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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

II. SCHIFF BASE FORMATION IN PHOSPHATIDYLETHANOLAMINE MONOLAYERS UPON PENETRATION BY RETINALDEHYDE

J. J. H. H. M. DE PONT, F. J. M. DAEMEN AND S. L. BONTING
Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)
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SUMMARY

1. Penetration of all-*trans*-retinaldehyde into phosphatidylethanolamine monolayers gives a much higher increase of film pressure than penetration of the same substance into phosphatidylcholine monolayers. No such difference was observed upon penetration of all-*trans*-retinol into monolayers of these two phospholipids.

2. The extra penetration effect into phosphatidylethanolamine monolayers only occurred at a subphase pH above 7 and was always accompanied by a shift towards shorter wavelengths of the absorption maximum of the monolayer material, as compared to the spectrum of retinaldehyde itself.

3. These observations indicate the formation of a Schiff base between retinaldehyde and phosphatidylethanolamine in the monolayer under the conditions mentioned.

4. These model experiments support a hypothesis on the mechanism of visual excitation, in which Schiff base formation plays an essential role.

INTRODUCTION

In studies aimed at elucidating the biochemical mechanism of the visual process BONTING[1] and BONTING AND BANGHAM[2] employed monomolecular films of rhodopsin at an air–water interface as a model for the structure of the rod-sac membrane. Illumination of the rhodopsin monolayer caused a small increase in film pressure, indicating an expansion of the molecules in the monolayer. It was believed that this effect might be due to penetration into the monolayer of retinaldehyde liberated during photolysis of the rhodopsin. When the penetration of retinaldehyde into monolayers of rhodopsin (bleached or unbleached) and of phospholipids was studied, a curious phenomenon was observed. With rising initial film pressure the increase in film pressure caused by penetration of retinaldehyde decreased in all cases. However, at film pressures of 40 dyne/cm, close to the collapse pressure of these films, the phosphatidylcholine–cholesterol monolayer became impenetrable to retinaldehyde, while a residual penetration of retinaldehyde occurred in monolayers of rhodopsin as well as of phosphatidylethanolamine.

It was postulated, that this residual penetration of retinaldehyde into phosphatidylethanolamine films might be due to Schiff base formation between the aldehyde group of retinaldehyde and the phospholipid aminogroup. The possibility of this reaction has already been suggested by KRINSKY[3]. The absence of an aminogroup in phosphatidylcholine and cholesterol would explain why no residual penetration occurred in the phosphatidylcholine–cholesterol films.

The purpose of the present study was to test this hypothesis. Three different experiments confirmed the hypothesis: retinol did not show a different penetration behavior in films of phosphatidylcholine and phosphatidylethanolamine; no residual penetration of retinaldehyde was observed in phosphatidylethanolamine films at a subphase pH below 6; the absorption spectrum of such a monolayer after penetration of retinaldehyde at pH above 7 indicated the presence of a Schiff base link.

MATERIALS AND METHODS

Phosphatidylethanolamine, containing 50 % palmitic acid, 30 % oleic acid and 20 % linoleic acid, as confirmed by gas chromatographic analysis, was synthetized according to DAEMEN[4]. Phosphatidylcholine was isolated from egg yolk according to PANGBORN[5]. Gas chromatographic analysis showed the following fatty acid composition: 30 % palmitic acid, 1 % palmitoleic acid, 14 % stearic acid, 26 % oleic acid, 12 % linoleic acid, 13 % linolenic acid and 4 % arachidonic acid. Both phospholipids were pure when examined by thin-layer chromatography. Stock solutions of the phospholipids (1.6 mM) in *n*-hexane (Merck) were stored at −20° under nitrogen. All-*trans*-retinol and all-*trans*-retinaldehyde were obtained from Distillation Products (Rochester, N.Y., U.S.A.); for penetration experiments, 4.6 mM solutions in absolute ethanol were prepared fresh each week and stored in the dark at −20°.

