Selective Inhibition of Protein Synthesis in Virus-Infected Mammalian Cells

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A number of translation inhibitors were tested for their effects on both control and encephalomyocarditis virus-infected mouse 3T6 cells. The virus-infected cells were specifically inhibited by gougerotin, edeine, and blasticidin S, whereas these drugs failed to penetrate into uninfected cells. Inhibition of infected cells by gougerotin became apparent when the synthesis of viral proteins commenced, suggesting that the latter process is accompanied by a permeability change in the cells that allows uptake of the drug. This permeability change was not observed in cells treated with cycloheximide soon after viral infection, although treatment with actinomycin D did not prevent inhibition by gougerotin. It is possible, therefore, that a specific viral protein is involved in the permeability change of the plasma membrane. Moreover, gougerotin was unable to inhibit protein synthesis in the presence of zinc ions, thus preventing gougerotin from entering into the infected cell. Membrane leakiness was not restricted to the encephalomyocarditis virus-3T6 system; it was also observed in mengovirus-infected 3T6 cells, Semliki Forest virus-infected BHK cells, and simian virus 40-infected CV1 cells at the time in which the synthesis of late proteins is maximal.

Normal cell metabolism is disrupted upon viral infection. Recently we proposed that this was caused by an alteration in membrane function resulting from viral proteins becoming integrated into the plasma membrane and altering its permeability properties (3, 5). Experiments which support this suggestion have shown that infection of mouse cells with encephalomyocarditis (EMC) virus makes the membranes leaky to monovalent ions, allowing entry of sodium into the cytoplasm with a release of potassium ions into the surrounding medium (9). Such changes in concentrations of ions in the cytoplasm might be responsible for many of the metabolic alterations observed after viral infection of susceptible cells (1, 3, 18). Furthermore, the in vitro synthesis of viral proteins requires ion concentrations different from those found to be optimal for efficient translation of cellular mRNA's (5).

Changes in the permeability of plasma membranes after viral infection allow not only ionic changes in the cytoplasm but also the redistri-

bution of other small metabolites (6, 17), and induction of membrane leakiness could be a general property shared by many viruses that infect either eucaryotic or procaryotic organisms (3, 6, 17). Using GppCH₂p, a nucleotide analog that inhibits protein synthesis in cell-free systems, we observed that the membrane of an infected cell became permeable at the onset of viral protein synthesis. Inhibition by GppCH₂p was specific for infected cells (4), favoring the interpretation that such cells become permeable to GppCH₂p and thus allow entry of this compound into the cytoplasm, where protein synthesis is then inhibited.

It remained to be demonstrated whether permeability of infected cells was specific only to GppCH₂p or whether any small compound, irrespective of its structural formula, could permeate cells infected by viruses known to induce the cytopathic effect (18). Furthermore, it was of interest to screen certain antibiotics known to preferentially affect protein synthesis in cell-free systems, to determine if these were also specific inhibitors of virus-infected cells. The experiments reported here were designed to answer these questions and to ascertain whether any relationship exists between the particular stage of viral development and the induction of membrane permeability in infected cells.

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MATERIALS AND METHODS

Materials. Radioactive compounds were purchased from The Radiochemical Centre, Amersham, England. GppCH$_2$p was from Boehringer; gougerotin was from Calbiochem; hygromycin B was a generous gift from Lilly; the other inhibitors were kindly given to us by D. Vázquez. Petri dishes were purchased from Nucantom, Denmark.

Cells and viruses. Baby hamster kidney cells (BHK), clone 21, mouse fibroblast 3T6 cells (3T6), and monkey cells (CV1), were propagated in 100-mm petri dishes containing 10 ml of Eagle medium as modified by Dulbecco (E$_4$D), supplemented with 10% fetal calf serum (E$_4$D$_{10}$). Chick embryo fibroblast cells (CEF) and Rous sarcoma virus (RSV)-transformed CEF were kindly provided by F. Tato.

Semliki Forest virus (SFV), EMC, mengovirus, and simian virus 40 (SV40) were grown on BHK, 3T6, 3T6, and CV1 cells, respectively. The fraction obtained after removal of cell debris by centrifugation was used as a source of the corresponding virus to infect the cells.

Conditions of infection and labeling with [^{35}S]methionine. (i) EMC virus-3T6 cell system. 3T6 cells were grown in 30-mm petri dishes containing 2 ml of E$_4$D$_{10}$. When the cell monolayer was confluent, the medium was removed, and samples were mock-infected or infected with EMC virus (1 to 2 PFU/cell). After incubation for 1 h at 37°C, the medium was removed, 1 ml of E$_4$D medium containing 5% calf serum (E$_4$D$_5$) was added, and the dishes were incubated for a further 4 h at 37°C. The medium was then replaced by 0.5 ml of E$_4$D medium without methionine and supplemented with 1% calf serum (E$_4$D$_1$-Met). The indicated concentration of inhibitor was then added, and incubation was immediately continued for 30 min at 37°C. After this time, 2 µl of [^{35}S]methionine (885 Ci/mmol, 2 mCi/ml) was added, and the dishes were further incubated for 1 h at 37°C.

