*EFFECTS OF STREPTOVITACIN A ON THE INITIAL EVENTS IN THE REPLICATION OF VACCINIA AND REOVIRUS**

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The sequence of events culminating in the intracellular release of DNA from vaccinia virus has been established by biochemical[1] and electron microscopic observations.[2, 3] It involves three defined stages: (*a*) adsorption to the cell surface and uptake of intact virus into phagocytic vacuoles, (*b*) a lysis of vacuolar and outer viral membranes, thereby releasing the genome-containing core into the cytoplasmic matrix, and (*c*) rupture of the core allowing extrusion of the DNA into the cytoplasm where foci of replication become established. Vaccinia passes phases (*a*) and (*b*) rapidly, in less than 20 min, but requires 1–2 hr to traverse phase (*c*), termed "uncoating" by Joklik.[1] The "uncoating" process is presumed to depend upon the induction or mobilization of specific cellular enzyme(s)[1, 3–5] produced as the result of the activation of a specific host gene by the infecting virus,[4] or through some other process. Experimental data indicate that extended (2–5 hr) pretreatment with such protein synthesis-inhibitors as puromycin decreases "uncoating,"[4] although the capacity of the cell to produce vaccinia resumes when the drug is removed.[6]

To test whether release of DNA from vaccinia actually requires short-term protein synthesis, in the present study use was made of streptovitacin A, one among a group of potent, rapidly acting inhibitors.[7] To provide a comparison with vaccinia, the action of this antibiotic was also tested on the development of reovirus, which appears to undergo a different intracellular sequence prior to release of its RNA genome.[8]

Materials and Methods.—Information about the L cell line, virus stocks, and nutrient media (NM) used in this study has already been published, as have been the procedures for inoculating cells with virus, sampling, assaying for infectivity, and preparing infected cells for counting and classifying cell-associated virus particles by electron microscopy.[3, 8] Rates of synthesis were determined by sampling 100-ml suspension cultures in spinner flasks[9] containing 5×10^5–10^6 cells/ml. Where appropriate, 10 μg/ml of streptovitacin A (Str) (Upjohn Co., lot 4931-RRH 7) was added to the NM.

Isotopic tracers were added to the cultures either simultaneously with the inhibitor or after pretreatment for about 2 hr and removal of the drug. To wash away the drug or remove extracellular tracer, the cells were subjected to 2 washing cycles by suspension in NM followed by centrifugation at 1000 rpm for 1–5 min.

Incorporation of DL-leucine-1-C^{14}, S.A. 28.6 mc/mM (New England Nuclear Co.), into material insoluble in 5 per cent trichloroacetic acid (TCA) was used as a measure of protein synthesis, and uptake of thymidine-methyl-H^3, S.A. 6.7 c/mM, into a fraction precipitated by 10 per cent TCA as a measure of DNA synthesis. Duplicate samples of TCA precipitates were dissolved in a few drops of formic acid and assayed for their content of C^{14} or H^3 in a Packard Tri-Carb scintillation counter. Hydrolysis of pellets of ruptured cells at 37° for 30 min with 1 mg/ml of

