The Structure and Synthesis of Influenza Virus Phosphoproteins*

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The synthesis and phosphorylation of influenza virus nucleoprotein and nonstructural protein were analyzed. The nucleoprotein (NP) was found to be phosphorylated in both infected cells and in isolated virions. The phosphate is in a monoaester linkage to a serine residue. Two-dimensional tryptic peptide maps of the 32P-labeled protein, as well as measurements of specific activity, suggest that NP is phosphorylated at one site per molecule. The viral nonstructural (NS1) protein is also phosphorylated, but on threonine residues. Up to a maximum of two sites per NS1 molecule could be so modified in infected cells, as demonstrated by two different methods of tryptic peptide analysis and by measurements of the ratio of 32P to 3H-amino-acids incorporated into NS1 protein species.

The NS1 protein is resolved into four major species of differing isoelectric point in a two-dimensional electrophoretogram. The most acidic species was found to have two phosphorylated sites per molecule, and the next most acidic species contained on the average one phosphate per molecule. Treatment of the phosphorylated species with bacterial alkaline phosphatase demonstrated that the level of phosphorylation is the only identifiable difference between the phosphorylated and unphosphorylated NS1 species. The distinction between the two unphosphorylated species could not be determined.

The distribution of the un-, mono-, and diphosphorylated NS1 species was characterized at different times after synthesis. These modifications were found to occur very rapidly after translation (30 to 60 s), after transport of the unmodified species from cytoplasm to nucleus of the infected cell. The phosphorylation of NP also takes place rapidly after its synthesis; the site within the cell of the NP phosphorylation has not been unambiguously determined.

Many polypeptides undergo posttranslational modifications in vivo; frequently, these modifications modulate the biological activity of a protein after its synthesis has been completed (1). Phosphorylation is a particularly common form of protein modification, and has been implicated as an important effector of regulation in a wide variety of cellular processes (2, 3). Phosphorylated proteins are also found in a wide variety of viruses, including vaccinia virus (4), certain rhabdoviruses (5), simian virus 40 (6), adenoviruses (7, 8), certain arboviruses (9), and avian and mammalian retroviruses (10-14). The avian sarcoma virus gene product is a phosphoprotein; it also possesses protein kinase activity (15, 16). Indeed, this kinase activity has been implicated in the transforming ability of the virus (10).

We have previously demonstrated that the influenza virus nucleoprotein and nonstructural protein 1 are phosphorylated (17, 18). In light of the important role phosphorylation plays in so many eukaryotic and prokaryotic systems, we have explored the phosphorylation of influenza viral proteins in greater detail. In this communication, we report the results of studies characterizing the nature of the phosphate-protein linkages, the number of phosphorylated sites per polypeptide chain, and the time course and intracellular location of these posttranslational protein modifications.

MATERIALS AND METHODS

Influenza Virus NP and NS1 Proteins are Phosphorylated in Infected Cells—In a previous publication, we reported that the nucleoprotein of influenza virions is phosphorylated (17). To characterize this phosphorylation in detail, we analyzed it in influenza virus-infected cells. Characterization of the cell-associated viral phosphoproteins by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis proved inadequate, due to a high background of host protein phosphorylation. We therefore turned to a modification of the O'Farrell two-dimensional protein electrophoresis system (18). The increased resolution afforded by this method has allowed us to distinguish the phosphorylation of the viral proteins from that of the cellular polypeptides.

Application of the modified O'Farrell technique to 14C-amino-acid-labeled influenza virus-infected cells is illustrated in Fig. 1a. The assignment of specific protein spots on the two-dimensional electrophoretic pattern to the known influenza viral proteins has been described previously (18); the proteins so identified are indicated in the figure. The viral nucleoprotein, nonstructural proteins, neuraminidase (NA), and hemagglutinin precursor (HAO) can be clearly seen in this autoradiogram. The influenza virus matrix (M) protein is also detectable, but due to its extremely basic isoelectric point,

1 Portions of this paper (including "Materials and Methods," portions of "Results," Table 1, and Figs. 2, 3, and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 8OM-1743, cite author(s), and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: NP, nucleoprotein; NS1, nonstructural protein 1; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; SDS, sodium dodecyl sulfate; 2 D, two-dimensional; BAP, bacterial alkaline phosphatase.
Influenza Virus Phosphoproteins: Structure and Synthesis

