Davis, Judith Fitzpatrick

NEURAMINIDASE OF INFLUENZA A VIRUS: STUDIES ON IMMUNOGENICITY AND INTERACTION WITH OTHER VIRAL PROTEINS AND LIPID

City University of New York PH.D. 1981

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NEURAMINIDASE OF INFLUENZA A VIRUS: STUDIES ON IMMUNOGENICITY AND INTERACTION WITH OTHER VIRAL PROTEINS AND LIPID

by

Judith Fitzpatrick Davis

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.

1981

APPROVAL PAGE

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.

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ABSTRACT

NEURAMINIDASE OF INFLUENZA A VIRUS: STUDIES ON IMMUNOGENICITY AND INTERACTION WITH OTHER VIRAL PROTEINS AND LIPID

by

Judith Fitzpatrick Davis

Advisor: Doris Bucher, Ph.D.

Development of an affinity isolation procedure for the neuraminidase (NA) of influenza A virus (Bucher, 1977), allowed purification of neuraminidase under conditions which preserved the native conformation of the protein, as assayed by enzyme activity. The advantage of enzyme activity as a sensitive index of protein conformation allowed NA to be used as a model for determining conformational requirements for immunogenicity of membrane glycoproteins.

Highly immunogenic preparations of purified neuraminidase were prepared by aggregation of the protein into protein micelles or by association with liposomes: non-aggregated neuraminidase was shown to be only poorly immunogenic. Specific activity of purified neuraminidase varied with

conformation but did not correlate with immunogenicity.

M protein was incorporated into a discrete population of heavy density (d 1.22gm/ml) liposomes in a manner that suggested cooperative interaction of M protein during liposomal formation, i.e. preferential association of protein with liposomes already containing protein as opposed to random association of protein with lipid which would produce a heterogeneous population of lighter liposomes. This suggested that the formation of M protein domains on the inner surface of influenza virus infected cells might function to provide recognition sites for the viral glycoproteins.

Neuraminidase was only poorly incorporated into liposomes carrying a negative charge, resulting in the formation of light density liposomes and aggregates that did not appear to contain any lipid. In the presence of M protein the incorporation of neuraminidase into liposomes was enhanced; heavy density liposomes which contained both NA and M protein were formed. The high specific activity of neuraminidase-M protein liposomes suggests that neuraminidase-M protein interaction affected neuraminidase conformation.

Hemagglutinin and the HA_2 polypeptide of hemagglutinin were both shown to be incorporated into lipid in a manner that was suggestive of cooperative interaction. In the presence of M protein

a discrete population of heavy density liposomes were formed with both proteins, indicating that both HA and HA_2 could associate with M to form HA-M protein liposomes and HA_2-M protein liposomes.

Liposomal systems containing one or more influenza virus proteins appear to offer a valuable tool for investigation of the mechanisms by which virus asembly proceeds, and may well prove valuable tools in the design and development of specific antiviral agents.

ACKNOWLEDGMENTS

I am most grateful to Dr. Doris Bucher for providing me with the opportunity to study the protein chemistry of the influenza virus and for her guidance and support during this time. I would like to thank Dr. Kilbourne for his advice and support. I am most appreciative of the discussions with Dr. Howie Lee, Dr. Michael Gallagher, and Dr. Robin Oshman. I would like to extend special thanks to Dr. Anthony Garro and Dr. Terry Krulwich for professional guidance. I am very grateful for the technical assistance provided by Dr. Robert Aaronson and Dr. Laura English, Ms. Barbara Porkorny and most especially that assistance and support provided by Ms. Tania Tomko.

Many thanks go to everyone in the Microbiology Department for making my five years at Mt. Sinai most enjoyable and stimulating.

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

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LIST OF ABBREVIATIONS

AHAb	anti-hemagglutinin antibody
AMPOX	p-aminophenyl oxamic acid
ANAb	anti-neuraminidase antibody
cmc	critical micelle concentration
CTAB	cetyl trimethyl ammonium bromide
DOC	ammonium deoxycholate
DTH	delayed type hypersensitivity
EDTA	disodium ethylenediamine-tetraacetate
FANA	2-deoxy-2,3dihydro-N-trifluoroaceteyl neuraminic acid
HA	hemagglutinin
HA_1	polypeptide formed when hemagglutinin is cleaved
HA_2	polypeptide formed when hemagglutinin is cleaved
HI	hemagglutination inhibition
i.m.	intra-muscularly
i.p.	intra-peritoneally
i.v.	intra-venously
M	membrane or matrix protein
PBS	phosphate buffered saline
NA	neuraminidase
NA_{TSS}	neuraminidase purified using TSS buffer
NI	neuraminidase inhibition
NP	nucleoprotein

NS	non-structural protein
P	polymerase proteins
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
SDS-PAGE	PAGE in the presence of SDS
SFV	Semliki Forest virus
T	T cell that provides helper function
TSS	buffer, pH 7, 0.02M Tris-HCl (Trihydroxymethyl aminomethane), 0.1%SDS, 0.05% sodium azide
TSTR	buffer, pH 7.4, 0.02M Tris-HCl (Trihydroxymethyl aminomethane), 0.1% Triton X-100, 0.05% sodium azide
VCN	Vibrio cholerae neuraminidase

CHAPTER 1. INFLUENZA

Influenza

"Influenza, an acute respiratory disease associated with....fever, chills, generalized aching (particularly muscular), headache, prostration, and anorexia...results from infection and destruction of cells lining the upper respiratory tract, trachea, and bronchi...Normally, influenza is a self-limited disease lasting 3-7 days. About 10% of patients with clinical influenza have small areas of lobular pulmonary consolidation. Although extensive pneumonia rarely develops, it accounts for most deaths from influenza. Severe influenza virus pneumonias without bacterial invasion occur, but secondary bacterial pneumonias are the major cause of death."

Ginsberg, H.S. in Davis, et al.(1973), pp.1322-23.

THE DISEASE

Influenza, named from the Italian, "influenza", because it was believed to be due to the influence of the stars (Beveridge, 1977), has probably been a disease of mankind since prehistory. An epidemic that appears to have been influenza was reported in 422 B.C. by Hippocrates and Livy (Beveridge, 1977). Epidemics identified as influenza were recorded during the Middle Ages. Probable influenza pandemics occurred in 1510 and again in 1580. It was not until 1729-30 that the first well-documented epidemic occurred: in London, where it was considered a "new disease", it was reported that only 1% of the population escaped disease and that during the peak of the epidemic, a thousand died each week. In the period between 1729-30 and the twentieth century, influenza pandemics occurred with a 10-30 year periodicity (see Beveridge for review). It would appear that the epidemiology of influenza has changed with modern times. Through the Middle Ages influenza occurred in pandemics and appeared not to have been frequent; that is, various pandemics are noted as being a "new disease" (Beveridge, 1977).

Starting in the eighteenth century, influenza caused pandemics every ten to twenty years. The correlation of the rise of influenza with the rise of international trading is so

obvious as to suggest that influenza may be one of the plagues of modern life (Kilbourne, 1975).

The influenza pandemic of 1918 was one of the greatest plagues in the history of mankind, and certainly the most lethal of the influenza pandemics. World wide mortality from influenza was estimated at 20 million, the greatest loss in human history for a single event (Beveridge, 1977). In the U.S., 0.5% (550,000) of the population died (Beveridge, 1977). In some places mortality was much higher: Somoa had 25% mortality and in India 5 million died (Beveridge, 1977).

While most of the mortality during the 1918 pandemic was due to secondary bacterial pneumonia (Kilbourne, 1975), it would appear that the increased susceptibility to bacterial infection was a function of the high virulence of the strain responsible for the pandemic (H1N1, formerly designated Hsw1N1, see Appendix V for revised nomenclature). The possibility of the emergence of an influenza strain with comparable virulence gives impetus to development of an effective method of prophylaxis against influenza A viruses.

While influenza-related mortality has been greatly reduced by the use of antibiotics, it is still a factor: 80,000 excess deaths resulted from the Asian Flu pandemic of 1957-58; 33,000 excess deaths from the mildest recent pan-

demic, that of Hong Kong Flu of 1968 (Osborn, 1977). This same epidemic is estimated by Kavet to have had a direct cost of Ω3.9 billion, of which only 20% was medically related costs (Kilbourne and Fox, 1973). In the United Kingdom during an eight year period, influenza accounted for 113 million days of lost work (Kilbourne and Fox, 1973). The inability of modern medicine to control influenza has resulted in its being aptly titled "the last great unconquered plague" (Kilbourne, 1963).

Influenza has not been satisfactorily controlled by immunization, as have been polio and smallpox, because of the ability of Influenza A viruses to modify their surface antigens in such a way that every 10-20 years there emerges a subtype to which the population has no immunity (for review, see Kilbourne, 1975). This ability is called antigenic shift and results in a pandemic.

Between pandemics the influenza glycoproteins undergo gradual changes which result in the emergence of antigenic variants, i.e., variants which are antigenically related but not identical (Laver et al., 1974). This gradual change in antigenicity is termed antigenic drift (Burnet, 1955) and is responsible for the occurrence of influenza epidemics in the periods between pandemics. The effects of antigenic shift and drift on vaccines will be discussed in Chapter 3.

THE VIRUS

In 1919, J.S. Koen, a veterinarian working in Iowa, observed a new disease in hogs, and noting that its appearance coincided with the outbreaks of Spanish influenza in humans and the influenza-like disease it caused, called it "flu" (Beveridge, 1977). Following up on these observations, Richard Shope was able to demonstrate the viral nature of the disease by showing that filtered nasal washings from infected pigs were able to transmit the disease when administered intranasally (Shope, 1931). In 1933 Smith, Andrews, and Laidlaw showed the viral nature of human influenza by demonstrating that filtered nasal washings from infected humans, when administered intranasally, reproduced influenza illness in ferrets.

Further confirmation of the viral nature of influenza was obtained by Francis in 1934 when he demonstrated that virus neutralizing antibodies were formed during influenza infection. This influenza virus was subsequently designated type A when, in 1940, Francis and Magill, independently, reported the

existence of an antigenically distinct influenza, type B. In 1949, a third type, influenza C, was recognized (Taylor, 1949; Francis et al., 1950).

The study of influenza A virus was greatly facilitated by two observations: 1). the virus could replicate to high titer in the allantoic sac of embryonated chicken eggs (Burnet, 1940); 2). the virus was able to agglutinate red blood cells and this technique could be used to titer influenza virus isolates (Hirst, 1941; McClelland and Hare, 1941).

The influenza viruses comprise the family orthomyxoviridae. On the basis of cross-reacting membrane(M) and nucleoproteins (NP), they are classified into three distinct types: A, B, and C (for review, see Schild and Dowdle, 1975). All three types of influenza viruses infect man but only influenza A appears to infect other species. Influenza C generally does not appear to cause significant disease (Davis et al., 1973). Influenza B is a major agent of disease but does not produce pandemics. The ability of influenza A to cause pandemics and epidemics resides in its ability to generate new immunologic identities for its surface proteins. During this century, three distinct antigenic subtypes have appeared: H1N1 (designations prior to 1980, HswlN1, 1918; HON1, 1930; H1NI, 1947) H2N2, 1957; H3N2, 1968 (Kilbourne, 1975), H1N1, 1977/78 (MMWR,

1978). Current classification of influenza viruses is listed in Appendix V. The appearance of a pandemic strain has been associated with a change in both HA and NA subtype, although a change in HA alone has been sufficient.

