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Protein Composition of the Structural Components of Vesicular Stomatitis Virus

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Digitonin, a sterol glycoside which complexes with cholesterol, stripped off the envelope of vesicular stomatitis (VS) virions and liberated two viral structural proteins, 83% of P6 and 53% of P4. Deoxycholate also disrupted VS virions but released nucleocapsid cores which could be identified by higher buoyant density, ratio of incorporated ^{3}H-uridine to ^{14}C-protein, and electron microscopy. The major nucleocapsid protein was P5 but varying amounts of the minor protein aggregate P2 were present, depending on the concentration of urea used for extraction. P2 appeared to be a polymer of P5. Two other minor structural proteins, P1 and P3, could not be located in the virion. From these data, we conclude that the three microscopically identifiable structures of VS virions are each composed primarily of a single major protein, as follows: P6 = envelope protein, P4 = protein of underlying "shell," and P5 = nucleocapsid protein.

In the preceding paper (10) the structural proteins of vesicular stomatitis (VS) viruses were characterized by polyacrylamide gel electrophoresis after dissolution of purified virions with acetic acid, sodium dodecyl sulfate (SDS), 0.5 M urea, and 2-mercaptoethanol. Three major proteins were identified and designated P4, P5, and P6. In addition, two consistent minor stainable bands and coincident peaks of radioactivity (P2 and P3) appeared to be aggregates of the major proteins on the basis of their susceptibility to 8 M urea. Another minor protein, P1, was resistant to fractionation by 8 M urea.

This report describes experiments designed to locate these VS viral proteins in the microscopically identifiable structural components of the virion.

MATERIALS AND METHODS

The materials and basic procedures have been described in detail in the preceding report (10) and are outlined below.

Production and purification of labeled virus. Stock preparations of the Indiana serotype of VS virus were grown in 10 monolayer cultures of L cells, $\sim 1.8 \times 10^{7}$ cells per monolayer. Virus yields in 17 hr were approximately 5×10^{10} plaque-forming units in 100 ml of medium. Viral proteins were labeled by adding to special basal medium, Eagle's (BME) 10 μc/ml each of ^{3}H-leucine (10.3 to 14.7 c/mmole) and ^{3}H-tyrosine (28.2 to 43 c/mmole) or 1.13 μc/ml of uniformly labeled ^{14}C-amino acids (54 mc/matom). In several experiments in which the proteins were labeled with ^{14}C-amino acids, viral RNA was labeled with 10 μc/ml of ^{3}H-uridine (20.0 c/mmole) added to the same culture media but containing actinomycin (0.33 μg/ml). Batches (100 ml) of labeled crude virus were partially purified by differential centrifugation and were concentrated to 0.5 or 1.0 ml. The concentrated virus was then purified by rate zonal centrifugation in 0 to 40% linear sucrose gradients and then by isopycnic sedimentation in CsCl (*see* 10).

Electron microscopy. Specimens were examined and photographed in Siemens-Halske Elmiskop I microscopes by John W. Greenawalt and Glenn Decker at Johns Hopkins University or by Carl A. Schnaitman at the University of Virginia. Purified virions or fractionated viral components were negatively stained with phosphotungstic acid (PTA), as previously described (3). In some cases the viral components were dialyzed overnight against 2% PTA in distilled water before being placed on Formvar-covered grids reinforced with carbon. Pelleted virions were fixed in glutaraldehyde and OsO_4, embedded in epoxy resin, sectioned with a diamond knife, and stained with uranyl acetate and lead citrate.

Polyacrylamide gel electrophoresis. The method of Maizel (5) was used with only minor modifications. In brief, viral proteins (2.5 to 3.5 mg/ml) were solubilized by addition of a tenth volume of glacial acetic acid and then made 0.5 or 8 M with respect to urea and 1% with respect to SDS. After incubation at 37 C for 1 hr, the suspension was then dialyzed at room temperature for 16 to 18 hr against 200 to 400 ml of 0.01 M phosphate buffer (*p*H 7.2) containing 0.1% SDS, 0.5 or 8 M urea, and 0.1% 2-mercaptoethanol. Protein samples of 0.1 to 0.2 ml with added bromophenol blue were layered with sucrose on 7.5% polyacrylamide gels containing 0.1 M phosphate (*p*H 7.2), 0.5 M urea, and 0.1% SDS. Electrophoresis was carried out at

