leaf pigments are adsorbed from benzene solution in columns of powdered sugar, the chlorophylls are relatively much more sorbed than the xanthophylls. If very small quantities of polar solvents are added to the benzene, the chlorophylls become relatively much less sorbed so that the chromatographic sequences are reversed.[31] This effect can now be ascribed, at least in part, to the disaggregation of the associated chlorophyll. In this connection, the existence of chlorophyll in various states of aggregation in solvents of different basicity explains many of the anomalous solubility relations of the pigments encountered in the preparation of the pure chlorophylls from plant material.

Brody and Brody[32] have deduced the presence of chlorophyll aggregates in ethanol solution by spectroscopic observations in the visible. We find, to the contrary, that ethanol has a strong disaggregating effect on chlorophyll, a conclusion supported by n.m.r. studies in methanol solution.[5] We have no reason to suppose that the chlorophyll aggregates in nonpolar solvents detected by infrared and n.m.r. spectroscopy occur in methanol or ethanol at chlorophyll concentrations of 10^{-2} M, and we are therefore at a loss to account for the conclusions of Brody and Brody. The nature of the chlorophyll aggregates in nonpolar solvents is further described in reference 5.

(31) H. H. Strain, *J. Phys. Chem.*, **57**, 638 (1953).

(32) S. S. Brody and M. Brody, *Nature*, **189**, 547 (1961); *Trans. Faraday Soc.*, **58**, 416 (1962).

Experimental

Materials.—**Chlorophylls** *a* **and** *b* were prepared from spinach by the procedure of Strain, *et al.*[4] **The methyl chlorophyllides** were prepared from cockleburr by the *in situ* reaction with methanol; details of this procedure will be described elsewhere. **Solvents** were of reagent grade.

Infrared spectra were measured on a Beckman IR-7 spectrophotometer, using microcells and a beam condenser. The spectrophotometer was calibrated for wave length and slit width. The solutions were prepared by dissolving 0.6 to 1 mg. of chlorophyll in about 10 μl. of solvent; the composition of the solution was determined by weighing, and the concentration was generally in the range of 0.07 to 0.1 M. The measurements were made in a Connecticut Instrument Co. 0.05-mm. Irtran microcell. This procedure proved very sparing of the difficultly-obtainable pure chlorophylls. Since relative peak heights were significant, spectra were run with various slit schedules and scanning speeds to minimize instrumental artefacts. Some of the spectra were checked on a Perkin–Elmer 421 spectrophotometer.

Molecular Weights.—A Mechrolab vapor pressure osmometer, Model 301A, was used. The measurements were made at 37°. Benzil was used as a standard to prepare calibration curves. The chlorophyll solutions were prepared by weight; generally about 1 mg. of material was used for each measurement. Although the solutions were prepared on a molal basis, the results are presented in terms of molarity, since the calibration solutions were prepared on a molar basis. On the basis of some experimental observations, we believe the error from this source to be less than the other errors inherent in the procedure. The spread of the data can be inferred from the results in Table I.

Acknowledgment.—Certain aspects of this investigation benefited greatly from the preparative work of Mr. Walter A. Svec.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF CHICAGO, CHICAGO 37, ILL., AND THE CHEMISTRY DIVISION, ARGONNE NATIONAL LABORATORY, ARGONNE, ILL.]

Nuclear Magnetic Resonance Spectra and Molecular Association of Chlorophylls *a* and *b*, Methyl Chlorophyllides, Pheophytins, and Methyl Pheophorbides[1]

BY G. L. CLOSS,[2] J. J. KATZ, F. C. PENNINGTON,[3] M. R. THOMAS, AND H. H. STRAIN

RECEIVED JULY 2, 1963

The n.m.r. spectra of methyl pheophorbides, pheophytins, methyl chlorophyllides, and chlorophylls of both *a*- and *b*-series are reported and resonances are assigned to the individual substituent protons. The spectra are extraordinarily sensitive to concentration and to the degree of aggregation of the compounds. The magnesium-free derivatives form weakly bonded aggregates, which may arise from π–π interactions, whereas the intermolecular forces involved in aggregate formation by the magnesium-containing compounds appear to be much stronger. Specific carbonyl–magnesium interaction is assumed to be responsible for the latter aggregation. Coordination of methanol with the magnesium atom is shown to be the cause of dissociation of the aggregates in methanol-containing solutions, and the dependence of the spectra on the methanol concentration is interpreted on the basis of competitive coordination.

Nuclear magnetic resonance (n.m.r.) spectroscopy has shown itself a powerful tool in the study of molecular structures and interactions. It is natural to consider the application of n.m.r. methods to the many still-unresolved problems of chlorophyll chemistry and function. Our interest derives in part from the availability of deuteriochlorophylls, in which all of the hydrogen is replaced by deuterium.[4] These substances may have special utility in n.m.r. studies because protons introduced into the chlorophyll molecule will have exceptional visibility. In this communication we wish to present and interpret the n.m.r. spectra of chlorophyll *a* (Ia) and *b* (Ib) and to describe in detail the unusual solvent and concentration dependence of these spectra. In the accompanying publication,[5] reinterpretation of existing data and evaluation of newly collected information on the infrared spectra of chlorophyll and its derivatives provided strong evidence for molecular association in solvents of low basicity. The n.m.r. data reported here not only are consistent with the association hypothesis, but also reveal some of the essential structural features of the aggregates. To assess the influence of the magnesium and the phytyl group on the n.m.r. spectra, the methyl chlorophyllides (IIa and IIb), pheophytins (IIIa and IIIb), and the methyl pheophorbides (IVa and IVb) of both the *a*- and *b*-series were included in this study. The nomenclature, structural formulas, and proton designations are shown in Table I.

Results

Chemical shift values for the proton resonances are reported in cycles per second (c.p.s.) from tetramethylsilane as internal standard with line positions at lower field designated as positive. Although some of the

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission. Part of the research was supported by National Institute of Health Grant USPHS-RG5841.

(2) A. P. Sloan Foundation Fellow.

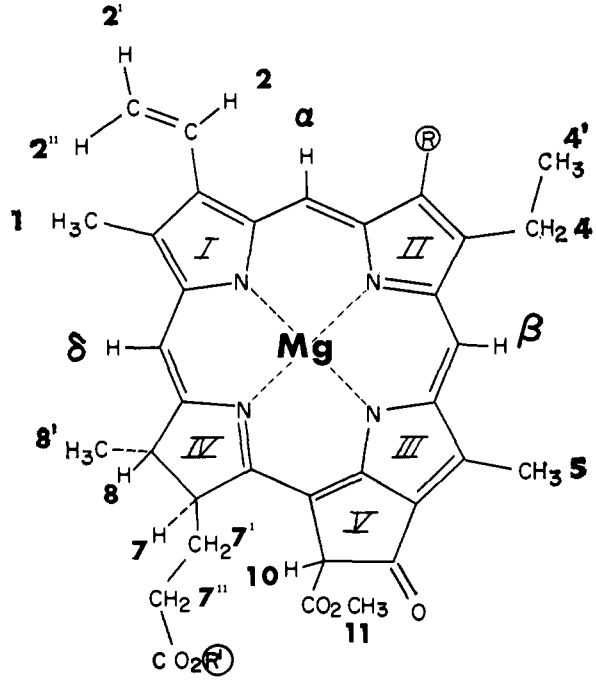
(3) Resident Research Associate, Coe College, 1962–1963, under the Associated Colleges of the Midwest-Argonne Semester Program.

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

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

TABLE I

NOMENCLATURE AND PROTON DESIGNATIONS



	Compound	Mg present[a]	R	Proton no. of R	R′	Proton no. of R′	Proton no. of >NH
Ia	Chlorophyll *a*	+	CH_3	3a	Phytyl[b]	..	...
Ib	Chlorophyll *b*	+	CHO	3b	Phytyl[b]	..	...
IIa	Methyl chlorophyllide *a*	+	CH_3	3a	CH_3	12	...
IIb	Methyl chlorophyllide *b*	+	CHO	3b	CH_3	12	...
IIIa	Pheophytin *a*	−	CH_3	3a	Phytyl[b]	..	13, 14
IIIb	Pheophytin *b*	−	CHO	3b	Phytyl[b]	..	13, 14
IVa	Methyl pheophorbide *a*	−	CH_3	3a	CH_3	12	13, 14
IVb	Methyl pheophorbide *b*	−	CHO	3b	CH_3	12	13, 14

[a] +, magnesium present; −, magnesium replaced by 2H. [b] The phytyl hydrogen atoms are not numbered.

spectra have been determined at 40-Mc. radiofrequency, all values reported here have been converted to the corresponding to 60-Mc. numbers. Low signal-to-noise ratios, encountered with dilute solutions of compounds of limited solubility, and line broadening arising from relatively high viscosities of the more concentrated solutions of other substances limited the accuracy of many line position measurements to ±1 c.p.s. With solutions containing aggregates, lines were broadened to an even greater extent.

Assignments of resonance values to the individual protons of the pigment molecules were made, in most cases, from considerations of the relative degree of deshielding caused by the ring current effect of the macrocyclic, fully conjugated ring, common to all chlorophyll derivatives. The deshielding of the substituent protons is a function of the coordinates in reference to the center and the plane of the ring, and of the magnitude of the ring current. Estimates of these parameters, together with considerations of neighboring group anisotropies, served as useful guides in establishing a qualitative sequence of anticipated chemical shifts into which the observed values could be fitted with a high degree of certainty.[6]

(6) For chemical shift assignments in the n.m.r. spectra of related porphins and chlorins, see: (a) R. J. Abraham, A. H. Jackson, and G. W. Kenner *J. Chem. Soc.*, 3468 (1961); (b) R. J. Abraham, *Mol. Phys.*, **4**, 145 (1961); (c) R. J. Abraham, A. H. Jackson, G. W. Kenner, and D. Warburton, *J. Chem. Soc.*, 853 (1963); (d) E. D. Becker and R. B. Bradley, *J. Chem. Phys.*, **31**, 1431 (1959); (e) J. Ellis, A. H. Jackson, G. W. Kenner, and J. Lee, *Tetrahedron Letters*, **2**, 23 (1960); (f) E. D. Becker, R. B. Bradley, and C. J. Watson, *J. Am. Chem. Soc.*, **83**, 3743 (1961); (g) W. S. Caughey and W. S. Koski, *Biochem.*, **1**, 923 (1962).

It will be advantageous to begin with the assignments of the resonances to the methyl pheophorbides (IVa, IVb), then to proceed to the pheophytins (IIIa, IIIb), and, finally, to discuss the magnesium complexes, methyl chlorophyllides (IIa, IIb), and chlorophylls (Ia, Ib).

