Inhibition of Influenza Virus Hemagglutination by Purified Plasma Mucoproteins.* (18600)

C. S. Stulberg, Ruth Schapira, Abner R. Robinson, Daniel H. Basinski, and Hugo A. Freund.

From the Research Laboratory, Children's Fund of Michigan, Detroit, Mich.

The adsorption of influenza virus to red cells, followed by red cell receptor destruction and subsequent elution of virus, have been suggestive of an enzymatic reaction(1). The probable substrates for such enzymatic activity have been shown to be mucopolysaccharides or mucoproteins present in red cell receptors(2,3). These mucoid substances, also found in plasma(4), in blood group substances(2), and in other biological materials (2,5), appear to act in competition with red cell receptors for virus in the virus hemagglutination reaction(6). The chemical nature of these mucoids remains obscure in spite of several investigations concerned with their isolation and identification(7-9). Recently, however, Winzler *et al.*(10) have isolated and tentatively characterized plasma mucoproteins with low isoelectric points, both chemically and electrophoretically.

This report describes the inhibition of influenza virus hemagglutination by an electrophoretically homogeneous mucoprotein.

Isolation of mucoprotein. The method of Winzler *et al.*(10), with slight modifications, was used for the preparation of mucoprotein from out-dated, blood-bank plasma. The plasma proteins were first removed by precipitation with 1.8 M perchloric acid. The filtrate then was dialyzed and adjusted to pH 4. Ammonium sulfate was added to the filtrate and the mucoprotein began to precipitate when the concentration was approximately 2/3 that of saturation. Infusorial earth (Johns-Manville hyflo super cel) was added to make a concentration of approximately 40 g per liter of mixture. After stirring for a few minutes, the mucoprotein was completely adsorbed on the infusorial earth. After filtration the mucoprotein was eluted with 5 100 ml portions of distilled water and the solution was saturated with ammonium sulfate, and refiltered. The re-precipitated mucoprotein was dissolved in a small amount of water and dialyzed. The salt-free mucoprotein was dried by lyophilization. The average yield was slightly more than 200 mg per liter of plasma. Two preparations, designated as A and B, were obtained in this manner; another preparation, C, was obtained by omitting the adsorption and drying the final product by evaporation at room temperature. Data from analyses of mucoprotein preparation A are given in Table I, and are consistent with unpublished analyses of several preparations. The total nitrogen content is in agreement with values reported by Weimer, *et al.* (11), but the tyrosine and carbohydrate values are somewhat lower than reported by those authors.

Electrophoretic characterization of muco-

* This report is a part of one phase of the arthritic research program initiated and directed by Hugo A. Freund, and supported, in part, by funds contributed by the National Foundation of Rochester, Michigan and the Michigan Chapter, Arthritis and Rheumatism Foundation. Blood samples for this work were obtained from the Children's Hospital of Michigan.

1. Hirst, G. K., *J. Exp. Med.*, 1942, v76, 195.

2. Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Aust. J. Exp. Biol. and Med. Sci.*, 1948, v26, 403.

3. Hirst, G. K., *J. Exp. Med.*, 1948, v87, 301.

4. Hirst, G. K., *J. Exp. Med.*, 1949, v89, 223.

5. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, v179, 1275.

6. Hirst, G. K., *J. Exp. Med.*, 1948, v87, 315.

7. McCrea, J. F., *Aust. J. Exp. Biol. and Med. Sci.*, 1948, v26, 355.

8. De Burgh, P. M., Pen-Chung, Y., Howe, C., and Bovarnick, M., *J. Exp. Med.*, 1948, v87, 1.

9. Howe, C., *J. Immunol.*, 1951, v66, 9.

10. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, v27, 609.

11. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *Fed. Proc.*, 1950, v9, 244.

TABLE I. Properties of Purified Plasma Mucoprotein (Preparation A).

Mobility, cm^2/sec./volt*	4.3×10^{-5}
Kjeldahl N %	10.9
Tyrosine %	1.7
Carbohydrate %	9.6-12.2
Inhibition titer†	4096-8192
μg mucoprotein at inhibition endpoint	0.3-0.15

* Acetate buffer pH 4.5, ionic strength 0.1.
† Reciprocal of dilution starting with 1.25 mg mucoprotein.

TABLE II. Inhibitory Effects of Purified Mucoprotein Preparations on Hemagglutination by Heated and Unheated Influenza Virus.

Mucoprotein (5 mg/ml)	Inhibition titer	
	Unheated virus	Heated virus
Preparation A	1024	4096- 8192
" B	1024-4096	4096- 8192
" C	variable	4096-16384

protein. The 3 preparations of isolated mucoprotein were studied electrophoretically at pH 4.5 as described by Mehl *et al.*(12), using acetate buffer, pH 4.5, ionic strength 0.1. The electrophoretic determinations were made with the Longsworth modification of the Tiselius apparatus. Fig. 1 shows the single component which appeared in each preparation and migrated toward the anode with a mobility of 4.3 x 10^{-5} cm^2/sec/volt.

