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Some Properties of Chlorophyll *a* at Hydrocarbon-Water Interfaces and in Black Lipid Membranes

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Summary. Chlorophyll *a* is very surface active in the system 2,2,4-trimethylpentane-water. The standard free energy of adsorption may be as high as 10.6 kcal/mole. However, chlorophyll adsorption at this interface is unable to stabilize black membranes. Black films formed from solutions of glyceryl monooleate and chlorophyll *a* exhibit a weak fluorescence which indicates that a small amount of pigment, ca. 1 to 2% by area, may be contained in the membranes. Calculations based on adsorption data show that inclusion of somewhat more chlorophyll *a* than this might be expected. However, interfacial tension data for mixed solutions do not support this expectation.

Whether or not they are illuminated, black lipid membranes formed from mixed solutions of chlorophyll *a* and glyceryl monooleate have electrical properties indistinguishable from those of films made in the absence of pigment.

As is well known, chlorophyll *a* is located in chloroplast lamellae of green algae and higher plants (Park, 1966). The precise arrangement of the chlorophyll molecules in this environment has not yet been determined. However, as the pigment contains both hydrophobic and hydrophilic groups, it has been postulated that the molecules may be arranged at interfaces within these membranes, or at the membrane surfaces (Menke, 1966; Weier, Stocking & Shumway, 1966). Such postulates have added interest and perhaps biological relevance to the study of chlorophyll *a* at air- and hydrocarbon-water interfaces.

The most extensive investigations have been carried out on insoluble monolayers of chlorophyll *a* at the air-aqueous solution interface (Chasovnikova, Nekrasov & Robozev, 1966; Ke, 1966; Trosper, Park & Sauer, 1968). Some studies of black lipid membranes formed from pigment-containing solutions have also been reported. In the earlier studies (Ting,

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Huemoeller, Lalitha, Diana & Tien, 1968; Hesketh, 1969) nothing was known concerning the adsorption of the chlorophyll *a* into the bilayer structures. More recently Läuger's group (Alamuti & Läuger, 1970; Steinemann, Alamuti, Brodmann, Marschall & Läuger, 1971) have reported extensive data for mixed films of chlorophyll *a* and lecithin, obtaining results which indicate that a small amount of pigment is included in the black membranes.

In the present investigation, attempts had been made to form black lipid membranes from saturated solutions of pure chlorophyll *a* in hydrocarbon solvents. The films generally did not drain properly, and the membranes obtained were not stable. Alteration of various experimental parameters did not improve this behavior, and we were led to question the interfacial activity of the pigment. In addition to its intrinsic interest, some knowledge of the thermodynamics of adsorption of chlorophyll *a* at a hydrocarbon-water interface is likely to be helpful in interpretation of the behavior of the pigment in natural and model membranes. With these considerations in mind, the adsorption studies here reported were undertaken.

Although chlorophyll *a* alone did not stabilize black membranes, it was possible to form these structures from mixed solutions of chlorophyll and glyceryl monooleate. Electrical properties of the mixed films gave no indication that a significant amount of pigment was included in them, although theoretical calculations based on the adsorption properties of the surfactant molecules predicted that some chlorophyll should be present. In an effort to resolve this difficulty, fluorescence measurements were performed on mixed black membranes. The results suggest that a very small percentage of the film molecules is indeed chlorophyll *a*. Alamuti and Läuger's (1970) fluorescence data and the absorption measurements of Steinemann *et al.* (1971) for mixed films of chlorophyll *a* and lecithin are consistent with our results.

Materials and Methods

Chlorophyll *a* was extracted from commercially obtained fresh spinach, *Spinacia oleracea*, and purified by column chromatography either on powdered polyethylene and icing sugar according to Anderson and Calvin (1964) or on a series of icing sugar columns as described by Strain, Thomas and Katz (1963), omitting the boiling step. The microcrystalline pigment suspension in wet 2,2,4-trimethylpentane (TMP) was stored in the dark at -10 °C. All experiments with chlorophyll were performed in dim green light or in the dark, unless otherwise stated. Pigments were extracted from washed, four-week-old pea seedlings by the method of Strain and Svec (1966), omitting the boiling

step. The lower, aqueous phase was retained, and the upper phase, after being dried over roasted sodium chloride, was fractionated on an icing sugar column, using petroleum ether with increasing amounts of *n*-propanol. Four fractions containing pigments of increasing polarity were collected.

