JOURNAL OF VIROLOGY, Jan. 1974, p. 113-124
Vol. 13, No. 1

Printed in U.S.A.

Protein Kinase and Phosphoproteins of Vesicular Stomatitis Virus

RICHARD L. IMBLUM AND ROBERT R. WAGNER

Department of Microbiology, The University of Virginia School of Medicine, Charlottesville, Virginia 22901

Received for publication 4 September 1973

Protein kinases of similar but not identical activity were found associated with vesicular stomatitis (VS) virions grown in mouse L cells, primary chicken embryo (CE) cells, and BHK-21 cells, as well as being present in VS virions grown in HeLa and *Aedes albopictus* cells. The virion kinase preferentially phosphorylated the nucleocapsid NS protein in vitro and to a lesser extent the envelope M protein. Other virion proteins were phosphorylated in vitro only after drastic detergent treatment. Partial evidence that the virion kinase is of cellular origin was obtained by finding reduced enzyme activity in virions released from cells pretreated with actinomycin D and cycloheximide. Selective detergent and detergent-salt fractionation of VS virions revealed that the kinase activity was present in the envelope but not the spikes. The virion kinase activity in a Triton-salt-solubilized envelope fraction could be separated from M and G proteins and partially purified by phosphocellulose column chromatography. Virions released from L, CE, and BHK-21 cells infected in the presence of [^{32}P]orthophosphate were labeled almost exclusively in the NS protein. Both soluble and nucleocapsid-associated NS phosphoprotein were present in cytoplasmic extracts of VS viral-infected L cells. The origin and function of the NS phosphoprotein remain to be elucidated.

Since the original observation of Strand and August (23) that protein kinase activity is present in preparations of Rauscher murine leukemia virus and two other enveloped viruses, avian myeloblastosis virus and vesicular stomatitis (VS) virus, this enzyme activity has been detected in a variety of other virus preparations (6, 7, 14, 16, 20, 22). This finding coupled with the discovery that specific viral-directed proteins are phosphorylated in vivo (18, 21, 22, 24) has led to speculation on the function and origin of the kinase and the significance of the phosphorylated viral proteins in the infective process.

Inasmuch as VS virus is an enveloped virus, which obtains its lipoprotein coat by budding from the host cell, it is likely that the virion contains host cell membrane proteins in addition to lipid (12). Thus, one has to consider the possibility that the protein kinase is an enzyme whose synthesis is directed by the cell genome and is captured by the virion as it buds from the cell.

In addition to a protein kinase, VS virus contains one protein, NS, which is phosphorylated in vivo (22). This protein, whose function is not known, is associated with the virion nucleocapsid as a minor component (13) but is a major protein in the cytoplasm of VS viral-infected cells (29).

In the results recorded below, we report the solubilization of the VS virion protein kinase and present evidence that the enzyme is envelope associated and cellular in origin. Our data also indicate selective in vitro phosphorylation of virion NS and M proteins and extend the findings of Sokol and Clark (22) on the in vivo phosphorylation of the NS protein.

MATERIALS AND METHODS

Chemicals and radiochemicals. ATP and vitamin-free casein were from Nutritional Biochemicals Corp., Cleveland, Ohio. Dithiothreitol (DTT) was from Calbiochem, La Jolla, Calif. Triton X-100, Lubrol WX, and cycloheximide were from Sigma Chemical Co., St. Louis, Mo. Nonidet P-40 (NP-40) was from Shell Chemical, Chicago, Ill. Actinomycin D was from Merck, Sharp & Dohme, West Point, Pa. [^{32}P]orthophosphate (carrier free) was from New England Nuclear Corp., Boston, Mass. γ-[^{32}P]ATP (10 to 20 Ci/mmol) was from New England Nuclear Corp., Boston, Mass., and Amersham/Searle, Arlington Heights, Ill. [^{3}H]leucine (50 Ci/mmol) and [^{3}H]tyrosine (50 Ci/mmol) were from Schwarz/Mann, Orangeburg, N.Y.

Vitamin-free casein was treated as described by Reimann et al. (17) prior to use as a substrate in protein kinase assays.

Cell cultures, virus, and media. The cultivation of L cells and primary chicken embryo (CE) cells has already been described (12). BHK-21 cells were grown in Falcon plastic flasks in Eagle BME with 10% fetal calf serum (FCS) and 10% tryptose phosphate broth. Confluent monolayers formed in 3 days at 37 C.

The Indiana strain of VS virus was used throughout (12). Infectivity was titrated by plating on L-cell monolayers under agar and the titers per milliliter were recorded as PFU.

Infection of cells and radioactive labeling of cells and virus. To study intracellular viral proteins, cells were infected at a multiplicity greater than 10. After 1 h the cells were washed twice with 0.9% NaCl and overlaid with 5 ml of phosphate-free medium which contained Hanks salts with phosphate replaced by 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.3), one-half BME amino acids, and BME glucose. After 2 h in the phosphate-free medium, carrier-free [^{32}P]orthophosphate (10 μCi/ml), [^{3}H]leucine (10 μCi/ml), and [^{3}H]tyrosine (10 μCi/ml) were added. After 3 additional hours cytoplasmic extracts of the cells were prepared as described below.

For the production of virions labeled with ^{32}Pi- or ^{3}H-amino acids, or both, cells were infected at a multiplicity of about 0.5. Virus was grown on cells in 5 ml of BME lacking unlabeled leucine and tyrosine with one-half the usual phosphate replaced with HEPES buffer (pH 7.3) at a final concentration of 12 mM and containing 10 μCi of carrier-free [^{32}P]orthophosphate per ml and 10 μCi each of [^{3}H]leucine and [^{3}H]tyrosine per ml. For production of virions labeled only with ^{3}H-amino acids, infected cells were overlaid with 5 ml of BME without unlabeled leucine and tyrosine containing [^{3}H]leucine (10 μCi/ml) and [^{3}H]tyrosine (10 μCi/ml).

Cell fractionation. Cytoplasmic extracts were prepared by the method of Penman et al. (15). The cells were washed twice with 5 ml of phosphate-buffered saline solution (PBS), scraped into 5 ml of cold Earle balanced salt solution (BSS), collected by centrifugation at 800 $\times$ *g* for 5 min, and washed twice by suspending in 5 ml of BSS and centrifuging at 800 $\times$ *g* for 5 min. The washed pellet was suspended in 0.5 to 1.0 ml of reticulocyte standard buffer (RSB) and allowed to stand for 20 min. The cells were disrupted with 20 strokes in a tight-fitting Dounce homogenizer. The nuclei in the homogenate were centrifuged out at 800 $\times$ *g* for 10 min. The supernatant fraction was retained as the cytoplasmic extract.

To separate soluble viral proteins from those already incorporated into sedimentable membranes and viral nucleocapsids, the cytoplasmic extract was centrifuged at 125,000 $\times$ *g* for 90 min in the Spinco SW50L rotor (29). The supernatant fraction was removed and the pellet was suspended in RSB equal to one-half the centrifuged volume.

