ELECTRO-OPTICAL MEASUREMENTS ON AQUEOUS SUSPENSION OF PURPLE MEMBRANE FROM *HALOBACTERIUM HALOBIUM*

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ABSTRACT The permanent dipole moment, polarizability, and the retinal angle of *Halobacterium halobium* purple membranes were determined at different pH values. All of the parameters have a maximum between pH 5 and 6. There is a reversal in the direction of the permanent dipole moment near pH 5. The value of permanent dipole moment was determined to be 60 D/protein at pH 6.6, and the value obtained for polarizability was 3×10^{-28} Fm^2/membrane fragment. The retinal angle of all-*trans* retinal was 0.8° smaller than that of the 13–*cis* conformation.

INTRODUCTION

Bacteriorhodopsin (BR) molecules embedded in the plasma membrane of *Halobacterium halobium* transport protons from the interior of the bacteria to the outside after absorbing light energy (Stoeckenius et al., 1979). Fragments containing BR are readily prepared from the bacteria and are composed of the single protein, bacteriorhodopsin, and membrane lipids. The first electro-optical measurements on these purple membranes (pm) in suspension were performed by Shinar et al. (1977) using short pulses of high electric fields ($\sim 10^4$ V/cm). Linear dichroism data indicate an ordering of the pm parallel to the electric field. Eisenbach et al. (1977) could produce oriented[1] pm layers immobilized in special gels using a direct current (DC) field of ~100 V/cm. Light-induced electric current indicated that a true orientation of the pm was obtained. The orientation of pm in suspension was studied by Keszthelyi (1980, 1982) who demonstrated that the pm have a large, permanent electric dipole moment perpendicular to the membrane plane. A small electric field (~20 V/cm) is enough to saturate the orientation of a large quantity of the membrane disks perpendicular to the electric field. An alternating electric field can also be used to order the membrane fragments through their induced dipole moment. The induced dipole moment is the result of the migration of counter ions along the membrane surface. The perpendicular relationship of the permanent and induced dipoles were clearly shown by Kimura et al. (1981) using linear dichroism and by Todorov et al. (1982) using light-scattering measurements. Tsuji and Rosenheck (1979), Tsuji and Neumann (1981), and Druckmann and Ottolenghi (1981) ordered the pm with short pulses of high electric field and confirmed the results of Shinar et al. (1977).

The values of the permanent and induced electric dipoles, as well as the angle of the retinal to the membrane plane normal, depend on different parameters including membrane fragment size, distance between the membrane fragments, and the ionic environment. Most of these factors influence the aggregation state of the membranes in suspension, which in turn masks the real orientation effects. In this paper we present a detailed study of the dependence of induced and permanent dipoles, as well as the retinal angle on pH, concentration, fragmentation, and light-dark adaptation. The pH dependence of the permanent dipole moment of the pm has already been reported by Kimura et al. (1981).

MATERIALS AND METHODS

Purple membrane fragments were prepared from *Halobacterium halobium* strain R$_1$M$_1$ using standard procedures (Oesterhelt and Stoeckenius, 1974) and were purified by washing in triple distilled water. Measurements were performed on samples with different pm concentrations, fragment sizes, and at various pH values. The pm concentration was adjusted by dilution with triple distilled water and the final concentration was determined by measuring the absorption at $\lambda = 575$ nm. The size of the membrane fragments was varied independently from concentration by altering the duration of ultrasonic fragmentation. The pH was changed either by the commonly used HCl or KOH (0.1/0.01%) system or by the

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[1] We use the expression orientation for true orientation, i.e., for cases where all the pm face one direction. Other cases are called alignment or ordering.

use of constant, low ionic strength ($I = 0.01$) buffers (Dawson et al., 1969). All measurements were performed at room temperature.