Sodium octadecyl sulfate (SOS) and cetyltrimethyl ammonium bromide (CTAB) were both highly pure, in that they exhibited neither a time dependent surface tension nor a minimum in the surface tension *vs.* concentration curve in the vicinity of the critical micelle concentration (cmc). Puriss grade cholesterol was obtained from Fluka. Glyceryl monooleate (GMO) and glyceryl dioleate were obtained from Sigma.

Analar acetone, methanol, and petroleum ethers were used for pigment isolations without further purification. Puriss grade *n*-butanol and *n*-octane were passed through alumina colums before use. Analar benzene and puriss grade TMP (Koch-Light) were distilled, passed through alumina columns, and saturated with buffered 0.1 N sodium chloride solution. Analar NaCl was roasted at 700 °C to remove organic impurities. Sodium mono- and dibasic phosphates, Analar grade, were used without further purification to buffer the electrolyte at pH 7.2, the final concentration of phosphate being 10^{-3} M. To ensure complete saturation of the hydrocarbons, the solvents were gently swirled over electrolyte solution in a closed vessel at room temperature for at least two days.

Water was twice distilled, first in a commercial still and then in a Pyrex still fitted with a quartz condenser and receiver.

Benzene solutions of chlorophyll *a* were prepared for interfacial tension measurements by dissolving a weighed amount of dried pigment in a weighed aliquot of the solvent already saturated with buffered electrolyte. The activity of the chlorophyll *a* in the benzene phase was determined with the aid of a Mechrolab Vapour Pressure Osmometer modified to give higher sensitivity (Cook, Redwood, Taylor & Haydon, 1968). N-hexadecane, 99.9% by gas-liquid chromatography, was used as the standard, and the benzene phase used as the reference.

Owing to the low solubility of the pigment in TMP the quantities of chlorophyll *a* required for tension experiments could not be weighed accurately. Instead, aliquots of TMP saturated with buffered electrolyte were allowed to stand over dried pigment in the dark for approximately 40 min. This preparation was centrifuged at maximum speed in a desk centrifuge (ca. 4,000 rpm) to remove suspended crystalline material. The supernatant was decanted, diluted to the desired concentration with the same solvent, and used immediately for tension measurements.

Mixed solutions of chlorophyll *a* and glyceryl monooleate were prepared by dissolving a weighed amount of the glyceride in a weighed aliquot of a saturated solution of chlorophyll in wet TMP, the final concentration of GMO lying above the cmc for bilayer experiments. The concentration of pigment monomer plus monomer-glyceryl monooleate complex was determined approximately from spectral data. Spectra were measured with a Unicam SP 500 or SP 800 spectrophotometer, with the appropriate electrolyte saturated hydrocarbon as a reference.

Interfacial tensions were determined by the drop volume method, all measurements being performed at 20 ± 0.05 °C. The correction factors of Harkins and Brown (1919) were used. The high optical densities of the more concentrated chlorophyll *a* solutions prevented direct observation of the drops. Under these circumstances, drop volumes were estimated from the micrometer reading when the drops hit the electrolyte-hydrocarbon interface in the tube. This method increased the uncertainty in surface tension values at the highest concentrations of pigment used.

Black lipid membranes were formed across a 0.1 or 0.15 cm diameter hole in a PTFE pot immersed in aqueous electrolyte. Vertical films were made using a brush technique (Mueller, Rudin, Tien & Wescott, 1963; Hanai, Haydon & Taylor, 1964), and horizontal

films formed using a manometer to control size and thinning (Duyvis, 1962). All electrical measurements were carried out on thick films and bilayers formed in the horizontal pot. Capacitances and conductances were obtained with instrumentation described previously (Hanai *et al.*, 1964, 1965) using small Pt:Pt electrodes. Bilayer areas were calculated from diameters observed under reflected dim green light.

