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Dextran sulfate inhibits the fusion of influenza virus with model membranes, and suppresses influenza virus replication in vivo

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Summary

The effect of dextran sulfate and related compounds on the fusion of influenza A virus with model membranes, composed of dioleylphosphatidyl-choline and cholesterol (1:0.5), was investigated by a fusion assay based on de-quenching of fluorescence of octadecyl-rhodamine-HCl (R18). Dextran sulfate samples of molecular weight of 500 000, 8000 and 5000 were found to be potent inhibitors of the virus-liposome fusion process. Polygalacturonic acid also showed anti-fusion activity, but to a lesser extent. Uncharged dextran, positively charged diethylaminoethyl-dextran, and the monomer glucosamin-1,6-disulfate were ineffective. It was shown that dextran sulfate interacts with the virus. Our results suggest that dextran sulfate binds to and inactivates the viral fusion protein.

Dextran sulfate; Influenza virus; Liposome; Fluorescence spectroscopy; Membrane fusion

Introduction

The antiviral effects of polyanions, such as dextran sulfate, heparin, polyacrylic acid and polymethacrylic acid, have been described by De Somer and his colleagues as early as 1968 (De Somer et al., 1968a,b). Based on these data, it was suggested that dextran sulfate may be inhibitory to the replication of human immunodeficiency virus (HIV) (De Clercq, 1986). The observations of Ito et al. (1987) and Ueno and Kuno (1987) proved that dextran sulfate is indeed a potent inhibitor of HIV in vitro (Ueno and Kuno, 1987). Several studies on the HIV inhibitory effects of dextran

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sulfate and related compounds have since been reported (Nakashima et al., 1987; Baba et al., 1988a,b; Chang et al., 1988; Mitsuya et al., 1988; Ito et al., 1989). According to Baba et al. (1988c) and Ito et al. (1989), dextran sulfate and mannan sulfate are also potent inhibitors of enveloped viruses other than HIV (i.e. herpes simplex virus, cytomegalovirus and vesicular stomatitis virus).

This apparently broad-spectrum antiviral activity, together with the low toxicity of the sulfated polysaccharides (used for decades as anticoagulant agents), make these compounds interesting candidates for long-term therapeutic treatment of viral diseases.

As to the mechanism of anti-HIV action of dextran sulfate, it appears that the polyanion interferes with the first step in virus infection, that is the attachment of the virus to the target cell membrane (Baba et al., 1988b; Mitsuya et al., 1988; Schols et al., 1989; Nakashima et al., 1989).

In the present study, the effect of dextran sulfate on the fusion of influenza A virus with receptor-free, zwitterionic liposomes was monitored by a fluorescence fusion assay. The results obtained show that dextran sulfate strongly inhibits the virus-induced fusion process. The polyanion appears to interact electrostatically with the virus, probably with the hemagglutinin component, which is positively charged at pH 7 and below (Skehel and Schild, 1971). It is well known that hemagglutinin undergoes a proton-induced conformational change at pH 5.0 (White et al., 1983; White and Wilson, 1987; Wharton, 1987; Ruigrok et al., 1988), which is essential for the fusogenic activity of influenza A virus. Our findings suggest that dextran sulfate interferes with this structural change, thereby inhibiting the fusion process.

It appeared of considerable interest to investigate whether dextran sulfate also interferes with the replication of influenza A virus. As shown by a hemagglutinin assay, dextran sulfate also inhibits the replication of influenza A virus in embryonated hen eggs.

Materials and Methods

Virus

Influenza virus (strain A Chile H_1/N_1) from the vaccine production seed, was from the Swiss Serum and Vaccine Institute Berne. The virus was grown in the allantoic cavity of hen eggs, and was purified twice by ultracentrifugation in a sucrose gradient, as described by Skehel and Schild (Skehel and Schild, 1971). For the fusion experiments, the virus suspensions were exhaustively dialyzed against phosphate-buffered saline (PBS), pH 7.5, and stored at −80°C until used.

