Interactions between Retinal and Phospholipids in Monomolecular Films at Acid pH

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The surface properties of mixed monomolecular films of retinal and phospholipids (p. lipids) are measured as a function of mole fraction at a nitrogen-water interface. An acid pH of 6.0 is maintained in the aqueous phase. Before irradiation the surface potential ΔV for 9-*cis* retinal, 11-*cis* retinal, phosphatidyl serine (PS) and phosphatidyl ethanolamine (PE), at $\pi = 12$ dyn/cm, are 490 mV, 645 mV, 548 mV and 375 mV, respectively. Before irradiation, A_0 for 9-*cis* and 11-*cis* are 58 Å^2 and 48 Å^2, respectively. Experimentally measured isotherms are compared with theoretically calculated isotherms. In case of mixed films of retinal and PS the experimental isotherms are greater than theoretical, while mixed films of retinal and PE are smaller than theoretical. A maximum value for the difference between theoretical and experimental areas are obtained at (retinal)/(p. lipid) = 0.1. Retinal and p. lipid do not appear to form a Schiff base, charge transfer or any other type of complex at pH 6. A eutectic type mixture between retinal and p. lipid may occur on the surface. A light induced change in ΔV of -130 mV is observed in the case of 11-*cis* and PE. The significance of these findings with respect to visual excitation is considered.

Introduction

Retinal is present in the outer rod segment of the retina, as the chromophore group of rhodopsin. The visually active forms of retinal are 11-*cis* and 9-*cis* retinal. One of the proposed mechanisms of binding between retinal and opsin is *via* a Schiff base. In general a Schiff base linkage involves the aldehyde group (of a retinal) and an amine group of lysine (Bownds [1]) or a phospholipid (Poincelot *et al.* [2], De Pont *et al.* [3]). Formation of a Schiff base is pH dependent; at pH 6.0 almost no Schiff base formation occurs (Morton and Pitt [4]). Beside Schiff base linkage formation, several other types of linkages are possible between retinal and protein, for example, sulfhydryl linkage (Wald and Brown [5]), charge transfer complex (Galindo [6]) or thiozolidine complex (Peskins and Love [7]). In the presence of borohydride, retinal has been observed to be bound to phosphatidyl ethanolamine in rhodopsin (Poincelot *et al.* [2], Kimbel *et al.* [8]). However, at this time the majority of evidence points to binding between retinal and lysine (Fager *et al.* [9], Abrahamson [10]), perhaps binding between retinal and phospholipid occurs after the photolysis of rhodopsin.

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Among the most widely distributed phospholipids (p. lipids) in nature are phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). Both PE and PS have been reported in rhodopsin rod outer segment (Bonting [11]). Phospholipids are released from outer rod segments after bleaching by light (Krinsky [12]; Zorn and Futterman [13]).

Studies of monolayers of 9-*cis* retinal (9-*cis*) and their interaction with amino acids at an air-water interface have been reported previously (Brockman and Brody [14]). The area per molecule of 9-*cis* extrapolated to zero pressure on a phosphate buffer at pH 6 was 56 Å^2, after irradiation the area decreased to 49 Å^2. Complexation between 9-*cis* retinal and β-mercapto-ethylamine, dissolved in the subphase, was also observed.

Reference added in proof: Interaction between retinal and phospholipids in monomolecular films at alkalien pH were reported by Yckowski and Brody [19].

In the present study, mixed monomolecular films of retinal and p. lipids were examined. All experiments were carried out at a nitrogen-water interface. The aqueous subphase was maintained at pH 6, where Schiff base linkage was unlikely to form. These experiments were carried out to test the possibility of interaction, other than Schiff base, between retinal and p. lipid. The effects of light on the mixed monolayers were also examined.

Materials and Methods

PS was obtained in chloroform solution from Applied Science Laboratories (State College, Pa.), PE from Mann Research Laboratories (Division of Schwartz Biochemicals N.Y., N.Y.). Both materials contain a distribution of fatty acids. 9-*cis* retinal was obtained from Sigma Chemical Company (St. Louis, Mo.), 11-*cis* retinal from Hoffman La Roche (Nutely, N.J.), prepurified nitrogen (99.995%) from Matheson Gas Products (Rutherford, N.J.), *n*-hexane (99% purity) and phosphate buffers from Fisher Scientific Company (N.J.). All chemicals were used without further purifications.

