

1-1-1969

# Inhibition of infectious bovine rhinotracheitis virus multiplication by thiosemicarbazones.

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INHIBITION OF INFECTIOUS BOVINE RHINOTRACHEITIS  
VIRUS MULTIPLICATION BY THIOSEMICARBAZONES

BY

T. WAYNE MUNRO

A Thesis  
Submitted to the Faculty of Graduate Studies through the  
Department of Biology in Partial Fulfilment  
of the Requirements for the Degree of  
Master of Science at the  
University of Windsor

WINDSOR, ONTARIO, CANADA

1969

UMI Number: EC52759

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## ABSTRACT

Isatin B-thiosemicarbazone (IBT) and its N-methyl derivative are antiviral drugs whose mode of action is not completely understood. Any insight into the antiviral action of these relatively simple molecules would prove valuable in the development of other antiviral agents. Since the spectrum of antiviral activity of the thiosemicarbazones has been limited to members of the poxvirus and adenovirus groups, the effectiveness of these drugs was tested against infectious bovine rhinotracheitis virus which belongs to the herpesvirus group. These drugs were found to be quite effective in inhibiting the multiplication of all virus isolates tested. Therefore, it appeared desirable to study their effects on some of the biochemical aspects of the multiplication of this virus. Radioisotope incorporation experiments showed that IBT inhibits the uptake of radioactive precursors into both viral RNA and DNA early in the viral growth cycle. However, this drug was found to be effective in inhibiting the production of infectious virus even if added late in the viral growth cycle when maturation of virus occurs.

## ACKNOWLEDGEMENTS

I am indebted to Dr. L.R. Sabina, Department of Biology, University of Windsor, for the scientific training and assistance I received during the course of this research and the writing of this thesis.

For critically reviewing this thesis, I wish to extend my sincere appreciation to Dr. A.H. Warner, Department of Biology and Dr. G.W. Kosicki, Department of Chemistry, both of the University of Windsor.

Special thanks are due to Miss D. Gail Morrow for her very able technical assistance, to G.M. Kouroupis for help with late night sampling, and to F.J. DePauli for collaborating in the development of a suitable overlay medium for virus assay.

I also wish to thank my wife, Barbara, for her moral support and for typing this thesis.

This investigation was supported by grant A 2904 from the National Research Council of Canada and by an Ontario Graduate Fellowship awarded to the author.

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## INTRODUCTION

Since the initial report on the antiviral activity of thiosemicarbazones in vivo (Hamre, Bernstein & Donovan, 1950), several reports have established that isatin B-thiosemicarbazone (IBT) and N-methylisatin B-thiosemicarbazone (N-methyl-IBT) are active against poxvirus infections in man and animals (Turner et al., 1965; Thompson et al., 1955; Bauer, 1965; Thompson et al., 1955; Bauer, 1955). As a result the mode of action of these antiviral compounds against various poxviruses *in vitro* has been extensively studied. Sheffield, Bauer & Stephenson (1960) have shown that IBT does not act as a contact agent nor does it affect virus absorption. Studies by Bach & Magee (1962 & 1965) and by Easterbrook (1962) indicated that inhibition of vaccinia virus by IBT occurred at the maturation phase late in the viral replication cycle. A later study by Woodson and Joklik (1965) supported these findings and showed further that IBT affects the ability of "late" vaccinia messenger-ribonucleic acid (mRNA) to express itself normally.

The present investigation was initiated to determine whether IBT and N-methyl-IBT could also inhibit the synthesis of infectious bovine rhinotracheitis (IBR) virus, which is a member of the herpesvirus group. Evidence is presented that these antiviral agents inhibit IBR multiplication. A study of the mode of action of IBT was

of special interest in view of the differences known about the cellular sites of IBR and poxvirus replication. Unlike poxviruses, in which replication is confined to the cytoplasm in cell cultures, IBR virus replicates in the nucleus. Our findings suggest that the mode of antiviral action of IBT on IBR virus may differ from the action on poxvirus reported by others.

## MATERIALS AND METHODS

### Cells and Media

The MDBK cell line of bovine kidney origin employed was obtained from Baltimore Biological Laboratory, Baltimore, Maryland. Medium E Lay-10FCS used for the growth of the cells was as described previously (Sabina & Parker, 1963).

### Viruses

Five isolates of infectious bovine rhinotracheitis (IBR) virus were used in this investigation. Isolates 1, 2 and 3 were generously supplied by Dr. A. Brown, Norden Laboratories, Lincoln, Nebraska. Isolate 4 was obtained from Dr. V.C.R. Walker, Connaught Medical Research Laboratories, Toronto, Ontario and isolate 5 was kindly furnished by Dr. A.S. Greig, Animal Diseases Research Institute, Hull, Quebec. For each isolate, virus pools were prepared as previously reported (Sabina & Parker, 1963) and stored at  $-70^{\circ}\text{C}$ . Such virus pools had infective titers from  $1.2 \times 10^6$  to  $2.3 \times 10^8$  plaque forming units per ml (p.f.u./ml.). All experiments with virus were done at a multiplicity of ca. 6 p.f.u./cell.