The average size of the membrane fragments was determined from light-scattering measurements using a 155 He-Ne laser ($\lambda = 632.8$ nm; Spectra-Physics Inc., Mountain View, CA). The degree of orientation was monitored by measuring the absorption of light polarized parallel and perpendicular to the field direction as a function of field strength. The pm in suspension were ordered using an alternating current (AC) field to align the induced dipoles or a low DC field that mainly influences the permanent dipoles. Voltage was supplied to platinized platinum electrodes and was controlled by a programmable switching unit (homemade). The frequency of the AC voltage was 6 kHz (no frequency effect was observed between 1 and 20 kHz). The light intensity was detected by a photomultiplier; the signal was amplified by Keithley 417 picoammeter (Keithley Instruments, Inc., Cleveland, OH) and stored in a multichannel analyzer (Central Research Institute for Physics, Hungary, smallest conversion time 40 μs, conversion range 256 quanta/channel, 1024 channel).

DATA EVALUATION

Aqueous suspensions of pm were exposed to alternating DC fields (0–60 V/cm) of 3-s duration separated by 6 s without field or an alternating electric field of 6 kHz of 0 to 350 V/cm (peak value). The intensity changes of transmitted light polarized parallel ($\Delta I_\parallel$) and perpendicular ($\Delta I_\perp$) to the electric field were measured at $\lambda = 575$ nm.

Fig. 1 *a* and *b* show the light intensity changes parallel to the electric field ($\Delta I_\parallel$) at various field strengths in both static and alternating electric fields. We see two clear differences between the two series of the curves. The first is that $\Delta I_\parallel$ is positive for a DC and negative for an AC field. This is due to the different spatial arrangement of the membrane fragments in the static and alternating electric fields. The second difference is in the shape of the curves at higher field strengths (discussed below).

Light intensity changes are related to the reduced dichroism (Fredericq and Houssier, 1973)

$$\frac{\Delta A}{A} = -\frac{1}{A}\lg\left(1 + \frac{\Delta I}{I}\right), \tag{1}$$

where ΔA is the corresponding absorbance change, and $A = \lg (I_o)/(I)$ is the absorbance in the absence of an electric field. The rotational symmetry around the direction of the electric field predicts that

$$\Delta A_\parallel = -2\Delta A_\perp. \tag{2}$$

Light scattering, however, disturbs the measured absorption and in some cases a correction is necessary. The change of light intensity due to excess scattering, ΔI_s, in ordered samples has the same sign as $\Delta I_\parallel$ in both static and alternating electric fields independent of the polarization of light. The same ΔI_s was added or substracted, respectively, from the measured $\Delta I_\parallel$ and $\Delta I_\perp$ values to fulfill Eq. 2. The corrections in ΔA values never exceeded 10%.

The reduced dichroisms at any field strength can be written as

$$\begin{aligned}\frac{\Delta A_\perp}{A} &= \left(\frac{3}{2}\sin^2\theta - 1\right)\Phi\,(\beta,\gamma)\\ \frac{\Delta A_\parallel}{A} &= (3\cos^2\theta - 1)\,\Phi\,(\beta,\gamma).\end{aligned} \tag{3}$$

The reduced dichroism value depends upon the angle (θ) between the membrane normal and the transition moment of the chromophore and upon the function, $\Phi\,(\beta,\gamma)$. $\beta = \mu E/kT$; μ, permanent dipole moment; E, electric-field strength; k, Boltzmann constant; T, temperature in degrees Kelvin; $\gamma = \alpha E^2/2kT$; and α, polarizability of the particles.