(ii) Mengovirus-3T6 cell system. Experimental conditions were as described above except that the multiplicity of infection was 5 PFU/cell. The dishes were incubated for 5 h at 37°C before E$_4$D$_5$ medium was replaced by E$_4$D$_5$-Met medium and then incubated for 1 h at 37°C after the addition of the inhibitors and before the addition of [^{35}S]methionine.

(iii) SFV-BHK cell system. BHK cells were grown and subsequently mock-infected or infected (50 PFU/cell) by SFV as described above, except that after virus absorption the medium was removed and replaced by 2 ml of E$_4$D medium containing 2 mg of bovine serum albumin per ml. The dishes were then incubated for 5 h at 37°C. Proteins were labeled by first incubating the cells in the presence of the inhibitor for 1 h and then labeling with 3 µl of [^{35}S]methionine (885 Ci/mmol, 2 mCi/ml) for another hour.

(iv) SV40-CV1 cell system. CV1 cells were grown in 30-mm petri dishes containing 2 ml of E$_4$D$_{10}$. Subconfluent cell monolayers were mock-infected or infected with SV40 (70 PFU/cell). After absorption for 2 h at 37°C (0 time in the infection process), the medium was removed, 2 ml of E$_4$D medium containing 5% fetal calf serum was added, and the dishes were incubated at 37°C. Protein synthesis was measured by

labeling the proteins with [^{35}S]methionine at 17 to 18 and 41 to 42 h postinfection. For this purpose, the medium was removed and replaced by 0.5 ml of E$_4$D$_1$-Met medium containing the inhibitor at the indicated concentration. The dishes were incubated for 1.5 h at 37°C. After this time, 4 µl of [^{35}S]methionine (885 Ci/ mmol, 2 mCi/ml) was added, and the incubation was continued for 1 h at 37°C.

Polyacrylamide gel electrophoresis. After the cells were incubated for 1 h at 37°C in the presence of [^{35}S]methionine (systems i, ii, iii, and iv above), the medium was removed, and the cell monolayer was washed with 2 ml of phosphate buffer. The cells were dissolved in 0.15 ml of 0.1 N NaOH containing 0.1% sodium dodecyl sulfate and 0.15 ml of sample buffer. Each sample was sonically disrupted to reduce the viscosity and heated to 90°C for 10 min. Samples of 10 µl were analyzed by polyacrylamide gel electrophoresis using 15% acrylamide gels. The gels were run overnight at 25 V. When the bromophenol blue tracker dye reached the bottom of the gels, electrophoresis was stopped and the gels were stained and destained. Fluorography with dimethyl sulfoxide–2,5-diphenyloxazole (20%, wt/wt) was then carried out, and the gels were dried. The gels were finally subjected to autoradiography (Kodak XH1 film), and the developed film was analyzed on a densitometer.

Measurement of protein synthesis. (i) EMC virus-3T6 cell system. 3T6 cells were grown and subsequently mock-infected or infected (1 to 2 PFU/cell) as described above. At 4 h after medium addition, the indicated amount of inhibitor was added directly to the medium, and the incubation was continued for 30 min. [3,4-^{3}H]proline (10 µl per dish; 41 Ci/mmol, 0.2 mCi/ml) was added, and after 1 h of incubation at 37°C the medium was removed. The cell monolayer was washed with 3 ml of saline phosphate buffer and 1 ml of 5% trichloroacetic acid. The precipitated cell monolayer was thoroughly washed three times with ethanol. The cell monolayer was dried under an infrared lamp, 0.5 ml of 0.1 N NaOH was added, and the mixture was kept for 15 min at room temperature. Samples of 0.3 ml were withdrawn and counted in a Packard scintillation spectrometer.

(ii) RSV-CEF cell system. CEF cells and Rous sarcoma virus (RSV)-transformed CEF cells were grown in 30-mm petri dishes containing 2 ml of E$_4$D$_{10}$. When the cell monolayer was subconfluent (4 × 10^5 cells per dish), the medium was removed and replaced by 1 ml of E$_4$D$_5$ containing the indicated concentration of inhibitor. The dishes were then incubated for 2 h or overnight (15 h) at 37°C. [^{35}S]methionine (10 µl; 885 Ci/mmol, 2 mCi/ml) was then added to the dishes, and the incubation was continued for 2 h at 37°C. Samples were processed, and radioactivity was estimated as described above.

