Chlorophylls

IV. Preparation and Purification of Some Derivatives of Chlorophylls a and b

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The reactions of pheophorbides a and b in methanolic potassium hydroxide were studied as functions of alkali and oxygen concentration. The derivatives formed as products of these reactions were separated utilizing the aqueous formic acid-chloroform-dimethylformamide (AFCD) solvent system. In the presence of atmospheric oxygen, boiling 30 % methanolic potassium hydroxide reacted upon pheophorbide a to yield purpurin 18 as the principal product, while chlorin e_6 was formed to a lesser extent. Under similar conditions, a 0.5 % methanolic solution of the alkali reacted upon pheophorbide a to yield a product which was spectroscopically identical to chlorin e_6. Partition fractionation revealed this product to be actually a mixture of various chlorin e_6 methyl esters. Hydrolysis of the ester mixture in 30 % methanolic potassium hydroxide under an argon atmosphere yielded nearly pure chlorin e_6, which was further purified utilizing the AFCD solvent system. Beginning with pheophorbide b, relatively good yields of rhodin g_7 were analogously (*i.e.* through methyl esters of rhodin g_7) prepared. These results indicate that, in dilute methanolic alkali solutions, solvolysis of the pheophorbide ring V is the principal reaction, even in the presence of oxygen. Conversely, concentrated methanolic alkali solutions result in the oxidative splitting of ring V as the predominant reaction in the presence of oxygen. A mechanism for the solvolysis of ring V is proposed and discussed.

The effects of solvent and temperature upon the saponification of methylpheophorbide a were studied by Conant and Moyer.[1] These authors reported that the formation of chlorin e_6 was completely suppressed when saponification was performed with an alcoholic solution of potassium hydroxide in which ethyl or propyl rather than methyl alcohol was employed as solvent. The same result was also claimed when methanolic potassium hydroxide was utilized, provided that saponification was carried out at a temperature of $-10°C$. Chlorin e_6 was reportedly the chief product when saponification was effected by means of boiling alcoholic potassium hydroxide in pyridine solution

(Willstätter's procedure [2]); no unstable chlorins were formed under these conditions.

Alkali concentration has since appeared to be of the utmost importance among the factors that determine the saponification products of the chlorophylls or derivatives thereof possessing an intact isocyclic ring V. Oster *et al.*[3] effected the saponification of chlorophyll *a* utilizing dilute methanolic potassium hydroxide (1 ml of 7 % potassium hydroxide in methanol was added to 50 ml of chlorophyll *a* solution). Employing partition chromatography as their analytical method, they found their product to consist of only one component, chlorophyllin *a* (Mg-chlorin e_6). Holt [4] reported that treatment of methylchlorophyllide *a* with 0.5 % methanolic potassium hydroxide in the presence of atmospheric oxygen results principally in the formation of Mg-chlorin e_6 trimethyl ester. When more concentrated (5 − 30 %) methanolic potassium hydroxide solutions were utilized, Mg-chlorin e_6 products were formed only if oxygen was excluded from the reaction mixture. These results were interpreted by Holt as signifying that, in dilute methanolic potassium hydroxide, the concentration of the phase test intermediate was so low that even under aerobic conditions methanolysis could proceed at a more rapid rate than oxidation.

In the present investigation, the author has compared the products formed from pheophorbide *a* as a result of two different saponification procedures. In one procedure, drastic conditions resembling those employed by Willstätter[2] were used, while in the other, the mild conditions of Holt [4] were utilized. It will be demonstrated that, in the former procedure, oxidative splitting [5] of ring V is the primary reaction, while in the latter, solvolysis of ring V occurs almost exclusively. As a result of the present study, methods will be described for the preparation, beginning with pheophorbides *a* and *b*, of chlorin e_6 and rhodin g_7 in relatively high yields. It will further be shown that the liquid-liquid partition technique, which has been previously [6,7] adapted to the separation of dicarboxylic porphyrins and hemins, may also be successfully applied to the fractionation of magnesium-free chlorophyll derivatives.

EXPERIMENTAL

Chlorophylls a and b were isolated from frozen soybean leaves using the liquid-liquid partition method previously [8] described.

