JOURNAL OF VIROLOGY, Feb. 1978, p. 675-684
0022-538X/78/0025-0675$02.00/0

Vol. 25, No. 2
Printed in U.S.A.

Inhibition of Transcription by Immunoglobulins Directed Against the Ribonucleoprotein of Homotypic and Heterotypic Vesicular Stomatitis Viruses

ANTHONY R. CARROLL AND ROBERT R. WAGNER*
Department of Microbiology, The University of Virginia School of Medicine, Charlottesville, Virginia 22901

Received for publication 6 October 1977

Specific antisera were raised by immunization of rabbits with purified nucleocapsids containing only RNA and N protein (ribonucleoprotein, RNP) obtained from vesicular stomatitis (VS) virions of the Indiana (VS_{Ind}) and the New Jersey (VS_{NJ}) serotypes. The specificity of anti-RNP_{Ind} serum was demonstrated by selective precipitation of homotypic RNP_{Ind} devoid of L and NS proteins; anti-RNP_{Ind} serum also selectively precipitated soluble N protein present in cytoplasm of infected cells, but co-precipitated a limited amount of contaminating soluble NS protein. Immunoglobulins prepared from each homotypic antiserum markedly inhibited in vitro transcription of VS_{Ind} and VS_{NJ} virions. Anti-RNP_{Ind} and anti-RNP_{NJ} immunoglobulins also exhibited cross-reactivity by inhibiting transcription of heterotypic virions, but only to a much lesser degree than in the homotypic reaction. Anti-RNP_{Ind} immunoglobulin did not inhibit transcription of the antigenically unrelated Chandipura rhabdovirus, but anti-RNP_{NJ} immunoglobulin did to a very limited extent. The transcription inhibitory activity of anti-RNP_{Ind} immunoglobulin was not dependent on RNP immunoprecipitation activity, which could be diluted out well before loss of antitranscriptase activity. Anti-RNP_{Ind} immunoglobulin appeared to exert its effect on transcription by blocking elongation rather than initiation or reinitiation of RNA transcripts.

Vesicular stomatitis (VS) virus and other rhabdoviruses contain a virion-associated, RNA-dependent RNA polymerase (transcriptase) (1, 4). This relatively simple complex is capable of the in vitro synthesis of at least four of the five VS virus mRNA's which are both capped and methylated at the 5′ end and polyadenylated at the 3′ end (7, 22, 23). It has also been recently suggested that the individual mRNA's are formed by processing of a larger transcript (2). This transcriptase activity is associated with the nucleocapsid of VS virus, and it has been demonstrated that both of the minor proteins L and NS plus the N protein-RNA template complex are essential for in vitro RNA synthesis (13, 15, 21).

The N protein is the major structural component of the VS virus nucleocapsid and protects the template RNA from the action of ribonucleases. It also seems likely that the N protein does more than merely protect viral RNA; L and NS proteins alone cannot attach to viral RNA devoid of N protein and cannot by themselves initiate the polymerase reaction (1, 12; Mellon and Emerson, personal communication). Efforts have been made to implicate translocation of N protein as an essential process for transcription of the underlying RNA template (5).

The production of specific antisera directed against the various components of the transcriptase complex may be useful as probes for studying the component parts of the VS virus transcription process. It has previously been reported that antiserum raised against intracellular NS protein is capable of terminating RNA synthesis directed by both virion and intracellular VS virus nucleocapsids (15).

The experiments reported here describe the production of antiserum directed against VS virus ribonucleoprotein (RNP) free of detectable L and NS proteins, and demonstrate that such an antiserum is an effective inhibitor of VS virion in vitro RNA synthesis. Viral RNP was used as the antigen to raise anti-N serum because N protein cannot be solubilized in its native form.

MATERIALS AND METHODS

Cell cultures and viruses. BHK-21 cells and L cells were cultivated as previously described (3). The methods of cultivating and purifying the San Juan strain of Indiana (VS_{Ind}) and the Concan strain of New Jersey (VS_{NJ}) serotypes of VS virus have been described (11). Chandipura virus received from D. H.

L. Bishop was propagated and purified as described for VS_{Ind} and VS_{NJ} viruses. All purified virus was resuspended in reticulocyte standard buffer (RSB: 10 mM KCl-1.5 mM $MgSO_4$-10 mM Tris-hydrochloride, pH 8.0) containing 15% glycerol. Protein concentration was estimated by the method of Lowry et al. (20).

Chemicals and radiochemicals. Nucleoside triphosphates were purchased from Calbiochem, La Jolla, Calif. Triton X-100 and dithiothreitol (DTT) were purchased from Sigma Chemical Co., St. Louis, Mo. Renografin was obtained from E. R. Squibb & Sons, Inc., Princeton, N.J. Freund adjuvant was purchased from Cappel Laboratories, Inc., Cochranville, Pa.

[^{3}H]uridine (28.1 Ci/mmol), ^{14}C-labeled protein hydrolysate (55 mCi/matom), and [^{35}S]methionine (410 Ci/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill. [^{3}H]UTP (12 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.Y.

Polyacrylamide gel electrophoresis. Proteins were analyzed using discontinuous Tris-glycine-buffered sodium dodecyl sulfate (SDS)-slab gels. The gel slabs (100 by 104 by 1.5 mm) were cast in a Hoefer Dual vertical slab gel electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) and consisted of a 10% resolving gel and a 5% stacking gel as described by Laemmli and Favre (18). Samples were mixed with or resuspended in gel sample buffer (6.25 mM Tris-hydrochloride [pH 6.8]-4% SDS-4% DTT-0.01% bromophenol blue) and heated at 100°C for 3 min. Gels were initially electrophoresed at 100 V/gel slab until stacking had occurred; the voltage was then increased to 200 V/slab gel, and electrophoresis was continued until the dye marker reached the bottom of the slab (~2.5 h).

Iodination of whole gels was performed as described by Christopher et al. (9). Fluorography and autoradiography were performed by previously described methods (6, 19).

Production of antisera. Anti-RNP_{Ind} serum was raised by immunization of rabbits with RNP free of detectable L and NS proteins and prepared as described in Results. The antigen (250 μg) was suspended in complete Freund adjuvant and injected intradermally. An equivalent antigenic booster suspended in saline was given intravenously at 7 weeks, and the rabbits were bled 1 week after. Antiwhole VS_{Ind} viral serum was prepared similarly by infection of rabbits with unfractionated VS_{Ind} virions (15). Anti-RNP_{NJ} serum (provided by S. U. Emerson) was raised in rabbits by intradermal and intravenous injections of purified VS_{NJ} RNP, as described for anti-RNP_{Ind} serum.

