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Infrared Spectra, Molecular Weights, and Molecular Association of Chlorophylls *a* and *b*, Methyl Chlorophyllides, and Pheophytins in Various Solvents¹

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The infrared spectra of chlorophylls *a* and *b* in carbon tetrachloride, chloroform, and benzene (nonpolar solvents) in the 1600–1750 cm^{-1} region are best interpreted on the basis of intermolecular aggregation involving coordination of ketone and aldehyde carbonyl oxygen atoms of one molecule with the central magnesium atom of another. In basic or polar solvents, the coordination unsaturation of the magnesium is satisfied by the solvent, and the chlorophylls exist predominantly in monomeric form. Direct molecular weight determinations confirm the relationship between the extent of molecular aggregation and the observed infrared spectra.

Introduction

In the course of characterizing the deuteriochlorophylls isolated from algae grown autotrophically in 99.8% D_2O , we examined the infrared spectra both of ordinary and fully deuterated chlorophylls. Our observations at that time indicated inconsistencies in the earlier interpretations of the infrared spectra in the carbonyl absorption region.⁴ Consequently, our investigations have now been extended to provide a more satisfactory interpretation of the infrared spectra. They have also included a detailed proton magnetic resonance study of chlorophyll and its derivatives, the results of which are described in the accompanying communication.⁵

The infrared spectra of chlorophylls *a* and *b* (Ia and Ib)⁶ in the 1550 to 1750 cm^{-1} region have been reported by Weigl and Livingston,⁷ Holt, Jacobs, and Morley,⁸ Sidorov and Terenin,⁹ and Karyakin and Chibisov.¹⁰ Where comparison is possible, the experimental observations are generally in good agreement.

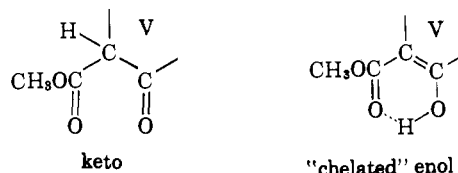
The most detailed analysis of the spectra, made by Holt and Jacobs,^{8a} is based on the assumption of extensive keto-enol tautomerism in chlorophyll *a* involving the β -keto ester in ring V. The results presented here suggest, however, that keto-enol tautomerism does not contribute significantly to the infrared absorption in this spectral region. The hitherto puzzling features of the spectra appear to result from molecular aggregation in nonpolar solvents.¹¹ This intermolecular association seems to be the result of coordination between the carbonyl groups and the central magnesium atom. The occurrence of molecular aggregation is indicated not only by the infrared spectra, but also by the molecular weight determinations described here. The n.m.r. studies⁵ likewise provide

convincing evidence for the existence of molecular aggregates in chlorophyll solutions in nonpolar solvents.

Infrared Spectra of Chlorophylls and Related Compounds.—A striking feature of the infrared spectra of CCl_4 solutions of chlorophylls *a* and *b* in the 1600–1750 cm^{-1} region is their similarity despite the presence of an additional carbonyl group in chlorophyll *b* (Fig. 1). Chlorophyll *a* in CCl_4 solution shows four bands. Three of these bands at 1736, 1695, and 1653 cm^{-1} are relatively strong, while the band at 1609 cm^{-1} is distinctly weaker. Chlorophyll *b* in CCl_4 has absorption bands at 1739, 1703, 1665, and 1608 cm^{-1} . All are relatively strong.

In tetrahydrofuran, tributyl phosphate, or triethylamine solutions, however, the two chlorophylls have very different spectra (Fig. 2). In these more basic solvents, chlorophyll *a* lacks the strong absorption peak near 1650 cm^{-1} characteristic of chlorophyll *a* in nonpolar solvents, whereas chlorophyll *b* shows the same number of absorption maxima that it does in nonpolar solvents. It is necessary, therefore, to account for (a) the similarity of the chlorophyll *a* and *b* spectra in nonpolar solvents, even though chlorophyll *b* should show additional absorption from its aldehyde carbonyl group, and (b) the differences between the spectral absorption properties of chlorophyll *a* in polar and nonpolar solvents.

Keto-Enol Tautomerism and the Interpretation of the Infrared Spectra.—Holt and Jacobs^{8a} postulated that the absorption band of chlorophyll *a* at 1640 cm^{-1} in the crystalline state and at 1652 cm^{-1} in CCl_4 and CS_2 was due to chelation of the ester carbonyl at C-10 with the $-\text{OH}$ formed by enolization.



This interpretation was based on a well-known study, by Leonard *et al.*,¹² of the infrared absorption spectra of cyclic β -keto esters; the presence of "extra" bands in the spectra of these compounds was accounted for by the chelated carbonyl group and the formation of new $\text{C}=\text{C}$ double bonds by enolization, and the absence of a strong OH absorption was explained by chelation of the $-\text{OH}$ to the ester carbonyl.¹³ While this interpretation

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(2) A. P. Sloan Foundation Fellow.

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(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).

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(8) (a) A. S. Holt and E. E. Jacobs, *Plant Physiol.*, **30**, 553 (1955); (b) A. S. Holt, "Infrared Spectroscopy of Chlorophyll and Derivatives" in "Research in Photosynthesis," H. Gaffron, Ed., Interscience Publishers, Inc., New York, N. Y., 1957; (c) A. S. Holt and H. V. Morley, "Recent Studies of Chlorophyll Chemistry" in "Comparative Biochemistry of Photoreactive Systems," M. B. Allen, Ed., Academic Press, Inc., New York, N. Y., 1960.

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(11) In this paper we use the term nonpolar to include solvents lacking pronounced basic properties, *i.e.*, carbon tetrachloride, benzene, chloroform, tetrachloroethylene, and heptane. The term polar is used to describe solvents that can function as electron donors such as water, methanol, ethanol, tetrahydrofuran, pyridine, triethylamine, or tributyl phosphate.