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis of influenza viral proteins synthesized in infected cells. MDCK cells (~10⁶) were either infected with influenza virus or mock-infected with medium alone. The cells were radiolabeled as indicated, and lysates were analyzed by two-dimensional electrophoresis, as described under "Materials and Methods." Autoradiograms of the resulting electrophoretograms are presented. a, virus-infected cells, labeled with [¹⁴C]-amino-acids (20 µCi, 5 to 5.5 h postinfection); b, mock-infected cells, labeled with [³²P]phosphate (500 µCi, 6 to 7 h postinfection); c, virus-infected cells, labeled with [³²P]phosphate (500 µCi, 6 to 7 h postinfection); d, extracts from cells in C, treated with 0.12 units of Escherichia coli alkaline phosphatase for 1 h at 37 °C before two-dimensional electrophoresis.

The majority of M protein does not focus within the pH range obtained in this separation. It is notable that the viral glycoproteins and nonstructural protein are heterogeneous in isoelectric point, forming constellations of interrelated protein spots. Fig. 1C displays an autoradiogram of the same two-dimensional electrophoresis technique applied to infected cells labeled with [³²P]PO₄. Three phosphate-labeled spots are seen in this figure which are absent in two-dimensional electrophoretograms of uninfected cells labeled in the same manner (Fig. 1b). This [³²P] label is associated with the positions of three of the influenza viral proteins visible in Fig. 1a: NP and the two most acidic species of the NS 1 protein. The phosphate label coincides exactly with the Coomassie blue-stained NP and NS 1 spots on the polyacrylamide gel itself (not shown).

These results suggest that the NP protein occurs in a phosphorylated state in infected cells as well as in virions. The viral NS 1 protein also appears to be phosphorylated in these cells. This phosphorylation is heterogeneous in expression: only a fraction of the total NS 1 protein present appears to be phosphorylated and the phosphate label is distributed between NS 1 protein species of two distinct isoelectric points.

Characterization of the Phosphate-Protein Linkages—The phosphorus found in the NS 1 and NP protein spots was resistant to the extensive RNase, DNase, and ethanol treatments employed in the sample preparation for electrophoresis (18), suggesting specific association with the viral polypeptides, rather than with superimposed nucleic acids or phospholipid. A detailed analysis of the sensitivities of the phosphorus-protein linkages to chemical and enzymatic hydrolyses, as well as a direct determination of the phosphorylated amino acids in these proteins, is presented in the miniprint supplement. These studies showed that cell-associated NP protein contains phosphoserine, the same amino acid residue previously demonstrated in virion-associated NP (17). In contrast, the major phosphorylated amino acid in NS 1 protein is phosphothreonine.

Number of Phosphorylated Sites per Viral Polypeptide: Two-dimensional Tryptic Peptide Mapping—A concept of the number of phosphates per polypeptide molecule was obtained by tryptic peptide analysis of radiolabeled NP and NS 1 proteins. The NP protein was studied first. Influenza virus-infected cells were labeled with [³²P]PO₄ and high levels of [³H]lysine and [³H]arginine. The lysed cells were analyzed by two-dimensional polyacrylamide gel protein electrophoresis, and the labeled NP protein spot was excised. The NP protein was then trypsinized directly within the polyacrylamide gel slice, and the eluted peptides were spotted onto a thin layer cellulose sheet. After electrophoresis and ascending chromatography, the resulting two-dimensional tryptic peptide map was autoradiographed. As seen in Fig. 4a, only one major [³²P]-labeled tryptic peptide was detected by this technique. To verify that the mapping procedure resolved the tryptic peptides, the same map was fluorographed to visualize the [³H]-amino-acid label (Fig. 4b; this fluorogram was first exposed 10 weeks after the autoradiogram in Fig. 4a to allow for the decay of the phosphorus-32). These results localize the site of phosphorylation of the NP protein to a single tryptic peptide within the 59,000-dalton polypeptide chain.
As shown in Fig. 5, the NS 1-d protein yielded two major phosphorylated tryptic peptides, suggesting that there are at least two distinct sites of phosphorylation on this molecule.

