Proteins of Vesicular Stomatitis Virus

I. Polyacrylamide Gel Analysis of Viral Antigens

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Infection of L cells with vesicular stomatitis virus results in the release, into the cell-free fluid, of four antigenic components separable by rate zonal centrifugation on sucrose gradients. The largest antigens are the infectious (B) particle and a shorter noninfectious, autointerfering (T) particle. The two small antigens are characterized by sedimentation coefficients of approximately 20S and 6S. Treatment of purified B or T particles with sodium deoxycholate results in the release from the particle of a nucleoprotein core which can be purified on sucrose gradient and which has a sedimentation coefficient characteristic of the virus from which it arose. Utilizing purified antigens labeled with ^{14}C -amino acids during growth, we examined the protein constituents of each antigen by acrylamide-gel electrophoresis. The proteins of B and T particles are identical, each containing one minor (virus protein 1) and three major (virus proteins 2, 3, and 4) proteins, numbered in order of increasing mobility. Virus protein 3 originates from the nucleoprotein core, whereas proteins 2 and 4 come from the coat. The origin of virus protein 1 is not known. The 20S antigen contains a single protein equivalent to virus protein 3, whereas the 6S antigen shows a single protein which is similar to, but probably distinct from, virus protein 2.

In 1956, Bradish, Brooksby, and Dillon (1) described four antigenic components of the vesicular stomatitis virus (VSV) system separable on the basis of sedimentation coefficient. The infectious, 625S component, a bullet-shaped particle approximately 65 nm in diameter and 180 nm long, has been named the B particle (12). A second, noninfectious particle, 330S, which appears to be a truncated form of the B particle, has been shown by Huang and Wagner (13) and Hackett (10) to be responsible for the autointerference phenomena originally described by Cooper and Bellett (9). This "transmissible interfering" component has been designated the T particle. Both the B and T particles contain single-stranded ribonucleic acid (RNA; reference 7) which is enclosed in a nucleoprotein core wound helically inside the viral coat or envelope (14). At least a portion of the viral coat appears to be derived from the host-cell membrane (8).

Two smaller antigens with approximate sedimentation coefficients of 20S and 6S are also produced. These antigens have been studied immunologically by Brown et al. (4-6), but relatively little is known of their functional origin or significance.

We purified these various antigens and, by utilizing the techniques of viral degradation and

acrylamide-gel electrophoresis described by Summers et al. (20), we compared the protein components of each antigen.

MATERIALS AND METHODS

Cells. A continuous-passage line of Earle's L cell designated L60 (14) was obtained from A. Howatson, Department of Medical Biophysics, Toronto. The cells were grown in suspension culture in Joklik modified minimal essential medium (MEM; Grand Island Biologicals Co., Grand Island, N.Y.) supplemented with 5% fetal calf serum. The cells were kept in constant exponential growth phase by daily twofold dilution.

Virus. A high-temperature-resistant strain of the Indiana serotype of VSV was obtained from T. Nakai of the Department of Medical Biophysics, Toronto. Stock virus was obtained by five successive passages of this virus, each at a multiplicity of 0.01 plaque-forming units (PFU) per cell. For virus passage, exponentially growing cells at 4×10^6 cells/ml were centrifuged at $400 \times g$ for 10 min. The cell pellet was suspended to 10^7 cells/ml in 10 ml of MEM containing 2% fetal calf serum and appropriately diluted virus. The virus was allowed to absorb in this volume by incubation for 45 min at 37°C in suspension culture. After this time, the infected culture was diluted in prewarmed MEM containing 2% fetal calf serum to a final cell concentration of 0.5×10^6 to 10^6 cells/ml. Incubation was continued in suspension culture at

37 C for 8 to 14 hr, at which time cells and cellular debris were removed by centrifugation at $400 \times g$ for 10 min; supernatant stock virus suspension was stored at -60 C until further use. The virus titer in the stock preparations ranged from 0.5×10^8 to 10^8 PFU/ml. Under these passage conditions, the stock virus contained approximately equal numbers of B and T particles as determined by sucrose gradient fractionation.

Assay for PFU and complement fixing (CF) activity. To determine virus plaque-forming titer, 0.1 ml of diluted virus was placed directly on a full L-cell monolayer, preformed in Falcon petri dishes (60 by 15 mm). The virus was allowed to absorb for 30 to 45 min at 37 C in an atmosphere of 5% CO_2 in air and 100% humidity. The infected cell sheet was then overlaid with 5 ml of solution containing MEM growth medium, 5% fetal calf serum, and 0.9% Noble agar (Difco). Plaques could be counted without staining after incubation for 20 to 24 hr at 37 C in a water-saturated, 5% CO_2 atmosphere.

