# *THE RELATIONSHIP OF THE INFLUENZA VIRUS INHIBITORY ACTIVITY OF GLYCOPROTEINS TO THEIR MOLECULAR SIZE AND SIALIC ACID CONTENT*

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*Abstract.*—Twenty-four different glycoproteins were investigated for their ability to inhibit hemagglutination by the A/PR8 and the B/Md influenza virus strains. A relationship between activity, the molecular size, and sialic acid content was found. This relationship was readily shown for the A/PR8 virus if the properties of the glycoproteins were compared with one another on a per cent basis. A proportion of approximately 1:1:1 for activity (weight basis) to moles sialic acid content to molecular weight existed for each inhibitory glycoprotein with more than 3 per cent sialic acid, on comparison with any other active glycoprotein.

A 1:3 correspondence between viral subunit and sialic acid residues of the inhibitor ovine submaxillary mucin was found experimentally and confirmed by calculation on a molecular model. The most potent inhibitors were the antigens of the human blood-group MN system and the Tamm-Horsfall urinary glycoprotein.

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The first direct evidence for the existence of cell receptors for influenza viruses was the observation that they agglutinate erythrocytes.[1, 2] Studies on the nature of influenza virus receptor substances, however, were first confined to the cell-receptor analogues found in body secretions. The potency of these analogues, which have been shown to be glycoproteins, is measured by their ability to inhibit hemagglutination by indicator influenza viruses (cf. ref. 3).

More recently a close relation between the M and N antigens of the second human blood-group system and the influenza virus receptors of human erythrocytes has been demonstrated. The activities of both are destroyed concomitantly by influenza viruses.[4] Influenza virus receptor glycoproteins possessing blood-group M and N specificity have been isolated from human erythrocyte stroma.[5–8] Sialic acid in terminal linkage has been implicated as the virus receptor site[3] and as the structure involved predominantly in blood-group M and N activity.[4] Influenza virus inhibitory activity of different glycoproteins could not be correlated with the quantity of sialic acid present nor with their molecular weight,[9, 10] except that the proteolytic cleavage of some inhibitors led to a considerable reduction of their activity.[3, 11]

In the present report, inhibitory activities of the erythrocyte receptors towards two influenza virus strains were compared with each other and with those of inhibitors from other sources in order to define more fully the receptor which is truly complementary to these viruses. Striking relationships were found between antiviral activity, molecular size, and sialic acid content.

*Materials and Methods.*—Blood-group and serum glycoproteins were prepared in our laboratories;[11-14] colominic acid from *Escherichia coli* K-235 was the gift of Prof. W. F. Goebel,[15] blood-group $Le^a$ sialomucopolysaccharide ($Le^a$ substance) † was given by Prof. W. T. J. Morgan, F.R.S.;[16] haptoglobin type 1-1 (Co.) by Prof. G. E. Connell and Miss D. Parr;[17] fresh submaxillary gland glycoproteins from sheep (OSM) and cattle (BSM) were obtained from Dr. M. I. Horowitz,[18] and T & H urinary glycoprotein preparations (cf. ref. 19) from Dr. L. Pape and Mr. K. Latham. The substances were >98% pure by immunochemical and physical criteria, except for haptoglobin 1-1 (Co.) which possessed <5% albumin. All but 2 glycoproteins were homogeneous, the inhomogeneities consisted of oligomers of the predominant molecular species; the physical properties of these preparations are given in Table 1. The substances were of human origin unless otherwise indicated. Only the M and N blood-group antigens possessed blood-group M or N specificity.

Diluent and solvent throughout was 0.10 molar aqueous NaCl containing 0.05 molar phosphate buffer pH 7.3 (PBS). However, the urinary glycoprotein, because of poor solubility in electrolytes, was dissolved in water before dilution with PBS. The inhibition of virus hemagglutination was determined, and the results were interpreted as previously described,[20] except that a microprocedure employing microtitrators and plastic U trays (Cooke Engineering Co.) was used.[21] All volumes were 25 $\mu$l. Chicken egg allantoic fluids, infected with either the PR8 strain of Type A or with the Maryland (Md) strain of Type B influenza virus stored at 1–2°C (with thimerosal 1:10,000 as preservative) were employed, after heating for 30 min at 56°C immediately before titration. Twofold geometrical dilutions of putative inhibitors were incubated with four agglutinating doses of virus for 2 hr at 23–26°C, and a 1% suspension of human blood-group O erythrocytes was then added. After an hour of incubation agglutinations were read by two individuals, one of whom did a "blind" reading. Each test included two standard inhibitors, a MM substance[13] and a meconium glycoprotein,[11] a titration series of the diluted virus plus red cells only, and a red cell suspension in PBS.