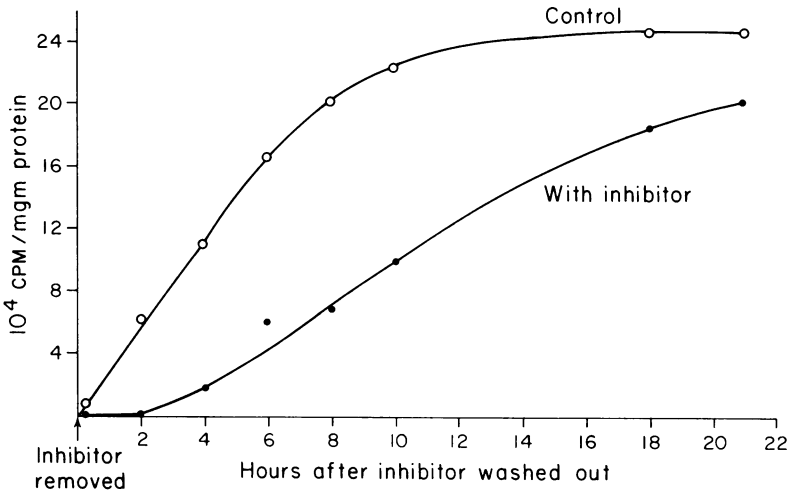


FIG. 1.—Rate of incorporation of DL-leucine-1-C^{14} by L cells in suspension culture. Following incubation in NM plus Str (10 μg/ml) for 2 hr and 15 min, the antibiotic was washed out, and cells were resuspended in warm NM containing 1.8 μg (0.25 μc)/ml of DL-leucine-1-C^{14}. Aliquots of 3 ml in duplicate were taken at the times indicated and prepared for counting and protein analysis.[11]

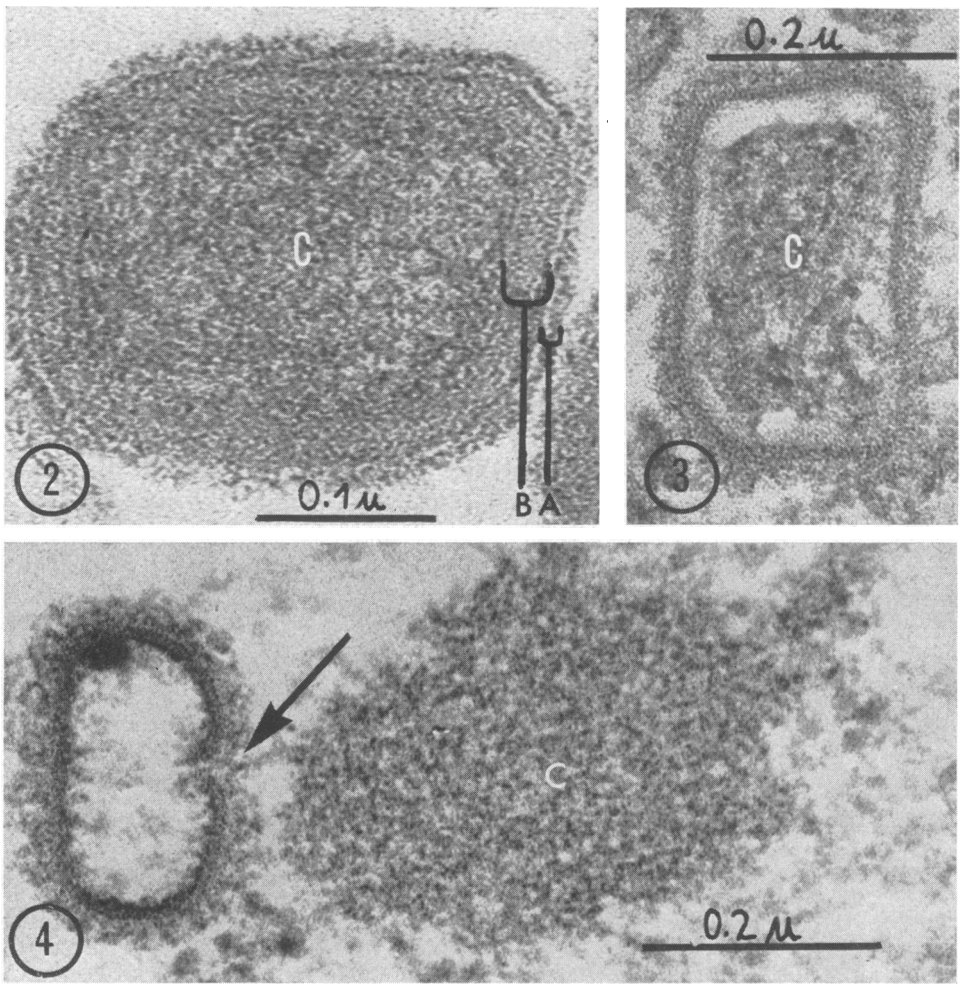
pancreatic deoxyribonuclease (Worthington, × 1 crystallized) released over 92 per cent of the H^3-thymidine label precipitable by TCA.