Complement-fixing titer was determined by using the microtiter technique described by Sever (17). Antiserum was prepared in rabbits. VSV was grown in human KB cells and partially purified by centrifugation at $400 \times g$ for 10 min to remove cells and by centrifugation at $41,000 \times g$ for 2 hr to pellet the virus; it was then suspended to 10^8 PFU/ml. Primary injections given intramuscularly and intraperitoneally contained 5×10^8 PFU with complete Freund's adjuvant. Three subsequent injections given weekly beginning 14 days after the primary injection contained 5×10^8 PFU intraperitoneal and 10^8 PFU intravenous without adjuvant. The rabbit was bled by exsanguination through a carotid catheter 1 week after the last injection. The serum was heat-inactivated at 56 C for 30 min.

Box titration was employed to determine optimal complement and hemolysin titers. The 50% hemolysis end point was determined visually. Antibody was standardized by box titration with a standard VSV lysate used as antigen and with one optimal unit of hemolysin and three units of complement. For the experimental CF determinations, four units of antibody were used.

Preparation of radioactively labeled viral components. Some 5×10^8 L cells were collected by centrifugation and resuspended in 25 ml of MEM with 2% fetal calf serum containing enough virus to give a multiplicity of infection (MOI) of 50 PFU/cell. Absorption was carried out in this volume in suspension culture at 37 C for 45 min; the culture was then diluted to 400 ml with prewarmed MEM containing 2% fetal calf serum. The time of dilution is taken as time zero of infection. After 3 hr of incubation at 37 C, the infected cells were pelleted by centrifugation at $400 \times g$ for 10 min and suspended in 200 ml of prewarmed medium which was identical to MEM except that it contained only one-twentieth the normal amino acid concentration and was supplemented with 2% fetal calf serum. After 30 min of incubation at 37 C, 70 μC of a ^{14}C -amino acid mixture (New England Nuclear Corp.) was added to the culture and incubation was continued for a further 5 to 6 hr. At this time, the

culture was harvested by centrifugation at $500 \times g$ for 15 min. The cell pellet resulting from this centrifugation was discarded, and only the supernatant fluid was carried through subsequent fractionations.

The cell-free virus fluid was centrifuged at $41,000 \times g$ for 2 hr. The pellet of this centrifugation, which contains almost all of the viral infectivity of the culture, is the $41,000 \times g$ pellet whose subsequent fractionation is described later. The supernatant fraction of this centrifugation contains the 20S and 6S viral antigens. These were precipitated from solution by the dropwise addition of 1.5 volumes of a saturated solution of ammonium sulfate as described by Brown et al. (4). Precipitation was allowed to occur at 4 C for a further 60 min. The precipitate was collected by centrifugation at $16,000 \times g$ for 20 min and washed once with 50% saturated ammonium sulfate solution by resuspension and further centrifugation. The precipitate was then dissolved in 1 ml of calcium and magnesium-free phosphate-buffered isotonic saline (PBS), pH 7.4, and dialyzed overnight against 1,000 ml of PBS. After dialysis, the antigens were fractionated by sucrose gradient centrifugation.

Preparation and analysis of sucrose gradients. Linear sucrose gradients from 5 to 30% sucrose in PBS were prepared in 5-, 30-, or 38-ml volumes, as required, by using a Buchler gradient maker. No more than 1.0 ml of material was layered on the 30- or 38-ml gradient, and no more than 0.2 ml on the 5-ml gradients. Gradients were usually collected by using an Isco model D fractionator. In the case of 30- and 38-ml gradients, successive 1-ml fractions were collected. If required, a small volume from each fraction was removed and analyzed for PFU or CF activity by the methods described above. Radioactivity determinations along the gradient were usually made by removing 0.05 to 0.1 ml from each fraction and placing this directly on glass fiber filter paper (2.5 cm diameter) in the bottom of a scintillation vial. The filters were dried by heating in an oven. After cooling, 5 ml of scintillation fluid consisting of 4 mg of 2,5-diphenyloxazole (PPO) and 0.3 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per ml dissolved in toluene was added. Counting was done in a Beckman scintillation counter. For fractions which may have contained free radioactive amino acids, the sample removed from the fraction was precipitated with cold 10% trichloroacetic acid onto Sartorius nitrocellulose filters (0.45 μm pore size) and washed with cold 5% trichloroacetic acid prior to drying and counting.

