JOURNAL OF VIROLOGY, Oct. 1968, p. 1028-1037
Vol. 2, No. 10

Inhibition of HeLa Cell Protein Synthesis by the Vaccinia Virion

BERNARD MOSS

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 19 July 1968

HeLa cell protein synthesis is rapidly suppressed after infection with purified vaccinia virus. This was measured in three ways. (i) In the presence of 5 μg of actinomycin D per ml, viral protein synthesis was prevented and the decline in host protein synthesis was measured directly. (ii) Virus particles irradiated with 800 ergs or more of ultraviolet (UV) light per mm² are defective in their ability to initiate viral protein synthesis, but they still inhibit host protein synthesis. After addition of UV-irradiated virus, the decline in host protein synthesis was measured. (iii) Polyacrylamide gel electrophoresis was used to distinguish between host- and virus-induced proteins. The following results were obtained. (i) The inhibition of HeLa cell protein synthesis begins within 20 min after infection with purified vaccinia particles. Greater than 95% inhibition occurs within 1 to 4 hr after infection, depending on the viral multiplicity used. (ii) The synthesis of viral ribonucleic acid or viral protein is not required for the inhibition of host protein synthesis. (iii) The ability of the virus particles to inhibit cell protein synthesis is lost after heat or detergent treatment. (iv) The ability of the virus particles to inhibit cell protein synthesis is retained after UV-irradiation. (v) Vaccinia viral protein synthesis in preinfected cells is resistant to the effects of superinfection with UV-irradiated vaccinia particles. (vi) Inhibition of cell protein synthesis is complete and does not involve the continued synthesis of small polypeptide fragments. (vii) A decrease in the size of host polyribosomes rapidly follows infection with vaccinia virus. The results are interpreted as a selective effect of some constituent of the vaccinia virus particle or virus-activated host enzyme on host protein synthesis at a level beyond that of transcription.

Many viruses dominate the metabolic activities of their host cells. In extreme cases, a complete block occurs in the synthesis of host macromolecules. Vaccinia inhibits host cell deoxyribonucleic acid (DNA) synthesis (13, 17, 18), the transport of ribonucleic acid (RNA) from the nucleus to the cytoplasm, and, eventually, nuclear RNA synthesis (2, 30) and cell protein synthesis (5, 11, 14, 22, 29, 32). Vaccinia inhibits host protein synthesis in the presence of actinomycin D (32) or of interferon (14). The latter findings have led to the suggestion that the virus particle or specific proteins within the particle block cell protein synthesis. These studies demonstrate that a functional viral genome is not required for the rapid and essentially complete inhibition of HeLa cell protein synthesis.

MATERIALS AND METHODS

Cell culture. HeLa S3 cells were maintained continuously in exponential growth in suspension with Eagle's modified medium (6) supplemented with 5% horse serum.

Virus. Vaccinia strain WR was grown in HeLa cells and purified by a sequence that included differential centrifugation and two zonal sucrose density gradient centrifugations (12, 28). Sucrose was removed by pelleting the virus twice; virus was stored in 1 mM tris(hydroxymethyl)aminomethane(Tris)-chloride (*p*H 8.8) at −70 C. A 30 to 40% recovery of plaque-forming units (PFU) was obtained. The number of virus particles was determined both from the protein concentration and from the absorbancy at 260 nm [primarily light scattering; (12, 28)]. Either method indicated a particle to PFU of 40 or 50:1. Plaques were counted on monolayers of either primary chick embryo cells or HeLa cells by use of an agar overlay.

Infection. Purified virus was sonically treated at 20 kc five times for 15-sec intervals at 0 C, and then added to concentrated (4×10^6 cells/ml) suspensions of HeLa cells at 37 C. Continuous mixing of virus and cells was accomplished with magnetic stirring bars. Approximately 50 to 60% of the PFU were adsorbed during a 30-min incubation. Untreated cells, as well as cells pretreated with actinomycin D (5 μg/ml, Cancer Chemotherapy Branch, NIH) or with cycloheximide (300 μg/ml, Upjohn Co., Kalamazoo, Mich.) adsorbed 40 to 50% of a purified

^{3}H-thymidine-labeled virus preparation in 30 min. At the end of the adsorption period, the cells usually were diluted 10-fold with fresh medium.

Ultraviolet (UV)-irradiation of vaccinia. Purified virus (1 to 2 $\times$ 10^9 PFU) in 1 ml of 1 mM Tris (*p*H 8.8) was irradiated with a General Electric G15T8 Sterilamp in open 60-mm plastic petri dishes. During the period of irradiation, the suspension was stirred continuously with a small magnetic bar.

Incorporation of labeled precursors. L-Phenyl alanine-^{14}C (350 to 355 mc/mm; Schwarz BioResearch Inc., Orangeberg, N.Y.) and mixed L-amino acids-^{14}C (New England Nuclear Corp., Boston, Mass.) were used. Unless specifically noted, the growth medium was modified by the use of dialyzed horse serum, and the concentration of phenylalanine was reduced to 0.01 mM, or the usual amount of essential amino acids (6) was reduced 10-fold. The kinetics of virus formation and the yields of virus obtained, using either of the modified media just described, were identical to those obtained with growth medium. All incubations were done while immersed in a 37 C water bath in stoppered tubes flushed with 5% CO_2 and air. Continuous mixing was maintained with magnetic stir bars. Incorporation of the labeled precursor was stopped by pouring the cells into cold isotonically buffered saline that contained an excess of the appropriate ^{12}C-amino acids. The chilled cells were centrifuged at 2 C, washed once, and then resuspended in 1 ml of the saline solution. The suspension was made 1 N in NaOH and incubated at room temperature for 15 min to release amino acids bound to transfer RNA. Unincorporated ^{14}C-amino acids were removed by three cycles of trichloroacetic acid precipitation, centrifugation, and dissolution of the precipitate in 0.1 N NaOH. Finally, duplicate 0.1-ml samples in 0.5 N NaOH were analyzed for protein (20) and counted in a low background gas-flow geiger counter. In one experiment, the trichloroacetic acid precipitates were collected and washed on membrane filters (B-6; Schleicher & Schuell Co., Keene, N.H.) for scintillation counting. In all experiments, zero-time samples were measured and the radioactivity was subtracted from the incorporation values.

