TERMINAL AMINO ACIDS OF RHODOPSIN

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Rhodopsin, the photosensitive pigment of rod vision, is a chromoprotein composed in equimolar parts of the protein opsin and the carotenoid retinene (1). Its absorption spectrum and molar extinction are known (2) and the kinetics of bleaching by light and of regeneration have been investigated (3). An upper limit of 40,000 has been set for its molecular weight (cattle) (1). Since rhodopsin is insoluble in water (4), but soluble in aqueous digitonin, it has been necessary to infer its properties from studies on aqueous solutions in which the solute is a high molecular weight protein-digitonin complex (1).

The present communication reports C-terminal and N-terminal analyses carried out on rhodopsin. For these to be significant, it is necessary that the protein be free from all interfering impurities. This was well demonstrated in the present investigation. Initially an N-terminal group was found, but after careful treatment of rhodopsin with mixed bed ion exchange resins, which alters none of its optical, photosensitive, or regenerative properties, C-terminal and N-terminal groups were found to be absent.

EXPERIMENTAL

Materials

Rhodopsin was prepared from cattle retinas essentially by procedures previously developed in this laboratory (5, 6).

Carboxypeptidase, three times crystallized, was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, and was crystallized once more in this laboratory by the method of Neurath and Schwert (7). This preparation showed 85 per cent activity toward carbobenzoxyglycyl-l-phenylalanine. An enzyme solution was prepared by washing the crystals four times with distilled water to remove residual amino acids and dissolving them in 1.5 M NaCl at 0°.

Methods

Purity of Rhodopsin—The optical properties of rhodopsin provide a useful index of purity. The absorption spectrum of the cattle pigment

* This research was supported in part by funds granted to Professor George Wald from the Rockefeller Foundation and the Office of Naval Research.
in aqueous digitonin solution has maxima at about 498 and 350 m\textmu, associated with the carotenoid chromophore, and a sharp peak at 278 m\textmu, associated with the protein opsin (8). A minimum occurs at about 400 m\textmu. Since most impurities tend to raise the absorption at short wave lengths, the lower the ratios of the optical densities at 278 and 400 m\textmu to the density at 500 m\textmu (278:500 and 400:500 ratios), the purer in general is the preparation. In the best preparations, these indices have the following values: 400:500 ratio, 0.22 to 0.26; 278:500 ratio, 2.0.

The concentration of cattle rhodopsin is determined from its optical density, its molar extinction coefficient at 500 m\textmu being 40,600 (2). The concentration of carboxypeptidase was obtained from the absorption at 278 m\textmu by assuming a molar extinction coefficient of 8.6 \times 10^4 (9).

**Bleaching and Regeneration**—Rhodopsin was bleached in orange (non-isomerizing) light. To regenerate, neo-b retinene in digitonin solution was added in excess, and the mixture was incubated in the dark at 25\degree, pH 6.5, until all changes were complete (3).

**The presence of small molecular weight peptides and amino acids** in rhodopsin was detected by permitting aliquots of rhodopsin solution to react with Dowex 50 ion exchange resin in the hydrogen form, 20 to 50 mesh, 4 per cent cross-linkage, for about 1 hour (10). The amino acids and peptides were eluted from the resin with 5 N NH₄OH and subjected to descending one-dimensional chromatography on Whatman No. 1 filter paper at 25\degree, with butanol-acetic acid-water (4:1:5) (11). The color was developed by using a spray consisting of 0.1 per cent ninhydrin in water-saturated butanol.

**Purification of Rhodopsin**—Small molecular weight peptides and amino acids were removed from rhodopsin solutions by chromatography on columns of mixed bed ion exchange resins. The ammonia-acetate cycle, hydrogen-hydroxyl cycle mixed bed column was prepared and operated essentially according to the method outlined by Dintzis (12). 1 per cent digitonin, previously purified by being passed through a mixed bed ion exchange column, was used as the eluent. The column and eluent were equilibrated at 4\degree overnight and run at 4\degree in the dark or red light.

**N-Terminal groups** were determined by the methods of Sanger (13) and Levy and Li (14). The DNP\textsuperscript{1} derivatives of several of the amino acids were prepared according to published methods (15) and served for comparison of \( R_F \) values. The chromatographic procedure of Blackburn (16) was used for separation and identification of the DNP amino acids. The paper chromatograms were developed on Whatman No. 4 filter paper at 25\degree in the dark. The \( R_F \) values obtained in this system were not con-

\textsuperscript{1} The following abbreviations are used in this paper: DFP, diisopropylfluorophosphate; FDNB, 1,2,4-fluorodinitrobenzene; DNP, 2,4-dinitrophenyl.
stant, and control spots of DNP amino acids were always run simul-
taneously. DNP-serine and DNP-glycine were separated and identified
according to the method of Levy (17).

