The Binding of Tissue-Specific Adhesive Molecules to the Cell Surface. A Molecular Basis for Specificity†

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ABSTRACT: Factors present in supernatants prepared from neural retina or cerebral lobe tissue cultures bind specifically to cells of the same type and promote cellular aggregation; the basis for the specificity of these factor–cell interactions has been investigated. Pronase digestion destroys binding of protein but not the carbohydrate portion of factors. Digestion with a mixture of protease-free glycosidases destroys both protein and carbohydrate binding. Purified β-*N*-acetylhexosaminidase reduces binding of retina factor by 80%. The enzymatic activity which destroys binding of cerebral lobe factor to cerebral cells appears to be α-mannosidase activity. Further, paper chromatography of the enzymatic digestion products of the binding factors reveals that *N*-acetylgalactosamine residues are released from the retina factor while mannosamine residues are released from the cerebral lobe factor. Inhibition of binding of factors to cells by monosaccharides is consistent with the enzyme data. *N*-Acetylgalactosamine maximally inhibits binding of factor to retina cells while mannosamine inhibits factor binding to cerebral cells. These data suggest that the specificity of cellular adhesion is determined, at least in part, by the sequence of sugars in an oligosaccharide residue of a complex glycoprotein.

The ability of embryonic cells to adhere selectively to cells of their own kind has been demonstrated by a variety of techniques (Roth, 1968; Roth *et al.*, 1971; Walther *et al.*, 1973). This selective adhesion is thought to play a role in *in vitro* aggregation and sorting out of embryonic cells as well as in vivo morphogenetic cell movements (see Lilien (1969) and Roth (1973) for reviews). Attempts to analyze the molecular mechanisms involved in specific cell adhesion have been hampered by an inability to prepare sufficient quantities of materials which mediate the process and by a lack of quantitative assays for these materials. Recently, our laboratory has developed both a simple procedure for obtaining quantities of cell culture supernatants which are able to promote cell aggregation and a quantitative assay for adhesive molecules in these supernatants which is based on their binding to the cell surface (Balsamo and Lilien, 1974a). In this report, we demonstrate that binding is mediated by carbohydrate residues. By means of competition experiments with simple sugars and enzymatic studies, we demonstrate that, for the two tissue types tested, binding is mediated by different sugars. These findings suggest that at least part of the specificity of cell adhesion is based on differences in the carbohydrate moieties of complex glycoproteins.

Experimental Section

Materials

Pronase (B grade), neuraminidase (*Vibriocholera*), and cytochalasin B were purchased from Calbiochem. Cytochalasin B was stored as a 1-mg/ml solution in dimethyl sulfoxide. "Rhozyme" was obtained from Rohm and Haas Corporation. β-Galactosidase (bovine liver) was a product from Sigma. The following products were obtained from Sigma or Pierce Chemical Company: D-glucose, D-galactose, L-fucose, D-mannose, D-glucosamine, *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetylneuraminic acid, glucuronic acid, and galacturonic acid. [^{3}H]Glucosamine (12.6 Ci/mmol) and [^{14}C]leucine (3.11 Ci/mol) were obtained from Amersham/Searle. [^{14}C]Glucosamine (50 Ci/mol) was a product of Nuclear Dynamics, Inc.

Methods

1. Preparation of Single Cells and Aggregation-Promoting Supernatants (APMs). Procedures for obtaining single cell suspensions and aggregation enhancing supernatants from neural retinas and from cerebral lobes were described in a previous publication (Balsamo and Lilien, 1974a); 10-day white leghorn chicken embryos were used in all cases. Labeled APMs were prepared by including either [^{3}H]glucosamine (1 μCi/ml.) or [^{14}C]glucosamine (2 μCi/ml.) or both [^{3}H]glucosamine and [^{14}C]leucine (1 μCi/ml.) in the tissue cultures. After 24 hr of culture, the medium was collected. Fresh medium without isotope was added for a further 24-hr period at which time it was collected. Both culture supernatants were centrifuged at 10,000*g* for 30 min and pooled. These culture supernatant solutions are called RAPM, for retina aggregation-promoting material, or CLAPM,[1] for cerebral lobe aggregation-promoting material.

