

Inhibition of the Multiplication of Enveloped RNA-viruses by Glucosamine and 2-Deoxy-D-Glucose

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SUMMARY

Glucosamine inhibited the formation of infectious fowl plague, Sindbis, and Semliki Forest virus but had little or no effect on the multiplication of vesicular stomatitis, Newcastle disease, and polio virus. 2-deoxy-D-glucose had a somewhat stronger effect than glucosamine. Only the production of virus glycoproteins seemed to be affected. Almost normal amounts of virus RNA and RNA polymerase were synthesized, and RNP-antigen activities reached control levels. After infection with fowl plague virus the nuclei and cytoplasm of cells incubated with glucosamine showed brilliant staining with fluorescent antibodies against RNP-antigen, whereas haemagglutinin-specific fluorescence was visible weakly in the cytoplasm. The virus-induced alterations of the cell surface, as measured by the agglutinability with Concanavalin A, were abolished by glucosamine.

INTRODUCTION

Little is known about the specificity and function of the carbohydrate components of animal viruses. In addition to glycolipids, which probably originate exclusively from host cell membranes, enveloped RNA-viruses contain carbohydrate components covalently linked to virus-specific proteins (Strauss, Burge & Darnell, 1970; Klenk, Caligiuri & Choppin, 1970; McSharry & Wagner, 1971; Burge & Huang, 1970). These carbohydrates are probably specified by cellular transferases (Burge & Strauss, 1970; Grimes & Burge, 1971; Compans *et al.* 1970; Schultze, 1970).

We have explored the function of the carbohydrates by feeding the host cells with different sugars or their derivatives. It was thought that disturbance of the normal carbohydrate metabolism might interfere with virus maturation and production of infectious particles.

It is known that amino sugars, or the corresponding deoxy sugars, are highly toxic for cells through interference with carbohydrate metabolism (Bekesi & Winzler, 1969; Keppler *et al.* 1970). In chick fibroblasts, the host cells mainly used in this paper, glucosamine is metabolized very actively, while galactosamine is less. *N*-acetyl glucosamine is less effective than glucosamine (Scholtissek, 1971).

METHODS

Tissue culture cells and virus strains. Primary chick fibroblasts, HeLa or BHK cells were used. The following virus strains were investigated: Fowl plague virus (FPV), strain ROSTOCK; Newcastle disease virus (NDV), strain ITALIEN and strain BEAUDETTE; Semliki Forest virus (SFV), strain OSTERRIETH; Sindbis virus (SV), strain AR 86; vesicular stomatitis virus (VSV), type INDIANA; polio virus, type I (Mahoney).

Virus multiplication. The cultures were infected at a multiplicity of 10 to 50 p.f.u./cell. The washed cells were incubated in 6 ml. per dish minimal medium (Eagle & Habel, 1956) at 37°. At the indicated times after infection (p.i.) the medium was removed for analysis. The cell layers were covered with the original volume of phosphate buffered saline and kept frozen until processed and analysed (Rott & Scholtissek, 1968).

Virus infectivity was determined by plaque assay in chick fibroblasts (Zimmermann & Schäfer, 1960).

Haemagglutination tests were performed in plastic plates using a 1% suspension of chick erythrocytes (Davenport, Rott & Schäfer, 1960). Haemagglutinating units (HAU) represent the reciprocal of the haemagglutination titre.

RNP-antigen was determined by the indirect haemagglutination inhibition test (Becht, 1968). The titres were expressed as the highest dilution of antigen inhibiting 4 agglutinating units of specific antiserum.

Neuraminidase activity was determined according to Drzeniek (1967) using bovine sialo-lactose as substrate. The amount of neuraminidase which liberated 1 μ mole sialic acid from the substrate per min at 37° was defined as one enzyme unit (EU).

Immunofluorescence was performed according to Breitenfeld & Schäfer (1957).

Synthesis of virus RNA in vivo was measured by incorporation of tritium-labelled nucleosides into RNA in the presence of actinomycin D. Further details are shown on the figures.