The samples were divided into four groups, and all in one group were tested in parallel. In addition, each sample was tested twice with other samples in two other test series. All glycoproteins were tested 7 to 14 times with each of the two virus strains and the arithmetic averages of the results reported. Strict comparison is possible only if all inhibitors are tested in parallel, since the results of serological titrations notoriously vary between different laboratories[22] and experimenters. Therefore, activities are given as percentages of that of the reference compound, blood-group MM glycoprotein Ca 979, the most active preparation with A/PR8 virus. It completely inhibited 4 hemagglutinating doses of A/PR8 virus at a concentration as low as 0.003 $\mu$g/0.1 ml, based on dilution before the addition of virus and red cells. This glycoprotein was three times more active with the A/PR8 virus than with the B/Md virus.

*Results.*—Blood-group MM antigen Ca 979, the urinary glycoprotein, the $Le^a$ substance, and the meconium glycoprotein were among the six most active inhibitors towards both viruses (Table 1).

*Studies with the A/PR8 virus:* Only the MM antigen Ca 979 and the urinary glycoprotein had similarly high activities; of the eight other glycoproteins with molecular weights >200,000, six showed activities, expressed on a weight basis, of between 1.5 and 7.5 per cent that of Ca 979. The $Le^a$ substance and BSM had the highest and equal activities among these six glycoproteins, although the former had only about one tenth of the molecular weight and two thirds of the sialic acid content of the latter. Gamma-A globulin had only a trace of activity, and $\alpha_2$-macroglobulin was inactive. OSM, the inhibitor with the highest sialic acid content (weight basis), had comparatively low activity; its

TABLE 1. *Sialoglycoproteins: Their molecular weights, sialic acid content, and antiinfluenza virus activities.*

| Glycoprotein | Molecular weight × $10^4$ | Sialic Acid Content: Weight[a] (%) | Sialic Acid Content: Moles/mole[b] glycoprotein | Antiviral Activity (% of Reference Substance Ca 979): Strain A/PR8: Activity, based on Weight | Strain A/PR8: Activity, based on Moles | Strain B/Md: Activity, based on Weight | Strain B/Md: Activity, based on Moles |
|---|---|---|---|---|---|---|---|
| Blood-group MM Ca 979 | 1,200 | 11.5 | 4,466 | 100 | 100 | 100 | 100 |
| Urinary glycoprotein (11/67) | 700 | 7.0 | 1,586 | 60.0 | 35 | 400 | 234 |
| γ-M Globulin #2645 | 696[c] | 1.8 | 405 | 1.5 | 0.870 | 25 | 14.51 |
| Submax. mucin, bovine (J+,2;60–70)[d] | 225 | 26.0 | 1,667 | 7.5 | 1.404 | 12.5 | 2.343 |
| $\alpha_2$-Macroglobulin #1067 | 82 | 1.8 | 48 | NA[e] | ... | 5 | 0.342 |
| Submax. mucin, ovine #3[d] | 75 | 28.0 | 680 | 3.0 | 0.188 | 33.3 | 2.085 |
| Blood-group NN Ca 825 | 59.5 | 16.2 | 312 | 4.17 | 0.207 | 12.5 | 0.622 |
| Meconium-Vg Ca 851 | 52 | 9.3 | 157 | 3.75 | 0.163 | 100 | 4.340 |
| γ-A Globulin #662 | 25.4[c] | 1.3 | 11 | 0.12 | 0.003 | 0.80 | 0.017 |
| Sialomucopolysacch. Le[a] #350 | 23.7 | 18.0 | 138 | 7.5 | 0.148 | 200 | 3.950 |
| Coeruloplasmin #2870 | 16 | 2.4 | 12 | NA | ... | 0.80 | 0.011 |
| Haptoglobin 1–1 (Co.) | 8.5 | 5.2 | 14 | 0.12 | 0.001 | 25 | 0.177 |
| Hemopexin #267 | 8.0 | 5.0 | 13 | NA | ... | 10 | 0.067 |
| $\alpha_{1x}$-Glycoprotein #1066 | 6.0 | 7.0 | 14 | 0.5 | 0.003 | 6.25 | 0.031 |
| Tryptophan-poor glycoprotein #267 | *ca.* 6.0 | 3.5 | 7 | NA | ... | 0.40 | 0.002 |
| $\alpha_1$-Antitrypsin #166 | 5.4 | 3.6 | 6 | 0.24 | 0.001 | 0.53 | 0.002 |
| Fetuin, bovine I/III | 4.5 | 6.0 | 9 | 0.24 | 0.001 | 12.5 | 0.047 |
| $\alpha_1$-Acid glycoprotein #2507 | 4.4 | 12.1 | 17 | NA | ... | 0.80 | 0.003 |
| Zn-$\alpha_2$-Glycoprotein #1267 | 4.1 | 4.7 | 6 | NA | ... | 0.80 | 0.003 |
| $\beta_2$-Glycoprotein I #466 | 4.0 | 4.5 | 6 | NA | ... | 1.67 | 0.006 |
| Blood-group MM subunit[f] | 3.4 | 12 | 13 | 1 | 0.003 | 1 | 0.003 |

