JOURNAL OF VIROLOGY, Feb. 1977, p. 584-600
Vol. 21, No. 2
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Herpes Simplex Virus Resistance and Sensitivity to Phosphonoacetic Acid

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Received for publication 13 September 1976

Phosphonoacetic acid (PAA) inhibited the synthesis of herpes simplex virus DNA in infected cells and the activity of the virus-specific DNA polymerase in vitro. In the presence of concentrations of PAA sufficient to prevent virus growth and virus DNA synthesis, normal amounts of early virus proteins (α- and β-groups) were made, but late virus proteins (γ-group) were reduced to less than 15% of amounts made in untreated infected cells. This residual PAA-insensitive synthesis of γ-polypeptides occurred early in the virus growth cycle when rates were identical in PAA-treated and untreated infected cells. Passage of virus in the presence of PAA resulted in selection of mutants resistant to the drug. Stable clones of mutant viruses with a range of drug sensitivities were isolated, and the emergence of variants resistant to high concentrations of PAA involved the sequential selection of mutants progressively better adapted to growth in the presence of the drug. Increased drug resistance of virus yield or plaque formation was correlated with increased resistance of virus DNA synthesis, γ-protein synthesis, and resistance of the virus DNA polymerase reaction in vitro to the inhibitory effects of the drug. PAA-resistant strains of herpes simplex virus type 1 (HSV-1) complemented the growth of sensitive strains of homologous and heterologous types in mixed infections in the presence of the drug. Complementation was markedly dependent upon the proportions of the resistant and sensitive partners participating in the mixed infection. Intratypic (HSV-1A × HSV-1B) recombination of the PAA resistance marker(s), P^r, occurred at high frequency relative to plaque morphology (*syn*) and bromodeoxyuridine resistance (B^r, thymidine kinase-negative phenotype) markers, with the most likely order being *syn*-B^r-P^r. Recombinant viruses were as resistant or sensitive to PAA as the parental viruses, and viruses recombinant for their PAA resistance phenotype were also recombinant for the PAA resistance character of the virus DNA polymerase. The results provide additional evidence that the herpesvirus DNA polymerase is the site of action of PAA and illustrate the potential usefulness of PAA-resistant mutants in genetic studies of herpesviruses.

Phosphonoacetic acid (PAA) is an effective inhibitor of herpes simplex virus (HSV) growth in tissue culture (31) and significantly reduces mortality and morbidity of experimentally infected animals (10, 22, 29, 31, 36). The compound is also active against a number of other herpesviruses but has little effect on representatives of the other groups of animal viruses tested, with the reported exception of vaccinia virus (R. G. Duff and L. R. Overby, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S161, p. 240).

In their initial studies, Overby et al. (31) reported a selective inhibition of virus DNA synthesis by PAA in WI-38 cells infected with HSV. Subsequently, the drug was shown to inhibit the in vitro activity of the virus-specific DNA polymerase of HSV, the herpesvirus of turkeys, and human cytomegalovirus (2, 20, 24, 27, 28). Although the β DNA polymerase activity of uninfected cells was unaffected by the drug, the α-polymerases from duck (24) and human (2) cells are inhibited. However, the available data are consistent with at least one of the actions of the drug in vivo being via the inhibition of the herpesvirus DNA polymerase.

Our interest in PAA arose from three considerations. First, the drug seemed to provide a means to re-examine the role of virus DNA synthesis in the regulation of herpesvirus protein synthesis. In experiments using hydroxyurea and cytosine arabinoside as inhibitors of HSV DNA synthesis, we showed that normal virus DNA synthesis was not a necessary precondition for the initiation of late (γ) protein synthesis (17). Previous investigators (25, 30)

had also shown significant synthesis of structural proteins in the absence of DNA synthesis, and recent studies of DNA-negative, temperature-sensitive (ts) mutants appear to confirm the view that normal DNA synthesis is not a precondition for the synthesis of significant amounts of late proteins (3, 33). However, other authors have claimed that inhibitors of DNA synthesis prevent late protein synthesis in HSV-infected cells (43). It seemed likely that these differences of interpretation might be resolved by a quantitative study of the effect of PAA on virus protein synthesis. Second, our early experiments and those of others (22) showed that virus mutants resistant to PAA could be isolated with relative ease. As well as providing appropriate controls for studies on the effects of the drug on protein synthesis, these resistant viruses also gave the opportunity to obtain independent biochemical and genetic evidence for the mode of action of the inhibitor. Finally, we wished to explore the genetics of resistance to PAA to provide some basis for the use of the resistance determinant(s) as an aid to the genetic mapping of herpesviruses.

MATERIALS AND METHODS

Solutions and chemicals. The culture medium for infected and uninfected cells was the Glasgow modification of Eagle medium with 5 or 10% calf serum and 10% tryptose phosphate broth. For labeling of infected and uninfected cells the medium was modified by lowering the serum concentration to 1 to 2%, omitting tryptose phosphate broth (labeling with [^{3}H]thymidine), and by using one-fifth (labeling intervals more than 1 h) or one-tenth (intervals of less than 1 h) of the normal levels of the amino acids used for labeling (methionine, valine, or arginine). Radioisotopes were purchased from the Radiochemical Centre, Amersham, Bucks., and were [*methyl*-^{3}H]thymidine (17 Ci/mmol), L-[U-^{14}C]arginine (324 mCi/mmol), L-[U-^{14}C]valine (280 mCi/mmol), and [^{35}S]methionine (5 Ci/mmol). Labeled precursors for DNA polymerase ([*methyl*-^{3}H]thymidine 5-triphosphate, 30 Ci/mol) and thymidine kinase (TK; [2-^{14}C]thymidine, 61 mCi/mmol) reactions were also from the Radiochemical Centre. Unlabeled deoxynucleoside triphosphates were purchased from Boehringer & Soehne, Mannheim. 5-Bromo-2-deoxyuridine (BUdR) was purchased from Sigma (St. Louis, Mo.), and the disodium salt of PAA was a gift from Lacy Overby of Abbott Laboratories (North Chicago, Ill.).

Cells and viruses. BHK-21 cells were used throughout for growth of virus inocula and for infectivity titrations as described previously (35, 44). BHK-21 cells were also used routinely for high-multiplicity infections. HEp-2 cells were used in some experiments for the purposes of comparison (see text). HSV type 1 (HSV-1) strains HFEM (44) and B2006 (7) were used for most of the experiments reported in this paper. Other strains of HSV-1 (Fl, MP, and 13; see Heine et al. [14]) and HSV-2 (G, see reference 9; and 3345, see reference 37) were used in some experiments, as noted in the text. HSV-1 (HFEM) forms large syncytial (*syn*) plaques on BHK-21 cells and is TK^{-} (BUdR resistant). This TK^{-} mutant lacks a recognizable thymidine kinase polypeptide and its associated antigenic activity (6, 18, 19).

Infection and labeling of infected cells with radioactive precursors. BHK-21 cells, either in suspensions of 5×10^{6} cells/ml or as confluent monolayers of 4×10^{6} to 6×10^{6} cells, and HEp-2 cell monolayers of 4×10^{6} to 6×10^{6} cells were infected at multiplicities of 5 to 50 PFU/cell. After incubation for 1 h at 37°C with constant agitation, the inocula (0.5 to 1.0 ml) were replaced with culture medium and incubation was continued at 37°C. At the required intervals the culture media were replaced with those containing labeled precursors. The time at which virus was added to the cells was designated 0 h.

Plaque assay under selective and nonselective conditions. Viruses were titrated by the suspension assay of Russell (35). Samples of 2.0 ml of appropriate dilutions of virus were mixed with suspensions of 8×10^{6} BHK-21 cells for 20 to 60 min at 37°C. The mixture was then dispensed into two 5.0-cm plastic petri dishes together with medium containing carboxymethyl cellulose. Under these conditions cells adhered and spread to form confluent monolayers within 4 h at 37°C, and large (1 to 2 mm) plaques were visible by 48 or 72 h. Monolayers were fixed with Formol-saline and stained with crystal violet or carbol-fuchsin after 48 or 72 h of incubation, and plaques were counted with the aid of a binocular microscope. Where appropriate (see Results), differential counts of plaques with syncytial and nonsyncytial morphology were taken.

For measurements of the resistance of plaque formation to PAA or BUdR the drugs were incorporated into the medium at a final concentration of 50 to 200 μg of PAA (see below) and 40 μg of BUdR per ml. In the presence of these concentrations of the inhibitors, BHK-21 cells adhere to and spread on plastic surfaces at 37°C and plaques formed by resistant viruses are not reduced in number, although they are normally somewhat smaller (ca. one-half to two-thirds the diameter of plaques formed in the absence of drugs). In the presence of 40 μg of BUdR per ml, TK^{+} viruses gave less than 0.4% of the number of plaques appearing in the absence of the drug, whereas TK^{-} viruses were unaffected. Although higher concentrations gave an increased ratio of TK^{-} to TK^{+} plaquing efficiencies, the efficiency of plaque formation by TK^{-} viruses was affected (e.g., with 100 μg/ml TK^{+} virus gave $\leq 0.1\%$ but TK^{-} vius gave 60% of the control plaque count). The ability to obtain selective plating of TK^{-} viruses in the presence of BUdR in normal (i.e., TK^{+}) BHK-21 cells is presumably a reflection of the very low levels of the cellular enzyme in confluent monolayers of these cells. Use of a TK^{-} derivative of BHK-21 cells (kindly provided by A. Buchan) did not confer

any advantage in this assay. The assay was not successful when used with TK+ HEp-2 cells, which contain much higher levels (ca. 20-fold) of cellular thymidine kinase as confluent monolayers.

