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Chlorophylls

II. Allomerization of Chlorophylls *a* and *b*

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Chloroplast pigments, obtained by extracting dry leaf powder with pyridine, were separated by multiple liquid-liquid partition using 70 fractionation tubes and employing petroleum ether-ethanol-formamide (PEF) as the solvent system. The longer the extraction time with pyridine, the more completely was chlorophyll *a* converted into a 10-ethoxy-lactone derivative. This observation was interpreted as supporting the concept, presented in Part I of this series, that allomerization of the chlorophylls is initiated by enolization.

The allomerization products of chlorophylls *a* and *b* in methanol were fractionated by multiple liquid-liquid partition utilizing 50 tubes and employing petroleum ether-benzene-methanol-formamide (PBMF) as the solvent system. The two chief products of chlorophyll *a* were a 10-methoxy-lactone derivative and 10-hydroxy-chlorophyll *a*. In addition to these, four minor components were detected: Mg-unstable chlorin methylphytyl ester, Mg-purpurin 7-(di)methylphytyl ester, Mg-chlorin e_6-dimethylphytyl ester and 10-methoxy-chlorophyll *a*. Evidence was presented demonstrating that chlorophyll *a* preparations isolated by means of conventional methods may be contaminated by the Mg-purpurin 7-triester. The principal allomerization product of chlorophyll *b* was a 10-methoxy-lactone derivative. The concentration profile of the fractionation revealed that this derivative was in slow equilibrium with another component, presumed to be Mg-*b*-purpurin 7-(di)methylphytyl ester. Mg-unstable rhodin methylphytyl ester was a third product of the *b*-series.

A reaction scheme for the allomerization of the chlorophylls was proposed. In addition, the formation of the 10-hydroxy- and 10-methoxy-chlorophylls, which were not included in the scheme, was briefly discussed.

In a previous investigation [1] concerning the fractionation of chlorophylls employing three different types of solvent system, the formation of the 10-alkoxy-lactone derivative from chlorophyll *a* during fractionation was observed to depend in a sensitive manner upon the polarity of the lower phase of the solvent system. When either petroleum ether-ethanol-formamide

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(PEF) or petroleum ether-benzene-methanol-formamide (PBMF) was employed as the solvent system, the alkoxy-lactone derivative did not form; in petroleum ether-methanol-formamide (PMF), however, such formation could not be avoided. This observation was interpreted as signifying that allomerization is preceded by enolization.

In the present article, further evidence will be forwarded in support of the reaction scheme previously presented by one of the authors.[1] This evidence derives from two fractionations in which pyridine was employed in the extraction of the pigments from the plant material. In addition, the allomerization products of chlorophylls *a* and *b* were separated by means of multiple liquid-liquid partition. The results of these studies will be compared with earlier investigations.[2–21]

EXPERIMENTAL

Extraction of the pigments from plant material. Extract 1. Five grams of dry powder prepared from soybean leaves were suspended in 250 ml of pyridine and the mixture was vigorously stirred manually. After 10 min, the suspension was filtered and the filtrate evaporated to dryness by means of a rotatory evaporator. The remaining residue was then dissolved in 10 ml of petroleum ether.

Extract 2. This extract was prepared in the same manner as Extract 1, except that the mixture was stirred by means of a magnetic stirrer for 1 h, rather than being stirred manually for 10 min.

Preparation of allomerized chlorophyll. Fifty milligrams of chlorophyll *a* (7 mg of chlorophyll *b*), isolated by multiple liquid-liquid partition,[1] were dissolved in 25 ml of methanol. The solution was then allowed to stand for 7 days in an Erlenmeyer flask provided with a loose glass stopper. At the conclusion of this time, the solution was evaporated to dryness and the residue dissolved in 15 ml of petroleum ether.

Preparation of some phyllin derivatives. Magnesium-free phyllin derivatives were prepared by treating a solution of the phyllin with 13 % hydrochloric acid. The phytyl group was removed by hydrolysis of the ester in 30 % hydrochloric acid. Methyl esters of the phyllins or their magnesium-free derivatives were prepared by means of diazomethane. The ethereal solution of diazomethane was prepared from nitrosomethylurea by saponification, as described by Eistert.[22] The magnesium-free derivatives were purified by partition between aqueous hydrochloric acid and diethyl ether.[3,23,24]

Equipment and solvents. Separations by multiple liquid-liquid partition were performed by means of the Hietala apparatus. Operating conditions were, in general, similar to those described previously.[1]

The PEF- and PBMF-solvent systems were prepared as earlier described.[1] The other solvents employed were of reagent grade and were used as commercially supplied.

