

EVIDENCE FOR CELL-SURFACE GLYCOSYLTRANSFERASES

Their Potential Role in Cellular Recognition

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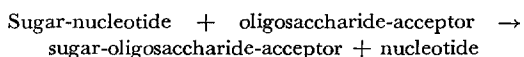
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ABSTRACT

Intact chicken embryo neural retina cells have been shown to catalyze the transfer of galactose-¹⁴C from uridine diphosphate galactose (UDP-galactose) to endogenous acceptors of high molecular weight as well as to exogenous acceptors. Four lines of evidence indicate that the galactosyltransferases catalyzing these reactions are at least partly located on the outside surface of the plasma membrane: (a) there is no evidence for appreciable uptake of sugar-nucleotides by vertebrate cells nor did unlabeled galactose, galactose 1-phosphate, or UDP-glucose interfere with the radioactivity incorporated during the reaction; (b) the cells remained essentially intact during the course of the reaction; (c) there was insufficient galactosyltransferase activity in the cell supernatants to account for the incorporation of galactose-¹⁴C into cell pellets; and (d) the intact cells could transfer galactose to acceptors of 10⁶ daltons, and the product of this reaction was in the extracellular fluid. Appropriate galactosyl acceptors interfered with the adhesive specificity of neural retina cells; other compounds, which were not acceptors, had no effect. These results suggested that the transferase-acceptor complex may play a role in cellular recognition.

Early theories on the mechanism of intercellular adhesion (11, 12) proposed that apposing cell surfaces contained complementary molecules which acted as a lock and key, or, more precisely, like an antigen-antibody reaction. As a result of studies on mouse teratoma (4) and chicken embryo neural retina cells (8), we have recently extended this idea (7), suggesting that the complementary molecules are enzymes and substrates on the apposing cell surfaces, specifically, the complex carbohydrates and glycosyltransferases.

The glycosyltransferases comprise several families of enzymes (6, 7), each of which catalyzes the following reaction:



Thus, chain elongation of the oligosaccharide units in the complex carbohydrates is effected by the addition of monosaccharide units through the action of different glycosyltransferases in a specific sequence. While all glycosyltransferases within one family, such as the sialyltransferases or the galactosyltransferases, utilize the same sugar-nucleotide as the glucose donor (e.g., cytidine monophosphate (CMP)¹-sialic acid or uridine diphosphate (UDP)-galactose), each of the enzymes is specific for the acceptor molecule or its analogues. Thus, different galactosyltransferases are required to add galactose to the oligosaccharide

¹ Abbreviations used in this paper: CMP, cytidine monophosphate; TCA, trichloroacetic acid; UDP, uridine diphosphate.

units in serum-type glycoproteins, in mucins, and in glycolipids. Where galactose occurs twice in one molecule, as in disialoganglioside, a different galactosyltransferase is required for each addition.

Previous experiments showed that removal of a terminal β -galactoside residue altered the adhesive recognition of chick embryo neural retina cells (8). Preliminary attempts were made to re-establish specificity in β -galactosidase-treated cells by transferring galactose- ^{14}C from UDP-galactose- ^{14}C to these intact cells with a crude homogenate as a source of galactosyltransferase. However, the homogenate-free control showed as much final incorporation of radioactivity into trichloroacetic acid-precipitable material as did the incubation with the added enzyme. Since it is unlikely that sugar-nucleotides enter animal cells, and because of little observed cell damage, the possibility of a surface transferase was suggested. Therefore a detailed examination of embryonic chicken neural retina cells for surface galactosyltransferases was undertaken.

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated commercial sources: uridine diphosphate-galactose- ^{14}C (298 mCi/mmole), New England Nuclear Corp., Boston, Mass.; uridine diphosphate-galactose, Calbiochem, Los Angeles, Calif.; α -galactopyranosyl-1-phosphate, *N*-acetylglucosamine, *N*-acetylgalactosamine, uridine diphosphate glucose, Sigma Chemical Co., St. Louis, Mo.; galactose, lactose, Pfanstiehl; phenyl α - and β -*N*-acetylgalactosaminides were the kind gifts of Dr. Bernard Weissman.

Media

All media were buffered as previously described (8).

NUTRIENT MEDIUM was prepared as previously described (8).

DISSOCIATING MEDIUM contained the following components: Ca^{++} , Mg^{++} , and glucose-free Hanks' basic salts solution plus Difco trypsin (Difco Laboratories, Inc., Detroit, Mich.) (1:250) 0.1%, w/v.

MEDIUM H consisted of Ca^{++} , Mg^{++} , and glucose-free Hanks' basic salts solution with 8 mM MnCl_2 and 10 mM NaN_3 .

Cell Preparation

Neural retinas were dissected from the eyes of 8-day old White Leghorn chick embryos and disso-

ciated as previously described (8). After dissociation the cells were washed twice in nutrient medium by centrifugation at 400 *g* for 5 min and resuspension. The cells were incubated at 37°C in a 25 ml DeLong flask on a gyratory shaker at 120 rpm. During this preincubation, the volume was adjusted so that the density of the suspension did not exceed 10^7 cells/ml. After preincubation, the cells were washed twice with the incubation medium to be used for the particular experiment (omitting the substrates).

Enzyme Assays

As described below, the products formed by incubation of neural retina cells with UDP-galactose appear to be predominantly glycoproteins and/or mucins and not glycolipids. The assays for the enzymes were therefore designed to determine UDP-galactose:glycoprotein and UDP-galactose:mucin galactosyltransferase activities with and without exogenous acceptors. The properties of galactosyltransferases from goat colostrum, pig and sheep submaxillary glands, and rat liver have been described (3, 6, 9, 10).

