Biochemical Effects of Isatin β-Thiosemicarbazone on Development of Vaccinia Virus. (27580)

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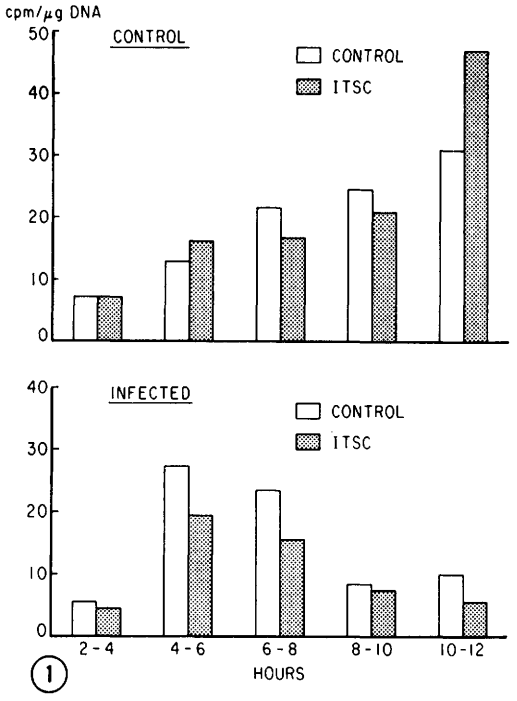
Isatin β-thiosemicarbazone (ITSC) and a large number of related compounds have been reported by Thompson *et al.*(1), Bauer(2), Bauer and Sadler(3,4), and Sheffield and Bauer(5) to afford protection against intracerebral infection of mice with various pox viruses, and also to inhibit the replication of these viruses in tissue culture(5). A remarkably high degree of specificity of the more active compounds was observed toward individual members of the pox group.

Little is known concerning the mode of action of ITSC except that it is not a contact agent nor does it affect virus absorption(5). For this reason it was of interest to examine the possible effect of ITSC on the biochemical stages in the intracellular development of vaccinia virus using the technics recently reported from this laboratory(6,7,8). The results presented below indicate that interference with virus multiplication occurs at a stage subsequent to deoxyribonucleic acid (DNA) biosynthesis and suggest the lability of the viral constituents made in the presence of the drug.

Methods. HeLa cells were grown as monolayers in a complete medium containing 20% calf serum(8,9), and vaccinia virus (originally obtained from the Rockefeller Institute, Nelson Strain) was passaged in HeLa cells and assayed on chick kidney monolayers as already described(8). Crude vaccinia virus was released from the cells by sonic disruption before use or prior to purification by centrifugation through a sucrose density gradient. The experiments with radioactive isotopes, the microautoradiography and the virus growth curves were carried out exactly as described(7,8). Thymidine-H^3 (0.36 c/mM) was obtained from Schwarz BioResearch Inc., Mt. Vernon, N. Y., and DL-valine-1-C^{14} (4.3 mc/mM) from Volk Radiochemical Co., Chicago, Ill. ITSC was dissolved in acetone (10 mg/ml) and added slowly with rapid stirring to medium at room temperature or 37° to give a solution with final concentration of 3.0 μg/ml. Controls with added acetone showed that the solvent at this concentration had no effect in any of the experiments.

Results and discussion. The temporal relationships of the synthesis of new DNA following infection have been determined from a study of the kinetics of thymidine-H^3 uptake into DNA(6,7) and by following the accumulation of DNA in the cytoplasm at sites for virus synthesis(7,8) in the presence of concentrations of ITSC sufficient to suppress completely the synthesis of new virus. Fig. 1 shows that there were no qualitative differences in the burst of DNA synthesis observed in infected cells in presence or absence of ITSC. Uptake of radioactivity into DNA fell to a low value in the infected cells by 8-10 hours indicating severe impairment of DNA synthesis later in the virus replication period, a result which was unaltered by the presence of ITSC. The increasing and parallel uptake by control and control plus ITSC cells showed that there was no effect of the drug on the normal incorporation of thymidine-H^3 into DNA. Results obtained by microautoradiography (not shown) also demonstrated DNA synthesis in treated cells, and further, showed that centers for virus production were set up in the cytoplasm in the presence of the drug since cytoplasmic DNA accumulated in these areas. The treated cells appeared indistinguishable from the previously described untreated cells(8).

These two experiments demonstrated that new, viral DNA was made in treated cells, and therefore the inhibitory action of ITSC must either (a) result from the production of non-functional DNA, or (b) take place at some later stage in the infection. The former mechanism was considered unlikely from the results of several additional experiments. ITSC was added to infected cells at various times following infection and the final yield of virus determined at 24 hours. No further



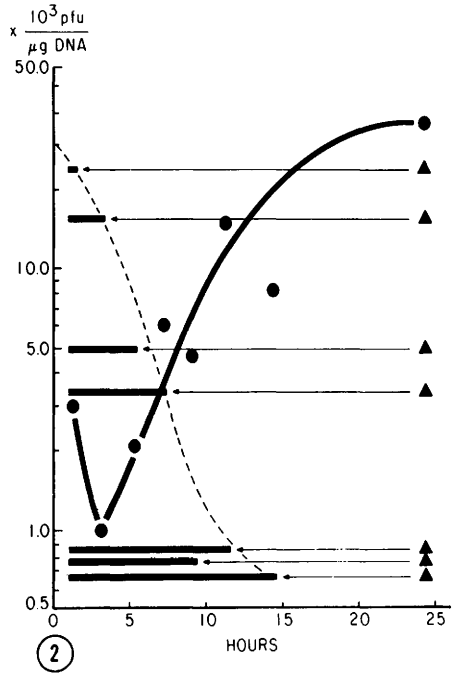


FIG. 1. Effect of ITSC on time course of thymidine-H^3 incorporation into control and infected HeLa cells. Results are avg of duplicate samples. To the cells in each 32-oz bottle containing 40 ml of medium 5 μc of thymidine solution was added at times shown post infection. Prior to analysis, DNA was solubilized by heating with 0.5 N $HClO_4$.

