BIOCHEMICAL INVESTIGATIONS OF RETINOTECTAL ADHESIVE SPECIFICITY

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ABSTRACT

The preferential adhesion of chick neural retina cells to surfaces of intact optic tecta has been investigated biochemically. The study uses a collection assay in which single cells from either dorsal or ventral halves of neural retina adhere preferentially to ventral or dorsal halves of optic tecta, respectively. The data presented support the following conclusions: (a) The adhesion of ventral retina to dorsal tecta seems to depend on proteins located on ventral retina and on terminal β-N-acetylgalactosamine residues on dorsal tecta. (b) The adhesion of dorsal retina to ventral tecta seems to depend on proteins located on ventral tecta and on terminal β-N-acetylgalactosamine residues on dorsal retina. (c) A double gradient model for retinotectal adhesion along the dorsoventral axis is consistent with the data presented. The model utilizes only two complementary molecules. The molecule suggested to be concentrated dorsally in both retina and tectum seems to require terminal β-N-acetylgalactosamine residues for adhesion. Its activity is not affected by protease. A molecule fitting these qualifications, the ganglioside GM2, could not be detected in a gradient, but lecithin vesicles containing GM2 adhered preferentially to ventral tectal surfaces. The second molecule, concentrated ventrally in both retina and tectum, is a protein and seems capable of binding terminal β-N-acetylgalactosamine residues. One enzyme, UDP-galactose:GM2 galactosyltransferase, has been found to be more concentrated in ventral retina than dorsal, but only by 30%.

A topographic selectivity characterizes the adhesion of neural retina cells to optic tecta. Cells from dorsal half-retina have been shown to adhere preferentially to ventral surfaces of optic tecta, while those from ventral half-retina prefer dorsal surfaces of tecta (2, 3, 25). Investigations of the biochemical mechanisms underlying this selectivity are reported here.

The basis for this study is an assay that compares the rates at which single retinal cells adhere to limited areas of tectal surface. In this assay, either dorsal or ventral halves of neural retina are labeled with 32P and dissociated with trypsin to form a single-cell suspension. Tecta are split into equal-sized dorsal and ventral halves and fixed to the bottom of a petri dish. The labeled retinal cells are added to the dish, and the dish is reciprocated. After a collection period, typically 1 h, the tectal halves are washed and counted individually in a scintillation counter to determine the numbers of radioactive retinal cells that adhered. Since the number of collisions between the labeled retinal cells and each of the tectal halves is equal, comparisons between the numbers of cells adhering to dorsal and ventral halves provide an operational definition of adhesive specificity.
With this assay, cells from dorsal retina were shown to adhere preferentially to ventral half-tecta, and cells from ventral retina to prefer dorsal half-tecta. Tecta that had never been innervated by retinal fibers were also used as collecting surfaces, and displayed selectivity similar to that shown by innervated tecta. The preferential adhesion could not, therefore, be attributed solely to the adhesion of retinal cell bodies to retinal axons on the surfaces of the tecta. Thus, a topographic selectivity in the adhesion between the two neural structures was established.

The accessibility of this adhesion assay to biochemical manipulation is exploited in this report. Data are presented that implicate two complementary molecules as the effectors of the dorso-ventral adhesive specificity. One of these is suggested to be a protein and the other to require terminal N-acetylgalactosamine residues for its activity. These molecules are proposed to be distributed in gradients of opposite polarity on both retina and tectum.

MATERIALS AND METHODS

**Materials**

Incubation medium (DECS) was prepared aseptically and contained the following components (vol/vol): Dulbecco's Modified Eagle's Medium (8), 90%, calf serum (Grand Island Biological Co., Grand Island, N. Y.), 10%, penicillin G (Parke, Davis & Co., Detroit, Mich.), 100 U/ml. Only 1/10 the normal amount of NaHCO₃ was included (0.035 g/liter). The medium was buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) at pH 7.2. Hanks' balanced salt solution (HBSS) was also buffered with 10 mM HEPES at pH 7.2. Labeling medium consisted of 4 ml of DECS containing 0.3 mCi of carrier-free ³²P-labeled inorganic PO₄. (International Chemical and Nuclear Corp.) Disso-
ciating medium contained 0.10% crude pancreatic trypsin extract (Difco Laboratories, Detroit, Mich., 1:250) in Ca ++-, Mg ++-, and glucose-free HBSS.

*Diplococcus pneumoniae* (strain 6301) and *Clostridium perfringens* (strain 13124) were purchased from the American Type Culture Collection.

Uridine diphosphate-[³²P]galactose (UDP-[³²P]Gal) was purchased from Amersham/Searle Corp. (Arlington Heights, III.). [³²P]N-acetylmannosamine ([³²P]ManNAc) was purchased from New England Nuclear (Bos-
ton, Mass.). [³²P]diioleyl phosphatidylcholine was the kind gift of Dr. Richard Pagano (Carnegie Institution of Washington).

Precast thin-layer chromatography (TLC) plates were purchased from Merck Chemical Div., Merck & Co., Inc. ( Rahway, N. J.). Three solvent systems were used to characterize gangliosides: chloroform/methanol/ water (60:35:8); chloroform/methanol/2.5 N ammonium hydroxide (60:35:8); n-propanol/water (70:30). Ganglioside and neutral glycolipid standards were the kind gift of Dr. Saul Roseman (The Johns Hopkins University). Additional GM₁ and GM₂ were purchased from Supelco, Inc. (Bellefonte, Pa.).

Dipalmitoyl lecithin and Azocoll were purchased from Calbiochem (San Diego, Calif.). Purified trypsin, purified chymotrypsin, cycloheximide, puromycin, dimethylphenyl-p-nitrophenyl-β-D-galactoside (pNp-Gal), pNp-β-D-α-N-acetylglucosamine (pNp-GlcNAc), pNp-β-D-N-acetylgala-
tosamine (pNp-GalNAc), GalNAc, GlcNAc, Gal, lactose, N-acetyllactosamine, N-acetylmannosamine, Tween 80, Triton CF-54, UDP-Gal, and adenosine 5'-monophosphate were all purchased from Sigma Chemical Co. (St. Louis, Mo.). Asialo-fetuin (AS-fetuin) and asialo-ovine submaxillary mucin (AS-OSM) were prepared from fetuin and OSM, respectively, by mild acid hydrolysis. Asialo-agalactofetuin (AS-AG-fetuin) was prepared from AS-fetuin by treatment with *Diplococcus β-galactosidase*.

**Methods**

The techniques of the retinotectal adhesion assay used in this laboratory have been described in detail previously.

**Preparation of Labeled Neural Retina Suspension:** Dorsal or ventral halves of neural retinas were dissected aseptically from both eyes of White Leghorn chick embryos, 7–9 days old. 4–6 retinal halves were incubated in 4 ml of labeling medium for 2–6 h at 37°C. The half-retinas were then rinsed in HBSS and incubated for 25 min at 37°C in dissociating medium. After this incubation, the half-retinas were triturated to a single-cell suspension with a small-bore Pasteur pipette, 4 ml of DECS was added, and the suspension was centrifuged at 170 g for 6 min. The cells were resuspended in DECS, counted in a hemocytometer, and diluted to 10⁶ cells/ml for most experiments. Aliquots were counted in a liquid scintillation counter to determine the specific activity of the cells.

**Preincubation:** In most experiments, the ³²P-labeled cells were preincubated in DECS before their exposure to tectal halves. Aliquots of 12 ml of cell suspension at 10⁵ cells/ml were reciprocated at 90 cycles/min in 50-mm petri dishes that contained a layer of paraffin on the bottom. After the desired preincubation time, 10-ml aliquots of cell suspension were transferred to similar dishes in which dorsal and ventral tectal halves were pinned.

**Preparation of Tectal Halves:** Intact optic tecta were dissected aseptically from 12-day chick embryos immediately before each experiment was to begin. Outer membranes including the pia mater were removed, and the tecta were split into dorsal and ventral halves (13) and trimmed of extraneous tissue. Since dorsal and ventral tectal halves can be unambiguously
distinguished by the geometries of their luminal surfaces, all halves were treated identically in the same vessels until scintillation counting.

**COLLECTION:** Six dorsal and six ventral tectal halves were pinned alternately to a paraffin layer in the bottom of a 50-mm petri dish (2). Collection was begun by transferring 10 ml of a labeled retinal cell suspension to each dish. The dish was covered and reciprocated at 90 cycles/min at 37°C on a reciprocald water bath shaker, model R76 (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). After the desired incubation period, each tectal half was removed from the dish, gently dipped five times in a large volume of HBSS, and counted individually in a scintillation counter.

Five dorsal and five ventral tectal halves were routinely used for each data point. Data are reported as the mean number of retinal cells adhering per tectal half ± the standard error of the mean. The significance of pairwise comparisons was determined using Student's t test.

**BACKGROUND DETERMINATIONS:** Corrections for absorption by the tectal halves of soluble, noncellular radioactivity were calculated individually for each experiment. Estimates of this correction were obtained by two independent methods. In the first of these, an additional aliquot of retinal cell suspension was reciprocated in the absence of tectal halves for one-half the collection time of the particular experiment. The suspension was then centrifuged at 250 g to remove the cells. 10 ml of supernate were reciprocated with tectal halves for the full collection time. The tecta were then washed as described above and counted for absorbed radioactivity. Since the leakage of soluble radioactivity by retinal cells and the absorbance of radioactivity by tectal halves are linear with both time and concentration (unpublished data), the levels of noncellular background radioactivity were determined.

The second method utilized tectal halves that had been incubated in the same vessel as the experimental halves, but it subjected the halves to be used for background determinations to a more vigorous washing procedure (2). The results obtained by the two methods were nearly identical.