Purification of released virions. The procedure for the partial purification of VS virus has previously been described (4). Eighteen hours postinfection virions were harvested from the media which were clarified by centrifugation at 800 $\times$ *g* for 10 min. The media were then centrifuged at 80,000 $\times$ *g* for 90 min in the Spinco SW27 rotor through a 2-ml pad of 50% glycerol in BSS. The pellets were suspended in BSS, sonicated, layered onto 0 to 40% linear sucrose gradients, and centrifuged for 90 min at 35,000 $\times$ *g* in the Spinco SW25.1 rotor. The visible band of B particles was removed by side puncture of the tube, diluted with BSS, and centrifuged at 80,000 $\times$ *g* for 60 min in the Spinco SW50L rotor. The pellet was suspended in 12 mM Tris-hydrochloride (pH 7.4), 1 mM $MgCl_2$, and 0.5 mM DTT. The protein content of each virion preparation was measured by the method of Lowry et al. (10).

Protein kinase assay. Protein kinase activity was assayed by measuring the incorporation of ^{32}P from γ-[^{32}P]ATP into trichloroacetic-precipitable material. A standard incubation mixture contained in a volume of 0.1 ml: 5 μmol of Tris-hydrochloride (pH 8.0), 0.4 μmol of $MgCl_2$, 0.05 μmol of DTT, 0.1 μliter of NP-40, 5 nmol of γ-[^{32}P]ATP (specific activity 100 to 400 counts per min per pmol), and 20 to 50 μg of viral protein. Incubations were for specified times at 31 C. The reactions were terminated by pipetting 50 μliters of the reaction mixture onto a 23-mm diameter disk of Whatman no. 3 filter paper and by immediately placing the disk in a beaker of constantly stirred 10% trichloroacetic acid containing 50 mM $Na_4P_2O_7$. The disks were processed as described by Bollum (1), and the radioactivity was counted in 10 ml of toluene-based scintillation fluor.

Polyacrylamide gel electrophoresis. Samples for gel electrophoresis were prepared in one of the following ways. For cytoplasmic extracts proteins were precipitated by the addition of 2 volumes of cold 95% ethanol. After standing overnight at 4 C, the ethanol precipitates were collected by centrifugation at 1,000 $\times$ *g* for 10 min and dissolved in 10 mM HEPES (pH 7.4), 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, and 8 M urea. ^{32}P-labeled virion pellets were dissolved directly in the above HEPES, SDS, mercaptoethanol, and urea buffer. Proteins from protein kinase incubations were precipitated after adding 50 μg of bovine plasma albumin by the addition of an equal volume of cold 20% trichloroacetic acid. After 15 min at 4 C, the precipitate was collected by centrifugation and washed once with 10 volumes of cold acetone. The washed pellet was dissolved in the HEPES, SDS, mercaptoethanol, and urea buffer. Samples were placed in a boiling-water bath for 2 min prior to placing on gels. As previously described (29), electrophoresis was on 7.5% acrylamide gels containing 0.1% SDS using 100 mM sodium phosphate (pH 7.4) at 5 mA per gel. After electrophoresis the gels were frozen and cut into 1.25-mm slices. Protein was extracted from each slice by incubation for 2 h at 50 C in 0.5 ml of solubilizer-water (9:1) (Nuclear-Chicago Corp.). Toluene-based scintillation fluor (10 ml) was added, and the samples were counted in a scintillation spectrometer.

RESULTS

Comparative protein kinase activity of VS virions grown in different cells. We initially

addressed ourselves to the question of whether the protein kinase in VS virions is of viral or cellular origin. Protein kinase activity was readily detectable in partially purified VS virions released from cells of five different animal species: mouse L cells, CE cells, BHK-21 cells, human HeLa cells, and mosquito *Aedes albopictus* cells. These data did not help to resolve the question of whether the protein kinase (s) is a cellular or viral protein. Another approach to this question was to compare the enzyme kinetics of protein kinase associated with VS virions grown in different cell lines.

VS virions grown in L, CE, and BHK-21 cells were partially purified by differential and rate zonal centrifugation. Equivalent amounts of each virion preparation were used to determine the K_m and V_{max} values for Mg-ATP of each virion enzyme. The enzyme assays were performed at pH 8.0 to ensure that essentially all the ATP was in the form of ATP^{-4} and sufficient Mg^{2+} to ensure that all ATP was complexed with magnesium ions.

Figure 1 shows the double reciprocal plots when Mg-ATP is the variable substrate of the kinase reaction catalyzed by enzyme in VS virions grown in L, CE, or BHK-21 cells. The Fig. 1 inset gives the figures calculated for the two kinetic parameters. The K_m values are quite similar for all three virion preparations and may reflect the intracellular concentration of ATP in the cells of origin. The V_{max} values are also similar but, on repeated determinations, we have consistently found the specific activities of kinases of VS virions from CE and BHK-21 cells to be 2 to 2.5 times greater than that of kinase of L-cell-grown virions. Preparations of virions obtained from HeLa or *A. albopictus* cells were inadequate for comparative kinetic analysis of their enzymes.

These data suggest, but do not prove, that the VS virion kinase is of cellular origin even though similar enzymes are found in virions produced by cells of five different animal species.

Protein kinase activity of VS virions grown in L cells pretreated with actinomycin and cycloheximide. If the protein kinase of VS virions is of cellular rather than of viral origin, exposing cells prior to infection with inhibitors of mRNA and protein synthesis should reduce the amount of enzyme found associated with virions grown in these cells.

Monolayer cultures of L cells were exposed for 12 h prior to infection to medium containing actinomycin D (2 μg/ml) and cycloheximide (50 μg/ml), or to actinomycin alone. This amount of actinomycin for 2 h resulted in 93% inhibition of trichloroacetic acid-precipitable [^{3}H]uridine incorporation; cycloheximide present for 2 h reduced by 96% the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable cellular extracts. Inhibitor-treated and control cells were then infected with VS virus at a multiplicity of 0.5 PFU/cell and incubated at 37 C in medium free of actinomycin or cycloheximide. Virions released into the medium were collected at 17 h postinfection, purified by rate zonal centrifugation, and assayed for kinase activity, protein content, and infectivity.