Fluorescent probe

Octadecylrhodamine-HCl, used for the labeling of influenza virus, was from Molecular Probes Inc.

Lipids and detergent

Dioleyl-phosphatidylcholine (DOPC) and cholesterol were from Sigma (99% pure). The detergent used for liposome preparation was sodium cholate, obtained by neutralization of twice recrystallized cholic acid with sodium hydroxide.

Polysaccharides

Dextran sulfate (molecular weight 500 000; 17% sulfur content) was from Pharmacia. The dextran sulfates of molecular weight 8000 and 5000, diethylaminoethyl dextran, polygalacturonic acid and glucosamine-1,6-disulfate (highest purity grade available) were from Sigma.

Fertilized hen eggs

The antiviral experiments were performed with eggs from Lohmann (Cuxhaven).

Liposome preparation

Unilamellar liposomes, composed of DOPC and cholesterol (1:0.5) were prepared by a detergent-dialysis method, described by Zumbühl and Weder (1981). DOPC, cholesterol and sodium cholate were dissolved in a 2:1 mixture of chloroform and methanol (p.a. grade), to obtain a DOPC:cholesterol ratio of 1:0.5, and a lipid: detergent ratio of 0.6. One ml of this solution was exhaustively dried by rotatory evaporation at 45°C for 1 h. The dry lipid film was suspended in 1 ml PBS buffer, pH 7.5. The resulting mixed-micelle solution was then dialyzed against a 1000-fold excess of PBS for 20 h at 40°C in a Mini-Lipoprep apparatus (Lightning Instruments). The resulting liposome preparation was routinely 3 mM in DOPC.

Fusion assay

The fusion assay used in the present study has been described in detail in the literature (Hoekstra et al., 1984). Intact virions were labeled with octadecylrhodamine-HCl (R 18) at self-quenching concentrations (8 mol %). Routinely, 6 μl of an ethanolic solution of R 18 (2 mM) were rapidly injected into 1 ml of virus suspension (0.75 mg protein·ml^{-1}). After 1 h of incubation at room temperature in the dark, excess R 18 was removed by repeated washing with PBS and centrifugation at 15 000 rpm for 15 min.* Then, 3 μl of the R 18-labelled virus preparation were incubated with 40 μl of the liposome suspension at 4°C for approx. 30 min. After this pre-binding period, the samples were diluted with pre-warmed PBS buffer to a final volume of 500 μl, and were used for fluorescence measurements at 37°C.

*Fluorescence control measurements with R 18-labelled virus showed that approximately 5–10% of the total fluorescence remained in the supernatant. The virus particles are thus sedimentable up to 90–95% under these conditions of centrifugation.

Unless noted otherwise, dextran sulfate and related compounds were added to the reaction mixture prior to the pre-binding period. Control samples without addition of inhibitor were included in each set of measurements.

The fluorescence of the samples was measured with a Perkin-Elmer model 3000 spectrofluorimeter, at 560 and 590 nm excitation and emission wavelengths, respectively. The instrument was equipped with a thermostatted cuvette-holder. After registration of the residual, initial fluorescence at pH 7.5 (F_0), the pH was adjusted to 5.0 by addition of 19 μl acetic acid (0.25 M). The pH of each sample was monitored in a Crisson pH meter 2000. The increase in fluorescence (F), resulting from the fusion of liposomal and viral membranes at pH 5.0, was monitored continuously for about 10 min. Then, 10 μl of Triton X-100 was added (0.2% v/v, final concentration), to obtain the fluorescence at 100% dequenching, or 'infinite' dilution of the fluorescent probe R 18 (F_t). The percentage of fluorescence-dequenching was calculated from equation (1) of Morris et al. (1989):

$$\% \text{ FDQ} = (F - F_0) / (F_t - F_0)\ 100 \qquad (1)$$

Hemagglutination tests

The influenza A virus hemagglutinin titer was determined as described by Palmer et al. (1975): Fresh chicken erythrocytes were washed 3 times with PBS, and suspended in 10 ml of buffer, pH 7.4. The packed cell volume was determined using a Clements hematocrit centrifuge. The erythrocytes were diluted with PBS to give a 0.5% v/v suspension. Of this suspension, 100 μl were incubated with the same volume of serial dilutions of the virus suspension. The extent of erythrocyte agglutination was determined in micro-titer wells, and was compared to the hemagglutinating activity of a hemagglutinin standard preparation (WHO).

