*THE INHIBITION OF VACCINIA VIRUS MULTIPLICATION BY ISATIN-β-THIOSEMICARBAZONE**

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Isatin-β-thiosemicarbazone (IBT, Fig. 1) inhibits the formation of mature vaccinia virus progeny.[1] Derivatives of IBT are also active and the nature of the chemical modification determines the antiviral spectrum, which for certain compounds includes not only poxviruses but even some enteroviruses.[2, 3] One of the derivatives, N-methyl-IBT, is currently being used in clinical smallpox control trials in India and Africa.

The manner in which IBT and its derivatives act is unknown. On the basis of the observation that some viral DNA and structural protein is synthesized in the presence of IBT, the suggestion had been made that the compound interferes with the maturation of viral progeny.[4, 5] There is no evidence that it acts as an amino acid or nucleic acid base analogue or that it is incorporated by covalent linkage into either protein or nucleic acid. Since the elucidation of the mode of antiviral action at the molecular level of a relatively small molecule could well have far-reaching implications for antiviral chemotherapy, we have initiated a search for the primary locus of action at which IBT inhibits the formation of mature progeny of vaccinia virus.

Materials and Methods.—Cells and virus: HeLa S3 cells were propagated in spinner culture as described.[6] The WR strain of vaccinia virus was grown in HeLa cells and purified according to the method of Joklik.[7] One highly purified preparation containing 2×10^{11} virus particles per ml[8] was used. The virus particle/PFU ratio was 20:1 when titrated on chick embryo fibroblasts.[9] Cells were infected by Procedure A as described by Becker and Joklik.[6] The techniques used for pulse-labeling, breaking open of cells, and the preparation of cytoplasmic fractions, as well as sucrose and sucrose-SDS density gradient analysis, have been fully described.[6, 10, 11]

Preparations of stock solutions of IBT: IBT was dissolved in *N* NaOH to give a 0.04 *M* solution. As soon as the compound had dissolved, this solution was diluted tenfold with distilled water. IBT slowly changes chemically in such a solution over a period of weeks as evidenced by complete loss of biological activity as well as spectral changes. A fresh solution was therefore made up for each experiment. Control cultures not receiving IBT were treated with an equivalent amount of 0.1 *N* NaOH. At the concentration used, the amount of alkali added did not change the pH of the medium by more than 0.2 pH units.

Materials: The specific activities of the labeled compounds used were: C^{14}-UR and C^{14}-dT, 20–30 mc/mmole; C^{14}-algal hydrolysate, 15 mc/mg; H^3-UR, 7 c/mmole; S^{35}-IBT, 196 mc/mmole. The nucleic acid precursors were purchased from Schwarz BioResearch, Inc.; the algal hydrolysate was from New England Nuclear Corp.; and S^{35}-IBT was custom-prepared by the Radiochemical Center, Amersham, Bucks, England.

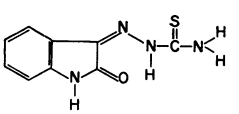

FIG. 1.

Results.—Viral growth: Figure 2 shows the dose-response curve relating concentration of IBT (added at time 0 as defined above) to viral yields at 24 hr. Most of the work was done with 15 μM IBT (3.3 μg/ml) which reduced the viral yield to 3 per cent of that in control cells. Certain experiments were done at 2 and 4 times this concentration. Figure 3 shows one-step growth curves in the presence and absence of 15 μM IBT. Virus found in the culture treated with IBT represents a true yield rather than residual inoculum.

Effect of IBT on uninfected host cells: 15 or 30 μM IBT had little effect on the growth of uninfected HeLa cells. At 60 μM IBT inhibition of growth was marked. This is in accord with the results reported by Sheffield.[12]

Effect on viral DNA replication: In the virus-cell system used here, 90 per cent of vaccinia virus DNA is formed between $1^1/_2$ and 4 hr after infection.[10] When HeLa cells were pulse-labeled with C^{14}-dT for periods of 10 min at various times following infection and the cytoplasmic fractions then examined for incorporated label (which represents newly formed viral DNA), there were no significant differences between cultures treated with 15μ M IBT from time 0 and control cells. Both the extent of viral DNA replication and its time course were unaffected by this concentration of IBT.