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

RESULTS AND DISCUSSION

The results of the distribution experiment performed on Extract 1 are presented in Fig. 1. PEF was employed as solvent system for this fractionation. The concentration profile of chlorophyll *a* revealed that a reaction of some type had occurred during fractionation. Judging upon the basis of spectroscopic and chemical characteristics, the deeply blue-green product D appeared to be Mg-purpurin 7-lactone-ethyl ether-methylphytyl ester (a 10-ethoxy-lactone derivative of chlorophyll *a*). These properties closely resembled those of Mg-purpurin 7-lactone-methyl ether-diester, which had been previ-

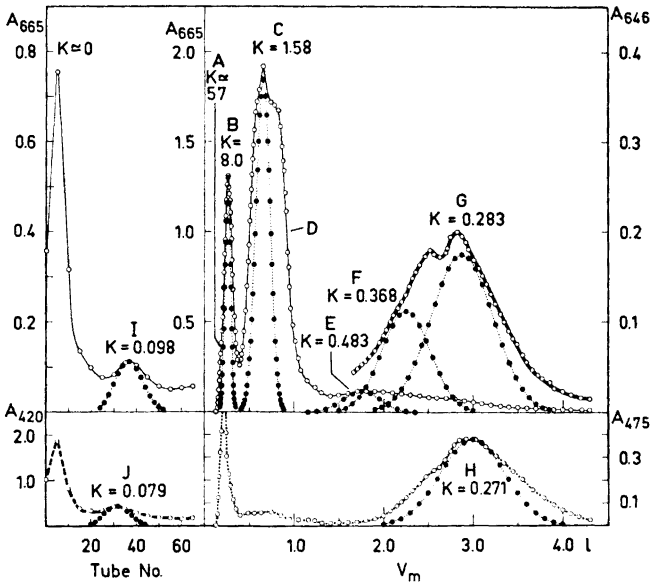

Fig. 1. Separation of chloroplast pigments employing Extract 1 and the PEF-system. The extract was sampled into tubes r = 0,..,3. Number of fractionation tubes utilized = $N = 70$. Average volume of the mobile phase in a partition unit = $v_m = 2.39$ ml; average volume of the stationary phase in a partition unit = $v_s = 11.11$ ml. Total volume of effluent eluted from the apparatus = $V_m = 4307$ 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_{646}, ··· = A_{475}, and ▪▪ = A_{420}. A = β-carotene, B = pheophytin *a*, C = chlorophyll *a*, D = Mg-purpurin 7-lactone-ethyl ether-methylphytyl ester, E = 10-hydroxy-chlorophyll *a*, F = chlorophyll *b*, G = Mg-*b*-purpurin 7-lactone-ethyl ether-methylphytyl ester, H = lutein, I = 10-hydroxy-chlorophyll *b*, J = violaxanthin, and K = chlorophyllide, *etc.*

ously characterized as the allomerization product of chlorophyll *a* when the PMF-system was utilized.[1] The material eluted at 2200 ml yielded a positive reaction to the Molisch phase test, whereas that eluted at 3000 ml appeared to react negatively. Components F and G were characterized, respectively, as chlorophyll *b* and Mg-*b*-purpurin 7-lactone-ethyl ether-methylphytyl ester (a 10-ethoxy-lactone derivative of chlorophyll *b*). The spectroscopic and chemical properties of the latter compound closely resembled those of the 10-alkoxy-lactone derivative found previously.[1] Only relatively small amounts of the 10-hydroxy-chlorophylls (components E and I) were detectable in the present case and these probably did not form during the fractionation. It appears more likely that they arose during extraction of the pigments from the plant material or during drying of the soybean leaves.