Preparation of immunoglobulin (Ig) fraction from serum. Because all sera contain RNase activity, it was necessary to prepare an Ig fraction to enable the effect of various antisera on virion transcriptase activity to be tested. The method of preparing an Ig fraction from whole serum was by ammonium sulfate precipitation and chromatography on *O*-(carboxymethyl)-cellulose and DEAE-cellulose, as described previously (15). Throughout the experiments described below, the Ig fraction was reconstituted in 10 mM sodium phosphate and 15 mM NaCl (pH 7.5) buffer.

Antibody titration of anti-RNP serum and Ig fraction. The anti-RNP serum and the Ig fraction of anti-RNP serum were titrated in a radioimmunoprecipitation assay using radioactively labeled RNP free of L and NS proteins. Serial twofold dilutions of serum or Ig fraction were made in 0.9% saline, and 10 μl of each dilution was pipetted into single-assay tubes. Control tubes received either 10 μl of saline or 10 μl of normal rabbit serum. For dilutions of greater than 1:8, 10 μl of 1/5 dilution of normal rabbit serum was added, and the other dilutions received 10 μl of saline. To each tube was added 10 μl of [^{3}H]uridine-labeled RNP, and the tubes were incubated at 37°C for 30 min. Following this incubation, 30 μl of goat anti-rabbit serum was added to dilutions greater than 1:8, and 60 μl was added to the other tubes. The tubes were incubated for a further 30 min at 37°C and then allowed to stand overnight at 4°C. Cold saline (0.5 ml) was added to each tube, which was then centrifuged at 800 × *g* for 30 min. The pellets were washed twice with 0.5 ml of cold saline and resuspended in 0.5 ml of 0.25 N acetic acid. The material was quantitatively transferred to vials by two rinses with 2.5 ml of Triton-toluene scintillation fluid for determination of precipitated radioactivity.

Immunoprecipitation of soluble viral proteins from VS_{Ind} virus-infected L cells. A total of 10^8 L cells infected with VS_{Ind} virus were labeled with ^{14}C-labeled amino acid mixture (20 μCi) 4 to 5 h postinfection. Cytoplasm was prepared by Dounce homogenization of infected cells suspended in 1 ml of RSB; nuclei and particulate material were removed by centrifugation at 150,000 × *g* for 2 h in an SW65 rotor using 0.6-ml insert tubes. Twenty-five-microliter amounts of the cytosol were incubated on ice with 10 μl of preimmune or immune antisera for 30 min. Secondary precipitation of antigen-antibody complexes was performed using A protein bearing *Staphylococcus aureus* (10, 17). One hundred-microliter suspensions of bacteria fixed with 10% formaldehyde in 0.15 M NaCl, 0.01 M Tris-hydrochloride (pH 7.4), and 0.25% Nonidet P-40 were added to each dilution, and incubation was continued on ice for 30 min. The bacterial immune complexes were collected by centrifugation at 800 × *g* for 5 min and washed three times with Nonidet P-40 buffer. The precipitates were resuspended in 200 μl of gel sample buffer and heated at 100°C for 3 min. The bacteria were removed by centrifugation at 800 × *g*, and the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis.

RNA polymerase assay. Transcriptase activity of virions was assayed as previously described (14). One volume of virus suspension in RSB–15% glycerol was treated with 1 volume of 2× Triton-high salt solubilizer for 2 min at 0°C and then mixed with 3 volumes of RSB–15% glycerol and 5 volumes of prereaction mixture consisting of 1.3 mM DTT, 8 mM magnesium acetate, and 50 mM Tris-hydrochloride (pH 8.0) containing 1.4 mM each of ATP, GTP, CTP, and 0.133 mM [^{3}H]UTP (230 μCi/μmole). Complete reaction mixtures were incubated in stoppered tubes at 31°C. At appropriate times, duplicate 100-μl samples were removed and placed into 0.6 ml of 67 mM sodium pyrophosphate containing 200 μg of yeast carrier RNA at 0°C. Ice-cold 25% trichloroacetic acid (0.5 ml) was added, and trichloroacetic acid-insoluble material was measured by scintillation spectroscopy, as previously

described (12). Where various Ig preparations were included in the transcriptase reaction mixtures, the following protocol was adopted. One volume of virus suspension in RSB was added to 1 volume of 2× Triton-high-salt stabilizer (HSS) and held on ice for 2 min. Two volumes of RSB-15% glycerol were then added, followed by 1 volume of Ig in 10 mM sodium phosphate-15 mM NaCl (pH 7.2). Control reactions received 1 volume of phosphate/NaCl buffer alone. Five volumes of prereaction mixture were then added, and reactions were then processed as described above.

RESULTS

Preparation and antigenic characterization of RNP antigen. A major objective of these experiments was to determine whether monospecific antiserum to the nucleocapsid N protein would inhibit in vitro transcription of VS virus. Previous experience by S. U. Emerson (personal communication) indicated almost insurmountable difficulties in solubilizing N protein, and N. F. Moore (quoted in reference 15) was not very successful in raising anti-N serum by injecting rabbits with N protein eluted from SDS-polyacrylamide gels. Therefore, in the present experiments, antiserum was raised by injecting rabbits with preparations of RNP containing, in addition to RNA, the full complement of N protein but devoid of detectable L and NS proteins. It also seemed likely that this specific anti-RNP serum would contain a population of antibody molecules reactive with N protein in its native configuration in association with viral RNA.

The following procedure was used to prepare RNP from purified VS_{Ind} virions. VS_{Ind} virus (4 mg) was diluted to 86 ml with RSB and mixed with 86 ml of 2× HSS (18.7% glycerol-3.74% Triton X-100-1.44 M NaCl-1.2 mM DTT-4.4 mM KCl-0.66 mM $MgSO_4$-4.4 mM Tris-hydrochloride, pH 8.0). After incubation at 37°C for 30 min, the material was placed into six 3.5-inch (ca. 88.9-mm) cellulose nitrate tubes containing 3 ml of 100% Renografin (undiluted, as supplied by Squibb) and 5 ml of 20% Renografin in HSS. The tubes were centrifuged in the SW27 rotor at 52,000 × *g* for 16 h at 4°C. The material appearing at the interface of the 20 and 100% Renografin was collected and diluted to 15 ml with RSB. To this was added 15 ml of 2× HSS, and the material was then resedimented onto a Renografin cushion in a single tube (3.5 by 1 inch [ca. 88.9 by 25.4 mm]) as described above. The material at the interface was again collected and dialyzed against 10 mM Tris-hydrochloride (pH 7.2) for 16 h. The dialyzed material was diluted to 4.63 ml with 10 mM Tris-hydrochloride (pH 7.2), and 1.46 g of CsCl was added. The material was placed in a polyallomer tube (2 by 0.5 inches [ca. 50.8 by 12.7 mm]) and centrifuged in an SW65 rotor at 115,000 × *g* for 48 h at 22°C. The visible band at an approximate density of 1.3 g/cm^3 was collected and dialyzed against 10 mM Tris-hydrochloride (pH 7.2) for 16 h.

