# Surface Properties of Monomolecular Films of Oxidized and Reduced Cytochrome *c* and *f*†

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ABSTRACT: The surface properties of monomolecular films of oxidized and reduced cytochromes *f* and *c* were measured at an air–water interface. Area/molecular ($A$) and surface potential ($\Delta V$) for oxidized and reduced forms of the cytochromes were measured as a function of pH. Oxidized cyt *f* has a *maximum* for both $A$ and $\Delta V$ at pH 7.5. At a surface pressure of 6 dyn/cm the maximum $A = 2600 \pm 50$ Å$^2$ and the maximum $\Delta V = 200 \pm 10$ mV. Reduced cyt *f* as a function of pH has a minimum value for both $A$ (2200 Å$^2$) and $\Delta V$ (95 mV). Oxidized cyt *c* as a function of pH has minima for $A$ (140 Å$^2$) and $\Delta V$ (188 mV) at pH 7.0 and 7.3, respectively. On the other hand, reduced cyt has *maximum* values for $A$ (220 Å$^2$) and $\Delta V$ (260 mV) at pH 7.0 and 7.3, respectively.

Models of molecular organization of the thylakoid membrane describe chlorophyll as oriented in a monomolecular film at a protein–lipid interface (Goedheer, 1957; Calvin, 1959; Kreutz, 1970). There is evidence that reaction center I contains chlorophyll *a* and cytochrome *f* (Butler, 1961; Vernon and Ke, 1966; Vernon *et al.*, 1966). On the basis of kinetic spectrophotometric changes at room temperature and in the solid state (77°K) it appears that chlorophyll and cytochrome are closely coupled (Witt *et al.*, 1965). Indeed from theoretical considerations it is proposed that in the reaction center chlorophyll and cytochrome, in close proximity to one another, constitute an energy sink (Clayton, 1965). Pigment monolayers on an aqueous surface closely simulate the *in vivo* state and serve as a useful model to study the physical ahd chemical properties of endogenous materials in an oriented, well-defined system.

Cytochrome *c* (cyt *c*) is associated with the mitochondrial membrane system *in vivo* where it functions as an electron transport carrier (Seligman *et al.*, 1968). While the properties of cyt *c* and cyt *f* in solution are well known, their properties in a membrane situation are not (Davenport and Hill, 1952).

The spreading properties of monomolecular films of reduced and oxidized cyt *c* were first reported by Jonxis (1939). More recently pressure–area curves of cyt *c* were reported by Aghion *et al.* (1969); however, they did not specify whether oxidized or reduced forms of cyt *c* were used nor did they determine the area/molecule. They did, however, demonstrate that there is an interaction between cyt *c* and chlorophyll in a mixed monolayer. The area/molecule of reduced and oxidized cyt *c* at pH 7.0, as well as the oxidation–reduction potential of a monomolecular film of cyt *c* at an air–water interface, were reported by Reinach and Brody (1972).

Surface properties of monomolecular films of oxidized and reduced cyt *c* and *f* are presented in this paper. Properties studied include area/molecule ($A$) and surface potential of the film ($\Delta V$) as a function of pH. Changes in $A$ and $\Delta V$ reflect conformational changes of the protein on the surface. Before interaction between cyt and chlorophyll can be evaluated, the surface properties of cyt monolayers must be determined.

## Materials and Methods

Monolayer studies are carried out using a Wilhelmy plate surface balance similar to that described previously (Brody, 1971). The entire apparatus is housed in an environmental chamber. The sensitivity of the balance is ±0.2 dyn/cm. A constant temperature of 15° is maintained throughout the experiments using a thermostatically controlled, water cooling system imbedded in the trough.

The concentration of the aqueous solution of cytochrome (cyt) is determined spectrophotometrically. For cyt *f* a molar extinction coefficient of $2.6 \times 10^4$ is used for the reduced form and $1.1 \times 10^4$ for the oxidized form. For cyt *c* a molar extinction coefficient of $2.9 \times 10^4$ is used for the reduced form and $0.84 \times 10^4$ for the oxidized form.

