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THE DIMERIZATION OF CHLOROPHYLL a, CHLOROPHYLL b AND BACTERIOCHLOROPHYLL IN SOLUTION

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# THE DIMERIZATION OF CHLOROPHYLL a, CHLOROPHYLL b AND BACTERIOCHLOROPHYLL IN SOLUTION

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# The Dimerization of Chlorophyll a, Chlorophyll b and Bacteriochlorophyll in Solution[1]

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## ABSTRACT

Analyses of the absorption spectra of three chlorophylls in carbon tetrachloride solution demonstrate the existence of monomer-dimer equilibrium in the concentration range from $10^{-6}$ to $10^{-3}$ mole-$l^{-1}$. The dimerization constants, $K_d = C_d/C_m^2$, are $(1.0 \pm 0.4) \times 10^4$ l-mole$^{-1}$ for chlorophyll a, $(0.8 \pm 0.3) \times 10^4$ l-mole$^{-1}$ for chlorophyll b and $(2.2 \pm 0.7) \times 10^4$ l-mole$^{-1}$ for bacteriochlorophyll at $24 \pm 2°C$, corresponding to standard free energies of dimer formation of -5.4, -5.3 and -5.8 kcal-mole$^{-1}$, respectively.

The absorption spectra of pure monomer and pure dimer in carbon tetrachloride are calculated for each pigment. For each of the chlorophyll dimers the long wavelength absorption band consists of a principal peak centered at approximately the position of the monomer absorption maximum and a shoulder to long wavelengths. The relative oscillator strengths of the split components indicate that the corresponding transition moments for the two molecules in each dimer are nearly

perpendicular to one another. The proton magnetic resonance spectrum of aggregated bacteriochlorophyll in $DCCl_3$ exhibits characteristic features similar to those previously reported for aggregated chlorophylls a and b. The evidence from these investigations leads to the conclusion that the structures of the dimers are nearly identical for the three chlorophyll molecules.

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The nature of the interaction of chlorophyll molecules with one another is of particular interest in relation to the organization of these molecules in photosynthetic systems. The presence of aggregated chlorophyll has frequently been invoked to account for complex features of the absorption spectra of plant chloroplasts and bacterial chromatophores. In a recent study evidence supporting an aggregated state of chlorophyll *in vivo* was obtained from measurements of the optical rotatory dispersion spectra of suspensions of chloroplast lamellar fragments.[3]

The formation of chlorophyll *a* dimers in concentrated solution in saturated hydrocarbons was first reported by Lavorel[4] and by Weber and Teale[5], based on comparisons of absorption spectra with action spectra for fluorescence. The dimer gave a broadened absorption spectrum, but did not contribute to the fluorescence of the solution. Further studies by Weber[6] of the fluorescence polarization and efficiency as a function of concentration led to the calculation of dimerization constants for chlorophyll *a* of 130 $l\text{-mole}^{-1}$ in liquid paraffin and 4.5 $l\text{-mole}^{-1}$ in ether. The latter value was based on data of Watson and Livingston;[7] the original authors had interpreted their own results as indicating the absence of appreciable concentrations of dimers. Brody and Brody[8] reported chlorophyll *a* dimer formation in concentrated ethanol solutions on the basis of absorption spectrum broadening; however, Stensby and Rosenberg[9] found evidence that in concentrated ethanol solutions the dimers are present in significant amounts only at temperatures well below room temperature. The only direct quantitative determination monomer-dimer equilibrium in solution is that of Aronoff[10]. From

measurements of the vapor pressure lowering as a function of chlorophyll a concentration in benzene, an equilibrium constant of 459 l-mole$^{-1}$ at 310°K was obtained.

The association of chlorophyll a, chlorophyll b and several of their derivatives has been studied using nuclear magnetic resonance, infrared spectrophotometry, and molecular weight measurements by Katz, and coworkers[11] and by Anderson and Calvin[12]. These investigators conclude that aggregation results chiefly from the interaction of the central magnesium of one chlorophyll molecule with a carbonyl functional group of the second. In each case the carbonyl group of ring V appears to be strongly involved; and in the case of chlorophyll b, the formyl substitutent of ring II also interacts with the magnesium, apparently leading to trimer formation at high concentrations.

In this paper we report studies on the monomer-dimer equilibrium of chlorophyll a, chlorophyll b and bacteriochlorophyll in carbon tetrachloride over a wide range of concentrations using the absorption spectra of the solutions as a measure of the species present. The methods are similar to those used to study the dimerization of methylene blue[13] and of cyanine dyes[14]. We have increased the sensitivity of the approach by measuring difference spectra between solutions of different concentrations with path lengths inversely proportional to the concentrations.

For each of the three chlorophylls, the evidence indicates that a simple monomer-dimer equilibrium exists throughout the concentration range from $10^{-6}$ mole-l$^{-1}$ to greater than $10^{-4}$ mole-l$^{-1}$. It is therefore possible to calculate the absorption spectra of pure monomer and pure

dimer for each of the chlorophylls in carbon tetrachloride. Analysis of the results suggests that the structures of the dimers are very nearly the same for the three pigments.

## Experimental

### Isolation of the Chlorophylls.

Chlorophylls a and b were obtained from spinach leaves using a modification of the procedure of Anderson and Calvin.[15] The mixed chlorophylls in an aqueous acetone extract are separated from the xanthophylls and other carotenoids using chromatography on polyethylene. Following the transfer of the chlorophylls to isooctane as solvent, the chlorophylls a and b are separated from one another by chromatography on powdered sugar. The separate isooctane solutions of chlorophylls a and b are each washed 5 times with water to remove contaminants (principally cornstarch) leached from the sugar. The chlorophylls precipitate from the isooctane solution upon standing overnight in the dark at -15°C. The solid material is collected by centrifugation, dried under vacuum and stored at room temperature in the dark.

Anal. Calc. for Chl a · $H_2O$:
C, 72.47; H, 8.18; N, 6.15. Found: C, 72.08; H, 8.04; N, 5.86.
Calc. for Chl b · $H_2O$: C, 71.38; H, 7.84; N, 6.05. Found: C, 71.31; H, 7.78; N, 5.9.

Bacteriochlorophyll was obtained from R. rubrum by extraction of the wet-packed cells with acetone. The pigment extract, diluted to a 70:30 acetone:water mixture, was separated chromatographically on polyethylene. Following chromatography, crystallization was induced by removing part of the acetone under vacuum. The bacteriochlorophyll

was then recrystallized from aqueous acetone. All operations were carried out in minimum light.

Anal. Calcd. for Bchl · $H_2O$: C, 71.06; H, 8.24; N, 6.03; Mg, 2.61. Found: C, 70.99; H, 7.83; N, 5.93; Mg, 2.60.

Preparation of Solutions

For each experiment, fresh stock solutions were prepared by dissolving a weighed sample of the hydrated chlorophyll in a known volume of carbon tetrachloride (ca. 4 mg/5 ml). Less concentrated solutions were then prepared by serial and/or parallel dilution. In order to prevent pheophytinization of the pigments, particularly bacteriochlorophyll, traces of impurities were removed from the carbon tetrachloride (Baker and Adamson, reagent grade) by the method of Fieser.[16] Ethanol, acetone and diethyl ether were each reagent grade and used without further purification. All solutions were prepared in dim green light and stored in the dark. No decomposition was observed, using high precision spectrophotometry as the test, for concentrated solutions standing throughout the day at room temperature or for several days at 0°C.

Absorption Spectra

Absorption spectra were recorded using a Cary 14R Spectrophotometer. Difference spectra were recorded directly using a dilute solution in a long path cuvette in the reference beam and a more concentrated solution in correspondingly shorter path length cuvette in the sample beam.[3] Cuvette path lengths were calibrated using potassium chromate solutions in 0.05 N KOH. Chlorophyll solutions were protected from room light

throughout in order to avoid bleaching. In general, solutions at concentrations less than $10^{-5}$ mole-$l^{-1}$ in carbon tetrachloride exhibited some bleaching during the recording of the spectra, and it was necessary to record them quickly to avoid substantial errors from this source. In each case both the sample and reference solutions were prepared by dilution of the stock solution just before the spectrum was recorded.

Nuclear Magnetic Resonance Spectra

NMR spectra were recorded using a Varian A-60 nuclear magnetic resonance spectrometer. 41 mg of bacteriochlorophyll was dissolved under nitrogen in 0.5 ml of $DCCl_3$ (99.7% enrichment; Isotopes Specialties Co., Burbank, Calif.), which had been distilled under vacuum and exhaustively evacuated at -78°C two times. The titration with $CD_3OD$ (99% enrichment, Bio-Rad Laboratories, Richmond, Calif.) was carried out by adding successive portions of the pure $d_4$-methanol using a precision microliter syringe (Hamilton Co., Whittier, Calif.). Chemical shifts were measured relative to the sharp resonance at 436 cps from the impurity of $HCCl_3$ present in the deuterated solvent. No tetramethyl silane was added in these studies.

## Results

The concentration dependence of the absorption spectra of chlorophyll b and bacteriochlorophyll are qualitatively similar to that reported previously for chlorophyll a.[3] In ethanol, acetone or diethyl ether the absorption spectra (in terms of molar absorptivities) are not appreciably affected by changes in concentration throughout the range $10^{-6}$ to $10^{-3}$ mole-$l^{-1}$. In carbon tetrachloride, on the other hand,

increasing concentration of each of the chlorophylls results in the attenuation of the principal absorption maxima and the formation of new bands (shoulders) at longer wavelengths. Difference spectra between solutions of different concentration, in cuvettes whose path lengths are in inverse proportion to the concentrations, have numerous maxima and minima throughout the visible and near infrared regions (Figs. 1-3; traces at the bottom of each figure).

Dimer Spectra

The presence of monomer-dimer equilibrium for the three chlorophylls in carbon tetrachloride is demonstrated from their spectrophotometric properties using a modification of established procedures.[14,13] Since the absorption spectrum of the dimer is different from that of the monomer, we can calculate concentrations of monomer and dimer present in each solution using: 1) the total concentration obtained from the initial weighing and the dilution factor; 2) the extinction coefficient of the monomer as measured in the most dilute solutions; and 3) various trial values for the extinction coefficients of the dimer at the same wavelength. The wavelengths chosen for the analyses were those of maxima or minima in the difference spectra.

