JOURNAL OF VIROLOGY, Nov. 1972, p. 969-978
Vol. 10, No. 5

Printed in U.S.A.

Mechanism of Mengo Virus-Induced Cell Injury in L Cells: Use of Inhibitors of Protein Synthesis to Dissociate Virus-Specific Events

F. D. COLLINS AND W. K. ROBERTS

Department of Microbiology, University of Colorado Medical School, Denver, Colorado 80220

Received for publication 28 July 1972

L cells were infected with Mengo virus in the presence of varying concentrations of protein synthesis inhibitors (azetidine-2-carboxylic acid, *p*-fluorophenylalanine, puromycin), and examined with respect to the effects of the inhibitors on several features of virus-induced cell injury. The virus-specific events in the cells could be dissociated into three groups, based on their sensitivity to the inhibitors: (i) viral ribonucleic acid (RNA) synthesis, bulk viral protein synthesis, and infectious particle production, all of which were prevented by low inhibitor concentrations; (ii) the cytopathic effect (CPE) and stimulation of phosphatidylcholine synthesis, which were sensitive to intermediate concentrations of the inhibitors; and (iii) the virus-induced inhibitions of host RNA and protein synthesis, which were resistant to the inhibitors of protein synthesis except at very high concentrations. It is concluded from this that the virus-induced CPE and stimulation of phosphatidylcholine synthesis are not consequences of the inhibition of cellular RNA or protein synthesis. Analysis of the virus-specific protein and RNA synthesized at several concentrations of azetidine and puromycin suggests that the CPE may be induced by a viral protein precursor. Virus-induced inhibition of host RNA and protein synthesis occurred at azetidine concentrations which blocked the synthesis of over 99.7% of the total viral RNA and over 99% of the viral double-stranded RNA (dsRNA). Calculations show that this would correspond to less than 150 dsRNA molecules per infected cell, resulting in a dsRNA-polysome ratio of less than 1:1,000; this indicates that host protein synthesis cannot be inhibited by an irreversible binding of dsRNA to polysomes.

Picornavirus infection of animal cells usually is accompanied by an inhibition of host cell ribonucleic acid (RNA), deoxyribonucleic acid, and protein synthesis (12, 15, 16, 19, 29, 34), followed by morphological changes within the cells and cell death (2, 8, 9). Because of the simplicity of the picornaviruses and the relative rapidity with which these virus-induced events take place, considerable work has been done with these systems in the hope of elucidating the mechanism(s) of virus-induced injury to animal cells. Despite the fact that much important information concerning the effects of viruses on cells has resulted from this work, the exact molecular mechanisms whereby viruses inhibit host macromolecular synthesis or cause cell degeneration remain obscure.

We have approached this problem by studying the effects of varying concentrations of protein synthesis inhibitors on several Mengo virus-induced events in L cells. The inhibitors selected for this investigation were puromycin, a general inhibitor of protein synthesis in animal cells, and the amino acid analogues *p*-fluorophenylalanine (FPA) and azetidine-2-carboxylic acid, which not only inhibit protein synthesis but also have been shown to selectively prevent the processing of certain viral protein precursors (20, 21). By carrying out infections in media containing increasing concentrations of inhibitors, we hoped to dissociate the virus functions from one another, and then to correlate these functions with the synthesis of specific viral proteins or protein precursors. The recent progress in our understanding of picornaviral protein synthesis and processing (6, 18, 20, 23) makes such an approach seem feasible. Also, the suggestions that virus-induced inhibition of host protein synthesis (10) and cytopathic effect (CPE) (7) may be caused by viral double-

stranded RNA (dsRNA) prompted us to investigate the effects of protein synthesis inhibitors on viral RNA synthesis.

MATERIALS AND METHODS

Virus and cell cultures. Mengo virus was grown in L cell monolayers. Virus stocks were stored in growth medium at −76 C. The L cells were from Flow Laboratories Inc., clone no. 929. They were grown at 37 C in a 5% CO_2 atmosphere, attached to plastic petri dishes (Falcon Plastics). The growth medium was Earle minimal essential medium (MEM), supplemented with 10% calf serum and 100 units of penicillin and streptomycin each/ml.

Chemicals and radioisotopes. Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; L-azetidine-2-carboxylic acid and DL-FPA were from Calbiochem, Los Angeles, Calif.; the actinomycin D was a generous gift from Merck Sharp and Dohme; the pancreatic ribonuclease and deoxyribonuclease were from Worthington Biochemical Corp., Freehold, N.J.; ^{3}H-uridine (27 Ci/mmole) and ^{14}C-choline (53 mCi/mmole) were from Amersham/Searle Corp., Arlington Heights, Ill.; and the ^{3}H-amino acid mixture (NET-250) was obtained from New England Nuclear Corp., Boston, Mass.

Infection of cell monolayers. Newly confluent monolayers of L cells were exposed to 150 plaque-forming units (PFU)/cell of Mengo virus, unless otherwise indicated. The inoculum was removed after 45 min at 37 C, and the cells were washed with MEM and replaced in growth medium. Any drug additions were present in the inoculum and again in the medium.

Plaque assay. Appropriate dilutions of a virus sample were spread on a just confluent monolayer of L cells for 1 hr. The inoculum was removed and the cells were overlayed with 1% agar (Difco certified) in MEM at 45 C. The agar was allowed to harden at room temperature for 30 min, and then growth medium was added. The cells were incubated for 3 days and then stained for 2 hr at 37 C with 0.01% neutral red.

Incorporation of radioactive precursors into acid-precipitable material. RNA, protein, and phosphatidylcholine synthesis were measured by the incorporation of radioactive uridine, amino acids, and choline into trichloroacetic acid-insoluble material. Cells were incubated in medium containing the radioactive precursor, the medium was removed, and the cells were extracted as a monolayer overnight in 10% trichloroacetic acid at 4 C. The monolayer was suspended with a rubber policeman and washed in cold 5% trichloroacetic acid and finally in absolute ethanol, except for measurements on phosphatidylcholine synthesis where the ethanol wash was omitted. The pellet was dissolved in a Nuclear-Chicago solubilizer and counted in a liquid scintillation counter.

