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Membrane Proteins Specified by Herpes Simplex Viruses

I. Identification of Four Glycoprotein Precursors and Their Products in Type 1-Infected Cells

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Polypeptide precursors to the major glycoproteins specified by herpes simplex virus type 1 were identified in immunoprecipitation experiments using antisera that reacted specifically with the viral glycoproteins and their precursors. The results demonstrate that the major glycosylated proteins detected in infected cells are derived from four antigenically distinct polypeptides. Three of these polypeptides become glycosylated in two discrete stages, yielding partially glycosylated intermediates and fully glycosylated products. The final products are the predominant species detected in cytoplasmic virions and in plasma membranes. The fourth polypeptide precursor appears to acquire very little carbohydrate and differs in several respects from the other three precursors.

Glycoproteins specified by herpes simplex virus (HSV) have been detected in the envelopes of virions (11, 19) and in purified membranes from infected cells (7, 18). Some or all of these proteins are probably essential for the envelopment and infectivity of virions (reviewed by P. G. Spear, *in* H. A. Blough and J. M. Tiffany [ed.], *Cell Membranes and Viral Envelopes*, in press), and they may also play a role in the altered social behavior of infected cells. A correlation has, in fact, been noted between the kinds of altered cell interactions observed (cell fusion or foci of cells having characteristic morphologies) and the spectrum of HSV glycoproteins detected in purified membranes from the cells (10).

The biochemical and biological properties of individual HSV glycoproteins have not yet been defined and, in fact, the actual number of glycoproteins specified by the virus is not known with certainty. Analyses of purified HSV-1 virions by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis revealed the presence of at least four major glycosylated species and several minor ones, most of which had very heterogeneous migration rates (6). The HSV-1 glycoproteins detected in purified plasma membranes are probably similar or identical to the virion glycoproteins, based on electrophoretic comparisons in SDS-acrylamide gels (7) and on immunological studies (13, 14). Recently, however, it has been noted that unfractionated HSV-1-infected cells contain some glycosylated viral polypeptides that are not present in virions or in plasma membranes (9). The purpose of the studies reported here was to characterize the polypeptide precursors to the HSV-1 glycoproteins and to determine whether some of the electrophoretically differentiable glycoproteins detected in infected cells might be related to each other in that they share all or part of the same polypeptide chain. Results obtained in immunoprecipitation experiments, using antisera directed against the HSV-1 glycoproteins, revealed that the major glycosylated proteins present in virions and in infected cells are derived from four antigenically distinct polypeptides and that three of these polypeptides can each exist in two glycosylated forms, one being an intermediate in the synthesis of the other.

MATERIALS AND METHODS

Cells and viruses. HEp-2 cells, obtained from Flow Laboratories, Inc., Rockville, Md., were grown in Dulbecco's modification of Eagle minimal essential medium. The virus strains used were HSV-1(F), which has been passaged a limited number of times in HEp-2 cells at low multiplicity and whose properties have been described (2), and the polykaryocyte-forming mutant designated HSV-1(MP) (2, 12).

Chemicals and radioactive precursors. Acrylamide was obtained from Bio-Rad Laboratories, Richmond, Calif.; *N-N'*-diallyltartardiamide (DATD) from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and *N,N,N',N'*-tetramethylethylenediamine was from Eastman Kodak Co., Rochester, N.Y. The detergents used in these studies (Nonidet-P-40 [NP-40], sodium deoxycholate [DOC] and purified SDS) were all sup-

plied by Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y. The radioactive precursors L-[^{35}S]-methionine (40 to 100 Ci/mmol) and D-[1-^{14}C]glucosamine-hydrochloride (5 to 10 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass.

Infection of cells and incubation with radioactive precursors. Monolayer cultures of HEp-2 cells in 32-ounce (ca. 0.946-liter) bottles were infected by the addition of virus at input multiplicities of 20 PFU/cell; medium 199 containing 1% fetal calf serum (199-V) was used to maintain the cells after infection. To label the carbohydrate moieties of viral glycoproteins, [^{14}C]glucosamine (at a final concentration of 1 μCi/ml) was introduced into the medium at 5 h postinfection, and the cells were harvested 19 h later. Viral polypeptides were labeled either by incubating infected cells from 5 to 24 h postinfection with 199-V containing 1/10 the usual concentration of methionine and [^{35}S]methionine at 1 μCi/ml of medium or by incubating infected cells for 15 min with 199-V lacking unlabeled methionine but containing [^{35}S]methionine at 10 μCi/ml.

Preparation of antisera. The antisera were produced in rabbits by the injection of NP-40 extracts of HSV-1(F) virions or similar extracts of membranes isolated from HEp-2 cells infected with HSV-1(MP). Briefly, HSV-1(F) virions or membranes from HSV-1(MP)-infected cells were purified according to previously reported procedures (18, 19). The virions or the membranes were then suspended in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.002 M $MgCl_2$, and 1% NP-40. After 20 min at 0 C, the suspensions were centrifuged at 100,000 $\times$ g (25,000 rpm in the SW27.1 rotor) for 2 h to remove the nucleocapsids or insoluble membrane proteins. The supernatants were mixed with complete Freund adjuvant for the first injection and with incomplete Freund adjuvant for one subsequent injection after an interval of 1 month. The rabbits were bled from the ear prior to beginning the immunization (preimmune sera) and were bled by cardiac puncture about 10 days after the last injection. The sera obtained were centrifuged for 2 h at 25,000 rpm in the SW27.1 rotor prior to use but were otherwise untreated, unless specified. For use in some experiments, the antiserum prepared against HSV-1(F) envelope proteins (no. 6) was absorbed with HSV-1(MP)-infected cell homogenates. The absorption was carried out by mixing 2 ml of antiserum with 2×10^8 infected cells that had been sonicated after suspension in 2 ml of 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.002 M $MgCl_2$, and 1% NP-40. After 24 h at 0 C, the mixture was centrifuged first at low speed and then three times at 25,000 rpm for 2 h in the SW27.1 rotor to recover the antibodies that did not precipitate with the HSV-1(MP) antigens.

Preparation of extracts for immune precipitation. Infected cell monolayers were washed three times with cold phosphate-buffered saline, and the cells were scraped into phosphate-buffered saline and collected by centrifugation. The cell pellets were suspended in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.002 M $MgCl_2$, 1% NP-40, and 1% DOC (4×10^7 cells/1.5 ml) and then sonicated. After removing the cell debris by low-speed centrifugation, the cell extracts were centrifuged for 2 h at 25,000 rpm in the SW27.1 rotor, using polycarbonate tubes. The supernatant fluids were stored at -20 C and used within 1 week of preparation. Immediately before use, each supernatant was thawed and centrifuged again for 2 h at 25,000 rpm in the SW27.1 rotor.

Preparation and washing of immune precipitates. Appropriate volumes of antiserum or normal rabbit serum were mixed with 0.1-ml aliquots of the supernatants obtained from detergent-solubilized infected cells. These mixtures were kept at 0 C for 12 to 18 h and then layered over 5-ml aliquots of 20% sucrose (prepared in 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% NP-40) in polycarbonate tubes. The tubes were then centrifuged for 1 h at 25,000 rpm in the SW27.1 rotor, after which the sample layer and 20% sucrose were carefully aspirated from the tube. The immune precipitates, recovered as pellets at the bottoms of the tubes, were dissolved in a solution containing 0.05 M Tris-hydrochloride, pH 7.0, 2% SDS, 5% 2-mercaptoethanol, 5% sucrose, and bromophenol blue in preparation for analyses by electrophoresis on SDS-acrylamide gels.

Electrophoresis on SDS-acrylamide gels. Aliquots of SDS-solubilized infected cell extracts and of the immune precipitates were subjected to electrophoresis on slabs of acrylamide cross-linked with DATD as previously described (6). The separation gels contained 8.6% acrylamide monomer and 0.23% DATD by weight. The stacker gels were cross-linked with methylenebisacrylamide in some experiments and with DATD in others. After electrophoresis the gels were fixed and stained with Coomassie brilliant blue (3), dried on filter paper, and placed in contact with Cronex X-ray film (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The autoradiograms obtained from some gels were scanned in a Gilford spectrophotometer, and the radioactivity associated with individual polypeptide bands was quantitated by computer-aided integration of the optical density of the bands.

RESULTS

Solubilization of the HSV-1 glycoproteins. Analyses of the HSV-1 glycoproteins by immune precipitation required that these proteins be extracted from infected cells in a soluble form. To determine the efficiency of the solubilization procedure described above, infected cells were extracted with the combination of NP-40 and DOC after incubation from 5 to 24 h postinfection in medium containing [^{14}C]-glucosamine. Under these conditions, only the viral glycoproteins are labeled because the bulk of host protein synthesis and processing has ceased (7, 20). Electrophoretic analyses of the pellet and supernatant fractions obtained during the extraction procedure (Fig. 1) revealed that all but one of the viral glycoproteins

were quantitatively solubilized and could be detected only in the 100,000 × g supernatant (S_2). One of the glycoproteins, however, appeared to be only partially solubilized in that it could be detected in both the final 100,000 × g supernatant (S_2) and in the combined pellets from the two high-speed centrifugations (P_2). The low-speed pellet fraction (P_1) was essentially devoid of viral glycoproteins.

