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The antiviral activity of 9-β-D-arabinofuranosyl adenine against vesicular stomatitis virus

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THE ANTIVIRAL ACTIVITY OF 9-β-D-ARABINOFURANOSYL ADENINE AGAINST VESICULAR STOMATITIS VIRUS

BY

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ABSTRACT

Previously, Ara-A has been found to be an effective antiviral agent against DNA virus infections. In this study, antiviral activity was shown against an RNA virus, vesicular stomatitis virus. The inhibitory effect of Ara-A was found to occur early during virus replication. Cycloheximide, puromycin, and radioisotope incorporation experiments showed that Ara-A seems to stimulate protein synthesis while depressing RNA synthesis. The exact mode of action of Ara-A remains unclear, however some speculation concerning this problem is reported herein.

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I wish to extend my thanks to Dr. L. R. Sabina, Department of Biology, University of Windsor, for his continued guidance, both personal and professional, throughout the course of this research and the writing of this thesis.

For their aid in reviewing this thesis, I am grateful to Dr. W. S. Cozine and Dr. A. H. Warner, both of the Department of Biology and to Dr. D. E. Schmidt, Department of Chemistry, all at the University of Windsor.

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I particularly wish to thank my wife Jane and my son Michael for their support and encouragement under often strained circumstances.

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INTRODUCTION

Although Lee *et al.* (1960) first synthesized 9-β-D-arabinofuranosyl adenine (Ara-A) with the objective of evaluating its effectiveness as an anticancer drug, subsequent studies have shown this drug to be a potentially useful antiviral. Privat de Garilhe and de Rudder (1964) found that Ara-A inhibited the production of infectious herpes simplex and vaccinia viruses in cell culture. The antiviral activity of Ara-A *in vitro* against vaccinia virus was confirmed by Freeman *et al.* (1965). Studies by Sidwell and co-workers (1967,1968) extended the range of antiviral activity of Ara-A in cell culture to include the cytomegaloviruses as well as experimental vaccinia virus infections in mice.

The expense of synthesis of Ara-A prevented extensive experimentation (Cohen, 1966) until this compound was isolated from a *Streptomyces antibioticus* (NRRL 3238) fermentation by Parke, Davis and Co. (1968). Thereafter, *in vivo* experimentation for evaluating the therapeutic effect of Ara-A was possible. Therapeutic activity of Ara-A against experimental keratitis (Sidwell *et al.*, 1969) and encephalitis (Schardein

and Sidwell, 1969) infections induced with herpes simplex virus in hamsters has been demonstrated. Sloan et al. (1969) employed a wide range of dose levels and routes of administration of Ara-A in mice infected intracerebrally with herpes simplex and observed that Ara-A was highly therapeutic and only slightly toxic regardless of the route of administration of the drug.

Miller et al. (1969) have carried out extensive screening of Ara-A against numerous DNA and RNA viruses in vitro. Their findings indicated the restriction of Ara-A inhibition to DNA virus infections. The only exception to their observations has been the controversial inhibition of Rous sarcoma virus specific foci. Freeman et al. (1965) reported no inhibition of Rous sarcoma virus, whereas Miller et. al. (1969) found Rous sarcoma foci to be inhibited by Ara-A. Inhibition of Rous sarcoma virus by Ara-A might be explained on the basis of the DNA requirement of this virus during replication (Bader, 1964,1966ab; Temin, 1964).

The present study was undertaken as a result of the finding that Ara-A inhibits the production of infectious vesicular stomatitis virus (an RNA virus)

grown in bovine kidney cell cultures.

Vesicular stomatitis virus (VSV) is a complex bullet-shaped virus about 68 x 175 nm in size (Howatson and Whitmore, 1962) which causes a serious disease in cattle and lacks a DNA requirement during its replicative cycle *in vitro* (Chamsey *et al.*, 1963 and Prevec *et al.*, 1963). Bradish and Kirkham (1966) have described the ultrastructure of VSV as consisting of four parts; a fringe of surface elements, a dense limiting envelope, an helical shell of capsomeres, and core material, with or without an axial hollow. In addition, they found that VSV generally exists in two states; bullet-shaped infectious particles (B) and truncated non-infectious (T) forms.

The nucleic acid core of VSV was found to be single stranded RNA having a molecular weight of 3×10^6 daltons (Brown *et al.*, 1967). Subsequent studies by Nakai and Howatson (1968) showed that the intact virion core existed as a ribbon-like strand of a series of regular rod-like subunits having an overall helical configuration.

Recently, Stampfer *et al.* (1969) demonstrated the complexity of VSV replication by isolating at least nine different species of virus-specific RNA

from infected cell cultures. Of these species, some were double stranded, some partially double stranded and some single stranded. When intact B and T particles were compared, it was found that B particles contained single stranded RNA sedimenting at 40S while T particles carried single stranded RNA sedimenting at 19S. Furthermore, Baltimore et al. (1970) have shown that VSV carries an RNA polymerase in the intact virion which is capable of synthesizing messenger RNA directly from the intact nucleocapsid as template without removal of protein (Baltimore, 1970).

Evidence presented here suggests a unique mode of action of Ara-A against an RNA virus (VSV) which appears to lack a DNA requirement during replication in a cell line more closely related to the natural host of the virus than those commonly employed by other workers.

MATERIALS AND METHODS

CELLS AND MEDIA

The MDBK bovine kidney cell line (Madin and Darby, 1958) used in these experiments was purchased from Baltimore Biological Laboratory, Baltimore, Maryland. Monolayer cultures were grown and maintained in medium ELay-10 FCS as previously described (Sabina and Parker, 1963). For experimental purposes, Eagle's minimum essential medium (MEM) (Eagle, 1959) was employed. All culture media were sterilized by passage through 0.2u porosity Cox filter membranes.

VIRUS

The Indiana serotype of vesicular stomatitis virus (VSV) used in this study was supplied by Dr. N.A. Labzoffsky, Ontario Department of Health, Toronto, Ontario. Stock virus was prepared by infecting MDBK monolayers with an exposure multiplicity of ca. 2 plaque forming units (p.f.u.) per cell. After adsorption for 1 hour at 37°C, medium ELay-10 FCS was added and cultures reincubated. At 24 hours postinfection, when the cytopathic effect was complete, all samples were subjected to three cycles of freezing and thawing

to release intracellular infectious virus. These suspensions were then centrifuged at 2000 r.p.m. for 20 minutes in a Sorvall SS-34 angle head rotor. The resulting infectious supernates were pooled and stored at -60°C. Titers of virus stocks ranged from 2.9×10^6 to 7.5×10^7 p.f.u./ml.

INFECTION OF CELLS

MDBK monolayers grown in 1 oz. Brockway bottles were generally used after 48 hours incubation at 37°C. The medium was poured off and virus added at an infecting multiplicity of ca. 15 unless otherwise stated. Adsorption was allowed to proceed for 1 hour at 37°C with occasional agitation. Thereafter, residual virus innoculum was removed and the cultures rinsed with Hank's Balanced Salt Solution (HBSS) prior to the addition of medium MEM or MEM containing test compounds.

Control cell monolayers were handled in a similar fashion but received only MEM in place of virus innoculum. Unless otherwise indicated, all experiments were carried out once using duplicate cultures and held at -60°C until assayed for infectivity or radioactivity.

VIRUS ASSAY

Infectivity was estimated by plaque formation in MDBK cell monolayers grown in 60-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, California). Replicate harvests of infectious virus from infected cultures were obtained in a similar fashion to that described under VIRUS. Log dilutions of the samples were made in medium MEM and 0.2 ml aliquots of appropriate dilutions were added to duplicate MDBK monolayers. After 1 hour at 37°C the cultures were overlaid with 5 ml of agar medium (Sabina and Munro, 1969). All dishes were then incubated at 37°C in a water saturated atmosphere of 5% CO_2 in air. The plaques were counted after approximately 24 hours.

ISOTOPES AND INHIBITORS

^{3}H-L-valine (specific activity, 1.5 Ci/mM), ^{3}H-L-leucine (specific activity, 1 Ci/mM) and ^{3}H-uridine (specific activity, 5.0 Ci/mM) were purchased from Amersham/Searle, Des Plaines, Illinois; cycloheximide (Acti-Dione) and puromycin dihydrochloride from Nutritional Biochemical Corp., Cleveland, Ohio; and actinomycin D from Sigma Chemical Co., St. Louis, Missouri. The 9-β-D-arabinofuranosyl adenine (micronized)

was made available by Dr. R. Brackett, Parke, Davis and Co., Detroit, Michigan.

Stock solutions of all chemicals were made in MEM just prior to use, except actinomycin D and Ara-A which were initially solubilized in ethanol and dimethyl sulphoxide (DMSO), respectively.

RADIOISOTOPE INCORPORATION STUDIES

VSV-infected monolayers were prepared as described under INFECTION OF CELLS. Radioactive precursors were added to monolayers at various times after infection for 0.5 or 1 hour pulses. Incorporation of radioisotopes into cells was stopped by pouring off the medium and washing twice with ice-cold HBSS. Cold 0.5 N perchloric acid (PCA) was added to the washed monolayers for storage at -20^{o}C until extraction procedures for RNA or protein were carried out. Uninfected cultures were treated similarly.

EXTRACTION AND DETERMINATION OF PROTEIN AND RNA

The extraction procedure for protein and RNA was carried out as follows. Frozen cell monolayers which had been exposed to radioactive precursors were thawed at room temperature and the acid-precipitated cells

scraped from the glass. Cell suspensions were transferred to centrifuge tubes and each culture bottle rinsed with cold 0.5 N PCA. Like suspensions were pooled and centrifuged. Each cell pellet was washed once with cold 0.5 N PCA and dissolved in 0.5 N NaOH. Separate aliquots of this solution were taken for radioactivity and protein (Lowry et al., 1951) determinations.

