Carbohydrate Composition of Bovine Rhodopsin*

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The carbohydrate content of bovine rhodopsin was investigated and found to be different from previously reported values.

Rod outer segments were isolated from dark-adapted bovine retinas by sucrose flotation and purified by sucrose density centrifugation. Rhodopsin was extracted with detergents and purified by chromatographic procedures involving calcium phosphate/cellulite chromatography followed by affinity chromatography on concanavalin A-Sepharose (or in some cases, gel filtration on agarose). Purified preparations of rhodopsin had A280/A490 ratios of 1.6 to 2.0. After treatment of the rhodopsin with chloroform/methanol (2/1) to remove lipids and detergents, the carbohydrate content was measured by gas-liquid chromatography, colorimetric and enzymatic analyses, paper chromatography, and electrophoresis. Rhodopsin was found to have about 9 mol of mannose and 5 mol of glucosamine per mol of visual pigment. A molar ratio of mannose/glucosamine of about 2 was also found in samples of rhodopsin obtained from two other laboratories. The amino acid analysis was similar to previously published values.

It has been estimated that visual pigment accounts for the bulk of the protein of the discs in the rod outer segments of the mammalian retina (1). Heller (2) and Heller and Lawrence (3) have reported that the bovine visual pigment, rhodopsin, is a glycoprotein containing mannose and glucosamine. Their analyses showed the presence of 3 mol of each of these two sugars per mol of rhodopsin. Apart from those reports, little information has been published concerning the carbohydrates of this molecule. Although the detailed structure of the carbohydrate groups in rhodopsin is not known, other glycoproteins that contain a single carbohydrate chain composed of this amount of mannose and glucosamine have not been described as yet. The present report is a reinvestigation of the carbohydrate content of bovine rhodopsin. While our amino acid analysis was similar to that reported previously (2, 4–6), the quantitative carbohydrate composition of the constituent sugars, mannose and glucosamine, was different, and is in accord with that described for other glycoproteins containing only these two sugars.

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MATERIALS AND METHODS

Dark-adapted retinas were obtained from George Hormel Co., Austin, Minn., and were placed in the dark at -20° until used. Emulphogene BC-720 (alkoxypoly(ethyleneoxy)ethanol) was donated by General Aniline and Film Corp., New York, N. Y., and Ammonyx LO (a 30% aqueous solution of alkylidimethylamine oxide) was a gift from the Onyx Chemical Co., Jersey City, N. J. Reagent grade chloroform and methanol were redistilled before use.

All operations for the isolation of visual pigment were performed at 4° under conditions of dim red light (Wratten 1A filters and red lights (General Electric type BAS)).

Isolation of Rod Outer Segments-For most of these studies rod outer segments were isolated by modifications of the procedure of Papernoster and Dreyer (7), while in early experiments the procedure of O'Brien et al. (8) was used. Fifty retinas were thawed, distributed into two Sorvall centrifuge tubes (10.5 x 3 cm), and homogenized gently in 25 ml of Solution A which contained 1.23 M sucrose/2 mM MgCl2/85 mM NaCl/5 mM Tris-acetate buffer, pH 7.4. Homogenization was performed in the Sorvall tubes by means of five strokes with a Teflon pestle rotating at slow speed. The homogenate was centrifuged at 3,000 x g (calculated at the bottom of the tube) for 5 min in a Sorvall SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.), and the supernatant fluid which contained the crude rod outer segments was removed. The pellet was rehomogenized and the homogenate centrifuged as above. The combined supernatant solutions were diluted 3-fold with 10 mM Tris acetate buffer, pH 7.4, and the mixture centrifuged for 20 min at 48,000 x g. The pellets from this centrifugation (containing the crude rod outer segments) were combined and washed by resuspension in a solution composed of 10 ml of Solution A and 20 ml of 10 mM Tris/acetate buffer, pH 7.4, and recentrifuged for 20 min at 48,000 x g. The crude rod outer segments were resuspended in 12 ml of 0.77 M sucrose containing 5 mM Tris/acetate buffer, pH 7.4, and 1 mM MgCl2 (Solution B) and homogenized manually with a Kontes conical glass homogenizer (Kontes Glass Co., Vineland, N. J.).