2. Assay for Binding of Radioactive Molecules in the Aggregation Enhancing Supernatants. Single cells prepared by trypsinization of neural retinas (10×10^6 cells/tube) or cerebral lobes (8×10^6 cells/tube) were incubated with 0.1–1.0 ml of labeled native or enzyme-treated RAPM or CLAPM; 100 μl. of horse serum and 50 mM Hepes buffer,

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[1] Abbreviations used are: RAPM, retina aggregation promoting material; CLAPM, cerebral lobe aggregation promoting material. This preparation was previously referred to as BAPM (Balsamo and Lilien, 1974a); BME, Eagles Basal medium; Hepes, *N*-2-hydroxythylpiperazinc-*N*′-2-ethanesulfonic acid.

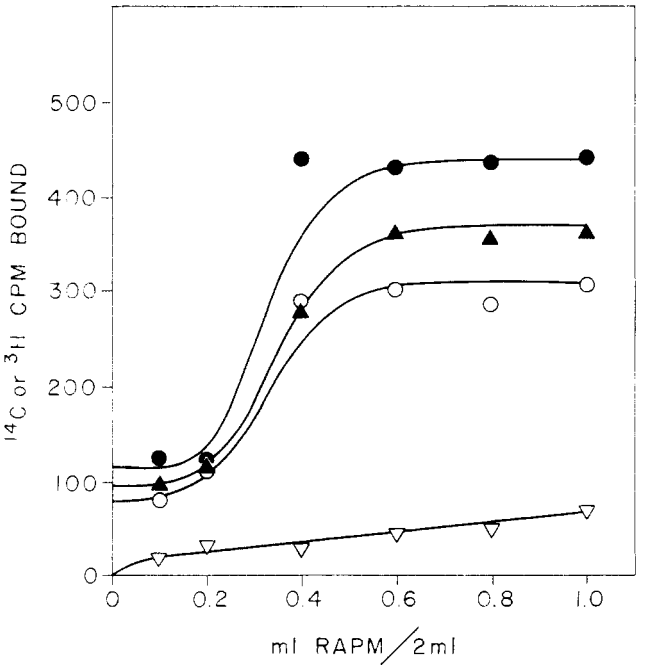

FIGURE 1: Binding of [^{14}C]leucine-[^{3}H]glucosamine double-labeled RAPM to homologous cells before (●, ○) and after (▲, ▽) Pronase digestion (100 μg/ml of Pronase, 37°, 3 hr). ^{3}H counts bound in native RAPM, ●; ^{14}C counts bound in native RAPM, ○; ^{3}H counts bound after Pronase digestion, ▲; ^{14}C counts bound after Pronase digestion, ▽.

pH 6.5 (RAPM) or pH 8.0 (CLAPM), were added to each assay tube and the volume was made 2 ml with Eagle's basal medium (BME). After a 10-min incubation at 4°, cells were harvested by centrifugation and 5% trichloroacetic acid containing glucosamine and leucine at a concentration of 30 μg/ml each was added. The precipitate was collected by centrifugation and washed once with 5% Cl_3CCOOH containing glucosamine and leucine. The final pellet was dissolved in 1.0 ml of 0.5 N NaOH and transferred to a scintillation vial. After neutralization with glacial acetic acid, 10 ml of Aquasol (New England Nuclear) was added to each vial and radioactivity measured in a liquid scintillation spectrometer (Beckman, LS 150). When double-labeled RAPM was analyzed, appropriate corrections were made for the overlap of ^{14}C and ^{3}H channels.