RESULTS

Inhibition of cell protein synthesis in the presence of actinomycin D. Vaccinia virus exerts rapid and profound effects on the metabolism of exponentially growing HeLa cells. The effect of purified virus on amino acid incorporation is shown in Fig. 1. The change in rate of amino acid incorporation results both from the inhibition of host protein synthesis and from the initiation of viral protein synthesis. The inhibition occurs more rapidly with higher multiplicities of virus. The change in the species of proteins synthesized by HeLa cells after vaccinia infection is illustrated by polyacrylamide gel electrophoresis of the radioactively labeled proteins (Fig. 2). The prominent peaks (Fig. 2b) have been identified as viral proteins by immunodiffusion (22). A detailed analysis, by polyacrylamide gel electrophoresis, of sequential protein synthesis after infection with vaccinia will be described elsewhere (22).

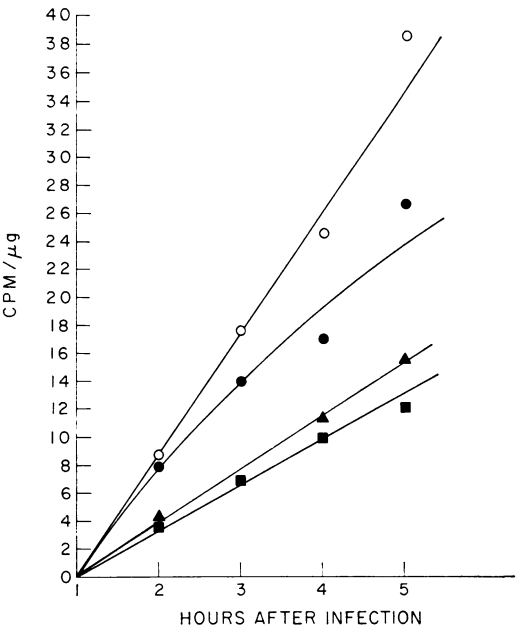

FIG. 1. *Effect of virus multiplicity on amino acid incorporation. HeLa cells were infected with purified vaccinia virus as described in Materials and Methods. After 30 min, the cells were diluted to 4 $\times$ 10^5 cells/ml and, after an additional 30 min, ^{14}C-amino acids were added. Cells were removed at indicated intervals and the counts/min per μg of trichloroacetic acid-precipitable protein was determined. Symbols:* ○, *no virus;* ●, *10 PFU/cell;* ▲, *50 PFU/cell;* ■, *100 PFU/cell.*

The change in protein synthesis after vaccinia infection is paralleled by the synthesis of viral RNA. This is shown by a burst of uridine incorporation (Fig. 3). The incorporation is reduced to a low level by incubating the cells in actinomycin D (5 μg/ml) for 30 min prior to and during infection (Fig. 3). Shatkin (32) found no significant synthesis of viral proteins in cells pretreated with 2 μg of actinomycin D per ml. Under these conditions, and by use of a crude virus preparation at a multiplicity of 10 to 20 PFU/cell, he made the interesting observation that net cell protein synthesis stops 4 hr after infection, whereas uninfected actinomycin D-treated cells continue to make protein for more than 12 hr. This inhibition of HeLa cell protein synthesis also occurs with highly purified virus in the presence of 5 μg of actinomycin D per ml (Fig. 4). The very rapid effect of the purified virus particles is illustrated by an experiment in which virus and

^{14}C-amino acids were added simultaneously to actinomycin D-treated cells (Fig. 5). The reduction in amino acid incorporation is approximately 40% in 10 to 20 min, and is nearly complete after 30 min. At this time and with the same concentration of actinomycin D, uridine incorporation can barely be detected (Fig. 3). Separate pretreatment of the virus with 5 μg of actinomycin D per ml for 30 min at 37 C before adding it to actinomycin D-treated cells did not significantly affect either the slight residual uridine incorpora-

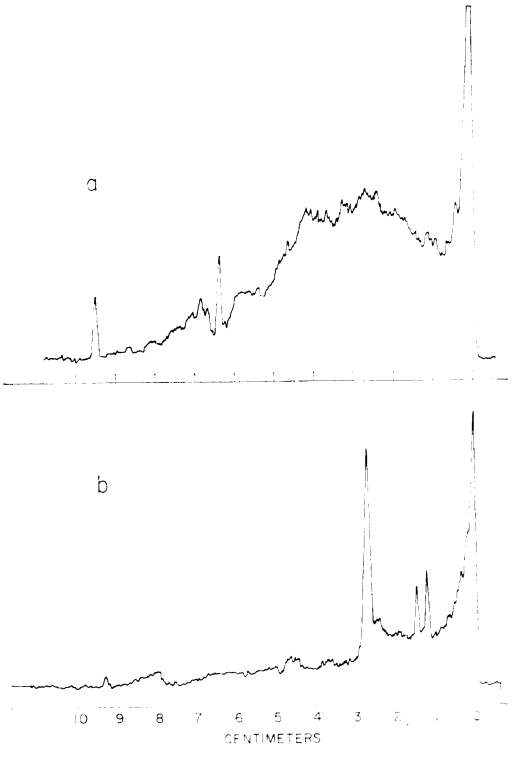

FIG. 2. *Changes in protein synthesis after vaccinia virus infection. HeLa cells in suspension were infected with 100 PFU of purified virus per cell. Three hours after infection, 1.6 × 10^7 cells in 40 ml were incubated for 60 min with 8 μc of ^{14}C-phenylalanine. The cells were washed and disrupted by sonic treatment. Particulate material was removed by centrifugation at 100,000 × g for 2 hr. Portions of the supernatant fluid, containing 25 to 30% of the acid-precipitable radioactivity, were fractionated into components by disc gel electrophoresis (22) by use of 7.5% polyacrylamide gels. The proteins contained in the gel were fixed for 18 hr in 7.5% acetic acid containing 1% amido-black. Excess stain and unincorporated ^{14}C-phenylalanine were removed by electrophoresis in 7.5% acetic acid. The gels were sliced longitudinally and dried as described by Fairbanks, Reeder, and Levinthal (8). Autoradiographs were made by exposure to X-ray film for 10 days. Optical density tracings were made with a Joyce-Loebl microdensitometer. (a) Uninfected cells. (b) Virus-infected cells.*