C-Terminal groups were determined with carboxypeptidase essentially
according to the method of Gladner and Neurath (18). DFP\(^2\) in \(10^{-4}\)
mmole per ml. of solution was added to inhibit any residual proteolytic
activity in the carboxypeptidase preparation, carboxypeptidase being
entirely inert toward this reagent.

Carboxypeptidase activity was determined by the method of Elkins-
Kaufman and Neurath, with carboxybenzoylglycyl-L-phenylalanine as
substrate (7).

Results

Peptide Contaminants—Rhodopsin, isolated in the usual manner and
exhaustively dialyzed against distilled water, upon examination for N-
terminal amino acids revealed the presence of DNP-glycine in almost
equimolar concentration. When such rhodopsin was further treated
with Dowex 50 resin, hydrogen form, the eluate from the resin contained
strong ninhydrin-positive material. This was later identified as peptide
or peptides containing DNP-glycine as the N-terminal residue (see below).
Evidently rhodopsin prepared in the usual manner is not pure, and the
equimolar N-terminal glycine which it contains is associated with a peptide
contaminant.

One consequence of the treatment with Dowex 50 resin is a change in
pH of the solution from neutrality to pH 2 or 3. This acidity denatures
and bleaches rhodopsin (19). A method was therefore sought which
would remove the adhering peptides without destroying the pigment (20).

Mixed bed ion exchange resins proved well suited for this purpose.
These provide an environment of minimal pH change, and remove salts,
amino acids, small molecular weight peptides, and fatty acids without
bleaching rhodopsin or altering its regenerability. The results of an
experiment with this procedure are shown in Table I. 14.1 mg. of rhodop-
sin were added to a mixed bed column, and samples were collected at
4\(^\circ\) in red light by using 1 per cent digitonin as eluent. The 400:500 ratio
of the rhodopsin initially was 0.245, the 278:500 ratio 2.31, and the maxi-
mal regeneration, 55 per cent. Table I shows that these values are not
changed significantly by this method of purification.

When samples of such purified rhodopsin were permitted to react with
Dowex 50 resin, hydrogen form, 4 per cent cross-linkage, 20 to 50 mesh,
for several hours and the NH\(_2\)OH effluents subjected to chromatography
in butanol-acetic acid-water (4:1:5), no ninhydrin-positive material was

\(^2\) Kindly supplied by Dr. Norman Krinsky.
detected. The source of this material in the original preparation had been removed efficiently by the mixed bed resin.

*N-Terminal Analysis of Purified Rhodopsin*—In the hope that N-terminal analyses could be carried out without destroying rhodopsin, the mildest possible conditions were employed for the reaction with FDNB. Even at 0°, however, and in the absence of ethanol, a typically insoluble, yellow powder was obtained. The rhodopsin clearly had been bleached by the procedure.

In a typical experiment a solution of rhodopsin containing $1.25 \times 10^{-7}$ mole in 2.5 ml. of 1 per cent digitonin and 0.1 M KCl at pH 8.0 was allowed to react at 25° with about 0.1 ml. of FDNB, with vigorous stirring in order to keep the solution saturated with the reagent. The pH was maintained at 8.0 by small additions of standard alkali and the reaction mixture mechanically shaken for 12 hours with white light excluded (14). The DNP rhodopsin, which precipitated as a yellow powder, was centrifuged and washed with water, ethanol, and ether, and was dried in air. From analogous treatment in digitonin controls it appeared that the water and ethanol washings had removed virtually all the digitonin, leaving the yellow DNP rhodopsin. This is insoluble in water and is not rendered soluble by digitonin, though it can be brought into solution in trichloroacetic acid.

The DNP protein thus obtained was divided into two portions (about $1.25 \times 10^{-7}$ mole) which were hydrolyzed for 4 and 8 hours in 5.7 N HCl

