

Synthesis and Processing of Glycoproteins gD and gC of Herpes Simplex Virus Type 1

GARY H. COHEN,^{1*} DEBORAH LONG,¹ AND ROSELYN J. EISENBERG²

Department of Microbiology and Center for Oral Health Research, School of Dental Medicine,¹ and Department of Pathobiology, School of Veterinary Medicine,² University of Pennsylvania, Philadelphia, Pennsylvania 19104

Herpes simplex virus type 1 (HSV-1) contains five glycoproteins, designated gA, gB, gC, gD, and gE. The present studies focused on the synthesis and processing of two of these, gC and gD. By using monoclonal antibody to gC, we demonstrated an antigenic and structural relationship between the precursor, pgC(110), and the product, gC(130). Tryptic peptide analysis showed that pgC and gC shared methionine peptides and that these molecules had the same fingerprint pattern as that of gC(130) extracted from the purified virion. These results suggested that post-translational processing of gC involved no major changes in methionine-containing tryptic peptides or in the cleavage sites required to generate those peptides. The syntheses of gC and gD were compared. We found that the glycoproteins were synthesized starting at different times in the infectious cycle; pgD was detected by 2 h postinfection, whereas pgC was first detected at 4 to 6 h postinfection. Both precursor molecules, pgC(110) and pgD(52), are basic glycopolypeptides, and in both cases processing involved changes in molecular weight and charge. These changes were detected by two-dimensional gel electrophoresis. Both glycoproteins exhibited heterogeneity, displayed as a series of spots (6 for gD and 15 to 20 for gC) of increasing negative charge and molecular weight. Neuraminidase treatment decreased the size, number, and acidic charge of the spots, suggesting that processing was due in part, but not entirely, to addition of sialic acid to pgD and pgC.

The glycoproteins of herpes simplex virus (HSV) are important structural components of the virion envelope and are involved in virus-induced alterations in infected mammalian cells. Our previous studies (3, 5, 8) have concentrated on the type-common glycoprotein, gD, which may be involved in adsorption of the virion to the cell (25). Pulse-chase experiments showed that glycoprotein gD exists in a precursor [pgD(52)] and product [gD(59)] form in the infected cell. The processing of gD appears to occur in the following manner: a 50,000-dalton (50K) core polypeptide (19) is glycosylated by the addition of an 1,800-dalton oligomannosyl residue (8), via a lipid dolichol-mediated transfer step, to form pgD(52). This molecule is then processed by the addition of carbohydrate and sialic acid to produce the product [gD(59)]. The alteration in molecular weight occurs concurrently with a change in molecular charge, and both changes are due, in part, to the addition of sialic acid to the oligomannosyl residue (8).

One of the goals of the present study was to extend our previous work by characterizing the events involved in the synthesis and processing of gD in greater detail. Our second goal was to

study another glycoprotein found in the virion envelope, which from the present work we believe to be gC (23).

It was previously shown that a 130,000-dalton complex (126,000 to 130,000) of glycoproteins could be immunoprecipitated from infected cell extracts by antiserum to the virion envelope (3, 8). Glycoproteins designated gA, gB, and gC are postulated to be in this complex (23). Because these glycoproteins are difficult to resolve by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), studies of their synthesis and processing have proven to be arduous. Spear (23) circumvented this problem in part by absorbing an antiserum which recognized all of the glycoproteins in the 130,000-dalton complex with an extract of cells infected with HSV type 1 (HSV-1) strain MP, a strain which lacks gC (10, 23). Thus, she obtained an antiserum which had the capacity to detect gC, but was restricted in its capacity to react with the other glycoproteins. Using this antiserum, she identified the putative precursor to gC as a 110K molecule. In our initial studies (8), we observed that a major component of the 130,000-dalton complex with a pI of 4.93 was selectively removed by detergent

treatment of virions. This molecule had a tryptic methionine fingerprint pattern similar to that of a 130K glycoprotein isolated from extracts of infected cells that had been pulse-labeled with [³H]methionine at 5 h postinfection (p.i.) and chased for an additional 5 h. This 130K glycoprotein was also shown to be unrelated structurally to gD (8).

In the present study, the virion glycoprotein gC was purified and an antiserum directed specifically against this component was prepared. We used this antiserum and tryptic peptide analyses to show that pgC(110) is antigenically and structurally related to gC(130). Synthesis and processing of glycoproteins gC and gD were studied. We found that these glycoproteins are synthesized at different times in the infectious cycle but that processing of both molecules appears to involve changes in both molecular weight and charge due to the addition of sialic acid.

MATERIALS AND METHODS

Cell cultures. Conditions for the growth and maintenance of KB cells and BHK cells have been previously described (5).

Virus preparation and titration. The procedures used for the preparation of virus stocks of HSV-1 (strain HF) and HSV-1 (strain MP), as well as the plaque assay, were described previously (2, 5). For infection, an input multiplicity of 20 PFU of HSV per cell was used. Radioactive labeling of HSV-1 was performed as previously described (3, 8). To solubilize the HSV envelope, purified virions were suspended in 0.02 M Tris buffer (pH 7.5) containing 0.05 M NaCl and 0.5% Nonidet P-40 (NP-40) and incubated at 37°C for 45 min. Tolylsulfonyl phenylalanyl chloromethyl ketone and *N*- α -tosyl-L-lysine chloromethyl ketone were added, each at a concentration of 0.1 mM, to inhibit proteolytic activity. The nucleocapsids were removed by centrifugation (4), and the supernatant fluids were made 1 mM with dithiothreitol and 1 M with urea (22). As a control, virions were extracted under these same conditions, and the extract was used to prepare antiserum. This antiserum was indistinguishable from anti-ENV-1 serum prepared as described previously (3).

Pulse-chase experiments. Experiments were performed by a modification of methods previously described (3, 8). To increase the incorporation of isotopically labeled methionine or arginine, the cells (35-mm plates) were overlaid after infection with minimal essential medium containing 1/10 the normal concentration of the appropriate amino acids. Pulse-labeling was carried out by incubating infected cells in 0.5 ml of Hanks salts containing one of the following radioisotopes: [³⁵S]methionine (specific activity, 600 Ci/mmol), 200 μ Ci; [methyl-³H]methionine (specific activity, 100 Ci/mmol), 250 μ Ci; and [2,3-³H]arginine (specific activity, 15 Ci/mmol), 125 μ Ci. Conditions for labeling are indicated in the appropriate figure.

Immunological procedures. Two antisera used in this study have been described previously (3). Anti-

CP-1 serum was used as a monoprecipitin antiserum against pgD and gD components. Anti-ENV-1 serum (3) prepared against the NP-40 extract of the virion envelope of HSV-1 was used to immunoprecipitate the envelope glycoproteins. Anti-130K serum (anti-gC serum) was prepared against the pooled fraction 2 obtained by Sephacryl S-200 gel filtration chromatography (see Fig. 1).

