Studies on Fragments of Rod Outer Segments from Bovine Retinas

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Monolayers of fragments of our rod segments were formed at an air-water interface. The area of these particles was measured as a function of the pH of the aqueous phase. A maximum area was measured at pH 6.5.

The film was characterised by measuring the decrease in surface pressure, after compression, and the half time required to reach constant pressure. At a pH between 6 and 7 the decrease in surface pressure was a minimum.

Irradiation of films of fragments resulted in a decrease in surface potential of between 30 and 40 mV. The largest decrease occured at a pH between 6 and 6.5. A small decrease in surface pressure was also observed upon irradiation.

Mixed films of fragments and phosphatidyl ethanolamine were examined. Irradiation of the mixed film resulted in a 70 mV decrease in surface potential.

I. Introduction

Many studies on rhodopsin have been reported. However, it is not yet clear how rhodopsin molecules are arranged on the disc membranes of rod outer segments or how rhodopsin functions in visual excitation [1–3]. In relation to these problems, we have studied surface isotherms and the effect of light on fragments of rod outer segments (ROS) at an air-water interface. The effect of pH and ionic strength of subphase on the film was examined. The stability of ROS films in the dark was also evaluated.

II. Materials and Method

Rod outer segments from cattle retinas were prepared mainly by the methods of Bitensky *et al.* [4] and Schichi [5]. All experiments were performed in a dark room with deep red light (15 W Kodak red safe lamp, Model A with Kodak filter # 2). Temperature of materials was kept around 5 °C for all experiments.

Cattle retinas (George Hormel and Co., Austin, Minn.) were vortexed with 47.6% sucrose solution for a few minutes using a vortex mixer. The vortexed mixture was centrifuged between a 47.6% sucrose solution and 66 mM phosphate buffer at $100\,000 \times g$ for 90 min at 5 °C. After centrifugation, the sucrose layer was removed with a syringe and the interface resuspended in the buffer layer. The buffer-interface suspension was centrifuged at $48\,000 \times g$ for 20 min at 5 °C and the pellet was resuspended in fresh 47.6% sucrose solution. Rod outer segments (ROS) in sucrose solution could be kept in the cold more than a month without denaturing. The optical density (OD) of ROS was measured using a Cary Model 14 spectrophotometer. Various ratios were calculated to assay the purity of ROS, $R_1 = A_{280}/A_{500}$; $R_2 = A_{400}/A_{500}$, where A_{280}, A_{400}, A_{500} represent OD at 280, 400 and 500 nm respectively.

About 1 ml of ROS prepared as above was sonicated using either a Raytheon Sonic Oscillator for 15 – 20 min or a Branson Sonic Cell Disruption, for three bursts of 2 – 3 sec. The sonicated suspension was centrifugated at $48\,000 \times g$ for 15 min at 5 °C. The optical density of supernatant was taken and the ratios R_1 and R_2 were calculated. The purity of the preparations used in this work were 60 – 80% unless otherwise noted. For comparison purposes the ratios used for 100% pure rhodopsin are $R_1 = 1.75$ and $R_2 = 0.21$ (Schichi [5]).

Details of the apparatus used in the present work were published previously [6]. In general a Whilhelmy plate film balance utilizing a Beckman microbalance (LM-500) was used to measure surface pressure of films of ROS fragments. Ni 63 was used as a β source for the surface potential measurements. The films were formed in a teflon coated trough (28″ × 8.5″ × 0.25″). All apparatus were enclosed in an environmental chamber and the temperature maintained at 15 °C. Various pH's and ionic strengths (I) of phosphate buffer were used as subphase.

The number of rhodopsin molecules spread on the aqueous surface is determined spectrophotometrically using a molar extinction coefficient of 4×10^4 at 500 nm. Using this value for the number of

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rhodopsin molecules in the film of ROS fragments, an area/molecule, A_π, is calculated at a particular film pressure π.

By electron microscopy it was observed that the largest fragments of ROS on the monolayer were between 500 and 300 Å in diameter. These large fragments comprised only about 5% of the film area. The majority of ROS fragments were smaller than 100 Å in diameter. A rhodopsin molecule has a diameter of the order of 50 Å.

III. Results and Discussion

1. *Surface isotherms of ROS fragments in dark at an air-water interface*

Pressure-area isotherms of ROS are observed to be of the liquid-expanded type as defined by Gaines [7]. In order to characterize these isotherms A is measured at 5.8 dyn/cm, $A_{5.8}$. It is observed that A varies with pH and I (see Table I). The largest A observed is at pH 6.5 and $I = 1.1$. For a given purity of ROS, A tended to be larger with higher ionic strength (see pH 6.5). The value of A depends on the purity of ROS fragments; the lower the purity of the ROS fragments, the smaller the value of A.