Polyacrylamide gel electrophoresis and densitometry of autoradiograms. Procedures for sample and gel preparation for sodium dodecyl sulfate-slab gel electrophoresis of labeled infected cell polypeptides and the staining and autoradiographic techniques were as previously described (14, 39). Absorbance measurements on autoradiographic images of separated polypeptides were made with a Pye Unicam SP1800 spectrophotometer equipped with an SP1809 gel-scanning attachment. The amount of label in separated bands was determined by planimetric analysis of the absorbance tracings. The rationale and application of these methods are described elsewhere (16, 17). Infected cell polypeptides (ICP) are numbered as described for HSV-1 (F1) (16, 17). There are some minor strain-specific differences in the electrophoretic mobility of certain ICPs of HSV-1 (HFEM) and HSV-1 (F1) (32; unpublished data), but they are not of significance for our present purposes.

Measurements of virus and cellular DNA synthesis. Procedures used for the preparation of [^{3}H]thymidine-labeled infected cell lysates and conditions for their separation on CsCl equilibrium density gradients into virus (ρ = 1.725 g/cm^3) and cellular (ρ = 1.7000 g/cm^3) DNA components were as described in detail by Halliburton and Timbury (11). The amounts of virus and cellular DNA synthesis in each sample were computed by dividing the measurement of the total incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material in proportion to the integrated counts under the two peaks of labeled DNA obtained from the fractionated CsCl gradient (11).

Immune sera and agar gel immunodiffusion tests. The preparation and properties of hyperimmune rabbit antisera to HSV-1 (HFEM)-infected RK-13 cells (general antisera) and their use in agar gel immunodiffusion tests with infected cell extracts have been described in detail (15, 39, 44). Antisera specific for the virus thymidine kinase (anti-TK antisera) were prepared by absorbing general antisera with an excess of cells infected with the TK$^-$ mutant virus HSV-1 (B2006), as described previously (6, 18, 19).

Other methods. Protein measurements were made by the method of Lowry et al. (26). The virus thymidine kinase was measured by the method of Klemperer et al. (23), and the virus and cellular DNA polymerase activities were measured as described by Keir et al. (21) or by the methods of Weissbach et al. (45). Thymidine kinase assays were performed on unfractionated homogenates of infected cells; assay mixtures contained 5 to 50 μg of infected cell protein. The assay was linear with time and amount of infected cell protein until 70% of the input [^{14}C]thymidine (0.1 to 0.2 μCi/assay) had been phosphorylated. DNA polymerase assays on homogenates of nuclear fractions from infected cells or homogenates of unfractionated infected cells contained 10 to 150 μg of infected cell protein. The assay was linear with time for at least 60 min and linear with amount of infected cell protein up to 150 μg.

RESULTS

Dose of PAA required to prevent the growth of infectious virus and effects of inhibitor on growth and plating of uninfected cells. A typical dose-response curve for the effect of PAA on the growth of HSV-1 strain HFEM in BHK-21 cells is shown in Fig. 1. An initial extracellular concentration of at least 200 μg/ml was required to prevent the production of infectious progeny in a high-multiplicity infection. Other strains of HSV-1 (e.g., F1, MP, B2006, and 13) and HSV-2 (G and 3345) gave similar results. Concentrations $\geq$200 μg/ml also prevented the growth of uninfected cells (Table 1), but BHK-21 cells were able to adhere and spread on plastic surfaces in the presence of up to 500 μg of the drug per ml. The effects of PAA on the growth of uninfected HEp-2 cells and of HSV-1 (HFEM) in HEp-2 cells were similar to data shown for infected and uninfected BHK-21 cells.

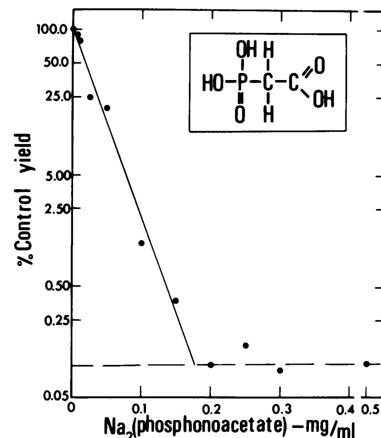

FIG. 1. *Concentration of PAA required to prevent the production of infectious virus. Replicate monolayer cultures of BHK-21 cells (5 × 10^6 cells/culture) were infected with 10 PFU of HSV-1 (HFEM) per cell. After an absorption period of 1.0 h, the inocula were replaced with growth medium containing 0 to 2 mg of disodium phosphonoacetate or 2.5 μg of actinomycin D per ml, and the cultures were incubated at 37°C for 24 h. The infected cultures were then disrupted by sonic vibration and assayed for their content of infectious virus. Cultures incubated in the absence of drug yielded a total of 200 PFU/cell (100%). The yield in the presence of 2.5 μg of actinomycin D per ml (0.1 to 0.2 PFU/cell, broken line parallel to abscissa) was taken as the control value in the absence of virus growth. Concentrations of disodium phosphonoacetate $\geq$200 μg/ml all resulted in yields of 0.1 to 0.3 PFU/cell. The structural formula of PAA is shown in the inset.*

TABLE 1. *Effect of PAA on the growth of uninfected BHK-21 cells*[a]

Concn of disodium phosphonoacetate (mg/ml)	No. of cells (n) per dish ($\times 10^{-5}$) at:		Fold increase in cell no. [$(n_{75} - n_{20})/n_{20}$]
	20 h	75 h	
0	4.6	39.3	7.5
0.05	5.0	40.7	7.1
0.10	4.2	26.7	5.3
0.15	4.9	12.4	1.5
0.20	4.7	4.7	0
0.50	4.4[b]	4.0	

[a] A total of 6×10^5 BHK-21 cells were seeded into each of 48 plastic petri dishes (5 cm in diameter) with 5.0 ml per dish of medium containing the range of phosphonoacetate concentrations indicated (8 dishes per concentration). Plates were incubated at 37°C, duplicate cultures were removed for each drug concentration at 20- to 30-h intervals for 96 h, and the cell number per plate was determined after trypsin-EDTA (Versene) suspension. Plates with 0 and 50 μg/ml were confluent at 75 h, and growth did not begin until after 20 h.

[b] Cells appeared morphologically normal by low-power microscopy of unstained preparations, and normal cell adhesion and spreading occurred in the highest concentration tested.

No surviving cells could be detected in cultures of 2×10^7 BHK-21 cells infected with 10 PFU of HSV-1 (HFEM-P0) per cell and maintained in the presence of 250 μg of the drug per ml for intervals of from 1 to 4 days and examined at daily intervals for 10 days.

Effects of immediate and delayed addition of PAA on growth of a sensitive virus. An experiment comparing the growth of a sensitive virus in untreated cultures and in cultures treated with 250 μg of PAA per ml for different intervals after infection is shown in Fig. 2. Additions before 1.5 h prevented significant production of infectious virus progeny, and additions after 3 h permitted progressively increased yields. The largest differential effect between the yield of infectious virus at the time of addition of the drug and the yield obtained at 18 h was seen when the drug was added at 4.5 or 6 h. At later times, when a significant yield of infectious virus had occurred before the addition of the drug, additions continued to depress the final yield. The effect of the drug on the production of infectious virus was slowly and inefficiently reversed even when it was removed at early times (e.g., −1.5 h, annotated triangular symbol in Fig. 2).

Selection of PAA-resistant viruses. Six independent passage series of sensitive isolates of HSV-1 were initiated in the presence of PAA. Each virus was subjected to multiple sequential rounds of limited growth in the presence of the drug so that the process of selection and the nature of the population changes during this process could be analyzed. Figures 3 and 4 show data on one such series with HSV-1 (HFEM).