The equilibrium constant for dimer formation is defined as

$$K_d = \frac{C_d}{C_m^{\,2}}$$

where $C_d$ and $C_m$ are the molar concentrations of dimer and of monomer, respectively. Figs. 4-6 are log-log plots of calculated concentrations based on the longest-wavelength maximum in a series of difference spectra for each compound. The data are fitted using the least-squares method

to a straight line with the theoretical slope 2.0, using the absorptivity of the dimer as the only adjustable parameter. The presence of species other than dimers would, in general, lead to curvature in the plots of data handled in this fashion. No such evidence is seen, even at the highest concentrations studied. Analysis of the data at other maxima and minima in the difference spectra give very similar plots. Measurements at the minima (corresponding to maxima in the absorption spectra) are especially sensitive to bleaching in the dilute reference solutions, and great care must be taken to avoid errors from this source.

Table I summarizes the parameters characterizing the plots shown in Figs. 4-6. Least-squares slopes and standard deviations are given for the final approximation made in each case. These slopes are not sufficiently different from 2.000 to justify further refinement. The equilibrium constant can be obtained from the intercept at log $C_m = 0$. As this is a fairly long extrapolation, it is especially sensitive to the exact value of the slope used. The $K_d$ values reported in Table I represent an interpolation to a slope identically equal to 2.000, based on the results of the several trial calculations for each compound. The same is true for the dimer extinction coefficients reported.

The spectra of the pure monomer and pure dimer for each chlorophyll in carbon tetrachloride are calculated using the corresponding equilibrium constant, a difference spectrum and an absorption spectrum of a dilute solution. Figs. 1-3 (upper curves) show the averages of two such calculations (three in the case of bacteriochlorophyll) for each of the chlorophylls. The monomer spectra are nearly identical to those of

Table I. Monomer-dimer Equilibrium Properties of Three Chlorophylls in Carbon Tetrachloride at 24 ± 2°C

| Compound | $\lambda$ (mμ) | Slope | $K_d$ (l-mole$^{-1}$) | $\Delta G^{\circ}_{297}$ [Kcal-(mole dimer)$^{-1}$] |
|---|---|---|---|---|
| Chlorophyll a | 682 | 2.006 ± 0.035 | $1.0 \times 10^4 \pm 0.4 \times 10^4$ | -5.4 ± 0.2 |
| Chlorophyll b | {664, 477} | 2.009 ± 0.037 | $0.8 \times 10^4 \pm 0.3 \times 10^4$ | -5.3 ± 0.2 |
| Bacteriochlorophyll | 810 | 1.995 ± 0.025 | $2.2 \times 10^4 \pm 0.7 \times 10^4$ | -5.8 ± 0.2 |

solutions containing $1 \times 10^{-6}$ mole-$l^{-1}$ (less than 4% dimer in each case). The dimer spectra exhibit broadening of each absorption band to longer wavelengths; however, each of the dimer bands retains a single maximum. The long wavelength dimer absorption band for each pigment has a distinct shoulder toward the red.

Absorption Spectra of the Chlorophylls

We have measured the absorption spectra of chlorophyll a, chlorophyll b and bacteriochlorophyll in carbon tetrachloride and in several other solvents. Table II summarizes the values for the wavelengths, millimolar absorptivities and, in some cases, the oscillator strengths for the principal absorption bands. Some relevant data from the literature are included, and a comparison shows that our observations are generally in good agreement with the more recent published values.

The literature data on bacteriochlorophyll are the least abundant and our observations merit some comment. Our spectrum in ether is in best agreement with that of Holt and Jacobs[20]; however, our absorptivities are higher by a few percent at each wavelength. The spectrum in acetone resembles that in ether, except that the secondary maximum at 392 mu in ether exists only as a weak shoulder in the acetone spectrum. The maximum absorptivities of bacteriochlorophyll in ethanol on the other hand, are appreciably less, the bands are broader and the transition near 600 mu is markedly red-shifted compared with the first two solvents.

Oscillator strengths for the two long wavelength electronic transitions, including both their 0-0 and 0-1 vibrational components, are calculated using absorptivities at 1 mu intervals and Simpson's

Table II. Summary of the Electronic Absorption Spectra of Three Chlorophylls. Absorptivities, $\varepsilon$(in l-millimole$^{-1}$-cm$^{-1}$); absorption maxima, $\lambda$(in m$\mu$) and oscillator strengths, f.

| Solvent | $\varepsilon$ | $\lambda$ | $\varepsilon_{blue}$ | $\lambda$ | $\varepsilon$ | $\lambda$ | f | $\varepsilon_{red}$ | $\lambda$ | f | $\frac{\varepsilon_{blue}}{\varepsilon_{red}}$ | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chlorophyll a** | | | | | | | | | | | | |
| Ether | 85.2 | (410) | 117.5 | (430) | 8.28 | (578) | | 90.1 | (662) | | 1.30 | (17) |
| (+5% $CCl_4$) | 69.2 | (410) | 110.9 | (429.5) | 7.15 | (578) | | 86.8 | (662) | | 1.28 | This study |
| $CCl_4$ | 47.0 | (415.0) | 67.7 | (432.9) | | | | 51.7 | (664.7) | | 1.31 | (18) |
| (Monomer) | 69.3 | (415) | 103.8 | (433) | 7.27 | (579) | 0.064 | 84.9 | (665) | 0.225 | 1.22 | This study |
| (Dimer)* | | | 83.7 | (434) | 8.50 | (588) | 0.060 | 59.5 | (668) | 0.257 | 1.41 | This study |
| **Chlorophyll b** | | | | | | | | | | | | |
| Ether | 57.0 | (430) | 158.5 | (455) | 11.5 | (595) | | 56.3 | (644) | | 2.82 | (17) |
| | 57.5 | (428.4) | 156. | (452.7) | | | | 52.1 | (642.4) | | 3.00 | (18) |
| (+0.1% $CCl_4$) | | | 161.4 | (452.4) | 10.9 | (594.5) | | 57.8 | (642.3) | | 2.80 | This study |
| $CCl_4$ ($10^{-5}$m-l$^{-1}$) | 50.9 | 431.8 | 139.9 | (456.5) | | | | 58.9 | (645.3) | | 2.86 | (18) |
| (Monomer) | | | 156.7 | (456) | 10.7 | (597) | 0.034 | 59.2 | (645) | 0.136 | 2.64 | This study |
| (Dimer)* | | | 108.7 | (464) | 13.2 | (598) | 0.038 | 45.6 | (648) | 0.151 | 2.39 | " " |

Table II (Continued)

| Solvent | Bacteriochlorophyll | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\varepsilon_{violet}$ | $\lambda$ | $\varepsilon$ | $\lambda$ | $\varepsilon$ | $\lambda$ | f | $\varepsilon_{red}$ | $\lambda$ | f | $\frac{\varepsilon_{red}}{\varepsilon_{violet}}$ | Reference |
| Ether | 85.5 | (358) | 52.8 | (391) | 22.1 | (575) | | 95.7 | (772) | | 1.12 | (19) |
| | 70.7 | (357) | 46.8 | (392) | 20.2 | (574) | | 93.4 | (767-70) | | 1.32 | (20) |
| | 73.4 | (358.5) | 48.1 | (391.5) | 20.9 | (577) | | 91.1 | (773) | | 1.24 | (17) |
| | 73.4 | (357) | 47.1 | (392) | 22.0 | (573) | 0.110 | 96.0 | (770) | 0.309 | 1.31 | This study |
| Ethanol | 58.5 | (365.5) | | | 15.2 | (607) | 0.123 | 62.0 | (773) | 0.307 | 1.06 | This study |
| Acetone | 65.7 | (358) | | | 19.4 | (576.5) | 0.127 | 69.2 | (770) | 0.300 | 1.05 | This study |
| $CCl_4$ (Monomer) | 61.7 | (361.5) | 35.5 | (398) | 20.1 | (580) | 0.108 | 88.0 | (780) | 0.280 | 1.42 | This study |
| (Dimer)* | 49. | (361) | | | 20.2 | (589) | 0.121 | 54. | (785) | 0.33 | 1.10 | |

*Absorptivities and oscillator strengths given per mole of monomer in the dimers.

approximation. As can be seen in Table II, the oscillator strengths for bacteriochlorophyll are not nearly so solvent dependent as are the absorptivities.

Brody and Brody[8b] report a red oscillator strength of 0.23 for chlorophyll a in ethanol, which is very close to our value in $CCl_4$. On the other hand, Jacobs, et al.[21] report values of 0.38, 0.28 and 0.79 for the red oscillator strengths of ether solutions of chlorophyllide a, chlorophyllide b and bacteriochlorophyllide, respectively. It is known that the absorption spectra of the chlorophyllides are virtually identical to those of the corresponding chlorophylls.[22] The discrepancies of the oscillator strengths is well outside the normal expected uncertainities. This is especially true for bacteriochlorophyll, where we have made measurements in ether and where the red absorption band is well resolved from higher electronic transitions.

Nuclear Magnetic Resonance Spectra of Bacteriochlorophyll

The nuclear magnetic resonance (NMR) spectra of chlorophyll a and chlorophyll b aggregates in $DCCl_3$ at high concentrations (ca. 0.1 $mole^{-1}l^{-1}$) have been reported by Closs, et al.[11b] The protons most affected by deshielding by the ring current effects in the aggregates were identified by comparison with the NMR spectra of the monomer molecules, obtained either upon dilution with $DCCl_3$ or by titration with $CD_3OD$, which effectively breaks up the chlorophyll aggregates.