3. Enzymatic Digestion of APMs. Pronase digestion (100 μg/ml) or [^{3}H]glucosamine-[^{14}C]leucine double labeled RAPM was carried out at 37°, pH 8.0, for 3 hr. This treatment rendered dialyzable 77% of the ^{14}C label and 26.7% of the ^{3}H label. The reaction was stopped by a 10-min incubation in a boiling water bath. A control RAPM sample, with no enzyme added, was incubated and heated in parallel with the experimental one. Binding assays were done in the presence of as much as 50 mg/ml of boiled Pronase as a further control. "Rhozyme" solutions were prepared by stirring 5 g of the crude enzyme preparation in 10 ml of deionized water, at 4°, for 1 hr. The suspension was centrifuged and the supernatant used as the enzyme source. This preparation was found to have very low proteolytic activity (equivalent to less than 50 National Formulatory Units of tryptic activity per ml). APM solutions were incubated with 0.1 or 0.5 volume of "Rhozyme" in 50 mM sodium citrate (pH 4.6) for 1 hr at 30°. The sample was assayed for binding as described in item 2. Controls consisted in APMs treated with heat-inactivated enzyme. β-*N*-Acetylhexosaminidase was purified from "Rhozyme" using the procedure of Bahl and Agrawal (1969) and its enzymatic activity was assayed as described by those authors. Digestion of APMs was carried out with 0.5 volume of the enzyme under the same conditions as described for "Rhozyme." α-Mannosidase activity was determined using the procedure described by Matta and Bahl (1971).

4. Assay for Inhibition of Binding by Monosaccharides. Single cells prepared by trypsinization were resuspended in a 250 mM sugar solution containing 10 μg/ml of cytochalasin B at pH 7.0 and the suspensions incubated at 37° for 15 or 30 min. At the end of this incubation, 0.2-ml aliquots containing 10 × 10^6 retina or 8 × 10^6 cerebral lobe cells and 50 μmol of monosaccharide were added to tubes containing 1 ml of [^{3}H]APM/2 ml and assayed as described in section 2 of Methods.

5. Assay for Stability of the Binding Factor-Cell Receptor Complex. To study the effect of single sugars on the stability of the APM-cell complexes, cells were incubated under standard conditions for binding assays with 1 ml of [^{3}H]APM. The cells were collected, resuspended in 1 ml of unlabeled APM or in a 250 mM sugar solution at pH 7.0, and further incubated in an ice-water bath for varying periods of time. At each time point, cells were harvested and assayed for bound radioactivity as described in section 2 of Methods.

6. Paper Chromatography. Descending chromatography of the dialyzed APMs or enzyme-digested APMs on Whatman no. 3 paper was carried out in the system described by Fischer and Nebel (1955) (pyridine-ethyl acetate-water-acetic acid, 5:5:3:1, v/v; a vessel containing pyridine-ethyl acetate-water, 11:40:6, v/v, is placed at the bottom of the tank); 10 μg of each of amino or *N*-acetylamino sugar were used as standards. Chromatography was continued for 14 hr; at this time the solvent was completely evaporated and the chromatograms were developed using the Elson-Morgan reaction as modified by Partridge (1948). For determination of the distribution of radioactivity on the paper chromatograms, lengthwise strips corresponding to each sample applied were cut out and cut transversely into 0.5-cm pieces. Each piece was placed in a vial and counted in toluene-2,5-diphenyloxazole (4 g/l.)-1,4-bis[2-(5-phenyloxazolyl)]benzene (100 mg/l.) scintillation solution.

Results and Discussion

1. The Effect of Proteolytic and Glycolytic Enzymes on Binding of Factors to Homologous Cells. The effect of broad spectrum proteolytic and glycolytic enzymes on the specific binding capacity of factors in the retina (RAPM) and cerebral lobe (CLAPM) aggregation promoting supernatants was investigated as a first attempt to identify the chemical nature of the molecules necessary for specific recognition of the cell surface sites. Figure 1 compares binding of native and Pronase-digested [^{3}H]glucosamine-[^{14}C]leucine double-labeled RAPM to retina cells. It is evident that binding of ^{3}H-labeled material is minimally affected by the enzyme digestion while binding of ^{14}C-labeled material is completely abolished. In a similar way, the ability of [^{3}H]glucosamine labeled CLAPM to bind to cerebral lobe cells is not destroyed by treatment with Pronase. These results allow the following alternative interpretations. (a) Protein and carbohydrate binding in the supernatants are independent. In this case taking into consideration previously reported evidence that in addition to bound carbohydrate, bound protein is also essential for specific cell aggre-

TABLE I: Inhibition of Binding of Factors to Homologous Cells: Digestion of APMs with Glycolytic Enzymes.[a]

