Preparation and Properties of Pyrochlorophyll a, Methyl Pyrochlorophyllide a, Pyropheophytin a, and Methyl Pyropheophorbide a Derived from Chlorophyll by Decarbomethoxylation[1]

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Chlorophyll a, methyl chlorophyllide a, pheophytin a, or methyl pheophorbide a when heated in pyridine at 100° give rise to a "pyro" series of compounds in which the carbomethoxy group has been eliminated from ring V. Methoxyl analyses, chromatographic behavior, and visible, infrared, and n.m.r. spectra support this interpretation. Such a reaction has previously been unknown for magnesium-containing chlorins. The n.m.r. and infrared data indicate that the magnesium-containing "pyro" compounds are aggregated in carbon tetrachloride solutions to a greater extent than the starting compounds and confirm the suggestion that keto–enol tautomerism does not play a decisive role in the genesis of the infrared spectra of the chlorophylls.

Introduction

In the course of a study on exchangeable hydrogen in chlorophyll,[3] we examined a variety of experimental conditions intended to accelerate exchange. One of these experiments yielded an unexpected result, and led to the preparation of a series of compounds closely related to chlorophyll but devoid of the ring V carbomethoxy group. These compounds have been designated as a "pyro" series in accordance with earlier usage,[4] although this nomenclature leaves something to be desired. The system of nomenclature used in this paper is given in Table I.

Infrared and n.m.r. spectroscopy have proved to be powerful tools in the elucidation of chlorophyll be-

havior,[5] and these methods have been similarly useful in the present work.

Of particular importance in these and related investigations is the preparation of pure chlorophylls and methyl chlorophyllides. Experience has shown that isolation methods must be chosen with great care if one is to obtain pigments that are free of colorless impurities.[6] Therefore, improved methods for the enzymatic formation and chromatographic isolation of the methyl chlorophyllides are described in the Experimental section.

Results

Pyrolysis.—Early studies by Conant and Hyde[4a] on the pyrolysis of methyl pheophorbide a (IV) and related porphyrins led to a series of pyro derivatives. The reaction was carried out in biphenyl, and CO_2 was identified as one of the products. Fischer and co-

Table I
Nomenclature and Proton Designations

Compound	Mg present	R	Proton no. of R	R'	Proton no. of R'	R''	Proton no. of R''
Chlorophyll a (Ia)	+	CH_3	3a	Phytyl	..[a]	CO_2CH_3	11
Pyrochlorophyll a (pyro-Ia)	+	CH_3	3a	Phytyl	..[a]	H	10
Methyl chlorophyllide a (IIa)	+	CH_3	3a	CH_3	12	CO_2CH_3	11
Methyl pyrochlorophyllide a (pyro-IIa)	+	CH_3	3a	CH_3	12	H	10
Pheophytin a (IIIa)	−	CH_3	3a	Phytyl	..[a]	CO_2CH_3	11
Pyropheophytin a (pyro-IIIa)	−	CH_3	3a	Phytyl	..[a]	H	10
Methyl pheophorbide a (IVa)	−	CH_3	3a	CH_3	12	CO_2CH_3	11
Methyl pyropheophorbide a (pyro-IVa)	−	CH_3	3a	CH_3	12	H	10

[a] Phytyl protons not numbered.

(1) This work performed under the auspices of the U. S. Atomic Energy Commission.

(2) Resident Research Associate, Coe College 1962–1963, under the Associated Colleges of the Midwest–Argonne Semester Program.
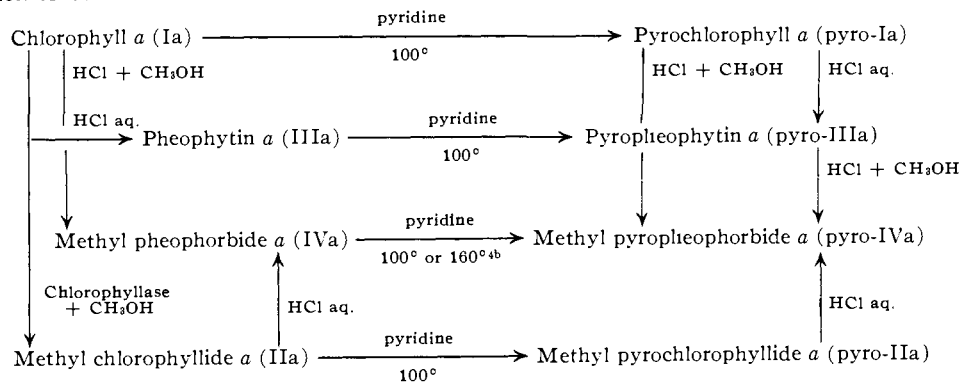
(3) J. J. Katz, M. R. Thomas, and H. H. Strain, J. Am. Chem. Soc., 84, 3587 (1962); J. J. Katz, M. R. Thomas, H. L. Crespi, and H. H. Strain, ibid., 83, 4180 (1961); J. J. Katz, R. C. Dougherty, F. C. Pennington, H. H. Strain, and G. L. Closs, ibid., 85, 4049 (1963).

(4) (a) J. B. Conant and J. F. Hyde, ibid., 51, 3668 (1929); (b) H. Fischer, L. Filser, W. Hagert, and O. Moldenhauer, Ann., 490, 1 (1931); (c) H. Fischer and A. Hinsdschil, Hoppe-Seylers Z. Physiol. Chem., 216, 57 (1933).

(5) (a) J. J. Katz, G. L. Closs, F. C. Pennington, M. R. Thomas, and H. H. Strain, J. Am. Chem. Soc., 85, 3801 (1963); (b) G. L. Closs, J. J. Katz, F. C. Pennington, M. R. Thomas, and H. H. Strain, ibid., 85, 3809 (1963).

(6) H. H. Strain, M. R. Thomas, H. L. Crespi, M. I. Blake, and J. J. Katz, Ann. N. Y. Acad. Sci., 84, 617 (1960).

TABLE II

THE PREPARATION OF VARIOUS "PYRO" COMPOUNDS AND THEIR CONVERSION TO AUTHENTIC METHYL PYROPHEOPHORBIDE a[4b]

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                           pyridine
Chlorophyll a (Ia) ─────────────────────────→ Pyrochlorophyll a (pyro-Ia)
   │                         100°
   │ HCl + CH₃OH                              │ HCl + CH₃OH    │ HCl aq.
   │                                          │               │
   │ HCl aq.              pyridine            ↓               ↓
   ├──────→ Pheophytin a (IIIa) ──────────→ Pyropheophytin a (pyro-IIIa)
   │           │            100°                               │
   │           │                                               │ HCl + CH₃OH
   │           │                                               │
   ↓           │          pyridine           ↓                ↓
Methyl pheophorbide a (IVa) ──────────────→ Methyl pyropheophorbide a (pyro-IVa)
                    ↑      100° or 160°[4b]                    ↑
 Chlorophyllase     │                                         │
  + CH₃OH           │ HCl aq.                                  │ HCl aq.
   ↓                │          pyridine
Methyl chlorophyllide a (IIa) ────────────→ Methyl pyrochlorophyllide a (pyro-IIa)
                               100°
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workers[4b] observed a similar pyrolysis in pyridine at 160° in sealed tubes. These pyrolysis reactions were all carried out on compounds lacking a central magnesium atom.

