INFRA-RED ABSORPTION SPECTRA OF CHLOROPHYLLS AND DERIVATIVES[1,2]

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We have measured the infra-red absorption spectra of chlorophyll and some of its derivatives in order to obtain information useful for eventual understanding of reversible transformations of this pigment. Such transformations are (a) Molisch phase test, (b) bleaching by $FeCl_3$, and (c) photochemical reduction by ascorbic acid—"Krasnovsky reaction" (10, 13, 16). Only in the case of the phase test is there evidence of the specific group involved (the cyclopentanone ring). Whether this ring is involved in the other reactions is not known. However, if the C=O bands in the spectrum of the parent substance are correctly assigned, any change in this ring should be apparent from the infra-red spectrum of the intermediate. To obtain unequivocal assignments of the C=O stretching bands we prepared and studied appropriate derivatives. In addition we studied the influence of solvent on the spectrum and found strong effects in the C=O region. These solvent effects were not studied by earlier investigators (18, 19).

The complexity of the spectra below 1600 cm^{-1} is so great that we have not attempted to assign the bands. Spectra are presented from 650 to 3800 cm^{-1} in order to make the data available for future comparisons (except in cases of chlorophyll a in ethyl ether and ethyl pheophorbide a-oxime in $CHCl_3$).

MATERIALS AND METHODS

Preparative techniques for most of the pigments studied have been described by us elsewhere (5, 6, 8). Those not described are as follows:

1. Pheophorbide a: Two-tenths gm of pheophytin a were incubated in a mixture of 80 % acetone (v/v, aqueous) and pigment-free meal obtained from leaves of *Ailanthus altissima*. Complete removal of phytol by chlorophyllase after 4 hours was demonstrated by transferring a test sample into ethyl ether and extracting with 0.01 *N* KOH (22). The entire pigment was then transferred into ether, dried, and adsorbed on a powdered sucrose column from 40 % $CHCl_3$ + 60 % petroleum ether mixture. The column was treated first with benzene, and then with 0.05, 1.0 and 2.0 % isopropanol in petroleum ether. The sugar bearing the main part of the pigment was removed and washed with petroleum ether to remove residual chloroform and benzene. The pigment was eluted from the sugar with acetone, transferred into ethyl ether, dried and subjected again to chromatographic purification. The product gave a positive Molisch phase test and had an absorption spectrum in the visible region identical with the known spectrum of ethyl pheophorbide a (5).

2. Pyropheophorbide a: This compound was prepared according to Fischer and Siebel (3). A solution of pheophorbide a in pyridine containing Na_2CO_3 was refluxed for 3 hours. The pigment was transferred into ethyl ether and extracted three times with 10 % HCl to remove phylloerythrin. A fraction was then extracted with 14 % HCl, transferred back into ethyl ether, dried in vacuo and adsorbed on a powdered sucrose column from 40 % $CHCl_3$ + 60 % petroleum ether. The column was treated first with benzene and then with 0.5 % isopropanol + 30 % chloroform in petroleum ether. The pigment-bearing zone was removed and the pigment was eluted and re-purified by chromatography.

3. Mono-2,4-dinitrophenylhydrazine derivatives of the following substances were prepared by a modification of the procedure of Iddles et al (7):

(a) Pheophorbide a: Two ml of 99.5 % ethanol saturated with 2,4-dinitrophenylhydrazine were acidified with 0.1 ml of 6 *N* HCl, and added to 20 mg of pheophorbide a dissolved in 2 ml of ethanol; the mixture was boiled for 2 minutes. A precipitate, which formed overnight at room temperature, was collected by centrifugation; it was washed twice with 75 % ethanol (aqueous) and dried in vacuo.

