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Inhibition of Herpes Simplex Virus Replication by Phosphonoacetic Acid

L. R. OVERBY, E. E. ROBISHAW, J. B. SCHLEICHER, A. RUETER, N. L. SHIPKOWITZ, AND J. C.-H. MAO

Abbott Laboratories, North Chicago. Illinois 60064

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Replication of herpes simplex virus in WI-38 cells was inhibited by phosphonoacetic acid, as measured by decreased virus cytopathogenic effect and incorporation of radiolabeled thymidine in virus-infected cells. The drug appeared to have no effect on adsorption, penetration, or release of the virus nor on the synthesis of ribonucleic acid or protein. It appeared to inhibit virus deoxyribonucleic acid synthesis.

Herpes simplex virus (HSV) is a frequent infection in man with degrees of severity from mild to severe discomfort (cutaneous lesions) to severe infection damaging the eye sight (herpes keratitis) to severe and life-threatening diseases (herpes encephalitis). It has been suggested recently that herpes-viruses may be directly or indirectly associated with cancer in several animals: frog renal adenocarcinoma (4), malignant lymphomas in chickens (Marek's disease) (11), rabbits (6), and monkeys (8). Herpes-type viruses have been indirectly associated with Burkitt's lymphoma, nasopharyngeal carcinoma (5), and cervical carcinoma (1) in humans. 5-Iodo-2-deoxyuridine and cytosine arabinoside are two well-known nucleoside analogs and are inhibitors of herpervirus replication. Both compounds are quite toxic (2, 12), and 5-iodo-2-deoxyuridine has been shown to be carcinogenic and teratogenic (10).

In earlier studies by Shipkowitz et al. (13), phosphonoacetic acid (PAA) was shown to be active against herpes dermatitis in mice and herpes keratitis in rabbits. We report here the efficacy of PAA against HSV in tissue culture, its effect on WI-38 cells, and studies related to the mechanism of action.

MATERIALS AND METHODS

Tissue culture. WI-38 cells (passages 22 to 30) were propagated in Eagle basal medium containing 10% fetal calf serum, 50 μg of neomycin per ml, and 50 U of polymyxin per ml. One milliliter of cell culture containing 10^5 to 10^6 cells was planted in test tubes (1.6 by 12.5 cm) or 96-well disposable trays (growth surface 1.76 cm^2 per well); or 20 ml was planted in 75-cm^2 Falcon flasks. The cells were grown to 90 to 100% confluency at 37 C in a 5% CO_2 incubator. For infection, the growth medium was decanted and replaced by maintenance medium (MM), consisting of no. 199 medium with Hanks balanced salt solution supplemented with Eagle basal medium vitamins, Eagle basal medium amino acids, 50 μg of meomycin per ml, 50 U of polymyxin per ml, and 2% of heat-inactivated fetal calf serum.

Virus stocks. Cultures of WI-38 cells in MM were infected with HVS type 1 (HSV-1) at a multiplicity of infection (MOI) of 10 mean tissue culture infective doses per cell. The cultures were harvested by three freeze-thaw cycles when maximum cytopathic effect (CPE) was observed. The crude lysates were frozen in vials at −70 C until used. Titers, expressed as mean tissue culture infective doses, ranged from 10^6 to 10^7 per ml.

Infected cells. Virus stock solutions were diluted in MM and were added to confluent WI-38 cells. The virus was allowed to adsorb for 1 h at 37 C. The medium and unadsorbed virus were removed by decanting and the cells were washed twice with MM. Cell cultures were then incubated at 37 C in MM.

Drug tests. PAA was prepared from sodium phosphite and sodium chloroacetic acid as described by Nylén (9). Stock solutions were prepared in MM at a concentration of 10 mg/ml. Portions were then added aseptically to cell cultures to give the desired final concentrations of PAA.

Ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein synthesis. Cultures (1 ml) of WI-38 cells were grown and infected in tubes. At various intervals during the incubation, the cultures were pulse-labeled for 30 min with [^{14}C]thymidine (0.5 μCi), [^{3}H]uridine (2.5 μCi), or ^{14}C-labeled amino acid mixtures (0.2 μCi). PAA was added at the time of HSV adsorption and was continued in the culture medium thereafter. After each 30-min labeling with radioactive materials, triplicate tubes were removed, and then the medium was decanted. The cell layers were washed twice with cold MM. Then, 4 ml of phosphate-buffered saline were added to each tube, and the cells were scraped from the tube wall. Trichloroacetic acid was added to a final concentration of 5%. The mixture was frozen, thawed once, and filtered on a membrane filter (0.45-μm pore). The filter was washed three times with 5% trichloroacetic

acid, dried, and counted in a liquid scintillation spectrometer. Counting efficiency for ^{14}C and ^{3}H was 80 and 20%, respectively.

