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Initiation of Vaccinia Virus Infection in Actinomycin D-pretreated Cells

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The early steps in vaccinia virus infection were studied in HeLa cells which had been treated with actinomycin D (1 μg/ml) and then incubated for several hours in fresh medium prior to infection. Initiation of infection occurred in such cells even though the synthesis of cellular ribonucleic acid and deoxyribonucleic acid (DNA) was severely depressed. Thymidine kinase was synthesized in amounts that exceeded those found in untreated, infected cells. The breakdown of viral "cores" to liberate viral DNA and the synthesis of viral specific DNA-polymerase also occurred but were somewhat delayed. A deoxyribonuclease resembling an exonuclease was made by the infected, pretreated cells. The time course for these events suggested that the genetic code for synthesis of thymidine kinase can be expressed before "cores" are broken down, but the DNA-polymerase can be synthesized only after liberation of the viral DNA. The amount of viral specific DNA-polymerase which was made after infection was proportional to the total number of virus synthesizing sites even beyond the point where all the cells were infected with one infectious particle. A similar relationship was observed for the amount of thymidine kinase formed and for the rate of viral DNA synthesis from ^{3}H-thymidine.

One of the most puzzling features of infection of mammalian cells with vaccinia virus has been that of the initial unmasking of virus nucleic acid. The studies of Joklik (10, 11) complemented by the electron microscopic observations of Dales (2, 4, 15) established many quantitative aspects of the process (for recent reviews, *see* 3, 12). Virus particles are engulfed into phagocytic vesicles where the outer protein coat of the virus is lost. The "core" that is left then must escape the vesicle, and the viral deoxyribonucleic acid (DNA) must be liberated and virus infection established. New protein synthesis was necessary for the release of viral DNA into a deoxyribonuclease-sensitive form (11). The process appeared coded for by DNA because "cores" accumulated quantitatively in the presence of actinomycin D. Pretreatment of cells immediately before infection with actinomycin D also depressed uncoating. These and other considerations led Joklik to postulate that the virus "core" was broken down by a cellular enzyme, the synthesis of which was induced by a protein of the invading virus. We have reported (using somewhat different conditions) that a pretreatment of cells with actinomycin D need not suppress initial reactions and that such pretreated cells could be used to determine the rate of viral DNA synthesis by measuring ^{3}H-thymidine uptake over a low cellular background (27). This paper presents the results of a more detailed study on the effect of actinomycin D pretreatment on early events and of the multiplicity of infection on early events. A brief report of some of these data has appeared (W. E. Magee and O. V. Miller, Federation Proc. **25**:652, 1966).

METHODS AND MATERIALS

HeLa cells were grown as monolayers in 32-oz (946-ml) prescription bottles in 40 ml of a complete medium containing 20% calf serum. The pretreatment with actinomycin D was performed by decanting the medium from 48-hr-old cells and replacing it with fresh medium containing actinomycin D (1 μg/ml). After 1.75 to 2 hr of incubation at 37 C, the drug-containing medium was decanted, and the monolayers were washed twice with Puck's saline. Fresh medium without the drug was added to the bottles which were then incubated for an additional 1.75 to 2 hr at 37 C. Control cells were treated similarly, except the medium did not contain the drug. For most of the experiments, the cells next were placed in suspension. The cell sheets were washed once with Eagle's basal medium without serum; then they were dislodged from the glass surface by treatment with 10 ml of 0.25% trypsin in Eagle's medium. They were collected by centrifugation, washed twice with Eagle's medium, and recovered by centrifugation for 2 min at 600 × *g*. The cells were resuspended (1.5 × 10^7/ml), and virus suspension was added (zero-hour). Infection was allowed to pro-

ceed for 30 min at 37 C with occasional swirling. The *p*H was adjusted with $NaHCO_3$ solution as needed. At the end of infection, the cells were collected by centrifugation, washed one or more times in medium, transferred into stoppered Erlenmeyer flasks at a level of 1.0×10^6 to 1.3×10^6 cells/ml, and incubated on a rotary shaker at 37 C.

Radioactive vaccinia virus was obtained by incubating infected cells in medium containing ^{3}H-thymidine for 18 to 20 hr. The radioactive virus was purified (W. E. Magee et al., Virology, *in press*). The final preparation contained 8 to 10 physical particles per plaque-forming unit (PFU), as determined by electron microscopic count and plaque assay on chick fibroblast cells.