CPE. Cell monolayers were exposed to 0.01% neutral red in growth medium for 1 hr. The cells were washed several times in phosphate-buffered saline, and the cell-bound dye was extracted in a mixture of equal parts ethanol and 0.1 M Na citrate buffer, *p*H 4.2. The dye concentration was measured at 540 nm.

Extraction of viral dsRNA from infected cells. Monolayers were dissolved in 0.15 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane (Tris) (*p*H 7.4), 10^{-3} M ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulfate. This material was extracted with an equal volume of buffer-saturated phenol and centrifuged at 20,000 × *g* for 15 min. The aqueous supernatant fluid was treated with an equal volume of 2-propanol, the solution was placed at −20 C overnight, and the precipitate was pelleted by centrifugation at 30,000 × *g* for 30 min. The pellet was reprecipitated twice, dissolved in a solution containing 0.1 M Tris (*p*H 7.4), 0.1 M $NaCl_2$, and treated with 10 μg of pancreatic deoxyribonuclease per ml and 1.0 μg of pancreatic ribonuclease per ml for 15 min at 37 C to degrade all nucleic acid except dsRNA.

RESULTS

Replication of Mengo virus in L cells. The effects of Mengo virus infection on L cell RNA synthesis, protein synthesis, and vital dye uptake are summarized in Fig. 1. Infection causes a

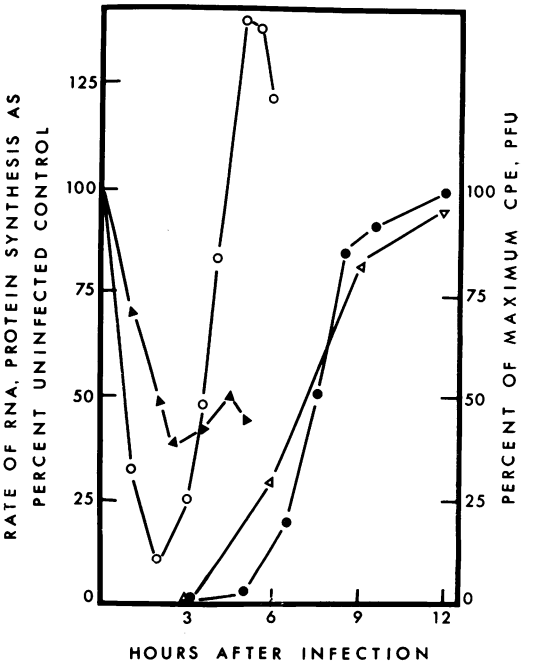

FIG. 1. *Replication of Mengo virus in L cells. Cells were exposed to 1 μCi of ^{3}H-uridine per ml in 30-min pulses, to measure RNA synthesis. Protein synthesis was measured analogously by using 0.5 μCi of ^{3}H-amino acids per ml in growth medium without amino acids or calf serum. PFU were assayed from the medium on L cell monolayers. CPE was assayed as in Materials and Methods. Symbols: ○, RNA synthesis; ▲, protein synthesis; ●, released PFU; and △, CPE.*

rapid and almost complete inhibition of host cell RNA synthesis, followed by a rapid increase in the synthesis of what can be shown to be viral RNA. There is a less rapid and less complete inhibition of host protein synthesis; analysis by polyacrylamide gel electrophoresis of the newly synthesized proteins indicates that host protein synthesis continues, although at a greatly reduced rate, during the peak of viral protein synthesis 4.5 to 6 hr after infection. These patterns of RNA and protein synthesis are similar to those originally described for this system by Franklin and Baltimore (12).

The titer of PFU begins to rise within the cell at the time of maximal viral RNA and protein synthesis to approximately 500 PFU/cell. These virions are not released into the cell-free medium, however, until several hours after the maximal intracellular titer has been reached. This delayed release occurs at the time of advanced cell degeneration and breakdown of the plasma membrane (1). Cell degeneration is accompanied by loss of the ability of the cell to concentrate the vital dye, neutral red, (Fig. 1). We have used this method to follow the virus-induced CPE.

Effects of FPA, azetidine, and puromycin on Mengo virus replication. Three groups of Mengo virus functions can be characterized by their relative resistance to inhibitors of protein synthesis (Fig. 2). In one group are virion production and the replication of viral RNA, eliminated by relatively low concentrations of the drugs. The CPE is more resistant and is inhibited only at higher drug concentrations. Finally, the virus-induced inhibition of host RNA and protein synthesis is relatively unaffected over the concentration range of inhibitors employed.

Several conclusions can be drawn from the patterns in Fig. 2. The fact that relatively high concentrations of protein inhibitors eliminate the CPE while permitting virus-induced inhibition of host RNA and protein synthesis shows that these are unrelated events. This means that the mechanism of inhibition of host RNA or protein synthesis, or both, does not concomitantly initiate the events leading to cell disintegration. Also, the observation that virus-induced CPE and inhibition of host RNA and protein synthesis occur at inhibitor concentrations that prevent the synthesis of detectable viral RNA leads to the less firm conclusion that viral RNA is not responsible for any of these events. An experiment presented later shows that azetidine inhibits the synthesis of viral dsRNA proportional to its inhibition of total viral RNA synthesis. This will be discussed in connection with the hypotheses that dsRNA causes the CPE (7) and virus-induced inhibition of protein synthesis (10).