In this and other passage series, growth in the presence of PAA resulted in the early emergence of resistant variants or mutants (Fig. 3). The proportions of resistant and sensitive viruses in a mixed population of virus from pas-

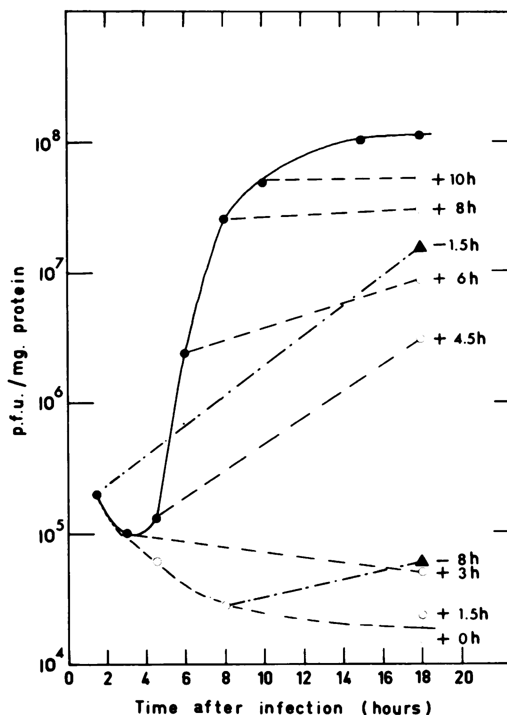

FIG. 2. *Effects of immediate and delayed addition and removal of PAA on the production of infectious virus in cultures of BHK-21 cells infected with a PAA-sensitive strain of HSV-1. Replicate monolayer cultures of 5×10^6 BHK-21 cells were infected with 20 PFU of HSV-1 (HFEM) per cell and incubated at 37°C in the absence of drug. Duplicate cultures were removed at intervals until 18 h after infection for the measurement of infectious virus (●). PAA at a final concentration of 500 μg/ml was added to three sets of duplicate cultures at 0 h and to further duplicate cultures of infected cells at 1.5, 3, 4, 5, 6, 8, and 10 h after infection. At 1.5 and 8 h, drug added at 0 h was removed from two sets of duplicate cultures, and these cultures were then reincubated in medium lacking the drug until 18 h, at which time they were removed and assayed for their content of infectious virus (▲, annotated with the time of removal of the drug). Cultures incubated from 0, 1.5, 3, 4, 5, 6, 8, and 10 h in the presence of the drug were also removed at 18 h and assayed for their content of infectious virus (○, annotated with the time of addition of the drug).*

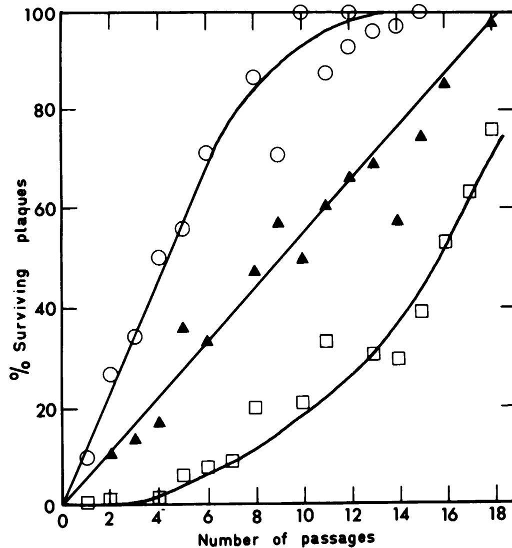

FIG. 3. Selection of PAA-resistant variants of HSV-1 (HFEM) during a passage series in the presence of the drug. Roller bottles of BHK-21 cells (6 × 10^8 cells/bottle) were infected with a PAA-sensitive virus, HSV-1 (HFEM-PO), at a multiplicity of 0.1 PFU/cell and incubated at 37°C for 48 h in the presence of 50 µg of PAA per ml. The progeny from this infection (passage 1) was used as the inoculum for a further passage under identical conditons, but with 100 µg of PAA per ml to produce passage 2. This in turn was used as inoculum to produce passage 3 in the presence of 300 µg of PAA per ml. All subsequent consecutive passages were incubated in the presence of 300 µg of PAA per ml with an input multiplicity of 0.1 PFU/cell. Samples of each passage were titrated in the absence of the drug and in the presence of 50 (○), 100 (▲), and 200 (□) µg of PAA per ml in the overlay medium. Plaques appearing after 48 to 60 h in the presence of the drug were scored, and the mean of the results from at least three independent titrations are recorded as a percentage of the plaques scored in the absence of the drug. The initial stock of sensitive virus (PO) titrated in the presence of 50 µg of PAA per ml gave less than 0.2% of the number of plaques recorded in the absence of the drug.

sage 10 were not altered by four passages in the absence of the drug, suggesting that resistant strains were not at a selective growth disadvantage in the absence of the drug (see also Table 4 and accompanying text). Passage of sensitive virus in the absence of PAA did not significantly affect the proportion of resistant viruses as measured by the plaque reduction assay (≤0.4% of plaques resistant to 50 µg/ml after 10 passages of HSV-1 [HFEM-P0] in the absence of the drug). More interestingly, in the presence of the drug viruses resistant to different concentrations of PAA arose and were selected at different rates. Thus, the relative proportions of plaques resistant to 50 and 100 µg/ml remained approximately constant, but resistance to 200 µg/ml appeared later in the passage series and did not increase in proportion to the increase in resistance to lower concentrations (Fig. 3). For example, at passages 2 and 6 the relative proportions of plaques resistant to 100 and 50 µg of PAA per ml were 0.4 and 0.46, whereas from passages 2, 6, 10, and 16 the relative proportions of plaques resistant to 200 and 100 µg/ml were 0.11, 0.23, 0.36, and 0.62, respectively.

Further information about some of the population changes selected during this passage series was obtained by cloning and by measurements of changes in the proportion of unselected markers in virus populations at the different passage levels. Ten clones of virus were isolated from each of passages 4, 10, and 18, and their resistance to 50, 100, and 200 µg of PAA per ml was analyzed by plaque reduction assay

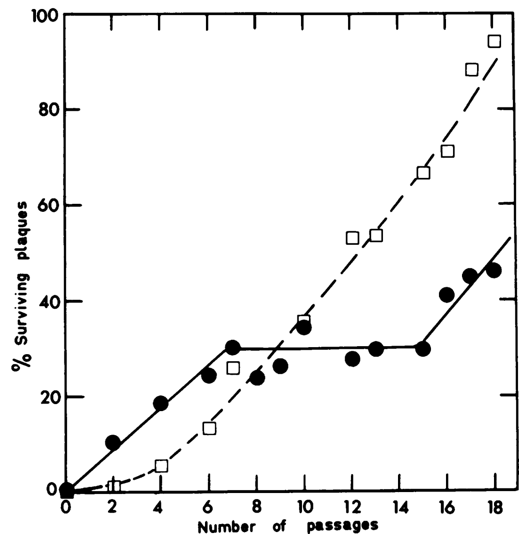

FIG. 4. Variations in the prevalence of an unrelated marker (BUdR resistance) during selection of PAA-resistant mutant viruses. Viruses from passages of the series illustrated in Fig. 3 were retitrated in the presence of 40 µg of BUdR per ml (●) or 160 µg of disodium phosphonoacetate per ml (□), and the number of plaques obtained was expressed as a percentage of those appearing on control plates incubated in parallel but without drugs in the overlay medium. Values shown are the means from at least three independent determinations. HSV-1 (HFEM) of passage 0 and other TK^+ viruses gave less than 0.4% of the control plaque count when in the presence of 40 µg of BUdR per ml, whereas the efficiency of plaque formation by TK^- viruses [e.g., HSV-1 (B2006)] was not significantly reduced by this concentration of the drug under our assay conditions (see Table 5).

(i.e., as for Fig. 3) and by comparing the yields of virus in the presence of these concentrations of the drug with those obtained in the absence of the drug. None of these clones was as sensitive as the parental virus, and in no case was growth dependent on the presence of the drug. However, stable clones with different levels of resistance were obtained. Clones completely resistant to 200 μg/ml were only obtained from passage 10 (1 of 10) and passage 18 (6 of 10). Measurements of the BUdR sensitivity of virus at different passage levels unexpectedly revealed a marked increase in the proportion of BUdR-resistant (i.e., TK^-) viruses from passages 0 to 6. At passage 6 some 30% of the virus population would form plaques under conditions that reduced plaque formation by TK^+ viruses to less than 0.4%. The proportion of BUdR-resistant viruses remained approximately constant from passages 6 to 15, whereas during these passages the proportion of viruses forming plaques in the presence of 160 μg of PAA per ml increased from 20 to 70% (Fig. 4). The proportion of BUdR-resistant virus then increased from passage 15 onwards. The appearance of a significant proportion of BUdR-resistant virus has only been observed in this passage series, and we emphasize that there is no necessary association between BUdR resistance and resistance or sensitivity to PAA. In the present context our interest in this apparently fortuitous occurrence is that it provides independent evidence for multiple successive waves of selection operating on mutations arising during the passage series. Thus, the early period of selection for the TK^- phenotype seems most plausibly explained by proposing that virus initially selected by 50 μg of PAA per ml was coincidentally TK^-. At about passage 5 it appears that a variant better adapted to growth in PAA arose in a TK^+ virus. The next phase of selection for the TK^- phenotype (from passage 15) presumably represents the emergence of a further variant with a growth advantage in the presence of PAA from the now significant population of TK^- viruses. Support for the conclusion that the variants resistant to high concentrations, which predominate in passage 18, are the result of multiple rounds of selection was also obtained from a comparison of the virus polypeptides in cells infected with resistant strains. Thus, one of the clones of virus from passage 18 (clone 1) resistant to more than 250 μg of PAA per ml produced an electrophoretically distinct form of the glycosylated virus protein (ICP12; for example, see Fig. 6), whereas the most resistant virus of passage 10 did not have this variant polypeptide. Clearly the resistant population of passage 18 was not produced by a simple enrichment of the most resistant members of the passage 10 population.