For immunoprecipitation, *Staphylococcus aureus* Cowan strain 1 (IgSorb, New England Enzyme Center) was used to collect antigen-antibody complexes. Each antiserum was titered to ensure that the maximal amount of antigen was precipitated. The precipitates were washed and the antigen-antibody complexes were dissociated as previously described (14, 19). Immunofluorescence was performed by a modification (4) of the indirect procedure described by Reed et al. (21). Serum neutralization titers were determined by the plaque reduction technique as previously described (20). The greatest dilution of serum causing a 50% reduction in titer was selected as the neutralizing titer.

Electrophoresis on SDS-PAGE. SDS-PAGE was carried out in slabs of 10% acrylamide cross-linked with 0.4% *N,N'*-diallyltartardiamide (DATD) or in slabs of 10% acrylamide cross-linked with 0.26% *N,N'*-methylenebisacrylamide as described previously (3, 8). After electrophoresis, the gels were stained with Coomassie brilliant blue. For autoradiography, the gels were dried on filter paper and placed in contact with Kodak X-Omat R (XR-5) film. For fluorography, the gels were impregnated with 2,5-diphenyloxazole (1) placed in contact with film and stored at -70°C. Protein standards ranging from 15,000 to 130,000 daltons were run on each gel. Two-dimensional (2-D) gel electrophoresis was performed by the technique of O'Farrell (17), using (i) a combined gradient consisting of a pH 5 to 7 and pH 3 to 10 mixture, (ii) a gradient of pH 3 to 10 alone, and (iii) nonequilibrium pH gradient electrophoresis (18).

Preparation of samples for tryptic peptide analysis. DATD gel slices were dissolved in 2% periodic acid according to the procedure described by Gibson (9), and the polypeptides were prepared for trypsinization by methods described previously (8, 28). Trypsinization and ion-exchange chromatography on Chromabeads P resin were carried out as previously described (8, 28).

Neuraminidase treatment. Neuraminidase (0.8 U/ml, type IX; Sigma Chemical Co.) was used to remove sialic acid from the glycoproteins. Two procedures were used. (i) Anti-ENV-1 serum was used to immunoprecipitate the glycoproteins from the cytoplasmic extract, with *S. aureus* used to collect the antibody-antigen complex. The complex was washed two times in enzyme buffer (0.1 M sodium acetate, pH 5.5, 2 mM CaCl₂, and 1 mM EDTA) and suspended in 0.2 ml of enzyme buffer containing neuraminidase. The complex was incubated for 1 h at 37°C, a second portion of enzyme was added, and incubation was continued for a second hour. (ii) Equal volumes of cytoplasmic extract and 2 \times enzyme buffer were mixed, and neuraminidase was added. At the end of the incubation period, anti-ENV-1 serum was added and the mixture was incubated for 20 min at 37°C. S.

aureus was added to precipitate the immune complex as previously described (14, 19). In both cases, control samples were incubated in the absence of enzyme. The washed pellets from procedures i and ii were resuspended in O'Farrell's lysis buffer (9.5 M urea, 2% NP-40, 1.6% Ampholine, pH 5 to 7 [LKB Instruments Inc.]) for isoelectric focusing or in disrupting buffer for SDS-PAGE.

RESULTS

Preparation of antiserum to a 130K polypeptide. Figure 1 shows the elution profile obtained when the NP-40 extract of [³⁵S]methionine-labeled virions (insert slot B) was subjected to gel filtration chromatography on Sephacryl S-200. The eluates under bars 1 through 4 were pooled separately, concentrated, and evaluated by SDS-PAGE. Pooled fraction 1 contained a 130K component and a 47K protein. On the basis of tryptic peptide analysis, we believe the

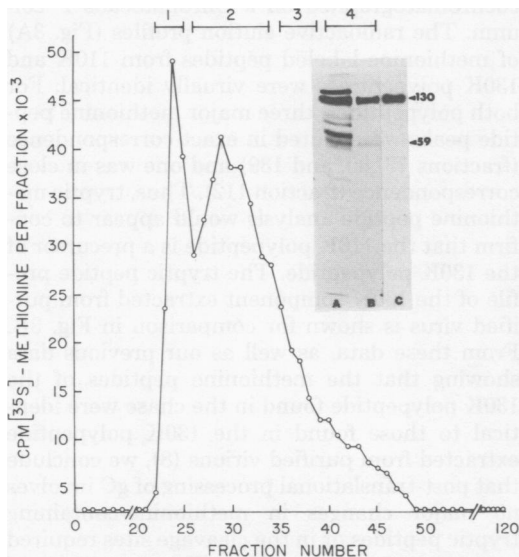


FIG. 1. Gel filtration chromatography of a detergent extract of purified herpes simplex virions. [³⁵S]methionine-labeled virions were extracted with 0.05 M Tris, pH 7.5, containing 0.5% NP-40 and 0.05 M NaCl. The extract was chromatographed on a column of Sephacryl S-200. For this experiment, a 0.5-ml sample was applied and the column was eluted with 0.05 M Tris, pH 7.5, containing 0.1% NP-40, 0.05 M NaCl, 1 M urea, and 1 mM dithiothreitol. The column size was 1.7 by 82 cm, the fraction size was 0.5 ml, and the flow rate was 40 ml/h. Recovery was 76% as determined by radioactivity. (Insert) Autoradiogram of virion polypeptides separated by SDS-PAGE. Track A, Purified virions labeled with [³⁵S]methionine; track B, polypeptides extracted with NP-40 from purified virions; track C, polypeptide from pooled fraction 2. The exposure time on X-ray film was five times longer for track C than for track B in order to detect any other components.

47K component to be a breakdown product of gD(59) (G. H. Cohen, D. Long, and R. J. Eisenberg, unpublished data). Pooled fraction 2 (Fig. 1, insert slot C) contained the 130K component and no detectable gD(59). However, long-term exposure of the gel to X-ray film did reveal a 65K molecule and a fourth band at 43K which we believe to be actin. Fractions 3 and 4 contained the 130K polypeptide(s) but in markedly reduced amounts. Antiserum to fraction 2 was prepared and will be referred to as anti-130K serum. Immunofluorescence studies showed that anti-130K serum gave a strong reaction against the plasma membrane of unfixed HSV-1-infected KB cells. In addition, the serum reacted against cytoplasmic and perinuclear components of infected fixed KB cells. No fluorescence was observed when this antiserum was reacted with either fixed or unfixed uninfected cells. These data suggested that we had prepared an antiserum that reacted with virus-induced membrane antigens. Anti-ENV-1 prepared against the virion envelope extract neutralized virus with a 50% titer of greater than 2,000 (Table 1). In contrast, anti-130K serum did not neutralize virus either in the presence or in the absence of added complement.

