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A Convenient Synthesis of Labelled Rhodopsin and Studies on its Active Site

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Digitonin solutions of labelled rhodopsin, containing ^3H in the retinyl moiety, were prepared by two related methods. Labelled rhodopsin was also prepared for the first time in cetyltrimethylammonium bromide and purified by column chromatography. It was shown that only certain rhodopsin preparations on denaturation in the dark and the reduction with sodium borohydride gave up to 60% of the radioactivity in a fraction characterized as *N*-retinylphosphatidylethanolamine. Such preparations also gave a lipid-linked retinyl moiety at the metarhodopsin-I stage, but, as expected, a protein-linked retinyl moiety at the metarhodopsin-II stage. Other preparations however, gave exclusively protein-bound radioactivity at the native-rhodopsin, metarhodopsin-I and metarhodopsin-II stages. It is therefore conceivable that the formation of *N*-retinylphosphatidylethanolamine is due to a non-enzymic reaction resulting from the transfer of the retinyl moiety from its native site to an amino group of a favourably oriented phospholipid molecule. The only firmly established aspect of the rhodopsin active site remains the demonstration in our previous work that at the metarhodopsin-II stage the retinyl moiety is linked to an ϵ-amino group of lysine. On the basis of chemical reactivity it is argued that the light-induced conversion of rhodopsin into metarhodopsin II involves a profound conformational change resulting in the dislocation of the retinylideneiminium chromophore from a non-polar environment in rhodopsin to a polar environment in metarhodopsin II.

It is well known that the visual system of bovine retinae comprises a membrane-bound protein, opsin, and a polyene aldehyde, retinal (Mathews, Hubbard, Brown & Wald, 1963). In the dark, opsin combines with 11-*cis*-retinal to form the light-sensitive enzyme–substrate complex rhodopsin, which photolyses through a number of unstable intermediates to form opsin and all-*trans*-retinal as shown below.

Rhodopsin ($\lambda_{max.}$ 498nm) $\xrightarrow{h\nu}$ pre-lumirhodopsin ($\lambda_{max.}$ 543nm) $\rightarrow$ lumirhodopsin ($\lambda_{max.}$ 497nm) $\rightarrow$ metarhodopsin I ($\lambda_{max.}$ 478nm) $\rightarrow$ metarhodopsin II ($\lambda_{max.}$ 380nm) $\xrightarrow{H_2O}$ all-*trans*-retinal ($\lambda_{max.}$ 387nm) and opsin (Mathews *et al.* 1963).

The presence of a Schiff-base linkage (R–C=N–R′) between retinal and the opsin molecule was first proposed by Pitt, Collins, Morton & Stok (1955); support for this hypothesis was provided by Bownds & Wald (1965) and by Akhtar, Blosse & Dewhurst (1965). The authors showed that although rhodopsin itself is resistant to attack by sodium borohydride, one of the derivatives, metarhodopsin II, readily reacts with sodium borohydride resulting in the formation of a reduced derivative of rhodopsin. The new derivative was formulated as dihydrometarhodopsin II (Akhtar, Blosse & Dewhurst, 1965, 1967, 1968). Degradative analysis of dihydrometarhodopsin II revealed that the retinyl moiety in this derivative was attached to the protein through an ϵ-amino group of a lysine residue (Akhtar *et al.* 1967, 1968). Parallel results were reported by Bownds (1967).

The question remained unanswered whether or not the retinal–opsin linkage identified above in metarhodopsin II was also present in native rhodopsin. Poincelot, Millar, Kimbel & Abrahamson (1969) reported that treatment of freeze-dried outer rod segments with methanol–hydrochloric acid resulted in the extraction of a phospholipid derivative of retinal, which was characterized as *N*-retinylphosphatidylethanolamine. Irradiation of rod segments to form metarhodopsin I before freeze-drying also yielded an extractable retinyl moiety; however, at the metarhodopsin II stage no such phospholipid-linked retinyl moiety was extractable.

The observations led these workers to suggest

that the active site of native rhodopsin contains the retinyl moiety linked to the amino group of phosphatidylethanolamine and that the conversion of rhodopsin into metarhodopsin II may be attended by the migration of the chromophore from lipid to ϵ-amino group of a lysyl residue in the backbone protein (Poincelot, Millar, Kimbel & Abrahamson, 1969, 1970; Kimbel, Poincelot & Abrahamson, 1970; Poincelot & Abrahamson, 1970). A qualified support for this view was provided by the work of Akhtar & Hirtenstein (1969) who demonstrated the isolation of some *N*-retinylphosphatidylethanolamine after the denaturation of native rhodopsin with trichloroacetic acid (Kito, Suzuki, Azuma & Sekoguti, 1968).