5 ma/gel for 5.5 hr. Gels stained with Coomassie blue were scanned for absorption peaks at 610 nm with the aid of a linear transport attachment to the Gilford 240 spectrophotometer. The stained gels were then sliced transversely into sections of 1.25 mm each; the gel slices were depolymerized with 30% H_2O_2, dissolved in *N*-chlorosuccinimide (NCS), and diluted in toluene-based fluors. The radioactivity was counted in a scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Structure of VS virions. Several groups of investigators have described the structural components of VS virions in some detail (3, 6, 7, 9). All microscopists appear to agree on the presence of a surface membranous structure or envelope with protruding spikes. In addition, an internal component, appearing as closely spaced striations in intact virions, can be released as a coiled or partially uncoiled helix, which probably represents the nucleoprotein core. There is some disagreement about the length and origin of the coils and about the presence or absence of a structure between the core and the envelope.

Figure 1A shows the structural components of negatively stained VS virions at various stages of disorganization. Three distinct structures can be seen. Figure 1B also reveals at least three concentric layers in virions sectioned transversely and stained with uranyl acetate and lead. These observations lead us to confirm that VS virions are cylinders composed of (i) an outer envelope with spikes, (ii) a rigid "shell" underneath the surrounding envelope, and (iii) an inner, packed nucleoprotein coil.

Fractionation of VS virions with digitonin. VS virions mature at the cell surface, bud from the cytoplasmic membrane, and appear to have a high content of lipid, particularly cholesterol, which is almost undoubtedly associated with the envelope (7, 9). James McSharry of this department (*unpublished data*) confirmed the presence of large amounts of cholesterol and observed that ^{3}H-cholesterol is rapidly incorporated during growth of VS virus; the ^{3}H label was retained by VS virions after extensive purification. These findings led us to believe that the envelope and its associated proteins could be stripped off by treatment with the sterol glycoside, digitonin. Digitonin complexes with cholesterol (2) and has been used successfully by Schnaitman et al. (8) to dissociate the outer and inner membranes of mitochondria. A similar technique was used by de-Thé (1) to remove the envelope of Rauscher leukemia virus from its nucleocapsid core.

Infectious B virions of VS virus labeled with ^{14}C-amino acids were purified by rate zonal and equilibrium sedimentation. By the procedure of Schnaitman et al. (8), crystalline digitonin (Calbiochem, Los Angeles, Calif.) was added to the viral suspension in a ratio of 0.4 mg of digitonin to 1 mg of viral protein. After incubation at 4 C for 10 min, the virus-digitonin mixtures were centrifuged at 40,000 rev/min (~130,000 × *g*) for 90 min in 1-ml tubes specially designed to fit the SW 65 rotor. The supernatant fluids were drawn off, the tubes were drained, and the visible pellets were suspended to their original volumes in distilled water or Earle's balanced salt solution.

Figure 2A shows an electron micrograph of digitonin-treated pelleted virus stained with PTA. Viral particles can be readily identified but show considerable distortion, flattening, and lack of PTA penetration compared with relatively intact virions (Fig. 1A). A prominent feature is that these "shells" of VS virions appear to be devoid of envelopes and characteristic spikes.

Samples of 100 μliters of supernatant fluids or pellets of digitonin-treated VS virions labeled with ^{14}C-amino acids were mixed with an equal amount of intact purified VS virions that had been labeled with ^{3}H-leucine and ^{3}H-tyrosine to provide viral protein markers. Proteins were extracted from these mixtures with acetic acid, 0.5 M urea, and 1% SDS; the solubilized ^{14}C- and ^{3}H-proteins were then subjected to coelectrophoresis on 7.5% polyacrylamide gels. The gels were stained for scanning and were sectioned for counting of both isotopes.

The results of one of these experiments are recorded in Fig. 3. As shown in Fig. 3A, six peaks of ^{14}C radioactivity can be identified in pelleted virus after treatment with digitonin; each of these peaks corresponds exactly to the six peaks of optical density (OD) at 610 nm and to the six ^{3}H-protein markers extracted from intact virions. Only P6 was appreciably reduced in amount.

Figure 3B illustrates the results of electrophoretic analysis of the supernatant-fluid proteins released from the same digitonin-treated virions labeled with ^{14}C-amino acids. Only P6 and P4 were present in the supernatant fluid in appreciable amounts. Table 1 summarizes these data by showing the relative proportions of ^{14}C counts present in proteins at each gel position corresponding to the six marker proteins. As noted, digitonin caused the release of 83% of P6 and 53% of P4 from VS virions but only 3.5% of P5 and insignificant amounts of P1, P2, and P3.

These data suggest that the primary envelope-associated protein is P6 but that digitonin also dissociates about 50% of P4, presumably from the surface of the virion.