Table I. Area as a function of pH and I.

pH	I	Å²/rhodopsin particle [b]
5.4	0.0038	568 [a]
6.1	0.10	3520
	0.40	871 [a]
6.5	0.14	1970
	0.28	2310
	1.1	7550
8.1	0.40	536 [a]
8.9	0.0038	618 [a]

[a] Indicates 40—50% purity of ROS fragments.
[b] Area measured at a surface pressure of 5.8 dyn/cm.

At a heptane-water interface [8] the value of A is much larger than at an air-water interface. At pH 6.5, $I = 0.1$, $A_{7.8} = 13\,600$ Å². This would indicate a very different orientation of the fragments at the two different interfaces.

2. *Stability of films of ROS fragments as a function of pH and I of subphase*

Stability of ROS fragments film in dark was examined by changing pH and I of subphase. Using a deep red safe light ROS fragments were put on the surface, the film was compressed and expanded once then compressed to a pressure π. The rest of the experiment was conducted in total darkness. The total area of the film was held constant. There is always a drift in π before a steady value is reached (see Fig. 3, part A). The magnitude of the change in π and the half time, $t_{1/2}$, to reach equilibrium appears to vary with pH, I and purity. The Table II shows the decrease in π and $t_{1/2}$ at a pressure of 10 dyn/cm as a function of pH and I.

Table II. Stability of ROS films as a function of pH and I.

pH	I	$\Delta\pi$	$\frac{\Delta\pi \times 10^3}{(\mathrm{OD} \times \mathrm{vol}) \times (2\text{-purity})}$	% purity [b]	$t_{1/2}$ [min]
5.4	0.0038	7.0	4.3	39	1.5
6.0	0.40	5.2	3.2	42	2.5
6.0	0.10	3.6	3.3	85	39
6.1	0.10	1.1	0.78	63	13
6.6	0.10	0.49	0.32	45	5.0
7.2	0.028	1.6	1.3	80.	0.8
8.1	0.40	5.5	3.3	35	3.5
8.9	0.0038	4.9	3.2	45	2.3
10.4 [a]	0.010	4.5	3.5	72	7.0

[a] Glycine buffer.
[b] Purity based of the R_1, see Materials and Methods section.

The magnitude in the change in π in dyn/cm ($\Delta\pi$) is adjusted both for purity of the ROS preparation and amount of material on the surface, *i. e.* $\frac{\Delta\pi}{\mathrm{OD} \cdot \mathrm{vol} \cdot (2\text{-purity})}$. This adjusted value as a function of pH is shown in Fig. 1, where OD is

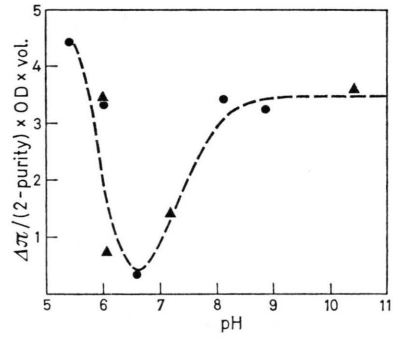

Fig. 1. Stability of films of ROS fragments as a function of pH, in the dark at 15 °C. Vertical axis gives total change in surface pressure (in dyn/cm) before equilibrium is reached after an initial compression to 10 dyn/cm. Constant area was maintained. The change in surface pressure is corrected for amount of material on the surface (OD x vol) and purity of the ROS. Purity of ROS is shown as follows: 40—50%; Δ 60—80%.

optical density and vol is volume of solution added to surfaces. The adjusted values of $\Delta\pi$ seem to be primarily a function of pH and not I. For example see Table II pH 6.0, $I = 0.4$ and 0.1, while the measured values of $\Delta\pi$ are markedly different, the values of the adjusted $\Delta\pi\, g$ are the same at the two values of I. Such agreement after adjusting $\Delta\pi$ was usually the case indicating that I is not a primary factor in affecting the drift of π.

In Fig. 2 are shown the variation of $t_{1/2}$, as a function of pH for samples of rhodopsin at high and low purity. Samples of higher purity appear to have a greater $t_{1/2}$.

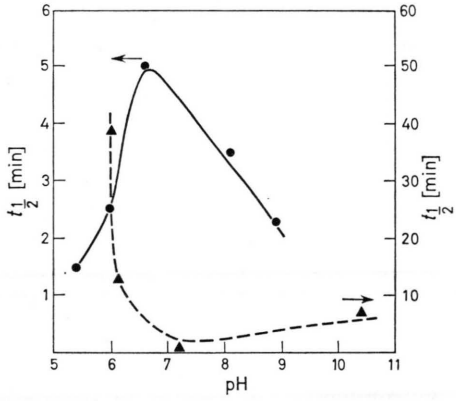

Fig. 2. Stability of films of ROS fragments as a function of pH. Vertical axis gives half-time required for the surface pressure to reach equilibrium after an initial compression to 10 dyn/cm. Constant area was maintained. Purity of the ROS is shown as follows: solid line, 40–50% left axis; broken line 60–80%, right axis.

