five different reduction products which could be separated on a sucrose column employing 2% PrOH in LP as the eluent (Fig. 8). The visible absorption spectra (Table I; 35–38) of the separated components suggest that components A and D represent the 10(*S*) and 10(*R*) configurations of Chl *b*-3-methanol, respectively; components E, F and G are three different diastereoisomers of DOCA *b*-3-methanol. This result is analogous to that obtained in the *a*-series. The stereochemical configurations of components E, F and G presumably are 9(*S*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*), respectively. The relative amounts of the last stereoisomer (G in Fig. 8) seems to be higher than the amount of the corresponding isomer in the *a*-series (I in Fig. 6). This slight difference may be accounted for by the potential presence of Chl *b'* in the Chl *b* preparation.

Stability of desoxo-chlorophyll alcohols

The DOCA do not show the reactions typical of phorbins possessing an intact β-keto ester system in ring V. Accordingly, they are not solvolysed, epimerized or allomerized in methanol. Further, they do not yield pyro-compounds (derivatives lacking the methoxycarbonyl group at C_{10}) on heating in pyridine[15] or collidine[16] and they give negative reactions to the phase test[15]. These properties of the DOCA can be explained by considering the fact that the enolization of the β-keto ester system is involved as a preliminary step in all of the above reactions. There is no

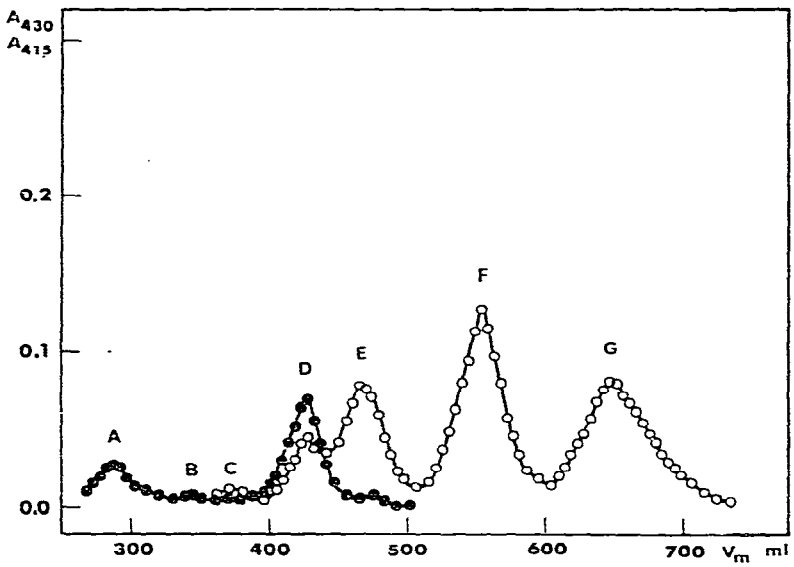

Fig. 8. Separation of the products from the $NaBH_4$ reduction of chlorophyll *b* for 30 min. h = 42.1 cm. Eluent = 2% 1-propanol in light petroleum (b.p. 60–80°). $\bar{u}$ = 0.9 ml/min. ○, A_{415}; ●, A_{430}. A = Chlorophyll *b*′-3-methanol; B and C = unknown impurities; D = chlorophyll *b*-3-methanol; E, F and G = 9(*S*) 10(*S*), 9(*R*) 10(*R*) and 9(*S*) 10(*R*) configurations of DOCA *b*-3-methanol, respectively.

possibility for enolization in the DOCA and, consequently, these compounds do not exhibit the reactions typical of chlorophylls and their magnesium-free derivatives.

In one respect, however, the DOCA seem to be more labile than their parent compounds, *viz.*, they lose the magnesium atom more readily than Chl *a* and *b*. Thus, as a result of prolonged standing in light petroleum, the DOCA were converted into the corresponding magnesium-free compounds as indicated by the visible absorption spectrum and separation on a sucrose column. The fact that standing in a polar solvent, *e.g.*, methanol, did not produce these magnesium-free pigments suggests that the formation of hydrogen bonds (Fig. 5) in DOCA causes additional strain and conformational alterations which affect the bonds between the magnesium atom and the nitrogen atoms.

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