Release of nucleocapsid from VS virions by deoxycholate. Deoxycholate disrupts VS virions

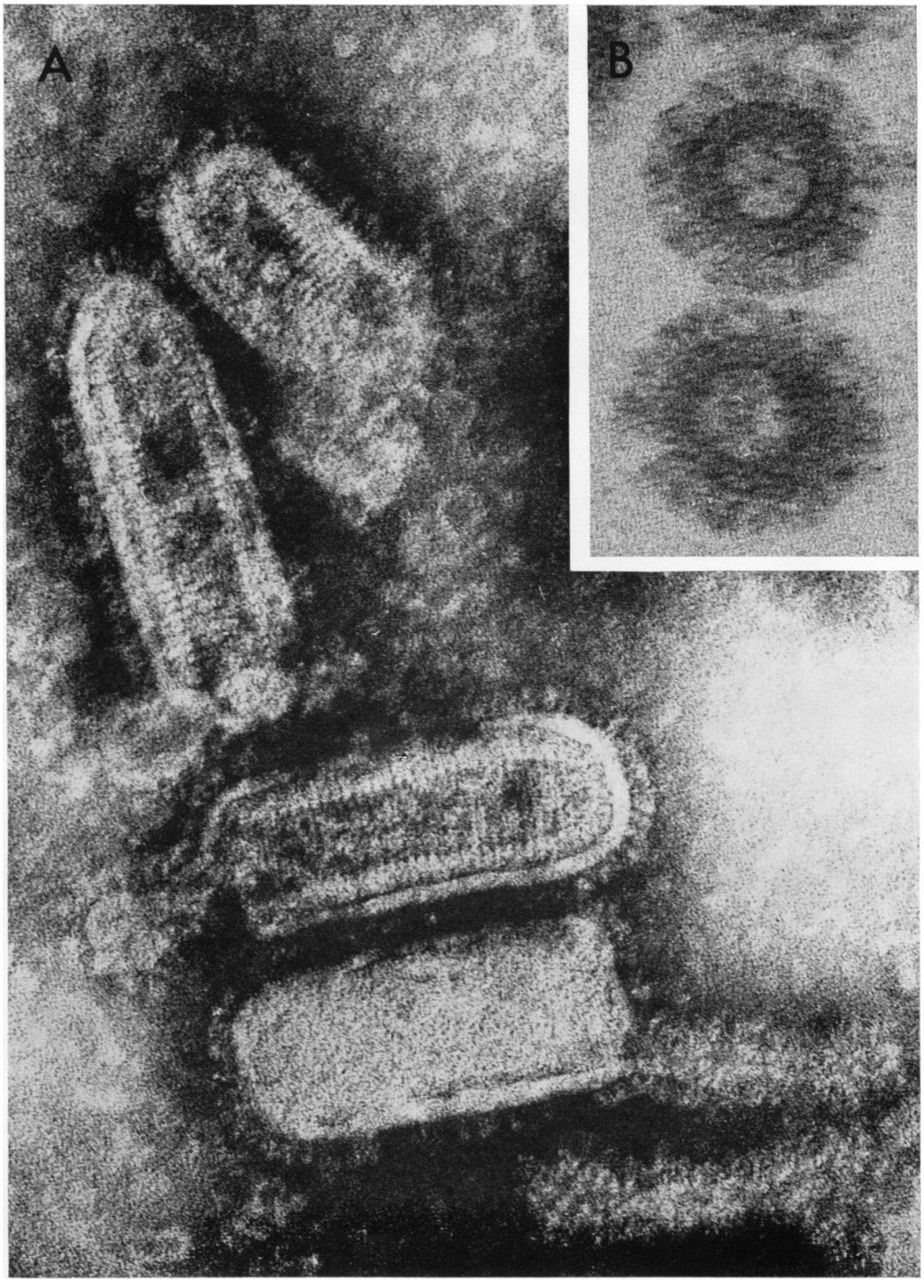

FIG. 1. *Structure of VS virions. (A) Purified and concentrated VS virions at various stages of disorganization after incubation in distilled water for 24 hr at 4 C were negatively stained with PTA. Note the external layer with spikes, the dense underlying layer or "shell", and the internal striations representing nucleoprotein coils. × 320,000. (B) Transverse section of pelleted, fixed, and epoxy-embedded VS virions stained with uranyl acetate and lead citrate. Note the fringe of spikes, an underlying electron-dense ring, and internal structure, presumably representing sectioned coils. × 400,000.*

