Inhibition of Echovirus-12 Multiplication by N-Carbobenzoxy-d-Glucosamine

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SUMMARY
The glucosamine derivative, N-carbobenzoxy-d-glucosamine (NCBZG) inhibits the multiplication of Echovirus-12 and the synthesis of both virus RNA and protein at a stage in the virus growth cycle after attachment and penetration. However, the compound does not inhibit virus multiplication after the appearance of progeny virus nor after virus RNA has accumulated. Incorporation of radioactive glucosamine and choline into infected and uninfected cultures is inhibited by NCBZG as is the virus-induced increase in choline incorporation. The compound also prevents the appearance of radioactive choline in isolated membranous structures. The compound did not alter significantly the cellular RNA or protein synthesis, plating efficiency of the cells, their growth over a period of several days, nor the virus-directed inhibition of cellular RNA and protein. These findings suggest that the compound inhibits virus multiplication by its effect on the initiation of biosynthesis which appears to require membrane synthesis.

INTRODUCTION
Infection with Echovirus-12, similarly to other picornaviruses, stimulates the incorporation of choline and the synthesis of cytoplasmic membranes (Halperen, 1974), and induces the formation of membrane-bound vesicles (Skinner, Halperen & Harkin, 1968). These facts coupled with the reports showing that newly synthesized smooth cytoplasmic membranes play a role in the synthesis of specific macromolecules of poliovirus (Caliguiri & Tamm, 1969; Caliguiri & Tamm, 1970a, b), and the finding that a cellular, glucosamine-containing constituent is included within Echovirus-12 capsids (Halperen, Stone & Korant, 1973), suggested that membrane synthesis is also essential for the multiplication of Echovirus-12.

In this report the results of experiments with a glucosamine derivative, N-carbobenzoxy-d-glucosamine (NCBZG), which inhibits the multiplication of Echovirus-12 and prevents the incorporation into cells of the membrane precursors, glucosamine and choline are described. Evidence presented here suggests that the synthesis of cytoplasmic membranes may be interdependent with the initiation of synthesis of Echovirus-12 macromolecules.

METHODS
Cell cultures. The LLCMK₂ continuous line of monkey kidney cells (Hull, Cherry & Tritch, 1962) was propagated as monolayers in plastic flasks or in 100 x 20 mm plastic
Petri dishes (Falcon Plastics) in a growth medium consisting of Eagle's minimal essential medium (MEM) containing 6 % calf serum and 100 μg/ml streptomycin and 100 units/ml penicillin. Trypsin (0.25 %) was used to disperse the cells for passage.

Virus. Echovirus-12 (Travis strain) used in these experiments was propagated in monolayers of LLCMK2 cells and maintained in serum-free MEM or in Hanks' balanced salt solution (HBSS). In all experiments to be described cells were infected by adsorption of virus to monolayers at 23 °C with multiplicities of 30 to 40 plaque forming units (p.f.u.) per cell. Virus haemagglutination and plaque titrations were performed as described previously (Halperen, Eggers & Tamm, 1964a). For storage, virus-infected cells from each plate were scraped into 0.5 ml of phosphate buffered saline (PBS), pH 7.2 to 7.4 and kept at -20 °C.

Chemicals. N-carbobenzoxy-D-glucosamine and p-fluorophenylalanine were purchased from Sigma Chemical Company, St Louis, Missouri. 2-(α-hydroxybenzyl)-benzimidazole (HBB) was obtained through the kindness of Dr H. J. Eggers, Giessen, West Germany. The compound was used at 219 μM concentration by shaking overnight at 37 °C in protein-free medium to dissolve it before use. Reticulocyte standard buffer (RSB) was prepared as described previously (Caliguiri & Mosser, 1971). Radioactive chemicals were purchased from New England Nuclear Corporation, Boston, Massachusetts. In all experiments 3H-glucosamine was used at 3 μc/ml (sp. act. > 500 mCi/mmol); 3H-leucine was used at 1 μc/ml (sp. act. 5 Ci/mmol); 3H-uridine was used at 1 μc/ml (sp. act. 30 Ci/mmol). 3H-choline methyl chloride was used at 3 μc/ml (sp. act. 5 Ci/mmol).