To obtain the results of Fig. 2, the drugs were

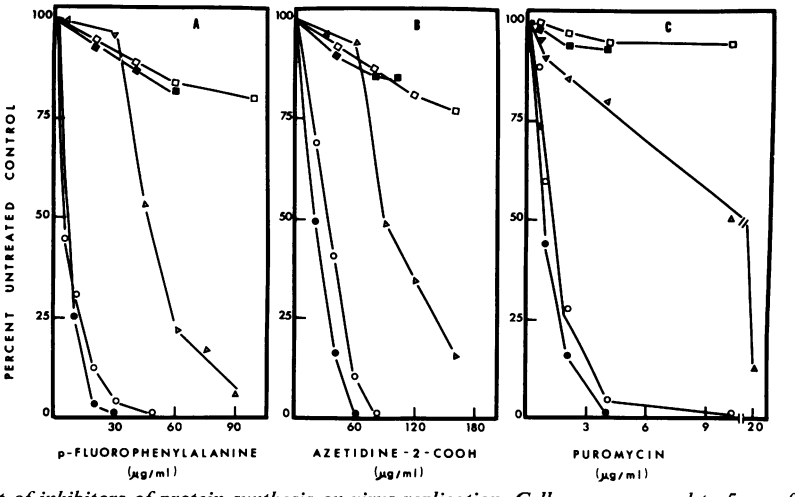

FIG. 2. *Effect of inhibitors of protein synthesis on virus replication. Cells were exposed to 5 μg of actinomycin D per ml from 2 hr before infection and then to 1 μCi of ^{3}H-uridine per ml 1 to 6 hr postinfection, to measure viral RNA synthesis. Cells in medium were freeze-thawed at 18 hr postinfection for the assay of total PFU. RNA and protein synthesis were measured as in Fig. 1 at 1.5 to 2.5 hr postinfection to calculate the viral inhibition of host cell RNA and protein synthesis, by using similarly labeled, uninfected controls treated with the same concentration of protein synthesis inhibitor. CPE was assayed 18 hr postinfection. The inhibitors of protein synthesis were added with the infecting virus. Symbols:* ○, *viral RNA synthesis;* ●, *PFU;* △, *CPE;* ■, *viral inhibition of cell protein synthesis; and* □, *viral inhibition of cell RNA synthesis.*

added at the time of infection. Since the inhibition of host RNA and protein synthesis occurs early in infection, it may be postulated that the failure of the drugs to prevent this inhibition was due to an insufficient intracellular concentration of the drugs early after infection. However, the inhibitors may be added 2 or 4 hr before infection without changing the results.

Viral RNA synthesis was measured between 1 and 6 hr after infection. It is possible that the protein inhibitors merely delayed this synthesis, and, therefore, our results are an exaggerated measurement of the inhibition of viral RNA replication by the drugs. Accordingly, the time between 1 and 13 hr postinfection, when the cells rupture, was divided into six 2-hr periods, and the effect of puromycin and azetidine on viral RNA synthesis, compared to infected untreated controls, was measured for each time period. Beyond 7 hr postinfection, the inhibitor effects were even more pronounced, and there was no delayed burst of RNA synthesis. Early viral RNA synthesis (1–3 hr postinfection) is inhibited to the same extent as later bulk synthesis.

Effects of azetidine and puromycin on viral protein synthesis. Both amino acid analogues and puromycin were selected for this investigation because of the possible differences that might be revealed concerning their effects on the synthesis of biologically active viral proteins. Puromycin, presumably, acts only to inhibit protein synthesis. Amino acid analogues also will block protein synthesis at high concentrations, but at lower concentrations they can become incorporated into newly synthesized protein molecules, causing a change in conformation and loss of biological activity of some of the protein species. This could affect virus-induced syntheses or inhibitions in infected cells by preventing the processing of certain viral protein precursors (20, 21) or by rendering completed viral proteins biologically inactive.

A comparison of the effects of puromycin and azetidine on total viral RNA and protein synthesis is shown in Table 1. Puromycin can be seen to inhibit viral RNA and protein synthesis to a similar extent. Presumably, this is a result of the effectiveness of the drug in inhibiting the synthesis of all viral proteins equally, including the RNA polymerase. In contrast, the suppression of viral RNA synthesis by low concentrations of azetidine is not accompanied by a concomitant decrease in the amount of viral protein synthesis. However, azetidine is a proline analogue (we found that a concentration ratio of proline-azetidine of 5:1 eliminates the effects of azetidine on virus replication), and apparently it can be incorporated into viral proteins in place of proline and prevents their normal function. In this way, the analogue appears to selectively destroy the activity of viral RNA polymerase and reduce viral RNA levels, without affecting those measurements of protein synthesis which fail to distinguish functional from nonfunctional polypeptides.

A difference can be seen between the shapes of the CPE inhibition curves shown in Fig. 2. The CPE is essentially unaffected at analogue concentrations which inhibit up to 90% viral RNA synthesis, whereas puromycin inhibits CPE at concentrations which only partially inhibit viral RNA synthesis. We interpret these results to mean that the analogues, unlike puromycin, selectively interfere with polymerase activity (or maturation) to a greater extent than the activity (or maturation) of the CPE effector protein.

Effect of azetidine on the time course of the CPE. Several lines of evidence indicate that the CPE observed in inhibitor-treated cultures is a typical, virus-induced cell death. The protein synthesis inhibitors alone caused no appreciable decrease in cell staining at the concentrations used, even after 24 hr. The notion that at intermediate concentrations the inhibitors may have potentiated cell death by virus is unlikely, be-

TABLE 1. *Effect of azetidine and puromycin on viral RNA and protein synthesis*

Puromycin concn (μg/ml)	Percent untreated control		Azetidine concn (μg/ml)	Percent untreated control	
	Viral RNA synthesis[a]	Viral protein synthesis[b]		Viral RNA synthesis[a]	Viral protein synthesis[b]
0.5	88	84	10	79	102
0.75	59	43	20	69	98
2	27	32	40	41	94
4	4	11	60	10	47

[a] Viral RNA synthesis measured as in Fig. 2.

[b] Protein synthesis measured as in Fig. 1. Viral protein synthesis is the amino acid incorporation 4.5 to 5.5 hr postinfection minus a background incorporation measured 1.5 to 2.5 hr postinfection.

cause at slightly higher concentrations there was no cell death. Finally, as shown in Fig. 3, the CPE appears within 1 hr of the expected time, at a concentration of azetidine sufficient to depress viral RNA synthesis by 90%.