Pheophorbides a and b were prepared from chlorophylls *a* and *b*, respectively, by shaking an ethereal solution of the chlorophyll with 30 % (w/w) hydrochloric acid for 5 min. The pheophorbide thus formed was transferred into the ether phase by diluting the acidic phase. The ether solution was then washed three times with distilled water. The spectroscopic properties of these pheophorbides are presented in Table 1, a and i.

Unstable chlorin 7 was produced from pheophorbide *a* by treating an ethereal solution of the pigment for 2 min with 25 % potassium hydroxide in 1-propanol [1,9] (Table 1, e).

Purpurin 7-lactone-methyl ether (Table 1, f) was prepared by shaking an ethereal solution of Mg-purpurin 7-lactone-methyl ether-methylphytyl ester [5] with 30 % (w/w) hydrochloric acid for 5 min. The magnesium-free pigment was transferred into diethyl ether by dilution of the acidic phase. The ether solution was then washed three times with distilled water.

Purpurin 18 was isolated from the saponification products of pheophorbide *a* by means of the partition fractionation described below (Table 1, g).

Table 1. Spectroscopic properties of some chlorophyll derivatives in ethyl ether.

Compound	I		II		III		IV		V		VI		Soret	
	nm	R	nm	R	nm	R	nm	R	nm	R	nm	R	nm	R
a. Pheophorbide a (1A, 3A) [a]	667.0	2.07	609.5	13.9	560.0	39.1	532.5	10.9	504.0	8.91	467.0	22.5	408.0	1.00
b. Chlorin e$_6$	666.0	2.82	610.5	29.2	560.0	76.5	530.0	30.1	500.0	11.3			400.0	1.00
c. Chlorin e$_6$ ester mixture	666.0	2.62	609.5	20.9	559.5	43.8	530.0	19.9	500.0	10.0			400.0	1.00
d. Chlorin k (?) (1B)	665.5	3.04	610.5	28.6	(557)[b]	(48.2)	529.5	25.3	499.0	10.8			399.5	1.00
e. Unstable chlorin 7(-mono- methyl ester)	668.5	2.63	611.0	18.6	560.0	42.8	529.5	15.9	499.5	10.7	(470)	(28.4)	399.0	1.00
f. Purpurin 7-lactone-methyl ether (-monomethyl ester)	668.5	2.50	611.0	18.7	561.0	47.8	528.0	14.9	498.5	10.0	(467)	(27.1)	399.0	1.00
g. Purpurin 18 (1C)	695.0	2.25	638.0	14.7	587.0	48.7	540.0	5.48	503.0	15.1	475.0	24.5	406.0	1.00
h. Chlorin p$_6$	670.0	2.80	615.0	24.8	565.0	63.2	529.0	21.5	498.0	11.7			399.0	1.00
i. Pheophorbide b	655.0	4.56	599.0	12.5	555.0	12.2	525.0	9.30					433.0	1.00
j. Rhodin g$_7$	652.0	6.92	597.5	25.7	560.0	20.3	524.0	15.5					427.0	1.00
k. Rhodin g$_7$ ester mixture	652.0	6.48	597.0	26.5	558.0	20.9	522.0	14.5					426.0	1.00

[a] Number in parentheses refers to figure number, while capital letter refers to component in that figure.
[b] Peak positions and ratios stated in parentheses are approximate.
R = quotient of absorbance at Soret band divided by absorbance at wavelength indicated.

Chlorin p_6 (Table 1, h) was produced by shaking an ethereal solution of purpurin 18 with 25 % methanolic potassium hydroxide for 10 min. [9] The pigments thus formed were transferred into the diethyl ether phase by neutralization with dilute hydrochloric acid. Chlorin p_6 was then extracted from the ether solution with 6 % hydrochloric acid. The derivative was transferred into fresh diethyl ether by diluting the acidic phase, and the ether solution was then washed three times with distilled water.

