DIFFERENT TRYPSIN SENSITIVITY OF THE HEMAGGLUTININ FROM ANTIGENICALLY SIMILAR RECOMBINANT INFLUENZA VIRUSES

by

ANN HART ERICKSON

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1977

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

3/28/77
date

Chairman of Examining Committee

3/29/77
date

Executive Officer

Supervisory Committee

The City University of New York

Abstract

DIFFERENT TRYPSIN SENSITIVITY OF THE HEMAGGLUTININ

FROM ANTIGENICALLY SIMILAR RECOMBINANT INFLUENZA VIRUSES

by

ANN HART ERICKSON

Adviser: Professor E. D. Kilbourne

The slow trypsin sensitivity of the hemagglutination activity of the recombinant influenza virus X-29 (H0N2) or its large-plaque variant X-29L differed from the rapidly trypsin-sensitive activity of X-12 (H0N2) or the parental virus A/NWS/33 (H0N1) and from the trypsin resistant activity of A/WSN/33 (H0N1) or the parental RI/5$^-$ (H2N2) virus. The antigenically similar recombinants X-12 and X-29L were studied to determine the molecular basis of the genetic marker of trypsin sensitivity and to examine the viral structure. The loss of hemagglutination activity of each virus correlated with the conversion of the $HA1_f$ subunit of the surface glycoprotein hemagglutinin into an electrophoretically slower migrating intermediate $HA1_s$. The different rate of loss of activity for X-12 (30 min) and X-29L (4 hr) is evidently due to two factors: (1) the trypsin-mediated conversion of $HA1_f$ into $HA1_s$ occurs more slowly for X-29L than for X-12, and (2) the $HA1_s$ component retains hemagglutination activity in X-29L but not in X-12. The trypsin-resistant surface glycoprotein neuraminidase was detected ultrastructurally as evenly distributed globular structures attached by thin filaments to the lipid bilayer. Since the amount of hemagglutinin protein and the amino acid compositions of the HA1 and HA2 subunits were the same for both viruses, the difference in their trypsin sensitivity is probably due to a point mutation affecting the availability of lysine or arginine residues, the position of bound carbohydrate or the protein conformation.

Acknowledgments

I am most grateful to Professor Edwin D. Kilbourne for guidance and encouragement, and for providing the opportunity to study the molecular biology of recombinant influenza viruses. Many thanks to my colleagues in the Department of Microbiology for lively discussions of this research project. I am particularly grateful to Dr. Doris J. Bucher for stimulating conversations, for assistance with column chromatography and for constructive criticism of this thesis, and to Dr. Peter Palese for advice and help with gel electrophoresis. In addition, I thank Ms. Barbara A. Pokorny for her cheerful and expert technical assistance.

The fellowship support from the Mount Sinai Medical Center during five years of my graduate studies is gratefully acknowledged.

I am especially grateful to my husband, Bruce, for his advice, faith and encouragement in moments of despair. Without his tolerance of long lab hours, midnight dinners and unironed clothes, this work would never have been completed.

This thesis is dedicated to my parents, Robert Lawrence Piper and Mary Ann Bodine Piper, who have provided me with constant support and encouragment throughout my formal education.

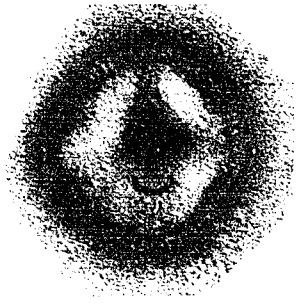

Table of Contents

List of Abbreviations

Abbreviation	Equivalent expression
BA	bovine albumin
BHK	baby hamster kidney
BIS	N,N'-methylene-bis-acrylamide
CAM	chorioallantoic membrane
CEF	chicken embryo fibroblast
cpm	counts per minute
DEAE	N,N-diethylaminoethyl
dpm	disintegrations per minute
DTT	1,4-dithiothreitol
EDTA	N,N,N',N'-ethylenediaminetetraacetic acid
EID_{50}	50% egg-infective dose
HA	hemagglutinin
HA1	larger hemagglutinin subunit
$HA1_f$	faster migrating HA1 component
$HA1_s$	slower migrating HA1 component
HA2	smaller hemagglutinin subunit
HABA	hemagglutinin-binding antigen
HAU	hemagglutination units
HI	hemagglutination inhibition
H0, H1, H2, H3	hemagglutinin serotypes
M	membrane protein
MDBK	Madin-Darby bovine kidney
MDCK	Madin-Darby canine kidney
NA	neuraminidase
NI	neuraminidase inhibition

Abbreviation	Equivalent expression
NP	nucleoprotein
NS	nonstructural protein
NTC	buffer containing NaCl, Tris, and $CaCl_2$ (see p. 99)
NTE	buffer containing NaCl, Tris, and EDTA (see p. 98)
N1, N2	neuraminidase serotypes
P	polymerase
PBS	phosphate-buffered saline
PFU	plaque-forming units
PI	plaque inhibition
PPO	2,5-diphenyloxazole
PSR	plaque-size reduction
PTA	phosphotungstic acid (sodium salt)
RDE	receptor destroying enzyme of *Vibrio cholera*
RNA	ribonucleic acid
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
Tris	2,2,2-*tris*-(hydroxymethyl)ethylamine

List of Tables

List of Figures

Chapter 1

Influenza: The Disease and the Virus

Influenza remains one of the few pandemic infectious diseases not controlled by immunization. The human disease was described as early as 1510, the causative virus was isolated in 1933, and a specific vaccine was prepared three years later, but it has not yet been possible to produce a vaccine that provides long-term protection in the manner of the smallpox and polio vaccines. Influenza virus induces an acute inflammation of the upper respiratory tract that is clinically characterized by fever, severe aching, pharyngitis and coughing. The incubation period is short and the disease is usually self-limiting in three to four days. Infection generally produces high morbidity and low mortality. Death mainly occurs in the old and infirm due to secondary bacterial infection.

History. The earliest detailed clinical account of influenza was the description by Short in 1749 (Thompson, 1852) of the 1510 epidemic in England, but descriptions of an epidemic disease that was probably influenza were recorded as early as the fourteenth century in Ireland (Thompson, 1852) and Italy (Crookshank, 1922). Thirty influenza pandemics and numerous lesser epidemics were described in the period between 1510 and 1930 (Francis and Maassab, 1965). During the pandemic of 1889-1892, Pfeiffer and Beck attempted to isolate the etiological agent and reported (Pfeiffer and Beck, 1892) that a bacillus, Hemophilus influenzae, was the causative agent of influenza. In 1933, however, Smith, Andrewes and Laidlaw (1933) demonstrated that bacteria-free nasal washings from patients with influenza produced a characteristic febrile

illness when inoculated intranasally into ferrets. The filterable agent or virus reisolated from infected ferrets transmitted the disease to other ferrets. Two years earlier, Shope (1931) had isolated a similar virus from swine that was essential for the transmission and production of swine influenza. The viral etiology of human influenza was confirmed by the demonstration by Francis (1934) that specific neutralizing antibodies against the virus developed during the course of the disease. Two years later, the first influenza vaccine was prepared (Chenoweth et al., 1936). These viruses were designated as type-A influenza when Francis (1940) and Magill (1940) independently isolated a second antigenically distinct etiological agent, type-B influenza. A third antigenically distinct virus, type C, was recognized in 1949 (Taylor, 1949; Francis et al., 1950).

Studies of influenza viruses were greatly aided by two important discoveries. First, Burnet (1940) reported that influenza viruses multiply to high titer in the allantoic cavity of an embryonated chicken egg, which provided a convenient and inexpensive culture system. Previous work with the virus had been confined to animal studies, mainly with swine, ferrets and mice (Andrewes et al., 1934; Francis, 1934). Second, Hirst (1941) and McClelland and Hare (1941) independently found that influenza viruses adsorb to and agglutinate chicken red blood cells. This provided a simple method for detecting and quantitating not only the influenza viruses but also the hemagglutination-inhibiting antibodies elicited by the virus.

Morphology. Influenza virions are pleomorphic, ranging from spherical particles with a diameter of about 100 nm to filamentous particles as long as 4 μm (Compans and Choppin, 1975) (Fig. 1.1).

Fig. 1.1. Morphology of influenza virus. The pleomorphic virions appear as globular to filamentous structures bearing an outer layer of spikes. Recombinant X-12 was magnified 160,000 times after negative staining with 4% sodium phosphotungstate at pH 7.0.

Electron micrographs of negatively stained virus preparations have revealed that the outer surfaces of the particles are covered with closely spaced projections 10-12 nm in length (Compans and Choppin, 1975). Two different structures compose this outer layer. The hemagglutinin (HA) spikes are rod-shaped molecules (Laver and Valentine, 1969) through which the virion attaches to N-acetylneuraminic acid on the cell surface. The more complex neuraminidase (NA) spikes have club-shaped heads that are attached to the virion by fibers terminating in small knobs (Laver and Valentine, 1969). These spikes enzymatically cleave terminal N-acetylneuraminic acid residues from glycoproteins and gangliosides, which causes elution of the virus from the cell surface.

The spikes are embedded in the lipid bilayer that the virus acquired during budding from the host cell plasma membrane. The major structural protein of the viral envelope, the membrane (M) protein, appears as an electron-dense layer closely juxtaposed to the inner surface of the electron-lucid lipid (Fig. 1.2). When stain penetrates disrupted virus particles, a helical internal component or nucleocapsid can be seen (Fig. 1.2). The nucleocapsid consists of the nucleoprotein (NP) and the single-stranded RNA genome. Three additional structural proteins, the polymerase (P) proteins, are probably also associated with the nucleocapsid.

VIRAL PROTEINS

Hemagglutinin. Hemagglutinin constitutes from 25% to 35% of the total virion protein (Schulze, 1973). Recent morphological studies of isolated hemagglutinin suggest that each HA spike is a trimer of three HA molecules (Laver, 1973; Griffith, 1975). This structure, which has a

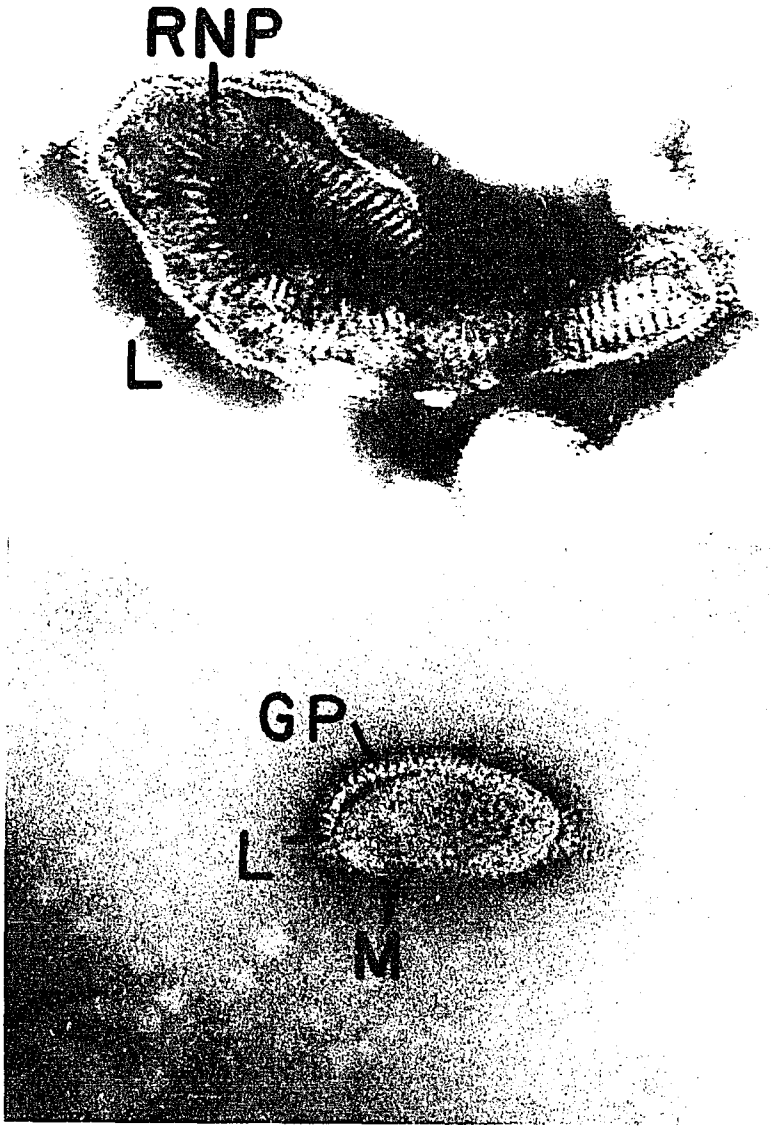

Fig. 1.2. Internal virion structure after virus disruption.
A. Tightly coiled ribonucleoprotein (RNP) is observed after penetration of stain into the virion. Surface glycoproteins are embedded in an electron-lucid lipid layer (L). X-12 was magnified 160,000 times after negative staining with 2% sodium phosphotungstate at pH 6.2.
B. Retention of virion shape by the interior membrane protein layer (M) after partial loss of the lipid layer (L) and surface glycoproteins (GP). Virus X-12 was magnified 160,000 times after negative staining with 1% ammonium molybdate at pH 7.9.

a molecular weight of about 210,000 daltons, adsorbs to but does not agglutinate erythrocytes. If detergent is removed from a preparation of isolated HA spikes, however, the spikes aggregate into clusters, presumably due to hydrophobic interaction of the ends of the molecules that were attached to the lipid bilayer. These multivalent structures do induce hemagglutination (Laver and Valentine, 1969).

The individual hemagglutinin molecules are synthesized as single polypeptide chains having a molecular weight of approximately 75,000 daltons. During virus maturation at the cell membrane, they are cleaved into two disulfide-bonded subunits. The HA1 subunit has a molecular weight of approximately 50,000 daltons and the HA2 subunit weighs about 25,000 daltons (see Chapter 3). The extent of this cleavage depends on the host cell and its culture medium, and the virus strain (Lazarowitz et al., 1971, 1973a,b; Rifkin et al., 1972; Skehel, 1972; Klenk and Rott, 1973, Stanley et al., 1973a, Klenk et al., 1975; Lazarowitz and Choppin, 1975). Complete cleavage usually occurs in the chick embryo, partial cleavage in continuous cell lines, and little or no cleavage in monolayers of primary chicken embryo fibroblast cells (see Fig. 3.11). Cleavage into HA1 and HA2 readily occurs during treatment of virions with trypsin (Schulze, 1970; Lazarowitz et al., 1971, 1973a; Klenk et al., 1975; Lazarowitz and Choppin, 1975) or plasmin (Lazarowitz et al., 1973b). Although cleavage is not required for the assembly of virus particles or for expression of hemagglutination activity (Lazarowitz et al., 1973a; Stanley et al., 1973a), it is essential for infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975).

The HA2 subunit is released from the carboxyl end of the hemagglutinin molecule (Skehel and Waterfield, 1975). The HA2 proteins

from five strains representative of human influenza A subtypes, one strain of influenza B (Skehel and Waterfield, 1975), and one recombinant influenza virus having an equine hemagglutinin (Bucher et al., 1976) were all found to share the same amino-terminal ten-residue sequence (H-Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile- . . .). This region contains a palindrome of seven amino acids (. . . -Phe-Gly-Ala-Ile-Ala-Gly-Phe- . . .) that was suggested by Skehel and Waterfield (1975) to be a recognition sequence for the proteases that cleave intact HA into HA1 and HA2.

HA2 appears to be the hemagglutinin subunit that interacts with the viral envelope. Exposure of X-31 virus (H3N2) (Brand and Skehel, 1972) or Bel virus (H0N1) (Skehel and Waterfield, 1975) to the protease bromelain released nonaggregating HA molecules. Sequence studies indicated that approximately 50 amino acid residues were missing from the carboxyl terminus of HA2 subunits of the proteolytically-released molecules. Since 21 of the missing amino acid residues are hydrophobic, the hydrophobic interaction of HA with the lipid bilayer probably occurs through the carboxyl terminus of the HA2 subunit.

If HA2 is the interior HA subunit, then HA1 must extend into the aqueous environment surrounding the virion and should contain the various site(s) that interact with erythrocytes, host cells, serum mucoproteins and antibodies. Experimental evidence in support of this assumption is limited. Eckert (1966, 1969, 1973) has isolated an HA1 dimer (HABA) that reacts with antibodies which inhibit hemagglutination. Brand and Skehel (1972) found that antisera against HA1 and against whole virus formed a single common precipitin line with bromelain-released hemagglutinin, but a precipitin line was observed with anti-HA2 sera only after the antigen was reduced. In addition, radio-iodination of the

virion with lactoperoxidase produced greater labeling of HA1 than HA2 (Stanley and Haslam, 1971), which suggests that the HA1 subunit is more exposed to the environment.

Hemagglutinin molecules undergo post-synthetic glycosylation during association with endoplasmic reticulum membranes (Compans, 1973; Hay, 1974). The sequence and composition of the carbohydrate moities are specified by the host cell (Compans and Choppin, 1975). Differences in the electrophoretic mobilities of glycoproteins from virions grown in different cell types suggest that the amount of carbohydrate incorporated into the glycoproteins may also be determined by the host cell (Haslam et al., 1970; Compans et al., 1970; Schulze, 1970). Approximately 24.4% of the weight of HA1 protein isolated from egg-grown H3 virus was contributed by carbohydrate, primarily N-acetylglucosamine, galactose, mannose and fucose (Ward and Dopheide, 1976). Carbohydrate, mainly N-acetylglucosamine, comprised only 4.7% of the weight of the HA2 polypeptide from this H3 virus.

Antibodies against hemagglutinin usually cause inhibition of erythrocyte agglutination as measured by the hemagglutination-inhibition (HI) assay and neutralization of infectivity as measured by the plaque-inhibition (PI) assay (Laver and Kilbourne, 1966). In contrast, antineuraminidase antibodies mainly inhibit cellular release of virions as measured by the plaque-size-reduction (PSR) assay (Jahiel and Kilbourne, 1966).

Neuraminidase. Although the quantity of the neuraminidase (NA) protein varies with both the host cell (Laver, 1963) and the virus strain (Laver and Kilbourne, 1966; Palese and Schulman, 1974; Mowshowitz and Kilbourne, 1975), this enzyme generally comprises only about 7% of the

total virion protein (Laver, 1963; Laver and Kilbourne, 1966; Skehel and Schild, 1971). Thus, the influenza virion bears about 550-600 surface spikes (Tiffany and Blough, 1970), but only 50-100 of these are neuraminidase (Bucher and Palese, 1975).

Comparison of the molecular weight of native neuraminidase spikes (200,000-250,000 daltons) with the weight of the dissociated NA polypeptide subunits (55,000-65,000 daltons) suggested that the spike is composed of four monomers (Bucher and Kilbourne, 1972; Kendal and Eckert, 1972; Lazdins et al., 1972; Wrigley et al., 1973). Ultrastructural studies of the NA protein have supported this view by revealing structures composed of four spheres in co-planar square array (Wrigley et al., 1973). Disulfide bonds are crucial for maintenance of the tetrameric structure but probably link dimers rather than all four subunits (Bucher and Kilbourne, 1972; Lazdins et al., 1972). Only the tetrameric structure possesses enzymatic activity (Bucher and Kilbourne, 1972; Kendal and Eckert, 1972).

Like hemagglutinin, neuraminidase is a glycoprotein, but little is known about the number or nature of the carbohydrate side chains. Kendal and Eckert (1972) have estimated that each NA molecule from egg-grown recombinant virus X-7(F1) contains about 5.7 glucosamine residues.

Influenza virus neuraminidase hydrolyzes 2→3 linkages between terminal N-acetylneuraminic acid residues and D-galactose or N-acetyl-D-galactosamine in glycoproteins and in small, soluble carbohydrates (Rafelson et al., 1966; Drzeniek, 1967). Although numerous physiological roles have been proposed for the viral enzyme, recent evidence indicates that neuraminidase functions in the release of virions from the host cell. The viral neuraminidase removes sialic acid residues

from budding virions (Klenk and Choppin, 1970; Klenk et al., 1970), thereby preventing virus aggregation and promoting the release of individual virions into the supernatant. Consistent with these results, mutants of the A/WSN/33 strain of influenza virus with a temperature-sensitive defect in their neuraminidase were found to have sialic acid residues on their surface and to form aggregates with each other and possibly with the cell surface upon release from the host cell (Palese et al., 1974).

Membrane protein. Several lines of evidence suggest that the 32,000 dalton membrane (M) protein forms the electron-dense layer seen in thin sections to be juxtaposed with the internal surface of the viral lipid (Apostolov and Flewett, 1969; Bächi et al., 1969; Compans and Dimmock, 1969; Schulze, 1972). First, the M protein, which comprises about 40% of the total virion protein (Choppin and Compans, 1975), is the only protein present in sufficient quantity to form such a structure. Second, removal of the glycoprotein spike layer leaves the M protein and the electron dense layer unchanged (Fig. 1.2) (Kendal et al., 1969; Schulze, 1972; Nermut, 1972). Third, the kinetics of iodination of M protein with chloramine-T (Stanley and Haslam, 1971) and fluorescent energy-transfer studies (Lenard et al., 1974) both indicate that M is internal to the lipid bilayer but external to the nucleocapsid.

The M protein probably maintains the structural integrity of the virion (Kendal et al., 1977) and may be responsible for the icosahedral symmetry observed ultrastructurally after freeze-etching and shadowing (Nermut and Frank, 1971). M protein may also provide a recognition site for the viral nucleocapsid during virus assembly at the cell membrane and may maintain a localized region within the membrane from which cellular

proteins are excluded (Choppin and Compans, 1975). Since M is the most plentiful protein in the virion but is found in relatively small amounts in infected cells, the synthesis of M may be a rate-limiting step that controls virus production (Lazarowitz et al., 1971).

Ribonucleoprotein. Negatively stained virions occasionally exhibit a large, rigid, tightly coiled structure inside the virus particles (Fig. 1.2) (Apostolov and Flewett, 1965; Almeida and Waterson, 1970; Schulze et al., 1970). After isolation from either purified virions or infected cells, this ribonucleoprotein (RNP) is recovered in several distinct size classes (Duesberg, 1969; Pons, 1971). Thus the viral genome exists as several segments of single-stranded RNA (Duesberg, 1968; Pons and Hirst, 1968; Bishop et al., 1971; Lewandowski et al., 1971; Skehel, 1971b).

Several lines of evidence indicate that the segmented RNA is the biologically functional form of the viral genome and not just the result of degradation. The high frequencies of genetic recombination observed with influenza virus (Burnet, 1951; Burnet and Lind, 1951a,b; Hirst and Gottlieb, 1955; Fraser, 1959; Simpson and Hirst, 1961) originally led to proposals by Burnet (1956) and Hirst (1962) that the RNA genome existed in segments and that recombination was the result of the random reassortment of the viral RNA segments. Further evidence for a segmented genome includes multiplicity reactivation following inactivation by ultraviolet light (Henle and Liu, 1951; Barry, 1961), differences in the kinetics of inactivation of the various viral functions by chemical agents (Scholtissek and Rott, 1964) or by ultraviolet light (Joss et al., 1969; Gandhi and Burke, 1970), and isolation after low-dilution serial passage of incomplete virus lacking or deficient in the largest RNA molecules (Duesberg, 1968; Pons and Hirst, 1969; Choppin and Pons, 1970). Finally,

biochemical analyses have established the segmented nature of the genome by showing that the different size classes of RNA each have pppAp at the 5'-terminus (Young and Content, 1971), an unphosphorylated 3'-terminus (Lewandowski et al., 1971), and distinct oligonucleotide patterns (Horst et al., 1972) and base sequences (Content and Duesberg, 1971).

Polyacrylamide gel analyses of viral RNA has revealed that the viral genome consists of eight pieces of RNA of appropriate sizes to code for the eight viral proteins (McGeoch et al., 1976; Palese and Schulman, 1976b; Pons, 1976; Ritchey et al., 1976; Palese et al., 1977), namely, the seven structural proteins (three P proteins, HA, NA, M and NP) and one nonstructural protein (NS) of unknown function found in infected cells (Lazarowitz et al., 1971). The existence of eight RNA segments is consistent with the finding that temperature-sensitive mutants of WSN virus fall into eight complementation groups (Hirst, 1973). Addition of the molecular weights of the eight RNA segments has allowed the total molecular weight of the RNA genome to be estimated as $5.7\text{-}5.9 \times 10^6$ daltons (McGeoch et al., 1976; Pons, 1976). The virion RNA is complementary to messenger RNA (Etkind and Krug, 1974; Glass et al., 1975) and is not covalently attached to poly-A (Etkind and Krug, 1974).

Each RNA piece is associated with the non-glycosylated nucleocapsid (NP) protein (about 60,000 daltons). The relative quantities of NP and RNA in the ribonucleoprotein segments indicate that one NP molecule is present for about every 20 nucleotides (Compans et al., 1972). The P polypeptides also appear to be associated with ribonucleoproteins isolated from virions (Bishop et al., 1972) or infected cells (Caliguiri and Compans, 1974). Their individual functions are not known, but they are probably all involved in virus-specific RNA synthesis (Compans et al.,

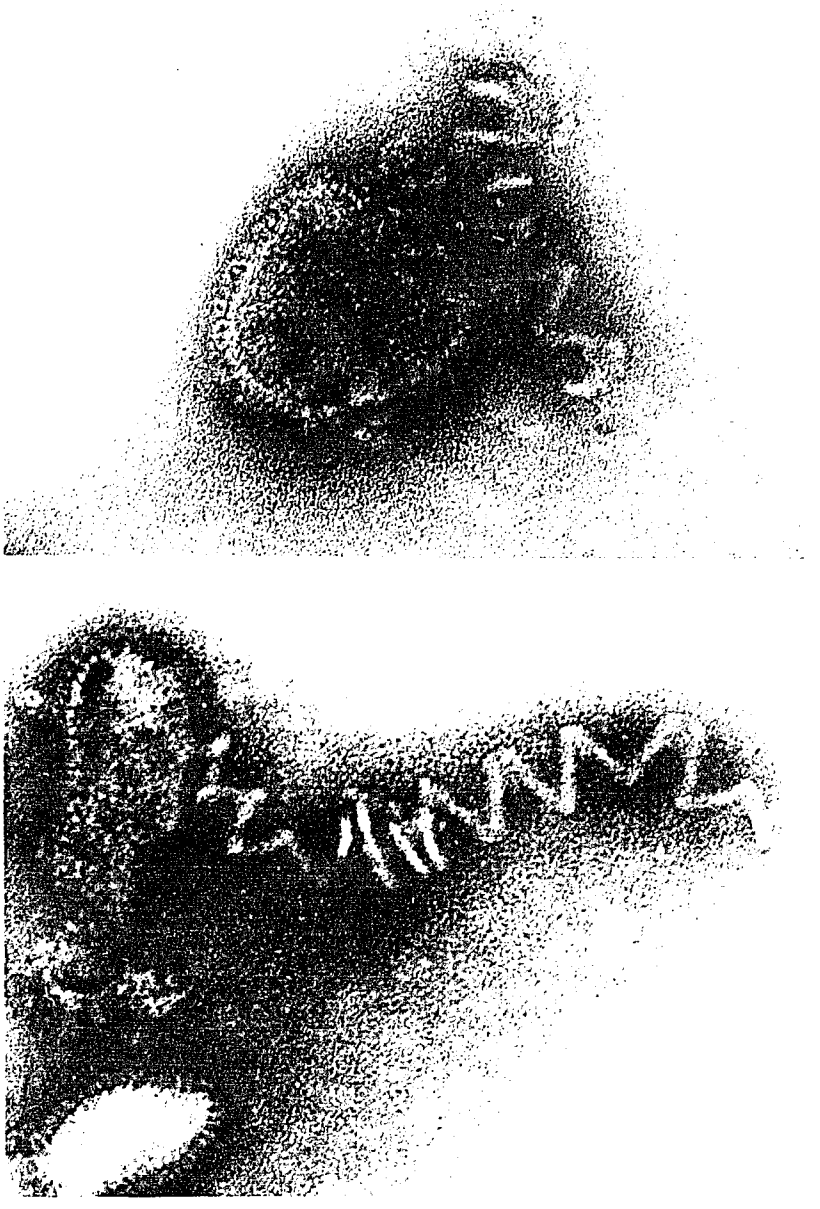

Fig. 1.3. Uncoiled ribonucleoprotein from influenza virus. Fortuitous disruption without detergent revealed the ribonucleoprotein emerging from the virion as a long, loose, continuous coil(s). Virus X-12 was magnified 160,000 times after negative staining with 1% ammonium molybdate at pH 7.9.

1970; Schulze, 1970; Skehel, 1971a; Bishop et al., 1972; Caliguiri and Compans, 1974). A virion-associated RNA-dependent RNA polymerase activity has been described (Bishop et al., 1971; Chow and Simpson, 1971; Penhoet et al., 1971; Skehel, 1971b) but has not been localized to a particular virion protein.

Infective virions must contain at least one copy of each ribonucleoprotein segment. Since there is no evidence to suggest physical linkage between the segments, such as overlapping complementary base pairing or a continuous protein backbone, the mechanism that insures inclusion of the proper number and type of segment during virus maturation is not clear. Almeida and Brand (1975) have reported that if virus is rapidly treated on a microscope grid with a non-ionic detergent, the ribonucleoprotein is seen as a continuous strand of 6 nm diameter arranged in the form of a double coil or helix. We have obtained similar preparations without detergent disruption showing a single strand in a greatly expanded helix emerging from virus particles (Fig. 1.3). The relationship of these structures to the shorter, wider structures seen in micrographs of purified RNP (Pons et al., 1969; Compans et al., 1972) is not understood. Almeida and Brand propose that the RNA exists in virions as a continuous double coil or helix which readily fractures, perhaps at specific points, into eight pieces on isolation. These results suggest that the RNA segments are initially separate in the host cell but are linked during virus assembly.

VIRAL GENETICS

Antigenic variation. Classification of influenza viruses was originally based on the antigenic distinctiveness of the nucleocapsid

protein. By 1949 three types of influenza viruses (A, B, C) were recognized by this criterion. Later studies revealed that viruses of the same type shared antigenically similar M as well as NP proteins (Schild, 1972). Of the three types, only the type-A viruses have been isolated from a variety of hosts other than man and have been associated with pandemic disease (Kilbourne, 1975).

The magnitude of the variation of influenza viruses was not appreciated until 1947, when a type-A virus appeared that was antigenically quite distinct from the viruses first isolated in 1933. The appearance of the new strain of influenza A virus was accompanied by disappearance from the human population of the old strain. These observations first suggested that previously acquired immunity to antecedent strains might be circumvented by the appearance of an antigenically distinct virus (Kilbourne, 1975).

The nature of the major antigenic changes was not understood until the viral neuraminidase was recognized as an antigenic component of the viral surface distinct from the hemagglutinin. This distinction arose from two types of evidence. First, degradation of virus particles with proteolytic enzymes (Mayron et al., 1961; Noll et al., 1962) or detergents (Laver, 1963) showed that it was possible to segregate the hemagglutination and neuraminidase activities. Second, isolation of stable, antigenically mixed hybrid viruses (Hirst and Gottlieb, 1955; Kilbourne and Schulman, 1965; Tumova and Pereira, 1965; Sugiura and Kilbourne, 1965) demonstrated that HA and NA segregate independently during recombination. Later studies showed that hemagglutination and neuraminidase activities were associated with two morphologically distinct spikes on the viral surface (Laver and Valentine, 1969; Webster and Darlington, 1969).

Introduction into the human population of an influenza A virus with antigenically different hemagglutinin and neuraminidase correlates with the major influenza epidemics (Table 1.1). Pandemic severity principally reflects the extent of antigenic change from preexisting virus. Although antigenic analyses have indicated that the hemagglutinin and neuraminidase antigens can and do vary independently (Schulman and Kilbourne, 1969), the most severe pandemic following the initial isolation of an influenza virus occurred in 1957 when both the HA and NA proteins underwent significant antigenic change.

Antigenic variation is the result of both minor and major changes in the viral surface proteins. During interpandemic periods, minor variation occurs due to genetic mutation, presumably under the selective pressures of antibodies in an immune population. These gradual antigenic changes, known as antigenic drift (Burnet, 1955), occur in type A and B viruses and in both the NA and HA proteins (Paniker, 1968; Schulman and Kilbourne, 1969; Meier-Ewert et al., 1970). Peptide maps of hemagglutinin proteins have revealed that these antigenic variants differ only in one or two tryptic peptides (Laver and Webster, 1968). In contrast, peptide maps of hemagglutinin proteins from different subtypes of A viruses indicate that the antigenic changes giving rise to pandemic disease correlate with major changes in the sequence of the hemagglutinin protein (Laver, 1964; Laver and Webster, 1972). These antigenic shifts are thought to result from genetic recombination in animals between human and animal influenza viruses (Rasmussen, 1964; Kilbourne, 1968). The resulting recombinants would possess the capacity to infect man but would have acquired new surface antigens against which the human population does not have antibody protection. Indeed, Kilbourne (1973) has proposed

TABLE 1.1

Major Antigenic Shifts in Type-A Influenza Virus Accompanying Influenza Epidemics of the Twentieth Century[a]

Year	Classification	Antigenic designation[b]	Severity
1918	SW (?)	$H_{SW}N_1$	pandemic ++++
1933	A_0	H_0N_1	epidemic
1947	A_1	H_1N_1	pandemic +
1957	A_2 (Asian)	H_2N_2	pandemic +++
1968	HK (Hong Kong)	H_3N_2	pandemic ++

[a]Adapted from Kilbourne (1973).

[b]H = hemagglutinin, N = neuraminidase.

that there are no true animal strains of influenza virus but only human viruses that have infected animals and de-adapted to man.

In contrast to the situation in humans, the appearance of new viruses in animals is not necessarily associated with the disappearance of previously recognized strains. This would increase the reservoir of antigenically distinct influenza viruses in animals. The introduction of new human influenza antigens by recombination between animal and human influenza viruses is supported by the in vitro isolation of antigenic hybrids between animal and human strains (Tumova and Pereira, 1965; Kilbourne, 1968; Easterday et al., 1969) and the in vivo detection of recombination between different animal virus strains (Webster et al., 1971; 1973a).

Genetic recombination. The proposals by Burnet (1956) and Hirst (1962) that influenza viruses possess segmented genomes that readily undergo gene reassortment led to numerous attempts to produce recombinant viruses in vitro. This work has been reviewed in detail by Sugiura (1975). These experiments were originally undertaken in an attempt to understand the mechanism of influenza virus mutation and to produce viruses of broader antigenicity for human immunization.

Recombination is achieved by simultaneously inoculating embryonated chicken eggs or tissue culture cells with parental viruses of two different serotypes. Identification of the recombinant viruses in the resulting mixed population is aided by inactivation of one of the parental viruses using heat or ultraviolet light. Two methods used to select against the remaining infective parental viruses are (1) neutralization with antiserum or serum glycoprotein inhibitors and (2) passage in a host or cell culture in which the infective parent cannot

multiply (Kilbourne, 1963). Based on recent knowledge of the two distinct viral surface antigens (HA, NA) and the effect on virus replication of specific antisera against either antigen, host cells have been inoculated with two infective parental viruses in the presence of antisera against the two surface antigens not desired in the recombinant virus. The resulting recombinant viruses can be purified by high-dilution passage in eggs or by plaquing on cell monolayers.

Numerous viral characteristics have been used as genetic markers to identify and characterize the recombinant viruses, including antigenicity (serotype), viral morphology, virulence, host range, plaque morphology, and sensitivity of the viral hemagglutination activity to heat, chemicals, or enzymes (Kilbourne, 1963). These characteristics were originally chosen as genetic markers because virus populations, both natural isolates and laboratory stock virus strains, were often found to be heterogenous with respect to these qualities. Most of these traits (virulence, host range, plaque morphology) are now thought to be under polygenic control (Sugiura, 1975). The studies described below suggest that stability of hemagglutination activity to heat, enzymes or chemicals is a property of the hemagglutinin protein which does not always segregate with serotype. Full understanding of these characteristics will require further elucidation of the molecular structure of the hemagglutinin. The antigenicity of the hemagglutinin or neuraminidase protein reflects the amino acid sequence of the protein and is controlled by a single gene. Thus immunological reactions of the two surface glycoproteins continue to be the method most commonly used to determine the derivation of these proteins from recombinant viruses. Recently, such biochemical techniques as polyacrylamide gel electrophoresis of viral RNA genome segments and the peptide mapping, amino acid analysis,

and sequencing of viral proteins have been used to determine the parental donor of both the internal and the surface proteins of recombinant viruses (see Chapters 3 and 6).

The study of recombinant influenza viruses has increased our understanding of influenza virus and disease, and has played an important role in attempts to control the disease by vaccination. The production of antigenically mixed viruses led to the understanding that hemagglutinin and neuraminidase are distinct antigens on the virus surface. Demonstration of the genetic compatibility between human and animal influenza strains led to proposals that antigenic shifts result from recombination between human and animal strains. Finally, controlled recombination has yielded viruses possessing both the growth characteristics of older, egg-adapted strains and the surface antigens of new natural isolates (Kilbourne and Murphy, 1960; Kilbourne, 1969a), which has not only greatly reduced the time required for production of vaccines against new variants but has also increased the yields of the vaccine viruses.

Chapter 2

Trypsin Sensitivity as a Genetic Marker

Trypsin, a protease from bovine pancreas that selectively catalyzes the hydrolysis of peptide bonds formed by the carboxyl groups of lysyl and arginyl residues (Bergmann et al., 1923), has been employed in several studies aimed at classifying influenza viruses and determining the structure of the virus particles. In early studies, viruses were treated with pancreatic extracts and the variable results were attributed to differences in the concentration of fatty acid and lecithin in the crude extracts and not to the enzymes themselves (Pirie, 1935). More reproducible results were obtained once crystalline preparations of the enzyme became available (Northrop and Kunitz, 1932). Merrill (1936) observed that the mouse infectivity of a strain of swine influenza was unaffected by incubation with 0.5% crystalline trypsin for 38 days at 4°C. Stone (1949) later found that incubation of B/Lee/42 virus with 0.25% trypsin for 4 hr at 37°C reduced the egg infectivity of the virus tenfold but had no effect on the hemagglutination activity of the virus. In contrast, Hoyle (1952) found that prolonged incubation with trypsin reduced the hemagglutination activity of A/PR/8/34 virus (H0N1).

Relation of trypsin sensitivity to serotype. Cleeland and Sugg (1960) found that the S1 strain of A/Se/53 (H1N1) lost all of its hemagglutination activity and nearly all of its egg infectivity on incubation with 0.25% crystalline trypsin for only 1 hr at 35°C. Repeated passage of the sensitive S1 virus at either high or low dilution caused no significant variation in its susceptibility to trypsin. A

second strain of A/Se/53, however, which was begun in 1956 with lyophilized allantoic fluid from the third egg passage of S1, was resistant to trypsin under the same conditions. The virus strains A/PR/8/34 (H0N1), A/CAM/46 (H1N1), and A/Japan 305/57 (H2N2), and three type-B influenza viruses were also resistant to trypsin. These results were substantiated by Gresser and Enders (1961).

In a more detailed study, Sugg and Cleeland (1962) compared the trypsin susceptibility of 36 strains of influenza viruses. When the viruses were assayed in allantoic fluid (4 hr, 37°C), trypsin sensitivity generally corresponded with the viral subtype. With the exception of A/PR/8/34 (H0N1), H0 and H1 viruses were trypsin-sensitive but H2 viruses were trypsin-resistant. Cleeland and Sugg (1960) suggested that trypsin sensitivity of the viral hemagglutination activity might serve as an easily recognized and stable marker for genetic studies. The feasibility of using trypsin sensitivity as a marker was first demonstrated by Kilbourne (1963). Recombination of inactivated, trypsin-sensitive NWS (H1N1) with infective trypsin-resistant RI/5^{+} (H2N2) produced two H1 viruses, one trypsin-sensitive and one trypsin-resistant. The trypsin resistance of the latter H1 virus was assumed to have been derived from the resistant parent, RI/5^{+}. In subsequent studies of recombinant viruses, the sensitivity of the viral hemagglutination activity segregated with the hemagglutinin subtype, H0 viruses being sensitive and H2 viruses resistant (Laver and Kilbourne, 1966; Kilbourne et al., 1967). In contrast, Hoyle and Almeida (1971) did not find a relationship between viral serotype and trypsin sensitivity of the viral hemagglutination activity. After treatment with trypsin for 4 hr at 37°C, the hemagglutination activity of the Burch (H1N1) and Swine viruses was

completely lost, the activities of A/PR/8/34 (H0N1), Bratislava (H1N1), Taiwan (H2N2) and England/67 (H2N2) were reduced, and both DSP (H0N1) virus and the Lee strain of type-B influenza showed a slight increase in hemagglutination activity.

Structural effects of trypsin treatment. The structure of the virus particle has also been explored using trypsin treatment. Valentine and Isaacs (1957a,b) disrupted influenza virus with the protease directly on microscope grids and observed that the viral spheres contain a ring of ribonucleoprotein enclosed within a protein coat. Mayron and co-workers (1961) reported that treatment of A/PR/8/34 (H0N1) and H2N2 strains with trypsin for 16 hr at 35°C released neuraminidase protein from virus particles in an enzymatically active form that lacked any hemagglutination activity. This result was one of the earliest demonstrations that hemagglutinin and neuraminidase are two different entities on the viral surface. More recent studies with trypsin (Schulze, 1970) revealed that treatment of A/WSN/33 (H0N1) virus destroyed neuraminidase but not hemagglutination activity and removed the uncleaved HA and NA proteins from the protein patterns seen by polyacrylamide gel electrophoresis. Studies with trypsin and with other proteases (Schulze, 1970; Compans et al., 1970; Nermut, 1972) demonstrated that the HA and NA proteins are exterior to the protease-resistant lipid layer on the virus particles.

Isolation of the recombinant viruses X-12 and X-29. A series of antigenically hybrid viruses was isolated from recombination between A/NWS/33 (H0N1) and A/RI/5/57 (H2N2) during studies that showed hemagglutinin and neuraminidase to be distinct and independent viral surface antigens. Two variants of RI/5 virus, $RI/5^{+}$ and $RI/5^{-}$, were used as parental viruses during the recombination experiments. Relative to

RI/5$^-$ virus, RI/5$^+$ virus was inhibited by a lower concentration of homotypic antibodies (Choppin and Tamm, 1959, 1960a), was more sensitive to neutralization by a serum glycoprotein (Choppin and Tamm, 1959, 1960a,b) and eluted more slowly or not at all from certain types of erythrocytes (Choppin and Tamm, 1959, 1960a). The increased stability of RI/5$^+$ virus cell--receptor complex was reflected in the viral growth characteristics of the two viruses. RI/5$^+$ multiplied in monkey kidney cells more rapidly and to a higher titer than RI/5$^-$ (Choppin, 1963). When these two RI/5 variants were compared by examining several genetic markers, including antigenicity, complement-fixing antigen (nucleoprotein), inhibition by serum glycoproteins, neuraminidase activity, morphology, trypsin sensitivity, plaque formation on Clone 1-5C-4 cells, and neurotropicity, they were identical by every marker except morphology and serum inhibitor sensitivity (Laver and Kilbourne, 1966). The molecular basis for the different inhibitor sensitivity and morphology of the viruses has not been elucidated.

The recombinant viruses were produced by double infection of embryonated chicken eggs with heat-inactivated NWS and infective RI/5 virus. Recombinant progeny were selected from the parental strains by inoculation of Clone 1-5C-4 cells. All plaque-forming viruses isolated were assumed to be recombinants, because the plaque-forming NWS parent was noninfective and the infective RI/5 parent does not produce plaques on these cells. Alternatively, when the RI/5$^+$ variant was used, the infective H2N2 parent was neutralized with glycoprotein inhibitor from horse serum. The resulting viruses were shown to be recombinants by examination of a series of genetic markers, including surface antigens, viral morphology, sensitivity to horse serum glycoprotein inhibitor,

sensitivity of their hemagglutination activity to inactivation by exposure to trypsin (40°C, 90 min), ability to form plaques on Clone 1-5C-4 cells, plaque morphology and quantity of neuraminidase activity. The derivation and characterization of a number of these recombinants has been previously described (Kilbourne et al., 1967).

Through this type of recombination experiment, the recombinant virus X-12 was isolated following double infection with NWS and RI/5$^-$ (Fig. 2.1). Analysis of the recombinant (see below) revealed that its hemagglutinin was derived from NWS (H0) and its neuraminidase from RI/5$^-$ (N2) and that it formed plaques on Clone 1-5C-4 cells.

In a later experiment, an attempt was made to segregate serotype from the genetic marker of sensitivity to serum inhibitor. The inhibitor-resistant X-12 virus was recombined with inhibitor-sensitive X-27 virus, a reciprocal recombinant that had derived its hemagglutinin gene from RI/5$^+$ (H2) and its neuraminidase gene from NWS (N1). Recombination involved double infection of eggs with the two infective parental viruses and subsequent passage of the mixed yield in eggs in the presence of NWS antiserum to inhibit viruses possessing the H0 hemagglutinin and N1 neuraminidase of NWS. The resulting virus was plaqued on Clone 1-5C-4 cells with NWS antiserum in the agar overlay. A small plaque, which was 0.5 mm in diameter as opposed to the 3 mm mean diameter of an NWS plaque, was picked and passaged at low dilution in eggs. Immunological characterization revealed that the resulting virus possessed an N2 neuraminidase but was inhibited in hemagglutination-inhibition (HI) assays by antisera to both NWS (H0N1) and X-9 (H2N1) viruses. Since this virus population may have been heterogeneous, the virus was again plaqued on Clone 1-5C-4 cells in the presence of NWS

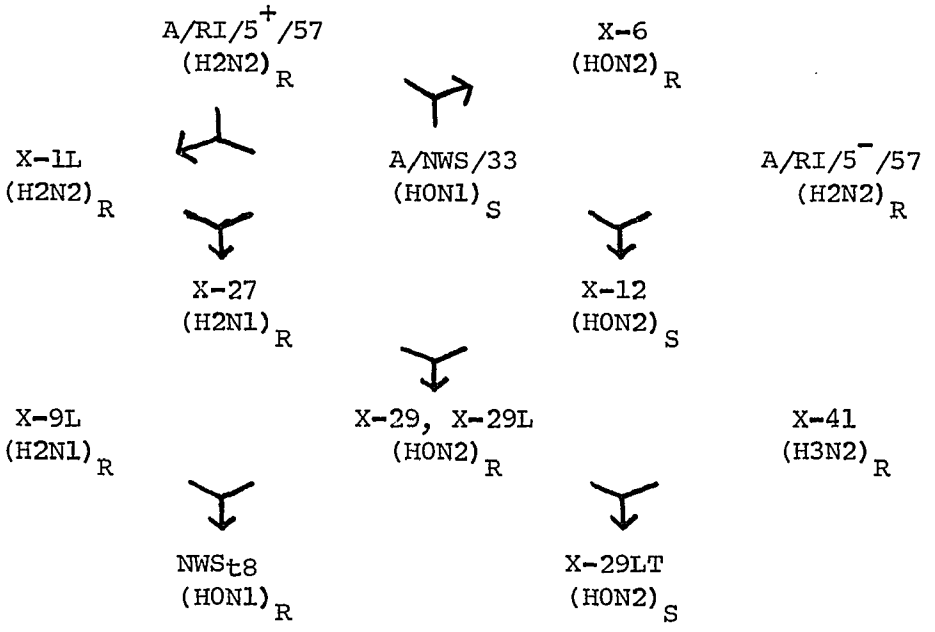

Fig. 2.1. Genetic derivation of five influenza recombinants of H0 serotype. The subscript denotes that the hemagglutination activity of the virus is resistant (R) or sensitive (S) to trypsin treatment for 90 min at 35°C (see Chapter 4).

antiserum. As before, a small plaque was picked and passed in eggs first at low dilution and subsequently at high dilution. The recovered virus was designated X-29. Recombinant virus X-29 was chosen for further study because, despite additional plaque-to-plaque passages on Clone 1-5C-4 cells and high dilution passages in eggs, it retained its sensitivity in HI assays to both NWS and X-9 antisera. This heightened sensitivity in HI assays to H2 antiserum is illustrated in Table 2.1, where X-29 is compared to two other H0 viruses, X-12 (H0N2) and NWS (H0N1). Recombinant X-29 was also found to differ from other H0 viruses in plaque-inhibition assays (Table 2.2). The virus proved to be less sensitive to inhibition of plaque formation by antisera directed against its H0 hemagglutinin. Thus, more anti-H0 antiserum was required to inhibit plaque formation by X-29 than was needed to inhibit plaque formation by three other H0 viruses, NWS, X-12 and X-7. Finally, X-29 was found to be more sensitive in plaque-size-reduction assays to antiserum directed against its N2 neuraminidase (Table 2.3). Less anti-N2 antibody was required to reduce by half the size of the plaques formed by X-29 than plaques formed by X-12 or X-7.

In an attempt to determine the cause of the unusual immunological reactions of X-29, the two recombinant H0N2 viruses, X-12 and X-29, were assayed for the genetic marker of trypsin sensitivity. Previous analysis had shown that NWS (H0N1) and RI/5 (H2N2), the original parents of both X-12 and X-27 and the grandparents of X-29, differed in the susceptibility of their hemagglutination activity to inactivation by trypsin (Laver and Kilbourne, 1966). The H0 virus NWS was found to lose its hemagglutination activity on exposure to trypsin for 90 min at 40°C, while the N2 viruses $RI/5^{+}$ and $RI/5^{-}$ retained their hemagglutination activity. Trypsin

TABLE 2.1

Antigenic Characterization of the Recombinant Viruses X-12 and X-29 as H0 Serotype by the Hemagglutination-Inhibition Assay[a]

Antiserum (serotype)	Virus (serotype)			
	NWS (H0N1)	X-12 (H0N2)	X-29 (H0N2)	X-27 (H2N1)
NWS (H0N1)	2048	2048	2048	
X-12 (H0N2)	8192	4096	8192	
X-9 (H2N1)	32	16	256	
X-27 (H2N1)	16	8	64	1024
RI/5^{+} (H2N2)		4	8	256
X-1L (H2N2)		4	16	1024

[a]Hemagglutination inhibition (HI) titers are expressed as the reciprocal of the highest antiserum dilution that completely inhibited hemagglutination.

TABLE 2.2

Sensitivity of the Recombinant Virus X-29 to H0 Antisera in the Plaque-Inhibition Assay[a]

Antiserum (serotype)	Virus (serotype)			
	NWS (H0N1)	X-7 (H0N2)	X-12 (H0N2)	X-29 (H0N2)
NWS (H0N1)	12800		25600	1600
X-7 (H0N2)		6400		< 800
X-29 (H0N2)	3200	6400		< 800

[a]Plaque-inhibition (PI) titers are expressed as the highest antiserum dilution that completely inhibited plaque formation (E. D. Kilbourne, unpublished results). PI is determined by the HA serotype (Jahiel and Kilbourne, 1966).

TABLE 2.3

Sensitivity to the Recombinant Virus X-29

to N2 Antisera in the Plaque-Size-Reduction Assay[a]

Antiserum serotype	Virus (serotype)		
	X-7 (HON2)	X-12 (HON2)	X-29 (HON2)
HeqlN2	1600	1600	6400
H3N2	6400	6400	25600

[a]Plaque-size-reduction (PSR) titers are expressed as the highest antiserum dilution that decreased by half the median plaque radius (E. D. Kilbourne, unpublished results). PSR is determined by the NA serotype (Jahiel and Kilbourne, 1966).

treatment of X-12 and X-29 revealed that the two recombinant viruses also differed in their sensitivity to the protease. X-12 resembled NWS, but the X-29 virus, which displayed heightened sensitivity to both H2 and N2 antibodies, resembled its H2N2 grandparent RI/5^{+} by retaining its hemagglutination activity on trypsin treatment (Table 2.4).

Trypsin treatment of additional recombinant viruses derived from NWS and RI/5^{+} or RI/5^{-} revealed that the majority of the viruses inheriting the NWS H0 hemagglutinin also acquired the trypsin-sensitivity genetic marker of that parent; likewise, the recombinants inheriting the H2 hemagglutinin from RI/5 were trypsin-resistant. X-29 virus, however, was not the only exception to this seemingly general observation. The recombinant virus X-6, which acquired its H0 hemagglutinin from NWS and its H2 neuraminidase from RI/5^{+} (Table 2.1), was also found to possess trypsin-resistant hemagglutination activity (Table 2.4). The demonstration that the H0N1 virus A/WSN/33 was trypsin-resistant (Table 2.4) further indicated that the trypsin-sensitivity marker did not necessarily segregate with hemagglutinin serotype.

Kilbourne has suggested that these observations could be explained if the two subunits of X-29 were derived from different parental viruses (Kilbourne et al., 1972). For example, the HA1 subunit might be derived from NWS and bear its antigenic determinants, while the HA2 subunit might be derived from RI/5^{-} and stabilize or shield the HA1--HA2 complex from tryptic hydrolysis. One mechanism suggested for the formation of such a hybrid hemagglutinin protein is low-frequency intragenic recombination between homologous RNA segments during double infection (Simpson and Hirst, 1968). This genetic mechanism would be distinct from the well-established high-frequency reassortment of entire RNA segments

TABLE 2.4

Hemagglutination Activity of Four H0 Viruses After Trypsin Treatment for 90 Minutes[a]

Virus (serotype)	Trypsin treated	Control
X-12 (H0N2)	< 4	512
X-29L (H0N2)	256	512
X-6 (H0N2)	128	64
WSN (H0N1)	16	16

[a]Virus was assayed in allantoic fluid for hemagglutination activity at room temperature after treatment with trypsin for 90 min at 35°C by method B (Chapter 4). Control virus in allantoic fluid was incubated for 90 min at 35°C with NTC buffer in place of trypsin. Hemagglutination activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 60 min at room temperature.

(Webster et al., 1973b). The unusual serological reactions and trypsin sensitivity observed for X-29 suggested that this recombinant might provide the first example of intragenic recombination for influenza virus. The studies described in this thesis were undertaken to determine the basis of the different serological reactions and trypsin sensitivity of the HON2 viruses X-12 and X-29. Comparison of these recombinants was also expected to define further the structure of the influenza hemagglutinin protein.

Chapter 3

Polyacrylamide Gel Electrophoresis of Viral Proteins

Polyacrylamide gel electrophoresis has proven to be a powerful technique for studying the structure of animal viruses. This procedure has been used to define the structural proteins of influenza virus, the relation of these proteins to one another and to the entire virus, and the time and place of their synthesis in infected cells (Kilbourne, 1975). In this study, the recombinant viruses X-12 and X-29L were compared to one another and to the parental viruses NWS and RI/5$^-$ and the recombinant viruses X-29LT and NWS$_{t8}$ through electrophoresis of their structural proteins. Both the molecular weights and the ratios of the various viral proteins were investigated. The resulting observations were useful in determining the parental donor of the surface proteins of the recombinant viruses, and in understanding the relationship of certain aspects of the virus structure to the trypsin susceptibility of the viral hemagglutinin protein.

MATERIALS AND METHODS

Virus. Virus strains NWS and RI/5$^-$ and the recombinants X-12, X-29L, X-29LT and NWS$_{t8}$ were cultivated in the allantoic sac of embryonated chicken eggs as described in Chapter 4. Radiolabeled X-12, X-29L and WSN were grown in chorioallantoic membrane (CAM) or chicken embryo fibroblast (CEF) cells as described in Chapter 4.

Polyacrylamide gel electrophoresis. Unless otherwise stated, samples for polyacrylamide gel electrophoresis were treated for 1 min at 100°C in 1% sodium dodecyl sulfate (SDS)--1% 2-mercaptoethanol. The

resulting dissociated and reduced proteins were subjected to electrophoresis in a discontinuous Tris--glycine--SDS system (Maizel, 1971). When double radiolabeled viruses were co-electrophoresed on the same gel, the two samples were mixed prior to dissociation and reduction. The 13% running gel was prepared by polymerizing a solution (A) of 13 wt% acrylamide, 0.35 wt% N,N'-methylene-bis-acrylamide (BIS), 0.1 wt% SDS, 0.052 wt% ammonium persulfate, and 0.05 vol% N,N,N',N'-tetramethylethylenediamine (TEMED) in 0.375 M Tris·HCl (pH 8.9) in 12 cm by 0.5 cm cylindrical glass tubes or between 12 cm by 16 cm glass plates. The 13% cylindrical gels with 40% plugs (13%/40%) were prepared by layering 7.5 cm of solution A on top of 2.5 cm of a modified solution A containing 40% acrylamide and 1.05% BIS and allowing both layers to polymerize at the same time. The 4.3% stacking gel was prepared by polymerizing a modified solution A containing 4.3% acrylamide and 0.11% BIS in 0.06 M Tris--phosphate (pH 6.7) on top of the 13% running gel. For each of the above gels, the ratio of acrylamide to BIS was 37:5:1. Continuous 6% to 20% gradient slab gels were prepared with a Canalco gradient maker.

The pH 8.5 electrophoresis buffer was a solution 0.05 M in Tris and 0.38 M in glycine, containing 0.1 wt% SDS. Disc gels were electrophoresed at 40 V for 15 hr; slab gels were run for 3 hr at 150 V. Protein bands were stained with 0.275 wt% Coomassie Brilliant Blue R 250 in a solution of 10% acetic acid, 40% water and 50% methanol for 30 min at 56°C. Excess stain was removed by incubating the gels in water containing 7% acetic acid and 5% methanol at 56°C. Disc gels were stored in aqueous 7% acetic acid at room temperature. The positions of the protein bands were recorded by densitometric tracing using a Model J

Canalco Micro Densitometer. Stained slab gels were photographed, shrunk in methanol for 5 min with constant agitation, and dried onto filter paper under vacuum at 100°C. When specified, samples of denatured and reduced protein were alkylated by adding one volume of 0.25 M 2-iodoacetamide to 5 volumes of sample and maintaining for 30 min at room temperature before electrophoresis.

For determination of radioactivity, gels were frozen on dry ice and cut into 1 mm slices with a Mickle gel slicer (Brinkmann Instruments). Slices were dissolved by incubation with 0.2 ml H_2O_2 for 4 hr at 70°C. After addition of Aquasol (New England Nuclear) or ScintiVerse (Fisher), radioactivity was measured in a Packard Tri-Carb Model 3320 scintillation counter. Counts per minute (cpm) were converted into disintegrations per minute (dpm) and corrected for spillover of ^{14}C counts into the ^{3}H channel using a computer program written by Dr. Roger Hand, The Rockefeller University, New York, New York.

Alternatively, radioactivity was detected by autoradiography. Dried gels containing ^{14}C-labeled proteins were exposed to Kodak No-Screen Medical X-Ray film at room temperature. Tritium-labeled proteins were visualized by the procedure of Bonner and Laskey (1974). Gels were dehydrated in dimethyl sulfoxide, soaked in 20% (w/w) 2,5-diphenyloxazole (PPO) in dimethylsulfoxide (22.2% w/v) for 3 hr, dried and exposed to Kodak RP Royal X-Omat Medical X-Ray film at -70°C.