Antiserum to 130K apparently recognizes glycoprotein gC. Figure 2A shows the results of immunoprecipitation studies using infected [³⁵S]methionine-labeled extracts. Anti-ENV-1 serum immunoprecipitated three major polypeptide bands from pulse-labeled HSV strain HF-infected cell extracts at 52,000, 110,000, and 126,000 daltons (track 1) and precipitated polypeptides of 59,000, 126,000 (which does not show up well in this figure), and 130,000 daltons from the chase (track 2). These results are in agreement with those previously reported (8). In contrast, anti-130K serum precipitated only the 110K polypeptide from the pulse (track 3) and the 130K polypeptide from the chase (track 4). These results are consistent with the idea first proposed by Spear (23) that the 110K and 130K components represent precursor and product forms of the same molecule and that anti-130K is a monoprecipitin antiserum capable of reacting with both forms. Evidence that the 130K polypeptide immunoprecipitated by anti-130K is gC is provided by the following experiment. BHK cells were infected with HSV-1 strain MP (which lacks gC) and labeled continuously with [³⁵S]methionine from 5 to 9 h p.i. Cytoplasmic extracts were prepared and immunoprecipitated with anti-ENV-1 serum (Fig. 2A, track 5) or anti-130K serum (Fig. 2A, track 6). It is evident that two polypeptide bands in the 130K complex, corresponding in electrophoretic mobility to gA and gB, were immunoprecipi-

TABLE 1. Serum neutralization of HSV-1 infectivity

Antiserum to:	Complement ^a	Neutralization titer
ENV-1	-	2,048 ^b
130K	-	0 ^c
130K	+	0 ^c

^a Source of complement was either non-heat-inactivated antiserum or heat-inactivated antiserum (56°C for 30 min) plus nonimmune rabbit serum.

^b Dilution of serum which leads to a 50% reduction in infectivity.

^c Undiluted antiserum failed to neutralize infectivity.

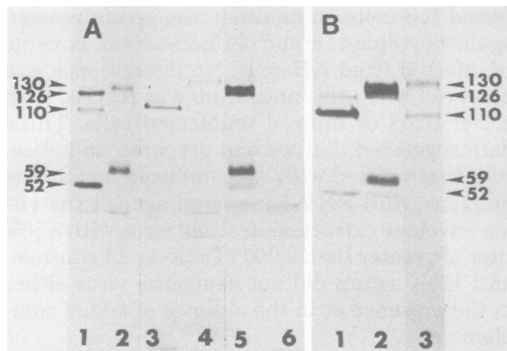


FIG. 2. SDS-PAGE analysis of anti-130K serum. (A) Autoradiogram of a 10% DATD cross-linked polyacrylamide gel of immune precipitates obtained from lysates of HSV-1-infected cells labeled with [³⁵S]methionine. Track 1, HSV-1 strain HF, pulse, anti-ENV-1 serum; track 2, strain HF, chase, anti-ENV-1 serum; track 3, strain HF, pulse, anti-130K serum; track 4, strain HF, chase, anti-130K serum; track 5, HSV-1 strain MP continuously labeled from 5 to 7 h p.i., anti-ENV-1 serum; track 6, same lysate as in track 5 but immunoprecipitated with anti-130K serum. (B) Fluorogram (SDS-PAGE as above) of immune precipitates from lysates of HSV-1 (strain HF)-infected cells labeled with [²⁻³H]mannose. Track 1, pulse, anti-ENV-1 serum; track 2, chase, anti-ENV-1 serum; track 3, cells continuously labeled from 5 to 7 h p.i., anti-130K serum.

tated by anti-ENV-1 serum (in addition to gD), whereas no corresponding polypeptide bands in the 130K region were immunoprecipitated by anti-130K serum.

Figure 2B shows that [²⁻³H]mannose was incorporated during a short pulse (track 1) into 52K, 110K, and 126K polypeptides. The carbohydrate label was chased (track 2) into molecules with molecular weights of 59,000, 126,000, and 130,000. These results are consistent with those of Fig. 2A, tracks 1 and 2. When HSV-1 strain HF was labeled continuously with [²⁻³H]mannose from 5 to 7 h p.i. and immunoprecipitated with anti-130K serum, two radioactively labeled bands with molecular weights of

110,000 and 130,000 were detected. These results demonstrate that anti-130K serum reacted only with two glycosylated proteins of 110,000 and 130,000 daltons. The results shown in Fig. 2A and 2B taken together indicate that the 130K serum is glycoprotein gC and that the 110K glycoprotein is probably the precursor pgC.

Tryptic peptide analysis of the 110K (pgC) glycoprotein and the 130K (gC) glycoprotein. Tryptic peptides were analyzed to verify that the 110K glycoprotein (pgC) was structurally related to the 130K glycoprotein (gC). Infected cells were labeled with [³H]methionine for 15 min at 5 h p.i. (pulse) or pulse-labeled with [³⁵S]methionine and followed by a 5-h chase. Cytoplasmic extracts were prepared, immunoprecipitated by anti-130K serum, and analyzed by SDS-PAGE. Bands corresponding to pgC(110) and gC(130) were cut out of the gel, dissolved in periodate, oxidized, trypsinized, and cochromatographed on a Chromabeads P column. The radioactive elution profiles (Fig. 3A) of methionine-labeled peptides from 110K and 130K polypeptides were virtually identical. For both polypeptides, three major methionine peptide peaks were eluted in exact correspondence (fractions 73, 80, and 139) and one was in close correspondence (fraction 112). Thus, tryptic methionine peptide analysis would appear to confirm that the 110K polypeptide is a precursor of the 130K polypeptide. The tryptic peptide profile of the 130K component extracted from purified virus is shown for comparison in Fig. 3B. From these data, as well as our previous data showing that the methionine peptides of the 130K polypeptide found in the chase were identical to those found in the 130K polypeptide extracted from purified virions (8), we conclude that post-translational processing of gC involves no major changes in methionine-containing tryptic peptides or in the cleavage sites required to generate those peptides. The next series of experiments was performed to study and compare some of the steps in the synthesis and processing of glycoproteins gC and gD of HSV-1.