Methyl Pheophorbides (IVa, IVb).—The spectra of methyl pheophorbide *a* (IVa) and methyl pheophorbide *b* (IVb) in deuteriochloroform solutions at the concentrations in moles per liter indicated in the legend, are presented in Fig. 1a and 1b, respectively. Table II summarizes the chemical shift assignments of particular protons as designated in Table I. The three low-field lines in the spectrum of IVa clearly belong to the methine bridge protons, α, β, and δ. The δ-proton, being flanked only by one pyrrole ring, must be the most shielded and is assigned the line at 510 c.p.s. No unambiguous distinction can be made between the α- and β-proton resonances. The tentative assignment of the β-proton to the lowest field line follows a suggestion made by Caughey and Koski[6] who reasoned that the slightly weaker shielding of the corresponding proton in chlorin e_6 trimethyl ester arises from the anisotropy of the carbonyl group at position 6. A somewhat less ambiguous analysis of the low-field resonances can be made for methyl pheophorbide *b* (IVb), in which the α-position is expected to be considerably deshielded by the paramagnetic effect of the aldehyde carbonyl. These considerations lead to the sequence of increasing shielding: 3b, α, β, δ in IVb, *vs.* β, α, δ in IVa.

The vinyl groups both in IVa and IVb are recognizable as ABX spin-splitting patterns. Analysis of the

TABLE II

CHEMICAL SHIFTS (C.P.S. FROM TETRAMETHYLSILANE) IN PHEOPHYTINS (III) AND METHYL PHEOPHORBIDES (IV)[a] IN $CDCl_3$

Proton	IIIa	IIIb	IVa	IVb
3b	...	635	...	635
α	548	585	549	585
β	559	535	559	533
δ	510	508	510	508
2	470	466	471	465
2′	363	367	362	365
2″	368	371	367	370
10	374	373	373	373
7			248[b]	249[b]
8			264[b]	267[b]
1	200	198	199	197
3a	184	...	183	...
5	218	209	217	208
7′				
7″	~148	~147	~147	~150
4			209[b]	202[b]
4′			96	89
8′			109	113
11	233	237	233	237
12	...	...	214	217
13			30	5
14	−110	−127	−105	−129
Phytyl[c]	308	310	...	...
Phytyl[d]	267	269	...	...

[a] Concentrations of solutions in deuteriochloroform (mole/liter): IIIa and IVa, 0.060; IIIb and IVb, 0.080. Values for other concentrations are available on request. [b] Values obtained from double resonance. [c] Olefinic hydrogen. [d] Oxygen bonded methylene.

spin–spin coupling by standard procedures[7] yields the chemical shifts as recorded in Table II and the coupling constants for $|J_{2,2'}|$, $|J_{2,2''}|$, and $|J_{2',2''}|$ of 18.7, 10.9, and 1.6 c.p.s. for IVa, and 18.3, 11.2, and 1.6 c.p.s. for IVb, respectively. The well documented observation of $|J_{trans}| > |J_{cis}|$ for vicinal couplings in olefins enables one to distinguish between the chemical shifts of the terminal vinyl protons. The sharp line (373 c.p.s.), coinciding in both spectra with the AB part of the vinyl resonance, must be attributed to the proton at position 10, which, in addition to being deshielded by the ring current effect, will be affected by the paramagnetic contributions of the two adjacent carbonyl groups. The low intensity multiplets centered around 260 c.p.s. in both spectra must arise from the protons 7 and 8. Direct evidence for this assignment will be presented below. The two methyl groups, 8′ and 4′, removed by one carbon atom from the conjugated ring, should give rise to signals at the high-field side of the spectrum. This assignment is made unambiguous by virtue of the spin coupling of 8′ and 4′ with vicinal protons, leading to doublets and triplets at 109 and 113, and 96 and 89 c.p.s., respectively. The poorly resolved multiplets in the vicinity of 150 c.p.s. arise from resonances of the methylene groups in the propionic acid side chains. The chemical shifts of 7′ and 7″ are very nearly the same because of the larger deshielding influence of the macrocyclic ring on the former group and the proximity of the carbonyl function to the latter.

Assignments of the intense lines located between 180 and 240 c.p.s. in both spectra to individual methyl groups can be made with a high degree of certainty. As the ester methyl (12) of IVa and IVb at the propionic acid side chain should exhibit a normal shielding value, and as this methyl is absent in the pheophytins (IIIa and IIIb), one must assign the peaks at 214 and 217

(7) J. A. Pople, H. J. Bernstein, and W. J. Schneider, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., Inc., New York, N. Y., 1959, p. 132.

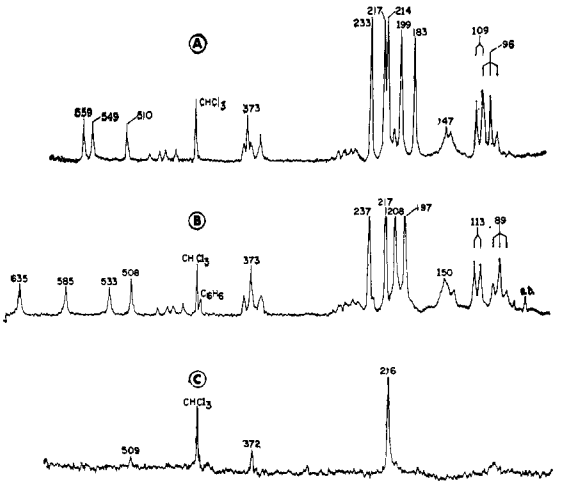


Fig. 1.—N.m.r. spectra in $CDCl_3$ of: A, methyl pheophorbide *a* (IVa), 0.060 mole/l.; B, methyl pheophorbide *b* (IVb), 0.080 mole/l.; C, methyl-(CH_3) deuteriopheophorbide *b*.

c.p.s. to methyl proton resonances in IVa and IVb, respectively. Further confirmation for this assignment is obtained from the spectrum of methyl-(CH_3) deuteriopheophorbide *b* (Fig. 1C). This compound was prepared from deuteriochlorophyll *b*[8] through treatment with aqueous hydrochloric acid, followed by esterification with methanol. The reaction conditions lead to exchange at the δ- and 10-position (signals at 509 and 372 c.p.s., respectively) and introduce a methyl group at the propionic ester chain. The strong signal at 216 c.p.s. arises from this ester methyl group. The distinction between the peaks at 214 and 217 c.p.s. in IVa (Fig. 1A) is based on the dilution shifts, to be discussed below, which indicate that the 214 c.p.s. signal arises from the ester methyl (12). The ester methyl of the β-keto acid function (11) should be somewhat less shielded and therefore will be the origin of the signals at 233 and 237 c.p.s. in IVa and IVb, respectively. The remaining lines must arise from the ring methyl groups 1, 3, and 5. The last is the least shielded because of the strong paramagnetic effect of the ketone carbonyl and is assigned to the peaks at 217 c.p.s. in IVa and 208 c.p.s. in IVb. This leaves the signal at 197 c.p.s. for methyl group 1 in IVb. Since this position should be shielded similarly in both IVa and IVb, the line at 199 c.p.s. in IVa is attributed to methyl group 1 as well, leaving the signal at 183 c.p.s. for methyl 3a. This assignment is highly compatible with the observed dilution shifts described below.

The chemical shift values for the remaining protons, 4, 7, and 8, were obtained from double resonance experiments.[9] Variation of the decoupling frequency by small increments while observing the collapse of the triplet structure of the 4′-methyl group resonance at 96 c.p.s. in IVa (Fig. 2b) led to the chemical shift value of 209 c.p.s. for methylene group 4. When the 209-c.p.s. region was observed using the same modulation frequency, a line was visible there although it partially overlapped with the methyl resonance at 114 c.p.s. Once the position of the methylene group 4 had been established, it was possible to attribute the low intensity peak at 206 c.p.s. to the high-field inner line of

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

(9) For a recent review on double resonance techniques see: J. D. Baldeschwieler and E. W. Randall, *Chem. Rev.*, **63**, 81 (1963). The specific technique adopted here is in close analogy to the method reported by S. L. Manatt and D. D. Elleman, *J. Am. Chem. Soc.*, **83**, 4095 (1961); see Experimental. Chemical shifts were calculated from the audiofrequency (Ω) and the radiofrequency amplitude (H_1) using the relationship $\delta = [\Omega^2 - (\gamma H_1/2\pi)^2]^{1/2}$.

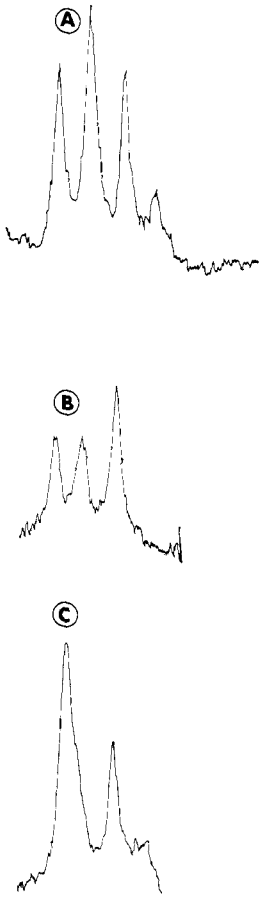


Fig. 2.—Proton resonance of groups 4′ and 8′ in methyl pheophorbide *a* (IVa): A, undecoupled spectrum; B, 4′ decoupled from 4; C, 8′ decoupled from 8.

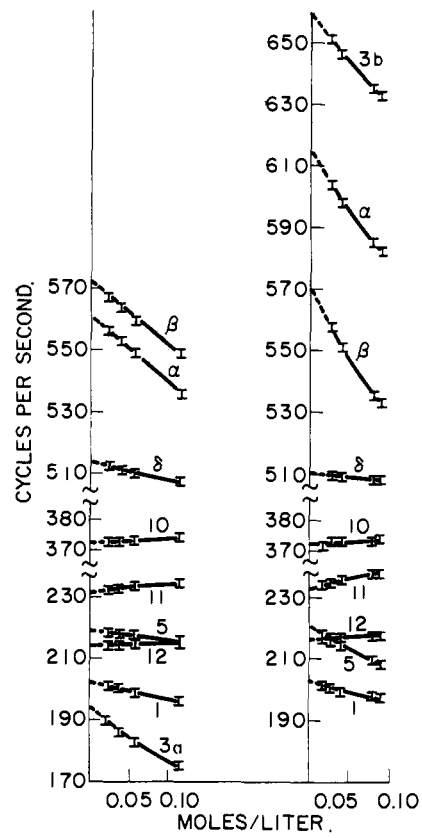


Fig. 3.—Dilution shifts in $CDCl_3$ of methyl pheophorbide *a* (IVa) (left) and methyl pheophorbide *b* (IVb) (right).

the expected quadruplet of group 4 in the undecoupled spectrum. More significant is the determination of the chemical shift parameters for protons 7 and 8 in IVa. Decoupling of proton 8 from methyl group 8′ converted the high-field doublet at 109 c.p.s. to a singlet as shown in Fig. 2C. Using the same modulation frequency the signal of proton 8 became visible as a somewhat broadened peak at 264 c.p.s. It was not possible to resolve this signal into the expected doublet. However, the observed line-width indicated a maximum possible coupling constant between protons 7 and 8 of 2.8 c.p.s. Similarly, the position of the resonance of proton 7 was found to be at 248 c.p.s. by irradiating the multiplet at 147 c.p.s. Again, no splitting into a doublet was observable, but the line width was similar to that of the decoupled proton 8. Decoupling experiments on compounds IVa and IVb gave the chemical shifts for the corresponding protons listed in Table II.