(b) Ethyl pheophorbide b: Twenty mg of ethyl pheophorbide b in 6 ml of 99.5 % ethanol were added to an equal volume of a saturated ethanol solution of 2,4-dinitrophenylhydrazine. The mixture was acidified with 0.04 ml of 6 *N* HCl and boiled for 2 minutes. After storage at room temperature for 3 hours, the precipitate was collected, washed twice with 75 % ethanol (aqueous) and dried. According to analysis this procedure gave only the mono-derivative:

	Percent		
	C	H	N
Calculated	62.8	5.31	13.7
Observed	63.5	4.96	13.8

(c) Pyropheophorbide a: This compound was prepared in the same manner as the derivative of pheophorbide a.

4. Ethyl pheophorbide a-oxime: This was prepared according to the method of Fischer et al (2). Forty-two mg of ethyl pheophorbide a were dissolved in 2.5 ml of pyridine containing 0.33 gm of $NH_2OH \cdot HCl$, and allowed to stand 8 days in darkness at room temperature. The product was transferred into ether, and the solution was extracted first with 10 % HCl, and then with 14 % HCl. The pigment extracted with 14 % HCl was transferred back into ether and

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dried. Its spectrum was measured without further purification.

The infra-red spectra were measured on a Perkin-Elmer Model 21 Double Beam Recording Spectrophotometer. Matched, sealed absorption cells with rock salt windows were used; the solvents were reagent grade. In the case of the phyllins no noticeable decomposition occurred during the measurement. This was shown by two methods: (a) measuring the infra-red absorption spectrum of a sample twice with an interval of 1 hour between measurements; and (b) by comparing the "purity ratios" (23) in the visible absorption spectra, before and after the measurement. The accuracy of the concentrations (w/v) indicated in the legends of the absorption curves was ± 5 %. Frequencies assigned to the bands in table I are correct to ± 2 cm^{-1} from 650 to 2000 cm^{-1} and to ± 10 cm^{-1} from 2000 to 3800 cm^{-1}.

Structural formulae of the compounds studied are those given by Fischer and Stern (4) and are indicated in figure 1.

Results and Interpretations

Table I presents a summary of the bands assigned.

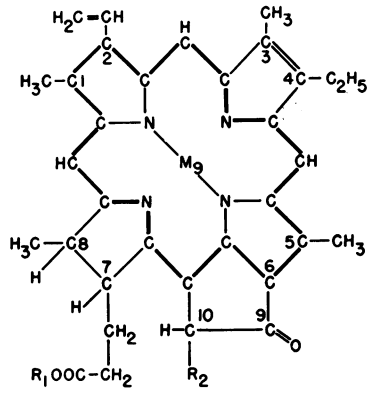
Spectra of Pheophytin and Its Derivatives: Pheophytin a and ethyl pheophorbide a contain three C=O groups—the ketone at C_9 in the cyclopentanone ring, and the propionate and carbmethoxy groups at C_7 and C_{10}, respectively. Two C=O stretching bands can be anticipated.

Ethyl pheophorbide a (in $CHCl_3$—fig 2), pheophorbide a (in $CHCl_3$—fig 3), and pheophytin a (in pyridine and in CCl_4—fig 4) show in fact two C=O bands. A third band in the double bond region, at 1618 to 1620 cm^{-1}, is due to a C=C vibration.

In figure 3, we see that the oxime of ethyl pheophorbide a has, as expected, only one C=O band, and that its maximum lies at 1737 cm^{-1}. The absence of the 1697 cm^{-1} band which appears in the spectrum of ethyl pheophorbide a confirms that this band is attributable to the ketone C=O. the C_7 and C_{10} ester C=O groups must account for the 1737 cm^{-1} band.

The spectrum of pheophorbide a in the C=O region differed from that of ethyl pheophorbide a. The ketone C=O band appeared broadened and shifted to 1702 cm^{-1}. This suggested that the C=O in the carboxyl group of the propionic acid absorbs on the high frequency side of the ketone band. The presence of the carboxyl group is confirmed by the broad OH stretching band between 2400 and 2800 cm^{-1} (1). In the spectrum of the phenylhydrazine derivative of pheophorbide a the most intense C=O band appears at 1728 cm^{-1}; it must be assigned to the one remaining ester group—the C_{10} carbmethoxy group. The shoulder at 1705 cm^{-1} must be due to the band of the carboxyl C=O of the C_7 propionic acid. This assignment agrees well with the observations of Jones et al (9) in studies of steroid acids.