Measurements of fluorescence were performed on horizontal films. The PTFE pot, in a glass-bottomed cell, was placed on a microscope stage. A high pressure mercury arc (Wotan HBO 200W) with suitable filters (Schott-Jena GB12 and GG4, and Farrand interference filter, 433 nm) served as the light source. The microscope substage condenser focused the image of a field stop in the hole in the PTFE pot. Exciting light collected by the 10× objective was blocked by a rejection filter (Optical Coatings Laboratory, Santa Rosa, California) and a red cutoff filter (Schott-Jena RG665 or RG695). The red sensitive photomultiplier (EMI 9558B) was mounted in a camera holder above the microscope eyepiece and was operated at 1,200 V dc. Fluorescence signals were observed as a shift in dc potential across the photomultiplier load resistance of 1.7×10^4 Ω when the camera shutter was opened. The differential amplifier of a Solartron oscilloscope, model no. CD 1183, provided adequate amplification of the signal. Values were corrected for a background signal owing to imperfect blocking of exciting light, obtained when no film was in the hole. Black film areas were calculated from capacitance measurements because the optical arragement precluded observation of the films by reflected light.

Results

Surface Activity of Chlorophyll a

The interfacial activity of chlorophyll *a* was investigated in liquid systems relevant to black lipid membrane studies. Interfacial tensions at the 0.1 N NaCl−TMP and 0.1 N NaCl−benzene interfaces were calculated from drop volume data obtained with various amounts of pigment in the hydrocarbon phase. Tensions were also measured in the former solvent system when GMO was present at concentrations above the cmc in the TMP phase. Interfacial tensions γ were calculated from drop volumes V, according to the equation

$$\gamma = \frac{V \Delta \rho g}{2\pi r} \phi \left(\frac{r}{V^{1/3}} \right) \tag{1}$$

where $\Delta\rho$ is the difference in density between the electrolyte and pigment solutions, g is the acceleration due to gravity, r the radius of the stainless steel tip used in the experiments and ϕ the correction factor of Harkins and Brown (1919). The tip had been calibrated with the systems air-water and decane-water, using interfacial tension values from International Critical Tables and Aveyard and Haydon (1965), respectively. The uncertainty in tip radius, $\pm 0.03_5$%, was negligible compared to the other experimental errors.

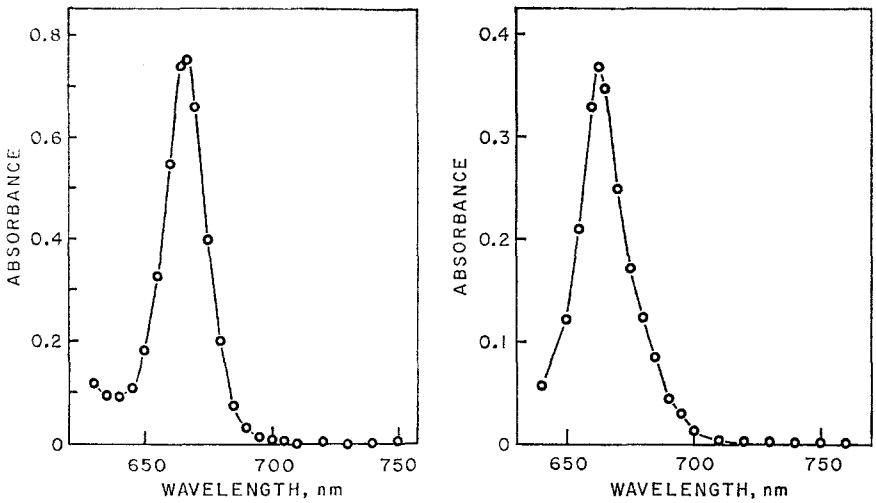

Fig. 1 (a) Absorption spectrum of chlorophyll *a* in electrolyte saturated benzene. (b) Absorption spectrum of the supernatant of a centrifuged, saturated solution of chlorophyll *a* in electrolyte saturated TMP

The surface excess of the chlorophyll *a* was obtained by means of the Gibbs equation

$$\Gamma^{(1)} = -\frac{1}{RT}\frac{d\gamma}{d\ln a} \tag{2}$$

where $\Gamma^{(1)}$ is the surface excess and a the activity of the chlorophyll in the bulk hydrocarbon phase. The determination of the activities of the pigment in the very dilute solutions employed was achieved by the indirect means described in the Appendix. For these calculations it was necessary to assume that a negligible amount of highly aggregated chlorophyll *a* was present in the hydrocarbon phases. Absence of far red absorption by the solutions used (Fig. 1) justifies this assumption.