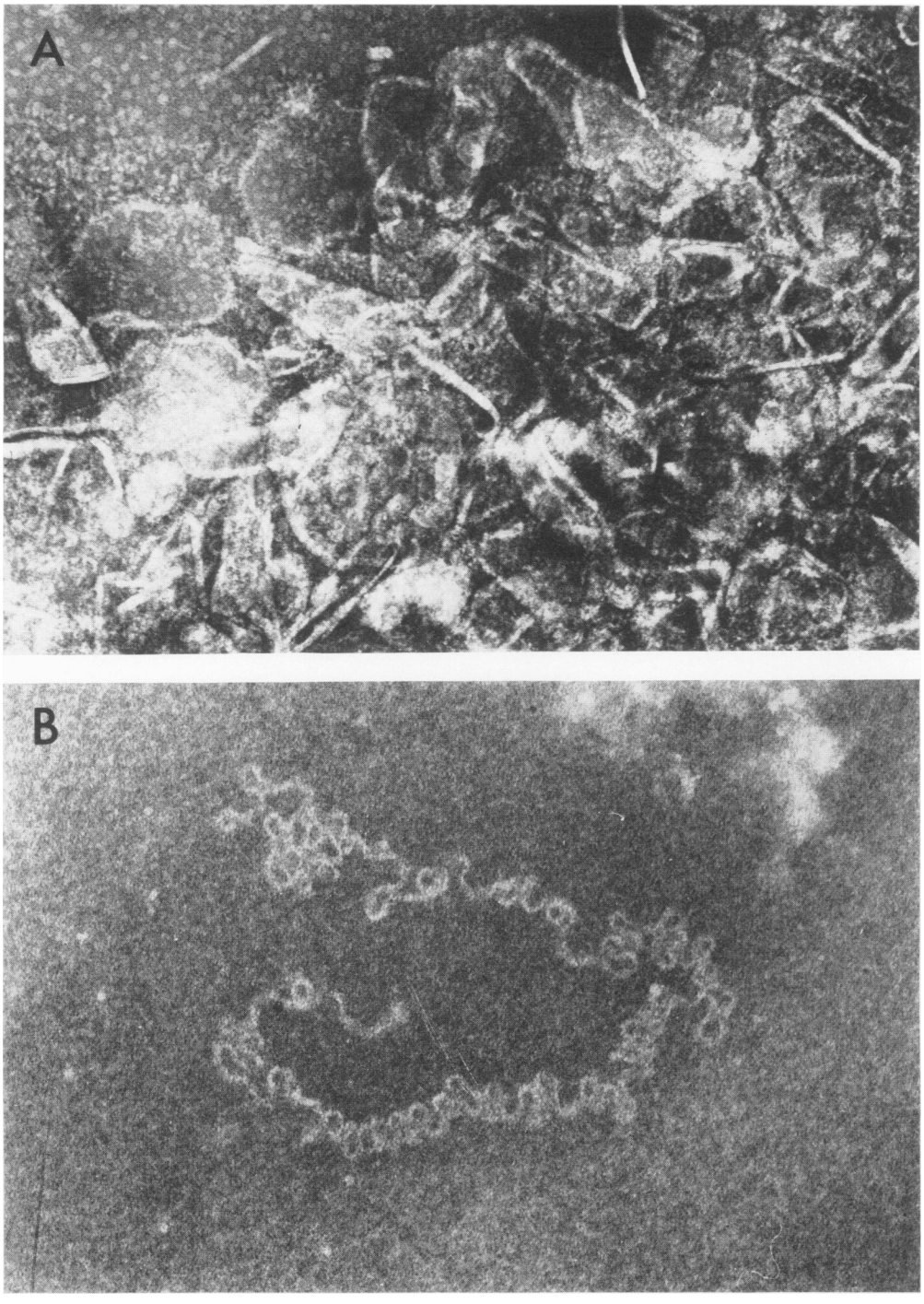

FIG. 2. *Degraded VS virions. (A) Shells of VS virions treated with digitonin, pelleted at 130,000 × g, and stained with PTA. Note the dense shells but the relative paucity of surrounding spikes. × 90,000. (B) Coils of VS virions released by treatment with deoxycholate, separated by banding in a CsCl gradient, and dialyzed against PTA in distilled water. × 160,000.*