Effects of mucoproteins on virus hemagglutination. The PR8 strain of influenza A virus was used in the hemagglutination tests. Pooled allantoic fluids, harvested 48 hours after inoculation of 10-day-old chick embryos with a 10^{-3} dilution of seed virus, were frozen or were refrigerated at 4°C. Aliquots of the same pools were heated at 56°C for 30 minutes. In a modified Salk pattern test, agglutination occurred consistently at a dilution of 1:5120 with the unheated virus, and of 1:2560 with the heated virus. For the virus hemagglutination-inhibition tests, the lyophilized mucoprotein samples were carefully weighed to an accuracy of 0.1 mg and diluted with 0.85% NaCl solution until they contained the desired concentration of mucoprotein. This mucoprotein solution then was diluted in serial two-fold dilutions in 0.25 ml volumes of physiological saline, and to each was added an equal volume of diluent containing 5 hemagglutination units of heated or unheated virus. After the mixture was incubated at room temperature for one hour, 0.5 ml of a 0.5% suspension of chicken red cells was added. The patterns of the sedimented cells were observed after incubation at 4°C, when agglutination of the cells was complete. The inhibition titer was arbitrarily read as the reciprocal of the highest dilution of mucoprotein which completely inhibited hemagglutination. The results of the inhibition tests are shown in Table II. It appears that all 3 mucoprotein preparations possess the capacity to inhibit hemagglutination by heat-inactivated virus. The inhibition titers of preparations A and B were approximately the same, which might be considered further evidence of the homogeneity of the purified product. With heat-inactivated virus the hemagglutination inhibition titers of preparations A and B ranged from 4096 to 8192, the minimal inhibition concentrations being approximately 0.15 to 0.3 μg of mucoprotein. Preparation C exhibited somewhat more variable inhibition titers, 4096 to 16,384. The mucoprotein preparations inhibited unheated, or active, virus hemagglutination at a lower and often variable dilution. The results with unheated virus paralleled the observations of previous investigators, who demonstrated that heat-inactivated virus does not destroy virus inhibitor, whereas unheated virus affects inhibitors to an extent which depends upon the experimental conditions employed.

Mucoproteins and hemagglutination-inhibition properties of heated serum. Both McCrea(7) and Hirst(4) have demonstrated the ability of virus hemagglutination inhibitor in plasma to withstand a temperature of 100°C. Both studied heat coagulation methods in attempts to identify this inhibitor as seromucoid. It was pertinent, therefore, to study the electrophoretic characterization at pH 4.5 and the agglutination-inhibition properties of heated human sera. Serum samples diluted approximately 1 to 3 with acetate buffer, pH 4.5, were heated at 100°C for 3 minutes. The

12. Mehl, J. W., Humphrey, J., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 106.

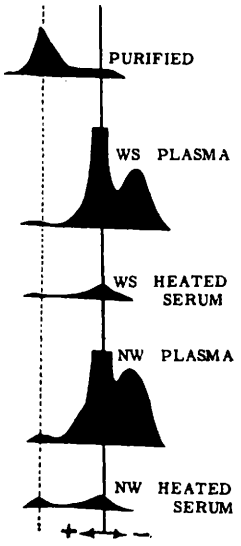

FIG. 1.

Electrophoresis patterns of preparations in acetate buffer, pH 4.5, ionic strength 0.1, showing the mucoprotein component (broken line) in purified solution, in plasma, and in heated serum.

uncoagulated supernate was prepared for electrophoretic examination according to the method of Mehl *et al.*(13). For the hemagglutination-inhibition tests, aliquots of the dialyzed samples used for electrophoresis were brought to pH 6.5-6.9 with NaOH and corresponding serial two-fold dilutions prepared with 0.85% saline.

Electrophoretic analyses of plasma and heated serum from 2 individuals representative of those with relatively low (WS) and high (NW) mucoprotein concentrations† are shown in Fig. 1. In the heat-treated specimens, a component appeared which had a mobility of 4.1-4.3 x 10^{-5} cm^2/sec/volt and was therefore considered to be a mucoprotein. These mobilities were in close agreement with that of the single component observed in the purified preparations and with the mobilities of similar components in unheated plasma from the same individuals. The relative mucoprotein concentrations of the plasma and heat-treated sera of WS and NW, together with their corresponding hemagglutination-inhibition titers, are shown in Table III. When more mucoprotein was present, higher inhibition titers were obtained, which indicates a relationship of the inhibitor to mucoprotein; however, these inhibition titers with unpurified substances may only indicate relative amounts of mucoprotein, for the differences in titers are not proportional to the differences in mucoprotein concentrations. From the electrophoretic patterns of the heated sera, it was evident that in addition to the mucoprotein, another component was present which had little mobility at pH 4.5, and this component may have affected the activity of the mucoprotein as an inhibitor.

Summary. Mucoprotein was isolated from human plasma by the perchloric acid filtrate method. This isolated mucoprotein was found to be electrophoretically homogeneous, and consistently inhibited hemagglutination by influenza virus in dilutions which contained 0.15 to 0.3 μg of mucoprotein. Hemagglutination inhibition with heated serum also indicated that the inhibitory substance was a mucoprotein.

TABLE III. Mucoprotein Concentrations of Human Plasma and Relative Mucoprotein Concentrations and Inhibition Titers of Heat-treated Human Sera.

Blood source	Plasma mucoprotein, mg/100 ml	Heated serum mucoprotein, mg/100 ml	Inhibition titer with heated virus
WS	77	84	16-32
NW	205	155	256-1024

Received February 26, 1951. P.S.E.B.M., 1951, v76

13. Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 110.

† In a study of plasma mucoproteins in rheumatoid arthritis, in this laboratory(14) the plasma of arthritic patients has been found to contain much higher concentrations of mucoprotein than that of normal individuals. In the present investigation, WS was normal while NW had rheumatoid arthritis.

14. Unpublished data.