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Transient Hyperglycosylation of Rhodopsin with Galactose

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Rhodopsin's oligosaccharide chains contain predominantly two types of sugar residues: mannose and *N*-acetylglucosamine. In the present work, bovine and rat rhodopsin were analysed biochemically for the presence of a third sugar, galactose. Treatment of bovine rod outer segments (ROS) with galactose oxidase followed by reduction with tritium-labeled sodium borohydride revealed the presence of existing molecules of galactose on rhodopsin. Rats injected intravitreally with [^{3}H]galactose and [^{14}C]leucine and maintained in darkness were killed 1 hr, 6 hr, 1, 3 or 5 days following the injection. Retinas were collected for subcellular fractionation and rhodopsin from each of the fractions was purified by ConA sepharose chromatography and SDS–PAGE. During the first 6 hr, galactose selectively labeled rhodopsin in the Golgi-enriched fraction resulting in increased [^{3}H]/[^{14}C] ratios in both Golgi and ROS. The data suggested that trimming was occurring at the transition from Golgi to ROS. Furthermore, a decrease in isotope ratio in the ROS between 6 hr and 1 day suggested further trimming of rhodopsin after membrane assembly in the ROS. Additional in vivo experiments demonstrated existing molecules of galactose on rhodopsin's oligosaccharide chain using lectin affinity chromatography. Rats injected intravitreally with [^{35}S]methionine were dark-adapted for 2 hr. Following subcellular fractionation of retinas, ConA purified rhodopsin from ROS was applied to one of two additional lectin columns: *Ricinus communis* agglutinin (RCA) or *Griffonia simplicifolia* I (GSA). Eight to nine per cent of the labeled rhodopsin was bound to and eluted from RCA, whereas none bound to GSA, indicating the presence of a β-galactoside. The RCA agarose eluted protein co-electrophoresed with a rhodopsin standard and was light sensitive. Galactose was shown to be the terminal sugar on this subset of rhodopsin and was not capped by neuraminic acid. Binding of rhodopsin's oligosaccharide to RCA was abolished by pre-treatment with β-galactosidase. Decreased binding of rhodopsin to RCA was observed following intravitreal injection of castanospermine but not swainsonine. Of those two inhibitors of glycoprotein trimming, only castanospermine would be expected to prevent the addition of galactose to the oligosaccharide. The association of galactose with rat rhodopsin appeared to be a transient one. At 2 hr, 8–9% of rhodopsin contained galactose, at 6 hr only 2·2% had galactose and by 24 hr less than 1% did. The galactose was trimmed from rhodopsin's oligosaccharide presumably after its role was complete. Separation of rhodopsin of the plasma membranes from rhodopsin of discs indicated that 75% of the galactose-containing rhodopsin was in the plasma membrane and only 25% was in the discs. These findings suggested a possible role for galactose in new disc formation with subsequent removal after the discs are sealed.

Key words: rhodopsin; rat; retina; glycosylation; galactose; lectin affinity chromatography; RCA; ricin.

1. Introduction

Rhodopsin contains two oligosaccharide chains linked to Asn_2 and Asn_{15} (Hargrave, 1977). For the most part, these chains contain only two types of sugar residues: mannose and *N*-acetylglucosamine (Heller, 1968; Shichi et al., 1969; Heller and Lawrence, 1970; Plantner and Kean, 1976). The synthesis of asparagine-linked glycoproteins typically involves the rough endoplasmic reticulum for the initiation of oligosaccharide synthesis and both the rough endoplasmic reticulum and Golgi complex for modification of the oligosaccharide chain (Kornfeld and Kornfeld, 1985). The oligosaccharide chain of rhodopsin is reminiscent of the 'core' oligosaccharide found in more complex glycoproteins prior to the addition of a terminal trisaccharide consisting of *N*-acetylglucosamine, galactose and neuraminic acid. Rhodopsin actually contains the first sugar of the terminal trisaccharide as demonstrated by glucosamine incorporation studies (O'Brien and Muellenberg, 1974; Bok, Hall and O'Brien, 1977). The remaining sugars, galactose and neuraminic acid have not usually been detected in the rhodopsin oligosaccharide chain. However, O'Brien (1976) demonstrated the direct transfer of galactose in vitro to bovine rhodopsin and showed chromatographically that the radiolabeled-galactose was incorporated as galactose since the radioactivity coincided with a galactose standard and was not converted to some other sugar (such as mannose). More recently, galactose was successfully incorporated into rat rhodopsin in vitro following incubation of whole retinas with [^{3}H]galactose (St Jules, Smith and O'Brien, 1990). Fukuda, Papermaster and Hargrave (1979) analysed bovine rhodopsin's

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amino terminal glycopeptide using gas chromatography and mass spectrometry and reported that in addition to the major oligosaccharide structure which contained three *N*-acetylglucosamine residues and three mannose residues, a minor variation of rhodopsin contained 0·1 mol galactose per three *N*-acetylglucosamine residues and three mannose residues. These data suggested that up to 10% of rhodopsin contained galactose.

Morphologic studies of carbohydrate-containing molecules in photoreceptor cells have invoked a different methodology utilizing lectins for detecting the presence of specific sugars. *Ricinus communis* agglutinin (RCA) is a lectin that specifically recognizes terminal galactose moieties associated with either glucosamine or *N*-acetylglucosamine in a β1–4 linkage (Baenziger and Fiete, 1979). Transmission electron microscopic studies of frog, bovine, monkey and rat retinas have shown binding of RCA to rod outer segments and in some cases rod inner segments (Nir and Hall, 1979; Hicks and Molday, 1985; Koide et al., 1986; Hicks and Barnstable, 1986). Likewise, similar results were obtained in fluorescent microscopic studies detecting RCA binding in frog, monkey, mouse, human, rabbit, and fish retinas (Bridges and Fong, 1979; Bridges, 1981; Uehara et al., 1983; Blanks and Johnson, 1984). Of great interest was the work by Hicks and Molday (1985) who published photomicroscopic evidence of post-embedding, direct labeling by RCA in the basal area of the ROS.

In the present study, we used biochemical techniques to characterize the subset of rhodopsin which appears to contain galactose. We present evidence to confirm the incorporation of galactose into rhodopsin in in vivo experiments. Additionally, we demonstrate the presence of galactose in bovine rhodopsin by radiolabeling existing moieties. Finally, using lectin affinity chromatography, an analytical procedure which is rapid, reliable and highly sensitive (Cummings, Merkle and Stults, 1989), we are able to separate the galactose-containing species of rhodopsin and study its transient association with the oligosaccharide chain.

2. Materials and Methods

Animals

For most experiments, Sprague–Dawley rats, 125–150 g (Taconic Farms, Germantown, NY) were used. One set of experiments utilized bovine retinas dissected from bovine eyes obtained fresh from a slaughterhouse.

Isotopic Labeling

For studies of in vivo labeling of rhodopsin with galactose, rats were anesthetized with ether and injected intravitreally with 2 μl per eye of a saline solution containing 2 μCi [^{14}C]leucine and 87·5 μCi [4–5-^{3}H]galactose (44·1 Ci mmol^{-1}). In other in vivo experiments, eyes were injected with 20 μCi [^{3}H]-leucine (60 Ci mmol^{-1}) or 3 μCi [^{35}S]methionine (1105 Ci mmol^{-1}) or 3 μCi [^{35}S]methionine and 20 μCi [^{3}H]glucosamine (44·8 Ci mmol^{-1}). The rats were maintained in darkness and at various intervals were killed with CO_2. The retinas were removed by proptosing the eye using a curved forceps, the prongs of which were ensheathed in polyethylene tubing. The cornea was slit with a scalpel blade causing the lens and vitreous humor to be extruded. The retina was lifted free of any other ocular material simply by squeezing and lifting the forceps upwards. The sclera remained attached to the optic nerve. This procedure takes only 10–15 s and the retinas are removed intact and ready for biochemical workup. No elaborate dissection is required.

