Arch Virol (1987) 94: 247–257

Archives of Virology

Induced Proteins in Cells Infected with Pseudorabies Virus

By

E. C. Hahn and Patricia S. Hahn

Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana-Champaign, Illinois, U.S.A.

With 3 Figures

Accepted November 7, 1986

Summary

New proteins appearing after infection of cultured L 929 cells with pseudorabies virus (PRV) were analyzed by SDS polyacrylamide gel electrophoresis. Analysis was facilitated by using a virus-cell system with marked inhibition of host protein synthesis after infection. Infected cells were pulsed during successive two hour periods through the infectious cycle with ^{35}S-methionine. Proteins were extracted with detergent and analyzed on high resolution reducing gels. Thirty-four protein bands were resolved on gels of different concentrations that varied from 7 to 15 percent. Calculated apparent molecular weights of the protein peaks were not dependent on gel concentration except for very large or small sized proteins. Eight glycoproteins were resolved after labeling with ^{14}C-glucosamine. The time course of incorporation of label was used as a measure of protein synthesis allowing the grouping of proteins according to the time of maximal synthesis. Several proteins shifted in MW during the course of infection, indicating possible post-translational cleavage or other minor modification.

Introduction

Pseudorabies virus (PRV) is a herpesvirus of pigs causing respiratory disease with frequent fatal infection of the central nervous system of young pigs (1, 6). Because of virulence of field strains, increased frequency of the disease, and latency, interest in the virus has grown. Although numerous publications have appeared that have characterized virion proteins (2, 23, 24) and certain viral proteins found within infected cells (3, 4, 13, 15, 18, 21), there has not been a systematic study of the intracellular proteins of this virus. This deficiency is, in part, due to technical difficulties in resolving viral

from cellular proteins, although PRV inhibits synthesis of cellular proteins (8). Often research groups differ in the molecular weight (MW) designation of particular proteins.

The number of PRV virion proteins that have been characterized has increased from 10 in early reports (23) to 20 with the application of techniques providing higher resolution (24). This number is still far below the theoretical coding capacity of PRV and below the number of virion proteins resolved for the related herpes simplex virus (10).

The importance of characterizing immunoreactive viral proteins and the current application of molecular cloning techniques to this virus (9, 16) require that the intracellular viral proteins must be better defined. We have examined the ^{35}S-methionine and ^{14}C-glucosamine labeled cytoplasmic proteins in PRV-infected mouse L 929 cells. In these cells PRV markedly inhibits cellular protein synthesis, facilitating designation of distinct intracellular viral protein peaks (ICPs). High resolution of proteins by SDS polyacrylamide gel electrophoresis was achieved by using N,N'-diallyltartardiamide as cross-linker and a mathematical method for calculating MWs as described elsewhere (7).

Materials and Methods

Virus and Cells

All cells were grown in Eagle's minimal essential medium (MEM) (GIBCO, Inc.) supplemented with 2 mM glutamine, 10 percent newborn calf serum, 200 units/ml penicillin, 0.2 μg streptomycin/ml, and buffered with sodium bicarbonate and HEPES. The Shope strain of pseudorabies virus (PRV) was replicated in porcine kidney (PK-15) cells. Stocks were prepared by infection of confluent monolayers at a multiplicity of infection (MOI) of 0.1 per cell. Virus was released from cells by freeze-thaw cycles and sonication. Stocks were clarified by centrifugation for 10 minutes at $1600 \times g$ and stored at −70° C. Titers of stocks which were used for these experiments were 1.11×10^8 and 9.1×10^7 $TCID_{50}$ per ml, calculated by the method of Reed and Muench (20).