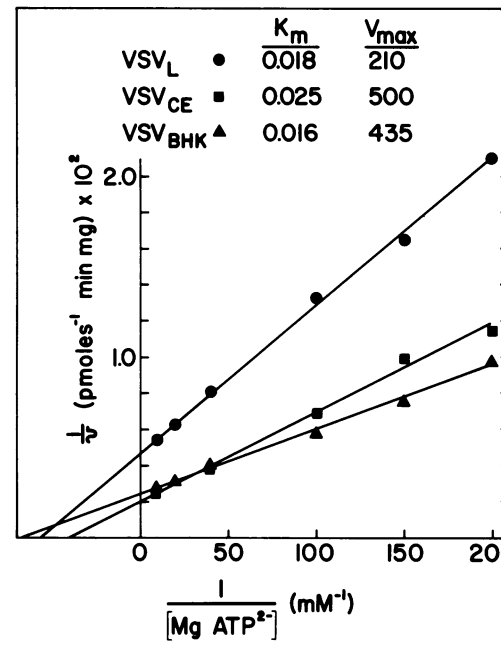

FIG. 1. *Lineweaver-Burke plots showing the effect of Mg-ATP concentration on the velocity of the protein kinase reaction for VS virions grown in different cells. Protein kinase activity of purified VS virions grown in L cells (●), CE cells (■), or BHK-21 cells (▲) was assayed in a standard reaction mixture containing excess Mg^{2+} (2.0 mM) but varying amounts of ATP to produce levels of Mg-ATP^{2-} of 0.005, 0.0067, 0.010, 0.025, 0.050, and 0.10 mM. The specific activity of γ-[^{32}P]ATP was 173 counts per min per pmol. Vitamin-free casein was present at a constant concentration of 5.0 mg/ml. Reaction mixtures were incubated for 5 min at 31 C. Acid-precipitable radioactivity was measured by pipetting 50-μliter samples of the incubation mixtures onto filter paper disks which were immediately placed into constantly stirring 10% trichloroacetic acid containing 50 mM $Na_4P_2O_7$. ^{32}P in processed disks was counted by scintillation spectrometry. Virion protein concentration was measured by the method of Lowry (10). The inset shows the calculated values of K_m (mM) and V_{max} (picamoles of ^{32}P incorporated per minute per milligram of protein) for virions grown in each cell type.*

The results of a typical experiment are shown in Table 1. It is apparent from this and another experiment that pretreatment of L cells with actinomycin or with actinomycin plus cycloheximide resulted in appreciable diminution of specific kinase activity. These data are reported as specific enzyme activity based on protein concentration of each preparation, which was roughly equivalent for purified virions released from inhibitor-pretreated or untreated cells. This effect of actinomycin supports the hypothesis that the protein kinase is of cellular origin.

More difficult to explain are the results shown in Table 1 of two- or three-fold reduction in specific infectivity of these purified virions released from inhibitor-pretreated cells. No evidence for excess quantity of defective T particles was found in sucrose density gradients. Similar reduced infectivity of VS virions caused by actinomycin has been reported (30). Conceivably, this effect on specific infectivity could be attributable to reduced concentration of protein kinase in the virion or, of course, to an effect on other cellular enzymes. Such a possibility has already been suggested for the protein kinase of frog polyhedral cytoplasmic deoxyribovirus (6).

Identification of VS viral phosphoproteins phosphorylated in vitro by the kinase of virions grown in different cells. Strand and August (23) reported that all five proteins of VS virions were phosphorylated in vitro by the endogenous protein kinase. They did not report the cells in which the VS virions were grown or details of the assay conditions, but we and F. Sokol (personal communication) have obtained similar results only when VS virions were completely disrupted by drastic treatment with detergents (see Table 2). It was of interest to determine whether virion proteins could be selectively phosphorylated when the kinase was activated gently by low concentrations of a nonionic detergent that should not disrupt the virions (9).

TABLE 1. *Protein kinase activity and infectivity of VS virions grown in L cells that were pretreated with actinomycin D or actinomycin D and cycloheximide*

Pretreatment of L cells[a]	Protein kinase specific activity (pmol of ^{32}P incorporated per min per mg)	Total protein[b] (mg)	Specific infectivity[c] (PFU/mg)
None	155	0.09	2×10^9
Actinomycin D	40	0.14	9×10^8
Actinomycin D + cycloheximide	73	0.07	7×10^8

[a] Confluent monolayer cultures of L cells were pretreated by incubation for 12 h in BME plus 2% FCS to which was added nothing (controls), actinomycin D (2 μg/ml), or actinomycin D (2 μg/ml) plus cycloheximide (50 μg/ml). Culture medium was then removed, the cell layers were washed twice with PBS, and the cells were infected with VS virus at a multiplicity of 0.5 PFU/ml. After further incubation for 18 h at 37 C, released virions in medium were collected and purified by differential and rate zonal centrifugation. Protein kinase activity of these virions was measured as described in Materials and Methods in the presence of casein (2.5 mg/ml). The specific activity of γ-[^{32}P]ATP was 230 counts per min per pmol.

[b] Determined by the Lowry method.

[c] Plaque assays performed on monolayers of L cells.

Purified VS virions labeled with ^3H-amino acids and released from L, CE, and BHK-21 cells were incubated for 30 min at 31 C in 0.1 ml of a standard kinase assay mixture containing 0.1% NP-40, 5 nmol of γ-[^{32}P]ATP, but no added protein substrate. The viral ^3H-labeled proteins were then precipitated, extracted with SDS and urea, and analyzed by electrophoresis on 7.5% acrylamide gels.

Figure 2 demonstrates the selectivity with which the endogenous kinase phosphorylated the viral proteins under these conditions. Only insignificant amounts of ^{32}P could be found associated with viral ^3H-labeled proteins N, G, and L. In contrast, proteins NS and M were heavily phosphorylated in virions derived from L cells (Fig. 2A), CE cells (Fig. 2B), and BHK-21 cells (Fig. 2C). Quite striking was the specific ^{32}P radioactivity of the minor nucleocapsid NS protein, which is hereby designated a phosphoprotein.

Similar results were obtained by in vitro phosphorylation of viral proteins NS and M in virions derived from *A. albopictus* cells. These data strongly suggest that the substrate specificity and perhaps the location within the virion of VS viral proteins are more important for their phosphorylation than is the cellular origin of the protein kinase.

Virion location of protein kinase and its separation from virus-specific proteins. VS virions are constructed of two major components, the nucleocapsid and the envelope (27). In addition to lipids and glycolipids, the envelope is composed of two of the five virion proteins: the matrix or M protein, which is the main structural element, and the glycoprotein (G), which forms the spikes that protrude from the envelope. The remaining three virion proteins, N, L, and NS, are complexed with the viral RNA to constitute the nucleocapsid.

