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# INTERACTIONS IN MIXED MONOLAYERS BETWEEN DISTEAROYL-L-PHOSPHATIDYLETHANOLAMINE, ROD OUTER SEGMENT PHOSPHATIDYLETHANOLAMINE AND ALL-*TRANS* RETINAL

## EFFECT OF pH *

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**The interactions in mixed monolayers between distearoyl-L-phosphatidylethanolamine, natural phosphatidylethanolamine purified from bovine rod outer segments and all-*trans* retinal have been studied at the nitrogen/water interface at 21.0 ± 0.5°C. Seven mixtures of each phospholipid with all-*trans* retinal, covering the whole range of molar fractions, were studied. The monolayers were spread on a $1 \cdot 10^{-3}$ M phosphate buffer subphase at three different pH values, 5.5, 7.1 and 8.2. The results for the two series of mixtures are strikingly different. The surface phase rule shows that all-*trans* retinal is miscible with the natural phospholipid at the interface. Small, negative deviations with respect to the additivity rule are observed in this case. The excess free energies of mixing were also calculated as a function of concentration for this system at four different surface pressures, 5, 7, 10 and 13 $mN \cdot m^{-1}$. They are negative for the four surface pressures considered and symmetrical with respect to the mole fraction. On the other hand, when distearoyl-L-phosphatidylethanolamine is mixed with all-*trans* retinal, the components are no longer miscible at the interface. This marked difference in behaviour between the two lipids reflects the importance of hydrophobic interactions in the mixed monolayers of phospholipids with retinals. Furthermore, for the two series of mixtures, the surface pressure isotherms do not show any significant shift when the subphase pH is changed from 5.5 to 8.2. This behaviour raises questions about the formation of a Schiff base between phosphatidylethanolamine and retinal at the interface. It is suggested that, owing to the nature of the disk membranes, such an effect would also be observed in vivo. The possible implications of this are discussed, particularly with respect to questions pertaining to the stability of the retinal chromophore.**

## Introduction

Phosphatidylethanolamine (PE) has always been considered as one of the most important component lipids found in the disk membranes, the functional units of the vertebrate photoreceptors. It accounts for about 40% of the phospholipids [1,2] present as the major class of lipids of the discal membrane and appears to be predominantly distributed on the outer (cytoplasmic) face of the

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Abbreviations: PE, phosphatidylethanolamine; DSPE, distearoyl-L-phosphatidylethanolamine; $PE_{ROS}$, phosphatidylethanolamine extracted from bovine rod outer segments; PC, phosphatidylcholine; DOPC, dioleoyl-L-phosphatidylcholine.

disk bilayer [3]. In order to grasp the importance of this phospholipid in the field of vision, one has to remember that in the early 1970's it was thought that in native rhodopsin the 11-*cis*-retinal chromophore was bound to PE as a Schiff base [4–6]. This idea was soon challenged and it is now well established that in rhodopsin the chromophore is attached through a Schiff base to a lysine residue of the protein [7]. At the present time PE is thought to be involved in the regeneration of rhodopsin. Shichi and Somers [8,9] have indeed found that PE stimulates the photoregeneration of rhodopsin from the opsin, via the formation of a Schiff base between PE and all-*trans* retinal. This chemical entity, retinylidene-PE, can act as an intramolecular non-enzymatic catalyst which, in its protonated form, can specifically isomerize the all-*trans* Schiff base to the 11-*cis* isomer under the influence of light [10].

From this evidence, it seems therefore apparent that PE plays an essential functional role in the regeneration process of rhodopsin, particularly owing to the formation of a Schiff base with the retinal isomers. In order to have a better understanding of the conditions prevailing to the formation of a Schiff base in a model system, this paper presents a monolayer study of the interactions between PE and all-*trans* retinal at the nitrogen/water interface. It represents a continuation of our previous papers [11,12] aiming at understanding the molecular interactions prevailing in mixed monolayers between rod outer segment components. We have therefore measured the surface pressure isotherms of the mixed monolayers of all-*trans* retinal with two different phosphatidylethanolamines. One was the natural highly unsaturated phospholipid extracted and purified from bovine rod outer segment disk membranes (and hereafter denoted $PE_{ROS}$), the other being the fully saturated distearoyl-L-phosphatidylethanolamine (DSPE). The present results show the importance of hydrophobic interactions in the thermodynamics of mixing of the retinals with phospholipids and establish that no Schiff base is formed between PE and the retinal molecules at the water interface. The possibilities for the same phenomena to occur within the disk membranes are examined and the consequences pertaining to the regeneration mechanism of rhodopsin are discussed.

