Effect of Polymerized Orosomucoid on some Strains of Influenza Virus

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1. Polymers of orosomucoid were produced in two molecular shapes, filamentous ('chain') and spherical ('ball'), by heating the sodium salt of the monomer in either water or high concentrations of sodium chloride. An 'intermediate' state containing both shapes in various proportions was found in preparations obtained by polymerizing orosomucoid in intermediate concentrations of sodium chloride. 2. The filamentous form of polymer was found to inhibit strongly the haemagglutination of some ('sensitive') strains but not of other ('insensitive') strains of influenza virus; the 'intermediate' form feebly inhibited haemagglutination by 'sensitive' strains. 3. The filamentous form agglutinated both 'sensitive' and 'insensitive' strains of virus; the other forms of polymer did not. It also inhibited multiplication of both 'sensitive' and 'insensitive' strains when inoculated into embryonated and de-embryonated eggs. 4. The 'intermediate' and spherical forms of the polymer had no effect on the virus multiplication. 5. Polymers of orosomucoid from which neuraminic acid had been split off had no detectable effect on influenza viruses.

It has long been known that many mucoproteins of high molecular weight inhibit haemagglutination and infectivity by myxoviruses. Orosomucoid, a mucoprotein of molecular weight 41 000, has little or no such activity. But when polymerized it has been found to be a powerful inhibitor of haemagglutination by some strains of influenza virus (and other myxoviruses) but not others (Whitehead, 1965; Whitehead, Flewett, Foster & Sammons, 1965).

The conditions for polymerization and the kinds of polymer produced have been further investigated, and the results are described in the preceding paper (Spragg, Halsall, Flewett & Barclay, 1969). The present paper describes various biological activities of polymerized orosomucoid: the inhibition of haemagglutination by some strains, but not others, of influenza virus and other myxoviruses, the inhibition of multiplication of some of these viruses, and the agglutination of viruses.

The activities of 'chain' and of 'ball' forms of polymer and the activity of polymerized orosomucoid protein without associated *N*-acetylneuraminic acid are described and discussed below.

METHODS

Preparation of orosomucoid and polymers. Orosomucoid was prepared as previously described (Easton, Hardwicke & Whitehead, 1962; Barker & Whitehead, 1963; Spragg *et al.*

1969). Polymers were made by heating 0.5% solutions of orosomucoid in sealed test tubes in a water bath, and examined in the electron microscope as described in the preceding paper (Spragg *et al.* 1969).

Influenza-virus strains used. Influenza-virus strains A/PR8, A/DSP, A/Persian Gulf Mill Hill 2408 2/55, A₁/England/55, an un-numbered A₂/England/65, an A₂/England/66, A/fowl plague Dutch strain, B/Lee, B/Taiwan 4/62 WIC 32367 L1, and the 1233 strain of influenza C were propagated by allantoic (or for the influenza C, amniotic) inoculation of 10-day-old embryonated eggs.

Indicator suspensions of the A/PR8 and A/DSP strains of influenza virus for use in haemagglutination-inhibition tests were prepared by diluting infected allantoic fluid 1:5 in borate-citrate buffer, pH 8.4 (Clarke & Lubs, 1917), and heating at 56° in a water bath for 30 min.

Haemagglutination-inhibition tests. Twofold serial dilutions of the polymer under investigation were made in 0.25 ml. of veronal-buffered saline, pH 7.2, and eight minimal haemagglutinating doses of indicator virus in a volume of 0.25 ml. were added to each dilution. After the samples had been kept for 30 min. at room temperature, 0.25 ml. of a 1% (v/v) suspension of fowl erythrocytes was added to each dilution and the tests were read after 60 min. at room temperature.

The haemagglutinating components were isolated from concentrated preparations of influenza virus that had been disrupted by diethyl ether. The concentrated virus was shaken with half its volume of ether for 10 min. and the ether then removed rapidly by a vacuum pump. Both procedures were carried out in a water bath at 35°. A 10 min. exposure to ether was selected in preference to a

longer period so as to preserve haemagglutinin activity (Choppin & Stoeckenius, 1964). In a further experiment the concentrated virus was disrupted by exposure to sodium deoxycholate for 10 min. at room temperature to produce much smaller sub-units than those produced by ether disruption (Laver, 1963). The appearances of the haemagglutinating components prepared by ether or deoxycholate treatment and the absence of intact virus particles were verified by electron microscopy of negative-stained preparations. The resulting suspension was purified by adsorption on and elution from fowl erythrocytes, and was converted into the indicator state by methods similar to those used for preparing whole indicator virus.

