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Sulfated Components of Enveloped Viruses

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The glycoproteins of several enveloped viruses, grown in a variety of cell types, are labeled with $^{35}SO_4^{-2}$, whereas the nonglycosylated proteins are not. This was shown for the HN and F glycoproteins of SV_5 and Sendai virus, the E_1 and E_2 glycoproteins of Sindbis virus, and for the major glycoprotein, gp69, as well as for a minor glycoprotein, gp52, of Rauscher leukemia virus. The minor glycoprotein of Rauscher leukemia virus is more highly sulfated, with a ratio of $^{35}SO_4$-[^{3}H]glucosamine about threefold greater than that of gp69. The G protein of vesicular stomatitis virus was labeled when virions were grown in the MDBK line of bovine kidney cells, although no significant incorporation of $^{35}SO_4^{-2}$ into this protein was observed in virions grown in BHK21-F line of baby hamster kidney cells. In addition to the viral glycoproteins, sulfate was also incorporated into a heterogeneous component with an electrophoretic mobility lower than that of any of the viral proteins in polyacrylamide gel electrophoresis. For virions doubly labeled with $^{35}SO_4^{-2}$ and [^{3}H]leucine, this component had a much greater ^{35}S-^3H ratio than any of the viral polypeptides and thus could not represent aggregated viral proteins. This material is believed to be a cell-derived mucopolysaccharide and can be removed from virions by treatment with hyaluronidase without affecting the amount of sulfate present on the glycoproteins.

A common property of enveloped viruses is the presence of glycoproteins on the exterior of their lipoprotein envelopes. Glycosylation has been shown to occur on cytoplasmic membranes (6, 10) and involves the stepwise addition of sugars which are probably added by cellular transferases in a cell-determined sequence. Often, the acidic sugar *N*-acetylneuraminic acid is present as a terminal residue on the carbohydrate side chains, thus contributing negative charge to the glycoproteins, and consequently to the virion surface (3, 16, 18). The myxo- and paramyxoviruses contain the enzyme neuraminidase as an integral component of their envelopes and thus do not contain any neuraminic acid (13, 14, 15, 23). Nonetheless, the exterior of the influenza virus is known to be anionic (20). We have recently reported that the influenza virion glycoproteins are sulfated and thus possess strongly acidic groups at their periphery (7a). The present results demonstrate that incorporation of $^{35}SO_4^{-2}$ into glycoproteins occurs as well with virions of the paramyxo, rhabdo, toga, and oncorna groups, and therefore seems to be a general characteristic of enveloped viruses. In all cases, as was observed previously for influenza virus, the label was also incorporated into a highly sulfated component with a lower electrophoretic mobility than any of the viral polypeptides, which has been tentatively identified as a host cell-derived sulfated polysaccharide which binds to and copurifies with the virions.

MATERIALS AND METHODS

Cells. The MDBK line of bovine kidney cells and the BHK21-F line of baby hamster kidney cells were grown according to previously described procedures (5, 11). JLS-V9 cells chronically infected with Rauscher leukemia virus (RLV) were obtained from J. J. McSharry and were grown in RPMI-1640 medium or in reinforced Eagle medium (2) supplemented with 10% calf serum. Primary chicken embryo fibroblasts were prepared from 10-day-old embryos and were grown in lactalbumin hydrolysate medium.

Viruses. SV_5 was grown in MDBK cells (24), as were Sendai virus (25) and the WSN strain of influenza virus (7). Vesicular stomatitis virus (VSV) was grown in BHK21-F cells (18) or in MDBK cells under similar conditions. Sindbis virus was grown in primary chicken cells by inoculating at a multiplicity of ~1 PFU/cell and harvesting the medium after 20 h. RLV was obtained from chronically infected JLS-V9 cells. All viruses were labeled in reinforced Eagle medium with 2% calf serum, containing 1 to 3 μCi of [^{3}H]leucine per ml and 25 to 50 μCi of $^{35}SO_4^{-2}$ per ml, for a period of 18 to 24 h, and purified as described previously, using precipitation with polyethylene glycol (PEG) and equilibrium zonal centrifugation in a potassium tartrate density gradient (7, 17). RLV was

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first purified on a continuous 15 to 60% sucrose gradient followed by a 5 to 40% tartrate gradient.