Virus RNA polymerase (RNA dependent) was determined using a homogenate of virus-infected cells from which the nuclei had been removed (Scholtissek & Rott, 1969).

Agglutinability with Concanavalin A was tested 6 hr after infection. The cells were washed twice with Ca-free phosphate-buffered saline and detached from the Petri dishes with 0.02% EDTA. After washing with Ca-free phosphate-buffered saline the cells were re-suspended in minimal medium (2×10^6 cells/ml.). Concanavalin A (1 mg./ml.) in Ca-free phosphate-buffered saline was diluted in a twofold series in the cups of a plastic tray so that 0.2 ml. remained to be mixed with 0.2 ml. of the cell suspension. The tray was shaken gently at room temperature and agglutination was read after 15 min. (Becht, Rott & Klenk, 1972).

Materials. [3 H]-uridine (24 c/m-mole) and [3 H]-guanosine (0.5 c/m-mole) were obtained from the Radiochemical Centre, Amersham, England. [3 H]-GTP (0.8 c/m-mole) was purchased from Schwarz BioResearch, Inc., Orangeburg, New York, U.S.A. The sugars and their derivatives were purchased from Sigma, St Louis, Missouri, USA and 1 M stock solutions at pH 7.2 were prepared and kept at -20°. Actinomycin D was a gift from Merck, Sharp and Dohme, New York, USA. Concanavalin A was obtained from Calbiochem, Los Angeles, California, USA.

RESULTS

Effect of glucosamine on the multiplication of various enveloped viruses

As shown previously (Scholtissek, 1971), 20 m-moles glucosamine has no significant effect on the synthesis of cellular RNA, although the rate of incorporation of [3 H]-leucine into protein is affected slightly.

After incubation of FPV with 50 m-moles glucosamine for 2 hr at room temperature there was no loss of infectivity as determined in the plaque test.

Glucosamine inhibited the production of infectious virus and of haemagglutinin by several enveloped viruses. Supernatant and cell-associated activities decreased to the same extent and indicated that the amino sugar had no significant effect on virus release.

As shown in Fig. 1, increasing doses of glucosamine had an increasing inhibitory effect

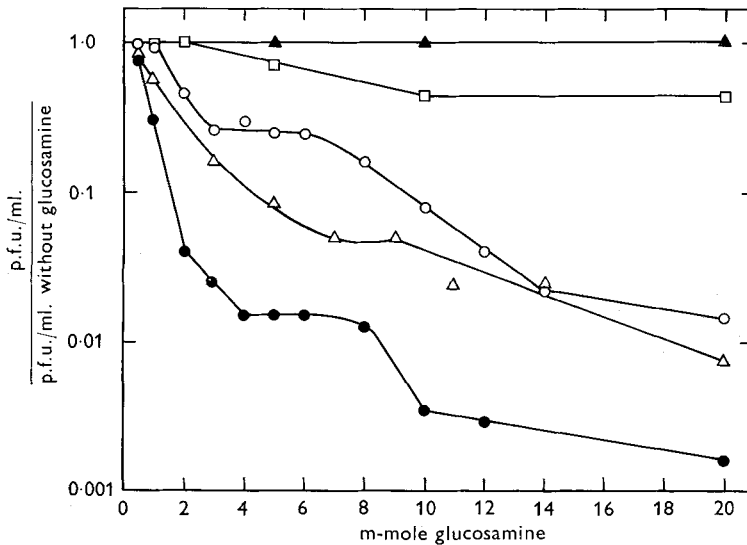


Fig. 1. Effect of glucosamine on the multiplication of several enveloped viruses. Different doses of glucosamine (abscissa) were added immediately after infection. The infectivity was tested 8 or 9 hr after infection. Δ , Fowl plague virus, cell-associated at 8 hr; \square , vesicular stomatitis virus, cell-associated at 8 hr; \circ , Sindbis virus, cell-free at 9 hr; \blacktriangle , Newcastle disease virus, cell-associated at 9 hr; \bullet , Semliki Forest virus, cell-free at 9 hr.

on the multiplication of FPV, SFV, SV and VSV. The biphasic shape of the curves suggested that inhibition occurred in two steps. The multiplication of NDV strain ITALIEN or strain BEAUDETTE was not significantly impaired. Polio virus in HeLa cells multiplied normally in the presence of glucosamine up to 50 m-moles.

