JOURNAL OF VIROLOGY, Oct. 1975, p. 944-950
Vol. 16, No. 5
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Virus-Induced Proteins in Pseudorabies-Infected Cells. II. Proteins of the Virion and Nucleocapsid

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Received for publication 6 May 1975

Analysis of purified naked and enveloped nucleocapsids of pseudorabies virus with high-resolution techniques has allowed a reassessment of their protein composition. Enveloped particles are shown to contain at least 20 proteins whose molecular weights are in the range 20,000 to 230,000. Naked nucleocapsids contain one major and seven minor proteins in the molecular weight range 20,000 to 155,000. Phosphorylation of at least one virion protein is shown to take place in vivo. These results demonstrate that pseudorabies virus is similar in its protein complement to other herpesviruses which have recently been examined.

Improved methods of preparation of virus and nucleocapsid together with the application of high-resolution electrophoresis techniques have allowed a reassessment of the structural protein content of herpes simplex virus (5, 11). Enveloped nucleocapsids were shown to contain at least 24 species of proteins, ranging in molecular weight from 25,000 to 275,000, while naked nucleocapsids contained four or five proteins. A similar structural complexity has also been demonstrated for equine herpes virus type 1 (10). I have applied such methods to the study of pseudorabies virus (pig herpes virus 1) and find that this virus is in general similar to the two previously mentioned and therefore more complex than previously reported (2).

MATERIALS AND METHODS

Cells. BHK-21 and HeLa cells were maintained in a modified Eagle medium (1) containing 100 units of penicillin and 100 μg of streptomycin per ml, and supplemented with 10% calf serum. Frequent pleuropneumonia-like organism checks were carried out and if detected the cultures were discarded.

Virus. Pseudorabies virus was originally derived from a stock preparation (8) and has subsequently been plaque purified three times. Virus stocks were prepared from infected BHK-21 monolayer cultures as previously described (3). Plaque assays were carried out on monolayer cultures of BHK-21 cells and normally gave titers of 10^9 to 10^{11} PFU/ml.

Purification of virus and nucleocapsid. The methods of purification were essentially those of Spear and Roizman (11) and Gibson and Roizman (5).

Virions and nucleocapsids were purified from either HeLa or BHK-21 cells infected for 18 to 24 h. Cultures were infected with virus at an input multiplicity of 20 PFU/cell in a small volume (25 ml) of medium and rolled for 1 h at 37 C to allow adsorption to take place. The inoculum was then replaced with 50 ml of fresh medium and the incubation was continued at 37 C. To label proteins, infected cells were incubated between 4 and 24 h postinfection in medium containing one fifth the usual concentration of methionine and 4 μCi of either [^{35}S]methionine or [*methyl*-^{3}H]methionine per ml.

The infected cells were scraped off the glass surface on which they were grown, collected by low-speed centrifugation, suspended in 2 volumes of 1 mM phosphate buffer, pH 7.4, and disrupted with four strokes of a Dounce homogenizer. Immediately afterwards, sufficient 60% (wt/wt) sucrose was added to yield a final concentration of 0.25 M. The cytoplasm was then separated from the nuclei by centrifugation at 500 × *g* for 10 min.

Samples (2 ml) of the supernatant were layered on 36-ml gradients (12 to 32%, wt/vol) of Dextran T10 (Pharmacia, Uppsala, Sweden) made up in 1 mM phosphate buffer. The dextran solutions were filtered through 0.2-μm membrane filters (The Nalge Co., Rochester, N.Y.) prior to use.

The gradients were centrifuged for 1 h at 20,000 rpm in the Beckman SW27 rotor. After centrifugation virions were found in a diffuse light-scattering band just above the middle of the tube. This partially purified virus was aspirated with a needle and syringe from just below the band.