### Table I

**Purification of Rhodopsin on Column of Mixed Bed Ion Exchange Resins**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Total effluent (ml.)</th>
<th>Ratio, $\frac{400}{500}$</th>
<th>Ratio, $\frac{278}{500}$</th>
<th>Regeneration per cent</th>
<th>Rhodopsin* mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original preparation</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>8.5</td>
<td>0.245</td>
<td>2.31</td>
<td>55</td>
<td>14.1</td>
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<tr>
<td>4</td>
<td>0.0</td>
<td>0.204</td>
<td>2.38</td>
<td></td>
<td>0.38</td>
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<tr>
<td>5</td>
<td>11.3</td>
<td>0.246</td>
<td>2.41</td>
<td>54</td>
<td>2.46</td>
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<tr>
<td>6</td>
<td>12.5</td>
<td>0.262</td>
<td>2.28</td>
<td>53</td>
<td>3.68</td>
</tr>
<tr>
<td>7</td>
<td>13.6</td>
<td>0.253</td>
<td>2.26</td>
<td>53</td>
<td>3.00</td>
</tr>
<tr>
<td>8</td>
<td>15.0</td>
<td>0.261</td>
<td>2.25</td>
<td>54</td>
<td>2.12</td>
</tr>
<tr>
<td>9</td>
<td>16.6</td>
<td>0.252</td>
<td>2.28</td>
<td>55</td>
<td>0.87</td>
</tr>
<tr>
<td>10</td>
<td>19.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Weight of rhodopsin calculated on the basis of a molar extinction of 40,600 (2) and a molecular weight of 40,000 (1). The total recovery from the column was 89 per cent.
at 110° in sealed tubes. The ether-extractable constituents of the 4 and 8 hour hydrolysates contained dinitrophenol and dinitroaniline, but no DNP-amino acid. The acid-soluble fraction of the 8 hour hydrolysate of the DNP protein contained only ε-DNP-lysine even when the fraction was hydrolyzed 20 hours longer. Neither DNP-proline nor DNP-hydroxyproline could be detected in the ether extract of the product of hydrolyzing the DNP protein in 12 N HCl for 24 hours. Equally negative results were obtained in experiments in which the purified rhodopsin was bleached before reacting with FDNB.

Because it omits ethanol from the reaction mixture, the conjugation of rhodopsin with FDNB, according to Levy (14), is milder than the procedure of Sanger (13). Ethanol in small concentrations at room temperature bleaches rhodopsin and denatures opsin. However, Sanger's method yielded the same results. It is concluded that, when properly purified, rhodopsin does not contain an available N-terminal amino acid.

C-Terminal Analysis of Purified Rhodopsin—It is possible to carry out C-terminal analyses under conditions which do not destroy rhodopsin. Purified rhodopsin, bleached and unbleached, was incubated with carboxypeptidase for various lengths of time, and the reaction mixtures were analyzed for liberated amino acids. To check whether the C-terminal portion of the rhodopsin molecule is involved in the formation of the chromophore, the 400:500 ratio was followed during the course of the incubation. Since, to keep the rhodopsin in solution, the reaction must be carried out in 1 per cent digitonin, the behavior of carboxypeptidase in this solvent must be investigated.

In a typical experiment, rhodopsin was first passed through the mixed bed ion exchange column. 1.9 × 10⁻² mole of rhodopsin (corresponding to about 8 mg. of protein) and carboxypeptidase were incubated for 3 hours at pH 7.8 at 25° at an initial substrate-enzyme ratio of 24:1. After 30 minutes incubation, an additional amount of carboxypeptidase was added to a final substrate-enzyme ratio of 12:1. A portion of the reaction mixture was bleached after 90 minutes incubation. Samples were removed at 1, 5, 10, 60, 120, and 180 minute intervals and analyzed for the 400:500 ratio, carboxypeptidase activity, and for liberated amino acids (18).

One-dimensional paper chromatography did not detect any traces of amino acids liberated from unbleached or bleached rhodopsin by the action of carboxypeptidase. No liberated amino acids were observed also in experiments in which the bleached and unbleached protein were incubated for 9 hours at a substrate-enzyme ratio of 10:1.

Carboxypeptidase retains its full activity in the presence of rhodopsin

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2 Dinitrophenol was bleached by exposure of the paper to HCl vapor. Dinitroaniline runs with the solvent front and was thus identified.

4 George Wald, unpublished observations.
and digitonin throughout a 3 hour incubation period. During such an incubation, the 400:500 ratio of the rhodopsin increased from an initial value of 0.245 to 0.293, indicating that some bleaching had occurred. Such changes were observed in several experiments. Rhodopsin controls showed no change in 400:500 ratio during this period. It should be pointed out that DFP, present in high concentrations during these incubations, may have contributed to the bleaching.

Identification of Peptides Adhering to Rhodopsin—A rhodopsin solution, containing about 3 mg. of protein, was dialyzed in the cold against distilled water, previously adjusted to pH 7 with 1 M NaOH. It was then treated with Dowex 50 ion exchange resin, in the hydrogen form. The resin was eluted with NH₄OH and the eluent chromatographed on paper. Two distinct ninhydrin-positive spots were observed, similar in Rₛ to lysine and glycine, of which the glycine-like spot was the more intense. Very faint spots similar in Rₛ to cysteine and alanine were also apparent.