Concentration, disruption, and electrophoresis of fractions. The location of purified radioactive viral antigen was determined in sucrose gradients as described above. The fractions containing each antigen were pooled separately and dialyzed against 500 volumes of distilled water overnight at 4 C to remove sucrose and other salts. The material was then concentrated to near dryness by flash evaporation at 30 C. The antigen was suspended in approximately 0.2 ml of a solution containing 1% sodium dodecyl sulfate (SDS), 10% acetic acid, and 0.5 M urea in 0.1 M phosphate buffer (pH 7.0) by the procedure of Summers et al. (20). After treatment for 1 hr at room

temperature, the mixture was dialyzed overnight at room temperature against 1,000 volumes of a solution containing 0.1% SDS, 0.1% 2-mercaptoethanol, and 0.5 M urea in 0.01 M phosphate buffer (pH 7.1). Samples of dialyzed protein were made 10% in sucrose by addition of finely powdered sucrose, and 0.3 ml of sample was layered on polymerized acrylamide gel columns.

Acrylamide gel columns (0.9 cm diameter by 16 cm long) were performed in plastic tubes. The final gel contained 5% acrylamide, 0.1% *N,N*-bis-methylene acrylamide, 0.1% SDS, 0.5 M urea, and 0.1 M sodium phosphate (pH 7.1). Ammonium persulfate at a final concentration of 0.07% and *N,N,N',N'*-tetramethylethylenediamine at 0.043% (v/v) were included in the gel as catalysts for polymerization. The electrophoresis running buffer, 0.1 M phosphate buffer (pH 7.1) containing 0.1% SDS, was carefully layered on the sample to fill the plastic tube. The tube containing the gel and sample was placed vertically in a Buchler-gel electrophoresis stand; running buffer, as described above, was placed in the reservoir chambers. The running buffer was maintained at temperatures slightly above 25°C to prevent precipitation of SDS. An initial field strength of 1 v/cm of gel was applied for 1 hr, and the voltage was then increased to 3 v/cm. The gel, after being freed from the walls of the plastic tube with slight water pressure, was sliced manually with a razor blade into discs approximately 0.16 cm in height. Each disc was placed directly into a scintillation vial containing 5 ml of Bray's scintillation fluid (3). The samples were counted in a Beckman scintillation counter.

RESULTS

Kinetics of infectivity and CF activity. Figure 1 illustrates the growth of viral infectivity and CF activity in an L-cell culture infected with VSV under the conditions used in our subsequent experiments. At various times postinfection, samples of 1-ml volume were removed. Of each sample, 0.5 ml was immediately frozen, then thawed and assayed for total PFU and CF activity. The other portion was centrifuged at $400 \times g$ for 10 min to remove cells, and the supernatant fraction was assayed for free CF and PFU activity. The infectious virus in the total culture begins to increase at 1 hr postinfection and increases exponentially until 6 hr postinfection (Fig. 1). Very little infectious virus remains cell-associated, supporting the hypothesis that virus matures and gains its infectivity on passage through the cell membrane (11). Total CF activity increases from 1 to 6 hr postinfection. In the early stages of infection, a large proportion of the CF antigen is cell-associated; however, by 10 hr postinfection, most of the CF activity is found in the extracellular fraction.

To ensure that the increase of CF activity with time after infection was in fact due to continued

FIG. 1. Kinetics of PFU and CF activity. L cells were centrifuged from an exponentially growing culture and suspended in 10 ml of VSV to give an MOI of 50. After absorption at 37°C for 30 min, the infected culture was diluted to 10^6 cells/ml with prewarmed MEM containing 2% fetal calf serum. Incubation was continued at 37°C. At indicated times post infection, samples were removed and PFU and CF were determined in total sample after freezing and thawing (total CF and PFU) and in cell-free sample (free CF and PFU).

synthesis of protein antigen, we performed the experiment shown in Fig. 2. The procedure was basically the same as that described above, except that the infected culture was split at 2 hr post-infection into two equal portions, and cycloheximide (final concentration, 20 μ g/ml) was added to one portion. Only the total PFU and CF activity were determined. The addition of cycloheximide, an inhibitor of protein synthesis (21), caused an immediate cessation in the increase of both PFU and CF activity (Fig. 2). We therefore conclude that CF activity in the infected culture results from de novo synthesis of virus-specific protein.

Purification of ¹⁴C-labeled VSV components. As ¹⁴C-labeled proteins were to be used in our subsequent analysis of viral proteins, it was necessary to ensure that there was a direct correspondence between radioactive components and the CF or PFU activity of the known VSV components.