RESULTS

Many antibiotics block protein synthesis (D. Vázquez, in H. V. R. Arnstein, ed., *MTP International Review of Biochemistry*, Series II; vol. 7: *Synthesis of Amino Acids and Proteins*, in press). We screened 19 drugs for their abilities

to inhibit specifically EMC-infected mouse 3T6 cells (Table 1). Certain compounds, including actinobolin, adrenochrome, blasticidin S, edeine A_1, edeine complex, gougerotin, polydextran sulfate, and pyrocatechol violet have almost no effect on translation in normal 3T6 cells. Since these compounds block protein synthesis in cell-free systems, it seems likely that they cannot cross the plasma membrane. In contrast, some compounds, like cycloheximide, pactamycin, emetine, and tubulosine, appear to be more pow-

TABLE 1. *Comparative effects of a number of inhibitors of protein synthesis on the incorporation of [3,4-^{3}H]proline by 3T6 cells and EMC-infected 3T6 cells*[a]

Inhibitor	Concn	Protein synthesis (% of control)	
		3T6	3T6 + EMC
Actinobolin	10^{-4} M	95	95
Adrenochrome	10^{-4} M	90	87
Amicetin	4×10^{-5} M	91	87
Amicetin	5×10^{-4} M	18	26
Anisomycin	5×10^{-8} M	59	54
Anisomycin	5×10^{-7} M	5	10
Aurintricarboxylic acid	7×10^{-4} M	99	88
Aurintricarboxylic acid	10^{-3} M	5	16
Blasticidin S	10^{-5} M	102	57
Blasticidin S	10^{-4} M	85	20
Chartreusin	10^{-5} M	88	85
Chartreusin	10^{-4} M	32	35
Cycloheximide	10^{-7} M	59	69
Cycloheximide	10^{-6} M	13	24
Edeine A_1	7.5 μg/ml	103	74
Edeine A_1	75 μg/ml	134	56
Edeine complex	750 μg/ml	124	75
Emetine	5×10^{-8} M	71	94
Emetine	5×10^{-7} M	32	61
Fusidic acid	5×10^{-5} M	80	89
Fusidic acid	5×10^{-4} M	3	16
Gougerotin	10^{-5} M	106	60
Gougerotin	10^{-4} M	100	20
Pactamycin	5×10^{-9} M	44	79
Pactamycin	5×10^{-8} M	2	6
Polydextran sulfate	10^{-6} M	96	98
Polydextran sulfate	10^{-5} M	94	112
Puromycin	5×10^{-6} M	84	100
Puromycin	10^{-4} M	3	10
Pyrocatechol violet	2×10^{-4} M	92	116
Sparsomycin	4×10^{-6} M	87	84
Sparsomycin	4×10^{-5} M	33	10
Tubulosine	4×10^{-8} M	79	86
Tubulosine	10^{-7} M	16	53
Tubulosine	4×10^{-7} M	4	13

[a] Protein synthesis was estimated by measuring incorporation of [3,4-^{3}H]proline as described in the text. Antibiotics were added to the culture medium dissolved in 10 μl of water (edeine, gougerotin, adrenochrome, puromycin, blasticidin S, actinobolin, anisomycin, fusidic acid, cycloheximide, emetine, polydextran sulfate, pactamycin), 10 μl of ethanol (pyrocatechol violet, aurintricarboxylic acid), or 5 μl of dimethyl sulfoxide (sparsomycin, tubulosine, amicetin, chartreusin). Either 8,600 or 2,600 cpm were incorporated in the controls with 3T6 cells and EMC-infected 3T6 cells, respectively.

erful inhibitors of protein synthesis in intact cells than in cell-free systems (10, 16). The protein-synthesizing apparatus in the cell may be particularly sensitive to inhibition by these antibiotics; alternatively, there may be preferential accumulation of these inhibitors by the intact cell (11). The latter possibility explains why these inhibitors are less active on virus-infected than on uninfected cells, because the transport of these compounds should be impaired in the former. Clearly, however, gougerotin, blasticidin S, and edeine cause a specific inhibition of protein synthesis in EMC-infected cells, as measured by the incorporation of [3,4-^{3}H]proline into protein, whereas actinobolin, adrenochrome, and pyrocatechol violet have little or no effect. However, these three latter compounds are relatively poor inhibitors of protein synthesis in vitro (L. Carrasco, Ph.D. Thesis, University of Madrid, 1974).

More detailed studies were then carried out on the action of gougerotin, blasticidin S, and edeine on EMC-infected 3T6 cells. Control and EMC-infected 3T6 cells were labeled with [^{35}S] methionine in the presence of various amounts of the required inhibitors, and the proteins synthesized were analyzed by polyacrylamide gel electrophoresis.

The infected cells were synthesizing viral proteins when membrane leakiness occurred, and the antibiotics blocked the synthesis of all proteins, both cellular and viral (Fig. 1). No inhibition of the synthesis of any particular protein of uninfected cells was detected. Of the three antibiotics tested, gougerotin showed the most clear discrimination between normal and infected cells, and consequently we chose this inhibitor for our subsequent studies.

Assuming that the inhibition of protein synthesis is caused by a change in permeability, it was important to determine at what time after infection this occurred. Figure 2 illustrates that, under our experimental conditions and using a low multiplicity of infection (1 to 2 PFU/cell), viral protein synthesis started at 4 h postinfection and continued for another 3 h. Inhibition by gougerotin became apparent at 5 h postinfection, at which time viral proteins were being synthesized, whereas total protein synthesis was decreasing. These results indicate that the synthesis of viral proteins occurs just prior to membrane leakiness, at least under conditions where a low multiplicity of infection is used.