**Aminex A-5 Ion Exchange Peptide Mapping**—We wished to make a detailed comparison of the relationship of the major NS 1 species. For this purpose, we turned to the Aminex A-5 ion exchange column peptide mapping technique of Weisblum et al. (25). This method results in extremely reproducible peptide elution profiles, and allows much more convenient use of dual radioisotopic labeling techniques than does two-dimensional thin layer mapping. Use of a column technique also allows convenient quantitation of radioactivity associated with each tryptic peptide.

**Fig. 4.** Two-dimensional thin layer mapping of NP protein tryptic peptides. Approximately 10⁶ MDCK cells were labeled from 2.5 to 5.5 h after influenza virus infection with 1 mCi of [³²P]phosphate and 100 μCi each of L-[5-'H]arginine and L-[4,5-'H]lysine, as described under "Materials and Methods." The labeled viral NP protein was isolated by two-dimensional protein electrophoresis and treated with trypsin. The resulting peptides were analyzed by thin layer cellulose two-dimensional peptide mapping. o indicates the origin. a, autoradiogram of two-dimensional thin layer map, visualizing [³²P]phosphate-labeled tryptic peptides; b, fluorogram of the same two-dimensional thin layer map as in a, visualizing the 'H-amino-acid-labeled tryptic peptides (the film was exposed 10 weeks after the autoradiogram in a).

**Fig. 5.** Two-dimensional thin layer mapping of NS-d protein tryptic peptides. Influenza viral NS-d protein was isolated separately from [³²P]phosphate-labeled, and from [¹C]-amino-acid-labeled, infected cells (~10⁶ cells each), as described under "Materials and Methods." The NS-d protein was treated with trypsin and the resulting peptides were mapped by the two-dimensional thin layer cellulose technique described in the legend to Fig. 4. o indicates the origin. a, map of [³²P]-labeled NS-d tryptic peptides, visualized by autoradiography; b, map of a mixture of [³²P]-labeled and [¹C]-label NS-d tryptic peptides, visualized by autoradiography; c, map in b autoradiographed again, 10 weeks later, to allow the [³²P] label to decay.
Fig. 6 demonstrates this technique applied to each of the four major NS 1 species. The radiolabeling of infected cells, isolation of the appropriate viral proteins, and preparation of tryptic peptides was identical in each case, and was performed as described under “Materials and Methods” in the miniprint supplement. It can be seen from this figure that the elution profile is extremely reproducible and is very similar for each of the NS 1 species; all four NS 1 proteins are closely related to one another.

Two major phosphate-labeled peaks (indicated by arrows) can be seen in the column profile of NS 1-d tryptic peptides (Fig. 6d), consistent with the two phosphopeptides detected with the two-dimensional mapping method described above. These two phosphate-labeled peaks contain roughly equal

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**Fig. 6.** Aminex A-5 column tryptic peptide maps of NS protein species. Approximately $10^6$ MDCK cells were labeled from 2.5 to 5.5 h after influenza viral infection with 2 mCi of ($^{32}$P)phosphate (carrier-free) and 100 μCi each of L-[5-$^3$H]arginine and L-[4,5-$^3$H]lysine (final specific activities, 2.5 Ci/mmol). The labeled viral NS protein species were isolated by two-dimensional polyacrylamide gel electrophoresis, and each species was trypsinized. The resulting peptides were mapped separately by the Aminex A-5 column technique described under “Materials and Methods.” For an explanation of the arrows and arrowheads, see text. a, NS-a tryptic peptide map; b, NS-b tryptic peptide map; c, NS-c tryptic peptide map. Radioactivity that eluted from the column in the 1M sodium citrate, pH 5, and the 1M NaOH washes (utilized in regenerating the column) was quantitated to demonstrate the complete elution of labeled peptides by the pyridine acetate gradient employed. d, NS-d tryptic peptide map.
amounts of $^{32}$P counts per min, and are coincident with $^3$H-labeled peaks representing lysine or arginine-containing peptides. The $^{32}$P/ $^3$H-amino-acid ratios are approximately equal for each peak (this would not be true if either phosphopeptide was generated by an incomplete tryptic cleavage of the other).

