

A MUCOPROTEIN DERIVED FROM HUMAN URINE WHICH
REACTS WITH INFLUENZA, MUMPS, AND
NEWCASTLE DISEASE VIRUSES

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Shortly after it was found that influenza viruses are capable of agglutinating chicken erythrocytes (1), it was shown that serum from normal ferrets inhibits such hemagglutination (2). Similar inhibition was obtained with serum from other animal species as well as with a variety of tissue suspensions (3-11). In addition, it was found (15) that after adsorption of influenza virus on erythrocytes spontaneous elution of the agent takes place and is accompanied by loss of the capacity of the erythrocytes to adsorb more virus. Subsequently, it was demonstrated that erythrocytes which had been treated with influenza virus have an electrophoretic mobility lower than that of untreated red blood cells (12-14). However, the properties of the eluted virus appeared to be unchanged (15). It was also shown that influenza viruses are capable of inactivating the inhibitory components derived from tissues (16, 17). After heating the virus at 56°C., the agent does not elute from erythrocytes and fails to inactivate inhibitory components (18-20). Moreover, virus so heated shows an increased susceptibility to inhibitors of hemagglutination (21).

The present view is that influenza viruses as well as mumps and Newcastle disease viruses are capable of altering enzymatically the structure of the surface of red blood cells and possess similar activity against inhibitors of hemagglutination. As a result of such viral action, the capacity of red blood cells or of inhibitory components to combine with these agents is lost. This concept was proposed originally by Hirst (2).

Several attempts to isolate, purify, and identify inhibitory components have yielded substances of high activity, but each has contained non-inhibitory contaminants (22, 23). In these investigations egg white was used as the starting material. In other less extensive studies various materials have been used including serum or plasma (4, 24, 25), erythrocytes (9, 26), and cow's milk (27).

The present study was undertaken because of interest in the enzymatic activity associated with influenza, mumps, and Newcastle disease viruses, and because the interaction between these agents and erythrocytes or inhibitory components may be analogous to certain phases of the interaction between viruses and susceptible host cells. Interest in the enzymatic activity of these

viruses derives from present ignorance of the mechanisms available to viruses during interaction with their environment, particularly during the process of multiplication. When the effects of viruses on the metabolic processes of host cells are considered, two hypothetical mechanisms appear to merit attention: the first involves effects mediated through viral enzymes; the second involves effects determined by genetic units of viruses but mediated through enzymes of the host cells. The analogy between the interactions of viruses with erythrocytes or hemagglutination inhibitors and host cells stems from the following observations: Influenza viruses adsorb on and elute from surviving or dead host cells in a manner similar to their interaction with red blood cells (28). Extracts of *Vibrio cholerae* abolish the capacity of erythrocytes, inhibitory components, and host cells to combine with viruses (17, 29, 30). Treatment of chick embryos or mice with such extracts renders these hosts temporarily less susceptible to influenza virus infection (31, 32).

In a previous communication (33) the inhibitory properties of normal human urine with respect to certain viruses were described and isolation of a mucoprotein responsible for the inhibitory capacity was reported. The presence in human urine of a highly active inhibitor of viral hemagglutination makes available an abundant source of this substance and one essentially free of proteins. Further work was carried out in the hope that the urinary mucoprotein might yield a single substance which would serve as a substrate for influenza viruses.

In this communication procedures for isolation and purification of urinary mucoproteins are described. It is shown that on purification a highly active compound is obtained which appears on the basis of physicochemical studies (34, 35) to be a single homogeneous substance. The substance appears to be mucoprotein in nature. A number of the biological, chemical, and physicochemical properties of this mucoprotein are presented. Certain features of the interaction between the substance and influenza, mumps, or Newcastle disease virus are demonstrated. The quantitative relationships and the kinetics of the interaction are delineated, and various biological and physicochemical properties of the mucoprotein altered by viral action are described.

Materials and Methods

Urinary Mucoproteins.—The term *urinary mucoproteins* applies to the material isolated from human urine by precipitation with sodium chloride under the conditions described below. The term *inhibitory mucoprotein* is used to designate a homogeneous component of urinary mucoproteins which is soluble in phosphate buffer. In most experiments solutions of mucoprotein in 0.025 M phosphate buffer at pH 6.8 were used. Solutions were prepared by dissolving frozen and dried mucoproteins. Because the substance dissolved slowly, it was usually necessary to hold the material for 16 hours at 4°C. and 8 hours at 25°C. The solutions were then centrifuged at 3,800 g for 30 minutes, the supernatants collected, and the concentration of mucoprotein was determined spectrophotometrically.

Viruses.—Lee, MB (influenza B virus strain isolated in 1950; third egg passage), PR8, FM1 (11th egg passage), NA and RA (influenza A virus strains isolated in 1951; NA: 2nd

and 7th egg passages; RA: 1st and 5th egg passages), swine, mumps, and Newcastle disease viruses were used. Each virus was inoculated into the allantoic sac of 10 day chick embryos and allantoic fluid was harvested 48 hours later. For most experiments allantoic fluid was dialyzed against 80 volumes of 0.025 M phosphate buffer, and then centrifuged at 3,800 g for 10 minutes. The supernates were stored at 4°C. In some experiments infected allantoic fluid was not dialyzed but was centrifuged at 3,800 g for 10 minutes, and then stored at -65°C.

V. cholerae Extract.—The Inaba strain of *V. cholerae* was grown at 37°C. for 16 hours in neopeptone broth. The bacteria were then sedimented in the centrifuge and the supernate was filtered through a Coors No. 2 candle. The filtrate was tested for sterility and for receptor-destroying enzyme (RDE) activity by the method of Stone (36). The filtrate was stored at 4°C. between experiments.

Red Blood Cells.—Blood from individual chickens was collected into ACD (37) and stored at 4°C. RBC were washed 3 times with 0.85 per cent NaCl buffered at pH 7.2 with 0.01 M phosphate. In preparing a 1 per cent suspension, a photometric procedure was used to measure RBC concentration.

Hemagglutination Tests.—Serial twofold dilutions of infected allantoic fluid were made in 0.4 ml. volumes of buffered saline and 0.4 ml. of RBC suspension was added to each tube (final concentration = 0.5 per cent). Tests were read after 1 hour except when Newcastle disease virus was used in which case a 20 minute period was employed because of rapid disagglutination of RBC. The last tube showing strong agglutination (2 to 3+) was considered as the end-point.

Hemagglutination-Inhibition Tests.—Serial twofold dilutions of inhibitory mucoprotein or urine were made in 0.2 ml. volumes of buffered saline. The dilution series was started with solutions containing not more than 100 µg. of mucoprotein per ml. With such concentrations good reproducibility of inhibition end-points was obtained. With higher concentrations the viscosity effect impaired the accuracy of twofold dilutions. Infected allantoic fluid, diluted with buffered saline so as to yield a concentration equivalent to 4 or more hemagglutinating units of virus in the final mixture, was added in 0.2 ml. amount to each tube. In most cases the virus was heated at 56°C. for 30 minutes; in certain instances, as with swine, mumps, and NDV, other temperatures were used, and in some cases untreated virus was employed. The mixtures were kept at room temperature for 1 hour, then 0.4 ml. of 1 per cent RBC suspension was added and the tests were read after another hour. The dilution showing complete inhibition of hemagglutination was taken as the end-point. The inhibition titers were expressed in terms of the amount of mucoprotein required for complete inhibition in micrograms per hemagglutinating unit of virus. In most instances inhibitory activity against Lee virus, heated at 56°C. for 30 minutes, *i.e.*, H. Lee, was determined.

Enzymatic Inactivation Experiments.—Equal volumes of mucoprotein solution and infected allantoic fluid, at desired concentration, were mixed and held at 37°C. or at other temperatures as specified below for various periods of time. The mixtures then were heated at 65°C. for 30 minutes to stop the reaction and to eliminate the virus. Hemagglutination-inhibition titrations were then carried out with Lee virus which had been heated at 56°C. for 30 minutes. When prolonged periods of incubation were employed, 1 to 2 drops of chloroform per ml. were added to the reaction mixture.