The general expression of the orientation function $\Phi(\beta,\gamma)$, which denotes the degree of orientation for disk-shaped particles having permanent and induced dipole moments perpendicular to each other, has been calculated by Shah (1963). Using this equation we computed and plotted some curves of $\Phi(E)$ at characteristic values of b^2/γ (Fig. 2). If $0 < \beta^2/\gamma < \infty$, $\Phi(E)$ shows a maximum depending on β^2/γ. The calculated values are represented

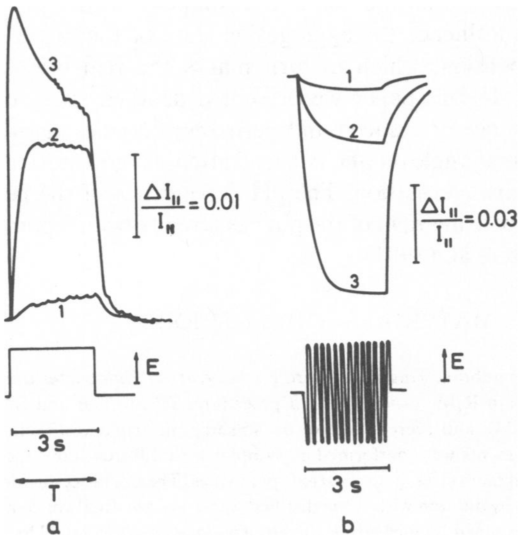

FIGURE 1 The light intensity changes (*a*) in a static electric field, $E_{1,2,3}$ = 1.7, 5.2, 15.8 V/cm, and (*b*) in alternating electric field, $E_{1,2,3}$ = 31, 77, 165 V/cm (peak values); $OD_{575nm} = 4.4$ at pH 8.8.

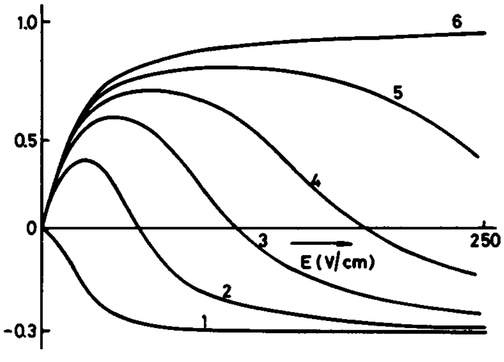

FIGURE 2 The calculated curves of $\Phi(E)$ at different values of β^2/γ. The curves 1, 2, 3, 4, 5, 6 denote β^2/γ = 0, 15, 30, 50, 90, and ∞, respectively.

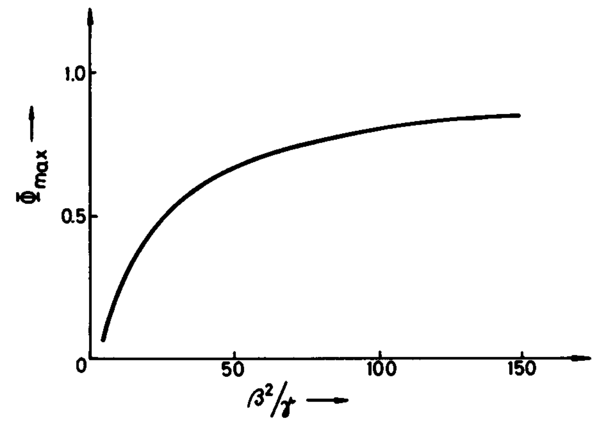

FIGURE 3 The calculated maximum of $\Phi(\beta,\gamma)$ at different values of β^2/γ.

in Fig. 3. Shah has suggested a simplified expression, if $\beta,\gamma \ll 1$, then

$$\Phi(\beta,\gamma) = \frac{1}{15}\beta^2 - \frac{2}{15}\gamma - \frac{\beta^4}{90} - \frac{2}{45}\gamma^2 + \frac{\beta^2\gamma}{30} \ldots \quad (4)$$

For high electric field

$$\begin{aligned} &\Phi(\beta, 0) \rightarrow 1 \\ &\Phi(0, \gamma) \rightarrow -1/2 \qquad (5) \\ &\Phi(\beta, \gamma) \rightarrow -1/2. \end{aligned}$$