In further experiments, infected cells were treated for 4 h with cycloheximide at a concentration high enough to block protein synthesis completely before addition of gougerotin. Subsequently the medium was removed and replaced by fresh medium. Under these conditions, membrane leakiness, as measured by the ability
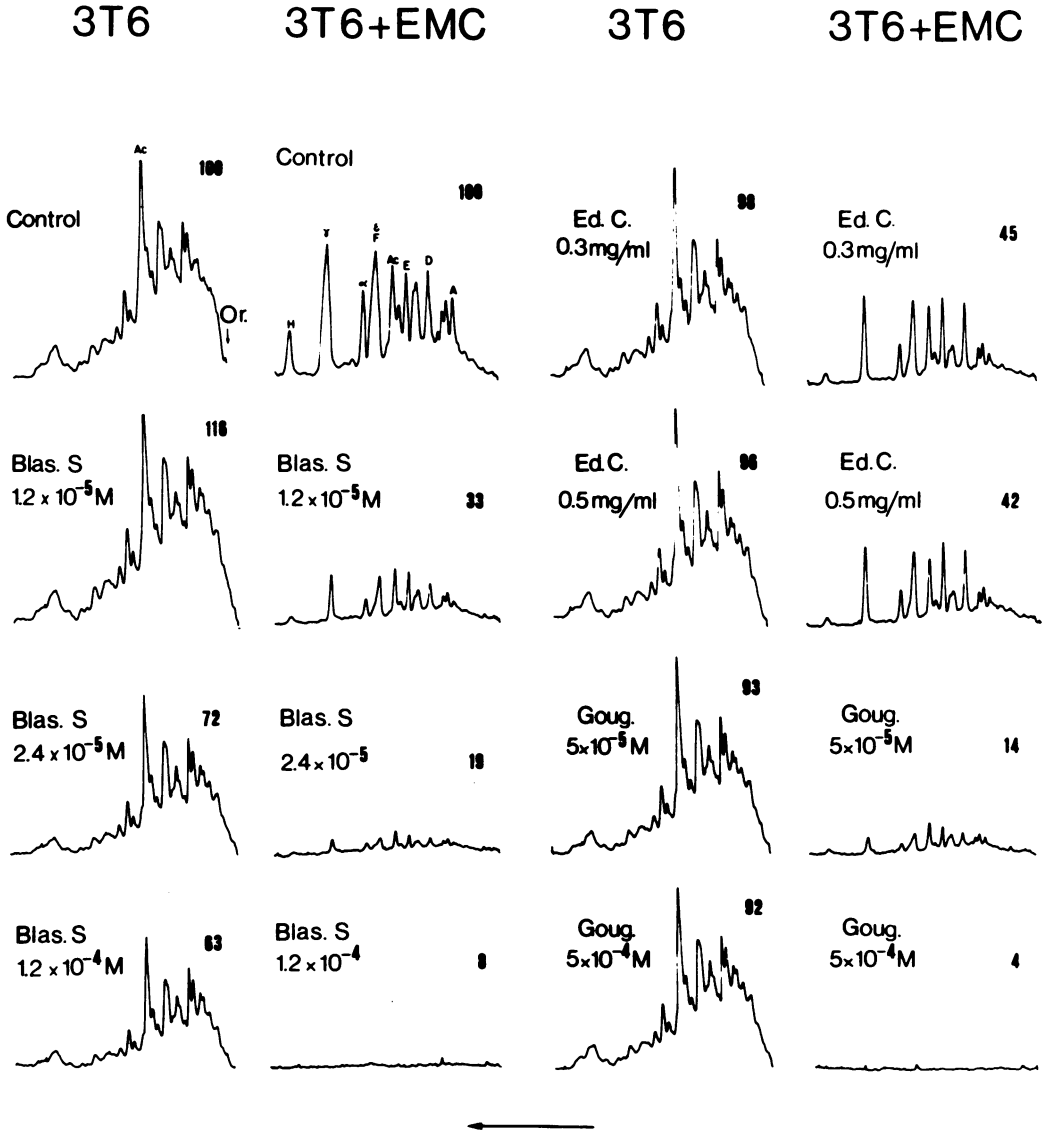
FIG. 1. *Membrane leakiness in EMC-infected 3T6 cells. Densitometric scans were carried out on the autoradiograms obtained as described in the text. The antibiotics used, along with the concentration employed in the corresponding culture medium, are shown beside each scan. The number on the right-hand side of each scan indicates the percentage of incorporation relative to the corresponding control. Or, Origin of sample application; Ac, actin; Blas. S, blasticidin S; Ed. C., edeine complex; Goug., gougerotin. Arrow indicates direction of migration.*

of gougerotin to inhibit translation, does not occur. Apparently, the permeability change is dependent on protein synthesis in the infected cell (Fig. 3). In contrast to this result, treatment of infected cells with actinomycin D did not suppress the development of permeability to gougerotin, further strengthening the possibility that the synthesis of viral proteins is necessary for modification of the membrane. Infected cells were also treated with zinc ions, which are known to inhibit protease activity (13), and this prevented inhibition by gougerotin (Fig. 3). It is possible, therefore, that membrane leakiness requires the synthesis of mature viral proteins or, alternatively, that zinc ions are able in some way to seal the leaks in the membrane.