Incorporation of progeny viral DNA into mature virions: Joklik and Becker[10] followed the fate of viral DNA molecules as they were incorporated into mature viral progeny. "Coating" of progeny viral DNA was measured by determining the loss of susceptibility to DNase of DNA synthesized at the time of maximum DNA replication. "Coating" commenced at 5–6 hr after infection and then continued steadily for about 10 hr until about 50 per cent of the progeny DNA was coated. Figure 4 shows that although some progeny DNA is coated in the presence of 15 μM IBT, the extent of coating is inhibited by at least 70 per cent.

Sucrose density gradients analysis of the fate of vaccinia progeny DNA labeled during the period from 120 to 130 min after infection showed that by 11 hr about 10 times more DNA is incorporated into mature virions in normal cells than in cells

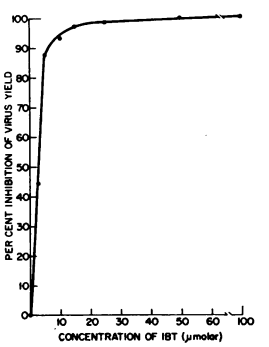

FIG. 2.—Effect of concentration of IBT on 24-hr virus yields; 1.6×10^8 HeLa cells were infected; the adsorbed multiplicity was 4 PFU per cell. At the end of the adsorption period the cells were washed once, resuspended at 5×10^5 cells/ml, and divided into eight equal parts each of which immediately received the appropriate amount of IBT. After incubation for 24 hr, the cells were disrupted by sonication and the virus was titrated as described above.

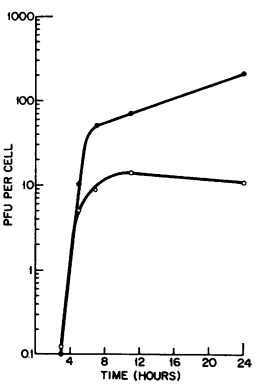

FIG. 3.—One-step growth curve of vaccinia strain WR in HeLa cells in the absence (*filled symbols*) and presence (*open symbols*) of 15 μM IBT. For details, see Fig. 2.

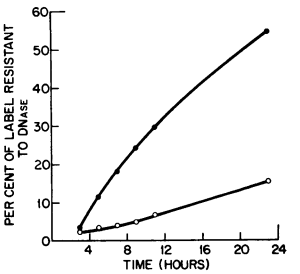

FIG. 4.—"Coating" of vaccinia progeny DNA in the absence (*filled symbols*) and presence (*open symbols*) of 15 μM IBT; 1.5×10^8 HeLa cells were infected as described in *Materials and Methods*. At the end of the adsorption period the culture was divided and one half made 15 μM with respect to IBT. At 2 hr both cultures were pulse-labeled with 5 μc C^{14}-dT for 10 min and then centrifuged and resuspended in fresh medium (with and without IBT, respectively). At intervals samples of 1×10^7 cells were removed, the cytoplasmic fractions prepared, and the amount of acid-insoluble label before and after incubation with 100 μg/ml DNase for 30 min at 37° was determined.

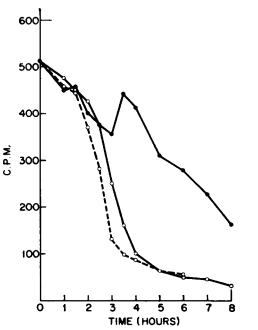

FIG. 5.—Effect of IBT on the incorporation of C^{14}-amino acids into cells infected in the absence of IBT (*filled symbols*), 15 μM IBT (*open symbols, continuous curve*), and 60 μM IBT (*open symbols, broken curve*). 15 μM IBT was added at time 0; 60 μM IBT was added 1 hr before each pulse. C^{14}-amino acid incorporation into whole cells after pulses of 10 min was measured. The increase between 3 and 5 hr was noted repeatedly and may represent synthesis of virus-specified protein.

continuously exposed to 15 μM IBT from time 0. No accumulation of DNA-containing immature viral forms could be detected.

