Retinylidene Schiff Bases in Phosphatidylcholine Reverse Micelles: Formation, Protonation and Stability

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All-*trans*-*N*-retinylidene-n-butylamine **3** has been formed in inverted micelles of phosphatidylcholine (PC)–hexane containing varying amounts of water ($[H_2O]/[PC] = 0–3$) and the formation, protonation and stability have been studied. The micelles have been found to catalyse the Schiff-base formation. The Schiff-base was found to be stable in the presence of structured water molecules bonded to the polar head groups of the micelles. A larger water-pool causes the decomposition of the Schiff-base. Schiff-base **3** intercalated in the inverted micelle was found to undergo protonation in the presence of 3-chloropropionic acid, the extent of which depended on the water-pool size. The results are discussed in terms of the formation, protonation and stability of retinylidene Schiff-base chromophores in rhodopsins.

The retinylidene chromophore has attracted much attention due to its unique photobiological properties in visual and halobacterial rhodopsins.[1,2] Further, the discovery of the presence of retinal in ciliate algae, such as *Chlamydomonas*[3] and of amoeba *Paramoecium*[4] has caused new interest in these photoactive chromophores. Retinylidene Schiff-bases have also recently been reported to exhibit non-linear optical properties.[5] One feature common to rhodopsins, and perhaps even to retinal proteins of other organisms, is the existence of a protonated retinal lysine Schiff-base chromophore (Fig. 1) in the protein. It is also believed that there are a few bonded water molecules at the reaction centre of rhodopsins. The three-dimensional structure of bacteriorhodopsin, for example, has revealed the presence of one or two structural water molecules[6] in the otherwise very hydrophobic channel above the Schiff-base chromophore. It is not clear however, how the Schiff-base is formed in the retinal pocket, how the protonated nitrogen is stabilized, or how the weak acidic residues of the protein are able to fully protonate the Schiff-base chromophore. The hydrophilicity of the retinal pocket is presumably very carefully controlled by the protein so that the labile Schiff-base chromophore can be optimally functional. To study these molecular details of the reaction centre of rhodopsins, an ideal model system could be a micellar system containing a retinylidene Schiff-base. Surfactant-solubilized water pools mimic the hydrophilic pockets of enzymes, whilst the alkyl chains of surfacant provide a hydrophobic domain.[7] Additionally, the reverse micelle formation and stabilization therein are dynamic processes; this behaviour is analogous to that of membrane bound proteins. Recently, it has been shown that the protonation of retinylidene Schiff-bases can be effected in surfactant solubilized water pools in non-polar solvents.[8,9]

Here, we report the intercalation of all-*trans*-*N*-retinylidene-n-butylamine **3** in the reverse micellar system of phosphatidylcholine (PC) in hexane. PC is similar to the natural system and also usually forms small water-pools. Formation of **3** by reacting retinal **1** and n-butylamine **2** in the micellar matrix has also been attempted. Furthermore, protonation of **3** by various acids in PC micelles has been studied.

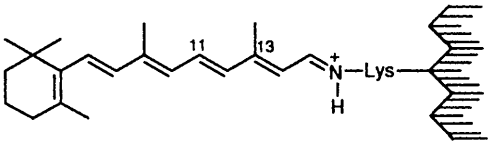
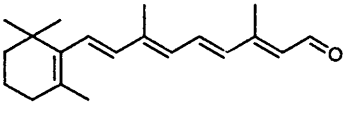


Fig. 1 The Schiff-base chromophore



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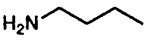

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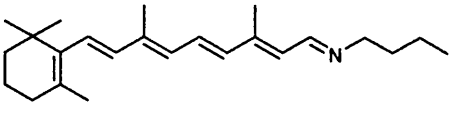

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Experimental

All-*trans*-retinal and phosphatidylcholine (PC) [Soyabean, type II-S] were obtained from Sigma. Hexane was from Spectrochem. Trifluoroacetic acid (TFA), trichloroacetic acid (TCA), chloroacetic acid (CAA), 3-chloropropionic acid (CPA) and propionic acid (PA) were from Aldrich. Double-distilled and deionized water was used for the micellar preparation. n-Butylamine (Aldrich) was distilled under nitrogen and stored over activated molecular sieves (4 Å). The PC was evacuated (10^{-4} Torr †) for 3–4 h before use and stored under nitrogen. The remaining chemicals were used as received. All procedures involving retinal and related compounds were performed under dim red light and under a nitrogen atmosphere. UV–VIS measurements were carried using a Shimadzu-260 UV–VIS spectrophotometer. All-*trans*-*N*-retinylidene-n-butylamine **3** was prepared as described previously,[9] it was stored in hexane solution at 4 °C in the dark under nitrogen.