Enzyme Preparation	β-*N*-Acetyl-glucosaminidase (units/ml)[a]	β-*N*-Acetyl-galactosaminidase (units/ml)[b]	α-Mannosidase (units/ml)[a]	APM	% Inhibition of Binding
Fresh "Rhozyme"	0.090	0.085	1.50	RAPM	87 ± 9
				CLAPM	61 ± 4
Frozen thawed "Rhozyme"[c]	N.D.	0.055	1.38	RAPM	24
				CLAPM	56
pH 4.0 "Rhozyme"[d]	N.D.	0.050	0.015	RAPM	30
				CLAPM	0
Purified β-*N*-acetyl-hexosaminidase	0.102	0.090	None detected	RAPM	78 ± 2
				CLAPM	0

[a] [^{3}H]Glucosamine labeled APM's were incubated with 0.5 volume of enzyme in 50 mM sodium citrate pH 4.6, for 1 hr, at 30°. The reported enzyme units are given as units per 1 ml of digestion mixture. [b] 1 unit of glycosidase is defined as 1 μmol of *p*-nitrophenyl released from the appropriate *p*-nitrophenyl glucoside per minute (β-*N*-acetylhexosaminidase) or per hour (α-mannosidase) at 30°. [c] The crude "Rhozyme" solution was frozen and thawed two times consecutively. [d] The crude "Rhozyme" solution was stored frozen at pH 4.0 for 48 hr prior to use.

gation (Balsamo and Lilien, 1974b), both binding reactions would be specific and inactivation of either moiety would not alter the binding of the other. (b) Binding of the protein is dependent upon carbohydrate binding; either the two moieties are linked in a complex macromolecule or binding of the carbohydrate effects a conformational change at the cell surface enabling the protein to bind.

Alternatives a and b were distinguished by digesting double-labeled RAPM with "Rhozyme," a semipurified extract of *Aspergillus niger* containing several different glycosidases (Bahl and Agrawal, 1969). Figure 2 shows that upon digestion of the carbohydrate moiety binding of both ^{14}C and ^{3}H label are lost. Thus, ^{14}C (protein) binding is dependent upon ^{3}H (carbohydrate) binding. As much as 90% inhibition of binding of [^{3}H]glucosamine-labeled APM is obtained after digestion of the factors with freshly prepared "Rhozyme" (Table I). Which of the various enzymatic activities present in "Rhozyme" is responsible for destroying the binding ability of the two APMs is distinguished in a number of ways. First, β-*N*-acetylhexosaminidase purified from "Rhozyme" destroys as much as 80% of RAPM factor binding activity but does not show any effect on the binding of CLAPM factor to cerebral lobe cells (Table I). Secondly, the activities responsible for destroying each of the factors are differentially stable to freezing and thawing. About 40% of the RAPM-inactivating ability is lost after freezing and thawing a "Rhozyme" preparation twice, while only 8% of the CLAPM destroying activity is lost. These figures parallel those obtained for inactivation of β-*N*-acetylgalactosaminidase and α-mannosidase, respectively (Table I). We also have taken advantage of the fact that α-mannosidase activity is unstable to storing at pH 4.0 (Matta and Bahl, 1971). We find that loss of α-mannosidase activity in "Rhozyme" is paralleled by a loss in CLAPM destroying activity (Table I). These results suggest that a terminal α-mannosidase sensitive bond is essential for the specific binding activity in CLAPM while a terminal β-*N*-acetylhexosamine residue is essential for binding of RAPM factor to retina cells.

Treatment of RAPM or CLAPM with β-galactosidase or neuraminidase did not reduce binding of either factor.

2. The Effect of Monosaccharides on Binding of Factors to Homologous Cells. A large number of single sugars were tested for their ability to inhibit binding of [^{3}H]glucosamine labeled factors in the APMs to homologous cells. The results are summarized in Table II. Reproducible inhibition of binding was observed only when trypsinized cells were incubated with a solution of the appropriate monosaccharide plus cytochalasin B (10 μg/ml) prior to the binding assay. The presence of cytochalasin B is required to prevent a loss of binding ability which is observed when trypsinized cells are maintained in culture at 37° prior to performing the binding assay.[2] (Control experiments have shown that

