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Reversible Inhibition of Herpes Simplex Virus Replication by Hydroxyurea

HERBERT S. ROSENKRANZ AND YECHIEL BECKER

Laboratory for Molecular Virology, Department of Virology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Hydroxyurea, at a concentration of 5×10^{-2} M, inhibits the replication of herpes simplex deoxyribonucleic acid (DNA) in the nuclei of infected cells. As a result, the synthesis of infectious virus progeny was prevented. The presence of parental viral DNA genomes in inhibited cells led to the synthesis of the viral structural peptides. The inhibitory effect of hydroxyurea was reversible; after washing the cells free from hydroxyurea, virus progeny appeared after a lag of 3 h. Upon resumption of viral DNA replication, the content of radioactive viral structural peptides gradually increased in parallel with the increase in mature virions. It is concluded that the information for the synthesis of viral structural peptides is transcribed from the parental DNA genomes.

The effect of hydroxyurea on the replication of herpes simplex virus (HSV) is due to the inhibition of HSV deoxyribonucleic acid (DNA) replication (8). Nii et al. (8) demonstrated by electron microscopy that empty viral capsids are synthesized in the nuclei of infected cells treated with hydroxyurea. With the aid of fluorescein-tagged antibodies, the synthesis of virus-specific antigens was demonstrable. It was therefore concluded that the parental viral DNA genomes code for structural capsid proteins (8). This finding is in agreement with our observation that in cytosine arabinoside-treated infected cells, in which viral DNA replication was fully inhibited, structural capsid proteins were synthesized (Becker and Olshevsky, in preparation). In the present study, the effect of hydroxyurea on the synthesis of viral DNA and proteins was studied. It was found that the inhibition of viral DNA replication by hydroxyurea abolished the synthesis of progeny virions, but the synthesis of the viral structural peptides was only partly affected. The inhibitory effect of hydroxyurea was reversible, and after the removal of the drug mature virions were formed.

MATERIALS AND METHODS

Virus and cells. The HF strain of herpes simplex virus was propagated in BSC_1 cells. Four-day-old BSC_1 monolayers were infected with 10 plaque-forming units/cell and incubated in Eagle's medium at 37 C. Under such conditions, the viral growth cycle is completed within 18 h after infection.

Infectivity determinations. Samples of infected cells were sonically disrupted and diluted in Eagle's medium supplemented with 5% calf serum. The virus content was determined by seeding samples on BSC_1 cell monolayers in plastic dishes (50 mm in diameter, NUNC, Denmark). The infected cultures were overlaid with agar and incubated for 3 days in a CO_2 incubator. The cultures were stained with neutral red and plaques were counted.

Isolation of herpesvirions. Infected cultures, untreated as well as exposed to different concentrations of hydroxyurea, were harvested by scraping 18 h after infection. The cell suspensions were sonically treated and centrifuged in 12 to 52% (wt/wt) sucrose gradients (1). This technique permits the isolation of the enveloped virions, nucleocapsids, and empty capsids in separate distinct bands (2). The amount of virions in the different preparations was determined by supplementing the infected cells with radioactive amino acids to label the viral proteins. The sucrose gradients were collected dropwise, and the radioactivity in each fraction was determined in a Packard scintillation counter after precipitation with trichloroacetic acid (5%, vol/vol).

CsCl gradients of DNA. Cells infected in the presence and absence of hydroxyurea (5×10^{-2} M) were incubated with ^{3}H-thymidine (1 μCi/ml) to label the DNA. The DNA molecules were released from the cells by treatment of the cells with sodium dodecyl sulfate (SDS, 1% wt/vol) and incubated overnight with Pronase (5 mg/ml) at 37 C. To each sample in 0.01 M tris(hydroxymethyl)-aminomethane (Tris), pH 8, containing 0.001 M ethylenediaminetetraacetate CsCl crystals were added and the density was adjusted to 1.69 g/ml. The tubes were centrifuged in the 50 Ti rotor of a Beckman preparative ultracentrifuge for 48 h at

40,000 rpm at 20 C. The gradients were collected from the bottom, and the refractive indices of selected fractions were determined in a Bausch & Lomb refractometer. Purified ^{14}C-labeled HSV DNA was used as a marker (14).