Preparation of PC Micelles and Intercalation of **3** *in the Micellar Matrix.*—The inverted micelles were prepared by shaking PC in hexane with the required amount of water. The solvent and the aqueous solutions were deoxygenated and all

† 1 Torr = 133.3 Pa.

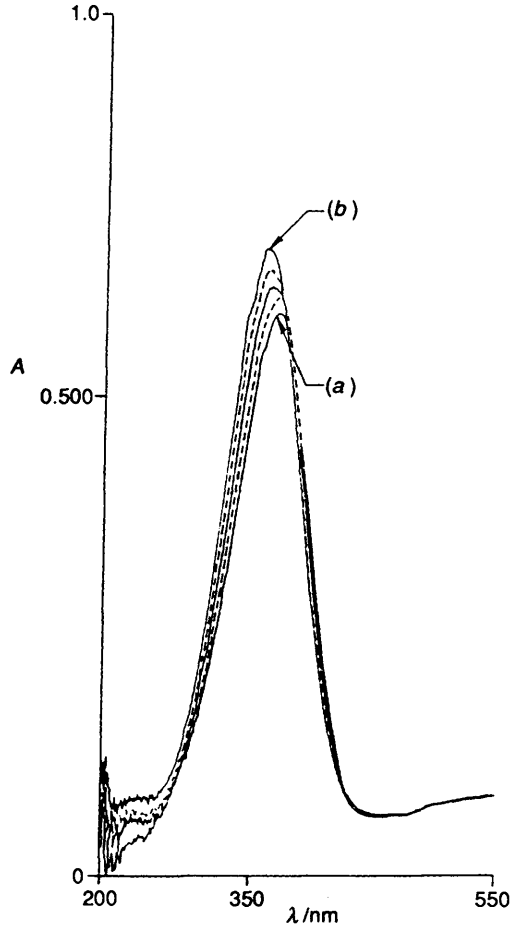

Fig. 2 Changes in the UV–VIS absorption spectrum of all-*trans*-retinal in the presence of n-butylamine in reverse micelles of PC–hexane (10^{-2} mol dm^{-3}): (*a*) all-*trans*-retinal, λ_{max} = 363 nm; (*b*) all-*trans*-*N*-retinylidene-n-butylamine Schiff-base, λ_{max} = 357 nm

the preparations were carried out under nitrogen, in order to exclude O_2 from the samples. Thus, PC was dried *in vacuo* (10^{-4} Torr) and then stored under nitrogen. Dry hexane was then added (by syringe) to obtain a 10^{-2} mol dm^{-3} PC suspension. Schiff-base **3** was added to obtain a final concentration of 1.4×10^{-5} mol dm^{-3}. The suspension was made clear by shaking, and the solution was stored under nitrogen in the dark at 4 °C.

Formation of **3** *in PC–Hexane Reverse Micelles.*—Equimolar amounts (1.7×10^{-5} mol dm^{-3}) of all-*trans*-retinal **1** and n-butylamine **2** were sonicated in the reverse micelles of PC (10^{-2} mol dm^{-3}) in hexane. The solution was kept in the dark at room temperature under nitrogen. The formation of **3** was followed by observing the growth of a new peak at 357 nm at different time intervals. In another experiment the formation of **3** was monitored by adding TFA to the micellar solution and observing the formation of protonated Schiff-base at different time intervals. A control experiment was carried out by placing the reactants in hexane alone. The rate of formation of **3** was calculated by plotting[10] $-\log(A_\infty - A_t)$ *vs.* t; where A_∞ is the absorbance at 357 nm at the end of the reaction and A_t is the absorbance at 357 nm at time t.