[^{3}H]Galactose, [^{3}H]glucosamine, [^{3}H]leucine and [^{14}C]leucine were purchased from New England Nuclear; [^{35}S]methionine was purchased from American Radiolabeled Chemicals, Inc.

Subcellular Fractionation and Purification of Rhodopsin

Isolated retinas were fractionated in dim red light by a method described previously (St Jules, Smith and O'Brien, 1990). Four to six retinas were suspended in 2 ml 40% sucrose containing 65 mM NaCl, 2 mM $MgCl_2$, and 5 mM Tris acetate pH 7·4. Rod outer segments were broken off with 15 3-sec bursts of a vortex mixer. After a 15-min centrifugation at 27000 *g* the floating ROS were removed with the supernatant and the retinal debris was treated again as above. The pooled crude ROS suspension was diluted with 3 volumes of buffered 10% sucrose, or in some cases 0·9% NaCl, and were sedimented by centrifugation at 27000 *g* for 15 min. Both the ROS and retinal debris were separately homogenized in 2 ml 20% sucrose made up in 65 mM NaCl, 2 mM $MgCl_2$, and 5 mM Tris acetate pH 7·4 using seven strokes of a Dounce tissue grinder (Wheaton 7 ml, B pestle with 0·0025–0·0035-inch clearance). These homogenates were layered over separate linear 50–25% sucrose gradients made up in the same buffer. After centrifugation for 2 hr in a Spinco SW 41 rotor at 35000 rpm, bands were removed with a Pasteur pipet, diluted with 10% sucrose and sedimented by centrifugation for 15 min at 27000 *g*. Two bands of unsealed and sealed ROS were near the top of the ROS gradient (Godchaux and Zimmerman, 1979). Below these was a zone of fine particles, enriched in Golgi. In the retinal debris gradient was a trace of the Golgi-enriched fraction and a prominent zone of coarse particles, including the rough endoplasmic reticulum, nuclei and synaptosomes. These fractions are described in more detail in St Jules and O'Brien (1986).

With the bovine retinas, discontinuous gradients were prepared by overlaying the bovine ROS sus-

pension with the following sucrose solutions: 2 ml of $d1{\cdot}15$, 5 ml of $d1{\cdot}13$ and 2 ml of $d1{\cdot}11$. The sucrose solutions were made up in 1 mM Tris acetate, pH 7·4, containing 0·1 mM $MgCl_2$. After centrifugation for 45 min at 35000 rpm in a Spinco SW 40Ti rotor, the ROS were removed from the interface at $d1{\cdot}11/d1{\cdot}13$ with a Pasteur pipette, diluted with 10% sucrose and sedimented by centrifugation for 15 min at 27000 *g*.

Purification of Rhodopsin

Rhodopsin-containing membranes from four to six rat retinas were extracted in dim red light for 1 hr at 0°C with 2 ml 1% Emulphogene BC 720 (General Aniline and Film) in 50 mM Tris acetate buffer, pH 6·9, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The extract was clarified by centrifugation at 27000 *g* for 15 min and applied to a 0·8 ml column of ConA sepharose (Pharmacia). After washing with 25 ml 0·1% Emulphogene in the same buffer, the rhodopsin was eluted with eight applications of 200 μl aliquots of 0·5 M α-methylmannoside (Sigma) also made up in the same buffer with 0·1% Emulphogene. Twenty microliters of each 200-μl fraction were sampled for radioactivity. Peak fractions were pooled and aliquots were adjusted to 2% sodium dodecyl sulfate (SDS) and 5 mM EDTA. Samples were incubated at room temperature (25°C) for 1 hr in dim red light. After the addition of one half volume of 50% glycerol containing 0·002% Bromophenol blue (Biorad) the samples were applied to 10% polyacrylamide disc gels with 3% stacking gels for electrophoresis by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue (Biorad) and cut into 1·2 mm slices with a Hoefer gel slicer and the slices containing the stained opsin were noted. Slices were digested overnight at 50°C in NCS protein solubilizer (Amersham) with 2·1% water. Slices were counted (two per vial) in Econofluor-2 (New England Nuclear, Boston, MA) using a Beckman LS 2800 scintillation counter. The mobility of radioactive opsin could be compared directly with that of the Coomassie blue stained mature opsin in the same cylindrical gel. Those experiments utilizing two isotopes (i.e. [^{3}H]galactose and [^{14}C]leucine) permitted determination of the ratio of these isotopes in the opsin band of the gels.

Labeling of Bovine ROS with Galactose Oxidase and Sodium Borohydride

To radiolabel existing galactose moieties on rhodopsin, bovine ROS purified by a discontinuous sucrose density gradient procedure, were subjected to enzymatic treatment with galactose oxidase followed by a reduction with tritium labeled sodium borohydride. In these experiments no external source of radioactive galactose was provided.

ROS from two bovine retinas were washed by centrifugation with PBS for 10 min at 27000 *g*. They were suspended in 1 ml PBS, an additional 1 ml of PBS containing 200 U of galactose oxidase (Worthington) was added to one ROS sample and 1 ml PBS without the enzyme was added to a second ROS sample as a control. Five microliters 0·1 M PMSF in ethanol were added to each sample. ROS were incubated at room temperature with occasional swirling for 30 min in the dark. ROS were centrifuged at 27000 *g* for 10 min, then resuspended in PBS and centrifuged for the same length of time and speed. ROS were suspended in PBS. To each sample was added 2·5 mCi of tritium labeled sodium borohydride (New England Nuclear) in 0·01 N NaOH made up in 1 ml PBS. After 30 min at room temperature, additional PBS was added and ROS were centrifuged at 27000 *g* for 10 min. Subsequently, ROS were washed twice by centrifugation in PBS. In a repeat of this procedure, one sample received pretreatment with 1 U neuraminidase from *Clostridium perfringens* (Sigma) at pH 5·3 for 1 hr at 37°C and the subsequent galactose oxidase incubation time was doubled. The ROS were solubilized and rhodopsin was analysed by SDS–PAGE as described above.

Lectin Affinity Chromatography

To further characterize the subset of rhodopsin which contains galactose (without introducing an external source of galactose) lectin affinity chromatography was used. In this method, ConA purified rhodopsin or in some cases the clarified extract of ROS from six rat retinas labeled with [^{35}S]methionine was applied to columns of either *Ricinus communis* agglutinin I (RCA) or *Griffonia simplicifolia* I (GSA), specific for β-linked or α-linked galactose residues, respectively. RCA agarose was purchased from U.S. Biochemical Corp., Cleveland, OH and GSA agarose was purchased from Biocarb Chemicals, Lund, Sweden. Columns were washed with 0·1% Emulphogene in 50 mM Tris acetate buffer, pH 6·9, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Samples were eluted from RCA agarose or GSA agarose columns with 200 μl aliquots of either 0·5 M β-methyl-D-galactopyranoside or α-methyl-D-galactopyranoside (U.S. Biochemical Corp.), respectively. Aliquots (20 μl) were analysed for radioactivity using a Beckman LS 2800 scintillation counter. Aliquots of the peak fractions were applied to SDS–polyacrylamide gels and analysed for radioactivity of opsin as described above.