Since the lactonization of chlorophyll *a* did not occur in the PEF-system when the pigments had been extracted from the frozen soybean leaves with either 80 % acetone or petroleum ether(3)-methanol(1),[1] it was therefore concluded that some reaction preceding lactonization had taken place in the

pyridine. In order to test the validity of this conclusion, a partition fractionation was performed on Extract 2. In this instance, the pyridine extraction was carried out for a considerably longer time than in the preparation of Extract 1. The resulting concentration profile revealed that chlorophyll *a* had now been transformed almost completely to the 10-alkoxy-lactone derivative (Fig. 2). Considering the fact that Extracts 1 and 2 both yielded clearly positive reactions to the Molisch phase test, the results described may best be interpreted by assuming that enolization proceeds with considerable velocity in pyridine and that it is this reaction which precedes the formation of the alkoxy-lactone derivative. These results appear to support strongly the reaction scheme previously presented for the allomerization.[1]

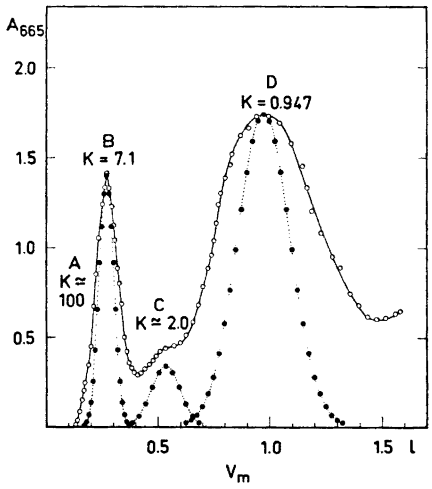

Fig. 2. Separation of chloroplast pigments employing Extract 2 and the PEF-system. The extract was sampled into tubes r = 0,..,3. $N = 70$. $v_m = 2.36$ ml; $v_s = 11.14$ ml. $V_m = 1580$ ml; flow rate = 2 ml/min. Theoretical (●) and experimental (O) values, the latter obtained by measuring A_{665} of the effluent fractions. A = β-carotene, B = pheophytin *a*, C = chlorophyll *a*, and D = Mg-purpurin 7-lactone-ethyl ether-methylphytyl ester.

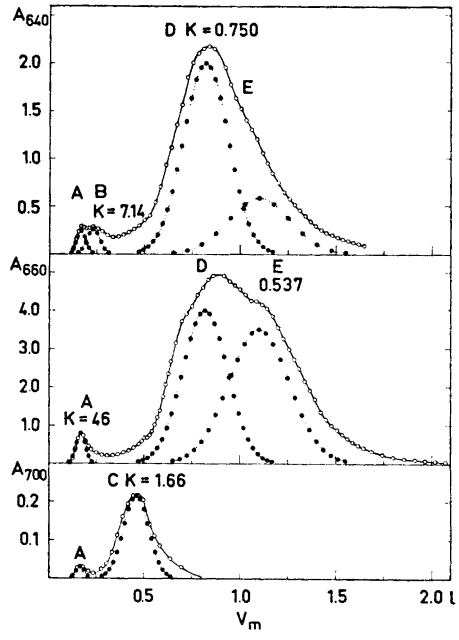

Fig. 3. Separation of the allomerization products of chlorophyll *a* employing the PBMF-system. The petroleum ether solution of the allomerization products of chlorophyll *a* was sampled into tubes r = 0,..,2. $N = 50$. $v_m = 3.14$ ml; $v_s = 10.36$ ml. $Vm = 2100$ ml; flow rate = 2 ml/min.. Theoretical (●) and experimental (O) values, the latter obtained by measuring the absorbances of the effluent fractions at three different wavelengths. A = 10-methoxy-chlorophyll *a*, B = Mg-chlorin e_6-dimethylphytyl ester, C = Mg-purpurin 7-(di)methylphytyl ester, D = Mg-purpurin 7-lactone-methyl ether-methylphytyl ester, and E = 10-hydroxy-chlorophyll *a*.

Fig. 3 presents the results obtained from the fractionation performed on the allomerization products of chlorophyll *a* in methanol. The components of the allomerization mixture were separated by means of the PBMF-system utilizing 50 fractionation tubes. This solvent system was selected in order to avoid the occurrence of further allomerization in the apparatus due to the possible presence of intact chlorophyll in the mixture. Five components were spectroscopically detected in the effluent series. The primary allomerization products of chlorophyll *a* were components D and E. Component D was spectroscopically and chemically very similar to the D of Fig. 2 and, therefore, undoubtedly represents Mg-purpurin 7-lactone-methyl ether-methylphytyl ester. Component E exhibited the properties of 10-hydroxy-chlorophyll *a*.[1]

The minor component A revealed, in diethyl ether, absorption maxima at 429 and 661 nm. When the ethereal solution of A was treated with 30 % hydrochloric acid, a product was formed which reacted negatively to the phase test, had a hydrochloric acid number greater than 15 and resembled pheophorbide *a* according to its visible absorption spectrum (maxima, in diethyl ether, at 667, 609, 560, 537, 504, and 408 nm).[25] These characteristics indicate that component A probably represents 10-methoxy-chlorophyll *a*.