Monolayer experiments were carried out in a Teflon trough of 20 cm × 5 cm × 1 cm. Surface tension was measured with a conventional torsion balance (V.D.F., United, 250 mg range, Nijmegen, The Netherlands) and as a Wilhelmy plate a microscopic coverslip (18 mm × 18 mm × 0.15 mm) attached to a platinum–iridium wire was used.

The pH of the subphase was varied by means of citric acid–phosphate buffer (0.12 M; pH 3.0), phosphate buffer (0.07 M; pH 5.3, 6.3 and 7.4) or borax–HCl buffer (0.06 M; pH 9.0). Twice-distilled water from an all-glass still was used throughout. Before each experiment the water surface was cleaned by moving the two teflon barriers towards each other, removing any surface film by suction and subsequently returning the barriers to their initial position. This procedure was repeated until the surface tension of the cleaned surface reached a value of 71–72 dyne/cm.

Force–area curves[6] of the phospholipids were measured as follows: with a micropipette, 5 μl of a 1.6 mM phospholipid solution were applied onto the subphase surface. After evaporation of the solvent the barriers were moved along the length of the trough in steps of 1 cm and the film pressure (surface tension of pure subphase *minus* surface tension of subphase with monolayer) was measured as a function of the area left for the phospholipid.

Penetration of retinaldehyde or retinol was studied by bringing with a microsyringe (Hamilton nr. 701 N) predetermined volumes of their ethanolic solutions beneath a phospholipid monolayer of preadjusted film pressure and by measuring the

resulting increase in film pressure. The addition was repeated once. The quantity of penetrating substance was adjusted according to the surface left between the barriers (normally between 25 and 50 cm^2). Per cm^2 0.05 μl of a 4.6 mM solution of retinaldehyde or retinol in absolute ethanol was applied each time. Application of solvent alone had no effect on the film pressure. Since the results of penetration experiments in dim red light were the same as those obtained under normal laboratory illumination (fluorescent light, 100 lux), all further experiments were carried out in the light.

The amount of retinaldehyde remaining in the aqueous subphase was determined by extracting 20 ml of the subphase with freshly distilled chloroform, evaporating the solvent and adding to the residue 500 μl propanol. In 300 μl of this solution retinaldehyde was determined by the thiobarbituric acid reaction using the method of FUTTERMAN AND SASLAW[7] at a tenfold reduction in scale. The extinction at 530 nm was corrected by the reading from an equal volume of subphase not penetrated by retinaldehyde. The measured amount was referred to the total subphase volume and was expressed as a fraction of the total amount of retinaldehyde used for penetration.

Absorption spectra of the monolayer materials were obtained in two ways. In the first procedure, the surface was compressed into the smallest possible area, removed with a 50-μl micropipette and mixed in a microcuvette with an equal volume of aqueous, neutralized 1 % digitonin solution. Digitonin was employed as a suitable non-ionic detergent. In an alternative procedure the absorption spectrum was obtained from a set of 25 parallel microscopic slides (held at 3-mm intervals in a slotted Teflon block) which had been dipped into the trough[8]. With both methods difference spectra of monolayers, either penetrated or non-penetrated by retinaldehyde, were obtained by means of a Zeiss PMQ II spectrophotometer. The coating technique was applied, because it would appear to offer less opportunity of Schiff base formation outside the monolayer subsequently to sampling in the procedure, whereby monolayer material is suspended in digitonin solution. With both techniques similar spectra of retinaldehyde were recorded, indicating that the results obtained are comparable.

RESULTS

The residual penetration of all-*trans*-retinaldehyde at high initial film pressure ($\pi = 40$ dyne/cm) in a phosphatidylethanolamine monolayer reported by BONTING AND BANGHAM[2] was confirmed. In six determinations the increase of film pressure ($\Delta\pi$) was 3.77 dyne/cm (S.E. 0.42), while for penetration of all-*trans*-retinaldehyde in a phosphatidylcholine monolayer $\Delta\pi$ was not significantly above zero ($\Delta\pi = 0.15$ dyne/cm, S.E. 0.15, 6 determinations).