Infectivity Titrations.—Serial tenfold dilutions of virus were made in buffered saline and dilutions inoculated allantoically into groups of 10 day old chick embryos, 0.1 ml. per embryo. Eggs were incubated at 35°C. for 48 hours. After chilling overnight at 4°C., the allantoic fluids were harvested and tested for hemagglutinating ability. E.I.50 (50 per cent embryo infectious end-point) was calculated in the usual manner.

Viscosity Determinations.—The viscosity of mucoprotein solutions was measured in Ostwald

viscosimeters at 37°C. or at 20°C. The viscosimeters were calibrated with distilled water as well as with 0.025 M phosphate buffer. In the calculation of the relative viscosity of a solution the calibration was chosen which corresponded to the solvent used.

EXPERIMENTAL

Precipitation of Mucoproteins from Urine with Sodium Chloride.—The striking effects of NaCl concentration on the activity and solubility of the inhibitor in urine were reported previously (33). Urinary mucoproteins were shown to be soluble in water but insoluble in solutions of NaCl of moderate strength. This property facilitated the development of a procedure for isolation and purification of the substances.

A more detailed study of conditions under which precipitation of urinary mucoproteins occurs was undertaken. It was found that at NaCl concentrations as low as 0.07 M (*i.e.*, one-half the physiological strength) reduced solubility of the mucoproteins was demonstrable. However, for rapid precipitation and maximal yield it was found to be desirable to use NaCl in a concentration of 0.58 M. Temperature and pH did not appear to be of critical importance within wide limits.

The procedure used for the isolation of urinary mucoproteins was as follows: Urine was collected from normal men into sterile bottles. Specimens were pooled and approximately 30 ml. of chloroform was added per liter. Pools were stored at 4°C. and the isolation of the mucoprotein was carried out at the same temperature. The pH of pools of urine was usually in the range of 6.0–7.0. Pooled urine was diluted with an equal volume of distilled water to dissolve precipitates consisting mainly of urates and phosphates. To the diluted urine, sodium chloride was added to a concentration of 0.58 M. After relatively complete sedimentation of the white flocculent precipitate, the supernatant was siphoned off. A 0.58 M solution of NaCl was added to the sediment for preliminary washing and the precipitate was again allowed to settle. After removal of the second supernatant, the precipitate was collected by centrifugation at 1900 R.P.M. for 30 minutes. Sediments were washed twice with 0.58 M NaCl and suspended in distilled water. Sodium chloride was removed by dialysis against approximately 120 volumes of distilled water over a period of 4 to 5 days. The dialyzed solution was centrifuged at 7,000 R.P.M. for 30 minutes. The small amount of sediment obtained was washed once with distilled water and the washing was added to the first supernatant; the sediment was discarded. The final supernatant was concentrated by preevaporation and then frozen and dried.

Two large pools of urine were used to prepare the mucoproteins employed in most of the experiments reported below. Table I summarizes certain features of these preparations. The specific gravity and pH of the two pools were closely similar. Comparison of the yields of urinary mucoproteins and of the inhibitory activities indicates that the precipitation procedure gave reproducible results.

Activity titrations showed that precipitation with sodium chloride under the conditions described above removed more than 99 per cent of the inhibitor from urine. The sediment discarded after centrifugation of the dialyzed material contained less than 0.1 per cent of the inhibitor. When quantitative procedures

were employed throughout, it was possible to obtain close to 100 per cent recovery of the inhibitory mucoprotein. Serial titrations yielded no indication that inactivation occurred during the process of isolation.

Separation of the Inhibitory Mucoprotein with Phosphate Buffer.—As was stated above, urinary mucoproteins are soluble in water but not in NaCl solutions of moderate concentration. For physicochemical studies as well as for interaction experiments with viruses it was necessary to find buffer solutions in which the inhibitory component was soluble.

Experiments showed that in 0.025 M phosphate buffer (ionic strength = 0.05) at pH 6.8, with mucoprotein concentrations from 0.5 to 6.0 mg. per ml., 20 to 80 per cent of the material was soluble depending on temperature of solution and age of the dried preparation. Dried

TABLE I
Starting Material and Yield for Two Preparations of Urinary Mucoproteins

	Preparation A	Preparation B
Urine, volume.....	54 liters	135 liters
specific gravity.....	1.015	1.015
pH.....	6.13	6.38
Mucoprotein, yield, total.....	1.4 gm.	3.2 gm.
yield/unit volume.....	0.025 gm./liter	0.024 gm./liter
activity*.....	0.008 μ g.	0.012 μ g.

* Expressed as amount of mucoprotein required for complete inhibition in micrograms per hemagglutinating unit of heated Lee virus (56°C. for 30 minutes).

mucoproteins required 18 to 24 hours to dissolve in this buffer at 4°C. and the process was accelerated by intermittent shaking. The insoluble portion was separated in the centrifuge at 3,800 g for 30 minutes. The concentration of mucoprotein remaining in the supernatant was determined spectrophotometrically. Comparison of the results of spectrophotometric determinations with the weight of dried aliquots indicated that the former were reliable within approximately ± 8 per cent.

Hemagglutination-inhibition titrations showed uniformly that the inhibitory activity was associated with the fraction soluble in phosphate buffer. Because of the large experimental error of the usual twofold dilution technique, a study was made to ascertain whether or not an amount of inhibitor smaller than 50 per cent of the total was insoluble in phosphate buffer. A photometric apparatus developed in this laboratory was employed in these experiments. The experimental error of the photometric technique used was found to be ± 9.5 per cent in a series of 50 duplicate hemagglutination titrations with PR8 and Lee virus.

The results are shown in Table II and indicate that the sedimentation of approximately 50 per cent of the total mucoproteins in phosphate buffer had no effect on the inhibitory activity of the solution. It appears, therefore, that

urinary mucoproteins obtained by salt precipitation in the manner described above are inhomogeneous and that only about 50 per cent of the total is inhibitory mucoprotein. When solution of the mucoprotein was carried out at 42°C. for 19 hours in the same buffer, 25 per cent loss of activity occurred. Despite this loss the active component remained soluble in phosphate buffer. These results indicate that separation of the inhibitory mucoprotein can be obtained by extraction of dried urinary mucoproteins at 4°C. with 0.025 M phosphate at pH

TABLE II
Separation of Inhibitory Mucoprotein from Urinary Mucoproteins with Phosphate Buffer

	Preparation dissolved at*	Centrifugation	Mucoprotein		Inhibitory activity		
			Concentration	Per cent of uncentrifuged control	Titer‡	Inhibitory mucoprotein, calculated§	Per cent of uncentrifuged control
	°C.		mg./ml.			mg./ml.	
Exp. 1	4	None	3.2	100	40,320	1.45	100
		Sup.	1.7	53	38,910	1.40	97
	42	None	3.2	100	26,115	0.94	63
		Sup.	2.5	78	29,640	1.07	74
Exp. 2	4	None	2.9	100	42,890	1.54	100
		Sup.	1.4	48	46,560	1.67	108
	42	None	3.0	100	30,990	1.12	72
		Sup.	2.2	73	29,790	1.07	69

* Solutions in phosphate buffer were held for 19 hours at the temperatures indicated.

‡ Determined photometrically with 4 units of heated Lee virus and expressed as the reciprocal.

§ Calculated on the basis of an inhibitory activity equal to 0.003 μ g. per unit of heated Lee virus.

|| Supernate after 3,800 g for 30 minutes.

6.8. As is shown in succeeding papers (34, 35), the inhibitory mucoprotein thus obtained appears to be a single homogeneous substance by both electrophoretic and ultracentrifugal criteria.

Chemical Properties.—Results of chemical analyses on urinary mucoproteins and on the inhibitory mucoprotein are given in Table III. Comparison of the results with values reported for a preparation of semipurified egg white inhibitor (22) indicates similarity in the elementary composition of the inhibitory mucoproteins obtained from urine and egg white.

Determinations of reducing sugars by Shaffer-Somogyi method and glucosamine by M. Sørensen method gave the following results:¹ Urinary mucoproteins, preparation A: reducing

¹ Kindly carried out by Dr. M. A. Jesaitis.

sugars (after hydrolysis with 1 N HCl for 14 hours) 19 per cent; glucosamine (after hydrolysis with 5 N HCl for 6 hours) 6.1 per cent. Inhibitory mucoprotein, separated from preparation B: reducing sugars (after hydrolysis with 5 N HCl for 6 hours) 33 per cent; glucosamine (after hydrolysis with 8 N HCl for 6 hours) 9.2 per cent.