Fractionation by discontinuous sucrose gradient centrifugation. Samples of cytoplasmic extracts were mixed with 60 % sucrose in reticulocyte standard buffer (RSB) to make the final sample preparation 30 % with respect to sucrose concentration, and 1.8 ml of sample was loaded into discontinuous sucrose gradients consisting of the following volumes and concentrations of sucrose in RSB: 0.5 ml 60 %, 0.9 ml 45 %, 0.8 ml 40 %, 1.8 ml sample in 30 %, 0.9 ml 25 %, 0.4 ml RSB. Samples were centrifuged at 27000 rev/min for 13 to 16 h and fractions were collected drop-wise after puncturing the bottom of the centrifuge tube. TCA-precipitates were collected on millipore filters and radioactivity determined in a scintillation counter.

RESULTS

Inhibition of virus multiplication with NCBZG

Initial studies of the effects of NCBZG on virus multiplication were carried out by incubating cultures with the compound for 19 h prior to infection, and keeping the compound on the cultures during the ensuing 8 h of a single cycle of virus multiplication. Under these conditions virus multiplication was completely inhibited. It was later found that 1 mmol of the compound could inhibit virus multiplication when added to cultures after virus had already been absorbed. A series of concentrations of the compound was then examined for virus-inhibitory activity. It was found that after incubating infected cultures with varying concentrations of the compound and examining the 8-h virus yields, 1 mm-NCBZG reduced the yield of virus progeny by greater than 99.9 %. Although 0.5 mm concentration reduced yields to less than 2 % of controls, there was variation among experiments with this concentration. However, since 1 mm concentration gave consistent inhibition of greater than 90 % of controls, this concentration was used to inhibit virus multiplication in all experiments to be described.

Phase microscopic examination of cultures revealed that up to 8 mm-NCBZG caused no gross morphological damage to cells in an 8 h period of incubation at 37 °C. At this concentration, however, in 19 h cells were loosened from the monolayers, while with
Fig. 1. Inhibition of Echovirus-12 when NCBZG was added to cultures at various times after infection. Replicate monolayer cultures (in 100 x 20 mm dishes) were infected and incubated at 37 °C. At the times indicated by the arrows duplicate dishes were drained and covered with 1 mM NCBZG in MEM and incubated until 8 h after infection at which time the dishes were drained and frozen. Also at the time of addition of the compound, duplicate dishes were drained and frozen. Subsequently, the monolayers were thawed, and virus was extracted and assayed by haemagglutination titration: △=△, 8-h yield in presence of NCBZG; ○—○, virus titre at times indicated.

4 mM NCBZG the monolayers remained intact for up to 48 h. When the compound was included in the medium during passaging of the cultures there was no reduction in the number of cells per dish for up to 72 h, in the presence of 4 mM, but there was a 40 to 60 % reduction with 8 mM in 48 h. The viability of the cells in the monolayers was also monitored by staining with neutral red and by counting trypsinized cells suspended in 1 % aqueous trypan blue. In all cases those cells that remained in the monolayers stained with neutral red and when trypsinized to remove them from the dish, were impermeable to the trypan blue.

Effect of NCBZG on various phases of the growth cycle of Echovirus-12

In order to define the stage(s) of virus reproduction sensitive to the action of NCBZG, the compound was added to cultures at varying times after infection. Cells were harvested after 8 h incubation and virus yields assayed by haemagglutination titration. It may be seen from the results summarized in Fig. 1 that the compound completely inhibited virus multiplication if added up to 3 h after infection but had almost no effect when added at later times. Addition at the fourth hour resulted in yields of 36 to 84 % maximum virus production (variation from three separate experiments).

Since the major antiviral effect of the compound occurs when the compound is present prior to infection, or at an early stage in the virus growth cycle, it was necessary to determine whether the compound could affect attachment of virus to susceptible cells or the haemagglutination assay procedure. Virus suspensions were incubated in the presence or absence of NCBZG for 4 h at 37 °C before dilutions were prepared for plaque titrations. From the results summarized in Table 1, it may be seen that NCBZG does not reduce the efficiency of plating of the virus or its ability to agglutinate erythrocytes.
Table 1. Lack of effect of NCBZG on the adsorption of Echovirus-12 to LLCMK_2 cells or to erythrocytes*

(a) p.f.u. adsorbed per plate

<table>
<thead>
<tr>
<th>Control</th>
<th>NCBZG</th>
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<td>19, 21, 22</td>
<td>20, 21, 22</td>
</tr>
</tbody>
</table>