Properties of resistant and sensitive viruses: correlation of PAA resistance of plaque formation, yield, and DNA synthesis in vivo and virus DNA polymerase in vitro. Figure 5 shows results of an experiment comparing the effects of PAA on the DNA polymerase activities of extracts made from cells infected either with a PAA-resistant virus or with a sensitive virus. Addition of 5 μg of the drug per ml inhibited the activity of the crude enzyme from extracts of the sensitive virus to about 10% of the control value, whereas the resistant virus was unaffected by up to 10 μg of the inhibitor per ml. Comparisons of the effects of the drug on plaque formation, yield of infectious virus,

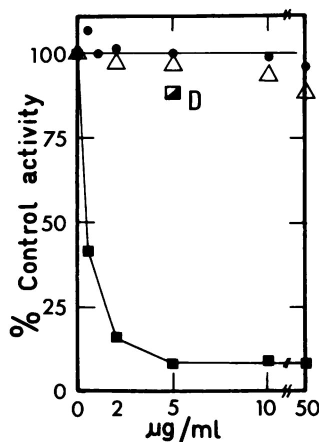

FIG. 5. *Effects of PAA on in vitro assays of the virus-specific DNA polymerase and thymidine kinase. Extracts of BHK-21 cells taken 12 h after infection with a PAA-sensitive virus, HSV-1 (HFEM-P0), or a resistant mutant, HSV-1 (HFEM-P18cl), were assayed for DNA polymerase activity and thymidine kinase activity in the presence of the indicated concentrations (micrograms per milliliter) of disodium phosphonoacetate in the assay mixtures. DNA polymerase activity of resistant and sensitive strains are represented by open triangles and filled squares, respectively, and thymidine kinase activity of HSV-1 (HFEM-P0) by filled circles. TK activity of both sensitive and resistant strains were unaffected by PAA, and only data for the sensitive strain are shown. A sample of the extract from cells infected with sensitive virus was treated with 5 µg of PAA per ml (30 min at 37°C) and was then dialyzed against 0.02 M phosphate buffer at pH 7.5, 0.1 N NaCl, and 1 mM dithiothreitol for 3 h at +4°C and assayed for residual DNA polymerase activity (partially filled square, annotated "D"). Incorporation in DNA polymerase reactions with resistant and sensitive extracts in the absence of the drug were* 2.0×10^4 *and* 2.5×10^4 *cpm, respectively, and* 4.4×10^4 *cpm of [*^{14}*C]TdR were phosphorylated in the thymidine kinase reaction in the absence of the drug.*

and DNA synthesis in vivo with the drug sensitivity of the virus DNA polymerase in vitro showed a correlation between these parameters. For example, in the presence of 250 μg of PAA per ml yields of virus from cells infected with 5 PFU of viruses from passage 0 (P0), clone 5 of passage 10 (P10c5), and clone 1 of passage 18 (P18c1) per cell were reduced to 3, 34, and 91%, respectively, of yields obtained in the absence of the drug. Virus DNA synthesis in cells labeled from 6 to 18 h after infection in the presence of 250 μg of PAA per ml was reduced to 3% (P0), 50% (P10c5), and 90% (P18c1) of the amounts made in infected cells incubated in the absence of the drug. DNA polymerase activities of extracts taken at 12 h after infection with viruses of P0, P10c5, and P18c1 were reduced to 10 to 15, 40, and 90 to 100% of the activities measured in the absence of the drug by 5 μg of PAA per ml.

Effects of PAA on the synthesis of virus-specific polypeptides and the appearance of virus-specific enzymes in cells infected with resistant and sensitive strains of HSV-1. Results of experiments to examine the inhibitory effects of PAA on the synthesis of virus proteins are presented in Fig. 6 and 7 and Tables 2 and 3. Increasing concentrations of the drug selectively reduced the synthesis of virus γ-polypeptides (Fig. 6, left: e.g.: ICP5, 10, 12, 21, 30–31, 40, 44, 45, 47, and 49), with no significant effects on the synthesis of α (e.g., ICP4)- or β (e.g., ICP6, 8, 36, and 39)-polypeptides. Protein synthesis by resistant strains was unaffected by the presence of up to 1 mg of the drug per ml (Fig. 6, right). However, significant levels of γ-protein synthesis by sensitive strains of the virus persisted even in the presence of very high concentrations of the inhibitor. This residual PAA-resistant γ-protein synthesis amounted to some 5 to 15% of the amounts made in untreated infected cells (Table 2). An analysis of the kinetics of γ-protein synthesis by sensitive and resistant strains in the presence and absence of PAA (Fig. 7, Table 3) showed that the residual γ-protein synthesis by sensitive strains in the presence of the drug was due to a distinct early period of synthesis which was unaffected by the inhibitor. Thus, at 3 to 3.5 h the rates of synthesis of ICP5 by sensitive strains did not differ in the presence or absence of the drug (Table 3). The rate of γ-protein synthesis at this time was some 20% of the maximum observed rate of synthesis. However, whereas rates of γ-protein synthesis continued to increase in the absence of the drug, in the presence of the drug rates of synthesis by sensitive viruses declined (Fig. 7), until at 7 to 7.5 h and 11.5 to 12 h they were 0.26 and 0.08 of the control rates. Rates of α (e.g., ICP4; Fig. 6 and 7, Table 3)- and β (e.g., ICP6; Fig. 7, Table 3)-protein synthesis by sensitive and resistant viruses were unaffected by PAA. Moreover, PAA had no effect on the synthesis of α-proteins after removal of a cycloheximide block (17) from cells infected with a resistant or a sensitive virus. In addition, α (e.g., ICP4, 0, 27)-and β (e.g., ICP6, 25, 39)-polypeptides of sensitive and resistant viruses were phosphorylated identically in the presence and absence of the drug.

The appearance of the virus-specific thymidine kinase activity in cells infected with resistant or sensitive viruses was entirely unaffected by up to 500 μg of PAA per ml, consistent with the identification of this activity as associated with a β-polypeptide (18; R. W. Honess, D. H. Watson, and B. Roizman, unpublished data). Measurements of virus DNA polymerase activity in dialyzed extracts of cells infected with resistant or sensitive viruses treated with 500 μg of PAA per ml from the time of infection likewise showed little effect of the drug on the synthesis of the virus DNA polymerase. Thus, from 50 to 80% of the activity observed in cultures incubated in the absence of the drug could be recovered from dialyzed extracts of cells infected with a sensitive virus incubated in the presence of PAA from the time of infection.

Complementation between resistant and sensitive strains in a mixed infection. The isolation of mutants unaffected by concentrations of PAA which prevented the growth of "wild-type" viruses allowed a study of complementation between resistant and sensitive viruses in mixed infections. In these experiments PAA-sensitive (P^s) and -resistant (P^r) strains of HSV-1 with syncytial (*syn*) plaque morphology were used to infect cells either singly or in mixed infections with nonsyncytial (syn^+), P^s strains of HSV-1 or -2. The yields of viruses of different plaque and resistance phenotypes from single and dual infections incubated in the presence and absence of the drug are summarized in Table 4.

In single infections the yield of the resistant virus was unaffected by 250 μg of PAA per ml (Table 4, line 3), and sensitive viruses were reduced to from 0.4 to 2.7% of the yields in the absence of the drug (lines 1, 2, 4). Yields of both viruses in the absence of the drug were similar. Mixed infections with two sensitive viruses were completely sensitive (lines 5, 8), whereas the resistant strains of HSV-1 complemented the growth of sensitive strains of both HSV-1 (cf. Table 4, lines 6 and 7 with line 5) and HSV-2 (cf. lines 8 and 9). The degree of complementa-