Since in FPV-infected chick fibroblasts many different virus activities can be measured, the effect of glucosamine in this system was studied in more detail. As shown in Figs. 2 and 3, there was little or no effect on the production of the virus RNP-antigen or RNA polymerase. Neuraminidase activity of FPV was significantly reduced. The virus RNA production *in vivo* was estimated after suppression by actinomycin D of the synthesis of cellular RNA. The formation *in vivo* of virus RNA polymerase activity of SFV was slightly affected (Fig. 3). The production of SFV-RNA (Fig. 4) as measured by the incorporation of [3 H]-guanosine into RNA in the presence of actinomycin D followed a different course in the presence of glucosamine from that in controls without the amino sugar. The total production of virus RNA shown by integration of the area under the curves was not altered significantly. If [3 H]-uridine was used as label, its incorporation into virus RNA in the presence of glucosamine was considerably higher than in untreated cells. This was due to a specific reduction of the UTP pool by the amino sugar (Scholtissek, 1971).

Virus multiplication in the presence of glucosamine applied at different times during the multiplication cycle

Pre-incubation of the host cells with glucosamine overnight and removal of the amino sugar immediately after infection did not affect the multiplication of FPV and SFV. As shown in Fig. 5, in order to be effective, glucosamine must be present at the time of haemagglutinin production and of virus maturation.

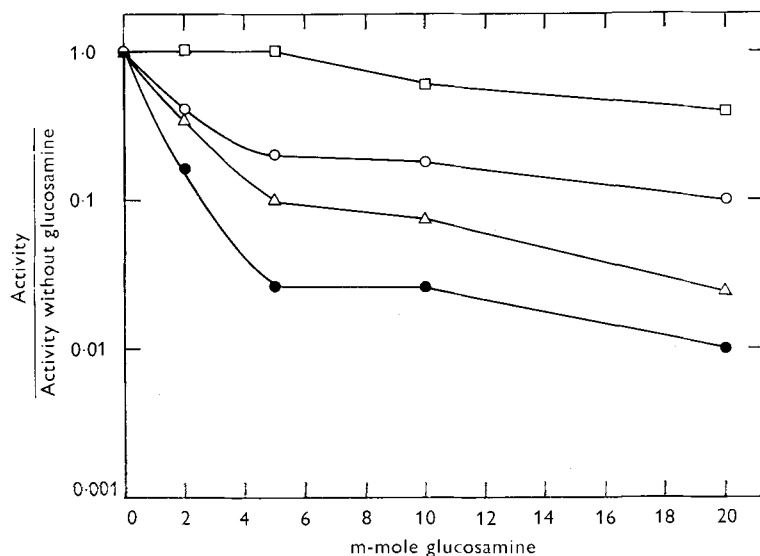


Fig. 2. Effect of glucosamine on the production of different components of fowl plague virus. Experimental details as in Fig. 1. The cell-associated activities were determined 8 hr after infection. □, RNP-antigen, titre; ○, neuraminidase (EU/ml.); △, haemagglutinin (HAU/ml.); ●, infectivity (p.f.u./ml.).