Purified virus, concentrated 20-fold by centrifugation and resuspension in water, was used in agglutination-inhibition tests. The virus suspension was disaggregated by ultrasonic vibration for 3 min. or longer, until the particles in samples examined by dark-ground microscopy were seen to be evenly distributed. Volumes of polymer, of pH 4.5, diluted 1:10, 1:50, 1:100, 1:500 and 1:1000 in sterile water and mixed with equal volumes of concentrated virus, were left for 2 hr. at room temperature and examined macroscopically. Agglutination was verified by dark-ground microscopy.

Inhibition of virus growth. 'Low-, medium- and high-salt' polymers (Spragg *et al.* 1969), sterilized by passage through a membrane filter, were inoculated allantoically into 10-day-old fertile eggs together with 10^3 egg-infectivity doses of virus in 0.1 ml. of Eagle's solution, pH 7.2, and the eggs were incubated for 42 hr. at 35° before being harvested. After incubation, allantoic fluids were titrated for the content of haemagglutinin and infective virus.

De-embryonated eggs were prepared by removing the contents of 10–12-day-old embryonated eggs, except for the chorioallantoic membrane; 10 ml. of Eagle's solution, 1 ml. of 'low-salt' polymer solution together with an inoculum of 10^3 egg-infective doses of virus were introduced, and the shell was sealed; in some experiments polymer was introduced 1 hr. or 3 hr. before the virus inoculum. The eggs were rotated at 35° for 42–46 hr. before being tested for haemagglutinin and infectivity as before.

RESULTS

Inhibition of haemagglutination. Electron microscopy revealed that the shape of polymer molecules of orosomucoid differed according to the salt concentration in which they were polymerized. Preparations made in water contained filamentous forms only (Plate 1a), and those made in 6% (w/v) sodium chloride contained predominantly spherical forms with a small proportion of short filaments (Plate 2c). Filamentous ('chain') and spherical ('ball') forms in approximately equal proportions were present in samples prepared in 3% (w/v) sodium chloride (Plate 1b).

Polymerized orosomucoid inhibited haemagglutination by influenza-virus strains A/PR8, A/Persian Gulf, A₂/England/66, B/Lee and B/Taiwan. It failed to inhibit haemagglutination by influenza-virus strains A/DSP, A₁/England/55, A₂/England/65, A/fowl plague or C (Table 1).

Table 1. *Inhibition of viruses by polymerized orosomucoid*

Most of these tests were carried out by two independent investigators; the titres did not differ by more than twofold, and in most cases were identical.

Influenza-virus strain	Inhibitor titre (reciprocal)
A/PR8	
Live	3840
Indicator	20480–40960
A/Persian Gulf	
Live	7680
Indicator	20480–40960
A ₁ /England/55	
Indicator	< 20
A/DSP	
Indicator	< 40
A/fowl plague	
Indicator	< 20
A ₂ /England/66	
Indicator	40960
A ₂ /England/65	
Indicator	< 10
B/Lee	
Live	< 160
Indicator	40
B/Taiwan	
Indicator	1280
C	
Indicator	< 5

Table 2. *Effect of sodium chloride concentration during polymerization on haemagglutination-inhibition by orosomucoid polymers of influenza virus A/PR8*

The polymers were made by heating 0.5% solutions of orosomucoid at 80° in sealed tubes in a water bath for 1 hr. in various concentrations of NaCl. Their effect on eight minimal haemagglutinating doses of influenza virus A/PR8 was tested.