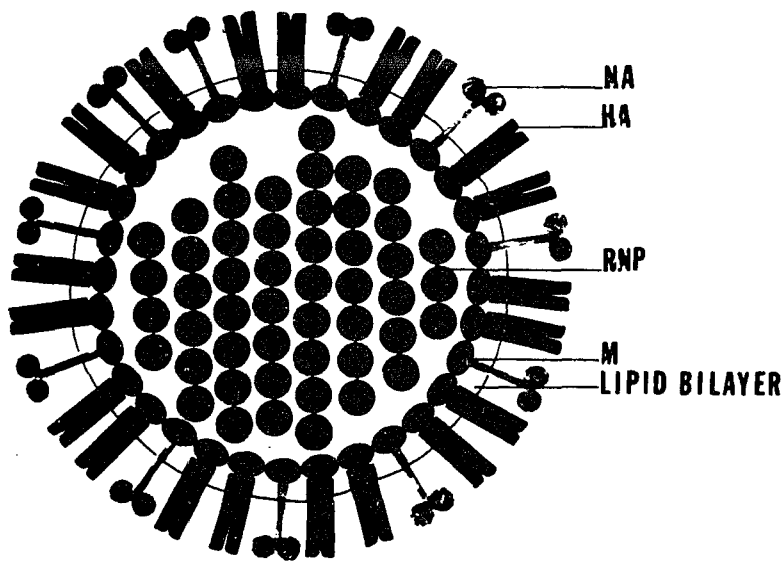
The basic organization of the influenza A virion as it is now understood from electron microscopic and biochemical studies is diagrammed in Fig. 1.1. The virion is approximately 100nm in diameter, has an outer lipid envelope into which are inserted the two viral glycoproteins, hemagglutinin(HA), and neuraminidase(NA). Internal to the envelope, forming a capsid-like structure on the internal surface of the lipid bilayer, is the M protein. The core of the virus contains the eight segments of the RNA genome in close association with the nucleoprotein(NP) and the three polymerase(P) proteins (for review, see Choppin and Compans, 1975).

The lipid membrane comprises 20-24% of the total viral mass and reflects the lipid composition of the host cell (for review, see Choppin and Compans, 1975). Approximately 75% of viral mass is protein: the three P proteins account for 1.5-2.7%; NP for 17-26%; M protein for 40% (Choppin and Compans, 1975); HA for 30% (Schulze, 1973); and NA for 2-10% (Bucher and Palese, 1975).

Viral RNA is estimated to comprise about 1 - 2% of viral weight. The genome consists of eight segments each of which has been shown to code for one of the eight viral proteins (Palese and Schulman, 1976; Ritchey et al., 1976; Palese et al., 1977). Until very recently it was believed that influenza A virus coded for only these eight proteins. Recently it has been demonstrated that two non-structural proteins (NS) are encoded by the NS gene (Inglis et al., 1979; Lamb and Choppin, 1979), and most recently it appears that the M gene may also code for two protein products (Winter and Fields, 1980).

The major emphasis of this treatise is on those proteins associated with the membrane; M, HA, and NA.

Figure 1.1. Diagrammatic representation of the influenza A virion. HA, hemagglutinin; NA, neuraminidase; M, M protein; RNP, ribonucleoprotein.


NA
HA
RNP
M
LIPID BILAYER

CHAPTER 2. HEMAGGLUTININ

Hemagglutinin is the major antigenic component of the influenza A virion. Antibodies to the HA alone are responsible for viral neutralization (Drzenik et al., 1966; Laver and Kilbourne, 1966). During antigenic drift, one or more of the antigenic determinants of the HA may change. This change can result in decreased antibody avidity or complete loss of cross-reactivity (Laver, 1974; Laver et al., 1979, a and b; Webster et al., 1979 b). When antigenic shift occurs the HA is replaced by a second HA with novel antigenic determinants; as result the population then has little or no viral neutralizing antibody to protect them against this virus bearing a novel hemagglutinin (for review, see Schulman, 1975).

MORPHOLOGY

The HA appears as a rod of ~120Å on the surface of the influenza virion (Oxford, 1981). When the viral membrane is disrupted by detergents, monomeric HA rods of ~140Å can be isolated which have the ability to attach to red blood cells, but not to agglutinate them. Removal of detergent results in aggregation of the HA by their hydrophobic bases into rosettes containing about 5 molecules, and restoration of hemagglutinating activity, i.e. hemagglutination is a function of a multivalent structure (Laver and Valentine, 1969; Webster and Darlington, 1969).

MOLECULAR STRUCTURE

The HA rod is composed of 3 HA molecules of ~80,000 daltons (Schulze, 1975). Approximately 19% of the molecular weight is contributed by carbohydrate (Ward and Dopheide, 1980; Verhoeyen et al., 1980). Most of the oligosaccharide units are located proximal to the membrane (Wilson et al., 1981). These oligosaccharide units do not contain sialic

acid, this moiety is apparently removed by the viral neuraminidase (Haukenes et al., 1966; Laver and Webster, 1966; Klenk et al, 1970).

Upon reduction, the 80,000 dalton HA yields two subunits, HA_1 and HA_2 of 58,000 and 26,000 daltons respectively (Laver, 1971; Skehel, 1972; Wilson et al., 1981). The HA_1 subunit contains the amino terminus; the HA_2 contains the hydrophobic carboxy terminus (Skehel and Waterfield, 1975).

The HA is translated on the rough endoplasmic reticulum of infected cells as a single polypeptide of 80,000 daltons (Waterfield et al., 1979 McCauley et al., 1980). Sequestration of the protein into the rough endoplasmic reticulum appears to follow the signal hypothesis for membrane proteins (Milstein et al., 1972; Blobel and Doberstein, 1975). Within the endoplasmic reticulum and Golgi apparatus the HA is glycosylated by host glycosidases (Schwarz et al., 1977; Lohmeyer and Klenk, 1979), and cleaved by proteases that remove an arginine containing peptide (Verhoeyen et al., 1980; Ward and Dopheide, 1980).

The sequence of many HA strains is now known (Porter et al., 1979; Ward and Dopheide, 1980; Dopheide and Ward, 1980; Sleigh et al., 1980; Gething et al., 1980; Min Jou et al., 1980; Verhoeyen et al., 1980; Threlfall et al., 1980). Pri-

mary sequence data has been correlated with data from monoclonal antibody studies to identify and characterize antibody binding sites (Laver et al, 1979; Webster et al., 1979; Both et al., 1980; Moss et al., 1980).

Most recently X-ray crystallographic study of the bromelain derived HA of A/Hong Kong/1/68 by Wilson, Skehel, and Wiley (1981) and identification of the antibody binding sites by Wiley, Wilson, and Skehel (1981), have greatly illuminated understanding of this molecule. These studies demonstrate the dramatic change in conformation of the molecule following cleavage to HA_1 and HA_2. Although originally synthesized as a single polypeptide with the C terminus of HA_1 leading into the amino terminus of HA_2, cleavage results in the carboxyl terminus of HA_1 and the amino terminus of HA_2 being distant from one another in the processed molecule. The HA_1 subunit was shown to carry six of the seven carbohydrate chains as well as the four major antigenic sites, as determined by analysis with monoclonal antibodies. Three sites are sufficiently close to the pocket which has been tentatively identified as the sialic acid binding site to suggest that binding of antibodies at these sites would sterically block the active site. As little as a single amino acid change in these antibody binding sites has been sufficient to cause a significant antigenic change (Both et al., 1980; Moss et al. 1980).

The highly conserved nature of the amino acids flanking the antigenic sites suggests that the conformation of the molecule is conserved during mutation and that changes in the antigenic sites do not affect conformation of the molecule.

FUNCTION

The HA serves two known functions: 1). attachment of the virion to the cell surface; and 2). penetration. The virus attaches to the cell surface via terminal sialic acid residues on the cell surface (for review, see Schulze, 1975). The ability of HA to bind to terminal sialic acid residues is the basis of the hemagglutination test in which viruses are titered by their ability to agglutinate red blood cells (Hirst, 1941, 1942 a,b). The sialic acid binding site has been tentatively identified by Wilson et al.(1981) as a pocket on the external surface of the HA_1 molecule.

It has been postulated that the HA functions in some later step in viral replication. Virions that had only uncleaved HA were not infectious though they possessed the ability to attach to cells: cleavage of the HA restored

infectivity (Lazarowitz and Choppin, 1975; Klenk et al, 1977). Recent evidence elucidates the role of HA_2 in penetration: the amino terminus of HA_2 is highly conserved and hydrophobic (Klenk, 1980). Liposomes that carry HA_2 have been shown to fuse with cellular surfaces (Rott, 1980). Further, it has been shown that tri-peptides with the same or similar sequence as the amino terminus of HA_2 could specifically block infection by influenza virus without blocking attachment (Richardson et al., 1980) suggesting the presence of a specific receptor on the cell surface for the amino terminus of the HA_2 molecule. These workers hypothesize that following attachment of the influenza virion to the cell surface, the HA_2 amino terminus is brought into contact with receptors on the cell surface which are capable of initiating penetration. The mechanism by which influenza virus penetrates the cell remains unclear. Rott (1980) has presented evidence that HA-NA bearing liposomes will fuse with cells. Oxford et al., (1981) did not observe fusion of HA-NA liposomes with cells but rather report entry of liposomes into cells by viropexis.

Helenius and colleagues (1980) have reported that although the observed route of entry for SFV is endocytosis, fusion of SFV with cellular membranes can be effected by lowering the pH. Huang and associates (1981) have reported that influenza A virus will fuse with cells when the pH is lowered.

Helenius et al. (1980) have demonstrated that low pH induced fusion is inhibited by lysosomotropic agents, known to increase lysosomal pH. Since these agents all inhibited an early step in SFV infectivity, they have suggested that a possible route of entry for SFV is via fusion of virus with the intracellular membrane of an intracellular vacuole with sufficiently low pH.

CHAPTER 3. NEURAMINIDASE

INTRODUCTION

Neuraminidase is the minor antigenic surface component of the influenza A virus. Neuraminidase is subject to antigenic drift as is hemagglutinin. During antigenic drift one or more of the antigenic determinants of NA may change (Paniker, 1968; Schulman and Kilbourne, 1969). When antigenic shift occurs, the current NA subtype may be replaced by one sharing no antigenic determinants (Paniker, 1968). However, this is not a requirement for emergence of a pandemic strain. There are nine neuraminidase subtypes, N1 - N9 (see Appendix V). Only N1 and N2 subtypes have been associated with human strains of influenza A virus during this century.

The NA activity of influenza virus was first reported by Hirst (1942), who observed disagglutination of red blood cells that had been agglutinated by influenza. Noting that the eluted virus could agglutinate fresh red blood cells but not those from which it had eluted, he reasoned that a viral enzyme had permanently modified the surface of the red blood cell. Only much later was it demonstrated that the hemagglutinating and enzymatic activities resided on separate proteins (Mayron et al., 1961; Noll et al., 1962; Laver, 1963). Absolute proof that the activities were on separate proteins came in 1966 when Laver and Kilbourne demonstrated by genetic recombination that the HA and NA segregated independently, and that there were antigenically and biochemically distinct neuraminidases.

NEURAMINIDASE CONTENT OF THE VIRION

NA is estimated to comprise 2 - 10% of the viral protein (Skehel and Schild, 1971; Laver and Kilbourne, 1966; Laver, 1963). However, the amount of NA in the virion has been found to vary widely depending on the virus strain and the host cell

(Laver, 1963; Laver and Kilbourne, 1966; Kilbourne et al., 1967; Webster et al., 1968; Palese and Schulman, 1974; Mowshowitz and Kilbourne, 1975). By electronmicroscopic methods, Wrigley (1979) estimated an HA:NA ratio of 5:1. Calculating 700-900 glycoprotein spikes/virion, he calculated 140-180 neuraminidase spikes per virion.