## Material and Methods

*Chemicals used and surface pressure work*

DSPE was a synthetic lipid obtained from P.L. Biochemicals (Milwaukee, WI), while $PE_{ROS}$ was extracted and purified from bovine rod outer segments according to the method described below. All-*trans* retinal was bought from Sigma (Saint Louis, MO). The compounds used were checked for purity by thin-layer chromatography using *n*-$C_6H_4/CH_3OH$ (18 : 1, v/v) for all-*trans* retinal and $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) for the two PEs. In all cases, a single spot was observed and no further purification was attempted.

The surface pressure isotherms were measured at the nitrogen/water interface on a $1 \cdot 10^{-3}$ M phosphate buffer. The temperature was kept at $21.0 \pm 0.5$°C throughout the study and the pH was varied from 5.5 to 8.2, the salts ($Na_2HPO_4$ and $NaH_2PO_4$, J.T. Baker Chemical Co., Phillipsburg, NJ) being used without further purification. The experimental conditions and precautions taken to ensure the consistency of the experimental results are described in Ref. 11. The solutions of all-*trans* retinal, DSPE and $PE_{ROS}$ used for the measurements of the surface pressure isotherms were prepared by weighing about 0.9 mg of all-*trans* retinal, 1.9 mg of DSPE and 1.5 mg of $PE_{ROS}$ on a Cahn electrobalance (Model RG 2000, Ventron Instrument Corp., CA). The total volume of the solution was 3.5 ml. Purified benzene [13] was used as spreading solvent for the mixed monolayers of all-*trans* retinal with $PE_{ROS}$, while a mixture of benzene and methanol (5 : 1, v/v) was used in the study of the interactions of all-*trans* retinal with DSPE. When all-*trans* retinal was used, the manipulations were carried out under a dim red light. The molar weights of all-*trans* retinal, DSPE and $PE_{ROS}$ used in the calculation of the molar fractions and molecular areas are 284, 748 and 800, respectively.

*Extraction and purification of $PE_{ROS}$*

Rod outer segments from bovine retinas are obtained from flotation of the disk membranes followed by a continuous gradient sucrose centrifugation according to Salesse et al. [14]. The purified membranes present a spectral ratio $A_{280}/\Delta A_{500} = 2.5$ to 3.0 after solubilization in an

Ammonyx LO (Onyx Chem. Co., Jersey City, NJ) buffer, as described by Raubach et al. [15]. The rod outer segments membrane fatty acid composition is in good agreement with the published values obtained for highly purified bovine rod outer segment membranes [16,17].

The total outer segment lipids are then extracted from the rod outer segment membrane suspension according to a procedure described by Miljanich [18]. All the solvents used were distilled and the glassware was cleaned from surface-active contaminants as reported by Tancrède et al. [13]. They were bubbled with Argon for at least 0.5 h before use. $PE_{ROS}$ was obtained from chromatography of the total rod outer segment lipids on DEAE-cellulose (Eastman Kodak Co., Rochester, NY) followed by a second chromatography on a silicic acid-celite column [14]. $PE_{ROS}$ was then checked for purity by thin-layer chromatography using $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) and showed only one spot. The purified sample was also analyzed for its fatty acid content [14]. The main fatty acid components for our purified PE sample, stearic acid (18 : 0, 24.6%) and docosahexaenoic acid (22 : 6($n-3$), 45.3%) together with the less abundant components were found in close agreement with those presented by Drenthe et al. [17] and Miljanich et al. [19].