Extending the measurements of the penetration of all-*trans*-retinaldehyde into monolayers of phosphatidylethanolamine and of phosphatidylcholine over the range $\pi = 15$ to 40 dyne/cm gave the curves shown in Fig. 1. The increase of film pressure over the entire range was higher for phosphatidylethanolamine monolayers than for phosphatidylcholine monolayers. Analysis of retinaldehyde present in the aqueous subphase showed that in no case more than 6 % of the retinaldehyde remained in the subphase.

When these two types of monolayers were penetrated by the same amounts of all-*trans*-retinol there was virtually no difference between the two phospholipids

(Fig. 2). Comparison of Figs. 1 and 2 shows moreover that the shape of the penetration curves for retinaldehyde and retinol were quite different.

It seemed desirable to study the effect of changing the subphase pH upon penetration, since formation of a Schiff base depends on pH. Alkaline pH favors

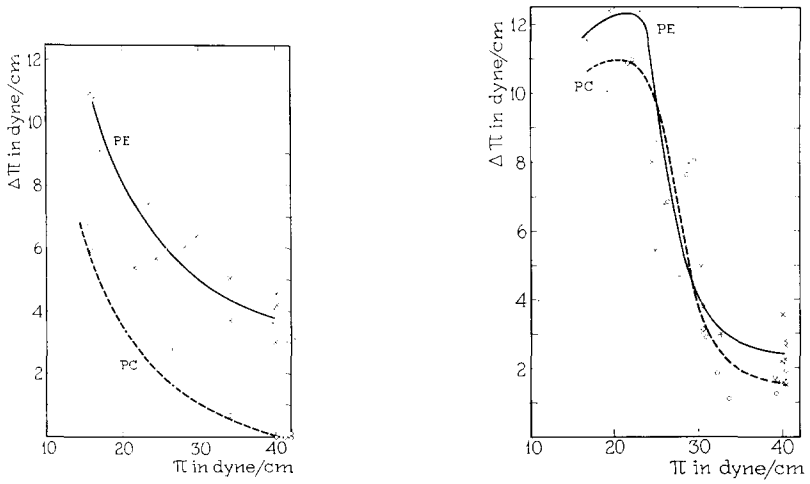

Fig. 1. Increase ($\Delta\pi$) of film pressure upon penetration of all-*trans*-retinaldehyde into monolayers of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) as a function of initial film pressure (π). The pH of the subphase was 7.4.

Fig. 2. Increase ($\Delta\pi$) of film pressure upon penetration of all-*trans*-retinol into phospholipid monolayers as a function of initial film pressure (π). The pH of the subphase was 7.4. For abbreviations see Fig. 1.

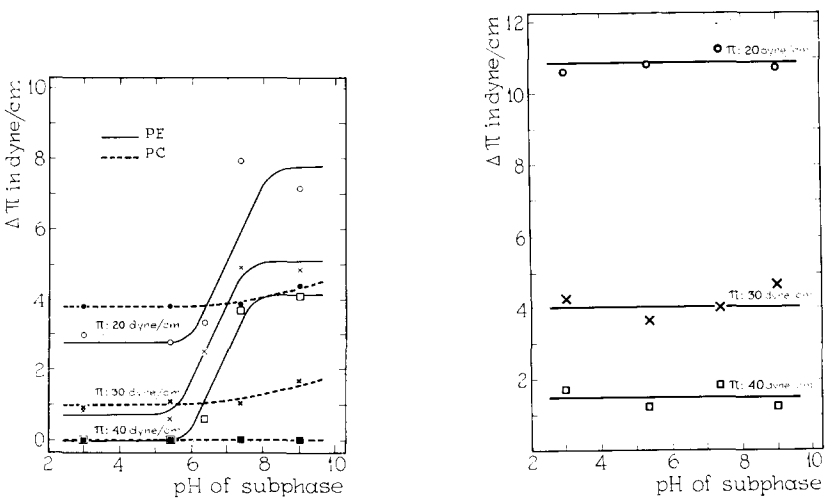

Fig. 3. Increase ($\Delta\pi$) of film pressure upon penetration of all-*trans*-retinaldehyde into phospholipid monolayers as a function of subphase pH at initial film pressure (π) of 20, 30 and 40 dyne/cm. For abbreviations see Fig. 1.