Fig. 6, a and b, represents the column profiles of similarly radiolabeled NS 1-a and NS 1-b tryptic peptides, respectively. Not unexpectedly, phosphate label is not associated with any NS 1-a or NS 1-b tryptic peptide peak. In addition, the tritiated peptides associated in Fig. 6d with the $^{32}$P-labeled peaks (positions indicated by arrows) have vanished, and two new $^3$H-peaks have appeared (indicated by arrowheads). This can best be explained if the tryptic peptides which are phosphorylated in NS 1-d are present, but unphosphorylated, in NS 1-a and b. The absence of the strongly acidic phosphate group would allow these peptides to bind more tightly to the anion exchanger, resulting in their retarded elution.

Fig. 6c displays the elution pattern of NS 1-c tryptic peptides. This pattern contains features of both the NS 1-b and the NS 1-d patterns: the same two peptides are phosphorylated in NS 1-c as NS 1-d (arrows), but are present in smaller relative amounts in the former. In addition, the two extra peptides seen in NS 1-a and b are also present in the NS 1-c pattern, though again in relatively reduced amounts.

The relationships of the various NS 1 species were further investigated by analyzing the Aminex A-5 patterns of tryptic peptides which had been treated with alkaline phosphatase (see the miniprint supplement). The tryptic peptide patterns of NS 1-c and NS 1-d became identical with that of NS 1-b following phosphatase treatment. The simplest interpretation of these results is that there are two sites at which the NS 1 protein can be phosphorylated. NS 1-d is hypothesized to be phosphorylated on both of these sites, and therefore possesses an acidic isoelectric point. The NS 1-c spot represents a superposition of two types of monophosphorylated NS 1 molecules: those phosphorylated only on site 1, and those phosphorylated only on site 2. This conclusion is supported by the observation that the phosphate to amino acid ratio of intact NS 1-d is twice that of NS 1-c (Fig. 2, miniprint supplement). We have been unable to characterize the difference between the unphosphorylated NS 1-a and NS 1-b proteins; the tryptic peptide maps of these two species are very similar, but not identical. Perhaps the distinction in isoelectric point of these two species is due to some undetected posttranslational and modification other than phosphorylation.

**Time Course and Intracellular Location of Viral Protein Phosphorylation**—The intracellular location of the phosphorylated influenza viral proteins was investigated. Influenza virus-infected MDCK cells were labeled to steady state levels with $^{32}$Pphosphate and $[^3]$Hleucine, and fractionated into nuclear and cytoplasmic fractions as described under "Materials and Methods" in the miniprint supplement. The proteins in these fractions were resolved separately by two-dimensional polyacrylamide gel electrophoresis and the radioactivity associated with the viral NP and NS 1 proteins in each fraction was quantitated. The results are displayed in Table II. As noted by other authors (27, 28), the majority of the $[^3]$Hleucine-labeled NS 1 protein (96%) was found in the nucleus under these conditions. A corresponding amount of $^{32}$P label was found in this experiment to be similarly associated with this nuclear NS 1 polypeptide. Distribution of the viral NP protein was slightly less unequal, with some 20% of the total $[^3]$Hlabeled NP polypeptide in the cytoplasmic fraction. The $^{32}$P/ $^3$H ratio of the nuclear NP protein is essentially identical with the $^{32}$P/ $^3$H ratio of the cytoplasmic NP protein. Phosphorylation, therefore, does not appear to affect appreciably the steady state distribution of the viral NP and NS 1 proteins in infected cells.

As discussed above, the only detectable difference between the NS 1-b, NS 1-c, and NS 1-d proteins is the level of phosphorylation of these species; the relative distribution of protein molecules among the NS 1 spots should therefore be indicative of the fraction of the NS 1 polypeptide in a particular phosphorylation state. By use of very short radioactive amino acid pulse labeling, followed by subcellular fractionation and two-dimensional protein electrophoresis, the synthesis and phosphorylation of the NS 1 protein might be studied by quantitation of the distribution of amino acid radiolabel among the different NS 1 polypeptide spots. This approach is conceptually similar to a method used by others to study posttranslational modifications of histones (29).

The results of such an experiment are shown in Fig. 8.