Hyaluronidase treatment of virions. Purified virions were dialyzed against 0.01 M sodium phosphate buffer (pH 7.2) and treated with bovine hyaluronidase at a final concentration of 1 mg/ml for 2 h at 37 C. The virions were repurified by banding on a tartrate gradient.

Triton fractionation of VSV. A modification of the procedure of Kelley et al. (12) was used. Purified virions were treated in 0.01 M phosphate buffer with 2% Triton X-100 for 20 min and pelleted at 30,000 rpm for 30 min. The glycoprotein was isolated from the supernatant by treatment with 10 volumes of *n*-butanol for 30 min followed by pelleting at 2,000 rpm for 20 min. The pellet was rinsed with anhydrous ether to remove residual *n*-butanol and was redissolved in phosphate buffer for analysis by polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide gel electrophoresis. Samples in 0.005 M sodium phosphate, 1% sodium dodecyl sulfate (SDS), and 1% β-mercaptoethanol were boiled for 1 min at 100 C (19). Ten percent acrylamide, 0.2% *N,N'*-methylenebisacrylamide gels containing SDS were used and were prepared as described by Caliguiri et al. (4). Processing of gels for determination of radioactivity was done as described previously (6).

Sugar assays. Sugar assays were performed as described by Dische (8). Hexose was assayed by reaction with indole and sulfuric acid, using glucose as the standard. Hexuronic acid was assayed by the carbazole-sulfuric acid method, using heparin as standard.

Chemicals and isotopes. [4,5-^{3}H]leucine and $^{35}SO_4^{-2}$ were obtained from Schwarz/Mann, Orangeburg, N.Y. [^{3}H]glucosamine was obtained from New England Nuclear, Boston, Mass. Components for polyacrylamide gels were obtained from Canal Industrial Corp., Rockville, Md. Hyaluronidase was obtained from Miles-Seravac, Berkshire, England. TPCK-treated trypsin was obtained from Worthington Biochemical Corp., Freehold, N.J. RPMI-1640 medium was obtained from GIBCO, Grand Island, N.Y.

RESULTS

Further characterization of host cell-derived sulfated components of influenza virions. We have previously reported that influenza virions grown in the presence of $^{35}SO_4^{-2}$ contain label incorporated into the viral glycoproteins as well as into a highly sulfated material of low electrophoretic mobility which has been tentatively identified as a cellular-derived sulfated polysaccharide (7a). The latter component can be removed from the virions by treatment with trypsin, whereas the sulfate incorporated into the HA_1 and HA_2 glycoproteins is unaffected. Treatment of virions with the enzyme hyaluronidase results in a simila. effect. After repurification on a tartrate gradient the virions obtained did not contain the sulfated polysaccharide, whereas the amount of sulfate present in the HA_1 and HA_2 glycoproteins did not change. The PAGE pattern of these virions appeared identical with that obtained for virions after trypsin treatment (Compans and Pinter, Virology, in press). However, when enzyme-treated virions were analyzed without repurification to examine the polyacrylamide gel patterns of the released polysaccharide products, a marked difference in the size of the fragments produced was found. Figure 1 shows the gel patterns obtained for untreated virions (A), virions treated with trypsin (B), and virions treated with hyaluronidase (C). Whereas the ^{35}S labeling pattern for the trypsin-treated virions is quite similar to that of the control, the pattern obtained for the hyaluronidase-treated virions differs in that essentially all of the sulfated polysaccharide has been removed from the gel. The label associated with the glycoproteins is unaffected, and otherwise the pattern appears identical to the one obtained by trypsin treatment. These results indicate that the sulfated polysaccharide is extensively degraded by hyaluronidase, but that the molecular weight of the polysaccharide is not markedly altered by trypsin treatment despite the fact that trypsin treatment, followed by rebanding, results in its removal from the virion (Compans and Pinter, Virology, in press).

After repurification of the trypsin or hyaluronidase-treated virions on tartrate density gradients the released polysaccharide is found at the top of the gradients and thereby can be isolated from the virions. The material released in this manner by trypsin treatment was analyzed for hexose and hexuronic acid, and the ratio of hexose to hexuronic acid was 10:1. These results indicate that hexuronic acid is only a minor component of the total carbohydrate.

