Oxidative Titration of Monomolecular Films of Cytochrome *c*-II and of Bacteriochlorophyll*

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ABSTRACT: Oxidation potentials, E_0, of monomolecular layers of bacteriochlorophyll (BChl) and cytochrome *c* (Cyt) are determined. The surface properties of these monolayers are measured using a Wilhelmy film balance during oxidative titration with ferricyanide (added to the aqueous subphase). The E_0's are determined from the midpoint of the change in surface pressure. The oxidative titrations of a monolayer of Cyt and of BChl appear to be one electron oxidation reactions. The values of E_0 for BChl and of Cyt are 438 and 362 mV, respectively. The E_0 for BChl is in close agreement with the oxidation potential measured *in vivo*. From measurements of the absorption spectra of BChl monolayers, it is found that the major product of the oxidative titrations of a BChl monolayer is 2-desvinyl-2-acetylchlorophyll a. Consideration of the reaction mechanism, changes in molecular area upon oxidation of BChl and calculation of relative absorption coefficients, allows inferences to be made about BChl orientation at the air–water interface. Some of the parameters that could affect E_0 of BChl and Cyt in monolayers are the electronegative potential due to oriented water at the surface, pigment–pigment interaction, and conformational state of the protein at an air–water interface.

In order to obtain more insight into the conditions that affect the chemical properties of bacteriochlorophyll (BChl)[1] and cytochrome *c* (Cyt) *in vivo* their oxidation properties are studied in monomolecular films at an air–water interface. Such a system provides a model for the pigment orientation and packing which occurs in membrane systems *in vivo*.

Determination of the oxidation potential, E_0 (*vs.* the hydrogen electrode), for the oxidation of a BChl monolayer at an air–water interface is relevant to the photosynthetic process. *In vivo*, chlorophyllous pigments are probably associated with membranes at a lipoid–protein interface (Rabinowitch and Govindjee, 1969); in such an environment its redox properties could be quite different from that which obtains in solution. *In situ* and E_0 of about 440 mV was obtained (by titrating the attenuation of the absorbancy change) for reaction center BChl (Kuntz *et al.*, 1964) and for P700 in green plants (Kok, 1961; Muller *et al.*, 1963). In aqueous methanol an E_0 of 550 mV was determined for the potential of BChl, by monitoring absorbancy changes upon titrating with ferric chloride (Goedheer *et al.*, 1958); in pure methanol an E_0 of 514 mV was determined (Fuhrhop and Mauzerall, 1969).

Since Cyt is also thought to be associated with membrane systems *in vivo*, it is of interest to measure the oxidation potential of a monomolecular film of Cyt *c*. In an aqueous system, titration of beef heart Cyt *c* gave a value for E_0 of 254 mV throughout the pH range 1.75–7.8 (Rodkey and Ball, 1950).

Experimental Section

Studies of monomolecular films were carried out using a Wilhelmy plate film balance. The entire apparatus was contained in an environmental chamber which had provision for evacuation and flushing with nitrogen. The sensitivity of the balance permitted measurements of film pressures with a precision of ±0.2 dyne/cm.

The films were formed in a wax-coated dish. With all films of BChl the aqueous subphase contained 2×10^{-2} M phosphate buffer (pH 7.8) and 10^{-4} M ferrocyanide (ferro). In the case of Cyt films the subphase contained 5×10^{-2} M phosphate buffer (pH 7.0), 0.3 M NaCl, 10^{-4} M ferro, and 10^{-7} M TMPD as a mediator. To determine E_0, the monomolecular film was titrated by slowly adding ferricyanide (ferri) to the subphase. So as not to disturb the film, ferri was added to the subphase through a wax-coated ring which was floating on the surface. The subphase was stirred slowly using a magnetic stirrer. The surface pressure of the film, π (at constant area), was plotted as a function of the potential of the subphase. From the midpoint value of π the value of E_0 was obtained. The potential of the subphase was determined from the mole ratio of ferri:ferro; the potential of the latter was measured in a separate experiment by using a combination electrode (Radiometer PK 149) and a Radiometer pH meter (Model TTT1C). The apparatus to measure the absorption spectrum of a monomolecular film was described elsewhere (Brody, 1971).