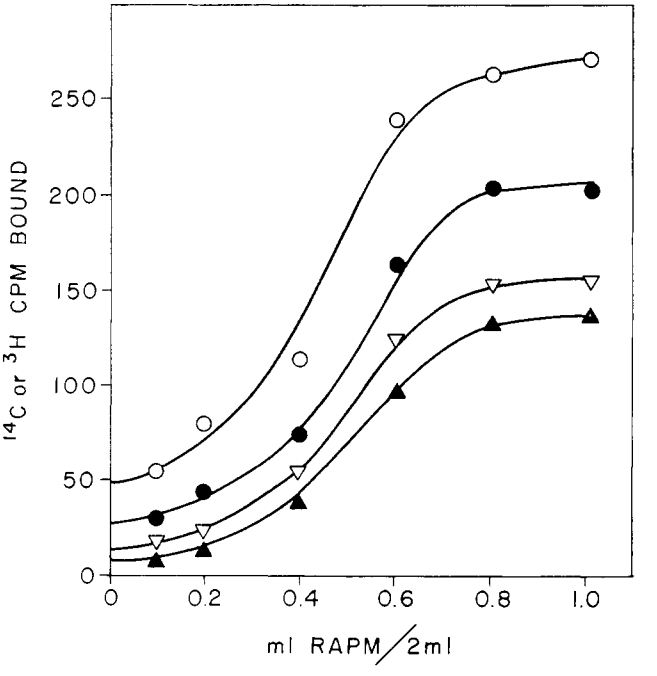

FIGURE 2: Binding of labeled RAPM factor to retina cells. Effect of "Rhozyme" digestion. [^{14}]Leucine-[^{3}H]glucosamine double labeled RAPM was incubated with 0.1 volume of freshly prepared "Rhozyme" solution in 50 mM sodium citrate buffer (pH 4.6) for 30 min at 30° (see Table I for activity of enzyme). ^{3}H counts bound in control, ●; ^{14}C counts bound in control, ○; ^{3}H counts bound after "Rhozyme" digestion, ▲, ^{14}C counts bound after "Rhozyme" digestion, ▽.

[2] This effect may be related to mobility of receptor sites in the cell membrane. A complete description of these data will be forthcoming.

TABLE II: Effect of Single Sugars on the Binding of Factors to Homologous Cells.

Sugar	% Inhibition of Binding: RAPM on Retina[a] 30 min	CLAPM on Cerebral Lobe[a] 30 min	CLAPM on Cerebral Lobe[a] 15 min
None	0	0	
Glucose	0	0	
Galactose	11 ± 1	7 ± 2	
Fucose	0	2 ± 1	
Mannose	0	2 ± 1	
Glucosamine	5 ± 5	23 ± 13	0
Galactosamine	15 ± 3	16 ± 6	
Mannosamine	5 ± 5	23 ± 9	16 ± 8
N-Acetylglucosamine	25 ± 6	11 ± 9	
N-Acetylgalactosamine	33 ± 3	11 ± 6	
N-Acetylmannosamine	17 ± 2	22 ± 9	3 ± 3
N-Acetylneuraminic acid	0	0	
Glucuronic acid	0	0	
Galacturonic acid	0	0	

[a] Results are means of five determinations.

TABLE III: Effect of Monosaccharides on the Dissociation of Factor–Cell Complex.[a]

Dilution Medium	Minimal Half-Life of Complex (min): RAPM Retina[b]	CLAPM Cerebral Lobe[b]
BME	2.6	4.6
Unlabeled APM	5.2	8.4
Glucosamine	3.0	5.5
Galactosamine	3.8	5.0
Mannosamine	2.5	8.2
N-Acetylglucosamine	3.4	5.4
N-Acetylgalactosamine	5.0	5.6
N-Acetylmannosamine	3.2	6.2

[a] Neural retina cells (10×10^6) and cerebral lobe cells (8×10^6) were incubated with 1 ml of [^{3}H]RAPM or [^{3}H]-CLAPM, respectively, for 10 min in an ice–water bath, as described in section 4 of Methods. The cells were resuspended in 1 ml of BME, unlabeled APM (0.6 ml of APM 0.4 ml of BME), or 250 mM sugar solution. After incubation for 5, 10, 20, 30, or 60 min in an ice–water bath, cells were collected and assayed for radioactivity as described in Methods. Radioactivity present in the cell pellet was calculated as per cent of radioactivity of time 0; the data were plotted as a function of time and the half-life was calculated as the time corresponding to 50% initial radioactivity. [b] Values are the results of three independent experiments carried out in triplicate.

