# Inhibition of Arc-like Fragments and Immature Forms of Vaccinia Virus by Methisazone[1] (36185)

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Methisazone is one of only a few antiviral agents with demonstrated effect not only in cultured cells and animals but also in human beings (1, 2). It is a methylated derivative of isatin β-thiosemicarbazone (IBT) and is more active than the nonmethylated compound with which most of the work has been done concerning the mechanism of action. Both compounds inhibit poxvirus infection of tissue cultures without affecting synthesis of viral DNA (3–6). However, there is some effect of these drugs on synthesis of poxviral proteins (6, 7).

In sections of cells infected with vaccinia virus, assembly of immature forms occurs when arc-like fragments of viral membrane enclose core material from the viral matrix (8, 9). The resulting immature forms consist of granular material surrounded by membranes and sometimes contain dense bodies or nucleoids. The mature forms are denser than immature forms and contain a dumbbell-shaped core. In one study of vaccinia-infected cells treated with IBT, numerous arc-like forms were found 24 hr after infection (10, 11), while another report indicated that immature forms sometimes with nucleoids were present in treated cells 38 hr after infection (4).

The purpose of the present work was to confirm the effect of methisazone on the maturation process and to decide whether methisazone blocks assembly of arc-like fragments or interferes with maturation of immature forms. Early intervals after infection were used so that stages of viral assembly and maturation would be as nearly synchronous as possible. After the work was begun, a third report indicated that no viral forms were found in methisazone-treated chorioallantoic membrane 24 hr after infection but that immature forms were present after 48 hr (12).

*Cell cultures.* HeLa and HEp 2 cells were grown as monolayers in bottles with Eagle's minimal esssenial medium, antibiotics, and 5% tryptose phosphate. Ten percent calf serum was used for growth of cells and 1% for maintenance.

*Viruses.* The WR strain of mouse neurotropic vaccinia virus (ATCC-VR119) was used except for preliminary experiments in which the Lederle chorioallantoic strain of vaccinia virus (ATCC-VR118) and adenoviruses types 2 and 7 were employed. Viral inocula and cell lines were cultured for mycoplasmas on standard medium (13), and contaminated inocula or cell lines were discarded.

*Solution of methisazone in culture medium.* Enough of the yellow powder was used to give a final concentration in the medium of 40 $\mu M$. The powder was dissolved in dimethylformamide (14), and this solution was dissolved drop by drop in water previously heated to 56°. Without allowing the solution to cool, it was autoclaved at 12 lb pressure for 10 min. After cooling to a temperature of 37° in a water bath, 9 vol of the solution were added to 1 vol of tenfold concentrated maintenance medium. For control, 9 vol of sterile water were added to 1 vol of tenfold concentrated maintenance medium. The medium was not allowed to cool below 37° and was used immediately. Only freshly prepared solutions of methisazone medium were used.[2]

The concentration of 40 $\mu M$ was used because it had been found to be nontoxic (14, 15). In the preliminary experiments of Table I, HeLa cells were infected with vaccinia virus (ATCC-VR118) or adenoviruses with or with-

[1] Supported by grant no. AI-08535 of the National Institutes of Health.

[2] Methisazone was kindly donated by Burroughs-Wellcome & Co., Inc. We are indebted to Dr. D. J. Bauer for helpful advice.

TABLE I. Effect of Methisazone on Multiplication of Vaccinia Virus and Adenoviruses.[a]

| No. of expt. | Virus | Methisazone in the medium: Absent | Methisazone in the medium: present |
|---|---|---|---|
| 1 | Vaccinia | 7.5[b] | 5[b] |
| 2 | | 6 | 2.5 |
| 3 | | 6 | 2 |
| 4 | Adenovirus 2 | 4.5 | 4 |
| 5 | | 5.5 | 5.5 |
| 6 | | 7 | 7 |
| 7 | | 7 | 7 |
| 8 | Adenovirus 7 | 4 | 4 |

[a] HeLa cells were infected with vaccinia virus or adenoviruses and incubated in medium with or without 40 $\mu M$ methisazone. After 48 hr, cells infected with vaccinia virus were frozen and thawed 3 times, and cells infected with adenovirus were sonicated. Titrations of the resulting viral suspensions were carried out by inoculating each of 2 tubes of HeLa cells with 0.2 ml of tenfold dilutions of the suspensions in maintenance medium. Cytopathic effects were observed daily for 4–6 days, and 50% tissue culture infectious doses ($TCID_{50}$) were determined.

[b] Log $TCID_{50}$ per ml.

out methisazone in a concentration of 40 $\mu M$ in the medium. Inhibition of growth of vaccinia virus was demonstrated readily. However, multiplication of the adenoviruses was not inhibited by methisazone, and this lack of inhibition indicated that the concentration of 40 $\mu M$ did not owe its antiviral action to a general deleterious effect on the cells.

In the experiments, the medium was removed, and the cells were covered with undiluted suspensions of virus. Two titrations of such inocula indicated multiplicities of 5 and 37 PFU per cell. After the viral suspensions had been in contact with the cells for 1 hr, the virus was removed, the cells were washed with balanced salt solution, and medium containing methisazone was added. Medium without methisazone was added to other bottles for control. Sections for electron microscopy were prepared at 6 and 9 hr after initial exposure of the cells to virus.

*Electron microscopy.* The cells were fixed with 5% glutaraldehyde, stained with 1% osmium tetroxide, and embedded in epoxy resin. Sections were stained for 2 min in alkalinized lead citrate (16). Approximately 1000 fields of sections of treated and untreated cells were examined and photographed. Autoradiography under the electron microscope with tritiated thymidine was done in cells 4 hr after initial exposure to virus by methods described previously (17).