Protonation of **3** *with Different Acids.*—Appropriate amounts of different acid solutions in hexane were added to the micellar solution of **3** (1.4×10^{-5} mol dm^{-3}). The resultant solution turned orange-yellow (depending upon the degree of protonation) and gave the protonated Schiff-base. The addition of acid was continued up to a ratio of [**3**]:[Acid] = 1:100.

Protonation of **3** *by CPA in PC–Hexane Reverse Micelles Containing Different Amounts of Water.*—To prepare micelles with varying w values ($w = [H_2O]/[PC]$) between 0–3, appropriate amounts of water were added to the reverse micelle solution. The protonation of **3** was carried out using CPA. The effect of acid concentration on protonation was studied by increasing the acid concentration at a fixed w value.

Stability of **3** *in PC–Hexane Reverse Micelles.*—Schiff-base **3** (2 cm^3, 1.4×10^{-5} mol dm^{-3}) in PC–hexane (w = 0–3) was placed in quartz cuvettes. These were septum sealed under a stream of nitrogen and left in the dark at 25 °C. The stability of the Schiff-base was checked by recording the UV–VIS spectra at varying time intervals.

Results and Discussion

Intercalation of **3** *in PC–Hexane Reverse Micelles.*—Upon incorporation in the PC–hexane system, synthetic **3** showed a major absorption band at 357 nm due to the *N*-retinylidene chromophore. A minor band (<2%) at 440 nm (due to the protonated form of **3**) was also observed when **3** (1.4×10^{-5} mol dm^{-3}) was shaken and stirred very vigorously in PC–hexane (10^{-2} mol dm^{-3}) containing 0.03 mol dm^{-3} (w = 3) water. However, this band was not seen in a micellar system with w < 2. When **3** was incorporated into a synthetic dipalmitoyl PC (DPPC) [DPPC–hexane (w = 3)] system, only the 357 nm band due to **3** was observed. There was no band at 440 nm corresponding to the protonated form of **3**. The protonation of **3** in the soya phospholipid may be due either to some acidic impurities in the commercial PC or to phosphoric acid residues of the lipid. As micellar structures are dynamic systems a few phospholipid molecules may become protonated, thus rendering the soya PC slightly acidic. Such minor acidic properties of PC were seen even after purifying the lipid on a chromatographic column. Schiff-base **3** in PC–hexane (w = 0–2) was found to be stable in the dark at ambient temperature. However, **3** incorporated in PC–hexane (w = 3) showed gradual decomposition and the 357 nm band due to **3** had completely disappeared after *ca.* 80 h at 25 °C in the dark. The expected retinal band, however, was not observed—suggesting destruction of the polyenic chromophore.

In-situ *Formation of* **3** *in PC–Hexane Reverse Micelles.*—All-*trans*-Retinal **1** reacted with n-butylamine **2** to give **3** in a PC reverse micellar matrix, as evidenced by UV–VIS absorption spectral data (Fig. 2). PC–hexane system containing retinal exhibited the UV–VIS band at 363 nm; the corresponding Schiff base **3** in the same micellar system showed a rather blue shifted spectrum with an absorption maximum at 357 nm. In a PC reverse micelle **3** was again found to show protonation, but it was never more than 0.5% protonated. With equimolar amounts of retinal **1** and n-butylamine **2**, the Schiff-base formation in the micellar medium was *ca.* 16–17 times faster compared to its formation in pure hexane. However, the UV–VIS absorption behaviour of **3** in hexane and in PC–hexane (w = 0 or 3) was similar (Fig. 3).

Effect of Water.—The rate of formation of **3** PC–hexane containing varying amounts of water was determined. The reaction was made pseudo first order with respect to amine concentration and values of k_{obs} and k_0 were obtained at w values 0–3, by plotting $-\log(A_\infty - A_t)$ *vs.* t (Table 1), where A_∞ is the absorbance of Schiff-base at the end of the reaction and A_t is the absorbance value at time t. The concentration (retinal = 5.4×10^{-6} mol dm^{-3}, n-butylamine = 5.4×10^{-5} mol dm^{-3}) of solutes in the micelle (10^{-2} mol dm^{-3}) were kept low in order to avoid structural distortions in the micelles.