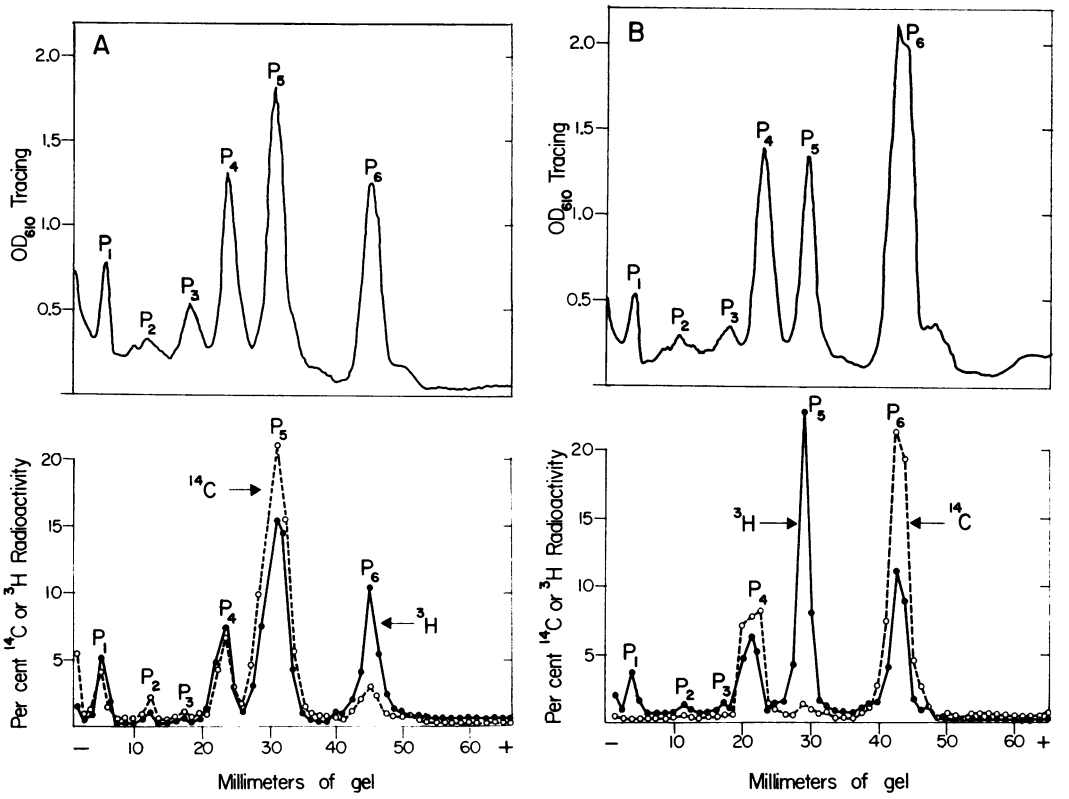

FIG. 3. *Electropherograms of ^{14}C-proteins extracted from pelleted and supernatant fractions of purified VS virions degraded with digitonin and separated by centrifugation at 130,000 × g for 90 min. The top panels represent tracings of gels stained with Coomassie blue and scanned at 610 nm in a Gilford spectrophotometer. The same sliced gels were analyzed for ^{14}C- and ^{3}H-protein markers extracted from intact virions. (A) Pelleted fraction of digitonin-degraded virions, 6,152 ^{14}C counts/min per 100 μliters. (B) Supernatant fraction of the same digitonin-degraded virions, 4,308 ^{14}C counts/min per 100 μliters.*

and releases internal coils (6, 7). We adopted this method for controlled degradation of VS virions. For purification of the nucleocapsid component, we took advantage of the fact that intact VS virions have a relative buoyant density in CsCl of approximately 1.20 g/ml (6, 10). We reasoned, correctly, that the lipid-free nucleocapsid should have a higher buoyant density and, therefore, should be readily separable from the enveloped virion by isopycnic sedimentation.

VS virions were grown in L cells in 1:50 BME without serum but containing ^{3}H-uridine (10 μc/ml) and ^{14}C-amino acids (1.13 μc/ml). The cells were exposed to actinomycin (0.33 μg/ml) in the medium throughout the period of infection. After incubation for 8 hr at 37 C, equal volumes of Eagle's minimal essential medium with cold amino acids and 2% calf serum were added to

TABLE 1. *Percentage of total ^{14}C radioactivity recovered from polyacrylamide protein fractions of VS virions treated with digitonin and centrifuged at 130,000 × g[a]*

Protein	Percentage of total ^{14}C	
	Supernatant fluid	Pellet
P1	0.2	3.5
P2	0.3	1.3
P3	0.2	0.9
P4	9.7	8.6
P5	1.1	29.8
P6	27.7	5.5
Total	39.2	49.6