Gottschalk (38) reported that a dialyzable split-product liberated from urinary mucoprotein as a result of enzymatic action of Lee virus contains isoglucosamine. The split-product was obtained in a yield of about 1 per cent of the mucoprotein used as substrate. The isoglucosamine would not contribute to the glucosamine values given above because this substance is destroyed on acid hydrolysis (38).

TABLE III
Results of Chemical Analyses

	Urinary mucoproteins		Inhibitory mucoprotein (phosphate-soluble fraction from preparation B)
	Preparation A	Preparation B	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Nitrogen.....	12.24*	11.83*	12.62*
Carbon.....	48.80	46.44	48.62
Hydrogen.....	6.65	6.84	6.26
Sulfur.....	3.09‡	1.82§	5.26‡
Phosphorus.....	None	None	—
Ash.....	2.99	1.69	3.75

All values are corrected for moisture and ash.

* Micro-Kjeldahl method.

‡ Parr bomb method.

§ Carius method.

Ultraviolet Absorption Spectra.—The ultraviolet absorption spectrum of urinary mucoproteins was determined with a Beckman model DU spectrophotometer. As shown in Fig. 1, the mucoproteins gave an absorption maximum at $\lambda = 277 \text{ m}\mu$. Beer's law, relating absorption to concentration, was found to hold at $\lambda = 277 \text{ m}\mu$ within the concentration range tested: 0.1 to 1.0 mg. of mucoprotein per ml. of 0.025 M phosphate buffer at pH 6.8. The linear relation obtained was used in determining the concentration of mucoprotein in various preparations. The validity of Beer's law was also tested at wavelengths of 245, 252, 290, and 305 $\text{m}\mu$, respectively, over a concentration range from 0.1 to 0.7 mg. per ml. in phosphate buffer. Linear relations between the negative logarithm of transmission and concentration were found in each case.

The ultraviolet absorption spectrum of the inhibitory mucoprotein, too, showed an absorption maximum at a wavelength of 277 $\text{m}\mu$ (Fig. 1). In order to establish whether any differences existed between the absorption spectra of urinary mucoproteins and the inhibitory mucoprotein, an aliquot of an uncentrifuged solution of mucoproteins was diluted so that the concentration corre-

sponded to that of the inhibitory mucoprotein solution. As can be seen in Fig. 1, the absorption spectra of the diluted urinary mucoproteins and of the inhibitory mucoprotein were qualitatively and quantitatively closely similar. These results were confirmed in a number of experiments. As shown above, chemical analyses of the inhibitory mucoprotein and the urinary mucoproteins also indicated chemical similarity between the two in spite of the fact that in certain other respects, including inhibitory activity and viscosity, the two fractions showed striking differences.

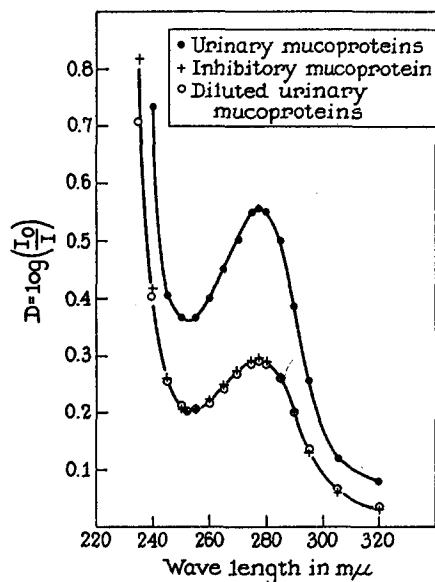


FIG. 1. Ultraviolet absorption spectra of urinary mucoproteins and of the inhibitory mucoprotein. 0.025 M phosphate buffer, pH 6.8, was used as solvent as well as blank in each case. The concentration of urinary mucoproteins was 0.48 mg. per ml. The concentration of diluted urinary mucoproteins and of the inhibitory mucoprotein was 0.23 mg. per ml. D = optical density.

Infrared Absorption Spectra.—Infrared absorption spectra of urinary mucoproteins as well as the inhibitory component were kindly determined by Dr. H. Jaffe. Both preparations showed three significant peaks at wavelengths of 6.5, 6.1, and 3.0 μ . The first two peaks appear to represent ketonic groupings ($C=O$) which may have either an amidic or acidic structure. The peak at $\lambda = 3.0 \mu$ corresponds with either $-OH$ or $-NH$ groups.

Stability of the Inhibitory Mucoprotein. Effect of pH.—Urinary mucoproteins were dissolved in a concentration of 5 mg. per ml. in each of the following buffers: sodium acetate, pH 5.4;

sodium vernal, pH 8.4, and pH 10.4. In each case the ionic strength was 0.025. The preparations were held at 4°C. for 3 days. A control consisting of mucoprotein dissolved in distilled water was handled identically.

The inhibitory activity of the solutions held at pH 5.4 and pH 8.4 was identical with that of the control (*i.e.* 0.016 μg . per unit H. Lee). The solution held at pH 10.4 showed a slight reduction in activity; less than a twofold decrease.

The pH stability of the mucoproteins also was tested at 25°C. The preparation employed was prepared by alcohol precipitation and fractionation at pH 3.0 as described previously (33).

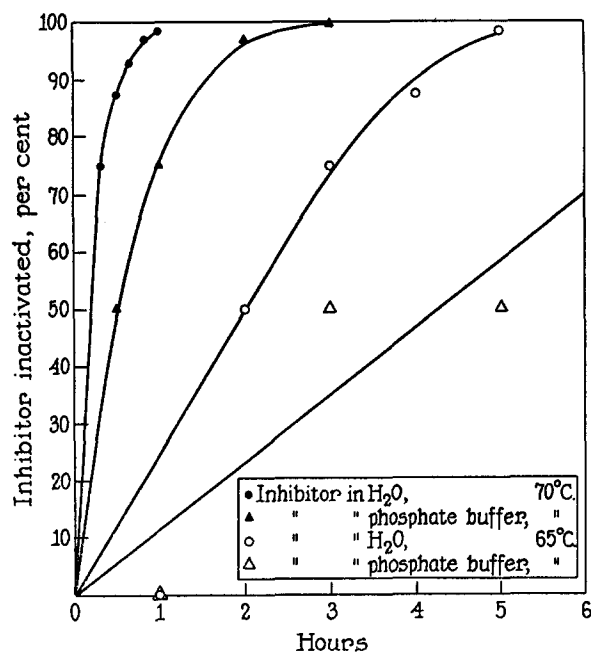


FIG. 2. Effect of heat on inhibitory activity of mucoprotein. The concentration of mucoproteins in water was 125 μg . per ml., and that of inhibitory mucoprotein in phosphate buffer was 42.5 μg . per ml. Control titrations showed the activity of mucoproteins in water to be 0.012 μg . per unit H. Lee and that of the inhibitory mucoprotein in phosphate buffer to be 0.0083 μg . per unit H. Lee.

Aliquots of a solution containing 1 mg. per ml. in water were diluted 1:4 in each of the following buffers: boric acid buffers, pH 10.9–8.2; phosphate buffer, pH 7.0; potassium phthalate buffers, pH 6.0–2.0; HCl, 0.1 N, pH 1.08. The pH determined before and after mixing buffer with the mucoprotein did not differ by more than 0.2 pH unit. Controls consisted of mucoprotein diluted similarly in each of the buffers, the pH of which had been readjusted to 6.5. The solutions were kept at 25°C. for 2.5 hours. The pH was then readjusted with 0.1 N HCl or 0.1 N NaOH to approximately 6.5 and the activity tested.

It is noteworthy that a visible precipitate appeared at pH 2.0 and 3.0, although none formed at pH 1.0 or 4.0. The precipitates dissolved promptly when the pH was readjusted to 6.5. The only specimen which showed decreased inhibitory activity was that at pH 1.08 and the

reduction in titer was marked: 99 per cent. From pH 2.0-10.9 no change in activity was observed. The inhibitory activity of these preparations was 0.012 μ g. per unit H. Lee.