For RNA determinations, the alkaline samples were incubated at 50°C for 20 minutes (Munro and Fleck, 1966) and then reacidified to 0.5 N with respect to perchloric acid. After centrifugation, aliquants of the acid soluble fractions were removed for radioactivity and RNA (Merchant et al., 1960) determinations.

RADIOACTIVITY ASSAY

Radioactivity was determined by the addition of samples to scintillation fluid (Warner and McClean, 1968). Counts were obtained in a Nuclear Chicago liquid scintillation counter, using either model 4643 or 6850. Specific activities are reported as counts per minute per milligram (c.p.m./mg) of RNA or protein.

RESULTS

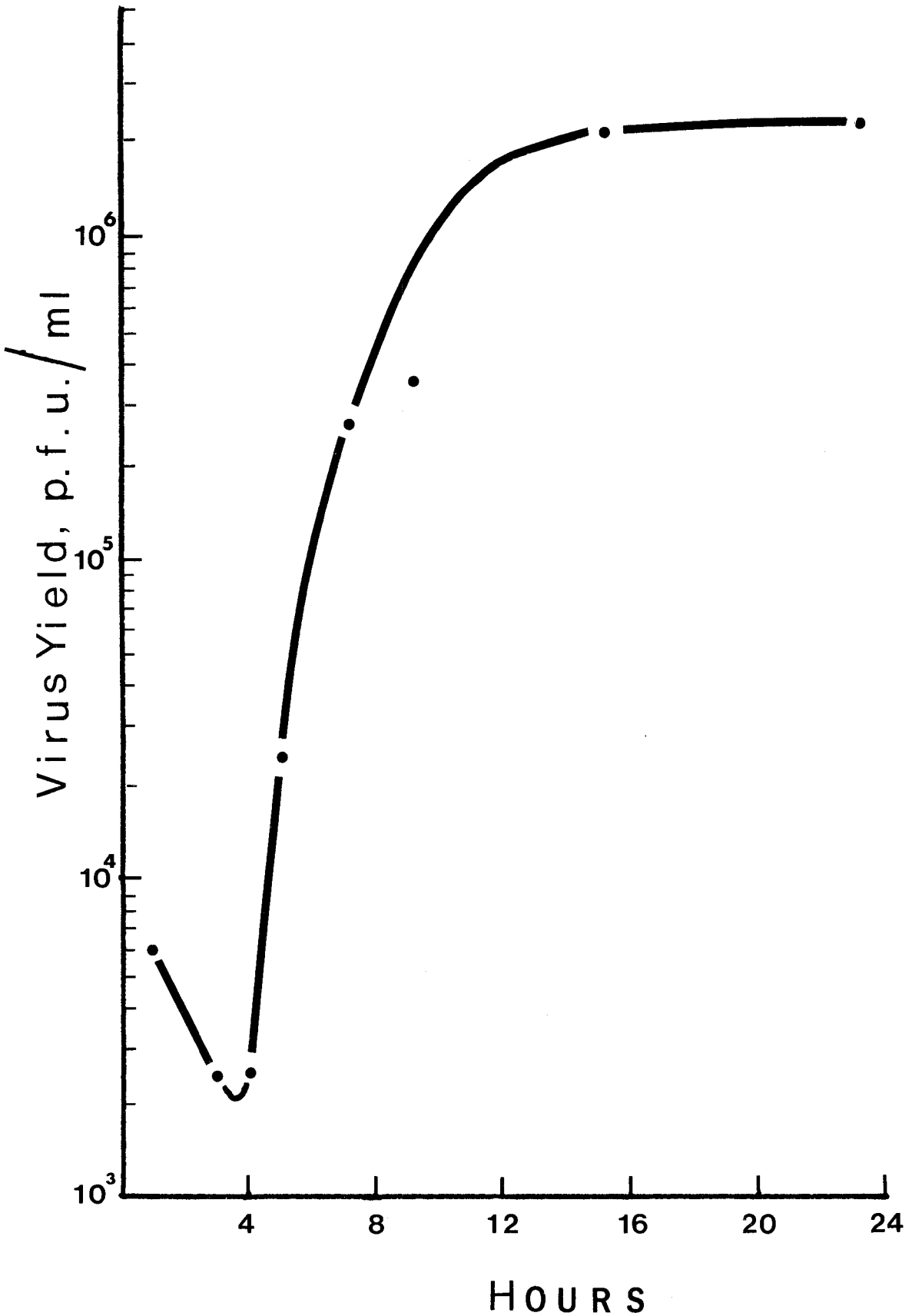
MULTIPLICATION OF VSV

In order to establish the time-course of VSV replication in MDBK cell monolayers, replicate infected cultures were removed at various times after infection and subsequently assayed for infectious virus. Figure 1 illustrates the rapid replication of VSV which reaches a maximum yield at about 16 hours postinfection. The latent phase is short and the duration of the logarithmic growth phase is about 6 hours.

EFFECT OF ARA-A ON UNINFECTED MDBK CELLS

The effect of Ara-A on the survival of MDBK cells was determined by setting up a series of cultures with different concentrations of the drug in medium MEM. Following 24 hours of incubation at 37°C, cultures were checked for gross cytotoxicity at a magnification of 400x using a Leitz inverted microscope. There was no evidence of cytotoxicity in cultures exposed to Ara-A in the range of 50 to 150 ug/ml when compared to untreated control cultures. However, in cultures exposed to 200 ug of Ara-A/ml, extensive rounding of

Fig. 1. Multiplication of vesicular stomatitis virus in MDBK cells. Duplicate infected cultures were sampled at the indicated times. Infectious virus yields are expressed as plaque forming units (p.f.u.)/ml.



cells and some free floating cells were observed.

EFFECT OF DMSO ON MDBK CELLS

Since DMSO has been shown to be deleterious to certain cell lines and viruses (Chang and Simon, 1968), it was important to establish its effect on MDBK cells and VSV replication. Replicate uninfected and VSV infected MDBK monolayers were exposed to varying concentrations of DMSO and allowed to incubate at 37°C until 24 hours postinfection. Similar cultures were observed in the absence of DMSO.

Microscopic examination of uninfected cultures with and without DMSO gave very similar appearances on the basis of gross morphology at a magnification of 400x. The titers of infected cultures are shown in Table 1. It appears that the highest concentration of DMSO tested (0.6%) did not result in any decrease in virus titer. The final concentration of DMSO used in the following experiments never exceeded 0.6%.

EFFECT OF ARA-A ON VSV INFECTIVITY

To evaluate the antiviral activity of Ara-A against VSV, the drug was applied to replicate infected cultures at levels of 50, 100 and 150 ug/ml. After 24 hours

Table 1.

The effect of different concentrations of DMSO on final infectious yields of VSV grown in MDBK cells.*

Concentration of DMSO (%)	Viral Yields (p.f.u./ml)
0	2.2×10^6
0.2	2.0×10^6
0.6	3.0×10^6

* Indicated DMSO concentrations were added after virus adsorption and samples titrated at 24 hours postinfection.

of incubation at 37°C, infectious titers were determined and per cent plaque reduction values calculated.

It can be seen (Figure 2) that per cent plaque reduction rises almost geometrically as the concentration of Ara-A increases, indicating a typical dose-response relationship. All further studies in the presence of Ara-A were carried out at a final drug concentration of 150 ug/ml.

EFFECT OF MULTIPLICITY OF INFECTION ON VIRAL YIELDS AND ARA-A INHIBITION

Because multiplicity of infection has been shown to affect final yields of infectious virus (Stampfer et al., 1969), an experiment was carried out to establish the optimal multiplicity of infection in this test system.

Table 2 shows that maximal yields of VSV in the absence of Ara-A were obtained at a multiplicity of 5.0. However, the inclusion of Ara-A resulted in a bicyclic pattern of inhibition. In order to evaluate the antiviral activity of Ara-A, it was pertinent to have the entire cell population infected, therefore, all subsequent experiments were begun with an infecting multiplicity of ca. 15.

Fig. 2. The effect of different concentrations of Ara-A on final yields of infectious VSV. Cultures were exposed to different concentrations of Ara-A as indicated after adsorption. Control cultures were incubated in MEM. Samples were titrated at 24 hours postinfection and per cent inhibition values calculated. Fig. 2 presents average per cent inhibition values from two experiments.