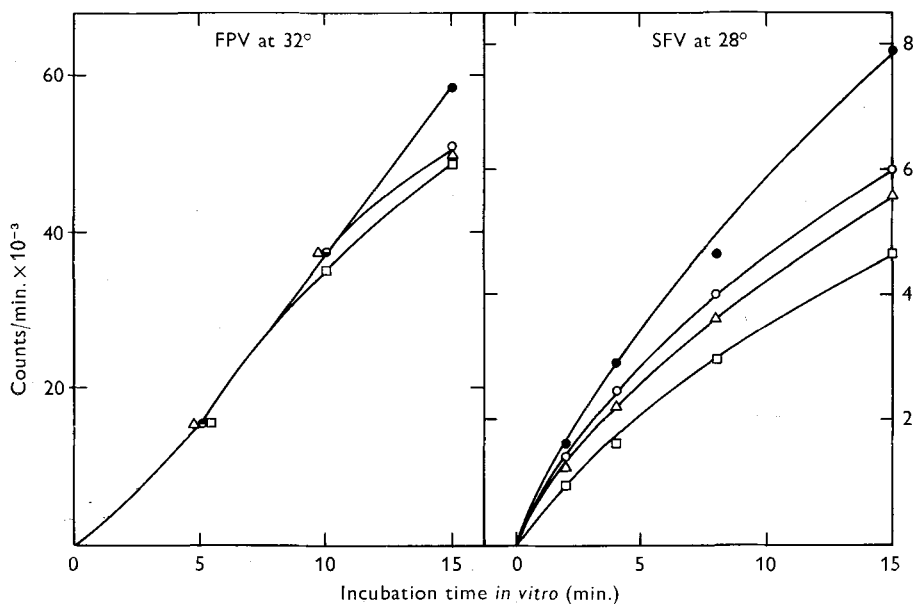


Fig. 3. Effect of glucosamine on the formation of RNA polymerase of fowl plague and Semliki Forest virus. Different doses of glucosamine were added immediately after infection. The cells were processed either 5 hr (FPV) or 6.5 hr (SFV) after infection. Cytoplasmic extracts were incubated for different times with $[^3\text{H}]$ -GTP plus co-factors (Scholtissek & Rott, 1969). FVP at 32°; the extracts contained 5.4 mg./ml. protein. SFV at 28°; the extracts contained 1.5 mg./ml. protein. Glucosamine at 20 m-moles (□), 10 m-moles (△), 5 m-moles (○) and absent (●).

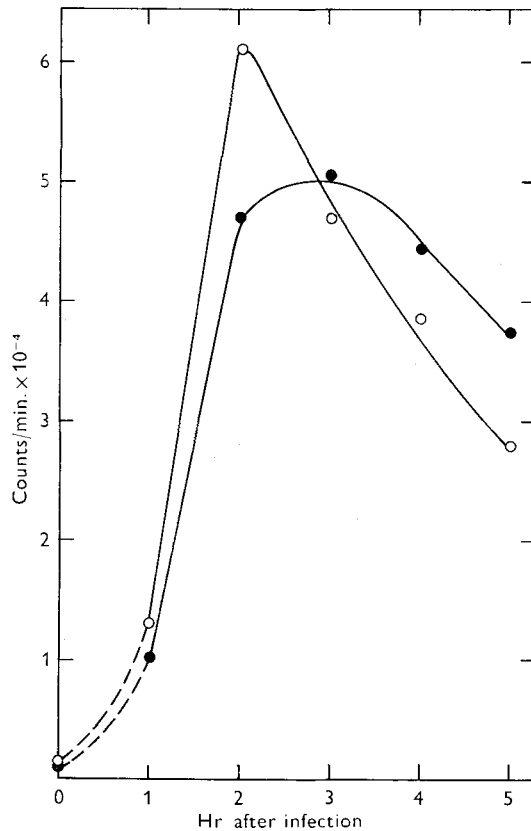


Fig. 4. Influence of glucosamine on the synthesis of Semliki Forest virus RNA *in vivo*. Immediately after infection, 0.5 $\mu\text{g./ml.}$ actinomycin D was added to all cultures. Half of the cultures received in addition 5 m-mole glucosamine. Pulses with 1.25 μC [^3H]-guanosine per culture were started at the times indicated on the abscissa. After 30 min. the cultures were washed four times with 6% trichloroacetic acid, twice with ethanol, and once with methanol. The dried cell layers were dissolved in 2 ml. of 0.1 M-NaOH at 60° for 10 min. They were transferred to the counting vessels and after addition of 10 ml. Bray-scintillation fluid they were counted in a Tricarb-scintillation counter. With (●) and without (○) 5 m-moles glucosamine.