Labeling of Viral Proteins

Confluent mouse L 929 cells in 100 mm Petri plates were infected with 1 ml sonicated PRV, diluted to achieve a MOI of 3. Adsorption was for 1 hour, after which the inoculum was removed. Medium MEM with 2 percent newborn calf serum was added, 5 ml/plate. At time = 0 hour one plate was decanted and MEM medium was added that contained one tenth of the usual concentration of methionine and 5 μCi/ml ^{35}S-methionine (Amersham, 1150 Ci/mmol). Pulses were made at 0–2, 2–4, 4–6, 6–8, and 2–8 hours after infection and 2–8 hours for uninfected cells. After a 2 hours pulse, cells were washed once with cold MEM and once with cold PBS. Cells were lyzed on the plate with triton X-100 lysis buffer: 1 percent triton X-100 in 0.05 M tris-HCl, pH 7.4, 0.1 M NaCl. Protease inhibitors (1 mM p-mercurobenzoate, 10 mM EDTA, and 1 mM 1-chloro-3-tosylamido-7-amino-L-2-heptanone) were present to prevent degradation of proteins as suggested (26). Lysis was for 5 minutes on ice with 0.2 ml followed by a similar wash with the same volume. Lysates were combined in 12 × 75 mm tubes and centrifuged for 10 minutes at $1500 \times g$ at 0° C. The supernatants were transferred to other tubes and 0.1 ml 10 percent SDS was added.

Samples received 25 μl of 2-mercaptoethanol and 50 μl of glycerol and were boiled for 2 minutes. For labeling of glycoproteins, cells were infected as above and incubated in MEM with 2 percent fetal bovine serum and half the usual glucose concentration. ^{14}C-glucosamine was added at a concentration of 2 μCi/ml (2.1 GBq/mmole) from 2 to 8 hours after infection.

Electrophoresis on DATD Gels

Samples were analyzed by high resolution SDS polyacrylamide gel electrophoresis on discontinuous slab gels (14). Complete description of the modified DATD gel system and the method of calculating MWs is provided in detail elsewhere (7).

Data Analysis

All univariate statistics, stepwise regression and analysis of variance within a linear regression model were performed with SAS 82.3 release (SAS Institute, Inc., Cary, NC) running on an IBM 4143 at the University of Illinois.

Results

Intracellular and Membrane PRV Proteins

Cytoplasmic and membrane-bound proteins were isolated at various times after infection of mouse L 929 cells with PRV. At the MOI of 3, intracellular virus appeared at 8 to 9 hours. All cells were uniformly susceptible. Intranuclear proteins were not analyzed because electrophoresis of these proteins is distorted by contaminating nucleoprotein. Proteins from control and infected cells at different times after infection were analyzed on gels with different concentrations of acrylamide (7 to 15 percent). The autoradiograph of a typical gel is shown in Fig. 1. The pattern of protein peaks from extracts of the uninfected cells (lanes 1 and 7) changed during the first hours of viral infection to a pattern that was largely comprised of new protein peaks (lane 2).

The viral inhibition of cellular protein synthesis was demonstrated by integrating areas of the more prominent control cell protein peaks throughout the PRV infectious cycle. The major contribution to this analysis was by cellular actin (MW 43 K). The results (data not shown) indicated that decay in cellular protein synthesis was exponential with a half-life of 2 hours. Regression analysis of the decay in synthesis of individual proteins indicated that PRV inhibited the major proteins in concert.

Analysis of PRV Proteins

A typical densitometric scan of PRV-infected cell proteins on SDS polyacrylamide gel electrophoresis is shown in Fig. 2. High MW proteins are on the left. Although not all proteins were resolved in this long labeling sample (2 to 8 hours post infection), the position of each is indicated.

A total of 33 lanes on 7 gels were analyzed for unique proteins after infection. Of all peaks analyzed, 34 were determined to be unique. The calculated MWs of the 34 new protein peaks are given in Table 1. Low frequencies for some proteins were attributable to their being found on only some of the lanes. The data include means, standard deviations of the means and coefficients of variation for the individual protein peaks from all gel concentrations at all times after infection. In general the coefficients of variation