This background typically amounted to ~15% of the radioactivity due to collected retinal cells. The levels of absorbed radioactivity were not affected by any of the degradative enzymes or by the addition of saccharides or protein synthesis inhibitors. Lowered temperature or the presence of general metabolic inhibitors during the collection period decreased the background. Ventral tectal halves consistently absorbed ~1.1 times as much soluble radioactivity as did dorsal tectal halves.

**PROTEASE EXPERIMENTS:** Protease activity was assayed using Azocoll, 5 mg/ml in phosphate-buffered saline (PBS), pH 7.8, by determining OD₅₉₀ after 15 min at 37°C. Crystalline trypsin and chymotrypsin were dissolved in PBS, pH 7.8, at concentrations of 0.009% and 0.016% (wt/vol), respectively. Dissociation with crystalline trypsin followed the procedures described above for dissociation with normal dissociating medium. Treatment of single retinal cells was accomplished by centrifuging the cells at 170 g for 6 min, resuspending in HBSS, centrifuging again, and resuspending in 10 ml of a protease solution. After 15 min at 37°C, 10 ml of DECS was added, and the cells were centrifuged and resuspended in 10 ml of DECS. Tectal halves to be treated were pinned to the paraffin layer of assay dishes, rinsed twice in HBSS, incubated in 10 ml of a protease solution for 15 min at 37°C, washed twice in DECS, and used in the same plates for collection experiments. Controls were incubated for 15 min in PBS, pH 7.8.

**GLYCOSIDASES:** Neuraminidase, β-galactosidase, and β-N-acetylhexosaminidase were partly purified from supernates of cultures of two strains of bacteria. Enzymes from *Diplococcus pneumoniae* were purified by the procedures of Hughes and Jeanloz (17, 18). Neuraminidase from *Clostridium perfringens* was purified by the procedure of Cassidy et al. (4). β-galactosidase and β-N-acetylhexosaminidase from *Clostridium* were purified by modifications of the procedures of McGuire et al. (27). The modifications introduced the initial steps of the Cassidy et al. procedure, such that all enzymes from *Clostridium* were prepared from the same cultures. *Clostridium* neuraminidase was assayed at pH 6.0 and *Diplococcus* neuraminidase at pH 6.5. Both assays used 5 mM sialyllactose as substrate in 0.5 ml of phosphate buffer. Enzyme samples and substrate were incubated together at 37°C for 15 min. Liberated sialic acid was detected with the thiobarbituric acid assay (47). β-galactosidase was assayed using 5 mM pNp-Gal as substrate in 0.5 ml of phosphate buffer, pH 6.5. Samples were incubated for 15 min at 37°C; the reaction was terminated by addition of 0.5 ml of 0.25 M sodium carbonate. The OD₅₉₀ was determined and the enzyme activity estimated using the molar extinction coefficient (18 x 10⁶) of p-nitrophenol at that wavelength. β-N-acetylhexosaminidase activity was determined using both pNp-GlcNAc and pNp-GalNAc as substrates at conditions identical to those described for β-galactosidase, except that the *Diplococcus* enzyme was assayed at pH 6.0.

Units of enzyme activity were defined as follows: neuraminidase—1 U of enzyme releases 1 μmol of sialic acid from 5 mM sialyllactose in 15 min at 37°C in a reaction volume of 0.5 ml; β-galactosidase—1 U of enzyme releases 1 μmol of galactose from 5 mM pNp-Gal in 15 min at 37°C in a reaction volume of 0.5 ml; β-N-acetylhexosaminidase—1 U of enzyme releases 1 μmol of GlcNAc from 5 mM pNp-GlcNAc in 15 min at 37°C in a reaction volume of 0.5 ml.

Retinal suspensions to be treated with a glycosidase were centrifuged at 170 g for 6 min, resuspended in HBSS, centrifuged again, and resuspended in PBS containing the desired glycosidase at 25 U/ml at the pH's defined above. The cells were incubated in 50-mm petri dishes with paraffin bottoms for 15 min at 37°C, centri-
fuged at 170 g for 6 min, and resuspended in DECS. Control retinal suspensions were treated with PBS at the same pH, but containing no glycosidase. Further controls contained the enzyme at the same concentration but previously inactivated by heating at 100°C for 15 min. Tectal halves were treated after having been pinned to the paraffin layers of assay dishes. The tectal halves were washed in HBSS, and incubated in 10 ml of PBS containing a glycosidase at the concentration and pH specified for retinal cells. After 15 min at 37°C, the tectal halves were washed twice in DECS and then used as collecting surfaces in the same petri dishes. Controls were treated with PBS at the same pH or with heat-inactivated enzyme.

**Collection Experiments with Sugars Present:** For experiments in which concentrations of sugars were 10 mM or less, the saccharide was added to DECS with no correction for the increase in osmolarity. Control incubations were done in the presence of additional NaCl at half the molarity of the added saccharide. In the experiments with higher concentrations of saccharide, DECS was prepared that was deficient in NaCl such that the osmolarity of the final medium was identical to that of normal DECS.

**Ganglioside Preparations:** Proportions of the individual gangliosides were determined using two methods, one reflecting cellular concentrations and the other, rates of synthesis. To determine concentrations, dorsal, middle, and ventral thirds of retinas were dissected from both eyes of 9- to 11-day chick embryos. Approx. 100 eyes were required for each experiment. Gangliosides were extracted by the procedures of Tettamanti et al. (46). The tissues were homogenized in 2.5 ml of 0.01 M potassium phosphate, pH 6.8, and aliquots were taken for protein determinations (23). After further homogenization in the presence of 20 ml of tetrahydrofuran, the suspensions were centrifuged at 600 g for 10 min. The supernate were decanted and the residues were extracted three more times with 8 ml of one part phosphate buffer, four parts tetrahydrofuran. The pooled supernates, after addition of 0.3 vol ethyl ether, were vigorously shaken for 2 min, then centrifuged at 600 g for 10 min. The upper organic phase, after addition of 0.1 vol deionized water, was shaken and centrifuged as before. The pooled aqueous phases were concentrated under N2, and dialyzed in 2 ml against deionized water for 2 days with three changes per day. The retentates were dried under N2 and dissolved in chloroform/methanol (2:1). The dissolved gangliosides were spotted on 20 × 20-cm TLC plates that had been activated at 120°C for 2 h. During the spotting, the bulk of the TLC plate was covered with a glass plate to inhibit hydration. The gangliosides, along with standards, were chromatographed in one of three solvent systems (listed in Materials) for 8–12 h. The plates were dried, and the individual gangliosides were quantitated by detection of bound sialic acid using the methods of Suzuki (43). Gangliosides were located and marked by exposing the plates to iodine vapors. After complete sublimation of the iodine, the areas of the individual gangliosides were scraped into small tubes. A few areas free of gangliosides were taken as blanks. To each sample, 0.25 ml of deionized water was added and the tubes were allowed to stand for 5 min. Then, 0.25 ml of resorcinol reagent (45) was added and each tube was vortexed vigorously for 1 min. The tubes were heated for 15 min in a boiling water bath, and the chromophores were extracted by the addition of 0.75 ml of butylacetate-butanol (85:15, vol/vol). The optical densities were read at 580 nm. With this procedure, 1 μg sialic acid yielded an OD of ~0.040.

To detect rates of synthesis, dorsal, ventral, and middle thirds from about 40 eyes of 8- to 11-day chick embryos were dissected aseptically and incubated in 20-ml De Long flasks (Becto, Vineland, N. J.) containing 5 ml of DECS supplemented with 15 mM NaHCO3 and 100 μCi [3H]ManNAc. The retinal thirds were incubated in open flasks for 12 h, with the pH being adjusted to 7.4 as needed. The retinas were washed in HBSS, resuspended in 0.5 ml of phosphate buffer, and extracted as described above. Dialysis retentate volumes were kept below 1.0 ml to prevent loss of gangliosides (21). After TLC as described above, the plate was divided into 67 bands of 3 mm each. In each of the lanes, the bands were scraped into individual scintillation vials and 0.3 ml of deionized water and 15 ml of Triton- tolueene counting solution were added. The vials were counted in a liquid scintillation system, and the distribution of radioactivity was plotted.

**Lechithin Vesicles:** Dipalmitoyl phosphati- dycholine (4 mg), 0.2 μCi [3H]dioloeil phosphatidyl- choline (50 μCi/mmol), and 80 μg of an individual ganglioside were mixed in organic solvents and dried under a stream of N2. The residues were suspended in 3 ml of PBS, pH 7.2, at 43°C, and sonicated at 43°C in an argon atmosphere for 15 min, using a sonifier cell disruptor at 50 W. After the sonication, which clarified the emulsion, 1 additional ml of PBS was added and the solution was transferred to an assay plate containing six dorsal and six ventral tectal halves. The assay plate consisted of a 50-mm glass petri dish with a layer of paraffin on the bottom as described above. However, embedded in the paraffin was a 35-mm Falcon tissue culture dish bottom (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) which itself contained a layer of paraffin. Tectal halves were pinned in the smaller dish in three rows of four, alternating dorsal and ventral halves. This arrangement allowed the experiment to be done in a suspension volume of 4 ml.

The assay plate containing tectal halves and labeled vesicles was reciprocated at 90 cycles/min at 37°C. After incubations ranging from 15 min to 2 h, tectal halves were removed from the plate, gently dipped five times in PBS, and placed in individual plastic scintillation vials. NCS tissue solubilizer (0.3 ml) was added and the tecta were incubated at 37°C
until solubilized. After addition of 50 μl of glacial acetic acid to neutralize the mixture and 10 ml of Triton-toluene counting solution, the vials were counted in a liquid scintillation system.