Results.—Inhibition of protein synthesis: Although compounds of the Str group have been shown to depress protein synthesis drastically, they inhibit DNA synthesis less and apparently do not slow down formation of RNA.[7, 10] L cells were exposed to Str ranging in amount from 1 to 100 μg/ml NM to ascertain the optimum concentration necessary for inhibiting incorporation of DL-leucine-1-C^{14}. At the chosen concentration of 10 μg/ml uptake of the C^{14} label was depressed 85–90 per cent within 15 min, and 95–98 per cent in less than 30 min. This virtually complete inhibition persisted during a 2-hr period of exposure to Str and for 2 hr after washing out the drug. During a period 2–10 hr following removal of the inhibitor, uptake of leucine-1-C^{14} proceeded at a linear rate which, however, equaled only about 50 per cent of the corresponding linear rate in the controls (0–6 hr on the time scale in Fig. 1).

In similar experiments testing the effect of this compound on thymidine-H^3 incorporation into DNA, it was found that Str reduced in less than 15 min the uptake to about 50 per cent of that observed in untreated cultures. Subsequently, the incorporation continued at the same depressed level during the 2-hr period of treatment, in agreement with published observations of others,[10] and for at least 15 hr after Str was removed (i.e., to the termination of the experiment).

In cultures examined by phase contrast microscopy 2 hr after Str was introduced, mitotic figures were not observed, but 15 hr after washing out the inhibitor mitoses again appeared. When 20 hr had elapsed from the time of reversal, the mitotic index was about 5 per cent, equivalent to the index in untreated companion cultures.

Blocking of DNA release from vaccinia cores: The sequence of stages through which vaccinia passes during penetration into L cells has already been described.[3]



FIGS. 2–4.—Thin sections of vaccinia virus or its components. Bracket *A* delimits the width of the outer lipoprotein coat, *B* indicates the width of the inner coat, and *C* designates the region occupied by DNA.

FIG. 2.—An intact particle viewed from its broad aspect.

FIG. 3.—A virial core possessing an intact coat and DNA within (cell from sample *B* of Fig. 6).

FIG. 4.—A shell remaining after the DNA passed from the core into the cytoplasmic matrix (cell from sample *D* of Fig. 6).

Electron micrographs (Figs. 2–4) illustrating only two of these stages are included to facilitate the reader's comprehension of data on particle counts, which follow. Figure 2 shows an intact vaccinia particle in which are evident a thin, outer lipoprotein membrane surrounding a thicker coat of the viral core. Figure 3 illustrates the appearance of an intact viral core enclosing the fibrous DNA within. The shell or ruptured coat of a core, shown in Figure 4, is devoid of internal dense material which was presumably released and became lodged in the cytoplasmic matrix. Aggregates of dense viroplasmic matrix, such as that shown in Figure 4, are the factories[12] or foci at which viral products accumulate.

