JOURNAL OF VIROLOGY, Nov. 1975, p. 1308-1326

Vol. 16, No. 5
Printed in U.S.A.

Proteins Specified by Herpes Simplex Virus

XIII. Glycosylation of Viral Polypeptides

ROBERT W. HONESS AND BERNARD ROIZMAN*

Departments of Microbiology, and Biophysics and Theoretical Biology, University of Chicago, Chicago, Illinois 60637

Received for publication 10 June 1975

In the course of herpes simplex virus 1 (HSV-1) replication in human epidermoid carcinoma no. 2 cells, the synthesis and glycosylation of host cell proteins ceases and is replaced by the synthesis and glycosylation of virus-specified polypeptides. Analyses of the synthesis of viral glycoproteins show that the glycosylation of viral polypeptides occurs late in the virus growth cycle and that certain of the precursors to major viral glycoproteins are members of the γ group of polypeptides, i.e., polypeptides synthesized at increasing rates until 12 to 15 h postinfection. Viral glycoproteins are formed by stepwise additions of heterosaccharide chains to completed precursor polypeptides. The precursor and the highly glycosylated product are separable by gel electrophoresis and are localized in different fractions of infected cells. Within 15 min of their synthesis, precursor polypeptides acquire heterosaccharide chains of about 2,000 molecular weight, which contain glucosamine but little or no fucose or sialic acid. Both precursor and product of this first stage of glycosylation are absent or present in low concentrations in the surface membranes of the infected cell and in the virion. The partially glycosylated product is then conjugated further in a slow, discontinuous process to form the mature glycoprotein of the virion and plasma membrane. These mature products bear large heterosaccharide units with molecular weights greater than 4,000 to 5,000; these contain fucose and sialic acid as well as glucosamine. Heterosaccharide chains from infected and uninfected cells are distributed among discrete size classes and the smallest chains consist of multiple saccharide residues.

Virions of herpes simplex virus consist of an icosahedral nucleocapsid composed of non-glycosylated polypeptides enclosed in a fibrillar layer with no recognizable symmetry (the "tegument") and surmounted by a membrane (the envelope) containing multiple glycosylated polypeptides (7, 24, 29, 35). The capsid is assembled in the nucleus of the infected cell and morphological evidence (5, 21, 22, 25) suggests that the virion acquires a glycoprotein-containing envelope at the nuclear membrane. Viral glycoproteins are also present on the plasma membrane (11) and smooth internal membranes of infected cells (27); glycoproteins at these sites may be distinct both in their composition and in their arrangement. Thus, virus-specified glycoproteins in the virion and plasma membrane of cells infected with the MP strain of herpes simplex virus 1 (HSV-1) differ qualitatively (submitted for publication) and, whereas the virion contains little or no host-derived protein (29), host and viral proteins are contiguous on vesicles derived from the plasma membrane (10).

We have shown previously that at least 49 infected cell polypeptides are synthesized after infection of human epidermoid carcinoma no. 2 (HEp-2) cells with the F1 strain of HSV-1 and that most of these polypeptides are virus specific (12). The virus-specific polypeptides could be arranged into four groups which differed in their temporal patterns of synthesis during the reproductive cycle and into three groups (α, β, and γ) on the basis of the factors regulating their synthesis (12, 13). For the purposes of this paper, the pertinent properties of these polypeptide groups are that α-polypeptides are made at highest rates from 3 to 4 h postinfection and β-polypeptides at maximal rates from 6 to 8 h, whereas the rates of synthesis of γ-polypeptides increase for at least 12 h. This paper deals with several aspects of synthesis and glycosyla-

tion of viral polypeptides in infected HEp-2 cells.

MATERIALS AND METHODS

Solutions and chemicals. Radiochemicals L-[^{14}C]leucine, isoleucine, and valine (250 to 300 mCi/mmol), and L-[^{3}H]leucine, isoleucine, and valine (7 to 15 Ci/mmol) were from Schwarz-Mann, Orangeburg, N.Y.; and D-[^{14}C]glucosamine (56.5 mCi/mmol), D-[^{3}H]glucosamine (3.6 Ci/mmol), L-[^{14}C]fucose (56.2 mCi/mmol), and L-[^{3}H]fucose (13.4 Ci/mmol) were from New England Nuclear Corp., Boston, Mass. [^{14}C]*N*-acetylglucosamine-6-P, [^{3}H]UDP-*N*-acetylglucosamine, and [^{14}C]GDP-mannose were the gifts of Alvin Markovitz, University of Chicago. Unlabeled stachyose, raffinose, and glucosamine, as well as cycloheximide (actidione) and Pronase, were purchased from Calbiochem; neuraminidase (*Clostridium perfingens*), ovalbumin, *N*-acetylneuraminic acid, galactosamine, mannosamine, fetuin, and porcine thyroglobulin from Sigma, and glucosamine-6-phosphate and puromycin from Nutritional Biochemicals, Inc.

Cells. HEp-2 cells were grown at 37 C in Eagle minimal essential medium supplemented with 10% calf serum, 0.001% ferric nitrate, and 1% sodium pyruvate.

Virus and virus infection. The origin and procedures for the propagation of strain HSV-1 (F1), as well as the electrophoretic properties of total and glycosylated polypeptides in the virion and in the plasma membrane of infected HEp-2 cells, have been described elsewhere (6, 11, 29). Cells were infected at a multiplicity of 20 PFU per cell.

Purification of enveloped virus particles (virions). Virions were labeled in vivo with D-[^{14}C]glucosamine and purified by the method of Spear and Roizman (29) as described by Heine et al. (9).

Purification of plasma membranes from infected and uninfected HEp-2 cells. Plasma membranes were purified from uninfected cells and 18-h infected cells by a modification of the method of Kamat and Wallach (15) described in detail by Heine et al. (11). Briefly, the procedure involves the separation of a microsomal fraction from cells disrupted by microcavitation in a nitrogen "bomb" by sedimentation through a barrier of Dextran 110. In the presence of divalent cations plasma membrane remained at the buffer-dextran interface, whereas most of the smooth internal membranes and microsomal components pelleted through the Dextran barrier (15). The plasma membrane fraction was then further purified by flotation through a discontinuous sucrose gradient. The extent of purification obtained by this procedure was estimated at about 30-fold (11) based on changes in the specific activities of 5'-nucleotidase (a plasma membrane marker) and reduced nicotinamide adenine dinucleotide-diaphorase (an enzyme associated with the endoplasmic reticulum).

Pronase digestion. Samples of labeled infected or uninfected cell lysates were first dialyzed exhaustively against at least three changes of 100 volumes of 0.05 M Tris-hydrochloride, pH 8.5, and 0.1% sodium dodecyl sulfate (SDS) to remove acid-soluble pools of labeled precursors prior to digestion. Dialyzed labeled preparations, as well as unlabeled glycoproteins with glycopeptides of known size and composition, were denatured and solubilized by heating at 80 C for 5 min in 2% SDS, 5% β-mercaptoethanol, and 0.05 M Tris-hydrochloride, pH 7.0. Samples were then suspended, at 0.5 to 2.0 mg/ml, in 0.05 M Tris-hydrochloride, pH 8.5, 0.1% SDS, and 0.01 M $CaCl_2$. A predigested solution of Pronase was then added to give an initial enzyme substrate ratio of 0.5 to 1.0. Additional enzyme was added during the 24- to 36-h incubation at 37 C. A drop of toluene was added to each incubation mixture to prevent microbial growth. Lysates of infected cells required 36 h of digestion under these conditions to yield a stable fraction of undigested material; however, the relative distribution of glycopeptides within the range 2,000 to 10,000 molecular weight did not change after 24 h of digestion.

Gel filtration chromatography. Glycopeptide samples were chromatographed on columns of Sephadex G50, fine (Pharmacia, Uppsala, Sweden), 1.5 by 80 or 1.0 by 70 cm, equilibrated, and eluted with 0.05 M Tris-hydrochloride, pH 8.5, and 0.1% SDS. Blue dextran 2000 (Pharmacia) was added to define the void volume (V_o), and either unlabeled raffinose or stachyose and labeled glucosamine were added routinely as other standards. Other markers were added as indicated in the text. The columns were calibrated by determining the elution volume (V_e) of glycopeptides and other compounds of known molecular weights, and a plot of log molecular weight versus V_e/V_o was linear for compounds having molecular weights from 500 to at least 4,400 (fetuin glycopeptide). Thyroglobulin, fetuin, and ovalbumin each yielded a single glycopeptide with relative elution volumes V_e/V_o of 1.66 to 1.68, 1.67 to 1.73, and 2.04 to 2.08, consistent with their molecular weights of 4,400, 4,100, and 1,550, respectively (32). Thyroglobulin glycopeptide (30) was detected by thiobarbituric acid assay of sialic acid after acid hydrolysis (34). Fetuin and ovalbumin glycopeptides were detected by the phenol-sulfuric acid assay, as were raffinose and stachyose (31).

The glycopeptide products of exhaustive Pronase digestion consist of both a heterosaccharide chain or chains linked to short peptide fragments, which are resistant to Pronase digestion because of steric hindrance by the heterosaccharide units. The peptide portion of such glycopeptides normally makes a minor contribution to the mass of the unit, which is therefore determined by the heterosaccharide moiety (32). Although the terms glycopeptide and heterosaccharide chain are used interchangeably in this paper, properties ascribed to heterosaccharide chains are deduced from the properties of glycopeptides.

Gel electrophoresis and quantitation of electrophoretically separated polypeptides. The procedures for sample preparation and electrophoresis on SDS-containing polyacrylamide gel slabs cross-linked with *N,N'*-diallyltartardiamide were as previously described (9). Labeled components were quantitated

by computer-aided planimetry of absorbance tracings of autoradiographic images of electrophoretically separated polypeptides. The methods used have been described and illustrated elsewhere (12).

In the text of this paper, "band," "polypeptide," and "protein" are used interchangeably in describing components separated by gel electrophoresis. It is one of the objects of this paper to show that a number of electrophoretically separated bands are related as precursor and product, and the use of the terms different band, polypeptide, or protein does not necessarily imply that these electrophoretically distinct components have unrelated amino acid sequences.

Determination of acid-soluble and -insoluble radioactivity. Monolayers of infected or uninfected HEp-2 cells were rinsed thoroughly with ice-cold phosphate-buffered saline to terminate incorporation and remove extracellular precursor and exchangeable intracellular radioactivity. The cells were then scraped from the flask and pelleted, and acid-insoluble radioactivity was measured by washing glass fiber strips, containing air-dried samples of cells, in cold 10% trichloroacetic acid. The strips were then washed with 80% ethanol, -2% potassium acetate, and finally with 80% ethanol, dried, and immersed in toluene-based scintillation fluid. Radioactivity in soluble pools of infected and uninfected cells was determined either by subtracting the acid-insoluble radioactivity from the total radioactivity in an air-dried sample, or by extracting the washed cell pellets with ethanol and drying an aliquot of the extract on glass fiber sheets. The two methods gave essentially similar results.