The fractions eluted within the interval 200 – 300 ml exhibited an absorption peak at approximately 640 nm. This was interpreted as indicating a small amount of Mg-chlorin e_6-dimethylphytyl ester (component B).[20] The presence of this compound was ascertained by isolating the magnesium- and phytyl-free derivative from two fractions eluted at about 250 ml. This isolation was effected by first treating the ethereal solution of the pigments from the fractions with 30 % hydrochloric acid. The pigments were then transferred into fresh ether by adding water to the acid phase. The pigment that was extracted from the ether solution into 5 % hydrochloric acid closely resembled chlorin e_6 according to its visible absorption spectrum (maxima, in diethyl ether, at 666, 609, 560, 529, 499, and 400 nm).[25]

Component C was characterized as Mg-purpurin 7-(di)methylphytyl ester upon the basis of the following properties: first, the visible absorption spectrum of C was quite similar to that of Mg-purpurin 7-triester, which Holt [20] has identified as being an allomerization product of methylchlorophyllide *a* in methanol; second, the magnesium-free derivative of C exhibited absorption maxima, in diethyl ether, at 680, 635, 542, 504, and 406 nm;[26] and third, the treatment of an ethereal solution of C with 25 % potassium hydroxide in propanol resulted in the formation of Mg-unstable chlorin.[11,24]

One additional pigment was also characterized as being among the allomerization products of chlorophyll *a*. This component (F) remained at the sampling end of the apparatus ($r_{max} = 4$, not indicated in Fig. 3), had a partition coefficient (K_F) equalling 0.024 and closely resembled the 10-methoxy-lactone derivative (D) according to its visible absorption spectrum.[1] When an ethereal solution of F was treated with diazomethane, the resulting product was Mg-purpurin 7-triester.[28] Upon the basis of these properties, F was characterized as representing Mg-unstable chlorin-methylphytyl ester.

The results obtained from the fractionation performed on the allomerization products of chlorophyll *b* in methanol are presented in Fig. 4. The principal allomerization product (D) was in this case also a 10-methoxy-lactone deriva-

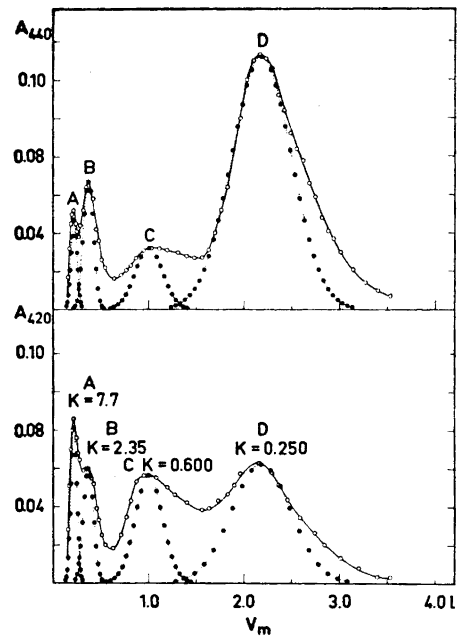

Fig. 4. Separation of the allomerization products of chlorophyll *b* employing the PBMF-system. The petroleum ether solution of the allomerization products of chlorophyll *b* was sampled into tubes r = 0,...,2. $N = 50$. $v_m = 3.14$ ml; $v_s = 10.36$ ml. $V_m = 2180$ ml; flow rate = 2 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring the absorbances of the effluent fractions at two different wavelengths. A and B = magnesium-free pigments, C = Mg-*b*-purpurin 7-(di)methylphytyl ester, and D = Mg-*b*-purpurin 7-lactone-methyl ether-methylphytyl ester.