The imine hydrogens (13 and 14) in both methyl pheophorbides gave signals at very high fields in agreement with previous observations on similar compounds.[6] It is remarkable, however, that the two protons cause two distinguishable signals in both IVa and IVb. The instantaneous and reversible disappearance of both peaks on shaking a $CDCl_3$ solution with deuterium oxide, and correspondence of the areas under each signal to one proton only, leaves little doubt about this assignment. The nonequivalence of the imine hydrogen signals indicates that a scrambling process which exchanges the protons with each other must be slow relative to the frequency separating the two peaks. Tentatively, the signal at lower field is assigned to the hydrogen that is mainly bonded to the nitrogen of ring III. The electron-withdrawing ability of the carbonyl function on ring V should render this nitrogen the least basic, and, consequently, the hydrogen attached to it the least shielded.[10]

Pheophytins (IIIa, b).—The spectra of pheophytin *a* (IIIa) and pheophytin *b* (IIIb), when measured with solutions of concentrations similar to those used with the pheophorbides, show the same general patterns as those of the pheophorbides. The information obtainable from the spectra of the pheophytins, however, is limited by the presence of the resonances of the numerous protons of the aliphatic phytyl group, which complicate certain portions of the spectrum. At the low-field side, all proton signals were identified and have chemical shift parameters which correspond closely to those of the pheophorbides. The assignments were carried out using the same principles discussed above and are listed in Table II. The absence of methyl resonances at 214 and 217 c.p.s. in the spectra of IIIa and IIIb, respectively, confirms the assignment of these peaks in the pheophorbide spectra to the propionic side-chain methyl group. The corresponding oxygen bonded methylene group of the phytyl chain causes a doublet at 267 and 269 c.p.s. in IIIa and IIIb, respectively. The olefinic proton, coupled to this group with a coupling constant of 6.9 c.p.s., shows the expected triplet at 308 and 310 c.p.s., respectively. The methyl resonances of the 4′ and 8′ groups are obscured by the intense signals from the phytyl methyl and methylene groups.[11]

Dilution Shifts.—The spectra of the methyl pheophorbides IVa and IVb and of the pheophytins IIIa and IIIb show a very strong dependence on concen-

(10) It should be pointed out that the argument presented here is based on the assumption that both imine hydrogens are equally exposed to the diamagnetic effect of the ring current. Because of the symmetry of the chlorin system this assumption may be valid if the hydrogens are mainly located at ring I and III as is usually assumed.

(11) For a copy of the n.m.r. spectrum of phytol see: "High Resolution NMR Spectra Catalog," Varian Associates, Palo Alto, Calif., 1962, spectrum 346.

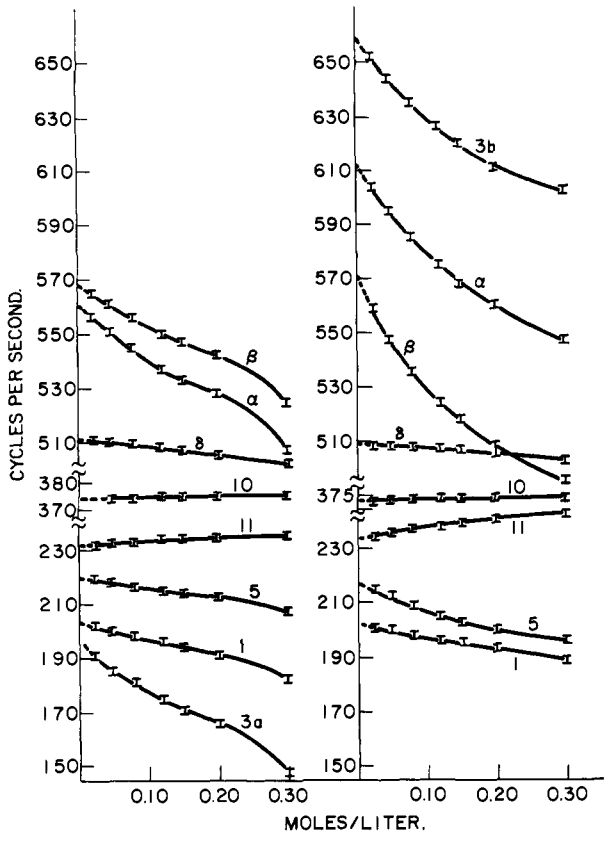


Fig. 4.—Dilution shifts in $CDCl_3$ of pheophytin *a* (IIIa) (left) and pheophytin *b* (IIIb) (right).

tration. The dilution shifts were measured against the *internal* standard tetramethylsilane. Because of the limited solubility of the methyl pheophorbides, the concentration ranges within which dilution shifts could be studied was quite limited. A greater concentration range was available with the pheophytins. Figures 3 and 4 present the chemical shift values of some of the more prominent groups as a function of concentration. As is evident from the figures, the magnitude of the dilution shifts are not the same for all protons. Particularly large paramagnetic shifts upon dilution are observed for the α- and β-methine protons in both the *a*- and *b*-series, as well as for the aldehyde (3b) proton in IIIb and IVb. The methyl groups bonded to the conjugated ring also undergo paramagnetic shifts upon dilution in all four compounds. Smaller displacements to lower field are observed for the δ-proton and, although not included in the figure, for the 4′-methyl group. In the spectrum of the methyl pheophorbide *a* (IVa) the signal of the latter group moved from 92 to 99 c.p.s. within the concentration range studied, and from 89 to 102 c.p.s. in methyl pheophorbide *b* (IVb). The concentration dependence of the vinyl proton resonances were more difficult to determine because of the low signal-to-noise ratios. But, the 2-proton, in particular, undergoes a paramagnetic dilution shift in all four compounds. The 8′-methyl group in IVa and IVb shows no displacement upon dilution larger than the experimental error, whereas diamagnetic shifts upon dilution were observed for the resonances of the 10-proton and the two ester methyl groups (11 and 12).

The dilution shifts of the corresponding pheophorbides and pheophytins are very nearly identical, and the shifts of the *a*- and *b*-series differ significantly only for the α- and β-protons of the methine bridges. These observations provide additional support for the resonance assignments of certain protons. For example,

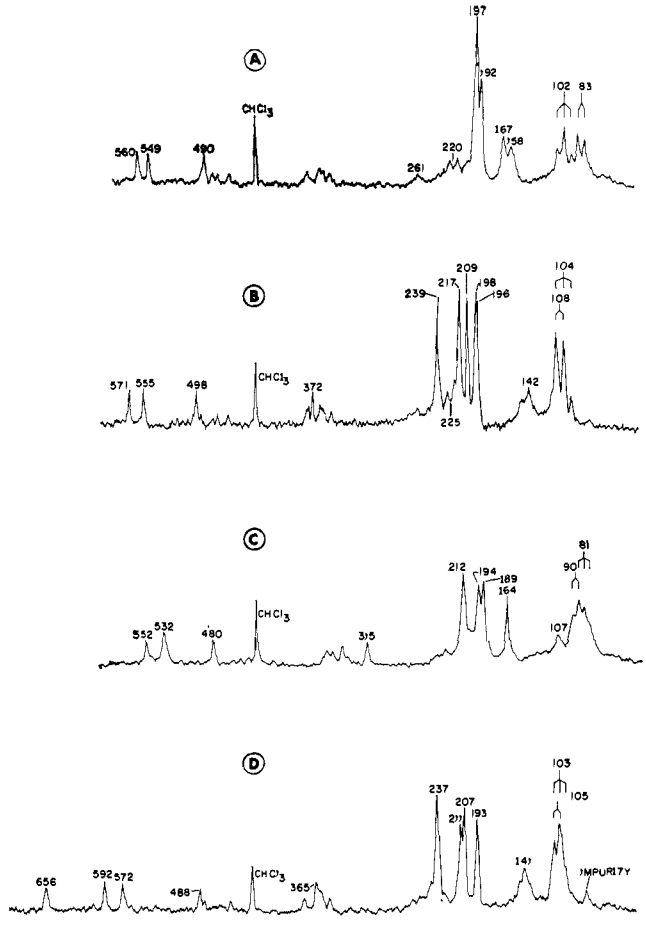


Fig. 5.—N.m.r. spectra of: A, methyl chlorophyllide *a* (IIa), in $CDCl_3$, 0.08 mole/l.; B, methyl chlorophyllide *a* (IIa) in $CDCl_3$–CD_3OD, 0.08 mole/l., approximately 3% CD_3OD; C, methyl chlorophyllide *b* (IIb) in $CDCl_3$–CD_3OD, 0.10 mole/l.; D, methyl chlorophyllide *b* (IIb), 0.10 mole/l., approximately 3% CD_3OD.

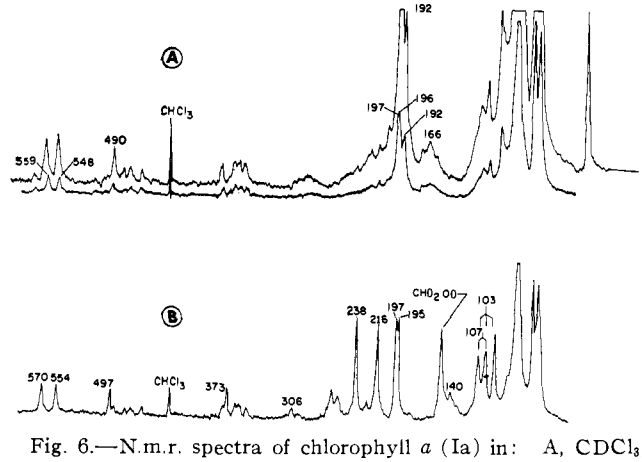


Fig. 6.—N.m.r. spectra of chlorophyll *a* (Ia) in: A, $CDCl_3$, 0.09 mole/l.; B, in $CDCl_3$–CD_3OD, 0.09 mole/l., approximately 3% CD_3OD.

the curve of the dilution dependence designated as 3a in Fig. 3 and 4, and the absence of an analogous curve for the *b*-series provides strong support for attributing this signal to the 3a-methyl group, present in the *a*-series but absent in the *b*-series. Furthermore, the assignment of the resonance of the propionic ester methyl (12) in the pheophorbide spectra is further strengthened by the absence of a line with similar dilution dependence in the pheophytin spectra. Finally, the previously mentioned distinction between α- and β-protons in the *a*-series, which was based upon the greater proximity of

the β-proton to the deshielding influence of the ketone carbonyl, becomes more meaningful when the chemical shifts are extrapolated to infinite dilution, and, as is observed here, the relative chemical shifts are maintained.