Unless stated otherwise, the incubation mixtures for detecting galactosyltransferase activity contained 100 μl of a suspension of intact neural retina cells in Medium H with 25 μM UDP-galactose- ^{14}C . The time of incubation and cell concentration are given for individual experiments. Incubation was conducted at 37°C in a shaking water bath. The reactions were stopped by rapid chilling to 0°C and the reaction mixtures were examined directly or were centrifuged in the cold at 200 *g* for 5 min so that the cells and supernatants could be examined separately. Samples (50 μl) of the total incubation mixtures or of the supernatant or cell pellet (after the addition of 50 μl of water) were transferred to Whatman No. 3MM paper saturated with 1% sodium tetraborate and subjected to electrophoresis at 62 v/cm for 25 min. Under these conditions, excess substrate UDP-galactose- ^{14}C , and its possible breakdown products (labeled galactose or glucose or their 1-phosphate esters), rapidly migrated from the origin while the labeled oligosaccharide products, endogenous and exogenous, remained at or very near the origin. Quantitative determination of the products was achieved by cutting the appropriate areas (2 inches in front of the origin and 1 inch behind) from the dried electrophoretograms and counting the strips in a toluene liquid scintillation system in a Packard liquid scintillation spectrometer. Alternatively, for incubations with only endogenous acceptor, reactions were stopped with 2 ml of cold 5% trichloroacetic acid (TCA) and the precipitate was filtered under suction through wetted Reeve Angel 934-AH glass fiber filters. The filters were washed with an additional 10 ml of cold 5% TCA and then with

10 ml of absolute ethanol, also at 0°C. The filters were then dried and counted as above. When testing for transferase activities with endogenous acceptors, TCA and electrophoretic assays gave the same results.

Identification of Galactose-¹⁴C

To identify the ¹⁴C-components in these products, acid and enzymatic (β -galactosidase) hydrolyses were used. For both hydrolyses, the cells from a 2 hr incubation were sonicated and the sonicate was dialyzed exhaustively at 4°C first against phosphate-buffered saline (0.14 M NaCl; 0.003 M KCl; 0.003 M Na₂HPO₄-KH₂PO₄ buffer at pH 7.2) and then against water. The retentate was divided and half was treated with 3.6 N H₂SO₄ for 4 hr in a boiling water bath. The other half was incubated for 4 hr at 37°C with 1800 units (9) of a partially purified β -galactosidase preparation from *Clostridium perfringens* in 0.5 ml of citrate-phosphate buffer, pH 6.5. The contents of each of the hydrolysis tubes were passed through an excess of mixed-bed ion exchange resin (equal volumes of a coarse mesh, Dowex 1 (HCO₃⁻) and Dowex 50 (H⁺), both 8% cross-linked). The resins were washed with water, and the combined filtrates of each hydrolysate were concentrated to syrups on a rotary evaporator. The syrups were then dissolved in 100 μ l of water and subjected to paper chromatography and electrophoresis in the presence of glucose and galactose standards.

The descending paper chromatographic system was butanol-pyridine-water (6:4:3), on Schleicher and Schüll Blue Ribbon chromatographic paper. Paper electrophoresis was in 1% sodium tetraborate as described above. Each of these systems can distinguish between glucose, galactose, D-fucose, and the pentoses.

RESULTS

Kinetics of Galactose Incorporation

Fig. 1 shows the incorporation of galactose-¹⁴C by intact neural retina cells as a function of time. When cells were allowed to "recover" from dissociation by previous incubation in nutrient medium they incorporated more galactose-¹⁴C than freshly dissociated cells, although the initial rates were similar. All further experiments were carried out with preincubated cells.

Fig. 2 shows that formation of ¹⁴C-labeled product from endogenous acceptor increases with increasing cell suspension density up to about 6×10^8 cells/ml.

The effect of increasing the UDP-galactose concentration on the rate of incorporation is shown in Fig. 3. The rate increased with the concentration of sugar-nucleotide, although saturation was not achieved. In order to conserve labeled sugar-nucleotide and maintain a high specific activity, reactions were conducted at subsaturating conditions with respect to UDP-galactose. At this concentration (25 μ M), under the conditions used, the rate of incorporation was proportional to cell number, and constant with time of incubation to about 2 hr. The effect of increasing MnCl₂ concentration is shown in Fig. 4. Apparent saturation in the absence of Ca⁺⁺ and Mg⁺⁺ was achieved at about 15 mM Mn⁺⁺. In the presence of these two divalent cations, approximately 25 mM Mn⁺⁺ was required for saturation. The experiments reported below were

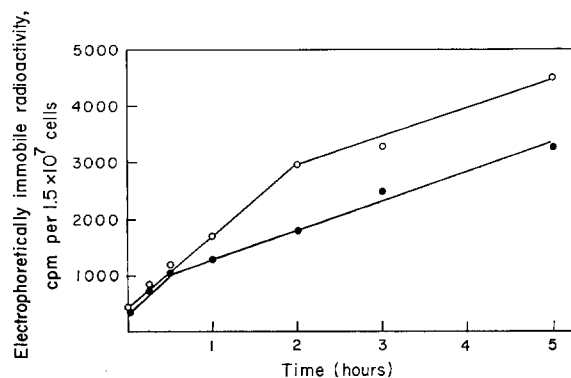


FIGURE 1 Effect of time of incubation on incorporation of galactose-¹⁴C into endogenous acceptors. Incubation mixtures were prepared as described in the text, and contained 1.5×10^8 cells/ml, and 25 μ M UDP-galactose-¹⁴C (specific activity, 10^8 cpm per μ mole). After incubating for the indicated times at 37°C, samples were assayed by the electrophoretic method as described in the text: O, cells preincubated for 30 min at 37°C in nutrient medium before assay; ●, cells assayed directly after dissociation.