## Results and Discussion

*Surface pressure isotherms of the pure components*

The surface pressure isotherms of the pure components of the present study, all-*trans* retinal (curve 1), DSPE (curve 2) and $PE_{ROS}$ (curve 3) are presented in Fig. 1. The isotherms were measured at the nitrogen/water interface at 21.0 ± 0.5°C on a $1 \cdot 10^{-3}$ M phosphate buffer at three different pH values: 5.5, 7.1 and 8.2. For each pH studied, the isotherm for the three compounds is the average of six different measurements done independently on two different batches of the pure components in the spreading solvent. The maximum deviation between the extremes in all cases was better than 2 Å$^2$/molecule. The surface pressure isotherms did not show any significant change when the pH was varied from 5.5 to 8.2, and could be superimposed throughout the whole range of surface pressures. This result was expected, since no group in either

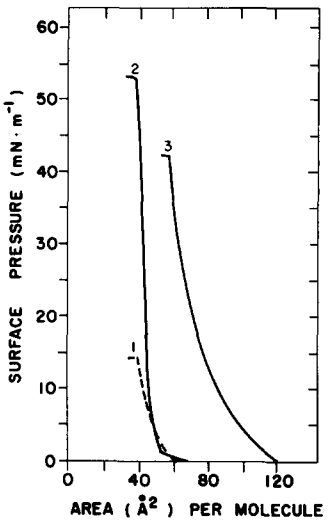


Fig. 1. Surface pressure-area isotherms of the pure components at the nitrogen/water interface at 21.0 ± 0.5°C. Curves 1, 2 and 3 are for all-*trans* retinal, DSPE and $PE_{ROS}$, respectively. The monolayers were spread from benzene for isotherms 1 and 3 and from benzene/methanol (5 : 1, v/v) for isotherm 2. The subphase was a $1 \cdot 10^{-3}$ M phosphate buffer. The isotherms shown are for three different pH values, 5.5, 7.1 and 8.2.

all-*trans* retinal or the phosphatidylethanolamines is chemically modified by changing the pH subphase within this range. The only modification that could be expected is deprotonation of the amino group in PE, but this is known to occur at a pH value of about 9 [20].

The isotherm presented here for the all-*trans* retinal isomer is almost identical to the one reported by us in an earlier study [11]. The collapse pressure and limiting surface area are 14.9 mN · m$^{-1}$ and 50 Å$^2$/molecule, respectively, as compared to 15.4 mN · m$^{-1}$ and 49 Å$^2$/molecule in Ref. 11. Except for the slight difference at the collapse pressure, the surface pressure isotherms are completely superimposable to within experimental error. Our isotherm for DSPE is also to within 4 Å$^2$/molecule identical to the one published by Phillips and Chapman [21] and Van Deenen et al. [22] for the same compound. Our extrapolated limiting area for DSPE is found to be 46 Å$^2$/molecule as compared to 42 Å$^2$/molecule and 43 Å$^2$/molecule in Ref. 21 and Ref. 22, respectively. In the former case, the subphase was almost the same as ours ($5 \cdot 10^{-4}$ M phosphate buffer, pH 7.4) while in the latter case, the mono-

layer was spread on a 0.1 M NaCl solution. The subphase has therefore very little effect on the surface characteristics of this compound. The spreading solvent is also without effect, since chloroform was used in Ref. 21 as compared to hexane/ethanol (4 : 1, v/v) in Ref. 22 and benzene/methanol (5 : 1, v/v) in the present study. This is also the case for the isotherm shown in Fig. 1, curve 3, for $PE_{ROS}$ spread on a $1 \cdot 10^{-3}$ M phosphate buffer at three different pH values, which is identical, to within 2 $Å^2$/molecule to the one presented by Salesse et al. [14] for the same compound spread on 0.1 M NaCl using hexane/ethanol (9 : 1, v/v) as spreading solvent. In both cases, the extrapolated limiting area and collapse pressures are found to be 78 $Å^2$/molecule and 43.0 $mN \cdot m^{-1}$, respectively. The much higher molecular areas found for $PE_{ROS}$ as compared to DSPE reflect the larger hydrophobic volume of the unsaturated fatty acyl chains present in natural PE. Indeed, about 45% of the total aliphatic chains in $PE_{ROS}$ are represented by docosahexaenoic acid (22 : 6($n-3$)).