[a] Determined and calculated as *N*-acetyl-neuraminic acid except bovine submaxillary mucin as *N,O*-diacetyl derivative.

[b] Nearest integer.

[c] Mean molecular weights calculated from the weight distributions of different molecular species. γ-M Globulin, molecular weights × $10^4$: 58, 100, 200, 1,100, 2,000; percentages of each were: 2, 41, 10, 34, 13. γ-A globulin, molecular weights × $10^4$: 16, 32, 48, 64; percentages of each were: 59, 28, 8, 5.

[d] Mean molecular weights, see text. Chemical data not corrected for ash and moisture.

[e] NA: <0.12 per cent inhibition for A/PR8 virus and < 0.4 per cent for B/Md virus, weight basis.

[f] Average values of two preparations tested.

activity was only twice that of $\gamma$-M globulin, the glycoprotein with one of the smallest amounts of sialic acid (weight basis) of all substances tested.

Activity generally depended on the molecular size, since all active glycoproteins with a molecular weight of <200,000 were of low potency, and the number of inactive compounds increased with decreasing molecular size. Expression of the activities of the inhibitors on a molar rather than a weight basis (Table 1, col. 6) underscored the high potency of Ca 979 and the urinary glyco-protein.

Quantitative comparison of the molecular weights and the number of sialic acid residues of the various glycoproteins was facilitated by expressing these properties for a given glycoprotein as per cent, on a molar basis, of the reference substance Ca 979. Such a comparison is shown in the second and third columns of Table 2. There was a remarkable parallelism between these two properties for all glycoproteins containing >3 percent sialic acid; if the percentage of molecular weight of a glycoprotein was divided by the per cent value of its sialic acid residues, a ratio approximating 1 was obtained for all glycoproteins, with maximal deviations up to a factor of 3. There was also a relation of these two parameters to activity which became most readily evident if a glycoprotein's activity was expressed on the basis of weight per cent of Ca 979. Table 2 depicts the relations of these properties to one another in the form of ratios for all the active substances with >3 per cent sialic acid. The calculated ratio of molecular weight to activity was very close to 1 for 10 of the 12 glycoproteins listed, that is, the decrease in activity was found to be proportional to the decrease in molecular size. The ratios of mole per cent sialic acid to weight per cent activity of the various glycoproteins also approached 1. They exceeded 3 only for the submaxillary mucins, for which no measured molecular weight was available and for which the figures given represent the mean of all possible molecular weights in the literature. If the lowest molecular weight possible for both substances, 500,000, was chosen instead, then the ratios approached 1 much more closely (Table 2). The mean ratio of mole per cent sialic acid over weight per cent activity for all substances compared in Table 2 was 1.6, as was also the ratio of per cent molecular weight over weight per cent activity for all substances. When the lower molecular weights for the submaxillary mucins were taken, the respective average ratios for all glycoproteins were 1.2 and 1.4.

If the activities of the inhibitors were expressed on a molar basis, as were the other properties, then a decrease of molecular size and sialic acid content in the order of one decadic logarithm was accompanied by a decline in the potency of the active substances by two decadic logarithms, that is, the activity decline proceeded approximately with the square of the decrease of molecular weight and sialic acid content. It follows that one can predict the degree of activity of a glycoprotein whose molecular weight and sialic acid content are known, and that reciprocal approximations can be made from the extent of the hemagglutination inhibition, provided that certain structural conditions are met by the glycoproteins (see *Discussion*). Thus, in spite of the appropriate size and sialic acid content, five substances listed in Table 1 containing >3 per cent sialic acid had no significant activity with A/PR8 virus. Remarkably, these five glycoproteins were active with the B/Md influenza virus.