Bacteriochlorophyll (BChl) and 2-desvinyl-2-acetylchlorophyll a (ox BChl) were prepared by the method described by Smith (Smith and Calvin, 1966). Much of the oxidant (chloranil) used in the latter preparation could be removed by hexane–acetone partitioning. Horse heart cytochrome *c* type II (Cyt) was purchased from Sigma Chemical Co. (St. Louis, Mo.), potassium ferricyanide and potassium ferrocyanide from Fisher Chemical Co., sodium ascorbate from Fluka, A. G. (Bucks, S. G.), and tetramethylphenylenediamine from Eastman Organic Chemical Division. Ferrocytochrome (Cyt^{2+}) was formed by the addition of excess ascorbate. Ferricytochrome (Cyt^{3+}) was formed by the addition of excess ferricyanide. All materials were used without further purification. Benzene solutions of BChl and ox BChl as well as aqueous solutions of Cyt^{2+} and Cyt^{3+} were added to the surface with a Hamilton gas-tight microliter syringe.

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[1] Abbreviations used are: BChl, bacteriochlorophyll; Cyt, cytochrome; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

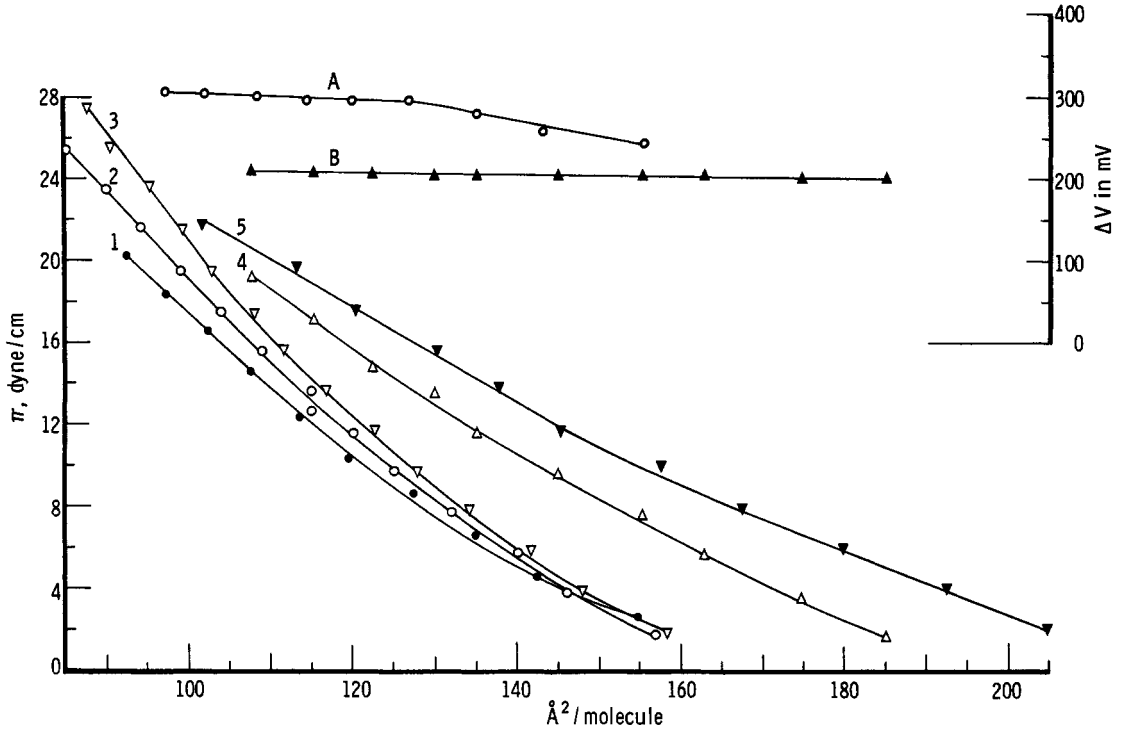

FIGURE 1: Surface isotherms of monomolecular films of bacteriochlorophyll (BChl) and oxidized BChl (ox BChl). In all cases the temperature of the subphase is 15° and contained 10^{-4} M ferrocyanide (ferro) and 2×10^{-2} M phosphate buffer (pH 7.8). Curve 1: BChl; curve 2: BChl with ferri:ferro = 2:1 in the subphase; curve 3: BChl with ferri:ferro = 7:1 in the subphase; curve 4: 2 desvinyl-2-acetylchlorophyll a (ox BChl) after partial removal of chloranil; curve 5: ox BChl with no chloranil removed. The surface potentials, ΔV, of BChl and ox BChl are shown by curves A and B, respectively.

The concentration of pigment in benzene was calculated from the optical density using a molar extinction coefficient of 75×10^3 l. M^{-1} cm^{-1} for BChl (at 780 nm) and 68×10^3 l. M^{-1} cm^{-1} for ox BChl (at 682 nm). Cyt concentration was calculated in a similar fashion using a molar extinction coefficient of 2.9×10^4 l. M^{-1} cm^{-1} for the reduced form and 8.4×10^3 l. M^{-1} cm^{-1} for the oxidized form (at 550 nm) (Van Gelder and Slater, 1962).