Early steps leading to release of vaccinia DNA can be quantitated by grouping

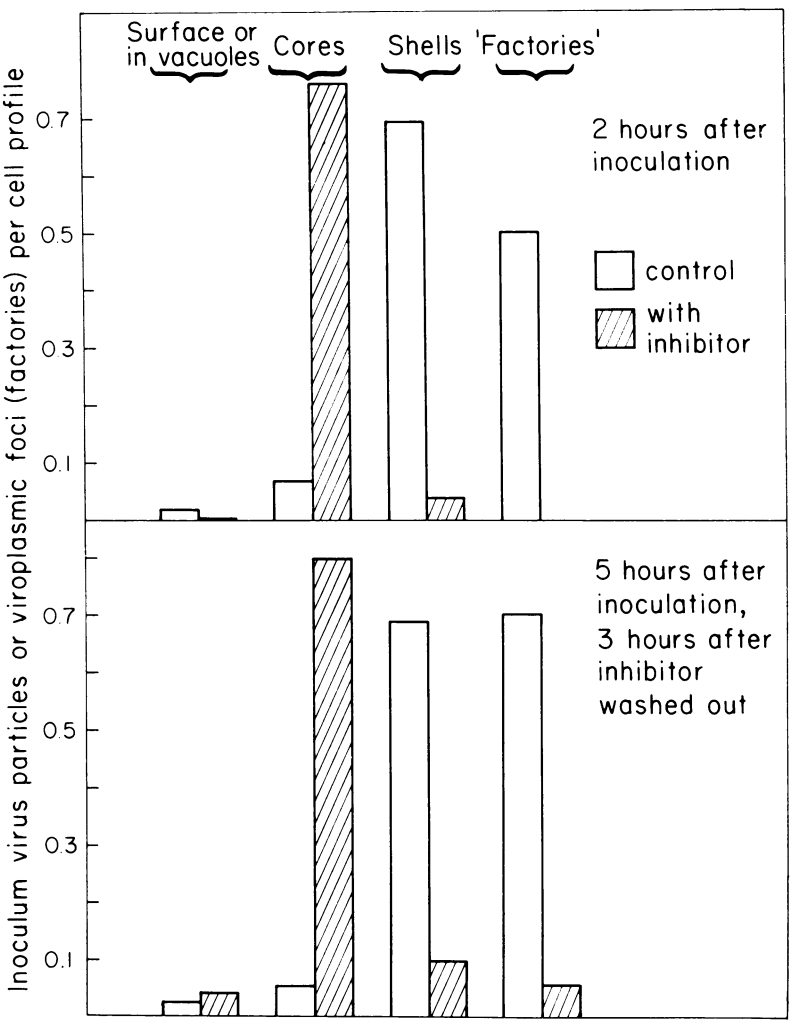


FIG. 5.—Penetration of vaccinia into L cells inoculated in the presence of Str. 1.1×10^7 cells suspended in 2 ml NM plus Str (10 μg/ml) were pretreated for 15 min, then inoculated at a multiplicity of 12 PFU/cell for 1 hr with Str present. Unadsorbed virus was washed away, and incubation in NM plus Str continued for another hour. After removing the inhibitor, cells were suspended in 60 ml warm NM, and a sample was removed for electron microscopy. Following further incubation for 3 hr in NM the second sample was taken. The average number of particles was calculated from counts made on about 100 cell profiles per sample.

inoculum particles, counted on cell profiles by electron microscopy, into the following four categories: (1) intact virus on the cell surface or engulfed and within phagocytic vacuoles, (2) virus degraded to cores, (3) shells or remnants of cores, and (4) factories in the cytoplasmic matrix. After inoculating vaccinia in the presence of Str penetration of particles to the core stage proceeded normally, but release of DNA from cores was stopped (upper part, Fig. 5; Fig. 6*B*). Once they had been blocked, the cores failed to release their genomes either at 3 hr or 7 hr after the inhibitor had been removed (lower part, Fig. 5; Fig. 6*C*) and protein synthesis had