In our investigations, we have found that the chlorophylls and their magnesium-containing derivatives, such as the deuteriochlorophylls and the methyl chlorophyllides, undergo comparable pyrolysis reactions. Presumably the relatively mild pyrolysis conditions (pyridine, 100°) are less vigorous than those used previously and are at least in part responsible for our observation that the chlorophylls themselves readily yield such derivatives in good yield.

The proof of the structures of pyrochlorophyll a (pyro-Ia), pyropheophytin a (pyro-IIIa), and methyl pyrochlorophyllide a (pyro-IIa) is based on chemical analyses, visible and infrared spectra, n.m.r. studies, chromatographic comparisons, and degradation to the known methyl pyropheophorbide a (pyro-IVa). The interrelationships of the pyro compounds are shown in Table II.

Analyses.—Methoxyl analyses summarized in Table III furnish proof of the loss of a methoxyl group on pyrolysis.

TABLE III

METHOXYL CONTENT OF PYRO COMPOUNDS[a] (% BY WEIGHT)

Compound	OCH₃, calcd.	OCH₃, obsd.
Ia	3.49	3.71
Pyro-Ia	0	0.40
IIa	9.87	9.13
Pyro-IIa	5.43	5.17
IIIa	3.56	3.76
Pyro-IIIa	0	0.48
IVa	10.23	10.56
Pyro-IVa	5.66	5.48

[a] Analyses by Clark Microanalytical Laboratory, Urbana, Ill.

Infrared Spectra.—Table IV summarizes the infrared data. Pyrochlorophyll a shows absorption bands in the carbonyl region similar to those observed in the spectra of chlorophyll a. In tetrahydrofuran (Fig. 1A) ester carbonyl absorption occurs at 1737 cm.$^{-1}$ and ketone carbonyl absorption at 1688 cm.$^{-1}$. The intensity of the ester carbonyl absorption in pyrochlorophyll a is approximately one-third that of the corresponding band in chlorophyll a (cf. Fig. 2A, ref. 5a). In CCl₄ (see Fig. 1B), CHCl₃, and in Nujol mulls, an additional absorption peak occurs in the range 1635–1660 cm.$^{-1}$.

Molecular weight measurements by vapor pressure osmometry[5a] show that pyrochlorophyll a is associated

in CCl₄ solution; a solution with a calculated molarity of 0.060 M had an observed molarity of only 0.023 M, indicative of extensive molecular aggregation. Previous experience suggests that molecular aggregation is responsible for the "extra" bands in the 1635–1660
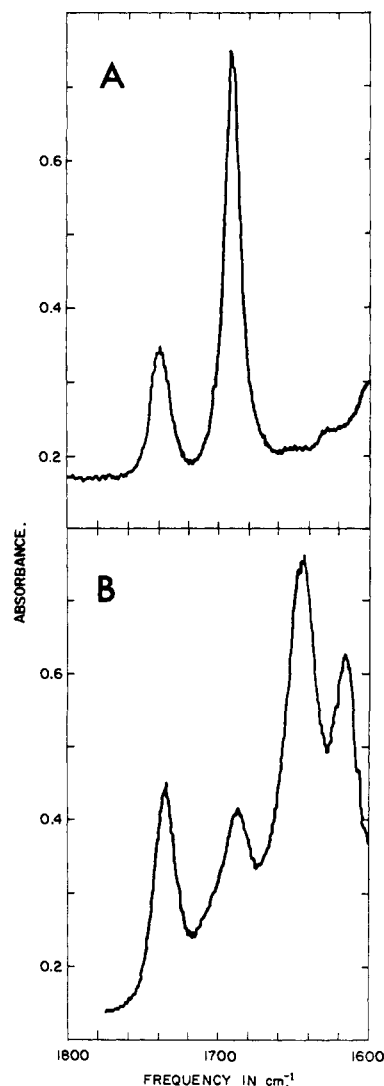

Fig. 1.—Infrared spectra of pyrochlorophyll a: A, in tetrahydrofuran; B, in CCl₄.

cm.$^{-1}$ region of the spectrum. The absorption peaks of pyro-Ia in this region thus appear to be entirely similar to the corresponding absorptions which have been observed in chlorophyll a spectra, and which have
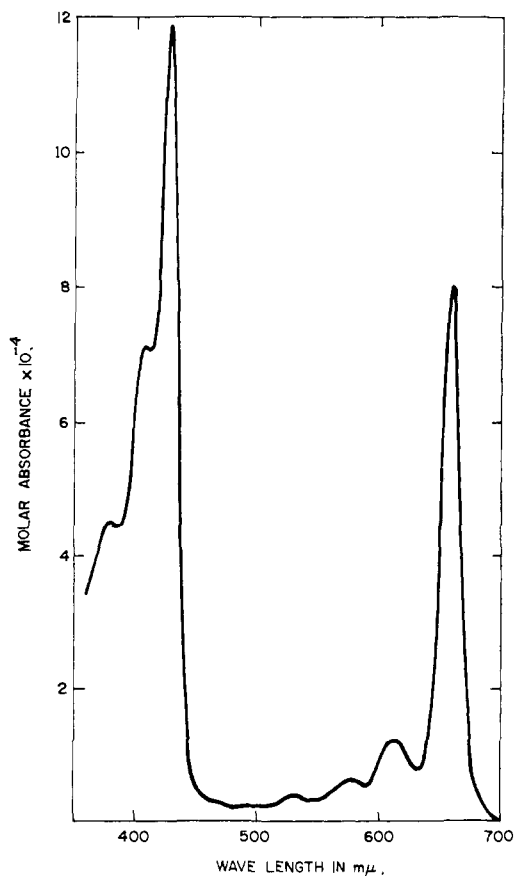
Fig. 2.—Visible spectrum of pyrochlorophyll a in ethyl ether—comparable to the spectrum of chlorophyll a except for the ratio of blue max. to red max.

TABLE IV

INFRARED ABSORPTION MAXIMA OF PYRO COMPOUNDS IN CARBON TETRACHLORIDE, CHLOROFORM, AND TETRAHYDROFURAN (THF) IN THE 1625–1750 CM.$^{-1}$ REGION

Compound	Solvent	Ester carbonyls, cm.$^{-1}$	Free ketone carbonyl, cm.$^{-1}$	Assoc. ketone carbonyl, cm.$^{-1}$
Chlorophyll a (Ia)	CCl₄	1736	1695	1653
Pyrochlorophyll a (pyro-Ia)	CCl₄	1735	1686	1643
Pyrochlorophyll a (pyro-Ia)	CHCl₃[a]	1728	1670	1635
Pyrochlorophyll a (pyro-Ia)	THF	1737	1688	. .
Pyrochlorophyll a (pyro-Ia)	Mull[b]	1740	~1695	~1650
Methyl chlorophyllide a (IIa)	CHCl₃[a]	1732	1680	1648
Methyl pyrochlorophyllide a (pyro-IIa)	CCl₄	1737	1700	~1640
Methyl pyrochlorophyllide a (pyro-IIa)	CHCl₃[a]	1729	1665	1637
Methyl pyrochlorophyllide a (pyro-IIa)	THF	1736	1683	. .
Methyl pyrochlorophyllide a (pyro-IIa)	Mull[b]	1735	~1678	~1641
Pheophytin a (IIa)	CCl₄	1743	1708	. .
Pyropheophytin a (pyro-IIIa)	CCl₄	1733	1694	. .
Pyropheophytin a (pyro-IIIa)	THF	1728	1695	. .
Methyl pheophorbide a (IVa)	THF	1739	1703	. .
Methyl pyropheophorbide a (pyro-IVa)	THF	1736	1696	. .