For this study, viral components were labeled with ¹⁴C-amino acids and partially purified by differential centrifugation. The $41,000 \times g$ pellet was suspended in 1 ml of PBS, sonically

FIG. 2. Effect of cycloheximide on PFU and CF activity. *L* cells were infected with VSV as in Fig. 1. At 2 hr postinfection, the culture was split in two portions, and cycloheximide (20 μ g/ml) was added to one portion. Samples were removed from the cultures at the indicated times and after freezing and thawing. PFU activity (A) and CF activity (B) were determined. Solid line: control culture; broken line: cycloheximide added.

treated for 15 sec with a Biosonik III microprobe to disaggregate the virus, and layered on 30 ml of a 5 to 30% sucrose gradient in PBS. After centrifugation for 1 hr at 4 C and $41,000 \times g$, successive 1-ml fractions of the gradient were collected by using an Isco model D fractionator. A small portion of each fraction was used to determine PFU, CF, and radioactivity. The distribution of these activities along the gradient is shown in Fig. 3.

Closest to the bottom of the gradient is the thin, visible band which corresponds to fractions 6 and 7 of the gradient. Associated with this band is some CF activity, a peak of radioactivity, and almost all the viral infectivity present in the gradient. This is the band of infectious B particles described by Huang et al. (12) and Hackett (10).

Higher up is the thicker band corresponding to fractions 11 and 12. This band shows CF activity and a peak of radioactivity, but no associated peak of infectivity. This is the T-particle band. We confirmed the designation of these bands as B particle and T particle, respectively, by electron-microscopic observation of material taken from these regions of the gradient. Under our conditions of high multiplicity of infection,

the yield of T particles was always as great or greater than the yield of B particles.

Material containing CF activity and radioactivity is also present at the top of the gradient depicted in Fig. 3. Although some of this material may result from viral breakdown during resuspension and sonic oscillation, a portion of it is undoubtedly due to contamination of the $41,000 \times g$ pellet with the 20S and 6S viral components.

From the correspondence of radioactivity, CF activity, and, in the case of the B particle, infectivity, with the visible bands in the sucrose gradient, we conclude that the ^{14}C -radioactivity associated with the B and T particles of VSV is in fact present in those particles and not in cosedimenting cellular contaminants.

In a method analogous to that described above, we analyzed the two smaller antigens of the VSV system. In this case, the viral proteins of the $41,000 \times g$ supernatant fraction were precipitated with 60% saturated ammonium sulfate and dialyzed as described in Materials and Methods. A volume of 1 ml of the protein concentrate was layered on a 5 to 30% sucrose gradient in PBS and centrifuged for 16.5 hr at $51,000 \times g$ and 4 C on the SW 50 rotor of a Spinco ultracentrifuge. Successive fractions (each 0.33 ml) were collected by dripping the gradient

FIG. 3. Sucrose gradient purification of B and T particles. VSV was grown in *L* cells in the presence of ^{14}C -amino acids. After removal of cells by centrifugation, the cell-free fluid was centrifuged at $41,000 \times g$ for 2 hr to pellet the virus. The viral pellet was suspended in PBS, sonically treated, and layered on 30 ml of a 5 to 30% sucrose gradient in PBS. Centrifugation was at $41,000 \times g$ for 1 hr. Successive 1-ml fractions were collected and samples removed for PFU, CF, and radioactivity determinations. The orientation of the tube and the visible bands in the gradient are shown in the inset. Symbols: Δ , infectivity; $\circ$, CF activity; $\bullet$, radioactivity.

FIG. 4. Sucrose gradient purification of 20S and 6S antigens. Radioactive virus was grown and partially purified as described in Fig. 3. The $41,000 \times g$ supernatant fraction was made 60% saturated in ammonium sulfate; the resultant precipitate was centrifuged and washed. The washed pellet was suspended in 1 ml of PBS and dialyzed against PBS; 0.2 ml was layered on a 5-ml 5 to 30% sucrose gradient in PBS. Centrifugation was at $51,000 \times g$ for 16.5 hr. Successive 20-drop fractions were collected through the bottom of the tube; a portion of each was used for determination of radioactivity (●) and CF activity (○); The bottom of the gradient tube is to the left.

through a hole in the bottom of the tube; each fraction was subsequently analyzed for CF activity and radioactivity (Fig. 4).

A peak of CF activity and radioactivity is present approximately halfway down the gradient. This corresponds to the 20S antigen described by Bradish et al. (1).

Material having both CF activity and radioactivity is present at the top of the gradient tube. This fraction probably contains both the 6S and 3S antigens described by Brown et al. (5, 6). We have not attempted to separate these two components, if they exist in our preparation, and shall therefore refer to this material as 6S VSV antigen.