The virus suspension was made 0.5 M with respect to urea by the addition of an appropriate amount of 5 M urea and was sonically treated for 5 s (M. S. E. Ltd., Crawley, England) to dissociate aggregates of virions and host membrane vesicles. This material was now made 50% (wt/wt) with respect to sucrose by the addition of solid sucrose. Samples (10 ml) of this solution were placed in 38-ml tubes and discontinuous sucrose gradients were formed by the successive layering of 40, 30, and 20% (wt/wt) sucrose (made up in 0.01 M Tris buffer) on top of the 50% sucrose-virus layer. These gradients were centrifuged at 25,000 rpm for 18 h in the SW27 rotor. The bulk of the virions floated to the 40 to 50% interface and were aspirated by needle and syringe. The virus suspension was

diluted fourfold with 0.01 M Tris buffer, layered over 10 ml of 10% sucrose in 2 M urea, 0.01 M Tris buffer in a 38-ml tube and centrifuged at 25,000 rpm for 2 h in the SW27 rotor. The virus pellets were suspended in a small volume of 0.01 M Tris buffer and frozen at −20 C.

For the purification of nucleocapsid the nuclear pellet from the low-speed spin after cell disruption was first washed three times in 1% Tween 80. This gave a clean preparation of nuclei with little cytoplasmic contamination, as judged by phase contrast microscopy. The nuclei were then suspended in 0.15 M NaCl, 0.01 M Tris, pH 7.2. To this was added sodium lauryl sulphate (SDS) to a concentration of 0.5% followed by deoxyribonuclease to 50 μg/ml. The lysate was incubated at 25 C for 10 min. Brij 58 and urea were then added to final concentrations of 0.5% and 0.5 M, respectively, and the extract was clarified by low-speed centrifugation.

Samples (1 to 2 ml) of the supernatant were layered on top of linear 10 to 40% (wt/wt) sucrose gradients in 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7.2, and centrifuged for 60 min at 23,000 rpm and 4 C in a Beckman SW27 rotor. In contrast to the report for herpes simplex virus (5) only one light-scattering band located near the middle of the tube could be detected. This was aspirated by a needle and syringe, diluted fourfold with 0.01 M Tris buffer and centrifuged at 25,000 rpm for 2 h in the SW27 rotor. The nucleocapsid pellet was resuspended in a small volume of 0.01 M Tris buffer and frozen at −20 C.

Acrylamide gel electrophoresis. Samples to be analyzed were heated for 2 min at 100 C in 3% SDS, 1% β-mercaptoethanol, and 50 mM Tris, pH 7.0.

Electrophoresis was carried out using a discontinuous system as described by Dimmock and Watson (4) and Laemmli (9).

The main gel contained 0.375 M Tris-hydrochloride, pH 8.8, 0.1% SDS, 0.03% (vol/vol) *N,N,N′,N′*-tetramethylethylene-diamine, 0.036% (wt/vol) ammonium persulfate with acrylamide concentrations in the range 7 to 14%, and a bisacrylamide to acrylamide ratio of 1:30. The stacker gel contained 0.125 M Tris-hydrochloride, pH 7.0, 0.1% SDS, 0.03% *N,N,N′,N′*-tetramethylethylene-diamine, 0.07% (wt/vol) ammonium persulfate, 3% acrylamide, and 0.1% bisacrylamide.

The electrode buffer was 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.5.

In some gels the bisacrylamide was replaced by *N,N′*-diallyltartardiamide (DATD); 1.5 g of DATD was substituted for each 1 g of bisacrylamide.

Samples (50 to 200 μl) were applied to disc gels of 6 mm inside diameter; samples (10 to 20 μl) were applied to slab gels 3 mm thick.

Gels were subjected to electrophoresis at a constant current of 3 mA/gel cylinder or 25 mA/gel slab. Bromophenol blue was used as a marker.

Processing of gels. Proteins in the gels were stained with Coomassie brilliant blue (0.5% [wt/vol]) in 40% methanol, 5% glacial acetic acid) and destained in 40% methanol, 50% glacial acetic acid, and 2% glycerol.

For autoradiography gel cylinders were cut in half longitudinally and gel slabs were dried onto filter paper and placed in contact with X-ray film.