Results

Fluorescence-dequenching versus time curves

Fig. 1 shows the increase in fluorescence (expressed in % FDQ) in the absence and presence of dextran sulfate (curves 1 and 2, respectively). The polyanion drastically reduced the rate of fusion between influenza A virus and liposomes.

In absence of inhibitor, the fluorescence at 30 min corresponded to 30% FDQ, which is in agreement with literature data for the same liposomal system (Nussbaum et al., 1987).

The initial rate of fusion was obtained from the tangent to the fusion curve at time 0, when the fusion was initiated by shifting the pH from 7.5 to 5.0.

Concentration-dependence of the dextran sulfate effect on fusion

Fig. 2 represents the relative initial rates of fusion as a function of dextran

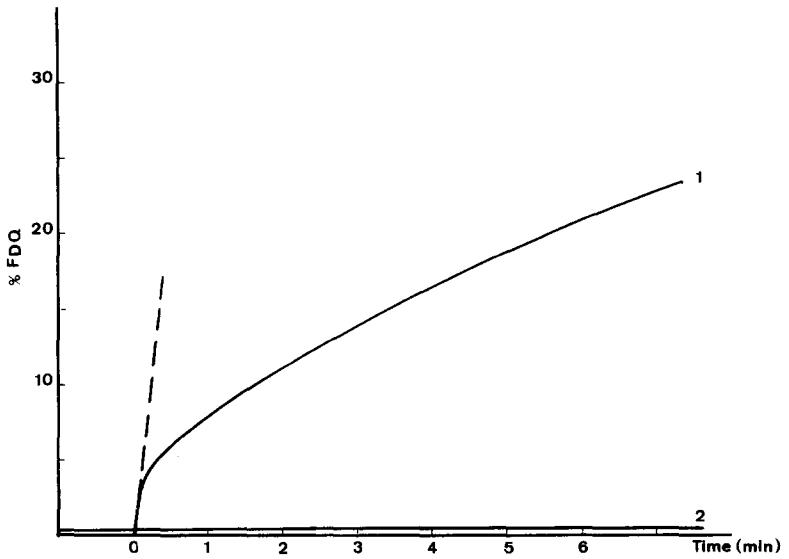

Fig. 1. Kinetics of fluorescence de-quenching of R 18-labeled influenza A virus with DOPC-cholesterol liposomes. The increase in fluorescence is expressed in % FDQ, calculated from equation (1) (see text). The initial fusion rates were obtained from the tangents to the fusion curves at time 0, when the fusion was initiated (dotted line in Fig. 1). The curves 1 and 2 correspond to fusion in the absence and presence of dextran sulfate, respectively. The concentration of dextran sulfate (MW 500 000) was 30 $\mu g \cdot ml^{-1}$.