MORPHOLOGY

The NA appears as a spike of ~120Å on the surface of the virion (Almeida and Hoyle, 1972; Laver and Valentine, 1969). When isolated by SDS disruption procedures, monomeric spikes of 160Å were observed (Laver and Valentine, 1969). These possessed a tetrameric head of 80 x 80 x 50Å attached to a 100Å stalk which terminated in a 40Å knob. When SDS was removed the NA molecules aggregated by the tails to form seeding dandelions or cartwheels (Laver and Valentine, 1969; Wrigley et al., 1973; Wrigley, 1979).

The NA of some strains can be isolated by proteolysis, using trypsin or pronase. Proteolytically derived NA possesses enzymatic activity but is unable to aggregate. This

suggests that the segment lost by proteolysis serves to anchor the protein in the membrane (Lazdins, 1972; Wrigley et al., 1973). The enzymatically active, proteolytically derived NA of B/Lee was seen to consist of a tetrameric head of 80 x 80 x 40Å (Wrigley et al., 1973).

STRUCTURE OF THE NEURAMINIDASE MOLECULE

The isolated NA macromolecule has a weight of 200,000 - 250,000 daltons: upon reduction the macromolecule dissociates into four polypeptides with molecular weights of 50,000 to 60,000 daltons (Webster, 1970,a,b; Skehel and Schild, 1971; Bucher and Kilbourne, 1972; Kendal and Eckert, 1972; Lazdins et al., 1972; Gregoriades, 1972). The NA tetramer is the only active form of the enzyme (Kendal and Eckert, 1972; Bucher and Kilbourne, 1972; Lazdins et al., 1972).

In the presence of SDS it is possible to dissociate the NA spike to dimers: in the presence of SDS and reducing agent the spike can be dissociated to monomers (Webster, 1970 a,b; Kendal and Eckert, 1972; Bucher and Kilbourne, 1972). Hence, it is proposed that the two monomers are held together by

disulphide bonds to form a dimer and that dimer-dimer interaction is probably based on hydrophobic interactions. Dissociation to the monomer is not necessarily irreversible, as reassociation to the active tetrameric form can occur (Bucher and Kilbourne, 1972).

Several investigators have reported the existence of two species of NA monomers (Webster, 1970 a; Skehel and Schild, 1971; Bucher and Kilbourne, 1972; Lazdins et al., 1972). Evidence for two equivalent NA subunits comes mainly from studies of proteolytically derived NA (Kendal and Eckert, 1972; Wrigley et al., 1973; Groome et al., 1977). However Gregoriades (1972) found evidence for only one NA subunit using non-proteolytically derived N1: based on predicted amino acid sequence from nucleotide sequencing of an N1 gene, Fields et al. (1981) predict only one type of subunit (see below).

Tryptic maps of proteolytically derived (^{14}C) carboxymethylated NA of A/RI/5+/57(H2N2), the N2 of X-7 (Kendal and Kiley, 1973, 1975) indicated the presence of only one NA. A repeat of this experiment using detergent purified NA (Kendal and Bucher, in Bucher and Palese, 1975) reported no difference in the peptide maps of NA isolated by either technique; both tryptic maps showed twenty spots. X-7 NA is reported to have 14 cysteines (Laver and Baker, 1972) and 21 cysteines (Kendal

and Kiley, 1973; Eckert, 1972) based on a molecular weight of 54,000. The predicted amino acid sequence of N1 (Field et al., 1981) shows the presence of 19 cysteines. (If labelled as above for tryptic maps proteolytically derived N1 would be predicted to yield about 9 (^{14}C) labelled tryptic fragments.)

If the peptide map data (20 spots) is correlated with the predicted amino termini sequences of N2 neuraminidases (Blok and Air, 1981) there would be at least two cysteines lost in the proteolytically derived NA. Proteolytically derived NA, therefore should not yield the same number of peptides as detergent derived NA unless the portions of the molecule containing the proteolytically derived fragment never appear on maps. In this case there is reason to believe that there are too many fragments on the peptide maps to be accounted for by only one NA, unless non-specific cleavage of NA occurred during proteolysis. Considering the recent evidence that the NS and M genes code for more than one protein (Inglis et al., 1979; Lamb and Choppin, 1979; Winter and Fields, 1980), it appears possible that the structure of NA may be found to be more complex than a simple tetramer comprised of four homologous subunits.

A possible explanation of the question of one or two NA polypeptides could be that the 50,000 and 34,000 dalton

proteins are both synthesized and form a dimer as proposed by Bucher and Kilbourne (1972) with the 50,000 dalton polypeptide providing lipid anchorage.

NUCLEOTIDE SEQUENCE

The nucleotide sequence of an N1 (the NA of A/PR/8/34) is complete (Field et al., 1981) and the sequencing of several other N1 and N2 strains has progressed into the amino terminus a significant distance (Blok and Air, 1980 a,b). Unfortunately this data has not been as helpful in elucidating primary NA structure as had been hoped, since no direct protein sequence information exists for NA.

The nucleotide sequencing of N1 has revealed the following (Field et al., 1981): the NA gene is 1423 nucleotides long and is predicted to code for a protein of 454 amino acids, molecular weight, 50,087 daltons excluding carbohydrate. There are five potential glycosylation sites (asn-X-ser/thr) which would predict about 20% carbohydrate. There is only one reading frame. However, the authors identify a second initiation site at nucleotide position 450. Translation

from the second initiation site would yield a protein of 34,000 daltons. The existence of the 34,000 dalton protein would present conceptual problems, as the authors postulate that the predicted NA molecule appears to have only one hydrophobic region which could be used for membrane insertion and this is at the amino terminus, a portion not translated in the 34,000 dalton protein. Another feature of the hypothetical 34,000 dalton protein is that it would contain only one carbohydrate attachment, as the other four sites are on the amino terminus.

One of the most interesting features of the inferred amino acid sequence is that the amino terminus of the NA appears to function as the membrane insertion site; that is, the leader sequence may not be removed but could remain to function in anchoring the molecule in the lipid bilayer. The usual synthetic pathway for membrane glycoproteins involves "anchoring" the newly synthesized glycoprotein in the membrane via the C-terminal hydrophobic region. However, the authors cite two intestinal enzymes, intestinal brush border isomaltase and amino peptidase which have the amino-terminus embedded in the lipid bilayer.

CHARACTERISTICS OF THE ENZYME

ENZYME SPECIFICITY

Influenza virus neuraminidase derives its name from its observed ability to hydrolytically cleave the glycosidic bond joining the keto group of a terminal N-acetyl neuraminic acid to D-galactose or D-galactosamine (Gottschalk, 1957; Blix et al., 1957). The viral NA will only cleave neuraminic acids with a free carboxyl group (Meindl and Tuppy, 1966a) and only the α linkage (Meindle and Tuppy, 1966 a,b). (The generic name for substituted neuraminic acids is sialic acid, hence NA is sometimes referred to as a sialidase.) While bacterial neuraminidases act on most α-ketosidic linkages (α, 2--3) (α, 2--4) (α, 2--6) and (α, 2--8), influenza NA preferentially cleaves α, 2--3 linkages (Drzeniek, 1967, 1972, 1973).

The Michaelis constants for various influenza neuraminidases have been published by Gottschalk and Drzeniek (1972). These values vary from a K_m of 1×10^{-3} M for PR8(N1) to 6.5×10^{-4} for X-7(N2) (Sedmak and Grossberg, 1973) The N2 neuraminidases generally show about five-fold greater substrate affinity than the N1 strains (for review, see Bucher and Palese, 1975).

PRODUCT INHIBITION

Neuraminidase is inhibited by its product N-acetyl neuraminic acid. However, the concentrations required are relatively high. Two N2 strains were found to have K_i's of 5 x 10^{-4}M and 4 x 10^{-4} (Walop et al., 1960; Rafelson et al., 1963 b). A/PR/8/34 (N1) had a K_i of 2 x 10^{-2}(Rafelson, 1963 b). The K_i's indicate that feedback inhibition of neuraminidase should not be a significant factor in the NA assay of Warren and Aminoff (Aminoff 1959,1961; Warren 1959,1963) where the maximal detectable concentrations of N-acetyl neuraminic acid are between 1 x 10^{-8}M and 3 x 10^{-8}M.

INHIBITORS OF NEURAMINIDASE ACTIVITY

A variety of substances will inhibit NA activity both specifically and nonspecifically (see Bucher and Palese, 1975 for review). Only those inhibitors relevant to this work will be presented here. It should be noted that the neuraminidase inhibitors discussed below do not inhibit hemagglutinin.

Antibody to the NA will inhibit NA activity and is the basis of the neuraminidase inhibition test for titering neuraminidase inhibiting antibodies in sera. This will be discussed under tests for enzymatic activity.

Based on Rafelson's report (1963) that N-acetyl neuraminic acid (with the relatively low K_i of 2 x x10^{-2}M to 5 x 10^{-3}M) was the most potent known inhibitor of influenza neuraminidase, Edmond et al., (1966) investigated the ability of structurally related compounds to inhibit NA activity. An investigation of substituted oxamic acids and phenyl glyoxal compounds revealed that a free carboxyl and an unsubstituted nitrogen were requirements: N-(p-aminophenyl) oxamic acid was the most potent inhibitor tested by their method.

Cuatrecasas and Illiano (1971) subsequently developed an affinity isolation technique for neuraminidases using N-(p-aminophenyl) oxamic acid coupled to agarose 4B columns by a glycyl-glycyl-tyrosine leash. This method was subsequently refined by Bucher (1977) and used to purify the neuraminidase of influenza A (See Appendix I for structures and details of affinity chromatography).

It should be noted here that it is not entirely clear that the affinity column retains NA through specific interaction of NA with N-(p-aminophenyl) oxamic acid; i.e. it may be that the column is not an "affinity" column but selectively retains NA though other interactions of the NA with the column. However, in support of the affinity theory is the observation that inactivated NA is not retained on the column (Bucher, 1977).

The most potent NA inhibitor is FANA (2-deoxy-2, 3 dihydro-N- trifluoroacetyl neuraminic acid), a derivative of deoxyneuraminic acid, developed by Meindl et al. (1974). With a K_i of 7.9×10^{-7} for A/Mel/35 (H1N1), it shows a 1000-fold higher affinity for neuraminidase than any known NA substrate. FANA has been shown to inhibit virus replication in tissue culture (Palese et al., 1974; Schulman and Palese, 1975), presumably by affecting virus release, as Palese et al. (1974) have reported that FANA does not affect penetration.

ENZYME STABILITY

The N2 neuraminidases have been found to exhibit greater stability than the N1 enzymes to three factors; heat, the Ca^{++} ion requirement, and detergent.

Neuraminidase is heat labile: the extent is strain dependent. The NA of A/PR/8/34 (N1) is inactivated by 30 minutes at $56^{o}C$, while N2 retained 67% of activity under these conditions (Rafelson et al., 1963a). Paniker (1968) reported that N2 strains retained full activity after one hour at $45^{o}C$, while N1 strains (NWS and WSE) both lost activity during this period.

A requirement for Ca^{++} for enzyme activity has been established. The calcium ion dependency is strain dependent. The N1 strains are more sensitive to Ca^{++} ion depletion than the N2 strains and require higher Ca^{++} ion levels to to maintain activity (Wilson and Rafelson, 1967). It has been shown that calcium ion depletion (by the addition of EDTA) increased heat sensitivity of both N1 and N2 strains (Boschman and Jacobs, 1965; Dimmock, 1971).