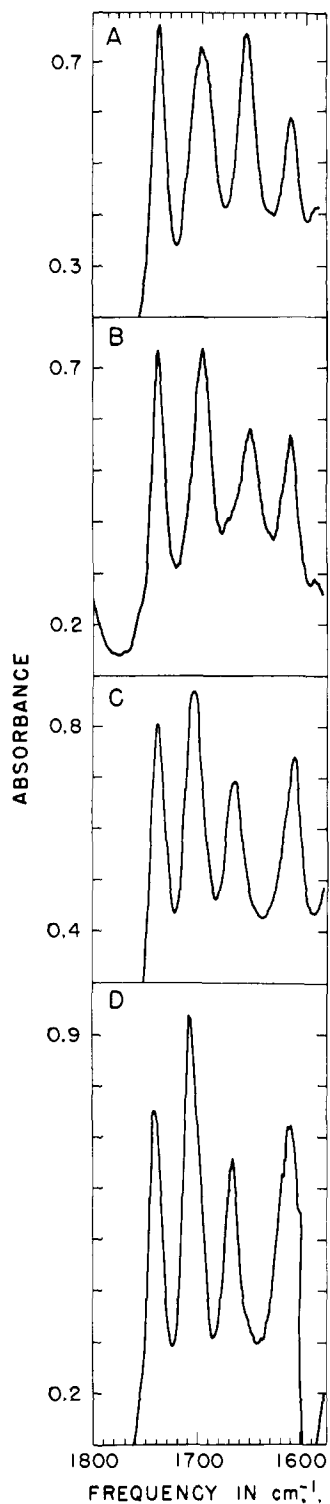


Fig. 1.—Infrared spectra of chlorophylls in nonpolar solvents: A, chlorophyll *a* in CCl_4 ; B, chlorophyll *a* in benzene; C, chlorophyll *b* in CCl_4 ; D, chlorophyll *b* in benzene.

accounts satisfactorily for the spectra of the β -keto esters, Holt and Jacobs were compelled to make a number of *ad hoc* assumptions in applying it to the chlorophyll spectra. To account for the solvent dependence of the chlorophyll spectra, Holt and Jacobs made three postulations; namely, chlorophyll *a* in CCl_4 , CS_2 , and in the crystalline state is enolized and chelated; in pyridine it is enolized but not chelated; and in chloroform and diethyl ether it is neither enolized nor chelated. The reputed existence of chlorophyll *a* in the keto form in CCl_4 but in the enol form in CHCl_3

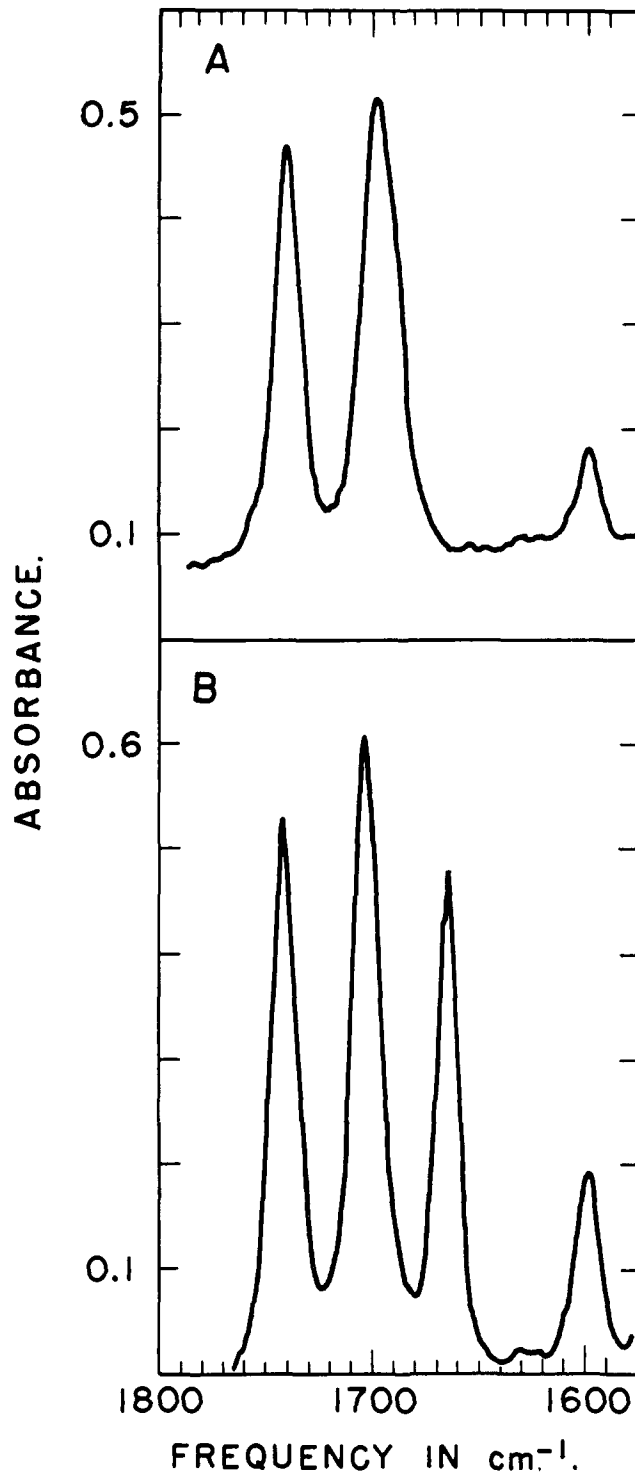


Fig. 2.—Infrared spectra of chlorophylls in tetrahydrofuran: A, chlorophyll *a*; B, chlorophyll *b*. The spectra in triethylamine and tributylphosphate are indistinguishable from the tetrahydrofuran spectra.

was attributed to the greater polarity of CHCl_3 . Holt and Jacobs also concluded that chlorophyll *b* does not enolize in CCl_4 , that pheophytins *a* and *b* (Ia and Ib, with Mg replaced by 2H) do not undergo enolization at all, except for pheophytin *a* in pyridine.

We believe that the foregoing interpretations are unsatisfactory for the following reasons: (1) the hydrogen exchange behavior of chlorophylls *a* and *b* are qualitatively alike, and suggests that both chlorophylls exist predominately in the keto form in CCl_4 solution; (2) small amounts of Lewis bases in nonpolar

solvents have a decisive and previously unrecognized effect on the spectra; (3) solvent effects on the spectra of cyclic β -keto esters are very different from those observed with chlorophyll; hence these compounds are not satisfactory models for chlorophyll behavior.

Hydrogen Exchange of Chlorophylls in Relation to Keto-Enol Forms.—The observed exchange of hydrogen between methanol and chlorophylls *a* and *b* is inconsistent with the hypothesis that either chlorophyll exists to a significant extent in the enol form in CCl_4 , or that there is a marked difference in the enolization behavior of the two chlorophylls. Katz, Thomas, Crespi, and Strain^{14,15} found that both chlorophyll *a* and *b* undergo only slow exchange of hydrogen with stoichiometric amounts of methanol in CCl_4 solution. Each chlorophyll possesses at least one exchangeable hydrogen atom, and both chlorophylls appear to behave in a qualitatively similar manner. From the slow rates of exchange the concentration of exchangeable $-\text{OH}$ must be low, for a very rapid exchange of hydrogen has been observed between methanol and the $-\text{OH}$ of lauryl alcohol under the chlorophyll exchange conditions. Thus the exchange observations make it unlikely that appreciable concentrations of enol exist in CCl_4 solutions of the chlorophylls.¹⁶ Our n.m.r. studies also strongly suggest that the chlorophylls exist for the most part in the keto form.⁵

