tures. The further addition of insulin markedly enhanced the growth pattern. Nissl and silver stains as well as electron microscopy studies supported the contention that these cells and fiber outgrowths were nerve cells and axons and not supporting or satellite cells. However, functional activity has not been proven.

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The Role of RNA in the Synthesis of Vaccinia Virus.* (29374)

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Recent studies have shown that several ribonucleic acid (RNA) containing animal viruses can replicate in spite of severe impairment of deoxyribonucleic acid (DNA)(1, 2) synthesis and DNA-dependent RNA synthesis(3). However, the multiplication of some DNA-containing animal viruses has been reported to be dependent upon the essential participation of RNA during the early stages of the infectious sequence(4,5,6,7). Agents such as ribonuclease or dichlororibofuranosylbenzimidazole(4,5) were found to inhibit the synthesis of DNA-containing viruses by specifically interfering with the metabolism of RNA within the infected cell. The present report supports this view. The pyrimidine anti-metabolite, 6-azauridine (6-AZU), was found to inhibit multiplication of vaccinia virus. Previous studies have indicated that the mode of action of this analogue is by interference with the conversion of orotic acid to uridine ribotide(8) and consequently with nucleic acid metabolism.

Materials and methods. The line of HeLa cells was originally obtained from Difco Laboratories, and routinely grown in Eagle's basal medium (EBM)(9) containing 10% calf serum (CaS). Except when stated, the basic experimental medium consisted of EBM and 2% CaS supplemented with thymidine (10^{-4} M) and deoxycytidine (10^{-4} M) in order to restrict the action of the analogue to RNA metabolism. To this medium was added either 6-AZU (10^{-4} M) alone, or with uridine (2×10^{-4} M). Cultures were infected with approximately 15 plaque-forming units (PFU) of centrifugally purified HeLa-passaged vaccinia virus per cell. After an adsorption period of 2 hours, the cultures were washed with Hanks' balanced salt solution (BSS) and fresh medium was introduced. Virus growth was assayed by the liquid overlay plaque technique(10). The preparation of immune sera to the vaccinial antigens (heat-labile-heat-stable and nucleoprotein) and the experiments with fluores-

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cent-antibody and microautoradiography were carried out as described(11,12). Thymidine-H^3 (6700 mc/mM) was obtained from New England Nuclear Corp., Boston, Mass., and 6-AZU was obtained from Calbiochem, Los Angeles, Calif.

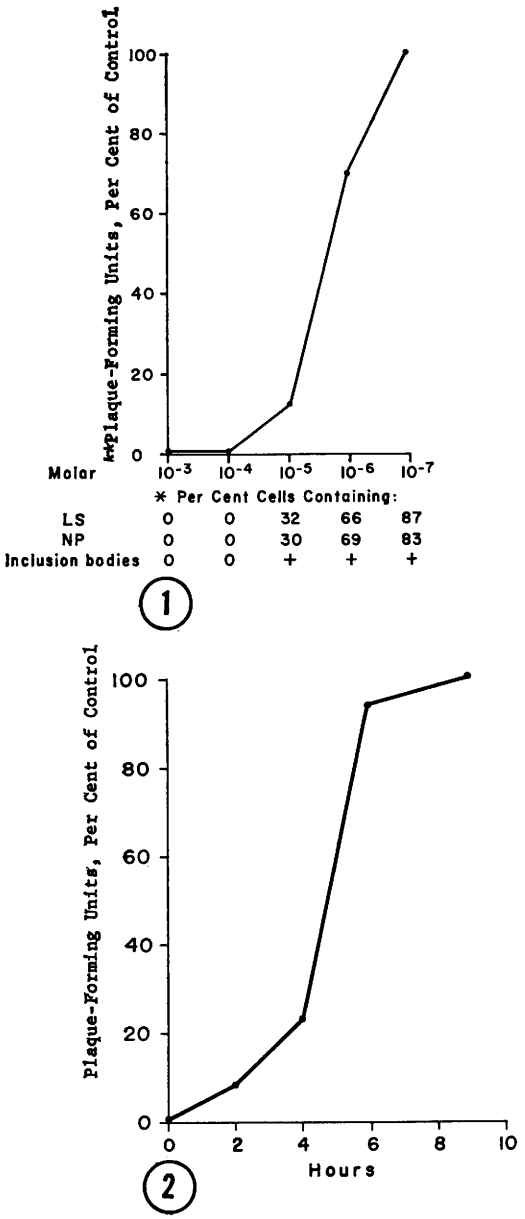

FIG. 1. Inhibition of vaccinia virus synthesis as a function of 6-azauridine (6-AZU) concentration.