### Virus Assay

IBR virus was assayed by plaque formation in monolayers of MDBK cells in 60-mm petri plates (Falcon Platin, Los Angeles, California). Adsorption of virus (0.5 ml/plate) was carried out for 1 hr at  $37^{\circ}\text{C}$ . The overlay

medium was the same as that used in a previous study (Sabina & Munro, 1969). After 72 hr incubation at 37°C in an atmosphere of 5% CO<sub>2</sub> in air, the agar-overlay was removed and the plaques counted.

#### Isotopes and Inhibitors

[<sup>3</sup>H] L-leucine (S.A. 1000 mc/m moles), [<sup>3</sup>H] cytidine (S.A. 1000 mc/m moles), and [<sup>3</sup>H] thymidine (S.A. 5000 mc/m moles) were obtained from Nuclear-Chicago Corp., Des Plaines, Illinois. Cycloheximide was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio and IBT was obtained from Mann Research Laboratories, New York, N.Y. N-methyl-IBT was kindly donated by Dr. L.E. Rhuland, The Upjohn Co., Kalamazoo, Michigan. FUdR (5-Fluoro-2'-deoxyuridine) was generously supplied by Dr. R.G. Brackett, Parke Davis Co., Detroit, Michigan.

#### Extraction of DNA, RNA and Protein

Following 1 hr pulsing with tritiated precursor, monolayer cultures in 16 oz. prescription bottles were drained and washed thrice with cold Hank's balance salt solution. Immediately cold 0.5 N HClO<sub>4</sub> was added to each bottle and the acid-precipitated cells stored at -25°C overnight. The procedure used to isolate the three biochemical fractions was essentially that of Schmidt & Thannhauser (1945). The cells were thawed at room temperature and scraped off the glass with a rubber policeman. Each bottle was then rinsed with cold 0.5 N HClO<sub>4</sub> and like suspensions pooled. After collection of cell pellets by

centrifugation they were extracted with cold 0.5 N HClO<sub>4</sub> for 30 min. and the acid-insoluble fraction was obtained by centrifugation. Two additional cold acid extractions of 10 minutes each were carried out. The acid-insoluble pellet was then suspended in a mixture of ethanol and ether (3:1) and steamed for 5 min. to solublize the lipids. To remove residual lipids the pellets were again extracted with the hot ethanol-ether mixture for 2 min. After centrifugation, alkali (0.5 N KOH) was added to the pellet, incubated for 20 min. at 50°C (Munro & Fleck, 1966) and a portion taken for protein analysis (Lowry, et al., 1951). The remaining protein and deoxyribonucleic acid (DNA) were precipitated with cold HClO<sub>4</sub> and the supernatant was used for ribonucleic acid (RNA) analysis (Mejbaum, 1939). The DNA was removed by extracting twice **with** acid (0.5 N HClO<sub>4</sub>) at 80°C for 15 min. Pooled supernatants were analyzed for DNA (Burton, 1956).

#### Isotope Incorporation Studies

Cultures of MDBK cells in 16 oz. prescription bottles were inoculated with IBR virus. The unabsorbed virus inoculum was washed off after 1 hr at 37°C and the cultures were covered with MEM medium (Eagle, 1959) or MEM containing IBT (20 µg./ml.). Uninfected cultures were treated similarly. To study the "rate" of synthesis of DNA, RNA, and protein, [<sup>3</sup>H] thymidine, [<sup>3</sup>H] cytidine, and [<sup>3</sup>H] L-leucine, respectively, were added directly to the medium at appropriate intervals (Fig. 1) for a 1 hr pulse. Duplicate cell cultures were

harvested and the DNA, RNA, and protein were extracted. Aliquots of 0.2 ml. were taken from the extracted material, added to 10 ml. of scintillation fluid (Warner & McClean, 1968) and assayed for radioactivity using a Nuclear-Chicago liquid scintillation system (Model 6850). The scintillation fluid was composed of toluene, ethylene glycol monomethyl ether, POP, and POPOP in the following composition: 1000 ml., 700 ml., 4 g. and 50 mg., respectively. Specific activity was expressed as counts/min./mg. of sample.

## RESULTS

### Effect of IBT on the growth of MDBK cells

In preliminary experiments, confluent MDBK cultures were exposed to media containing concentrations of IBT ranging from 5 to 20  $\mu\text{g./ml.}$  Following a 24-hr incubation period, the cells were washed and subcultured in the absence of IBT. No cytotoxic effects were observed at this time nor in subsequent subcultures. However, if 20  $\mu\text{g.}$  per ml. of IBT were added to the growth medium, both the generation time and the cytoplasmic volume of the cells increased. After the third subculture, it was found that IBT-treated cells lose their ability to divide.

### Influence of IBT and N-Methyl-IBT on the multiplication of IBR isolates

To determine whether IBT or its derivative N-methyl-IBT had any effect on the multiplication of IBR virus, duplicate MDBK cultures were inoculated with IBR isolates. After incubation at  $37^{\circ}\text{C}$  for 1 hr, the cultures were washed, covered with MEM medium containing either 0, 5, 10, or 20  $\mu\text{g./ml.}$  of IBT, and the 24-hr virus yields titrated. The results in Table 1 show that the yield of progeny virus of all isolates tested was effectively inhibited. When N-methyl-IBT was substituted for IBT in MEM media, the inhibitory capacity of the former was found to be slightly less for all isolates except number 3 (Table 2). For this reason, IBT was used in subsequent experiments.