We have performed a similar titration for bacteriochlorophyll. Fig. 7 shows the NMR spectra of bacteriochlorophyll in $DCCl_3$ (upper curve) and the same solution with an excess of $CD_3OD$ (lower curve). The shifts in the positions of the various resonances throughout the titration are

illustrated in Fig. 8, and some of the resonances that can be assigned are tabulated in Table III. As in the case of chlorophylls a and b, the largest change that occurs upon breaking up bacteriochlorophyll aggregates is for the C-10 proton. Following the reasoning of Closs, et al.,[11b] we interpret this as indicating the participation of the C-9 carbonyl of ring V in the aggregate formation. The interaction is presumed to be primarily with the central magnesium of a second bacteriochlorophyll molecule. A confirmation of the assignment of the C-10 proton resonance was obtained somewhat inadvertently. A small amount of oxygen was introduced to the nitrogen purged solution of bacteriochlorophyll during the titration with $CD_3OD$. Over the course of a week at 0°C in the dark, the sample allomerized in the presence of the alcohol. The initial effect on the spectrum was the complete disappearance of the resonance at 361 cps and the formation of a new band at 507 cps. No other changes occurred during this initial interval, although evidence of much further decomposition appeared upon additional standing. Allomerization is thought to involve an oxidation of ring V (e.g., see Aronoff[23]).

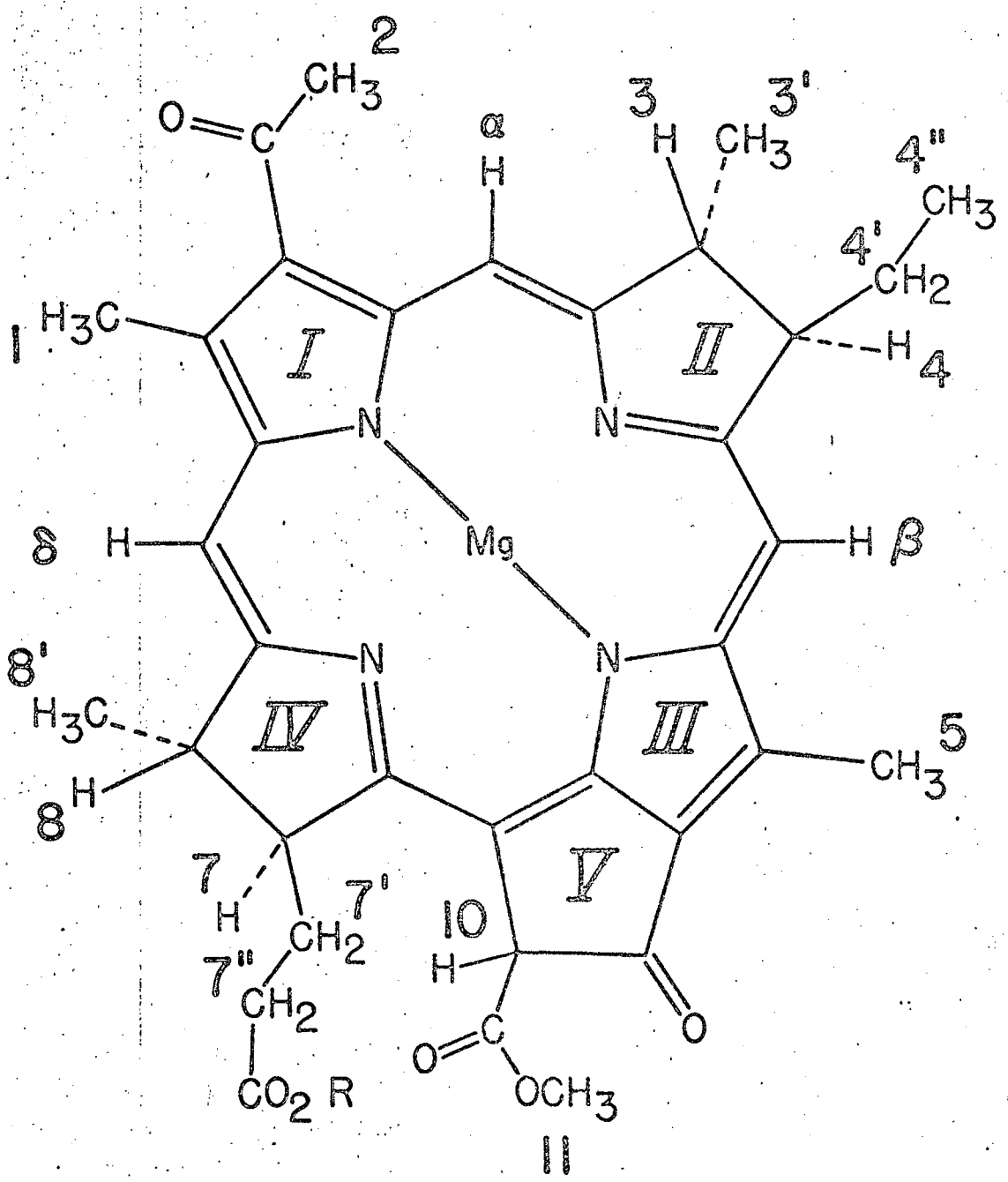
Chlorophyll b aggregation appears to involve strong interactions with the formyl carbonyl at position 3 as well as with the C-9 carbonyl.[11b] This gives rise to higher aggregates than dimers at concentrations in excess of $10^{-2}$ mole-$l^{-1}$.[11a] Chlorophyll a does not possess a second active carbonyl group and forms only dimers up to a concentration of 0.1 mole-$l^{-1}$. The NMR data on bacteriochlorophyll strongly suggest that the acetyl carbonyl at position 2 is also involved in aggregate

Table III. Chemical Shifts (cps from Tetramethylsilane Position) for Bacteriochlorophyll dissolved in $DCCl_3$ plus $CD_3OD$*

| Proton | $DCCl_3$ | $DCCl_3 + CD_3OD$ | Difference |
|---|---|---|---|
| α | 500 | 524 | + 24 |
| β | 501 | 501 | 0 |
| δ | 488 | 490 | + 2 |
| 10 | (307) | 361 | + 54 |
| 11 | 207.5 | 233 | + 25.5 |
| 5 or 1 | 192 | 200.5 | + 8.5 |
| | 175 | 196 | + 21 |
| 2 | 154 | 180 | + 26 |

*Concentrations: bacteriochlorophyll, 0.09 mole-$l^{-1}$; $CD_3OD$, 1.0 mole-$l^{-1}$.

(Note to editor: Place the structure of bacteriochlorophyll MUB-8686-A at the bottom of this table.)


2
CH3
O
C
α
H
3
H
3'
CH3
4''
CH3
4'
CH2
1
H3C
I
II
H
4
N
N
δ
H
Mg
H
β
8'
H3C
N
N
IV
III
5
CH3
8
H
7
H
7'
CH2
V
10
H
7''
CH2
O
C
O
CO2 R
OCH3
11

MUB-8686-A

formation in this high concentration range. The methyl protons of the acetyl function, and the α proton adjacent to it are both shifted significantly downfield when the dimers are broken up. The resonances of the β and δ methine bridge protons, by contrast, are virtually unaffected.

A complete assignment of the NMR spectrum of bacteriochlorophyll is not possible on the basis of our present evidence. For example, the methyl substituents at positions 1 and 5 are in very nearly equivalent locations in bacteriochlorophyll. Each is two carbons removed from a ketone carbonyl and adjacent to a saturated pyrrole ring on the other side. The two resonances assigned to these methyl protons, at 196 and 200.5 cps in excess methanol-$d_4$, cannot be distinguished without further studies. They are of interest, however, since the former is shifted upfield 21 cps and the latter only 8.5 cps in the aggregates.

## Discussion

Dimer Structures. Changes in the long wavelength absorption bands of chlorophyll a as a function of concentration in carbon tetrachloride were reported by Anderson and Calvin.[12] Their spectra showed virtually identical behavior to those reported here, but were interpreted to result from a chlorophyll a-chlorophyll a interaction of a non-specific type, leading to a lowering of the energies of the electronic transitions and a red shift in the absorption spectra. Livingston, Watson and McArdle[24] have likewise rejected the presence of simple monomer-dimer equilibrium in accounting for the absence of chlorophyll fluorescence in solutions in rigorously dry benzene.

These solutions become fluorescent upon the addition of one of a wide variety of nucleophilic "activator" molecules; however, the concentration dependence of the increased fluorescence is at variance with an equilibrium involving dimers of chlorophyll.

Analysis of our results demonstrates that the true situation in carbon tetrachloride solutions involves both monomer-dimer equilibrium and energy lowering of the transitions in the dimer relative to the monomer. A quantitative treatment of the absorption data is consistent with the presence of well-defined chlorophyll dimers in carbon tetrachloride. Furthermore, the component molecules are rather strongly coupled to one another. At the same time the center of the red transition of chlorophyll a shifts by 192 $cm^{-1}$ from 15040 $cm^{-1}$ in the monomer to 14848 $cm^{-1}$ in the dimer. As seen in Table IV, similar red shifts are observed for chlorophyll b and bacteriochlorophyll. Additional confirmation of the presence of monomer-dimer equilibrium of chlorophyll a in carbon tetrachloride is obtained from the effect on the absorption spectra of concentrated solutions which are titrated with small amounts of nucleophilic reagents.[25] Analysis of the results, particularly for strong complex formers such as pyridine or ethanol, is entirely consistent with an equilibrium involving chlorophyll monomers, chlorophyll dimers and complexes formed with a single molecule of the nucleophile. These observations apply to solutions in carbon tetrachloride only; the situation may be quite different in the case of aromatic solvents like benzene.

A number of similarities in the properties of the dimers of chlorophyll a, chlorophyll b and bacteriochlorophyll suggest that,

at least in carbon tetrachloride or chloroform as solvent, they result from similar molecular interactions: (1) The equilibrium constants for dimerization are all within a factor of three of one another. The free energies of dimerization are $\Delta G^{\circ} = -5.4$, $-5.3$ and $-5.8$ Kcal-mole$^{-1}$ at 297°K for chlorophyll a, chlorophyll b and bacteriochlorophyll, respectively. (2) The similar splittings of the long wavelength absorption bands for the dimers indicate that the relative orientations of the long wavelength electric dipole transition moments of the two molecules in the dimer are nearly the same for the three pigments (see below). (3) The proton magnetic resonance spectrum of bacteriochlorophyll aggregates exhibits shifts analogous to those reported previously for chlorophylls a and b.