Distilled water was prepared by passing tap water through a Barnstead ion exchange column then distilling from a permanganate solution in an all glass distillation apparatus (Corning, model AG-2, Corning, N.Y.). Before an experiment all glassware and utensils were cleaned with ethanol, then rinsed in distilled water.

A Wilhelmy film balance enclosed in an environmental chamber similar to that previously described by Aghion *et al.* [15] was used to measure surface isotherms. The atmosphere above the surface was flushed and maintained with nitrogen. A Beckman microbalance (model LM-500) was used to measure the surface pressure (π). The trough was a teflon coated aluminum tray (60 cm × 17 cm × 1 cm). Teflon coated glass barriers were used. The aqueous subphase contained 10^{-2} M phosphate buffer, pH 6.0. The temperature of the aqueous phase was thermostatically maintained at 15 ± 0.05 °C throughout the experiment. The area/molecule (A) at a particular π is given as A_π.

Surface potential measurements were made using a radio-active tungsten electrode, about 1/2 cm above the surface and a Ag-AgCl electrode in the subphase. An electrometer (Keithley model 610) was used to measure the surface potential of the clean surface (V_{H_2O}), and the surface with the film (V). The surface potential of the film ΔV is equal to $(V - V_{H_2O})$. The sensitivity of the balance permits measurement of π with a precision of ± 0.08 dyn/cm, and ΔV ± 5 mV.

Retinal stock solutions were prepared in a dim red light using *n*-hexane as the spreading solvent. Concentration of the retinal solution was determined spectrophotometrically, using a Cary model 14R recording spectrophotometer. The extinction coefficient used for 9-*cis* and 11-*cis* retinal in *n*-hexane is 3.36×10^4 and 2.63×10^4 l/mol/cm, respectively, at the absorption maximum (Brody [16]).

Films of various compositions were prepared by mixing together in the same solution known concentrations of p. lipid and retinal. The molecular weights of p. lipids used for calculating the number of molecules on the surface are 792 for PS and 800 for PE. The mixed films were spread on the surface using a 100 microliter gas tight Hamilton glass syringe. The solvent was removed by alternate evacuating and flushing the environmental chamber with nitrogen 4 – 5 times.

Surface isotherms and potentials were measured before and after illumination, in a nitrogen atmosphere. Irradiation of the film was carried out using a 100 low pressure Hg arc lamp without any filters. The light intensity on the surface was about 2×10^3 ergs/cm^2 sec. Unless otherwise noted the light induced change in ΔV given in this work was determined by first measuring ΔV in the dark at $\pi =$ 12 dyn/cm (ΔV_{12}), then irradiating in the compressed state while maintaining the area constant. After irradiation the film was expanded and the surface isotherm and ΔV were measured repeatedly until there were no further changes in ΔV or area/molecule.

The values for theoretical area/molecule and surface potential were calculated using the following expressions: Theoretical $\Delta V = N_a \Delta V_a + N_b \Delta V_b$; theoretical area $= N_a A_a + N_b A_b$, where N_a and N_b are mole fractions, ΔV_a and ΔV_b are surface potentials, A_a and A_b are area/molecule of retinal and p. lipid, respectively.

Results

Individual isotherms: Surface isotherms (π-A curves) for 9-*cis*, 11-*cis*, PS and PE at a nitrogen-

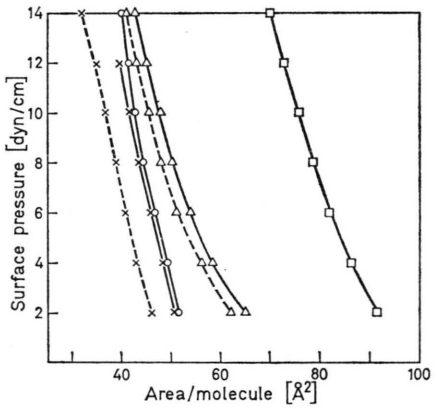

Fig. 1. Surface isotherms of 9-*cis* retinal are shown by triangles (△△△), 11-*cis* retinal by crosses (×××), phosphatidyl serine by circles (○○○), and phosphatidyl ethanol amine by squares (□□□). The isotherms and measures before and after irradiation are shown by solid lines and dashed lines, respectively. The subphase contained 10^{-2} M phosphate buffer at pH 6, temperature of the subphase was 15 °C.