Treatment of VS virions under different conditions promotes the selective release of each of

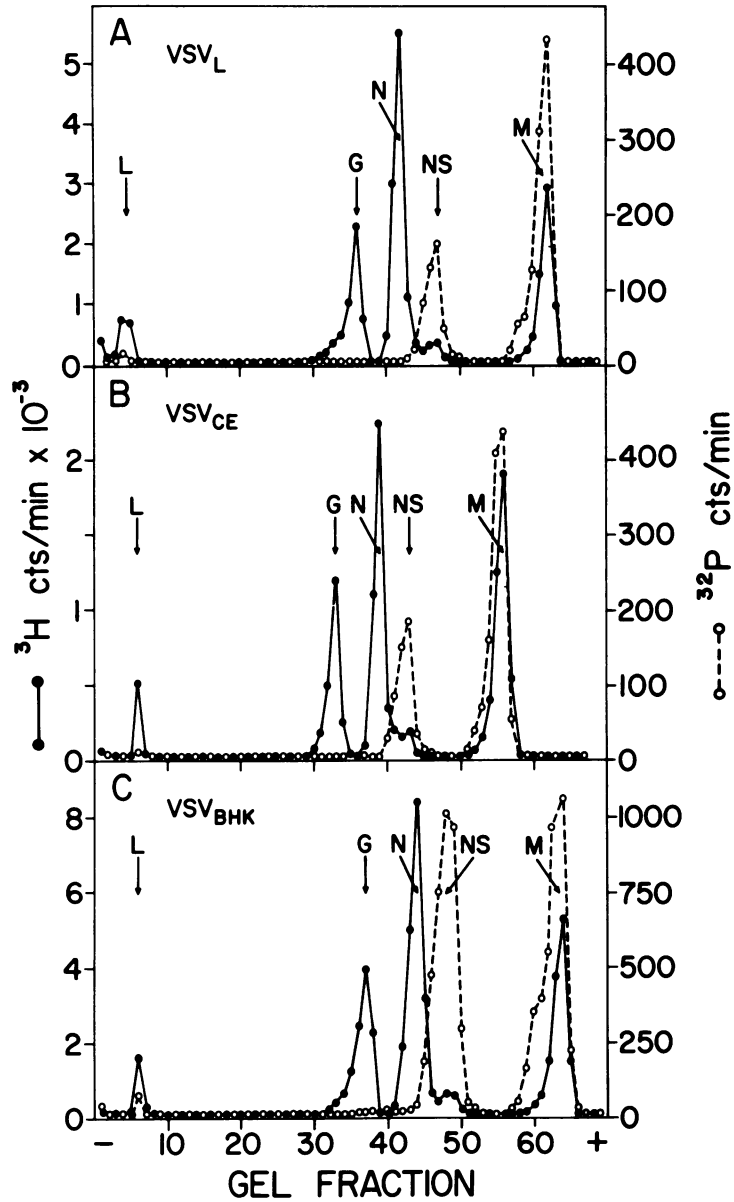

FIG. 2. *Phosphorylation in vitro of VS viral proteins by the protein kinase of purified VS virions grown in L, CE, and BHK-21 cells. Virions that had been labeled with ^{3}H-amino acids were incubated at 31 C for 30 min in the standard protein kinase assay mixture containing γ-[^{32}P]ATP (5 nmol, 300 counts per min per pmol) but without added casein substrate. After incubation, the viral ^{3}H-labeled proteins were precipitated with cold 10% trichloroacetic acid, washed with cold acetone and dissolved in 10 mM HEPES buffer (pH 7.4) containing 1% SDS, 1% 2-mercaptoethanol, and 8 M urea. After boiling the mixture, the proteins were subjected to electrophoresis for 9 h at 5 mA on 7.5% polyacrylamide gels. Gel slices of ~1.25 mm were dissolved in solubilizer and toluene-based fluors (Nuclear-Chicago Corp.) and counted in a scintillation spectrometer. Arrows mark the positions of VS virion proteins L, G, N, NS, and M.*

the virion proteins. Exposure to the nonionic detergent, Triton X-100, in a very low-salt buffer solubilizes almost all the G protein but almost none of the other four virion proteins (9). Increase in salt concentration to 0.3 M causes release of M protein as well as G but leaves the

nucleocapsid intact (4). Other detergents, particularly ionic detergents, produce more drastic disruption of the virion (28).

With these data in mind, a number of different detergent mixtures were tested as solubilizers of VS virion protein kinase and of virion proteins labeled with ^{3}H-amino acids. After treatment with each detergent for 30 min at 4 C, the virions were separated into pellet and supernatant fractions, each of which was then assayed for kinase activity. In addition, the supernatant and pellet fractions were tested by polyacrylamide gel electrophoresis for their content of ^{3}H-labeled protein and ^{32}P-labeled protein.

Table 2 summarizes the relative capacity of the various detergents to solubilize virion kinase and ^{3}H-labeled proteins. Quite obviously, solubilization of G protein does not result in significant release of kinase activity from the virion. Significant kinase activity could be found in the supernatant fractions only when some of the M protein was liberated by detergent action. The most effective solubilizer of protein kinase was Triton X-100 in the presence of 0.3 M NaCl. NS and M proteins were the best (or most available) substrates for phosphorylation by the endogenous virion kinase, but the G protein could also be phosphorylated when virions were disrupted with Lubrol-WX.

TABLE 2. *Capacity of different detergents to solubilize protein kinase of VS virions correlated with the viral proteins solubilized and phosphorylated*

Detergent treatment[a]	Percent kinase[b]		VS viral proteins[c]	
	Supernatant	Pellet	Solubilized	Phosphorylated
0.5% Triton	9	85	G	NS, M
0.5% Triton + 0.3 M NaCl	65	25	G, M	NS, M
0.5% BRIJ 58	0	75		
0.1% BRIJ 58 + 0.1% DOC	4	90	G	NS, M
0.5% Lubrol-WX	0	65	G	G, NS, M
0.5% Lubrol-WX + 0.2% digitonin	0	92	G(1/3)	G, NS, M
0.1% NP-40	15	70	G, M	NS, M

[a] VS virions labeled with ^{3}H-amino acids were purified by rate zonal centrifugation and suspended in 12 mM Tris-hydrochloride (pH 8.0), 1 mM $MgCl_2$, and 0.5 mM DTT. Virions at a protein concentration of ~2 mg/ml were treated with various detergent solubilizers with constant stirring for 30 min at 4 C, except that treatment with 0.1% NP-40 was for 10 min at 30 C. DOC, Deoxycholate.

[b] After treatment with detergents, the VS virions were separated into supernatant and pellet fractions by centrifugation at 125,000 × *g* for 90 min. The pellet fraction was suspended in the original buffer to the original volume. The pellet and supernatant fractions were assayed for protein kinase activity in the presence of added casein. Percent activity recovered is based on kinase activity of control unfractionated virions.

[c] Samples of supernatant and pellet fractions used for kinase assay were analyzed after extraction for their composition of VS viral ^{3}H- and ^{32}P-labeled proteins by electrophoresis on 7.5% SDS-polyacrylamide gels. VS viral proteins recorded as phosphorylated represent all those labeled with ^{32}P in gel sections of electrophoresed supernatant and pellet of each detergent-treated preparation. No gels were analyzed for the 0.5% BRIJ 58 preparation.