The experimental data for the benzene-electrolyte systems are plotted as γ *vs.* $\log_{10} a$ in Fig. 2, where $a = [C_m] + [C_d]$ (*see* Appendix). The vertical error bars represent the standard deviations in γ calculated from the volumes of at least six drops. A cmc, which would be indicated by a flattening of the curve at high concentrations, was not found in the concentration range under investigation.

The surface excess of chlorophyll *a* at the benzene-electrolyte interface has been computed at various interfacial tensions using Eq. (2) and the data of Fig. 2. For a substance as surface active as chlorophyll *a* it is permissible to equate surface excess to surface concentration, and thus interfacial areas

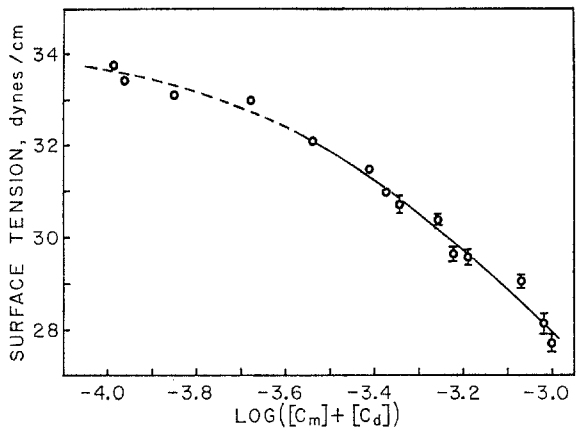

Fig. 2. Interfacial tension as a function of $\log_{10} a$ for the system chlorophyll *a* in benzene... 0.1 N NaCl, where $a = [C_m] + [C_d]$, and $[C_m]$ and $[C_d]$ are bulk concentrations of monomer and dimer, respectively. (*See* Appendix)

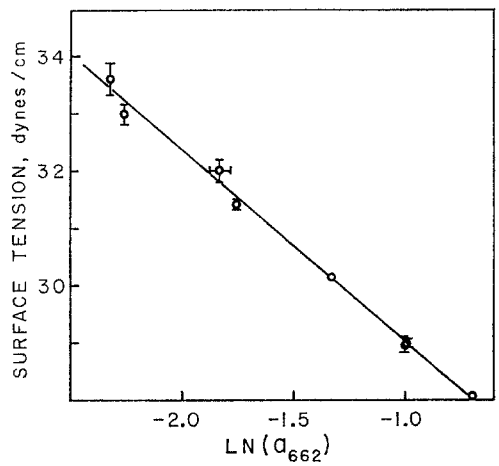

Fig. 3. Interfacial tension as a function of $\ln(\mathscr{A}_{662})$ for the system chlorophyll *a* in TMP... 0.1 N NaCl

per molecule have been calculated from the inverse of the surface excess. In Table 1 these areas are given for various surface pressures π, where

$$\pi = \gamma_0 - \gamma,$$

and γ_0 is the interfacial tension in the absence of chlorophyll *a*.

Tensions at the TMP-electrolyte interface are plotted in Fig. 3 as a function of the natural logarithm of the chlorophyll *a* solution absorbance at the red maximum, 662 to 663 nm (*see* Appendix). The points are best

Table 1. Area per molecule of chlorophyll *a* adsorbed at the benzene-0.1 N NaCl interface, at various surface pressures[a]

π (dynes/cm)	A (Å^2)	π (dynes/cm)	A (Å^2)
1.14	400	3.82	112
1.32	367	4.17	107
1.64	284	4.79	101
2.26	186	5.36	98
2.93	146	6.31	95
3.51	122		

[a] $\pi = \gamma_0 - \gamma$, where $\gamma_0 = 34.15$ dynes/cm.

fitted by a straight line. The area per chlorophyll molecule at the interface throughout the concentration range investigated is found to be 120 ± 10 Å^2 from Eq. (A.1). The interfacial pressure range over which this area is applicable is 16.6 to 22.2 dynes/cm.