water interface are shown in Fig. 1. In dark ΔV_{12} for 9-*cis*, 11-*cis* retinal, PS and PE, are 490 mV, 645 mV, 548 mV and 375 mV, respectively. The A_{12} for PS and PE are 42 Å^2 and 73 Å^2, respectively. The isotherms of PS and PE are uneffected by irradiation. The small area for PS compared to PE suggests that important differences in saturation exist. This may have some important implications for the interaction between retinal and p. lipid.

Illumination of retinal films results in a decrease in area. The A_0 for 9-*cis* and 11-*cis* before irradiation is 58 Å^2 and 48 Å^2; the A_{12} is 45 Å^2 and 40 Å^2, respectively. After irradiation A_0 decreases to 56 Å^2 and 43 Å^2, the A_{12} to 44 Å^2 and 36 Å^2, respectively. Illumination at constant area results in a decrease of ΔV for 9-*cis* and an increase of ΔV for 11-*cis* (Fig. 2). When the experiment was carried out as described in the material and methods then 9-*cis* showed a 20 mV increase in ΔV.

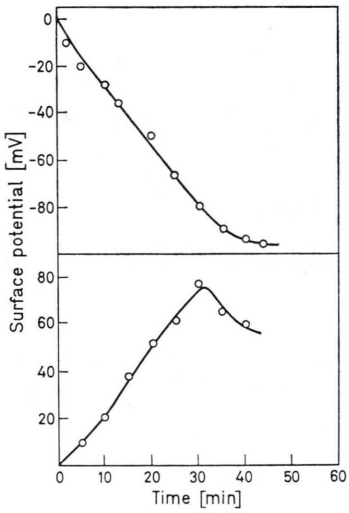

Fig. 2. Time dependent change in surface potential, ΔV, during irradiation of a monomolecular film of 9-*cis* and 11-*cis* retinal, at a constant area. Irradiation was carried out using a low pressure Hg arc lamp, the intensity on the surface was about 2×10^3 ergs/cm^2 sec. Upper curve is for 9-*cis*, the light induced change in ΔV is -95 mV; lower curve is for 11-*cis*, the light induced change in ΔV appears to be $+80$ mV.

9-*cis* retinal and PE: Surface isotherms of mixed monomolecular films of 9-*cis* and PE are measured as a function of the mol ratio (retinal)/(p. lipid). The experimentally measured (EXPER) isotherms are compared with the theoretically calculated (THEOR) isotherms, as done previously (Brody [17]). The THEOR areas are calculated from the individual isotherms of 9-*cis* and PE (in cm^2), and the number of molecules of each component added to the surface. The EXPER areas (in cm^2) at $\pi = 12$ dyn/cm are always found to be significantly smaller than the THEOR areas (Fig. 3). The minimum value for the difference between EXPER and THEOR (*i. e.* EXPER – THEOR) occurs at a mole ratio (9-*cis*)/(PE) $= 0.1$. Irradiation of the film decreases the EXPER area about 7% in all cases studied, the data for ΔV as a function of the ratio (retinal)/(p. lipid) were too scattered to indicate any definite trend.

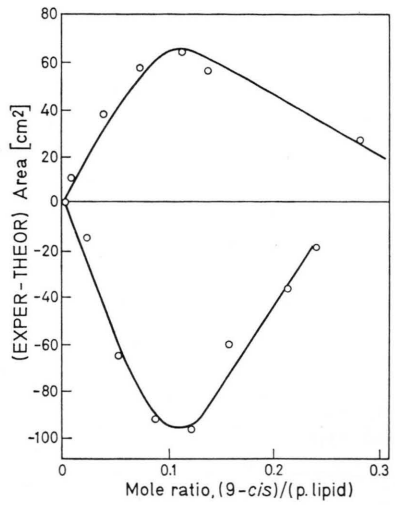

Fig. 3. The difference between the experimentally measured (EXPER) and theoretically calculated (THEOR) isotherms of a mixed film as a function of the molar ratios of retinal and phospholipid in the film. The difference between the two isotherms in cm^2 are measured at a surface pressure of $\pi = 12$ dyn/cm. Upper curve is for a mixed film of 9-*cis* retinal and phosphatidyl serine. Lower curve is for 9-*cis* retinal and phosphatidyl ethanolamine. Subphase is the same as in Fig. 1.