Neuraminidase Treatment

To determine if the galactose of rhodopsin was capped with a neuraminic acid, rat ROS labeled with [^{35}S]methionine were incubated with 1 U neuraminidase from *Vibrio cholerae* (Boehringer-Mannheim) at 37°C for 2 hr. A second ROS sample was incubated with no enzyme. The incubated suspension was centrifuged for 15 min at 27000 *g*. The ROS were extracted for 1 hr and the clarified extracts were

applied to RCA agarose columns. Aliquots of the peak fractions were applied to SDS–polyacrylamide gels and analysed for radioactivity of opsin as described above.

β-*Galactosidase Treatment of Rhodopsin's Oligosaccharide*

ConA purified rhodopsin labeled with [^{35}S]methionine and [^{3}H]glucosamine was incubated at 37°C for 42 hr with 20 U of *N*-glycanase (Genzyme, Boston, MA) in 0·15% SDS, 10 mM β-mercaptoethanol, 100 μM PMSF, 15 mM EDTA, 1·25% NP–40, pH 7·8. At the end of the incubation the pH of the sample was adjusted to 6·0 by the addition of acetic acid. The solution was buffered with MES (Sigma). β-Galactosidase (200 mU) from *Diplococcus pneumoniae* (Boehringer-Mannheim) were added to half of the sample. Both the enzyme treated sample and the control were incubated at 37°C for 24 hr. The samples were applied to separate RCA agarose columns and subsequently eluted with 200-μl aliquots of β-methyl-D-galactopyranoside as described above. The amount of radioactivity eluted from RCA after β-galactosidase treatment was compared to that eluted from RCA without the enzyme treatment.

In vivo Treatment with Castanospermine or Swainsonine

To determine the consequences of the interruption of glycoprotein processing on the galactosylation of rhodopsin, two inhibitors of the glycoprotein processing pathway, castanospermine and swainsonine (Boehringer-Mannheim) were used in in vivo experiments. The concentration of the stock solution of castanospermine was 100 mg ml^{-1}. It was prepared by dissolving 5 mg of the inhibitor in 50 μl dimethyl sulfoxide (DMSO, Sigma). The concentration of the stock solution of swainsonine was 1 mg ml^{-1}. It was prepared by dissolving 500 μg of the inhibitor in 500 μl water. Rats were injected intravitreally with [^{35}S]methionine as a control or [^{35}S]methionine and either castanospermine (10 μg per eye) or swainsonine (0·17 μg per eye). Since the castanospermine preparation utilized DMSO at a final concentration of 0·08 μl per eye the same amount of DMSO was also injected into the eyes of all other rats to control for any effects of DMSO on the galactosylation of rhodopsin. After 1 hr of dark adaptation rats were killed, retinas were collected for subcellular fractionation. Rhodopsin was purified from the ROS fraction and the Golgi-enriched fraction by ConA sepharose chromatography. A 100-μl fraction of the ConA eluate was saved for SDS–PAGE and the remainder was applied to RCA agarose columns for elution with β-methyl-D-galactopyranoside as described above. The RCA eluates were then applied to SDS–PAGE gels. The percentage of ConA purified rhodopsin which contained galactose was determined from the radioactivity of the rhodopsin band of the gels of the RCA eluate as compared to the ConA gels for each treatment group. The data from the three repetitions of this experiment were analysed via a one-way analysis of variance for the ROS samples and for the Golgi samples. The test of significance used was Tukey's multiple comparison procedure.

Light-sensitivity Experiments

ROS labeled with [^{35}S]methionine and purified on a linear sucrose gradient were extracted with 1·0% Emulphogene as described above. Prior to purification of rhodopsin over ConA sepharose, half of the clarified extract was exposed to light for 10 min at 4°C. Each sample was then applied to a ConA sepharose column and chromatographed as described above. The eluted samples were each applied to separate RCA agarose columns and eluted according to the previously described procedures. Selected eluted fractions were applied to SDS polyacrylamide gels along with a [^{3}H]rhodopsin standard for determination of the amount of radioactivity present in opsin under the conditions of light exposure and darkness.

Plasma Membrane Preparation

In order to determine whether the galactose-containing rhodopsin was present in photoreceptor discs or plasma membrane a modification of the method of Molday and Molday (1987) was used. [^{35}S]methionine-labeled ROS from 18 rats were prepared using the linear sucrose gradient procedure described previously. ROS were suspended in 1 ml homogenizing buffer consisting of 20 mM Tris acetate, pH 7·4, 2 mM $MgCl_2$, 20% sucrose. Neuraminidase (0·1 U) from *Anthrobacter ureafaciens* (Boehringer-Mannheim) was added to the ROS suspension and incubated for 2 hr at 4°C. After centrifugation at 27000 ***g*** for 15 min, the pellet was suspended in 1 ml of homogenizing buffer to which ricin agarose (U.S. Biochemical Corp.) was added. This mixture was incubated for 2 hr at 4°C. Ricin agarose was used rather than ricin–gold–dextran because it is sufficiently dense to pellet through the sucrose gradient (Boesze-Battaglia and Albert, 1989). The ROS were pelleted in homogenizing buffer by centrifugation at 27000 ***g*** for 15 min, hypotonically lysed for 30 min in 0·02 M Tris buffer, pH 7·2, and washed twice with 0·02 M Tris buffer pH 7·2 (containing 2 mM EDTA) by centrifugation at 27000 ***g*** for 15 min. The sample was treated with trypsin (Boehringer-Mannheim) for 30 min at 4°C at a final concentration 0·2 μg ml^{-1}. Soybean trypsin inhibitor (Boehringer-Mannheim) was then added to a final concentration of 1 μg ml^{-1}. After two additional 30 min washings with 0·2 M Tris buffer at 27000 ***g***, the ROS membranes were layered on linear sucrose gradients consisting of 9 ml of a 50–25% (w/v) sucrose underlaid with 1 ml 60% (w/v) sucrose. All sucrose solutions were made up

in 65 mM NaCl, 2 mM $MgCl_2$, and 5 mM Tris acetate pH 7·4. After centrifugation for 3 hr in a Spinco SW 41 rotor at 35000 rpm, the band of discs was drawn off using a Pasteur pipet and the plasma membranes at the bottom of the tube were also removed. These two fractions were separately centrifuged at 27000 *g* for 15 min followed by the addition of 1·0% Emulphogene to each pellet to extract rhodopsin. Extracts were applied to RCA agarose columns, washed with 0·1% Emulphogene, and eluted according to the previously described procedures. Selected eluates were applied to SDS–polyacrylamide gels and the amount of radioactivity present in rhodopsin was determined for the discs and plasma membrane.

3. Results

Labeling of Bovine ROS with Galactose Oxidase and Sodium Borohydride

In the experiments using bovine retinas, purified ROS were treated with galactose oxidase to oxidize the sixth carbon hydroxyl group of galactose to a carbonyl group. This treatment was followed by exposure to tritium labeled sodium borohydride which chemically reduced the carbonyl group of galactose back to a hydroxyl with the introduction of a tritium atom (Gahmberg and Hakomori, 1973; Carubelli and Wen, 1990).

Figure 1 shows that without galactose oxidase treatment there was tritium incorporation into rhodopsin. There was a twofold increase in label following the galactose oxidase treatment. The galactose oxidase-dependent label tended to be in a species that migrated slower than rhodopsin as is indicated in the figure by the dashed line representing the difference between the two treatment groups.