Enzymatic assays were carried out as described for thymidine kinase (27) and DNA-polymerase (26, 28).

Deoxyribonuclease activity was determined by measuring the release of radioactivity from labeled DNA. The radioactive DNA was prepared with a scaled-up DNA-polymerase reaction mixture and with a particulate enzyme from infected cells (28) and salmon sperm DNA as primer. The enzyme was removed from the reaction mixture at the end of 30 min of incubation by high-speed centrifugation, and the DNA was denatured by heating in a boiling-water bath for 5 min followed by rapid cooling. The reaction mixture for the enzymatic assay of deoxyribonuclease was the same as that used for DNA-polymerase without the radioactive deoxynucleotide triphosphate. After 30 min of incubation at 37 C, the samples were chilled and 0.05 ml of 4 mg/ml bovine serum albumin was added to each tube, followed by 0.05 ml of 2.5 N $HClO_4$ containing celite. The samples were centrifuged, a portion of the supernatant fluid was withdrawn and heated for 30 min at 50 C, and the radioactivity was counted.

Virus, "cores," and viral DNA from cell homogenates were separated from one another by centrifugation through linear gradients consisting of 10 to 40% sucrose in 0.01 M tris(hydroxymethyl)aminomethane buffer, *p*H 7.4 (11). The gradients were centrifuged at 15,000 rev/min for 40 min with an SW 25.1 swinging bucket rotor. Twenty-three to twenty-five fractions were collected from each tube, carrier DNA was added, and acid-insoluble radioactivity was determined for each.

RESULTS

Conditions for pretreatment of cells with actinomycin D. Radioactive actinomycin D (1 μg/ml) was used to determine the amount of drug which became firmly bound to HeLa cells (Table 1). Approximately 9% of the added antibiotic was taken up by the cells in 1.75 hr. During the subsequent incubation period with normal medium, 39 to 46% of the cell-associated radioactivity eluted from the cells, indicating the effectiveness of this procedure to remove loosely bound antibiotic. An additional 23% of the total was still susceptible to methanol and cold trichloroacetic acid extraction.

Cellular nucleic acid metabolism was severely depressed by actinomycin D and did not recover after the drug was removed. Ribonucleic acid (RNA) synthesis was followed by measuring ^{3}H-cytidine incorporation. In one experiment, the uptake of isotope was 28% of normal by the second hour of actinomycin D treatment, 14% of normal during the first 1-hr interval after adding normal medium, and 5 to 8% of normal during the subsequent 2 to 5 hr. Table 2 gives ^{3}H-thymidine uptake into DNA by control and infected cells that were treated with actinomycin D in various ways. Cellular DNA synthesis was very low after exposure to 0.5 μg/ml of the drug. No viral DNA could be made in infected cells if 0.5 μg/ml or more of actinomycin D was present prior to and during infection. Viral DNA was

TABLE 1. *Uptake and elution of radioactive actinomycin D by HeLa cells*[a]

Fraction	Sample 1		Sample 2	
	Counts/min	Percentage of total	Counts/min	Percentage of total
Total added	7.13×10^4		7.13×10^4	
Total taken up by cells	6.24×10^3	100	6.50×10^3	100
Eluted into normal medium	2,210		2,820	
Eluted into wash	225		169	
Total eluted	2,435	39	2,989	46
Extracted from cells				
Methanol 1	793		—	
Methanol 2	172		—	
TCA	490		—	
Total extracted	1,455	23	—	
Alkaline digest	2,350	38	3,520	54

[a] ^{3}H-actinomycin D (0.99 μg/ml; 7.20×10^3 counts per min per μg) was incubated with approximately 5×10^6 HeLa cells in monolayers for 1.75 hr. The cells were washed four times with Puck's saline and incubated in unlabeled medium for 1.75 hr, washed, and harvested. Cells from sample 1 were extracted as indicated; cells from sample 2 were digested immediately in alkali.