A reconstruction experiment was carried out to determine whether sulfated macromolecules secreted by MDBK cells would bind to virions in vitro. Influenza virus, grown in MDBK cells and labeled with [^{3}H]leucine, was mixed before purification with culture fluids of uninfected MDBK cells labeled for the same period with $^{35}SO_4^{-2}$. After incubation for 3 h at 37 C the virions were purified by PEG or ammonium sulfate precipitation followed by banding in a tartrate gradient. The resulting virus band contained both ^{3}H and ^{35}S labels. Analysis by PAGE showed that the ^{35}S label was associated with a heterogeneous material migrating throughout the first 30 fractions of the gel, which appears similar to the sulfated polysaccharide associated with virions grown in the presence of

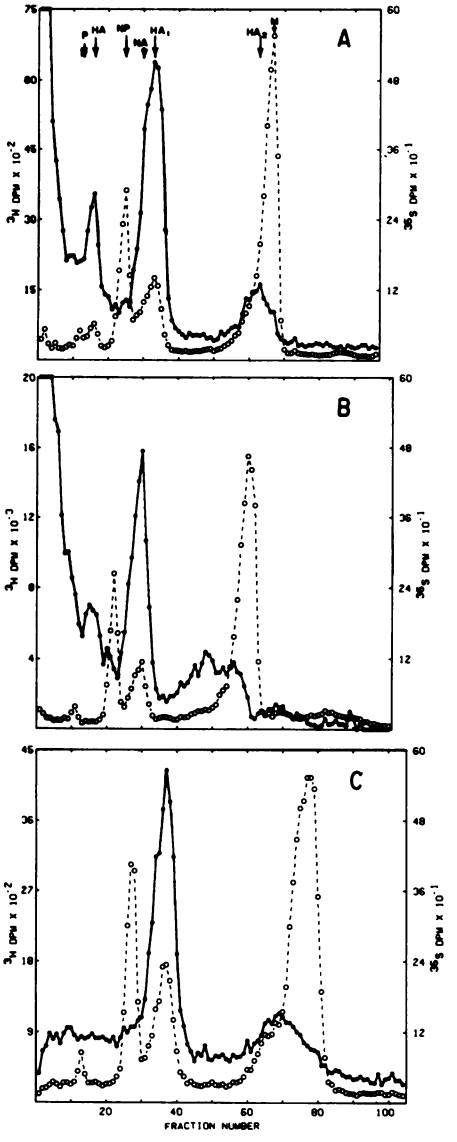

FIG. 1. *PAGE patterns of SDS-dissociated influenza virions labeled with 2 μCi of [4,5-^{3}H]leucine per ml (○) and 50 μCi of $^{35}SO_4^{-2}$ per ml (●). In all figures migration is from left to right. (A) Control virions. (B) Virions treated with 100 μg of trypsin per ml for 1 h at 37 C. (C) Virions treated with 1 mg of hyaluronidase per ml for 2 h at 37 C. The variation in the ratios of ^{3}H to ^{35}S occurs because a different virus preparation was used in B.*

$^{35}SO_4^{-2}$ label. This result suggests that the polysaccharides associated with virions are similar to components produced by normal cells which are secreted into the culture medium and which can bind rapidly to the virions in vitro.

However, the material which associates with virions in vitro does not appear to be bound as firmly as are the molecules found on virions grown in the presence of $^{35}SO_4^{-2}$. In the former case, purification of the virions by high-speed pelleting in place of PEG or ammonium sulfate precipitation resulted in the removal of 90% of the ^{35}S counts, whereas in the latter case pelleting does not remove the polysaccharide.

