The regeneration of rhodopsin following the removal of detergent. NORMAN T. FELBERG.

The visual pigment, rhodopsin, is a membrane-bound glycoprotein. Detergent-solubilized rhodopsin retains lipid from rod outer segments (ROS) during extraction, much of which can be removed, but only at the expense of the chemical and physical properties of rhodopsin. Certain detergents can also modify the properties of rhodopsin. For example, photobleached rhodopsin cannot be regenerated with exogenous 11-cis-retinal, its prosthetic group, after solubilization with cetyltrimethylammonium bromide (CTAB), Triton X-100, or Emulphogene BC-720. Two laboratories have reported that the removal of detergent restores certain properties to rhodopsin. Zorn and Futterman described the inability to regenerate rhodopsin after the removal of Triton X-100 unless supplemented by phospholipid. Chabre and co-workers report the formation of lamellar structures which undergo light-induced changes after the removal of Triton X-100 from detergent-solubilized preparations.

This report describes the ability of Triton X-100 and CTAB-solubilized rhodopsin preparations to regenerate in the presence of 11-cis-retinal after the partial removal of detergent.

**Detergent extraction.** ROS were prepared from frozen bovine retinas (G. A. Hormel & Co.) according to Heller, and stored as a pellet in liquid nitrogen. Thawed ROS pellets were suspended in phosphate buffer and washed with 1/15 M sodium phosphate buffer, pH 7.0. Following centrifugation, the ROS pellets were suspended in 1 per cent Triton X-100 or 40 mM CTAB in phosphate buffer and mixed. Triton X-100 extracts were centrifuged immediately (40,000 × g, 20 minutes, 5° C.), while CTAB extracts were maintained at 4° C. for 4 hours prior to centrifugation.

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**Table I. Triton X-100-solubilized rhodopsin** was incubated with Bio-Beads SM-2 as described in methods. Aliquots were removed at the times indicated and rhodopsin content determined from the decrease in A500 nm. after photobleaching. Excess 11-cis-retinal was added to the photobleached sample and the degree of regeneration was calculated from the increase of A500 nm. The amount of photopigment formed by regeneration was calculated by measuring the decrease in A500 nm. after the regenerated sample was relabeled.

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>Recovered</th>
<th>Rhodopsin regenerated (%)</th>
<th>Re-bleached</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>21.5</td>
<td>2.2</td>
</tr>
<tr>
<td>30</td>
<td>94.5</td>
<td>94.0</td>
<td>60.9</td>
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<tr>
<td>60</td>
<td>94.0</td>
<td>104.0</td>
<td>59.3</td>
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<tr>
<td>90</td>
<td>77.9</td>
<td>84.1</td>
<td>56.1</td>
</tr>
<tr>
<td>120</td>
<td>76.4</td>
<td>90.5</td>
<td>49.4</td>
</tr>
</tbody>
</table>

**Rhodopsin content** was determined spectrophotometrically on a Gilford Model 2400S from the difference in A500 nm. before and after photobleaching assuming a molar extinction coefficient of 40,600 M⁻¹ cm⁻¹.

**Rhodopsin regeneration** was performed by adding an excess of 11-cis-retinal (Hoffman-LaRoche) dissolved in acetone, to photobleached preparations of rhodopsin. Some samples were regenerated in the absence of detergent but assayed after extraction with 1 per cent Triton X-100 in order to reduce light-scattering effects. Regeneration was determined from the increase in A500 nm. As a final control, all regenerated samples were photobleached again and the loss of A500 nm. was determined to assure measurement of a light-sensitive species.

The purity of ROS preparations can be characterized by the spectral ratio, A500 nm./A600 nm., of the detergent-solubilized rhodopsin in the preparation. The lowest spectral ratio (i.e., highest purity) obtained directly from ROS was reported by DeGrip, Daemen, and Bonting as 0.20. The ROS preparations employed in this study are characterized by spectral ratios (A500 nm./A600 nm.) of 0.40 in Triton X-100. The lack of great purity in this preparation should not interfere with these experiments or their interpretation. The ability of the rhodopsin in this preparation to regenerate
has no absorbance at 600 nm.). The data in this indicates that it is not photopigment. In contrast, as much as ½ of the material regenerated after partial removal of detergent was rebledashed. Similar experiments with CTAB incubated for 70 minutes with Bio-Beads SM-2, showed a 91.5 per cent recovery of rhodopsin, all of which could be regenerated. Prior to the removal of 40 mM CTAB, less than 10 per cent regeneration was obtained, and little of this could be rebledashed.

Turbidity was observed during the removal of detergent as evidenced in Fig. 1 as the increase in A\text{\wedge}00 nm. (presumably due to light-scattering, since rhodopsin has no absorbance at this wavelength). There was no change in A\text{\wedge}00 nm. after photobleaching rhodopsin samples partially free of Triton X-100, while samples partially free of CTAB demonstrated increased light-scattering after photobleaching.

It is generally accepted that certain detergent-solubilized preparations of photobleached rhodopsin cannot be regenerated even with 11-cis-retinal. It has become routine to study regeneration in either ROS or digitonin extracts, since they readily permit regeneration with exogenous 11-cis-retinal or enzymatic and chemical systems which can produce it.

The removal of Triton X-100 and CTAB from solubilized preparations, as described in this paper, provides a new substrate for regeneration studies.