Electronic absorption spectra provide information relevant to the structure of the dimers of chromophoric molecules. Several theoretical treatments based on the interactions of point dipoles have been reported in the literature.[26-28] On the basis of the conclusions of the theoretical arguments the relative orientations and the distance of separation of the transition dipoles can be deduced from the relative oscillator strengths, $D_+$ and $D_-$, of the split components, the dimer band splittings, $\nu_+ - \nu$, and, in the case of optically active molecules such as the chlorophylls, from the rotational strength of the dimer. Although the theoretical model, based on point dipole interactions, represents a great oversimplification for molecules as complex as the chlorophylls, it is nevertheless instructive to consider the model as a first approximation.

The analysis of the long wavelength dimer absorption bands of the chlorophylls is difficult, owing to the appreciable overlap of the two broad components. Furthermore, the corresponding monomer transitions are appreciably asymmetric when they are plotted on an energy scale. In order to synthesize the dimer spectra, we first assume that the two split components have identical shapes. The two components differ only in their amplitudes and in the frequencies of their respective maxima. Each component is approximated as a compound Gaussian function. The requisite asymmetry is introduced by assigning a smaller half-bandwidth to the Gaussian at frequencies less than that at the maximum, and a larger half-bandwidth at frequencies greater than that at the maximum (e.g., see Jørgensen[29]). The same halfwidth parameters are assigned to both components of the dimer band, however. The two compound Gaussian curves are adjusted, with the aid of a computer, so that their sum approximates the dimer spectrum. The success of this approach can be best judged by the result for bacteriochlorophyll shown in Fig. 9. The fit is reasonably close in the region of the center of the transition; however, poorer agreement obtains in the wings, where the Gaussian functions fall off too rapidly. The 0-1 vibrational components were also included as separate compound Gaussians, but with greater half-bandwidths than for the corresponding 0-0 components. The parameters for the "best-fit" synthesis for the 0-0 bands for the three chlorophylls are summarized in Table IV, where the subscripts + and - refer to the high and low frequency components, respectively.

Unfortunately, the rotational strengths cannot be calculated so simply. The optical rotatory dispersion spectra and circular dichroism

spectra of each of the chlorophylls do not exhibit local conservation of rotational strength in the regions of individual transitions (see Sauer[3] for the ORD spectrum of chlorophyll a dimers in carbon tetrachloride). This probably results from static field interactions of each component of the dimer on the electronic configuration of the other. The static field effects are probably also responsible for the hyperchromism observed for the dimers (Table IV). We hope to present a treatment of the ORD spectra of the chlorophyll dimers in a future publication.

The zero-order theory for the interaction of point electric dipole transition moments in dimers predicts that the ratio of the dipole strengths of the two split components, $D_+/D_-$, is sensitive only to the angle between the two transition moments.[28] The angular dependence is given by

$$\frac{D_+}{D_-} = \frac{1 + \cos \alpha}{1 - \cos \alpha} ,$$

where $\alpha$ is the angle between the two transition dipole moments. The angles calculated from the data presented here are given in Table IV. They lie between 73 and 82° for the long wavelength oscillators of the three chlorophylls. Thus, these pairs of absorption oscillators are nearly perpendicular to one another in the three dimers. The similarities apply, of course, only to the dimers in relatively dilute solutions ($\sim 10^{-4}$ mole-l$^{-1}$), and not necessarily to the higher aggregates apparently present in chlorophyll b and bacteriochlorophyll solutions at the higher concentrations ($10^{-2}$ - $10^{-1}$ mole-l$^{-1}$) used in the NMR studies.

In order to characterize completely the geometry of the dimers

Table IV. Peak Frequencies and Dipole Strengths for the Long Wavelength Absorption Bands of Monomers and Dimers of Three Chlorophylls in Carbon Tetrachloride (Data are presented for the 0-0 vibrational bands only.)

| Compound | Chlorophyll a | Chlorophyll b | Bacterio-chlorophyll |
|---|---|---|---|
| Monomers | | | |
| $\lambda$, mμ | 665 | 645 | 781 |
| $\tilde{\nu}$, $cm^{-1}$ | 15040 | 15504 | 12805 |
| D, $debye^2$ | 24.65 | 16.91 | 37.60 |
| Dimers | | | |
| $\tilde{\nu}_+$, $cm^{-1}$ | 15029 | 15458 | 12810 |
| $\tilde{\nu}_-$, $cm^{-1}$ | 14666 | 15060 | 12320 |
| $(\tilde{\nu}_+ - \tilde{\nu}_-)$, $cm^{-1}$ | 363 | 398 | 490 |
| $1/2\ (\tilde{\nu}_+ + \tilde{\nu}_-)$, $cm^{-1}$ | 14848 | 15259 | 12565 |
| $\tilde{\nu} - 1/2\ (\tilde{\nu}_+ + \tilde{\nu}_-)$, $cm^{-1}$ | 192 | 245 | 240 |
| $D_+$, $debye^2$ | 32.6 | 26.3 | 49.0 |
| $D_-$, $debye^2$ | 22.1 | 14.5 | 37.1 |
| $D_+/D_-$ | 1.48 | 1.81 | 1.32 |
| $\alpha$, degrees | 78.2 | 73.8 | 81.9 |
| Hyperchromism (%) $100 \times \left(\frac{1/2(D_+ + D_-)}{D} - 1\right)$ | 11.0 | 20.6 | 14.5 |

we need to specify three additional parameters, such as the angle between the normals to the porphyrin planes, the distance between the centers of the planes, and the angle between one of the normals and a line joining the centers. The dimer splitting and the rotational strengths of the corresponding dimer transitions give us two relationships involving these parameters, but still permit one degree of freedom. In principle, we may be able to remove the remaining degree of freedom and obtain checks on the structure by applying a similar analysis to another transition oriented differently with respect to the chlorophyll molecular axes. The two electronic transitions next highest in energy in each chlorophyll are thought to lie in the porphyrin plane but to be perpendicular to the long wavelength transition moment directions.[30] Unfortunately, the first of these near 600 mμ is very weak and the second, the Soret band in the blue, is strongly overlapped by another transition of different orientation. The best case for this analysis is the well-resolved transition at 580 mμ in bacteriochlorophyll. Here, however, the dimer spectrum shows no evidence of splitting, merely a shift to the red. It may be that one component has nearly zero oscillator strength, or that both are appreciable but the splitting is small. Comparison with the monomer spectrum might suggest that the short wavelength component is missing and lead to the conclusion that the transition moments are nearly anti-parallel to one another ($\cos \alpha \cong -1$); however, we cannot rule out the possibility of a pronounced red shift of the center of the transition in the dimer. There is no reason to take the red shift of the long wavelength transition as a measure of this effect, since different excited states are involved.

Brody and Brody[8b] have reported a spectrum and dimer geometry for chlorophyll a at high concentrations ($2.9 \times 10^{-2}$ moles-$l^{-1}$) in ethanol. Their results are entirely different from ours. We believe that the source of this discrepancy lies in several erroneous assumptions in their approach. Their calculation of the dimer spectrum assumes that the dimer has no absorption at the wavelength of maximum absorption of the monomer, at 665 mμ in the red in ethanol. Not only is this entirely inconsistent with their corollary assumption that the absorbance at 665 mμ does not change when one converts a substantial part of the monomer to dimers, but it is in complete disagreement with dimer spectrum shown in Fig. 1 of the present paper. In their measurement of the difference spectra no attempt was made to assure that the product of concentration times path length was the same in both the sample and reference cuvettes. As a consequence, their "absorption spectrum" for the dimers resembles our difference spectra for concentrated versus dilute solutions, and bears no similarity to a true dimer absorption spectrum. The dimer splitting they calculate is too large by a factor of two and the dimer oscillator strengths have no foundation whatsoever. In addition, we feel that their arbitrary use of the model of McRae and Kasha,[27] in which the transition moments of the two molecules of the dimer are assumed to be coplanar, has no foundation for the case of the chlorophylls. Additional objections to the work of Brody and Brody have been raised by Stensby and Rosenberg,[9] who were unable to observe spectrophotometric evidence for chlorophyll dimer formation in ethanol at similar concentrations at room temperature, and by Katz, et al.[11a] and Closs, et al.,[11b] who found strong disaggregating effects of small

amounts of methanol or ethanol added to concentrated solutions containing chlorophyll a dimers in non-polar solvents. Our spectral observations have confirmed the strong complexing ability of ethanol at the expense of dimerization.[3] Stensby and Rosenberg have suggested that the broadened absorption observed by Brody and Brody may have resulted from undissolved chlorophyll present in suspension at the high concentrations.

On the basis of similarities in electronic absorption spectra, proton magnetic resonance spectra and free energies of formation, we conclude that the dimers of chlorophyll a, chlorophyll b and bacteriochlorophyll in carbon tetrachloride are probably very similar in structure. This similarity must result from a corresponding similarity in the forces responsible for dimerization; hence, we feel that dimerization in relatively dilute solutions results from features which the three molecules have in common. The interaction of the C-9 carbonyl of one chlorophyll molecule with the magnesium of the second molecule in the dimer (Closs, et al.;[11b] Katz, et al.;[11a] Anderson and Calvin[12]) is a reasonable proposal for these dimerization interactions. The additional interactions involving the formyl substituent of chlorophyll b and the acetyl carbonyl of bacteriochlorophyll must be weaker, and they apparently only become important at much higher concentrations of the pigments.

The long wavelength transition moments of the two molecules making up chlorophyll dimers are nearly perpendicular to one another; however, our present data do not permit a decision as to whether the molecules in the dimer are oriented with their porphyrin planes parallel to one

another with one of them skewed 80° with respect to the other, whether the two planes are mutually perpendicular, or whether the structure is somewhere between these two extremes.

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## Figure Captions

Fig. 1. Absorption spectra of chlorophyll a in carbon tetrachloride (upper curves) calculated for pure monomer (solid curve) and pure dimer (dashed curve). Absorptivities are given per mole of monomer in the dimer. The lower curve (dashed) shows a difference spectrum measured directly between two solutions at different concentrations and for path lengths inversely proportional to the concentrations.

