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Synthesis of Proteins in Cells Infected with Herpesvirus, VI. Characterization of the Proteins of the Viral Membrane*

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Abstract. The envelope of pseudorabies virions can be removed by treatment with Triton X-100, leaving the nucleocapsid intact. The viral envelope removed in this fashion contains all the glycoproteins and about 40 per cent of the proteins of the virions. Analysis by polyacrylamide gel electrophoresis revealed that the envelope proteins migrate as four distinct peaks, all of which contain glycoproteins. The proteins of the nucleocapsids migrate in the gels as six distinct peaks.

Introduction. Viruses of the herpes group, of which pseudorabies virus is a typical member, are composed of three different types of morphological structures—(1) the inner core, which contains the genome of the virus, (2) the capsid, which is composed of 162 capsomeres that surround the core, and (3) the outer envelope.[1] We have been analyzing pseudorabies virions in an effort to characterize the proteins in each of these structures.[2–6]

Electrophoretic analysis in polyacrylamide gels of pseudorabies virions, disassembled with sodium dodecyl sulfate, revealed that this virus is composed of proteins which migrate as seven distinct peaks.[4] The proteins in these peaks differ with respect to their molecular weight and amino acid composition[4] and four of these peaks contain glycoproteins.[6] Glycoproteins associated with the nuclei of pseudorabies virus-infected cells show a pattern of electrophoretic migration in polyacrylamide gels similar to that of the viral glycoproteins.[6] Since the outer viral membrane is acquired by the virion from the nuclear membrane,[7–10] it seemed likely that the two types of glycoproteins are identical and that the glycoproteins found associated with the virions constitute part of its outer envelope. Evidence for the latter hypothesis is provided by the experiments in this paper.

Materials and Methods. Virus and cell culture: The properties of pseudorabies virus and cultivation of rabbit kidney monolayer cultures were described previously.[1,11] Primary rabbit kidney cells were grown in 90-mm Petri dishes in EDS.

Media and solutions: *EDS:* Eagle's synthetic medium[12] plus dialyzed bovine serum (3%). *EDS/2:* The same as EDS, except that the concentration of amino acids was reduced half the normal amount. *TBS:* A buffer containing the same salts as PBS, except that the phosphate is replaced by Tris-HCl, 0.01 M, pH 7.5. *TBSA:* The same as TBS plus 1% crystalline serum albumin.

Chemicals: ^{14}C-leucine (spec. act. 316 mCi/mmole), ^{3}H-leucine (spec. act. 40

Ci/mmol), ^{14}C-arginine (spec. act. 220 mCi/mmol), and ^{3}H-arginine (spec. act. 45 Ci/mmol) were purchased from Schwarz BioResearch. ^{3}H-thymidine (spec. act. 5 Ci/mmol) was purchased from the New England Nuclear Corp., and ^{3}H-glucosamine (spec. act. 1.9 Ci/mmol) was purchased from Tracerlab. Triton X-100 was a gift from Rohm and Haas Co.

Labeling of pseudorabies virus: Confluent monolayers of rabbit kidney cells were infected (adsorbed multiplicity, 5) and incubated for 16 hr in EDS/2 containing one of the following: ^{14}C-leucine (2 μCi/ml), ^{3}H-leucine (20 μCi/ml), ^{14}C-arginine (2 μCi/ml), ^{3}H-arginine (20 μCi/ml), ^{3}H-thymidine (10 μCi/ml), ^{3}H-glucosamine (30 μCi/ml), or a combination of one ^{14}C- and one ^{3}H-labeled compound, as indicated in each experiment.

Purification of pseudorabies virus: This was done as described previously,[5] with minor modifications. The culture medium, containing extracellular virus, was collected, clarified by centrifugation at 5000 rpm for 10 min in a Sorvall SS-1 rotor, and the virus was sedimented on a 70% sucrose cushion by centrifugation at 13,500 rpm for 1 hr in the Spinco SW-25 rotor. The virus was then collected and further purified by centrifugation in sucrose gradients as described previously.[5]

Treatment with Triton X-100: The purified virus obtained from the sucrose gradients was diluted with TBSA, so that the concentration of sucrose was decreased to 10% and the pH was adjusted to 8.5. Triton was added, final concentration 0.2% (0.5% Triton produced the same result), and the samples were incubated at 45°C for 10 min. Controls without Triton were treated identically. The samples were then centrifuged in 15–30% sucrose gradients, in TBSA, pH 8.5, as described previously.[5]