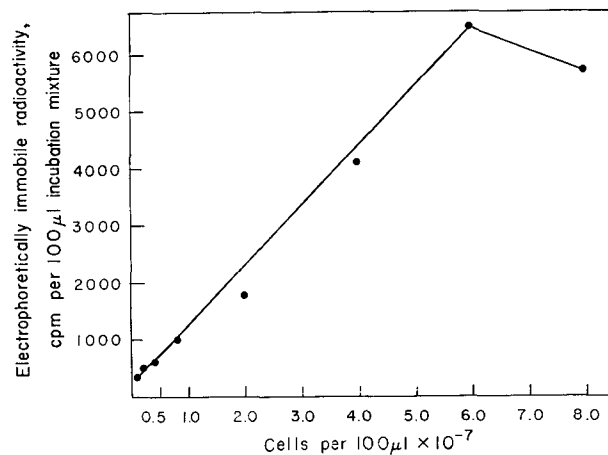


FIGURE 2 Effect of cell concentration on incorporation of galactose-¹⁴C into endogenous acceptors. Mixtures were prepared as described in the text and in Fig. 1, except for the cell densities which were varied as indicated. After incubating for 105 min at 37°C, 50-μl samples of the suspensions were assayed by the electrophoretic method.

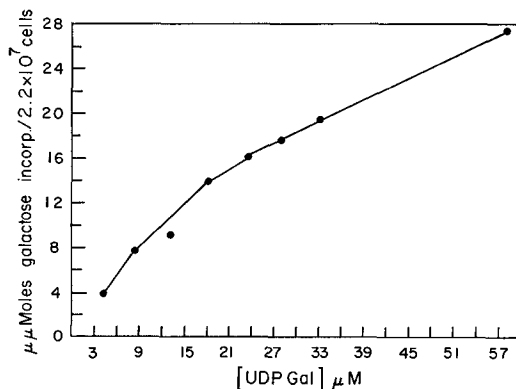


FIGURE 3 The effect of the concentration of UDP-galactose-¹⁴C on galactose-¹⁴C incorporation into endogenous acceptors. Incubation mixtures were prepared as described in the text with the indicated concentrations of labeled UDP-galactose (specific activity, 10⁸ cpm per μmole), and 3 × 10⁸ cells/ml. After 90 min at 37°C, 75-μl samples were subjected to the electrophoretic assay.

all conducted in the presence of 8 mM MnCl₂ and in the absence of Ca⁺⁺ and Mg⁺⁺.

Effect of Incubation Conditions on Cell Viability

A preincubated cell suspension of 3 × 10⁷ neural retina cells/ml was incubated in Medium H but without added UDP-galactose. At various

times up to 4 hr, samples were removed, diluted 100-fold in the same incubation medium, and examined two ways: (a) with a 0.1% solution of trypan blue to determine the percentage of cells which fail to exclude this dye after 15 min at room temperature, and (b) with a Coulter electronic particle counter which counts cells on the basis of their ability to exclude electrolytes from the surrounding medium (5). Before being exposed to the incubation medium, less than 5% of the cells examined failed to exclude trypan blue. After 4 hr under the incubation conditions described, however, between 10 and 20% of the cells took up the dye. No decrease in actual cell number could be detected with this visual method. In the Coulter counter study, duplicate cell samples were taken for each time point and triplicate counts were made for each sample. Under these conditions, no decrease in cell number could be detected during the 4 hr incubation period.

Effect of Potential Competitors of UDP-Galactose

Although transport of nucleotide sugars has not been reported in vertebrate cells, it is possible that UDP-galactose does in fact enter the neural retina cells under these conditions and is utilized internally. If this is the case, then rapid equilibration of UDP-galactose with internal UDP-glucose,

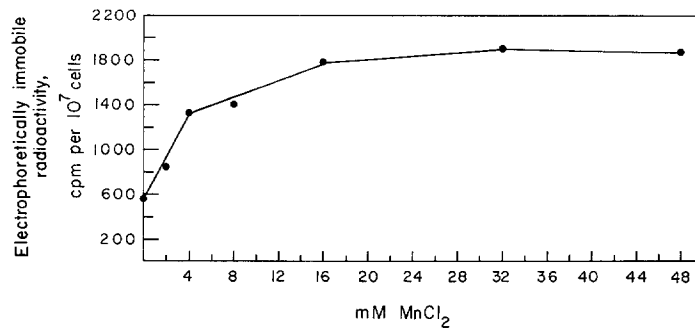


FIGURE 4 The effect of MnCl_2 concentration on the incorporation of galactose- ^{14}C into endogenous acceptors. Incubation mixtures were prepared as described in the text with the indicated concentrations of MnCl_2 , using 10^8 cells/ml, and $25 \mu\text{M}$ UDP-galactose- ^{14}C (specific activity, 6×10^7 cpm per μmole). After 90 min at 37°C , $100\text{-}\mu\text{l}$ samples were subjected to the electrophoretic assay.

catalyzed by UDP-glucose 4'-epimerase, would be expected. Table I shows that a fivefold molar excess of unlabeled UDP-glucose did not decrease the amount of radioactivity incorporated. Another possibility was that labeled UDP-galactose was degraded externally, and one or more of the labeled products was taken up by the cells and then used internally as the galactosyl donor. To test this possibility, unlabeled galactose and galactose-1-phosphate were added to the incubation mixtures. Table I shows that excess amounts of these potential hydrolytic products of UDP-galactose had no effect on incorporated radioactivity, with the possible exception of galactose-1-phosphate.