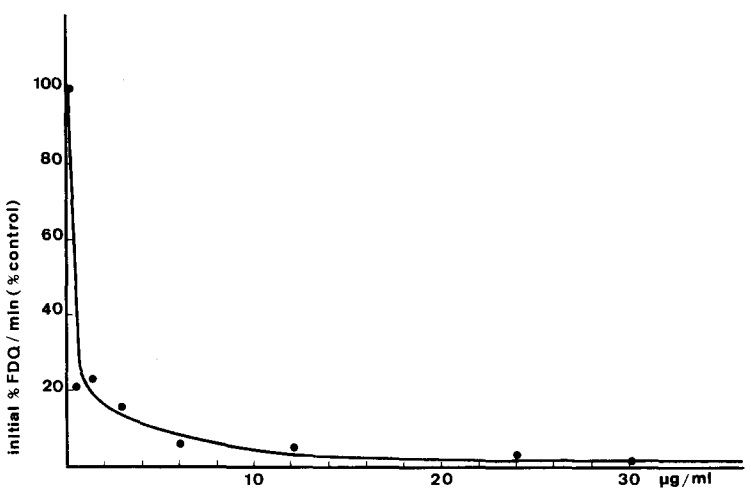

Fig. 2. Effect of the concentration of dextran sulfate on the relative initial fusion rate of influenza A virus with liposomes. The initial fusion rates are given in % of control values, obtained in the absence of the polyanion. The concentration of dextran sulfate (MW 500 000) is given in μg per ml of the reaction mixture.

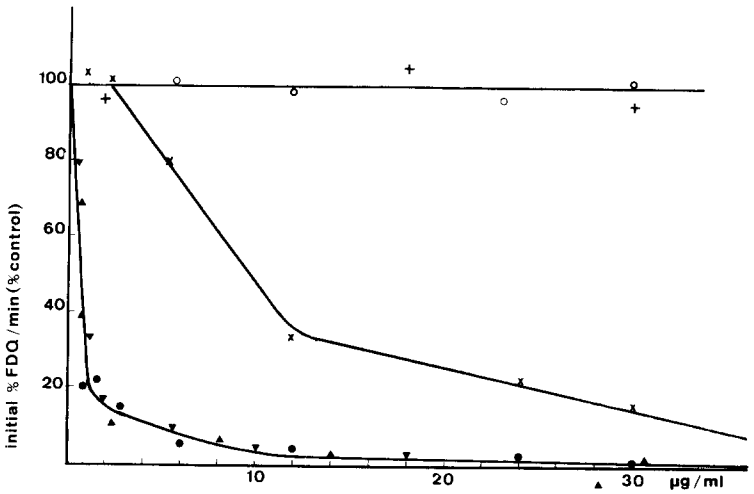

Fig. 3. Effect of molecular weight and negative charge of various polysaccharides on the relative initial fusion rate of R 18-labeled influenza A virus with DOPC-cholesterol liposomes. The initial fusion rates are given in % of control values, as in Fig. 2. The polysaccharide concentrations are given in μg per ml of the reaction mixture. Symbols: •, dextran sulfate (MW 500 000); ▼, dextran sulfate (MW 8000); ▲, dextran sulfate (MW 5000); ○, glucosamin-1,6-disulfate; ○, dextran (MW: 70 000); X, polygalacturonic acid; +, diethylaminoethyldextran.

sulfate concentration. At 0.5 $\mu g \cdot ml^{-1}$, the polyanion reduced the initial fusion rate by 50%. At 10 $\mu g \cdot ml^{-1}$, dextran sulfate achieved a complete suppression of the fusion process.

Effect of molecular weight and electric charge of the polysaccharide

The results obtained with dextran sulfate samples of varying molecular weight, and with polysaccharides of different negative charge are presented in Fig. 3. The dextran sulfate samples with molecular weight 500 000, 8000 or 5000 caused the same decrease in the initial fusion rates as a function of polysaccharide concentration. The monomer glucosamin-1,6-disulfate did not affect the virus-induced fusion process. Nor did the uncharged dextran or the positively charged diethylaminoethyl dextran. Polygalacturonic acid reduces the initial fusion rate. The concentration required to reduce the FDQ values to 50% was 10 $\mu g \cdot ml^{-1}$, i.e. 20-fold higher than the corresponding value for dextran sulfate.

