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Dissociation of Vesicular Stomatitis Virus and Relation of the Virion Proteins to the Viral Transcriptase

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A procedure has been developed for the sequential removal and purification of the glycoprotein and membrane protein of vesicular stomatitis virus (VSV). Neither of these proteins exhibited transcriptase activity. All of the activity was recovered in the ribonucleic acid (RNA)-ribonucleoprotein complex of VSV, which also has four other minor proteins associated with it. During transcription of 41% of the RNA of a virus preparation, no dissociation of the ribonucleoprotein from the viral RNA was observed.

Vesicular stomatitis virus (VSV) is a bullet-shaped rhabdovirus which contains a virion ribonucleic acid (RNA)-dependent RNA polymerase (3). The virus particles have been shown to possess three major proteins: a glycoprotein, G; a ribonucleoprotein, N; and a membrane protein, M (7, 9-15, 18, 23, 24). The glycoprotein, which is the sole protein containing carbohydrate, is located on the particle surface (12); the ribonucleoprotein is associated with the virion RNA genome and can be observed by electron microscopy to be in the form of a helical structure (20). The membrane protein is supposed to be internal to, or part of, the inner membrane of the virus. Viral "cores" can be obtained by differential centrifugation of detergent- and ether-treated particles, and these have been shown to possess either the N and M proteins or only the N protein, depending on the treatment involved (8-14, 23, 24).

In addition to the three major protein components of the virus, there is a high-molecular-weight protein, L, which is regularly observed on sodium dodecyl sulfate (SDS)-gel electrophoresis of disrupted virions (13, 23, 24). Another virion protein, M-S, present in relatively small quantities, has been observed by Mudd and Summers (18). We have recently observed two other minor virion protein components, A and B, which migrate on gel electrophoresis very close to the N protein.

It is not known which, if any, of the proteins so far observed constitute the virion transcriptase. In the investigation described here, the identification of the virion transcriptase protein has been followed by its RNA polymerase activity. It is clear that neither the glycoprotein (G) nor the membrane protein (M) is part of the transcriptase enzyme, although any of the other five proteins could be components of it.

We have previously shown that transcription of VSV RNA is repetitive and complete (4-6, 19). In a population of virus particles, about 30 to 50% of the total RNA is involved in transcription at 31 C as judged by the ribonuclease resistance of the viral RNA after phenol extraction (4, 19). Since a greater mass of product RNA is synthesized in comparison with the amount of template transcribed (4), two questions can be asked concerning the fate of the ribonucleoprotein or other RNA-associated proteins, during the transcription process. Are they liberated from the RNA during transcription? Do they associate with the product RNA?

We have attempted to answer these questions by following the fate of the ribonucleoprotein and other proteins during transcription of 41% of the mass of viral RNA. We have used two approaches: polyethyleneglycol (PEG)-dextran phase separation (2), and 4% agarose exclusion column chromatography. By either approach, no dissociation of the ribonucleoprotein or other minor proteins from the viral RNA was observed, nor was there any observed association of these proteins with the product RNA.

A procedure has been developed for the sequential removal and purification of the glycoprotein and membrane proteins from VSV virus preparations.

MATERIALS AND METHODS

Preparation of virus, transcriptase reaction conditions, and purification of transcription product RNA. The purification of ^{3}H-uridine-labeled VSV through PEG precipitation, and successive equilibrium and velocity sucrose gradient centrifugations has been described (1, 4, 5, 19). The virus preparation contained no defective particles. After passage through Sephadex G-25 to remove sucrose, the virus was used to prime a 100-fold, 12.50-ml reaction mixture (prewarmed to 31 C), containing ^{32}P-α-uridine triphosphate (UTP) to label the product RNA species. At 0, 1, 2, 3, 4, and 6 hr, 1.5-ml samples were removed from the incubation mixture and the residual solution was taken at 7 hr. A second reaction mixture (1.25 ml), containing no guanosine triphosphate (GTP) or adenosine triphosphate (ATP) was similarly incubated with ^{3}H-VSV for 6 hr and processed in conjunction with the other 6-hr time point. From each time point, 0.5 ml was subtracted, extracted for total RNA (^{3}H and ^{32}P species), and purified from triphosphates; samples were digested with ribonuclease, with or without an annealing pretreatment (4–6, 19).

Phase separation of the reaction product nucleic acids and virion proteins. Amounts of 1 ml of the remaining samples were mixed at room temperature with 0.3 ml of 4 M NaCl and 1.2 ml of an aqueous mixture of PEG 6000-dextran T 500 (Pharmacia, Uppsala, Sweden), followed by another 0.3 ml of 4 M NaCl. The PEG-dextran mixture consisted of 6 g of PEG, 4 g of dextran, and 50 g of water. After shaking for 1 min, the mixture was cooled to 4 C, and the upper PEG phase was separated from the lower dextran phase by centrifugation at 1,000 $\times$ g for 2 min at 4 C. Each phase was carefully and totally separated from the other, and a portion was subtracted to determine the content of RNA (^{3}H and ^{32}P species) by precipitation with cold 5% (w/v) trichloroacetic acid. The remainder of each phase was precipitated with three volumes of cold 5% trichloroacetic acid in an SW41 Spinco centrifuge tube, and the precipitate was collected by centrifugation at 20,000 $\times$ g for 20 min at 4 C with the use of a swinging-bucket Sorvall HB4 rotor. The precipitate was washed by centrifugation with 3 ml of cold 5% trichloroacetic acid to remove residual PEG or dextran, followed by two 3-ml washes with cold absolute alcohol to remove the trichloroacetic acid and Triton. After drying, the protein species in each sample were determined by SDS-polyacrylamide gel electrophoresis.