In the course of measuring isotherms of mixed films it is observed that there is a time dependent change of π before a steady state value is reached. At lower values of π, the time required to reach steady state is a few seconds, at higher values of π it may take several minutes to reach equilibrium (Fig. 4).

11-*cis* retinal and PE: Surface isotherms and potentials of mixed films of 11-*cis* and PE are measured as a function of the mole fraction (11-*cis*)/(PE). The surface isotherms of such films are very reproducible. The EXPER areas are always less than THEOR areas. A minimum value for the difference (EXPER – THEOR) is obtained at a mole ratio of (11-*cis*)/(PE) $= 0.1$ (Fig. 5). Irradiation of the films increases the EXPER area about 6%. The EX-

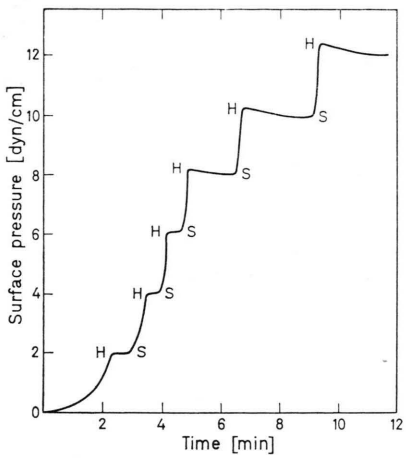

Fig. 4. Typical data used to determine the surface pressure—area isotherm of retinal. Compression of the film is halted (H) periodically and the surface pressure measured as a function of time. When the surface pressure reaches a steady value, compression is started again (S). It can be seen that there is a time dependent change of surface pressure after each compression. At low pressures the time required to reach equilibrium is a few seconds, at higher pressures it takes one to two minutes.

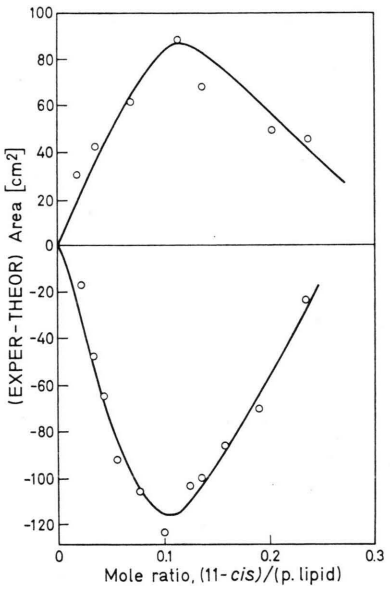

Fig. 5. The difference between the experimentally measured (EXPER) and theoretically calculated (THEOR) isotherms of a mixed film as a function of the molar ratios of retinal/p. lipid in the film. The difference between the two isotherms in cm^2 are measured at a surface pressure of $\pi = 12$ dyn/cm. Upper curve is for 11-*cis* retinal and phophatidyl serine. Lower curve is for 11-*cis* retinal and phosphatidyl ethanolamine. Subphase is the same as in Fig. 1.

PER ΔV is larger than the THEOR ΔV (in dark). Illumination results in a large decrease in the ΔV of the mixed film (*e. g.* -130 mV).

9-*cis* retinal and PS: With mixed films of 9-*cis* and PS (in contrast to the data of 9-*cis* and PE) the EXPER isotherms are larger than the THEOR isotherms. A maximum value for the difference (EXPER – THEOR) is obtained at a mole ratio (9-*cis*)/(PS) = 0.1 (Fig. 3). The EXPER ΔV is smaller than the THEOR ΔV.

Irradiation decreases the EXPER area about 11%. In general, the light induced changes in ΔV are negligible (about 6 mV). Films are exposed alternately to light and dark periods; during the light period a decrease in ΔV is often observed while during the dark period an increase in ΔV is often observed (Fig. 6).