Analysis of labeled proteins. The infected cultures were labeled with ^{3}H-leucine (1 μCi/ml) during infection. The cells were then scraped off the glass and dissolved in SDS buffer (0.005 M Tris, 0.1 M NaCl, 0.5% SDS, pH 7.3). The radioactive proteins were analyzed by electrophoresis in acrylamide gels by the technique of Maizel (6) as described previously (10). The gels were sliced and the radioactivity in each slice was determined. The total radioactivity in each gel and the radioactivity present in each peptide band were calculated.

Reversion of hydroxyurea effect. The hydroxyurea-treated cells were washed twice with medium and reincubated at 37 C in the presence of the required radioactive precursors. Samples of infected cultures were removed at different time intervals, and the radioactivity and infectious virus content were determined.

Materials. Hydroxyurea was obtained from Sigma Chemical Co., St. Louis, Mo. Thymidine-methyl-^{3}H (specific activity, 12.0 Ci/mmol) and ^{3}H-leucine (specific activity, 390 mCi/mmol) were from the Radiochemical Centre, Amersham, England. CsCl was purchased from B.D.H. Chemicals, Poole, England, and Pronase was from Calbiochem AG, Switzerland. Materials for gel electrophoresis were as reported (11).

RESULTS

Effect of hydroxyurea on the synthesis of herpesvirions. HSV-infected BSC_1 cells received different concentrations of hydroxyurea (ranging from 10^{-3} to 5×10^{-2} M) immediately after infection. It was found (Fig. 1) that 10^{-3} M hydroxyurea only partially affected the synthesis of infectious virus, whereas in the presence of 5×10^{-2} M hydroxyurea the formation of infectious progeny was inhibited. A concentration of 5×10^{-3} M hydroxyurea, which reduced the infectious virus yield by 2.5 logs, also prevented the formation of mature enveloped virions but allowed the synthesis of the viral nucleocapsids (Fig. 2). In the presence of 5×10^{-2} M hydroxyurea, the formation of the viral capsids was markedly affected, but radioactive capsids still were detected in the sucrose gradient of infected treated cells. These results demonstrated that hydroxyurea inhibits the synthesis of mature infectious herpesvirus progeny, in agreement with the electron microscopy studies (8).

Effect of hydroxyurea on viral DNA and protein synthesis. Analysis of the DNA synthesized in herpesvirus-infected BSC_1 cells by CsCl density gradient centrifugation (Fig. 3) distinguished between two DNA species: the

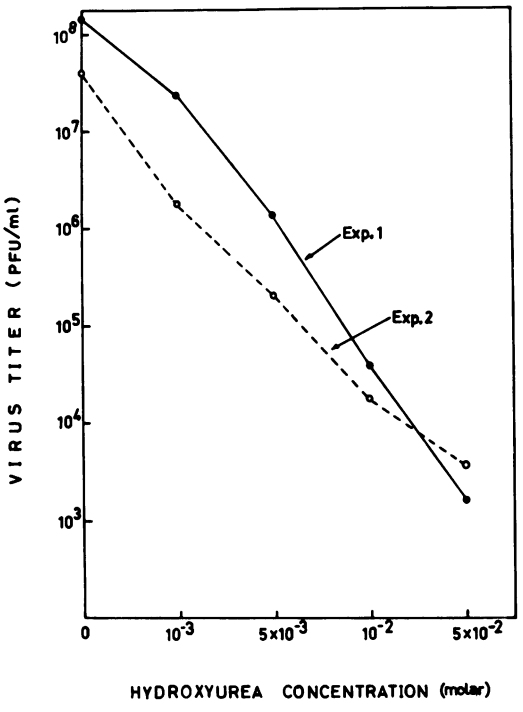

FIG. 1. *Effect of hydroxyurea on the formation of infectious herpesvirus progeny in infected cells. BSC_1 cells were infected with HSV and treated with different concentrations of hydroxyurea. Samples were obtained from infected cells at the end of the virus growth cycle and sonically treated; the yield of infectious virus was then determined by the plaque assay.*

cellular DNA which banded at a density of 1.696 g/ml and the viral DNA which banded at a density of 1.718 g/ml (Fig. 3A). Treatment of infected cells with 5×10^{-2} M hydroxyurea abolished the synthesis of viral DNA (Fig. 3B) and markedly affected the synthesis of cellular DNA.