The area/molecule $A_\pi$ and surface potential $\Delta V_\pi$ are measured at a given surface pressure, $\pi$ dyn/cm. The accuracy of $A_\pi$ is ±2% unless otherwise noted. Surface potential, $\Delta V$, measurements are made as described previously (Brody, 1971). The accuracy of $\Delta V_\pi$ is ±10 mV.

$\pi$–$\Delta V$ and $\pi$–$A$ curves are measured repeatedly until reproducible values are obtained. Usually after three isotherms, consistent values are obtained.

Since the $\pi$–$A$ curves for cyt do not have a linear portion an extrapolation to determine $A$ at $\pi = 0$ dyn/cm is very uncertain, therefore $A_\pi$ is measured at $\pi = 6$ dyn/cm. $\Delta V$ is equal to the difference in potential of the surface with the film ($V$) and the clean surface ($V_{H_2O}$) *i.e.*, $V - V_{H_2O}$. More reproducible values of $\Delta V$ are obtained by calculating $V_{H_2O}$ from $V$ as a function of concentration of molecules on the surface, see results (Brody, 1973). $\Delta V$ is related to the perpendicular component of the dipole moment of the molecule by the expression $\Delta V = 37.7\mu_\perp/A$.

The cyt *f* is a gift from Professor N. Bishop of the University of Oregon, Corvallis. Horse heart cyt *c* type II is obtained from Sigma Chemical Co. (St. Louis, Mo.). Chlorophyll *a* is prepared as described by Aghion *et al.* (1969). Addition of excess sodium ascorbate (Nutritional Biochemical Corporation, Cleveland, Ohio) is used to completely reduce the cytochromes. Addition of excess potassium ferricyanide is used to completely oxidize the cytochromes (Fisher Chemical Co.). Phosphate buffer, ionic strength 0.6, is used

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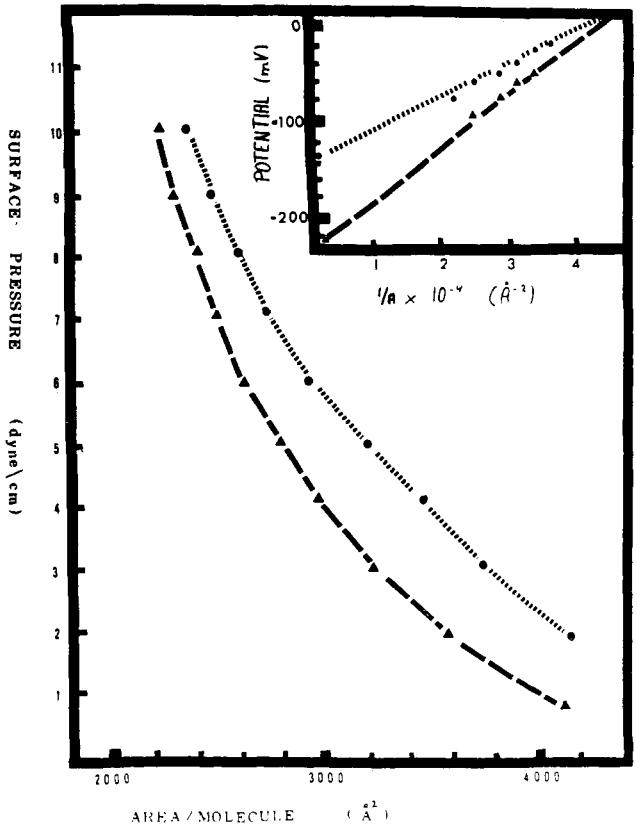


FIGURE 1: Area/molecule of oxidized (— —) and reduced (●) cytochrome *f* as a function of surface pressure. The insert shows $\Delta V$ as a function of molecules/Å$^2$ of oxidized (— —) and reduced (●) cytochrome *f*. The aqueous subphase contained phosphate buffer (pH 7.8), ionic strength 0.6 at 15°.

