ACTINOMYCIN *D* AND VACCINIA VIRUS INFECTION OF HELA CELLS

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INHIBITION of synthesis of DNA by 5-fluorodeoxyuridine[1] or of formation of RNA by actinomycin *D* (C_1)[2] prevents the replication of vaccinia virus in tissue culture cells. It has been reported that, when DNA synthesis is blocked, viral protein formation proceeds[3]. It seemed of interest, then, to examine the effect of RNA suppression by actinomycin on viral protein synthesis.

The procedures utilized for the growth and infection of HeLa *S*3-1 cells with strain *WR* vaccinia virus, the plaque assay for infectious virus[4], and the indirect immunological precipitation of radioactive viral protein[3,5,6] have already been described.

The formation of infectious vaccinia virus in suspension cultures of HeLa *S*3-1 cells is more than 99 per cent inhibited by 0·1γ actinomycin/ml. added 1½ h after infection (Table 1). Furthermore, treatment of cells prior to infection with 2γ actinomycin/ml., a concentration which limits RNA synthesis within 1 h to less than 2 per cent the normal rate, results in an almost complete block in viral protein synthesis (Fig. 4). Conversely, when RNA formation is similarly blocked in uninfected cells, protein synthesis continues at an optimal rate for 5 h followed by a diminished rate for at least another 7 h (Fig. 1). To examine whether renewal of RNA in infected cells is necessary for continued viral protein synthesis, 2γ actinomycin/ml. was added to a suspension culture at 5 h after infection, a time when only viral protein synthesis is proceeding rapidly. Within 1 h after addition of actinomycin, protein synthesis stopped (Fig. 2).

Table 1. ACTINOMYCIN INHIBITION OF INFECTIOUS VIRUS SYNTHESIS

Actinomycin concentration (γ/ml.)	Virus titre (PFU/ml.)
—	3×10^8
0·01	$2{\cdot}8 \times 10^8$
0·1	2×10^3
0·5	$< 10^3$

Actinomycin at the concentrations shown was added 1½ h after infection to replicate suspension cultures containing 2 × 10^5 HeLa cells per ml. Virus titres were determined after 19 h. The control culture contained 2×10^3 PFU/ml. at 3 h after infection.
PFU, plaque-forming units.

It has been reported previously that the synthesis of protein(s) less than 1 h prior to virus maturation is necessary for infectious virus formation[4]. Therefore, the interruption of viral protein synthesis by actinomycin might be expected to interrupt whole virus synthesis within this interval. As seen in Table 2, virus production is blocked within 1 h when actinomycin is added 5 h following infection. The maturation process remains sensitive to the antibiotic throughout the infectious cycle (Fig. 3). Addition of actinomycin at 3½ h after infection, 1 h before the onset of maturation, blocks subsequent infectious virus formation completely. Inhibition also occurs within 1 h when actinomycin is added at 11 h after infection. At this time a full yield of viral DNA[1] and most of the viral protein[3] have been synthesized.

Table 2. INTERRUPTION OF VIRUS MATURATION BY ACTINOMYCIN

Hours after infection	Virus titre (PFU/ml.) Control	Actinomycin
3	$3{\cdot}2 \times 10^4$	$3{\cdot}2 \times 10^4$
5	$1{\cdot}1 \times 10^6$	$1{\cdot}1 \times 10^5$
5½	—	$1{\cdot}5 \times 10^5$
6	—	$2{\cdot}7 \times 10^5$
7	$7{\cdot}6 \times 10^5$	$2{\cdot}7 \times 10^5$
8½	2×10^6	$2{\cdot}4 \times 10^5$
12	4×10^6	$2{\cdot}2 \times 10^5$

At 5 h after infection, the suspension culture was divided, and one of the resulting cultures received 2γ actinomycin/ml. At the indicated times, samples were removed from each and assayed for infectious virus titre.

The formation of cell proteins diminishes following vaccinia virus infection of HeLa cells (Fig. 4). During the first 4 h of infection the rate of protein synthesis is rapid, and viral proteins comprise 30–40 per cent of the total protein synthesized. From the fourth hour on, the total protein synthetic rate parallels that observed for viral protein formation, and after this time in the infectious cycle no net increase in cell protein occurs. The inhibition of cell protein formation by infection is not dependent on the continued synthesis of viral products. Net cell protein synthesis stops at 4 h after infection even when the formation of RNA, viral proteins, and viral DNA[2] has been blocked by treatment of the cells with actinomycin beginning 1 h prior to infection (Fig. 4).