A preparation of ^{3}H-uridine-labeled VSV in 2 ml of 0.15 M NaCl and 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) was mixed at 20 C with 0.6 ml of Triton N101 (3 mg per ml, final concentration) and 2.4 ml of an aqueous mixture of PEG 6000 and dextran T 500. The mixture was cooled to 4 C and then centrifuged in a narrow tube at 1,000 $\times$ g for 2 min at 4 C. A parallel mixture lacking virus was treated similarly. The upper PEG phase (3 ml) of the virus preparation was carefully removed and reextracted with the lower dextran phase of the second mixture. After centrifugation, the PEG phase was again carefully removed, and a portion was mixed with three volumes of cold 5% (w/v) trichloroacetic acid (to determine the protein species) and stored at 4 C (see Table 1 and Fig. 1, line b). Transcriptase enzyme assays (with or without added VSV RNA) and the presence of labeled viral RNA were determined on portions of the PEG phase (see Table 1, line b). The lower dextran phase (2 ml) of the original virus extract was mixed with the PEG phase of the mixture lacking virus and was similarly reextracted. Protein, RNA, and transcriptase enzyme assays (with or without added viral RNA) were also performed on portions of the dextran phase (see Results and Table 1, Fig. 1, line b).

Agarose gel column chromatography. The residual 2 ml of the 7-hr reaction sample was mixed with sodium deoxycholate (1 mg/ml, final concentration), NaCl (1 M, final concentration), and 0.2 ml of glycerol, and was then loaded on a column of 4% agarose in 0.15 M NaCl, 0.01 M Tris-hydrochloride (pH 8.0), 0.005 M 2-mercaptoethanol, 3 mg of Triton N101 per ml, and 0.01 M $MgCl_2$. The column (90 by 1 cm) was then eluted, at 4 C, with the same solution, and fractions were collected and monitored for acid-insoluble radioactivity (^{3}H and ^{32}P). The position where the labeled triphosphates eluted was determined by a hand geiger counter. Fractions were also monitored for their protein content by gel electrophoresis after two trichloroacetic acid precipitations, followed by two ethanol washes (as described above). Transcriptase enzyme assays were run on each fraction of the column by taking 0.1 ml of the fraction and 0.025 ml of a mixture of the four ribonucleoside triphosphates (containing ^{32}P-α-UTP having the same specific activity as that used in the original incubation mixture). These enzyme reaction mixtures were incubated at 31 C, and samples (50 μliters) were removed at intervals to determine the rate of enzyme activity. Duplicate assays containing 0.5 μg of ^{3}H-VSV RNA per reaction mixture were also performed on each fraction.

Protein gel electrophoresis (22). Virus preparations (1 to 5 mg) were precipitated in 5% (w/v) trichloroacetic acid; the precipitate was recovered by centrifugation at 10,000 $\times$ g for 20 min at 3 C and was washed with absolute alcohol by a similar centrifugation. The pellet was dissolved in 0.2 ml of 1% (w/v) SDS, 0.1% (w/v) 2-mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7.0), and 1.0 M urea, by incubating in a sealed tube at 60 C for 30 min. The solution was mixed with 0.01 ml of 0.05% (w/v) bromophenol blue in water and 0.1 ml of glycerol, and a sample (0.01 to 0.05 ml) was loaded on an 8% polyacrylamide gel. These gels (0.5 cm in diameter, 11 cm long) were prepared as follows. To 13.36 ml of 30% (w/v) acrylamide-0.8% (w/v) N,N'-methylene-bis-acrylamide were added: 5 ml of 1.0 M sodium phosphate buffer (pH 7.0), 0.5 ml of 10% (w/v) SDS, 3 g of urea, and distilled water to a final volume of 49 ml. After addition of 1 ml of catalyst solution containing 0.025 ml of N,N,N',N'-tetramethylethylenediamine and 0.05 g of ammonium persulfate, samples of the mixture were pipetted into glass electrophoresis tubes and allowed to polymerize under a layer of carefully distilled water. Protein electrophoresis was performed at 4 ma per gel for 18 hr with the use of an electrophoresis buffer containing 0.1 M sodium phosphate (pH 7.0). 1.0 M urea, and 0.1% (w/v) SDS. After extrusion of the gel, pro-

teins were stained at 37 C for 40 min in 15 ml of 0.275% (w/v) Coomassie brilliant blue, 50% v/v methanol, and 10% (v/v) glacial acetic acid in water. After removal of the stain, excess dye was eluted from the gel by incubating at 60 C in 30 ml of 7% (v/v) glacial acetic acid and 5% (v/v) methanol in water. The elution buffer was changed four times at 2-hr intervals, and final traces of dye were removed by overnight elution in the same solution. Gels were stored in 7% (v/v) glacial acetic acid in water and were scanned at 640 nm by use of an automatic double-beam spectrodensitometer (model SD3000, Schoeffel Instr. Corp., Westwood, N.J.).

RESULTS

Dissociation of VSV by Triton N101 and sodium chloride. A procedure has been devised for the dissection of VSV based on the separation of Triton N101-treated virion components between PEG and dextran phases (see Materials and Methods). After separation, the PEG phase was found to contain only the glycoprotein (G); all other virion proteins, as well as RNA and endogenous polymerase activity, were recovered in the dextran phase (Table 1, Fig. 1, line b). No templated or endogenous polymerase activity was recovered in the PEG phase, and addition of viral RNA depressed the endogenous polymerase activity in the dextran phase (Table 1).