Table I illustrates another experiment in which the galactose oxidase incubation time was doubled and the amount of radioactivity in the galactose oxidase treated ROS was 6·8-fold greater (over 70000 dpm) than the samples not treated with the enzyme. Pretreatment with neuraminidase did not increase the labeling. The non-specific labeling of opsin in the samples not treated with the enzyme was due perhaps to reductions of the retinyl-lysine Schiff's base linkage. These experiments demonstrate the presence of galactose in the oligosaccharide chains of native rhodopsin in ROS plasma membranes and basal folds. Since the enzyme does not have access to the carbohydrate chains within the sealed discs, it is not known whether there are any galactose residues in the disc membrane rhodopsin.

In Vivo Labeling of Rat Rhodopsin with Galactose

Rats injected intravitreally with [^{3}H]galactose and [^{14}C]leucine were maintained in darkness. At various times (1 hr, 6 hr, 1, 3 or 5 days) following the injection, four retinas were collected for subcellular fractionation. Rhodopsin from each of the fractions was purified by ConA sepharose chromatography and SDS–PAGE. Table II provides the ratios of [^{3}H] derived from galactose to [^{14}C]leucine in rhodopsin from the subcellular fractions enriched in either rough endoplasmic reticulum, Golgi or ROS. The labeling pattern was complex. Galactose was probably converted to glucose and mannose residues of the core

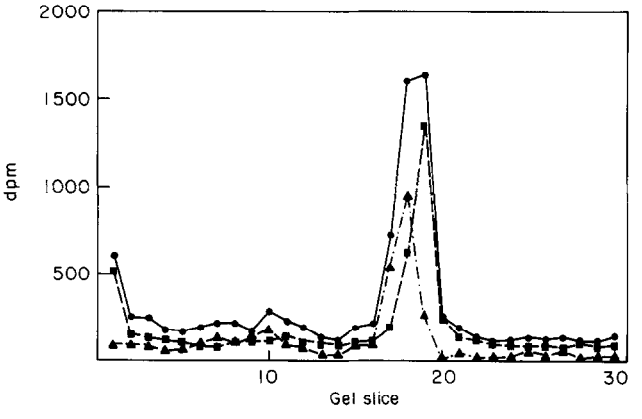

FIG. 1. Composite radioactivity profiles of the opsin regions of sodium dodecyl sulfate-polyacrylamide (SDS–PAGE) gels of bovine ROS. The ROS were purified by discontinuous sucrose density gradients and were incubated 30 min with (●) or without (■) galactose oxidase. Both samples were then incubated with tritium labeled sodium borohydride. Aliquots representing the ROS of 0·125 retinas were solubilized and applied to gels for electrophoresis. Although there was incorporation of tritium into rhodopsin without galactose oxidase treatment, there was a considerable increase in label following the galactose oxidase treatment. The galactose oxidase-dependent label tended to be in a species that migrated slower than rhodopsin as indicated by the dashed line (▲) representing the difference between the two treatment groups. The Coomassie blue-stained opsin band on a parallel gel coincided with the radioactive profile of the untreated sample.

TABLE I

Reduction of bovine ROS with tritium-labeled sodium borohydride

Pre-treatment		
Neuraminidase	Galactose oxidase	dpm in opsin
–	–	9006
–	–	12287
–	+	74809
+	+	70674

Bovine ROS were pre-incubated for 1 hr at 37°C with neuraminidase added where indicated. Subsequently, the ROS were incubated at room temperature for 1 hr with galactose oxidase added where indicated. Finally, all ROS samples were reduced with [^{3}H]sodium borohydride, solubilized and an aliquot representing the ROS from 0·05 retina analysed by SDS–PAGE. The gels were sliced and counted and the opsin zone located by comparison with a standard on a parallel gel.

TABLE II

Ratio of [^3H] derived from galactose to [^{14}C]leucine in rat rhodopsin from major cell fractions

Post-injection time	Sub-cellular fraction		
	Rough ER	Golgi	ROS
1 hr	4·8	8·3	2·5
6 hr	3·6	4·9	2·9
1 day	4·2	2·6	0·9
3 day	5·6	2·7	0·8
5 day	3·4	2·5	1·2

Rats were injected intravitreally with [^{3}H]galactose and [^{35}S]-methionine. At the indicated times four retinas were taken for subcellular fractionation. Rhodopsin, extracted from each fraction was purified on ConA sepharose and analysed by SDS–PAGE. The dual radioactive opsin peaks all migrated slower than the Coomassie Blue stained mature opsin except in the ROS samples at 1, 3 and 5 days where they all coincided.

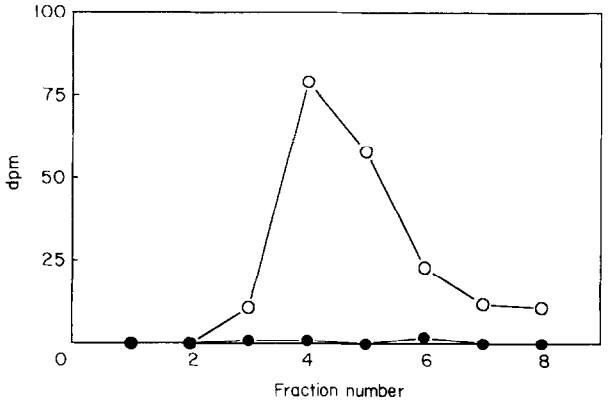

FIG. 2. Elution profiles of ConA purified rhodopsin applied to one of two lectin columns: RCA (○—○) or GSA (●—●). Six rats were injected intravitreally with [^{35}S]-methionine and were killed 2 hr later at which time retinas were collected for subcellular fractionation and rhodopsin purification by ConA sepharose chromatography. Half of the sample was applied to the RCA agarose column and half to the GSA agarose column. The washed RCA agarose column was eluted with 0·5 M β-methyl-D-galactopyranoside and the washed GSA agarose column was eluted to 0·5 M α-methyl-D-galactopyranoside beginning at fraction 1 in both cases. At 2 hr post-injection, 8–9·8% of the labeled rhodopsin bound to and was eluted from the RCA column, whereas none bound to GSA.

oligosaccharide in the rough endoplasmic reticulum. During the first 6 hr, however, galactose selectively labeled rhodopsin in the Golgi-enriched fraction resulting in increased [^{3}H]/[^{14}C] ratios in both Golgi and ROS. By day 1 the galactose pool was exhausted and carbohydrate labeling that occurred in the rough endoplasmic reticulum-enriched fraction was probably due to glycogen turnover. The decreasing isotope ratios at each time point suggest that trimming was occurring at the transition from Golgi to ROS. Furthermore, the decrease in isotope ratio in the ROS between 6 hr and 1 day suggests further trimming of

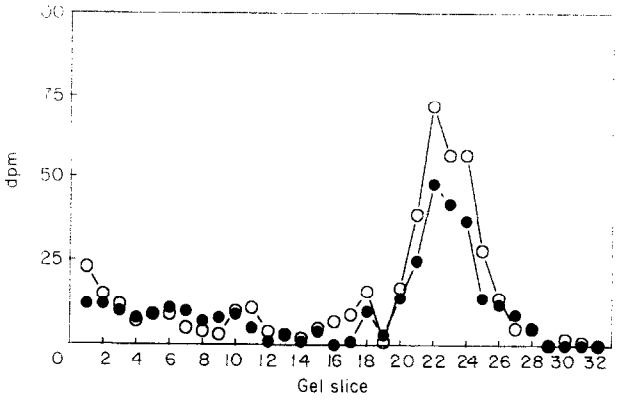

FIG. 3. Radioactivity profile of the SDS–PAGE gels of the RCA agarose-eluted sample depicted in Fig. 2. The protein eluted from RCA agarose (○—○) co-electrophoresed with a rhodopsin standard (●—●) that had been labeled with [^{3}H]leucine in vivo, for 2 hr. Gels were stained with Coomassie blue and cut into 1·2 mm slices for counting. Migration was from left to right. The radioactivity profiles were slightly to the left of the Coomassie blue-stained opsin band, as expected (see Fig. 1) of an opsin having a larger oligosaccharide.

rhodopsin after membrane assembly in the ROS. Some of this lost carbohydrate may have been galactose, particularly in the ROS.

