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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
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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.

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

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INTRODUCTION

Since the initial report on the antiviral activity of
thiosemicarbazones _in vivo_ (Hamre, Bernstein & Donovick,
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

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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 ELay-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°C. Such virus pools had infective titers from 1.2×10^6 to 2.3×10^8 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 Plastin, Los Angeles, California). Adsorption of virus (0.5 ml/plate) was carried out for 1 hr at 37°C. The overlay

medium was the same as that used in a previous study

(Sabina & Munro, 1969). After 72 hr incubation at

$37^{\circ}C$ in an atmosphere of 5% CO_2 in air, the agar-overlay

was removed and the plaques counted.

Isotopes and Inhibitors

$[^3H]$ L-leucine (S.A. 1000 mc/m moles), $[^3H]$ cytidine

(S.A. 1000 mc/m moles), and $[^3H]$ 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 $HClO_4$ was added to each

bottle and the acid-precipitated cells stored at $-25^{\circ}C$

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 $HClO_4$ and like

suspensions pooled. After collection of cell pellets by

centrifugation they were extracted with cold 0.5 N HClO₄
for 30 min. and the acid-insoluable fraction was obtained
by centrifugation. Two additional cold acid extractions
of 10 minutes each were carried out. The acid-insoluable
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 HClO₄ and the supernatant was used
for ribonucleic acid (RNA) analysis (Mejbaum, 1939). The
DNA was removed by extracting twice with acid (0.5 N HClO₄)
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,
[³H] thymidine, [³H] cytidine, and [³H] 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 μg./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 μg. 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 $37^{o}C$ for 1 hr, the cultures were washed, covered with MEM medium containing either 0, 5, 10, or 20 μg./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 ($\mu g./ml.$)	Titers of IBR isolates ($p.f.u./ml.$)				
	1	2	3	4	5
0	1.1×10^7	1.0×10^7	2.3×10^8	7.4×10^6	1.8×10^7
5	6.1×10^4	3.4×10^4	4.5×10^7	4.0×10^3	7.2×10^5
10	1.0×10^4	2.2×10^2	2.6×10^7	1.6×10^3	8.9×10^4
20	0	0	1.8×10^6	0	1.1×10^3

TABLE 2

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

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

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 $[^3H]$ 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, $[^3H]$ 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 $[^3H]$ L-leucine incorporation in control and IBT-treated cultures. However, the differences that occurred were relatively small.

When $[^3H]$ 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 $[^3H]$ cytidine incorporation at an early stage (4-5 hr) of IBR infection. In similar cultures, incorporation of $[^3H]$ cytidine was decreased