The adsorption data for the benzene system obey the Langmuir model in so much as they may be fitted quite closely to the adsorption isotherm

$$\frac{A_0}{A - A_0} = x e^{-\Delta\mu^0/kT}$$

where x is the mole fraction of surface active solute, if the co-area, A_0, is assumed to be 78 Å^2 and the standard free energy of adsorption, $\Delta\mu$, to be -4.9 kcal/mole. The data for the TMP system are not sufficiently extensive for a similar analysis to be carried out. However, if the same adsorption model and the same co-area are assumed to hold, the standard free energy of adsorption is calculated to be -10.6 kcal/mole. The standard free energy of adsorption of GMO in the TMP system is ca. -7.92 kcal/mole (Andrews, 1970). If, therefore, it is assumed that from mixed solutions of chlorophyll *a* and GMO in TMP the relative adsorption is determined by the Boltzmann distribution, the composition of the adsorbed monolayer, and hence also of the black membranes (Cook, Redwood, Taylor & Haydon, 1968) may be calculated.

For example, the concentration of monomeric chlorophyll *a* in the GMO solutions used for bilayer fluorescence measurements was estimated from spectroscopic data, using $\varepsilon \simeq 7 \times 10^4$ liters/mole-cm at 663 nm [1], to be approximately 7×10^{-6} moles/liter. The monomeric GMO concentration was 3×10^{-3} moles/liter. On this basis, ca. 18% of the polar lipid

1 The extinction coefficient of monomeric chlorophyll *a* in wet aliphatic hydrocarbons has not been determined accurately. This value represents a reasonable estimate (e.g., Seely & Jensen, 1965).

molecules in the membrane should have been chlorophyll *a*. The calculation is obviously quite rough, and the errors are difficult to assess. In theory it should also be possible to determine adsorption in the bilayer from measurements of the contact angle for lenses in horizontal films (Haydon and Taylor, 1968). Such measurements were not undertaken, however. The chemical potentials of the two adsorbing species could not be varied sufficiently to provide enough data for a more accurate calculation.

In contrast to the above prediction, addition of chlorophyll *a* to GMO solutions caused negligible change in the interfacial tension of the latter. This result suggested that only an estimated 1% or less of the interfacial area was occupied by pigment.

Stability of Membranes Formed from Solutions Containing Chlorophyll a

Films formed from a saturated solution of chlorophyll *a* in wet TMP did not generally drain to the black state. When they did so, they burst immediately. Silvery or colored films formed frequently and were often quite stable. Such films occasionally developed small black patches, so that the films gave the appearance of a lace doily. Sometimes small crystals, presumably of the pigment, were also visible in the films. This type of behavior has been observed previously for thin films formed of chlorophyll *a* and other pigments (Tien, Huemoeller & Ting, 1968). No electrical measurements were performed on these films, as it was very difficult to measure the black areas accurately.

Various attempts were made to obtain stable black films containing chlorophyll *a*, without adding surfactants which themselves form stable bilayers. None of the following modifications enhanced black film formation or stability: 1) variation of NaCl concentration in the aqueous phase from 10^{-3} N to 2 N; 2) addition of cholesterol up to a concentration of approximately 10^{-2} M; 3) use of *n*-octane, or benzene, or 30% (by volume) butanol in TMP as the hydrocarbon phase; 4) ultrasonic dispersion of chlorophyll in the solvent; 5) addition to the hydrocarbon phase of β-carotene or oleyl alcohol or glyceryl dioleate, none of which stabilize black membranes when they are the only surfactant present; or 6) presence in the aqueous phase of the water soluble surfactants CTAB or SOS at concentrations either below or above the cmc.

These negative results strongly suggest that chlorophyll *a* does not function as an adequate stabilizer of black lipid membranes. However, glyceryl monooleate in TMP does form stable bilayer membranes, and the addition of chlorophyll *a* to these solutions does not affect membrane

stability. Bilayers formed from these solutions appeared no different from those made from pure GMO solutions, unless sufficient excess aggregated pigment was present to collect at the interfaces.