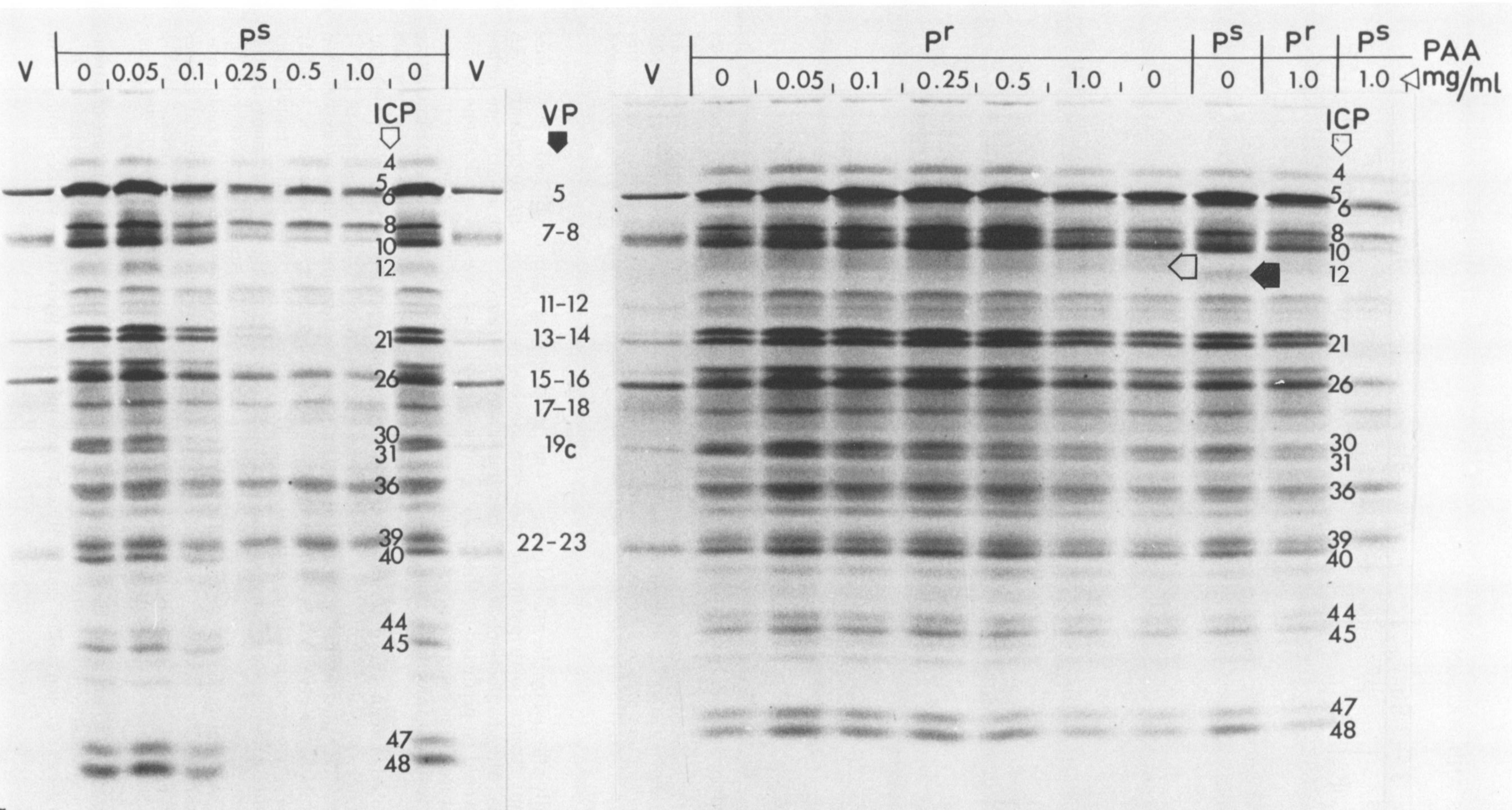

FIG. 6. *Autoradiograms of labeled polypeptides separated on 10% polyacrylamide gel slabs from lysates of HEp-2 cells taken 22 h after infection with 50 PFU of PAA-sensitive (P^s) and -resistant (P^r) variants of HSV-1 (HFEM) per cell. Replicate cultures of 4 × 10^6 infected cells were labeled with 0.2 μCi of [^{14}C]arginine and [^{14}C]valine per ml from 1.5 to 22 h after infection in the presence of from 0 to 1.0 mg of sodium phosphonoacetate per ml. The sensitive virus was HSV-1 (HFEM) of passage 0 and the resistant virus was a clone of HSV-1 (HFEM) passage 18, P18c1. Virion polypeptides (VP) separated from purified labeled virions (V) of HSV-1 (HFEM) passage 0 subjected to electrophoresis in peripheral positions of the slab gels are annotated as previously described (14, 38), and selected ICPs are indicated as detailed elsewhere (16, 17). An electrophoretic variant of ICP12 present in extracts of cells infected with P18c1 is indicated by the open arrow between sample positions 8 and 9, and ICP12 of the parent virus is indicated by the filled arrow between sample positions 9 and 10 of the gel on the right-hand side of the figure.*

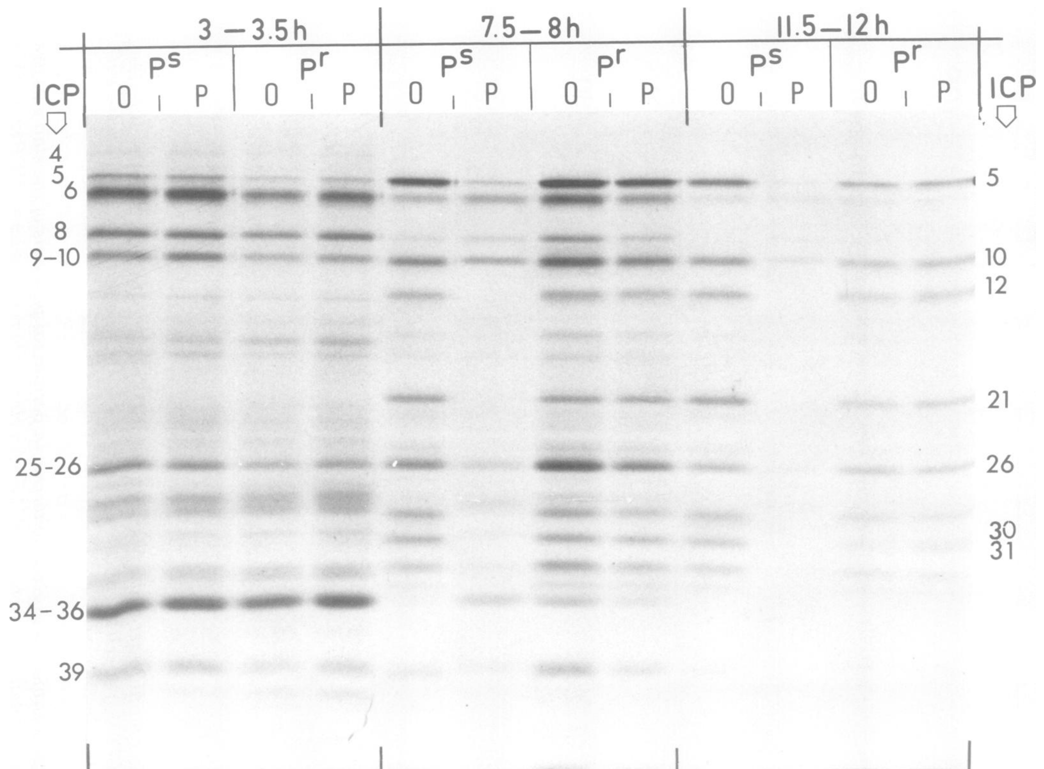

FIG. 7. *Autoradiogram of labeled polypeptides separated on an 8.5% polyacrylamide gel slab from lysates of HEp-2 cells labeled with 2 μCi of [^{35}S]methionine per ml from 3 to 3.5, 7.5 to 8, and 11.5 to 12 h after infection with 20 PFU of PAA-resistant (P^r) or -sensitive (P^s) variants of HSV-1 (HFEM) per cell infected and maintained in the presence of 500 μg of sodium phosphonoacetate (P) per ml or in the absence of the drug (0). Samples were removed and prepared for electrophoresis at the end of the labeling interval. The sensitive variant was passage 0 of HSV-1 (HFEM) and the resistant variant was clone 1 of passage 18 (Fig. 3). Selected ICPs are annotated.*

tion was markedly affected by the ratio of sensitive and resistant strains participating in the mixed infections. Mixed infections were more sensitive than would be predicted on the basis of a simple mixture of appropriate proportions of sensitive and resistant infections (lines 6 and 7).

Recombination between the determinants for PAA resistance, plaque morphology, and BUdR resistance (TK$^-$ phenotype). Resistance to PAA was a stable property of virus clones in the absence of selective pressure. This, and the fact that resistance could be scored in a simple plating test, made it possible to look for recombination of the PAA resistance phenotype with other virus markers. The objectives of this series of experiments were: (i) to establish that recombination occurs between PAA resistance and other markers and to measure its frequency (see below); (ii) to examine the pattern of segregation of resistance phenotype among recombinant viruses (i.e., partial or complete) and the cosegregation of different resistance parameters; and, finally, (iii) to order the PAA resistance marker(s) (P^r) relative to other markers and illustrate the applications of this resistance marker for recombination mapping of herpesvirus genes. For these experiments we chose a combination with two other markers that are easily and accurately scored and that can be obtained in any desired strain of HSV-1 with little difficulty and with some expectation of a degree of genetic equivalence or identity (see Discussion), i.e., the plaque morphology variants syncytial (*syn*) and nonsyncytial (*syn*$^+$) and the TK$^+$ and TK$^-$ phenotypes (as determined by BUdR sensitivity or resistance, B^s or B^r). The combination of parental viruses was therefore a *syn*.B^s.P^r virus, HSV-1 (HFEM-P18 clone 1), and a *syn*$^+$.B^r.P^s virus, HSV-1 (B2006).