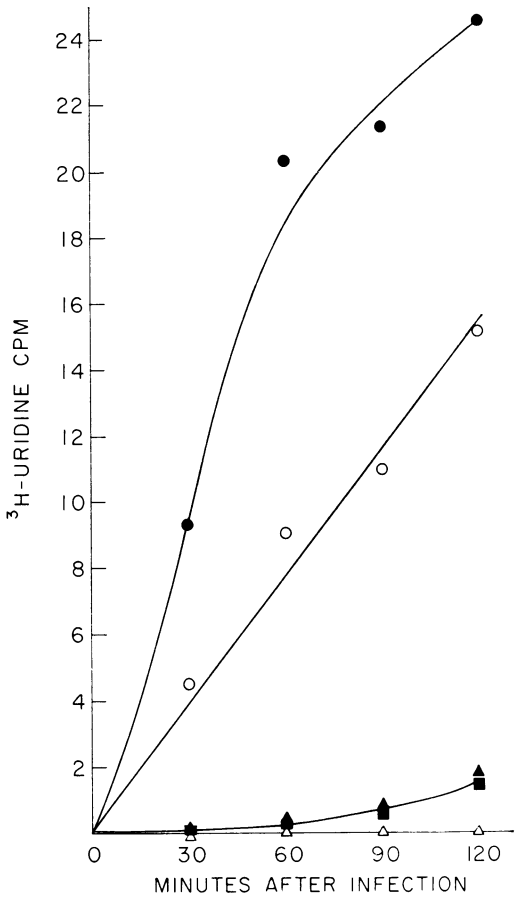

FIG. 3. *Effect of vaccinia virus and actinomycin D on uridine incorporation by HeLa cells. HeLa cells were treated with actinomycin D (5 μg/ml) for 30 min at 37 C. The cells were centrifuged and resuspended at 4 × 10^6 cells/ml in fresh actinomycin D medium containing 0.1 mM uridine. Purified virus (50 PFU/cell) and uridine-5-^{3}H (17 μc/ml) were added. In one case, the virus was pretreated with 5 μg of actinomycin D per ml for 30 min at 37 C. Cells not treated with actinomycin D were also infected, and then incubated with isotope. Uninfected cells were treated identically. At intervals, triplicate 0.1-ml samples were added to 10^6 carrier cells in 8 ml of cold phosphate-buffered saline containing 1 mM ^{12}C-uridine. The cells were centrifuged at 2 C and resuspended in 1 ml of cold, distilled water. One set of samples was made 0.5 N in KOH and incubated for 18 hr at 37 C. The other sets were stored frozen during this time. All samples were precipitated with cold 5% trichloroacetic acid; then collected and washed on filters. After drying, the filters were placed in scintillation vials and counted. Incorporation into RNA was calculated from the trichloroacetic acid-precipitable counts minus the remaining trichloroacetic acid-precipitable counts after alkaline hydrolysis. The latter correction was small. Symbols: ○, no virus; ●, 50 PFU/cell; △, actinomycin D-treated cells; ▲, actinomycin D-treated cells + 50 PFU/cells; ■, actinomycin D-treated cells + 50 PFU of actinomycin D-treated virus per cell.*

tion (Fig. 3), or the inhibition of cell protein synthesis.

Concentrations of virus required for rapid inhibition of host protein synthesis do not disrupt HeLa cells in the presence of actinomycin D (Table 1), and they lead to the production of viral RNA, viral proteins, and infectious virus in the absence of actinomycin D.

A trivial cause for the decrease in amino acid incorporation after vaccinia infection, failure of ^{14}C-amino acids to penetrate the cell, was investigated. At 1 and 2 hr after infection, actinomycin D-treated cells were incubated for 30 min with ^{14}C-amino acids. The cells were washed rapidly three times with cold, buffered saline. The trichloroacetic acid-precipitable and trichloroacetic acid-soluble radioactivity were determined. Expression of amino acid incorporation as trichloroacetic acid-precipitable radioactivity (counts/min) divided by acid-soluble radioactivity corrects for differences in entry of ^{14}C-amino acids. The results in one experiment, expressed in this manner, was a 91% inhibition

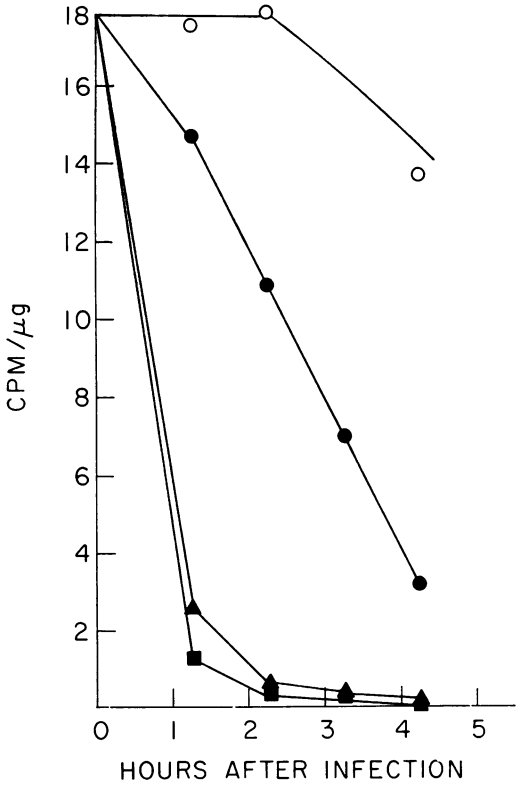

FIG. 4. *Inhibition of HeLa cell protein synthesis by purified virus in presence of actinomycin D. HeLa cells were treated with actinomycin D (5 μg/ml) for 30 min. The cells were resuspended at 4 × 10⁶/ml in fresh medium containing actinomycin D and 0.05 mM phenylalanine. Purified virus was added; after 30 min, the cells were diluted in the same medium to a concentration of 4 × 10⁵/ml. At indicated times, 5-ml portions were incubated for 30-min intervals with 1.25 μc of L-phenylalanine-^{14}C. The counts/min per μg of trichloroacetic acid-precipitable protein was determined. The values are plotted at the midpoint of each incubation period. Symbols: ○, no virus; ●, 25 PFU/cell; ▲, 50 PFU/cell; ■, 100 PFU/cell.*