Elution of the glycine-like spot, followed by hydrolysis in a sealed tube in 5.7 N HCl at 110° for 24 hours, indicated the presence of lysine, serine, glycine, alanine, and glutamic acid in equimolar amounts and traces of proline, valine, phenylalanine, and leucine (Fig. 1). Elution and hydrolysis of the spot corresponding in Rₛ to lysine gave the identical pattern, except that the traces of proline, valine, phenylalanine, and leucine were absent.

To identify the N-terminal amino acids of these two peptides, the lysine-like and glycine-like spots (associated originally with 6 mg. of rhodopsin) were eluted and allowed to react with FDNB according to the method of Sanger and Thompson (21). The DNP peptides thus obtained were each divided into two portions and hydrolyzed for 4 and 8 hours, respectively, in 5.7 N HCl at 110° in sealed tubes. The yellow hydrolysates were diluted with twice their volume of water and the DNP amino acids extracted with ether. Both ether extract and aqueous solution were evaporated to dryness in vacuo.

The ether-extractable constituents of the 4 and 8 hour hydrolysates of both the lysine-like and glycine-like peptides contained DNP-glycine, dinitrophenol, and dinitroaniline. Very faint traces of DNP-valine, DNP-phenylalanine, and DNP-leucine were also present. No DNP amino acids were found in the aqueous layer.

From these results it can be concluded that the ninhydrin-positive spots corresponding in Rₛ to lysine and glycine are peptides, similar in amino acid content, and both having glycine as the N-terminal amino acid. This is evidently the source of the N-terminal glycine found in our rhodopsin preparations before treatment with ion exchange resins.

It has already been mentioned that such glycine was found in amounts roughly equal in molarity to rhodopsin, implying that the glycine-terminal
peptides are associated with rhodopsin roughly mole for mole. This point is sufficiently interesting to merit a review of the procedures involved. The results are summarized in Table II.

Samples of DNP rhodopsin, which had not been treated with ion ex-

![Figure 1](http://example.com/figure1.png)

**Fig. 1.** Paper chromatogram of hydrolysate of peptide removed from cattle rhodopsin by treatment with ion exchange resins (left); compared with a known mixture of amino acids (right). This is the peptide that before hydrolysis displayed an R_f like glycine. Descending chromatogram on Whatman No. 1 filter paper, at 25°. Solvent butanol-acetic acid-water (4:1:5).

change resins, were hydrolyzed in sealed tubes for 4, 8, and 24 hours, in 5.7 or 12 N HCl at 110°. The ether-extractable constituents of the 8 hour hydrolysate contained only DNP-glycine, dinitrophenol, and dinitroaniline. DNP-glycine was separated and distinguished from DNP-serine by chromatography of the ether extract in 1.5 M phosphate (17). The acid-soluble fraction of the hydrolysate of the DNP protein contained only ε-DNP-lysine. Neither DNP-proline nor DNP-hydroxyproline
could be detected in the ether extract of a 24 hour hydrolysate of the DNP protein in 12 N HCl. The 4 hour hydrolysate in 5.7 N HCl gave a prominent spot for DNP-glycine. Examination of a hydrolysate of the DNP protein oxidized with performic acid indicated the complete absence of DNP-cysteic acid (22).

DNP-glycine was therefore the only N-terminal amino acid found in preparations not treated with resins. Its quantity was determined as follows. The quantity of rhodopsin prior to hydrolysis was determined by measuring its extinction, before conjugation with FDNB. The FDNB reaction and washings were carried out in one test tube, and all precautions were taken to prevent loss of material. The DNP rhodopsin was hydrolyzed in the same test tube and extracted with ether. The ease with which DNP-glycine is destroyed on acid hydrolysis made it necessary to carry out the quantitative determination after different periods of hydrolysis. Controls containing DNP-glycine alone and together with denatured rhodopsin were similarly treated. The ether-extractable materials were chromatographed, eluted from the paper with 1 per cent NaHCO₃, and made up to volume. The concentration was determined by measuring the optical density at 350 m\(\mu\); the molar extinction coefficient of DNP-glycine at this wave length was found to be 1.69 \(\times\) 10⁴. Table II (last column) shows that the glycine N-terminal peptides are associated with rhodopsin in a roughly equimolar amount.