[a] The supernatant-fluid ^{14}C radioactivity applied to the gel contained 4,308 counts/min per 100 μliters, and the pellet radioactivity contained 6,152 counts/min per 100 μliters. Data are abstracted from experiment shown in Fig. 3.

each culture. Media were harvested and pooled 17 hr after infection, and the virions were concentrated and purified. The procedure adopted for controlled degradation of VS virions was to add 1.25 mg of sodium deoxycholate (Matheson, Coleman and Bell, Cincinnati, Ohio) to 0.25 ml of the labeled viral suspension at a protein concentration of approximately 1.5 mg/ml. Higher concentrations of deoxycholate resulted in lower yields of nucleocapsids. The viral suspension was stirred constantly for 2 hr at room temperature, then was mixed with 4.5 ml of CsCl in 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.8 (starting specific gravity, 1.3 g/ml), and was centrifuged for 72 hr at 38,000 rev/min in the SW 50 rotor. Two clearly visible and sharp light-scattering bands could be seen consistently, but only a single band could be seen in control viral suspensions not exposed to deoxycholate.

Figure 4 shows the distribution of radioactivity in a representative CsCl gradient after centrifugation of deoxycholate-degraded purified virions labeled with ^{3}H-uridine and ^{14}C-amino acids. Coincident sharp peaks of ^{3}H and ^{14}C disintegrations were evident at densities equivalent to 1.32 and 1.20 g/ml. As noted, only 20% of the counts in RNA and 14% of the counts in protein were present in the high-density peak, suggesting that most of the nucleoprotein was not dissociated from lipid-containing virions. On repeated determinations, the amount of deoxycholate-dissociable ^{3}H-RNA ranged from 10 to 30%. Nevertheless, the ^{3}H:^{14}C ratio (RNA:protein) was always greater in the high-density region of the gradient. A minor error is implicit in these calculations because some ^{14}C-amino acids are incorporated into RNA.

The nucleoprotein released from VS virions by deoxycholate was completely resistant to ribonuclease (40 μg of crystalline pancreatic ribonuclease per 100 μliters of degraded virion suspension). Ribonuclease treatment did not affect the buoyant density of the nucleoprotein or its ^{3}H-uridine and ^{14}C-protein content. The high-density nucleoprotein fraction was not infectious when plated on L cells.

Fraction 13 ($\rho \cong 1.32$ g/ml) from the CsCl gradient shown in Fig. 4 was dialyzed overnight against 2% PTA in distilled water and was examined by electron microscopy. Figure 2B shows one representative field of this preparation, which reveals the characteristic coiled nucleoprotein of VS virus (7, 9). Other fields and other similar preparations often revealed aggregated coils, but only rare intact virions, always markedly distorted, were encountered. The top band, fraction 28 (Fig. 4), $\rho \cong 1.20$ g/ml, contained large numbers of recognizable intact virions, usually aggregated and distorted, but only rare coils which seemed to be attached to fragments of viral debris.

Identification of nucleocapsid protein. The high- and low-density fractions from CsCl gradients after isopycnic centrifugation of deoxycholate-degraded VS virions labeled with ^{14}C-amino acids were analyzed for their protein constituents. ^{3}H-proteins extracted from intact virions were used as markers.

Figure 5 shows the electrophoretic profiles of ^{14}C-proteins of fraction 13 ($\rho \cong 1.32$) and 28 ($\rho \cong 1.20$) of the CsCl gradient depicted in Fig. 4. These proteins were extracted with and dialyzed against 0.5 M urea as well as acetic acid, SDS, and 2-mercaptoethanol. The nucleoprotein coils collected from fraction 13 contained two primary proteins that migrated in SDS-gels to the same position as P2 and P5 of the ^{3}H-proteins used as