Sulfate incorporation into paramyxoviruses. To determine whether sulfated macromolecules were present in other enveloped viruses, we analyzed virions of other major groups by PAGE. The paramyxovirus SV_5 contains five major polypeptides, two of which are glycoproteins (13, 24). The gel pattern obtained for SV_5 virions doubly labeled with [^{3}H]leucine and $^{35}SO_4^{-2}$ is shown in Fig. 2A. All five polypeptides are resolved, and the only polypeptides which are labeled by $^{35}SO_4^{-2}$ are the two glycoproteins HN and F (24). The other

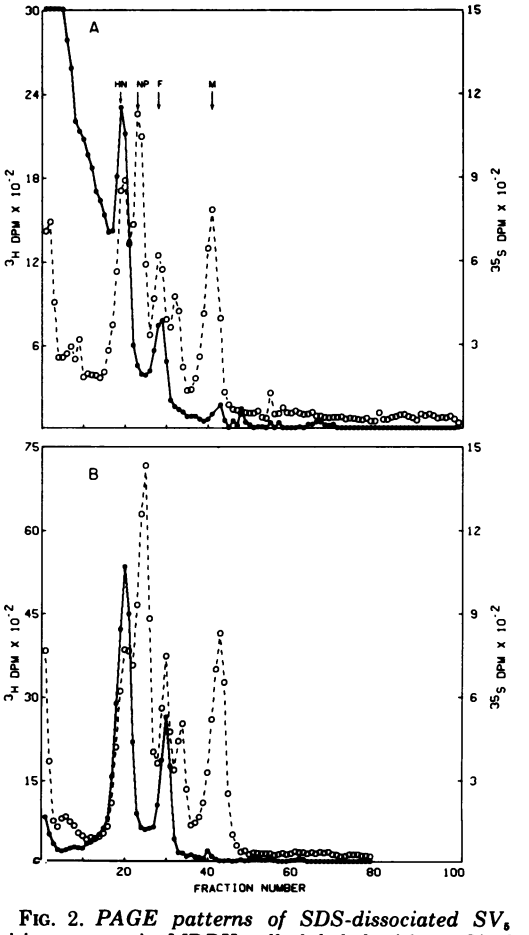

FIG. 2. *PAGE patterns of SDS-dissociated SV_5 virions grown in MDBK cells labeled with 1 μCi of [4,5-^{3}H]leucine (○) and 50 μCi of $^{35}SO_4^{-2}$ (●) per ml. (A) Untreated virions. (B) A different preparation of virions following treatment with 1 mg of hyaluronidase per ml and rebanding on 5 to 40% potassium tartrate density gradients.*

three nonglycosylated polypeptides do not contain any of the sulfate label. For SV_5, as with influenza virus, a large amount of the sulfate label was present in a heterogeneous peak found near the origin of polyacrylamide gels. This material contained very small amounts of amino acid label and appears similar to the sulfated polysaccharide present on influenza virions (Compans and Pinter, Virology, in press). Treatment of purified SV_5 virions with hyaluronidase, followed by repurification on a tartrate gradient, resulted in the quantitative removal of this material (Fig. 2B). The [^{3}H]leucine pattern is unchanged and the sulfated material near the origin of the gel is absent, whereas the sulfate label remains associated with glycoproteins.

Similar results were obtained for Sendai virus grown in MDBK cells. The sulfate label was associated with glycoproteins HN and F (25), whereas the nonglycosylated proteins did not contain any sulfate label. A significant amount of sulfate was found associated with the material migrating near the origin of the gel as was found for SV_5 and influenza virions, and this too was removed by hyaluronidase.

Sindbis virus. The polypeptides of Sindbis virus grown in primary chicken embryo cells are shown in Fig. 3. Two peaks labeled with [^{3}H]leucine are resolved, the smaller one corresponding to the nonglycosylated nucleocapsid protein and the larger one to the two envelope proteins, E_1 and E_2 (26). Again, a large amount of sulfate label is found associated with the material remaining near the origin of the gel. As was the case with the myxo- and paramyxoviruses, this fraction contained very little [^{3}H]leucine label.

VSV. We have investigated the incorporation of $^{35}SO_4^{-2}$ into VSV grown in both BHK21-F cells and in MDBK cells. VSV contains three major proteins, a glycoprotein of molecular weight 69,000 which constitutes the surface spikes, the nucleoprotein of molecular weight 50,000, and the membrane protein of molecular weight 29,000. There are also two minor proteins, L and NS, which are also nonglycosylated (29).