**GALACTOSYLTRANSFERASE ASSAYS:** Dorsal, middle, and ventral retinal thirds were dissected from both eyes of 2–10 chick embryos, aged 4–10 days. The tissues were centrifuged free of the HBSS, in which they were dissected, and suspended in 0.5–1.0 ml of 62.5 mM cacodylate buffer, pH 6.8. The tissues were disrupted with three 5-s bursts from a Bronwill Biosonic Sonicator at half power. Aliquots were removed for protein determinations (23). Volumes of detergent solution equal to one-fourth the volumes of the sonicates were added. The detergent solution contained 10 mg/ml Triton CF-54 and 5 mg/ml Tween 80. Cell sonicates (50 μg/50 μl) were added to small incubation tubes to start the reaction. These tubes each contained 150 nmol AMP (30), 500 nmol Mn++, 10 nmol UDP-[αH]Gal (25 mCi/mmol), and exogenous acceptor as indicated. All of these latter components had previously been added to the tubes and evaporated to dryness in a rotary evaporator. After incubation at 37°C, the reactions were terminated by adding 10 μl of 250 mM EDTA, pH 6.8, and chilling on ice. Glycosylated product was measured by subjecting 50 μl of the stopped reaction mixture to high-voltage electrophoresis on 1% borate-impregnated paper. The origins were counted in toluene-based fluor in a liquid scintillation system. Breakdown of the labeled precursor to free galactose or galactose-1-PO₄ was determined by descending chromatography in 95% ethanol/1.0 M ammonium acetate (5:2) (6).

**RESULTS AND DISCUSSION**

**The Effect of General Metabolic Inhibitors**

To determine whether metabolic energy was necessary for retinotectal adhesion, collection experiments were performed in the presence of two inhibitors of metabolic activity, azide and dinitrophenol, and at 2°C. Preincubation of dorsal or ventral retinal cells took place at 37°C with no inhibitors present, except during the last half hour when either an inhibitor was added or the temperature lowered. Each of the experimental conditions decreased the numbers of retinal cells adhering (Table I). Lowered temperature, however, was the most effective inhibitor and the only experimental condition in which no specificity was exhibited.

The two chemical inhibitors decreased adhesion by about 60–75% at the concentrations used. Whether the residual adhesion is due to incomplete blockage of metabolism or is energy-independent is difficult to determine since extensive metabolic poisoning may lead to cell death. However, energy-dependent processes seem necessary for maximal adhesion rates.

When the temperature was lowered to 2°C, adhesion was nearly abolished. This inhibition may be due to more complete blockage of metabolism, but a second possibility is an alteration in the physical states of the cell membranes. Decreasing temperature causes a decrease in the lateral mobility of some membrane-associated molecules (11), and such mobility has been suggested to be necessary for intercellular adhesion (9, 42).

**The Effects of Proteases**

An early observation with this assay was that cells dissociated from ventral retina did not adhere preferentially to dorsal tectal halves immediately after their exposure to crude trypsin and, in fact, exhibited a slight preference for ventral tectal halves. Incubation in nutrient medium was required before ventral retina ac-

| Labeled retinal suspension | Tectal halves | No inhibitor, 37°C | Azide (10 mM) | Dinitrophenol (2 mM) | 2°C |
|----------------------------|--------------|-------------------|--------------|---------------------|-----|
| Dorsal§ | Dorsal | 2,060 ± 140 | 680 ± 120* | 550 ± 40* | 80 ± 30† |
| Ventral | 3,430 ± 460 | 1,380 ± 90* | 840 ± 70* | 100 ± 20‡ |
| Ventral† | Dorsal | 4,140 ± 630 | 950 ± 140* | 820 ± 70* | 110 ± 50‡ |
| Ventral | 1,930 ± 220 | 670 ± 70* | 620 ± 30* | 140 ± 70‡ |

* Significantly different from control, P < 0.1.
† Significantly different from control, P < 0.1, and significantly different from other treatments of the same suspension, P < .01.
§ 8 day, 1.1 x 10⁶ cells/ml, 0.22 cpm/cell, 4-h preincubation with inhibitors present only for final 30 min.
† 7½ day, 1.0 x 10⁶ cells/ml, 0.35 cpm/cell, 4-h preincubation with inhibitors present only for final 30 min.
quired its preference for dorsal half-tecta. Similar lag periods after trypsinization have commonly been found to precede the intercellular adhesion of embryonic cells (42). In contrast to this, cells from dorsal retina immediately preferred ventral tectal halves and maintained this preference even after lengthy preincubation. This asymmetry in the effects of trypsin between dorsal and ventral retina prompted the proposal that molecules necessary for the specific binding of dorsal retinal cells to ventral tecta were less susceptible to net removal or alteration by the proteolytic action of trypsin than were those that mediate the binding of ventral retina to dorsal tecta. Incubation in DECS was proposed as being necessary to replace the latter class of molecules (2, 35).

An alternative to the initial interpretation stems from the fact that the trypsin solution used for dissociation was a crude pancreatic preparation containing degradative enzymes other than trypsin. The binding molecules on ventral retina might not be susceptible to proteolysis, but rather to degradation by other classes of enzymes. The following experiments rule out this possibility.

With Azocoll, an artificial proteolytic substrate, it was determined that, on a weight basis, purified trypsin was 11 times more active a protease than the pancreas preparation. At a proteolytic activity identical to that of the usual dissociating medium, purified trypsin was used to dissociate retina. Suspensions containing some clumps of cells resulted and were used in collection assays. Again, immediately after dissociation, dorsal retinal cells adhered preferentially to ventral half-tecta, and ventral retina cells showed a slight preference for ventral half-tecta (24). Experiments in which retinal cells were preincubated supported the initial hypothesis but were difficult to quantitate precisely because of impractical amounts of retinal cell clumping (24).

This difficulty was overcome by the data reported in Table II. Labeled dorsal and ventral retina were dissociated with crude trypsin and preincubated for 3 h. Aliquots of the single cells were tested for adhesive specificity while the remainders were washed free of serum and treated with either crystalline trypsin or chymotrypsin. Aliquots of these cells were either assayed immediately for adhesive specificity to-

### Table II

| Labeled retinal suspension | Tectal halves | Retinal cells adhering/tectal half in 1 h | 3 h after trypsin treatment |
|----------------------------|--------------|------------------------------------------|----------------------------|
|                            |              | After 3-h preincubation                  | After preincubation and trypsin treatment | 3 h after trypsin treatment |
| Dorsal††                    | Dorsal       | 2,750 ± 280                             | 2,060 ± 450                   | 2,220 ± 240                 |
|                            | Ventral      | 4,440 ± 320                             | 4,070 ± 480                   | 3,920 ± 330                 |
| Ventral**                   | Dorsal       | 5,130 ± 670                             | 3,030 ± 420                   | 4,270 ± 320†                |
|                            | Ventral      | 2,780 ± 110                             | 2,620 ± 610                   | 2,520 ± 160                 |

* Significantly different from nontreated, \( P < 0.01 \).
† Significantly different from treated, \( P < 0.05 \).
¶ 0.009% (wt/vol), 37°C, 15 min.
|| 0.017% (wt/vol), 37°C, 15 min.
¶ 8 day, \( 1.2 \times 10^6 \) cells/ml, 0.38 cpm/cell.
** 7 day, \( 1.0 \times 10^6 \) cells/ml, 0.45 cpm/cell.
‡‡ 8 day, \( 1.0 \times 10^6 \) cells/ml, 0.13 cpm/cell.
‡‡ 8 day, \( 0.9 \times 10^6 \) cells/ml, 0.20 cpm/cell.
ward tectal halves or allowed to recover from the protease treatment for 3 additional h and then assayed for adhesive specificity toward freshly dissected tectal halves. Table II demonstrates that single cells from ventral retina, shown to be capable of adhering preferentially to dorsal tectal halves, lost this preference when treated with either pure protease. After 3 h of recovery from this second treatment, the preference was restored. Dorsal retina maintained specificity for ventral half-tecta throughout.

Table III contains results of experiments in which puromycin or cycloheximide, both shown to inhibit protein synthesis effectively in chick retinal cells (22), was added to the preincubation medium but removed before the collection assay. Each of these agents prevented the expression of ventral retina's adhesive preference for dorsal half-tecta but had little effect on the adhesive behavior of dorsal retina. Preincubation at 2°C, followed by a 1-h collection at 37°C, yielded similar results.

Taken together, these experiments support the initial hypothesis (2, 35) that there are proteins on the surface of ventral retina that are necessary for its preferential adhesion to dorsal tecta. These molecules seem to be susceptible to cleavage by exogenous proteases and require 3 h of incubation in nutrient medium and protein synthesis to be replaced.

While this result by itself is unremarkable, its significance is emphasized by the contrasting results with dorsal retina. The molecules on the surface of dorsal retina that participate in its preferential adhesion to ventral tecta do not seem to be susceptible to cleavage by exogenous proteases. The molecules may be nonprotein or may be proteins that are sterically or chemically insensitive to the proteases used. Alternatively, the molecules may be cleaved by the proteases but replaced on the cell surface extremely rapidly. In any case, the molecules seem to differ from those on the surface of ventral retina.

As a complement to these experiments, tecta were treated lightly with crystalline trypsin or chymotrypsin before collection experiments. The treatments were not so extensive as to disrupt the physical integrity of the tectal halves, although collection periods were necessarily restricted to 30 min. Retinal cells that had been allowed to recover from dissociation were incubated with the treated tecta. The numbers of retinal cells adhering to the tectal halves were generally decreased by ≈15% (Table IV). However, the adhesion of dorsal retinal cells to ventral half-tecta was consistently reduced threefold.

Again, the asymmetry of these results is suggestive. Proteins on the surface of ventral tecta may be necessary for optimal adhesion of dorsal retinal cells. In contrast, the relatively mild effects of proteases on the adhesion of ventral retina to dorsal tecta suggest that the specificity-conferring molecules on dorsal tectal surfaces are relatively insensitive to the proteases used, or replaced extremely rapidly.