For scintillation counting gel cylinders were sliced laterally into 1-mm segments and dissolved in 0.5 ml of concentrated hydrogen peroxide (about 30% [wt/vol]). Scintillant (10 ml; 0.5% 2,5-diphenyloxazole in toluene mixed in a ratio of 60:40 with methoxyethanol) was added, and the radioactivity was measured.

The absorbance of bands in stained gels and autoradiograms was measured with the Gilford gel scanner attachment and Gilford recording spectrophotometer at 600 nm.

RESULTS

Enveloped virus. The procedure described in Materials and Methods was found to give a clearly defined virion band both on the dextran and discontinuous sucrose gradients exactly as has been described by Spear and Roizman (11) for herpes simplex virus.

An electron micrograph of a sample of virus after final pelleting and resuspension is shown in Fig. 1a. Some loss of integrity of the envelope attends this process. Normally no naked nucleocapsids were observed in such preparations and there was little contamination with other material. Figure 1b shows a preparation of naked nucleocapsids.

It was found that good preparations of virus could be obtained in this way both from infected BHK-21 cells and infected HeLa cells. Comparision of the proteins present in these should allow minor contamination by host material which might be expected to vary from one cell line to another to be identified and discounted.

This expedient of using two cell lines to characterize virus together with electron micrographs of the purified material seems not unreasonable in view of the difficulties which attend the interpretation of $^{3}H/^{14}C$ ratios in purifying virus labeled with one isotope from cells prelabeled with the other. In common with the report for herpes simplex (11), I find that in experiments of this latter type the ratio changes approximately 20-fold in the case of pseudorabies. This, however, only indicates the purification of virus-induced proteins relative to cellular proteins and underestimates the purification of virion proteins. Similarly changes in infectivity due to the preparation procedure render estimates of purification based on PFUs uncertain (11).

In Fig. 2 Coomassie blue-staining profiles of polyacrylamide gels of the proteins of virus particles from BHK and HeLa cells are illustrated. These profiles could be reproducibly

