synthesis. Although a drug does not exhibit a specificity *in vitro* for viral nucleic acid, the intracellular milieu of the host may protect its own DNA from binding while leaving the viral DNA exposed. For example, chloroquine is antagonized by the binding of cations to DNA(4), and the binding of actinomycin D was shown recently to be antagonized by nucleoprotein (11).

Summary. Chloroquine, a 4 aminoquinoline 'antimalarial' which binds to DNA, has been shown to inhibit replication of a DNA bacteriophage of *E. coli* under conditions where growth of the host continues. It is suggested that the ability of the drug to bind to DNA may account for this effect, but a more general metabolic effect cannot be ruled out.

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Protein Leakage from Mengovirus-Infected Cells.* (32205)

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Many viruses cause a rapid destruction of mammalian cells cultured *in vitro*. While the occurrence of cytopathic alterations affords a dramatic visualization of host cell-virus interaction the basic mechanism(s) responsible for these untoward effects is obscure. One of the manifestations of virus-induced cellular injury is the leakage of enzymes and proteins from cells(1,2). We have found the leakage of acid-insoluble proteins from mengovirus-infected cells to be a reliable and quantitative measure of cellular injury. The present investigation was undertaken to examine the kinetics of protein leakage from mengovirus-infected cells, and by the use of selective inhibitors of protein synthesis an attempt was made to delineate some of the factors which underlie the protein leakage phenomenon. The experiments presented here suggest that cellular protein leakage is conditioned by viral protein synthesis and not the suppression of host cell functions by the viral genome.

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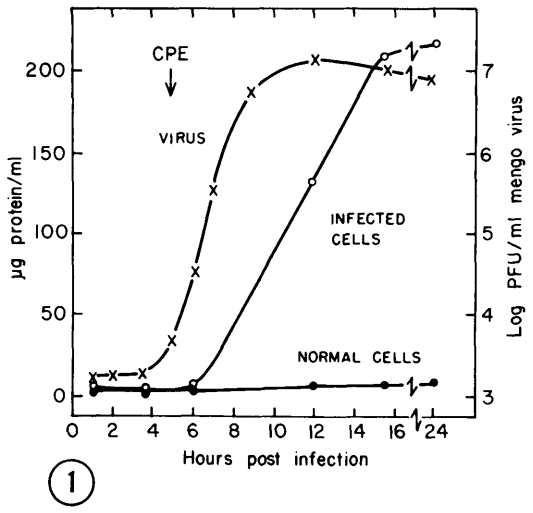
Materials and methods. Cells and virus. An established line of guinea pig spleen cells (GPS) was cultivated in suspension in Eagle's medium (Grand Island Biological Co., MEM F-15) supplemented with 10% calf serum and 0.5% lactalbumin hydrolysate. Monolayers were prepared by plating out an appropriate cell suspension onto 6.0 cm plastic culture dishes (Falcon).

The mengovirus used in this study was obtained from E. Swim (Cleveland) and passaged several times on GPS cells.

Plaque titrations were carried out by plating 0.3 ml of an appropriate virus dilution onto GPS monolayers, and the subsequent addition of an agar overlay containing 200 μg/ml of protamine sulfate. After 48 hours 1:5000 neutral red, containing 0.01% acetic acid, was added to the dishes and the plaques were counted at 60 hours.



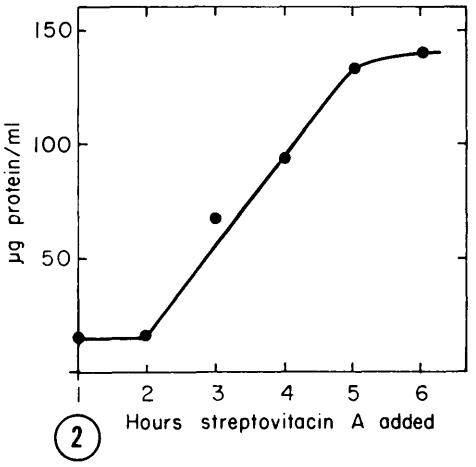


FIG. 1. Temporal relationship between the appearance of extracellular virus (x———x) and protein in mengovirus-infected (○———○) and normal (●———●) GPS cell cultures.

FIG. 2. Protein leakage from mengovirus-infected, streptovitacin A-treated GPS cells. Streptovitacin A (10 µg/ml) was added at hourly intervals, for 6 hr, following virus addition. The inhibitor remained in contact with cells until 18 hr post-infection, at which time the culture fluids were collected and examined for protein content.