The neuraminidases of the N1 strains are inactivated by SDS (Gregoriades, 1972; Laver and Baker, 1972). The N2 strain enzymes appear to be relatively insensitive to SDS and have been isolated with activity using SDS extraction procedures (Laver, 1963; Rott et al., 1970; Bucher and Kilbourne 1972). Low levels of non-ionic detergents do not appear to adversely affect either N1 or N2 activity (Gregoriades, 1972; Bucher, 1977; Gallagher, personal communication).

THE NEURAMINIDASE ASSAY

The standard assay used for quantitating NA activity is that developed by Warren (1959, 1963) as modified by Aminoff (1959, 1961). This assay measures the amount of neuraminic acid liberated by neuraminidase from a substrate such as fetuin or sialyllactose; liberated N-acetyl neuraminic acid is cleaved by periodate to yield β-formylpyruvic acid which then reacts with 2-thiobarbituric acid to yield a chromophore with maximal absorbance at 549nm. The procedure as it is performed in this laboratory is described in detail in Appendix II.

THE NEURAMINIDASE INHIBITION (NI) ASSAY

Antibodies to the neuraminidase have been detected by several techniques: inhibition of neuraminidase enzyme activity (NI) (Fazekas de St. Groth, 1962, 1963; Aymard-Henry et al., 1973); immunoprecipitation (Easterday et al., 1969; Schild and Pereira, 1969; Palese et al., 1973); reduction in plaque size (Jahiel and Kilbourne, 1966); and the mouse titration test (Schulman et al., 1968).

The NI test measures inhibition of neuraminidase activity in the Warren-Aminoff assay using fetuin as substrate, as developed by Fazekas de St. Groth (1962, 1963). Rafelson et al. (1963 a,b) showed that inhibition of activity by antibody was substrate concentration independent and that antibody appeared to inhibit activity by sterically blocking the enzyme site. Antibody inhibition was observed with large substrates such as fetuin (48,000 daltons), but there was little or no inhibition with small substrates such as sialyllactose.

Details of the NI Assay used in this paper are included in Appendix II. Briefly, antisera (a.s.) to be titered are appropriately diluted and incubated with a standardized dilution of virus overnight at $37^{o}C$: controls are the standardized virus dilution with no added antisera, and antisera with no virus. The % NA activity present at any antisera dilution is calculated from the formula.

$$\% \text{ NA Activity} = O.D._{549} \text{ of sample} / O.D._{549} \text{ of virus control}$$

The % activity of various dilutions of the same antiserum are plotted on semi-log paper and the inverse of the dilution at which 50% inhibition of activity is predicted is recorded as the NI titer of that serum.

FUNCTIONS OF THE INFLUENZA NEURAMINIDASE

The neuraminidase of influenza evidently serves several functions, in addition to the "possible physiological role of removing neuraminic acid from mucins, which are hemagglutination inhibitors" (Bucher and Palese, 1975).

It has been demonstrated that removal of sialic acid from the viral glycoproteins during viral synthesis facilitates proteolytic cleavage of the HA presumably by exposing the proteolytic site (Schulman and Palese, 1977; Nakajima and Suguira, 1980). Proteolytic cleavage of the HA is essential for infectivity as discussed in Chapter 2.

Neuraminidase appears to facilitate viral release from infected cells (Kilbourne et al., 1968) by preventing self-aggregation of the virus via viral HA attaching to sialic acid residues on neighboring virions and by preventing attachment of virus to sialic acid residues of the host cell membrane. Evidence for this function comes from study (Palese et al., 1974; Palese and Compans, 1975) of a temperature sensitive (ts) NA mutant which at the non-permissive temperatures results in newly synthesized virus aggregating at the cell surface of infected cells: treatment with Vibrio cholerae neuraminidase allowed disaggregation of the virus. Viral aggregation was

also seen if the NA mutant was grown at the permissive temperature but in the presence of FANA, a potent influenza neuraminidase inhibitor.

Recent evidence from Rott's (1980) experiments with liposomes carrying viral proteins suggests that NA may be required for viral penetration. He observed that liposomes carrying only HA would attach to but not fuse with cell membranes, but that liposomes carrying both HA and NA would both attach to and fuse with cell membranes. In these experiments HA liposomes could be shown to fuse with cells if Vibrio cholerae neuraminidase was substituted for influenza NA. It has been reported that FANA did not affect infectivity (Palese et al., 1974; Schulman and Palese, 1975). It has also been demonstrated that anti-neuraminidase antibodies do not affect penetration (Seto and Rott, 1966; Jahiel and Kilbourne, 1966; Webster and Laver, 1967; Kilbourne et al., 1968).

A hypothetical role for NA in the fusion observed by Rott (1981), is that release of HA from sialic acid receptors on the periphery of the cell would allow the HA to reattach to receptors that are closer to the lipid bilayer. This could function to bring the HA_2 amino terminus closer to its receptor which is presumably on the cell membrane.

PURIFICATION OF NEURAMINIDASE

Neuraminidase has been purified from influenza virus using a variety of separation techniques. The choice of separation technique depends on the purpose for which NA is to be used and the degree of purity required. Generally it can be said that harsh extraction techniques are less complex procedurally and have better yields but do not result in retention of biological activities of the protein (Tanford and Reynolds, 1976).

Purification of neuraminidase involves two steps: 1). solubilization of the NA and then; 2). separation of the NA from other viral proteins by electrophoretic, centrifugal, or chromatographic techniques. There are two methods for solubilization of the NA: proteolytic treatment of the virion as first developed by Noll and colleagues in 1962; and detergent solubilization of the virus as first used by Laver in 1963.

SOLUBILIZATION OF NA BY PROTEOLYTIC ENZYMES.

Noll et al. (1962) trypsin treated influenza virus which solubilized the NA activity and then purified NA by gradient centrifugation. Kendal and Kiley (1975) reported 50-100% NA

recovery following proteolysis using nagarse. Proteolysis using trypsin, nagarse, or pronase followed by purification by chromatographic or centrifugation techniques has been used for many studies of neuraminidase structure (Kendal and Eckert, 1971; Rott et al., 1972; Lazdins et al., 1972; Wrigley et al., 1973; Kendal and Kiley, 1975; Allen et al., 1977).

As discussed earlier, proteolytically derived NA is enzymatically active but is incapable of aggregation, having lost its hydrophobic membrane insertion site. It exists as a tetramer with each subunit weighing from 30,000 to 50,000 daltons. In addition a substantial amount of carbohydrate has been lost .

Attachment of carbohydrate to the region of the stalk proximal to the membrane has been further supported by sequence data (Field et al., 1981) which shows four of the five potential carbohydrate attachment sites in that portion of the predicted molecule which is the purported membrane insertion site.

SOLUBILIZATION OF NEURAMINIDASE BY DETERGENTS

Laver (1963) separated NA from N2 strains of influenza using SDS disruption of the virus and SDS electrophoresis on

cellulose acetate. This procedure isolates an intact enzymatically active neuraminidase which is capable of forming aggregates when detergent is removed (Laver and Valentine, 1969). The N1 neuraminidases are SDS sensitive (Gregoriades, 1972; Laver and Baker, 1972) and show loss of activity on exposure to SDS.

To isolate the N1 neuraminidase of NWS, Gregoriades (1973) used solubilization by 1% nonidet P-40 in the presence of 0.5M urea: this procedure resulted in 60-80% loss of activity. NA was purified from solubilized virus by glycerol gradient centrifugation followed by chromatography on DEAE cellulose; 1% nonidet P-40 concentration was maintained throughout to prevent reaggregation of viral proteins. NA eluted from the DEAE cellulose column with an apparent molecular weight of 58,000 as determined by SDS polyacrylamide gel electrophoresis.

Bucher and Kilbourne (1972) characterized the NA of X-7 using SDS solubilization. Following solubilization of virus with 2% SDS at 37^{o}C in the presence of reducing agent, the sample was chromatographed on Bio-Gel A-5 in the presence of low concentrations of SDS and reducing agent. NA eluted with a molecular weight of 53,000 in the presence of reducing agent. On polyacrylamide gels, however, the neuraminidase

which eluted at 53,000, migrated with an apparent molecular weight of 240,000 daltons indicating reaggregation. When the 240,000 component was reduced in the presence of SDS and re-electrophoresed,two components of 66,000 and 58,000 in a 1:1 ratio were seen. Only the tetrameric form of NA was active.

Bucher (1977) solubilized the influenza strains X-7 (H1N1), Aichi (H3N2), and X-38(H7N2) using a combination of ionic and non-ionic detergents. Virus was disrupted with 1% SDS for 15 minutes at 37^{o}C followed by solubilization in Triton X-100. The virus solution was dialyzed at 4^{o}C overnight and chromatographed on an NA affinity column N-(p-aminophenyl) oxamic acid (Cuatrecasas and Illiano, 1971) as described in Appendix I. Using this technique, 1.6% of viral protein was recovered and this accounted for 123% of activity. The specific activity of neuraminidase purified by affinity chromatography was 189 nM NANA/min/mg, a 77-fold increase over the specific activity of the solubilized virus.

ANTIBODY TO NEURAMINIDASE

Neuraminidase is aptly described as the minor antigenic component of the virus for the following reasons:

1. There is three to thirty - fold less NA present on the virion than HA (for review see Schulze, 1975; Bucher and Palese, 1975).

2. There is now considerable evidence to suggest that in the human response to influenza A virus there is antigenic competition between the HA and NA antigens and that NA is the weaker antigen in the primed population (Kilbourne, 1976; Kendal et al., 1977).

3. Antibodies to neuraminidase (ANAb) are not neutralizing, i.e. antibody to NA does not affect infectivity of the virus (Seto and Rott, 1966; Jahiel and Kilbourne, 1966; Webster and Laver, 1967; Kilbourne et al., 1968). Nor does ANAb generally prevent hemagglutination of red blood cells by virus (Brown and Laver, 1968), though at high titers HI has been observed (Kilbourne, 1968; Schulman and Kilbourne, 1969).

Antibody to neuraminidase has been shown to inhibit NA activity by sterically blocking the enzyme site (Fazekas de St. Groth, 1962, 1963; Rafelson et al., 1963 b). This is the basis of the NI test discussed earlier.

Antibody to NA has been shown to prevent release of virions and in the presence of anti-neuraminidase antibody, newly synthesized virus is seen aggregated on the surface of infected cells (Seto and Rott, 1966; Webster and Laver, 1967; Kilbourne et al., 1968; Compans et al., 1969).

It is not clear whether anti-neuraminidase antibody prevents release of virus by inhibition of enzyme activity, which has been shown to cause aggregation (Palese et al., 1975) or by bridging of the virions by antibody.

The use of monovalent antibodies to answer this question has not given decisive answers. Becht et al (1971) found that monovalent anti-neuraminidase antibodies inhibited enzyme activity but did not block release of virus during a single replicative cycle. Kilbourne et al. (1974) found reduction of plaque size in the plaque reduction assay which measures inhibition over a multicycle period and hence could be expected to be more sensitive. It is probable that anti- neuraminidase antibodies inhibit viral release by virtue of the fact that both inhibition of enzyme activity and bridging of virions by bivalent antibody contribute to viral aggregation.

IMMUNOGENICITY OF NEURAMINIDASE

Reports of the immunogenicity of purified NA are difficult to evaluate because criteria for immunogenicity (i.e. absolute quantity of antigen, specific activity, and use of adjuvants) have not been established.

Kendal and Kiley (1975) report using proteolytically purified NA from several strains as antigen and reported a good NI response when 50-100 µg of purified NA was injected in complete Freunds adjuvant. Webster and Laver (1967) reported that the NA purified from X-7 virus by electrophoresis was poorly immunogenic; a subsequent report found the X-7 NA immunogenic, however, the amount of NA used for immunization was not reported (Kilbourne et al., 1968).