Tectal halves that were treated with trypsin and then preincubated before exposure to retinal cells failed to recover their original adhesive properties (24). This apparent inability to regenerate molecules necessary for selective adhesion may reflect the possible acellular na-

### Table III

| Labeled retinal suspension | Tectal halves | No inhibitor | Cycloheximide (10 μg/ml) | Puromycin (5 μg/ml) | 2°C |
|---------------------------|--------------|--------------|--------------------------|-------------------|-----|
| Dorsal                    | Dorsal       | 3,240 ± 180  | 3,090 ± 220              | 2,850 ± 310       | 2,780 ± 160 |
|                           | Ventral      | 6,120 ± 1,010| 5,430 ± 390              | 5,220 ± 620       | 4,930 ± 320 |
| Ventral                   | Dorsal       | 7,440 ± 1,610| 3,720 ± 170*             | 3,130 ± 160*      | 3,120 ± 240* |
|                           | Ventral      | 4,240 ± 330  | 4,060 ± 190              | 3,580 ± 320       | 3,360 ± 440 |

All collections at 37°C in absence of all inhibitors.
* Significantly different from control, *P* < 0.01.
‡ 8 day, 1.0 × 10⁶ cells/ml, 0.18 cpm/cell.
§ 9 day, 0.9 × 10⁶ cells/ml, 0.24 cpm/cell.
### TABLE IV

**Retinotectal Adhesion after Protease Treatment of Tectal Halves**

| Labeled retinal suspension | Tectal halves | PBS-treated tecta | Trypsin-treated tecta | Chymotrypsin-treated tecta |
|----------------------------|--------------|-------------------|-----------------------|---------------------------|
| Dorsal                     | Dorsal       | 1,060 ± 190       | 890 ± 110             | 910 ± 40                  |
|                            | Ventral      | 1,850 ± 290       | 620 ± 70*             | 670 ± 110*                |
| Ventral*                   | Dorsal       | 1,790 ± 150       | 1,530 ± 270           | 1,410 ± 220               |
|                            | Ventral      | 920 ± 90          | 820 ± 100             | 740 ± 60                  |

* Significantly different from control, $P < 0.01$.
† 0.09% Crystalline trypsin, 15 min, 37°C.
‡ 0.17% Crystalline chymotrypsin, 15 min, 37°C.
§ 7 day, $1.0 \times 10^6$ cells/ml, 4-h preincubation, 0.36 cpm/cell.
¶ 7 day, $1.0 \times 10^6$ cells/ml, 4-h preincubation, 0.42 cpm/cell.

ture of the tectal elements to which the retinal cells bind (2).

The juxtaposition of experiments in which retinal cells were treated with proteases and experiments in which tecta were treated, accentuates the following complementarity. The adhesion of dorsal retina to ventral tectum seems to depend on proteins present on ventral tectal surfaces, while the molecules on dorsal retina that may participate in this binding seem protease-resistant. In contrast, the adhesion of ventral retina to dorsal tectum seems to depend on proteins located on ventral retina, while participating molecules on dorsal tectal surfaces seem protease-resistant.

The simplicity of these data is consistent with a model for retinotectal adhesive specificity presented previously (25). The model is an outgrowth of ideas put forward by Sperry (40) regarding retinotectal synaptic specificity. Two molecules, which possess lock-and-key complementarity toward each other, are proposed as determining retinotectal adhesive specificity along the dorsoventral axis. Specificity is achieved through a double-gradient distribution of the respective molecules, as depicted in Fig. 1. Ventral retina, rich in locks, would adhere preferentially to dorsal tectum, which is rich in keys. Similarly, dorsal retina, rich in keys, would prefer ventral tectum, rich in locks.

The results obtained with the collection assay after protease treatment are explicable by such double gradients along the dorsoventral axis. If treatment with protease resulted in a net removal of the locks of the model but not the keys, ventral retina and ventral tectum would be especially sensitive. On the other hand, dorsal retina and tectum would remain relatively unaffected. These hypothetical predictions are thus consistent with the observed experimental data. The slight preference of ventral retina for ventral tectum that is observed immediately after trypsinization of retina would also be predicted, for trypsinization would render ventral retina relatively rich in keys, and thus favor its adhesion to lock-rich ventral tectum.

**The Effects of Glycosidases and of Saccharide Haptens**

In an attempt to further characterize the
molecules involved in retinotectal adhesion, specific glycosidases were prepared from the extracellular secretions of two bacteria, *Diplococcus pneumoniae* (17, 18) and *Clostridium perfringens* (4, 27). Table V gives data on the degree of purity and cross-contamination of the enzymes used. Because these enzymes were not purified to strict homogeneity, two sources were used so as to lower the probability that any effects on adhesion were due to unknown contaminants.

Either retina or tecta were pretreated with a glycosidase before a collection experiment. The adhesion of the treated tissue was compared to that of tissue exposed for an equal time to PBS at the same pH, or to that of tissue treated with heat-inactivated preparations of the glycosidase.

Table VI shows the results of pretreating retinal cells or tectal surfaces with *Diplococcus* or *Clostridium* neuraminidase. There were no significant effects on the numbers of retinal cells collected in any of the experiments.

Both of the β-N-acetylhexasaminidases used in this study are capable of cleaving terminal β-N-acetylgalactosaminides and β-N-acetylgalactosaminides from oligosaccharides. When retinal cells or tectal surfaces were treated with such enzymes, a general decrease in the numbers of adherent retinal cells resulted (Table VII). As with the proteases, certain combinations were especially

### Table V

*Degradative activities of Diplococcal and Clostridial Glycosidases*

| Units of activity toward: | pNP-β-Gal* | pNP-α-Gal* | pNP-β-GlcNAC* | pNP-β-GalNAC* | pNP-β-Glc* | Sialyllectose* | Azocoll† |
|--------------------------|------------|------------|---------------|---------------|-----------|--------------|---------|
| D. P. neuraminidase, pH 6.5 | 0.1        | 0.0        | 0.0           | 0.0           | 0.0       | 25           | 0.0     |
| C. P. neuraminidase, pH 6.0 | 0.0        | 0.0        | 0.0           | 0.0           | 0.0       | 25           | 0.0     |
| D. P. β-galactosidase, pH 6.5 | 25         | 0.4        | 0.0           | 0.1           | 0.0       | 0.0          | 0.0     |
| C. P. β-galactosidase, pH 6.5 | 25         | 0.0        | 0.8           | 0.9           | 0.1       | 0.0          | 0.0     |
| D. P. β-N-Ac-Hexase, pH 6.0 | 0.1        | 0.0        | 25            | 17.9          | 0.2       | 0.0          | 0.0     |
| C. P. β-N-Ac-Hexase, pH 6.5 | 0.6        | 0.0        | 25            | 13.6          | 0.2       | 0.0          | 0.0     |

* All substrates 5 mM; 1 U = 1.0 μmol cleaved in 15 min at 37°C.
† Protease substrate: 5 mg/ml; 1 U = 0.01 ΔOD520 in 60 min at 37°C.
D. P., *Diplococcus pneumoniae*; C. P., *Clostridium perfringens*.

### Table VI

*Retinotectal Adhesion after Treatment with Bacterial Neuraminidase*

| Enzyme source | Labeled retinal suspension | Tectal half | Neither tissue treated | Retinal cells treated | Tectal halves treated |
|---------------|----------------------------|-------------|-----------------------|-----------------------|----------------------|
| *Diplococcus pneumoniae* | Dorsal† | Dorsal | 2,450 ± 130 | 2,560 ± 210 | 2,380 ± 70 |
| | | Ventral | 4,100 ± 280 | 4,020 ± 190 | 3,960 ± 340 |
| | Ventral∥ | Dorsal | 5,190 ± 650 | 4,740 ± 350 | 4,980 ± 240 |
| | | Ventral | 3,090 ± 410 | 3,220 ± 210 | 3,520 ± 260 |
| *Clostridium perfringens* | Dorsal‡ | Dorsal | 3,720 ± 150 | 3,890 ± 220 | 3,970 ± 170 |
| | | Ventral | 6,350 ± 620 | 6,580 ± 240 | 6,120 ± 390 |
| | Ventral** | Dorsal | 4,720 ± 350 | 4,890 ± 610 | 4,650 ± 210 |
| | | Ventral | 2,530 ± 90 | 2,470 ± 330 | 2,490 ± 190 |

* 25 day, U/ml in PBS, pH 6.5, 15 min, 37°C; controls treated with PBS.
† 25 day, U/ml in PBS, pH 6.0, 15 min, 37°C; controls treated with PBS.
∥ 7 day, 0.9 × 10⁶ cells/ml, 0.36 cpn/cell, 3-h preincubation.
‡ 7 day, 1.1 × 10⁶ cells/ml, 0.19 cpn/cell, 4-h preincubation.
§ 7 day, 1.0 × 10⁶ cells/ml, 0.24 cpn/cell, 4-h preincubation.
** 7 day, 1.1 × 10⁶ cells/ml, 0.27 cpn/cell, 4-h preincubation.