Identification of the Labeled Endogenous Product

When cells were incubated in Medium H with labeled UDP-galactose and the incubation mixture was divided three ways, equivalent amounts of radioactivity were TCA precipitable, electrophoretically immobile, and nondialyzable under the conditions described under Materials and Methods. The amount of radioactivity detected by these methods was unaffected by sonication of the incubation mixture after the incubation was completed. If a sample of a sonicated and dialyzed incubation mixture was centrifuged at $100,000 g$ for 60 min, 80% of the radioactivity was recovered in the pellet fraction.

For identification of the labeled product, a 1 ml incubation mixture with 3×10^8 whole cells and $25 \mu\text{M}$ UDP-galactose at 5×10^8 cpm per μmole was incubated for 2 hr. After this time, the mix-

ture was sonicated and then dialyzed, first against three 1 liter volumes of phosphate-buffered saline, pH 7.4, and then against three 1 liter volumes of distilled water, all at 4°C . The retentate (64,000 cpm) was divided in half and one-half was acid-hydrolyzed while the other was treated with β -galactosidase, as described under Materials and Methods. The hydrolysates were desalted by passage through mixed-bed resins and each filtrate contained at least 85% of the counts initially applied. The acid-hydrolyzed sample was concentrated and contained one detectable radioactive spot (100% of applied radioactivity) which migrated with authentic galactose in the electrophoretic and paper-chromatographic systems used. After similar treatment, the enzymatically hydrolyzed sample showed that only 44% of the radioactivity was released and that it migrated with authentic galactose. The remainder of the label was immobile in both systems.

In order to determine whether or not the endogenous products were predominantly glycolipid in nature, a sonicated and dialyzed reaction mixture was tested for its solubility in 7 volumes of chloroform-methanol, 2:1, and subsequently washed according to the method of Folch (2). Using this procedure, 6% of the nondialyzable radioactivity was soluble in chloroform-methanol, suggesting that only a small amount of labeled, neutral glycolipid was present.

Utilization of Exogenous Acceptors

For these experiments, potential galactosyl acceptors were added to the reaction mixtures with the whole cells. After the incubations, the reaction

TABLE I

Effect of Potential Inhibitors on Whole Cell Transferase Activity toward Endogenous Acceptors

For Experiment I, incubation mixtures containing 10^7 cells/ml, $21 \mu\text{M}$ UDP-galactose- ^{14}C (specific activity, 5×10^7 cpm per μmole), and the compounds indicated were prepared as described in the text. After 2 hr at 37°C , $50\text{-}\mu\text{l}$ samples were subjected to the paper electrophoretic assay. The mixture without UDP-glucose or galactose-1-P exhibited 541 cpm of galactose- ^{14}C incorporated into the endogenous acceptors.

In Experiment II, incubation mixtures were prepared as described in the text, contained 3×10^8 cells/ml, and $25 \mu\text{M}$ UDP-galactose- ^{14}C (specific activity, 5×10^7 cpm per μmole). After 90 min at 37°C , $50\text{-}\mu\text{l}$ samples were subjected to paper electrophoresis; the mixture without added galactose incorporated 2061 cpm of galactose- ^{14}C into the endogenous acceptors.

Potential competitor	Concentration	% of control activity
	μM	
Experiment I		
None		100
UDP-glucose	25	91
UDP-glucose	50	96
UDP-glucose	125	99
Galactose-1-P	25	110
Galactose-1-P	50	87
Galactose-1-P	125	81
Experiment II		
None		100
Galactose	25	104
Galactose	50	113
Galactose	75	112
Galactose	100	91

mixtures were centrifuged at $200 g$ for 5 min and $50 \mu\text{l}$ of the supernatants were subjected to paper electrophoresis. Controls contained no added acceptor. Table II summarizes the results of two experiments in which large and small molecular weight compounds were tested. The active, low molecular weight acceptors with whole neural retina cells were *N*-acetylglucosamine, phenyl β -*N*-acetyl-D-glucosaminide, and phenyl α -*N*-acetyl-D-galactosaminide. Sialidase- and β -galactosidase-treated orosomucoid (α_1 -acid serum glycoprotein) and sialidase-treated ovine submaxillary mucin were also active acceptors.

Supernatant Galactosyltransferase Activity

The activity observed might conceivably have resulted from the secretion of galactosyltransferases into the medium rather than from the activity of cell surface enzymes.

TABLE II

Relative Efficiency of Whole Cell Transferase Activity toward Various Acceptors

For Experiment I, the incubation tubes were prepared as described in the text and contained 0.1 ml of a whole cell suspension (2×10^8 cells/ml), $25 \mu\text{M}$ UDP-galactose (10^8 cpm per μmole), and the compounds indicated. After incubation for 2 hr at 37°C , each tube was centrifuged at $200 g$ for 5 min, and $50 \mu\text{l}$ of the supernatant solution were removed and subjected to paper electrophoresis as described. In Experiment II, the conditions were identical except that the concentration of cells was $3 \times 10^8/\text{ml}$ and the incubation time was 3 hr. The enzyme-treated orosomucoid and mucin concentrations were 5.0 and $2.5 \text{ mg}/0.1 \text{ ml}$, respectively.