Laver and Webster (1976) separated the hemagglutinin and neuraminidase from other viral components of A/Port Chalmers/1/73(H3N2) by ammonium deoxycholate (DOC) disruption and electrophoretic separation and reported that the subunits were as effective as equivalent amounts of whole inactivated virus when injected into rabbits in saline; however, they were not immunogenic in hamsters. The quantities used are difficult to evaluate since they calculated the dose by hemagglutinating units (see Appendix III) which are not directly proportional

to protein concentration. Using single radial diffusion, they calculated that equivalent amounts of the subunit preparation contained about 70% of the HA and NA of the intact virus. Only at a dose of 16,000 HAU units was a positive response observed to the NA antigen in either the subunit vaccine or intact virus. Bucher (personal communication) has calculated that 20,000 HAU units contain roughly 1 mg of protein (X-7 virus strain). Using this estimate to calculate protein, suggests that Laver and Webster (1976) found an NI response only at the level of 0.8mg of intact virus. As X-7 is estimated to be 10% NA, this would mean that 80 ug of NA in whole virus or 50-60 ug of NA in the subunit preparation was the threshhold for response. This is a relatively large antigen dose.

Desselberger (1977) reported the results of experiments to determine if method of preparation of an antigen could affect the ability of antibody to establish antigenic relationships between neuraminidases of influenza. He found that virus preparation procedures influenced both the quantity and quality of the antibody formed. Purified NA was not used.

ENHANCEMENT OF THE IMMUNE RESPONSE BY NEURAMINIDASE

There have been several reports indicating that neuraminidase is capable of enhancing the immune response. Vibrio cholerae neuraminidase (VCN) treatment has been shown to:

1. Increase the immunogenicity of a variety of tumor cells: tumors were excised, VCN treated, and used to vaccinate their recipients (Sanford, 1967; Currie and Bagshawe, 1969; Bekesi et al., 1971; Simmons et al., 1971; Sedlacek et al., 1975). Results were similar when VCN was injected simultaneously with tumors.

2. Stimulate phagocytosis by macrophages (Weiss et al., 1966; Lee, 1968; Knop et al., 1977).

3. Increase cell mediated and antibody mediated cytotoxicity (Kedar et al., 1973).

4. Enhance the in vitro lymphocyte reaction (Han, 1973).

5. Increase antibody and plaque forming cell responses to various antigens if they were administered with VCN intramuscularly, or intraperitoneally, but not when administered intravenously (Knop et al., 1978).

6. Reduce the induction of tolerance by high dose levels of unaggregated bovine serum albumin (Knop et al., 1978).

The mechanisms for neuraminidase enhancement of the immune system are not known. As NA activity is necessary for the effect, the enhancement is presumed to be due to enzymatic alteration of the antigenic surface, or the cellular surfaces of immune cells which in turn is postulated to alter either immune recognition or processing.

It has been shown that sugars play a significant role in physiological recognition processes. Gesner and Ginsberg (1964) showed that glycosidase treated lymphocytes when reintroduced to the circulation appeared to be preferentially taken up by the liver rather than lymph nodes, which is where non-glycosidase treated lymphocytes appear after reintroduction to the circulation. Ashwell and Morell (1974) have reported that removal of serum glycoproteins from circulation by reticulo-endothelial cells of the liver is a function of specific recognition of galactose residues exposed when sialic acid is removed. Aminoff et al. (1977) have demonstrated that removal of sialic acid from the surface of red blood cells results in their removal from circulation by reticulo-endothelial cells of the liver and spleen. The authors suggest that recognition of sialidase treated red blood cells may be a

function of specific recognition of now terminal galactose residues exposed upon removal of sialic acid.

ROLE OF ANTI-NEURAMINIDASE ANTIBODIES IN INFECTION

It has been demonstrated (Schulman and Kilbourne, 1969) that anti-neuraminidase antibodies (ANAb) can alter the course of infection with influenza A virus in the mouse: lower viral titers and less extensive lung damage were observed in the lungs of mice having ANAb, but no relevant AHAb, than in the lungs of mice receiving no immunization. Similar results were reported by Allan et al. (1971) and Rott et al. (1974).

Anti-neuraminidase antibodies may also offer protection to man. The influenza pandemic of 1967-68 (H3N2) was milder in comparison with the pandemic of 1958 (H2N2); this has been attributed to the fact that while the HA changed the NA remained an N2 subtype. It has also been shown that ANAb levels correlate with protection from infection; at high NA levels the effect is that of neutralization (Dowdle et al., 1973, Greenberg et al., 1974). Further, it has been observed that those with detectable ANAb who become infected with influenza

A show reduced shedding of virus, thereby reducing the probability that they will spread disease (Murphy et al., 1972; Couch et al., 1974; Kim et al., 1976).

On the basis of the observations that ANAb could modify disease, Kilbourne and colleagues in 1971 proposed a neuraminidase—specific or infection permissive vaccine for protection against type A influenza viruses (see page 51).

IMMUNE RESPONSE TO THE NA ANTIGEN

There is a large body of information documenting the correlation of HI titers with immunity to influenza A virus infection (See Schulman, 1975 for review). The lack of early data on the significance of ANAb in disease led to a relative neglect of the study of ANAb response.

Evidence of the increased immunogenicity of NA when the population is not primed to the HA comes from a study on the NA-specific vaccine (Kilbourne, 1976). In this study, those vaccinated with H7N2 had a greater ANAb response than those receiving H3N2 vaccine (H3N2 circulated 1968 - present). Kil-

bourne suggested that there was antigenic competition between the HA and NA antigens. This observation was borne out in a study by Kendal and colleagues (1977) in which they compared the ANAb response relative to the AHAb response. This group analyzed data from vaccine trials that were part of the National Influenza Immunization Program of 1976 which aimed to vaccinate against an anticipated H1N1 (A/New Jersey/11/76) pandemic. Concurrently, there was immunization to the H3N2 strain, A/Victoria/3/75. H3N2 viruses had been circulating for 8 years; the majority of the population was unprimed to H1N1 (the swine strain circulating from 1918-1929 and the N1 having been out of circulation since 1958). For immunization with the H1N1 strain they report the following: of those under 18 years of age ~23% had a detectable ANAb response while only ~12% responded to the HA; in the age group between 18-24, ~36% had a ANAb response and ~38% had an AHAb response; in those over 24 years of age, 54% had an ANAb response while 90% responded to the HA. They also noted that with 2 doses of H1N1, 70% responded to NA whereas only 41% of those receiving one dose of A/Vic/3/75 developed ANAb even though there was demonstrable priming to the N2 in the population.

This was interpreted as evidence of antigenic competition between the HA and NA antigens, i.e., the unprimed population responded as well or better to NA than the HA antigen. This

suggests that it is not poor immunogenicity of the NA per se that accounts for it being less immunogenic in adults. The relatively poor response to NA is a result of preferential recognition of HA by the primed immune system. This hypothesis is substantiated by the fact that from 1963 to 1968, H2N2 vaccines induced ANAb in less than a third of the vaccinees while in 1968-69, vaccines to the new H3N2 strain induced ANAb response in 66-96% of vaccinees (Henessey et al., 1972; Mostow et al., 1975).

THE NEURAMINIDASE-SPECIFIC VACCINE

OVERVIEW OF CURRENT INFLUENZA VACCINES

As discussed earlier, the inability to control influenza by vaccination resides in the ability of influenza virus to undergo antigenic shift and drift. These two factors greatly complicate vaccination programs, necessitating the almost constant construction and manufacture of new vaccine strains.

Currently available inactivated whole virus vaccines have 50-90% efficacy (Liebovitz et al., 1971). In the adult population the efficacy is 70-90% (Davenport, 1961, 1973; Kilbourne et al., 1974). Yearly vaccination is recommended because of the brevity of protection, although studies have indicated that protection can last longer (Kilbourne et al., 1974).

Influenza vaccines currently in use are basically similar to the whole inactivated vaccine first developed by Francis. Although improvements in viral purification techniques have reduced the reactivity of the vaccine (Tyrrell and Smith, 1979), such vaccines are still reactive, especially in the very young (Kilbourne et al, 1974). Reactivity is reduced in the disrupted or "split" virus vaccines. These vaccines appear almost as effective as formalin inactivated whole virus in effecting seroconversion in the primed population but they are 50% less effective than whole virus vaccine in effecting seroconversion in an unprimed population (Barry et al., 1974; Parkman et al., 1976). The main difficulties with current vaccines are the relatively short period of immunity they confer (1-2 years) and their reactogenicity. Various strategies have been proposed for overcoming these deficits.

It is not possible to discuss the live virus vaccines (Murphy et al., 1976; Richman et al., 1977) within this thesis. Suffice it to say that the genetic instability of mutant strains would appear to be of special concern for influenza because of its well documented ability to stably mutate its proteins and because of the high probability of a recombinational event. Murphy and associates (1976) have shown that revertants of the temperature sensitive (ts) mutants do occur. Influenza A virus has been shown to recombine in in-vivo infections (Webster et al., 1971, 1973). Since 1978, both H1N1 and H3N2 subtypes circulated (MMWR, 1978) clearly demonstrating that more than one subtype of virus may be circulating at any one time. Thus, it is probable that some vaccinated individuals may be subclinically infected. Coinfection of humans with H1N1 and H3N2 viruses has been observed (Yamane et al. 1978). Young and Palese (1979) have shown that some of the H1N1 serotypes circulating in 1978 and 1979 had aquired genes in common with the prevalent H3N2 virus strains; they have suggested that these H1N1 strains arose by recombination. As there is no feasible way to predict the virulence of a recombinant, the danger of a recombinational event would appear to present an unacceptable risk associated with live virus vaccines.

NEURAMINIDASE-SPECIFIC VACCINE TRIALS

In 1972, Kilbourne and colleagues proposed the use of a neuraminidase-specific vaccine that would take advantage of the ability of ANAb to limit disease. Such a vaccine would specifically immunize an individual only to the neuraminidase antigen. The development of antibodies to NA would permit infection by influenza A virus on challenge, but limit viral replication, thus preventing clinical illness. Replication of the virus would allow the host's immune system to mount long-lasting immunity. Several clinical trials of the NA-specific vaccine have been conducted. These trials of neuraminidase-specific vaccines have employed whole virus vaccine utilizing recombinants containing an equine hemagglutinin which was considered antigenically "irrelevant".

1. In 1976, Kilbourne reported the results of a vaccine trial which compared the efficacy of a neuraminidase-specific vaccine with that of a conventional influenza A vaccine. Two recombinant viruses were employed, X-37, A/England/42/72 (H3N2), and X-38(H7N2). Vaccines were comparable with respect to antigenic potency of the neuraminidase as determined by the antigenic extinction test in rabbits. The vaccines also had equivalent neuraminidase activity and CCA

(chick cell agglutinating) units. They found the NA-specific vaccine superior to the conventional vaccine in raising ANAb titers; significant rises in ANAb titers were seen in 25% of those receiving X-37 and in 69% of those receiving X-38. Vaccinees were not challenged with live virus so there is no information correlating ANAb titers with protection.

Those individuals immunized with H7 showed significant rise in titers to H3. This was believed to be a result of an anamnestic response since all vaccinees had been previously exposed to the H3 antigen. However, rabbits immunized with the H7 strain also produced antibody which cross-reacted with H3. Persons vaccinated with H3 did not produce antibody cross-reactive with H7N1.

It was suggested that the greater ANAb response in those receiving the "irrelevant" HA was due to "differences in the immunological processing" of the two vaccines; i.e. the anamnestic response to H3 might compete with the ANAb response. The anamnestic response to the H3 antigen by vaccination with H7 raises questions as to the "irrelevance" of an irrelevant HA.

