PREPARATION OF AN INHIBITOR OF VIRAL HEMAGGLUTINATION FROM HUMAN ERYTHROCYTES*

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During the course of our work on the interaction of influenza virus with glycoproteins, it became desirable to isolate highly potent inhibitors of viral hemagglutination in order to compare their properties and chemical composition with other glycoproteins with which the viruses react but which are not hemagglutination inhibitors. One of the most active of such inhibitors can be demonstrated in erythrocytes. It appears likely that this inhibitor may be the surface component with which the viruses combine to produce hemagglutination. Several investigators including de Burgh (1), McCrea (2), Green and Wooley (3), Howe *et al.* (4), Klenk *et al.* (5, 6), and Kathan *et al.* (7, 8) have prepared materials from erythrocytes possessing inhibitory activity to viral hemagglutination. The present paper describes an improved and convenient procedure which consistently yields a product of very high inhibitory titer. This material is usually homogeneous according to the criteria which we have applied including paper and moving boundary electrophoresis at several pH values and ultracentrifugal analysis. Chemical studies indicate that this material is a glycoprotein.

Materials and Methods

Virus.—Influenza virus, PR-8 and Lee strains, were propagated by inoculating 10-day-old embryonated chicken eggs with 0.1 ml. of a 1:1000 dilution of infective chorioallantoic fluid. The chorioallantoic fluid was harvested after incubation for 48 hours at 35° C. Indicator virus was made by heating the fluid at 56° C. for 30 minutes. This was titered for hemagglutination by the Salk method (9) and diluted with 0.9 per cent saline buffered at pH 7.4 with 0.15 M phosphate buffer (PBS) to 16 hemagglutination units/ml. and retitered before use. One hemagglutination (HA) unit is defined as that amount of virus which will just cause the hemagglutination of 0.5 ml. of 0.5 per cent human type 0, Rh-positive red cells in a final volume of 1 ml.

Hemagglutination Inhibition Titration.—The hemagglutination inhibitory titer was determined by making serial twofold dilutions of aqueous solutions of inhibitors in PBS. To 0.25 ml. of the inhibitor solutions was added 0.25 ml. of the indicator virus suspension (4 HA units), the mixture shaken, and kept at 4° C. for 10 minutes. One-half ml. of 0.5 per cent red

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cell suspension was added to each tube. The tubes were shaken and then stored for at least 4 hours at 4° C. before reading the pattern in the conventional manner. One hemagglutination inhibition (HAI) unit is defined as that amount of inhibitor which will just prevent the agglutination of 0.5 ml. of 0.5 per cent human red cells by 4 HA units of indicator virus.

Analytical Procedures.—Sialic acid was determined by Bial's orcinol reaction and by the direct Ehrlich's reaction as given by Werner and Odin (10), and by the thiobarbituric acid method of Warren (11).

Hexosamine was determined by the method of Morgan and Elson (12) as modified by Bogoch (13).

Protein-bound hexose and fucose was determined by the methods described by Winzler (14) modified by omitting those steps necessary to precipitate the proteins.

Nitrogen was determined by the method of Lang (15) involving nesslerization of diluted sulfuric acid digests. The digestion mixture was that recommended by Campbell and Hanna (16).

Phosphorus was determined by a modification of the method of Gericke and Kurmies (17).

Chromatography of amino acids, hexoses, hexosamines, and sialic acid was performed using a solvent system of ethyl acetate, pyridine, and water in volume proportions of 10:4:3. Occasionally, for confirmation, a solvent system of methyl ethyl ketone, acetone, water, and formic acid in the volume ratio of 30:10:10:1 was used as recommended by Högström (18).

Descending chromatography on Whatman 3MM paper for 16 to 20 hours was employed. Staining of amino acids was accomplished with a spray of 0.4 per cent ninhydrin in acetone, or the buffered ninhydrin of Moore and Stein (19), followed by heating at 105° C. in an oven until color developed. Staining of hexoses and hexosamines was accomplished by spraying the separated constituents with aniline oxalate, as used by Partridge (20) or by ammoniacal silver nitrate (21), and heating in the oven until color developed. Sialic acid was stained with Ehrlich's reagent modified as follows: 4 gm. of paradimethylaminobenzaldehyde was dissolved in 40 ml. concentrated hydrochloric acid and 160 ml. methyl cellosolve.