Methyl Chlorophyllides (IIa and IIb) and Chlorophylls (Ia and Ib).—Figure 5 shows the spectra of methyl chlorophyllide *a* (IIa) and methyl chlorophyllide *b* (IIb) in deuteriochloroform with and without added methanol–CD_3OD. The corresponding spectra of the chlorophylls *a* (Ia) and *b* (Ib) are shown in Fig. 6 and 7. The spectra of all four compounds respond to a

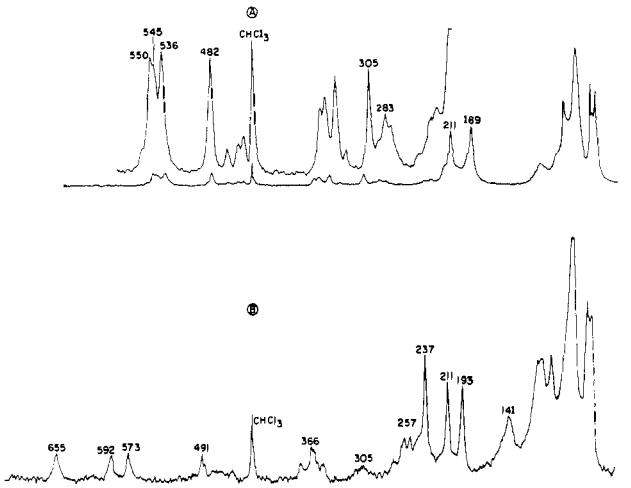


Fig. 7.—N.m.r. spectra of chlorophyll *b* (Ib) in: A, $CDCl_3$, 0.12 mole/l.; B, in $CDCl_3$–CD_3OD, 0.12 mole/l., approximately 3% CD_3OD.

remarkable degree to the addition of small amounts of methanol. In contrast, the same concentrations of methanol-d_4 have almost no effect on the spectra of the methyl pheophorbides. In the latter, none of the resonance lines shift by more than 3 c.p.s. compared to the methanol-free solutions.

As will be justified in the Discussion, the spectra of the methanol-containing solutions are more closely related to the monomeric chlorophyllides and chlorophylls and are therefore better suited for the initial line assignment. A comparison of the spectra of the magnesium-free compounds (IIIa, IIIb, IVa, and IVb) with those of the magnesium derivatives in $CDCl_3$–CD_3OD solutions shows that the magnesium produces only minor changes in the chemical shifts of substituent protons.

The line assignments as given in Table III are made on the basis of the same considerations as discussed for the methyl pheophorbides. Again, no absolute distinction can be made between the α- and β-methine protons in the spectra of Ia and IIa and the assignment adopted is based upon the same reasoning as outlined above. Furthermore, the proton resonances of the 1- and 3a-methyl groups are so closely spaced that their assignment to either of the methyl groups becomes somewhat arbitrary. The coupling constants of the vinyl groups $|J_{2,2''}|$, 18.1 and 18.0; $|J_{2,2'}|$, 11.2 and 11.6; $|J_{2',2''}|$, 1.5 and 1.7 c.p.s. for Ia and IIa, respectively, are, within experimental error, the same as those of the magnesium-free derivatives. The chemical shifts of the methylene protons 4′ in Ia and IIa were determined by double resonance of the same type as described above. Attempts to measure the chemical shift of the 7- and 8-protons *via* double resonance were unsuccessful because the signal-to-noise ratio was too low.

TABLE III

CHEMICAL SHIFTS (C.P.S. FROM TETRAMETHYLSILANE) IN CHLOROPHYLLS (I) AND METHYL CHLOROPHYLLIDES (II) DISSOLVED IN $CDCl_3$(A) and $CDCl_3$ PLUS METHANOL-d_4 (B)[a,b]

Proton	Ia		Ib		IIa		IIb	
	A	B	A	B	A	B	A	B
3b	...	...	550	655	...	...	552	656
α	548	554	536	592	549	555	532	592
β	559	570	545	573	560	571	532	572
δ	490	497	482	491	490	498	480	488
2	476	475	447	471	477	476	446	471
2′	359	358	350	359	360	358	347	358
2″	370	368	354	369	371	369	350	369
10		373	305	366	261	372	315	365
1	196	197	189	193	197	198	189	193
3a	192	195	...	...	192	196	...	...
5	166	216	189	211	167	217	194	211
7′								
7″		~140		~141		~142	~107	~141
4		225			220	225		
4′		103			102	104	81	103
8′		107			83	108	90	105
11	197	238	211	237	197	239	212	237
12	...	...	...	...	158	209	164	207
Phytyl[c]		306	283	305	...	...	...	...
Phytyl[d]		258		257	...	...	...	...

[a] Concentrations of compounds (mole/liter): Ia, 0.09; Ib, 0.12; IIa, 0.08; IIb, 0.10. Values for other concentrations are available on request. [b] Methanol concentration approximately 3% by weight. [c] Olefinic hydrogen. [d] Oxygen-bonded methylene.

Solvent and Concentration Effects.—As shown by Fig. 5 to 7, the addition of methanol-d_4 to chloroform solutions of the chlorophylls and methyl chlorophyllides results in marked changes in the chemical shift parameters. In the *a*-series these are particularly noticeable in the methyl resonance region and in the behavior of the C-10 proton signal. Indeed, the C-10 proton can scarcely be seen in the spectra of the alcohol-free solutions. Similar changes are observable in the *b*-series. Here, in alcohol-free media the aldehyde proton, 3b, has completely lost its identity. As a basis for correlation, a series of spectra was measured in which incremental additions of methanol-d_4 were made to solutions of chlorophylls and methyl chlorophyllides in chloroform under conditions such that the concentration changes of the pigments were kept at a minimum (not larger than 7%).

The results of these measurements for Ia and IIa are shown in Fig. 8 and 9. The largest paramagnetic shifts with increasing methanol concentration were observed for the signal of the C-10 proton in the spectra of both compounds. The methyl group resonances of protons 5 and 11 in Ia and IIa and of protons 12 in IIa also show large paramagnetic shifts. Relatively minor changes occur in the positions of the methine bridge protons while the resonances of the methyl groups 1 and 3a seem to be very insensitive to the medium. Although not displayed on the graphs, some paramagnetic shifts with increasing mole ratios of CD_3OD are observed for the resonances of the 8′-methyl group and to a very small extent for the 4-methylene protons. The latter shift is only observable in IIa. Although no accurate numbers can be given for the solvent shifts of the 7′- and 7″-methylene protons, a paramagnetic shift of the order of 30 to 40 c.p.s. is apparent, since these signals are obscured in the methanol-free solutions by the multiplets of groups 4′ and 8′. Practically no solvent changes are observed for the chemical shifts of the vinyl protons and the 4′-methyl group. Changes in the positions of the signals of protons 7 and 8 could not be determined because of low signal-to-noise ratios.

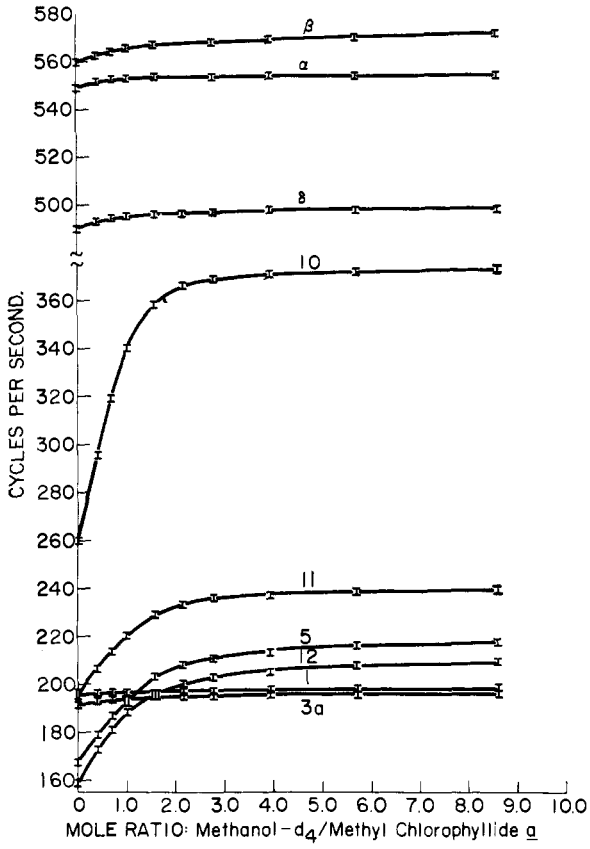


Fig. 8.—Methanol titration curve in $CDCl_3$ of methyl chlorophyllide *a* (IIa), 0.09 mole/l.

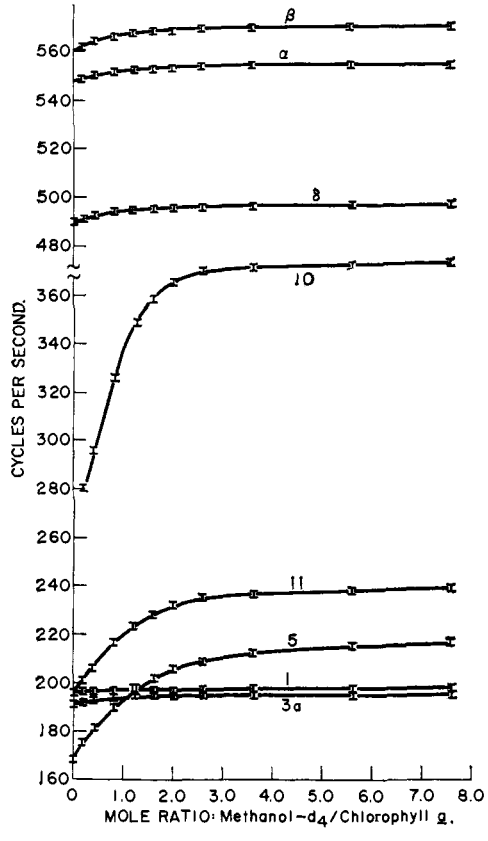


Fig. 9.—Methanol titration curve in $CDCl_3$ of chlorophyll *a* (Ia), 0.15 mole/l.

On the basis of these experiments, the signal assignments are straight forward (Table III).

Figure 10 shows the effect of methanol-d_4 addition on

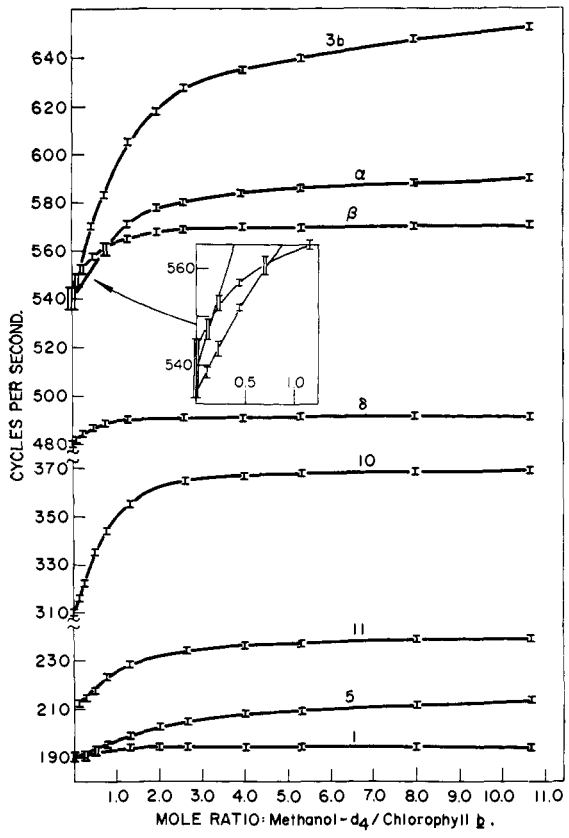


Fig. 10.—Methanol titration curve in $CDCl_3$ of chlorophyll *b* (Ib), 0.15 mole/l.

the chlorophyll *b* resonances. Here the largest paramagnetic shift is found for the aldehyde (3b) proton. Sizable low-field shifts are also observed for the α- and β-methine bridge protons as well as for the C-10 hydrogen. At low methanol concentrations the α-, β-, and 3b-protons show very similar solvent dependences; substantial line broadening prevents accurate position measurements. The curves for this region are therefore somewhat ambiguous, and in particular, the existence of a crossover of chemical shifts of the α- and β-protons at mole ratio 0.7 is uncertain. Consequently, the curves have been drawn through the measured points to give the maximum continuity of the chemical shifts. The relative magnitudes of the slopes of the curves in this region corresponds well with those at higher mole ratios. Other paramagnetic shifts, not included in the figure, are observed for the signals of the vinyl protons, the methylene protons 7′ and 7″, and of the oxygen-bonded methylene group and the olefinic proton of the phytyl chain.