Specificity of the antisera. The specificity of rabbit antiserum no. 6, prepared against envelope proteins from purified HSV-1(F) virions, was tested in immunoprecipitation experiments, using detergent-solubilized extracts from [^{35}S]methionine-labeled infected and uninfected HEp-2 cells. The results presented in Table 1 and Fig. 2 show that this antiserum efficiently precipitated a few labeled proteins that were present only in infected cell extracts and also reacted to a limited extent with two minor proteins from uninfected cells (Fig. 2). These cellular proteins did not comigrate with any of the viral proteins recognized by the antiserum and, in fact, were not detectable in immune precipitates obtained from infected cell extracts, presumably because of the rapid inhibition of host cell protein synthesis. Serum obtained from the rabbit prior to immunization had no activity against infected or uninfected cell proteins (Table 1 and Fig. 2), further demonstrating the specificity of the reactions observed with the serum obtained after immunization.

The results shown in Fig. 3 demonstrate that the viral proteins precipitated by antiserum no. 6 are glycosylated. In this experiment, the immune precipitates were obtained from extracts of cells that had been incubated from 5 to 24 h postinfection with either [^{14}C]glucosamine or [^{35}S]methionine. The electrophoretic comparisons in Fig. 3 reveal that the precipitated species had incorporated both radioactive precursors. Although the antigen preparation injected into the rabbit contained nonglycosylated envelope proteins as well as the virion glycoproteins (4), the glycoproteins were evidently the predominant immunogens. Figure 3 also shows that antiserum no. 6 reacted with all of the major glycosylated species present in the infected cell extract, in spite of the fact that some of these species are not detectable in virions (9) and were not represented in the immunizing antigen (for comparison, [^{14}C]glucosamine-labeled virion proteins were included in the electrophoretic separations shown in Fig. 3). This observation suggested that some of the glycosylated species in infected cells might be physically differentiable from,

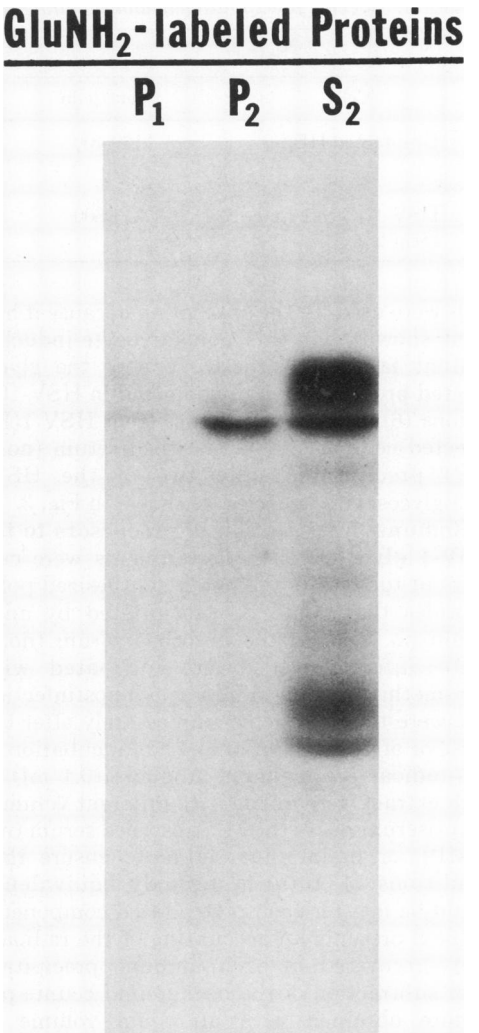

FIG. 1. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of HSV-1 glycoproteins that were present in each subcellular fraction obtained during the extraction procedure described in the text. In this figure and also in Fig. 2 through 5, the separation gel contained 8.6% acrylamide cross-linked with DATD and the stacking gel was cross-linked with bisacrylamide. Direction of migration was from top to bottom. S_2, The final 100,000 × g supernatant that was used in immunoprecipitation experiments; P_2, combined 100,000 × g pellets; P_1, the low-speed pellet containing large cell debris. GluNH$_2$, Glucosamine.*

but antigenically related to, virion glycoproteins, a hypothesis that is supported by other experiments reported here.

Although antiserum no. 6 reacted only with the viral glycoproteins, it proved useful to further restrict its reactivity by appropriate absorptions. Disrupted HSV-1(MP)-infected

TABLE 1. *Specific precipitation of labeled viral proteins by antiserum no. 6 [anti-HSV-1 (F) envelope proteins]*

Extract		Serum	Precipitates	
Cells	Counts/min		Counts/min	% of input
Uninfected HEp-2	139,400	preimmune	1,300	0.93
		postimmune	2,000	1.43
HSV-1(F)-infected HEp-2	114,600	preimmune	600	0.52
		postimmune	26,700	23.30

cells were used for the absorption because it had been shown that this polykaryocyte-inducing mutant fails to synthesize one of the glycosylated proteins normally detected in HSV-1(F) virions (6) and in membranes from HSV-1(F)-infected cells (10). The absorbed serum (no. 6 MP) precipitated only two of the HSV-1(F)-glycosylated species, as shown in Fig. 3.

Immune precipitation of precursors to the HSV-1 glycoproteins. Experiments were carried out to identify the newly synthesized polypeptides that could be precipitated by antiserum no. 6 or by the absorbed serum (no. 6 MP). Infected cells were incubated with [^{35}S]methionine for 15 min at 5 h postinfection and were then extracted immediately after the pulse or after an additional 4 h of incubation in non-radioactive medium. Aliquots (0.1 ml) of each extract were mixed with different volumes of antiserum no. 6, the MP-absorbed serum (no. 6 MP), or preimmune serum to ensure that conditions of antigen-antibody equivalence would be met for each precipitated component. Table 2 presents an accounting of the radioactivity recovered in each immune precipitate after subtraction of the background counts per minute obtained with an equal volume of preimmune serum. Aliquots (20 μl) of each immune precipitate were analyzed by SDS-gel electrophoresis (not shown). Fortuitously, it was found that, although some variations were observed in the minimal volume of antiserum required to precipitate individual components, all labeled species were maximally precipitated by a single quantity of antiserum. These quantities were 100 μl of antiserum no. 6 for the pulse-labeled extract and 200 μl for the pulsed-chased extract (Table 2); the twofold difference in amount of antiserum required for maximal precipitation before and after the chase was probably due to the fact that significant amounts of unlabeled protein would be made during the chase from 5.25 to 9.25 h postinfection and would add to the total antigen pool.

The autoradiogram in Fig. 4 shows the electrophoretic separation of the labeled proteins precipitated by both the absorbed and unabsorbed antiserum under optimal conditions. From the extract obtained immediately after the pulse, antiserum no. 6 precipitated four polypeptides, designated pA, pB, pC, and pD, whereas no. 6MP reacted only with pC. Although pB and pC are not resolved on the gel slab shown in Fig. 4, evidence for the existence of the two species comes from findings that the absorbed antiserum precipitated only the polypeptide in the leading edge of the pB-pC doublet and that, under different electrophoretic conditions, the two species could be resolved, as described below.

After the 4 h of incubation that followed the pulse of [^{35}S]methionine, seven electrophoretically differentiable species could be precipitated by antiserum no. 6 (the species designated C_1 is barely detectable in Fig. 4). Three of these species (B_1, C_1, and D_1) had electrophoretic mobilities similar to those of the precursors pB, pC, and pD, respectively, although less radioactivity was associated with the bands detected after the chase than with the electrophoretically similar bands detected after the pulse. Concomitant with the loss of radioactivity from the electrophoretic positions of these three precursors during the chase, there was the appearance of three new labeled species (C_2, B_2, and D_2) whose electrophoretic mobilities differed from those of any of the precursors. Furthermore, these new species yielded quite diffuse bands on SDS-acrylamide gels, which is characteristic of the electrophoretic behavior of most HSV-1 glycoproteins present in virions and in plasma membranes (7, 19). Of the three new species detected after the chase, only one (C_2) was precipitated by the MP-absorbed antiserum.