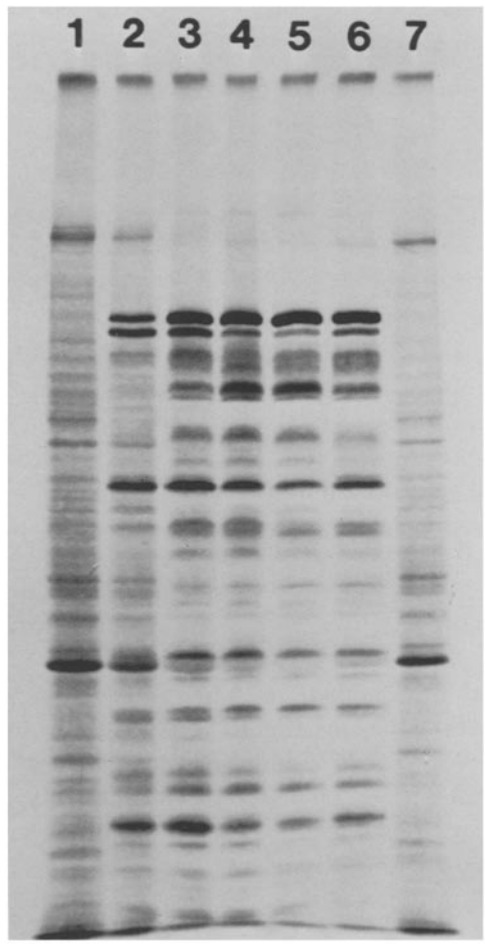

Fig. 1. Autoradiograph of PRV proteins separated by SDS polyacrylamide gel electrophoresis. Mouse L 929 cells were infected or mock infected with PRV at a multiplicity of 3 and labeled during various time periods with ^{35}S-methionine. Soluble cytoplasmic and membrane proteins were extracted in triton X-100 buffer and electrophoresed on a 9 percent acrylamide gel. *1* and *7* control cell proteins labeled for 6 hours. *6* contains proteins after PRV infection labeled for the same period. *2* through *5* pulse labeled proteins at 0–2, 2–4, 4–6, and 6–8 hours after infection

were between 2 and 5 percent of the means except at the extreme top and bottom of the MW scale. This variation was largely a result of gel effects related presumably to protein conformation and minor protein processing that occurred with time during the infectious cycle.

Relative migration of proteins should be a constant function of the concentration of acrylamide in the gels. The contribution of the gel concentration to the variation in apparent MW was largely seen with the very large and very small proteins. This relationship was analyzed for all the 34 presumed viral proteins. All proteins except ICP 8 behaved as expected under changing gel concentrations. The free mobility ($-100 \log 100\ R_f$ extrapolated to 0 percent acrylamide) of all proteins had a mean of 223.5 ± 7.4. Some of the variation seen in the calculated MWs (Table 1) could be related to slight changes in the MWs of particular protein peaks during the