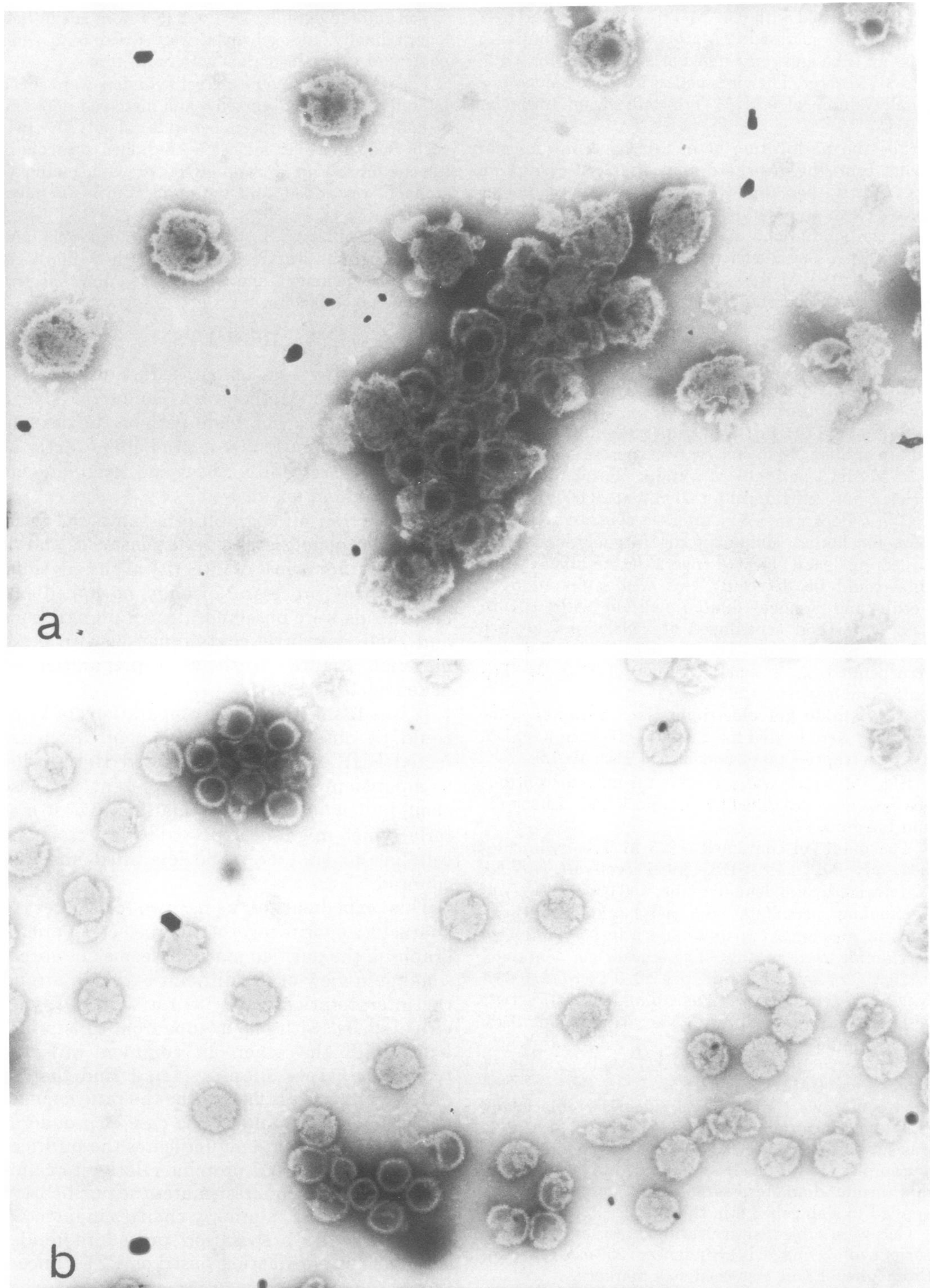

FIG. 1. *Electron micrographs of enveloped (a) and naked (b) nucleocapsids. Some loss of structural integrity is apparent principally due to the final pelleting and resuspension of the particles. No cross contamination of enveloped and naked particles was found.*

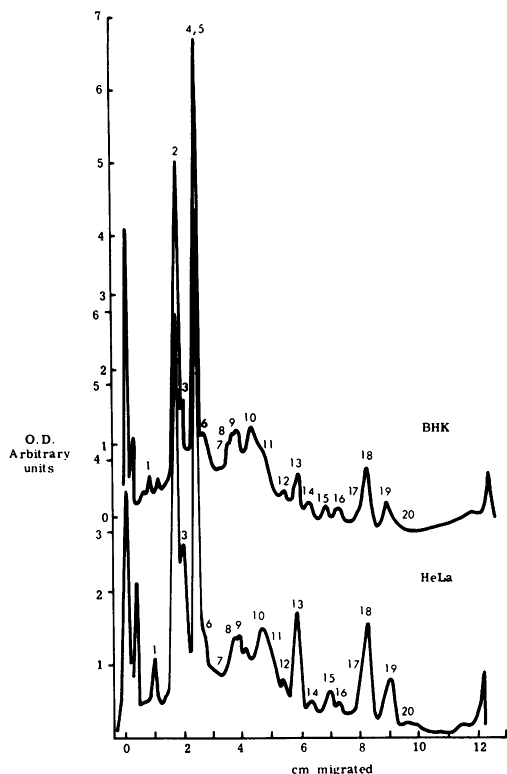

FIG. 2. *Electropherograms of mature virions prepared from infected BHK-21 cells (upper trace) and infected HeLa cells (lower trace). Virions were prepared from cells harvested 24 h after infection at an input multiplicity of 20 PFU/cell. Virions were solubilized in 3% SDS-1% β-mercaptoethanol and subjected to electrophoresis in 10% acrylamide-0.3% bisacrylamide disc gels. The profiles show the absorbance of Coomassie blue-staining material.*

obtained from the two cell types and allowed identification of 20 virion polypeptides. The scan of an autoradiograph of a gel of a [^{35}S]methionine-labeled preparation from HeLa cells is shown in Fig. 3.