In repeated experiments, Triton X-100 in the presence of 0.3 M NaCl solubilized 60 to 75% of the protein kinase activity without solubilizing any N, L, or NS proteins from the sedimentable nucleocapsid. Therefore, it seems reasonable to assume that the virion kinase activity is not associated with nucleocapsid N, L, or NS proteins, nor is the G protein responsible for kinase activity. The most logical hypothesis was that the VS virion protein kinase was a membrane protein associated with the spikeless virion envelope. The kinase solubilized by Triton-0.3 M NaCl could be the viral M protein or a nonviral membrane component. To distinguish between these possibilities, VS virions grown in L cells and labeled with ^{3}H-amino acids were treated with Triton X-100 in 0.3 M NaCl and pelleted by centrifugation at 125,000 × *g*. The resulting supernatant was fractionated by adsorption on a phosphocellulose column and eluted by 0.5 M NaCl. The column effluent was assayed for kinase activity and for ^{3}H-labeled protein, which were subsequently characterized by disc gel electrophoresis.

Figure 3 shows the elution profiles of solubilized virion kinase and envelope ^{3}H-labeled protein from a phosphocellulose column. Most of the enzyme activity eluted late in association with only a small amount of trailing ^{3}H-labeled protein. In contrast, almost all the ^{3}H-labeled protein came off the column in two earlier peaks with only a slight leading shoulder of enzyme activity.

The first phosphocellulose peak was free of enzyme and contained only protein G. The viral ^{3}H-labeled proteins in fraction 20 and 29 of the phosphocellulose column (Fig. 3) were also analyzed by polyacrylamide gel electrophoresis. Fraction 20, which contained 58% of the protein and 9% of the kinase activity, consisted of 30% protein G and 67% protein M. Fraction 29, which represented 91% of the kinase activity, contained no protein G and about 3% of the amount of protein M found in peak 20. When the major kinase peak 29 was rechromatographed on phosphocellulose, most of the en-

zyme eluted in the same position with almost no contaminating ^{3}H-labeled protein.

These data indicate that the protein kinase in VS virions is not one of the identifiable viral proteins but is presumably a cellular enzyme that is solubilized by treatment of virions with Triton-0.3 M NaCl. The presence of some kinase activity associated with proteins G and M eluted from the phosphocellulose column could be due to the tendency of these proteins to aggregate under these conditions (S. U. Emerson, personal communication), with resultant entrapment of the enzyme. The possibility exists, of course, that the earlier eluting protein kinase is a different species of enzyme from the major component. Rechromatographing the kinase associated with proteins G and M resulted in elution in the same position. However, the same pattern of kinase and viral ^{3}H-labeled protein elution from phosphocellulose was obtained with the Triton-0.3 M NaCl-solubilized fraction of VS virions grown in BHK-21 cells.

In vivo phosphorylation of proteins in VS virions released from infected L, CE, and BHK cells. If any significance is to be attached to the demonstrated presence of a VS virion protein kinase capable of phosphorylating viral proteins, at least minimal evidence must be obtained for the existence of viral phosphoproteins in infected cells which can also be incorporated into released virions. It was also essential to determine whether in vitro selective phosphorylation by virion kinase of certain VS viral proteins, particularly NS protein, also occurs in vivo.

Cultures of L, CE, and BHK-21 cells were infected with VS virus and incubated in medium containing carrier-free [^{32}P]orthophosphate and ^{3}H-amino acids. Virions in medium harvested 18 h after infection were concentrated and partially purified. Proteins extracted from these virions with SDS, urea, and 2-mercaptoethanol were analyzed on SDS-acrylamide gels assisted by a double-label program for ^{32}P and ^{3}H.

Figure 4 shows conclusively that NS phosphoprotein is the principal viral protein phosphorylated in vivo, and that this phosphoprotein is incorporated into VS virions. Roughly equivalent amounts of ^{32}P were incorporated into the NS protein of virions grown in L (Fig. 4A), CE (Fig. 4B), and BHK-21 (Fig. 4C) cells, indicating again that the substrate potential of the NS protein is more important for phosphorylation than is the activity of the cellular enzyme. It is also evident in Fig. 4 that the major envelope protein M is also phosphorylated in vivo to a

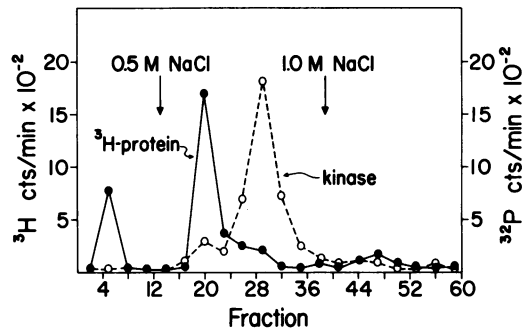

FIG. 3. *Fractionation by phosphocellulose column chromatography of solubilized protein kinase and ^{3}H-labeled proteins of VS virions. VS virus grown in L cells labeled with ^{3}H-amino acids was partially purified by rate zonal centrifugation and then disrupted by incubation for 15 min at 4 C in 12 mM Tris-hydrochloride (pH 8.0), 1 mM $MgCl_2$, 0.5 mM DTT, 0.5% Triton X-100, and 0.3 M NaCl. The virus suspension was then centrifuged for 90 min at 115,000 × g; the supernatant fraction containing viral proteins G and M was dialyzed twice for a total of 3 h against 100 ml of 50 mM Tris-hydrochloride (pH 7.4), 25% glycerol, 0.1% Triton X-100, and 0.1 mM DTT. This material was applied to a 4-ml phosphocellulose column that had been equilibrated with the dialysis buffer. The column was washed with two column volumes of the dialysis buffer, and then with four column volumes of dialysis buffer containing 0.5 M NaCl, and finally with dialysis buffer containing 1.0 M NaCl. Fractions were assayed for protein kinase by the standard procedure with casein (2.5 mg/ml) as substrate. Viral ^{3}H-labeled protein was assayed as acid-precipitable radioactivity. The specific activity of γ-[^{32}P]ATP was 154 counts per min per pmol.*

very limited extent, not unlike the phosphorylation of M protein in vitro by the virion kinase. In contrast, proteins G and N appear to have little acceptor capacity for phosphate. Conclusions about protein L are not so clear because of the very large amount of ^{32}P radioactivity at the top of the gel, which is undoubtedly due to [^{32}P]RNA. When this RNA was digested with RNase before electrophoresis, it was more apparent that L protein is slightly or not at all phosphorylated.