Effect of azetidine on the synthesis and cleavage of viral polypeptides. A mechanism of viral protein synthesis involving the cleavage of large precursor polypeptides has been established for poliovirus (20, 23), encephalomyocarditis virus (6), and Mengo virus (18). The analogues azetidine and FPA, among others, have been found to block the cleavage of some of the poliovirus precursors (20, 21). Presumably, the analogue is incorporated into the precursor, making it an unsuitable substrate for the cleavage enzyme. We were interested in determining the nature and amounts of the viral proteins synthesized in infected cells at analogue concentrations which prevent the appearance of active viral RNA polymerase but permit the virus-induced CPE. By comparing this pattern with that of the viral proteins synthesized at higher analogue concentrations (preventing CPE but permitting virus-induced inhibition of host RNA and protein synthesis), one might hope to make a preliminary identification of a protein responsible for the CPE. Accordingly, we examined the sodium dodecyl sulfate-polyacrylamide gel patterns of intracellular virus proteins made at several azetidine concentrations.

Figure 4A presents the gel pattern of proteins

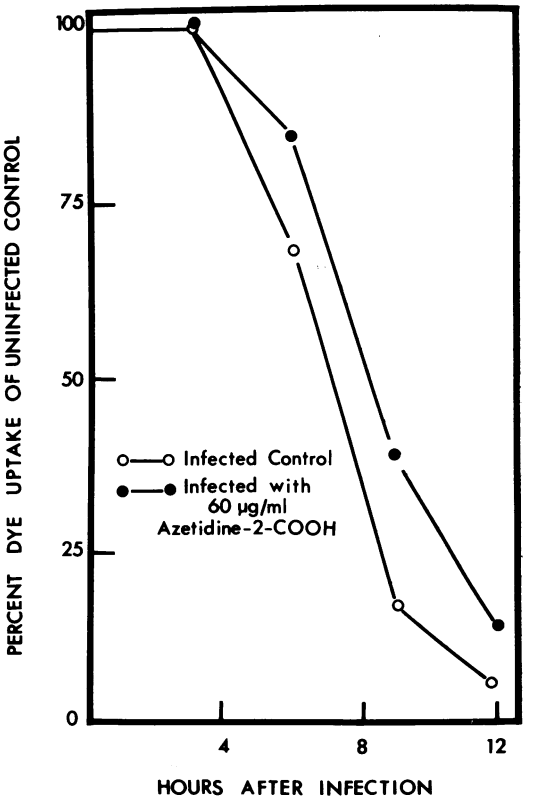

FIG. 3. *Effect of azetidine on the time course of viral CPE.*

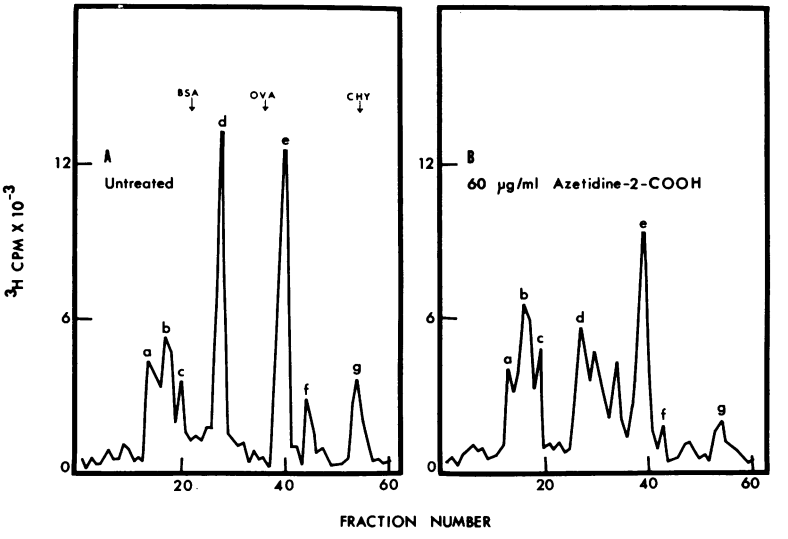

FIG. 4. *Effect of azetidine on the synthesis of viral proteins. Infected monolayers were exposed to 12.5 µCi of ^{3}H-amino acids per ml 4.5 to 6 hr after infection. Cell extracts were prepared by the method of Butterworth et al. (6) and run on 10% polyacrylamide-sodium dodecyl sulfate gels by the procedure of Summers et al. (30). Molecular weight standards: BSA is bovine serum albumin at 67 × 10^3; OVA is ovalbumin at 45 × 10^3; and CHY is chymotrypsinogen A at 25 × 10^3.*

made in infected cells between 4.5 and 6 hr postinfection. Before this time, the patterns are a mixture of the complex pattern of uninfected cells and the gradually emerging viral proteins. Only by 4.5 hr have the host proteins been reduced sufficiently for the viral proteins to stand out as clearly as shown here. This pattern is basically similar to that for Mengo virus presented by Holland and Kiehn (18). These authors have shown by pulse-chase experiments that several of the proteins in size group a, b, and c are precursors of smaller proteins. The large amounts of these precursor proteins which are found after a relatively long period of labeling indicate that the kinetics of precursor cleavage with Mengo virus are slower than for encephalomyocarditis virus, where barely detectable amounts of precursor proteins are observed after a 1.5-hr labeling period (6). Peaks d and e would be expected to contain stable noncapsid proteins (6), whereas the capsid proteins should include peaks f and g and part of peak e (28). Figure 4B shows that the effect of azetidine is to increase the amount of the precursor proteins, especially b and c, while reducing the amount of all other proteins. Also, two protein peaks appear between the normal peaks at d and e. These may represent improper cleavage products. This is consistent with a role for azetidine in impairing the cleavage of certain Mengo virus precursor proteins.

The patterns at lower azetidine concentrations, not presented here, show that the analogue interferes with precursor cleavage over the same concentration range that it depresses viral RNA synthesis. This may be associated with inactivation of the viral polymerase. At higher analogue concentrations, when CPE is affected, there is too little viral protein synthesized to clearly distinguish individual peaks above background on the gels.