A sample of the RNP preparation was dissociated in 2% SDS-2% DTT at 100°C for 3 min, and the proteins were analyzed by electrophoresis in an SDS Tris-glycine-buffered 10% polyacrylamide slab gel. After electrophoresis, the whole gel was iodinated as described by Christopher et al. (9) to increase sensitivity and the number of protein bands detected by autoradiography.

Figure 1 shows the autoradiogram of the RNP preparation and also a co-electrophoresed sample of whole VS_{Ind} virus. The RNP contained only a single polypeptide comigrating with the viral N protein. No other minor bands appeared upon prolonged exposure of the X-ray film.

Assays of anti-RNP_{Ind} serum by radioimmunoprecipitation. The antibody activity of anti-RNP_{Ind} serum was analyzed in two ways: first, by its ability to precipitate purified RNP containing only N protein and RNA; and second, by the degree to which it would precipitate soluble viral proteins from cytoplasmic extracts of infected cells.

Indirect radioimmunoprecipitation of RNP free of detectable L and NS proteins was performed using serial dilutions of the anti-RNP_{Ind} serum. The result demonstrates that this serum was capable of precipitating RNP and that when using 10 μg of RNP_{Ind} antigen, the serum had a 50% precipitation end point at a dilution of approximately 1:240 (Fig. 2). In the experiments described in Fig. 2, RNP labeled either in the protein with [^{35}S]methionine or in the RNA with [^{3}H]uridine gave essentially the same result.

In another experiment [^{3}H]uridine-labeled whole virus, disrupted with 2% Triton and 0.72 M NaCl, was used as antigen in titrating the anti-RNP_{Ind} serum. Again, a similar 50% end point was obtained at a dilution of 1:200 of the antiserum (data not shown). The anti-RNP_{Ind} serum was therefore equally capable of precipitating RNP in the absence or presence of all other virion components. This would suggest that this serum had considerable anti-RNP activity (presumably directed against the N protein) and that if it did contain antibodies against other virion components, they did not interfere with immunoprecipitation of virion RNP.

Indirect precipitin tests using A protein bearing *Staphylococcus aureus* (10, 17) for co-precipitation of antigen-antibody complexes were also used to determine which viral proteins in extracts of infected cell cytoplasm formed complexes with either anti-RNP_{Ind} serum or anti-

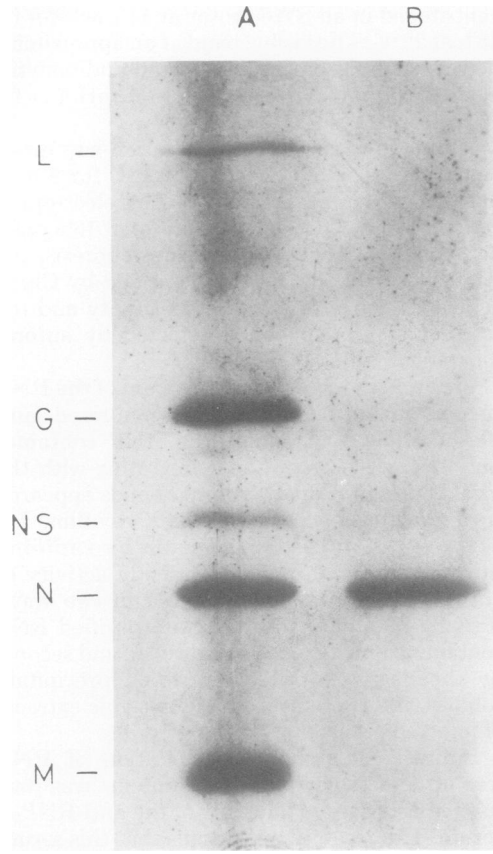


FIG. 1. *Autoradiogram of iodinated VS viral proteins following electrophoresis on a 10% polyacrylamide-SDS slab gel of (A) 30 μg of purified VS_{Ind} virus and (B) 10 μg VS_{Ind} virus RNP purified by HSS and CsCl centrifugation. Samples for electrophoresis were mixed with equal volumes of electrophoresis sample buffer containing 4% SDS, 4% DTT, 0.065 M Tris (pH 6.8), 20% glycerol, and 0.01% bromophenol blue tracking dye and heated at 100°C for 3 min. Electrophoresis using an SDS Tris-glycine buffering system was performed at 200 V after stacking for approximately 2 h and continued until the tracking dye reached the end of the gel. The gel was fixed, stained, and then iodinated as described by Christopher et al. (9). The dried iodinated gel was subjected to autoradiography for 16 h using Kodak SB-54 X-ray film. Note the absence of L and NS protein in RNP.*

whole VS_{Ind} viral serum.

L cells infected with VS_{Ind} virus were labeled with ^{14}C-amino acids, and a supernatant fraction (150,000 × *g*) of the cytoplasmic extract was prepared from 5-h cultures. Anti-RNP_{Ind} or antiwhole viral serum was incubated with the infected cytoplasmic supernatant fraction for 30 min at 0°C. The resulting immune complexes were precipitated using A protein bearing *S. aureus*. The precipitates were washed and dissolved in gel sample buffer by heating at 100°C for 3 min and analyzed for ^{14}C-labeled proteins by SDS-polyacrylamide gel electrophoresis and fluorography.

Figure 3(I) shows the fluorogram of the proteins precipitated by anti-RNP_{Ind} (B) and antiwhole VS_{Ind} viral serum (C). No viral proteins were precipitated by preimmune rabbit serum (D). The total ^{14}C-labeled proteins present in an equivalent sample of supernatant cytoplasm are also shown for comparison (A).