Saponification of pheophorbide a with boiling 30 % methanolic potassium hydroxide. An ethyl ether solution of pheophorbide *a*, containing about 10 mg of the pigment, was evaporated to dryness at reduced pressure. Eighty milliliters of boiling 30 % (w/w) methanolic potassium hydroxide were then added to the residue, thereby causing the yellow-brown colour of the phase test intermediate to appear. After 1 min, the solution was poured into a separatory funnel containing 200 ml of diethyl ether and 700 ml of distilled water. The pigments were then transferred into the ether phase by neutralization with hydrochloric acid. The phases were separated and the lower phase was extracted twice with 100 ml of diethyl ether. The combined ether extracts were then washed three times with distilled water. A visible absorption spectrum indicated that the product consisted of several components.

Saponification of pheophorbide a with 0.5 % methanolic potassium hydroxide. One hundred milliliters of 0.5 % (w/w) methanolic potassium hydroxide were added, in the presence of atmospheric oxygen and at room temperature, to a solution of 10 mg pheophorbide *a* in 50 ml ethyl ether. The characteristic colour of the phase test intermediate appeared within a second following the addition of the alkali solution. After 10 min, 500 ml of distilled water were added to the reaction mixture and the derivatives were then transferred into the diethyl ether phase by neutralizing with hydrochloric acid. The separated ether layer was washed three times with distilled water. The visible absorption spectrum of the ethereal solution closely resembled that of chlorin e_6 (Table 1, c). A partition fractionation, described below, indicated that the product consisted of various methyl esters of chlorin e_6.

Preparation of free chlorin e_6. The methyl esters obtained from the saponification of pheophorbide *a* with 0.5 % methanolic potassium hydroxide were transferred into 70 ml of ethyl ether. The solution was then treated for 1 h with 80 ml of 30 % (w/w) methanolic potassium hydroxide at room temperature and under an argon atmosphere. The reaction was stopped by adding 700 ml of distilled water to the mixture, and the derivatives were then transferred into diethyl ether by neutralization with hydrochloric acid. The ether solution of the saponification products exhibited an absorption spectrum which was very similar to that of chlorin e_6.

Preparation of free rhodin g_7. Pheophorbide *b* (5 mg) was first treated with dilute (0.5 %) methanolic potassium hydroxide in a manner similar to that described above for pheophorbide *a*. The absorption spectrum of the products of this saponification closely resembled that of rhodin g_7 (Table 1, k). The methyl esters were then treated with 30 % methanolic potassium hydroxide in a way analogous to that described above for the preparation of free chlorin e_6. An absorption spectrum of the saponification products indicated slight contamination by at least one impurity.

Fractionation by multiple liquid-liquid partition. Partition fractionations were performed utilizing the Hietala apparatus [10] and the aqueous formic acid-chloroform-dimethylformamide (AFCD) [7] solvent system. A phase ratio of about 0.3 was employed; in other respects, however, experimental conditions were similar to those previously used. [7] In order to effect more rapid separation, advantage was taken of stepwise pH-gradient elution. [6]

Spectroscopic data. The absorption spectra of the derivatives were recorded utilizing a Cary Model 15 spectrophotometer. Single absorbances were measured by means of a Beckman DU spectrophotometer.

RESULTS

Fig. 1 presents the results of the separation of the products formed on treating pheophorbide *a* briefly with boiling 30 % methanolic potassium hydroxide in the presence of atmospheric oxygen. Component A, which was
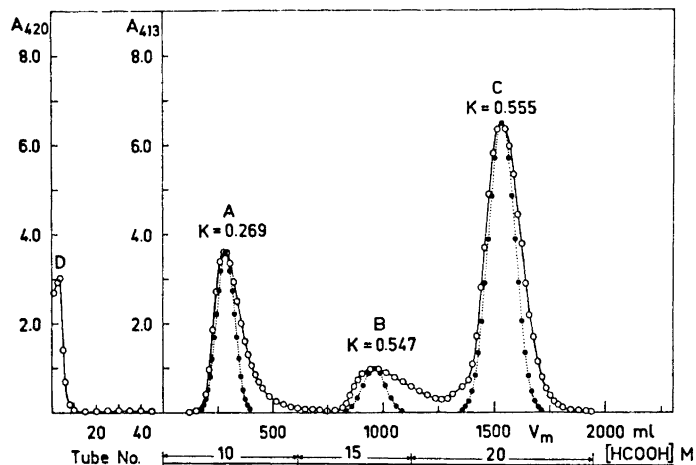
Fig. 1. Separation of chlorophyll *a* derivatives. Solvent system: HCOOH (of varied molarity)/CHCl$_3$(25) − DMF(1). The ether solution of the products formed upon the saponification of pheophorbide *a* with boiling 30 % methanolic potassium hydroxide was evaporated to dryness at reduced pressure. The residue was dissolved in 2 ml of dimethylformamide (DMF), to which were then added 50 ml of CHCl$_3$ + 8 ml of H$_2$O + 5 ml of HCOOH. The mixture was sampled into tubes r = 0, ...,4. Number of tubes used in the fractionation = N = 50. Average volume of mobile phase (acid) in a partition unit = v_m = 3.00 ml; average volume of stationary phase (CHCl$_3$ − DMF) in a partition unit = v_s = 10.50 ml. Total volume of effluent eluted from the apparatus = V_m = 1940 ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring $A_{413\ nm}$ of the effluent fractions and $A_{420\ nm}$ of the lower phases in the tubes. A = chlorin e$_6$, B = chlorin k (?), C = purpurin 18, and D = 10-methoxy-pheophorbide *a* (?).