Table 1 Rates of formation of all-*trans*-*N*-retinylidene-n-butylamine in PC reverse micelles (10^{-2} mol dm^{-3}) with varying water-pool size

Water content in reverse micelles/mol dm^{-3}	$k_{obs}/10^{-3}$ s^{-1}	$k_0/10^{-2}$ dm^3 mol^{-1} s^{-1}
0	5.43	0.97
0.01	5.63	1.03
0.02	6.00	1.06
0.03	8.66	1.11

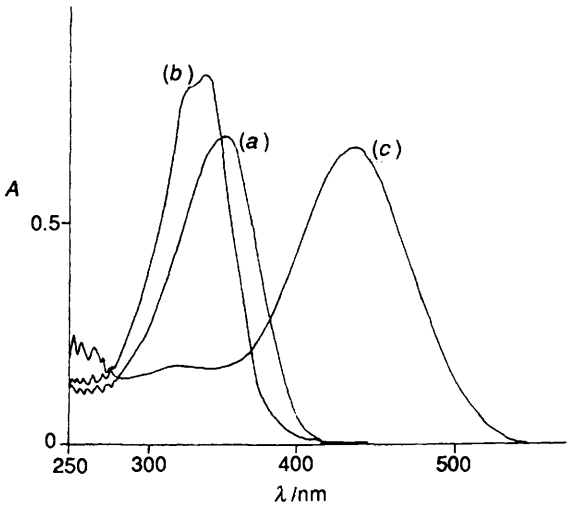

Fig. 3 UV–VIS spectra (2×10^{-5} mol dm^{-3}): (*a*) all-*trans*-retinal; (*b*) all-*trans*-*N*-retinylidene-n-butylamine; (*c*) all-*trans*-*N*-retinyldene-n-butylammonium in PC–hexane (10^{-2} mol dm^{-3}) reverse micelles

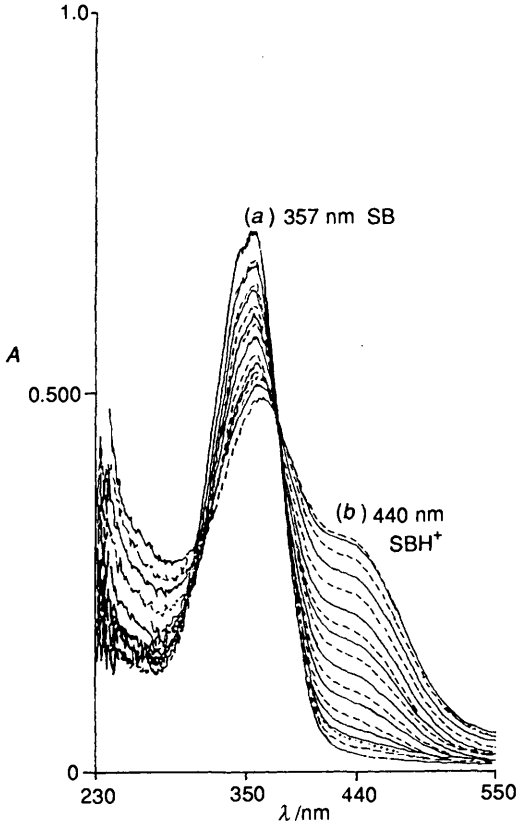

Fig. 4 UV–VIS absorption changes during protonation of all-*trans*-*N*-retinylidene-n-butylamine in PC–hexane reverse micelles by CPA

Table 2 Extent of protonation of all-*trans*-*N*-retinylidene-n-butylamine in PC reverse micelles (10^{-2} mol dm^{-3}) with different acids

Acid	pK_a (H_2O)	Protonation (%)
TFA	0.30	65
TCA	0.89	50
CAA	2.85	36.56
CPA	4.06	29.5
PA	4.87	19.8