The data shown in Fig. 1 were taken on samples that were varied in pH, dilution, and particle size. To obtain values of μ and α the following procedures were used. Values of $\Delta A/A$ were plotted as a function of field strength. (Representative curves are displayed in Fig. 4 *a* and *b*.) Because only the induced moment participates in the orientation of the fragments in an AC field, whose period is much greater than the relaxation time of the fragments, β can be considered zero. Evaluation of γ is straightforward. θ is obtained from the saturation value of the reduced dichroism, where $\Phi(0,\gamma)_s = -1/2$. Using this θ value, we determine $\Phi(0,\gamma)$ for any initial value of the $\Delta A/A$ data. Then γ is calculated with the help of Eq. 4. When a DC field is used, both the permanent and induced dipole moments influence the orientation. The saturation at low fields is only apparent (Fig. 2). Therefore, $\Phi(\beta,\gamma)$ and from it β were calculated using Eqs. 3 and 4 with θ and γ determined in AC measurement.

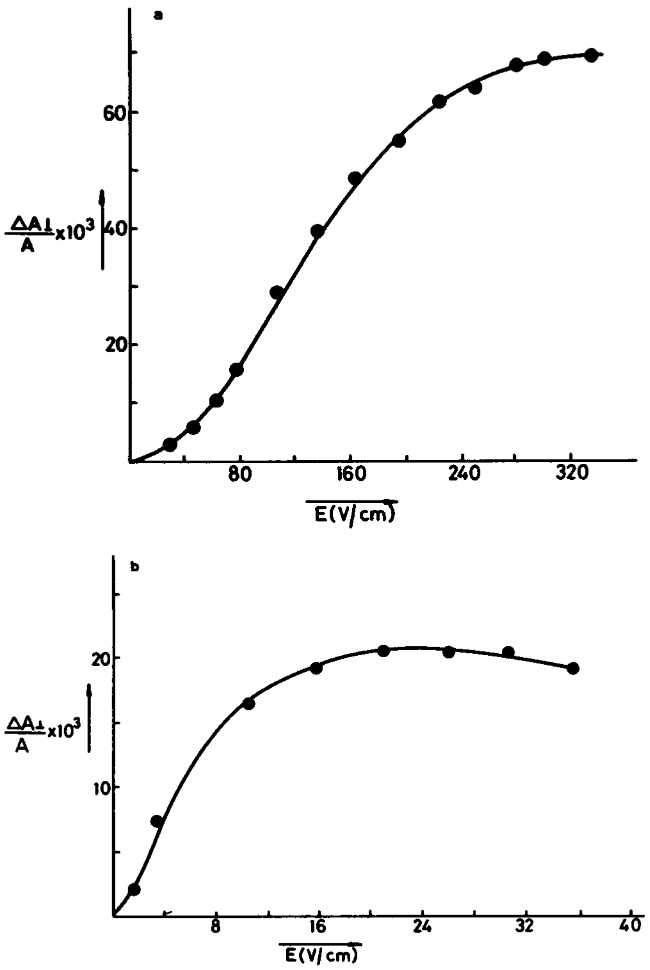

FIGURE 4 The reduced dichroism as a function of field strength for the (*a*) alternating electric field and for the (*b*) static field $OD_{575nm} = 4.4$ at pH 8.8.

Because experience showed that pH influenced the aggregation state of the pm, corrections were needed for the average fragment size, which was evaluated from the light-scattering measurements. The angular dependence (2–30°) of the intensity of scattered light was measured and the average particle size was determined from the equation of Kratky and Porod (1949) given for disk-shaped particles. The average particle diameter is nearly constant in the pH range 5–10 (Fig. 5), but at pH ≤ 4 a pronounced aggregation appears. We considered the average size as a relative value because the literature does contain another equation for the light-scattering yielding values for the diameter that differ by a constant factor (Doty and Edsall, 1951).

