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# Immunochemical Studies with Gangliosides. II. Investigations of the Structure of Gangliosides by the Hapten-Inhibition Technique

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The hapten-inhibition technique has been used to obtain further information about the chemical structure of gangliosides. The most striking findings were obtained with antibodies produced against a ganglioside derivative (asialoganglioside) from which *N*-acetylneuraminic acid had been cleaved by mild acid hydrolysis. The results obtained in the present study are consistent with the sequence of hexoses and *N*-acetylgalactosamine proposed by Svennerholm (1962), and by Kuhn and Wiegandt (1963). Studies with a variety of sugars and sugar derivatives indicated that inhibition is encountered only with a glycosidically bound β-D-galactopyranose or 2-acetamido-2-deoxy-β-D-galactopyranose moiety.

Although there is at present general agreement about the number and type of carbohydrate residues in brain gangliosides (Svennerholm, 1962; Kuhn and Wiegandt, 1963; Wagner *et al.*, 1963), the exact order and configuration of the linkages of these molecules has remained a controversial subject. Conflicting results have been obtained by acid hydrolysis (Klenk and Gielen, 1961), permethylation (Klenk and Gielen, 1960; Egge, 1960; Karkas and Chargaff, 1960), oxidation with periodate (Klenk *et al.*, 1962), or reduction with borohydride (Kanfer and Brady, 1963). A reproducible method was developed in this laboratory (Yokoyama *et al.*, 1963) for the production of antibodies against gangliosides and asialoganglioside, the glycosphingolipid residue of gangliosides from which the *N*-acetylneuraminic acid had been removed by mild acid hydrolysis (Trams and Lauter, 1962). Because of the availability of these specific antibodies, it was thought desirable to utilize the hapten-inhibition technique (Landsteiner, 1945; Kabat, 1958) in an attempt to obtain further insight into the structure and configuration of gangliosides. This procedure is based on the observation that the presence of a low molecular weight compound which resembles the structure of a particular antigen is able to block the reaction between the antigen and its specific antibody. The validity of the procedure rests upon the assumption that in a comparison of a series of inhibitors the substance exhibiting inhibition at the lowest concentration has a structure most similar to the determining group on the antigen (Kabat and Mayer, 1961). The method has been used with considerable success to elucidate the structure of the antigenic group of dextran (Kabat, 1957) and blood group substance A antigen (Schiffman *et al.*, 1962).

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## Experimental Procedure

*Materials.*—Gangliosides were prepared from beef brain by the method of Trams and Lauter (1962). Other samples were purchased from the Sigma Chemical Co. (lot 81B-623). When gangliosides were hydrolyzed under mildly acid conditions, two major glycolipid products were obtained. Analyses of these materials revealed that both contained 1 molecule each of sphingosine, fatty acid, glucose, and *N*-acetylgalactosamine. The compound designated as asialoganglioside contained, in addition to these components, 2 molecules of galactose. The other material, referred to as aminoglycolipid (Bogoch, 1961), had only 1 additional molecule of galactose.

A sample of Tay-Sachs' ganglioside was generously furnished to us by Dr. Joel A. Dain of Rhode Island University. Red-cell stroma globoside was prepared by Mr. Joseph V. Formica in this laboratory by the method of Makita and Yamakawa (1962). This material cochromatographed with authentic globoside kindly supplied by Dr. Yamakawa. Cytolipin H was a gift from Dr. Maurice M. Rapport. The following materials were the gifts of the respective persons mentioned: samples of *N*-acetylglucosamine, Dr. Roger W. Jeanloz; samples of anhydro derivatives of galactose, Dr. Nelson K. Richtmyer; various β-galactosides, Dr. Elizabeth Neufeld; samples of raffinose and stachyose, Dr. Dexter French; galactobiose, Dr. Roy L. Whistler; 4-*O*-β-D-galactosyl-*N*-acetylglucosamine, Professor Richard Kuhn, a sample of colominic acid, Dr. G. T. Barry; and samples of methyl galactosides, Dr. Hewitt G. Fletcher. Other sugars and oligosaccharides were purchased from commercial sources.

*Methods.*—Antiganglioside and antiasialoganglioside antibodies were produced in rabbits by the method of Yokoyama *et al.*, (1963). The rabbit antiserum ob-

tained by the injection of ganglioside-coated cells was specific for gangliosides and did not cross-react with cells coated with either asialoganglioside or aminoglycolipid in the hemagglutination test system (Yokoyama *et al.*, 1963). The antibody produced by the injection of cells coated with asialoganglioside cross-reacted with cells coated with aminoglycolipid, but not with ganglioside-coated cells. The antibody titre was determined with the hemagglutination technique developed by Yokoyama *et al.* (1963), with the following minor modifications. For coating the red cells, 1 volume of washed packed cells was suspended in 20 volumes of a solution containing 1 mg/ml of ganglioside in 0.01 M potassium phosphate–buffered isotonic saline (*p*H 6.4). Asialoganglioside and aminoglycolipid were dissolved in the same buffer to which 0.01% Triton X-100 was added. The same solution containing Triton X-100 was used for tests with cytolipin H, and the suspension was sonicated and clarified by the addition of 0.1% of bovine serum albumin.

