Newcastle Disease Virus-induced Plasma Membrane Damage

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SUMMARY

Chick embryo fibroblasts infected by Newcastle disease virus release cellular enzymes into the overlay medium. The kinetics of release were determined for two cytoplasmic enzymes, lactate dehydrogenase (LDH) and glutamic oxaloacetic transaminase, and a lysosomal enzyme, betaglucuronidase. Permeability studies showed that this release was accompanied by an alteration in the permeability of the plasma membrane of infected cells. Both [14C]-sucrose and [14C]-dextran entered infected cells at times when release of enzymes was observed, 6 h post-infection, while sucrose entered cells by 4 h post-infection, before release of enzymes could be detected. The addition of cycloheximide or 2-deoxy-D-glucose to infected cells that were already leaking LDH showed that glycoprotein synthesis is required for the virus-induced release of LDH. Stabilization of membrane permeability was accompanied by stabilization of protein synthesis after virus-induced inhibition, and by a decrease in the virus antigens in the plasma membrane. It is hypothesized that the structural alterations of the plasma membrane, indicated by the presence of new virus antigens, are responsible for the functional alterations observed.

INTRODUCTION

Infection of chick embryo fibroblasts (CEF) with the Texas strain of Newcastle disease virus (NDV) results in cell death. Some of the pathogenic events observed are an inhibition of both protein synthesis and RNA synthesis (Wilson, 1968), the degradation of cellular RNA (Huo & Wilson, 1969), the activation of cellular lysosomes (Wilson, 1972), and the release of cellular enzymes into the overlay medium (Wilson, 1972). Wilson (1972) has shown that when NDV is grown in chloroquine treated cells, it will replicate without an inhibition of cellular protein synthesis or the degradation of cellular RNA. It appears, therefore, that these events are not necessary for the growth of the virus. It thus seems unlikely that all of the observed pathogenic events are independent, primary virus events. One might explain all of these pathogenic effects, however, as being caused by a primary virus event of either lysosomal activation or damage to the plasma membrane of the host cell.

The release of enzymes from virus-infected cells has been noted previously (Gilbert, 1963; Blackman & Bubel, 1969; Wilson, 1972). Blackman & Bubel (1969) also observed that in poliovirus-infected Hep-2 cells, extralysosomal betaglucuronidase appeared approx. 2 h

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before extracellular enzymes were detected. They suggested that lysosomal enzymes were responsible for damage to the cell membrane.

We wanted to see whether the release of enzymes from NDV-infected cells was a reflection of permeability changes in the plasma membrane, gross cell lysis, or escape of cellular contents during virus budding. Permeability studies were therefore designed to investigate the penetration of certain solutes into infected cells. We also investigated the possibility that lysosomal hydrolases or altered structural components of the plasma membrane might be responsible for enzyme leakage from NDV-infected cells.

**METHODS**

**Materials.** Uniformly labelled $[^{14}\text{C}]-\text{L}-\text{leucine (0.26 Ci/mmol), [6-}^{3}\text{H}]-\text{D}-\text{glucosamine (6.6 Ci/mmol), [5-}^{3}\text{H}]-\text{uridine (28 Ci/mmol), [8-}^{3}\text{H}]-\text{guanosine (7.7 Ci/mmol), [}^{3}\text{H}]-\text{water (25 Ci/mmol), uniformly labelled [}^{14}\text{C}]-\text{D}-\text{succrose (5.2 Ci/mmol) and uniformly labelled [}^{14}\text{C}]-\text{dextran (> 60 mCi/mmol, 60,000 to 90,000 mol. wt.) were obtained from New England Nuclear Corporation. Silicione oil (versiluble F-50) was a gift from the General Electric Company.**

**Cells and virus.** All experiments were performed on primary chick embryo fibroblasts. Culture methods have been described previously (Wilson & LoGerfo, 1964). The Texas (GB) strain of NDV was grown for 44 h in 11-day-old fertile chicken eggs. Virus was centrifuged from allantoic fluid for 60 min at 30,000 g and resuspended in phosphate-buffered saline (PBS). Cells were infected with approx. 250 p.f.u./cell. After a 30 min adsorption period at 39 °C monolayers were washed with PBS, fresh Eagle's medium was added, and the cells were incubated at 39 °C in a 5 % CO$_2$ atmosphere. Zero time for the infection was taken as the time of addition of fresh Eagle's medium.