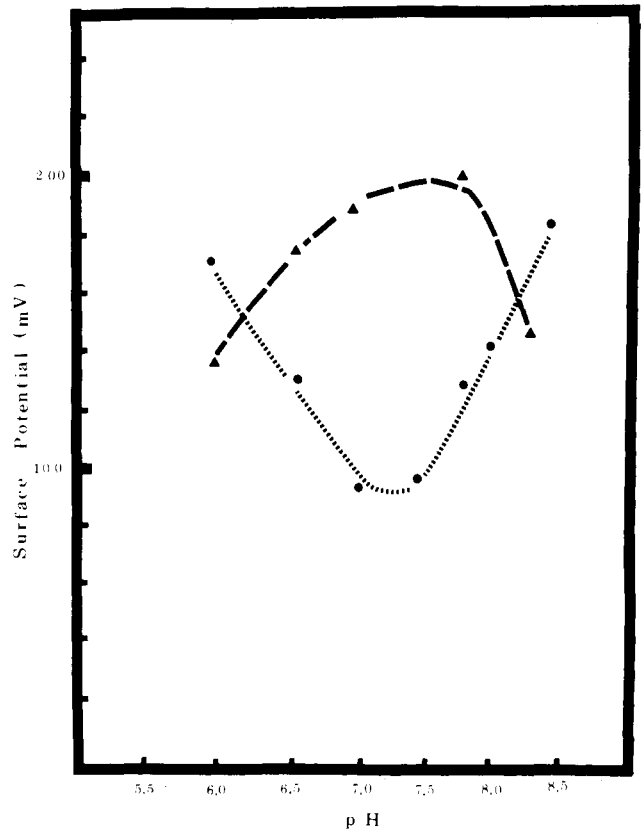


FIGURE 2: The surface potential of oxidized (— —) and reduced (●) cytochrome *f* as a function of pH. Subphase contained phosphate buffer ionic strength 0.6 at 15°. The surface pressure is 6 dyn/cm.

in all the experiments. These experiments are performed in air and under fluorescent light.

## Results

*Cytochrome f.* Surface isotherms for oxidized and reduced cyt *f* at pH 7.8 are shown in Figure 1; $A_6$ for oxidized cyt *f* is 2640 Å$^2$ and for reduced cyt *f* is 2900 Å$^2$.

A graph of $\Delta V$ as a function of $1/A$ (molecules/Å$^2$) at pH 7.8 is shown in Figure 1. Extrapolating to $1/A$ equal to zero gives the potential of water under the film, *i.e.*, $V_{H_2O}$; for reduced cyt *f*, $V_{H_2O}$ is −137 mV, for oxidized cyt *f* it equals −231 mV. These values are used to calculate $\Delta V$.

The $\Delta V_6$ and $A_6$ of oxidized and reduced cyt *f* as a function of pH are shown in Figures 2 and 3. Oxidized cyt *f* has a maximum for both $\Delta V_6$ and $A_6$ at about pH 7.5. The maximum value for $\Delta V_6$ is 200 mV, the maximum for $A_6$ is 2600 Å$^2$.

Reduced cyt *f*, on the other hand, had a minimum for both $\Delta V_6$ and $A_6$ at about pH 7.3. The minimum value for $\Delta V_6$ is 95 mV; the minimum $A_6$ is 2200 Å$^2$.

The curves for oxidized and reduced cyt *f* as a function of pH have equal areas at pH of 6.8 and 7.8 (Figure 3); they have equal $\Delta V$'s at pH of 6.2 and 8.2 (Figure 4). Once the film stabilizes on the surface, constant reproducible isotherms can be measured for several hours. There is no evidence of time-dependent denaturation.

*Cytochrome c.* The surface isotherms of oxidized and reduced cyt *c* at pH 7.8 are shown in Figure 4. $\Delta V$ as a function of $1/A$, for oxidized and reduced cyt *c*, is shown in Figure 4; by extrapolation $V_{H_2O}$ is −160 and −201 mV, respectively.

