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Reactivity with lectins of the saccharide components of rhodopsin in reconstituted membranes.

Orientation of the carbohydrates

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Rhodopsin-containing liposomes may provide a model for investigating the interaction of intrinsic membrane glycoproteins in biological systems. As part of the characterization of this preparation, the surface orientation of the carbohydrates of rhodopsin, assembled from purified bovine rhodopsin and egg phosphatidylcholine was examined, and is the topic of this report. The major tool used in these studies was the interaction with the carbohydrate-specific reagents, plant lectins. Two techniques were used: (1) lectin-mediated aggregation of the liposomes, as measured by light scattering; (2) the binding of ^{125}I-labeled succinylated concanavalin A, and Scatchard analysis as a measure of affinity. The preparation most extensively examined had a mole ratio of rhodopsin:phospholipid of 1:100. Among a variety of lectins which were examined, only concanavalin A, succinylated concanavalin A, and wheat germ agglutinin were able to mediate the aggregation of rhodopsin-containing liposomes, as expected. The aggregation with concanavalin A was prevented by the presence of sugars having the α-D-glucopyranosyl configuration, and that brought about with wheat germ agglutinin, by *N*-acetylglucosamine (GlcNAc). In addition, the aggregation with concanavalin A was reversed with methyl α-D-mannoside, and with wheat germ agglutinin, by GlcNAc, suggesting that membrane fusion did not take place. On a molar basis, wheat germ agglutinin brought about a greatly reduced extent of aggregation as compared to concanavalin A, suggesting the relative inaccessibility of GlcNAc residues in the liposomes as compared to mannose. The initial rate of the aggregation, however, were similar with either lectin. The first-order rate constants were unaffected by wide variation in the concentrations of concanavalin A and wheat germ agglutinin, and by variation in the mole ratios of rhodopsin in the liposomes from 0.2 to 19 moles per 100 moles of egg lecithin. Rhodopsin-liposomes were also prepared from a total lipid extract of rod outer segments instead of egg lecithin. Similar kinetic properties were exhibited by this preparation as were obtained with the liposome prepared with the purified phospholipid. Scatchard analysis of the binding of ^{125}I-labeled succinylated concanavalin A by rhodopsin liposomes indicated the presence of a single class of binding site as the preferred fit, with an apparent K_d of $2.8 \cdot 10^{-7}$ M. The binding was destroyed or extensively interfered with by trypsinization and by periodate treatment.

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Introduction

In view of the complexity of natural membranes, phospholipid vesicles are being widely used as models in studies of membrane interactions in a variety of biological systems. The incorporation of glycoproteins into the lipid bilayer is potentially a powerful tool for investigating the influence of these molecules on surface reactions. One of the most extensively characterized intrinsic membranous glycoproteins is the molecule, rhodopsin. It is the major protein component of the discs of the rod outer segment [1], and can be obtained in relatively high yield in a purified, delipidated state [2]. A variety of reports have described the preparation of and many of the properties of phospholipid vesicles (liposomes) which have been prepared with this molecule [3–7]. In the recent period considerable attention has been given to the possibility that glycoproteins may act as receptors for cellular interactions by virtue of their carbohydrate components. Within this context it was felt that rhodopsin-liposomes might provide a convenient model for studies of this nature. However, relatively little information is available concerning the reactivity of the carbohydrate components of rhodopsin in this type of preparation. The carbohydrate groups of rhodopsin, made up of mannose and *N*-acetylglucosamine, comprise about 7% of its weight [2,8]. The carbohydrate groups of rhodopsin in the native membranes face the interior of the disc, not accessible to surface reactions [9]. By studying the proteolysis of reconstituted membranes, Fung and Hubbell [10] demonstrated that a portion of the rhodopsins in the reconstituted vesicles were inverted as compared to the orientation in the native discs. This alteration should also occur with the carbohydrate groups. This aspect of the topography of rhodopsin in reconstituted membranes, although inferred [10], has not previously been directly demonstrated. The present report documents this orientation utilizing the capacity of lectins to interact with carbohydrate groups exposed on the surface of the rhodopsin-liposomes. A detailed characterization of this aspect of the surface properties of this model membrane system is provided in terms of the kinetics, and stoichiometry of the interactions with the plant lectins, concanavalin A, succinylated concanavalin A (succinyl-con A), and wheat germ agglutinin.

Experimental Procedures

Liposome preparation

Rhodopsin-liposomes were prepared by the procedures described by Hong and Hubbell [3,4]. Solutions of egg phosphatidylcholine (70 μmol) in chloroform were evaporated to dryness on a rotary evaporator and then maintained at room temperature for 2 h under vacuum. The phospholipid was dissolved in 8 ml of 610 mM tridecyltrimethylammonium bromide (TriTab) in 15 mM sodium phosphate buffer (pH 6.6), containing 1 mM dithiothreitol. The mixture was maintained at room temperature under an atmosphere of argon, with occasional shaking until clarified, after which it was filtered through a 0.22 μm membrane (Millex, Millipore Corp., Bedford, MA). The filtrate was then mixed with rhodopsin using various ratios of phospholipid to rhodopsin. These and all subsequent preparative procedures, unless indicated otherwise, were carried out under dim red light at 4°C. The mixture was stored overnight under argon, after which it was dialyzed against one liter of 10 mM Hepes buffer (pH 7.0), containing 1 mM dithiothreitol. Dialysis was carried out for 3 to 4 days with 2 changes of dialysis buffer per day, bubbled continuously with N_2. After dialysis, the cloudy suspension of liposomes was removed from the dialysis bag and homogenized gently with a teflon Potter-Elvehejm homogenizer. The rhodopsin and phosphate content of the preparation was determined as described below. The liposome preparations were stored under argon at 4°C in light-sealed vials. Control liposomes were prepared in the same manner, but in the absence of rhodopsin.

Liposomes were also prepared from a total lipid extract of purified bovine rod outer segments instead of egg lecithin. The rod outer segments were prepared as described previously [2]. The suspension of rod outer segments from 150 bovine eyes was homogenized under argon with 20-fold volumes of chloroform/methanol (2 : 1, v/v) using a conical shaped Kontes all glass homogenizer (Kontes Glass Co., Vineland, NJ). After centrifugation (500 × *g*, International centrifuge), the extract was filtered through a 0.5 μm solvent-re-

sistant membrane (Millex-SR, Millipore Corp., Bedford, MA) and evaporated to dryness on a rotary evaporator. The extract was stored at −70°C in the presence of 0.5 μmol of α-tocopherol under argon. Rhodopsin-liposomes were prepared from this preparation by the same procedures as described above.

Rhodopsin preparation; detergent preparation

Rod outer segments, isolated from dark-adapted frozen retinas (George Hormel Co., Austin, MN) were extracted with TriTab (0.1 M in 0.015 M sodium phosphate uffer (pH 6.6), containing 1 mM dithiothreitol) using 10 ml of detergent-buffer per 75 retinas. The extract was centrifuged for 20 min at 39000 × *g*, and the supernatant solution chromatographed on hydroxyapatite (DNA grade, Bio-Gel HTP, Bio-Rad Laboratories, Richmond, CA) [4]. The rhodopsin-containing fractions were pooled and the concentration of rhodopsin determined spectrophotometrically, as described previously [2]. The yield of rhodopsin from this treatment varied from 75 to 82% and the A_{278}/A_{498} ratio varied from 1.8 to 1.9 for ten preparations.

The detergent, tridecyltrimethylammonium bromide, was synthesized by reacting trimethylamine with 1-bromotridecane as described by Hong and Hubbell [4]. The product was recrystallized three times from acetone/methanol (90 : 10, v/v) (m.p., 222–228°C).

