"GANGLIOPROTEIN AND GLOBOPROTEIN": THE GLYCOPROTEINS REACTING WITH ANTI-GANGLIOSIDE AND ANTI-GLOBOSIDE ANTIBODIES AND THE GANGLIOPROTEIN CHANGE ASSOCIATED WITH TRANSFORMATION*

Yasuto Tonegawa and Sen-itiroh Hakomori

Biochemical Oncology, Fred Hutchinson Cancer Research Center and University of Washington, 1124 Columbia, Seattle, Washington 98104

Received March 17,1977

Summary- The cell surface-labeled glycoproteins that are reactive to anti-glycolipid antibodies were solubilized by zwitterionic or non-ionic detergents and separated by specific anti-glycolipid antibodies through double immune precipitation or specific absorption on *Staphylococcus aureus*. The patterns of these glycoproteins ("ganglioprotein" or "globoprotein") as compared to glycolipids were examined on SDS-acrylamide gel electrophoresis. The major "globoprotein" of human erythrocytes was not in "glycophorin" or "PAS 1" but in the "PAS 2 or 3" region. The GM_1-ganglioproteins of 3T3 cells were all deleted in KiMSV transformants, whereas the intense protein peaks reactive to anti-asialo GM_2 (ganglio-N-triosylceramide) appeared in these transformants.

Recently a number of studies have indicated that glycolipid composition and metabolism undergo alterations associated with transformation (1), cell contact (2) and the cell cycle (3). Various glycolipids have been identified as blood group (4) and Forssman antigens (5). Gangliosides have been implicated as receptors for cholera toxin (6), thyrotropic hormone (7), serotonin (8), interferon (9) and Sendai virus (10). In these studies glycolipids isolated from cells were comparatively analyzed, and used as inhibitors of antigen antibody reactions or ligand-cell interactions. There is the possibility, however, that the same carbohydrate structures as found in the major glycolipids of cells, such as gangliosides and globoside, could also occur in oligo-

*This investigation was supported by National Institute of Health Research Grant GM23100 and CA20026.

Abbreviations and glycolipid designations: RBC: red blood cells, SDS: sodium dodecyl sulfate, Buffer saline: 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM sodium phosphate, pH adjusted as indicated, BSA: bovine serum albumin, 3T3KiMSV: 3T3 cells transformed with murine sarcoma virus Kirsten strain, TRIS: tris-(hydroxymethyl)aminomethane, EDTA: ethylenediaminetetraacetic acid, NET: NaCl (150 mM)-EDTA(5 mM)-Tris(50 mM) pH 7.0 containing 0.05% NP-40, GM_1:Galβ1→3 GalNAcβ1→4[NeurAc2→3]Galβ1→4Glc→ceramide
ganglio-N-triosylceramide (asialo GM_2): GalNAcβ1→4Galβ1→4Glc→ceramide
globoside: GalNAcβ1→3Galα1→4Galβ1→4Glc→ceramide
paragloboside: Galβ1→4GlcNAcβ1→4Galβ1→4Glc→ceramide

ISSN 0006-291X

saccharides linked to proteins, and could function as antigens or receptors. The present paper describes the result of studies to explore such possibility by using anti-GM_1, anti-asialo GM_2, anti-globoside, and anti-paragloboside antibodies. This preliminary note describes the "globoprotein" pattern of human erythrocytes and also a remarkable change of "ganglioproteins" of 3T3 cells which results from viral transformation.

MATERIAL AND METHODS

Cells and Surface-Labeling: Cloned Balb/c 3T3 cell lines (A-31) and the "non-productive" transformed cells with Kirsten murine sarcoma virus (3T3KiMSV; clone K-234) (11) were obtained from Dr. M. Hatanaka, Frederick Cancer Research Institute, Frederick, Maryland. These cells were cultured in Dulbecco modified Eagle's medium (12; GIBCO, Santa Clara, CA.) supplemented with 10% fetal calf serum in 5% carbon dioxide atmosphere. Human erythrocytes were obtained from laboratory personnel. Cells were labeled by the procedure as previously described (13).

