1985

# Analysis of influenza a virus polypeptides by limited proteolysis in SDS-polyacrylamide gels.

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# ANALYSIS OF INFLUENZA A VIRUS POLYPEPTIDES BY LIMITED PROTEOLYSIS IN SDS-POLYACRYLAMIDE GELS

by

Mark Andrew Parrington

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Biology in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

WINDSOR, ONTARIO, CANADA

1985

DEDICATION

To my father G. E. Parrington

ABSTRACT

ANALYSIS OF INFLUENZA A VIRUS POLYPEPTIDES BY LIMITED PROTEOLYSIS IN SDS-POLYACRYLAMIDE GELS

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Purified influenza A viruses, A/PR/8/34 (H1N1), A/FM/1/47 (H1N1), A/USSR/90/77 (H1N1), A/Singapore/1/57 (H2N2), and A/Hong Kong/8/68 (H3N2), disrupted under reducing and non-reducing conditions were subjected to electrophoresis in SDS-polyacrylamide gels. Only the surface glycoprotein hemagglutinin (HA) and two internal polypeptides, the nucleoprotein (NP) and matrix protein (M) were isolated for study. Such gel-isolated polypeptides were subjected to limited proteolysis in SDS-polyacrylamide gels (14%) using the technique described by Cleveland et al., (1977). Of the proteases studied, subtilisin, pronase P, and proteinase K were the most effective in hydrolyzing virus polypeptides. Hydrolysis fragments generated by the digestion of the M polypeptide were similar regardless of the virus used. Whereas the HA and NP polypeptides yielded different digestion patterns for each virus tested. From the results it appears that comparison of polypeptide digestion patterns is a simple alternative method for identifying alterations in influenza polypeptides between and within subtypes.

ACKNOWLEDGMENTS

I would like to extend my sincere thanks to Dr. L. R. Sabina, Department of Biology, University of Windsor, for his inspiration and guidance throughout the course of this research. The help he has given me in writing this thesis is greatly appreciated.

For critically reviewing this thesis, I am grateful to Dr. M. J. Dufresne, Department of Biology and Dr. N. F. Taylor, Department of Chemistry, both of the University of Windsor.

A note of thanks is extended to Mrs. Margit Palnau for typing this thesis.

To my wife, Linda, a very special thanks for understanding, patience and encouragement given during this research.

This investigation was supported by a grant from the National Sciences and Engineering Council of Canada.

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## LIST OF ABBREVIATIONS

EDTA - disodium ethylenediamine tetraacetate

Endo-D - endoglycosidase D

Endo-H - endoglycosidase H

NaCl - sodium chloride

PBS - phosphate buffered saline

PAGE - polyacrylamide gel electrophoresis

RNA - ribonucleic acid

SDS - sodium dodecyl sulfate

TEMED - N,N,N',N'-tetramethylethylenediamine

Tris - tris (hydroxymethyl) methylamine

HA - monomer form of the influenza virus hamagglutinin polypeptide

HA (m) - monomer form of the influenza virus hemagglutinin polypeptide

HA (t) - trimer or spike form of the influenza virus hemagglutinin polypeptide

NA - influenza virus neuraminidase glycoprotein

M - influenza virus matrix polypeptide

NP - influenza virus nucleoprotein

PR - A/PR/8/34 (H1N1)

FM - A/FM/1/47 (H1N1)

USSR - A/USSR/90/77 (H1N1)

Singapore - A/Singapore/1/57 (H2N2)

Hong Kong - A/Hong Kong/8/68 (H3N2)

# INTRODUCTION

Influenza has plagued humans for centuries and has been described as an unvarying disease caused by a varying virus (Kilbourne, 1975). It frequently affects large portions of the population regardless of age or previous infection history and remains today as a poorly controlled disease despite the fact that the infectious agent, influenza virus was first isolated 50 years ago (Beveridge, 1977).

Influenza viruses are members of the orthomyxovirus family of RNA viruses. They possess an internal helical ribonucleoprotein, which is surrounded by an inner protein matrix and an outer lipo-protein envelope (Compans et al., 1972). The influenza virus genome is composed of eight segments of negative, single-stranded RNA (Palese, 1977). Influenza particles which are highly pleomorphic can be identified in electron micrographs by the presence of surface glycoprotein spikes. These hemagglutinins and neuraminidases project from the lipid bilayer of the envelope to form the characteristic fringe (Rott, 1977).

Influenza viruses are divided into three antigenic types A, B and C. The types are separated according to the nature of their internal ribonucleoprotein (NP) antigen which does not cross-react between types (Assaad et al., 1979). To date all virus strains associated with pandemic influenza have been influenza A viruses isolated from animals, birds and humans (Ward, 1981). On the basis of the antigenic

character of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (N), influenza A viruses are classified into different subtypes. Both glycoproteins have the ability to undergo extensive antigenic variation (Laver, 1978). Two types of antigenic variation recognized are antigenic drift and shift. The former type is caused by a number of successive random point mutations in the hemagglutinin gene which lead to minor changes in the primary amino acid sequence of the hemagglutinin polypeptide (Laver and Webster, 1968). Such antigenic drifting also occurs in the neuramínidase polypeptide. The evolution of new unrelated influenza A strains are the result of antigenic shift. Apparently the shift process is facilitated by genetic recombination between influenza strains which infect animals, birds or humans producing progeny (Laver and Webster, 1973). Over a period of time through antigenic drifting and shifting, variants are constantly evolving which have characteristic hemagglutinin and neuraminidase polypeptides. Webster and Bean (1978) suggested that variation is not only associated with viral genes coding for H and N surface antigens, but changes also occur in other genes controlling virulence and transmissibility of influenza viruses. Influenza A viruses are recognized on the antigenic basis of their hemagglutinins and neuraminidases. Antigenic variation occurs mainly within influenza viruses classified as type A, three major subtypes affecting man are H1N1, H2N2 and H3N2 (Assaad et al., 1980).

Isolated hemagglutinin spikes are composed of three hemagglutinin molecules (Wiley et al., 1977; Wilson et al., 1980, 1981). Each hemagglutinin monomer consists of two smaller polypeptide chains $HA_1$ and $HA_2$ (Lazarowitz et al., 1971; Klenk et al., 1972; Skehel, 1972) which are held together by disulfide bonds (Laver, 1971).

In 1979 the World Health Organization (Assaad et al., 1980) suggested that subtype relationships between influenza A viruses might be based on the stability of the hemagglutinin polypeptide to physical and chemical agents, including proteases. This suggestion initiated the following biochemical study to evaluate the enzyme susceptibility of structural proteins of influenza A strains. Initially the effect of mercaptoethanol, SDS and several proteases on polypeptides of the influenza PR strain was examined in SDS-polyacrylamide gels. Once proteases were found that hydrolyze the HA, M and NP influenza polypeptides, proteolytic digestion patterns of these polypeptides from several influenza reference strains were compared in gels. By these studies possible correlations between digestion patterns obtained with specific influenza subtypes could be investigated.

## MATERIALS AND METHODS

### Viruses

Influenza virus strains A/PR/8/34 (H1N1), A/FM/1/47 (H1N1), A/USSR/90/77 (H1N1), A/Singapore/1/57 (H2N2), and A/Hong Kong/8/68 (H3N2) were provided by D. A. McLeod, Laboratory Centre for Disease Control, Ottawa, Canada. These strains were grown for 48 hours at 34°C in 10-day old embryonated eggs inoculated allantoically (Rovozzo and Burke, 1973) with an input of 0.8 to 2.6 hemagglutination (HA) units of virus. After 48 hours of incubation, all virus-infected eggs were refrigerated at 4°C overnight. Allantoic fluids were clarified by centrifugation at 3,000 xg for 10 minutes at 4°C. To pellet viruses, infectious allantoic fluids were centrifuged for 1 hour at 65,000 xg (Beckman Model L2-65B; rotor 60Ti). The sedimented viruses were then resuspended in Dulbecco's (1954) phosphate buffered saline (PBS) (minus $Ca^{++}$ and $Mg^{++}$ salts).

### Virus Purification

The PR and FM strains of influenza viruses were purified by rate zonal centrifugation in preformed discontinuous gradient columns of sucrose in PBS. Viruses were spun at 90,000 xg for 1.5 hours in a Beckman SW41 rotor. Opalescent bands harvested from the 32.5 - 37.5% zone were recentrifuged through a 2-step 10 - 20% sucrose gradient. The virus

pellet was then resuspended in a small volume of PBS. Singapore and Hong Kong strains were purified similarly. However, virus bands were removed from the 30 - 37.5% sucrose zone of centrifuged gradient tubes. Similar centrifugation conditions were used to purify concentrated virus samples of USSR except that the preformed discontinuous gradient columns had a sucrose cushion of 45%. In comparison to other virus harvests, USSR virus particles were recovered as a pellet rather than as a virus band.

Virus Envelope Extraction

To extract the envelope proteins, 200 ul of purified virus sample (200 ug of protein) was mixed with an equal volume of 3% Triton X-100 prepared in flu buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6). This mixture was layered on a cushion of 10% (w/v) sucrose in flu buffer containing 2% Triton X-100. Centrifugation was carried out in a Beckman SW41 rotor at 90,000 xg for 1.5 hours at 4°C. The material that remained on top of the sucrose cushion was removed and kept at 4°C, until examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE).

Hemagglutination (HA) Assay

The hemagglutinating activity of influenza viruses was determined with rooster red blood cells by the method of Palmer et al., (1975).

Protein Assay

All protein determinations were assayed by the Bradford (1976) procedure with bovine serum albumin as standard.

Chemicals

Acrylamide, bis crosslinker, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, sodium dodecyl sulfate (SDS), mercaptoethanol, Coomassie brilliant blue (R-250), low and high molecular weight standard kits for SDS-PAGE, and Triton X-100 were purchased from Richmond, California; trypsin (EC 3.4.21.4) (2X crystalline) from bovine pancreas, subtilisin Carlsberg (EC 3.4.21.14) from a strain of Bacillus subtilis, proteinase K (EC 3.4.21.14) from Tritirachium album, pronase P (type V1) from Streptomyces griseus, Aspergillus acid proteinase (EC 3.4.23.6) from Aspergillus saitoi, alpha-chymotrypsin (EC 3.4.21.1) from bovine pancreas, carboxypeptidase B (EC 3.4.17.2) from porcine pancreas, and alpha-lactalbumin from bovine milk were purchased from Sigma Chemical Co., St. Lous, Mo.; cathepsin C (EC 3.4.14.1) from beef spleen, bromelain (EC 3.4.22.4) from Ananas sativus, and ficin (EC 3.4.22.3) from Ficus carica were obtained from Boehringer Mannheim, West Germany; Tris (hydoxymethyl) methylamine, sucrose (special density gradient grade), glycine, methanol, hydrochloric acid, glacial acetic acid, sodium dihydrogen orthophosphate (monobasic), and disodium hydrogen orthophosphate (dibasic) from BDH Chemicals, Toronto,

Ontario, Canada; Tris-HCl, citric acid (monohydrate), and disodium ethylenediamine tetraacetate (EDTA) from Fisher Scientific Co., Fair Lawn, New Jersey; endoglycosidase-D (endo-D) from Diplococcus pneumoniae and endoglycosidase-H (endo-H) from Escherichia coli obtained from Miles Scientific Co., Naperville, Il.

Glycosidase Treatment of Glycopeptides

PR influenza virus (565 ug of protein) was suspended in 300 ul of citrate-phosphate buffer (0.05 M, pH 6.5) containing 0.04 U each of endo-H and endo-D. The mixture was continuously stirred throughout the 20 hour incubation period at 37°C. For detecting hydrolyzed glycoproteins, samples were mixed with SDS, glycerol and bromophenol blue, and then applied to an SDS-polyacrylamide gel.

SDS-Polyacrylamide Gel Analysis

Viruses and isolated polypeptides of influenza A strains were analyzed by electrophoresis on polyacrylamide gel slabs in a Protean cell (Bio Rad Laboratories, Richmond, California) using the discontinuous buffer system of Laemmli (1970). The electrophoresis buffer consisted of 0.025 M Tris, 0.19 M glycine and 0.1% SDS. Virus polypeptides were isolated in separating gels containing a final concentration of 10% acrylamide, 0.27 bisacrylamide, 0.023% TEMED, 0.045% ammonium persulfate, and 0.1% SDS in 0.38 M Tris buffer (pH 8.8). The stacking gel had 3% acrylamide, 0.08% bisacrylamide, 0.05% TEMED, 0.1% ammonium persulfate, and

0.1% SDS in 0.123 M Tris buffer (pH 6.8). Treatment of virus samples varied, however, all virus samples after dilution in the appropriate buffer contained 10% glycerol, 0.007% bromophenol blue and 1.6 mg of viral protein/ml. Gels were run at 19 mA until the tracking dye (bromophenol blue) reached the bottom of the separating gel. Bands were identified in the gel after staining with 0.1% Coomassie blue, 50% methanol, and 10% acetic acid for 25 minutes, and destained for 55 minutes in 5% methanol and 10% acetic acid. After rinsing the gel with cold water, individual bands were sliced out with a scalpel, and allowed to preequilibrate for 30 minutes with occasional swirling in 10 ml of sample buffer containing final concentrations of 0.125 M Tris-HCl (pH 6.8), and 0.1% SDS.

Limited proteolysis of viral polypeptides was done essentially by the procedure of Cleveland et al., (1977). The composition of the stacking gel was identical to that used to isolate virus polypeptides. Also, the composition of the separating gel was as described for 10% gels except that acrylamide and bisacrylamide were prepared at concentrations of 14 and 0.3% respectively (Appendix A). Gel slices were inserted into the sample wells of the slab. Spaces around the slices were filled with 30% glycerol in sample buffer. Fifty ul of enzyme preparation in sample buffer with 10% glycerol was loaded into appropriate wells (Appendix B). An electric current of 30 mA was applied until the Coomassie blue stain had migrated approximately

2 cm into the separating gel. Electrophoresis was continued at 20 mA and the current was turned off when the stain reached the bottom of the gel. The gel was stained for 1 hour in 0.25% Coomassie blue, 50% methanol, and 10% acetic acid, and destained by diffusion in 5% methanol and 10% acetic acid.

# RESULTS

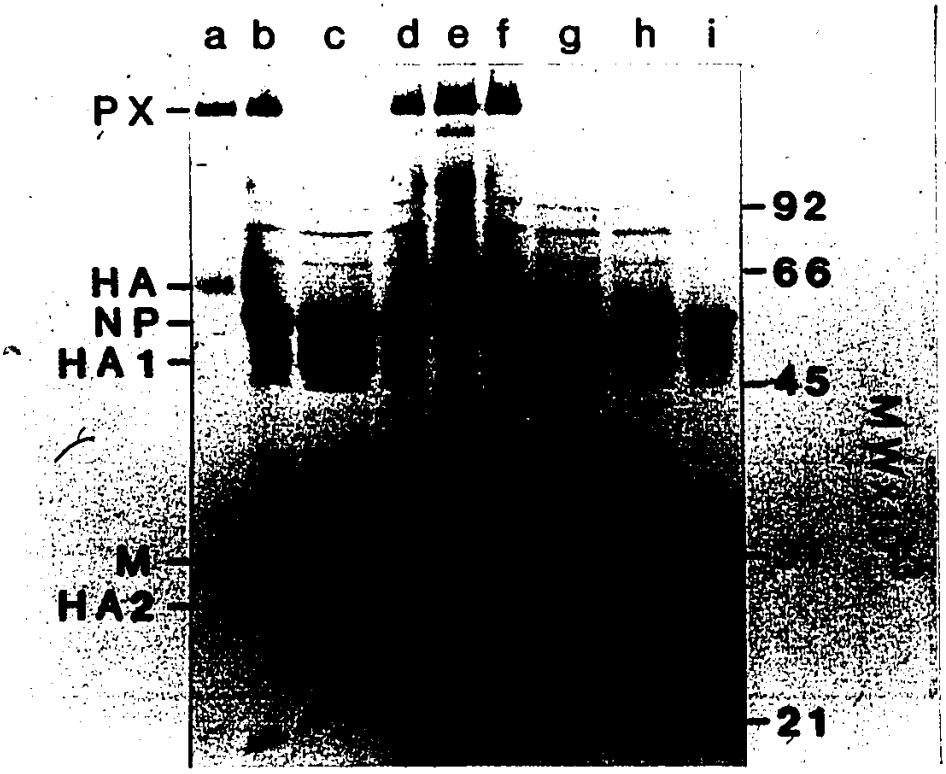
## Polypeptide Profiles of PR Virus Dissociated Under Different Conditions

To isolate specific surface and internal polypeptides for limited proteolysis studies, the dissociation of intact PR viruses with SDS alone or SDS in combination with mercaptoethanol and heat treatment was first examined by SDS-PAGE. The results of this experiment are shown in Fig. 1.

As others have found (Schulze, 1970; Compans et al., 1970; Klenk et al., 1973) the dissociation of influenza A viruses under reducing conditions (SDS, mercaptoethanol and heat) resulted in the appearance of all the characteristic polypeptide species including the hemagglutinin subunits ($HA_1$ and $HA_2$) (Fig. 1, c, g, h and i). By omitting mercaptoethanol and heat treatment in the preparation of the virus sample, a different SDS-PAGE profile was obtained (Fig. 1, e). Although the polypeptide bands NP and M appear unaffected, both the $HA_1$ and $HA_2$ bands are absent. A major alteration in the gel pattern was the detection of two new bands. One band (PX) is located near the top of the gel and another directly above the NP band with an apparent molecular weight of 57 K. Both polypeptides were identified as envelope proteins. This was demonstrated by extracting the envelope of the PR strain from intact viruses as described under Materials and Methods. Gel electrophoresis

Fig. 1. Polypeptide profiles of PR virus dissociated under different conditions. Preparations of PR virus: (b, d, e and f) under non-reducing conditions, (c, g, h and i) under reducing conditions. Preparation of PR virus envelope proteins: (a) under non-reducing conditions.