TABLE 2. *Comparison of ^{3}H-thymidine incorporation by control and infected cells treated in various ways with actinomycin D*[a]

Treatment	Amt of drug (μg/ml)	Counts per min per mg of DNA	
		Control	Infected
Cells pretreated with actinomycin D 1.75 hr, followed by incubation for 1.75 hr, without drug	1.0	0.14×10^4	3.36×10^4
Same but no actinomycin D used	0	—	2.80×10^4
Actinomycin D added at 2 hr postinfection	1.0	0.81×10^4	1.20×10^4
Actinomycin D added 1 hr prior to infection and present during and after infection	0	4.01×10^4	3.84×10^4
	0.5	0.22×10^4	0.51×10^4
	1.0	0.11×10^4	0.55×10^3
	2.0	0	0

[a] ^{3}H-thymidine uptake into DNA was measured in all samples at 4.5 to 5 hr postinfection. Control cells received the same medium changes, etc., as the infected cells. Maximal uptake of ^{3}H-thymidine into infected, nontreated cells occurs between 2 and 3 hr postinfection.

made in cells which received the actinomycin D in a pretreatment only. This observation was amply confirmed by microradioautography, since radioactivity was found concentrated in viral inclusion areas (22). A partial inhibition of DNA synthesis was observed when the drug was added at 2 hr postinfection.

Early events in actinomycin D-pretreated cells. Increases in thymidine kinase and DNA-polymerase after infection were compared in normal and actinomycin D-pretreated cells (Fig. 1). Initiation of the synthesis of thymidine kinase was normal, but there was a delay in its "shut off" that caused elevated levels of the enzyme in the pretreated cells (Fig. 1A). In contrast, first increases in DNA-polymerase were delayed somewhat, and synthesis proceeded at a slower rate than normal (Fig. 1B). Actinomycin D (1 μg/ml) in the pretreatment resulted in the highest level of thymidine kinase and permitted 70 to 90% of normal increase in DNA-polymerase by 6 hr postinfection (Fig. 3). Kit et al.

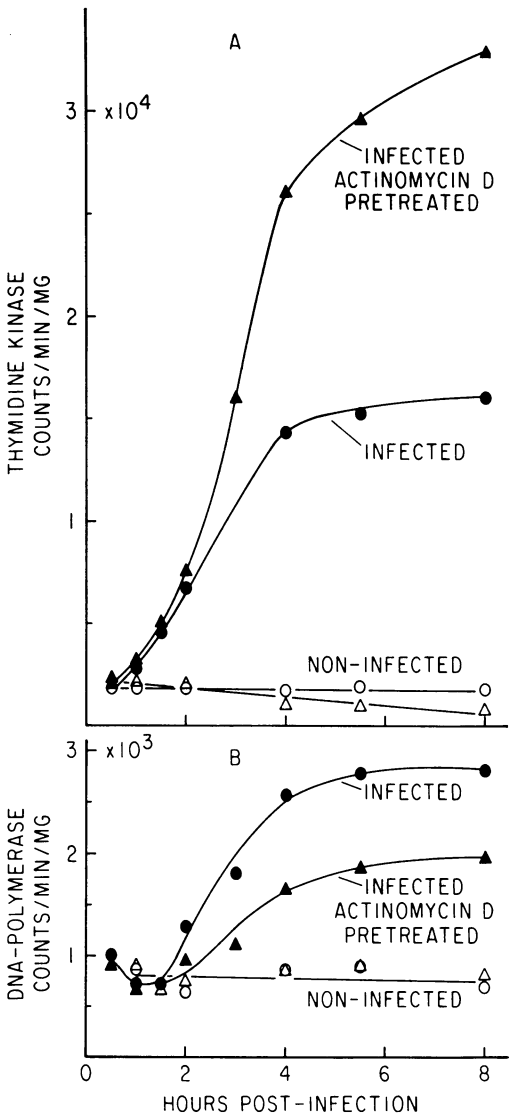

FIG. 1. *Thymidine kinase (A) and DNA-polymerase (B) activities after infection of normal and actinomycin D-pretreated cells. Closed symbols represent infected cells, and open symbols, the corresponding noninfected cells. Actinomycin D (1 μg/ml) was used in the pretreatment. The cells were infected in suspension with an adsorbed multiplicity of approximately 5 PFU/cell.*

(21) also observed synthesis of thymidine kinase in infected, actinomycin D-pretreated cells, but less than normal amounts were found.