**Titration of virus.** Virus solutions were diluted in sterile PBS and inoculated on confluent monolayers of CEF. After 30 min at 39 °C, the plates were overlayed with 5 ml of a solution made of one part agar 0.8 % with neutral red (0.1 mg/ml) and one part 2 × Eagle's medium. The plates were then incubated at 39 °C in a 5 % CO$_2$ atmosphere, and the plaques were counted 3 to 4 days later.

**Radioactive labelling.** The rate of protein synthesis was measured by labelling the cells with Hanks's medium containing 0.05 μCi/ml $[^{14}\text{C}]-\text{leucine for 30 min. TCA precipitation and counting procedures have been described previously (Bolognesi & Wilson, 1966). The rate of total RNA synthesis was measured by labelling the cells for 30 min with either $[^{3}\text{H}]-\text{guanosine or }[^{3}\text{H}]-\text{uridine, both at 0.1 Ci/ml in Hanks's medium. To measure virus RNA synthesis specifically, actinomycin D at 2 μg/ml was added to the labelling medium, and the label was added at the time of infection. The rate of glycoprotein synthesis was measured by labelling the cells with $[^{3}\text{H}]-\text{glucosamine at 5 μCi/ml in Hanks's medium. The label was added at the time of infection and remained for the duration of the experiment.**

**Haemadsorption.** Red blood cells were sedimented from fresh citrated chicken blood, washed in citrate saline, and resuspended in PBS at 0.3 % (v/v). The medium was removed from the culture plates and the monolayers were washed with cold PBS. Then the RBC suspension (4 ml) was overlaid and allowed to adsorb at 4 °C for one h. The overlay was then replaced with fresh PBS and the plates were rotated slowly on a shaker platform for 5 min. The cells were then gently washed in fresh PBS to remove unadsorbed RBC. The monolayers were dissolved in an 0.8 % saline solution containing 0.1 % Triton X-100 to induce hemolysis. This solution was clarified by sedimentation, and the haemoglobin estimated from $E_{550}$. 


Enzyme assays. Lactate dehydrogenase (LDH) was measured by observing the decrease in $E_{340}$ as NADH$_2$ is oxidized. The reaction mixture contained 0.1 ml 0.01 M-sodium pyruvate, 0.1 ml 0.002 M-NADH$_2$ at pH 8.0, 2.7 ml 0.03 M-phosphate buffer at pH 7.4, and 0.1 ml enzyme. β-glucuronidase was assayed as described by Allison & Sandelin (1963). For LDH a unit of activity has been defined as an initial rate of oxidation of 1 μmol of NADH$_2$ per minute at 25 °C; for β-glucuronidase as the cleavage of 1 μmol of phenolphthalein glucuronide per min at 37 °C.

Glutamic oxaloacetic transaminase (GOT) 2.6.1.1) was assayed using Worthington Biochemical Corporation determatube SGO. For GOT, one unit of activity was defined as a decrease in $E_{290}$ of 0.001 per min at 25 °C.

Silicone technique. The permeability of CEF plasma membrane for sucrose and dextran was measured using the silicone layer technique. Cell monolayers were scraped into 2 ml Hanks’s medium and incubated at 20 °C with [3H]-H$_2$O at 2.5 μCi/ml and [14C]-sucrose or [14C]-dextran, both at 0.3 μCi/ml. At various intervals up to 30 min, 0.2 ml samples were removed and placed in a Beckman 152 minifuge, and the cells were centrifuged rapidly through a layer of silicone oil on to 0.1 ml of 16 % perchloric acid. Then 0.05 ml of the upper layer was removed and counted for [3H] and [14C]. The upper portion of the minitube was then washed three times and 0.08 ml of the lower layer was removed through the silicone and also counted for [3H] and [14C].