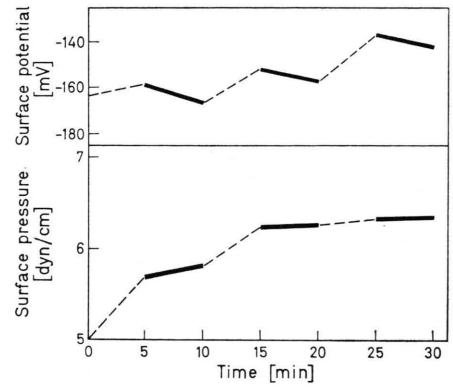

Fig. 6. The mixed films of retinal and phospholipids are exposed alternately to light and dark periods of time, while the area of the film is held constant. Changes in surface pressure and surface potential are recorded. Dark periods are shown by solid lines and illumination periods by dotted lines. The upper curve is for a mixed film of 9-*cis* and phosphatidyl serine (PS), mol ratio of 0.15. The lower curve is for a mixed film of 11-*cis* and PS mole ratio of 0.10.

11-*cis* retinal and PS: With the mixed films of 11-*cis* and PS (in contrast to the data of 11-*cis* and PE) the EXPER areas are larger than the THEOR areas. A maximum value for the difference (EXPER – THEOR) is obtained at a mol ratio (11-*cis*)/(PS) = 0.1 (Fig. 5). In the case of ΔV the EXPER value is smaller than the THEOR. In general, there are negligible changes in ΔV and areas upon irradiation. In some experiments a time dependent decrease in π is observed during illumination, while in the dark π is rather constant (Fig. 6). All data are summarized in Table I.

Discussion

The A_{12} for 9-*cis* retinal reported in this work is in fair agreement with that reported previously by

Table I. Summary of surface properties of retinal and p. lipid films.

Film	A_{12} [Å] Dark	A_{12} [Å] Light	ΔV_{12} [mV] Dark	ΔV_{12} [mV] Light	Light induced changes in ΔV_{12} [mV]
9-*cis*	45	44	500	520	+20
11-*cis*	40	36	510	590	+80
PS	42	42	548	548	—
PE	73	73	375	375	—

	Total film area [cm²] Dark	Total film area [cm²] Light	ΔV_{12} [mV] Dark	ΔV_{12} [mV] Light	Light induced changes Area [cm²]	Light induced changes ΔV_{12} [mV]
(9-*cis*)/(PE) =0.1	521	486	310	320	−35	+10
(9-*cis*)/(PS) =0.1	454	404	198	192	−50	−6
(11-*cis*)/(PE) =0,09	381	404	495	365	+23	−130
(11-*cis*)/(PS) =0.1	612	609	68	58	−3	−10

Brockman and Brody [14]. However, our values of A_{12} for 9-*cis* and 11-*cis* retinal are smaller than those reported by Brody [16] for A_{10}. The difference in A might be related to small differences in technique or variation in the purity of the material. The smaller areas for retinal are probably correct, the larger areas could result from impurities in the retinal or solvent. There are several critical stages where errors can arise. A relatively long time (several minutes of stirring) is required to completely dissolve retinal in *n*-hexane. It also requires a long time for the film to stabilize on the surface and to reach its minimum area. It can take an hour or more before reproducible isotherms of retinals can be measured.

Our ΔV's for 11-*cis*, 9-*cis* and for the mixed films are, within experimental error, close to those reported previously (Brody [17], Brockman and Brody [14]). While the isotherms of the mixed films of 9-*cis*/PE and 11-*cis*/PE were quite reproducible, those of 9-*cis*/PS and 11-*cis*/PS were not. The origin for this lack of reproducibility may originate in the solubility of chloroform, used as the solvent for PS, in the water phase. Variability in the purity of PS might also be one of the factors limiting the precision of these results.