(b) Haemagglutination titre before dilution for plaque titration†

<table>
<thead>
<tr>
<th>Control</th>
<th>NCBZG</th>
</tr>
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<tbody>
<tr>
<td>640</td>
<td>640</td>
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* One-tenth ml virus containing 1·1 × 10^6 p.f.u./ml was mixed with an equal volume of 4 mm-NCBZG dissolved in HBSS and incubated for 6 h at 37 °C; control virus was incubated with HBSS alone. Dilutions were then prepared and 0·5 ml volumes were allowed to adsorb to triplicate, washed and drained monolayer cultures (in 50 × 10 mm Petri dishes) for 30 min at 23 °C with rocking. Cultures were then covered with agar overlay and incubated till plaques appeared (3 to 5 days at 37 °C). Samples of dilution for the plaque assays were further diluted in 0·5 ml volumes of PBS for haemagglutination titrations.

† Mean titre from three separate experiments.

Fig. 2. Antiviral activity of NCBZG after reversal of long-term HBB-inhibition. Replicate cultures (in 100 × 20 mm dishes) were infected and incubated for 19 h with 219 μM-HBB in MEM. Dishes were then washed three times with ice-cold MEM and further incubated at 37 °C. At the indicated time intervals duplicate dishes were drained, covered with 1 mm-NCBZG and incubated until 5 h after HBB-reversal, at which time they were drained and frozen. As controls, duplicate cultures were also drained and frozen at the time of the addition of NCBZG. Subsequently, all dishes were thawed, scraped up into 0·5 ml volumes of PBS, frozen and thawed three times, clarified by centrifugation at 2000 g, and assayed for haemagglutinating activity. The bars represent 5 h yields when NCBZG was added at the times indicated. Control titres are shown by ○—○.
Fig. 3. Inhibition of virus protein and RNA synthesis by NCBZG. Replicate cultures were incubated in the presence of 219 µM-HBB for 19 h. After reversal of HBB by washing, duplicate cultures were incubated with either fresh HBB, with 1 mM-NCBZG or with HBSS. To duplicate cultures ³H-leucine or ³H-uridine was added and, at the indicated time intervals, cultures were extracted with TCA and radioactivity counted as previously described (Halperen, Stone & Korant, 1973). (a) ³H-leucine: ○—○, in HBSS; ×—×, in 1 mM-NCBZG; •—•, in 219 µM-HBB. (b) ³H-uridine: ○—○, in HBSS; ×—×, in 1 mM-NCBZG; •—•, in 219 µM-HBB.

The addition of NCBZG to cultures after infection, as described above, left open the possibility that multiple early virus-specific events might occur before adding or during incubation with the compound. To determine whether NCBZG inhibits processes essential for virus multiplication that occur after penetration, advantage was taken of the fact that 219 µM-HBB prevents virus multiplication while permitting virus attachment and penetration (Eggers & Tamm, 1962). Therefore, NCBZG was added to infected cultures after they had been incubated for 19 h in the presence of HBB and subsequently washed with MEM to reverse the virus inhibitory effects of the HBB.

From Fig. 2 it may be determined that after reversal of the inhibitory effect of the HBB, virus multiplication proceeds with a lag period of 1.5 to 2.0 h, approx. 1.25 to 1.5 h less than the usual latent period. When cultures were incubated for 19 h with HBB before reversing the inhibitory effect, NCBZG could suppress virus multiplication if added no later than 30 min after reversal. These results indicate that NCBZG probably inhibits virus multiplication at a stage after penetration. Although multiplication was prevented under these conditions it was not known whether either virus-specific RNA or protein could have...
been synthesized in the presence of the compound. To examine this question replicate cultures were incubated with HBB and then, after HBB reversal, incubated with and without NCBZG, together with either radioactive uridine or leucine. Since virus-induced inhibitions of the synthesis of cellular RNA and protein continue in the presence of HBB (Halperen, 1967; Bablanian, 1972) the accumulation of TCA-precipitable radioactive uridine and leucine would represent the virus-specific RNA and protein synthesis in the virtual absence of cellular RNA and protein synthesis.