TABLE 1.

Effect of IBT on the multiplication of IBR isolates

Conc. of IBT ( $\mu\text{g./ml.}$ )	Titers of IBR isolates (p.f.u./ml.)				
	1	2	3	4	5
0	$1.1 \times 10^7$	$1.0 \times 10^7$	$2.3 \times 10^8$	$7.4 \times 10^6$	$1.8 \times 10^7$
5	$6.1 \times 10^4$	$3.4 \times 10^4$	$4.5 \times 10^7$	$4.0 \times 10^3$	$7.2 \times 10^5$
10	$1.0 \times 10^4$	$2.2 \times 10^2$	$2.6 \times 10^7$	$1.6 \times 10^3$	$8.9 \times 10^4$
20	0	0	$1.8 \times 10^6$	0	$1.1 \times 10^3$

TABLE 2

Effect of N-methyl-IBT on the multiplication of IBR isolates

Conc. of N-methyl-IBT ( $\mu\text{g./ml.}$ )	Titers of IBR isolates (p.f.u./ml.)				
	1	2	3	4	5
0	$1.1 \times 10^7$	$1.0 \times 10^7$	$2.3 \times 10^8$	$7.4 \times 10^6$	$1.8 \times 10^7$
5	$2.1 \times 10^6$	$1.9 \times 10^6$	$4.9 \times 10^7$	$7.7 \times 10^5$	$5.1 \times 10^6$
10	$3.8 \times 10^4$	$7.0 \times 10^2$	$1.3 \times 10^5$	$2.9 \times 10^3$	$8.8 \times 10^5$
20	0	0	$5.2 \times 10^3$	0	$1.6 \times 10^3$

Effect of IBT on incorporation of labeled compounds by control and IBR-infected cultures

To obtain some information on cellular metabolism in uninfected and IBR-infected MDBK cells in the presence and absence of IBT, pulse experiments with labeled compounds were performed as described in Methods.

The rates of [ $^3\text{H}$ ] thymidine incorporation in DNA shown in Fig. 1 indicate that no qualitative differences occur when uninfected cultures treated with IBT are compared with untreated controls. However, when IBT was added to infected cultures, the incorporation of label was appreciably inhibited as early as the 4-5 hr pulse. This inhibition was found to be about 65% at the 10-11 hr pulse when compared with untreated controls.

As can be seen from Fig. 2, [ $^3\text{H}$ ] L-leucine was incorporated into protein at similar rates by uninfected cultures in the presence and absence of IBT. With infected cultures, there was some variability in [ $^3\text{H}$ ] L-leucine incorporation in control and IBT-treated cultures. However, the differences that occurred were relatively small.

When [ $^3\text{H}$ ] cytidine was used in uninfected cultures in the presence of IBT, the rates of incorporation of label into RNA were essentially the same as in the absence of the drug (Fig. 3). On the other hand, the results showed that IBT markedly affects [ $^3\text{H}$ ] cytidine incorporation at an early stage (4-5 hr) of IBR infection. In similar cultures, incorporation of [ $^3\text{H}$ ] cytidine was decreased

FIG. 1. Rate of [ $^3\text{H}$ ] thymidine incorporation into control and IBR-infected cells in the presence and absence of IBT. General conditions of infection and exposure to tritium label were as given in Methods. All values are the mean of 2 experiments. Horizontal bars represent IBT (20  $\mu\text{g./ml.}$ ) treated cells and solid bars represent untreated cells.