Effect of Bases on the Spectra in Nonpolar Solvents.—Holt and Jacobs^{8a} observed a startling difference between the spectra of chlorophyll *a* in CCl_4 and in CHCl_3 , which they attributed to a difference in solvent polarity and a consequent effect on the extent of enolization and chelation. Similar spectral differences due to another variation of these solvents are illustrated by the infrared spectra of methyl chlorophyllide *a* reported in Fig. 3. (Methyl chlorophyllides *a* and *b*, which have spectra that closely resemble those of the parent chlorophylls, are used here to indicate that the spectral variations with solvent, as in Fig. 1, 2, and 3, are independent of the presence of the phytol group.) In Fig. 3A, methyl chlorophyllide *a* in anhydrous, alcohol-free chloroform has the 4-banded spectrum observed in nonpolar solvents such as carbon tetrachloride (Fig. 1). In Fig. 3B, methyl chlorophyllide *a* in chloroform plus alcohol has the 3-banded spectrum observed in polar solvents. This variation of the spectrum arises from the presence of very small amounts of ethanol, as small as those usually incorporated by the manufacturer to stabilize the chloroform. When as little as 0.5% ethanol is added to chloroform, an amount about that usually added as a stabilizer, and an amount that provides at least one mole of ethanol per mole of chlorophyll, the band near 1650 cm^{-1} disappears with a concurrent increase in the intensity of the ketonic carbonyl absorption near 1700 cm^{-1} . If chloroform stabilized with ethanol is washed with water and then dried, the spectrum shown in Fig. 3A is observed. In this destabilized chloroform, the spectra either of chloro-

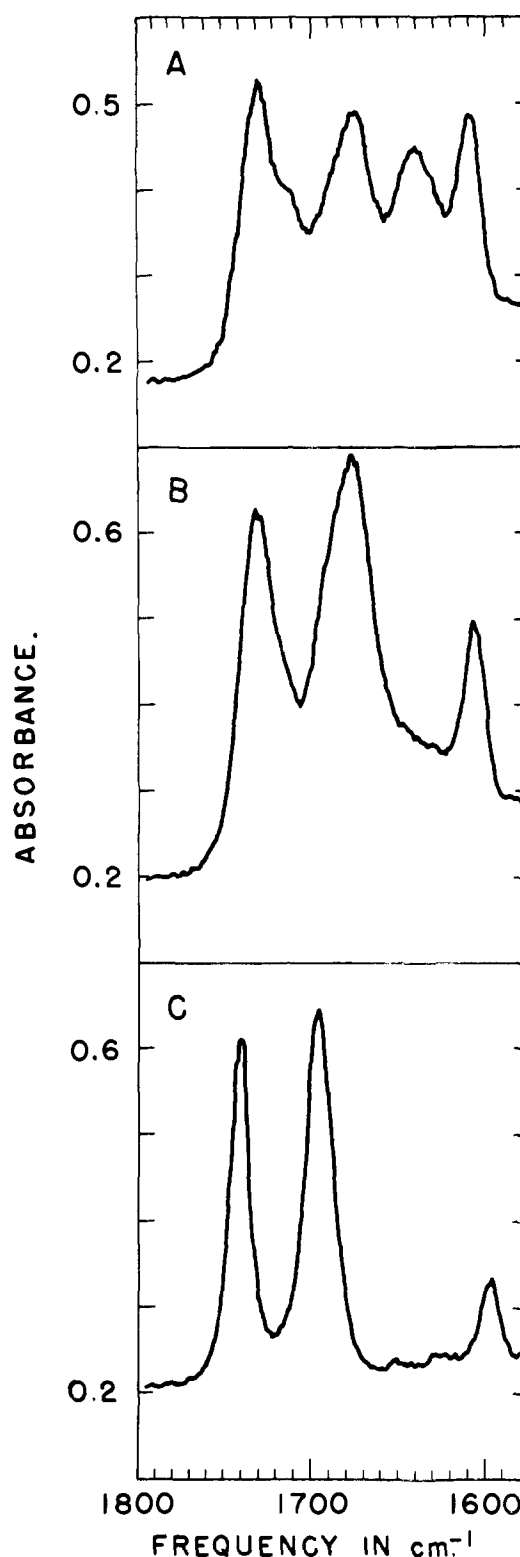


Fig. 3.—Infrared spectra of methyl chlorophyllide *a* in polar and nonpolar solvents: A, in destabilized chloroform; B, in chloroform containing 0.5% ethanol; C, in tetrahydrofuran.

phyll *a* or methyl chlorophyllide *a* are similar to those observed in CCl_4 .

Figure 4 shows the spectra of methyl chlorophyllide *b* in stabilized and destabilized chloroform and in tetrahydrofuran. These spectra are entirely comparable to those of chlorophyll *b* in the same solvents.

As shown in Fig. 5A and 5B, the addition of ethanol to carbon tetrachloride also causes the chlorophyll *a* bands to shift. Higher concentrations of ethanol

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(16) When chlorophyll *a* is treated with one equivalent of CH_3OD in CCl_4 solution, exchange is slow and one hydrogen atom undergoes exchange.¹⁴ At the time these studies were carried out, the effects of methanol on the state of aggregation of chlorophyll was not recognized. The n.m.r. studies on hydrogen exchange in chlorophyll *a*¹⁵ were carried out with a considerable excess of methanol, and the exchange conditions were therefore quite different. It was also not recognized that the signal of the C-10 hydrogen in methanol-free solutions is shifted to very much higher fields and broadened to such an extent as to render it undetectable. Exchange at the C-10 position thus could not be detected under the conditions then used. Preliminary n.m.r. data now indicate that both the C-10 and δ -hydrogen atoms are labile in both chlorophylls, with the C-10 exchange the faster. The exchange behavior of the chlorophylls is therefore under renewed investigation.