We have in this paper examined a number of independently prepared samples of rhodopsin and our results now show that a phospholipid-linked retinyl derivative can be isolated only from certain types of preparations. It is therefore conceivable that the formation of *N*-retinylphosphatidylethanolamine is due to a non-enzymic reaction resulting through the transfer of the retinyl moiety from its native site to an amino group of a favourably orientated phospholipid molecule. This transfer shows a remarkable degree of specificity and, when operative, occurs only in the denaturation of either rhodopsin or metarhodopsin I and not in that of metarhodopsin II. The relevance of this specificity to the biochemistry of vision is discussed. A new convenient method for the preparation of tritiated rhodopsin, and its purification by gel filtration in cetyltrimethylammonium bromide is also described.

RESULTS

Preparation of rhodopsin by method (a). Labelled rhodopsin containing tritiated retinyl moiety at the active site was prepared by two apparently related methods. The first method is based on the original procedure of Wald & Brown (1956), for the reaction

$$\text{opsin} + 11\text{-}cis\text{-retinal} \rightarrow \text{rhodopsin}$$

and later developed for radiochemical synthesis by Akhtar *et al.* (1967, 1968).

Method (a). This involves the grinding of bovine retinae, flotation and bleaching of rod outer segments, extraction of opsin and regeneration of rhodopsin with tritiated 11-*cis*-retinal. The details

Table 1. *Rhodopsin preparation A: distribution of radioactivity between methanol-soluble and protein-bound fractions after denaturation and reduction with sodium borohydride*

Expt. 1. Rhodopsin solution (1.0–3.0 ml) in 2% digitonin–sodium phosphate (0.067 M, pH 7.0) was treated with 20% (w/v) trichloroacetic acid (TCA) in the dark to a final concentration of 0.1 M. The solution was then reduced with 5 mg of $NaBH_4$/ml, freeze-dried and extracted with 2 × 4 ml of methanol. The precipitate was dissolved in 3.0 ml of 1.0 M-Hyamine hydroxide in methanol. Expt. 2. Rhodopsin (1.0–3.0 ml) was adjusted to pH 7.8 with NaOH, then irradiated for 5 min at 0°C with a light of 500 nm to generate metarhodopsin I. It was then treated as in Expt. 1. Expt. 3. Rhodopsin (1.0–3.0 ml) was adjusted to pH 6.2 with HCl, then irradiated for 10 min at 7°C with a light of 500 nm to generate metarhodopsin II. It was then treated as in Expt. 1. Expt. 4. Rhodopsin (1.0–3.0 ml) was treated with 5 mg of $NaBH_4$/ml in the dark, then irradiated for 15 min at 20°C, generating metarhodopsin II *in situ*, which was reduced to dihydrometarhodopsin II by the $NaBH_4$ present. The solution was freeze-dried and extracted with methanol as in Expt. 1. Expt. 5. Rhodopsin (1.0–3.0 ml) was treated with 5 mg of $NaBH_4$/ml in the dark and then methanol was added dropwise to a final concentration of 45% (v/v). The solution was freeze-dried and extracted with methanol as in Expt. 1. Expt. 6. Rhodopsin (1.0–3.0ml) was treated with HCl in the dark to a final concentration of 0.1 M. It was then treated as in Expt. 1. Expt. 7. Rhodopsin (1.0–3.0 ml) was freeze-dried in the dark, then treated with 2 × 4 ml of methanol containing 0.1 M-trichloroacetic acid. The precipitate was dissolved in Hyamine as in Expt. 1. Expts. 8, 9 and 10. The solutions were treated as in Expts 1, 2 and 3, aged preparations being used as described in the text. All radioactivity measurements were corrected for quenching to a constant efficiency.

Expt. no.	Solution	Treatment	Percentage of radioactivity: Methanol-soluble	Percentage of radioactivity: Protein-bound
1	Rhodopsin	TCA/$NaBH_4$	70.9	29.1
2	Metarhodopsin I	TCA/$NaBH_4$	70.3	29.7
3	Metarhodopsin II	TCA/$NaBH_4$	29.3	70.7
4	Metarhodopsin II (*in situ*)	$NaBH_4/h\nu$	19.4	80.6
5	Rhodopsin	$NaBH_4$/methanol	42.3	57.7
6	Rhodopsin	HCl/$NaBH_4$	45.6	54.4
7	Rhodopsin (freeze-dried)	Methanol/TCA	74.1	25.9
8	Aged rhodopsin	TCA/$NaBH_4$	75.6	24.4
9	Aged metarhodopsin I	TCA/$NaBH_4$	75.7	24.3
10	Aged metarhodopsin II	TCA/$NaBH_4$	25.2	74.8

of this preparation are given in the Experimental section. This particular preparation is referred to as 'rhodopsin preparation A'.