Fig. 1



under non-reducing conditions revealed the presence of the PX and 57 K bands (Fig. 1, a). The 57 K polypeptide banded at a position in the gel slab corresponding to monomeric hemagglutinin (HA) molecules.

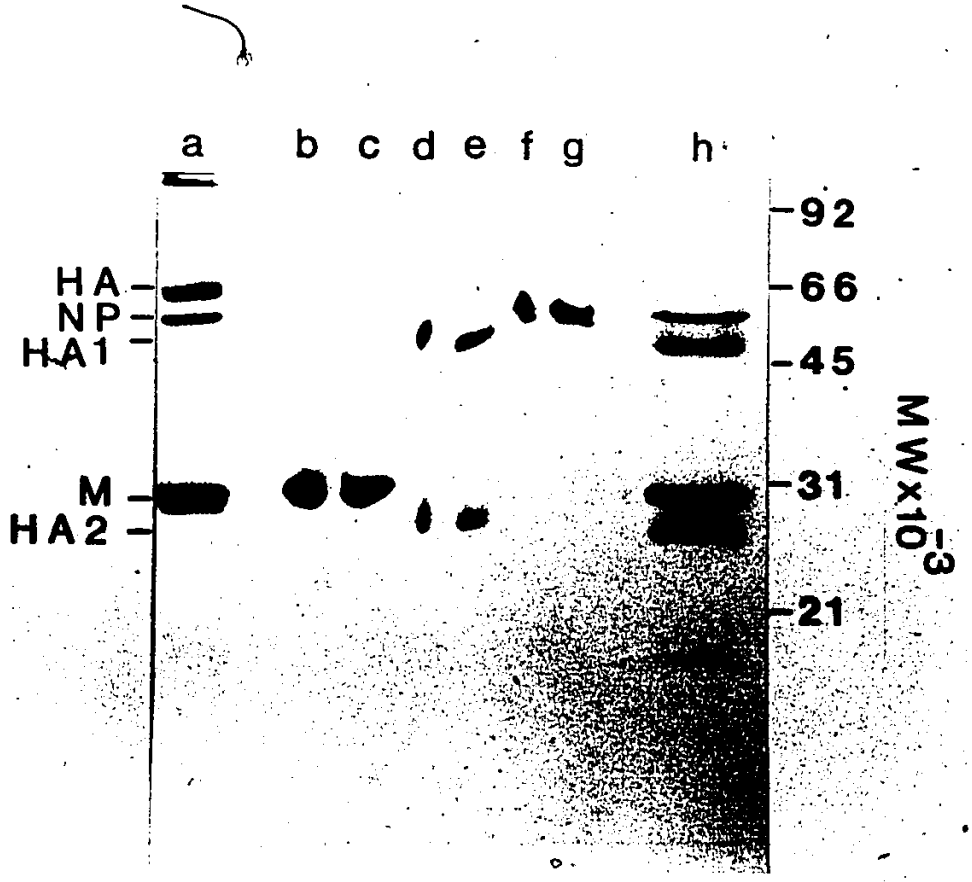
Since monomeric HA molecules of influenza viruses are converted into the $HA_1$ and $HA_2$ subunits under reducing conditions, the SDS-PAGE patterns seen in Fig. 1, b, d and f were unexpected. In this experiment, PR virus preparations were not treated with mercaptoethanol or heat before loading into gel sample wells adjacent to those with viruses dissociated under reducing conditions. After electrophoresis, the only major polypeptide band noticeably altered, possibly by diffusing mercaptoethanol, was the 57 K band. This finding in conjunction with the appearance of $HA_1$ and $HA_2$ polypeptide bands strongly suggested that the 57 K polypeptide represents monomeric HA molecules.

Effect of Mercaptoethanol on Isolated Polypeptide Bands

To determine the effect of mercaptoethanol on gel-isolated polypeptide bands, PR virus samples dissociated with only SDS were subjected to electrophoresis. The 57 K (HA), NP and M polypeptide bands were sliced out of the gel. Gel slices were preequilibrated in both sample buffer with and without 10% mercaptoethanol for 30 minutes. All gel slices were re-electrophoresed in another polyacrylamide gel. The results of this experiment are shown in Fig. 2.

Fig. 2. Effect of mercaptoethanol on isolated PR virus polypeptide bands. Polypeptide bands exposed to mercaptoethanol: (c) M, (e) HA, (g) NP. Control polypeptide bands: (b) M, (d) HA, (f) NP. Preparations of PR virus: (a) under non-reducing conditions, (h) under reducing conditions.

Fig. 2



As in the case of mercaptoethanol treatment of whole viruses (Fig. 1), the NP and M polypeptide isolates were unaffected by preequilibration in the presence of the reducing agent (Fig. 2, c and g). However, the 57 K (HA) polypeptide was cleaved into two polypeptides by exposure to mercaptoethanol (Fig. 2, e). These two polypeptides migrate to positions that correspond to $HA_1$ and $HA_2$ polypeptides of virus samples prepared under reducing conditions (Fig. 2, h). This data along with the results of Fig. 1 clearly demonstrates that the 57 K polypeptide is the monomeric form of the HA polypeptide.

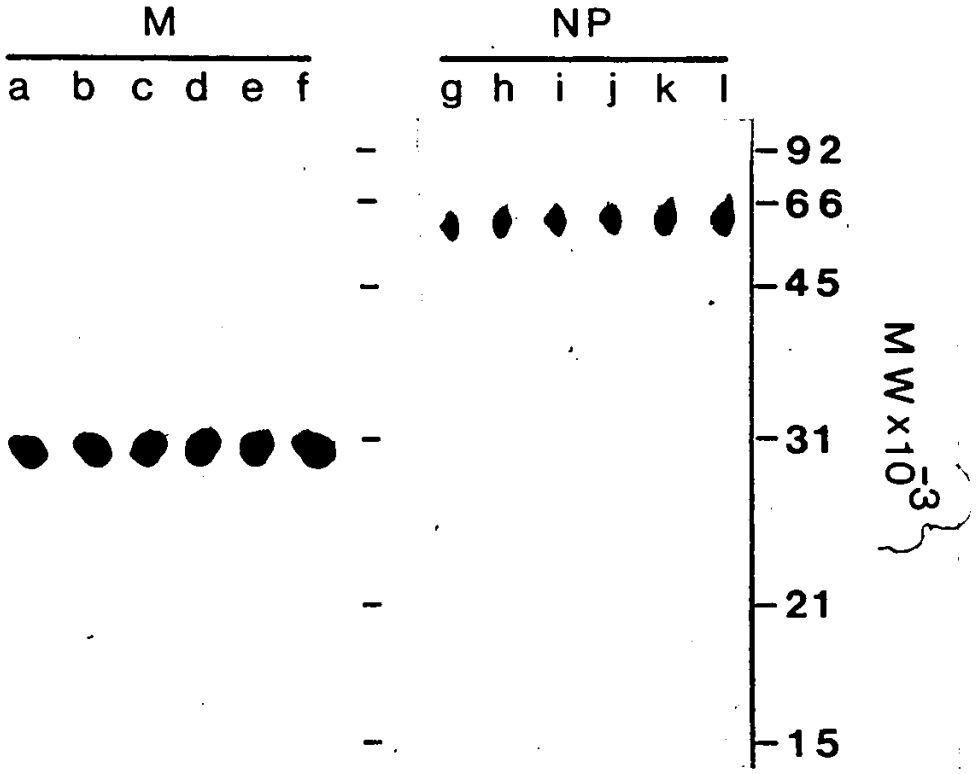
Determination of Conditions for Hydrolysis of Gel-Isolated Polypeptides by Proteases

To determine an effective method for subjecting gel-isolated virus polypeptides to limited proteolysis, ficin was tested for its ability to diffuse into gel slices. In this case, gel slices of M and NP polypeptides of PR virus were preequilibrated for 30 minutes in sample buffer containing various concentrations of ficin, and then electrophoresed on a 14% polyacrylamide gel. The results of this experiment are shown in Fig. 3.

Irrespective of the ficin concentration used during preequilibration of the gel slices, no hydrolysis occurred (Fig. 3, b, c, d, e, f, g, h, i, j, and k). With untreated control slices of M and NP polypeptides, degradation was not evident after preequilibration and electrophoresis of the

Fig. 3. Preequilibration of gel slices of PR virus M and NP polypeptides in the presence of ficin. M polypeptide bands exposed to ficin concentrations (ug/ml) of: (b) 1, (c) 5, (d) 10, (e) 50, (f) 100. NP polypeptide bands exposed to ficin concentrations (ug/ml) of: (g) 100, (h) 50, (i) 10, (j) 5, (k) 1. Control polypeptide bands: (a) M, (l) NP.

Fig. 3



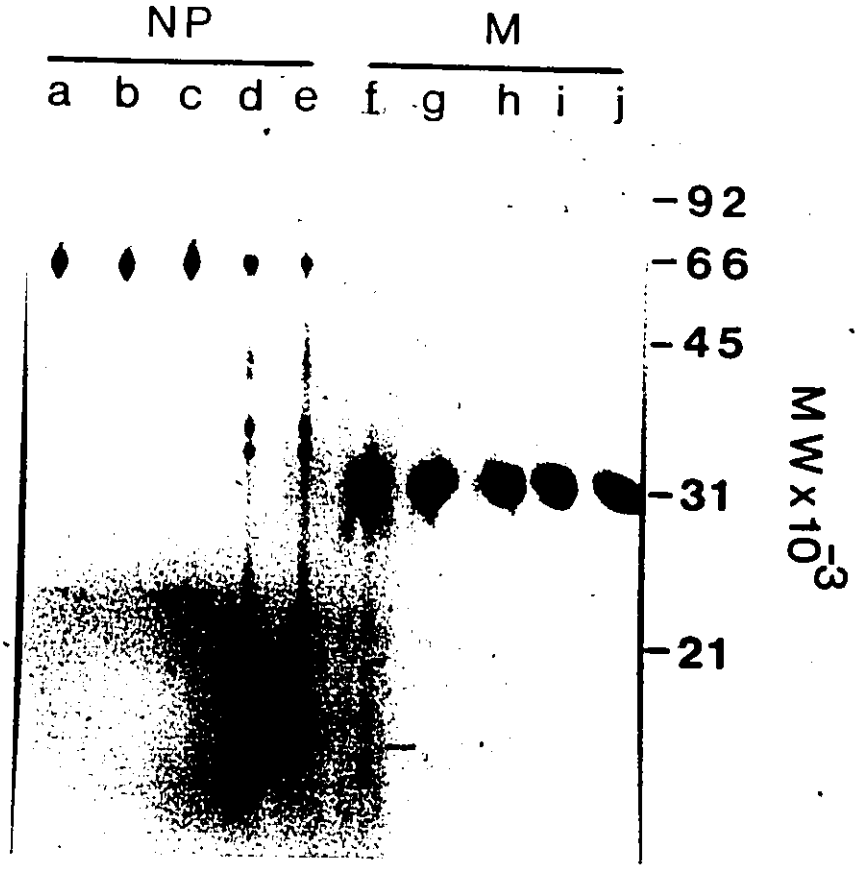
two polypeptides (Fig. 3, a and 1).

The failure of ficin to hydrolyze the NP and M polypeptides suggests that the enzyme was unable to penetrate the polyacrylamide slice within the 30 minute preequilibration time. Or, the cleavage sites of NP and M polypeptides were inaccessible to attack by ficin. Further experiments were done to establish the effect of ficin and trypsin electrophoresed through gel slices of isolated PR virus polypeptides. Gel-isolated NP and M polypeptides were preequilibrated for 30 minutes in sample buffer, loaded into sample wells of a polyacrylamide gel, overlaid with either 100 or 300 ug/ml of enzyme and electrophoresed. Figure 4 shows the results.

Preequilibration and electrophoresis conditions had no effect on untreated controls of gel-isolated NP and M polypeptides (Fig. 4, b and h). Ficin was able to hydrolyze both the NP and M polypeptide bands at concentrations of either 100 or 300 ug/ml. Hydrolysis fragments of NP polypeptide bands are seen in gels as two main bands with approximate molecular weights of 36 K and 34 K, and several less intensely stained bands ranging between 18 K to 23 K daltons (Fig. 4, d and e). Minimal hydrolysis of M polypeptide bands by ficin was observed with the appearance of two very lightly stained bands having peptides of 15 K and 16 K daltons (Fig. 4, f and g). Some of the NP and M polypeptide bands remained unhydrolyzed by the ficin concentrations used.

Fig. 4. Effect of electrophoresing trypsin or ficin through gel slices of PR virus NP and M polypeptides. NP polypeptide bands exposed to trypsin concentrations (ug/ml) of: (a) 100, (c) 300. NP polypeptide bands exposed to ficin concentrations (ug/ml) of: (d) 100, (e) 300. M polypeptide bands exposed to ficin concentrations (ug/ml) of: (f) 300, (g) 100. M polypeptide bands exposed to trypsin concentrations (ug/ml) of: (i) 100, (j) 300. Control polypeptide bands: (b) NP, (h) M.

Fig. 4



Neither trypsin concentration (100 or 300 ug/ml) tested had the ability to hydrolyze the NP or M polypeptide bands (Fig. 4, a, c, i and j).

The results from the previous experiments with ficin indicate that preequilibration of the gel-isolated polypeptides with enzyme prior to electrophoresis, was ineffective in achieving limited proteolysis of virus polypeptides. Therefore, all other limited proteolysis experiments were carried out by electrophoresing enzyme through gel slices of viral polypeptides.

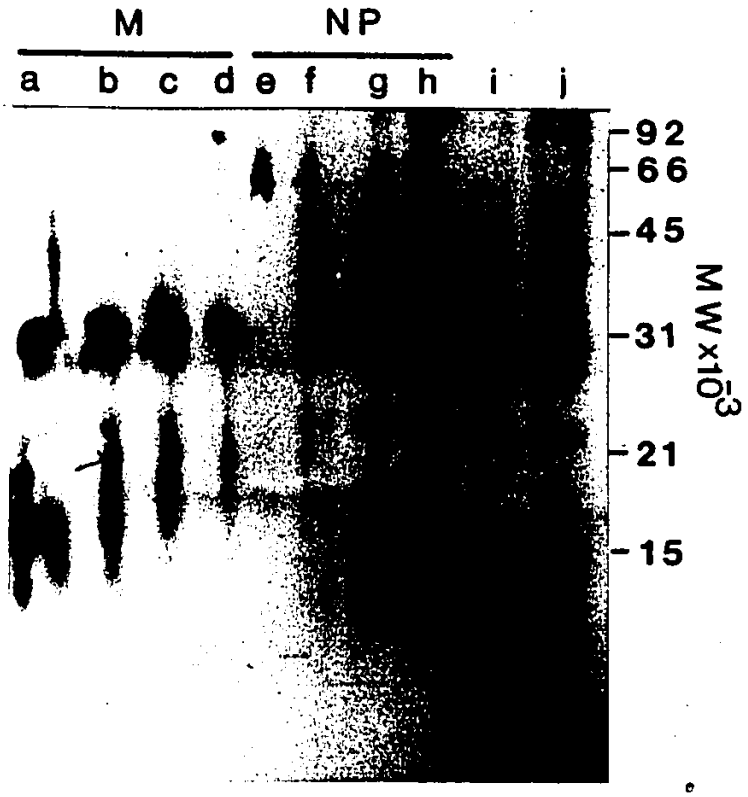
Variation of Ficin Concentrations on Hydrolysis of NP or M Polypeptide Bands

To ascertain whether total hydrolysis of NP and M polypeptide bands was possible, gel-isolated polypeptides of PR virus placed in sample wells with varying concentrations of ficin were electrophoresed. Similarly, untreated control gel slices of NP and M polypeptides were run to ensure that these polypeptides remained stable throughout the procedure used (Fig. 5, d and e). As shown in Fig. 5 (h), complete hydrolysis of the NP polypeptide band was obtained only at a concentration of 1000 ug/ml. However, at this concentration the enzyme is visible in the gel (Fig. 5, j) and tends to interfere with the identification of some hydrolysis fragments.

Incomplete hydrolysis of the added M polypeptide bands by ficin was noted even when tested at a concentration

Fig. 5. Variation of ficin concentrations on hydrolysis of NP or M polypeptide bands. M polypeptide bands exposed to ficin concentrations (ug/ml) of: (a) 1000, (b) 500, (c) 250. NP polypeptide bands exposed to ficin concentrations (ug/ml) of: (f) 250, (g) 500, (h) 1000. Control polypeptide bands: (d) M, (e) NP. Control ficin at concentrations (ug/ml) of: (i) 500, (j) 1000.

Fig. 5



of 1000 ug/ml (Fig. 5, a, b and c). Furthermore, the addition of increasing concentration levels of ficin to hydrolyze M polypeptide bands resulted in a corresponding increase in hydrolysis fragments (Fig. 5, c, b and a).