RESULTS

Viral polypeptide patterns. The typical protein pattern for reduced influenza virus polypeptides resolved by our gel system is illustrated in Fig. 3.1. Eight structural polypeptides of influenza virus grown in ovo

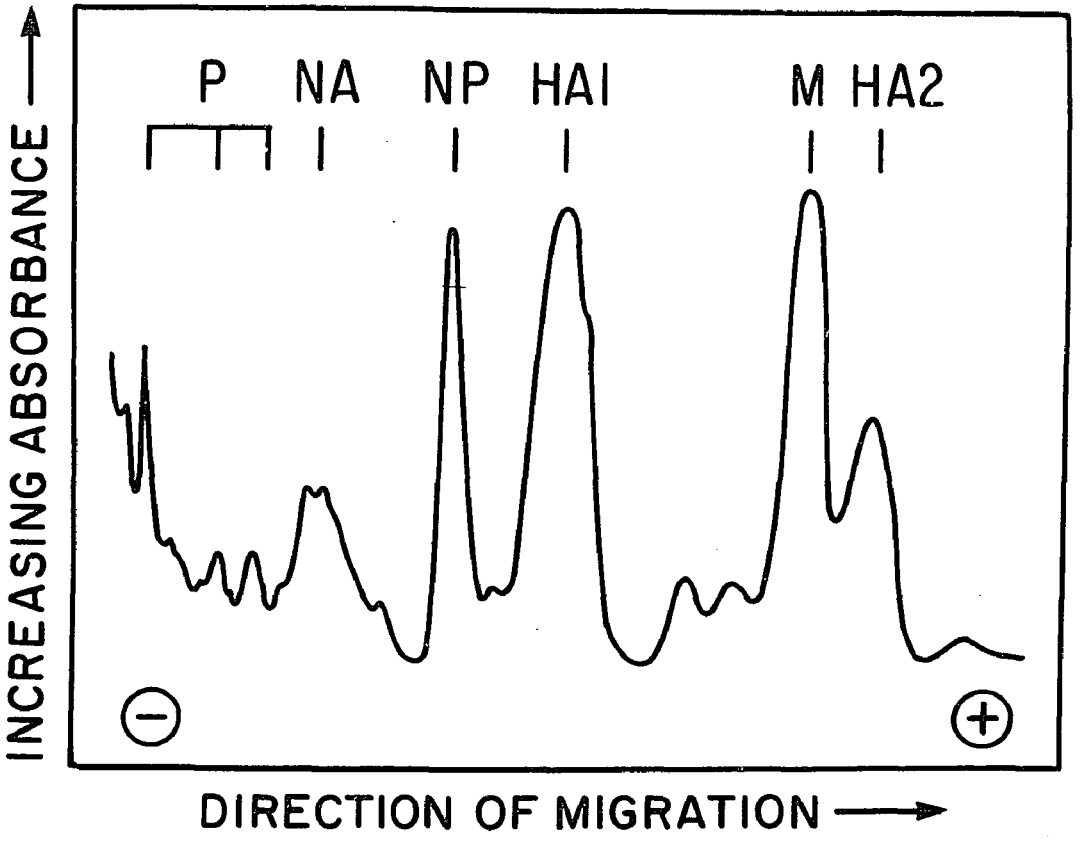

Fig. 3.1. Typical protein pattern for influenza virus grown in ovo. RI/5⁻ virus was dissociated in the presence of SDS and reduced with 2-mercaptoethanol, and the structural proteins were resolved by electrophoresis on a 13% polyacrylamide disc gel. The densitometric tracing of the Coomassie blue-stained protein bands is shown.

can be readily detected. There is now general agreement as to the identity of these proteins (Compans et al., 1970; Haslam et al., 1970; Schulze, 1970; Lazarowitz et al., 1971, 1973a; Skehel and Schild, 1971; Klenk et al., 1972; Palese and Schulman, 1974; Ritchey et al., 1977). Since the three P proteins have high molecular weights and are present in virus particles in small quantities (Ritchey et al., 1977), they are often difficult to detect in purified virus preparations. When influenza virus is cultivated in certain cell types (e.g. MDBK, MDCK, CEF) the HA protein is not totally cleaved into the disulfide-linked subunits HA1 and HA2 (Lazarowitz et al., 1973a; Klenk et al., 1975) and migrates above the NP protein.

Due to the anomalous binding of SDS by glycoproteins (Bretscher, 1971; Segrest et al., 1971) and to the variations seen in glycosylation due to host cell type (Compans et al., 1970; Haslam et al., 1970; Schulze, 1970), the proteins NA, HA1 and HA2 migrate differently in various gel systems (e.g. Compans et al., 1970; Palese and Schulman, 1974). Therefore, the identity of the bands corresponding to these proteins should be established for each system. The appearance of glycoproteins is characteristic on stained SDS--polyacrylamide gels (Fig. 3.1). The attachment of varying amounts of carbohydrate produces a heterogeneous family of proteins, which migrate together as a broad, diffuse band. Proteins containing carbohydrate can also be identified by differentially radiolabeling carbohydrate and amino acids during virus cultivation (Compans et al., 1970; Schulze, 1970; Palese and Schulman, 1974). Thus, after electrophoretic separation of the proteins, the polyacrylamide gels were sliced and counted for both radiolabels. The protein patterns of Fig. 3.2 and 3.3 revealed that labeled carbohydrate

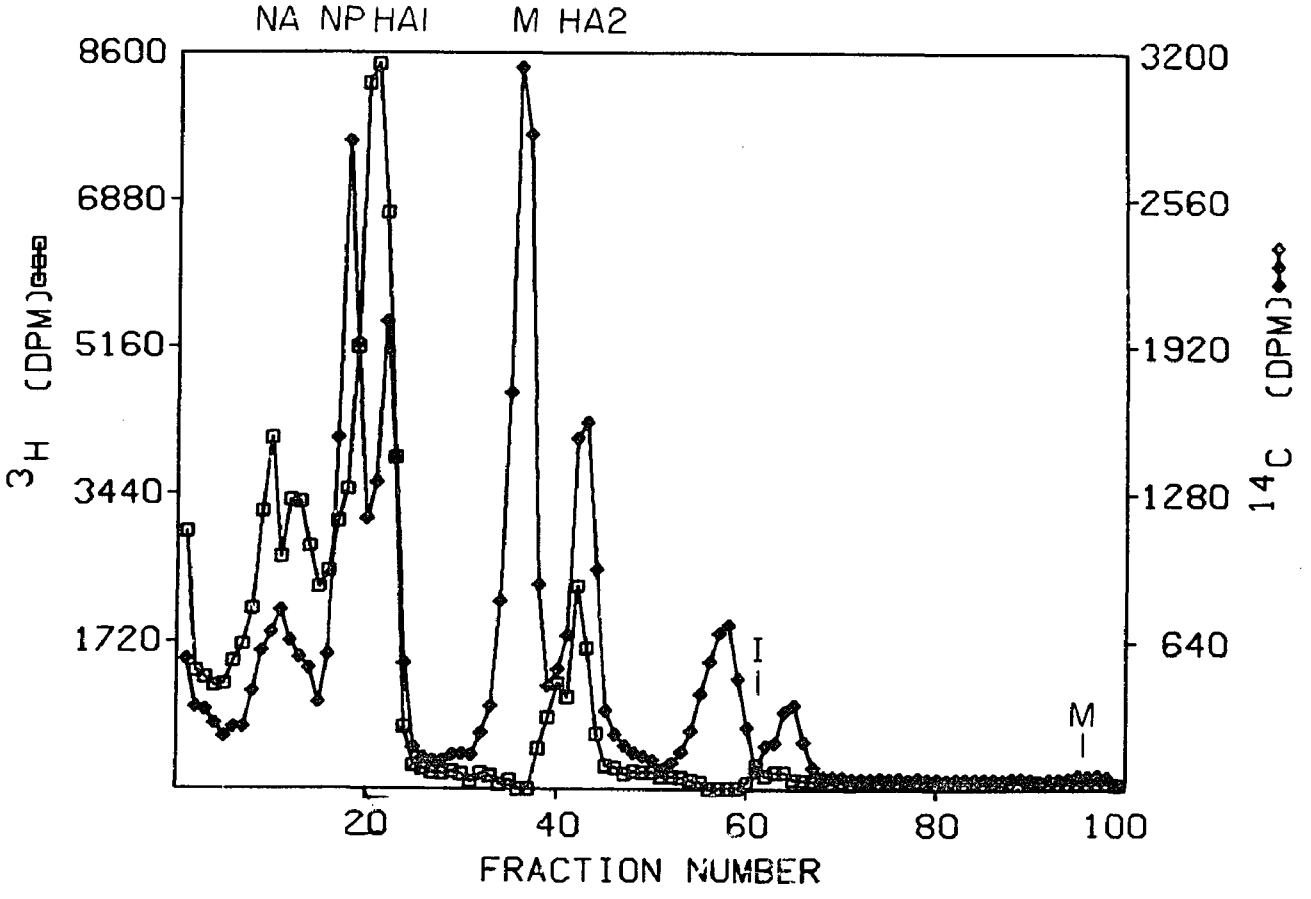

Fig. 3.2. Glycoproteins of X-12 virus grown in CAM cells. Virus labeled with [3H]glucosamine and virus labeled with a mixture of [^{14}C]amino acids were co-electrophoresed on a 13% acrylamide gel terminating with a 40% acrylamide plug. The acrylamide interface (I) and the position of the marker dye (M), bromphenol blue, are indicated. The direction of migration is from left to right.

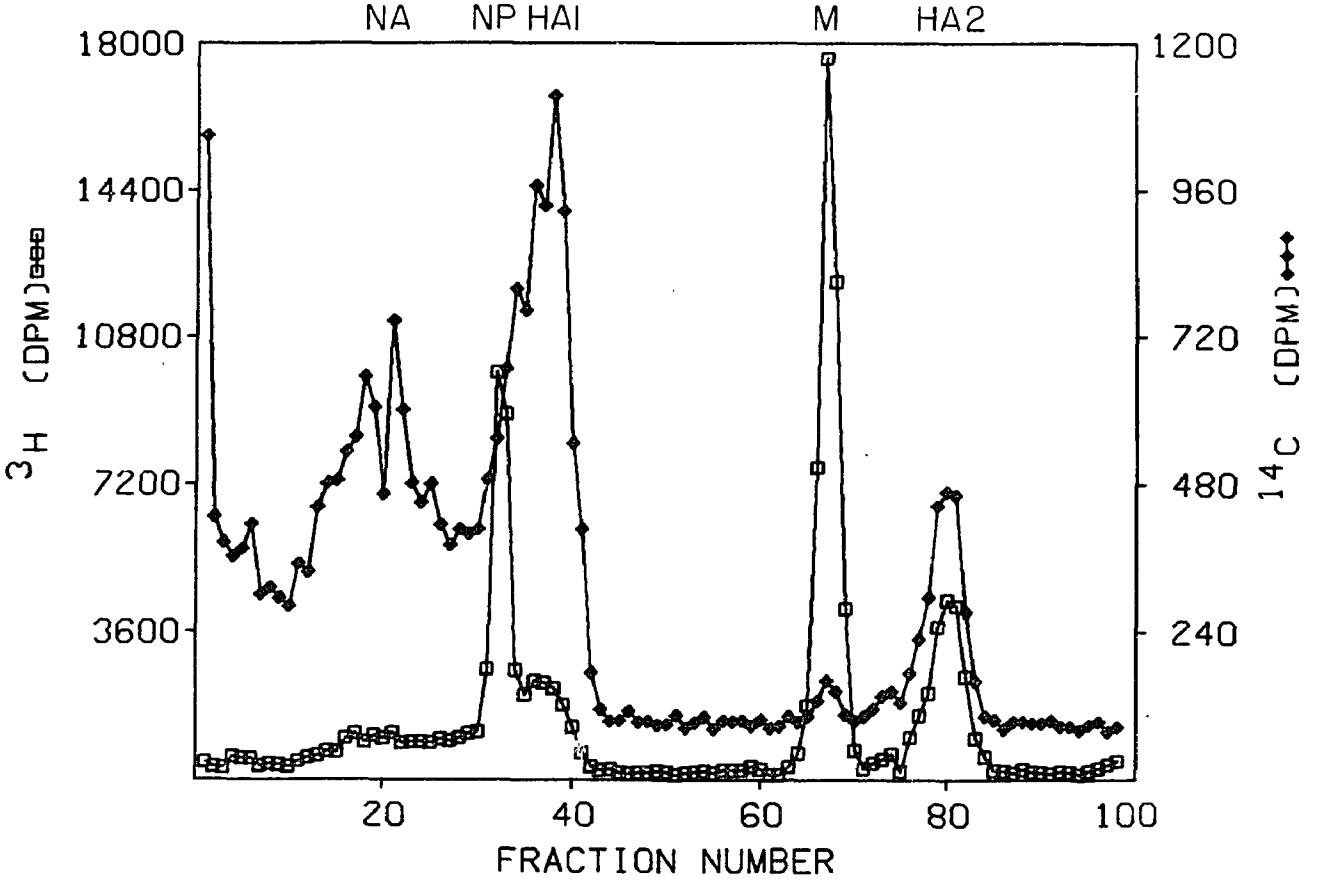

Fig. 3.3. Glycoproteins of X-29L virus grown in CAM cells. Virus labeled with [^{14}C] glucosamine and virus labeled with a mixture of [^{3}H] amino acids were co-electrophoresed on a 13% acrylamide gel. The direction of migration is from left to right.

was incorporated into three viral proteins: (1) the neuraminidase protein, which migrated as a double peak midway between the gel origin and the non-glycosylated NP polypeptide, (2) the HA1 subunit, which ran after NP, and (3) the HA2 subunit, the smallest structural polypeptide of the virus.

The NA glycoprotein can be distinguished from uncleaved HA by comparing the migration of the viral proteins in polyacrylamide gels in the presence or absence of 2-mercaptoethanol. In the absence of this reducing agent, the hemagglutinin subunits remain disulfide bonded and co-migrate with uncleaved HA (Laver, 1971; Lazarowitz et al., 1971; 1973a) at a position above NP. Upon reduction, the cleaved hemagglutinin separates into the subunits HA1 and HA2, which migrate after NP and M, respectively. Reduction shifts the neuraminidase protein from a very high molecular weight band near the gel origin to a single band or a close pair of bands migrating above NP (Webster, 1970a, b; Kendal and Eckert, 1972; Bucher and Kilbourne, 1972; Skehel and Schild, 1971; Lazdins et al., 1972). Fig. 3.4B shows the electrophoretic migration patterns of oxidized X-12 and X-29L proteins. During electrophoresis 2-mercaptoethanol in the sample to the left of the X-12 lane diffused into the X-12 sample and partially reduced the hemagglutinin protein. Thus, in the left third of the X-12 lane the HA band disappeared and two new bands appeared in the positions of the HA1 and HA2 subunits. The neuraminidase and the P protein bands were very faint in these lanes.

Comparison of viral protein patterns. The electrophoretic migration patterns of the structural proteins of the parental viruses NWS and RI/5$^-$ and their recombinant viruses X-12 and X-29L were compared on a 13%

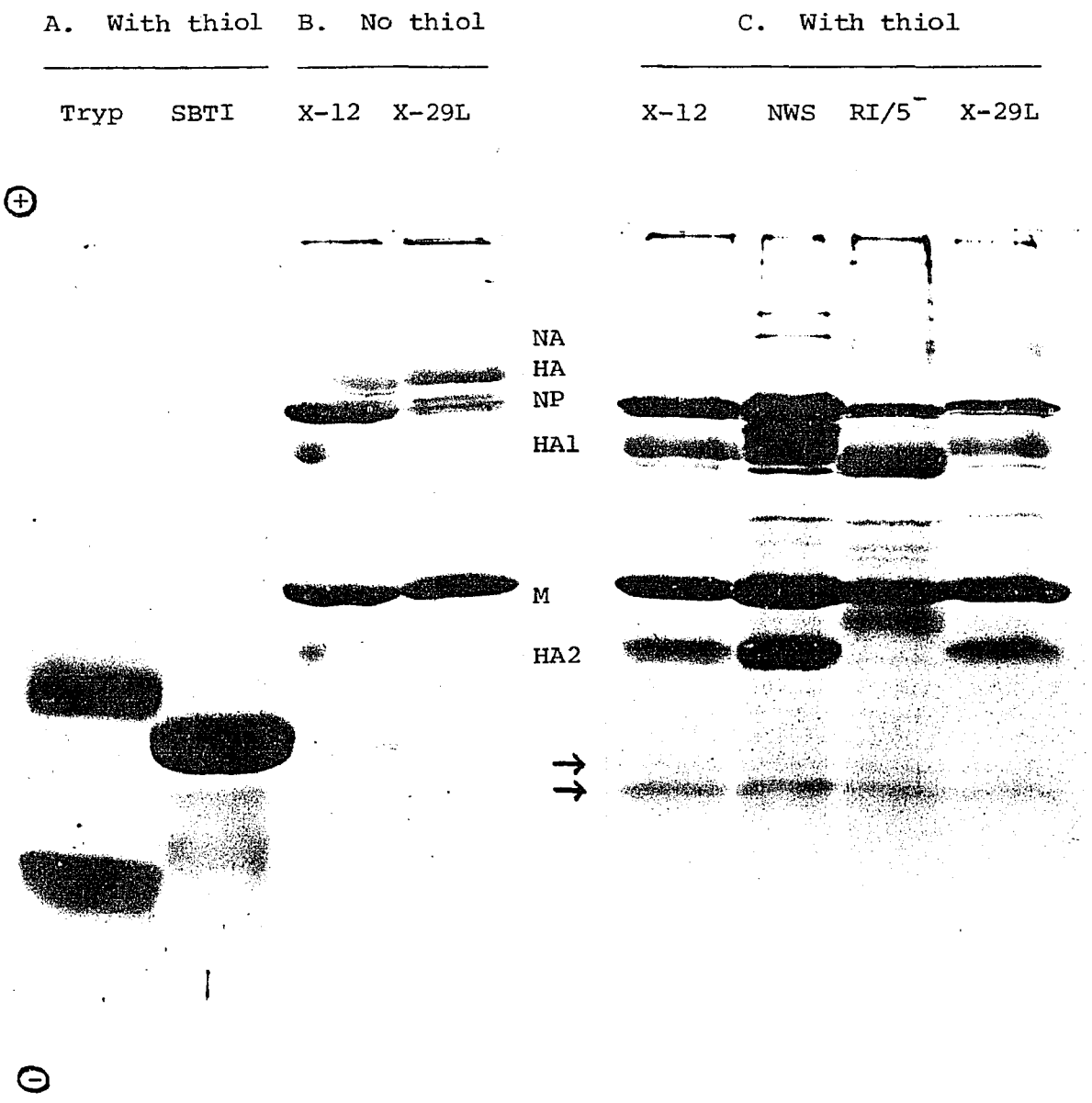

Fig. 3.4. Electrophoretic patterns of stained proteins from X-12, NWS, RI/5⁻ and X-29L. Proteins were resolved by electrophoresis on a 13% polyacrylamide slab gel containing SDS and stained with Coomassie blue. (A) Trypsin and soybean trypsin inhibitor were dissociated and reduced in the presence of SDS and 2-mercaptoethanol. (B) X-12 and X-29L were dissociated by SDS in the absence of 2-mercaptoethanol. Diffusion of reducing agent from lane 2 into lane 3 resulted in partial reduction of the X-12 hemagglutinin protein. (C) X-12, NWS, RI/5⁻ and X-29L were each dissociated and reduced in the presence of SDS and 2-mercaptoethanol. Two small polypeptides are indicated by solid arrows.

polyacrylamide slab gel (Fig. 3.4C). No differences were detected in the parallel migration of the NP, HA1, M and HA2 proteins of the three H0 viruses. The two hemagglutinin polypeptides from the H2 virus RI/5$^-$, however, had different electrophoretic mobilities. The HA1 polypeptide from the H2 parent ran slightly faster than its H0 counterpart, while the HA2 polypeptide of the H2 virus migrated considerably slower than the HA2 proteins of the three H0 viruses. Comparison of protein patterns for the four viruses also revealed that the NA doublet band was prominent in both the X-29L and RI/5$^-$ patterns but was very faint in the X-12 and NWS lanes, even though the density of the staining of the other protein bands of these latter viruses indicated that the total viral protein in the X-12 and NWS lanes was as great or greater than that in the RI/5$^-$ and X-29L lanes. Thus X-29L and RI/5$^-$ evidently possessed more neuraminidase protein than X-12 or NWS.

The altered migration of the RI/5$^-$ hemagglutinin proteins and the presence of a more prominent NA band in X-29L and RI/5$^-$ protein patterns was also seen in densitometric tracings of stained disc gels (Fig. 3.5 and 3.6). Comparison of gels run in the presence and absence of 2-mercaptoethanol clearly distinguished the NA doublet from the uncleaved hemagglutinin protein. Similar protein patterns for the recombinant viruses X-29LT and NWS_{t8} (Fig. 3.7 and 3.8, respectively) revealed that the X-29LT virions contained more neuraminidase protein than RI/5$^-$ or X-29L, and that the NWS_{t8} virions contained approximately the same amount of NA protein as NWS and X-12.

The electrophoretic migration of the high molecular weight proteins from six viruses was also studied. Comparison of the slow densitometric tracings shown in Fig. 3.9 revealed that the three major proteins in the high molecular weight region appeared in one pattern for viruses of N1

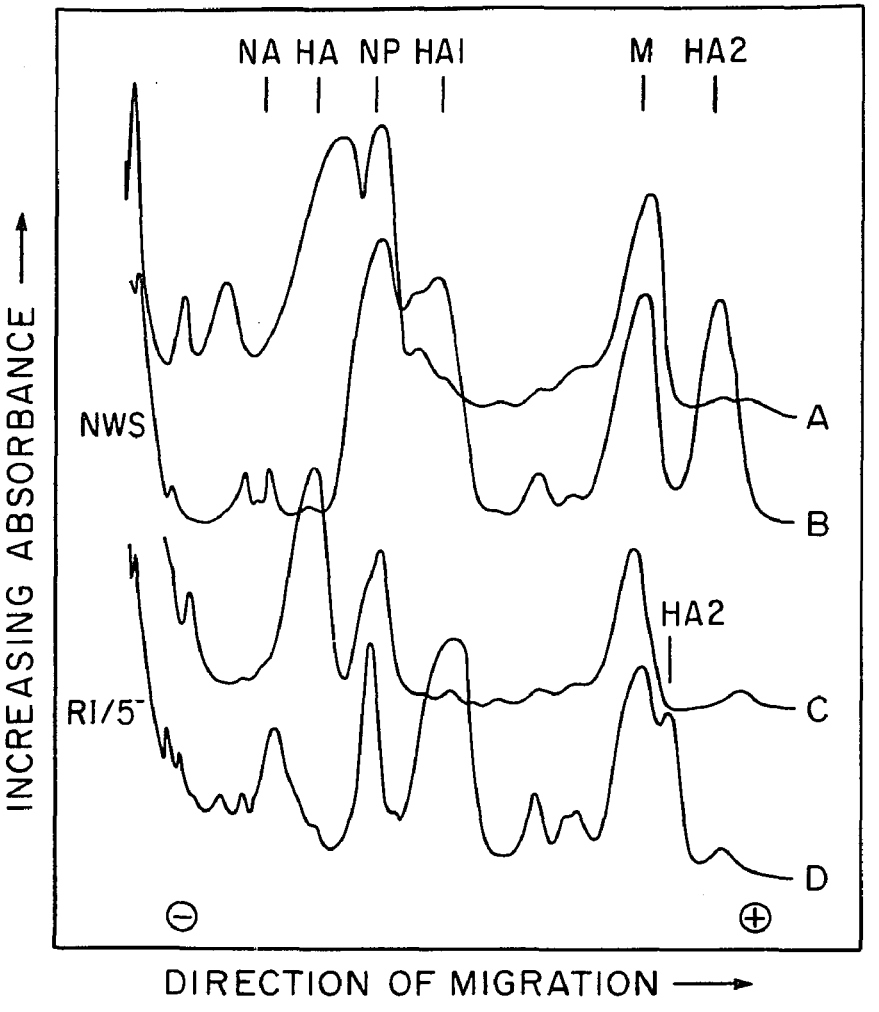

Fig. 3.5. Comparison of the protein patterns of parental viruses NWS and RI/5⁻. Egg-grown virus was dissociated at 100°C in the absence (A, C) or presence (B, D) of 2-mercaptoethanol and the polypeptides were separated by electrophoresis on 13% polyacrylamide--SDS disc gels. Densitometric tracings of the Coomassie blue-stained protein bands are shown.

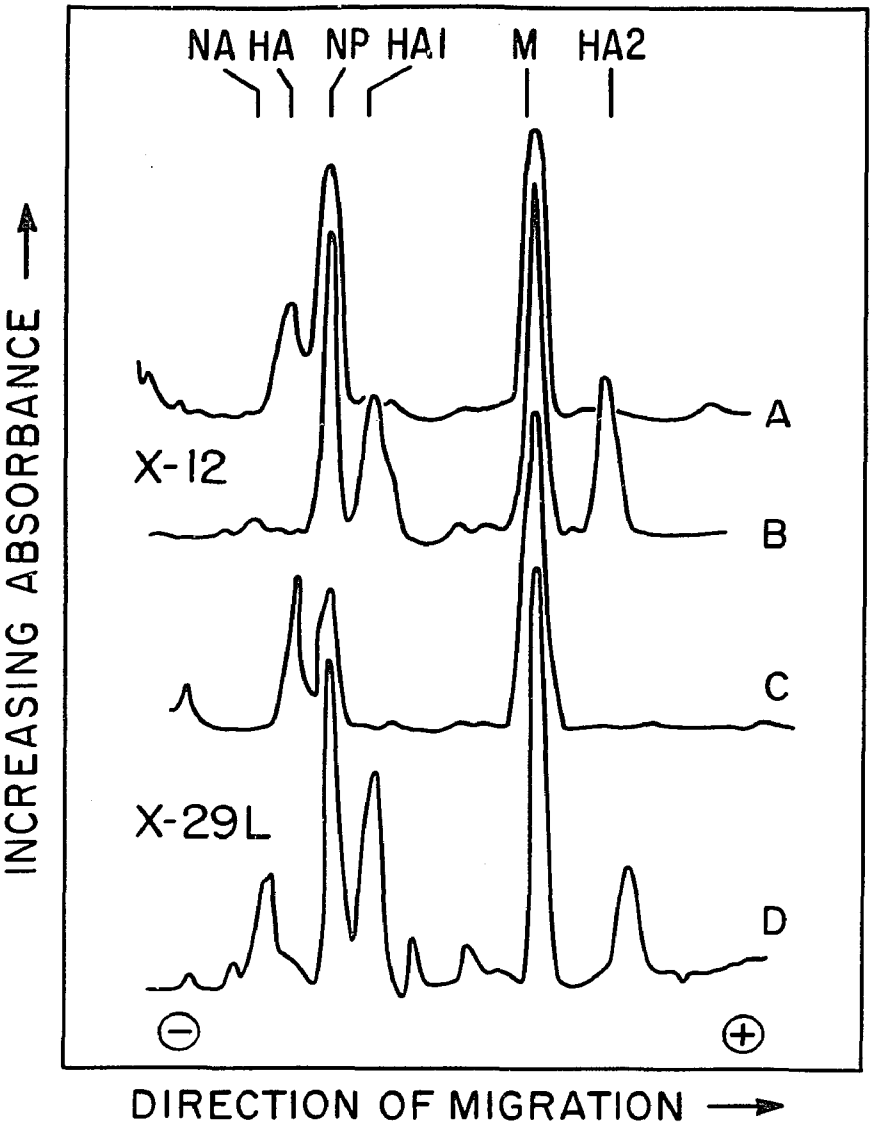

Fig. 3.6. Comparison of the protein patterns of recombinant viruses X-12 and X-29L. Egg-grown virus was dissociated at 100°C in the absence (A, C) or presence (B, D) of 2-mercaptoethanol and the polypeptides were separated by electrophoresis on 13% polyacrylamide--SDS disc gels. Densitometric tracings of Coomassie blue-stained protein bands are shown.

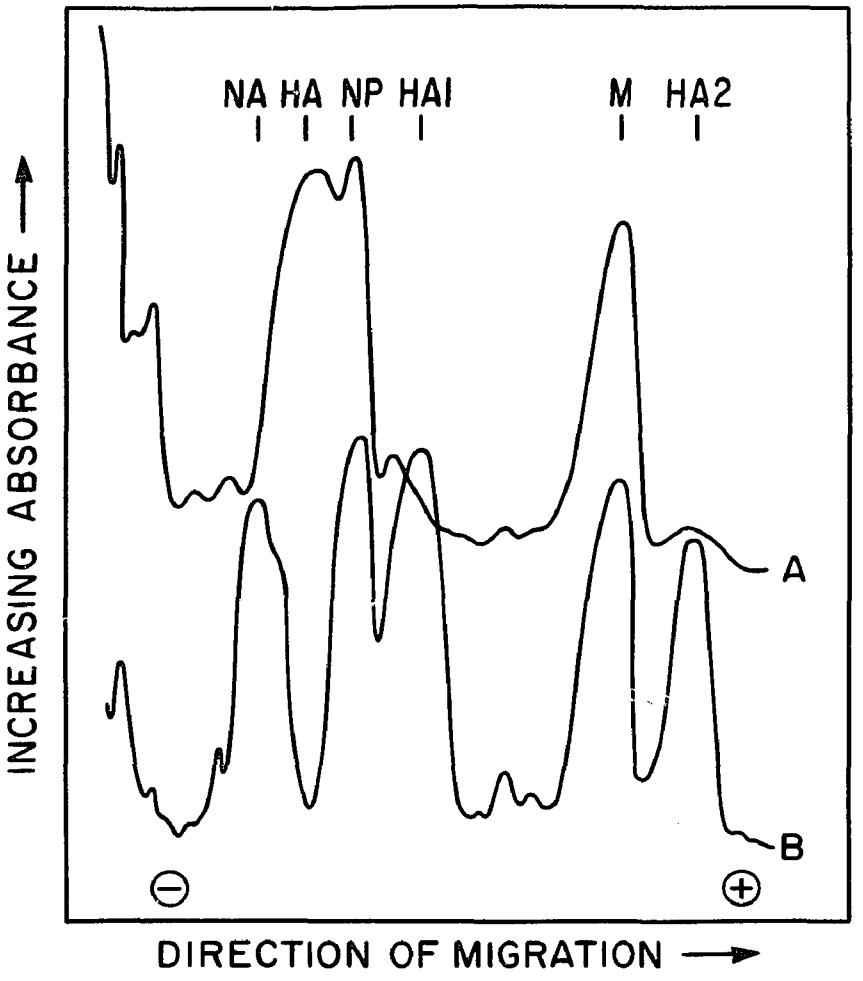

Fig. 3.7. Reduced and unreduced protein patterns of recombinant virus X-29LT. Virus was dissociated in the absence (A) and presence (B) of 2-mercaptoethanol. The structural proteins were resolved by electrophoresis on SDS--polyacrylamide disc gels. Densitometric tracings of the Coomassie blue-stained protein bands are shown.

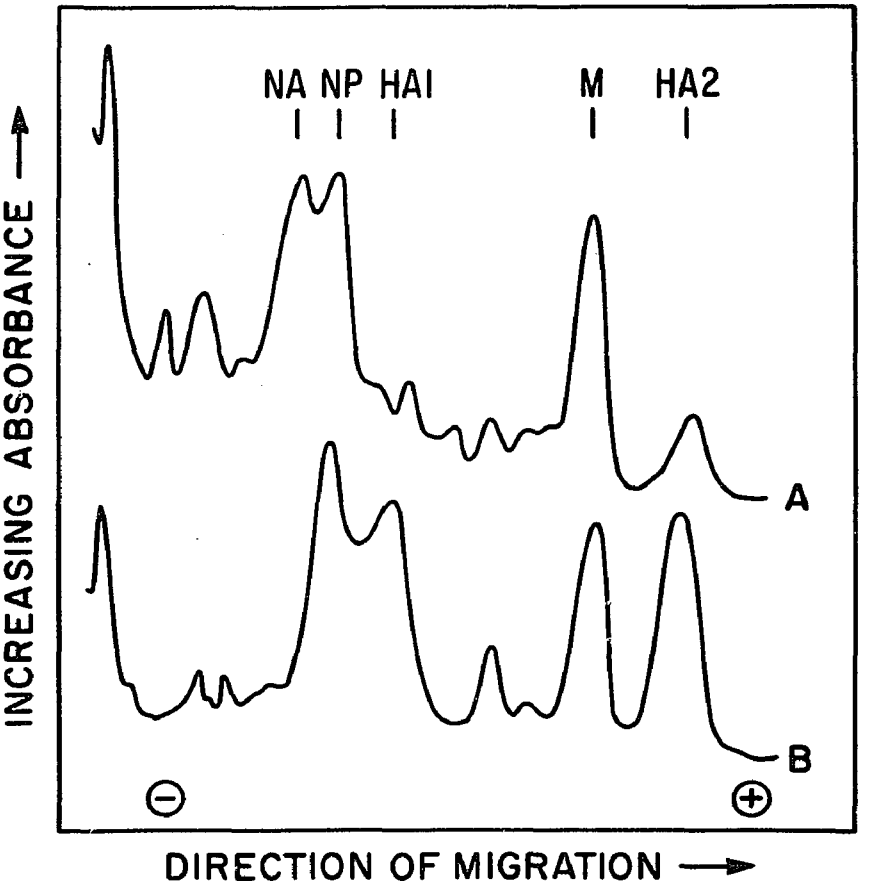

Fig. 3.8. Reduced and unreduced protein patterns of recombinant virus NWS_{t8}. Virus was dissociated in the absence (A) and presence (B) of 2-mercaptoethanol. The structural proteins were resolved by electrophoresis on SDS--polyacrylamide disc gels. Densitometric tracings of the Coomassie blue-stained bands are shown.

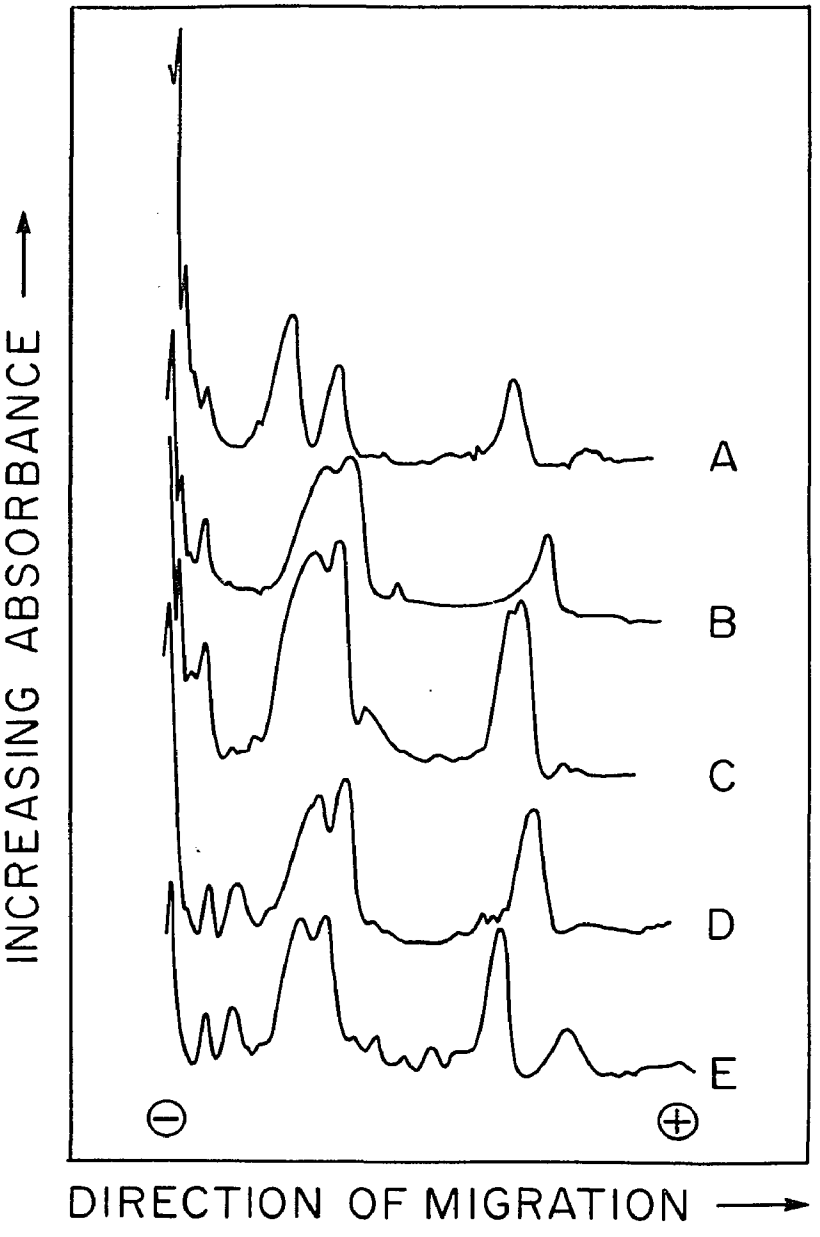

Fig. 3.9. Comparison of unreduced, high-molecular-weight viral proteins on polyacrylamide gels. Three N2 viruses, RI/5⁻ (A), X-12 (B), and X-29LT (C), and two N1 viruses, NWS (D) and NWS_{t8} (E), were dissociated with SDS and electrophoresed on 13% polyacrylamide disc gels. The densitometric tracings of the Coomassie blue-stained protein bands are shown.

serotype (NWS and NWS_{t8}) and in another pattern for viruses of N2 serotype ($RI/5^{-}$, X-12 and X-29LT). The protein pattern for X-29L was identical to that of X-29LT. The first major band migrated at the same rate for each virus. The second band from the N1 viruses and the third band from the N2 viruses also migrated simultaneously. But the second major band from the N2 viruses was not seen in the gels for the N1 viruses, and the relatively wide third major band from the N1 viruses was not present on the N2 gels. These patterns were consistent with the possibility that the latter bands are the neuraminidase protein, although glycoprotein staining or carbohydrate labeling would be necessary to distinguish the unreduced NA protein from the three P proteins.

Hemagglutinin protein concentration. The quantity of the hemagglutinin protein in the recombinant viruses X-12 and X-29L was determined by growing each virus in CEF cells (Chapter 4) in the presence of a mixture of radiolabeled amino acids (^{14}C for X-12 and ^{3}H for X-29L) added 2 hr after infection. Polyacrylamide gel co-electrophoresis of the proteins of the differentially labeled viruses revealed that the hemagglutinin protein was not cleaved into the HA1 and HA2 subunits during virus cultivation in CEF cells (Fig. 3.10 and Fig. 3.11C). The cpm ratio of HA to NP plus M was 0.69 for X-12 and 0.70 for X-29L.

Small polypeptides. Two small radiolabeled polypeptides have been consistently observed after electrophoresis of viral proteins on several types of gels. The separation of the major viral proteins on a 13% acrylamide gel was normally enhanced by prolonging the electrophoresis time until the marker dye and any polypeptides smaller than about 25,000 daltons had migrated off the gel. The patterns in Fig. 3.1 and 3.3 are typical. When 13% gels were run for a sufficiently shorter period,

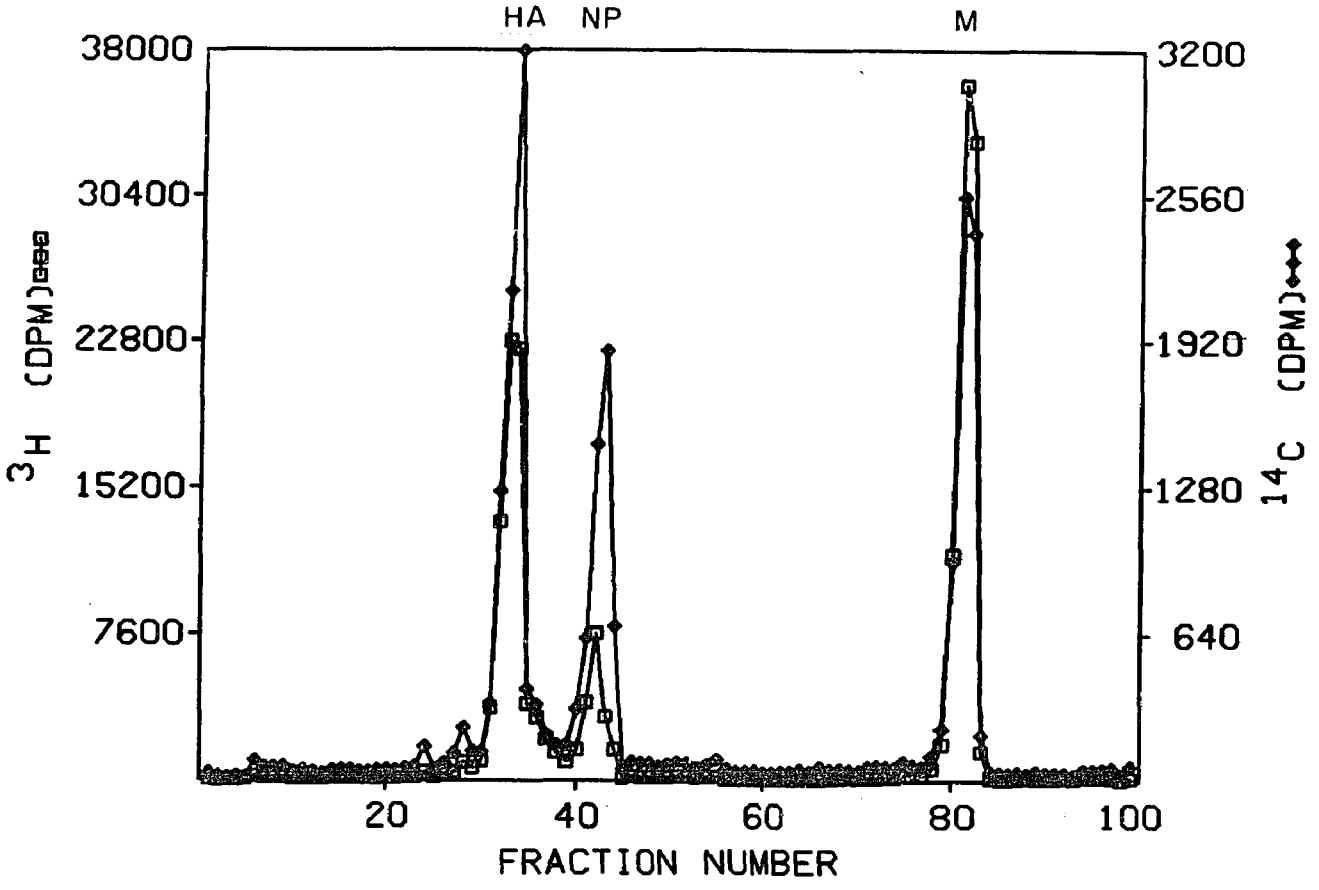

Fig. 3.10. Electrophoretic profile of the proteins of X-12 and X-29L grown in CEF cells. A mixture of [^{14}C] amino acids (X-12) or [^{3}H] amino acids (X-29L) was added to the virus growth medium 2 hr after infection. The proteins of the purified viruses were resolved on a 13% polyacrylamide disc gel.

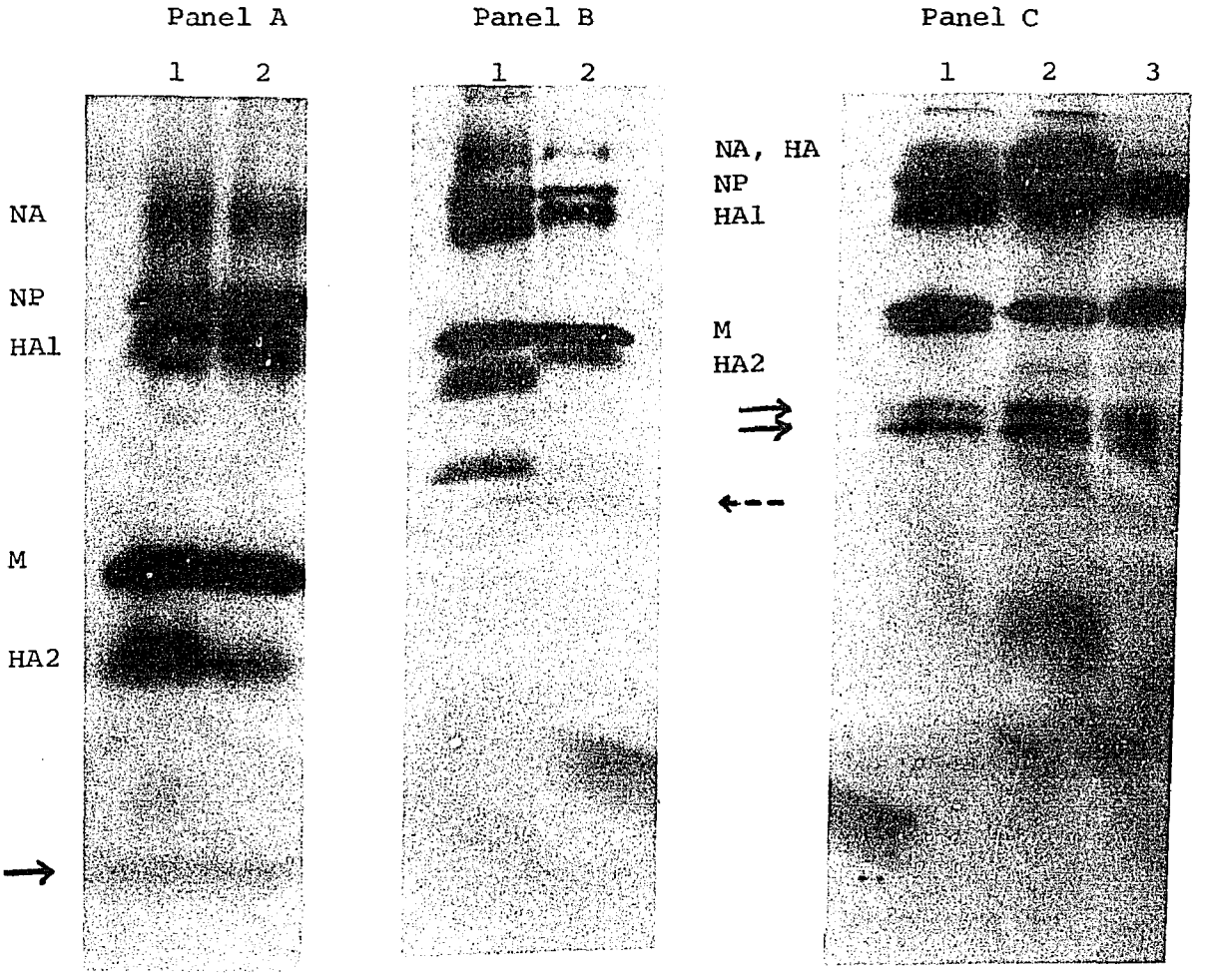

Fig. 3.11. Autoradiographic detection of two small polypeptides after electrophoresis in three gel systems. Panel A shows a 13% polyacrylamide slab gel containing X-29L grown in CAM cells and labeled with [^{14}C]lysine (lane 1) or [^{14}C] arginine (lane 2). Panel B shows a slab gel with a 13% acrylamide upper region and 40% acrylamide lower region containing X-12 grown in CAM cells (lane 1) and WSN grown in MDBK cells (lane 2). Panel C shows a slab gel with a 15% to 30% acrylamide gradient containing X-12 grown in CAM cells (lane 1), X-12 grown in CEF cells (lane 2), and WSN grown in MDBK cells (lane 3). Two small polypeptides are indicated by solid arrows and the 13%/40% acrylamide interface by a dashed arrow. Viral proteins shown in Panels B and C were labeled with a mixture of [^{14}C] amino acids.

however, small radiolabeled polypeptides could also be seen. For example, a faint, diffuse band was seen well below the HA2 band and near the bottom of lane two in Fig. 3.11A. Viral proteins have also been resolved on slab gels containing an upper 13% acrylamide region and a lower 40% acrylamide region. The higher cross-linking of the lower region was designed to slow the migration of any polypeptide degradation products formed during trypsin treatment of the virus. When the marker dye had migrated nearly to the bottom of a 13%/40% gel, the major viral proteins were resolved and the two small polypeptides appeared as diffuse bands above the faint band at the 13%/40% acrylamide interface (Fig. 3.11B). When viral proteins were run on a 15% to 30% gradient slab gel, the major proteins were less well resolved but both small polypeptides appeared as sharp, well defined bands (Fig. 3.11C).

The presence of the two small polypeptides was studied as a function of host cell and virus strain. X-12 grown in CAM cells and in CEF cells and WSN virus grown in MDBK cells were electrophoresed in adjacent lanes of a 15% to 30% gradient gel (Fig. 3.11C). The pattern of the small polypeptides was similar in all three cases. The small polypeptides were detected in the trypsin-resistant viruses RI/5^{-}, WSN, and X-29L and the trypsin-sensitive viruses X-12 and NWS shown in Fig. 3.4 . For each of these five virus strains, the faster polypeptide migrated at the same rate and thus had the same molecular weight. The same was true for the slower polypeptide. Both polypeptides were still present after egg-grown X-12 or X-29L was purified by centrifugation on two consecutive sucrose gradients. The smaller, more prominent polypeptide was also present in X-12 or X-29L when the viral proteins were resolved under nonreducing conditions (Fig. 3.4). One or both polypeptides were also present in egg-grown Sendai virus.

The molecular weights of the small polypeptides were estimated by parallel slab gel electrophoresis of the viral proteins from X-12, X-29L and Sendai virus with several marker proteins of low molecular weight: insulin (6,000 daltons), ribonuclease (13,700 daltons), tobacco mosaic virus coat protein (17,000 daltons), chymotrypsin (11,000 and 13,000 daltons) and pepsin (35,000 daltons). The results from two stained slab gels indicated that the small polypeptides have molecular weights of roughly 20,000 daltons.

The polypeptides have been partially characterized by two differential radiolabeling experiments. Fig. 3.11A shows the radiolabeled protein patterns observed for X-29L virus grown in CAM cells with [^{14}C] lysine or [^{14}C] arginine. Both small polypeptides incorporated arginine and the smaller one took up lysine, but the larger polypeptide incorporated little or no lysine. Furthermore, Fig. 3.2 shows the protein patterns observed for X-12 virus grown in CAM cells with [^{3}H] glucosamine or a mixture of [^{14}C] amino acids. The small polypeptides, which were observed as a single unresolved peak between the HA2 peak and the peak at the 13%/40% interface, did not incorporate the radiolabeled glucosamine.

Neuraminidase activity. The possibility that the greater quantity of neuraminidase protein seen in polyacrylamide gels of X-29L versus X-12 corresponded to increased neuraminidase activity was examined through the determination of the ratio of neuraminidase activity to hemagglutination activity for each virus. Indeed, the neuraminidase activity of the viruses correlated with the quantity of neuraminidase protein present. The neuraminidase activity of X-29L was about 1.7 times that of X-12 when normalized to hemagglutination activity (Table 3.1) and the

TABLE 3.1

Comparison of the Neuraminidase Activites of the Recombinant Viruses X-12, X-29L, and X-29LT

Ratio	Experiment	Relative neuraminidase activity	
		X-29L/X-12	X-29LT/X-29L
NA/HA[a]	1	1.8	2.0
	2	1.6	
	3	1.7	
	Average	1.7	
NA/Protein[b]	1	1.4	
	2	2.1	
	Average	1.7	

[a]Both assays were performed on fresh allantoic fluid. Neuraminidase activity, determined by the method of Aminoff (1961) using a 12 hr incubation period, was expressed as the milligrams of N-acetylneuraminic acid released from the substrate fetuin by 1 ml of virus suspension in 1 hr. The assay temperature was 37°C in Experiments 1 and 2, and 30°C in Experiment 3. Hemagglutination activity was determined as described in Chapter 4 and was expressed as the reciprocal of the highest virus dilution showing complete hemagglutination in 60 min at room temperature.

[b]Both assays were performed on purified virus. Neuraminidase activity was determined at 37°C as described above. Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard and was expressed in milligrams per milliliter of virus suspension.

neuraminidase activity of X-29LT was twice that of X-29L. When the incubation temperature of the neuraminidase assay was lowered from 37°C to 30°C in order to minimize heat inactivation, the enzyme activities of both X-12 and X-29L were reduced by about 60%. Thus the ratio of their neuraminidase activities remained the same. Allantoic fluid from infected eggs was used for these assays to avoid the clumping of viral particles that usually occurs to some extent during virus purification. Since the virus was not purified, however, it was not possible to determine the protein content and thus normalize the enzyme activities to protein content. The hemagglutination activity of X-12 is trypsin sensitive and partial degradation of the HA1 protein probably occurs either during release of the virions into the allantoic fluid or during virus purification (see below). Thus normalization of the neuraminidase activity of each virus to its hemagglutination activity may be an unreliable measure of relative neuraminidase activity. Therefore, in another set of experiments, X-12 and X-29L virions were purified by sucrose gradient centrifugation and their neuraminidase activities were normalized to their protein content (Table 3.1). Although virus clumping can greatly alter the hemagglutination titers, it probably has little effect on the neuraminidase assay in the presence of excess enzyme substrate. These assays indicated that the neuraminidase activity of X-29L was approximately 1.7 times greater than that of X-12, which was consistent with the ratio observed (1.7) when the neuraminidase activities were normalized to the hemagglutination activity.

Molecular weight determinations. The approximate molecular weights of the structural proteins of X-12 and X-29L were determined on polyacrylamide gels by comparison with the migration distances of proteins with known molecular weights (Weber and Osborn, 1969). The

range and average values determined for the proteins of each virus are given in Table 3.2. Molecular weights were not determined for the three P proteins since marker proteins larger than 63,000 daltons were not included. Using the slab gel pictured in Fig. 3.4 and the calculated molecular weights for the X-29L proteins, the molecular weightsof the RI/5$^-$ HA1 and HA2 proteins were estimated to be about 48,000 and 29,000 daltons, respectively. These values are slightly different from the calculated values of 50,000 daltons for the HA1 subunit and 26,500 daltons for the HA2 subunit of the H0 viruses.

Approximate apoprotein molecular weights for the hemagglutinin subunits (Table 3.2) were calculated by assuming that 24.4% of the observed weight of HA1 and 4.7% of the HA2 observed weight was contributed by carbohydrate. These carbohydrate values were determined by Ward and Dopheide (1976) for an H3 hemagglutinin and thus may not be exactly correct for the H0 and H2 proteins. The apoprotein molecular weight for the HA1 subunit was calculated to be about 38,000 daltons for the H0 viruses and 36,000 daltons for the H2 viruses. The apoprotein molecular weights for the HA2 subunit were about 25,500 daltons for the H0 serotypes and about 28,000 daltons for the H2 serotypes.

Ratio of HA1 to HA2. Based on these molecular weights, the weight ratio of the HA1 apoprotein to the HA2 apoprotein was calculated to be 1.3 for RI/5$^-$ and 1.5 for X-12 and X-29L. As a control, the well-studied H0N1 virus WSN, which possesses trypsin-resistant hemagglutination activity (see Chapter 4), was cultivated in the presence of radiolabeled amino acids in CAM cells and purified by sucrose density gradient centrifugation. Following polyacrylamide gel electrophoresis, the areas of the WSN HA1 and HA2 peaks were measured by integrating the total

TABLE 3.2

Molecular Weights of Proteins from X-12 and X-29L Viruses Grown in Embryonated Chicken Eggs

Virus	Viral protein	Observed molecular weight		Estimated apoprotein molecular weight[b]
		Mean[a]	Range	
X-12	NP	59,000	57,500-60,000	
	HA1	49,000	47,000-50,500	37,000
	M	32,000	31,500-33,000	
	HA2	26,000	23,000-29,000	25,000
X-29L	NP	60,000	58,000-62,000	
	HA1	51,000	50,000-51,500	39,000
	M	32,000	31,000-34,000	
	HA2	27,000	25,000-30,000	26,000

[a]The values given are the means of three values for each protein, to the nearest 500 daltons. Molecular weights were determined by the method of Weber and Osborn (1969) using bovine serum albumin, ovalbumin, soybean trypsin inhibitor, chymotrypsinogen, trypsin and myoglobin as marker proteins.

[b]Apoprotein molecular weights were calculated to the nearest 500 daltons by assuming that carbohydrate consistutes 24.4% of HA1 and 4.7% of HA2 by weight (Ward and Dopheide, 1976). M and NP proteins lack carbohydrate (Choppin and Compans, 1975).

radioactivity in each peak. The area of the HA1 peak was found to be 1.7 times that of the HA2 peak, close to the weight ratio expected for the H0 viruses. The HA1 to HA2 weight ratios observed for the H0 viruses of this study, however, were frequently less than the calculated ratio, even in the absence of exogenous trypsin. When gel patterns of three different preparations of radiolabeled X-12 virus were integrated, the HA1 to HA2 weight ratio was 1.14 ± 0.19 (mean ± standard deviation; n = 9), or 76% of the calculated value. For example, the ratio was 1.0 for the amino acid profile in Fig. 3.2. Based on three different preparations of radiolabeled virus, X-29L showed a HA1 to HA2 weight ratio of 0.77 ± 0.14 (mean ± standard deviation; n = 8), or only 51% of the expected ratio. For example, the total radioactivity in the amino acid-labeled HA1 peak of the X-29L protein pattern in Fig. 3.3 was only 0.6 of that in the HA2 peak. The corresponding HA1 to HA2 molar ratios are 0.76 ± 0.13 for X-12 and 0.51 ± 0.09 for X-29L.

In addition, the HA1 to HA2 ratios measured by densitometric tracings of the stained proteins of egg-grown viruses were qualitatively consistent with the ratios measured for radiolabeled virus grown in CAM cells. Trypsin-resistant RI/5$^-$ always had a higher HA1 to HA2 ratio than did X-12, X-29L or NWS, even after allowance for apoprotein molecular weight differences. For example, based on four determinations, the HA1 to HA2 ratio for NWS was only 48% of the expected value or 55% of that for RI/5$^-$ (see also Table 4.5). Similarly, densitometric tracings of the stained proteins from egg-grown virus in Fig. 3.6 showed that the HA1 to HA2 ratio for X-12 was only 36% of the expected value, while the HA1 to HA2 ratio for X-29L was normal. Thus the HA1 to HA2 ratio varied considerably for different preparations of the same virus. Nevertheless,

regardless of the cell type used to cultivate the virus or the method used to quantitate the hemagglutinin peaks, HA1 was frequently found in reduced quantity relative to HA2 for the H0 serotype viruses X-12, X-29L and NWS, but not for the H2 serotype virus RI/5^{-}.

DISCUSSION

Electrophoretic pattern of viral proteins. The identity of the influenza virus protein peaks resolved by polyacrylamide disc gel electrophoresis was established by labeling protein and carbohydrate with different radioisotopes (^{14}C, ^{3}H). Radiolabeled amino acids were incorporated into one preparation of a virus and radiolabeled glucosamine into another preparation of the same virus. When these samples were combined for electrophoresis, the identity of the peaks corresponding to the viral glycoproteins NA, HA1 and HA2 was confirmed by the presence of the glucosamine label (Fig. 3.2 and 3.3).

The structural proteins of two recombinant viruses, X-12 and X-29L, were then compared on polyacrylamide gels. The molecular weights calculated for their structural proteins on the basis of electrophoretic mobility were identical within experimental error. The proteins from X-12 and X-29L showed coincident bands when electrophoresed in adjacent lanes of a 13% slab gel or a 7-20% acrylamide gradient slab gel. In addition, when X-12 was radiolabeled with one isotope (^{3}H or ^{14}C) and X-29L with the other, their proteins showed coincident radioactive peaks when co-electrophoresed on a 13% disc gel.

Neuraminidase. In our gel system, the reduced N2 neuraminidase protein migrated as two close bands of equal intensity above the position of the uncleaved HA protein. Other investigators have found a double

band for the N2 neuraminidase, but there has been disagreement about the ratio of the two components observed (Webster, 1970a; Bucher and Kilbourne, 1972; Skehel and Schild, 1971; Lazdins et al., 1972). Kendal and Eckert (1972) found only one component for proteolytically derived N2 neuraminidase. In addition, only one subunit has been seen for neuraminidases from the serotypes N1 (Gregoriades, 1972) and B/Lee (Lazdins et al., 1972; Laver and Baker, 1972; Wrigley et al., 1973). It is not known if the two bands represent (A) two neuraminidase subunits specified by different genome segments, (B) two disulfide-bonded subunits proteolytically-derived from a single neuraminidase protein, or (C) two neuraminidase subunits having identical apoproteins with different carbohydrate components. Since electrophoretic separation of influenza RNA genome segments on polyacrylamide gels has revealed only one segment coding for neuraminidase (Palese and Schulman, 1976b), the two observed components are probably derived from a single gene product.