[a] The ethanol usually present as a stabilizer was removed by washing the chloroform repeatedly with water; the chloroform was then dried.   [b] In Nujol mull.

been attributed to coordination of ketone carbonyl oxygen to the central magnesium atom of another chlorophyll molecule. When the relative intensities of the uncoordinated ketone absorption (1686 cm.$^{-1}$) and the coordinated ketone absorption (1643 cm.$^{-1}$) in pyrochlorophyll a are compared (Fig. 1B), the latter absorption is found to be much more intense. However, in chlorophyll, itself, the relative intensities are roughly the same. Furthermore, the intensity of the coordinated ketone band is much greater in the pyro compound than in the corresponding band in chlorophyll a (see Fig. 1A, ref. 5a). This implies that pyrochlorophyll a may be more highly aggregated than chlorophyll a in nonpolar solvents and clearly indicates that the carbomethoxy group is not essential for such coordination to occur. Absence of the carbomethoxy group appears, in fact, to diminish the steric difficulties in aggregate formation.

Similar observations were made on the spectra of methyl pyrochlorophyllide a (pyro-IIa). In tetrahydrofuran, the spectra of pyro-Ia and pyro-IIa in the carbonyl region are virtually identical; the aggregation peaks of both compounds have vanished in this basic solvent. The disaggregating effects of electron donor solvents thus imply that the coordination properties of the central magnesium atom in the pyro compounds is essentially similar to that of the magnesium in ordinary chlorophyll.

Finally, pyropheophytin a (pyro-IIIa) in CCl₄ solution has an infrared spectrum very similar to that of pheophytin a (IIIa), i.e., there is no "extra" absorption peak at 1640 cm.$^{-1}$. However, the ester carbonyl

absorption of the pyro compounds has a much lower intensity relative to the ketone carbonyl than do corresponding absorptions in pheophytin a, as would be expected from a compound lacking one ester group.

The infrared spectra thus support the conclusion that pyro compounds lack the ring V carbomethoxy group present in chlorophyll a and methyl chlorophyllide a. Further, the "extra" bands in pyrochlorophyll a cannot arise from chelated ester carbonyl absorption, as was suggested by Holt and Jacobs[7] to be the source of the "extra" band in chlorophyll a. Enol formation in ring V of pyrochlorophyll a must surely occur to a very much smaller extent than in chlorophyll a itself. The present infrared observations thus tend to confirm the conclusions of Katz and co-workers[5a] that the "extra" band in chlorophyll a is due to molecular aggregation and arises in nonpolar solvents from the coordination of ketone oxygen with magnesium.

Absorption Spectra in the Visible Region.—The spectral absorption properties of the pyro compounds and those of the parent substances were measured in ethyl ether and dimethylformamide (Table V). The molecular absorption coefficients for chlorophyll a are those reported by Strain, Thomas, and Katz.[8] The values for pheophytin a in ether are close to the value 59,000 reported by Zscheile and Comar[8b] and to the 63,700 reported by Smith and Benitez.[8c] A value of 45,600 at 663 mμ for the absorption coefficient of methyl pyrochlorophyllide a in dimethylformamide solution re-

(7) A. S. Holt and E. E. Jacobs, *Plant Physiol.*, **30**, 553 (1953).
(8) (a) H. H. Strain, M. R. Thomas, and J. J. Katz, *Biochim. Biophys. Acta*, **75**, 306 (1963); (b) F. P. Zscheile and C. L. Comar, *Botan. Gaz.*, **102**, 463 (1941); (c) J. H. C. Smith and A. Benitez in "Modern Methods of Plant Analysis," Vol. IV, K. Paech and M. V. Tracey, Ed., Springer-Verlag, Berlin, 1955, p. 142.

TABLE V

SPECTRAL PROPERTIES OF CHLOROPHYLL a AND ITS DERIVATIVES IN ETHYL ETHER AND DIMETHYLFORMAMIDE (DFA)[a]

Compound	Molecular weight	Solvent	λ_{max}, mμ (red)	λ_{max}, mμ (blue)	Ratio of abs. blue/abs. red	Molecular absorption coefficient at "red" max.
Magnesium chelate series						
Chlorophyll a	893.48	Ether	660.5	428.5	1.295	86,300
Methyl chlorophyllide a	629.00	Ether	660.5	427.5	1.30	83,000
Pyrochlorophyll a	835.44	Ether	659.5	429.0	1.49	80,000
		DFA	663.0	432.5	1.44	64,000
Methyl pyrochlorophyllide a	570.96	Ether	659.0	428.0	1.52	72,000
		DFA	663.0	432.0	1.45	60,000
Magnesium-free series						
Pheophytin a	871.17	Ether	667.0	409.0	2.09	61,000
Methyl pheophorbide a	606.69	Ether	667.0	408.5	2.07	59,200
		DFA	667.0	412.5	2.15	45,000
Pyropheophytin a	813.14	Ether	667.0	409.0	2.09	49,000
Methyl pyropheophorbide a	548.66	Ether	667.0	408.5	2.09	52,000
		DFA	667.0	412.0	2.36	45,000

[a] Absorption spectra were measured on a Cary 14 recording spectrophotometer and checked on another instrument.

TABLE VI

CHEMICAL SHIFTS (C.P.S. FROM TETRAMETHYLSILANE) OF "PYRO" COMPOUNDS

Compound	Solvent	10	11	1	3	5	12	α	β	δ	13, 14
Chlorophyll a (Ia, 0.09 M)[b]	CDCl₃	...[a]	197	196	192	166	...	548	599	490	...
Pyrochlorophyll a (pyro-Ia, 0.094 M)	CDCl₃	...	...	195	191	154	...	546	553	486	...
Chlorophyll a (Ia, 0.09 M)[b]	CDCl₃ + CD₃OD[c]	373	238	197	195	216	...	554	570	497	...
Pyrochlorophyll a (pyro-Ia, 0.077 M)	CDCl₃ + CD₃OD[d]	302	...	198	195	210	...	552	567	496	...
Methyl chlorophyllide a (IIa, 0.08 M)[b]	CDCl₃ + CD₃OD[c]	372	239	198	196	217	209	555	571	498	...
Methyl pyrochlorophyllide a (pyro-IIa, satd.)	CDCl₃	...[a]	...	194	191	167	177	546	554	488	...
Methyl pyrochlorophyllide a (pyro-IIa, 0.09 M)	CDCl₂ + CD₃OD[e]	302	...	197	194	212	210	551	568	496	...
Pheophytin a (IIIa, 0.06 M)[b]	CDCl₃	375	233	200	184	218	...	548	559	510	−110
Pyropheophytin a (pyro-IIIa, 0.1 M)	CDCl₃	306	...	199	181	212	...	546	552	508	∼−117
Methyl pheophorbide a (IVa, 0.06 M)[b]	CDCl₃	373	233	199	183	217	214	549	559	510	−105
Methyl pyropheophorbide a (pyro-IVa)	CDCl₃	308	...	201	188	215	215	552	559	510	∼−111

[a] Unresolved multiplets with low signal-to-noise ratios. [b] These data were obtained from G. L. Closs and co-workers.[5b] [c] 3% CD₃OD. [d] 12% CD₃OD. [e] 11% CD₃OD.

ported by Corwin and Wei[9] is significantly lower than our values. Furthermore, we do not observe a maximum at 667 mμ, as they report. This suggests that the compound of Corwin and Wei, prepared by introduction of magnesium into methyl pyropheophorbide a, is not as pure as the product obtained by pyrolysis. Differences also exist in the absorption spectra of methyl pyropheophorbide a, for we do not observe the pronounced maximum at 663 mμ reported by Corwin and Wei. On the other hand, the absorption coefficient of 50,400 at 667 mμ for methyl pyropheophorbide a in dimethylformamide observed by Corwin and Wei[9] is greater than our measured values.