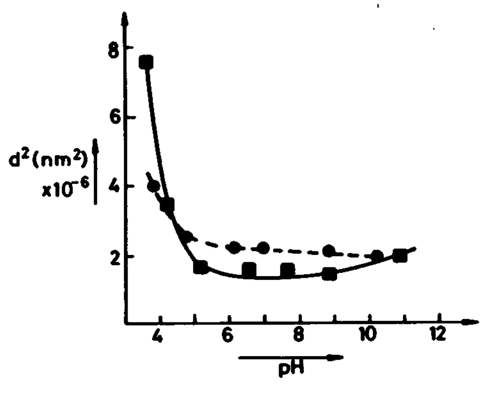

FIGURE 5 The pH dependence of the diameter squared of pm, pH was adjusted by HCl/KOH system (——) or by low ionic strength buffer (---); $OD_{575nm} = 4$ at pH 6.6.

TABLE I
RELAXATION TIMES (τ), PERMANENT DIPOLE MOMENTS (μ), AND THE CALCULATED VALUES OF R FOR FRAGMENTED PM

τ	μ*	$R = \sqrt{\mu}/\sqrt[3]{\tau}$
s		
1	2.5	1.6
0.02	0.25	1.8
0.005	0.05	1.2

*Arbitrary unit.

RESULTS AND DISCUSSION

Effect of Fragmentation and Dilution

Sonication breaks the pm into smaller fragments. Table I contains the values of the permanent dipole moments (in arbitrary units) and the relaxation times (in a DC field) for samples sonicated for different lengths of time. The expectation that $\mu \sim r^2$ and $\tau \sim r^3$ is fulfilled during these experiments, and this is indicated by the near constancy of $R = \sqrt{\mu}/\sqrt[3]{\tau}$ (Table I). In another experiment it was found that the values for the polarizability (α) and the permanent dipole moment (μ) decrease by the same factor with increased fragmentation (Table II). Dilution of the pm suspension did not affect α and μ in the same way. As in shown in Table III, α had a minimum while μ continuously decreased with dilution. The latter result is easily explained because the degree of aggregation decreases with greater dilution. Variation in the value of α reflects the contribution of two opposing effects. According to the data in Table II, α increases with the size of the particles and also increases with dilution, i.e., with increasing distances between pm (McLaughlin, 1977).

pH Dependence of Polarizability and Permanent Dipole Moment

The polarizability (α) and the permanent dipole moment (μ) were determined varying pH both in a HCl/KOH system and in buffered solutions. Because of the slight change in the average size of pm at different pH's, a normalization by area was done. As it was mentioned previously, we are considering the measured diameters as relative values only and therefore, the curves in Fig. 6 *a* and *b* represent the relative pH dependency.

Both α and μ displayed a maximum, but they did not occur at the same pH. Fig. 6 *b* shows that μ changes its sign near pH 5. This result could not be concluded using the linear dichroism data because it does not discriminate between the directions of absolute orientation (between dipoles parallel and antiparallel to the orienting field). The absolute orientation of the pm was determined by observing proton movement through the membrane after flash excitation (Keszthelyi and Ormos, 1980). Protons will move from the interior to the exterior face of the plasma membrane. The direction of the induced current relative to the orienting field indicates the membrane direction. Fig. 7 shows that the electric signal changes sign around pH 5. This change is the consequence of the sign change of the permanent dipole moment. At pH > 5 the negative charge of the dipole is at the internal side of the membrane. A sign change in the dipole moment of the pm around pH 5 was also observed by electron-microscopic methods (Fisher et al., 1977).

The pH dependence of μ is in a good agreement with Kimura's results (Kimura et al., 1981), in spite of the fact that the pm suspension was more dense in our case (OD ~ 4 compared with OD = 0.3 in Kimura's measurement). Our intention was to measure the parameters in high concentration, similar to the circumstances that are favorable for the study of the electric response signals in oriented pm suspension (Keszthelyi and Ormos, 1980).

TABLE II
DEPENDENCE OF THE POLARIZABILITY (α) AND PERMANENT DIPOLE MOMENT (μ) ON FRAGMENTATION

	α*	μ*
	2.5	1.2
Fragmentation	1.2	0.6
	0.7	0.3

*Arbitrary unit.