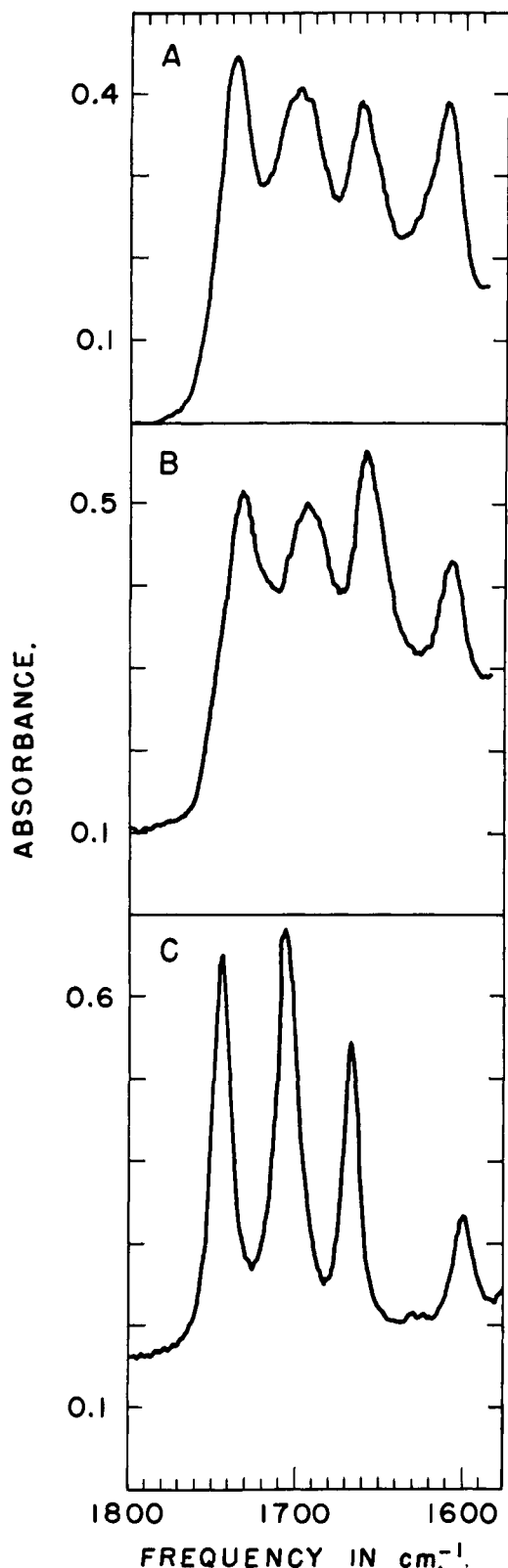


Fig. 4.—Infrared spectra of methyl chlorophyllide *b* in polar and nonpolar solvents: A, in destabilized chloroform; B, in chloroform containing 0.5% ethanol; C, in tetrahydrofuran.

are necessary in CCl_4 than in CHCl_3 to shift the 1653 cm^{-1} peak to higher frequencies. A marked shift is already observed at 1% ethanol concentration (Fig. 5A), and in a 10% ethanol- CCl_4 solution only a broad band at 1680 cm^{-1} is observed (Fig. 5B). The broadening of the ketone absorption and the splitting of the ester peaks probably is the result of hydrogen bonding

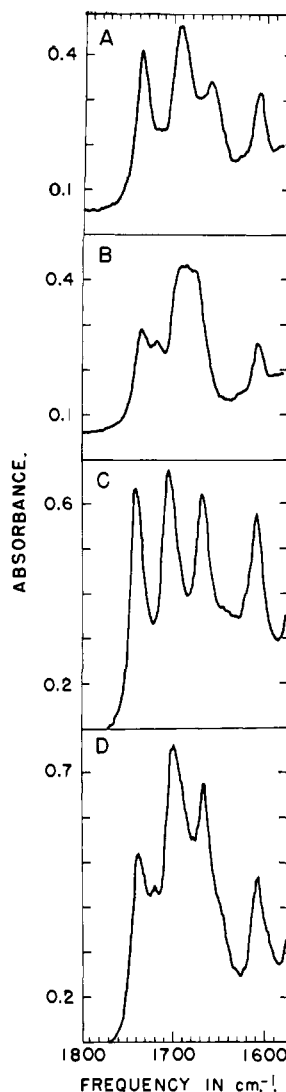


Fig. 5.—Effect of ethanol on the infrared spectra of the chlorophylls in carbon tetrachloride solution: A, chlorophyll *a*, 1% ethanol; B, chlorophyll *a*, 10% ethanol; C, chlorophyll *b*, 1% ethanol; D, chlorophyll *b*, 10% ethanol.

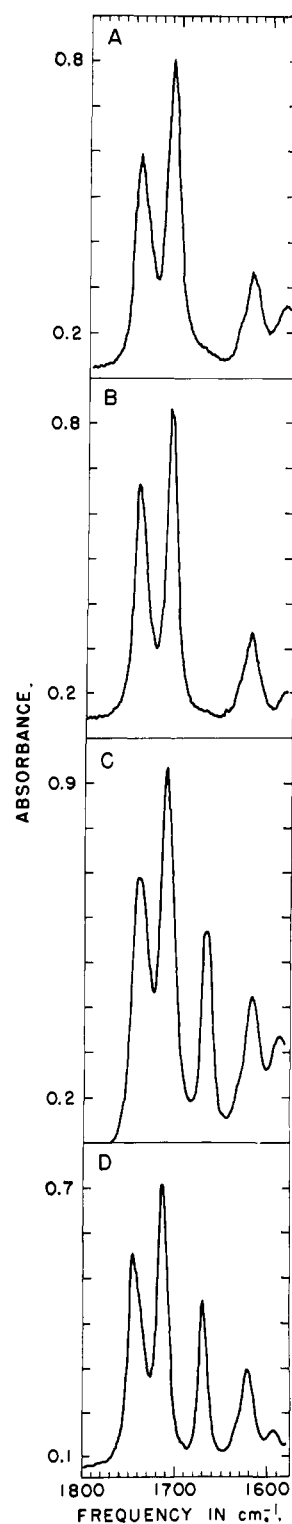


Fig. 6.—Infrared spectra of the pheophytins in nonpolar and polar solvents: A, pheophytin *a* in CCl_4 ; B, pheophytin *a* in tetrahydrofuran; C, pheophytin *b* in CCl_4 ; D, pheophytin *b* in tetrahydrofuran.

with the hydroxyl group of the ethanol. It thus appears that the presence or absence of alcohol (or of other Lewis bases, such as pyridine) is critical for the presence of an absorption band near 1650 cm^{-1} in chlorophyll *a* (or methyl chlorophyllide *a*) solutions in

nonpolar solvents. Figures 3C and 4C, showing the spectra of methyl chlorophyllides *a* and *b* in tetrahydrofuran, emphasize the similarity between spectra observed in nonpolar solvents plus alcohols and those observed in pure solvents with strong electron-donor properties. The same sensitivity to Lewis bases is evident also in the proton magnetic resonance spectra of the chlorophylls and is described in the accompanying communication.⁵

The effect of ethanol upon the spectra of chlorophyll *b* dissolved in CCl₄ is shown in Fig. 5C and 5D. The relative intensity of the ketonic absorption is increased and that of the peak near 1600 cm.⁻¹ diminished by the addition of ethanol. The effects of ethanol in the chlorophyll *b* spectra are primarily in the relative intensities of the peaks and, thus are not as dramatic as for chlorophyll *a*. They confirm, however, the far-reaching effects of hydroxylic solvents on the spectra of the chlorophylls.