Analytical procedures

The rhodopsin concentration of extracts and of liposomes was measured spectrophotometrically in the presence of 0.02 M hydroxylamine, as described previously [2], and also by radioimmunoassay, as described by Plantner, Hara, and Kean [11]. Phosphate was measured after acid hydrolysis [12].

In the present studies the mole ratio of rhodopsin in the recombinants was varied. The several rhodopsin-liposome preparations will be designated in terms of '*x*R', where *x* refers to the mole ratio of rhodopsin to 100 moles of egg lecithin. Thus, for example, '1R' indicates a liposome with the mole ratio of rhodopsin to phospholipid of 1:100, and 0.2R refers to a liposome composed of 0.2 mole rhodopsin:100 moles of phospholipid. Since the former relationship is similar to that present in retinal rod outer segments, the 1R-liposome was the preparation most extensively examined in the present study. Most of the rhodopsin-liposomes which were formed closely retained the initial ratio of rhodopsin to phospholipid that was present in the reaction mixture used for their preparation, consistent with previous observations [3,5,7] concerning the formation of liposomes when using alkyltrimethylammonium detergents. An exception was the 19R liposome which was formed although the molar ratio of rhodopsin to egg lecithin which was present in the reaction mixture was 10 : 100. Rhodopsin-liposomes (1R) were also prepared using the lipid extract from rod outer segments instead of egg lecithin. The amount of the extract which was used in the preparations was based upon an analysis for total phosphate.

Aggregation assay

The aggregation of liposomes was measured turbidimetrically by following the increase in absorbance at 360 nm. Immediately before measurement, the liposomes were sonicated in an ice bath for 10 s using the needle probe of a Bronwill Biosonik III sonicator (Bronwill Scientific, Rochester, NY) at its lowest setting. Measurements were carried out at room temperature (23°C) using a Beckman model 25 double-beam recording spectrophotometer or a Gilford model 252 photometer (Gilford Instrument Laboratories, Oberlin, OH) mounted on a Beckman DU monochromator. Aliquots of rhodopsin-liposomes containing 1 nmol rhodopsin were mixed with solutions of lectins in 0.01 M sodium phosphate buffer (pH 7.0), containing 0.14 M NaCl (phosphate-buffered saline) or other reagents as indicated, in a total volume of 1.0 ml. (In control experiments, similar turbidities were obtained in the presence of phosphate-buffered saline containing 0.1 mM $CaCl_2$ and $MnCl_2$.) The absorbance was measured as a function of time after mixing under conditions of usual room fluorescent lighting or under dim red light. Identical measurements were carried out on mixtures that contained liposomes and buffer alone (controls). Measurements were recorded for periods from 0.2 to 120 min.

Kinetic analysis

The rates of aggregation of rhodopsin-liposomes brought about by the presence of concanavalin A and wheat germ agglutinin were investigated under a variety of conditions. In all of these studies, the concentration of the rhodopsin-liposome was maintained at 1 nmol/ml based on the rhodopsin concent of the liposome. The first order rate constants were calculated by the relationship:

$$\ln(\Delta A_\infty - \Delta A_t) = -kt + \ln(\Delta A_\infty - \Delta A_0)$$

A_∞ is the experimentally observed maximal absorbance defined operationally as that obtained by 120 min, by which time no further increase in absorbance was seen. In cases where decreases in absorbance with incubation time occurred due to settling of aggregates, the maximal absorbance was utilized. A_t refers to the absorbance at time t. A_0 refers to the absorbance at time zero, defined here as the 0.2 min recording after mixing. The delta values refer to the differences in absorbance between those obtained with the suspension of liposomes in the presence and absence of lectins. The first-order rate constants were calculated from the slopes of the lines generated after subtracting the slower process from the more rapid phase, the latter defined here as that occurring over the period from 0.2 to 1.0 min after mixing (see Fig. 4B).

Iodinated succinyl-con A; Scatchard analysis

Succinylated concanavalin A was iodinated by the lactoperoxidase procedure using $Na^{125}I$, as described previously [13]. Binding assays of iodinated lectin to the liposome were carried out in a manner similar to that described by Chicken and Sharom [14]. ^{125}I-succinyl-con A, present over a concentration range of 0.02 mg/ml to 4.0 mg/ml (0.36 μM to 72 μM) was incubated with a suspension of 0.5 nmol of rhodopsin-liposomes (1R) in Dulbecco's phosphate-buffered saline in a total volume of 0.5 ml at room temperature. After 1.5 h, an equal volume of cold, 25% (w/v) polyethylene glycol-1000 (PEG 1000) was added. After standing 15 min in ice, the mixture was filtered through 0.2 μm Durapore filters (Millipore), and the filters washed with the 25% polyethylene glycol solution. Identical incubations were carried out in the presence of 0.1 M methyl α-D-mannoside to control for non-specific binding. After washing, the filters were dried, transferred to tubes, and the radioactivity measured on a gamma counter. Binding data, in the form of bound/free vs. bound were analyzed by computer using the 'Ligand' program of Munson and Rodbard [15].

Modifications of the liposomes

Trypsinization. Liposomes were incubated at room temperature for 30 min with 0.75% trypsin in phosphate-buffered saline. After the addition of trypsin inhibitor, the liposomes were recovered by centrifugation, washed once in phosphate-buffered saline by suspension and recentrifugation, and finally resuspended in phosphate-buffered saline.

Detergent treatment. Liposomes were incubated for 30 min in the cold in the presence of 0.05% taurodeoxycholate in phosphate-buffered saline, as described by Campbell et al. [16]. The liposomes were recovered by centrifugation, and washed once. The rhodopsin content of the liposome was measured by the RIA. The same procedures were also carried out on rhodopsin liposomes prepared in the presence of ^{125}I-labeled bovine serum albumin. The latter regent was prepared by the chloramine-T procedure, as described previously [13]. When using these liposomes, the radioactivity released into the supernatant solution, after the detergent incubation, was measured as an indication of the permeability caused by the detergent treatment.

Periodate oxidation. 1R-Liposomes were incubated in the presence of 10 mM sodium periodate in 10 mM Hepes buffer (pH 7.0), at 4°C in the dark. After 5 h, by which time there was no further change in the absorbance at 223 nm, the reaction was terminated by the addition of an 850 molar excess of ethylene glycol. After dialysis against a 200-fold volume excess of Hepes for 2 days with four changes in medium, the liposomes were recovered by centrifugation. Controls were prepared by subjecting the liposomes to the same procedures, now using a mock oxidation mixture prepared by pre-treating the periodate with ethylene glycol.

Freezing and thawing. 1R-Liposomes were subjected to several cycles of rapid freezing and thawing by imersion in polypropylene tubes into a dry-ice acetone bath. Glass tubes were avoided due to binding of the liposomes.

Materials

The foloowing materials were purchased from the indicated sources: concanavalin A, succinylated concanavalin A, wheat germ agglutinin, polyethylene glycol-1000, from Sigma Chemical Co., St. Louis, MO; *Limulus polyhemus, Maclura pomifera, Ricinus communis I, Ulex europaeus* I, from EY Laboratories, San Matea, CA.; L-α-lecithin (egg) (phosphatidylcholine), from Avanti Biochemicals, Inc., Birmingham, AL. (This material migrated as one spot when examined by thin-layer chromatography in the solvent system composed of chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, v/v) on Silica gel 60 plates (EM Laboratories, Elmsford, NY).); trimethylamine and 1-bromotridecane, from Eastman Kodak Co., Rochester, NY: $Na^{125}I$ in 0.1 M NaOH, from New England Nuclear Corp, Boston, MA. All other reagents were the highest grade commercially available.