RESULTS

Uptake and incorporation of labeled glucosamine into infected and uninfected HEp-2 cells. Glucosamine is the precursor of choice for a general study of the biosynthesis of glycoproteins. It is incorporated almost exclusively into glycoprotein and glycolipid with relatively little metabolic interconversion to nonsaccharides (3, 33), and as the *N*-acetylated hexose it is a major component of almost all heterosaccharide chains in both proximal and internal positions (32). However, glucosamine is converted into a nucleotide sugar derivative (17) before being incorporated into a glycoprotein or glycolipid. One objective of this study was to compare several aspects of glycoprotein biosynthesis in infected and uninfected cells and to examine the course of glycosylation at different times after infection. To be able to differentiate real differences in the molar rate of glucosamine incorporation from differences attributable to variations in the uptake and equilibration of the labeled precursor, we undertook a study of the uptake and incorporation of labeled glucosamine into infected and uninfected cells. Results of these experiments are presented in Fig. 1 and 2.

Figure 1 shows the rate of uptake and of incorporation of D-[^{14}C]glucosamine and ^{3}H-labeled amino acids into cells during 4-h intervals beginning at two different times after infection or mock infection. The pool of labeled glucosamine in infected cells increased linearly for at least 4 h independent of the time of addition of the precursor, whereas the rate of uptake into uninfected cells decreased progressively with time (Fig. 1a and d). The rate of incorporation of glucosamine into acid-insoluble material increased with time in both infected and uninfected cells, in contrast to the nearly constant rate of incorporation of labeled amino acids (Fig. 1b, e and c, f). The rate of uptake of glucosamine into infected cells also increased during the virus growth cycle (Fig. 1a, d), suggesting that the elevated incorporation into acid-insoluble material (Fig. 1b, e) may be explained by increases in the specific activity of the glucosamine pool in infected cells (also see below). The overall patterns of uptake and incorporation of glucosamine throughout the virus growth cycle (Fig. 2) were obtained by combining the results of a large number of experiments such as that shown in Fig. 1. The permeability of infected cells to glucosamine (Fig. 2a) increased 2.5- to 3-fold during the first 10 to 12 h and thereafter remained relatively constant. The incorporation of labeled glucosamine (Fig. 2b) remained unchanged for approximately 6 h after infection and thereafter it increased at a linear rate attaining values 2.5- to 3.0-fold higher at 18 to 24 h postinfection.

Assuming that the specific activity of the glucosamine pool remains constant, but that the size of the pool increases throughout infection, the data in Fig. 1 and 2 directly reflect differences in the relative molar rates of glucosamine incorporated between uninfected cells and cells at different times after infection. However, an alternative interpretation of the data shown in Fig. 1 and 2 is that increased permeability to extracellular glucosamine occurs after infection without any change in the size of the total intracellular pool of glucosamine. Increased rates of uptake of labeled glucosamine therefore would be reflected in a proportionate increase in the specific radioactivity of the pool. On the basis of this assumption, the relative molar rates of glucosamine incorporation could be obtained by correcting the observed incorporation of labeled glucosamine for differences in rate of uptake of the labeled precursor shown in Fig. 2. Thus, a measure of the real molar rates of glucosamine incorporation can be obtained by dividing the

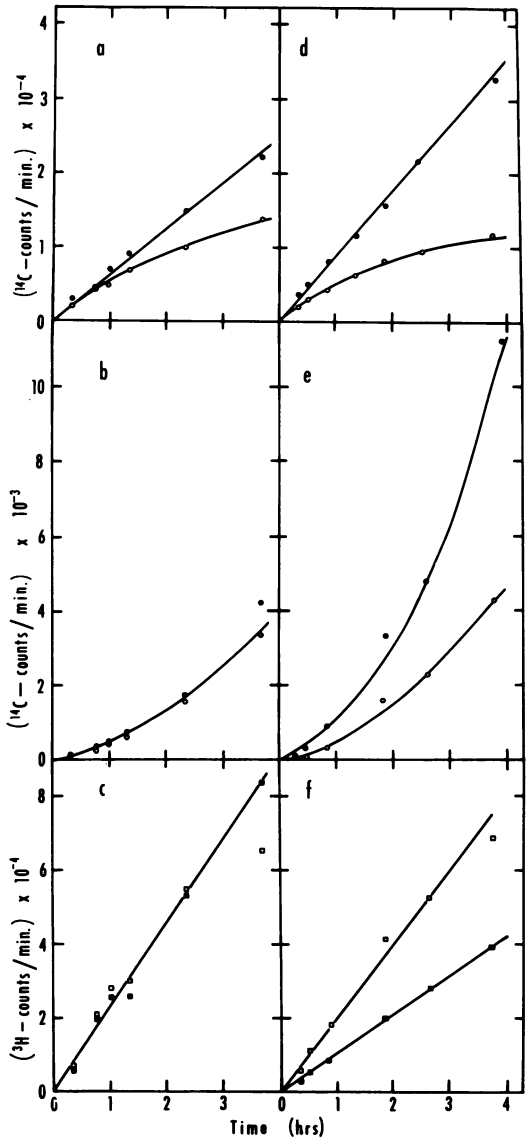

FIG. 1. *Kinetics of uptake of* D-[^{14}C]*glucosamine into the acid-soluble pools (a, d) of infected (solid symbols) and mock-infected HEp-2 cells (open symbols) and incorporation of* D-[^{14}C]*glucosamine (b, e) and* [^{3}H]*leucine, isoleucine, and valine (c, f) into acid-insoluble material. At 1.75 h (panels a, b, and c) or at 18.5 h (panels d, e, and f) after infection with HSV-1 (F1) or after mock infection, medium containing* [^{14}C]*glucosamine (0.2 μCi/ml, 56.6 mCi/mmol) and* [^{3}H]*leucine, isoleucine, and valine (2 μCi of an equimolar mixture of the three amino acids per ml of medium) was added to replicate cultures of infected and uninfected cells. Samples of 10^6 cells were removed at intervals for about 4 h after the addition of medium containing labeled precursors, and acid-soluble and -insoluble radioactivity was measured. Each point in the figure represents the mean of triplicate determinations. The initial glucose concentration in the medium was 1.1 mM.*

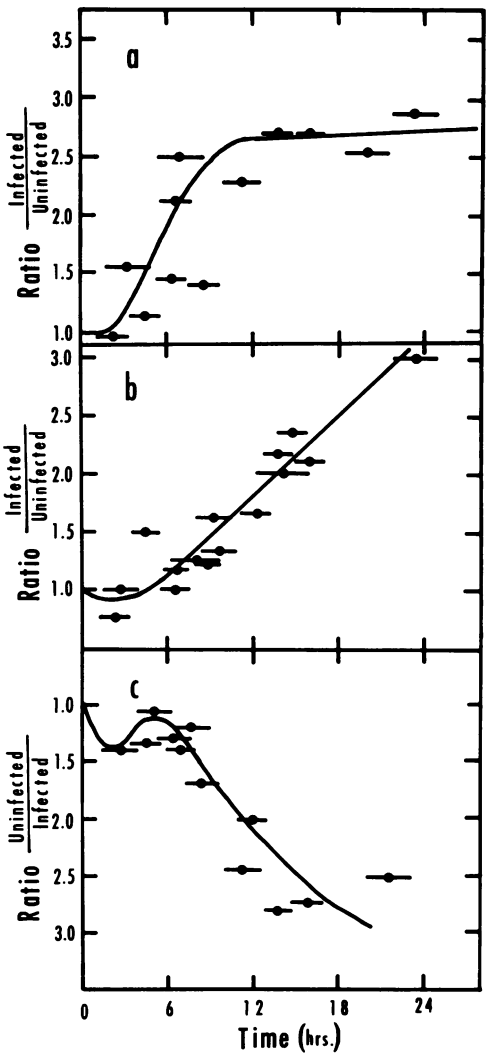

FIG. 2. *Changes in (a) rates of uptake and (b) the rates of incorporation of labeled glucosamine or (c) incorporation of labeled amino acids after infection of HEp-2 cells with HSV-1. The data are derived from curves such as those shown in Fig. 1. Each bar represents the ratio of labeled glucosamine in the acid soluble pools (a), or glucosamine label in acid-insoluble material (b) or amino-acid incorporation into acid-insoluble material (c), measured over 2- to 3-h intervals for infected and uninfected cells. The points are located at the midpoint of the observation interval. Uptake of labeled glucosamine by infected cells was linear for at least 4 h after the addition of labeled presursor. Although the rates of uptake into uninfected cells were not constant (Fig. 1) both uptake and incorporation approximate a linear relationship for the first 2 to 3 h after the addition of labeled precursor. The data were obtained by fitting straight lines to data such as that shown in Fig. 1. This assumption does not introduce a significant error; the scatter in points from different experiments was greater than the error introduced by assuming linearity for glucosamine uptake and incorporation during the first 2 to 3 h.*

relative rates of incorporation (Fig. 2, panel b) by the relative rates of uptake (Fig. 2, panel a). The rate of glucosamine incorporation obtained in this fashion declined from 0 to 10 h postinfection to a value 1.7 times lower than that of uninfected cells. Thereafter the rate of incorporation into infected cells increased linearly until by 20 h it was equal to that observed in uninfected cells.

Rates of incorporation of labeled amino acids during the virus growth cycle are shown in Fig. 2c. The overall rate of protein synthesis declines late in infection due to declining rates of host and early viral protein synthesis. As we have shown in detail elsewhere (12, 13) the major structural polypeptides of the virus are members of the γ-group, synthesized at gradually increasing rates until late in infection. The increasing rates of glucosamine incorporation late in infected cells parallels the rate of synthesis of γ-polypeptides, consistent with the identification of certain of the major glycoprotein precursors as members of the γ-group of viral polypeptides (see below).

The ratio of the rates of uptake of labeled glucosamine into infected and uninfected cells was independent of the concentration of glucosamine or of glucose in the medium in the range of 0.4 to 40 μM glucosamine and 0.55 to 5.55 mM glucose. Absolute rates of uptake, K, were sensitive to variations in either parameter. For example, measurements from 6 to 9 h after infection or mock infection with an initial concentration of 0.7 μM D-[^{3}H]glucosamine gave initial rates of uptake of 152 femtomol/min per 10^6 cells and 36.2 femtomol/min per 10^6 cells for infected cells, and 58 femtomol/min per 10^6 cells and 14.4 femtomol/min per 10^6 cells for uninfected cells for glucose concentrations of 0.55 and 5.55 mM, respectively. Thus, for infected cells the ratio ($K_{0.55\,mM}/K_{5.55\,mM}$) was 4.2 and for uninfected cells this ratio was 4.05, and the ratio (K infected/K uninfected) for 0.55 mM glucose was 2.6 and for 5.5 mM it was 2.48.

Chemical form of the labeled precursor in acid-soluble pools of infected and uninfected cells. Analyses of acid-soluble pools of infected and uninfected cells exposed to labeled glucosamine showed that about 80% of the radioactivity co-chromatographed with UDP-*N*-acetylglucosamine. The remainder was present as glucosamine, *N*-acetylglucosamine, and glucosamine-6-P. The results of a typical analysis of acid-soluble pools from infected cells are shown in Fig. 3. Analyses of pools extracted from infected cells labeled for 1 or 3 h did not differ significantly, and neither glucosamine uptake or conversion to UDP-*N*-acetylglucosamine was affected by a 3-h treatment with puromycin or with cycloheximide.