eluted from the apparatus with 10 M formic acid, was characterized as chlorin e$_6$ upon the basis of its spectroscopic properties (Table 1, b) [11] and low hydrochloric acid number (SZ = 3).[9] The next component, B, was eluted with 15 M formic acid and possessed the following characteristics: (1) spectroscopic properties (Table 1, d) similar to those of chlorin e$_6$ but distinctly different from those of unstable chlorin 7 and purpurin 7-lactone-methyl ether (Table 1, e and f); (2) a hydrochloric acid number of 12; (3) neither treatment with diazomethane [12] nor standing in methanol saturated with hydrochloric acid resulted in the formation of purpurin 7-trimethyl ester or purpurin 5-dimethyl ester, thus excluding the possibility of component B representing one of the "unstable" chlorins (chlorin 7 and chlorin 5) [9,13,14] or their methyl esters; (4) after standing for 6 days in methanolic hydrochloric acid in the presence of atmospheric oxygen, the pigment was partially converted into a porphyrin exhibiting absorption maxima, in diethyl ether, at 630, 580 and 550 nm (these values are in accord with the spectroscopic properties of rhodoporphyrin-γ-hydroxymethyl-lactone-methyl ester [9,15]); (5) standing in concentrated hydrochloric acid for 5 min did not alter the spectroscopic properties of the pigment; and (6) hydrolysis with boiling 25 % methanolic potassium hydroxide for 10 min converted part of the pigment into purpurin 18, as evidenced by ab-

sorption maxima, in diethyl ether, at 695, 637, and 540 nm. Upon the basis of these properties, it is likely that fraction B eluted within the range 830 − 1180 ml consisted of chlorin k, *i.e.* rhodochlorin-γ-hydroxymethyl-lactone, which is known to be spectroscopically very similar to chlorin e_6.[16]

The principal component, C, was eluted from the apparatus with 20 M formic acid and was identified as purpurin 18 upon the basis of the following properties: (1) a visible absorption spectrum very similar to that of purpurin 18 (Table 1, g);[9] (2) a hydrochloric acid number of 18;[1] (3) shaking of a diethyl ether solution of the pigment with 25 % methanolic potassium hydroxide for 10 min resulted in the formation of chlorin p_6 (chlorin a; Table 1, h); and (4) heating with pyridine-sodium carbonate for 10 min partially converted the pigment into rhodoporphyrin-γ-carbonic acid anhydride,[17] as indicated by absorption maxima, in diethyl ether, at 655, 597 (broad band), and 550 nm.