Added acceptor	Concentration	Electrophoretically immobile radioactivity*
	μM	
Experiment I		
None		180
Phenyl α - <i>N</i> -acetylglucosaminide	1	253
Phenyl β - <i>N</i> -acetylglucosaminide	1	1163
Phenyl α - <i>N</i> -acetylgalactosaminide	1	1691
Phenyl β - <i>N</i> -acetylgalactosaminide	1	185
<i>N</i> -Acetylglucosamine	1	1062
<i>N</i> -Acetylglucosamine	5	2177
<i>N</i> -Acetylglucosamine	10	1672
<i>N</i> -Acetylgalactosamine	1	188
<i>N</i> -Acetylgalactosamine	5	309
<i>N</i> -Acetylgalactosamine	10	254
<i>N</i> -Acetylmannosamine	1	150
Experiment II		
None		417
Sialidase- and galactosidase-treated orosomucoid		4750
Sialidase-treated ovine submaxillary mucin		5653

* cpm per $50 \mu\text{l}$ of cell-free supernatant.

Accordingly, the exogenous acceptors *N*-acetylglucosamine and sialidase-treated ovine submaxillary mucin were tested both with cell suspensions and with supernatant fluids derived from these suspensions. For determining the activity of cell suspensions incubations were performed as described in the section on utilization of exogenous acceptors, and 50- μ l samples of the supernatants obtained by centrifugation of the incubation mixtures were used directly for electrophoresis. For testing the activity of cell supernatants, the suspensions were first incubated in Medium H at 37°C for the time specified. The suspensions were centrifuged and 100 μ l of each supernatant were added to tubes containing the labeled UDP-galactose and the acceptor, both of which had been previously dried under vacuum. After the specified incubation times, 50 μ l of these solutions were spotted directly for electrophoresis. The results of these experiments show (Figs. 5 and 6) that whole cell suspensions can catalyze the transfer of galactose to sialidase-treated ovine submaxillary mucin and *N*-acetylglucosamine and that most of this activity resides on the cells.

Activity of Whole Cell Preparations Compared to Homogenates

If the fraction of enzymatic activity detected in intact cells compared to total activity in cell homogenates was very small, a low percentage of damaged cells could have been responsible for the the observed results. Incubations were therefore conducted with whole cell preparations in the presence and absence of exogenous acceptors, and with homogenates prepared by sonic vibration. Incubation mixtures with sonicates were prepared with and without detergent (Triton X-100), which has been shown to exert a considerable stimulatory effect on these galactosyltransferases (9). In the absence of detergent, the whole cell preparations incorporated 75% as much galactose-¹⁴C into endogenous acceptor as the homogenate. When the mucin was added to the incubation mixtures, the whole cell preparations were 92% as active as the homogenates. In the presence of detergent, homogenates showed a twofold increase in activity towards endogenous acceptors and more than a sixfold increase in activity towards exogenous acceptors. Table III shows that intact cells have 39% of the endogenous activity of homogenates plus detergent. Intact cells show 14% of the activity toward exogenous acceptors

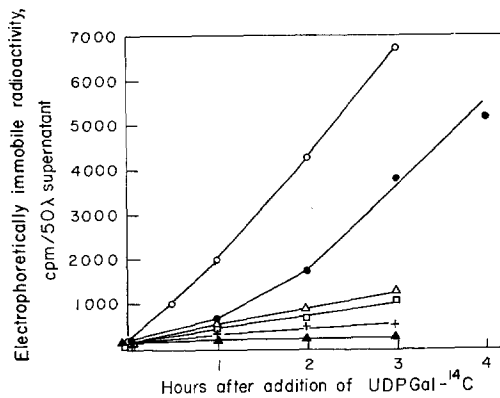


FIGURE 5 UDP-galactose:mucin galactosyltransferase activity in cell suspensions and in supernatant fluids. Incubation mixtures were prepared as described in the text with 25 μ M UDP-galactose (10^8 cpm per μ mole), and 0.25 mg of sialidase-treated ovine submaxillary mucin, and where present, intact cells at 1.3×10^8 cells/ml. The enzyme sources for each incubation were the following: \circ , whole cell suspensions which had been preincubated in Medium H at 37°C for 3 hr; \bullet , whole cell suspensions incubated directly with UDP-galactose and mucin; Δ , \square , $+$, supernatants obtained by centrifugation at 200 *g* for 6 min of cells preincubated for 4, 2, and 1 hr, respectively, in Medium H; \blacktriangle , supernatant obtained after 2 hr preincubation above, and incubated with UDP-galactose-¹⁴C but without mucin.

Incubations containing cells were terminated by centrifuging at 200 *g* for 3 min and chilling the supernatant fluids to 0°C. Incubations containing supernatant fluids were terminated by chilling to 0°C. All supernatants were subsequently subjected to paper electrophoresis.

compared to homogenates plus detergent. The ratios of whole cell to homogenate activities in the absence of detergent were much higher, as indicated above. These results, together with our inability to detect significant soluble galactosyltransferase activity in the cell supernatant fluids, suggest that cell lysis and cell damage alone cannot account for the observed activities with the whole cell preparations.