Isolation of DNA. WI-38 cells were grown to confluency in 75-cm² Falcon flasks. At 4, 10, and 16 h postinfection (MOI = 10), the MM was decanted; 20 ml of fresh MM containing 10 μCi of [^{14}C]thymidine was added to untreated infected cells, and 10 μCi of [^{3}H]thymidine were added to another flask containing infected cells treated with PAA (100 μg/ml). After a 2-h incubation, DNA was isolated from each culture according to the Marmur procedure (7) in which the cells were removed from the flask wall, treated with a 1% sodium dodecyl sulfate-1 M sodium perchlorate mixture, and then treated with a chloroform-isoamyl-alcohol (24:1; vol/vol) mixture. Strands of DNA were precipitated by swirling with 2 volumes of ethanol. The DNA was collected and suspended in 0.1 SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.3).

Isolated DNA was treated with ribonuclease (50 μg/ml) for 6 h at room temperature and then was mixed with CsCl dissolved in 0.015 tris(hydroxymethyl)aminomethane (pH 9.0). The density of the solution was adjusted to 1.70 g/cm³, and then the solution was centrifuged in a Spinco SW65 rotor at 38,000 rpm for 40 h. Fractions of five drops from a 23-gauge needle were collected. Portions of each fraction were precipitated with trichloroacetic acid at a final concentration of 2.5%. After standing at 4 C for 1 h, the mixture was filtered on a membrane filter, and radioactivity was determined by a liquid scintillation spectrometer.

Radioisotopes and reagents. [^{3}H]uridine (specific activity, 1 Ci/mmol), [^{3}H]thymidine (1 Ci/mmol), and [^{14}C]thymidine (50 Ci/mol) were obtained from International Chemical and Nuclear Corp. The ^{14}C-labeled amino acid mixture (1.5 Ci/g) was purchased from New England Nuclear Corp. Phosphate-buffered saline contained 8.766 g of NaCl, 11.795 g of $NaHPO_4 \cdot 7\ H_2O$, and 21.526 g of $NaH_2PO_4 \cdot H_2O$ per liter. Cesium chloride (optical grade) was obtained from Schwarz/Mann.

RESULTS

Prevention of cytopathology by PAA. A typical culture of WI-38 cells after a 24-h infection with HSV-1 is shown in Fig. 1a. The cells became rounded, clumped, and multinucleated. However, in an identical culture in the presence of PAA (100 μg/ml), this characteristic CPE was absent after a 24-h infection (Fig. 1b). The treated, infected cells could not be distinguished from normal WI-38 cells (Fig. 1c); however, on some occasions, marginal CPE was observed in the infected cultures treated with PAA. This may be related to growth conditions of the cells or to the strength of the virus stock solution, but it is quite clear that PAA always suppressed CPE.

Reduction in infectious virus yield. PAA caused a delay in the appearance of progeny virus as well as a marked reduction in total virus produced (Fig. 2). At 12 h postinfection, HSV titer was decreased by 10^2 mean tissue culture infection doses and there was no further production of progeny virus in the presence of 200 μg of PAA per ml.

In another experiment, infectious virus present in the medium and in the cells of the PAA-treated and untreated samples were compared. Although the total amount of virus was reduced in the PAA-treated cultures, about one-third of the virus was in the medium after 24 h of infection whether in the presence or absence of the drug. These results suggested that PAA had little effect on the release of virus from infected host cells.

Relationship of MOI and effective dosage of PAA. Various concentrations of PAA were tested against cultures infected with virus at MOI varying from 0.001 to 35. CPE was scored on the third day after infection. The minimum inhibitory concentration was defined as the lowest drug concentration that inhibited CPE in one of the duplicate samples (2). The effective dosage of PAA varied with MOI (Table 1). The minimal inhibitory concentration of the drug was 20 to 40 μg/ml at a MOI of 0.001, but it increased to 200 μg/ml at a MOI of 35.