Preparation and Properties of Anti-Glycolipid Antibodies: Anti-globoside, anti-asialo GM_2, anti-paragloboside, and anti-GM_1 ganglioside antisera were prepared in rabbits according to the procedure as described previously (14,15). Since these antisera contained anti-bovine serum albumin (BSA), the antisera were treated with BSA-Sepharose column (16). The titer of antigloboside (for trypsinized human RBC), of anti-GM_1 (for GM_1-coated RBC), of anti-asialo GM_2 (for trypsinized guinea pig RBC), and of anti-paragloboside (for sialidase treated human RBC) were 1:256, 1:512, 1:32, and 1:32, respectively. The specificities of these antisera have been extensively studied as previously described (14,15).

Glycolipid Samples: Globoside and paragloboside were prepared from human RBC (17), ganglio-N-triosylceramide (asialo GM_2) from guinea pig RBC (18), and GM_1 ganglioside from human brain according to the procedure described by Momoi *et al* (19). These preparations were homogeneous on thin-layer chromatography as free state and as acetate. GM_1 ganglioside was labeled by tritiation of sphingosine double bond (PtO_2 catalysis) and the "globosidic acid" was prepared by oxidative ozonolysis of the olefinic bond of sphingosine and coupled to BSA by the carbodiimide method (20).

Extraction of Cells: Quantitative extraction of both glycoproteins and glycolipids was carried out by a modified combination of methods with zwitterionic detergent (21,22), non-ionic detergent NP-20 (23) and 3 M KCl (24). 1% "zwitterionic" detergent (Empigen BB, Albright and Wilson Ltd., Marchon Division, Whitehaven, England) in Tris buffer pH 8.0, or 1% "Tergitol" NP-40 (Sigma Chemical Co., St. Louis, Mo) in 3 M KCl were found satisfactory. These conditions were chosen as these detergents do not interfere with subsequent antigen-antibody reactions. 1) The surface-labeled erythrocytes (0.5 ml) were lysed in 30 ml of ice-cold 20 milliosmolar phosphate buffer (pH 7.4), and centrifuged at 27,000 xg for 20 minutes. The pellet was washed 3 times with the same buffer. The final pellet was then suspended in 0.5 ml of 1% "Empigen" in 50 mM Tris buffer, pH 8.0 and allowed to stand for 1 hour in ice with occasional shaking. The suspension was centrifuged at 27,000 xg for 30 minutes, followed by 100,000 xg for 60 minutes. The supernatant was filtered through a packed "glass-wool" filter to eliminate insoluble floating material. The filtrate was immediately reacted with anti-glycolipid antibody as described in the next section.
2) Surface-labeled 3T3 Cells (four 15 cm plates; 3.8×10^7 cells) or the surface-labeled 3T3KiMSV cells (two 15 cm plates; 8×10^7 cells) were pelleted, and mixed with 0.5 ml of 1% NP-40 3 M KCl in buffer saline (pH 7.0). The