Results

Bacteriochlorophyll. A monolayer of BChl is stable in the dark, under a nitrogen atmosphere. The pigment's stability is verified in two ways. First, the surface tension of a compressed film remains constant as a function of time. Second, there is no evidence of a change in the spectral properties of BChl after being on the surface for 2 hr. The latter is determined both by recovering the film from the surface, dissolving it in a benzene solution, and examining it in a spectrophotometer, as well as by monitoring the absorption spectrum of the monomolecular film.

The surface isotherms for BChl and for ox BChl (produced by chloranil oxidation) are shown in Figure 1 (curves 1, 4, and 5). The area/molecule of BChl, A_0, extrapolated to $\pi = 0$ dyne/cm is 147 Å²; for ox BChl the A_0 is 180 Å². The isotherm of BChl varies with the mole ratio of ferri:ferro in the subphase; in Figure 1, curves 2 and 3, are isotherms of BChl when ferri:ferro is 2:1 and 7:1, respectively. The isotherms of ox BChl before and after partitioning to remove chloranil are also shown in Figure 1 (curves 5 and 4, respectively); the A_0's at $\pi = 10$ dynes/cm in the two cases are 154 and 142 Å², respectively.

A known amount of BChl (1.06×10^{14} molecules/cm²) is added to the aqueous surface of the wax-covered dish so as to give a π of 18.8 dynes/cm; this surface pressure is found to give the best results in the titration experiment. When a small amount of ferri is added to the subphase π decreases to a steady value within a few seconds. Additional aliquots of ferri are added until there is no further change in π. The total decrease of π is about 4 dynes/cm. In Figure 2, curve A, is shown π for BChl as a function of the oxidation potential (of the ferri:ferro couple) in the subphase. From Figure 2, curve A, the midpoint for the titration of a BChl monolayer is found to be at $\pi = 17.0 \pm 0.2$ dynes/cm, and the corresponding E_0 is 438 ± 1 mV. A monolayer of ox BChl cannot be oxidized further in the range used in this work (Figure 2, curve B). The absorption spectra of expanded films of BChl and ox BChl are shown in Figure 3 as curves A and C, respectively.