Since a fluorographic procedure was used, the distribution of radioactivity in the sample can be accurately represented by microdensitometry of the image obtained on presensitized X-ray film (19). Microdensitometer scans were made from the fluorogram shown in Fig. 3(I), and the percentage of each viral protein precipitated by the various sera was calculated (Fig. 3[II]). These data demonstrate the specificity of the anti-RNP_{Ind} serum for N protein. The low level of precipitation of NS protein could be due to a small amount of anti-NS activity present in the

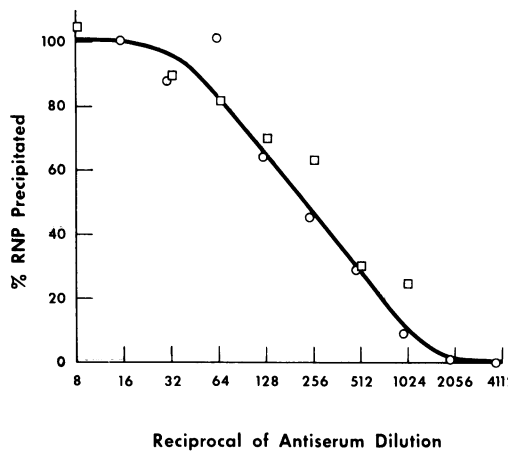
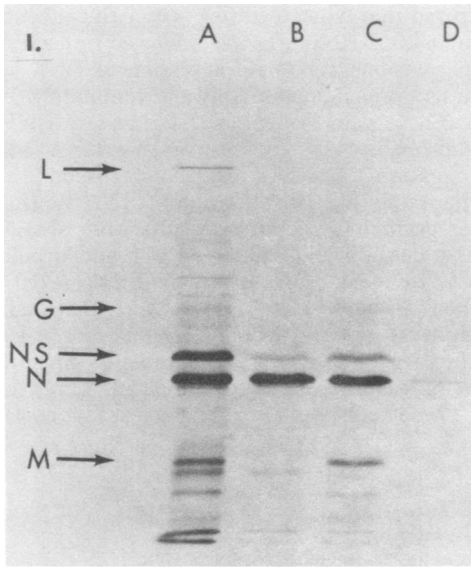


FIG. 2. *Titration of anti-RNP_{Ind} serum by radioimmunoprecipitation of VS_{Ind} RNP free of detectable L and NS proteins and labeled with either [^{35}S]methionine (○) or [^{3}H]uridine (□) in separate experiments. Purified RNP distributed in 10-μl volumes, each containing 10 μg of protein, was mixed with serial twofold dilutions of anti-RNP_{Ind} serum followed by incubation with hyperimmune goat anti-rabbit serum, as described in Materials and Methods. The immune precipitates were collected by centrifugation and washed three times before determination of precipitated radioactivity by scintillation counting. Calculation of the percentage of immunoprecipitation was based on values of 2,575 ^{35}S cpm and 1,650 ^{3}H cpm of trichloroacetic acid-precipitable RNP. Subtracted from these calculations were 450 ^{35}S cpm and 278 ^{3}H cpm of RNP nonspecifically precipitable by control preimmune rabbit serum.*



II.

	B	C	D
L	nd	23	nd
G	nd	67	nd
NS	11	30	nd
N	92	73	4
M	nd	70	nd

% of each polypeptide precipitated

FIG. 3. *Fluorogram of polyacrylamide slab gel (I) and densitometry quantitation of viral proteins (II) following indirect radioimmunoprecipitation by anti-RNP_{Ind} and antiwhole VS_{Ind} virion sera of soluble ^{14}C-labeled VS_{Ind} virus proteins from cytoplasmic extracts of infected cells. As described in more detail in Materials and Methods, the cytoplasm of VS_{Ind}-infected L cells, labeled from 4 to 5 h postinfection with a ^{14}C-amino acid mixture, was centrifuged at 150,000 × g for 2 h. The supernatant cytoplasm (25 μl) was incubated with (B) anti-RNP_{Ind} serum, (C) antiwhole VS_{Ind} virus, and (D) preimmune rabbit serum followed by A protein-bearing staphylococci. The precipitated proteins were analyzed on a 10% SDS slab gel along with an equivalent sample (25 μl) of unprecipitated cytoplasm to determine total VS viral L, G, NS, N, and M proteins present (see track A). The slab gel was analyzed by fluorography, and the fluorogram was further analyzed by scanning densitometry to calculate the percentage of each viral protein precipitated by each antiserum. nd, Not detected.*

anti-RNP_{Ind} serum or, more likely, to co-precipitation of NS protein in nucleocapsids or N-NS complexes. In this respect it should be remembered that any residual intracellular nucleocapsids present in the supernatant cytoplasmic extract would presumably have associated NS protein, and these nucleocapsids would be precipitated by the anti-RNP_{Ind} serum. As additional evidence that co-precipitation of NS protein was not due to contaminating NS antibody was the finding (data not shown) that a 1:5 dilution of the anti-RNP_{Ind} serum, rather than precipitating less NS protein, co-precipitated the same 10 to 11% of NS protein as did the undiluted anti-RNP_{Ind} serum (see Fig. 3).

Effect of anti-RNP_{Ind} on in vitro transcriptase activity of VS_{Ind} and Chandipura viruses. Because of the presence of RNase in normal or immune whole rabbit serum, it was necessary to produce an RNase-free Ig fraction. This was done by $(NH_4)_2SO_4$ precipitation and chromatography on *O*-(carboxymethyl)-cellulose and DEAE-cellulose as described previously (15). The anti-RNP_{Ind} Ig was tested in a standard transcription assay (see Materials and Methods) using purified whole virus. The Ig in 10 mM sodium phosphate–15 mM NaCl buffer (pH 7.2) was incorporated into the reaction mixture as 10% of the final volume, and controls received an equal volume of phosphate buffer.

Figure 4 shows the comparative transcriptase activities of VS_{Ind} and Chandipura viruses in the presence and absence of anti-RNP_{Ind} Ig. At a concentration of 50 μg of Ig/0.1 ml of VS_{Ind}, transcription was inhibited to greater than 95% of the control activity. Inhibition was proportionately reduced by progressive dilution of the anti-RNP_{Ind} Ig preparation. In contrast, 50 μg Ig/0.1 ml had no effect on the transcriptase activity of Chandipura virus. Furthermore, an Ig fraction prepared from normal rabbit serum had no significant effect on in vitro transcription by VS_{Ind} virus (see Table 1).

Effect on RNA synthesis of anti-RNP_{Ind} Ig added after initiation of transcription. It was of interest to compare the antitranscriptase activity of anti-RNP_{Ind} Ig with that of anti-NS Ig, which appears to act on RNA transcript chain elongation (15). One would anticipate that if anti-RNP Ig acts at the site of initiation, RNA synthesis should continue for a time, owing to elongation of already-initiated chains. If, however, anti-RNP_{Ind} Ig acts like anti-NS Ig only on chain elongation, one would expect rapid switch-off of all RNA synthesis when anti-RNP Ig is added during any stage of transcription.