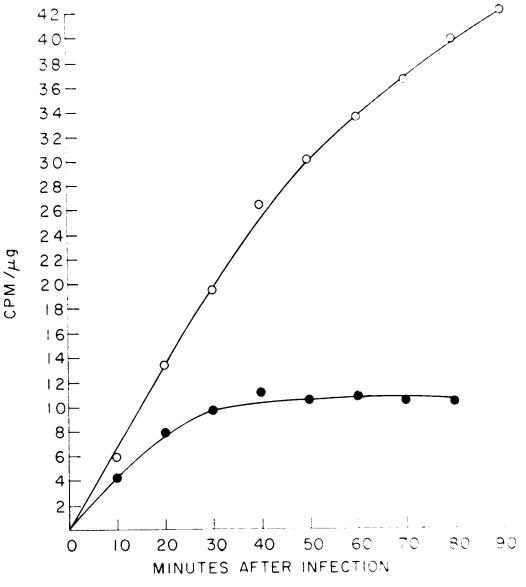

FIG. 5. *Amino acid incorporation after infection of HeLa cells in the presence of actinomycin D. HeLa cells were treated with actinomycin D (5 μg/ml) for 30 min at 37 C. The cells were resuspended at 4 × 10⁶/ml in fresh growth medium containing actinomycin D. Purified virus (50 PFU/cell) and ^{14}C-amino acids were added. At 10-min intervals, 4-ml portions were removed and rapidly chilled with cold phosphate-buffered saline containing excess ^{12}C-amino acids. They were then washed and incubated with 1 N NaOH and trichloroacetic acid-precipitated. The precipitates were collected on filters and counted in a liquid scintillation spectrometer. Symbols: ○, no virus; ●, 50 PFU/cell.*

TABLE 1. *Stability of actinomycin D-treated HeLa cells after infection with vaccinia virus*

Hours after infection	Determination of protein (μg) at virus multiplicities (PFU/cell) of			
	0	10	50	100
1.5	64	64	61	64
2.5	64	64	64	66
3.5	64	64	56	57
4.5	58	54	57	58

[a] Portions containing approximately 2 × 10⁶ cells were removed at indicated times, washed, precipitated with trichloroacetic acid, and dissolved in 0.5 ml of 0.5 N NaOH. Lowry protein determination was done on 0.1 ml with crystalline bovine serum albumin as a standard.

of cell protein synthesis 1 hr after infection with 50 PFU of vaccinia per cell and a 96% inhibition 2 hr after infection.

The possibility existed that after vaccinia infection of actinomycin D-treated cells, short chain polypeptides (not precipitable with trichloroacetic acid) were made. This possibility was ruled out by passage of the trichloroacetic acid-soluble material through a column (1 by 100 cm) of G-15 Sephadex equilibrated with 1% formic acid. The presence of small peptides was not detected; the elution profile was identical to that of the ^{14}C-amino acids.

Inhibition of cell protein synthesis by UV-irradiated virus. The inhibition of cell protein synthesis by vaccinia in the presence of actinomycin D suggests that a functional viral genome is not required for this effect. This was tested by irradiating the virus with UV light. The infectivity (measured by plaque assay on chick embryo monolayers) of the virus could be reduced by nearly 10^{-6} with little loss of its ability to inhibit cell protein synthesis in the presence of actinomycin D (Table 2). Even after much greater irradiation, this ability is largely retained by the virus particles (Fig. 6a).

Viral protein synthesis requires a functional viral genome. The prediction was made, therefore, that after a large amount of UV-irradiation, the ability of the virus particles to initiate viral protein synthesis could be blocked without affecting the ability of the particles to inhibit cell protein synthesis. The inhibition of cell protein synthesis by UV-irradiated vaccinia in the absence of actinomycin D is shown in Fig. 6b. Net amino acid incorporation by cells infected with unirradiated virus results from the inhibition of cell protein synthesis and the synthesis of viral proteins. The significant finding is that virus irradiated with 2,700 ergs/mm^2 inhibits cell protein synthesis, but synthesizes little or no viral proteins. After much more irradiation, the ability of the virus particle to inhibit cell protein synthesis does decline. The decline is similar to that which occurs with irradiated vaccinia in the *presence* of actinomycin D (Fig. 6a); in view of the high dose required, the decline may be an effect on the viral protein rather than on the viral DNA.

Specificity of the inhibition. If the inhibitory effect of vaccinia is specific for host cell protein

TABLE 2. *Effect of UV irradiation on ability of virus to inhibit cell protein synthesis*

UV (ergs/mm^2)[a]	Survival[b]	Amino acid incorporation (% of uninfected cells) at hours after infection[c]			
		1 to 1.5	2 to 2.5	3 to 3.5	4 to 4.5
0	1	56.7	10.3	5.3	2.8
135	3×10^{-3}	61.5	18.2	6.3	2.8
270	4×10^{-4}	67.5	18.8	5.2	4.7
810	2×10^{-6}	75.4	20.2	8.8	3.9

[a] Purified virus (2×10^9 PFU/ml) in 1 mM Tris (*p*H 8.8) was irradiated in 60-mm plastic petri dishes.

[b] Survival was measured by plaque assay on primary chick embryo monolayers.

[c] Actinomycin D-treated cells (5 μg/ml) were infected with the same multiplicity of virus particles equivalent to 25 PFU/cell of unirradiated virus. After 30 min the cells were diluted from a concentration of 4×10^6/ml to 4×10^5/ml. At the indicated times, ^{14}C-amino acids were added to 5-ml portions. After 30 min, the reactions were terminated. Uninfected cells were treated in an identical manner. The counts/min per mg of trichloroacetic acid-precipitable protein was determined and the percentages were calculated.