**Table II. Steady state distribution of NP and NS 1 proteins: nuclear versus cytoplasmic fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>NP</th>
<th>NS 1 (all species)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$^3$H counts/min</td>
<td>$^{32}$P counts/min</td>
</tr>
<tr>
<td>Nuclear</td>
<td></td>
<td>6,492</td>
<td>5,734</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td>1,959</td>
<td>1,723</td>
</tr>
</tbody>
</table>

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The results of such an experiment are shown in Fig. 8.

Influenza virus-infected cells were pulse-labeled with [35S]methionine for 30 s, and the cells were either immediately separated into nuclear and cytoplasmic fractions, or first "chased" with unlabeled methionine for the time indicated. The nuclear and cytoplasmic proteins were analyzed separately by two-dimensional gel electrophoresis. The results were quantitated by excising the appropriate viral protein spots from the two-dimensional electrophoretograms and counting the incorporated radiolabel by an NCS/Omnifluor/toluene liquid scintillation fluor technique (21). The distribution of label among the various NS 1 protein species versus length of chase was plotted (Fig. 8).

Even in this very short pulse period, a significant percentage of the [35S]methionine label appeared in the nuclear fraction NS 1 protein in the absence of any chase period. This would indicate that translocation of the NS 1 protein from cytoplasmic ribosomes to the nuclei is extremely rapid. Chase periods of increasing duration resulted in the decline of radiolabel in cytoplasmic NS 1 protein and a concomitant increase in the appearance of radioactivity in the nuclear fraction NS 1 polypeptide.

The first form of NS 1 protein to be labeled in these pulse-chase experiments, in either cytoplasm or nucleus, was the NS 1-b species. This protein appears to represent the primary translation product of the NS 1 gene. If the 30-s pulse was followed in turn by a chase period greater than 1 min in length, [35S]methionine radiolabel began to appear also in the positions of the nuclear fraction NS 1-a, NS 1-c, and NS 1-d protein species. Radiolabeled NS 1-a, c, and d protein species have never been detected in the cytoplasm of these infected cells before they appear in the nuclear fraction. The hetero-
The high resolution afforded by the two-dimensional electrophoresis technique has allowed phosphorylation of influenza viral proteins to be studied during the infectious cycle, despite a background of host cell phosphorylation events. In addition, the ability to resolve the NP 1 polypeptide on two-dimensional gels into distinct species of differing phosphorylation states has allowed an analysis of this posttranslational modification that could not otherwise be achieved by use of a simple one-dimensional separation technique.

The phosphorus associated with both the influenza viral NP and NS 1 proteins appears to be in the form of terminal phosphomonoesters, linked to serine residues in the case of the NP polypeptide, and to threonine residues in the case of the NS 1 polypeptide. These phosphorus-protein associations clearly represent phosphorylation, and not ADP-ribosylation. ADP-riboseyl groups linked to polypeptides are labile to hydrolysis by phosphodiesterases, and resistant to bacterial alkaline phosphatase (26), whereas the opposite sensitivities were found for the viral protein-phosphorus linkages. The site of phosphorylation on the viral NP protein was localized to a single tryptic peptide, whereas the NS 1 protein appears to be phosphorylated on at least two distinct sites.

The NS 1 protein could be resolved by the two-dimensional electrophoretic technique into four major species with distinct isoelectric points (NS 1-a, NS 1-b, NS 1-c, and NS 1-d). It is unlikely that this observed heterogeneity is artifactual. Caution was taken during sample preparation to avoid extremes of temperature or pH that might result in carboxymylation or deamidation. Loading the protein samples on either the basic or acidic end of the isoelectric focusing dimension failed to alter the general pattern of nonstructural protein heterogeneity. At least part of the NS 1 protein charge heterogeneity could be attributed to phosphorylation: no phosphate was associated with either NS 1-a or NS 1-b species, whereas NS 1-c appeared to represent molecules phosphorylated at only a single site, and NS 1-d to represent molecules phosphorylated at two sites. The reason for the differing isoelectric points of the NS 1-a and NS 1-b species could not be elucidated in these experiments; perhaps some form of posttranslational modification other than phosphorylation is involved.