The effect of addition of anti-RNP_{Ind} Ig to actively transcribing VS_{Ind} virions was examined using two concentrations of Ig. First, a concentration of Ig was used which, when added prior to initiation of transcription, would result in complete inhibition. Secondly, a concentration

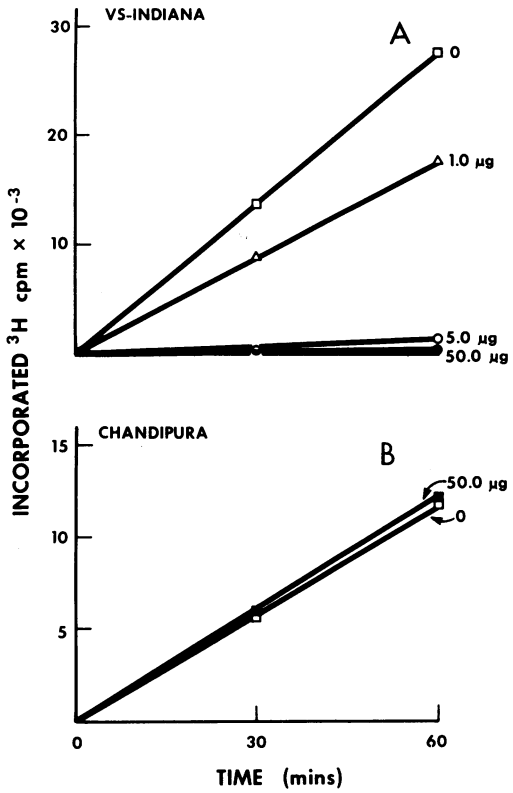


FIG. 4. *Effect of anti-RNP$_{Ind}$* Ig on VS$_{Ind}$ *(A) and Chandipura (B) virion transcriptase activity. Reaction mixtures were prepared (see Materials and Methods) containing 20 µg of viral protein plus the indicated amounts (µg of protein) of anti-RNP$_{Ind}$ Ig per 0.1 ml. Reactions were incubated at 31°C in stoppered reaction tubes. Duplicate 0.1-ml samples were removed at 0, 30, and 60 min, and incorporation of ^{3}H[UMP] into trichloroacetic acid-insoluble material was determined.*

of Ig was used which, when added prior to initiation, would cause approximately 50% inhibition.

Standard in vitro transcriptase reaction mixtures were prepared containing 15 µg of VS$_{Ind}$ virus per 0.1 ml but minus 1 volume of RSB–15% glycerol. The reactions were incubated at 31°C, and 1 volume of anti-RNP$_{Ind}$ Ig was added at time 0 or 1 volume of anti-RNP$_{Ind}$ Ig or phosphate buffer was added at the times indicated. Final concentrations of anti-RNP$_{Ind}$ Ig were either 50 µg/100 µl (for complete inhibition of transcription at time 0) or 2.5 µg/100 µl (for partial inhibition of transcription at time 0). Samples were removed at various times and analyzed for trichloroacetic acid-insoluble [^{3}H] RNA.

The results of these experiments demonstrated that when a sufficiently high concentration of anti-RNP$_{Ind}$ Ig was added to transcribing VS$_{Ind}$ virions, further progression of RNA synthesis stopped immediately and completely (Fig. 5). These data would suggest that anti-RNP$_{Ind}$ Ig probably acts by interrupting RNA chain elongation. Furthermore, under conditions where only partial inhibition of RNA synthesis was occurring, the same results were obtained regardless of whether the Ig was added before or after initiation of RNA synthesis. That is, when a low level of Ig was added 1 h after initiation, RNA synthesis continued for at least a further 3 h at a rate comparable to that when the Ig was added prior to initiation.

These data would further suggest that specific

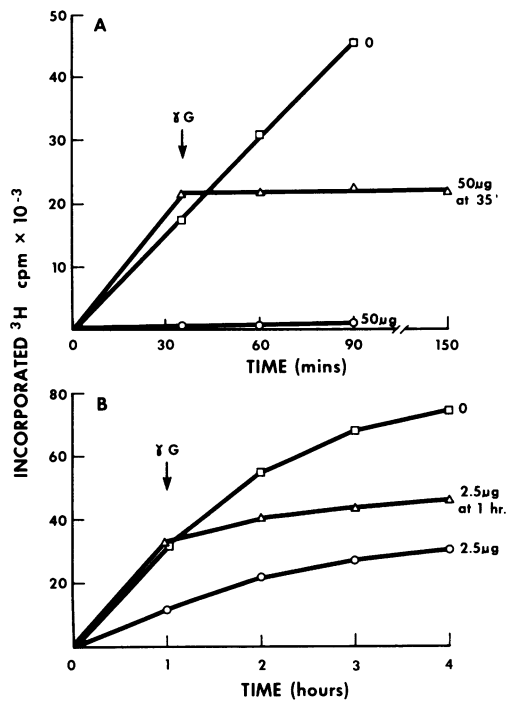


FIG. 5. *Effect of addition of anti-RNP$_{Ind}$ Ig to VS$_{Ind}$ virus before or after (arrow) initiation of transcription. (A) Sufficient anti-RNP$_{Ind}$ Ig (50 µg) to completely inhibit in vitro transcriptase activity, when present at zero time, was added to actively transcribing virions 35 min post-initiation; a separate standard transcriptase reaction mixture served as a control and received phosphate buffer at 35 min. (B) Sufficient anti-RNP$_{Ind}$ Ig to partially inhibit (approximately 50%) transcriptase activity, when present at zero time, was added to actively transcribing virions 1 h post-initiation; phosphate buffer was added to a control reaction mixture 1 h post-initiation. Duplicate 0.1-ml samples were removed at the indicated times, and incorporation of [^{3}H]UMP into trichloroacetic acid-insoluble material was determined.*

anti-RNP Ig inhibits chain elongation rather than exerting its primary effect on re-initiation.

Comparative effects of anti-RNP Ig on transcriptase inhibition and immunoprecipitation of virion RNP. In an initial attempt to determine the mechanism of action of anti-RNP_{Ind} Ig, the relationship between transcriptase inhibition and direct immunoprecipitation of RNP was compared quantitatively. Transcriptase inhibition was measured (as previously described) in 0.1 ml of reaction mixtures containing either 30 or 3 μg of [^{3}H]uridine-labeled VS_{Ind} virus (3,396 cpm/30 μg). Concentrations per 0.1 ml of anti-RNP_{Ind} Ig were selected for virus concentrations of 30 or 3 μg which gave either >95% inhibition of transcription (50 or 2.5 μg, respectively) or approximately 50% inhibition of transcription (5 or 0.5 μg, respectively).