Studies to Determine the Nature of the Linkage of Galactose to Rhodopsin

Rats injected intravitreally with [^{35}S]methionine were maintained in darkness for 2 hr at which time 12 retinas were collected for subcellular fractionation and rhodopsin purification by ConA sepharose chromatography. The ConA-purified rhodopsin was then applied to one of two lectin columns: RCA or GSA. RCA specifically recognizes β-linked galactose moieties (Baenziger and Fiete, 1979) and GSA is specific for α-linked galactose residues (Blake and Goldstein, 1980). At 2 hr, 8–9·8% of the labeled rhodopsin bound to and was eluted from the RCA column, whereas none bound to GSA, thus indicating the presence of a β-galactoside (Fig. 2). The protein eluted from RCA was shown to be rhodopsin by co-electrophoresis with rhodopsin that had been labeled with [^{3}H]leucine in vivo for 2 hr (Fig. 3).

Experiments to Determine Whether the Galactose of Rhodopsin is Capped by Neuraminic Acid

Rats injected intravitreally with [^{35}S]methionine were maintained in darkness for 2 and 4 hr at which time 12 retinas were collected for subcellular fractionation. ROS were incubated with and without neuraminidase. The amount of rhodopsin bound to and eluted from the RCA agarose columns was not increased by treatment with neuraminidase. The total dpm in the opsin region of SDS–polyacrylamide gels for the two conditions and time points are shown in

TABLE III

Effect of neuraminidase treatment of ROS on binding of rhodopsin to RCA agarose

Post-injection time (hr)	Neuraminidase	dpm in opsin
2	+	1137
2	−	1132
4	+	721
4	−	729

Two groups of six rats were injected intravitreally with [^{35}S]-methionine and killed at the indicated times. ROS were prepared and half of each sample incubated with neuraminidase. After extraction, the rhodopsin samples were purified on ConA sepharose and subsequently chromatographed on Ricin agarose.

Table III. These data suggest that although complex asparagine-linked oligosaccharides often have a terminal trisaccharide containing *N*-acetylglucosamine linked to galactose and capped with neuraminic acid (Kornfeld and Kornfeld, 1985), rhodopsin lacks neuraminic acid.

Demonstration that Binding of the Rhodopsin Oligosaccharide to RCA can be Decreased with β-galactosidase Treatment

Rats were injected intravitreally with [^{35}S]methionine and [^{3}H]glucosamine to provide markers for the polypeptide and the oligosaccharide, respectively. They were maintained in darkness for 2 hr at which time 24 retinas were collected for subcellular fractionation and rhodopsin purification by ConA sepharose chromatography. The ConA-purified rhodopsin was treated initially with *N*-glycanase to hydrolyze the asparagine-linked oligosaccharides from rhodopsin. This treatment was followed by incubation of half of the sample with β-galactosidase which specifically hydrolyses terminal galactose residues that are β1–4 linked to *N*-acetylglucosamine. As shown in Fig. 4, the amount of radioactivity eluted from RCA after β-galactosidase treatment was significantly less than that of the sample eluted from RCA without the enzyme treatment. These same results were obtained in repetitions of this experiment and suggest that removal of galactose from the rhodopsin oligosac-

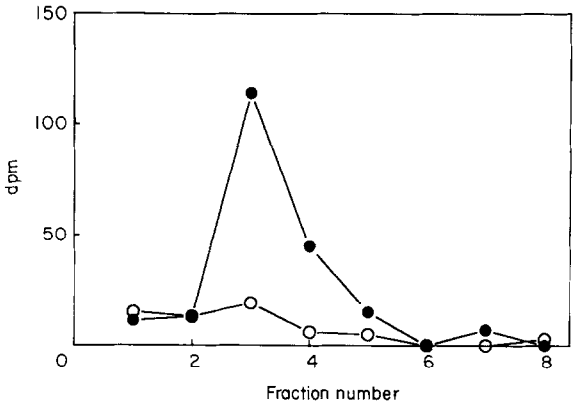

FIG. 4. Elution profiles from RCA agarose columns of the cleaved oligosaccharide of rhodopsin which had either been treated with β-galactosidase (○—○) or not (●—●). Twelve rats were injected intravitreally with [^{35}S]methionine and [^{3}H]glucosamine and kept dark for 2 hr, at which time retinas were collected for subcellular fractionation and rhodopsin purification by ConA sepharose chromatography. The rhodopsin was incubated at 37°C for 42 hr with *N*-glycanase to hydrolyze the ^{3}H-labeled oligosaccharide. β-Galactosidase was added to half of the sample. Both the β-galactosidase treated sample and the control were incubated at 37°C for 24 hr. Each sample was applied to an RCA agarose column which was washed and subsequently eluted with β-methyl-D-galactopyranoside beginning at fraction 1. The amount of radioactivity (dpm ^{3}H) eluted from RCA agarose after β-galactosidase treatment was significantly less (0·6%) than that of the sample eluted from RCA agarose without the enzyme treatment (3·5%).

TABLE IV

Effect of swainsonine or castanospermine on the fraction of ConA purified rhodopsin that binds to RCA

	Inhibitor		
	Control	Swainsonine	Castanospermine
Percentage of ConA purified rhodopsin from ROS that binds RCA*	3·30%±0·12	5·77%±2·0	0·64%±0·3
Percentage of ConA purified rhodopsin from Golgi that binds RCA	4·06%±1·80	4·26%±1·1	1·08%±0·3

Rhodopsin was labeled for 1 hr following simultaneous intravitreal injection of [^{35}S]methionine and swainsonine or castanospermine, or neither in three groups of six rats.

* Data are expressed as the mean percentages of three experiments ± S.D. One-way analysis of variance for the ROS and Golgi samples indicated that there was a significant difference among the three groups (ROS: $F = 11·7$, $P = 0·01$; Golgi: $F = 21·5$, $P = 0·003$). Tukey's paired comparisons test confirmed that the percentages obtained for the castanospermine treated group differed significantly from the control or swainsonine treated group, but these latter two groups did not differ significantly ($P < 0·05$).