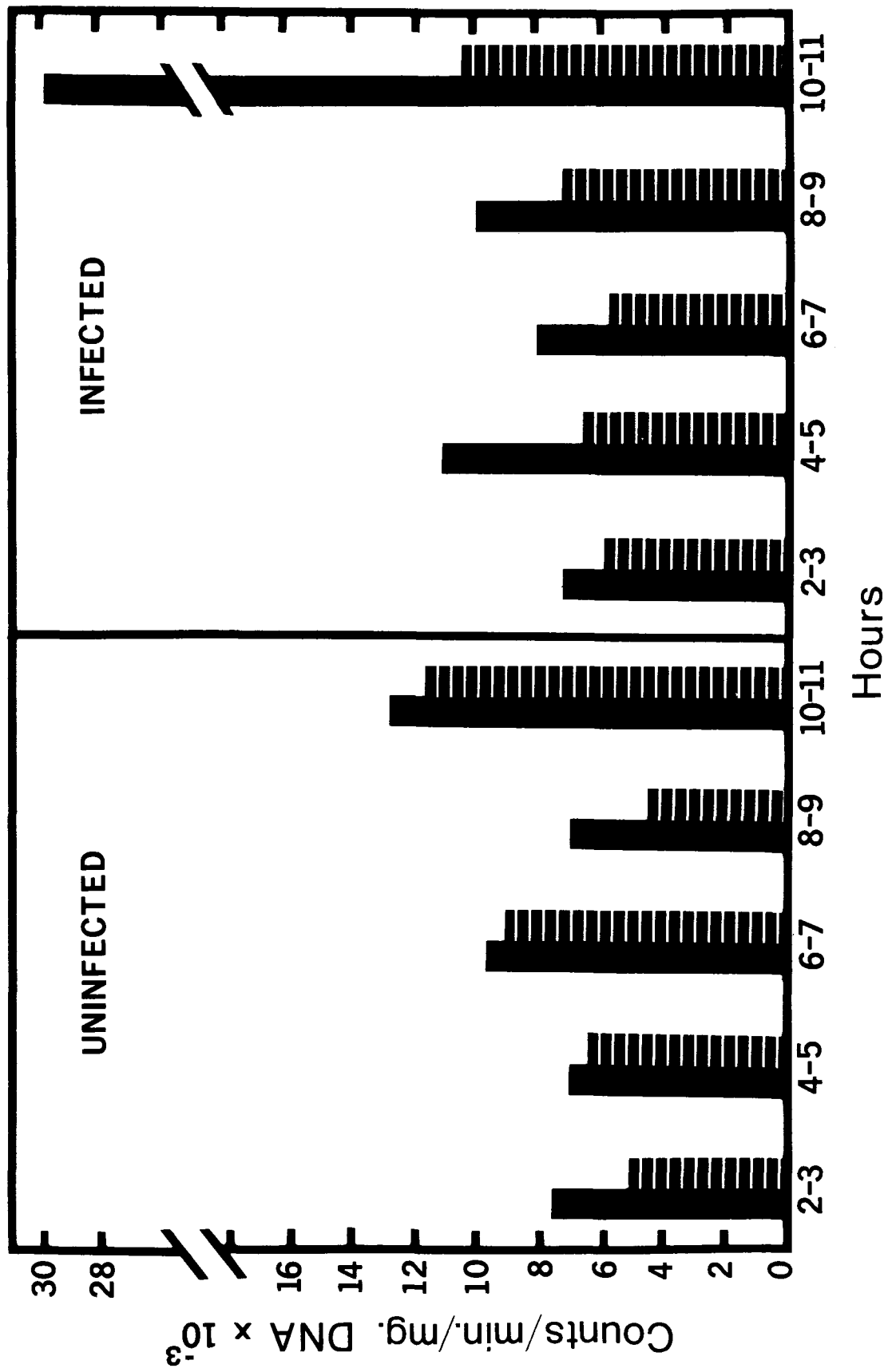


FIG. 2. Rate of  $[^3\text{H}]$  L-leucine incorporation into control and IBR-infected cells in the presence and absence of IBT. Legend as for Fig. 1.