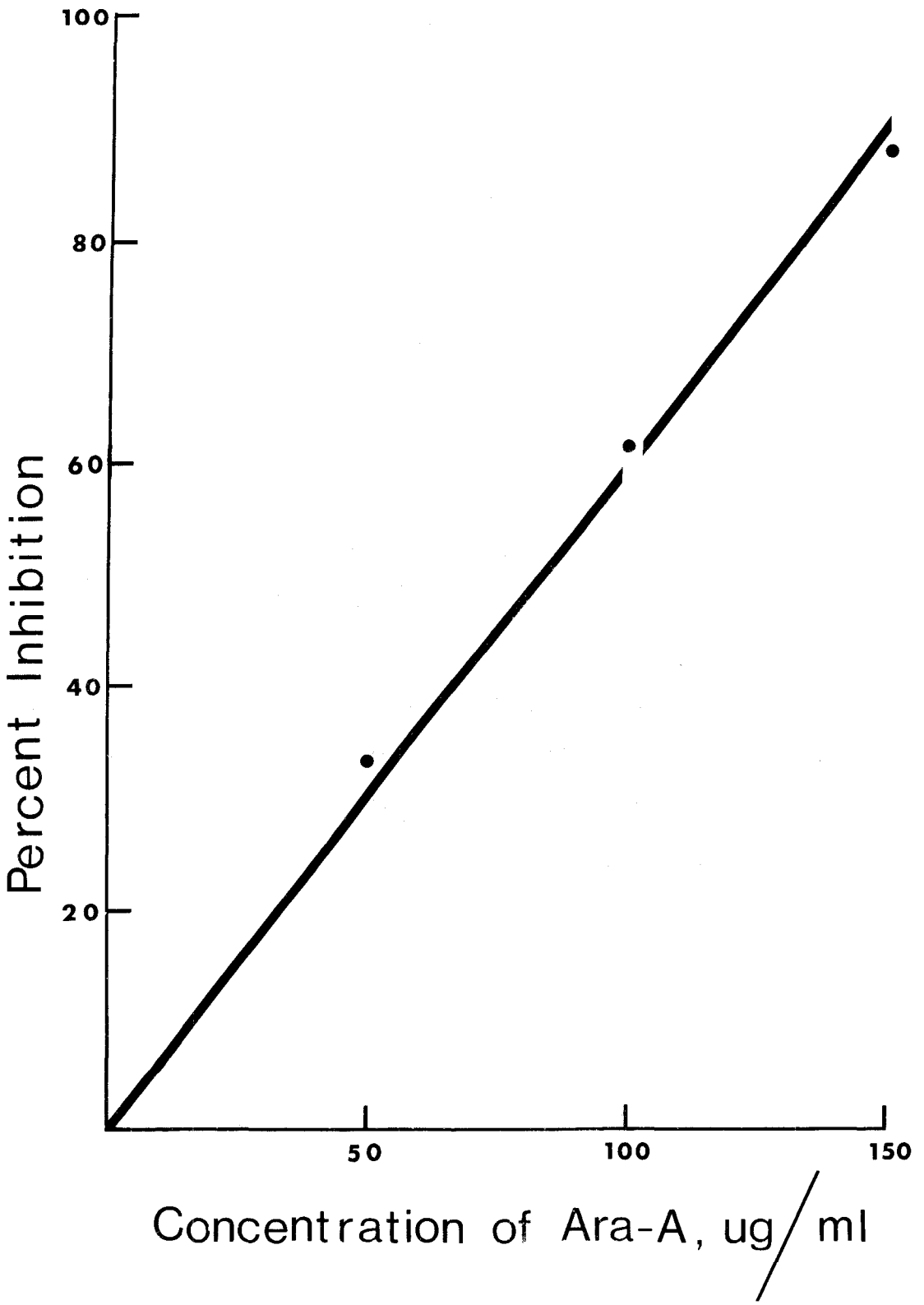


Table 2.

The effect of multiplicity of infection on viral yields and Ara-A inhibition.*

Multiplicity of infection (p.f.u./cell)	Virus Yield (p.f.u./ml)	
	MEM	MEM + Ara-A
0.01	5.7×10^5	1.0×10^5
0.1	4.0×10^6	6.0×10^5
1.0	5.2×10^6	3.8×10^6
3.4	4.4×10^6	1.7×10^6
5.0	8.7×10^6	2.2×10^6
15.0	8.7×10^6	1.0×10^6

* Duplicate cultures were exposed to the indicated infecting multiplicities. Ara-A (150 ug/ml), when present, was added after adsorption. Virus yields were determined at 24 hours postinfection.

MULTIPLICATION OF VSV IN THE PRESENCE OF ARA-A

To obtain information about the inhibitory effect of Ara-A upon VSV replication in MDBK cells, an experiment was performed similar to that described for the infectivity curve of VSV except that Ara-A was incorporated into medium MEM. The experimental results are shown in Figure 3.

Comparison of the infectivity curves of untreated cultures (Figure 1) and Ara-A treated cultures (Figure 3) showed that Ara-A caused a rapid drop in virus yield beginning about 9 hours postinfection. With untreated cultures, the exponential rate of virus multiplication was extended until maximal steady levels were reached after 12 hours of infection.

ADDITION OF ARA-A TO INFECTED CELLS

Further definition of the period of Ara-A activity in MDBK infected cultures was studied by adding the drug at different intervals after infection for varying periods of time. Control infected cultures were allowed to incubate in medium MEM for the 24 hour test period. Infectious titers of the samples were determined and expressed as the per cent of the 24 hour control culture (Figure 4).

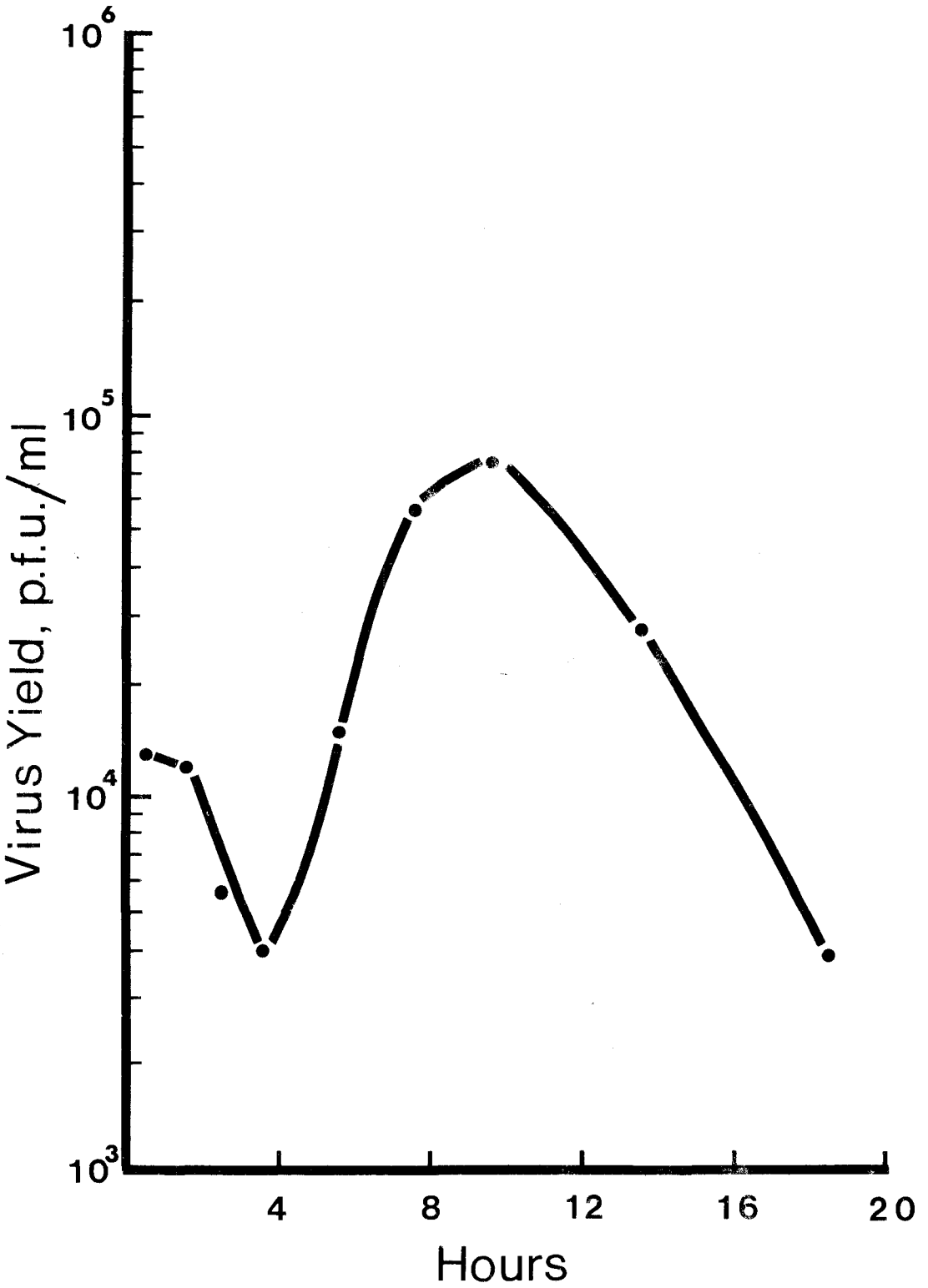
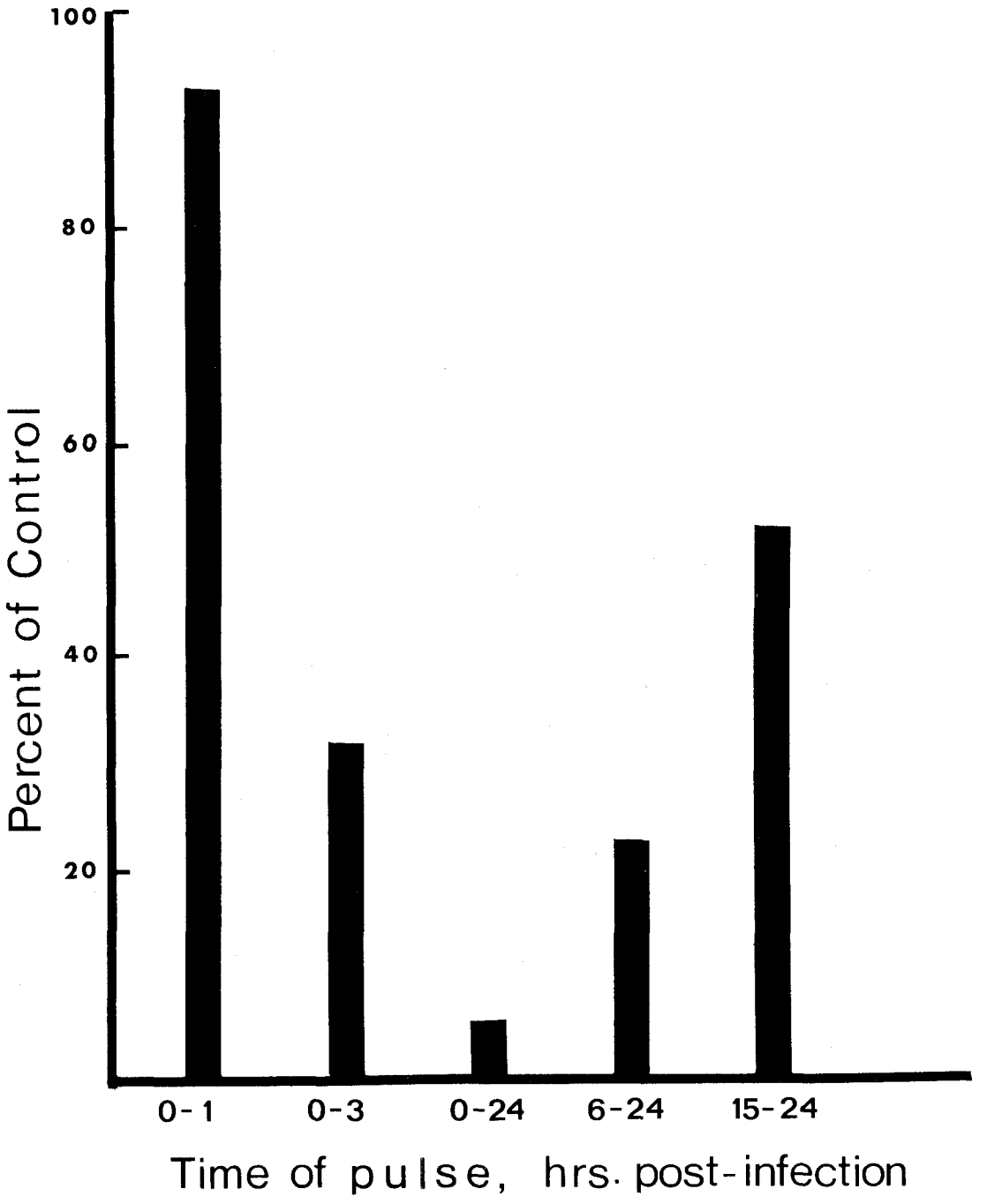