Treatment of rhodopsin, metarhodopsin I and metarhodopsin II with denaturants followed by reduction with sodium borohydride. Three solutions, containing rhodopsin, metarhodopsin I and metarhodopsin II, were separately treated with trichloroacetic acid to give derivatives with extinction maxima at 440nm. Each solution was separately treated with sodium borohydride, freeze-dried and then extracted with methanol. Table 1 shows that whereas rhodopsin and metarhodopsin I gave 70% of the original radioactivity extractable into methanol, metarhodopsin II gave predominantly protein-bound radioactivity (Expts. 1, 2 and 3 of Table 1). When metarhodopsin II generated *in situ* was trapped with sodium borohydride, once again most of the radioactivity was present in the protein fraction and only 20% was soluble in methanol (Expt. 4 of Table 1). The denaturation of rhodopsin was studied with other denaturants such as methanol, hydrochloric acid, and methanol–trichloroacetic acid. In all these experiments (Expts. 5, 6 and 7 of Table 1) varying amounts of methanol-soluble radioactive material were obtained. However, detailed analysis was only carried out on methanol extracts obtained from trichloroacetic acid denaturation of rhodopsin (Expt. 1 of Table 1).

T.l.c. analysis of methanol-soluble radioactive material. Table 2 (Expt. 1) shows that when analysed by t.l.c., 55% of the original methanol-soluble radioactivity from the trichloroacetic acid denaturation of rhodopsin (Expt. 1 of Table 1) ran in the region of synthetic *N*-retinylphosphatidylethanolamine. That the main radioactive component in this fraction was in fact *N*-retinylphosphatidylethanolamine was established by unambiguous degradations described in the Experimental section.

The original solution of rhodopsin prepared by method (*a*), when stored in the dark at −65°C for 6 months, showed no change in its spectroscopic or bleaching properties or its ability to form derivatives such as metarhodopsin I and metarhodopsin II. When an aged solution of rhodopsin was processed under the conditions of Expts. 1, 2 and 3 of Table 1, expected distributions of predominantly methanol-soluble radioactivity from rhodopsin and metarhodopsin I, and mostly protein-bound radioactivity from metarhodopsin II, were obtained (Expts. 8, 9 and 10 of Table 1).

The methanol extracts from Expts. 7 and 8 of Table 1 on t.l.c. analysis, however, revealed that radioactivity was now distributed in several zones (Expts. 2 and 3 of Table 2). The distribution pattern was similar to that obtained when hydrolysis products of phosphatidylethanolamine form Schiff-base derivatives with retinal. It seemed highly unlikely that lipids, hitherto considered essential to the active site, could undergo hydrolysis during storage without affecting the spectroscopic integrity of the rhodopsin.

We therefore undertook fresh preparations of rhodopsin by a method which, for technical reasons, was somewhat modified.

Preparation and properties of rhodopsin prepared by method (b). This method relied on homogenization of retinae to separate rod outer segments, which after flotation in sucrose were hardened with

Table 2. *Radioactivity distributions on t.l.c. analysis of methanol extracts of trichloroacetic acid-denatured samples of freshly prepared rhodopsin, aged rhodopsin and aged metarhodopsin I (preparation A)*

The methanol extracts obtained from fresh rhodopsin, aged rhodopsin and aged metarhodopsin I (Expts. 1, 8 and 9 of Table 1) were neutralized with acetic acid, evaporated to dryness under N_2 and extracted into chloroform or ethyl acetate. Samples of these extracts were analysed by t.l.c. in chloroform–methanol (75:11, v/v). The chromatograms were developed for 16cm, examined under u.v. light, and then 2cm bands were scraped off the plates for radiochemical analysis. The positions of known standards are shown in the table.