Studies on the synthesis and processing of gC and gD. KB cells were infected with HSV-1 and pulse-labeled for 15 min with [²⁻³H]mannose (or [³⁵S]methionine) at 10 min, 2 h, 4 h, 6 h, 8 h, and 10 h p.i. Our initial studies showed that both of these radioactive labels could be detected in the precursor molecules pgD(52) and pgC(110) within 2 to 5 min after the addition of label (data not shown). Cytoplasmic extracts were immunoprecipitated with anti-ENV-1 serum and analyzed by SDS-PAGE. Since the results using both radioactive labels were essentially the same, only the data using

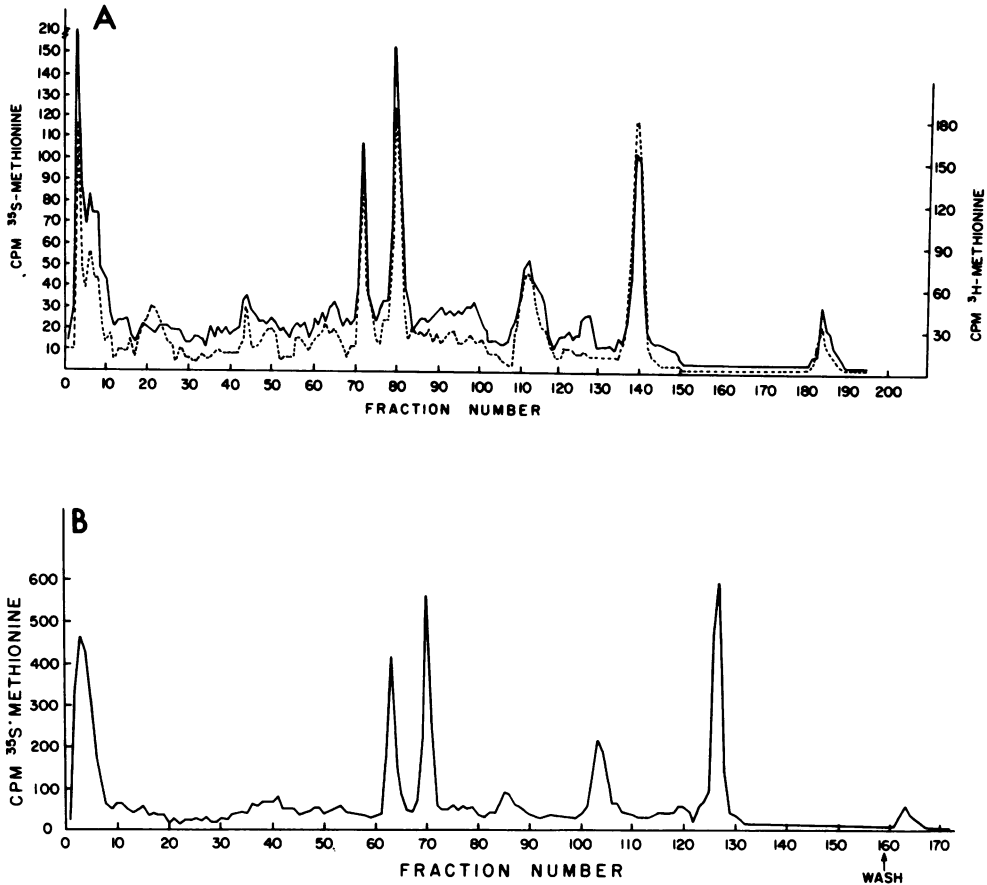


FIG. 3. Tryptic fingerprint analysis of pgC and gC of HSV-1. (A) 130K glycoprotein (—) isolated from the [³⁵S]methionine-labeled chase by immunoprecipitation with anti-130K serum and SDS-PAGE was trypsinized and cochromatographed on a column of Chromabeads P with [³H]methionine-labeled 110K glycoprotein isolated from a pulse-labeled cytoplasmic extract (---). (B) 130K glycoprotein was extracted from [³⁵S]-methionine-labeled purified virions with NP-40, isolated by SDS-PAGE, trypsinized, and chromatographed on Chromabeads P.

[2-³H]mannose are shown (Fig. 4). Synthesis of pgD(52) as well as another glycosylated polypeptide at 126,000 daltons was detected by 2 h p.i., whereas pgC(110) was first detected by 6 h p.i. The time course of synthesis of each glycoprotein was estimated by cutting out each labeled polypeptide band and determining radioactivity by scintillation counting. The incorporation of [³H]mannose into pgD(52) as well as the 126K polypeptide reached a maximum between 4 and 6 h p.i. and then remained constant. In contrast, incorporation of label into pgC(110) was still increasing at 10 h p.i. Identical results for pgD(52) were obtained when monoprecipitin anti-CP-1 serum was used to immunoprecipitate pgD from the corresponding cytoplasmic extracts (shown as open boxes in Fig. 4B). Previous experiments showed that viral DNA synthesis begins in this system at 2.5 h p.i. (6). Regulation

of synthesis of gC and gD is apparently different, since pgD is synthesized before and pgC is synthesized after DNA synthesis begins.

Processing of precursor glycoproteins to product forms. These experiments were performed to determine the time interval(s) required to process pgD(52) to gD(59) and pgC(110) to gC(130). Infected KB cells were pulse-labeled with [2-³H]mannose for 10 min at 6 h p.i. The label was removed, cycloheximide was added in unlabeled medium to stop further protein synthesis (12, 23), and the label was chased for the times indicated in Fig. 5. The processing step(s) involved in the conversion of pgC(110) to gC(130) occurred within 5 min after the beginning of the chase period or 15 min after the addition of [2-³H]mannose. The processed form gD(59) was first detected 10 min after the start of the chase. These results are similar to