The absorption spectrum of a film of BChl when the subphase contains ferri:ferro = 7:1 is shown in Figure 3, curve B. The absorption maxima, peak ratios and the absorption coefficients of the red absorption bands of the chlorophyllous monolayers are listed in Table I. In an expanded monolayer, BChl has maxima at 787 and 584 nm (the blue bands were not measured), and a shoulder at 690 nm; the BChl titrated with ferri:ferro = 7:1 has maxima at 772, 688, 586, and 440 nm. The bands of ox BChl are 691 and 438 nm where the subphase contains ferri:ferro = 7:1; in the absence of ferri:ferro the red absorption maximum is at 683 nm.

While BChl films are readily oxidized by ferri:ferro couples, BChl in solution cannot be oxidized by any ferri:ferro couple. To oxidize BChl in solution requires a stronger oxidant such as $FeCl_3$ (Holt and Jacobs, 1954). These observations are in accord with the finding that the E_0 of BChl in a monolayer is lower than in solution.

Cytochrome c-II. Stable monomolecular films of Cyt *c* are

TABLE I: Spectral Data and the Areas/Molecule of Chlorophyllous Monolayers.

	$\epsilon_s \times 10^{-6}$ cm²/mole[c]	$\epsilon_m \times 10^{-6}$ cm²/mole[d]	Absorbance max. (nm)	Blue:Red[e]	A_0 (Å²)	β[f] (deg)
Bacteriochlorophyll	75	58	*787*, 584		147	47
2-Desvinyl-2 acetyl-Chl a[a]	68	67	*691*, 438	1.5	180	36
Bacteriochlorophyll oxidized by ferri:ferro = 7:1		34	772, *688*, 586, 440	1.3		
Chlorophyll a[b]	79.5	66	*672*	1.25	122	41

[a] Subphase contains ferri:ferro = 7:1, chloranil removed by partitioning. [b] From data presented elsewhere (Brody, 1971); measurements made at π = 8 dynes/cm. [c] ϵ_s and ϵ_m are absorption coefficients in benzene solution and in a monolayer, respectively. [d] Red absorption bands used for calculation of ϵ are in italic type. [e] Ratio of absorption maxima in a monolayer. [f] Calculated from $3\epsilon_s \cos^2\beta = 2\epsilon_m$.

readily formed at an air–water interface. The procedure to obtain the film is to gently add small amounts (40 μl) of a fairly concentrated sample of Cyt (10^{-3} M) at several loci on the surface of water. In order to keep the Cyt on the surface an ionic strength of at least 0.3 is required. From surface isotherms it is seen that oxidation of Cyt results in an increase in area/molecule of 30 Å² (at π = 8 dynes/cm). Isotherms of Cyt^{2+} and Cyt^{3+} are given in Figure 4, curves A and B, respectively. The presence of any oxygen in the system is apparently of no consequence since Cyt *c* does not combine with molecular oxygen (Harbury *et al.*, 1965). Since Cyt^{3+} has a larger area/molecule than Cyt^{2+} on the surface, the oxidation of Cyt^{2+}, at constant film area, results in an increase of π.

The results of a typical oxidative titration are shown in Figure 5, curve A. The midpoint for the titration is at π = 8.6 dynes/cm, the corresponding E_0 is 362 ± 2 mV. Titration of a Cyt^{3+} monolayer shows no change in π (Figure 5, curve B). Starting with a film of Cyt^{3+} and a film of Cyt^{2+}, each containing the same protein density, the final value of π after titrating is the same in both cases (Figure 5). Thus it appears that the product of this titration is Cyt^{3+}.