Effect of PAA on normal WI-38 cells. There was little or no toxicity in the contact-inhibited WI-38 cells treated with PAA. The confluent cell layer (Fig. 1d) was incubated with 200 μg of PAA per ml for 2 days and still appeared normal when observed by microscope. There were no significant decreases in the number of viable cells treated with 100 μg of PAA per ml for a period of 96 h. The drug also appeared not to affect RNA synthesis in confluent cell cultures, as judged by radioactive uridine and thymidine uptakes (see Fig. 4 and 5). Similarly, protein synthesis was unaffected, as judged by ^{14}C incorporation into amino acids. However, growing cells appeared to be less tolerant to PAA. In dividing WI-38 cultures, there was a decrease in the number of cells in the culture with 50 and 100 μg of PAA per ml. No changes in the number of cells were observed at 10 μg/ml (Fig. 3).

Effect of time for addition or withdrawal of PAA on cytopathology. PAA was added to confluent cultures during and after HSV-1 adsorption. Virus titer was determined 24 h after infection. The results indicated that PAA could be added as late as 6 h postinfection and still significantly inhibit the production of progeny virus (Table 2). These results implied that the inhibitory effects of PAA were not related to early events in the virus growth cycle, such as adsorption or penetration, but did inhibit some later event.

It was also of interest to note whether PAA-

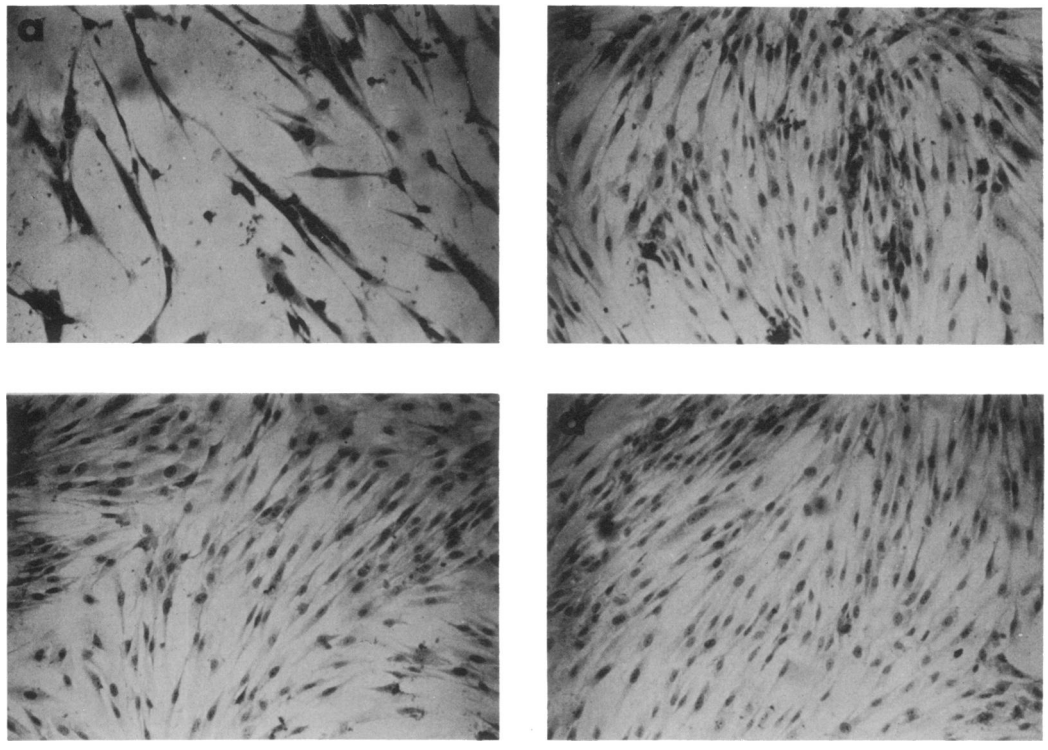

FIG. 1. *Effect of PAA on HSV-1 and host cells. WI-38 cells were grown on cover slips. (a) HSV-infected WI-38 cells (MOI = 1) at 24 h postinfection. (b) HSV-infected WI-38 cells (MOI = 1), treated with 100 μg of PAA per ml, at 24 h postinfection. (c) Control WI-38 cells. (d) WI-38 cells incubated with 200 μg of PAA per ml for 2 days. These cells were stained with Giemsa stain.*

protected infected cells would resume virus replication after removal of the drug. For this work, PAA was present at the time for infection and was then removed at various times thereafter. The results (Table 3) indicate that CPE appeared 3 days after infection in the untreated culture. However, this period was extended in cells treated with PAA and seemed to follow a pattern up to 10 days of treatment, so that the longer the treatment with PAA, the longer the period before CPE became visible. Thus, it appeared that even after prolonged treatment with PAA, functioning virus genomes remained and could be expressed after removal of the drug.