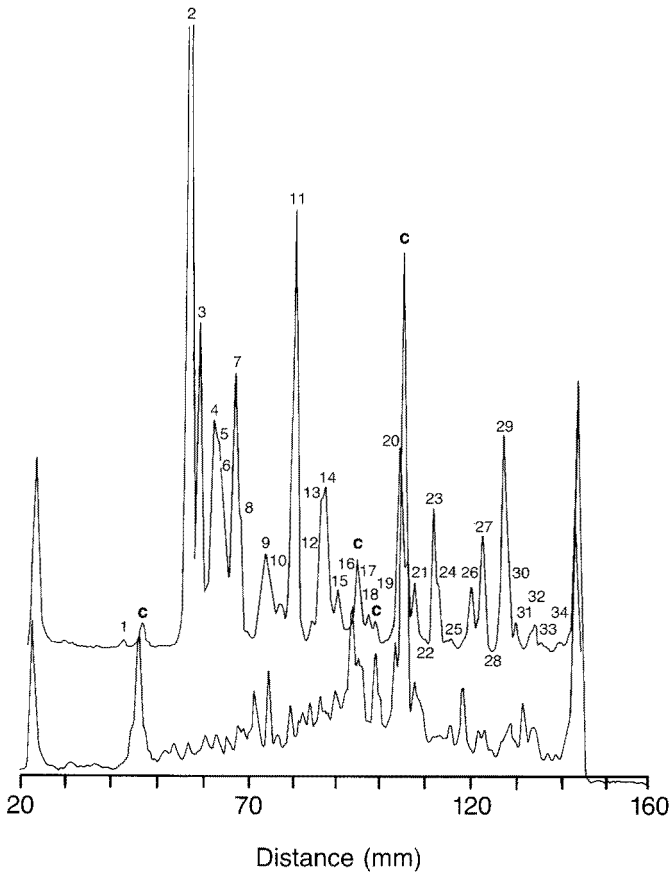

Fig. 2. PRV protein peaks. Scans of autoradiographs of infected and control wells are superimposed. Labeling was from 2–8 hours for controls and 4–6 hours for infected samples. Numbered viral proteins indicate the position of viral protein peaks on this and other gels

Table 1. *Intracellular proteins (ICP) after PRV infection*[a]

ICP	Freq.[b]	MW mean	STD[b]	CV[b]	Min	Max
1	19	272.1	18.2	6.7	232.5	300.6
2	28	146.0	8.7	5.9	137.0	172.6
3	23	134.8	4.3	3.2	128.7	144.4
4	27	121.1	2.6	2.1	114.8	125.6
5	25	116.3	2.6	2.2	111.0	120.1
6	10	113.4	3.5	3.1	109.7	120.5
7	29	106.3	2.2	2.1	103.0	110.5
8	25	101.0	3.6	3.5	94.0	107.7
9	32	87.4	2.5	2.8	82.7	93.1
10	29	80.8	2.1	2.6	76.4	84.5
11	33	74.5	2.2	3.0	70.6	78.2
12	27	68.0	3.7	5.5	61.6	73.5
13	30	65.4	3.8	5.9	58.0	71.3
14	21	64.6	3.1	4.8	57.7	69.5
15	29	61.0	3.1	5.0	54.2	65.3
16	16	58.1	2.9	5.0	51.1	62.6
17	19	55.0	1.7	3.1	51.7	58.0
18	28	53.7	1.5	2.9	50.4	56.0
19	17	49.5	1.1	2.1	47.3	51.0
20	31	47.0	1.3	2.9	44.5	49.3
21	32	45.0	1.7	3.8	40.5	48.2
22	25	44.0	1.3	3.0	41.9	46.6
23	33	41.9	1.3	3.0	38.7	44.0
24	27	40.9	1.0	2.5	39.4	42.4
25	24	38.8	0.9	2.4	37.0	41.2
26	28	36.1	0.6	2.1	34.7	37.8
27	27	34.4	0.9	2.5	33.0	36.2
28	10	33.5	0.8	2.3	31.8	34.5
29	23	31.4	0.8	2.4	30.4	33.2
30	21	29.8	0.9	3.0	27.9	31.7
31	23	27.4	1.2	4.3	23.9	29.5
32	18	25.0	1.7	6.8	21.1	27.3
33	18	23.6	1.7	7.9	20.4	26.0
34	20	22.1	1.8	8.1	19.6	25.3

[a] Mean apparent molecular weights based on measurements of relative migration of proteins on all gels and all times after infection. Summary data from all gels

[b] *Freq.* number of observations; *STD* standard deviation of the mean; *CV* coefficient of variation

infectious cycle. Because of the number of observations for each protein peak, it was possible to analyze the changes in MW during the infectious cycle by linear regression. Major changes in MW, determined by unbalanced analysis of variance ($p < .05$), indicated a significant shift in MW for ICP 8 (96.4 to 102.4). Other proteins that changed significantly were ICPs 5, 18, 21, and 24. In general, changes reflected early increases in MW followed by losses of MW in the range of 1000 to 2000 late in infection.

Integration of viral protein peaks during infection reflected the kinetics of synthesis. The proteins could be described by three patterns. Most proteins were maximally synthesized throughout the latter three-fourths of the infectious cycle. A second group of proteins reached maximal synthesis during the 2–4 hours time period and then stayed at that level. A small number of proteins were found early and then decreased with time (ICPs 12, 16–19, 21, 22, 25). Three ICPs (2, 7, 34) were synthesized maximally at 6–8 hours.