Our results show that substitution of methyl for phytyl in the magnesium chelate series (pyro-Ia vs. pyro-IIa) or in the magnesium-free series (pyro-IIIa vs. pyro-IVa) has little effect upon the spectral properties in the visible region, as has been reported in earlier work with the chlorophylls and the chlorophyllides.[8] By contrast, removal of the magnesium has a pronounced effect on the spectra of both the chlorophylls and the pyrochlorophylls (pyro-Ia vs. pyro-IIIa). The removal of the carbomethoxy group by pyrolysis has very little effect upon the spectra (see Fig. 2 and Table V). So similar are the spectra of chlorophyll a and pyrochlorophyll a that it would be very difficult to ascertain solely from an examination of the absorption

(9) A. H. Corwin and P. E. Wei, J. Org. Chem., **27**, 4285 (1962).

spectrum in the visible region whether or not pyrochlorophyll a is present in nature as a normal constituent of the chloroplast.

N.m.r. Studies.—Proton magnetic resonance measurements yielded convincing evidence for the elimination of the carbomethoxy group on pyrolysis. Table VI summarizes the n.m.r. data for the pyro compounds. For comparison, Table VI also includes chemical shifts for the related unpyrolyzed compounds.

The n.m.r. spectra were measured on a Varian A-60 instrument, and all chemical shift values are reported in cycles per second from tetramethylsilane as an internal standard, with chemical shifts to lower fields positive.

The spectra were measured in CDCl₃ and CDCl₃–CD₃OD solutions. The spectra of the magnesium complexes (pyro-Ia and pyro-IIa) showed remarkable changes in chemical shift values when alcohol was added (Fig. 3 and 4). These observations are entirely consistent with those of Closs and co-workers.[5b] The resonances assigned to the C-10 protons and to the methyl protons at position 5 exhibit the greatest paramagnetic shifts on disaggregation.

The assignments for the pyro compounds listed in Table VI are based on a correlation with the n.m.r. spectra of chlorophyll reported by Closs and co-workers.[5b] The greater extent of molecular aggregation of pyrochlorophyll a in nonpolar solvents as com-
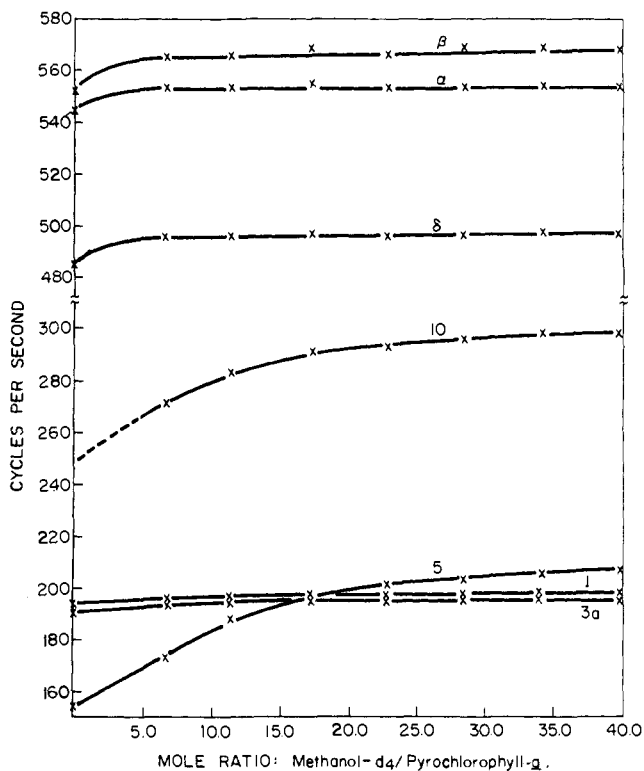
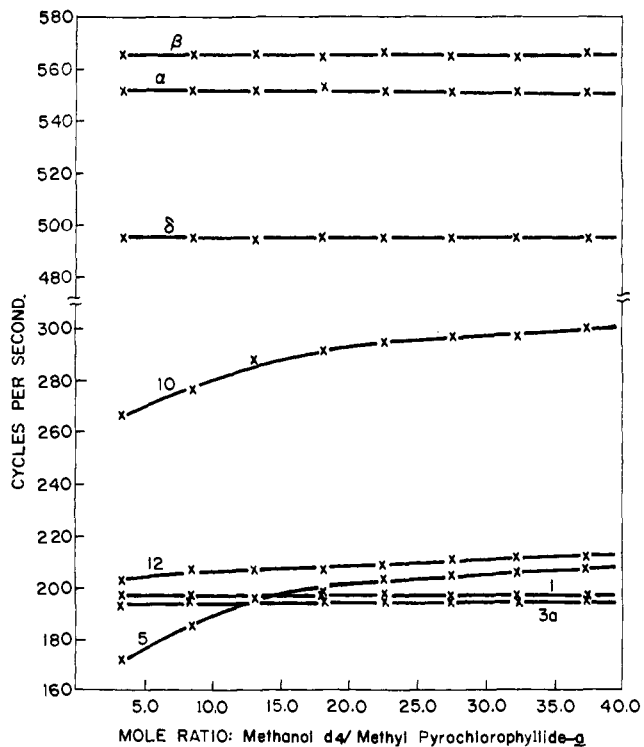Fig. 3.—Methanol titration curve in CDCl₃ of pyrochlorophyll *a* (∼0.08 *M*).

Fig. 4.—Methanol titration curve in CDCl₃ of methyl pyrochlorophyllide *a* (∼0.09 *M*).

Fig. 5.—Dilution shifts of methyl pyropheophorbide *a* in CDCl₃. For clarity the dilution shifts are shown to be linear functions of concentration although some deviation from linearity is to be expected.