Effect of Heat.—Urinary mucoproteins dissolved in distilled water and inhibitory mucoprotein in phosphate buffer were heated at 65° or 70°C. for various periods of time and the inhibitory activity then was determined. Fig. 2 gives the results of a typical experiment. The inhibitor tolerated heating better in phosphate buffer at pH 6.8 than in distilled water at pH 6.3. These results indicate that the inhibitory capacity of mucoproteins separated from urine is less stable on heating than certain other inhibitors (10, 25). In fact, the purified inhibitory mucoprotein appeared to be more unstable than the original material in urine (33).

TABLE IV
Inactivation of Inhibitor by Heat

Inhibitor				Temperature, °C.			
				65		70	
Material	Medium	pH	Activity	Time, min.			
				30	120	30	120
Inhibitor inactivated, per cent							
Urine*	Water	6.0	512‡	0	0	0	50
	0.025 M phosphate buffer	6.8	512‡	0	0	0	50
Urinary inhibitor	Water	6.3	0.008§	0	50	75	>98
	0.025 M phosphate buffer	6.8	0.005§	0	0-50	50	97

* Dialyzed against distilled water.

‡ Determined with 8 units of heated Lee virus and expressed as the reciprocal of urine dilution.

§ Determined with 8 units of heated Lee virus and expressed as μ g. per unit of virus.

For purposes of comparison dialyzed urine and inhibitor preparations isolated from urine were heated simultaneously under identical conditions and the extent of reduction in activity determined. The results given in Table IV show that the inhibitor in urine was inactivated by heat more slowly than was the inhibitor in urinary mucoproteins or the purified inhibitory mucoprotein.

During this study virus was eliminated frequently from mucoprotein-virus mixtures in phosphate buffer at pH 6.8 by heating at 65°C. for 30 minutes. The results described above show that such heating did not cause appreciable reduction in inhibitory activity.

The results of the heat inactivation experiments appear of particular interest when correlated with viscosity measurements and with the results of ultracentrifugation studies. Heating the mucoproteins in water at 70°C. for 30 minutes reduced the activity by 75 per cent and the relative viscosity of a 0.1 per cent solution from 1.8 to 1.04. After such treatment, ultracentrifugation studies (35) showed the presence of two components, whereas the unheated preparation

showed but one. The new component had a lower sedimentation constant and was present in greater concentration than the original.

Physicochemical Properties of the Inhibitory Mucoprotein.—In succeeding papers (34, 35) the results of studies on the inhibitory mucoprotein in the electrophoresis apparatus and in the ultracentrifuge are described. Both investigations showed the substance to be a single homogeneous compound.

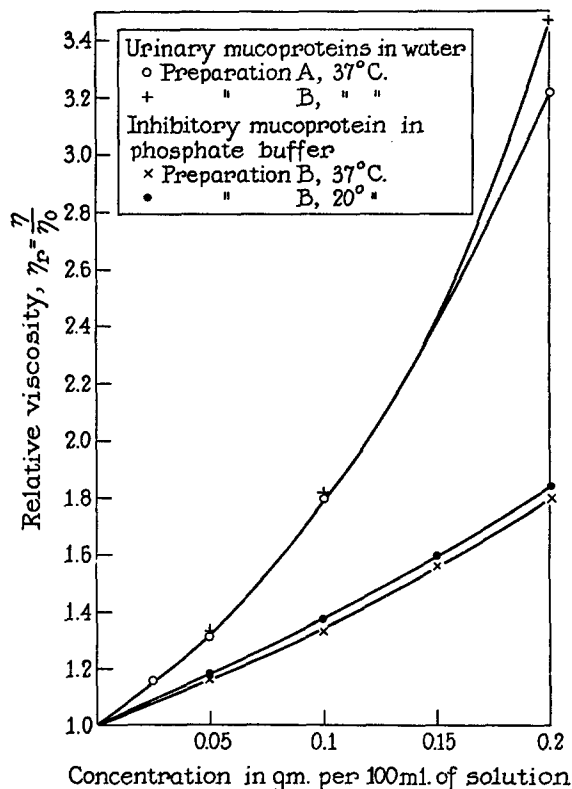


FIG. 3. Dependence of viscosity of urinary mucoproteins and inhibitory mucoprotein on concentration.

Viscosity.—The relation between concentration and viscosity was determined, as described above, with both urinary mucoproteins and inhibitory mucoprotein. With urinary mucoproteins two preparations: A and B, were studied at 37°C. in water. With the inhibitory mucoprotein one preparation was examined at 20° and at 37°C. in 0.025 M phosphate buffer at pH 6.8. Fig. 3 gives the results of representative experiments. The urinary mucoproteins were extraordinarily viscous. However, the inhibitory mucoprotein, although giving solutions of marked viscosity, did not contribute as much to the total viscosity

of urinary mucoproteins as did the non-inhibitory fraction. The viscosity of urinary mucoproteins was measured in water whereas that of the inhibitory mucoprotein was determined in phosphate buffer. When the viscosity of the inhibitory mucoprotein was determined in water, somewhat higher values were obtained.

The viscosity data for the inhibitory mucoprotein at 20°C. (Fig. 3) were used to estimate the degree of particle asymmetry in terms of the axial ratio, a/b , of an ellipsoid of revolution, where a = major axis and b = minor axis. The relation of intrinsic viscosity, defined as the value of the specific viscosity at infinite dilution, to axial ratio as formulated by Simha (39) and computed by Mehl *et al.* (40) was used. A value for ν which is a function of the axial ratio, was obtained by dividing specific viscosity by volume fraction of the solute and extrapolation to zero concentration. The results of the computation showed that $\nu = 540$ which corresponds to an axial ratio, a/b , of 95.

Diffusion.—The diffusion coefficient of the inhibitory mucoprotein at 1°C. was kindly determined by Dr. L. G. Longworth with the aid of the Rayleigh interference method (41–43). Although the solute concentration was only 0.24 per cent and the Kahn-Polson siphoning procedure (44) was used to form the initial boundary in the modified Tiselius cell, a zero time correction (45) of 3200 seconds was observed, indicating, possibly, that the high viscosity and slow diffusion of the material interfered with the formation of a sharp boundary. A diffusion coefficient of 1.77×10^{-8} was obtained by a procedure corresponding to the so called height-area method. On correction to 20° this becomes 3.25×10^{-8} cm.² per sec.: the value used below in connection with the sedimentation studies (35).

The diffusion of the material was not ideal, however, the gradient of refractive index being skew with the maximum displaced to the solvent side. If the Boltzmann relation (46) is used to compute a differential diffusion coefficient at the concentration corresponding to the 20 fringes in the pattern, the results can be represented approximately by the relation $D = 2 \times 10^{-8} + 17 \times 10^{-8} \cdot C$ (47). Here C is the concentration of the solute in grams of dry weight per 100 ml. of solution. In view of the probable concentration dependence of the diffusion coefficient noted here, the provisional nature of the molecular weight and shape factor reported below will be recognized.

The sedimentation constant at zero concentration was found to be 29.5×10^{-13} (35) and on the assumption that the specific volume is 0.685 (48–50), the molecular weight is computed as 7.0×10^6 , and the f/f_0 as 5.71. The axial ratio fitting the diffusion and sedimentation data was calculated on the basis of Perrin's equation (51) and was found to be 202.

Reacting Proportions of Inhibitory Mucoprotein and Viruses.—To determine the relation between the quantity of inhibitory mucoprotein and the quantity of heated virus inhibited, hemagglutination-inhibition titrations were carried

out in which the two were varied with respect to each other. The results of representative experiments are shown in Fig. 4. As can be seen, a linear relation holds over a wide range when 4 or more units of heated virus is used. However,

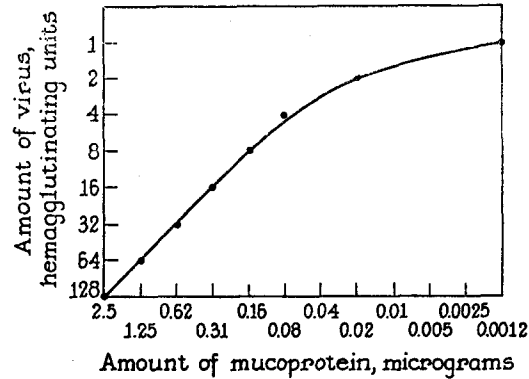


FIG. 4. Quantitative relation between inhibitor and virus. Urinary mucoproteins, dissolved in 0.025 M phosphate buffer, pH 6.8, and heated Lee virus, dialyzed against the same buffer, were employed in the concentrations indicated.