PRV Glycoproteins

The pattern of intracellular proteins that were labeled late in infection with ^{14}C-glucosamine is shown in Fig. 3. Five major and three minor peaks were resolved. The MWs of these eight protein peaks and the corresponding proteins from ^{35}S-methionine labeling experiments are given in Table 2.

The pattern of the glycoproteins corresponds with particular protein peaks labeled with ^{35}S-methionine in both position and amount as reflected in the area of the peaks with one exception. GP 6, a minor protein appearing

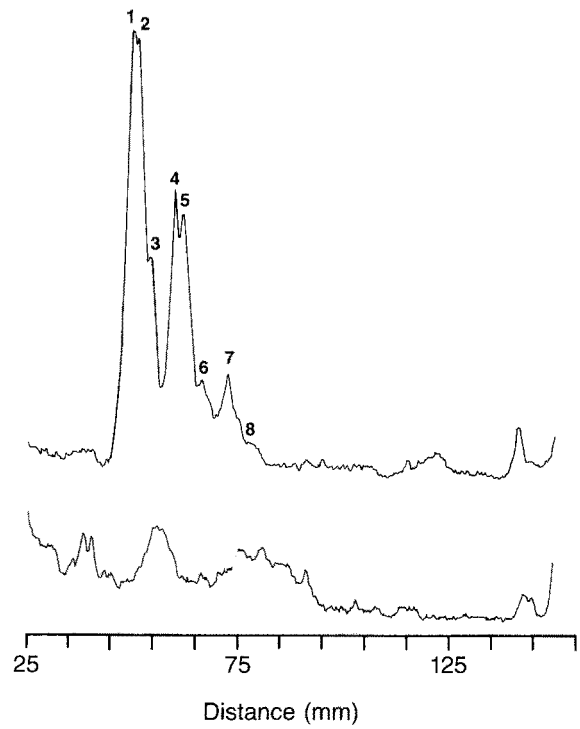

Fig. 3. Pseudorabies glycoproteins. Infected and control cells were labeled from 2–8 hours with ^{14}C-glucosamine. Proteins were extracted as above and electrophoresed on 11 percent gels. The scans of autoradiographs of both infected and control cells (below) scans are superimposed

Table 2. *PRV glycoproteins*[a]

Glycoprotein	GPMW[b]	ICP[c]	MW_{11}[c]	MW_{a11}[c]
GP 1	118	4	122.0	121.1 ± 2.6
GP 2	113	5	114.0	116.0 ± 2.6
GP 3	102	7	104.0	106.0 ± 2.2
GP 4	86	9	85.8	87.4 ± 2.5
GP 5	82	10	79.6	80.8 ± 2.1
GP 6	73	11	73.8	74.5 ± 2.2
GP 7	64	14	64.7	64.6 ± 3.1
GP 8	57	16	59.0	58.1 ± 2.9

[a] Infected and control cells were labeled from 2–8 hours with ^{14}C-glucosamine. Extracted proteins were electrophoresed on 11 percent gels. Glycoproteins were designated according to peaks indicated in Fig. 4

[b] Molecular weight as determined from ^{14}C-glucosamine gel

[c] Corresponding ICPs with mean molecular weights based on ^{35}S-methionine labelled proteins: (MW_{11}), from 11 percent gels (n = 5), and (MW_{a11}), from all gels (n = 27) (from Table 1)

after glucosamine labeling, corresponds to a major peak (ICP 11, 74.5 K) appearing with methionine labeling. The smallest glycoprotein, GP 8, is present in Fig. 3 as a shoulder. In other experiments with asynchronous labeling, this peak is more prominent. It corresponds in MW to ICP 16, an early protein.