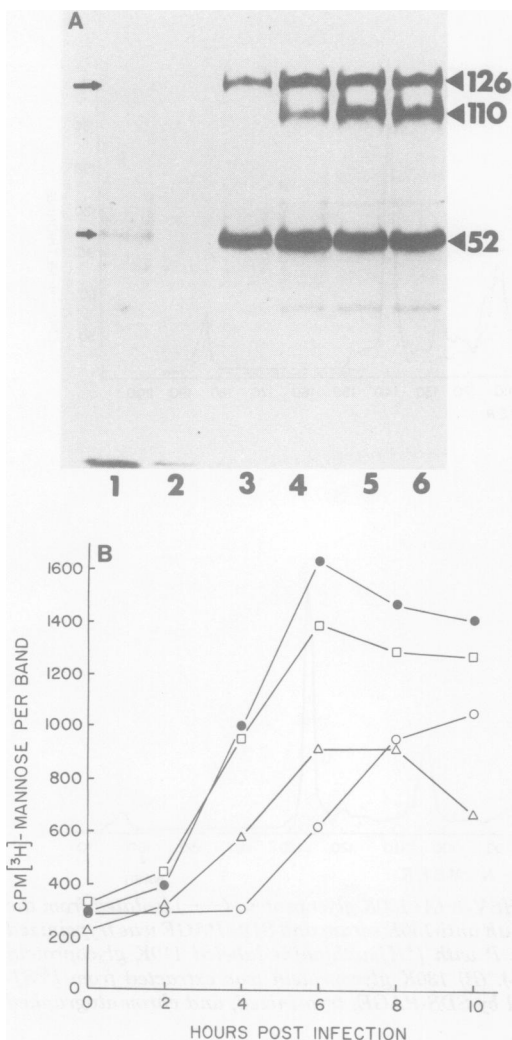


FIG. 4. (A) Time course of synthesis of HSV-1 glycoproteins. Fluorogram of a 10% DATD cross-linked polyacrylamide gel of cell lysates immunoprecipitated by anti-ENV-1 serum. Cells were pulse-labeled for 15 min with $[2\text{-}^3\text{H}]\text{mannose}$ at the times indicated below, and the cytoplasmic extracts were immunoprecipitated. Track 1, 2 h p.i. Track 2, 10 min after addition of virus (this should be equivalent to a zero time control); the 2-h pulse was separated on the gel from the 4 h sample to avoid possible spillover of labeled polypeptides. Track 3, 4 h p.i. Track 4, 6 h p.i. Track 5, 8 h p.i. Track 6, 10 h p.i. (B) Quantitative estimation of the relative rates of synthesis of individual HSV glycoproteins. Each of the radioactive polypeptides in panel A was cut out of the dried fluorogram and counted by scintillation counting. Symbols: ●, pgD(52) anti-ENV-1 serum; □, pgD(52) anti-CP-1 serum (pattern not shown in panel A); ○, pgC anti-ENV-1 serum; △, glycoprotein 126K anti-ENV-1 serum.

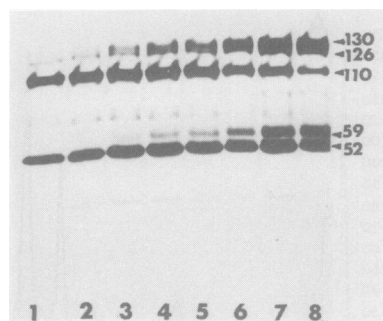


FIG. 5. Time required for processing of HSV-1 glycoproteins. Fluorogram of SDS-PAGE of immune precipitates obtained by addition of anti-ENV-1 serum to lysates of infected cells. Cells were pulse-labeled with $[2\text{-}^3\text{H}]\text{mannose}$ for 10 min at 6 h p.i. and harvested immediately for track 1. Duplicate samples were washed and incubated for various times in unlabeled medium containing 50 μg of cycloheximide per ml. Track 2, 5-min chase; track 3, 10-min chase; track 4, 15-min chase; track 5, 25-min chase; track 6, 45-min chase; track 7, 60-min chase; track 8, 100-min chase (under these conditions of cycloheximide treatment, protein synthesis was less than 1% of the non-treated control infected cells).

those reported previously (8, 23) with $[^{35}\text{S}]\text{methionine}$ as the label to follow processing. In Fig. 5, the precursor molecules appeared to be converted directly to the higher-molecular-weight form without going through an intermediate, as previously shown (8, 12, 23).

The next series of experiments used 2-D gel electrophoresis to enhance the resolution of the processed molecules. Previously (8) we showed that the conversion of pgD(52) involved a change in molecular charge as well as molecular weight, and we postulated that the molecular weight shift was due, at least in part, to the addition of sialic acid. In these earlier studies, we found a 130K component (pI 4.93) in addition to gD(59) (pI 6.4) in detergent extracts of purified virions. We will present further evidence here that this 130K component is gC.

Cytoplasmic extracts from the previous experiment were subjected to isoelectric focusing (IEF) in the first dimension and SDS-PAGE (methylene bisacrylamide) in the second. Two conditions for IEF were used: (i) a combined gradient (pH 5 to 7 and 3 to 10) (17) and (ii) a pH 3 to 10 gradient (17). The extract of pulse-labeled cells contained three $[2\text{-}^3\text{H}]\text{mannose}$ -labeled precursor molecules which were basic in nature, corresponding in molecular weight to pgD(52), pgC(110), and 126K (Fig. 6A; compare with Fig. 5, track 1). With this IEF system, the basic end of the pH gradient was flattened and as a result all three precursors appear to be

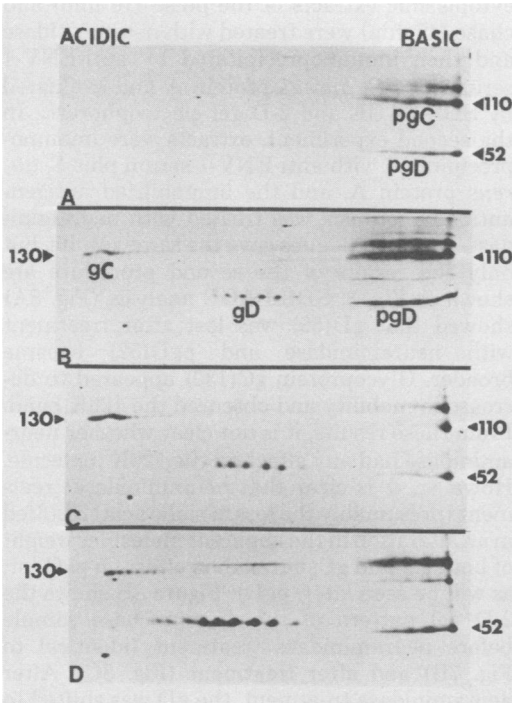


FIG. 6. Fluorogram showing the separation of [$2\text{-}^3\text{H}$]mannose-labeled glycoproteins by 2-D gel electrophoresis (pH 5 to 7). Cytoplasmic extracts obtained from the experiment shown in Fig. 5 were subjected to IEF in the first dimension and SDS-PAGE (cross-linked with methylenebisacrylamide) in the second. (A) 10-min pulse (see Fig. 5, track 1); B, 10-min chase (see Fig. 5, track 3); C, 45-min chase (see Fig. 5, track 6); D, 100-min chase (see Fig. 5, track 7).

somewhat streaked. All of the samples shown in Fig. 5 were evaluated on 2-D gels, but only three representative chase times (10, 45, and 100 min) are shown in Fig. 6B through 6D. Several points are notable. First, as early as 10 min after the beginning of the chase, the processed form of pgD(52) was detectable and appeared as a series of spots which increased in charge (with isoelectric points ranging from 5.9 to 6.6) as they increased in molecular weight. Second, the number of unit charges of gD(59) appeared to be constant; i.e., the same number of spots and the relative proportion of label in each spot was found in the 10-min or the 100-min chase. Third, processing of pgC(110) into gC(130) was detected within 5 to 10 min after the beginning of the chase, and gC(130) appeared also as a series of spots of more than 20 components with isoelectric points ranging from 4.93 to 6.4. The proportion of mannose label in each spot as well as the positions of the spots in the pH gradient also appeared to be constant with time of chase.