FIG. 1. Rate of $[^3H]$ 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 µg./ml.) treated cells and solid bars
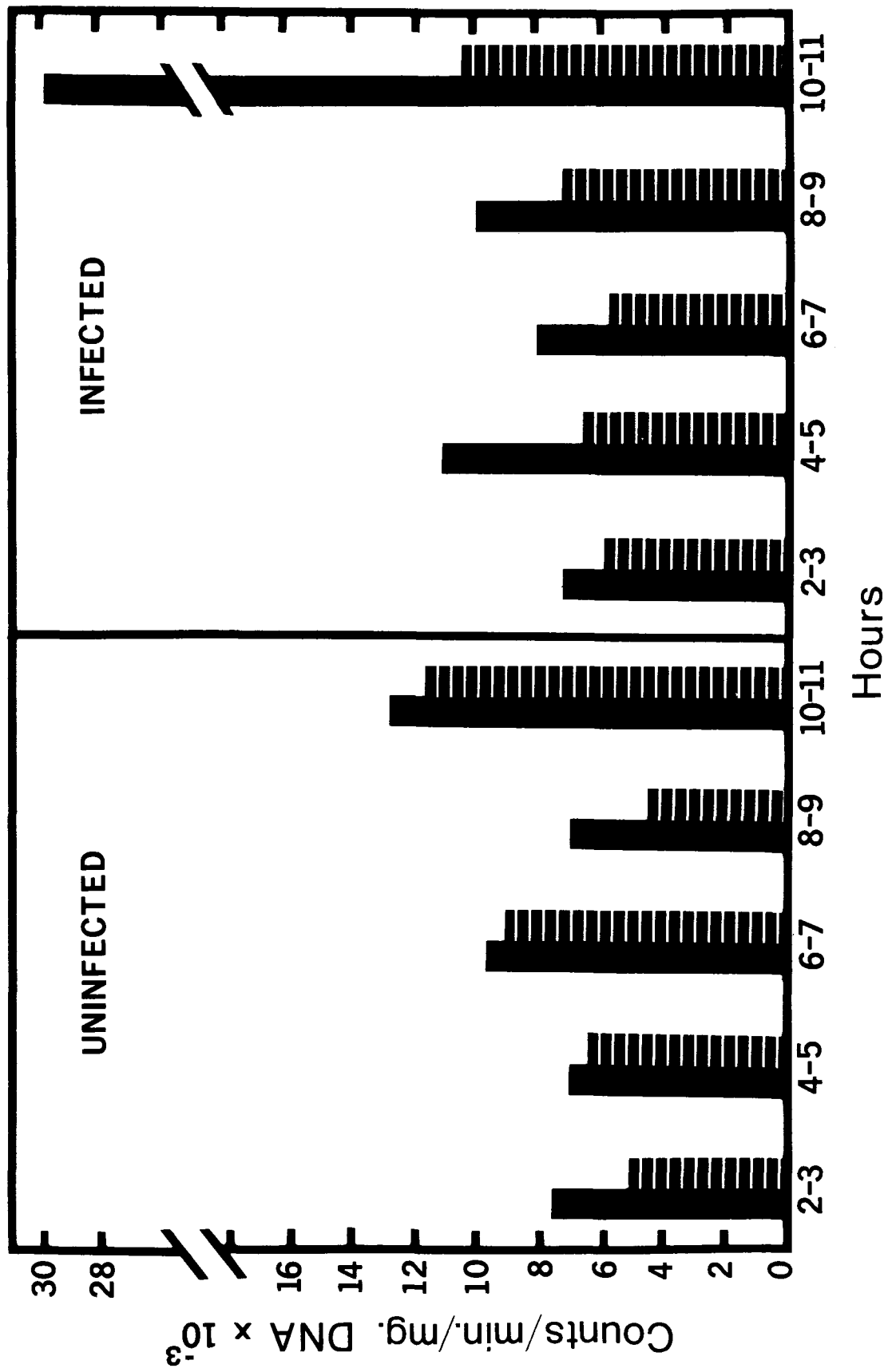represent untreated cells.