Protein leakage from cells was measured in the following manner: GPS cell monolayers containing 3.0×10^6 cells were prepared in plastic culture dishes. After infection, the cells were washed thrice with phosphate buffered saline (pH 7.0), and 5.0 ml of culture medium (MEM-F 15) lacking serum were added to each dish. At selected intervals following infection, the supernatant fluids were removed and centrifuged at 5000 $\times$ *g* for 10 minutes. Cold perchloric acid (PCA) was added to the chilled supernatant fluids to a final concentration of 0.5 M. The resulting precipitates were washed twice with cold 0.5 M PCA and examined for protein content by the method of Lowry *et al*(3) using bovine serum albumin as the protein standard.

Results. Kinetics of virus and protein release. GPS cell monolayers were infected with mengovirus at an input multiplicity of 10 plaque forming units (PFU) per cell. At selected intervals after infection supernatant fluids were withdrawn and examined for the presence of extracellular virus and PCA-insoluble protein. Replicate uninfected GPS cell monolayers were included in all experiments. The kinetics of protein leakage relative to the appearance of extracellular virus are shown in Fig. 1. It is noteworthy that the appearance of extracellular virus preceded protein leakage by approximately 1 hour. Furthermore, while maximal extracellular virus titers were obtained by the 11th hour of infection, less than 50% of the total protein leakage had occurred. These data suggest that significant quantities of mengovirus may be released from GPS cells without materially affecting the permeability of the cell membrane to intracellular proteins. The earliest manifestations of cytopathic effects (CPE) were clearly distinguishable at the onset of virus release and prior to the appearance of extracellular protein. Early CPE was recognized as a rounding of cells accompanied by nuclear prominence. Shedding of cytoplasm had not occurred by this time.

Inhibitors of protein synthesis were examined next for their effects on mengovirus-induced protein leakage. Streptovitacin A (kindly supplied by Dr. G. M. Savage, Upjohn Co.) and actinomycin D were selected for this purpose since these compounds allowed differentiation between host cell and virus-directed protein synthesis. Streptovitacin A belongs to a group of rapidly-acting protein inhibitors(4) and has been shown

TABLE I. Protein Leakage and Mengovirus Synthesis in Actinomycin D and Streptovitacin A-Treated Cells.

Inhibitor added (hr post infection)	Treatment	Protein released (μg/ml)*	Total virus (PFU/ml)*	CPE*
0	none	10	—	0
0	virus	143	2.3×10^7	4†
1	AcD‡	16	—	1§
2	"	13	—	1
3	"	31	—	1
5	"	23	—	1
1	virus + AcD‡	135	2.0×10^7	4
2	"	133	2.2×10^7	4
3	"	124	2.0×10^7	4
4	"	123	1.9×10^7	4
5	"	128	2.0×10^7	4
1	StA‖	12	—	0
2	"	8	—	0
3	"	9	—	0
4	"	7	—	0
5	"	9	—	0
6	"	9	—	0

* Samples for protein and virus assays were collected and microscopic observation of cells was performed 18 hr post infection.

† Degrees of cytopathic effect were ranked as follows: rounding of cells, 1-2+; nuclear prominence and shedding of cytoplasm, 3+; complete cellular degeneration and detachment from the surface of the culture dish, 4+.

‡ Actinomycin D, 2 μg/ml of culture fluid.

§ Rounding of cells with diffuse cytoplasmic granularity. This reaction was clearly distinguishable from mengovirus-induced CPE.

‖ Streptovitacin A, 10 μg/ml of culture fluid.

to suppress the synthesis of both RNA and DNA viruses(5,6). Actinomycin D interferes with DNA-dependent RNA synthesis (7) but does not inhibit the synthesis of mengovirus(8).