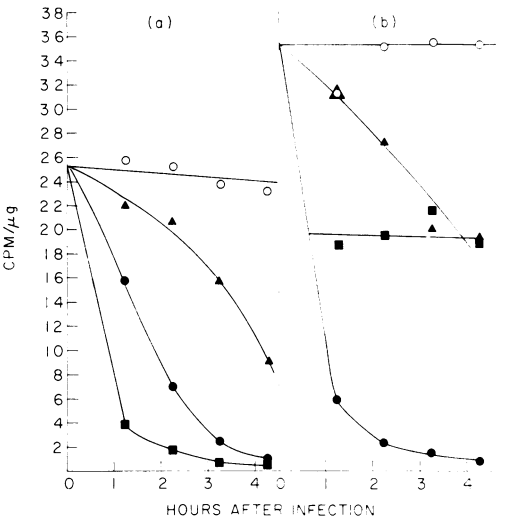

FIG. 6. *Inhibition of HeLa cell protein synthesis by UV-irradiated virus in the presence and absence of actinomycin D. Purified virus (2×10^9 PFU/ml) in 1 mM Tris (pH 8.8) was irradiated with a germicidal lamp as described. Separate portions of HeLa cells in 0.1 amino acid medium were infected with the same multiplicity of virus particles equivalent to 25 PFU/cell of unirradiated virus. After 30 min, the cells were diluted from a concentration of 4×10^6 cells/ml to 4×10^5 cells/ml. At the indicated times, 5-ml portions were incubated for 30 min with 1.25 μc of ^{14}C-amino acids. Uninfected cells were treated in an identical manner. The counts/min per μg of trichloroacetic acid-precipitable protein was determined. The experiment was repeated with actinomycin D-treated cells. (a) Actinomycin D. (b) No actinomycin D. Symbols: ○, no virus; ■, unirradiated virus; ●, irradiated (2,700 ergs/mm^2) virus; ▲, irradiated (8,100 ergs/mm^2) virus.*

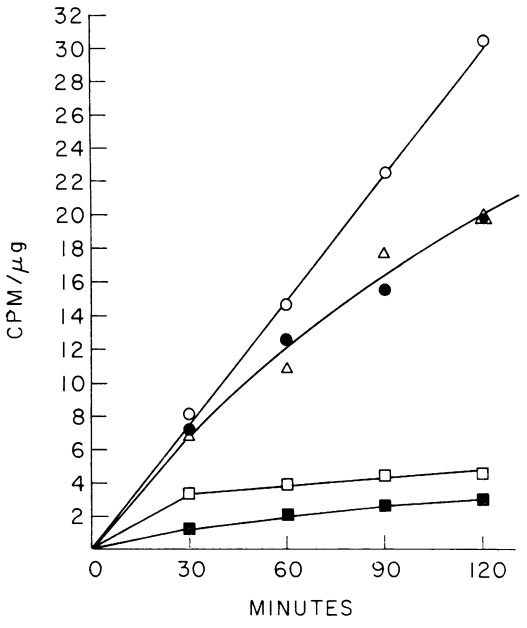

FIG. 7. *Effect of UV-irradiated virus on amino acid incorporation in infected and uninfected HeLa cells. A suspension of HeLa cells was divided into several portions. One portion was infected at zero-time with 25 PFU of virus per cell; another irradiated (800 ergs/mm²) was infected with the same number of virus particles. The remaining cultures were not infected. After 30 min, all the cells were washed and resuspended in fresh medium. At this time, infection or superfinection was performed with either unirradiated or irradiated virus. After an additional 30 min, the cells were diluted to a concentration of 4 × 10⁵/ml and 1.25 μc of ¹⁴C-amino was added. Samples were removed at 30-min intervals and the counts/min per μg of trichloroacetic acid-precipitable protein was determined. Symbols: ○, no virus; △, unirradiated virus added 30 min after zero-time; □, irradiated virus added 30 min after zero-time; ●, unirradiated virus added at zero-time and irradiated virus 30 min after zero-time; ■, irradiated virus added at zero-time and unirradiated virus 30 min after zero-time. The time scale on the abscissa refers to minutes after addition of ¹⁴C-amino acids.*

synthesis, UV-irradiated vaccinia should not affect the synthesis of viral proteins in cells preinfected with unirradiated virus. Accordingly, HeLa cells were infected with 25 PFU of live vaccinia per cell. After 30 min, the cells were washed and then challenged with the same amounts of irradiated virus. Amino acid incorporation was measured following the latter addition. Irradiated virus inhibited cell protein synthesis in uninfected cells, but had no effect on viral protein synthesis in preinfected cells (Fig. 7). This experiment shows the specificity of the effect on uninfected HeLa cells, but it does not indicate the level at which infected cells have achieved immunity. If cell protein synthesis is first blocked by treatment with irradiated virus, superinfection with live virus does not stimulate protein synthesis (Fig. 7). Possible explanations include (i) the failure of the superinfecting virus to properly penetrate the cell, (ii) a requirement of host protein synthesis for the initiation of viral protein synthesis, and (iii) sensitivity of very early viral protein synthesis to the inhibitory process.

Effects of protein denaturing agents. Incubation of vaccinia at 56 C causes a progressive loss in the ability to inhibit HeLa cell protein synthesis (Table 3). Virus heated at 56 C for 30 min has completely lost the ability to rapidly inhibit cell protein synthesis in the presence or absence of actinomycin D. Virus treated with a 0.05% concentration of the detergent NP-40 (Shell, Ltd., England) for 60 min has also lost the ability to block cell protein synthesis (Table 4). These methods of denaturation have been reported (4, 7) to create particles that are readily taken up by host cells, but that fail to leave the phagocytic vacuoles. There they are gradually destroyed. The fate of the particles has not been verified by electron microscopy in these experiments.