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TABLE VII

Retinotectal Adhesion after Treatment with Bacterial β-N-Acetylhexosaminidase

| Enzyme source                  | Labeled retinal suspension | Tectal half | Neither tissue treated | Retinal cells treated | Tectal halves treated |
|--------------------------------|-----------------------------|-------------|-----------------------|-----------------------|----------------------|
| *Diplococcus pneumoniae*$      | Dorsal                      | Dorsal      | 2,430 ± 210           | 1,860 ± 90*           | 2,070 ± 260          |
|                                |                             | Ventral     | 4,560 ± 620           | 2,990 ± 140$          | 4,240 ± 390          |
|                                | Ventral                     | Dorsal      | 6,170 ± 390           | 5,850 ± 610           | 2,820 ± 140$         |
|                                |                             | Ventral     | 3,990 ± 470           | 3,740 ± 210           | 2,810 ± 310*         |
| *Clostridium perfringens*      | Dorsal                      | Dorsal      | 2,860 ± 350           | 2,450 ± 210           | 2,230 ± 270          |
|                                |                             | Ventral     | 5,320 ± 390           | 2,730 ± 240$          | 4,820 ± 300          |
|                                | Ventral**                   | Dorsal      | 4,250 ± 610           | 3,970 ± 390           | 1,960 ± 210$         |
|                                |                             | Ventral     | 2,290 ± 100           | 2,060 ± 240           | 1,720 ± 260*         |

* Significantly different from controls, $P < 0.05$.
$ Significantly different from controls, $P < 0.01$.
§ 25 U/ml in PBS, pH 6.0, 15 min, 37°C; controls treated with PBS.
|| 25 U/ml in PBS, pH 6.5, 15 min, 37°C; controls treated with PBS.
* 8 day, 1.0 × 10⁶ cells/ml, 0.09 cpm/cell, 4-h preincubation.
** 7 day, 1.0 × 10⁶ cells/ml, 0.24 cpm/cell, 4-h preincubation.
†† 7 day, 0.9 × 10⁶ cells/ml, 0.23 cpm/cell, 4-h preincubation.
††† 7 day, 1.0 × 10⁶ cells/ml, 0.19 cpm/cell, 4-h preincubation.

Sensitive. When tecta were treated, the most precipitous decrease occurred in the binding of ventral retina to dorsal tecta. When retinas were treated, dorsal retina's adhesion to ventral tecta was most affected. Heat-inactivated preparations caused no deviation from controls.

These results are precisely complementary to the effects of proteases on retinotectal adhesion. The adhesion of dorsal retina to ventral tectum seems to depend on terminal, N-acetylated hexosamine residues located on retina and on proteins located on tectum. In contrast, the adhesion of ventral retina to dorsal tectum seems to depend on proteins located on retina and terminal, N-acetylated hexosamine residues located on tectum.

Further information was obtained when retinal cells or tectal halves were treated with β-galactosidase. A general increase in the numbers of adherent retinal cells resulted (Table VIII). The most pronounced increases occurred in the adhesion of ventral retina to ventral tectum when either retina or tectum was treated. Again, heat-inactivated preparations caused no deviation from PBS controls.

Further experiments were performed in which retina or tecta were treated with combinations of the glycosidases. In all of these experiments, the effects of β-N-acetylhexosaminidase were dominant. That is, the overall effect was a general decrease in the numbers of retinal cells collected. Again, the adhesion of treated, dorsal retina to ventral tecta and the adhesion of ventral retina to treated, dorsal tecta were most affected (24).

As an independent test for the involvement of terminal, acetylated hexosamine residues in the binding, and as a potential method for further description, certain mono- and disaccharides were included in collection assays. Table IX records the results of six separate experiments. Of the sugars tested, only GlcNAc and GalNAc decreased to any extent the numbers of retinal cells adhering to tecta, GalNAc being somewhat more effective than GlcNAc. Neither sugar affected the preferences exhibited by the retinal cells.

The results with glycosidases and saccharide haptens are consistent with the model for retinotectal adhesion described above, and suggest further refinement. N-acetylated hexosamines, perhaps GalNAc residues, seem to be involved in the adhesion process. The asymmetric results of the glycosidase experiments suggest that these residues are located primarily on dorsal retina and dorsal tectum. This is consistent with the distribution of keys discussed above, and suggests that they may be recognized through terminal GalNAc residues. A prediction of the model would thus be that this particular GalNAc-containing molecule be resistant to removal by exogenous proteases, or...
TABLE VIII
Retinotectal Adhesion after Treatment with Bacterial β-Galactosidase

| Enzyme source | Labeled retinal suspension | Tectal half | Neither tissue treated | Retinal cells treated | Tectal halves treated |
|---------------|------------------------|------------|----------------------|----------------------|----------------------|
| **Diplococcus pneumoniae**† | Dorsal | Dorsal | 2,850 ± 360 | 3,130 ± 140 | 3,070 ± 290 |
| | | Ventral | 4,640 ± 210 | 4,820 ± 260 | 4,780 ± 410 |
| | Ventral‡ | Dorsal | 5,190 ± 430 | 5,970 ± 360 | 5,860 ± 610 |
| | | Ventral | 2,860 ± 360 | 4,640 ± 130* | 5,130 ± 470* |
| **Clostridium perfringens**‡ | Dorsal | Dorsal | 3,620 ± 260 | 3,970 ± 380 | 3,920 ± 210 |
| | | Ventral | 6,170 ± 390 | 6,820 ± 850 | 6,350 ± 470 |
| | Ventral† | Dorsal | 4,890 ± 410 | 5,130 ± 290 | 5,470 ± 410 |
| | | Ventral | 2,720 ± 290 | 4,420 ± 350* | 4,430 ± 280* |

* Significantly different from controls, P < 0.01.
† 25 U/ml in PBS, pH 6.5, 15 min, 37°C; controls treated with PBS.
‡ 25 U/ml in PBS, pH 6.5, 15 min, 37°C; controls treated with PBS.
§ 8 day, 10 × 10⁶ cells/ml, 0.23 cpm/cell, 3-h preincubation.
¶ 8 day, 0.9 × 10⁶ cells/ml, 0.19 cpm/cell, 3-h preincubation.
** 7 day, 1.0 × 10⁸ cells/ml, 0.29 cpm/cell, 4-h preincubation.
*** 7 day, 1.1 × 10⁸ cells/ml, 0.21 cpm/cell, 4-h preincubation.

be replaced at a very rapid rate after such a treatment.

Furthermore, the alterations caused by treatment with β-galactosidase suggest that the above molecule might also exist with its N-acetylhexosamine as the penultimate residue to a β-linked galactoside. Treatment with a β-galactosidase might then expose the previously cryptic hexosamines and thereby increase the rates of adhesion. That this effect is more pronounced in ventral-ventral interactions may point to an uneven distribution of the galactoside-terminating molecule or may simply reflect the normal paucity of the hexosamine-terminated molecule in the ventral areas.

The Distribution of Gangliosides
Along the Dorsoventral Axis
of Neural Retina

The above studies with degradative enzymes and sugar haptens suggested three properties of the molecule proposed to be more concentrated dorsally than ventrally: (a) proteases did not produce a net removal or alteration of the molecule, (b) β-N-acetylhexosaminidase and sugar hapten data point toward a terminal N-acetylated hexosamine, possibly GalNAc, (c) β-galactosidase data suggest that the molecule can exist with a terminal galactoside. One glycoconjugate that possesses all of these properties and, furthermore, is found in neuronal membranes, is the monosialyl ganglioside, GM₂ (Svennerholm notation [44]). The structure of this molecule is:

\[
\text{sialic acid} \quad \text{GalNAc} \quad \text{glucose} \quad \text{ceramide.}
\]

This molecule terminates in GalNAc, would not be affected by protease treatment, and forms the monosialyl ganglioside GM₁ with the addition of a β-galactoside to the GalNAc. Because of this correspondence, the distribution of the gangliosides was determined as a function of position in the dorsoventral axis of the retina. This was accomplished in two ways, both of which measured the amount of sialic acid present in individual bands after the use of TLC to separate the gangliosides. One method chemically determined the amount of sialic acid present in each band using the resorcinol assay (45). The other measured rates of incorporation of [³H]ManNAc, a precursor relatively specific for sialic acid.

Chemical detection required dissection of about 100 retina into dorsal, middle, and ventral thirds in order to obtain accurately measurable levels of the gangliosides, especially GM₂ since it contains
### Table IX

**Effects of Saccharides on Retinotectal Adhesion**

| Labeled cell suspension | Added saccharide         | Conc (mM) | To dorsal tecta (no. of cells adhering in 1 h) | To ventral tecta (no. of cells adhering in 1 h) |
|-------------------------|--------------------------|-----------|-----------------------------------------------|-----------------------------------------------|
| Dorsal retina§          | None                     |           |                                               |                                               |
|                         | N-acetylglucosamine      | 5.0       | 2,140 ± 180                                   | 3,830 ± 610                                   |
|                         | N-acetylgalactosamine    | 5.0       | 1,630 ± 90*                                   | 3,040 ± 400                                   |
|                         | Galactose                | 5.0       | 1,240 ± 180†                                  | 2,120 ± 220*                                  |
| Dorsal retina‖          | None                     |           |                                               |                                               |
|                         | N-acetylactosamine       | 5.0       | 2,590 ± 310                                   | 4,200 ± 160                                   |
|                         | N-acetylmannosamine      | 5.0       | 2,780 ± 290                                   | 4,020 ± 310                                   |
|                         | N-acetylgalactosamine    | 5.0       | 1,240 ± 180                                   | 2,930 ± 200†                                  |
| Dorsal retina§          | None                     |           |                                               |                                               |
|                         | N-acetylglucosamine      | 10        | 1,150 ± 160*                                  | 3,010 ± 210                                   |
|                         | N-acetylglucosamine      | 50        | 990 ± 320†                                    | 2,120 ± 360*                                  |
|                         | N-acetylgalactosamine    | 10        | 920 ± 70†                                     | 1,530 ± 210†                                  |
|                         | N-acetylgalactosamine    | 50        | 670 ± 110†                                    | 1,320 ± 80†                                   |
| Dorsal retina‖          | None                     |           |                                               |                                               |
|                         | N-acetylmannosamine      | 100       | 2,750 ± 410                                   | 4,840 ± 250                                   |
|                         | N-acetylglucosamine      | 100       | 2,930 ± 240                                   | 4,860 ± 210                                   |
|                         | N-acetylgalactosamine    | 100       | 1,750 ± 180*                                  | 3,130 ± 310†                                  |
|                         | N-acetylgalactosamine    | 100       | 1,020 ± 210†                                  | 2,240 ± 360†                                  |
| Ventral retina‡         | None                     |           |                                               |                                               |
|                         | Galactose                | 10        | 4,650 ± 450                                   | 2,730 ± 70                                    |
|                         | N-acetylgalactosamine    | 10        | 4,920 ± 240                                   | 2,580 ± 210                                   |
|                         | N-acetylglucosamine      | 10        | 2,900 ± 310†                                  | 1,360 ± 130†                                  |
|                         | N-acetylglucosamine      | 10        | 3,400 ± 160*                                  | 2,020 ± 240†                                  |
| Ventral retina§§        | None                     |           |                                               |                                               |
|                         | N-acetylactosamine       | 20        | 6,850 ± 610                                   | 3,920 ± 320                                   |
|                         | N-acetylmannosamine      | 20        | 6,720 ± 450                                   | 3,860 ± 160                                   |
|                         | N-acetylgalactosamine    | 20        | 7,100 ± 1,020                                 | 4,060 ± 210                                   |
|                         | N-acetylgalactosamine    | 20        | 3,350 ± 390†                                  | 1,980 ± 280†                                  |