Fig. 2. Absorption spectra of chlorophyll b in carbon tetrachloride (upper curves) calculated for pure monomer (solid curve) and pure dimer (dashed curve). Absorptivities are given per mole of monomer in the dimer. The lower curve (dashed) shows a difference spectrum measured directly between two solutions at different concentrations and for path lengths inversely proportional to the concentrations.

Fig. 3. Absorption spectra of bacteriochlorophyll in carbon tetrachloride (upper curves) calculated for pure monomer (solid curve) and pure dimer (dashed curve). Absorptivities are given per mole of monomer in the dimer. The lower curve (dashed) shows a difference spectrum measured directly between two solutions at different concentrations and for path lengths inversely proportional to the concentrations.

Fig. 4. Log-log plot of the concentration of dimer versus concentration of monomer for chlorophyll a in carbon tetrachloride. Solid line drawn with theoretical slope 2.000. Calculation based on an assumed dimer absorptivity of $\varepsilon_{680} = 3.95 \times 10^4$ l-(mole monomer)$^{-1}$ - cm$^{-1}$ and a measured monomer absorptivity of $\varepsilon_{680} = 0.80 \times 10^4$ l-mole$^{-1}$ - cm$^{-1}$. See Table III for least-squares analysis of points shown.

Fig. 5. Log-log plot of the concentration of dimer versus concentration of monomer for chlorophyll b in carbon tetrachloride. Solid line drawn with theoretical slope 2.000. Calculation based on an assumed dimer absorptivity of $\varepsilon_{664} = 2.29 \times 10^4$ l-(mole monomer)$^{-1}$ - cm$^{-1}$ and a measured monomer absorptivity of $\varepsilon_{664} = 0.56 \times 10^4$ l-mole$^{-1}$ - cm$^{-1}$. See Table III for least-squares analysis of points shown.

Fig. 6. Log-log plot of the concentration of dimer versus concentration of monomer for bacteriochlorophyll in carbon tetrachloride. Solid line drawn with theoretical slope 2.000. Calculation based on an assumed dimer absorptivity of $\varepsilon_{810} = 3.45 \times 10^4$ l-(mole monomer)$^{-1}$ - cm$^{-1}$ and a measured monomer absorptivity of $\varepsilon_{810} = 0.47 \times 10^4$ l-mole$^{-1}$ - cm$^{-1}$. See Table III for least-squares analysis of points shown.

Fig. 7. Nuclear magnetic resonance spectra (60 megacycles) of bacteriochlorophyll in $DCCl_3$ (upper) and with a 10-fold excess of $CD_3OD$ in $DCCl_3$ (lower). Traces at left are run at 3-fold higher sensitivity than those at right. Chemical shifts are given relative to TMS (not present in the sample), based on a shift of 436 cps for $HCCl_3$.

Fig. 8. Chemical shifts for proton magnetic resonance absorptions of bacteriochlorophyll in $DCCl_3$ as a function of increasing concentration of $CD_3OD$. Assignments of some of the resonances to specific protons are given at the right.

Fig. 9. Decomposition of the long wavelength electronic transition of bacteriochlorophyll dimers in carbon tetrachloride into two similar asymmetric Gaussian components (dashed curves). The sum of these components (solid curve) is compared with the experimental absorptivities (per mole of monomer present in the dimers) (O). Parameters are summarized in Table IV.

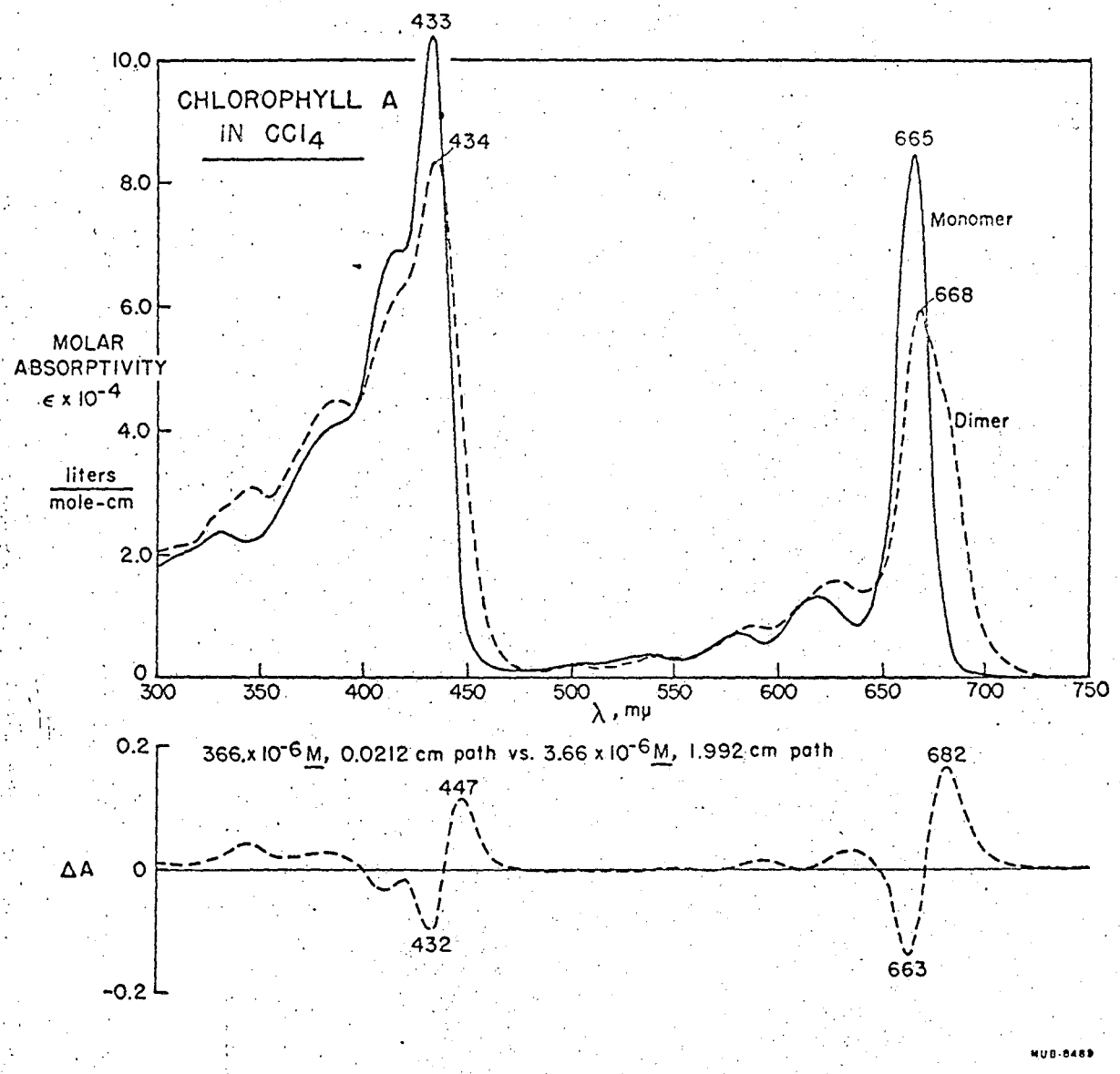

CHLOROPHYLL A
IN CCl4
433
434
665
668
Monomer
Dimer
MOLAR
ABSORPTIVITY
ε x 10^-4
liters
mole-cm
10.0
8.0
6.0
4.0
2.0
0
300
350
400
450
500
550
600
650
700
750
λ, mμ
366.x 10^-6 M, 0.0212 cm path vs. 3.66 x 10^-6 M, 1.992 cm path
0.2
ΔA
0
-0.2
447
432
682
663
MUB-6469