Stable lipid bilayer membranes formed from solutions of chlorophyll *a* and carotenoids, or of plant pigment extracts have been reported (Tien, 1968; Ting *et al.*, 1968; Hesketh, 1969). In an effort to determine which pigments conferred stability on these films, a pigment extract from pea seedlings was fractionated as described above. Each of the four eluates was washed to remove polar solvent and any water soluble material that was eluted from the icing sugar. The eluates and the original aqueous phase were flushed with nitrogen gas and stored at -10 °C. Aliquots of each fraction were evaporated to dryness and resuspended in electrolyte-saturated TMP for use in forming thin lipid films. In no case was all material observed to go into solution in the wet hydrocarbon. Only the solutions of the aqueous phase and of the last, most polar, eluate formed stable films. Films obtained with the resuspended aqueous phase material did not drain properly to the black state, but those obtained with the eluate gave large black films stable for several minutes. Spectroscopy and thin-layer chromatography on mannitol using 2% methanol in TMP (v/v) as developing solvent revealed that chlorophylls were absent from both these solutions. They did, however, contain large amounts of carotenoids, having composite long wavelength absorption maxima below 475 nm. The material proved labile when stored at -10 °C in the dark under nitrogen. The other three eluates, which contained most of the chlorophylls, did not form thin films. The use of *n*-octane rather than TMP for resuspension gave similar results.

Electrical Properties of Films Containing Chlorophyll a

The average capacitance of black films formed from mixed solutions of GMO and chlorophyll *a* in electrolyte-saturated TMP was 0.393 ± 0.003 $\mu F/cm^2$ at a frequency of 1 kHz. This value is within experimental error of that observed for bilayers of pure GMO in TMP (Andrews, 1970), and indicates that film thickness was not altered by the presence of a small amount of the pigment.

dc conductances were measured with the films irradiated with dim green light or with white light (36 W tungsten microscope lamp). No special precautions were taken to avoid heating of the films or electrolyte by this small source. With the apparatus used for these measurements, the light source could not be focused on black film alone. Rather an area of the

PTFE pot slightly larger than the hole was illuminated. Thus, any conductance changes observed could not be attributed to effects occurring in the bilayer alone upon illumination.

The conductance of thick films of chlorophyll *a* and GMO in TMP was not changed by exposure to white light, and was about the same as that of the thick films of GMO in TMP in the absence of pigment, $R=8$ to 10×10^8 Ω cm^2. A thick film of chlorophyll *a* alone in TMP, however, was an order of magnitude less conducting, $R=2.4\times10^{10}$ Ω cm^2, and the resistivity of these films was very reproducible. The presence of saturating amounts of β-carotene in place of, or in addition to, chlorophyll *a* in these thick films had no effect on conductance.

Black films formed from GMO solutions containing either or both pigments were, in dim green light, an order of magnitude more conducting than thick films, $R=0.4$ to 1.0×10^8 Ω cm^2. No significant, reproducible change in conductance was detected for such films when they were irradiated with white light. In some cases, the illuminated bilayers became about two or three times more conducting than they had been in dim green light, but in other instances the opposite effect, or no effect at all, was observed upon illumination. A very slight average increase in conductance occurring upon white light illumination could, in part, be accounted for by thermal effects, as discussed by Hesketh (1969). Also, the films were generally short-lived, bursting within about 20 min of draining to the black stage, and it is often observed that bilayer conductances increase markedly shortly before films break. It is possible that such an increase in some cases coincided with illumination, for the small conductance changes, when they occurred, were frequently not reversible.

Film conductances were also measured when 9×10^{-6} M cytochrome *c* was present in the aqueous phase. Presence of the protein made black films very unstable, but did not appear to alter their conductance properties. If the PTFE support became coated with cytochrome, both thick and bilayer films were highly conducting, most probably owing to border leaks along the edge of the hole. There was no evidence of any effect on film electrical properties owing to interaction between cytochrome and pigment.

Fluorescence from Chlorophyll a *in Black Lipid Membranes*

When fluorescence measurements were performed, care was taken to avoid direct illumination of the thick meniscus of film-forming solution surrounding the black membranes. Field stops were always chosen to have

image areas considerably less than that of the hole in the pot. Nonetheless, some scattered light most probably reached the meniscus, which then fluoresced, because fluorescence generally continued to exhibit an inverse dependence on black film area as the latter exceeded the area of the field stop image. However, for sufficiently large black membrane areas, fluorescence was frequently observed to reach a low value independent of further changes in film area. This residual fluorescence was attributed to chlorophyll molecules included in the bilayer membrane.