*Results.* In control cells without methisazone, arc-like fragments and immature forms of viral particles were found easily 6 and 9 hr after infection (Fig. 1). After 9 hr, in addition to arc-like fragments and immature forms, dense mature virions were numerous. Many viral particles were concentrated in particular areas of cytoplasm so that individual viral particles and fragments were identified readily. In the cytoplasm of methisazone-treated cells, neither arc-like fragments nor viral particles were found at 6 or 9 hr after infections. In some of the treated cells, dense bodies occurred and are illustrated in Fig. 2. These bodies differed from the viral particles in control cells and usually occurred singly.

Autoradiography under the electron microscope with tritiated thymidine revealed labeled foci of DNA synthesis 4 hr after infection not only in controls but also in cells treated with methisazone and confirmed similar observations of others with light microscopic autoradiography (3, 4).

A few plaque titrations were carried out to compare the viral content of cells after incubation for 6 hr with and without methisazone but only small differences were found. Nevertheless, electron microscopy revealed a striking difference between treated and untreated cells in that neither viral particles nor arc-like forms were found in methisazone-treated cells.

*Discussion.* The results were unexpected in that accumulations of arc-like fragments and immature viral particles were not found in cells treated with methisazone. If the action of the drug was to inhibit assembly of viral fragments or to inhibit maturation of virions, one would expect larger numbers of fragments or immature forms than were present in controls.

We speculate that a major reason for the difference between our findings and others may be that IBT and methisazone are relatively insoluble compounds and that precipitation after 24 hr or more may result in incomplete anti-

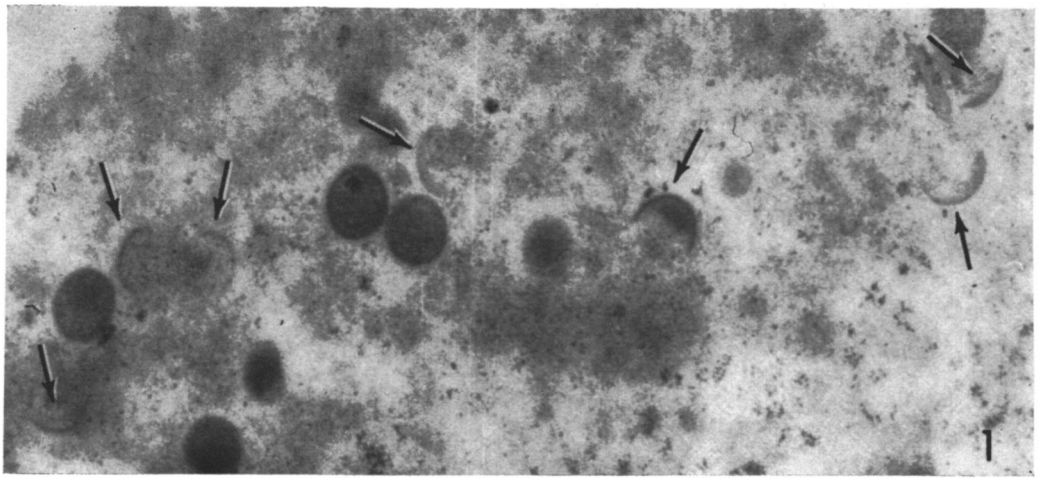

FIG. 1. Arc-like fragments (arrows) and immature forms of vaccinia virus in the cytoplasm of a HeLa cell 6 hr after inoculation (untreated with methisazone) approx. 30,000×.

viral action and resumption of viral replication. An additional point suggesting the possibility of incomplete antiviral action is that levels of IBT of 10 or 20 $\mu M$ and 20 $\mu M$ methisazone were used in the experiments done by others. Our experiments utilized 40 $\mu M$ methisazone and yet susceptibility of the cells to adenoviruses indicated no general deleterious effect of this concentration of methisazone on the cells.

Our results indicate that methisazone acts at a stage of viral development that follows synthesis of viral DNA and yet precedes the formation of visible viral fragments or immature particles. Thus, the action of methisazone on vaccinial infection differs from that of rifampicin since recent evidence indicates that the latter drug allows the formation of early viral membranes but inhibits assembly and maturation of the viral particle (18, 19).

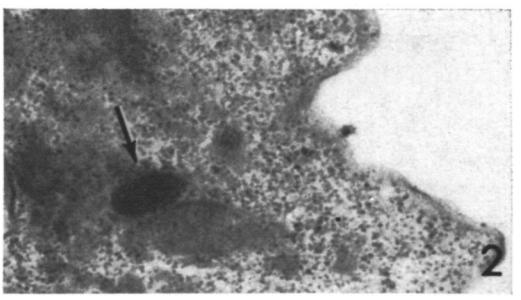

FIG. 2. Dense body (arrow) in the cytoplasm of a HeLa cell 9 hr after inoculation with vaccinia virus (treated with methisazone) approx. 30,000×.

*Summary.* HeLa and HEp 2 cells were infected with vaccinia virus for 1 hr and then treated with methisazone in a concentration of 40 $\mu M$. Electron microscopic observations of the cells were carried out 6 and 9 hr after infection. In controls without methisazone, arc-like fragments of virus and immature virions were found at 6 hr after infection. After 9 hr, in addition to fragments and immature forms, there were numerous clusters of mature viral particles. In cells treated with methisazone, fragments, immature forms, and mature virions were not found. These observations indicate that the antiviral action of methisazone occurs at a stage preceding visible viral fragments or particles.

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Received Oct. 1, 1971. P.S.E.B.M., 1972, Vol. 139.