Effect of Potential Acceptors on Selective Adhesion

Previous results (8) suggested that β -D-galactopyranosyl end groups were partially responsible for the specific intercellular adhesion of embryonic chicken neural retina cells. On the basis of this theory the transferases most likely to be involved,

i.e. which would require β -D-galactopyranosyl end groups, are the sialyltransferases or fucosyltransferases, rather than the galactosyltransferases. Nevertheless, in light of the studies reported above,

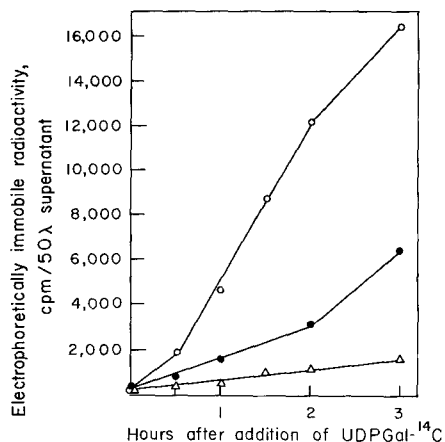


FIGURE 6 UDP-galactose: *N*-acetylglucosamine galactosyltransferase activity in cell suspensions and in supernatant fluids. Incubation mixtures were prepared as described in the text; where present, intact cells were used at 4×10^8 cells/ml. The specific activity of the UDP-galactose-¹⁴C ($25 \mu\text{M}$) was 10^8 cpm per μmole , and *N*-acetyl-D-glucosamine was used at 5 mM. The following enzyme sources were tested: O, a cell suspension incubated in Medium H at 37°C for 2 hr; ●, a cell suspension used without preincubation in Medium H; Δ, the cell-free supernatant obtained as described in Fig. 5 from a cell suspension preincubated in Medium H for 2 hr. All samples were subjected to centrifugation at 200 *g* for 3 min, and the supernatant fluids were assayed by paper electrophoresis.

which suggest the presence of cell surface galactosyltransferases, an attempt was made to prevent or inhibit intercellular adhesion using exogenous substrates known to interact with these galactosyltransferases. Using the previously described modification of the collecting-aggregate assay for specific adhesion (8), several potentially active, low molecular weight compounds were tested for their ability to interfere with the collection of ³²P-labeled neural retina cells by unlabeled neural retina aggregates. The results of four similar experiments are summarized in Table IV. A small inhibition is consistently observed with *N*-acetylglucosamine, *N*-acetylgalactosamine, and β -linked phenyl *N*-acetylglucosaminide. No evidence for significant inhibition was observed with α -linked phenyl *N*-acetylglucosaminide, *N*-acetylmannosamine, lactose, or *N*-acetylglucosamine.

A single, similar experiment was conducted using the low molecular weight analogues of the mucin-type acceptors, methyl and phenyl α -*N*-acetylgalactosaminides, (and their β -linked analogues). The small amounts of all of these compounds allowed only one experimental flask for each. However, both α -linked compounds showed marked stimulation of collection when compared to effects of the β -linked compounds, which did not differ significantly from the control flasks.

Proportion of Cells Catalyzing the Endogenous Reaction

Since the quantity of labeled galactose incorporated into either endogenous or exogenous ac-

TABLE III
Comparison of Galactosyltransferase Activities toward Endogenous and Exogenous Acceptors in Intact Cells and Cell Sonicates

The incubations were conducted as described in the text with $25 \mu\text{M}$ UDP-galactose (10^8 cpm per μmole), and cell suspensions (or sonicates from such suspensions) at 2×10^8 cells/ml. Where added, the detergent Triton X-100 (1% solution in water) was used at a concentration of 10 $\mu\text{l}/100 \mu\text{l}$ of incubation mixture. For exogenous activity, 0.75 mg of sialidase-treated ovine submaxillary mucin was added as acceptor.

Enzyme source	Endogenous activity (no acceptor)*	Ratio of endogenous activity in intact cells to activity in sonicate	Exogenous activity (plus acceptor)*	Ratio of exogenous activity in intact cells to activity in sonicate
Intact cells	992	1.00	1,919	1.00
Cell sonicate	1,237	0.80	2,097	0.92
Cell sonicate plus detergent	2,569	0.39	13,732	0.14

* Electrophoretically immobile radioactivity as cpm per 50 μl of incubation mixture.

TABLE IV
Effect of Potential Galactosyl Acceptors on the Collection of Labeled Neural Retina Cells by Unlabeled Neural Retina Aggregates

These experiments were carried out as described previously (8). Unlabeled aggregates of neural retina cells (diameter \cong 0.5mm) were circulated in freshly prepared suspensions of ^{32}P -labeled neural retina cells.* After collection, the aggregates were harvested, washed gently, and dried on paper. The paper slips were then counted in a toluene scintillation mixture in a liquid scintillation spectrometer.