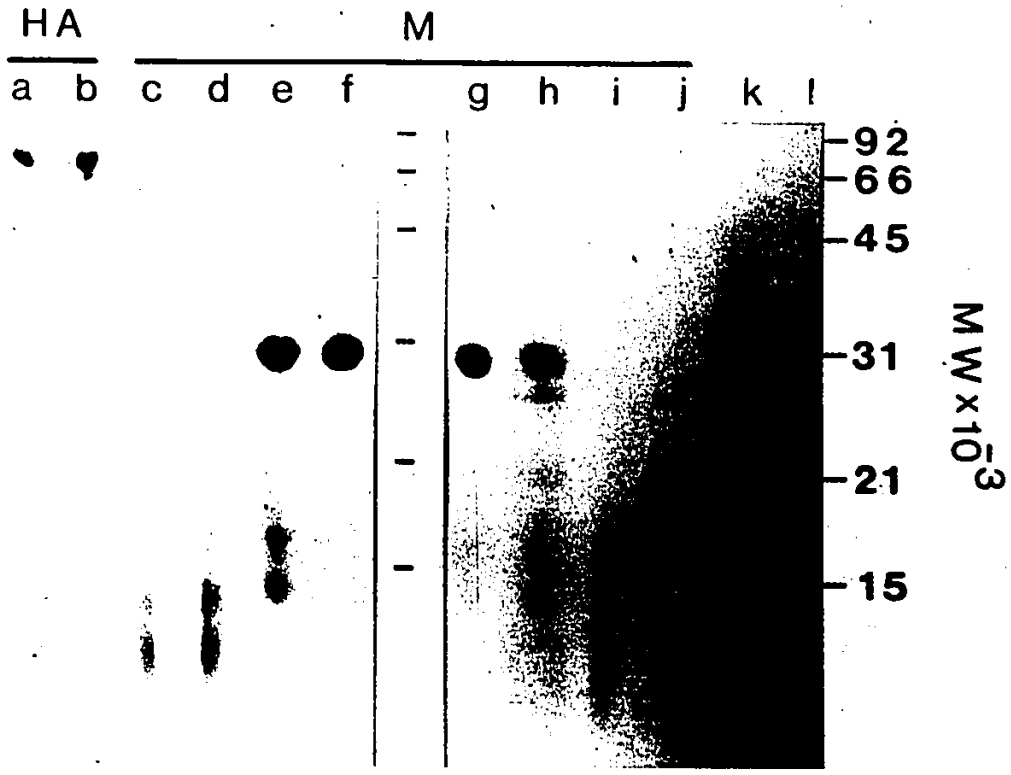
Effect of Ficin and Pronase P on Different Polypeptide Bands of PR Virus

In view of the preceeding results, modification of the PAGE assay procedure was necessary to eliminate resolvable components of control ficin preparations (1000 ug/ml) which interfere with the assay of hydrolysis fragments of M polypeptide band and, also to determine if the M polypeptide band can be completely degraded. Thus the effect of reducing the amount of PR virus M polypeptide on the degree of hydrolysis by a lesser concentration of ficin was examined.

Half or full size gel slices were loaded into sample wells and overlaid with 250 ug/ml of ficin. Control half and full size gel slices of M polypeptide were tested under the same conditions. Figure 6 (f and g) shows no evidence of fragmentation of the control M polypeptide bands. Although the two sizes of gel slices treated with ficin yielded similar hydrolysis fragments (Fig. 6, e and h), the half slice amount of M polypeptide was not totally degraded by 250 ug/ml of enzyme (Fig. 6, h). However, fragments of hydrolysis obtained with a full gel slice

Fig. 6. Effect of ficin and pronase P on half or full gel slices of PR virus M polypeptides and susceptibility of PR virus HA polypeptide band to ficin. Full gel slices of M polypeptide exposed to pronase P concentrations (ug/ml) of: (c) 500, (d) 250. Full gel slice of M polypeptide exposed to a ficin concentration (ug/ml) of: (e) 250. Half gel slices of M polypeptide exposed to pronase P concentrations (ug/ml) of: (i) 250, (j) 500. Half gel slice of M polypeptide exposed to a ficin concentration (ug/ml) of: (h) 250. HA polypeptide band exposed to a ficin concentration (ug/ml) of: (a) 250. Control polypeptide bands: (b) HA, (f) full gel slice of M, (g) half gel slice of M. Control ficin at a concentration (ug/ml) of: (k) 250. Control pronase P at a concentration (ug/ml) of: (l) 500.

Fig. 6



were easier to detect than those from a half gel slice (Fig. 6, e and h).

When M polypeptide was treated with pronase P (250 or 500 ug/ml) as described above, the M polypeptide band was completely hydrolyzed (Fig. 6, c, d, i and j). Their fragments were identified in the 15 K to 10 K region of the gel (Fig. 6, c, d, i and j). As with ficin, the fragments of a hydrolyzed full gel slice were more readily identified. Pronase P in contrast to ficin was not detectable on the gel at a concentration of 500 ug/ml (Fig. 6, l).

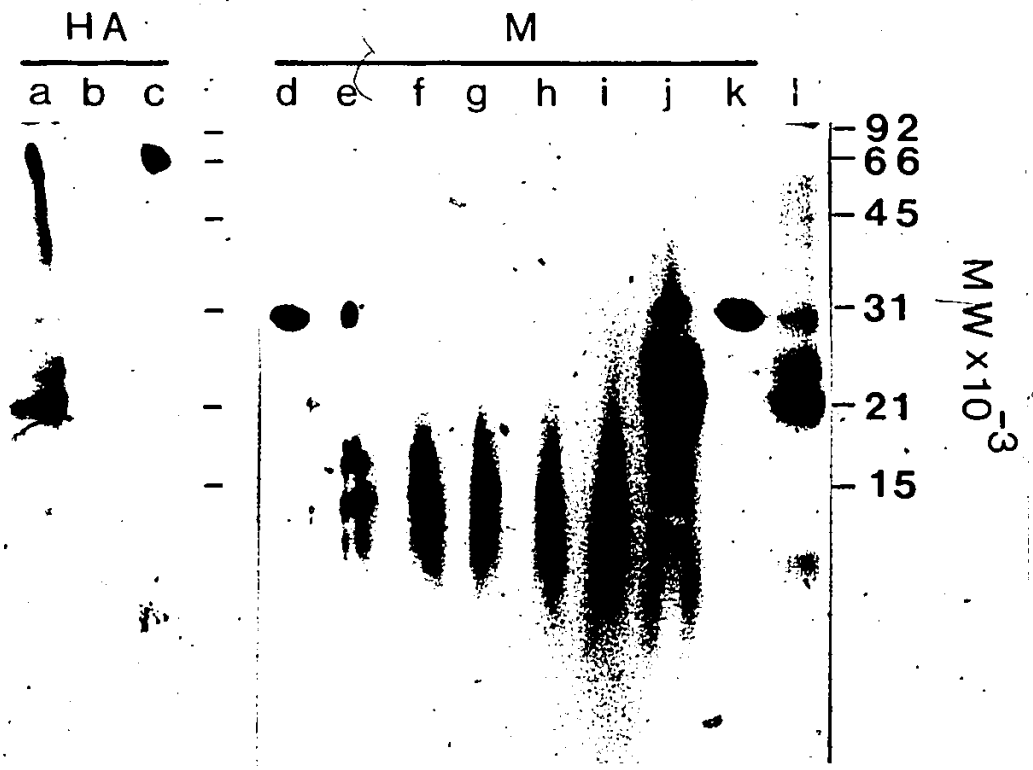
An experiment was done to determine if the HA polypeptide of PR virus was susceptible to ficin at a concentration of 250 ug/ml. The failure of ficin to hydrolyze the HA polypeptide band is seen in Fig. 6 (a).

Susceptibility of M and HA Polypeptide Bands of PR Virus to Pronase P and Cathepsin C

Since it was demonstrated earlier that M polypeptide bands were hydrolyzed completely by 250 ug/ml of pronase P, similar gel slices were used to establish whether less enzyme could comparably hydrolyze M polypeptide bands. It is evident (Fig. 7, e, f, g, h and i) that complete hydrolysis of M polypeptide bands was obtained for all concentrations of pronase P tested except at 5 ug/ml. The hydrolysis fragments appeared similar irrespective of the enzyme concentration. However, if the minimum amount of pronase P

Fig. 7. Susceptibility of M and HA polypeptide bands of PR virus to pronase P and cathepsin C. HA polypeptide band exposed to a cathepsin C concentration (units/ml) of: (a) 2. HA polypeptide band exposed to a pronase P concentration (ug/ml) of: (b) 100. M polypeptide bands exposed to pronase P concentrations (ug/ml) of: (e) 5, (f) 10, (g) 50, (h) 100, (i) 100. M polypeptide band exposed to a cathepsin C concentration (units/ml) of: (j) 2. Control polypeptide bands: (c) HA, (d) M. Control cathepsin C at a concentration (units/ml) of: (l) 2.

Fig. 7



(5 ug/ml) is used, the resolution of the digestion pattern was best.

Treatment of the HA polypeptide with 100 ug/ml of pronase P resulted in complete hydrolysis of the HA polypeptide band (Fig. 7, b). The hydrolysis fragments generated were found in the 16 K to 5 K region of the gel.

With cathepsin C (2 units/ml), both the HA and M polypeptide bands were hydrolyzed to a limited extent (Fig. 7, a and j). However, the enzyme is visible in the gel (Fig 7, l) at the concentration used and interferes with identification of hydrolysis fragments.

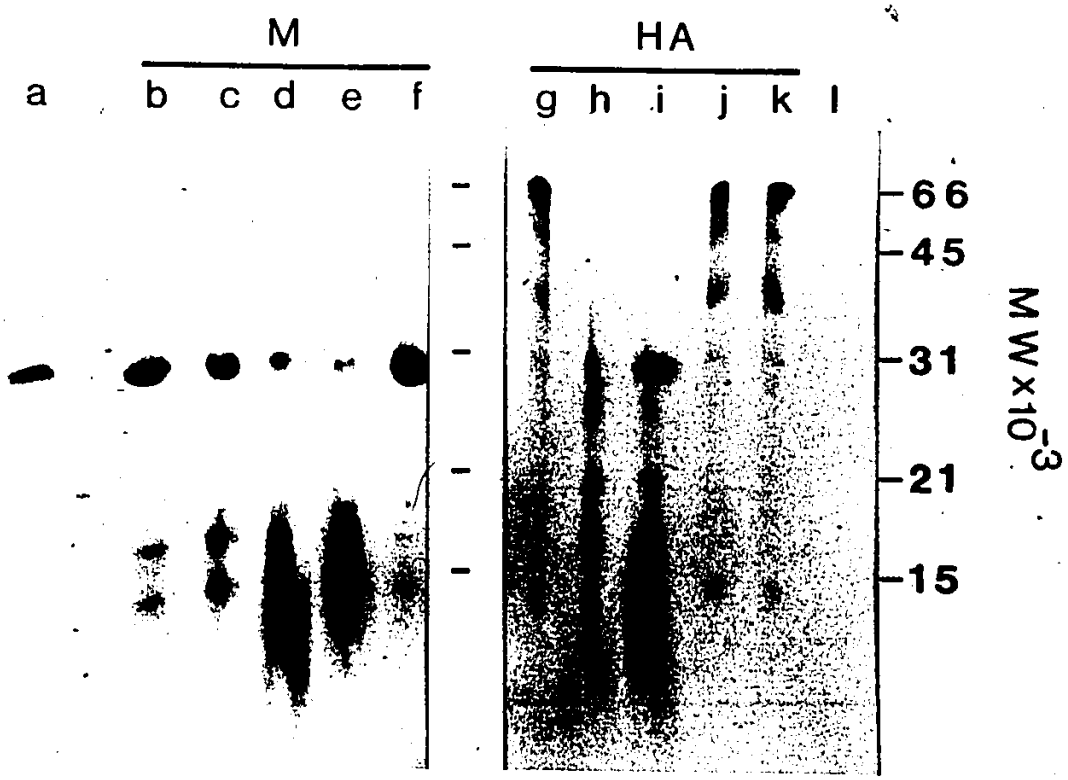
Susceptibility of HA and M Polypeptide Bands of PR Virus to Proteinase K and Aspergillus Acid Proteinase

An experiment was performed to determine the degree of susceptibility of HA and M polypeptide bands to proteolysis by proteinase K or Aspergillus acid proteinase.

Partial hydrolysis of M polypeptide bands with Aspergillus acid proteinase yielded two major fragments (Fig. 8, b and c). Their apparent molecular weights are 18 K and 14 K. The extent of degradation of HA polypeptide bands treated with Aspergillus acid proteinase (Fig. 8, j and k) appears to be the same as that of the HA control (Fig. 8, g). This result suggests that HA molecules are refractory to hydrolysis by Aspergillus acid proteinase. There is no evidence for masking of any hydrolysis fragments, since the protease was not visible in the gel at

Fig. 8. Susceptibility of HA and M polypeptide bands of PR virus to proteinase K and Aspergillus acid proteinase. M polypeptide bands exposed to Aspergillus acid proteinase concentrations (ug/ml) of: (b) 500, (c) 100. M polypeptide bands exposed to proteinase K concentrations (ug/ml) of: (d) 500, (e) 100. HA polypeptide bands exposed to proteinase K concentrations (ug/ml) of: (h) 100, (i) 500. HA polypeptide bands exposed to Aspergillus acid proteinase concentrations (ug/ml) of: (j) 100, (k) 500. Control polypeptide bands: (f) M, (g) HA. Enzyme controls at concentrations of 500 ug/ml: (a) proteinase K, (l) Aspergillus acid proteinase.

Fig. 8



the highest concentration tested (Fig. 8, 1).

It is apparent (Fig. 8, a) that proteinase K (500 ug/ml) is readily visible in the gel. Based on this finding, M polypeptide bands were considered to be completely hydrolyzed by using 100 or 500 ug/ml of proteinase K. The molecular weights for the fragments produced ranged from 19 K to 10 K (Fig. 8, d and e). Complete hydrolysis of HA polypeptide bands with proteinase K produces several hydrolysis fragments. Their electrophoretic mobilities indicated molecular weights ranging between 26 K and 12 K (Fig. 8, h and i).

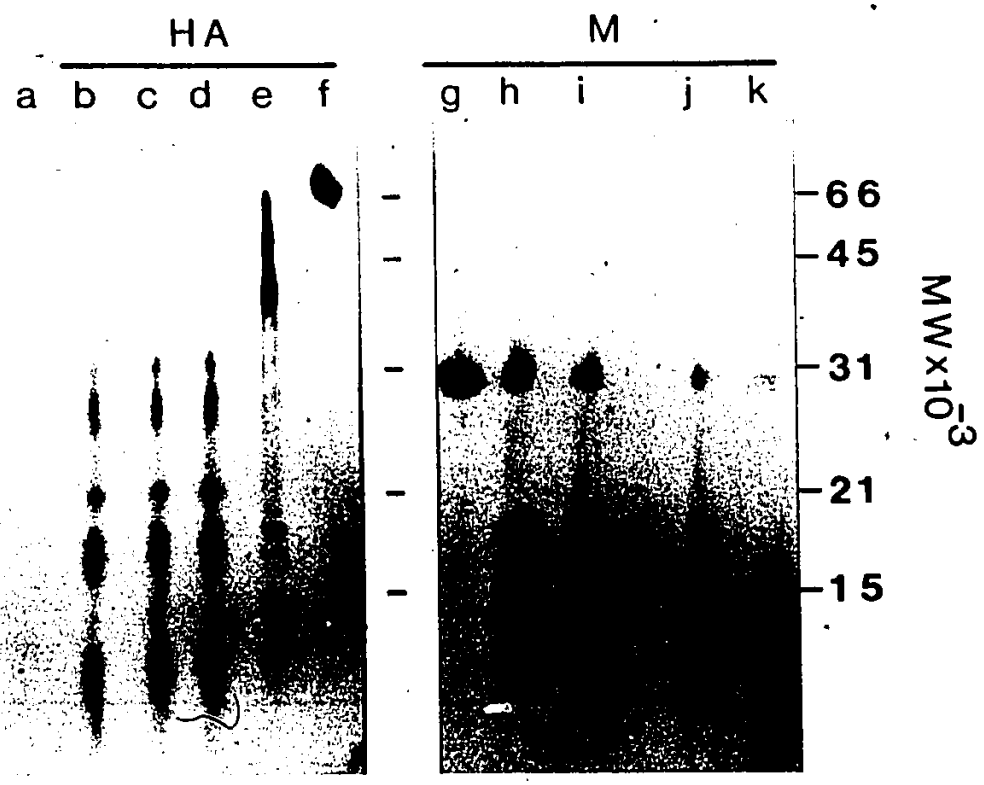
In order to determine the minimum amount of proteinase K required for complete hydrolysis of M and HA polypeptide bands of PR virus, the enzyme was used at levels of 1, 10, 50 and 100 ug/ml. The fragments resulting from complete hydrolysis of the HA polypeptide bands were identical (Fig. 9, b, c and d). Only a proteinase K concentration of 1 ug/ml was incapable of completely hydrolyzing the HA polypeptide band (Fig. 9, e). Decreasing the amount of proteinase K below 50 ug/ml resulted in only partial hydrolysis of M polypeptide bands (Fig. 9, h and i).

Effect of Trypsin on HA and M Polypeptide Bands of PR Virus

The HA and M polypeptides of influenza A strains have previously been digested by exposure to trypsin (Laver and Webster, 1968; Laver and Downie, 1976; Laver et al., 1980;

Fig. 9. Effect of concentration levels of proteinase K on PR virus HA and M polypeptide bands. HA polypeptide bands exposed to proteinase K concentrations (ug/ml) of: (b) 100, (c) 50, (d) 10, (e) 1. M polypeptide bands exposed to proteinase K concentrations (ug/ml) of: (h) 1, (i) 10, (j) 50, (k) 100. Control polypeptide bands: (f) HA, (g) M. Control proteinase K at a concentration (ug/ml) of: (a) 100.