Cyclic β -Keto Esters as Model Compounds.—We have re-examined the infrared spectra, in CCl₄, of two cyclic β -keto esters, 2-carbethoxycyclopentanone and 2-carbethoxycyclohexanone, with results that confirm those of Leonard, *et al.*¹² The addition of ethanol or pyridine to the CCl₄ solutions does not shift, eliminate, or appreciably alter the intensity of the band at 1660 cm.⁻¹, assigned by Leonard, *et al.*, to chelated ester carbonyl in the enolic form. These compounds thus do not respond to solvent changes in the same way as do the chlorophylls. Further, Kabachnik, Yoffe, and Vatsuro¹⁷ have shown that the percentage of enol in 2-carbethoxycyclopentanone is low, of the order of 2–8%, and is about the same in both polar and nonpolar solvents. For this amount of enolization, hydrogen exchange in 2-carbethoxycyclopentanone is surprisingly slow¹⁴; consequently the concentration of the enol may be even lower, and the rate of enolization must also be slow. The molecular weight of these compounds, measured by a vapor pressure osmometer, shows them to be monomeric in chloroform solution. From these observations, cyclic β -keto esters are not particularly appropriate models for the properties of ring V in chlorophylls.

Yates and Williams¹⁸ have noted that the infrared spectrum of 3,4-diphenyl-2-cyclopenten-1-one is anomalous. In addition to a band at 1720 cm.⁻¹, another absorption peak of comparable intensity is observed at 1695 cm.⁻¹. The "extra" peak is explained by Fermi resonance between the carbonyl vibration and a closely lying overtone of the out-of-plane bending vibrations of the ethylenic hydrogen. When the ethylenic hydrogen is replaced by deuterium, the bending mode of the ethylenic hydrogen is displaced and only one absorption peak is evident in the carbonyl-stretching region. With fully deuterated chlorophyll, we have explored the possibility that the extra absorption peak in chlorophyll *a* might be due to Fermi resonance with a vibrational mode of the C-10 proton. Protio- and deuterio-chlorophyll *a* exhibited very similar spectra in the 1600–1800 cm.⁻¹ region. From this result, the extra peak in the chlorophyll *a* spectra cannot be explained in terms applicable to the cyclopentenones of Yates and co-workers.

Role of Magnesium in the Genesis of the Spectra.—The coordination properties of magnesium have not been considered in previous interpretations of the infrared spectra of the chlorophylls and their near derivatives. Magnesium usually has a coordination

number of four and normally assumes a tetrahedral structure in coordination compounds. In the chlorophylls and the methyl chlorophyllides, magnesium presumably has a planar configuration, and Parry¹⁹ and Eichhorn²⁰ have considered some possible consequences of the distortion of the usual magnesium structure. In the chlorophylls, however, the unusual planar configuration of the magnesium is not the only factor that needs to be taken into consideration. Thus, numerous compounds of magnesium are known in which magnesium exhibits a coordination number of six; magnesium halides form such compounds with alcohols, ethers, ketones, esters, and amides.²¹ Magnesium in phthalocyanin complexes shows a strong tendency to solvation by water,²² and Wei, Corwin, and Arellano²³ have found that magnesium-containing porphyrins form stable dipyrindine complexes similar to hemichromes, in which the magnesium exhibits octahedral coordination. Chlorophyll itself has long been known to coordinate water.²⁴ Perhaps the most convincing evidence for the coordinative unsaturation of the magnesium in the chlorophylls has been provided by Livingston,²⁵ who finds that chlorophylls *a* and *b* form stable monosolvates with Lewis bases such as water, alcohols, amines, ketones, and ethers, and that the chlorophylls are strongly fluorescent when solvated (in polar solvents) and nonfluorescent in the unsolvated state. Although it was originally postulated that solvation occurred by hydrogen bonding to the keto-oxygen of ring V, Evstigneev, *et al.*,²⁶ found that the magnesium-free pheophytins did not show changes in fluorescence intensity such as occur with the chlorophylls. It was, therefore, concluded that coordination with Lewis bases does not depend on the integrity of ring V but occurs only by way of the central magnesium atom. The evidence for coordinative unsaturation of the magnesium thus seems to be quite well established.

The important role of magnesium in the origin of the spectra in nonpolar solvents is clearly indicated by the behavior of various magnesium-containing and magnesium-free derivatives of chlorophyll. Figure 6 shows the spectra of the magnesium-free pheophytins *a* and *b* in carbon tetrachloride and in tetrahydrofuran solutions. The peak near 1650 cm.⁻¹, so prominent in the chlorophyll *a* spectrum in CCl₄, is missing in pheophytin *a*. The pheophytin spectra are essentially independent of solvent polarity, very different from the situation with the chlorophylls themselves. Similarly, the magnesium-free methyl pheophorbides *a* and *b* (not shown) have spectra that are also free of pronounced solvent effects. In a Nujol mull, methyl pheophorbide *a* shows no absorption near 1650 cm.⁻¹. The methyl chlorophyllides, on the contrary, show exactly the same solvent dependence as do the chlorophylls (Fig. 3). It is, therefore, difficult to avoid the conclusion that the peak near 1650 cm.⁻¹ in the chlorophyll *a* spectra in nonpolar solvents is related to the presence of magnesium.

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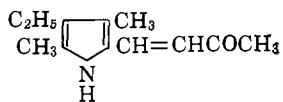
⁽²⁵⁾ R. Livingston and S. Weil, *Nature*, **170**, 750 (1952); R. Livingston, *Quart. Rev. (London)*, **14**, 174 (1960).

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⁽¹⁷⁾ M. I. Kabachnik, S. T. Yoffe, and K. V. Vatsuro, *Tetrahedron*, **1**, 317 (1957).

⁽¹⁸⁾ P. Yates and L. L. Williams, *J. Am. Chem. Soc.*, **80**, 5896 (1958); P. Yates, N. Yoda, W. Brown, and B. Mann, *ibid.*, **80**, 202 (1958).

We have carried out a few experiments designed specifically to detect coordination between magnesium and a carbonyl group. Magnesium tetraphenylporphyrin has been used as the magnesium chelate. In the presence of this substance, the C=O absorption peak of acetone is unaffected, as is also the case for N-methyl-2-pyrrole aldehyde. However, when a more highly conjugated ketone is employed, a shift in the C=O frequency is observed in the presence of the magnesium chelate. The acetone condensation product of cryptopyrrole aldehyde



in the presence of magnesium tetraphenylporphyrin shows a C=O absorption peak at 1605 cm.⁻¹; the peak is strongly diminished in intensity and the shift to lower frequencies (40 cm.⁻¹) is comparable to that observed in chlorophyll *a*. However, this is a very basic ketone and both of the compounds may not be good models for the chlorophyll situation.