The material (D) that remained at the sampling end of the apparatus primarily exhibited absorption peaks, in diethyl ether, at 668, 610, 535, 505, and 408 nm. These values are rather similar to the spectroscopic properties of pyropheophorbide *a* monomethyl ester.[11] However, since the hydrochloric acid number of component D must have been greater than that of purpurin 18, it appears likely that the material consisted principally of either 10-methoxy-pheophorbide *a* or 10-methoxy-pyropheophorbide *a*. The slight absorption peaks observed, in diethyl ether, at 632, 577, and 555 nm, suggest that the material contained a small quantity of chloroporphyrin e_7-lactone-methyl ester.[9]
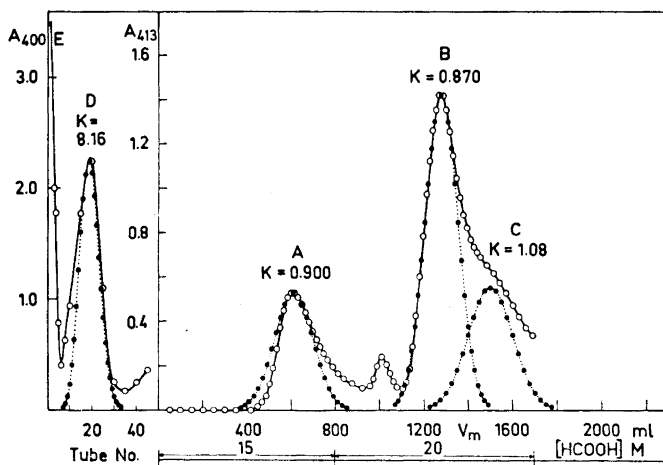

Fig. 2. Separation of chlorin e_6-methyl esters. Solvent system: HCOOH (varied molarity)/CHCl$_3$(25) − DMF(1). The ether solution of the products formed upon the saponification of pheophorbide *a* with 0.5 % methanolic potassium hydroxide was evaporated to dryness at reduced pressure. The residue was dissolved in 2 ml of DMF, to which were then added 50 ml of CHCl$_3$ + 6 ml of H$_2$O + 8 ml of HCOOH. The mixture was sampled into tubes r = 0,...,4. N = 50. v_m = 3.04 ml; v_s = 10.46 ml. V_m = 1680 ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring $A_{413 nm}$ of the effluent fractions and $A_{400 nm}$ of the lower phases in the tubes. A − E = various methyl esters of chlorin e_6.

The saponification of pheophorbide a with 0.5 % methanolic potassium hydroxide in the presence of atmospheric oxygen resulted in the formation of a product which spectroscopically appeared to be homogeneous and very similar to chlorin e_6 (Table 1, c). However, when the material was subjected to partition fractionation (Fig. 2), it was revealed to consist of a complex mixture of several components (A − E), all of which were spectroscopically identical. Fraction A is believed to consist of monomethyl ester(s), while fractions B − D are thought to be various dimethyl esters of this compound (there are three possibilities). The last component, E, is presumably chlorin e_6 trimethyl ester. The slight peak apparent between fractions A and B is probably due to a premature molarity change which has caused crowding of the tail of fraction A.

The methyl esters of the fractionation presented in Fig. 2 were combined and saponified with 30 % methanolic potassium hydroxide while carefully avoiding exposure of the reaction mixture to oxygen (refer to experimental). The fractionation of the resulting saponification products is presented in Fig. 3. Free chlorin e_6 was obtained in good yield (> 90 %), thus demonstrating in a convincing manner that the components of Fig. 2 indeed were various methyl esters of chlorin e_6.
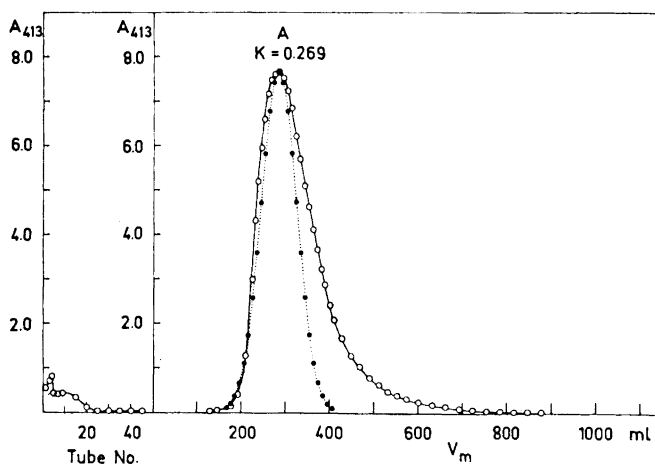

Fig. 3. Isolation of free chlorin e_6. Solvent system: $HCOOH(10 M)/CHCl_3(25) - DMF(1)$. The ether solution of the products formed upon the saponification of the chlorin e_6-methyl esters of Fig. 2 was evaporated to dryness at reduced pressure. The residue was dissolved and sampled into the apparatus as described in Fig. 1. N = 50. $v_m = 3.00$ ml; $v_s = 10.50$ ml. $V_m = 888$ ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring $A_{413 nm}$ of the effluent fractions and of the lower phases in the tubes. A = chlorin e_6.