Data from plating a simple mixture of parental viruses and a sample of the yield from a mixed infection with these viruses is shown in

TABLE 2. *Effects of PAA on the synthesis and accumulation of virus-specific polypeptides representative of α, β, and γ control groups in cells infected with a PAA-sensitive strain of HSV-1*[a]

Control group	Infected cell polypeptide (ICP)	Relative amt (%) of polypeptide accumulated in the presence of disodium phosphonoacetate (mg/ml):					
		0	0.05	0.10	0.25	0.5	1.0
α	ICP4	100	110	100	105	89	110
β	ICP6	100	100	111	107	120	140
	ICP39	100	105	120	110	118	120
γ	ICP5	100	96	80	26	14	14
	ICP12	100	97	90	19	≤19	≤15
	ICP21	100	91	96	22	13	≤14
	ICP40	100	75	64	27	≤15	≤12
	ICP47	100	104	125	15	4	≤10
	ICP48	100	115	140	19	8	≤14

[a] Viral polypeptides labeled from 1.5 to 22 h after infection of BHK-21 cells with HSV-1 (HFEM) were quantitated by planimetry of absorbance profiles from autoradiograms such as those shown in Fig. 6. The amounts of each polypeptide made in the presence of the drug are expressed as percentages of the amounts made in untreated cultures.

Table 5 to illustrate the assays involved in analyzing the quantitative aspects of the recombination process. Recombination frequencies obtained from a number of complete experiments are summarized in Table 6. It may be seen that appropriately controlled plating tests in the presence of these drugs readily revealed high frequencies of recombination between plaque morphology markers and BUdR and PAA resistance determinants, the average frequencies being about 8% between *syn* and $B^{r/s}$ and about 18 to 20% between syn^{+} and $P^{r/s}$ (Table 6).

Analysis of recombinant clones: cosegregation of resistance parameters, segregation of unselected markers, and likely gene order. The relatively high frequency of presumptive recombinant types made it possible to undertake further analysis, both to confirm their recombinant character and to determine the pattern of segregation of unselected resistance markers and the cosegregation of properties with the resistance phenotype. For this purpose three clones of each parental type ($syn.B^{s}.P^{r}$ and $syn^{+}.B^{r}.P^{s}$) were "picked" from plates of a simple mixture, and 15 clones of each recombinant type ($syn.B^{r}$ and $syn^{+}.P^{r}$) were "picked" from the yields of a mixed infection plated under selective media. Viruses from these primary plaque purifications were grown and plaque-purified again by end point dilution and titration in nonselective media. Working stocks of each virus were then prepared. Of the initial "plaques," 6/6 parental clones and 10/15 ($syn^{+}.P^{r}$) and 8/15 ($syn.B^{r}$) recombinant clones were obtained with clonally uniform plaque morphology and in a sufficiently high titer for use in the following experiments.

Appropriate dilutions of each virus were first replated in a plaque reduction assay (i.e., in the absence of drugs and in the presence of 40 μg of BUdR and 160 μg of PAA per ml), and the plaque number and morphology were noted (Table 7). All plaques of a given clone were of the same morphology, and all presumptive $syn^{+}.P^{r}$ and $syn.B^{r}$ clones were resistant on replating with an efficiency that did not differ significantly from the resistant parentals tested in parallel (Table 7). In each recombinant class one clone with stable "mixed" plaque morphology was obtained, i.e., syn^{+}-*syn* (clone 3 of the $syn^{+}.P^{r}$ class) and *syn*-syn^{+} (clone 1 of the $syn.B^{r}$ class). When the recombinants were scored for the segregation of the unselected resistance marker it was found that 2/10 of the $syn^{+}.P^{r}$ clones were also fully resistant to BUdR (clones 4 and 15), the remainder being fully sensitive. All (8/8) $syn.B^{r}$ clones were P^{s} (Table 7). The most likely order for the three markers is therefore: syn-B^{r}-P^{r}. This order is

TABLE 3. *Kinetics of PAA-resistant γ-protein synthesis in cells infected with PAA-sensitive and -resistant viruses*

Labeling interval (h postinfection)	Infecting virus[a]	Rate of synthesis in presence of PAA/rate of synthesis in untreated cultures[b]		
		ICP4 (α)	ICP5 (γ)	ICP6 (β)
3–3.5	P^{s}	1.30	1.06	1.10
	P^{r}	0.91	0.96	1.00
7–7.5	P^{s}	0.90	0.26	1.40
	P^{r}	1.00	0.86	1.00
11.5–12	P^{s}	–[c]	0.08	0.80
	P^{r}	–	1.17	0.94

[a] P^{s}, PAA-sensitive virus, HSV-1 (HFEM) passage 0; P^{r}, PAA-resistant virus, HSV-1 (HFEM) passage 18, clone 1.

[b] Amounts of each polypeptide synthesized during the labeling intervals shown were quantitated by planimetry of absorbance tracings from autoradiograms such as those shown in Fig. 7. The amount of each polypeptide made in the presence of the inhibitor is expressed as a fraction of that made in the absence of the inhibitor. Absolute rates of synthesis by sensitive and resistant virus strains in the absence of the inhibitor did not differ significantly.

[c] –, Rates of synthesis in the presence and absence of inhibitor were too low to be measured reliably.

TABLE 4. *Homo- and heterotypic complementation of the growth of PAA-sensitive virus in a mixed infection with a resistant virus*[a]

Sample	Infecting virus(es)	Plaque morphology and resistance phenotype of infecting viruses	Input multiplicity (PFU/cell)	Yield (*syn* and/or *syn*$^+$, pfu/cell) from cultures incubated with: 0 μg/ml		250 μg/ml		% of control yield obtained in presence of drug	
				syn	*syn*$^+$	*syn*	*syn*$^+$	*syn*	*syn*$^+$
1	HSV-1(HFEM-PO)	*syn*.P^s	20	30		0.11		0.4	
2	HSV-1(B2006)	*syn*$^+$.P^s	20		23		0.62		2.7
3	HSV-1(HFEM-P18c1)	*syn*.P^r	20	36		44		≥100	
4	HSV-2(3345)	*syn*$^+$.P^s	20		28		0.15		0.54
5	HSV-1(HFEM-PO)+ HSV-1(B2006)	*syn*.P^s *syn*.$^+P^s$	20 +5	30	13	0.13	0.04	0.43	0.3
6	HSV-1(HFEM-P18c1)+ HSV-1(B2006)	*syn*.P^r *syn*$^+$.P^s	20 +5	42	7	25	2.5	60	36
7	HSV-1(HFEM-P18c1)+ HSV-1(B2006)	*syn*.P^r *syn*$^+$.P^s	16 +20	11.3	18.5	1.72	1.24	15.2	6.7
8	HSV-1(HFEM-PO)+ HSV-2(3345)	*syn*.P^s *syn*$^+$.P^s	20 +2	27	2.5	0.28	≤0.07	1.02	≤2.6
9	HSV-1(HFEM-P18c1)+ HSV-2(3345)	*syn*.P^r *syn*$^+$.P^s	20 +2	26.5	1.2	30.5	1.3	≥100	≥100

[a] Samples of 10^7 BHK-21 cells were infected in suspension with syncytial (*syn*) or nonsyncytial (*syn*$^+$), PAA-sensitive (P^s) or -resistant (P^r) strains of HSV-1 and HSV-2 either singly or in the combinations indicated. After an absorption period of 1 h, each infected cell suspension was divided into two samples of 5 × 10^6 cells, one sample being plated in medium without PAA (0 μg/ml) and the other in medium containing 250 μg of disodium phosphonoacetate per ml. Infected cultures were incubated at 37°C for 24 h, and at the end of this time the cells were harvested and disrupted by sonication, and the yield of infectious virus was titrated in BHK-21 cells in the absence of the drug. Syncytial (*syn*) and nonsyncytial (syn$^+$) plaques were scored independently.

TABLE 5. *Frequency of parental and apparent recombinant plaque types in the yield from a mixed infection and in a simple mixture of (syn.B^s.P^r) and syn$^+$.B^r.P^s) viruses*[a]

Sample	Plaque morphology	No. of plaques 0	+PAA	+BUdR	PFU/cell
Inoculum	*syn*	196	169	0[c]	20
Mixture	*syn*$^+$	222	0[c]	221	22
Yield	*syn*	194	234	32[c]	20
	syn$^+$	206	24[c]	216	20

[a] Parental viruses: *syn*.B^s.P^r, HSV-1(HFEM-P18c1); *syn*.$^+B^r$.P^s, HSV-1(B2006).

[b] Number of plaques on two 5.0-cm-diameter dishes of BHK-21 cells infected with appropriate dilutions of virus inoculum or yield and incubated with growth medium and carboxymethyl cellulose containing no drugs (0) or with PAA at a final concentration of 160 μg/ml or BUdR at a final concentration of 40 μg/ml.

[c] Plaques with presumptive recombinant phenotype.

consistent with the location of P^r further from *syn* than the distance of B^r to *syn* (Table 6).

In the next experiment virus of selected clones (see Table 7) was used to infect replicate samples of 4 × 10^8 to 6 × 10^8 cells at a multiplicity of 5 PFU/cell. Cell extracts were taken 14 to 18 h after infection and analyzed in gel diffusion tests for the presence of material reacting with anti-TK antiserum and for the PAA resistance or sensitivity of the virus DNA polymerase reaction in vitro. Results from these experiments are also summarized in Table 7. All the B^s recombinant clones tested reacted with anti-TK antiserum and all B^r clones failed to react, thus sharing the antigenic deficiency expected of the parental *syn*$^+$.B^r.P^s virus. More significantly, for all recombinant clones tested resistance or sensitivity of the virus DNA polymerase activity cosegregated with the resistance phenotype of the virus as measured by the plaque reduction assay (Table 7). Examination of selected *syn*$^+$.P^r clones by gel electrophoresis of labeled infected cell polypeptides showed that the variant polypeptide ICP12 (Fig. 6) of the resistant parental virus did not segregate with the resistance phenotype.