Band A, precipitated after the chase, had an electrophoretic mobility identical to pA and had as much, or usually more, radioactivity associated with it than did pA (Fig. 4). It seems likely on the basis of other data to be presented that all of pA was recovered after the chase as A. There are at least two explanations for the finding that greater quantities of the A polypep-

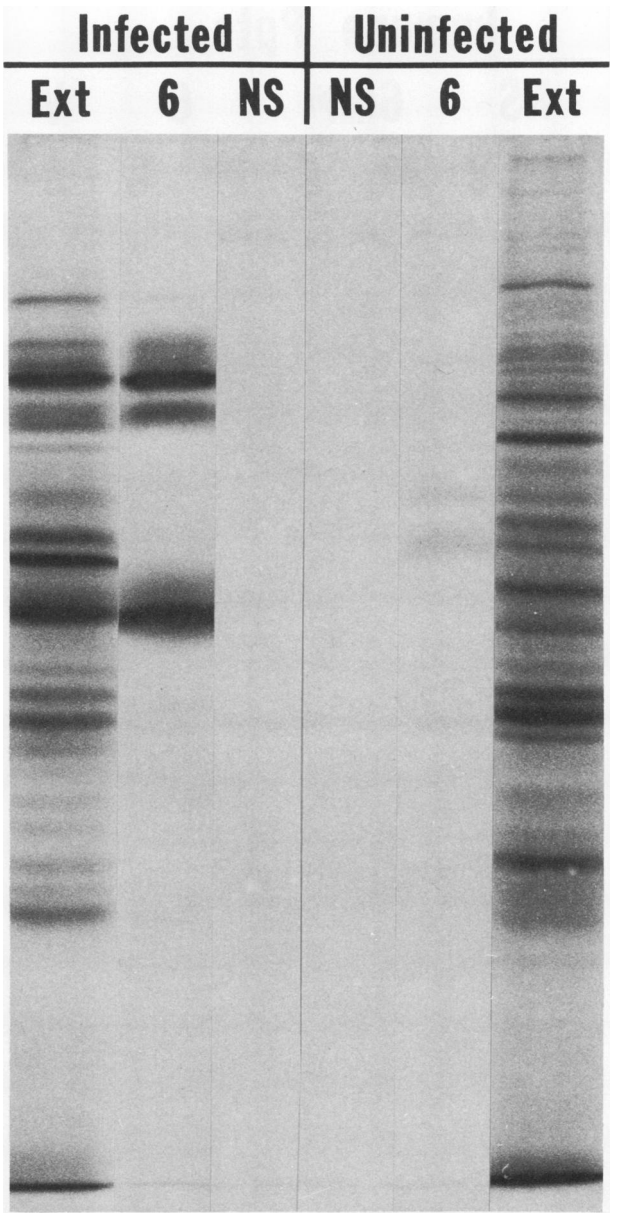

FIG. 2. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of [^{35}S]methionine-labeled proteins present in the detergent-solubilized extracts (Ext) from infected or uninfected HEp-2 cells and in the precipitates obtained from these extracts, using antiserum no. 6 or the serum obtained prior to immunization (NS). All samples were subjected to electrophoresis on the same gel slab, but relevant tracks of the autoradiogram were cut out and rearranged for presentation in this figure.*

tide were usually recovered in immune precipitates obtained after a chase than immediately after a pulse. Inasmuch as A is probably identical to the glycoprotein that cannot be quantitatively solubilized by NP-40 and DOC (see Fig. 1), the possibility exists that the newly synthesized form of A is even less soluble than the fully glycosylated form. Electrophoretic comparisons of extracts obtained from pulse-labeled cells before and after the chase support this hypothesis, although quantitation is difficult because of the number of labeled proteins that migrate near these species. In addition, the fully glycosylated form of A may be antigenically more

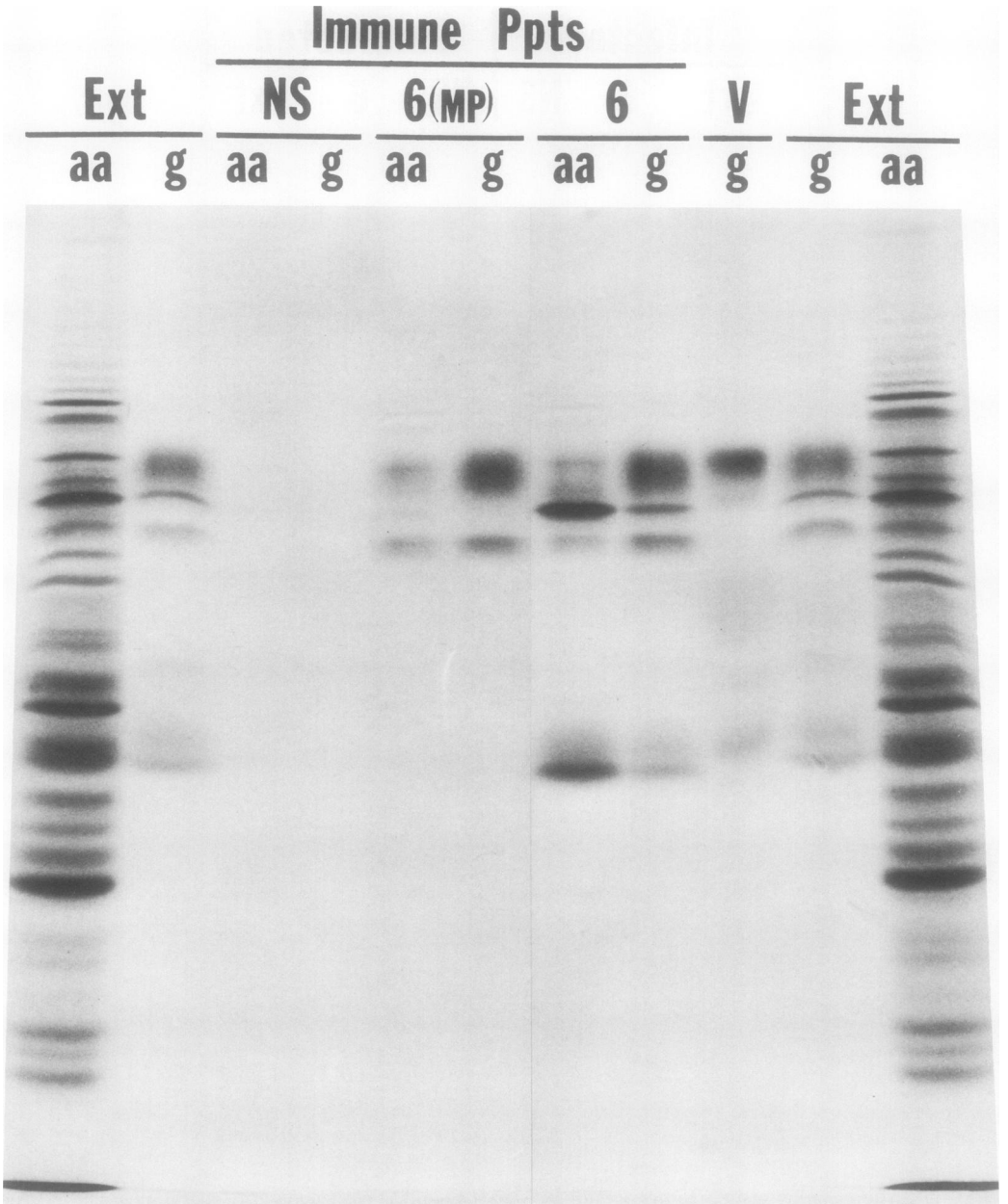

FIG. 3. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of [^{35}S]methionine (aa)-labeled and [^{14}C]glucosamine (g)-labeled proteins present in detergent-solubilized extracts (Ext) from infected HEp-2 cells and in the immune precipitates obtained from these extracts, using antiserum no. 6, MP-absorbed no. 6 [6(MP)], or preimmune serum (NS). A sample of [^{14}C]glucosamine-labeled HSV-1(F) virion proteins (V) was also included in this analysis for comparison.*

reactive than the newly synthesized form. It should be noted that the difference in total counts per minute precipitated by antiserum no. 6 before and after the chase (Table 2) is due almost exclusively to the greater amounts of label recovered in A than in pA and that this phenomenon occurs even when protein synthesis is inhibited by cycloheximide during the chase, as will be demonstrated in a later section.

A pulse-chase experiment identical to the one just described was also performed at 14 h postinfection (Table 3 and Fig. 5). The results

TABLE 2. *Radioactivity recovered in immune precipitates after a pulse label or pulse chase beginning at 5 h postinfection*

Extracts		Antiserum		Net counts/min in precipitates[a] ($\times 10^{-3}$)
Labeling regime	Counts/min per 100 μl ($\times 10^{-3}$)	Designation no.	Volume (μl)	
Pulse label at 5 h postinfection	1,048	6	25	82.1
			50	101.5
			100	110.6
			200	98.8
		6 (MP)[b]	100 (1:2)	18.6
			200 (1:2)	22.4
			400 (1:2)	20.3
Pulse label at 5 h postinfection plus 4-h chase	970	6	25	54.3
			50	110.3
			100	125.6
			200	142.4
		6 (MP)	100 (1:2)	13.8
			200 (1:2)	23.7
			400 (1:2)	21.8

[a] Counts per minute in immune precipitates after subtraction of the background counts per minute obtained with an equal volume of preimmune serum.