The water space, $W$, defined as the total volume of water that has come through the oil, is related to the amount of tritium in the upper and lower layer by

$$\frac{T}{0.08} = \frac{T_0 W}{0.1 + W} \text{ ct/min,}$$

$T = [3H]$ counted in lower layer and $T_0 = [3H]/ml$ in upper layer. Therefore, we may calculate $W$:

$$W = \frac{0.1 T}{0.08 T_0 - T} \text{ ml.}$$

The sucrose space, $S$, defined as the volume of water that has come through the oil that is available to sucrose, is similarly related to the amount of [14C] in the upper and lower layers by:

$$\frac{C}{0.08} = \frac{C_0 S}{0.1 + W} \text{ ct/min,}$$

where $C = [14C]$ in lower layer and $C_0 = [14C]/ml$ in upper layer. Therefore, we may calculate $S$:

$$S = \frac{C(0.1 + W)}{0.08 C_0} \text{ ml.}$$

In some experiments [14C]-dextran was used in place of sucrose and the dextran space was calculated accordingly. Since sucrose penetrates cells very slowly the ratio $S/W$ is usually much less than one. Increases in $S/W$ during an experiment indicate that the sucrose is penetrating into the cells.

RESULTS

Kinetics of the release of cellular proteins

When monolayers reached confluence, they were infected with NDV, and at various times after infection the overlay medium was removed and assayed for the two soluble cytoplasmic enzymes, LDH and GOT, and for a lysosomal enzyme, β-glucuronidase.
It can be seen from Fig. 1(a), (c) that release of LDH and β-glucuronidase from infected cells begins at 5 to 6 h post-infection (p.i.). The time of the beginning of release of GOT is not seen as clearly (Fig. 1(b)), but it appears that by 6 h p.i. this release has begun. At 10 h p.i. the percentage of enzyme in the overlay medium compared to the total enzyme present in the cells plus medium is 74% for LDH, 62% for β-glucuronidase and 27% for GOT. It can also be seen that once the release of these cellular proteins has begun, the rate of release continues to increase.

In this same experiment the kinetics of virus-induced inhibition of protein synthesis was also assayed. In Fig. 1(d) protein synthesis in infected cells is plotted as the percentage of protein synthesis in control cells. It is seen that protein synthesis inhibition also begins at 5 to 6 h p.i., and that by 9 h p.i. protein synthesis is down to 2 to 3% of that in control cells.

To correlate the growth cycle of the virus with the release of cellular enzymes, we examined the time course of virus maturation, virus RNA synthesis, and haemadsorption. Virus maturation can be followed in the growth curve of p.f.u. (Fig. 2a). In this experiment 10^7 cells were infected with 2.7 x 10^7 p.f.u., of which 1.5 x 10^7 p.f.u. were adsorbed to the
cells. Virus production begins between 4 and 6 h, and continues to increase steadily during the times assayed. In Fig. 2(b), (c) it can be seen that both virus RNA synthesis and haemadsorption have begun by 4 h. Haemadsorption is a reflexion of the presence of virus-antigens in the plasma membrane of the host cell. At zero time there is a slight haemadsorption reaction which disappears by 2 h. The positive haemadsorption reaction at zero time is most likely a reflection of input virus membranes, and the disappearance at 2 h would reflect the replacement of these virus antigens with cellular antigens. By 4 h, however, virus antigens are being produced since a large rise in haemadsorption is observed.

It is seen then, that virus maturation, virus-induced inhibition of protein synthesis, and enzyme release all seem to begin between 4 and 6 h, while virus RNA synthesis and virus protein synthesis have already begun by 4 h.