Electrophoresis was carried out on paper or in the Antweiler apparatus at several pH values ranging from 4.5 to 8.6 in 0.05 molar acetate, phosphate, or veronal buffers. High voltage paper electrophoresis was accomplished at 44 volts/cm. on Whatman No. 1 paper moistened with pyridine acetate buffer at pH 5.2 (100 volumes pyridine, 40 volumes glacial acetic acid, and 860 volumes water) suspended in heptane at 10° − 15° C. Sedimentation studies were made in a Spinco analytical ultracentrifuge with automatic temperature control at 59,780 R.P.M. Determinations were made in PBS at pH 7.4 and the sedimentation constants were corrected to 20° C. in water.

RESULTS

Preparation of the Inhibitor.—

Six or more pints of type 0 Rh-positive blood were centrifuged, the plasma decanted, and the "buffy coat" of white cells skimmed off. The cells were washed three times with PBS by centrifugation to eliminate any residual plasma or white cells since both of these also contain inhibitor material. The packed cells were then hemolyzed overnight with ten times their volume of distilled water at 4° C. The lysed cells were recovered by centrifugation at 700 *G* for 1 hour. Residual hemoglobin was removed by washing the stroma with distilled water several times by centrifugation until no hemaglobin could be detected in the supernatant. The pH of the stromal suspension was adjusted to 5.5 and the suspension centrifuged at 690 *G* for 30 minutes. The packed stroma was then frozen in this condition to serve as source material for further work up. Frozen pooled stroma, of mixed blood types, prepared in a

similar manner was generously supplied by Merck, Sharpe and Dohme, Inc.[1], West Point, Pennsylvania.

As shown in Fig. 1, 30 gm. of the frozen material was thawed, 50 ml. of distilled water added, and the cold suspension homogenized in a Waring blendor for 5 minutes. The finely divided suspension was adjusted to pH 8.0 with dilute NaOH and centrifuged for 2 hours at 700 *G*. It was found that the "fresh" (unfrozen) stroma source would not sediment easily at these centrifuge speeds, whereas the material once frozen for several days, sedimented easily. The insoluble residue was transferred to a beaker containing 700 ml. of 50 per cent phenol (by volume) maintained at 68° – 70° C. and stirred for 10 minutes. The mixture was permitted to cool to room temperature and was centrifuged at 700 *G* for 60 minutes. The upper aqueous phase was decanted, dialyzed against running tap water until free of phenol, and lyophilized.

Frozen Human Stroma
↓ to 30 gm., add 50 ml. distilled H_2O, homogenize, adjust to pH 8, centrifuge 700 *G*
Insoluble Elinin
↓ add 700 ml. 50 per cent phenol, 68°, stir 10 minutes cool to room temperature ,centrifuge at 700 *G*
Phenol Layer | Aqueous Layer
↓ dialyze against tap H_2O lyophilize
Crude Inhibitor (yield about 200 mg.)
↓ to 30 mg., add 10 ml. $CHCl_3$ – MeOH, 2:1, shake 10 minutes add 10 ml. distilled H_2O, shake 10 minutes centrifuge at slow speed
Chloroform Layer | Aqueous Layer
↓ centrifuge at 105,000 *G* decant solution, dialyze against distilled H_2O lyophilize
Pure Inhibitor (yield about 12 mg.)

FIG. 1. Method of preparation of inhibitor.

The yield was about 200 mg. To 30 mg. aliquots of this material was added 10 ml. of a chloroform-methanol solution (2 to 1 volume/volume) and the mixture shaken for 10 minutes. An additional 10 ml. of distilled water was added, and the mixture again shaken for 10 minutes. Following centrifugation at 700 *G*, the upper aqueous phase containing the inhibitor was decanted and recentrifuged at 105,000 *G* for 60 to 90 minutes. The supernatant solution was then dialyzed against cold distilled water and lyophilized. The yield was 12 to 14 mg.

The material thus obtained has a titer, of 8,000 to 16,000 HAI units/mg. against 4 HA units of influenza PR-8 indicator virus and 45,000 to 60,000 HAI units/mg. against 4 HA units of the Lee strain of influenza virus in the indicator state. The titer decreases upon standing in the dry state–both at refrigerated temperatures and at room temperature. The inhibitory titer of the discarded residues rarely exceeded 64 to 128 HAI units/mg.