From this residual fluorescence signal normalized to unit illuminated area, and that given by a sample of film-forming solution of known chlorophyll concentration and volume, an approximate number of pigment molecules in the black film was calculated. In this calculation it was assumed that the fluorescent yield of chlorophyll *a* is the same in the bilayer as in solution; i.e., that quenching was negligible. A solution of absorbance 0.71 at 663 nm, optical path length 0.1 cm, and illuminated volume $1.8 \pm 0.2_5 \times 10^{-4}$ cm^3 gave a corrected fluorescence signal of 52.8 mV. Using the approximate extinction coefficient for chlorophyll *a* in electrolyte saturated TMP (*see above*) it was calculated that $1.1 \pm 0.2 \times 10^{13}$ molecules of pigment were in the direct path of the exciting light. The corrected residual fluorescence signal detected from bilayers, 0.025 mV, would thus be caused by emission from $5 \pm 1 \times 10^9$ pigment molecules in the illuminated film area, which was $3.6 \pm 0.5 \times 10^{-3}$ cm^2 for the two faces. For the interfacial area of ca. 120 Å^2 per chlorophyll *a* molecule, this number corresponds to a chlorophyll density of $1.4 \pm 0.3 \times 10^{12}$ molecules/cm^2 or between 1 and 2% coverage. The average center-to-center pigment separation would be roughly 85 ± 10 Å. This result is significantly lower than that predicted by the calculations based on interfacial adsorption, but is not unreasonable in light of the mixed solution interfacial tensions or the capacitance data.

Discussion

It is clear from the interfacial tension experiments that chlorophyll *a* is strongly adsorbed at the TMP – 0.1 N NaCl interface. This adsorption is nevertheless unable to stabilize black membranes, possibly because the area per phytyl chain is over 100 Å^2, whereas in bilayer membranes of GMO or lecithin the area per hydrocarbon chain is in the region of 30 Å (Andrews, Manev & Haydon, 1970).

The stability reported in the literature of bilayers obtained from chlorophyll-containing solutions of plant extracts (Ting *et al.*, 1968; Hesketh,

1969) is thus probably owing to the other surfactants – xanthophylls or other fat soluble chloroplast lipids present in the crude pigment extracts – in the film forming solutions employed. The extent to which chlorophyll *a* may be present in bilayers formed from such mixtures cannot be estimated unless the adsorption of the other components is known.

The free energy change upon adsorption of chlorophyll *a* at the TMP – 0.1 N aqueous NaCl interface relative to that upon adsorption of GMO in the same system indicates that a few area percent of chlorophyll *a* may be expected to remain in a black film after draining. However, replacement of as much as 18% of $C_{18:1}$ oleate moieties in the hydrocarbon phase by phytyl, which is effectively $C_{16:1}$ beyond the carbonyl group, should result in an increase of film capacitance of up to 2.5% owing to decreased thickness of the hydrophobic part of the bilayer. A deviation of this size is significantly larger than experimental error and would have been detected. That somewhat less than 18% of the polar lipids in the black film were chlorophyll *a* is further supported by the adsorption characteristics from mixed solutions and the fluorescence results.

The conclusion drawn from these latter data, that chlorophyll *a* occupies only 1 to 2% of the area of the black films, is not particularly reliable if the limitations of the measurements and the assumptions used in the calculations are considered. However, Alamuti and Läuger's (1970) recent accurate fluorescence measurements on the system chlorophyll *a* and dioleyl lecithin provide some support for our results. At bulk chlorophyll concentration of 10^{-4} M they found 6.5×10^{11} chlorophyll molecules per cm^2 of bilayer, or ca. 0.8% area coverage if a pigment molecule occupies 120 $Å^2$ in the interface. The rough correspondence between this datum and that reported here may indicate that the latter result is more reliable than had been supposed. A more direct comparison cannot be made without some knowledge of the adsorption of the chlorophyll *a* in the lecithin system.

Cherry, Hsu, and Chapman (1971) have also reported absorption spectra of lecithin-chlorophyll bilayers. They are able to detect the order of 2% chlorophyll in the membranes, when the bulk chlorophyll *a* concentration is roughly 10^{-3} M and the bulk lecithin/chlorophyll ratio 5:1. Interestingly, in this system the relative adsorption of chlorophyll *a* is distinctly less than that of the phospholipid, whereas in the glyceryl monooleate-chlorophyll *a* system the pigment appears to be more strongly adsorbed than the neutral lipid.