Discussion

The results reported have described 34 new proteins, not found in uninfected cells. Although some of these may be induced cellular proteins, the large coding capacity of PRV and the inhibition of host protein synthesis would strongly suggest that the new protein peaks are coded by the virus. All but 8 of the new proteins increased in synthesis with time after infection in the face of the decline in synthesis of cellular proteins. Of particular interest as a precursor protein is ICP 1 (272 K). It is not one of the immediate-early proteins of which the largest is 185 K (21) or found in purified virions (24). The precision in the estimates of the MW of the new proteins is due in part to the high resolving capacity of the DATD cross-linked gels that form gradients during polymerization (7) and also due to the method of calculation. The majority of the error in MW estimates is due to the behavior of non-globular proteins on gels with high acrylamide concentration. It must be cautioned that MW determination by sieving technique results in what are apparent MWs. The 34 peaks that have been resolved represent only the more prominant proteins.

The validity of determining apparent MWs rests on an assumption that both unknowns and standards interact with the gel matrix equivalently. It is

generally accepted that conformity of both standards and unknowns to the Ferguson equation and similar free mobilities for standards and unknowns support this assumption (17, 22). All viral proteins except ICP 8 satisfied these criteria. The mean free mobility for the viral proteins (220 ± 8.1) was similar to that determined independently for the standards (223.5 ± 7.4) (7).

In earlier studies of PRV proteins it was indicated that cellular protein synthesis was reduced as reflected in a shift in amino acid composition from cellular to viral composition predicted by base ratio of viral nucleic acid (8). Our results indicate that cellular protein synthesis in L 929 cells decayed exponentially. Reexamination of data concerning inhibition of total cellular glycoprotein synthesis after infection (2) also yielded a linear regression of log (percent of control) on time after infection ($r = .928$, $p < .01$).

The discrepancy between the relative size of ICP 11 and GP 6 may reflect that there are two polypeptides with MWs close to 74 K, one minor, glycosylated protein and a major non-glycosylated polypeptide. Alternatively, the difference in the size of these peaks could be due to a difference in the ratio of glucosamine to methionine of this peak relative to other glycoproteins.

In a systematic study of PRV virion proteins using DATD gels (24), 20 proteins were designated. Many of these virion proteins are similar in MW to the intracellular proteins described above. Using SDS polyacrylamide gel electrophoresis where gels were cross-linked with N,N′-methylenebisacrylamide, early studies (2, 9) have characterized four viral glycoproteins. However, analysis by crossed-immunoelectrophoresis with cell culture-derived PRV has demonstrated 20 reactive proteins including eight glycosylated polypeptides (24). More recently, seven glycoprotein monomers have been described for PRV (9). Differences in estimated MW of all but the three smallest of these were apparent when our results were compared. The related bovine herpesvirus-1 produces at least 11 glycoproteins (25).

Analysis of the time of maximal synthesis of each protein indicated three periods of expression in agreement with results obtained with herpes simplex virus (11). Our data do not allow classification of PRV protein synthesis into five classes as has been achieved by DEATLY and BEN-PORAT (5).

The observed changes in MW of certain proteins during the infectious cycle might be due to either proteolysis or post-translational modification. An argument for the latter concept is that the MW of several proteins (ICPs 4, 17, 19, 26, 29, 34) was very constant throughout the cycle.

The ability to reproducibly resolve intracellular viral proteins of PRV will be an important tool for studies of protein processing, application of monoclonal reagents and molecular pathogenesis.

Acknowledgements

The authors thank Marion Sarmiento for her suggestions in the early development of this work. This study was supported in part by grants from the Illinois State Department of Agriculture and Project No. ILLU 70-0974 of the of Agricultural Experiment Station, University of Illinois at Urbana-Champaign.