TABLE V
Comparison of Activity of Inhibitory Mucoprotein Relative to Various Viruses

Virus	Inhibitor activity	
	Determined with heated virus at 25°C.	Determined with unheated virus at 4°C.
	$\mu\text{g.}^*$	$\mu\text{g.}^*$
Lee.....	0.007	0.24
MB, 3rd egg passage.....	0.007	0.028
PR8.....	0.0035	0.0035
FM1.....	0.007	0.014
NA, 2nd egg passage.....	0.0035	0.014
" 7th " ".....	0.007	0.014
RA, 1st " ".....	0.007	0.014
" 5th " ".....	0.007	0.014
Swine.....	0.0018	0.0035
NDV.....	—‡	0.45
Mumps.....	0.014	0.007

* Expressed as $\mu\text{g.}$ per unit of virus.

‡ Rapid disagglutination of erythrocytes occurred.

when less than 4 units is used, this relation no longer holds. Therefore, whenever quantitative comparisons of the inhibitory activity of various preparations were made, 4 or more units of virus was used.

In a series of inhibition titrations with various preparations of urinary muco-

proteins carried out over many months, the average activity was found to be 0.010 μg . per unit H. Lee. After separation of the inhibitory mucoprotein, this component showed activity 2.5 to 5 times greater, *i.e.*, 0.002 to 0.004 μg . per unit H. Lee. Comparative titrations with other strains of influenza viruses, as shown in Table V, indicated that the inhibitory activity of the substance did not vary markedly in respect to the 6 different strains used, 3 of which were freshly isolated (1st to 7th egg passages).

In these titrations, 8 units of virus was used. The desired concentration of allantoic fluid was determined separately for the heated viruses at 25°C. and for the unheated viruses at 4°C. Lee, MB, PR8, FM1, NA, and RA viruses were heated at 56°C. for 30 minutes. Swine virus was heated at 52°C. for 30 minutes, NDV at 50°C. for 30 minutes, and mumps virus at 46°C. for 20 minutes.

In Table V, comparison is also made between inhibitory activity determined with heated viruses at 25°C. and that determined with untreated viruses at 4°C. All activity values fell within the same range with the striking exceptions of the low values found with untreated Lee and Newcastle disease viruses at 4°C. These are discussed below.

The infectivity titer of Lee virus is approximately $E.I.50 = 10^{-8.5}$ with an inoculum of 0.1 ml. On the basis that at the infectivity end-point there are 10 viral particles in the inoculum (52), it can be shown that infected allantoic fluid contains $10^{10.5}$ viral particles per ml. The hemagglutination titer of such fluid usually is 1,000 indicating that at the end-point there are $10^{7.5}$ viral particles per ml. Therefore, 0.003 μg . of inhibitory mucoprotein appears to be capable of reacting with $10^{7.5}$ viral particles. The molecular weight of the mucoprotein has been found to be approximately 7.0×10^6 . On the basis of these values, it can be computed that the ratio between viral particles and mucoprotein molecules at the end-point in routine titrations is of the order of 1:8. It was reported previously that the time of interaction between inhibitor and heated virus is a factor of importance in activity measurements (33). Although inhibitor titers showed a rapid rise during the first hour of interaction (the period used in routine titrations as well as in the experiments summarized in Fig. 4), maximal titers were not reached until after a 12 hour period of interaction. This was found to be true for inhibitor in dialyzed urine as well as for purified inhibitor preparations. Between the 1st and the 12th hour the titer increased 4 to 8 times. Therefore, it appears likely that after equilibrium has been reached the ratio between viral particles and inhibitory mucoprotein molecules at the titration end-point is close to 1.

Inactivation of the Inhibitory Mucoprotein by Viruses.—In the present study, the process of inactivation of the inhibitory mucoprotein by influenza viruses was found to show characteristics compatible with certain kinetic features of enzyme reactions. In experiments reported below, the effects of the concentration of virus and of mucoprotein on the rate of inactivation are described. The capacities of different viruses to inactivate the inhibitory mucoprotein are compared. A comparison is also made of the activities of various virus preparations stored under different conditions for various periods of time. In addition, the effect of *V. cholerae* extract on the activity of inhibitory mucoprotein is described.

Effect of Concentration of Virus on the Rate of Inactivation of the Inhibitory Mucoprotein.—

Mixtures containing 114 μg . of inhibitory mucoprotein and various amounts of Lee virus were held for 105 minutes at 37°C. The mucoprotein was dissolved in 0.025 M phosphate buffer at pH 6.8. The virus was dialyzed against and diluted with the same buffer. The reaction was stopped by heating at 65°C. as described above and the amount of inhibitor remaining was determined with 8 units of heated Lee virus. The control solution of mucoprotein had, both before and after the experimental period, an inhibition activity of 0.0035 μg . per unit H. Lee.

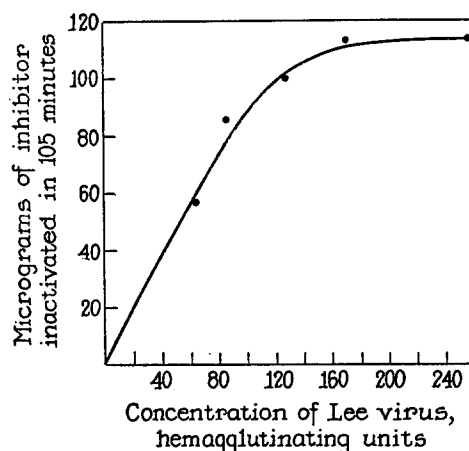


FIG. 5. Rate of inactivation of inhibitory mucoprotein as a function of the concentration of Lee virus. The amount of mucoprotein in each mixture was 114 μg .; the reaction time was 105 minutes; the temperature 37°C. The amount of inhibitor remaining after the reaction had been stopped was measured with heated Lee virus.

Fig. 5 depicts the results of a representative experiment with viral concentration as the variable. As is evident, the first part of the curve approaches a straight line indicating that when conditions were properly chosen, direct proportional dependence of rate of inactivation on viral concentration was demonstrable. With high concentrations of virus, the changes in rate became progressively smaller. The characteristics of the curve are compatible with well known features of enzyme kinetics which have been demonstrated repeatedly in analogous experiments with various enzymes and substrates (53).

Inactivation of the Inhibitory Mucoprotein by Virus as a Function of Time.—

Mixtures containing 58 μg . of inhibitory mucoprotein and various amounts of Lee virus were held at 37°C. for periods up to 30 minutes. Both reagents were in phosphate buffer, as in the preceding experiments. After stopping the reaction by heating, the amount of inhibitor was measured with heated Lee virus. The control mucoprotein solution had an activity of 0.0017 μg . per unit H. Lee throughout the experiment.

In the experiment summarized in Fig. 6, the course of inactivation of the inhibitory mucoprotein was followed with three viral concentrations. As can be seen, with 256 or 128 units of Lee virus, the reaction seemed to follow essentially a logarithmic course indicating a rapidly decreasing velocity when the concentration of virus relative to the concentration of mucoprotein was high. However, at a viral concentration of 64 units, the inactivation appeared to follow a linear course during the short period of time studied. Comparison of the rates obtained during the initial phase of the reaction with each quantity of

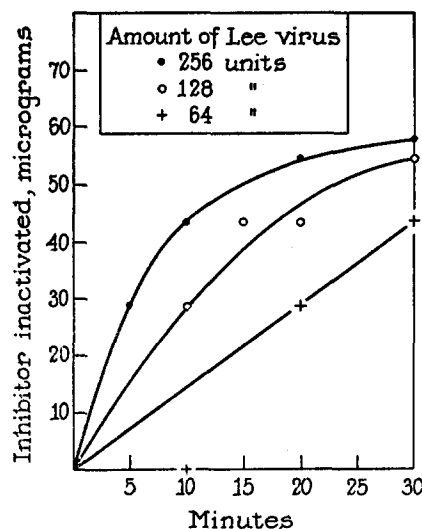


FIG. 6. Inactivation of inhibitory mucoprotein by various amounts of Lee virus as a function of time. The amount of mucoprotein was 58 μg .; the temperature 37°C. The residual inhibitor was determined with heated Lee virus.

virus used supports the conclusion that under proper conditions the rate of inactivation is directly proportional to the viral concentration.