[b] Antiserum no. 6 [anti-HSV-1(F) envelope proteins] after absorption with HSV(MP)-infected cells. The absorption procedure resulted in a twofold dilution of the antiserum.

obtained were similar in that the same four precursors were detected and seven products could be differentiated after the chase, although some differences were observed in the relative quantities of products detected late, as compared with early, in infection (compare Fig. 4 and 5). Specifically, late in infection greater quantities of radioactivity accumulated in species C_1 and D_1 and lesser amounts in C_2 and D_2. Moreover, less of pB, relative to the other precursors, was synthesized late in infection than was made early, and proportionately less radioactivity accumulated in B_1 and B_2. In Fig. 5, B_2 and C_2 from the precipitate obtained with antiserum no. 6 form one diffuse band, but B_2 can be recognized as the labeled material in that band that was not precipitated by the MP-absorbed antiserum.

The alphabetic designations assigned to the pulse-labeled polypeptides precipitated by antiserum no. 6 before and after a chase (Fig. 4 and 5) reflect the proposed precursor-product relationships and the hypothesis that B_1, C_1, and D_1 are partially glycosylated intermediates of the fully glycosylated B_2, C_2, and D_2, respectively. Support for this scheme of glycoprotein processing comes from antigenic and kinetic analyses and from comparisons of glucosamine-labeled and amino acid-labeled stable products.

Antigenic relationships between individual precursors and products. The findings that the absorbed antiserum (no. 6 MP) precipitated only one precursor (pC) and two products (C_1 and C_2) and that the amount of radioactivity in pC was approximately equal to the radioactivity in C_1 plus C_2 (Fig. 4 and 5, Tables 2 and 3) strongly suggest that these species all share the same polypeptide chain. Several comments should be made here about the complex appearance of band pC in both Fig. 4 and 5. Although it was noted consistently that pC exhibited a diffuse leading edge and a sharp trailing edge, the available evidence suggests that this is due to heterogeneity in the initial stages of glycosylation of the C polypeptide rather than to the existence of two polypeptides in that band. Specifically, the radioactivity in pC could be quantitatively converted to one species (C_2) early in infection, even though this label accumulated in two species (C_1 and C_2) late in infection (Fig. 4 and 5). As will be demonstrated in a later section, both C_1 and C_2 are glycosylated and C_1 is probably an intermediate in the synthesis of C_2. Inasmuch as C_1 comigrates with the leading edge of pC (Fig. 5), the possibilities exist that the first carbohydrate residues added may slightly increase the electrophoretic mobility of the C polypeptide in SDS gels or that removal of a small peptide by proteolysis may accompany the early stages of glycosylation.

To obtain evidence for antigenic relationships

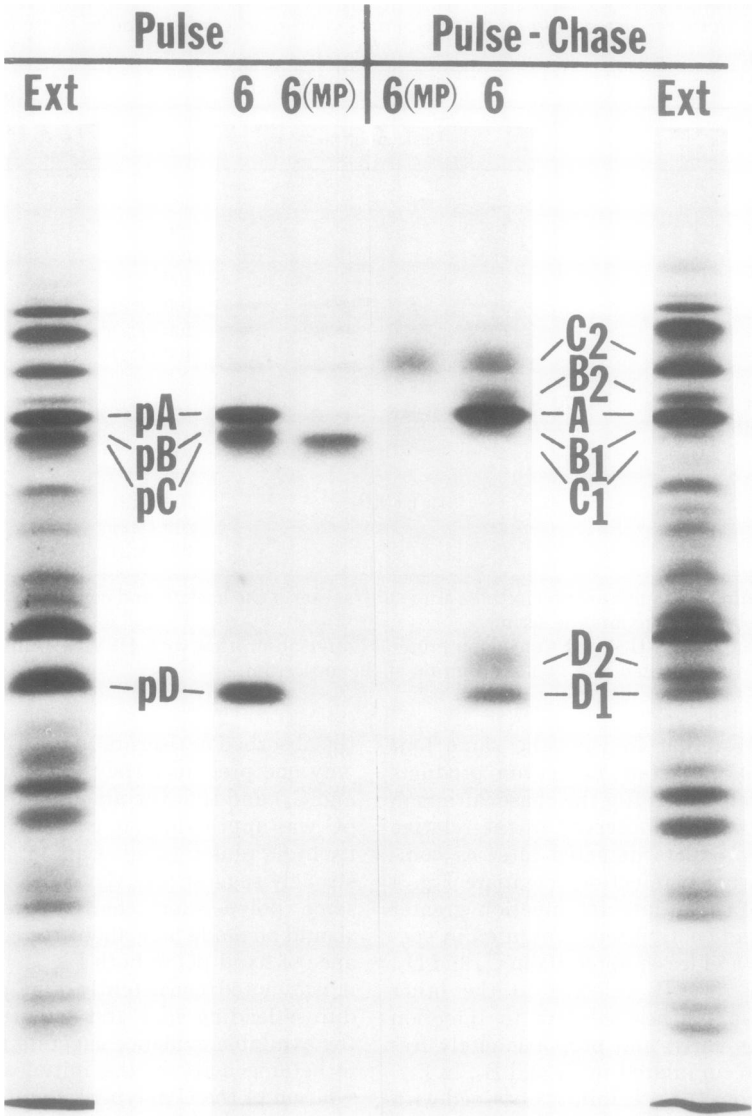

FIG. 4. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of labeled proteins that were precipitated by antiserum no. 6 or by MP-absorbed no. 6 [6(MP)] from cell extracts prepared immediately after a 15-min pulse with [^{35}S]methionine at 5 h postinfection (pulse) or after an additional 4 h of incubation in non-radioactive medium (pulse-chase). Aliquots of the cell extracts (Ext), equivalent to the amount from which the precipitates were obtained, were analyzed on the same gel slab and are shown for comparison.*

among other components, immunoprecipitations of the labeled precursors and products were carried out in the presence of increasing amounts of unlabeled competing protein. In this experiment, infected cells were pulse labeled at 5 h postinfection, as before, and were extracted immediately after the pulse or after an additional 4 h of incubation in non-radioactive medium. Cycloheximide (50 μg/ml) was incorporated into the medium during the chase, however, because it was important for the design of this experiment to keep the total amount of glycoprotein antigen constant during the chase, which could be done by inhibiting new protein synthesis after the pulse. It had been shown previously that glycosylation proceeds normally under these conditions (9). The experimental procedure was to mix aliquots of

TABLE 3. *Radioactivity recovered in immune precipitates after a pulse label or pulse chase beginning at 14 h postinfection*

Extracts		Antiserum		Net counts/min in precipitates[a] ($\times 10^{-3}$)
Labeling regime	Counts/min per 100 μl ($\times 10^{-3}$)	Designation no.	Volume (μl)	
Pulse label at 14 h postinfection	362.0	6	100	19.1
			200	20.7
			400	21.0
		6 (MP)[b]	400 (1:2)	5.5
Pulse label at 14 h postinfection plus 4-h chase	317.5	6	100	29.1
			200	28.9
			400	11.4
		6 (MP)	400 (1:2)	6.3

[a] Counts per minute in immune precipitates after subtraction of the background counts per minute obtained with an equal volume of preimmune serum.

[b] Antiserum no. 6 [anti-HSV-1(F) envelope proteins] after absorption with HSV(MP)-infected cells. The absorption procedure resulted in a twofold dilution of the antiserum.

each labeled extract with different quantities of unlabeled, infected cell extract prior to the addition of antiserum no 6, to determine the amount of competitor required to inhibit the precipitation of each individual labeled precursor or product. This protocol could aid in identifying species that share the same polypeptide chain because the quantity of unlabeled extract required to inhibit precipitation of each labeled species depends upon the titer of antibodies directed against each polypeptide, as well as on the total amount of each antigen in the competing extract. The unlabeled extract was prepared from cells that had been incubated from 5 to 9 h postinfection in cycloheximide so that the competing preparation was free of newly synthesized precursors. To maintain the same protein concentration and volume in each reaction mixture, appropriate amounts of unlabeled, uninfected cell extract were also added prior to addition of the antiserum.

The autoradiogram in Fig. 6 shows the electrophoretic separation of the labeled species precipitated in the absence or presence of varying amounts of unlabeled competitor. (Note that in Fig. 6 and 8, in contrast to Fig. 4 and 5, pC is well separated from pB; the use of DATD in the stacking gel causes pC to migrate faster than it does when the stacking gel is cross-linked with bisacrylamide.) Figure 7 presents quantitation of the results shown in Fig. 6, obtained by integrating densitometer scans of the autoradiogram for each species of interest; it should be noted that the photograph in Fig. 6 was overexposed so that the weakly labeled bands could be visualized, but the optical density of the autoradiogram itself was proportional to the radioactivity in each band, as determined by quantitating different exposures of the same gel. The data shown in Fig. 7 demonstrate that the precipitation of pC and C_2 was inhibited to the same extent by 4 mg of competing extract, but not at all by lesser amounts, consistent with the antigenic relationship detected by use of the MP-absorbed antiserum. Component C_1 was not evident in this experiment, presumably because the radioactivity in pC was quantitatively converted to C_2 during the chase. The extent to which precipitation of pD, D_1, and D_2 was inhibited by different amounts of competing protein, in the absence of any effects on the precipitation of other species, strongly suggests that all of the D components share the same polypeptide chain. Under the particular conditions of this experiment, precipitation of the labeled A and B components was not affected although their precipitation could be inhibited if a smaller volume of antiserum was used. This result is not unexpected because, in titration experiments, it was demonstrated that antiserum no. 6 contained higher titers of antibodies to the A and B species than to the C and D components. These titration experiments are not shown because they yielded the same results as shown in Fig. 6 (decreasing volume of antiserum from left to right). The experiments demonstrating competitive inhibition of precipitation of the A and B species are not shown either because it was not possible to differentiate these species by the amount of unlabeled competitor required for inhibition.