Electron microscopy

Rhodopsin-liposomes were negatively stained with 1% phosphotungstic acid on collodion coated copper grids, after which electron microscopy was carried out with a JEOL 100c microscope. Magnification was calibrated with a catalase standard.

Results

Size, density, spectral properties, electron microscopy

Rhodopsin-liposomes (1R) were examined by equilibrium sucrose density centrifugation. 1 ml of a liposome suspension (26 nmol of rhodopsin) was layered on the top of a linear 10 to 40% (w/w) sucrose gradient and centrifuged overnight at $96\,300 \times g$ (r_{av}) in a Beckman SW-27 rotor using a model L5-75 ultracentrifuge (Beckman Instruments, Palo Alto, CA). After centrifugation, fractions were recovered by aspiration from the bottom of the tube, and the absorbance measured at 498 nm. The refractive index of each fraction was measured with an Abbe refractometer. The rhodopsin-liposomes banded in a sharp peak at a density of 1.11 g/ml, as reported previously [3,7].

When examined by electron microscopy, the 1R-liposomes appeared mainly as unilaminar vesicles. When these figures (about 200) were analyzed by a computerized image analyzer (Cambridge Instrument Co., Monsey, NY), the diameter was calculated to be 898 ± 31 Å (mean ± S.D.) (Fig. 1). In some preparations, however, a much greater range of sizes was detected. Thus, for example, in a preparation where about 90% of the liposomes were in the range of 1000 Å, about 1% were 10-fold greater in size.

Specificity of aggregation; inhibition; reversal

In accord with the presence of mannose and *N*-acetylglucosamine as the only sugars present in rhodopsin concanavalin A, succinyl-con A, and wheat germ agglutinin were the only lectins among several which were examined in whose presence aggregation of rhodopsin-liposomes was detected. The following lectins, at the indicated concentrations, had no effect on the light scattering of 1R-liposomes: *Ulex europaeus* (170 μg/ml); *Ricinus communis* I (50 μg/ml); *Maclura pomifera* (50 μg/ml); *Limulus polyhemus* (50 μg/ml). There was relatively little difference in aggregation when the reactions were performed in the light or dark. There was also little effect on the aggregation of liposomes after freezing and thawing. In contrast to the findings of Van der Bosch and McConnell [17], aggregation did not occur with control liposomes, i.e., liposomes prepared from egg phos-

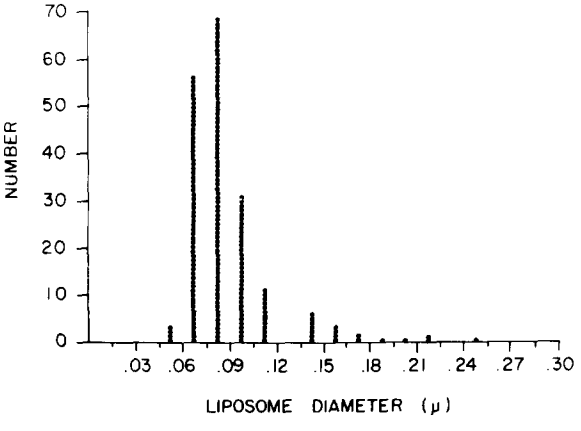


Fig. 1. Size distribution of 1R rhodopsin-liposomes. After electron microscopy, the images were analyzed by a computerized image analyzer (Cambridge Instrument Co., Monsey, NY). The average diameter was calculated to be 898 ± 31 Å (0.0898 ± 0.0031 μm).

phatidylcholine in the absence of rhodopsin. However, the concentration of phospholipid vesicles used in the present studies was much lower (27–130-fold in terms of phosphate concentration), as well as the concentration of concanavalin A (the lowest concentration used by Van der Bosch and McConnell was 10-fold higher than that used here). In addition, the presence of control liposomes at concentrations up to 4-fold greater than that of the rhodopsin liposomes had little or no influence on the aggregation of the 1R-liposome which occurred in the presence of concanavalin A.

Consistent with the specificity of concanavalin A, the aggregation process was inhibited by the presence of sugars containing an α-D-glucopyranosyl configuration, as seen in Table I. The sequence of inhibitory capacity of the monosaccharides is almost identical to that described previously by Goldstein et al. [18] for the inhibition of the Con A-dextran precipitation reaction. In accord with the specificity of wheat germ agglutinin, *N*-acetylglucosamine (0.1 M) completely inhibited the aggregation of 1R-liposomes that occurred in the presence of 300 μg/ml of this lectin. In addition, the aggregation of 1R-liposomes which occurred in the presence of concanavalin A (100 μg/ml) was reversed over 80% upon the addition of 0.1 M methyl α-D-mannoside (Fig. 2). In other experiments there was complete reversal. Essentially identical results were obtained using the divalent derivative, succinyl-con A (data not shown). Similarly, the aggregation which occurred in the presence of wheat germ agglutinin (300 μg/ml) was rapidly reversed by about 65% by the addition of 0.1 M GlcNAc. As with the other studies, there were essentially no differences between experiments performed in the light or dark. Prior treatment of the rhodopsin-liposome with periodate (10 mM), followed by incubation with concanavalin A, resulted in a decrease of about 80% in the aggregation which was induced by concanavalin A in the controls.

TABLE I

INHIBITION BY SUGARS OF THE AGGREGATION OF RHODOPSIN-LIPOSOMES INDUCED BY CONCANAVALIN A

Rhodopsin-liposomes (1 rhodopsin : 100 egg lecithin, 1 nmol/ml) were incubated in the presence of the indicated sugars for 30 min in the presence of concanavalin A (100 μg/ml). The percent inhibition refers to the percent difference in absorption at 360 nm obtained in the absence of the added sugars.

Sugars (0.3 M)	% Inhibition
Methyl α-D-glucoside	92
D-Mannose	77
D-Fructose	55
D-Glucose	27
N-Acetylglucosamine	23
N-Acetylgalactosamine	5.6
L-Fucose	4.2
N-Acetylmannosamine	3.3
D-Galactose	0.4
D-Glucosamine	0

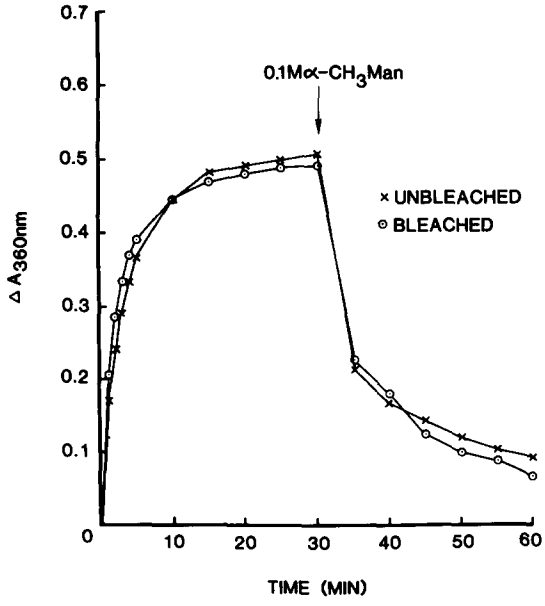


Fig. 2. Reversibility of aggregation of rhodopsin-liposomes. Aggregation of 1R-liposomes (1 nmol/ml) in phosphate-buffered saline was induced by the presence of concanavalin A (100 μg/ml) and the increase in light scattering followed at 360 nm at room temperature. After 30 min, methyl α-D-mannoside was added to a final concentration of 0.1 M, and the effect on the absorbance followed for an additional 30 min. Shown on the ordinate is the difference in absorbance obtained with a suspension of 1R-liposomes incubated in the presence and absence of concanavalin A. (×), carried out under dim red light conditions; (○), under usual laboratory fluorescent lighting.