2. In 1974, Couch and associates reported on the results of a vaccine trial using X-32 (H7N2), an influenza A recombi-

nant of X-31 (H3N2) and A/equine/Prague/1/56 (H7N7): inactivated influenza B vaccine was used as control. They demonstrated that the neuraminidase-specific vaccine was infection-permissive and significantly reduced clinical illness upon infection. Inapparent illness correlated with serum ANAb. The immunity provided by natural infection of vaccinees was complete six months later.

3. In 1977, Vonka and associates conducted an NA-specific vaccine trial with 1200 persons using inactivated whole virus (H1N2). They reported the following: increase in titer to H1 in a majority of subjects; rise in ANAb in slightly more than half the vaccinees; only rare rises in antibody to H3. Following a natural outbreak of H3N2 Victoria-like strain, they noted approximately two-fold less morbidity among vaccinated subjects than among controls.

4. In the fall of 1974, a field trial of neuraminidase-specific vaccine was undertaken (Ogra et al, 1977; Beutner et al., 1976). Vaccinees were 300 schoolchildren divided into three equal groups and vaccinated with X-41 (H3ChN2), X-42 (H7N2Ch), or placebo. A natural outbreak of H3ChN2 virus subsequently occurred (1974, 1975). Infection was ascertained serologically. They found the NA-specific vaccine to be infection permissive, but reported that a greater proportion

of those receiving the neuraminidase-specific vaccine had inapparent infections and when infected, clinical disease was somewhat milder. The neuraminidase-specific vaccine induced mean ANAb titers three to four times higher than the conventional vaccine---clinical infection correlated with low ANAb titers. However, disease rates among the NA-specific vacinees was significantly higher than for those receiving conventional vaccines.

In 1975-76, a natural outbreak of the Victoria strain (H3N2) occurred. In this outbreak, protection against illness in the two groups was very similar, 80% for those immunized with H3ChN2, 73% for those immunized with the H7N2 (Beutner et al., 1979). The authors concluded that the basic premise of the NA-specific vaccine was sound, but that in practice the NA- specific vaccine offered no advantages over conventional vaccines.

This conclusion may be premature. The vaccinees receiving the NA-specific vaccine did have inapparent or mild infections and the rationale of the vaccine is that the immunity from these would be long-lasting---so that in order to assess the efficacy one would really need data to the present date, i.e., the protection afforded by the conventional vaccine would be expected to fall off after the first year or two and

then those protected by natural infection should begin to outnumber those protected by conventional vaccination. Indeed, a long term study by Hoskins et al., (1979) showed that over a four year period during which H3N2 strains were circulating, conventional vaccines, even if administered yearly, gave vaccinees no cumulative advantage over non-vaccinees.

The use of purified neuraminidase as antigen in the NA-specific vaccine would appear to offer several potential advantages. This vaccine would contain only one protein, the viral neuraminidase. Theoretically, it should be less reactogenic (Neurath and Rubin, 1971) as inactivated whole virus vaccine. It should eliminate the problem of antigenic competition, i.e. the immune response to the "irrelevant" hemagglutinin interfering with the immune response to neuraminidase (for review see Aurora, 1979).

CHAPTER 4. THE M PROTEIN

The M (membrane or matrix) protein is the major structural component of type A influenza virus: it has a molecular weight of about 25,000 daltons and accounts for about 40% of total virion protein (Choppin and Compans, 1975). The M proteins from all type A influenza viruses are cross-reactive, and are considered type specific. (Schild and Dowdle, 1975).

MORPHOLOGY

The precise position of M in the influenza virion is still not clear. Compans et al. (1970), Schulze (1970), and Klenk et al. (1972) have reported that M protein in intact virus is not accessible to proteases. It is not labelled by techniques which are known to label external proteins (Stanley and Haslam, 1971; Rifkin et al., 1972).

Recently Reginister and associates (1979) have shown that proteolytic treatment of the virion results in binding of anti-M antibodies, suggesting that removal of glycoprotein spikes exposes M on the virion surface. Reginister and colleagues (1979) have also shown that non-proteolytically treated Singapore 1957 strain (H2N2) virions can bind anti-M antibody and that anti-M antibody reduces the infectivity of this strain ten-fold; anti-M antibody was also shown to have HI and complement fixing activities against H1N1, H2N2 and H3N2 strains. These authors concluded that M was exposed on the virion surface and that neutralizing and HI antibodies probably were having their effect through steric hindrance. These authors previously demonstrated that proteolytic treatment of influenza virions decreased by half the amount of M in intact virions (Reginister et al., 1975/76), suggesting that removal of glycoprotein spikes exposes M on the virion surface.

The M protein has been described as forming a protein shell independent of, but directly beneath the lipid bilayer of the virion. The evidence for this rests mainly on the electron microscopic observations of Bachi (1969) and Compans and Choppin (1973) and the biochemical studies of Schulze (1972). The studies of Reginister and colleagues (1979) sug-

gest that M is accessible at the virion surface and they question the assumption that M is an internal component of the virion.

Using a fluorescent lipid probe, Lenard et al. (1974) showed that M was not more than 11Å from the membrane and did not rule out its being partially intramembranous. When the influenza virion is subjected to mild detergent disruption, the M protein is found to be insoluble, indicating a hydrophobic conformation. Gregoriades (1973) isolated the M protein from both virions and infected cells using acidic chloroform-methanol. The amino acid composition (Gregoriades, 1973) does not predict a hydrophobic protein and so it is assumed that the hydrophobicity of M is a function of conformation.

This laboratory's procedure for isolation of the M protein, disruption by 10% SDS and sonication (Bucher et al., 1976, 1980), produces an opalescent suspension on removal of SDS, supporting the observation that the hydrophobicity of M is due to conformation. The recent evidence of Gregoriades (1980) and Bucher et al.(1980) that M protein can be incorporated into the lipid bilayer of liposomes will be discussed in Chapter 8.

FUNCTION OF THE M PROTEIN.

The M protein appears to be associated with virion assembly and stability, virus yield, amantadine sensitivity, and perhaps even with infectivity.

Stability. Kendal et al (1977 b) reported that when influenza A viruses were replicated at elevated temperatures, there was an increase in the proportion of particles with lowered buoyant density, as well as a decrease in the infectivity of the yield. The lowered infectivity was due to the increased numbers of low density particles and these particles had greatly reduced M protein content. The low density particles were reported to be more fragile.

Assembly. Kendal et al (1977 c) showed that the ability of a cold adapted mutant to replicate at the lower temperature was a function of the M protein. The wild type virus appeared to have normal transcription and translation of M, but did not form virions at the colder temperatures.

Both papers by Kendal and colleagues indicate that there appears to be an optimal temperature for incorporation of M into the virion. Above that temperature, M is poorly incorporated and the result is lowered infectivity. The poor incor-

poration of M into virions at the colder temperature is suggestive of an active role of lipid fluidity in virion formation.

Amantadine Sensitivity. Lubeck et al. (1978) and Hay et al. (1979) have both implicated the M protein in the varying sensitivity of influenza A strains to amantadine hydrochloride. In the presence of amantadine no transcription of the viral genome occurs but this inhibition is reversed when amantadine is removed (Skehel et al., 1977). While the mechanism by which amantadine hydrochloride inhibits viral replication is not clear, it is known from studies of Dourmashkin and Tyrrell (1974), that it does not appear to affect virus attachment or uptake, nor does it visibly affect the process by which the virion enters the cytoplasm, i.e., gross uncoating.

Infectivity. Kendal et al. (1977) showed that virus grown at elevated temperatures had reduced infectivity and that the reduction in infectivity was due to reduced M protein content. Along the same lines, studies of amantadine sensitivity indicate that the M protein plays some role in establishing infectivity, i.e. amantadine sensitivity is a function of the M protein and involves an early step in infection.

Virus Yield. Schulman and Palese (1978) have shown association of the M as well as the NP proteins with high yield when virus is propagated in eggs.

IMMUNOLOGY OF M PROTEIN

Anti-M antibody has not been associated with protection from infection and appears to have no neutralizing effect. Thus, although M proteins of all influenza A viruses share common antigenic determinants, this does not serve to prevent the emergence of new subtypes to which the population has no significant immunity. Further, it has been shown that specific immunization to M protein, while it modified the course of the disease in mice, did not prevent infection (Webster and Hinshaw, 1977).

It has been observed that M protein poorly stimulates the production of antibody. Webster and Hinshaw (1977) immunized mice to purified M and saw no M antibody response by single radial diffusion. Even after natural infection, which could be expected to boost a low antibody response, only half the animals had M antibody detectable by single radial diffusion. Cretescu et al., (1978) reported that M antibody was detected by single radial diffusion in post convalescent sera of <7%

and most of these had very severe infections. The conclusion that M protein induced a weak antibody response was based on what now appears to be a very insensitive assay. The development of the enzyme linked immunosorbent assay(ELISA) for evaluation of M antibodies, utilizing purified M-protein antigen adsorbed on microtiter plates permits re-examination of the antibody response to M protein (M.W. Khan, personal communication).

M has been detected on the surface of infected cells (Ada and Yap, 1979; Biddison et al., 1977). The detectable M on the surface of infected cells was not believed to be in close contact with the HA as AHAb did not affect the binding of M antibody (Ada and Yap, 1979). However, if M were in close contact with HA it may be sterically blocked and hence undetectable, or it may occupy a different and inaccessible space in the lipid bilayer, i.e., interaction of M and HA could change the position and/or conformation of M protein.

Hackett and colleagues (1979) used well characterized monoclonal antibodies to M protein and demonstrated that while M was detectable on the surface of infected cells, the amounts detected were 2 - 3 orders of magnitude lower than those reported by Ada and Yap (1979). Yewdell and associates (1981)

used a diverse panel of monoclonal anti-M antibodies with results that supported the observations of Hackett et al. (1979).

It has been shown that cytotoxic T cells specific for influenza M protein will lyse HLA compatible influenza A infected cells (Braciale, 1977). Webster and Hinshaw (1977) reported that mice immunized to purified M, while showing no detectable antibody response, gave a strong DTH response to M protein---the DTH to an equivalent amount of whole virus was weaker indicating that conformation of M in the virus may differ from that of solubilized M. In the same study, it was noted that mice immunized to M showed enhanced viral clearance from the lung though lung lesions were as severe as in controls.

The results of Webster and Hinshaw (1977) are reminiscent of the demonstration by Schulman and Kilbourne (1965) of heterotypic immunity induced by influenza A infection: when mice previously infected by H1N1 strains were challenged with an H2N2 strain, they had lower viral titers and decreased lung lesions and mortality than controls. They also noted an earlier response to the H2 antigen than was seen in controls.

CHAPTER 5. LIPOSOMES

OVERVIEW

As Tanford and Reynolds (1976) point out, the study of membrane proteins is in its infancy: the same can be said for the study of membranes. In fact, until recently, membranes were viewed as rather inert, uninteresting cellular components. The last decade has seen increased interest in lipids and membrane structure and the dynamics of lipid-protein interaction. Liposomes have been an invaluable tool in the study of lipid-protein interaction. Liposomes are discrete, closed lipid structures formed when lipid exists in an aqueous environment (Bangham, 1978). Since the earliest studies of Bangham and Horne (1964), much has been learned about the structure of lipid in an aqueous environment. When in aqueous solution, lipids aggregate by their hydrophobic alkyl chains

into a bilayer. Depending on the lipid to water ratio, the bilayer exists in sheets (at low water concentrations), or as closed micellar structures (at high water concentrations) (Bangham et al., 1967).