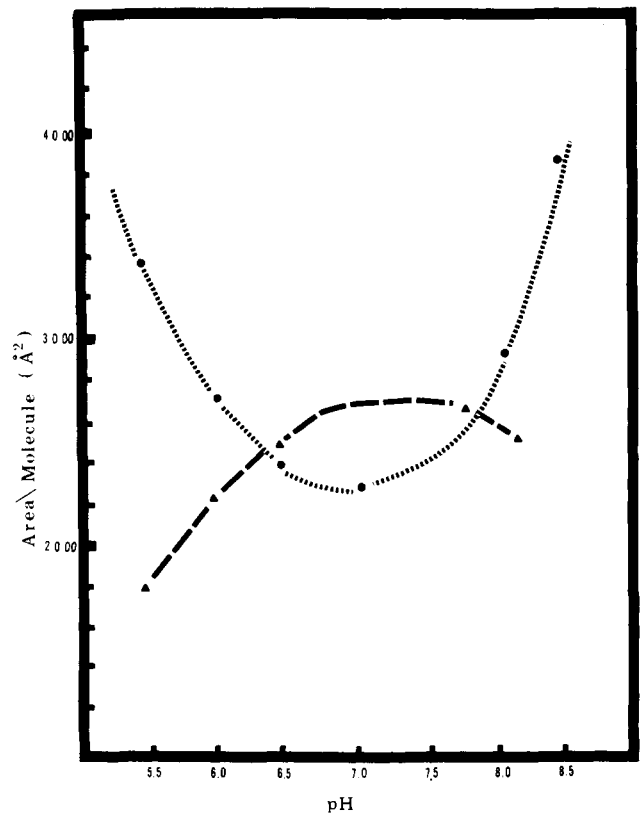


FIGURE 3: Area/molecule of oxidized (— —) and reduced (●) cytochrome *f* at surface pressure of 6 dyn/cm as a function pH. Subphase same as in Figure 2.

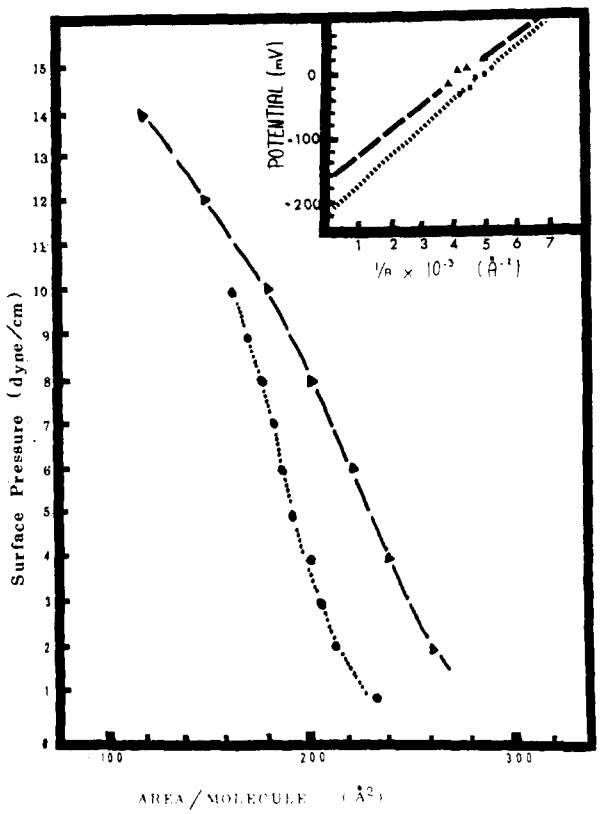


FIGURE 4: Area/molecule of oxidized (. . .) and reduced (▲) cytochrome *c* as a function of surface pressure. The insert shows $\Delta V$ as a function of molecules/Å$^2$ of oxidized (— —) and reduced (●) cytochrome *c*. Subphase same as Figure 1.