*The mixed monolayers of $PE_{ROS}$ with all-trans retinal*

The surface pressure isotherms of the mixed monolayers of the all-*trans* retinal isomer with $PE_{ROS}$ have also been studied as a function of composition at three different pH values, 5.5, 7.1 and 8.2, respectively. The experimental results are presented in Fig. 2, which shows the isotherms of seven mixtures of all-*trans* retinal with $PE_{ROS}$, covering the whole range of molar fractions. In this figure, curves 1–7 represent the isotherms of the mixtures for which the molar fraction of $PE_{ROS}$ is 0.100, 0.200, 0.400, 0.500, 0.600, 0.800 and 0.900, respectively. Curves a and b represent the surface pressure isotherms of the pure components, all-*trans* retinal and $PE_{ROS}$, respectively. For each pH, the curve is the average of three different determinations done on two separate batch solutions of the pure components in benzene, the spreading solvent used with this series of mixtures.

In similarity to the results already found for the mixtures of DOPC with all-*trans* retinal [11] or 11-*cis* retinal [12], the isotherms for all the mixed monolayers show a true collapse pressure around 43 $mN \cdot m^{-1}$, very close to the collapse pressure of $PE_{ROS}$, together with an apparent collapse pressure varying as a function of composition. The apparent collapse pressure is very clearly observed at 16, 18, 24 and 29 $mN \cdot m^{-1}$ for $x_{PE_{ROS}} = 0.100$, 0.200, 0.400 and 0.500, respectively. It is not observed for the higher molar fractions. As already discussed extensively in our previous works [11,12], the existence of these apparent collapse pressures is associated with the rejection of one of the components from the monolayer upon the increase of the surface pressure. In the case under study here, the all-*trans* retinal molecules would be rejected from the monolayer to interact hydrophobically with the aliphatic chains of the phospholipids, thereby maximizing this type of interaction. The apparent collapse pressure represents the onset pressure for this rejection to occur. A similar behaviour was observed when DOPC was mixed with either all-*trans* retinal [11] or 11-*cis* retinal [12]. Furthermore, the fact that these apparent collapse pressures vary with composition provides evidence for the miscibility of the components at the interface. Indeed, if one applies the surface phase rule [11,23] to the present system, keeping the temperature and pressure constant and assuming the components miscible at the interface imply that the collapse pressure should vary with composition of the film-forming substances (the system possesses two degrees of freedom). This behaviour is observed with the apparent collapse pressures, the values of which vary with composition. If the monolayer is compressed further, the all-*trans* retinal molecules are ejected from the monolayer and the collapse pressure should not vary with composition (the system possesses only one degree of freedom). This situation is found at the true collapse pressure which, in Fig. 1, is shown to be constant at 43 $mN \cdot m^{-1}$ for all the molar fractions studied.

The analysis of the present results as well as the monolayer results presented in our earlier studies [11,12] therefore lead to the conclusion that the retinal molecules, the 11-*cis* or all-*trans* isomer are expelled from the polar water interface to interact hydrophobically with the aliphatic chains of the phospholipid molecules. By extrapolating these results to the *in vivo* system, it has been suggested [11] that the retinal isomers, by considering their apolar nature, would also be expelled from the

polar interfaces of the disk membranes to interact hydrophobically with the alkyl part of the lipid molecules. This is consistent with the prevailing ideas about the localization of the retinal chromophore in the disk membrane. Recently, Thomas and Stryer [24] from fluorescence energy transfer experiments have indeed shown that the retinal chromophore in rhodopsin is located near the centre of the hydrophobic core of the disk membrane. Upon bleaching, it is likely that all-*trans* retinal could be rejected within the hydrophobic region of the lipid bilayer. Our conclusion has also received further support in a recent NMR study by Inoue et al. [25]. These authors have studied, by means of carbon-13 and proton NMR spectroscopies, the effect of adding the retinal isomers and vitamin A (retinol) on the dynamic structure and stability of egg phosphatidylcholine bilayers. Their main conclusion, which confirms ours, is that the retinal isomers are incorporated within the aliphatic portion of the bilayer, hydrophobically interacting with the hydrocarbon chains at the inner core of the membrane. The retinal isomers, being less polar than vitamin A, are found to be intercalated more deeply within the bilayer.