Considerable variation was seen in the quantity of neuraminidase protein associated with the three egg-grown HON2 viruses. The same observation has been reported for other influenza virus strains (Seto, 1964; Laver and Kilbourne, 1966; Palese and Schulman, 1974; Mowshowitz and Kilbourne, 1975). The factor controlling the heritable ratio of the two surface proteins incorporated into mature virions has not been determined. In addition, the quantity of neuraminidase can vary with the cell line in which the virus was grown (Laver, 1963) and with the multiplicity of infection employed (Lenard and Compans, 1975). All of the viruses compared in this study, however, were cultivated *in ovo* using a relatively dilute inoculum of approximately 10^2 EID_{50} per egg.

The finding that the trypsin-resistant viruses X-29L and RI/5$^-$ both have large quantities of neuraminidase protein relative to the trypsin-sensitive viruses X-12 and NWS suggested that the amount of neuraminidase protein on the virus particles might determine the trypsin-sensitivity of the viral hemagglutination activity. For example, an increased quantity of NA protein might sterically hinder enzymatic degradation of the hemagglutinin protein. The quantity of NA protein in the trypsin-sensitive recombinant virus X-29LT, however, argued against this possibility. Polyacrylamide gel electrophoresis revealed that the X-29LT virus possessed a very large quantity of neuraminidase. Whereas the NA peak comprised approximately 4% of the X-29L protein, based on densitometry of stained gels, it was about 18% of the total X-29LT protein. Part of the large X-29LT peak, however, could be uncleaved HA protein. Similarly, the neuraminidase activity of the recombinant X-29LT was twice that of X-29L, when normalized to hemagglutination activity. Thus the quantity of neuraminidase on the virus particles does not appear to correlate with trypsin sensitivity.

The ratio of NA activity to hemagglutination activity was 1.7 times greater for X-29L than for X-12, which is consistent with the detection of more NA protein in the X-29L virus. This relative neuraminidase activity was the same whether the neuraminidase assay was conducted at 30°C or 37°C. The neuraminidase enzyme activity of N1 serotype viruses is heat-labile but the activity of the N2 viruses is relatively heat stable (Rafelson et al., 1963; Paniker, 1968). Heat inactivation is observed after 30 min at 55°C or after 12 hr at 37°C. If either virus possessed a heat-sensitive neuraminidase, the ratio of its neuraminidase to hemagglutination activity would have increased at the lower incubation

temperature. Since the ratio for both viruses decreased about 60% at the lower temperature, neither virus possessed heat-labile neuraminidase. This result confirmed the serological evidence that neither virus inherited the N1 neuraminidase from NWS. In addition, if the high molecular weight protein whose migration shifted with NA serotype were the neuraminidase protein, the observation that this protein from X-12 and X-29L migrated identically with the RI/5$^-$ protein rather than the NWS protein also confirmed that the two recombinants acquired their neuraminidase protein from RI/5$^-$.

Negligible neuraminidase activity was detected for the N1 viruses NWS and NWS_{t8} because the NA protein was heat inactivated during the conventional incubation overnight at 37°C. The neuraminidase activity of these viruses might be measured by limiting the incubation period to 1 hr at a lower temperature. But since the peaks observed for the NA proteins of NWS and NWS_{t8} were very small, the neuraminidase activity of these viruses is probably low relative to the activity of RI/5$^-$ or X-29LT.

Hemagglutinin. Comparison of the protein patterns of the parental viruses revealed that the hemagglutinin subunits of the H2 virus RI/5$^-$ migrated at different rates from the HA subunits of the H0 virus NWS. Viruses of these serotypes cultivated in CAM cells have also been observed to migrate differently by Skehel and Waterfield (1975). Presumably the hemagglutinin genes of NWS and RI/5$^-$ code for protein molecules of about the same size. The calculated apoprotein molecular weights for the total hemagglutinin molecule (HA1 plus HA2) were the same for the H0 serotype (63,500 daltons) and the H2 serotype (64,000 daltons). Thus the observed differences in the molecular weights of the individual hemagglutinin subunits from these serotypes could be due

either to the presence of different amounts of carbohydrate or to cleavage of a different peptide bond during conversion of HA into HA1 and HA2. Since recent sequence studies have shown that the first 11 amino acids at the amino terminus of HA2 are identical for five different serotypes (Skehel and Waterfield, 1975; Bucher et al., 1976), the cleavage site is probably constant and the molecular weight differences are most likely due to different carbohydrate contents.

The hemagglutinin subunits of the two recombinant viruses X-12 and X-29L were found to have the same electrophoretic mobilities as the hemagglutinin subunits of NWS but different mobilities from the subunits of RI/5$^-$. Thus, by the criterion of electrophoresis, both recombinants inherited their hemagglutinin genome segment from NWS. This result is consistent with the HO serological typing of these viruses.

When grown in CAM cells in vivo or in vitro, the viruses NWS, RI/5$^-$, X-12, X-29L, NWS_{t8} and WSN gave protein patterns showing little or no uncleaved HA protein. Occasionally a small shoulder was seen on the NA protein peak (for example, see Fig. 3.1), but it was not clear that this represented uncleaved HA. The radiolabel for the glucosamine-labeled viral proteins did not drop to the baseline between the NA doublet and the HA1 peak, which may have been due to the presence of a small amount of the uncleaved HA glycoprotein. The extent of HA cleavage depends on the virus strain, the host cell, the extent of viral cell damage, and the presence or absence of serum in the medium (Lazarowitz et al., 1971, 1973a,b; Klenk and Rott, 1973; Stanley et al., 1973a). Lazarowitz and co-workers (1973a) detected uncleaved HA in RI/5$^-$ virus but not in WSN virus when each virus was grown in embryonated eggs. Klenk and co-workers (1975) found uncleaved HA in egg-grown A/chick/Germany/49 (Hav2Neq1) but not in A/FPV/Rostock (Hav1N1).

When grown in CEF cells, the viruses X-12 and X-29L contained mainly uncleaved HA and little or no HA1 or HA2 (Fig. 3.10). After this result was obtained, Klenk and co-workers (1975) independently reported that the HA protein is not cleaved when either PR/8 or Swine virus is grown in CEF cells. Since they found that fowl plague hemagglutinin was completely cleaved during growth in these cells, the cleavage is a specific property of the virus strain and is not due to an inherent lack of proteolytic enzyme activity in CEF cells.

When X-12 or X-29L virus was grown in eggs or in CAM cells and the viral proteins were examined by gel electrophoresis, the relative molar amount of the HA1 subunit present in either virus was variable but was consistently less than the amount of the HA2 subunit. Thus part of the HA1 subunits (and possibly the HA2 subunits) had been digested without the addition of trypsin or other exogenous proteases. Since the amount of HA1 lost was variable, it was impossible to calculate accurately what proportion of the total viral protein was hemagglutinin protein. In CEF cells, however, little or no cleavage of the peptide bond between the HA1 and HA2 regions of the hemagglutinin occurred. Since this sensitive bond was not cleaved, it is unlikely that significant digestion of the HA protein occurred. Therefore the HA content determined for CEF-grown virus probably represents the inherent HA content of the virus. The HA proteins of X-12 and X-29L grown in CEF cells were found to co-migrate and to amount to approximately 70% of the sum of the M and NP proteins of each virus. Assuming that NA comprises about 7% (Bucher and Palese, 1975) and the P proteins about 3% (Schulze, 1973) of the total viral protein, the HA protein comprised about 37% of the total viral protein for both X-12 and X-29L. This result is consistent with the reports that hemagglutinin represents 25% to 35% of the virion protein, depending on

the virus strain, host cell and polyacrylamide gel system (Schulze, 1973, 1975). Thus the trypsin resistance of X-29L does not appear to be due to a greater amount of HA protein. The unlikely possibility that neuraminidase co-migrates with hemagglutinin would not significantly affect this result, since the amount of NA protein in the virus is only 7-10% of the viral protein (Laver and Kilbourne, 1966; Kendal et al., 1968; Skehel and Schild, 1971).

Weight ratio of HA1 to HA2. Based on the carbohydrate content determined by Ward and Dopheide (1976) for an H3 hemagglutinin, and on the protein molecular weights calculated in this study for the two hemagglutinin subunits, the expected weight ratio of HA1 apoprotein to HA2 apoprotein was 1.3 or 1.5, for the H2 and H0 serotypes, respectively. Since we found that the hemagglutinin proteins from X-12 and X-29L were sensitive to degradation by exogenous trypsin, it is not surprising that the HA1 to HA2 ratio for these two viruses was less than 1.5 (1.14 for X-12 and 0.77 for X-29L). The cleavage of HA into HA1 and HA2 occurs when trypsin is added to virus preparations (Lazarowitz et al., 1973a; Klenk et al., 1975). Since virus grown in embryonated eggs or in cultured chorioallantoic membrane cells has all of its hemagglutinin cleaved into HA1 and HA2 subunits (Klenk et al., 1975; Stanley et al., 1973a), trypsin-like enzymes are active in the allantoic fluid. Degradation of HA1 may also occur during virus purification through the release of proteolytic enzymes from contaminating cellular components or bacteria. But since little or no uncleaved HA protein was observed after isolation and purification of CEF-grown X-12 virus, trypsin-like enzymes are not very active during routine virus purification procedures. Despite the apparent insensitivity of the X-29L hemagglutination activity to trypsin, these observations appeared to confirm that hemagglutinin

protein from X-29L resembled the hemagglutinin from X-12 rather than that from RI/5$^-$ in its sensitivity to proteolytic attack.

Several other strains of influenza evidently contain less HA1 than HA2. Schulze (1973, 1975) has estimated the number of HA1 and HA2 molecules present in several viruses. The weight ratios of HA1 to HA2 can be estimated by multiplying the molar ratio by the calculated equimolar weight ratio (1.5 for H0 serotype, 1.3 for H2 serotype). Thus the estimated HA1 to HA2 weight ratio is 1.24 for A/Bel/40 (H0N1) based on data of Skehel and Schild (1971), 1.12 for A/WSN/33 (H0N1) using results of Compans and co-workers (1970), and 0.55 for X-31 (H2N2) based on data of Skehel (1971b). Schulze (1975) has noted that if the estimated deficiency in HA1 is the result of errors in molecular weight determinations, the errors are systematic because HA2 is invariably in molar excess. Schulze (1973) has also pointed out that the presence of fewer molecules of HA1 than HA2 in a virus particle can result from incorporation of incomplete hemagglutinin molecules into the virus or removal of some HA1 subunits after virus assembly, for example, by reduction of disulfide bonds.

It is unlikely that the loss of HA1 is due to reduction of the disulfide bonds linking HA1 and HA2. In several experiments, no reduction in hemagglutination activity was detected on treatment of X-12 or X-29L with dilute dithiothreitol (final concentration 0.05 M). Similar results were reported by Hoyle (1969) who found that when eight strains of influenza virus were treated with dithiothreitol (final concentration reported as 1/800) hemagglutination activity was only destroyed when virus was treated with the reducing agent in combination with a denaturing agent, such as urea or guanidine hydrochloride.

Finally, Grossgebauer (1973) observed that A2/Singapore/1/57 lost its hemagglutination activity only when reduction with mercaptoethanol (0.2-0.5 M) was followed by alkylation with iodoacetamide or sodium iodoacetate. It seems unlikely that the virus is exposed during isolation procedures to both a high concentration of reducing agent and denaturing or alkylating agents.

Another possible explanation for the presence of more HA2 molecules than HA1 molecules in X-12 and X-29L is that initial cleavage of HA1 generates two polypeptide fragments that co-migrate with HA2. This possibility implies that the uncleaved hemagglutinin molecule consists of three regions (or domains; Edelman and Gall, 1969) of roughly equal size. After the initial proteolytic cleavage of HA into the two-domain subunit HA1 and the one-domain subunit HA2, the HA1 subunit is cleaved into two smaller subunits (HA1a, HA1b) that each represent one domain. Proteolytic cleavage within each of the domains would presumably be slower than fragmentation between the domains. This scheme is consistent with the amino acid compositions of the HA1 and HA2 subunits (see Chapter 6). The close compositional relatedness of HA1 and HA2 is readily explained if HA1 consists of two domains, HA2 comprises one domain, and all three domains are sequentially similar. A hemagglutinin polypeptide chain consisting of three similar domains could have arisen by evolution of HA from one domain through internal gene duplication.

Small polypeptides. During this study two small polypeptides (each about 20,000 daltons) were consistently observed in preparations of influenza virus. For a variety of reasons, the presence of these polypeptides in preparations of influenza virus has not been previously reported. They migrate as diffuse bands well ahead of HA2 on 13% gels

and are probably even less obvious on gels containing less acrylamide. The sharpness of these polypeptides bands on 13% gels varies considerably with the electrophoresis time and voltage, more diffusion occurring during long electrophoresis at low voltage. In several gel systems, the conditions used to enhance the resolution of the major viral proteins cause these polypeptide bands to be electrophoresed off the end of the gel. Most investigators who have noticed these bands probably assumed that they represented contaminating host cell proteins.

The source of these small polypeptides is unknown. Since each of the eight segments of the influenza genome has already been assigned a protein-coding function (Palese and Schulman, 1976b; Ritchey et al., 1976; Palese et al., 1977), it is unlikely that the small polypeptides are the sole product(s) of a viral gene. Alternatively, they could be host-cell contaminants not separated from virus particles during purification, host-cell polypeptides incorporated into virus particles, or by-products of virus-coded proteins.

First, the small polypeptides could be contaminants from the host cell. But this possibility is decreased by several experimental observations. The polypeptides were observed to be present and to migrate identically in several types of virus preparations, including four different H0 influenza viruses, one H2 influenza virus and Sendai virus (a paramyxovirus). They were also seen to migrate identically in virus preparations grown in several types of host cells, including embryonated eggs and chicken chorioallantoic membrane cells, chicken embryo fibroblast cells and Madin-Darby bovine kidney cells. They were visualized by protein staining and were radiolabeled when labeled amino acids were added to infected CAM cells as late as 4 hr after infection. During viral infection cellular synthesis of influenza virus-specific

proteins replaces synthesis of host-specific protein. The viral-mediated reduction in host cell protein synthesis reduces the incorporation of radiolabel into host cell proteins. Thus less contaminating protein is observed in preparations of radiolabeled virus than in preparations of viral proteins visualized by staining (compare Fig. 3.4 and Fig. 3.11). The time required after infection to produce maximal inhibition of host protein synthesis varies with the host cell and is not known for CAM cells. Nevertheless, the two polypeptides were prominent in patterns of radiolabeled viral proteins visualized either by gel autoradiography or by scintillation counting of gel slices. Attempts to remove the small polypeptides from virus preparations by centrifugation through a second sucrose gradient were unsuccessful.

Second, the small polypeptides could be host-cell polypeptides that were incorporated into the virus particles. The polypeptides were not glycosylated as measured by incorporation of radiolabeled glucosamine. Thus if they are host membrane proteins, they are probably not exposed on the cell surface. Influenza and other enveloped viruses are reported to have ATPase and nucleoside triphosphate phosphotransferase activities (Roy and Bishop, 1971) which are presumably of host origin. These activities have not yet been correlated with any virion polypeptides. Compans and Pinter (1975) have described a host cell-derived sulfated mucopolysaccharide that migrates near the gel origin and can be removed from virus particles by mild trypsin treatment. They propose that the mucopolysaccharide may be loosely bound to the glycoproteins or attached to the viral lipid directly via a peptide portion of the molecule. It is unlikely, however, that this peptide is related to the two small polypeptides described here, which migrate ahead of HA2 in the absence of trypsin.

Third, the small polypeptides could be by-products of virus-coded proteins. If they are disulfide-bonded subunits of one of the viral proteins, they should not be seen in patterns of viral proteins that have been denatured and electrophoresed in the absence of reducing agent. A faint band that could correspond to the small polypeptides was seen in the pattern of unreduced viral proteins (Fig. 3.4), but it is not certain that this band is the same as that seen in patterns of reduced viral proteins. This band may be due to a contaminating protein of the appropriate molecular weight, as is seen in the preparation of soybean trypsin inhibitor (Fig. 3.4). The release of a 20,000-dalton polypeptide subunit from a viral protein during reduction would cause a major change in the migration of the nonreduced viral protein. Such a shift has not been described for the influenza proteins.

Alternatively, the two small polypeptides could be by-products of post-translational modification of virus-coded proteins. They may be cleaved from a virus protein at the viral maturation sites in the cell membrane and thus be incorporated into the lipid bilayer of the virus particle. For example, the small polypeptides could be signal peptides. The two viral surface glycoproteins, hemagglutinin and neuraminidase, are probably transferred to the exterior surface of the cell membrane in the manner described for excreted cellular proteins (Blobel and Dobberstein, 1975). The two small polypeptides are not present in equal concentrations, which is the pattern expected if the larger, less abundant one were cleaved from neuraminidase and the small, more abundant one were split from hemagglutinin. Since Sendai virus also buds from the host cell membrane (Compans et al., 1966; Choppin et al., 1971) and possesses two surface glycoproteins, two signal peptides might also be expected in this

virus. Signal peptides for excreted host cell proteins have not yet been isolated. They may be degraded in the cell after removal from the protein or they may be difficult to detect among the numerous cell membrane proteins. If the latter situation holds, it may be possible to detect the viral signal peptides in membranes from which host cell proteins have been excluded during virus maturation (Scholtissek and Klenk, 1975). The molecular weight (about 20,000 daltons) of the observed polypeptides, however, makes it unlikely that they are signal peptides. The signal peptide regions characterized to date for host cell proteins are only 20 to 25 amino acids long (Devillers-Thiery et al., 1975), which would correspond to a peptide weighing only 2000 to 3000 daltons. The two polypeptides could be the by-products of other yet undescribed post-translational modifications of viral proteins that occur at or within the cell membrane. Except for the cleavage of HA into HA1 and HA2, however, there is no evidence that influenza virus-specific polypeptides are generated by cleavage of larger precursors (Taylor et al., 1969; Lazarowitz et al., 1971; Skehel, 1972; Klenk and Rott, 1973). In addition, the gene products of each of the eight RNA segments have molecular weights that are in reasonable agreement with the coding capacity determined for their corresponding viral RNA segments (Ritchey et al., 1976).

SUMMARY

No differences were detected in the molecular weights of the structural proteins of the recombinant viruses X-12 and X-29L as measured by electrophoretic migration of the proteins on SDS--polyacrylamide gels. Comparison of the protein patterns of the recombinants with those of the parental viruses NWS (HON1) and RI/5$^-$

(H2N2) indicated that X-12 and X-29L acquired their hemagglutinin subunits HA1 and HA2 but not their neuraminidase from NWS, which is consistent with their serological typing (H0N2). Although the trypsin-resistant viruses X-29L and RI/5$^-$ were found to possess more neuraminidase protein and neuraminidase activity than the trypsin-sensitive viruses X-12 and NWS, trypsin sensitivity did not correlate with neuraminidase content because the trypsin-sensitive recombinant X-29LT was found to have more NA protein and activity than X-29L. Protein patterns revealed that X-12 and X-29L had an HA1 to HA2 weight ratio lower than the 1.3 to 1.5 expected based on apoprotein molecular weights. Degradation of HA1 protein may have occurred within the allantoic fluid or during virus purification. Finally, two small polypeptides of unknown origin having molecular weights of about 20,000 daltons were consistently observed in the electrophoretic patterns of viral proteins from several influenza strains grown in several cell types.

Chapter 4

Trypsin Treatment of Influenza Viruses

Ten years ago, a series of influenza A viruses was derived by genetic recombination between the wild-type viruses NWS (H0N1) and RI/5$^+$ (H2N2). These recombinant viruses were characterized by a number of criteria, including the type and size of plaques formed on Clone 1-5C-4 cells, sensitivity to antibody inactivation in hemagglutination-inhibition, plaque-inhibition and plaque-size-reduction assays, susceptibility of infectivity to neutralization by serum inhibitors, and the sensitivity of hemagglutination activity to inactivation by the pancreatic protease trypsin (Kilbourne et al., 1967). This last criterion was shown to be a heritable characteristic that appeared to correlate with the viral serotype (Kilbourne, 1963). Viruses bearing the NWS-derived H0 hemagglutinin protein on their surface lost their hemagglutination activity in approximately 30 min when suspended in allantoic fluid and exposed to trypsin (0.5-0.6 mg/ml) at 35-40°C. These viruses were termed trypsin sensitive. Viruses bearing the RI/5$^+$-derived H2 hemagglutinin protein retained their hemagglutination activity for at least 90 min under the same conditions and were described as trypsin resistant. The trypsin sensitivity and source of 25 influenza viruses are shown in Table 4.1.

The recombinants X-6 and X-29, which contained the H0 hemagglutinin gene of NWS and the N2 neuraminidase gene of RI/5$^-$, appeared to be exceptions to this general rule (entries 7 and 12 of Table 4.1). This chapter describes the results of a study to define the molecular basis of the trypsin resistance of X-29 and its large-plaque variant, X-29L, by comparison with the trypsin-sensitive recombinant X-12 (H0N2).

TABLE 4.1

Trypsin Sensitivity of 25 Egg-Grown Influenza A Viruses

Entry	Virus (serotype)	Viral source	Trypsin sensitivity[a]
*1.	A/NWS/33 (H0N1)	Natural isolate	Sensitive
*2.	A/WSN/33 (H0N1)	Natural isolate	Resistant
*3.	A/PR/8/34 (H0N1)	Natural isolate	Resistant
4.	X-3 (H0N1)[b]	A/NWS/33 x A/RI/5$^+$/57	Sensitive
5.	X-4 (H0N1)[b]	A/NWS/33 x A/RI/5$^+$/57	Sensitive
6.	X-5 (H0N2)[b]	A/NWS/33 x A/RI/5$^+$/57	Sensitive
7.	X-6 (H0N2)[b]	A/NWS/33 x A/RI/5$^+$/57	Resistant
8.	X-7 (H0N2)[b]	A/NWS/33 x A/RI/5$^+$/57	Sensitive
9.	X-7(F1) (H0N2)[b]	A/NWS/33 x A/RI/5$^+$/57	Sensitive
10.	X-8 (H0N2)[b]	A/NWS/33 x A/RI/5$^+$/57	Sensitive
*11.	X-12 (H0N2)	A/NWS/33 x A/RI/5$^-$/57	Sensitive
*12.	X-29 (H0N2)	X-12 (H0N2) x X-27 (H2N1)	Resistant
*13.	X-29L (H0N2)	X-12 (H0N2) x X-27 (H2N1)	Resistant
*14.	X-29LT (H0N2)	X-29L (H0N2) x X-41 (H3N2)	Sensitive
*15.	NWS_{t2} (H0N1)[c]	X-29 (H0N2) x X-9L (H2N1)	Resistant
*16.	NWS_{t8} (H0N1)	X-29 (H0N2) x X-9L (H2N1)	Resistant
17.	X-9L (H2N1)[b]	A/NWS/33 x A/RI/5$^-$/57	Resistant
18.	X-27 (H2N1)[c]	A/NWS/33 x A/RI/5$^-$/57	Resistant
*19.	A/RI/5$^+$/57 (H2N2)	Natural isolate	Resistant
*20.	A/RI/5$^-$/57 (H2N2)	Natural isolate	Resistant
21.	X-1 (H2N2)[b]	A/NWS/33 x A/RI/5$^-$/57	Resistant
22.	X-1L (H2N2)[b]	A/NWS/33 x A/RI/5$^-$/57	Resistant

TABLE 4.1, continued

Entry	Virus (serotype)	Viral source	Trypsin sensitivity[a]
23.	X-2 (H2N2)[b]	A/NWS/33 x A/RI/5^{-}/57	Resistant
24.	X-28 (H2N2)[c]	X-12 (H0N2) x X-27 (H2N1)	Resistant
25.	X-41 (H3N2)	A/PR/8/34 x A/Pt. Chalmers/1/73 (H3N2)	Resistant

[a]A virus is defined as trypsin sensitive (or resistant) if its hemagglutination activity decreased (or did not decrease) to zero or at least a thousand-fold (2^{10}) after exposure to trypsin for 90 min using method A or B (see Materials and Methods). Viruses marked with an asterisk (*) are discussed below.

[b]Kilbourne et al., 1967.

[c]E. D. Kilbourne, unpublished results.

MATERIALS AND METHODS

Virus. Influenza virus strains employed in this study include A/Neurotropic Wilson Smith (NWS)/33 (H0N1), A/Wilson Smith Neurotropic (WSN)/33 (H0N1), A/RI/5$^-$/57 (H2N2), A/RI/5$^+$/57 (H2N2), A/PR/8/34 (H0N1), A/Port Chalmers/1/73 (H3N2), and the recombinant viruses NWS_{t2} (H0N1), NWS_{t8} (H0N1), X-3 (H0N1), X-6 (H0N2), X-9L (H2N1), X-12 (H0N2), X-29 (H0N2), X-29L (H0N2), X-29LT (H0N2) and X-41 (H3N2). NWS and WSN are both neurotropic variants of the WS strain, the first influenza virus isolated from humans (Smith et al., 1933).

Isolation of the recombinant viruses NWS_{t2}, NWS_{t8}, and X-29L. Eleven-day-old embryonated chicken eggs were doubly infected with equal EID_{50} (50% infectivity dose) quantities of X-29 (H0N2) and X-9L (H2N1). Eggs were also separately infected with X-29 and X-9L at the same virus concentration. After incubation for 40 hr, the allantoic fluids were harvested separately and used to inoculate new 10-day-old eggs at 1:10 dilution in the presence of heat-inactivated H2N2 antiserum raised against RI/5$^+$. After two egg passages in the presence of this antiserum, the fluids from the doubly infected eggs, from the X-29 eggs, and from the X-9L eggs were pooled separately and used to infect Clone 1-5C-4 cell monolayers at six dilutions. The infected cells were overlayed with agar containing H2N2 antiserum treated with the Receptor Destroying Enzyme (RDE) of Vibrio cholera (Burnet et al., 1946); an equal number of infected control plates were overlayed with agar containing RDE-treated normal rabbit serum. On the fifth day following infection, virus from three large plaques was passaged in eggs at high dilution: (A) a possible recombinant virus plaqued in the presence of H2N2 antiserum; (B) a possible recombinant virus plaqued in the presence of normal rabbit

serum; and (C) a large-plaque variant of X-29 (X-29L) plaqued in the presence of normal rabbit serum. Virus from plaques A or B was again plated on Clone-1-5C-4 cell monolayers in the presence of H2N2 antiserum. On the third day after inoculation, one large plaque from the passage of plaque A (NWS_{t2}) and one large plaque originating from plaque B (NWS_{t8}) were picked and passaged at high dilution in eggs. The new possible recombinant viruses were then typed antigenically by the hemagglutination-inhibition (HI), neuraminidase-inhibition (NI), plaque-inhibition (PI) and plaque-size-reduction assays and tested for trypsin sensitivity.

Isolation of the recombinant virus X-29LT. Ten-day-old embryonated eggs were doubly infected with equal EID_{50} quantities of X-29L (H0N2) and X-41 (H3N2). At the same time, X-29L and X-41 were also passaged separately in eggs at the same virus concentration. After incubation for approximately 40 hr, the allantoic fluids were harvested separately and inoculated into new 10-day-old eggs at a 1:10 dilution in the presence of RDE-treated antiserum against A/Port Chalmers/1/73 (H3N2). After three egg passages in the presence of serum, fluids from the doubly infected eggs were pooled and passaged at high dilution in eggs in the presence of the Port Chalmers antiserum. Fluids from the two eggs having the highest titers at the highest dilution at which the virus grew were characterized antigenically by the HI and NI assays.

Stock virus. With the exception of WSN virus, virus stocks were prepared by inoculating the allantoic cavity of 10-day or 11-day-old embryonated chicken eggs with 10^2 to 10^3 EID_{50} of virus diluted in buffered saline (0.85% NaCl in 0.01 M phosphate buffer, pH 7.3) containing penicillin (10^5 units/l.) and streptomycin (0.1 g/l.). Following incubation at 37°C for 42-48 hr, the eggs were chilled and the

allantoic fluid was individually harvested from each egg. The fluids were assayed for hemagglutination activity and tested for sterility by streaking on blood agar plates. The serile fluids having the highest hemagglutination activities were pooled, clarified by low speed centrifugation at 500 g for 10 min at 4°C and stored frozen at -95°C. WSN virus cultivated in Madin-Darby bovine kidney (MDBK) cells (Ditchfield and Grinyer, 1965) or in embryonate eggs was kindly provided by Dr. P. Palese and Dr. J. Schulman, respectively, Mount Sinai School of Medicine, New York.

Cells. Cells from Clone 1-5C-4 of the Wong-Kilbourne variant of the aneuploid Chang human conjunctival cell line were cultivated in Medium 199 (Microbiological Associates) containing 10% heat-inactivated fetal calf serum as described previously (Wong and Kilbourne, 1961; Sugiura and Kilbourne, 1965; Kilbourne, 1969b). (B) Primary cultures of chicken embryo fibroblasts (CEF) were prepared by repeated trypsinization of 8-day to 11-day-old embryos with 0.25% trypsin in buffered saline after excision of head, wings, and legs and removal of internal organs. Cells were grown to confluency in Medium 199 containing 10% heat-inactivated fetal calf serum. (C) Chorioallantoic membrane pieces (CAM) were prepared under sterile conditions by cutting the shells of 11-day-old embryonated chicken eggs approximately 5 cm below the air pocket and removing the membranes covering the air pockets. Each membrane piece was cut into three pieces. Two membrane pieces, each from a different egg, were placed in 25 x 150-mm culture tubes with 1 ml of a buffered salt solution (pH 7.28) containing glucose but lacking amino acids (Tamm et al., 1952). (D) MDBK and MDCK (Madin-Darby canine kidney) cell monolayers were provided by Dr. M. Ueda, Mount Sinai School of Medicine, New York. Baby

hamster kidney cells (BHK) were obtained from Dr. R. Compans, The Rockefeller University, New York.

Virus infection. Confluent cell monolayers were washed twice with phosphate-buffered saline (PBS; Dulbecco and Vogt, 1954) containing 0.2% bovine albumin (PBS--BA) and inoculated with 0.1 ml of a virus solution in PBS--BA. After adsorption for 30 min at 37°C, monolayers were washed twice with PBS--BA. Clone 1-5C-4 and CEF cells were incubated with Medium 199 containing 0.2% bovine albumin. Infected MDBK, MDCK and BHK cells were incubated with reinforced Eagle medium (Bablanian et al., 1965) containing $NaHCO_3$ (1.5 mg/ml), 0.2% bovine albumin, and 0.001% diethylaminoethyl-dextran (DEAE--dextran). CAM cells were infected by addition of 0.1 ml of undiluted stock virus to the 1 ml of medium in which the membrane pieces were suspended; approximately 1 hr after the initial infection, an additional 0.1 ml of undiluted virus was added to each culture tube. Virus was harvested 18-24 hr after infection.

The growth media for radioactive virus were supplemented with radioisotopes at the following concentrations: [^{3}H] amino acids, 50 μC/ml for CAM and 30 μC/ml for CEF; [^{14}C] amino acids, 25-30 μC/ml for CAM and 7-10 μC/ml for CEF; [^{3}H] glucosamine, 50 μC/ml; [^{14}C]-glucosamine, 25 μC/ml; [^{3}H] lysine or arginine, 25-50 μC/ml, [^{14}C]-lysine, arginine or leucine, 5-8 μC/ml. The radiolabel was added to the virus growth medium 2-4 hr after the initial infection.

Virus purification. After a slow spin (2000 g for 15 min) at 4°C to pellet cellular debris, virus was pelleted from allantoic fluid at 100,000 g for 2 hr either directly or onto a cushion of 75% sucrose in 0.02 M Tris-HCl buffer (pH 7.4) containing 0.001 M EDTA and 0.1 M NaCl (NTE). Concentrated virus was layered on a linear 30-60% sucrose

gradient in NTE. CAM culture fluid containing virus was layered on a sucrose gradient directly after clarifying. To ensure visibility of gradient bands containing radiolabeled virus, unlabeled virus prepared at the same time and in the same manner was mixed with the radiolabeled virus prior to gradient centrifugation. After centrifugation at 100,000 g for 2 hr, the visible viral band was collected with a syringe by puncturing the side of the centrifuge tube. Following dilution in NTE, the virus was either pelleted at 100,000 g for 1 hr or dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) overnight at 4°C. Pellets were suspended in water, PBS, or 0.02 M Tris-HCl buffer (pH 7.5) containing 0.01 M $CaCl_2$ and 0.1 M NaCl (NTC).

Virus assay. Plaque assays were performed on Clone 1-5C-4 or CEF cells as described previously (Sugiura and Kilbourne, 1965; Kilbourne, 1969b). Monolayers in 60-mm petri dishes were washed twice with PBS--BA and inoculated with 0.1 ml of 10-fold serially diluted virus in PBS--BA. After a 30 min adsorption period at 37°C, the inoculum was removed, and the monolayers were washed twice with PBS--BA and overlayed with 0.6% agar in Medium 199 containing 0.01% DEAE--dextran. After incubation at 35°C for 3 days, CEF plates were overlayed with 0.6% agar in Medium 199 containing 0.004% neutral red, and the plaques were counted on the fourth and fifth days. On the fourth or fifth day, the agar was removed from Clone 1-5C-4 cell monolayers, the cells were stained with an aqueous solution containing 0.1% crystal violet in 20% ethanol, and the plaques were counted.

Egg infectivity was determined by inoculating 0.1 ml of 10-fold serially diluted virus into the allantoic cavity of 10-day or 11-day-old embryonated chicken eggs. Allantoic fluids were harvested 42-48 hr after

inoculation and assayed for hemagglutination activity; the 50% infectivity endpoints (EID_{50}) were calculated by the Reed-Muench equation (Lennette, 1964).

Hemagglutination assay. Hemagglutination titration was performed in glass tubes or, when specified, in plastic microtiter plates, by adding equal volumes of 0.5% human Type-O red blood cells in PBS to serial two-fold dilutions of virus in PBS. When specified, precision hemagglutination assays were performed using fractional dilutions of virus (Horsfall and Tamm, 1953). After 60 min at 22 to 26°C, or 2-2.5 hr at 4°C, the titration end points were determined by the pattern technique (Salk, 1944). Titers were expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination.

Neuraminidase assay. Neuraminidase activity was determined by the method of Aminoff (1961) using an overnight incubation at 37°C with the enzyme substrate fetuin.

Hemagglutination-inhibition assay. The hemagglutination-inhibition (HI) assay was performed as described by Casals (1967). The hemagglutination titer of a virus suspension was determined as described above. The virus suspension was adjusted to contain 20 hemagglutination units per ml and equal volumes were added to twofold serial dilutions of antisera, which had been treated overnight at 37°C with the Receptor Destroying Enzyme (RDE; General Biochemicals) of Vibrio cholera (Burnet et al., 1946) to destroy nonspecific inhibitors. After incubation at room temperature for 1 hr, an equal volume of 0.5% human red blood cells was added to each antiserum dilution and the assay was again incubated for 1 hr at room temperature. The HI titer was expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

Neuraminidase-inhibition assay. The neuraminidase-inhibition (NI) assay was performed as described by Downie (1970). The neuraminidase activity of a virus preparation was determined by addition of the neuraminidase substrate fetuin, incubation overnight at 37°C, and measurement of the absorbance of the N-acetylneuraminic acid liberated at 549 nm in a 1-cm cell with a Beckman DU-2 spectrophotometer. The viral preparation was then diluted so that sufficient N-acetylneuraminic acid was liberated during the incubation period to give an absorbance between 0.3 and 0.9. This constant dilution of virus was incubated for 30 min at room temperature with equal volumes of serial dilutions of antiserum that had been heat-inactivated at 56°C for 30 min. The controls contained the same amount of virus as measured by neuraminidase activity plus serial dilutions of normal rabbit serum. The NI titer of an antiserum was expressed as the highest dilution of the antiserum which inhibited 50% of the neuraminidase activity of the control containing the same dilution of normal rabbit serum.

Plaque-inhibition and plaque-size-reduction assays. Plaque assays were performed as described above except that the agar overlay contained dilutions of RDE-treated antiserum. RDE-treated normal rabbit serum was incorporated into the overlay of infected control plates. After approximately four days, the agar was removed and the plates were stained with crystal violet. Plaques were counted and their diameters were measured. The plaque-size-reduction titer (PSR) was expressed as the reciprocal of the highest serum dilution that decreased the median plaque radius by 50%. The plaque-inhibition titer (PI) was expressed as the reciprocal of the highest serum dilution that decreased the number of plaques per plate by 50%.

Protein assay. Protein was estimated by the method of Lowry et al., (1951) using bovine serum albumin as the protein standard.

Trypsin assay. The activities of commercial trypsin samples were determined by the method of Hummel (1959). The rate of hydrolysis of the synthetic substrate N^{α}-p-toluenesulfonyl-L-arginine methyl ester (TAME) was measured as the increase in absorbance at 247 nm. One unit of enzyme activity hydrolysed one micromole of TAME per minute at 25°C and pH 8.1 in the presence of 0.01 M calcium ion. Unless specified otherwise, the reaction was performed at 25°C in 0.02 M Tris buffer (pH 8.0) containing 0.01 M $CaCl_2$ and 0.1 M NaCl.

Treatment of virus with trypsin. Two procedures were followed, as specified. (A) Virus in allantoic fluid or purified virus suspended in PBS was mixed with an equal volume of 0.12% trypsin (Worthington TRTPCK) in PBS and incubated at 40°C. The final concentration of trypsin was 0.6 mg/ml. At several time points, an aliquot was removed and mixed with a half volume of 0.12% soybean trypsin inhibitor (Worthington) in PBS that was precooled to and maintained at 0°C in an ice bath. (B) Purified virus suspended in NTC was mixed with an equal volume of trypsin (1 mg/ml) in NTC. The final concentration of trypsin was 0.5 mg/ml. Following incubation at 35°C for the specified time, an aliquot was added to an equal volume of NTC containing soybean trypsin inhibitor (1 mg/ml) that was precooled to and maintained at 0°C in an ice bath. With either procedure, a control virus preparation was incubated at 35°C with PBS or NTC but without trypsin for the appropriate time before addition of soybean trypsin inhibitor.

After the trypsin reaction was quenched, the samples were assayed for hemagglutination activity and/or neuraminidase activity and then treated in one of the following three ways. (A) Virus was pelleted to

remove trypsin and inhibitor by centrifugation for 1 hr at 52,000 g. Each pellet was resuspended in a small volume of NTC, assayed for protein and submitted to electrophoresis on polyacrylamide gels (Chapter 3). (B) When trypsin-treated virus preparations were sufficiently concentrated, samples for polyacrylamide gels were immediately denatured, reduced and alkylated. (C) For electron microscopy, each virus sample was layered onto a 4-ml linear 30-60% sucrose gradient prepared in NTE immediately after the trypsin reaction was quenched. After centrifugation at 100,000 g for 2.5-3 hr, the virus band was collected with a needle inserted directly into the band through the tube wall. The virus was dialyzed overnight at 4°C against 0.02 M Tris buffer (pH 7.5).

Treatment of virus with pronase, chymotrypsin, pepsin, IgA protease, or bromelain. α-Chymotrypsin (Worthington), pronase (Calbiochem) and pepsin (Worthington) were each dissolved at a concentration of 1 mg/ml in NTC, except that the pepsin solution lacked the 0.01 M $CaCl_2$. Equal volumes of enzyme and purified virus in NTC were mixed at 35°C. At the specified times, aliquots were cooled in an ice bath and assayed for hemagglutination activity at 25°C. IgA protease was a gift of Dr. A. G. Plaut, Tufts-New England Medical Center, Boston, Massachusetts; it was dissolved at a concentration of 2.5 mg/ml in 0.02 M Tris buffer (pH 7.5) and mixed with an equal volume of purified virus in PBS at 37°C. At specified time points, aliquots were cooled in an ice bath and assayed for hemagglutination activity at 25°C. Bromelain treatment was performed by the method of Compans et al. (1970).

Electron microscopy. For ultrastructural studies, purified virus was dialyzed against 0.02 M Tris (pH 7.5) and negatively stained in one of three ways. Virus was mixed with (A) an equal volume of 4% sodium

phosphotungstate (PTA), pH 7.0, (B) 2% PTA, pH 6.2, or (C) 1% ammonium molybdate, pH 7.9, and a drop placed on a grid covered with a carbon-coated Parlodion film. Preparations were examined with an AEI 801 transmission electron microscope.

RESULTS

Determination of trypsin activity. The enzymatic activity of trypsin under various experimental conditions was determined using the synthetic substrate $\underline{N}^{\alpha}$-$\underline{p}$-toluenesulfonyl-L-arginine methyl ester (TAME). The trypsin used in these experiments was a new sample from Worthington Biochemicals reported to have an activity of 240 units/mg when assayed in 0.046 $\underline{M}$ Tris (pH 8.1) containing 0.01 $\underline{M}$ $CaCl_2$. When assayed in 0.02 $\underline{M}$ Tris buffer (pH 8.0) containing 0.1 $\underline{M}$ NaCl and 0.01 $\underline{M}$ $CaCl_2$, an activity of 209 units/mg was observed. Lowering the pH to the physiological range of 7.2 to 7.5 reduced the activity against the synthetic substrate to 158 units/mg. The pH optimum of trypsin has been reported to vary with the substrate between pH 7.5 and 9.5 (Northrop and Kunitz, 1932). Omitting the calcium ions at pH 7.5 further reduced the activity to 127 units/mg. If a solution of trypsin at pH 7.5 was stored for 4 days at 4°C without calcium, the enzyme activity dropped to 99 units/mg. When the enzyme was stored in the presence of calcium, however, the activity only fell to 145 units/mg. This observation was consistent with reports that calcium, although not obligatory for tryptic activity (Green and Neurath, 1953), stabilizes the enzyme against autolysis (Gorini, 1951; Bier and Nord, 1951).

Calcium ions also reduced the sensitivity of trypsin to heat. Enzyme activity fell to zero when trypsin was incubated at 35°C for 75

min in the absence of calcium. In the presence of calcium, however, enzymatic activity dropped only 50%. Incubation of a small volume of a trypsin solution containing calcium in a boiling water bath proved to be an ineffective means of quenching enzyme activity. After one minute at 100°C, the enzyme activity was unchanged. After 5 min the activity fell 21%, and by 10 min the activity had dropped only 31%. Sodium azide, which is frequently added to buffers to prevent bacterial growth, was omitted from the virus preparations because it reduced trypsin activity by 50%.

Conditions for trypsin treatment of virus. A final trypsin concentration of 0.5-0.6 mg/ml was used for all virus treatments. This concentration had the same effect on the hemagglutination activity of NWS or other trypsin-sensitive viruses either when the virus and contaminating protein were suspended in allantoic fluid or when purified virus was suspended in PBS or Tris buffer containing NaCl and $CaCl_2$ (NTC buffer). The concentration of trypsin present during treatment of viral suspensions was generally the same, although the amount of trypsin present varied with the volume of the viral suspension. Since the amounts of virus and contaminating protein present were not usually known but varied considerably from one viral preparation to another, the ratio of substrate to trypsin varied accordingly. The final concentration of trypsin selected for these studies (0.5 mg/ml) was sufficiently high that trypsin was generally in excess.

Although trypsin treatments were conducted at both 40°C and 35°C, the lower incubation temperature was generally used to minimize heat inactivation of the viral hemagglutination and neuraminidase activities. In one experiment, incubation for 8 hr at 35°C without trypsin reduced

the hemagglutination activity of X-29L 50% and the activity of NWS 75%. Such temperature controls, which contained buffer in place of the trypsin solution, were included during each trypsin treatment to distinguish between heat and proteolytic inactivation of the viral hemagglutination activity.

At several time points during trypsin treatment, soybean trypsin inhibitor, which inhibits trypsin mole for mole (Wu and Scheraga, 1962; Frattali and Steiner, 1968), was added to aliquots of the virus at a final concentration equal to that of the enzyme. Since the half-life of the reversible trypsin--soybean inhibitor complex is only about 7 min (Mihalyi, 1972), the reaction mixture was transferred to an ice bath after addition of the inhibitor. At the zero time point, trypsin was added while the virus suspension was in an ice bath. An aliquot was immediately removed and mixed with soybean trypsin inhibitor in the ice bath. The efficiency of this quench procedure in preventing further trypsin digestion was examined by incubating virus with trypsin for 4 hr at 35°C or at 4°C, with or without inhibitor. The results (Table 4.2) indicated that either addition of inhibitor at 35°C or incubation at 4°C was sufficient to prevent trypsin digestion of hemagglutinin as measured by loss of viral hemagglutination activity. This is a relatively insensitive means of assaying enzyme inhibition, however, because half of the initial hemagglutination activity must be lost before enzyme activity is detected.

Comparison of the weight ratio of HA1 to HA2 for a virus not exposed to trypsin with the ratio for a virus exposed to trypsin for "0 min" indicated that trypsin was active even at 0°C. Some HA1 protein digestion had occurred at the zero time point even though no change in

TABLE 4.2

Hemagglutination Activity of X-12 or X-29L after Trypsin Treatment: Influence of Temperature or Soybean Trypsin Inhibitor[a]

	Trypsin, 35°C		Trypsin, 4°C		Trypsin plus inhibitor, 35°C	
Virus	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr
X-12	4096	< 4	4096	4096	2048	4096
X-12	512	< 4	512	256	512	512
X-29L	512	128			512	512

[a]Virus in allantoic fluid was assayed after treatment by method B for 0 hr and 4 hr with trypsin at 35°C or 4°C or with trypsin and soybean trypsin inhibitor at 35°C. Hemagglutination activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 60 min at room temperature.

hemagglutination activity was detected. Before exposure to trypsin, the weight ratio of HA1 to HA2 was 1.14 ± 0.19, n = 9 (mean and standard deviation, number of observations) for X-12 and 0.77 ± 0.14, n = 8 for X-29L. At the zero time points, these values were 0.79 ± 0.08, n = 4 for X-12 and 0.47 ± 0.44, n = 3 for X-29L. Thus about 30-40% of the HA1 protein was lost before the zero time point samples were quenched.

Electrophoretic patterns of trypsin and soybean trypsin inhibitor. The electrophoretic mobilities of trypsin and soybean trypsin inhibitor relative to the viral proteins was determined to distinguish these exogenous proteins from viral degradation products. Trypsin and inhibitor were electrophoresed in separate lanes of a 13% polyacrylamide slab gel next to four different influenza viruses (Fig. 3.4). Reduced trypsin (23,985 daltons, Mihalyi, 1972) migrated as two components, the larger of which ran slightly faster than HA2 (26,000-27,000 daltons; Chapter 3) and the soybean inhibitor (21,500 ± 800 daltons; Wu and Scheraga, 1962; Frattali and Steiner, 1968) migrated between the two trypsin components. The slower-migrating trypsin band had the molecular weight reported for the active enzyme. The second, faster-migrating band appeared to consist of several smaller components, which were probably inactive forms of enzyme that had undergone autolysis (Sipos and Merkel, 1970).

When trypsin-treated virus was pelleted prior to electrophoresis, trypsin and its inhibitor remained in the supernatant and were not detected on gels electrophoresed for 15 hr (Fig. 4.2, 4.3 and 4.20). When the concentrations of trypsin and inhibitor were high, the virus was not pelleted prior to electrophoresis, and electrophoresis was terminated when the marker dye bromphenol blue reached the bottom of the gel, HA2

protein was obscured by the larger trypsin component (Fig. 4.8). When radiolabeled virus was trypsin-treated and the viral proteins were resolved on a polyacrylamide gel, trypsin and soybean inhibitor were not detected during liquid scintillation counting of the gel slices as only radioactivity was monitored. The trypsin--inhibitor complexes were denatured by SDS during preparation of the sample for polyacrylamide gel electrophoresis. The complexes, which appeared during electron microscopy as globular structures that obscured details of the virus ultrastructure, could be separated from trypsin-treated virus by density gradient centrifugation.

Virus yields from several types of host cells. The yield of virus obtained from several types of host cell was determined prior to choosing a cell type for culture of radioactive virus. The virus yields, which were measured by determining the hemagglutination activity per milliliter of culture medium, are shown in Tables 4.3 and 4.4. The highest virus yields were obtained by recovering virus from embryonated eggs 42 to 48 hr after infection. One egg normally gave 6 to 10 ml of allantoic fluid containing about 1500 hemagglutination units (HAU) per milliliter. When virus was recovered after 24 hr from chicken chorioallantoic membrane (CAM) pieces infected twice at a 30-minute interval with 0.1 ml of undiluted virus per ml of medium, the virus yield was 20-25% of the yield from eggs. When the CAM medium was infected with a virus suspension diluted a thousand-fold, no progeny virus was detected in 24 hr. Even after an infection period of 72 hr, the virus yields were only about one-fifth of those for undiluted virus after 24 hr.

The virus yields from infection of chicken embryo fibroblast (CEF) monolayers made from 8-day eggs were lower after 24 hr for X-12

TABLE 4.3

Yields of Viruses X-12 and X-29L

Grown in CAM, CEF, and Clone 1-5C-4 Cells

Host cell, conditions	Infection time, hr	Hemagglutination activity per ml[a]			
		X-12		X-29L	
		Mean	Range	Mean	Range
CAM in vivo, 11-day eggs	42-48	1500	256-8192	1600	256-8192
CAM in vitro, undiluted virus	24	400	128-1024	300	128-1024
	48	600	512-1024	330	256-512
CAM in vitro, virus diluted $1:10^3$	24	< 4		< 4	
	48	60	32-128	50	16-64
	72	90	32-128	50	16-64
CEF in vitro, 8-day eggs	24	100	64-128	450	256-512
CEF in vitro, 9-day eggs	24	20	8-32	150	64-256
CEF in vitro, 10-day eggs	24	40	16-64	256	
Clone 1-5C-4, 5 PFU per cell	24	25	16-64	50	32-64
	48	64	16-64	256	64-512
	72	32		64	
Clone 1-5C-4, 10 PFU per cell	24	64	16-64	60	16-128
	48	100		240	32-512
	72	64		100	32-128

[a]Hemagglutination titers per ml of culture medium or allantoic fluid are expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 60 min at room temperature.

TABLE 4.4

Yields of Several Egg-Grown Viruses from Infection of MDBK, BHK and MDCK Cell Lines

Cell line	Virus (serotype)	Hemagglutination activity per ml[a]		Infection of eggs with virus from first cell line passage, EID_{50}
		First virus passage in cell line	Second virus passage in cell line	
MDBK	X-12 (H0N2)	138	< 4	$10^{5.5}$
MDBK	X-29L (H0N2)	1024	< 4	$10^{4.5}$
MDBK	NWS (H0N1)	512	< 4	$10^{4.5}$
MDBK	NWS_{t8} (H0N1)	8192	< 4	$10^{3.8}$
MDBK	X-3 (H0N1)	128	< 4	$> 10^{4.0}$
BHK	X-12	512	< 4	$> 10^{4.0}$
BHK	X-29L	512	< 4	$> 10^{4.0}$
BHK	NWS_{t8}	1024		
BHK	X-3	32		
MDCK	X-12	32		
MDCK	X-29L	64	< 4	
MDCK	NWS_{t8}	128		
MDCK	X-3	32		

[a]Hemagglutination activity per ml of culture fluid is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 60 min at room temperature. The value shown is the average hemagglutination activity of two tissue culture dishes.

(100 HAU/ml) than for X-29L (450 HAU/ml). Since the infectivity of the stock viruses was not generally determined by plaquing on CEF cells, undiluted virus was used to achieve the highest available multiplicity of infection. When virus was recovered after 48 hr from Clone 1-5C-4 cell monolayers infected at high multiplicity (5 to 10 PFU per cell), the virus yield was about 10% of the yield from eggs. Yields were slightly lower at shorter or longer infection periods.

Three continuous kidney cell lines were examined in another experiment. The yields of both X-12 and X-29L were higher from Madin-Darby bovine kidney (MDBK) cells or baby hamster kidney (BHK) cells than from Madin-Darby canine kidney (MDCK) cells. The EID_{50} titers for virus isolated from the first MDBK cell passage were low, averaging about 10^5 compared to 10^7 for egg-grown virus. Attempts to infect fresh MDBK or BHK cells with virus isolated from the same cell type were unsuccessful.

Trypsin treatment of the resistant parental virus $RI/5^-$. The hemagglutination activity of $RI/5^-$ (H2N2) was not changed by treatment with trypsin for as long as 4 hr (Table 4.5). The hemagglutination activities detected in the supernatants after pelleting of the trypsin-treated virus were also constant for 4 hr (Table 4.5). The activity of the closely related strain $RI/5^+$ (H2N2) was also unchanged after trypsin treatment (see Table 4.12). In addition, the neuraminidase activity of $RI/5^-$ was not affected by trypsin treatment for 4 hr (Fig. 4.1).

The densitometric patterns of the viral proteins during trypsin treatment of purified egg-grown $RI/5^-$ are shown in Fig. 4.2, and the corresponding relative peak areas are given in Table 4.5. After exposure

TABLE 4.5

Trypsin Sensitivity of the Parental Viruses RI/5$^-$ and NWS

Virus	Time (min)	Relative peak area,[a] % NP/M	HA1/NP+M	HA2/NP+M	HA1/HA2	HA activity[b] Supernatant	Pellet
RI/5$^-$	0	100	100	100	100	2048	16384
	60	105	96	106	91	2048	16384
	240	107	86	88	98	2048	16384
NWS	0	100	100	100	100	512	16384
	30	71	55	78	71	4	512
	60	66	46	77	59	0	4
	240	79	24	73	33	0	0

[a]Peak areas were measured by cutting out and weighing tracings of the peaks of densitometric scans of Coomassie blue-stained disc gels. The electrophoretic profiles are shown in Fig. 4.2 and 4.3. The ratio at the time indicated was divided by the ratio at 0 min.

[b]Hemagglutination activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 60 min at room temperature.

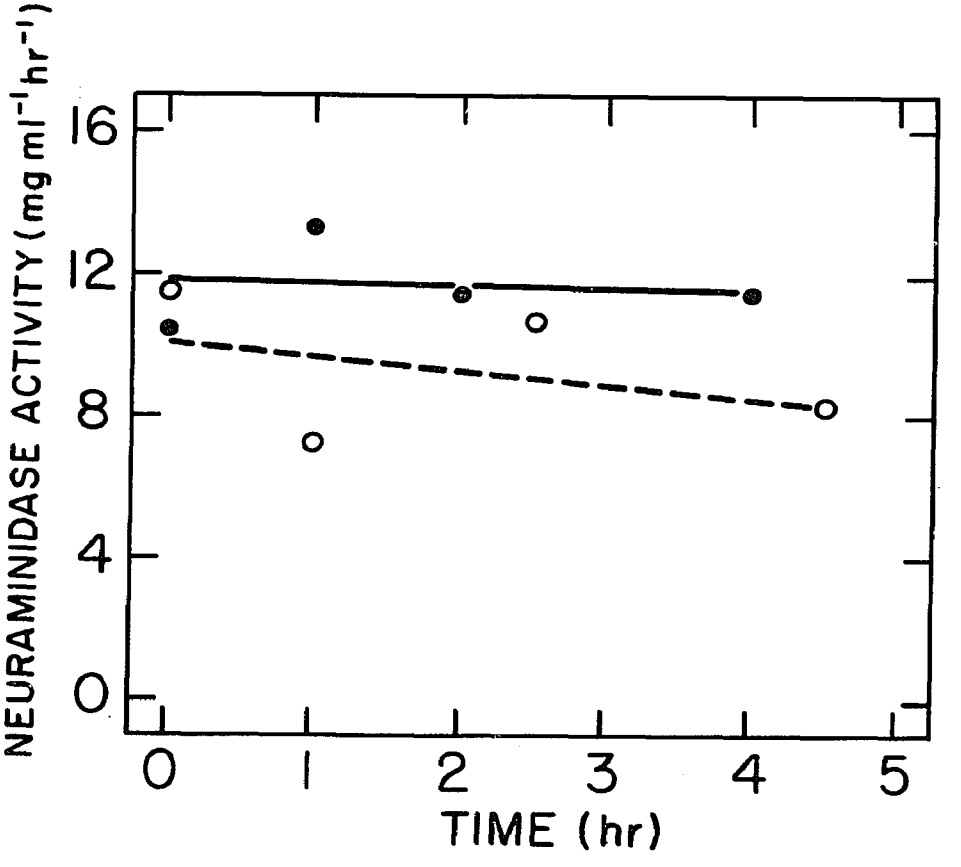

Fig. 4.1. Neuraminidase activity during trypsin treatment of RI/5⁻. Purified virus was treated with trypsin by method B and equal volumes of virus suspension were assayed with fetuin at the indicated time points. Two separate experiments (O , ●) and the least squares lines of best fit are shown. The trypsin sensitivity of the N1 neuraminidase of NWS was not determined because this enzyme is heat-sensitive (Rafelson et al., 1963 ; Paniker, 1968), and it is difficult to distinguish between trypsin and heat inactivation.

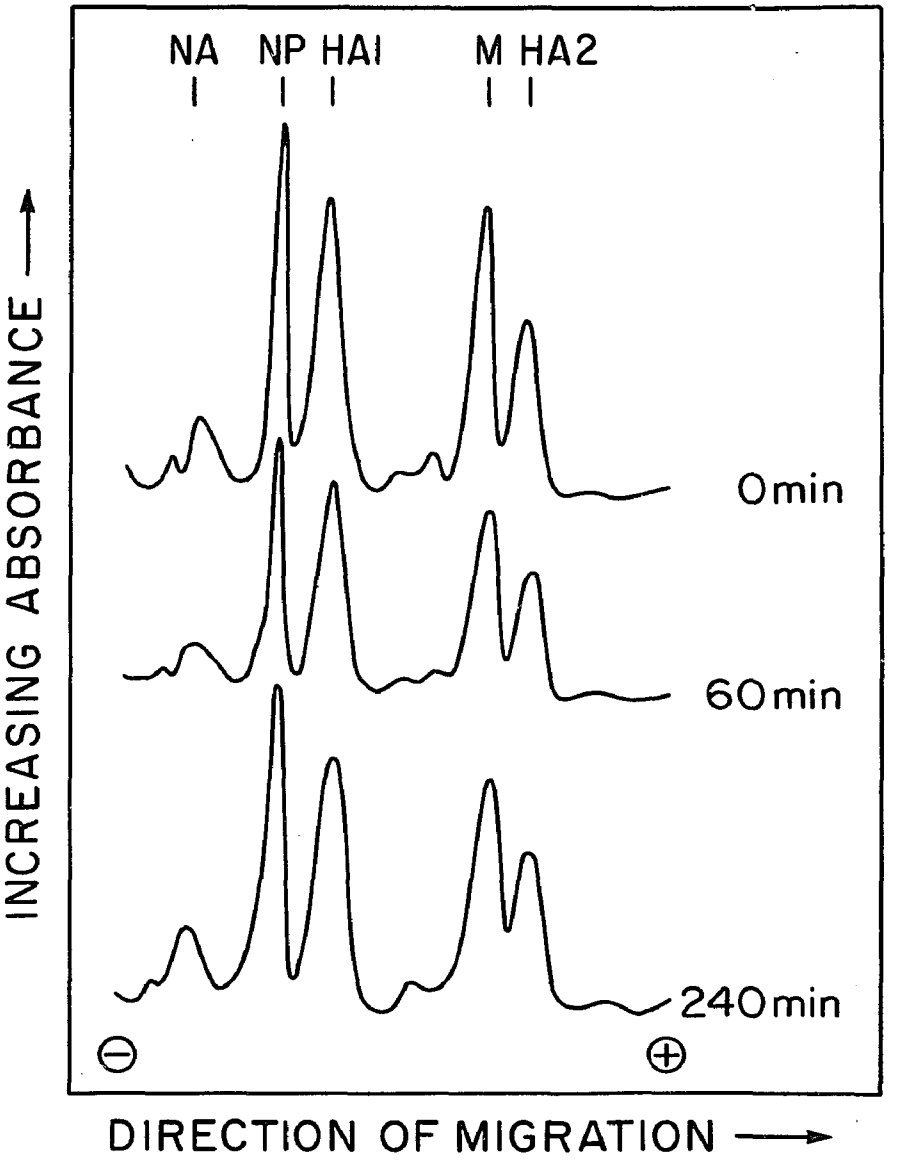

Fig. 4.2. Electrophoretic profile of RI/5^{-} proteins during trypsin treatment. Purified virus was treated by method B. Densitometric tracings of 13% polyacrylamide gels stained with Coomassie blue are shown. The relative peak areas are shown in Fig. 4.5.

to trypsin for 4 hr, the relative amount of HA1 decreased only about 10% and the amount of HA2 was essentially unchanged.