Fig. 9



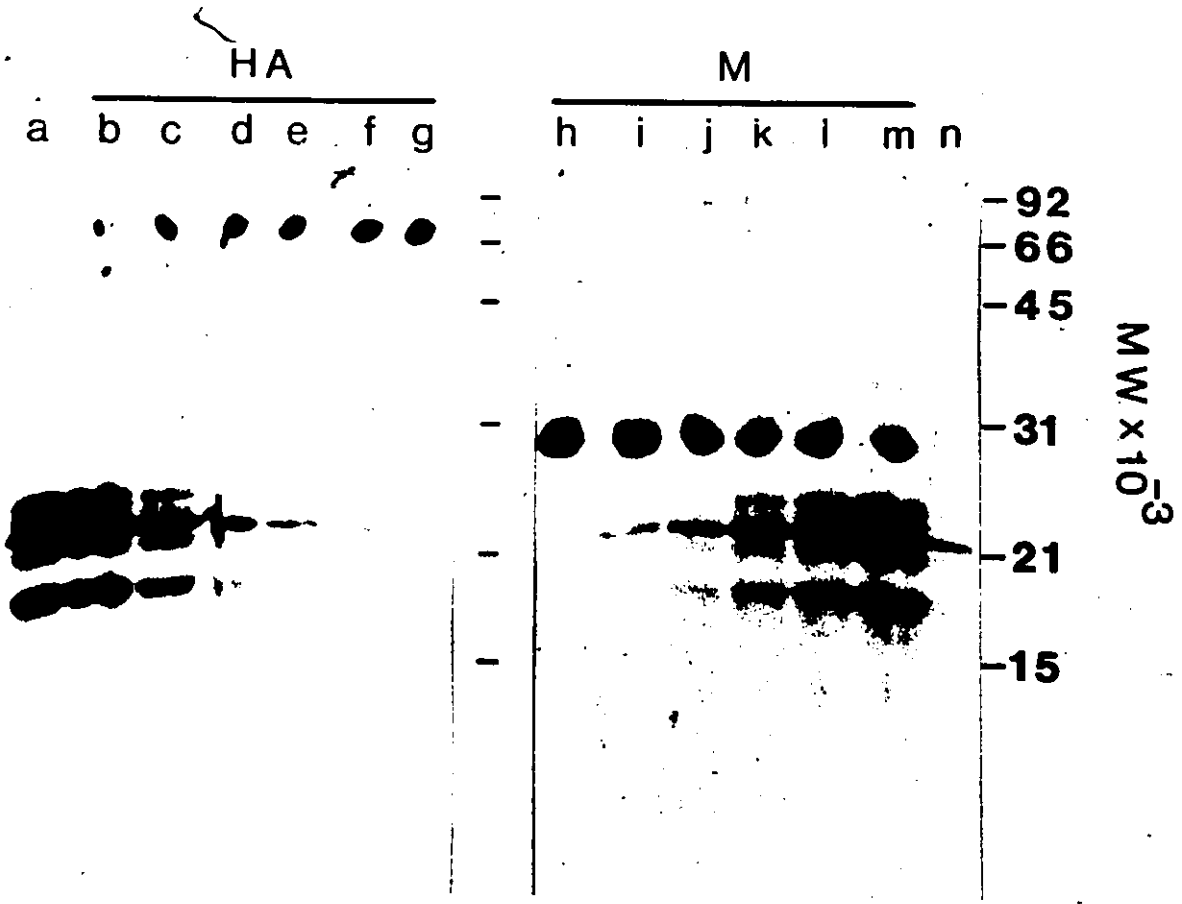
Basak et al., 1981). However, there are strain-specific variations in the susceptibility of the HA polypeptide to trypsin (Klenk et al., 1972a; Lazarowitz et al., 1973a; Stanely et al., 1973). Therefore the effect of trypsin on the HA and M polypeptide bands was determined.

When the HA polypeptide band was exposed to trypsin concentrations of 500 and 1000 ug/ml a single hydrolysis fragment was observed with an apparent molecular weight of 48 K (Fig. 10, c and b). Because the HA polypeptide band was almost completely hydrolyzed by trypsin, it is possible that other hydrolysis fragments were obscured by a heavy background of enzyme proteins (Fig. 10, b). Under the electrophoretic conditions used, the trypsin preparation was resolved into a complex pattern of bands at concentrations higher than 250 ug/ml (Fig. 10). The result indicates that HA polypeptide bands from PR virus were relatively unaffected by trypsin concentrations of about 500 ug/ml.

Treatment of M polypeptide bands with trypsin at varying concentrations, resulted in no apparent hydrolysis fragments (Fig. 10, i, j, k, l, m and n). If any hydrolysis fragments were generated with the highest test concentration of trypsin, they would likely be obscured by the background of trypsin bands (Fig. 10, m).

Fig. 10. Effect of trypsin on the HA and M polypeptide bands of PR virus. HA polypeptide bands exposed to trypsin concentrations (ug/ml) of: (b) 1000, (c) 500, (d) 250, (e) 100, (f) 50. M polypeptide bands exposed to trypsin concentrations (ug/ml) of: (i) 50, (j) 100, (k) 250, (l) 500, (m) 1000. Control polypeptide bands: (g) HA, (h) M. Control trypsin at concentrations (ug/ml) of: (a) 1000, (n) 100.

Fig. 10



Effect of Carboxypeptidase B, Bromelain, Alpha-Chymotrypsin and Subtilisin on the HA and M Polypeptide Bands of PR Virus

Other proteases, carboxypeptidase B, bromelain, alpha-chymotrypsin and subtilisin were studied to establish their ability to hydrolyze HA and M polypeptide bands of PR virus.

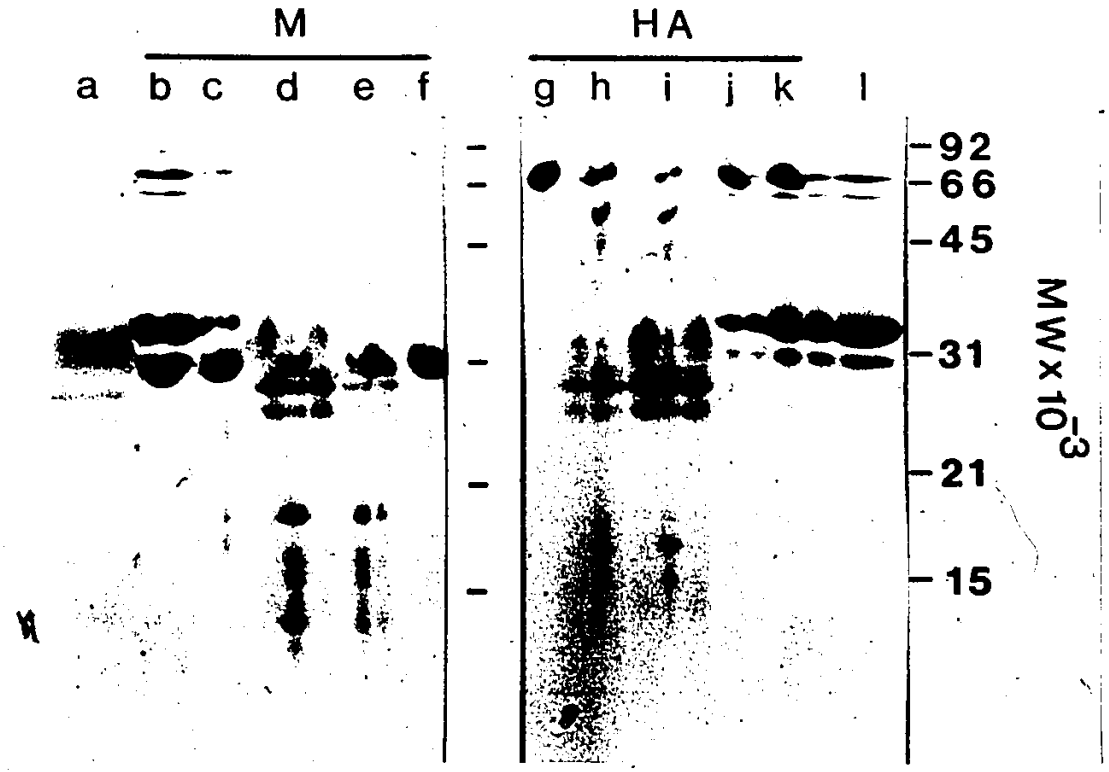
Hydrolysis of HA and M polypeptide bands with the highest concentration of carboxypeptidase B was not evident even though the enzyme preparation could be resolved by electrophoresis into a number of bands (Fig. 11, b, c, j, k and l).

Bromelain however, was capable of hydrolyzing both the M and HA polypeptide bands. The extent of hydrolysis of M polypeptide bands indicated partial and almost complete hydrolysis at bromelain concentrations of 100 and 500 ug/ml (Fig. 11, d and e). Hydrolysis fragments had molecular weights ranging from 18 K to 11 K (Fig. 11, d and e). Electrophoresis of HA polypeptide bands with different concentrations of bromelain yielded similar results (Fig. 11, h and i). The hydrolysis fragments generated had apparent molecular weights of 53 K, 16 K and 14 K (Fig. 11, h and i). The data in Fig. 11 (h and i) demonstrate that the visible bromelain bands produced could mask other hydrolysis fragments of the HA polypeptide band in the 42 K to 24 K (Fig. 11, a) region of the gel.

Figure 12 shows that control HA (b and g) and M (h and m) polypeptide bands were considerably hydrolyzed

Fig. 11. Effect of carboxypeptidase B and bromelain on the HA and M polypeptide bands of PR virus. M polypeptide bands exposed to carboxypeptidase B concentrations (ug/ml) of: (b) 500, (c) 100. M polypeptide bands exposed to bromelain concentrations (ug/ml) of: (d) 500, (e) 100. HA polypeptide bands exposed to bromelain concentrations (ug/ml) of: (h) 100, (i) 500. HA polypeptide bands exposed to carboxypeptidase B concentrations (ug/ml) of: (j) 100, (k) 500. Control polypeptide bands: (f) M, (g) HA. Control enzymes at concentrations of 500 ug/ml: (a) bromelain, (l) carboxypeptidase B.

Fig. 11



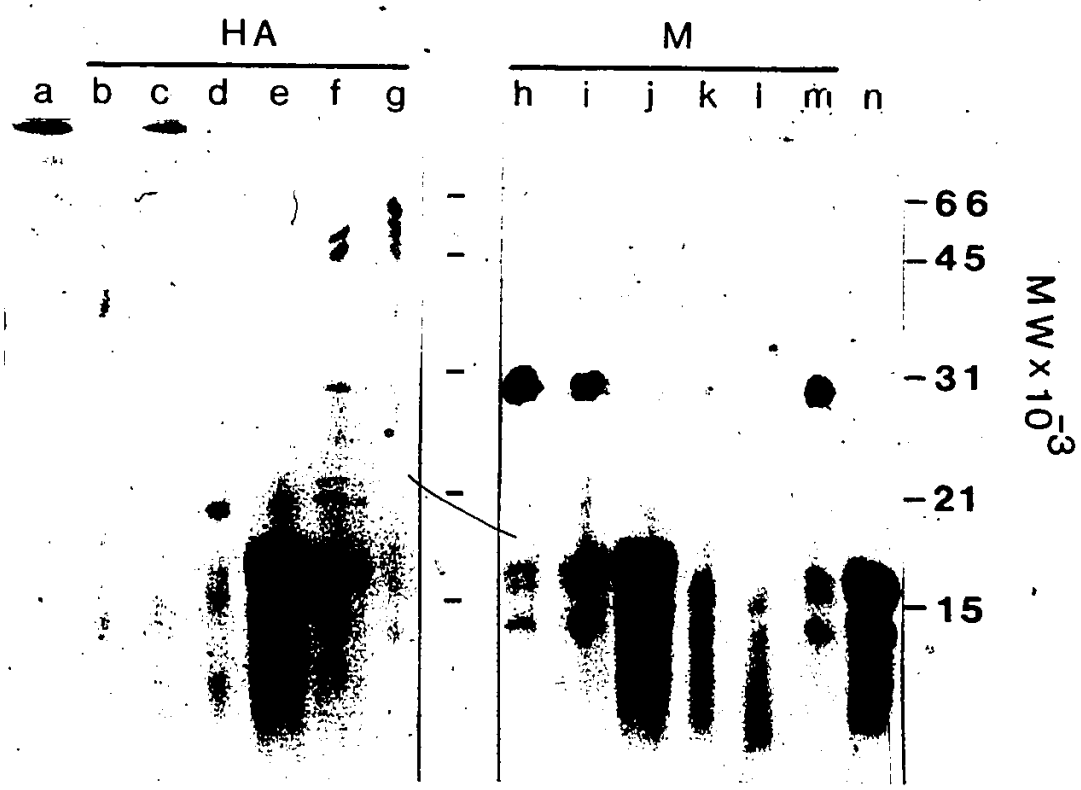
by subtilisin or alpha-chymotrypsin that leaked from adjacent sample wells. At a concentration of 500 ug/ml, alpha-chymotrypsin was able to completely hydrolyze the HA and M polypeptide bands (Fig. 12, e and j). However, hydrolysis fragments generated were obscured by the visible enzyme bands in the 18 K to 10 K region of the gel (Fig. 12, n).

Complete hydrolysis of HA polypeptide bands was obtained with either concentration of subtilisin used (Fig. 12, c and d). The lower concentration of subtilisin yielded a clearer resolution of hydrolysis fragments with molecular weights between 20 K and 11 K (Fig. 12, d). Similarly, the M polypeptide band was also degraded completely by subtilisin at the concentrations tested. As with the HA polypeptide, the lower enzyme concentration generated clearer hydrolysis fragments with molecular weights between 16 K and 10 K (Fig. 12, k). Because subtilisin bands occur near the top of the gel at a concentration of 500 ug/ml, hydrolysis fragments were not masked by enzyme bands (Fig. 12, a).

Based on the foregoing results shown in Fig. 3 to Fig. 12, three enzymes were selected for use in further limited proteolysis studies of influenza A viruses. The three enzymes chosen were pronase P, proteinase K and subtilisin.

Fig. 12. Effect of subtilisin and alpha-chymotrypsin on the HA and M polypeptide bands of PR virus. HA polypeptide bands exposed to subtilisin concentrations (ug/ml) of: (c) 500, (d) 100. HA polypeptide bands exposed to alpha-chymotrypsin concentrations (ug/ml) of: (e) 500, (f) 100. M polypeptide bands exposed to alpha-chymotrypsin concentrations (ug/ml) of: (i) 100, (j) 500. M polypeptide bands exposed to subtilisin concentrations (ug/ml) of: (k) 100, (l) 500. Control polypeptide bands: (b and g) HA, (h and m) M. Enzyme controls at a concentration of 500 ug/ml: (a) subtilisin, (n) alpha-chymotrypsin.

Fig. 12



Characterization of the Envelope PX Polypeptide Associated with PR Virus

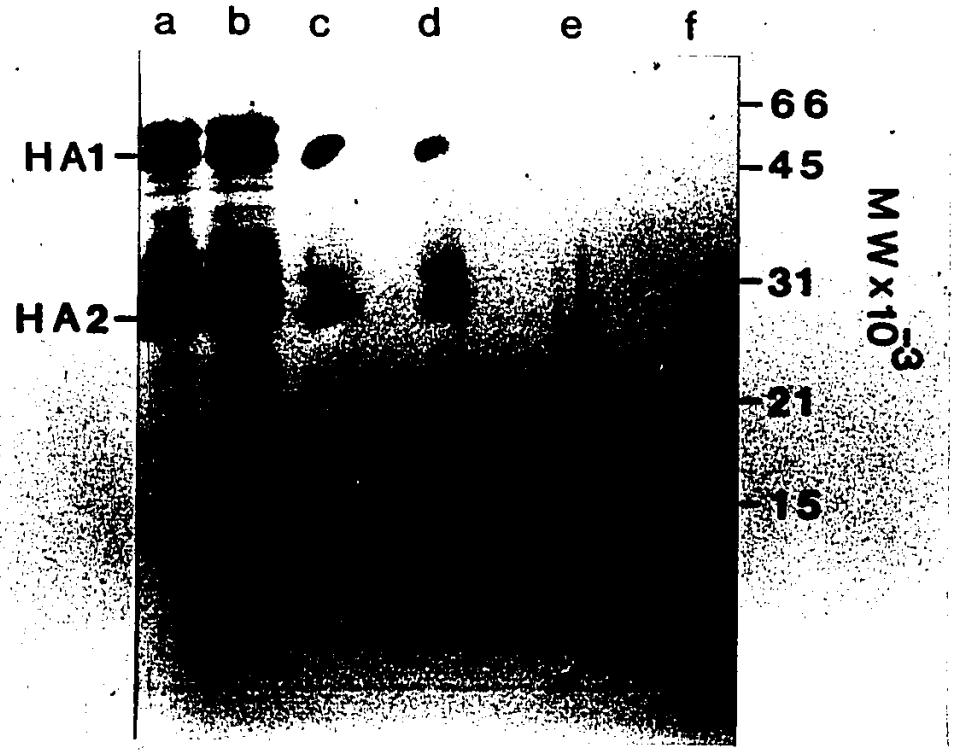
Gel slices of the high molecular weight envelope polypeptide previously labelled as PX (Fig. 1, a) and the HA polypeptide were re-electrophoresed in a second polyacrylamide gel after preequilibration in sample buffer with or without 10% mercaptoethanol for 30 minutes. Mercaptoethanol treatment split both PX and HA preparations into two bands (Fig. 13, c and d). These bands had electrophoretic mobilities comparable to $HA_1$ and $HA_2$ polypeptide bands observed with PR virus samples prepared under reducing conditions (Fig. 13, a and b). Subtilisin treatment of HA and PX polypeptides generated identical digestion patterns in the gel (Fig. 13, e and f). These results demonstrated that the PX polypeptide was a multimeric form of the HA polypeptide. The PX polypeptide is most likely the trimer form of the HA polypeptide because of its high molecular weight (approximately 200 K) and the fact that HA polypeptides exist as trimers on the envelope surface of influenza viruses.

Identification of HA Polypeptide Bands of Hong Kong and Singapore Viruses

After electrophoresis of Singapore and Hong Kong viruses under non-reducing conditions, the banding patterns of polypeptides obtained had only a faint staining band for the

Fig. 13. Characterization of the PX polypeptide associated with the PR virus envelope. Polypeptide bands exposed to mercaptoethanol: (c) HA, (d) PX. Polypeptide bands exposed to 50 ug/ml of subtilisin: (e) HA, (f) PX. Preparations of PR virus: (a and b) under reducing conditions.

Fig. 13



monomer form of the HA polypeptide in comparison to PR virus samples treated similarly. However, the Singapore strain had two bands in the region of the gel where the trimer form of the HA polypeptide should migrate whereas a single band was found with Hong Kong virus (data not shown).