The hemagglutination-inhibition tests were performed in the following manner. A solution of the sugar or oligosaccharide to be examined was mixed with an equal volume of the antiserum and allowed to react for 15–20 minutes at 23°. Serial dilutions from 2- to 32-fold of these mixtures were made with saline, and cells coated with the appropriate antigen were added. Most substances were tested for inhibition at an initial concentration of 0.05 M. When inhibition was observed with the test material at this level, diminishing concentrations of the inhibitory compound were examined until inhibition no longer occurred. The lowest concentration which caused inhibition was used for examination with serially diluted antibody in which the ratio of antibody to inhibitor was kept constant (Boyd, 1956). Positive control tests were run simultaneously with appropriately diluted antisera. Negative controls were performed similarly with uncoated cells. In the present experiments, hapten inhibition was considered positive only when blocking of the hemagglutination occurred in all dilutions of the antiserum which showed agglutination in the absence of inhibitor. All instances of inhibition were thoroughly checked on subsequent occasions utilizing other batches of coated cells.

## Results

The specificity of the antiganglioside and antiasialoganglioside antibodies is illustrated by the fact that the antibody was absorbed only by the respective hapten, (Table I). It is apparent, however, that sera from animals immunized with asialoganglioside cross-reacted with cells coated with aminoglycolipid. The antigenic determinant for ganglioside antibody appears to include the *N*-acetylneuraminic acid residue since the antiganglioside system was inhibited only by free *N*-acetylneuraminic acid and colominic acid, a polymeric compound of *N*-acetylneuraminic acid molecules joined in a 2 → 8 ketosidic linkage. In addition to *N*-acetylneuraminic acid, the terminal galactose molecule of ganglioside seems to be a part of the antigenic determining site of the ganglioside molecule since the ganglioside-antiganglioside system was not inhibited by Tay-Sachs' ganglioside. The latter material lacks the terminal molecule of galactose which is present in the normal ganglioside molecule (Svennerholm, 1962; Wagner *et al.*, 1963).

A strong inhibition of the antiasialoganglioside system by β-galactosides was demonstrated in the present study. Lactose was the most potent inhibitor followed by lactobionic acid, *o*-nitrophenyl-β-D-galactopyranoside, 6-*O*-β-D-galactopyranosyl-D-galactose, 4-*O*-β-D-galactosyl-*N*-acetylglucosamine, 3-*O*-β-D-galactopyranosyl-D-arabinose, 3-*O*-β-D-galactopyranosyl-D-glucose, methyl β-D-galactopyranoside, and methyl 2-acetamido-2-deoxy-β-D-galactopyranoside. No monosaccharide or α-linked disaccharide was inhibitory.

In contrast with these findings were the observations that much higher concentrations of lactose were required to inhibit the cross-reacting aminoglycolipid-coated cells with the asialoganglioside antibody. The most potent hapten inhibitor of the aminoglycolipid system appeared to be methyl 2-acetamido-2-deoxy-β-D-galactopyranoside, whereas the α anomer was ineffective in this regard even at considerably higher concentrations. Furthermore, this cross-reacting system was inhibited by relatively high concentrations of monosaccharides such as D-galactose and *N*-acetyl-D-galactosamine.

## Discussion

The present state of the development of the hapten-inhibition technique permits several conclusions to be drawn with regard to the structural configuration of gangliosides. The results with the antiasialoganglioside system indicated that lactose, 4-*O*-β-D-galactopyranosyl-D-glucose, was the most effective inhibitor and presumably reflects most accurately the structure of the asialoganglioside hapten. Other galactose-containing disaccharides also inhibited, but a higher concentration than that for inhibition with lactose was required. In no case was inhibition observed with an α-linked oligosaccharide.

It is apparent that relatively high concentrations of monosaccharides inhibited the cross-reaction between cells coated with aminoglycolipid and asialoganglioside antibody, whereas monosaccharides were ineffective inhibitors in the antiasialoganglioside system. These findings are not too surprising in view of the limitations imposed on the hapten-inhibition technique in a cross-reacting system (Kabat, 1958). If one assumes that the strength of binding of the cross-reacting aminoglycolipid to the antiasialoganglioside antibody is less than for the asialoganglioside, then weakly bound inhibitors such as monosaccharides would compete more effectively against aminoglycolipid than asialoganglioside.