Studies with fluorescent antibodies

Studies with fluorescent antibodies specific to the RNP-antigen and haemagglutinin of FPV (Breitenfeld & Schäfer, 1957) showed that 10 m-moles glucosamine did not affect the appearance of RNP-antigen in the nucleus or cytoplasm of infected cells. However, haemagglutinin-specific staining in the cytoplasm was very faint.

Effect of glucosamine on the agglutinability of FPV-infected cells with Concanavalin A

A variety of cells infected with different enveloped RNA-viruses can be specifically agglutinated with Concanavalin A. The production of envelope components seems to be essential for the agglutinability of myxovirus-infected cells (Becht *et al.* 1972). It was therefore of interest to study the influence of glucosamine on the agglutinability by Concanavalin A of FPV-infected cells.

Infected cells without glucosamine were readily agglutinated 6 hr after infection by a

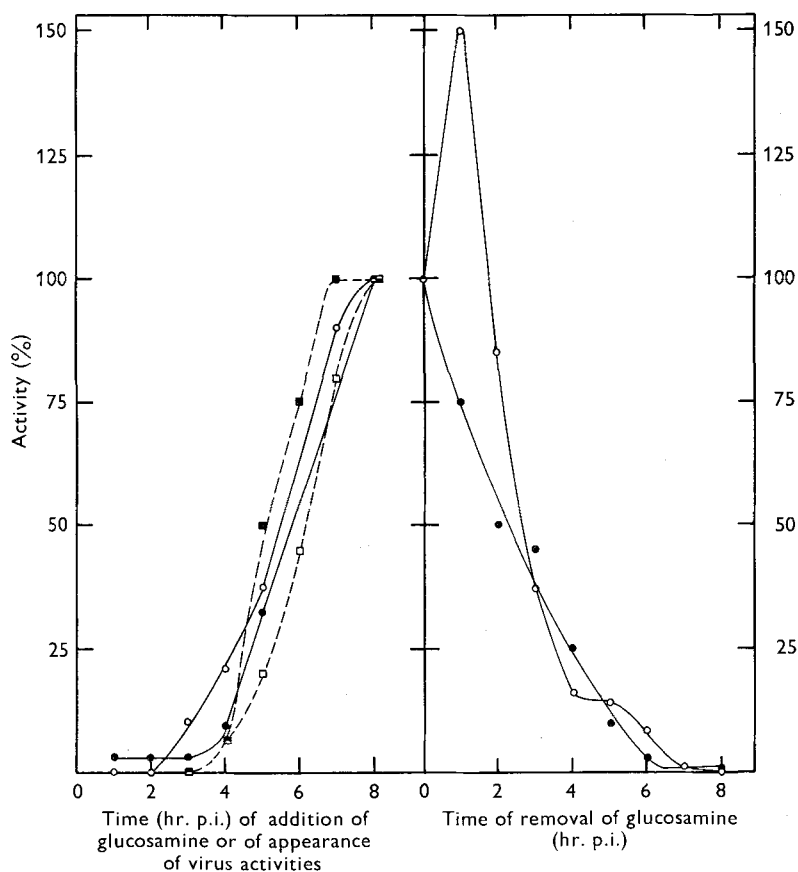


Fig. 5. Effect on fowl plague and Semliki Forest virus multiplication of glucosamine present at different times of the multiplication cycle. Left: glucosamine was added to the infected cultures at the times after infection indicated. The virus yields were determined 8 (FPV) or 9 (SFV) hr after infection. Right: glucosamine was added immediately after infection. The cells were washed with phosphate buffered saline and covered with fresh minimal medium at the times indicated. The cells were harvested 8 hr after infection. ●, FPV, haemagglutinin (HAU/ml.), cell-associated (10 m-moles glucosamine). ○, SFV, infectivity (p.f.u./ml.), cell-free (5 m-moles glucosamine). The appearance after infection of FPV haemagglutinin (HAU/ml., ■) and of SFV infectivity (p.f.u./ml., □) are also shown on the left.