The data shown in Fig. 1 demonstrate that the rate of entry of labeled glucosamine into the acid-soluble pool of both infected and uninfected cells was much greater than its rate of withdrawal. Thus, after a 3-h labeling interval only 10 to 15% of the total intracellular radioactivity was acid precipitable. Moreover, the fact that the most of the soluble pool was UDP-*N*-acetylglucosamine (Fig. 3) suggested that this large pool would not exhange with the medium to permit an effective "chase." Direct evidence for the stability of the pool emerged from an experiment (Fig. 4) in which the loss of labeled precursor from the acid-soluble pool of infected cells was monitored after removal of labeled glucosamine from the medium. After 3 h of incubation the pool was still about 70% of its initial value. This observation places limitations on interpretation of pulse-chase experiments with labeled glucosamine.

Glycosylated polypeptides of infected and uninfected cells. A comparison of the glycosylated polypeptides synthesized by mock-infected cells and by cells 6.4 to 15.75 h after infection with HSV-1 (F1) is shown in Fig. 5. The synthesis of glycosylated polypeptides characteristic of the uninfected cell was no longer detected by 4 to 5 h after infection with 20 plaque-forming units per cell and infected cells thereafter synthesized a novel set of glycosylated polypeptides. Evidence that these glycoproteins are virus specified is as follows: (i) polypeptides are electrophoretically distinct from those of uninfected cells (11, 27; Fig. 5); (ii) a number of the glycosylated polypeptides are structural components of the virus particle and are uniquely labeled with radioactive amino acids added after infection (29); (iii) the electrophoretic mobility and number of glycosylated polypeptides vary with the virus type and even with different strains of the same type (16; Heine and Roizman, manuscript in preparation); (iv) glycosylated polypeptides are precipitable by antisera prepared against infected rabbit kidney cells or against purified virions, but not by anti-uninfected cell sera (14; P. G. Spear, Proc. Conf. Herpesvirus Oncogenesis, in press; unpublished observations).

Comparison of glycosylated polypeptides and of glycopeptides from infected and uninfected cells. The purpose of these experiments was to compare the electrophoretic profiles of the glycoproteins from the total infected cell

with those of the infected cell plasma membrane and of the virion, and to examine the size distribution and composition of heterosaccharide chains in these samples. The results of

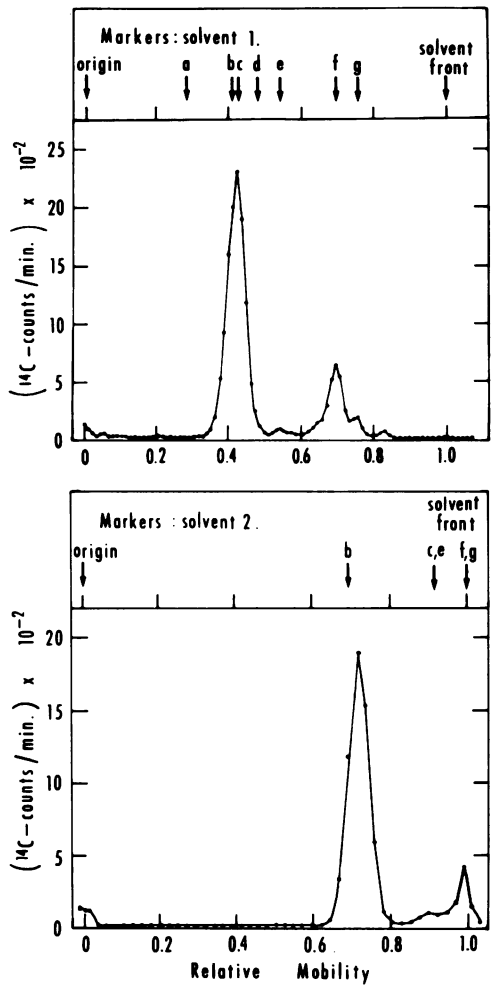

FIG. 3. *Paper chromatography (Whatman 3MM) of the acid-soluble pool from infected cells labeled with* D-[^{14}C]*glucosamine. Samples of acid-soluble pool from infected cells were chromatographed together with marker compounds (below) by descending development with either solvent 1 (3 volumes of 1 M ammonium acetate, pH 5.0; 7 volumes of 95% ethanol) or solvent 2 (79 volumes of saturated ammonium sulphate; 19 volumes of 0.05 M phosphate buffer, pH 6.0; 2 volumes of isopropyl alcohol) for 13 to 18 h at 25 C. The positions of marker compounds chromatographed on the same paper in each of the two solvent systems are indicated by the annotated arrows at the top of each frame of the figure. These markers were: (a) ADP-glucose; (b) UDP-N-acetylglucosamine; (c) glucosamine-6-P; (d) GDP-mannose; (e) N-acetylglucosamine-6-P; (f) glucosamine; (g) N-acetylglucosamine.*

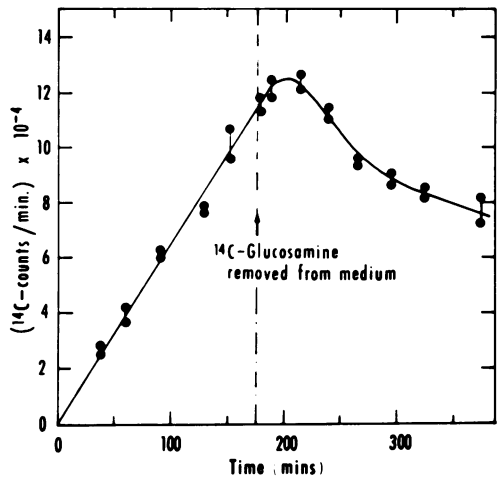

FIG. 4. *Influx and efflux of labeled glucosamine from the acid-soluble pool of infected cells. Medium containing 0.5 μCi of* D-[^{14}C]*glucosamine (1.11 mM glucose) per ml was added to replicate culture of 10^6 HEp-2 cells at 5.5 h after infection, and the uptake into the acid-soluble intracellular pool was measured at intervals thereafter for a total of 180 min. The medium containing labeled precursor was then removed (arrow) and the cell monolayers were first rinsed with medium lacking radioactive precursors and then replenished with fresh unlabeled medium. Incubation and sampling were then continued for an additional 180-min interval.*

some of these comparisons are shown in Fig. 6 and 7. The virus-specific glycoproteins contained in the virion and in the plasma membrane of cells infected with this strain of HSV-1 were similar both qualitatively and quantitatively (Fig. 6). However, there were marked differences between the electrophoretic profiles of these glycoproteins and those of the glycosylated polypeptides contained in the total infected cell lysate. Thus glycosylated polypeptides *a* and *g*, which co-migrated with virion glycoproteins VP 8 and VP 18, respectively, were prominent in both the virion and plasma membrane but were present in relatively low concentrations in the total infected cell. Conversely, glycosylated polypeptide *b*, *c*, *d* (not resolved in this electropherogram), and *h*, which were absent or present in relatively low concentrations in the virion or the plasma membrane of infected cells, were prominent components of the population of total infected cell glycoproteins. The cross-hatched region of Fig. 6 indicates those components which predominated in the total infected cell but were present in low concentration, or were absent from the virion and plasma membrane.

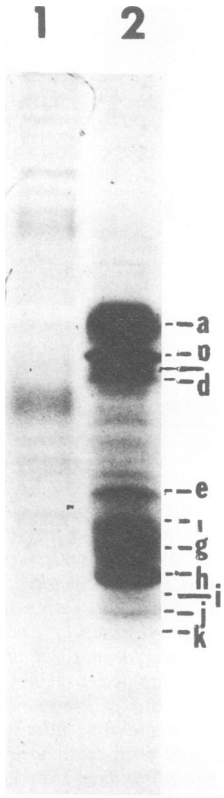

FIG. 5. *Autoradiograms of labeled polypeptides electrophoretically separated on polyacrylamide gel slab from infected and uninfected HEp-2 cells. Uninfected HEp-2 cells or HEp-2 cells infected with 20 plaque-forming units per cell of HSV-1 (F1) were labeled with 0.5 μCi of D-[^{14}C]glucosamine per ml from 6.25 to 15.75 h after infection or mock infection. Samples of the total infected (slot 2) and uninfected (slot 1) cell lysates were then separated by electrophoresis on an 8.5% polyacrylamide gel slab. The apparent molecular weights of glycosylated bands in the infected cell lysate range from about 130,000 for band a to 60,000 for band g and 45,000 for band k. The anode side of the gel is to the bottom of this figure and to the right in subsequent figures showing absorbance tracings of electrophoretically separated polypeptides.*

In Fig. 7 comparisons of the size distributions of Pronase-derived glycopeptides from glucosamine-labeled total infected and uninfected cells (Fig. 7a), infected cell plasma membranes (Fig. 7b), and purified virions (Fig. 7c) are shown together with glycopeptides from infected cells labeled for 8 and 23 h (panel d). All these samples contained a similar set of size classes of glucosamine-labeled glycopeptides (peaks II–VI; see legend to Fig. 7) and differed in the distribution of label among these classes. The average chain length of glucosamine-labeled glycopeptides in uninfected cells was greater than that of infected cells labeled during an 8-h interval (panel a), but with longer labeling intervals approximating the cell generation time, the average chain length of infected cell glycopeptides was similar to that of an unsynchronized population of uninfected cells (compare Fig. 7, panels a and d). More significantly, however, the results illustrated in panels b and c of Fig. 7 show that the glycopeptide chain length in the glycoproteins of the virion and plasma membrane were, on the average, greater than those of the total infected cell glycoproteins. For example, glycopeptides in peak II, with an estimated molecular weight of 2,000, were prominent components of the infected cell digests but minor components of virion and plasma membrane digests. Conversely glycopeptides in peaks III and IV with molecular weights of about 4,300 and 6,800, respectively, were the major species in digests of the virion and plasma membrane and relatively minor components of digests from total infected cell glycoproteins. Labeled glycopeptides from purified plasma membrane of infected and uninfected cells showed a similar size distribution even though, as previously reported (11), the glycoproteins from which they were derived are specified by different genomes.

The data presented so far show that the infected cells contain glycosylated polypeptides which give rise to small heterosaccharide chains and are differentiated from those present in the virion and the plasma membrane on the basis of their electrophoretic mobility.

(i) Size of glycopeptide chains. The molecular weights of Pronase-resistant glycopeptides were estimated by their elution volumes (V_e/V_o) from columns of Sephadex G50 relative to glycopeptides of known size from fetuin, thyroglobulin, and ovalbumin. Sucrose, the trisaccharide raffinose, the pentasaccharide stachyose, and amino sugars *N*-acetylneuraminic acid and glucosamine were used as additional calibrating standards. The molecular weights of the glycopeptide peaks estimated from these data were: peak II, 1,800 to 2,500; peak III, 4,000 to 4,600; peak IV, 6,400 to 7,200; peak V, 8,400 to 9,200; and peak VI, 10,000 to 12,000. The estimated sizes of glycopeptides in peaks II and III should be more reliable than those with molecular weights greater than 5,000, largely because of absence of well characterized glycopeptide markers of greater than 5,000 molecular weight.