Added compound	Concentration	Mean No. of cells collected per aggregate [‡]
<i>mM</i>		
Experiment I		
None		121 (12) [‡]
<i>N</i> -Acetylglucosamine	1.0	59 (8)
<i>N</i> -Acetylglucosamine	5.0	71 (8)
Experiment II		
None		175 (12)
<i>N</i> -Acetylglucosamine	5.0	129 (8)
<i>N</i> -Acetylglucosamine	10.0	139 (12)
<i>N</i> -Acetylgalactosamine	5.0	92 (12)
Phenyl α - <i>N</i> -acetylglucosaminide	2.5	146 (12)
Phenyl β - <i>N</i> -acetylglucosaminide	2.5	108 (12)
Experiment III		
None		130 (12)
<i>N</i> -Acetylgalactosamine	0.1	132 (12)
<i>N</i> -Acetylgalactosamine	0.5	94 (12)
<i>N</i> -Acetylgalactosamine	1.0	111 (6)
<i>N</i> -Acetylgalactosamine	5.0	110 (12)
<i>N</i> -Acetylmannosamine	1.0	146 (12)
<i>N</i> -Acetylgalactosamine plus <i>N</i> -acetylglucosamine	1.0 each	112 (6)
Experiment IV		
None		17 (8)
<i>N</i> -Acetylglucosamine	0.25	13 (8)
Lactose	0.25	17 (8)
<i>N</i> -Acetyllactosamine	0.25	19 (8)

* Experiment I was conducted for 150 min with 2.5×10^5 labeled cells/ml. Experiment II was conducted for 195 min with 1.7×10^5 labeled cells/ml. Experiment III was conducted for 180 min with 1.0×10^5 labeled cells/ml. Experiment IV was conducted for 120 min with 1.1×10^5 labeled cells/ml.

[‡] Number in parentheses represents number of aggregates used.

ceptors was relatively small, it appeared possible that only a few cells in the large population used for these studies might be active. To test this, a standard incubation was performed except that tritiated UDP-galactose was used at 44 μM and 16 $\mu\text{Ci}/\mu\text{mole}$. A control tube contained no manganese. After incubating, the cells were pelleted, fixed in Carnoy's fixative, embedded in

paraffin, sectioned, and exposed to Kodak NTB2 Liquid Photographic Emulsion in light-proof boxes at 4°C for 3 wk. Examination of the slides showed that at least 90% of the cells were labeled. In the manganese-free control, many fewer cells demonstrated grains, and those which did had a much lower number of grains per cell than in the preparations with manganese.

DISCUSSION

Complex carbohydrates appear to be involved in intercellular adhesion of mouse teratoma cells (4) and embryonic chicken neural retina cells (8). The present studies were designed to test the hypothesis that glycosyltransferases are located on cell surfaces, and that they may play a role in intercellular adhesion. The enzymes studied were galactosyltransferases.

Embryonic chicken neural retina cells were incubated with labeled UDP-galactose in the presence and absence of exogenous acceptors. The cells transferred galactose to endogenous acceptors, and kinetic studies were conducted to determine optimal conditions. The galactose-¹⁴C-labeled products obtained in this manner appeared to be mainly high molecular weight compounds and were not in the neutral glycolipid class. Studies with exogenous acceptors suggested the presence of two galactosyltransferases, one which acts on serum-type glycoproteins containing terminal *N*-acetylglucosamine residues (5), and another which transfers galactose to *N*-acetylgalactosamine linked to the polypeptide core of mucins (9).

The following results suggest that galactosyltransferases are located on the surfaces of the chicken embryonic neural retina cell.

(a) The supernatant fluids obtained after incubating the cells contained little galactosyltransferase activity. In addition, Figs. 5 and 6 show that the enzymes are not unstable under the conditions used. Therefore, it is unlikely that the results can be explained by enzyme released into the medium.

(b) Incorporation of radioactivity was not affected when the incubations were conducted in the presence of excess quantities of unlabeled galactose or galactose-1-P. Therefore, it is unlikely that the labeled sugar nucleotide was hydrolyzed externally, and the resulting labeled galactose or galactose-1-P then utilized after transport through the cell membrane. In addition, the incubations were conducted in the presence of azide which makes it less likely that the cell could synthesize the sugar nucleotide internally. The possibility that UDP-galactose could penetrate the cell membrane and could be utilized internally seems improbable since the incorporation of radioactivity was not affected by the presence of UDP-glucose.

(c) The cell preparations not only utilized low molecular weight acceptors such as *N*-acetyl-

glucosamine, but also the high molecular weight (about 10⁶ g/mole) mucin and the serum-type glycoprotein. The labeled products were found in the extracellular fluid. It appears unlikely that the proteins would be absorbed by the cell, galactosylated, and then secreted. Also, there is no evidence for the uptake of *N*-acetylglucosamine or the other active acceptor hexosamine derivatives by animal cells.

(d) In the absence of detergent, incorporation of galactose into both endogenous and exogenous acceptors by whole cells ranged between 75 and 92% of the activities observed with the homogenates. When detergent was added to the homogenates, the activity of the whole cells was 39% of that of the homogenates with respect to incorporation into endogenous acceptors, and 14% of that of the homogenates into exogenous acceptor (mucin). The results indicate that a substantial fraction of the total cellular activity is exhibited by the whole cell preparations, and that the small degree of lysis which might have gone undetected could not have accounted for this activity.

Using two different methods to detect lysis after incubation of the intact cells with the substrates, little cell damage was detected. One tentative (but improbable) explanation is that a small proportion of the cells everted in such a fashion that the enzymes were exposed and highly active while the resulting particle sedimented at 200 *g* for 5 min. This small population could have acted on all the other cell surfaces as well as the exogenous acceptors. This possibility could account for the observed results.