These gels were all of 10% acrylamide with bisacrylamide as the cross-linking agent. The use of DATD has been reported to confer certain advantages in the handling of gels and better resolution of the sample applied (6, 11). Using DATD for these purposes it was found that the migration of some virion proteins was affected and that better resolution of proteins 2 to 6 in particular could be obtained. A scan of an autoradiogram of virion polypeptides prepared from HeLa cells and separated on a DATD cross-linked gel is shown in Fig. 4. The resolution obtained in this way is superior to that obtained using bisacrylamide as cross-linker.

Electrophoresis was carried out on 7, 10, and 14% acrylamide gels with bisacrylamide as cross-linker and on 10% acrylamide with DATD as cross-linker with a view to determining molecular weights. Standard proteins used were β-galactosidase (130,000), bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen (26,000), and cytochrome *c* (13,500).

Molecular weights of virus proteins calcu-

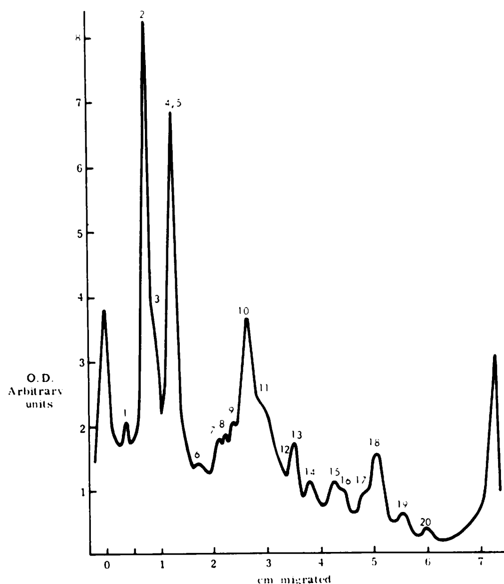

FIG. 3. *Autoradiogram of mature virion proteins labeled with [^{35}S]methionine from 4 to 24 h postinfection. Electrophoresis was carried out on a 10% acrylamide-0.3% bisacrylamide disc gel.*

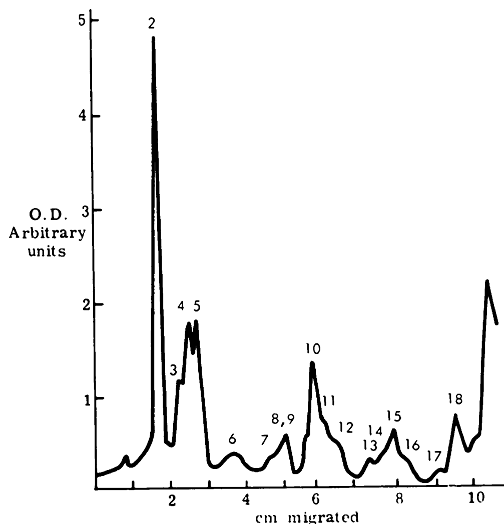

FIG. 4. *Autoradiogram of mature virion proteins labeled with [^{35}S]methionine from 4 to 24 h postinfection. Electrophoresis was carried out on a 10% acrylamide-0.45% DATD disc gel.*

TABLE 1. *Molecular weights of viral proteins calculated from their migration on acrylamide gels relative to that of standard proteins*

Protein no.	Mol wt	Virion	Nucleo-capsid
1	230,000	+	
2	150,000	+	+
3	140,000	+	
4	120,000	+	+
5	115,000	+	
6	110,000	+	
7	92,000	+	
8	85,000	+	
9	82,000	+	+
10	72,000	+	
11	63,000	+	+
12	56,000	+	
13	45,000	+	
14	41,000	+	+
15	36,500	+	
16	32,000	+	+
17	29,000	+	
18	27,000	+	+
19	22,500	+	+
20	20,500	+	

lated on the basis of relative migration in these gels are given in Table 1.