In Fig. 3(a) is shown the result of accumulation of radioactive leucine, after HBB reversal, in infected cells incubated with fresh 219 μM-HBB, with 1 mM-NCBZG, or with HBSS with neither HBB nor NCBZG. Fig. 3(b) shows the result of accumulation of uridine under the same experimental conditions. It is evident from this data that NCBZG prevents virus protein synthesis after virus penetration, since in the presence of NCBZG, leucine accumulation was the same as that in the continued presence of HBB. However, approx. 38% of the control virus RNA was synthesized in the presence of the NCBZG. The reduced radioactivity noted in the cultures not treated with NCBZG is probably due to loss of cells from the dishes.

To determine whether NCBZG could arrest virus multiplication after virus RNA synthesis was already in progress, the compound was added to infected cultures that had been
first incubated in the presence of p-fluorophenylalanine (FPA) for 6 or 7 h before the cultures were washed free of the compound and incubation continued in the presence or absence of NCBZG. In the presence of 4 mM-FPA infected cultures produced 50 to 80% of the virus RNA that is normally synthesized in the absence of the compound, while neither virions nor other viral haemagglutinating particles are produced (Halperen, Eggers & Tamm, 1964b). The results of these experiments, depicted in Fig. 4, show that on reversal of the inhibitory effect of FPA, virus production is initiated with virtually no lag period and the kinetics of synthesis is the same in the presence or absence of NCBZG. These results taken together with the early time in the virus growth cycle after which NCBZG no longer arrests virus multiplication (see Fig. 2) suggests that the effect of the compound is on a stage immediately preceding or involved in the initiation of virus biosynthesis.

Lack of effect of NCBZG on virus-induced inhibition of synthesis of cellular protein and RNA, and lack of effect on cellular protein and RNA synthesis

Similarly to other picornaviruses, Echovirus-12 infection causes the inhibition of host cell protein and RNA synthesis (Halperen, 1967; Bablanian, 1972). To examine whether NCBZG could prevent or modify this virus-induced effect, infected and ‘mock’ infected cultures were pulsed for 15 min with radioactive uridine or with an amino acid mixture after intervals of incubation at 37 °C, in the presence or absence of 1 mM-NCBZG. That NCBZG does not change this virus-specific function may be seen from the results summarized in Fig. 5 and 6. The compound is also seen not to have any pronounced effect on the synthesis of RNA and protein in uninfected cultures.
Fig. 6. Lack of effect of NCBZG on virus-induced inhibition of cellular RNA synthesis and lack of effect on RNA synthesis in uninfected cells. Cultures were infected and treated identically to those used for the experiments described in Fig. 6, except that $^3$H-uridine was used instead of leucine; △—△, uninfected without NCBZG; □——□, uninfected with NCBZG; ○—○, infected with NCBZG; ●—●, infected without NCBZG.

Inhibition of choline incorporation into uninfected and infected cultures by NCBZG

The ability to stimulate choline incorporation and to induce the development of membrane-bound vesicles in infected cells is another property shared by Echovirus-12 with other picornaviruses (Skinner et al., 1968; Halperen, 1974). Cellular membranes are known to contain derivatives of glucosamine as well as choline, and radioactive choline has been used to label specifically cytoplasmic membranes in virus-infected cells (Amako & Dales, 1967b). Furthermore, while choline incorporation is stimulated by Echovirus-12 and inhibited by NCBZG (Fig. 7), the rate of incorporation of glucosamine was found to remain constant (Halperen et al., 1973). It was therefore of special interest to determine whether NCBZG would have a similar or different effect on the incorporation of glucosamine. Replicate cultures were infected, incubated in the presence of radioactive glucosamine, and duplicate cultures were examined for TCA-precipitable radioactivity at hourly intervals. Table 2 summarizes the results of experiments showing the effects of NCBZG on the incorporation of glucosamine into infected cells. It is clear from this data in Table 2 and Fig. 7 that NCBZG inhibits the incorporation of both membrane precursors, glucosamine and choline, into TCA-precipitable material in these cultures, although the greater effect appears to be on the incorporation of glucosamine.