tive (Mg-*b*-purpurin 7-lactone-methyl ether-methylphytyl ester), being spectroscopically and chemically very similar to the alkoxy-lactone derivative of chlorophyll *b* described earlier.[1] Component C exhibited the red band (I) at 645 nm and the Soret band (S) at 422 nm and yielded an A_S/A_I ratio of 2.96. Based upon the concentration profile presented in Fig. 4, it may be deduced that a slow equilibrium has existed between components C and D. The interchangeability of these components was also demonstrated by the fact that, upon standing in the effluent, C converted to D. These characteristics appear to indicate that fraction C consisted of Mg-*b*-purpurin 7-(di)methylphytyl ester. Components A and B, apparently magnesium-free pigments, were not further studied due to their incomplete resolution and small amounts.

One additional component (E) also remained at the sampling end of the apparatus in the fractionation performed on the allomerization products of chlorophyll *b*. This component was spectroscopically similar to Mg-*b*-purpurin 7-lactone-methyl ether-methylphytyl ester and was identified as Mg-unstable rhodin methylphytyl ester, a result analogous to that obtained in the *a*-series. No hydroxy-chlorophyll *b* was detectable in the *b*-series. Furthermore, neither Mg-rhodin g_7-triester nor 10-methoxy-chlorophyll *b* were identified among the allomerization products of chlorophyll *b*. Small amounts of these latter compounds, if present, may well have been obscured by the magnesium-free pigments.

The results of the fractionations presented in Figs. 3 and 4 reveal the utmost importance of avoiding allomerization during the extraction of the pigments from plant material. Preparations of isolated chlorophyll are other-

wise liable to become contaminated by small amounts of impurities, which have been observed as minor allomerization products in the above fractionations. For example, chlorophyll *a* may become contaminated by component C of Fig. 3. The separation of this component ($K_C = 1.66$) from chlorophyll *a*, which has a partition coefficient of 2.83 [1] in the same solvent system, would be difficult. Brody and Broyde [27,28] found small amounts of a chlorophyll derivative in chlorophyll *a* isolated by means of conventional chromatographic methods. This impurity could be separated from the chlorophyll *a* by washing with petroleum ether, since the derivative had a higher solubility in nonpolar solvents. Brody and Broyde have suggested that this pigment could be a form of chlorophyll *a* which is reactive in photosynthesis. The present authors believe, however, that this pigment is actually an allomerization product of chlorophyll. Evidence for this belief derives from the fact that the difference spectrum presented by Broyde and Brody [28] for the pigment referred to as F 698 is remarkably similar to the absorption spectrum of Mg-purpurin 7-triester (component C of Fig. 3). This similarity concerns not only the positions of the Soret band and band I (at approximately 420 and 670 nm, respectively), but extends also to the ratio of their peak heights. A ratio of 2.6 is obtained from the spectrum given by Broyde and Brody, whereas the corresponding ratio as determined by the present authors is 2.7.

Fischer and Pfeiffer [12] identified Mg-purpurin 7-lactone-ethyl ether-diester as a product of the allomerization of ethylchlorophyllide *a* in ethanol. According to the mechanism postulated by the above authors, the C-10 carbon atom is first attacked by oxygen, thus resulting in the formation of a hydroperoxide at the C-10 position. This causes the isocyclic ring to become labile, and the ring is thereafter split hydrolytically. A free carboxyl group is then introduced at C-6 and simultaneously a disproportionation of the oxygens at C-10 occurs. Subsequently, one of the newly-formed hydroxyl groups at C-10 reacts with the solvent to form the ether, while the other hydroxyl group at C-10 reacts with the C-6 carboxyl group and produces the lactone bridge.

The results obtained in this laboratory do not support the above mechanism proposed by Fischer and Pfeiffer for the formation of the lactone derivative. The present authors propose the mechanism illustrated in Fig. 5 for the formation of Mg-purpurin 7-lactone-alkyl ether-diester (V), Mg-purpurin 7-triester (X) and Mg-unstable chlorin-diester (VII). According to this reaction scheme, chlorophyll (I) is first enolized. Due to the double bond formed between C-9 and C-10, the enolate ion (II) is labile and the double bond undergoes oxygen cleavage, thus resulting in the formation of Mg-purpurin 7-monomethylphytyl ester (III). The instability of this compound arises from the activation of C-10 by the methoxycarbonyl group (the authors are not aware of compound III having been either isolated or synthesized [24]). This component is rapidly solvated by methanol to give Mg-purpurin 7-lactone-methyl ether-methylphytyl ester (V), with IV as a possible intermediate. In the presence of water or hydroxyl ions, however, compound III yields Mg-unstable chlorin-monomethylphytyl ester (VII), with VI as a possible intermediate. Purpurin 18 (IX) may be obtained from VII by hydrolysis (yielding VIII), followed by the splitting-off of formic acid. As a third possibility, compound III can be esterified, thus resulting in the formation of the Mg-purpurin 7-triester (X).