* Cells containing either LS- or NP-antigens at 15 hr post-infection. Average of 500 cells counted.

** Yield at 24 hr post-infection. 6-AZU added at zero time of infection.

FIG. 2. Effect of 6-AZU added at different intervals of the infectious sequence. 6-AZU (10^{-4} M) added at times indicated. Virus yields determined at 24 hr post-infection.

Results. Inhibition as a function of 6-AZU concentration. The effects of varying concentrations of 6-AZU added at the time of infection on vaccinia virus multiplication in HeLa cells are shown in Fig. 1. The effect of the inhibitor on production of inclusion bodies and viral antigens is also included. Concentrations of 6-AZU of 10^{-3} and 10^{-4} M, but not 10^{-5} M, completely suppressed the production of infectious vaccinia virus. The inhibitory effect was progressively lost with lower concentrations of the inhibitor. Examination of cover-slip preparations of these cultures for production of inclusion bodies and viral antigens (LS- and NP-antigens) also showed complete inhibition at concentrations of 10^{-4} M or greater. Viral antigens and inclusion bodies were partially produced with lower concentrations of the inhibitor. Since a concentration of 6-AZU of 10^{-4} M was found completely to inhibit viral antigens, inclusion bodies, and infectious virus production, this dose was selected for further experiments.

Reversal of 6-AZU effect with uridine. To ascertain whether the inhibitory effect of 6-AZU in presence of thymidine and deoxycytidine is restricted to RNA metabolism, the effect of added uridine on the inhibitor-treated infected cultures was determined. The inhibition of infectious virus production was completely reversed by addition of uridine (2×10^{-4} M) even as late as 6 hours post-infection (Table I). Results obtained by acridine orange staining and fluorescent-antibody procedures (not shown) also showed that such cultures yielded approximately the same number of cells containing inclusion bodies and viral antigens as the infected controls.

In another experiment the effect of pre-treatment with 6-AZU on the cell's ability to support virus production after removal of the inhibitor was examined. A series of HeLa cell cultures was exposed to 6-AZU for various periods. After 12 and 4 hours pre-treatment, the inhibitor was removed and the cell cultures washed with Hanks' BSS before in-

TABLE I. Reversal with Uridine.

Culture	PFU/ml	% inhibition
Virus	2.0×10^8	—
" + AZU	2.3×10^4	99.86
" + " + U0	2.3×10^8	0
" + " + U2	2.7×10^8	0
" + " + U6	2.0×10^8	0

Cell cultures were exposed to approximately 15 PFU per cell of vaccinia virus for 2 hr at 37°C, then washed with Hanks' BSS to remove unadsorbed virus, and reincubated after addition of experimental medium. Virus at 2 hr = 2.0×10^4 PFU /ml. Experimental medium = EBM98 CaS2 plus thymidine (10^{-4}M) and deoxycytidine (10^{-4}M). AZU = 6-azauridine (10^{-4}M) added at time of infection. U = uridine (2×10^{-4}M) added at times indicated. Virus yields determined at 24 hr post-infection.

fection. Total virus was determined 24 hours after initiation of infection.

Pre-treatment of cell cultures with 6-AZU for as long as 12 hours, and its subsequent removal, did not impair the ability of the cell cultures to support virus multiplication (Table II). Virus production from both 12 and 4 hour pre-treatment cultures is comparable to that produced by non-treated cultures.

Effect of 6-AZU added at different intervals of the infectious sequence. In this experiment the temporal relationship between addition of the analogue and virus production was examined. 6-AZU (10^{-4} M) was added to a series of HeLa cell cultures at intervals after infection with vaccinia virus. Untreated infected cultures inoculated with BSS served as controls. Virus yields were determined at 24 hours post-infection.

In these experiments, summarized in Fig. 2, it is apparent that complete inhibition of virus production occurred only when 6-AZU is added at time of infection (0 times). Inhibition decreased beyond that point and addition of the analogue to infected cultures at 6 hours or more after infection failed to reduce the final yield of virus below that in untreated infected controls. It can be inferred from the data that synthesis of precursor RNA for virus synthesis began approximately at time of infection and that sufficient RNA was made by 6 hours after infection to allow almost maximal production of infectious particles.

TABLE II. Effect of Pre-Treatment with 6-AZU.

Culture	PFU/ml
Virus	2.0×10^8
AZU— 4	2.4×10^8
AZU—12	2.1×10^8

Cell cultures were exposed to 6-AZU (10^{-4}M) for varying periods. After 4 and 12 hr pre-treatment, the analogue was removed, the cell cultures washed with BSS before infection. Total virus was determined at 24 hr post-infection.