The data presented indicate that all glycosides tested containing a terminal galactose unit in β- but not α-glycosidic linkage were found to inhibit the hemagglutination reaction. The current interpretation of such tests is that the terminal residues of antigenic materials make the predominating contributions to the energy of binding of ligands to antibodies (Kabat and Mayer, 1961). The present findings suggest that the terminal sugar in asialoganglioside is galactose held in β-glycosidic linkage. It might be construed, since lactose and 2 of its derivatives were the most potent inhibitors, that the terminal galactose is linked 1 → 4 to the next sugar, not necessarily glucose.

It was found that the hemagglutination reaction was inhibited by β- but not by α-methyl-*N*-acetylgalactosamine or by *p*-nitrophenyl-β-D-*N*-acetylglucosamine. These findings are consistent with the concept that *N*-acetylgalactosamine is a portion of the antigenic determining site of the asialoganglioside molecule. An alternative interpretation is that the inhibition by β-methyl-*N*-acetylgalactosamine is due to its mimicking a β-galactoside rather than acting as a β-galactosaminiside. However, since disaccharides containing galactose and *N*-acetylgalactosamine were not available, this point could not be further pursued.

TABLE I

HAPTEN INHIBITION OF AGGLUTINATION OF CELLS COATED WITH GLYCOSPHINGOLIPIDS[a]

| Inhibitor | Antigen-Antibody System | | |
|---|---|---|---|
| | Ganglioside[b] | Asialoganglioside[c] | Aminoglycolipid[c] |
| 1,6-Anhydro-α-D-galactofuranose | (−) 0.050 M | (−) 0.050 M | (−) 0.050 M |
| 1,6-Anhydro-β-D-galactopyranose | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| Aminoglycolipid | (−) 1 mg/ml | (+) 1 mg/ml | (+) 1 mg/ml |
| Asialoganglioside | (−) 1 | (+) 1 | (+) 1 |
| Cellobiose | (−) 0.050 M | (−) 0.050 M | (−) 0.050 M |
| Colominic acid | (+) 2.0 mg/ml | [d] | [d] |
| Cytolipin H | (−) 1 mg/ml | (−) 1 mg/ml | (−) 1 mg/ml |
| Floridoside | (−) 0.050 M | (−) 0.050 M | (−) 0.050 M |
| Galactinol | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| D-Galactose | (−) 0.050 | (−) 0.050 | (+) 0.050 |
| Galactosylarabinose | (−) 0.025 | (+) 0.025 | (−) 0.025 |
| 4-*O*-α-D-Galactopyranosyl-D-galactose | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| 6-*O*-β-D-Galactopyranosyl-D-galactose | (−) 0.050 | (+) 0.015 | [d] |
| D-Galactopyranosyl-β-1-3-D-glucose | (−) 0.050 | (+) 0.050 | [d] |
| 4-*O*-β-D-Galactosyl-*N*-acetylglucosamine | [d] | (+) 0.020 | [d] |
| Ganglioside | (+) 1 mg/ml | (−) 1 mg/ml | (−) 1 mg/ml |
| Gentiobiose | (−) 0.050 M | (−) 0.050 M | (−) 0.050 M |
| Gentianose | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| Globoside | (−) 1 mg/ml | (−) 1 mg/ml | [d] |
| Lactobionic acid | (−) 0.050 M | (+) 0.003 M | (−) 0.050 M |
| Lactose | (−) 0.050 | (+) 0.0015 | (+) 0.050 |
| Maltose | (−) 0.050 | (−) 0.050 | [d] |
| Melibiose | (−) 0.050 | (−) 0.050 | [d] |
| Mellezitose | (−) 0.050 | (−) 0.050 | [d] |
| Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside | [d] | (−) 0.060 | (−) 0.060 M |
| Methyl 2-acetamido-2-deoxy-β-D-galactopyranoside | [d] | (+) 0.060 | (+) 0.010 |
| Methyl α-D-galactopyranoside | (−) 0.050 M | (−) 0.050 | (−) 0.050 |
| Methyl β-D-galactopyranoside | (−) 0.050 | (+) 0.050 | (−) 0.050 |
| *N*-Acetyl-D-galactosamine | (−) 0.050 | (−) 0.050 | (+) 0.050 |
| *N*-Acetyl-D-glucosamine | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| *N*-Acetyl-D-mannosamine | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| *N*-Acetylneuraminic acid | (+) 0.050 | (−) 0.050 | (−) 0.050 |
| Neuraminlactose | (−) 0.020 | (−) 0.020 | (−) 0.020 |
| *o*-Nitrophenyl-β-D-galactopyranoside | (−) 0.050 | (+) 0.010 | (−) 0.050 |
| *p*-Nitrophenyl-*N*-acetyl-β-D-2-deoxy-2-aminoglucoside | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| Planteose | (−) 0.025 | (−) 0.025 | (−) 0.025 |
| Raffinose | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| Stachyose | (−) 0.050 | (−) 0.050 | (−) 0.050 |
| Tay-Sachs' ganglioside | (−) 2 mg/ml | (−) 2 mg/ml | (−) 2 mg/ml |
| Trehalose | (−) 0.050 M | (−) 0.050 M | [d] |
| Turanose | (−) 0.050 | (−) 0.050 | [d] |