It is possible that much of the radioactivity present at the top of the gradient, although it occupies the same region as 6S viral antigen, is, in fact, due to contaminating cellular protein. As a control for this possibility, we prepared a ^{14}C -labeled fraction from uninfected cells which had been treated, labeled, and fractionated by methods identical to those used for infected cultures. No results of this experiment are shown since, in this case, no label was present in the $41,000 \times g$ pellet; although some radioactivity was present in the $41,000 \times g$ supernatant fraction, none was precipitated with 60% saturated ammonium sulfate. The value of this particular

type of control is dubious, since cells infected with virus are undoubtedly more fragile and capable of leaking their own proteins to the culture medium than are growing, uninfected cells. Later we shall present another experiment in which cells were prelabeled with ^{14}C -amino acids and subsequently infected in the absence of label.

From the above experiment, we would conclude that the radioactivity associated with 20S and 6S antigen did not result from nonviral material cosedimenting with the viral antigens.

Because we had purified labeled B particles, T particles, 20S VSV antigens, and 6S VSV antigens, we were in a position to investigate the protein components of each of these fractions. Each purified, concentrated fraction was treated with SDS, urea, and acetic acid, followed by dialysis against SDS, urea, and mercaptoethanol. The resultant proteins were analyzed by acrylamide-gel electrophoresis.

Proteins of purified B and T particles. Purified radioactive B and T particles taken from a gradient similar to that shown in Fig. 6A were disrupted and analyzed as described. The resultant distribution of radioactivity along the gel for each particle is shown in Fig. 5. At least three major peaks of radioactivity and one minor peak are seen in both B- and T-particle preparations. Starting with the minor peak, we numbered the fractions 1, 2, 3, and 4 in order of increasing mobility. Both B and T particles showed the same distribution of labeled proteins on the acrylamide gel (Fig. 5). Quantitative differences in the relative amount of radioactivity in any of the protein peaks between B and T particles were not reproducible.

Coelectrophoresis of a mixture of B- and T-particle proteins yielded the same pattern shown in Fig. 5, thus proving that the electrophoretic mobility of the proteins from both B and T particles are identical.

The possibility remains that some of the viral proteins are not recovered in the gel. This was examined in two ways. First, electrophoresis of viral proteins under conditions of reversed polarity showed that in this case no detectible radioactivity entered the gel. Secondly, we calculated the recovery within the gel of the radioactivity applied to the gel. Under our conditions of gel slicing and subsequent counting of radioactivity, there was a loss of radioactivity owing either to quenching or to self-absorption in the gel. To determine the proper correction factor, we mixed a known amount of protein sample uniformly with acrylamide. After the gel had solidified into a cylinder approximately 1 cm long, the gel

FIG. 5. Acrylamide analysis of B- and T-particle proteins. B and T particles containing ^{14}C -labeled proteins were purified as shown in Fig. 6A. The viral particles, after pelleting at $41,000 \times g$ for 2 hr, were suspended in SDS, urea, and acetic acid, followed by dialysis against SDS, urea, and mercaptoethanol to degrade the particles to their constituent proteins. The samples were placed on 5% vertical acrylamide gels (16 cm in length) and run for 14 hr at 3 v/cm. The gels were then sliced into 0.16-mm discs and radioactivity was determined by using Bray's scintillation fluid and a Beckman scintillation counter. The sample was applied at the cathode side (left). (T) Proteins of T particles (48,000 counts/min applied to gel); (B) proteins of B particles (28,000 counts/min applied to gel) labeled 1, 2, 3, and 4, in order of increasing mobility.

was sliced and counted in the usual manner. From this experiment, we determined that the counting efficiency for radioactivity in the gel slices was 12.0% of that obtained by the filter paper method. Using this correction factor, we can calculate that over 90% of the applied radioactive material is accounted for in our gel.

On the basis of these results we conclude that no major differences exist in the protein compositions of B and T particles.

Since VSV is composed of both a nucleoprotein core and an outer envelope (2, 14, 19), we were interested in determining which of the viral

proteins was associated with the core and which with the envelope or coat.

Purification of viral nucleoprotein cores. Nakai and Howatson (14) described the release of viral cores from B and T particles treated with sodium deoxycholate (DOC). We utilized the DOC treatment of virus followed by sucrose gradient centrifugation to obtain pure core particles from either B or T particles (Fig. 6).