### Table II

<table>
<thead>
<tr>
<th>Preparation*</th>
<th>Extinction ratios before FDNB treatment</th>
<th>Hydrolysis in 5.7 N HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio, 400</td>
<td>Ratio, 278</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>0.280</td>
<td>1.93</td>
</tr>
<tr>
<td>‡</td>
<td>0.266</td>
<td>2.51</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.248</td>
<td>2.23</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.216</td>
<td>2.29</td>
</tr>
</tbody>
</table>

* Sanger's method was used in all the preparations except when otherwise stated.
† The values presented are those of denatured and hence bleached pigment, not native rhodopsin.
‡ Twice carried through FDNB conjugation.
§ FDNB reaction according to Levy and Li (14).
DISCUSSION

Once rhodopsin has been carefully freed from small molecular weight impurities, N-terminal analyses performed according to the method of Sanger and C-terminal analyses with carboxypeptidase indicate a complete absence of either type of group.

It is unfortunate that rhodopsin is insoluble in water in the absence of agents solubilizing. This has prevented the application of experimental procedures ordinarily used to test protein homogeneity. Up to the present, the optical properties of rhodopsin have been its most reliable index of purity. The 278:500 ratio provides an acceptable index of relative purity of different samples if one is concerned only with impurities such as proteins or peptides containing aromatic amino acids. However, this index cannot be taken as a measure of absolute purity, since the correct ratio for "pure" rhodopsin is not known, and the ratio is insensitive to non-absorbing impurities, including non-aromatic amino acids and their peptides. Indeed the peptide impurities which were removed in the mixed bed resin contained no aromatic amino acids, and hence could not affect this ratio. With regard to protein impurities present in rhodopsin solutions, it seems unlikely that such impurities, if present, would be unreactive toward carboxypeptidase and would show no N-terminal groups with FDNB; that is, the absence of terminal groups after purification is itself good evidence that probably no foreign proteins are present.

The use of carboxypeptidase for C-terminal analysis has been carefully considered by Gladner and Neurath (18). Recent work by Niu and Fraenkel-Conrat (23) has emphasized the importance of the use of chemical methods for C-terminal analysis rather than reliance solely upon the use of carboxypeptidase. Such chemical methods would have to be employed to establish more rigorously the absence of C-terminal groups in rhodopsin. However, all of them involve conditions which would destroy the pigment. For the present, it is possible only to conclude that rhodopsin, bleached or unbleached, contains no C-terminal amino acids available to carboxypeptidase.

The slight increase of the 400:500 ratio during the incubation of rhodopsin with carboxypeptidase is probably caused by traces of the proteolytic enzymes, trypsin and chymotrypsin, present in carboxypeptidase preparations and not completely inhibited by DFP. It has been observed in this laboratory that trypsin and chymotrypsin bleach rhodopsin with consequent increase of the 400:500 ratio.6

The N-terminal analysis, since it employs the small molecular reagent

6 George Wald and C. M. Radding, unpublished observations.
FDNB rather than an enzyme, yields a more reliable result. Even here, however, the possible presence of interfering components such as lipides or sugars does not permit a final judgment. Negative results found with the DNP method have been interpreted to mean lack of N-terminal residues in several other proteins such as ovalbumin (24, 25), tropomyosin, and myosin (26).

We have referred to the glycine N-terminal peptides which accompany rhodopsin before treatment with ion exchange resins as "contaminants." That they may have some more particular significance is suggested by the observations (1) that they are associated with rhodopsin so intimately as not to be removed by drastic preextraction of retinal tissue with aqueous solvents or by exhaustive dialysis of rhodopsin solutions, and (2) that they are present in a quantity roughly equimolar with rhodopsin (Table II).

The analysis of N-terminal and C-terminal residues of rhodopsin was originally undertaken in the hope that the findings, if positive, would serve as a logical starting point for sequence investigation and would perhaps yield some insight into the nature of the linkage between retinene and opsin. For these, one must evidently turn to other methods.

It is a pleasure to express gratitude to Professor George Wald for many valuable and constructive criticisms during the preparation of the manuscript.

SUMMARY

Cattle rhodopsin, prepared by standard methods and allowed to react with 1,2,4-fluorodinitrobenzene, was found to contain dinitrophenylglycine, in approximately equimolar concentration, as the N-terminal amino acid. However, this proved to be the terminal group of small molecular peptide (or peptides), strongly adsorbed to rhodopsin, but removed from the protein by treatment with mixed bed ion exchange resins. This treatment did not alter the optical properties of rhodopsin, its photosensitivity or regeneration after bleaching. After such treatment, cattle rhodopsin was found to contain no N-terminal or C-terminal amino acids available to 1,2,4-fluorodinitrobenzene or carboxypeptidase.

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TERMINAL AMINO ACIDS OF RHODOPSIN
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