[a] The details of the test procedures are given in the text. A (+) sign indicates complete inhibition of all dilutions of the antiserum and the figure following indicates the minimum concentration of hapten required for inhibition. A (−) sign indicates a lack of inhibition and the figure following indicates the highest concentration of the test material used. [b] Serum from rabbits immunized with gangliosides (Yokoyama *et al.*, 1963) was used as the source of antibody. [c] Serum from rabbits immunized with asialogangliosides was used in these tests. [d] Not determined.

sphingosine-glucose-galactose-galactose-*N*-acetylgalactosamine
|
fatty acid

Scheme I.

sphingosine-glucose-galactose-*N*-acetylgalactosamine-galactose
|
fatty acid

Scheme II.

Our immunochemical findings, therefore, could lend support to the structure for asialoganglioside proposed by Svennerholm (1962) and Kuhn and Wiegandt (1963) as shown in Scheme II, with a terminal β-galactose linked to a penultimate β-*N*-acetylgalactosamine. This is further corroborated by the lack of inhibition by globoside. This compound contains the same type and number of hexose residues as asialoganglioside (Yamakawa *et al.*, 1962) and the sequence of the components proposed by these investigators is illustrated in Scheme I.

One might have suspected that cytolipin H (ceramidelactose) might have been an effective inhibitor in this system. It is possible that an insufficient amount of material was employed in the present experiments. Also its low solubility in an aqueous medium may have prevented the attainment of a sufficiently high concentration to show inhibition.

It is quite apparent that the primary question remaining to be resolved is the location of the molecules of *N*-acetylneuraminic acid in the intact ganglioside molecule. Since neuraminlactose was not an inhibitor

at the concentration employed, it is reasonable to assume that the *N*-acetylneuraminic acid linkage in monosialoganglioside is different from that in neuraminlactose. Several alternative possibilities may be visualized; viz., (1) the *N*-acetylneuraminic acid might be linked to *N*-acetylgalactosamine; (2) the *N*-acetylneuraminic acid may be linked to a different carbon of galactose; or (3), as suggested by studies of the reduction of intact gangliosides with borohydride (Kanfer and Brady, 1963), it might not be ketosidically linked. So far we have not been able to obtain a sufficient quantity of *N*-acetylneuraminyl-*N*-acetylgalactosamine or the various other possible isomers of neuraminlactose or neuramingalactose to test as inhibitors.

Finally, hapten-inhibition assays were carried out with whole antiserum and different inhibitors used in the present study may be bound to different extents to serum albumin. However, the high concentrations of hapten required for inhibition make it seem unlikely that major difficulties due to binding with albumin would be encountered in these experiments.

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# Lipid Alterations in *Euglena gracilis* Cells During Light-induced Greening*

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Etiolated cells of *Euglena gracilis* were subjected to constant illumination in a mineral medium. After a short lag period the cells produced chlorophyll in three successive stages. Sulfolipids accumulated before measurable amounts of chlorophyll. Sulfolipid synthesis occurred in stages corresponding to those of chlorophyll synthesis. In contrast, the cellular galactolipid level increased at a linear rate with the onset of illumination, independently of chlorophyll level. Upon illumination of the cells, the major fatty acids of the well-nourished etiolated cell, $C_{13}$ and $C_{14}$, rapidly disappeared. The green cell replenished its fatty acid complement by the synthesis of unsaturated fatty acids mainly of the 16- and 18-carbon series.

A study of the lipids of photosynthesizing organisms may result in new information about photosynthetic processes. We have previously reported an accumulation of unsaturated fatty acids, galactolipids, and sulfolipids in photosynthesizing *Euglena gracilis* cells (Rosenberg, 1963). The present experiments were designed to trace these three lipid components during stages of the greening of etiolated cells. Greening was induced by constant illumination of the cells. Chlorophyll content was taken as an index of photosynthetic activity. The cells were examined for a relationship between their chlorophyll content and the levels of newly synthesized lipids. Conditions were arranged to limit the extraneous factors imposed by growth and division of the cells.

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## MATERIALS AND METHODS

Cells of *Euglena gracilis* were grown in the dark on a synthetic medium (Wolken, 1961) and harvested. The harvested cells were washed in distilled water. Approximately 4-g batches were suspended in 100 ml