DOC treatment of B particles or T particles produces particles separable from each other by gradient centrifugation and characteristic of the virus particle from which they were produced (Fig. 6B and C). These particles are nucleoproteins and contain RNA having the same sedimentation coefficient as RNA extracted from the parent virus particle (*unpublished observations*). We call these particles viral cores and determined their sedimentation coefficients by linear interpolation on sucrose gradients using published sedimentation coefficients of cellular polyribosomes as standards (15). Values of 100S and 140S were obtained for T-particle cores and B-particle cores, respectively. It should be pointed out that DOC treatment of virus is relatively inefficient

FIG. 6. Purification of cores from B and T particles. B and T particles were prepared as described in Fig. 3. Centrifugation on a 5 to 30% sucrose gradient in PBS at $41,000 \times g$ for 1 hr resulted in the radioactive peaks shown in A. B particles and T particles were taken from fractions indicated by the bars in A. After pelleting by centrifugation at $41,000 \times g$ for 2.0 hr, each was separately suspended in 0.5% DOC and 0.5% BRIJ (detergent obtained from Atlas Chemical Industries) in PBS. After treatment of 1 hr at room temperature, the material was layered on 5 to 30% sucrose gradients in PBS and centrifuged at $51,500 \times g$ for 2.5 hr. This resulted in the purification of T particle cores with a sedimentation coefficient of 100S (B) and B particle cores of 140S (C) from the dissociated strip, or coat proteins of each seen at the top of the gradient tube. The top of the gradient tube is to the left in all diagrams.

in the production of these particles. Some of the virus, after treatment, sediments at a position not greatly different from that of whole virus and probably resembles the tightly coiled internal component described by Brown et al. after Tween-ether treatment of VSV (6).

The radioactivity at the top of the gradients shown in Fig. 6B and C presumably represents viral envelope protein and perhaps some core protein arising from complete disruption of a small percentage of virus particles. We shall refer to this low-molecular-weight protein fraction, obtained after DOC treatment of virus, as viral "strip" protein.

Proteins of viral core and coat. The purified radioactive core and "strip" protein fractions were analyzed by acrylamide gel electrophoresis in a manner analogous to that described for whole viral particles. As a control marker for protein mobility in all subsequent experiments, we utilized, for convenience, the material of the $41,000 \times g$ pellet, rather than purified virus. This pellet contains chiefly B and T particles, with some contaminating low-molecular-weight material (Fig. 3), and yields, after disruption, a protein pattern on acrylamide gel qualitatively the same as that shown in Fig. 5. We refer to this material as "total virus."

Figure 7 shows the result of DOC treatment of "total virus." In this experiment, a small portion of the total virus sample was incubated with DOC under conditions identical to those used to prepare core and "strip" protein from virus. The material was then, without further fractionation, treated with SDS, urea, acetic acid, and mercaptoethanol, as described, and run on acrylamide gel. A parallel non-DOC-treated sample was also analyzed. No difference in protein mobility as a result of DOC treatment is seen in comparing Fig. 7a with 7a'. This experiment ensured that preparation of core protein and strip protein by DOC treatment would have no effect on the electrophoretic mobility of these proteins. Virus protein peak 2 appears to have a component which appears as a shoulder on the higher mobility side of this radioactive peak (Fig. 7a and a'). We have been unable to determine if this does in fact represent a separate protein or is an artifact of our technique. One possibility will be discussed later when we consider the 6S antigen.

Core and strip protein were prepared by DOC treatment of purified B and T particles followed by purification on sucrose gradients. Figure 7b shows the distribution of ^{14}C -radioactivity on acrylamide gel obtained from the electrophoresis of 60,000 counts/min of disrupted core protein. Only one electrophoretically homogeneous species

FIG. 7. Acrylamide gel analysis of VSV core and coat proteins. Radioactive total virus (obtained by using the pellet of the $41,000 \times g$ centrifugation described in Fig. 3) was used as a mobility standard in these experiments. (a) The radioactive profile on acrylamide of 40,000 counts/min of disrupted total virus protein. (a') An identical sample exposed to 0.5% DOC and 0.5% BRIJ (see legend to Fig. 6) prior to disruption. Viral core and strip proteins were prepared and purified as shown in Fig. 5. Electrophoresis of 60,000 counts/min of disrupted core protein gave the pattern shown in b; a mixture of 60,000 counts/min of core protein and 40,000 counts/min of total protein gave the pattern shown in b'. Similarly, the pattern obtained from 60,000 counts/min of disrupted strip protein (c) and a mixture of 60,000 counts/min of strip protein plus 40,000 counts/min of total protein (c') are shown. Electrophoresis was for 19 hr from left to right.

of protein was observed. To determine which viral protein corresponded to core protein, we coelectrophoresed 60,000 counts/min of disrupted core protein and 40,000 counts/min of total protein. The total protein was identical to that used to obtain Fig. 7a (note changes in radioactivity scale). The coelectrophoresis resulted in a large increase in radioactivity asso-

ciated with virus protein peak number 3 (Fig. 7b). We therefore conclude that virus protein 3 arises from the protein of the viral nucleoprotein core.