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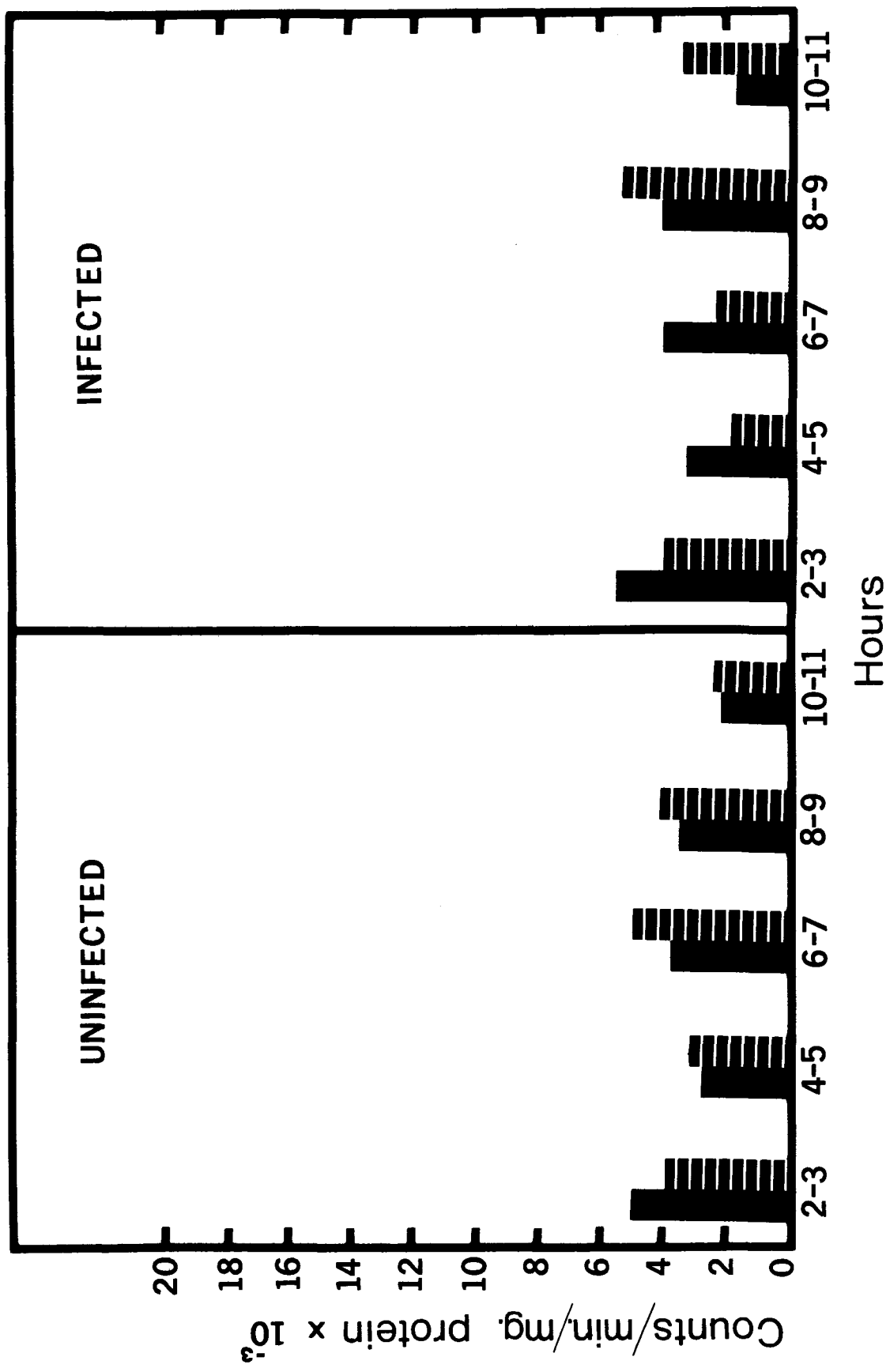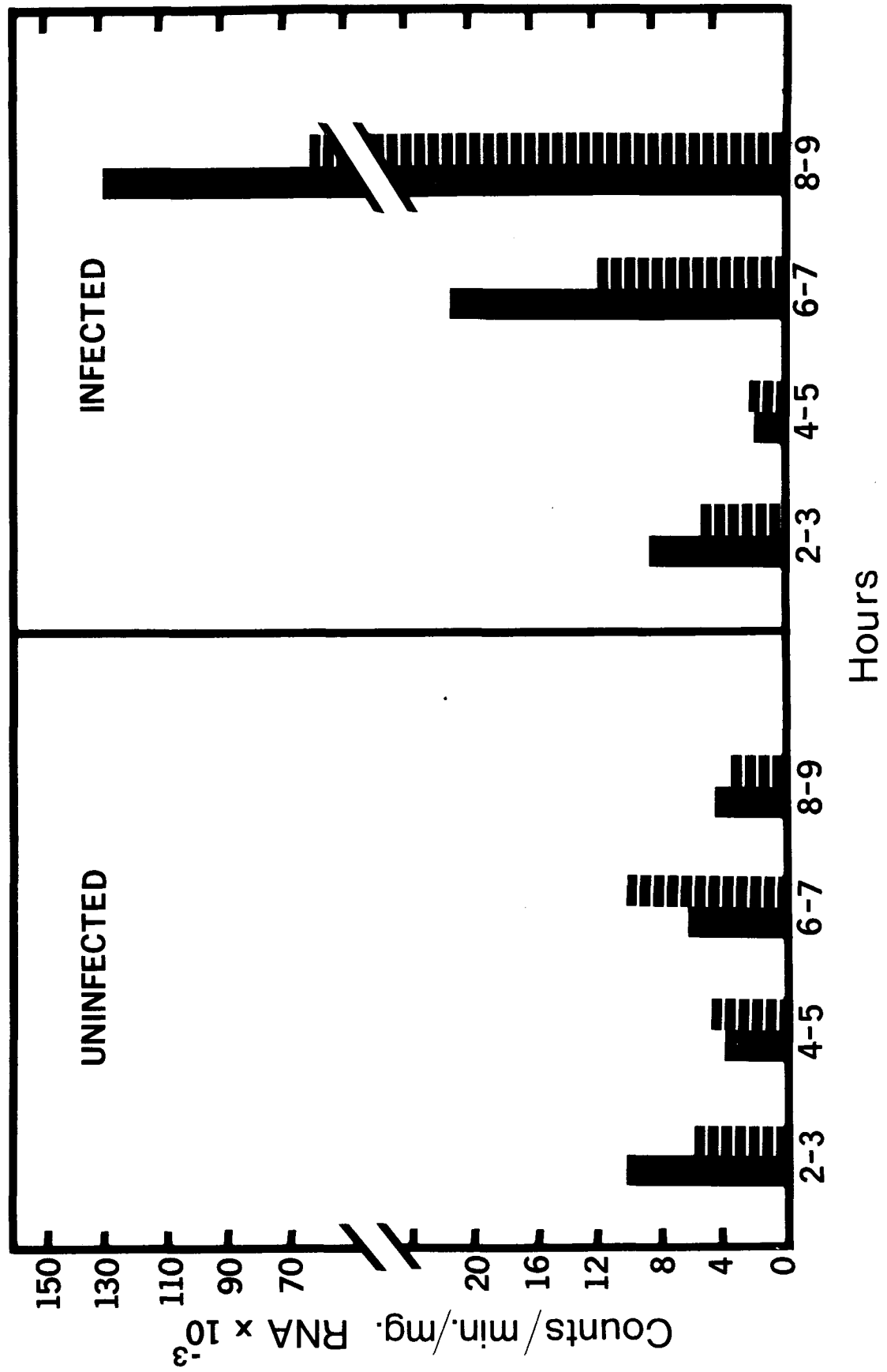presence and absence of IBT. Legend as for
Fig. 1.