Trypsin treatment of the sensitive parental virus NWS. Exposure of the virus NWS (H0N1) to trypsin resulted in substantial loss of hemagglutination activity in 30 to 60 min (Table 4.5). The effect of trypsin on the neuraminidase activity of NWS was not measured because this activity is heat-labile for viruses of N1 serotype (Rafelson et al., 1963; Paniker, 1968).

The densitometric patterns of the viral proteins during trypsin treatment of purified, egg-grown NWS are shown in Fig. 4.3, and the corresponding relative peak areas are presented in Table 4.5. The loss of hemagglutination activity was accompanied by a loss of HA1 protein. About 55% of HA1 was lost in 30 min and essentially all of HA1 was lost in 4 hr. The HA2 protein was also degraded by exposure to trypsin, although at a much slower rate than HA1. About 25% of the HA2 protein was lost in 30 min, but the amount of HA2 then remained constant for the next 3.5 hr. An active HA component was not released into solution by trypsin cleavage because the hemagglutination activity detected in the supernatant after pelleting of the trypsin-treated virus was low and decreased during trypsin treatment in a manner similar to the hemagglutination activity of the pelleted virus. The supernatant protein concentrated by acetone precipitation or ultrafiltration (Amicon, PM-10 membranes) gave a faint gel pattern containing all of the viral proteins and substantial amounts of trypsin and soybean trypsin inhibitor, but no other proteins larger than HA2 were seen.

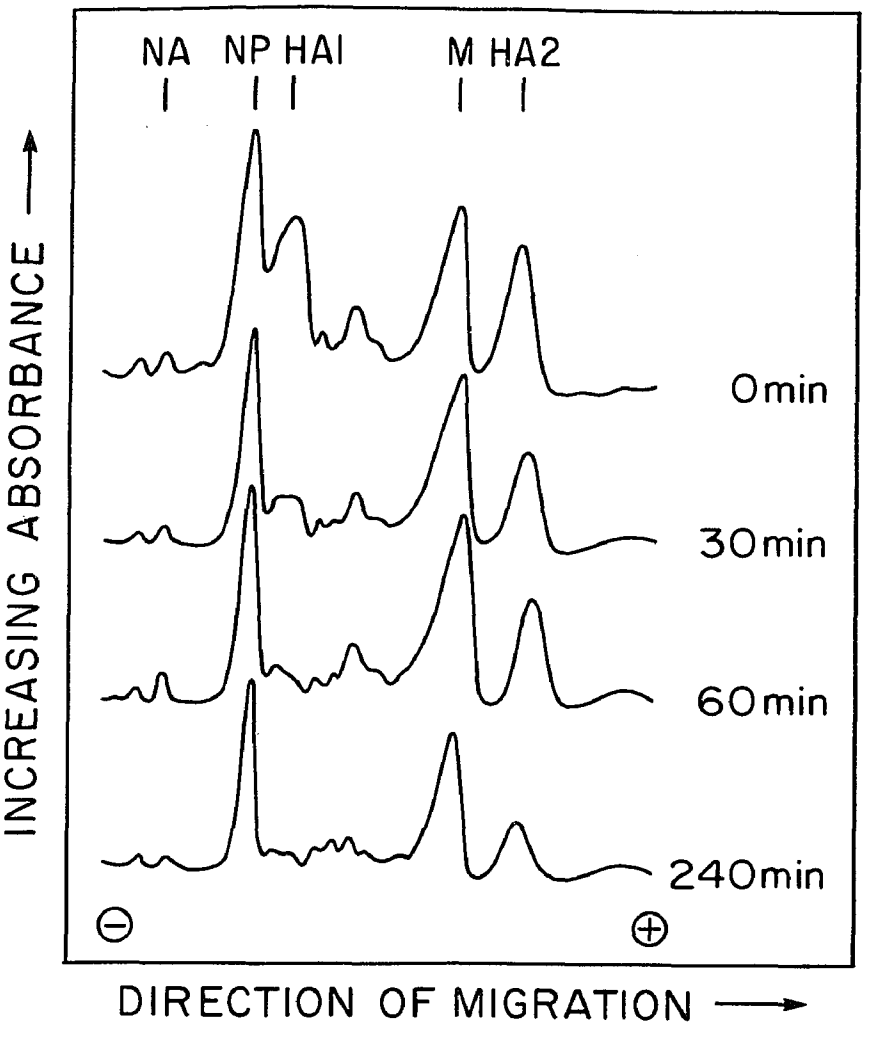

Fig. 4.3. Electrophoretic profile of NWS proteins during trypsin treatment. Purified virus was treated by method B. Densitometric tracings of 13% polyacrylamide gels stained with Coomassie blue are shown. The relative peak areas are shown in Table 4.5.

Trypsin treatment of the recombinant viruses X-12 and X-29L.

A. Hemagglutination activity. The recombinant X-12 (HON2) is defined as "trypsin-sensitive" because it loses almost all of its hemagglutination activity on trypsin treatment for 90 min (Chapter 2). The recombinant X-29 (HON2) and its large-plaque variant X-29L (HON2) are described as being "trypsin-resistant" because they lose little or no hemagglutination activity under those conditions. In general, the same results were obtained using a virus suspension in allantoic fluid (Tables 2.4 and 4.6; Fig. 4.4) or a suspension of purified virus in PBS or Tris buffer containing NaCl and $CaCl_2$ (Fig. 4.5 and 4.6). Precision assay of hemagglutination activity or prolonged treatment showed that X-29 and X-29L are not totally resistant to trypsin. For example, during trypsin treatment for 2 hr X-29L progressively lost 40% of its initial activity after correction for heat inactivation (Fig. 4.6). In addition, after a 1-hr period of apparent resistance, X-29 slowly lost 97% of its initial activity over the next 6 hr (Fig. 4.5).

B. Infectivity. The effect of trypsin treatment on viral infectivity was not as dramatic as the effect on hemagglutination activity. In a typical experiment, the hemagglutination activity of X-12 dropped three orders of magnitude in 30 min but the viral infectivity, as measured by plaque formation of Clone 1-5C-4 cells, decreased only one order of magnitude (Fig. 4.4).

C. Neuraminidase activity. The neuraminidase activity of these N2 recombinants was not affected by exposure to trypsin. The neuraminidase activity for both X-12 and X-29L remained relatively constant during trypsin treatment for 2 hr (Fig. 4.7). The variations observed in activity with time were probably due to slight differences in the quantity of virus assayed.

TABLE 4.6

Hemagglutination Activity of Viruses X-12, X-29L, WSN and NWS_{t8} during Trypsin Treatment for 240 Minutes[a]

Time (min)	X-12 (HON2)	X-29L (HON2)	NWS_{t8} (HON1)	WSN (HON1)
0	1024	1024	8192	1024
15	2048	1024 (512)	2048	1024
30	2048	1024	4096	1024
60	256 (0)	1024	4096	1024
90	< 4	1024	4096	2048 (512)
120	< 4	2048 (512)	4096	2048 (1024)
180	4	2048	4096 (2048)	1024 (512)
240		4096	4096 (2048)	1024 (512)

[a]Hemagglutination activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 2.5 hr at 4°C. Values in parentheses show the change after the assay tubes were kept at room temperature for 2 hr. The viruses were assayed in allantoic fluid, except that WSN was grown in MDBK cells and assayed in the culture fluid.

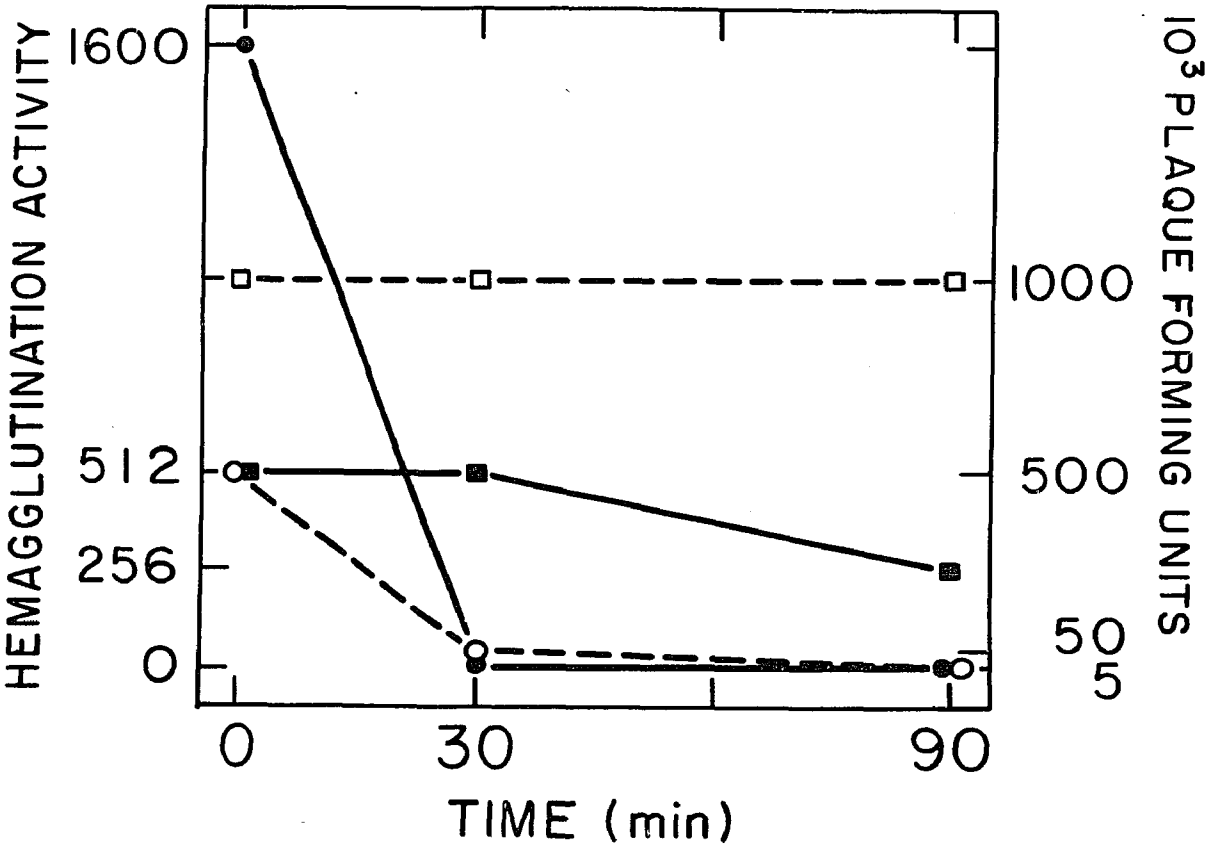

Fig. 4.4. Hemagglutination activity and viral infectivity during trypsin treatment of X-12 and X-29. Virus in allantoic fluid was treated with trypsin by method A and portions (0.01 ml for X-12 and 0.1 ml for X-29) were bioassayed. Hemagglutination activity is expressed as the reciprocal of the highest dilution of X-12 (●) or X-29 (■) that gave complete hemagglutination. Viral infectivity is expressed as the number of plaque-forming units detected by plaquing of X-12 (○) or X-29 (□) on Clone 1-5C-4 cells. (E. D. Kilbourne, unpublished results).

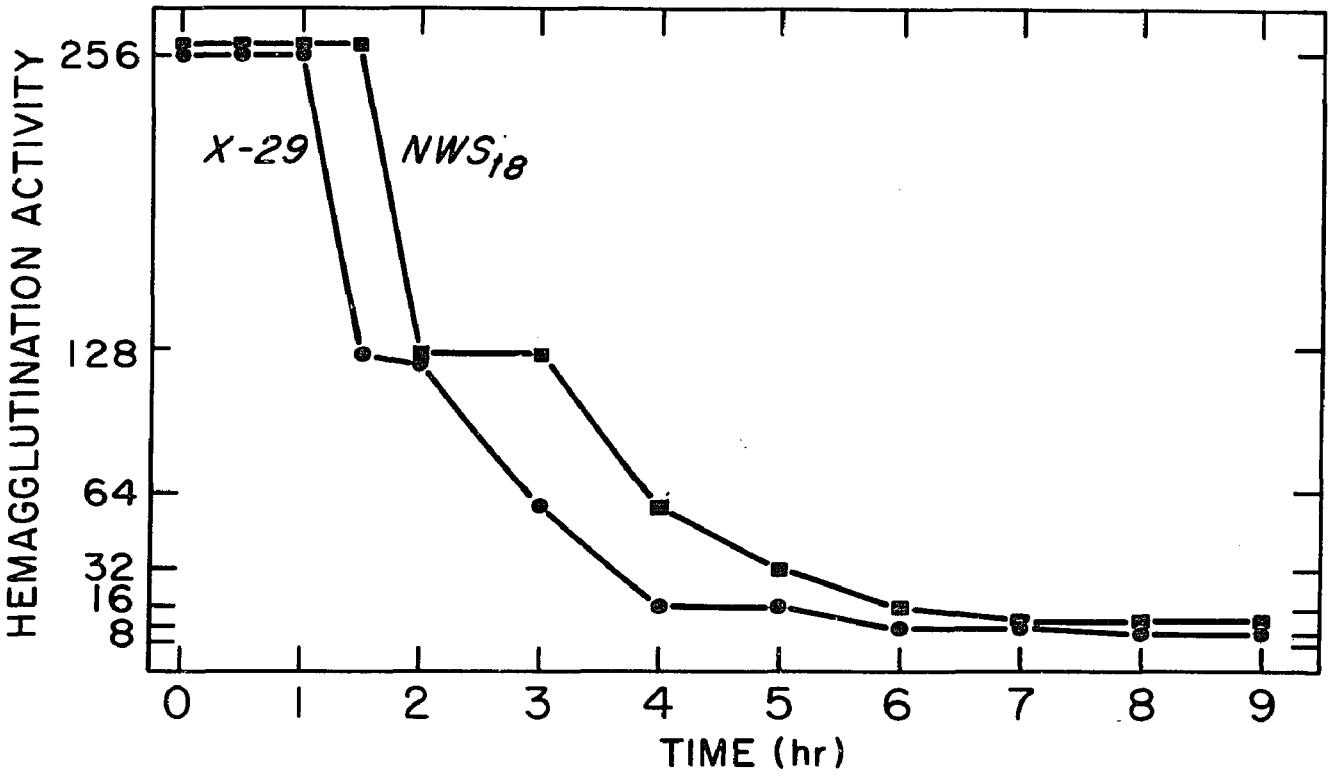

Fig. 4.5. Hemagglutination activity of the purified viruses X-29 and NWS_{t8} during prolonged treatment with trypsin. Activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination after 1 hr at room temperature: X-29 (●) and NWS_{t8} (■).

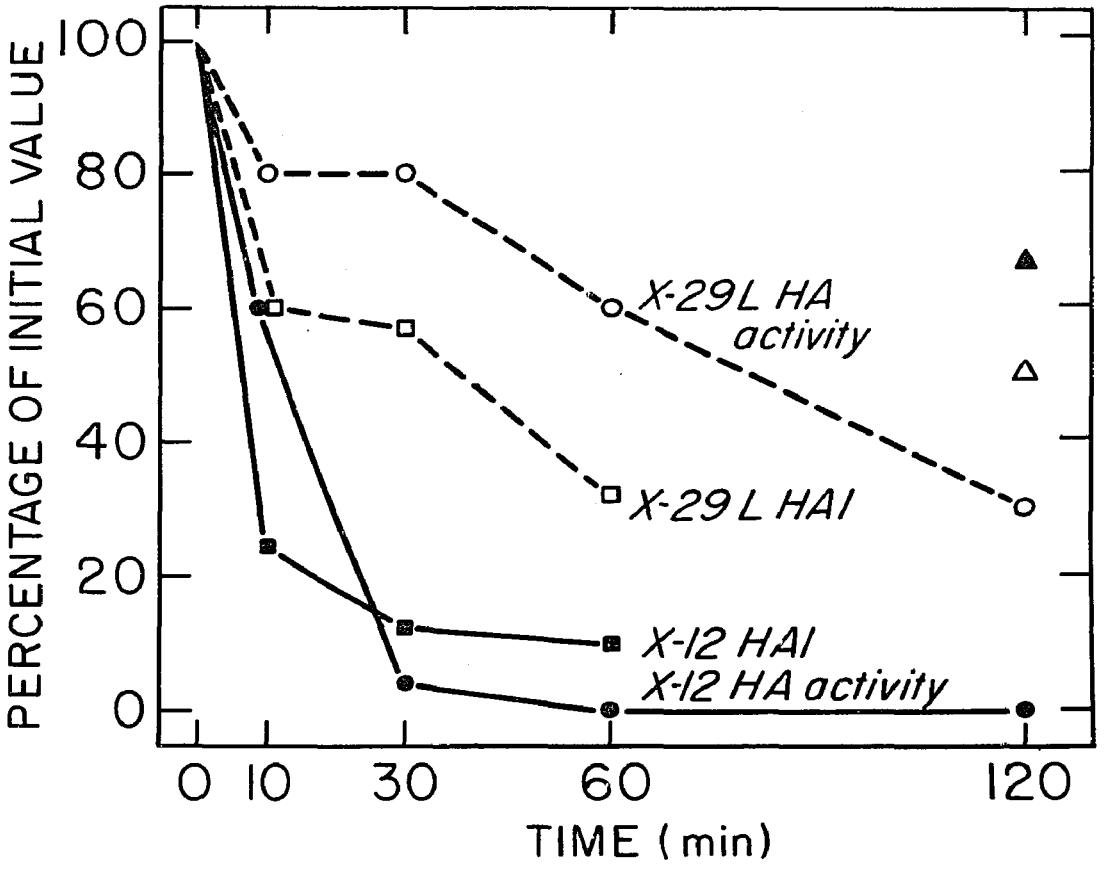

Fig. 4.6. Relative amount of HA1 protein and hemagglutination activity of viruses X-12 and X-29L during trypsin treatment. Polyacrylamide gels and details of these experiments are shown in Fig. 4.8. The amount of the HA1 protein for X-12 (■) and X-29L (□) is normalized to the amount of the M protein, where the amounts were measured by integration of densitometric tracings of stained polyacrylamide gels. Hemagglutination activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 2.5 hr at 4°C and is normalized to the initial value (5120 for both viruses). Activities are shown for X-12 with (●) and without (▲) trypsin, and X-29L with (○) and without (△) trypsin.

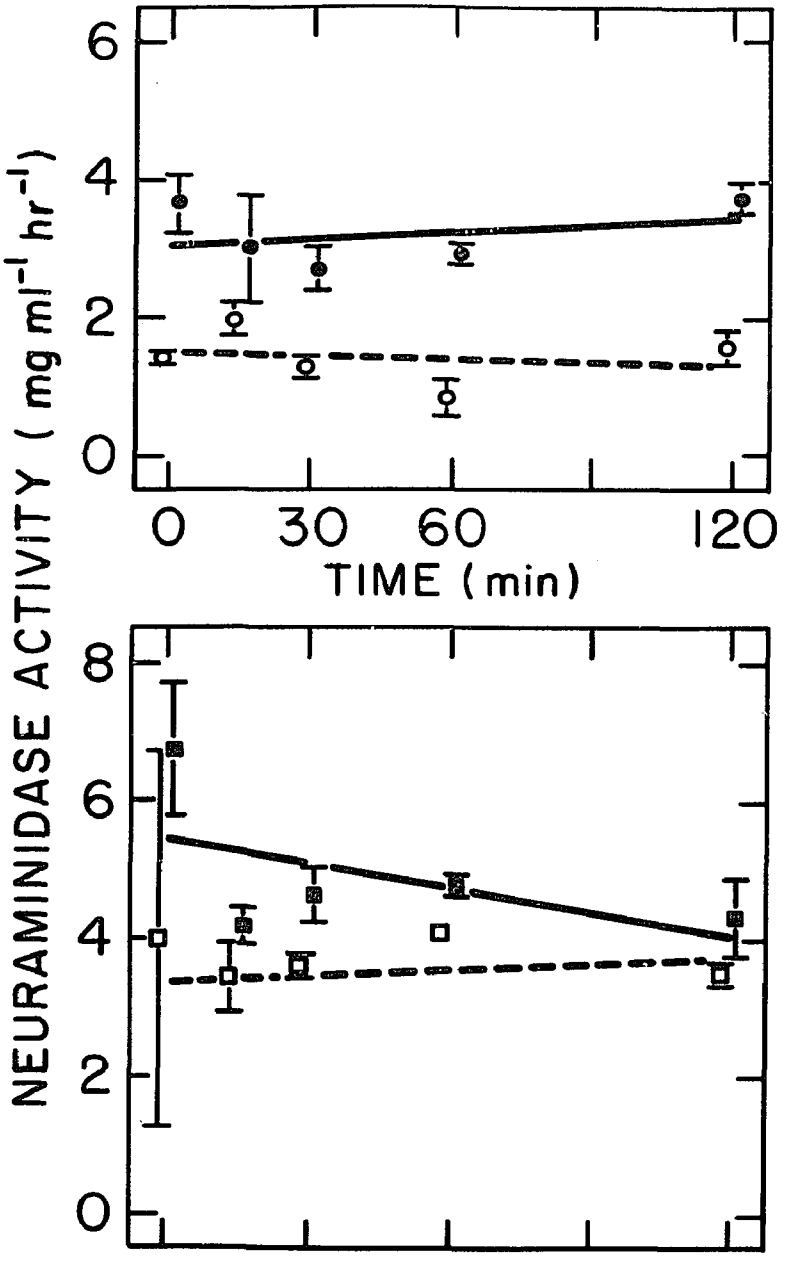

Fig. 4.7. Neuraminidase activity during trypsin treatment of X-12 and X-29L. Purified virus was treated with trypsin by method B and equal volumes of virus suspension were assayed with fetuin at the indicated time points. Assay values were not normalized to hemagglutination activity or total protein because both decreased with time. Two separate experiments are shown for X-12 (○ , ●) and for X-29L (□ , ■). The symbols and error bars indicate the mean and standard deviation of four assays per time point. The lines are the least squares lines of best fit for all 20 assays per experiment.

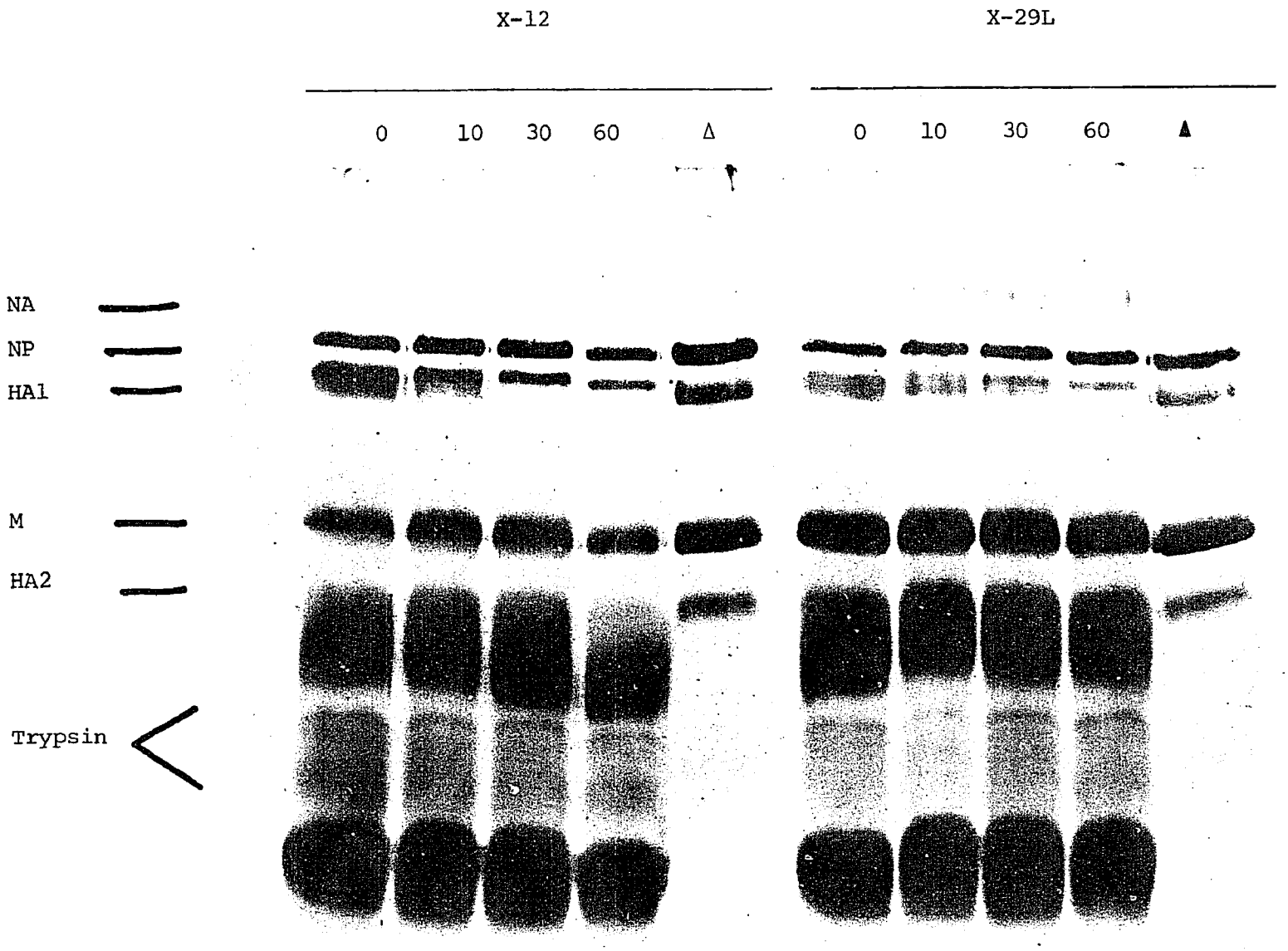

Fig. 4.8. See next page for legend.

Legend for Fig. 4.8. Figure is shown on the previous page.

Fig. 4.8. Stained polyacrylamide gels of egg-grown purified viruses X-12 and X-29L during trypsin treatment. Viral suspensions containing equal final concentrations of viral protein (1.9 mg/ml) were treated by method B, except that the final trypsin concentration (2.0 mg/ml) was increased fourfold to compensate for the larger concentration of viral protein. The hemagglutination activity of each virus at the zero time point was 5120. At time points, one aliquot of each reaction mixture was immediately denatured, reduced, and alkylated (see Chapter 3) for gels, and a second aliquot was mixed in an ice bath with 50 equivalents of soybean trypsin inhibitor and serially diluted for precision hemagglutination assay at 4°C. Shown above for both viruses are the proteins present at 0, 10, 30 and 60 min plus a 120-min control without trypsin (Δ , ▲) after electrophoresis on a 13% polyacrylamide slab gel and staining with Coomassie blue. Electrophoresis was continued for 30 min after the marker dye bromphenol blue reached the bottom of the gel to enhance the resolution of HA2 from the upper trypsin component. The relative amount of HA1 protein and hemagglutination activity are shown in Fig. 4.6. Densitometric patterns of HA1 protein are shown in Fig. 4.9, and the change with time of the slower and faster HA1 components relative to the hemagglutination activity are shown in Fig. 4.10.

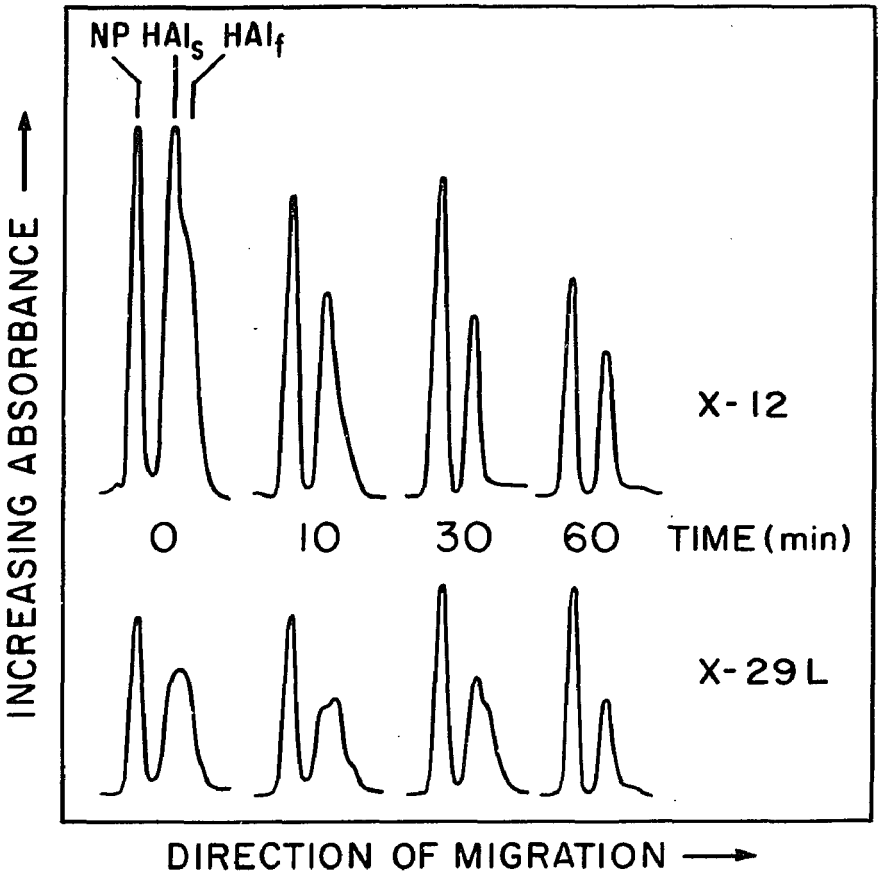

Fig. 4.9. Densitometric patterns of the HA1 protein during trypsin treatment of X-12 and X-29L. See Fig. 4.8 for a photograph of the slab gel and the experimental details. The NP peak and the HA1 peak are shown at four time points for each virus. The change with time of the relative areas of the slower HA1 component (HAl_s) and the faster component (HAl_f) are shown in Fig. 4.10.

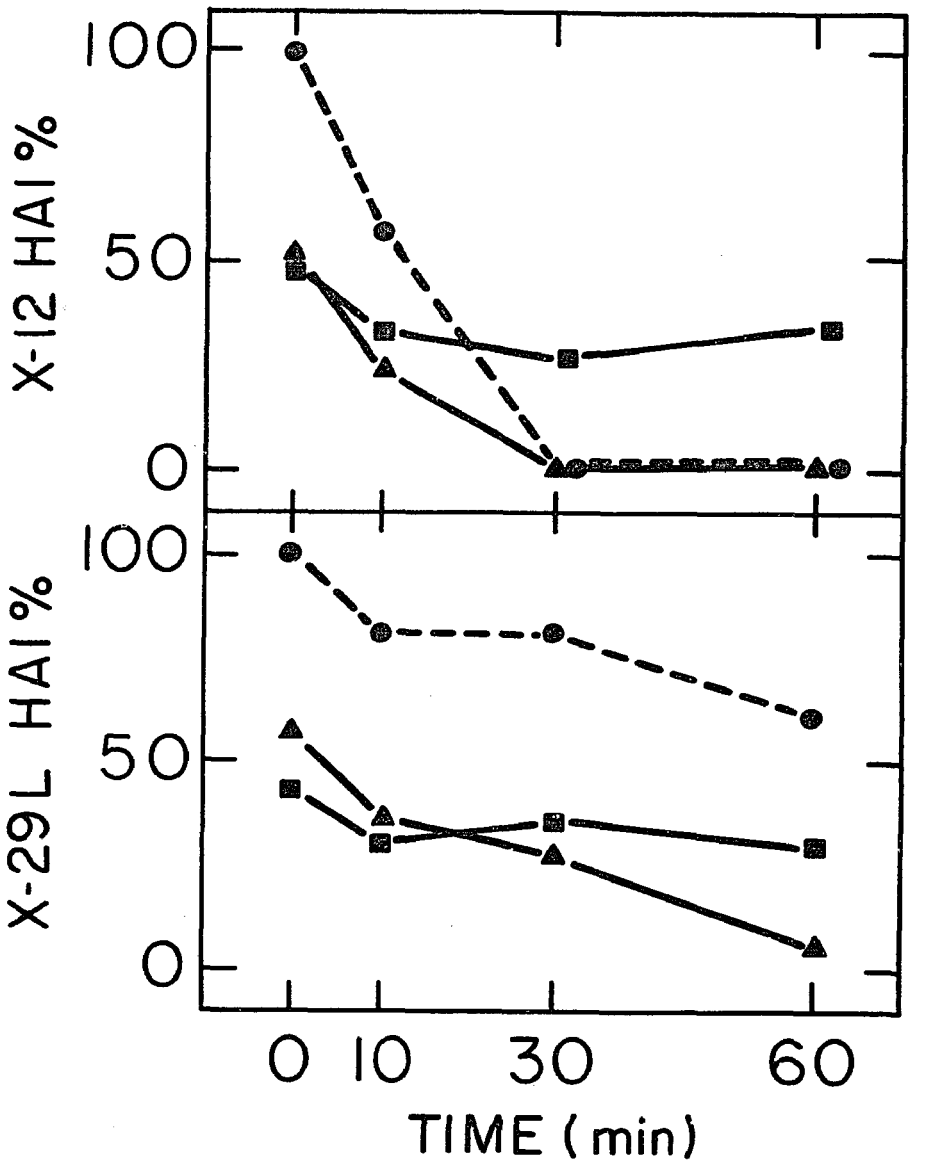

Fig. 4.10. Relationship of hemagglutination activity to the relative amounts of the slower and faster HA1 components present during trypsin treatment of X-12 and X-29L. The slab gel and the experimental details are given in Fig. 4.8; densitometric patterns of HA1 protein are shown in Fig. 4.9. Hemagglutination activity (●--●) is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination in 2.5 hr at 4°C and is normalized to the initial value (5120 for both viruses). At the zero time point, the total HA1 protein ($HA1_t$) is assumed to consist initially of a slower migrating component ($HA1_s$) and a faster migrating component ($HA1_f$). The area of the $HA1_s$ peak was estimated by multiplying the densitometric area of the NP peak by the height ratio of the $HA1_s$ and NP peaks. The amount of $HA1_s$ (■) is expressed as the area ratio of the $HA1_s$ and NP peaks at the time indicated divided by the corresponding ratio at 0 min. The area of the $HA1_f$ peak was estimated by subtracting the estimated area of the $HA1_s$ peak from the area of the $HA1_t$ peak. The amount of $HA1_f$ (▲) is expressed as the area ratio of the $HA1_f$ and NP peaks at the time indicated divided by the corresponding ratio at 0 min.

of $HA1_f$. Exposure to the protease, even for the short period required to quench the zero time point sample, resulted in the disappearance of nearly 50% of the $HA1_f$ component and appearance of an equal quantity of $HA1_s$.

Since the viruses were not concentrated by centrifugation prior to electrophoresis, the HA2 protein was not clearly resolved from trypsin. Thus it was impossible to determine if the HA2 subunit was also affected by trypsin. None of the viral proteins underwent a shift in molecular weight with time, and no new protein bands appeared above HA2 as trypsin treatment progressed.

The hemagglutination activity of both viruses at each time point is compared in Fig. 4.6 to the quantity of HA1 protein present as determined by densitometric scans of the stained gel. In this 2-hr experiment, the hemagglutination activity of X-29L decreased with trypsin treatment, but at a much slower rate than for X-12. Although the hemagglutination activity of the X-29L control virus, which was held at 35°C for 2 hr in the absence of trypsin, fell to half of the initial value, the sample of X-29L treated with trypsin for 2 hr had dropped significantly lower. In the first 10 min of trypsin treatment of X-12 and in the first 60 min for X-29L, the amount of the HA1 protein dropped faster than did hemagglutination activity.

E. Electrophoretic patterns of radiolabeled proteins. The trypsin treatments were repeated using X-12 and X-29L viruses grown in CAM cells in the presence of ^{14}C- and ^{3}H-labeled amino acids, respectively. Sufficient virus was provided for hemagglutination assays by adding the radiolabeled virus to a larger amount of unlabeled virus grown in the same cell type. Virus was then purified and trypsin treated by method B.

At each time point, an aliquot of virus was mixed with soybean trypsin inhibitor in an ice bath. A portion of each aliquot was assayed for hemagglutination activity and scored after standing overnight at 4°C. The remaining trypsin-treated virus was pelleted, denatured, reduced and electrophoresed on a 13% polyacrylamide disc gel containing SDS. The gels were sliced and counted. For each gel slice the background counts were subtracted and the net counts per minute (cpm) were converted into disintegrations per minute (dpm). The amount of each viral protein was estimated by adding the dpm under each peak. The amounts of HA1 and HA2 were normalized to the sum of the amounts of NP and M by dividing the total dpm in the HA1 and HA2 peaks by the sum of the dpm in the M and NP peaks. The consistency of the recovery of NP and M from one gel to another was determined by calculating the ratio of the areas of these peaks.

The electrophoretic patterns of CAM-grown, ^{3}H-labeled X-12 virus during trypsin treatment are shown in Fig. 4.11. These results confirmed the results obtained using unlabeled X-12 grown in embryonated eggs (i.e. CAM cells in vivo). The quantity of HA1 protein decreased with increasing time of exposure to the protease (Fig. 4.12). The amount of HA2 protein also decreased, but more slowly than HA1. After loss of nearly half of the HA2 protein during the first 30 min, the quantity of HA2 remained relatively constant for the next 90 min. The percentage loss of HA1 protein is compared with the percentage loss of hemagglutination activity in Fig. 4.12. From 30 to 120 min the hemagglutination activity was lost faster than viral-associated HA1 protein was lost.

After the hemagglutination assay tubes were scored, the tubes for each time point were pooled and centrifuged to measure the efficiency of viral adsorption to the erythrocytes (Table 4.7). The amount of

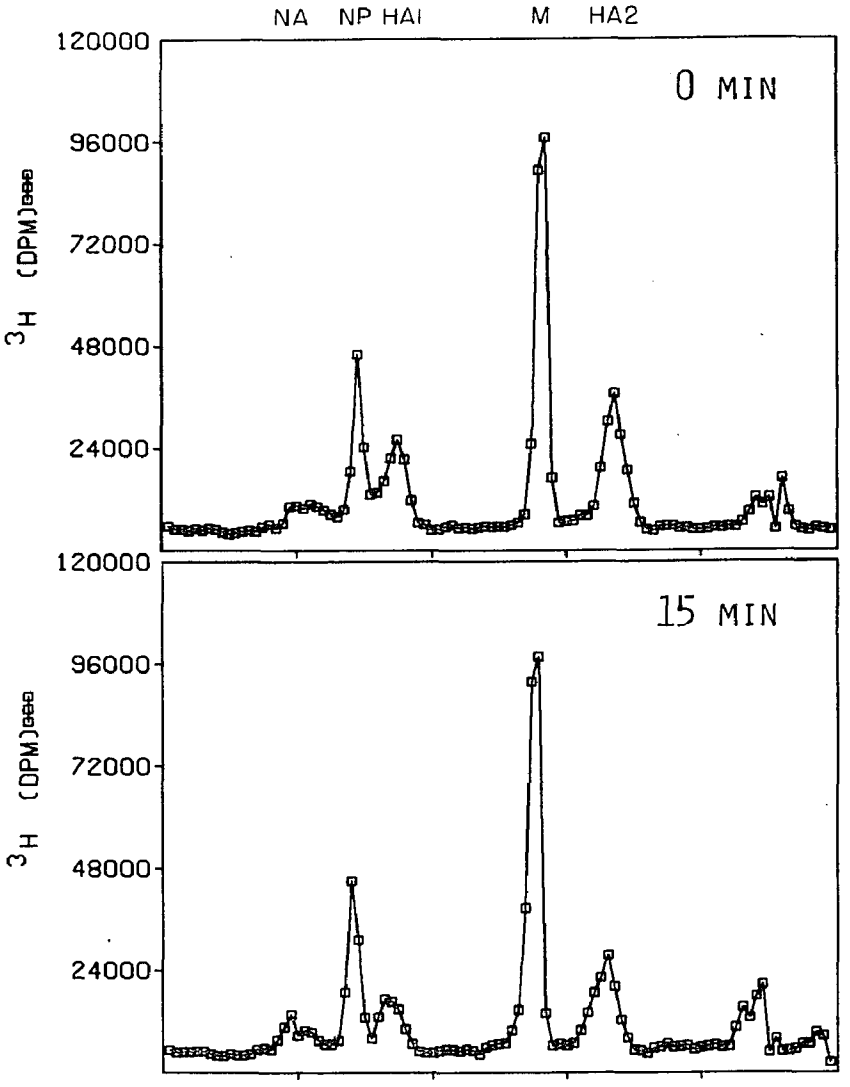

Fig. 4.11. [This figure is continued on the next page.]

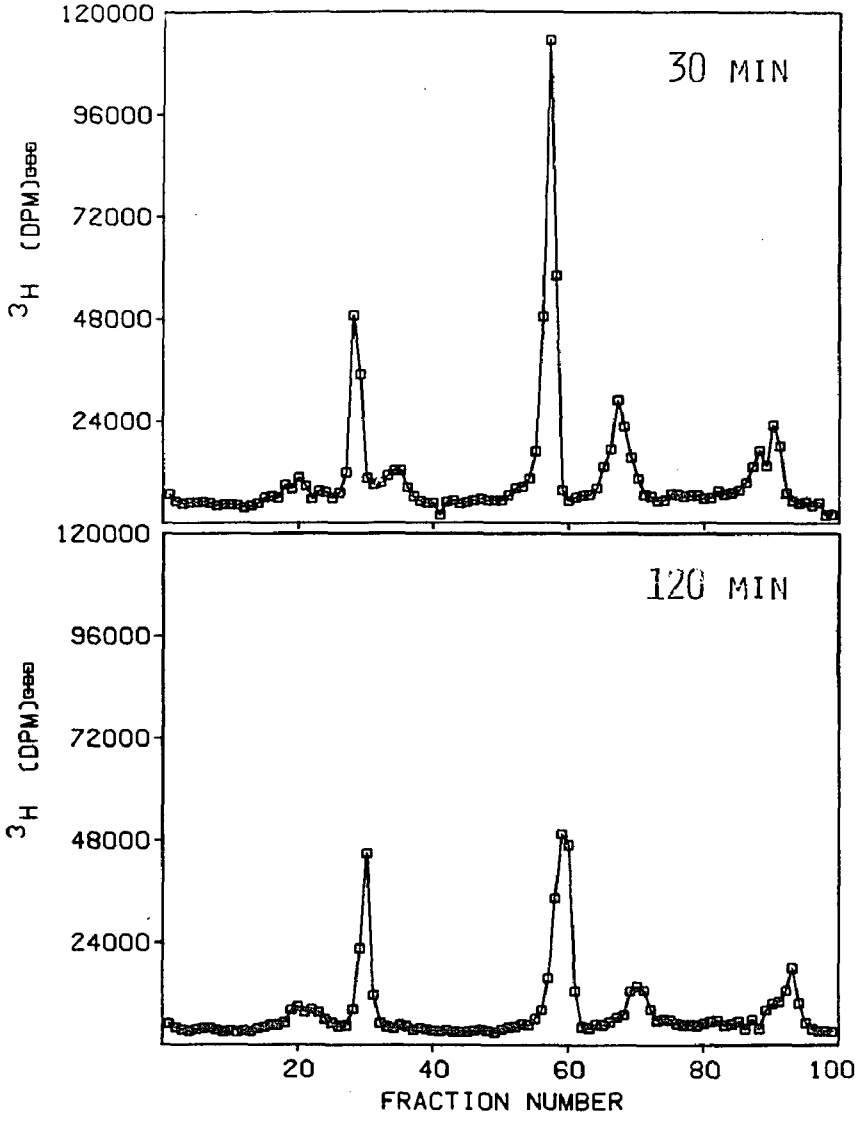

Fig. 4.11. Electrophoretic patterns of radiolabeled viral proteins during trypsin treatment of X-12. Purified virus was treated by method B and assayed for hemagglutination activity (Fig. 4.12). Reduced virus samples were electrophoresed on 13% polyacrylamide gels for 15 hr (about 4 hr past elution of the marker dye). The dpm observed for the ^{3}H-labeled viral proteins are shown.

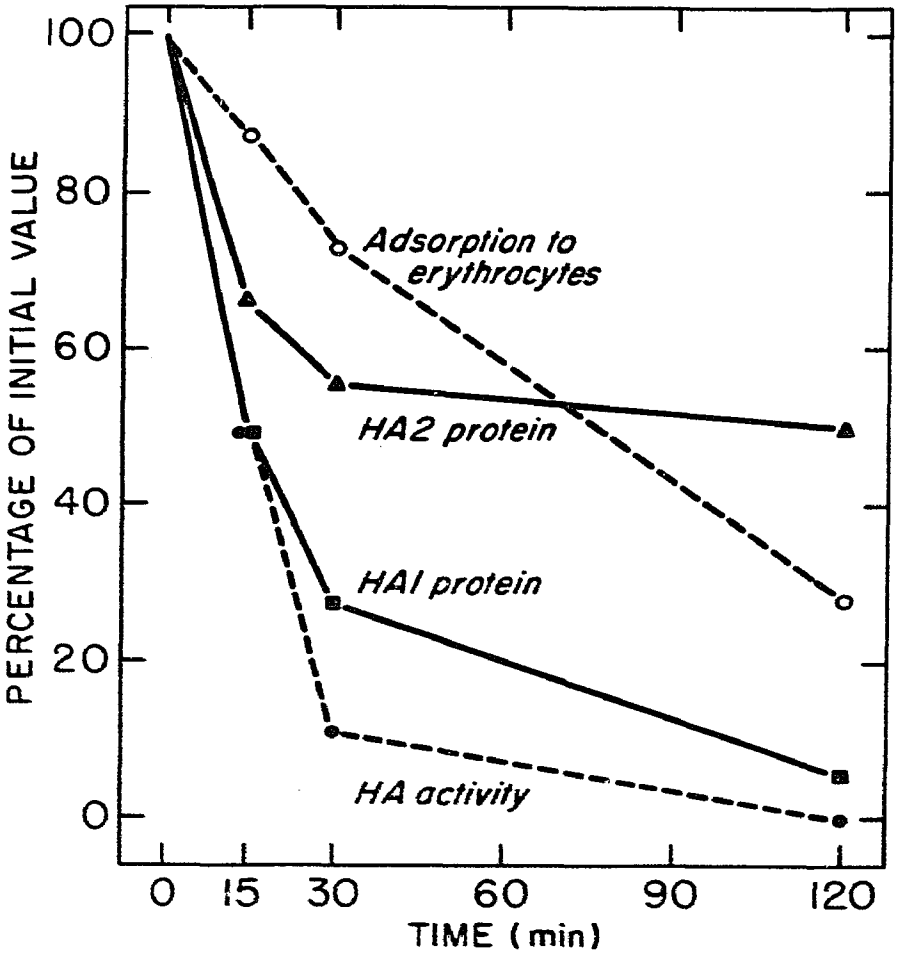

Fig. 4.12. Relative amounts of HA1 and HA2 protein, hemagglutination activity, and erythrocyte adsorption of X-12 during trypsin treatment. Amounts of HA1 (■—■) and HA2 (▲—▲) were measured by integration of the radioactive protein peaks in Fig. 4.11, and are normalized to the sum of the areas of the NP and M peaks. The dpm ratio of NP/M was relatively constant for the four time points (mean ± standard deviation: 0.41 ± 0.09). Hemagglutination activity (●--●) and erythrocyte adsorption (○--○) are taken from Table 4.7.

TABLE 4.7

Interaction of Trypsin-Treated, Radiolabeled X-12 Virus with Erythrocytes:

Relative Efficiency of Adsorption and Cross-Linking

	Adsorption of virus to erythrocytes[a]			Cross-linking of erythrocytes		
Time of trypsin treatment, min	Virus free in supernatant, cpm (%)	Virus adsorbed in pellet, cpm (%)	Relative adsorption efficiency[b]	Hemagglutination activity[c]	Relative cross-linking efficiency[d]	Ratio of adsorption to cross-linking[e]
0	337 (30%)	789 (70%)	1.00	2048	1.00	1.0
15	501 (40%)	766 (60%)	0.86	1024	0.50	1.7
30	637 (49%)	672 (51%)	0.73	256	0.125	5.9
120	1132 (80%)	286 (20%)	0.29	16	0.0078	37

[a]The first eight hemagglutination assay tubes (three tubes beyond the last tube that gave complete hemagglutination) were pooled and layered over 1 ml of 1% sucrose in PBS. After slow centrifugation to pellet the erythrocytes, the ^{14}C-labeled virus in the pellet and supernatant was measured by scintillation counting. The cpm values were corrected for quenching (20% in the supernatants, 52% in the pellets) through multiplication by quench correction factors (1.26 ± 0.05 for the supernatants, 2.08 ± 0.06 for the pellet suspensions) determined by addition of [^{14}C] amino acids. Shown in parentheses is the percentage of radiolabeled virus in the supernatant or pellet, where the sum is taken as 100%.

[b]The percentage of adsorbed virus at the time indicated was divided by the percentage at 0 min. The results are graphically compared with the amounts of HA1 and HA2 protein in Fig. 4.12.

TABLE 4.7, continued

[c]Hemagglutination activity was assayed after trypsin treatment by method B for the time indicated and is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination after overnight incubation at 4°C.

[d]The hemagglutination activity at the indicated time was divided by the activity at 0 min. The results are graphically compared with the amounts of HA1 and HA2 in Fig. 4.12.

[e]Relative adsorption efficiency was divided by the relative cross-linking efficiency.

radiolabeled viral proteins adsorbed to the erythrocyte pellet decreased with time but at a slower rate than the loss of hemagglutination activity (Fig. 4.12). After 120 min, the ratio of viral adsorption to erythrocytes versus viral cross-linking of erythrocytes was 37 times the initial ratio (Table 4.7).

The electrophoretic patterns of X-12 and X-29L during trypsin treatment were directly compared by co-electrophoresis of samples of ^{14}C-labeled X-12 and ^{3}H-labeled X-29L removed at the same time from parallel trypsin digestions (Fig. 4.13-4.15). The hemagglutination activity of each virus was determined by the precision technique at eight time points during the 3 hr trypsin treatment. The assays were performed in an ice bath and were read after standing at 4°C for 18 hr, after standing an additional 2 hr at 22°C, and after standing another 6 hr at 22°C (Fig. 4.14). When assayed at 4°C, the hemagglutination activity of X-12 disappeared after 90 min of trypsin treatment, while the activity of X-29L only dropped 50% in 3 hr. Warming the hemagglutination assay to room temperature accelerated the drop in activity, but the difference in the rate of loss of hemagglutination activity by the two viruses was retained. Control virus preparations incubated at 35°C for 3 hr without trypsin did not lose hemagglutination activity even after the assay tubes had stood for 8 hr at room temperature. This effect of temperature during incubation of the hemagglutination assay mixtures for trypsin-treated virus was seen when replicate assays were incubated at 22°C and at 4°C and when an assay was read first at 4°C and again after standing at room temperature for at least 2 hr (Fig. 4.14; Table 4.6).

At each time point, the aliquots of each virus not used for the hemagglutination assay were pelleted, resuspended in distilled water,

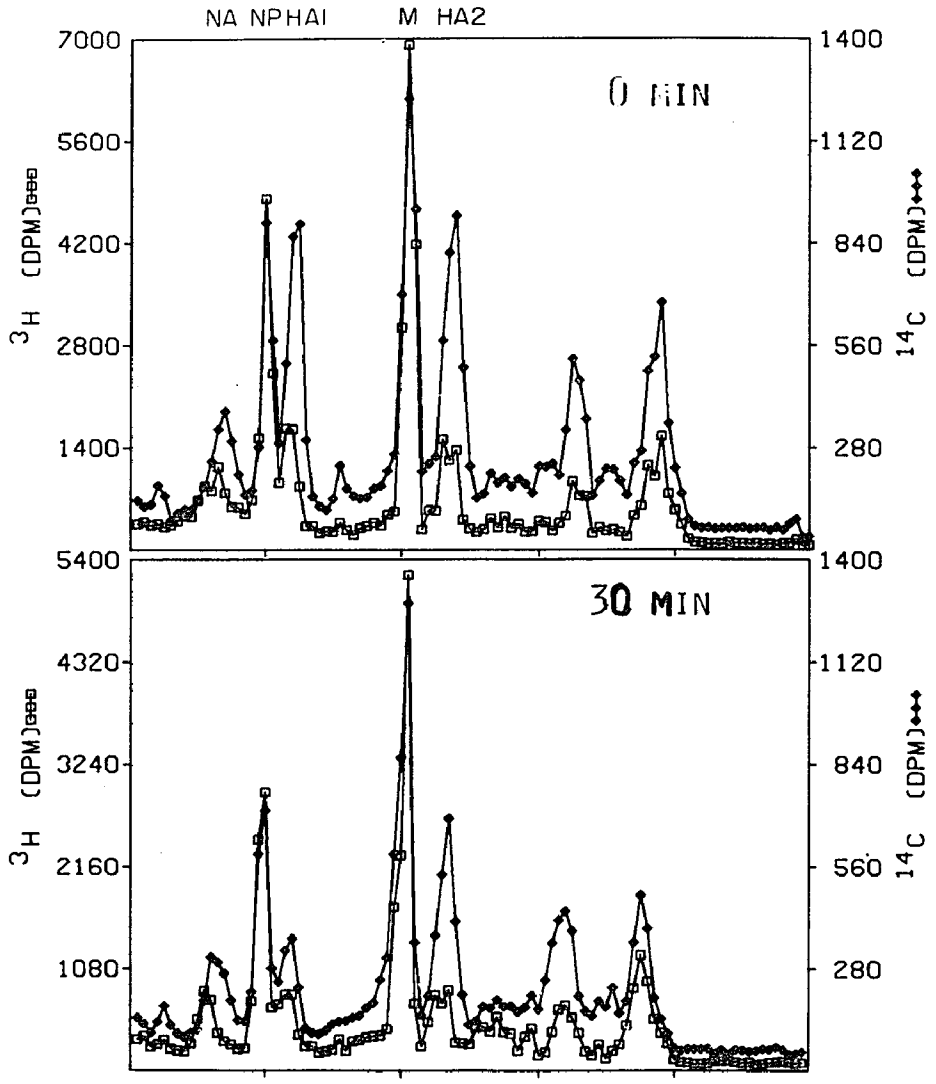

Fig. 4.13. [This figure is continued on the next page.]

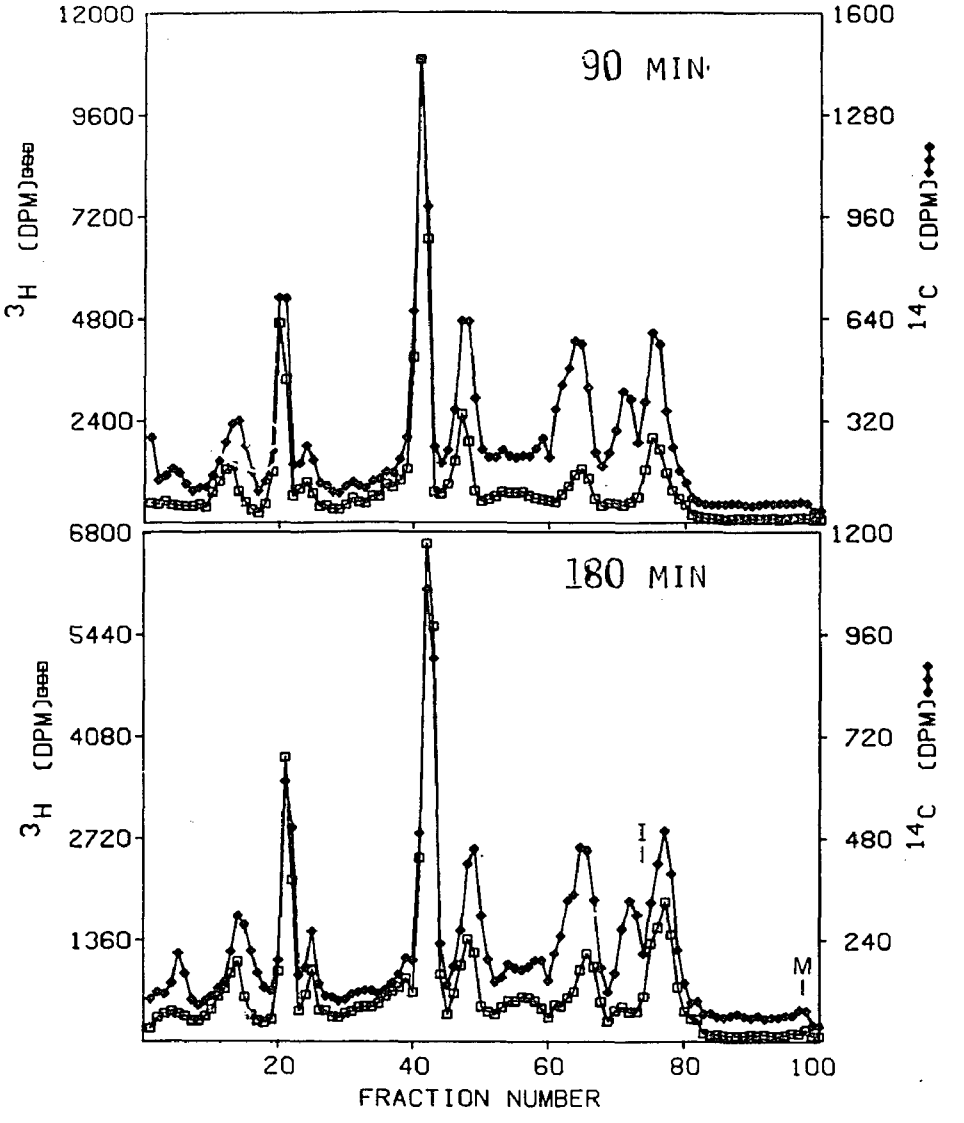

Fig. 4.13. Electrophoretic patterns of radiolabeled viral proteins during trypsin treatment of X-12 and X-29L. Purified viruses were separately treated with trypsin by method B and assayed for hemagglutination activity (Fig. 4.14). Samples of each virus treated for the same time were combined, reduced, and electrophoresed on 13%/40% polyacrylamide gels for 10 hr until the marker dye nearly reached the bottom of the disc gels. The positions of the 13%/40% interface (I) and the marker dye (M) were recorded and the gels were sliced and counted. The dpm observed for the ^{14}C-labeled X-12 proteins (◆) and the ^{3}H-labeled X-29L proteins (◻) are shown. The amount of HA1 or HA2 relative to NP plus M was calculated from the peak areas (Fig. 4.15). The ratio of NP to M (mean ± standard deviation (n = 8): 0.55 ± 0.13 for X-12, 0.48 ± 0.07 for X-29L) was relatively constant.