Effect of dextran sulfate on the pH dependence of the fusion activity of influenza A virus

Fig. 4 shows the initial fusion rates of R 18-labelled virus with liposomes in the pH range of 7.5 to 4.0. Curves 1 and 2 correspond to data, obtained in the absence and presence of dextran sulfate, respectively. In the absence of the polyanion, the initial fusion rate shows a pronounced maximum at pH 5.0, which is characteristic

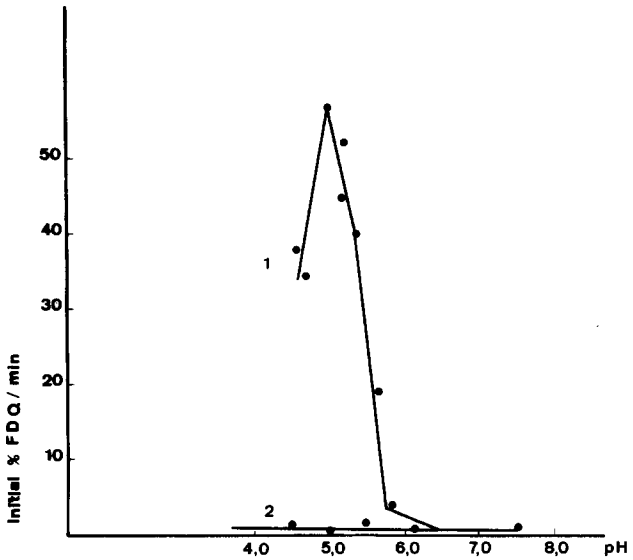

Fig. 4. Effect of dextran sulfate on the pH-dependence of the fusion activity of influenza A virus. The fusion activities are expressed as initial fusion rates, given in % FDQ min^{-1}. Curves 1 and 2 correspond to the pH-dependence of the initial fusion rates in the absence and presence of dextran sulfate, respectively. The concentration of dextran sulfate (MW 500 000) was 30 $\mu g \cdot ml^{-1}$.

for influenza virus-induced fusion processes. In the presence of dextran sulfate, the initial fusion rates are close to zero, irrespective of the pH.

Dextran sulfate pre-treatment of the virus

Of the R 18-labelled virus suspension 15 μl were incubated with 5 μl of a dextran sulfate solution (3 $mg \cdot ml^{-1}$) at pH 7.5 and 4°C for 30 minutes. Unbound dextran sulfate was removed by repeated washing with a 150-fold volume of cold buffer, and centrifugation for 5–8 min at 15 000 rpm in the cold. The pretreated virus suspension was then used for fusion assays, as described. In each set of experiments, controls without dextran sulfate addition were run, under the same experimental conditions as used for the pre-treatment procedure. Table 1 shows the results obtained in a triplicate experiment. If the virus is pre-treated, the inhibitory effect of dextran sulfate is identical to the effect observed with pre-treatment of the virus-liposome mixtures alone, where dextran sulfate was not removed prior to the fusion assay.

Effect of the time of dextran sulfate addition relative to the pH shift from 7.5 to 5.0

Virus and liposomes were pre-incubated at 4°C for 30 min in the absence of dextran sulfate. The samples were then diluted with PBS, warmed to 37°C, and

TABLE 1
Pretreatment of influenza A virus with dextran sulfate

Pretreatment 30 min/4°C	Concentration of dextran sulfate	% FDQ min^{-1} (% control)
Virus alone	0	100
Virus alone*	100 μg ml^{-1}	3
Virus + liposomes**	100 μg ml^{-1}	1

Fusion assay in the absence (*) and presence (**) of free dextran sulfate.

placed in the thermostatted cuvettes of the spectrofluorimeter. At defined times (t) before and after the pH shift, dextran sulfate was added. The fusion rates were determined from the tangents to the fluorescence versus time curves at times t, in the absence (control) and presence of dextran sulfate. Fig. 5 shows the relative rates of fusion in function of t. If dextran sulfate is added prior to (negative t values in Fig. 5), or simultaneously with the pH shift (t = 0), the same pronounced inhibition of the fusion process is observed. Addition of the polyanion after the pH shift reduces the fusion rate only if dextran sulfate was added within a time interval of no more than 20–30 s. Thereafter, the addition of dextran sulfate remained without effect.