At a concentration of 60 μg/ml, azetidine reduces viral RNA synthesis by 90% without any significant effect on the CPE (Fig. 2B). At this concentration, there is a distortion in the profile of the viral proteins being synthesized; however, all the viral proteins present in the untreated control cells are also detected in the analogue-treated cells (Fig. 4), preventing a qualitative statement being made concerning absent proteins not involved in the CPE.

Protein synthesis requirement for the inhibition of host RNA synthesis. The results shown in Fig. 2 could be interpreted as indicating that protein synthesis is not required for virus-induced inhibition of host RNA and protein synthesis. However, Franklin and Baltimore have shown that 100 μg of puromycin per ml largely prevents the Mengo virus-induced inhibition of L cell RNA synthesis (12), and Verwoerd and Hausen have found that 100 μg of FPA per ml prevents the ME virus-induced inhibition of RNA synthesis in the same cells (32). We also find that high concentrations of protein synthesis inhibitors can prevent virus-induced inhibition of host RNA synthesis, but that the effect of the inhibitors is dependent upon the multiplicity of infection (MOI). These results are summarized in Table 2.

At a high MOI, 100 μg of FPA per ml has little effect on the virus-induced inhibition of host RNA synthesis. However, at the lower MOI, this drug concentration does block the virus-induced inhibition, bringing our results into accord with those of Verwoerd and Hausen (32). Puromycin, at the high concentration used here, prevents the inhibition at both MOI levels.

Effect of azetidine on the synthesis of viral dsRNA. Recently, it has been suggested that viral dsRNA made during picornavirus infections is responsible for the CPE (7) and the inhibition of host protein synthesis (10). Our results (Fig. 2B) show that, at a concentration of azetidine where viral RNA synthesis falls to background levels (0.3% of the infected control), the CPE and the inhibition of host RNA and protein synthesis remain at 60 and 85% of normal, respectively. However, dsRNA represents about only 5% of the total RNA made during Mengo virus infection, and it is possible that this small component could remain at nearly normal levels and escape notice amid a great reduction in total viral RNA. Therefore, we measured the effect of azetidine on the synthesis of dsRNA, characterizing the latter by its ribonuclease resistance and migration on a sucrose density gradient (Fig. 5). Total viral RNA, as well as dsRNA, was determined in the infected azetidine-treated cells, and we found

TABLE 2. *Influence of MOI on the effectiveness of protein synthesis inhibitors in preventing Mengo virus-induced inhibition of L cell RNA synthesis*

Multiplicity of infection (PFU/cell)	Percent of normal L cell RNA synthesis remaining after infection,[a] in the presence of:	
	100 μg of FPA per ml[b]	100 μg of puromycin per ml[b]
50	88	94
150	6	91

[a] Measured 1.5 to 2.5 hr postinfection.

[b] The drugs were present from the time of infection.

that azetidine inhibits the synthesis of dsRNA to the same extent as total viral RNA: 60 μg of azetidine per ml inhibits dsRNA by 93% and total viral RNA by 89%; 80 μg/ml inhibits dsRNA by at least 99% and total viral RNA by 99.7%. Since virus-induced CPE and inhibition of host RNA and protein synthesis occur at azetidine concentrations greater than 80 μg/ml, we conclude that, if viral RNA or dsRNA are causing these effects in our system, then extremely low concentrations of the RNA must be sufficient to produce these effects.

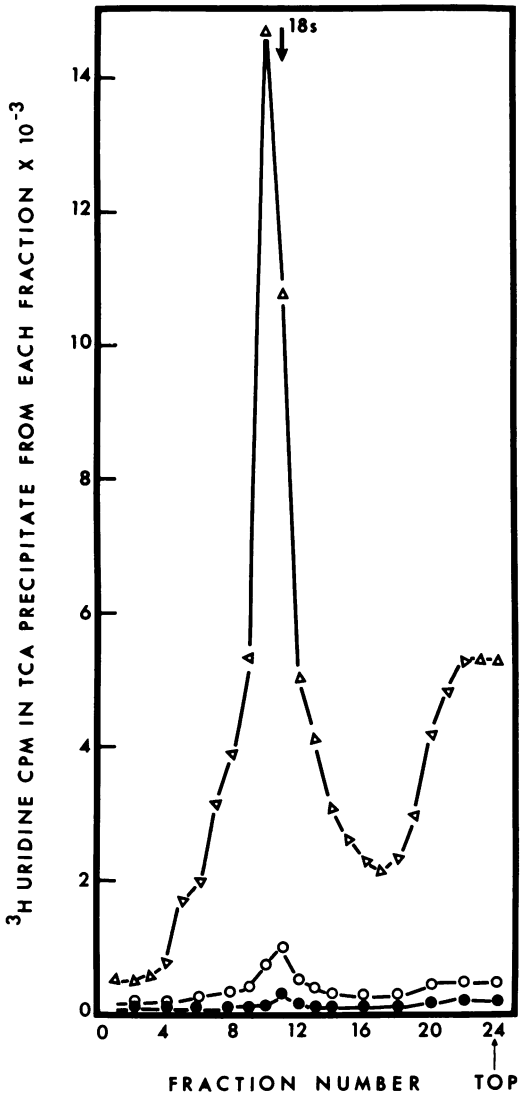

FIG. 5. *Effect of azetidine on the synthesis of viral dsRNA. Actinomycin D-treated infected cells were exposed to 10 μCi of ^{3}H-uridine per ml at 1 to 6 hr post-infection. dsRNA was prepared as in Materials and Methods and centrifuged through a 5 to 20% sucrose gradient at 23,000 rev/min for 18 hr in the SW 25.1 rotor, with ribosomal RNA as a sedimentation marker as previously described (27). Symbols: △, no azetidine; ○, 60 μg of azetidine per ml; ●, 80 μg of azetidine per ml.*

Effect of puromycin on virus-induced membrane synthesis. Picornavirus infection causes an increase in choline incorporation into cell lipids (2, 24, 26). In the case of Mengo virus-infected L cells, this choline has been shown to go into membranes which can be seen in the electron microscope to proliferate in infected cells (2). Thus, the increase in choline uptake is a measure of virus-induced membrane hyperplasia.