**Penetration studies**

Penetration experiments were designed to determine whether or not the release of cellular enzymes from infected cells reflected a change in the permeability of the plasma membrane. At various times after infection the medium was removed and replaced with 2 ml Hanks’s medium. The cells were then scraped and incubated at 20 °C with [3H]-H₂O and
Table 1. Effect of infection on the water space

<table>
<thead>
<tr>
<th></th>
<th>W (ml)</th>
<th>% S/W</th>
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</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>1.21</td>
<td>23</td>
</tr>
<tr>
<td>Infected cells (8 h)</td>
<td>1.12</td>
<td>63</td>
</tr>
</tbody>
</table>

Fig. 3. Penetration of dextran and sucrose.

[14C]-sucrose or [14C]-dextran. Since scraping the monolayers yielded a suspension with large clusters of cells, differing numbers of cells were assayed at each point. To ensure that we were not assaying a very limited and special population of infected cells, an experiment was performed using vials with approx. 1/9 the surface area of normal culture dishes. At 8 h p.i. the medium was removed and 0.2 ml Hanks's medium was added. The entire monolayer was then scraped, incubated in [3H]-H2O and [14C]-sucrose for 4 min, and then spun through silicone. As seen in Table 1, the water vol. of the infected monolayer was 93% of the vol. of the control monolayer. Since the total water vol. of control and infected cells is nearly the same, all subsequent results are reported as the percentage of the water space occupied by the sucrose or dextran (% S/W or D/W), with no respect to absolute values of vol.

In most experiments scraped cells were incubated in sucrose or dextran for varying lengths of time and then 0.2 ml samples were removed and spun through silicone oil. When cells were observed after 1 min of incubation in [3H]-H2O and [14C]-dextran, all the infected and control cultures had approximately the same value for the % D/W, regardless of the time after infection. Since it is assumed that even infected cells would not allow significant amounts of dextran to penetrate in the first min of incubation, this shows that the extracellular water space does not vary significantly as the infection progresses. When the incubation period in dextran was extended to 20 min, significant increases were seen in the % D/W. These increases must have been the result of dextran penetration into cells since there was no increase in the amount of extracellular water centrifuging through the silicone oil.

When the kinetics of penetration of sucrose or dextran were analysed, it was found that
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Fig. 4. Effect of NDV on the distribution of the lysosomal enzyme, betaglucuronidase. At various times post-infection the medium was removed from control and infected plates and saved for analysis. The monolayers were scraped into 2 ml of sucrose-EDTA (0.25 M-sucrose, 0.0005 M-EDTA, pH 7) and homogenized in a loose fitting dounce homogenizer (20 strokes). The homogenate was centrifuged at 20000 g for 20 min to obtain a soluble cytoplasmic fraction and a particulate lysosomal fraction. The pellet was resuspended in 2 ml of 0.8 % saline solution, which contained 0.1 % Triton X. All three fractions, medium, cytoplasm, and lysosomes, were assayed for β-glucuronidase.

at all times p.i. the % S/W increased linearly until 20 min of incubation in radioisotope. The % S/W and % D/W after 20 min of incubation in radioisotopes are shown in Fig. 3(a), (b) for the various time p.i. It can be seen that both sucrose and dextran enter the cell at times after infection when release of cellular enzymes has been noted, and that sucrose and dextran have different rates of penetration. It should also be noted that as the infection progresses, the rate of penetration is continually increasing. In addition, it appears that the smaller molecule, sucrose, enters the infected cells at 4 h p.i., before release of cellular proteins is observed. A similarity to the haemadsorption curve (Fig. 2c) can also be seen. At zero time there is a slight positive reaction which then returns toward controls by two hours and then begins to increase by 4 h.