The PAGE patterns obtained for VSV doubly labeled with [^{3}H]leucine and $^{35}SO_4^{-2}$ in BHK21-F and MDBK cells were similar. Virions from both cell lines had a similar ratio of total $^{35}SO_4^{-2}$ to [^{3}H]leucine, and, in both cases, the vast majority of ^{35}S counts migrated near the origin of polyacrylamide gels in a peak that was very intense and broad and interfered with the resolution of the glycoprotein. Hyaluroni-

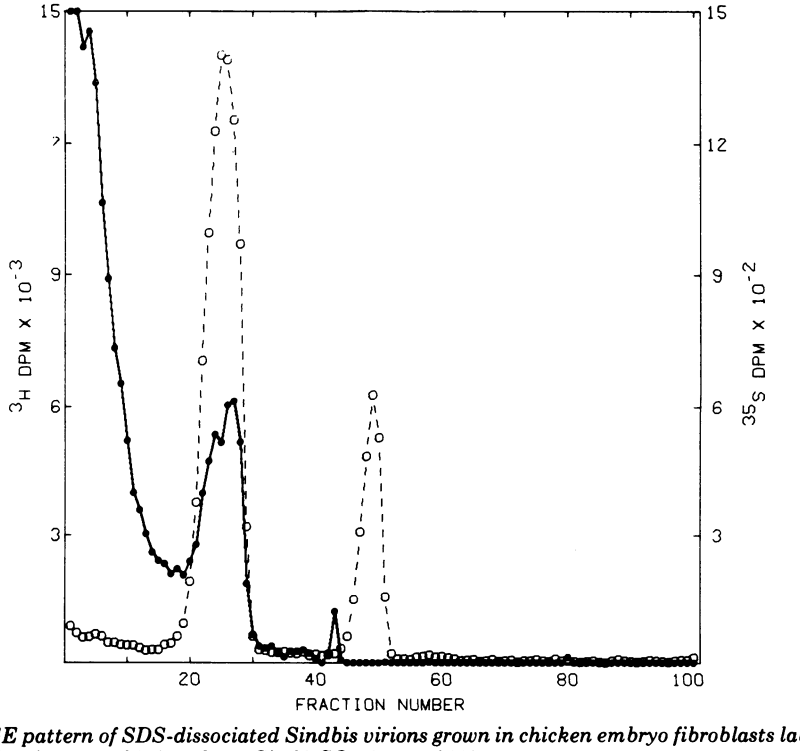

FIG. 3. *PAGE pattern of SDS-dissociated Sindbis virions grown in chicken embryo fibroblasts labeled with 1 μCi of [4.5-^{3}H]leucine per ml* (○) *and 50 μCi of $^{35}SO_4^{-2}$ per ml* (●).

dase treatment resulted in the removal of this material; however, it also resulted in the loss of a significant fraction of the glycoprotein molecules, apparently due to the sensitivity of the VSV glycoprotein to a protease impurity present in the hyaluronidase preparation. To determine whether in fact $^{35}SO_4^{-2}$ was incorporated into the VSV glycoprotein it was necessary to isolate the glycoprotein before analysis by PAGE.

The glycoprotein was purified using a modification of a published procedure involving extraction with Triton X-100 followed by pelleting. (12). The resulting supernatant contained only the glycoprotein, whereas the other viral proteins, as well as the cellular-derived sulfated material, were found in the pellet. Figure 4 shows the PAGE patterns obtained for the purified glycoproteins. For virus grown in MDBK cells, an appreciable amount of sulfate