Properties of the Inhibitor.—The inhibitor gave a single narrow zone when subjected to paper electrophoresis at pH values of 4.5, 6.2, and 8.6, and a

[1] We wish to thank Dr. Benjamin Sanders for his cooperation in providing us with this preparation.

single peak in the Antweiler electrophoresis at the same pH values. At pH 4.5 and 6.2 the protein moved toward the negative electrode while at pH 8.6, it moved to the positive electrode. Treatment of the inhibitor with active influenza virus resulted in a single band with a decreased electrophoretic mobility at pH 8.6. A single peak was obtained by ultracentrifugation over a period of 2 hours at 59,780 R.P.M. (Fig. 2). Occasionally, a higher molecular weight contaminant ($S^{H_2O}_{20} > 17.0$) appeared to the extent of about 4 per cent. The sedimentation constant, extrapolated to zero concentration, and corrected to 20°C. and with water as solvent, was determined to be 2.16S. The diffusion

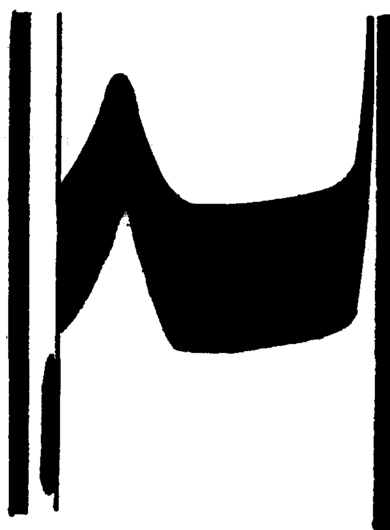

FIG. 2. Ultracentrifugal pattern of inhibitor. Speed 59,780 R.P.M., 85 minutes, 40° bar angle, 15 mg./ml.

constant, corrected similarly for temperature and solvent, was determined to be 0.575×10^{-6} cm.²/sec. By employing the equation $M = \frac{RTs}{D\,(1\text{-}V\rho)}$ and assuming the product of V (partial specific volume) and ρ (density) to be 0.71–a likely value within the range of most proteins–the molecular weight was calculated to be 31,400.

Chromatography of the products resulting from hydrolysis under various concentrations of acid (6 N, 1.2 N, 0.12 N, 0.012 N, 0.0012 N, distilled water) and for varying time periods (15, 60, and 150 minutes, and 20 hours) showed the presence of the following substances: lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, proline, valine, histidine, and leucine, isoleucine, methionine, threonine, galactosamine, galactose, sialic acid, and pos-

sibly glucosamine. Glucose, mannose, as well as other amino acids, were absent or were present in only trace amounts. The presence of lysine, arginine, aspartic acid, and glutamic acid were confirmed by high voltage electrophoresis. Histidine was confirmed by high voltage electrophoresis and by the sulfanilic acid color test as described by Mann (22).

Chemical analysis gave the data indicated in Table I. Application of the Liebermann-Burchard reaction showed the absence of cholesterol and the Dische carbazole test (23) was negative for uronic acids.

When aliquots of some of the hydrolysates mentioned above were titered for hemagglutination inhibitory activity, the results shown in Table II were obtained. It appeared that loss of inhibitory activity paralleled the appearance

TABLE I

Chemical Analysis

Constituent	Dry weight
	per cent
Nitrogen	10.1
Protein bound hexose (as galactose)	12.4
Hexosamine (as glucosamine)	12.1
Phosphorus	0.25
Fucose	1.12
Sialic acid (Ehrlich)	22.0
(Bial)	24.8
(thiobarbituric acid)	21.4

of free sialic acid during mild acid hydrolysis. Most of the sialic acid occupies a terminal position, since it is almost completely removed from the protein by acid hydrolysis with 0.012 N hydrochloric acid at 100°C. for 1 hour or 0.1 N sulfuric acid at 80°C. for 1 hour, without the release of any other substituent. The role of sialic acid in inhibitory activity was also established in another way. A sample of the inhibitor weighing 2.1 mg. was incubated with one-half ml. of 0.005 molar sodium periodate for 4 hours at pH 6 in the dark at room temperature. At the end of this period, 25 microliters of ethylene glycol was added to exhaust the remaining periodate. Aliquots were titered and shown to possess no inhibitory activity. To other aliquots of the periodate treated preparation were added equal volumes of 1.2 N HCl or 0.12 N HCl and these were hydrolyzed at 100°C. for 1 and 3 hours. Under these conditions, sialic acid can be demonstrated in 0.06 N HCl hydrolysates and galactose and hexosamine in the 0.6 N HCl hydrolysates of untreated inhibitor. However, the periodate-treated samples did not liberate any sialic acid while the other carbohydrates appeared in the usual amounts. The inhibitory activity of the protein

was totally destroyed when incubated with trypsin (1.2 mg./100 mg. protein; 1 hour), with active influenza virus (256 HA units/mg. protein; 3 hours), or with crude RDE from *Vibrio cholera* (3 hours). Lysozyme had no effect on activity. Dr. Georg F. Springer kindly tested our preparation for M and N blood group activity and found that it inhibits agglutinins anti-M and anti-N to a rather high degree. We have found no other blood group activity associated with this material.