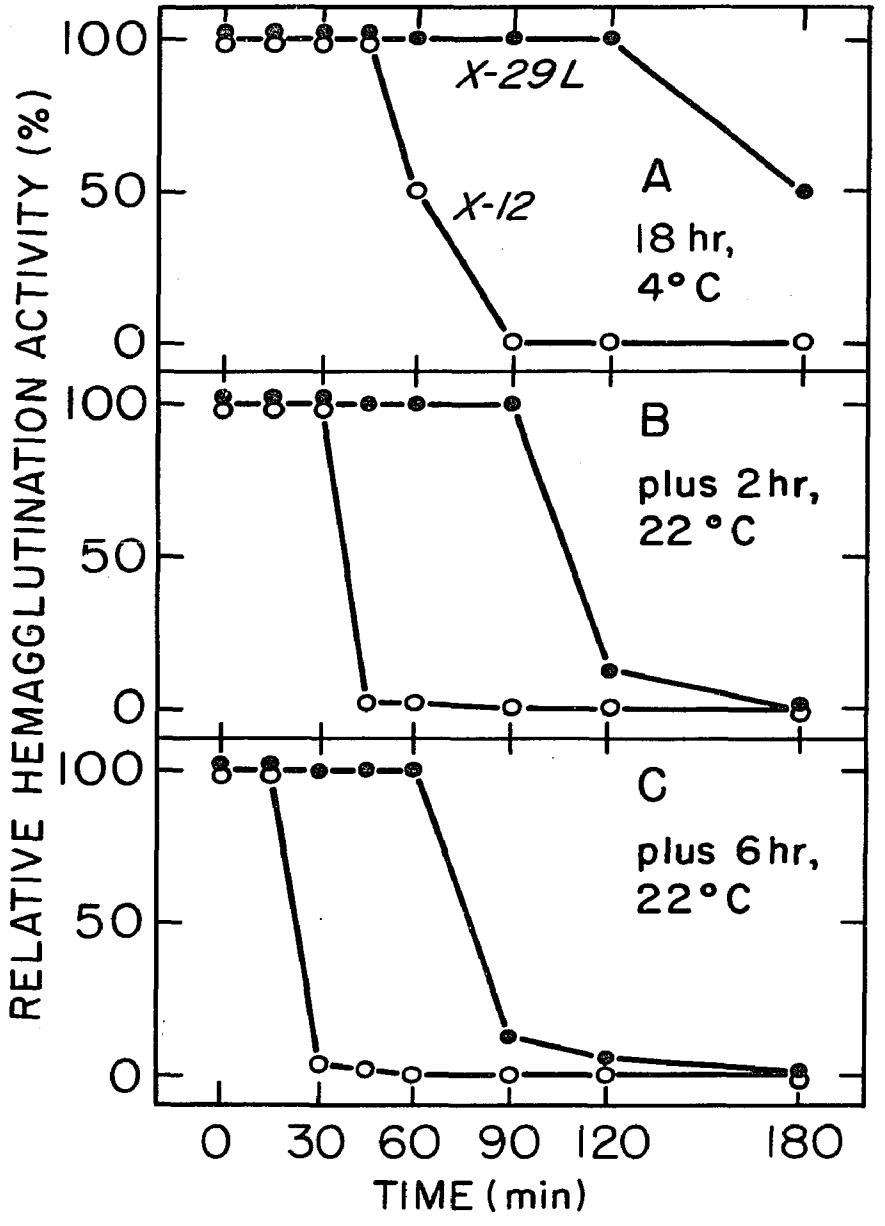

Fig. 4.14. Relative hemagglutination activity during trypsin treatment of X-12 (O) and X-29L (●). See Fig. 4.13 for details. Hemagglutination assays were read three ways for each time point: initially after standing for 18 hr at 4°C (Panel A), then after standing for an additional 2 hr at 22°C (Panel B), and finally after standing for 6 hr longer at 22°C (Panel C).

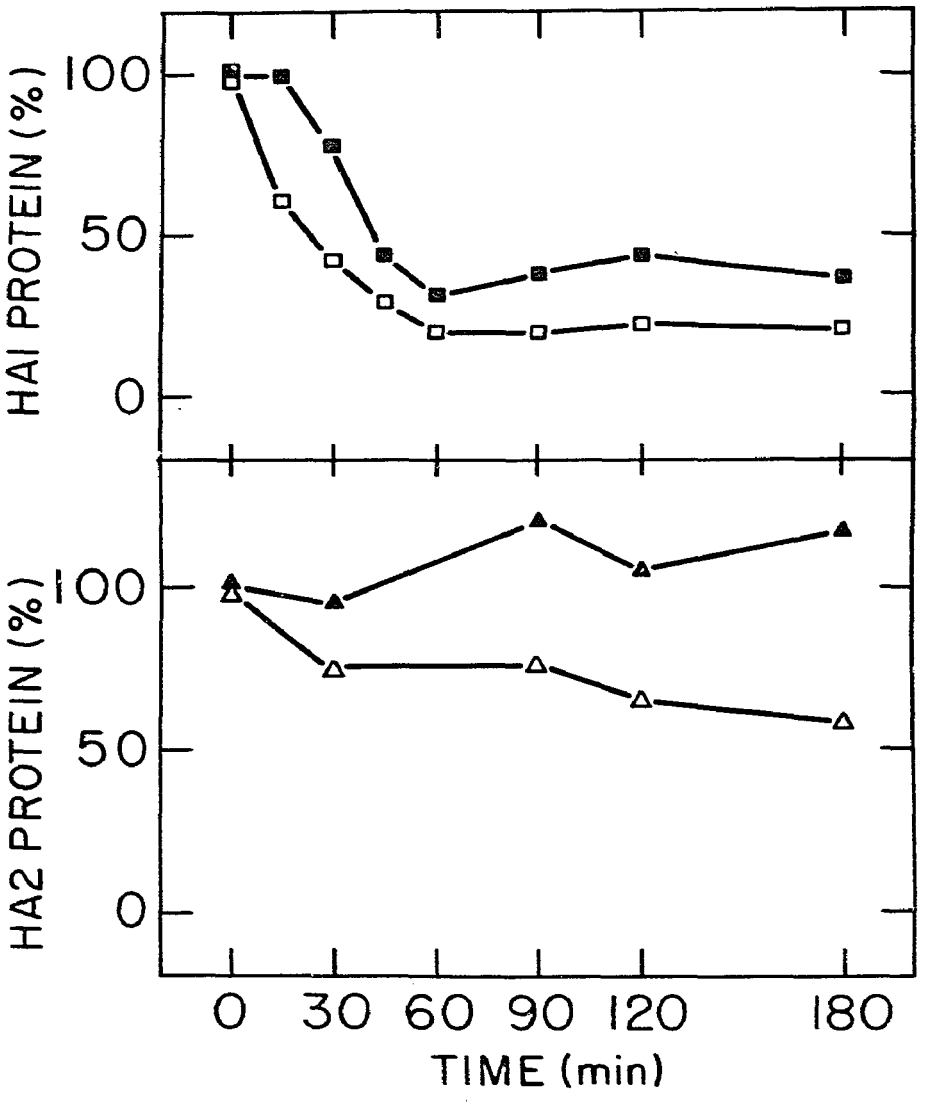

Fig. 4.15. Relative amount of HA1 and HA2 protein present during trypsin treatment of X-12 and X-29L. See Fig. 4.13 for details. The amount of HA1 for X-12 (□) and X-29L (■) is expressed as the dpm ratio of HA1 to NP plus M at the time indicated divided by that at 0 min. The amount of HA2 for X-12 (△) and X-29L (▲) is expressed analogously.

combined and co-electrophoresed on 13% disc gels containing 40% acrylamide plugs. Four of the eight gels are illustrated in Fig. 4.13. The relative peak areas for the HA1 and HA2 proteins during trypsin treatment are shown in Fig. 4.15.

The quantity of the HA1 subunit in the trypsin-sensitive virus X-12 gradually decreased during the first hour and remained constant at about 20% of the initial value for the next two hours. The decrease in HA1 during the first 45 min was not accompanied by a corresponding decrease in the hemagglutination activity (Fig. 4.14A). During the second 45-min period, however, loss of 8% of the initial HA1 protein was accompanied by complete loss of hemagglutination activity. The HA1 protein of the "trypsin-resistant" virus X-29L also decreased gradually over the first hour of trypsin treatment and remained constant at nearly 40% of the initial value for the next two hours. This loss of HA1 contrasted with the maintenance of full hemagglutination activity for X-29L during the first two to three hours of treatment (Fig. 4.14A).

The HA2 protein of X-12 slowly fell over 3 hr to 60% of the initial value, while the HA2 of X-29L was relatively constant (Fig. 4.15). In other experiments not shown, however, the loss of HA2 from X-29L closely paralleled the loss from X-12. In one experiment, after an initial drop, the amount of HA2 was constant at about 60% of the initial value for both viruses. In another case, the loss of HA2 was more gradual for both X-12 and X-29L. Thus the data from several experiments indicated that the amount of HA2 for both viruses generally decreased to about 60% of the initial value after trypsin treatment for 2 hr.

F. Initial rates for loss of HA1 protein. During trypsin treatment, the HA1 subunit was generally lost faster from X-12 than from

X-29L. This trend is evident from the plot of HA1 versus time shown in Fig. 4.15 and from similar graphs of other experiments not shown. To calculate the initial rate of loss of HA1, the amount of HA1 protein is expressed as the mole fraction (χ), which is defined as one at 0 min ($\chi_0 \equiv 1$). Since the enzyme is in excess, the loss of the substrate should be first-order in HA1. The integrated form of the apparent first-order rate equation is:

$$kt = \ln(\chi_0/\chi_t),$$

where χ_0 and χ_t are the mole fractions of HA1 present initially and at time t, respectively, and k is the apparent first-order rate constant. When the logarithm of the mole fraction ratio was plotted versus time, straight lines were obtained for both X-12 and X-29L (Fig. 4.16). Thus the initial loss of HA1 from both X-12 and X-29L was shown to be first-order in HA1 during trypsin treatment. Composite rate constants were calculated from the slopes of the least-squares lines of best fit through seven data points from three experiments for X-12 and six data points from the three experiments for X-29L (Table 4.8). The initial apparent first-order rate constant for loss of HA1 from X-12 [$(6.7 \pm 2.6) \times 10^{-4}$ sec^{-1}] was 2.3 times greater than the corresponding rate constant for X-29L [$(2.9 \pm 1.4) \times 10^{-4}$ sec^{-1}].

G. Ultrastructural studies. The effect of trypsin treatment on the ultrastructure of X-12 and X-29L was examined by electron microscopy of negatively stained virus preparations. When trypsin and soybean inhibitor were not removed from the virus preparations, the structural details were obscured by globular structures spread uniformally over the surface of the specimen grid. Examination of negatively-stained solutions of trypsin, soybean trypsin inhibitor, and a mixture of these

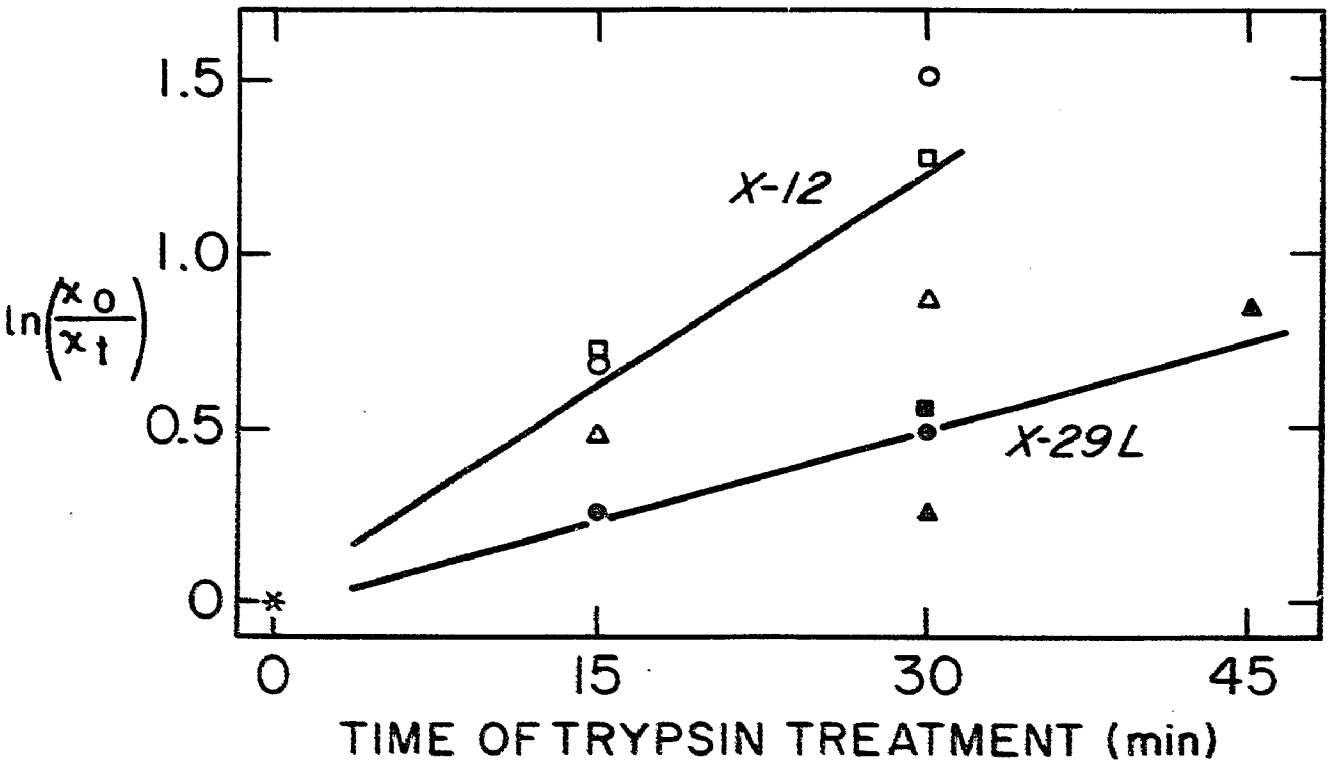

Fig. 4.16. Initial first-order rate of loss of EA1 protein from X-12 and X-29L during trypsin treatment. Rate constants are the slopes of the least-squares lines of best fit for seven data points from three experiments for X-12 (✱ , □ , ○ , △) and for six points from three experiments for X-29L (✱ , ● , ▲ , ■); see Table 4.8.

TABLE 4.8

Initial First-Order Rate Constants for Loss of HA1 Protein from X-12 and X-29L During Trypsin Treatment[a]

Virus	Expt	Data points	Symbol	Apparent first-order rate constant k, 10^{-4} sec^{-1} Least-squares slope	90% confidence interval
X-12	1	3	□	7.1	5.5-8.6
	2	3	○	8.4	7.0-9.8
	3	3	△	5.1	4.0-6.1
	1-3	7[b]		6.7	4.1-9.4
X-29L	4	3	●	2.7	2.5-3.0
	5	3	▲	2.6	1.6-3.6
	6	2	■	3.1	
	4-6	6[b]		2.9	1.5-4.3

[a]The apparent first-order rate constant for loss of HA1 was calculated from the slope k of the least-squares line of best fit for the data points using Olivetti-Underwood statistical programs (Williams, 1969) and the equation $kt = \ln(\chi_0/\chi_t)$, where χ_t is the mole fraction of HA1 at time t. The 90% confidence interval is the range of rate constants that contains the correct rate constant with 90% confidence. The composite lines are shown in Fig. 4.16.

[b]The composite rate constant was calculated using only one data point at x = 0 min and y = 0 min.

two proteins revealed that the globular structures were trypsin--inhibitor complexes. Therefore, trypsin-treated virus was purified by density gradient centrifugation and dialyzed against Tris buffer prior to electron microscopy.

Comparison of untreated virus (Fig. 4.17) with trypsin-treated virus (Fig. 4.18 and 4.19) revealed that the protease treatment reduced the density of the glycoprotein spikes on the virus surface. Spikes remaining after 2 hr had a knob-like appearance and were distributed evenly on the surface of the virus particles.

Attempts to distinguish X-12 from X-29L ultrastructurally at various time points during trypsin treatment were generally unsuccessful. Differences in the density of the surface spikes were only detected when the hemagglutination activities of the two viruses were considerably different. Preparations of X-12 and X-29L were indistinguishable before trypsin treatment (Fig. 4.17). After trypsin treatment for 30 min, the two viruses also appeared identical even though the hemagglutination activity of the X-12 preparation (4 HAU) was 32 times lower than the activity of the X-29L virus (128 HAU) (Fig. 4.18).

Assay of the neuraminidase activity of trypsin-treated virus indicated that the activity of both X-12 and X-29L remained essentially unchanged throughout the period of exposure to the protease (Fig. 4.7). When the hemagglutination activity was less than 4 HAU and the number of surface projections was decreased considerably, the neuraminidase activity was essentially unchanged and knob-like structures were seen on the viral surface. For example, after trypsin treatment of the viruses for 2 hr (Fig. 4.19), the X-12 virus had 109% and the X-29L virus had 88% of the initial neuraminidase activity. The remaining surface projections

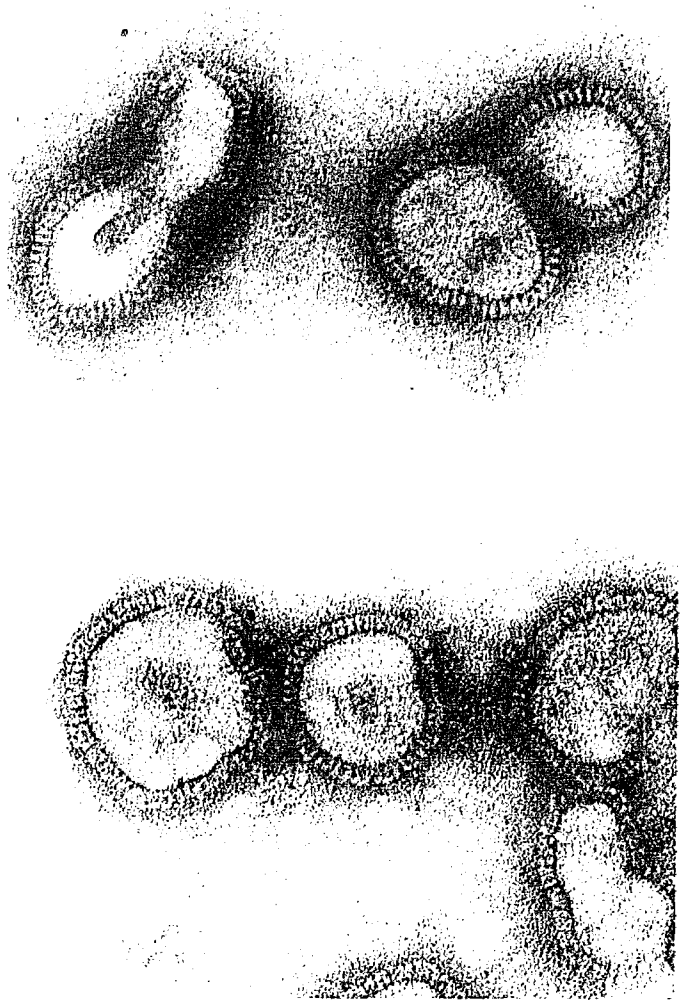

Fig. 4.17. Ultrastructure of X-12 and X-29L virus before trypsin treatment. These typical electron micrographs of X-12 (above) and X-29L (below) show the virus particles at 160,000 magnification after negative staining with 4% sodium phosphotungstate at pH 7.0.

Fig. 4.18. Ultrastructure of X-12 and X-29L viruses after trypsin treatment for 30 minutes. Knob-like surface projections are seen on both X-12 (above) and X-29L (below) after trypsin treatment by method B and purification by density gradient centrifugation. The viruses were magnified 160,000 times after staining with 4% sodium phosphotungstate at pH 7.0.

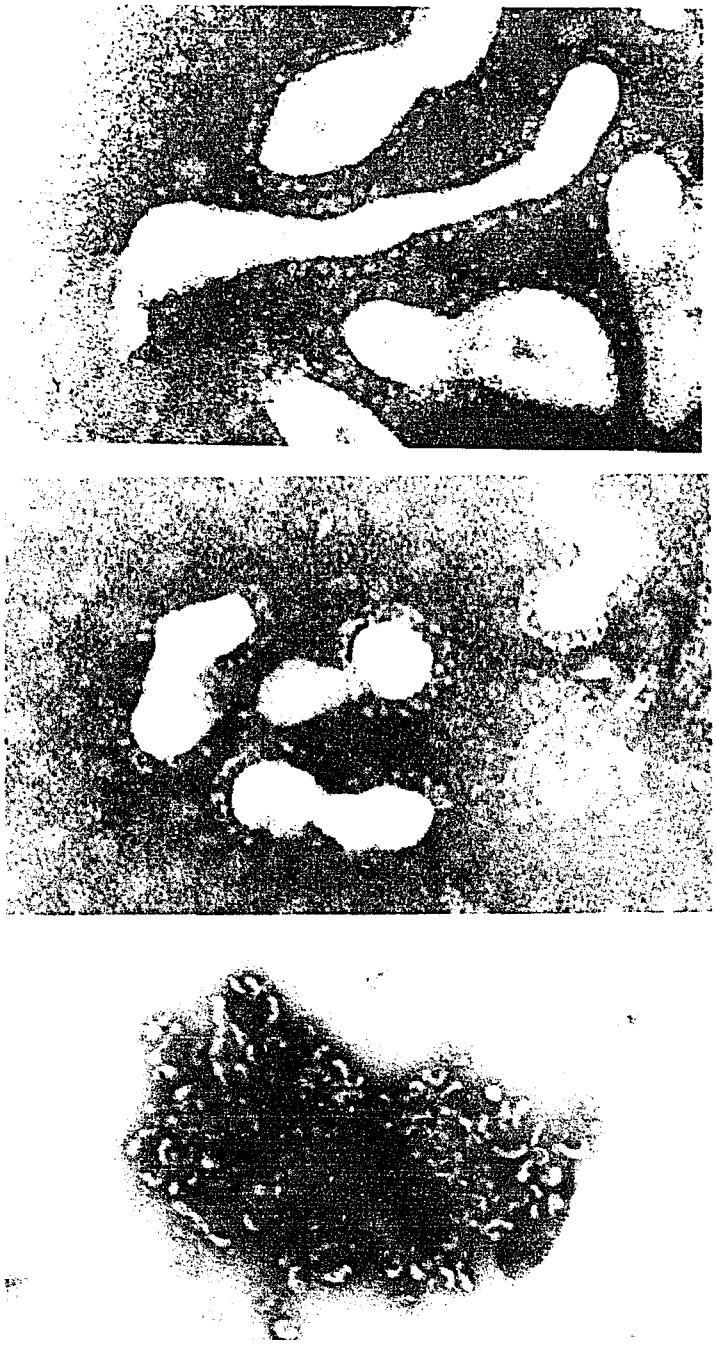

Fig. 4.19. Ultrastructure of X-12 and X-29L viruses after trypsin treatment for two hours. Magnified 160,000 times, the surface projections of X-12 (above) and X-29L (middle) appear as globular structures bound to the viral surface by narrow fibers. At 25,000 times magnification (below), X-29L virions that have lost most of their surface projections aggregate in large clumps. Viruses were negatively stained with 4% sodium phosphotungstate at pH 7.0.

appeared as globular structures attached to the viral surface by thin filaments (Fig. 4.18 and 4.19). Preparations of either virus that had lost the majority of their surface spikes showed large clumps of aggregated virus particles (Fig. 4.19).

H. Absence of large degradation products of HA. No decrease in the molecular weights of the HA1 and HA2 proteins was detected by polyacrylamide gel electrophoresis during trypsin treatment. The quantities of HA1 and HA2 decreased, but partially digested molecules still attached to the virus particles were not observed. Two small polypeptides that migrated faster than HA2 were observed (Fig. 4.11). They probably correspond to the two polypeptides weighting about 20,000 daltons seen in gel patterns of virus not exposed to trypsin (Chapter 3). The area of the larger polypeptide peak seen in Fig. 4.11 remained constant relative to the sum of the areas of the NP and M proteins over two hours of trypsin treatment. The area of the smaller peak, however, increased to 5.5 times the initial value relative to the sum of the areas of the NP and M peaks. This increasewas not observed, however, during other trypsin treatments of radiolabeled virus.

The electrophoresis conditions were modified to detect small radiolabeled proteins that pelleted with trypsin-treated virus by adding a plug of 40% acrylamide to the bottom of the 13% gels and stopping the electrophoresis when the marker dye, bromphenol blue (670 daltons), reached the bottom of the gel. Peaks due to several small polypeptides were routinely detected near the 13%/40% polyacrylamide interface. The peaks seen in Fig. 4.13 were typical. The areas of the three small proteins showed no consistent trend with the time of trypsin digestion.

These peaks did not appear to be homogenous.

When unlabeled, trypsin-treated virus was not centrifuged to separate free polypeptides from proteins associated with the virus but was directly applied to the gel, the two diffuse trypsin bands obscured much of the lower half of the gel and the background was high between the two bands (Fig. 4.8). One band that migrated slightly faster than the larger trypsin component was not evident in the control virus preparations not exposed to trypsin. Although trypsin was not electrophoresed alone on this gel, this band is probably a degradation product of trypsin, an enzyme that readily undergoes autolysis (Gorini, 1951; Bier and Nord, 1951). A very faint band with a similar mobility can be detected in the trypsin preparation shown in Fig. 3.4 of Chapter 3. In a similar experiment using radiolabeled virus, the background of radiolabeled polypeptides migrating ahead of HA2 was very high but no distinct peaks that increased with time were seen. Trypsin and its breakdown products were not detectable because they were not radiolabeled.

The possibility that tryptic cleavage released the HA1 or HA2 proteins as intact or nearly intact subunits was examined by electrophoresis of the protein remaining in the supernatant after pelleting of trypsin-treated virus. Since the supernatants averaged 2-3 ml in volume, the 0.1-ml samples applied to the gels were very dilute. The only major bands detected by protein staining migrated with mobilities equal to those of trypsin (23,800 daltons) and soybean trypsin inhibitor (about 21,500 daltons). Occasionally a very faint pattern identical to that of the total viral protein was discerned.

Any proteins that were present in the supernatant were concentrated by ultrafiltration using Amicon membranes that retained proteins larger

than about 10,000 daltons (PM-10). When the concentrated supernatants were examined by electrophoresis, no degradation products of the HA1 subunits were seen to appear with time (Fig. 4.20). The supernatant gel pattern after 240 min of trypsin treatment was virtually identical with the control pattern for a mixture of purified trypsin and soybean trypsin inhibitor. Based on separate electrophoresis of trypsin and inhibitor (Fig. 3.4), the middle peak was identified as inhibitor and the outer peaks as trypsin. The protein background was higher for the gels of the viral supernatants than for the gel of the enzyme and inhibitor, but no major peaks were evident. Supernatants concentrated by acetone precipitation gave the same gel pattern as supernatants concentrated by ultrafiltration. The supernatants from radiolabeled virus were not electrophoresed on gels because the radioactivity was too low.

I. Lack of host-cell effect on trypsin sensitivity. The influence of the host cell on the trypsin sensitivity of X-12 and X-29L was examined by cultivation of these viruses in Clone 1-5C-4 cells. Since virus yields from Clone 1-5C-4 cells were very low (Table 4.3), the harvested virus was concentrated by pelleting, but it was not purified on sucrose gradients. X-12 virus grown in Clone 1-5C-4 cells was trypsin-sensitive, while X-29L virus from Clone 1-5C-4 cells remained trypsin-resistant. For example, the hemagglutination activity of the X-12 virus fell from 512 HAU initially to 4 HAU after 30 min of exposure to trypsin. In contrast, the hemagglutination activity of X-29L virus from Clone 1-5C-4 cells remained at 1024 HAU even after 5 hr of trypsin treatment. These results are identical to those observed for these viruses grown in allantoic membrane cells. Host cell also had no effect on the trypsin resistance of WSN (HON1). The virus remained

trypsin-resistant when grown in MDBK cells (Table 4.6) or in embryonated chicken eggs (Table 2.4).

Antigenic characterization and trypsin treatment of the recombinants NWS_{t2} and NWS_{t8}. Recombination between X-29 (HON2) and X-9L (H2N1) produced the viruses NWS_{t2} and NWS_{t8} (E. D. Kilbourne, unpublished results). Derivation of the hemagglutinin and neuraminidase genes of these recombinant viruses was established by antigenic characterization. Highly diluted antiserum against NWS (HON1) inhibited the hemagglutination activity of both NWS_{t2} and NWS_{t8}, while RI/5^{+} (H2N2) antiserum had little effect (Table 4.9). The latter serum also had no effect on the size of the plaques produced by NWS or by the two recombinants. Antiserum against an H2N1 recombinant virus which derived its hemagglutinin from A/Japan 305/57 (H2N2) and its neuraminidase from A/NWS/33 (HON1) reduced the plaque size but did not inhibit plaque formation of the three viruses, while antisera against either X-12 or NWS (both HO) inhibited plaque formation by NWS_{t2} and NWS_{t8}. Thus the latter viruses both exhibited the HON1 serotype.

Trypsin treatment confirmed that NWS_{t2} and NWS_{t8}, which acquired the HO gene from X-29, also inherited its trypsin resistance (Table 4.10). Virus NWS_{t8} retained its hemagglutination activity in the presence of the protease when the assay mixtures were incubated at either 4°C or at room temperature (Table 4.6). Like X-29, the trypsin resistance of the NWS_{t8} hemagglutination activity was only relative. During exposure to trypsin for 9 hr, the NWS_{t8} hemagglutination activity gradually fell to zero at a rate very similar to that for the loss of the hemagglutination activity of X-29 (Fig. 4.5). Polyacrylamide gels of trypsin-treated NWS_{t8} (Fig. 4.20) revealed that the quantity of HA1 protein decreased during trypsin

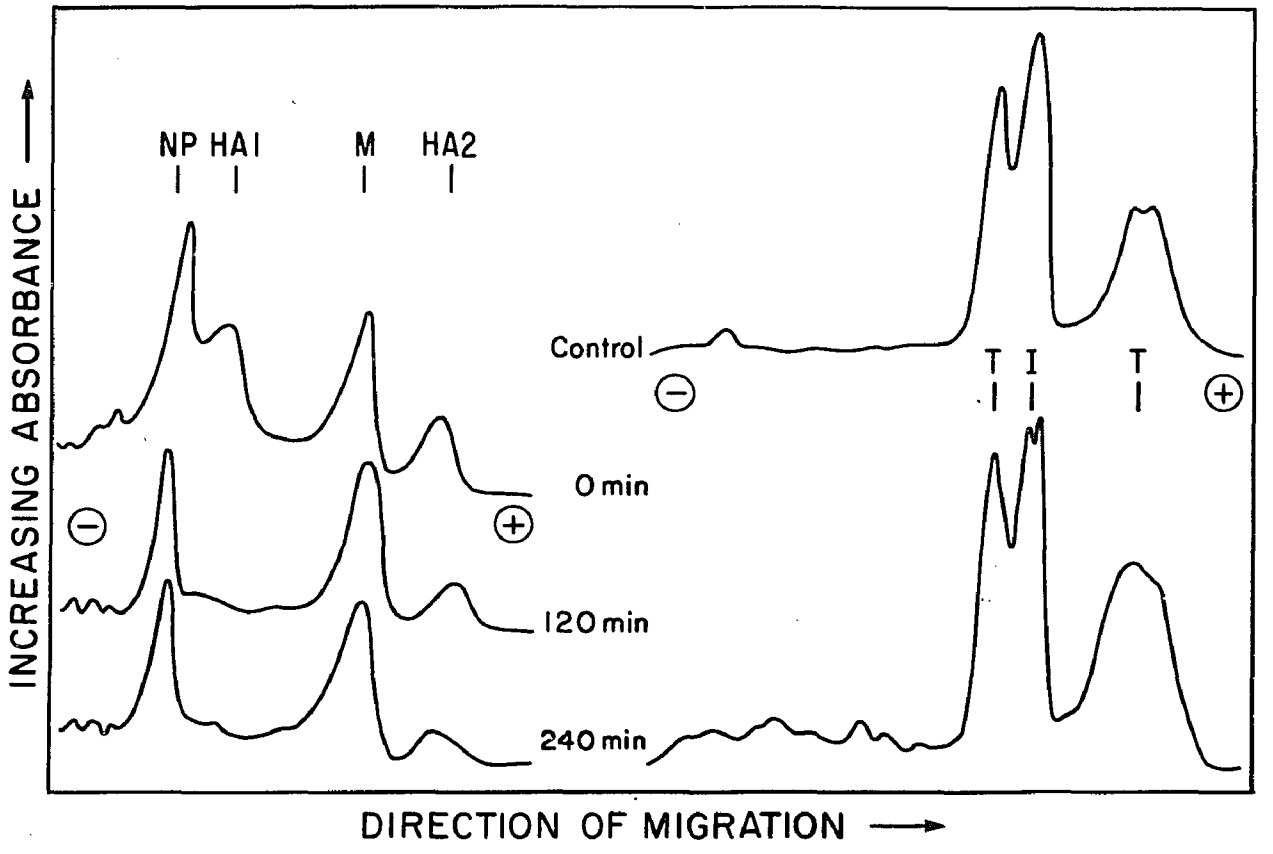

Fig. 4.20. Electrophoretic profile of NWS_{t8} proteins during trypsin treatment. Purified virus was treated by method B. Densitometric tracings of 13% polyacrylamide gels stained with Coomassie blue are illustrated. The left panel shows the proteins of the pelleted virus; the right panel shows the proteins of the supernatant after concentration by Amicon ultrafiltration. The control was a mixture of trypsin and soybean trypsin inhibitor.

TABLE 4.9

Antigenic Characterization of the Recombinant Viruses NWS_{t2} and NWS_{t8} as HONl Serotype

Antiserum (antigen serotype):	NWS (HONl)		X-12 (HON2)		(H2Nl)[c]		RI/5+ (H2N2)	
Assay:[a]	HI	PI	PI	PSR	PI	PSR	HI	PSR
Determinant serotype:[b]	(HO)	(HO)	(HO)	(N2)	(H2)	(Nl)	(H2)	(N2)
Virus (serotype)								
A/NWS/33 (HONl)		64000	25600	0	0	4000		0
NWS_{t2}	81920	102400	25600	0	0	1600	40	0
NWS_{t8}	81920	400		0	0	1600	40	0

[a]Hemagglutination-inhibition (HI) titers are expressed as the reciprocal of the highest antiserum dilution that completely inhibited hemagglutination. Plaque-inhibition (PI) titers are expressed as the highest antiserum dilution that decreased by half the number of plaques per plate. Plaque-size-reduction (PSR) titers are expressed as the highest antiserum dilution that decreased by half the median plaque radius.

[b]HI and PI are determined by the HA serotype, whereas PSR is determined by the NA serotype (Jahiel and Kilbourne, 1966).

[c]This recombinant virus derived its hemagglutinin from A/Japan 305/57 (H2N2) and its neuraminidase from A/NWS/33 (HONl).

TABLE 4.10

Hemagglutination Activity of NWS_{t2} (H0N1) and NWS_{t8} (H0N1) After Trypsin Treatment for 90 Minutes[a]

Virus (serotype)	Trypsin-treated	Control
X-12 (H0N2)	< 4	4096
NWS (H0N1)	32	8196
NWS_{t2} (H0N1)[b]	8192	4096
NWS_{t8} (H0N1)[b]	8192	4096
X-29 (H0N2)	8192	4096

[a]Virus in allantoic fluid was mixed with an equal quantity of a 0.5% solution of trypsin (Difco) in PBS, incubated at 40°C for 90 min, and assayed for hemagglutination activity. Control virus was incubated for 90 min at 40°C without trypsin. Hemagglutination titers are expressed as the reciprocal of the highest virus dilution giving complete hemagglutination in 60 min at room temperature. E. D. Kilbourne, unpublished results.

[b]Viruses NWS_{t2} and NWS_{t8} were derived from recombination between X-29 (H0N2) and X-9L (H2N1).

treatment and was barely evident after 4 hr even though the hemagglutination activity (512 HAU) remained unchanged.

Antigenic characterization and trypsin treatment of the recombinant X-29LT. Double infection of eggs with X-29L (H0N2) and the high-yielding virus X-41 (H3N2) produced the recombinant X-29LT, an H0N2 virus with the growth characteristics of the X-29L parent. The hemagglutination activity of the new X-29LT stock virus was 2048, while that of the parental X-29L stock virus was 4096. In hemagglutination inhibition assays, X-29LT appeared identical to the X-29L parental virus (Table 4.11). Although endpoints were not reached in the neuraminidase inhibition test because the serum was not serially diluted a sufficient number of times, preliminary results suggested X-29LT derived its neuraminidase gene from X-29L. Like X-29L, X-29LT was inactivated by the IgG fraction of antiserum prepared against purified X-7(F1) (H0N2) neuraminidase. Antiserum against A/Port Chalmers/1/73 (H3N2) inhibited the neuraminidase activity of X-41, which derived its N2 neuraminidase gene from the Port Chalmers strain, but had little inhibitory effect on the neuraminidase activity of either X-29L or X-29LT.

Trypsin treatment indicated that, unlike its antigenically identical parent X-29L, X-29LT was trypsin sensitive (Table 4.12). Both before and after cloning by high-dilution passage in eggs, the virus rapidly lost its hemagglutination activity on exposure to trypsin. Incubation of X-29LT at 35°C for 4 hr with buffer did not alter its hemagglutination activity, indicating that the inactivation of hemagglutination activity was due to trypsin and not to heat. In contrast, the two parental viruses also passaged in eggs at the same concentration retained their resistant character.

Treatment of virus particles with other proteases. The effect of several other proteases on the hemagglutination activity of X-12 and X-29L was also studied. After incubation for 4 hr at 35°C with pronase at a final concentration of 0.5 mg/ml, the hemagglutination activity of each virus fell from 1024 HAU to zero. In contrast, after incubation for 4 hr at 35°C with chymotrypsin, pepsin or IgA protease, each at a final concentration of 0.5 mg/ml, the hemagglutination activity of the two viruses was unchanged. Three separate treatments of purified X-12 and X-29L with bromelain for 4 hr failed to produce any loss of hemagglutination activity. Similar treatment of WSN virus in tissue culture medium resulted in a 75% decrease in activity.

DISCUSSION

Trypsin treatment of the parental viruses NWS and RI/5^{-}. The hemagglutination activity of the H0N1 virus NWS was very sensitive to inactivation by trypsin. The activity was completely lost in about 30 min when NWS was treated in allantoic fluid or after purification. The protein patterns of trypsin-treated virus on polyacrylamide gels revealed that the loss of hemagglutination activity was accompanied by partial loss of the HA1 protein. About 28% of the HA1 protein was already lost by the time the zero time point sample was quenched. The initial weight ratio of HA1 to HA2 (1.08) was only 72% of the 1.5 ratio calculated from approximate apoprotein molecular weights of the hemagglutinin subunits. The quantity of HA2 protein also decreased during trypsin treatment but at a slower rate than for HA1. After an initial drop of 22% in the first 30 min, the quantity of HA2 remained relatively constant for 4 hr, although the HA1 protein disappeared completely.

TABLE 4.11

Antigenic Characterization of the Recombinant Virus X-29LT as H0N2 Serotype

Antiserum (antigen serotype):	NWS (H0N1)	X-7 (H0N2) IgG anti-NA	Port Chalmers (H3N2)	
Assay:[a]	HI	NI	HI	NI
Determinant serotype:	(H0)	(N2)	(H3)	(N2)[b]
Virus				
X-29L (H0N2)	20480	> 400	< 40	< 10
X-29LT Clone 1	20480	> 400	40	< 10
X-29LT Clone 2	10240	> 400	< 40	< 10
X-41 (H3N2)	640	> 400	5120	> 100

[a]Hemagglutination inhibition (HI) titers are expressed as the reciprocal of the highest antiserum dilution that completely inhibited hemagglutination. Neuraminidase inhibition (NI) titers are expressed as the highest dilution of antiserum containing a constant quantity of virus that exhibited half of the neuraminidase activity of a control containing normal rabbit serum at the same dilution and the same quantity of virus.

[b]Although the neuraminidase antigens from X-29L, which was derived from A/RI/5⁻/57, and from X-41, which was derived from A/Port/Chalmers/1/73, are both described as N2 serotype, the NI titer distinction seen with the Port Chalmers antiserum is probably due to antigenic drift between 1957 and 1973 (Schulman and Kilbourne, 1969).

TABLE 4.12

Hemagglutination Activity of X-29LT

during Trypsin Treatment for 240 Minutes

Virus (serotype)	Time (min)			
	0	60	90	240
X-12 (H0N2)	1024		< 4	< 4
X-29 (H0N2)	2048			4096
X-29L (H0N2)[b]	128		256	128
X-29LT (H0N2)[b]	64		4	< 4
X-29LT (H0N2)[c]	256		< 4	< 4
X-29LT (H0N2)[c]	512	512		< 4
X-29LT,[c] no trypsin	1024			1024
X-41 (H3N2)[b]	64		64	32
A/PR/8/34 (H0N1)	1024	2048		2048
A/RI/5^{+}/57 (H2N2)	64	128		128

[a]Virus in allantoic fluid was treated with trypsin using method B. Activity is expressed as the reciprocal of the highest virus dilution that gave complete hemagglutination.

[b]This virus was passed twice in eggs in the presence of 10-day antiserum directed against A/Port Chalmers/1/73 (H3N2).

[c]This virus was recovered after one high-dilution passage in eggs.

In contrast, the hemagglutination activity of RI/5$^-$ and RI/5$^+$ was completely stable for at least 4 hr in the presence of trypsin. The HA1 protein of RI/5$^-$ was relatively stable to trypsin, dropping only 14% during treatment for 4 hr. Similarly, the amount of HA2 decreased only 12% in 4 hr. These changes were too small to be reflected in the hemagglutination activity, which appears constant in the binary dilution assay until about half of the activity is lost. The weight ratio of HA1 to HA2 for RI/5$^-$ was constant for 4 hr; the ratio at 4 hr was 98% of the ratio at the zero time point. The latter ratio (1.7) is higher than the 1.3 ratio calculated from the estimated apoprotein molecular weights. The calculated value was based on the uptake of Coomassie blue dye by the protein portions of the HA1 molecule. If the HA1 carbohydrate also adsorbs some dye molecules, the observed ratio would be higher than the calculated ratio. The HA1 to HA2 ratio measured by the incorporation of radiolabeled amino acids in each subunit was also higher for the trypsin-resistant HON1 virus WSN (1.7) than the calculated weight ratio for the HO viruses (1.5). Thus the calculated and observed ratios are only approximate numbers.

Some hemagglutination activity was detected in the supernatants after either virus was pelleted. This supernatant activity was affected by trypsin in the same manner as the hemagglutination activity in the pellets. Thus it is likely that the supernatant activity was due to a small amount of whole virus that either failed to pellet or was washed off the pellet when the supernatant was decanted. The activity in the NWS supernatants did not increase during trypsin treatment as would be expected if tryptic cleavage released an active hemagglutinin component from the virus particles.

The N2 neuraminidase of RI/5$^-$ was also insensitive to trypsin.

Neuraminidase activity remained relatively constant during trypsin treatment for 4 hr. Hoyle and Almeida (1971) have also reported that the N2 neuraminidase is almost completely resistant to trypsin. The trypsin sensitivity of the N1 neuraminidase of NWS was not determined because this enzyme is heat sensitive (Rafelson et al., 1963; Paniker, 1968), and it is difficult to distinguish between trypsin and heat inactivation. Several investigators have reported that the N1 neuraminidases are sensitive to trypsin degradation to varying degrees. Hoyle and Almeida (1971) found that the PR/8 and Swine neuraminidases are partially resistant to trypsin. Schulze (1970) and Lazarowitz and co-workers (1973a) have reported that the WSN enzyme is readily removed from virus particles by mild trypsin treatment.

Trypsin treatment of the recombinant viruses X-12 and X-29L. During the 1971 NIH workshop on influenza polypeptides and antigens, Kilbourne described the trypsin sensitivity of the recombinants X-12 and X-29 (Kilbourne et al., 1972). Although both viruses shared the H0 serotype of the parental virus NWS, X-29 was found to differ from X-12 and NWS in that its hemagglutination activity was not sensitive to treatment with trypsin under standard conditions (90 min at 40°C).

A. Hemagglutination activity. The H0 viruses NWS and X-12 lost essentially all of their hemagglutination activity after treatment with trypsin (0.5-0.6 mg/ml) for 30 to 90 min at 35-40°C. Under these conditions the activity of the H2 parental virus RI/5$^-$ was fully resistant to trypsin for at least 4 hr. The hemagglutination activity of X-29 remained unchanged for the first hour and then slowly disappeared over the next seven hours. In one experiment the activity was 25% of the initial value after 3 hr, 6% after 4 hr, and less than 1% after 7 hr

of trypsin treatment (Fig. 4.5). The activity of X-29 was initially resistant to trypsin treatment but ultimately fully sensitive. Thus the hemagglutination activity of X-29 (and its large-plaque variant X-29L) is slowly trypsin sensitive rather than trypsin resistant. This trypsin response of X-29 and X-29L resembles the trypsin sensitivity of X-12, NWS, and other H0-serotype viruses more than the trypsin resistance of RI/5$^-$ and other H2-serotype viruses.

The apparent hemagglutination activity observed for a sample of trypsin-treated virus varied with the assay temperature. In one experiment, for example, the hemagglutination activity of NWS disappeared after a 60-min exposure to trypsin when assayed at 22°C but only after a 90-min exposure when assayed in parallel at 4°C. In addition, the hemagglutination activity of X-12 and X-29L appeared to be lost slowly when read at 4°C, faster when subsequently read after 2 hr at 22°C, and faster still when read after 8 hr at 22°C (Table 4.6, Fig. 4.14). This effect was not observed for the control viruses not exposed to trypsin. The faster loss of hemagglutination activity when the assay mixtures were read at room temperature is probably due either to the slow action of residual trypsin on the HA spikes or to the action of the viral neuraminidase on the cellular HA receptors. Elution of virus particles bearing a full complement of HA spikes is not seen but elution was observed when prior trypsin treatment removed most of the active HA spikes.

B. Neuraminidase. The N2 neuraminidase of both X-12 and X-29L was evidently resistant to trypsin because the neuraminidase activity of these viruses did not decrease significantly during trypsin treatment (Fig. 4.7). This resistance was expected because the neuraminidase

activity of RI/5$^-$, the parent from which the N2 gene was derived, was also trypsin-resistant (Fig. 4.1). Calcium ions, included in the NTC buffer to stabilize the trypsin against autolysis, probably also stabilized the viral neuraminidase (Bucher and Palese, 1975), resulting in little or no heat inactivation.

The spikes remaining on the viral surface after total loss of the hemagglutination activity appeared to have knob-like heads attached to the virus surface by thin fibers. This appearance, coupled with the retention of neuraminidase activity of these virus preparations, suggested that the spikes were neuraminidase molecules. Negatively stained, isolated neuraminidase spikes have a cubic head, composed of four tetrameric units, attached to a fiber which terminates in a small, round, hydrophobic structure (Laver and Valentine, 1969; Wrigley et al., 1973). In contrast, isolated hemagglutinin spikes are rod-shaped (Laver and Valentine, 1969). Similar knob-like structures were observed on bromelain-treated virus particles by Nermut (1972) and tentatively identified as neuraminidase protein.

The knob-like structures were evenly dispersed on X-12 and X-29L virus particles. No differences were detected in either the number or distribution of the structures on X-12 and X-29L virus particles, although the latter virus has about twice as much neuraminidase protein and activity as X-12 (Chapter 3). In contrast, Nermut found that the knobs were clustered on the virus particles. Compans and co-workers (1969) and Kendal and Madeley (1970) observed that anti-neuraminidase antibodies appeared to be localized in patches on the virus surface, suggesting that neuraminidase proteins may exist in discrete areas. This clumping of neuraminidase antibodies may actually be an artifact

reflecting the fluidity of membranes and the tendency of antibodies reacted with cell membrane proteins to form patches and caps.

C. Amounts of HA1 and HA2 protein. The patterns of the viral proteins after polyacrylamide gel electrophoresis were visualized either by staining the gels with Coomassie blue dye or by liquid scintillation counting of slices of gels containing radiolabeled virus. Densitometric scanning of the stained gels revealed finer details of the individual protein bands but also visualized cellular protein contaminants, trypsin, and soybean trypsin inhibitor. Scintillation counting of sliced, radiolabeled gels only visualized proteins synthesized after viral infection. It also permitted co-electrophoresis of one ^{14}C-labeled virus and another ^{3}H-labeled virus. But the counting of sliced gels gave only a gross idea of the shapes of the protein peaks because each protein was distributed over only a few slices.

Since the influenza virion binds to cellular surfaces by interaction of the HA1 hemagglutinin subunit with cellular neuraminic acid, the loss of hemagglutination activity during trypsin treatment was expected to correlate with some alteration of HA1 by trypsin. Indeed, the most prominent change in the protein patterns during trypsin treatment of a sensitive virus was the loss of HA1 protein. This loss was apparent on both stained gels (Fig. 4.3 and 4.8) and radiolabeled gels (Fig. 4.11 and 4.13). In addition, electron microscopy revealed a dramatic decrease in the density of spikes visible on the viral surface by negative staining. Hoyle and Almeida (1971) have also noted that treatment of the HO virus DSP with trypsin reduced the number of viral surface projections. An exact correlation between hemagglutination activity and spike density was not possible. Two virus preparations could only be distinguished

ultrastructurally when they differed considerably in hemagglutination activity. Preparations having titers of 4 and 128 HAU had approximately the same appearance (Fig. 4.18). Control preparations of X-12 and X-29L were indistinguishable, as were trypsin-treated preparations having similar hemagglutination activities.

Loss of spikes was accompanied by a tendency of the virus particles to aggregate in large clumps (Fig. 4.19), presumably due to the hydrophobic nature of the HA2 protein remaining attached to the viral surface after trypsin treatment. Spikeless WSN particles which retain HA2 protein after bromelain treatment also form large aggregates (Compans et al., 1970). Thus the isolated particles seen on specimen grids may not have been representative of the whole trypsin-treated virus population, but may have been the virus particles which had not lost as much surface protein as the particles in clumps.

During two trypsin treatments of X-12, the amount of HA1 decreased as the hemagglutination activity decreased (Fig. 4.6 and 4.12). In a third experiment, the amount of HA1 steadily decreased to about 20% of the initial value during the first 60 min of trypsin treatment and remained constant for the next 120 min of trypsin treatment (Fig. 4.15). The corresponding hemagglutination activity was unchanged for the first 45 min but was lost by 90 min (Fig. 4.14). In general, the amount of HA1 protein decreased faster than the hemagglutination activity, which in turn decreased faster than viral infectivity (Fig. 4.4).

Although an influenza virion is estimated to bear 300-450 HA spikes (Schulze, 1973, 1975), only two HA spikes per virion are theoretically required for hemagglutination and only one for infectivity. In reality, however, several HA spikes per virion are probably required for

hemagglutination. To cross-link two erythrocytes, the spikes need to be sufficiently separated on the virion surface. Several spikes must probably be present on the average to insure the proper spike distribution required for lattice formation. Thus a certain minimal number of hemagglutinin spikes is required to produce visible agglutination of erythrocytes. When the number of spikes falls below this number, no hemagglutination will be observed even though some HA protein may still be present. This situation is probably achieved for X-12 in 30-90 min because at this time the percentage of HA1 protein remaining is greater than the percentage of hemagglutination observed. Hoyle and Almeida (1971) have noted that ". . . changes in haemagglutinin titre of virus after trypsin treatment do not accurately reflect the action of the enzyme on the haemagglutinin protein . . ." They found that although trypsin treatment did not change hemagglutination titers, disruption of trypsin-treated virus with ether reduced the hemagglutination activity to less than 1% of the original value, suggesting that fewer multivalent virus fragments were produced from trypsinized virus than from untreated virus.

Influenza virus can interact with erythrocytes either by binding to one cell or by cross-linking two cells. The ability of X-12 virus to cross-link two erythrocytes was measured by the hemagglutination activity, and the ability of X-12 to adsorb to one or two erythrocytes was measured by determining the amount of radioactive virus bound to pelleted erythrocytes (Table 4.7). The efficiency of hemagglutination decreased much faster during trypsin treatment than the efficiency of erythrocyte adsorption (Fig. 4.12). After treatment for 2 hr, only about 1% of the virus was able to cross-link red cells, whereas 29% was still adsorbed to the cells.

Since the hemagglutination activity of X-29L was usually constant during the first 30 to 90 min of trypsin treatment, the amount of HA1 protein was also expected to be reasonably constant during this period. In reality, however, the HA1 protein of X-29L decreased much faster than the activity. In one experiment, HA1 protein gradually decreased to about 40% of the initial value in 60 min and remained at that level for the next 120 min (Fig. 4.15), but the hemagglutination activity did not change during the first 120 min (Fig. 4.14A).

During trypsin treatment, the total amount of HA1 protein generally decreased more rapidly for X-12 than for X-29L (Fig. 4.6, 4.15). The initial loss of radiolabeled HA1 protein from both viruses was first-order in HA1 (Fig. 4.16). The apparent first-order rate of loss of total HA1 protein during the first 30 min was 2.3 times faster for X-12 than for X-29L (6.7 vs 2.9 x 10^{-4} sec^{-1}; see Table 4.8).

Careful examination of the stained protein patterns of virus during trypsin treatment revealed that the loss of 30-40% of the HA1 protein that occurred before the zero time point samples were quenched (see above) was accompanied by a slight decrease in the electrophoretic mobility of about 55% of the HA1 protein band (Fig. 4.8). The decrease in mobility may have been due to a trypsin-mediated conformation change. Comparison of the HA1 band of the control virus with that of the zero time point sample indicated that prior to exposure to trypsin, HA1 migrated as a single component having a mobility equal to that of the faster of the two HA1 components ($HA1_f$) seen after addition of the protease. At the zero time point, both viruses contained almost equal amounts of the fast and slow components ($HA1_f + HA1_s = HA1_t$), but with increasing time of trypsin treatment, the faster migrating component

($HA1_f$) decreased more rapidly than the slower migrating component ($HA1_s$) (Fig. 4.10). The $HA1_s$ component for both viruses appeared to drop from 50% to 40% of the initial $HA1_t$ during the first 10 min and then to remain at 40% for the next 50 min. In contrast, the $HA1_f$ components of X-12 and X-29L were removed by trypsin at different rates. The $HA1_f$ of X-12 steadily dropped to zero in 30 min, while $HA1_f$ of X-29L slowly disappeared over 60 min. Thus, on exposure of virus to trypsin, HA1 protein appears to be lost as follows:

$$HA1_f \xrightarrow{\text{fast}} HA1_s \xrightarrow{\text{slow}} \text{no HA1}$$

The initial rate of loss of $HA1_t$ for both viruses, which mainly consisted of loss of $HA1_f$, was consistent with the apparent first-order rate constants for loss of radiolabeled $HA1_t$.

Comparison of the losses of $HA1_s$ and $HA1_f$ with the loss of hemagglutination activity was even more revealing (Fig. 4.10). The relative activity of X-12 observed after 10 and 30 min (60% and 3%, respectively) correlated well with the relative amount of $HA1_f$ remaining at 10 and 30 min (47% and about 0%, respectively). In contrast, the hemagglutination activity of X-29L only dropped 40% in 60 min, while the $HA1_f$ component was almost completely lost in that time. After 120 min of trypsin treatment, the X-29L activity had only decreased to 60% of the initial value. These results suggest that in the absence of the $HA1_f$ component, the $HA1_s$ component of X-12 was essentially inactive but the $HA1_s$ component of X-29L still retained substantial hemagglutination activity.

The exterior HA1 subunit rather than the interior, membrane-bound HA2 subunit is generally believed to be the structural protein that determines hemagglutination activity and infectivity (Schulze, 1975).

Comparison of protein weight ratios before trypsin treatment and after very brief treatment (0-min time point) showed that although HA1/HA2 decreased 30-40%, HA2/(NP + M) did not change. Thus HA1 but not HA2 was lost during this very brief exposure to trypsin. In contrast to the different rates of loss of HA1 from X-12 and X-29L, the amount of the HA2 subunit lost during trypsin treatment was similar for both viruses. The amount of radiolabeled HA2 decreased to 60-80% of the initial value during the first 30 min and to 40-60% during the first 2 hr. These losses did not correlate with the loss of hemagglutination activity, but may represent digestion of HA2 subunits left unprotected by the HA1 subunits lost initially.

The initial description of the recombinant X-29 (H0N2) as exhibiting trypsin-resistant hemagglutination activity led to the suggestion that this resistance was inherited from the resistant parent RI/5$^-$ (H2N2) rather than from the sensitive parent NWS (H0N1), with which it share the same hemagglutinin serotype (Kilbourne et al., 1972). During this study, however, X-29 and X-29L have been shown to be slowly trypsin-sensitive. These recombinants more closely resemble the trypsin-sensitive viruses NWS, X-12 and other NWS-derived H0 viruses rather than the fully trypsin-resistant parental virus RI/5$^-$. In addition, the amount of HA1 protein of RI/5$^-$ was not changed during exposure to trypsin for 4 hr (Fig. 4.2 and Table 4.5), but the HA1 of X-29L decreased to about 40% in only 1 hr (Fig. 4.10 and 4.15). X-29L also differs from RI/5$^-$ in the weight ratio of HA1 to HA2. The ratio observed for the radiolabeled proteins of X-29L (0.77 ± 0.14; $n = 8$) was considerably lower than the ratio of 1.5 calculated from the H0 apoprotein molecular weights. In contrast, the ratio observed for the stained

proteins of RI/5$^-$ (1.7) is larger than the ratio of 1.3 calculated for H2 viruses. By this criterion, the HA1 subunit of RI/5$^-$ is also much more resistant to proteases during growth and isolation than the HA1 subunit of X-29L.

Contrary to published reports (Sugg and Cleeland, 1962), the trypsin sensitivity of human influenza viruses does not correlate with the hemagglutinin serotype. As previously noted (Hoyle and Almeida, 1971), PR/8, a standard HON1 strain, was fully trypsin-resistant for 4 hr (Table 4.12). Likewise, WSN (HON1) was resistant when grown in either embryonated eggs (Table 2.4) or MDBK cells (Table 4.6). But since the NWS-derived recombinants X-29 and X-29L have been shown to be slowly trypsin-sensitive and X-6 is expected to behave similarly, it is generally true that all HO-serotype viruses derived from NWS are sensitive to trypsin.

D. Hemagglutinin degradation products. During trypsin treatment of X-12 and X-29L, the quantities of the HA1 and HA2 proteins associated with the virus particles decreased with time, but no changes were detected in the molecular weights of the two proteins. Fragments smaller than about 20 residues may have been removed, however, which would have produced molecular weight changes too small to be detected on polyacrylamide gels. Since no large hemagglutinin cleavage products were detected either associated with or released from the virus, an initial rate-limiting cleavage of HA1 was evidently followed by rapid cleavage of the fragments to produce small peptides. The rate-limiting cleavage may have changed the secondary structure of HA1, since substrate denaturation generally increases the rate of tryptic digestion (Mihalyi, 1972). Digestion to peptides could occur either while HA1 is disulfide-bonded to

HA2 or after HA1 is released from the virus as a nearly intact, readily unfolded subunit. Since little HA2 was lost until HA1 was almost gone, degradation of HA1 occurs more readily than degradation of HA2. After cleaving one HA1 subunit, trypsin preferentially attacked another HA1 subunit rather than attack the HA2 subunit. This result probably reflects the accessibility of the two hemagglutinin subunits.

The relatively high background seen on stained gels of the protein released to the supernatant (Fig. 4.20) was probably due to HA degradation products and to the breakdown of some virus particles. Content and Duesberg (1970) found that glycopeptides released from pronase-treated virus had lower electrophoretic mobilities than the intact viral glycoproteins. They suggested that this may result from a reduced negative charge due to failure of SDS to bind to the carbohydrate residues of the glycopeptides. This could explain why the background in gels of supernatant protein is high the whole length of the gel and not just in the low molecular weight region. No major peaks other than those of trypsin and soybean trypsin inhibitor were detected in the supernatant protein, but is possible that hemagglutinin degradation products were co-migrating with these proteins.