(ii) Distribution of fucose among the vari-

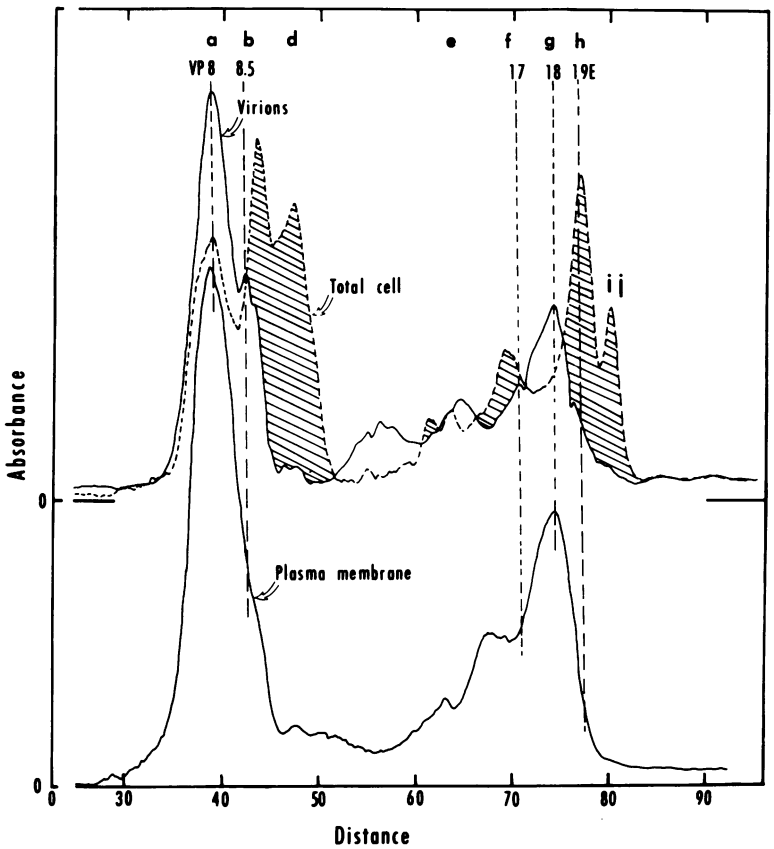

FIG. 6. *Absorbance tracing of autoradiograms of* D-[^{14}C]*glucosamine-labeled polypeptides separated by electrophoresis on an 8.5% polyacrylamide gel slab. Upper tracings (solid line), purified virions of HSV-1 (F1); upper tracings (broken line), total lysate of infected HEp-2 cells; lower tracing, purified plasma membranes from HSV-1 (F1)-infected cells. The cross-hatched region between the upper tracings indicates those glycosylated bands that were present in high concentrations in the total infected cell lysate, but that were absent or present in much lower concentrations in the virion or the plasma membrane from infected cells.*

ous size clones of glycopeptides. In a previous publication (9) we showed that the glucosamine-labeled proteins in the virion were also labeled with fucose, and moreover, the ratio of fucose/glucosamine label did not differ markedly for the different bands of glycosylated polypeptides in the virion. Other experiments (unpublished data) showed that, although the fucose/glucosamine ratio of viral glycoproteins in the plasma membrane was relatively constant, it was higher than that for the total infected cell glycoproteins (11). For the purposes of the present paper we wished to determine the relative fucose content of different glycopeptides. The chromatographic elution profiles of glycopeptides from Pronase digests of infected and uninfected cells doubly labeled with [^{14}C]fucose and [^{3}H]glucosamine (Fig. 8) showed that the fucose/glucosamine ratio of different size classes of glycopeptides was not uniform; it was at least threefold higher in glycopeptides in peaks III and IV than in peak II of both infected and uninfected cells. The lower average ratio of fucose/glucosamine label in infected cells could be explained by a higher specific activity of the glucosamine pool in infected cells (Fig. 1 and 2). The higher fucose content of longer glycopeptide chains (III and IV) would be consistent with the typically distal location of fucose on heterosaccharide chains. As shown below the decline in ratio for the largest glycopeptides (V and VI) may be due to both the addition of side chains containing glucosamine and the presence of label from glucosamine as terminal sialic acid residues.

(iii) Presence of labeled sialic acid in the virion and large glycopeptides of infected cells labeled with glucosamine. Although we

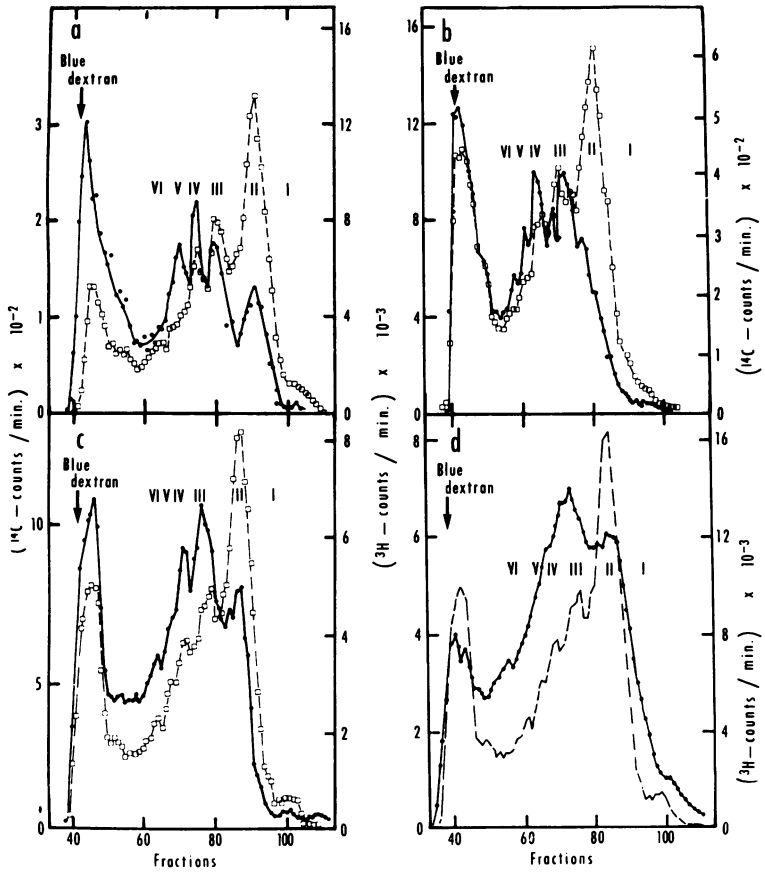

FIG. 7. *Sephadex G50 gel filtration chromatography of glycopeptides obtained by Pronase digestion of: (a) a total lysate of uninfected cells labeled with* D-[^{14}C]*glucosamine from 4 to 12 h after mock infection (filled circles, solid line) and a total lysate of HSV-1 (F1)-infected cells labeled with* D-[^{3}H]*glucosamine from 4 to 12 h postinfection (open squares, broken line); (b) purified plasma membrane fraction from HSV-1 (F1)-infected cells labeled with* D-[^{14}C]*glucosamine (filled circles, solid line) and a total infected cell lysate as in (a) (open squares, broken line); (c) virions purified from HSV-1 (F1)-infected cells labeled with* D-[^{14}C]*glucosamine (filled circles, solid line) and a total infected cell lysate as in (a) (open squares, broken line); (d) a total lysate of HSV-1 (F1)-infected cells labeled with* D-[^{3}H]*glucosamine from 4 to 27 h postinfection (filled circles, solid lines) and a total infected cell lysate labeled from 4 to 12 h postinfection, as shown in (a) (broken line). Samples shown in a, b, and c were mixed prior to Pronase digestion and co-chromatographed. The infected cell lysate labeled with* D-[^{3}H]*glucosamine from 4 to 27 h postinfection shown in d was chromatographed alone; the profile of infected cells labeled from 4 to 12 h postinfection and shown in d was taken from an independent run on the same column. Roman numerals I to VI refer to reproducible peaks of Pronase-resistant material observed in digests of glucosamine-labeled infected and uninfected cells. Peak I was identified as residual UDP-N-acetylglucosamine by subsequent paper chromatography of the isolated material. Peaks II–VI were not interconvertible by further digestion with Pronase and rechromatographed as distinct species. The material eluting with blue dextran in the void volume was in part due to incomplete digestion; however, a fraction of the material could not be converted to II–VI by further digestion with Pronase. This material did not contain fucose.*

have noted that there is negligible incorporation of labeled glucosamine into nonsaccharide components in HSV-1-infected cells (9, 16), we have not previously examined in detail possible conversions to other sugars. The conversion of labeled UDP-*N*-acetylglucosamine to CMP neuraminate and its incorporation as a terminal *N*-acetylneuraminic acid residue was a likely conversion of significance to experiments described below. Since terminal sialic acid residues are susceptible to either neuraminidase or mild acid hydrolysis, we examined the sensitivity of radioactivity in glucosamine-labeled virions, glycoproteins, and purified glycopeptides

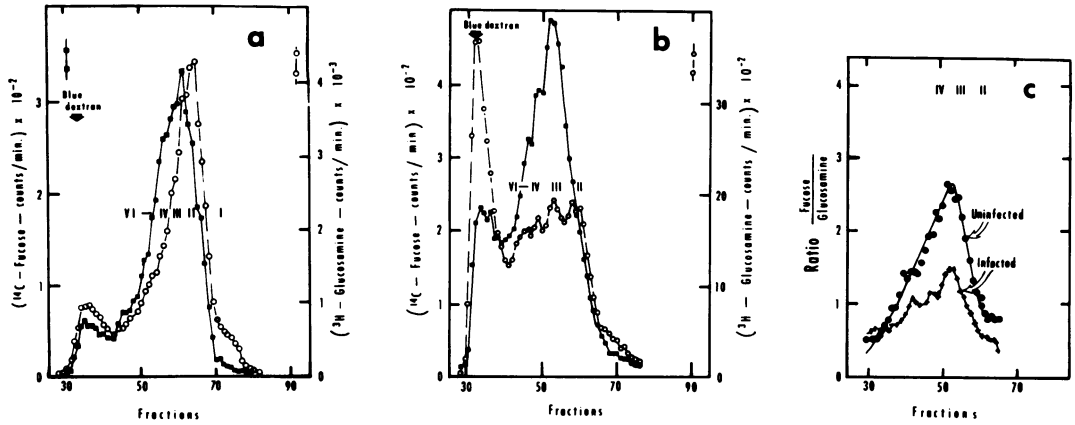

FIG. 8. *Sephadex G50 gel filtration chromatography of glycopeptides from infected (a) and uninfected (b) HEp-2 cell lysates labeled with* L-[^{14}C]*fucose and* D-[^{3}H]*glucosamine. Cells were labeled from 10.5 to 13.25 h after infection or mock infection with 2 μCi of* L-[^{14}C]*fucose and 10 μCi of* D-[^{3}H]*glucosamine per ml of medium. Lysates of labeled cells were then digested with Pronase and the glycopeptides were separated on a column of Sephadex G50 together with stachyose and blue dextran markers. The ratio of fucose to glucosamine label ([fucose counts/min]/[glucosamine counts/min]) × 10 across the fractions from columns shown in a and b is shown in frame c.*

to these treatments. Results of these experiments, summarized in Fig. 9–12, showed the following: first, neuraminidase digestion or mild acid hydrolysis of purified virions removed up to 30 to 40% of label derived from glucosamine (Fig. 9). The labeled material released by this treatment co-chromatographed with an *N*-acetylneuraminic acid marker on paper (Fig. 10) and on gel filtration chromatography (Fig. 11). Second, mild acid hydrolysis selectively reduced the radioactivity associated with larger glycopeptides and reduced the average molecular weight of glycopeptides containing glucosamine (Fig. 11). The only low-molecular-weight product detected appeared to be *N*-acetylneuraminic acid, suggesting that these changes were attributable solely or mainly to its removal rather than to partial hydrolysis of other internal linkages. Finally, examination of the content of acid-labile glucosamine in purified glycopeptides showed that, whereas longer glycopeptides (V to VI) contained a substantial fraction (30 to 50%) of their labeled glucosamine as sialic acid, there was no detectable release of sialic acid from the shortest (peak II) glycopeptides (Fig. 12).