To relate pgC(110) and gC(130) as studied by SDS-PAGE (one dimensional) with the components seen in the 2-D gel system, we altered the conditions of IEF. This second system (17) was designed to increase the resolution of the basic region. To identify gC, we used anti-130K serum in addition to anti-ENV-1 for immunoprecipitation. Figure 7A shows the pattern obtained for the 10-min pulse and Fig. 7B shows the pattern for the 45-min chase, using anti-ENV-1 serum to immunoprecipitate each extract. The precursor pgD(52) was resolved into two components, pI 7.55 and 7.45. The second, more acidic compo-

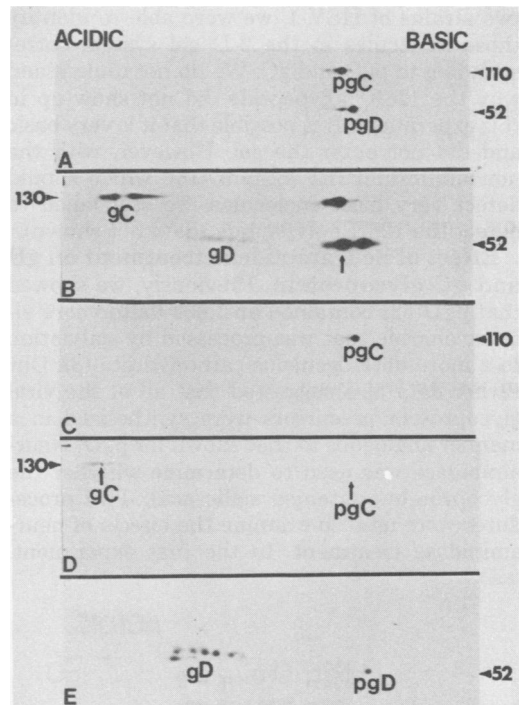


FIG. 7. Two-dimensional gel electrophoresis (pH 3 to 10 gradient) of HSV-1 glycoproteins. Cytoplasmic extracts of infected cells were immunoprecipitated with anti-ENV-1 serum (A, B, and E) or with anti-130K serum (C and D). (A) HSV-1 (HF)-infected cells pulse-labeled with [$2\text{-}^3\text{H}$]mannose for 10 min at 6.5 h p.i., anti-ENV-1 serum (see Fig. 5, track 1, and Fig. 6A); (B) HSV-1 (HF)-infected cells pulse-labeled with [$2\text{-}^3\text{H}$]mannose for 10 min at 6.5 p.i. and then chased for 45 min in the presence of cycloheximide, anti-ENV-1 serum (see Fig. 5, track 6, and Fig. 7C). The arrow points to a second spot for pgD with a pI of 7.65. (C) HSV-1 (HF)-infected cells pulse-labeled as in (A), anti-130K serum; (D) HSV-1 (HF)-infected cells labeled as in (B), anti-130K serum; (E) HSV-1 (MP)-infected cells continuously labeled with [^{35}S]methionine from 5 to 9 h p.i., anti-ENV-1 serum (see Fig. 2A, track 5).

ment was barely apparent in the pulse but was prominent in the 45-min chase. The 110K component appeared as one spot (pI of 7.40), and the 126K band was missing. Figures 7C and 7D show the results when anti-130K serum was used to immunoprecipitate the same extracts. The 110K band in Fig. 7C (pI 7.40) corresponds to pgC in Fig. 6A, and the 130K band in Fig. 7D corresponds to gC in Fig. 6B. Figure 7E shows the pattern obtained when extracts of HSV-1 strain MP were immunoprecipitated with anti-ENV-1 serum and analyzed in the 2-D gel system. Both pgD and gD were resolved, but no molecules corresponding to pgC, gC, or 126K were detected. Thus, by using the two antisera and the two strains of HSV-1, we were able to identify those molecules in the 2-D gel system corresponding to pgC and gC. We do not understand why the 126K polypeptide did not show up in this experiment. It is possible that it is very basic and did not enter the gel. However, with the nonequilibrium IEF system (18), which should detect very basic molecules, we still failed to detect the 126K polypeptide (data not shown).

Effect of neuraminidase treatment on gD and gC glycoprotein. Previously, we showed that pgD(52) contained an 1,800-dalton core oligosaccharide that was processed by sialylation to a more heterogeneous carbohydrate (8). Our earlier data also suggested that all of the viral glycoprotein precursors were synthesized in a manner analogous to that shown for pgD. Neuraminidase was used to determine whether the glycoprotein contained sialic acid. Two procedures were used to examine the effects of neuraminidase treatment. In the first experiment,

cytoplasmic extracts of the pulse (10 min) and chase (45 min) were treated with neuraminidase and then immunoprecipitated by anti-ENV-1 serum plus *S. aureus* protein A and evaluated by SDS-PAGE and 2-D gel electrophoresis. In the second experiment, extracts were immunoprecipitated with anti-ENV-1 serum plus *S. aureus* protein A, and the immobilized antigen-antibody complex was treated with neuraminidase. Both techniques gave the same results, but only the results of the second procedure are shown in Fig. 8. SDS-PAGE analysis (Fig. 8A) showed that gD(59) was lost after treatment with neuraminidase and pgD(52) became broader. Glycoprotein gC(130) appeared to decrease in mobility and obscured the 126K band. From these results, it is not clear whether neuraminidase had any effect on the 126K molecule. However, it is clear that neuraminidase treatment (presumably the loss of sialic acid) resulted in an alteration in the apparent molecular weight of both gD and gC, but had no effect on pgC (or, as will be seen later, pgD). Figure 8B shows the 2-D gel pattern of the 45-min chase sample before neuraminidase treatment (identical to Fig. 7B) and after treatment (Fig. 8C). After neuraminidase treatment, the gD was shifted to a position corresponding to a more basic pI, the number of spots was altered, and the apparent molecular weight of gD was reduced (see arrow, Fig. 8C). Neuraminidase treatment of gC resulted in similar alteration of charge and molecular weight. There was no change in the molecular weight or charge of either precursor molecule.