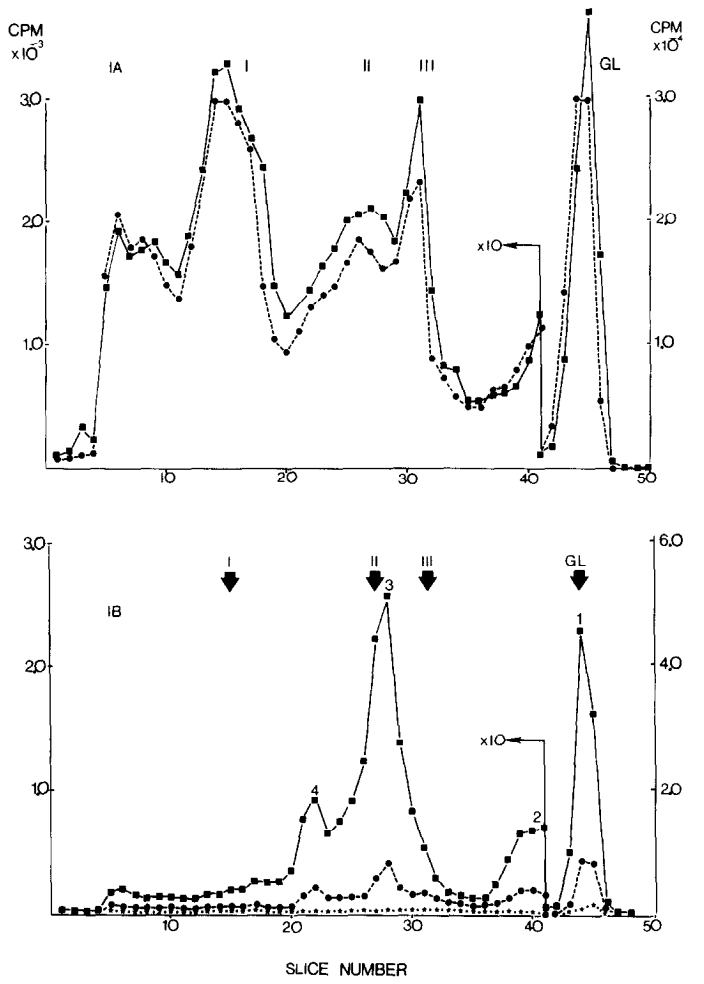

Fig. 1. Protein and Lipid Profile that React with Anti-Globoside and Anti-Paragloboside Antibodies.

The upper panel (1A) shows SDS gel electrophoresis pattern of surface-labeled human erythrocyte ghosts. ■——■——■ Total solubilized membrane in SDS on heating. ●---●---● Extract with "Empigen-Tris" buffer pH 8.0. From the front of gel; the first peak (GL) is glycolipid, the second, third and fourth are corresponding to bands of PAS 3, PAS 2 and PAS 1 or to bands "g", "e", and "c" of Itaya *et al* (27). The lower panel (1B) shows SDS gel electrophoresis pattern of the fraction reactive to anti-globoside and anti-paragloboside isolated from "Empigen-Tris" extract of the surface-labeled ghost. ■——■——■ activity with anti-globoside, ●---●---● activity with anti-paragloboside, ★ • • ★ • • ★ activity with preimmune serum. The peak 1 is globoside and paragloboside; note that the major peak is corresponding to the PAS 2 band or "band e" (27). No peak is seen which corresponds to "PAS-1" or "band c". The right ordinate indicates the activity for glycolipid, and the left ordinate indicates the activity for other peaks.

suspension was kept in an ice bath for 2-3 hours with occasional shaking. The extract was separated in the same way as in the "Empigen" extract.
Reaction of the Extract with Anti-Glycolipid Antibody and Separation of Antigen-Antibody Complex: 1) "Empigen"-Tris extract was mixed with anti-glycolipid. The antigen-antibody complex was separated on "*Staphylococcus aureus*" that carries "Protein A", according to the method described by Kessler (25) with a slight modification. 380 µl of NaCl-EDTA-Tris (NET) pH 7.0 containing 0.05% NP-40 and 0.1% BSA was mixed with 100 µl of the "Empigen" extract, followed by addition of 20 µl of antiserum. After standing for 60 minutes in an ice-water bath, and 400 µl of a 10% suspension of *Staphylococcus aureus* in NET was added, mixed well and incubated for 30 minutes at 4° C. The mixture was centrifuged at 5,000 rpm for 5 minutes, followed by washing of the bacterial pellet with NET buffer 3 times. The final pellet was mixed with 100 µl of the "sample buffer" for SDS gel electrophoresis (26), heated in a boiling water bath for 2 minutes, and centrifuged at 5,000 rpm for 5 minutes. The supernatant was subjected to SDS gel electrophoresis. 2) 100 µl of NP-40-KCl extract was diluted with 360-380 µl buffer saline (pH 7.0) and then mixed with 40-20 µl of antibody solution, incubated for 1 hour in ice-water, 200 µl of anti-rabbit γ-globulin goat antiserum was added. The reaction mixture was incubated on ice for 2-3 hours and then overlayed onto 1 ml of 10% sucrose and 3% NP-40 in buffer saline (pH 7.0) in a silicon-coated centrifuge tube and spun at 3,000 rpm (1,800 xg) for 30 minutes (27). The precipitate was suspended in 1 ml of 0.5% NP-40 in buffer saline and centrifuged at 3,000 rpm for 10 minutes. This washing procedure was repeated 3 times. The precipitate was finally dissolved in "sample buffer" (26) with heating and subjected to SDS gel electrophoresis.
Gel Electrophoresis in SDS-Acrylamide: The final extract with "sample buffer" was placed onto a disc of acrylamide gel according to the method of Weber and Osborn (26), disc gels were sliced in 2 mm thickness using "Gilson Gel Slicer" (Gilson Instr. Co.), and the radioactivity of each slice was counted.