pared to chlorophyll *a*, already indicated by the infrared spectra, is confirmed by the fact that it is necessary to add much more alcohol to pyrochlorophyll *a* dissolved in CDCl₃ in order to effect a maximum shift in the resonances of the C-10 protons and the 5-methyl protons than is required under comparable conditions with chlorophyll *a*.
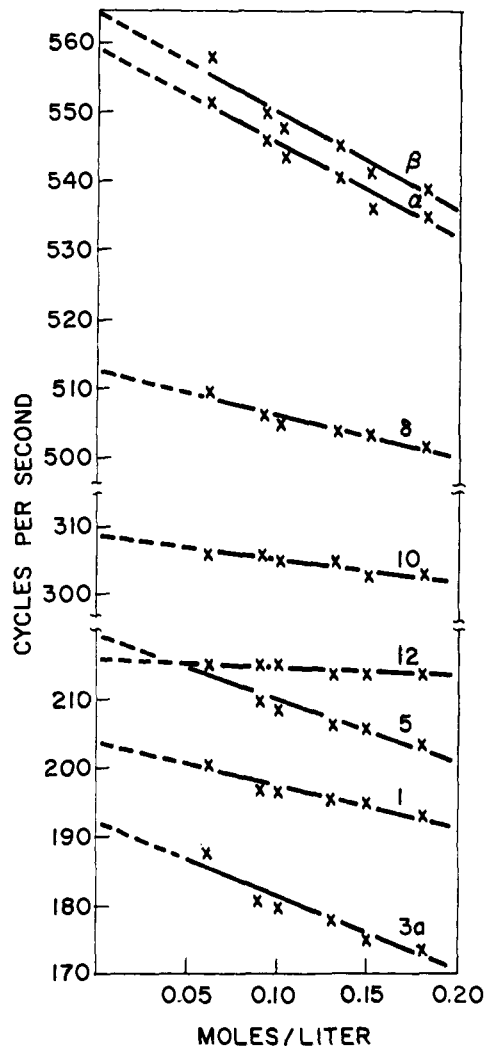
Closs and co-workers[5b] have also shown that n.m.r. spectra of the magnesium-free derivatives (III and IV) are strongly concentration dependent. We have observed significant dilution shifts in the n.m.r. spectra of methyl pyropheophorbide *a* (pyro-IVa) (Fig. 5). The largest dilution shifts were noted for the 3a-methyl and the α- and β-protons. Contrary to the findings of Abraham and co-workers[10] on some porphyrin compounds, in the magnesium-free pyro compounds the imine protons do not exhibit the largest chemical shifts on change in concentration.

Direct comparison of the n.m.r. spectra in Table VI shows that the 11-methyl group in the carbomethoxy group is no longer present in the pyro compounds. Closs and co-workers[5b] have shown that resonances at 238 c.p.s. in chlorophyll *a*, at 239 c.p.s. in methyl chlorophyllide *a*, and at 233 c.p.s. in pheophytin *a* and methyl pheophorbide *a* (see Table VI for concentrations) arise from the 11-methyl protons. These methyl resonances are absent in the pyro compounds, affording strong support for the suggestion that the carbomethoxy group has been eliminated in the pyro compounds.

(10) R. J. Abraham, P. A. Burbidge, A. H. Jackson, and G. W. Kenner, *Proc. Chem. Soc.*, 134 (1963).

It is interesting to note that in pyrochlorophyll a there are two protons at the C-10 position. Not only does this confirm the absence of the carbomethoxy group, but it also clearly indicates that no appreciable amount of the enol form is present. The C-10 resonances occur at higher field than the single C-10 proton in chlorophyll a, which is to be expected since the carbomethoxy group deshields the C-10 proton in the β-keto esters.

Mathewson, Richards, and Rapoport[11] carried out n.m.r. studies on a pyropheophorbide derived from *Chlorobium* chlorophyll 660 and assigned resonances at δ 4.92 in $CDCl_3$ and δ 5.33 in $CDCl_3$-CD_3OD to the two C-10 protons. Their value for the chemical shift of the C-10 protons in $CDCl_3$-CD_3OD is very close to that reported in Table VI. However, direct comparison with our data is difficult because Mathewson, Richards, and Rapoport in this publication do not specify the concentrations either of the pigment or of the CD_3OD, variables that Closs and co-workers[5b] have shown may greatly affect the chemical shifts.

The δ-H of the pyro compounds proved to be exchangeable in $CDCl_3$-CD_3OD solutions. In 11% CD_3OD solutions, more than half of the δ-H in pyro-IIa was exchanged in 1 week at room temperature. The C-10 proton resonance intensities of pyro-IIa were not markedly reduced in this same solution after 2.5 months. The δ-hydrogen in pyro-Ia under the same conditions is also exchanged. The exchange observations so far made on the pyro compounds are consistent with the conclusion that enolization involving the C-10 protons occurs to only a very small extent, and the δ-methine hydrogens are exchangeable, as in the case of the related compounds possessing a C-10 carbomethoxy group.

In addition, when pyro-IIIa was heated with a mixture of pyridine and D_2O, the C-10 protons were exchanged, while the δ-H was hardly affected. Exchange at C-10 will be facilitated by either acid or base, but exchange at the δ-position probably proceeds primarily by electrophilic substitution; the inertness of the δ-hydrogen to pyridine-D_2O supports this view. The imine hydrogens also exchanged in pyridine-D_2O. A more quantitative assessment of hydrogen exchange in the pyro compounds is in progress.

Chromatographic Comparison of the Chlorophylls and Their Derivatives.—All the pigments listed in Table II were compared by adsorption in columns of powdered sugar with petroleum ether plus 0.5% 1-propanol as the development liquid. To facilitate the identification of these pigments in natural sources, the chlorophyll derivatives were also adsorbed with the common carotenoids found in leaves. The chromatographic adsorption sequence is shown in Table VII. Pheophytin b was adsorbed just below chlorophyll b', but its position relative to methyl pyrochlorophyllide a was not established. Obviously, most of the pigments, except those enclosed by brackets, may be separated easily by chromatography.

It is noteworthy that the pyro compounds containing magnesium are more sorbed than the parent magnesium-containing pigments. The magnesium-free pyro compounds are slightly less sorbed than the magnesium-free parent substances. This effect may be

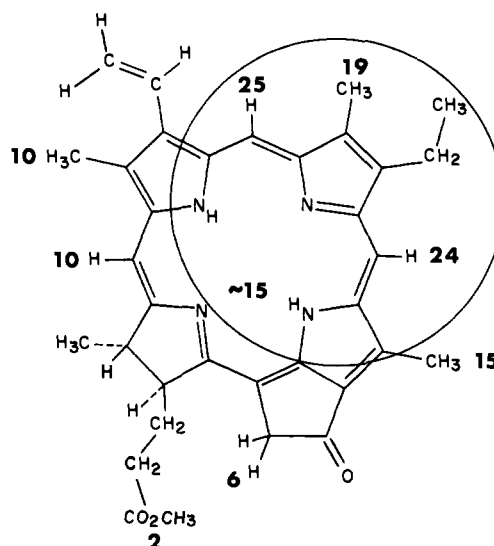(11) J. W. Mathewson, W. R. Richards, and H. Rapoport, *J. Am. Chem. Soc.*, **85**, 364 (1963).

Fig. 6.—Aggregation map of methyl pyropheophorbide a based on dilution shifts in $CDCl_3$ in the concentration range from 0.18 M to extrapolated values at zero concentration.

attributed to the greater tendency to aggregation exhibited by the magnesium-containing pyro compounds in nonpolar solvents.

The reaction scheme shown in Table II indicates that each of the seven substances may be converted, by standardized procedures, to authentic methyl pyropheophorbide a. Conversely, the isolation of methyl pyropheophorbide a, after the reactions indicated in Table

TABLE VII

CHROMATOGRAPHIC SEQUENCES OF CHLOROPHYLLS, THEIR PYRO DERIVATIVES, AND SOME CAROTENOID PIGMENTS[a]

Neoxanthin
Pyrochlorophyll b
Violaxanthin
Chlorophyll b
[Chlorophyll b'
Methyl pyrochlorophyllide a [lutein +
Methyl chlorophyllide a zeaxanthin
Methyl chlorophyllide a'
Methyl pheophorbide a
Methyl pyropheophorbide a
Pyrochlorophyll a
Chlorophyll a
Chlorophyll a'
Pheophytin a
Pyropheophytin a
Carotene

[a] Adsorbent, powdered sugar; solvent, petroleum ether plus 0.5% 1-propanol.