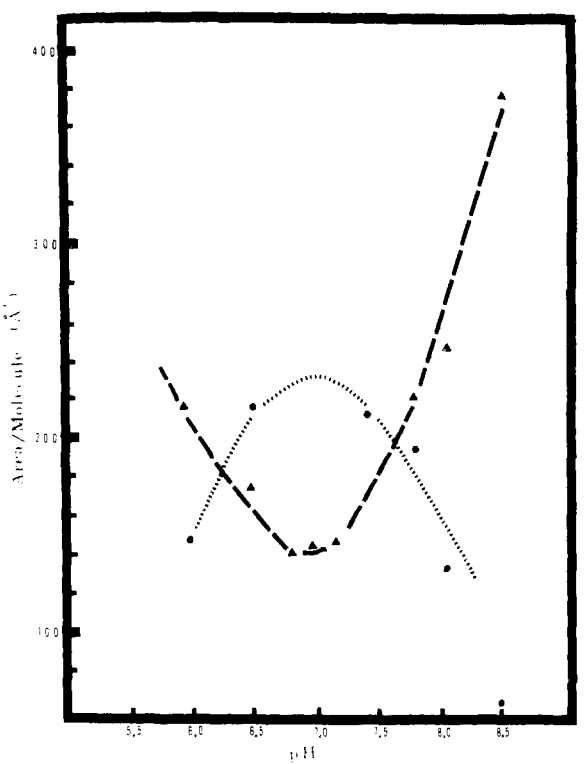


FIGURE 5: Area/molecule of oxidized (— —) and reduced (●) cytochrome *c* as a function of pH, at a surface pressure of 6 dyn/cm. Subphase same as in Figure 2.

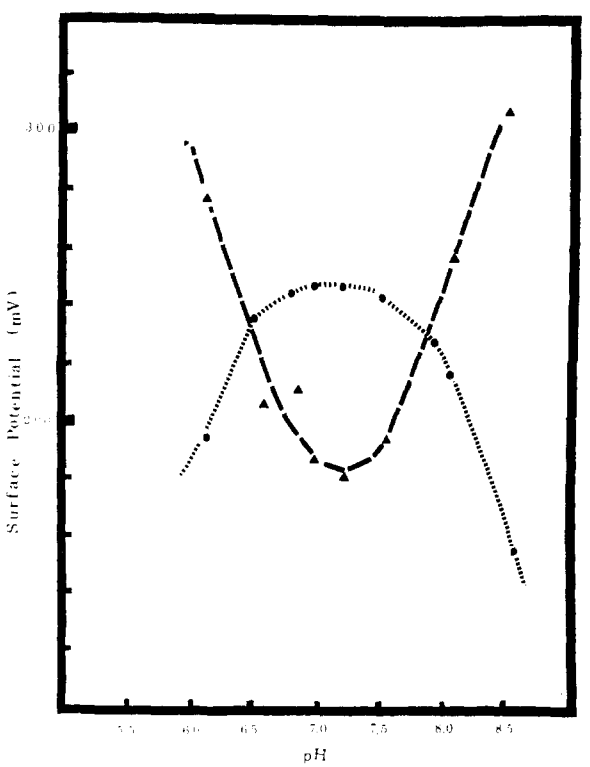


FIGURE 6: Surface potential of oxidized (- -) and reduced (●) cytochrome *c* as a function of pH, at a surface pressure of 6 dyn/cm. Subphase same as in Figure 2.

The $A_6$ for oxidized cyt *c* as a function of pH exhibits a minimum at about pH 7.0, see Figure 5. The minimum for $A_6$ is 140 Å$^2$. The $\Delta V_6$ as a function of pH also exhibits a minimum at about pH 7.3 (see Figure 6). The minimum for $\Delta V_6$ is 188 mV. It takes about 1 hr for the film to stabilize and give reproducible isotherms.

Reduced cyt *c* appears to be very unstable on the surface. Because of the large uncertainty for $A_6$ the graph shown in Figure 5 has only qualitative significance. While $A_6$ varied greatly, the variation of $\Delta V_6$ is only ±25 mV. The surface isotherm for reduced cyt *c* at pH 7.8 is shown in Figure 4. $\Delta V$ as a function of $1/A$ is shown in Figure 4 (by extrapolation $V_{H_2O} = -203$ mV). A graph of $\Delta V_6$ as a function of pH is shown in Figure 6. Both $A_6$ and $\Delta V_6$ as a function of pH exhibit maxima. The maximum for $A_6$ occurs about pH 7.0 and is 220 ± 42 Å$^2$, the maximum for $\Delta V_6$ occurs at pH 7.3 and is 260 mV. The curves for oxidized and reduced cyt *c* as a function of pH have equal areas at pH 6.3 and 7.7 (Figure 5); they have equal $\Delta V$'s at pH 6.7 and 7.8 (Figure 6).