In the spectrum of pyropheophorbide a (which lacks the C_{10} carbmethoxy group) a shoulder at 1704 cm^{-1}, superimposed upon the more intense ketone band at 1685 cm^{-1}, must again be attributed to the propionic acid carboxyl C=O. In the phenylhydrazine derivative of the same compound, only the C=O of propionic acid remains. Unfortunately, this deriva-



COMPOUND	Mg	R_1	R_2
CHLOROPHYLL a	+	$C_{20}H_{39}$	$-C(=O)OCH_3$
PHEOPHYTIN a	−	"	"
ETHYL CHLOROPHYLLIDE a	+	C_2H_5	"
ETHYL PHEOPHORBIDE a	−	"	"
PHEOPHORBIDE a	−	H	"
PYROPHEOPHORBIDE a	−	H	H
b COMPOUNDS SAME AS ABOVE EXCEPT C_3 METHYL GROUP = $-C(=O)H$			
C_2 VINYL = $-C(=O)CH_3$, $\Delta^{3,4}$ SATURATED			
BACTERIOCHLOROPHYLL	+	$C_{20}H_{39}$	$-C(=O)O-CH_3$
METHYL BACTERIOCHLOROPHYLLIDE	+	CH_3	"

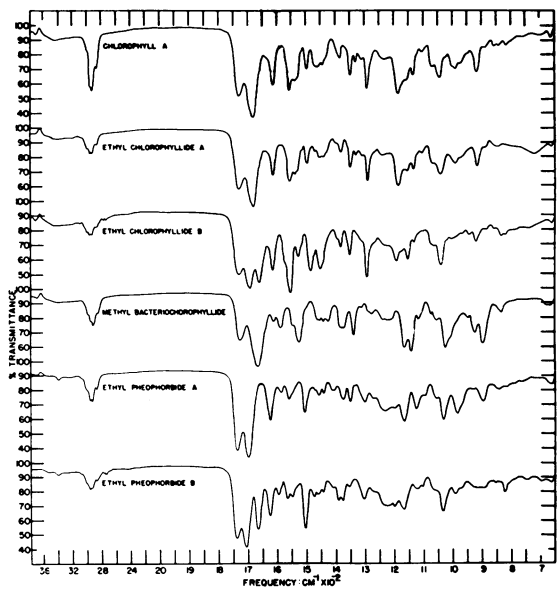


Fig. 1 (*above*). Structure of chlorophylls and derivatives.

Fig. 2 (*below*). Infra-red absorption spectra of chlorophyll and derivatives. Solvent—$CHCl_3$. Concentration—2 %. Cell depth—0.2 mm.

tive was not sufficiently soluble in $CHCl_3$, and its spectrum had to be measured from Nujol (liquid petrolatum) mull. The carboxyl C=O band was found broadened and shifted to 1725 cm^{-1}, probably due to the solid state (1).

The assignment of the 1610 cm^{-1} band to semi-isolated or vinyl C=C (19), is not confirmed by the spectra of the phenylhydrazine derivatives. The intensified absorption at 1613 to 1618 cm^{-1} with these compounds is due to conjugated C=C bonds in the phenyl group. This supports the assignment of this band in the spectra of the phyllins and phytins to conjugated C=C, since the same band is seen also in the spectrum of bacteriochlorophyll (fig 6).