By using VS virus labeled in the RNA it was possible to determine simultaneously the degree of immunoprecipitation of RNP by measuring the extent of [^{3}H]RNA precipitated. It had previously been found that the immunoprecipitin titers of anti-RNP serum were the same for purified RNP containing only N protein and RNA as they were for whole disrupted virus. This indicates that the other viral proteins do not influence the precipitation of RNP by anti-RNP serum. To determine the degree of [^{3}H]-RNP immunoprecipitation, standard transcriptase reactions were prepared which lacked [^{3}H]UTP. For reactions containing 30 μg of virus per 0.1 ml, volumes of 0.1 ml were used, whereas for reactions containing 3 μg of virus per 0.1 ml, reaction volumes of 1 ml were used. The previously determined levels of anti-RNP_{Ind} Ig were added (see above) to the reactions, and after incubation at 31°C for 60 min the extent of immunoprecipitation of RNP was determined.

The results shown in Table 1 demonstrate that there was no direct relationship between immunoprecipitation of virion RNP by anti-RNP Ig and inhibition of transcriptase activity. Under conditions where transcription was inhibited by >95%, only approximately 50% of the RNP was precipitated by the same concentration of Ig. Moreover, at approximately 50% inhibition of transcription by anti-RNP Ig, no precipitation of RNP occurred.

It would appear, therefore, that inhibition of transcription by anti-RNP Ig is not simply a result of precipitation of the template RNP. However, it is not possible to determine from these data the relative proportions of antibody molecules that inhibit transcription or result in formation of precipitable or nonprecipitable antibody-RNP complexes.

Effect of anti-RNP_{Ind} Ig on VS_{NJ} virus transcriptase. Although the type-specific surface antigens (glycoproteins) of VS_{Ind} and VS_{NJ} viruses are antigenically unrelated, cross-reactivity of the core RNP antigens (N proteins) has been reported (8, 16). We therefore tested the effect of anti-RNP_{Ind} Ig on the in vitro transcriptase activity of VS_{NJ} virus.

TABLE 1. *Relative degree of VS_{Ind} virus transcriptase inhibition and immunoprecipitation produced by different concentrations of anti-RNP_{Ind} Ig*

Anti-RNP_{Ind} Ig (μg)	Transcriptase activity[a] (% of control VS_{Ind} virus) at virus concn of:		Immunoprecipitation[b] (% of total ^{3}H-RNA) at virus concn of:	
	0.3 mg/ml	0.03 mg/ml	0.3 mg/ml	0.03 mg/ml
0[c]	95[c]	100	5.4	5.0
50	0		57	
5				70
2.5	57	3	5.0	53
0.5		57		3.6

[a] Duplicate 100-μl standard reaction mixtures containing either 30 or 3 μg of [^{3}H]uridine-labeled VS_{Ind} virus (3,396 cpm/30 μg) were incubated with 0, 2.5, or 50 μg of anti-RNP_{Ind} or normal rabbit serum Ig for 60 min at 31°C. The incorporation of ^{3}H[UMP] into trichloroacetic acid-insoluble material was determined, and the inhibition was calculated as a percentage of controls.

[b] To determine immunoprecipitation, identical reaction mixtures were prepared which lacked [^{3}H]UTP. For reactions containing 3 μg of VS_{Ind} virus per 100 μl, reaction volumes of 1 ml were used. Amounts of the various Ig fractions identical to those used in the transcriptase inhibition study were added to the reactions, and the samples were incubated at 31° for 60 min. Precipitable complexes were sedimented at 2,000 × *g* for 15 min and washed 3 times with phosphate-buffered saline (1 ml). The precipitates were dissolved in 0.5 ml of 0.5 N acetic acid and transferred to a Triton/toluene scintillant for the determination of precipitated radioactivity. Total trichloroacetic acid-precipitable radioactivity was also determined, and the immunoprecipitable radioactivity was expressed as a percentage of the total.

[c] Control contained 50 μg of Ig from normal rabbit serum.

Preliminary experiments indicated that anti-RNP_{Ind} Ig was far less inhibitory against VS_{NJ} than against VS_{Ind}. To obtain significant levels of inhibition, it was necessary to use low concentrations of VS_{NJ} virus and high concentrations of Ig. Reaction volumes of 250 μl were used, containing 5 μg of VS_{NJ} virions and up to 125 μg of anti-RNP_{Ind} Ig.

The data shown in Table 2 demonstrate that under these conditions anti-RNP_{Ind} Ig inhibited VS_{NJ} transcription by 80%; progressively lower amounts of Ig resulted in lower levels of inhibition. Table 2 also shows that under these con-

ditions transcription by VS_{Ind} virus was almost completely (>95%) inhibited by approximately 5 μg of anti-RNP_{Ind} Ig.

To rule out nonspecific effects at such high concentrations of Ig and low concentrations of virus, Chandipura virus was tested under comparable conditions, and its transcriptional activity was found to be unaffected by anti-RNP_{Ind} Ig (Table 2).

It would appear, therefore, that anti-RNP_{Ind} Ig had limited but significant activity in inhibiting in vitro transcription of heterotypic VS_{NJ} virus. However, the antibody was approximately 50 times more efficient in the homotypic reaction as compared with the heterotypic reaction, which may reflect the extent of immunogenic cross-reactivity of the RNP of VS_{Ind} and VS_{NJ} viruses.

Comparative effects of anti-RNP_{NJ} Ig on in vitro transcription of VS_{NJ}, VS_{Ind}, and Chandipura viruses. We were interested to know if antisera prepared specifically against VS_{NJ} virus RNP would be as efficient an inhibitor of VS_{NJ} virus transcriptase as anti-RNP_{Ind} Ig was of VS_{Ind} virus transcriptase. Furthermore, was the low level of cross-reactivity of anti-RNP_{Ind} Ig against VS_{NJ} virus conversely true for anti-RNP_{NJ} Ig against VS_{Ind} virus transcriptase?

Antiserum against VS_{NJ} RNP was raised by the same method as described above for anti-RNP_{Ind} serum. A ribonuclease-free Ig fraction was prepared (as described in Materials and Methods) and tested against the transcriptase activities of VS_{NJ}, VS_{Ind}, and Chandipura viruses in a standard transcriptase assay.