Fig. 1

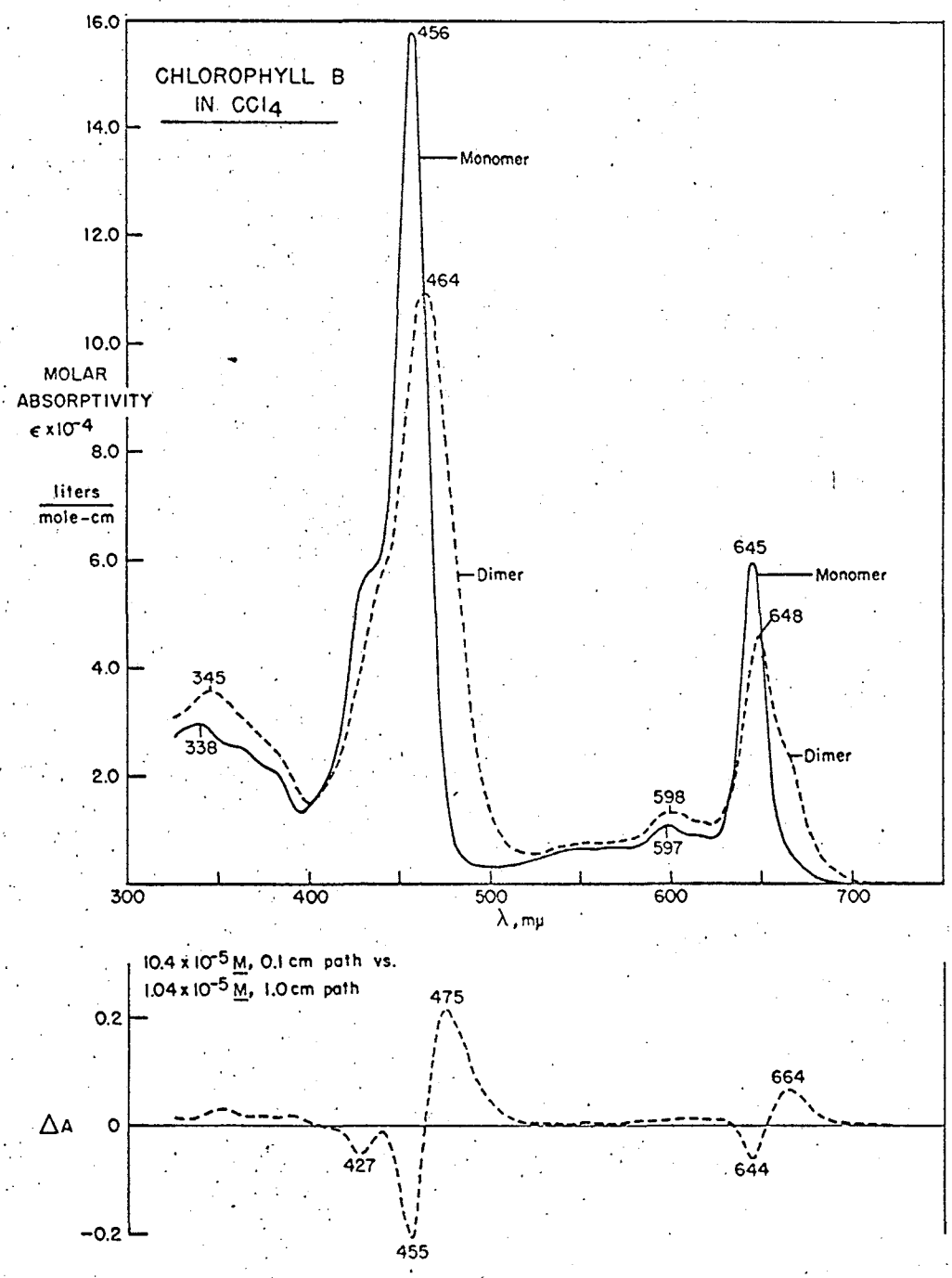

CHLOROPHYLL B
IN CCl4
MOLAR
ABSORPTIVITY
ε x10⁻⁴
liters / mole-cm
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
456
Monomer
464
Dimer
345
338
598
597
645
Monomer
648
Dimer
300
400
500
600
700
λ, mμ
10.4 x 10⁻⁵ M, 0.1 cm path vs.
1.04 x 10⁻⁵ M, 1.0 cm path
ΔA
0.2
0
-0.2
475
427
455
664
644

Fig. 2

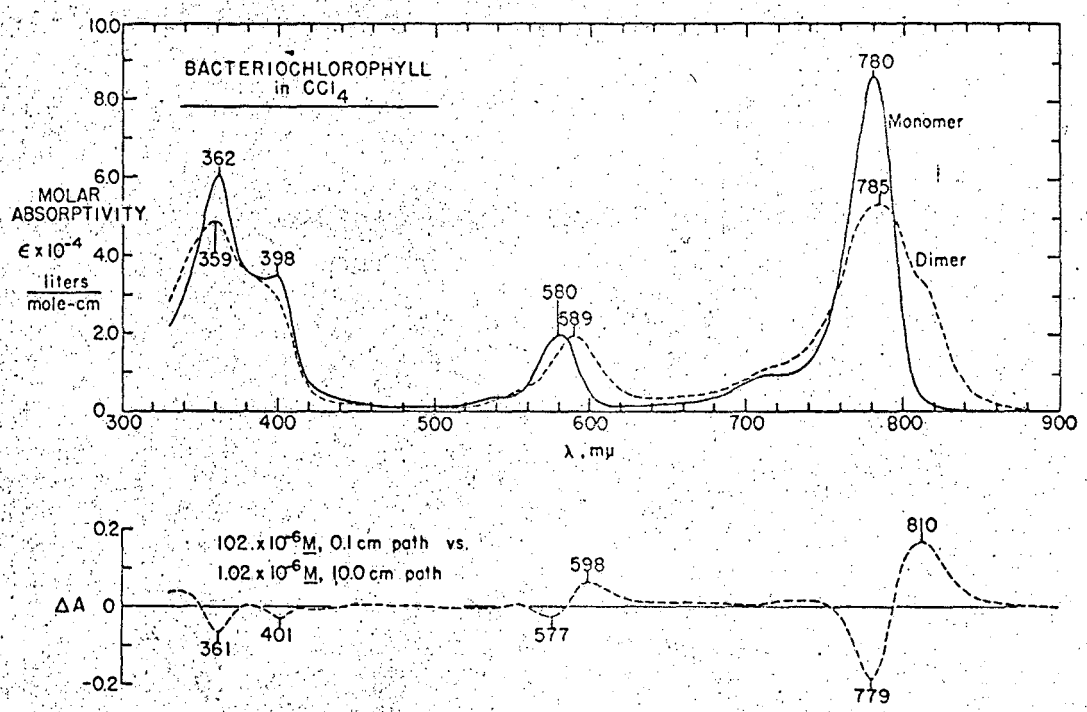
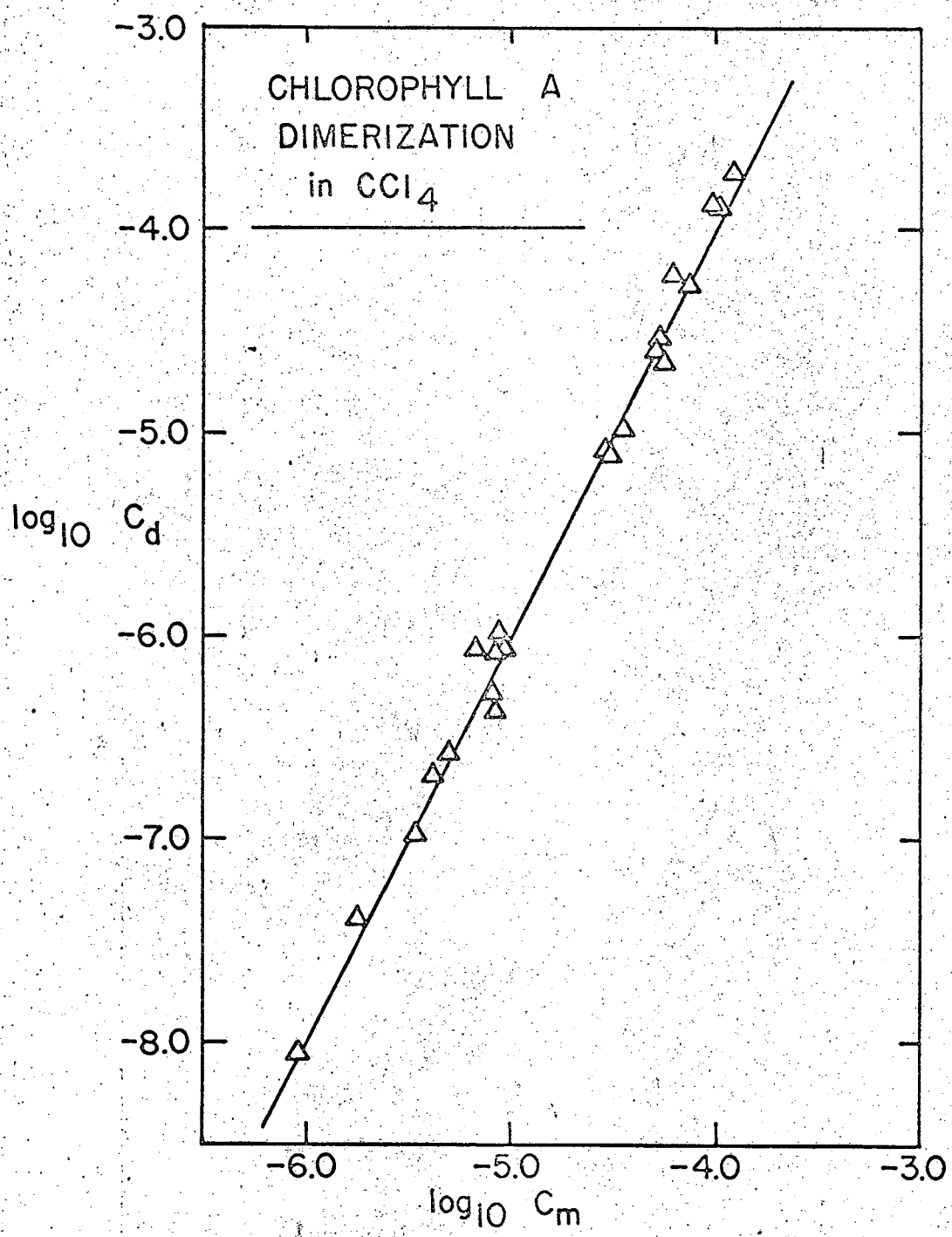

BACTERIOCHLOROPHYLL
in $CCl_4$
MOLAR ABSORPTIVITY
$\epsilon \times 10^{-4}$ $\frac{\text{liters}}{\text{mole-cm}}$
10.0
8.0
6.0
4.0
2.0
0
362
359
398
580
589
780
785
Monomer
Dimer
300
400
500
600
700
800
900
λ, mμ
0.2
ΔA 0
-0.2
$102 \times 10^{-6}$ M, 0.1 cm path vs.
$1.02 \times 10^{-6}$ M, 10.0 cm path
361
401
577
598
779
810