When the dextran phase was reextracted with PEG, after pretreatment with 1 M NaCl, all of the membrane (M) protein was recovered in the new PEG phase (Table 1, Fig. 1, line d). No RNA or RNA polymerase activity (with or without added RNA) could be demonstrated in this PEG phase (Table 1). The remaining proteins, RNA, and endogenous RNA polymerase activity were all recovered in the new dextran phase (Table 1, Fig. 1, line d). The addition of RNA depressed the endogenous RNA polymerase activity to the same extent as in the previous experiment (see footnote to Table 1).

It can be concluded from these results that neither the glycoprotein nor the membrane protein is part of the virion transcriptase. Both proteins can be prepared free from other virion proteins by this relatively simple technique. Which of the other proteins constitute the virion transcriptase is not known.

It has been found that the same results can be obtained with Triton X100 used in lieu of the

TABLE 1. *Distribution of proteins, RNA, and polymerase activity of detergent-disrupted VSV after polyethylene glycol-dextran extraction*[a]

Treatment	RNA	Polymerase	Protein species						
			L	G	N	A	B	M-S	M
a. Virus, detergent-treated	+	+	+	+	+	+	+	+	+
b. Detergent and then PEG-DEX extractions	DEX (96%)	DEX (110%)	DEX	*PEG*	DEX	DEX	DEX	DEX	DEX
c. Detergent and 1 M NaCl; then PEG-DEX extractions	DEX (96%)	DEX (95%)	DEX	*PEG*	DEX	DEX	DEX	DEX	*PEG*
d. To DEX of b, add NaCl; then PEG-DEX extractions	DEX (96%)	DEX (95%)	DEX	—	DEX	DEX	DEX	DEX	*PEG*

[a] A preparation of ^{3}H-uridine-labeled VSV was dissociated by Triton N101 (3 mg per ml), and the presence of RNA, RNA polymerase (1,100 pmoles per hr per 10^4 counts per min of ^{3}H-RNA), and virion proteins (see Fig. 1, line a) was determined (line a). A similar preparation was treated at 20 C with detergent and then extracted with a mixture of polyethylene glycol 6000 (PEG) and dextran T500 (DEX). After cooling to 4 C, the PEG and DEX phases were separated by centrifugation, recovered, and reextracted (see text for details). The presence in each phase of ^{3}H-RNA and RNA polymerase, as related to the original preparation, was determined, and the phase where they were principally recovered and the amount are given (line b). The presence in either phase of the principal amount of each virion protein is also given. The relative amounts of the various protein species in each phase are shown in Fig. 1, line b. The endogenously templated RNA polymerase activity of the dextran phase (1,210 pmoles per hr per 10^4 counts per min of ^{3}H-RNA) was depressed to 850 pmoles per hr per 10^4 counts per min of ^{3}H-RNA by the addition of 0.5 μg of VSV RNA per reaction mixture. No endogenous, or templated, RNA polymerase was detected in the PEG phase. A similar extraction after detergent dissociation in 1 M NaCl is given in line c (see Fig. 1, line c). No endogenous or templated RNA polymerase activity was detected in the PEG phase. A sample of the DEX phase of line b was treated with NaCl and then extracted with the PEG-DEX mixture; the results are given in line d (see also Fig. 1, line d). Again, no endogenous or templated RNA polymerase activity was detected in the PEG phase.