Fig. 4. Increase ($\Delta\pi$) of film pressure upon penetration of all-*trans*-retinol into phosphatidylethanolamine monolayers as a function of subphase pH at initial film pressure (π) of 20, 30 and 40 dyne/cm.

Schiff base formation, because this reaction requires a non-protonated aminogroup. Therefore the pH of the subphase was varied between 3.0 and 9.0. Fig. 3 indicates that only at pH > 7.5 the extra penetrating effect of retinaldehyde into monolayers of phosphatidylethanolamine occurs maximally. This applied for initial surface pressures of 20, 30 and 40 dyne/cm. For monolayers of phosphatidylcholine there was no dependence of penetration upon the pH of the subphase (Fig. 3). In this case $\Delta\pi$ was about the same as for the penetration of retinaldehyde in phosphatidylethanolamine monolayers at pH < 6. This effect is not due to a greater pH dependence of the phosphatidylethanolamine monolayer itself as compared to phosphatidylcholine. This could be concluded from the following observations: first, penetration of retinol into phosphatidylethanolamine monolayers was not pH dependent (Fig. 4), and secondly, force–area curves of this phospholipid coincided over a subphase pH range of 3.0–9.0.

It is known that Schiff base formation displaces the absorption maximum of retinaldehyde to shorter wavelengths. Therefore, difference spectra of phosphatidylethanolamine, penetrated with retinaldehyde, as compared to the corresponding non-penetrated material, were obtained from suspensions of monolayer material in an equal volume of 1 % aqueous, neutralized digitonin (Fig. 5). After penetration on a subphase of pH = 5.3 a difference spectrum was obtained with a maximum between 385 and 390 nm which coincides with the maximum for free retinaldehyde in aqueous digitonin solution. Similar difference spectra, after penetration on a subphase of pH = 7.4, gave an absorption maximum between 370 and 375 nm. After penetration of retinaldehyde into phosphatidylcholine films the difference spectrum, independent of the subphase pH, again coincided with the spectrum for free retinaldehyde. A similar shift in absorption maximum after penetration of retinaldehyde into a

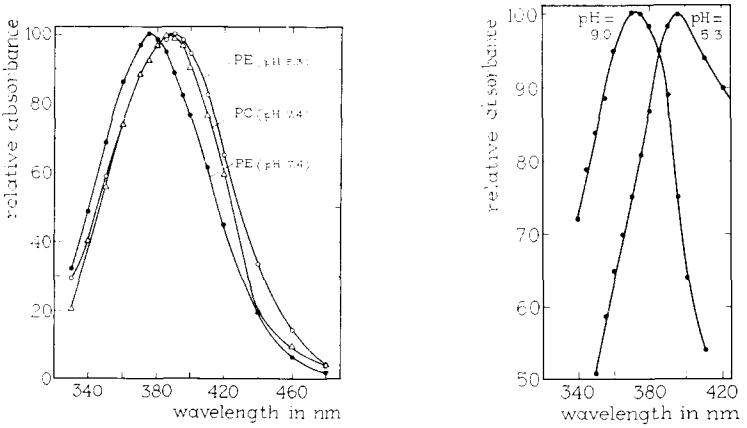

Fig. 5. Absorption spectra of phospholipid monolayer material (dissolved in equal volume of 1 % digitonin) after penetration of all-*trans*-retinaldehyde on subphases of pH 5.3 and 7.4. The spectra shown are difference spectra of penetrated and non-penetrated material. Absorbance at the absorption maxima is arbitrarily set at 100, absorbance at 500 nm at 0. For abbreviations see Fig. 1.

Fig. 6. Absorption spectra of phosphatidylethanolamine monolayer material (coated on parallel glass slides) after penetration of all-*trans*-retinaldehyde on subphases of pH 5.3 and 9.0. Difference spectra, obtained as described in Fig. 5.

phosphatidylethanolamine monolayer at a subphase pH of 9.0 was obtained after coating the monolayer material on a set of parallel glass slides (Fig. 6).