Fig. 3




MUB-8418

Fig. 4

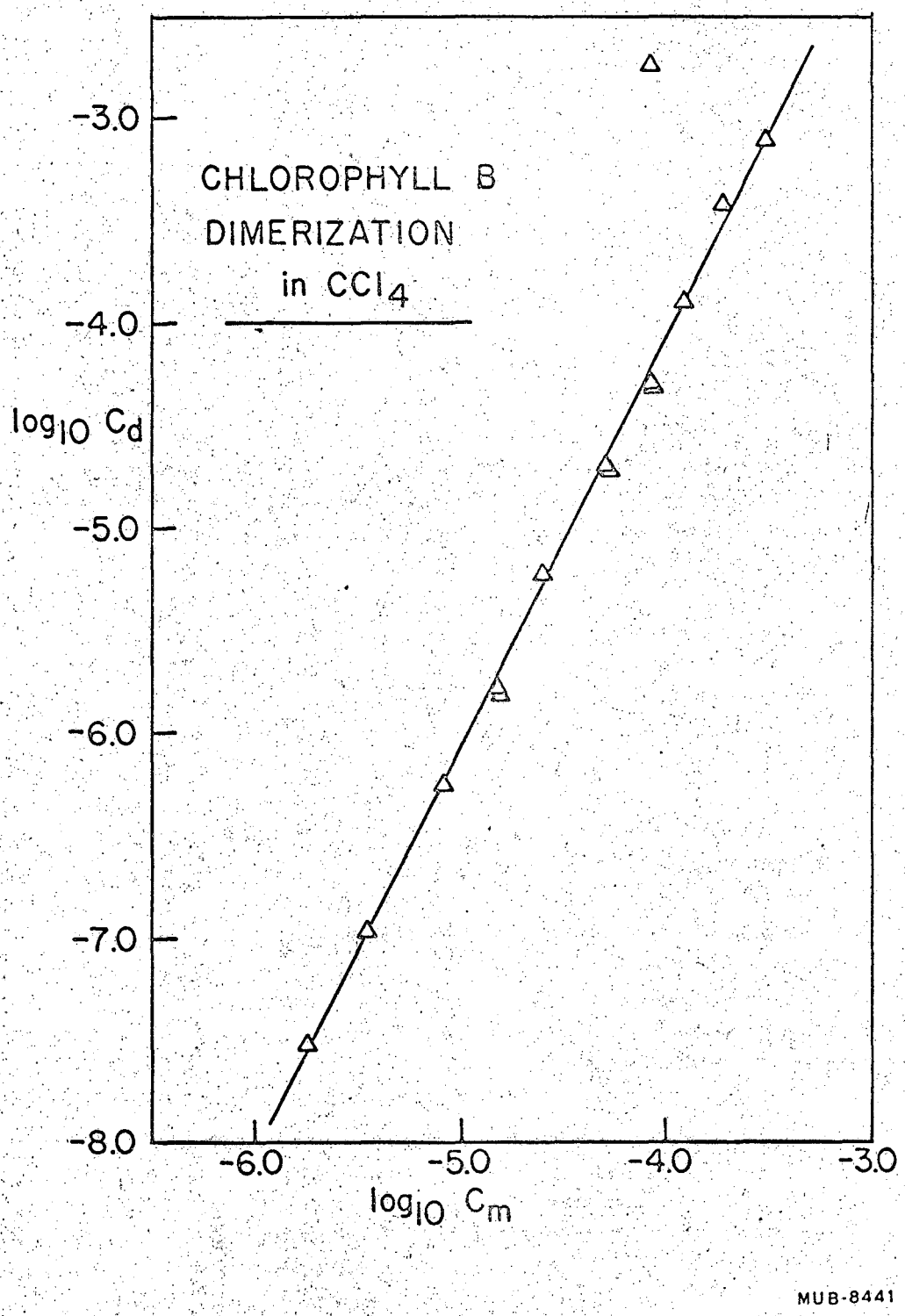
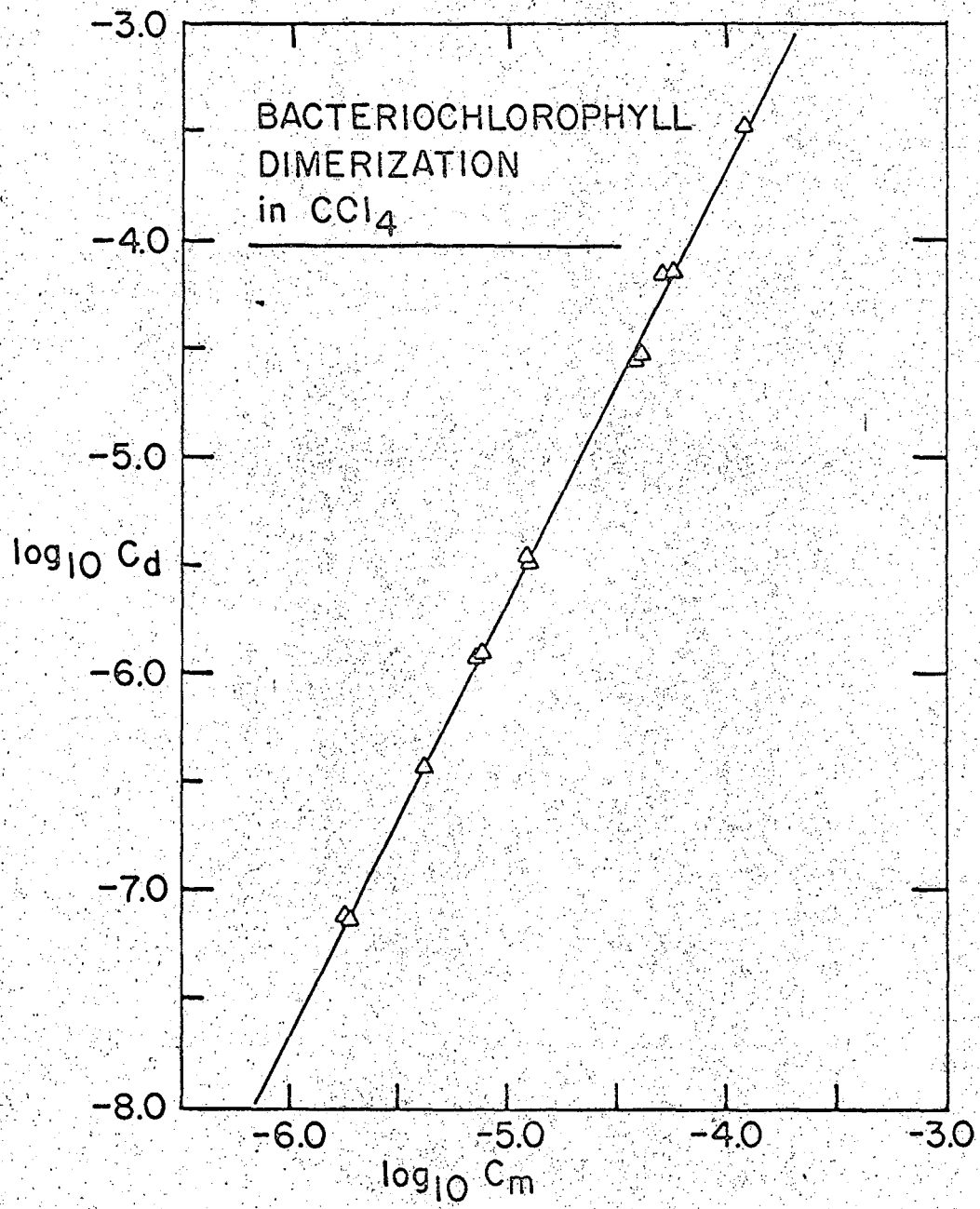
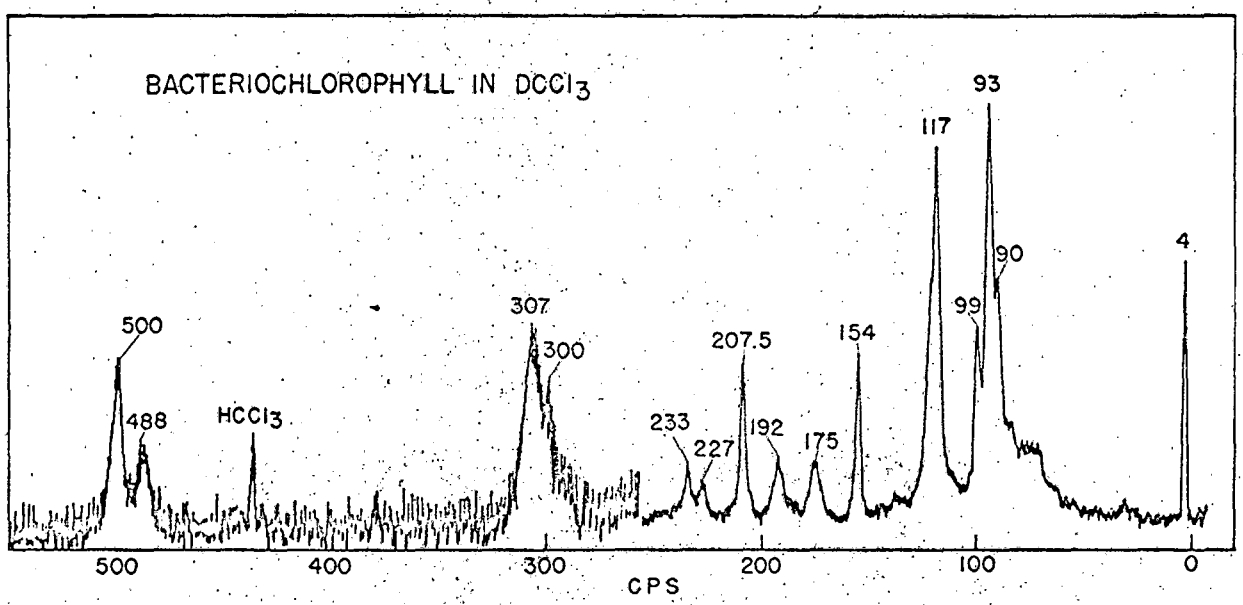

CHLOROPHYLL B
DIMERIZATION
in $CCl_4$
-3.0
-4.0
-5.0
-6.0
-7.0
-8.0
$\log_{10} C_d$
-6.0
-5.0
-4.0
-3.0
$\log_{10} C_m$
MUB-8441