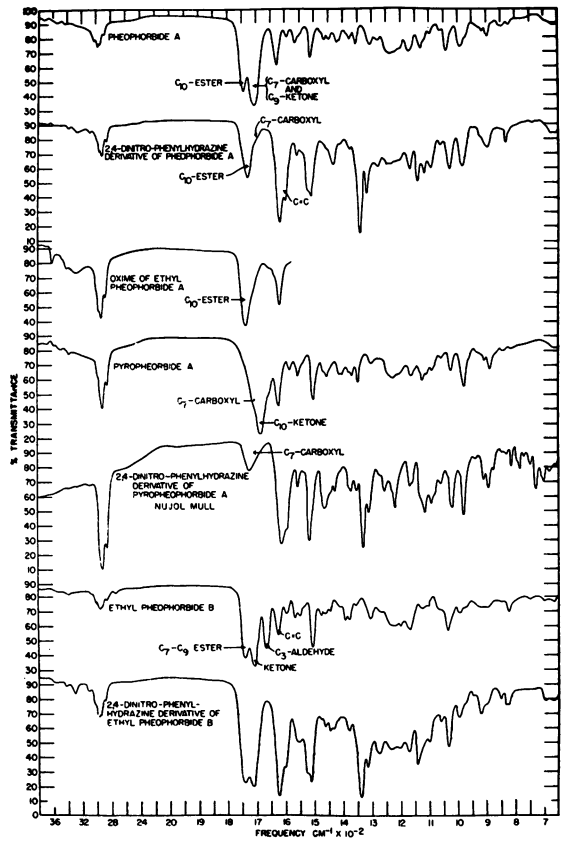
The spectra of pheophytin a in the two solvents CCl_4 and pyridine, are quite similar in the double bond stretching region, but are very different in the higher frequencies. In pyridine (fig 4) we note a broad band between 3700 and 3100 cm^{-1} with a more narrow band superimposed on it at 3382 cm^{-1}. We attribute this double band to vibrations of two different groups—the N—H groups of pyrrole nuclei accounting for the sharp maximum at 3382 cm^{-1}, and a H-bonded or otherwise associated OH group accounting for the broad band. An associated OH group can result from enolization between C_9 and C_{10} and strong bonding of the enolic OH at C_9 with N atom of pyridine (compare with the interpretation of the chlorophyll a spectrum in CCl_4 or CS_2 below). In the spectrum of pheophytin a in CCl_4 (fig 4) the weak band at 3392 cm^{-1} can be attributed to N—H stretching in agreement with the assignment of Weigl and Livingston (19). Phytol causes the strong C—H stretching band at 2920 cm^{-1}.

The band of the C_3 aldehyde group of ethyl pheophorbide b absorbs at 1663 cm^{-1} as proved by its disappearance from the spectrum of the phenylhydrazine derivative and its absence from the spectra of pheophorbide a and ethyl pheophorbide a (fig 2, 3). The characteristic weak C—H stretching band at 2720

TABLE I

INFRA-RED ABSORPTION BANDS OF CHLOROPHYLLS AND DERIVATIVES. FREQUENCIES CORRECTED (cm^{-1})