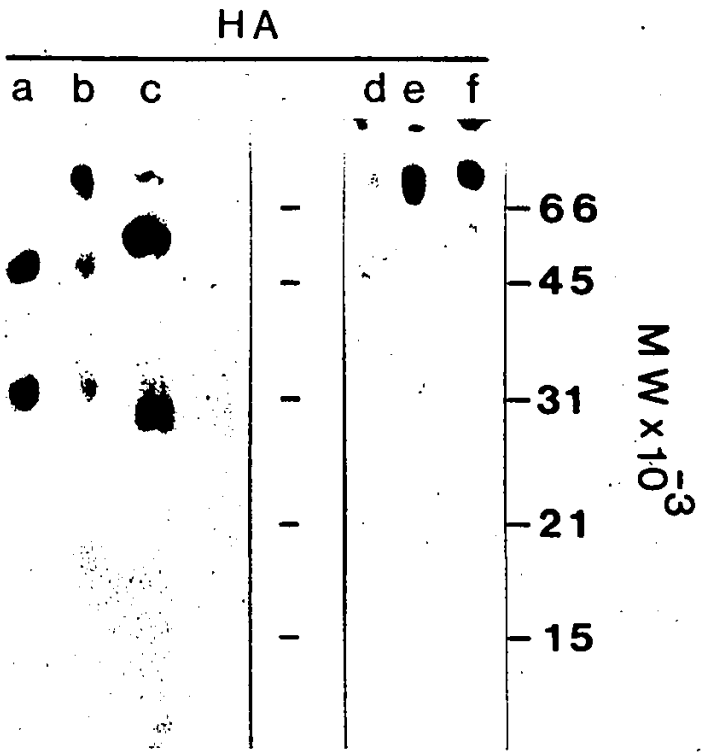
To determine if bands in the trimer region were composed of HA polypeptides, gel slices of these bands were preequilibrated in sample buffer with or without 1% mercaptoethanol for 30 minutes. Gel-isolated bands exposed to reducing conditions resulted in the appearance of two fragments after re-electrophoresis. The relative position of the two Singapore fragments corresponded to apparent molecular weights of 48 K and 31 K (Fig. 14, a and b). Whereas, polypeptide fragments of 53 K and 25 K were observed with the Hong Kong strain (Fig. 14, c). Such molecular weight values are consistent with what would be expected for $HA_1$ and $HA_2$ subunits. This strongly suggests that all the bands tested are composed of HA polypeptides.

Effect of SDS on HA Polypeptides of PR Virus

Although PAGE of Singapore and Hong Kong virus preparations show the presence of heavy staining bands of the trimer form of the HA polypeptide [HA (t)], the gel patterns of PR virus shows a fainter HA (t) band. In order to more accurately compare the HA polypeptides of Singapore, Hong Kong and PR viruses in gels, it was necessary to maximize the amount of recoverable HA (t) from PR virus.

Fig. 14. Identification of the HA polypeptide bands of Hong Kong and Singapore viruses. HA polypeptide bands exposed to mercaptoethanol: (a) Singapore virus (faster migrating species), (b) Singapore virus (slower migrating species), (c) Hong Kong virus. Control HA polypeptide bands: (d) Singapore virus (faster migrating species), (e) Singapore virus (slower migrating species), (f) Hong Kong virus.

Fig. 14



It was found that the amount of trimer recoverable was dependent upon the concentration of SDS present in the virus sample. When a PR virus sample with SDS omitted was electrophoresed, the gel pattern indicated that the amount of HA polypeptide remaining in the trimer form was considerable (Fig. 15, a). Conversely, the addition of 6% SDS to the virus sample resulted in relatively little residual HA polypeptides in the trimer form (Fig. 15, b). However, heavy staining of the monomer form of the HA polypeptide band was observed, presumably resulting from increased amount of hemagglutinin molecules. The amount of SDS in the sample also influenced the width of the polypeptide bands in the gel.

Comparison of the HA and M Polypeptides of PR, Hong Kong and Singapore Strains of Influenza

When the M polypeptide bands of the PR, Hong Kong and Singapore viruses are observed in a polyacrylamide gel, it appears they all have the same approximate molecular weight (Fig. 16, a, b and c). The digestion patterns generated when M polypeptides of each virus were hydrolyzed by pronase P are almost identical (Fig. 16, d, e and f). Only with the M polypeptide band of PR virus, was a difference in the digestion pattern observed. An additional hydrolysis fragment of about 14 K daltons was detected (Fig. 16, d).

Fig. 15. Effect of SDS on HA polypeptides of PR virus. Preparation of PR virus under non-reducing conditions: (a) without SDS, (b) with SDS.

Fig. 15

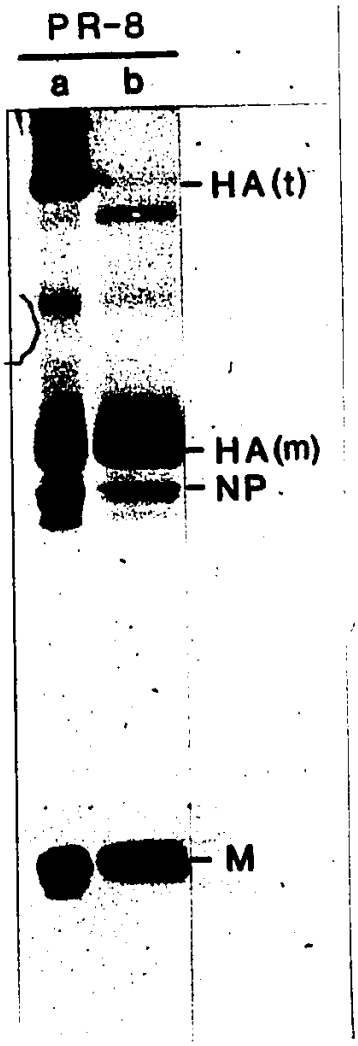
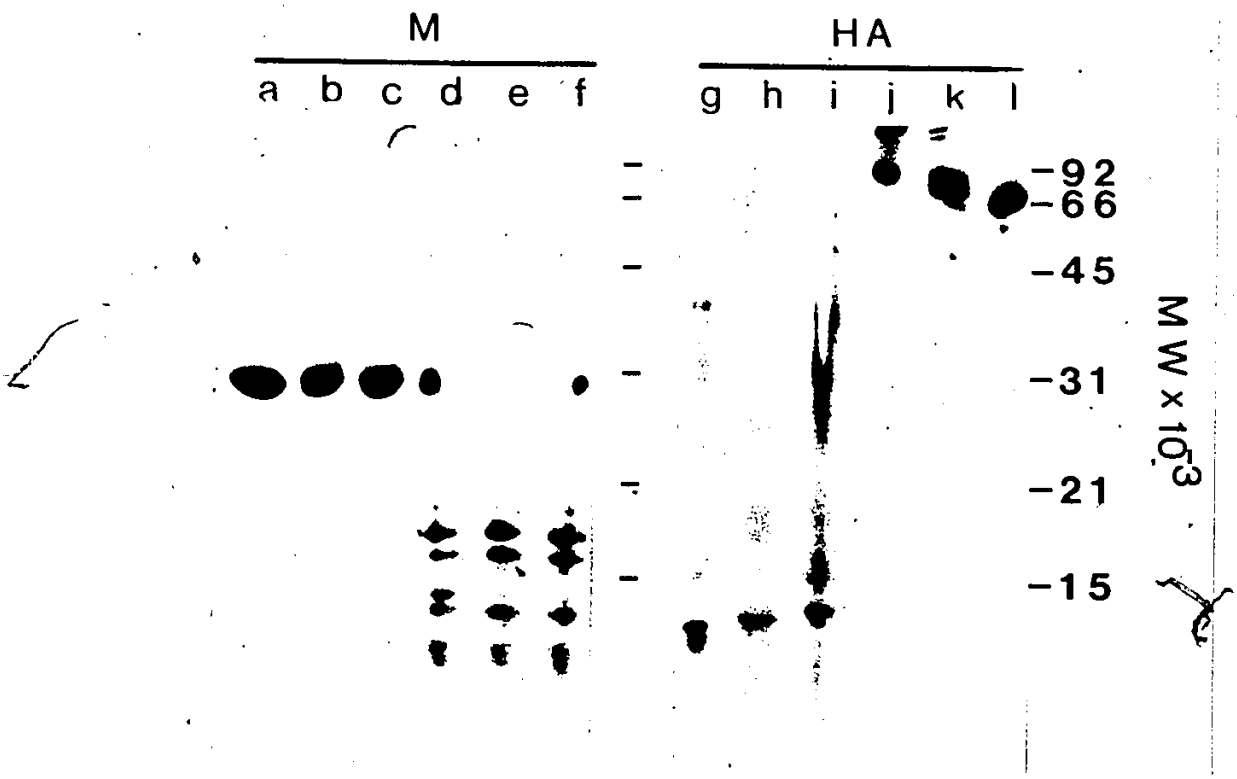


Fig. 16. Comparison of the HA and M polypeptides of PR, Hong Kong and Singapore viruses treated with pronase P. M polypeptide bands exposed to 5 ug/ml of pronase P: (d) PR virus, (e) Singapore virus, (f) Hong Kong virus. HA polypeptide bands exposed to 5 ug/ml of pronase P: (g) Hong Kong virus, (h) Singapore virus, (i) PR virus. Control M polypeptide bands: (a) PR virus, (b) Singapore virus, (c) Hong Kong virus. Control HA polypeptide bands: (j) Hong Kong virus, (k) Singapore virus, (l) PR virus.

Fig. 16



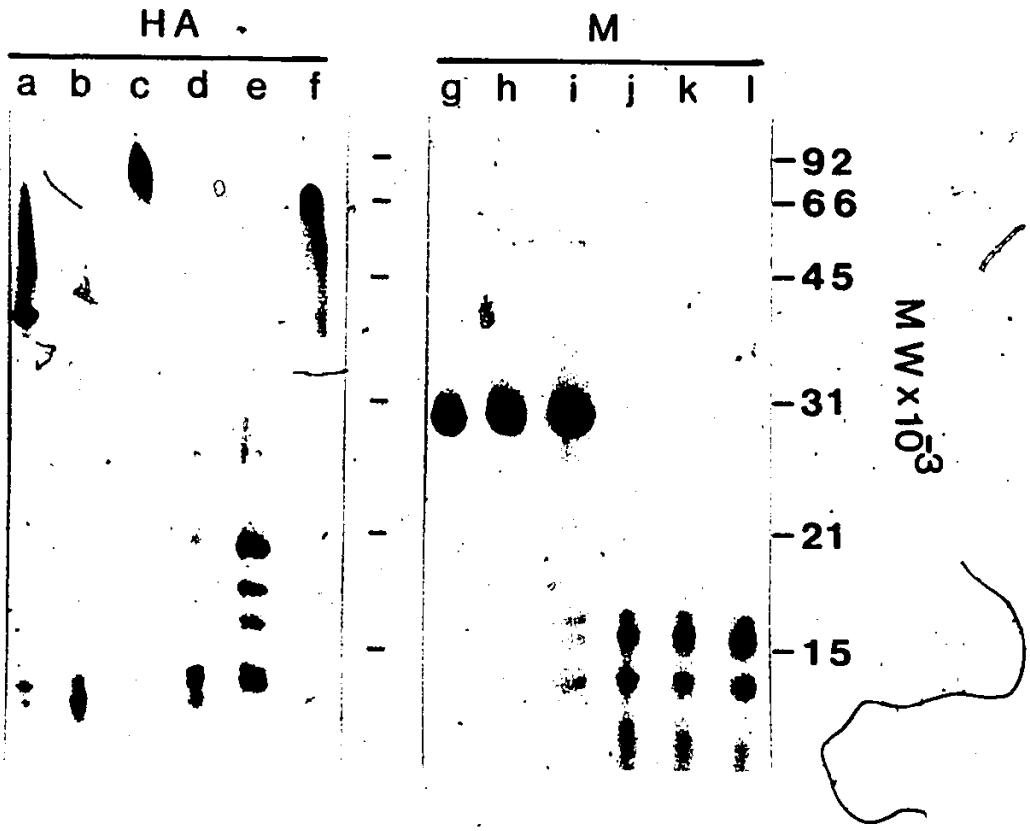
Undigested HA polypeptide bands of all three viruses showed differing electrophoretic mobilities in the gel (Fig. 16, j, k and l). Hence, the decreasing order in molecular weight of the HA polypeptide bands was Hong Kong, Singapore and PR virus (Fig. 16, j, k and l). Pronase P hydrolysis of the HA polypeptide bands isolated from each virus resulted in three different digestion patterns (Fig. 16, g, h and i). Comparison of the digestion patterns generated, showed that hydrolysis of the PR virus HA polypeptide band yielded the largest number of distinct fragments (Fig. 16, i). Only the PR virus pattern had a 16 K dalton fragment (Fig. 16, i). A 15 K dalton fragment found in digestion patterns of HA polypeptide of both PR and Hong Kong viruses was absent from the fragments obtained with Singapore virus (Fig. 16, h). Irrespective of the above differences, HA polypeptide digestion patterns of all three viruses contained a 13 K dalton fragment (Fig. 16, g, h and i). An additional major hydrolysis fragment slightly smaller (about 12 K daltons) was observed in only the digestion pattern of Hong Kong virus (Fig. 16, g).

Complete hydrolysis of M polypeptide bands of these viruses resulted in identical digestion patterns when exposed to subtilisin (Fig. 17, j, k and l).

Three different digestion patterns of the test viruses were obtained after hydrolysis of their HA polypeptides by subtilisin (Fig. 17, b, d and e). Again the largest number of distinct bands were found in the digestion

Fig. 17. Comparison of the HA and M polypeptides of PR, Hong Kong and Singapore viruses treated with subtilisin. HA polypeptide bands exposed to 100 ug/ml of subtilisin: (b) Hong Kong virus, (d) Singapore virus, (e) PR virus. M polypeptide bands exposed to 100 ug/ml of subtilisin: (j) PR virus, (k) Singapore virus, (l) Hong Kong virus. Control HA polypeptide bands: (a) Hong Kong virus, (c) Singapore virus, (f) PR virus. Control M polypeptide bands: (g) PR virus, (h) Singapore virus, (i) Hong Kong virus.

Fig. 17



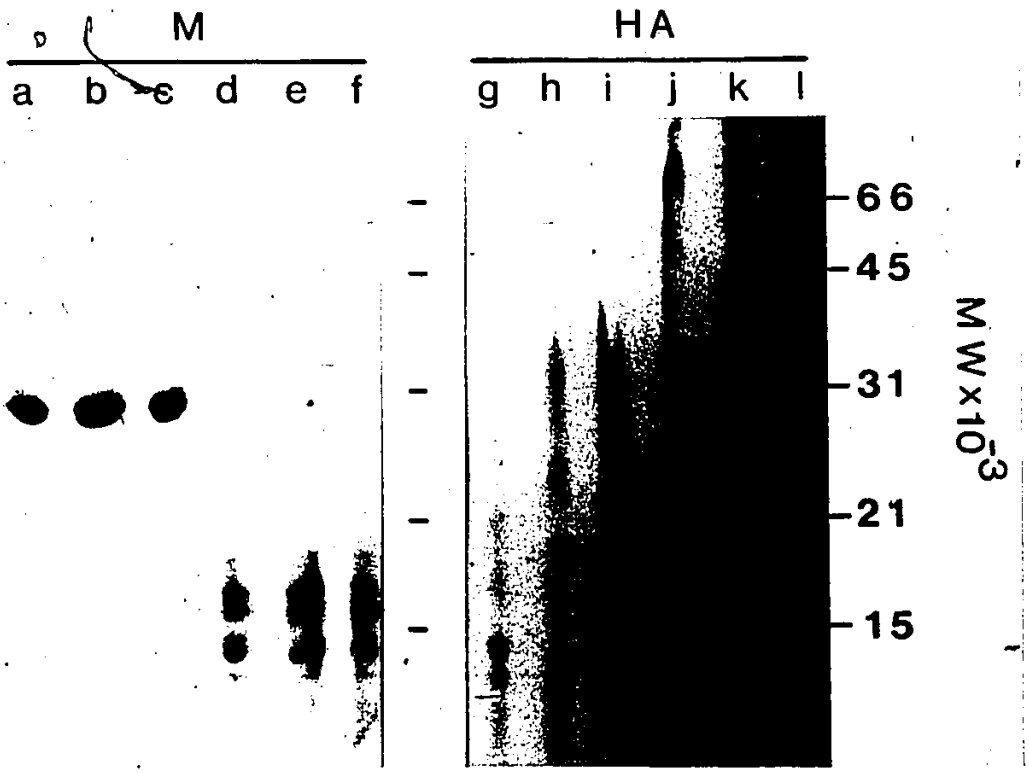
pattern of PR virus (Fig. 17, e). When subtilisin hydrolyzed HA polypeptide bands of Singapore and PR viruses were compared, the former had several fragments with molecular weights corresponding to PR virus fragments (Fig. 17, d and e). Their digestion patterns showed that PR and Singapore viruses contained additional fragments with molecular weights of about 16 K/11 K (Fig. 17, e) and 12 K (Fig. 17, d), respectively. Hydrolysis of the Hong Kong virus HA polypeptide band yielded a digestion pattern with only two clearly resolved fragments (Fig. 17, b). Their apparent molecular weights of 14 K and 13 K, correspond to the molecular weights of hydrolysis fragments found in PR and Singapore digestion patterns (Fig. 17, b, d and e).

After exposure of the M polypeptides of PR, Singapore and Hong Kong viruses to proteinase K the digestion patterns generated were identical (Fig. 18, d, e and f).

When the HA polypeptides of these viruses were hydrolyzed by proteinase K, three markedly different digestion patterns were generated (Fig. 18, g, h and i). Hydrolysis of PR virus yielded nine discrete fragments (Fig. 18, i). This digestion pattern contained a major 18 K dalton fragment which was absent in the digestion patterns obtained from the other two viruses (Fig. 18, i). Fewer hydrolysis fragments were obtained with the Singapore and Hong Kong viruses (Fig. 18, g and h).

Fig. 18. Comparison of the HA and M polypeptides of PR, Hong Kong and Singapore viruses treated with proteinase K. M polypeptide bands exposed to 50 ug/ml of proteinase K: (d) PR virus, (e) Hong Kong virus, (f) Singapore virus. HA polypeptide bands exposed to 50 ug/ml of proteinase K: (g) Singapore virus, (h) Hong Kong virus, (i) PR virus. Control M polypeptide bands: (a) PR virus, (b) Hong Kong virus, (c) Singapore virus. Control HA polypeptide bands: (j) Singapore virus, (k) Hong Kong virus, (l) PR virus.