Choline was previously shown to specifically label the membrane-bound vesicles which developed in mengovirus-infected cells (Amako & Dales, 1967b). Also, when choline-labelled cytoplasmic membranes were fractionated from poliovirus-infected cells by discontinuous sucrose gradient centrifugation, distinct classes of membranes were found to be associated
NIZBZG Echovirus-12 inhibition

Fig. 7. Inhibition of choline incorporation into virus-infected and uninfected cultures. Virus infected or 'mock' infected cultures were incubated in the presence or absence of 1 mM-NCBZG. At the indicated times, \(^{3}H\)-choline was added for 30 min before the cultures were chilled on crushed ice and TCA-precipitable radioactivity determined: \(\triangle \)---\(\triangle\), infected without NCBZG; \(\square \)---\(\square\), infected with NCBZG; \(\bigcirc \)---\(\bigcirc\), uninfected without NCBZG; ------, uninfected with NCBZG.

Table 2. Effect of NCBZG on glucosamine incorporation*

<table>
<thead>
<tr>
<th>Hours after infection</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>(^{3}H)-glucosamine</td>
<td>190</td>
<td>430</td>
<td>860</td>
<td>1975</td>
</tr>
<tr>
<td>(^{3}H)-glucosamine + NCBZG</td>
<td>50</td>
<td>90</td>
<td>140</td>
<td>290</td>
</tr>
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</table>

* Infected cultures were treated similarly to those used for the experiments described in Fig. 7 with the exception that \(^{3}H\)-glucosamine was incubated with the cultures for each of the indicated periods in the presence or absence of 1 mM-NCBZG. TCA-precipitable radioactivity was determined as in the previous experiments.
Fig. 8. Groups of six infected or 'mock' infected monolayer cultures (in 100×20 mm plastic Petri dishes) were incubated in the presence or absence of 1 mM-NCBZG for 6 h at 37 °C. 3H-choline was added to each culture 30 min before the end of the incubation period. Cultures were then pressed into crushed ice, washed twice with ice-cold PBS, once with RSB, and the cells were then scraped up into 5×10⁻⁵ M-MgCl₂ in which the cells were allowed to swell before disrupting with a glass Dounce homogenizer. Nuclei and cell debris were separated by centrifuging at 900 g, and the mitochondria removed by centrifuging at 11000 g. The supernatant fluid was then fractionated by discontinuous sucrose gradient centrifugation and TCA-precipitable radioactivity determined as described in the Methods: ×-×, uninfected without NCBZG; ○-○, infected without NCBZG; △-△, uninfected with NCBZG; ●-●, infected with NCBZG.

with the synthesis of specific virus macromolecules (Caliguiri & Tamm, 1969). Experiments were therefore undertaken to examine the effects of Echovirus-12 infection on the synthesis of cytoplasmic membranes and to determine whether NCBZG could modify the choline-labelling of cytoplasmic membranes extracted and isolated from virus-infected or 'mock'-infected cultures. Cytoplasmic extracts, prepared as described in Methods, were made up to a final concentration of 30 % sucrose (v/v) in reticulocyte standard buffer, and then layered into discontinuous sucrose gradients before centrifugation.

TCA-precipitable radioactive counts of fractions collected gave the patterns depicted in Fig. 8. In the figure are represented three classes of choline-labelled structures isolated from uninfected and Echovirus-12-infected cultures. While choline labels the most rapidly
sedimenting membranes in uninfected cells, the lighter membranes show more labelling with choline after infection. It may also be seen that in all cases incubation with NCBZG abolishes the choline labelling of cytoplasmic membranes in both uninfected and infected cultures. Although not shown here, treatment of cytoplasmic extracts with 0.9% DOC before dialysis against 500 volumes of RSB and processing for fractionation by discontinuous sucrose gradient centrifugation yielded a considerable loss of TCA-precipitable radioactivity and a change to more polydisperse labelling in the lighter membrane region.

**DISCUSSION**

The virus-inhibitory activity of N-carbobenzoxy-D-glucosamine is characterized by the following features. The compound inhibits virus multiplication when added to cultures before infection, or after attachment and penetration, but not after newly synthesized RNA had accumulated nor after the appearance of progeny virus. NCBZG also inhibits the incorporation of the membrane precursors glucosamine and choline into infected and uninfected cultures, arrests the virus-directed, increased choline incorporation and prevents the appearance of radioactive choline in isolated membrane fractions.