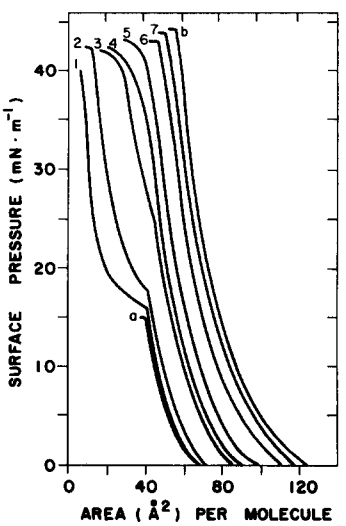


Fig 2. Surface pressure-area isotherms of the mixed monolayers of all-*trans* retinal with $PE_{ROS}$ at three different pH values, 5.5, 7.1 and 8.2. Curves 1–7, the molar fractions of $PE_{ROS}$ are 0.100, 0.200, 0.400, 0.500, 0.600, 0.800 and 0.900, respectively. Curves a and b are for all-*trans* retinal and $PE_{ROS}$. The monolayers were spread at the nitrogen/water interface using benzene as spreading solvent. The subphase was $1 \cdot 10^{-3}$ M phosphate buffer.

The surface pressure isotherms presented in Fig. 2 also present the striking feature of being completely invariant upon changing the pH from 5.5 to 8.2. The isotherms presented in Fig. 2 were found to superimpose to within experimental error (2 Å$^2$/molecule) over the range of pH values covered here. These results are in contrast with those reported by De Pont et al. [26], which have shown that a Schiff base can be formed in the monolayer between the aldehyde group of all-*trans* retinal and the amino group of PE. The formation of the Schiff base corresponded to an increase in surface area of about 5 to 8 Å$^2$/molecule and occurred at pH values above 7. This discrepancy between the two sets of results may find origin in the difference between the experimental procedures underlying the two series of results. De Pont et al. [26] measure, for various surface pressures, the increase of the film pressure occurring by penetration of all-*trans* retinal into the PE monolayer. Therefore, they spread a monolayer of PE on the water interface, compress it up to a given surface pressure and inject ethanolic solutions of the retinal isomer underneath the monolayer, in the subphase. The hydrophobic retinal molecules diffuse to the interface and on their way, the aldehyde group can interact with the amino group of PE to form the Schiff base. On the other hand, our methodology is completely different. In our case, the monolayers are carefully deposited at the nitrogen/water interface from a mixture of the two components in an organic solvent, benzene being used for this series of mixtures. In such a solvent, the retinal molecules are free and not linked to PE, as the maximum absorption read for each solution ($\lambda_{max}$ = 385–390 nm) indicates. The fact that the surface pressure isotherms for any of the mixtures do not shift significantly (within 2 Å$^2$/molecule) upon changing pH in the range studied implies that no Schiff base is formed between PE and all-*trans* retinal at the water interface, at least under our experimental conditions. This conclusion is also consistent with the behaviour of Schiff bases in aqueous environments, which are known to be easily hydrolyzed to their amine and aldehyde components [27].

This conclusion is further enhanced in Fig. 3, which shows the mean molecular areas, $A_{12}$, for

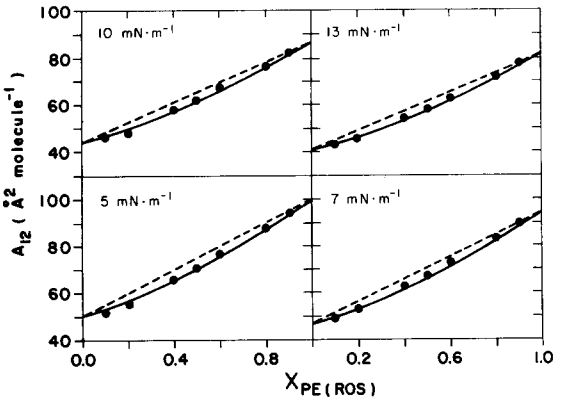


Fig. 3. The mean molecular area of the mixed monolayers of all-*trans* retinal with $PE_{ROS}$ as a function of the mole fraction of $PE_{ROS}$ at different surface pressures. Dashed curves, calculated results assuming the additivity rule. Solid circles, the experimental values at pH 5.5, 7.1 and 8.2 taken from Fig. 2.