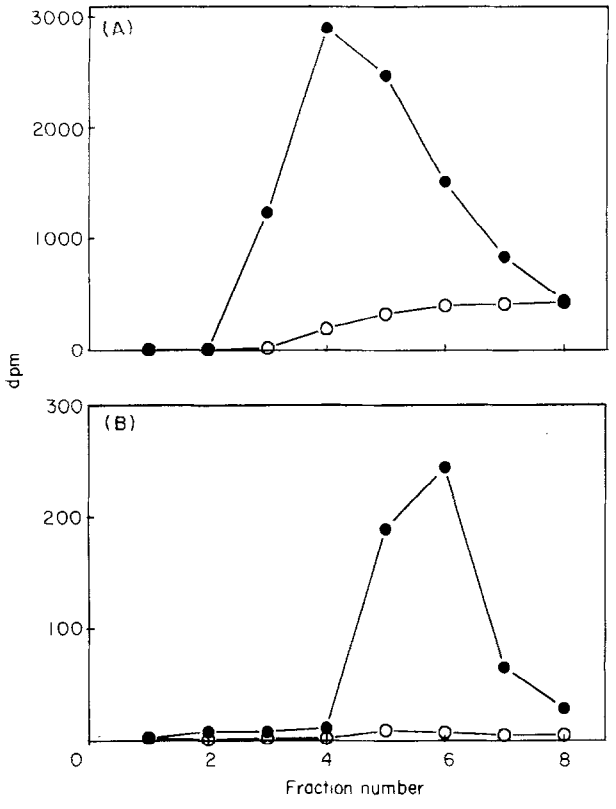

FIG. 5. Light sensitivity experiment showing the elution patterns from ConA sepharose columns (A) and RCA agarose columns (B). Six rats injected intravitreally with [^{35}S]-methionine were maintained in darkness for 2 hr at which time retinas were collected for subcellular fractionation. Half of a sample of detergent extracted purified ROS labeled in vivo with [^{35}S]methionine was exposed to light for 10 min while the remainder of the sample was kept dark. Each sample was chromatographed on ConA sepharose and eluted with α-methyl mannoside. The ConA sepharose column eluates were applied to RCA agarose which was washed with buffer and then eluted with β-methyl-D-galactopyranoside beginning at fraction 1. As would be expected, the dark sample bound to and was eluted from ConA sepharose. 7·0% of that sample was bound to and eluted from RCA agarose. In contrast, considerably less radioactivity was eluted when the light-exposed sample was chromatographed on ConA. What little was eluted did not bind to RCA. (●—●) Dark; (○—○) light.

charide using this highly specific enzyme dramatically reduces the binding of the oligosaccharide to RCA. These data provide further support for the presence of galactose on the oligosaccharide of rhodopsin.

Demonstration that Binding of Rhodopsin to RCA is Abolished by Treatment with Castanospermine But Not Swainsonine

Rats in groups of six injected intravitreally with [^{35}S]methionine only or [^{35}S]methionine and either catanospermine or swainsonine were dark adapted for 1 hr at which time retinas were collected for subcellular fractionation. Rhodopsin was purified from the ROS- and Golgi-enriched fractions by ConA sepharose chromatography and the eluates were applied to RCA agarose columns. Both the RCA agarose eluates and a fraction of the ConA Sepharose eluates were applied to SDS–PAGE gels. The data in Table IV, representing three replicates of this experiment, show that the mean percentage of ConA purified rhodopsin from ROS which bound to RCA was greatly reduced in the castanospermine exposed group. A similar effect was seen in the Golgi-enriched fraction. The statistical analysis of these data indicated that the percentages obtained in the castanospermine treated ROS and Golgi samples were significantly different from the control or swainsonine treated group. These data demonstrate that castanospermine, which inhibits glucosidase I, disrupted the binding of rhodopsin to RCA, whereas swainsonine, which inhibits Golgi mannosidase II, did not. Since RCA is specific for β-galactose residues, it appears likely that in the presence of castanospermine, galactose was never linked to the oligosaccharide chain of rhodopsin.

Light-sensitivity Experiments

Rats injected intravitreally with [^{35}S]methionine were maintained in darkness for 2 hr at which time 12 retinas were collected for subcellular fractionation. Half of a sample of detergent extracted purified ROS labeled in vivo with [^{35}S]methionine was exposed to light while the remainder of the sample was kept dark. Each sample was chromatographed on ConA sepharose and eluted with α-methyl mannoside. When rhodopsin is bleached it binds to ConA, but is not eluted from the lectin by α-methyl mannoside. The ConA sepharose column eluates were applied to RCA agarose columns and eluted with β-methyl-D-galactopyranoside. The elution patterns from the ConA sepharose columns and the RCA agarose columns are shown in [Fig. 5(A) and (B)], respectively. As would be expected, the dark sample bound to and was eluted from ConA. Furthermore, 7·0% of that sample was bound to and eluted from RCA. In contrast, much less radioactivity was eluted when the light-exposed sample was chromatographed on ConA. What little was eluted did not bind to RCA. SDS–polyacrylamide gels of the material eluted from RCA indicated that the dark sample coincided with a rhodopsin standard labeled with [^{3}H]leucine, whereas there was virtually no radioactivity detected on the gel of the light exposed sample (Fig. 6). These data indicate that the ConA purified material that binds to RCA is light sensitive and is therefore rhodopsin.

Determination of which Photoreceptor Cellular Compartments have Galactose-containing Rhodopsin

N-linked oligosaccharides are typically assembled in the endoplasmic reticulum on dolichylphosphate and are then transported by means of vesicles to the Golgi membrane (Kornfeld and Kornfeld, 1985). In the trans Golgi, galactose may be added to a terminal *N*-

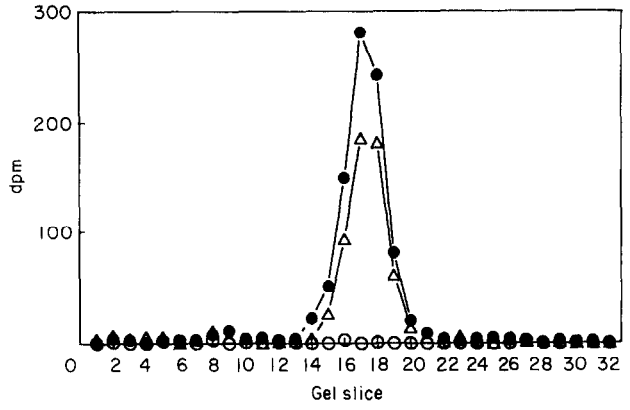

FIG. 6. SDS–polyacrylamide gels of the material eluted from RCA agarose [described in Fig. 5(B)]. The dark sample coincided with a rhodopsin standard labeled in vivo with [^{3}H]leucine, whereas there was virtually no radioactivity detected on the gel of the light-exposed sample. As before (Fig. 3) the radioactive opsins migrated slightly slower than the Coomassie blue-stained mature opsin. (○—○) Light; (●—●) dark; (△—△) rhodopsin standard.

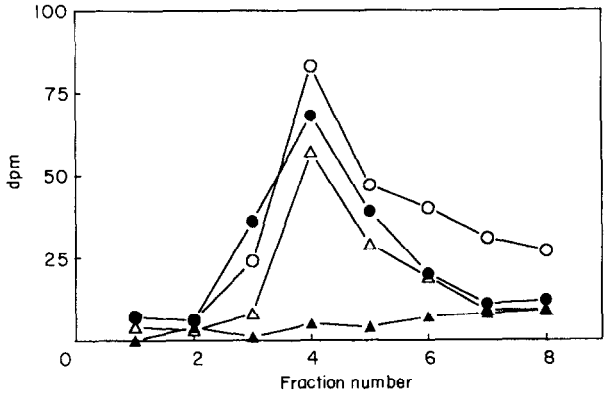

FIG. 7. Elution profiles from RCA agarose columns of bands separated from a linear 50–25% sucrose gradient over which had been layered a crude ROS suspension. The ROS suspension was prepared from retinas of six rats which had been intravitreally injected with [^{35}S]methionine and dark adapted for 2 hr. This procedure typically yields four bands on the gradient: a faint uppermost band 1·5–1·8 cm from the top of the gradient, two bands of unsealed and sealed ROS (1 and 2, respectively) and a zone of fine particles, enriched in Golgi. Pellets of each of these bands were detergent extracted and applied to separate RCA agarose columns which were then washed. As shown, the Golgi-enriched fraction and both ROS fractions (ROS 1 and ROS 2) bound to and were eluted from RCA with β-methyl-D-galactopyranoside. The uppermost band did not demonstrate RCA binding. Thus rhodopsin appears to have acquired galactose residues before leaving the Golgi. (○—○) Golgi; (●—●) ROS 2; (△—△) ROS 1; (▲—▲) band 1·5–1·8.