The rate of choline uptake compared to uninfected cells doubles during infection (Fig. 6). A concentration of puromycin, which depresses viral protein synthesis by 90%, is shown to decrease the virus-stimulated choline uptake by only 40%. This concentration of puromycin has no effect on choline uptake by uninfected cells. Over 98% of the ^{14}C-choline in the acid-insoluble precipitates was extractable with

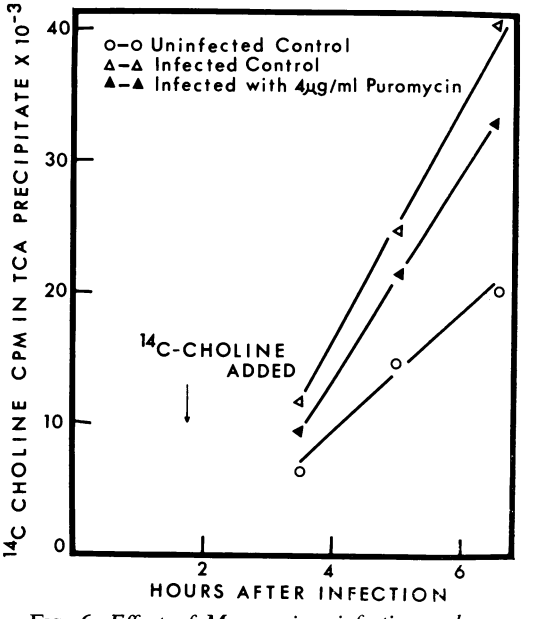

FIG. 6. *Effect of Mengo virus infection and puromycin on phosphatidylcholine synthesis. Cells were incubated in 10% rather than 5% CO_2, to enhance choline uptake (24), and actinomycin D (5 μg/ml) was added to the growth medium at 0 time to make the virus-induced stimulation of choline incorporation more apparent (2). ^{14}C-choline (1 μCi/ml) was added to the medium at the time indicated, and the incorporation into acid-precipitable material was determined at various times, as described in Materials and Methods.*

chloroform-methanol (3:1), showing that the ^{14}C-choline was incorporated exclusively into the lipid fraction.

Figure 7 presents a more complete study, comparing the effects of puromycin on viral RNA synthesis, increased membrane synthesis, and cell death. The viral RNA curve (Fig. 7) can be used as an estimate of viral protein synthesis (Table 1). One can conclude that a protein responsible for membrane hyperplasia has the same sensitivity to puromycin as a CPE effector protein, that both proteins are required in far less than normal amounts to achieve their effects, and that equal amounts of either protein produce equal effects. This suggests that the same viral protein induces membrane hyperplasia and cell death.

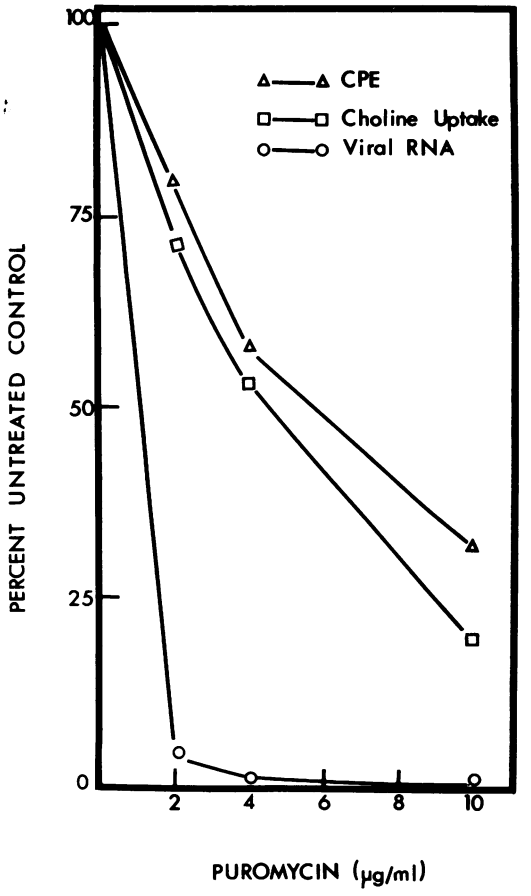

FIG. 7. *Comparative effect of puromycin on viral RNA synthesis, CPE, and virus-induced choline uptake. Viral RNA synthesis and CPE were measured as in Fig. 2. Choline uptake was measured as in Fig. 6 from 1.75 to 6.5 hr postinfection. Virus-induced choline uptake is the difference between the incorporation in infected cells and uninfected controls treated with the same concentration of puromycin.*

DISCUSSION

The virus-specific events that we have been concerned with in this study fall into three classes, depending upon their sensitivity to inhibition by puromycin, azetidine, and FPA. These classes are probably a reflection of the amount of viral protein synthesis required to produce the various virus-induced effects. Viral RNA synthesis, bulk viral protein synthesis, and PFU formation require the most viral protein synthesis for detection and, therefore, are the most sensitive to the protein synthesis inhibitors. Much less viral protein synthesis is required to produce the CPE and the stimulation of choline uptake, and these two events form a second sensitivity class. The least amount of viral protein synthesis is required to induce the inhibition of host RNA and protein synthesis, and very high concentrations of protein synthesis inhibitors are necessary to eliminate these effects. The fact that very little viral protein synthesis is required to establish the CPE and the virus-induced inhibitions of host RNA and protein synthesis is also indicated by the observations that high concentrations of interferon do not abolish these effects (13, 14), and that the inhibitions occur in poliovirus-infected cells which have been treated with sufficient guanidine to block virus replication (3, 17, 22, 25).