minimal concentration ($5 \mu\text{g./ml.}$) of Concanavalin A. No agglutination was observed, even with 0.5 mg./ml. of Concanavalin A, if the infected cells were incubated with 20 m-mole glucosamine. Glucosamine at up to 20 m-moles *in vitro* had no effect on the agglutinability of FPV. Cells infected with NDV and incubated with 20 m-moles glucosamine showed the same agglutinability with Concanavalin A at 8 hr after infection as infected cells not treated with the amino sugar.

Other sugars

The following sugars in concentrations up to 10 m-moles were without effect on the multiplication of FPV and SFV: D-mannose; D-galactose; L-fucose; 2-deoxy-D-galactose; D-ribose; D-arabinose; D-xylose, D-sorbose, D-fructose, 2-deoxy-D-ribose, L-rhamnose, glucuronic acid lactone, glucosaminic acid, D-galactosamine, α -methyl-D-glucopyranoside.

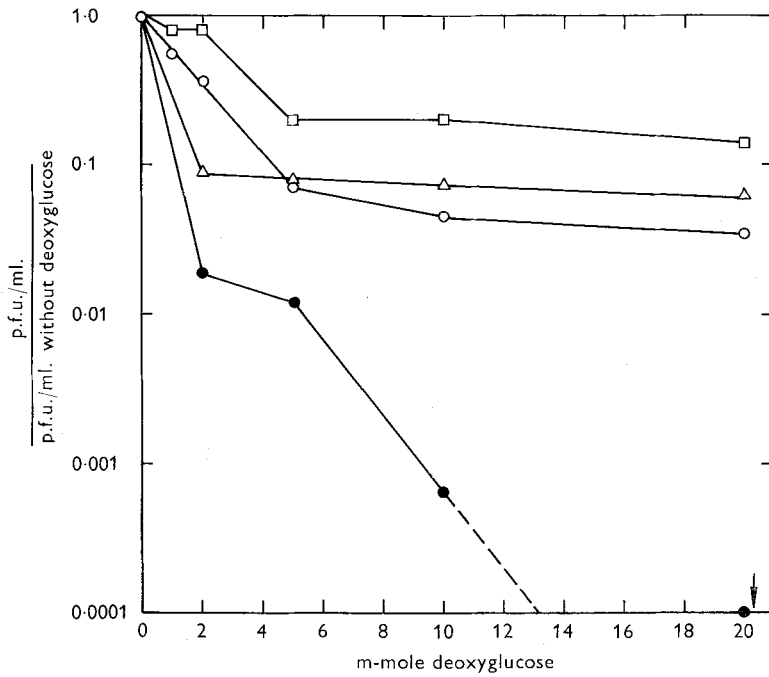


Fig. 6. Effect of 2-deoxy-D-glucose on the multiplication of different enveloped viruses. Deoxyglucose at the concentrations indicated was added immediately after infection. The infectivity was determined 9 hr after infection. □, Vesicular stomatitis virus, cell-associated; ○, Sindbis virus, cell-free; ●, Semliki Forest virus, cell-free; △, Newcastle disease virus, cell-associated.