Kinetics of polypeptide conversion from precursors to products. Infected cells were incubated for 15 min with [^{35}S]methionine at 5

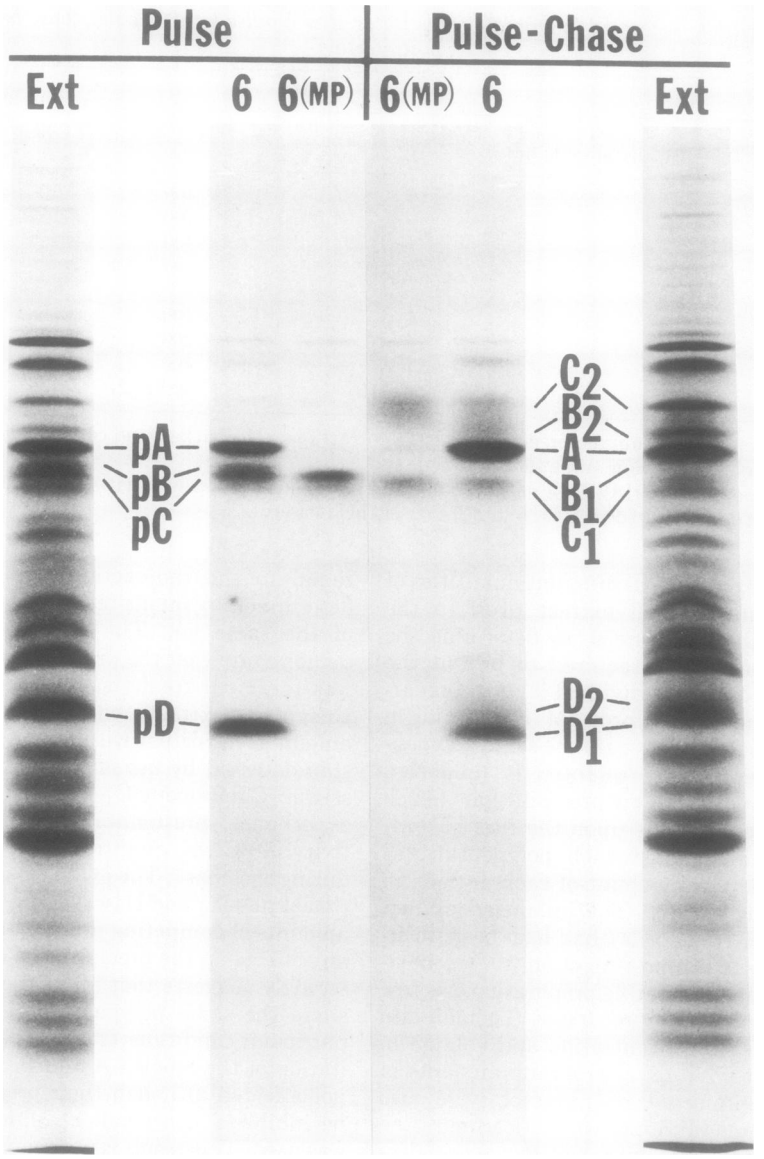

FIG. 5. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of labeled proteins that were precipitated by antiserum no. 6 or by MP-absorbed no. 6 [6(MP)] from cell extracts prepared immediately after a 15-min pulse with [^{35}S]methionine at 14 h postinfection (pulse) or after an additional 4 h of incubation in non-radioactive medium (pulse-chase). Aliquots of the cell extracts (Ext), equivalent to the amount from which the precipitates were obtained, were analyzed on the same gel slab and are shown for comparison.*

h postinfection and then extracted immediately after the pulse or after 20, 60, 150, and 240 min of incubation in non-radioactive medium containing cycloheximide. The autoradiogram in the left half of Fig. 8 shows the immune precipitates obtained with optimal quantities of antiserum no. 6, using the extracts prepared at the times indicated. The right half of Fig. 8 shows immune precipitates obtained from the same extracts with an antiserum prepared against purified membranes from HSV-1(MP)-infected cells. All components recognized by the anti-HSV-1(F) serum were also precipitated by the anti-HSV-1(MP) serum, except pC, C_1, and C_2, a further confirmation of their proposed precursor-product relationship.

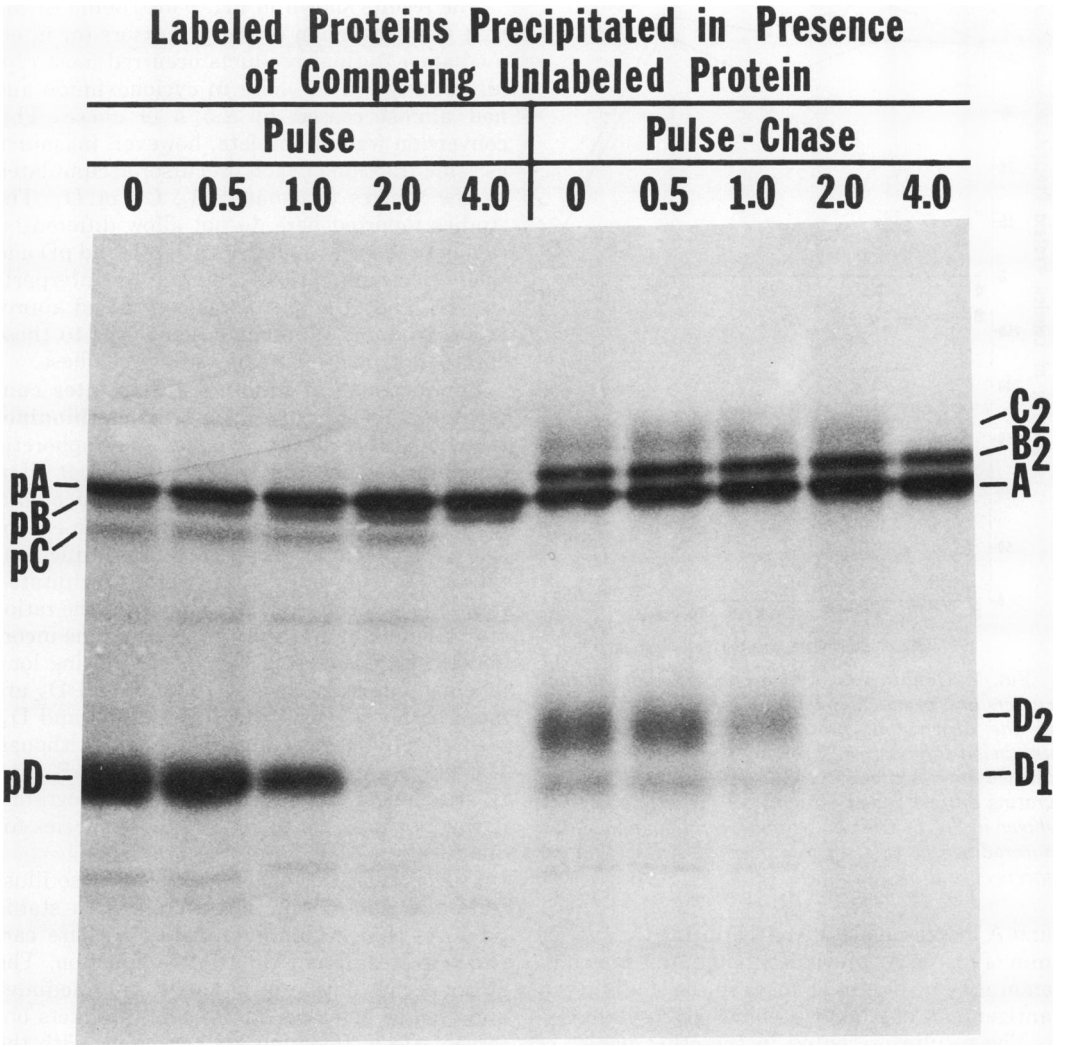

FIG. 6. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of labeled viral proteins precipitated by antiserum no. 6 in the absence or presence of 0.5 to 4.0 mg of unlabeled infected cell protein. The radiolabeled extracts were prepared from cells either immediately after a 15-min incubation with [^{35}S]methionine at 5 h postinfection (pulse) or after an additional 4 h of incubation in non-radioactive medium containing cycloheximide at 50 μg per ml (pulse-chase). In this figure and in Fig. 8, the stacking gel was cross-linked with DATD.*