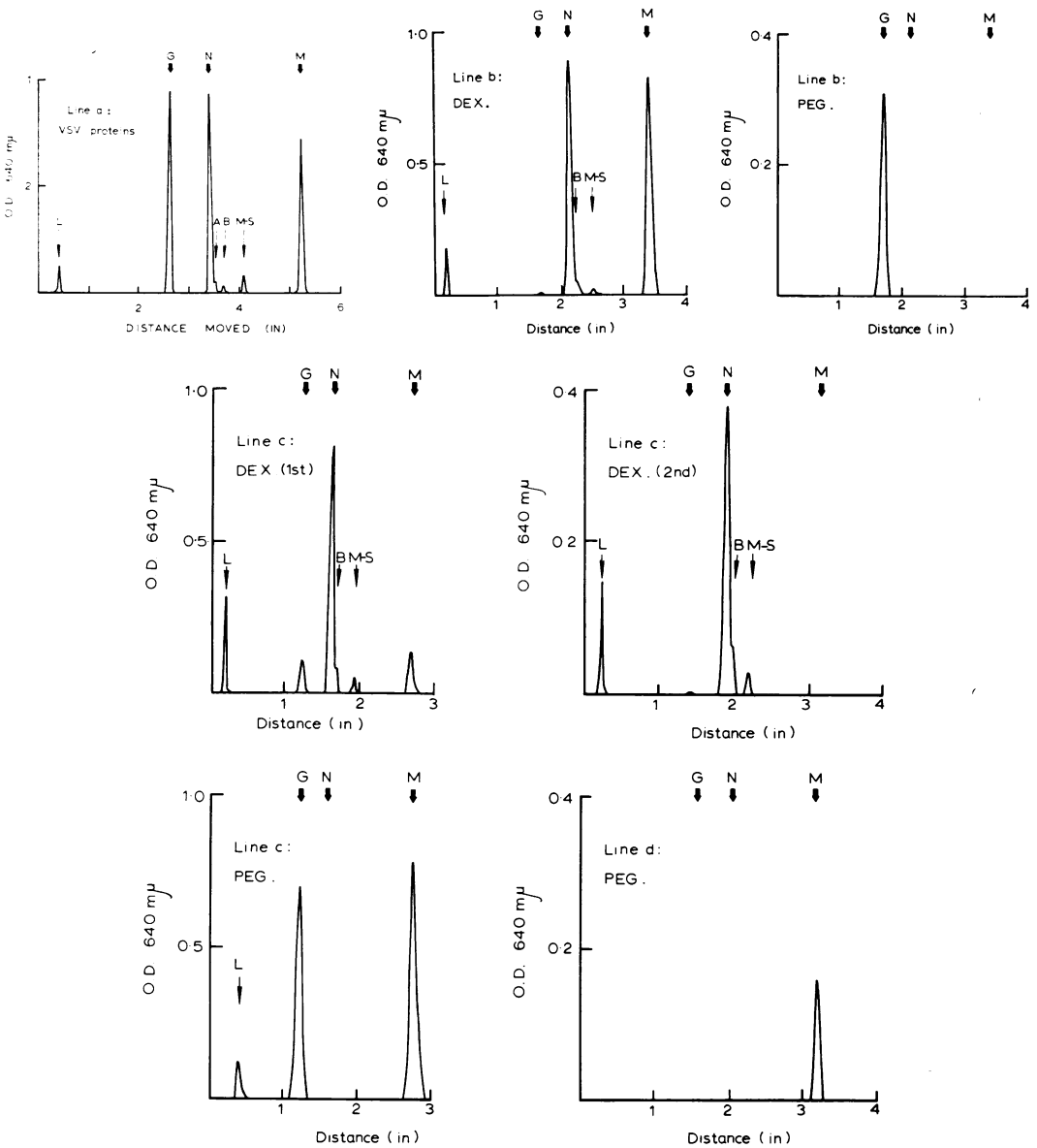

FIG. 1. *Fractionation of VSV proteins by polyethylene glycol-dextran (PEG-DEX) extraction. Preparations of VSV were dissociated with detergent and extracted with PEG-DEX mixtures as described in Table 1. Proteins were obtained from the extracts by trichloroacetic acid and ethanol precipitations (see Materials and Methods), dissociated in SDS-urea, and subjected to electrophoresis in 8% polyacrylamide gels as described in Materials and Methods. All profiles refer to the extractions described in Table 1 (lines a–d). The complete virus is given in the line a profile. The DEX and PEG phases of Triton-dissociated virions (Table 1) are given in the line b profiles. The first and second DEX and pooled PEG extractions of NaCl-detergent dissociated virions are given in the line c profiles. The PEG phase from NaCl reextraction of the DEX phase of the line b profile is given in the line d profile. The corresponding dextran phase of line d was similar to that of line c DEX (2nd) and is not given. The identities of the various proteins (left to right: L, G, N, A, B, M-S, and M) are indicated and were determined from parallel electrophoreses of SDS-dissociated complete virus (see line a). The presence of A protein is not always shown, although on extended electrophoresis it was always present in the fractions which also contained the N, B, and M-S proteins.*

Triton N101. Moreover, if the initial virus dissociation is performed in 1 M NaCl before PEG-dextran extraction and the dextran phase is reextracted by more PEG, all of the G and M proteins are recovered in the PEG phases. A small amount of the L protein is also recovered in the PEG phase, but all of the other proteins, as well as the RNA and transcriptase activity, are recovered in the dextran phase (Table 1, Fig. 1, line c). In Fig. 1, line c, are shown the protein species of the first and second dextran phases to demonstrate the value of the reextraction process by PEG (see Fig. 4).

It has been shown that, in 1 M NaCl, nucleic acids possess a partition coefficient which segregates them into the dextran phase of a PEG-dextran mixture (2). Most free proteins under such conditions are segregated into the PEG phase (2). This suggests that the ribonucleoprotein (N) and other VSV virion proteins recovered in the dextran phase are tightly bound to the virion genome. The reason that the M protein is only liberated from the virion by salt treatment is not known, although it could reflect a strong hydrogen-bonded association with the inner membrane of the virus particle.

RNA content of VSV. From the ratio of protein to RNA in a purified virus preparation, the number of daltons of virion protein can be determined if one knows the molecular weight of the RNA and assumes that there is one RNA molecule per virion. A preparation of ^{3}H-uridine-labeled virus containing no detectable defective particles was found to have 6.44×10^5 counts/min of acid-insoluble ^{3}H-RNA and 2.2 mg of protein, as determined by a Lowry protein assay. The RNA, after extraction and removal of phenol, was found to possess a specific activity of 2.7×10^4 acid-insoluble counts per min per μg of RNA, as determined from the optical density at 260 nm. Therefore, it was calculated that the original virus preparation contained 24 μg of RNA, and the protein to RNA ratio was 92:1. If VSV preparations have 64% of their mass as protein (16), then this RNA represents about 0.7% of the virion mass. Based on the relative electrophoretic mobility of VSV RNA, we have calculated that the VSV genome has a molecular weight of 4.4×10^6 daltons (5). Therefore, if there is only one RNA molecule per virion, then the virion protein is equivalent to 405×10^6 daltons.

Association of the virion ribonucleoprotein with the viral RNA during transcription. To determine the number of N protein molecules per RNA, it is necessary to know the daltons of N protein per virus particle. Based on the calculation in the previous section indicating that there are 405×10^6 daltons of protein per virion genome and the percentage of the total protein as N protein (Table 2), as well as its reported molecular weight of 52,000 (18), we calculated that there are about 2,300 molecules of N protein per virion (Table 2) or one N protein molecule per six nucleotides (see Discussion). The values for the other virion proteins are also given in Table 2.

Transcriptase product RNA is complementary to the virion RNA (1, 3); therefore, we can conclude that the transcription process involves hydrogen-bonding of triphosphates to template nucleotides, and this process is presumably mediated by a transcriptase enzyme (whose composition and mass are at present unknown). Since the template RNA molecules on phenol extraction become ribonuclease-resistant as a result of transcription (4–6), they are therefore (in their extracted form) in a double-stranded structure with product molecules. This suggests that before extraction some product molecules are in juxtaposition to and (or) hydrogen-bonded with their template RNA counterpart.