These data confirm the finding of Sokol and Clark (22) that the NS protein is selectively phosphorylated in vivo and extend this observation to show that the pattern of phosphorylation is not dependent on the cell used to propagate the virus. Moreover, when L cells were exposed to actinomycin D (2 μg/ml) and cycloheximide (50 μg/ml) for 12 h prior to infection with VS virus, the amount of ^{32}P incorporated into NS protein of released virions was approximately

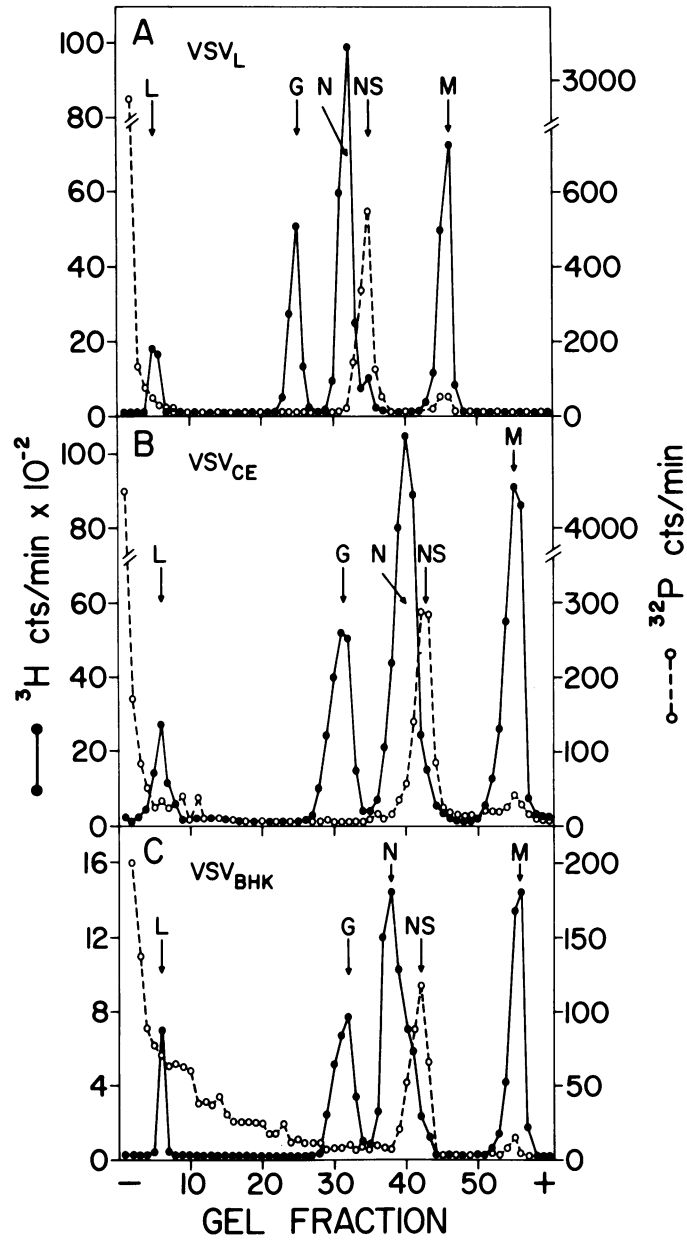

FIG. 4. *Phosphorylation in vivo of VS viral protein in virions released from cells infected in the presence of [^{32}P]orthophosphate. Cultures of L, CE, and BHK cells were infected with VS virus at a multiplicity of 0.5 PFU/cell and incubated in BME which contained 10 μCi of carrier-free [^{32}P]orthophosphate per ml and 10 μCi each of [^{3}H]leucine and [^{3}H]tyrosine per ml. VS virions in the medium 18 h after infection were partially purified by rate zonal centrifugation. The proteins extracted with 1% SDS, 0.1% 2-mercaptoethanol, and 8 M urea were subjected to electrophoresis on 7.5% polyacrylamide gels. Arrows mark the positions of VS virion proteins L, G, N, NS, and M.*

the same as that in NS protein from virions of cells not exposed to RNA and protein inhibitors. This result possibly indicates that the cellular kinase that phosphorylates the NS protein in vivo may be different from the actinomycin-sensitive kinase incorporated into the VS virion.

Phosphorylation of intracellular VS viral proteins. Although the NS protein is only a

minor component of the VS viral nucleocapsid (13, 26), a soluble protein with identical electrophoretic mobility is present as a major constituent in the cytoplasm of infected cells (29). Therefore, we sought to determine whether the soluble NS-like protein was also preferentially phosphorylated as the nucleocapsid NS phosphoprotein. We also hoped to shed some light on the question of precursor-product relationship of the soluble and nucleocapsid NS proteins.

These experiments were performed by infecting monolayer cultures of L cells at a multiplicity of 10 PFU/cell and then labeling at 3.5 h postinfection with [^{32}P]orthophosphate and ^{3}H-amino acids in medium free of carrier inorganic phosphate. At 6 h postinfection, the cells were disrupted and the cytoplasm was separated into supernatant and pellet fractions by centrifugation at 125,000 $\times$ g for 90 min. The pellet and supernatant fractions, as well as unfractionated cytoplasm, were analyzed for ^{3}H-labeled proteins and phosphoproteins by SDS-acrylamide gel electrophoresis.

Figure 5 reveals the presence of a major ^{32}P-labeled protein peak, the migration of which corresponds to that of NS ^{3}H-labeled protein, in unfractionated cytoplasm (Fig. 5A), as well as the cytoplasmic supernatant fraction (Fig. 5B) and cytoplasmic pellet fraction (Fig. 5C) of VS viral-infected L cells. As expected, the concentration of NS ^{3}H-labeled protein was greater in the supernatant fraction than in the pellet, but the specific ^{32}P radioactivity was equivalent for the soluble and nucleocapsid NS protein. The fact that this ratio of ^{32}P-^{3}H was about the same for the supernatant and pellet NS proteins suggests that there is not a large pool of unphosphorylated NS protein and that the NS protein is phosphorylated before incorporation into nucleocapsid.

It was not possible to determine whether the M protein (or any other except NS) is phosphorylated in the infected L cell. The M protein is always a minor constituent of the pelleted membrane fraction (29), as noted by ^{3}H-labeled protein electropherograms in Fig. 5C. The high background of ^{32}P, probably caused by slow migrating cellular phosphoproteins and fast migrating phospholipids, makes impossible determination of low level phosphorylation of other virion proteins. The same patterns of phosphorylation of NS protein were also present in extracts of VS viral-infected CE and HeLa cells, although the background levels of ^{32}P in these gels were even higher than in those of infected L cells, which made interpretation even more difficult.

Pulse-chase experiments were attempted with VS viral-infected L cells in an attempt to determine if the soluble NS phosphoprotein is a precursor of the nucleocapsid NS phosphoprotein. These studies were not successful because both [^{32}P]orthophosphate and ^{3}H-amino acids were always chased into rather than out of protein. Pulse times as short as 5 or 10 min with ^{32}P did not consistently label protein. By using longer pulses of 30 or 60 min, the NS protein of nucleocapsid pellets was never labeled with ^{32}P before the NS protein supernatant fraction. This result is consistent with the thesis that the NS protein is phosphorylated before incorporation into nucleocapsids, but much better data are required to establish this point.

An attempt was also made to study the phosphorylation of intracellular NS protein in L cells which had been pretreated with actinomycin D (2 μg/ml) and cycloheximide (50 μg/ml), or with actinomycin D alone, for 12 h prior to infection with VS virus. The proteins of these infected cells were analyzed by polyacrylamide gel electrophoresis by the protocol described in the legend to Fig. 5. The results (not shown) indicate that substantial, but perhaps slightly reduced, phosphorylation of the NS protein still occurs despite reduced protein synthesis in the cell. These data must be interpreted with some caution because the effect of actinomycin and cycloheximide on intracellular pool sizes is not known.