To characterize the viral proteins synthesized in the infected cells in the absence of viral DNA replication, we labeled untreated and hydroxyurea-treated (5×10^{-2} M) infected cells with ^{3}H-leucine; the cells were harvested at the end of the virus growth cycle, and the labeled proteins were analyzed by electrophoresis on acrylamide gels. In both untreated and hydroxyurea-treated infected cells, viral structural peptides were synthesized (Fig. 4). The main difference between the two preparations was that less radioactivity, 50% of the untreated control, was incorporated into the viral peptides V and VI in the hydroxyurea-treated cells (Fig. 4A). The synthesis of peptides II (the major capsid peptide) and III (envelope glycopeptide) in the hydroxyurea-

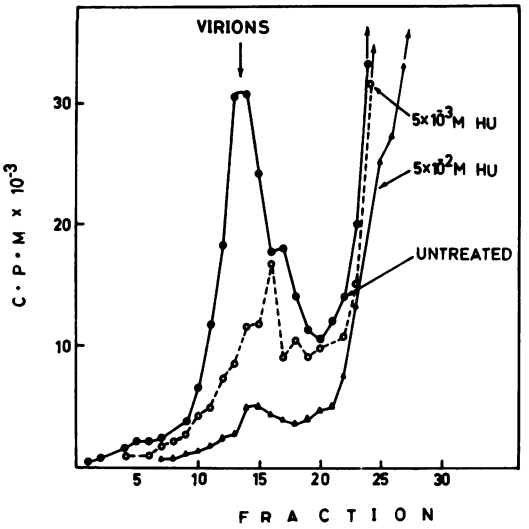

FIG. 2. *Effect of hydroxyurea on the synthesis of herpes simplex virions. Herpes simplex virus-infected cells were incubated in the presence of hydroxyurea and ³H-leucine. At the end of the virus growth cycle, the untreated and hydroxyurea-treated infected cells were harvested and sonically treated; the homogenates were centrifuged in sucrose gradients (12 to 52%, wt/wt). The gradients were collected and the radioactivity in each fraction was determined.*

treated cells (Fig. 4B) indicated that viral structural peptides are coded by HSV parental genomes. This finding extends the observation of Nii et al. (8) that viral nucleocapsids (seen by electron microscopy) were present in hydroxyurea-treated cells. This result is in agreement with the finding (Becker and Olshevsky, in preparation) that viral structural peptides were synthesized in cytosine arabinoside-treated herpesvirus-infected cells.

Reversibility of hydroxyurea inhibitory effect. The inhibitory effect of hydroxyurea on DNA synthesis is reversed upon removal of the drug (15). It was therefore of interest to determine the time course of HSV replication after removal of the inhibitor. HSV-infected BSC_1 cells, treated with 5×10^{-2} M hydroxyurea, were washed with two changes of fresh medium and reincubated at 37 C; then samples were withdrawn for determinations of the synthesis of the infectious virus progeny. We found (Fig. 5) that mature virions were formed with a lag period of 3 to 5 h after the removal of the inhibitor. Maximal virus yield was obtained at 12 h after the removal of the inhibitor.

The time course of herpes simplex virus synthesis, after the removal of the drug, was determined by isolation of ³H-leucine-labeled