TABLE 3. *Effect of heating at 56 C on ability of virus to inhibit cell protein synthesis*

Time at 56 C (min)[a]	Survival[b]	Amino acid incorporation (% of uninfected cells) at hours after infection[c]			
		1 to 1.5	2 to 2.5	3 to 3.5	4 to 4.5
0	1	22	9	4	3
2.5	1	46	25	13	7
5.0	6×10^{-1}	62	40	24	18
10.0	5×10^{-2}	77	70	37	33
30.0	$<10^{-4}$	117	115	84	100

[a] Purified virus, suspended in growth medium containing 5% horse serum, was sealed in glass vials and totally immersed in a 56 C water bath for the indicated times. Inactivation was stopped by placing the vials on ice.

[b] Survival was measured by plaque assay on primary chick embryo monolayers.

[c] Actinomycin D-treated cells (5 μg/ml) were infected with the same multiplicity of virus particles equivalent to 30 PFU/cell of untreated virus. After 30 min, the cells were diluted from a concentration of 4×10^6/ml to 4×10^5/ml. At the indicated times, ¹⁴C-amino acids were added to 5-ml portions. After 30 min, the reactions were terminated. Uninfected cells were treated in an identical manner. The counts/min per μg of trichloroacetic acid-precipitable protein was determined and the percentages were calculated.

Irreversible inhibition of cell protein synthesis by vaccinia in the presence of cycloheximide. Even purified preparations of vaccinia may contain small amounts of RNA (12, 28). These experiments have not ruled out the possibility that this RNA may be used to synthesize specific proteins that are involved in the inhibition of cell protein synthesis. The following experiments were designed to test whether viral protein synthesis is required prior to the inhibition of cell protein synthesis. The experiments depend on the reversibility of cycloheximide as an inhibitor of protein synthesis. Cells were infected with vaccinia in the presence of both actinomycin D and cycloheximide; after intervals of time, the latter drug was washed out. After washing, the ability of the cells to synthesize protein was measured. If the viral inhibitor is associated with the virion, the removal of cycloheximide should not lead to the resumption of cell protein synthesis. Alternatively, if the inhibitor is a protein synthesized after virus infection, this protein could not have been synthesized in the presence of cycloheximide. In the latter case, removal of cycloheximide should permit cell protein synthesis to continue until the hypothetical inhibitor is synthesized and accumulates within the cell.

TABLE 4. *Effect of NP-40 detergent on ability of virus to inhibit cell protein synthesis*

Treatment	Amino acid incorporation[c] (% of uninfected cells) at hours after infection			
	1 to 1.5	2 to 2.5	3 to 3.5	4 to 4.5
None	22.8	10.2	4.8	2.0
NP-40[a]	95.8	91.6	76.9	87.3
Heat[b]	90.2	105.0	98.2	72.6

[a] Purified virus (2.5×10^9 PFU/ml) was treated with .05% NP-40 detergent in 1 mM Tris (*p*H 8.8) for 1 hr at 37 C. The virus was pelleted and resuspended. Survival was less than 10^{-4}.

[b] Purified virus (2.5×10^9 PFU/ml) in 1 mM Tris (*p*H 8.8) was heated for 30 min in a sealed vial totally immersed in a 56 C water bath. Survival was less than 10^{-4}.

[c] Actinomycin D-treated cells (5 μg/ml) were infected with the same multiplicity of virus particles equivalent to 33 PFU/cell of untreated virus. After 30 min, the cells were diluted from 4×10^6/ml to 4×10^5/ml. At indicated times, 5-ml portions were incubated with ^{14}C-phenylalanine. After 30 min, the reactions were terminated. Uninfected cells were treated in an identical manner. The counts/min per μg of trichloroacetic acid-precipitable protein was determined and the percentages were calculated.

Preliminary experiments confirmed that concentrations of cycloheximide from 20 to 300 μg/ml reversibly inhibited amino acid incorporation in HeLa cells from 91 to 98%, respectively, within 15 min. Cells pretreated with actinomycin D and cycloheximide, (Table 5) were infected with 25 PFU of vaccinia per cell. After 2 hr, some of the cells were washed and resuspended in cold medium that was free of cycloheximide. Other cells were washed and resuspended in cold media that contained the original concentration of cycloheximide. Uninfected cells were treated in a similar manner. Care was taken to keep the cells at 2 C during the entire washing procedure to prevent protein synthesis from occurring prior to the addition of ^{14}C-amino acids. Only after the

TABLE 5. *Inhibition of cell protein synthesis by vaccinia in the presence of actinomycin D and cycloheximide*[a]

Culture no.	Actinomycin D (μg/ml)	Cycloheximide (μg/ml)	Virus (PFU/cell)	Cycloheximide (μg/ml) in wash	Trichloroacetic acid-precipitable protein (counts/min per mg) at time (min)			
					20	40	60	80
1	5	0	0	0	12,702	31,441	44,268	66,985
2	5	300	0	300	303	833	1,250	1,407
3	5	300	0	0	8,939	20,428	31,823	41,137
4	5	0	25	0	969	1,770	2,561	2,982
5	5	300	25	300	959	934	1,214	1,227
6	5	300	25	0	1,390	2,403	2,862	3,233

[a] HeLa cells were treated with actinomycin D or actinomycin D + cycloheximide. After 30 min, three cultures were infected with purified virus. After 30 min of adsorbtion, all cells were resuspended to a concentration of 4×10^5/ml. The initial concentration of actinomycin and cycloheximide (0 or 300 μg/ml) was maintained. After infection for 2 hr, all cells were washed three times at 2 C. Some cultures were washed free of cycloheximide, but in others the wash medium contained 300 μg/ml of cycloheximide. The cells were resuspended in fresh cold medium of the same composition used for the wash. ^{14}C-amino acids (0.33 μc/ml) was added and the cells were returned to the 37 C water bath. At 20-min intervals, samples were removed and the counts/min per mg of trichloroacetic aid-precipitable protein was determined.

addition of ^{14}C-amino acids were the cells returned to the 37 C water bath. Cells were removed at timed intervals, and amino acid incorporation was measured. The results are presented in Table 5. The three upper lines of Table 5 show (i) amino acid incorporation by uninfected cells in the presence of actinomycin D, (ii) the inhibition of protein synthesis by cycloheximide, and (iii) the ability of uninfected cells to resume protein synthesis after washing out cycloheximide. The three lower lines are the identical experiments conducted simultaneously with virus-infected cells. They show (i) the inhibition of cell protein synthesis by vaccinia in the presence of actinomycin D, (ii) the inhibition of protein synthesis by cycloheximide in virus-infected cells, and (iii) the failure of the virus-infected cells to resume protein synthesis after washing out cycloheximide. Comparison of lines 3 and 6 is particularly important. This experiment demonstrates that, after vaccinia infection, the synthesis of RNA or protein is not required for the viral inhibition of cell protein synthesis. This experiment was repeated, using lower concentrations of cycloheximide, with the identical conclusion.