DISCUSSION

Penetration of retinol into monolayers of phosphatidylcholine and phosphatidylethanolamine gives approximately equal increases ($\Delta\pi$) in film pressure (Fig. 2). Replacing retinol by retinaldehyde, *i.e.* changing an alcohol by an aldehyde group, gives a much higher value for $\Delta\pi$ upon penetration into a monolayer of phosphatidylethanolamine than upon penetration into a phosphatidylcholine film (Fig. 1). An interaction between the two polar end groups, *viz.* the aldehyde group of retinaldehyde and the amino group of phosphatidylethanolamine, could explain this phenomenon. The most likely type of interaction would be the formation of a Schiff base between the aldehyde group of retinaldehyde and the amino group of phosphatidylethanolamine. The quarternary ammonium group of phosphatidylcholine, which replaces the amino group of phosphatidylethanolamine, cannot form a Schiff base.

Spectroscopic evidence for the formation of a Schiff base upon penetration of retinaldehyde in phosphatidylethanolamine film was obtained as follows. From the work of PITT *et al.*[9] it is known that a retinylidene Schiff base has, depending upon the medium used, an absorption maximum at 360–365 nm, about 25 nm below the absorption maximum of free retinaldehyde (385–390 nm). Measurement of the absorption spectra of monolayers, penetrated with retinaldehyde at a subphase pH of 7.4 and 9.0, showed a similar shift in the case of phosphatidylethanolamine, but not of phosphatidylcholine. This result was obtained whether the monolayer material was present in 0.5 % digitonin in a cuvette (Fig. 5), or as a coat on a set of parallel glass slides (Fig. 6). Acidification of the suspension in digitonin returned the absorption maximum to 390 nm in the case of phosphatidylethanolamine. This is due to the instability of the retinylidene Schiff base at acid pH (refs. 9–10).

Further evidence in favor of Schiff base formation follows from Fig. 3, where $\Delta\pi$ for the penetration of retinaldehyde into a phosphatidylethanolamine monolayer is plotted as a function of the pH of the subphase. This figure shows that the film pressure began to increase at subphase pH > 6, reaching a maximal value at subphase pH > 7.5. In accordance with this observation no spectral shift was noticed after penetration of retinaldehyde into phosphatidylethanolamine monolayers at a subphase of pH 5.3 (Figs. 5 and 6). Neither of these pH effects occurred with phosphatidylcholine monolayers (Fig. 3). MORTON AND PITT[10] showed that a retinylidene Schiff base can only be formed with a non-protonated amino group, which requires a pH > 6. Therefore, we must conclude that Schiff base formation cannot occur in a monolayer on a subphase with pH < 6, which explains the absence of the extra increase in film pressure at subphase pH < 6 and the absence of a shift in the absorption maximum at pH 5.3.

At first sight the apparent pK of about 7, indicated by Fig. 3 for the Schiff base formation, might seem in contradiction to the apparent pK of 9 (ref. 11) for the protonation of the amino group of phosphatidylethanolamine. The explanation for this apparent discrepancy must be that the removal of non-protonated phospholipid by retinaldehyde shifts the equilibrium between the protonated and non-protonated forms of the phospholipid to the latter form.

An alternative explanation for the effect of subphase pH upon retinaldehyde penetration in a phosphatidylethanolamine monolayer could be a change in packing of the phospholipid molecules due to shifting the subphase pH. However, this explanation is quite unlikely, since neither the force–area curves for phosphatidylethanolamine films, nor the penetration of retinol into these films (Fig. 4) were affected by the subphase pH. Moreover, in the absence of Schiff base formation deprotonation of the phospholipid amino group and a related change in packing would occur at a pH of about 9, rather than at pH 7. On the basis of these observations we may conclude that upon penetration of retinaldehyde into phosphatidylethanolamine monolayers at neutral pH a retinylidene Schiff base is formed, and that this reaction can explain the residual penetration of retinaldehyde in these monolayers at high film pressures.