Liposomes are commonly formed by two methods. In the chloroform-methanol method, lipids are dissolved in chloroform—methanol and organic solvents are evaporated off slowly while lipid is deposited as a thin film on the walls of a flask. The lipid is slowly hydrated and the mixture is then sonicated (Bangham, 1978). To form liposomes by dialysis, lipids are solubilized in a detergent solution. This results in the formation of mixed micelles, that is, micelles which contain both lipid and detergent. Detergent is then dialyzed away. The use of the detergent, octylglucoside , which is easily dialyzable, has made this method most efficient (Helenius et al., 1977). Liposomes formed by both of these methods are heterogeneous populations consisting of small and large unilamellar vesicles and small and large multilamellar vesicles. Sonication increases the proportion of unilamellar vesicles.

Liposomal conformation and permeability can be affected by several parameters, these include: the solvent, ionic strength of the solvent, pH, lipid composition, protein composition and concentration of the bilayer, ions such as Ca^{++}, temperature, chaotropic agents, mechanical manipulations, such as sonication, and some anaesthetics (for review, see Hill, 1978; Chapman et al., 1978; Paphadjopoulos et al., 1978; Bangham, 1978). Comparison of experiments must take into account the considerable variation in the aforementioned factors.

Our interest in liposomes stems from our interest in studying conformation of a membrane protein in a state as close to that of the virion as possible, and the effect of liposomal incorporation of an antigen on its interaction with the immune system. There have been reports that association of an antigen with liposomes enhanced the immune system response to a specific antigen. The mechanisms of this enhancement have not been clear.

Several possibilities exist: 1). liposomes may have an adjuvant effect (Manesis et al., 1979); 2). liposomes may enhance through aggregation of protein, which enhances immunogenicity (Neurath and Ruben, 1971); 3). liposomes may facilitate antigen processing by the immune system.

In the last few years there have been several studies on the interaction of influenza proteins with liposomes to form virosomes. These will be discussed in some detail.

VIROSOMES

Almeida and co-workers (1975) disrupted influenza with nonidet P-40 and isolated membrane proteins by density gradient centrifugation. The detergent solubilized viral glycoproteins were added to preformed, sonicated liposomes (lecithin, dicetyl phosphate in a weight ratio of 9:1) and sonicated for 15 minutes. They termed these protein bearing liposomes, virosomes.

Virosomes had a size range of 20-100 nm and possessed a fringe of spikes giving them an appearance not unlike that of the influenza virion. The biological activity of these virosomes was not reported. Hyper-immune sera to intact homologous influenza virus aggregated the liposomes. It was stated that preliminary studies indicated that such liposomes were immunogenic but not pyrogenic; however, no data were presented and this has not been confirmed.

Huang et al., (1979) prepared HA-NA preparations by disrupting virus with 7% octylglucoside and removing most of the internal viral components by centrifugation. Octylglucoside solubilized lipid preparations were added to the solubilized HA-NA preparations and liposomes were formed by dialysis; virosomes were isolated by pelleting. Virosomes contained HA and small amounts of NP and M (SDS PAGE): NA was not seen on gels but its presence was confirmed by enzymatic activity.

They reported that NA could be absorbed onto the surface of preformed liposomes but that HA could not. HA appeared to be integrated into the lipid bilayer. In the virosomes, regardless of lipid composition, there was a protein to lipid ratio of 0.09:1.

Their HA-NA virosomes were capable of several viral-type functions: they agglutinated red blood cells (pure lecithin liposomes being the least successful), and the NA appeared to be cleaving sialic acid off red blood cells, as disagglutinated erythrocytes could not be reagglutinated indicating loss of sialic acid receptors, (Chapter 2). Subsequently this laboratory (Rott, 1980), reported that liposomes containing HA only would absorb to cell surfaces, but that when NA activity was present, virosomes could be observed fusing with the cell membrane.

Oxford et al. (1981) investigated the interaction of HA with phospholipid vesicles. Virus was disrupted with 2% Triton X-100 (or octylglucoside) and viral proteins "purified" by sucrose density gradient centrifugation. Liposomes, using various lipids and lipid ratios, were preformed by the chloroform-methanol method and sonication. Virosomes were prepared by mixing preformed liposomes with protein at a protein to lipid ratio of 1:50 and sonicating for 20 min. Virosomes showed the presence of spikes oriented in both directions from the lipid bilayer. They noted that HA attached more readily to negative liposomes than to positively or neutrally charged liposomes. Average liposome size was 70-500 nm.

Freeze fracture techniques showed no evidence of HA deeply penetrating the lipid bilayer: measurements of HA spike length before and after incorporation into liposomes indicated that only 1 nm of the 14 nm spike was embedded in lipid. Virosomes could attach to Vero cells and were observed being taken up by viropexis. The immunogenicity of virosomes was excellent: 2 μg of liposomal HA was immunogenic in guinea pigs (protein content was estimated by rocket immuno-electrophoresis). Liposomal HA was ten-fold more immunogenic than the isolated subunits: the isolated subunits appear (in electron micrographs) as a mixture of monomer and aggregate forms.

Separate injection of liposomes and protein subunits did not result in increased immunogenicity, indicating that liposomes did not have adjuvant activity.

Shortly after the work presented in this thesis had begun, the results of a project very similarly conceived were reported by Morein, Helenius, and Simons (1978). Morein et al. (1978) solubilized the membrane proteins of Semliki Forest virus (SFV) with 2% Triton X-100 and separated the viral glycoprotein spikes from other viral components by sucrose density gradient centrifugation in the presence of Triton X-100. It had been previously shown (Simons et al., 1973) that glycoproteins isolated by this method existed in the monomeric form, had a sedimentation coefficient of 4.5S, bound 75 molecules of Triton X-100, and contained E1 and E2 polypeptides in a 1:1 ratio. Removal of detergent by chromatographic techniques resulted in aggregation into an octomeric form with a sedimentation coefficient of 29S. The octomeric complex appeared as a protein micelle by electron microscopy and had HA activity.

Virosomes were prepared by mixing octylglucoside solubilized SFV spike proteins with octylglucoside solubilized lecithin and dialyzing (Helenius et al., 1977). Liposomes formed by this method possessed HA activity, were heterogeneous in

size, and consisted of two populations, a protein-rich and a protein-poor population. The spike protein was reported to be oriented outwardly as in the virion, since it was accessible to proteases.

Mice were vaccinated with 10 µg of protein in monomer, octomer, or virosomal form (Morein et al., 1978). Multimeric protein forms (octomer, virosomes) induced good protection against infection with SFV: monomer was only poorly immunogenic even when injected with Freunds adjuvant.

Recently, this same laboratory (Balcarova et al., 1981) has correlated antibody titer with protection. Two doses of 1 µg each were administered to mice. There was little or no primary response to any form. Secondary immunization resulted in high antibody titers to octomer and virosomal forms, but a 20-fold lower response was evidenced by those receiving the monomer form. The protection afforded by monomer was 100-fold less than that seen with either of the multimeric protein forms (octomers and virosomes had equivalent efficacies). They recommended the use of octomer for immunization because of the instability of lecithin liposomes. Unfortunately, these studies do not compare the efficacy of antigen forms with that of whole inactivated virus.

Gregoriades (1980) demonstrated that the M protein of influenza interacted with phosphotidylcholine vesicles. She was able to demonstrate that under her conditions, about 5000 daltons of the 25,000 dalton protein remained trypsin insensitive when M was associated with lipid. Liposomes were formed either by dialysis of M protein with sodium deoxycholate or by addition of M protein to preformed liposomes and vortexing.

Two independent studies using purified proteins of hepatitis B as vaccine antigen have been reported. Hepatitis B surface antigen was purified by $CsCl_2$ density gradient centrifugation and incorporated into liposomes (Manesis et al., 1979). Immunization was by multiple injections of 1.5 and 10µg quantities of antigen into guinea pigs and efficacy was tested by delayed type hypersensitivity (DTH) and antibody titer. Titers to the liposomal form of antigen were greater than 100-fold higher than to the monomer form of the antigen. Use of adjuvant did not enhance the immune response to liposomal or monomer protein. A primary response to liposomal antigen was observed; response to monomer occurred only after secondary and tertiary immunization. Little or no DTH was seen with monomer, whereas half the animals immunized with liposomal antigen had a delayed type hypersensitivity (DTH) reaction.

Hepatitis B protein micelles were used as immunogen by Skelly and colleagues (1981). 5 and 10 µg doses of either intact virus particles or protein micelles were used. Good immunogenicity for both was reported. The protein micelles resulted in significantly higher antibody titers than inact 22nm particles. However, both antigen forms were absorbed to alum for injection.

The virosome system provides a model for dissecting the replication cycle of the virus: penetration, uncoating, and assembly. The virosome also has promise as an immunological tool for dissecting the immune response to an antigen alone or in the presence of other antigens which might affect immunogenic conformation or processing. One of the potential uses of liposomes is for vaccines. One would predict that the protein in liposomes would be in a conformation very similar to that in the virus and thus overcome the low immunogenicity of purified proteins.

CHAPTER 6. STUDIES OF PURIFICATION PROCEDURES FOR NEURAMINIDASE

Our eventual goal was to purify neuraminidase for use as a vaccine (see Chapter 7). Our first step was to attempt to vary the conditions of purification in such a way as to produce a purified NA product that was an FDA injectable (acceptable for vaccine use), and to optimize conditions so as to provide the maximal NA yield from virus. In the process of investigating methods by which we could accomplish these ends, we enhanced our knowledge of the requirements of the purification procedure.

INTRODUCTION

The interactions of membrane proteins with lipids and detergents have been extensively characterized (Reynolds and Tanford, 1970a, b; Makino et al., 1973; Tanford, 1974; Robin-

son and Tanford, 1975; Helenius and Simons, 1975; Gulik-Krzywicki, 1975; le Maire et al., 1976; Tanford and Reynolds, 1976; Simons et al., 1978).

Integral membrane proteins possess a hydrophobic portion of the molecule which is embedded in the lipid bilayer. It has been generally noted that membrane proteins, when removed from the lipid environment are susceptible to denaturation. Non-ionic detergents may prevent denaturation of membrane proteins by simulating the lipid environment of the lipid bilayer (for review see Gulik-Kryzwicki, 1975; Helenius and Simons, 1975). Detergents are amphiphiles with a structure similar to phospholipids, i.e. they have long hydrocarbon tails and polar head groups: in aqueous solutions above the critical micelle concentration (cmc) they form micelles -- the hydrophobic tails aggregate and the polar head groups interact at the aqueous interface (Tanford and Reynolds,1976). The cmc is a measure of the concentration below which the detergent exists in the monomer form and as such is a measure of the stability of the micellar form and indicates the ease with which the detergent can be dialyzed -- only monomers are dialyzable.

Ionic detergents such as SDS (sodium dodecyl sulfate) and CTAB (cetyl trimethyl ammonium bromide) are more disruptive of

lipid-protein interactions than are non-ionic detergents (Tanford and Reynolds, 1976). SDS disrupts membranes by inserting in the lipid bilayer and lowering the surface tension. As the SDS to lipid ratio increases, the lipid bilayer breaks up forming mixed lipid-SDS-protein micelles. Most ionic detergents denature proteins (Tanford and Reynolds, 1976; Gulik-Kryzwicki, 1975). Reynolds and Tanford (1970a,b) have shown that SDS binds to proteins in a cooperative process and have suggested that alteration in conformation induced by SDS binding is responsible for denaturation.