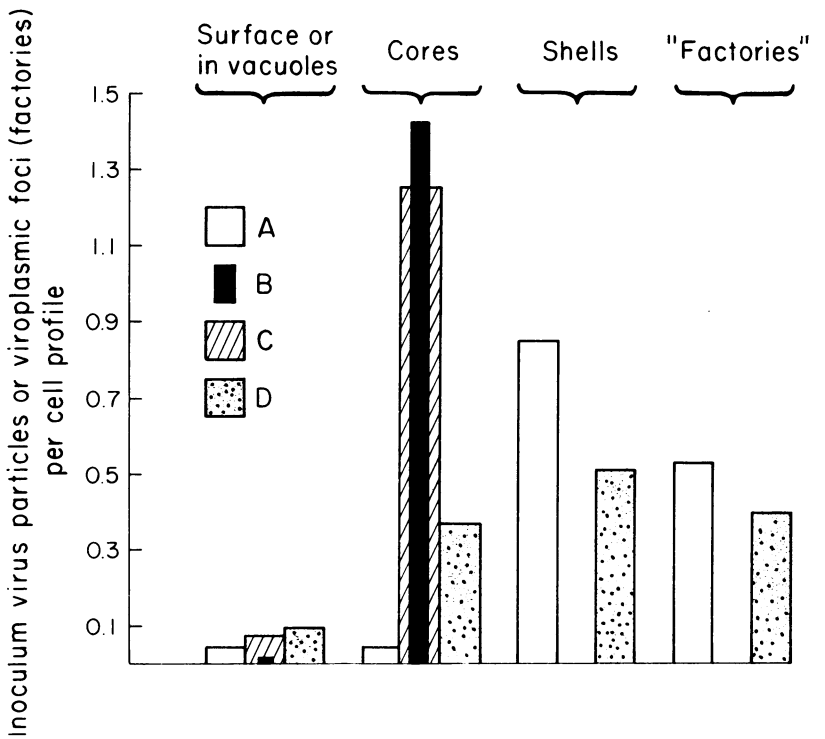


FIG. 6.—Penetration of vaccinia into L cells inoculated during or after treatment with Str. Conditions for infecting cells and sampling as in Fig. 5. (*A*) Controls inoculated at the same time as *D*, sampled 2 hr later. (*B*) Inoculated in the presence of Str, sampled 2 hr later. (*C*) Inoculated as in *B*, sampled 9 hr later or 7 hr after removing Str. (*D*) Inoculated 5 hr after removing Str, sampled 2 hr after infection.

resumed (Fig. 1). However, inoculum introduced 5 hr after removal of the drug proceeded through all 3 phases mentioned (Fig. 6*D*). The system retains, therefore, its capacity to release viral DNA.

Brief exposure of L cells to Str depressed protein synthesis and stopped release of viral DNA. Once this block had been effected, it became irreversible under conditions that allowed resumption of synthesis.

Effect of streptovitacin on replication of vaccinia and reoviruses: The above data from particle counting can indicate only whether the genetic component of vaccinia has been released in the cytoplasmic matrix where it initiates its functions. They cannot indicate whether subsequently virus components are synthesized or not. To measure the capability of L cells for producing infectious progeny, monolayers[15] of cells in Str-containing NM were inoculated with vaccinia, and assays were made on samples taken at selected intervals after washing out the inhibitor. As had been anticipated, L cells infected under conditions which prevented release of the DNA failed to yield progeny (Table 1, col. 2). Fusion of cells to form multinucleate giants, which is evident by phase contrast microscopy in infected sedentary cultures,[2] was absent in this case.

If inoculation was carried out several hours after resumption of protein synthesis,

TABLE 1

EFFECT OF STREPTOVITACIN A ON MULTIPLICATION OF VACCINIA VIRUS*

Hr after inoculation	Controls (1)	Treated during inoculation (2)†	Inoculated after inhibitor removed (3)‡
4	2.4	5.0	1.8
12	270	0.7	26
18	410	5.0	210
24	510	8.5	270

Multiplicity of inoculum 6 PFU/cell.
* Titer 10^5 PFU/ml.
† In (2) monolayers of cells were pretreated for 15 min with Str, then inoculated for 1 hr in the presence of Str. Unadsorbed virus was removed and incubation continued for 1 hr more with Str in the NM.
‡ In (3) cells were pretreated with Str for 2 hr, 15 min. The inhibitor was washed out and cultures were incubated 5 hr in NM without Str, then were inoculated for 1 hr in the usual manner.

TABLE 2

EFFECT OF STREPTOVITACIN A ON THE MULTIPLICATION OF REOVIRUS*

Hr after inoculation	Controls (1)	Treated during inoculation (2)
4	4.8	7.0
8	20.0	3.2
12	450	88
16	2800	470
18	3300	590
20	4800	1300
24	5100	2100

Multiplicity of inoculum 1.5 PFU/cell. In (2), cells were pretreated for 15 min with Str, then incubated with reovirus in the presence of Str for another 2 hr. The inhibitor and unadsorbed virus were washed away and cells suspended in fresh NM.
* Titer 10^4 PFU/ml.

virus was formed at a slower rate than in the controls but ultimately almost a full yield was produced (Table 1, cols. 1 and 3).