Preparation of samples for electrophoresis: The purified Triton-treated and untreated particles were layered on sucrose gradients (15–30%) and centrifuged at 15,000 rpm for 2 hr in the SW-25 rotor. The pelleted particles were resuspended in TBS and the proteins prepared for electrophoresis, as described previously.[4] The proteins remaining at the top of the gradients after treatment of particles with detergent were collected and were concentrated with Carbowax before dialysis. Electrophoresis, fractionation of the gels, and the assay of radioactivity were performed as described earlier.[4]

Electron microscopy: The viral particles were centrifuged in a sucrose gradient (15–30%; TBSA, pH 8.5) to the bottom of the tubes and, after the pellets were washed with distilled water to remove the sucrose, the particles were resuspended in distilled water and stained with phosphotungstic acid, according to the method of Brenner and Horne.[13]

Results. Effect of Triton on pseudorabies virions: Successful isolation of the nucleocapsids after removal of the membrane depends on two factors—the composition and the pH of the medium. Unless optimal conditions are used, the virions seem to disintegrate after treatment with the detergent. The optimal conditions for stabilization of the nucleocapsid were determined as described in Table 1. Virus labeled with ^{3}H-thymidine was treated with Triton under various conditions and the amount of label that sedimented in sucrose gradients to a position close to that of intact virions was determined. (In all the gradients, counts were found only in this position and at the top of the gradients.)

It is clear that the nucleocapsids are unstable at low pH and that the presence of both sucrose and albumin in the suspending medium contributes to the stabilization of these structures. In all subsequent experiments, therefore, virus was suspended in sucrose dissolved in TBSA buffered at pH 8.5 (see *Materials and Methods*).

^{3}H-leucine-labeled pseudorabies virions, purified as described in *Materials and Methods*, were treated with Triton and mixed with ^{14}C-leucine-labeled untreated virions and their behavior in sucrose gradients was compared. Figure

TABLE 1. *Effect of treatment with Triton under various conditions on the integrity of the nucleocapsid.*

Medium	pH	Triton-treated (cpm/sample)	Untreated
TBSA	6.5	220	1354
10% sucrose	7.0	495	ND*
	7.5	679	ND
	8.0	965	ND
	8.5	1395	1430
TBS	8.5	690	1322
10% sucrose			
TBSA	7.5	595	ND
	8.5	1195	1464
TBS	7.5	320	ND
	8.5	590	1295

^{3}H-thymidine-labeled, partially purified pseudorabies virus was suspended in the indicated medium, treated with Triton, and sedimented in a sucrose gradient, as described in *Materials and Methods*. The number of counts sedimenting in the gradient to a position close to that of infectious virus was determined.

* ND, not done.

1 shows that after treatment with Triton, the movement of the particles through the gradients was altered significantly; the treated particles moved more slowly than the untreated ones. A relatively large amount (approx. 50%) of the viral proteins was solubilized by treatment with Triton and remained at the top of the gradient.

Figure 2 shows the results of an experiment in which virions doubly labeled with either ^{3}H-glucosamine and ^{14}C-leucine or with ^{3}H-thymidine and ^{14}C-leucine were treated with Triton and then centrifuged in sucrose gradients. Practically all the glycoproteins (glucosamine is incorporated mainly into the glycoproteins of the virions[6]) were removed from the particles by treatment with Triton (Fig. 2*b*); the viral DNA was, however, unaffected and remained associated with the particles (Fig. 2*d*). Thus, treatment with Triton removes from the viral particles all the glycoproteins, about half the proteins, and none of the DNA.

The exact amount of viral protein removed by treatment with Triton can be calculated from the ratio of ^{14}C-leucine to ^{3}H-thymidine in the untreated and in

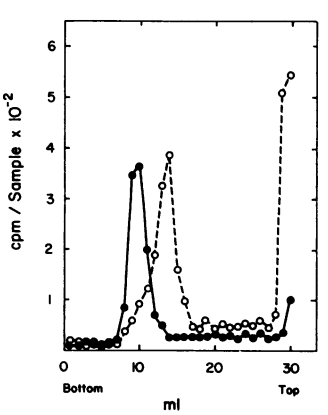

FIG. 1.—Sedimentation in sucrose gradients of Triton-treated and untreated virions. ^{3}H-leucine-labeled, purified pseudorabies virions were prepared and treated with Triton, as described in *Materials and Methods*. The sample was chilled, mixed with a preparation of ^{14}C-leucine-labeled virions, and centrifuged in a sucrose gradient. Fractions were collected and the radioactivity in each was determined. (—○—), triton-treated; (—●—), untreated.