Inhibitory effect of dextran sulfate on viral hemagglutinin production

To assess the influence of dextran sulfate on the multiplication of influenza

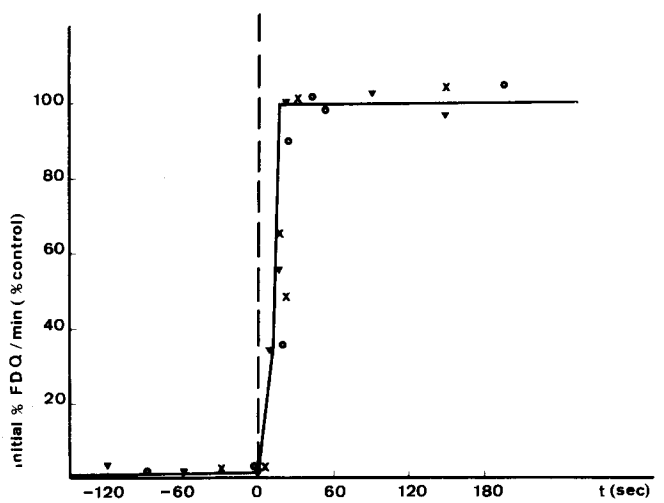

Fig. 5. Effect of time of dextran sulfate addition, relative to the pH shift from 7.5 to 5.0, on the initial fusion rate. At time t = 0, the pH of the reaction mixture was adjusted to 5.0. Negative and positive t values indicate times at which the polyanion was added before and after the pH shift, respectively. The relative initial fusion rates are expressed in % of control values, and the concentration of dextran sulfate (MW 500 000) was 30 μg per ml of the reaction mixture. The symbols O, ▽ and X represent data from three separate experiments.

virus in fertilized chicken eggs, the following experiments were performed. Three groups of 10 embryonated eggs were inoculated in the allantoic cavity with 0.1 ml of influenza virus suspension (10 egg infective doses, EID_{50}). Of a dextran sulfate solution (10 $mg \cdot ml^{-1}$), 0.1 ml was injected into the infected eggs at 0, 2 and 4 h after infection. After a 48-h incubation period at 35°C, the hemagglutinin concentration in the allantoic liquid was determined by the hemagglutination test. The standard deviations were ± 24 and ± 12 $IU \cdot ml^{-1}$ for the positive control group (virus only), the dextran sulfate-treated groups, respectively. The results, which represent the mean values for three separate experiments are shown in Fig. 6. Dextran sulfate, when added at time 0, achieved a complete suppression of the influenza A virus hemagglutinin activity. When added at 2 or 4 h after infection, hemagglutinin activity was only partially suppressed. In control experiments it was verified that dextran sulfate does not interfere with the hemagglutinin titration assay.

Discussion

Dextran sulfate was shown to be a potent inhibitor of the fusion of influenza A virus with receptor-free, zwitterionic liposomes. A comparison of different polysaccharides indicated that only negatively charged, especially sulfated, polysaccharides have an inhibitory effect on the fusion process.

The sulfate ion increases the hydration of proteins (Bull and Breeze, 1970, 1976). The sulfate groups may thus act by enhancing the hydration barrier, a major force to be overcome in a membrane fusion process.

The experiments, in which only the virus was pre-treated with dextran sulfate, suggest that the polyanion binds to, and inactivates, the virus. Influenza virus bears two surface glycoproteins, hemagglutinin and neuraminidase. Both proteins are positively charged at pH 7 and below (Skehel and Schild, 1971). Polyanionic compounds may be expected to interact with these positively charged glycoproteins. Neuraminidase exhibits no fusion activity. It thus appears that the glycoprotein with which dextran sulfate interacts is hemagglutinin, the viral fusion protein.

Hemagglutinin is known to undergo a conformational change at pH 5.0, which is essential for the fusion activity of influenza virus (Skehel et al., 1982; White and Wilson, 1987; Wharton et al., 1986, 1988). This structural change is very rapid, and is irreversibly completed after approximately 15 seconds (Morris et al., 1989).

The pH-dependence of the fusion activity of influenza virus (Fig. 4, curve 1) directly reflects this change in hemagglutinin structure. In the presence of dextran sulfate, virus fusion activity at pH 5.0 is completely abolished (Fig. 4, curve 2). This result strongly suggests that dextran sulfate interferes with the conformational change in hemagglutinin, and thereby inhibits the fusion process.