Evidence for post-translational addition of heterosaccharide chains to viral glycoproteins and the interconversion of glycosylated viral polypeptides: Electrophoretic profiles of polypeptides labeled with glucosamine and ICP labeled with radioactive amino acids. The electrophoretic profiles of infected cell polypeptides (ICP) labeled with ^{14}C-labeled

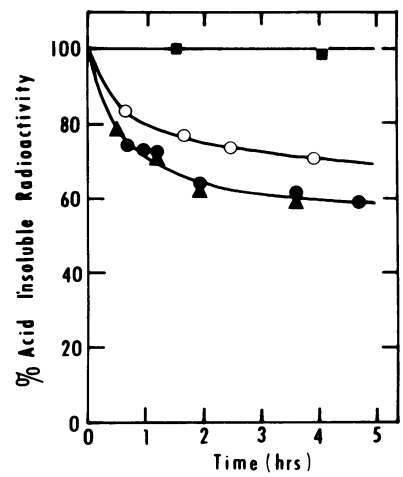

FIG. 9. *Effects of neuraminidase or mild acid hydrolysis on acid-insoluble radioactivity in purified virions of HSV-1 (F1). Symbols:* ■, *mild acid hydrolysis (0.025 M* H_2SO_4*/75 C) of virions labeled with* ^{14}*C-labeled amino acids;* ● *and* ▲, *mild acid hydrolysis of virions labeled with* D-[^{14}C]*glucosamine (two separate experiments);* ○, *neuraminidase digestion of virions labeled with* D-[^{14}C]*glucosamine (9.5 mg of neuraminidase per ml in 0.05 M Tris-hydrochloride, pH 6.6, 0.1 M NaCl, 2 mM* $CaCl_2$ *at 37 C).*

amino acids and bands of glucosamine-labeled polypeptides are shown in Fig. 13. Seven major glucosamine labeled bands (*a–h*), and a number of minor bands (between *d* and *e* and traveling slightly faster than *h*), were detected in the infected cell lysate. However, only three of these

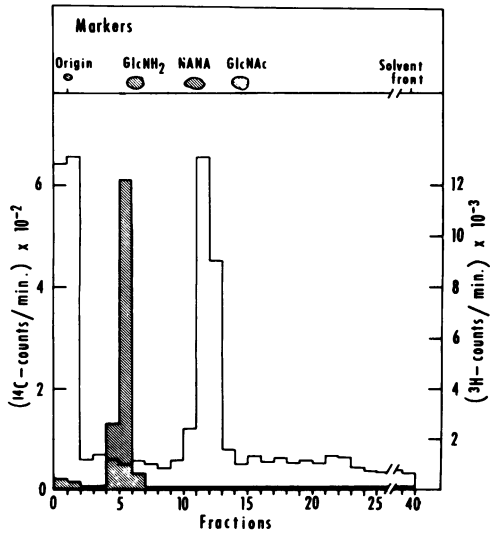

FIG. 10. *Paper chromatography of the products of mild acid hydrolysis of* D-[^{14}C]*glucosamine-labeled virions. A sample of purified virions was hydrolyzed with 0.025 N* H_2SO_4 *at 75 C for 1 h. The hydrolysate was then mixed with a sample of* D-[^{3}H]*glucosamine and separated by descending chromatography on Whatman 3MM paper with a solvent consisting of isoamylacetate/acetic acid/water (3/3/1). Samples of unlabeled N-acetylglucosamine (GLcNAc), N-acetylgalactosamine, N-acetylneuraminic acid (NANA),* D*-glucosamine (*$GLcNH_2$*),* D*-galactosamine, and* D*-mannosamine were applied to neighboring positions on the same sheet of paper. The solvent front was allowed to travel about 40 cm and positions of unlabeled marker compounds were then located with ninhydrin or alkaline silver nitrate reagent. Positions of marker compounds are indicated at the top of the figure; N-acetylgalactosamine migrated with N-acetylglucosamine in this solvent and* D*-galactosamine and* D*-mannosamine with* D*-glucosamine. Labeled components were located by scintillation counting of eluates from 1-cm strips of the chromatogram; the open histogram shows radioactivity derived from* D-[^{14}C]*glucosamine-labeled virions, and the cross-hatched histogram the* D-[^{3}H]*glucosamine marker.*

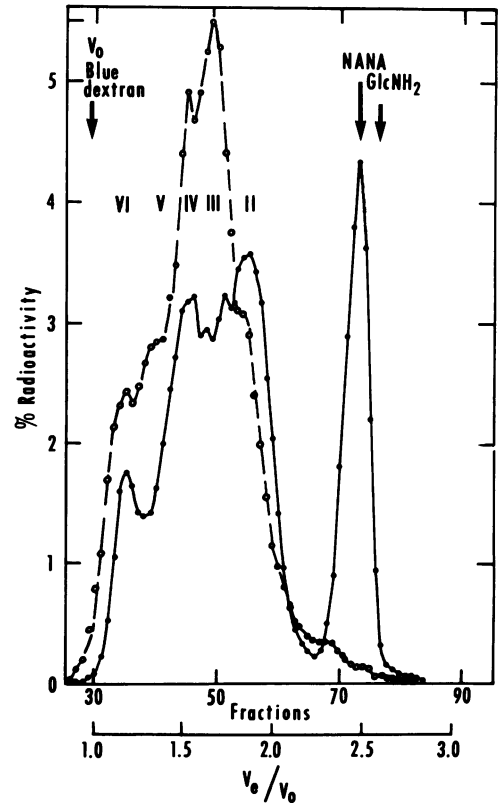

FIG. 11. *Sephadex G50 gel filtration chromatography of glycopeptides from a Pronase digest of purified virions before and after mild acid hydrolysis. A sample of purified virions labeled with* D-[^{14}C]*glucosamine was exhaustively digested with Pronase, and the digest was separated on a column of Sephadex G50 (open circles and broken line) together with N-acetylneuraminic acid (NANA) and* D*-glucosamine (*$GLcNH_2$*) markers. After sampling for determination of radioactivity fractions 29–60 were pooled, concentrated, and hydrolyzed with acid (0.025 N* H_2SO_4 *at 75 C for 1 h). The hydrolysate was neutralized, mixed with NANA and* $GLcNH_2$*, and rechromatographed on the same column (filled circles, solid line). NANA and* $GLcNH_2$ *(arrows) eluted in identical positions in the two runs.*

bands, *b*, *d*, and *h*, have electrophoretic mobilities indistinguishable from ICP labeled during a short pulse with ^{14}C-labeled amino acids. Thus, ICP 10 co-migrated with peak *b* and the ratio of glucosamine/amino acid label across the peak was constant; similarly, ICP 12 and band *d* and ICP 30 and band *h* were coincident. Although other ICP labeled during a short pulse with radioactive amino acids migrate in close proximity to glycosylated bands (e.g., ICP 8, 21, 25, 26), not only is the ratio of glucosamine to amino acids not constant across peaks but the glycosylated and unglycosylated bands in those regions are affected differently by changes in gel strength, and they are synthesized and accumulate with different kinetics and partition independently during cell fractionation. ICP 12 and 30 are members of the γ-group of virus-specified proteins (12, 13) which are made at increasing rates late in infection, and the major bands of *a*, *d*, and *h* of glucosamine-labeled polypeptides (e.g., *a*, *d*, and *h*) are also made at rates which increase progressively late in infection (e.g., compare Fig. 2 and 16). In the subsequent sections of this paper, we will present data which suggest that the polypeptides in *d* and *h*

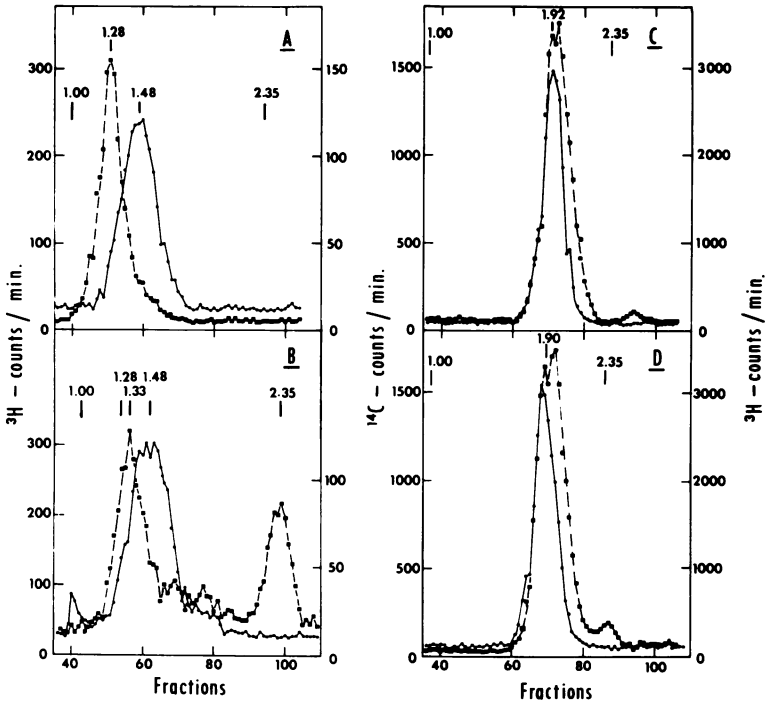

FIG. 12. *Sephadex G50 gel filtration chromatography of purified glycopeptides before and after mild acid hydrolysis. (A and B) Co-chromatography of purified [^{14}C]glucosamine-labeled glycopeptides V (filled circles, solid line) and [^{3}H]glucosamine-labeled glycopeptides (VI) (filled squares, broken line) untreated (A) or after mild acid hydrolysis of glycopeptide (VI) (B). (C and D) Co-chromatography of [^{14}C]glucosamine-labeled glycopeptides II (filled circles, solid line) untreated (C) or after mild acid hydrolysis of [^{3}H]glucosamine-labeled glycopeptides II (D). Note that there are at least two size classes of glycopeptides within the region denoted as II in this and other figures; the higher-molecular-weight region was from the [^{14}C]glucosamine-labeled glycopeptides, whereas the entire region of glycopeptides II was purified from [^{3}H]glucosamine-labeled glycopeptides. The markers within each panel indicate the elution position (V_e/V_o) of glycopeptides. An unlabeled sialic acid marker eluted at V_e/V_o of 2.35 to 2.36 in these columns.*

are precursors to more extensively glycosylated proteins forming other major bands of glycosylated polypeptides.

