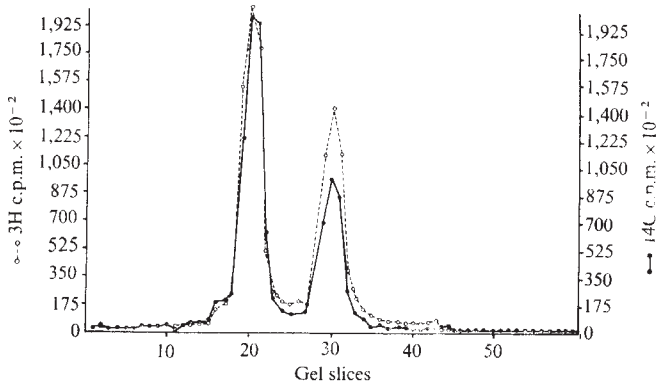


**Fig. 4** Formamide gels were prepared according to the method of Staynov *et al.*[14]. All other details of the electrophoretic procedures are the same as described in Fig. 1 except the E buffer used in the electrophoresis chambers was adjusted to *p*H 9.0 rather than 7.8. *B. subtilis*, – – – –; *E. coli*, ——.

sequence. Therefore, rRNAs with a higher GC content presumably retain more stable double-stranded loops, pack tighter, and behave as smaller (lighter) particles during electrophoresis or sedimentation.

We believe that the electrophoretic differences we observed in the 1/3E buffer may be due to small differences in the GC content of the rRNAs of these particular bacterial species. Determinations of their respective GC contents are in progress.

Owing to the extended length of the labelling period, we do not believe any of the species of rRNA described are precursor RNAs. Morris and Schuap have separately reported multiple conformational forms of both 23S and 16S rRNAs from *E. coli*[8,9]. These forms were only seen, however, when special conditions such as a *p*H gradient[8] or electrophoresis for 7.5 h at 0 °C (ref. 9) were used. We have not seen any multiple peaks in our electrophoresis conditions.

To test our assumption that the differences we observe in non-denaturing gels were due primarily to conformational difference, we ran the rRNA samples on formamide gels. Formamide essentially destroys all secondary bonding and reduces migration to a dependency on the primary polynucleotide sequence[10]. In all cases we found coelectrophoresis of the different bacterial rRNAs. A single example is shown in Fig. 4. Thus, no molecular weight differences were seen between the respective 23S and 16S rRNA of the different bacterial species studied.

Using a combination of lowered ionic strength in our gels and double labelling of rRNAs we were able to show major migrational differences in the electrophoretic mobility of non-denatured rRNA from various bacterial species. Extension of this work could lead to differential rRNA profiles of bacteria; which might be useful in the identification of non-cultivable pathogens, or to help establish phylogenetic relationships between different bacterial species.

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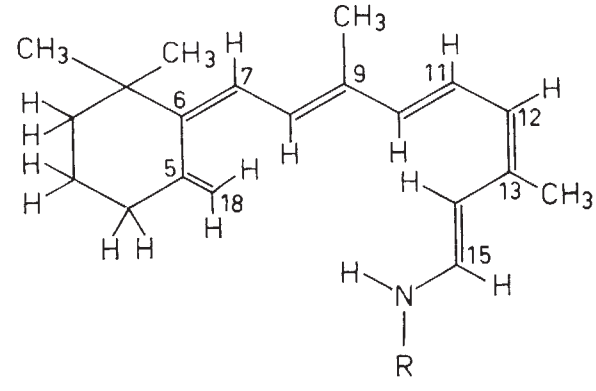
# Structure of the chromophoric group in bathorhodopsin

In the photolysis of the visual pigment rhodopsin the intermediate first observed is bathorhodopsin[1,2] (formerly called prelumirhodopsin). It is generally held that isomerisation of 11-*cis* to all-*trans* retinal occurs during this initial step. On theoretical grounds, however, an hexaene–amine structure (Fig. 1) has been proposed as an alternative for the chromophore of bathorodopsin (ref. 3 and unpublished results of K. Van der Meer, J. J. C. Mulder and J.L.). This chromophore has an exomethylene double bond between the atoms C5 and C18 and the nitrogen of its ene–amine moiety derives from the ε-amino group of a lysine residue of opsin. It is a retrotautomer of the chromophoric group in native rhodopsin which is a protonated Schiff base of 11-*cis* retinal. We now present two lines of evidence which support the structure depicted in Fig. 1: first, the interpretation of a recently published laser resonance Raman spectrum of bathorhodopsin[4], and second, experiments which establish hydrogen (deuterium) exchange during photolysis of rhodopsin.

The laser resonance Raman spectrum of bathorhodopsin differs from both rhodopsin and isorhodopsin by having additional peak at 1,539, 920, 877 and 856 $cm^{-1}$. The 1,539 $cm^{-1}$ absorption is consistent with both the N–H ene–amine structure derived from a primary amine and a protonated Schiff base structure. The 877 $cm^{-1}$ absorption corresponds with the wagging vibration of 1.1-dialkyl substituted ethene[5], that of 1.1-dimethylethene is found at 883 $cm^{-1}$ (ref. 6). Terpenes with an exomethylene bond (for example, sabinene, nopinene and camphene) show three strong peaks in the Raman spectrum around 920, 877 and 856 $cm^{-1}$ (refs 7 and 8). For comparison we recorded the laser Raman spectrum of vitamin $D_3$ (in $CCl_4$), which has a conjugated triene structure with an exomethylene group. The Raman spectrum (Cary 81, He–Ne laser) shows peaks 930, 894, 880 and 863 $cm^{-1}$. These data strongly suggest that bathorhodopsin, in contrast to native rhodopsin, contains an exomethylene double bond and that the chromophoric group in bathorhodopsin has the structure shown in Fig. 1.