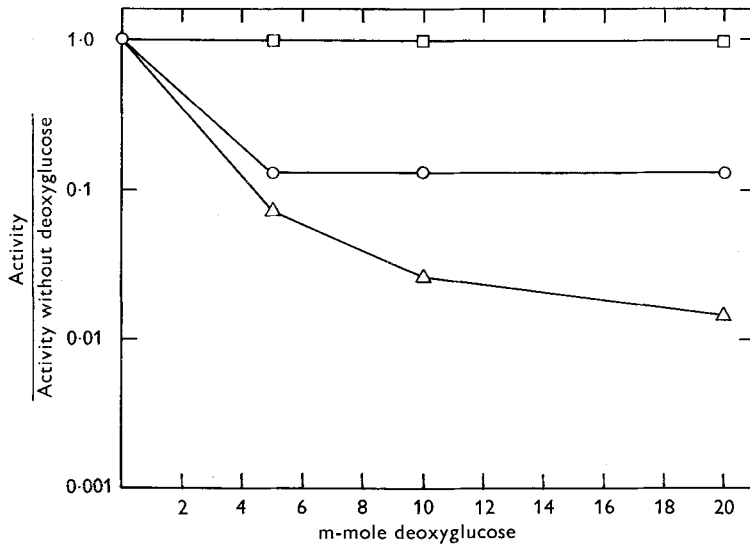


Fig. 7. Effect of 2-deoxy-D-glucose on the production of different components of fowl plague virus. Experimental details as in Fig. 1. The cell-associated activities were determined 8 hr after infection. △, Haemagglutinin (HAU/ml.); ○, neuraminidase (EU/ml.); □, RNP-antigen (titre).

N-acetyl glucosamine was without effect even at a concentration of 50 m-moles. None of these compounds reversed the effect of glucosamine.

The only sugar with a pronounced activity was 2-deoxy-D-glucose (Figs. 6, 7). In contrast to glucosamine, it also had some inhibitory effect on the production of NDV. Deoxyglucose had the same influence on host RNA and protein synthesis as glucosamine (unpublished).

Other cells

The multiplication of SFV in BHK cells and of FPV or SV in HeLa cells was inhibited by glucosamine as described for chick fibroblasts. As mentioned before, polio virus multiplied normally in HeLa cells treated with up to 50 m-moles glucosamine.

DISCUSSION

We have shown that glucosamine inhibits strongly the replication of several enveloped viruses (influenza A (FPV), a rhabdovirus (VSV), arboviruses (SFV, SV)), but has no significant influence on the reproduction of a paramyxovirus (NDV) and a polio virus.

Glucosamine is incorporated into carbohydrates, probably as *N*-acetyl derivative, since phosphorylation and *N*-acetylation of the amino sugar occur prior to the formation of the UDP-derivative (Molnar, Robinson & Winzler, 1964), the final precursor of the glycoprotein. As shown previously (Scholtissek, 1971), *N*-acetyl glucosamine is metabolized only half as efficiently as glucosamine. This may be the reason why the acetyl derivative does not inhibit significantly the multiplication of virus.

All the enveloped viruses tested contain glucosamine or one of its derivatives, since they can be labelled with the radioactive amino sugar. It is interesting that glucosamine does not impair significantly the multiplication of NDV, although deoxyglucose has a small effect.

Glucosamine obviously interferes with the production of the carbohydrate-containing subunits which are part of the virus envelope. As previous studies have shown (Scholtissek, 1971), glucosamine is metabolized more efficiently in chick fibroblasts than several other sugars. Therefore it is possible that UTP is used preferentially to form UDP-*N*-acetylglucosamine and is no longer available in sufficient quantity for the activation of other sugars which are also constituents of the virus envelope.

It is not known whether glucosamine and 2-deoxy-D-glucose also act via glycolipids, which are derived from the host cell membranes (Klenk & Choppin, 1970). In the presence of these compounds the membrane of FPV-infected cells is not modified to an extent which leads to the exposure of Concanavalin A-reactive sites.

D. O. White and his colleagues (in preparation) also found that 2-deoxy-D-glucose specifically inhibited the synthesis in HeLa cells of the haemagglutinin of influenza virus, strain BEL.

Note added in proof. After this manuscript was sent for publication a paper by E. D. Kilbourne (Inhibition of influenza virus multiplication with a glucose anti-metabolite (2-deoxy-D-glucose) (1959) *Nature, London*, **183**, 271) came to our attention, in which the inhibition of influenza multiplication in embryonated eggs by 2-deoxy-glucose is described.

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