the various mixtures of $PE_{ROS}$ with all-trans retinal, plotted as a function of the mole fraction of $PE_{ROS}$. The mean molecular areas were taken from Fig. 2 at four different surface pressures, 5, 7, 10 and 13 $mN \cdot m^{-1}$. The dashed lines in Fig. 3 were calculated assuming the additivity rule, i.e., $A_{12} = x_1 A_1 + x_2 A_2$, where $x_1$ and $x_2$ are the mole fractions of all-*trans* retinal and $PE_{ROS}$ and $A_1$ and $A_2$ the molecular areas in the two pure component films at the same surface pressures. It is clear from Fig. 3 that small negative deviations from ideality are observed for the four surface pressures studied, the mean molecular area in the mixtures being smaller than those calculated assuming the additivity rule, the maximum of the effect being located around $x_{PE_{ROS}} = 0.5$. These small negative deviations with respect to ideality, of the order of 5 $Å^2$/molecule, are comparable to those observed in our earlier works for DOPC mixed with either all-*trans* retinal [11] or 11-*cis* retinal [12]. They are therefore much more likely to be due to hydrophobic interactions rather than to the formation of a Schiff base, the magnitude of the effect observed for $PE_{ROS}$ being the same as for DOPC, for which no amino group is present to form a Schiff base.

It is therefore possible, owing to the hydrophobic nature of the all-*trans* retinal molecules and to the conformation of the hydrophilic ethanolamine group of PE at the water interface, that the reaction between the aldehyde group of retinal and the amino group of PE does not occur. However, this conclusion is only valid considering the specific conditions prevailing at the water interface as a result of the nature of the constituents involved here. It does not imply that when vesicles (e.g., mixed vesicles of PE and PC) are prepared from suspensions of the constituents in water, a Schiff base cannot be formed. In this case it is most likely that during the preparation of the sample, the groups involved in the Schiff base formation come close enough for the reaction to occur. For the same reason, in biochemical extractions and purifications involving the disk membranes, formation of a Schiff base can be easily induced if the membrane entity is destroyed. This experimental artefact could then yield to erroneous conclusions when the *in vivo* system is concerned.

Further considerations about the lack of existence of a Schiff base between PE and retinal at the water/nitrogen interface can be given by calculating the excess free energies of mixing, $\Delta G^{\pi}_{xs}$, of the components in the monolayer. The surface pressure isotherms of the pure components and the mixtures, presented in Fig. 2, can be used to evaluate this parameter. A detailed description of the calculation technique is presented in Ref. 11. Fig. 4 shows the results obtained for $\Delta G^{\pi}_{xs}$ at four

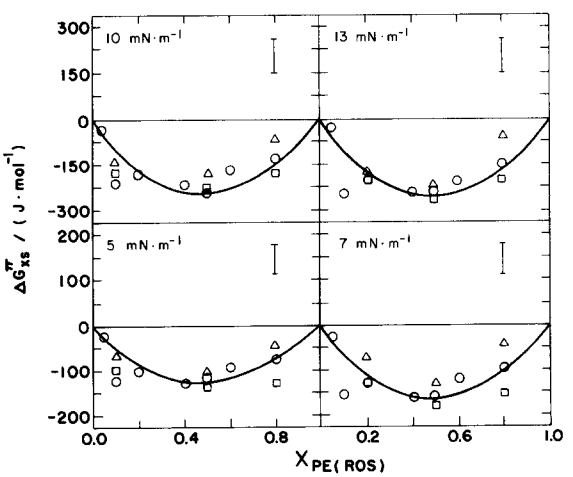


Fig. 4. The excess free energies of mixing all-*trans* retinal with $PE_{ROS}$ as a function of the mole fraction ($X$) of $PE_{ROS}$ at different surface pressures and pH values: △, 5.5, ○, 7.1 and □, 8.2. The bars at the upper right represent the experimental error on the excess free energies.

different surface pressures, 5, 7, 10 and 13 mN · $m^{-1}$, plotted as a function of $x_{PE_{ROS}}$. The excess free energies of mixing are negative for the four surface pressures studied, the minimum being centred around $x_{PE_{ROS}} = 0.500$. Furthermore, the points in Fig. 4 represent the values of $\Delta G^{\pi}_{xs}$ for the three pH values studied. The results show that the $\Delta G^{\pi}_{xs}$ values are not significantly different, to within experimental error (the error bars on $\Delta G^{\pi}_{xs}$ are represented at the upper right corner for each surface pressure) for the range of pH covered. If a Schiff base between all-*trans* retinal and PE had formed at pH values above 7, as found by De Pont et al. [26], a corresponding change would have been observed in the $\Delta G^{\pi}_{xs}$ values. Fig. 4 shows that this is clearly not the case.