The results shown in Figures 2 and 5 are replotted using the Nernst equation, $E = E_0 + 0.06/n \log$ (ox:red), in Figure 6, where E is the measured electrode potential, E_0 the oxidation potential, n the number of electrons transferred and ox:red the ratio of oxidized to reduced material, respectively. It is assumed that in the films containing both oxidized and reduced species there is no interaction between species and that there is a direct relationship between π and the ratio of ox:red species. For Cyt the ratios of ox:red species are obtained from the expression $(\pi - 6.24):(10.97 - \pi)$; for BChl the expression used is $(18.8 - \pi):(\pi - 15.3)$. Constants in the latter ratios are π's at the beginning and end of titration, respectively. From the slope of the graph, the number of electrons transferred in the oxidative titration of Cyt is 0.8 and for BChl it is approximately 0.9. It appears that the titration is in close agreement with a one electron-transfer reaction in both cases.

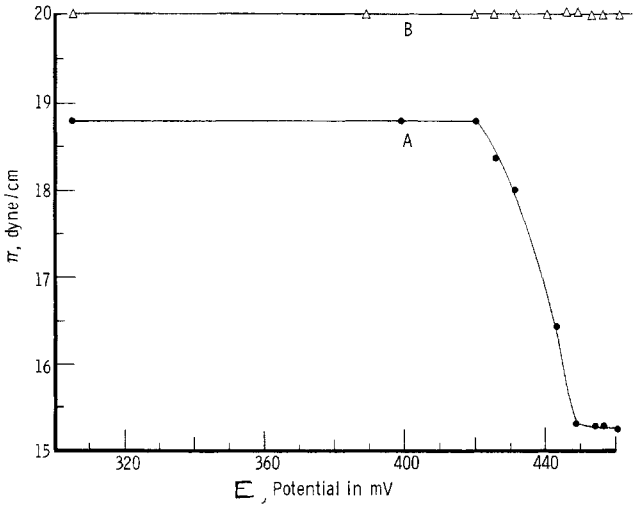

FIGURE 2: Oxidative titration of BChl and ox BChl monolayers at an air–water interface, at constant film area. In both cases the subphase contained 10^{-4} M ferro and 2×10^{-2} M phosphate buffer (pH 7.8). Curve A: surface pressure, π, of a monolayer of BChl as a function of the oxidation potential, E, generated by a ferri:ferro couple in the subphase. Curve B: surface pressure of a monolayer of ox BChl as a function of the oxidation potential in the subphase.

Discussion

At an air–water interface the molecules are oriented with their polar groups anchored in the water. The molecules are in a negatively charged environment resulting from the oriented water at the interface. The effect of this negative environment on the oriented molecules could well modify the electron affinity and therefore E_0 of Cyt and BChl from that observed in solution. Pigment–pigment interaction could also modify E_0. In the case of Cyt there may be a change in conformation upon forming a monolayer which might also be reflected in changing the value of E_0.

In a monolayer the E_0 of BChl has a value of 438 mV which is about 110 mV lower than the 550 mV measured in aqueous methanol (Goedheer *et al.*, 1958) and about 76 mV lower than the 514 mV (270 mV *vs.* calomel) measured in 100% methanol (Fuhrhop and Maurzerall, 1969).

The relevance of BChl monolayers as a model for *in vivo* phenomena gains support by the agreement between the E_0's for the chemical oxidation of BChl monolayers and for the attenuation of light-induced absorbancy changes in chromatophores (*i.e.*, 440 mV) using ferricyanide (Kuntz *et al.*, 1964).

From the surface isotherms of BChl and ox BChl (Figure 1) one would expect to observe an increase in π during the chemical oxidation of a BChl monolayer by ferri. Nevertheless,