COMPOUND	SOLVENT	NH	OH	C—H PHYTOL	C—H ALDEHYDE	C=O ESTER	C=O KETONE	C=O CARBOXYL C_7	C=O ALDEHYDE	C=O CHELATE	C=C
Ethyl chlorophyllide a	$CHCl_3$	....	....	....	....	1733	1678	....	....	....	1610
Chlorophyll a	Crystals in Nujol	3605?	3440+ 3252	2920	....	1727+ 1740	1694	....	....	1640	1604
Chlorophyll a	CCl_4, CS_2	....	3350 (weak)	2916	....	1735	1691	....	....	1652	1610
Chlorophyll a	Ethyl ether	....	....	....	....	1738	1698	....	....	....	1608
Chlorophyll a	Pyridine	....	3400 (strong)	....	....	1740	1683	....	....	....	....
Ethyl chlorophyllide b	$CHCl_3$	....	....	....	2720	1730	1689	....	1657	....	1610
Chlorophyll b	$CHCl_3$	....	....	2920	2720	1728	1688	....	1655	....	1610
Chlorophyll b	Crystals in Nujol	....	3360	2924	2710	1738	1697	....	1655	....	1607
Chlorophyll b	CCl_4, CS_2	....	3350	2924	2710	1738	1701	....	1664	....	1609
Pheophytin a	CCl_4	3392	....	2930	....	1740	1705	....	....	....	1618
Pheophytin a	Pyridine	3382	3100-3700	2920	....	1736	1700	....	....	....	1617
Ethyl pheophorbide a	CS_2	3375	....	....	....	1743	1706	....	....	....	1618
Ethyl pheophorbide a	$CHCl_3$	3380	....	....	....	1736	1697	....	....	....	1620
Pheophorbide a	$CHCl_3$	....	2400-2800	....	....	C_{10}: 1741	1702	1702	....	....	1622
Pheophorbide a-2,4-dinitrophenyl-hydrazone	$CHCl_3$	....	....	....	....	C_{10}: 1728	....	1705	....	....	1613
Pyropheophorbide a	$CHCl_3$	....	....	....	....	....	1685	1704	....	....	1620
Pyropheophorbide a-2,4-dinitrophenyl-hydrazone	Nujol mull	....	....	....	....	....	....	1725	....	....	1610
Pheophytin b	CCl_4	3410	....	2930	2720	1740	1709	....	1665	....	1618
Ethyl pheophorbide b	$CHCl_3$	3380	....	....	2720	1737	1704	....	1663	....	1620
Ethyl pheophorbide b-2,4-dinitrophenyl-hydrazone	$CHCl_3$	....	....	....	....	1740	1710	....	....	....	1618
						ester	*ketone*		*acetyl*		
Bacteriochlorophyll	CCl_4	....	3350	2916	....	1735	1683		1655		1610
Methyl bacteriochlorophyllide	$CHCl_3$	....	....	....	....	1727	1662		1662		1610



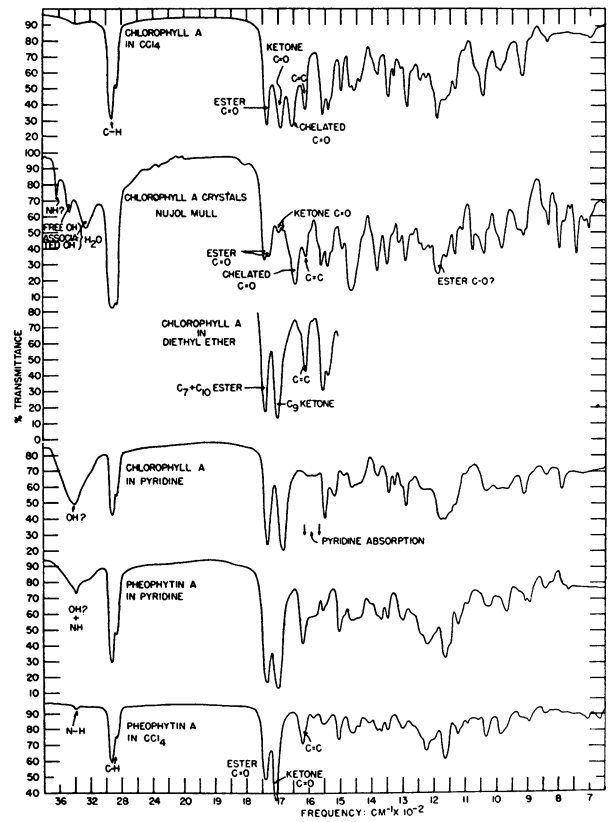


FIG. 3 (*left*). Infra-red absorption spectra of chlorophyll derivatives. Solvent—$CHCl_3$. Cell depth—0.1 mm. Concentrations (starting with uppermost curve)—4 %, 4 %, 3.5 %, 4 %, Nujol mull, 2 %, 6 %.