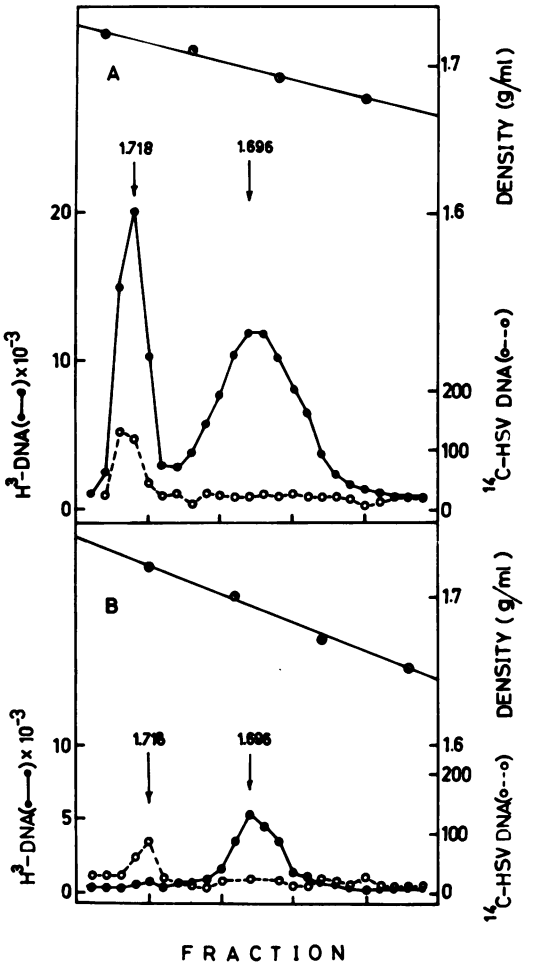

FIG. 3. *CsCl gradient analysis of the DNA species synthesized in the absence and presence of hydroxyurea in infected cells. Two cultures were infected with herpes simplex virus (HSV) and labeled with ³H-thymidine. To one culture, hydroxyurea (5×10^{-2} M) was added; the other served as a control. The cultures were incubated for 18 h; the DNA was then extracted and analyzed by centrifugation in CsCl gradients. To each gradient, HSV ¹⁴C-DNA was added as a marker. (A) DNA from HSV-infected cells. (B) DNA from HSV-infected hydroxyurea-treated cells. Symbols: ●, ³H-DNA; ○, ¹⁴C-HSV DNA marker.*

virions in sucrose gradients (Fig. 6). It was found that more than 3 h were needed for the detection of the initial progeny of virions. The amount of labeled virions gradually increased thereafter, in a manner similar to the increase in infectious virus, and reached maximal yield 15 to 18 h after the removal of the inhibitor. Only a slight increase in the radioactivity in the virions band was found at 24 h.