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The homogenate was then passed through a 26 gauge needle and the volume of the effluent adjusted to 16 ml by the addition of Solution B. The suspension was divided into two 0.5 ml portions and each added to the top of a discontinuous sucrose gradient. The gradient was prepared by layering 8 ml portions of 1.14 M, 1.0 M, and 0.84 M sucrose solutions, all containing 0.1 mM MgCl₂ and 5 mM Tris/acetate, pH 7.4. The concentration of the sucrose solutions was monitored with an Abbé refractometer and adjusted as necessary. The tubes were centrifuged at 90,000 × g for 45 min in a SW 25.1 rotor (Beckman Instruments, Palo Alto, Calif.). The material separated into bands as previously described (7). The bulk of the rod outer segments were present at the 0.84/1.0 M sucrose interface, with additional outer segments present in the 1.0/1.14 M sucrose interface. Only the material from the upper band was used for the preparation of rhodopsin. The material at the interface was recovered and diluted 3-fold with 10 mM Tris/acetate bufer, pH 7.4, and the rod outer segments sedimented by centrifugation at 48,000 × g for 20 min. They were then washed twice by resuspension in the same buffer and recentrifugation. The final pellet of purified material was stored at -70° in the dark.

**Carbohydrate Composition of Rhodopsin**

Preparation of Rhodopsin—Several alternative procedures for preparing rhodopsin were investigated. Table I shows the analyses at various steps in a representative experiment involving several established techniques for the isolation. The yield of rhodopsin is based on the amount extracted from purified rod outer segments. Evaluations that are based on the A₂₅₀ of crude fractions, such as the crude homogeneous or crude rod outer segments are not regarded as reliable due to high backgrounds and distortions in the spectra. The A₂₅₀/A₄₉₈ ratio is a widely used index to assess purity of rhodopsin, the lower the ratio, the greater the purity. Although a wide range in this ratio has been reported, preparations having ratios of up to about 2.5 have been regarded as indicating "purified" rhodopsin (20), ratios as low as 1.6 have been obtained (7, 8).

Chromatography on columns of calcium phosphate/celite has been used extensively in the preparation of rhodopsin (5, 7, 8, 21). The recovery of rhodopsin from this adsorbent ranged from 70% to 80%, and the A₂₅₀/A₄₉₈ varied from 1.6 to 1.8 (Table I).

The presence of covalently bound mannosse makes rhodopsin susceptible to fractionation by affinity chromatography on Con A-Sepharose (Fig. 1). This technique has been used previously by Steinemann and Stryer (22). No A₄₉₈-absorbing material and variable amounts of A₂₅₀-absorbing material were observed during elution with buffer alone. When 0.25 M α-methyl-d-glucoside was added to the buffer, rhodopsin was eluted from the column. The recovery varied from 70% to 80% of the amount applied to this column, and the A₂₅₀/A₄₉₈ varied from 1.6 to about 2.1. The increase in ratio was thought to indicate some bleaching during the procedures, but more likely was the result of spectral contributions from concentration of the detergents, as has been suggested previously (2). The overall yield of rhodopsin through these steps ranged from about 45% to 60%. These procedures resulted in an enrichment of rhodopsin of about 2-fold when compared to the concentration of rhodopsin in purified rod outer segments, and about 4-fold
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TABLE I
Analyses of rhodopsin during purification

| Fraction                        | $A_{280}$/$A_{400}$ | Rhodopsin$^a$ | Protein recovery | Mannose$^b$ | Glucosamine$^c$ | Mannose/glucosamine |
|--------------------------------|---------------------|---------------|-----------------|-------------|-----------------|---------------------|
|                                | %                   | g mol/100 mg protein | %              | %           | %               | %                   |
| Crude rod outer segments$^d$   | 13                  | 5.3           | 100             | 17          | 6.7             | 2.5                 |
| Purified rod outer segments    | 2.7                 | 100$^e$       | 100             | 100         | 6.6             | 1.7                 |
| Crude rhodopsin                | 2.7                 | 100           | 97              | 80          | 8.8             | 4.4                 |
| CaPO$_4$/celite                | 1.7                 | 69            | 30              | 46          | 7.7             | 3.9                 |
| Con A-Sepharose                | 2.1                 | 49            | 28              | 36          | 8.9             | 5.4                 |
| Final concentrate              | 2.1                 | 45            | 22              | 34          | 8.6             | 5.0                 |

$^a$ Rhodopsin concentration was based on $A_{400}$ with the use of an extinction coefficient of 40,600 (19).