DISCUSSION

Despite the fact that a protein kinase is present in VS virions grown in five widely divergent cell types, and despite the fact that the same virion proteins are selectively phosphorylated regardless of the cell of origin, these kinases are most likely of cellular rather than of viral origin. In support of the contention that the virion kinases are cellular enzymes are: (i) comparative kinetic data on enzymes of different virions, (ii) evidence for deficient enzyme activity in virions grown in actinomycin-pretreated cells, and (iii) separation of viral proteins from virion kinase by phosphocellulose column chromatography. That viral infection does not induce the synthesis of cellular kinase is suggested by the finding that purified kinase from virions grown in the presence of ^{3}H-amino acids was essentially free of cochromatographed ^{3}H-labeled protein. Further evidence that the virion incorporates preformed rather than newly synthesized kinase is provided by the finding that pretreatment of cells with actinomycin or cycloheximide did not eliminate kinase activity

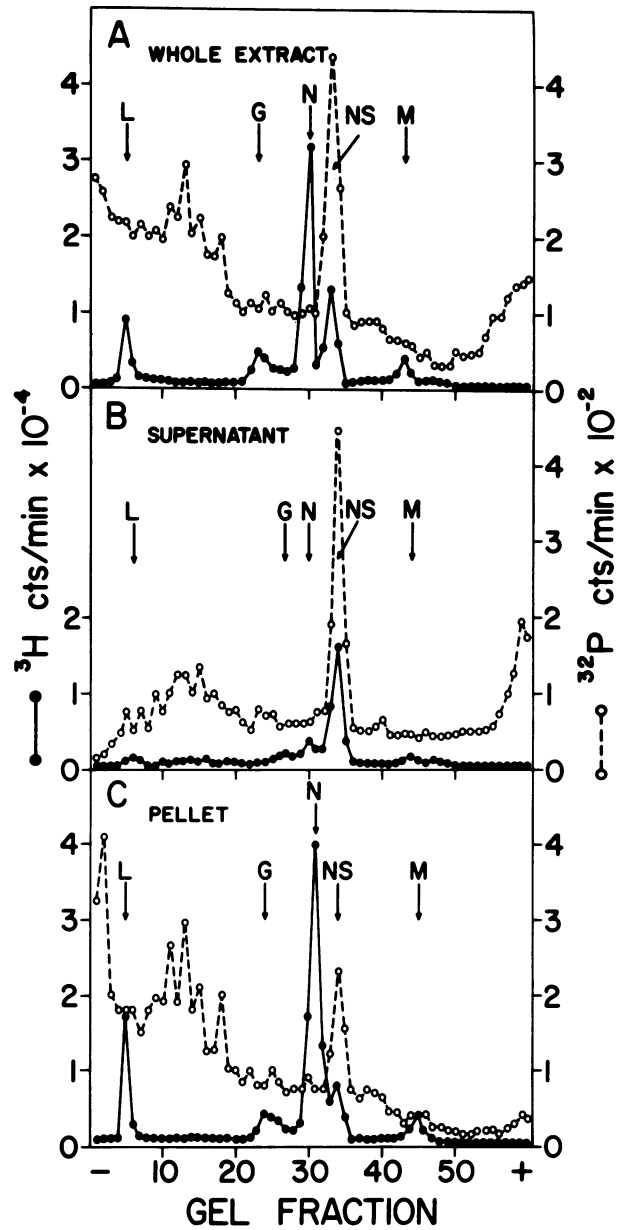

FIG. 5. *Phosphorylation of VS viral L-cell-associated proteins. Cultures of L cells were infected with VS virus at a multiplicity > 10 PFU/cell and labeled from 3.5 to 6 h postinfection in a medium which contained 10 μCi of carrier-free [^{32}P]orthophosphate per ml and 10 μCi each of [^{3}H]leucine and [^{3}H]tyrosine per ml. Cytoplasmic extracts of the cells, prepared by Dounce homogenization at 6 h postinfection, were centrifuged for 90 min at 125,000 × g. Portions of the unfractionated cytoplasmic extract (A), and of the supernatant (B) and pellet (C) fractions, were subjected to electrophoresis on 7.5% polyacrylamide gels. Arrows mark the positions of virion proteins L, G, N, NS, and M.*

in subsequently released virions. These experiments do not exclude the possibility that viral infection modifies the host protein kinase.

No final interpretation can be offered for the consistent finding of two separable peaks of enzyme activity on phosphocellulose chromatography of VS virion kinase. The linear kinetics for double reciprocal plots of kinase

activity in virions grown in three different cell types suggests the presence of a single enzyme in each preparation. It seems likely that the minor enzyme peak seen eluting from phosphocellulose columns is due to absorption and trapping of kinase by aggregated viral G and M proteins. However, the possible existence of two virion kinases has not been ruled out. The possibility that the protein kinases are not part of the virion can not be discounted completely but seems very unlikely. Even after extensive purification by the method of McSharry and Wagner (12), which eliminates 99.9% of contaminating host cell material, the kinase activity was still associated with the infectious virion (unpublished data).

Controlled detergent disruption of purified VS virions provided some basis for locating the protein kinase in the virion. Little or no enzyme was solubilized by treatment with nonionic detergents that liberated virtually all of spike protein G. Little residual enzyme activity was present in nucleocapsids devoid of envelope. Most of the kinase activity could be recovered in a fraction solubilized by Triton X-100 in the presence of 0.3 M NaCl, which also strips off the envelope protein M. The assumption seems reasonable, therefore, that VS virion protein kinase is a membrane enzyme derived from cell membrane. Smooth membrane of uninfected or infected L cells banded in a sucrose density gradient showed a considerable amount of protein kinase activity (unpublished data). Other evidence for membrane location of the VS virion kinase is based on selective phosphorylation in vitro of M protein, as well as NS protein, after activation by very small amounts of NP-40. The likely explanation for phosphorylation of matrix protein M under these conditions is its accessibility as a substrate in the virion membrane. An extension of this logic might also apply to the selectivity of in vitro phosphorylation of NS protein by the virion kinase. Structural models of the VS virion have been proposed in which the nucleocapsid is wound up in juxtaposition to the envelope and particularly to the M protein (2). The NS protein presumably occupies an exterior position on the nucleocapsid because it can be readily removed merely by a hypertonic environment (4, 5). Conceivably, therefore, the NS phosphoprotein serves to bind the nucleocapsid to the viral membrane, possibly through some noncovalent linkage with the M protein. In this position, both the NS and M protein could be phosphorylated by the cell membrane-derived protein kinase.

