Chlorophyll a and Cytochrome c at a Heptane-Water Interface

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The surface pressure was measured as a function of area/molecule for chlorophyll a (Chl) and cytochrome c (Cyt) at a heptane-water interface. At a surface pressure of 6 dyn/cm the area per molecule of Chl ($A_{Chl} = 113 \pm 6 \AA^2$) for reduced Cyt c (red. Cyt) the $A_{red. Cyt} = 5000 \pm 300 \AA^2$ and for oxidized Cyt c (ox. Cyt) $A_{ox. Cyt} = 4100 \pm 250 \AA^2$. Cyt appears to denature at the interface. Irradiation results in a decrease of the $A_{Chl}$ for Chl to $A_{Chl} = 100 \pm 5 \AA^2$. There appears to be interaction between Chl and red. Cyt in a mixed film no interaction is observed between Chl and ox. Cyt.

Introduction

While there has been considerable work with biological pigments at an air-water interface, no studies have been carried out at a lipid-water interface. The latter has more relevance to the biological membrane system. Recent technical advances facilitate studies at lipid-water interfaces.

The parallels between chlorophyll a (Chl) monolayers at air-water interfaces and the orientation of Chl in the chloroplast have been recognized for a long time. Reports of the action of light on Chl monolayers at an air-water interface have been made by Bellamy et al. and Aghion et al. As a possible model for studies of the photoproperties of Chl in vivo we decided to test the ability of Chl to carry out a light reaction at a heptane-water interface. This interface more nearly coincides with the basic model of the cell membrane by allowing the Chl molecules to align at a liquid-liquid interface rather than at a gas-liquid interface.

Insofar as cytochrome c (Cyt) is associated with membrane systems in vivo, it is of interest to measure the area/molecule of reduced cytochrome c (red. Cyt) and oxidized cytochrome c (ox. Cyt). Furthermore, in vivo reaction center I Chl is associated with a c type cytochrome (Cyt f) ; tests are conducted to ascertain if there is an interaction between Cyt and Chl at a heptane-water interface.

Materials and Methods

All experiments are carried out in an electrically shielded, temperature controlled chamber. The experimental chamber is thermostated by circulating water from a constant temperature bath (20 ± 0.05 °C) through a heat exchanger. The interfacial surface pressure, $\pi$, is measured with a sand blasted platinum Wilhelmy blade in conjunction with a torsion balance. The Brooks frame used in this work is similar to that used by Brooks and Pethica. The main difference is that the frame used in this work is fabricated of stainless steel instead of glass. The barriers used for compression of the monolayer are made of ordinary glass. Frame and barriers are coated with dimethylchlorosilane.

All components to be coated are thoroughly cleaned by washing in distilled water, soaking for an hour in acetone, soaking in distilled water for one hour, soaking in clean heptane overnight, washed with distilled water, and washed with absolute ethanol. A large dissecting dish is filled with a 2% solution of dimethylchlorosilane (Eastman Kodak) in carbon tetrachloride and the components are immersed in the solution for 24 hours. Coated barriers and Brooks frame are washed with pure n-heptane to remove excess silane solution and allowed to sit overnight in n-heptane, they are then washed with distilled water and are ready to use. Appropriate parts of the Brooks frame and barriers are made hydrophillic as described by Brooks and Pethica.

Reagent grade n-heptane purchased from Eastman Kodak (b.p. 96 – 97 °C) is purified by fractional distillation using a reflux column (approximately 3 ft. long). This purified heptane is used for the heptane phase of the system. All barriers, syringes and auxiliary components used for work at the interface are stored in a glass Soxhlet apparatus and continually washed with the purified heptane. The aqueous phase of the heptane-water interface used in these experiments is phosphate buffer, pH 7.8, ionic strength 0.6. Stock solutions are prepared from Na$_2$HPO$_4$ (Sigma Chem. Co.,
St. Louis, Mo.) NaH₂PO₄ (Sigma Chem. Co.), and NaCl (Mallinckrodt, N. J.). Distilled water of satisfactory purity is obtained by deionizing with a Barnstead standard ion-exchange column then distilling from permanganate in an all glass still (Corning, N. Y., Model A.G.-2). Chl is prepared as described by Aghion et al.⁵. This Chl measured at an air-water interface has an area 4% larger than the smallest published values (of 122 Å²). The Cyt used in our experiments is Cyt type III from horse heart, free of ammonium sulfate and NaCl (Sigma Chem. Co.). According to Sigma's assay the Cyt is 98% oxidized. The Cyt is treated either with excess potassium ferricyanide (Fisher Certified Reagent, N. J.) to yield ox. Cyt or with excess sodium ascorbate (National Biochemical Corporation, Cleveland, Ohio) to yield red. Cyt.