These findings taken together with the following lines of evidence suggest that virus-directed synthesis of cytoplasmic membranes and the initiation of synthesis of Echovirus-12 macromolecules are interdependent processes. Firstly, it has been established that in cells infected with several picornaviruses there is a close temporal relationship between the initiation of virus RNA biosynthesis and the formation of smooth cytoplasmic membrane-bound vesicles (Dales et al. 1965; Amako & Dales, 1967a, b; Caliguiri & Tamm, 1969; 1970a, b; Mosser, Caliguiri & Tamm, 1972). It was also shown that poliovirus RNA synthesis occurs in membranous structures (Girard, Baltimore & Darnell, 1967). Data presented here and previously reported indicates that similar relationships exist for Echovirus-12 infected cultures (Skinner et al. 1968). Furthermore, special sedimentation classes of membranes appear also to serve as specific sites for the synthesis of virus RNA and virus protein (Caliguiri & Tamm, 1970b; Roumiantzeff, Summers & Maizel, 1971), and recent evidence suggests that at least the association with membranes, if not membrane synthesis per se, is required for the assembly of virions (Yin & Knight, 1975). Secondly, it was previously shown that the latest time after infection at which the addition of HBB to infected cultures still prevented the development of membrane-bound vesicles (3.0 h post infection) was the same time that the compound could still also prevent the synthesis of capsid protein (Skinner et al. 1968). Thirty minutes later, addition of HBB resulted in the complete development of membrane-bound vesicles along with a limited yield of virus capsids. These findings, together with the present data showing that NCBZG antiviral activity is virtually lost when the compound is added to cultures later than 30 min after reversal of long term virus inhibition by HBB, plus the finding that virus protein synthesis is more consistently sensitive to inhibition by NCBZG than virus RNA synthesis, along with the report that poliovirus-induced increase in choline incorporation may proceed in the absence of poliovirus RNA synthesis, but not without virus protein synthesis (Penman, 1965) suggests that the ‘triggering’ of membrane synthesis and capsid protein synthesis are under the control of an HBB-inhibitable process. This process could be the synthesis of the early virus ‘polyprotein’ of Echovirus-12 (Korant, Lonberg-Holm & Halperen, 1970; Korant & Halperen, 1975) or the synthesis of the virus RNA polymerase.

Since virus inhibitory concentrations of NCBZG had little effect on the plating efficiency, early culture growth, or cellular RNA and protein synthesis, it is probable that only a small
proportion of the total cytoplasmic membranes are altered in response to virus infection, and this may account for the reported multiplication of poliovirus in the presence of the inhibitors of mitosis, vinblastine and colcemide (Kuhn, St John & Wolff, 1975). NCBZG also had no effect on the virus-induced inhibitions of the synthesis of cellular RNA and protein, indicating that the membrane synthesis-stimulating function of Echovirus-12 is different from that responsible for causing the inhibitions of cellular metabolism. It should also be mentioned that virus-inhibitory concentrations of NCBZG were within the same order of magnitude as that used with guanidine hydrochloride for the inhibition of picornavirus multiplication (Crowther & Melnick, 1961). Guanidine hydrochloride, similarly to HBB and NCBZG, is also ineffective in preventing virus-induced inhibitions of the synthesis of cellular macromolecules (Bablanian, 1972).

In experiments not detailed in this report NCBZG also was found to have a marked antiviral effect on equine rhinovirus, while poliovirus multiplication in the same culture type was less sensitive. Further specificity of the compound was shown by the findings that less than antiviral concentrations of NCBZG for Echovirus-12, which showed no effect on LLCMK_2 cells, were quite toxic to HeLa cells, causing marked cellular degeneration within an 8 h period of incubation.

These findings are consistent with the previously reported findings of different sedimentation classes of cytoplasmic membranes that can be isolated from different types of cell cultures, and the differences in classes of membranes synthesized in response to infection with different viruses or due to changes in incubation temperature (Halperen, 1974). These studies also indicate that NCBZG or other possible selective inhibitors of membrane synthesis may be used as tools for examining the regulation of virus-specific functions, as well as for the potential characterization of relationships among cell cultures.

Kasia W. Aist and Angelina Lombardo provided fine technical assistance for these experiments. I wish to thank Dr James Kauer of the Central Research Department of E.I. Du Pont de Nemours, Wilmington, Delaware for suggesting the use of the glucosamine derivative NCBZG, and to express my appreciation to the virology group in the Central Research Department, at whose facility these experiments were performed.

REFERENCES


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