The bilayer membranes formed from the mixed solutions of GMO and chlorophyll *a* showed no increase in their conductance over those of GMO

alone, whether or not they were illuminated. This might have been because the percentage of chlorophyll *a* present was undoubtedly quite small. Nevertheless, in the absence of an appropriate electron acceptor it is scarcely to be expected that the chlorophyll *a* would have rendered the bilayers electronically conducting. Furthermore, the method of illumination was not sufficiently precise to ensure that light absorption occurred on one side of the bilayer only, and also, it is extremely unlikely that the small amount of chlorophyll *a* present would have been so aggregated as to promote solid-state electronic conductance. The results of Trissl and Läuger (1970) for a similar system are in accord with these observations. Tien and Verma (1970) have also recently reported negligible photoeffects in films formed from chloroplast materials, in the absence of asymmetric conditions.

The large conductance increases reported for bilayer systems containing chlorophyll *a* and other photosynthetic pigments in an apparently symmetric environment (Tien, 1968; Hesketh, 1969) may in part have been owing to interactions of the various excited chromophores upon illumination, as well as to the much higher bulk pigment concentrations used. Indeed, the only published action spectrum (Tien, 1968) suggests that accessory and bulk pigments are involved in the observed photoconductivity.

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Appendix

In wet hydrocarbon, chlorophyll *a* usually exists in the form of dimers and higher aggretates as well as hydrated monomers (Ballschmiter, Truesdell & Katz, 1969). In wet benzene, only hydrated monomers and dimers occur at the concentrations used in the present experiments. The absence of higher aggregates was confirmed by the absence of far red absorption (Fig. 1a). For the monomer-dimer equilibrium

$$2C_m \cdot H_2O \rightleftharpoons C_d \cdot (H_2O)_2$$

the concentrations are sufficiently small for the activity coefficients of the individual species to be reasonably neglected, and hence the dimerization constant K_d may be

written

$$K_d = [C_d]/[C_m]^2.$$

The only available value for the hydrated chlorophyll *a* dimerization constant in benzene, $K_d = 459$ liters/mole, was obtained by Aronoff (1962), who compared vapor pressure lowering by pigment solutions with that by known monomeric and dimeric materials in the solvent. Using a somewhat more sensitive instrument, we obtained the activity of chlorophyll *a* in electrolyte-saturated benzene for the highest concentration used here (4.5 mM) and from this calculated that $K_d = 250 \pm 30$ liters/mole. From this value the monomer and dimer concentrations were calculated at lower concentrations. The activity required for the Gibbs equation was then assumed to be $([C_m] + [C_d])$. In fact, the correction for dimerization was not very appreciable at the lower concentrations. Also, the seemingly large uncertainty in K_d did not introduce more than 1.5% uncertainty in the activities.

The chlorophyll *a* solutions in TMP were too dilute, even at the highest concentrations, for activities to be measured with the vapor pressure osmometer. The determination of the concentrations of the chlorophyll *a* by spectrophotometry was also not possible because the extinction coefficient of the pigment in wet TMP is not known. Aggregates as well as hydrated monomers and dimers may be present in these solutions. The aggregates absorb in the far red, with a band maximum located at 748 nm (Strain *et al.*, 1963). However, centrifugation of solutions prepared as described above effectively removed the aggregates, as shown by the absence of far red absorption by the supernatant (Fig. 1*b*). The slight shoulder on the long wavelength side of the main red band suggests that only a small (and this is assumed negligible) amount of dimer was present. If, therefore, it is assumed that to a first approximation, absorbance at the red maximum, 662 to 663 nm, is proportional to the hydrated monomer concentration, the absorbance is written

$$\mathscr{A}_{662} = \text{constant} \times [C_m]$$

from which

$$\ln(\mathscr{A}_{662}) = \ln(\text{constant}) + \ln[C_m].$$

Eq. (2) of the text becomes

$$\Gamma^{(1)} = -\frac{1}{RT}\frac{d\gamma}{d\ln(\mathscr{A}_{662})} \tag{A.1}$$

Thus, surface excess of chlorophyll *a* can be obtained from a plot of γ *vs.* $\ln(\mathscr{A}_{662})$ for the TMP solutions.

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