RESULTS

Distribution Pattern of Globoside and Paragloboside Groups in Glycolipids and Glycoproteins of Human Erythrocytes. The pattern of the surface-labeled globoside, trapped with anti-globoside, consists of 4 fractions separated on gel electrophoresis (see Fig. 1). The fastest migrating fraction (peak 1), coincident with the glycolipid band, showed the highest activity. The second, third and fourth peaks were not glycolipids as all the glycolipid should complex with SDS and migrate at the position of "1" (see "Discussion" and Fig. 3). Although the activities of peaks "2", "3", and "4" were much weaker than "1", they are consistently present in many separate analyses. The "2" corresponds to a minor surface-labeled "band k" as previously described (28). The "3", the major "globoprotein", corresponds to the band "PAS-2 or 3" (29), and the "4" corresponds to "PAS-4" (29). Significantly, none of these "globoproteins" correspond to "PAS-1" or "glycophorin" (30) (Fig. 1A and B). The glycoprotein peaks reacting with anti-paragloboside exactly matched the "globoprotein" peaks,

TABLE I. RELATIVE QUANTITIES OF THE SURFACE-LABELED GLYCOPROTEINS AND GLYCOLIPIDS THAT ARE REACTIVE TO ANTI-GLOBOSIDE ANTIBODY

The total [^{3}H]-activity of cell surface labeled carbohydrates, solubilized on heating in SDS	100%
"Empigen"-Tris extract	93
Total fraction separated by reaction with anti-globoside	20
Fractions after SDS-gel electrophoresis	
globoside	16.6
globoprotein 2	0.4
globoprotein 3	2.0
globoprotein 4	0.6

Numbers indicate % of total activity, solubilized by SDS

and preimmune serum did not give any peak (Fig. 1A and B). The relative proportion of these "globoproteins" as compared to globoside and total surface-labeled carbohydrates is shown in Table I.

Distribution Pattern of GM_1-Ganglioside and Ganglio-N-Triosyl (Asialo GM_2) Group in Glycolipids and Glycoproteins of Balb/3T3 Cells and their Transformants. The extract of surface-labeled 3T3 cells was double immune precipitated by anti-GM_1 rabbit antibody and anti-rabbit Ig goat antibody, and showed at least 5 components on SDS gel electrophoresis. The predominant peak 1 obviously corresponds to GM_1-ganglioside, but peaks 2-5 may represent glycoproteins having the carbohydrate structure as similar to that of GM_1 ganglioside (Fig. 2A). A similar experiment with anti-ganglio-N-triosylceramide (asialo GM_2) did not show any peaks under the same condition (Fig. 2A). This corresponds with the fact that this glycolipid is virtually absent in 3T3/Balb cells (15).