Nucleocapsid. Nucleocapsid was prepared as described in Materials and Methods. On the 10 to 40% sucrose gradient only one band could be detected after centrifugation. This is in contrast to the report for herpes simplex virus (5) and also to a previous report for pseudorabies virus (2). In these cases an upper band was found which consisted mainly of particles containing little or no DNA.

The differences in protein profile after electrophoresis reported for these two capsid forms were only quantitative in the case of pseudorabies virus with all species present in one also present in the other. In the case of herpes simplex one new protein was present in the DNA-containing particles which was absent in empty particles. My failure to find two bands may reflect a lack of empty particles but even if a mixture is present all nucleocapsid proteins should be observed though not in the correct relative proportions. The reported DNA-to-protein ratio for herpes simplex capsid forms are 0.014 for capsids with little DNA and 0.136 for capsids presumed to contain the full complement of DNA (5). The capsids of pseudorabies described in this paper were found to have a DNA-to-protein ratio of 0.123 when analyzed by the same methods used by Gibson and Roizman (5) for herpes simplex virus. This suggests that the particles examined must be predominantly DNA containing.

In Fig. 1b is shown an electron micrograph of pelleted and resuspended material from the sucrose gradient. This demonstrates that these nuclear particles are naked nucleocapsids.

The Coomassie blue-staining profile (Fig. 5) shows that there is one major band (which co-migrates with protein 2 of the enveloped particles). The remaining seven bands are all present in relatively small amounts. The pattern given by an autoradiograph is almost identical to that of stain. A long exposure autoradiograph of a 14% acrylamide gel is shown in Fig. 6. This allows unequivocal demonstration of the presence of the protein corresponding to protein 19 of the virion.

Incorporation of ^{32}P. Gibson and Roizman (6) found that ^{32}P was incorporated into at least one capsid protein and into a number of virion proteins. In the case of pseudorabies (Fig. 7) no reproducible incorporation into any capsid protein could be demonstrated. Only a low level of ^{32}P was incorporated, though there was always some activity associated with protein 4. In mature virion there was clear incorporation into protein 4 with small amounts of activity again present at other parts of the gel.

DISCUSSION

It has been demonstrated in this report that the structural protein complements of naked and enveloped nucleocapsids of pseudorabies

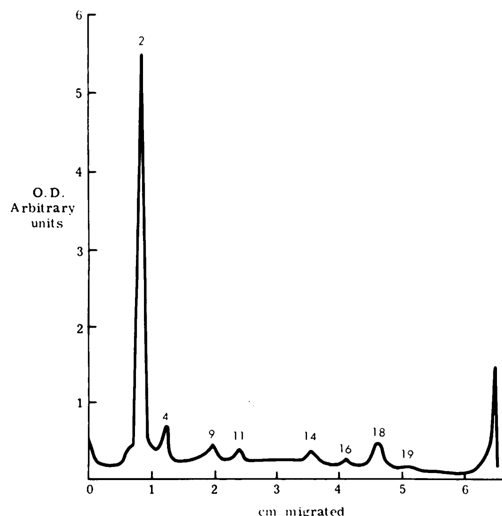

FIG. 5. *Electropherogram of naked nucleocapsid proteins prepared from infected HeLa cells 24 h postinfection. Electrophoresis was in 10% acrylamide-0.3% bisacrylamide. The profile shows the absorbance of Coomassie blue-staining material.*