Several small polypeptides of unknown derivation pelleted with trypsin-treated virus. At least two of these were also associated with virus not exposed to trypsin (Chapter 3). After digestion of the exposed or susceptible portions of the HA2 molecule, a non-glycosylated fragment may remain embedded in the membrane, as occurs after bromelain cleavage of the hemagglutinin protein of some virus strains (Brand and Skehel, 1972; Skehel and Waterfield, 1975). It is unlikely, however, that one of the 19,000 dalton polypeptides associated with untreated virus is a

by-product of hemagglutinin digestion by proteases. First, the polypeptides were also associated with RI/5$^-$ virus, which possesses an HA1 protein that is relatively insensitive to trypsin. Secondly, with the exception of the X-12 treatment shown in Fig. 4.11, the 19,000-dalton polypeptides did not increase regularly with increasing time of trypsin treatment. Instead, the amount of the several polypeptides migrating ahead of HA2 varied with time (Fig. 4.13); they probably are degradation intermediates that remained associated with the virus during pelleting. Identification of a membrane-bound hemagglutinin fragment would require gradient centrifugation of the trypsin-treated virus and resolution of the radioactive, virus-associated proteins on high-percentage acrylamide gels.

It is possible that large hemagglutinin degradation products remained attached to the virus but co-migrated on polyacrylamide gels with M or HA2. If an HA1 fragment migrated with M, an increase in the amount of M protein should be evident with time; the NP to M ratio was relatively constant, however, during the period of trypsin treatment. The presence of HA1 fragments that migrate under M protein could be more accurately detected by treating glucosamine-radiolabeled virus with trypsin and examining the M-protein peak for a co-migrating glycoprotein. The presence of a small amount of an HA1 fragment co-migrating with HA2 would be obscured by HA2, especially if the HA1 fragment increased as HA2 decreased. The existence of a co-migrating HA1 fragment was neither confirmed nor ruled out by the gradual decrease of the HA2 peak during trypsin treatment (Fig. 4.15). Since substantial amounts of galactose, mannose, and fructose are present in HA1 but only trace amounts in HA2 (Ward and Dopheide, 1976), incorporation of one of these radiolabeled

sugars into the viral glycoproteins and examination of the HA2 peak for increasing amounts of this sugar during trypsin treatment would demonstrate the presence of an HA1 fragment that co-migrates with HA2.

E. Lack of host-cell effect on trypsin sensitivity. The host cell has two major effects on the viral hemagglutinin protein. First, the carbohydrates found in the host-cell membrane determine the composition of the viral carbohydrate moieties (Choppin and Compans, 1975). Secondly, proteases present in the host cell membrane determine whether or not the HA protein is cleaved to HA1 and HA2 (Lazarowitz et al., 1973a; Klenk and Rott, 1973; Stanley et al., 1973). Both of these host characteristics might affect the trypsin sensitivity of the viral hemagglutination activity. Altering the carbohydrate content and composition of the hemagglutinin molecules could affect the accessibility of the peptide bonds cleaved by trypsin. Cleavage of HA into its subunits is probably required to expose other susceptible peptide bonds and to permit further digestion of the molecule. Addition of trypsin to a virus with trypsin-sensitive hemagglutination activity but uncleaved HA should cause cleavage of HA into HA1 and HA2 (Lazarowitz et al., 1973a; Klenk et al., 1975) followed by digestion of the HA1 subunits in the same manner as observed for viruses possessing completely cleaved HA. In contrast, a hemagglutinin protein that cannot be cleaved into HA1 and HA2 by trypsin is probably resistant to tryptic digestion.

Assay of virus grown in a limited number of host cell types suggested that the host cell does not determine the trypsin sensitivity of viral hemagglutination activity. X-12 and X-29L retained their characteristic sensitivities when grown in Clone 1-5C-4 cells or CAM

cells in vivo (embryonated eggs) or in vitro (isolated membrane pieces). The extent of cleavage of hemagglutinin in Clone 1-5C-4 cells is not known for any strain of influenza virus. WSN virus was trypsin-resistant whether grown in MDBK cells in vitro or in CAM cells in vivo. When grown in MDBK cells, WSN contained only a small amount of HA1 and HA2, but when cultured in CAM cells in vivo, the HA was completely cleaved to HA1 and HA2. Yet this difference in the extent of HA cleavage apparently did not affect the resistance of the hemagglutination to loss of activity on exposure to trypsin.

Growth of radiolabeled virus in CAM cells. Although the greatest virus yields were obtained using embryonated eggs, CAM cells were chosen for culture of radiolabeled virus because the virus recovered was less contaminated with non-viral proteins and less radiolabel was required to incorporate sufficient radioactivity into the virus. Virus recovered from CAM cells was as concentrated or more concentrated, as measured by HAU/ml, than that produced by infection of CEF cells or a variety of continuous cell lines. The HA protein of virus grown in CAM cells is completely cleaved into the HA1 and HA2 subunits, while no cleavage occurs in CEF cells and only a portion of the HA protein undergoes cleavage in MDBK cells grown in the presence of serum (Klenk et al., 1975). Thus exposure of CAM-grown virus to trypsin results in only one trypsin-mediated phenomenon, digestion of the hemagglutinin subunits. In contrast, trypsin treatment of virus grown in CEF or MDBK cells would result in two trypsin-mediated events, cleavage of HA into HA1 and HA2 and digestion of HA1 and HA2. In addition, unlike the virus-growth medium for other cell types, the CAM medium does not contain amino acids. Thus added radiolabeled amino acids are not diluted by cold amino acids

in the medium. This situation allows more efficient incorporation of radiolabel into the newly synthesized virus. Since the CAM cells are maintained in only 1 ml of medium per tube and as the virus yields per milliliter of medium are relatively high, less radiolabeled amino acids are required to incorporate a given amount of radioactivity into the virus than with cell monolayers maintained in 8-10 ml of medium.

Although ten 1-ml tubes of CAM cells are required to yield the same volume of virus-containing medium as one infected 10-ml bottle of CEF or MDBK cells, the CAM cells were easier to work with for several reasons. (1) CAM cells do not require weekly or bi-weekly division as do the continuous cell lines. (2) CAM membranes can be infected immediately and do not require overnight growth like CEF cells. (3) Since CAM cells are not maintained in the presence of serum, they do not have to be removed from the maintenance medium and washed prior to infection. As the infecting virus is added directly to the cell maintenance medium, the latter does not have to be added back to the cells following virus adsorption. (4) Since the virus recovered from infected membranes is relatively concentrated (high HAU/ml) and is not as contaminated by non-viral proteins as is egg-grown virus, it can be layered directly onto sucrose gradients without prior concentration and the visible bands recovered. Thus CAM cells were chosen for the culture of radiolabeled virus destined for treatment with trypsin. A limited quantity of radiolabeled virus was also grown in CEF cells.

Trypsin treatment of the recombinants NWS_{t2} and NWS_{t8}. Double infection of eggs with X-29 (H0N2) and X-9L (H2N1) theoretically yielded viruses of four serotypes (H0N1, H0N2, H2N1, H2N2) through reassortment of the viral RNA genes. Passage of this mixed yield in the presence of

antiserum to RI/5^{+} (H2N2) suppressed the replication of viruses bearing the H2 hemagglutinin and reduced the yields of viruses having an N2 neuraminidase. The serotypes of the resulting viruses (H0N1 or H0N2) were distinguished by the size of the plaques formed on Clone 1-5C-4 cells. NWS and other H0N1 viruses form large plaques and H0N2 viruses form small plaques on these cells. By addition of the anti-H2N2 serum during replication of the mixed yield from the double infection and by subsequent selection of large plaques formed on Clone 1-5C-4 cells, two recombinant viruses (NWS_{t2} and NWS_{t8}) were isolated that were expected to have H0N1 serotype.

Antigenic characterization of these viruses by hemagglutination-inhibition, plaque-inhibition, and plaque-size-reduction assays confirmed that NWS_{t2} and NWS_{t8} both derived the H0 hemagglutinin gene from X-29 and the N1 neuraminidase gene from X-9L (Table 4.9). The hemagglutination activity of NWS_{t2} and NWS_{t8} was resistant to trypsin for 90 min (Table 4.10). Thus the H0N2 parental virus X-29 passed its trypsin resistance to recombinants with a different neuraminidase serotype. The production of NWS_{t2} and NWS_{t8} (H0N1) from X-29 (H0N2) and X-9L (H2N1) resembled the production of X-12 (H0N2) from NWS (H0N1) and RI/5^{-} (H2N2), since in both cases, the trypsin sensitivity of the viral hemagglutination activity segregated with the H0 hemagglutinin protein when associated with either an N1 or an N2 neuraminidase.

Trypsin treatment of the recombinant X-29LT. Double infection of eggs with X-29L (H0N2) and X-41 (H3N2) followed by passage in the presence of A/Port Chalmers/1/73 antiserum (H3N2) produced the recombinant virus X-29LT, which was antigenically identical to X-29L. The high-yielding parental virus X-41 had previously been selected from recombination

between A/Port Chalmers/1/73 and the high-yielding virus A/PR/8/34 (HON1) (E. D. Kilbourne, unpublished results). The sensitivity of the hemagglutination activity of X-29LT to inactivation by the H0-directed NWS antiserum and insensitivity to the H3-directed Port Chalmers antiserum indicated that X-29LT had inherited the H0 hemagglutinin gene from X-29L (Table 4.11). Inhibition of the neuraminidase activity of X-29LT by the X-7 antiserum but not by the Port Chalmers antiserum (Table 4.11) indicated that X-29LT had also acquired the N2 neuraminidase gene from X-29L, which like X-7, was derived from RI/5$^-$. Although Port Chalmers and X-7 both possess a neuraminidase protein of N2 serotype, these results indicate that the two proteins differ antigenically. The drift in the N2 antigen between its characterization in 1957 and the present is well established (Schulman and Kilbourne, 1969). Since the anti-H3N2 serum was raised and assayed against virus of the same serotype, it is possible that the observed inhibition of the X-41 neuraminidase activity was due to steric hinderance by the homologous hemagglutinin protein (Schulman and Kilbourne, 1969). The derivation of the X-29LT neuraminidase would be conclusively established if the activity of the Port Chalmers antiserum were demonstrated against a virus with the Port Chalmers N2 neuraminidase and a heterologous (non-H3) hemagglutinin.

Unlike the two parental viruses, X-29LT exhibited trypsin-sensitive hemagglutination activity (Table 4.12). Thus recombination between the trypsin-resistant Port Chalmers virus and the slowly sensitive virus X-29L yielded a trypsin-sensitive virus. An explanation for the reversion of the hemagglutination activity from the slow sensitivity of X-29L to the more rapid sensitivity of most NWS-derived H0 viruses is not readily apparent.

The only structural difference observed between X-29L and X-29LT was the quantity and activity of the neuraminidase protein. X-29LT possessed approximately four times as much NA protein as X-29L and twice as much neuraminidase activity (Table 3.17). It is unlikely, however, that the quantity and activity of the viral NA protein determines the trypsin susceptibility of viral hemagglutination activity. X-12, which is trypsin sensitive like X-29LT, has less neuraminidase activity and protein than X-29L (Table 3.1). Similarly, the H0N2 recombinants X-7 and X-7(F1) differ in their neuraminidase content (Webster et al., 1968) but are both trypsin-sensitive (E. D. Kilbourne, unpublished results).

Treatment of influenza virus with other proteolytic enzymes. The different effect of trypsin on the hemagglutination activity of X-12 and X-29L was not observed using five other proteolytic enzymes. Pronase, a protease from Streptomyces griseus that exhibits the broadest specificity of any known proteolytic enzyme (Smyth, 1967), destroyed the hemagglutination activity of both recombinants. In another study, the hemagglutinin proteins of both A and B influenza virus types and four subtypes of A virus were readily digested by pronase but varied greatly in resistance to trypsin (Hoyle and Almeida, 1971). In addition, pronase inactivated the hemagglutination activity of NWS and two recombinant viruses possessing the NWS hemagglutinin (Schloer, 1971) and removed all the glycoprotein spikes from WSN virus (Schulze, 1970).

Chymotrypsin, which preferentially catalyzes the hydrolysis of peptide bonds involving the L-isomers of tryosine, phenylalanine and tryptophan (Green and Neurath, 1954; Desnuelle, 1960), did not inactivate the hemagglutination activity of either virus. Under more severe conditions (overnight incubation with 1 mg of enzyme per ml of virus

suspension), chymotrypsin reduced the hemagglutination activity of WSN virus by a factor of 10^3-10^4 (Schulze, 1970). Thirty-one strains of influenza virus retained complete hemagglutination activity, however, for 5 hr with 2.5 mg of chymotrypsin per ml of viral suspension (Sugg and Cleeland, 1962).

The hemagglutination activity was not inactivated by pepsin at pH 7.5. This result was not surprising because generally only denatured proteins are degraded by this enzyme.

IgA protease, a highly specific extracellular enzyme elaborated by Neisseria gonorrhoeae and Neisseria meningitidis cleaves a proline-threonine peptide bond in the heavy chain subclass A1 of the principal mucosal antibody IgA (Plaut et al., 1975). The hemagglutination activities of both X-12 and X-29L were resistant to this protease for 4 hr. Thus a proline-threonine bond is not exposed on the surface of the hemagglutinin protein, the enzyme requires a specific substrate conformation or a specific carbohydrate group for activity, or cleavage of such a bond does not affect the hemagglutinin-erythrocyte binding site.

Stem bromelain, a pineapple protease of broad specificity that preferentially attacks basic residues (Murachi, 1970), destroyed the hemagglutination activity and removed the HA, HA and HA1 proteins of purified WSN (H0N1) virus grown in MDBK cells (Compans et al., 1970). In contrast, the protease released all but a 3,000-dalton fragment of the hemagglutinin protein from X-31 (H3N2) (Brand and Skehel, 1972) and A/Bel/42 (H0N1) (Skehel and Waterfield, 1975) without further digestion, evidence that the viral glycoproteins show strain-specific differences. Bromelain has also been reported to destroy the neuraminidase protein and

reduce the hemagglutination activity of purified, egg-grown A/Bel/42 by 80% in 2-3 hr (Nermut, 1972). In this study, however, the hemagglutination activity of purified X-12 and X-29L, viruses which also possess an H0 hemagglutinin, did not decrease during treatment with bromelain for 4 hr. The same enzyme preparation reduced the activity of WSN virus in tissue culture fluid only 75% in 4 hr, suggesting that either the efficiency of the protease against the virus was reduced because of competing protein contaminants in the culture fluid, or that the activity of this particular enzyme preparation was not high. In either case, the hemagglutinin protein of X-12 and X-29L was relatively resistant to cleavage by bromelain.

SUMMARY

The influenza viruses NWS and RI/5$^-$ are distinguishable immunologically and by their sensitivity to trypsin. In contrast to the H2N2 virus RI/5$^-$, the HON1 virus NWS lost both its HA1 protein and its hemagglutination activity on exposure to trypsin for 30 min. Most viruses deriving their H0 hemagglutinin from NWS lost their hemagglutination activity within 90 min of trypsin treatment. The recombinant HON2 viruses X-6 and X-29, however, resembled RI/5$^-$ by retaining their hemagglutination activity for 90 min. Whereas RI/5$^-$ was completely stable to trypsin for at least 4 hr, X-29 or its large-plaque variant X-29L showed apparent trypsin resistance for about 2 hr but lost about 90% of its initial activity after 4 hr. Unlike RI/5$^-$, X-29L lost about one-third of its HA1 protein during brief trypsin treatment. Thus X-29 and X-29L are not trypsin-resistant like RI/5$^-$ but are slowly trypsin-sensitive.

The rapid trypsin sensitivity of recombinant X-12 (H0N2) was compared with the slow sensitivity of the antigenically similar X-29L (H0N2) in several ways. The initial loss of HA1 protein correlated with the loss of hemagglutination activity for X-12 but not for X-29L. The apparent first-order rate of loss of radiolabeled HA1 protein was about 2.3 times faster for X-12 than for X-29L. Electron microscopic examination of trypsin-treated virus particles showed that both viruses bore distinctive knob-like surface projections attributable to the trypsin-resistant neuraminidase protein.

Examination of stained slab gels revealed that the HA1 proteins of both X-12 and X-29L initially migrated as one component. Upon very brief exposure to trypsin, the HA1 protein appeared as two electrophoretically distinct proteins, a slower migrating component ($HA1_s$) and a faster migrating protein ($HA1_f$) coincident with the initial HA1. The amount of the $HA1_s$ protein of both viruses was relatively constant for at least 60 min of trypsin treatment. The $HA1_f$ component of X-12 was lost in about 30 min, which correlated with the loss of hemagglutination activity. Loss of the $HA1_f$ component of X-29L was nearly complete in 60 min, which was consistent with the 2.3-fold slower loss of HA1 seen with radiolabeled virus. But this loss of $HA1_f$ did not correlate with the much slower loss of X-29L hemagglutination activity.

The initial tryptic cleavage of the active $HA1_f$ protein of X-12 evidently produced the inactive $HA1_s$ protein, whereas cleavage of the $HA1_f$ of X-29L gave an $HA1_s$ protein that still possessed hemagglutination activity. The different trypsin sensitivity of X-12 and X-29L is evidently due to two factors: (1) the trypsin-mediated conversion of $HA1_f$ into $HA1_s$ occurs more slowly for X-29L than for X-12, and (2) the

$HA1_s$ component retains hemagglutination activity in X-29L but not in X-12. This implies that a structural difference in the hemagglutinin molecules of X-12 and X-29L affects the initial cleavage of the two molecules by trypsin.

The slow trypsin sensitivity of X-29L is a heritable characteristic that is independent of the neuraminidase protein. Recombination of X-29 (H0N2) with X-9L (H2N1) provided NWS_{t8} (H0N1), which showed the same slow loss of hemagglutination activity during trypsin treatment seen for X-29, even though the two viruses bear serotypically different neuraminidase proteins. Recombination of X-29Lwith trypsin-resistant X-41 (H3N2), however, furnished X-29LT (H0N2), which was rapidly sensitive to trypsin although it had acquired both its hemagglutinin and neuraminidase from X-29 L. The trypsin-sensitive recombinant X-29LT was found to have twice as much neuraminidase activity and protein as the resistant parent X-29L. Thus, although the two trypsin-resistant viruses $RI/5^-$ and X-29L possess approximately twice as much neuraminidase protein and activity as the two sensitive viruses NWS and X-12, the content of neuraminidase protein and activity apparently does not determine the trypsin sensitivity of the viral hemagglutination activity.

Finally, the differential loss of hemagglutination activity produced by trypsin was not observed using five other proteases. The hemagglutinin proteins of both X-12 and X-29L were rapidly inactivated by pronase, and were resistant to bromelain, chymotrypsin, IgA protease, and pepsin.

Chapter 5

Antisera Against Isolated Viral Proteins

The recombinant viruses X-12 and X-29 were originally distinguished by antigenic characterization. Although both exhibit the H0N2 serotype, they differ in their reactivity in plaque-inhibition, hemagglutinin-inhibition, and neuraminidase-inhibition assays (Chapter 2). Compared to X-12, X-29 showed a reduced reactivity with antiserum directed against the H0 hemagglutinin and an increased sensitivity to a range of H2 antisera. These antigenic characteristics were possibly due to differences in the structures of their hemagglutinin proteins. Thus the hemagglutinin subunits of X-12 and X-29 were isolated and purified in large quantities for antisera preparation and amino acid analysis.

MATERIALS AND METHODS

Virus. The recombinant viruses X-12, X-29 and X-29L were grown in 10-day or 11-day old embryonated chicken eggs and purified as described in Chapter 4.

Purification of isolated viral proteins. The viral proteins HA1, HA2, M and NP were purified by gel filtration as described by Bucher and colleagues (1976). Purified virus (approximately 60 mg) was pelleted and resuspended in 4.8 ml of 0.02 M Tris·HCl (pH 7.4) containing 0.1% SDS and 0.05% sodium azide (Buffer A). After disruption with 10% SDS (1.2 ml), the virus preparation was sonicated with a Biosonik Ultrasonicator (Bronwill) for about 2 min and then heated at 56°C for 60 min. After brief centrifugation (30 min at 16,500 rpm) to pellet insoluble material, the viral protein solution was pumped into the bottom of a column

(2.5 x 90 cm) containing Bio-Gel A-5m (Bio-Rad) equilibrated in Buffer A. The top of this column was connected to the bottom of an identical column. Fractions (about 4 ml) were eluted at room temperature from the top of the second column with Buffer A at an upward flow rate of 12-15 ml/hr, collected with an LKB 2000 fraction collector, and monitored for absorbance at 260 nm with an LKB Uvicord I ultraviolet monitor. The peak fractions were pooled, and the pool containing the co-eluting viral proteins HA and NP was concentrated to about 4.8 ml by pressure ultrafiltration using an Amicon Model 52 apparatus equipped with PM-10 membranes. After addition of 1.0% SDS (1.2 ml), this sample was re-chromatographed on the same columns under the same nonreducing conditions.

The fractions containing HA and NP were again pooled and concentrated. The hemagglutinin protein was then reduced to furnish the subunits HA1 and HA2 by addition of dithiothreitol (final concentration: 0.1 M). Following addition of SDS (final concentration: about 2%), the sample was chromatographed on a second pair of columns (each 2.5 x 90 cm) containing Bio-Gel A-5m equilibrated in 0.02 M Tris-HCl (pH 7.4) containing 0.1% SDS, 0.05% sodium azide and 0.01 M sodium thioglycolate (Buffer B). Fractions were eluted with Buffer B at an upward flow rate of 12-15 ml/hr and were monitored at 260 nm. The peak fractions were pooled, and the HA1, HA2 and NP pools were each individually re-chromatographed on the same pair of columns under the same reducing conditions. The fractions were pooled, diluted with acetone (final concentration: 80%), and kept overnight at -20°C. The precipitate was pelleted by centrifugation (2000 rpm for 20 min at 4°C), resuspended in distilled water and re-precipitated with acetone as before. The pellet

was suspended in 1 ml of distilled water containing 1% SDS for amino acid analysis or in PBS for antibody production.

Polyacrylamide gel electrophoresis (Chapter 3) was used to characterize the proteins eluted from the columns and to assess their purity.

Antisera production. Rabbits were injected with antigen and bled by Mrs. W. Haab, Pocono Rabbit Farm and Laboratory, Canadensis, Pennsylvania. Following an initial test bleeding, each of the following samples was injected into two male rabbits: purified HA1 protein from X-12, purified HA2 protein from X-12, purified HA1 from X-29L, purified HA2 from X-29L, intact X-12 virus, or intact X-29L virus. For each initial injection and the first booster injection, about 25 μg of purified hemagglutinin protein was suspended in 0.5 ml of PBS or 170-190 μg of whole virus in 0.5 ml of PBS. The quantity of protein injected was increased for later inoculations (Table 5.1). On Day 0, each of the above samples was mixed with Freund's adjuvant and injected into the rabbit footpad. Test bleedings were taken on Days 14 and 28. On Day 42 a booster injection of viral protein was administered intravenously without adjuvant. Following test bleedings on Days 57 and 62, a booster injection with adjuvant was given through the footpad on Day 73. Test bleedings were taken on Days 92 and 106. On Day 119 a booster of viral protein with adjuvant was given through the footpad and sample bleedings were taken on Day 134. Intravenous booster injections without adjuvant were given on Day 153 and test bleedings were taken on Day 162. The above rabbits and three new control rabbits of approximately the same age were each injected intravenously with 50 μg of purified A1/Cornell/56 virus (H1N1) without adjuvant on Day 190. Test bleedings were taken on

TABLE 5.1

Amounts of Viral Antigens from X-12 and X-29L Used for Rabbit Immunization

Virus, antigen	Amount of protein injected (μg) Day: 0	42	52	73	119	153	Total protein (μg)
X-12, HA1	26	26	52	26	52	26	208
X-12, HA2	19	19	38	19	72	72	239
X-29L, HA1	25	25	50	36	36	72	244
X-29L, HA2	25	25	34	9	0[a]	0[a]	93
X-12, whole	191	191	382	1092	1092	1092	4040
X-29L, whole	167	167	334	954	954	954	3530

[a]Lack of antigen precluded booster injections of HA2 from X-29L on Days 119 and 153.

Days 196, 199, 202, 205 and 210, and on Day 224 each rabbit was bled from the heart.

Antisera assays. Antisera were heated at 56°C for 30 min and treated with RDE (Chapter 4) prior to assay. Hemagglutination-inhibition (HI), neuraminidase-inhibition (NI), plaque-inhibition (PI) and plaque-size-reduction (PSR) assays were performed as described in Chapter 4.

RESULTS

Preparation of purified viral proteins. Isolation of virus from 600 infected embryonated eggs yielded about 60 mg of purified viral protein. After disruption of the virus with SDS, the viral proteins were separated by gel filtration on agarose columns, as illustrated in Fig. 5.1A. The viral proteins present in each peak were identified by comparison of their polyacrylamide gel electrophoresis protein patterns with the pattern of whole virus (Fig. 5.2). Peak 1 at the void volume, which contained partially disrupted virus and the viral RNA, varied in magnitude from run to run. The location of the viral neuraminidase in Peak 3 was established both by the polyacrylamide gel protein patterns and by neuraminidase assay of the eluted column fractions. Peak 4 contained the P proteins and some hemagglutinin protein. The majority of the HA protein co-eluted with NP in peak 5, and Peak 6 primarily contained the M protein. More than half of the protein applied to the column was recovered as HA and NP in peak 5.

The peak fractions corresponding to Peak 5, which contained HA and NP, were pooled and re-chromatographed on the same pair of columns under the same nonreducing conditions. As illustrated in Fig. 5.1B, the HA + NP

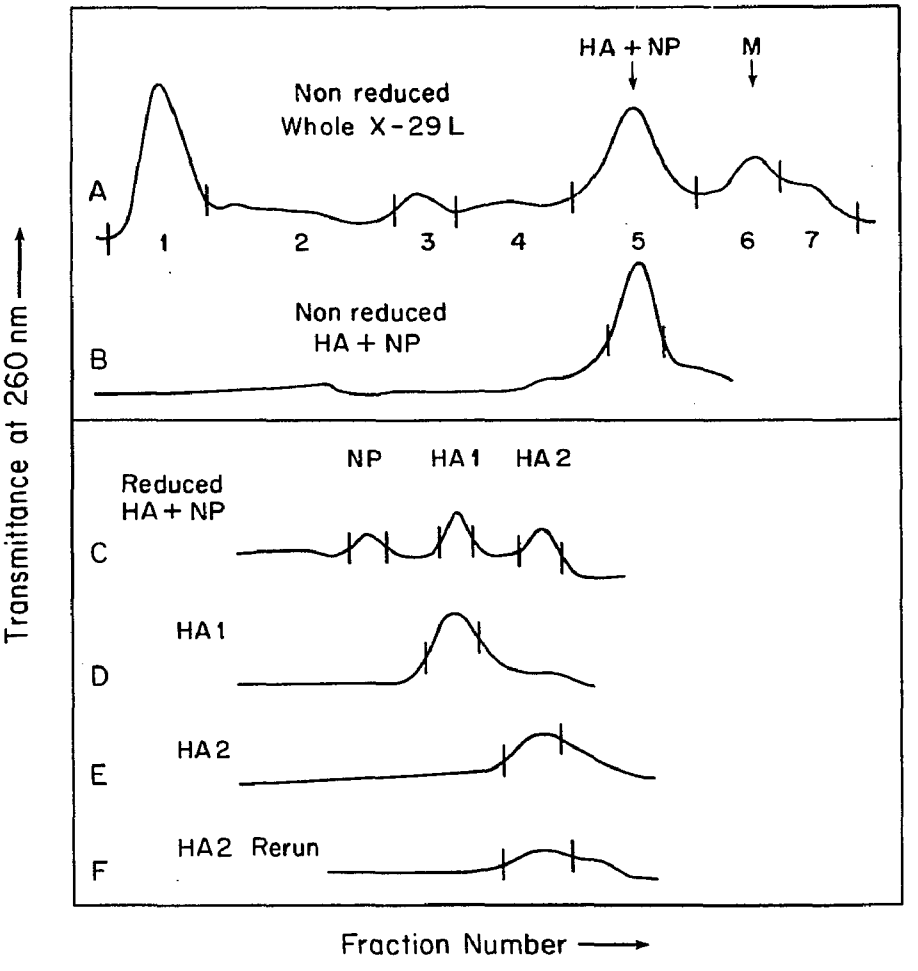

Fig. 5.1. Purification of X-29L hemagglutinin subunits by SDS gel filtration before and after reduction. X-29L virus (60 mg) was disrupted in SDS and the resulting proteins were gel filtered on Bio-Gel A-5m in Buffer A under nonreducing conditions (Panel A). Peak 5 was rerun (Panel B) under the same conditions. The HA plus NP peak (23 mg) was reduced with dithiothreitol and gel filtered (Panel C) in Buffer B, which contained sodium thioglycolate. The HA1 peak was rerun (Panel D) under these reducing conditions to give 0.84 mg of purified HA1 protein. The HA2 peak was rerun twice (Panels E and F) to furnish 0.37 mg of purified HA2 protein. The vertical lines indicate the fractions pooled. Gel patterns of the proteins in the initial peaks (Panel A) are shown in Fig. 5.2 and patterns of the purified proteins are shown in Fig. 5.3.

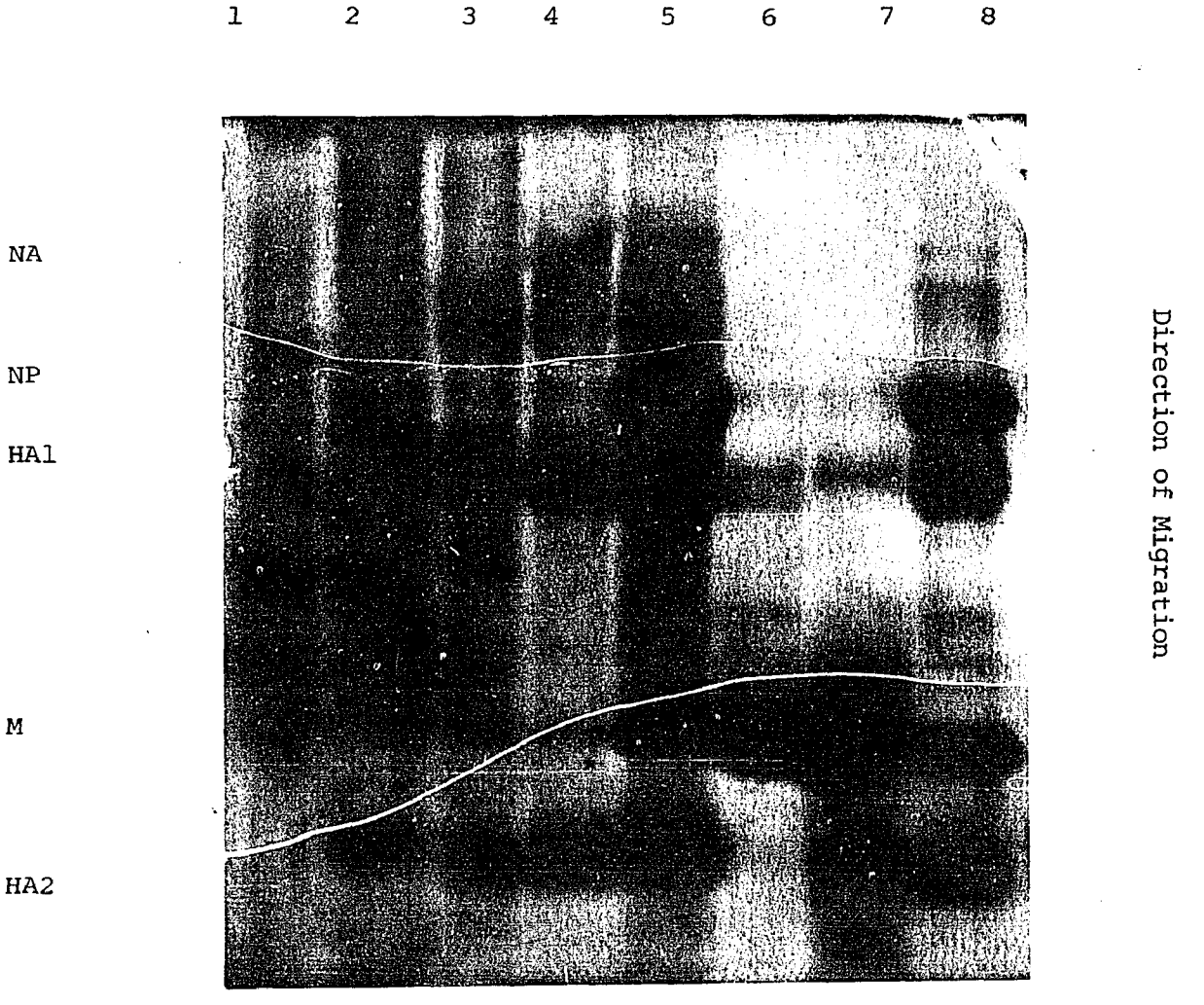

Fig. 5.2. Polyacrylamide slab gel electrophoresis of X-29L viral proteins after SDS gel filtration. The origin of the first seven protein samples is shown in Fig. 5.1. Sample 8 was purified X-29L virus. The proteins were visualized by staining with Coomassie blue.

peak was sharper after the second column run. Approximately 30% of the original viral protein was recovered in this peak. The HA and NP proteins were separated by reduction of the hemagglutinin protein. The HA1 and HA2 subunits were both smaller than NP and eluted after the NP peak (Fig. 5.1C). Each of these three proteins was purified by further gel filtration under the same reducing conditions. Rechromatography of HA1 once (Fig. 5.1D) provided 0.84 mg of purified HA1 protein. Rechromatography of HA2 twice (Fig. 5.1E and 5.1F) afforded 0.37 mg of purified HA2 protein. The M protein was also purified by additional chromatography.

The purity of the isolated viral proteins was measured by polyacrylamide gel electrophoresis. Fig. 5.3 shows the protein patterns observed for the HA1 and HA2 proteins obtained from the separations shown in Fig. 5.1.

Assay of antisera. Test bleedings were assayed for immunological activity by hemagglutinin-inhibition (HI; Table 5.2) and plaque-inhibition (PI; Table 5.3). Both assays indicated that few antibodies were elicited in response to the purified hemagglutinin subunits, except in one rabbit that received the HA2 protein from X-12. The best antibody response was seen on Day 62, when the HI titer was only 40 and the PI titer was only about 10. Repeated inoculations with the purified proteins intravenously or into the footpad with or without adjuvant had no effect.

In contrast, the preparations of whole virus elicited a significant antibody response. The highest HI response was also observed on Day 62 after the initial and two booster injections (Table 5.2). Whole virus antibodies were also detected by PI assay; the activity against the parental virus NWS (HON1) was slightly greater than against the recombinant X-7 (HON2) (Table 5.2).

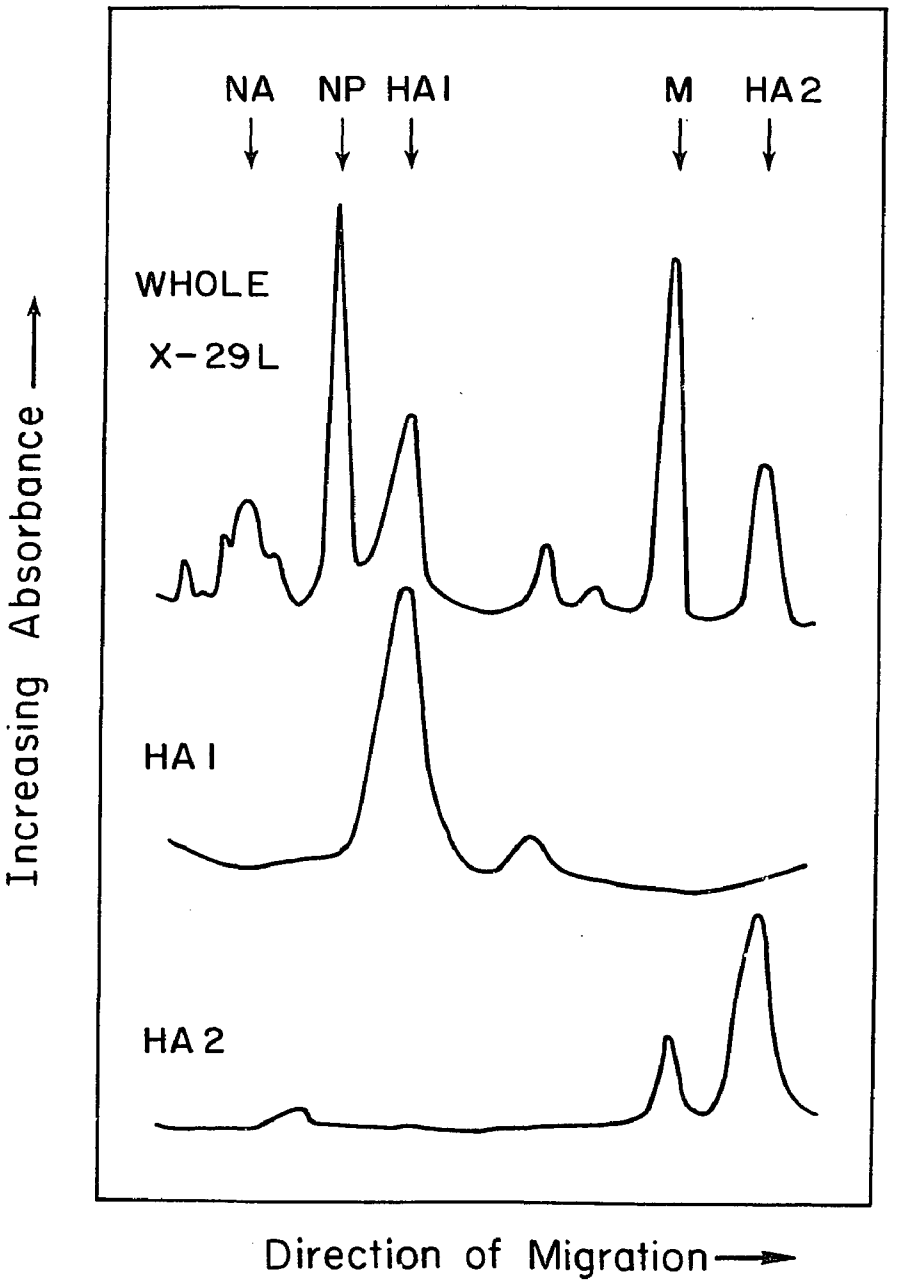

Fig. 5.3. Polyacrylamide gel electrophoresis of purified hemagglutinin subunits from X-29L virus. HA1 and HA2 were purified by gel filtration on Bio-Gel A-5m (Fig. 5.1). Densitometric tracings of the Coomassie blue-stained protein bands are shown.

TABLE 5.2

Hemagglutination-Inhibition Activity of Rabbit Antisera

Elicited by Purified Hemagglutinin Subunits from X-12 and X-29L

Virus, antigen	Day: / Event[b]:	HI activity against whole X-12 (or X-29L) virus[a]													
		0 AI	14 TB	28 TB	42 AI	52 AI	62 TB	73 AI	106 TB	119 AI	134 TB	153 AI	162 TB	190 AI	224 TB
X-12 HA1			0	0					0(0)		0		0		
X-12, HA1			0	0			40(0)		0(0)		0		0		40
X-12, HA2			0	0			20480(10240)		640(160)		320		320		
X-29L, HA1			0	0					0(0)		0		0		
X-29L, HA1			0	0			40(0)		0(0)		0		0		40
X-29L, HA2			0	0					0(0)		0		0		
X-29L, HA2			0	0			40-80(0)		0(0)		0		0		40
X-12, whole			0	40					2560(1280)		10240		20480		
X-12, whole			160	160			40960(20480)		2560(1280)		2560		2560		320
X-29L, whole			40	160					1280(1280)		5120		2560		
X-29L, whole			80	80			10240(10240)		1280(2560)		5120		10240		$\geq$ 640

See the next page for footnotes.

[Footnotes for Table 5.2.]

[a]Hemagglutination-inhibition activity is expressed as the reciprocal of the highest serum dilution that completely inhibited the hemagglutination activity of X-12 virus (or X-29L virus).

[b]Antigen injections (AI) or test bleedings (TB) were performed on the days indicated. The antigen injected at Day 190 was whole A1/Cornell/56 (H1N1) virus.

TABLE 5.3

Plaque-Inhibition Activity of Rabbit Antisera Elicited by Purified Hemagglutinin Subunits from X-12 and X-29L

	PI activity against NWS or X-7 virus[a]					
Day:	62	62	92	92	92	224
Virus, antigen / Virus:	NWS[b]	X-7[b]	NWS	NWS[b]	X-7	X-7[b]
X-12, HA1			< 400	< 400	< 400	
X-12, HA2			> 1600	160-320	> 1600	
X-12, whole			> 6400			
X-29L, HA1	< 10	< 10	< 400		< 400	20
X-29L, HA2	13	10			< 400	40
X-29L, whole	6400	2800				1600

[a]PI activity is expressed as the reciprocal of the highest antiserum dilution that decreased the number of plaques by half.

[b]The mixture of antiserum and plaquing virus was incubated for 30 min at 4°C before inoculation of the Clone 1-5C-4 cells.

The weak antibody response to the purified hemagglutinin subunits was verified by examining the response of the immunized rabbits and naive rabbits to an antigenically heterologous virus (H1N1). The kinetics of the antibody responses for four representative rabbits is illustrated in Fig. 5.4. In all cases, rabbits previously exposed to the purified viral protein gave an immune response more rapidly than the naive animals. After injection of the heterologous H1N1 antigen on Day 190, a slight increase in the titer against the homologous antigen was detected by both the HI and PI assays (Tables 5.2 and 5.3). Day-62 serums did not cross-react with H1N1 virus, as measured by HI assays, except for the serum from one rabbit inoculated with whole X-12 virus, which had a titer of 80 against the heterologous antigen. The immune response detected 100 days later, however, was less specific. Day-162 sera just prior to injection of H1N1 virus had HI titers of 40 when assayed against the heterologous antigen. Thus antisera sampled shortly before injection of the H1N1 virus showed slight cross-reactivity with the heterologous H1N1 virus.

One rabbit that received the HA2 protein from X-12 virus gave a definite immune response as shown by both HI and PI assays. On Day 62, the HI titer of serum from this rabbit was equivalent to that of sera from rabbits receiving whole virus, but the sera from later bleedings gave lower HI titers than sera against whole virus. When serum from this rabbit was assayed for neuraminidase-inhibition (NI) of RI/5$^-$ virus and plaque-size-reduction (PSR) activity against the recombinant virus X-1L (H2N2), titers of 32 and 800, respectively, were obtained. Unfortunately, the second rabbit that received the HA2 protein from X-12 died very early in the experiment.

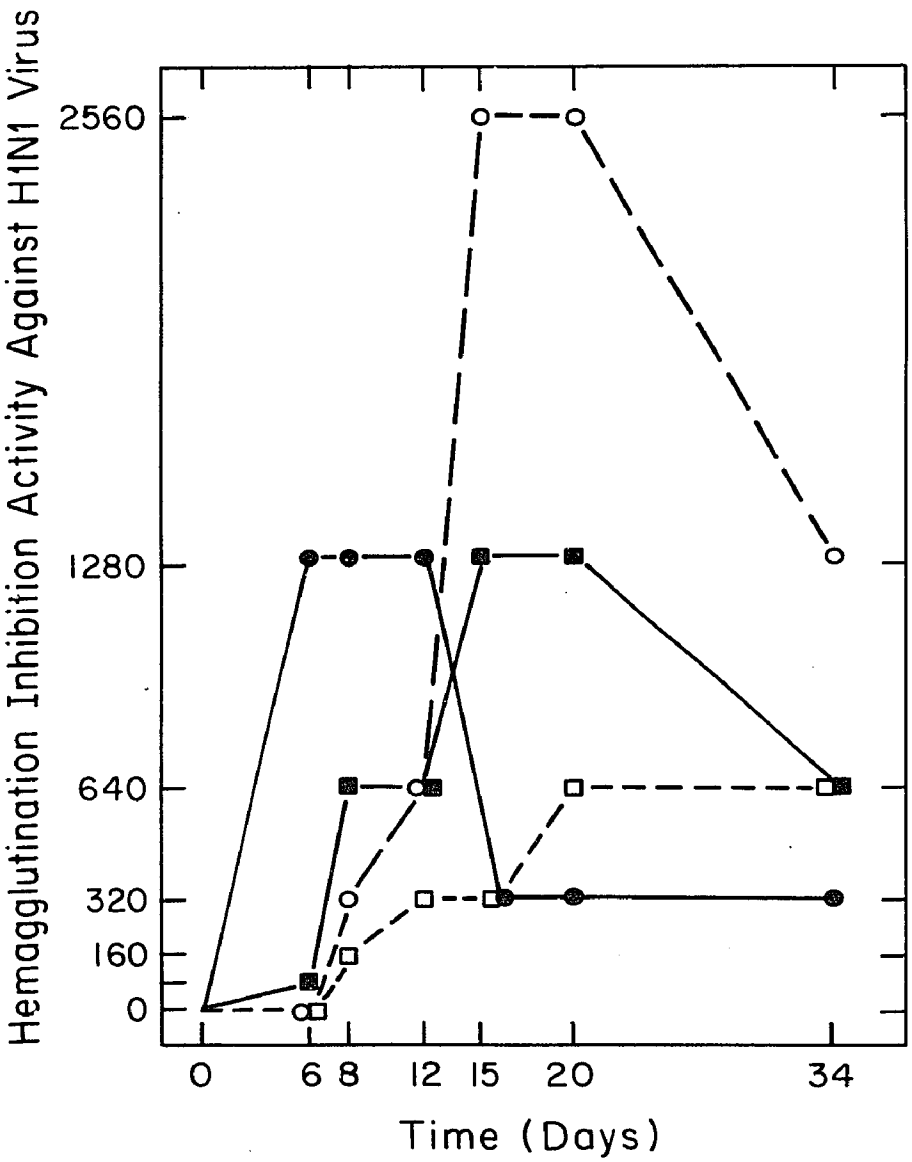

Fig. 5.4. Kinetics of immune response of H0N2-immunized and naive rabbits to H1N1 virus. On Day 0 each rabbit was injected intravenously without adjuvant with 50 μg of purified A1/Cornell/56 (H1N1) virus. The HI activity is expressed as the reciprocal of the highest serum dilution that completely inhibited hemagglutination. The rabbits were previously immunized with whole viral protein (●) or purified HA1 subunits (■) from X-29L (H0N2) or were immunologically naive (○ , □).

DISCUSSION

The different sensitivity of the hemagglutination activity of X-12 and X-29L to trypsin (Chapter 4) and the reactivity of the two viruses in immune reactions (Chapter 2) suggested that the major structural difference between the two viruses would probably be found in their hemagglutinin proteins. Thus the viral proteins NP, HA1, HA2 and M were isolated in relatively pure form by the gel filtration procedure used by Bucher and co-workers (1976) to isolate large quantities of influenza viral proteins for sequencing studies. Although the viral NA and P proteins were detected in certain column fractions, no attempt was made to isolate and purify these proteins because they constitute so little of the total viral protein that their final yields would have been small. Purified hemagglutinin subunits have previously been isolated on a large scale by electrophoresis of SDS-disrupted virus on cellulose acetate (Laver, 1964). This procedure cannot be used for all virus strains, however, because it requires that the hemagglutinin remain active while the neuraminidase protein is denatured on exposure to SDS. Hemagglutinin has also been isolated in milligram quantities by chromatography of viral proteins on DEAE-cellulose in the presence of the non-denaturing detergent sodium lauroyl sarcosinate (Sarkosyl) (Stanley et al., 1973b).

The hemagglutinin subunits isolated by gel filtration in the presence of SDS and DTT produced a low antibody response in rabbits. Although 200-250 μg of purified hemagglutinin protein was injected per rabbit over a period of 224 days, the plaque-inhibition and hemagglutinin-inhibition titers of sera were never greater than 40 and were usually less.

The immune response observed on injection of the heterologous antigen, H1N1 virus, provided evidence that an immune response, although

weak, had been elicited by the H0N2 antigens. The response of those rabbits previously exposed to the H0N2 antigens was characterized by a shorter lag phase than that of the naive rabbits receiving the H1N1 virus (Fig. 5.4). Thus the H0N2-primed rabbits exhibited not a primary but a secondary immune response. Since the secondary response was elicited by an influenza virus of a different subtype, the primed animals had previously responded to a related antigen, a phenomenon known as original antigenic sin (Francis et al., 1953). This effect is ascribed to cross-stimulation of an enlarged population of cross-reactive B memory cells remaining after the primary antigenic stimulation (Eisen et al., 1969). Since antisera samples taken before injection of the H1N1 virus showed slight cross-reactivity with this heterologous antigen, part of the reactivity observed after H1N1 injection was probably due to cross-reactivity between the H0N2 antigen used in the HI and PI assays and antibodies elicited in a primary response to the H1N1 virus.

The low production of antibodies active against the native HA antigen was probably the result of denaturation of the hemagglutinin protein during isolation. Because of the hydrophobic nature of HA2 and NA and the tendency of HA and NA to form mixed aggregates in the absence of detergent (Laver and Valentine, 1969), it was necessary to isolate the proteins in the presence of the detergent SDS. Denaturation by this detergent may have destroyed crucial conformational antigenic determinants on the hemagglutinin subunits. Similarly, Webster (1970b) found that HA1 isolated by gel filtration after denaturation with guanidine hydrochloride and mercaptoethanol failed to block antibodies in the hemagglutination-inhibition test. This result suggests that the antigenic determinants are not composed of adjacent amino acids on a single polypeptide chain.

The sensitivity of an antigen to detergent denaturation or the importance of conformational determinants probably varies with the protein. In contrast to our results with the HA subunits, antibodies are formed against non-histone chromatin proteins exposed to 1% SDS and injected into rabbits in the presence of 0.1% SDS (Stumph et al., 1974). Also, detection of neuraminidase activity in the third column peak indicated that the isolated N2 enzyme remained at least partially active in the presence of detergent.

Alternatively, the weak production of antibodies active against the native viral HA antigen might be due to the reduction of the disulfide bond(s) linking HA1 and HA2. Because the NP and HA proteins co-migrate during gel filtration on agarose, it was necessary to reduce HA to give the subunits HA1 and HA2, which were separated from NP by further gel filtration. The antigenic determinants of the native hemagglutinin molecule may require interaction of the disulfide-bonded HA subunits. Laver and co-workers (1974) found that one of the multiple antigenic determinants of hemagglutinin is missing from bromelain-released hemagglutinin and suggested that some antigenic sites may overlap with or reside entirely within the HA2 glycoprotein.

It is also possible that the major antigenic sites of native hemagglutinin result from the interaction of two or more hemagglutinin molecules. Electron micrographs have revealed that purified hemagglutinin spikes are triangular in shape, suggesting that each spike is composed of three HA molecules (Laver, 1973; Griffith, 1975). Despite acetone precipitation and resuspension in PBS, sufficient SDS may have remained in our preparation of HA1 to prevent functional interaction of the subunits. By extraction of PR/8 virus with chloroform--methanol followed

by treatment with guanidine and dithiothreitol, Eckert (1966, 1969, 1973) isolated a hemagglutinin-binding antigen (HABA), a dimer of the HA1 subunit (see Chapter 6) that removes anti-HA antibodies from serum prepared against whole virus and induces the formation of hemagglutination-inhibiting and neutralizing antibodies.

One of seven rabbits gave a significant immunological response to an isolated hemagglutinin subunit, producing neutralizing and hemagglutination-inhibiting antibodies in response to injection of HA2 protein from X-12 virus. The magnitude of this response, especially in response to the HA2 subunit, and the detection of low anti-neuraminidase activity suggest that this rabbit may have accidentally received an injection of whole virus. This possibility cannot be ruled out by the response of the second rabbit receiving the same antigen because that rabbit died early in the experiment. Alternatively, the rabbit may have been hyper-responsive and produced specific antibodies to the HA2 protein, which weakly inhibited neuraminidase activity through steric hinderance. Consistent with this possibility, the two rabbits injected with the HA2 protein from X-29L also produced serum with HI and PI titers as high or higher than the titers of sera from rabbits injected with the HA1 protein from X-29L.

The contribution of the HA2 subunit to the immunogenicity of the hemagglutinin protein is not fully understood. Bromelain-released hemagglutinin lacks one of the antigenic determinants of the intact molecule (Laver et al., 1974), which suggests that at least one antigenic determinant is partly or completely on HA2. Brand and Skehel (1972) demonstrated by immunodiffusion that antibodies could be elicited against a 30-μg sample of HA2 protein recovered from SDS--polyacrylamide

gels. This HA2 antiserum, however, only reacted with reduced hemagglutinin molecules, while antisera against whole virus, HA or HA1 all reacted with nonreduced bromelain-released HA to give one common precipitin line. This suggested that the functional antigenic site of the intact hemagglutinin is located on HA1 but that dissociation of HA1 and HA2 exposes an additional site on HA2. In contrast, our preliminary results with low-titer antisera suggest that antibodies elicited by HA2 protein may block the biological activity of the hemagglutinin protein. This result could be due to involvement of HA2 in the hemagglutinin active site, to steric hinderance of the active site by the HA2 antibodies, or to common determinants on HA1 and HA2 (see Chapter 6).

Denatured, isolated hemagglutinin subunits probably elicit antibodies against some antigenic determinants that are far from the hemagglutinin active site or are not exposed on the native molecule. These determinants should be detectable by immunological assays not involving blocking of a biological activity, such as immunodiffusion. Preliminary attempts to characterize the antisera raised against the isolated hemagglutinin subunits by double immunodiffusion were not very successful. The bands obtained were extremely faint, which precluded the analysis of reactions of antigenic identity such as those required for immunologic comparison of the subunits from X-12 and X-29L. Schild and Dowdle (1975) have reported that assay for antibodies to hemagglutinin by double immunodiffusion is considerably less sensitive than HI tests because precipitin reactions are generally only observed with sera with HI titers of several hundred.

SUMMARY

Purified hemagglutinin subunits HA1 and HA2 and proteins M and NP were isolated from the recombinant viruses X-12 and X-29 in milligram quantities by gel filtration in the presence of SDS and dithiothreitol. All four viral proteins were analyzed by amino acid analysis (Chapter 6). In addition, the purified hemagglutinin subunits were injected into rabbits for antisera production.

Purified HA1 and HA2 proteins elicited few antibodies capable of blocking the biological activity of the hemagglutinin molecule. Despite the injection of 200-250 μg of purified protein, the highest hemagglutination-inhibition titer observed was only 40 on Day 62; the corresponding plaque-inhibition titer was about 10. The HA2 protein elicited an immunological response which was equal to or better than that seen with the HA1 subunits. The poor immunogenicity of the hemagglutinin subunits was probably due to denaturation of the antigen during isolation. Production of subunit vaccines by immunization with isolated hemagglutinin would be a significant medical advance. The HA1 and HA2 subunits isolated by treatment of virus with SDS and thiol, however, are poorly suited for this purpose.

Chapter 6

Amino Acid Compositions of Viral Proteins

Double infection with trypsin-sensitive and trypsin-resistant parental viruses has yielded both trypsin-sensitive and trypsin-resistant recombinant viruses of the same serotype. One explanation for this observation is that the trypsin sensitivity of the viral hemagglutination activity is an inherited characteristic. Thus, although trypsin-resistant X-29 appears serotypically identical to trypsin-sensitive X-12 (both H0N2), X-29 may have inherited some characteristic not detected by serological typing that renders its hemagglutination activity more resistant to inactivation by trypsin.

Trypsin susceptibility may correlate with the primary structure of the hemagglutinin molecule. Since the trypsin-resistant parent RI/5$^-$ is serotypically H2 and the trypsin-resistant recombinant X-29 is serotypically H0, the hemagglutinin molecule is not identical for these viruses. The two hemagglutinin subunits, HA1 and HA2, of X-29, however, might be derived from different parents. For example, the X-29 hemagglutinin may consist of an exterior HA1 polypeptide possessing the H0 antigenic determinants of its NWS parent and an interior HA2 polypeptide possessing the trypsin resistance of its RI/5$^-$ parent. Since the hemagglutinin protein is synthesized as a single polypeptide that is cleaved at the cell surface into the two subunits (Lazarowitz et al., 1971), the HA1 and HA2 proteins are not normally inherited independently. Hemagglutinin genes from the sensitive and resistant parents, however, might undergo crossover in a doubly infected cell. The hybrid genes created by this intragenic recombination may code for the serological

markers of one parent and the trypsin-sensitive site from the other. The RNA regions exchanged could be small gene fragments or large regions sufficient to code for complete hemagglutinin subunits.

Alternatively, another viral protein may determine the proteolytic susceptibility of the hemagglutination activity. For example, variation in M protein might affect the positioning and exposure of the hemagglutinin molecules on the surface of the virus. Since serotyping only characterized the external antigenic determinants, nothing was known about the derivation of the internal proteins of the recombinant viruses.

Thus, we were interested in determining if trypsin susceptibility correlated with the inheritance of a particular viral protein. Several methods employed to identify the parental donors of recombinant virus proteins are discussed below.

Immunological reactions are most commonly used to determine the derivation of the proteins of recombinant influenza viruses (Kilbourne et al., 1967). The serotype of a new recombinant virus is established by quantitating the reaction of the surface proteins, hemagglutinin and neuraminidase, with antisera prepared against the known parental serotypes. Although this assay indicated that both X-12 and X-29 inherited the hemagglutinin protein from NWS, the proportion of the hemagglutinin protein recognized by the serotypic antibodies is not known. Presumably some changes in amino acid composition do not affect the antigenic determinants.

Antibodies to the internal viral proteins M and NP have been detected in whole-virus antisera by complement-fixation or immunodiffusion assays (Pereira, 1969; Schild, 1972). These tests have shown that the NP and M antigens are type-specific. This result is the

basis for the antigenic characterization of influenza viruses as type A, B or C. Immunological reactions have not demonstrated characteristic differences in the internal proteins of different viral serotypes. Thus these procedures have not been employed to follow inheritance of the internal viral proteins.

Peptide mapping, a sensitive means of assessing protein homology, involves exhaustive trypsin digestion of the protein followed by resolution of the tryptic fragments by two-dimensional chromatography--electrophoresis. This method has been used by Laver and Downie (1976) to identify the parental donor of the membrane protein of recombinant viruses. Differences in the hemagglutinin proteins from different viral serotypes have also been detected by this technique (Laver and Webster, 1972).

The recent development of polyacrylamide gel electrophoresis sytems capable of resolving the RNA genome segments of influenza virus (McGeoch et al., 1976; Palese and Schulman, 1976a; Pons, 1976) has made it possible to determine which parental virus has donated each recombinant-virus gene. This procedure has been employed to determine the genetic composition of the high-yielding influenza virus recombinant used as the vaccine strain against "swine" influenza (Palese et al., 1976). No correlation is available, however, between the change in electrophoretic migration of an RNA gene and the corresponding changes in amino acid composition of the protein for which it codes. In addition, phenotypic characteristics of similar viruses are not necessarily reflected in their RNA gel patterns. For example, the antigenically identical recombinants X-53 and X-53a showed similar RNA gel patterns but differed in several biological properties, including the size of plaques formed on MDCK cells and virus

yields from embryonated eggs (E. D. Kilbourne, unpublished results).

Determination of the amino acid sequence of parental and recombinant viral proteins can firmly establish protein inheritance patterns. The limited availability of large quantities of purified proteins has so far precluded the sequencing of a complete influenza virus protein, although short amino-terminal sequences of HA have been reported (Skehel and Waterfield, 1975; Bucher et al., 1976). These data suggest that the amino termini of HA1 and especially HA2 are relatively conserved. Further sequence analysis will be necessary to determine the sequence characteristic for each serotype.