Although the phosphorylated forms of NS 1 protein could readily be resolved from the unphosphorylated forms by twodimensional electrophoresis, the same was not true of the viral NP protein. The NP polypeptide formed a single spot on the equilibrium isoelectric focusing dimension, and the position of this spot was not visibly altered by removal of the NP protein-associated phosphate by alkaline phosphatase. This is not unexpected: 1) the extremely alkaline isoelectric point of the NP protein resulted in its migration at the very edge of the resolving space; 2) the larger number of potentially ionizable amino acyl side chains in the NP protein (due to its higher molecular weight—59,000 versus 23,000 for the NS 1 polypeptide) would result in a smaller change in the pI of this protein for a given change in charge on any one amino acyl group.

The NS 1 protein was found to be synthesized, as expected, in the cytoplasmic fraction of infected cells, but appeared to be very rapidly transported into the nucleus. This result is consistent with the work of Krug and Etkind (27) and Krug and Soeiro (28), demonstrating the association of this viral protein with the cell nucleus. The only form of NS 1 protein to be labeled in these cells during a short pulse (30 s) of [35S]methionine was the NS 1-b species. The heterogeneity
characteristic of the "mature" form of this protein did not arise until after a 1-min chase period, only subsequent to the appearance of the NS 1-b polypeptide in the nuclear fraction. Therefore, modification of this protein, i.e. phosphorylation, probably occurs within the nucleus. After very long chase periods or under steady state labeling conditions, very low amounts of NS 1-a, NS 1-c, and NS 1-d species could be found in the cytoplasmic fraction; whether these cytoplasmic forms of NS 1 protein are of physiological significance, or are simply due to leakage from the nucleus during the cell fractionation procedure, could not be determined. Under steady state labeling, all forms of cytoplasmic NS 1 protein represented less than 5% of the total NS 1 polypeptide in these cells.

As in any cellular fractionation scheme, the definition of "nuclear" versus "cytoplasmic" location must be viewed with caution. The technique utilized in the experiments reported here appeared to yield a fairly clean separation of nuclei and cytoplasm. The efficacy of this procedure was determined by inspection of the Coomassie blue-stained electrophoretograms derived from these separate fractions: very abundant host proteins visible in the cytoplasmic fractions were completely absent from the nuclear fraction, and vice versa (electrophoretograms not shown). The viral glycoproteins (known to be associated with cellular cytoplasmic and plasma membranes (30-33)) were also absent from the nuclear fractions.

The role of phosphorylation in influenza virus replication remains to be elucidated. Recent observations (34) indicate a role for NS 1 proteins in the synthesis of virion RNA from cRNA templates. The distribution of phosphorylated forms we have elucidated for NS 1 might represent intermediates in an NS 1-catalyzed reaction involving phosphate (such as kinase or phosphatase) or alternatively may represent modifications responsible for modulation of NS 1 function.

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REFERENCES
Influenza Virus Phosphoproteins: Structure and Synthesis

Supplement to
The Structure and Synthesis of Influenza Virus Phosphoproteins
Martin L. Prusiner and Edward E. Behravan

MATERIALS AND METHODS

Virus and Cells:
A plaque purified stock of the A/MX/31 strain of influenza virus (originally a gift of Dr. George Schachter) was used. Cells obtained from the Madin-Darby Canine Kidney (MDCK) line, obtained from the American Type Culture Collection, were employed for all experiments. Cells were maintained in 15% horse serum (Hyclone Laboratories, Utah) supplemented with 10% fetal bovine serum.

Infectious Radiolabeled Labeling:
2,3) A suitable filter containing 0.5% WBC cells was used for radiolabeled labeling. Infectious virus was then added per bottle. After allowing 6 h for viral infection, the cells were washed twice with PBS buffer containing 30 mM sodium phosphate, 140 mM NaCl, and 3 mM MgCl2. The cells were then labeled with [3H]thymidine for 90 min at 4°C, lysed with 0.1% Triton X-100, and the lysates were used for Western blot analysis.

Characterization of the Phosphoprotein: Protein Phosphatase-1 Kinase Activity

Characterization of the Phosphorylated NS-1 Protein Phosphatase activity was determined by incubating the NS-1 protein with 32P-labeled ATP and phosphatase inhibitors. The phosphorylated protein was then isolated by SDS-PAGE and visualized by autoradiography.