Percentage distribution of radioactivity in methanol extracts derived from				
Rhodopsin (fresh)	Rhodopsin (aged)	Metarhodopsin I (aged)	Positions of known standards	R_F
2.0	0.9	7.4	.....	1.0
16.7	10.1	7.6	Retinal	0.76
1.6	13.0	17.2	.....	0.60
55.8	22.5	22.7	*N*-Retinylphosphatidylethanolamine	0.43
11.6	4.7	17.5	*N*-Retinylphosphatidylserine	0.35
3.6	9.2	20.2	Hydrolysis products	0.28
5.0	18.9	5.8	*N*-Retinylethanolamine	0.20
3.7	20.7	1.6	Hydrolysis products	0.00

Table 3. *Rhodopsin preparation B: distribution of radioactivity between methanol-soluble and protein-bound fractions after denaturation and reduction with sodium borohydride*

Expts. 1–7. Experimental procedure was as in Expts. 1–7, Table 1. Expts. 8 and 9. Cetyltrimethylammonium bromide extracts of rhodopsin were purified by gel filtration (see the Experimental section). Fractions (2.0ml) of the purified solution were denatured as described in Expts. 1 and 4 (Table 1) respectively. TCA, trichloroacetic acid; *hν*, irradiated with white light for 15min at 20°C.

Expt. no.	Solution	Treatment	Percentage of radioactivity	
			Methanol-soluble	Protein-bound
1	Rhodopsin	TCA/$NaBH_4$	16.7	83.3
2	Metarhodopsin I	TCA/$NaBH_4$	13.1	86.9
3	Metarhodopsin II	TCA/$NaBH_4$	24.1	75.9
4	Metarhodopsin II (*in situ*)	$NaBH_4$/*hν*	18.4	82.6
5	Rhodopsin	$NaBH_4$/methanol	12.6	87.4
6	Rhodopsin	HCl/$NaBH_4$	13.6	86.4
7	Rhodopsin (freeze-dried)	Methanol/TCA	13.3	86.7
8	Rhodopsin (purified)	TCA/$NaBH_4$	6.4	93.6
9	Metarhodopsin II (*in situ*, purified)	$NaBH_4$/*hν*	10.2	89.8

alum and exposed to light of 500±5nm. The last process photolysed the rhodopsin, yielding all-*trans*-retinal and opsin *in situ*; however, there was no regeneration of rhodopsin, as the all-*trans*-retinal released did not absorb light at 500nm to give the 11-*cis*-isomer. Limited amounts of tritiated 11-*cis*-retinal were added to give about 60% regeneration of rhodopsin, and after incubation in the dark for 3h the regenerated rod segments were washed, freeze-dried and extracted with light petroleum (b.p. 40–60°C) to remove retinal and lipid impurities. Rhodopsin was then extracted by homogenization in either digitonin or cetyltrimethylammonium bromide. The extracts of rhodopsin made by method (*b*) are referred to as 'rhodopsin preparation B'. Digitonin extracts of rhodopsin preparation B were subjected to the same denaturation conditions as in Expts. 1–7 (Table 1), and these results are shown as Expts. 1–7 (Table 3).

Cetyltrimethylammonium bromide extracts of rhodopsin were purified by agarose gel filtration (see the Experimental section) and subjected to the same conditions as in Expts. 1 and 4 (Table 1). The results are shown as Expts. 8 and 9 (Table 3). In all the experiments in Table 3 the radioactivity was found to be predominantly bound to the protein fraction after denaturation and reduction with sodium borohydride. Six samples of labelled rhodopsin prepared by method (*b*) gave results identical with those displayed in Table 3. Further preparations of rhodopsin by method (*a*), however, revealed that only 50% of the preparations gave distributions as in Table 1; the other 50% gave results similar to Table 3.

DISCUSSION

Poincelot *et al.* (1969, 1970) subjected freeze-dried rhodopsin, metarhodopsin I and metarhodopsin II to extraction with methanol–hydrochloric acid, and found that with rhodopsin and metarhodopsin I a phospholipid-linked retinyl moiety could be extracted. The retinyl moiety remained bound to the protein in metarhodopsin II. These experiments led these workers to suggest that the active site of rhodopsin contains phosphatidylethanolamine linked to the polyene moiety. A qualified support for this view was provided by our previous work (Akhtar & Hirtenstein, 1969).

We now have two types of labelled rhodopsin preparations, having identical spectroscopic properties and both, at the metarhodopsin-II stage, reacting with sodium borohydride to give predominantly protein-bound radioactivity. However, at the native-rhodopsin stage, the preparations made by method (*a*) gave *N*-retinylphosphatidylethanolamine on denaturation with trichloroacetic acid followed by reduction with sodium borohydride, whereas the rhodopsin preparation B and rhodopsin purified by column chromatography, when subjected to the same treatment in parallel experiments, gave protein-bound radioactivity at the rhodopsin, as well as at the metarhodopsin-II, stage. Since preparation B, which has all the characteristics of rhodopsin, gave no phospholipid-linked retinyl moiety, it is doubtful whether phosphatidylethanolamine is directly involved in the visual protein.