Variation in liposome concentration and lectin concentration

The extent of aggregation in the presence of concanavalin A (100/μg ml) using 1R-liposomes was linear over a range from 0.35 to 1.4 nmol rhodopsin/ml (Fig. 3). When the concentration of concanavalin A was varied, an increase in turbidity was seen with incresing concentration of lectin (Fig. 4A). Similar results were obtained using succinyl-con A (data not shown). Although wheat germ agglutinin brought about much less turbidity than equimolar concentrations of concanavalin A (see below), wheat germ agglutinin also mediated the aggregation of rhodopsin-liposomes, as can be seen in Fig. 5.

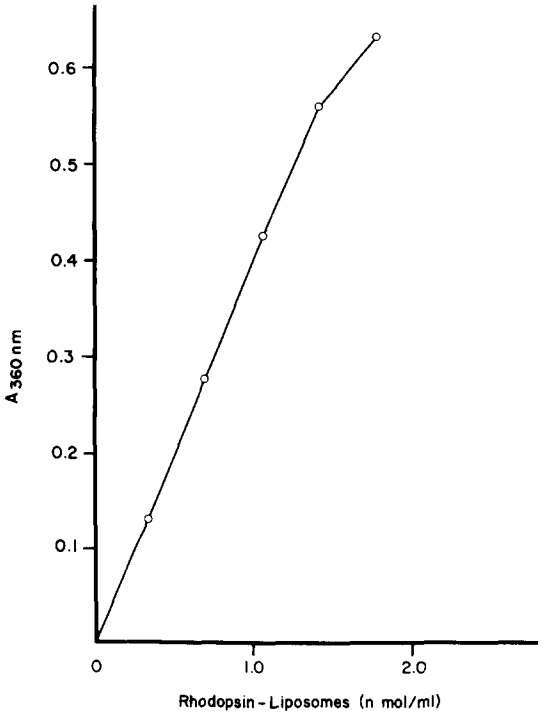


Fig. 3. Effect of variation in rhodopsin-liposome concentration on aggregation in the presence of concanavalin A. The aggregation of 1R-liposomes (1 rhodopsin: 100 egg phosphatidylcholine) was followed by measuring the increase in light scattering at 360 nm after the addition of concanavalin A (100 μg/ml). The concentration of the liposome, in terms of the rhodopsin concentration, was varied as indicated on the abscissa, and the aggregation by the difference in absorbance at 360 nm between that obtained in the presence and absence of the lectin. The values plotted are those obtained after incubating for 30 min at 23°C.

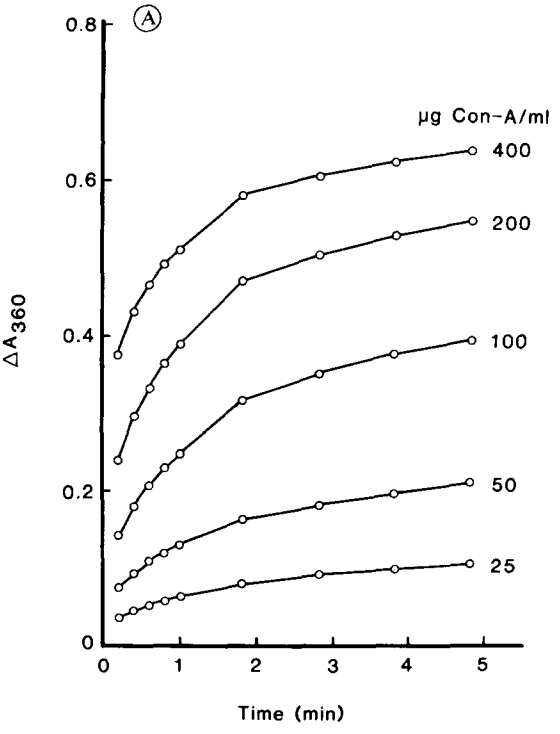
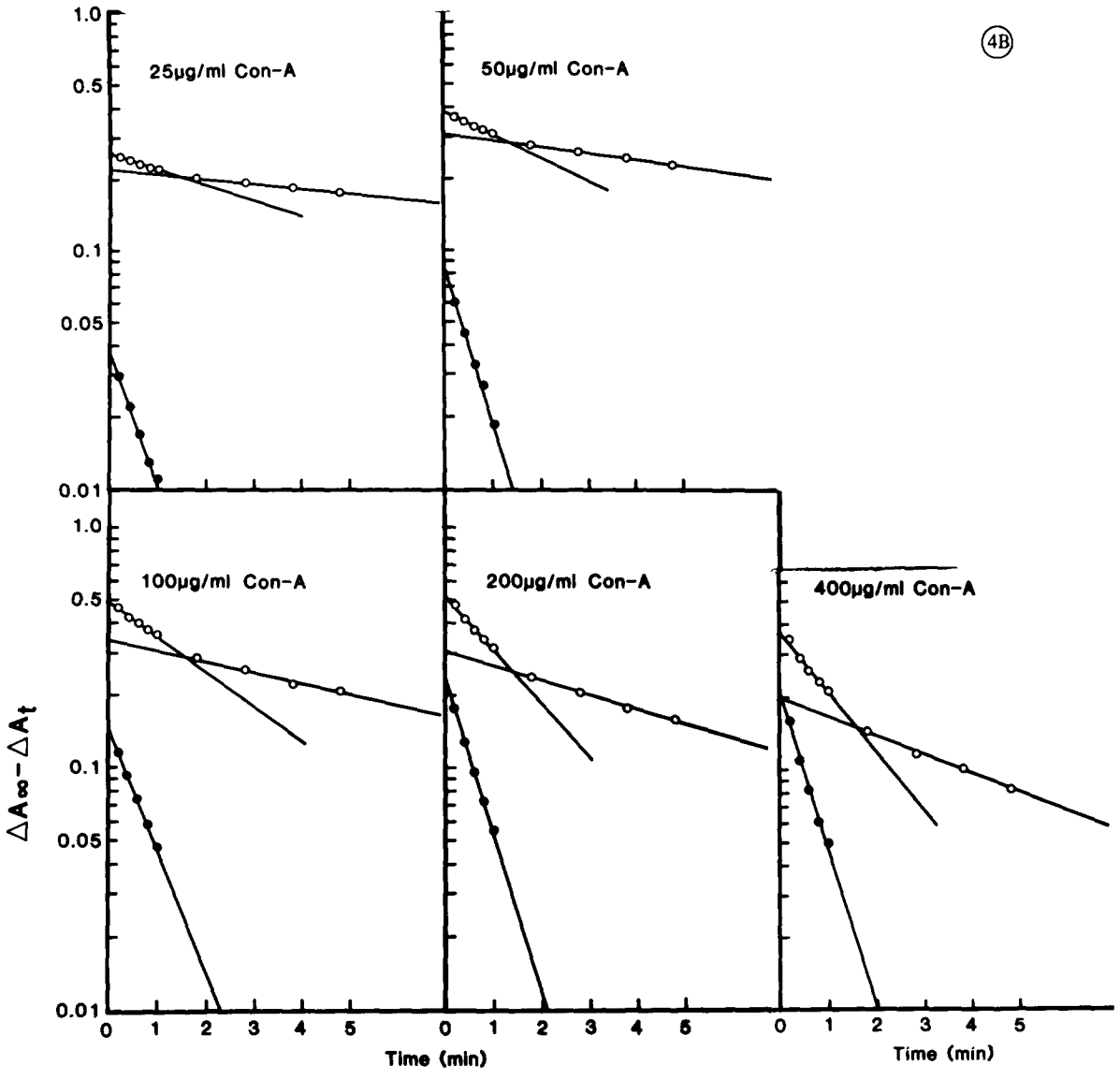