Effect of Concentration of Inhibitory Mucoprotein on Rate of Inactivation by Virus.—

Mixtures containing 128 units of Lee virus and 29, 58, or 116 μg . of inhibitory mucoprotein were held at 37°C. for periods up to 30 minutes. Both reagents were in phosphate buffer, as in the preceding experiments. After stopping the reaction by heating, the amount of inhibitor was measured with heated Lee virus. The control mucoprotein solutions had an activity of 0.0017 μg . per unit H. Lee.

Fig. 7 shows the results of a representative experiment of this type. It can be seen that the rates of inactivation during the initial phase of the reaction were closely similar, and thus appear to be independent of the concentration of in-

hibitory mucoprotein. The decrease in rate during the later phases of reaction became apparent first with the lowest mucoprotein concentration. These findings also are in agreement with the characteristics of enzyme reactions.

Comparison of the Enzymatic Activity of Various Viruses Relative to Inhibitory Mucoprotein.—

The enzymatic activities of Lee, PR8, and FM1 viruses against the inhibitory mucoprotein at 37°C. were compared in the following manner: 128 units of the desired virus was mixed with 58 μg . of inhibitory mucoprotein. In addition, 256 units of Lee virus was mixed with 58

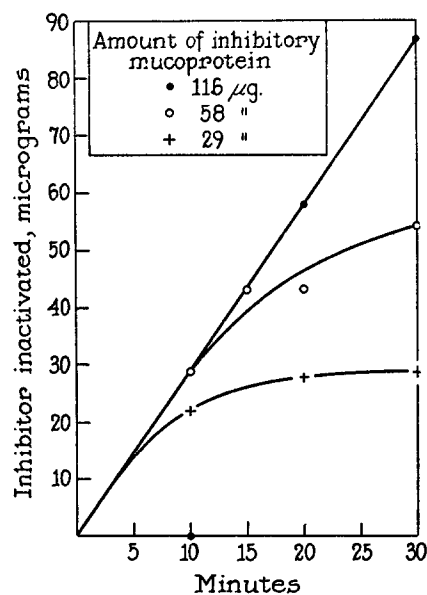


FIG. 7. Rate of inactivation of inhibitory mucoprotein by Lee virus as a function of mucoprotein concentration. The amount of Lee virus was 128 units; the temperature was 37°C. The residual inhibitor was determined with heated Lee virus.

μg . of the substance. The virus preparations were undialyzed allantoic fluids diluted appropriately with 0.025 M phosphate buffer at pH 6.8. The mucoprotein was dissolved in the same buffer. The mixtures were held at 37°C. for periods up to 80 minutes. Aliquots were removed at intervals, heated at 65°C. for 30 minutes and the inhibitory activity of each aliquot measured. For this purpose each of 3 viruses was employed. All had been heated at 56°C. for 30 minutes and a final concentration of 8 units was used in every instance. The mucoprotein solution used had, at the beginning of the experiment, an inhibitory activity of 0.0071 μg . per unit H. Lee, H. PR8, or H. FM1, respectively. At the end of the experiment the control solution showed no diminution in activity.

The results of this experiment are given in Fig. 8. As can be seen, Lee virus inactivated the mucoprotein more rapidly than PR8 which had a more rapid action than FM1. However, the difference between the rates obtained with

256 and 128 units, respectively, of Lee virus was of the same order of magnitude as the difference in the rates obtained with 128 units of Lee or PR8. Because of lack of information regarding the heat stability of freshly recovered strains of influenza viruses and because NDV, mumps, and swine influenza viruses are less heat stable than PR8 or Lee viruses, similar experiments were carried out with these agents at 25°C.

The procedure employed was identical with that described above with the following exceptions: 64 units of each virus was mixed with 58 μg . of inhibitory mucoprotein. The mixtures were held at 25°C. for periods up to 320 minutes. Inhibitory activity was measured with heated Lee virus.

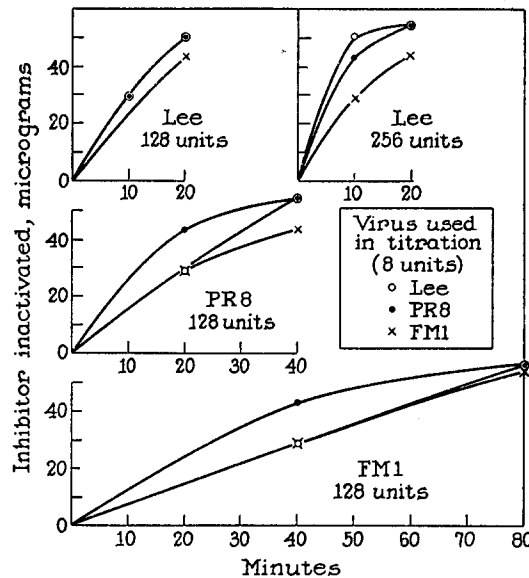


FIG. 8. Comparison of the rates of inactivation of inhibitory mucoprotein by various viruses at 37°C. 58 μg . of mucoprotein was mixed with either Lee, PR8, or FM1 virus and held for the time indicated. After the reaction had been stopped by heating at 65°C., the amount of inhibitor remaining in each mixture was measured with heated Lee, PR8, and FM1.

The results of these experiments are shown in Fig. 9. As can be seen, differences in the rates of inactivation by certain of the viruses appear definite. At 40 minutes Newcastle disease and Lee viruses had inactivated 87 and 75 per cent, respectively, of the inhibitory mucoprotein, whereas after 320 minutes there was no demonstrable inactivation by either swine or mumps virus under the experimental conditions employed.

Comparison of the Enzymatic Activity of Viruses Stored under Different Conditions.—In the inactivation experiments described above, freshly dialyzed viral preparations or undialyzed allantoic fluids, which had been stored at

$-65^{\circ}\text{C}.$, were used. In the present experiments, various preparations of Lee or PR8 virus which had been stored for different periods at either 4 or $-65^{\circ}\text{C}.$ were employed.

In each case 128 units of each virus preparation and $58\ \mu\text{g}.$ of inhibitory mucoproteins were used. To obtain the desired concentration of virus, the preparations were diluted with $0.025\ \text{M}$ phosphate buffer at pH 6.8. The mixtures were held at $37^{\circ}\text{C}.$ for 20 or 60 minutes. Aliquots removed after these intervals were heated at $65^{\circ}\text{C}.$ for 30 minutes and inhibition titrations were performed with 4 units final of heated Lee virus. The control mucoprotein solutions had an activity of $0.0035\ \mu\text{g}.$ per unit H. Lee throughout the experiment.

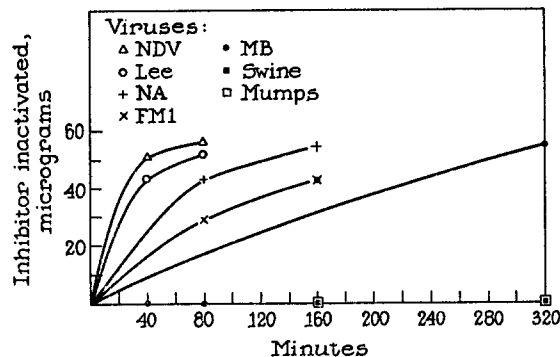


FIG. 9. Comparison of the rates of inactivation of inhibitory mucoprotein by various viruses at $25^{\circ}\text{C}.$ $58\ \mu\text{g}.$ of mucoprotein was mixed with 64 units of the viruses indicated. NA represents 7th egg passage, 1951 influenza A virus. MB represents 3rd egg passage, 1950 influenza B virus. In each instance the amount of inhibitor remaining in the mixture was measured with heated Lee.