Effect of IBT on protein synthesis: Since progeny DNA is coated predominantly by protein, failure of DNA to become coated suggests interference with the synthesis of structural viral protein. The ability of cells infected in the absence and presence of IBT to incorporate C^{14}-amino acids was therefore compared (Fig. 5). Cells infected in the presence of 15 μM IBT synthesized protein at the same rate as normally infected cells for about $2^1/_2$ to 3 hr but then suffered a drastic loss in this ability; at 2 hr there was no inhibition of protein synthesis, whereas at 4 hr inhibition was 80 per cent and at 6 hr 90 per cent. In the presence of 60 μM IBT the inhibition occurred about half an hour earlier.

Effect of IBT on viral mRNA synthesis: Inhibition of protein synthesis may be due to inhibition of mRNA synthesis or to interference with the function of mRNA. The effect of IBT on the synthesis of vaccinia mRNA was determined as described by Becker and Joklik.[6] In brief, this method depends on the fact that in the uninfected HeLa cell all RNA is synthesized in the nucleus, whereas in cells infected with vaccinia virus viral mRNA is transcribed in the cytoplasm, since it is there that the viral genome becomes established.

It was found that the rate of viral mRNA synthesis was unaffected by the presence of 15 μM IBT. Neither the extent of synthesis nor its time course was affected for up to 7 hr after infection.

Effect of IBT on polyribosomes: In view of this result the effect of IBT on the incorporation of viral mRNA into polyribosomes was determined at various times after infection.

(*a*) Figure 6 shows the polyribosome distribution as determined both by measurement of optical density and by the presence of labeled viral mRNA in

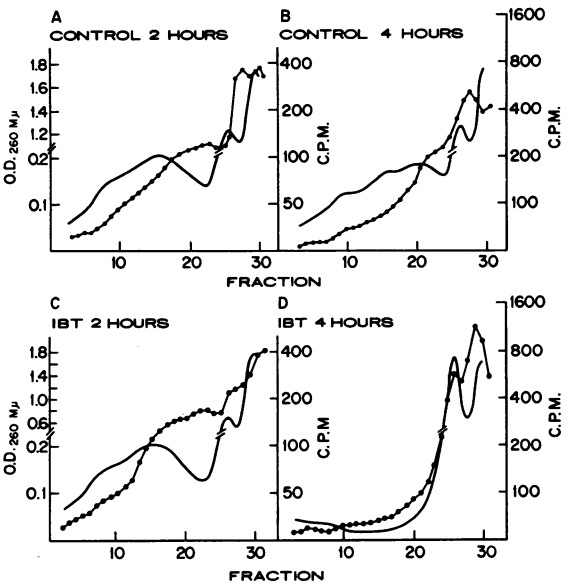

FIG. 6.—Optical density and radioactivity profiles in 15–30% sucrose density gradients (centrifuged 2 hr at 25,000 rpm) of cytoplasmic fractions of HeLa cells infected for 2 and 4 hr in the absence (*A* and *B*) and presence (*C* and *D*) of 15 μM IBT. For each gradient the cytoplasmic fraction of 4 × 10^7 cells pulse-labeled with C^{14}-UR for 10 min was used.

cytoplasmic fractions of cells infected in the presence and absence of 15 μM IBT at 2 and 4 hr after infection. Whereas at 2 hr there was no difference between the profiles, there were very few polyribosomes present at 4 hr in cells infected in the presence of IBT, and there was much less viral mRNA in the polyribosome region. This pattern was not changed if IBT was added for up to 5 hr before infection. The implication is that when IBT is present, viral mRNA synthesized during the first 2–3 hr after infection is capable of forming stable polyribosomes, but that mRNA synthesized after this time is not. For convenience, we will refer to the polyribosomes during the first 2–3 hr of the infection cycle as being resistant to IBT and after this time as being susceptible to IBT. However, we do not wish to imply that IBT itself disrupts polyribosomes.

(*b*) In this experiment the rate of breakdown of susceptible polyribosomes was measured after administration of IBT. Cells infected for 4 hr were exposed to 15, 30, 60, and 120 μM IBT, and 30 and 60 min later the number of 74*S* ribosomal monomers (single ribosomes) was measured by density gradient analysis. Measurement of the increase in the number of single ribosomes is the most sensitive measure of the breakdown of polyribosomes, since, even at 4 hr after infection, at least 70 per cent of the total cellular 74*S* ribosome complement is still present in polyribosome form. Figure 7 shows the relative increases in the number of single 74*S* ribosomes caused by the various concentrations of IBT. Fifteen μM IBT had relatively little effect within 1 hr, while 60 and 120 μM IBT caused all the 74*S* ribosomes that could be released from polyribosome form by IBT to be released within 1 hr. However, even at 120 μM IBT, not all but only about 80 per cent of ribosomes in polyribosome form were released.