Fig. 4. Effect on infectious yields of pulsing MDBK cells with Ara-A. Ara-A (150 ug/ml) was added to duplicate cultures for the indicated time periods. Control infected cultures were incubated in MEM. All samples were allowed to incubate for 24 hours. When MEM containing Ara-A was removed, the cultures involved were washed twice with HBSS prior to the addition of MEM alone for reincubation.

Fig. 3. Multiplication of VSV in the presence of Ara-A. Legend as for Fig. 1 except that Ara-A (150 ug/ml) was added to all cultures at the time of infection.



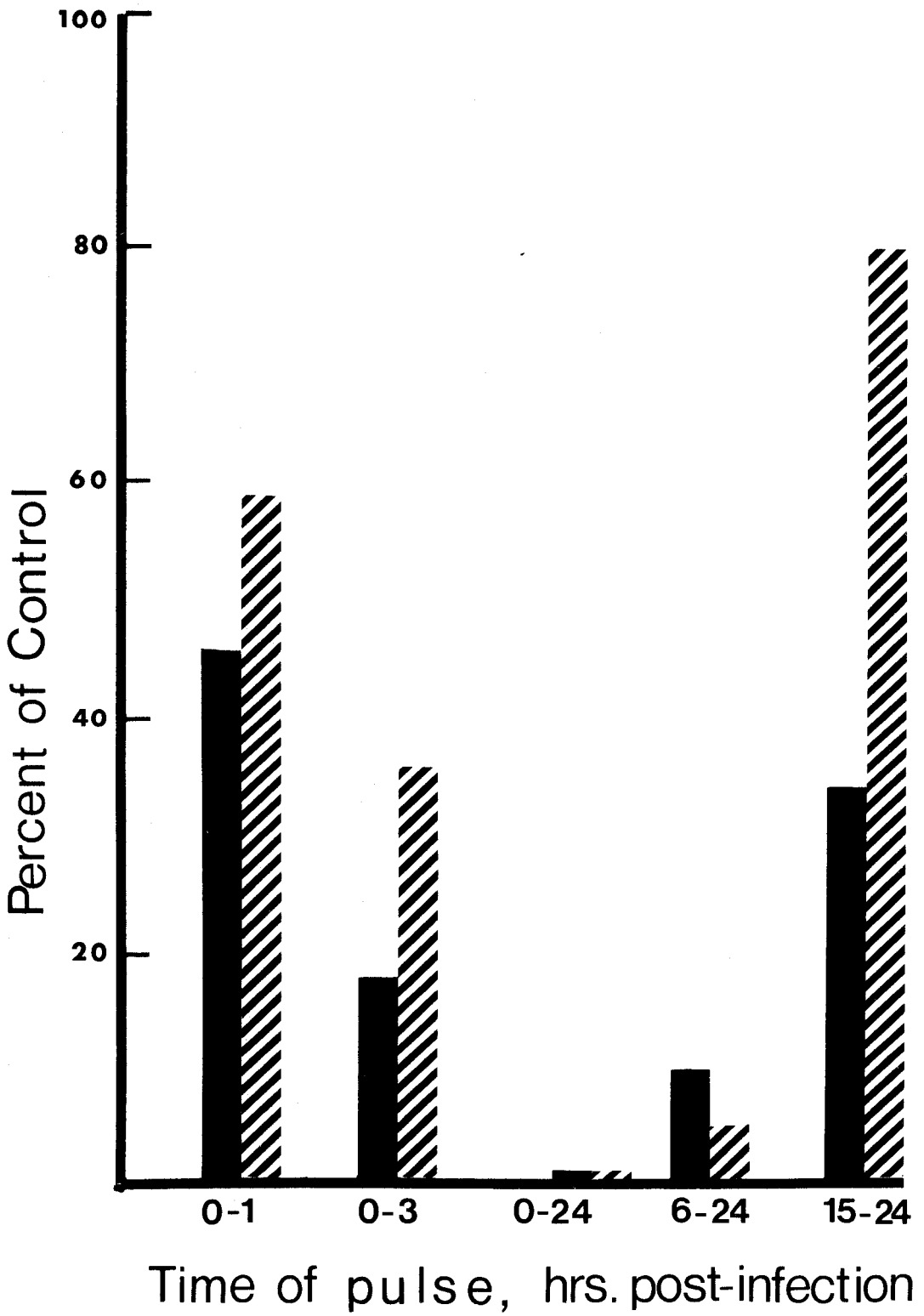
The addition of Ara-A during the first 3 hours of infection resulted in about 70% inhibition whereas only a slight increase in inhibitory activity was evident when the drug was present from 6 to 24 hours postinfection. It is also clear that the presence of Ara-A during the first hour of infection did not effectively inhibit virus development. These effects suggest that Ara-A is most active early during viral replication but after the first hour of infection.

ADDITION OF PUROMYCIN AND ARA-A TO INFECTED CELLS

To determine whether Ara-A inhibition was dependent on protein synthesis, puromycin at a final concentration of 20 ug/ml was added to infected monolayers in combination with and without Ara-A. Virus-infected control cultures were incubated in medium MEM.

As shown in Figure 5, when puromycin was used for the first 3 hours after infection, the presence of puromycin and Ara-A showed less inhibition than did puromycin alone. During the 6 to 24 hour and 0 to 24 hour periods, differences were relatively small. Apparently, Ara-A relieves puromycin inhibition rather than acting synergistically.

Fig. 5. The effect on infectious yields of pulsing monolayers with puromycin (20 ug/ml) and puromycin (20 ug/ml) plus Ara-A (150 ug/ml) for the indicated time periods. Conditions were similar to those for Fig. 4. Solid bars indicate the effect of puromycin alone. Cross-hatched bars indicate the effect of puromycin plus Ara-A.



ADDITION OF CYCLOHEXIMIDE AND ARA-A TO INFECTED CELLS

To ascertain the influence of a different protein inhibitor in combination with Ara-A on VSV-infected cells, cycloheximide at a level of 20 ug/ml was tested as described in the preceding experiment. Since cycloheximide is reported to act by binding only to eukaryotic ribosomes (Brock, 1970), it was expected that only cellular protein synthesis would be affected in this experiment. The results are illustrated in Figure 6. The difference in inhibition induced by cycloheximide and puromycin was found to be only 10 to 15%. Also, the addition of combined cycloheximide and Ara-A was considerably less inhibitory to virus production than cycloheximide alone.

INCORPORATION OF ^{3}H-L-VALINE INTO INFECTED AND UNINFECTED CELLS WITH AND WITHOUT ARA-A

Since Ara-A had been shown to relieve inhibition of VSV replication by the protein inhibitors tested, it was of interest to discover whether Ara-A stimulated protein synthesis. Replicate cultures were pulsed for 1 hour at various times after infection with ^{3}H-L-valine as described under RADIOISOTOPE INCORPORATION STUDIES.

Fig. 6. The effects of cycloheximide and cycloheximide plus Ara-A on VSV infectious yields. Legend as for Fig. 5 except cycloheximide (20 ug/ml) was substituted for puromycin.