Concn. of NaCl (%, w/v)	Haemagglutination-inhibition titre	
	Original	After 4 weeks at 4°
0	1:10240	1:10240
0.25	1:1280	1:10240
0.375	1:2560	1:5120
0.75	1:1280	1:5120
1.25	1:80	1:2560
3.0	1:10	1:40
3.5	Nil	Nil
6.0	Nil	Nil

Optimum salt concentrations for the production of the haemagglutination inhibitor lay between 0 and 1% (w/v) in samples heated at 80° for 60 min. Activity declined rapidly in samples prepared in

Table 3. *Effect of time of polymerization on haemagglutination-inhibition by orosomucoid polymers of influenza virus A/PR8*

The polymers were made by heating 0.5% solutions of orosomucoid in 1% (w/v) NaCl at 80° in sealed tubes in a water bath for various times. Their effect on eight minimal haemagglutinating doses of influenza virus A/PR8 was tested.

Time (min.)	Haemagglutination-inhibition titre	
	Original	After 4 weeks at 4°
5	Nil	Nil
10	1:5	1:10
20	1:80	1:1280
30	1:320	1:5120
45	1:81920	1:81920
60	1:327680	1:327680

Table 4. *Effect of temperature of polymerization on haemagglutination-inhibition by orosomucoid polymers of influenza virus A/PR8*

The polymers were made by heating 0.5% solutions of orosomucoid in 1% (w/v) NaCl for 1 hr. in sealed tubes in a water bath at various temperatures. Their effect on eight minimal haemagglutinating doses of influenza virus A/PR8 was tested.

Temperature	Haemagglutination-inhibition titre	
	Original	After 4 weeks at 4°
35°	Nil	Nil
45	Nil	Nil
55	Nil	Nil
65	1:80	1:80
72.5	1:160	1:640
80	1:20480	1:20480
85	1:20480	1:20480
90	1:2560	1:10240
95	1:5120	1:5120

sodium chloride concentration between 1% and 3% (w/v) and was absent from polymers prepared in sodium chloride concentrations above 3% (w/v) (Table 2).

The time required for maximum production of inhibition was determined by taking samples at intervals during polymerization at 80° in 1% sodium chloride. No inhibitor had formed in early samples. After 10 min. a gradual increase in haemagglutination-inhibitor titre was found in successive samples, reaching a maximum at 1 hr. (Table 3). No further increase took place if heating was continued for more than 1 hr.

Samples of monomer in 1% (w/v) sodium chloride were heated for 1 hr. at different temperatures: some haemagglutination inhibitor appeared at 65°; the maximum yield was at 80° and 85°; at 90° and 95° less inhibitor was produced (Table 4).

Thus maximum inhibitory activity was found in

Table 5. *Agglutination of influenza-virus strain A/DSP by 'low-salt' polymer of orosomucoid*

For experimental details, see the text.

Dilution of polymerized orosomucoid	Amount of agglutination
1:10	Nil
1:50	Partial
1:100	Complete
1:500	Partial
1:1000	Nil

monomer heated for 60 min. at 80–85° in water or 1% sodium chloride.

Certain samples of polymer, mainly those prepared in medium salt concentrations, increased in haemagglutination-inhibition titre after storage for 4 weeks at 4° (Table 2), thus confirming the conclusion reached on other grounds, that 'balls' can form 'chains' (Spragg *et al.* 1969).

The results of haemagglutination-inhibition tests with haemagglutinating components of virus disrupted either by ether or by sodium deoxycholate treatment were similar to those with whole indicator virus.

Agglutination of virus suspensions. Indicator virus was unsatisfactory for agglutination tests because auto-agglutination occurred. The auto-agglutination took a longer period to appear (3 hr.) than agglutination produced by 'low-salt' polymer (1–2 hr.), but eventually masked agglutination by the polymer. Agglutination of virus by polymer did not occur at pH values above 5.0. At dilutions between 1:50 and 1:500 'low-salt' polymer at pH 4.5 agglutinated both influenza-virus strains A/PR8 and A/DSP. Large clumps of virus were formed. Agglutination became increasingly less pronounced at higher and lower dilutions of polymer until, dependent on the concentration of virus, at a polymer concentration of 1:10 it was usually insignificant. A typical example is given in Table 5.