Uncoating of the invading virus was followed with virus labeled in DNA by ^{3}H-thymidine. Virus, "cores," and viral DNA were separated on sucrose gradients according to the procedures of Joklik (11). Figure 2A shows the quantitative

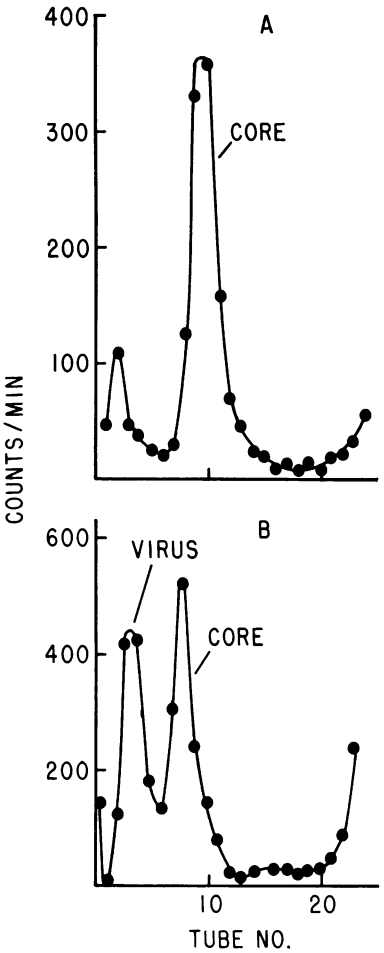

FIG. 2. *Separation of virus, "cores," and viral DNA on sucrose density gradients. (A) "Cores" accumulated by 3 hr postinfection in the presence of actinomycin D. (B) Normal infection 45 min after exposure of cells to virus. Vaccinia virus labeled in DNA with ^{3}H-thymidine was used. Adsorbed multiplicity was approximately 90 particles per cell.*

accumulation of "cores" in cells which were treated with actinomycin D throughout infection (11). During a normal infection, "cores" appear and disappear very rapidly if high multiplicities of infection are used. Approximately equal amounts of "cores" and virus were present at 45 min postinfection of normal cells (Fig. 2B). By 1.5 hr after infection, only small amounts of either intact virus or "cores" remained, and viral DNA was found in the top of the gradient after sonic treatment of the cells or in the bottom attached to particulate material if cells were processed by Dounce homogenization (28).

Gradients were run to establish a time course for the appearance and disappearance of "cores" in normal and pretreated cells (Fig. 3). In normal cells, "cores" were broken down rapidly and about 30 min in advance of new DNA-polymerase synthesis (Fig. 3A). The time course shown is more rapid than that published by Joklik (10, 11) because of the higher multiplicity of infection used. In actinomycin D-pretreated cells, "cores" were broken down, but their disappearance was delayed and proceeded at a slower rate. The increase in DNA-polymerase correlated well with this time course. In contrast to the data for thymidine kinase, these experiments indicated that liberation of viral DNA from the "core" had to be well under way before new DNA-polymerase was synthesized.

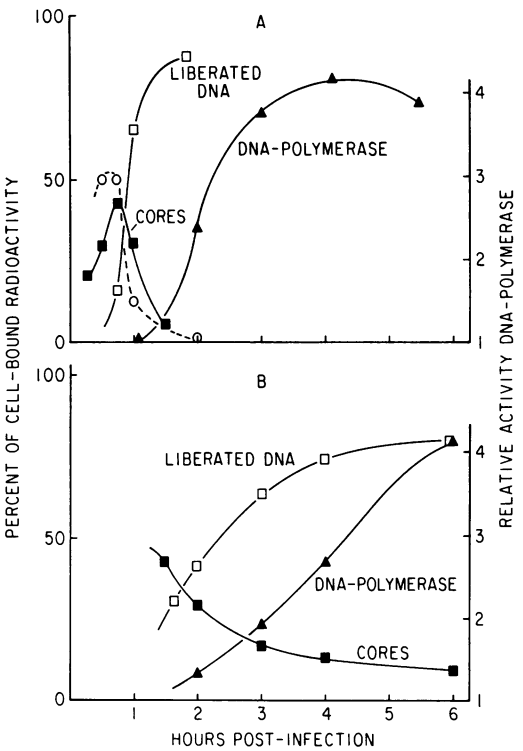

FIG. 3. *Time course for the breakdown of "cores" and the appearance of new DNA-polymerase in (A) normal and (B) actinomycin D-pretreated cells. The percentage of radioactivity in "cores" was determined from that recovered from sucrose density gradients using virus labeled with ^{3}H-thymidine. Liberated DNA was measured as radioactivity which became acid-soluble after incubation of samples of the homogenate with deoxyribonuclease (10). Two experiments are shown for core breakdown in (A) (solid and dashed lines). DNA-polymerase activity is shown as relative to that of control cells equal to 1.0. Adsorbed multiplicity of infection was 60 particles per cell.*