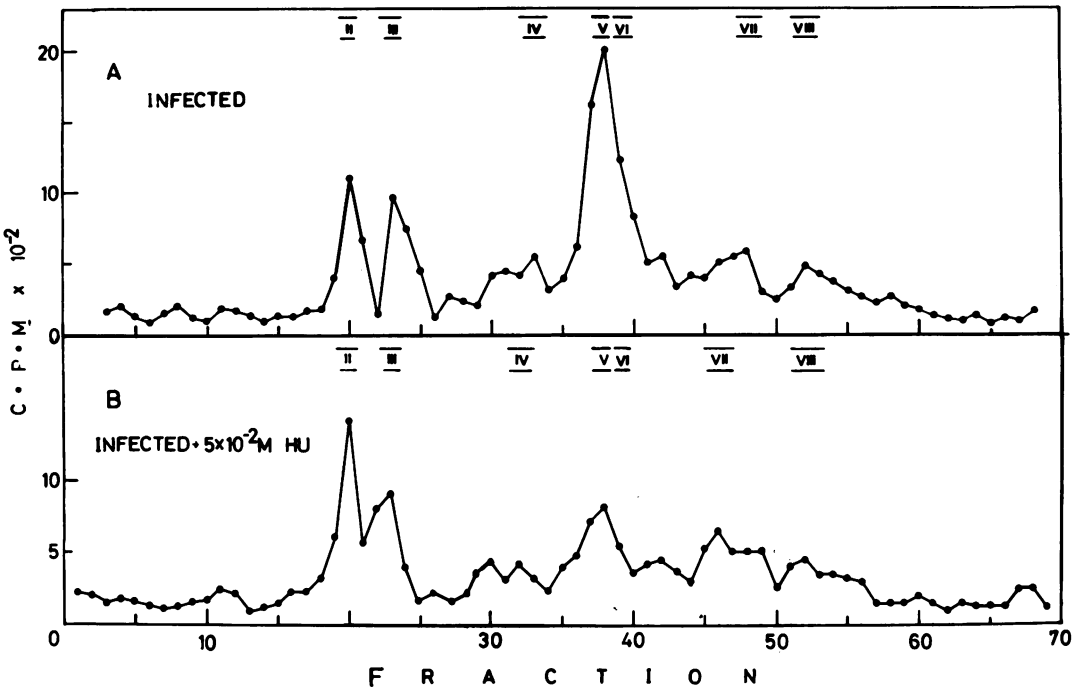

FIG. 4. *Analysis of viral structural peptides synthesized in herpes simplex virus (HSV)-infected cells in the presence of hydroxyurea. Two cultures were infected with HSV and were labeled with ^{3}H-leucine. One culture was also treated with 5×10^{-2} M hydroxyurea, and the second served as a control. After incubation for 18 h, cells were harvested and solubilized with a solution of 1% sodium dodecyl sulfate. The preparations were analyzed by electrophoresis in acrylamide gels, and the position in the gels of the viral structural peptides are indicated by the Roman numerals. (A) Infected cells. (B) Infected, hydroxyurea (5×10^{-2} M)-treated cells.*

The synthesis of viral structural peptides in the infected cells was studied at different time periods after the removal of hydroxyurea by continuously labeling the cells with ^{3}H-leucine. The synthesis of all of the detectable radioactive viral structural peptides was found to increase gradually after the removal of the inhibitor (Fig. 7). A marked increase was found at 9 h after reversal (Fig. 7), and a maximal content was reached 18 h after the removal of the antibiotic (not shown). It is of interest that high-molecular-weight proteins were detected at the top of the gel at 3 and 6 h after the removal of the inhibitor but not at 9 and 18 h. These peptides are of a molecular weight higher than 200,000 daltons and might be precursors of the viral peptides. A quantitative analysis of the viral peptides is given in Table 1. The decrease in content of the radioactive "precursor" and the increase in the content of the viral peptides is shown. This result demonstrated that the synthesis of the viral DNA makes possible the synthesis of viral structural peptides and allows the completion of the processes which lead to the assembly of the viral structural peptides into virions. The peptides which are responsible for the maturation of herpesvirions were synthesized after the initiation of viral DNA replication and might be gene products of the viral progeny DNA. It is of interest that, although viral peptides were synthesized in the absence of viral DNA synthesis, the virus cycle after removal of the inhibitor resembled the virus growth cycle in uninhibited HSV-infected cells (11).

DISCUSSION

Hydroxyurea was found to inhibit the formation of DNA viruses by inhibiting the replication of the viral parental genomes in the infected host cells. However, hydroxyurea does not interfere with the synthesis of messenger ribonucleic acid (RNA) species by the DNA-dependent RNA polymerases and does not directly affect the synthesis of functional proteins (15, 17). As a result of its properties, hydroxyurea inhibits a large number of mammalian DNA viruses (vaccinia [12, 13, 16], frog kidney virus [18], African swine fever virus [3], infectious bovine rhinotracheitis

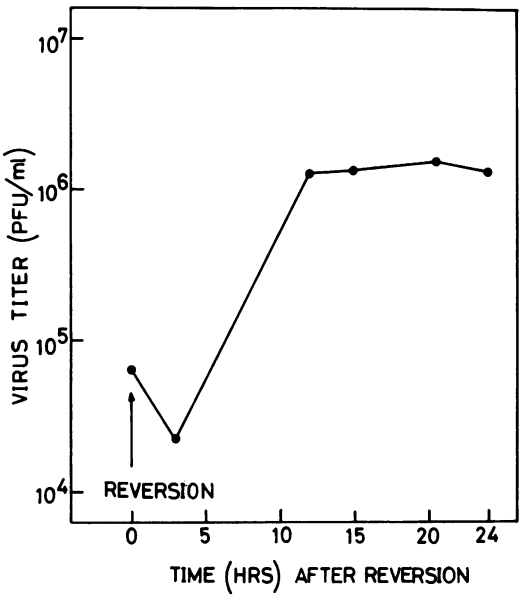

FIG. 5. *Synthesis of infectious virus progeny after removal of hydroxyurea. Infected cultures were incubated for 18 h with hydroxyurea (5×10^{-2} M). The inhibitor was removed by washing the infected cells with two changes of medium, and the cultures were reincubated at 37 C in fresh medium. Samples were removed and infectious virus was determined.*