In a similar manner, we examined the strip protein fraction obtained after DOC treatment of whole virus. Two major peaks of radioactivity with a less highly radioactive peak between them was obtained (Fig. 7c). By coelectrophoresis with total protein (Fig. 7c) the major peaks corresponded to viral protein peaks 2 and 4. Some protein moving with the electrophoretic mobility of viral protein 3 was also present in the strip protein. Whether this represents a species different from that found in viral core or whether it results from contamination of strip protein by core material disrupted by DOC treatment is not known. We have noted, however, that the amount of protein 3 relative to proteins 2 and 4 present in strip protein preparations is variable, suggesting that the second explanation may be more likely.

From these experiments we conclude that viral protein 3 comes from the core of the virus, whereas proteins 2 and 4 come from the viral coat or envelope. We did not detect virus protein 1 in either core or strip-protein preparations. The reason for this is unknown.

Proteins of 20S and 6S VSV antigens. Having identified electrophoretically the proteins of viral core and coat, we next examined the proteins present in the 20S and 6S antigens. These antigens were prepared and purified by sucrose gradient fractionation; each was separately examined in acrylamide gels (Fig. 8). The 20S antigen contains one major protein component. The presence of two distinct peaks of lower mobility in this particular experiment was undoubtedly due to incomplete disruption, since in three other runs (not shown) only the major protein peak was present. Coelectrophoresis of this material with total virus showed (Fig. 8a') that the protein of the 20S antigen corresponded to viral protein 3. The mobility of the protein from the 20S antigen is therefore identical to the protein of the viral core. Some implications of this finding are considered in the discussion.

Electrophoresis of the 6S viral antigen also yielded a single protein (Fig. 8b). On coelectrophoresis with total protein (Fig. 8b'), the 6S protein has a mobility similar to that of virus protein 2. By varying gel concentrations from 4 to 7.5%, and under various conditions of electrophoresis, we have been unable to resolve the 6S protein from viral protein 2. However, in all these runs, the 6S protein appears to be displaced to the high mobility side of viral protein 2 and may, in fact, correspond to the shoulder observed

FIG. 8. Acrylamide gel analysis of 20S and 6S antigens. Radioactive 20S and 6S antigens were prepared and purified as shown in Fig. 4. Each isolated fraction, after dialysis, concentration, and disruption, was analyzed by electrophoresis for 19 hr at 3 v/cm. Total virus standards were identical to those used in Figure 7; (a) 54,000 counts/min of disrupted 20S antigen; (a') 54,000 counts/min of disrupted 20S antigen plus 60,000 counts/min of total virus; (b) 60,000 counts/min of disrupted 6S antigen; (b') 60,000 counts/min of disrupted 6S antigen plus 40,000 counts/min of total virus.

on the anode side of virus protein 2 (Fig. 7a and 7a').

Proteins of prelabeled cells. Cartwright and Pearce (8) reported that VSV carries with it a cellular antigen, presumably arising from the cell membrane, which appears to form part of the viral envelope on emergence of the infectious particle from the cell (11). We attempted to determine the nature of this cell-specific antigen, assuming it to be a protein, by the following experiment.

An exponentially growing culture of L cells (250 ml at 3×10^6 cells/ml) was labeled with ^{14}C -amino acids (0.1 mc/ml) for 24 hr. At the end of this time, the cells were collected by centrifugation at $400 \times g$ for 10 min and suspended in 150 ml of prewarmed unlabeled MEM. After a 7-hr incubation at 37 C, the cells were again collected as above and infected with virus at a multiplicity of 50 PFU/cell; the cell-free viral fluid was harvested at 12 hr postinfection in the usual manner. Any radioactivity present in viral components should therefore represent primarily cellular protein incorporated in the particle. Total

FIG. 9. Protein released from prelabeled infected cells. Cells were incubated with ^{14}C -amino acids for one generation time and for a further 7 hr in the presence of nonradioactive growth medium. They were then infected with virus at a $\text{MOI} = 50$ and the viral fluid was harvested. The only radioactive protein present in the cell-free fluid was precipitated from the $41,000 \times g$ supernatant fraction with ammonium sulfate and prepared for electrophoresis. Electrophoresis was for 15 hr at 3 v/cm from left to right.

virus was prepared by centrifugation at $41,000 \times g$, and the pelleted virus was purified by sucrose gradient centrifugation. Although large quantities of B and T particles could be visibly detected on the gradients, no radioactivity above background could be detected in either fraction. We cannot calculate the amount of radioactivity we might have expected to find in virus under these conditions, making the significance of this result uncertain.