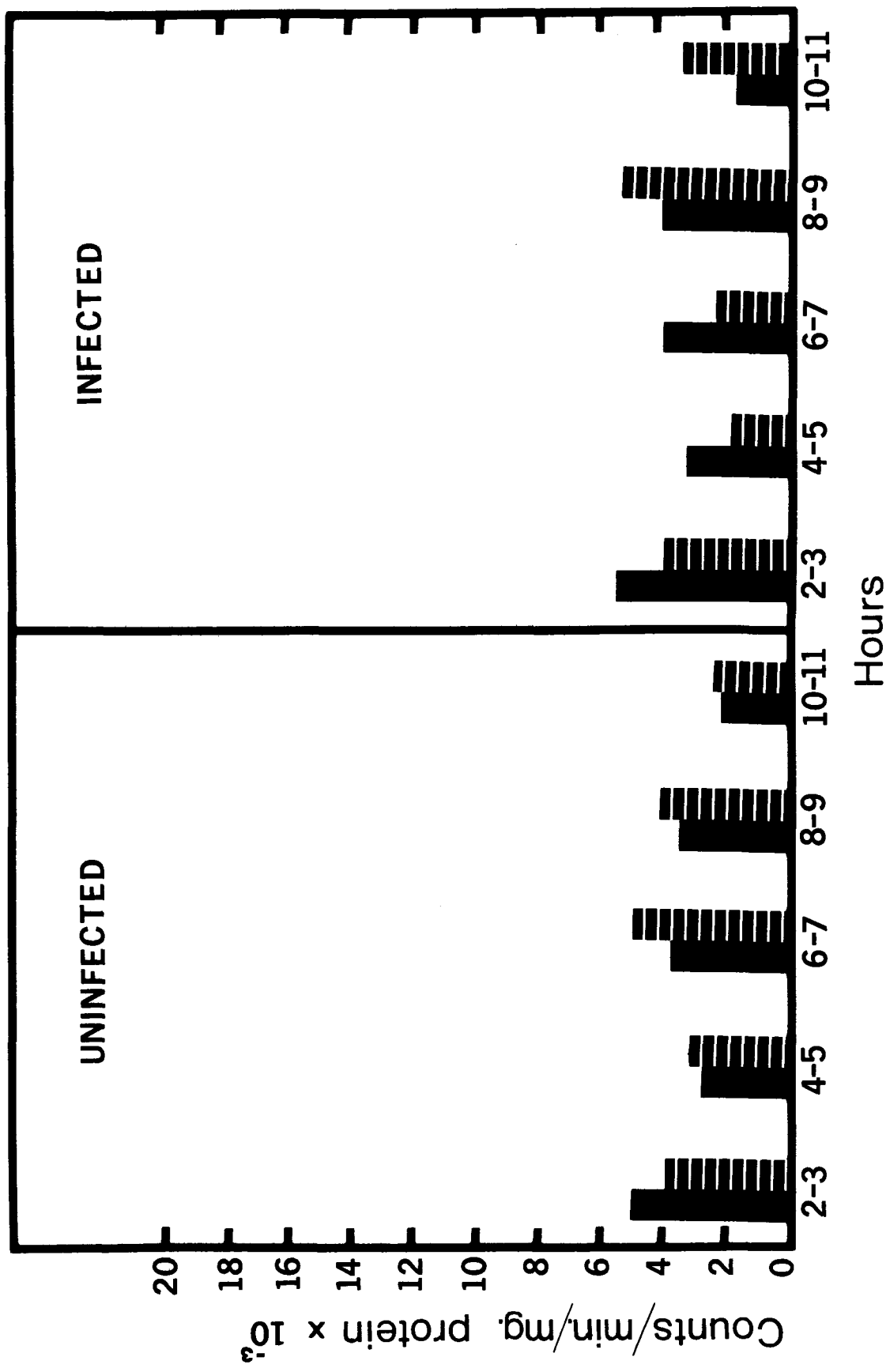
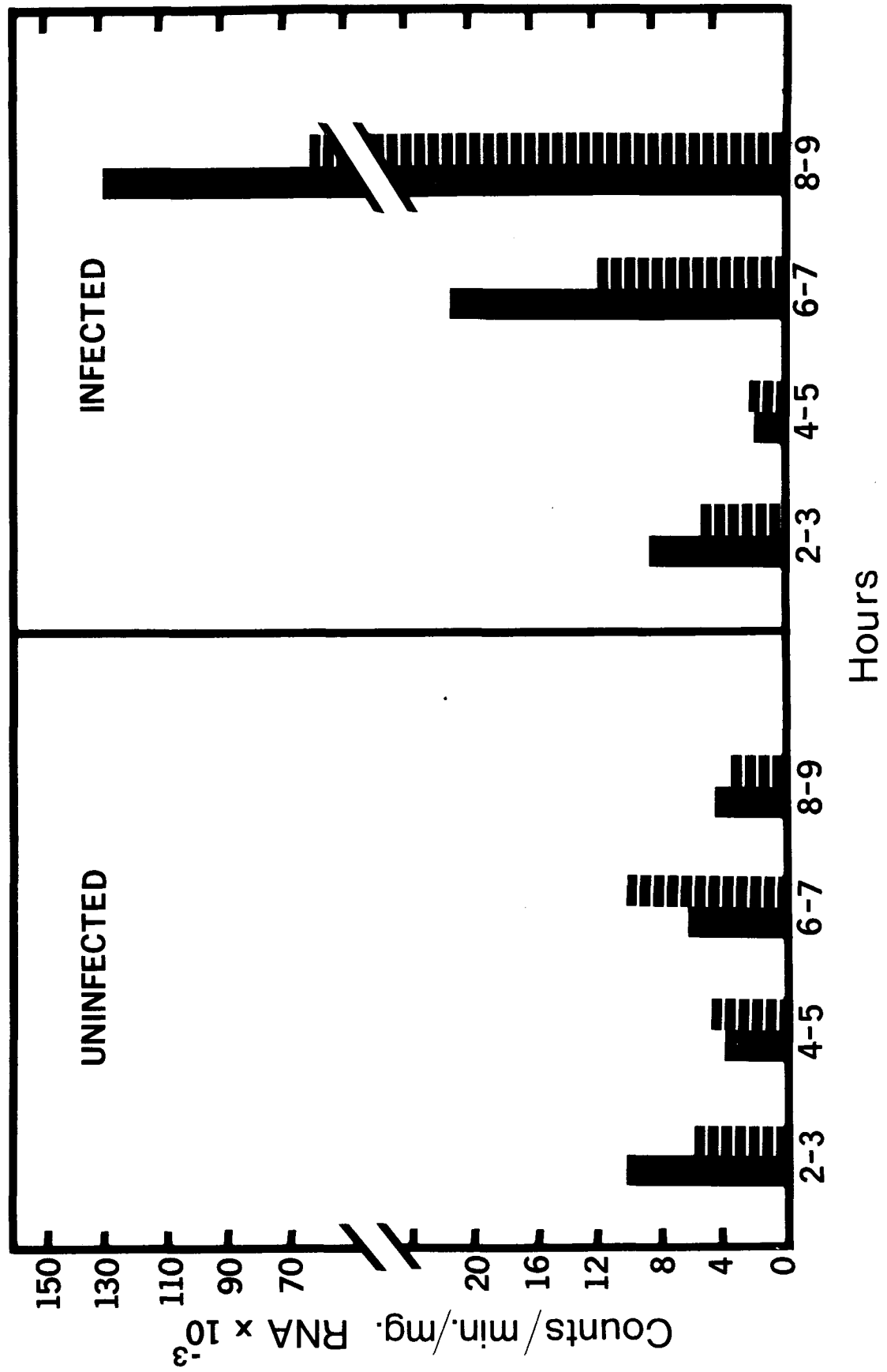


FIG. 3. Rate of [ $^3\text{H}$ ] cytidine incorporation into control and IBR-infected cells in the presence and absence of IBT. Legend as for Fig. 1.