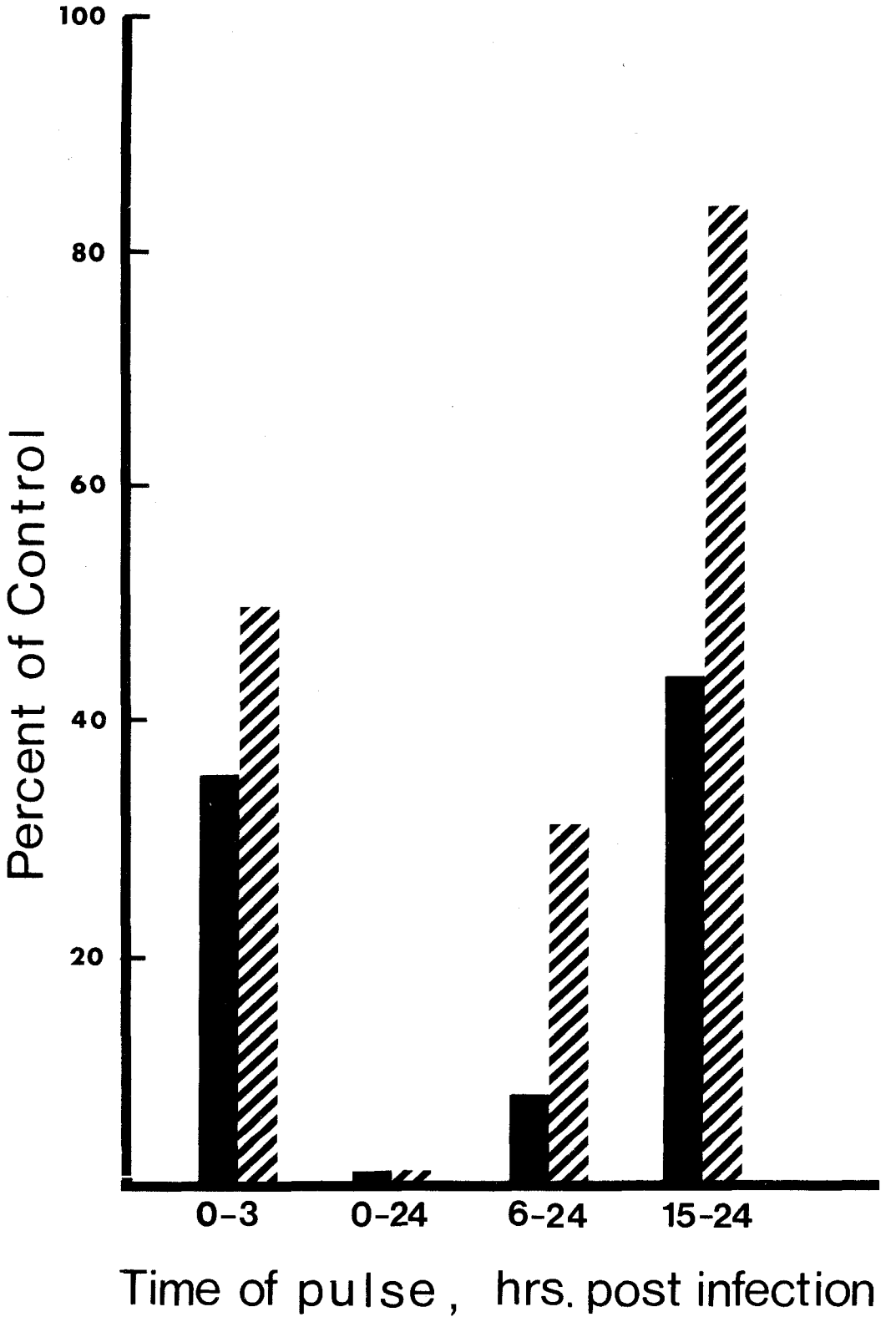
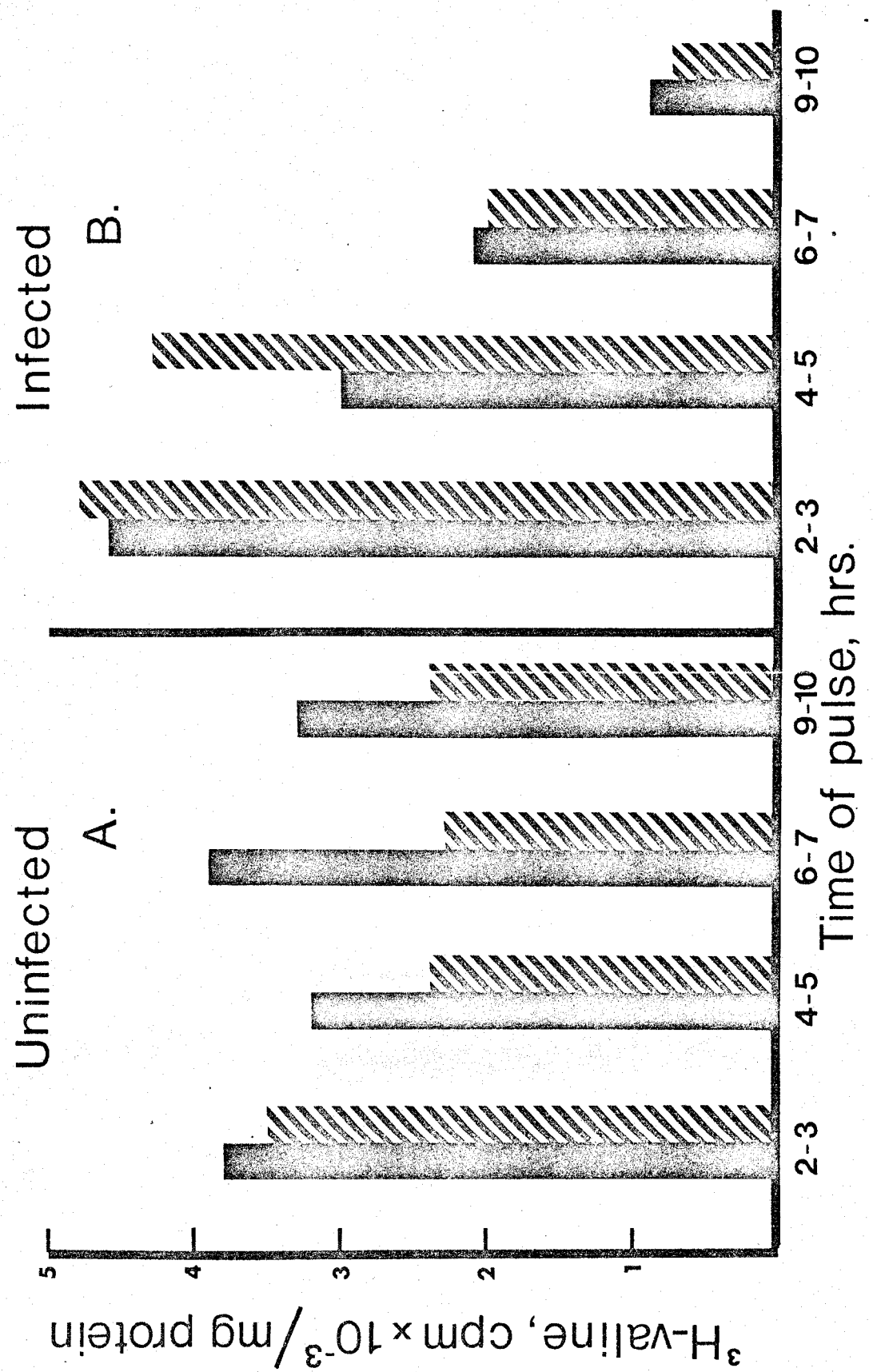


Figure 7A showed that Ara-A had a slight depressing effect on ^{3}H-valine incorporation into uninfected cultures. With virus-infected cultures (Figure 7B), the rate of incorporation of ^{3}H-valine into both Ara-A treated and untreated cultures was found to be gradually inhibited throughout the 10-hour test period. The magnitude of inhibition varied since cultures exposed to Ara-A up until 5 hours postinfection showed somewhat more radioactivity than untreated cultures at this time. Prolonged treatment with Ara-A caused similar inhibition of radioactivity incorporation compared to untreated cultures.

INCORPORATION OF ^{3}H-L-LEUCINE IN THE PRESENCE OF ACTINOMYCIN D INTO INFECTED AND UNINFECTED CELLS WITH AND WITHOUT ARA-A

In order to preclude masking of viral events by normal cellular protein synthesis, actinomycin D at a final concentration of 3 ug/ml was added to all cultures. In these experiments, duplicate infected cultures were exposed to ^{3}H-L-leucine for 0.5 hour pulses beginning at various times after infection. When Ara-A was present, the drug was added at the time of infection.

Fig. 7. ^{3}H-L-valine incorporation into uninfected (A) and infected (B) cells in the presence and absence of Ara-A. General conditions for pulsing with ^{3}H-L-valine (4 uCi/culture), harvesting, extraction and counting procedures were described in MATERIALS AND METHODS. Ara-A (150 ug/ml) when used, was applied at the time of infection. Solid bars indicate the absence of Ara-A, cross-hatched bars its presence.



The radioactivity incorporation profiles of uninfected cultures containing actinomycin D without and with Ara-A were essentially the same (Figure 8). When similarly treated infected cultures were compared, there was a greater rate of incorporation in Ara-A treated samples except during the 0.5 to 1 hour pulse (Figure 9). Although infected cultures with Ara-A incorporated ^{3}H-leucine at a greater rate, the pattern of incorporation was comparable to infected controls. Compared to the ^{3}H-valine experiment, the general trend of incorporation of ^{3}H-leucine after infection in the presence of actinomycin D was upward. This finding is consistent with the synthesis of viral directed proteins.

INCORPORATION OF ^{3}H-URIDINE IN THE PRESENCE OF ACTINOMYCIN D INTO INFECTED AND UNINFECTED CELLS WITH AND WITHOUT ARA-A

It was necessary to observe the influence of Ara-A on viral RNA synthesis during VSV replication. Therefore, an experiment similar to the preceding one was carried out using ^{3}H-uridine. When specific activities for uninfected cultures were obtained

Fig. 8. ^{3}H-L-leucine incorporation in the presence of actinomycin D into uninfected cultures without and with Ara-A. General conditions were as described for Fig. 7 except ^{3}H-L-leucine (6 uCi/culture) was substituted for ^{3}H-L-valine and actinomycin D (3 ug/ml) was present in all cultures.