Lysosomal activation and permeability changes

Several investigators using different virus-cell systems have attempted to relate lysosomal activation to different types of virus-induced cell damage (Allison & Sandelin, 1963; Wolff & Bubel, 1964; Flanagan, 1966; Blackman & Bubel, 1969; Huo & Wilson, 1969; Bienz, Egger & Wolf, 1973). Blackman & Bubel (1969) observed that Hep-2 cells infected with poliovirus, extralysosomal betaglucuronidase appeared approx. 2 h before extra-
Table 2. Effect of 2-deoxy-D-glucose on glycoprotein synthesis

<table>
<thead>
<tr>
<th></th>
<th>ct/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>162</td>
</tr>
<tr>
<td>Infected</td>
<td>196</td>
</tr>
<tr>
<td>Control + drug</td>
<td>65</td>
</tr>
<tr>
<td>Infected + drug</td>
<td>57</td>
</tr>
</tbody>
</table>

At zero time p.i. 2-deoxy-D-glucose (2 mg/ml) was added to a control and an infected culture. All cultures were incubated in [3H]-glucosamine at 5 μCi/ml in Hanks's medium. After 8 h, the cells were washed twice with 5% TCA and prepared for counting.

Table 3. Effect of 2-deoxy-D-glucose on haemadsorption

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>E550-infected</th>
<th>E550-infected + drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.028</td>
<td>0.020</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.010</td>
</tr>
<tr>
<td>4</td>
<td>0.197</td>
<td>0.010</td>
</tr>
<tr>
<td>6</td>
<td>0.438</td>
<td>0.114</td>
</tr>
<tr>
<td>8</td>
<td>0.524</td>
<td>0.175</td>
</tr>
</tbody>
</table>

At zero time p.i. 2-deoxy-D-glucose (2 mg/ml) was added to half of the infected cultures. At various times p.i. the medium was removed and the monolayers were assayed for haemadsorption as described in Methods.

Table 4. Protective effect of 2-deoxy-D-glucose against virus-induced membrane damage and inhibition of protein synthesis

<table>
<thead>
<tr>
<th></th>
<th>Betagalactosidase enzyme units</th>
<th>LDH enzyme units</th>
<th>Protein synthesis (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.166</td>
<td>7.2</td>
<td>774</td>
</tr>
<tr>
<td>Infected</td>
<td>0.865</td>
<td>50.4</td>
<td>74</td>
</tr>
<tr>
<td>Control + drug</td>
<td>0.126</td>
<td>4.6</td>
<td>609</td>
</tr>
<tr>
<td>Infected + drug</td>
<td>0.126</td>
<td>6.4</td>
<td>350</td>
</tr>
</tbody>
</table>

At zero time p.i. 2-deoxy-D-glucose was added to half of the control cultures and half of the infected cultures. At 10 h p.i. the medium was removed from the plates and analysed for extracellular LDH and betagalactosidase as an indication of membrane damage, while monolayers were assayed for the rate of protein synthesis as described in Methods.

cellular protein was released. In addition, Allison, Magnus & Young (1966) have shown that it is possible to photosensitize lysosomes in tissue culture cells. Upon exposure to light, these lysosomes release lysosomal enzymes, and this is followed by a loss of the functional integrity of the plasma membrane.

Experiments were therefore performed to see if extralysosomal β-glucuronidase could be observed before the leakage of extracellular protein. At varying times post-infection the overlay medium was removed and saved for analysis. The cells were then scraped into 0.2 M-sucrose-EDTA, homogenized, and centrifuged to obtain a soluble cytoplasmic fraction and a particulate lysosomal fraction. The results of such an experiment are shown in Fig. 4. It should be noted that the loss of lysosomal bound β-glucuronidase in infected cells occurs at 6 to 7 h p.i. and that this lysosomal activation is reflected by the appearance of β-glucuronidase in the overlay medium. That is, there is no time interval between lysosomal activation and the leakage of β-glucuronidase into the medium. In addition, at no time during the infection, does there appear to be an increase above control levels of cytoplasmic β-glucuronidase.
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Effect of cycloheximide and 2-deoxy-D-glucose on the kinetics of leakage of LDH

At 6 h after infection with NDV either cycloheximide (20 μg/ml) or 2-deoxy-D-glucose (2 mg/ml) was added. At various times the supernatant medium was removed and assayed for LDH.