Previous investigations concerning the CPE in picornavirus-infected cells have suggested that the CPE is induced by a "late" viral protein (1, 13), and that the CPE might be caused by hydrolytic enzymes released from the lysosomes of infected cells (11, 33). There has been no evidence for virus-specific proteins synthesized late, but not synthesized early, in picornavirus infections (5, 31), and it seems probable that a "late" viral protein is simply one in which virus replication must proceed for a fairly long period of time to permit synthesis of the protein in sufficient concentration to produce a detectable effect. Thus, a viral protein precursor is as likely to induce the CPE as a capsid protein, and an indication that this might be the case is the difference in CPE inhibition profiles observed between puromycin and the amino acid analogues (Fig. 2). One interpretation for the lag in CPE inhibition observed with increasing concentrations of the analogues, but not puromycin, is that puromycin inhibits the synthesis of all viral proteins equally, whereas low concentrations of the analogues may inhibit the synthesis or maturation of certain viral proteins without

affecting the CPE effector protein. Since 60 μg of azetidine per ml reduces the concentration of all viral proteins except the precursors a, b, and c (Fig. 4), these, then, would be the more likely inducers of the CPE. A similar argument would suggest that virus-specific RNA does not induce the CPE: analogue sufficient to reduce viral RNA and dsRNA synthesis to 10% or less has little effect on the CPE, whereas a concentration of puromycin which reduces viral RNA synthesis to 10% produces a small but significant inhibition of the CPE (Fig. 2).

The similar sensitivity to puromycin shown by the virus-induced stimulation of choline uptake and CPE (Fig. 7) indicates that these two effects are induced by similar concentrations of a virus-specific product(s). This is preliminary evidence that these events may be related, which is what one would expect if leakage of hydrolytic enzymes from lysosomes induces both the stimulation of choline uptake and the CPE (2), or if membrane proliferation is a necessary prerequisite for the CPE.

The experiments presented in Fig. 2 and 7 show that inhibitors of protein synthesis can prevent the CPE and stimulation of choline incorporation at concentrations that permit the virus-induced inhibition of host cell RNA and protein synthesis. This is evidence that the inhibition of host RNA and protein synthesis, which occur early in infection, are not sufficient to cause the membrane proliferation and CPE, which occur later in the viral replicative cycle. It has been recognized for a number of years that inhibition of cellular RNA or protein synthesis per se will not produce a rapid cell disintegration similar to the virus-induced CPE. However, most of this work was done with inhibitors such as actinomycin (12) or puromycin (4, 13), and the possibility was not excluded that the mechanism of action of the virus-induced inhibition of cell RNA or protein synthesis is such that it concomitantly initiates events leading to an eventual CPE. Evidence against this possibility was provided in poliovirus-infected cells where guanidine, an inhibitor of poliovirus replication, was used to dissociate the CPE from the virus-induced inhibitions of host RNA and protein synthesis (4). We have corroborated these results in another system, by using inhibitors of protein synthesis to dissociate these virus-induced effects. In addition, we have extended the results to include membrane proliferation.

The poliovirus-induced inhibition of HeLa cell RNA and protein synthesis can be prevented if the RNA in the virion is inactivated with proflavine (17) or ultraviolet light (25). This suggests that picornavirus-induced inhibition of host RNA and protein synthesis is mediated by virus-specific protein or RNA copied from intact viral genomes. Very little synthesis of viral protein or RNA must be necessary to initiate these inhibitions, however, since blocking all detectable virus replication with interferon (13, 14), guanidine (3, 17, 25), or inhibitors of protein synthesis (Fig. 2) does not prevent these effects. It is apparent from Fig. 2 that the virus-induced inhibitions of host RNA and protein synthesis occur at concentrations of FPA and azetidine greater than those required to inhibit all detectable (99.9%) viral RNA synthesis. It might be expected, therefore, that most, if not all, of the viral products needed to induce these inhibitions can be synthesized from parental RNA, provided that the concentration of parental RNA in the infected cell is sufficiently large. The necessity of introducing a large amount of parental RNA into the cells would be an explanation for the observation that under conditions restricting viral RNA synthesis, a high MOI is required to inhibit host RNA and protein synthesis (reference 17; Table 2).

The nature of the virus-specific products which induce the inhibition of host RNA and protein synthesis and the CPE is of interest, particularly in view of the findings that dsRNA can kill animal cells (7) and inhibit in vitro protein synthesis (10). We have attempted to identify the viral products synthesized at azetidine concentrations that inhibit virus replication but permit these virus-induced effects. Qualitative (Fig. 4) and quantitative (Table 1) estimates of viral protein synthesized at high analogue concentrations were not possible because of relatively high backgrounds of host protein synthesis. Host RNA synthesis can be selectively inhibited with actinomycin D, however, and this permits sensitive measurements of the amounts of viral RNA and dsRNA synthesized at various analogue concentrations. Azetidine, at a concentration of 80 μg/ml, inhibited the synthesis of over 99.7% of the viral RNA and over 99% of the dsRNA (Fig. 5). Based on the fact that the RNA in each centrifugation in Fig. 5 was extracted from 5×10^7 cells, and that the peak fraction 11 from the 0 azetidine sample had an absorbance at 260 nm of 0.04, one can calculate that each infected cell at 0 azetidine should contain approximately 15,000 dsRNA molecules. In the presence of 80 μg of azetidine per ml, each infected cell would contain a maximum of 150 dsRNA molecules, a number which should be even lower at the higher azetidine concentrations which also permit the virus-induced inhibition of host RNA and protein synthesis. In our

hands, analysis of L cell homogenates by zonal centrifugation shows a ribosome concentration of approximately 5×10^6 ribosomes per L cell, about 20% of the ribosomes being present in polysomes. Therefore, L cells infected in the presence of 80 μg of azetidine per ml would contain a ratio of dsRNA-polysomes of approximately 1:1,000, a figure much less than the estimate of 1:1 needed to inhibit protein synthesis in the in vitro system (10). This would mean that dsRNA could not inhibit protein synthesis in our system by simply binding to the polysomes, but must inactivate the protein-synthesizing system in a catalytic fashion or by inducing the formation of an inhibitor, modes of action more commonly associated with proteins than with RNA. The types and concentrations of viral proteins synthesized at high analogue concentrations are unknown, but they would be expected to include a high proportion of viral protein precursors (Fig. 4).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant CA 10641 from the National Cancer Institute.