Fig. 4. Kinetics of aggregation of rhodopsin-liposomes in the presence of various concentrations of concanavalin A (con A). Rhodopsin-liposomes (1R), present at a concentration of 1 nmol rhodopsin/ml, were incubated at room temperature in the presence of various concentrations of concanavalin A, as indicated. (A) The increse in absorbance at 360 nm as a fucntion of time of incubation. On the ordinate is the difference in absorbance between that obtained in the presence and absence of the lectin. The change in absorbance over the first five minute period is shown for reactions which were carried out for up to 120 min. (B) Kinetic plots of the data. ΔA_∞ is the experimentally observed maximal absorbance as described under Experimental Procedures; A_t, the absorbance at time t; A_0, the absorbance at time zero, defined as the absorbance measured at 0.2 min after mixing. The delta vallues refer to the differences in absorbance in the presence and absence of the indicated concentration of concanavalin A. The first-order rate constants were calculated from the slopes of the lines (●) generated after subtracting the slow phase from the rapid phase (0.2 min to 1.0 min).

Kinetics of aggregation

After plotting the data in terms of first-order kinetics, as described in Experimental Procedures, it is apparent that the aggregation with concanavalin A was composed of more than one phase; a rapid component followed by one or more slower components (Fig. 4B). A similar pattern was obtained with wheat germ agglutinin



(plot not shown). After the first minute, the results fell approximately onto a second straight line of different slope. The values for the latter (the slow component) were subtracted from the initial rapid component for times up to one minute. The differential values are plotted on the same graph,and the first order rate constant of the rapid phase was then calculated from the slope of the line generated in this manner.

In Fig. 4B are kinetic plots of the aggregation observed in the presence of various concentrations of concanavalin A, from which first-order rate constants were calculated. Data of this type are summarized in Table II. As can be seen, relatively little variation in the first-order rate constants for the aggregation of 1R-liposomes was observed over an 80-fold range in the concentration of concanavalin A.

Although the extent of aggregation mediated by wheat germ agglutinin was much less than that achieved in the presence of concanavalin A (see below) the initial rates of aggregation of rhodopsin-liposomes by the two lectins were similar, as can be seen by the data summarized in Table III.

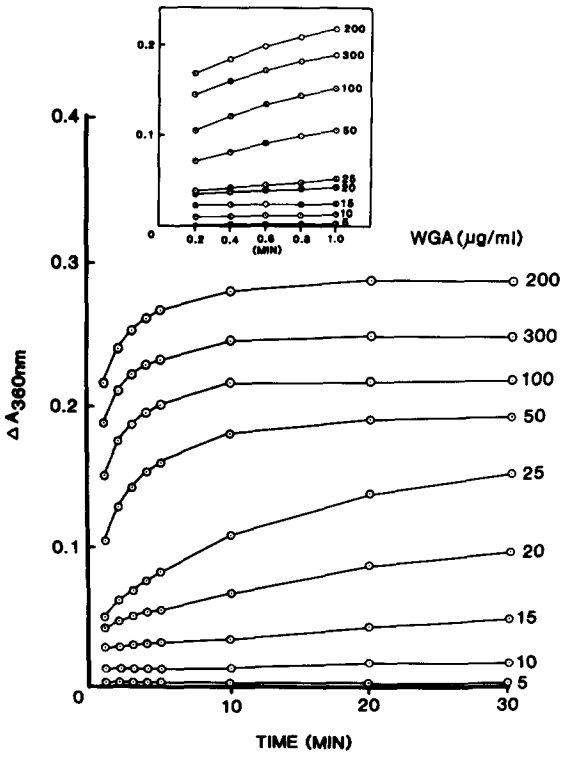


Fig. 5. Aggregation of rhodopsin-liposomes in the presence of various concentrations of wheat germ agglutinin (WGA). The aggregation of 1R-liposomes (1 nmol/ml) was followed as described in Experimental Procedures and in Fig. 4 in the presence of the indicated concentrations of wheat germ agglutinin in a total volume of 1 ml. In the inset is shown the time period of the reaction from which the initial rates were calculated.

The characteristics of the kinetic plots with wheat germ agglutinin were similar to those obtained with concanavalin A (data not shown). Reflecting the low reactivity with wheat germ agglutinin, it was not possible to calculate first-order rate constants of aggregation at concentrations of wheat germ agglutinin below 20 μg/ml.

Extent of reaction

Although the initial rates of aggregation of 1R-liposomes were similar in the presence of either concanavalin A or wheat germ agglutinin, the extent of the reactions, as indicated by the maximal turbidity achieved was greatly different with the two lectins. Defining a 'reactivity index' as the ratio: $\Delta A_\infty/M$, where M refers to the molarity of

TABLE II

FIRST-ORDER RATE CONSTANTS FOR THE AGGREGATION OF RHODOPSIN-LIPOSOMES IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF CONCANAVALIN A

Rhodopsin-liposomes (1R) were incubated in the presence of the indicated concentrations of concanavalin A (con A), and the first-order rate constants calculated from the increase in light scattering at 360 nm, as described in Experimental Procedures and in Fig. 4B. N, number of determinations. Figures are presented as means ± S.D.

Concn. of Con A		First-order rate constant (min^{-1})	N
μg/ml	μM [a]		
5	0.0455	2.1 ± 0.42	3
10	0.0909	2.7 ± 0.29	3
15	0.136	2.1 ± 0.40	3
20	0.182	1.9 ± 0.15	3
25	0.227	1.8 ± 0.33	4
50	0.455	1.6 ± 0.13	4
100	0.909	1.6 ± 0.24	5
200	1.82	1.7 ± 0.16	4
400	3.64	2.3 ± 0.75	4

[a] A molecular weight of 110000 for the tetramer was used in calculations.

TABLE III

FIRST-ORDER RATE CONSTANTS FOR AGGREGATION OF RHODOPSIN LIPOSOMES BY WHEAT GERM AGGLUTININ

1R-liposomes were incubated in the presence of wheat germ agglutinin (WGA) at room temperature for up to 2 h. The first-order rate constants were calculated from plots of the data shown in Fig. 5, and as described in Experimental Procedures. Figures are presented as means ± S.D. N, number of determinations.

Concn. of WGA		First-order rate constant (min^{-1})	N
μg/ml	μM [a]		
20	0.556	1.5 ± 0.20	3
25	0.694	1.2 ± 0.21	3
50	1.39	1.4 ± 0.05	3
100	2.78	1.6 ± 0.11	3
200	5.56	1.8 ± 0.29	3
300	8.33	1.6 ± 0.04	3

[a] A molecular weight of 36000 for the dimer of wheat germ agglutinin was used in the calculations.

the lectin, the variation in this value as a function of the concentration of concanavalin A and wheat germ agglutinin is seen in Fig. 6. The reactivity index with concanavalin A was relatively constant up to a concentration of about 0.5 μM. At about 0.6 μM, the response was wheat germ agglutinin reached an optimum value which was about one-third of that obtained with concanavalin A. At this point the molar concentrations of the lectins were about half of that of the rhodopsin-liposomes present in the reaction mixture (1 μM). The reactivity index then decreased with increasing lectin concentration. Since the spectral measurements were made of relatively stable suspensions, it is suggested that the decrease in reactivity index was due to the excess in lectin concentrations over that of the liposomes as a consequence of their multivalency.