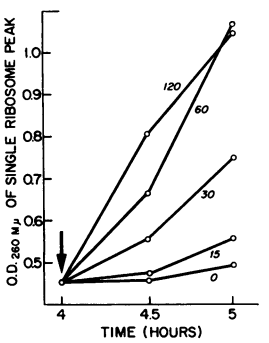

FIG. 7.—Effect of IBT concentration on the release of single 74*S* ribosomes from polyribosomes. At 4 hr after infection a cell culture was divided. One sample served as control and IBT was added to five others to give final concentrations of 0, 15, 30, 60, and 120 μM. Samples were removed for analysis 30 and 60 min later. All samples contained 4×10^7 cells. The cytoplasmic fractions were placed onto 15–30% sucrose density gradients, centrifuged for 2 hr at 25,000 rpm, and the optical density of the 74*S* single ribosome peak was measured.

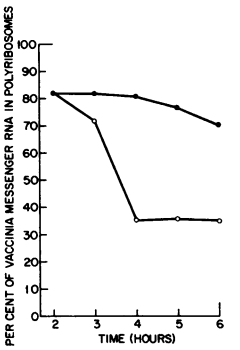

FIG. 8.—The effect of IBT on the percentage of vaccinia mRNA in polyribosomes. For details, see text. Density gradient analysis: Cytoplasmic extracts of 4×10^7 cells were layered onto 15–30% sucrose density gradients, centrifuged for 15 hr at 20,000 rpm, and the percentage of labeled viral mRNA in the pellet, that is, in polyribosomes, calculated. *Filled symbols:* no IBT. *Open symbols:* IBT added 1 hr before each time point.

(*c*) Further evidence for the concept that polyribosomes formed by viral mRNA during the first 2–3 hr after infection are not susceptible to the action of IBT, whereas those formed after this time are, was provided in the following experiment. Cells were infected in the absence of IBT. At various times after infection samples of the culture were made 60 μM with respect to IBT, a concentration which, as shown in the previous experiment, induces within 1 hr far-reaching destruction of susceptible polyribosomes formed by viral mRNA. After 60 min, the cells were then exposed to UR-C^{14} for 10 min and the proportion of viral mRNA in polyribosomes was determined by density gradient analysis. The results are shown in Figure 8. When IBT was added at 1 or 2 hr after infection, about 80 per cent of the viral mRNA formed during the 10-min period following the 60-min exposure to IBT was found to be in polyribosome form. However, when IBT was added at 3, 4, or 5 hr, a rapidly decreasing proportion of the viral mRNA synthesized during the 10-min pulse 1 hr later was present in polyribosome form. This result was obtained even if IBT was added as early as 5 hr before or as late as 1 hr after infection. Under all conditions tested, viral mRNA synthesized in the presence of IBT during the first 3 hr of the infection cycle forms stable polyribosomes, but mRNA synthesized after this time does not.

(*d*) Reversibility of the action of IBT was also tested. Two cultures of cells were exposed to 60 μM IBT 1–2 and 2–3 hr after infection, respectively, and then pulse-labeled for 10 min with C^{14}-UR at 3, 4, and 5 hr after infection. In each case the proportion of labeled viral mRNA in polyribosome form was determined by density gradient analysis. Even when IBT was present only from 1 to 2 hr after infection, these proportions were less than when cells had not been exposed to IBT at all; and this effect was more marked when IBT was present from 2 to 3 hr. The washing procedure used to remove IBT at 2 and 3 hr, respectively, reduced the intracellular IBT concentration to less than 1 per cent. It seems that IBT triggers a reaction which requires very little if any IBT for its continuance.