If the retroretinal structure of Fig. 1 were an inter-

**Fig. 1** Structure of the chromophoric group in bathorhodopsin.

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mediate in the rhodopsin photolysis in the process of reconstituting the 18-$CH_3$ group in all-*trans* retinal, the ultimate product of rhodopsin photolysis *in vitro*, a hydrogen atom (proton) should be reattached to the 18-$CH_2$ group in one of the thermal reactions following the formation of bathorhodopsin. Then, hydrogen exchange between chromophore and apoprotein during photolysis might be detected by means of deuteration (or tritiation) of the latter and analysis of the chromophore. In spite of the negative outcome of an earlier attempt to detect tritium exchange during rhodopsin photolysis[3], various considerations led us to reinvestigate this approach in a slightly modified manner.

In a first attempt, rhodopsin, isolated as rod outer segments from 60 bovine eyes by a sucrose gradient technique[9], was washed repeatedly with $D_2O$ and lyophilised. After incubation in $D_2O$ in various conditions, the sample was irradiated with orange light for 30 min. The suspension was brought to 60% (v/v) ethanol and extracted with hexane. The retinal was isolated (thin-layer and high pressure liquid chromatography) in pure form. The samples were placed in an AEI MS-902 mass spectrometer through the direct, heatable introduction system. Spectra were taken, slowly heating the sample from 40 to 120 °C. From the retinal isolated, eight complete mass spectra, each at probe temperatures of 40, 60, 90, 100 and 120 °C, were obtained. At 100 and 120 °C, 20 spectra in the parent peak region were taken. The mass spectra thus obtained closely resemble those published for all-*trans* retinal[10]. In three independent experiments in which the incubation conditions with $D_2O$ are varied (0 °C, 14 h; 0 °C, 18 h; 20 °C, 18 h), however, no deuterium incorporation was found in the chromophore.

This result might be explained by the possibility that $D_2O$ has no access to the region of direct protein–chromophore interactions in rhodopsin. Chances for more complete deuteration of the apoprotein could be better in opsin, where the chromophore is absent. Therefore, opsin, isolated as rod outer segments in room light[9], was washed repeatedly with $D_2O$ and lyophilised. The preparation was resuspended in $D_2O$ and after incubation for various times a fivefold molar excess of 11-*cis* retinal was added. After 1.5 h rhodopsin regeneration, the preparation was centrifuged and the pellet was extracted three times with hexane to remove the excess of retinal. The residue containing regenerated rhodopsin was resuspended in $D_2O$. Samples of illuminated and unilluminated rhodopsin were extracted, purified and submitted to mass spectrometry as described above. The results are presented in Table 1.

In five independent experiments, deuterium incorporation to the extent of $6 \pm 1\%$ of one D atom was found in the all-*trans* retinal extracted from illuminated samples, as determined from the peaks of $m/e = 284$, 285 and 286, calculated according to Beynon and Williams[11]. In the control experiments, the chromophore extracted from unilluminated samples (largely 11-*cis* retinal as shown by thin-layer chromatography) did not show any deuterium incorporation. This demonstrates that deuterium is incorporated in the chromophore as a result of illumination.

The accepted mechanism for the photolysis of rhodopsin is based on a photochemical *cis-trans* isomerisation of the chromophoric group[12]. Since retinal does not show H-D exchange, in either thermal or photochemical conditions, our observation of a light-induced H-D exchange in the chromophore of rhodopsin is difficult to explain by such a model. We have proposed another mechanism, in which a hydrogen shift is introduced as the photochemical step, resulting in a hexaene-amine moiety as structure for early photointermediates of rhodopsin photolysis (unpublished results of K. Van der Meer, J. J. C. Mulder and J. L.). This model leaves two possibilities for the exchange of hydrogen between chromophore and apoprotein. The hydrogen donated to the apoprotein is either not that which is returned in reconstituting the chromophore or it is later reattached to the chromophore.

**Table 1** Percentage of deuterium incorporation in retinal extracted from rhodopsin obtained by regeneration from $D_2O$-incubated opsin and 11-*cis* retinal

| Opsin incubation with $D_2O$ (°C, h) | 11-*cis* Retinal from rhodopsin before illumination | all-*trans* Retinal from rhodopsin after illumination |
|---|---|---|
| 20, 1 | — | 6 |
| 20, 5 | — | 5 |
| 20, 14 | 0 | 5 |
| 20, 18 | 0 | 7 |
| 30, 14 | — | 7 |

In the first case, 100% monodeuteration could be expected if the hydrogen-donating site on the apoprotein is completely deuterated. But our observation that the percentage of deuterium exchange is low and independent of the time during which opsin is incubated with $D_2O$ (Table 1), suggests that such a hydrogen-donating site may not exist. In the second case, one would not necessarily observe H–D exchange; exchange might occur during the time the shuttling hydrogen spends on the protein. We suggest that the very short lifetimes of the photointermediates, bearing a hexaene–amine as chromophore, are responsible for the relatively low deuterium incorporation in retinal during photolysis.

In conclusion, the laser resonance Raman spectrum of bathorhodopsin and the occurrence of hydrogen exchange between chromophore and apoprotein during photolysis support a hydrogen shift as the primary event in the photolysis of rhodopsin.

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## Crystal structure of cholesterol monohydrate

Cholesterol, almost water insoluble, normally occurs in the body bound to lipoprotein, or incorporated in lipid aggregations such as bile micelles, or within certain biomembranes. When cholesterol levels are abnormally high, single crystals of the monohydrate tend to deposit; in bile, clumps of these crystals form gallstones[1]. Crystals of cholesterol monohydrate also

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