The small, negative values obtained for the excess free energies of mixing $PE_{ROS}$ with all-*trans* retinal are also consistent with the small contraction of the mixed monolayer observed in Fig. 3 and discussed above. Furthermore, these values are almost similar to the $\Delta G^{\pi}_{xs}$ values obtained when DOPC was mixed with 11-*cis* or all-*trans* retinal. For example, at 10 mN · $m^{-1}$, the values obtained for DOPC mixed with the 11-*cis* and all-*trans* isomer [11,12] are $-280 \pm 35$ and $-340 \pm 35$ J · $mol^{-1}$, respectively, as compared to $-240 \pm 35$ J · $mol^{-1}$ for $PE_{ROS}$ mixed with the all-*trans* isomer. The fact that the excess free energies of mixing all-*trans* retinal with DOPC or $PE_{ROS}$ are so slightly different is a strong indication that the thermodynamics of mixing of these compounds is mainly governed by hydrophobic interactions, the polar interactions playing only a minor role, if any.

The experimental finding that all-*trans* retinal does not form a Schiff base with PE at the water interface together with the importance of hydrophobic forces in the interactions of the retinal isomers with phospholipids are both of particular interest when extrapolations to the in vivo system, the disk membrane, are analyzed. As discussed above, if the retinal molecules are expelled from the polar interface to interact hydrophobically with the acyl moiety of the lipid bilayer, it is likely, as suggested by the present results, that under such conditions a Schiff base does not form between PE and the retinal. This is a mere consequence of both the apolar nature of the retinal molecules and their localization within an organized lipid matrix. If the Schiff base is not formed in vivo the retinal isomers, particularly 11-*cis* retinal, are no longer susceptible to the isomerization reaction and the degradation process observed by Groenendijk et al. [28] on incubating at pH 7.2 retinoids (retinal, retinol etc.) with phospholipid and membrane suspensions. These authors have indeed shown that the retinal isomers, particularly 11-*cis* retinal, were led to substantial isomerization and to partial degradation when incubated in darkness with membrane extracts or lipid suspensions. The presence of PE and the formation of a Schiff base with retinal are important factors for the isomerization to occur. We agree that under such experimental conditions (incubation of the retinals with phospholipids) a Schiff base may be formed between PE and the retinal. However, it does not imply that this is necessarily the case in vivo, considering the organization of the constituents within the disk membrane. The present results indicate that the retinal molecules can simply interact hydrophobically with the aliphatic chains of the phospholipids, without any interaction with their polar heads. The retinal molecules would indeed not be susceptible to isomerization or partial degradation, no Schiff base being involved. This interpretation therefore suggests that the retinal isomers are protected in vivo simply by their localization within the aliphatic portion of the bilayer. There is no necessity, as suggested by Groenendijk et al. [28] to evoke binding of the retinal molecules to proteins to achieve protection from their environment. However, more experimental evidence on the exact localization of the retinal molecules after photobleaching of rhodopsin in the disk membrane (within the aliphatic portion of the bilayer, as suggested by the present work, or at the interface) is required to assess this point further. Furthermore, the present results bring further support to the hypothesis proposed in our previous work [11] suggesting that the unit responsible for the regeneration of 11-*cis* retinal from all-*trans* retinal would be an intrinsic protein or a protein with an hydrophobic anchor containing the active site. The regeneration mechanism of rhodopsin, following this line of reasoning and in the light of the experimental results presented here, is therefore more likely to be an intramembranous enzymatic process.