The autoradiogram of the immune precipitates obtained with antiserum no. 6 was scanned in a densitometer, and the radioactivity associated with each designated species was quantitated by integration of the appropriate regions in each track of the autoradiogram. By summing the peak areas of species believed to share the same polypeptide chain, i.e., those assigned the same alphabetic designation, the results shown in the upper left quadrant of Fig. 9 were obtained. Note that the sums of radioactivity recovered in all C components and in all D components remained constant throughout the entire chase period. The sum of all B components also remained relatively constant, with a slight decrease at 20 min and 1 h of incubation after the pulse. Because B_1 and B_2 migrate on either side of band A, their quantitation is less reliable than that of all other species, which may account for the slight nonconservation of radioactivity. Alternatively, some partially glycosylated forms of the B polypeptide may comigrate with band A. The amount of radioactivity that migrated in the position of pA

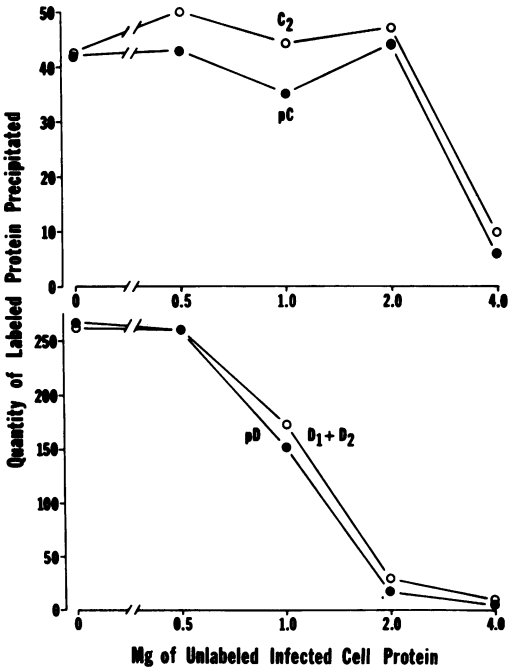

FIG. 7. *Quantities of [^{35}S]methionine-labeled precursors and products precipitated by antiserum no. 6 in the absence or presence of 0.5 to 4.0 mg of unlabeled infected cell protein. The amount of each labeled species precipitated was determined by integrating optical density tracings of the autoradiogram shown in Fig. 6. The photographic reproduction of this autoradiogram (Fig. 6) was overexposed so that all species could be visualized.*

and A increased significantly during the first 20 min of chase. As previously noted, this phenomenon may be due to changes in the solubility or antigenicity of A as it becomes glycosylated.

The results presented in the other panels of Fig. 9 reveal that the radioactivity lost from the regions of pB + B_1, pC + C_1, or pD + D_1 was almost exactly compensated by increases in radioactivity in the proposed final products, all of which migrated slower than the respective precursors. Moreover, the kinetics of accumulation of each final product were slightly different, and the levels of radioactivity detected throughout the chase in each set of related species (the B, C, and D components, respectively) were significantly different (note that each panel in Fig. 9 is drawn to a different scale). All of these observations, coupled with antigenic relationships demonstrated for the C components and suggested for the D components, argue strongly for the proposed scheme of precursor-product relationships and exclude most others that might be considered.

The results shown in Fig. 9 also demonstrate that the conversion of the precursors (or intermediates) to final products occurred most rapidly during h 1 of chase in cycloheximide and had almost ceased by 2.5 h of chase. This conversion was incomplete, however, inasmuch as some fraction of each precursor accumulated as the species designated B_1, C_1, or D_1. The studies reported here do not allow differentiation between the precursors pB, pC, and pD and their electrophoretically similar counterparts (B_1, C_1, and D_1), but it was considered appropriate to apply different designations to those species detected before and after the chase.

Comparisons of immune precipitates containing glucosamine-labeled and methionine-labeled stable products. The electrophoretic comparisons shown in Fig. 10 reveal that all of the pulse-labeled polypeptides that accumulated during a chase (with the possible exception of B_1) comigrated with glucosamine-containing polypeptides that were precipitated from infected cell extracts. Moreover, the ratios of [^{14}C]glucosamine and [^{35}S]methionine incorporated into several of the species during long labeling intervals suggest that C_2 and D_2 are more highly glycosylated than are C_1 and D_1, respectively (Fig. 3 and Table 4). Although [^{14}C]glucosamine could be detected in B_1 and B_2 after longer exposures of the autoradiograms, it was not possible to resolve these species for quantitation.

The comparisons shown in Fig. 10 also illustrate the electrophoretic differences in stable products that accumulate during a chase carried out at different times after infection. The larger accumulation of proposed intermediates and greater heterogeneity of final products observed late in infection are consistent with the hypothesis that glycosylation proceeds less efficiently late in the infectious cycle than early. The stable products detected after a long labeling interval with [^{35}S]methionine resemble the late pattern more than the early pattern, as might be expected. In spite of the fact that significant amount of the intermediates accumulate in infected cells, they are not readily detectable in virions (Fig. 10).

Apparent molecular weights of the glycoprotein precursors. The electrophoretic mobility of each glycoprotein precursor was estimated on 8.6% SDS-acrylamide gels cross-linked with DATD, using a stacking gel cross-linked either with bisacrylamide or with DATD and using HSV-1(F) virion polypeptides as molecular weight standards. The results obtained with both kinds of stacking gel were identical for all

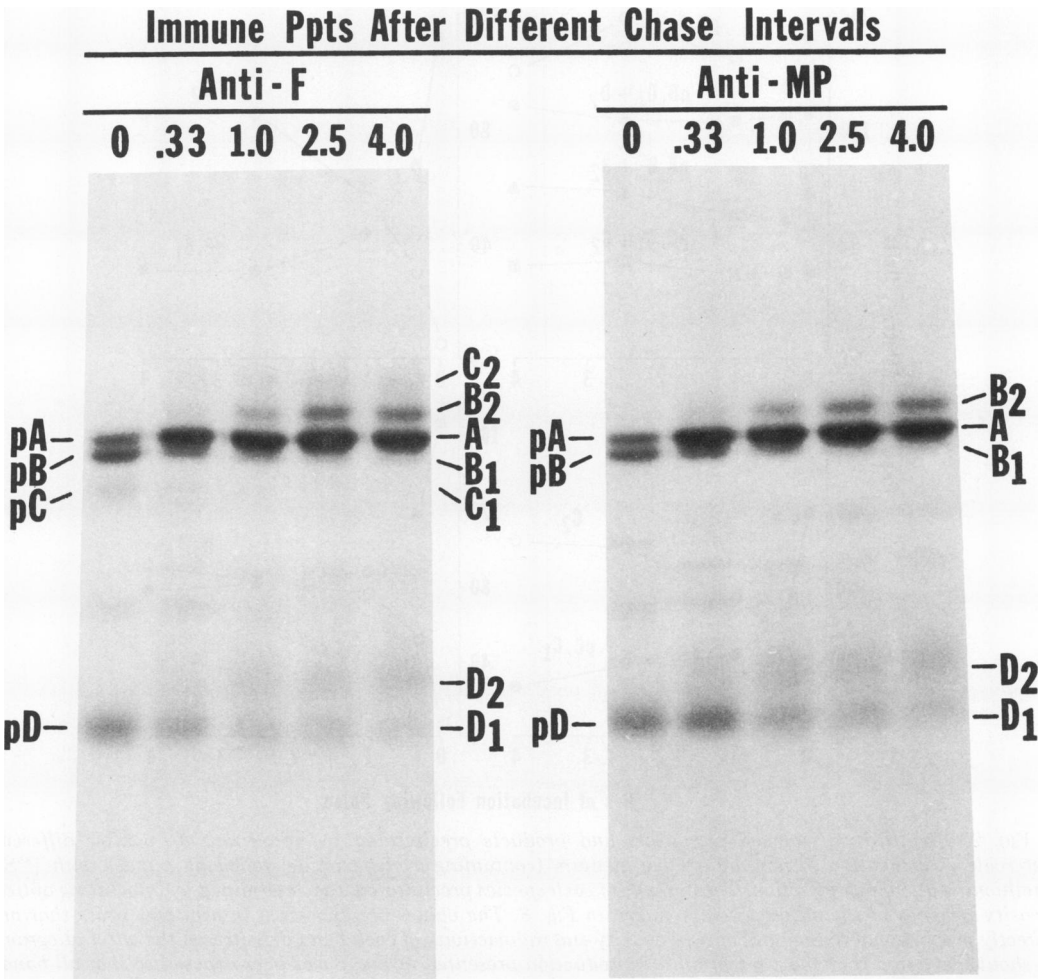

FIG. 8. *Autoradiogram of an SDS-acrylamide gel slab showing the electrophoretic separation of proteins precipitated from cell extracts prepared immediately after a 15-min incubation with [^{35}S]methionine at 5 h postinfection or after varying intervals of incubation (0.33 to 4.0 h) in non-radioactive medium containing cycloheximide following the pulse. The immune precipitates were obtained by mixing aliquots of the cell extracts either with antiserum no. 6 (anti-F) or with an antiserum prepared against membrane proteins from HSV-1 (MP)-infected cells (anti-MP).*

components except pC. Figure 11 shows the electrophoretic mobilities of the marker proteins and precursors as a function of molecular weight, with the apparent molecular weights for each precursor presented in Table 5. Some uncertainties exist as to the reliability of these values because the precursors may be partially glycosylated and may bind SDS anomalously, resulting in migration rates that are not strictly inversely proportional to the logaritham of molecular weight (5). In fact, glycosylation may explain the variations in electrophoretic mobility of pC relative to marker proteins under different conditions of electrophoresis. The electrophoretic mobilities of B_2, C_2, and D_2 are also shown in Fig. 11, in relation to those of their respective precursors; the molecular weights of these fully glycosylated products cannot be estimated by SDS-acrylamide gel electrophoresis, but it is instructive to visualize the change in mobility that occurs as a result of glycosylation.