Can this phenomenon also be invoked to explain the effect of light upon rhodopsin monolayers[2], and, more importantly, can it occur *in vivo* in the rod-sac membranes? In the bovine retinal rod outer segment there are approx. 1000 rod sacs regularly stacked. The membranes of these rod sacs consist of molecular complexes of phospholipids (30% of dry weight of rod outer segments) closely associated with the visual pigment rhodopsin[3]. These phospholipids consist for 52% of the amino group-containing phospholipids phosphatidylethanolamine and phosphatidylserine (R. M. BROEKHUYSE, F. J. M. DAEMEN AND S. L. BONTING, unpublished results). Calculation* shows that per retinaldehyde residue approx. 68 phospholipid amino groups are present in the rod-sac membrane. The molecular complex of phospholipid and rhodopsin, prepared from bovine rod outer segments, spreads easily in a monomolecular layer, and then behaves, even after extraction by hexane, like a phospholipid monolayer, *e.g.*, in showing the same collapse pressure of approx. 46 dyne/cm and in the absence of a hysteresis effect upon compression and decompression as the latter. Therefore, the increase in film pressure upon illumination of a rhodopsin monolayer could be explained by assuming that the retinaldehyde released during photolysis of rhodopsin would penetrate between the phospholipid molecules and form a Schiff base with their amino groups.

Two alternative explanations of the light effect in rhodopsin monolayers, namely *cis–trans* isomerization and protein conformational change, appear to be less likely. *Cis–trans* isomerization in films of free retinaldehyde leads to a decrease in film pressure, rather than an increase[14]. Moreover, considering the small size of the retinaldehyde group relative to the opsin moiety the mere *cis–trans* isomerization would be expected to have little, if any, effect on the packing of the rhodopsin molecules in the film. Protein conformational changes during photolysis of rhodopsin, as observed by CRESCITELLI, MOMMAERTS AND SHAW[15] and by KITO AND TAKEZAKI[16], would also be expected to have little effect on the film pressure of a monolayer with predominant phospholipid character unless the arrangement of the phospholipids in the phospholipid–rhodopsin complex would thereby be considerably altered.

* Assuming an average phospholipid molecular weight of 750, and a total phospholipid content of 30% of dry weight, of which 52% consists of the amino group-containing phospholipids, there would be 205 μmoles phospholipid amino groups per g dry weight of rod outer segments. Determination of the retinaldehyde contents of this preparation by means of the thiobarbituric acid method[7] and by decrease of extinction after bleaching (assuming a molecular extinction coefficient of 40600 (ref. 12) and a molecular weight of 40000 (ref. 13) for rhodopsin) gave values of 2.7 and 3.2, averaged 3.0 μmoles retinaldehyde per g dry weight. Hence, per retinaldehyde residue approx. 68 phospholipid amino groups are present in the rod-sac membrane.

Since formation of a Schiff base between retinaldehyde and a phospholipid amino group has been shown to occur in a phosphatidylethanolamine monolayer at room temperature and neutral pH, it seems reasonable to assume that the same reaction can occur *in vivo* in a rod-sac membrane upon photolysis of rhodopsin. However, if this reaction is to be involved in visual stimulation, as proposed by BONTING AND BANGHAM[2], then it must occur in a time span of about 1 msec. The monolayer techniques employed in the present study cannot record such fast changes in surface tension, hence are not able to confirm or deny this point. All that can be said at the moment is that this Schiff base would have to be formed at the metarhodopsin II stage of the photolytic process, and that the observed range for the absorption maximum ($\lambda = 370$–375 nm) approaches the maximum commonly reported for metarhodopsin II ($\lambda = 380$ nm). Blocking of the amino group would make the surface charge of the rod-sac membrane locally more negative, increasing the passive permeability for sodium and potassium, as demonstrated in model experiments by BONTING AND BANGHAM[2]. The resulting cation exchange in this view would cause a depolarizing current, leading to stimulation of the synapse with the bipolar cell.

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