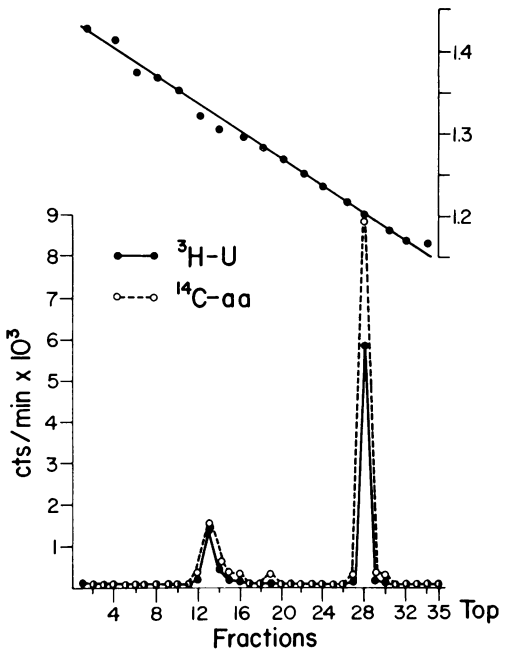

FIG. 4. *Fractionation in CsCl density gradient of VS virion components degraded by deoxycholate. The virus was grown in L cells in the presence of ^{3}H-uridine (10 μc/ml) and ^{14}C-amino acids (1.13 μc/ml), purified by differential, rate zonal and equilibrium centrifugation, then disrupted with sodium deoxycholate, and mixed with 4.5 ml of CsCl (starting specific gravity, 1.30 mg/ml). Centrifugation was carried out for 72 hr at 38,000 rev/min in the SW 50 rotor. Fractions of about 0.15 ml each were collected from the bottom, and 50-μliter samples were assayed for density and radioactivity. Symbols: ●, ^{3}H (RNA); ○, ^{14}C (protein).*

markers (Fig. 5A). There was no P6 and less than 10% of P4 in the nucleocapsid fraction. The slowest moving ^{14}C peak could not be identified and may be a polymer. In addition, a large amount of ^{14}C-protein did not enter the gel. These results suggested that a considerable amount of nucleocapsid protein aggregated under the conditions of the experiment. Previous experiments (10) had shown that P2 is a polymer or aggregate of one or several of the major proteins.

Figure 5B reveals that, in contrast to the nucleocapsid, P4 and P6 were the predominant proteins in deoxycholate-degraded virions that band at a density of 1.20 g/ml. Although there was some P5 in the low-density fraction, it was markedly reduced compared to P5 present in intact virions labeled with ^{3}H-amino acids.

Extraction with 8 M urea provided a more critical test of the identity of the nucleocapsid protein. The above experiments were repeated with virions labeled with ^{14}C-amino acids, degraded with deoxycholate, and separated by CsCl centrifugation into two fractions of densities 1.32 and 1.19 g/ml. Both fractions were extracted with and dialyzed against 8 M urea as well as acetic acid, SDS, and 2-mercaptoethanol prior to electrophoresis on separate gels along with ^{3}H-protein markers extracted from intact virions.

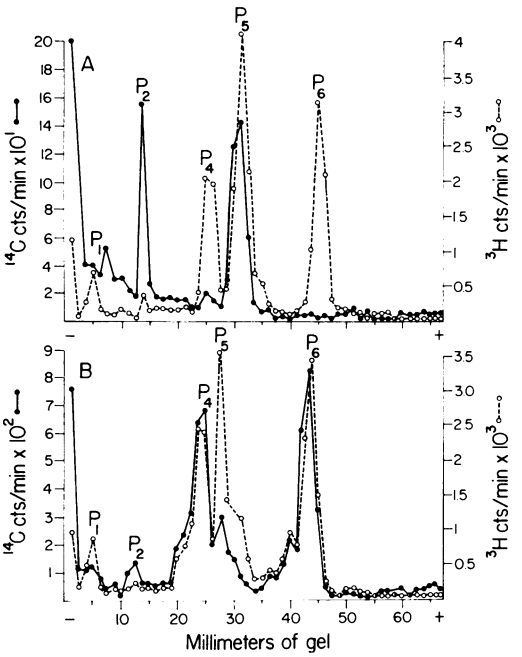

FIG. 5. *Electropherograms of ^{14}C-proteins extracted from VS virions degraded with deoxycholate and fractionated by equilibrium centrifugation. The ^{14}C-proteins of each fraction were extracted with acetic acid, 0.5 M urea, SDS, and 2-mercaptoethanol along with ^{3}H-protein markers from intact purified virions and were co-electrophoresed on 7.5% polyacrylamide gels in the presence of 0.5 M urea and 0.1% SDS. (A) Deoxycholate fraction 13 ($\rho \cong 1.32$ g/ml) shown in Fig. 4. (B) Deoxycholate fraction 28 ($\rho \cong 1.20$ g/ml) shown in Fig. 4.*