Variation in mole ratio of rhodopsin

The effect of varying the mole ratio of rhodopsin in the liposomes on the aggregation brought about by concanavalin A and wheat germ agglutinin was examined. No ordered pattern in the extent of light scattering was seen with the liposomes whose rhodopsin composition varied from 0.2R to 19R in the presence of concanavalin A, as seen in Fig. 7. Similar results were obtained with wheat germ agglutinin (data not shown). A summary of the first-order rate constants calculated for liposomes containing the various mole ratios of rhodopsin in the presence of 100 μg/ml of concanavalin A or 300 μg/ml of wheat germ agglutinin is seen in Table IV. There were little differences in the rates of aggregation of these preparations with either lectin.

When examined by electron microscopy, the appearance of the liposomes having mole ratios varying from 0.2R to 2R was similar to that of the

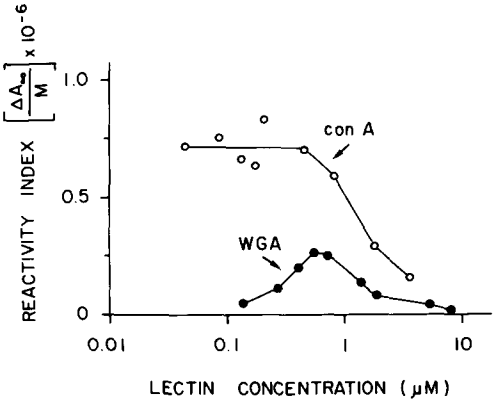


Fig. 6. Extent of aggregation of rhodopsin-liposomes by concanavalin A (Con A) and wheat germ agglutinin (WGA). Rhodopsin-liposomes (1R, 1 nmol/ml) were incubated at room temperature in the presence of the indicated concentrations of concanavalin A (○) or wheat germ agglutinin (●). The increase in absorbance at 360 nm was followed until a constant level was reached. The ΔA_∞ refers to the difference between this value and that obtained by the liposome in the absence of lectin. Plotted on the ordinate is this value divided by the molar concentration, a ratio defined as the 'reactivity index'.

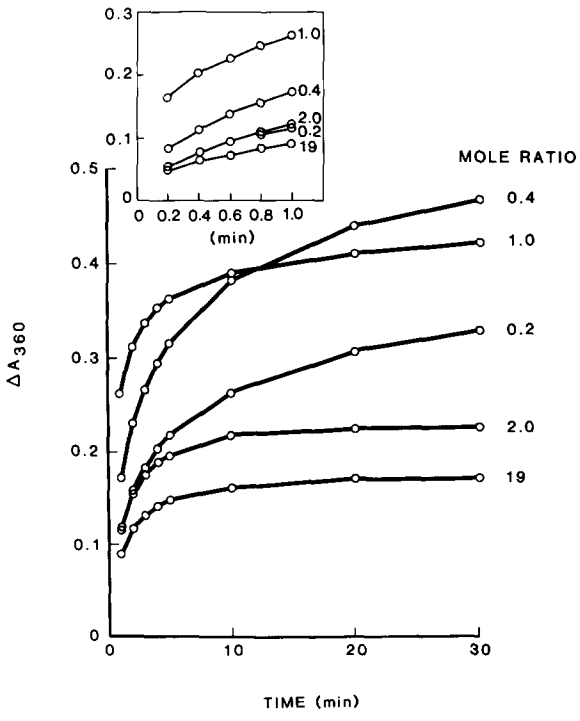


Fig. 7. Aggregation induced by concanavalin A of rhodopsin-liposomes containing various mole ratios of rhodopsin. Rhodopsin-liposomes were formed using egg lecithin and various concentrations of purified bovine rhodopsin, as described in Experimental Procedures. The mole ratios refer to the ratio of the moles of rhodopsin to 100 moles of lecithin. The concentration of rhodopsin during the incubations was 1 nmol/ml in all cases, and the aggregation was carried out in the presence of 100 μg/ml of concanavalin A over the time period indicated on the abscissa. In the inset is shown the phase of the reaction used for the calculation of the initial rates of aggregation.

TABLE IV

FIRST-ORDER RATE CONSTANTS FOR THE AGGREGATION BY CONCANAVALIN A AND WHEAT GERM AGGLUTININ OF RHODOPSIN-LIPOSOMES CONTAINING VARIOUS MOLE RATIOS OF RHODOPSIN TO EGG LECITHIN

Rhodopsin-liposomes containing the indicated mole ratios of rhodopsin to phospholipid, were incubated in the presence of 100 μg/ml of concanavalin A (Con A) or 300 μg/ml of wheat germ agglutinin (WGA) and the increase in light scattering measured at 360 nm. The first-order rate constants of aggregation were calculated as described iN Experimental Procedures. The figures are presented as means ± S.D. *N*, number of experiments.

Mole ratio [a]	Con A		WGA	
	Rate constant (min^{-1})	*N*	Rate constant (min^{-1})	*N*
19	1.4 ± 0.12	4	not measurable	3
2	1.7 ± 0.15	4	not measurable	3
1	1.6 ± 0.24	5	1.4 ± 0.22	3
0.4	1.5 ± 0.08	4	1.5 ± 0.03	3
0.2	1.5 ± 0.21	4	1.3 ± 0.14	3

[a] Mole rhodopsin: 100 mole egg lecithin.

1R-liposome. The 19R-product, however, had completely lost the round vesicle shape and appeared as an amorphous sheet of membranes.

Rhodopsin-liposomes reconstituted from rod outer segment lipids

The aggregation of rhodopsin-liposomes formed with purified rhodopsin and a total lipid extract of rod outer segments was also investigated. Shown in Fig. 8 are plots of the increase in absorbance obtained with 1R-liposomes made in this manner incubated in the presence of various concentrations of concanavalin A. Similar studies were carried out with wheat germ agglutinin (plots not shown). When the aggregation was examined as previously, similar kinetic responses were seen as when using rhodopsin-liposomes prepared with egg lecithin. A summary of the first-order rate constants calculated for the aggregation induced by concanavalin A and wheat germ agglutinin are seen in Tables V and VI, respectively. The rate constants with both lectins were similar to one another and to those obtained using the liposome prepared with purified egg lecithin.

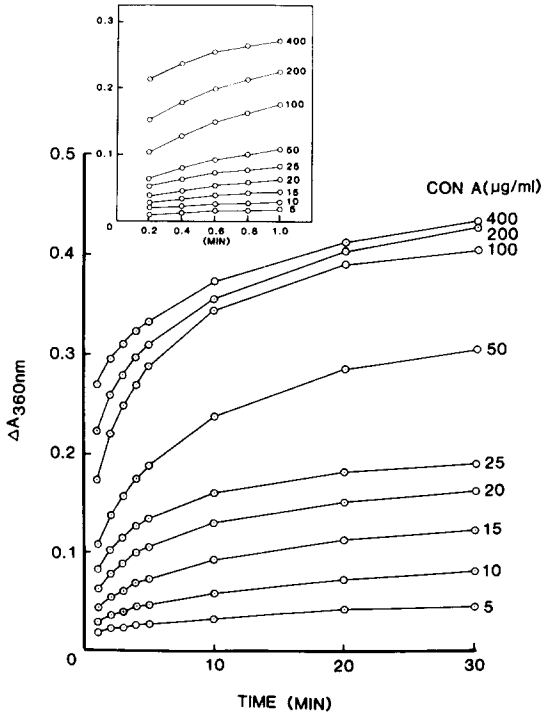


Fig. 8. Aggregation by concanavalin A of rhodopsin-liposomes reconstituted from purified bovine rhodopsin and a total lipid extract of rod outer segments. Bovine rod outer segments, isolated by sucrose density centrifugation, were extracted with chloroform/methanol (2 : 1, v/v) under an atmosphere of argon, then filtered and maintained under argon in the presence of 0.5 μmol of α-tocopherol. Rhodopsin-liposomes were prepared using this extract by the same procedures as previously. The mole ratio of rhodopsin to phospholipid in the reconstituted liposome was 1:100. The increase in light scattering brought about by the presence of various concentrations of concanavalin A as a function of incubation time is shown. The concentration of rhodopsin during the incubation was 1 nmol/ml. In the inset the increases in turbidity are shown for the time periods used in the calculations of the initial rate of aggregation, as described in Experimental Procedures.