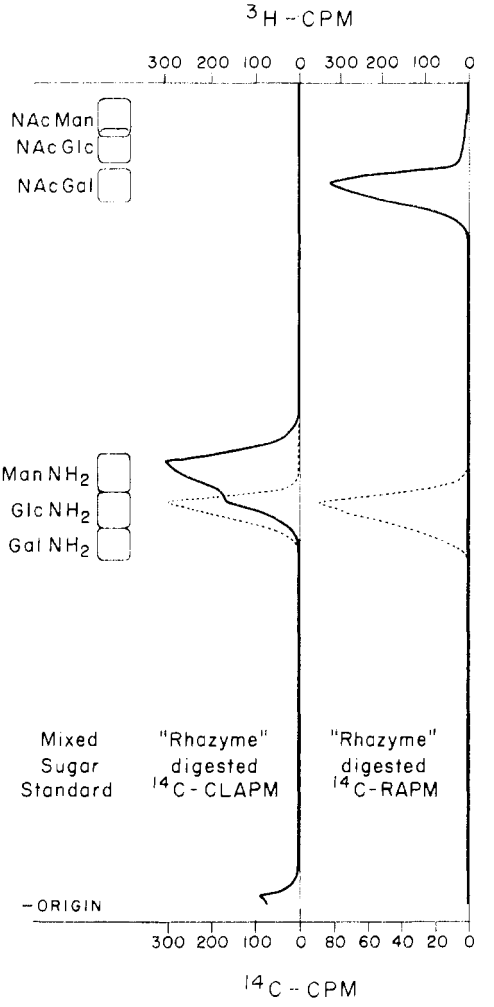

FIGURE 3: Paper chromatography of "Rhozyme" digested APMs. [^{14}C]Glucosamine labeled RAPM and CLAPM were extensively dialyzed against a 0.01 M phosphate–0.15 M NaCl solution (pH 7.2) containing 30 μg/ml of cold glucosamine. The factors were digested with 0.1 volume of enzyme solution and samples of 200 μl were applied to the paper (see Table I for specific activities of enzyme). [^{3}H]Glucosamine was also applied to the same spot as an internal control (- -). Glucosamine, galactosamine, and mannosamine and their N-acetylated derivatives were either included in the same spot or run in parallel. The procedures for development, staining, and counting of ^{14}C-labeled monosaccharides released are described in Materials and Methods.

including cytochalasin B in the assay vials does not affect binding.) While in both cases more than one monosaccharide exhibits some inhibition the relative effectiveness of specific sugars differs in the two cases (Table II). In particular, mannosamine is among the most effective inhibitors of CLAPM factor binding and has little or no effect on RAPM factor binding; conversely *N*-acetylgalactosamine is the most effective inhibitor of RAPM factor binding and has relatively little effect on CLAPM factor binding. These differences are consistent with the loss of binding ability after enzymatic digestion (Table I).

It is not clear at present what interpretation should be ascribed to the finding that more than one monossacharide exhibits some degree of reproducible inhibition. *N*-Acetylgalactosamine exhibits the greatest ability to inhibit binding of RAPM factor to retina cells, but *N*-acetylglucosamine, *N*-acetylmannosamine, and galactosamine are also partially inhibitory. Inhibition of binding of CLAPM factor to cerebral lobe cells was more difficult to interpret. After a 30-min preincubation period, mannosamine, glucosamine, and *N*-acetylmannosamine each have a reproducible effect (about 23% inhibition). However, if the preincubation time is reduced to 15 min, mannosamine is the only monosac-

charide showing any significant inhibition (Table II). Glucosamine and mannosamine appear to inhibit binding *via* interaction with the same binding site. This is apparent since preincubation of cerebral lobe cells with equal concentrations of both sugars together (250 mM final concentration) leads to a degree of inhibition equal to that obtained with mannosamine alone (125 mM).