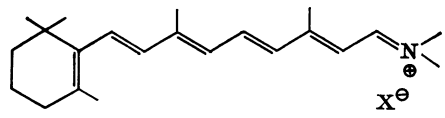
The presence of an ϵ-amino group of lysine at the active site of metarhodopsin II has been firmly

established (Akhtar *et al.* 1967, 1968; Bownds, 1967). The active-site linkage in native rhodopsin, however, is not amenable to stabilization by direct reactions, and had to be approached only after treatment with denaturants, which may induce a shift of the retinyl moiety from its original site to a new group. Confusion about the active site of native rhodopsin must therefore remain until the possibility of such a migration has been completely eliminated.

The present work, however, suggests that the type of evidence on which the presence of a phospholipid at the rhodopsin active site was originally proposed must be treated with caution. Indirect evidence against the involvement of a phospholipid in the rhodopsin active site has been presented by Hall & Bacharach (1970). Bearing in mind the qualification made above, an alternative possibility that can be drawn from the present work is as follows. Preparations of rhodopsin that yield *N*-retinylphosphatidylethanolamine may contain membrane lipid fragments bound to protein, which may participate in 'non-enzymic' (or 'non-physiological' or 'chemical') transamination reactions under denaturation conditions. If this is so, then it is remarkable that when operative, the transfer of the retinyl moiety from the active site to a lipid amino group occurs only in rhodopsin and metarhodopsin I. The transfer is not observed in metarhodopsin II. This may be explained by assuming that the retinyl moiety in rhodopsin and metarhodopsin I is located in a lipophilic (non-polar) environment where membrane fragments, when present, are also attached and that a suitably orientated amino group of such a membrane fragment participates in a non-enzymic transamination reaction during denaturation. The lack of involvement of the lipid amino group in transamination at the metarhodopsin II stage could then be attributed to the presence of the retinyl moiety in the derivative in a hydrophilic (polar) environment. This pattern of reactivity will be in accordance with the fact that sodium borohydride, a reagent expected to be readily accessible to hydrophilic regions, reacts only with metarhodopsin II. No reaction is observed with rhodopsin or metarhodopsin I (Akhtar *et al.* 1965; Bownds & Wald, 1965).

These results, therefore, are consistent with the notion that light-induced conversion of rhodopsin into metarhodopsin II involves a profound conformational change resulting in the dislocation of the retinylideneiminium chromophore (structure of type I) from a non-polar environment in rhodopsin into a polar environment in metarhodopsin II.

The presence of the retinylideneiminium chromophore (structure of the type I) in a non-polar micro environment in rhodopsin ($\lambda_{max.}$ 500nm) and in a polar micro environment in metarhodopsin II



Retinylideneiminium chromophore

Structure of type I

($\lambda_{max.}$ 380nm) may help to explain the absorption spectra of these species. In model studies it has been shown that the chromophore of the type I generally absorbs at higher wavelengths in non-polar than in polar solvents (Akhtar *et al.* 1968; Dewhurst, 1967; Akhtar, 1970; Irving, Byers & Leermakers, 1970).

EXPERIMENTAL

Phosphatidylethanolamine, phosphatidylserine, 5% rhodium on charcoal and Hyamine hydroxide (1M, in methanol) were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Bio-Gel A was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Interference filters of band-width 9nm were obtained from Grubb–Parsons Ltd., Newcastle upon Tyne, U.K. All other chemicals and apparatus were as previously described (Akhtar *et al.* 1968).

The preparation of 11-*cis*-retinal, tritiated 11-*cis*-retinal, t.l.c. techniques, and radioactivity measurements were as previously described (Akhtar *et al.* 1968).

Purification of tritiated 11-cis-*retinal.* Tritiated 11-*cis*-retinal, synthesized as described previously (Akhtar *et al.* 1968) was purified by preparative t.l.c. in light petroleum (b.p. 40–60°C)–acetone (20:1, v/v). Spectroscopic analysis indicated that the product was 85–90% pure. The tritiated 11-*cis*-retinal was evaporated to dryness and dissolved in Tween 80 (10mg/ml) buffered to pH6.4 with 0.067M-sodium phosphate, and used immediately.