Characteristics of mRNA formed in the presence of IBT: (a) *Lack of attachment to ribosomal particles:* Vaccinia mRNA becomes attached to 40*S* subribosomal

particles within about 1 min after its formation and the resulting complexes can be chased into polyribosomes.[11] The band of labeled complexes is readily demonstrable on suitable pulse-labeling and density-gradient centrifugation, as is shown in Figure 9*A* for cells infected for 4 hr. There is no labeled RNA between the 40*S* and 4*S* regions. In the presence of IBT, however, viral mRNA synthesized at 4 hr accumulates in the region between 40*S* and 4*S* (Fig. 9*B*). In the absence of IBT, about 80 per cent of the total viral mRNA synthesized in a 10-min period at 4 hr after infection was associated with polyribosomes and the rest with the 40*S* band, but in the presence of the compound only 35 per cent was associated with polyribosomes and the rest was in the region between 40*S* and 4*S*. The accumulation there was so marked that it was difficult to determine whether any of it was associated with the 40*S* band.

At 2 hr after infection, the profiles were similar to those in Figure 9*A*, irrespective of the presence or absence of IBT.

The situation was further investigated using pulses of varying duration. Cells infected for 4 hr in the absence and presence of IBT were pulse-labeled with C^{14}-UR for $3^1/_2$, 8, and 13 min, and the distribution of labeled material with a sedimentation coefficient smaller than 60*S* was examined by density-gradient analysis. Whereas after a pulse of $3^1/_2$ min most of the viral mRNA not in the pellet was associated with the 40*S* band both in the presence and absence of IBT (profiles similar to Fig. 9*A*), after 8 min of pulse-labeling only the mRNA formed in the absence of IBT was so associated; mRNA formed in the presence of IBT was then already predominantly in the region between 40*S* and 4*S* (profile similar to Fig. 9*B*). The profiles after 13-min pulsing were qualitatively similar to those after 8 min.

In summary, vaccinia mRNA synthesized throughout the infection cycle in the absence of IBT, and in the presence of IBT that synthesized during the first $2^1/_2$–3 hr, is associated almost exclusively with ribosomal particles, either ribosomes in the form of polyribosomes or with 40*S* subribosomal particles in the form of complexes. However, vaccinia mRNA synthesized *after* the first $2^1/_2$–3 hr of the infection cycle in the presence of IBT is distributed in this manner only during the first 5 min or so after its formation; after this time most of it behaves in density gradients as one would expect free mRNA to behave.

(b) *Size of mRNA formed in the presence of IBT*: During the period 2–4 hr after infection, the median sedimentation coefficient of vaccinia mRNA molecules is approximately 16*S*.[6] This value is the same whether determined after a labeling period of $3^1/_2$, 8, or 13 min (Fig. 10*A*). Similarly, the *S* value of the viral mRNA formed in the presence of IBT at 2 hr after infection is also about 16*S*, as determined either by $3^1/_2$- or 13-min pulses. However, the mRNA synthesized at 4 hr after infection in the presence of IBT behaves differently. If the labeling period is $3^1/_2$ min, that is, if one examines the RNA very soon after it is synthesized, its sedimentation coefficient is indeed 16*S*, suggesting that mRNA of normal size is being transcribed. However, after 8 or 13 min of labeling, the median sedimentation coefficient is considerably smaller than 16*S*, in the neighborhood of 8*S* (Fig. 10*B*).

Preliminary experiments have shown that cytoplasmic fractions of cells infected in the presence of IBT for 4 hr do not change the sedimentation coefficient of normal vaccinia mRNA synthesized at 4 hr after infection when the two are mixed *in vitro* and incubated for 1 hr at 37°. Moreover, the sensitivity to ribonuclease of normal

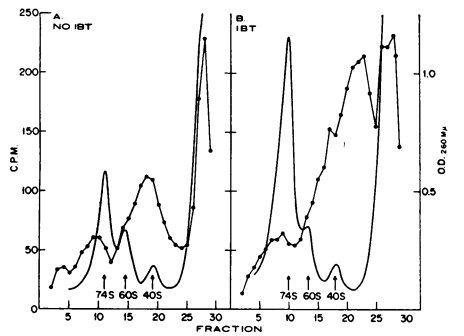

FIG. 9.—Effect of IBT on the distribution of viral mRNA in sucrose density gradients. For (*B*) cells were exposed to 30 μM IBT at 2 hr after infection and pulse-labeled with C^{14}-UR for 10 min 2 hr later; 4×10^7 cells were used for both (*A*) and (*B*). The cytoplasmic extracts were centrifuged into 15–30% sucrose density gradients for 15 hr at 18,000 rpm.