The next step we pursued was to check membrane leakiness in other virus-cell systems. GppCH$_2$p, blasticidin S, edeine complex, and gougerotin inhibited protein synthesis in men-
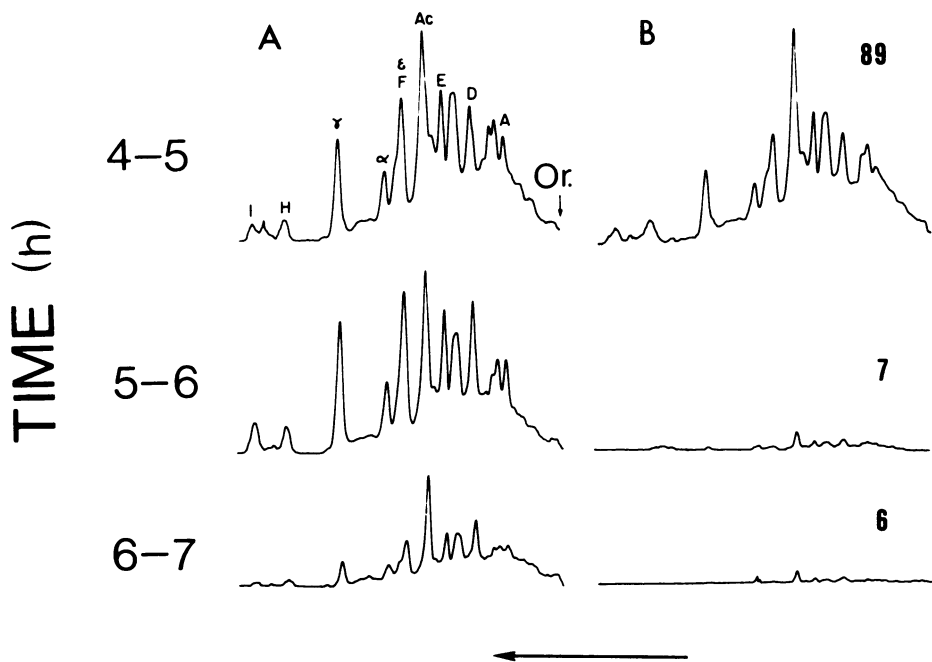
FIG. 2. *Time course of the inhibition by gougerotin of protein synthesis in EMC-infected 3T6 cells. 3T6 cells were grown and infected with EMC virus as described in the text. After virus absorption for 1 h at 37°C, the medium was removed and replaced by 1 ml of E_4D_5 medium, this moment corresponding to 0 time on the figure. Every hour, the medium was removed and replaced by 0.5 ml of E_4D_1-Met medium containing 2 µl of [^{35}S]methionine (885 Ci/mmol, 2 mCi/ml) and, where indicated, 0.1 mM gougerotin. The dishes were further incubated for 1 h at 37°C. Samples of 10 µl were withdrawn, analyzed by polyacrylamide gel electrophoresis, and processed as described in the text. The times show the labeling period with [^{35}S]methionine. Other conditions were as indicated in the legend to Fig. 1. Column A, Control cells; column B, cells treated with 0.1 mM gougerotin. Arrow indicates direction of migration.*

govirus-infected 3T6 cells, but their effect in control cells was much less potent (Fig. 4).

SFV belongs to the *Togaviridae* family (18). Hygromycin B and edeine complex inhibited protein synthesis in SFV-infected BHK cells, whereas their effect in control cells was much less potent (Fig. 5). On the other hand, gougerotin and blasticidin S had little or no effect on control cells, but their inhibitory effect upon SFV-infected BHK cells was smaller than in the case of 3T6 cells infected by EMC virus (Fig. 1) or mengovirus (Fig. 4), probably indicating that the altered permeability to drugs of togavirus-infected mammalian cells is less acute than in the case of picornavirus-infected cells.

SV40 belongs to the *Papovaviridae* family and is unrelated to picornaviruses and togaviruses from the philogenetic point of view. Infection by SV40 of a susceptible cell can produce either lysis or transformation, depending on the host cell (19). We analyzed SV40-promoted membrane leakage using the permissive CV1 cell line that gives rise to productive infection and cell lysis. Because SV40 development takes place over a few days, membrane leakage was mea-

sured at 17 and 41 h postinfection. Edeine complex, gougerotin, and hygromycin B had little or no effect on control CV1 cells, whereas they blocked protein synthesis during the late phase of infection, when the synthesis of the viral coat protein VP1 was maximal (Fig. 6). No effect was observed at the beginning of the late phase, indicating that the alteration of the membrane was brought about late in infection. These results are in agreement with recent observations in SV40-infected CV1 cells (15).