We conclude that conversion of pgD(52) to

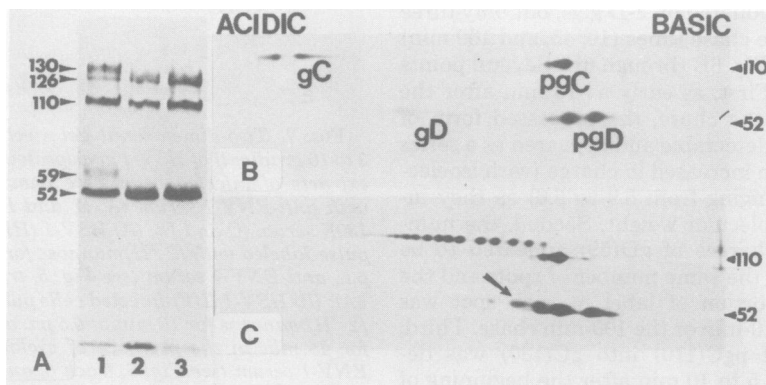


FIG. 8. Neuraminidase treatment of HSV glycoproteins analyzed by SDS-PAGE (A) and 2-D gel electrophoresis (B and C). In each case, the immune precipitates were obtained by using anti-ENV-1 serum. (A) Track 1, immunoprecipitates of [^3H]mannose-labeled cells after a 45-min chase similar to the experiment shown in Fig. 6; track 2, cell extracts were immunoprecipitated and treated with neuraminidase (0.04 U/ml for 1 h); track 3, same as in track 2 except that the concentration of enzyme and incubation times were doubled. (B) Same immunoprecipitate as in (A), track 1. Same photograph as in Fig. 7B. (C) Same immunoprecipitate as in (A), track 3. Arrow indicates location of gD after neuraminidase treatment.

gD(59) and pgC(110) to gC(130) involves a change in electric charge as well as molecular weight. Both of these alterations in properties are due in part to addition of sialic acid.

DISCUSSION

The purpose of the present study was to continue our investigations of the synthesis and processing of HSV-1 glycoproteins. By using monoprecipitin antiserum to undenatured gD as an immunological probe, we previously demonstrated the structural relationship between the precursor and product forms of this molecule (8). The results presented here extend our knowledge of gD and characterize a second virion glycoprotein of HSV-1. We purified this glycoprotein from the envelope of purified virions by methods gentle enough to avoid denaturation of the protein and used it to prepare anti-130K serum. The following evidence indicates that anti-130K serum reacts specifically with glycoprotein gC(130) and its precursor, pgC(110). First, anti-130K serum immunoprecipitated a 130K component from cytoplasmic extracts of cells infected with strain HF of HSV-1 but not from extracts of cells infected with strain MP. The latter strain lacks glycoprotein gC (10, 23). Second, a 110K molecule was immunoprecipitated from cytoplasmic extracts of cells that had been infected with strain HF and pulse-labeled with either [³⁵S]methionine or [2-³H]mannose at 5 h p.i. A 130K molecule was immunoprecipitated from cells that had been pulse-labeled at 5 h p.i. and then chased for 5 h. Third, tryptic peptide analysis firmly established a structural relationship between the 110K and 130K molecules. From the present data as well as our previous study, where it was shown that the methionine peptides of the gC(130) found in the chase were identical to those found in the 130K polypeptide extracted from purified virions, we conclude that there are no major alterations in the primary structure of gC between its synthesis [pgC(110)], its processed form [gC(130)], and its final incorporation into the mature virion. These results confirm and extend the original observations of Spear (23) and Honess and Roizman (12).

Although anti-130K serum immunoprecipitated the appropriate molecules from infected cell extracts and reacted with cells by immunofluorescence, the serum did not neutralize virus infectivity either in the presence or in the absence of added complement. In terms of neutralizing activity and the role of complement, these results differ from those of other investigators. Courtney and Powell (7) prepared antiserum to a glycosylated polypeptide, called 123K, isolated from SDS-PAGE and corre-

sponding to the gC region. This antiserum to the SDS-denatured polypeptide neutralized virus but only in the presence of complement (7). Vestergaard and Norrild (27) prepared antiserum to an immunoprecipitate called AG-6, shown to correspond to gC(130) (16). This serum neutralized HSV-1, and added complement had no effect. Spear (23) prepared a monoprecipitin antiserum to gC by absorbing an HSV-1 antiserum with an extract of cells infected with HSV-1 strain MP, but did not report whether this serum was capable of neutralizing virus infectivity. L. Pereira (personal communication) has prepared monoclonal antibody to gC which neutralizes virus in the presence or absence of complement. Thus, although all of these sera, including anti-130K, are capable of immunoprecipitating gC, they appear to differ in their capacity to neutralize virus and in their biological response to complement. Differences in the strain of virus used as well as in the methods of preparation of antigen and antisera could account for discrepancies in the properties of the antisera.

The evidence derived from this paper extends our knowledge of the synthesis of gD and can be summarized as follows. As early as 2 h p.i. a 50K, putative unglycosylated precursor polypeptide (19) is glycosylated, probably as a cotranslational event, by the addition of an 1,800-dalton oligomannosyl residue (8) via a dolichol-mediated step (19). Two-dimensional gel electrophoresis studies suggest that pgD(52), with a pI of 7.55, is converted (within a 45-min period) to a second, more acidic form with a pI of 7.45. This shift is apparently not due to the addition of sialic acid, since neuraminidase had no effect on the molecule. The reason for the shift is unknown and may reflect an intermediate step in processing such as clipping of the high-mannose chain before addition of further carbohydrate (13, 26). This clipping might result in a conformational change in the protein which would expose or bury charged groups. It is noteworthy that this processing step was observed only when IEF was carried out in a pH 3 to 10 gradient (17). Resolution of this intermediate does not appear to be dependent upon conditions used, i.e., loading of the sample at the basic end of the gradient, since identical results were obtained with the nonequilibrium IEF technique (18), whereby the sample is applied at the acidic end of the gradient. Processing of pgD(52) to the product form gD(59) occurs within 15 min and includes an increase in molecular weight and charge. In 2-D gels, gD appears as a series of spots (approximately six) which increase in charge (from pI 5.9 to 6.6) as they increase in molecular weight; i.e., the most acidic member of the series was the largest molecule. The num-

ber of spots as well as the relative intensity of each spot was constant regardless of the time of chase. These results may be interpreted in one of two ways. One possibility is that one spot is the precursor of another in the series. For example, pgD(52) could be converted to spot 1, spot 2, spot 3, etc., and finally to gD(59), provided that the final product turned over at a rate similar to that of the input of pgD(52) into the sequence of reactions. In this case, one might expect to find only the final processed form in the virion. However, all six spots were found when detergent extracts of purified virions were analyzed by 2-D gel electrophoresis (Cohen and Eisenberg, unpublished data). The second possibility is that the smallest, least charged spot is not a precursor of the largest, most charged spot. In other words, processing from one or both precursor spots to the series of spots that constitute gD(59) may occur in one step and may involve a conformational alteration in the protein as a consequence of sialylation of the oligosaccharide residue(s).