II, provides a proof of structure of the several parent substances, provided all the preparations of the methyl pyropheophorbide a were identical. By chromatography in columns of powdered sugar, all the preparations were homogeneous. Upon adsorption together, they yielded but one zone and were, therefore, chromatographically identical.

Discussion

It is clear from our studies that certain unusual physical and chemical properties associated with chlorophyll a are also observed with pyrochlorophyll a, e.g., the formation of aggregated species in chloroform and carbon tetrachloride solutions and the disaggregating effects of alcohol. Aggregation–disaggregation
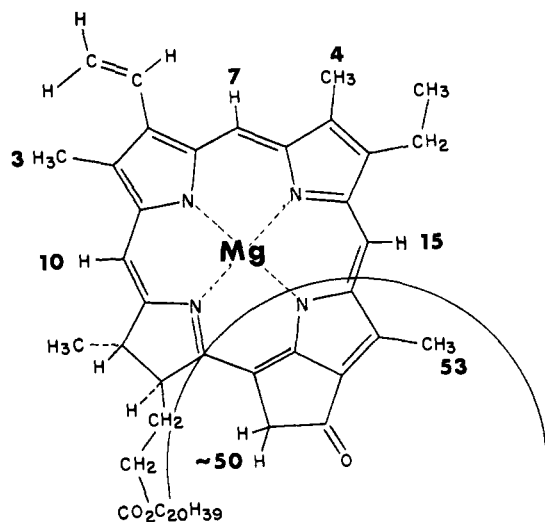
Fig. 7.—Aggregation map of pyrochlorophyll *a* based on methanol titration data in $CDCl_3$.

appears to depend on the presence of magnesium in the molecule and is not appreciably altered by the absence of the carbomethoxy group in ring V.

Aggregation maps for methyl pyropheophorbide *a* (see Fig. 6) and pyrochlorophyll *a* (see Fig. 7) were prepared from the n.m.r. data on dilution shifts (0.18 *M* to infinite dilution, Fig. 5) and methanol titration (Fig. 3). The maps are similar to those reported by Closs and co-workers.[5b] Surprisingly, the carbomethoxy group appears to have little effect on the relative orientation of the macrocycles.

On the other hand, the exchangeability of the proton at C-10 in chlorophyll *a* is much greater than of the corresponding protons at C-10 in pyrochlorophyll *a*. This is to be expected since the carbomethoxy group should facilitate enolization, even though the extent of enolization is small.

Pyrochlorophyll *a* does not allomerize under conditions that lead to extensive allomerization of chlorophyll *a*. There was no alteration of the pigment in a saturated methanol solution exposed to the air for 3 days. Nor does pyrochlorophyll *a* give a positive phase test. This confirms earlier conclusions that the phase test and allomerization require the presence of a β-keto ester and a C-10 proton in ring V.

Because the pyro compounds have no asymmetry at C-10, they provide pertinent information with regard to isomerization of the chlorophylls, *i.e.*, chlorophylls *a* and *a'*. In our chromatographic studies there was no indication that any of the pyro compounds isomerized in a manner analogous to the isomerization of chlorophyll. This finding furnishes support for the conclusion that chlorophyll *a* and *a'* differ by spatial arrangement of the two substituent groups at C-10.[5b,12] Closs has pointed out to us that a measurement of the chemical shifts of the 7- and 8-protons in the pyro series would be very valuable for establishing the stereochemistry of chlorophyll *a*,[5b] and such measurements are in progress.

Pyrochlorophyll in Nature?—The possibility that pyrochlorophyll may be a normal component of the chloroplast deserves comment. The isolation of pyro

compounds from natural sources is well established. Pyropheophorbide[4c] and phylloerythrin[13] arise from chlorophyll in the process of digestion, presumably by enzymatic decarbomethoxylation; the enzymes are considered to be of bacterial origin. Holt and co-workers[14] consider *Chlorobium* chlorophylls-650 and -660 to be derivatives of pyropheophorbide, a view concurred in by Mathewson, Richards, and Rapoport.[11] That large amounts of pyrochlorophyll are present in chloroplasts is unlikely because of the ease with which ordinary and pyrochlorophyll can be separated chromatographically; if pyrochlorophyll were a major component it hardly would have escaped detection. Whether pyrochlorophyll might be normally present in small amounts is not so clear. Although the likelihood is small that pyrochlorophyll is a normal component of the chloroplast, a search is probably justified, particularly in the light of the widely held view that only a very small fraction of the chlorophyll in the chloroplast is photosynthetically active.[15]

It is interesting to note that Rabinowitch[16a] has speculated about the existence of a reversible carboxylation cycle involving chlorophyll as possibly important in the photoreduction of CO_2. Pyrochlorophyll *a* would obviously lend itself to such a cycle. Rabinowitch,[16b] however, emphasizes that carboxylation does not necessarily constitute reduction. There is no reason to suppose that appropriate enzymes are present to catalyze a reversible carboxylation cycle involving C-10, and, indeed, the decarboxylation may be spontaneous and essentially irreversible. Nevertheless, it may be useful to acquire some data on the fluorescence, phosphorescence, and photochemical behavior of the pyro compounds.

Experimental

Preparation of Chlorophylls.—The chlorophylls were isolated from spinach by chromatographic and precipitation procedures described before.[6] The several steps in these procedures were designed to provide pure substances free of other leaf constituents and impurities derived from the solvents and the chromatographic columns.[17]

Preparation of the Methyl Chlorophyllides.—The preparation of the methyl chlorophyllides by enzymatic methanolysis in leaves presented many unanticipated difficulties. Mallow leaves, which had been collected in California and found to be a rich source of chlorophyllase,[12c] were not available here in the Chicago area. Hollyhock leaves, collected in California, had been found to contain chlorophyllase,[12c] but such leaves, collected here, contained oxidative enzyme systems in addition to chlorophyllase. Consequently these leaves, collected here in the autumn and allowed to react with methanol, yielded most of each chlorophyll in an oxidized form. As indicated by the absorption spectra and the chromatographic sequences, these oxidized chlorophylls had previously been prepared from barley leaves by an enzymatically induced oxidation.[12c] Oxidized *a*, adsorbed above chlorophyll *a*

(12) (a) W. M. Manning and H. H. Strain, *J. Biol. Chem.*, **151**, 1 (1943); (b) H. H. Strain and W. M. Manning, *ibid.*, **146**, 275 (1942); (c) H. H. Strain, *J. Agr. Food Chem.*, **2**, 1222 (1954).

(13) R. Hill in "Comprehensive Biochemistry," M. Florkin and E. H. Stotz, Ed., Vol. 9, Elsevier Publishing Co., Amsterdam, 1963, p. 90.

(14) A. S. Holt and H. V. Morley, *J. Am. Chem. Soc.*, **82**, 500 (1960); A. S. Holt and D. W. Hughes, *ibid.*, **83**, 499 (1961); A. S. Holt D. W. Hughes, H. J. Kende, and J. W. Purdie, *ibid.*, **84**, 2835 (1962); H. V. Morley and A. S. Holt, *Can. J. Chem.*, **39**, 755 (1961); D. W. Hughes and A. S. Holt, *ibid.*, **40**, 171 (1962).

(15) A. Müller, B. Rumberg, and H. T. Witt, *Proc. Roy. Soc.* (London), **B157**, 313 (1963).

(16) E. I. Rabinowitch, "Photosynthesis," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1945: (a) p. 454; (b) p. 187.