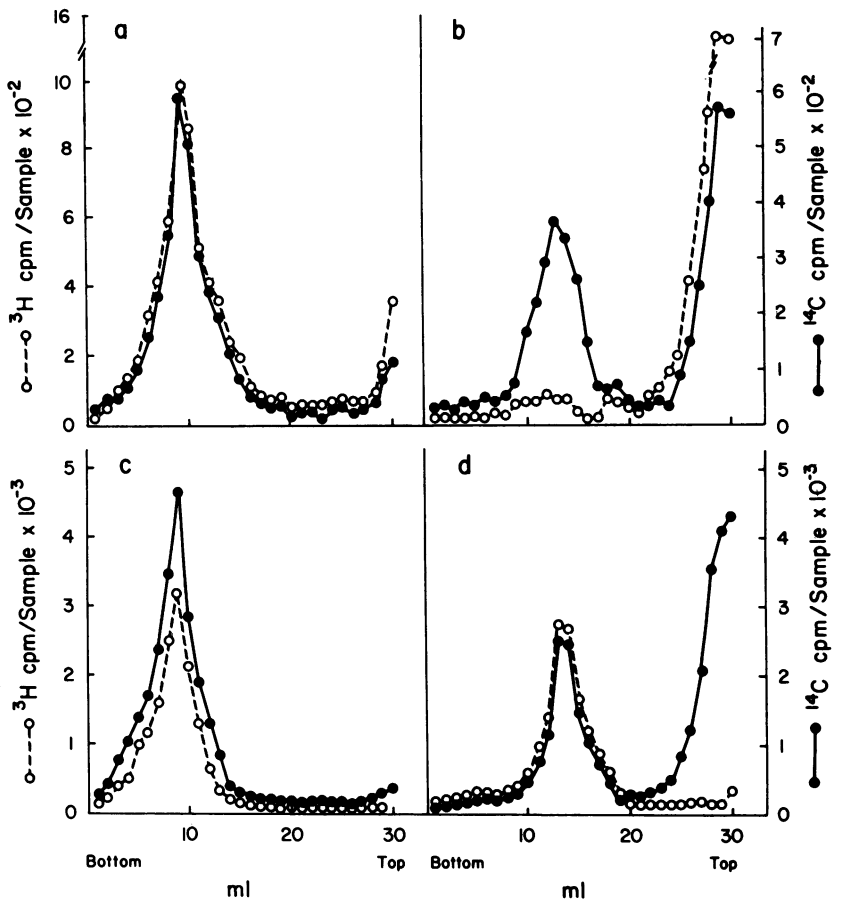

FIG. 2.—Amount of DNA, protein, and glycoprotein that remains associated with viral particles after treatment with Triton. Purified particles of pseudorabies virus labeled with ^{3}H-glucosamine and ^{14}C-leucine (panels *a* and *b*) or with ^{3}H-thymidine and ^{14}C-leucine (panels *c* and *d*) were prepared as described in *Materials and Methods*. Part of the samples was treated with Triton (*b* and *d*), and part was not (*a* and *c*). The samples were centrifuged in sucrose gradients, and the amount of radioactivity in the various fractions of the gradient was determined.

the Triton-treated virions. This ratio (^{14}C/^{3}H) in the untreated particles was 1.46 (Fig. 2*c*) and in the treated particles, 0.92 (Fig. 2*d*). Since no DNA (^{3}H) was lost from the particles upon treatment with Triton, it can be deduced from the difference in ratios that 37% of the viral proteins was removed from the virions by Triton.

Electron microscopy of Triton-treated and untreated viral particles: Figure 3 provides visual evidence that the viral membrane has been removed from the virions by Triton. In both the untreated and Triton-treated preparations, some of the particles appeared empty after being stained with phosphotungstic acid. However, this is probably an artifact resulting from the staining technique; thin sections of pellets obtained by high-speed centrifugation of the same viral