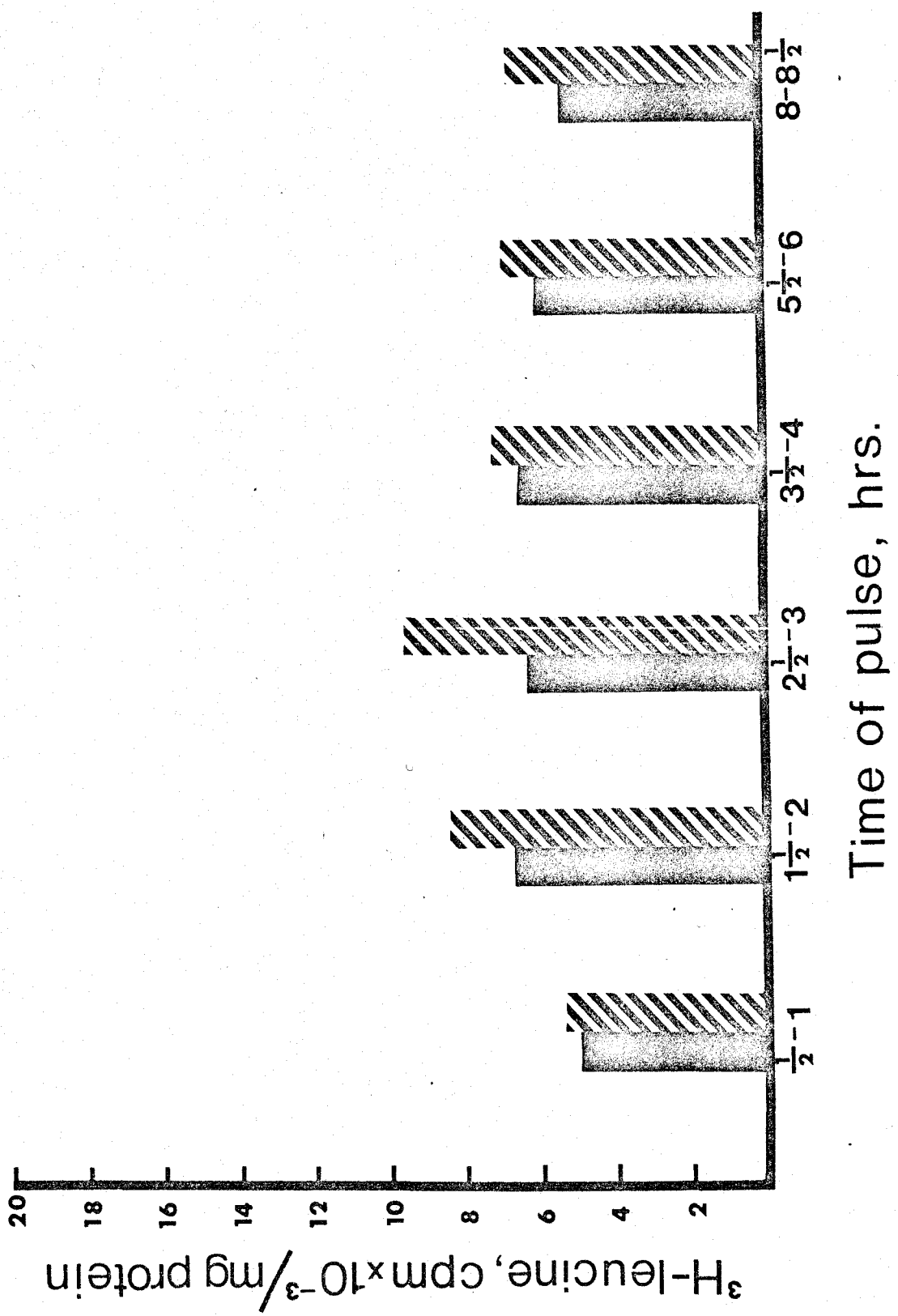


Fig. 9. ^{3}H-L-leucine incorporation in the presence of actinomycin D into infected cells without and with Ara-A. Legend as for Fig. 8 except infected cultures were used.

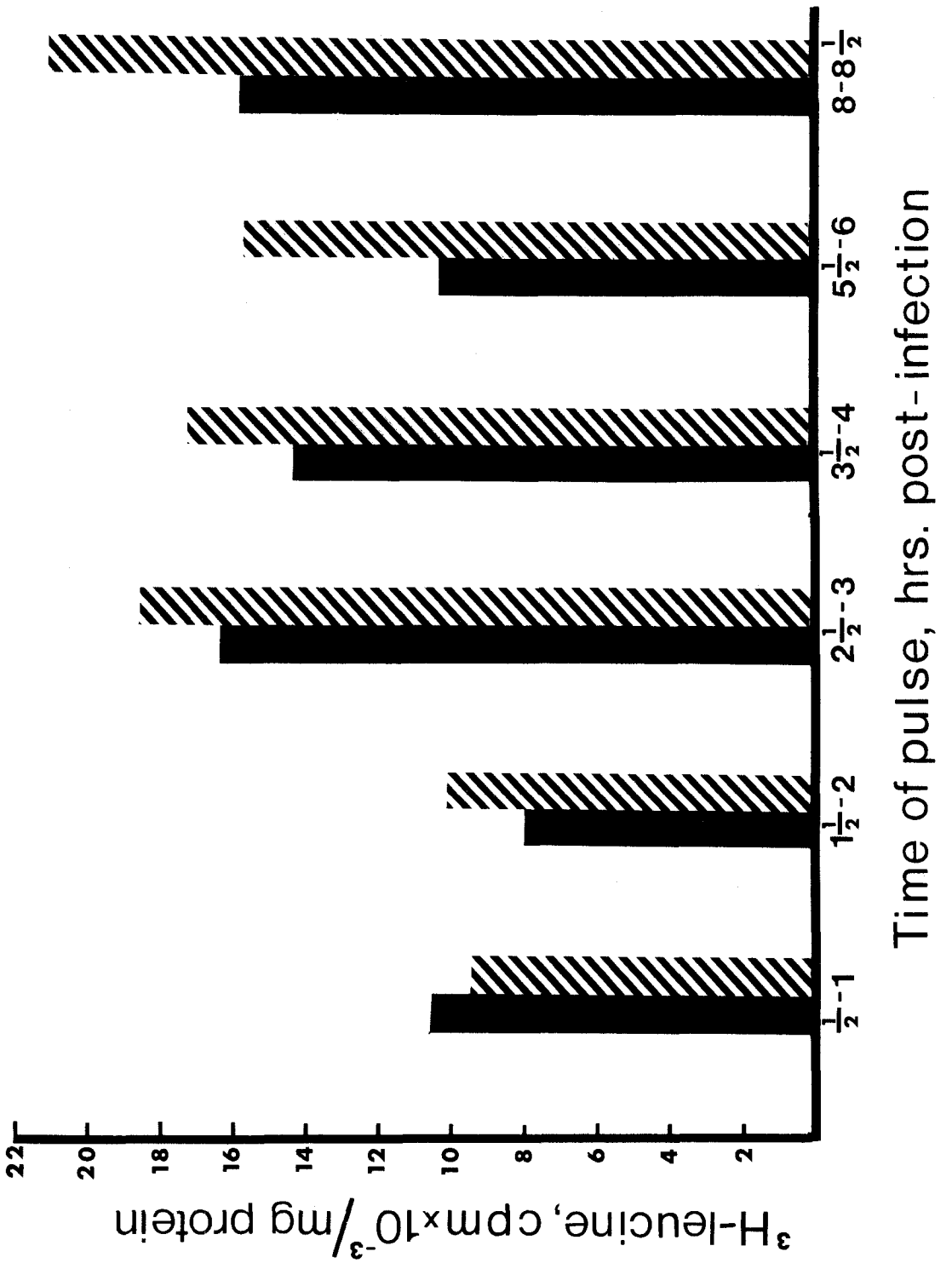
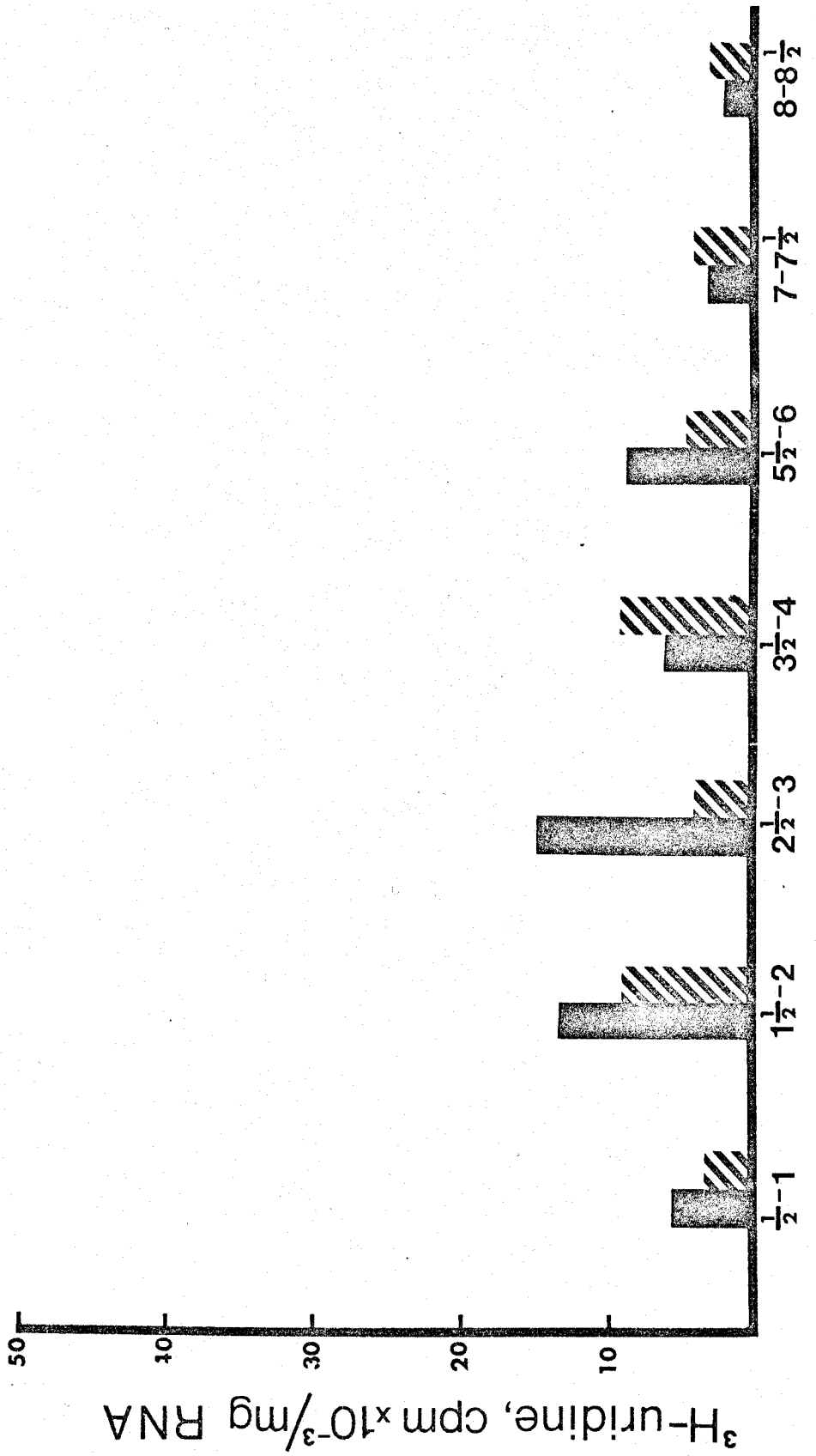