Determination of the Absolute Value of μ and α

For the determination of the absolute value of μ and α we used a diluted (OD = 0.3) and slightly sonicated sample at pH 6.6. The value of permanent dipole moment (μ) related to a membrane fragment is ~2.9×10^6 Debye (D), which is close to the data in the literature (2.4×10^6 D; Kimura et al., 1981). The permanent dipole moment of the pm is the sum of individual dipole moments, generally related to the number of BR molecules, which is proportional to the area of pm. Since the determination of particle diameter by light scattering is uncertain, we measured the area of fragments electromicroscopically (Fig. 8). Calculating

TABLE III
EFFECT OF DILUTION OF PURPLE MEMBRANE SUSPENSION ON THE POLARIZABILITY (α) AND PERMANENT DIPOLE MOMENT (μ)

A*	α‡	μ‡
1.58	1.5	8.7
0.74	1.15	4.5
0.21	2.4	2.7
0.13	2.4	2.65

*A is the absorbance of the solution.
‡Arbitrary unit.

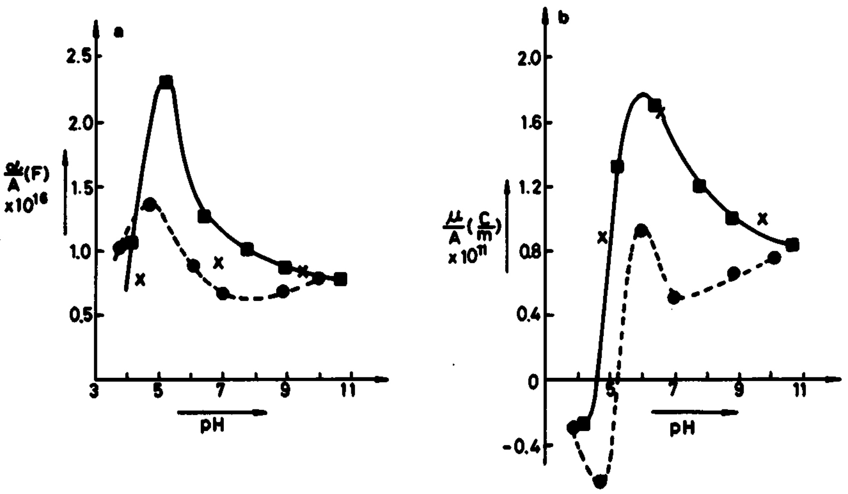

FIGURE 6 (*a*) The pH dependence of the polarizability per unit surface area. pH was adjusted by HCl/KOH system (—) or by low ionic strength buffer (---); OD$_{575nm}$ = 4 at pH 6.6; crosses (x) denote the values for higher dilution, OD$_{575nm}$ = 0.3 in HCl/KOH system. (*b*) The pH dependence of the permanent dipole moment per unit surface area. pH was adjusted by HCl/KOH system (—) or by low ionic strength buffer (---); OD$_{575nm}$ = 4 at pH 6.6; crosses (x) denote the values for higher dilution, OD$_{575nm}$ = 0.3 in HCl/KOH system.

with the average size $\mu(\mathrm{BR}) \simeq 140$ D is obtained, which agrees with the data of other authors (Kimura et al., 1981; Tsuji and Neumann, 1981). However, particles of different size contribute to the measured change of absorbance differently; therefore, the size distribution must be taken into account.

Using Eq. 4 and neglecting γ at low field strength

$$\Delta A_i \sim S_i \mu_i^2, \tag{6}$$

where ΔA_i is the absorbance change caused by a fragment with a size S_i and a permanent dipole moment μ_i. With the help of this relation the permanent dipole moment (μ_o) of a pm of a standard unit area (S_o) can be written as

$$\mu_o = \mu \left(\frac{\sum_i m_i n_i^3}{\sum_i m_i n_i} \right)^{-1/2}, \tag{7}$$

where μ is the measured dipole moment, and m_i is the number of fragments with a size n_i expressed in S_o units. From this (with $S_o \simeq 1\ \mu\mathrm{m}^2$) $\mu_o = 1 \times 10^{-23}$ Cm (coulomb meter) and $\mu(\mathrm{BR}) = 60$ D.