For all of these reasons, it can be suggested that the transcription process, involving enzyme, triphosphates, template, and product RNA at the growing site of RNA synthesis, could cause a displacement of the N protein from the virion genome. Although these are not obligatory reasons (because we do not know whether the N protein

TABLE 2. *Proteins of vesicular stomatitis virus*[a]

Protein species	Percentage of total protein	Molecular wt	Daltons of protein per virion	No. per virion
L.....	2.5 ± 0.3	175,000	10×10^6	60
G.....	34.2 ± 1	67,000	139×10^6	2,100
N.....	29.8 ± 1	52,000	121×10^6	2,300
A.....	0.8 ± 0.2	50,000	3.3×10^6	65
B.....	1.6 ± 0.2	48,000	6.5×10^6	140
M-S...	2.3 ± 0.2	40,000	9.3×10^6	230
M.....	28.8 ± 1	25,000	117×10^6	4,700

[a] The percentage of total protein is based on a computation of the amount of Coomassie brilliant blue strain bound per protein species (average of three determinations). The molecular weights (except for A and B) are the data of Mudd and Summers (18). The molecular weights of A and B were calculated from Fig. 1, line a. The daltons of protein per virion were calculated as described in the text from the virion protein to RNA ratio (92:1), assuming that the viral RNA has a molecular weight of 4.4×10^6 daltons (5). The number of daltons of protein in each species was then calculated from the percentage of total protein in that species. The number of protein molecules per virion was calculated by dividing the daltons of a protein species by its respective molecular weight.

has a role in the process), displacement and freedom of N protein can be tested by suitable experiments. Moreover, if the N protein is displaced but reassociates with RNA after the active site of RNA synthesis has moved along the genome, any reassociation with the excess of *product RNA* present can also be sought. Experiments were therefore designed to examine the fate of the N protein during transcription of a substantial proportion (41%) of the virion RNA.

The PEG-dextran phase separation described in a previous section is a convenient method for separating the ribonucleoprotein-RNA complex from the two other major virion proteins. Consequently, we decided to determine the fate of the N protein during transcription with regard to whether the process of transcription was sufficient to free it from the RNA so that it could thereafter be recovered in the PEG phase.

A reaction mixture containing ^{3}H-uridine-labeled VSV and ^{32}P-α-UTP, to label the product RNA species, was incubated at 31 C, and samples were removed at intervals to determine the ^{3}H-RNA ribonuclease resistance and the partition of RNA and proteins after 1 M NaCl treatment with the use of *one* PEG-dextran phase extraction (see Table 1, Fig. 1, line c, 1st dextran extract). A control reaction lacking ATP and GTP was incubated for 6 hr at 31 C and then similarly extracted.

It was found that there was an increase in the ^{3}H-RNA ribonuclease resistance from 1.3% for the zero-time sample (unincubated sample) to about 41% for the 180-min and remaining samples (Fig. 2). The ribonuclease resistance of the viral RNA in the control reaction was 1.5% after 6 hr of incubation. After annealing of the reaction product nucleic acids, greater than 90% of the ^{3}H-RNA was rendered ribonuclease-resistant for the 4-hr and subsequent samples. Since the product ribonuclease resistance of the unannealed samples decreased from 24 to 4% for the 1- to 7-hr samples and from 36 to 9% for the corresponding annealed samples, three conclusions can be drawn: (i) 40% of the viral RNA was involved in transcription; (ii) virion RNA was completely transcribed by the enzyme activity; and (iii) more product RNA was made, on a mass basis, than the total amount of viral RNA present.

During PEG-dextran phase separation, for all time points examined, almost all (97%) of the RNA (both product and template species) was recovered in the dextran phase. Almost all of the G and M proteins and a small amount of the L protein were recovered in the PEG phase (see Fig. 3). All of the N protein and minor proteins, together with some of the G and M proteins, were recovered in the dextran phase (Fig. 3). No