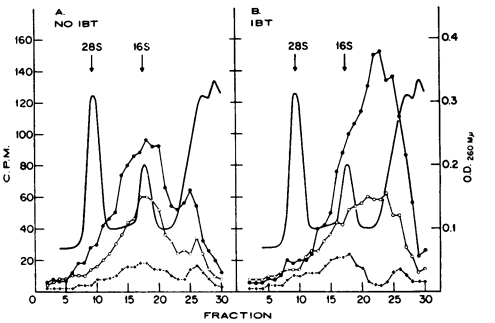

FIG. 10.—Effect of IBT on the size of viral mRNA. IBT was administered as for Fig. 9. Cells were labeled with H^3-UR for $3^1/_2$ (x), 8 (○), and 13 (●) min. The cytoplasmic fractions derived from 10^7 cells were treated with SDS as described and centrifuged for 16 hr at 24,000 rpm in 15–30% sucrose-SDS density gradients.

vaccinia mRNA and the 8*S* material accumulating in the presence of IBT in the later part of the infection cycle is the same. Further work is proceeding and will be reported later (Joklik and Woodson, to be published).

Experiments with S^{35}-IBT: It was shown by using S^{35}-IBT that there is no difference in the extent to which IBT is taken up by normal and infected cells. In both cases the compound was concentrated about fivefold when present in the medium at a concentration of 15 μM. Density gradient analysis of cells infected in the presence of S^{35}-IBT gave no indication that IBT was bound to any particular cell constituent. If such an association exists, its demonstration will necessitate IBT with a considerably higher specific activity.

Discussion.—The results presented here lead to the conclusion that IBT affects the ability of "late" vaccinia mRNA, that is, mRNA synthesized after the first 3 hr of the infection cycle, to express itself normally. The precise mechanism responsible is not yet clear. However, it seems that in the presence of IBT, mRNA of the normal size is synthesized and that this mRNA can be incorporated into polyribosome form. However, within a very short period after its formation, probably not exceeding on the average 5 min, the sedimentation coefficient of this mRNA decreases from the normal 16*S* to about 8*S*. It is uncertain at the moment whether this change represents a change in configuration, breakage of molecules by a highly specific mechanism, or the first stage of a slow endonucleolytic attack. However, it is clear that the net effect of the presence of IBT from 3 hr after infection on, is a reduction in the functional half life of viral mRNA from at least 15 min and probably more than 30–60 min to less than 5 min. As a result, the synthesis of proteins programed to be translated later than 3 hr after infection is drastically reduced and in consequence little if any mature viral progeny is formed.

"Early" viral mRNA, on the other hand, appears to function normally in the presence of IBT; not only is it associated with polyribosomes in the normal manner but proteins programed to be formed during the first part of the infection cycle,

such as "early" enzymes and some structural viral proteins (unpublished results), are synthesized as usual.

The time around $2^1/_2$–3 hr thus appears to be critical. This period coincides with the most active period of viral DNA replication.[11] It appears possible that the "early" stable mRNA represents mRNA coded from parental genomes while the "late" unstable mRNA is coded from progeny genomes; or alternately, that there is coded from progeny genomes a factor which in the presence of IBT converts "late" viral MRNA to the nonfunctional 8*S* form. It has not yet been determined whether this change affects "early" as well as "late" mRNA, that is, whether "early," stable viral mRNA becomes susceptible to IBT at 3 hr. Work on this problem is continuing.

Summary.—IBT inhibits the formation of vaccinia virus progeny. IBT does not affect viral DNA replication or viral mRNA synthesis, or the functioning of "early" viral mRNA. However, in the presence of IBT the sedimentation coefficient of "late" viral mRNA, i.e., viral mRNA synthesized after the first 3 hr of the infection cycle, decreases from the normal value of 16*S* to about 8*S* within about 5 min of synthesis. As a result, the number of polyribosomes formed by viral mRNA is greatly reduced and viral proteins programed to be translated from then on are not formed.

Abbreviations: dT, thymidine; UR, uridine; IBT, isatin-β-thiosemicarbazone; PFU, plaque-forming unit; SDS, sodium dodecyl sulfate; mRNA, messenger RNA.

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