The enzymatic digestion and sugar inhibition data taken together suggest that RAPM factor binding to retina cells is mediated by a terminal *N*-acetylgalactosamine residue. Destruction of the ability of CLAPM factor to bind to cerebral lobe cells parallels α-mannosidase activity. Although this enzyme has not been reported to release terminal mannosamine residues, it has been found to release the acetylated derivative indicating that it has a broad spectrum of activities for mannose derivatives (Matta and Bahln 1971). In addition, mannosamine has the greatest effect as an inhibitor of binding. Glucosamine may play a secondary role in binding; possibly as another component sugar of the CLAPM factor binding site.

3. Chromatographic Identification of Labeled Sugars Released from Glycosidase-Treated [^{14}C]APMs. To further characterize the terminal sugar residue present in APM factors, ^{14}C-labeled APMs were treated with "Rhozyme" and the released ^{14}C-labeled monosaccharides were identified on paper chromatography. In Figure 3 we present the pattern of spots of a standard sugar mixture and the correspondent radioactive tracings obtained after "Rhozyme" treatment of both CLAPM and RAPM. No radioactive monosaccharide could be detected in undigested controls run in parallel. The radioactive peak observed in the "Rhozyme"-digested [^{14}C]RAPM chromatogram corresponds to *N*-acetylgalactosamine. The same pattern was observed when [^{14}C]RAPM was treated with purified β-*N*-acetylhexosaminidase. Both [^{14}C]mannosamine and [^{14}C]glucosamine are liberated after treatment of [^{14}C]CLAPM with "Rhozyme," mannosamine in the higher amount.

4. Effect of Monosaccharides on the Stability of the Factor-Cell Receptor Complex. One further indication that the monosaccharides identified by enzymatic digestion and sugar inhibition experiments are significant for the recognition of a cell surface receptor is the fact that these sugars have a reproducible effect on the stability of the factor-receptor complex (Table III). In examining the factor binding data, we have suggested that nonlinearity of the curve obtained upon plotting cpm bound *vs.* APM concentration may indicate cooperative interactions between factor-binding sites (Balsamo and Lilien, 1974a). Our interpretation of the data is that filling sites produce receptor–receptor interactions which increase the association constant for subsequent binding events. One way of analyzing this positive cooperativity is to study the rate of dissociation of the factor–cell receptor complex in the presence of a cooperative agent. In Table III we show half-lives obtained by monitoring the dissociation of [^{3}H]RAPM factor–retina cell receptor complex in the presence of BME, unlabeled RAPM, or any one of a series of monosaccharides. In the presence of either cold RAPM or *N*-acetylgalactosamine, the half-life of the complex, [^{3}H]RAPM factor–cell receptor is increased by a factor of 2. The [^{3}H]CLAPM factor–cerebral lobe cell complex was also analyzed in a similar way (Table III). In this case mannosamine is the most effective sugar in increasing the half-life of the complex.

Conclusion

We have previously proposed that binding of a factor in the aggregation enhancing supernatants is mediated by a carbohydrate moiety; in addition a bound protein moiety is essential for the function of a third component, possibly a ligator, to establish a complete adhesion (Balsamo and Lilien, 1974b). Now, we have succeeded in demonstrating that the specificity of binding of factors to cells is based on the type of sugar residues in the carbohydrate moiety of what is probably a complex glycoprotein molecule. The specificity of binding of RAPM factor to retina cells appears to depend on a terminal *N*-acetylgalactosamine residue while CLAPM factor binding to cerebral cells most likely depends on a terminal mannosamine residue.

The evidence that terminal amino sugar residues impart a degree of specificity to intercellular adhesion raises several further problems of biological interest. Chief among these is to understand how such specificities are brought into being and integrated into the morphogenetic sequences of which they are a likely part. Well-known work with blood group specificities suggests by analogy that the regulation of glycosyl transferases will be a particularly worthwhile avenue to explore. In this context the biochemical model for cell adhesion proposed by Roseman (1970) and Roth *et al.* (1971) suggesting a role for glycosyl transferases and their oligosaccharide acceptors is of obvious interest.

Acknowledgments

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