* Significantly different from controls, $P < 0.05$.
† Significantly different from controls, $P < 0.01$.
§ 8 day, 1.1 × 10⁶ cells/ml, 0.08 cpm/cell, no preincubation.
‖ 8 day, 1.0 × 10⁶ cells/ml, 0.13 cpm/cell, no preincubation.
¶ 7 day, 0.86 × 10⁶ cells/ml, 0.16 cpm/cell, 3-h preincubation.
** 8 day, 1.0 × 10⁶ cells/ml, 0.13 cpm/cell, 4-h preincubation.
†† 8 day, 1.0 × 10⁶ cells/ml, 0.09 cpm/cell, 3-h preincubation.
‡‡ 8 day, 0.90 × 10⁶ cells/ml, 0.20 cpm/cell, 3-h preincubation.

<1% ganglioside sialic acid. Protein, total ganglioside sialic acid, and molar percentages of the individual ganglioside species after TLC in either of two solvent systems are reported in Table X. The values for each of the retinal thirds agree well with the data for whole retina reported by Dreyfus et al. (7). There were no significant differences in the values of any of the gangliosides as a function of position. This includes GM₂, the species of primary interest. Almost all (92–98%) of the sialic acid applied to the plates was accounted for.

The other method, i.e., measuring rates of incorporation of [3H]ManNAc, yielded similar results. The incorporation into sialic acid seemed specific since no radioactivity co-chromatographed with neutral glycolipids or phospholipids, although iodine vapors indicated their presence near the top of the TLC plates. However, one radioactive, nondialyzable, nonganglioside component was detected. It chromatographed near GM₄ in chloroform/methanol/water (60:35:8), near GM₁ in chloroform/methanol/2.5 NH₄OH (60:35:8), and...
TABLE X

Molar Percentages of the Gangliosides of Neural Retina

| Ganglioside | Exp 1: TLC in CHCl₃/CH₃OH/H₂O (60:35:8) | Exp 2: TLC in CHCl₃/CH₃OH/2.5 N NH₄OH (60:35:8) |
|-------------|-----------------------------------------|-----------------------------------------------|
|             | Dorsal third | Middle third | Ventral third | Dorsal third | Middle third | Ventral third |
| GM₃         | 10           | 9.7          | 9.2           | 11.6         | 10.1         | 11.5          |
| GM₂         | 1.5          | 1.4          | 1.4           | 1.6          | 1.4          | 1.5           |
| GM₁         | 4.8          | 5.1          | 4.9           | 5.3          | 6.0          | 6.4           |
| GD₃         | 33           | 36           | 36            | 31           | 30           | 31            |
| GD₁₄        | 23           | 22           | 22            | 24           | 25           | 22            |
| GD₁₉        | 16           | 14           | 14            | 13           | 15           | 13            |
| GT₁         | 7.5          | 6.9          | 7.9           | 8.5          | 8.7          | 9.1           |
| GQ₁         | 2.1          | 2.0          | 2.1           | 2.8          | 2.1          | 2.1           |
| GQ'         | 2.2          | 2.4          | 2.2           | 2.4          | 2.1          | 2.3           |
| Homogenate  | 76           | 81           | 74            | 85           | 88           | 79            |
| Protein (mg)| 88           | 103          | 84            | 108          | 112          | 91            |
| Total ganglioside sialic acid (µg) | 88           | 103          | 84            | 108          | 112          | 91            |

slightly ahead of GM₃ in n-propanol/water (70:30). Its mobility was not affected by 0.2 N H₂SO₄ at 80°C for 1 h, conditions sufficient for hydrolysis of sialic acid from gangliosides (10), and it did not co-chromatograph with [³H]ManNAc.

A profile of the distribution of radioactivity as a function of mobility after chromatography in n-propanol/water (70:30) is shown in Fig. 2. The results correspond well with those of the chemical analysis. No significant differences in the relative levels of the individual gangliosides were detectable among the retinal thirds. Chromatography in the other two solvent systems defined above gave similar results. All of the radioactivity applied to the plate was recoverable.

Thus, although an oligosaccharide sequence similar to that of GM₂ was implicated by the glycosidase and hapten data, no asymmetry either in the overall concentration of GM₂ or in its rate of net synthesis was detectable. This may indicate that another glycoconjugate possessing an oligosaccharide analogous to the terminal saccharide sequence of GM₂ exists in a gradient and is responsible for the selective adhesion. If this were the case, this molecule would be predicted to be insensitive to removal by protease or to be replaced very rapidly after the treatment. A second possibility is that a functional gradient of GM₂ does exist at the cell surface but that the analytical methods employed were unable to detect it. This gradient could result from asymmetric compartmentalization and transport of GM₂ to the cell surface, even though overall concentration levels were uniform.

The Adhesion of Lecithin Vesicles to Tectal Halves

The double gradient model in the dorsoventral axis presented above might predict that retinal cells whose surfaces were denuded of all molecules, other than the proposed recognition molecule terminating in GalNAc, would still be capable of adhering to tecta and would adhere preferentially to ventral halves. Although this experiment is impossible, it might be approximated using phospholipid vesicles containing the ganglioside GM₂.

When phospholipids in aqueous suspension are sonicated, small lamellar vesicles result (16). Such vesicles have been shown to interact with animal cell plasma membranes (29). Furthermore, low levels of gangliosides are readily incorporated into lamellae of the phospholipid lecithin (15). Although the distribution of the ganglioside incorporated into the lamellae was not determined, it was assumed for these studies that at least some of the carbohydrate chains would be free to interact with tecta. Therefore, the role of terminal, β-linked GalNAc residues in the adhesion to tectal surfaces was tested by preparing vesicles that included 2% GM₂. The ganglioside-containing vesicles were included with dorsal and ventral tectal halves in a collection assay. As controls for specificity, vesicles containing other gangliosides or solely lecithin were also prepared.

Dipalmitoyl lecithin was the principal component of the lipid mixture, which also contained 200,000 cpm of [³H]dimyristoyl lecithin and 2% of a ganglioside as indicated.
Fig. 2 Net synthesis of gangliosides along the dorsoventral axis of 10-day neural retina. Retinal thirds were incubated for 12 h in [3H]ManNAc and then extracted for gangliosides. The gangliosides from each third were chromatographed in n-propanol/water (70:30), and the radioactivity in 3-mm bands was determined. The profiles for dorsal retinal thirds (−) and ventral retinal thirds (−) are shown. Middle thirds yielded a similar profile. Positions of standards and an unidentified component are also shown.

Fig. 3 shows a time-course for the adhesion of vesicles containing either 2% GM₁ or 2% GM₂. A control in which the lecithin was sonicated with no ganglioside present, and then to which 2% GM₂ was added, is also shown.

Vesicles formed from only lecithin or from lecithin and GM₁ adhered at equal rates and showed little preference in their adhesion to dorsal and ventral tectal halves. Vesicles formed from lecithin and GM₂, however, adhered about twice as rapidly and displayed a distinct preference for ventral tectal halves.

Table XI presents the results of three other experiments in which vesicles with the indicated additions were incubated with six dorsal and six ventral tectal halves for 1 h. Consistently, vesicles containing GM₂ adhered to a greater extent than did vesicles of other compositions. Furthermore, only the GM₂-containing vesicles displayed a preference for ventral tectal halves.

These experiments thus mimic the adhesive selectivity obtained with cell suspensions prepared from dorsal retina. They provide independent support for the suggestion that the preferential binding of dorsal retina to ventral tecta is mediated by β-GalNAc-terminated molecules on dorsal retina and by molecules complementary to GalNAc on tecta. They also support the suggestion that the latter recognition molecules are also present on dorsal tecta, but are more concentrated ventrally than dorsally.

Galactosyltransferase Activities

The experiments with β-galactosidase showed an increase in the numbers of retinal cells collected by the treated tecta. A simple interpretation of these data is that cleavage of terminal β-galactosides increases the number of molecules responsible for adhesion. This could occur if the recognition molecule that terminates in β-GalNAc could also exist with GalNAc as the penultimate residue to a terminal galactoside. This galactoside would be added to the proposed adhesion molecule through the action of a galactosyltransferase. This enzyme would catalyze the transfer of a galactose residue from sugar donor, UDP-Gal, to the proposed binding molecule.