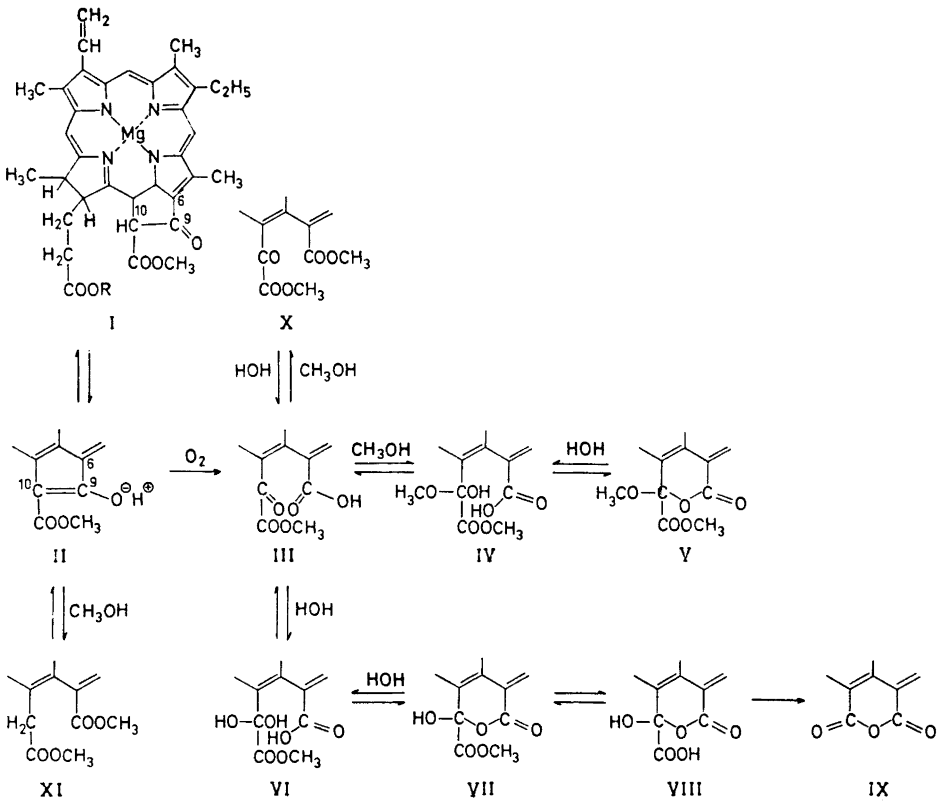

Fig. 5. Proposed mechanism for the allomerization of chlorophyll *a*. R = phytyl.

A reaction competing with the oxidation of the enolate ion (II) is that of methanolysis,[29] which yields the Mg-chlorin e_6-triester (XI). It should be noted, however, that such methanolysis is reversible, while the oxidation is irreversible.

The reaction scheme presented in Fig. 5 is in general agreement with the studies of Holt [20] and of Conant *et al.*[3,4] Holt concluded that the formation of the 10-alkoxy-lactone derivative in alcohols involves the oxidation of trace amounts of phase test intermediate, which was assumed to be the enolate anion. Conant *et al.* characterized unstable chlorin and its monomethyl ester as the allomerization products of pheophorbide *a* in the presence of hydroxide. Upon standing, these compounds were reported to yield purpurin 18 and purpurin 7-trimethyl ester. When esterified with diazomethane, both unstable chlorin and its monomethyl ester were converted into purpurin 7-trimethyl ester.