Fig. 3 shows the values of the orientation function (Φ) calculated from Shah's equation vs. β^2/γ. The polarizability of a membrane fragment was determined 3×10^{-28} Fm^2; therefore, $\beta^2/\gamma = 2\mu^2/\alpha kT \simeq 160$. Φ_{max}, corresponding to $\beta^2/\gamma = 160$, is between 0.85 and 0.9 which is contrary to the measured value ~0.2. This strong discrepancy shows that besides the contribution of the induced moment, there must be another disorienting effect in DC field. This problem will be discussed in detail in the Electrophoretic Flow Effect section.

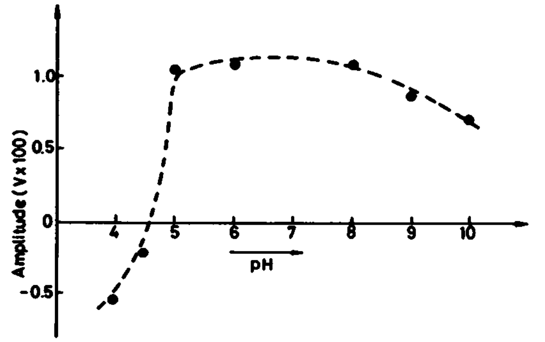

FIGURE 7 The pH dependence of the amplitude of the first component of the electric response signal after flash excitation. Field strength for orientation was 15 V/cm; OD$_{575nm}$ = 8 at pH 6.0.

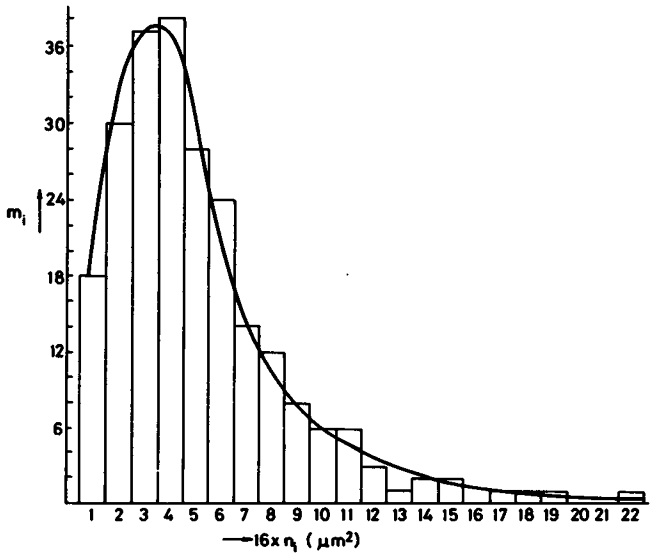

FIGURE 8 The size distribution of pm (OD$_{575nm}$ = 0.3 at pH 6.6; n_i, the surface area of a membrane fragment, m_i, the frequency of n_i).

Chromophore Angle θ

The chromophore angle, θ, can be determined according to Eq. 3 from the saturation value of the reduced dichroism. Because saturation due to the permanent dipole moment is only apparent, it is more accurate to use the saturation values of AC measurements to calculate θ. Results for θ vs. pH are shown in Fig. 9. We obtained higher values ($\theta \sim 69$–$70°$) with more dilute samples.