Much additional data are required to prove the relationship, if any, of the virion kinase to the specificity of intracellular phosphorylation of VS viral proteins. Although in vivo phosphorylation is almost exclusively of the NS protein, it is difficult to visualize the cellular site of this chemical reaction, to say nothing of its purpose. The cytoplasmic-soluble NS protein is phosphorylated at least as readily as the nucleocapsid NS protein, and there is no evidence that either is membrane associated. A possible hypothesis, therefore, might be that the intracellular NS protein(s) is not phosphorylated by the cell membrane-associated protein kinase. Proteins of non-enveloped viruses devoid of kinase activity are still phosphorylated in vivo (21, 24). Conceivably, the protein kinase of enveloped viruses does not function in viral replication but serves in some way to promote penetration by fusion of the virion envelope with the cytoplasmic membrane of the host cell (8).

The function of the VS viral NS phosphoprotein is completely unknown (4, 5). Because of its association with the nucleocapsid, some speculation has centered about a conceivable role in regulating the fidelity of transcription catalyzed by the L-protein transcriptase (5). The relationship of the VS virion kinase to the virion phosphotransferase described by Roy and Bishop (19) also remains to be investigated. Since VS viral RNA synthesis also demands the existence of an RNA replicase, and only NS protein of the five VS viral proteins has not been assigned a function, the NS phosphoprotein is also a candidate for the viral RNA replicase. Much speculation has revolved around the role of phosphoproteins interacting with nucleic acids as a basis for control of cellular transcription and replication (3, 11, 25), but firm evidence for these functions is also lacking. Similar possibilities exist for investigating the functions of the many viral phosphoproteins that have been and will be described.

ACKNOWLEDGMENTS

This research was supported by grant VC-88 from the American Cancer Society, by grant GB-35257X from the National Science Foundation, and by Public Health Service grant AI-11112 from the National Institute of Allergy and Infectious Diseases. R. L. Imblum is a fellow of the American Cancer Society.

We thank Suzanne Emerson who developed several of the techniques used in these experiments and Ruth Snyder for technical advice.

LITERATURE CITED

1. Bollum, F. J. 1966. Filter paper disk techniques for assaying radioactive macromolecules, p. 296-300. *In* G. L. Cantoni and D. R. Davies (ed.), Procedures in nucleic acid research. Harper and Row, New York.
2. Cartwright, B., C. J. Smale, F. Brown, and R. Hull. 1972. Model for vesicular stomatitis virus. J. Virol. 10:256-260.

3. Elgin, S. C. R., S. C. Froehner, J. E. Smart, and J. Bonner. 1971. The biology and chemistry of chromosomal proteins, p. 1-57. *In* E. J. DuPraw (ed.), Advances in cell and molecular biology, vol. 1. Academic Press Inc., New York.
4. 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.
5. Emerson, S. U., and R. R. Wagner. 1973. L protein requirement for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **12**:1325-1335.
6. Gravell, M., and T. L. Cromeans. 1972. Virion-associated protein kinase and its involvement in nongenetic reactivation of frog polyhedral cytoplasmic deoxyribovirus. Virology **48**:847-851.
7. Hatanaka, M., E. Twiddy, and R. V. Gilden. 1972. Protein kinase associated with RNA tumor viruses and other budding RNA viruses. Virology **47**:536-538.
8. Heine, J. W., and C. A. Schnaitman. 1971. Entry of vesicular stomatitis virus into L cells. J. Virol. **8**:786-795.
9. Kelley, J. M., S. U. Emerson, and R. R. Wagner. 1972. The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. J. Virol. **10**:1231-1235.
10. 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.
11. Martelo, O. J., S. L. C. Woo, E. M. Reimann, and E. W. Davie. 1970. Effect of protein kinase on ribonucleic acid polymerase. Biochemistry **9**:4807-4813.
12. McSharry, J. J., and R. R. Wagner. 1971. Lipid composition of purified vesicular stomatitis virus. J. Virol. **7**:59-70.
13. Mudd, J. A., and D. F. Summers. 1970. Protein synthesis in vesicular stomatitis virus-infected HeLa cells. Virology **42**:328-340.
14. Paoletti, E., and B. Moss. 1972. Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. J. Virol. **10**:417-424.
15. Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger RNA. Proc. Nat. Acad. Sci. U.S.A. **49**:654-662.
16. Randall, C. C., H. W. Rogers, D. N. Downer, and G. A. Gentry. 1972. Protein kinase activity in equine herpes virus. J. Virol. **9**:216-222.
17. Reimann, E. M., D. A. Walsh, and E. G. Krebs. 1971. Purification and properties of rabbit skeletal muscle adenosine 3',5'-monophosphate-dependent protein kinase. J. Biol. Chem. **246**:1986-1995.
18. Rosemond, H., and B. Moss. 1972. Phosphoprotein component of vaccinia virions. J. Virol. **11**:961-970.
19. Roy, P., and D. H. L. Bishop. 1971. Nucleoside triphosphate phosphotransferase. A new enzyme activity of oncogenic and non-oncogenic "budding" viruses. Biochim. Biophys. Acta **235**:191-206.
20. Rubenstein, A. S., M. Gravell, and R. Darlington. 1972. Protein kinase in enveloped herpes simplex virus. Virology **50**:287-290.
21. Russell, W. C., J. J. Skehel, R. Machado, and H. G. Pereira. 1972. Phosphorylated polypeptides in adenovirus infected cells. Virology **50**:931-934.
22. Sokol, F., and H. F. Clark. 1973. Phosphoproteins, structural components of rhabdoviruses. Virology **52**:246-263.
23. Strand, M., and J. T. August. 1971. Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. Nature N. Biol. **233**:137-141.
24. Tan, K. B., and F. Sokol. 1972. Structural proteins of simian virus 40: phosphoproteins. J. Virol. **10**:985-994.
25. Teng, C. S., C. T. Teng, and V. G. Allfrey. 1971. Studies of nuclear acidic proteins. Evidence for their phosphorylation, tissue specificity, selective binding to deoxyribonucleic acid, and stimulatory effects on transcription. J. Biol. Chem. **246**:3597-3609.
26. Wagner, R. R., M. P. Kiley, R. M. Snyder, and C. A. Schnaitman. 1972. Cytoplasmic compartmentalization of the protein and ribonucleic acid species of vesicular stomatitis virus. J. Virol. **9**:672-683.
27. Wagner, R. R., L. Prevec, F. Brown, D. F. Summers, F. Sokol, and R. MacLeod. 1972. Classification of rhabdovirus proteins: a proposal. J. Virol. **10**:1228-1230.
28. Wagner, R. R., T. C. Schnaitman, R. M. Snyder, and C. A. Schnaitman. 1969. Protein composition of the structural components of vesicular stomatitis virus. J. Virol. **3**:611-618.
29. Wagner, R. R., R. M. Snyder, and S. Yamazaki. 1970. Proteins of vesicular stomatitis virus: kinetics and cellular sites of synthesis. J. Virol. **5**:548-558.
30. Yamazaki, S., and R. R. Wagner. 1970. Action of interferon: kinetics and differential effects on viral functions. J. Virol. **6**:421-429.