RSV belongs to the *Retroviridae* family, and its development does not kill the host cell. Moreover, it does not induce shutoff of host cell protein synthesis (19); hence, it is not included in the membrane leakage model (3). However, it has been proposed that transformed cells could have a membrane with a few and regulable small pores (8), and, in fact, it was found that transformed cells show a number of changes in their plasma membrane as compared with normal cells (14). Among these changes in permeability there is an increase in sugar and amino acid transport (12, 20). Moreover, the membrane of transformed cells becomes leaky to some en-
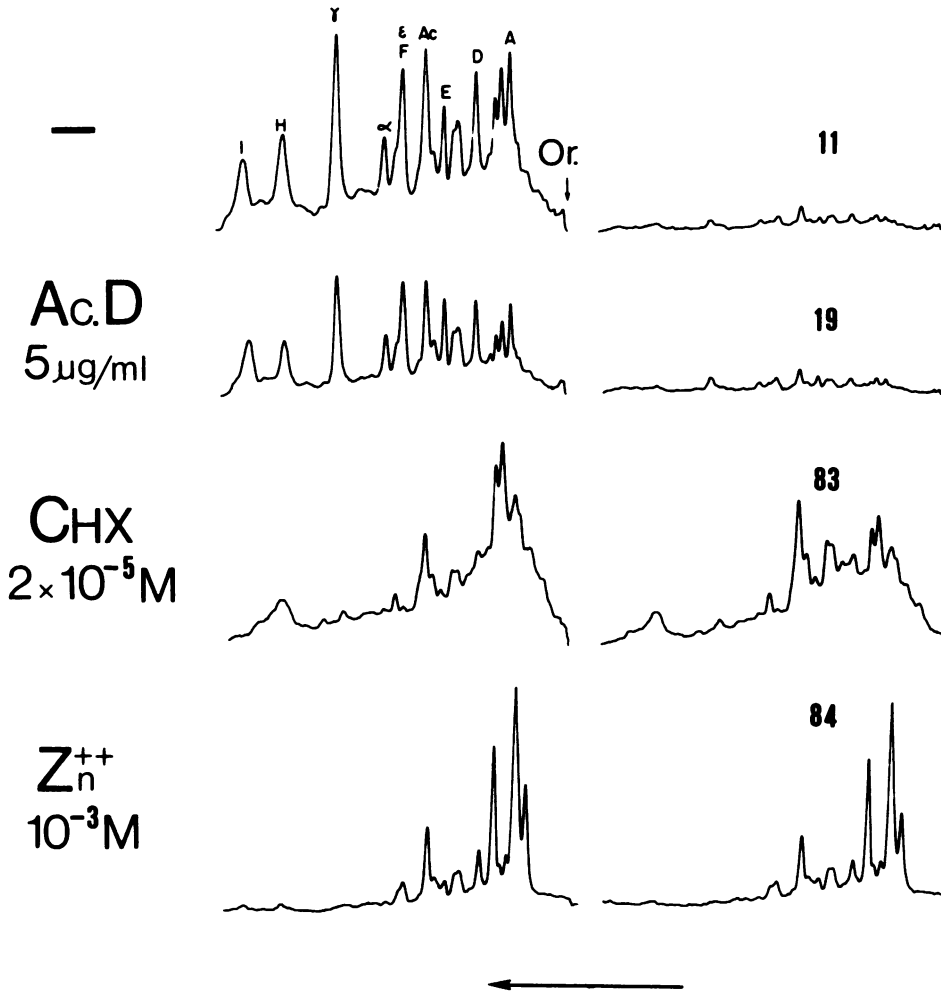
FIG. 3. *Gene expression requirement for gougerotin inhibition. 3T6 cells were infected with EMC virus as described in the text. After virus absorption for 1 h at 37°C, the medium was removed and replaced by 1 ml of E_4D_5 containing the indicated concentration of actinomycin D (Ac.D), cycloheximide (CHX), or $ZnCl_2$. After 4 h at 37°C, the medium was removed and replaced by 0.5 ml of E_4D_1-Met medium containing 2 µl of [^{35}S]methionine (885 Ci/mmol, 2 mCi/ml) and, where indicated, 0.1 mM gougerotin. Other conditions were as described in the legend to Fig. 2. Left column, Control cells; right column, cells treated with 0.1 mM gougerotin. Arrow indicates direction of migration.*

zymes (20), and a number of drugs preferentially accumulate in cancerous tissues in naturally occurring malignancies (7).

Therefore, a number of drugs, including actinobolin, adrenochrome, amicetin, anisomycin, aurintricarboxylic acid, blasticidin S, chartreusin, chlortetracycline, cycloheximide, deoxycycline, edeine complex, emetine, fusidic acid, gougerotin, GppCH₂p, oxytetracycline, pactamycin, polydextran sulfate, puromycin, pyrocatechol violet, sparsomycin, and tubulosine, were tested for their inhibitory effect on protein synthesis in control and RSV-transformed CEF cells. A wide range of concentrations from 10^{-3} to 10^{-9} M of the above-mentioned inhibitors and different incubation conditions were tested in these experiments. However, under these particular conditions no preferential effect with any drug was found on RSV-transformed 3T6 cells (conditions were as described under Materials and Methods; results not shown).