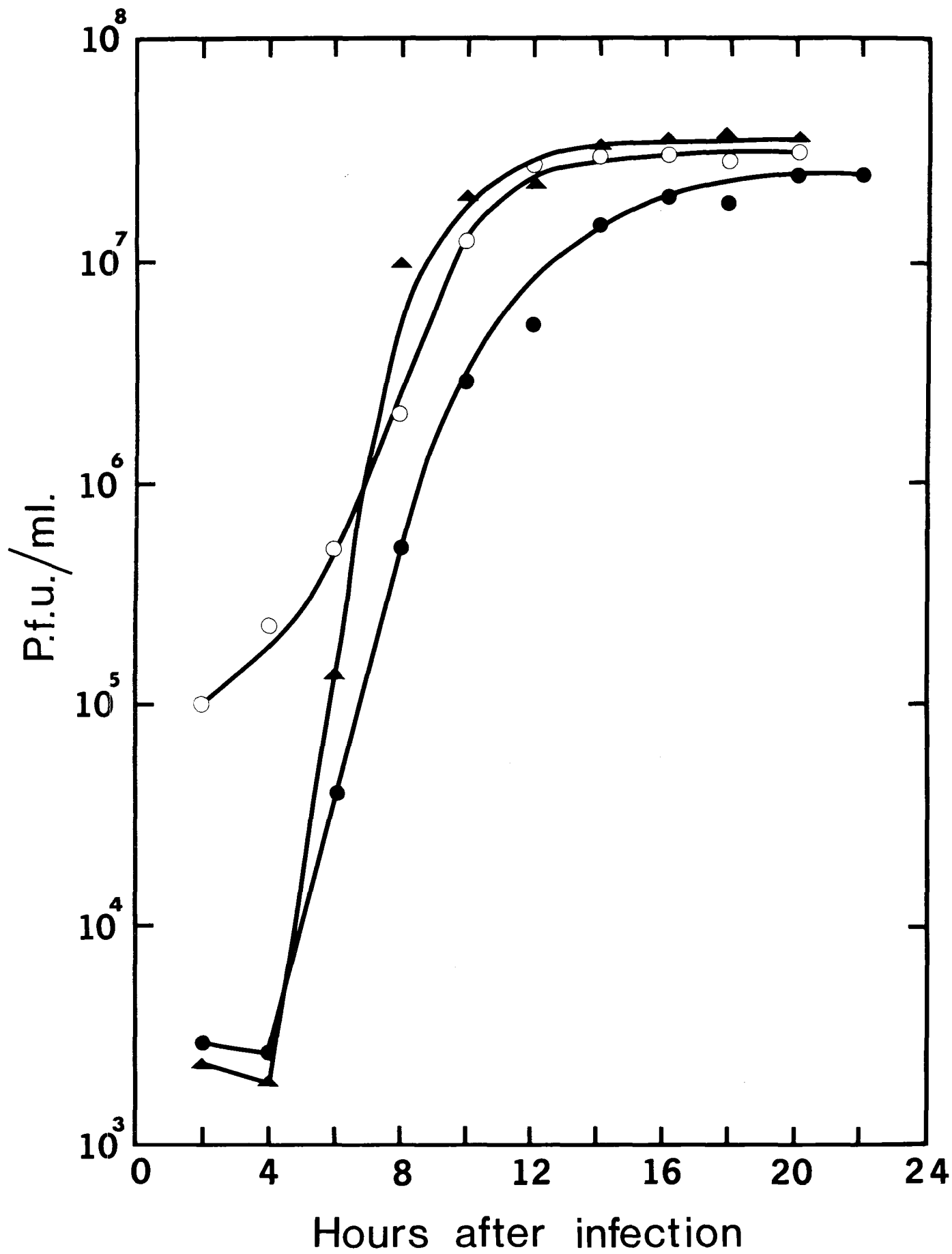
more than 50% in IBT-treated cultures when compared with control cultures.

#### Time course of synthesis of viral DNA and protein

Before studying the mode of action of IBT, it was important to establish the time course of events that occur during virus formation. Thus the rate of synthesis of viral DNA and protein was determined and compared with the maturation of infectious virus. For these determinations, FUdR (16 ug./ml.) and cycloheximide (20 µg./ml.) were used to block the synthesis of viral DNA and protein, respectively. Inhibitor was added to duplicate IBR-infected cultures at 2-hr intervals and total virus assayed at 22 hr postinfection. The rate of infectious virus formation was determined by sampling duplicate infected cultures at 2-hr intervals and assaying for infectious progeny.

Results of these experiments are shown in Fig. 4. The onset of viral DNA synthesis occurred between 2 and 4 hr after infection. Although sufficient viral DNA was made by 12 hr to give a full yield of virus, the portion of the eventual yield of mature virus assembled was about 20% at this time. Furthermore, it appears that viral DNA synthesis preceded the formation of infectious virus by 3 to 3.5 hr. Formation of viral protein began at 4 to 6 hr and was completed by about 14 hr postinfection.