We did, however, obtain radioactivity in the $41,000 \times g$ supernatant fraction which was precipitable with 60% saturated ammonium sulfate. This material was treated in the usual manner and analyzed by gel electrophoresis. The result (Fig. 9) revealed an electrophoretically diffuse material with one predominant peak of radioactivity. By comparison with other gels of total virus run under identical conditions at the same time, we would locate this peak between viral proteins 3 and 4. This cellular protein appears to be distinct from the viral proteins we previously identified, and we conclude that, under our conditions, we failed to identify a cell-specific, viral-carried antigen.

DISCUSSION

Our results indicate that both B and T particles of VSV contain four protein components when examined by acrylamide gel electrophoresis. In order of increasing mobility, we have called these viral proteins 1, 2, 3, and 4. The complete

correspondence of proteins in each of these particles was not unexpected, since B and T particles appear to be antigenically similar (13) and, except for their length, are morphologically identical when observed by electron microscopy (12).

The approximate molecular weight of each of the viral proteins can be calculated according to the procedure described by Shapiro et al. (18). Using the monomer and dimer of bovine serum albumin as standards, we obtained molecular weight estimates of 230,000, 84,000, 64,000, and 47,000 daltons for virus proteins 1, 2, 3, and 4, respectively.

Virus protein 3 appears to be derived principally from the nucleoprotein core of the virus. Nakai and Howatson (14) described the core as a chain of repeating subunits (90 by 30 by 30 nm) strung together along the viral RNA strand. Assuming a protein density of 1.30 g/cc, we can calculate that a protein of this size would have a molecular weight of 63,000 daltons. The excellent agreement between this calculated value and the estimate from polyacrylamide columns suggests that each physical subunit, as defined by Nakai and Howatson, contains one molecule of virus protein 3.

From our analysis of protein removed from the viral core by DOC treatment, we conclude that viral proteins 2 and 4 are wholly derived from the viral envelope or coat. Some protein similar in electrophoretic mobility to viral protein 3 may also originate in the viral coat but this is considered unlikely. Bradish and Kirkham (2) described the morphology of the viral coat as seen by electron microscopy. The coat appears to consist of three distinct layers, including a fringe of surface projections. It seems unlikely that such a complex structure is composed of only two distinct proteins. We can consider the following alternatives.

(i) Viral protein peaks 2 and 4 are composed of a number of electrophoretically similar proteins. We have already described the possibility of a second protein corresponding to the protein of the 6S antigen which is not readily resolved from virus protein 2. We are currently investigating other techniques for protein separation which may help resolve this problem.

(ii) Some of the viral coat proteins may be derived from cellular membrane proteins formed prior to infection and thus remaining unlabeled, and therefore undetected, in our experiment. In this case, we may have expected the cells, whose protein had been prelabeled for one full generation time prior to infection, to transfer some of this label to viral progeny. Although we failed to detect such transfer, the question remains un-

resolved, since we are unable to calculate the amount of transferred label we may have expected to detect.

(iii) Finally, one of the viral coats may be composed entirely of lipid material. It is known that the virus contains a large proportion of phospholipid (16). Simpson and Hauser (19) showed that the outer viral coat can be disrupted with phospholipase C, with the release of structures which they suggest are composed of cholesterol.

We failed to identify the structural origin of virus protein 1. This protein has an estimated molecular weight of 230,000 daltons. Although it was present in small amounts in all preparations of purified or total virus, it is possible that it represents a polymer of any of the viral proteins 2, 3, or 4 which is particularly resistant to degradation under our procedures.

One of our most unexpected results was the finding that the protein of the 20S antigen had a mobility characteristic of that of the viral nucleoprotein fraction. Relatively little is known about the 20S antigen. Brown and Cartwright (5) described an antigen with a similar sedimentation coefficient which had a rosette-like appearance in the electron microscope and which they suggest is probably derived from the viral coat. In this case, we might have expected the protein of the 20S particle to be identical to either virus protein 2 or virus protein 4. It is possible that the 20S antigen we examined is, in fact, derived from the envelope and is represented by the small amount of protein 3 present in DOC-prepared strip protein. A more intriguing possibility rests on the early observation of Bradish et al. (1) that infectivity is associated with the 20S antigen. In this case, the 20S antigen would be a nucleoprotein; this possibility is currently under investigation.

The origin and functional significance of what we call the 6S antigen remains undecided. Our results suggest that this fraction has a mobility similar to but probably not identical with viral protein 2. Whether this particular antigen represents a viral coat component such as the surface projection, which is either easily lost from the completed virus or released from the infected cell, remains to be determined.

This study of VSV antigens has suggested a number of biologically interesting relationships between the viral antigens. We are currently engaged in a more detailed characterization of each of the identified protein components.

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