Table VI shows the stability of influenza virus enzymes active against inhibitory mucoprotein on storage under various conditions. Freshly dialyzed allantoic fluids and undialyzed fluids stored at $-65^{\circ}\text{C}.$ for as long as 20 months were equally active. Dialyzed Lee virus which had been stored at $4^{\circ}\text{C}.$ for 3 to 6 months showed a definite loss in activity without an accompanying reduction in hemagglutination titer. Dialyzed PR8 virus, after storage at $4^{\circ}\text{C}.$ for 12 months showed markedly reduced activity although the hemagglutination titer was within the usual range. Thus, on prolonged storage at $4^{\circ}\text{C}.$, both Lee and PR8 appear to become similar to viruses which have been heated at $56^{\circ}\text{C}.$ for 30 minutes in that they lose much of their enzymatic activity but retain the ability to agglutinate red blood cells.

Effect of V. cholerae Extract on the Inhibitory Mucoprotein.—Inhibitory mucoprotein in phosphate buffer was mixed with an equal volume of *V. cholerae* extract. The mixture was held at $37^{\circ}\text{C}.$ for different periods of time and then heated at $65^{\circ}\text{C}.$ for 30 minutes. The remaining inhibitor activity was measured with heated Lee virus. It was found that 99 per cent of the inhibitor was in-

activated within 1 hour. The concentration of mucoprotein used was 72 $\mu\text{g.}$ per ml., and the control titer of the substance mixed with heated *V. cholerae* extract (65°C. for 30 minutes) was 0.0029 $\mu\text{g.}$ per unit H. Lee virus.

Properties of the Inhibitory Mucoprotein Altered by Viral Action.—The electrophoretic and ultracentrifugal characteristics of the inhibitory mucoprotein altered by influenza virus are described in succeeding papers (34, 35). No change

TABLE VI
Effect of Storage on the Enzymatic Activity of Lee and PR8 Viruses

Virus preparation used as enzyme*				Time of enzymatic action at 37°C.				
Virus	Storage		Hemagglutination titer†	20 min.		60 min.		
	Temperature	Time		Inhibitor activity	Inhibitor inactivated	Inhibitor activity	Inhibitor inactivated	
—	°C.	—	—	$\mu\text{g.}$	per cent	$\mu\text{g.}$	per cent	
—	—	—	—	0.0035	0	0.0035	0	
Lee	Dialyzed, 0.1 M phosphate buffer pH 7.14	4	6 days	512	0.056	94	>1.8	>99.8
		"	6 mos.	1024	0.014	75	0.22	98.4
	Dialyzed, 0.025 M phosphate buffer pH 6.8	4	6 days	512	0.056	94	>1.8	>99.8
		"	3 mos.	1024	0.014	75	0.11	97
Undialyzed	-65	6 days	512	0.11	97	>1.8	>99.8	
	"	20 mos.	1024	0.22	98.4	>1.8	>99.8	
PR8	Dialyzed, 0.1 M phosphate buffer pH 7.14	4	12 mos.	1024	0.0035	0	0.007	50
	Undialyzed	-65	20 mos.	1024	0.026	87.5	0.23	98.4

* 128 units of each viral preparation was used.

† Expressed as reciprocal of dilution.

in viscosity of the altered substance was observed in repeated experiments. This finding correlates well with the results of studies in the ultracentrifuge (35) which showed no evidence of extensive splitting or aggregation of the altered mucoprotein molecules.

Beard *et al.*, working with ovomucoid, observed both a decrease in viscosity (54) and a decrease in sedimentation constant (55) after alteration of their inhibitor by influenza viruses. It should be pointed out that the inhibitor in egg white has not been obtained in the form of a single homogeneous component. The best preparations showed three components on electrophoresis and the largest of these was found to be inactive (56).

The ultraviolet and infrared absorption spectra of the altered inhibitory mucoprotein were found not to differ appreciably from those of the native substance. Experiments have failed to show differences in the precipitability, with sodium chloride, of the altered mucoprotein.

The results obtained in this study correlate satisfactorily with the findings of Gottschalk (38) who recovered a dialyzable split-product in a yield of no more than about 1 per cent from urinary mucoprotein which had been treated with Lee virus. The nature of this split-product has been extensively studied and Gottschalk has ascribed an isoglucosamine structure to the compound which he has defined as *N*-1-deoxy-1-ketose aminoacid (or peptide).

Activity of Inhibitory Mucoprotein against Untreated or Heated Viruses.—Previous observations, made with the urinary inhibitor (33) and representative strains of influenza, mumps, and Newcastle disease viruses, confirmed and extended results obtained with inhibitors derived from other sources (10, 57). It was found that at room temperature inhibition titers were low when untreated viruses were used. The only two untreated viruses which gave high inhibition titers at room temperature were swine influenza and mumps (33). The hypothesis has been entertained that untreated viruses which give low inhibition titers at room temperature are enzymatically active against mucoid inhibitors and that inactivation takes place while the titration is in progress. In the enzymatic experiments reported above, it was shown that swine influenza and mumps viruses were the only viruses among those tested which were incapable of reducing the inhibitory capacity of urinary mucoprotein in 5 hours at room temperature.

Assuming that at 4°C. the rate of enzymatic activity of viruses is greatly reduced, it would be expected that untreated viruses should be, to a high degree, inhibitable by urinary mucoprotein at this temperature. It was shown in Table V that this was generally the case but that Lee and Newcastle disease viruses afforded striking exceptions. A similar observation pertaining to Lee virus was made by Anderson (17).

The following experiments were done to determine the mechanism of this peculiar reaction with Lee virus:—

In Experiment 1 various amounts of mucoprotein were mixed at 4°C. with 16 units of untreated Lee virus, held for 1 hour, and RBC added. No inhibition of hemagglutination occurred when the quantity of mucoprotein was as much as 3.5 $\mu\text{g.}$, *i.e.*, 0.22 $\mu\text{g.}$ per unit of virus. In Experiment 2, 40 units of the same unheated virus preparation was mixed at 4°C. with 3.5 $\mu\text{g.}$ of mucoprotein and held for 1 hour. The mixture was then heated at 65°C. for 30 minutes and the inhibitory activity of the mucoprotein determined with heated Lee virus. There was no reduction in the activity of the inhibitor even though the ratio between mucoprotein and virus in the original mixture was 0.085 $\mu\text{g.}$ per unit, *i.e.*, 2.6-fold less than in Experiment 1.

It appears that these seemingly conflicting results are dependent upon the difference in the way in which inhibitor was measured in the two experiments. In Experiment 1 the activity of the inhibitor was determined with untreated

virus. On the other hand, in Experiment 2, after heating the mixture at 65°C., the activity of the inhibitor was determined with heated virus in the usual manner.

A modified hemagglutination-inhibition experiment was devised in order to determine whether, under the conditions of Experiment 1, the low inhibition titer with untreated virus at 4°C. was due to inactivation of the inhibitor.

After holding dilutions of inhibitor with 16 units of untreated Lee virus at 4°C. for 1 hour, the mixtures were heated at 65°C. for 30 minutes and then cooled to 4°C. A constant amount, 16 units, of heated Lee virus was added to all tubes, and after 1 hour at 4°C. RBC were added. It was found that the activity of the inhibitor so treated was equal to that of the control, 0.007 $\mu\text{g.}$ per unit H. Lee, which had been held with heated Lee virus under the same conditions.

These results show that low inhibitory titers obtained with untreated Lee virus at 4°C. are not due to inactivation of the inhibitor. Moreover, they suggest that the low inhibitory activity at this temperature may be due to a greater affinity of untreated Lee virus for chicken erythrocytes than for inhibitory mucoprotein; that after heating the virus the relationship is changed so that affinity for mucoprotein is increased. This point of view is in agreement with the hypothesis offered by Anderson *et al.* (58).

The question can be raised whether or not untreated Lee virus combines at all with inhibitory mucoprotein at 4°C. An affirmative answer was obtained in an experiment in which untreated Lee virus and inhibitory mucoprotein were mixed and held at 4°C. for 1 hour. The mucoprotein was then precipitated with 0.58 M NaCl and removed by centrifugation. Controls showed that such a procedure did not reduce the virus titer in the absence of the mucoprotein. However, in the presence of 0.28 $\mu\text{g.}$ of mucoprotein per unit of Lee virus, 92 per cent of the virus was precipitated with the mucoprotein indicating that the two were associated at 4°C.