LITERATURE CITED

1. Amako, K., and S. Dales. 1967. Cytopathology of mengovirus infection. I. Relationship between cellular disintegration and virulence. Virology 32:184–200.
2. Amako, K., and S. Dales. 1967. Cytopathology of mengovirus infection. II. Proliferation of membranous cisternae. Virology 32:201–215.
3. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. Virology 26:100–113.
4. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. II. The relation between poliovirus growth and virus-induced morphological changes in cells. Virology 26:114–121.
5. Baltimore, D. 1969. The replication of picornaviruses, p. 101–176. *In* H. B. Levy (ed.), The biochemistry of viruses. Marcel Dekker, New York.
6. Butterworth, B. E., L. Hall, C. M. Stoltzfus, and R. R. Rueckert. 1971. Virus-specific proteins synthesized in encephalomyocarditis virus-infected HeLa cells. Proc. Nat. Acad. Sci. U.S.A. 68:3083–3087.
7. Cordell-Stewart, B., and M. W. Taylor. 1971. Effect of double-stranded viral RNA on mammalian cells in culture. Proc. Nat. Acad. Sci. U.S.A. 68:1326–1330.
8. Dales, S., H. J. Eggers, I. Tamm, and G. E. Palade. 1965. Electron microscopic study of the formation of poliovirus. Virology 26:379–389.
9. Dales, S., and R. M. Franklin. 1962. A comparison of the changes in fine structure of L cells during single cycles of viral multiplication, following their infection with the viruses of mengo and encephalomyocarditis. J. Cell Biol. 14:281–302.
10. Ehrenfeld, E., and T. Hunt. 1971. Double-stranded poliovirus RNA inhibits initiation of protein synthesis by reticulocyte lysates. Proc. Nat. Acad. Sci. U.S.A. 68:1075–1078.
11. Flanagan, J. F. 1966. Hydrolytic enzymes in KB cells infected with poliovirus and herpes simplex virus. J. Bacteriol. 91:789–797.
12. Franklin, R. M., and D. Baltimore. 1962. Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. Cold Spring Harbor Symp. Quant. Biol. 27:175–198.
13. Gauntt, C. J., and R. Z. Lockart, Jr. 1966. Inhibition of Mengo virus by interferon. J. Bacteriol. 91:176–182.
14. Haase, A. T., S. Baron, H. Levy, and J. A. Kasel. 1969. Mengo virus-induced cytopathic effect in L-cells: protective effect of interferon. J. Virol. 4:490–495.
15. Hausen, P., and D. W. Verwoerd. 1963. Studies on the multiplication of a member of the Columbia SK group (ME virus) in L cells. III. Alteration of RNA and protein synthetic patterns in virus infected cells. Virology 21:617–627.
16. Holland, J. J. 1963. Depression of host-controlled RNA synthesis in human cells during poliovirus infection. Proc. Nat. Acad. Sci. U.S.A. 49:23–28.
17. Holland, J. J. 1964. Inhibition of host cell macromolecular synthesis by high multiplicities of poliovirus under conditions preventing virus synthesis. J. Mol. Biol. 8:574–581.
18. Holland, J. J., and D. Kiehn. 1968. Specific cleavage of viral proteins as steps in the synthesis and maturation of enteroviruses. Proc. Nat. Acad. Sci. U.S.A. 60:1015–1022.
19. Homma, M., and A. F. Graham. 1963. Synthesis of RNA in L cells infected with mengovirus. J. Cell. Comp. Physiol. 62:179–192.
20. Jacobson, M. F., J. Asso, and D. Baltimore. 1970. Further evidence on the formation of poliovirus proteins. J. Mol. Biol. 49:657–669.
21. Jacobson, M. F., and D. Baltimore. 1968. Polypeptide cleavage in the formation of poliovirus proteins. Proc. Nat. Acad. Sci. U.S.A. 61:77–84.
22. Leibowtiz, R., and S. Penman. 1971. Regulation of protein synthesis in HeLa cells. III. Inhibition during poliovirus infection. J. Virol. 8:661–668.
23. Maizel, J. V., and D. F. Summers. 1968. Evidence for large precursor proteins in poliovirus synthesis. Proc. Nat. Acad. Sci. U.S.A. 59:966–971.
24. Penman, S. 1965. Stimulation of the incorporation of choline in poliovirus-infected cells. Virology 25:148–152.
25. Penman, S., and D. Summers. 1965. Effects on host cell metabolism following synchronous infection with poliovirus. Virology 27:614–620.
26. Plagemann, P. G. W., P. H. Cleveland, and M. A. Shea. 1970. Effect of mengovirus replication on choline metabolism and membrane formation in Novikoff hepatoma cells. J. Virol. 6:800–812.
27. Roberts, W. K., J. F. E. Newman, and R. R. Rueckert. 1966. Synthesis of Maus-Elberfeld viral RNA in ascites tumor cells. J. Mol. Biol. 15:92–101.
28. Rueckert, R. R. 1971. Picornaviral architecture, p. 255–306. *In* K. Maramarousch and E. Kurstak (ed.), Comparative virology. Academic Press Inc., New York.
29. Salzman, N. P., R. Z. Lockart, and E. D. Sebring. 1959. Alterations in HeLa cell metabolism resulting from poliovirus infection. Virology 9:244–259.
30. Summers, D. F., J. V. Maizel, and J. E. Darnell. 1965. Evidence for virus-specific non-capsid proteins in poliovirus-infected HeLa cells. Proc. Nat. Acad. Sci. U.S.A. 54:505–513.
31. Summers, D. F., J. V. Maizel, and J. E. Darnell. 1967. The decrease in size and synthetic activity of poliovirus polysomes late in the infectious cycle. Virology 31:427–435.
32. Verwoerd, D. W., and P. Hausen. 1963. Studies on the multiplication of a member of the Columbia SK group (ME virus) in L cells. IV. Role of "early proteins" in virus induced metabolic changes. Virology 21:628–635.
33. Wolff, D. A., and H. C. Bubel. 1964. The disposition of lysosomal enzymes as related to specific viral cytopathic effects. Virology 24:502–505.
34. Zimmerman, E. F., M. Heeter, and J. E. Darnell. 1963. RNA synthesis in poliovirus-infected cells. Virology 19:400–408.