The spectrum of methyl chlorophyllide *b* was measured only at mole ratios of CH_3OH to IIb of 0 and 8 (Table III). The same general methanol dependence of the spectrum was noted, and additional values were obtained for the shifts of methyl groups 4′ and 8′, both of which are obscured in the spectrum of Ib.

It should be noted that the spectra of compounds Ia, Ib, IIa, and IIb are also sensitive to small amounts of water. Although no quantitative study has yet been made, it appears that the chemical shift changes in $CDCl_3$ with water are similar to those caused by methanol.

The methanol dependence of the chemical shift parameters of the spectra of the magnesium derivatives was made more self-consistent by determining the dilution shifts both in methanol-containing and in methanol-free solutions. In contrast to the findings in the

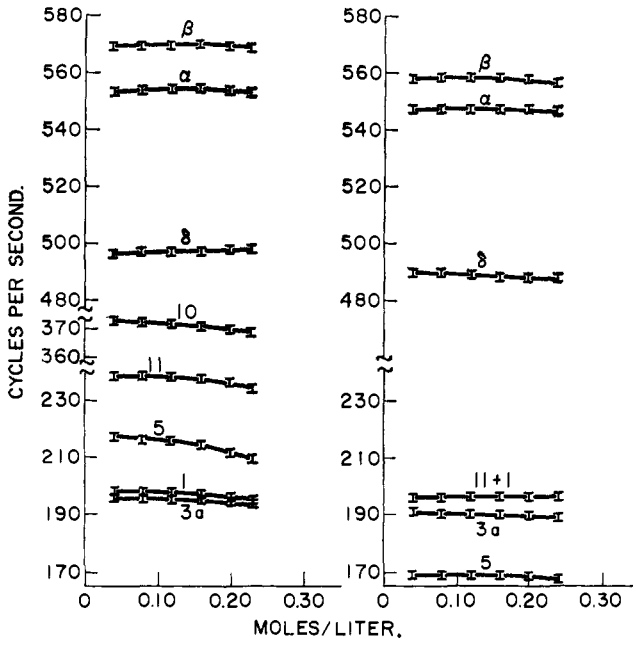


Fig. 11.—Dilution shifts of chlorophyll *a* (Ia) in $CDCl_3$ (right) and $CDCl_3$–3.2% w./w. CD_3OD (left).

magnesium-free series, the dilution shifts for methyl chlorophyllides *a* and chlorophyll *a* in both solvent systems are very small. The results of the measurements for some prominent groups of Ia are plotted in Fig. 11. Within the much smaller concentration range available for IIa, almost no dilution shift was detectable in either solvent system.

The dilution behavior of the methanol-containing and methanol-free solution of chlorophyll *b* is displayed in Fig. 12. In contrast to chlorophyll *a*, relatively large dilution shifts are noticeable in both solutions. The relatively complex behavior of the α-, β-, and 3b-protons in the methanol-free solutions is most apparent. Because the signals of these protons are broadened and are partially overlapping, the interpretation of the concentration dependence of the individual protons is not without ambiguities. Therefore, curves have been drawn through the measured points to give the maximum continuity in chemical shifts. The choice made here, which assigns the α-proton to signal at highest field at concentrations less than 0.23 *M* is based upon the methanol dependence of the signals of the α- and β-protons. As shown in Fig. 10, the α-proton appears to be more shielded in methanol-free solutions than the β-proton.

Discussion

Stereochemical Implications.—The observed chemical shifts of the substituent protons of the magnesium-free derivatives III and IV and of the magnesium chelates I and II in methanol-containing solutions, are generally in line with published data of similar porphin and chlorin compounds.[6] Of special interest are the chemical shifts and spin coupling constants of the 7- and 8-protons in methyl pheophorbides *a* and *b*. The significance of these values is connected with the problem of the stereochemistry of chlorophyll derivatives. The degradation studies of Linstead[12] provided evidence for a *trans* relationship of the propionic ester chain and the 8-methyl group. The observed maximum of 2.8 c.p.s. for spin coupling between the 7- and 8-proton confirms this assignment for IVa and IVb, since this limit includes the generally observed coupling

(12) R. P. Linstead, U. Eisner, G. E. Ficken, and R. B. Johns, Special Publication No. 3, The Chemical Society, London, 1955, p. 83.

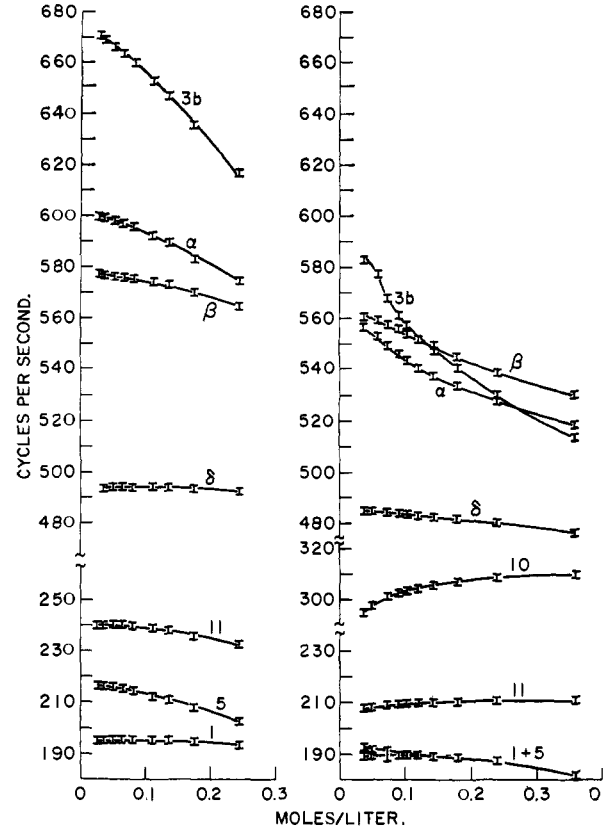


Fig. 12.—Dilution shifts of chlorophyll *b* (Ib) in $CDCl_3$ (right) and $CDCl_3$–3.2% CD_3OD (w./v.) (left).

constant of *trans* vicinal protons on five-membered rings, but is too small for a *cis* relationship.[13] However, no experimental evidence is available regarding the configuration of the C-10 carbomethoxy group in chlorophyll and its derivatives. It appears that the *trans* relationship of this function with the propionic ester chain is energetically favored for steric reasons. Since equilibration of the β-keto ester group can occur through enolization (*vide infra*) it can be argued that this *trans* configuration most probably represents the stereochemistry of chlorophyll. However, experimental evidence in support of this argument is certainly desirable.[14] Neglecting the influence of substituents (including ring V) the π-system of the chlorin skeleton has a plane of symmetry normal to the plane of the ring and bisecting rings II and IV. This symmetry leads to identical contributions of the ring current to the magnetic environment of the 7- and 8-protons. Therefore, it is necessary to attribute any observed chemical shift differences between these protons to neighboring group anisotropies. Because the 8′-methyl and the propionic ester chain can be expected to provide similar shielding at both positions, one is left with the assymmetry created by ring V and its substituents as main contributing factors to the differences in magnetic environment of the 7- and 8-protons. The observed higher shielding of proton 7 by 16 and 18 c.p.s. in IVa and IVb, respectively, appears to support the above-mentioned *trans* configuration. Molecular

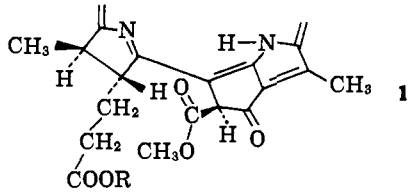
(13) A value of 6–9 c.p.s. would be expected for the coupling constant of the *cis* oriented protons; see M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).

(14) The total synthesis of chlorophyll *a*[15] does not constitute such evidence, since closure of ring V was accomplished by a Claisen condensation proceeding with unknown stereochemistry.[16] At best, it can be expected that the most stable isomer is formed, since equilibration under the reaction conditions is highly probable.

(15) R. B. Woodward, *et al.*, *J. Am. Chem. Soc.*, **82**, 3800 (1960).

(16) A. Stoll and E. Wiedemann, *Fortschr. Chem. Forsch.*, **2** (3), 538 (1952).

models indicate that conformation 1 of the carbomethoxy group has the least nonbonded interactions.[17]



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In this conformation, in which the carbonyl oxygen points toward the ring, the 7-hydrogen is located in the diamagnetic cone of the carbonyl group if their mutual orientation is *cis*. Although this argument cannot be regarded as experimental *proof* for the stereochemistry, it certainly strongly supports the assignment.

In this connection renewed attention should be drawn to the chlorophyll *a*–chlorophyll *a'* relationship. It has been suggested that these compounds are epimeric at C-10.[18] Although no assignment of configuration has been made, the observations reported here are consistent with this proposal.

The possibility of keto–enol tautomerism in chlorophyll derivatives is of interest in connection with previous interpretations of the infrared spectra of these compounds.[19] The n.m.r. results reported here do not provide evidence for the presence of any significant enol fraction in compounds I–IV under the conditions studied. This statement is supported by the fact that hydroxylic protons undergo instantaneous deuterium exchange when treated with deuterated alcohols. If the signal which has been attributed to the C-10 proton (or any other proton for that matter) were actually arising from an enol hydroxyl hydrogen, it should disappear upon shaking the pigment solution with deuterium oxide or upon adding methanol-d_4. In fact, in all the compounds studied, even a large excess of methanol-d_4 caused only relatively slow decrease of signal intensity.[20] Furthermore, the chemical shift of this signal is different from that expected for an enol hydrogen. Naturally these results do not exclude the presence of small amounts of enol in a relatively slowly established equilibrium with the keto form.

Dilution of Shifts of Pheophytin and Methyl Pheophorbides.—Most remarkable are the observed highly selective dilution shifts of some signals in the spectra of the pheophytins and methyl pheophorbides and the strong methanol dependence of the spectra of the chlorophylls and methyl chlorophyllides. Related concentration and solvent effects of n.m.r. spectra have been studied by numerous investigators.[22] In general, four mechanisms are known to contribute to solvent and concentration shifts of the spectra. These are: (a) changes in volume susceptibility, (b) the solution anisotropy effect, (c) van der Waals interactions between molecules distorting the diamagnetic electronic circulation, and (d) effects due to dipolar reaction fields. If chemical shifts are measured against internal standards, contributions from mechanism (a) and nonspecific contributions from (b) and (c) are internally compensated even when the reference molecule differs substantially in shape from the molecules under investigation.[22h] The observed solvent and concentration dependence of the spectra of I–IV therefore must be attributed to specific interactions existing between solvent and solute, or to associations of the solute molecules themselves. The chemical shifts will be affected in the resulting complexes through mechanisms (b), (c), and (d). Contributions from (c) are usually assumed to be quite small[22e] and may be safely neglected in a qualitative discussion of relatively large solvent and concentration shifts. The relative importance of the remaining mechanisms (b) and (d) will be largely determined by the anisotropy of the associated molecules and by their dipole moments. For the chlorophyll derivatives, the exceptionally large anisotropy of the macrocyclic ring assures a strong contribution from mechanism (b) which probably will outweigh heavily any effects of dipolar reaction fields.