Effect of Mucoprotein on the Infectivity of Influenza Viruses.—The effect of urinary mucoprotein on the infectivity of Lee and RA (1st egg passage) strains of influenza B and A viruses, respectively, as well as swine influenza virus, was investigated in the chick embryo. The quantity of mucoprotein, the time it was injected, the amount of virus inoculated, and the duration of incubation were varied. No effect on the infectivity of Lee virus was observed when up to 300 $\mu\text{g.}$ of urinary mucoproteins was injected per embryo with various quantities of virus, the dilutions of virus having been prepared in buffered saline. 600 $\mu\text{g.}$ per embryo reduced the infectivity end-point of Lee virus from $10^{-7.5}$ to 10^{-6} . The infectivity of RA virus was not affected by 14.5 $\mu\text{g.}$ of inhibitory mucoprotein per embryo. However, when dilutions of virus were prepared in the inhibitor solution, 300 $\mu\text{g.}$ of inhibitor per embryo reduced the infectivity end-point of Lee virus from $10^{-7.5}$ to $10^{-5.7}$, and a reduction of greater than 10^5 -fold with swine influenza virus was noted. This apparent effect probably is attributable

to combination of the virus and mucoprotein while the dilution series was being prepared.

When 200 μg . of inhibitory mucoprotein was injected per embryo 2 hours before Lee virus, a reduction in infectivity titer of the order of 1 to 2 log units was observed in three different experiments. In addition, hemagglutination titrations of individual allantoic fluids from eggs which had received mucoprotein showed 4- to 16-fold lower titers than allantoic fluids from eggs which had not received mucoprotein. However, hemagglutination-inhibition titrations showed that the apparent low virus yields were associated with demonstrable inhibitory activity in the infected allantoic fluids. Infected allantoic fluids from control eggs showed no inhibitory activity, as was found also by other workers (10, 59).

In view of the ability of influenza viruses to inactivate the inhibitory capacity of mucoprotein, it is not surprising that the substance showed no marked effect on infectivity titrations with these agents in the chick embryo except when dilutions of virus were made in inhibitor solution.

Antigenic Properties of the Urinary Mucoprotein.—Antibodies were prepared in rabbits against urinary mucoproteins as well as against the inhibitory mucoprotein.

20 mg. of urinary mucoproteins or 10 mg. of inhibitory mucoprotein was injected intravenously per rabbit in divided doses over the course of several weeks and blood collected 2 weeks after the last injection.

Precipitation Tests.—The capillary precipitation technique (60) was employed. Experiments with urinary mucoproteins and the inhibitory mucoprotein showed that 2 to 10 μg . per ml. of either was capable of yielding precipitates when mixed with antiserum against urinary mucoproteins. After treatment of either mucoprotein preparation with *V. cholerae* extract, which resulted in greater than 99 per cent loss of inhibitory capacity, the altered mucoprotein still gave precipitates with antiserum against native urinary mucoproteins. In experiments with antiserum against the inhibitory mucoprotein as little as 0.04 μg . per ml. of inhibitory mucoprotein was sufficient to give precipitates. Inactivation of inhibitory mucoprotein by *V. cholerae* extract did not alter its capacity to give precipitates with the homologous antiserum.

Effect of Antiserum on Inhibitory Capacity.—

Dilutions of normal rabbit or antisera against urinary mucoproteins were mixed with a constant amount of inhibitory mucoprotein. The sera used had been absorbed with chicken erythrocytes to remove agglutinins against such RBC and the *serum inhibitor* was inactivated by treatment with PR8 virus and subsequent heating at 65°C. for 30 minutes. Ten minutes after the mixtures were prepared, 8 units of heated Lee virus was added and 1 hour later chicken erythrocytes were added as in the usual hemagglutination-inhibition test. Controls for the various reagents were set up in parallel.

Immune rabbit serum, at a dilution of 1:80, was found to be capable of preventing the inhibitory effect of 1.2 μg . of inhibitory mucoprotein. In terms of control titrations of inhibitor and virus, this quantity represented 8 times the amount of inhibitor required to inhibit the virus present in the mixture (8 units final).

Although antibodies against the inhibitory mucoprotein are capable of obliterating inhibitory activity, there is no reason to suppose that such antibodies combine with those groupings of the molecule which react with viruses. The fact that mucoprotein altered by the action of *V. Cholerae* extract reacts with antibodies against the native substance in undiminished degree suggests that other groupings are involved.

In other experiments no evidence was obtained that antibodies against urinary mucoproteins were capable of agglutinating either chick or human erythrocytes. Moreover, such antibodies did not prevent infection of the chorioallantoic membrane with swine influenza virus.

DISCUSSION

The finding that mucoproteins isolated from human urine by means of precipitation with sodium chloride yield, on extraction with phosphate buffer, a fraction which consists of a single homogeneous substance, according to electrophoretic (34) and ultracentrifugal (35) criteria, is of interest because this fraction appears to be responsible for all the hemagglutination-inhibiting activity of such urine. The methods employed in isolation and purification are both simple and gentle, and the product obtained is characterized by high molecular weight, high degree of molecular asymmetry, and biological activity of a very high order. A homogeneous substance which reacts with influenza, mumps, and Newcastle disease viruses thus becomes available. At the present time the ability of this substance to inhibit hemagglutination by heated viruses is the most accessible of its biological properties and one which can be measured with some precision. In causing inhibition of hemagglutination, mucoprotein molecules appear to react with influenza viral particles in a proportion which is close to 1:1. This correlates well with the estimate of Friedewald and Pickels (52) that the ratio of influenza viral particles to red blood cells at the 50 per cent hemagglutination end-point is approximately 1.

Of chief interest is the finding that inhibitory mucoprotein appears to act as a substrate for enzymes associated with influenza, mumps, and Newcastle disease viruses. A detailed and quantitative study of influenza virus-mucoprotein interaction has provided further evidence in favor of the hypothesis that these viruses are capable of altering inhibitory mucoprotein in an enzymatic manner. The combined evidence emerging from this study, the work reported in succeeding papers (34, 35), and Gottschalk's investigations (38) indicate that the loss of the ability of the mucoprotein to combine with influenza,

mumps, and Newcastle disease viruses, brought about by the action of enzymes associated with these agents, is not due to extensive alteration in the molecular structure of the substance, but is the result of the splitting off of a small portion of the molecule.

Certain features of experiments on enzymes associated with influenza, mumps, and Newcastle disease viruses require comment. Among these are the following: the number and nature of the enzymes are not known; the uncertainties involved in estimating the concentration of enzymes are marked, making comparison of the activity of different preparations unreliable; and the enzymatic action is measured in terms of loss of inhibitory activity of the mucoprotein which, with conventional techniques, does not permit measurement of small changes. The problem of the purity of preparations of animal viruses bears directly on enzymatic experiments with these agents. Although there is strong evidence that enzymatic activity against inhibitory mucoprotein is associated with the viral particles, it has been shown (61) that highly purified preparations of influenza virus contain host material in considerable amount and, as a consequence, could contain enzymes derived from infected host cells. There is, however, no evidence that normal allantoic fluid or chorioallantoic membrane from embryonated eggs contains enzymes capable of inactivating inhibitory mucoprotein. Reservations similar to those stated above have been expressed previously by other workers (16, 17).

SUMMARY

A mucoprotein, present in normal human urine, has been isolated and obtained in a state of a high degree of purity. A number of the biological, chemical, and physicochemical properties of the substance have been studied. From the results obtained in the present investigation and those reported in succeeding papers (34, 35) it appears that the mucoprotein has a high molecular weight, *i.e.*, of the order of 7.0×10^6 , consists of thread-like molecules which have axial ratios of approximately 100, and is specifically antigenic. This substance, which appears to be free of contaminating material, possesses in extraordinary degree the capacity to react with influenza, mumps, and Newcastle disease viruses. At equilibrium, with influenza virus, the minimal amount of the substance capable of giving a demonstrable reaction with one hemagglutinating unit of virus appears to be about $0.0003 \mu\text{g}$. The mucoprotein is altered by preparations of influenza viruses and its capacity to react with these agents or others is lost. The kinetics of the inactivation process brought about by influenza viruses is in accord with those of well known enzyme-substrate systems. With the exception of the capacity to react with viruses, altered mucoprotein did not differ from the native substance relative to any of the properties examined in the present study. That certain physicochemical properties of the altered mucoprotein are different from those of the native substance is demonstrated in succeeding papers (34, 35).

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