FIG. 2. Effect of time of removal of ITSC on final virus yield. Lengths of horizontal bars indicate duration of exposure to ITSC and their positions along the ordinate correspond to final virus titer (▲). Virus growth curve from untreated cells (●). Points represent avg virus titer from cells in duplicate sets of 60-mm petri plates, 2 plates being used for each analysis. Infection was for 1 hr with approximately one infectious dose of virus per cell. ITSC (3 μg/ml) was added with medium at 1 hr post infection, replaced with control medium at times shown, and cells harvested at 24 hr.

increases in virus titer were observed after addition of the drug regardless of the time at which it was added. Thus the synthesis of virus stopped even when ITSC was added after DNA synthesis was completed. These results are in contrast to those observed by Salzman(10) who studied the effect of 5-fluorodeoxyuridine, a specific inhibitor of DNA synthesis. This drug was ineffective if added later than 6 hours post infection.

In another experiment ITSC was added immediately following infection and removed at various times thereafter (Fig. 2). The dashed line indicates that the residual capacity to synthesize virus after treatment with ITSC approximated the reciprocal of the normal virus growth curve. The interval remaining after the last removal of the drug and before harvest would have been sufficient for synthesis of a complete yield of virus had the cells retained their capability for viral replication.

An additional experiment was performed which indicated that new viral DNA synthesized in presence of ITSC was capable of being incorporated later into new virus. Infected cells were incubated from hour 1 to 4 post infection in medium containing thymidine-H^3 and ITSC. The cells were washed and normal medium replaced until harvest at 12 hours. The virus titer was 28% of that from untreated cells. The virus was purified by exhaustive digestion with DNase. The specific activity of the virus in counts/min/infectious unit was approximately 70% of the virus from untreated cells.

Uptake of valine-1-C^{14} into protein was depressed following infection (Table I). Values in the presence of ITSC were similar to those of the corresponding untreated cells. The virus (harvested at 12 hours) was purified by centrifugation through a density

TABLE I. Effect of ITSC on Incorporation of Valine-1-C^{14} into Infected and Control HeLa Cells.*

Additions	Counts/min/μg protein x 10^{-4}	
	Control	Infected
None	4.45	2.72
ITSC (3 μg/ml)	3.98	1.98

* Exposure to valine-1-C^{14} (0.15 mg/ml, 16 ml/bottle) was for 3 hr commencing 4 hr post infection, in a medium consisting of Hanks' salts, valine-free Eagle's amino acid mix, vitamins, glutamine, and 20% calf serum. The radioactive medium was replaced by ordinary growth medium supplemented with 0.1 mg/ml DL-valine. Harvest was at 15 hr post infection.

gradient. The purified virus recovered from untreated cells was radioactive but no radioactivity could be demonstrated in the small virus peak from the inhibited cells. This indicated that there was no assembly of intact, but non-infectious particles. Further experiments are under way to try to detect the presence of specific viral protein in the treated cells.

Certain conclusions can be drawn from the above experiments. ITSC did not influence the initial stages of viral infection including penetration, synthesis of a functional viral DNA, and localization of this DNA at characteristic cytoplasmic sites. The time course of exposure experiments suggested that the drug was active during all parts of the virus replication cycle even though previous work clearly demonstrated a temporal separation between the various processes. The virus killed the invaded cells as evidenced by the decline in their ability to synthesize DNA and the failure to obtain viable clones from the treated, infected cells(11). These results, together with the observation of the progressive loss in the potential of cells for making virus during incubation with ITSC, emphasize the existence of two processes which are initiated upon infection of a cell by vaccinia. One of these processes results in production of more virus, and the second in eventual death of the cell. It would appear that by the use of ITSC it is possible to dissociate these two processes, in that appearance of infectious units can be suppressed completely without any demonstrable delay in the reactions which herald the death of the host cell.

Summary. The effect of ITSC, a highly specific antiviral agent, on some of the biochemical parameters of vaccinia virus replication in HeLa cell monolayers has been studied. At concentrations which completely inhibited infectious virus production this compound did not affect the previously observed enhancement of thymidine-H^3 incorporation, and the inhibition of valine-C^{14} incorporation in infected cells, nor did it affect these reactions in control cells. There was a typical cytoplasmic accumulation of thymidine-H^3 at virus-synthesizing sites in presence of the drug. In kinetic experiments, ITSC prevented virus formation only in proportion to the part of the virus replication cycle during which it was present, and independent of the absolute time in the cycle at which it was first added. The implications of these results on the mode of action of ITSC are discussed.

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