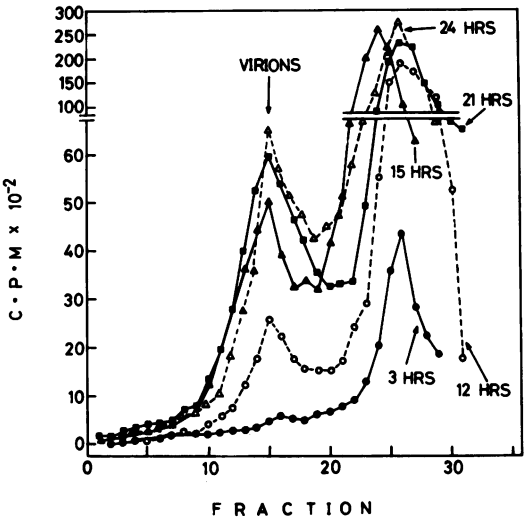

FIG. 6. *Synthesis of labeled herpesvirions in hydroxyurea-treated cultures after the removal of hydroxyurea. A series of infected cultures treated with 5×10^{-2} M hydroxyurea were incubated for 18 h. The cultures were then carefully washed and reincubated in fresh medium in the presence of ^{3}H-leucine. At different time intervals (3, 12, 15, 21, and 24 h after the removal of the drug), cultures were withdrawn, sonically treated, and centrifuged in a sucrose gradient under conditions which permit the isolation of herpesvirions. The gradients were collected and the trichloroacetic acid-precipitable radioactivity was determined in each fraction. The results of five different gradients are shown in the figure.*

virus [4], herpesvirus [8], adenovirus [5], polyoma virus [9], and bacterial viruses T4 [7] and T5 [19]). The mode of action of hydroxyurea is not yet known.

The most striking feature of hydroxyurea inhibition of HSV replication is the synthesis of viral structural peptides and empty viral capsids (8) in the absence of viral DNA synthesis. These findings indicate that the parental viral DNA genomes are transcribed in the infected cells and the viral messenger RNA molecules, transcribed by an unknown RNA polymerase, and translated by the host-cell protein-synthesizing apparatus to yield the structural peptides. The peptides II and VIII (the constituents of the viral capsid [10]) assemble into capsids (8) in the absence of progeny DNA. Further assembly processes are inhibited in the absence of viral DNA synthesis. The viral glycopeptides III, IV, and V (10) were also synthesized in the presence of hydroxyurea, indicating that the messenger RNA species transcribed from the parental DNA genomes contain the information for various structural peptides. Further studies to characterize the messenger RNA species synthesized in the hydroxyurea-treated cells are in progress.

After removal of hydroxyurea, the parental viral DNA genomes are replicated, and the increase in the number of viral DNA molecules per cell leads to the synthesis of additional messenger RNA species responsible for further synthesis of radioactive viral structural peptides in the infected cells. At least 3 h elapse between the removal of the inhibitor from the infected cells and the formation of mature herpesvirions. It was previously reported (11) that 2 h are needed for the formation of mature virions. Thus, it is possible that the delay in formation of virions is due to processes which precede viral DNA replication. Preliminary studies revealed that the viral peptides synthesized in the hydroxyurea-treated cells were utilized for the formation of virions after the removal of the inhibitor (not shown). However, it is also possible that these peptides were not assembled into empty capsids in the presence of hydroxyurea and that they function only upon the removal of the inhibitor. These results suggest that the progeny DNA is packed into the preformed empty nucleocapsids which assemble independently of the viral DNA. Only the viral nucleocapsids which contain the genomes are capable of association with cellular