Infrared Spectra of Chlorophyll *a*.—Our interpretation of the chlorophyll *a* spectra proceeds from the assumption that chlorophyll *a* in nonpolar solution exists in aggregated form, and that the peak near 1650 cm.⁻¹ is a consequence of this intermolecular aggregation. In solutions where the coordination unsaturation can be satisfied by the solvent or by added bases, this peak is absent. The extensive chemical work of Holt and Jacobs then provides a sound basis for the assignment of the other peaks in this region.

In polar solvents (tetrahydrofuran, triethylamine, tributyl phosphate), or in nonpolar solvents containing aliphatic alcohols or amines, the following assignments can be made: (1) the strong band at 1735 cm.⁻¹ results from the two ester carbonyl absorptions²⁷; (2) the strong band near 1700 cm.⁻¹ originates from the C-10 ketone carbonyl; (3) the relatively weak absorption peak near 1610 cm.⁻¹ originates from C=C or C=N skeletal vibrations.

In nonpolar solvents, however, the coordination unsaturation of the magnesium will be satisfied by intermolecular coordination with the ketone oxygen (or in the case of chlorophyll *b*, the aldehyde carbonyl). This results in a shift of the C-10 ketone absorption to lower frequencies by about 40 cm.⁻¹, and leads to the appearance of a new strong peak near 1650 cm.⁻¹. We shall refer to this absorption band as an *aggregation peak*. If disaggregation is favored, as by addition of alcohol, then the aggregation peak disappears with a simultaneous increase in the ketone absorption at 1700 cm.⁻¹ (relative to the ester peak at 1735 cm.⁻¹). Similarly, in polar solvents, the relative intensity of the ketone peak is markedly increased, and this may be taken as evidence for the participation of the ketone in molecular aggregation.

In carbon tetrachloride solution, the ketone peak is always visible. Since it is established by the molecular weight determination (see below) that chlorophyll *a* is predominantly a dimer in CCl₄ at the concentrations used for the infrared measurements, about one-half of the carbonyl groups are involved in aggregation if the dimer involves only one Mg··O=C< interaction. The degree of aggregation does not appear to be strongly concentration dependent; over a 40-fold concentration range, no significant differences in

the intensity of the aggregation peak are noted in CCl₄ solution.

Aggregation of chlorophyll *a* cannot only be reduced, it can also be enhanced. In the less-favorable solvent tetrachloroethylene, chlorophyll *a* shows a very strong aggregation peak (Fig. 7A). When hexane, in which chlorophyll is insoluble, is added to a CCl₄ solution of chlorophyll *a*, the chlorophyll undergoes further aggregation, as shown in Fig. 7B. Here the ketone peak has practically disappeared, and the spectrum closely resembles that of chlorophyll *a* crystals in a Nujol mull. In CCl₄ solution, the aggregation peak appears at 1653 cm.⁻¹; in 60% hexane-CCl₄, the peak appears at 1640 cm.⁻¹, which is also its position in the spectrum of solid chlorophyll *a* in a Nujol mull (Fig. 8A). We believe that the 1653 cm.⁻¹ peak is characteristic of the dimer and that the one at 1640 cm.⁻¹ is related to higher degrees of aggregation, of which the solid state represents the upper limit.

The effect of water on the spectra has been examined. Chlorophyll preparations often show broad absorption bands at 3400 cm.⁻¹ that probably originate from the presence of water. Jacobs, Vatter, and Holt²⁸ indicated that water was essential for the crystallization of chlorophyll, and our preparations have generally been crystallized by similar procedures in the presence of water. We believe, therefore, that the absorption at 3400 cm.⁻¹ arises from this cause, rather than from enolic OH as suggested by Holt and Jacobs.²⁸ This conclusion is reinforced by the observation of Siderov and Terenin⁹ that the 3400 cm.⁻¹ absorption peak is absent in a carefully dried mixture of chlorophyll *a* and *b* in pyridine solution.

Water and other volatile bases can be readily removed from chlorophyll by codistillation with carbon tetrachloride on the vacuum line. This does not of course imply that the coordination of water (or of carbonyl oxygen) is weak. Rather, the difference in bond

energies between magnesium and water, $\text{Mg} \cdots \text{OH}_2$, and between magnesium and carbonyl oxygen, $\text{Mg} \cdots \text{O}=\text{C}$, must be small, a circumstance that accounts for the ease with which chlorophyll aggregates are dissociated by bases, as well as the relative ease with which the bases can subsequently be removed.

The extent to which water acts as a disaggregating base depends on the solvent. In destabilized chloroform saturated with water, chlorophyll *a* shows the three-banded spectrum characteristic of disaggregating solvents; this observation is entirely consistent with the disaggregating effects of water on the n.m.r. spectra.⁵ In carbon tetrachloride, however, water does not have a pronounced effect, since the spectra of chlorophyll *a* solutions in CCl₄ are essentially unchanged when these solutions are shaken with water.

Infrared Spectra of Chlorophyll *b*.—The chlorophyll *b* situation is considerably more complicated than the *a*. In pheophytin *b* the peak at 1663 cm.⁻¹ is clearly, on the basis of Holt's work, to be assigned to the aldehyde carbonyl absorption. In polar solvents, therefore, the following assignments, based for the most part on the work of Holt and Jacobs, can be made: (1) the 1735 cm.⁻¹ peak arises from the ester absorptions; (2) the ketone of ring V absorbs near 1700 cm.⁻¹; (3) the peak near 1660 cm.⁻¹ is assigned to the aldehyde carbonyl; (4) the peak near 1600 cm.⁻¹ is due to C=C or C=N skeletal vibrations.

(27) The attribution of this peak to the ester carbonyl vibrations is supported by the work of H. R. Wetherell, M. J. Hendrickson, and A. R. McIntyre, *J. Am. Chem. Soc.*, **81**, 4517 (1959).

(28) E. E. Jacobs, A. E. Vatter, and A. S. Holt, *Arch. Biochem. Biophys.*, **53**, 228 (1954).