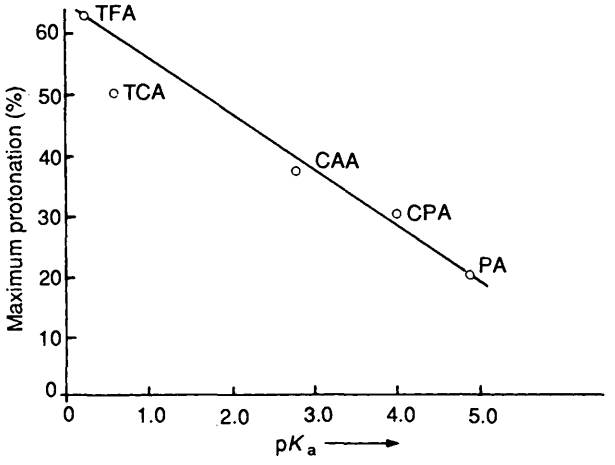

Fig. 5 Maximum protonation (%) of **3** in PC–hexane ($w = 0$) reverse micelles *vs.* pK_a of acids

It is evident that the water-pool size does not influence the Schiff-base formation significantly. The faster formation of **3** in a micellar matrix is therefore mainly due to microphysical effects of micellar structure–solute compartmentalization, organization and orientation. In addition the Schiff-base can be pulled into the hydrophobic core and the water formed during Schiff-base formation is taken up in the water-pool. Thus, inverted micelles of PC act as *in situ* dehydrating agents.

Effect of Acid.—All-*trans*-*N*-retinylidene-n-butylamine (**3**) incorporatated in PC–hexane ($w = 0$) reacted with different acids such as TFA ($pK_a = 0.3$ in H_2O), TCA ($pK_a = 0.69$ in H_2O), CAA ($pK_a = 2.85$ in H_2O), CPA ($pK_a = 4.06$ in H_2O) and PA ($pK_a = 4.87$ in H_2O) to give the protonated form of **3**. Fig. 4 shows changes in the UV–VIS absorption of the micellar Schiff-base upon addition of CPA. The protonated Schiff-base in inverted micelles showed an UV–VIS absorption maximum at 440 nm. The percentage of protonation was calculated from the absorption decreases at 357 nm, taking $\varepsilon = 4.96 \times 10^4$ dm^3 cm^{-1} mol^{-1} for **3** and assuming quantitative protonation of **3** (Table 2). As expected, stronger acids were able to protonate **3** to the maximum extent. The extent of protonation depended linearly on the pK_a values of the acids (Fig. 5). Addition of these acids caused no unusual change in the micellar solution of **3** or its protonated form, except for the change in the colour (yellow–orange), the intensity of which increased with the amount of acid added.

Furthermore, the protonation in PC–hexane containing different amounts of water ($w = 0$–3) was studied. The protonation was effected with CPA since it has a pK_a value (4.06 in H_2O) similar to those of natural acidic residues (glutamic acid $pK_a = 4.85$ and aspartic acid $pK_a = 3.56$ in H_2O) in proteins (Table 3). It is observed that an increased amount of co-solubilized water causes an increase in the extent of protonation. Thus, relatively low concentrations of CPA (equimolar to **3**) caused a *ca.* 25-fold increase in the protonated form in the presence of 0.03 mol dm^{-3} water. It was noted that

Table 3 Extent of protonation of all-*trans*-*N*-retinylidene-n-butylamine with equimolar amounts of CPA (1.4×10^{-6} mol dm^{-3}) in PC–hexane reverse micelles[a] containing varying amounts of water

Water content in reverse micelle[a]/mol dm^{-3}	Protonation (%)
0.0	0.6
0.01	7.2
0.02	10.8
0.03	15.3

[a] PC–hexane system, 10^{-2} mol dm^{-3}.