Fig. 4. Time course of infectious virus formation and synthesis of viral DNA and viral protein as determined by FUdR and cycloheximide inhibition. MDBK cultures were infected, washed twice after 1 hr at 37°C and covered with MEM medium. FUdR (16 µg./ml.) or cycloheximide (20 µg./ml.) was added at 2-hr intervals to duplicate cultures. Incubation was continued until 22 hr postinfection. Each point in the viral DNA curve (O) and viral protein curve (▲) represents total yield of virus at 22 hr. Points in the infectious virus curve (●) represent virus synthesized at this time in untreated cultures.

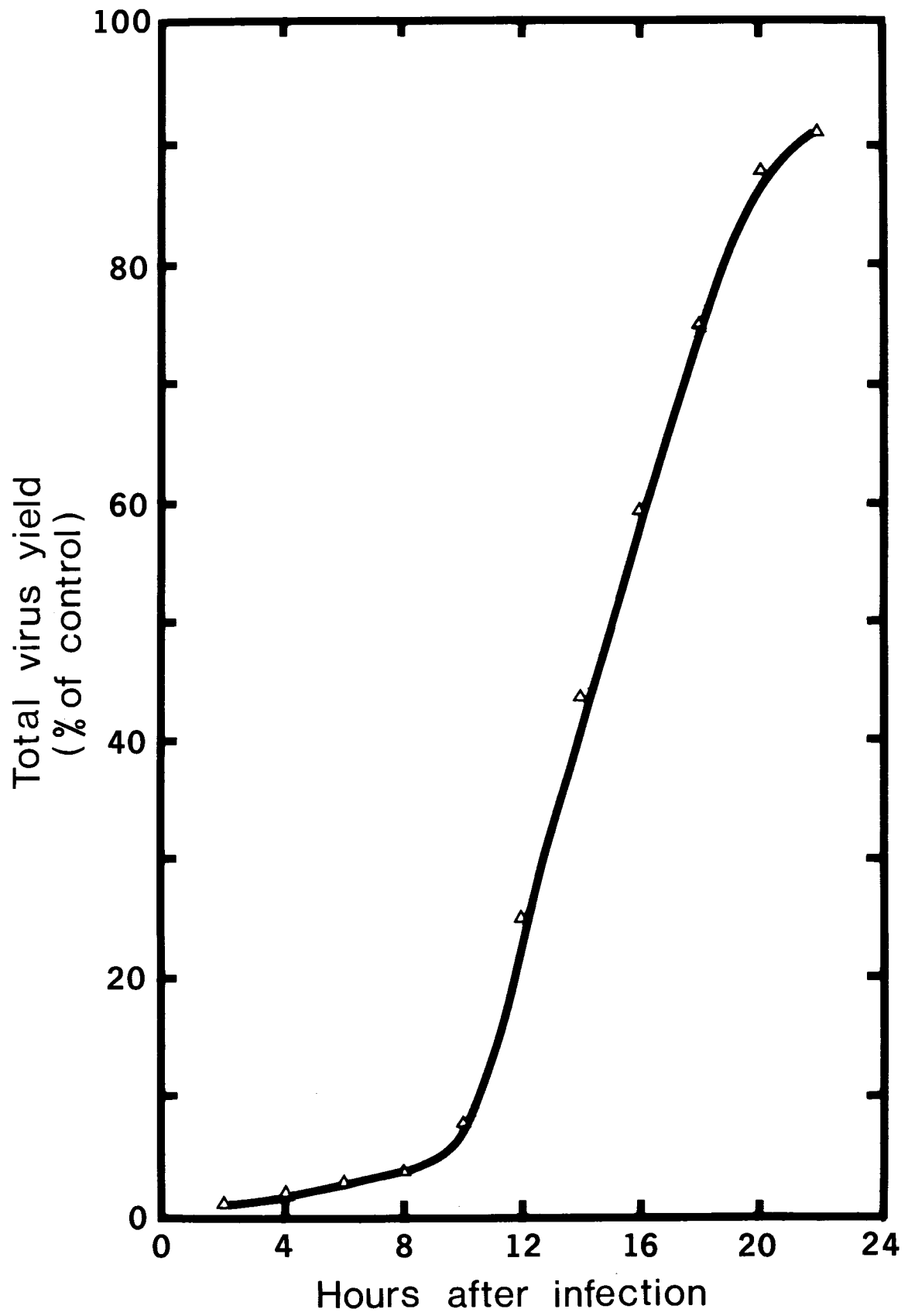


Inhibition of IBR virus multiplication by addition of IBT  
at various times after infection

Since IBT interfered with DNA and RNA synthesis prior to 12 hr postinfection (Fig. 1, 3), at which time adequate viral DNA had been synthesized for maximum yield of virus (Fig. 4), it was of interest to know its influence on the formation of infectious virus during the growth cycle. Cultures were infected and IBT (20 µg./ml.) was added at 2-hr intervals to duplicate cultures after infection. Total yield of virus after 24 hr were titrated and expressed as a percentage of the 24-hr yield from untreated controls. The results of such an experiment are shown in Fig. 5. The addition of IBT at 10 hr postinfection was effective in reducing the final yield of infectious virus, but had little effect when it was added between 10 and 14 hr after virus infection.