Previous observations, suggesting that intracellular release of RNA from reovirus may be dependent upon pre-existing cellular enzymes,[8] led to the prediction that inhibition with Str during inoculation should, in contrast with vaccinia, not prevent completion of reproductive cycles, once inhibition of protein synthesis had been reversed. This could be demonstrated, as shown by the data in Table 2. In the reversed cultures, infectious progeny was produced more slowly than in the controls, as was to be expected from the gradual resumption of protein synthesis (Fig. 1), but after 24 hr almost full yields were made. Consistent with the delay in reovirus multiplication was a 2–4-hr lag in development of characteristic aggregates of progeny particles, generally in the perinuclear zone of infected L cells.

Discussion.—Experiments with Str have provided additional information about two aspects of the early cell-virus interaction. They have shown that the host must synthesize protein at the time of inoculation for effecting release of DNA from cores, so as to initiate the replicative cycle of vaccinia. They have also shown that, in contrast, the infectious cycle of reovirus is not terminated by inhibition of protein synthesis during inoculation.

Stoppage of DNA release following short exposure to Str is presumably equivalent to an inhibition of "uncoating" which results from pretreatment of long duration with other suppressors of protein synthesis, such as puromycin.[4] The observed action of Str on release of DNA from vaccinia can be reconciled also with Abel's evidence regarding induction of "decoating" enzyme(s). The "decoating" enzyme, which could be extracted from infected host cells, was shown to be most active 3–7 hr after inoculation in releasing DNA from poxviruses in a cell-free system.[5]

The evidence indicating a dependence on newly synthesized protein for the rupture of the inner coat of vaccinia focuses attention on possible mechanisms which act to induce such synthesis. The fact that a block to the release of DNA, once it has been established by Str, becomes irreversible (Figs. 5, 6*B*, and 6*C*) suggests that factors involved in the induction process function briefly, at the maximum for only several hours. One such factor might be the specific RNA template postulated by Joklik,[4] who assumes that it is synthesized after a component of the invading

poxvirus particle penetrates to the nucleus and derepresses some specific locus of the genome of the host. However, our previous experiments with inhibitors of nucleic acid synthesis, particularly with actinomycin D, indicate that synthesis of new RNA may not be obligatory for releasing the DNA from vaccinia.[13] The issue remains unsettled: Joklik reported inhibition of uncoating at higher actinomycin concentrations,[4] while in our previous experiments "uncoating" occurred at lower concentrations of the antibiotic which, nevertheless, depressed cellular RNA synthesis over 90 per cent. This raises the possibility that induction of an "uncoating" factor involves a mechanism not controlled by a *newly* formed host template or message.

It has been postulated from our electron microscopic studies on the penetration of reovirus into L cells that its RNA is released asynchronously from dense intracytoplasmic inclusions, tentatively identified as lysosomes.[8] The prediction was made that lytic enzymes concentrated in such inclusions prior to inoculation are capable of stripping the protective coats of reovirus. With this virus, inhibition of protein synthesis during inoculation would, therefore, not stop replication. This prediction was fulfilled by experiments with Str (Table 2).

A distinction in the action of this inhibitor on the two agents examined here emphasizes the differences observed in the interactions between the various animal viruses and host cells. Such differences, described in detail elsewhere,[14] suggest that each virus type interacts with cells in a characteristic manner, as if events leading to replication are regulated not only by the host but also by the virus, according to the chemical composition and construction of the protective coats of the latter.

Summary.—Streptovitacin A rapidly inhibits protein synthesis in L cells and irreversibly stops release of the DNA from vaccinia virus, thereby blocking the infectious cycle. By comparison multiplication of reovirus is only temporarily arrested.

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[15] Infectivity experiments were conducted with monolayer cultures because Str-treated and vaccinia-infected L cells maintained in suspension showed a tendency to premature lysis.