Effect of PAA on RNA, protein, and DNA synthesis in normal and HSV-infected cells. The time-course of [^{3}H]uridine incorporation (Fig. 4) fell into two groups: HSV-infected cells with and without PAA (100 μg/ml), and the uninfected cells in the absence and presence of PAA. The HSV-infected cells showed an increased RNA synthesis compared to uninfected cells. RNA synthesis was not affected by PAA in either case. Similar results were observed for protein synthesis represented by ^{14}C-amino acid incorporation.

The effect of PAA on DNA synthesis of HSV-infected cells was very striking. The rate of [^{14}C]thymidine incorporation into infected cultures was much higher than in uninfected cells (Fig. 5). In the presence of 100 μg of PAA per ml, DNA synthesis in infected cells was markedly reduced. On the other hand, PAA had no significant effect on DNA synthesis of host cells.

To differentiate the effect of PAA on host and viral DNA synthesis, DNA of infected cells in the presence or absence of PAA was isolated and separated by isopycnic banding in CsCl. The thymidine incorporated at 6 h postinfection corresponded to cellular DNA only (ρ = 1.700 g/cm^3; Fig. 6). By 12 h postinfection, a new peak of DNA (ρ = 1.727 g/cm^3) which corresponded to HSV DNA (3) appeared, but it was absent in the infected cultures treated with PAA. By 18 h after infection, an increased proportion of viral DNA synthesis was found in the infected cul-

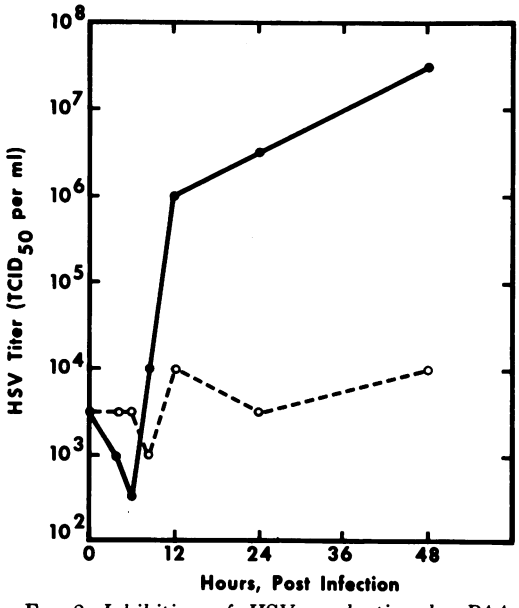

FIG. 2. *Inhibition of HSV production by PAA. HSV-infected cells (MOI = 3.2) after incubation for various times were scraped from the tube wall. The mixture was frozen and thawed three times. Virus titer was determined by the end-point method based on CPE. PAA was added at various adsorptions and was re-installed after washing the unadsorbed virus. Symbols: ●, HSV-infected cells; and ○, HSV-infected cells with 0.2 mg of PAA per ml.*

TABLE 1. *Effective dosages of PAA at various MOI*

MOI	Minimum inhibitory concn (μg/ml)
0.001	20 to 40
0.01	40 to 60
0.1	60 to 80
1	100
35	200

[a] WI-38 cells were grown to confluency in 96-well disposable trays. The growth medium was removed, MM containing various concentrations of PAA and HSV-1 was added, and incubation was continued for 3 days. The tests were run in duplicate. The minimum inhibitory concentration is the lowest concentration of drug which inhibited CPE in one of the two wells.

ture, but viral DNA in the culture treated with PAA was absent.

DISCUSSION

PAA was shown to inhibit HSV-1 replication in tissue culture. The effective dosage for suppressing progeny virus was related to the virus MOI. The estimated minimal inhibitory concentration was 20 to 40 μg/ml at a MOI of 0.001, but it increased to 200 μg/ml at a MOI of 35. A similar relationship between MOI and dosage was reported by Fiala et al. (2) in studies of cytosine arabinoside inhibition of HSV. PAA is a strong acid with three ionizable groups ($^{-}O_3P$-CH_2-COO^{-}). The relatively low activity of this compound in tissue culture could be associated with its ionic character and with the difficulty of transport of charge molecules through cell membranes.