Another means of assessing protein relatedness is comparison of amino acid composition. This technique has not been used to identify the parental donors of recombinant virus proteins. If the composition of a given structural protein from one parent is sufficiently different from that of the other parent, it should be possible to determine which parent donated this protein to any recombinant virus. Specifically, if one of two recombinant viruses possesses a hybrid hemagglutinin gene, this situation might be detected by comparing the amino acid compositions of their HA1 and HA2 proteins. A direct correlation between amino acid composition and sequence has been demonstrated for several protein families (hemoglobin, immunoglobulin light chain, cytochrome c) for which extensive sequence data are available (Marchalonis and Weltman, 1971). Thus composition comparisons for viral proteins should be in qualitative agreement with relationships deduced by more rigorous methods. Since amino acid compositions are relatively easy to measure, we have used this technique to compare the proteins of the parental and recombinant influenza viruses.

The amino acid composition data available for influenza A proteins are limited. Three groups have published compositions for the H0 hemagglutinin (Laver and Baker, 1972; Eckert, 1973; Skehel and Waterfield, 1975) which differ from the composition of an H3 hemagglutinin (Ward and Dopheide, 1976). The N2 neuraminidase has been analyzed twice (Kendal and Eckert, 1972; Laver and Baker, 1972), but the composition published by Kendal and Eckert was not complete, being performed on proteolytically-derived protein. The only M and NP proteins which have been analyzed were isolated from the A0/Bel/42 virus (Laver and Baker, 1972). Laver and Baker (1972) also determined compositions of five influenza B proteins. A quantitative comparison of the variations in these amino acid compositions has not been made. Earlier reports of amino acid compositions for influenza virus proteins (Knight, 1947; Hoyle and Davies, 1961) appeared before the viral protein preparations had been adequately demonstrated to be pure polypeptides.

In an attempt to determine if the trypsin-resistant hemagglutination activity of X-29 was inherited from the trypsin-resistant parent, RI/5^{+}, the amino acid compositions of four proteins from the recombinant viruses X-12 and X-29 were compared with those of the parental viruses NWS and RI/5^{-}.

MATERIALS AND METHODS

Isolation of viral proteins. The parental viruses NWS and RI/5^{-} and the recombinants X-12 and X-29 were grown in eggs and purified by density gradient centrifugation as described previously (Chapter 4). For analysis of the X-29 proteins HA2 and M, the samples were isolated from a large-plaque variant, X29L. The individual viral proteins were isolated

by two different procedures that both avoided the use of proteolytic enzymes. The recombinant-virus proteins were separated by gel filtration and their purity was demonstrated by polyacrylamide gel electrophoresis (Chapter 5). The parental viral proteins were isolated directly from 13% acrylamide gels which contained SDS and were electrophoresed in Tris--glycine buffer. After electrophoretic dialysis (115 mA, 3 h) against 5% acetic acid to remove free glycine (T. E. Hugli, personal communication), the stained protein bands were cut from the gels.

Acid hydrolysis of viral proteins. Recombinant virus proteins were hydrolyzed at 115°C for 24 h *in vacuo* either by 4 N methanesulfonic acid containing 0.2% tryptamine (Simpson *et al.*, 1976) or 6 N HCl (Moore and Stein, 1963). In certain cases, cysteine was determined as cysteic acid by oxidation of the protein with 3% performic acid for 4 h at 0°C followed by hydrolysis with 6 N HCl for 24 h at 115°C (Moore, 1963). Parental virus proteins in gel slices were hydrolyzed with 6 N HCl for 24 h at 110°C by the method of Houston (1971). During acid hydrolysis, asparagine is converted into aspartic acid and glutamine into glutamic acid. Serine and threonine values were not corrected for destruction during hydrolysis.

Amino acid analysis of viral proteins. Analysis of the recombinant virus proteins was performed by Dr. Paul L. Fletcher, Jr. and Dr. Stanford Moore, The Rockefeller University, New York, New York on a Durrum D-500 analyzer. Parental virus proteins were analyzed by Dr. Tony E. Hugli, Scripps Clinic and Research Foundation, La Jolla, California, on a Beckman 120 C analyzer equipped with 3-mm microbore columns.

Calculation formulas.

A. Amount of each amino acid analyzed. As the amino acid residue types are resolved by ion-exchange chromatography, they are recorded as peaks which are integrated by the amino acid analyzer. The nanomoles of amino acid represented by a peak area is determined from analyses (run before or after the sample) of a standard amino acid mixture containing either 1 or 10 nmol of each amino acid. The specific relationship is:

$$Mol_X = \left(\frac{Area_X}{Area_{SX}} \right) Mol_{SX} \qquad (1)$$

where X is a given type of amino acid, Mol_X is the amount of X in the sample analyzed (nmol), $Area_X$ is the area of the X peak from the sample analyzed (mm^2), $Area_{SX}$ is the area of the X peak from the standard amino acid mixture analyzed (mm^2), and Mol_{SX} is the amount of X in the standard amino acid mixture analyzed (nmol). The Beckman analyzer calculated and recorded the nanomoles of each amino acid per milliliter of protein hydrolysate solution.

When the viral proteins were isolated from gels, background levels of amino acids in the gel were subtracted. A blank polyacrylamide gel was electrophoresed and a slice was hydrolyzed and submitted for amino acid analysis. Low levels of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine and leucine were detected and subtracted as follows:

$$Bkg_X = \left(\frac{Wt_P}{Wt_B} \right) \left(\frac{Vol_B}{Vol_P} \right) Area_{BX} \qquad (2)$$

where X is a given type of amino acid, Bkg_X is the background level of X, Wt_P is the weight of the protein gel slice, Wt_B is the weight of the blank gel slice, Vol_B is the volume of amino acid hydrolysate from the

blank gel slice (ml), Vol_P is the volume of amino acid hydrolysate from the protein gel slice (ml), and $Area_{BX}$ is the area of the X peak from the blank gel slice analyzed (mm^2).

B. Mole percentage of each amino acid. The number of residues of each amino acid type per 100 residues (mole percent) was calculated as follows:

$$\chi_X = \frac{Mol_X}{\sum_{i=1}^{n} Mol_{X_i}} \tag{3}$$

where X is a given type of amino acid, χ_X is the mole percentage of X (mol %), Mol_X is the amount of X in the sample protein analyzed (nmol), and n is the number of types of amino acid residues considered. Mole percentage calculations were based on 14 types of amino acids (omitting proline, glycine, cysteine and tryptophan) for the HA1, HA2 and NP proteins and on 15 types of amino acids for the M proteins.

C. Number of residues per protein molecule. The number of residues of each amino acid type per protein molecule was calculated as follows:

$$Res_X = \frac{(\chi_X)\ (MW_P)}{\sum_{i=1}^{n} \left[(\chi_{X_i})\ (MW_X)\right]} \tag{4}$$

where X is a given type of amino acid, Res_X is the number of residues of X in one protein molecule (based on viral protein molecular weights shown in Table 3.2), χ_X is the mole percentage of X in the protein (mol %), MW_P is the molecular weight of the protein (daltons), n is the number of types of amino acid residues considered, and MW_X is the molecular weight of one residue of X (daltons).

For this calculation, values for cysteine, proline, and glycine were

included when at least one determination was available for the protein. When no value was available, an average of the mole percentages obtained for that protein isolated from the other viruses was substituted in the calculation. For HA1 and HA2 proteins, the tryptophan mole percentages reported by Ward and Dopheide (1976), but recalculated on the basis of 14 amino acid types, were used for the calculation. These values may be low due to the presence of carbohydrate, which reduces tryptophan recoveries even during hydrolysis with methanesulfonic acid (Moore, 1972). The mole percentage of cysteine reported by Skehel and Waterfield (1975) for A0/Bel/42 HA2 was recalculated on the basis of 14 amino acid types and used for calculation of the number of residues in all four HA2 proteins. For M and NP, cysteine and tryptophan values were unknown and thus were omitted from the calculation. Cysteine values reported for NP and M by Laver and Baker (1972) were not obtained using performic acid oxidation and are probably low; tryptophan was not determined by these workers. Thus for the NP and M proteins, the numbers of residues calculated are high. Since cysteine and tryptophan together probably comprise less than 5% of the residues, the error in the calculated values is small.

For all four viral proteins, serine values were increased 10% and threonine values 5% to correct for destruction during hydrolysis. These correction values were not determined experimentally but represent the typical correction required by destruction during hydrolysis with 6 N HCl at 110°C for 20 to 24 hr (Moore, 1972). Cysteine values were increased by 8% as conversion into cysteic acid is only 90 to 95% efficient (Hirs, 1972).

The HA1 apoprotein molecular weights used for the calculation of number of residues were 38,000 daltons for X-12, X-29L and NWS, and 39,300 daltons for RI/5^{-} (Table 3.2). The HA2 apoprotein molecular

weights used were 25,000 daltons for the three H0 viruses and 28,600 daltons for RI/5$^-$ (Table 3.2). In addition, the number of residues was also calculated using the apoprotein molecular weights reported by Ward and Dopheide for an H3 virus, 35,500 daltons for HA1 and 28,300 daltons for HA2. The molecular weights were corrected for carbohydrate content by reducing the observed HA1 molecular weights by 24.4% and the HA2 values by 4.7% (Ward and Dopheide, 1976).

D. Amount of protein recovered from one polyacrylamide gel. The quantity of viral protein isolated from each stained band of a polyacrylamide gel was calculated by:

$$Mol_G = \frac{(Vol_P)\ (Conc_X)}{Res_X} \tag{5}$$

where Mol_G is the amount of protein recovered from one stained polyacrylamide gel band (nmol), Vol_P is the volume of the hydrolysis solution containing the amino acids from the protein gel slice (ml), X is a given type of amino acid, $Conc_X$ is the concentration of X in the protein hydrolysis solution (nmol/ml), and Res_X is the number of residues of X in one molecule of the protein (based on viral protein molecular weights shown in Table 3.2).

E. Amount of protein analyzed. The total picomoles of protein analyzed was calculated by:

$$Mol_A = \frac{Mol_X}{Res_X} \tag{6}$$

where Mol_A is the amount of protein analyzed (nmol), X is a given type of amino acid, Mol_X is the amount of X in the sample analyzed (nmol), and Res_X is the number of residues of X in one molecule of the protein (based on viral protein molecular weights shown in Table 3.2).

Comparison of the amino acid compositions for a protein from two viruses.

A. Percentage of different amino acids. When two or more analyses of a given protein were available for both of two viruses, the compositions of this protein from the two viruses were compared by the t-test for determining the statistical significance of the difference between two sample means. The Olivetti-Underwood desk calculator program 5.2, entitled "t-Test for the Significance of the Difference between Two Sample Means" (Williams, 1969) was used to calculate for each amino acid (1) the mean mole percentage (2) the estimated standard deviation of the population, (3) the absolute value of the difference between the mean mole percentages, and (4) the t-statistic. The several mole percentage values determined for a given amino acid of a given protein can be considered a statistical population. The t-statistic is based on the difference of the means of two populations and the estimated standard deviation of each population. Values for P, the level of significance, were interpolated from Student's table that correlates values of P and t (Croxton, 1959). The value (1 - P) is commonly interpreted as the confidence level that the two populations are different.

When only one analysis of a given protein is available for a certain virus, the standard deviation of the population cannot be calculated and the t-test is not applicable. As an alternative, the correlation between the absolute value of the difference between two sample means, $|X_1 - X_2|$, and the value of (1 - P) was investigated. Using data from six comparisons of analyses of HA1 protein (see for example Tables 6.1 and 6.2), $|X_1 - X_2|$ was graphed versus (1 - P) for all amino acid pairs where $0.01 < |X_1 - X_2| < 1.2$ (Fig. 6.1). As the scatter of the points was not random, the least-squares line of best fit for these points was

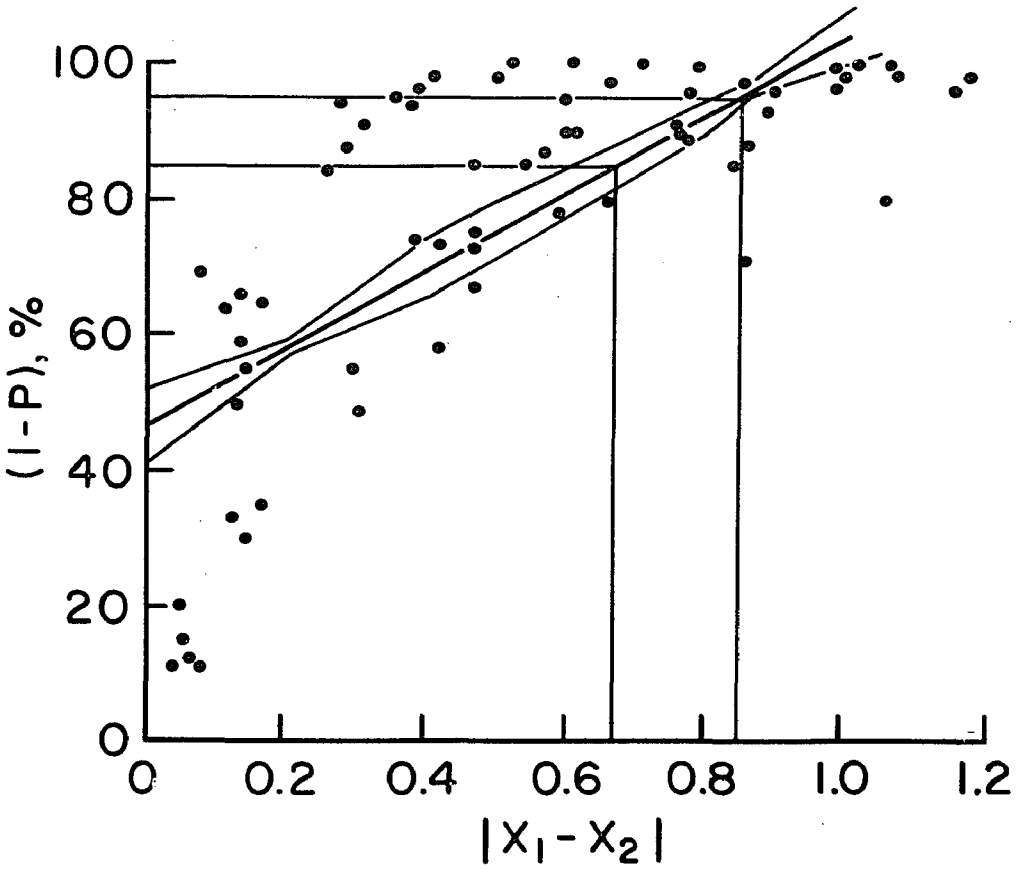

Fig. 6.1. Correlation of $|X_1 - X_2|$, the difference of the mean mole percentages, with (1 - P), the confidence level that the means are different. Data from six comparisons of composition for four HA1 proteins were graphed for each pair of amino acids having $0.01 < |X_1 - X_2| < 1.2$, which is equivalent to $10\% < (1 - P) < 100\%$. The least-squares line of best fit for the graphed points (slope = 0.57 ± 0.14) was calculated using Olivetti-Underwood statistical programs 2.14, 2.15, and 2.51 (Williams, 1969). The biconvex curves represent the 95% confidence interval for the regression line. When only one analysis was available for either of two proteins being compared, (1 - P) was estimated for each amino acid by calculating the difference in the (mean) mole percentages and obtaining the corresponding value of (1 - P) from the least squares line. Each pair of amino acids were scored as probably different if $(1 - P) > 95\%$, difference uncertain if $85\% < (1 - P) < 95\%$, and probably not different if $(1 - P) < 85\%$.

calculated using Olivetti-Underwood programs 2.14 (Data Reduction--Moments about Origin and Means), 2.15 (Data Reduction--Correlation and Simple Linear Regression, and 2.51 (Plotting a Regression Line and Its Confidence Interval)(Williams, 1969). When only one analysis of a given protein was available for one or both of the viruses to be compared, (1 - P), the confidence level that the mole percentage values are different, was estimated for each amino acid by calculating the difference in the (mean) mole percentage values, and graphically obtaining the corresponding value of (1 - P) from Fig. 6.1.

Each of 14 amino acid types of the proteins from the two viruses compared were scored as probably different if (1 - P) was greater than 95%, difference uncertain if (1 - P) was between 85% and 95%, and probably not different if (1 - P) was less than 85%. The number of amino acid residue types scored as probably different were counted for each virus pair and the corresponding percentage of the 14 amino acids compared was calculated. This percentage of different amino acids was then compared to that calculated for other virus pairs.

B. SΔQ relatedness index. A modified form of the SΔQ relatedness index of Marchalonis and Weltman (1971) was calculated as follows:

$$S\Delta Q = \sum_{i=1}^{14} \left(\chi_{A_i} - \chi_{B_i} \right)^2 \qquad (7)$$

where χ_{A_i} is the mole percentage of the i-th amino acid of protein A and χ_{B_i} is the corresponding mole percentage for protein B. The SΔQ values were calculated for a given protein from two viruses using mean mole percentage values based on fourteen types of amino acids (omitting glycine, proline, cysteine and tryptophan).

RESULTS

Amount of protein analyzed. Amino acid compositions were obtained by hydrolysis and analysis of picomolar quantities of purified viral proteins. One polyacrylamide gel usually contained sufficient amounts of the HA1, HA2, M, and NP proteins for one analysis of each protein. The amount of protein recovered from one gel varied from 300-600 pmol for HA1, 150-250 pmol for HA2, 250-500 pmol for M, and 50-200 pmol for NP.

Amino acid compositions. Thirty-three amino acid analyses were obtained on the HA1, HA2, M and NP proteins of the parental viruses NWS and RI/5$^-$ and the recombinant viruses X-12 and X-29. The mole percentage values calculated from the relative molar quantities of amino acids for the HA1 proteins of X-12, X-29 and X-29L are listed in Appendix Tables 6.13 and 6.14. The mole percentage data obtained for the four viral proteins from the four viruses are summarized in Appendix Tables 6.15 to 6.18. When more than one analysis was obtained on a given protein, the mean mole percentage value and the standard deviation are shown for each amino acid.

Cysteine values were omitted from the calculation because recoveries were often low due to partial oxidation. For one analysis of the HA1 protein of each recombinant, cysteine was oxidized with performic acid and determined as cysteic acid. Proline values were not included in the mole percentage calculations for the HA1, HA2, and NP proteins because the Durrum analyzer was not always equipped with the 440-nm photometer required to detect the yellow proline--ninhydrin dye. Despite electrophoretic dialysis of Tris--glycine buffer from the polyacrylamide gel slices, the glycine values obtained for the proteins of the parental viruses were exceptionally high and were therefore omitted. Finally,

tryptophan was omitted from the calculation of mole percentages. The recovery of tryptophan after methanesulfonic acid hydrolysis of the hemagglutinin proteins was not quantitative due to the presence of carbohydrate (Moore, 1972). During HCl hydrolysis of the parental proteins, tryptophan was completely destroyed (Moore, 1972).

Number of residues per protein. The number of residues calculated for the HA1, HA2, M and NP proteins from X-12, X-29, NWS and RI/5$^-$ are shown in Appendix Tables 6.19 to 6.24. The values for HA1 and HA2 were calculated two ways. Tables 6.19 and 6.21 are based on molecular weight determinations described in Table 3.2 for the specific viruses, while Tables 6.20 and 6.22 are based on the molecular weights calculated by Ward and Dopheide (1976) for the subunits of an H3 hemagglutinin. The molecular weights used to calculate the average number of residues of M and NP proteins were those determined for these particular viruses (Chapter 3).

Comparisons of amino acid compositions. The mean mole percentage values determined for each protein from the four viruses were compared using the t-test for statistical significance. As an example, the comparison of the HA1 proteins of X-12 and X-29 is given in Table 6.1 and the comparison of the HA1 proteins of NWS and RI/5$^-$ in Table 6.2. When the two proteins being compared were each analyzed more than once, the difference of the means, the calculated t-statistic, the level of significance (P), and the confidence level that the means are different (1 - P) were tabulated. When either of the proteins being compared was only analyzed once, the difference of the mole percentage values and the estimated confidence level were listed. The confidence level was estimated from the difference of the mole percentages by using the approximate correlation shown in Fig. 6.1. For each comparison of two

TABLE 6.1

Comparison of Amino Acid Compositions for

HA1 Protein: X-12 versus X-29

Amino acid[a]	Difference of means (mol %)	t, calculated Student's statistic	P, calculated level of significance[b]	Confidence level that the means are different (%)
Asp	0.68	1.19	0.29[c]	71
Thr	0.0005	0.0017	> 0.90	< 10
Ser	0.47	1.04	0.33	67
Glu	0.04	0.15	0.89	11
Ala	0.90	2.64	0.04	96
Val	0.57	1.74	0.13	87
Met	0.86	3.37	0.03[d]	97
Ile	0.31	0.70	0.51	49
Leu	0.54	1.66	0.15	85
Tyr	0.60	2.05	0.10[c]	90
Phe	0.15	0.80	0.45	55
Lys	0.05	0.26	0.80	20
His	0.14	1.02	0.34	66
Arg	0.26	1.61	0.16	84

[a]Since proline, glycine, cysteine and tryptophan were not usually determined, these residue types were omitted from the comparison.

[b]Since t was calculated from nine (n) analyses, P is based on seven (n - 2) degrees of freedom.

[c]This value is based on five degrees of freedom from seven analyses.

[d]This value is based on four degrees of freedom from six analyses.

TABLE 6.2

Comparison of Amino Acid Compositions for HA1 Protein: NWS versus RI/5[-]

Amino acid[a]	Difference of means (mol %)	t, calculated Student's statistic	P, calculated level of significance[b]	Confidence level that the means are different (%)
Asp	2.38	6.50	0.02	98
Thr	1.57	9.67	0.01	99
Ser	2.74	10.96	0.009	99.1
Glu	0.42	1.01	0.42	58
Ala	0.61	2.97	0.10	90
Val	1.18	8.30	0.02	98
Met	1.16	5.61	0.04	96
Ile	0.66	6.46	0.02	98
Leu	2.04	9.12	0.02	98
Tyr	1.06	1.88	0.20	80
Phe	0.66	1.92	0.20	80
Lys	0.31	3.23	0.09	91
His	0.08	1.36	0.31	69
Arg	0.29	2.69	0.12	88

[a]Since proline, glycine, cysteine and tryptophan were not usually determined, these residue types were omitted from the comparison.

[b]Since t was calculated from four (n) analyses, P is based on two (n - 2) degrees of freedom.

viruses, the residue types were scored as probably different, difference uncertain, or probably not different based on the magnitude of the confidence level.

The amino acid compositions of protein pairs were also compared using the $S^{\Delta}Q$ relatedness index. In this thesis, $S^{\Delta}Q$ has been calculated on the basis of 14 types of amino acids (omitting glycine, proline, cysteine and tryptophan) rather than the 16 types (omitting cysteine and tryptophan) used by Marchalonis and Weltman (1971). The effect of reducing the number of amino acid types compared was determined by calculating $S^{\Delta}Q$ values for six nonviral proteins in seven pairs using mole percentage values based on either 14 or 16 amino acid types. The results, which are presented in Table 6.3, are typical for comparison of related and non-related proteins (Marchalonis and Weltman, 1971). Reducing the number of amino acid types on which the mole percentage values are based from 16 to 14 increased the $S^{\Delta}Q$ values by about 30%, independent of the size or relatedness of the proteins being compared.

Experimental variations in amino acid analyses can produce different results for two samples of the same protein. This variation was measured by comparing two groups of replicate analyses for the HA1 protein of recombinant virus X-12 (Table 6.4). When the analyses were assigned to the two groups in five different ways, the percentage of different amino acids ranged from 0% to 21%, and the $S^{\Delta}Q$ values varied from 1 to 6.

The average amino acid compositions determined for the HA1 proteins isolated from X-12, X-29, NWS and RI/5^{-} are compared in Table 6.5 by both methods: percentage of different amino acids and the $S^{\Delta}Q$ relatedness index. The HA2, M and NP proteins are similarly compared in Tables 6.6, 6.7 and 6.8, respectively.

TABLE 6.3

Correlation of SΔQ Values Based on 14 or 16 Types of Amino Acid Residues

First protein[a]	Second protein[a]	SΔQ value[b]		Ratio[c]
		14 Residues	16 Residues	
Human hemoglobin β-chain (15,900)	Monkey hemoglobin β-chain (16,000)	8.8	7.5	1.17
Human hemoglobin β-chain (15,900)	Bovine trypsinogen (24,000)	474	352	1.35
Human Hemoglobin β-chain (15,900)	Pig GPD (35,700)	189	142	1.33
Monkey hemoglobin β-chain (15,900)	Lobster GPD (35,700)	200	150	1.33
Bovine trypsinogen (24,000)	Bovine chymotrypsinogen (25,700)	113	87	1.30
Bovine trypsinogen (24,000)	Pig GPD (35,700)	208	152	1.47
Pig GPD (35,700)	Lobster GPD (35,700)	22.4	17.3	1.29

[a]The molecular weight of the protein in daltons is shown in parentheses. Glyceraldehyde-phosphate dehydrogenase is abbreviated GPD. Amino acid compositions are taken from Dayhoff (1972).

[b]The SΔQ calculation based on 16 types of amino acid residues omits cysteine and tryptophan. The calculation based on 14 types of amino acid residues omits, in addition, proline and glycine.

[c]The value shown is the ratio of SΔQ values based on 14 types of amino acid residues to SΔQ values based on 16 types of amino acid residues.

TABLE 6.4

Comparison of Replicate Analyses:

Range of Values Expected for Relatedness Indices

Analysis Runs[a]		Relatedness Indices	
Group 1	Group 2	% Different	SΔQ
1, 2, 3	4, 5	21	6
2, 3, 4	5, 1	0	1
3, 4, 5	1, 2	14	6
4, 5, 1	2, 3	0	1
5, 1, 2	3, 4	14	3

[a]Data for amino acid analysis runs 1 to 5 are presented in Appendix Table 6.13. When a mole percent value was missing, an average of the values for that amino acid in the remaining four analyses was substituted in the calculation.

TABLE 6.5

Comparison of Amino Acid Compositions for HA1 Protein by Two Methods, Percentage of Different Amino Acids and SΔQ Relatedness Index

% \ SΔQ[a]	X-12	X-29	NWS	RI/5⁻
X-12		3	8	45
X-29	14		8	36
NWS	36	43		25
RI/5⁻	64	64	50	

[a]Percentages of different amino acids are shown below the diagonal; the SΔQ relatedness indices (see text) are shown above.

TABLE 6.6

Comparison of Amino Acid Compositions for HA2 Protein by Two Methods, Percentage of Different Amino Acids and SΔQ Relatedness Index

% \ SΔQ[a]	X-12	X-29L	NWS	RI/5⁻
X-12		5	5	12
X-29L	21		6	18
NWS	21	14		8
RI/5⁻	36	29	29	

[a]Percentages of different amino acids are shown below the diagonal; the SΔQ relatedness indices (see text) are shown above.

TABLE 6.7

Comparison of Amino Acid Compositions for M Protein by Two Methods, Percentage of Different Amino Acids and SΔQ Relatedness Index

% \ SΔQ[a]	X-12	X-29L	NWS	RI/5
X-12		2	7	6
X-29L	8		6	6
NWS	31	20		2
RI/5	31	27	27	

[a]Percentages of different amino acids are shown below the diagonal; the SΔQ relatedness indices (see text) are shown above.

TABLE 6.8

Comparison of Amino Acid Compositions for NP Protein by Two Methods, Percentage of Different Amino Acids and SΔQ Relatedness Index

% \ SΔQ[a]	X-12	X-29L	NWS	RI/5⁻
X-12		21	6	22
X-29	50		22	13
NWS	21	64		17
RI/5⁻	29	50	29	

[a]Percentages of different amino acids are shown below the diagonal; the SΔQ relatedness indices (see text) are shown above.

The amino acid compositions of the HA1, HA2, M and NP proteins isolated from the same virus were compared by calculating SΔQ values for the six possible comparisons of the four viral proteins. The SΔQ values for each pairwise comparison of the X-12, NWS and RI/5$^-$ proteins are given in Table 6.9. Also shown is the average SΔQ value for each protein pair. Since more than one analysis was not usually available for the X-29 proteins, comparison among these analyses was not considered statistically significant and is not included.

DISCUSSION

The inheritance of parental proteins by recombinant viruses and the relationship between composition and serotype have been studied by quantitative comparison of amino acid compositions of influenza virus proteins. Only a few amino acid analyses have been previously reported for influenza virus proteins because of the problem of purifying the proteins in sufficient quantity for analysis. This study involved the comparison of 33 analyses obtained for 16 influenza virus proteins isolated by gel filtration or by polyacrylamide gel electrophoresis. Although the amount of protein generally obtained from one band of a single polyacrylamide gel (about 300 pmol) was substantially less than the quantity that can be obtained by gel filtration, it was much larger than the amount needed for amino acid analysis using microbore columns (about 30 pmol).

Analyses for this study were obtained on protein samples isolated, hydrolyzed and analyzed by two different procedures. Proteins from the parental viruses (NWS and RI/5$^-$) were isolated by polyacrylamide gel electrophoresis and hydrolyzed with HCl, whereas proteins from the

TABLE 6.9

Comparison of the Amino Acid Compositions of Different Proteins from the Same Virus by the SΔQ Relatedness Index

Comparison[a]	SΔQ relatedness index[a]			
	X-12	NWS	RI/5^{-}	Average
HA1 vs HA2	45	40	76	54
HA1 vs M	118[b]	126	73	106
HA1 vs NP	97	86	64	82
HA2 vs M	139[b]	146	131	139
HA2 vs NP	112	103	70	95
M vs NP	54[b]	70	49	58

[a]Indices were calculated from the mole percent data of Appendix Tables 6.15 to 6.18. For these comparisons, mole percent values for M protein are based on 14 amino acid types.

[b]For the mole percentages of aspartic acid and methionine of M protein, the X-12 values were estimated as the average of the values for X-29, NWS, and RI/5^{-}.

recombinant viruses (X-12 and X-29) were isolated by gel filtration and hydrolyzed with methanesulfonic acid. Although some of the experimental variation in composition might be attributed to these procedural differences, the close agreement observed between the three HA2 proteins of H0 serotype (NWS, X-12, X-29) indicates that both procedures give very similar results. Observed differences in composition between two proteins could be due to real differences or to the statistical variation expected in replicate analyses of the same protein. Thus two different procedures were used to compare protein compositions, namely, percentage of different amino acids and the $S\Delta Q$ relatedness index.

Calculation of the percentage of different amino acids in two proteins was based on the t-test, a standard statistical method for comparing two numerical populations. Since calculation of the t-statistic requires the standard deviation, a measure of the scatter of replicate analyses, two analyses of each protein were necessary and more were desirable. For several of the 16 proteins analyzed, however, duplicate analyses were not obtained. When either of the proteins being compared was only analyzed once, (1 - P), the confidence level that the mole percentage values were different, was estimated from the magnitude of the difference of the mole percentages using the approximate correlation shown in Fig. 6.1. Since this estimated value of (1 - P) does not take into account the statistical scatter expected for replicate analyses, it is not statistically rigorous and should be interpreted qualitatively rather than quantitatively. Mole percentage values were considered to be different only if (1 - P) was 95% or higher.

The $S\Delta Q$ relatedness index (Marchalonis and Weltman, 1971), a statistic specifically developed for comparing amino acid compositions,

has frequently been used to measure the relatedness of two proteins. Among the many enzymes and structural proteins compared by the SΔQ index have been D-galactose dehydrogenase from Pseudomonas species (Blachnitzky et al., 1974), isocitrate lyase from Neurospora and Pseudomonas (Johanson et al., 1974), and various feather keratins (Brush, 1974). For the hemoglobins, the cytochromes, and the immunoglobulin light chains, protein families for which extensive sequence data are available, Marchalonis and Weltman (1971) found a statistically significant correlation between SΔQ values and actual differences in amino acid sequence. In 98% of 5000 protein pairs studied, unrelated protein pairs gave SΔQ values above 100. In this study, SΔQ values were based on mole percentages calculated from 14 rather than 16 types of amino acids. In this situation, the SΔQ values are generally 30% higher (Table 6.3) and the value observed for unrelated proteins should be 130 or greater.

Differences detected in the compositions of two proteins by amino acid analysis were reflected in the viral protein pattern obtained when the viruses were grown in the presence of individual, radiolabeled amino acids of the same specific activity. Examination of Panel A of the autoradiograph shown in Fig. 3.11 suggested that HA1 and HA2 both contain more lysine than arginine, HA2 contains less arginine than HA1, NP protein is relatively rich in arginine, and M protein contains approximately equal quantities of these positively charged amino acids. These visual observations are completely consistent with mole percentages determined for lysine and arginine by amino acid analysis (Appendix Tables 6.15 - 6.18).

Comparison of HA1 proteins. The HA1 proteins from the recombinant viruses X-12 and X-29 are very closely related. Comparison of their

amino acid compositions showed that only 14% of the molar percentage values for their amino acids were probably different and that their SΔQ relatedness index was 3 (Table 6.5). These comparisons are not distinguishable from the several comparisons of the X-12 HA1 protein with itself, as shown in Table 6.4.

Two proteins with very similar amino acid compositions are not necessarily identical. Three genetic events that are not excluded by these comparisons are (1) that either HA1 gene acquired a limited number of point mutations; (2) that recombination of the HA genes of NWS and RI/5$^-$ produced the HA gene for X-29 in which a very small region of the RI/5$^-$ gene crossed over into the NWS gene; or (3) that recombination of the parental HA genes produced recombinants in which one large region or several smaller regions of the HA gene crossed over but that the average amino acid compositions coded for by the exchanged regions were very similar. The HA1 protein of the three H0 viruses have quite similar compositions, but each is significantly different from the HA1 protein of the H2 parent RI/5$^-$ (Table 6.5). Thus it is unlikely that the X-29 gene for HA was generated by crossover of a large region of the RI/5$^-$ gene coding for the trypsin-resistant site into the NWS gene.

Comparison of the HA1 proteins from X-29 and its large plaque variant X-29L indicates that these proteins have very similar compositions. Only 21% of the mole percentages for the 14 amino acids were estimated to be different at the 95% confidence level, and the calculated SΔQ value was 7. Both values fall within the range determined for replicate analyses of the same protein (Table 6.4).

The molecular basis for variation in plaque size is not known. No difference can be seen by electrophoretic migration on polyacrylamide

gels between the RNA hemagglutinin genes coding for plaque-size variants of the same serotype (J. Schulman, personal communication). This finding is consistent with the great similarity in amino acid composition of X-29 and X-29L proteins and suggests that the increased plaque size of X-29L might be due to one or a few point mutations in the hemagglutinin protein or in another viral protein. Comparison of the single analysis for HA1 from X-29L with the mean composition for HA1 from X-29 indicates that these proteins probably differ in their content of alanine, methionine and leucine (Appendix Table 6.14). The X-29L protein appears to contain less alanine and methionine and more leucine than the X-29 protein. If this compositional difference is correct, the X-29L variant may produce larger plaques because it contains a few more hydrophobic residues, which would enhance the binding of the viral hemagglutinin molecule to neuraminic acid on the cell surface and thus increase the efficiency of viral infection. This hypothesis might be tested by comparing the ability of the two viruses to elute from erythrocytes at 4°C, a temperature at which neuraminidase is inactive.

The amino acid compositions determined for the HA1, HA2, M and NP proteins from X-12, X-29, NWS and RI/5$^-$ were compared to the compositions of these proteins from A/Bel/40 virus (Laver and Baker, 1972; Skehel and Waterfield, 1975) and an H3N1 recombinant virus (Ward and Dopheide, 1976). The literature data were made compatible with the present data by recalculating the mole percentage values on the basis of 14 amino acids (Appendix Tables 6.25 and 6.26). Although the HA1 proteins from X-12, X-29 and NWS appear at first glance to be more similar to one another (Table 6.5) than to the HA1 proteins of Bel virus (Table 6.10), the two reported compositions of the Bel viral proteins are as different from

TABLE 6.10

Comparison of Amino Acid Compositions of the Same Proteins from Different Viruses by the SΔQ Relatedness Index

Comparison[b]	SΔQ relatedness index[a]			
	HA1	HA2	M	NP
$Bel_{L\&B}$ vs $Bel_{S\&W}$	18	9		
$Bel_{L\&B}$ vs X-12	14	8	34	41
$Bel_{S\&W}$ vs X-12	16	3		
$Bel_{L\&B}$ vs X-29	15	11	36	52
$Bel_{S\&W}$ vs X-29	17	3		
$Bel_{L\&B}$ vs NWS	6	8	22	35
$Bel_{S\&W}$ vs NWS	8	8		
$Bel_{L\&B}$ vs RI/5^{-}	28	20	39	19
$Bel_{S\&W}$ vs RI/5^{-}	33	18		
H3N1 vs X-12	46	37		
H3N1 vs X-29	48	50		
H3N1 vs NWS	37	38		
H3N1 vs $Bel_{L\&B}$	36	37		
H3N1 vs $Bel_{S\&W}$	32	42		
H3N1 vs RI/5^{-}	78	36		

[a]Indices were calculated from the mole percentage data of Appendix Tables 6.15 to 6.18, 6.25, and 6.26.

[b]Subscripts on Bel virus refer to Laver and Baker (1972) or Skehel and Waterfield (1975). The H3N1 data are from Ward and Dopheide (1976).

each other as each is from the NWS, X-12, and X-29 proteins. Thus the actual differences in amino acid composition between Bel and other H0 viruses is also probably limited. Minor differences between the hemagglutinin proteins from the NWS virus isolated in 1933 and the Bel virus isolated in 1940 have been detected immunologically (Schulman and Kilbourne, 1969). This variation due to antigenic drift should be reflected in the amino acid composition.

The SΔQ index shows that the compositions of the HA1 proteins from the four H0 viruses are as different from the H2 virus as from the H3 virus (33 vs 39), but, based on a single comparison, the H2 and H3 proteins are considerably more different from one another (SΔQ = 78), as shown in Table 6.11. In addition, the mole percentage values for certain "diagnostic" amino acids can be used to qualitatively assign an HA1 protein to a specific serotype. Thus, aspartic acid is low for H2 (11.1%), moderate for H0 (13.7%) and high for H3 (16.3%); similarly, leucine is low for H3 (8.3%), moderate for H0 (10.8%), and high for H2 (12.6%). Other amino acids show a distinctly different value for one serotype than for the other two serotypes. Thus, threonine is low for H0 (7.3% vs 9.4 or 10.7%), serine is low for H2 (9.0% vs 11.4 or 12.1%), and isoleucine is high for H3 (8.2% vs 5.8 or 6.3%). The H0 values cited here are an average of the values for NWS, X-12 and X-29 (Appendix Table 6.15). Thus HA1 proteins of the H0, H2 and H3 serotypes can be distinguished by their amino acid compositions. These serotypes might also be distinguishable by comparing peptide maps of their HA1 protein. For example, Laver and Webster (1972) have observed differences in the peptide maps of the HA1 subunit from H2 and H3 viruses.

TABLE 6.11

Comparison of the Amino Acid Compositions for the Subunits of Hemagglutinin Proteins from Viruses with Different Serotypes

Serotypes compared[a]	SΔQ relatedness index[b] (mean ± standard deviation)	
	HA1	HA2
H0 vs H0	11 ± 5	7 ± 3
H0 vs H2	33 ± 8	15 ± 5
H0 vs H3	39 ± 7	41 ± 6
H2 vs H3	78	36

[a]Viruses Bel, NWS, X-12 and X-29 share the serotype H0 and RI/5^{-} is serotype H2.

[b]The values shown are the average of the SΔQ indices listed in Tables 6.5, 6.6 and 6.10.

Comparison of HA2 proteins. The amino acid composition of the HA2 proteins is more conserved than the composition of the HA1 proteins (Table 6.11). No significant difference is observed between the HA2 proteins of Bel, X-12, X-29L and NWS, the four H0 viruses ($S\Delta Q = 7$). A slight difference is seen between the HA2 proteins from H0 and H2 viruses ($S\Delta Q = 15$), and a much greater difference is evident between the HA2 subunits from an H3 virus and either the H0 or H2 viruses ($S\Delta Q = 41$ or 36). These results are consistent with the peptide maps published by Laver and Webster (1972; 1973) which showed that although the maps of the HA2 proteins from H0, H2 and H3 viruses were characteristically different, they varied less than the peptide maps for HA1. In addition, the limited sequence data available for the HA2 proteins (Skehel and Waterfield, 1975; Bucher et al., 1976) indicate that the amino terminal sequences for HA2 are more conserved than those for HA1.

The relative conservation of the HA2 proteins can be explained in several ways. Unlike the external HA1 subunit, the internal HA2 subunit probably does not possess antigenic determinants and thus is not subjected to selective immunological pressures (Brand and Skehel, 1972; Eckert, 1973). Alternatively, the structural requirements may be more stringent for the interior hemagglutinin subunit. Although only half as large as HA1, HA2 must maintain a hydrophobic interaction with the lipid bilayer, must conserve its steric compatibility with the HA1 subunit, and perhaps maintain the integrity of a part of the cell receptor binding site.

Even though the two parental HA2 subunits have more similar compositions than the parental HA1 subunits, the X-29 recombinant probably did not acquire its HA2 subunit from RI/5$^-$. Both comparison methods indicate that the HA2 protein from X-29L is more similar to the

NWS protein than to the RI/5$^-$ protein (Table 6.6).

Comparison of the internal proteins M and NP. The internal proteins of the H0 viruses are as different from each other as they are from the H2 internal proteins (Tables 6.7 and 6.8). Since serotypic classification is not based on the M and NP proteins, no correlation was expected. Both the very low SΔQ values of 2 to 7 and the low 8% to 31% different amino acids suggest that the M proteins of X-12, X-29, NWS and RI/5$^-$ are very closely related. Since the internal M protein is not exposed to selective immunological pressures, this result is not unexpected. In addition, the M protein is a type-specific antigen (Schild, 1972) and these are all type-A viruses. Finally, Laver and Downie (1976) have found that peptide maps of the M proteins from H0N1, H3N2 and Hav6Nav5 viruses are almost identical.

Comparison of the compositions of the NP proteins showed 21% to 64% different amino acids and SΔQ values of 6 to 22 (Table 6.8). Even though M and NP are both internal, type-specific proteins, the variation of composition among the NP proteins is greater than that among the M proteins. Early complement fixation assays could not distinguish if the ribonucleoproteins of type A viruses were of human, porcine, equine or avian origin (Lennette and Horsfall, 1941; Lief et al., 1958; Lief, 1963; Pereira et al., 1965). Davenport and co-workers (1960), however, found differences between the NP proteins from several strains of influenza A virus by using checkerboard complement-fixation tests. Recently, the use of high-resolution gradient-polyacrylamide gels has shown differences in the electrophoretic migration of the NP proteins from H0 and H3 viruses (Ritchey et al., 1977). But no difference was seen for the M proteins from viruses of these serotypes, which is consistent with our results.

The conservation of the amino acid compositions for both parental and both recombinant viruses suggests that the M protein does not determine the trypsin sensitivity of the viral hemagglutination activity. Similarly, the compositions of the NP proteins were not expected to correlate with the trypsin susceptibility. The variation detected between NP proteins more likely reflects differences in the RNA sequences of the several genome segments. Indeed, both methods of comparing compositions suggest that the NP proteins of X-12 and NWS are probably the most closely related pair.

Number of residues. The number of residues of each amino acid type per protein molecule was calculated to determine the number of tryptic or homo-serine lactone-containing peptides expected following treatment with trypsin or cyanogen bromide (Appendix Tables 6.19 to 6.24). Since this calculation is based on not only the mole percentages for the 18 hydrolysate amino acids but also on the protein molecular weight and carbohydrate content, the numbers of residues calculated for glycoproteins are only approximate. Thus protein comparisons were based on the more exact mole percentages rather than on the calculated number of residues. In addition to the molecular weights determined in this study, the molecular weights determined by Ward and Dopheide (1976) for an H3N1 virus were used to calculate the number of residues for the hemagglutinin proteins because these weights were corrected for the effect of the acrylamide concentration on protein migration (Segrest et al., 1971). It seems unlikely that the differences in electrophoretic migration observed between the H0, H2 and H3 hemagglutinin subunits are due to actual differences in protein size. Therefore, molecular weights for the hemagglutinin apoproteins should be nearly identical and independent of

serotype. The migrational differences detected on polyacrylamide gels are probably due to differences in carbohydrate content. The only precise carbohydrate determinations for hemagglutinin subunits, however, are those by Ward and Dopheide (1976) for an H3 hemagglutinin. Although these values are only estimates for the H0 and H2 proteins, they were used to calculate the apoprotein molecular weights for the parental and recombinant hemagglutinin subunits.

The two relatedness indices generally gave similar results. The closest agreement between the two methods was seen for comparisons of the amino acid compositions for the HA1 subunit, the viral protein for which the most analyses were available. Although the SΔQ relatedness index was quicker and easier to calculate, it was based on the averages of the available analyses and ignored the statistical derivation from the averages. The data for two proteins being compared was weighted equally, even when one was the result of one determination and the other was the average of several. The percentage of different amino acids provided a statistical measure of similarity and the corresponding level of confidence for each amino acid, while the SΔQ method yielded only a single number to relate the two compositions. When two or more analyses were available for each of the two proteins being compared, the statistical procedure probably yielded more reliable data. When several analyses were not available for both proteins being compared, calculation of the SΔQ relatedness index was preferable.

Comparison of different proteins. The compositional relatedness of different structural proteins from the same influenza virus was explored using the SΔQ index. In their initial study of protein relatedness using SΔQ, Marchalonis and Weltman (1971) reported that of more than 5000

pairs studied, protein pairs having no known sequence homology gave SΔQ values above 130 in 98% of the cases and values above 65 in every case. These SΔQ values have been increased by 30% to correct for calculation of mole percentages on the basis of 14 rather than 16 amino acid types (Table 6.3). Based on these correlations, the SΔQ values shown in Table 6.9 suggest that the HA1 and HA2 subunits of the hemagglutinin protein are sequentially homologous, and that the proteins M and NP are also homologous. At first glance, these results are surprising. Even though HA1 and HA2 comprise the first two-thirds and the last third, respectively, of the single polypeptide chain of uncleaved hemagglutinin, no aspect of their function requires them to be sequentially homologous. The low SΔQ values for HA1 versus HA2, however, would be expected if HA evolved from a gene about one-third its present size by internal gene duplication through unequal crossover. The present HA1 protein would thus contain about two copies and HA2 roughly one copy of the initial gene product. The compositions of the HA1 and HA2 subunits from A/Bel/42 are also very similar, except that HA1 contained about nine times as much proline as HA2 (Laver, 1971). Similarly, the unexpected demonstration of possible sequence homology between M and NP suggested by their low SΔQ values could be explained by a gene about the size of the M gene undergoing gene doubling and then internal gene duplication to give both M and NP genes. Alternatively, the close composition relatedness of the M and NP proteins may only reflect the fact that each probably possesses a hydrophilic region which faces the lipid bilayer or the RNA, respectively. Interestingly, the fact that both M and NP have type-specific antigenic determinants is consistent with their possible sequence homology.

Compositional identification of a hemagglutinin protein. In 1973 Eckert described the isolation of a protein from A/PR/8/34 (H0N1) influenza virus that did not agglutinate erythrocytes but did bind hemagglutination-inhibiting antibodies. In an attempt to characterize this HABA antigen, its amino acid composition was compared with the compositions published by Laver (1971) for the HA1 and HA2 proteins from Bel virus. It was not possible, however, to determine the identity of HABA from its amino acid composition alone. Since HA1 has a higher proline content than HA2 (Laver, 1971), HABA was presumed to be related to HA1. Molecular weight determinations and immunological data supported the characterization of HABA as a dimer of the HA1 subunit.

To test the feasibility of using the SΔQ relatedness index to characterize unknown proteins, the molar ratios reported for HABA were recalculated as mole percentages based on 14 amino acid types (Appendix Table 6.26) and compared to the mean mole percentages determined for HA1 and HA2 proteins from the H0 viruses Bel, NWS, X-12 and X-29 (Table 6.12). Comparison of HABA with the HA1 proteins gave an average SΔQ value of 15 ± 4, while comparison to the HA2 proteins gave 38 ± 6. Since comparison of the HA1 proteins with one another gave a mean SΔQ value of 11 ± 5 (Table 6.11) and comparison of an HA1 protein with an HA2 protein gave a mean SΔQ value of 42 + 2 (Table 6.9), HABA is more closely related to HA1 than to HA2. Thus the SΔQ values alone are quantitatively consistent with the HABA protein being an HA1 protein from an H0 virus.

TABLE 6.12

Comparison of the HABA Protein with the HA1 and HA2 Proteins from H0 Serotype Viruses[a]

Comparison[b]	SΔQ relatedness index
$Bel_{L\&B}$ HA1 vs HABA	17
$Bel_{S\&W}$ HA1 vs HABA	21
NWS HA1 vs HABA	11
X-12 HA1 vs HABA	12
X-29 HA1 vs HABA	14
Average[c] for H0 HA1 vs HABA	15 ± 4
$Bel_{L\&B}$ HA2 vs HABA	42
$Bel_{S\&W}$ HA2 vs HABA	34
NWS HA2 vs HABA	43
X-12 HA2 vs HABA	41
X-29 HA2 vs HABA	29
Average[c] for H0 HA2 vs HABA	38 ± 6

[a]HABA is a purified influenza virus antigen that binds hemagglutination-inhibiting antibodies directed towards H0 serotypes (Eckert, 1973).

[b]Subscripts on Bel virus refer to Laver and Baker (1972) or Skehel and Waterfield (1975).

[c]The value given is the mean ± the standard deviation.

SUMMARY

Thirty-three amino acid analyses have been obtained for four different influenza virus proteins (HA1, HA2, M and NP) from three H0 viruses (NWS, X-12, X-29) and one H2 virus (RI/5$^-$). Comparison of these and published analyses by two different methods, calculation of the percentage of different amino acids and the SΔQ relatedness index, has revealed that the HA1 proteins from viruses of different serotypes were readily distinguishable on the basis of composition, while the HA2 proteins were more conserved among the serotypes. No relationship was detected between the viral serotype and the compositions of the internal viral proteins M and NP. All of the M proteins analysed appeared to be very closely related, but compositions of the NP proteins varied considerably from virus to virus.

Comparison of the compositions of different proteins from the same virus revealed a striking similarity between the HA1 subunit and the HA2 subunit and between the M and NP proteins. These relationships may reflect a common genetic origin or common structural characteristics.

Based on the amino acid compositions of viral proteins HA1, HA2, M and NP, the slowly trypsin-sensitive H0 virus X-29 is more closely related to the trypsin-sensitive H0 viruses X-12 and NWS than to the trypsin-resistant H2 virus RI/5$^-$. Thus it is unlikely that X-29 acquired its relatively trypsin-resistant character from RI/5$^-$ through inheritance of one of these four structural proteins.

APPENDIX

1. Tables 6.13 and 6.14 show the mole percentage values determined for each analysis of the HA1 protein from the viruses X-12, X-29 and X-29L.

2. Tables 6.15 to 6.18 contain the average amino acid composition in mole percentage for the proteins HA1, HA2, M and NP from the viruses X-12, X-29, NWS and RI/5^{-}.

3. Tables 6.19 to 6.24 show the number of residues calculated for the proteins HA1, HA2, M and NP from the viruses X-12, X-29, NWS and RI/5^{-}.

4. Tables 6.25 and 6.26 present recalculated mole percentage values based on data of Laver and Baker (1972), Skehel and Waterfield (1975), Eckert (1973), and Ward and Dopheide (1976).

TABLE 6.13

Amino Acid Compositions for HA1 Protein from X-12

Amino acid[a]	Mole percentage[b]				
	Run 1 (150 pmol)	Run 2 (25 pmol)	Run 3 (305 pmol)	Run 4 (110 pmol)	Run 5 (440 pmol)
Asp	14.5	13.8	c	14.9	13.8
Thr	6.6	6.6	6.8	7.2	7.9
Ser	13.4	12.6	13.2	11.1	12.5
Glu	11.6	11.8	11.6	12.1	12.0
Ala	8.0	6.6	7.7	6.8	6.5
Val	5.4	5.2	6.2	6.5	6.4
Met	d	0.9	1.8	1.7	0.8
Ile	7.1	7.6	5.8	5.8	5.5
Leu	10.7	11.8	10.7	11.1	11.5
Tyr	d	5.8	4.6	5.0	5.0
Phe	3.3	4.1	3.5	3.8	3.6
Lys	5.8	6.4	6.1	6.3	6.5
His	2.7	2.8	2.9	2.8	3.4
Arg	4.5	4.1	4.9	4.8	4.6

[a] Proline, glycine, cysteine and tryptophan were not usually determined.

[b] Mole percentage is based on the 14 types of amino acids listed. Runs 1 - 3 are replicative analyses of the same protein preparation. Samples were hydrolyzed with 4 N methanesulfonic acid for 24 h at 115°C except for Run 1, which was oxidized with performic acid and hydrolyzed with 6 N HCl for 24 h at 110°C. The picomoles of protein analyzed was calculated assuming an apoprotein molecular weight of 38,000 daltons.

[c] Aspartic acid was not resolved from methionine sulfoxide.

[d] Methionine and tyrosine were partially destroyed during performic acid oxidation.

TABLE 6.14

Amino Acid Compositions for HA1 Protein from X-29 and X-29L

Amino acid[a]	Mole percentage[b]				
	X-29 Run 1 (230 pmol)	X-29 Run 2 (150 pmol)	X-29 Run 3 (410 pmol)	X-29 Run 4 (180 pmol)	X-29L Run 1 (360 pmol)
Asp	14.5	13.4	c	12.8	14.0
Thr	6.8	7.4	6.9	6.9	7.6
Ser	12.5	11.9	12.4	11.6	11.5
Glu	11.6	12.7	11.5	11.7	11.6
Ala	8.1	7.5	8.2	8.3	6.2
Val	6.1	6.9	6.4	6.7	6.8
Met	d	e	2.1	2.2	1.2
Ile	6.1	5.9	5.8	6.3	6.0
Leu	10.7	9.9	10.9	11.0	11.5
Tyr	d	4.2	4.6	4.7	5.1
Phe	3.6	4.2	3.7	3.7	3.8
Lys	5.7	6.4	6.2	6.2	6.5
His	2.8	2.7	2.8	2.9	3.4
Arg	4.8	4.8	5.0	4.9	4.8

See next page for footnotes.

Footnotes for Table 6.14

[a]Proline, glycine, cysteine and tryptophan were not usually determined.

[b]Mole percentage is based on the 14 types of amino acids listed. X-29 Runs 1 - 4 are replicative analyses of the same protein preparation. For X-29 Run 1, the sample was oxidized with performic acid and hydrolyzed with 6 N HCl for 24 h at 110°C; the Run 2 sample was hydrolyzed similarly but without prior performic acid oxidation. Samples for the remaining runs were hydrolyzed with 4 N methanesulfonic acid at 115°C for 24 h (X-29 Run 3 and X-29L Run 1) or 48 h (X-29 Run 4). The picomoles of protein analyzed was calculated assuming an apoprotein molecular weight of 38,000 daltons.

[c]Aspartic acid was not resolved from methionine sulfoxide.

[d]Methionine and tyrosine were partially destroyed during performic acid oxidation.

[e]Methionine was partially oxidized during hydrolysis.

TABLE 6.15

Average Amino Acid Composition of HA1 Protein: Mole Percentage

Amino acid[a]	Mole percentage[b] (mean ± standard deviation)			
	X-12 (n = 5)	X-29 (n = 4)	NWS (n = 2)	RI/5 (n = 2)
Asp	14.2 ± 0.6[c]	13.6 ± 0.9[c]	13.5 ± 0.3	11.1 ± 0.4
Thr	7.0 ± 0.6	7.0 ± 0.3	7.8 ± 0.2	9.4 ± 0.2
Ser	12.6 ± 0.9	12.1 ± 0.4	11.7 ± 0.3	9.0 ± 0.2
Glu	11.8 ± 0.2	11.9 ± 0.6	12.3 ± 0.3	12.7 ± 0.5
Ala	7.1 ± 0.7	8.0 ± 0.4	6.0 ± 0.0	6.6 ± 0.3
Val	6.0 ± 0.6	6.5 ± 0.3	7.5 ± 0.1	3.7 ± 0.2
Met	1.3 ± 0.5[c]	2.2 ± 0.1[c]	1.2 ± 0.2	2.3 ± 0.2
Ile	6.4 ± 1.0	6.0 ± 0.2	6.4 ± 0.1	5.8 ± 0.0
Leu	11.2 ± 0.5	10.6 ± 0.5	10.6 ± 0.1	12.6 ± 0.3
Tyr	5.1 ± 0.5[c]	4.5 ± 0.3[c]	4.3 ± 0.4	3.3 ± 0.7
Phe	3.6 ± 0.3	3.8 ± 0.3	3.9 ± 0.5	3.3 ± 0.1
Lys	6.2 ± 0.3	6.1 ± 0.3	6.9 ± 0.1	7.2 ± 0.1
His	3.0 ± 0.3	2.8 ± 0.1	3.4 ± 0.0	3.3 ± 0.1
Arg	4.6 ± 0.3	4.8 ± 0.1	4.4 ± 0.0	4.7 ± 0.2

[a]Proline, glycine, cysteine and tryptophan were not usually determined.

[b]Mole percentage is based on the 14 types of amino acids listed; n is the number of analyses.

[c]The X-12 aspartic acid, methionine and tyrosine values are each an average of four analyses. The X-29 aspartic acid and tyrosine values are each an average of three analyses. The X-29 methionine value is an average of two analyses.

TABLE 6.16

Average Amino Acid Composition of HA2 Protein: Mole Percentage

Amino acid[a]	Mole percentage[b] (mean ± standard deviation)			
	X-12 (n = 2)	X-29L (n = 1)	NWS (n = 2)	RI/5⁻ (n = 1)
Asp	16.4 ± 0.1	15.2	15.4 ± 0.2	15.3
Thr	5.2 ± 0.2	6.0	5.2 ± 0.2	4.1
Ser	8.4 ± 0.4	9.4	7.8 ± 0.0	6.3
Glu	14.0 ± 0.2	13.6	13.7 ± 0.1	14.3
Ala	6.3 ± 0.0	6.4	6.3 ± 0.2	6.4
Val	6.2 ± 0.2	7.1	7.4 ± 0.1	7.6
Met	2.8 ± 0.9	2.6	2.5 ± 0.1	3.6
Ile	6.7 ± 0.4	6.0	7.0 ± 0.1	6.5
Leu	10.4 ± 0.2	10.2	10.9 ± 0.2	10.2
Tyr	5.4 ± 0.1	5.5	4.8 ± 0.0	4.9
Phe	4.5 ± 0.2	4.3	4.4 ± 0.1	5.1
Lys	8.8 ± 0.2	8.9	9.4 ± 0.2	9.3
His	1.9 ± 0.2	1.8	2.3 ± 0.0	2.2
Arg	3.1 ± 0.2	3.0	3.2 ± 0.3	4.3

[a]Proline, glycine, cysteine and tryptophan were not usually determined.

[b]Mole percentage is based on the 14 types of amino acids listed; n is the number of analyses.

TABLE 6.17

Average Amino Acid Composition of M Protein: Mole Percentage

Amino acid[a]	Mole percentage[b] (mean ± standard deviation)			
	X-12 (n = 1)	X-29 (n = 1)	NWS (n = 2)	RI/5⁻ (n = 2)
Asp	c	9.8	8.0 ± 0.3	8.0 ± 0.4
Thr	7.1	6.8	7.4 ± 0.7	6.9 ± 0.2
Ser	7.4	7.0	6.6 ± 0.2	6.8 ± 0.7
Glu	14.0	13.3	14.1 ± 0.2	14.4 ± 0.2
Pro	4.6	4.5	3.7 ± 0.3	4.0 ± 0.0
Ala	10.4	10.2	11.3 ± 0.1	11.5 ± 0.2
Val	6.4	6.9	7.9 ± 0.3	7.6 ± 0.1
Met	c	4.4	4.6 ± 0.2	4.3 ± 0.3
Ile	4.8	4.9	4.8 ± 0.1	4.5 ± 0.1
Leu	10.1	11.0	11.5 ± 0.4	11.3 ± 0.1
Tyr	2.9	2.8	2.0 ± 0.3	2.0 ± 0.0
Phe	3.2	3.5	3.1 ± 0.4	3.0 ± 0.4
Lys	6.1	6.2	5.9 ± 0.2	6.2 ± 0.0
His	2.1	2.0	2.1 ± 0.1	1.9 ± 0.1
Arg	6.8	6.7	7.0 ± 0.0	7.7 ± 0.3

[a]Proline, glycine, cysteine and tryptophan were not usually determined.