GPS monolayers were infected with mengovirus at an input multiplicity of 10 PFU per cell. At hourly intervals thereafter, streptovitacin A (10 μg/ml) or actinomycin D (2 μg/ml) was added to replicate infected and normal GPS cell monolayers. (These inhibitor concentrations suppressed the incorporation of C^{14}-labelled amino acids into cellular proteins by greater than 95%.) The sequential addition of inhibitors was discontinued after 6 hours. Eighteen hours post infection, the cultures were examined microscopically for evidence of CPE and the supernatant fluids were removed for total virus and protein determinations. The results of these experiments are summarized in Table I and Fig. 2. While actinomycin D addition resulted in a minor degree of protein leakage and a diffuse granularity of GPS cells, streptovitacin A had no measurable effect of GPS cell cultures. Thus it is clear that the extended (18 hours) suppression of cellular protein synthesis did not result in pronounced cellular injury as measured by the appearance of extracellular protein. This observation assumes some importance when viewed from the standpoint that one of the earliest manifestations of mengovirus infection is the inhibition of host cell macromolecule synthesis(9). It is unlikely therefore, that the suppressive function of the infecting viral genome is instrumental in initiating the protein leakage reaction. The presence of actinomycin D in no way interfered with the virus-induced reactions which lead to protein leakage or synthesis of virus (Table I).

The interruption of viral protein synthesis by streptovitacin A during the early portion of the virus growth cycle rapidly arrested damage to the host cell. The amount of protein released from infected, streptovitacin A-treated cells, was related to the time of inhibitor addition (Fig. 2). These data also revealed that the amount of protein leakage from mengovirus-infected cells was directly related to the amount of virus synthesis which had occurred by the time of inhibitor addition. Furthermore, it is clear that the reactions involved in protein leakage were initiated between the second and third hours and were completed by the fifth hour of infection. Data summarized in Table II show that streptovitacin A is effective in arresting mengovirus synthesis and the de-

TABLE II. Mengovirus Synthesis and Cytopathic Effects in Streptovitacin A-Treated Cells.

StA added (hr post-infection)*	Total virus (PFU/ml)†	Cytopathic effect†
0	2.5×10^7	4‡
1	5.0×10^3	0
2	5.1×10^3	0
3	4.0×10^3	1
4	1.0×10^4	2
5	4.5×10^5	3
6	1.9×10^7	4

* Streptovitacin A, 10 μg/ml of culture fluid.

† Samples for virus assay collected and determination of the degree of CPE made 18 hr post-infection.

‡ Degrees of CPE, see Table I.

velopment of CPE when added early during the infectious cycle.

Discussion. There is reason to believe that the leakage of protein from mengovirus-infected GPS cells is a reliable and quantitative measure of cellular injury. The data presented here suggest that protein leakage is conditioned by the appearance of virus-specific proteins, and not merely the suppression of host cell protein synthesis by the viral genome. This is inferred by the experiments which demonstrated that actinomycin D and streptovitacin A failed to induce a significant amount of protein leakage in normal GPS cells. Furthermore, the occurrence of protein leakage in virus-infected, actinomycin D-treated cells emphasized the role of virus-induced reactions leading to this event. The ameliorating effect of streptovitacin A on virus-induced protein leakage is best explained from the standpoint that virus-specific proteins are involved in the leakage phenomenon. In this respect these experiments support the conclusions of Bablanian *et al*(10,11) who suggested that poliovirus-induced cytopathology resulted from the direct action of viral proteins. The streptovitacin A experiments also showed that the amount of protein released by infected cells and the degree of cytopathic alteration are proportional to the amount of virus synthesized (Fig. 2 and Table II). It is significant that the appearance of extracellular virus preceded protein leakage. This would suggest that mengovirus may gain exit from the cell without materially affecting the permeability of the host cell membrane. This interpretation, however, is tempered by the fact that the assay for viral infectivity may be more sensitive than the measurement of extracellular protein.

The exact mechanism(s) by which virus-specific products effect protein leakage is obscure. The activation of lysosomal hydrolases as a consequence of virus infection is well substantiated, and it has been suggested that these enzymes are responsible for the cytopathic alterations which accompany virus infection(12,13,14). In view of these findings it is conceivable that lysosomal hydrolases, released in response to virus infection, may injure the cell membrane with the resultant leakage of cytoplasmic protein. An examination of the temporal relationship between the activation of lysosomal enzymes and the onset of protein leakage might provide some evidence for such a mechanism.

Summary. Infection of cells with mengovirus resulted in the leakage of protein which may be taken as a reliable and quantitative measure of cellular injury. Experiments with actinomycin D and streptovitacin A suggested that virus-specific proteins are the cause of protein leakage, and tended to rule out the suppressive action of the viral genome as a primary factor. The appearance of extra-cellular virus and the earliest manifestation of CPE preceded protein leakage by approximately 1 hour.

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