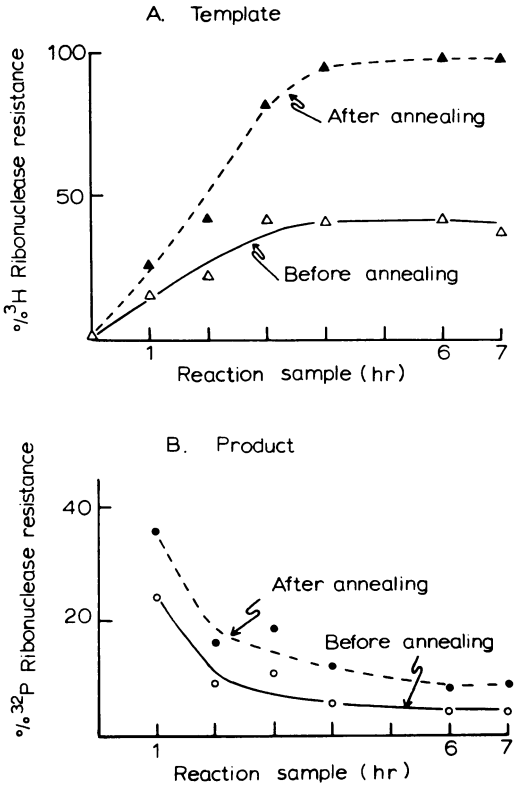

FIG. 2. *Ribonuclease resistance of the RNA from a VSV transcriptase reaction. A reaction mixture containing ^{32}P-α-UTP, to label the product species, was primed by a preparation of ^{3}H-VSV (4). The rate of UMP incorporation was linear through 7 hr of incubation at 31 C and was equivalent to 1,100 pmoles of UMP per hr per 10^4 counts per min of ^{3}H-RNA. Samples were subtracted from the reaction mixture at intervals, and the reaction product nucleic acids were purified as described previously (4). RNA (concentration: 10 μg of ^{3}H-RNA per ml) in 0.01 M phosphate buffer-0.005 M EDTA (pH 7.0) was annealed at 60 C in the presence of 0.4 M NaCl for 120 min. The ribonuclease resistance of the ^{3}H and ^{32}P species (with or without prior annealing) was determined in 0.4 M NaCl by digesting samples with ribonuclease A and T_1 (10 μg of each per ml) for 30 min at 37 C. The percentage of residual acid-insoluble RNA is given as a function of the reaction sample.*

change in protein patterns could be distinguished between any of the samples or with the 6-hr control reaction.

It can be concluded, therefore, that either the ribonucleoprotein was still attached to RNA (viral or product species) or its partition coefficient was such that it segregated, even in 1 M NaCl, with the dextran phase.

Agarose column chromatography of VSV reaction products. In the experiment described in the previous section, the rate of transcriptase en-

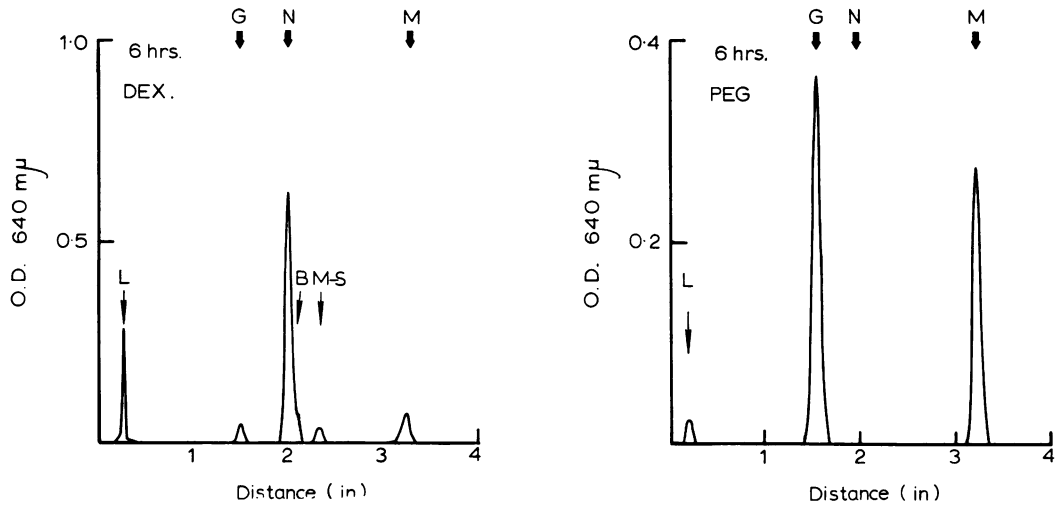

FIG. 3. *Fractionation of viral proteins from a reaction mixture, after polyethylene glycol-dextran (PEG-DEX) extraction. Samples of the reaction mixture described in Fig. 2 were subjected to PEG-DEX extraction and the protein species in each phase (PEG and DEX) were determined (see Table 1, Fig. 1, and Materials and Methods for details). Since only one extraction was used, some residual solution of the PEG phase was present with the DEX layer and accounted for the presence of G and M proteins in that sample (see Fig. 1, line c, DEX (1st). The patterns of protein species of each reaction time point (0 to 7 hr) as well as a 6-hr control reaction, lacking ATP and GTP, were identical to the 6-hr sample shown. The identification of the protein species (L, G, N, B, M-S, and M) was determined from parallel electropherograms of SDS-disrupted complete VSV.*

zyme activity was linear through 7 hr of incubation. It was decided therefore to take the remainder of the reaction mixture (2 ml) and determine whether the ribonucleoprotein (N) was associated with the template or product RNA species by use of another method of analysis. Since the viral RNA is larger than the product RNA species, agarose gel column chromatography was used. In 4% agarose, the RNA-ribonucleoprotein complex of VSV is excluded from the gel beads and elutes in the void fraction of the column (*see below*). Free product RNA species, being smaller (5), elute later in the included fractions of the column.

The 7-hr VSV reaction products were loaded, and then resolved, on a column of 4% agarose (Fig. 4). Each eluant fraction was monitored for acid-insoluble RNA (^{3}H-template and ^{32}P-product species) and RNA polymerase enzyme activity (with or without added VSV RNA). As indicated in Fig. 4, all of the ^{3}H-viral RNA with some ^{32}P-product species and all of the endogenously primed RNA polymerase (expressed as picomoles per hour per 10^{4} counts/min of ^{3}H-RNA) were recovered in the excluded volume of the column. The amount of polymerase activity recovered in these fractions represented 110% of the amount loaded on the column, and its activity was linear through at least 2 hr of further incubation. Templated or endogenously primed polymerase activity was not detected through the rest of the column and is not shown (Fig. 4). Addition of RNA to the excluded fractions of the column depressed the endogenous activity. Most of the ^{32}P-product RNA was recovered throughout the rest of the column—but before the ^{32}P-UTP which was detected by a hand geiger counter.

The indicated column eluants (Fig. 4) were pooled and precipitated with 5% (w/v) trichloroacetic acid; the precipitate was collected by centrifugation and was washed (by centrifugation) with trichloroacetic acid and twice with alcohol prior to being dissolved in SDS-urea for protein analysis. The resulting patterns of protein species in these agarose column fractions are shown in Fig. 5.