The observed dilution shifts (relative to internal tetramethylsilane) of certain resonances of the pheophytins and methyl pheophorbides in deuteriochloroform must result primarily from intermolecular interactions. Specific solvent–solute interactions certainly do exist, since the π-system of the chlorin skeleton can be expected to function as acceptor for the hydrogen bond donor, chloroform. The shift of the residual chloroform proton resonance relative to tetramethylsilane upon dilution of the pheophytin solution by a maximum of 6 c.p.s. to lower field can be accepted as evidence for such interaction, but changes of this interaction with respect to the environment of the pheophytin molecules upon dilution will be small, because the chloroform is in large excess at all concentrations measured. Furthermore, the association of chloroform with the pheophytins seems to have relatively minor influence on the chemical shifts of the substituent protons. This is borne out by the observation that carbon tetrachloride solutions of pheophytin *a* show almost identical chemical shifts when extrapolated to zero concentration.[23] It seems reasonable, therefore, to attribute the major fraction of the dilution shift to complex formation among the solute molecules themselves. Direct evidence for such associations is available in the form of molecular weight determinations of IIIa and IIIb in carbon tetrachloride. The data show partial molecular association even at the concentrations used in vapor phase osmometry ($\sim 5 \times 10^{-2}$ mole/l.).[5]

As Fig. 4 shows, the dilution shifts of the individual substituent protons are not identical. This behavior can be explained satisfactorily if it is assumed that at higher concentrations an appreciable fraction of the

(17) Rotation of the carbomethoxy group in **1** through 90° in either direction leads to serious crowding of the oxygen atoms with the 7-hydrogen, while the conformation obtained through rotation of 180° places restrictions on the free rotation of the methoxy group. The latter conformation is also incompatible with the observed chemical shift of protons 11, since a diamagnetic shift should result from the location of those protons above the macrocyclic ring.

(18) H. H. Strain, *J. Agr. Food Chem.*, **2**, 1222 (1954).

(19) A. S. Holt and E. E. Jacobs, *Plant Physiol.*, **30**, 553 (1953).

(20) In a previous communication it was claimed that the deuterium exchange in chlorophyll *a* occurs at the δ-position.[21a] While this observation is undoubtedly correct C-10 deuterium exchange occurs as well and is even faster. At that time it was not recognized that in methanol-free solutions the signal of the C-10 hydrogen is shifted to very much higher field and broadened to become undetectable in dilute solutions. As the exchange work was carried out on deuteriochlorophyll *a* and as the resulting solutions were dried before the n.m.r. spectra were determined, the second proton introduced at the C-10 evaded detection. The whole subject of exchangeable hydrogen in chlorophyll is under renewed investigation in these laboratories.

(21) (a) J. J. Katz, M. R. Thomas, and H. H. Strain, *J. Am. Chem. Soc.*, **84**, 3587 (1962); (b) J. J. Katz, M. R. Thomas, H. L. Crespi, and H. H. Strain, *ibid.*, **83**, 4180 (1961).

(22) *Cf.* (a) A. A. Bothner-By and R. E. Glick, *J. Chem. Phys.*, **26**, 1651 (1957); (b) J. R. Zimmerman and M. R. Foster, *J. Phys. Chem.*, **61**, 282 (1957); (c) W. G. Schneider, H. J. Bernstein, and J. A. Pople, *J. Chem. Phys.*, **28**, 601 (1958); (d) W. G. Schneider, H. J. Bernstein, and J. A. Pople, *J. Am. Chem. Soc.*, **80**, 3497 (1958); (e) A. D. Buckingham, T. Schaefer, and W. G. Schneider, *J. Chem. Phys.*, **32**, 1227 (1960); (f) J. V. Hatton and R. E. Richards, *Trans. Faraday Soc.*, **57**, 28 (1961); (g) P. Diehl and R. Freeman, *Mol. Phys.*, **4**, 399 (1961); (h) R. J. Abraham, *ibid.*, **4**, 369 (1961); (i) R. Kaiser, *Can. J. Chem.*, **41**, 430 (1963).

(23) The only proton resonance which deviates by more than the experimental error in carbon tetrachloride from that observed for chloroform solutions at infinite dilution is that of the C-10 hydrogen. The larger deshielding of this proton in chloroform by 10 c.p.s. may arise from hydrogen bonding of chloroform with the carbonyl group at ring V.

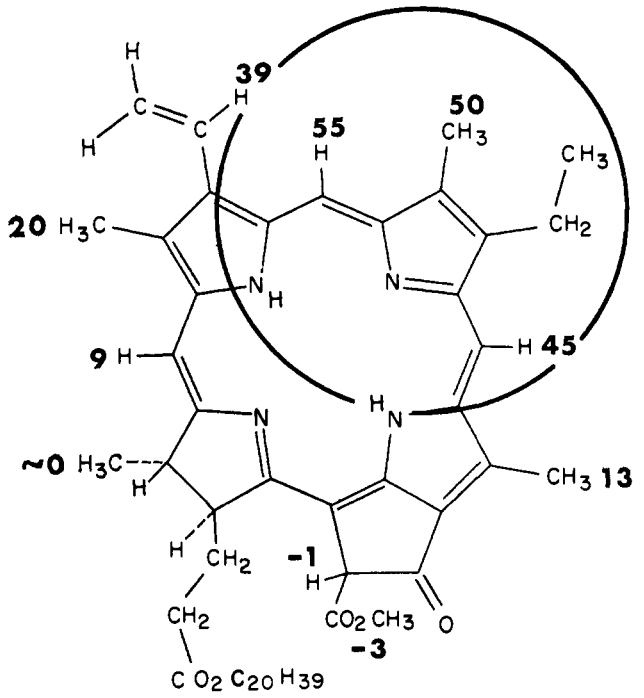

Fig. 13.—Aggregation map of pheophytin *a* (IIIa) based on dilution shifts in $CDCl_3$ in the concentration range from 0.3 mole/l. to extrapolated values at zero concentration.

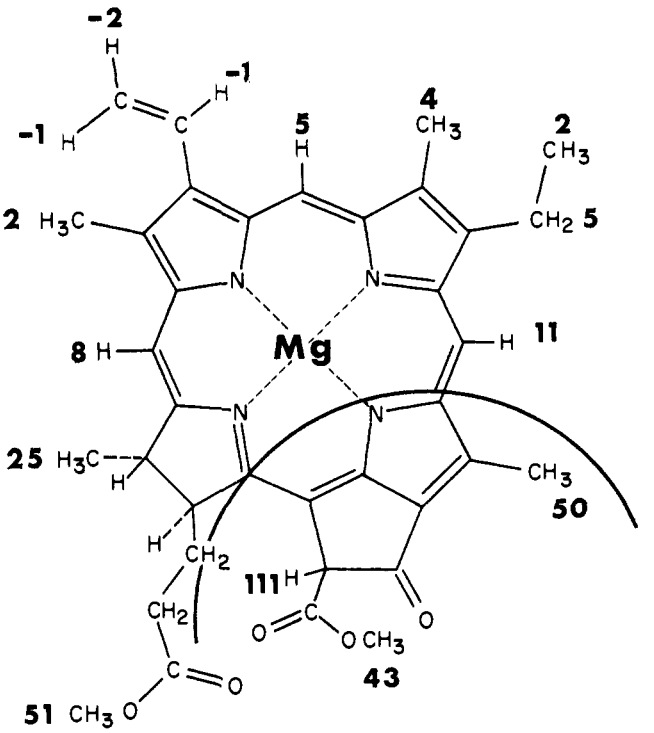

Fig. 14.—Aggregation map of methyl chlorophyllide *a* (IIa) based on methanol titration data in $CDCl_3$.

molecules is present as a dimer in which the mutual diamagnetic shielding of the monomer units differs at the various substituent protons.[24] A particularly favorable structure meeting this requirement is a sandwich-type arrangement in which the projections of the two centers of the disks are somewhat displaced from one another. A schematic presentation of this structure is shown in Fig. 13, in which the circle represents the π-electron system of a second pheophytin molecule. In this orientation, those protons located at positions of mutual overlap of the two rings will experience a significant diamagnetic shielding. The total dilution shifts exhibited by the respective protons of IIIa upon dilution from 0.3 *M* to zero concentration are presented as the numbers in Fig. 13. The largest shifts are found for protons in the vicinity of ring II. This region should then correspond to the area of maximum overlap of the monomer units in the dimer. Presumably all possible orientations will occur in the equilibrium, but the configuration indicated is the most populated one. The measured spectrum is the weighted average of all species in solution when the equilibrium is very mobile. The relatively sharp line width at all concentrations shows that the mean lifetime of the associated species must indeed be very short. Probably, the resistance to the formation of a true sandwich in which the projections of the rings coincide is due to steric factors. In such a structure the substituents on rings IV and V, which deviate from the plane would inhibit formation of a parallel orientation of the ring planes.

The dilution shifts of pheophytins *b* can be explained on the same basis. The resulting structure of the most probable configuration of the dimer is very similar to that of IIIa.

Although the presence of higher aggregates cannot be ruled out, it appears reasonable to assume that the dilution shifts of both IIIa and IIIb at low concentrations are mainly determined by a monomer–dimer equilibrium. In principle, the equilibrium constant can be obtained from the relationship $(d\delta/dc)_{c\to 0} = \Delta 2K$, where $(d\delta/dc)_{c\to 0}$ is the limiting slope of the dilution curves at infinite dilution and Δ stands for the chemical shift differences of the individual protons between monomer and dimer. Since the chemical shifts of the pure dimers are not known, K can only be estimated. If Δ is arbitrarily assumed to be twice the chemical shift differences between 0.3 *M* solutions and infinite dilution, values of 0.9 and 1.6 l./mole are obtained for the monomer–dimer equilibrium constants for IIIa and IIIb, respectively. These values, of course, cannot represent anything more than the correct order of magnitude. The fact that the deviation from these numbers is not larger than $\pm 10\%$, depending which dilution curve is chosen for the computation, indicates the fundamental correctness of this treatment. In this connection it is significant that the limiting slopes of the dilution curves in carbon tetrachloride are approximately twice as steep, indicating considerably stronger association in that solvent. Molecular weight data are in line with this observation.[5]

Within the much smaller range available for study, the dilution shifts for all protons of the methyl pheophorbides are identical with those obtained for the pheophytins. The phytyl chain, therefore, appears to have no influence on the association equilibrium in the solvent investigated.[25]

Methanol Dependence and Dilution Shifts of Methyl Chlorophyllides and Chlorophylls.—The striking shifts in the spectra of the chelates I and II upon adding methanol-d_4 to the chloroform solutions must again be attributed to specific interactions among the solute molecules, methanol being part of the solute. As outlined in the preceding paper, the infrared spectra are best interpreted on the basis of strong molecular associations of I and II in nonpolar solvents. Addition of small amounts of ethanol were postulated to break up these complexes as evidenced by profound changes in the infrared spectra and molecular weights. The methanol dependence of the n.m.r. spectra provides

(24) *Cf.* W. G. Schneider, H. J. Bernstein, and J. A. Pople, *J. Am. Chem. Soc.*, **80**, 3497 (1958).