The major effect of PAA on HSV replication did not appear to be on adsorption or penetration of virus, since PAA added 6 h after virus inoculation still protected the cells. This drug also had no effect of the release of virus from the host cells, since the ratio of virus in the medium and in the cells was similar among infected samples in the presence or absence of the drug. When the drug was removed from the infected cells, there was a delay of the appearance of CPE for several days. This fact suggests that action of PAA was virostatic in nature. PAA apparently had no significant effect on the synthesis of viral RNA or protein. The most dramatic effect was inhibition of viral DNA

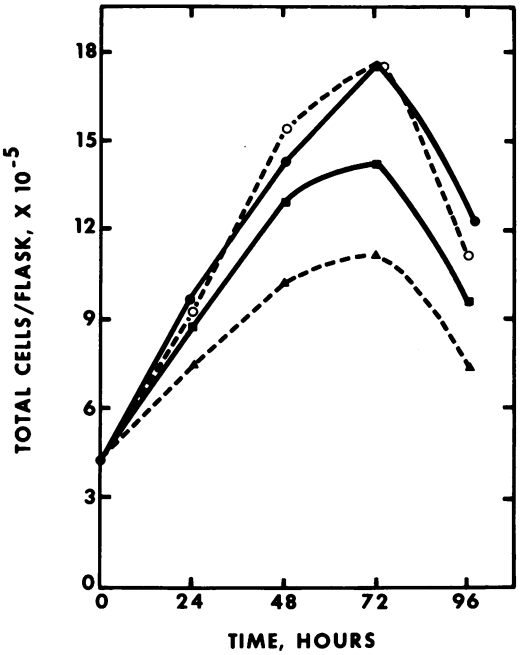

FIG. 3. *Effect of PAA on dividing WI-38 cells. WI-38 cells were planted at 25 × 10⁴ cells in 5 ml in 30-cm² Falcon flasks. PAA was added at the time cells were 50% confluent, and cells were incubated at 37 C in growth medium. After various timed intervals, flasks were withdrawn in triplicate, and cells were counted. Symbols: ○, 0.01 mg/ml; ■, 0.05 mg/ml; ▲, 0.1 mg of PAA per ml; and ●, no PAA added.*

TABLE 2. *Effect of addition time of PAA on the infectious virus yield*[a]

Postinfection (h)	$TCID_{50}$/0.1 ml	
	+ PAA	- PAA
0	2.0	6.0
0.5	3.0	7.0
1	2.5	5.5
2	2.0	5.5
4	3.0	5.5
6	3.5	5.5
8	5.5	5.5

[a] WI-38 cells were grown to confluency in tubes. Growth medium was removed and MM containing PAA (200 μg/ml) was added at various times during and after 1-h HSV-1 adsorption (MOI = 10). Cells were incubated at 37 C for 24 h after the addition of PAA. Then, the cells were frozen, and the virus titer was determined by the end-point method based on CPE. Each test was done in triplicate, and the three tubes were pooled for titration. $TDIC_{50}$, Mean tissue culture infective dose.

TABLE 3. *Effect of withdrawal time of PAA on cytopathology*[a]

Removal of PAA (days postinfection)	Appearance of CPE (days after PAA removed)
3	4 to 6
5	5 to 8
10	10 to 16
17	9 to 11
-PAA	1 to 3

[a] WI-38 cells were grown to confluency in 96-well disposable trays. PAA (60 μg/ml) was present at the time of HSV adsorption (MOI = 0.01). After various lengths of time, the MM without PAA was added, incubation was continued, and cells were observed daily for CPE. Medium was changed every 3 to 4 days to maintain the cells.

synthesis. HSV-infected cells showed an increased rate of DNA synthesis, as shown in Fig. 5. However, in the presence of PAA, the rate of DNA synthesis was greatly decreased. Since cellular DNA synthesis is not significantly altered by PAA, the inhibited DNA synthesis must be of viral origin. This fact is more convincingly shown in Fig. 6, in which viral and cellular DNA were separated on the basis of buoyant density in CsCl. Viral DNA (ρ = 1.727 g/cm^3) was detected at 12 h postinfection and an increased amount was found after 18 h of infection, whereas in the presence of 100 μg of PAA per ml, viral DNA was undetectable in both samples. This experiment also confirmed that PAA had little effect on the synthesis of cellular DNA (ρ = 1.700 g/cm^3). Further work is