It seems likely that the failure of high concentrations of polymer to agglutinate virus suspensions occurred because under these conditions each virus particle was completely surrounded by large numbers of polymer molecules. The polymer does not itself tend to aggregate. Therefore virus particles would not be stuck to each other. Plate 2(d) illustrates virus particles whose virus surface had been completely occupied by large numbers of polymer molecules to the extent that the normal surface structure could not be discerned by negative staining. If the polymer solutions were diluted from 1:50 to 1:1000, conditions favourable to agglutination occurred. Plates 3(e), 3(f) and 4(g) illustrate

Table 6. *Effect of 'low-salt' polymer on the growth of influenza-virus strains A/PR8 and A/DSP in embryonated eggs*

Additions of 1 ml. of 'low-salt' polymer solution and 10^3 egg-infectivity doses of virus/egg were made. In the haemagglutination test, the control results are each from one egg with virus only, and the test results are each from one egg with virus and polymer. In the egg-infectivity tests, the allantoic fluid from the one egg with virus and polymer was taken and subjected to serial tenfold dilutions; each dilution was tested on four eggs, and each result shown is the mean of the four eggs.

Influenza-virus strain	Haemagglutination titre		Egg-infectivity titres of allantoic fluid from test egg
	Control	Test	
A/PR8	1:2560	Nil	Nil
	1:5120	Nil	$10^{4.5}$
	1:5120	Nil	Nil
	1:5120	Nil	$10^{3.75}$
A/DSP	1:1280	Nil	$10^{2.5}$
	1:1280	Nil	$10^{3.25}$
	1:1280	Nil	$10^{4.5}$

particles connected to each other by strands of polymer. In very high dilutions there was insufficient polymer to cause appreciable agglutination.

Dilutions of 'high- and medium-salt' polymer failed to agglutinate virus strains A/PR8 and A/DSP.

Inhibition of virus multiplication. Preliminary tests with 10-day-old embryonated eggs established that: (1) allantoic inoculation of 1 ml. of 'low-salt' polymer, sterilized by filtration, was harmless to the embryo; (2) 1 ml. of 'low-salt' polymer inoculated almost simultaneously with 10^3 egg-infective doses of the influenza-virus strain A/PR8 into the allantoic cavity inhibited viral growth; (3) smaller doses of polymer did not prevent viral growth; (4) infectivity was not inhibited if virus was inoculated 1 hr. either before or after the polymer.

A sample (1 ml.) of 'low-, medium- or high-salt' polymer solution (0.5%, polymer) was therefore used as a standard inoculum to determine the effect on the multiplication of 10^3 egg-infective doses of virus strains A/PR8 and A/DSP. The results are given in Table 6.

The 'low-salt' polymer completely inhibited multiplication of the influenza-virus strain A/PR8 in two tests; both haemagglutinin and infectivity were absent from the allantoic fluid of inoculated eggs after 48 hr. incubation. In the other two tests, polymerized orosomucoid suppressed viral multiplication and the allantoic fluid lacked haemagglutinin but was infective; this was also the case in three tests with strain A/DSP (Table 6).

Introduction of 'medium-salt' and 'high-salt'

polymer had no effect on the growth of virus in embryonated eggs.

Similar results were obtained when the experiments were repeated in the chorioallantoic membranes of de-embryonated eggs. Both 'sensitive' and 'insensitive' strains were inhibited. In the four tests carried out the membranes failed to produce haemagglutinin, but 0.1 ml. of the culture was infective to embryonated eggs. In most experiments polymerized orosomucoid and virus were introduced to the de-embryonated eggs at approximately the same time. But, in contrast with the findings with embryonated eggs, if polymerized orosomucoid was added either 1 hr. or 3 hr. before the virus, the results were the same as when the orosomucoid and virus were introduced together. After 48 hr. incubation, the de-embryonated eggs failed to produce haemagglutinin but contained infective virus.

DISCUSSION

Inhibition of haemagglutination. It has been shown that polymers of orosomucoid actively inhibit haemagglutination by certain strains of influenza virus (Whitehead *et al.* 1965).

In the range of polymers investigated in the present paper, the 'low-salt' ('chain') polymer of filamentous form was the most efficient inhibitor of viral haemagglutination and multiplication. It was also the only form of polymer to agglutinate virus suspensions. However, it failed to inhibit haemagglutination by either whole or fractionated virus of certain strains.