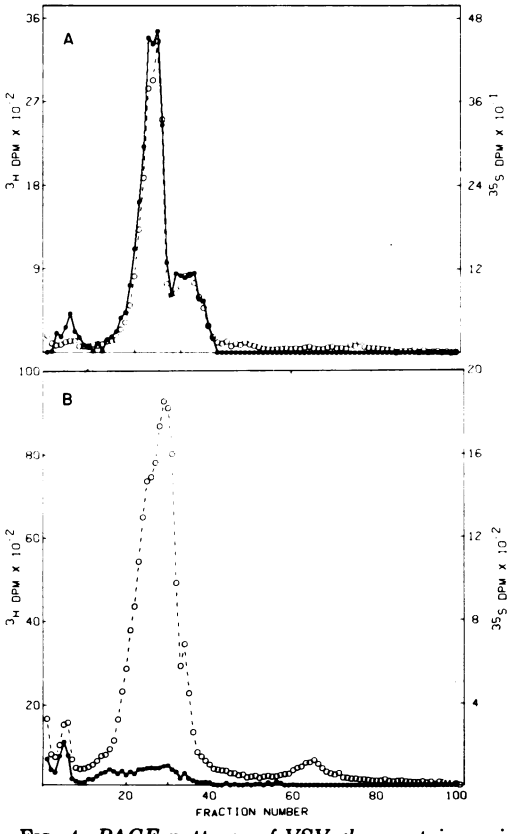

FIG. 4. *PAGE patterns of VSV glycoprotein purified by Triton X-100 fractionation. Virions were labeled with 2 μCi of [4,5-^{3}H]leucine per ml (○) and 50 μCi of $^{35}SO_4^{-2}$ per ml (●). (A) Glycoprotein of VSV grown in MDBK cells. (B) Glycoprotein of VSV grown in BHK21-F cells. The small peak in A preceding the glycoprotein probably represents a degradation product of the glycoprotein.*

was found associated with the glycoprotein, whereas for virus grown in BHK21-F cells no significant incorporation was found. In contrast, significant incorporation of sulfate into the glycoproteins was previously found (Compans and Pinter, Virology, in press) for influenza virions grown in BHK21-F cells.

Rauscher leukemia virus. The PAGE patterns obtained for RLV isolated from JLS-V9 cells were similar to previously published patterns (reviewed in Tooze [28]). Bands corresponding to major viral proteins gp69, p30, p15, p12, and p10 were obtained using Coomassie blue-stained gels or virus labeled with ^{3}H-labeled amino acids. For virus labeled with $^{35}SO_4^{-2}$ two sulfate-containing proteins were observed (Fig. 5); the major sulfated protein corresponded to the major glycoprotein of RLV, gp69, and the minor sulfated component corresponded to a smaller polypeptide. A similar component has been identified in some previous reports as a minor glycoprotein by virtue of its incorporation of [^{3}H]glucosamine, and estimates of its molecular weight have varied in the literature from values of 42,000 (21) to 60,000 (22). A value for the molecular weight of this component of 52,000 ± 2,000 was arrived at by coelectrophoresis on 10% polyacrylamide gels of $^{35}SO_4^{-2}$-labeled RLV with [^{3}H]leucine-labeled WSN strain influenza virus. The molecular weight of the larger glycoprotein was estimated to be 69,000 ± 1,000, in agreement with other reports (1).

When RLV samples labeled separately with [^{3}H]glucosamine and $^{35}SO_4^{-2}$ were analyzed by coelectrophoresis, the glucosamine and the sulfate-containing peaks were coincident (Fig. 6). The percentage of sulfate versus glucosamine was ~2.5-fold greater for gp52 than for gp69. This could be an indication of a different carbohydrate composition for the two glycoproteins. For RLV, as was found with the other viruses studied, an appreciable amount of the sulfate label was found associated with the material migrating near the origin of the polyacrylamide gels.

DISCUSSION

We have previously demonstrated that the glycoproteins of influenza virions are sulfated, whereas the nonglycosylated components are not (Compans and Pinter, Virology, in press). The present results demonstrate that sulfation of glycoproteins appears to be a general characteristic of enveloped viruses. Members of five different major groups have been studied, and in each instance the glycoproteins are labeled with $^{35}SO_4^{-2}$, whereas the carbohydrate-free