TABLE 2. *Comparative effects of anti-RNP_{Ind} Ig on in vitro polymerase activity of homotypic VS_{Ind} virus and heterotypic VS_{NJ} and Chandipura viruses*[a]

Anti-RNP_{Ind} Ig (μg)	Transcription (cpm/0.25 ml per 90 min)		
	VS_{NJ} virus	VS_{Ind} virus	Chandipura virus
0	3,170	12,725	3,320
5		455	
12.5	3,030		
42	2,100		
125	660		3,420

[a] Duplicate 0.25-ml standard RNA polymerase reaction mixtures were prepared, containing 5 μg of protein of VS_{NJ}, VS_{Ind}, or Chandipura virus. The indicated amounts of anti-RNP_{Ind} or normal rabbit Ig were added, and the reactions were incubated at 31°C for 90 min. The incorporation of [^{3}H]UMP into trichloroacetic acid-insoluble material was determined as described in Materials and Methods. Ig from normal rabbit serum (125 μg) resulted in incorporation by VS_{Ind} virus of 12,675 cpm/0.25 ml in 90 min.

TABLE 3. *Comparative effects of anti-RNP_{NJ} Ig on VS_{NJ}, VS_{Ind}, and Chandipura virus in vitro RNA polymerase activity*[a]

Anti-RNP_{NJ} Ig (μg/0.1 ml)	Transcription (cpm/0.1 ml per 120 min)			
	VS_{NJ} virus (30 μg)	VS_{Ind} virus (30 μg)	VS_{Ind} virus (2 μg)	Chandipura virus (2 μg)
0	13,500	43,080	5,030	2,070
0.5	12,400			
1.0	7,550			
5	4,950			
50	850	42,700	2,604	800

[a] Standard RNA polymerase reaction mixtures were prepared, containing the indicated amounts (micrograms of protein per 0.1 ml) of VS_{NJ}, VS_{Ind}, or Chandipura virus. The indicated amounts of anti-RNP_{NJ} Ig were added, and the reactions were incubated at 31°C. Duplicate 0.1-ml samples were removed at 120 min, and the incorporation of ^{3}H[UMP] into trichloroacetic acid-insoluble material was determined as described in Materials and Methods.

Table 3 illustrates that, in 0.1-ml reactions containing 30 μg of VS_{NJ} viral protein, the anti-RNP_{NJ} Ig exhibited considerable inhibitory activity against the in vitro transcriptase. At a concentration of 50 μg/0.1 ml, anti-RNP_{NJ} caused a 95% inhibition in RNA synthesis by VS_{NJ} virions. The levels of antitranscriptase activity were comparable to the activity of anti-RNP_{Ind} against VS_{Ind} virus (compare Table 3 with Fig. 4).

Under the same conditions (30 μg of virus and 50 μg of Ig per 0.1 ml) anti-RNP_{NJ} Ig had no effect on the in vitro transcriptase activity of VS_{Ind} virus (Table 3). However, as previously noted (Table 2), to demonstrate inhibition of VS_{NJ} virus transcriptase by anti-RNP_{Ind} Ig, it was necessary to use low concentrations of virus and high concentrations of Ig. A similar experiment was therefore performed using anti-RNP_{NJ} against VS_{Ind} and Chandipura viruses.

Standard reaction mixtures were prepared containing 2 μg of either VS_{Ind} or Chandipura virus and 0 or 80 μg of anti-RNP_{NJ} Ig. The extent of RNA synthesis was determined after 2 h at 31°C. The results illustrate that, under these conditions, anti-RNP_{NJ} Ig was active to a limited extent in inhibiting transcription of both VS_{Ind} and Chandipura viruses (Table 3). Since Chandipura virus was assumed to be antigenically unrelated to VS_{NJ} virus and was included in these experiments as a control against nonspecific effects of the antisera, the significance of this result is unclear. The Ig fraction of normal rabbit sera was tested under a variety of condi-

tions (up to 50 μg/0.1 ml) and was never found to have any effect on the in vitro transcription of VS_{Ind}, VS_{NJ}, or Chandipura virus (data not shown).

DISCUSSION

The enzymology of the VS virion transcriptase has been worked out in some detail by Emerson and her colleagues (4, 12, 13). It is clear that both the L and NS proteins are required to be associated with the nucleocapsid template before transcription can occur. Enzymatic activity is lost when the L protein alone or L and NS proteins together are stripped off the nucleocapsid in a high-salt environment (12, 13). Transcriptase activity is restored by reconstitution of RNP with L and NS proteins. Recent data reveal that homotypic NS protein can bind independently to stripped nucleocapsids of VS_{NJ} virus, but binding of L protein to trigger transcriptional events requires prior binding of NS protein to RNP (Mellon and Emerson, in press). The transcriptase complex is specific for each rhabdovirus serotype; L and NS proteins of VS_{Ind} or VS_{NJ} virus cannot restore transcriptase activity to the heterotypic nucleocapsid (4).

The N protein of the nucleocapsid also appears to play an important role in the transcriptase complex, above and beyond its structural function in enclosing and protecting the RNA genome. The N protein almost undoubtedly provides one or more sites for binding of the tightly attached NS protein. The N protein forms an impermeable sheath around the template RNA and is so tightly intertwined that it is difficult to dissociate and almost impossible to solubilize in anything milder than SDS (Emerson, personal communication). Active transcription does not dislocate the N protein enough to make the virion RNA accessible to digestion by ribonuclease (5). It is not unlikely that the N protein possesses an enzymatic function or at least serves as an essential cofactor for the NS and/or L protein. However, no specific functions have been assigned to any of the three nucleocapsid proteins. In fact, the VS viral transcription process has not been successfully dissociated into its component reactions of initiation, chain elongation, termination, polyadenylation, and capping. The only known inhibitors of specific stages of the transcription reaction are nonspecific reagents such as aurintricarboxylic acid and polyethylene sulfonate which appear to act at or near the transcription initiation site (14) and antibodies to NS protein (15) and RNP, as reported in this paper, which inhibit chain elongation.

The data reported here represent another attempt to probe the transcriptase functions of the various nucleocapsid proteins by the use of specific antisera to perturb the transcriptional events. Antiserum was raised by injecting rabbits with RNP complexes of N protein and RNA devoid of L and NS proteins; it was not possible to obtain N protein monomers in their native state to use as an immunogen. The specificity of anti-RNP serum for N protein could be demonstrated reasonably satisfactorily by radioimmunoprecipitation of homotypic RNP and soluble N protein present in VS virus-infected cell cytoplasm. It seem likely that the limited degree of co-precipitation of NS protein is due to cytoplasmic complexes of N-NS proteins as well as residual nucleocapsid complexes containing NS protein. However, minor contamination of anti-RNP serum with NS antibody cannot be ruled out completely until the N and NS proteins can be purified to homogeneity. Our data also indicate that the antitranscriptase activity of anti-RNP Ig is not due simply to nucleocapsid precipitation; anti-RNP Ig diluted beyond its capacity to precipitate nucleocapsids still retained much of its antitranscriptase activity.