## Discussion

As can be seen in Figures 2, 3, 5, and 6, there are two pH's where both the area/molecule and surface potential are approximately the same for the oxidized and reduced forms of cyt. At these pH's a redox reaction (of cyt) might be carried out with minimal change in protein conformation or charge distribution.

At the isoelectric point a maximum or minimum area for a protein is expected. The present study was not carried out at the isoelectric points of cyt *c* and *f* which occur at a pH of 10.6 and 4.7, respectively. The isoelectric point, as usually determined, is an average neutral charge for the entire surface of the protein molecule. In the present study for cyt *c* and *f*, there is an effective isoelectric point measured for that portion of the protein surface in contact with, or sub-

Table I: Summary of Results.[a]

| | pH max | | at pH max | |
|---|---|---|---|---|
| | for $A_6$ | for $\Delta V_6$ | $A_6$,Å$^2$ | $\Delta V_6$, mV |
| Ox cyt *f* | 7.5 | 7.5 | 2600 | 200 |
| Red cyt *c* | 7.0 | 7.3 | 220[b] | 260 |
| | pH min | | at pH max | |
| Red cyt *f* | 7.3 | 7.3 | 2200 | 95 |
| Ox cyt *c* | 7.0 | 7.3 | 140 | 188 |

[a] Except as noted $\Delta V$ is ±10 mV and $A_6$ is ±2%. [b] ±42 Å$^2$.

merged in, the aqueous phase. The fact that the pH maximum and minimum are about the same for the reduced and oxidized cyt *f* (or cyt *c*) indicates that the same portions of the cyt molecule are in contact with the water in the two redox states. That is, the same group of amino acids are being titrated.

In the physiological range of pH used for this study cyt *c* has a net negative charge and cyt *f* a net positive charge. This opposite charge of the two cyt's may be related to the opposite behavior observed for $A$ and $\Delta V$ as a function of pH (Figures 2, 3, 5, and 6). The $\Delta V$'s for cyt *c* in both the oxidized and reduced forms are more positive than the same redox states in cyt *f*. Reduction of cyt *f* results in a decrease of $\Delta V$ at a pH of 7.5 (see Table I). This is the direction of change to be expected for $\Delta V$ upon addition of an electron. On the other hand, reduction of cyt *c* results in an increase of $\Delta V$ at a pH of 7.3. In the case of cyt *c*, perhaps reduction results in a release of anions, thereby producing a reorientation of charge on the protein surface (Schejter and Margalet, 1970).

When outside the physiological pH range of 6–8 there is a reversal in the relative size and potential of the oxidized and reduced forms of cyt (Figures 3 and 6). *In vivo*, these changes in $A$ and $\Delta V$ could well be related to the position or orientation of a particular redox state of cyt in the membrane which in turn could determine its role in electron transfer processes. Changes in shape of the protein molecule as a result of redox state have previously been proposed (Butt and Keilin, 1962; Hagihara *et al.*, 1958).

Previous reports for the area of oxidized and cyt *c* by Reinach and Brody (1972) gave an area larger than reported here. The origin for this difference may be, in part, the different ionic strengths used in the two experiments. Also, in the previous work the subphase contained high concentrations of ferricyanide or ascorbate to generate the required redox state of cyt *c*.

A much larger area/molecule is measured for the cyt *f* than for the cyt *c*. This is probably related to the difference in molecular weight of the two proteins. The molecular weight for cyt *c* is 12,400 and for cyt *f*, 110,000. There is about a tenfold difference in molecular weights and in the area/molecule, suggesting that the thickness of the molecular film is similar for cyt *c* and *f*.

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