Fig. 5. Effect of cycloheximide and 2-deoxy-D-glucose on the kinetics of leakage of LDH. At 6 h after infection with NDV either cycloheximide (20 μg/ml) or 2-deoxy-D-glucose (2 mg/ml) was added. At various times the supernatant medium was removed and assayed for LDH.

Effect of 2-deoxy-D-glucose on virus-induced membrane damage

It has been reported that 2-deoxy-D-glucose inhibits the formation of influenza virus glycoproteins (Kaluza, Scholtiszek & Rott, 1972; Klenk, Scholtiszek & Rott, 1972). In Table 2 it can be seen that when [3H]-glucosamine incorporation is used to assay total glycoprotein synthesis in normal or infected CEF, there is a marked inhibition of synthesis when 2-deoxy-D-glucose is added at a concentration of 2 mg/ml. When haemadsorption is assayed, a similar inhibition is observed (Table 3). When other growth characteristics were followed, it was found that 2-deoxy-D-glucose had a significant inhibitory effect on p.f.u. formation but only a minor effect on virus RNA synthesis.

The latter result indicates that 2-deoxy-D-glucose does not inhibit the synthesis of the virus proteins which are necessary for virus RNA synthesis (Wilson & LoGerfo, 1964). Although this drug markedly inhibits glycoprotein synthesis, it has only a small effect on total cell protein synthesis (Table 4). Table 4 also indicates that 2-deoxy-D-glucose prevents any virus-induced enzyme leakage and has some protective effect against the virus-induced inhibition of cell protein synthesis.

Effect of cycloheximide and 2-deoxy-D-glucose on the kinetics of leakage of LDH

To further explore the mechanism of virus-infected membrane damage, either cycloheximide or 2-deoxy-D-glucose was added to infected cell cultures that were already leaking enzymes. Both of these drugs had the same effect on the kinetics of leakage of LDH (Fig. 5). As noted previously, infected cultures show a continual increase in the rate of leakage of LDH with time. The infected cultures with drug added at 6 h, however, exhibited a
Fig. 6. Effect of 2-deoxy-D-glucose on the kinetics of leakage of β-glucuronidase. At 6 h p.i., the medium was removed from some cultures and replaced with Eagle’s medium containing 2-deoxy-D-glucose (2 mg/ml). At various times the medium was removed from the cultures and assayed for β-glucuronidase.

Fig. 7. Effect of 2-deoxy-D-glucose on haemadsorption. Cells were infected with approx. 30 p.f.u./cell. At 6 h p.i. the medium of some cultures was replaced with Eagle’s medium containing 2-deoxy-D-glucose (2 mg/ml). At various times the monolayers were assayed for haemadsorption.
**NDV-induced membrane damage**

Fig. 8. Effect of 2-deoxy-D-glucose on NDV-induced inhibition of protein synthesis. At 6 h p.i. the medium of some cultures was replaced with Eagle's medium containing 2-deoxy-D-glucose (2 mg/ml). At various times the monolayers were assayed for the rate of protein synthesis.

slight decrease in their rate of leakage of LDH compared to the rate at the time of addition of the drug. When β-glucuronidase was examined in an experiment employing only 2-deoxy-D-glucose, the same results were obtained (Fig. 6).

In Fig. 7 haemadsorption is followed in this type of experiment. When 2-deoxy-D-glucose is added to infected cell cultures at 6 h p.i. a steady decrease in haemadsorption is seen over time. Therefore, when 2-deoxy-D-glucose is added to infected cells after leakage has begun, there is both a decrease in the rate of leakage and a decrease in virus antigens in the plasma membrane.