A concentrated benzene (Mallinckrodt Analytical Reagent) solution of Chl, usually less than 10 µl (at OD ≈ 250) is delivered to the clean heptane-water interface using a gas tight 100 µl Hamilton Syringe. The movable barrier is then compressed and expanded repeatedly. After about 1½ hours reproducible isotherms are measured (see Fig. 3). Area per molecule (A) in Å² is measured as a function of surface pressure (π) in dyn/cm. The value of A at a specific π is expressed as A₈.

An aqueous solution of Cyt (about 10 µl) is added to the interface using a 100 µl Hamilton gas tight syringe. Films are irradiated with white light from a 500 watt slide projector. The incident light intensity at the interface is 1.3 x 10⁴ ergs/cm² sec.

**Results and Discussion**

**Chlorophyll**

In Fig. 1 are shown typical isotherms of Chl. For unirradiated Chl A₁₀ = 88 Å² and A₆ = 113 Å². After irradiation A₁₀ = 81 Å² and A₆ = 100 Å². The accuracy of these measurements are ± 5%, one of the main errors introduced is the measurement of material added to the interface.

It is useful to compare these results at the heptane-water interface with those published for an air-water interface. Aghion et al.⁵, show isotherms of Chl in the dark where A is larger than that reported in Fig. 1. At an ionic strength of 0.1 from the paper by Aghion et al.⁵, A₁₀ = 92 Å². In the present work an ionic strength of 0.6 is used, perhaps the difference in A₁₀ may arise in part from the difference in ionic strength. Another difference in the experimental conditions is temperature. The experiments described here are conducted at 20 °C while those of Aghion et al.⁵ were at 15 °C. There may be a small effect of temperature on A, however, this has not been investigated systematically.

By comparing the slope of the Chl isotherm in Fig. 1 with the slope shown in the paper by Aghion et al.⁵, it can be seen that solid films are formed at air-water interfaces and liquid films at heptane-water interfaces. At an oil-water interface the molecules of oil can penetrate between the hydrocarbon chains (here the porphyrin rings) and remove interchain attraction. This effect can moderate the internal cohesion of the porphyrin rings that occurs at the air-water interface. In this way a smaller A can result at the heptane-water interface.
In Fig. 2 is shown the change of $A_6$ as a function of time at $\pi = 6$ dyn/cm, both in the dark and during irradiation. In this experiment it can be seen that $A_6$ stabilized in the dark after 90 min. The $A_6$ of Chl increases about 15% in the dark during the 90 min it takes to stabilize the film. This is probably the time required to form a stable film at the interface although it is possible that small amounts of dust filter through the heptane and accumulate at the interface. However, if it were simply dust $A$ would not reach an equilibrium value so quickly. Furthermore, leakage of the film would result in a decrease of $A$, so leakage could not account for the time required for stabilization.

Irradiation does not start until $A$ is constant in the dark for 15 min. During irradiation there is a continuous decrease in $A_6$ for 60 - 80 min, after which a steady value is obtained. The $A_6$ decreases 11% before a steady state is reached. This light induced decrease in $A_6$ indicates that a photoreaction of Chl is occurring. The quantum yield for this reaction is quite low, the estimated lower limit for the yield is $2 \times 10^{-3}$. At an air-water interface the quantum yield for photooxidation of Chl is $60 \times 10^{-3}$.

Aghion et al. have shown a light reaction of Chl at the air-water interface. They concluded that this reaction is a photooxidation resulting in an increase of $A$ (opposite to the observation at a heptane-water interface). They suggested that the oxidation site is at the porphyrin-air rather than the porphyrin-water interface, perhaps at the vinyl substituent. Aghion et al. proposed that the oxidized state of the pigment is more hydrophillic, so that the angle between the plane of the porphyrin and water surface would be smaller, resulting in a larger $A$. Our results, on the other hand, show that illumination results in a decrease of $A$ at the heptane-water interface. If a photooxidation is occurring at the heptane-water interface it could be with oxygen dissolved either in the heptane or aqueous phase. Since the change of $A$ here is opposite to that observed at the air-water interface, perhaps at the heptane-water interface, oxidation occurs at the hydrophillic rather than hydrophobic portion of the porphyrin. In general the effect of light on Chl causes a rearrangement of charge in the porphyrin ring or a substituent group thereby increasing the internal cohesion of the film or the angle of the porphyrin at the heptane-water surface.