All of the N protein was recovered with the ^{3}H-RNA in the excluded fraction of the column (Fig. 5, fraction 1), together with all four minor proteins (although two of them are not resolved from under the N protein in the profile shown). The next four fractions contained no detectable protein. The virion glycoprotein (G) was recovered in fractions 6 through 10, and the membrane (M) protein, in fractions 9 through 11. Some of the largest minor protein (L) was recovered in fractions 7 and 8. The amount this represented, as determined by computation, was about 20 to 30% of the total recovered from the column (fractions 1, 7, and 8). None of the other

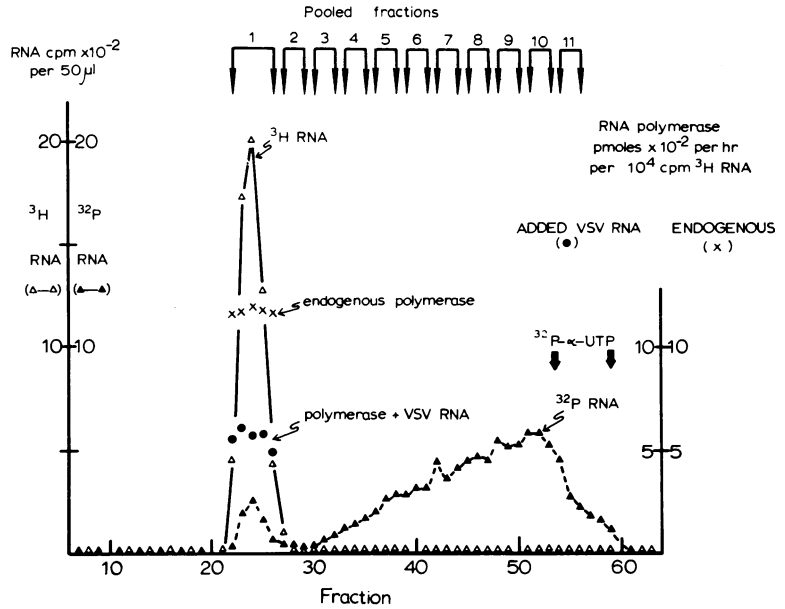

FIG. 4. *Agarose gel column chromatography of VSV reaction products. The residual solution from the 7-hr reaction sample (Fig. 2) was mixed with sodium deoxycholate (pH 7) to give a concentration of 1 mg per ml, NaCl, and glycerol, and was loaded; it was then resolved on a column of 4% agarose (see Materials and Methods for details). Eluant fractions were monitored for their content of acid-insoluble RNA (^{3}H and ^{32}P species), and the RNA polymerase activity was determined (endogenous or with added VSV RNA). The enzyme activity was detected only in the column fractions which contained ^{3}H-RNA and is expressed in these fractions in terms of the picomoles of uridine monophosphate incorporated per hour per 10^{4} counts per min of ^{3}H-RNA. No enzyme activity was obtained in any other eluant fraction and is therefore not shown. Samples of each fraction were combined as indicated (pools 1–11), and proteins were extracted and resolved by polyacrylamide gel electrophoresis (Fig. 5). The elution of ^{32}P-UTP as determined by a hand geiger counter is indicated.*

three minor proteins was detected in fractions 6 through 12.

In conclusion, although 41% of the viral RNA had been transcribed, forming a greater mass of product then template RNA present, none of the N protein was found in association with free product RNA species. All of the N protein, three of the minor proteins, and 70 to 80% of the large minor protein (L) were recovered in the void fractions, which also contained all of the ^{3}H-RNA and all of the endogenously templated RNA polymerase activity. These results suggest, therefore, that these proteins are quite tightly associated with the virion RNA and that some, or all, of them constitute the virion transcriptase. No evidence for displacement of N from the ribonucleoprotein-RNA complex during transcription was obtained.

DISCUSSION

Proteins of VSV. In calculating the number of molecules of each protein species per virion, we have used the protein molecular weights published by Mudd and Summers (18). Their values are in agreement with our unpublished observations with influenza virion proteins for comparison (data not shown). We found that the large minor protein (L) migrates slowly in 8% polyacrylamide gels and is recovered near the gel origin. Since the front of the gel is the surface which is polymerized under water, discrepancies in gel concentration can occur at this surface (hence the value of "loading" gels—of 3% polyacrylamide). No loading gels were used in the analyses described here so that there is some observed variation in the electrophoretic mobility of the L protein relative to the other proteins (see Fig. 1). Using longer times for electrophoresis whereby the migration of L into the gel is greater, or using the other end of the gel for loading, we found that the L protein migrates as if it has a molecular weight of between 165,000 and 145,000. These values are somewhat lower than those of Mudd and Summers (18).