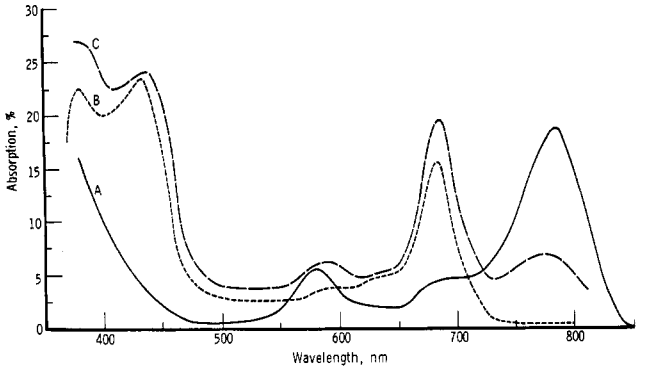

FIGURE 3: Curve A: absorption spectrum of an expanded monolayer of BChl. Curve B: absorption spectrum of an expanded monolayer of ox BChl. Ox BChl was made by the addition of chloranil to an acetone solution of BChl. Curve C: absorption spectrum of an expanded monolayer of BChl over a subphase containing ferri:ferro = 7:1. (To compare this curve with curves A and B multiply the vertical scale by 0.5.) In all cases, the subphase contained 2×10^{-2} M phosphate buffer (pH 7.8) and 10^{-4} M Fe^{2+}. The per cent absorption shown in the figure is for light passing through the monolayer 18 times.

an opposite effect is observed, *i.e.*, π decreases (Figure 2). A similar unexplained hysteresis effect was found by Aghion (Aghion *et al.*, 1969) for a photooxidation of a compressed monolayer of Chl.

While there are similarities between the absorption spectrum of ox BChl and the product (or one of the products) formed by titrating a BChl monolayer, it is not yet certain if they are identical. Since the absorption maxima and peak ratios of the same substance in solution and in monolayers are quite different, it is not possible to compare their absorption spectra. (Also, in a monolayer the area/molecule, or π, has an important effect on the absorption spectrum.) Therefore, in order to provide a basis of identification, spectra were

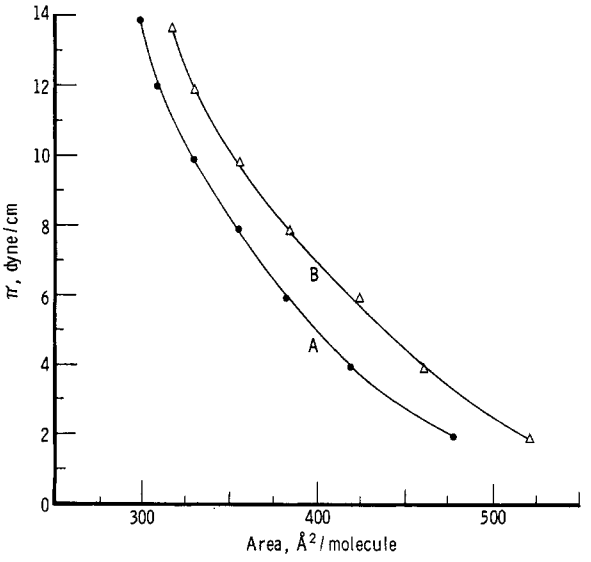

FIGURE 4: Surface isotherms of monomolecular films of reduced and oxidized horse heart cytochrome *c*-II (Cyt^{2+} and Cyt^{3+}, respectively). In both cases the subphase contained 5×10^{-2} M phosphate buffer (pH 7.0) and 0.3 M NaCl. Curve A: Cyt^{2+} reduced by excess ascorbate; curve B: Cyt^{3+} oxidized by excess ferri.