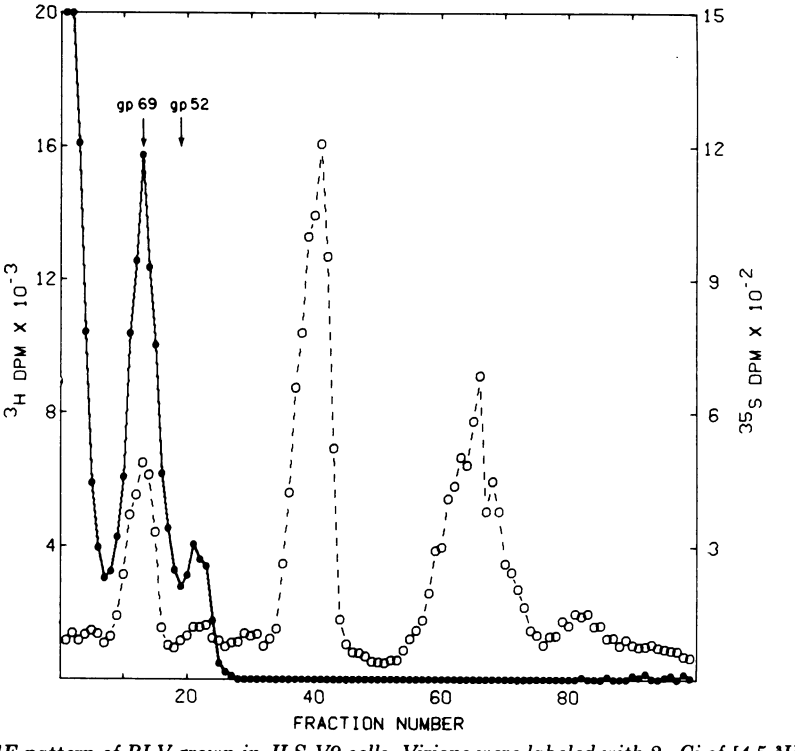

FIG. 5. *PAGE pattern of RLV grown in JLS-V9 cells. Virions were labeled with 2 μCi of [4,5-^{3}H]leucine per ml* (○) *and 50 μCi of $^{35}SO_4^{-2}$ per ml* (●).

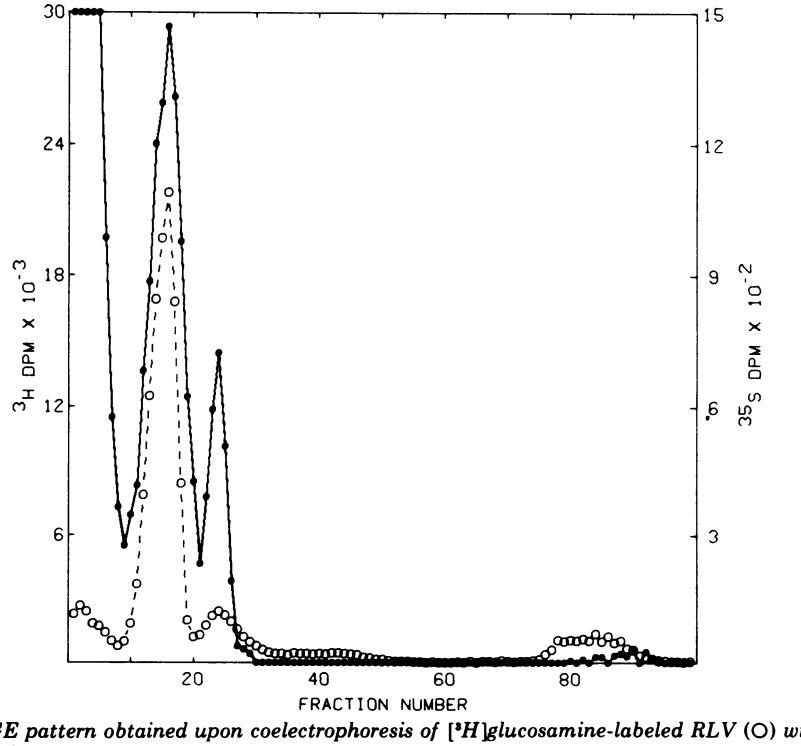

FIG. 6. *PAGE pattern obtained upon coelectrophoresis of [^{3}H]glucosamine-labeled RLV* (○) *with $^{35}SO_4^{-2}$-labeled RLV* (●).

proteins are unlabeled. A major nonstructural glycoprotein in herpesvirus-infected cells has also been shown to contain sulfate (9). The extent of sulfation varies for different cell types; more sulfate is incorporated into the glycoproteins of influenza virions when grown in MDBK cells than when grown in BHK21-F cells (Compans and Pinter, Virology, in press), and no appreciable $^{35}SO_4^{-2}$ is found in VSV grown in BHK21-F cells. However, sulfation occurred to an appreciable extent in the other cases studied.