Effects of inhibitors of protein synthesis on the incorporation of glucosamine and fucose into viral glycoproteins. The experiments in this section were designed to determine the approximate time of course of glycosylation of completed polypeptide chain by measuring the incorporation of glucosamine and fucose into glycoproteins after complete inhibition of viral protein synthesis with puromycin (100 μg/ml; Fig. 14). Although this concentration of the drug completely precluded amino acid incorporation within 1 to 2 min after its addition, there was no measurable effect on incorporation of glucosamine (Fig. 14, panel a) or fucose (panel b) for at least 12 and 80 min, respectively. Both fucose and glucosamine were incorporated into viral glycoproteins in the presence of the inhibitor and less than 10% of the label was incorporated into glycolipid (results not shown). It should also be noted that the same results were obtained using cycloheximide (50 μg/ml) in place of puromycin. Estimates of the time taken to affect the addition of glucosamine were not significantly influenced by the incorporation as sialic acid. For the first 2 h after adding cycloheximide or puromycin, only 10 to 14% of total radioactivity incorporated from labeled glucosamine was released by mild acid hydrolysis. However it is possible that at later times incorporation as sialic acid does form a significant fraction of total incorporation of labeled glucosamine.

Therefore, we interpret these results to mean that the average polypeptide precursor enters the glycosylating system within 12 to 15 min after its synthesis, and that 60 to 70 min later the bulk of the fucose has already been added to the heterosaccharide chain.

Changes in the distribution of glucosamine

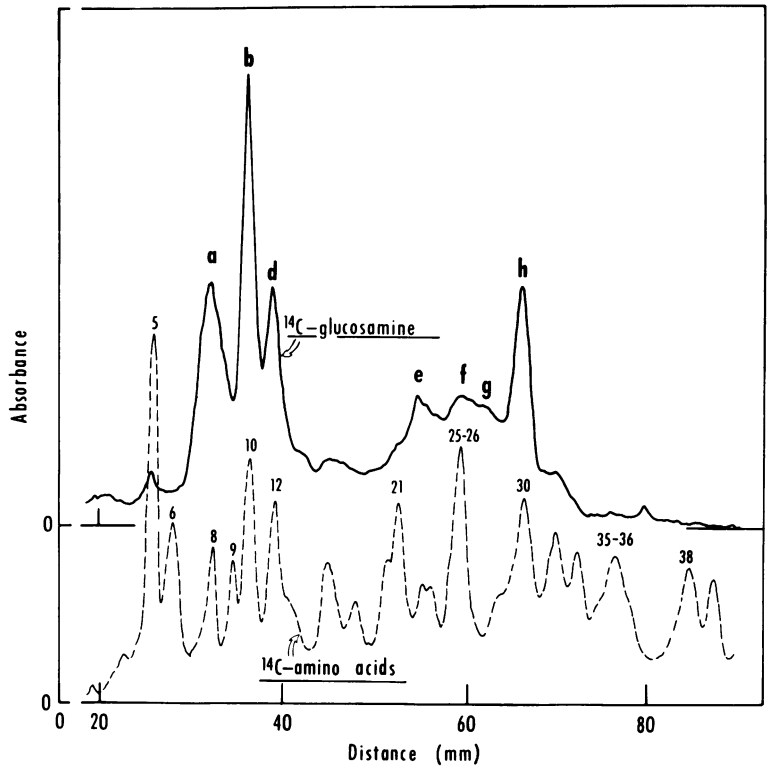

FIG. 13. *Absorbance profiles of autoradiograms of labeled polypeptides electrophoretically separated from total infected cell lysates labeled with* D-[^{14}C]*glucosamine (upper tracing) or* ^{14}C*-labeled amino acid (lower tracing). Separate cultures of infected cells were labeled with* D-[^{14}C]*glucosamine from 6 to 10 h after infection and with* ^{14}C*-labeled amino acid from 9.5 to 10 h. Glycosylated polypeptides are designated by letters (see Fig. 5 and 6); the ICP labeled with* ^{14}C*-labeled amino acids are numbered as previously described (12, 13). Samples were run in adjacent positions of an 8.5% polyacrylamide gel slab.*

label throughout glycosylated viral polypeptides in the presence of an inhibitor of protein synthesis. Since glycosylation continues in the absence of protein synthesis, changes in the distribution of glucosamine label throughout the electrophoretically distinct forms of viral glycoproteins under these circumstances should reflect an increasing preponderance of more completely processed molecules. Such changes may be due simply to the conversion of one electrophoretic species to another by glycosylation, or may also involve other processing steps such as cleavage or phosphorylation. The results of an experiment designed to examine these changes are shown in Fig. 15 and 16. In this experiment replicate cultures of infected cells were labeled with [^{14}C]glucosamine from 6.4 to 8.4 h postinfection and were then incubated in the presence of cycloheximide (50 μg/ml). Treated cultures were harvested at 8.4 h and at intervals until 15.75 h after infection. Untreated infected cells were also labeled for 2-h intervals at different times after infection and continuously from 6.4 to 15.75 h after infection. The glycosylated polypeptides contained in these total infected cell lysates were electrophoretically separated on a polyacrylamide gel slab (Fig. 15). The distribution of glucosamine label among separated polypeptides from treated and untreated cultures was quantitated by computer-aided planimetry of absorbance tracings of the autoradiographic images. The results, shown in part in Fig. 15 and 16, revealed significant changes in the distribution of label among the separated bands. Thus band *d* (Fig. 15 and 16) almost entirely disappeared, and the percentage of total label migrating with band *b* decreased, whereas the percentage of label in components *a* and *g* increased and a new band *c*, not detected in 6.4- to 8.4-h sample, appeared in the presence of the inhibitor. The changes in distribution of label probably reflect the normal consequences of the maturation of glycosylated polypeptides since

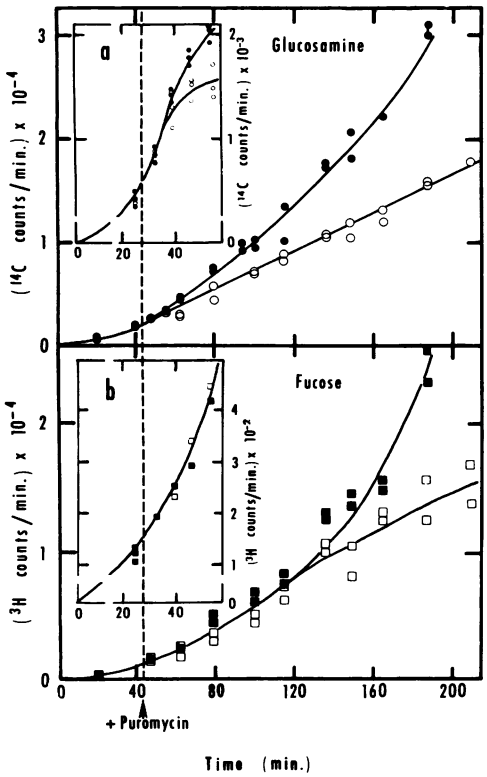

FIG. 14. *The effect of puromycin on the incorporation of glucosamine (upper panel and inset a) and fucose (lower panel and inset b) into acid-insoluble material in infected cells. Replicate cultures of 10^6 HEp-2 cells were infected with 20 plaque-forming units of HSV-1 (F1) per cell. At 9.5 h after infection (zero time, upper and lower panels) medium containing 0.2 μCi of D-[^{14}C]glucosamine per ml and 5.0 μCi of L-[^{3}H]fucose per ml was added to the cultures. After 45 min puromycin was added to half the cultures to give a final concentration of 100 μg/ml and the incubation was continued. Duplicate samples of treated (open circles, glucosamine; open squares, fucose) and untreated (filled circles, glucosamine; filled squares, fucose) cultures were removed at the intervals shown and the incorporation of glucosamine and fucose into acid-insoluble material was measured. The insets show data from a similar experiment in which isotopes were added to cultures 6 h after infection and triplicate cultures were removed at 10-min intervals for 40 min after the addition of puromycin to measure more exactly the time at which glucosamine (a) and fucose (b) incorporation was affected by the exposure to puromycin in concentrations to inhibit protein synthesis.*

alternative methods for obtaining populations enriched for more fully processed polypeptides, e.g., by increasingly longer labeling intervals (compare samples labeled from 6.4 to 8.4 h and 6.4 to 15.75 h, Fig. 15), show similar alterations in the distribution of label among the different glycosylated polypeptides. Comparisons of glycosylated polypeptides in untreated infected cells labeled for 2-h intervals at different times after infection (over the period from 6 to 16 h) did not show any major changes in the distribution of label throughout different polypeptides (Fig. 16).

It seems significant that those species which decrease in the presence of the inhibitor (e.g., *d*, *e*, and *h*) are absent, or present in low concentrations, in the plasma membrane and the virion, whereas those species which increase in the presence of the inhibitor (e.g., *a*, *g*) are the major glycosylated species in the plasma membrane and the virion (compare Fig. 6 and 15).

Changes in average glycopeptide chain lengths in the presence of an inhibitor of protein synthesis. In a previous section we presented some data on the characterization of glycopeptides from the total infected cell, the virion, and the plasma membrane and showed that the fraction of glucosamine label in high-molecular-weight glycopeptides was related to the length of the labeling interval (Fig. 7 and associated text). Figure 17 shows comparisons of the size distribution of [^{14}C]glucosamine-labeled glycopeptides from lysates of untreated infected cells labeled from 6.4 to 8.4 h and 6.4 to 15.75 h (left panel), or from 6.4 to 8.4 h, and then incubated in the presence of cycloheximide to 15.75 h (Fig. 17, right panel). The results show that glucosamine-labeled glycopeptides increased in average size in the presence of cycloheximide and that an increase was also observed during the long labeling interval in the absence of the drug. (Compare, for example, the change in the fraction of label contained in the smallest glycopeptide [peak II] in the sample labeled from 6.4 to 8.4 h and in that labeled from 6.4 to 8.4 h and incubated in the presence of cycloheximide until 15.75 h [Fig. 17, right panel].)

We conclude that glycopeptides increase in size with time and that at least a portion of the short glycopeptide chains, present in peak II, are precursors to one or more of the longer chains of glycopeptides in peaks III–VI. It is noteworthy that labeling intervals as short as 0.5 h failed to reveal glycopeptides smaller than those present in peak II.