Vaccinia virus infection of HeLa cells leads to a sharply decreased, although continued, rate of incorporation of

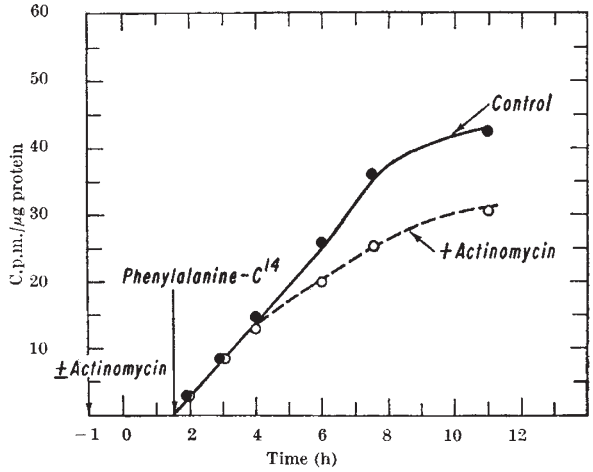

Fig. 1. Continued protein synthesis in uninfected cells in the presence of actinomycin. Actinomycin, 2γ/ml., was added to one of two suspension cultures of 2×10^5 cells/ml. After 1 h at 37° C, the cells were treated as described in the legend to Fig. 4, but without the addition of virus. Following phenylalanine-^{14}C addition (0·01 mM, 2 μc./μM), samples were removed, extracted twice with cold 5 per cent trichloroacetic acid, defatted and hydrolysed with 1 N potassium hydroxide. An aliquot of the hydrolysate was counted and another used to determine total protein[9]

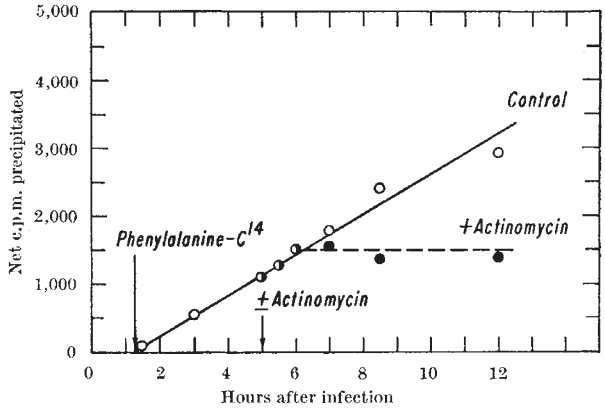

Fig. 2. Inhibition of viral protein synthesis by actinomycin. A suspension culture received phenylalanine-^{14}C at 1½ h after infection. At 5 h the culture was divided and 2γ actinomycin/ml. added to one of the resulting cultures. The incubation was continued, and samples removed from each at the indicated times were assayed for viral protein by immunological precipitation

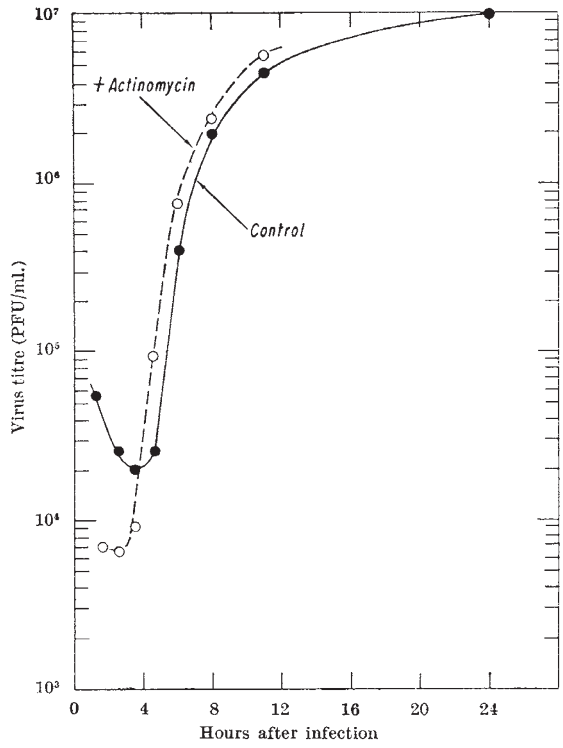

Fig. 3. Inhibition of infectious virus formation by actinomycin. A series of replicate cultures of infected cells was established at a cell concentration of 2×10^5/ml. One culture was sampled at increasing intervals after infection to obtain the infectious virus growth curve which is designated 'control'. At the indicated time after infection, one culture of the series received 0·5γ actinomycin/ml. At 24 h after infection, all inhibited cultures were assayed for infectious virus. The curve labelled 'actinomycin' represents the 24-h virus yields obtained when actinomycin was added at the times indicated by the position of the points on the curve

uridine into acid-insoluble material (Table 3)[7]. The observed reduction in total RNA synthesis may reflect a decrease in cell RNA formation together with an increase in the synthesis of virus-related RNA. Both the base composition and site of synthesis of the RNA made by vaccinia-infected cells are of interest in view of the cytoplasmic formation of both viral DNA and proteins[8]. A decrease in cell RNA synthesis would not by itself explain the cessation of cell protein synthesis in vaccinia-infected

Table 3. Reduction in RNA Synthesis after Vaccinia Infection

Hours after infection	Total CPM incorporated	% of maximum
Uninfected	2,310	100
2	2,110	91
4	1,400	61
7	730	32

At the indicated times after infection, one of a series of replicate suspension cultures each containing 100 ml. of cells at a concentration of 2×10^5/ml. received 2×10^{-5} M uridine-^{14}C (specific activity = 0·56 μc./μM). After 30 min the culture was chilled and the amount of uridine-^{14}C incorporated into RNA was determined[10].