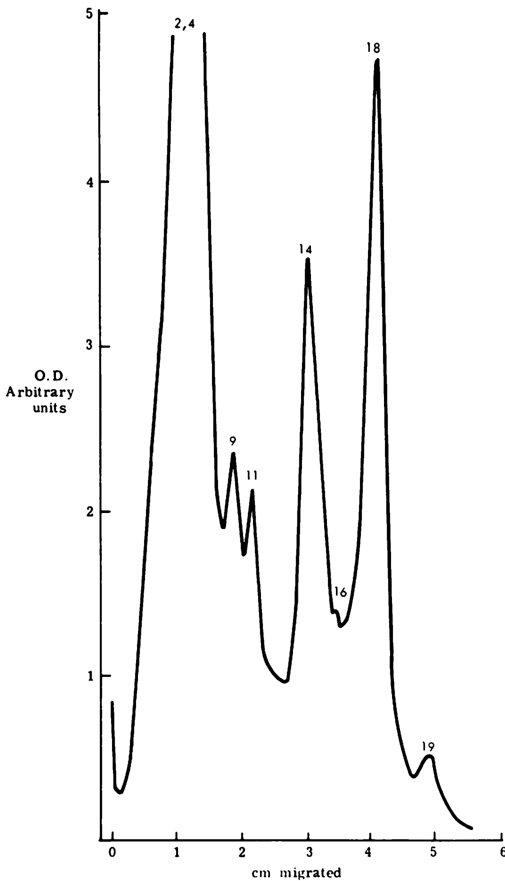

FIG. 6. *Long exposure autoradiogram of naked nucleocapsid proteins labeled with [^{35}S]methionine from 4 to 24 h postinfection. Electrophoresis was carried out in a 14% acrylamide-0.47% bisacrylamide disc gel.*

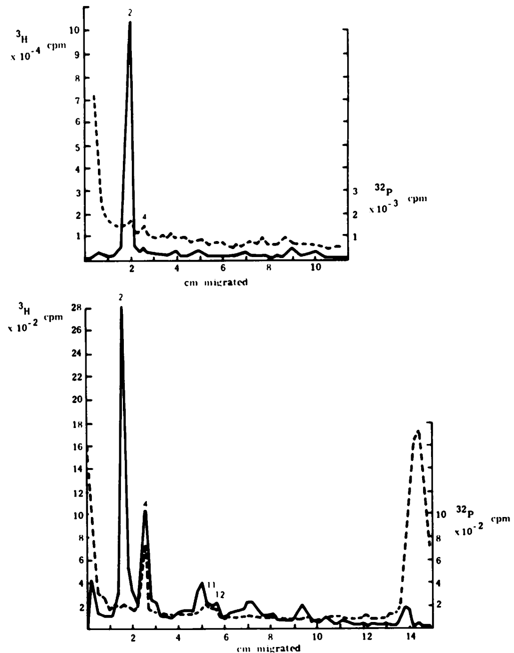

FIG. 7. *Electropherograms of naked nucleocapsid (a) and mature virus (b) proteins labeled with [methyl-^3H]methionine and ^{32}P-labeled inorganic phosphate from 4 to 24 h postinfection. The medium was phosphate free in addition to containing the normal reduced amount of methionine during the period of labeling. Samples were treated in 0.5% SDS to disrupt the particle structure then treated with DNase. After this treatment SDS was added to 3% and the samples were processed in the normal way. Electrophoresis was on 10% acrylamide-0.3% bisacrylamide. The electropherogram shows the radioactivity, as determined by scintillation counting, of 1-mm slices of the gels.*

virus are similar to those of other herpes viruses (5, 10, 11). The methods used have allowed better resolution of virion proteins than had previously been reported, and the simple pattern found for naked nucleocapsid also appears to be more in agreement with recent work.

One difference here is that only one protein with molecular weight greater than 160,000 was found. No combination of acrylamide and cross-linker concentration among the many that were used was able to resolve this species any further. It was noted that small amounts of unlabeled, Coomassie blue-staining proteins did occur in this area from time to time, suggesting nonspecific contamination with host material. The spectrum of such species varied from one preparation to another and from one cell line to another.

The significance of phosphorylated protein species in herpes virus preparations is not clear but is of some interest for purposes of comparison with the report of a similar finding for herpes simplex virus (6) that pseudorabies virus also shows some incorporation of ^{32}P in vivo.

ACKNOWLEDGMENTS

I thank R. M. S. Smellie for the provision of facilities, D. Mease for technical assistance, and I. Montgomery for assistance with the electron microscopy.

I am grateful to the Medical Research Council for financial support.

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