FIG. 3. Rate of $[^3H]$ 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.
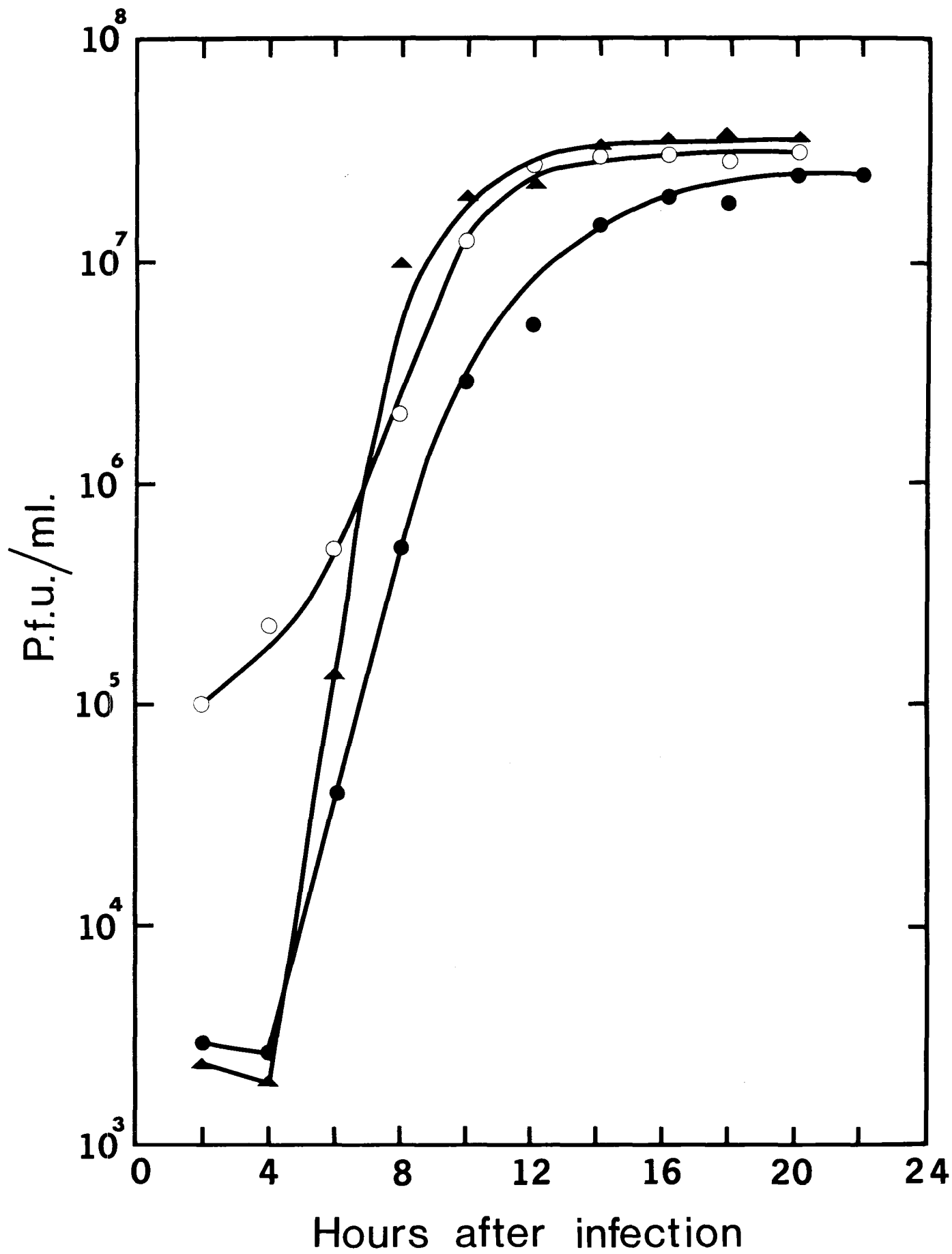
Inhibition of IBR virus multiplication by addition of IBT
at various times after infection