TABLE 2. *Molecular weight and sialic acid content in relation to inhibitory activity of A/PR8 influenza virus.*

| Glycoprotein | Molecular wt.[a] | Sialic acid[b] | Activity[c] | Molecular wt.[a] / Activity[c] | Sialic acid[b] / Activity[c] |
|---|---|---|---|---|---|
| Blood-group MM Ca 979 | 100 | 100 | 100 | 1.0 | 1.0 |
| Urinary glycoprotein (11/67) | 58.33 | 35.5 | 60.0 | 0.97 | 0.59 |
| Submax. mucin, bovine (J+,2;60–70) | 18.75 (4.17)[d] | 37.3 (8.29) | 7.5 | 2.50 (0.56) | 4.97 (1.10) |
| Submax. mucin, ovine #3 | 6.25 (4.17) | 15.2 (10.1) | 3.0 | 2.08 (1.39) | 5.07 (3.37) |
| Blood-group NN Ca 825 | 4.96 | 7.0 | 4.17 | 1.19 | 1.68 |
| Meconium-Vg Ca 851 | 4.33 | 3.52 | 3.75 | 1.15 | 0.94 |
| Sialomucopolysacch. Le[a] #350 | 1.98 | 3.09 | 7.50 | 0.26 | 0.41 |
| Haptoglobin 1–1 (Co.) | 0.71 | 0.31 | 0.12 | 5.92 | 2.58 |
| $\alpha_{1x}$-Glycoprotein #1066 | 0.50 | 0.31 | 0.50 | 1.0 | 0.62 |
| $\alpha_1$-Antitrypsin #166 | 0.45 | 0.13 | 0.24 | 1.89 | 0.54 |
| Fetuin, bovine I/III | 0.38 | 0.20 | 0.24 | 1.58 | 0.83 |
| Blood-group MM subunit | 0.28 | 0.29 | 1.0 | 0.28 | 0.29 |

[a] Per cent of blood-group MM glycoprotein Ca 979.
[b] Per cent of Ca 979 on molar basis.
[c] Per cent of Ca 979 on weight basis.
[d] Figures in parentheses based on lowest possible molecular weight for these mucins; see text.

*Studies with the B/Md virus:* All glycoproteins were more active with the B/Md virus, in relation to Ca 979, than with the A/PR8 virus. They had higher activity also on an absolute basis except for BSM and $\alpha_1$-antitrypsin #166 both of which were slightly more active with the A/PR8 virus. Also, with the B/Md virus the urinary glycoprotein and the $Le^a$ substance were more active on a weight basis than was the reference compound. Meconium-Vg antigen was as active as Ca 979, although it had only 4.3 per cent of the molecular weight of the latter.

Table 1 shows that all nine glycoproteins having a molecular weight of >60,000 and a sialic acid content of >3.5 per cent were highly active. Except for fetuin, which was much more potent, the activities of substances with molecular weights <60,000 were *ca.* 1 per cent of that of the reference glycoproteins. A ratio of 1:1:1 for mole per cent of sialic acid, per cent molecular weight, and weight per cent activity, was approached by the following inhibitors of B/Md virus: BSM, NN glycoprotein, tryptophan-poor glycoprotein, $\alpha_1$-acid glycoprotein, and the blood-group MM subunit. For $\gamma$-M globulin, $\alpha_2$-macroglobulin, $\gamma$-A globulin, coeruloplasmin, $\alpha_1$-antitrypsin and Zn-$\alpha_2$-glycoprotein a ratio of molecular weight to activity of 1:1 was calculated, and that of sialic acid to activity was Ca 0.3. The activity of the remaining nine glycoproteins in Table 1 was much higher than would be the case if a 1:1 ratio of activity to sialic acid or activity to molecular size existed. Therefore, on the basis of mole per cent sialic acid and per cent molecular weight these nine compounds had a higher activity than reference substance Ca 979.

Toward B/Md virus the ratios of activity to moles of sialic acid and molecular weight, when expressed as per cent of Ca 979, were largest for haptoglobin and $Le^a$ substance; with sialic acid as the denominator these ratios were 81 and 65 respectively and with the molecular weight as the denominator they were 35 and 101. These ratios exceeded those of the most active compound, the urinary glycoprotein, manyfold (8 and 10). Such calculations on the other glycoproteins showed that with the B/Md virus the most active compounds relative to their size and sialic acid content were those with molecular weights between 45,000 and 500,000.