DISCUSSION

The present results add evidence to the membrane leakage model indicating that the membrane of infected cells becomes modified during
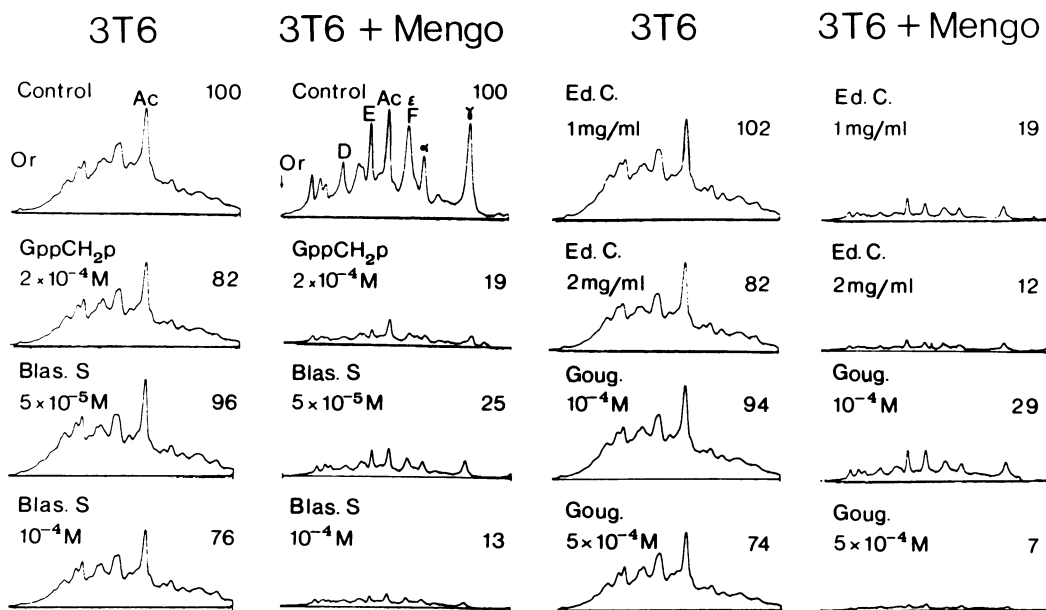
3T6

Control   Ac   100

Or

GppCH$_2$p
2 × 10^{-4}M   82

Blas. S
5 × 10^{-5}M   96

Blas. S
10^{-4}M   76

3T6 + Mengo

Control   Ac E   100
           E   F   Y

Or   D

GppCH$_2$p
2 × 10^{-4}M   19

Blas. S
5 × 10^{-5}M   25

Blas. S
10^{-4}M   13

3T6

Ed. C.
1 mg/ml   102

Ed. C.
2 mg/ml   82

Goug.
10^{-4}M   94

Goug.
5 × 10^{-4}M   74

3T6 + Mengo

Ed. C.
1 mg/ml   19

Ed. C.
2 mg/ml   12

Goug.
10^{-4}M   29
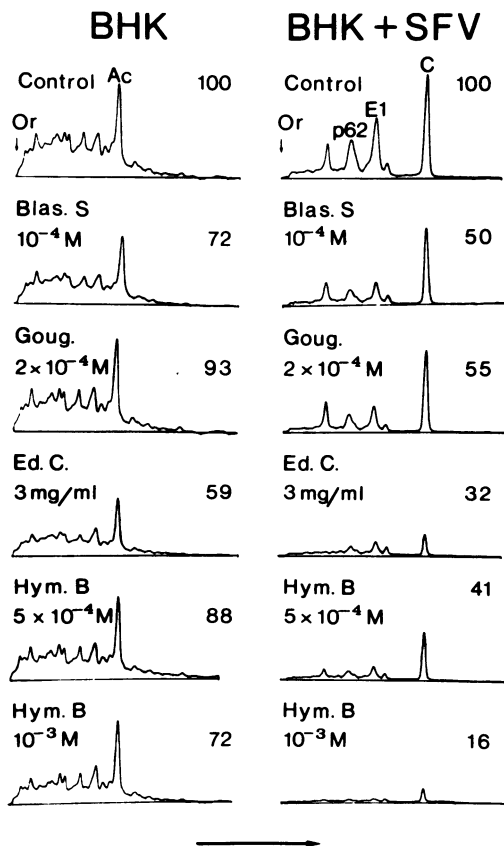
Goug.
5 × 10^{-4}M   7

FIG. 4. *Membrane leakiness in mengovirus-infected 3T6 cells. The conditions were as described in the text and the legend to Fig. 1. Arrow indicates direction of migration.*

BHK

Control   Ac   100

Or

Blas. S
10^{-4}M   72

Goug.
2 × 10^{-4}M   93

Ed. C.
3 mg/ml   59

Hym. B
5 × 10^{-4}M   88

Hym. B
10^{-3}M   72

BHK + SFV

Control   C   100
         E1
Or   p62

Blas. S
10^{-4}M   50

Goug.
2 × 10^{-4}M   55

Ed. C.
3 mg/ml   32

Hym. B
5 × 10^{-4}M   41

Hym. B
10^{-3}M   16

viral infection at the time in which the synthesis of late viral proteins occurs. This modification implies an unspecific change that allows the passage through the membrane of molecules, very different from the chemical point of view. This phenomenon does not seem to be dependent on the cell line employed, since it occurred with 3T6, BHK, and CV1 cells, nor specific for a particular virus, since it could be promoted by viruses as different as EMC and mengo (picornaviruses), SFV (a togavirus), and SV40 (a papovavirus).