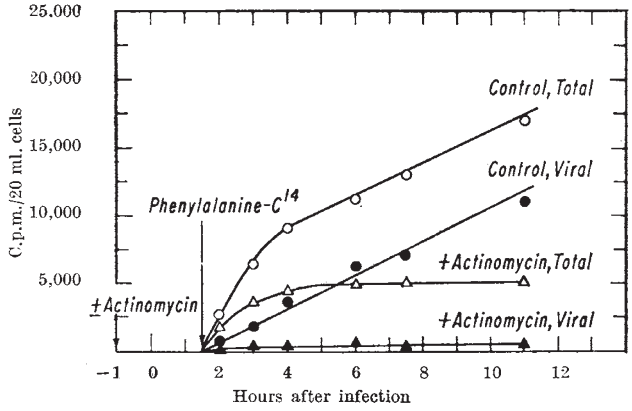

Fig. 4. Inhibition of cell protein synthesis following virus infection. One of two suspension cultures containing 2×10^5 cells/ml. was treated with 2γ/ml. actinomycin for 1 h at 37°. Each was then concentrated ten-fold, infected with an input multiplicity of 10–20 PFU/cell, washed twice in phenylalanine-deficient Eagle's medium[11] containing 20 per cent dialysed human serum, and resuspended to 2×10^5 cells/ml. in deficient medium plus 5 per cent dialysed horse serum. Actinomycin was present throughout. At 1½ h after infection, 0·01 mM phenylalanine-^{14}C (specific activity = 2 μc./μM) was added and the incubation continued at 37° C. Samples were removed at time-intervals and centrifuged. Total radioactivity incorporated into protein was determined following cold 5 per cent trichloroacetic acid extraction and solubilization in 1 N potassium hydroxide of an aliquot of each sample. Viral protein was measured by immunological precipitation of a second aliquot

cells, since protein synthesis in uninfected cells continues for 12 h or longer when RNA formation has been 98 per cent inhibited by actinomycin (Fig. 1). The finding that synthesis of cell protein decreases even when viral products are not formed in infected cells suggests that the infecting virus inhibits the ability of the RNA synthesized prior to infection to support cell protein formation. In the presence of the antibiotic only the inhibition of cell protein synthesis may occur. In its absence, the synthesis of virus-related RNA could proceed and might then direct the synthesis of viral proteins.

The foregoing observations suggest that a vaccinia virus DNA-directed RNA is synthosized in infected HeLa cells and that its synthesis throughout infection is necessary for continued viral protein and infectious virus production. Information now being sought concerning the cellular site of synthesis and chemical nature of this RNA may provide a better understanding of the processes by which virus re-directs the metabolism of cells.

[1] Salzman, N. P., *Virology*, **6**, 150 (1960).
[2] Reich, E., Franklin, R. M., Shatkin, A. J., and Tatum, E. L., *Proc. U.S. Nat. Acad. Sci.*, **48**, 1238 (1962).
[3] Shatkin, A. J., *Virology*, **20**, 292 (1963).
[4] Salzman, N. P., Shatkin, A. J., and Sebring, E. D., *Virology*, **19**, 542 (1963).
[5] Scharff, M., and Levintow, L., *Virology*, **19**, 491 (1963).
[6] Shatkin, A. J., *Biochim. Biophys. Acta*, **61**, 310 (1962).
[7] Kit, S., and Dubbs, D. R., *Virology*, **18**, 274 (1962).
[8] Cairns, J., *Virology*, **11**, 603 (1960).
[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
[10] Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
[11] Eagle, H., *Science*, **130**, 432 (1959).

A POSSIBLE CASE OF GENETIC ASSIMILATION OF BEHAVIOUR

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CLUTTERBUCK and Beardmore[1] recently reported that if *Drosophila melanogaster* is reared on a medium adulterated with a substance to which they are aversive, such as peppermint oil, then the behaviour of the flies may alter so that they become less aversive to the adulterant. These authors found that flies reared on peppermint-flavoured medium for six generations became less averse to that substance, both measured by the relative numbers of eggs laid on it compared with the number laid on a normal medium, and also in terms of the number of flies