Fig. 4 presents the isolation of rhodin g_7 (component A). The yield in this case was not as high as in the isolation of chlorin e_6. The spectroscopic properties of fraction A (Table 1, j) closely resembled those previously described for rhodin g_7 trimethyl ester.[9,18] The material that remained at the sampling end
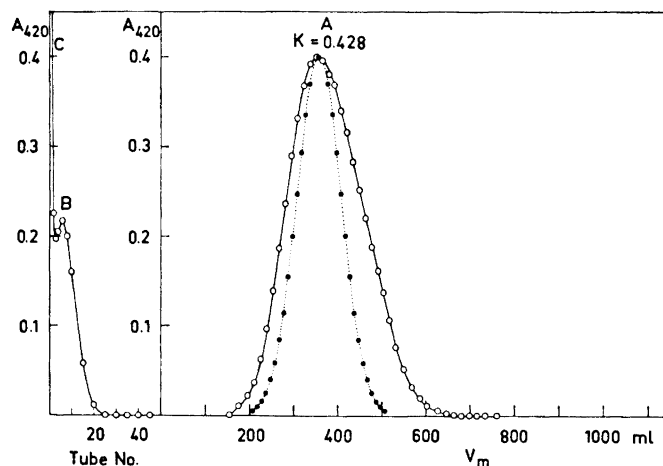
Fig. 4. Isolation of free rhodin g_7. Solvent system: HCOOH(13 M)/CHCl$_3$(25) – DMF(1). The ether solution of the products formed upon the saponification of rhodin g_7-methyl esters was evaporated to dryness at reduced pressure. The residue was dissolved in 2 ml of DMF, to which were then added 50 ml of CHCl$_3$ + 7 ml of H$_2$O + 7 ml of HCOOH. The mixture was sampled into tubes r = 0, ... ,4. N = 50. v_m = 2.89 ml; v_s = 10.61 ml. V_m = 760 ml; flow rate = 1.5 ml/min. Theoretical (●) and experimental (○) values, the latter obtained by measuring $A_{420\ nm}$ of the effluent fractions and of the lower phases in the tubes. A = rhodin g_7, B = a second rhodin, and C = b-purpurin 18.

of the apparatus appeared to consist of two components (B and C). Fraction B resembled rhodin g_7 regarding its visible absorption spectrum and is probably a second rhodin. Fraction C exhibited absorption maxima, in ethyl ether, at 671.5, 620, 557, 518, and 431 nm. These values are in accord with the spectroscopic properties of b-purpurin 18.[9]

DISCUSSION

The fact that purpurin 18 was the primary product when pheophorbide a was saponified with a boiling concentrated solution of methanolic potassium hydroxide indicates that, in this case, oxidative splitting of ring V[5] is the principal reaction in the presence of oxygen. The results of Fig. 2 confirm the argument presented by Holt [4] that saponification with dilute methanolic potassium hydroxide causes the solvolysis of ring V as the predominant reaction, even in the presence of oxygen.