In striking contrast, several remarkable glycoprotein peaks were demonstrated with anti-ganglio-N-triosylceramide antibody in 3T3KiMSV cells (Fig. 2B). Only a minimum activity was found with anti-GM_1 antibody. These results correspond with the accumulation of ganglio-N-triosylceramide (asialo GM2) in

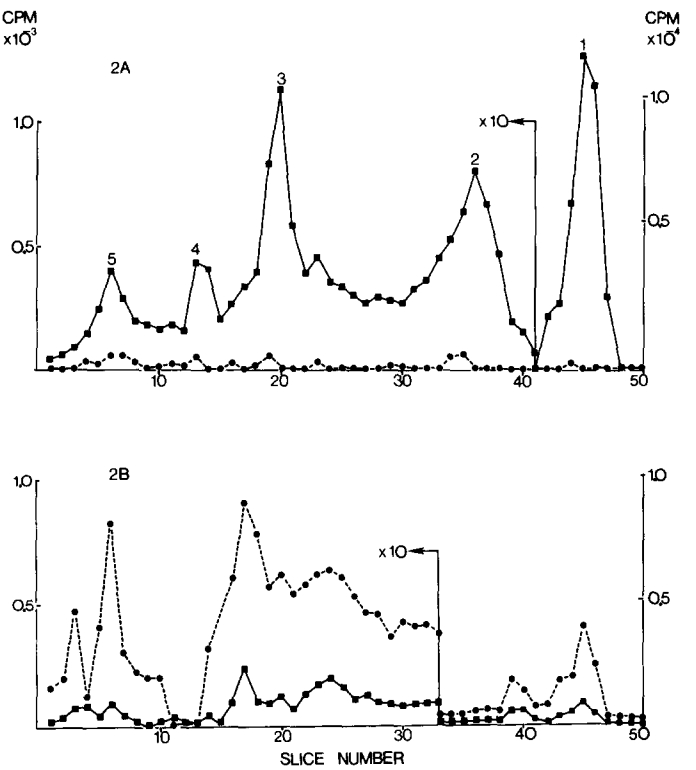

Fig. 2. Protein and Lipid Profile that React with Anti-GM_1 and Anti-Asialo GM_2 Antibodies.

The upper panel (2A) shows SDS gel electrophoresis pattern of the fraction reactive to anti-GM_1 ganglioside and anti-asialo GM_2 (anti-ganglio-N-triosyl-ceramide), isolated from NP40 3M KCl extract of the surface-labeled 3T3 Balb/c cells. ■——■——■ activity with anti-GM_1 ganglioside, ●– –●– –● activity with anti-asialo GM_2. The lower panel (2B) shows the SDS gel electrophoresis pattern of the same fraction as above, but isolated from 3T3KiMSV cells. The ordinate designition is the same as in Fig. 1. The pattern is based on the value substracted by the activities with preimmune sera.

Kirsten tumor cells (15), whereas GM_1 ganglioside was greatly reduced/or deleted in these cells (1).

DISCUSSION

The results indicate the presence of cell surface glycoproteins that carry the same carbohydrate chain as those found in the major glycolipid such as globoside and GM_1 ganglioside. There is however, a remote possibility that some cell surface protein may form a "complex" with glycolipid which may be difficult to dissociate upon heating in SDS. If so, such protein-glycolipid