Inhibitory effects of dextran sulfate are observed only if the polyanion is present at the time the pH was shifted to 5.0. If dextran sulfate is added 20 s after the pH shift, its inhibitory effect on the virus fusion activity is no longer maintained (Fig. 5). This result adds further evidence to the assumption that dextran sulfate prevents the conformational change in hemagglutinin.

The mechanism of action of dextran sulfate and related compounds has been mainly investigated in experiments with HIV (Baba et al., 1988b; Mitsuya et al., 1988; Schols et al., 1989; Nakashima et al., 1989). It is interesting that dextran sulfate also suppresses the fusion, i.e. syncytium formation, between HIV-infected and uninfected target cells (De Clercq, 1989).

The results presented above were obtained with a model-system under in vitro conditions. If they are also applicable to the in vivo situation, dextran sulfate may be a potent antiviral agent. This premise seems to be borne out, as suggested by the results obtained in ovo (Fig. 6). Dextran sulfate significantly reduced hemagglutinin production in the allantoic fluid of influenza A virus-infected eggs. If dextran sulfate is added at the time of virus infection, the hemaglutinin expression is completely suppressed. Addition of inhibitor 2 or 4 h after infection results in an increase of approximately 30% of the positive control value. These results can be interpreted as follows: the concentration of hemagglutinin, produced within the 48 h incubation period, is proportional to the number of infected cells, which are committed to encode hemagglutinin and other viral proteins. Addition of dextran sulfate together with influenza virus, completely suppresses the infection of allantoic cells. Two to 4 h after infection, the adsorption and penetration of the first virus cycle in the host cells is completed. Addition of the inhibitor at this stage of viral replication prevents infection of new allantoic cells. The cells infected prior to addition of the inhibitor remain unaffected by dextran sulfate. This behavior suggests that the dextran sulfate acts extracellularly.

Our findings suggest that dextran sulfate inhibits the in vivo replication of

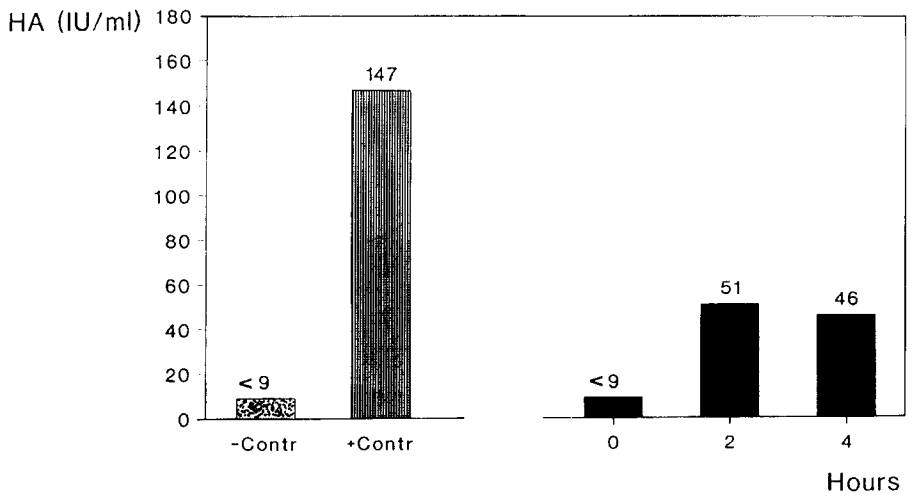

Fig. 6. Effect of dextran sulfate on the production of influenza A virus hemagglutinin in embryonated eggs. Hemagglutinin (HA) production was measured after a 48-h incubation period, and is expressed in international units (IU) per ml^{-1}. The positive virus control is marked by ▥, the negative control by ▨, and the dextran sulfate-treatment/virus groups by ■ Dextran sulfate was added at either 0, 2 or 4 h after infection.

influenza virus, and that this antiviral activity is presumably based on inhibition of the virus-induced fusion process.

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