The mechanism of this solvolysis (methanolysis, hydrolysis, or aminolysis) has not as yet been elucidated in detail. An important consideration is whether or not the methanolysis of ring V occurs through a mechanism similar to that proposed by Weller and Livingston [19] for the aminolysis of this ring. These investigators did not observe the characteristic colour of the phase test intermediate during the course of the amine reaction and they therefore concluded that this reaction differs in many respects from that of the phase test. They proposed a mechanism whereby the amine attacks carbon-9 of the all-keto form of the chlorophyll, thus resulting, through a transition state possessing

an onium-structure, in a carbanion with a free electron pair at carbon-10. The carbanion was then assumed to react rapidly with the solvent (amine) to yield a chlorin 6-carboxamide.
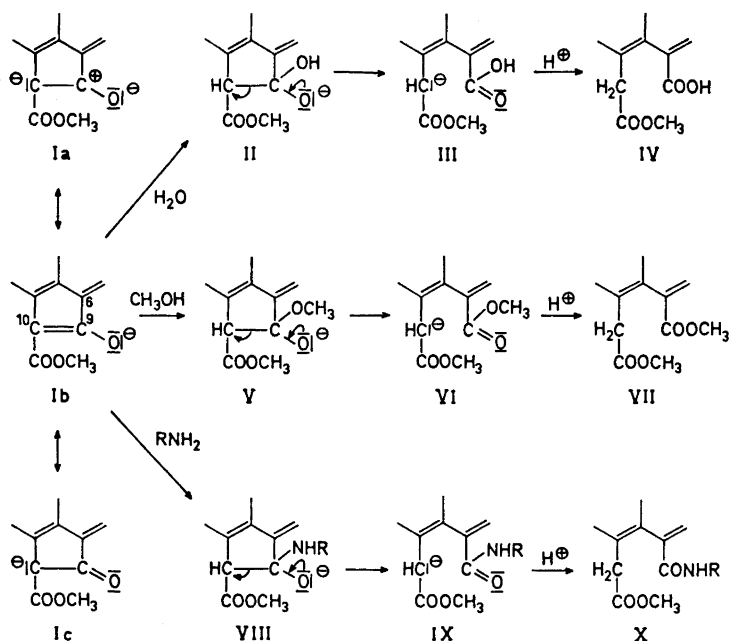

Fig. 5. Proposed mechanism for the solvolysis of ring V of the chlorophylls and their derivatives.

The present author now proposes a general mechanism, shown in Fig. 5, for the solvolysis of ring V. According to the reaction scheme, the enolate ion (I) is the key intermediate of this solvolysis.[5] The solvent (water, methanol or amine) presumably reacts with the enolate ion, through addition across the carbons-9 and -10 double bond, to yield the unstable intermediate (II, V or VIII, respectively). Such addition may be understood upon assuming that the enolate ion is a resonance hybrid to which form Ia makes a considerable contribution. The unstable intermediate is converted by rearrangement to the respective carbanion (III, VI or IX), which then reacts rapidly with the solvent to give either chlorin e_6 monomethyl ester (IV), chlorin e_6 dimethyl ester (VII) or chlorin 6-carboxamide (X). The following evidence supports the mechanism illustrated in Fig. 5:

1. Shortly after the addition of dilute methanolic potassium hydroxide to pheophorbide *a*, a brown colour was clearly observed. It is therefore probable that solvolysis proceeds *via* the phase test intermediate.

2. Fischer and Riedmair[20] concluded that methanolysis requires an activated hydrogen at the C-10 postition, since pyropheophorbide did not undergo this reaction.

3. The investigations of Fischer and Oestreicher [21] concerning phorbin synthesis (a reversed reaction to that of methanolysis) suggest the C-10 carbanion as a probable intermediate. The required presence of a strong base in the synthesis of phorbin is understandable when it is assumed that the base initiates the reaction by extracting a C-10 proton from compound VII.

4. The lack of drastic colour change during aminolysis is no proof that the reaction does not proceed through the phase test intermediate (enolate ion). If the intermediate is consumed as rapidly as it is formed, then no impressive colour change is to be expected. This situation is analogous to that of the allomerization reaction.[4,5]

5. The investigations of Pennington and co-workers [22] support the concept of aminolysis proceeding through the phase test intermediate. These researchers followed the amine reaction by means of NMR spectra in tetrahydrofuran-d_8 and observed that the C-10 proton was eliminated during the course of the reaction and that a new resonance, associated with two protons, appeared upfield. They interpreted this as confirming the deduction that ring V had been opened and that a chlorin 6-carboxamide had been formed. They also observed that a comparable amine reaction did not occur with pyropheophorbide a and interpreted this to signify that the β-keto ester system is essential for the cleavage of ring V.

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