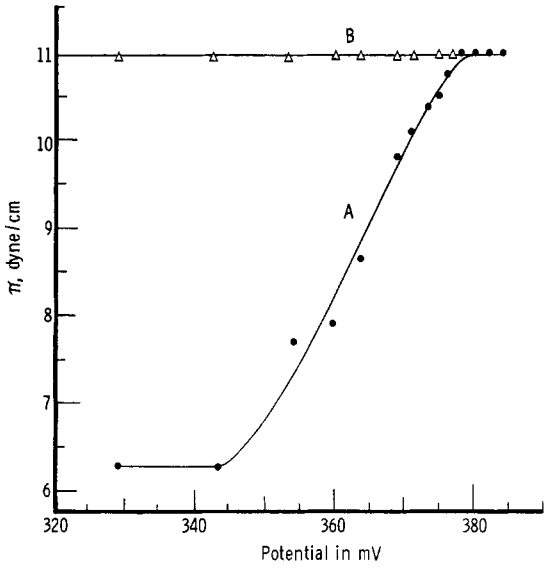

FIGURE 5: Oxidative titration of Cyt^{2+} and Cyt^{3+} monolayers at an air–water interface, at constant film area. In both cases the subphase contained 5×10^{-2} M phosphate buffer (pH 7.0), 0.3 M NaCl, and 10^{-7} M TMPD. Curve A: surface pressure, π, of a monolayer of Cyt^{2+} as a function of the oxidation potential, E, generated by a ferri:ferro couple in the subphase. Curve B: surface pressure of a monolayer of Cyt^{3+} as a function of the oxidation potential in the subphase.

measured for monolayers of BChl, ox BChl and a partially titrated film of BChl (Figure 3).

In the absorption spectrum of the partially titrated film of BChl (Figure 3, curve C) the bands with maxima at 688 and 438 nm probably correspond to ox BChl. The absorption

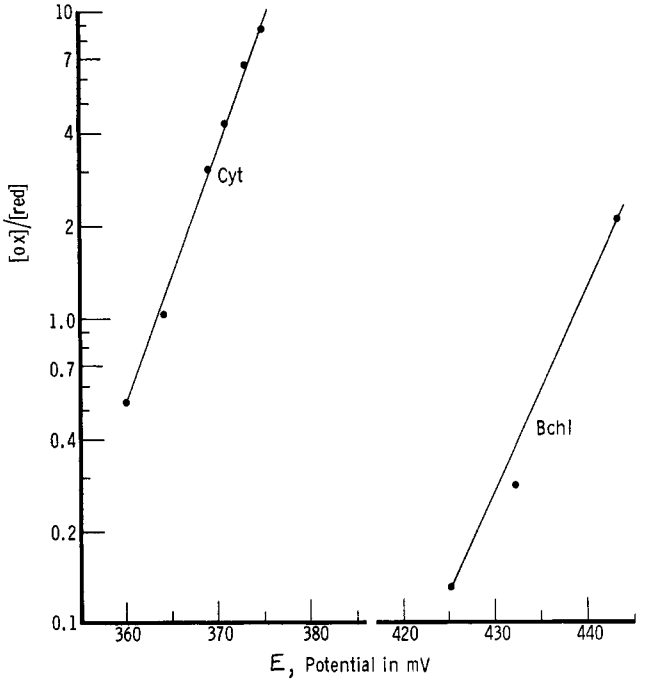

FIGURE 6: The data in Figures 2 and 5 are replotted using the Nernst equation ($E = E_0 + 0.0g/n$ log (ox:red)) so as to obtain an estimate of the number of electrons, n, transferred in the oxidative titration of BChl, and of Cyt^{2+}. From the slopes n is 0.9 and 0.8, respectively.

bands at 586 and 772 nm are not readily attributable to either BChl or ox BChl. The red absorption bands of BChl and ox BChl are at different wavelengths, *i.e.*, 787 and 691 nm, respectively. Furthermore, the width of the BChl band (measured at 0.75 max) is 38 nm, while the width of the 772 band (at 0.75 max) is about 60 nm. The 586-nm band cannot be attributed solely to BChl since ox BChl has only a shoulder at 586 nm. When the ratio of absorption of the partially titrated film at 772 nm:586 nm (Figure 3, curve C) is compared to that for BChl at 787 nm:584 nm (Figure 3, curve A), it is seen that the absorption at 586 nm is too high to be attributed entirely to BChl.