The marked difference in the interaction of PS and PE with retinal is shown by the opposite behavior of (EXPER − THEOR) (Figs. 3, 5). The values of (EXPER − THEOR) are proportional to the free energy of mixing and reaction on the surface (Davies and Rideal [18]). The negative value for (EXPER − THEOR) shows a spontaneous system for retinal in the presence of PE. However, a positive value shows a nonspontaneous system for retinal in the presence of PS. The type of interaction between retinal and p. lipid depends upon the charge and polarity, as well as, the structure of the p. lipid. The ionic properties of PS and PE are dependent upon the pH of the subphase. At pH 6.0, PE has no net charge, while PS has a negative charge. That retinal and p. lipid interact is indicated by the minimum and maximum values for (EXPER − THEOR) (Figs 3, 5). Since the minimum and maximum values for (EXPER − THEOR) occur at a ratio of (retinal)/(p. lipid) = 0.1, the possibility of complex formation is unlikely. Therefore, it is unlikely that at pH 6, there is any charge transfer, Schiff base or any other type of binding between retinal and p. lipid. Perhaps the (retinal)/(p. lipid) ratio of 0.1 is indicative of the formation of an eutectic mixture in the two dimensional solution containing lipid and retinal. The interaction between p. lipid and retinal could depend to a large extent on the fatty acid residues of the p. lipids. Alternatively, the ratio of 0.1 might indicate the relative concentration of p. lipid containing those fatty acid residues which facilitate interaction with retinal. While well defined p. lipids would be valuable for these model systems they were not available for this study.

In the case of 11-*cis* and PE the light induced change in ΔV is −130 mV; this value is in agreement with that reported previously by Brody [16]. For the other three types of mixed films the effect of light on ΔV is negligible.

In the case of monomolecular films of pure 9-*cis*, previous workers reported a light induced change of ΔV of −55 mV (Brockman and Brody [14]) and −30 mV (Brody [16]). These values compare favorably with the time dependent change shown in Fig. 2, where ΔV is −95 mV. However, from the ΔV's measured before and after irradiation (see Materials and Methods), for some inexplicable reason, negligible changes in ΔV are observed.

In the case of films of pure 11-*cis*, previous workers reported a negligible light induced change in ΔV (Brody [16]). In the present study, however, an 80 mV increase of ΔV is observed both in time dependent measurements and usual method (see Materials and Methods). The experimental conditions used in the present study differed significantly from those used previously by Brody [16] and could account for the different results. In the present work the monolayers are exposed to 100 W Hg lamp without any filter and all experiments are carried out in a nitrogen atmosphere. Brody [16] used a blue filter in conjugation with his lamp so that a lower light intensity was used. Furthermore, all Brody's experiments were carried out in air.

From the values of ΔV for retinal films with and without p. lipids it appears that the orientation of retinal on the surface is greatly modified by the presence of p. lipids. Films of pure 11-*cis* give a light induced change in ΔV of +80 mV. While −130 mV is observed for films of 11-*cis* and PE. Furthermore, for retinals in the presence of PS, there is no significant light induced change in ΔV.

As can be seen in Fig. 1, illumination results in an isotherm with smaller area. Thus, if the area is held constant, as the film is illuminated, one would expect π to decrease. Nevertheless, during irradiation a small increase in π is observed in the case of 11-*cis* retinal (Fig. 6). Such anomalous behavior was reported previously by Aghion *et al.* [15].

The properties of rhodopsin appear to be dependent upon the associated p. lipids (PE in particular) (Zorn and Futterman [13]). If, as commonly presumed, the carbonyl group of retinal is complexed to lysine, perhaps the rest of the retinal molecule is "dissolved" in the p. lipid fraction of the lipoprotein. The absence of complexation between retinal and p. lipid at pH 6.0 would argue against the retinal-p. lipid interaction in rhodopsin and for the retinal-lysine complex. Evidence showing the occurrance of the latter complex in monomolecular films at pH 6.0 was presented previously (Brody [16]).

Perhaps visual excitation might occur as a direct result of the primary action of light on rhodopsin in situ. The primary photo reaction, in the visual process is a photoisomerization of 11-*cis* retinal. In the present study it was shown that irradiation of a film of 11-*cis* can result in about 100 mV change in potential (see Fig. 5 and Table I). This is about the same potential as is usually associated with membranes. It is proposed that visual excitation occurs as the result of the *cis-trans* photoisomerization which produces a change in potential of the visual membrane. The role of opsin in this model would be to maintain the 11-*cis* in the proper orientation with respect to the membrane so that a maximum change in membrane potential results upon photoisomerization. With this view all the other stages in the "visual cycle" (*lumi*, *meta* I, II, *para* etc.) are simply part of the mechanism of regeneration of rhodopsin.

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