Fig. 5




MUB-7620

Fig. 6


BACTERIOCHLOROPHYLL IN DCCl3
93
117
90
4
99
307
500
300
207.5
154
488
HCCl3
233
227
192
175
500
400
300
200
100
0
CPS

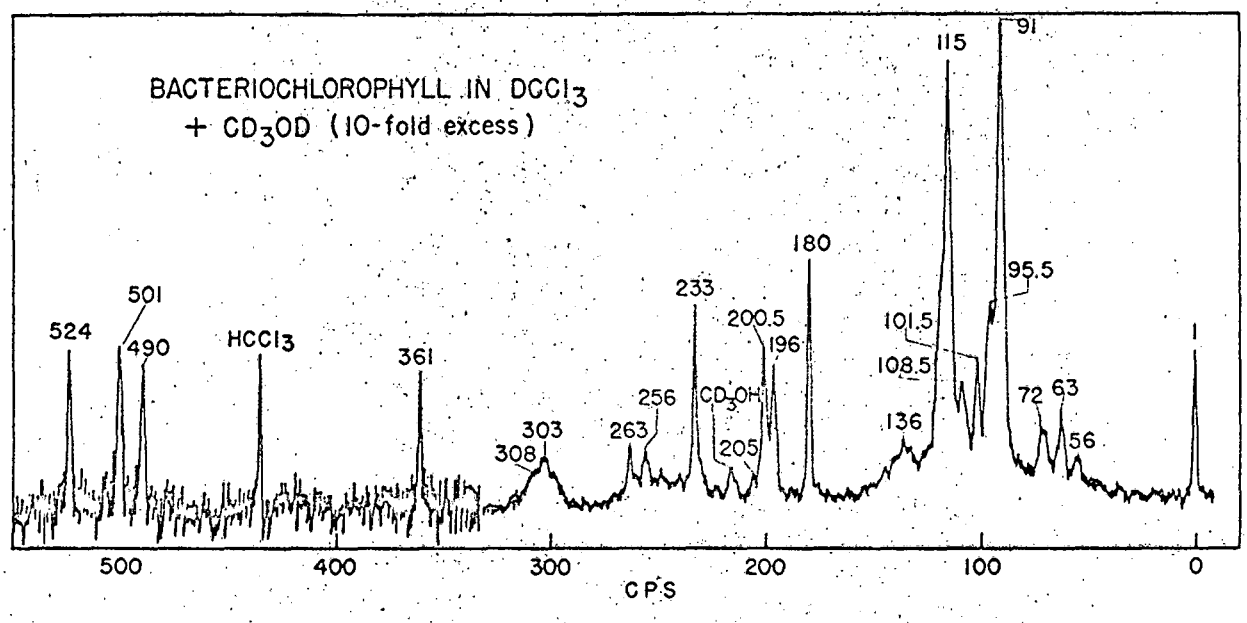
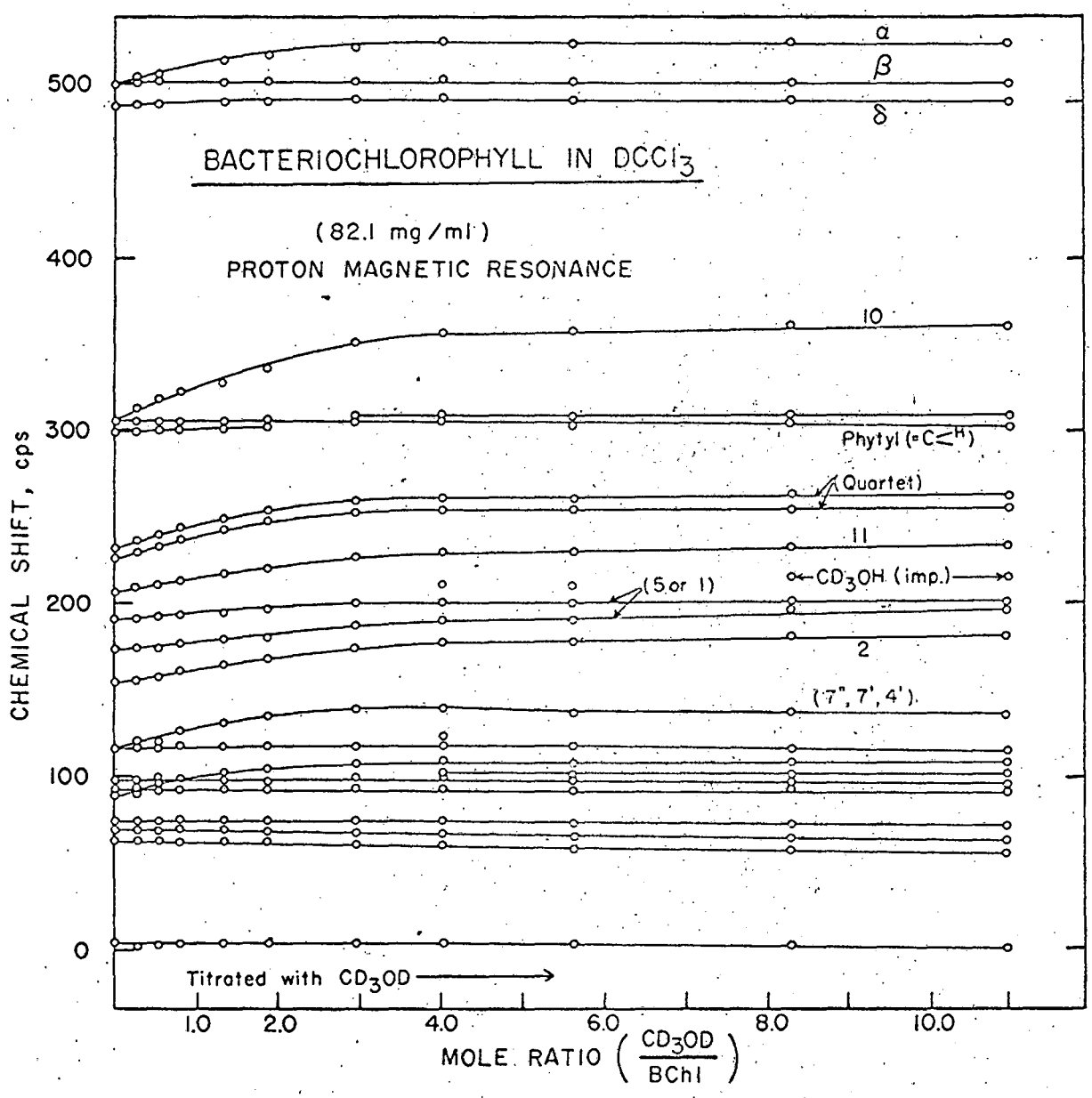

BACTERIOCHLOROPHYLL IN DCCl3
+ CD3OD (10-fold excess)
115
91
180
95.5
233
501
200.5
101.5
524
490
HCCl3
196
1
361
108.5
256
CD3OH
72
63
303
263
136
308
205
56
500
400
300
200
100
0
CPS

Fig. 7




MUB-8498

Fig. 8

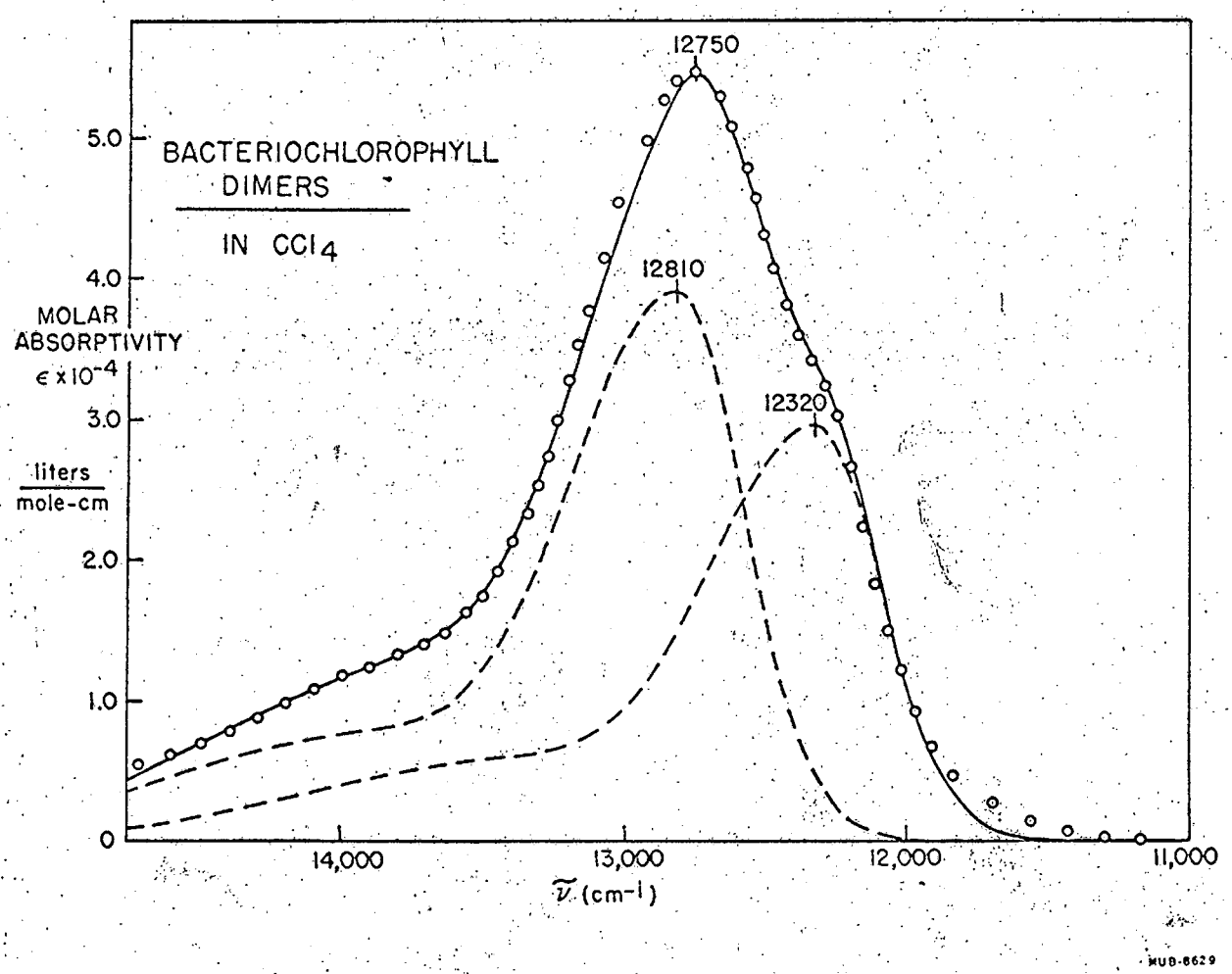

BACTERIOCHLOROPHYLL
DIMERS
IN $CCl_4$
MOLAR
ABSORPTIVITY
$\epsilon \times 10^{-4}$
liters
mole-cm
5.0
4.0
3.0
2.0
1.0
0
12750
12810
12320
14,000
13,000
12,000
11,000
$\tilde{\nu}$ (cm-1)
MUB-8629

Fig. 9

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