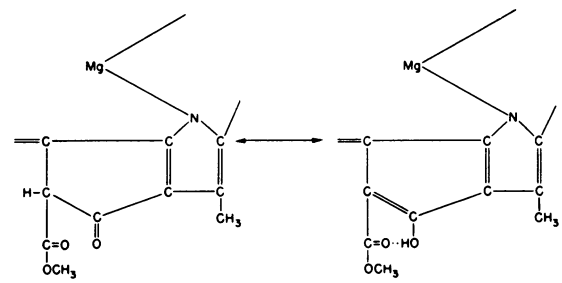
FIG. 4 (*right*). Infra-red absorption spectra of chlorophyll a and pheophytin a in different solvents. Cell depth—0.1 mm. Concentrations—Chlorophyll a (CCl_4—6 %; ethyl ether—6 %; pyridine—3 %), pheophytin a (CCl_4—6 %; pyridine—6 %).

cm^{-1}, seen in figure 5 (15) is further confirmation of the presence of an aldehyde group.

SPECTRA OF PHYLLINS AND DERIVATIVES: Except at 2920 cm^{-1} where C—H groups of phytol absorb strongly, the spectra of chlorophyll a and its ethyl derivative are quite similar (fig 2). As with pheophytin a in $CHCl_3$ the ester C=O groups absorb at 1735 to 1740 cm^{-1}. The ketone band is, however, shifted to 1678 cm^{-1} (a shift of 19 cm^{-1} to lower frequencies) and the C=C band to 1610 cm^{-1} (a shift of 7 to 8 cm^{-1} to lower frequencies).

The aldehyde C=O of ethyl chlorophyllide b absorbs at 1657 cm^{-1}; other double bond bands lie at 1730, 1689 and 1610 cm^{-1} (see fig 5 for spectrum of chlorophyll b in $CHCl_3$).

In figure 4 are the spectra of chlorophyll a in CCl_4, pyridine and ethyl ether; the spectrum of crystalline chlorophyll a dispersed in Nujol is also included. We note that in CCl_4 solution four bands occur between 1735 and 1610 cm^{-1}, in contrast to only three in $CHCl_3$ or ether solutions (only two are visible in pyridine solution, since the band at ca 1610 cm^{-1} is blocked by the intense absorption of pyridine itself). Pheophytin a in CCl_4 (fig 4) shows only three bands. We believe this extra band, which appears only when chlorophyll a (or ethyl chlorophyllide a), but not pheophytin a, is dissolved in non-polar solvents (CS_2, CCl_4), results from enolization and chelation, as follows:



Precedent for this interpretation is found in studies of Leonard et al (11) dealing with chelation in β keto esters of cyclopentanone, e.g., diethyl cyclopentanone-2-5-dicarboxylate. In the spectrum of this compound an additional band of medium intensity was observed at 1671 cm^{-1} and assigned to chelated C=O. The absence of an enolic OH band in the spectra of keto