Anti-RNP_{Ind} and anti RNP_{NJ} Ig exhibited minor but quite significant capacity to inhibit transcription of heterotypic virions. This antitranscriptase cross-reactivity is consistent with the evidence of immunological relatedness between the core (N protein) antigens of VS_{Ind} and VS_{NJ} virions (8). The slight ability of anti-VS_{NJ} serum (in contrast to anti-RNP_{Ind} serum) to neutralize the transcriptase activity of the presumably antigenically unrelated Chandipura virus is more difficult to explain without further immunological data.

Comparison of these data on anti-RNP serum are generally consistent with the earlier studies of Imblum and Wagner (15) on anti-NS_{Ind} immunoglobulin, but certain discrepancies are evident. Imblum and Wagner (15) raised antibody by injecting rabbits with NS protein isolated from the cytoplasm of cells infected with VS_{Ind} virus. Anti-NS_{Ind} Ig also was quite effective in inhibiting transcription of VS_{Ind} virus which, in a manner indistinguishable from the action of anti-RNP_{Ind} Ig, appeared to result from termination of RNA chain elongation. The anti-NS_{Ind} Ig appeared to react specifically with NS-protein antigen by immunodiffusion and radioimmunoprecipitation, but it was not technically possible to rule out a certain limited degree of immunological cross-reactivity with soluble N protein (or N-NS protein complexes) in infected cytoplasmic extracts. Therefore, we cannot rule out completely the possibility that anti-RNP and anti-NS Ig both inhibit VS viral transcription by

reacting with the same nucleocapsid component to terminate RNA chain elongation.

The previously described anti-NS_{Ind} Ig (15), unlike anti-RNP_{Ind} Ig, exhibited no capacity to inhibit transcription by heterotypic VS_{NJ} virus. The most likely explanation for this difference is that the antigenic cross-reactivity of VS_{Ind} and VS_{NJ} viruses (8) is due only to group specificity of the major N-protein antigen and does not apply to the minor NS protein. It must be recalled, however, that heterotypic inhibition of transcription by anti-RNP_{Ind} and anti-RNP_{NJ} Ig required the use of reaction mixtures containing only 2 to 5 μg of virion proteins and as much as 50 to 125 μg of anti-RNP Ig. Imblum and Wagner (15) found that 75 μg of anti-NS_{Ind} Ig did not inhibit transcription by 10 μg of VS_{NJ} virions, but they did not test their anti-NS Ig preparations against lower concentrations of heterotypic virions.

It seems likely from these studies that anti-RNP and anti-NS Ig have different antigenic specificity in their reaction with transcribing complexes of VS virus but that their mode of antitranscription action may be similarly directed against termination of RNA chain elongation. It also can be assumed that the N protein plays a significant role as a cofactor in transcription of VS virions, presumably in concert with the NS protein.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI-11112 from the National Institute of Allergy and Infectious Diseases, grant BMS 72-02223 from the National Science Foundation, and grant VS-88 from the American Cancer Society.

We thank Suzanne Emerson and Margaret Mellon for antisera and many helpful suggestions.

LITERATURE CITED

1. **Baltimore, D., A. S. Huang, and M. Stampfer.** 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci. U.S.A. **66**:572-576.
2. **Banerjee, A. K., G. Abraham, and R. J. Colonno.** 1977. Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. **34**:1-8.
3. **Barenholz, Y., N. F. Moore, and R. R. Wagner.** 1976. Enveloped viruses as model membrane systems: microviscosity of vesicular stomatitis virus and host cell membranes. Biochemistry **15**:3563-3570.
4. **Bishop, D. H. L., S. U. Emerson, and A. Flammand.** 1974. Reconstitution of infectivity and transcriptase activity of homologous and heterologous viruses: vesicular stomatitis (Indiana serotype), Chandipura, vesicular stomatitis (New Jersey serotype), and Cocal viruses. J. Virol. **14**:139-144.
5. **Bishop, D. H. L., and P. Roy.** 1972. Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. J. Virol. **10**:234-243.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.
7. **Both, G. W., S. A. Moyer, and A. K. Banerjee.** 1975. Translation and identification of the mRNA species synthesized *in vitro* by the virion-associated RNA polymerase of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **72**:274-278.
8. **Cartwright, B., and F. Brown.** 1972. Serological relationships between different strains of vesicular stomatitis virus. J. Gen. Virol. **16**:391-398.
9. **Christopher, A. R., M. L. Nagpal, A. R. Carroll, and J. C. Brown.** 1978. Chloramine T mediated radioiodination of proteins on intact polyacrylamide slab gels. Anal. Biochem. **85**:421-427.
10. **Cullen, S. E., and B. D. Schwartz.** 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. J. Immunol. **117**:136-142.
11. **Dubovi, E. J., and R. R. Wagner.** 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. **22**:500-509.
12. **Emerson, S. U., and R. R. Wagner.** 1972. Dissociation and reconstitution of the transcriptase and template activities of vesicular stomatitis B and T virions. J. Virol. **10**:297-309.
13. **Emerson, S. U., and Y. Yu.** 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **15**:1348-1356.
14. **Hunt, D. M., and R. R. Wagner.** 1975. Inhibition by aurintricarboxylic acid and polyethylene sulfonate of RNA transcription of vesicular stomatitis virus. J. Virol. **16**:1146-1153.
15. **Imblum, R. L., and R. R. Wagner.** 1975. Inhibition of viral transcriptase by immunoglobulin directed against the nucleocapsid NS protein of vesicular stomatitis virus. J. Virol. **15**:1357-1366.
16. **Kang, C. Y., and L. Prevec.** 1970. Proteins of vesicular stomatitis virus. II. Immunological comparisons of viral antigens. J. Virol. **6**:20-27.
17. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617-1624.
18. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. J. Mol. Biol. **80**:575-599.
19. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{2}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335-341.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
21. **Naito, S., and A. Ishihama.** 1976. Function and structure of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. **251**:4307-4314.
22. **Rhodes, D. P., S. A. Moyer, and A. K. Banerjee.** 1974. *In vitro* synthesis of methylated messenger RNA by the virion-associated RNA polymerase of vesicular stomatitis virus. Cell **3**:327-333.
23. **Villarreal, L. P., and J. J. Holland.** 1973. Synthesis of poly(A) *in vitro* by purified virions of vesicular stomatitis virus. Nature New Biol. **246**:17-19.