Fig. 18



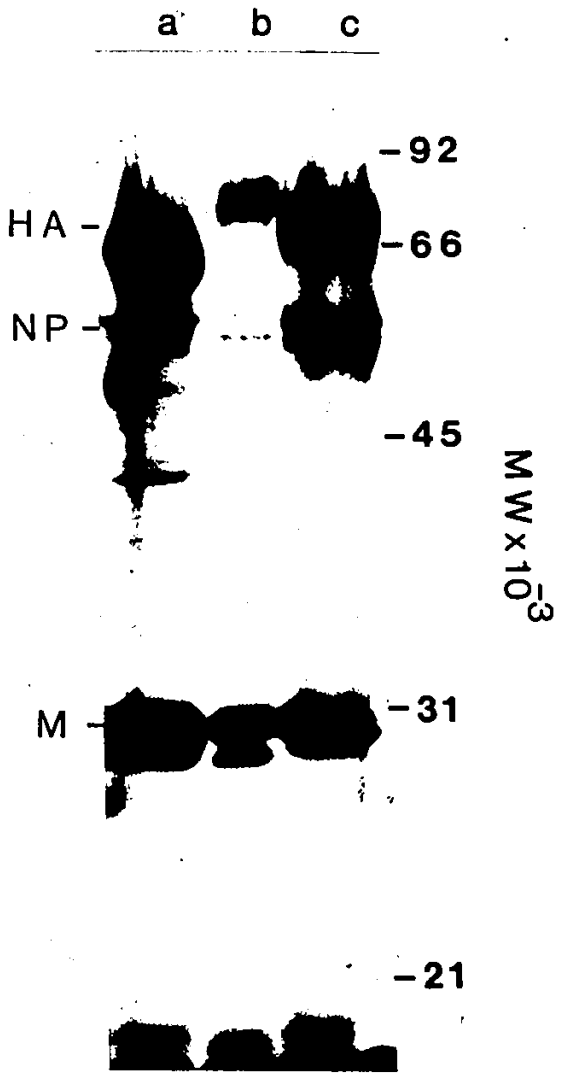
In the preceding experiments (Fig. 16 - Fig. 18) on hydrolysis of polypeptides of different influenza A subtypes, each strain chosen was one of the first viruses isolated in that subtype group having major changes in surface antigens indicative of antigenic shift. Since differences were found between the HA polypeptide digestion patterns of different subtype strains, studies were done to compare polypeptides (HA, M and NP) of viruses from the same subtype.

SDS-PAGE Profiles of H1N1 Influenza Viruses

Three H1N1 influenza viruses (PR, FM and USSR) prepared under non-reducing conditions were compared by SDS-PAGE. Results (Fig. 19, b and a) show that the molecular weight of the HA polypeptide of USSR virus was the largest and that of PR virus was the smallest. The M polypeptides of all three viruses appeared to have similar molecular weights (Fig. 19, a, b and c). FM virus had two major bands in the region of the gel where NP polypeptides were expected to migrate (Fig. 19, c). Under reducing conditions, unexpectedly these two bands appeared in the same location of the gel when electrophoresed in the absence of reducing agents (data not shown). This finding hindered identification and isolation of the FM virus NP polypeptide.

Fig. 19. SDS-PAGE profiles of H1N1 influenza viruses. Preparation of viruses under non-reducing conditions: (a) PR virus, (b) USSR virus, (c) FM virus.

Fig. 19



Comparison of the HA, M and NP Polypeptides of PR, USSR and FM Strains of Influenza

When M polypeptides of PR, FM and USSR viruses were hydrolyzed with pronase P, similar gel patterns of fragments were obtained (Fig. 20, j, k and l).

HA polypeptides of PR, FM and USSR viruses were hydrolyzed into different digestion patterns by pronase P (Fig. 20, a, b and c). Digestion patterns of all three HA polypeptides contained major fragments with apparent molecular weights of 27 K and 14 K. Hydrolysis of the HA polypeptide bands of PR and FM viruses generated additional fragments of 15 K daltons and 19 K daltons, respectively (Fig. 20, a and b). Further observations of their digestion patterns indicate that the few stained fragments taken together, are not representative of an intact HA polypeptide. Presumably the fragments are only those portions of the HA molecules of each virus resistant to complete degradation by this non-specific protease.

After the M polypeptides of these viruses were hydrolyzed with proteinase K, the digestion patterns appeared quite similar (Fig. 21, g, h and i).

H1N1 viruses HA polypeptides were hydrolyzed differently by proteinase K (Fig. 21, d, e and f). HA polypeptide digestion patterns of PR, FM and USSR viruses contained several fragments in the 31 K to 25 K region of the gel as well as fragments with apparent molecular weights of 21 K,

Fig. 20. Comparison of the HA and M polypeptides of PR, USSR and FM viruses treated with pronase P. HA polypeptide bands exposed to 5 ug/ml of pronase P: (a) PR virus, (b) FM virus, (c) USSR virus. M polypeptide bands exposed to 5 ug/ml of pronase P: (j) USSR virus, (k) FM virus, (l) PR virus. Control HA polypeptide bands: (d) PR virus, (e) FM virus, (f) USSR virus. Control M polypeptide bands: (g) USSR virus, (h) FM virus, (i) PR virus.

Fig. 20

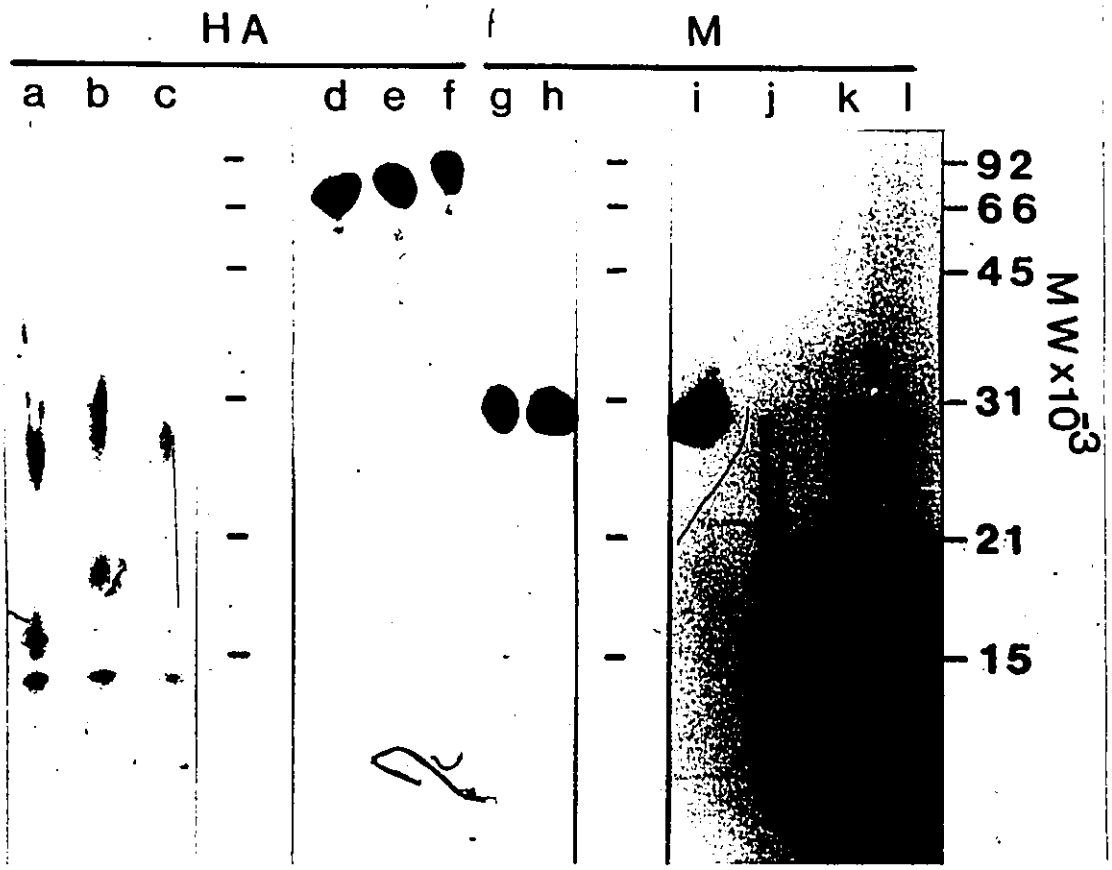
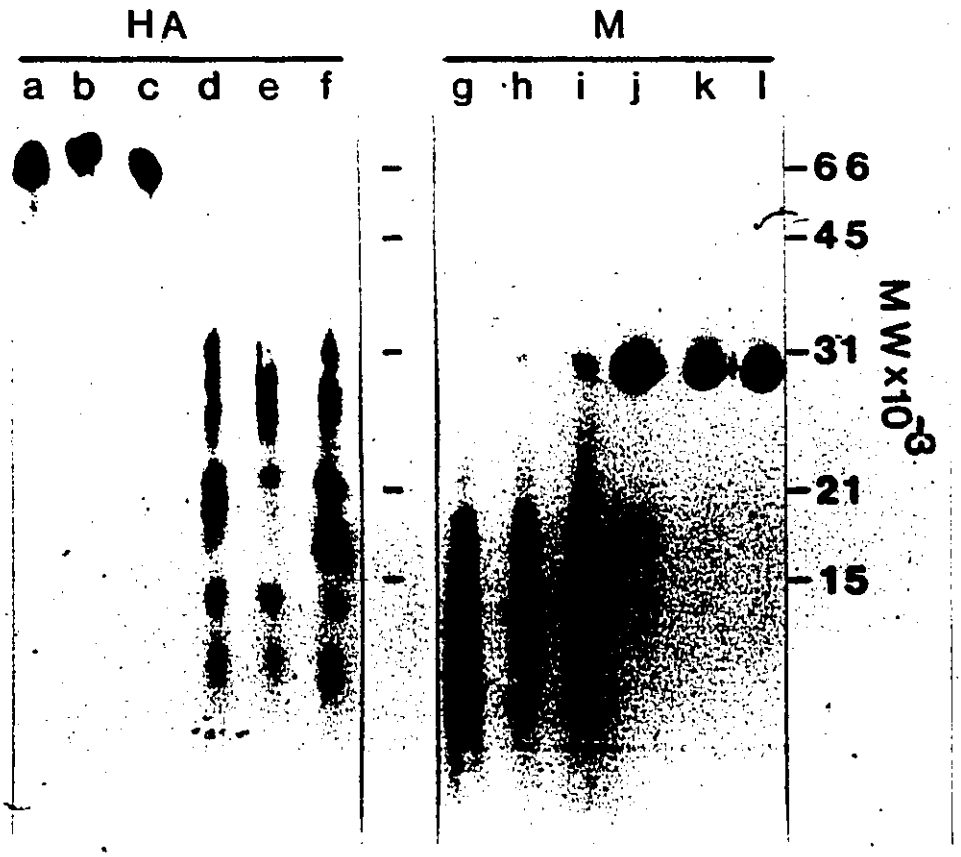


Fig. 21. Comparison of the HA and M polypeptides of PR, USSR and FM viruses treated with proteinase K. HA polypeptide bands exposed to 50 ug/ml of proteinase K: (d) FM virus, (e) USSR virus, (f) PR virus. M polypeptide bands exposed to 50 ug/ml of proteinase K: (g) PR virus, (h) USSR virus, (i) FM virus. Control HA polypeptide bands: (a) FM virus, (b) USSR virus, (c) PR virus. Control M polypeptide bands: (j) PR virus, (k) USSR virus, (l) FM virus.

Fig. 21



14 K and 11 K (Fig. 21, d, e and f). In addition the hydrolysis of hemagglutinins of PR and FM viruses generated a 19 K dalton fragment (Fig. 21, d and f). However, this fragment was more heavily stained in the FM virus pattern (Fig. 21, d). Unique to the PR virus gel pattern were two more major fragments with molecular weights of about 18 K and 17 K (Fig. 21, f).

Comparison of M polypeptides of PR, FM and USSR viruses in a gel after hydrolysis by subtilisin showed the digestion patterns were almost identical (Fig. 22, g, h and i).

Digestion patterns generated by hydrolysis of the HA polypeptides of H1N1 viruses by subtilisin were different for each virus irrespective of certain similarities (Fig. 22, d, e and f). Hydrolysis of HA polypeptides of PR, FM and USSR viruses generated fragments with apparent molecular weights of 21 K, 14 K and 12 K. PR and FM viruses HA polypeptide digestion patterns contained additional fragments of 19 K daltons and 17 K daltons (Fig. 22, d and f). A 16 K dalton fragment was observed only in the PR virus HA polypeptide digestion pattern (Fig. 22, d). The data shown in Fig. 23 (a, b and c) illustrates the reproducibility of the results obtained for hydrolysis of PR, FM and USSR viruses HA polypeptides by subtilisin.

NP polypeptides of PR, FM and USSR viruses were hydrolyzed by subtilisin into a large number of fragments Fig. 24, e, f, g and h). Most fragments were located in the 21 K to 11 K region of the gel. However, the exact

Fig. 22. Comparison of the HA and M polypeptides of PR, USSR and FM viruses treated with subtilisin. HA polypeptide bands exposed to 100 ug/ml of subtilisin: (d) PR virus, (e) USSR virus, (f) FM virus. M polypeptide bands exposed to 100 ug/ml of subtilisin: (g) FM virus, (h) USSR virus, (i) PR virus. Control HA polypeptide bands: (a) PR virus, (b) USSR virus, (c) FM virus. Control M polypeptide bands: (j) FM virus, (k) USSR virus, (l) PR virus.

Fig. 22

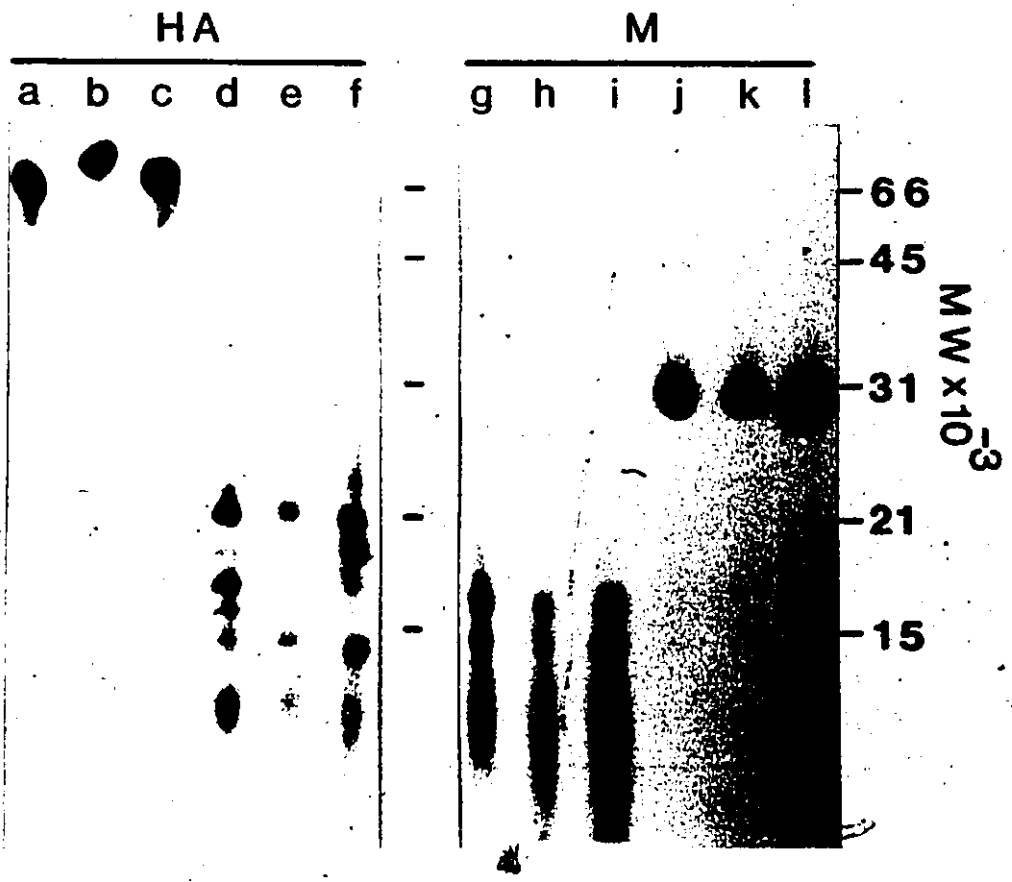


Fig. 23. Comparison of the HA polypeptides of PR, USSR and FM viruses treated with subtilisin. HA polypeptide bands exposed to 100 ug/ml of subtilisin: (a) PR virus, (b) USSR virus, (c) FM virus. Control HA polypeptide bands: (g) FM virus, (h) USSR virus, (i) PR virus.