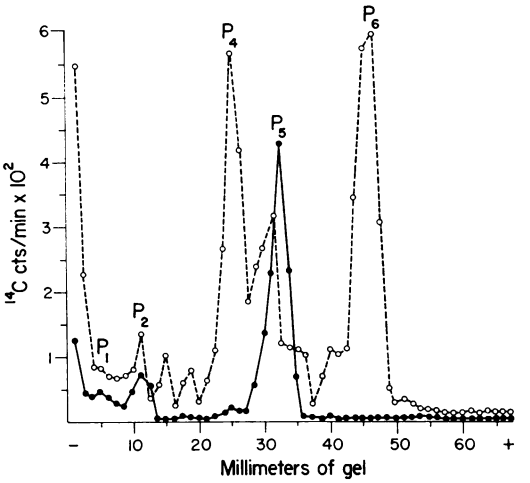

FIG. 6. *Electropherograms of ^{14}C-proteins extracted with 8 M urea from VS virion components degraded with deoxycholate and fractionated by CsCl density centrifugation under the same conditions as in Fig. 5 except for the use of 8 M urea for extraction of proteins from the two virion fractions. Each fraction was electrophoresed concurrently on separate gels. The positions of the peaks (P1, P2, P4, P5, and P6) correspond to ^{3}H-proteins extracted from intact VS virions and run on the same gels. Symbols: ●, deoxycholate fraction of $\rho \cong 1.32$ g/ml; ○, deoxycholate fraction of $\rho \cong 1.19$ g/ml.*

Figure 6 compares the electrophoretic profiles of the two deoxycholate-degraded virion fractions solubilized with 8 M urea. Clearly, P5 was the predominant polypeptide in the nucleocapsid fraction, although about 15% of recoverable ^{14}C counts still migrated with P2. No other proteins were detected in the nucleocapsid. In contrast, P4 and P6 predominated in the deoxycholate product that banded at density 1.19 g/ml; residual P5 was also present in the low-density fraction in an amount equivalent to that in the nucleocapsid fraction but considerably less than that expected in intact virions.

DISCUSSION

As previously reported (10), the molecular weights of Indiana serotype VS virion proteins were estimated as follows: P6 $\cong$ 34,500, P5 $\cong$ 59,500, P4 $\cong$ 81,500, P3 $\cong$ 140,000, P2 $\cong$ 186,000, and P1 $\cong$ 275,000. Similar values for four of

these proteins were obtained by Kang and Prevec (4). Only P6 extracted from the antigenically distinct New Jersey serotype VS virus turned out to have a different molecular weight, a finding which led to the suggestion that P6 is the surface protein (antigen) responsible for neutralization of each of the two viruses with only type-specific antiserum (10). The studies reported herein lend support to this contention. P6 is the primary protein split off VS virions exposed to digitonin, which presumably complexes with cholesterol in the virion envelope. Virions pelleted after digitonin treatment had a correspondingly reduced content of P6 and appeared by electron microscopy to be devoid of envelopes and spikes. However, about 50% of P4 was also solubilized after exposure of virions to digitonin. It seems probable, therefore, that P4 is the structural protein next most accessible to digitonin and may represent the shell protein underneath the envelope. Only insignificant amounts of P5 and the three minor proteins were released from virions by action of digitonin.

The experiments on degradation of VS virions with deoxycholate revealed that 10 to 30% of ribonucleocapsid was released as determined by banding of fractionated virions in CsCl gradients centrifuged to equilibrium. The nucleocapsid was identified on the basis of its high buoyant density, its increased ratio of ^{3}H-RNA to ^{14}C-protein, and visualization of its characteristic coils by electron microscopy. Essentially two proteins, P5 and P2, were present in the nucleocapsid fraction after extraction in 0.5 M urea. A comparison of the molecular weights of these two proteins raises the question whether P2 is a polymer of P5, possibly a trimer. Even after extraction with 8 M urea, about 15% of the nucleocapsid protein migrates in SDS-gels containing 0.5 M urea in the region of P2. The most likely interpretation of these findings is that the nucleocapsid protein is composed of a single polypeptide, P5, which has a marked tendency to aggregate into polymeric forms, particularly after purification. Such an hypothesis is consistent with our earlier finding that P2 present in intact virions was substantially reduced in amount by extraction in 8 M urea. Kang and Prevec (4) also identified the same protein (numbered 3 in their system) in nucleocapsid cores of VS virions.

Electron microscopy has revealed that cylindrical virions of VS virus are composed of at least three structural components: an envelope with spikes, an underlying "shell", and an internal coiled nucleocapsid core. The studies on partial degradation with digitonin and deoxycholate indicate that the envelope protein is P6 and the core protein is P5. The partial contamination of both solubilized envelope and separated cores with P4 is consistent with the hypothesis that P4 is the shell protein which lies between the envelope and the nucleocapsid. Data presented previously (10) suggest that the minor proteins P2 and P3 are aggregates or polymers of the major proteins. The position and function of the large polypeptide, P1, are unclear.

ACKNOWLEDGMENTS

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