acetylglucosamine of the oligosaccharide chain. In an effort to determine if the Golgi apparatus, as well as the ROS, of rod photoreceptor cells contained galactose, subcellular fractionation of ROS was performed. Rats were injected intravitreally with [^{35}S]methionine and were maintained in darkness for 2 hr at which time 12 retinas were collected and crude ROS preparations were made using the vortexing procedure described. Linear 50–25% sucrose gradients of crude ROS suspensions which have been centrifuged for 2 hr typically have four bands. A very faint uppermost band is approximately 1·5–1·8 cm from the top of the gradient. Two bands of unsealed and sealed ROS are below the 1·5 cm band (Godchaux and Zimmerman, 1979). Below these is a zone of fine particles, enriched in Golgi. Pellets of each of these bands were detergent extracted and applied to separate RCA columns. As shown in Fig. 7, the Golgi-enriched fraction and both ROS fractions (ROS 1 and ROS 2) bound to and were eluted from RCA. The uppermost band did not demonstrate RCA binding. SDS–PAGE of these fractions showed that the Golgi and ROS fractions co-electrophoresed with a rhodopsin standard. Although the Golgi-enriched fraction is not entirely free of ROS, the data suggest that the galactose residue of rhodopsin is present on the oligosaccharide in the Golgi as it is in other glycoproteins containing N-linked oligosaccharides.

Time-course of the Trimming of Galactose from Rhodopsin

The results of the in vivo experiments in which [^{3}H]galactose was used to label rhodopsin over several days (described above) suggested that galactose might be trimmed from the oligosaccharide of rhodopsin in the transition from Golgi to ROS as well as in ROS over time. To test this, a time course experiment was conducted. Rats were injected with [^{35}S]methionine and [^{3}H]galactose and groups of 6 animals were killed 2, 6 and 24 hr following injection. Retinas were collected for subcellular fractionation, ROS were extracted and rhodopsin was purified by ConA sepharose chromatography. A small fraction of the ConA eluate was applied to SDS–polyacrylamide gels and the remainder was applied to RCA. Table V provides the data obtained from gel electrophoresis of the ConA- and RCA-eluted samples. Electrophoresis of the RCA agarose eluates revealed that the percentage of the ConA-purified rhodopsin that bound to RCA decreased over time. At 2 hr, 8·69% of the rhodopsin bound to RCA, after 6 hr 2·17% was bound and by 24 hr only 0·89% was bound. Electrophoresis of the ConA-purified rhodopsin showed that the ratio of the isotopes (that is the ratio of [^{3}H]galactose to [^{35}S]-methionine) decreased over time from 0·40 at 2 hr to 0·15 by 24 hr. Clearly, trimming of galactose from rhodopsin had occurred suggesting that galactose may be an early component of rhodopsin, but as the molecule progresses through the ROS the galactose appears to be lost. In contrast, the ratio of the isotopes ([^{3}H]galactose to [^{35}S]methionine) in the rhodopsin that bound to RCA was higher than in the total pool eluted from ConA and did not change significantly over time. Thus, the ratio changes were due to removal of galactose residues.

TABLE V

The percentage of ConA-purified rhodopsin that binds to RCA and the ratio of [^3H] to [^{35}S] in gel electrophoresis of the purified rhodopsin

	Hours post-injection		
	2	6	24
Percentage of ConA purified [^{35}S] rhodopsin that binds RCA	8·69%	2·17%	0·89%
Isotope ratio in ConA purified rhodopsin	0·40	0·29	0·15
Isotope ratio in ConA purified rhodopsin that binds to RCA	0·77	0·71	0·93

Three groups of six rats were injected intravitreally with [^3H]galactose and [^{35}S]methionine. Retinas were collected at the indicated times, ROS were prepared and rhodopsin was purified on ConA sepharose. Aliquots were analysed by SDS–PAGE and the rest chromatographed on ricin agarose. Ricin eluates were also analysed by SDS–PAGE.

Plasma Membrane Preparation

It was of interest to determine whether the galactose-containing rhodopsin was present in photoreceptor discs or plasma membrane. A modification of the method of Molday and Molday (1987) was used in which nine rats were injected intravitreally with [^{35}S]methionine and were maintained in darkness for 2 hr, at which time ROS were prepared using the linear sucrose gradient procedure described previously. After treatment with neuraminidase ROS were incubated with ricin-agarose. Subsequent to lysis and trypsin treatment, the ROS membranes were layered on linear 50–25% sucrose gradients underlaid with 60% sucrose. Following centrifugation, discs and the plasma membranes were removed. Detergent extracts of these two components were applied to RCA agarose columns. Elution patterns from RCA revealed significant binding of plasma membrane extracts and a small amount of binding of disc extracts. SDS–polyacrylamide gels of the eluates showed that the total amount of radioactivity present in rhodopsin of the plasma membrane (1149 dpm) was 3·6 times greater than the total amount of radioactivity present in disks (318 dpm). From these experiments it appears that at 2 hr post-injection, 74·5% of the labeled galactose-containing rhodopsin is in the plasma membrane and 25·4% is in discs.

4. Discussion

Although rhodopsin's oligosaccharide chains have been thought to contain mainly two types of sugar residues [mannose and *N*-acetylglucosamine (Heller, 1968; Shichi et al., 1969; Heller and Lawrence, 1970; Plantner and Kean, 1976)], there is evidence that a small fraction of rhodopsin also may contain galactose. Fukuda et al. (1979) found that 10% of the oligosaccharides in bovine rhodopsin contained galactose. Furthermore, O'Brien (1976) showed that bovine ROS preparations support the transfer of galactose from UDP-galactose to rhodopsin. St Jules et al. (1990) demonstrated the in vitro incorporation of galactose into rhodopsin and its subsequent removal after rhodopsin had reached the outer segment. These findings suggested that galactose may in fact be associated with rhodopsin of the ROS, if only for a short period of time. Perhaps the transient nature of its association explains its rather elusive detection by some biochemical techniques. In the current experiments, efforts were focused on demonstrating the presence of galactose by methods which could detect existing molecules, rather than solely by methods to show incorporation of exogenous galactose.

Galactose molecules were shown to exist on bovine rhodopsin using the galactose oxidase–sodium borohydride treatment. The results clearly demonstrated that there were galactose molecules present on rhodopsin which were altered by the highly specific galactose oxidase treatment and were then reduced by the sodium borohydride with the introduction of radiolabel. The bovine model does not permit in vivo studies of oligosaccharide synthesis, however, so it became necessary to investigate the incorporation of galactose in the rat model. The studies in which galactose was injected intravitreally in rats and monitored over several days demonstrated a selective labeling during the first 6 hr in the Golgi-enriched and the ROS fractions. Thus, even though conversion of galactose to other sugars commonly found on rhodopsin's oligosaccharide chain probably occurred, the initial pulse of galactose was apparently incorporated as galactose in the Golgi where galactosyl transferase is known to be localized. The results strongly suggested that the galactose had been added to rhodopsin and then trimmed over time.