(25) Since these measurements involve only the macrocyclic rings, it is possible that micelle formation involving the phytyl chains only and leaving the rings relatively far apart would remain undetected.

strong support for this interpretation. The chemical shifts displayed as functions of methanol concentrations in Fig. 8–10 show that the addition of the first two moles of methanol brings about most of the chemical shift changes. The magnitude of this "methanol shift" is so large for some protons that methanol–pigment interaction alone can hardly be visualized as the sole factor. Furthermore, the fact that the methanol-free solutions exhibit abnormally high shielding values whereas relatively normal values are observed for the methanol-containing solutions is strong indication for specific interaction among the pigment molecules in the absence of bases.

The maximum "methanol shifts" from mole ratio 0 to 8 of methyl chlorophyllide *a* are mapped in Fig. 14. In contrast to the dilution shifts of the magnesium-free derivatives the maximum shifts are observed in the vicinity of ring V. A very similar figure though less complete can be constructed for the "methanol shifts" of chlorophyll *a*. The methanol shifts of both compounds indicate that in the methanol-free solution there are aggregates, which have regions of mutual overlap in the vicinity of ring V. If the aggregates are mainly dimers, as inferred from the molecular weight determinations, a satisfactory structure may be represented schematically by Fig. 14 in which the second monomer unit is represented by the semicircle. Bonding of the magnesium atom of one monomer unit with the carbonyl of ring V of the other is believed to be responsible for the preferred geometry. Such a model also agrees best with the infrared data since it was shown that the carbonyl stretching frequency undergoes the largest changes upon addition of methanol. Finally, dissociation of the dimer upon addition of methanol can be easily understood on the basis of this structure, since complexing of the methanol with the magnesium will displace the carbonyl function from its position as the fifth ligand. As can be seen from models, the arrangement of the monomers in the dimer can hardly be in parallel planes for steric reasons. Furthermore, the relatively large methanol shift of the 8′-methyl group can be explained better if it is assumed that the ring-planes intersect at a small angle. Somewhat surprising is the large methanol shift of the propionic ester methyl (12) in IIa; this seems to indicate considerable coiling of the propionic ester chain into a preferred arrangement, which, in methanol-free solutions, places the ester methyl into the diamagnetic shielding zone of one of the two rings.

The dilution behavior of both the methanol-free and methanol-containing solutions of Ia (Fig. 11) indicates little change in the structure of the species in both solutions over the concentration range studied.[26] No further aggregation to higher polymers seems to occur at higher concentrations and little evidence can be found for dissociation of the dimer within the concentration range studied in methanol-free solutions. This finding is in sharp contrast to the behavior of the magnesium-free derivatives III and IV, and emphasizes the different nature of the intermolecular bonding. Certainly, chelation of the magnesium with carbonyl functions can be expected to lead to stronger association than the π–π interactions responsible for the aggregate formation in III and IV. The absence of any significant dilution shift in the methanol-containing solutions of Ia indicates the absence of association among the methanol–pigment complexes. Probably, the coordination of the methanol with the magnesium provides a steric barrier to the close approach of two macrocycles.

The binding of methanol to the magnesium atom is very clearly demonstrated in the behavior of the methanol CH_3 resonance as function of the methanol–methyl chlorophyllide *a* ratio. At approximately equimolar concentration the methanol methyl group is displaced by 102 c.p.s. to higher field, indicating the location of the nuclei in a strongly shielded region such as is provided near the magnesium atom.[27] When a second mole of methanol is added, the methoxy protons of the alcohol are still displaced by 87 c.p.s., pointing to the possibility that two moles of methanol can be bound to the magnesium as the fifth and sixth ligand.[29]

The more complex behavior of the dilution shifts of chlorophyll *b* can be associated with the bifunctionality of the *b*-series. As molecular weight data suggest,[5] chlorophyll *b* has a larger tendency to aggregate. This finding is confirmed by the "methanol titration" data presented in Fig. 10, which indicate that higher methanol concentrations are needed to break up these aggregates. The dilution shifts of the methanol-containing solutions (Fig. 12, left) result from the increasing methanol/chlorophyll *b* mole ratio with decreasing concentration of Ib. The concentration dependence of the chemical shifts in the methanol-free solutions (Fig. 12, right) probably result from a dimer–trimer equilibrium. Molecular weight data do indeed indicate that higher aggregates are formed in the *b*-series. In Fig. 15 the methanol shifts of 0.08 *M* solutions are mapped out. It can be seen that essentially two highly shielded regions exist in the aggregates. They are located in the vicinities of both carbonyl functions. This may be rationalized by assuming the existence of two dimers of comparable stability. It is proposed that both structures involve carbonyl oxygen–magnesium chelation, and that they differ in regard to which carbonyl group is engaged in bonding. It is likely that at higher concentrations trimers occur in which a molecule binds with both its ketonic and

(26) After this work was completed, R. J. Abraham, P. A. Burbidge, A. H. Jackson, and G. W. Kenner (*Proc. Chem. Soc.*, 134 (1963)) called attention to the large dilution shifts to be expected for the n.m.r. spectra of porphyrin derivatives. However, the implication that all of the compounds cited in their reference 1 are subject to a large dilution shift is only partially correct. Chlorophyll *a* shows only a slight dilution shift in $CDCl_3$, and specific structural features can enhance, or practically abolish, dilution shifts.

(27) This result should be compared with the recently reported n.m.r. spectra of N-alkyl substituted porphyrins.[28] The chemical shift of the methyl protons of the N-ethyl group in N-ethyletioporphyrin experiences an additional shielding of approximately 200 c.p.s. from the ring current of the macrocycle. The larger shift of the latter protons, compared to the methoxy protons in the methanol complex of IIa, arises in part from their closer proximity to the center of the ring. Furthermore, the 102 c.p.s. refers to methanol in deuteriochloroform. A more meaningful comparison would result from using a magnesium–methanol chelate in which a diamagnetic ring current effect is absent as a reference. The methyl resonance of such a molecule would probably occur at lower field than in methanol, thus increasing the diamagnetic shift value for the methoxy protons in IIa–methanol complex.

(28) W. S. Caughey and P. K. Iber, *J. Org. Chem.*, **28**, 269 (1963).

(29) The problem of calculating equilibrium constants from the data has been considered. It appears that at least for chlorophyll *a* the complexing of one methanol molecule suffices to break up the dimer, because a spectrum at equimolar methanol–chlorophyll concentration shows more than half of the total methanol shift (Fig. 9). If the dissociation process could be described by the equation 2MeOH + dimer = 2 monomer·MeOH the equilibrium constant should be given by

$$K = \frac{2C_0(\delta/\Delta)^2}{(1 - \delta/\Delta)(C_m - C_0\delta/\Delta)^2}$$

where

C_0 = nominal chlorophyll concentration
C_m = methanol concentration
Δ = total methanol shift
δ = methanol shift at C_0 and C_m

If this equation is applied to a number of points of Fig. 9, the agreement among values obtained from different protons is poor; deviations are as great as 50%. At best, an order of magnitude estimate can be obtained this way, and a value of $K \sim 10^2$ l./mole appears probable. Several reasons can be advanced for the failure of this treatment, among them the inadequate description of the dissociation process in terms of only two species.

aldehydic carbonyl function one chlorophyll *b* molecule. Such binding must be weak, since substantial dissociation to dimers occurs in the concentration range available for study.

The question of the detailed geometry of the dimers and the possible trimer cannot be answered with the data at hand. An arrangement with parallel planes is spacially possible for the dimer involving the aldehyde function, particularly when the carbonyl group is rotated out of the ring plane. As already pointed out in connection with chlorophyll *a*, the dimer involving the ketone carbonyl can hardly be visualized to have a parallel geometry.

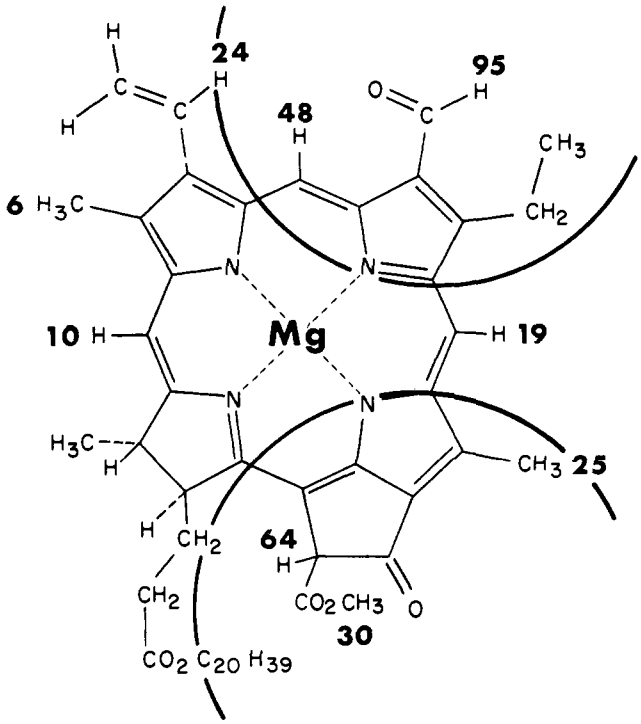

Fig. 15.—Aggregation map of chlorophyll *b* (Ib) based on methanol titration data in $CDCl_3$.

There has been considerable discussion in the recent literature on the possible existence of aggregated chlorophyll both *in vivo* and in solution, based primarily on observations of electronic spectra.[30] While our results support the possibility of aggregate formation in nonpolar solvents, we have no reason to suppose that chlorophyll aggregates exist to any appreciable extent in basic solvents. We feel constrained to point out a seeming incompatability between our results and those of Brody and Brody.[30e,30f] According to these workers, dimers exist in ethanol solution in an equilibrium that is almost temperature independent; from the formula on p. 418 of reference 30f, the dimer/monomer ratio for a 10^{-2} *M* solution of chlorophyll *a* in ethanol at room temperature is calculated to be 0.38. We find that the more strongly associated chlorophyll *b* has an n.m.r. spectrum in neat methanol-d_4 essentially identical with that seen in chloroform–methanol mixtures. This strongly suggests that dimers do not form in methanol at the concentrations of chlorophyll studied by the Brodys. We do not believe the differences in disaggregating power between methanol and ethanol is significant; indeed, the infrared data[5] show ethanol to be a disaggregating base. We, therefore, consider it unlikely that dimers of the type described here will persist in disaggregating solvents such as methanol or ethanol at room temperature. Our conclusions thus seem to agree with those of Stensby and Rosenberg[30g] who, from spectral measurements, have also concluded that dimerization is essentially absent in ethanol solution at room temperature.