Other transferases that have been examined have been shown to be specific for both the sugar they transfer and for the acceptor to which the transfer occurs (31). The latter recognition implies a molecular complementarity between transferase and acceptor and has prompted the hypothesis by Roseman (32) that such enzyme-substrate interac-
Adhesion of \([\text{H}]\)lecithin vesicles to dorsal and ventral tectal halves. The adhesion to dorsal tecta (\(\bullet\)) and to ventral tecta (\(\times\)) are shown for vesicles of three different compositions: (\(-\)), vesicles containing lecithin and 2% GM\(_2\); (\(-\)), vesicles containing lecithin and 2% GM\(_3\); (\(\cdots\)), vesicles formed by sonication of lecithin alone, 2% GM\(_3\) being added after the sonicating process.

Figure 3 Adhesion of \([\text{H}]\)lecithin vesicles to dorsal and ventral tectal halves. The adhesion to dorsal tecta (\(\bullet\)) and to ventral tecta (\(\times\)) are shown for vesicles of three different compositions: (\(-\)), vesicles containing lecithin and 2% GM\(_2\); (\(-\)), vesicles containing lecithin and 2% GM\(_3\); (\(\cdots\)), vesicles formed by sonication of lecithin alone, 2% GM\(_3\) being added after the sonicating process.

Adhesions could provide the lock-and-key mechanisms by which cells might selectively adhere to other cells. He suggests that transferases on the plasma membrane of one cell bind only to apposed cells whose surfaces possess substrates appropriate for the transferases, and that this recognition provides for specificity in intercellular adhesion. This theory has been supported by a growing literature that localizes these enzymes in the extracellular portions of various intact cells (39), including chick neural retina (37), and by myriad observations that link such enzyme-substrate interactions to recognition and morphogenetic phenomena (33).

For these reasons, galactosyltransferase activity was determined for sonicates from dorsal, ventral, and middle thirds of neural retina. Activities toward endogenous acceptors and toward seven exogenously added acceptors are presented in Table XII. In six of the eight comparisons, no differences among the retinal thirds were detected. However, both free GalNAc and GM\(_2\), which terminate with a \(\beta\)-linked GalNAc, gave small but significant asymmetries. In both cases, activities in ventral retinal sonicates were about 30% higher than those in dorsal retinal sonicates, while activities from middle-third sonicates were intermediate.

All of the activities listed were studied under one set of experimental conditions. These conditions have been determined to be optimal for transfer to GM\(_2\) and within the linear range of protein (unpublished data). The concentrations of exogenous acceptor are saturating in the cases of GM\(_2\), GlcNAc, GalNAc, ceramide-lactose, and ceramide-glucose.

To determine whether asymmetric degradation of the nucleotide sugar donor, UDP-[\({\text{H}}\)]Gal, to free [\(\text{H}\)]Gal or to [\(\text{H}\)]Gal-1-phosphate contributed to these results, aliquots of the reactions were chromatographed in 95% ethanol-1.0 M ammonium acetate (6). The extent of breakdown was found to be equal among the retinal thirds (24),
**Table XI**

The Adhesion of Lecithin Vesicles to Tectal Halves in 1 h

| Vesicle composition | [3H]Lecithin adhering to tectal half | To dorsal half | To ventral half |
|---------------------|-------------------------------------|---------------|---------------|
|                     | cpm                                 | dorsal half   | ventral half  |
| Lecithin            | 440 ± 30                            | 450 ± 20      | 450 ± 20      |
| Lecithin + 2% GM₂   | 680 ± 60*                           | 780 ± 30*     | 430 ± 20      |
| Lecithin + 2% GM₃   | 410 ± 30                            | 430 ± 20      | 450 ± 30      |
| Lecithin (GM₂ added after sonification) | 440 ± 30 | 450 ± 30 | 450 ± 30 |
| Lecithin            | 560 ± 30                            | 590 ± 40      | 590 ± 30      |
| Lecithin + 2% GM₂   | 630 ± 20                            | 680 ± 30*     | 600 ± 20*     |
| Lecithin + 2% ceramide-glucose | 520 ± 10 | 550 ± 20 | 680 ± 30* |
| Lecithin + 2% GD₁₃₈| 520 ± 40                            | 540 ± 30      | 540 ± 30      |
| Lecithin            | 420 ± 20                            | 430 ± 40      | 600 ± 30      |
| Lecithin + 2% GM₂   | 580 ± 20*                           | 670 ± 30*     | 670 ± 30*     |
| Lecithin + 2% ceramide-lactose | 400 ± 20 | 390 ± 30 | 870 ± 30* |
| Lecithin + 2% GM₁  | 400 ± 10                            | 420 ± 20      | 420 ± 20      |

* Significantly greater than the value with lecithin alone, P < 0.05.
‡ Significantly greater than the corresponding dorsal value, P < 0.05.
§ 200,000 cpm and 4 mg lecithin in 4 ml PBS per assay plate.

and small when assays were performed in the presence of AMP (30). Also, no evidence was found for asymmetric degradation of glycosylated product since β-galactosidase activity toward pNp-Gal was found to be equal among the retinal thirds (24).

As further controls, levels of activity among the retinal thirds were determined for five additional classes of glycosyltransferases using both endogenous and selected exogenous acceptors. Various degradative enzymes, and one enzyme of general metabolism, were also assayed. All of these activities were found to be identical across the dorsoventral axis (24). In addition, saturation levels of binding for five iodinated lectins were determined and found to be equal among the retinal thirds (24).

One of the molecules suggested to participate in retinotectal adhesion was proposed to be more concentrated ventrally than dorsally, to have a binding site for an acetylated hexosamine, probably β-linked GalNAc, and to be a protein. If GM₂:galactosyltransferase were present on the outside surfaces of the retinal cells, it would fulfill these criteria. However, in a linear model such as the one proposed, the expected ratio between the number of adhesion molecules in ventral retina and the number on dorsal retina would be predicted to be threefold. The difference detected here is in the right direction, but only 30%.

Several possible explanations exist. If only a subpopulation of retinal cells were capable of adhering to the tecta, pronounced asymmetries among these cells could be diminished in an assay of the entire population of retinal cells. Alternatively, more than one class of transferases might be capable of transferring galactose to GM₂ and to free GalNAc. One of these might be responsible

**Table XII**

Some Galactosyltransferase Activities of Sonicated, 7- to 8-Day Neural Retina as a Function of Position along the Dorsoventral Axis

| Added acceptor | Dorsal third | Middle third | Ventral third |
|----------------|-------------|-------------|--------------|
| tGM₂ (0.2 mM) | 2.31        | 2.76        | 3.02         |
| GalNAc (5 mM) | 1.96        | 1.87        | 1.89         |
| tGM₂ (0.2 mM) | 2.04        | 2.36        | 2.65         |
| GalNAc (5 mM) | 0.11        | 0.12        | 0.15         |
| GalNAc (5 mM) | 2.01        | 2.06        | 2.04         |
| tGM₂ (0.2 mM) | 2.04        | 2.41        | 2.41         |
| GalNAc (5 mM) | 0.07        | 0.09        | 0.12         |
| GalNAc (5 mM) | 2.15        | 2.12        | 2.07         |
| tGM₂ (0.2 mM) | 2.01        | 2.44        | 2.68         |
| GalNAc (5 mM) | 0.09        | 0.10        | 0.12         |
| GalNAc (5 mM) | 1.78        | 1.70        | 1.67         |
| Cer-glucose (0.2 mM) | 1.47 | 1.49 | 1.41 |
| Cer-lactose (0.2 mM) | 0.76 | 0.82 | 0.74 |
| AS-AG-fetuin (5 mg/ml) | 4.41 | 4.31 | 4.36 |
| (terminal β-GalNAc) | 6.31 | 6.08 | 6.21 |
| AS-OSM (6.8 mg/ml) | 6.31 | 6.08 | 6.21 |
| (terminal α-GalNAc) | 6.31 | 6.08 | 6.21 |

* In all assays in which exogenous acceptors were added, the activity toward endogenous acceptors was subtracted.
‡ Averaging the four experiments in which GM₂ was the added acceptor: dorsal < middle, P < 0.05; middle < ventral, P < 0.05; dorsal < ventral, P < 0.01.
for retinotectal adhesive specificity and exist in a gradient on the cell surface; the others might be distributed symmetrically. The resultant activity could differ between ventral retina and dorsal retina by only 30%. Another possibility is that the 30% difference in activity might be magnified by asymmetric compartmentalization or transport of the enzyme to the cell surface, and in this way determine specificity.

The asymmetry may play a less direct role in the adhesion process. One mechanism for regulating the concentration of the proposed GalNAc-containing recognition molecule might be to inactivate varying amounts of the molecule by the addition of terminal galactose residues. The 30% asymmetry in enzyme levels detected might be responsible for greater inactivation in ventral retina than in dorsal retina. Lastly, the enzyme asymmetry may be completely independent of recognition function. However, considering the variety of other data that implicate a terminal, β-linked GalNAc residue as a recognition site for retinotectal specificity, such a coincidence seems improbable. The asymmetry observed in activity levels more likely provides further support for a double gradient model similar to the one discussed, and may reflect the utilization of a galactosyltransferase as a binding molecule.

The possibility that GM2:galactosyltransferase might serve as a recognition molecule was further investigated by determining its activity as a function of embryonic age. Barbera (2), in studying the development of retinotectal adhesion, found no evidence for the preferential adhesion of ventral retinal cells to dorsal tectal halves before embryonic day 7. He suggested (2) that the recognition molecule proposed by Marchase et al. (25) to be concentrated in ventral retina might not be present in quantities sufficient for recognition before this time. Because of this, galactosyltransferase activities toward GM2 and GlcNAc were assayed as a function of embryonic age from 4 to 10 days (Fig. 4).