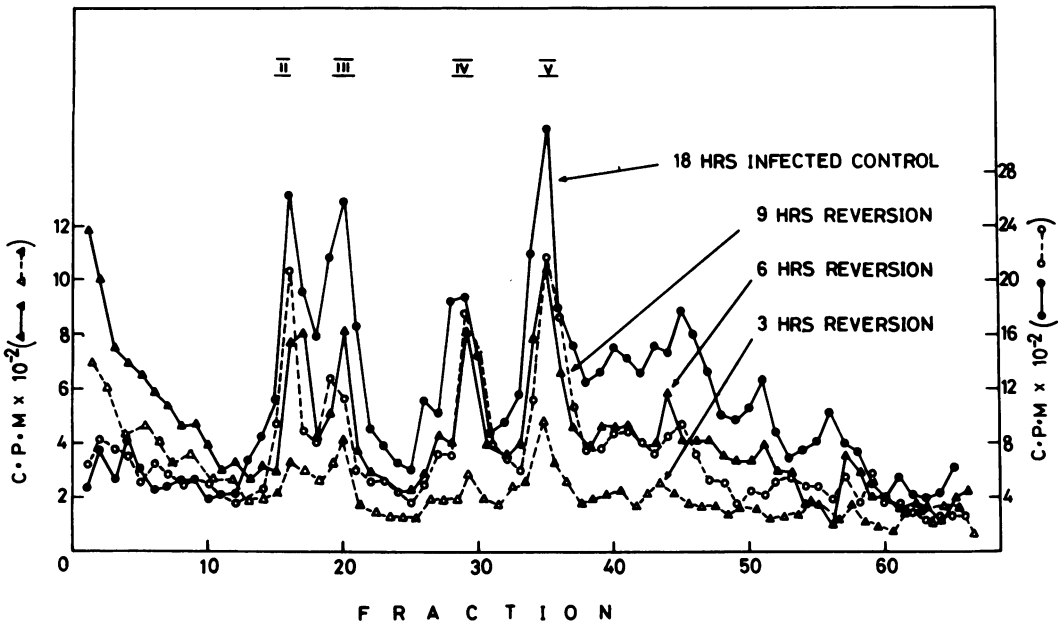

FIG. 7. *Characterization of peptides synthesized in herpes simplex virus (HSV)-infected cells after removal of hydroxyurea. The experiment was carried out as described in Fig. 6. Samples obtained from the hydroxyurea-treated HSV-infected cells were harvested at different time periods (3, 6, 9, and 18 h) after the removal of the drug. HSV-infected cells, similarly labeled, were harvested at 18 h postinfection and were used as a control for viral protein synthesis. The cells were sonically treated, and a sample from each preparation was analyzed by electrophoresis in acrylamide gels after treatment with SDS, urea, and mercaptoethanol. The gels were sliced and the radioactivity in each slice was determined. The analysis of four samples (3, 6, 9 [reversal] and 18 h [control]) were plotted in the figure. The 18-h reversal sample resembled the untreated control. Note different scales of radioactivity.*

TABLE 1. *Synthesis of herpes simplex virus structural peptides in infected cells after the removal of hydroxyurea*

Viral peptide no.	Percentage of total radioactivity in the gel						
	Expt 1				Expt 2		
	3 h[a]	12 h	15 h	21 h	3 h	6 h	9 h
"Precursors"[b]	65.5	48.0	20.5	29.6	13.5	24.2	13.1
II[c]	2.6	3.8	6.9	9.1	6.2	7.4	10.8
III[c]	5.9	6.2	8.1	8.0	7.2	9.1	8.7
IV[c]	2.7	8.7	11.5	10.6	4.3	10.7	14.5
V + VI[c]	8.2	28.0	26.0	26.6	12.7	14.3	17.9

[a] Time after removal of hydroxyurea.

[b] "Precursors" were the peptides which banded in the first centimeter of the gel.

[c] The area below all the radioactive bands in each acrylamide gel was determined and compared with bands from similar untreated, infected cells which were taken as 100%. Bands VII and VIII were less than 3% of the total radioactivity.

membranes, modified by the viral glycopeptides, to form mature virions.

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