Therefore, it is clear that the titrated film contains at least one more substance other than BChl and ox BChl. Since ox BChl is stable to further oxidation by ferricyanide, it probably is the final product in this titration. The substance (or substances) giving rise to the absorption at 772 and 586 nm is probably some oxidation intermediate between BChl and ox BChl. A possibility for such an intermediate would be a one electron oxidation product of BChl; ox BChl is a two electron oxidation product. Such a possibility is in agreement with the results shown in Figure 6 where it is found that the titration is in close agreement with a one electron-transfer reaction. Ox BChl may be formed either from a dismutation of the one electron intermediate or by further oxidation of the intermediate.

From the results of the oxidative titration of BChl, it appears that the orientation of BChl monolayers may be somewhat different from that proposed for Chl. Chl is probably oriented at the water surface with the ester linkages and the carbonyl group (of ring V) being anchored to the water while the hydrophobic portions of the porphyrin ring and phytol are in the air phase (Alexander, 1937; Hughes, 1936). If BChl were to have an orientation similar to that of Chl, it is unlikely that ferricyanide could oxidize specifically C-3,C-4 to give the oxidation product ox BChl. Perhaps in the case of BChl the acetyl group of ring I is anchored to the water in addition to, or instead of ring V, thereby bringing it into closer proximity with the water surface to allow the reaction between ferri and C-3,C-4. Such an orientation would project a larger cross-sectional area on the surface than if the water anchorage were only through the polar groups of ring V and the phytol ester. Indeed the area/molecule, A_0, of BChl (147 Å^2) is larger than that of Chl (123 Å^2). A rotation of the plane of the BChl molecule, relative to Chl, would be consistent with measurements of the area/molecule and relative absorption coefficients of monolayers (see below).

The geometrical relationship between the absorption coefficients of a pigment in solution ϵ_s and in a monolayer ϵ_m is $3\epsilon_s \cos^2 \beta = 2\epsilon_m$, where β is the angle between the transition dipole and the surface of the water (Tweet *et al.*, 1964a,b). In order to calculate an approximate value for β which can be used for comparison purposes the experimentally determined value of ϵ_m and the ϵ_s of BChl in benzene is used in the above equation.

In Table I are given the values of ϵ_m for expanded monolayers ($\pi = 0$) of BChl and ox BChl oxidized by ferri:ferro = 7:1 in the subphase. For comparison the angle β is also calculated for a Chl monolayer using data published previously (*i.e.*, $\epsilon_m = 66 \times 10^6$ and $\epsilon_s = 79 \times 10^6$ cm^2 per mole) (Brody, 1971). From a study of the angular dependence of fluorescence Tweet *et al.* estimated $\beta \leq 20°$ for Chl (Tweet *et al.*, 1964a,b).

On the basis of the absorption coefficients and β (see Table I) it appears that the transition dipole of BChl is oriented in a slightly more vertical fashion on the surface than it is in Chl. This would result in a smaller area/molecule, A_0, for BChl than Chl. Actually the A_0 for BChl is larger than for Chl. The observations of ϵ_m, A_0 and the chemical titration are compatible if BChl is attached to the surface at ring I and the phytol ester, rather than at ring V and the phytol ester (as in Chl). Such a rotation could allow the plane of the porphyrin to project a larger area on the surface, as well as, increase the angle between the water surface and the absorption transition dipole. In the case of ox BChl, the angle β decreases, relative to BChl, so that a larger A_0 is expected and is indeed the case.

In the case of Cyt there is no information on its conformational state in monomolecular films. Consequently, it is not possible to relate protein conformation with its different oxidation potentials (Loeb, 1968; Malcom, 1962). When horse heart Cyt is bound to phospholipid membranes (Kimelberg and Lee, 1970), E_0 is 225 mV which is different than the E_0 in solution (Rodkey and Ball, 1950) and in monolayers (362 mV). That there probably are differences in Cyt conformation between solution and monolayers arises from the perturbing effect of the air–water interface. The different E_0's of Cyt in solution and in monolayers may then be related either to the protein conformation state or the highly negative nature of the oriented water at the interface.

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