References

1. Baskerville A, McFerran JB, Dow C (1973) Aujeszky's disease in pigs. Vet Bull 43: 465–480
2. Ben-Porat T, Kaplan AS (1970) Synthesis of proteins in cells infected with herpesvirus V. Viral glycoproteins. Virology 41: 265–273
3. Ben-Porat T, Kervina M, Kaplan AS (1975) Early functions of the genome of herpesvirus V. Serological analysis of "immediate-early" proteins. Virology 65: 355–362
4. Ben-Porat T, Shimono H, Kaplan AS (1970) Synthesis of proteins in cells infected with herpesvirus IV. Analysis of the proteins in viral particles isolated from the cytoplasm and nucleus. Virology 41: 256–264
5. Deatly AM, Ben-Porat T (1985) Relation between the levels of mRNA abundance and kinetics of protein synthesis in pseudorabies virus-infected cells. Virology 143: 558–568
6. Gustafson DP (1975) Pseudorabies. In: Dunne HW, Leman AD (eds) Diseases of swine, 4th edn. Iowa State University Press, Ames
7. Hahn EC, Hahn PS (1986) Properties of acrylamide gels cross-linked with low concentrations of N,N'-diallyltartardiamide. J Virol Methods 15
8. Hamada C, Kaplan AS (1965) Kinetics of synthesis of various types of antigenic proteins in cells infected with pseudorabies virus. J Bacteriol 89: 1328–1334
9. Hampl H, Ben-Porat T, Ehrlicher L, Habermehl K-O, Kaplan AS (1984) Characterization of the envelope proteins of pseudorabies virus. J Virol 523: 583–590
10. Heine JW, Honess RW, Cassai E, Roizman B (1974) Proteins specified by herpes simplex virus XII. The virion polypeptides. J Virol 143: 640–651
11. Honess RW, Roizman B (1974) Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol 14: 8–19
12. Kaplan AS, Shimono H, Ben-Porat T (1970) Synthesis of proteins in cells infected with herpesvirus III. Relative amino acid content of various proteins formed after infection. Virology 40: 90–101
13. Kerr CL, Pennington TH (1984) The effect of monensin on virion production and protein secretion in pseudorabies virus-infected cells. J Gen Virol 65: 1033–1041
14. Lämmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T 4. Nature 27: 680–685
15. Ladin BF, Ihara S, Hampl H, Ben-Porat T (1982) Pathway of assembly of herpesvirus capsids: an analysis using DNA^+ temperature-sensitive mutants of pseudorabies virus. Virology 116: 544–561
16. Mettenleiter TC, Lukacs N, Rziha H-J (1985) Mapping of the structural gene of pseudorabies virus glycoprotein A and identification of two non-glycosylated precursor polypeptides. J Virol 53: 52–57
17. Neville DM Jr. (1971) Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J Biol Chem 246: 6328–6334
18. Pennington TH, McCrae MA (1971) Processing of a pseudorabies virus-induced protein which is glycosylated, sulfated and excreted. J Gen Virol 34: 155–165

19. Platt KB (1984) The porcine humoral response to detergent extracted Aujeszky's disease (pseudorabies) virus antigens. Vet Microbiol 9: 35–51
20. Reed L, Muench H (1938) A simple method of determining 50 percent endpoints. Am J Hyg 27: 493–497
21. Rixon FJ, Feldman LT, Ben-Porat T (1980) Expression of the genome of defective interfering pseudorabies virions in the presence or absence of helper functions provided by standard virus. J Gen Virol 46: 119–138
22. Rodbard D, Chrambach A (1970) Unified theory for gel electrophoresis and gel filtration. Proc Natl Acad Sci USA 65: 970–977
23. Shimono H, Ben-Porat T, Kaplan AS (1969) Synthesis of proteins in cells infected with herpesvirus I. Structural viral proteins. Virology 37: 49–55
24. Stevely WS (1975) Virus-induced proteins in pseudorabies-infected cells II. Proteins of the virion and nucleocapsid. J Virol 16: 944–950
25. van Drunen Littel-van den Hurk S, Babiuk LA (1986) Synthesis and processing of bovine herpesvirus 1 glycoproteins. J Virology 59: 401–410
26. Zezulak KM, Spear PG (1984) Limited proteolysis of herpes simplex virus glycoproteins that occurs during their extraction from Vero cells. J Virol 50: 258–262

Authors' address: Dr. E. C. Hahn, College of Veterinary Medicine, Department of Veterinary Pathobiology, University of Illinois, 2001 South Lincoln Avenue, Urbana, IL 61801, U.S.A.

Received September 9, 1986