Evidence that the sulfate is covalently bound to the glycoproteins is provided by the fact that for all of the viruses studied the $^{35}SO_4^{-2}$ label migrates with the glycoproteins after a variety of dissociation and purification techniques. This was true as well for different viruses grown in the same cell type. We have previously shown for influenza virus that the sulfate label on the glycoproteins can be recovered after acid hydrolysis as free sulfate (Compans and Pinter, Virology, in press) indicating that it is in the form of a sulfoester, presumably of one or more of the sugar components of the carbohydrate side chains. The relative amounts of $^{35}SO_4^{-2}$ to [^{3}H]glucosamine for the various glycoproteins of the myxo- and paramyxoviruses remained fairly constant. This was not the case for the two RLV glycoproteins, as gp52 was found to contain a two- to threefold greater $^{35}SO_4^{-2}/^3$H-glucosamine ratio than did the major glycoprotein. This difference in extent of sulfation may be an indication of a different carbohydrate composition and structure for the two glycoproteins, or it may indicate that the orientation of the glycoprotein relative to the viral surface plays a role in determining to what extent sulfation occurs.

The fact that VSV grown in BHK21-F cells did not incorporate detectable amounts of $^{35}SO_4^{-2}$ into the viral glycoprotein, while the same glycoprotein did incorporate the label when the virus was grown in MDBK cells, indicates that even for proteins containing the appropriate receptor sites sulfation need not always occur. Thus, under the labeling conditions described, the absence of $^{35}SO_4^{-2}$ incorporation does not conclusively indicate the lack of carbohydrate on a given protein. The specific transferases involved in sulfation are probably cellular enzymes, and thus one might expect considerable variation in the activities of these factors in different cell lines. Furthermore, sulfation may require certain carbohydrate components or sequences, which may be absent for some glycoproteins in a given cell line. A direct relationship between sulfation and the presence or absence of *N*-acetylneuraminic acid residues was not observed, since sulfate was incorporated into viruses which contained this sugar as well as into those which did not.

Our results also indicate that the highly sulfated component found near the origin of SDS-polyacrylamide gels of influenza virus is present in all viruses from a variety of cell lines studied. The material has been tentatively identified as a cellular polysaccharide with a high affinity for the virions, and the present work further corroborates that description. We have previously shown that this material can be labeled before viral infection and that it is located on the virion suface where it is accessible to proteases (Compans and Pinter, Virology, in press). The association of this sulfated polysaccharide with virions is quite strong and it was present in virus preparations purified by a variety of means, including PEG or ammonium sulfate precipitation followed by banding on either sucrose or potassium tartrate gradients. The reconstruction experiments described in this paper indicate that a component with electrophoretic properties similar to the sulfated polysaccharide is produced by uninfected cells and binds rapidly to the virions.

The sulfated polysaccharide found with Sendai virus may be identical with an acidic, glucosamine-containing component of low electrophoretic mobility which has recently been isolated from Sendai virions (27).

Treatment of the virions with hyaluronidase followed by rebanding resulted in the removal of the sulfated polysaccharide. The effect of hyaluronidase is distinct from that of trypsin, since the electrophoretic mobility of the polysaccharide does not differ appreciably after trypsin treatment, whereas after hyaluronidase treatment the polysaccharide has been degraded to small fragments which migrate off the gel. These fragments are not dialyzable, and they have a SDS-polyacrylamide migration rate similar to or greater than that of a protein of ~15,000 molecular weight.

We have shown by chemical analysis that the amount of uronic acid present in the polysaccharide constitutes less than 10% of the total hexose content of the molecule. This may be enough uronic acid to account for the susceptibility of the polysaccharide to hyaluronidase, although the preparation of hyaluronidase used for these experiments was not homogeneous, and it is possible that one of the minor components present contributes to the degradation of the polysaccharide. However, more highly purified preparations produced similar results (A. Pinter, unpublished data).

The role that the sulfate groups of the glycoproteins play is currently under investigation. The possibility exists that they may have activity in the binding of virions to cellular receptors, and they may contribute to some of the antigenic determinants of the viral glycoproteins. They also may protect the viral glycoproteins from degradation by exoglycosidases, thus increasing the extracellular lifetime of the virions and consequently their infectivity. The function of the polysaccharides present on the surfaces of the various viruses is also unknown. We have previously suggested that they may be inhibitors of viral infectivity (Compans and Pinter, Virology, in press), and they may introduce new antigenic determinants at the virion surfaces.

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