Fig. 1. Rhodopsin (4.6 nMoles) in 2 ml. of 1 per cent Triton X-100 in 1/15 M sodium phosphate buffer, pH 7.0, were mixed with 0.6 Gm. of moist Bio-Beads SM-2 and mixed by inversion. Aliquots were removed at the times indicated and light-scattering determined as A\text{\wedge}00 nm. (rhodopsin has no absorbance at 600 nm.). The data in this figure and in Table I were obtained in the same experiment.

was confirmed in a 1 per cent digitonin extract of the ROS.

Table I shows the recovery of rhodopsin during Bio-Beads SM-2 treatment and the ability to regenerate after partial removal of Triton X-100. It is important to note in Table I, last column, that the material regenerated in 1 per cent Triton X-100 (time, 0 minutes) could not be rebledashed, indicating that it is not photopigment. In contrast, as much as ½ of the material regenerated after partial removal of detergent was rebledashed. Similar experiments with CTAB incubated for 70 minutes with Bio-Beads SM-2, showed a 91.5 per cent recovery of rhodopsin, all of which could be regenerated. Prior to the removal of 40 mM CTAB, less than 10 per cent regeneration was obtained, and little of this could be rebledashed.

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The ability of rhodopsin to regenerate after the removal of detergent was reported by Zorn and Futterman. In their experiments, in order to obtain regeneration in a detergent-free non-enzymatic system (dithiothreitol and all-trans-retinal), phospholipid had to be added as a supplement. From this, it was concluded that phospholipid was essential for the regeneration of rhodopsin with 11-cis-retinal. The procedure described here does not require any supplements and suggests that after detergent removal protein-lipid interactions have been restored.

Another detergent-free rhodopsin preparation has been described. This method removed Triton X-100 with toluene containing lecithin and yielded a protein-lipid aggregate demonstrating a lamellar structure. Bio-Beads SM-2 removal of detergent also resulted in aggregate formation (Fig. 1). Chabre and co-workers did not attempt to regenerate their detergent-free rhodopsin.

Bio-Beads SM-2 removal of detergent is rapid and reportedly does not lead to the loss of such proteins as albumin or those in microsomes. Holloway has indicated that detergent removal closely approaches the critical micelle concentration only after 120 minutes in this procedure. The Triton X-100 concentration after a 30 minute treatment with Bio-Beads SM-2 is more than 0.2 per cent. This would suggest that regeneration can occur in appreciable concentrations of Triton X-100 (see Table I).

In light of the previous results it is possible to speculate that membrane-bound rhodopsin exists as a highly organized phospholipid-protein complex, in which the phospholipid plays an important role in maintaining the protein conformation. When phospholipid is removed, chemical and physical changes are observed. Thus it would appear that solubilization of ROS by detergents that inhibit regeneration act by disrupting phospholipid-protein interaction. These detergents, however, do not necessarily change other chemical and physical properties and may interact with rhodopsin to maintain these conformational attitudes. Removal of detergents allows the reorganization of protein-phospholipid in an aggregated complex, which presumably restores the missing conformational attitude since the newly formed aggregate can be regenerated with 11-cis-retinal. In all probability, following treatment with Bio-Beads SM-2, detergent remains in the sample below the critical micelle concentration. This amount of detergent does not prevent regeneration and, therefore, it is assumed that loss of the ability to regenerate is due to physical separation of phospholipid from rho-
dopsin in a micellar species, which results in a modified conformation.

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REFERENCES


Retrobulbar injection of bupivacaine (Marcaine) for anesthesia and akinesia. R. Brian Smith and Jay G. Linn, Jr.

Bupivacaine (Marcaine), 1-n-butyl-DL-piperidine-2-carboxylic acid-2, 6-dimethylamidine hydrochloride, is a new long-acting anilide local anesthetic introduced into clinical practice in the United States in 1973. It was synthesized in 1957 by AF Ekenstam, Egner, and Pettersson.1 Bupivacaine is closely related structurally to mepivacaine (Carbocaine). It differs from mepivacaine in that a butyl group was substituted for a methyl group. The drug has been extensively used in other countries for pain relief, epidural, and regional anesthesia.2-4.

Because of its unique long action, retrobulbar blocks were performed using bupivacaine for various ophthalmologic procedures on seven patients. Bupivacaine is available in concentrations of 0.25 per cent, 0.5 per cent, and 0.75 per cent with and without epinephrine 1:200,000. A concentration of 0.75 per cent is recommended for motor block. Patients were premedicated with hydroxyzine, 50 mg. intramuscularly, 45 minutes preoperatively. In the operating room 2.5 mg. increments of intravenous diazepam were administered to a total ranging from 5 to 10 mg. A 30 ml. 0.75 per cent solution of bupivacaine was mixed with 150 international units of hyaluronidase (Wydase). Two milliliters of this solution was used for retrobulbar block. A facial nerve block using a modified Van Lint technique was performed using between 5 and 7 ml. of solution. Tetracaine (Pontocaine) 0.5 per cent was applied topically to the cornea in all cases prior to the retrobulbar block. In two patients tetracaine was repeated once during the procedure.

The results are shown in Table I. Onset of akinesia was between 10 and 15 minutes. In Patient 1, the retrobulbar block was repeated because of incomplete akinesia.

Patients 1 and 2 were observed for return of eye movement. The first detectable movements returned in 130 and 135 minutes and complete movements returned in 240 and 285 minutes, respectively.

Patient 6 was a 35-year-old female who was 11 weeks pregnant. Local anesthesia was chosen because of pregnancy.