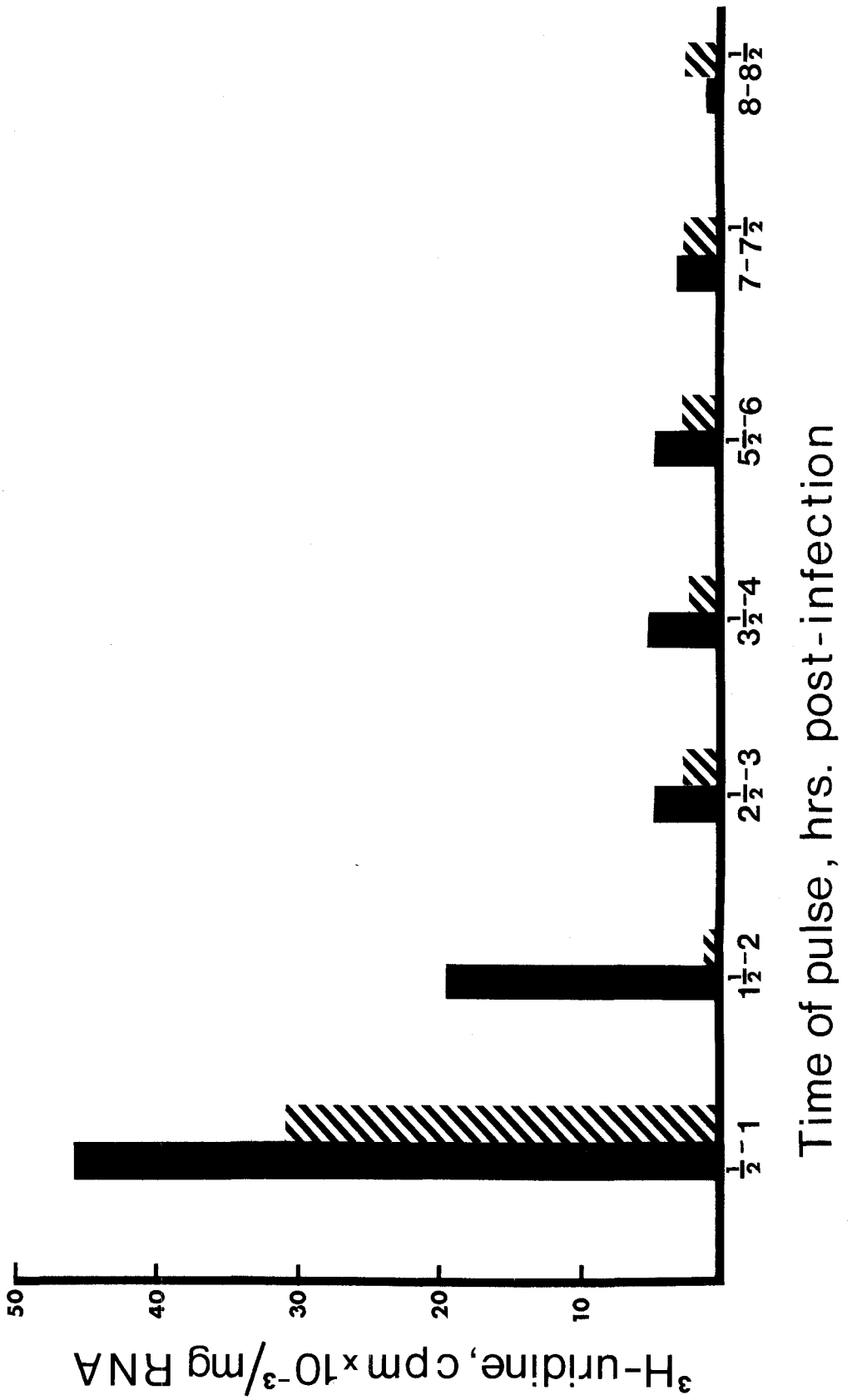
Fig. 10. ^{3}H-uridine incorporation in the presence of actinomycin D into uninfected cells with and without Ara-A. Legend as for Fig. 8 except ^{3}H-uridine (3 uCi/culture) was used. Solid bars indicate the absence of Ara-A, cross-hatched bars its presence.



(Figure 10), there was little change in the amount of incorporation of ^{3}H-uridine throughout the experiment compared to infected cultures.

In infected cultures (Figure 11), the presence of Ara-A resulted in considerably lower amounts of incorporation during the first two pulses with little effect during the remaining pulses. The overall trend in the presence or absence of Ara-A showed a marked drop in the incorporation of radioisotope with time. Since actinomycin D had previously been shown not to affect VSV replication (Huang and Wagner, 1966; Black and Brown, 1968), these results were puzzling. The virus stock used in these experiments was checked for infectivity in order to exclude inactivation as a cause for such results. The stock virus suspension was found to be fully active. Shortly after the conclusion of these experiments, Yamazaki and Wagner (1970) reported similar findings with VSV in rabbit kidney cells. Even under such adverse conditions as described above, Ara-A still caused a reduced rate of incorporation of ^{3}H-uridine compared to the infected control as early as the 0.5 to 1 hour pulse.

Fig. 11. ^{3}H-uridine incorporation in the presence of actinomycin D into infected cells with and without Ara-A. Legend as for Fig. 8 except ^{3}H-uridine (3 uCi/culture) and infected cultures were used.



INCORPORATION OF ^{3}H-URIDINE INTO INFECTED AND UNINFECTED CELLS WITH AND WITHOUT ARA-A

Since actinomycin D was found unsuitable to study VSV RNA synthesis, the preceding experiment was duplicated in full without the addition of actinomycin D. These results are reported in Figures 12 and 13. The incorporation rates for uninfected cultures (Figure 12) show little variation throughout the course of the experiment. Ara-A exhibited a slight depressing effect on incorporation in uninfected cultures. Infected cultures (Figure 13) without Ara-A demonstrated increased rates of incorporation of ^{3}H-uridine over control values during pulses beginning at 0.5, 3.5, 5.5 and 7 hours after infection. Comparison of Ara-A treated infected samples with uninfected controls showed the greatest increase in rate of incorporation during the first pulse. Specific activities for infected cultures with and without Ara-A (Figure 13) show generally lower rates of incorporation in the presence of Ara-A. In addition, the greatest differences were noted during the 0.5 to 1 and 5.5 to 6 hour pulses,where values for cultures lacking Ara-A were considerably higher.

Fig. 12. ^{3}H-uridine incorporation into uninfected cells with and without Ara-A. Legend as for Fig. 7 except ^{3}H-uridine (3 uCi/culture) was substituted for ^{3}H-L-valine.