Scatchard analysis

The aggregation studies described above do not provide information concerning the number of lectin binding sites or their affinity. In order to obtain this information, a Scatchard analysis was carried out using ^{125}I-succinyl-con A. (As described above, succinyl-con A reacted with the rhodopsin liposome in the same manner as did concanavalin A in the aggregation assays.) The results of the binding studies are seen in Fig. 9. While apparently a nonlinear Scatchard plot was

TABLE V

FIRST-ORDER RATE CONSTANTS FOR THE AGGREGATION OF RHODOPSIN-LIPOSOMES (1R)[a] RECONSTITUTED FROM ROD OUTER SEGMENT LIPIDS IN THE PRESENCE OF CONCANAVALIN A

Liposomes were constructed with purified bovine rhodopsin and the total lipid extract from bovine rod outer segments in the mole ratio of 1 rhodopsin:100 phospholipid. Aggregation was measured at 360 nm in the presence of the indicated concentrations of concanavalin A (Con A), and the first-order rate constants calculated. The figures are presented as means ± S.D. *N*, number of determinations.

Concn. of Con A		Rate constant (min^{-1})	*N*
μg/ml	μM		
5	0.0455	2.4 ± 0.42	3
10	0.0909	1.5 ± 0.15	3
15	0.136	1.7 ± 0.16	3
20	0.182	1.8 ± 0.17	3
25	0.227	1.8 ± 0.16	4
50	0.455	1.5 ± 0.25	4
100	0.909	1.7 ± 0.06	4
200	1.82	1.8 ± 0.09	4
400	3.64	2.0 ± 0.04	3

[a] The mole ratio of rhodopsin:phospholipid was 1:100.

obtained, a computer analysis of the data employing the 'Ligand' [15] program indicated a slight preference ($p = 0.1$) for a model having a single class of binding sites containing also about 0.4% of a non-specific or non-saturated component, as compared to a two class binding site model. The apparent dissociation constants (K_d) for the single site model or for the high-affinity site of the two site model, were identical, $2.8 \cdot 10^{-7}$ M. The number of binding sites were also similar. For the single site model there were calculated to be $(4.03 \pm 0.27) \cdot 10^{-7}$ mol/l of binding sites in a suspension containing 1 nmol of rhodopsin liposomes per ml. Presented also in Fig. 9 is the plot calculated for the single-site model. Thus, about 0.4 mole of divalent succinylated con A was bound per mole of rhodopsin by the IR-liposome.

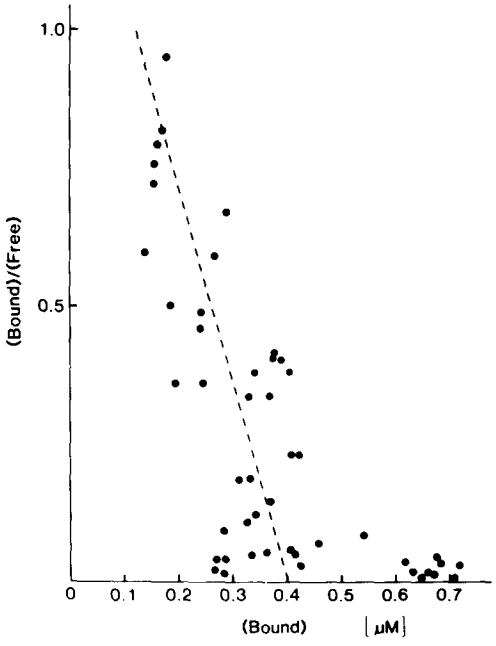


Fig. 9. A Scatchard plot of the data of the binding by rhodopsin-liposomes (1R) of ^{125}I-labeled succinylated con A. Specific binding was obtained, and the data analyzed by the 'Ligand' program [15], as described in Experimental Procedures. The dotted line indicates the Scatchard plot calculated by the computer for a model having a single class of binding sites.

TABLE VI

FIRST-ORDER RATE CONSTANTS FOR THE AGGREGATION OF RHODOPSIN-LIPOSOMES RECONSTITUTED FROM ROD OUTER SEGMENT LIPIDS IN THE PRESENCE OF WHEAT GERM AGGLUTININ (WGA)

Liposomes were constructed with purified bovine rhodopsin and the total lipid extract from bovine rod outer segments in the mole ratio of 1 rhodopsin:100 phospholipid and aggregation measured as described in Table V. Figures are presented as means ± S.D. *N*, number of determinations.

Concn. of WGA		First-order rate constant (min^{-1})	*N*
μg/ml	μM		
50	1.39	1.7 ± 0.83	3
100	2.78	1.2 ± 0.11	3
200	5.56	1.7 ± 0.42	3
300	8.33	1.2 ± 0.19	3
400	11.1	1.1 ± 0.19	3

In these studies, the non-specific binding, i.e., the binding of ^{125}I-succinyl-con A which occurred in the presence of methyl α-D-mannoside, accounted for about 12% of the total binding. Consistent with the observations described previously, control liposomes, i.e., liposomes prepared in the absence of rhodopsin, examined over a range in concentra-

tion of the iodinated lectin from 0.04 mg/ml to 0.4 mg/ml, bound less than 1.0% of that bound by 1R-liposomes. It is suggested that the low affinity second site which is evident in Fig. 9 at concentrations greater than 14.5 μM of succinyl-con A (0.4 μM bound) may reflect the influence of secondary, non-specific hydrophobic interaction of the lectin with the phospholipid matrix of the liposome, not resolved by methyl-α-D-mannoside, as pointed out by Goldstein and Hayes [19].

Modified liposomes

A summary of the effects of the various treatments of the 1R-liposomes on their ability to bind ^{125}I-succinyl-con A is seen in Table VII. Extensive loss in binding activity resulted from treating the liposomes with trypsin and periodate. The latter effect is consistent with the destruction of terminal α-mannose residues of the rhodopsin oligosaccharide chains. The residual reactivity in the presence of this reagent could reflect the interaction with internal α-mannose residues of rhodopsin which are resistant to oxidation by periodate (Liang et al. [20], and Kean et al. [21]).

After treatment with deoxycholate, about 87% of the rhodopsin of the liposome was recovered, indicating little solubilization of the glycoprotein.

TABLE VII

^{125}I-LABELED SUCCINYLATED CONCANAVALIN A BINDING BY RHODOPSIN-LIPOSOMES AFTER VARIOUS TREATMENTS

^{125}I-Succinyl con A (200 μg) was incubated with 0.5 nmol of rhodopsin-liposomes (1R), as described in Experimental Procedures. The 100% value was 21.2±0.6 (7) μg bound/nmol rhodopsin. The values are the means±S.D. where applicable. *N*, number of determinations.

Treatment	Binding of succinyl-con A (% of control)	*N*
None (control)	100	7
Freezing and thawing	99 ±14	3
Trypsin	2.2± 2.8	7
Taurodeoxycholate	104 ± 7	5
Trypsin followed by taurodeoxycholate	13 ± 6	2
Taurodeoxycholate followed by trypsin	0	2
Sodium periodate	55 ± 2	5
Mock sodium periodate	107 ± 8	2

Under these conditions, about 36% of the entrapped ^{125}I-labeled bovine serum albumin was released. While these conditions might have allowed for the lectin to penetrate the vesicle and interact with internal sites, no increase in binding of the lectin was observed.