However, the most reasonable explanation for all of the data is that the galactosyltransferase (mucin and glycoprotein) activities described in this report are in fact located primarily on the surfaces of embryonic chicken neural retina cells. These results are also consistent with previous studies on these two galactosyltransferases in crude homogenates of embryonic chicken brain (1). Altogether, seven glycosyltransferases were studied and were found to be located primarily in the membrane fraction of nerve-ending particles (synaptosomes). The present investigation suggests that in the neural retina some galactosyltransferases are located on the external surface of the plasma membrane.

Studies with rat liver glycosyltransferases, including the galactosyltransferase which utilizes α_1 -acid glycoprotein, showed that the enzymes

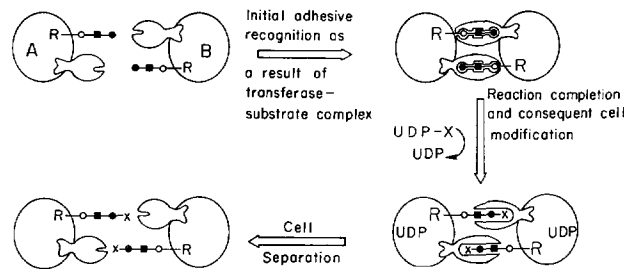


FIGURE 7 Schematic representation of the possible role of surface glycosyltransferases and their substrates in intercellular adhesive recognition. Cells *A* and *B* have both substrates (*R*-containing groups) and enzymes with active sites exposed. The theory allows for surface modification when the reaction is completed; this could provide a possible explanation for some contact phenomena such as contact inhibition and induction.

were probably bound to membranes located in the Golgi-rich fraction (9). Since the Golgi membrane has been postulated to fuse with the plasma membrane, this suggests one way by which transferases can be incorporated on the surface membranes.

Transferases present on the outer surface of these cells may be responsible for synthesizing part of the external, complex carbohydrate-rich coating found on most cells. In addition, they may act as "locks" in a lock-and-key model for intercellular adhesive recognition. The glycosyl acceptors would be the "keys." Whole batteries of transferases and their appropriate acceptors differing in specificities and in number or distribution could account for the tissue-specific recognition often described in embryonic cells. This type of model is illustrated in Fig. 7 and is meant to apply only to earlier adhesions where contact specificity has been seen and not to later adhesions characterized by desmosomes, tight junctions, etc.

In the accompanying paper, β -galactosyl end groups were implicated in the process of specific adhesion. The enzymes which utilize such substrates are generally not galactosyltransferases but other glycosyltransferases. Nevertheless, data presented in Table IV suggest that substrates which combine with galactosyltransferases have some inhibitory effect on specific adhesion. This apparent contradiction might be the result of a constellation of enzymes, i.e., a multiglycosyltransferase system, being required for specific adhesion. For example, terminal galactose residues might be required by sialyltransferases, and their interaction could be largely responsible for cell recognition in this system. This could explain why removal of galactose decreases selectivity. Also, if galactosyltransferases

were minor components, the effect of their competitors would also be small.

The transferase-acceptor model is attractive because it allows cells to detach merely by completing the glycosyltransferase reaction and because, upon reaction completion, each cell has changed its surface chemistry. These properties could be partially responsible for the poorly understood contact phenomena which apparently control much of morphogenesis, metastasis, growth, and inductive effects.

Studies are now in progress to determine whether other cells contain surface glycosyltransferases, and if so, the degree to which these enzymes are involved in the process of intercellular communication.

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BIBLIOGRAPHY

1. DEN, H., B. KAUFMAN, and S. ROSEMAN. 1970. Properties of some glycosyltransferases in embryonic chicken brain. *J. Biol. Chem.* **245**:6607.
2. FOLCH, J., M. LEES, and G. H. SLOANE STANLEY. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**:497.
3. MCGUIRE, E. J., G. W. JOURDIAN, D. M. CARL-

- SON, and S. ROSEMAN. 1965. Incorporation of D-galactose into glycoproteins. *J. Biol. Chem.* **240**:4113.
4. OPPENHEIMER, S. B., M. EDIDIN, C. W. ORR, and S. ROSEMAN. 1969. An L-glutamine requirement for intercellular adhesion. *Proc. Nat. Acad. Sci. U. S. A.* **63**:1395.
 5. ORR, C. W., and S. ROSEMAN. 1969. Intercellular Adhesion. I. A quantitative assay for measuring the rate of adhesion. *J. Membrane Biol.* **1**:124.
 6. ROSEMAN, S. 1968. In *Biochemistry of Glycoproteins and Related Substances*. Proceeding 4th International Conference of Cystic Fibrosis of the Pancreas (Mucoviscidosis), Karger, New York.
 7. ROSEMAN, S. 1970. The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. In *Chemistry and Physics of Lipids*. North Holland Publishing Co., Amsterdam.
 8. ROTH, S., E. J. MCGUIRE, and S. ROSEMAN. 1971. An assay for intercellular adhesive specificity. *J. Cell. Biol.* **51**:525.
 9. SCHACHTER, H., J. Inderjit, R. HUDGIN, L. PINTERIC, E. J. MCGUIRE, and S. ROSEMAN. 1970. Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.* **245**:1090.
 10. SCHACHTER, H., and E. J. MCGUIRE. 1968. Galactose transferase from pig submaxillary gland. *Fed. Proc.* **22**:345.
 11. TYLER, A. 1946. An auto-antibody concept of cell structure, growth and differentiation. *Growth*. **10**:Symposium 6, 7.
 12. WEISS, P. 1947. The problem of specificity in growth and development. *Yale J. Biol. Med.* **19**:235.