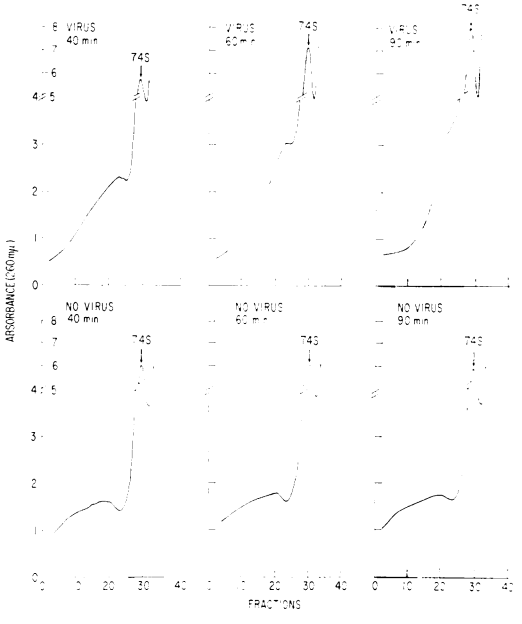

FIG. 8. *Effect of vaccinia virus on host polyribosomes. HeLa cells (4 × 10⁶/ml) were treated for 20 min with 5 μg of actinomycin D per ml. The cells were divided into two portions; one was infected with 25 PFU of vaccinia virus per cell. After 30 min, the cells were diluted to 4 × 10⁵/ml in the same medium. At 40, 60 and 90 min after infection, 8 × 10⁷ cells were rapidly centrifuged, and then resuspended in cold, Earle's solution. The cells were washed and the cytoplasm was obtained by Dounce homogenization in 1.2 ml of 10 mM Tris-chloride, 10 mM KCl, 1.5 mM $MgCl_2$ (pH 7.4). The nuclei were removed by low speed centrifugation and the supernatant fluid was layered on top of a preformed 15 to 30% linear sucrose gradient in the latter buffer. Centrifugation was at 63,580 × g for 105 min in the SW 25.1 rotor. The gradient was collected by piercing the bottom of the tube and pumping through a 2-mm Gilford flow cell.*

Effect of vaccinia virus on polyribosomes. The effect of vaccinia virus on host cell polyribosomes was examined. HeLa cell polyribosomal protein synthesis decays with a half-life of 3 to 4 hr after treatment with actinomycin D (26). The stability of HeLa cell polyribosomes is demonstrated in Fig. 8. If virus is added to HeLa cells in the presence of actinomycin D, an enhanced rate of breakdown occurs. A loss of heavy polyribosomes and an increase in single ribosomes is apparent 40 min after infection with 25 PFU of purified vaccinia per cell (Fig. 8). The decrease in polyribosome size is progressive.

DISCUSSION

Mengo, polio, Newcastle disease, and herpes virus have been shown to inhibit host macromolecular synthesis. With these viruses, the inhibitor responsible for the suppression of host protein synthesis is synthesized after infection (1, 27, 34, 35). In the case of adenovirus, the inhibition does not occur until viral antigen production has started 16 to 20 hr after infection (3), although inhibition has been attributed to proteins that are structural components of the virus (19). The inhibition of host protein synthesis by vaccinia virus is quite different. The very rapid inhibition of HeLa cell protein synthesis after vaccinia infection, the multiplicity effect, the ability to establish the block in the presence of actinomycin D and cycloheximide, and the retention of this ability by irradiated virus unable to support significant viral protein synthesis indicate that a functional viral genome is not required for inhibition of host protein synthesis. Because all experiments reported here were carried out with highly purified vaccinia preparations, the large, complex vaccinia particle itself must be responsible for the block in cell protein synthesis. It has not been established, however, whether specific proteins or other constituents of the virus are directly responsible for the block, or whether cell enzymes that are released after virus penetration are involved. At least 17 to 20 polypeptides can be resolved by polyacrylamide gel electrophoresis of dissociated vaccinia virions (10, 22), and at least two enzyme activities are associated with the virion (16, 23, 24).

The rapid inhibition of cell protein synthesis may be partly responsible for other viral effects such as cytopathic changes (9), the block in the transfer of nuclear RNA to the cytoplasm (2, 30), and the block in cell DNA synthesis (13, 17, 18). The latter effect has also been reported to occur in the presence of a protein inhibitor (13).

The rapidity with which protein synthesis is inhibited indicates that the block must occur at the level of translation of messenger RNA (mRNA) and not at the level of transcription. The progressive reduction in the size of cell polyribosomes after vaccinia infection, observed when viral RNA synthesis is prevented, is consistent with either degradation of host mRNA or interference with ribosome attachment to mRNA. Joklik and Merigan (14) reported a breakdown of host polyribosomes in vaccinia-infected cells that were pretreated with interferon. Under the latter condition, viral RNA is synthesized, but apparently it cannot associate with ribosomes.

It is possible that the viral translation process, in contrast to the host translation process, is immune to the inhibition caused by the vaccinia particle. Alternatively, the large amounts of viral RNA that are synthesized may obscure a relative inhibition. It is known that early viral mRNA is relatively stable (15, 21, 25, 31), although this has not been tested as a function of the multiplicity of infection. Experiments are in progress to distinguish between various mechanisms of inhibition and to determine the nature of this selectivity.