Preparation of tritiated rhodopsin by method a. Opsin solution in 2% (w/v) digitonin in 0.067M-sodium phosphate buffer, pH6.4, was prepared as described previously (Akhtar *et al.* 1968); to this was added limiting quantities of purified tritiated 11-*cis*-retinal in the dark, and the mixture was incubated at 20°C for 3h to effect maximum incorporation of the retinyl moiety into rhodopsin. The final solution showed E_{500} 0.5, and contained 7.0×10^7 c.p.m./μmol. This solution was stored in 3ml fractions in the dark at −65°C.

Preparation of tritiated rhodopsin by method b. All operations were carried out in near-total darkness, at a temperature of 4°C whenever possible. The buffer solutions were 0.067M-sodium phosphate, pH values as indicated below.

Dark-adapted retinae were homogenized for 5min in a loose-fitting piston-barrelled glass homogenizer (clearance 1.0mm), then suspended in 45% (w/v) sucrose in the buffer, pH7.0 (2ml/retina). The suspension was centrifuged at 24000*g* for 45min and the floated rod tissue removed by

decantation, washed with 3 vol. of the buffer, pH 7.0, and centrifuged at 38000*g* for 30 min to precipitate the rod tissue. This precipitate was homogenized as before and suspended in 40% (w/v) sucrose in the buffer, pH 7.0 (1 ml/retina), and centrifuged at 38000*g* for 45 min. The surface layer of rod tissue was removed and washed three times with water, with centrifugation at 38000*g* after each washing. The solid was then lightly homogenized in 25 ml of 4% (w/v) potassium alum and left for 30 min. The suspension was then diluted with 3 vol. of water and centrifuged at 38000*g* for 10 min. The precipitated rod segments were then washed twice with water, and twice with the buffer, pH 7.0, with centrifugation at 38000*g* for 10 min after each washing. Finally the rod tissue was homogenized in 20 ml of 0.067 M-sodium phosphate buffer, pH 6.4, irradiated with light of 500±5 nm for 20 min and left for a further 20 min in dim red light. Tritiated 11-*cis*-retinal in Tween 80 was added in limiting quantities, to facilitate not more than 70% regeneration of the original rhodopsin. After a 3 h incubation in the dark, the suspension was centrifuged and the precipitated rod tissue was washed twice with water and once with the buffer, pH 7.0, with centrifugation at 38000*g* for 10 min after each washing. The rod tissue was then freeze-dried and homogenized with 3×20 ml of light petroleum (b.p. 40–60°C) with centrifugation at 38000*g* for 10 min after each extraction. Excess of petroleum was removed under vacuum, and the tissue was washed twice with water. Tritiated rhodopsin was extracted by homogenization in either 2% (w/v) digitonin in 0.067 M-sodium phosphate buffer, pH 7.0, or into 1.47% (w/v) cetyltrimethylammonium bromide in 0.067 M-sodium phosphate buffer, pH 7.0. In both cases, the homogenized suspension was shaken vigorously in the dark for 90 min at 15°C, centrifuged at 38000*g* for 30 min at 12°C and the clear supernatants were retained.

Purification of cetyltrimethylammonium bromide extracts of tritiated rhodopsin. These extracts of tritiated rhodopsin were purified by the method of Heller (1968) and Hall, Bok & Bacharach (1968) by using Bio-Gel A (1.5 M, 100–200 mesh agarose) in 1.47% (w/v) cetyltrimethylammonium bromide in 0.067 M-sodium phosphate buffer, pH 7.0. Fractions (5.0 ml) of tritiated rhodopsin extracts were applied to a column (2.0 cm × 50 cm) in the dark, and the flow rate was maintained at 30 ml/h. Fractions (3 ml) were collected and analysed spectroscopically and radiochemically. The appearance of radioactivity exactly followed the 500 nm extinction peak as shown in Fig. 1, indicating that the tritiated retinal was in the active site of native rhodopsin. The fractions corresponding to 73–78 ml elution volume were pooled and used for further experiments.

Denaturation of tritiated rhodopsin. The original radioactive rhodopsin preparation A was diluted with unlabelled rhodopsin in some experiments to facilitate spectroscopic analysis. In all experiments the solutions were diluted to give E^{1cm}_{500} 0.8–1.0. Although the absolute total radioactivity used in each experiment varied (Expts. 1–10, Table 1, 4.5×10^4–10.5×10^4 c.p.m.; Expts. 1–9, Table 3, 10.5×10^4–30×10^4 c.p.m.), more than 90% of the original radioactivity in each experiment was accounted for in the methanol-soluble and protein-bound fractions. Digitonin extracts of tritiated rhodopsin prepared by methods *a* and *b* displayed identical spectroscopic properties (Fig. 2).