(17) H. H. Strain, M. R. Thomas, H. L. Crespi, and J. J. Katz, *Biochim. Biophys. Acta*, **52**, 517 (1961).

but below chlorophyll b, exhibited λ_{max} 427.5 and 660 mμ in ether, 432.5 and 665 mμ in methanol. Oxidized b, adsorbed above chlorophyll b, exhibited λ_{max} 450 and 641 mμ in ether, 467 and 652.5 mμ in methanol. The same products were obtained from leaves treated with methanol or with acetone. Earlier experiments had shown these products to be chromatographically identical with corresponding pigments formed along with others in the allomerization or nonenzymatic oxidation of the chlorophylls in methanol.[12c] The formation of the oxidation products was retarded, and the formation of the methyl chlorophyllides predominated when the leaves were exposed to methanol in a nitrogen atmosphere. Preliminary n.m.r. observations on the oxidized a indicate the introduction of an hydroxy group at C-10.

Because of the long, cold winter, green leaves rich in chlorophyllase, including the *Ailanthus* leaves recommended by Holt and Jacobs,[18] were unavailable outdoors for a period of nearly 6 months. Moreover, the chromatographic methods that had been employed for the chromatographic separation of very small quantities of the chlorophyllides[12c] were not suitable for the isolation of the quantities needed for our current measurements.

Fortunately, the common cocklebur (*Xanthium pennsylvanicum*), was grown in our greenhouses for studies of photoperiodism, especially the flowering response. Leaves of this plant are rich in chlorophyllase. After preliminary isolation of the chlorophyllide mixture, adequate quantities of the chlorophyllides could be separated by chromatography on powdered sugar using benzene and benzene plus propanol as the wash liquids.

For preparation of the chlorophyllides, cocklebur leaves (about 1 kg.) were cut into small sections with scissors and placed in a 4-l. Pyrex bottle. Nitrogen was passed through the bottle for 1.5 hr. to displace the air; 2 l. of methanol was added, and the stream of nitrogen was continued for 1.5 hr. Then the bottle was stoppered and rotated at 14 r.p.m. for about 16 hr. By this time, the leaves had turned from green to yellow due to the "crystallization," in the leaves, of the methyl chlorophyllides. The light green liquid was discarded, and the pigments were extracted by the addition of methanol (1 l.) plus ethyl ether (800 ml.), followed by additional rotation of the bottle for 0.5 hr. All the liquid was then poured out, and the extraction was repeated with methanol (1 l.), ethyl ether (500 ml.), and petroleum ether (300 ml., b.p. 30–60°), with a rolling period of 0.5 hr. A third extraction was made with methanol (1 l.), ethyl ether (400 ml.), and petroleum ether (400 ml.) with a rolling period of 0.5 hr. This extract was poured out, and a fourth extraction was made with methanol (1 l.), ether (200 ml.), and petroleum ether (600 ml.), again with a rolling period of 0.5 hr. The extracts were filtered through a thin layer of cotton and combined. The pigments were transferred to the ether plus petroleum ether by addition of an excess of strong salt solution. The ether layer was washed with water and evaporated to about 100–150 ml. in a water bath. Petroleum ether (about 100 ml., b.p. 20–40°) was added to the deep green solution. The solution was allowed to stand in a Thermos jar with solid carbon dioxide for 1–2 days. Crystals that had formed were collected by centrifugation of the green solution. These crystals, contaminated with much colorless material, were washed with petroleum ether, dried under vacuum, and preserved in evacuated ampoules. The yield was about 0.9 g.

Substantiating our earlier observations, small quantities of the chlorophyllides were readily separable by chromatography on powdered sugar (columns 8 cm. in diameter by 32–35 cm. in length) with petroleum ether plus 1-propanol (0.5%) as the wash liquid. But with larger quantities of the chlorophyllides, it was necessary to employ ether plus petroleum ether as the solvent. Even with this solvent mixture, some of the pigments often separated from the solution and collected on top of the sugar. Moreover, additional quantities of the chlorophyllides crystallized in the sugar when the narrow initial zone of the adsorbed mixture was washed with petroleum ether plus propanol.

Crystallization of the pigments in the sugar columns was avoided by the use of benzene as the solvent. For example, the mixture of the chlorophyllides (about 0.9 g.) was dissolved in warm benzene (150–300 ml.). This solution was drawn into three columns of powdered sugar (8 by 35 cm.). The adsorbed pigments were washed with warm benzene, which produced two contiguous green zones, similar in color to those of chlorophylls a and b, preceded by a wide yellow zone due to the xanthophylls. Only traces of chlorophylls were present. The a and the b zones

(18) A. S. Holt and E. E. Jacobs, *Am. J. Botany*, **41**, 710 (1954).

were removed separately, except for the overlapping region, and packed into chromatographic tubes where the pigments were eluted with benzene plus about 3% ethanol. For convenience, the eluates were treated with water (about 300 ml.) and permitted to stand overnight in the refrigerator. Each eluate was then washed twice with water to remove the residual alcohol. If crystals had formed, the solutions were warmed to redissolve the pigments.

For further purification, the methyl chlorophyllide a dissolved in benzene was readsorbed on two columns of powdered sugar (8 by 30 cm.), and the sorbed pigments washed with warm benzene plus 0.5 % 1-propanol, which carried the methyl chlorophyllide a well below the remaining b. Traces of the less sorbed a' appeared below the a zone. The a zones were removed from each column and packed into chromatographic tubes where the pigment was eluted with benzene plus ethanol (3%). The eluates were combined and extracted with 200-ml. portions of aqueous methyl alcohol (50, 60, 70, and 80% methanol). The benzene was then extracted several times with water and evaporated to dryness at reduced pressure. The residue was transferred to a small flask with about 20 ml. of benzene where it was diluted with about 90 ml. of methanol and 70 ml. of n-heptane. Crystals of the methyl chlorophyllide a separated as the methanol was washed out with water. These crystals were collected by centrifugation, washed with petroleum ether, and dried under vacuum (10^{-3} mm.) at 100° for 4 hr. Molecular absorption coefficients indicated some contamination by colorless substances; therefore, the preparation was dissolved in benzene, which was filtered, concentrated to a small volume, and diluted with cyclohexane. The crystals that separated at room temperature were collected and vacuum dried. The glistening purple-black crystals weighed 49.7 mg. The molecular absorption coefficient in ether at 660.5 mμ was 76,000. The absorption curve was identical with that for chlorophyll a.

The mother liquor from the crystals was cooled with solid carbon dioxide. Additional crystals that formed were collected by centrifugation and dried under vacuum at 100°. These weighed 18.9 mg., molecular absorption coefficient 81,000, absorption curve identical with that for chlorophyll a (Table V).

The methyl chlorophyllide b, after elution from the three chromatographic columns, was readsorbed from benzene in two columns of powdered sugar (8 by 35 cm.). It was washed with a little warm benzene and then with benzene plus 1-propanol (0.5 and 1.0%). Only small amounts of b' and a formed zones below the b zone. The b zones were removed and packed into a chromatographic tube, and the pigment was eluted with benzene plus ethanol or propanol (3%). The eluate was washed with water (three times with 300 ml.) and then evaporated almost to dryness. Upon the addition of petroleum ether (50–80 ml., b.p. 20–40°) the methyl chlorophyllide b separated rapidly. It was collected, dissolved in a little acetone, and crystallized by the addition of petroleum ether. The glistening purple-black crystals did not contain visible contaminants. The yield was usually about 50 mg. The spectral absorption curve was identical with that for chlorophyll b (Table V).