$^b$ Mannose and glucosamine were assayed by GLC after the fractions were treated as described under "Materials and Methods."

$^c$ Molar ratio of mannose/glucosamine.

$^d$ Rod outer segments were isolated by the procedure of Papermaster and Dreyer (7), and rhodopsin was purified as described under "Materials and Methods."

$^e$ The 100% value was 0.63 g mol/50 retinae.

when compared to the crude preparation. These values are minimal since they refer only to detergent-extractable protein.

When Con A-Sepharose is used, there appears to be little advantage in the use of the calcium phosphate/celite step. In addition to removing extraneous protein, the affinity column also removed non-rhodopsin carbohydrate components (Table I) that were not separated from rhodopsin by calcium phosphate/celite.$^2$

Absorption Spectra, SDS Polyacrylamide Gel Electrophoresis, Purity—Bovine rhodopsin, purified by these procedures, had the typical absorption spectrum (6) in the native state and after bleaching (Fig. 2). These same spectral properties were observed in all of the fractions from the purified rod outer segments through the remaining steps in the fractionation procedure outlined in Table I. SDS polyacrylamide gel electro-

$^2$ In studies dealing with the cell-free incorporation of mannose into glycoproteins of the retina, other non-rhodopsin components which contained mannose were not separated from rhodopsin by calcium phosphate/celite columns, but were separated from rhodopsin by chromatography on Con A-Sepharose (E. L. Kean and J. J. Plantner, manuscript in preparation).

phoresis of rhodopsin purified by these procedures showed patterns similar to that described previously for purified rhodopsin (7). Under the conditions used$^3$ a molecular weight of about 35,000 can be calculated for rhodopsin. The criteria for the purity of the rhodopsin used in these studies (obtained by well established procedures of rod purification, extraction of rhodopsin, and chromatographic techniques) were: the low $A_{370}$/$A_{400}$ ratios, the spectral properties before and after bleaching, and the results of SDS-polyacrylamide gel electrophoresis. The procedures used here produced a product whose purity was as high as or higher than that published elsewhere for bovine rhodopsin.

Chloroform/Methanol Treatment; Amino Acid Analysis—It was not possible to analyze the carbohydrate content of rhodopsin directly by GLC because of interference by the detergents present in the buffers and the lipids associated with this molecule. The interfering substances were effectively removed by treating rhodopsin preparations with 20 volumes of

$^3$ Samples containing 0.25 to 1.0 nmol of rhodopsin were preincubated with 2.5% SDS (7) and applied to gels containing 5.6% acrylamide. Standard proteins (cytochrome c, myoglobin, chymotrypsinogen, ovalbumin, and bovine serum albumin) were treated similarly.
chloroform/methanol (2/1). Rhodopsin is insolubilized by this process. The distribution of protein was determined by a complete amino acid analysis of the starting material (purified rhodopsin, in the presence of detergent lipids, and buffer) and of the soluble and insoluble fractions after treatment with chloroform/methanol (9/1). Glucosamine was also measured in these same fractions by means of the amino acid analyser. As can be seen in Table II, there was essentially complete recovery of protein and glucosamine in the precipitate that was formed.

The amino acid analysis of rhodopsin purified by these techniques is presented in Table III, and is similar to that reported by several other laboratories (2, 4–6). The data presented in Table III are average values from hydrolyses carried out for 24, 48, and 72 hours, not extrapolated to zero time since an increase of about 9% in the yield of total amino acids was observed over this period of time (data not shown). Presented also are calculations based on residues per 100 residues. There is good agreement between these values and the average value of several reports in the literature. Tryptophan was not determined but a value of 5 residues/mol has been reported elsewhere (2).