In another experiment in which cell cultures were pretreated for 6 hr before virus infection with growth medium containing IBT (20 µg./ml.), there was no decrease in the final viral yield when the drug was withdrawn immediately before infection. However, if the drug was also present throughout the growth cycle, the virus titer was slightly lower than the 2 hr yield obtained in the previous experiment (Fig. 5).

Fig. 5. Effect of the addition of IBT during the viral growth cycle. The drug was added to duplicate cultures at the times indicated. Each point on the curve represents total yield of virus at 24 hr.



## DISCUSSION

Previous studies on the mode of action of IBT and its N-methyl derivative on virus synthesis have, thus far, been limited to members of the poxvirus and adenovirus groups (Easterbrook, 1962; Bauer & Apostolov, 1966). Our study extends the spectrum of antiviral activity associated with IBT, since it was clearly shown that the synthesis of IBR virus which belongs to the herpesvirus group could be effectively inhibited by this drug. The results concerning the mode of action of IBT on IBR replication tend to support the view that the drug may interfere not only with the function of "late" viral mRNA as suggested by Woodson & Joklik (1965) with vaccinia virus, but also "early" viral mRNA.

As in previous studies (Woodson & Joklik, 1965; Bauer & Apostolov, 1966) it was found that concentrations of IBT sufficient to inhibit viral replication had no cytotoxic effect on host cells. Cultures pretreated with IBT prior to virus infection had the potential to produce maximal yields, a finding similar to that observed by others (Appleyard, Hume & Westwood, 1965; Bauer & Apostolov, 1966). The evidence that IBT may exert its effect at a late stage in the replicative cycle was provided by the finding that the addition of the drug 10 hr after infection could prevent the synthesis of infectious virus. This late effect on the maturation of virus has also been demonstrated



in other virus-host cell systems. Appleyard, Hume & Westwood (1965) found that the addition of IBT by 3 hr postinfection was necessary for complete inhibition of rabbit poxvirus replication, whereas the drug added at 13 hr postinfection effectively inhibited the replication of adenovirus type 11 (Bauer & Apostolov, 1966). These results seem likely when comparing different types of viruses which differ considerably in their growth cycles.

Bach & Magee (1962, 1965), studying the inhibitory effects of IBT and N-methyl-IBT on vaccinia virus, showed major differences when a low or high level of drug was tested. Although low concentrations of either drug suppressed virus yields and did not interfere with [ $^3\text{H}$ ] thymidine uptake into DNA, the drugs at high levels did suppress uptake of label into both control and infected cells. In contrast we found that IBT at a concentration of 20  $\mu\text{g./ml.}$  reduced the incorporation of [ $^3\text{H}$ ] thymidine into IBR-infected cells but not into control cells. Also, during the early stage of the IBR growth cycle, the uptake of radioactivity into RNA of infected cells was depressed. These results indicate that IBT may have some effect quite early in the eclipse period as previously suggested (Appleyard, Hume & Westwood, 1965).

Woodson & Joklik (1965) have shown that by 4 hr after infection IBT severely limits the formation of stable polyribosomes by vaccinia-mRNA. They suggested that the

drug caused a change in conformation or degradation of the viral mRNA and thus inhibited the formation of "late" viral proteins necessary for viral maturation.

It should be pointed out here that vaccinia and IBR virus differ considerably in their replication rates. With vaccinia the synthesis of viral DNA begins at 1 to 1.5 hr after infection and is essentially complete by 4 to 5 hr (Woodson, 1968) which enables a maximum yield of virus to be produced by 8 hr (Woodson & Joklik, 1965). However, IBR-DNA synthesis was not complete until 12 hr postinfection and full yields of virus were obtained only after 15 hr. It is then conceivable that the early effect we observed of IBT on viral RNA would cause an inhibition not only of the production of "late" viral proteins as with vaccinia virus but also of the "early" proteins required for viral DNA synthesis (Hamada, Tomoya & Kaplan, 1966) which was shown by the decreased radioactive incorporation into viral DNA. This early effect of IBT would appear to be related to the length of the growth cycle of this virus since the IBR-RNA would be exposed to the IBT for a longer time period allowing for degradation of the RNA to occur prior to viral DNA synthesis.

## SUMMARY

The growth and appearance of MDBK cells after a 24 hr period were found to be relatively insensitive to the presence of 20  $\mu\text{g./ml.}$  of IBT in the growth medium. Both IBT and its N-methyl derivative effectively inhibited the multiplication of different IBR isolates. When cell cultures were pretreated for 6 hr before virus infection with medium containing IBT there was no effect on the final yield of virus. The addition of IBT 10 hr after infection by which time sufficient viral DNA and viral proteins had been synthesized severely suppressed the formation of infectious virus. Radioisotope experiments showed that IBT inhibited the incorporation of tritium label into both viral RNA and DNA early in the viral growth cycle. It is suggested that this antiviral agent can inhibit not only the formation of "late" viral proteins used in the maturation phase but also "early" viral proteins necessary for replication of the viral genome.

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