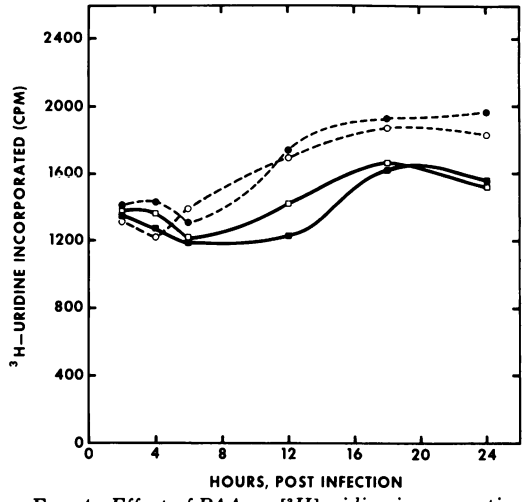

FIG. 4. *Effect of PAA on [^{3}H]uridine incorporation into normal and HSV-infected cells. The confluent WI-38 cells and HSV-infected cells (MOI = 4) were pulse-labeled for 30 min at intervals with 2.5 μCi of [^{3}H]uridine per ml. Radioactivity of trichloroacetic acid-insoluble material was determined. The 2% fetal calf serum was omitted from the MM. Symbols: ■, normal WI-38 cells; □, WI-38 cells in the presence of 0.1 mg of PAA per ml; ●, HSV-infected WI-38 cells; and ○, HSV-infected WI-38 cells in the presence of 0.1 mg of PAA per ml.*

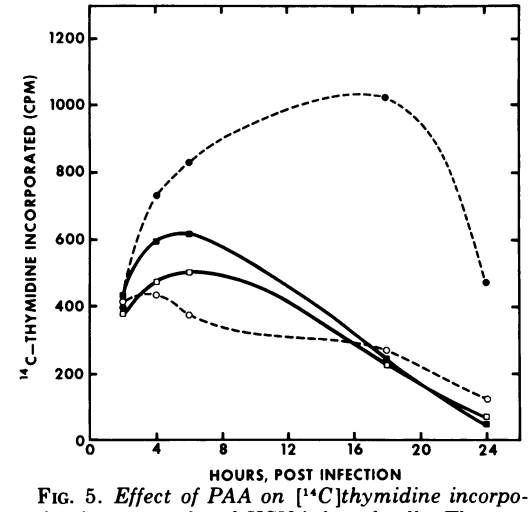

FIG. 5. *Effect of PAA on [^{14}C]thymidine incorporation into normal and HSV-infected cells. The confluent WI-38 culture and HSV-infected culture (MOI = 4) were pulse-labeled for 30 min at intervals with 0.5 μCi of [^{14}C]thymidine per ml. Radioactivity of trichloroacetic acid-insoluble material was determined. The 2% fetal calf serum was omitted from the MM. Symbols: ■, normal WI-38 cells; □, WI-38 cells in the presence of 0.1 mg of PAA per ml; ●, HSV-infected cells; and ○, HSV-infected cells in the presence of 0.1 mg of PAA per ml.*

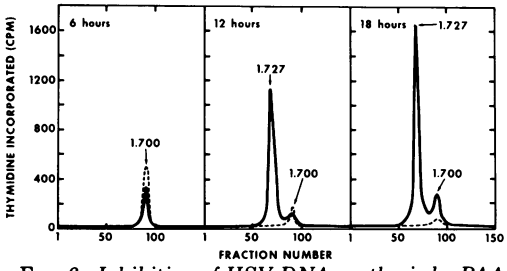

FIG. 6. *Inhibition of HSV-DNA synthesis by PAA. The HSV-infected cells in the presence of PAA (0.1 mg/ml) were pulse-labeled for 2 h with 10 μCi of [^{3}H]thymidine (-----), and those infected in the absence of PAA were pulse-labeled with 10 μCi of [^{14}C]thymidine (——) in 20 ml of MM. The purified DNA was subjected to CsCl centrifugation. Fractions were collected from the bottom of the tube. Radioactivity of trichloroacetic acid-precipitable material was determined.*

underway to establish whether this represented a direct effect or an indirect action on a specific viral enzyme system. Preliminary results have indicated that PAA specifically inhibits the viral DNA-dependent DNA polymerase.

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