Sensitive and resistant strains can be found among influenza viruses that are antigenically very similar, e.g. A₂ strains. We can only speculate about the differences between the strains that determine whether they are sensitive or resistant to poly-

EXPLANATION OF PLATES 1-4

(a) Orosomucoid polymerized by heating a solution in water. Filamentous ('chain') molecules negatively stained with uranyl formate can be seen. (b) Both filamentous ('chain') and spherical ('ball') forms of polymer can be seen. The stain was uranyl formate. (c) Spherical molecules of orosomucoid polymerized in 6% NaCl. The stain was uranyl formate. (d) Influenza virus completely covered by excess of filamentous orosomucoid polymer. The normal surface structure of the virus is completely obscured. The stain was phosphotungstate. (e) and (f) Influenza virus mixed with filamentous polymerized orosomucoid. Filamentous molecules can be seen overlying the surface projections (spikes) of the virus and linking adjacent virus particles (at the arrows). The stain was phosphotungstate. (g) A chain of influenza particles connected together by filamentous orosomucoid. The stain was phosphotungstate. The dimension marks indicate 0.1 μ (100 nm.).

merized mucoprotein. Hoyle & Hana (1966) showed that there appeared to be differences in the amino acid groupings antigenically most important on the surface of the A/DSP and B/Lee influenza viruses, groupings containing tryptophan being especially important for virus A and those containing histidine being more important for virus B. Possibly some such difference, insufficient to affect the serological activity of the viruses, may affect their sensitivity to orosomucoid polymers.

It has for some time been believed (Hoyle, Horne & Waterson, 1961; Noll, Aoyagi & Orlando, 1962; Choppin & Stoeckenius, 1964), that haemagglutinin is situated on the spikes projecting from the surface of influenza-virus particles. These spikes are about 7.0 nm. apart, a distance too long to be bridged by orosomucoid monomer but capable of being bridged by chain polymer. We wondered whether there might not be some simple mechanical explanation for the inhibitory activity of the chain polymer but not of the ball polymer or the small monomer. But this attractive theory was disproved as, after disruption by ether or further degradation by sodium deoxycholate, the isolated haemagglutinating component of the virus behaved like whole virus, being just as sensitive to inhibitor.

Inhibition of infectivity. 'Low-salt' polymer inhibited the multiplication of virus strains, irrespective of their sensitivity to haemagglutination inhibition in the allantoic cells of both embryonated and de-embryonated eggs. Almost simultaneous inoculation of polymer and virus was necessary for the suppression of growth in embryonated eggs. If virus was inoculated 1 hr. before polymer, or polymer 1 hr. before virus, infectivity was not inhibited.

This result is in contrast with the findings of Lobodzinska, Morowiecki, Zielinska & Skurska (1966), who found that influenza virus was neutralized if inoculated into tissue cultures 3 hr. after polymerized orosomucoid prepared by cross-linking orosomucoid polymer in the presence of acetaldehyde. They inferred that cells subjected to the action of polymer reacted to virus differently from normal cells. It may be that structural differences between orosomucoid polymerized by our method and by theirs may account for the

differences, or that polymer is rapidly destroyed in the normal allantoic cavity.

Although great differences in sensitivity of haemagglutinin to inhibition were found among otherwise similar strains of virus, these differences were not reflected in the sensitivity of infectivity to polymerized orosomucoid; and all strains tested were agglutinated by it at a suitable hydrogen-ion concentration. Presumably therefore the polymer is active against two sites on the virus: haemagglutinin and some other site related to infectivity.

Polymers from which neuraminic acid had been removed (Spragg *et al.* 1969) were biologically inactive. This suggests that neuraminic acid imparts a property to the molecule that is essential for the attachment of the inhibitor to the virus. The loss of activity might possibly be due to chemical changes in the molecule, such as the conversion of *N*-terminal glutamine into pteroyl-glutamic acid (Ikenaka, Bammerlin, Kaufmann & Schmid, 1966) or alteration in the steric relations of amino acids in the polypeptide chain resulting from the considerably altered charge distribution in neuraminic acid-free polymer compared with native polymer.

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