When the buffer pH is between 6.0 – 7.0, the $\Delta\pi$ is at a minimum and $t_{1/2}$ is a maximum. This is the pH range for optimum rhodopsin activity. The magnitudes of the surface potential ΔV varied widely and could not be correlated with pH of the subphase.

A mixed film of ROS fragments (~88% pure) phosphatidylethanolamine (mol ratio 1:2, respectively) was examined at pH 6.7, $I = 0.1$. The stability test in the dark gave a $\Delta\pi = 0.33$ dyn/cm with a $t_{1/2}$ of 4.9 min. This value of $\Delta\pi$ is less than that observed without PE (Table II). No change in ΔV was observed during the stability test. During irradiation a $\Delta\pi \approx 0.06$ dyn/cm and $t_{1/2} \approx 3$ min was observed; the largest change observed in ΔV was -70 mV.

3. *Light effect on ROS films*

After a few surface isotherms of the ROS fragments were measured in the dark the film was compressed to 10 – 15 dyn/cm and allowed to stabilize. The film was irradiated at constant film area and π measured as a function of time. Very small light induced changes in film pressure were observed, see Fig. 3. In Table III are shown the values measured for the change of film pressure in light and $t_{1/2}$ required to reach equilibrium during continuous irradiation at various pH's and I.

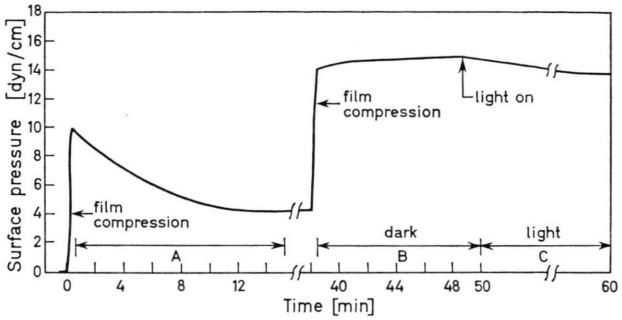

Fig. 3. Time-dependent changes of surface pressure of a film of ROS fragments in the dark and during irradiation. A. Change in film pressure in the dark after initial compression. B. Surface pressure of the film before irradiation. C. Time-dependent change of surface pressure during irradiation.

Table III. Light induced changes in surface pressure and $t_{1/2}$ as a function of pH.

pH	I	$\Delta\pi \times 10^3$ / OD × vol	$\Delta\pi \times 10^3$ / OD × vol × (2-purity)	purity [b]	$t_{1/2}$ [a]
5.4	0.0038	0.61	0.38	0.39	15
6.0	0.10	1.2	1.0	0.9	7
6.0	0.40	0.78	0.49	0.42	26
6.5	0.10	0.68	0.62	0.9	20
6.6	0.10	0.20	0.13	0.45	11
7.2	0.028	0.43	0.36	0.8	31
8.1	0.40	**1.0**	0.63	0.35	25
8.9	0.0038	**0.59**	0.38	0.45	14
10.4	0.01	0.61	0.47	0.72	33

[a] Half time in min required for surface pressure to reach equilibrium.

[b] Purity based on the ratio R_1, see Materials and Methods section.

The difference in ΔV measured at a film pressure of 5.9 dyn/cm before (ΔV_D) and after (ΔV_L) irradiation ($\Delta V_L - \Delta V_D$), are shown in Table IV. While the light induced changes in ΔV varied considerably the largest changes were observed between pH 6 and 6.5. The magnitude of the change in ΔV

Table IV. Light induced changes in surface potential as function of pH and I.

pH	I	$(\Delta V_L - \Delta V_D)$ [mV]
5.4	0.0038	−11
6.1	0.10	−40
	0.40	−22
6.5	0.14	−36
	0.29	−25
8.1	0.40	− 6
8.9	0.0038	− 6

ΔV_L, surface potential after irradiation.
ΔV_D, surface potential in dark, before irradiation.
Surface potentials measured at 5.9 dyn/cm.

does not appear to depend on I. After irradiation, ΔV usually decreased.

In the course of photo-decomposition of rhodopsin to opsin and retinal only minor changes in surface area are observed. It has been suggested [9] that phospholipids in the membrane may stabilize opsin so that large conformational changes do not occur upon irradiation. While light induced changes in A are small, changes in ΔV are quite significant. Whether these changes in ΔV arise from the photo-isomerisation of retinal [10] or the conformational changes of opsin or both is yet to be resolved.

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