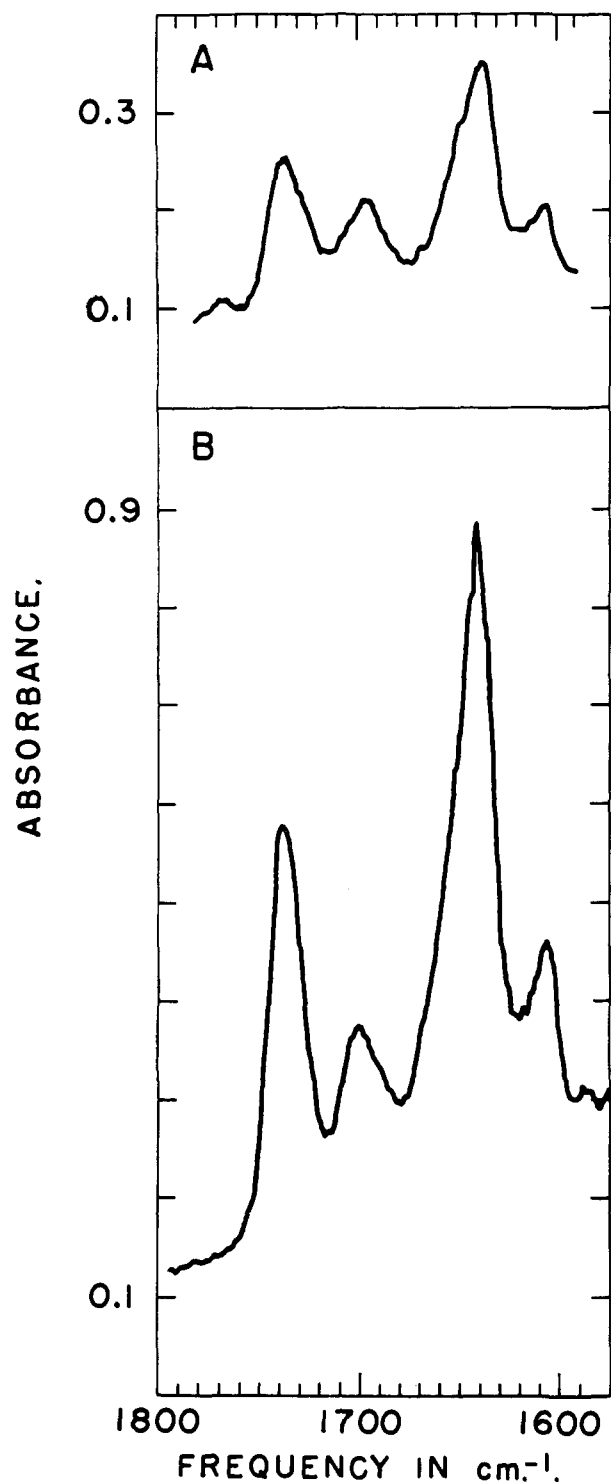


Fig. 7.—Infrared spectra of chlorophyll *a* in solvents that permit aggregation: A, tetrachlorethylene; B, in 60% hexane- CCl_4 .

The interpretation of the spectra of chlorophyll *b* in nonpolar solvents is much less definitive than for chlorophyll *a*. Both the aldehyde and ketone carbonyl oxygens can participate in aggregate formation, with the aldehyde carbonyl probably giving rise to the stronger coordination. There are thus two sources of aggregation peaks. Further, the aggregation peak originating from coordination by the ketone carbonyl oxygen is close in wave length to the absorption originating from the uncoordinated aldehyde carbonyl. It appears likely that the spectral consequences of coordination of the aldehyde carbonyl oxygen are to

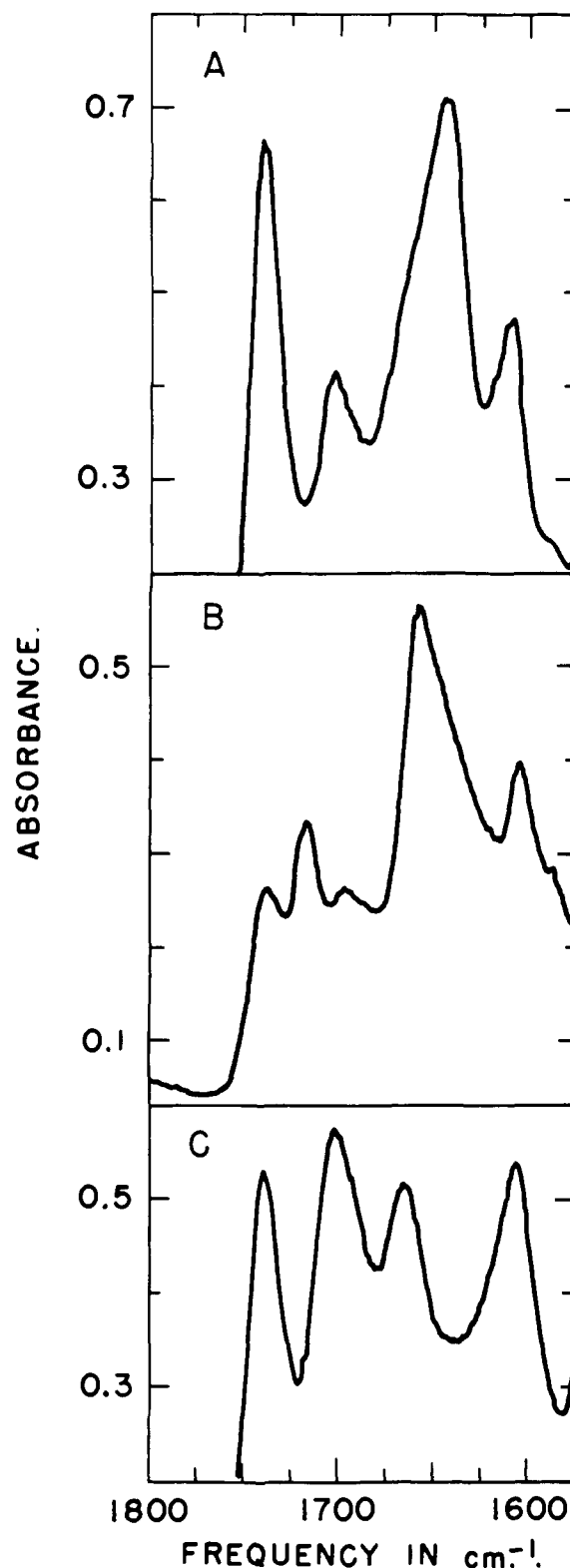


Fig. 8.—Infrared spectra of solid chlorophylls in Nujol mulls: A, chlorophyll *a*; B, methyl chlorophyllide *a*; C, chlorophyll *b*.

be found very near the 1600 cm^{-1} skeletal vibrations absorption. Thus, we consider the 1660 and 1600 cm^{-1} peaks to be composite in nonpolar solvents: (1) the peak near 1660 cm^{-1} is partially due to coordinated ketone oxygen and partially due to uncoordinated aldehyde oxygen; (2) the peak near 1600 cm^{-1} is partially due to skeletal vibrations but also contains a contribution from coordinated aldehyde oxygen. When a Lewis base is added to a chlorophyll *b* solution in CCl_4 , or if the chlorophyll *b* is dissolved in a polar