It is not known whether the other minor proteins (A, B, M-S) are constituents of the L protein, separate entities, or degradation products of the N protein. Since they are associated with the RNA-ribonucleoprotein complex of VSV, we conclude that they are not part of the outer con-

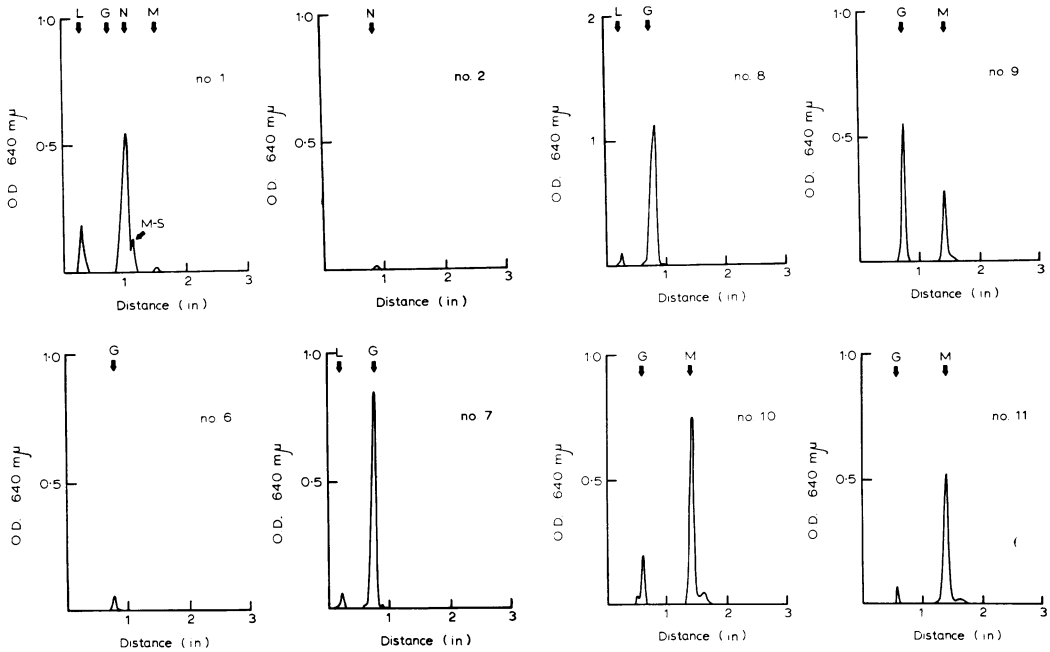

FIG. 5. *Polyacrylamide gel electrophoresis of the proteins from agarose column chromatography of VSV reaction products. Proteins were purified by trichloroacetic acid and ethanol precipitates of the pooled eluants of the agarose chromatogram described in Fig. 4 (see Materials and Methods for details). After dissociation in SDS-urea, the protein species were resolved by polyacrylamide gel electrophoresis. No protein was detected in fractions 3 to 5, and therefore the profiles are not shown. The identification of the various protein species (L, G, N, B, M-S, and M) were obtained by parallel electropherograms of SDS-disrupted VSV virions.*

stituents of the virion, although they could be hydrogen-bonded to any of the outer parts, e. g., M, G, or the lipid layers. The percentages of the total protein present in the minor proteins are not very reliable owing to their small quantities (0.8 to 2.5%). Therefore, the calculation of their number per virion is only approximate (Table 2). Attempts are being undertaken to purify the L and other minor proteins to determine whether they possess any relationship with each other or with the N protein.

We have indicated that the A minor protein segregates through the dextran-PEG extraction and agarose column chromatography with the RNA-ribonucleoprotein complex of VSV. The A protein migrates very close to the N protein (Fig. 1, line a) and can be discerned on long electrophoretic separations under the conditions conditions described. In most of the experiments described in this communication, we have used short periods of electrophoresis and have not demonstrated in such runs the presence of the A protein (Fig. 1, 3, and 5). The use of short electrophoresis periods was intended because we did not want to lose the M protein. The presence or absence of the A protein has been ascertained by subsequent longer electrophoresis (data not shown), and the results are indicated in the text. As can be seen from Fig. 1 and 3, the B protein, after these short electrophoretic separations, appears as a leading edge to the N protein (although it is clearly visible as a distinct band to the eye).

The dalton equivalents of protein in VSV virions have been calculated by reference to the amount of viral RNA per total viral protein. The figure obtained is equivalent to 0.7% RNA per virion based on the determination that the virus has 64% of its mass as protein (16, 17). The percentage of VSV RNA is quite close to the value obtained for rabies virus, and the amount of N protein per virion RNA is also quite close to that of rabies (20). The figures for the molecular weights of each protein species are based on those reported by Mudd and Summers (18), and the values for A and B were calculated by relation to the other proteins (see Fig. 1, line a). The percentage of the total protein in an individual species of VSV protein was determined by computing the amount of Coomassie brilliant blue stain bound by the proteins after separation on polyacrylamide gel electrophoresis (Table 2). Although this calculation assumes that all proteins bind the

stain in proportion to their protein mass, this assumption has been validated by comparing dye-binding results to those involving the distribution of proteins labeled by ^{3}H-amino acids (compare reference 21). We do not know the effect of the carbohydrate moiety of the glycoprotein on its dye-binding capability or electrophoretic mobility, and therefore the figures for the number of G molecules per virion are tentative.

The virion RNA (4.4×10^6 daltons) is composed of approximately 14×10^3 nucleotides (based on an average nucleotide molecular weight of 340). Therefore, we calculated that there are about six nucleotide residues per N protein molecule (Table 2), i.e., 2,000 daltons of RNA per 52,000 daltons of protein. These calculations suggest, therefore, that the protein molecules are probably quite tightly packed along the RNA genome of the virus.

Ribonucleoprotein-RNA complex of VSV. By two approaches (PEG-dextran extraction and agarose gel chromatography), we have been unable to separate VSV RNA from the minor proteins and N protein of the virus. All of the RNA polymerase enzyme activity is recovered, by either approach, with this RNA-ribonucleoprotein complex. Clearly, neither the G nor M protein is part of this RNA polymerase, although any, or all, of the other proteins could be part of the enzyme. The process of transcription apparently does not release the N protein from the complex, and none is recovered with the product RNA. How the N protein remains attached to the RNA through the transcription process is an interesting and challenging problem, and its relationship is being further investigated.

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