Table 4 Stability of all-*trans*-*N*-retinylidene-n-butylamine Schiff-base (9×10^{-6} mol dm^{-3}) in PC–hexane (10^{-2} mol dm^{-3}, $w = 3$)

t/h	A[a]	Decomposition (%)
0	0.370	0
20	0.364	3
40	0.344	13
60	0.320	17
80	0	100

[a] $\lambda = 357$ nm.

the extent of protonation of **3** in inverted micelles of PC–hexane is much lower than the extent of protonation observed for **3** when incorporated in sodium bis(2-ethylhexyl)sulphosuccinate (AOT) micelles.[8] Inverted micelles of used PC took up water to a maximum value of $w = 5$. On the other hand, AOT acts almost like a sponge and can take up large amounts of water (up to $w \approx 50$). Thus, it is clear that the availability of protons, rather than the acid strength, is of overriding importance in Schiff-base protonation. Proton availability depends on the extent of acid dissociation. This, in turn, is favoured by the availability of water in higher concentrations in the inverted micelles. It is interesting to note that the shift in the absorption maximum of **3** in the PC micelle upon addition of TFA is similar to its shift in methanolic solution. Thus, when TFA is added to a solution of **3** either in methanol or in PC micelles, the resulting protonated Schiff-base shows an absorption band at 440 nm. The spectral shift of the protonated Schiff-bases derived from long conjugated polyenes are the consequence of a comparatively small delocalization of their positive charge in the ground-state compared to the excited state. Furthermore, the absorption shift is controlled by the distance between the centres of cationic and anionic charges.[11,12] Similar absorption shifts in PC micelles and methanol indicates that the counter-ion is CF_3COO^- and that the Schiff-base is predominantly located in the hydrocarbon domain of the reverse micelle.

Role of Water.—It is well established that the water inside the micelle is distributed in hydration layers around the phospholipid polar heads and at least two types of water have been observed: one associated with the polar groups, while the other is relatively free.[13,14] In appropriately dried PC there will be no bulk water-pool. If there is any water left it will only be associated with the polar head groups. It has been reported[15] that egg lecithin has *ca.* 3–4 such water molecules. Apparently, the highly associated water molecules do not cause the added acids to dissociate significantly. The acid (*e.g.* CPA) added to the PC containing **3** only dissociates when there are free water molecules in the water-pool.

Schiff-base **3** incorporated in PC inverted micelles in the dark underwent gradual decomposition, the extent of which depended on the amount of co-solubilized water (Table 4). Thus, a solution of **3** [10^{-5} mol dm^{-3} in PC–hexane ($w = 0$)] showed no significant decrease at 357 nm during the first 60 h. On the other hand, **3** incorporated into PC–hexane ($w = 3$), showed a *ca.* 17% decrease in its 357 nm UV–VIS band during the same period, and this band totally disappeared in *ca.* 80–90 h. The expected band of retinal formed due to possible hydrolysis of **3** by the phosphoric residue of PC was observed, but its intensity was very weak. This indicated the destruction of the polyene chromophore in the inverted micelles of PC. Similar destruction of **3** was also seen when it was left in micellar solution for a long time (106 h) even in the absence of added water. In the case of PC inverted micelles, it has been found that the main effect of the incorporation of the solutes into the micelle is to alter the distributions and the organization of the water molecules.[16] The solutes may reduce the number of water molecules available per polar head, building up their own hydration shells; they may also break, or instead extend, the network of hydrogen bonds. All these effects probably co-exist, but at this stage it is not possible to state which is predominant.

Conclusion

It has been found that the Schiff-base formation in an organized environment of phospholipids is feasible and the membrane structure of phospholipid helps in driving the reaction [eqn. (1)]

$$R^1CHO + R^2NH_2 \rightleftharpoons R^1CH{=}N{-}R^2 + H_2O \quad (1)$$

towards the right by removing water. The Schiff-base can be protonated to a significant extent with relatively weak acids in membrane-like structures. The extent of this protonation depends on the availability of protons rather than upon the acid strength. The retinylidene Schiff-base can remain stable in a hydrophilic environment, provided the water molecules are strongly bound. The behaviour of rhodopsins (particularly bacteriorhodopsin) is mimiced in terms of Schiff-base formation, protonation and stability in the PC–hexane–H_2O reverse micelle. However, extension of the results to real membrane and rhodopsin is not warranted at this time. Further studies to determine the locations of the retinylidene chromophore in inverted micelles, and important factors influencing micellar parameters such as acid–base properties of the water-pool and the effect of solutes on the micellar structures are required.

Acknowledgements

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