DISCUSSION

In this paper we have presented observations on some of the effects of PAA on HSV growth and virus protein synthesis in tissue culture. We have considered aspects of the selection of viruses resistant to the drug and have used these resistant viruses to provide additional biochemical and genetic evidence for the mode of action of the drug. Finally, we have given preliminary data on the genetics of PAA resistance and an illustration of the use of the resistance marker in the mapping of the herpesvirus

genome. Some of these data merit further comment.

To prevent virus growth and DNA synthesis in BHK-21 or HEp-2 cells infected at high multiplicities with sensitive strains of HSV-1, it was necessary to add at least 250 μg (1.44×10^{-3} M) of the disodium salt of PAA per ml to the culture medium before 3 h after the addition of virus to the cells. Infected cells maintained in the presence of the drug did not survive. Concentrations of PAA necessary to prevent virus growth also severely affected growth and division of uninfected cells, although these concentrations did not prevent cell adhesion or spreading and were not directly toxic to confluent monolayers of uninfected cells. Similar findings have been reported by Overby et al. (31) and by Huang (20). It is noteworthy that the inhibitory effects of the drug are slow to take effect and are inefficiently reversed and that there is a large (≥20-fold) discrepancy between the extracellular concentrations of PAA necessary to prevent virus growth and DNA synthesis and the concentrations required to inhibit the virus DNA polymerase in vitro (13, 27, 28, 31; Fig. 1, 2, 5). This suggests that (i) the permeability of cells to the inhibitor is low, (ii) the in vitro sensitivity of the DNA polymerase reaction is not a reliable measure of the sensitivity of its function in vivo, or (iii) the inhibitory action of the drug on virus replication involves an effect on a function other than, or in addition to, the virus DNA polymerase. We will consider further our data showing that different degrees of resistance of virus growth correlate with different degrees of resistance of the virus DNA polymerase in vitro and other evidence from recombinant viruses that a resistant DNA polymerase is probably necessary and possibly a sufficient condition for complete resistance in vitro. These latter observations clearly indicate that alternative (iii) does not apply and that there is at any rate a proportionality, if not equality, between the in vitro and in vivo drug sensitivity of the virus DNA polymerase. Although the structure of the acid (see Fig. 1) might lead us a priori to favor the first of the above alternatives, there is presently little information on factors governing the relationship between extracellular and intracellular concentrations of PAA. Differences between cell lines and growth conditions could affect this relationship. Detailed investigations of the permeability of cells to the inhibitor and factors affecting it will be aided by the synthesis of a radioactive derivative.

Effects of PAA on virus-specified protein synthesis and its control. Concentrations of PAA sufficient to prevent the production of infectious virus progeny and the synthesis of virus DNA reduced the amounts of all known polypeptides of the γ-group (17) to 5 to 15% of the amounts made in untreated infected cells. Differences in the response of different γ-polypeptides to intermediate concentrations of PAA (cf. ICP40 and 48 with 100 μg of PAA per ml) are presently unexplained. The drug had no significant effect on the synthesis of α- or β-polypeptides or on the synthesis of the virus-specific DNA polymerase or thymidine kinase. We note here that a selective reduction in the expression of the "viral capsid antigen" occurs

TABLE 6. *Summary of recombination frequencies between syn, B^r, and P^r in two- and three-factor crosses*

Cross[a] and expt no.	No. of *syn*.B^r plaques (y_1)	% Recombination between *syn* and B^r (x_1)[b]	Cross[a] and expt no.	No. of *syn*$^+$. P^r plaques (y_2)	% Recombination between *syn*$^+$ and P^r (x_2)[b]
A			B		
1	54	10.1	8	125	19.1
2	30	6.7	9	88	14.7
3	19	10.6	10	36	9.6
4	88	8.1			
C			C		
5	70	6.0	5	297	26.0
6	27	7.5	6	79	21.6
7	50	9.0	7	99	18.0

$\bar{x}_1$ = simple mean = 8.3% $\sigma_1 = \pm 1.7\%$ ($\bar{x}_{w_1}$ = "weighted" mean = 8.1%)[c]

$\bar{x}_2$ = simple mean = 18.2% $\sigma_2 = \pm 5.6\%$ ($\bar{x}_{w_2}$ = "weighted" mean = 21.3%)[c]

[a] Cross A: $syn^+.B^r \times syn.B^s$. Cross B: $syn^+.P^s \times syn.P^r$. Cross C: $syn^+.B^r.P^s. \times syn.B^s.P^r$.

[b] % Recombination = (number of recombinant plaques × 200)/total plaques.

[c] $\bar{x}_1 = \frac{\Sigma x_1}{n}$; $\bar{x}_{w_1} = \frac{\Sigma(x_{1a}y_{1a} + x_{1b}y_{1b} \cdots + x_{1n}y_{1n})}{\Sigma(y_{1a} + y_{1b} \cdots + y_{1n})}$ $: \sigma_1 = \pm \sqrt{\frac{\Sigma(x_1 - \bar{x}_1)^2}{n-1}}$.

TABLE 7. *Segregation of unselected resistance markers and cosegregation of B^r with TK^- and P^r with Pol^r phenotype in recombinant clones*

Virus	Virus clone	Plaques obtained in the presence of: 0	+P	+BUdR	Classification of unselected marker[a]	Reaction with anti-TK antiserum[b]	Resistance or sensitivity of viral DNA polymerase in vitro[c]
Parental viruses	*syn*.P^r-1	150	130	0	B^s	+	Pol^r
	syn.P^r-2	300	220	1	B^s	+	Pol^r
	syn.P^r-3	400	260	1	B^s	ND	ND
	syn$^+$.B^r-1	201	0	186	P^s	−	Pol^s
	syn$^+$.B^r-2	131	0	119	P^s	−	Pol^s
	syn$^+$.B^r-3	82	0	67	P^s	ND	ND
PAA-resistant recombinants	*syn*$^+$.P^r-1	414	236	0	B^s	+	Pol^r
	syn$^+$.P^r-2	120	129	1	B^s	+	Pol^r
	syn$^+$-*syn*.P^r-3	182	153	0	B^s	+	Pol^r
	syn$^+$.P^r-4	508	400	433	$\underline{B^r}$	−	Pol^r
	syn$^+$.P^r-5	133	85	0	B^s	+	Pol^r
	syn$^+$.P^r-6	577	327	1	B^s	ND	ND
	syn$^+$.P^r-8	160	120	0	B^s	ND	ND
	syn$^+$.P^r-11	75	68	0	B^s	ND	ND
	syn$^+$.P^r-13	208	160	0	B^s	ND	ND
	syn$^+$.P^r-15	80	106	67	$\underline{B^r}$	−	Pol^r
BUdR-resistant recombinants	*syn*-*syn*$^+$.B^r-1	141	0	102	P^s	ND	ND
	syn.B^r-2	201	0	186	P^s	−	Pol^s
	syn.B^r-4	90	0	97	P^s	−	Pol^s
	syn.B^r-8	78	0	94	P^s	−	Pol^s
	syn.B^r-9	113	0	149	P^s	−	Pol^s
	syn.B^r-11	105	4	98	P^s	ND	ND
	syn.B^r-12	154	0	128	P^s	ND	ND
	syn.B^r-13	120	0	110	P^s	ND	ND

[a] Classification on the basis of plaque reduction data.

[b] Cytoplasmic fractions from Dounce-homogenized infected cells were tested in immunodiffusion reactions against anti-TK antiserum: +, positive gel diffusion test; −, negative gel diffusion test; ND, not done.

[c] Nuclei from Dounce-homogenized infected cells were sonicated, and samples of sonicated nuclei were incubated for the measurement of DNA polymerase activity in the presence of 5 μg of disodium phosphonoacetate per ml and in the absence of the drug. Resistant DNA polymerase (Pol^r) gave $\geq$70% of the activity in the absence of the drug; sensitive DNA polymerases (Pol^s) gave $\leq$20% of the activity in the absence of the drug.

together with the loss of nonintegrated copies of the Epstein-Barr virus genome from producer cell lines cultured in the presence of PAA (40, 46). The expression of the Epstein-Barr virus "early antigen" was not affected by the drug (40). Experiments of the present paper indicate that the observed reduction in HSV-1 "late" protein synthesis is attributable to the failure of PAA-treated cells to sustain the synthesis of γ-polypeptides and not to an effect on the "switch on" of γ-protein synthesis. Rates of synthesis were identical in treated and untreated cultures until about 4 h after infection (Fig. 7, Table 3) and thus reached more than 20% of the maximum rate of γ-protein synthesis observed in untreated cells (17). In the presence of PAA, rates of γ-protein synthesis in cells infected with sensitive strains of the virus declined after 6 h together with the synthesis of β-polypeptides. In the absence of the drug (or with resistant virus strains in the presence of the drug, Fig. 7) rates of γ-protein synthesis continued to increase. These results support the view that normal synthesis of virus DNA is not a requirement for the initiation of γ-protein synthesis (see Introduction). To sustain the contrary interpretation (43), it is essential to show that the methods used are capable of differentiating between a reduced accumulation of late proteins in the absence of DNA synthesis and an obligate requirement for DNA synthesis before any late protein can be synthesized.