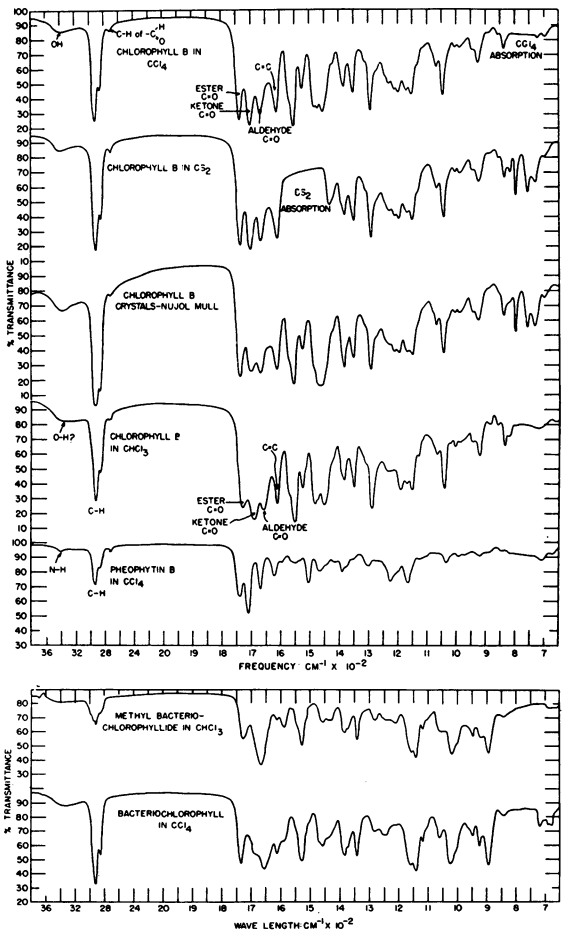


FIG. 5 (*above*). Infra-red absorption spectra of chlorophyll b and pheophytin b in different solvents. Cell depth—0.1 mm. Concentrations: Chlorophyll b (CCl_4—6 %; CS_2—7.4 %; $CHCl_3$—6 %), pheophytin b—3 %.

FIG. 6 (*below*). Infra-red absorption spectra of methyl bacteriochlorophyllide in $CHCl_3$ (2 %) and bacteriochlorophyll in CCl_4 (5 %). Cell depth—0.1 mm.

esters of cyclohexanone also was noted by these workers, and interpreted as a confirmation that the enol existed chiefly in the chelated form. The weak broad OH band at ca 3350 cm^{-1} seen in the spectrum of chlorophyll a may indicate the presence of free enolic OH, but may also be due to bound or associated H_2O molecules. This possibility is suggested by our earlier conclusion that water is necessary for crystallization of chlorophyll a, b and bacteriochlorophyll (8), and also by the presence of bands of free and associated OH in the spectrum of crystalline chlorophyll a dispersed in Nujol. From the much greater intensity of absorption by chelated C=O relative to that by the ester or ketone C=O groups, we infer that in the crystalline state, chlorophyll a exists almost entirely in the chelated form. A sharp band at 3605 cm^{-1}, which is absent in the spectra of solutions, is noticeable also in the spectrum of the crystals. We have tentatively assigned it to N—H groups in the pyrrole nuclei, but cannot explain the shift from 3392 cm^{-1} where the N—H band is located in solutions of pheophytin a; nor can we account for its increased intensity.

In pyridine, chlorophyll a shows evidence of a very strong associated OH band, and no evidence of a chelated C=O band. It appears that enolization has occurred, but that chelation of the β keto ester has been hindered by strong bonding between the enolic OH group at C_9 and the nitrogen atom in pyridine.

Figure 5 shows the spectra of crystals of chlorophyll b in Nujol, and of chlorophyll b dissolved in CCl_4, CS_2, and $CHCl_3$. In all spectra we note four bands in the region 1735 to 1600 cm^{-1}, which, from the evidence given above are characteristic respectively of ester, ketone, and aldehyde C=O groups and of C=C bonds. We note also that the spectrum of crystals in Nujol is very similar to the solution spectra. This is in contrast to the case of chlorophyll a for which the spectrum of crystals is quite different, in the C=O region, from the spectra in polar solvents. We found but one sample of chlorophyll b with an additional band that may be ascribed to chelated C=O; this band was located at 1645 cm^{-1}. On further chromatographic purification and recrystallization (shown by X-ray diffraction) this band no longer appeared. Twenty subsequent spectra gave no evidence of this band. The consistency of these results suggests that chlorophyll b does not enolize or chelate, in CCl_4, CS_2 or when crystallized. Because an OH bending vibration occurs at 1642 cm^{-1} we first surmised that the extra band seen with the earlier sample was due to water (17). However, while the addition of water to solutions of chlorophyll b in CCl_4 increased the absorption at this frequency, it did not produce the originally observed sharp band.