Fig. 5 does not account for the formation of the 10-hydroxy- and 10-methoxy-chlorophylls. No clear conclusions can be drawn, upon the basis of the results presented above, regarding the mechanism of the formation of these

allomerization products. However, it appears likely that, in the light of these results and of those previously reported,[1] the 10-hydroxy-chlorophylls are formed independently of the allomerization products illustrated in Fig. 5. Evidently, the formation of 10-hydroxy- or 10-methoxy-chlorophylls does not require enolization as a preliminary step. They may well arise directly through the action of hydroxyl or methoxyl ions upon the C-10 atom of chlorophyll. Earlier investigations [7,12,15,17,20] in which quinone, potassium permanganate or iodine were utilized as the oxidizing agent, rather than oxygen, suggest a second possible mechanism: The proton at C-10 is first abstracted by base (OH^-, OCH_3^-, *etc.*) to give a carbanion, which is subsequently oxidized by the oxidant (quinone, I_2, $KMnO_4$, O_2, *etc.*), thus yielding a carbonium ion. This carbonium ion thereafter reacts rapidly with either methoxyl or hydroxyl ions to produce, respectively, 10-methoxy- or 10-hydroxy-chlorophyll.

REFERENCES

1. Hynninen, P. H. and Ellfolk, N. *Acta Chem. Scand.* **27** (1973) 1463.
2. Seely, G. R. In Vernon, L. P. and Seely, G. R., Eds., *The Chlorophylls*, Academic, New York–London 1966, p. 91.
3. Conant, J. B. and Moyer, W. W. *J. Am. Chem. Soc.* **52** (1930) 3013.
4. Conant, J. B., Hyde, J. F., Moyer, W. W. and Dietz, E. M. *J. Am. Chem. Soc.* **53** (1931) 359.
5. Fischer, H. and Riedmair, J. *Ann. Chem.* **506** (1933) 107.
6. Fischer, H. and Hagert, W. *Ann. Chem.* **502** (1933) 41.
7. Fischer, H. and Heckmaier, J. *Ann. Chem.* **508** (1934) 250.
8. Fischer, H., Heckmaier, J. and Hagert, W. *Ann. Chem.* **505** (1933) 209.
9. Fischer, H. and Kahr, K. *Ann. Chem.* **531** (1937) 209.
10. Fischer, H. and Scherer, T. *Ann. Chem.* **519** (1935) 234.
11. Fischer, H., Süs, O. and Klebs, G. *Ann. Chem.* **490** (1931) 38.
12. Fischer, H. and Pfeiffer, H. *Ann. Chem.* **555** (1944) 94.
13. Fischer, H. and Spiegelberger, G. *Ann. Chem.* **510** (1934) 156.
14. Fischer, H. and Spiegelberger, G. *Ann. Chem.* **515** (1935) 130.
15. Fischer, H., Filser, L. and Plötz, E. *Ann. Chem.* **495** (1932) 1.
16. Fischer, H., Heckmaier, J. and Scherer, T. *Ann. Chem.* **510** (1934) 169.
17. Strell, M. *Ann. Chem.* **550** (1942) 50.
18. Strain, H. H. *Agr. Food Chem.* **2** (1954) 1222.
19. Johnston, L. G. and Watson, W. F. *J. Chem. Soc.* **1956** 1203.
20. Holt, A. S. *Can. J. Biochem. Physiol.* **36** (1958) 439.
21. Pennington, F. C., Strain, H. H., Svec, W. A. and Katz, J. J. *J. Am. Chem. Soc.* **89** (1967) 3875.
22. Eistert, B. In *Newer Methods of Preparative Organic Chemistry*, Interscience, New York 1948, p. 513.
23. Willstätter, R. and Mieg, W. *Ann. Chem.* **350** (1906) 1.
24. Fischer, H. and Stern, A. *Die Chemie des Pyrrols*, Akad. Verlag. Leipzig 1940, Vol. II/2.
25. Stern, A. and Wenderlein, H. *Z. physik. Chem. (Leipzig)* **A 174** (1935) 81.
26. Stern, A. and Pruckner, F. *Z. physik. Chem. (Leipzig)* **A 180** (1937) 321.
27. Brody, S. S. and Broyde, S. B. *Nature* **199** (1963) 1097.
28. Broyde, S. B. and Brody, S. S. *Biochem. Biophys. Res. Commun.* **19** (1965) 444.
29. Fischer, H. and Oestreicher, A. *Ann. Chem.* **546** (1940) 53.

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