Samples (1.0–3.0 ml) of rhodopsin in 2% (w/v) digitonin in 0.067 M-sodium phosphate buffer, pH 7.0 (E^{1cm}_{500} = 0.8–1.0), were treated in the dark with 20% (w/v) trichloroacetic acid to a final concentration of 0.1 M. Spectroscopic analysis showed an instantaneous change in $\lambda_{max.}$ from 500 nm to 440 nm. The solution was transferred to a centrifuge tube containing 5 mg of $NaBH_4$/ml and centrifuged at 0°C for 10 min to minimize foaming. Spectroscopic analysis of this solution showed a new $\lambda_{max.}$ at 333 nm. This reduced product was freeze-dried in a 50 ml

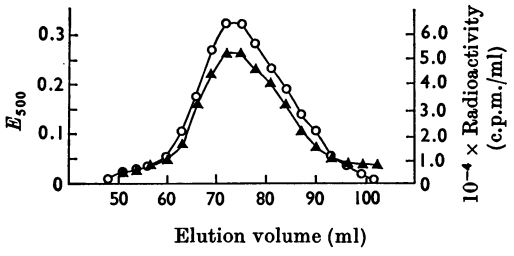


Fig. 1. Elution pattern of a sample (5.0 ml) of radioactive rhodopsin, in 1.47% cetyltrimethylammonium bromide–0.067 M-sodium phosphate, pH 7.0. Chromatography was in the dark on a column (2 cm × 50 cm) of Bio-Gel A (1.5 M, 100–200 mesh). The temperature was maintained at 19°C; flow rate was 30 ml/h. Fractions (3.0 ml) were collected and analysed spectroscopically and radiochemically. ▲, E_{500}; ○, radioactivity.

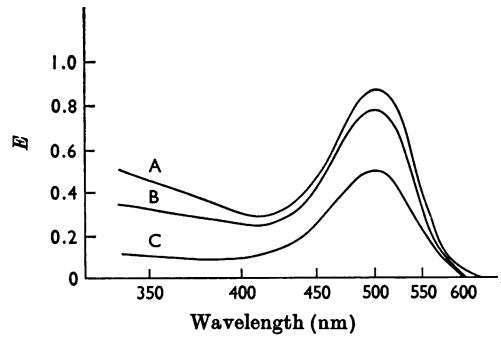


Fig. 2. Spectroscopic properties of rhodopsin preparations. Curve A, native rhodopsin in 2% digitonin in 0.067 M-sodium phosphate buffer, pH 7.0, (E_{400}/E_{500} = 0.36). Curve B, tritiated rhodopsin preparation A, in 2% digitonin in 0.067 M-sodium phosphate buffer, pH 7.0 (E_{400}/E_{500} = 0.32). Curve C, tritiated rhodopsin in 1.47% cetyltrimethylammonium bromide in 0.067 M-sodium phosphate buffer, pH 7.0, purified by gel filtration on Bio-Gel A (1.5 M, 100–200 mesh agarose) (E_{400}/E_{500} = 0.20). The curve obtained for rhodopsin preparation B in digitonin is identical with curve B, and is therefore not included.

round-bottomed flask, and the powder was extracted with 2×4.0ml of methanol at 20°C, with centrifugation after each extraction. The protein precipitate was dissolved in 3.0ml of Hyamine hydroxide (1M in methanol). Samples (0.1ml) of the methanol-soluble and protein fractions were assayed for tritium by standard liquid-scintillation techniques. Treatment of cetyltrimethylammonium bromide extracts of rhodopsin with trichloroacetic acid caused the formation of a gel that could not be analysed spectroscopically; however, addition of $NaBH_4$ to this gel gave a clear solution with $\lambda_{max.}$ 333nm. Denaturation of rhodopsin solutions with methanol or HCl, and the extraction of freeze-dried rhodopsin with methanol containing 0.1M-trichloroacetic acid, were performed as described in Table 1.

Generation of metarhodopsin I and metarhodopsin II. Metarhodopsin I and metarhodopsin II were formed by a method related to that of Mathews *et al.* (1963). Rhodopsin (1.0–3.0ml) was adjusted to pH7.8 by addition of 0.5M-NaOH in the dark. Irradiation of this solution at 0°C with light of 500±5nm generated an equilibrium mixture containing 80% metarhodopsin I ($E^{1cm}_{480} = 0.70$) and 20% metarhodopsin II ($E^{1cm}_{380} = 0.38$).