The need to demonstrate the presence of galactose without directly incorporating it into rhodopsin lead to the use of lectin affinity chromatography. This powerful technique allows for the separation of glycoproteins based on the affinity for certain sugar moieties (Cummings et al., 1989). ConA, which has an affinity for mannose residues, has long been used in the purification of rhodopsin. In the present work, a portion of the ConA-purified rhodopsin was shown to bind to the lectin RCA which specifically recognizes terminal galactose moieties associated with either glucosamine or *N*-acetylglucosamine in a β1–4 linkage (Baenziger and Fiete, 1979), whereas no rhodopsin bound to GSA which has an affinity for α-linked galactose (Blake and Goldstein, 1980). Methionine was injected intravitreally into rats in order to label the polypeptide chain of rhodopsin. That the

radiolabeled protein which bound to RCA was indeed rhodopsin was verified by light sensitivity experiments. In addition, the binding of the rhodopsin oligosaccharide to RCA could be decreased significantly by cleaving the galactose moiety with the highly specific enzyme β-galactosidase. On the other hand, treatment with neuraminidase, which specifically hydrolyses neuraminic acid from oligosaccharide chains, neither increased nor decreased the binding of rhodopsin to RCA. These experiments indicated that the galactose of rhodopsin was not capped with neuraminic acid as is often the case with complex asparagine-linked oligosaccharides whose terminal trisaccharides contain *N*-acetylglucosamine linked to galactose capped with neuraminic acid (Kornfeld and Kornfeld, 1985).

The galactose of terminal trisaccharides of N-linked oligosaccharides are typically added to *N*-acetylglucosamine in the *trans*-Golgi (Kornfeld and Kornfeld, 1985). In the present work, it was of interest to determine if galactose was associated with rhodopsin purified from the Golgi-enriched fraction as well as ROS. It was determined by subcellular fractionation and subsequent RCA chromatography that the rhodopsin of the Golgi-enriched fraction did indeed contain galactose. Furthermore, it was shown that the addition of galactose to the *N*-acetylglucosamine could be inhibited by in vivo exposure to castanospermine which specifically inhibits Glucosidase I. Glucosidase I is a rough endoplasmic reticulum enzyme that cleaves the terminal glucose from the $Glc_3Man_9GlcNAc_2$ (Repp et al., 1985). With this early step in the processing of the oligosaccharide disrupted, the subsequent steps in the processing pathway could not continue. The addition of galactose to *N*-acetylglucosamine was prevented because the oligosaccharide chain was never processed to the point where galactose is typically added to *N*-acetylglucosamine. By way of contrast, the addition of galactose to *N*-acetylglucosamine was not affected by swainsonine which inhibits the Golgi mannosidase II. This enzyme typically cleaves two mannose residues from one of the branches of the oligosaccharide chain (Elbein, 1983). The processing of the other branch of the oligosaccharide chain can continue normally. It is to this other chain, which contains a mannose and an *N*-acetylglucosamine, that galactose is typically added. However, it should be noted that a negative result with swainsonine is of limited significance since there is no assurance that it reached and inhibited the target enzyme. To the best of our knowledge, these experiments provide the first evidence in a mammalian system that glycoprotein processing can be modified in rhodopsin following in vivo exposure to the appropriate inhibitory agent. Chambers et al. (1986) reported that intraocular injection of frogs with up to 250 μg of castanospermine and swainsonine resulted in neither a decrease in rhodopsin content nor a change in the length of photoreceptor outer segments. Fliesler, Rayborn and Hollyfield (1986a) demonstrated that in vitro exposure of *Xenopus* retinas to castanospermine resulted in the hyperglycosylation of opsin. The opsin underwent normal intracellular transport and was utilized for the biogenesis of ROS membranes having normal disc morphology.

The 1 hr to 5 day in vivo galactose labeling studies of rhodopsin purified from rough endoplasmic reticulum-, Golgi-enriched fractions and ROS, suggested that the amount of galactose-derived label associated with rhodopsin was not constant. It appeared from this work that trimming had occurred in the ROS over the course of several hours and also had occurred in transit from one subcellular compartment to another, namely, the Golgi-enriched fraction to ROS. We chose to investigate the trimming phenomenon which was apparently occurring in the ROS using lectin affinity chromatography. Two hours after the injection, approximately 8–9% of the labeled rhodopsin bound to RCA suggesting that about 8–9% of newly synthesized rhodopsin contains a β-galactose residue. The percentage of rhodopsin which contained galactose decreased by more than half within 6 hr and by 24 hr of the injection less than 1% of rhodopsin contained galactose. One might surmise that if the galactose concentration is greatest within 2 hr of injection, perhaps it constitutes a transient component of rhodopsin's oligosaccharide chain. One possibility is that it is associated with the rhodopsin of the plasma membrane and basal folds of the ROS and is removed as discs are sealed and move apically. The work of Hicks and Molday (1985) emphasized the presence at the basal area of the outer segments of ricin-binding compounds.

The possible function of a galactose on rhodopsin at this one area is open to speculation. It has been shown that the oligosaccharide of rhodopsin or some other ROS protein such as peripherin is essential for the process of disc morphogenesis by Fliesler and Basinger (1985) and by Fliesler, Rayborn and Hollyfield (1985) who successfully disrupted disc formation with tunicamycin, an inhibitor of oligosaccharide synthesis. Mechanisms have been proposed (Fliesler, Rayborn and Hollyfield, 1986b; Fliesler, 1988) showing how the oligosaccharide of rhodopsin could play an important role in disc formation. As new discs are formed, presumably the opposite faces of a newly forming disc must be aligned and brought into close apposition. It might be that the oligosaccharide plays some role in the process in much the same way that a lectin binds a ligand or a hydrolase interacts with an oligosaccharide substrate. If there were an enzyme–substrate interaction, perhaps a carbohydrate residue such as galactose on one surface might be cleaved by a hydrolase such as a galactosidase on the opposing surface. During this process the two membrane surfaces would come into close apposition with the exclusion of extracellular matrix allowing fusion of the new disc. This hypothesis led to the experiment in which ROS plasma membranes were separated from

discs for the purpose of determining in which compartment the galactose-containing rhodopsin was most prevalent. The experiments showed that the greatest preponderance of the galactose-containing rhodopsin was in the plasma membrane component. This finding lends support to the hypothesis that a transiently present galactose is important in new disc formation in bovine and rat retinas because the galactose appears to be associated with the plasma membrane until the discs are finally fused. This proposed mechanism would not specifically require galactose as the substrate, only that the components of the oligosaccharide, however they may vary among species, be the substrates of the appropriate hydrolases.

In the summary, the present work has shown through biochemical techniques that a subset of rhodopsin contains galactose. In fact, shortly after it is synthesized, approximately 8–9% of the rhodopsin in the ROS contains this sugar residue. The galactose appears to be added in the Golgi complex in the same way that a terminal trisaccharide is assembled on a complex oligosaccharide chain. The galactose is not capped, however, with neuraminic acid. We presented evidence to confirm the incorporation of galactose into rhodopsin in in vivo experiments. Additionally, we demonstrated the presence of galactose in bovine rhodopsin by radiolabeling existing moieties. Finally, using lectin affinity chromatography, we were able to separate the galactose-containing species of rhodopsin and study its transient association with the oligosaccharide chain. We determined that galactose is trimmed from rhodopsin over the course of about 24 hr, presumably after it has served its function which may be related to new disc formation.

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