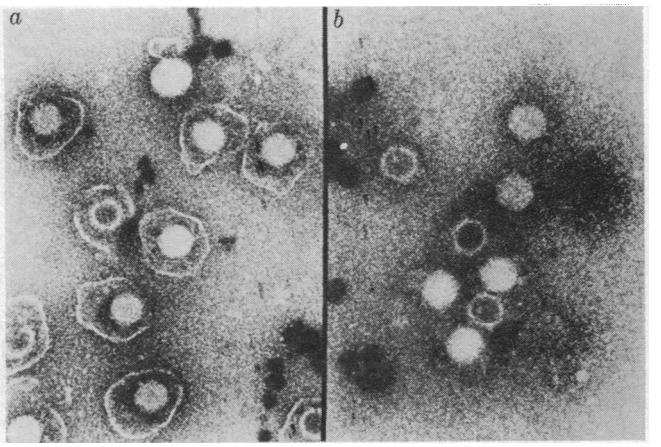

FIG. 3.—Electronmicrographs of Triton-treated and untreated viral particles.
(*a*) Untreated.
(*b*) Triton-treated.

preparations were examined in the electron microscope and were found to be composed of particles containing an electron-dense core. We conclude, therefore, that under the conditions used, treatment with Triton removes selectively the envelope proteins and leaves the nucleocapsids intact.

Polyacrylamide gel electrophoretic analysis of the proteins of the envelope and of the nucleocapsids: Figure 4 shows an electrophoretogram of the proteins remaining at the top of the gradients after treatment of ^{3}H-leucine-labeled purified virions with Triton (envelope proteins). In contrast to intact ^{14}C-labeled virus (added to the sample as an internal marker), which contains proteins migrating as seven distinct peaks, only proteins migrating as peaks 3, 4, 4*a*, and 6 are found in the preparation containing the envelope proteins.

Figure 5 shows the pattern of electrophoretic migration of proteins present in intact virions and in the nucleocapsids. As shown previously,[6] four of the seven protein peaks present in intact virus (Fig. 5*b*) contain glycoproteins (peaks 3, 4, 4*a*, and 6). The nucleocapsids (Fig. 5*a*) do not contain any appreciable amounts of glycoproteins (note the difference in the ^{3}H scale between panels *a* and *b*). The amounts of leucine-labeled proteins migrating to positions 3, 4*a*, and 6 also seem to be reduced relative to the proteins migrating as peaks 2, 7, and 8; how-

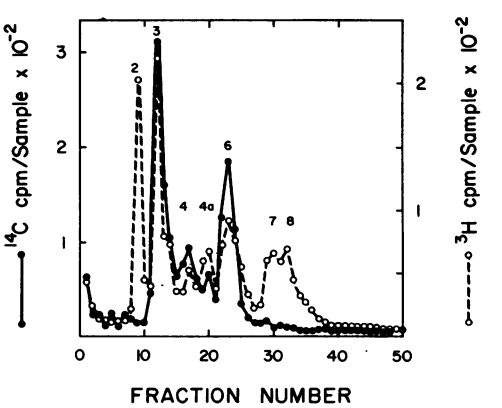

FIG. 4.—Electrophoretogram of the envelope proteins. Purified pseudorabies virions labeled with ^{14}C-arginine were treated with Triton and centrifuged in sucrose gradients. The proteins remaining on the top of the gradient were collected, concentrated, mixed with the proteins of intact ^{3}H-arginine-labeled virions, and electrophoresed in polyacrylamide gels.

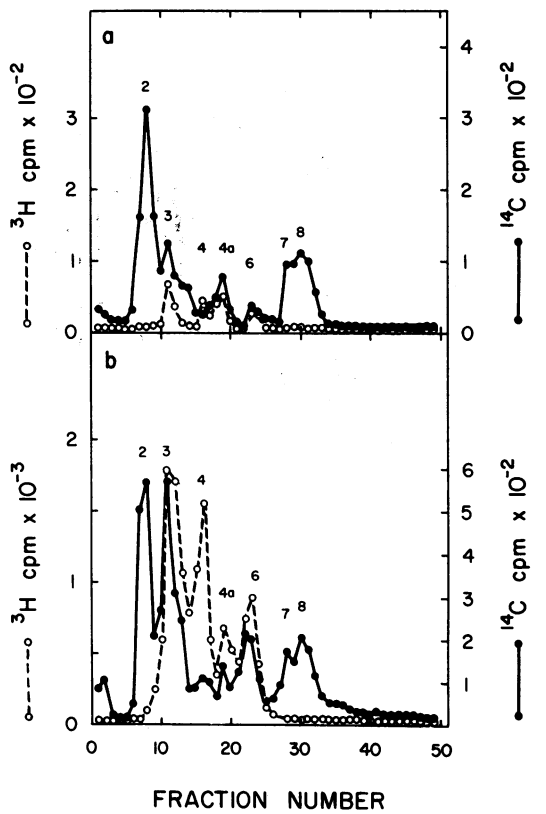

FIG. 5.—Electrophoretograms of the proteins in the nucleocapsids. Purified pseudorabies virions labeled with ^{3}H-glucosamine and ^{14}C-leucine were treated with Triton (*a*) or were not treated (*b*). The particles were collected after centrifugation in sucrose gradients, and the proteins were electrophoresed in polyacrylamide gels.

ever, a significant amount of these proteins is still present. Peak 4 is not detectable in the ^{14}C-leucine-labeled nucleocapsids.