Sonicates from dorsal retina transferred galactose to GlcNAc at the same rate as sonicates from ventral retina at each age. The rates decreased markedly as the tissue matured. However, transfer to GM2 rose appreciably at day 7. Sonicates from dorsal and ventral retina younger than this contained equal levels of activity toward GM2, but an asymmetric increase resulted in a 30% enrichment in ventral retina activity compared to dorsal retina activity after this time. This asymmetric increase thus correlates with the developmental appearance of ventral retina's adhesive preference for dorsal tectum and provides further support for the involvement of GM2:galactosyltransferase in the recognition process.

The Relation of These Data to Previous Studies of Retina-Retina Adhesion

The symmetries between retina and tectum obtained in the studies with degradative enzymes suggested that only two molecules participate in retinotectal adhesion and that both are present on retina and on tectum. Therefore, previous studies, in which whole retina was dissociated and retina-retina adhesion was studied, might involve the same molecular mechanisms that have been studied here in a retinotectal context. Support for this contention and for the double gradient model was provided by Gottlieb et al. (12). Confluent monolayers composed of retinal cells from limited areas of retina were constructed and used as collecting surfaces in an adhesion assay. 32P-labeled cell suspensions from either dorsal or ventral retina were allowed to adhere to the monolayers. Gottlieb et al. (12) found not only that dorsal retina preferred ventral retina monolayers and vice versa, but also that, by constructing monolayers from sixths of retina, an actual gradient in preference along the dorsoventral axis could be detected.

![Figure 4](https://example.com/fig4.png)

**Figure 4** Galactosyltransferase activity in sonicates of dorsal (●) and ventral (×) halves of neural retina as a function of embryonic age. (−), transfer to 0.2 mM GM2; (−), transfer to 5 mM GlcNAc.
Publications from five laboratories have presented evidence relating to the biochemistry of retina-retina adhesion. Studies by Roth et al. (36, 37) measured the effects of degradative enzymes and simple sugars on the adhesion of single retinal cells to preformed retinal aggregates. They found that trypsinization of single cells to nontrypsinized aggregates caused only a short, irreproducible lag in the rate of adhesion that varied from 0 to 15 min. They also found that treatment of aggregates with β-galactosidase caused a substantial increase in the numbers of retinal cells adhering to aggregates. When simple sugars were included in the assay, both GalNAc and GlcNAc inhibited the collection of retinal cells, while other sugars were ineffective. Each of these results is consistent with results obtained in this study and would be predicted by the double gradient model.

The other four groups have isolated factors from retina or retina culture supernates that either inhibit or stimulate the aggregation of retinal cells (1, 14, 28, 38). One of these factors, isolated by Balsamo and Lilien (1), has been characterized as a glycoprotein that requires terminal N-acetylgalactosamine residues for aggregation-promoting activity and for binding to retinal cells. Treatment with β-N-acetylgalactosaminidase from Aspergillus niger liberated GalNAc from the preparation and severely decreased its ability to bind to retina and to promote aggregation. When simple sugars were included in assays that measured the binding of this factor to cells, GalNAc inhibited 33% of the binding and GlcNAc 25%. These studies, therefore, also implicate terminal GalNAc residues as being important for retinal cell adhesion. A correspondence between this factor and the molecule postulated here to be concentrated dorsally in retina and tectum is an untested possibility.

The Relation of These Data to Neuronal Specificity

The stimuli for investigating the adhesion preferences of retinal cells for tectal surfaces were Sperry’s ideas (40, 41) concerning the mechanisms determining neuronal interconnections, specifically, the topographically ordered projection of the retina onto the optic tectum. In forming this projection, axons from the most nasal area of retina innervate solely the posterior extreme of tectum, while axons from the most temporal area of retina innervate solely the anterior extreme of tectum. Between these limits, a continuous spatial representation of retina maps across the tectal surface. In a similar fashion, dorsal retina connects exclusively with ventral tectum and ventral retina with dorsal tectum (13).

Sperry’s hypothesis of neuronal specificity suggested that retinal ganglion cells exhibit this selectivity because of unique batteries of cell surface molecules that serve as identification tags for the individual neurons. Interactions between these molecules and similar recognition molecules on the tectum were proposed to result in the stereotopic pattern of synapses, “each axon linking only with certain neurons to which it becomes selectively attached by specific chemical affinities (41).”

The retinotectal adhesion assay was designed as an attempt to detect in vitro the “specific chemical affinities” that Sperry proposed as determinants of retinotectal synaptic specificity. In fact, the preferences demonstrated by dissociated retinal cells for tectal surfaces do mimic the pattern of synaptic connections along the dorsoventral axis. However, important differences between the adhesion experiments and the in vivo situation require a cautious interpretation of this correspondence.

First, the in vivo retinotectal map is created by interactions between the axonal tips of the retinal ganglion cells and the tectal surface, while the adhesion assay detects selectivity using retinal cell bodies. If both these specificities are the result of a common mechanism, then cell bodies in the retina would necessarily possess cell surface molecules similar to the recognition molecules present on axonal tips.

Second, the experiments reported here have been carried out with tecta that had previously been innervated by retinal fibers in vivo, although similar results were obtained with tecta that had never been innervated by retinal axons. Thus, the natural complement of innervating retinal fibers does not cause the recognition sites for cells in the adhesion assay to become occupied and unavailable. This does not imply, however, that the mechanisms are independent, since Hunt and Jacobson (20) have shown in Xenopus that a tectum can accommodate retinotopic innervation from more than one eye at a time.

Third, and most important, cell suspensions for the adhesion assay are prepared from whole neural retina, and thus contain cell types other than just ganglion cells. In a typical adhesion experiment, a total of less than one half of 1% of the retinal cells adhere to the tectal halves. The selective adhesion exhibited by this small percentage of
retinal cells could reflect the preferences of all the cells present in the retina, or could be due solely to the ganglion cells or to another subpopulation having no relevance to synaptic specificity. Attempts to test for selective adhesion among larger numbers of retinal cells using the assay described here included increasing the numbers of tectal halves from 12 to 36 and lengthening the times of collection. With these changes, the rates at which single cells adhered and the topographic specificity that they exhibited remained constant, but the total numbers of cells collected could be increased to only ~5%. However, using large numbers of aggregates prepared from halves of optic tecta as collecting surfaces, McClay et al. (26) have corroborated the preferential adhesion of dorsal and ventral retinal cells for tectal tissue. In their experiments, >50% of the input retinal cells adhered to aggregates made from the proper halves of tecta. Also, in the measurements of retinal-retinal adhesion made by Gottlieb et al. (12) discussed above, dorsal-ventral preferences were exhibited by as many as 70% of the cells.

These results thus support the assertion (2, 3, 25) that retinal cells other than ganglion cells participate in the adhesion experiments and display specificity toward tectal surfaces. If the mechanisms utilized for in vivo synaptic specificity are the same as those responsible for adhesion, then the retinotectal projection may result from a more global system of establishing polarity and positional information within the developing embryo. In previous work Hunt and Jacobson (19), using transplantation techniques in Xenopus, determined that cues responsible for positional information within the retina are also present in the flank. Chung and Cooke (5) also found evidence for such generalized cues and raised the possibility that influences initially involved in morphological determination are subsequently reutilized at a finer level for the establishment of specific patterns of synapse formation. A potential advantage to having many cells of the retina possess the recognition molecules has been discussed by Roth (34).

These results are also important for the interpretation of experiments in which biochemical measurements are done on retinal homogenates. If only a small percentage of the retinal cells were to possess the molecules responsible for recognition, then potential biochemical asymmetries among this subpopulation might be masked in assays of whole retina. Since this does not seem to be the case, biochemical measurements on whole retina should provide more easily assessable data.

Thus, although the selectivity detected in the adhesion assay and the selectivity that establishes the retinotectal projection in vivo may be independent, it is also plausible that they reflect the same molecular mechanisms. The accessibility of the adhesion assay to biochemical manipulation may provide a strategy for determining whether this correspondence does exist: to characterize the molecules responsible for selectivity in the adhesion assay and then to attempt to verify a role for the same molecules in synaptic specificity. If this correspondence were established, it would not only support Sperry's hypothesis, but would also recommend the study of in vitro adhesion as a useful tool for the understanding of neuronal specificity in particular and morphogenesis in general.

CONCLUSIONS

The data presented support the following arguments: (a) Inhibitors of general metabolism decrease the rates of retinal cell adhesion to tecta. Low temperature is especially effective. (b) The adhesion of ventral retina to dorsal tecta seems to depend on proteins located on ventral retina. Terminal β-N-acetylglactosamine residues on dorsal tecta also seem to be necessary. (c) The adhesion of dorsal retina to ventral tecta seems to depend on proteins located on ventral tecta. Terminal β-N-acetylglactosamine residues on dorsal retina also seem to be necessary. (d) One of the proposed dorsoventral recognition molecules may exist in an inactive form, masked by a terminal β-galactoside. (e) Vesicles containing the ganglioside GM2, which terminates in β-GalNAc, adhere preferentially to ventral tectal surfaces in a manner predicted by the previous data. (f) A double gradient model for retinotectal adhesive specificity is consistent with the data presented here. The model utilizes only two molecules that are proposed to possess lock-and-key complementarity. The molecule suggested to be concentrated dorsally in both retina and tectum seems to require terminal β-N-acetylgalactosamine residues for adhesion. Its activity is not affected by protease. A molecule fitting these qualifications, the ganglioside GM1, could not be detected in a gradient. The second molecule, concentrated ventrally in both retina and tectum, is a protein and seems capable of binding terminal β-N-acetylgalactosamine residues. One enzyme, UDP-galactose:GM1 galactosyltransferase, has been found to be more concen-
trated in homogenates of ventral retina than of dorsal retina, but only by 30%. Its activity in retina has been found to increase at the developmental age at which ventral retina first exhibits preferential adhesion for dorsal tectum.

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