It has been recognized for some time that fluorescence and luminescence of chlorophyll are solvent dependent. Livingston and co-workers[31] found that strictly dry chlorophyll *a* or *b* in a "dry" solvent such as benzene has no fluorescence, but that the addition of polar substances such as water, alcohols, acids, or amines activates the fluorescence. It has generally been considered that it is only the chlorophyll-addition compound that is capable of fluorescence.[32] However, since both the n.m.r. and infrared studies[5] strongly suggest that at least in the concentration range used in these studies the magnesium always has a coordination number greater than 4, in polar solvents by coordination with the solvent and in nonpolar media by coordination with other chlorophyll molecules, it appears reasonable that the fluorescence behavior is rather to be interpreted on the basis of the degree of aggregation of the chlorophyll. Similarly, it has been postulated that the relative positions of the $\pi-\pi$ and $n-\pi$ first excited singlet levels are reversed in polar and nonpolar solvents. In nonpolar solvents free of traces of moisture the $n-\pi$ level has been considered to lie below the $\pi-\pi$ singlet level, whereas the $\pi-\pi$ level is the lower in polar solvents or in nonpolar solvents containing small amounts of bases.[33] Here, also, the demonstrated effects of the solvent on the degree of aggregation of the chlorophyll needs to be taken into account for a satisfactory interpretation of the electronic spectra.

Finally, most photochemical studies on chlorophyll have been in the 10^{-4}–10^{-6} *M* range, and the question of whether molecular aggregates occur in this concentration is important. Our studies do not provide an answer to this question, since no equilibrium constants could be obtained for dissociation in methanol-free solutions. The resulting binding from carbonyl–magnesium interactions may be strong enough in nonpolar solvents to allow substantial aggregation even at these low concentrations. However, we believe that the concentrations used in our work provide results of greater significance in connection with the possible state of chlorophyll in chloroplasts, because the concentration range of chlorophyll in this environment is very close to that employed in this study (0.06–0.2 mole/l.).[34] For an interpretation of the properties of chlorophyll in nature, it is clearly desirable to acquire data on the electronic spectra of chlorophyll at high concentrations in solvents in which the state of aggregation of the chlorophyll is known.

(30) (a) R. Livingston, *Quart. Rev.* (London), **14**, 174 (1960); (b) J. Lavorel, *J. Phys. Chem.*, **61**, 1600 (1957); *J. Chem. Phys.*, **55**, 905 (1958); (c) G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **53**, 646 (1957); (d) S. S. Brody, *Science*, **128**, 838 (1958); (e) S. S. Brody and M. Brody, *Nature*, **189**, 547 (1961); (f) S. S. Brody and M. Brody, *Trans. Faraday Soc.*, **58**, 416 (1962); (g) P. S. Stensby and J. L. Rosenberg, *J. Phys. Chem.*, **65**, 906 (1961).

(31) R. Livingston, W. F. Watson, and J. McArdle, *J. Am. Chem. Soc.*, **71**, 1542 (1949).

(32) (a) J. R. Platt, in "Radiation Biology," A. Hollaender, Ed., Vol. III, McGraw-Hill Book Co., Inc., New York, N. Y., 1956, p. 115; (b) V. B. Evstigneev, V. A. Gavrilova, and A. A. Krasnovski, *Doklady Akad. Nauk SSSR*, **70**, 261 (1950).

(33) See J. Franck, J. L. Rosenberg, and C. Weiss, Jr., in "Luminescence of Organic and Inorganic Materials," H. P. Kollmann and G. M. Spruch, Ed., John Wiley and Sons, Inc., New York, N. Y., 1962, p. 16 *et seq.*; R. S. Becker and M. Kasha, in "The Luminescence of Biological Systems," F. H. Johnson, Ed., American Association for the Advancement of Science, Washington, D. C., 1955, p. 30 *et seq.*

(34) E. Rabinowitch, "Photosynthesis," Vol. 1, Interscience Publishers, Inc., New York, N. Y., 1945, p. 411.

Experimental[35]

Materials.—Chlorophylls *a* and *b* were prepared from spinach by the procedure of Strain, *et al.*[4] The methyl chlorophyllides *a* and *b* were prepared from cockleburr by the *in situ* reaction with methanol; details of this procedure will be described elsewhere. Pheophytins *a* and *b* and methyl pheophorbide *b* were prepared from the corresponding chlorophylls following well-described procedures.[36] Methyl pheophorbide *a* was kindly donated by Professor R. B. Woodward. Deuteriochloroform and methanol-d_4 were commercial samples (Volk) and were used without further purification.

N.m.r. Spectra.—The n.m.r. spectra were recorded on Varian spectrometer systems DP 60, DP 40, and A60. Chemical shifts were measured by the conventional side-band techniques using Hewlett–Packard oscillators and frequency counters. Chemical shifts of the spectra determined on the A60 spectrometer were read off the calibrated charts. No particular care was taken to control the temperature of the samples; accordingly, temperatures varied from 28–37°.

Spin decoupling experiments were carried out using wide-line equipment of the DP spectrometers. Field modulation was applied with a Hewlett–Packard 200CD oscillator connected to the Varian V-4250A sweep unit. Phase sensitive detection was carried out with the lock-in amplifier V-4270A. The spectra were recorded as the audiofrequency modulation side bands, while the main radiofrequency field H_1 served as the decoupling field.

(35) The complete numerical chemical shift values of compounds I–IV at the various concentrations studied and the methanol shift values of compounds I and II are available upon request.

(36) H. Fischer and H. Orth, "Die Chemie des Pyrrols," Vol. II$_2$, Akademische Verlagsgerellschaft, Leipzig, 1940.

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Studies on Polynucleotides. XXIV.[1] The Stepwise Synthesis of Specific Deoxyribopolynucleotides (4).[2] Protected Derivatives of Deoxyribonucleosides and New Syntheses of Deoxyribonucleoside-3′ Phosphates[3]

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The following protected derivatives of the four major deoxyribonucleosides have been prepared: 5′-O-mono-*p*-methoxytrityl- and 5′-O-di-*p*-methoxytritylthymidine from thymidine and the appropriate trityl chloride; N-benzoyl- and N-anisoyldeoxycytidine and from these the corresponding 5′-O-trityl-, 5′-O-mono-*p*-methoxytrityl-, and 5′-O-di-*p*-methoxytrityl- derivatives; N,N,O$^{3'}$,O$^{5'}$-tetrabenzoyldeoxyadenosine and N-benzoyldeoxyadenosine; and from the latter, the corresponding 5′-O-mono-*p*-methoxytrityl- and 5′-O-di-*p*-methoxytrityl-derivatives; 3′,5′-di-O-acetyldeoxyguanosine, N-di-*p*-methoxytrityl-O$^{3'}$,O$^{5'}$-diacetyldeoxyguanosine, N-di-*p*-methoxytritydeoxyguanosine, and N,O$^{5'}$-bis-di-*p*-methoxytritydeoxyguanosine. Phosphorylation of 5′-O-di-*p*-methoxytritylthymidine, of N-benzoyl-5′-O-di-*p*-methoxytrityldeoxycytidine, of N-benzoyl-5′-O-di-*p*-methoxytrityldeoxyadenosine, and of N,O$^{5'}$-di-*p*-methoxytrityldeoxyguanosine with a mixture of β-cyanoethyl phosphate and dicyclohexylcarbodiimide followed by removal of the protecting groups gave excellent yields of thymidine-3′, deoxycytidine-3′, deoxyadenosine-3′, and deoxyguanosine-3′ phosphates, respectively. A sensitive colorimetric method for the estimation of compounds containing the di-*p*-methoxytrityl group is described.

General Introduction.—Of the two possible approaches to the synthesis of the naturally occurring internucleotidic linkage from two protected nucleoside or nucleotide components, the first is that in which a protected deoxyribonucleoside-5′ phosphate is condensed with the 3′-hydroxyl group of a second suitably protected component.[2a,2b,4,5] In the second approach a protected nucleoside-3′ phosphate is condensed with the 5′-hydroxyl group of a second suitably protected nucleoside, nucleotide, or an oligonucleotide.[2c,4] Both approaches have previously been investigated for the synthesis of deoxyribo-oligonucleotides[2,4] and the first approach was concluded to be superior.[6–8] Therefore this approach has formed the basis of further systematic studies of the synthesis of deoxyribopolynucleotides, which are the subject of the present series of papers.

In the first phase of this work the preparation of suitably protected derivatives of the major deoxyribonucleosides was undertaken. This is the main theme of the present paper. The next phase involved study of the internucleotide bond formation between different nucleosides and nucleotides, since differences in reactivities could have been expected. This phase of the study is reported in the following paper.[9] Two subsequent papers[10,11] report on the synthesis of deoxyribo-oligonucleotides containing different nucleosides in known sequences. Brief reports of portions of this work have already appeared.[12,13]

(1) Paper XXIII: W. Fiers and H. G. Khorana, *J. Biol. Chem.*, **238**, 2789 (1963).

(2) Previous papers which deal directly with this topic: (a) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958); (b) **81**, 4647 (1959); (c) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962).

(3) This work has been supported by grants from the National Science Foundation, Washington, D. C., the National Cancer Institute of the National Institutes of Health, Bethesda, Md., and the Life Insurance Medical Research Funds, New York, N. Y.

(4) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 5.

(5) H. G. Khorana in "The Nucleic Acids," Vol. III, E. Chargaff and J. N. Davidson, Ed., Academic Press, Inc., New York, N. Y., 1960, p. 105.

(6) The considerations leading to this conclusion are: (1) It is rather impractical to prepare suitably protected deoxyribonucleoside-3′ phosphates with alkali-labile groups on the amino groups and acid-labile groups such as di-*p*-methoxytrityl group on the 5′-position. For example, as demonstrated in the Experimental section, the di-*p*-methoxytrityl group in N-benzoyl-5′-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate is extremely labile to acid and yet complete removal of the group cannot be carried out selectively prior to removal of the N-benzoyl group since the glycosyl bond in the N-benzoyldeoxyadenosine moiety is also extremely sensitive to acid. (2) The yields of internucleotide bonds using stoichiometric amounts of the two components were lower than usual when bulky substituents are present in the nucleotide component as, for example, 5′-O-tritylthymidine-3′ phosphate (ref. 2c, 8, and 9). (3) A component bearing the free 5′-hydroxyl group and a preformed diester bond is not completely stable in the presence of the carbodiimide reagent; for example, thymidylyl-(3′ → 5′)-3′-O-acetylthymidine reacted in dry pyridine with dicyclohexylcarbodiimide to form small amounts of new products.[2c] Furthermore, triester formation was detected by interaction of an activated diester with the primary hydroxyl group of 3′-O-acetylthymidine.[2c] Side reactions of this type have not been encountered with components which bear the secondary 3′-hydroxyl group. (4) The ready availability of deoxyribonucleoside-5′ phosphates is of practical significance.

(7) It should be emphasized that the analysis described[6] applies to work in the deoxyribonucleotide series. In the stepwise synthesis of ribo-oligonucleotides the favored and, in fact, the only, practical approach has been shown to be that which starts from protected ribonucleoside-3′ phosphates: D. H. Rammler and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 3112 (1962); D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 1363, 3852 (1963).

(8) G. Weimann and H. G. Khorana, *ibid.*, **84**, 4329 (1962).

(9) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963).

(10) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963).

(11) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).