Effect on thymidine-H³ incorporation into viral and cellular DNAs. Since the preceding data have implied the participation of RNA in the biosynthesis of vaccinia virus, the effect of 6-AZU on incorporation of tritiated thymidine into treated infected and non-treated infected cultures was examined. Both treated and non-treated infected HeLa cells grown on coverslips in Leighton tubes, were exposed for 30 minutes to tritiated thymidine (0.25 μc/ml) at various time periods during the infectious cycle. Autoradiograms on the washed, acid extracted cells were made with Kodak NTB3 liquid emulsion as described(12).

TABLE III. Effect of 6-AZU on Incorporation of Thymidine-H³.

Exp media	*% of cells containing thymidine-H³ in the cytoplasm 2 hr	4 hr	6 hr	8 hr
dC, V.	0	32	46	74
6-AZU, dC, V.	0	0	0	0
6-AZU, dC, U, V.	0	34	54	68

Coverslip cultures of HeLa cells were exposed to approximately 15 PFU per cell of vaccinia virus for 2 hr at 37°C, then washed with Hanks' BSS to remove unadsorbed virus, and reincubated after addition of experimental media. Thymidine-H³ (0.25 μc/ml) was added for the last half hour of each period studied. Experimental media: EBM98-CaS2 with dC = deoxycytidine (10^{-4}M); 6-AZU = 6-azauridine (10^{-4}M); U = uridine (2×10^{-4}M).

* Avg of 1000 cells containing thymidine-H³ in cytoplasmic inclusion bodies.

6-AZU added at zero time of infection completely suppresses the incorporation of tritiated thymidine into the cytoplasmic centers of virus synthesis in inhibitor-treated infected cultures (Table III). Whereas at 8 hours post-infection 74% of infected cells exhibit label-containing cytoplasmic centers of virus synthesis, no label is observed in the cytoplasm of inhibitor-treated infected cell cultures. As observed before, the inhibitory

effect of 6-AZU was specifically reversed by uridine. Inhibitor-treated infected cultures which contained uridine yielded approximately the same number of cells containing cytoplasmic label as the non-treated infected cultures. Although not shown here, as was found previously, a decline in the number of label-containing nuclei in infected cultures occurred. This decrease was also observed in the inhibitor-treated infected cultures reversed with added uridine.

Discussion. This study describes the effect of 6-AZU, an inhibitor of uridylic acid synthesis and, therefore, of RNA production, on multiplication of the DNA-containing vaccinia virus. The data clearly indicated the essential participation of RNA in the production of infectious virus. Furthermore, this participation extends to the production of virus-specific antigens (LS- and NP-antigens) and viral DNA. That 6-AZU interferes with new RNA production in the infected cell is clearly indicated by the evidence that added uridine will reverse the effect.

The inhibition of infectious virus synthesis by 6-AZU at varying times after infection suggests that the involvement of new RNA for fabrication of infectious virus commences early during the infectious sequence and is completed at about 6 hours after infection. Complete inhibition is obtained when the analogue is added at time of infection, and is progressively less effective until at 6 hours after infection no inhibition is observed. When such data are compared with the temporal studies of the production of vaccinial antigens, inclusion bodies, and infectious virus(11), it is clear that the participation of this new RNA precedes the appearance of these biological units.

The participation of an RNA component in the synthesis of the DNA-containing bacterial viruses has been described(13). Thus, the RNA synthesized in T2 phage-infected *E. coli* has been found to resemble in many of its properties the hypothetical messenger RNA. Whether such "messenger" RNA components are involved in production of vaccinia virus can only be inferred from the present data. It was recently reported that the polypeptide antibiotic Actinomycin D, an inhibitor of the DNA-dependent RNA system, completely suppresses the production of vaccinia virus through interference with new RNA synthesis(14).

Preliminary examination of the ribonucleic acids existing in normal and vaccinia virus-infected cells have yielded a number of rapidly-labelled RNA fractions possessing sedimentation values of from 6-25S and appearing in infected cultures at about 3 hours after infection (manuscript in preparation). Further characterization of these fractions is in progress.

Summary. 6-azauridine, an inhibitor of RNA metabolism, has been found to inhibit the synthesis of the DNA-containing vaccinia virus. The inhibition can be specifically removed by addition of uridine. The essential participation of RNA occurs early during the infectious sequence and is completed at about 6 hours after infection. Studies on vaccinial antigens and DNA production indicated that these biologic units are all dependent upon the production of this early RNA component.

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