TABLE II

Hemagglutination Inhibition Titers (PR-8 Indicator Virus) Following Hydrolysis under Stated Conditions

	15 min.	60 min.	150 min.
Distilled H_2O—37°C.	8000	—	—
Distilled H_2O—100°C.	4400	4400	—
0.0012 N HCl—100°C.	160	0*	0
0.012 N HCl—100°C.	0*	0*	0
0.12 N HCl—100°C.	0	0	0
1.2 N HCl—100°C.	0	0	0

Original titer was 8000 HAI units/mg.

* Only sialic acid identified chromatographically under these conditions of hydrolysis.

DISCUSSION

A procedure resulting in the isolation of a material from human erythrocytes possessing high inhibitory activity against influenza virus hemagglutination has been described. It is felt likely that this material is the receptor substance with which the influenza virus reacts although this has not been conclusively established. However, some evidence can be submitted in support of this hypothesis. The material isolated is the only potent inhibitor found in the various fractions obtained by our procedure, and when the method is carefully executed, the highest titer found in other fractions rarely exceeds 64 to 128 HAI units/mg. Secondly, pretreatment of intact red cells with active influenza virus followed by preparation of the inhibitor in the usual manner results in a material possessing no inhibitory titer against the same strain of virus.

The preparation described in this paper differs in several ways from others previously isolated from erythrocytes in that (*a*) it has a much higher inhibitory titer, (*b*) it has a higher sialic acid content than others analyzed for this constituent, (*c*) it is more homogeneous than other preparations for which electrophoretic or ultracentrifugal data are available. Other minor differences also exist in terms of amino acid composition, and molar ratios of hexose to hexosamine to sialic acid. The values here reported indicate molecular ratios of

sialic acid, hexose, hexosamine, and fucose of approximately 10:10:10:1. A minimum molecular weight based on fucose analysis would be in the vicinity of 14,500, a value essentially one-half of the value of 31,400, obtained from ultracentrifugal and diffusion studies.

The fact that the inhibitor described here possesses M and N blood group activity deserves further emphasis since recently Stalder and Springer (24) and Baranowski (25) have reported on purified blood group antigens M and N with properties very similar to materials which are described here. It has also been reported that M and N activity is destroyed by neuraminidase (26, 27). Should these materials be identical, it is possible that M and N blood group activity is associated with the site with which influenza viruses react.

Although many glycoproteins have been reported to contain sialic acid (28) not all of these are hemagglutination inhibitors even though their sialic acid residues are removed by neuraminidase. Thus orosomucoid is not at all inhibitory to viral hemagglutination but its sialic acid may be completely removed by viral neuraminidase. There must, therefore, be structural differences between inhibitors and non-inhibitors permitting both to act as substrates but only certain types (29) to combine firmly with influenza virus.

Studies on structural similarities and differences between glycoproteins which are inhibitory and non-inhibitory to viral hemagglutination are in progress employing the stromal inhibitor here described.

SUMMARY

A material, derived from human erythrocytes and believed to be identical with the receptor site for the myxoviruses, has been obtained in homogeneous form. The method of preparation involves pH adjustment of the stroma, hot phenolic extraction, chloroform-methanol treatment, and ultracentrifugation. The material so obtained possesses a high inhibitory titer to viral hemagglutination (45,000 to 60,000 inhibitory units/mg. against 4 hemagglutination units of Lee strain of influenza virus) and appears to be a glycoprotein containing 22 to 24 per cent sialic acid, 12 per cent hexose, 12 per cent hexosamine, 1 per cent fucose in addition to at least eleven amino acids. The sialic acid probably occupies a terminal position in an oligosaccharide chain extending from the protein peptide chain. The molecular weight is near 30,000—an unusually low value for substances possessing this biological activity. M and N blood group activity also seems to be associated with this protein.

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