McAuslan recently has shown increases in deoxyribonuclease activities after infection with vaccinia virus (23, 24), and we observed that a deoxyribonuclease increase is demonstrable after infection of either normal or actinomycin D-pretreated cells (Fig. 4A). The enzyme was most active with a heat-denatured substrate, and the activity was at least 20-fold greater when a DNA-polymerase product was used rather than DNA which had been randomly labeled with ^{3}H-thymidine. These observations suggested that the enzyme was an exonuclease able to hydrolyze nucleotides from that portion of a DNA chain which had been synthesized recently by the DNA-polymerase. It thus resembles the "acid" nuclease described by Eron and McAuslan (5, 25). Like the DNA-polymerase, this enzyme was found in the particulate fractions (Fig. 4B), and its activity was somewhat lower than normal in actinomycin D-pretreated cells.

Multiplicity of infection and initial reactions. The increase in viral specific DNA-polymerase with increasing multiplicity of infection is shown in Fig. 5. The first steep rise in enzyme activity was proportional to the number of cells infected, and one infectious unit equaled 8 to 10 adsorbed virus particles. When all cells were infected, total DNA-polymerase activity appeared to be about 3.3 times that found in the control cells. Thereafter, the DNA-polymerase continued to increase at a slower rate with increasing numbers of virus adsorbed per cell. It was reasoned that this continued increase might be proportional to the number of functioning inclusions in the infected cells since viral specific DNA-polymerase and new viral DNA can be shown to be associated with particulate fractions from the cytoplasm (M. K. Bach, Federation Proc. **22**:645, 1963; 13, 14, 28). A comparison was made between ^{3}H-thymidine uptake and the number of inclusion areas that could be scored by microradioautographic examination of infected cells (Table 3). A good correspondence was seen among the multiplicity of infection, the rate of ^{3}H-thymidine uptake, and the number of inclusions in the cells. This result could be expected from the investigation of Cairns (1), who showed that each infectious particle could establish its own center of DNA synthesis. The upper limits for this relationship could not be examined because cells in the monolayers fused together in large masses when very high multiplicities were used. A similar correspondence could be shown between thymidine kinase levels (20) and ^{3}H-thymidine uptake (Table 4). We have previously shown a close correspondence between ^{3}H-thymidine uptake and DNA-polymerase (27) so that it is apparent that the magnitudes of all these early reactions are related directly to the number of inclusion areas.

DISCUSSION

The presence of actinomycin D during vaccinia infection completely suppresses virus synthesis (8, 31). Conditions were sought for the pretreatment that would provide little or no free drug present at the time of virus addition. This was achieved by incubating cells in normal medium for several hours after removal of actinomycin D,

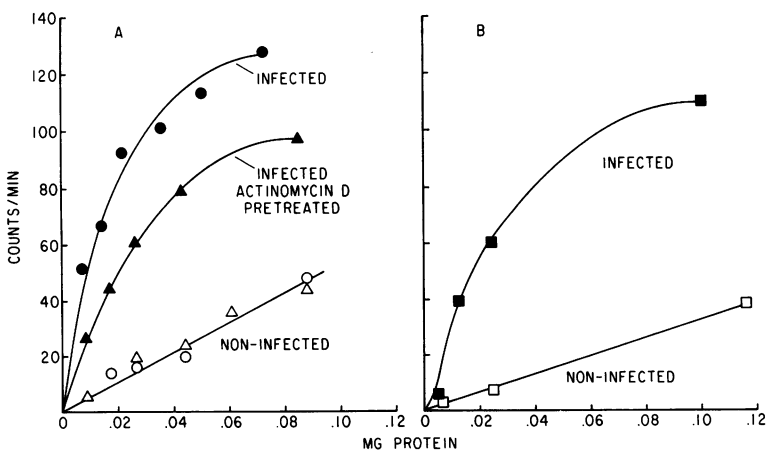

FIG. 4. *Assay of deoxyribonuclease in cytoplasmic extracts (A) and in a high-speed pellet fraction from the cytoplasm (B). The cells were ruptured by Dounce homogenization at 4 hr postinfection, and the nuclear fraction was removed by low-speed centrifugation. The substrate was radioactive DNA prepared by the DNA-polymerase reaction.*