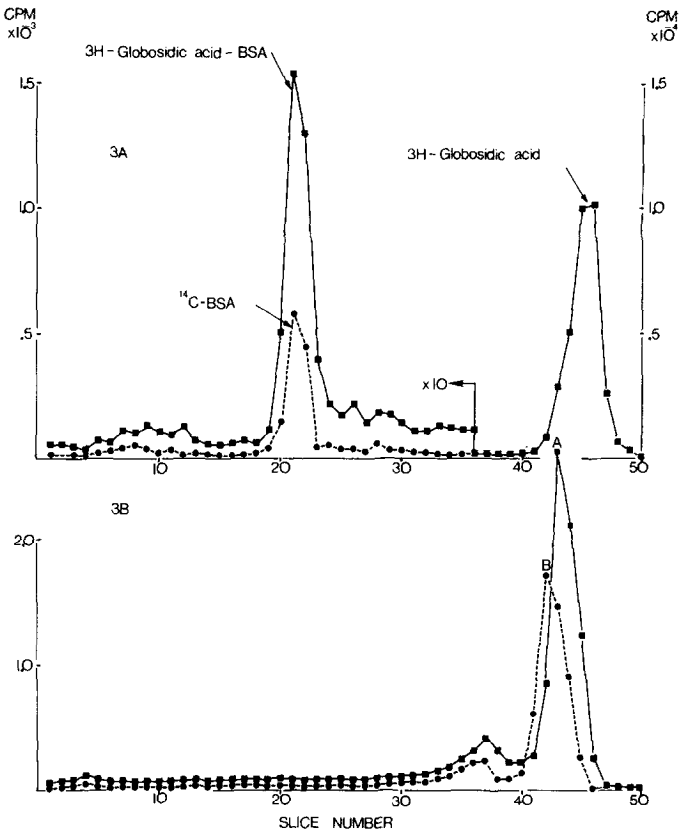

Fig. 3. SDS Gel-Electrophoretic Mobility of Free and Protein-Bound Glycolipid.

The upper panel (3A) shows the electrophoretic pattern of free [^{3}H]-globoside derivative and that covalently bound to BSA. The lower panel (3B) shows the electrophoretic pattern of [^{3}H]-GM_1 ganglioside incorporated into 3T3 cells, and separated on SDS gel electrophoresis. "A" represents the value with [^{3}H]-GM_1 added in serum free medium, "B" represents the value with [^{3}H]-GM_1 added in trypsinized cells. A small peak close to the glycolipid peak could be an artifact caused by "slicing error", as this peak was not detected in separate experiments (data not shown).

complexes could be detected as a glycoprotein peak on gel electrophoresis. Such possibility has been excluded by the following experiments: 1) 3T3 cells were grown in medium containing [^{3}H]-labeled GM_1 ganglioside for 24 hours; gangliosides were incorporated into plasma membranes (31). The cells were harvested, extracted and analyzed as described under "Methods". [^{3}H]-ganglioside was recovered in the glycolipid fraction but not in the glycoprotein

fractions (Fig. 3B). 2) [^{3}H]-globoside covalently linked to BSA (through "globosidic acid") was recovered in BSA fraction on SDS gel electrophoresis, and free [^{3}H]-"globosidic acid" was separated in glycolipid fraction (Fig. 3A). We believe therefore that those protein peaks reacting with anti-glycolipid antibodies must represent the glycoproteins bearing the same or similar oligosaccharides as those present in glycolipids. Recently, the sialosyl 2→8 sialosyl residue was found in brain glycoprotein (32), this same residue is the well known structure in brain gangliosides.

Reflecting the deletion of GM_1 ganglioside and accumulation of asialo GM_2 in Kirsten-transformed 3T3 cells (15), the activity of GM_1 "ganglioprotein" was virtually absent, but the activity of the protein reacting with anti-asialo GM_2 was greatly enhanced in these transformed cells. It may be that a similar kind of change in carbohydrate structure associated with transformation may occur both in ganglioside and in "ganglioprotein". Both globoside and paragloboside groups are mainly present in the PAS 2 or 3 fraction and are absent in PAS 1 ("glycophorin"). This indicates that each glycoprotein is characterized by a specific structure and only a few share specific structure with glycolipids. It has been stated that "PAS 1" is related to "PAS 2" (29), the result of this study suggests that "PAS 1" and "PAS 2" may not be related in their carbohydrate structure, or "globoprotein 3" may be a different molecule from "PAS 2 or 3" although it migrates to the same position as "PAS 2 or 3".

Acknowledgement

We thank Dr. Robert Nowinski for his technical advice and allowing us to use his automatic gel slicer.