DISCUSSION

The results presented in this study demonstrate that the major HSV-1 glycoproteins are derived from four antigenically distinct polypeptides. Thus, the virion glycoproteins desig-

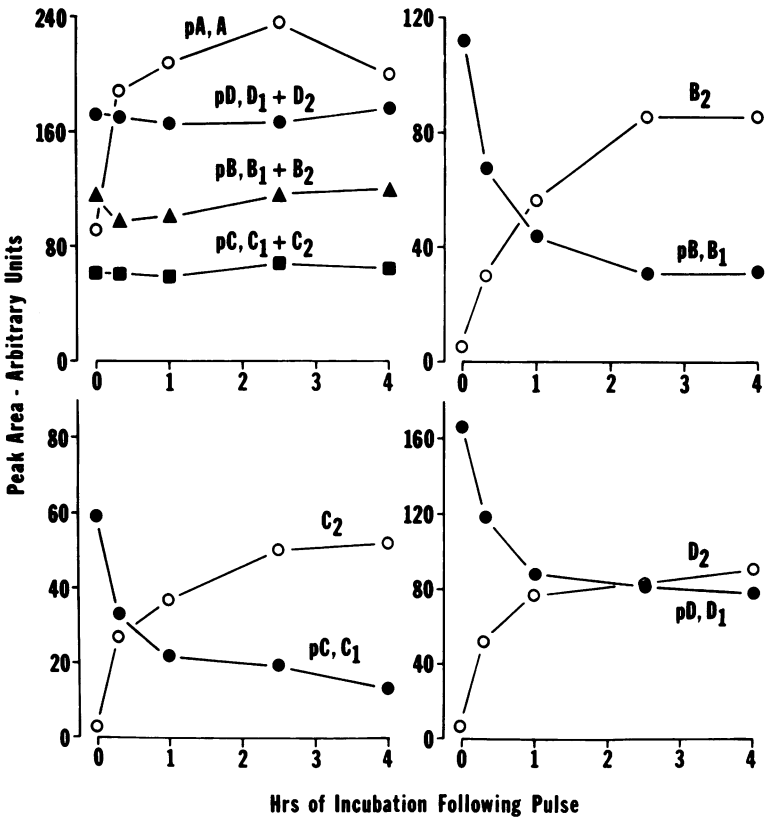

FIG. 9. *Quantities of labeled precursors and products precipitated by antiserum no. 6 after different intervals of incubation in non-radioactive medium (containing cycloheximide) following a pulse with [^{35}S]-methionine at 5 h postinfection. The amount of each species precipitated was determined by integrating optical density tracings of the autoradiogram shown in Fig. 8. The values are expressed in arbitrary units that are directly proportional to the total optical density and radioactivity of each band detected on the autoradiogram. It should be noted that the photographic reproduction presented in Fig. 8 was over-exposed so that all bands could be visualized.*

nated VP8, VP7, VP8.5, and VP18 (6) correspond to the fully glycosylated forms of polypeptides C, B, A, and D, respectively (Fig. 10). Two additional glycosylated species are prominent in unfractionated infected cells, as noted also by Honess and Roizman (9); these species correspond to C_1 and D_1 (Fig. 10), which are antigenically related to C_2 and D_2, respectively, and probably are partially glycosylated intermediates to the fully glycosylated forms. At least two minor glycoproteins can also be detected in infected cell extracts (Fig. 1 and 3) and do not appear to be related to the four polypeptides that were characterized in this study. The properties of these proteins are now under investigation.

Identification of the glycosylated products that were derived from each polypeptide precursor was based on several considerations, including: (i) the precipitation of only one precursor (pC) and two products (C_1 and C_2) by the MP-absorbed antiserum (Fig. 4 and 5); (ii) the radioimmunoprecipitation competition experiments (Fig. 6 and 7), which provided evidence for the antigenic relatedness of all the D components; (iii) the kinetics with which individual pulse-labeled precursors (or intermediates) were converted to fully glycosylated products (Fig. 8 and 9); and (iv) the finding that significantly different levels of radioactivity were associated with, and conserved in, each set of related species (Fig. 9). These results provide strong evidence for the proposed scheme of precursor-product relationships and rule out most other possibilities.

Two points should be made regarding the post-translational processing of the B, C, and D polypeptides. First, two electrophoretically differentiable glycosylated forms are derived from each polypeptide. Second, several lines of evi-

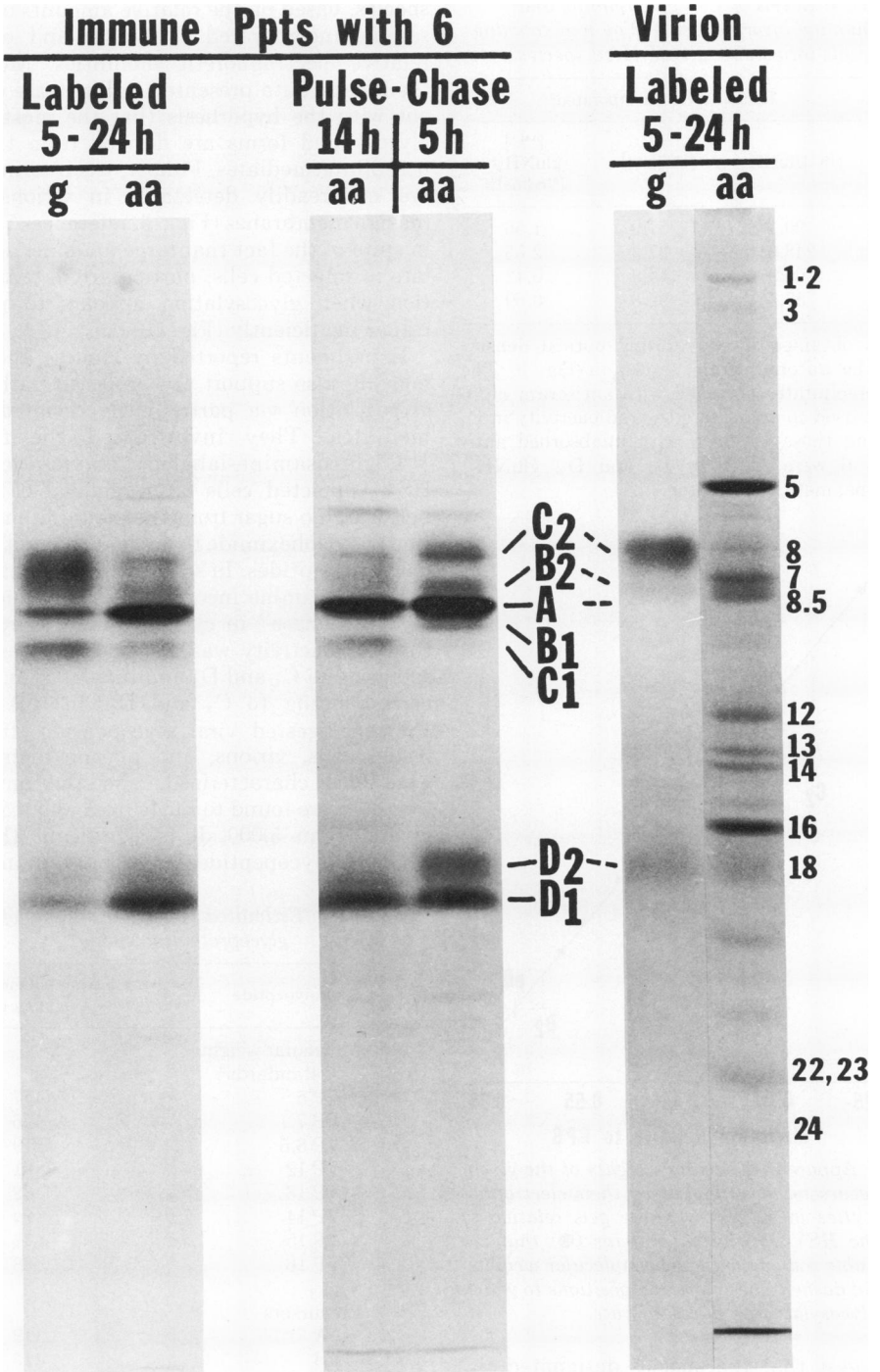

FIG. 10. *Autoradiogram of an SDS-acrylamide gel slab, comparing the electrophoretic mobilities of proteins precipitated by antiserum no. 6 from extracts of cells that had been incubated from 5 to 24 h postinfection with [^{14}C]glucosamine (g) or [^{35}S]methionine (aa) or from extracts of cells pulse labeled with [^{35}S]methionine at 5 and 14 h postinfection and then incubated for 4 h in non-radioactive medium. HSV-1(F) virion proteins, labeled either with [^{14}C]glucosamine or [^{35}S]methionine, are shown for comparison.*