Fig. 23

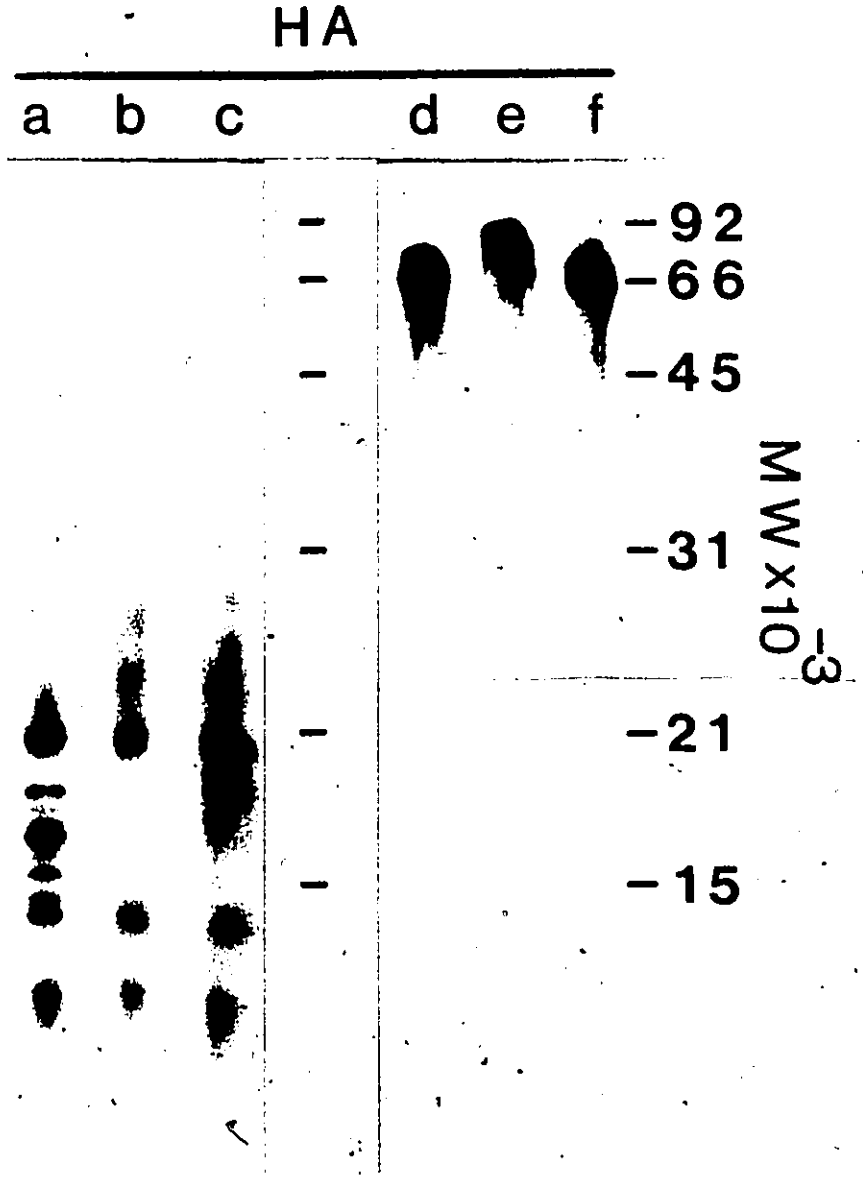
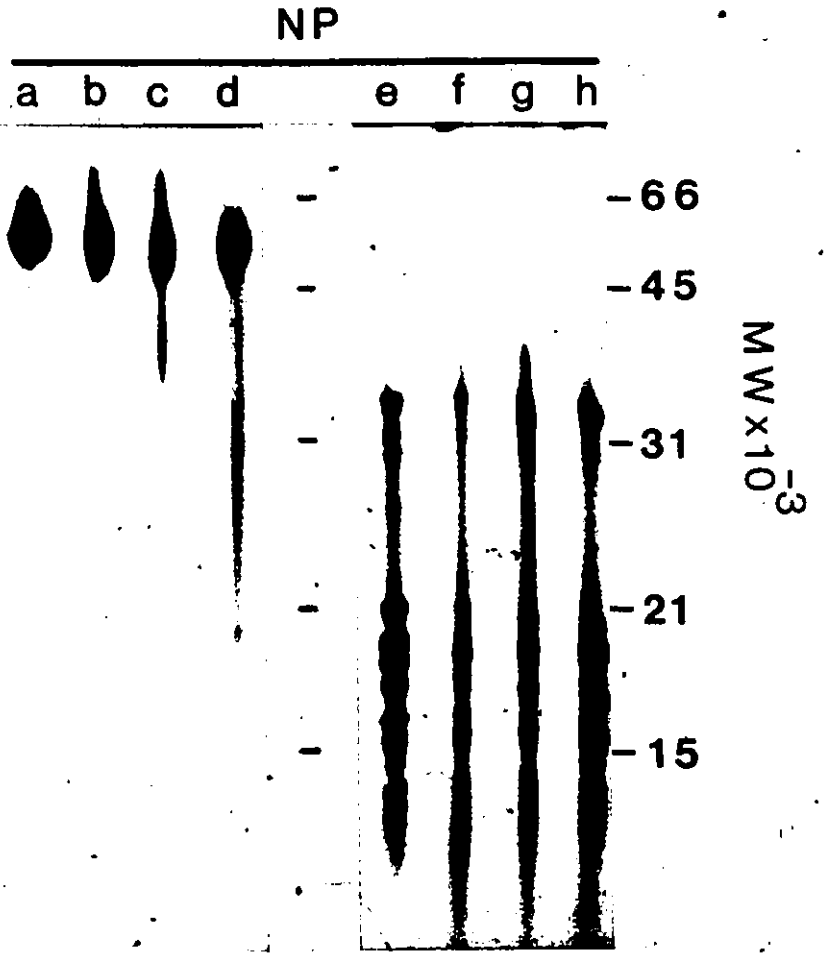


Fig. 24. Comparison of the NP polypeptides of PR, FM and USSR viruses treated with subtilisin. NP polypeptide bands exposed to 100 ug/ml of subtilisin: (e) PR virus, (f) FM virus (slower migrating species), (g) FM virus (faster migrating species), (h) USSR virus. Control NP polypeptide bands: (a) PR virus, (b) FM virus (slower migrating species), (c) FM (faster migrating species), (d) USSR virus.

Fig. 24



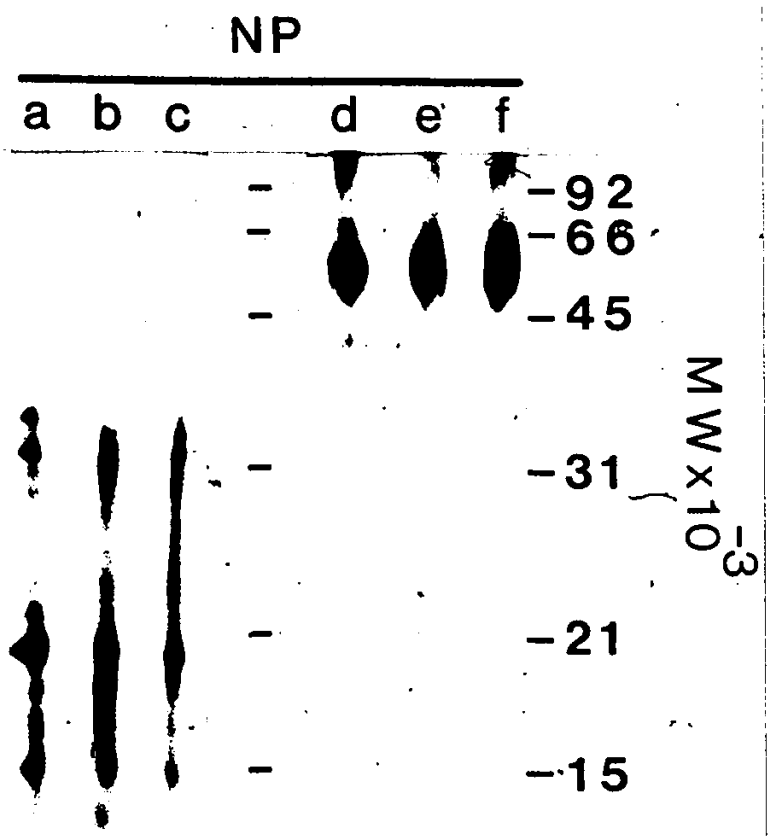
number of fragments obtained and their molecular weights appeared to differ between H1N1 viruses. The two previously observed NP polypeptide bands of FM virus (Fig. 19, c) yielded distinct digestion patterns (Fig. 24, f and g). The slower migrating NP polypeptide band of FM virus had a similar molecular weight as NP polypeptides of PR and USSR viruses. However, the digestion pattern of the faster migrating NP polypeptide band of FM virus appeared similar to that of the USSR virus NP polypeptide (Fig. 24, g and h).

Hydrolysis of the slower migrating NP polypeptide of FM virus and the NP polypeptides of PR and USSR viruses by pronase P appeared to generate distinguishable digestion patterns (Fig. 25, a, b and c). However, all three digestion patterns contained fragments with approximate molecular weights of 35 K, 32 K, 20 K and 15 K (Fig. 25, a, b and c), although staining intensity of these fragments demonstrated some strain-specific variations.

PR and USSR viruses NP polypeptides hydrolyzed by proteinase K also generated dissimilar digestion patterns (Fig. 26, a and b). The PR virus gel-pattern contained a larger number of fragments than that of USSR virus (Fig. 26, a and b). A major 19 K dalton fragment was found in both gel-patterns.

Fig. 25. Comparison of the NP polypeptides of PR, FM and USSR viruses treated with pronase P. NP polypeptide bands exposed to 10 ug/ml of pronase P: (a) PR virus, (b) USSR virus, (c) FM virus (slower migrating species). Control NP polypeptide bands: (d) PR virus, (e) USSR virus, (f) FM virus (slower migrating species).

Fig. 25



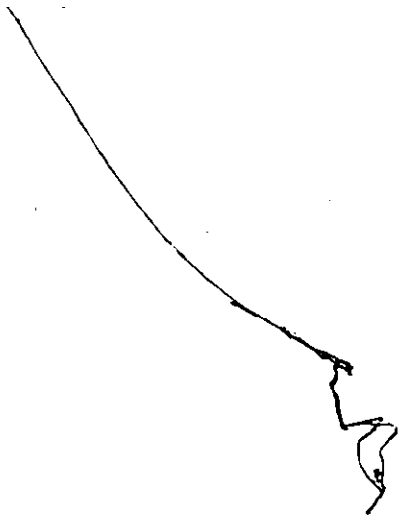
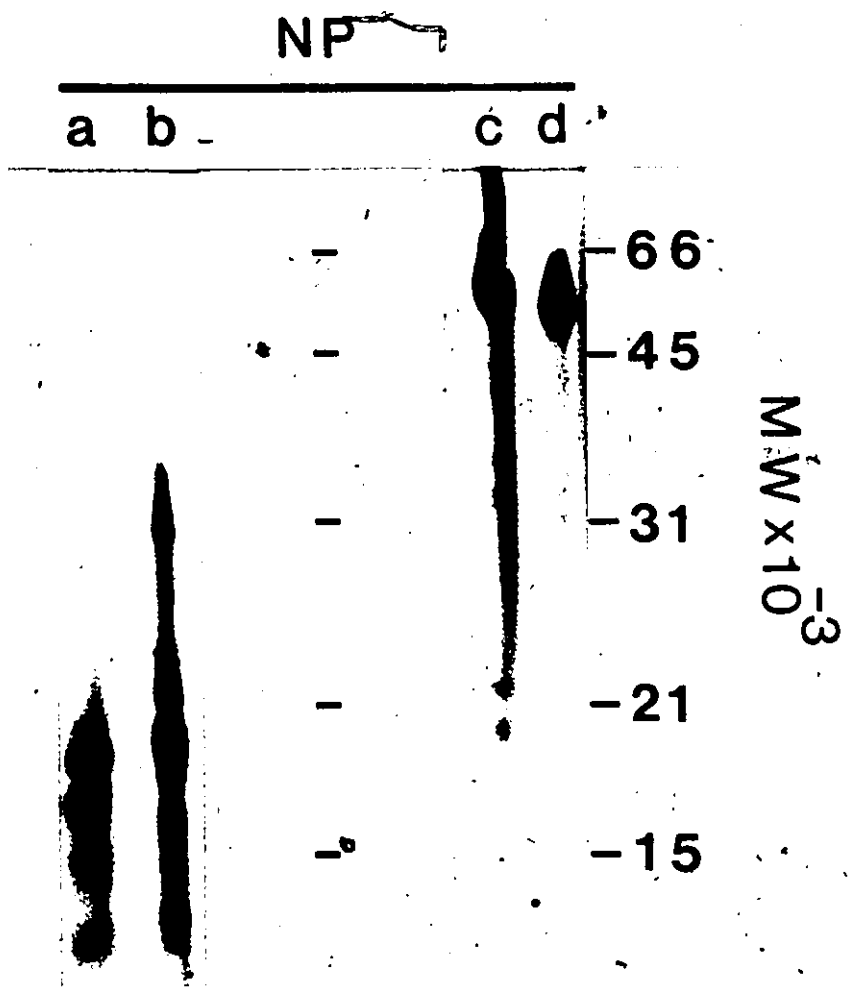


Fig. 26. Comparison of NP polypeptides of PR and USSR viruses treated with proteinase K. NP polypeptide bands exposed to 50 ug/ml of proteinase K: (a) USSR virus, (b) PR virus. Control NP polypeptide bands: (c) PR virus, (d) USSR virus.

Fig. 26

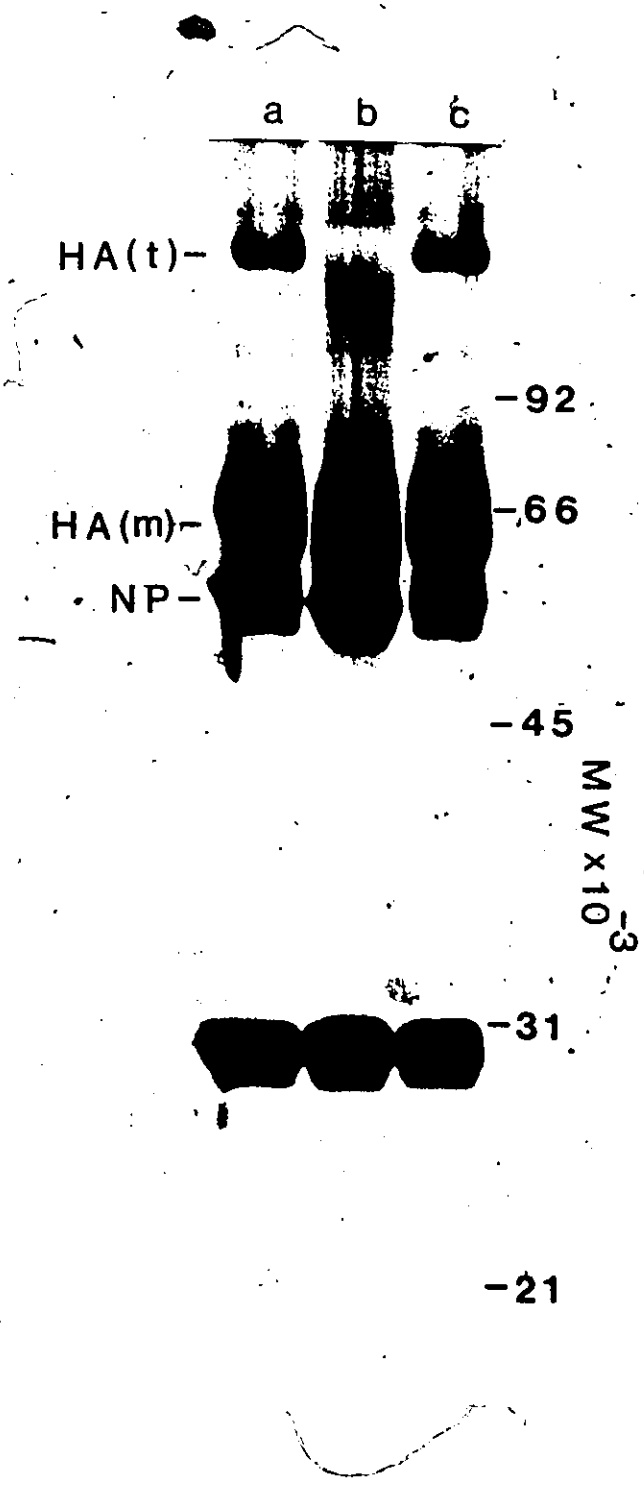


Effect of Endoglycosidase Treatment on PR Virus Polypeptides

PR virus treated with endoglycosidase as described under Materials and Methods, was prepared under non-reducing conditions and electrophoresed. The only polypeptide noticeably affected, was the HA polypeptide (Fig. 27, b). Figure 27 (b) shows that the trimer form of the HA polypeptide, [HA (t)] was totally eliminated. The molecular weight of some of the monomer form of the HA polypeptide [HA (m)] appears to have been reduced (Fig. 27, b). Unfortunately this unglycosylated HA polypeptide comigrated with the NP polypeptide making its isolation from the gel impossible (Fig. 27, b).

Fig. 27. Effect of endoglycosidase treatment on PR virus polypeptides. PR virus incubated for 20 hours at 37°C in the presence of: (a and c) 0.05 M citrate-phosphate buffer (pH 6.5), (b) 0.05 M citrate-phosphate buffer (pH 6.5) containing 0.04 Units/ml of endoglycosidase-H and endoglycosidase-D.

Fig. 27



# DISCUSSION

Influenza A viruses have escaped control by vaccination because of frequent changes in the antigenic character of HA or NA surface glycoproteins. Variations in the HA are considered more important because HA is quantitatively the predominant surface glycoprotein (White, 1974). Also major pandemics of the recent past have been associated with antigenic shift in the HA with or without NA shift. Therefore, methods capable of detecting these changes are important.

In this study digestion patterns of the HA polypeptides of several different influenza A viruses were easily distinguishable. This occured whether the HA polypeptides were isolated from viruses within the same subtype or in different subtypes. Therefore, limited proteolysis studies in SDS-gels show potential as a method for detecting changes in HA polypeptides.

Comparison of hydrolyzed influenza virus polypeptides and identification of enzymes that these polypeptides were susceptible to, was possible by performing limited proteolysis studies in SDS-polyacrylamide gels as described by Cleveland et al., (1977). The original procedure outlined by Cleveland was modified slightly for this study. Gel solutions and sample buffer were prepared without the addition of EDTA. Also a shorter stacking gel (28 mm) than recommended (50 mm) was used and current was reduced

but not shut off during the run.

Several types of proteases were tested for their suitability in limited proteolysis studies with HA and M polypeptides of PR virus. Selection of proteases was dependent on their ability to meet three criteria. First, a protease had to be capable of totally hydrolyzing the HA and M polypeptide bands at a concentration of 500 ug/ml or less. Secondly, the enzyme must not produce visible band(s) in the gel, after staining, in a region where masking of hydrolysis fragments could occur. Thirdly, a digestion pattern generated by complete hydrolysis of a viral polypeptide band had to remain constant over at least a 5-fold increase in the protease concentration. This last criteria is particularly important since quantities of each viral polypeptide can vary slightly between strains.