The drug, 2-deoxy-D-glucose, can also be shown to have a stabilizing effect on protein synthesis in infected cells. The effect on protein synthesis of adding the drug at 6 h p.i. can be seen in Fig. 8. Once protein synthesis has stabilized, no further inhibition occurs over the time period assayed.

**DISCUSSION**

It has been shown previously that CEF infected with NDV release some of their cellular enzymes into the overlay medium (Wilson, 1972). In the experiments described in this paper, it can be seen that the release of three different cellular enzymes begins at approx. 6 h p.i. and that the kinetics of their release indicates that as the infection progresses there is a progressive increase in the rate of release of cellular material. Using the silicone technique it has been shown that the plasma membranes of infected cells are damaged with respect to their permeability properties.

As has been seen with enzyme leakage, once penetration of dextran or sucrose has begun, the rate of penetration continues to increase as the infection progresses. It should also be noted that these curves in Fig. 3(a), (b) closely resemble the haemadsorption curve (Fig. 2c).
As the infection progresses there is neither a decrease in the water vol. of infected monolayers (Table 1), nor is there an increase in the extracellular vol. of infected cells assayed by the silicone technique. Assuming that there are no significant increases in the individual cell water vol. late in infection, we can conclude that no cell lysis is occurring. Whereas escape of enzymes during virus budding and increased blebbing cannot be ruled out, it is reasonable to conclude that a permeability change occurs in the plasma membranes of virus-infected cells, and that enzyme leakage is a consequence of this permeability change.

Two hypotheses have been proposed for the mechanism of the virus-infected permeability changes in the plasma membrane. The first is a lysosomal hydrolase mechanism. In our experiments we have seen that there is no time delay between lysosomal activation and plasma membrane leakage, such as has been seen with poliovirus (Blackman & Bubel, 1969). In addition, at no time during the infectious cycle is there an increase of soluble, cytoplasmic β-glucuronidase above control levels. It appears that there is no cause and effect relationship between lysosomal activation and plasma membrane leakage.

A second hypothesis for the mechanism of the observed permeability changes is that the alterations of the structural components of the plasma membrane result in altered functional properties. Because the new membrane antigens in infected cells are virus glycoproteins, experiments were designed using the glycoprotein synthesis inhibitor, 2-deoxy-d-glucose (Kaluza et al. 1972; Klenk et al. 1972). If 2-deoxy-d-glucose is present at the time of infection, the drug prevents any virus-induced damage to the plasma membrane and provides some protection against the virus-induced inhibition of protein synthesis (Table 4). Since production of virus is also inhibited, the interpretation of this experiment is not unambiguous. Experiments were therefore performed to study the effect of 2-deoxy-d-glucose when added to virus-infected cultures that were already leaking enzymes. Infected cell cultures release enzymes at progressively greater rates as the infection proceeds. If cycloheximide or 2-deoxy-d-glucose are added to infected cells at 6 h p.i., it is seen that the response to both drugs is identical (Fig. 5); that is, the rate of leakage decreases. These kinetics are inconsistent with an enzymatic hydrolysis mechanism for plasma membrane damage. Unless the hydrolases are extremely short-lived, it would be expected that the rate of leakage would continue to increase even if production or activation of the hydrolases were stopped. The cycloheximide data indicate that protein synthesis is required to alter the permeability properties of the membrane, and the 2-deoxy-d-glucose data indicate that glycoprotein synthesis specifically, is required.

When the presence of virus antigens in the membrane is assayed (Fig. 7), it is seen that after addition of 2-deoxy-d-glucose at 6 h p.i., the amount of haemadsorption does not continue to increase, but shows a decline with time. The stabilization of leakage concomitant with the decrease in virus antigens in the plasma membrane and the similarity between the curves for the kinetics of appearance of haemadsorption (Fig. 2c) and the penetration of the small molecule sucrose (Fig. 3b) are consistent with the hypothesis that the permeability changes are a result of an altered structural make-up of the plasma membrane.

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