In conclusion, we wish to emphasize that our earlier experiments have shown that a control

function of α- and/or β-polypeptides is necessary to switch on γ-protein synthesis (17). The present results do not exclude the possibility that this control function is normally performed by an enzyme of DNA metabolism provided that such a function is insensitive to PAA and does not operate via the production of stoichiometrically normal amounts of virus DNA. As with all similar experiments, we cannot presently exclude an obligate requirement for small amounts of de novo DNA synthesis which persist in the presence of the inhibitor.

Selection and properties of PAA-resistant mutants of HSV-1. The frequency of occurrence of resistant viruses and the processes operating during their selection were of obvious relevance to applications of the drug in vivo. Moreover, knowledge of the biochemical and genetic properties of resistant viruses was of interest both to studies on the mode of action of the inhibitor and as a prerequisite for the exploitation of the resistance marker in more general analyses of herpesvirus genetics.

The pertinent results from the present paper may be summarized as follows. (i) Viruses resistant to PAA were invariably selected during passage in the presence of the drug. We presently have no accurate estimates of the frequency of occurrence of mutations conferring resistance to different levels of the drug.

(ii) Resistant viruses do not appear to be at a selective disadvantage in tissue culture in the absence of PAA. Natural or artificial mixtures of resistant and sensitive viruses maintained the same composition during passages in the absence of the drug. Resistant viruses reached the same titer as sensitive viruses in single infections.

(iii) Stable clones of virus mutants with different degrees of resistance to PAA could be isolated from virus populations subjected to different numbers of passages in the presence of the drug. In all six of the passage series we have so far performed, the appearance of resistance to low levels of PAA (50 μg/ml) preceded the appearance of resistance to high levels (200 μg/ml). In at least one series (Fig. 3 and 4), the emergence of variants resistant to 200 μg/ml involved sequential selection operating on mutations arising during the passage series in a member of a population resistant to lower concentrations of the drug. Changes in relative proportions of viruses resistant to different levels of PAA and in unselected markers for components of the virus population clearly show that in at least this one passage series the emergence of highly resistant viruses was a multistage process. The effects of different regimes of selection have not been investigated.

(iv) Although the characteristics of the selection process outlined above infer that multiple mutations are required to confer resistance to high concentrations of the drug, all measured parameters of resistance changed concurrently. Thus, increased resistance of clones measured by plaque formation and yield were correlated with increased resistance of γ-protein synthesis, virus DNA synthesis, and the virus DNA polymerase. Hay and Subak-Sharpe (13) have also shown that mutants of HSV-1 and -2 resistant to 100 μg of PAA per ml in a plaque reduction assay have increased resistance of in vivo virus DNA synthesis correlated with increased resistance of DNA synthesis in nuclei isolated from infected cells and of the virus DNA polymerase reaction in vitro. These correlations may be misleading to some extent since the initial criterion for considering a virus "resistant," both here and elsewhere (13), was plaque formation in the presence of the drug. Whereas the resistance of the DNA polymerase is apparently a necessary condition for resistance of plaque formation and yield, our only evidence that it is a sufficient condition is derived from studies of recombinant viruses (see below).

(v) No clones of virus with a PAA-dependent phenotype have yet been obtained, although one clone of passage 18 reproducibly grows to approximately twofold higher titers in high-multiplicity infections incubated in the presence of 250 μg of PAA per ml than in cultures without the drug. It appears that mutation to PAA dependence is an extremely rare event or that mutations are at a selective disadvantage to those conferring resistance. Further passages of resistant strains may result in the emergence of drug-dependent viruses, and deliberate regimes of selection can obviously be devised.

Co-dominance of resistance and sensitivity in mixed infections of sensitive and resistant viruses. A clone of HSV-1 (HFEM) fully resistant to concentrations of PAA that would prevent the growth of "wild-type" viruses was able to complement the growth of sensitive strains of homologous (HSV-1) or heterologous (HSV-2) type in dual infections in the presence of the drug (Table 4). The proportion of recombinant viruses in the presence of the drug in progeny of homotypic mixed infections did not differ significantly from the proportion observed in the absence of the drug (see below), and thus the effect observed was complementation and not rescue of the plaque morphology marker of the sensitive parental virus by recombination. An obvious inference from inter- or heterotypic complementation of growth in the presence of PAA is that the resistant HSV-1 polymerase

was able to replicate HSV-2 DNA in a mixed infection. Complementation and recombination between ts mutants of HSV-1 and HSV-2 have been reported (9, 42), and in their interesting analysis Esparza et al. (9) showed that HSV-1 would complement a DNA-negative HSV-2 mutant with a suspected ts lesion in the virus DNA polymerase. Our own results also showed that in mixed infections of PAA-sensitive and -resistant strains not only did growth of the sensitive partner become more resistant, but growth of the resistant partner became more sensitive than in the single infection. That is, resistance and sensitivity were co-dominant. The observed resistance of the mixed infections was also significantly less than would be predicted from a simple proportionality related to the multiplicities of resistant and sensitive partners. This finding and some more general aspects of the effects of gene dosage are presently under more detailed investigation. However, one possible interpretation of the observation that addition of sensitive virus had a disproportionate effect on the sensitivity of a mixed infection is that the PAA-sensitive function is multimeric and that the addition of a minority of sensitive subunits to a pool of resistant subunits renders resultant multimers of mixed composition fully sensitive.

Genetics of PAA resistance and the utility of the resistance marker. Recombination of the PAA resistance marker(s) occurs at high frequency with respect to plaque morphology and BUdR resistance (TK gene) markers. Measurements of recombination frequency and analysis of the segregation of the unselected marker in recombinant viruses indicate that the PAA resistance marker(s) is located further from *syn* than is the TK gene and on the same "side" of *syn* as the TK gene. The sources of the rather large standard deviations for the mean estimates of recombination frequency are presently under investigation, but the variability observed does not seem to be atypical (4, 5, 41). This problem was recognized by Brown et al. (5), and they have used an unselected plaque morphology marker to assist in ordering ts mutations. However, the number of ts markers showing sufficient differences in linkage to *syn* for its segregation to be informative is obviously limited. The PAA resistance marker(s), which is well separated from *syn*, should provide a valuable additional aid to this form of analysis. We should note here that the syncytial marker we have used in this work cannot be assumed to be genetically identical to that of Brown et al. (5). The *syn* and syn^+ properties of the viruses we have used are stable, whereas the markers used in their previous work show a rather high "reversion frequency," variously estimated at from 10^{-4} (4) to 10^{-6} (5). Whereas it seems likely that such frequencies imply a single gene change, this merely means that the syn/syn^+ character may be determined by a single gene, not that it is exclusively and invariably determined by this gene. In view of the multiplicity of glycoproteins specified by HSV (38, 39), it would not be surprising if multiple loci could be involved in determining this property. The isolation of recombinant viruses with stable "mixed" plaque morphology indicates that more than one gene is involved in determining the difference between the plaques of the *syn* and syn^+ viruses used in these studies. Crosses between recombinants with mixed plaque morphology should prove informative.

The observations of this paper that (i) recombinant viruses were either fully resistant or fully sensitive and that (ii) the resistance character of recombinant viruses was correlated with the drug resistance of the virus DNA polymerase reaction in vitro are clearly consistent with the enzyme as the sole site of action of PAA. However, further investigation of the resistance marker is essential before its equivalence to the structural gene(s) for the virus DNA polymerase can safely be assumed. In particular, it is necessary to analyze recombinants with markers located close to and on either side of the the resistance marker(s) in order for cosegregation to provide convincing evidence of identity. Such an analysis is presently being undertaken (R. W. Honess, A. Buchan, and I. W. Halliburton, unpublished data). It may be noted here that ts mutants with suspected lesions in the virus DNA polymerase (1, 12, 34), i.e., *ts pol*, should constitute an appropriate test for the identity of the PAA resistance marker. Thus, in a cross $tsX.ts\ pol^+.P^r$ with $tsX^+.ts\ pol.P^s$ all the ts^+ progeny should be P^r, and in the reciprocal cross all ts^+ progeny should be P^s if P^r is identical to pol^r or is located beyond *pol* with respect to the *tsX* site. Performance of this test with PAA-resistant forms of ts mutants on either side of the suspected polymerase mutation should be capable of providing convincing evidence for identity or nonidentity of P^r and pol^r.

ACKNOWLEDGMENTS

The present work was begun by one of us (R. W. H.) in Bernard Roizman's laboratory in Chicago and was continued in Leeds. The work was begun after the communication of the preliminary unpublished data of Lacy Overby and his collaborators at Abbott Laboratories (North Chicago, Ill.), and we gladly acknowledge both this and the subsequent gifts of PAA from Abbott Laboratories. We are grateful to the Medical Research Council for a project grant. Technical assistance in Leeds was provided by S. Pullen and A. Reader.

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