The relative amount of proteins migrating as peaks 3, 4*a*, and 6 that remains associated with the nucleocapsid was ascertained by coelectrophoresing intact ^{14}C-leucine-labeled virions with ^{3}H-leucine-labeled nucleocapsids. The ratio of ^{3}H/^{14}C in the proteins in peak 2 (proteins which are not removed from the particles by treatment with Triton (see Fig. 4)) was taken as 1 and the ratios in the other protein peaks was expressed as a fraction of that ratio. The results (Table 2) show that about 20–30% of the proteins of both peaks 3 and 6 and about 70% of the proteins of peak 4*a* remain associated with the nucleocapsid. The fact that the nucleocapsids contain leucine-labeled proteins migrating as peaks 3, 4*a*,

TABLE 2. *Distribution among the different peaks of the proteins of nucleocapsids and of intact virions.*

	Peak Number					
Expt.	2	3	4*a*	6	7	8
1	1.00	0.28	0.70	0.27	1.00	0.95
2	1.00	0.31	0.78	0.22	1.05	1.00

Intact virions labeled with ^{14}C-leucine and nucleocapsids labeled with ^{3}H-leucine were prepared as described in *Materials and Methods*. The two were mixed and the proteins prepared for polyacrylamide gel electrophoresis. The ratio of ^{3}H/^{14}C in the proteins migrating to peak 2 was taken as unity. The ratios in the other protein peaks were expressed as a fraction of the ratio in peak 2.

and 6, but none of the glycoproteins migrating to the same positions in the gel, indicates that the viral proteins contain at least two proteins that migrate to these positions; one of the two proteins in each peak is a glycoprotein and a component of the viral envelope.

Discussion. The experiments in this paper show that the outer membrane of pseudorabies virions is a complex structure containing at least four different glycoproteins. This confirms our previous conclusion,[6] based on indirect evidence, that these proteins are components of the membrane. There is increasing evidence that the glycoproteins of animal cells are found principally in membranes.[14] It is interesting that all four of the protein peaks isolated from the viral membrane contain glycoproteins. Whether the membrane also contains other nonglycosylated proteins migrating coincidentally with the glycoproteins is at present unknown.

Electron microscopic evidence suggests that the outer membrane of the herpes viruses is derived from the nuclear membrane.[7-10] However, experiments with ferritin-tagged antibody showed that both the nuclear and cytoplasmic membranes of herpes simplex virus-infected cells contain viral antigens.[15] The glycoproteins associated with the membranes of the virions are present in the cytoplasm of infected cells in small amounts only, if at all.[6] It is clear, therefore, that the antigenically altered membranes in the cytoplasm of the infected cells are not identical with the structural membranes of pseudorabies virus. This finding differs from that reported recently,[16] so that in this respect it seems that infection with pseudorabies virus may differ from infection with some strains of herpes simplex virus.

The role of the viral membrane in the biology of the herpes viruses has been a subject of discussion for some time.[1] The availability of a method which removes the membrane and leaves the nucleocapsids intact should allow us to acquire clear-cut information on this issue.

We have shown previously[4] that when the proteins of pseudorabies virions are separated by sodium dodecyl sulfate-gel electrophoresis, seven distinct peaks of proteins are obtained. The proteins in these peaks differ from each other in molecular weight and in amino acid composition. The present paper shows that three of these peaks contain at least two proteins; this raises the minimal number of structural viral proteins to 10. We are now in a position to attempt to assign these proteins to the different viral structures. We know now that the viral envelope contains proteins migrating to the peaks designated as 3, 4, 4*a*, and 6. A comparison of the proteins in coreless particles which can be isolated from the nucleus[5,17] and the proteins in the nucleocapsids suggests that the proteins migrating to peaks 4*a*, and probably also 3 and 6, consist at least partially of core proteins. We are now in the process of attempting to purify the viral cores and to identify the characteristics of the proteins associated with these structures.

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