Transferrin (molecular weight 90,000, sialic acid 1.5%) and $\alpha_2$-HS-glycoprotein (molecular weight 49,000, sialic acid 4.1%) did not inhibit either virus. $Beta_2$-glycoprotein III (molecular weight 35,000, sialic acid 5.5%) showed no activity for one sample (#167) and traces for two others (#1, #3); these low activities are likely due to contaminants. The homopolymer colominic acid (molecular weight 3,000–4,000) which has no terminal repeating units[15, 23] was inactive, even after opening of the internal ester bonds by alkali.

*Discussion.*—The virus-inhibiting activity of sialoglycoproteins as measured with two influenza virus strains was shown to depend on both molecular size and sialic acid content of the glycoproteins. The inhibiting activity of the glycoproteins toward the PR8 strain of Type A influenza virus increased in proportion to their sialic acid content and molecular size. On the other hand, with the Maryland strain of the Type B influenza virus the relatively highest inhibition was given by glycoproteins with a molecular size of 45,000–500,000. This,

together with the generally higher potency of all inhibitors toward the B/Md virus, indicates that on the surface of the B/Md virus either fewer combining sites need to be neutralized to prevent red cell agglutination, or smaller, terminal, repeating three-dimensional units on the inhibitory glycoproteins that carry the determinant structures may fulfill the requirements of complementary fit between B/Md virus and inhibitors. Such smaller structures might be expected to occur more frequently, and this in turn would explain why so many more glycoproteins are active with the B/Md virus than with the A/PR8 virus. Lack of properly spaced repeating units or their inaccessibility would account for the inactivity of some sialoglycoproteins.

The inhibitors have indeed been shown to possess repeating subunits terminating in sialic acid.[3, 11] Thus, over the surface of the OSM molecule (molecular wt. of $1 \times 10^6$) 800 sialyl-$\alpha$-*N*-acetyl-galactosaminoyl residues are distributed.[3] The activity of an inhibitor is probably dependent on the number of sialic acid subunits which can simultaneously contact complementary points on the virus surface. It is pertinent therefore that the envelope of myxoviruses is composed of identical repeating subunits; these, from center to center, are approximately 70 Å apart.[24] We have calculated that on the OSM molecule sialyl residues can occur at points *ca.* 70 Å apart. The 800 sialyl-$\alpha$-*N*-acetyl-galactosaminoyl units are linked O-glycosidically to seryl and threonyl residues, which amount to about 1,300 per molecule (cf. ref. 25). Thus, a sialyl-$\alpha$-*N*-acetyl-galactosaminoyl residue is bound to approximately three of every five hydroxy amino acid residues. If an even distribution of the amino acids of the OSM molecule is assumed (for which no structural proof exists), then the distance between two hydroxy amino acids on a fully stretched peptide chain (OSM seems to be a random coil[3]) amounts to 14.54 Å, and the distance between 5 such "identity periods" corresponds to that between 2 repeating subunits on the myxovirus surface. However, since two sialic acids are in excess for each sialic acid required to give a 1:1 correspondence between viral subunit and sialic acids some cluster formation of the hydroxy amino acids and thier substituents would also be permissible. This calculation of a 3:1 excess of sialic acid over that needed for combination with the virus agrees with the experimental observation of a three-fold excess of sialic acid over the observed activity of the OSM of minimal molecular size (Table 2). A five-fold surplus of sialic acid exists when the mean molecular size of this glycoprotein is taken instead.

Comparison of the peptide backbones of the various glycoproteins indicated no direct relation to activity, nor was there an obvious difference in the distribution pattern of amino acids between active and inactive compounds.

A correlation similar to that found for glycoproteins inhibiting the A/PR8 virus exists for the human erythrocyte blood-group M and N antigens, of which we have obtained fractions ranging in molecular size from $3.0 \times 10^4$ to $1.2 \times 10^7$, when their blood-group activity is determined with human sera.[11–13] It is tempting to speculate that, because of their high activities, the MN blood-group antigens and the T & H urinary glycoprotein are *the* myxovirus receptors in man, especially in view of their reported occurrence and origin in epithelial

structures.[19, 26, 27] However, extrapolation to other strains of influenza viruses must await additional experiments.

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† $Le^a$ substance, $Le^a$ sialomucopolysaccharide; OSM, ovine submaxillary mucin; BSM, bovine submaxillary mucin; PBS, phosphate-buffered saline.

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