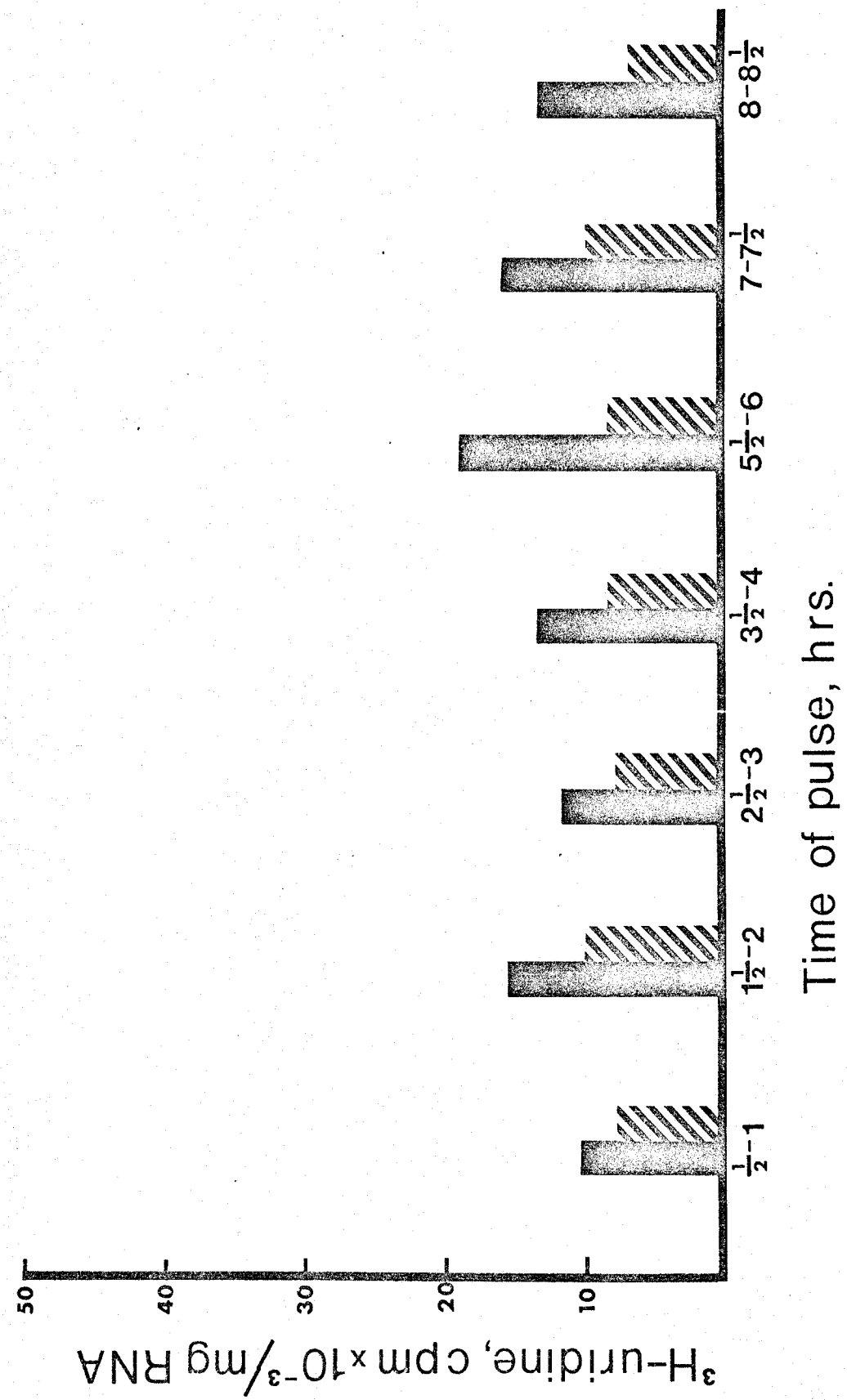
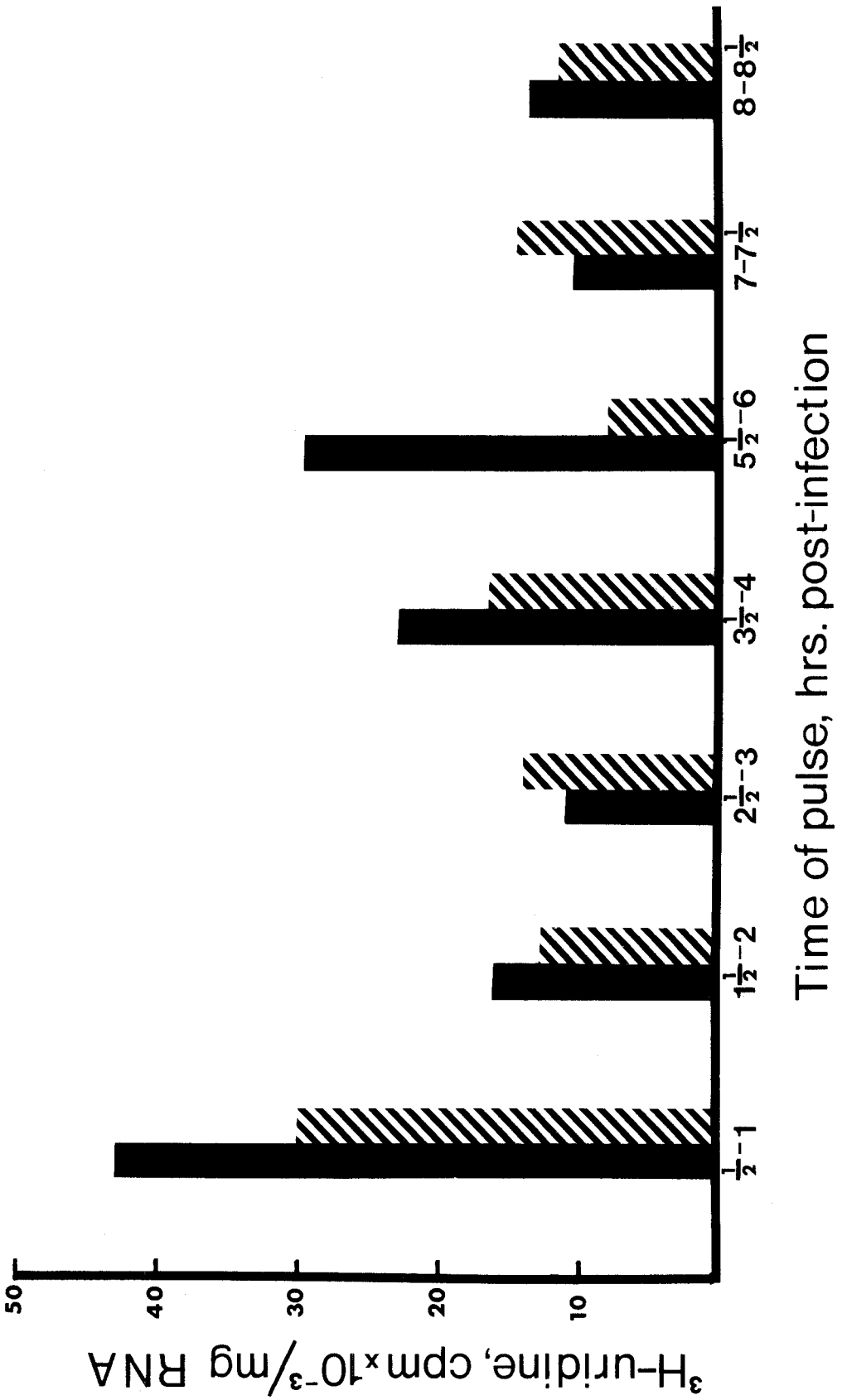


Fig. 13. ^{3}H-uridine incorporation into infected cells with and without Ara-A. Legend as for Fig. 7 except ^{3}H-uridine (3 uCi/culture) and infected cultures were used.



DISCUSSION

Previously, studies of the antiviral activity of Ara-A have shown high therapeutic activity against DNA viruses but not against RNA viruses. This study reports the first incidence of Ara-A activity against an RNA virus (VSV) other than Rous sarcoma virus which has been shown to be dependent on DNA synthesis for replication (Bader, 1964, 1966 ab; Temin, 1964). Ara-A antiviral activity against VSV has been demonstrated to occur early during replication confirming the findings of Miller et al. (1969) in connection with DNA viruses.

Although Ara-A has been shown to be an effective antiviral agent (Miller et al., 1969; Sidwell et al., 1967, 1968, 1969; Sloan et al., 1969) its exact mode of action remains unknown. On the basis of ^{3}H-L-leucine incorporation in the presence of actinomycin D, evidence has been presented here indicating a lack of inhibition of viral protein synthesis by the drug. The relief by Ara-A of puromycin and cycloheximide inhibition of VSV infectivity may imply association of the drug with ribosomes and/or transfer RNA. This association, if it exists, does not seem to inhibit protein synthesis

but in fact appears to stimulate the incorporation of ^{3}H-L-leucine in the presence of actinomycin D. This stimulation was apparently masked in the ^{3}H-L-valine incorporation experiment which probably reflects the trend of cellular protein synthesis during VSV infection. The decrease in incorporation of ^{3}H-L-valine as VSV infection progresses confirms recent findings by Wertz and Younger (1970).

Early viral RNA synthesis is partially inhibited by Ara-A as demonstrated by ^{3}H-uridine incorporation but does not seem extensive enough to account for the inhibition of later viral RNA synthesis. Baltimore et al. (1970) have shown that early viral RNA synthesis in VSV-infected cells is directed by the intact infecting nucleocapsid and may be carried out by an RNA polymerase carried in the intact virion. Furthermore, Baltimore (1970) has hypothesized that early viral RNA formation and replication of the viral genome are separate events. Therefore, the evidence presented here seems to indicate direct inhibition of the replication of the viral genome.

Lucas-Lenard and Cohen (1966) have reported that the 5'-diphosphate derivative of Ara-A (Ara-ADP) inhibits the action of the enzyme polynucleotide

phosphorylase isolated from Escherichia coli. This enzyme (Edmonds and Abrams, 1960; Ventkataraman and Mahler, 1963) and one of its products, polyadenylic acid (Poly A) (Hadjivassiliou and Brawerman, 1966), have been isolated from mammalian systems. Ara-A has also been shown to inhibit DNA synthesis in TA_3 ascites cells (Brink and LePage, 1966) and to inhibit DNA polymerase in mammalian cells (Furth and Cohen, 1967).

Polynucleotide phosphorylase has been shown to be capable of synthesizing RNA (Grunberg-Manago and Ochoa, 1955) and cellular RNA synthesis has been shown to be shut off rapidly after VSV infection (Huang and Wagner, 1965; Wertz and Younger, 1970). Therefore, one might speculate that this enzyme is similar to the RNA-dependent RNA polymerase carried in the VSV virion. Also, the comparatively little inhibition of early viral RNA formation may be a reflection of a protective effect due to complexing of the enzyme with the nucleocapsid.

As yet, there have been no reports dealing with radioisotope labelled Ara-A in viral infections. In vitro studies of L cells by Cohen (1966) failed to show incorporation of tritium-labelled Ara-A into

DNA, RNA or nucleotides. York and LePage (1966) found Ara-ATP inhibition of DNA polymerase in TA_3 ascites cells to be non-competitive. These findings seem to indicate that Ara-A may inhibit VSV replication by affecting the enzyme responsible for the replication of the VSV genome although the drug does not seem to bind to the active site of similar enzymes.

In conclusion, although the exact mode of action of Ara-A against VSV remains to be determined, the drug does not appear to be a protein synthesis inhibitor but appears to block viral RNA synthesis in some manner.

SUMMARY

Ara-A has been shown to be effective in inhibiting the replication of vesicular stomatitis virus (VSV) in MDBK cells as early as the first three hours of infection. Ara-A was shown to follow a typical dose-response relationship in this test system. When used in combination with the protein synthesis inhibitors puromycin and cycloheximide, Ara-A seems to relieve inhibition of infectivity rather than acting synergistically. Radioisotope incorporation studies showed that VSV infection shuts off protein synthesis in MDBK cells and that Ara-A did not affect this process. When incorporation of ^{3}H-L-leucine in the presence of actinomycin D was studied, Ara-A was shown to stimulate incorporation. Incorporation experiments utilizing ^{3}H-uridine demonstrated inhibition of viral RNA synthesis during the period of expected genome replication.

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