ACKNOWLEDGMENTS

I thank N. P. Salzman for advice and encouragement, A. J. Shatkin for reading the manuscript, and Carol Uhlendorf for technical assistance.

LITERATURE CITED

1. Baltimore, D., Franklin, R. M., and Callender, J. 1963. Mengovirus-induced inhibition of host ribonucleic acid and protein synthesis. Biochim. Biophys. Acta **76**:425–430.
2. Becker, Y., and Joklik, W. K. 1964. Messenger RNA in cells infected with vaccinia virus. Proc. Natl. Acad. Sci. U.S. **51**:577–585.
3. Bello, L. J., and Ginsberg, H. S. 1967. Inhibition of host protein synthesis in type 5 adenovirus-infected cells. J. Virol. **1**:843–850.
4. Dales, S., and Kajioka, R. 1964. The cycle of multiplication of vaccinia virus in Earle's strain L cells. I. Uptake and penetration. Virology **24**:278–294.
5. Dubbs, D. R. and Kit, S. 1964. Isolation and properties of vaccinia mutants deficient in thymidine kinase inducing activity. Virology **22**:214–225.
6. Eagle, H. 1959. Amino acid metabolism in mammalian cell culture. Science **130**:432–437.
7. Easterbrook, K. B. 1966. Controlled degradation of vaccinia virions in vitro: an electron microscopic study. J. Ultrastruct. Res. **14**:484–496.
8. Fairbanks, G., Jr., Levinthal, C., and Reeder, R. H. 1965. Biochem. Biophys. Res. Commun. **20**:393–399.
9. Hanafusa, H. 1960. Killing of L-cells by heat and UV-inactivated vaccinia virus. Biken's J. **3**:191–199.
10. Holowczak, J. A., and Joklik, W. K. 1967. Studies on the structural proteins in vaccinia virus. I. Structural proteins of virions and cores. Virology **33**:717–725.
11. Holowczak, J. A., and Joklik, W. K. 1967. Studies on the structural proteins of vaccinia virus. II. Kinetics of the synthesis of individual groups of structural proteins. Virology **33**:726–739.
12. Joklik, W. K. 1962. The preparation and characteristics of highly purified radioactively labeled poxvirus. Biochim. Biophys. Acta **61**:290–301.
13. Joklik, W. K., and Becker, Y. 1964. The replication and coating of vaccinia DNA. J. Mol. Biol. **10**:452–474.
14. Joklik, W. K., and Merigan, T. C. 1966. Concerning the mechanism of action of interferon. Proc. Natl. Acad. Sci. U.S. **56**:558–565.
15. Jungwirth, C., and W. K. Joklik. 1965. Studies on "early" enzymes in HeLa cells infected with vaccinia virus. Virology **27**:80–93.
16. Kates, J. R., and McAuslan, B. R. 1967. Poxvirus DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. U.S. **58**:134–141.
17. Kato, S., Ogowa, M., and Miyamoto, H. 1964. Nucleocytoplasmic interactions in poxvirus infected cells. I. Relationship between inclusion formation and DNA metabolism of the cells. Biken's J. **7**:45–56.
18. Kit, S., and Dubbs, D. R. 1962. Biochemistry of vaccinia-infected mouse fibroblasts (strain L-M). I. Effects on nucleic acid and protein synthesis. Virology **18**:274–285.
19. Levine, A. J., and Ginsberg, H. S. 1967. Mechanism by which fiber antigen inhibits multiplication of type 5 adenovirus. J. Virol. **1**:747–757.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21. McAuslan, B. R. 1963. The induction and repression of thymidine kinase in the poxvirus infected HeLa cell. Virology **21**:383–389.
22. Moss, B., and Salzman, N. P. 1968. Sequential protein synthesis following vaccinia virus infection. J. Virol. **2**:1016–1027.
23. Munyon, W., Paoletti, E., and Grace, J. T., Jr. 1968. RNA polymerase activity in purified infectious vaccinia virus. Proc. Natl. Acad. Sci. U.S. **58**:2280–2287.
24. Munyon, W., Paoletti, E., Ospina, J., and Grace, J. T., Jr. 1968. Nucleotide phosphohydrolase in purified vaccinia virus. J. Virol. **2**:167–172.
25. Oda, K., and Joklik, W. K. 1967. Hybridization and sedimentation studies on "early" and "late" vaccinia messenger RNA. J. Mol. Biol. **27**:395–419.

26. Penman, S., Scherrer, K., Becker, Y., and Darnell, J. E. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger-RNA. Proc. Natl. Acad. Sci. U.S. **49**:654–662.
27. Penman, S., and Summers, D. 1965. Effects on host cell metabolism following synchronous infection with poliovirus. Virology **27**:614–620.
28. Planterose, D. N., Nishimura, C., and Salzman, N. P. 1962. The purification of vaccinia virus from cell culture. Virology **18**:294–301.
29. Salzman, N. P., and Sebring, E. D. 1967. Sequential formation of vaccinia virus proteins and viral deoxyribonucleic acid replication. J. Virol. **1**:16–23.
30. Salzman, N. P., Shatkin, A. J., and Sebring, E. D. 1964. The synthesis of a DNA-like RNA in the cytoplasm of HeLa cells infected with vaccinia virus. J. Mol. Biol. **8**:405–416.
31. Sebring, E., and Salzman, N. P. 1967. Metabolic properties of early and late vaccinia virus messenger ribonucleic acid. J. Virol. **1**:550–558.
32. Shatkin, A. J. 1963. Actinomycin D and vaccinia virus infection in HeLa cells. Nature **199**:357–358.
33. Shatkin, A. J. 1963. The formation of vaccinia virus protein in the presence of 5-fluorodeoxyuridine. Virology **20**:292–301.
34. Sydiskis, R. J., and Roizman, B. 1967. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virology **32**:678–686.
35. Wheelock, E. F. 1962. The role of protein synthesis in the eclipse period of Newcastle disease virus multiplication in HeLa cells as studied with puromycin. Proc. Natl. Acad. Sci. U.S. **48**:1358–1366.