Removal of sialic acid from gD(59) by neuraminidase reduced the molecular weight of the molecule to approximately 53,000, reduced the number of differently charged spots that were resolved, and shifted the charge of all the spots toward the basic end of the gradient but not back to the precursor forms. These studies confirm and extend our earlier observation that mild acid treatment of gD(59) oligosaccharide reduced its molecular weight considerably, but not back to the size of the core oligosaccharide (8). The failure of neuraminidase treatment to shift the molecule back to its precursor size and charge might be explained in several ways, including: (i) the presence of additional carbohydrate on the processed form of the molecule (12), (ii) the presence of sialic acid residues that are resistant to neuraminidase or acid treatment, (iii) an irreversible conformational change in the molecule which occurs as a result of sialylation and exposes more acidic residues, or (iv) some other post-translational modification. In this latter regard, neither sulfate (as $^{35}\text{SO}_4$) nor $^{32}\text{PO}_4$ was incorporated into gD or gC (Cohen, Eisenberg, and Pizer, unpublished data).

Synthesis and processing of gC is, in general terms, similar to that of gD. However, there appears to be a significant difference in the time course of appearance of pgD(52) and pgC(110), since pgD(52) is detected by 2 h p.i. and pgC(110) does not appear before 4 to 6 h p.i. This observation suggests that gD may be an early or β protein and that gC may be a late or γ protein (15). Further experiments are required to fulfill the operational definition for assignment of these glycoproteins to different kinetic

classes (11). Beginning at 6 h p.i. and within 10 min after synthesis begins, an 1,800-dalton oligomannosyl residue (8) is transferred to a polypeptide precursor of pgC, via a dolichol-mediated step (19). Experiments with tunicamycin (19) suggest that this unglycosylated precursor may have a molecular weight of approximately 85,000. Processing of pgC(110) to gC(130) occurs within 10 min after the chase and leads to a dramatic shift in apparent molecular weight, pI, and number of charged spots. The precursor pgC(110) has a pI of 7.55, and the major components (5 out of 20 total) of gC(130) have an average pI of 4.93. These alterations are due in large part but not completely to the addition of sialic acid to pgC(110). Removal of sialic acid reduces the size, number, and acidic charge of the spots. Further experiments are needed to clarify why the gC molecules do not revert back to the size or charge of the precursor, pgC, when sialic acid is removed.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant DE-02623 from the National Institute of Dental Research. R.J.E. was supported in part by a special grant from the University of Pennsylvania.

We thank William Lawrence, Joel Rosenbloom, and Wesley Wilcox for their help in preparation of this manuscript. We also thank Manuel Ponce de Leon, Cassie Hydrean-Stern, and Madeline Cohen for their excellent technical assistance.

LITERATURE CITED

- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
- Cohen, G. H., M. N. Factor, and M. Ponce de Leon. 1974. Inhibition of herpes simplex virus type 2 replication by thymidine. *J. Virol.* 14:20-25.
- Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg. 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. *J. Virol.* 27:172-181.
- Cohen, G. H., M. Ponce de Leon, H. Diggelmann, W. C. Lawrence, S. K. Vernon, and R. J. Eisenberg. 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. *J. Virol.* 34:521-531.
- Cohen, G. H., M. Ponce de Leon, and C. Nichols. 1972. Isolation of a herpes simplex virus specific fraction which stimulates the production of neutralizing antibody. *J. Virol.* 10:1021-1030.
- Cohen, G. H., R. K. Vaughan, and W. C. Lawrence. 1971. Deoxyribonucleic acid synthesis in synchronized mammalian KB cells infected with herpes simplex virus. *J. Virol.* 7:783-791.
- Courtney, R. J., and K. L. Powell. 1975. Immunological and biochemical characterization of polypeptides induced by herpes simplex virus types 1 and 2, p. 63-73. *In*, G. de-Thé, M. A. Epstein, and H. Zur Hausen (ed.), *Oncogenesis and herpes viruses II*. International Agency for Research on Cancer, Lyon.
- Eisenberg, R. J., C. Hydrean-Stern, and G. H. Cohen. 1979. Structural analysis of precursor and product forms of type-common envelope glycoprotein D (CP-1 antigen) of herpes simplex virus type 1. *J. Virol.* 31:608-620.

9. Gibson, W. 1974. Polyoma virus proteins. A description of the structural proteins of the virion based on polyacrylamide gel electrophoresis and peptide analysis. *Virology* **62**:319-336.
10. Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman. 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. *J. Virol.* **14**:640-651.
11. Honess, R. W., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* **14**:8-19.
12. Honess, R. W., and B. Roizman. 1975. Proteins specified by herpes simplex virus. XIII. Glycosylation of viral polypeptides. *J. Virol.* **16**:1308-1326.
13. Hunt, L. A., J. R. Etchison, and D. F. Summers. 1978. Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* **75**:754-758.
14. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcus protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617-1624.
15. Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer. 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 \times HSV-2 recombinants. *J. Virol.* **26**:389-410.
16. Norrild, B., S. L. Shore, and A. J. Nahmias. 1979. Herpes simplex virus glycoproteins: participation of individual herpes simplex virus type 1 glycoprotein antigens in immunocytolysis and their correlation with previously identified glycopolypeptides. *J. Virol* **32**:741-748.
17. O'Farrell, P. 1975. High resolution two-dimensional gel electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
18. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**:1133-1142.
19. Pizer, L. I., G. H. Cohen, and R. J. Eisenberg. 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. *J. Virol.* **34**:142-153.
20. Ponce de Leon, M., H. Hesse, and G. H. Cohen. 1973. Separation of herpes simplex virus-induced antigens by concanavalin A affinity chromatography. *J. Virol.* **12**:766-774.
21. Reed, C. L., G. H. Cohen, and F. Rapp. 1975. Detection of a virus-specific antigen on the surface of herpes simplex virus transformed cells. *J. Virol.* **15**:668-670.
22. Roizman, B., and P. R. Roane, Jr. 1963. Demonstration of a surface difference between virions of two strains of herpes simplex virus. *Virology* **19**:198-204.
23. Spear, P. G. 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. *J. Virol* **17**:991-1008.
24. Spear, P. G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. *J. Virol.* **9**:143-159.
25. Spear, P. G., M. Sarmiento, and R. Manservigi. 1978. The structural proteins and glycoproteins of herpes viruses: a review, p. 157-167. In G. de-Thé, W. Henle, and F. Rapp (ed.), *Oncogenesis and herpesviruses III, part I*, IARC Scientific Publication no. 24. International Agency for Research on Cancer, Lyon.
26. Tabas, I., S. Schlesinger, and S. Kornfeld. 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. *J. Biol. Chem.* **253**:716-722.
27. Vestergaard, B. F., and B. Norrild. 1978. Crossed immunoelectrophoresis of a herpes simplex type 1-specific antigen: immunological and biochemical characterization. *J. Infect. Dis.* **138**:639-643.
28. Vogt, V. M., R. Eisenman, and H. Diggelmann. 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. *J. Mol. Biol.* **96**:471-493.