Carbohydrate Analyses: GLC, Enzymatic, Colorimetric—After rhodopsin was treated with chloroform/methanol, mannose and glucosamine were the only sugars detected in the insoluble residue. Table I shows analyses of a representative study from various stages in the purification of rhodopsin. About 34% of the total mannose and glucosamine and 45% of the rhodopsin present in the purified rod outer segments were recovered in the final purified product. At this stage there were approximately 9 mol of mannose and 5 mol of glucosamine per mol of rhodopsin. A molar ratio of mannose to glucosamine of about 2 was observed also at each step after the isolation of the purified rod outer segments. Additional fractionation of rhodopsin on columns of agarose (see under “Materials and Methods”) did not alter the carbohydrate analysis (data not shown).

A summary of carbohydrate analyses of rhodopsin purified by chromatography on columns of calcium phosphate/celite followed by Con A-Sepharose is presented in Table IVA. Given also in this table are analyses for mannose and glucosamine by means of different techniques of measurement. Relatively good agreement was obtained among the several diverse methods, indicating the general reliability of the measurements. Data from GLC (a procedure common for both of the sugars) showed the presence of about 9 mol of mannose and 3 mol of glucosamine per mol of bovine rhodopsin. As a control, analysis of the carbohydrate content of ovalbumin by these techniques showed the presence of mannose and glucosamine in amounts similar to those reported by other authors (23).

Heterogeneity of the carbohydrate components of glycoproteins is a widely observed phenomenon, and may explain some of the variation observed in these investigations. The data in Table IVA were obtained for rhodopsin purified by a rigorously controlled procedure, described under “Materials and Methods.” In preliminary experiments, many variations were tried, such as the nature of the buffers, types and concentrations of detergents, different procedures for isolating the rod outer segments, and additions and deletions to the purification process. When the GLC data for all of these preparations (some of which had A280/A450 ratios greater than the preparations presented in Table IVA) were combined with those presented in Table IV the following values were calculated from 13 separate preparations of rhodopsin (moles/mol of rhodopsin ± S.E.): mannose, 7.7 ± 0.59; glucosamine, 4.1 ± 0.38. The molar ratio of mannose/glucosamine was 1.9 ± 0.08. It is perhaps not surprising that these procedural variations as well as the use of different batches of eyes over a period of over 2 years from cattle not reared under controlled laboratory conditions may have resulted in rhodopsin molecules showing somewhat different amounts of components present in relatively minor concentrations. However, in spite of these differences, samples of rhodopsin obtained by a variety of isolation and purification procedures contain amounts of mannose and glucosamine that differ substantially from the values reported previously (2, 3) with respect to both the absolute amounts of these two sugars and their molar ratio.

Purified rhodopsin was also obtained from two other labora-
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| Component       | Moles/mol rhodopsin |
|-----------------|---------------------|
| Mannose         | 8.8 ± 0.60 (5)      |
| GLC             | 7.1 ± 0.37 (2)      |
| Anthrone        | 8.3 ± 0.39 (2)      |
| Glucosamine     | 5.1 ± 0.50 (5)      |
| Elson-Morgan    | 4.5 ± 0.15 (3)      |
| Amino acid analyzer | 5.9 ± 0.31 (4) |

B. Molar ratios based on GLC

| Source of rhodopsin | Mannose/glucosamine |
|---------------------|----------------------|
| This laboratory*    | 1.1 ± 0.11 (b)       |
| Dr. P. J. O'Brien   | 2.1                  |
| Dr. E. W. Abrahamson| 2.0                  |

* Rhodopsin was purified by calcium phosphate/celite chromatography and affinity chromatography on Con A-Sepharose from rod outer segments isolated by the procedure of Papermaster and Dreyer (7) as described under "Materials and Methods." The calculation of moles of rhodopsin was based on A_m values with the use of an extinction coefficient of 40,600 (19).

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