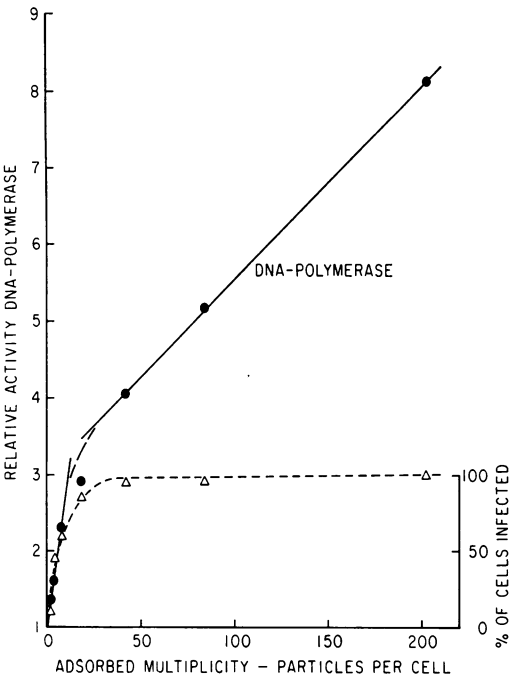

FIG. 5. *Relationship between new DNA-polymerase activity and the adsorbed multiplicity of infection. The number of virus particles adsorbed to cells in suspension was determined directly by the use of virus labeled with ^{3}H-thymidine. DNA-polymerase was measured 5 hr postinfection. Percentage of cells infected was determined by plating samples of infected cells on cover slips, labeling with ^{3}H-thymidine 2 to 6 hr postinfection, and counting the cells with labeled inclusions by microradioautography. The dashed line shows the theoretical curve for 10 adsorbed particles equaling one infectious unit.*

during which time the loosely bound drug dissociated from the cells. The profound inhibition of uncoating observed by Joklik (11), with actinomycin D-pretreated cells, most likely was due to infection of cells immediately after treatment with the drug. By using our described regimen, cellular DNA and RNA synthesis was inhibited very severely by actinomycin D, yet initiation of infection occurred. Therefore, if any transcription of host cell DNA is necessary (e.g., in uncoating), it would have to take place either on sites that are extremely resistant to the action of actinomycin D, or on sites from which the drug is easily dissociated after infection. Breakdown of the viral "cores" and the increase in DNA-polymerase were delayed by approximately 45 min in the pretreated cells. Thymidine kinase synthesis, however, was initiated without delay and, in addition, failed to "shut off" at the usual time. Apparently, the viral genome must be fully exposed to code for DNA-polymerase, but this

TABLE 3. *Correlation between DNA synthesis and the number of inclusion areas in infected cells*

Adsorbed multiplicity[a] (PFU/cell)	^{3}H-thymidine uptake[b] (counts per min per mg of DNA)	Avg no. of inclusions per cell
2.2	8.2×10^4	3.24
1.2	2.9×10^4	1.31
0.45	0.7×10^4	0.54
0.1	0.3×10^4	0.03
None	0.2×10^4	0

[a] Adsorbed multiplicity was calculated from the proportion of cells showing one or more labeled inclusions by microradioautography. ^{3}H-thymidine was added from 2 to 3 hr postinfection.

[b] ^{3}H-thymidine uptake was measured in companion monolayers which were pretreated with actinomycin D. Exposure to ^{3}H-thymidine was from 3.5 to 4 hr postinfection.