Of the enzymes tested only pronase P, proteinase K and subtilisin met each of the conditions. Proteinase K and subtilisin are serine proteases while all three enzymes are non-specific and capable of completely hydrolyzing proteins and peptides. In consideration of this, the finding that these proteases, over a wide concentration range did not completely hydrolyze PR virus polypeptides but left sizable fragments (greater than 10 K) remains to be clarified. Furthermore, Sabina et al., (1981) also noted this result when treating influenza viruses with pronase P or subtilisin.

Two other proteases, bromelain and alpha-chymotrypsin also showed potential for use in this study.

Unfortunately however, both enzymes produce visible bands in the gel, that mask hydrolysis fragments. Bromelain is a non-specific protease that can hydrolyze polypeptides especially at bonds involving leucine or glycine. Previously bromelain has been used to cleave hemagglutinin spikes from the surface of intact influenza viruses (Compans et al., 1970; Brand and Skehel, 1972). It was demonstrated that bromelain was releasing the HA by cleaving the molecule 50-90 residues from the C-terminal end of the $HA_2$ subunit (Skehel and Waterfield, 1975; Waterfield et al., 1979). In this study however, bromelain cleaved the HA polypeptide into three major fragments. This would indicate at least two more cleavage sites on the molecule. A possible explanation for this finding is that bromelain cleavage sites on individual HA molecules are unavailable when the HA is in the trimer (spike) form.

Alpha-chymotrypsin hydrolyzes peptides, especially at bonds involving the carboxyl group of aromatic amino acids. Approximately 7% of the amino acids in HA molecules are tyrosine or phenylalanine. Therefore, there appear to be potential sites for attack by this protease. This could account for the ability of alpha-chymotrypsin to completely digest the HA polypeptide band of PR virus.

As mentioned, bromelain and alpha-chymotrypsin were not used in this study because of masking difficulties. However, both enzymes show potential in limited proteolysis studies where the use of radiolabelled virus and

autoradiograms would eliminate this problem.

Previous research has clearly demonstrated that influenza virus HA and M polypeptides could be effectively hydrolyzed by trypsin (Laver and Webster, 1968; Laver and Downie, 1976; Laver et al., 1980; Basak et al., 1981). Therefore, the failure of trypsin to be more effective at hydrolyzing these polypeptides in this study was unexpected. Possibly this could be due to the low pH in the stacking gel (6.8), where hydrolysis occurs. This is far below the optimum pH range for trypsin (7.5-8.5).

According to Ewasyshyn (1983), the allantoic fluid of eggs infected with influenza viruses contain inhibitors to subtilisin activity but have high levels of carboxypeptidase B activity. The finding in this study that the HA and M polypeptides of PR virus were sensitive to subtilisin but refractory to carboxypeptidase B activity is consistent with this observation.

In this study the HA polypeptide of PR virus was found to have an apparent molecular weight of 57 K. This is much smaller than the accepted molecular weight of approximately 75 K (Dopheide and Ward, 1978 b). However, when the HA polypeptide is cleaved by mercaptoethanol, the $HA_1$ and $HA_2$ subunits have apparent molecular weights of 48 K and 28 K. These molecular weights are consistent with accepted values (Ward and Dopheide, 1976).

Detection of HA trimer [HA (t)] molecules in SDS-gels, has apparently not been previously demonstrated. However,

resistance of Hong Kong virus (H3N2) HA molecules to dissociation by SDS was reported (Laver, 1973). Increased resistance of Hong Kong (H3N2) and Singapore (H2N2) viruses HA (t) molecules to SDS as compared with those of H1N1 viruses (PR, FM, USSR) could have potential for subtype differentiation. If further testing of influenza virus hemagglutinin trimers demonstrates this is a consistent finding between subtypes, then a virus could be assigned to the H1N1 subtype on this basis. In the limited proteolysis experiments it was important for comparative purposes that HA polypeptides should be hydrolyzed when the entire population of hemagglutinins is in the monomer or trimer form. This was done in case the tertiary structure of the HA trimer (spike) affects digestion. The ability to alter the amount of PR virus hemagglutinins in the trimer form by varying the SDS concentration allowed these studies to be carried out.

Digestion patterns of HA polypeptides isolated from PR (H1N1), Singapore (H2N2) and Hong Kong (H3N2) viruses differed whether hydrolyzed by subtilisin, proteinase K or pronase P. The results showed that HA polypeptides of each virus were susceptible to hydrolysis to varying degrees. Therefore, it can be concluded that limited proteolysis studies are sensitive enough to detect alterations in HA polypeptides resulting from antigenic shift.

The digestion patterns of HA polypeptides were also found to differ if the HA polypeptides are isolated from

viruses within the same subtype. When HA polypeptides of three H1N1 viruses (PR, FM and USSR) were compared, strain-specific differences were observed. Irrespective of these differences however, analogous digestion patterns of HA polypeptides of PR, FM and USSR viruses always contained some fragments with corresponding molecular weights. Furthermore, there was a correlation between the number of fragments observed in HA polypeptide digestion patterns and the year of virus occurrence throughout the drifting of H1N1 strains. The most recent isolate tested, USSR virus (1977) always generated the least number of hydrolysis fragments whereas the earliest isolate PR virus (1934) yielded the greatest number. This result tends to indicate that antigenic drifting is making H1N1 virus HA polypeptides more susceptible to complete degradation by subtilisin, proteinase K or pronase P. Fragments of corresponding size seen in all digestion patterns of the USSR virus HA polypeptide, are also observed in the PR and FM virus patterns. Therefore, possibly these fragments represent conserved regions in the HA molecules of H1N1 viruses. The results of these experiments demonstrated that although HA polypeptides of the H1N1 viruses tested are similar, they can still be differentiated using limited proteolysis studies. This indicates that this method is sensitive enough to detect changes in HA polypeptides caused by antigenic drift.

Pronase P routinely hydrolyzed HA polypeptides of viruses to a greater extent than subtilisin or proteinase K.

Presumably pronase P digestion of virus HA polypeptides generated fewer stable fragments, greater than 10 K daltons, than the other proteases.

Of interest was the finding that HA polypeptide digestion patterns of the five influenza viruses always contained a hydrolysis fragment with an approximate molecular weight of 13 K to 14 K when treated with subtilisin, pronase P or proteinase K. Whether these fragments are similarly composed or occupy similar locations in the HA polypeptide is unknown at this time. This fragment may however, represent a highly conserved portion of all influenza A virus HA polypeptides.

Although it is difficult to account for the differences noted in the HA polypeptide digestion patterns, there are a number of possible explanations. Changes in the primary amino acid sequence of the HA polypeptide caused by antigenic drift or shift, could be adding or eliminating sites available to enzymatic attack. Also strain-specific differences in glycosylation of HA polypeptides could be affecting the ability of certain enzymes to hydrolyze an otherwise susceptible portion of the HA molecule.

There are considerable strain-dependent variations in the number, location and types of oligosaccharide chains linked to the HA polypeptide (Schwarz and Klenk, 1981; Basak and Compans, 1983). Such variations can occur in HA polypeptides isolated from viruses without or within the same subtype (Gething et al., 1980; Hiti et al., 1981).

An attempt to elucidate the role of oligosaccharides in digestion of PR virus HA polypeptides, proved unsuccessful since unglycosylated HA polypeptides comigrated with the NP polypeptide band.

In this study the digestion patterns of M polypeptides were almost identical for all five viruses tested. Immunological studies with M polypeptides of influenza A viruses showed that no antigenic differences existed (Schild, 1972). These results indicate that unlike the HA and NA polypeptides, the M polypeptide is highly conserved. In contrast however, Laver and Downie (1976) found that irrespective of many similarities there were clearcut differences in tryptic peptide maps of M polypeptides from several influenza A viruses. A possible explanation for the apparent contradiction between this study and that of Laver and Downie is that they were able to use trypsin to hydrolyze the M polypeptide. It may be that the specificity of trypsin allowed detection of changes in the M polypeptide that the non-specific proteases were unable to detect.

Apparent differences found between digestion patterns of NP polypeptides isolated from H1N1 viruses (PR, FM, USSR) were unexpected and are difficult to account for. The antigenic relatedness of NP polypeptides is used to classify influenza viruses as type A, B or C (Pereira, 1969). Possibly the antigenic sites in this polypeptide are more highly conserved than other portions of the molecule. Further limited proteolysis studies on NP polypeptides of

additional influenza viruses, may clarify these apparent differences.

Presently serological testing is the major method used to classify influenza A viruses and to detect changes in the HA or NA polypeptides. However, inspite of their many advantages some problems have occurred when antibodies are used. Immunological relationships originally indicated that influenza A viruses infecting man were divided into four subtypes (HO, H1, H2 and H3). Later, gene segment homology showed that the HO and H1 subtypes were closely related and should be categorized under a single subtype (H1) (Scholtissek, 1978a). Use of monoclonal antibodies has allowed detection of a single amino acid substitution in an antigenic site of the HA polypeptide (Sleigh et al., 1981). However, variants whose HA polypeptides differ by amino acids not in epitopes were not distinguishable with the monoclonal antibody preparations (Webster et al., 1979). Because the production of antibodies is time consuming and because they can miss detection of amino acid changes outside of antigenic sites, alternative detection methods are required.

Tryptic peptide mapping in one or two dimensions of influenza HA and M polypeptides has shown differences between virus strains (Laver and Webster, 1968; Laver and Downie, 1976; Basak et al., 1981). One difficulty in this type of study is that virus polypeptides are compared on their susceptibility to only one enzyme. A change in the amino acid sequence of the polypeptide not affecting a

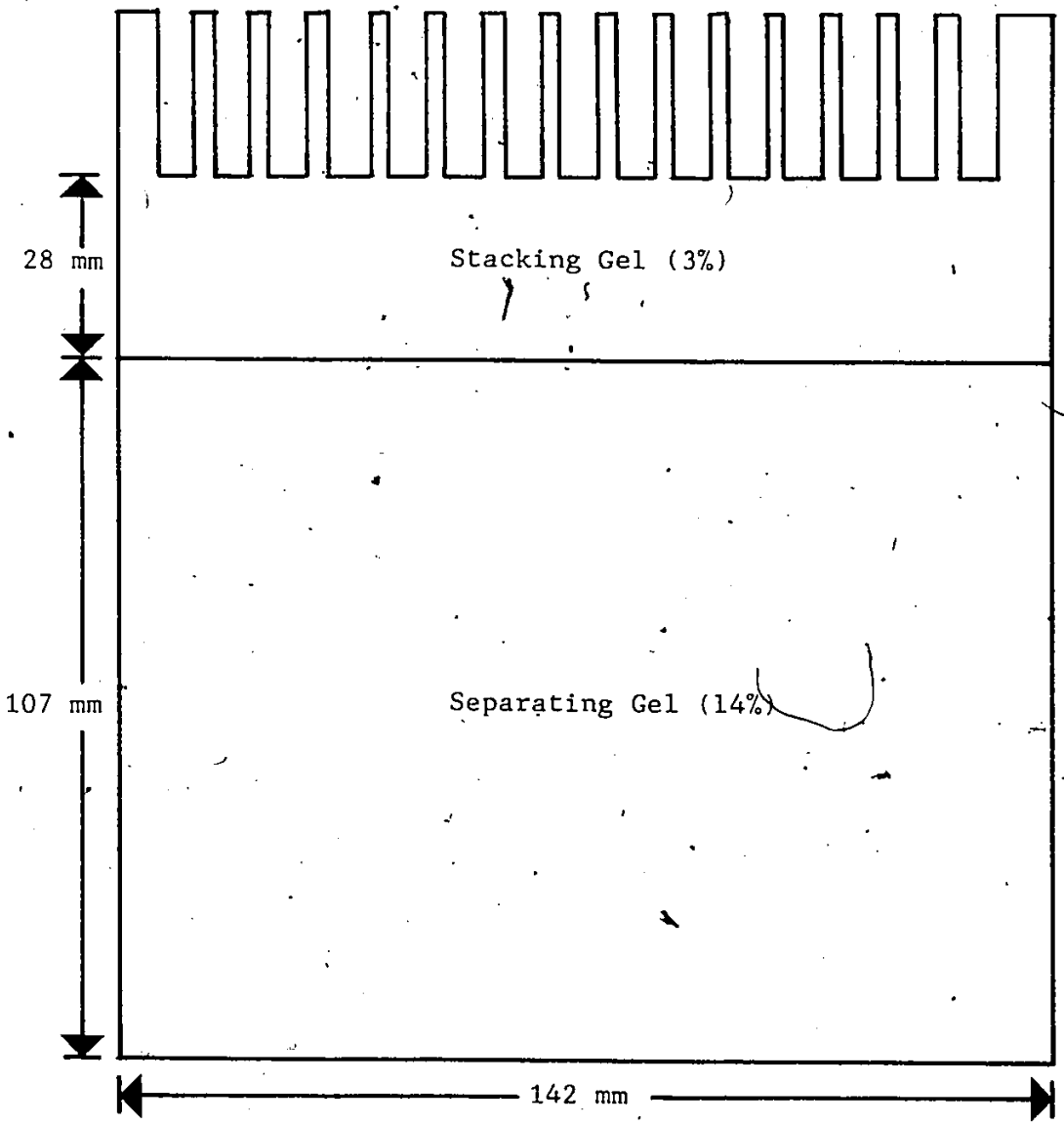
trypsin cleavage site(s) could escape detection.

One of the best methods for detecting changes in HA polypeptides involves the determination of the amino acid sequence. Recently several groups have done sequencing of HA polypeptides of several influenza A viruses (Verhoeyen et al., 1980; Gething et al., 1980). Sequencing detects changes in amino acid composition at specific sites in the HA polypeptide. However, the sequencing procedure is time consuming and more specific than necessary for categorizing an influenza virus in one of the subtypes.

Experiments in this study demonstrate that limited proteolysis of hemagglutinin molecules of influenza A viruses in SDS gels shows potential as an alternative method in subtyping strains. Furthermore, this technique could be useful in supplementing current methods to detect antigenic drifting of hemagglutinins.

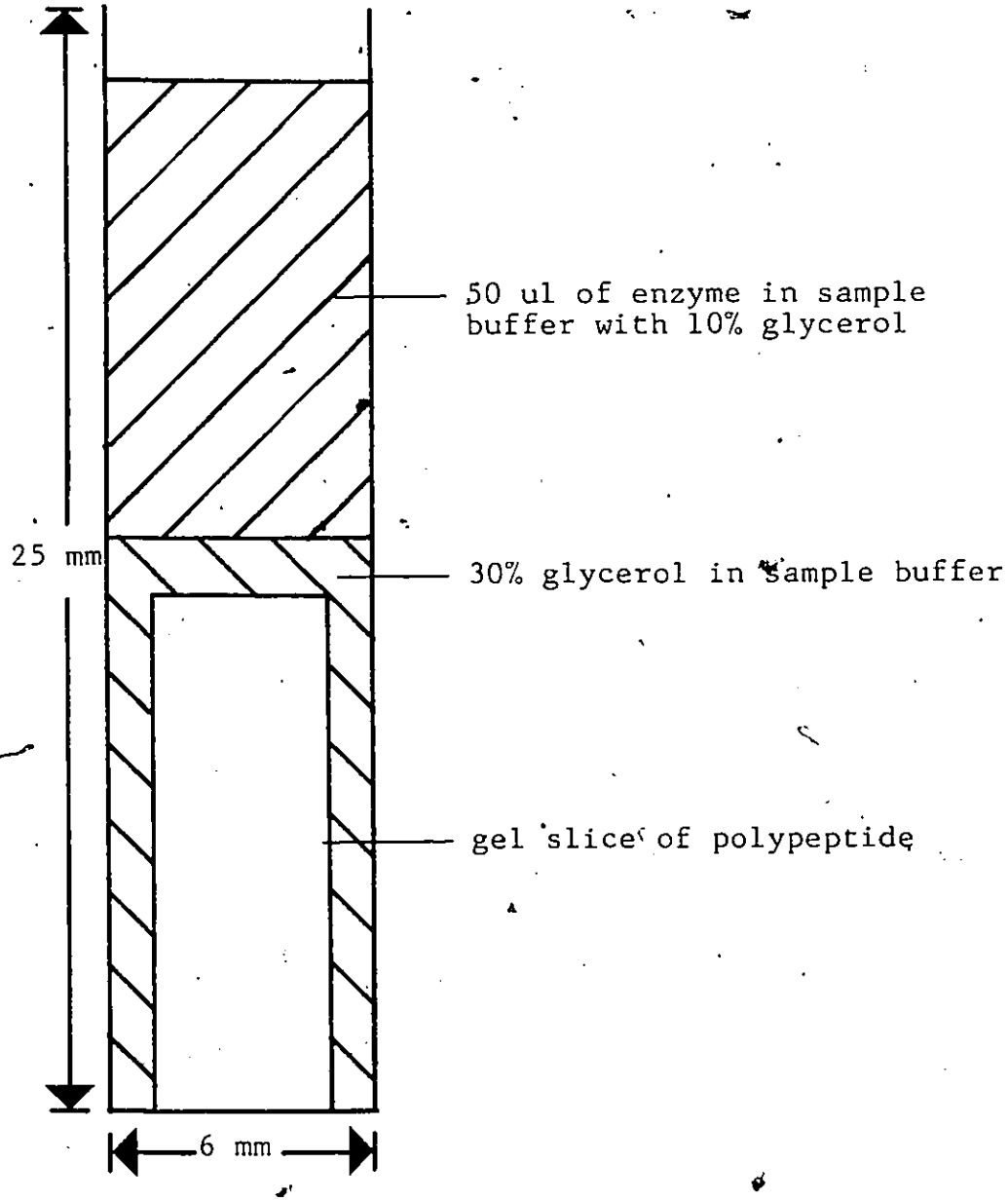
APPENDIX A

Gel for Hydrolysis of Influenza Polypeptides



APPENDIX B

Gel-Isolated Polypeptide in Sample Well



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VITA AUCTORIS

Mark Andrew Parrington

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