The synthesis of viral macromolecules involves in many cases the use of host cell enzymes. This applies to the synthesis of viral proteins, which are made by the same overall mechanism used by uninfected cells (18). It was earlier postulated that inhibitors acting on the protein synthesis apparatus, or blocking the synthesis of macromolecules in general, could not be used to interfere specifically with viral development (2). With the finding that viruses can produce profound alterations in the host cell membrane during their development it became worthwhile, at least theoretically, to explore the possibility of finding an inhibitor of protein synthesis that could selectively penetrate and thus

FIG. 5. *Membrane leakiness in SFV-infected BHK cells. Hym. B, Hygromycin B. Otherwise, the conditions were as described in the text and the legend to Fig. 1. Arrow indicates direction of migration.*
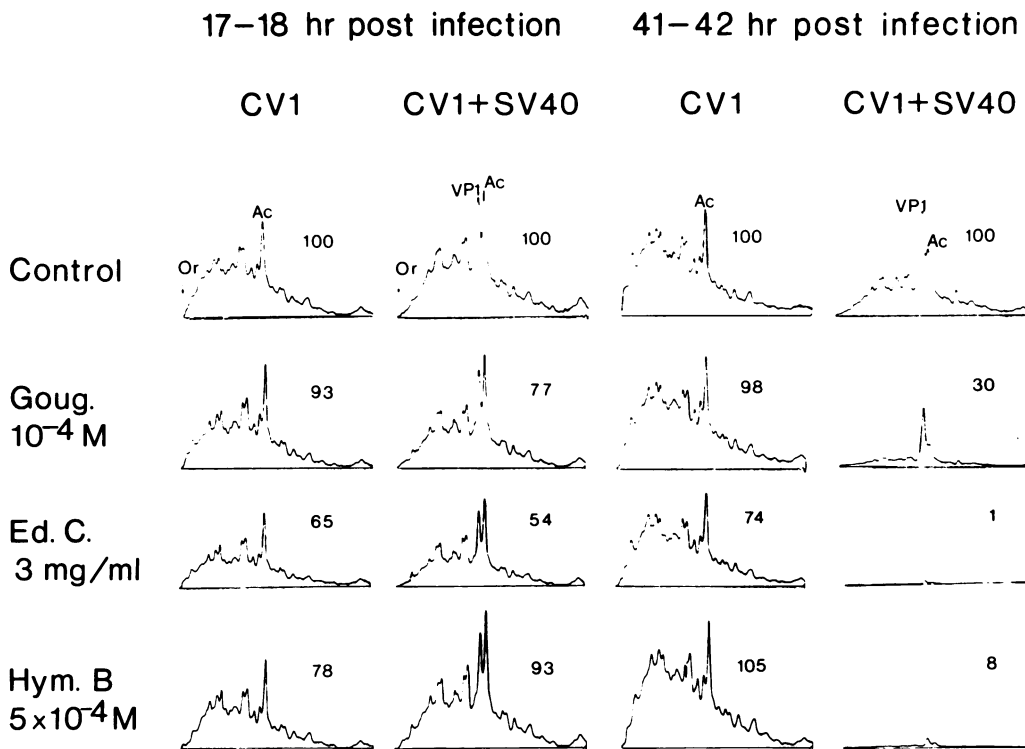
FIG. 6. *Membrane leakiness in SV40-infected CV1 cells. Protein synthesis was estimated at 17 or 41 h postinfection. Hym. B, Hygromycin B. Otherwise, the conditions were as described in the text and the legend to Fig. 1. Arrow indicates direction of migration.*

inhibit virus-infected cells (3, 4). The data shown in this paper indicate that such inhibitors exist and also suggest that many drugs might fulfill this specific inhibitory function, since 5 out of the 21 antibiotics tested were effective.

Our work suggests that it may be possible to design specific antiviral agents that interfere with macromolecular synthesis. Such an inhibitor must have a high inhibitory activity in vitro and must not penetrate into uninfected cells. We believe in addition that it is likely to be a natural or semisynthetic product. Clearly, more naturally occurring inhibitors should be screened, and those with the strongest in vitro activity should be selected. Many of the known antibiotics do not permeate into mammalian cells, and those that do enter could be chemically modified by either introduction of a polar group or removal of the part of the molecule that allows the compound to diffuse through the lipidic bilayer in normal cells. Any chemically modified compound must of course retain its inhibitory properties. An inhibitor with the above characteristics could be a wide-spectrum antiviral agent, since membrane leakiness occurs in several virus-cell systems (4; and Fig. 1 and 4 to 6).

These results also illustrate how membrane leakiness can be measured in a very simple way. Thus, in a heterogeneous population of cells synthesizing different kinds of protein (e.g. uninfected and virus-infected cells), it is possible to determine what percentage of both populations have their membrane leaky at any moment.

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