TABLE 4. *Ratios of [^{14}C]glucosamine and [^{35}S]methionine incorporated during long labeling intervals into stable glycosylated species*

Glycosylated species	Radioactivity incorporated[a]		
	[^{14}C]gluNH$_2$	[^{35}S]meth	[^{14}C] gluNH$_2$/ [^{35}S]meth
C_1	90.1	57.9	1.56
C_2	248.6	97.3	2.55
D_1	60.9	144.8	0.42
D_2	57.5	82.8	0.69

[a] Values obtained by integrating optical density scans of the autoradiogram shown in Fig. 3. The immune precipitates obtained with antiserum no. 6 (MP) were used to quantitate the radioactivity in C_1 and C_2, and those obtained with unabsorbed antiserum no. 6 were used for D_1 and D_2. gluNH$_2$, Glucosamine; meth, methionine.

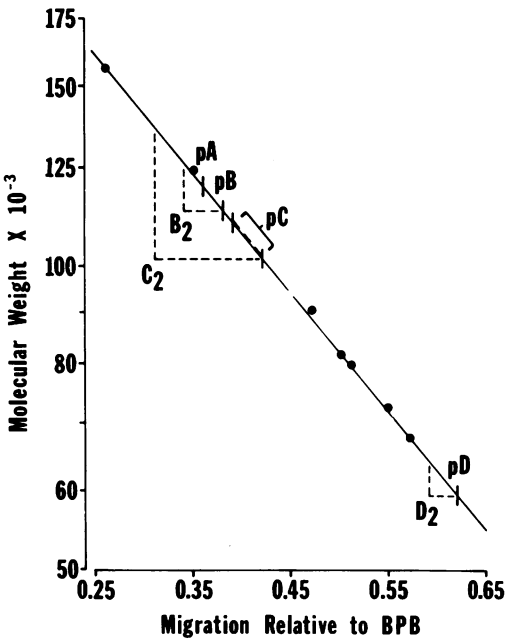

FIG. 11. *Apparent molecular weights of the glycoprotein precursors as estimated by their electrophoretic mobilities in SDS-acrylamide gels relative to those of the HSV-1(F) virion proteins (●) that are listed in Table 5 along with their molecular weights. The vertical dashed lines mark the positions to which the fully glycosylated products migrate.*

dence suggest that the species designated B_1, C_1, and D_1 are partially glycosylated intermediates to the final products B_2, C_2, and D_2, respectively. Each of the latter species appears to contain more carbohydrate than its antigenically related counterpart among the former species, based on the relative amounts of glucosamine incorporated (Table 4) and on their relative electrophoretic mobilities. Moreover, the kinetic data presented in Fig. 9 are consistent with the hypothesis that the most highly glycosylated forms are derived from the proposed intermediates. Finally, the intermediates are not readily detectable in virions or in plasma membranes (Fig. 10, references 7 and 9), in spite of the fact that large amounts accumulate in infected cells, particularly late in infection when glycosylation appears to proceed rather inefficiently (Fig. 10).

Experiments reported by Honess and Roizman (9) also support the proposed pathway of glycosylation via partially glycosylated intermediates. They investigated the fate of [^{14}C]glucosamine-labeled polypeptides in HSV-1-infected cells after removal of the labeled amino sugar from the medium and addition of cycloheximide to prevent the synthesis of new polypeptides. In spite of the fact that some [^{14}C]glucosamine incorporation continued during the "chase" in cycloheximide, they found that radioactivity was lost from species corresponding to C_1 and D_1 and increased in species corresponding to C_2 and D_2. In this study, Pronase-digested viral glycopeptides from infected cells, virions, and plasma membranes were also characterized, and the molecular weights were found to range from about 2,000 to greater than 5,000. It is significant that the smallest glycopeptides were present in much

TABLE 5. *Estimated molecular weights of the glycoprotein precursors*

Polypeptide	Estimated molecular weight ($\times 10^{-3}$)
Molecular weight standards[a]	
VP 5	157
VP 7	126
VP 8.5	119
VP 12	91
VP 13	82
VP 14	80
VP 15	73
VP 16	68
Precursors	
pA	119
pB	113
pC	102-110
pD	59

[a] Estimated molecular weights of the HSV-1(F) virion proteins (VP 5 to VP 16) were taken from studies by Heine et al. (6).

greater quantities in unfractionated infected cells than in virions and plasma membranes, suggesting that these glycopeptides may have been derived from the partially glycosylated intermediates. Analyses of the carbohydrate chains from individual intermediates and products, as well as tryptic peptide comparisons, are now in progress.

It should be emphasized that the two electrophoretically differentiable products derived from each precursor apparently define two discrete stages of glycosylation. Studies of Sindbis virus glycoproteins have also revealed discrete stages in the carbohydrate addition to these proteins (17) although possible intermediates were not electrophoretically differentiable from the products on SDS gels, as has been observed in the present study of the HSV-1 glycoproteins. On the basis of the results presented here, it is not possible to determine when the initial stages of glycosylation occur because the newly synthesized polypeptides (which may have some carbohydrate associated with them) and the stable intermediates have similar electrophoretic mobilities. The second stage of glycosylation, which yields C_2, B_2, and D_2, occurs within 20 to 30 min of polypeptide synthesis (Fig. 9), however, so that the initial stages of glycosylation must occur very rapidly, perhaps even before release of the nascent proteins from polysomes. Recent studies support the hypothesis that the HSV-1 glycoproteins acquire some glucosamine within a few minutes of polypeptide synthesis, inasmuch as the addition of cycloheximide to infected cells caused a decreased rate of glucosamine incorporation after 12 to 15 min (9). It is rather striking that the electrophoretic mobilities of the intermediates and products are so different, especially those of C_1 and C_2 (Fig. 4 and 5). Moreover, at various times during a 4-h chase period, the conversion of the pulse-labeled polypeptides from intermediates to final products yielded discrete species, suggesting that a large mass of carbohydrate may be added all at once, possibly from preformed oligosaccharide donors (1), during the second stage of glycosylation.

The question arises as to the apparent inefficiency of the second stage of glycosylation. That is, why do significant amounts of the polypeptides remain in the form of partially glycosylated intermediates, even early in infection (Fig. 9)? Preliminary experiments (E. Schechter and P. G. Spear, manuscript in preparation) indicate that the intermediates tend to accumulate in nuclear membranes of the infected cell, suggesting that intracellular localization of the polypeptides may determine whether the glycosylation can proceed to completion.

It should be pointed out that the intracellular processing of the HSV-1 glycoproteins does not appear to involve any proteolytic cleavages, as has been reported for some of the RNA-enveloped viruses (15, 16), unless small peptide fragments are removed concomitant with the addition of carbohydrate. Moreover, there is no evidence that any of the four precursors arise by proteolytic cleavage of even larger translation products or polyproteins, although the pulse-labeling intervals used here might not have been short enough to detect them. Previous attempts to detect the synthesis of polyproteins in HSV-1-infected cells, however, were not successful, even with the use of potent inhibitors of proteolytic enzymes (8).

The properties of the glycoprotein designated A differentiate it from the other three glycoproteins that have been characterized in this study. Whereas B_2, C_2, and D_2 are all readily dissociable from membranes by the action of NP-40 and DOC, A is only partially solubilized by these detergents, suggesting that it may interact not only with lipids in membranes but also with some other components. In addition, glycoprotein A incorporates much less glucosamine (Fig. 3 and 10) and probably has much less total carbohydrate than the other species. Moreover, glycosylation has no apparent effect on the electrophoretic mobility of this polypeptide, suggesting that the nature and mass of its carbohydrate chains may differentiate it from all the other glycoproteins. It should also be noted that, although large amounts of the A polypeptide are synthesized in infected cells, little of it is incorporated into virions, relative to the other glycoproteins (Fig. 10). It will be important to further characterize these two types of HSV-1 glycoproteins, both for investigations of their roles in herpesvirus infection and to correlate the chemical properties and functions of glycoproteins in general.

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