The spectrum of bacteriochlorophyll in CCl_4 is shown in figure 6 with that of methyl bacteriochlorophyllide in $CHCl_3$. Here again we see considerable difference between spectra in the two solvents. Two bands in the double bond region can be assigned with assurance—the ester C=O band at 1735 cm^{-1} (CCl_4), 1727 cm^{-1} ($CHCl_3$) and the C=C band at 1610 cm^{-1}. In $CHCl_3$ the intense band at 1662 cm^{-1} must be assigned to the ketone groups at C_9 and C_2. In CCl_4, we note a separation of this band into two bands, the more intense one lying at 1655 cm^{-1} and the other broadened one, lying at 1683 cm^{-1}. Unfortunately, we did not have sufficient pigment to prepare derivatives which would permit us to distinguish between the cyclopentanone and the acetyl C=O groups. Tentatively, we assign the band at 1685 cm^{-1} to the cyclopentanone C=O, and the more intense band at 1657 cm^{-1} to the conjugated acetyl C=O. However, the reverse assignments may also be possible, in which case bacteriochlorophyll in CCl_4 would have the chelated-enol form. Further work must be done to clarify the assignments of the C=O bands of this compound.

Discussion

From the spectra described above we see that the β keto ester of the cyclopentanone ring of chlorophyll a is enolized and chelated, both in the crystalline state and in CCl_4 and CS_2. This is not the case when the same pigment is dissolved in other solvents, e.g., $CHCl_3$ or ether. In pyridine it appears that the pigment is enolized but not chelated. The spectrum of pheophytin a in CCl_4 shows that the removal of magnesium from chlorophyll a prevents enol formation, except in basic solvents such as pyridine. This result is interesting and invites speculation. Since magnesium is less electronegative than the hydrogen by which it is replaced in pheophytin (14), it follows that the C_9 keto oxygen will be more negative when magnesium is present, and enolization will be facilitated.

The reason why chlorophyll b does not enolize and chelate in CCl_4 may be because the electronegative C_3 aldehyde group can reduce the ability of magnesium to induce a negative charge on the C_9 keto oxygen.

It is also known that in anhydrous alcohol chlorophyll a is allomerized more rapidly than chlorophyll b, and that pheophytins a and b are not allomerized (4). Such results would be expected if formation of the enol is prerequisite to production of the unstable intermediate which reacts with oxygen. Weller and Livingston (20, 21) hypothesize that this intermediate is produced as the result of traces of base present in the alcohol and that it is actually the phase-test intermediate.

The fact that chlorophyll b does not chelate in CCl_4 may also be due to hydrogen bonding between traces of polar substances, e.g., H_2O and the C=O groups of the β keto ester. Livingston et al (12) have suggested that such an addition is responsible for the activation of chlorophyll fluorescence. The keto form is presumed to be fluorescent, and the chelated-enol form to be non-fluorescent. This hypothesis could now be tested by measurement of the infra-red spectrum of chlorophyll b dissolved in the specially purified benzene used by these workers. A chelated C=O band should appear if the non-fluorescent form is a chelated enol.

Summary

1. C=O, C=C, C—H and N—H groups in chlorophylls and their derivatives have characteristic infra-red absorption bands. In the double bond stretching region the absorption bands of the following groups have been assigned: C_7 and C_{10} ester C=O; C_9 ketone C=O; C_7 carboxyl C=O of the free acid; C_3 aldehyde C=O of the b series; conjugated C=C.

2. When chlorophyll a is dissolved in the polar solvents $CHCl_3$ and ethyl ether, the β keto ester of the cyclopentanone ring exists in the keto form. In pyridine, the enol form is present as shown by a strong associated OH stretching band. In the non-polar solvents CCl_4 and CS_2, and when the pigment is crystalline, the enol form is chelated, as shown by the appearance of a chelate C=O stretching band.

3. Pheophytin a shows neither a chelate C=O band nor an enolic OH band when dissolved in CCl_4 or CS_2. An enolic OH band appears in pyridine solution.

4. Chlorophyll b does not enolize and chelate when dissolved in $CHCl_3$, CCl_4 or CS_2, nor when it crystallizes.

5. It is suggested that the C_9 keto oxygen is more negatively charged (thus facilitating enolization) when magnesium is present than when it is absent as in pheophytin, and that it is more negatively charged in chlorophyll a than it is in chlorophyll b.

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