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.
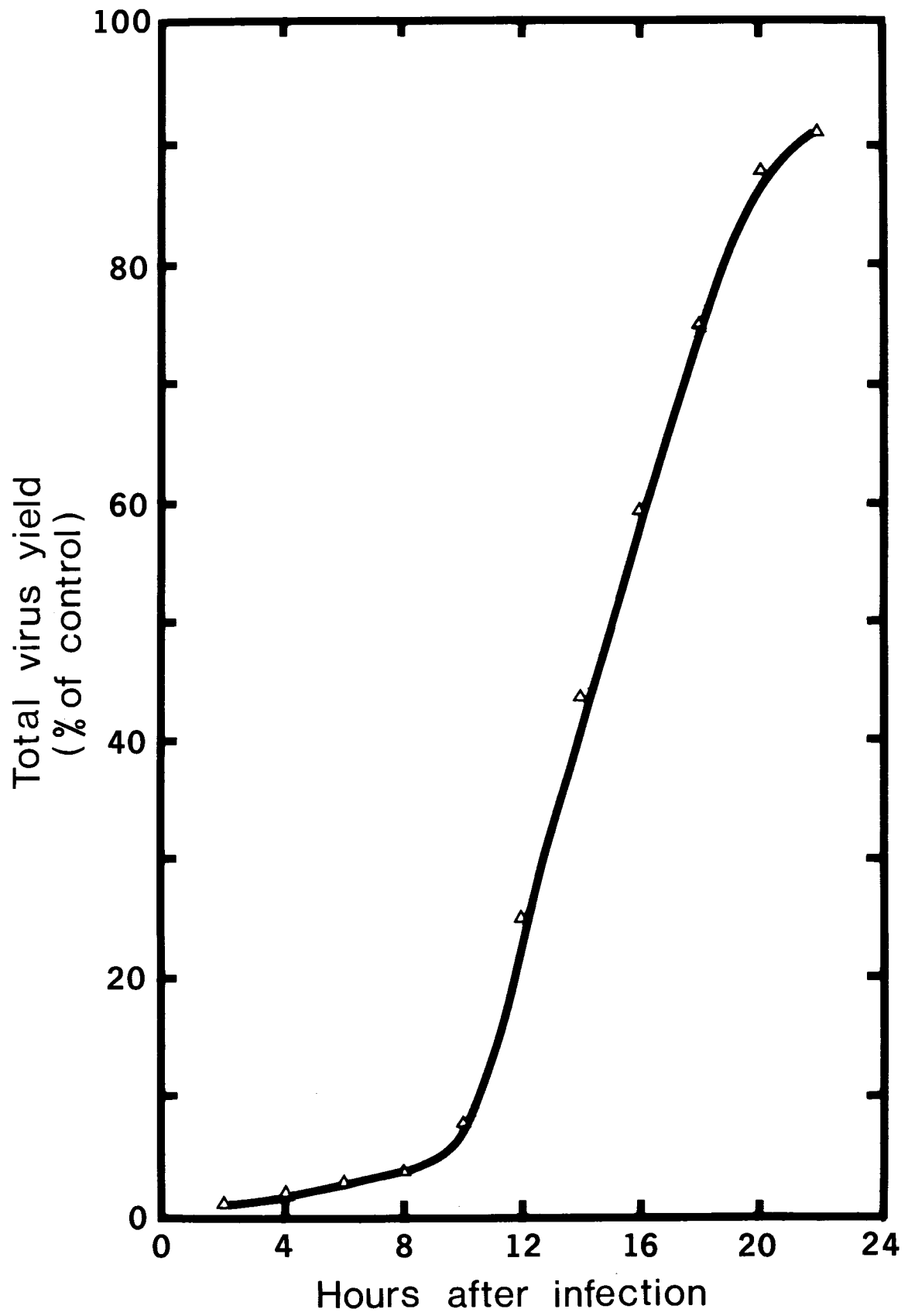
DISCUSSION

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 $[^3H]$ 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 µg./ml. reduced the incorporation of $[^3H]$ 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 μg./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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