Freezing and thawing of the liposome caused no change in the net binding of the lectin, suggesting that similar randomization which might have resulted from the latter treatments was already present in the reconstituted membrane vesicels. Trypsinization resulted in almost complete loss of binding. This is in contrast to the effect of similar treatments of rod outer segment discs which were unsealed with detergents, as described by Clark and Molday [9]. Thus, the orientation of the amino terminus of rhodopsin in the phospholipid membrane of the liposome may not be the same as the structural arrangement in ntive disc membranes.

Discussion

Consistent with the presence of terminal α-D-mannosyl groups in its sugar chains [20–22], rhodopsin in disc membranes, as well as purified rhodopsin, has been shown to bind to concanavalin A [2,9,23]. The presence of a terminal GlcNAc residue as well as an internal *N*,*N*-diacetylchitobiosyl residue in rhodopsin should provide also for an interaction with wheat germ agglutinin [24]. The binding of wheat germ agglutinin with disc membranes has been reported [9,25,26], although detailed information concerning the interaction of purified rhodopsin itself with this lectin is not available. The greatly reduced extent of aggregation of the vesicles which was obtained with wheat germ agglutinin as compared to concanavalin A suggests the relative crypticitity of some of the GlcNAc-residues of rhodopsin in the liposomes as compared to mannose.

SImilar to the results obtained with liposomes constructed with glycolipids [27–29], with glycophorin [30,31], and with the concanavalin A receptor from human erythrocytes [14], the aggregation of rhodopsin-liposomes brought about by concanavalin A and wheat germ agglutinin was reversed by the addition of sugars which compete with the sugars in the rhodopsin-liposome for binding by the lectin. These observations indicate

that the increase in turbidity brought about by concanavalin A did not involve vesicle fusion. With wheat germ agglutinin, fusion was probably not a major factor.

Unlike most other reports dealing with similar processes [27–31], in the present studies the kinetic analyses showed that the aggregation of rhodopsin-liposomes mediated by lectins was at least biphasic, readily separable into a fast initial first order process and a slower, higher order process which may represent a composite of several time constants. A multiphasic process was also detected by Orr et al. [28], although unlike the present situation, the initial phase was slow. The presence of a rapid initial phase was pointed out by Chicken and Sharom [14] for the concanavalin A induced agglutination of vesicles constructed with the concanavalin A receptor of erythrocytes, but was not described further. In the present work, attention was directed only to the initial rapid component. This is the first description of these processes in rhodopsin-containing membranes, either natural or synthetic. Although the extent of aggregation was influenced by the concentration of concanavalin A and wheat germ agglutinin, the first-order rates of aggregation were relatively constant over a wide range in the concentration of these lectins. The rate constants for the aggregation of rhodopsin-liposomes which contained different mole ratios of rhodopsin, each examined at the same concentration (1 nmol rhodopsin/ml), in the presence of concanavalin A or wheat germ agglutinin were also relatively constant, unlike the findings with glycolipid-liposomes [28]. This observation suggests that the surface density of rhodopsin in the liposome may not play a role in the initial rate of aggregation, but only the number of sites available for reaction with the lectin.

Differences were revealed in these studies in the orientation of the carbohydrates of rhodopsin in the liposomes as compared to this property of disc membranes as described in previous reports. It is of interest that while most of the rhodopsin is located in the disc membranes of the rod outer segment, the plasma membrane has been observed to contain about 1.5% of the total rhodopsin of the outer segment [32]. Unlike the orientation in the intact discs, the carbohydrate groups of rhodopsin in the plasma membrane face the extracellular space [32], perhaps in a manner analogous to the orientation observed here for the liposomes. The detailed characterization presented in this report of the availability and reactivity of the carbohydrate groups of rhodopsin in synthetic vesicles prepared with this integral membrane glycoprotein may provide a basis for further studies utilizing this preparation in experiments dealing with cell-membrane interactions.

Acknowledgements

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References

1 Heitzmann, H. (1972) Nature New Biol. 235, 114
2 Plantner, J.J. and Kean, E.L. (1976) J. Biol. Chem. 251, 1548–1552
3 Hong, K. and Hubbell, W.L. (1972) Proc. Natl. Acad. Sci. USA 69, 2617–2621
4 Hong, K. and Hubbell, W.L. (1973) Biochemistry 12, 4517–4523
5 O'Brien, D.F., Costa, L.F. and Ott, R.A. (1977) Biochemistry 16, 1295–1303
6 Montal, M. (1979) Biochim. Biophys. Acta 559, 231–257
7 Jackson, M.L. and Litman, B.J. (1982) Biochemistry 21, 5601–5608
8 Heller, J. and Lawrence, M.A. (1970) Biochemistry 9, 864–869
9 Clark, S.P. and Molday, R.S. (1979) Biochemistry 18, 5868–5873
10 Fung, B.K.-K. and Hubbell, W.L. (1978) Biochemistry 17, 4403–4410
11 Plantner, J.J., Hara, S. and Kean, E.L. (1982) Exp. Eye Res. 35, 543–548
12 Chen, P.S., Jr., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
13 Lentrichia, B.B., Plantner, J.J. and Kean, E.L. (1980) Exp. Eye Res. 31, 1–8

14 Chicken, C.A. and Sharom, F.J. (1983) Biochim. Biophys. Acta 729, 200–208
15 Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
16 Campbell, C.H., Miller, A.L. and Rome, L.H. (1983) Biochem. J. 214, 413–419
17 Van der Bosch, J. and McConnell, H.M. (1975) Proc. Natl. Acad. Sci. USA 72, 4409–4413
18 Goldstein, I.J., Hollerman, C.E. and Smith, E.E. (1965) Biochemistry 4, 876–883
19 Goldstein, I.J. and Hayes, C.E., (1978) Adv. Carbohydr. Chem. Biochem. 35, 127–340
20 Liang, C.-J., Yamashita, K., Muellenberg, C.G., Shichi, H. and Kobata, A. (1979) J. Biol. Chem. 254, 6414–6418
21 Kean, E.L., Hara, S., Mizoguchi, A., Matsumoto, A. and Kobata, A. (1983) Exp. Eye Res. 36, 817–825
22 Fukuda, M.N., Papermaster, D.S. and Hargrave, P.A. (1979) J. Biol. Chem. 254, 8201–8207
23 Steinemann, R. and Stryer, L. (1973) Biochemistry 12, 1499–1502
24 Nicolson, G.L. (1974) Int. Rev. Cytol. 39, 89–190
25 Nir, I. and Hall, M.O. (1979) Exp. Eye Res. 29, 181–194
26 Bridges, C.D.B. (1981) Invest. Ophthalmol. Vis. Sci. 20, 17–23
27 Redwood, W.R. and Polefka, T.G. (1976) Biochim. Biophys. Acta 455, 631–643
28 Orr, G.A., Rando, R.R. and Bangerter, F.W. (1979) J. Biol. Chem. 254, 4721–4725
29 Curatolo, W., Yau, A.O., Small, D.M. and Sears, B. (1978) Biochemistry 17, 5740–5744
30 Redwood, W.R., Jansons, V.K. and Patel, B.C. (1975) Biochim. Biophys. Acta 406, 347–361
31 Goodwin, G.C., Hammond, K., Lyle, I.G. and Jones, M.N. (1982) Biochim. Biophys. Acta 689, 80–88
32 Kamps, K.M.P., DeGrip, W.J. and Daemen, F.J.M. (1982) Biochim. Biophys. Acta 687, 296–302