TABLE I
 AGGREGATION OF CHLOROPHYLLS AND PHEOPHYTINS IN VARIOUS SOLVENTS FROM MOLECULAR WEIGHT DATA

Solvent	Chlorophyll <i>a</i>			Chlorophyll <i>b</i>			Pheophytin <i>a</i>			Pheophytin <i>b</i>		
	Molarity		<i>R</i> ^b	Molarity		<i>R</i> ^b	Molarity		<i>R</i> ^b	Molarity		<i>R</i> ^b
	Calcd.	Obsd.		Calcd.	Obsd.		Calcd.	Obsd.		Calcd.	Obsd.	
Benzene	0.035	0.020	1.8	0.047	0.016	2.9						
	.065	.033	2.0	.066	.025	2.6						
	.106	.54	2.0	.103	.032	3.2						
Carbon tetra- chloride	.034	.016	2.1									
	.042	.024	1.9	.048	.016	3.0	0.051	0.040	1.3	0.040	0.034	1.2
	.063	.030	2.1				.059	.056	1.1	.054	.032	1.7
	.092	.046	2.0	.081	.022	3.7				.065	.042	1.5
Chloroform ^a	.045	.023	2.0	.042	.022	1.9	.030	.033	0.91	.023	.020	1.2
	.071	.041	1.7	.062	.028	2.2	.052	.055	.95	.050	.049	1.0
				.073	.039	1.9						
Chloroform + 0.5% ethanol	.036	.038	0.95	.043	.037	1.2	.039	.042	.93	.052	.052	1.0
	.057	.058	.98	.055	.052	1.1	.055	.057	.97			
	.075	.079	.95	.067	.060	1.1						

^a Destabilized by washing with water and drying. ^b Ratio of molarity calculated as monomer to the observed molarity.

solvent, disaggregation occurs at both loci. The C-10 ketone peak near 1700 cm.⁻¹ markedly increases in intensity, and the aggregation peak near 1660 cm.⁻¹ now becomes purely the aldehyde carbonyl absorption. From molecular weight measurements the possibility exists that coordination of the aldehyde to magnesium is not completely reversed by the polar solvent systems used here. However, an increase in the intensity of the 1660 cm.⁻¹ peak is indeed noted under polar conditions, and a distinct decrease in the intensity of the 1600 cm.⁻¹ band is also observed (Fig. 2B). Although the spectrum of solid chlorophyll *a* in a Nujol mull is different from that of the substance in benzene or carbon tetrachloride solution (see Fig. 8A), that of a chlorophyll *b* mull (Fig. 8C) is not, at first sight, radically different from the solution spectra. The most prominent difference between the spectra of the solid and the dissolved chlorophyll *b* is in the height of the peak near 1600 cm.⁻¹. In the solutions, this absorption peak is always weaker than the ester absorption at 1735 cm.⁻¹. This is in marked contrast to the situation with mulls of chlorophyll *a* or methyl chlorophyllide *a* (Fig. 8A and 8B) where the absorption peaks near 1600 cm.⁻¹ have almost the same relative intensity as in spectra taken in solution. In the solid chlorophyll *b*, the 1600 cm.⁻¹ peak is as strong as the ester absorption. We believe that this is confirmation for the view already expressed that coordination by the aldehyde carbonyl makes a contribution to the 1600 cm.⁻¹ peak in nonpolar solvents.

Molecular Weights.—The molecular weights of the chlorophylls and their derivatives were measured in CCl₄, benzene, destabilized CHCl₃, and CHCl₃ containing 0.5% ethanol using a vapor pressure osmometer (Table I). Aronoff²⁹ measured the molecular weight of chlorophyll *a* in benzene, and proposed a monomer-dimer equilibrium. Our results in benzene are in satisfactory agreement with those of Aronoff, and the vapor pressure osmometer appears to provide a useful method for the measurement of molecular aggregation in chlorophyll solutions.

Chlorophyll *a* in benzene, CCl₄, and destabilized CHCl₃ in the concentration ranges studied is found to be aggregated and to exist at least as a dimer. In CHCl₃ containing ethanol, however, chlorophyll *a* is monomeric, confirming the disaggregating effects of small amounts of alcohol.

The molecular weight measurements on chlorophyll *b* are especially informative, since our interpretation of the infrared spectra are based on considerations of the

relative intensities of composite bands. The measurements consistently showed that chlorophyll *b* is more highly aggregated than is chlorophyll *a*. In benzene solutions chlorophyll *b* is in fact nearly trimeric. As in the case of chlorophyll *a*, however, small amounts of alcohol cause nearly complete conversion of the chlorophyll *b* aggregate to monomer.

Methyl chlorophyllide *a*, as expected, is found to be aggregated in destabilized chloroform. The absence of a phytol chain should make it easier to form aggregates, and the general behavior of the chlorophyllides indicates this to be so. The low solubility of the methyl chlorophyllides precluded extensive measurements in other nonpolar solvents.

In general, the magnesium-free chlorophyll derivatives show only a small tendency to aggregation in nonpolar solvents. Pheophytin *a* is predominantly monomeric in CCl₄ or CHCl₃, as well as in CHCl₃ containing alcohol. Pheophytin *b* appears to form significant amounts of aggregates in CCl₄, but is monomeric in pure CHCl₃, as well as in CHCl₃ containing small amounts of alcohol. Molecular aggregation in nonpolar solvents of the magnesium-free compounds is thus markedly less than for the chlorophylls themselves. It thus appears that there is a close connection between the presence of "extra bands" in the infrared spectra and the degree of molecular aggregation. Molecular aggregation is found to have an equally pronounced effect in the proton magnetic resonance spectra of the chlorophylls.⁵ While it is perhaps premature to extrapolate the present findings to the green leaf, nevertheless molecular aggregation must certainly be taken into account when the physical chemical behavior of the chlorophylls is considered.

Molecular Aggregation and Properties of Chlorophyll.—The observations on molecular weights and on infrared spectra are pertinent to interpretation of the effect of solvents upon the spectral properties of chlorophylls in the visible region. The solid chlorophylls are but slightly soluble in petroleum ether. They are non-fluorescent, and their absorption maxima occur at very long wave lengths in the red region. Upon the addition of even a little polar solvent (*e.g.*, 0.5% 1-propanol) to petroleum ether, the chlorophylls become very much more soluble. The solutions become strongly fluorescent, and the absorption maxima are shifted to shorter wave lengths.³⁰

The current observations are also of significance to interpretation of the effect of solvents upon the chromatographic behavior of the chlorophylls. When the

(29) S. Aronoff, *Arch. Biochem. Biophys.*, **98**, 344 (1962).

(30) H. H. Strain, *Science*, **116**, 174 (1952).

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 Brody³² 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 cocklebur 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 μ l. 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.

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[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 Pheophorbides¹

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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 reinter-

pretation 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 phytol 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

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(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).