At acid pH's pheophytinization of Chl occurs in the dark at an air-water interface; pheophytinization is accompanied by a decrease of $A$. The direction of change of $A$ is similar to that observed in this work. However, at a pH of 7.8 films of Chl are stable, therefore, in our experiments pheophytinization is not likely to occur.

**Cytochrome**

Fig. 3 shows the isotherm for red. Cyt after being on the surface for 25 min and 135 min. Here, as for Chl, the main error is the measurement of material added to the interface. When red. Cyt is added to the interface there is a continual increase of $A$ with time. Fig. 4 illustrates the increase of $A_6$ of...
red. Cyt as a function of time. A steady value of $A_6$ is reached after about 140 min. The $A_6$ for red. Cyt = 5000 ± 250 Å². In Fig. 3 is shown the isotherm for ox. Cyt after being on the surface for 25 min. With Cyt there are great variations in $A$ on a day to day basis. Whether these fluctuations arise from technique, environmental factors (dust, solvent purity), or heterogeneity of the Cyt sample was not determined.

Reinach and Brody⁹ reported an $A$ for Cyt at the air-water interface of roughly one tenth the area measured at the heptane-water interface. The difference in pH and ionic strength used in the two experiments might account in part for some of the difference in $A$, but it is insufficient to account for an order of magnitude. The origin for the difference in $A$ is more likely, that Cyt is unraveling at the heptane-water interface. Such a proposal would be compatible with the data in Fig. 4 which shows a large increase of $A$ for red. Cyt as a function of time.

Apparently when Cyt is spread at the heptane-water interface, the lipid penetrates between the hydrocarbon chains and removes interchain attraction. Additionally, the lipid penetration could destroy the quaternary and tertiary structure, thereby causing Cyt to lose its structural integrity and thus occupy a greater area. Since Cyt is almost totally insoluble in the heptane phase, as denaturation proceeds the Cyt would lie fully extended on the water phase allowing additional contact between the heptane and Cyt. This would further hasten the loss of quaternary and tertiary structure.

**Mixed Monolayers**

There may be a little interaction between Chl and ox. Cyt in a mixed film. The experimentally determined average value for $A_6$ overlaps the theoretically determined value when the concentration of Chl and ox. Cyt are approximately equal.

However, when there is excess ox. Cyt to Chl (2.3:1) then the experimental $A_6$ is less than the theoretical values (see Table I). While there is a small increase of $A$ during irradiation, it is not clear whether this is the result of a photoreaction or simply reflects a slow time dependent increase of $A$.

There is an interaction between Chl and red. Cyt in a mixed film. At a mole ratio of 3 Chl : 1 red. Cyt the experimentally measured $A$ is significantly smaller than the theoretically calculated $A$ (see Table I). Interaction between Chl and red. Cyt may involve interdigitation, as well as, complexation between the two molecules. While preliminary data indicate small increase in $A$ during illumination it remains to be established if it is significant.

The observation that there is a large interaction between Chl and red. Cyt and a small, if any, interaction with ox. Cyt is particularly interesting. In a primary photoreaction of photosynthesis (system I) there is an oxidation of Cyt by a Chl¹⁰. The specific complexation between Chl and red. Cyt observed at the heptane-water interface may be a significant model for studying the *in vivo* reaction.

### Table I. Mixed films of Chl and Cyt.

<table>
<thead>
<tr>
<th>Film ratio</th>
<th>Experimental $A_6$**</th>
<th>Theoretical $A_6$ from Fig. 3* and Fig. 1 [Å²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Chl: 1 red Cyt c</td>
<td>570 ± 34</td>
<td>1235 ± 75</td>
</tr>
<tr>
<td>3 Chl: 1 red Cyt c</td>
<td>683 ± 41</td>
<td>1235 ± 75</td>
</tr>
<tr>
<td>1 Chl: 2.3 ox. Cyt c</td>
<td>1984 ± 119</td>
<td>2690 ± 160</td>
</tr>
<tr>
<td>1 Chl: 1.3 ox. Cyt c</td>
<td>2165 ± 130</td>
<td>2365 ± 140</td>
</tr>
</tbody>
</table>

* Using smallest $A_6$ in Fig. 3.

** Experimental $A_6$ = total area/total number of molecules at interface.

Theoretical $A_6 = n_1 A_1 + n_2 A_2$ where $n =$ mole fraction and $A = A_4$ for each component.