REFERENCES

1. Hakomori, S. (1975) Biochim. Biophys. Acta 417, 55; Brady, R.O., and Fishman, P.T. (1974) Biochim. Biophys. Acta 335, 121.
2. Hakomori, S. (1970) Proc. Nat. Acad. Sci. USA 67, 1741; Sakiyama, H., Gross, S.K., and Robbins, P.W. (1972) Proc. Nat. Acad. Sci. USA 69, 872; Critchley, D.R., and Macpherson, I. (1973) Biochim. Biophys. Acta 246, 145.
3. Chatterjee, S., Sweeley, C.C., Velicer, L.F. (1973) Biochem. Biophys. Res.

Commun. 54, 585; Gahmberg, C.G., and Hakomori, S. (1975) J. Biol. Chem. 250, 2438.
4. Hakomori, S., and Kobata, A. (1974) The Antigens Vol. II. pp. 79-140. Ed. M. Sela. Academic Press, New York.
5. Siddiqui, B., and Hakomori, S. (1971) J. Biol. Chem. 246, 5766.
6. Cuatrecasas, P. (1973) Biochemistry 12, 3547.
7. Mullin, L.D., Fishman, P.H., Lee, G., Aloj, S.M., Ledley, F.D., Winard, R.J., Kuhn, L.D., and Brady, R.O. (1976) Proc. Nat. Acad. Sci. USA 73, 842.
8. Woolley, D.W., and Gommi, B.W. (1965) Proc. Nat. Acad. Sci. USA 53, 959.
9. Besancon, F., Ankel, H., and Basu, S. (1976) Nature 259, 576.
10. Haywood, A.M. (1974) J. Mol. Biol. 83, 427.
11. Aaronson, S.A., and Weaver, C.A. (1971) J. General Virol. 13, 245.
12. Dulbecco, R., and Freeman, G. (1959) Virology 8, 396.
13. Gahmberg, C.G., and Hakomori, S. (1973) Proc. Nat. Acad. Sci. USA 70, 3329.
14. Koscielak, J., Hakomori, S., and Jeanloz, R.W. (1968) Immunochem. 5, 441; Naiki, M., Marcus, D.M., and Ledeen, R.W. (1974) J. Immunol. 113, 84.
15. Rosenfelder, G., Young, W.W., Jr., and Hakomori, S. Cancer Research in press.
16. Cuatrecasas, P., and Anfinsen, C.B. (1971) Method Enzymol. 22, 345.
17. Laine, R.A., Stellner, K., and Hakomori, S. (1974) Methods in Membrane Biol. Vol. 2, pp. 205-247. Ed. E. Korn. Plenum Press, New York.
18. Seyama, Y., and Yamakawa, T. (1974) J. Biochem. 75, 837.
19. Momoi, T., Ando, S., and Nagai, Y. (1976) Biochim. Biophys. Acta 441, 488.
20. Laine, R.A., Yogeeswaran, G., and Hakomori, S. (1974) J. Biol. Chem. 249, 4460.
21. Allen, J.C., and Humphries, C. (1975) FEBS Letters 57, 158.
22. Carter, W., and Sharon, N. Arch. Biochem. Biophys. in press.
23. Nathenson, S.G., and Cullen, S.E. (1974) Biochim. Biophys. Acta 344, 1.
24. Reisfeld, R.A., and Kahan, B.D. (1970) Fed. Proc. 29, 2034.
25. Kessler, S.W. (1975) J. Immunol. 115, 1617.
26. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406.
27. Ledbetter, J., Nowinski, R.C., and Emery, S. J. Virol. in press.
28. Itaya, K., Gahmberg, C.G., and Hakomori, S. (1975) Biochem. Biophys. Res. Commun. 64, 1028.
29. Steck, T.L. (1974) J. Cell Biol. 62, 1.
30. Marchesi, V.T., Tillack, T., Segrest, J., and Scott, R. (1972) Proc. Nat. Acad. Sci. USA 69, 1445.
31. Keenan, T.W., Schmidt, E., Franke, W.W., and Wiegandt, H. (1975) Exp. Cell Res. 92, 259.
32. Finne, J., Krusius, T., and Rauvala, H. (1977) Biochem. Biophys. Res. Commun. 74, 405.