TABLE 4. *Relationship between adsorbed multiplicity, thymidine kinase and DNA-synthesis*[a]

Adsorbed multiplicity (PFU/cell)	^{3}H-thymidine uptake (counts per min per mg of DNA)	Thymidine kinase (counts per min per mg of protein)
12.0	21.4×10^4	28.2×10^3
2.0	9.7×10^4	17.2×10^3
0.3	2.2×10^4	6.1×10^3
0.05	0.4×10^4	5.4×10^3
None	0.3×10^4	3.5×10^3

[a] Adsorbed multiplicity was calculated from the number of virus particles added using adsorption efficiencies determined from similar experiments with radioactive virus. ^{3}H-thymidine uptake was measured in actinomycin D-pretreated cells from 3.5 to 4 hr postinfection. Thymidine kinase was determined in the same experiment using both normal and actinomycin D-pretreated cells (average values). The cells were infected in suspension.

requirement is unnecessary for synthesis of thymidine kinase (16). These findings are in accordance with recent reports showing that viral specific RNA could be made even if "core" breakdown was prevented by inhibition of protein synthesis (16, 29, 32). Furthermore, Kates and McAuslan (17) described a DNA-dependent RNA-polymerase present on the "core" itself that catalyzed this synthesis. They suggest that the early messenger RNA may code for a variety of early virus proteins, perhaps including thymidine kinase and an uncoating enzyme. When uncoating was prevented by inhibition of protein synthesis, the amount of viral specific RNA synthesized was increased (16); this action provided evidence that "shut off" of early message takes place only

after liberation of the bulk of the viral DNA from the "cores." If increased RNA synthesis could be shown after infection of the actinomycin D-pretreated cells as a result of the short delay in "core" breakdown, an explanation would be found for the increased amounts of thymidine kinase that we observed.

The cause of the disruption of later events in virus maturation in actinomycin D-pretreated cells is not known. The yield of infectious virus is less than 10% of the normal (8, 27, 31). Kajioka et al. (15) observed an altered inclusion area in infected, actinomycin D-pretreated cells where polysomes failed to appear. The simplest explanation would be that small amounts of bound antibiotic gradually dissociate from cellular components and reassociate with the viral DNA to disrupt normal transcription. We attempted to detect this directly with microradioautography using radioactive actinomycin D, but without success. The ^{3}H-actinomycin D was localized over the nucleus (7), and no transfer of label to cytoplasmic inclusion areas was observed. The sensitivity was low in this experiment, however, due to the small amount of radioactivity incorporated.

The amount of viral DNA-polymerase synthesized correlated well with the total virus adsorbed. At low multiplicities of infection, the enzyme level was directly proportional to the observed number of infected cells. Upon multiple infection, the relationship changed so that a four- to five-fold increase in adsorbed particles was necessary to obtain a doubling of enzyme activity. The same type of relationship apparently holds for thymidine kinase, although the reason for this is not clear. If the code for thymidine kinase is read before the "core" is broken down, then the amount of enzyme should be proportional to the total number of "cores" reaching the cell cytoplasm, unless further limitations are posed by the cell on the rate of RNA or protein synthesis. Coding for DNA-polymerase appears to occur only after the viral DNA has been released from the "core," depends upon "initiation," and is proportional to the number of inclusion areas in the cells.

The quantitative relationships involved in uncoating should not be overlooked since Joklik (10) showed that once uncoating begins most of the intracellular virus takes part, and Dales (4, 15) showed many empty "cores" in the vicinity of an inclusion area by electron microscopy. Thus, an inclusion may contain more than one viral genome, and uncoating could provide a means of rapidly salvaging viral DNA even from "cores" that are noninfectious, provided they are in the vicinity of the inclusion. Many of these genomes then could be expected to participate in coding for new DNA-polymerase which would permit an early synchronous burst of enzyme synthesis, sufficient to be readily detectable.

Green et al. (9) provided evidence that the DNA-polymerase is rate limiting for synthesis of new viral DNA. We found that stimulated ^{3}H-thymidine uptake was difficult to detect in untreated cells unless the cells were multiply infected (27). Obviously, multiple infection is not necessary to obtain full virus yields. This condition probably explains the differences reported in the literature in which some laboratories were unable to detect increases in ^{3}H-thymidine incorporation after infection unless resting or cold-shocked cells were used (18, 19). The overall rate of ^{3}H-thymidine uptake in infected cells depends not only on the rate of cellular DNA synthesis but also on the precise multiplicity of infection.

Our data give additional support for the idea that the new DNA-polymerase is coded for by the virus (28), since a derepression of a host function would not be expected to continue to increase upon multiple infection of the cells. All of these reactions, which might be considered early viral functions, are sensitive to interferon inhibition, including synthesis of viral DNA, DNA-polymerase (22), thymidine kinase (6, 30), and uncoating (W. E. Magee et al., Virology, *in press*).

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