When 1.0–3.0ml of rhodopsin, adjusted to pH6.2 by addition of 0.5M-HCl in the dark, was irradiated at 7°C with light of 500±5nm for 10min the equilibrium mixture formed contained 85% metarhodopsin II ($E^{1cm}_{380} = 0.78$) and 15% metarhodopsin I ($E^{1cm}_{480} = 0.26$). These solutions were denatured, reduced and extracted as for rhodopsin.

Preparation of N-*retinylidene derivatives of phosphatidylethanolamine, phosphatidylserine, ethanolamine and serine, and reduction to the* N-*retinyl derivatives.* The amino compound (10μmol) and all-*trans*-retinal (10μmol) were dissolved in methanol (3ml), and adjusted to pH8.5 with triethylamine. The solutions were shaken in the dark for 2h at 25°C under N_2. Formation of Schiff-base derivatives was determined as described previously (Akhtar *et al.* 1968). The *N*-retinylidene compounds were reduced with $NaBH_4$ (10–20mg) at 4°C. Decolorization of the solution indicated complete reduction.

Separation and purification of N-*retinyl derivatives of phosphatidylethanolamine, phosphatidylserine, ethanolamine and serine.* The solutions were neutralized with acetic acid and evaporated to dryness under N_2. The residues were extracted into either chloroform or ethyl acetate, and purified by preparative t.l.c. Chromatography in chloroform–methanol (75:11, v/v) separated *N*-retinylphosphatidylethanolamine (R_F 0.43) and *N*-retinylphosphatidylserine (R_F 0.36). Chromatography in chloroform–methanol–water (85:25:2, by vol.) separated *N*-retinylethanolamine (R_F 0.45) and *N*-retinylserine (R_F 0.16). All these compounds were fluorescent under u.v. light.

Hydrogenation of N-*retinyl to perhydroretinyl derivatives.* *N*-Retinyl derivatives in 10ml of ethyl acetate were stirred under H_2 at 20°C for 1h with 2mg of 5% rhodium on charcoal as catalyst. The catalyst was removed by filtration and the solution evaporated for analysis by t.l.c. Chromatography in chloroform–methanol (75:11, v/v) separated perhydroretinylphosphatidylethanolamine (R_F 0.47) and perhydroretinylphosphatidylserine (R_F 0.38). Chromatography in chloroform–methanol–water (85:25:2, by vol.) separated perhydroretinylethanolamine (R_F 0.45) and perhydroretinylserine (R_F 0.16). These compounds showed no fluorescence under u.v. light, but could be identified by exposure to I_2 vapour.

Hydrolysis of perhydroretinylphosphatidylethanolamine. Perhydroretinylphosphatidylethanolamine was heated with 6M-HCl at 110°C in a sealed tube for 2h. After cooling, the solution was neutralized with 6M-NaOH at 4°C and extracted with 6×3.0ml of diethyl ether. The ether extract was evaporated and analysed on t.l.c. in chloroform–methanol–water (85:25:2, by vol.). The sole extracted hydrolysis product was indistinguishable from synthetic perhydroretinylethanolamine (R_F 0.45).

Analysis of methanol extracts from Expt. 1 *of Table* 1. The methanol extracts were neutralized with acetic acid and evaporated to dryness under N_2. The chloroform or ethyl acetate extract of the residue was analysed on t.l.c. in chloroform–methanol (75:11, v/v). Expt. 1 of Table 2 shows a typical distribution of radioactivity obtained in this analysis. The band corresponding to *N*-retinylphosphatidylethanolamine was eluted with chloroform–methanol (2:1, v/v), and synthetic *N*-retinylphosphatidylethanolamine was added as carrier. It was then subjected to hydrogenation as described above. Analysis of the product on t.l.c. in chloroform–methanol (75:11, v/v) showed that 57% of the radioactivity was associated with a single band (R_F 0.47) corresponding to synthetic perhydroretinylphosphatidylethanolamine. This band was eluted in chloroform–methanol (2:1, v/v), and evaporated to dryness under N_2. After hydrolysis in 6M-HCl as described above, the ether extract was analysed on t.l.c. in chloroform–methanol–water (85:25:2, by vol.) and 70% of the radioactivity applied to the plate ran in a single band (R_F 0.45) which was indistinguishable from that of synthetic perhydroretinylethanolamine. It was therefore concluded that a significant proportion of the original methanol-soluble radioactivity was in *N*-retinylphosphatidylethanolamine.

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