Light-Adaptation and Dark-Adaptation

All of the above measurements were performed on light-adapted pm. In some experiments low intensity green light ($\sim 2 \times 10^{-9}$ W/cm^2) was used during the measurements. The orientation function (Φ) was measured using a DC field for both dark-adapted (DA) and light-adapted (LA) pm. No difference (<5%) was found in the orientation function. The saturation value that is characteristic for the angle of the transition dipole moment θ is, however, smaller in the LA form. The calculated difference of angles is 0.4 ± 0.1°, which is independent of pH in the range 6.5–10. The DA form contains 50% all-*trans* and 50% 13-*cis* isomer of retinal. Because Eq. 3 can be approximated by linear function in small angular range, we may state that the angle between the transition dipole moments of all-*trans* and 13-*cis* isomer is 0.8 ± 0.2°. This is in agreement with the value found by Bogomolni et al. (1977).

Electrophoretic Flow Effect

The changes in light intensities, $\Delta I_{\parallel}$ and $\Delta I_{\perp}$ (data not shown), decrease for large static electric fields (E), with a time constant different from the time constant of rise and fall (Fig. 1 *a*). Such effects have already been described by Keszthelyi (1980) and were thought to be the result of tilting of the membrane disks due to electrophoretic movement. This tilting of the disk from the perpendicular direction to the electric field decreases the values of $\Delta I_{\parallel}$ and $\Delta I_{\perp}$.

The decrease of excess light intensity was found in the DC field measurement and not in AC signals of the same samples. This observation favors an electrophoretic flow effect that is surely absent in AC field. On the other hand, one would expect the tilting effect to be independent from the concentration of pm. The measurements show, however, that the falling parts in Fig. 1 *a* do not appear at low concentration (OD = 0.3). Because of this concentration dependence electrophoretic flow ordering is improbable.

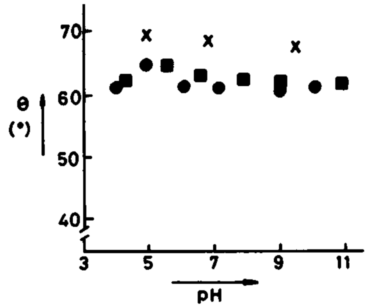

FIGURE 9 The pH dependence of the angle of the retinal to the surface normal. pH was adjusted by HCl/KOH system (●) or by low ionic strength buffer (■); $OD_{575nm} = 4$ at pH 6.6; crosses (x) denote the values for higher dilution, $OD_{575nm} = 0.3$ in HCl/KOH system.

A light microscopic study was performed to investigate the effects appearing in electric fields. A reversible clustering of membrane fragments occurred when a static electric field was applied. The clusters appeared immediately after switching on the electric field and moved electrophoretically towards the anode. The clusters disintegrated when the DC field was switched off. Clustering was more pronounced in dense suspensions and at high electric fields. (In a dense suspension [OD = 20] the clusters were ~2 μm diam and moved with a velocity of 20 μm/s at 10 V/cm). On the other hand, even in high AC fields such an effect could not be observed.

The appearance of clusters is probably due to the nonuniform electrophoretic movement of the different pm. The increased interaction between the particles involved in the clusters can produce disorientation. Thus the lower maximum value of the orientation function (see Determination of the Absolute Value of μ and α) becomes understandable. The decrease of $\Delta I_{\parallel}$ *and* $\Delta I_{\perp}$ in DC field (Fig. 1 *a*) can be attributed to a secondary effect, possibly a slow build up of larger clusters from the suddenly appearing smaller clusters that were also observed in dense solutions.

CONCLUSION

The variation of α and μ with pH is apparently the consequence of changing charges on the membrane surface. Presently it is not possible to decide, with certainty, whether the permanent dipole moment has its origin in the BR molecules or in the membrane lipids. A more detailed study is needed with specific modifications either in the BR or the plasma membrane lipid molecules. It is important that the ratio of permanent to induced dipole moments at low field strength is large and in this way a real orientation of the pm fragments can be obtained. The orientation of pm even in concentrated suspension is quite sufficient for more extensive experimentation because the angle θ does not scatter more than ≃ 10° even in large clusters. It is probable that other membrane systems have characteristics similar to the BR system and are therefore appropriate for the similar studies of charge movements.

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