Preparation of Pheophytin a.—Ordinary pheophytin a was prepared from chlorophyll a by the addition of about 0.5 ml. of concentrated hydrochloric acid to about 100 ml. of an ether solution containing 50 to 100 mg. of chlorophyll. After 1 to 2 min., the acid was removed by washing with water. The ether was diluted with a little petroleum ether and evaporated. The residue was suspended in a small amount of low-boiling petroleum ether (20–40°), cooled with Dry Ice overnight, collected by centrifugation, and dried under vacuum at 100° for 1 hr. The spectral absorption curve was the same as that already reported for pheophytin a[8b,c] (Table V). Methoxyl analysis is given in Table III.

Preparation of Methyl Pheophorbide a.—The methyl pheophorbide a was prepared in two ways, as indicated in Table II. By a modification of Fischer's method,[4b] chlorophyll a, 0.1 g., was dissolved in methanol (10 ml.). Then 1 ml. of a 22% solution of dry HCl in methanol was added, and the solution was boiled under reflux for 1 hr. The solution was cooled, diluted with ethyl ether, washed with water, and extracted twice with 10% aqueous HCl. Then the pigment was extracted into 18% HCl and retransferred to fresh ether by dilution with water. The ether layer was concentrated and diluted with petroleum ether. Crystals of the methyl pheophorbide a that separated were collected and dried under vacuum at 100° for 1 hr.

Methyl pheophorbide a was also prepared by removal of magnesium from methyl chlorophyllide a. To this end, the methyl chlorophyllide was dissolved in methanol. A few drops of concentrated hydrochloric acid were added at room temperature, and, after a few minutes, ether was added, and the methanol and acid were washed out with water. The pheophorbide was then crystallized by the addition of petroleum ether, collected by centrifugation, and dried under vacuum at 100° for 1 hr.

The products from the two procedures were chromatographically identical in columns of powdered sugar. The spectral absorption curves were also identical and were the same as those of pheophytin a, Table V. A typical methoxyl analysis is given in Table III.

Pyrolysis Conditions.—When attempts were made to promote the exchange of the deuterium of deuteriochlorophyll a with the hydrogen of water by heating chlorophyll solutions in pyridine plus 20% water at 100°, the chlorophyll underwent a series of changes that were followed chromatographically. After only a few minutes, chlorophyll a' was formed. This pigment then increased in quantity until, after 20–30 min., it represented about 20% of the mixture. Thereafter, both the a and the a' decreased as a third pigment, pyrochlorophyll a, appeared, isolated as a green zone above the chlorophyll a in the sugar columns. After a heating period of 24 hr., all but traces of the a and a' had disappeared, and the pyrochlorophyll a was the principal colored product. After a heating period of less than 24 hr., it was necessary to separate the pyrochlorophyll a from the residual chlorophyll a and a' by chromatography; with longer heating periods, which produced complete pyrolysis, the pyrolysis product could be isolated free of other pigments without resorting to chromatographic separations. The same sequence of reactions observed in aqueous pyridine was also observed in pyridine that had been freshly distilled over potassium hydroxide. As the temperature employed in these exploratory experiments was much lower than the 160° employed by Fischer and co-workers[4b] for the pyrolysis of methyl pheophorbide a to its pyro derivative in pyridine, all the pyro compounds in Table II were prepared at 100°, but for confirmation of the reaction products, the methyl pyropheophorbide a was also prepared at 160°.

For all these transformations, the pigments, 50–100 mg., were dissolved in about 10 ml. of pyridine. This was placed in a constricted tube which was then cooled, evacuated with a water aspirator, and sealed. The sealed tubes were heated in a water bath, at 100° for 48 hr., or in a muffle furnace, at 160°, for 2 hr. After cooling, the tubes were opened, the pyridine was evaporated from flasks at reduced pressure and the residue taken up in a little petroleum ether. All the pyro compounds except pyropheophytin a separated in crystalline form. The pyropheophytin a was obtained as a residue by evaporation of the solution.

Analyses.—We encountered considerable difficulty in obtaining satisfactory elementary analyses, particularly of the magnesium-containing compounds. This was due to difficulties in attaining complete combustion, from the tendency of the magnesium chelates to sorb water or other bases, and from the small amounts of material available. Analyses are by Mr. William Saschek of the Department of Chemistry, University of Chicago.

Anal. Calcd. for $C_{53}H_{70}O_3N_4Mg \cdot H_2O$ (pyrochlorophyll a, pyro-Ia): C, 74.58; H, 8.50; Mg, 2.85. Found: C, 74.68; H, 8.24; Mg, 2.65. Calcd. for $C_{53}H_{72}O_3N_4$: (pyropheophytin a, pyro-IIIa): C, 78.28; H, 8.93; N, 6.89. Found: C, 78.16; H, 9.05; N, 6.19.

Pyrolysis Products.—As indicated by the horizontal arrows in Table II, four pyrolysis products were formed from four different substances in the a series. As shown by chromatography, each of the pyrolysis products was homogeneous, a single substance. In addition to methoxyl analyses and visible, infrared, and n.m.r. spectra, these pyro compounds exhibited other characteristic properties. All of them gave negative phase tests whereas the unpyrolyzed compounds gave positive phase tests when the solutions in ether were treated with methanol solutions of potassium hydroxide.

The pyrochlorophyll a could not be shaken from solution in petroleum ether as an emulsion with water, whereas ordinary chlorophyll a was removed completely by this procedure.[6]

As indicated by the vertical arrows at the right of Table II, three of the pyro compounds were converted to methyl pyropheophorbide a. The reaction conditions were the same as those employed for the corresponding reactions at the left of Table II and described in the preceding sections. The preparations of methyl pyropheophorbide a obtained from these reactions and by methyl pheophorbide a at 100° for 48 hr. and at 160° for 2 hr. were chromatographically and spectroscopically identical. These observations demonstrate that the pyrolysis reaction proceeds in the same way after the phytyl group is substituted by methyl, after the magnesium is substituted by two hydrogens, and after both the magnesium and the phytyl are substituted by hydrogen and methyl.

Some preparations of methyl pyropheophorbide a prepared by Fischer's method gave low methoxyl analyses. Presumably, some hydrolysis occurred during the isolation.

Pyrolysis of deuteriochlorophyll a in pyridine produced a deuteriopyrochlorophyll a. This product was adsorbed above the parent deuteriochlorophyll a in sugar columns. It exhibited spectral absorption properties similar to those of chlorophyll a, and it gave a negative phase test when treated with alkali.

When chlorophyll b (Ib) and methyl chlorophyllide b were pyrolyzed under the same conditions employed with the a series, compounds of the pyro-b type were isolated in the chromatographic columns. Like the pyro-a compounds these pyro pigments were more sorbed on sugar than the parent b compounds and gave negative phase tests.

Chromatography.—Virtually all the chromatographic separations and comparisons were made in columns of powdered sugar. The sugar was packed dry without any preliminary treatment.

For determination of the chromatographic sequences, two or three of the pigments were adsorbed in columns of powdered sugar (1 cm. by about 20 cm.). The adsorbed pigments were then washed with petroleum ether containing 0.5% 1-propanol until the zones had migrated 10–15 cm. This adsorption procedure was repeated with various pigments until the sequence was established.