DISCUSSION

In this paper we presented data on the uptake and incorporation of labeled glucosamine into infected cells, a characterization of the glycosylated polypeptides, and glycopeptide chains found in the total infected cell, the plasma

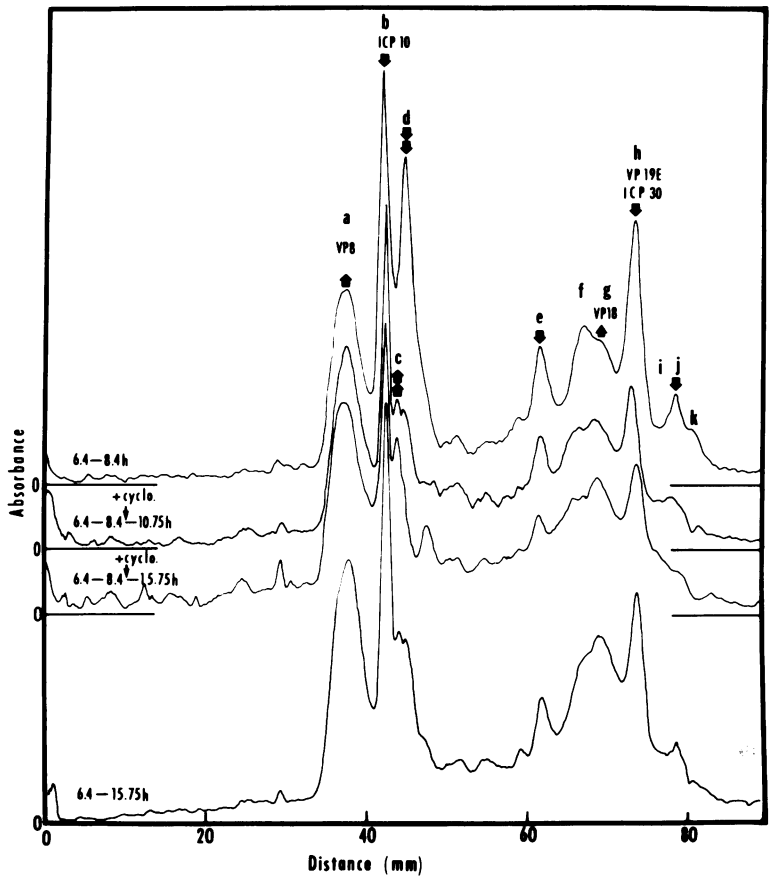

FIG. 15. *Absorbance tracing of autoradiograms of electrophoretically separated polypeptides from lysates of cells infected with HSV-1 (F1) and labeled with* D-[^{14}C]*glucosamine from 6.4 to 8.4 h (top tracing) and then incubated in the presence of cycloheximide (50 μg/ml) until 10.75 or 15.75 h postinfection, or labeled continuously from 6.4 to 15.75 h postinfection. Major glycosylated polypeptides of the total infected cell are designated by letter as shown in Fig. 5. The positions of selected glycosylated virion polypeptides (VP) and ICP are also shown. Arrows directed upward or downwards over a number of glycosylated polypeptides indicate those species whose fraction of total increases or decreases, respectively, during the chase period in the presence of cycloheximide (see Fig. 16).*

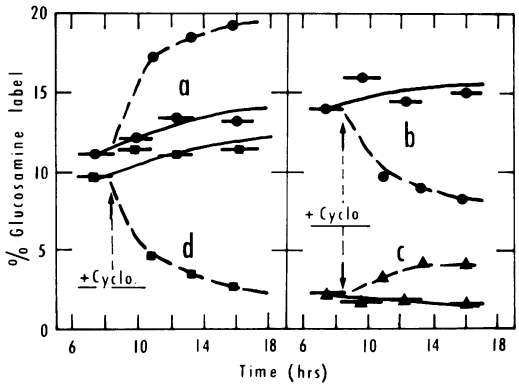

FIG. 16. *Changes in the distribution of glucosamine label in selected electrophoretically separated viral glycoproteins (a–d, Fig. 15) labeled for 2-h intervals at different times during the virus growth cycle in untreated cells (horizontal bars with symbols interconnected by solid lines) or in cells labeled from 6.4 to 8.4 h and then treated with cycloheximide for different intervals (symbols interconnected by broken lines). The data were obtained by planimetric analysis of autoradiogram absorbance tracings such as those shown in Fig. 15. The arrow (+ cyclo) shows the time of addition of cycloheximide to treated cultures. The different symbols in each frame show data for the separate glycoproteins in treated and untreated cultures. For example, band a (filled circles in left hand frame) accounts for some 11 to 13% of total glucosamine label during 2-h labeling periods at different times after infection (horizontal bars with filled circles, interconnected by solid line) but increases to about 17% after a 2-h treatment with cycloheximide and to almost 20% after 7 h of treatment with cycloheximide (filled circles, interconnected by broken line). Note that the pairing of a with d and b with c is for convenience only; the figure is not meant to imply specific precursor-product relationships.*

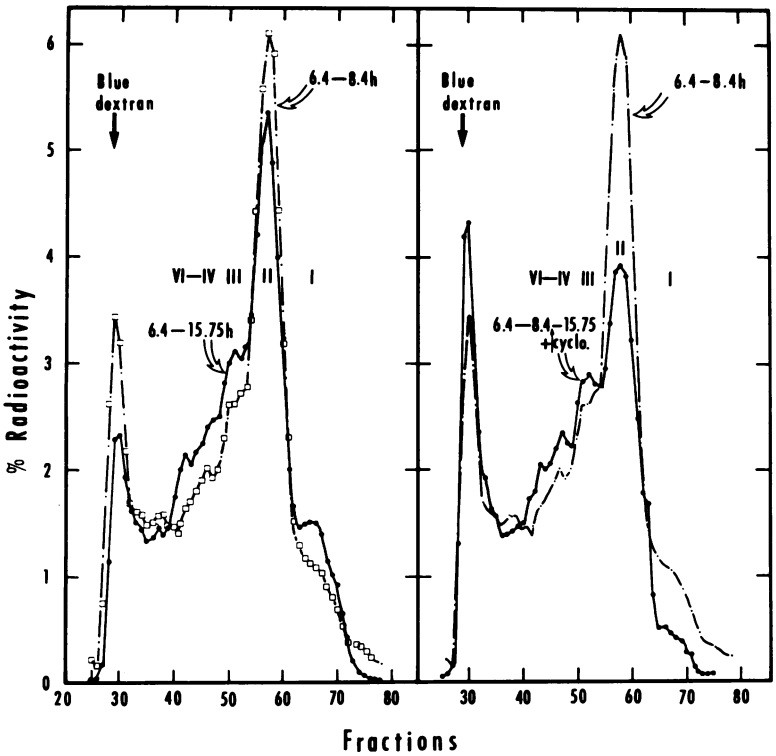

FIG. 17. *Sephadex G50 gel filtration chromatography of glycopeptides from lysates of infected cells labeled with* D-[^{14}C]*glucosamine. Left panel:* □, *cells labeled from 6.4 to 8.4 h;* ●, *cells labeled from 6.4 to 15.75 h. Right panel: broken line, cells labeled from 6.4 to 8.4 h;* ●, *cells labeled from 6.4 to 8.4 h and then incubated from 8.4 to 15.75 h with 50 μg of cycloheximide per ml. All samples were analyzed in successive runs on the same column and each sample was co-chromatographed with stachyose and* D-[^{3}H]*glucosamine as well as blue dextran as interval markers.* D-[^{3}H]*glucosamine eluted in fractions 76–77 (V_e/V_o = 2.63–2.66) and stachyose in fractions 70–71 (V_e/V_o = 2.43) in all columns.*

membrane, and the virion. We also presented data indicating that the addition of heterosaccharide chains is a multistep process which begins some 15 min after the synthesis of the primary precursor polypeptides and that a slow process of further glycosylation then converts this glycosylated secondary precursor into the highly glycosylated mature products found in the virion and plasma membrane.

Changes in permeability of infected cells to extracellular glucosamine. The gradual increase in the rate of uptake of labeled glucosamine during the first 12 h of infection could be simply a nonspecific alteration in the permeability of infected cells because of a failure to replace host cell membrane proteins or other components regulating uptake, or it may be due to a specific rearrangement of host proteins or a transport function of a virus-specified protein. Other workers have noted increases in the permeability of animal cells to labeled sugars and amino sugars during the normal cell cycle and after virus infection or transformation (8, 20). Although we do not know the mechanism responsible for increased uptake and accumulation of the labeled precursor by infected cells, one of its consequences may be to increase the apparent rate of glucosamine incorporation into glycoproteins. Measurements of the relative rates of glycoprotein synthesis therefore should take into account possible changes in precursor pools.

It should be noted that we have not yet determined whether the size of the intracellular pool or merely its specific radioactivity is changed by herpesvirus infection. However, irrespective of the correction employed it is clear that glycosylation of herpesvirus proteins is predominantly a late event in the replication of the virus. Continued glycosylation in the presence of inhibitors of DNA synthesis (23) is not incompatible with conclusion; we have shown elsewhere that in cells infected with nondefective populations of herpesvirus particles, γ-protein synthesis is only reduced by ~50% in the presence of high concentrations of

inhibitors of DNA synthesis (13).

Characterization of glycosylated polypeptides and Pronase-derived glycopeptides from infected and uninfected cells and from the virion and plasma membrane of infected cells. Previous work from this (27) and other laboratories (2) has shown that herpesviruses induce the synthesis of novel set of glycoproteins in infected cells. Keller et al. (16) also demonstrated that the pattern of glycosylated polypeptides varied with the virus type or strain infecting the cell, and more recently (Heine and Roizman, manuscript in preparation) it was shown that the virion and purified plasma membrane of HEp-2 cells infected with certain virus strains (HSV-1 [MP] and HSV-1 [13vB4]) differed in their glycoprotein composition. The comparative studies presented in this paper clearly show that the infected cell contains a number of glycosylated, virus-specified polypeptides which are absent or present in relatively low concentrations in the virion or in the plasma membrane of infected cells (e. g., bands *b*, *d*, *h*). Furthermore, the glycoproteins from these different fractions give rise to different populations of glycopeptides when subjected to Pronase digestion. The glycopeptides derived from the total infected-cell glycoproteins are characterized by a lower average chain length and a relatively high concentration of the shortest recognized glycopeptide chains (glycopeptides II) which were present as minor species in the virion and the plasma membrane. Very similar observations were recently reported by Sefton and Keegstra (26) on Sindbis virus glycoproteins in the infected cell and the virion.

A number of other properties of the glycopeptides from herpes simplex-specific glycoproteins are worthy of comment.

(i) The glycopeptides in limit Pronase digests were distributed discontinuously among a small number of size classes, II–VI, with estimated modal molecular weights ranging from about 2,000 for glycopeptides of peak II to about 12,000 for glycopeptides of peak VI. The observation that polypeptides from infected and uninfected cells labeled with glucosamine for as little as 0.5 h did not contain glycopeptides smaller than 2,000 molecular weight (peak II) is most readily interpreted by proposing that glycopeptides in peak II are transferred intact from a nonprotein intermediate. The discrete size classes of more complex glycopeptides likewise indicates that later steps in glycosylation are discontinuous. This discontinuity may be the consequence of partitioning glycosyl transferases or the result of stepwise additions of preformed oligosaccharide chains. With respect to the second alternative there is increasing experimental support for the existence of transfers from lipid carrier intermediates as the mechanism for heterosaccharide chain addition to glycoproteins in animal cells (1).