RESULTS

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The chemical and enzymatic sensitivity of the phosphorus-protein linkage for the NP protein isolated from infected cells were identical to those previously reported for the phosphorylated NP protein found in purified virus (17). The association of phosphorus with the intact NP protein was found to be refractory to all chemical and enzymatic treatments performed, including boiling.

The polypeptide nature of the phosphorus-containing NP protein was confirmed by prior treatment with phosphatase C to produce a phosphopeptide (Fig. 7). This peptide was resistant to alkaline phosphatase treatment. A similar resistance to alkaline phosphatase and acid phosphatase observed for NMP and NSP proteins suggests that the NP protein contains a phosphorus-protein linkage that can be digested by alkaline phosphatase (25). The results obtained from the enzymatic treatments of NP protein must be viewed cautiously. The accessibility of a protein group to a proteolytic enzyme may be limited by glycosylation or other factors. We therefore attempted to identify the nature of the phosphorus-protein linkages by a different approach. 2-5-P(labeled NP and NS proteins were purified from infected cells by 2-5-P protein electrophoresis and acid hydrolysis. The phosphorlated amino acids were then separated by this method, and their P values compared to those of authentic phosphoprotein acid standards. The results are illustrated in Fig. 3. It is notable that the only 2-5-P(labeled amino acid found in NP protein isolated from infected cells is phosphoserine. This is the same phosphoserine acid found in NP protein isolated from purified virus (17). In contrast, the major phosphorlated amino acid found in the NS protein either NMP or NSP) is phosphothreonine.

![Diagram](https://example.com/diagram.png)

**Fig. 3.** Thin layer electrophoretic identification of phosphoprotein acids present in viral-associated NP and NS proteins. Acid hydrolysates of 2-5-P(labeled influenza virus NP, NMP, and NSP proteins species were prepared as described in Materials and Methods, and the phosphorylated components were resolved by thin layer electrophoresis. Autoradiograms of the resulting electrophoretic patterns are presented. The positions of authentic phosphoprotein and phospholipid standards, run as internal standards and visualized by ultraviolet radiation, are indicated.

**Alkaline Phosphatase Treatments of NP Trp Pptides**

The sensitivity of 2-5-P(labeled NP and NSP proteins to alkaline phosphatase treatment was tested. Aliquots of the tryptic peptide samples were treated with alkaline phosphatase and applied to an alkaline 4.5 column. The resulting elution profiles for NP and NSP are presented in Fig. 7. (Identical results were obtained for NSP).

**Fig. 7.** Alkaline 4.5 column map of alkaline phosphatase-treated NP or NSP trypic peptides. An aliquot of the NP or NSP trypic peptides sample in Fig. 6 was adjusted to pH 11.0 in Tris buffer, pH 9.0, and was treated with 0.1 unit of alkaline phosphatase (Worthington Biochem., RAM grade) for 1 h at 37°C. One milligram of bovine serum albumin trypic peptides was then added as carrier, the sample was lyophilized, redissolved with 5% formic acid, and the peptides were applied to an alkaline 4.5 column. The column was subsequently eluted by the protocol described in Materials and Methods for Fig. 6.

The analysis clearly shows that the 2-5-P(labeled tryptic peptide is susceptible to removal by phosphatase, although the 2-5-P(labeled in the intact protein is not. This result indicates that the phosphorus in tryptic peptide must be true proteolitic membranes. This in turn implies that the phosphorus in the intact NP protein is also in the free of terminal phosphomonoesters which are protected from enzymatic hydrolysis. Phosphoprotein acids that would be expected to be labile to alkaline phosphatase, but which are inaccessible to enzymatic hydrolysis in the intact protein, have been reported for other peptides.

The Alkaline 4.5 pattern of the alkaline phosphatase-treated NP protein trypic peptides (Fig. 7) is identical in every detail to that of the untreated NP protein (Fig. 6). The same is true of the alkaline phosphatase-treated NSP protein trypic peptide pattern (column profile not shown). The only detectable difference among the NP, NSP, and NSP protein species, therefore, is the relative level of phosphorylation of the phosphopeptides.