*The mixed monolayers of DSPE with all-trans retinal*

In order to assess further the importance of hydrophobic interactions in the thermodynamics of mixing of all-*trans* retinal with phospholipids, the surface pressure isotherms of the mixed monolayers of DSPE with all-*trans* retinal, covering the whole range of mole fractions, have been measured at three different pH values, 5.5, 7.1 and 8.2. The results for the various mixtures are presented in Fig. 5. Similarly to the mixtures involving $PE_{ROS}$, the isotherms presented in Fig. 5 are the average of three different measurements done on two batches of the pure components in the spreading solvent. The isotherms in the two series were superimposable to within 2 $Å^2$/molecule over the surface pressure and pH range covered.

The results presented in Fig. 5 show that the surface pressure isotherms are not changed significantly upon changing pH from 5.5 to 8.2. This situation is analogous to the one found when $PE_{ROS}$ was mixed with all-*trans* retinal and enhances our conclusion that the retinal molecules do not form a Schiff base with the PE molecules at the interface. However, the surface pressure isotherms for the mixed monolayers of DSPE with all-*trans* retinal are strikingly different from those involving $PE_{ROS}$, shown in Fig. 2. The apparent collapse pressure in the latter case varied as a function of composition, indicating miscibility of the components in the mixed monolayer, as the discussion based on the miscibility rule presented above has shown. However, keeping the same phosphatidylethanolamine polar head but changing the aliphatic chains (e.g., substituting the highly unsaturated acyl chains in $PE_{ROS}$ to saturated chains in DSPE) changes the miscibility pattern completely. This results from the fact that the apparent collapse pressure for the mixtures involving DSPE is no longer a function of composition, as Fig. 5 reveals. On the contrary, the apparent collapse pressure is located, for all the molar fractions studied, at a surface pressure value of about 16 $mN \cdot m^{-1}$, close to the collapse pressure of all-*trans* retinal (15 $mN \cdot m^{-1}$). When analyzed in terms of the surface phase rule, the fact that the apparent collapse pressure is no longer a function of composition implies that DSPE is not miscible with all-*trans* retinal at the water interface. This probably results as a consequence of the absence of double bonds in the acyl chains of DSPE which allows the phospholipid molecules to get closer to each other and, by so doing, to maximize the attractive short-range London dispersion forces between them. This lack of miscibility also implies that patches of phospholipid molecules and retinal molecules would be found at the interface, but surface potential measurements are required to give support to this assertion.

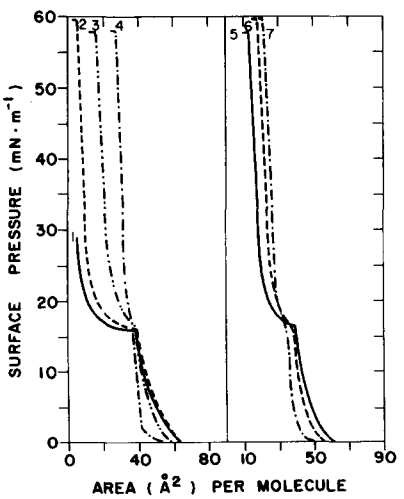


Fig. 5. The surface pressure-area isotherms of the mixed monolayers of all-*trans* retinal with PE(18:0) at three pH values, 5.5, 7.2 and 8.1. Curves 1–4, the mole fraction of DSPE is 0.100, 0.200, 0.500 and 0.900. Curves 5–7, the mole fraction of DSPE is 0.400, 0.600 and 0.800. The experimental conditions are as described in the legend of Fig. 1, except for the spreading solvent benzene/methanol (5:1, v/v) being used here for all the mixtures.

In summary, the present work has established the miscibility of $PE_{ROS}$ with all-*trans* retinal at the nitrogen/water interface. On the other hand, when DSPE is mixed with the retinal isomer, the components are no longer miscible at the interface. We have shown that this striking difference in behaviour provides evidence for the importance of hydrophobic interactions in the thermodynamics of mixing of the retinals with phospholipids. Our work has also shown that, under the present experimental conditions, no Schiff base is formed between PE and all-*trans* retinal at the interface. The present work, based on our conclusions that the retinal molecules interact mainly hydrophobically with the phospholipid molecules

and that no Schiff base is formed at the water interface therefore raises questions about the existence of a Schiff base in vivo. However, admittedly, further experimental work pertaining to the exact localization of the all-*trans* retinal molecules in the disk membrane after photobleaching of rhodopsin is required to assess this point further.

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