(ii) The glycopeptides differed in their monosaccharide content, specifically the ratio of fucose/glucosamine label was highest for glycopeptides III and IV in both infected and uninfected cells and in the total infected cell, the plasma membrane, and the virion. This ratio for glycopeptides III and IV was at least three times higher than that for glycopeptides II. In addition glycopeptides II contained no detectable glucosamine-derived labeled sialic acid, whereas the longer glycopeptides (III–VI) of the plasma membrane and the virion contained a significant fraction of their total glucosamine-derived label as sialic acid. The incorporation of labeled glucosamine as sialic acid has been noted previously for animal cells and for glycoproteins of other enveloped animal viruses (18, 19). We have found that up to 40% of glucosamine label in herpes virions was released by mild acid hydrolysis and co-chromatographed with *N*-acetylneuraminic acid on gel filtration and on paper chromatography. Treatment of purified glycopeptides VI from the virion or the total infected cell with mild acid converted it from a component with a molecular weight of about 12,000 (V_e/V_o = 1.275) to one with a molecular weight of about 10,500 (V_e/V_o = 1.325) with the appearance of 30% of the glucosamine-derived radioactivity as free sialic acid (V_e/V_o = 2.35; molecular weight 310). Presuming that the pool of CMP-*N*-acetylneuraminate is derived directly from the pool of UDP-*N*-acetylglucosamine and that counts per minute per mole of *N*-acetylneuraminic acid = counts per minute per mole of *N*-acetylglucosamine, then there are some 3 to 4 mol of *N*-acetylglucosamine/mol of sialic acid in the longest glycopeptides (V and VI) and a total of some 3 to 5 mol of sialic acid/mol of glycopeptide ([12,000-10,500]/310) and thus 9 to 20 mol of *N*-acetylglucosamine/mol of glycopeptide VI. The fact that the largest glycopeptides contain multiple sialic acid residues but were not reduced to smaller units by further Pronase digestion could mean either that these units are branched or that multiple unbranched chains are tightly clustered on the polypeptide chain so that they form a single Pronase-resistant unit. The latter hypothesis seems less likely since it implies that the probability of the occurrence of multiple clustered copies of a single type of

heterosaccharide is higher than that of having a single copy of the same chain. However, differentiation between these alternatives will require further analyses of glycopeptide structure.

Stages in the biosynthesis of herpesvirus glycoproteins. Populations of glycosylated polypeptides in the total infected cell and in the infected cell plasma membrane differ with respect to both their electrophoretic properties and their heterosaccharide chain lengths. This observation may mean that the infected cell produces two unrelated sets of glycosylated polypeptides, only one set of which is present in the virion and plasma membrane, or that the set of glycosylated polypeptides present in high concentrations in the total infected cell and absent from the virion and plasma membrane are the precursors to those more completely glycosylated polypeptides. Studies by Spear (manuscript in preparation) suggest that the latter alternative is correct inasmuch as antisera prepared against purified virion envelope proteins precipitate all the major glycosylated viral proteins from infected cell extracts. We presented data which support the latter explanation and also give some insight into the steps involved in the glycosylation of herpesvirus-specific polypeptides.

The experiments with the inhibitors of protein synthesis indicate that the majority, if not all, of the glucosamine and fucose incorporation occurred after completion of the polypeptide chain and that there is a considerable pool of nonglycosylated precursor polypeptides in cells infected at high multiplicities. These observations are in general agreement with those previously presented by Spear and Roizman (28), and further show that the average time from the completion of the polypeptide chain to the first measurable addition of glucosamine is approximately 12 to 15 min and that fucose incorporation does not occur for an additional 60 to 70 min. Since we detect no polypeptides or glycopeptides containing fucose which do not also contain glucosamine, this observation alone provides indirect general evidence for precursor-product relationships. The analysis of changes in the distribution of glucosamine label between different classes of glycopeptide chains and the bands of glycosylated polypeptides separated after short and long labeling intervals or after the addition of an inhibitor of protein synthesis provides more explicit support. Thus, we have shown that, with increases in the duration of the labeling interval or after the addition of puromycin or cycloheximide, the average glycopeptide chain length increases. Under the same conditions we observed decreases in the glycosylated polypeptides in bands *d*, *e*, and *h* and increases in bands *a* and *g*, which co-migrate with highly glycosylated polypeptides of the virion and the plasma membrane.

Although our observations demonstrate the existence of precursors and their more completely glycosylated products, they do not permit any direct deductions as to which polypeptides are so related. More promising approaches to this aspect of the problem have recently been made by Spear (in press; manuscript in preparation) utilizing antisera of restricted specificity to demonstrate the presence of shared antigenic determinants between electrophoretically distinct bands of glycosylated polypeptides.

ACKNOWLEDGMENTS

We would like to thank Patricia Spear for communicating her results in advance of publication.

This work was done under the auspices of University of Chicago Cancer Center (CA 14599) of the National Cancer Institute and were aided by grants from the National Cancer Institute (CA 08494), American Cancer Society (VC 103), and the National Science Foundation (NSF BMS73-06940).

LITERATURE CITED

1. Behrens, N. H., H. Carminatti, R. J. Staneloni, L. F. Lelois, and A. I. Cantarella. 1973. Formation of lipid bound oligosaccharides containing mannose. Their role in glycoprotein synthesis. Proc. Natl. Acad. Sci. U.S.A. **70**:3390–3394.
2. Ben-Porat, T., and A. S. Kaplan. 1970. Synthesis of proteins in cells infected with herpesvirus. V. Viral glycoproteins. Virology **41**:265–273.
3. Burge, B. W., and J. H. Strauss, Jr. 1970. Glycopeptides of the membrane glycoprotein of Sindbis virus. J. Mol. Biol. **47**:449–466.
4. Bussell, R. H., and W. S. Robinson. 1973. Membrane proteins of uninfected and Rous sarcoma virus-transformed avian cells. J. Virol. **12**:320–327.
5. Darlington, R. W., and L. H. Moss. 1969. The envelope of herpes virus. Prog. Med. Virol. **11**:16–45.
6. Ejercito, P. M., E. D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effect on social behaviour of infected cells. J. Gen. Virol. **3**:357–364.
7. Gibson, W., and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10**:1044–1052.
8. Hatanaka, M. 1974. Transport of sugars in tumor cell membranes. Biochim. Biophys. Acta **355**:77–104.
9. 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.
10. Heine, J. W., and B. Roizman. 1973. Proteins specified by herpes simplex virus. IX. Contiguity of host and viral proteins in the plasma membrane of infected cells. J. Virol. **11**:810–813.
11. Heine, J. W., P. G. Spear, and B. Roizman. 1972. Proteins specified by herpes simplex virus. VI. Viral proteins in the plasma membrane. J. Virol. **9**:431–439.
12. Honess, R. W., and B. Roizman. 1973. Proteins specified by herpes simplex virus. XI. Identification and relative

molar rates of synthesis of structural and nonstructural herpesvirus polypeptides in the infected cell. J. Virol. **12**:1347-1365.
13. 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.
14. Honess, R. W., and D. H. Watson. 1974. Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J. Gen. Virol. **22**:171-185.
15. Kamat, V. B., and D. F. H. Wallach. 1965. Separation and partial purification of plasma membrane fragments from Ehrlich ascites carcinoma microsomes. Science **148**:1343-1345.
16. Keller, J. M., P. G. Spear, and B. Roizman. 1970. Proteins specified by herpes simplex virus. III. Viruses differing in their effects on the social behavior of infected cells specify different membrane glycoproteins. Proc. Natl. Acad. Sci. U.S.A. **65**:865-871.
17. Kornfeld, S., and V. Ginsberg. 1966. The metabolism of glucosamine by tissue culture cells. Exp. Cell Res. **41**:592-600.
18. Lai, M. M. C., and P. H. Deusberg. 1972. Differences between the envelope glycoproteins and glycopeptides of avian tumor viruses released from transformed and from non-transformed cells. Virology **50**:359-372.
19. Lawford, G. R., and H. Schachter. 1966. Biosynthesis of glycoproteins by liver. The incorporation of ^{14}C glucosamine into protein bound hexosamine and sialic acid of rat liver subcellular fractions. J. Biol. Chem. **241**:5408.
20. Le Breton, E., and Y. Moule. 1961. Biochemistry and physiology of the cancer cell, p. 497-544. *In* J. Brachet and A. E. Mirsky (ed.). The cell, vol. 5. Academic Press Inc., New York.
21. Nii, S., C. Morgan, and H. M. Rose. 1968. Electron microscopy of herpes simplex virus. II. Sequence of development. J. Virol. **2**:517-536.
22. Nii, S., C. Morgan, H. M. Rose, and K. C. Hsu. 1968. Electron microscopy of herpes simplex virus. IV. Studies with ferritin conjugated antibodies. J. Virol. **2**:1172-1184.
23. Roizman, B. 1972. The biochemical features of herpesvirus-infected cells, particularly as they related to their potential oncogenicity, p. 1-17. *In* P. M. Biggs, G. de Thé, and L. N. Payne (ed.), Oncogenesis and herpesviruses. IARC, Lyon.
24. Roizman, B., and D. Furlong. 1974. The replication of Herpes viruses, p. 229-403. *In* H. Fraenkel-Courat and R. R. Wagner (ed.), Comprehensive virology, vol. 3. Plenum Press, New York.
25. Schwartz, J., and B. Roizman. 1969. Concerning the egress of herpes virus from infected cells. Electron microscope observations. Virology **38**:42-49.
26. Sefton, B. M., and K. Keegstra. 1974. Glycoproteins of Sindbis virus: preliminary characterization of the oligosaccharides. J. Virol. **14**:522-530.
27. Spear, P. G., J. M. Keller, and B. Roizman. 1970. Proteins specified by herpes simplex virus. II. Viral glycoproteins associated with cellular membranes. J. Virol. **5**:123-131.
28. Spear, P. G., and B. Roizman. 1970. Proteins specified by herpes simplex virus. IV. Site of glycosylation and accumulation of viral membrane proteins. Proc. Natl. Acad. Sci. U.S.A. **66**:730-737.
29. Spear, P. G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:143-159.
30. Spiro, R. G. 1965. The carbohydrate units of thyroglobulin. J. Biol. Chem. **240**:1603-1610.
31. Spiro, R. G. 1966. Analysis of sugars found in glycoproteins, p. 3-26. *In* E. F. Neufeld and V. Ginsberg (ed.), Methods in enzymology, vol. VIII. Academic Press Inc., New York.
32. Spiro, R. G. 1973. Glycoproteins. Adv. Protein Chem. **27**:349-467.
33. Strauss, J. H., Jr., B. W. Burge, and J. E. Darnell. 1970. Carbohydrate content of the membrane protein of Sindbis virus. J. Mol. Biol. **47**:437-448.
34. Warren, L. 1959. The thiobarbitaric acid assay of sialic acids. J. Biol. Chem. **234**:1971-1975.
35. Wildy, P., W. C. Russell, and R. W. Horne. 1960. The morphology of herpes virus. Virology **12**:204-222.