[b]Mole percentage is based on the 14 types of amino acids listed; n is the number of analyses.

[c]Values were not determined for aspartic acid and methionine, since methionine underwent partial oxidation to methionine sulfoxide, which was not resolved from aspartic acid.

TABLE 6.18

Average Amino Acid Composition of NP Protein: Mole Percentage

Amino acid[a]	Mole percentage[b] (mean ± standard deviation)			
	X-12 (n = 2)	X-29 (n = 1)	NWS (n = 2)	RI/5 (n = 2)
Asp	13.0[c]	13.3	11.5 ± 0.3	12.2 ± 0.5
Thr	6.1 ± 0.2	6.4	6.4 ± 0.1	6.3 ± 0.0
Ser	8.4 ± 0.8	9.3	8.8 ± 0.4	9.7 ± 0.8
Glu	14.2[c]	12.1	13.7 ± 0.5	13.6 ± 0.0
Ala	8.9 ± 0.4	9.1	8.7 ± 0.1	9.8 ± 0.3
Val	5.2 ± 0.1	5.2	6.4 ± 0.2	6.3 ± 0.2
Met	4.3 ± 0.2	2.6	3.5 ± 0.0	3.8 ± 0.1
Ile	6.3 ± 0.6	5.4	7.1 ± 0.3	6.0 ± 0.0
Leu	8.1 ± 0.2	9.8	8.2 ± 0.2	8.2 ± 0.4
Tyr	3.5 ± 0.3	3.9	3.0 ± 0.1	2.8 ± 0.3
Phe	4.7 ± 0.7	4.6	4.4 ± 0.8	3.4 ± 0.1
Lys	4.8[c]	6.9	5.3 ± 0.0	7.5 ± 0.5
His	1.5 ± 0.1	2.2	1.8 ± 0.1	2.1 ± 0.0
Arg	11.1 ± 0.3	9.0	11.1 ± 0.2	8.3 ± 0.1

[a] Proline, glycine, cysteine and tryptophan were not usually determined.

[b] Mole percentage is based on the 14 types of amino acids listed; n is the number of analyses.

[c] This value was obtained from a single determination.

TABLE 6.19

Average Amino Acid Composition of HA1 Protein: Number of Residues based on an Apoprotein Molecular Weight of 38,000 Daltons[a]

Amino acid	Number of residues[b] (mean ± standard deviation)			
	X-12 (n = 5)	X-29 (n = 4)	NWS (n = 2)	RI/5 (n = 2)
Asp	39.6 ± 1.6[c]	38.2 ± 2.4[d]	37.9 ± 0.9	32.2 ± 1.2
Thr[e]	20.5 ± 1.6	20.8 ± 0.8	23.0 ± 0.5	28.6 ± 0.5
Ser[f]	38.4 ± 2.5	37.5 ± 1.3	36.2 ± 0.9	28.6 ± 0.6
Glu	32.8 ± 0.6	33.4 ± 1.6	34.5 ± 0.8	36.8 ± 1.5
Pro	20.2 ± 0.4[g]	23.0 ± 1.2	21.8 ± 0.6	22.0 ± 0.1
Gly	27.0 ± 0.7	26.0 ± 1.5	26.6[h]	27.5[h]
Ala	19.8 ± 1.8	22.6 ± 1.0	17.0 ± 0.1	19.3 ± 0.8
Cys	12.0[i]	10.2[i]	11.1[h]	11.5[h]
Val	16.5 ± 1.7	18.4 ± 1.0	21.1 ± 0.2	25.2 ± 0.6
Met	3.6 ± 1.4[c]	6.1 ± 0.2[j]	3.3 ± 0.6	6.7 ± 0.5
Ile	17.6 ± 2.6	17.0 ± 0.6	18.0 ± 0.4	16.7 ± 0.1
Leu	31.0 ± 1.3	30.0 ± 1.4	29.7 ± 0.4	36.6 ± 0.8
Tyr	14.2 ± 1.4[c]	12.7 ± 0.7[d]	12.2 ± 1.2	9.5 ± 1.9
Phe	10.1 ± 0.8	10.7 ± 0.7	11.1 ± 1.4	9.5 ± 0.1
Trp	4.1[k]	4.1[k]	4.1[k]	4.2[k]
Lys	17.2 ± 0.8	17.3 ± 0.8	19.4 ± 0.2	20.9 ± 0.4
His	8.2 ± 0.8	7.9 ± 0.2	9.6 ± 0.1	9.7 ± 0.2
Arg	12.8 ± 0.9	13.7 ± 0.3	12.5 ± 0.1	13.7 ± 0.4

See next page for footnotes.

Footnotes for Table 6.19

[a]This value is based on molecular weight determinations for these specific viruses from this study (Table 3.2), assuming a carbohydrate content of 24.4% (Ward and Dopheide, 1976). For RI/5$^-$, a molecular weight of 39,300 daltons (Table 3.2) is used for the calculation.

[b]n is the number of analyses performed.

[c]The X-12 aspartic acid, methionine and tyrosine values are each an average of four analyses.

[d]The X-29 aspartic acid and tyrosine values are each an average of three analyses.

[e]Threonine values were increased by 5% to correct for loss during hydrolysis.

[f]Serine values were increased by 10% to correct for loss during hydrolysis.

[g]The X-12 proline value is an average of two analyses.

[h]Since glycine and cysteine values were not available for these viruses, the average of the mole percentages observed for X-12 and X-29 was used for this calculation.

[i]Cysteine values were increased by 8% to correct for incomplete oxidation to cysteic acid. One analysis employing performic acid oxidation was available.

[j]The X-29 methionine value is an average of two analyses.

[k]Tryptophan values are based on the mole percent determined by Ward and Dopheide (1976) for an H3N1 influenza virus.

TABLE 6.20

Average Amino Acid Composition of HA1 Protein: Number of Residues based on an Apoprotein Molecular Weight of 35,500 Daltons[a]

Amino acid	Number of residues[b] (mean ± standard deviation)			
	X-12 (n = 5)	X-29 (n = 4)	NWS (n = 2)	RI/5 (n = 2)
Asp	37.2 ± 1.5[c]	35.9 ± 2.3[d]	36.3 ± 0.9	29.1 ± 1.1
Thr[e]	19.2 ± 1.6	19.5 ± 0.7	22.0 ± 0.5	25.8 ± 0.4
Ser[f]	36.1 ± 2.6	35.2 ± 1.2	34.6 ± 0.9	25.8 ± 0.6
Glu	30.8 ± 0.5	31.4 ± 1.5	33.0 ± 0.7	33.3 ± 1.4
Pro	19.0 ± 0.4[g]	21.6 ± 1.1	20.8 ± 0.5	19.9 ± 0.1
Gly	25.4 ± 0.7	24.4 ± 1.4	25.5[h]	24.8[h]
Ala	18.6 ± 1.7	21.2 ± 0.9	16.2 ± 0.0	17.4 ± 0.8
Cys	11.2[i]	9.5[i]	10.6[h]	10.4[h]
Val	15.5 ± 1.6	17.3 ± 0.9	20.2 ± 0.2	22.7 ± 0.5
Met	3.4 ± 1.3[c]	5.7 ± 0.2[j]	3.1 ± 0.6	6.1 ± 0.5
Ile	16.6 ± 2.5	16.0 ± 0.6	17.3 ± 0.4	15.1 ± 0.1
Leu	29.2 ± 1.2	28.1 ± 1.3	28.4 ± 0.4	33.0 ± 0.7
Tyr	13.3 ± 1.3[c]	12.0 ± 0.7[d]	11.7 ± 1.1	8.6 ± 1.8
Phe	9.5 ± 0.8	10.0 ± 0.7	10.6 ± 1.3	8.6 ± 0.1
Trp	3.8[k]	3.9[k]	3.9[k]	3.8[k]
Lys	16.2 ± 0.8	16.3 ± 0.8	18.6 ± 0.2	18.9 ± 0.3
His	7.7 ± 0.7	7.4 ± 0.2	9.2 ± 0.1	8.8 ± 0.2
Arg	12.0 ± 0.9	12.8 ± 0.3	11.9 ± 0.1	12.4 ± 0.4

See next page for footnotes.

Footnotes for Table 6.20

[a]This is the apoprotein molecular weight determined by Ward and Dopheide (1976) for an H3N2 influenza virus.

[b]n is the number of analyses performed.

[c]The X-12 aspartic acid, methionine and tyrosine values are each an average of four analyses.

[d]The X-29 aspartic acid and tyrosine values are each an average of three analyses.

[e]Threonine values were increased by 5% to correct for loss during hydrolysis.

[f]Serine values were increased by 10% to correct for loss during hydrolysis.

[g]The X-12 proline value is an average of two analyses.

[h]Since glycine and cysteine values were not available for these viruses, the average of the mole percentages observed for X-12 and X-29 was used for this calculation.

[i]Cysteine values were increased by 8% to correct for incomplete oxidation to cysteic acid. One analysis employing performic acid oxidation was available.

[j]The X-29 methionine value is an average of two analyses.

[k]Tryptophan values are based on the mole percent determined by Ward and Dopheide (1976) for an H3N1 influenza virus.

TABLE 6.21

Average Amino Acid Composition of HA2 Protein: Number of Residues based on an Apoprotein Molecular Weight of 25,000 Daltons[a]

Amino acid	Number of residues[b] (mean ± standard deviation)			
	X-12 (n = 2)	X-29L (n = 1)	NWS (n = 2)	RI/5 (n = 1)
Asp	30.9 ± 0.2	28.4	29.0 ± 0.4	33.0
Thr[c]	10.2 ± 0.3	11.8	10.3 ± 0.3	9.4
Ser[d]	17.4 ± 0.8	19.2	16.2 ± 0.1	14.9
Glu	26.4 ± 0.5	25.4	25.8 ± 0.2	30.8
Pro	4.9 ± 1.6	7.0	3.6 ± 0.4	3.6
Gly	19.7 ± 0.4	19.6	19.8[e]	22.6[e]
Ala	11.8 ± 0.1	12.1	11.9 ± 0.4	13.9
Cys	4.3[f]	4.3[f]	4.4[f]	5.0[f]
Val	11.6 ± 0.4	13.2	14.0 ± 0.1	16.3
Met	5.2 ± 1.6	4.9	4.8 ± 0.2	7.7
Ile	12.6 ± 0.8	11.3	13.3 ± 0.2	14.0
Leu	19.6 ± 0.4	19.1	20.6 ± 0.5	22.0
Tyr	10.2 ± 0.1	10.3	9.0 ± 0.0	10.5
Phe	8.4 ± 0.4	8.0	8.2 ± 0.2	11.0
Trp	3.2[g]	3.2[g]	3.3[g]	3.7[g]
Lys	16.5 ± 0.4	16.7	17.8 ± 0.3	20.2
His	3.6 ± 0.4	3.4	4.3 ± 0.0	10.4
Arg	5.8 ± 0.4	5.6	6.0 ± 0.5	9.2

See next page for footnotes.

Footnotes for Table 6.21

[a]This value is based on molecular weight determinations for X-12, X-29L and NWS from this study (Table 3.2), assuming a carbohydrate content of 24.4% (Ward and Dopheide, 1976). For RI/5$^-$, a molecular weight of 28,600 daltons (Table 3.2) is used for the calculation.

[b]n is the number of analyses performed.

[c]Threonine values were increased by 5% to correct for loss during hydrolysis.

[d]Serine values were increased by 10% to correct for loss during hydrolysis.

[e]Since glycine values were not available for these viruses, the average of the mole percentages observed for X-12 and X-29L was used for this calculation.

[f]Cysteine values are based on the mole percent determined by Skehel and Waterfield (1975) for A/Bel/42 (H0N1) influenza virus.

[g]Tryptophan values are based on the mole percent determined by Ward and Dopheide (1976) for an H3N1 influenza virus.

TABLE 6.22

Average Amino Acid Composition of HA2 Protein: Number of Residues based on an Apoprotein Molecular Weight of 28,300 Daltons[a]

Amino acid	Number of residues[b] (mean ± standard deviation)			
	X-12 (n = 2)	X-29L (n = 1)	NWS (n = 2)	RI/5 (n = 1)
Asp	35.2 ± 0.3	32.3	33.0 ± 0.5	32.7
Thr[c]	11.6 ± 0.3	13.4	11.7 ± 0.3	9.3
Ser[d]	19.8 ± 0.9	21.9	18.5 ± 0.1	14.8
Glu	30.0 ± 0.5	28.9	29.4 ± 0.3	30.5
Pro	5.6 ± 1.8	7.9	4.1 ± 0.4	3.6
Gly	22.4 ± 0.4	22.3	22.5[e]	22.4[e]
Ala	13.4 ± 0.1	13.7	13.6 ± 0.5	13.8
Cys	4.9[f]	4.9[f]	4.9[f]	4.9[f]
Val	13.2 ± 0.5	15.1	15.9 ± 0.1	16.2
Met	5.9 ± 1.9	5.5	5.5 ± 0.2	7.6
Ile	14.3 ± 0.9	12.8	15.2 ± 0.2	13.9
Leu	22.3 ± 0.4	21.7	23.4 ± 0.5	21.8
Tyr	11.6 ± 0.1	11.8	10.3 ± 0.0	10.4
Phe	9.6 ± 0.4	9.1	9.4 ± 0.2	10.9
Trp	3.7[g]	3.7[g]	3.7[g]	3.7[g]
Lys	18.8 ± 0.5	19.0	20.3 ± 0.3	20.0
His	4.1 ± 0.5	3.8	4.9 ± 0.0	4.8
Arg	6.6 ± 0.5	6.4	6.9 ± 0.6	9.2

See next page for footnotes.

Footnotes for Table 6.22

[a]This is the apoprotein molecular weight determined by Ward and Dopheide (1976) for an H3N2 influenza virus.

[b]n is the number of analyses performed.

[c]Threonine values were increased by 5% to correct for loss during hydrolysis.

[d]Serine values were increased by 10% to correct for loss during hydrolysis.

[e]Since glycine values were not available for these viruses, the average of the mole percentages observed for X-12 and X-29L was used for this calculation.

[f]Cysteine values are based on the mole percent determined by Skehel and Waterfield (1975) for A/Bel/42 (HONl) influenza virus.

[g]Tryptophan values are based on the mole percent determined by Ward and Dopheide (1976) for an H3Nl influenza virus.

TABLE 6.23

Average Amino Acid Composition of M Protein: Number of Residues based on a Protein Molecular Weight of 32,000 Daltons

Amino acid[a]	Number of residues[b] (mean ± standard deviation)			
	X-12 (n = 1)	X-29L (n = 1)	NWS (n = 2)	RI/5⁻ (n = 2)
Asp	23.2	26.3	21.6 ± 0.8	21.6 ± 0.9
Thr[c]	20.0	19.1	20.9 ± 2.0	19.3 ± 0.6
Ser[d]	21.8	20.6	19.4 ± 0.6	20.0 ± 2.1
Glu	37.5	35.6	37.8 ± 0.6	38.6 ± 0.6
Pro	12.3	12.1	9.9 ± 0.8	10.8 ± 0.1
Gly	23.8	19.8	21.8[e]	21.8[e]
Ala	28.0	27.2	30.3 ± 0.2	30.8 ± 0.5
Val	17.2	18.4	21.2 ± 0.7	20.3 ± 0.2
Met	11.9	11.8	12.2 ± 0.7	11.6 ± 0.8
Ile	12.8	13.1	12.9 ± 0.3	12.0 ± 0.4
Leu	27.1	29.4	30.9 ± 1.0	30.2 ± 0.2
Tyr	7.7	7.6	5.3 ± 0.8	5.4 ± 0.1
Phe	8.6	9.5	8.4 ± 1.1	7.9 ± 1.1
Lys	16.4	16.6	15.9 ± 0.5	16.6 ± 0.1
His	5.7	5.3	5.7 ± 0.3	5.1 ± 0.2
Arg	18.3	17.8	18.7 ± 0.1	20.6 ± 0.8

See next page for footnotes.

Footnotes for Table 6.23

[a]Cysteine and tryptophan were not determined.

[b]n is the number of analyses.

[c]Threonine values were increased by 5% to correct for loss during hydrolysis.

[d]Serine values were increased by 10% to correct for loss during hydrolysis.

[e]Since glycine values were not available for these viruses, the average of the mole percentages observed for X-12 and X-29L was used for this calculation.

TABLE 6.24

Average Amino Acid Composition of NP Protein: Number of Residues based on a Protein Molecular Weight of 58,000 Daltons

Amino acid[a]	Number of residues[b] (mean ± standard deviation)			
	X-12 (n = 2)	X-29 (n = 1)	NWS (n = 2)	RI/5 (n = 2)
Asp	58.2[c]	60.7	51.8 ± 1.3	55.7 ± 2.1
Thr[d]	28.7 ± 0.8	30.5	30.5 ± 0.4	30.4 ± 0.2
Ser[e]	41.5 ± 3.8	46.6	43.6 ± 1.8	48.8 ± 4.2
Glu	63.6[c]	55.2	61.9 ± 2.4	62.2 ± 0.2
Pro	21.6 ± 0.7	22.1	22.4 ± 0.2	21.9[c]
Gly	42.1 ± 7.4	37.1	40.5[f]	41.1[f]
Ala	40.0 ± 1.8	41.4	39.3 ± 0.4	44.7 ± 1.5
Val	23.2 ± 0.5	23.8	29.0 ± 1.0	29.1 ± 0.9
Met	19.3 ± 0.9	11.9	15.9 ± 0.0	17.4 ± 0.6
Ile	28.4 ± 2.8	24.8	32.0 ± 1.3	27.4 ± 0.2
Leu	36.3 ± 0.8	44.7	37.2 ± 0.8	37.6 ± 2.1
Tyr	15.7 ± 1.3	17.9	13.5 ± 0.5	12.8 ± 1.6
Phe	21.1 ± 3.1	21.1	19.8 ± 3.7	15.8 ± 0.4
Lys	21.8[c]	31.3	24.0 ± 0.0	34.4 ± 2.2
His	6.6 ± 0.5	10.0	8.3 ± 0.4	9.8 ± 0.1
Arg	49.8 ± 1.3	40.9	49.9 ± 0.7	38.1 ± 0.5

See next page for footnotes.

Footnotes for Table 6.24

[a]Cysteine and tryptophan were not determined.

[b]n is the number of analyses.

[c]This value was obtained from a single determination.

[d]Threonine values were increased by 5% to correct for loss during hydrolysis.

[e]Serine values were increased by 10% to correct for loss during hydrolysis.

[f]Since glycine values were not available for these viruses, the average of the mole percentages observed for X-12 and X-29 was used for this calculation.

TABLE 6.25

Amino Acid Compositions for Proteins from Influenza Virus A/Bel/42 (H0N1)[a]

Amino acid	Laver & Baker, 1972				Skehel & Waterfield, 1975	
	HA1	HA2	M	NP	HA1	HA2
Asp	13.6	15.8	8.1	9.8	13.8	16.0
Thr	8.4	5.1	7.0	6.4	8.3	5.3
Ser	10.7	9.0	6.2	7.8	10.1	9.4
Glu	13.2	15.2	17.4	17.4	12.8	14.0
Pro	-	-	3.6	-	-	-
Ala	6.4	6.4	11.1	9.2	6.1	6.0
Val	7.0	6.8	6.9	6.2	6.7	6.8
Met	0.6	0.9	2.2	1.6	0.8	2.6
Ile	7.7	6.8	5.2	7.2	7.7	6.9
Leu	10.4	11.0	12.9	10.0	9.7	9.6
Tyr	4.0	4.1	1.1	2.0	5.1	5.7
Phe	3.8	4.9	2.8	4.9	3.9	4.6
Lys	6.8	9.1	6.0	6.5	6.6	8.4
His	2.3	1.8	1.9	1.5	3.1	2.0
Arg	5.0	3.1	7.7	9.5	5.3	2.7

[a]Data were recalculated to generate mole percentage values based on 14 types of amino acid residues for proteins HA1, HA2 and NP. Mole percentage values for M protein are based on 15 types of amino acids.

TABLE 6.26

Amino Acid Compositions for Hemagglutinin Proteins from H0N1 and H3N1 Influenza Viruses[a]

Amino acid	H0N1 HABA	H3N1 HA1	H3N1 HA2
Asp	14.9	16.3	15.2
Thr	6.8	10.7	5.9
Ser	12.5	11.4	6.2
Glu	12.8	9.5	15.7
Ala	5.9	5.2	7.1
Val	6.8	7.8	4.9
Met	2.4	1.5	1.8
Ile	6.6	8.2	10.0
Leu	10.4	8.3	8.9
Tyr	3.6	4.1	3.8
Phe	1.9	4.0	5.2
Lys	7.4	5.6	6.4
His	2.8	2.1	2.6
Arg	5.2	5.2	5.4

[a]Data for the H0N1 protein HABA from A/PR/8/34 (Eckert, 1973) and the HA1 and HA2 proteins from an H3N1 recombinant (Ward and Dopheide, 1976) were recalculated to generate mole percentage values based on 14 types of amino acid residues.

Chapter 7

Comparison of the Recombinant Viruses X-12 and X-29

The classic phenotypic marker of influenza viruses has been their antigenicity as measured by serological typing. One of several other markers used to characterize the series of recombinant viruses derived from A/NWS/33 and A/RI/5$^-$ was the sensitivity of the viral hemagglutination activity to trypsin. Most viruses of the H0 serotype were sensitive to trypsin, as measured by loss of hemagglutination activity, within 90 min. In contrast, viruses of the H2 serotype suffered no loss of activity under these conditions. Two exceptions to this general rule were the recombinant viruses X-6 and X-29, which were trypsin-resistant despite the H0-characterization of their hemagglutinin protein.

During this study, the trypsin-sensitive recombinant X-12 (H0N2) was compared with the antigenically similar but trypsin-resistant recombinant X-29. Several of the properties examined are summarized in Table 7.1. Three goals of this study were to define the biochemical basis of trypsin sensitivity, to explore the structure of the virion through trypsin treatment, and perhaps to learn more about the spontaneous generation of phenotypic variation.

Differences in trypsin sensitivity. Since X-29 was derived by recombination of a trypsin-sensitive and a trypsin-resistant virus, it seemed reasonable that its trypsin resistance was acquired from the trypsin-resistant parent. Comparison of X-29 with the H2 virus RI/5$^-$ and the H0 viruses NWS and X-12 revealed, however, that by the criteria of serotype, the HA1 to HA2 ratio, and the sensitivity of both the HA1 protein and the hemagglutination activity to trypsin, X-29 resembled

TABLE 7.1

Comparison of the Influenza Viruses X-12 and X-29L

Experimental property	Comparison	Chapter
Viral serotype	Same	2
Molecular weights of HA1, HA2, M, and NP proteins	Same	3
Amino acid compositions of HA1, HA2 and M proteins	Same	6
Amount of HA protein in virions from CEF cells	Same	3
Initial ratio of HA1 to HA2 from CAM cells	X-12 > X-29L	4
Sensitivity to anti-H0 serum	X-12 > X-29L	2
Sensitivity to anti-H2 serum	X-29L > X-12	
Amount of neuraminidase activity	X-29L > X-12	3
Amount of NA protein	X-29L > X-12	3
Retention of neuraminidase activity during trypsin treatment	Same	4
Rate of loss of hemagglutination activity during trypsin treatment	X-12 > X-29L	4
Rate of loss of HA1 protein during trypsin treatment	X-12 > X-29L	4
Independence of trypsin sensitivity on host cell	Same	4

the sensitive H0 viruses and not the resistant H2 virus. Although X-29 was initially described as trypsin resistant, closer examination has shown that its hemagglutination activity is slowly trypsin-sensitive. The activity of X-12 or NWS was rapidly lost in about 30 min and the activity of RI/5$^-$ was stable for 4 hr, yet the activity of X-29 or its large-plaque variant X-29L was only slightly sensitive during the first two hours but was lost by 4 hr. In addition, although the observed HA1 to HA2 ratio for RI/5$^-$ was similar to the calculated ratio, the observed ratio for X-29 resembled the ratios for X-12 and NWS in being about half of the calculated ratio. Unlike the H2 virus, all three of the H0 viruses apparently suffered partial loss of the HA1 subunit during virus culture or purification. Loss of hemagglutination activity during trypsin treatment of any of these H0 virses was accompanied by loss of the HA1 subunit from the virus particles. In contrast, the loss of the HA2 subunit was much slower and the neuraminidase activity remained constant during trypsin treatment.

The initial first-order rate for loss of radiolabeled HA1 protein during trypsin treatment was about 2.3 times faster for X-12 than for X-29L. Before treatment, both viruses showed a single HA1 component on stained gels. During trypsin treatment, however, both viruses contained not only a faster migrating component $HA1_f$, which corresponded to the initial HA1 protein, but also a slower migrating $HA1_s$ protein. Since no other new proteins were detected by gel electrophoresis, the initial $HA1_f$ protein was evidently converted into the $HA1_s$ protein, which was subsequently degraded to small peptides. The $HA1_f$ protein of X-12 was lost within 30 min and that of X-29L was degraded in somewhat over 60 min. For both viruses, however, the amount of the $HA1_s$ component was about equal to the amount of the $HA1_f$ component after brief treatment and

remained nearly constant for at least 60 min. Loss of hemagglutination activity of X-12 correlated well with the disappearance of $HA1_f$, but the activity of X-29L was only decreased by half when $HA1_f$ was essentially gone. Thus the difference in the rate of loss of hemagglutination activity is apparently due to the $HA1_s$ component of X-29L still being active while the $HA1_s$ of X-12 is inactive.

Explanations for trypsin sensitivity. Several explanations for the difference between the rapid trypsin sensitivity of X-12 and the slow trypsin sensitivity of X-29L were considered and rejected. The type of neuraminidase on the viral surface is not important because NWS_{t8} was slowly trypsin-sensitive even though it was serotypically N1, not N2. The amount of neuraminidase protein is unimportant because X-12 had only half as much and X-29LT had twice as much neuraminidase as X-29 and both were rapidly sensitive. The amount of hemagglutinin protein was also not important because X-12 and X-29L viruses grown in CEF cells contained the same amount of uncleaved HA. The internal membrane protein probably plays little role in orienting the hemagglutinin protein with respect to the neuraminidase or the external environment because neither surface protein is believed to extend through the lipid bilayer. In addition, the membrane proteins from both viruses showed very similar amino acid compositions.

The initial explanation (Kilbourne et al., 1972) proposed for the different trypsin sensitivity of these antigenically similar viruses was intragenic recombination between RNA segments during double infection. For example, intragenic recombination between RNA segments separately coding for the H0 and H2 hemagglutinin proteins might produce a hybrid RNA segment coding for most of the HA1 subunit from H0 and the rest of

HA1 and all of HA2 derived from the H2 segment. The corresponding hybrid HA1--HA2 protein might thus possess the H0 serotype and the H2 resistance to trypsin. This would explain why X-29 shows greater reactivity than X-12 with anti-H2 serum. Were this scheme correct, it would be a striking observation because no example of intragenic recombination has yet been described for influenza viruses. In fact, this scheme seems unlikely on two accounts. First, the electrophoretic mobility and thus the molecular weights of the HA1 and HA2 subunits from X-29L were identical to those from NWS and X-12 (H0) and clearly different from those from RI/5$^-$ (H2). Second, the amino acid compositions of the HA subunits from X-29 were very similar to those from NWS and X-12 and less similar to those from RI/5$^-$. These results cannot rule out, however, the intragenic exchange of a region of HA similar or small enough to escape detection.

The most plausible explanation for the different trypsin sensitivity of X-12 and X-29 is the presence of different amino acids at one or a few positions due to point mutation. This proposal is consistent with the very similar amino acid compositions for the hemagglutinin subunits of NWS, X-12 and X-29. Several types of amino acid changes might be consistent with the loss of $HA1_f$ being slower and the $HA1_s$ being more active for X-29L than for X-12. First, a readily accessible lysine or arginine residue in or near the erythrocyte-binding site of X-12 might have been replaced by a nonbasic residue in X-29L. Rapid cleavage of the $HA1_f$ from X-12 at this basic residue would produce an inactive $HA1_s$, but the slower initial cleavage of $HA1_f$ from X-29L at a less accessible basic residue could preserve the integrity of the erythrocyte-binding site and thus hemagglutination activity. Second, an equivalent result

might be achieved by replacing a conformationally important residue in or near the binding site of X-12 by a dissimilar residue in X-29L. Plausible candidates for the conformationally important residue include proline and glycine, which favor β-turns and disfavor α-helical polypeptide structure; phenylalanine or tryptophan, which tend to bury in the hydrophobic interior of a protein; and cysteine, which uniquely forms the disulfide bonds that stabilize protein structure. Such a change might make an accessible basic residue in X-12 inaccessible in X-29L. Third, replacement of a residue in X-12 by a serine, threonine or aspartic acid residue in X-29L might permit additional glycosylation and thus block the access of trypsin to a previously exposed basic residue. Extra carbohydrate attached to X-29L might also affect the protein interactions within the hemagglutinin trimers. One or a few changes of important amino acid residues cannot usually be detected in proteins the size of HA1 and HA2 (25,000 to 50,000 daltons) by amino acid composition. Even peptide mapping may not be adequate for this task. Possibly only the amino acid sequence of the HA1 proteins from both viruses would suffice.

Trypsin sensitivity as a phenotypic marker. Recent reviewers (Schulze, 1975; Sugiura, 1975) have cited the reported trypsin resistance of X-29 (Kilbourne et al., 1972) as proof that the trypsin sensitivity of H0 viruses derived from A/NWS/33 (H0N1) can be segregated from the hemagglutinin serotype and thus is not exclusively determined by the hemagglutinin protein. The present study has shown, however, that the X-29 hemagglutination activity is not trypsin-resistant but slowly trypsin-sensitive and is evidently solely determined by the structure of the hemagglutinin protein. Thus for NWS-derived viruses, the general

distinction between trypsin sensitivity and trypsin resistance does correlate with the hemagglutinin serotype. But the specific kinetic distinction between rapid and slow trypsin sensitivity is not revealed by the hemagglutinin serotype even though it is solely determined by the nature of the hemagglutinin protein. Therefore influenza viruses can differ phenotypically in a biologically important way that is not detected by normal serological classification using subtype-specific antisera.

The sensitivity to trypsin does not correlate with the H0 hemagglutinin serotype but does correlate with the NWS-derived H0 hemagglutinin. The H0N1 viruses A/WSN/33 and A/PR/8/34 resemble A/ RI/5$^-$/57 (H2N2) in suffering no loss of hemagglutination activity after trypsin treatment for 4 hr. In addition, WSN also resembles RI/5$^-$ but not X-12 or X-29L because the observed HA1 to HA2 ratio is close to the calculated ratio.

The phenotypic property of slow trypsin sensitivity can be genetically inherited because the recombinant NWS_{t8} (H0N1) derived from X-29 (H0N2) and X-9L (H2N1) was also slowly trypsin-sensitive. In addition to X-29, X-29L and NWS_{t8}, the NWS-derived H0 recombinants X-6 and NWS_{t2} may also be slowly trypsin-sensitive. Recombination of slowly trypsin-sensitive X-29L and trypsin-resistant X-41 (H3N2) produced the rapidly trypsin-sensitive virus X-29LT (H0N2). This example evidently shows that a slowly trypsin-sensitive virus can revert to being rapidly trypsin sensitive and suggests that the kinetic distinction is controlled by a subtle and readily reversible modification of the hemagglutinin protein. A substantially larger number of NWS-derived recombinants would have to be isolated and characterized before the frequencies of

interconversion between the slowly and rapidly trypsin-sensitive phenotypes could be established.

Viruses that share the same serotype can exhibit a number of different biological properties. This study has shown that the antigenically similar H0N2 recombinant viruses X-12 and X-29 differ in the trypsin sensitivity of their hemagglutination activity and infectivity and in the amount of their neuraminidase activity and NA protein. The viruses were also distinguished by two other phenotypic properties. First, the infectivity of X-29 was less sensitive than that of X-12 to anti-H0 sera but the X-29 hemagglutination activity was more sensitive to anti-H2 sera. The reasons for these antigenic distinctions are not known but may be related to structural changes in the hemagglutinin protein the same as or similar to the point mutation(s) that could explain the difference in trypsin sensitivity. For example, an altered protein conformation or a different glycosylation pattern might hide certain H0-specific antigenic sites and expose other sites common to all subtypes. Second, the HA1 to HA2 ratio before trypsin treatment was 50% greater for X-12 than for X-29L. The apparently greater loss of HA1 from X-29L might expose more HA2 subunits and thus render the virus more sensitive to antisera directed against the compositionally more conserved HA2 subunit.

Structural information from trypsin treatment. Examination of the properties of trypsin-treated virus particles has provided several insights into the structure of the influenza virion. The classic example is the discovery that the host-dependent cleavage of intact HA protein into its HA1 and HA2 subunits is mediated by trypsin, plasmin and other naturally occurring serine proteases. Two other examples resulting from this study are ultrastructural visualization of the

neuraminidase protein on the virion and the compositional relatedness of influenza proteins.

Although isolated neuraminidase tetramers have been examined by electron microscopy, the structure and distribution of the neuraminidase protein are not readily apparent on the intact virion because of the much larger amount of hemagglutinin protein on the viral surface. The ultrastructure of the neuraminidase spikes was readily visible, however, after trypsin treatment of X-12 or X-29L. The residual spikes appeared as globular heads attached to the lipid bilayer by a long narrow fibers, thus resembling in vivo the neuraminidase structures previously seen in vitro. These surface projections were evenly distributed over the viral surface. Since the presence of these structures correlated with the retention of neuraminidase protein and activity after trypsin treatment but not with the loss of hemagglutinin protein and hemagglutination activity, the knob-like projections evidently represent neuraminidase glycoprotein.

The amino acid compositions of four proteins (HA1, HA2, M, NP) from four viruses (NWS, X-12, X-29, RI/5$^-$) were compared by two statistical methods. The compositions of the HA1 and HA2 proteins correlate with the H0, H2 and H3 serotypes. Thus the amino acid composition of either subunit could be used to predict the serotype of a new virus. Alternatively, the parental donor of an isolated HA protein could be identified through its composition. Comparison of proteins of different serotypes showed that the HA2 composition is generally more conserved than the HA1 composition. Since the HA1 subunit is more exposed to solvent and is the major antigenic subunit, it is more sensitive to the natural

pressure of antigenic selection. The internal M and NP proteins from different serotypes are as compositionally different from one another as either is from HA2. The M proteins from different serotype viruses were very similar but the NP proteins showed definite differences. Comparison of the different proteins from a single virus revealed that HA1 and HA2 subunits had very similar compositions and similarly the internal proteins M and NP are quite similar. These results suggest that either due to common ancestry or similar functional constraints HA1 and HA2 on one hand and M and NP on the other may be sequentially homologous.

Bibliography

Almeida, J. D., and Brand, C. M. (1975). J. Gen Virol. 27, 313.

Almeida, J. D., and Waterson, A. P. (1970). In "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 27-51. Academic Press, New York.

Aminoff, D. (1961). Biochem. J. 81, 384.

Andrewes, C. H., Laidlaw, P. P., and Smith, W. (1934). Lancet 2, 859.

Apostolov, K. and Flewett, T. H. (1965). Virology 26, 506.

Apostolov, K. and Flewett, T. H. (1969). J. Gen. Virol. 4, 365.

Bablanian, R., Eggers, H. J., and Tamm, I. (1965). Virology 26, 100.

Bächi, T., Gerhard, W., Lindenmann, J., and Mühlethaler, K. (1969). J. Virol. 4, 769.

Barry, R. D. (1961). Virology 14, 398.

Bergmann, M., Brand, E., and Weinmann, F. (1923). Hoppe-Seyler's Z. Physiol. Chem. 131, 1.

Bier, M., and Nord, F. F. (1951). Arch. Biochem. Biophys. 33, 320.

Bishop, D. H., Obijeski, J. F., and Simpson, R. W. (1971). J. Virol. 8, 66.

Bishop, D. H. L., Roy, P., Bean, W. J., Jr., and Simpson, R. W. (1972). J. Virol. 10, 689.

Blachnitsky, E.-O., Wengenmayer, F., and Kurz, G. (1974). Eur. J. Biochem. 47, 235.

Blobel, G., and Dobberstein, B. (1975). J. Cell Biol. 67, 835.

Bonner, W. M., and Laskey, R. A. (1974). Eur. J. Biochem. 46, 83.

Brand, C. M., and Skehel, J. J. (1972). Nature (London) New Biol. 238, 145.

Bretscher, M. S. (1971). Nature (London) New Biol. 231, 229.

Brush, A. H. (1974). Comp. Biochem. Physiol. 48B, 661.

Bucher, D. J., and Kilbourne, E. D. (1972). J. Virol. 10, 60.

Bucher, D. J., and Palese, P. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 83-123. Academic Press, New York.

Bucher, D. J., Li, S. S.-L., Kehoe, J. M., and Kilbourne, E. D. (1976). Proc. Nat. Acad. Sci. U.S. 73, 238.

Burnet, F. M. (1940). Aust. J. Exp. Biol. Med. Sci. 18, 353.

Burnet, F. M. (1951). J. Gen. Microbiol. 5, 46.

Burnet, F. M. (1955). "Principles of Animal Virology." Academic Press, New York.

Burnet, F. M. (1956). Science 123, 1101.

Burnet, F. M., and Lind, P. E. (1951a). J. Gen. Microbiol. 5, 59.

Burnet, F. M., and Lind, P. E. (1951b). J. Gen. Microbiol. 5, 67.

Burnet, F. M., McCrea, J. F., and Stone, J. D. (1946). Brit. J. Exp. Pathol. 27. 228.

Caliguiri, L. A., and Compans, R. W. (1974). J. Virol. 14, 191.

Casals, J. (1967). In "Methods in Virology" (K. Maramorosch and H. Koprowski, ed.), Vol. 3, pp. 113-198. Academic Press, New York.

Chenoweth, A., Waltz, A. D., Stokes, J., Jr., and Gladen, R. G. (1936). Am. J. Dis. Child. 52, 757.

Choppin, P. W. (1963). Virology 21, 342.

Choppin, P. W., and Compans, R. W. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 15-51. Academic Press, New York.

Choppin, P. W., and Pons, M. W. (1970). Virology 42, 603.

Choppin, P. W., and Tamm, I. (1959). Virology 8, 539.

Choppin, P. W., and Tamm, I. (1960a). J. Exp. Med. 112, 895.

Choppin, P. W., and Tamm, I. (1960b). J. Exp. Med. 112, 921.

Choppin, P. W., Klenk, H.-D., Compans, R. W., and Caliguiri, L. A. (1971). In "Perspectives in Virology" (M. Pollard, ed.), Vol. 7, pp. 127-158. Academic Press, New York.

Chow, N.-L., and Simpson, R. W. (1971). Proc. Nat. Acad. Sci. U.S. 68, 752.

Cleeland, R., and Sugg, J. Y. (1960). J. Immunol. 85, 539.

Compans, R. W. (1973). Virology 55, 541.

Compans, R. W., and Choppin, P. W. (1975). Compr. Virol. 4, 179.

Compans, R. W., and Dimmock, N. J. (1969). Virology 39, 499.

Compans, R. W., and Pinter, A. (1975). Virology 66, 151.

Compans, R. W., Holmes, K. V., Dales, S., and Choppin, P. W. (1966). Virology 30, 411.

Compans, R. W., Dimmock, N. J., and Meier-Ewert, H. (1969). J. Virol. 4, 528.

Compans, R. W., Klenk, H.-D., Caliguiri, L. A., and Choppin, P. W. (1970). Virology 42, 880.

Compans, R. W., Content, J., and Duesberg, P. H. (1972). J. Virol. 10, 795.

Content, J., and Duesberg, P. H. (1970). J. Virol. 6, 707.

Content, J., and Duesberg, P. H. (1971). J. Mol. Biol. 62, 273.

Crookshank, F. G. (1922). In "Influenza: Essays by Several Authors" (F. G. Crookshank, ed.), pp. 64-80. William Heinemann, London.

Croxton, F. E. (1959). "Elementary Statistics with Applications in Medicine and the Biological Sciences." Dover, New York.

Davenport, F. M., Rott, R., Schafer, W. (1960). J. Exp. Med. 112, 765.

Dayhoff, M. O. (1972). "Atlas of Protein Sequence and Structure 1972, Vol. 5." National Biomedical Research Foundation, Washington D. C.

Desnuelle, P. (1960). In "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), Vol. 4, pp. 93-118. Academic Press, New York.

Devillers-Thiery, A., Kindt, T., Scheele, G., and Blobel, G. (1975). Proc. Nat. Acad. Sci. U.S. 72, 5016.

Ditchfield, J., and Grinyer, I. (1965). Virology 26, 504.

Downie, J. C. (1970). J. Immunol. 105, 620.

Drzeniek, R. (1967). Biochem. Biophys. Res. Commun. 26, 631.

Duesberg, P. H. (1968). Proc. Nat. Acad. Sci. U.S. 59, 930.

Duesberg, P. H. (1969). J. Mol. Biol. 42, 485.

Dulbecco, R., and Vogt, M. (1954). J. Exp. Med. 99, 167.

Easterday, B., Laver, W. G., Pereira, H. G., and Schild, G. C. (1969). J. Gen. Virol. 5, 83.

Eckert, E. A. (1966). J. Bacteriol. 92, 1430.

Eckert, E. A. (1969). J. Immunol. 102, 1105.

Eckert, E. A. (1973). J. Virol. 11, 183.

Edelman, G. M., and Gall, W. E. (1969). Annu. Rev. Biochem. 38, 415.

Eisen, H. N., Little, J. R., Steiner, L. A., and Simms, E. E. (1969). Isr. J. Med. Sci. 5, 338.

Etkind, P. R., and Krug, R. M. (1974). Virology 62, 38.

Francis, T., Jr. (1934). Science 80, 457.

Francis, T., Jr. (1940). Science 92, 405.

Francis, T., Jr., and Maassab, H. F. (1965). In "Viral and Rickettsial Infections of Man" (F. L. Horsfall, Jr., and I. Tamm, eds.), pp. 689-740. J. B. Lippincott, Philadelphia.

Francis, T., Jr., Quilligan, J. J., and Minuse, E. (1950). Science 112, 495.

Francis, T., Jr., Davenport, F. M., and Hennessy, A. V. (1953). Trans. Assoc. Amer. Physicians 66, 231.

Fraser, K. B. (1959). Virology 9, 178.

Frattali, V., and Steiner, R. F. (1968). Biochemistry 7, 521.

Gandhi, S. S., and Burke, D. C. (1970). In "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 675-683. Academic Press, New York.

Glass, S. E., McGeoch, D., and Barry, R. D. (1975). J. Virol. 16, 1435.

Gorini, L. (1951). Biochim. Biophys. Acta 7, 318.

Green, N. M., and Neurath, H. (1953). J. Biol. Chem. 204, 379.

Green, N. M., and Neurath, H. (1954). In "The Proteins" (H. Neurath and K. Bailey, eds.), Vol. 2, Part B, pp. 1057-1198. Academic Press, New York.

Gregoriades, A. (1972). Virology 49, 333.

Gresser, I., and Enders, J. F. (1961). Virology 13, 420.

Griffith, I. P. (1975). In "Negative Strand Viruses" (R. D. Barry and B. W. J. Mahy, eds.), Vol. 1, pp. 121-143. Academic Press, New York.

Grossgebauer, K. (1972). Zentralbl. Bakteriol. Parasitenk. Infektionsk. Hyg. Abt. 1: Orig. Reihe A 223, 56.

Haslam, E. A., Hampson, A. W., Egan, J. A., and White, D. O. (1970). Virology 42, 555.

Hay, A. J. (1974). Virology 60, 398.

Henle, G., Liu, C. (1951). J. Exp. Med. 94, 305.

Hirs, C. H. W. (1967). Methods Enzymol. 11, 59.

Hirst, G. K. (1941). Science 94, 22.

Hirst, G. K. (1962). Cold Spring Harbor Symp. Quant. Biol. 27, 303.

Hirst, G. K. (1973). Virology 55, 81.

Hirst, G. K., and Gottlieb, T. (1955). Virology 1, 221.

Horsfall, F. L., Jr., and Tamm, I. (1953). J. Immunol. 70, 253.

Horst, J., Content, J., Mandeles, S., Fraenkel-Conrat, H., and Duesberg, P. (1972). J. Mol. Biol. 69, 209.

Houston, L. L. (1971). Anal. Biochem. 44, 81.

Hoyle, L. (1952). J. Hyg. 50, 229.

Hoyle, L. (1969). J. Hyg. 67, 301.

Hoyle, L., and Almeida, J. D. (1971). J. Hyg. 69, 461.

Hoyle, L., and Davies, S. P. (1961). Virology 13, 53.

Hummel, B. C. W. (1959). Can. J. Biochem. Physiol. 37, 1393.

Jahiel, R. I., and Kilbourne, E. D. (1966). J. Bacteriol. 92, 1521.

Johanson, R. A., Hill, J. M., and McFadden, B. A. (1974). Biochim. Biophys. Acta 364, 341.

Joss, A., Gandhi, S. S., Hay, A. J., and Burke, D. C. (1969). J. Virol. 4, 816.

Kendal, A. P., and Eckert, E. A. (1972). Biochim. Biophys. Acta 258, 484.

Kendal, A. P., Madeley, C. R. (1970). Arch. Gesamte Virusforsch. 31, 219.

Kendal, A. P., Biddle, F., and Belyavin, G. (1968). Biochim. Biophys. Acta 165, 419.

Kendal, A. P., Apostolov, K., and Belyavin, G. (1969). J. Gen. Virol. 5, 141.

Kendal, A. P., Galphin, J. C., and Palmer, E. L. (1977). Virology 76, 186.

Kilbourne, E. D. (1963). Progr. Med. Virol. 5, 79.

Kilbourne, E. D. (1968). Science 160, 74.

Kilbourne, E. D. (1969a). Bull. WHO 41, 643.

Kilbourne, E. D. (1969b). In "Fundamental Techniques in Virology" (K. Habel and N. P. Salzman, eds.), pp. 146-160. Academic Press, New York.

Kilbourne, E. D. (1973). J. Infect. Dis. 127, 478.

Kilbourne, E. D. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 1-14. Academic Press, New York.

Kilbourne, E. D., and Murphy, J. S. (1960). J. Exp. Med. 111, 387.

Kilbourne, E. D., and Schulman, J. (1965). Trans. Assoc. Amer. Physicians 78, 323.

Kilbourne, E. D., Lief, F. S., Schulman, J. L., Jahiel, R. I., and Laver, W. G. (1967). In "Perspectives in Virology" (M. Pollard, ed.), Vol. 5, pp. 87-106. Academic Press, New York.

Kilbourne, E. D., Choppin, P. W., Schulze, I. T., Scholtissek, C., and Bucher, D. J. (1972). J. Infect. Dis. 125, 447.

Klenk, H.-D., and Choppin, P. W. (1970). Proc. Nat. Acad. Sci. U.S. 66, 57.

Klenk, H.-D., and Rott, R. (1973). J. Virol. 11, 823.

Klenk, H.-D., Compans, R. W., and Choppin, P. W. (1970). Virology 42, 1158.

Klenk, H.-D., Rott, R., and Becht, H. (1972). Virology 47, 579.

Klenk, H.-D., Rott, R., Orlich, M., and Blödorn, J. (1975). Virology 68, 426.

Knight, C. A. (1947). J. Exp. Med. 86, 125.

Laver, W. G. (1963). Virology 20, 251.

Laver, W. G. (1964). J. Mol. Biol. 9, 109.

Laver, W. G. (1971). Virology 45, 275.

Laver, W. G. (1973). Adv. Virus Res. 18, 57.

Laver, W. G., and Baker, N. (1972). J. Gen. Virol. 17, 61.

Laver, W. G., and Downie, J. C. (1976). Virology 70, 105.

Laver, W. G., and Kilbourne, E. D. (1966). Virology 30, 493.

Laver, W. G., and Valentine, R. C. (1969). Virology 38, 105.

Laver, W. G., and Webster, R. G. (1968). Virology 34, 193.

Laver, W. G., and Webster, R. G. (1972). Virology 48, 445.

Laver, W. G., and Webster, R. G. (1973). Virology 51, 383.

Laver, W. G., Downie, J. C., and Webster, R. G. (1974). Virology 59, 230.

Lazarowitz, S. G., and Choppin, P. W. (1975). Virology 68, 440.

Lazarowitz, S. G., Compans, R. W., and Choppin, P. W. (1971). Virology 46, 830.

Lazarowitz, S. G., Compans, R. W., and Choppin, P. W. (1973a). Virology 52, 199.

Lazarowitz, S. G., Goldberg, A. R., and Choppin, P. W. (1973b). Virology 56, 172.

Lazdins, I., Haslam, E. A., White, D. O. (1972). Virology 49, 758.

Lenard, J., and Compans, R. W. (1975). Virology 65, 418.

Lenard, J., Wong, C. Y., and Compans, R. W. (1974). Biochim. Biophys. Acta 332, 341.

Lennette, E. H. (1964). In "Diagnostic Procedures for Virus and Rickettsial Disease" (E. H. Lennette and N. J. Schmidt, eds.), pp. 1-66. American Public Health Assoc., New York.

Lennette, E. H., and Horsfall, F. L. (1941). J. Exp. Med. 73, 581.

Lewandowski, L. J., Content, J., and Leppla, S. H. (1971). J. Virol. 8, 701.

Lief, F. S. (1963). J. Immunol. 90, 172.

Lief, F. S., Fabiyi, A., Henle, W. (1968). J. Immunol. 80, 53.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, K. J. (1951). J. Biol. Chem. 193, 265.

Magill, T. P. (1940). Proc. Soc. Exp. Biol. Med. 45, 162.

Maizel, J. V., Jr. (1971). In "Methods in Virology" (K. Maramorosch and H. Koprowski, H., eds.), Vol. 5, pp. 179-246. Academic Press, New York.

Marchalonis, J. J., and Weltman, J. K. (1971). Comp. Biochem. Physiol. 38B, 609.

Mayron, E. W., Robert, B., Winzler, R. J., and Rafelson, M. E., Jr. (1961). Arch. Biochem. Biophys. 92, 475.

McClelland, L., and Hare, R. (1941). Can. J. Public Health 32, 530.

McGeoch, D., Fellner, P., and Newton, C. (1976). Proc. Nat. Acad. Sci. U.S. 73, 3045.

Meier-Ewert, H., Gibbs, A. J., and Dimmock, N. J. (1970). J. Gen. Virol. 6, 409.

Merrill, M. H. (1936). J. Exp. Med. 64, 19.

Mihalyi, E. (1972). "Application of Proteolytic Enzymes to Protein Structure Studies." CRC Press, Cleveland.

Moore, S. (1963). J. Biol. Chem. 238, 235.

Moore, S. (1972). In "Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium" (J. Meienhofer, ed.), pp. 629-653. Ann Arbor Science Publishers, Ann Arbor, Michigan.

Moore, S., and Stein, W. H. (1963). Methods Enzymol. 6, 819.

Mowshowitz, S., and Kilbourne, E. D. (1975). In "Negative Strand Viruses" (B. W. J. Mahy and R. D. Barry, eds.), Vol. 2, pp. 765-775. Academic Press, New York.

Murachi, T. (1970). Methods Enzymol. 19, 273.

Nermut, M. V. (1972). J. Gen. Virol. 17, 317.

Nermut, M. V., and Frank, H. (1971). J. Gen. Virol. 10, 37.

Noll, H., Aoyagi, T., and Orlando, J. (1962). Virology 18, 154.

Northrop, J. H., and Kunitz, M. (1932). J. Gen. Physiol. 16, 295.

Palese, P., and Schulman, J. (1974). Virology 57, 227.

Palese, P., and Schulman, J. L. (1976a). J. Virol. 17, 876.

Palese, P., and Schulman, J. L. (1976b). Proc. Nat. Acad. Sci. U.S. 73, 2142.

Palese, P., Tobita, K., Ueda, M., and Compans, R. W. (1974). Virology 61, 397.

Palese, P., Ritchey, M. B., Schulman, J. L., Kilbourne, E. D. (1976). Science 194, 334.

Palese, P., Ritchey, M. B., and Schulman, J. L. (1977). Virology 76, 114.

Paniker, C. K. J. (1968). J. Gen. Virol. 2, 385.

Penhoet, E., Miller, H., Doyle, M., and Blatti, S. (1971). Proc. Nat. Acad. Sci. U.S. 68, 1369.

Pereira, H. G. (1969). Progr. Med. Virol. 11, 46.

Pereira, H. G., Tumova, B., and Law, V. G. (1965). Bull. WHO 32, 855.

Pfeiffer, R., and Beck, M. (1892). Dtsch. Med. Wochenschr. 18, 465.

Pirie, A. (1935). Brit. J. Exp. Pathol. 16, 497.

Plaut, A. G., Gilbert, J. V., Artenstein, M. S., and Capra, J. D. (1975). Science 190, 1103.

Pons, M. W. (1971). Virology 46, 149.

Pons, M. W. (1976). Virology 69, 789.

Pons, M. W., and Hirst, G. K. (1968). Virology 34, 585.

Pons, M. W., and Hirst, G. K. (1969). Virology 38, 68.

Pons, M. W., Schulze, I. T., and Hirst, G. K. (1969). Virology 39, 250.

Rafelson, M. E., Schneir, M., and Wilson, V. W. (1963). Arch. Biochem. Biophys. 103, 424.

Rafelson, M. E., Gold, S., and Priede, I. (1966). Methods Enzymol. 8, 677.

Rasmussen, A. F., Jr. (1964). In "Newcastle Disease Virus: An Evolving Pathogen" (R. P. Hanson, ed.), pp. 313-325. Univ. of Wisconsin Press, Madison, Wisconsin.

Rifkin, D. B., Compans, R. W., and Reich, E. (1972). J. Biol. Chem. 247, 6432.

Ritchey, M.B., and Palese, P. (1976). Virology 72, 410.

Ritchey, M. B., Palese, P., and Schulman, J. L. (1976). J. Virol. 20, 307.

Ritchey, M. B., Palese, P., and Schulman, J. L. (1977). Virology 76, 114.

Roy, P., and Bishop, D. H. L. (1971). Biochim. Biophys. Acta 235, 191.

Salk, J. E. (1944). J. Immunol. 49, 87.

Schild, G. C. (1972). J. Gen. Virol. 15, 99.

Schild, G. C., and Dowdle, W. R. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 315-372. Academic Press, New York.

Schloer, G. M. (1971). Fed. Proc. 30, 413.

Scholtissek, C., and Klenk, H.-D. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 215-242. Academic Press, New York.

Scholtissek, C., and Rott, R. (1964). Virology 22, 169.

Schulman, J. L., and Kilbourne, E. D. (1969). Proc. Nat. Acad. Sci. U.S. 63, 326.

Schulze, I. T. (1970). Virology 42, 890.

Schulze, I. T. (1972). Virology 47, 181.

Schulze, I. T. (1973). Adv. Virus Res. 18, 1.

Schulze, I. T. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 53-82. Academic Press, New York.

Schulze, I. T., Pons, M. W., and Hirst, G. K. (1970). In "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 324-346. Academic Press, New York.

Segrest, J. P., Jackson, R. L., Andrews, E. P., and Marchesi, V. T. (1971). Biochem. Biophys. Res. Comm. 44, 390.

Seto, J. T. (1964). Biochim. Biophys. Acta 90, 420.

Shope, R. E. (1931). J. Exp. Med. 54, 373.

Simpson, R. W., and Hirst, G. K. (1961). Virology 15, 436.

Simpson, R. W., and Hirst, G. K. (1968). Virology 48, 528.

Simpson, R. J., Neuberger, M. R., and Liu, T.-Y. (1976). J. Biol. Chem. 251, 1936.

Sipos, T., and Merkel, J. R. (1970). Biochemistry 9, 2766.

Skehel, J. J. (1971a). J. Gen. Virol. 11, 103.

Skehel, J. J. (1971b). Virology 45, 793.

Skehel, J. J. (1972). Virology 49, 23.

Skehel, J. J., and Schild, G. C. (1971). Virology 44, 396.

Skehel, J. J., and Waterfield, M. D. (1975). Proc. Nat. Acad. Sci. U.S. 72, 93.

Smith, W., Andrewes, C. H., and Laidlaw, P. P. (1933). Lancet 2, 66.

Smyth, D. G. (1967). Methods Enzymol. 11, 214.

Stanley, P., and Haslam, E. A. (1971). Virology 46, 764.

Stanley, P., Gandhi, S. S., and White, D. O. (1973a). Virology 53, 92.

Stanley, P., Crook, N. E., Streader, L. G., and Davidson, B. E. (1973b). Virology 56, 640.

Stone, J. D. (1949). Aust. J. Exp. Biol. Med. Sci. 27, 229.

Stumph, W. E., Elgin, S. C. R., and Hood, L. (1974). J. Immunol. 113, 1752.

Sugg, J. Y., and Cleeland, R. (1962). J. Immunol. 88, 369.

Sugiura, A. (1975). In "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 171-213. Academic Press, New York.

Sugirua, A., and Kilbourne, E. D. (1965). Virology 26, 478.

Tamm, I., Folkers, K., and Horsfall, F. L., Jr. (1952). Yale J. Biol. Med. 24, 559.

Taylor, R. M. (1949). Am. J. Public Health 39, 171.

Taylor, J. M., Hampson, A. W., and White, D. O. (1969). Virology 39, 419.

Thompson, T. (1852). "Annals of Influenza or Epidemic Catarrhal Fever in Great Britain from 1510 to 1837." The Sydenham Society, London.

Tiffany, J. M., and Blough, H. A. (1970). Virology 41, 392.

Tumova, B., and Pereira, H. G. (1965). Virology 27, 253.

Valentine, R. C., and Isaacs, A. (1957a). J. Gen. Microbiol. 16, 195.

Valentine, R. C., and Isaacs, A. (1957b). J. Gen. Microbiol. 16, 680.

Ward, C. W., and Dopheide, T. A. A. (1976). FEBS (Fed. Eur. Biochem. Soc.) Lett. 65, 365.

Weber, K., and Osborn, M. (1969). J. Biol. Chem. 244, 4406.

Webster, R. G. (1970a). In "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 53-74. Academic Press, New York.

Webster, R. G. (1970b). Virology 40, 643.

Webster, R. G., and Darlington, R. W. (1969). J. Virol. 4, 182.

Webster, R. G., Laver, W. G., and Kilbourne, E. D. (1968). J. Gen. Virol. 3, 315.

Webster, R. G., Campbell, C. H., and Granoff, A. (1971). Virology 44, 317.

Webster, R. G., Campbell, C. H., and Granoff, A. (1973a). Virology 51, 149.

Webster, R. G., Laver, W. G., and Granoff, A. (1973b). In "Virus Research" (C. F. Fox and W. S. Robinson, eds.), pp. 513-524, Academic Press, New York.

Williams, J. B. (1969). "Statistical Analysis for Programma 101." Olivetti Underwood Corp., New York.

Wong, S. C., and Kilbourne, E. D. (1961). J. Exp. Med. 113, 95.

Wrigley, N. G., Skehel, J. J., Charlwood, P. A., and Brand, C. M. (1973). Virology 51, 525.

Wu, Y. V., and Scheraga, H. A. (1962). Biochemistry 1, 698.

Young, R. J., and Content, J. (1971). Nature (London) New Biol. 230, 140.