One strategy for purification of a biologically active membrane protein is to disrupt the membrane with an ionic detergent and subsequently solubilize and stabilize the protein in non-ionic detergent. One must employ a sufficiently high concentration of non-ionic detergent such that each protein molecule is in a separate micelle (Tanford and Reynolds, 1976). Ionic detergent levels are then reduced by dialysis.

After purification of protein it is possible to dialyze away detergent to form protein micelles which contain several protein subunits (Simons et al.,1978). Laver and Valentine (1969) removed SDS from purified HA and NA preparations by acetone precipitation, and showed formation of protein micelles by electron microscopy. Neuraminidase aggregates

appeared as "seeding dandelions", each aggregate contained about 12 spikes; hemagglutinin aggregates had a star-like appearance and contained about 5 spikes.

Proteins solubilized in detergent can be incorporated into lipid by dialysis (Helenius et al., 1977; Huang et al., 1979). Detergent solubilized lipids and protein are mixed. As detergent is dialyzed away, lipid vesicles containing protein molecules are formed. Liposomes formed by dialysis have been observed to bear the protein in the same outward orientation in which it is seen on the cell surface: in contrast when lipid-protein interaction is effected by sonication, proteins have been observed having the spike oriented toward the interior as well as exterior of the liposome (Oxford et al., 1981).

Development of an affinity isolation procedure for purifying the NA of influenza provided a method of obtaining purified NA while maintaining the enzyme in a conformation which simulated that of the enzyme in the virus -- as assayed by enzyme activity which is considered a very sensitive indicator of the conformation of the protein (Tanford and Reynolds, 1976).

The procedure for purification of NA by affinity chromatography is outlined in Appendix II. Briefly it involves

three steps: 1). disruption of the virus; 2). solubilization of the NA; 3). purification of NA by affinity chromatography.

As the affinity isolation technique used in this laboratory allowed recovery of ~2% of viral protein (Bucher, 1977), and NA was reported to constitute about 10% of X-7 viral protein (Bucher and Kilbourne, 1972), our first step was to investigate the efficacies of various detergents and detergent concentrations on protein purity and yield. It was also our hope to modify the affinity isolation procedure by substituting Tween 80 for Triton X-100. (Tween 80 is an FDA approved injectable for vaccine administration while Triton X-100 is not.)

MATERIALS AND METHODS

Virus. X-31B(H3N2), a recombinant of A/Aichi/2/68(H3N2) and A/PR/8/34(H1N1) used as vaccine strain, was grown in the allantoic sac of embryonated chick eggs and purified as described in Appendix IV.

Assay for Stability of Neuraminidase in Various Detergents. Whole virus was diluted to 1mg/ml by the Lowry assay (Lowry et al., 1951), and disrupted with 1%, 0.5% or 0.17% SDS, or 1% or 0.5% CTAB and mixed for 15 minutes at room temperature. Disrupted samples were brought to final concentration of 10% Triton X-100, or 10% or 20% Tween 80 and dialyzed overnight at $4^{o}C$ against pH 5 absorbing buffer (0.05M sodium acetate, 0.001M $CaCl_2$, 0.1mM EDTA and 0.1% Triton X-100 or 0.1% Tween 80). Protein concentrations and NA activity were assayed as described in Appendix II on the following day.

Affinity Chromatography. Whole virus was diluted to 1mg/ml as above and disrupted with 1% or 0.17% SDS or 1% CTAB and solubilized with 10% Tween 80. Solubilized virus was dialyzed for 24-48 hours against pH 5 absorbing buffer and then centrifuged at 20,000 rpm for one hour in a Beckman JA 21 rotor at $4^{o}C$. Pelleted material was solubilized in calcium acetate buffer (0.05M acetate, 0.002M $CaCl_2$ at pH 7) and assayed for protein and NA activity and then stored at $-70^{o}C$. Affinity chromatography followed the procedures outlined in Appendix I. Briefly, supernatant (sup) was applied to the affinity column equilibrated with absorbing buffer. One ml fractions were collected and sample absorbance at A_{260nm} was monitored by a Uvicord I.

Material not absorbing to the column was assayed for NA activity and protein and subsequently pooled (protein pool). Fractions eluting at a pH >7 were neutralized with 1N HCl and assayed for protein and NA activity and for purity by PAGE before pooling (enzyme pool).

Protein was assayed by the method of Lowry (Lowry et al., 1951) with the addition of 0.25% SDS to facilitate reading in the presence of non-ionic detergent. NA activity was assayed by the method of Aminoff (1961) as described in Appendix II.

RESULTS

Virus was disrupted with various concentrations of CTAB, a positively charged ionic detergent, or SDS, a negatively charged ionic detergent and then solubilized with either 10% Triton X-100, or 10 or 20% Tween 80. Detergent treated samples were dialyzed overnight. The stability of neuraminidase to the various detergent treatments was assayed by comparing the specific activities of the various samples.

As Fig. 6.1 shows, specific activity increases 2-4 fold over that of whole virus when virus is solubilized. 1% SDS appeared to offer no advantage over the lower concentration of SDS. 1% CTAB resulted in better stabilization of NA activity than 0.5% CTAB. 10% Tween 80 appeared as effective in stabilizing NA activity as either 10% Triton X-100 or 20% Tween 80.

On the basis of the stabilization data, we evaluated the effect of the following detergent treatments on the yield and purity of NA recovered from affinity isolation: 0.17% SDS-10% Tween 80; 1% SDS-10% Tween 80; and 1% CTAB- 10% Tween 80.

Analysis of protein distribution during affinity chromatography (Fig. 6.2) shows that 1% and 0.17% SDS were equally effective in disrupting the virus: both treatments solubilized ~60% of the viral protein. 1% CTAB solubilized about 80% of viral protein. The superiority of CTAB in solubilizing viral protein was not an intrinsic advantage; it was hoped that this first step would selectively solubilize the surface proteins.

1% SDS resulted in a very poor protein yield in the enzyme pool (Fig. 6.2), indicating that it was denaturing the NA -- the column does not absorb denatured NA. High levels of protein recovery in the enzyme pool were seen with both 0.17% SDS and 1% CTAB treated samples. The sample treated with

0.17% SDS showed 10% of viral protein in the enzyme pool which is in good agreement with the estimate of 10% NA: the 20% recovery in the 1% CTAB treated sample suggested that there might be contaminating proteins. Analysis of the enzyme samples by PAGE gels confirmed the presence of more than 50% contamination by HA and NP in the 1% CTAB solubilized pool (data not shown). The enzyme pool derived from virus treated with 0.17% SDS appeared pure on SDS PAGE (Fig. 6.3).

Fig. 6.4 shows the specific activity of NA in the various pools. The specific activity of the samples treated with 1% SDS was low following disruption, indicating that such high levels of SDS denature the NA of X-31B. The superiority of 1% CTAB in solubilizing NA from the virus can be seen in the enrichment for NA activity seen in the supernatant. In contrast in the 0.17% SDS treated sample, the enzyme activity of the supernatant was only about two-fold higher than that of the pellet.

The specific activities of the 1% CTAB and 0.17% SDS treated sample enzyme pools are roughly equivalent. Since the CTAB enzyme pool contained at least 50% other viral proteins this indicates that the CTAB treated NA had at least two-fold higher specific activity than the 0.17% SDS treated sample. This was disturbing as it indicated possible denaturation of the X-31B NA by even low concentrations of SDS.

When total neuraminidase activity is examined (Fig. 6.5) it can be seen that solubilization with 1% SDS resulted in loss of activity and that less than 5% of viral NA activity was evident in the enzyme pool. 0.17% SDS and 1% CTAB both resulted in about three-fold enhancement of NA activity following virus disruption (supernatant + pellet). 0.17% SDS and 1% CTAB resulted in 60 and 90% recovery, respectively. However, if this figure is corrected to account for the three-fold increase in specific activity seen on solubilization then recovery is 20% and 30% respectively for the 0.17% SDS and 1% CTAB treatments.

As can be seen in Fig. 6.4a the highest fold purification obtained by 0.17% SDS or 1% CTAB was 6-9 fold. If the specific activity of NA prepared by these purification techniques is compared with the ~40 fold purification of X-7 NA seen with 1% SDS-10% Triton X-100 (Fig. 6.4b) one can appreciate our concern over possible denaturation of the X-31B NA during purification.

The specific activity of X-7 neuraminidase, purified by 1% SDS-10% Triton X-100, from a batch of X-7 that had been stored at -20^{o}C for two years is shown in Fig. 6.4b for comparison with other purification procedures. The specific activity scale has been adjusted to account for the initially

higher specific activity of X-7 NA. As can be seen, the specific activity of X-7 neuraminidase, purified by 1% SDS -10% Triton X-100, is about 40 fold higher than that of the enzyme in the virus. This increase in specific activity is much higher than that seen for any of the purification procedures investigated.

DISCUSSION AND CONCLUSIONS

It is now accepted that the immunogenicity of a protein can be affected by the method of isolation. One of the main advantages of using NA was that enzyme activity provided an extremely sensitive tool for monitoring changes in the molecule during purification.

Purification of NA by affinity isolation (Bucher, 1977), results in a forty-fold increase in specific activity of the enzyme. If this figure is corrected for the enhancement of viral NA specific activity seen in the presence of detergents, then it means an actual 10-20 fold enhancement of specific activity which is in keeping with the 5-10% estimate of NA per virion. These figures indicate that we are reliably able to isolate the enzyme while maintaining it in a conformation that is sufficiently similar to that of whole virus (enzymatic activity is maintained).

Initial efforts to use Tween 80 as the non-ionic detergent for purification of antigenic NA failed. The Tween could not be removed by dialysis (Tween 80 has a very low cmc), and Tween 80 solubilized neuraminidase was not immunogenic in rabbits.

The affinity isolation method for neuraminidase involves solubilization of the virus in 10% Triton X-100: at lower concentrations it is impossible to separate the two surface glycoproteins HA and NA, as they appear to exist in mixed micelles. At 10% Triton X-100 the HA passes through the affinity column in high Triton X-100 concentrations, the NA is adsorbed and elutes on elevation of pH, at a Triton X-100 concentration similar to that of the eluting buffer, 0.1%. After

concentration of the eluted fractions, the storage Triton X-100 concentrations are between 2-3%. These Triton X-100 concentrations can be reduced to below detectable levels by dialysis for one week (Markovitz and Bucher, unpublished).

Originally we had hoped to isolate NA from a batch of outdated Aichi(H3N2) vaccine(inactivated with 0.01% formalin). We were unable to purify NA from the Aichi vaccine batches using affinity chromatography. The viral proteins in this Aichi vaccine preparation were shown to be extensively cross-linked (Chen and Bucher, unpublished). PAGE gels of formalin treated virus showed that large amounts of viral protein were unable to enter a 7% gel presumably because of cross-linking.

We had planned to use X-31B as the viral strain of choice since it bears an NA which was closely related antigenically to the NA of the strain in circulation at that time. However, our results indicated that the NA of X-31B is inactivated by 1% SDS, and even at the 0.17% SDS level there was also evidence of denaturation. CTAB proved an unacceptable alternative as an ionic detergent; the enzyme pool was heavily contaminated with other viral proteins. It may be that CTAB does not effectively disrupt protein-lipid and protein-protein interactions so that subsequent solubilization with non-ionic detergent does not result in micelles carrying only one molecule.

When NA purified with Tween 80 was used for immunization of rabbits little or no NI antibodies were produced. This was in contrast to results obtained with Triton X-100 solubilized NA samples (Markovitz and Bucher, unpublished). Prior to immunization, it was laboratory procedure to dialyze for several days. This procedure lowered Triton X-100 levels but did not affect Tween 80 levels. We hypothesized that the superior immunogenicity of Triton X-100 samples was due to the aggregated state of the protein when detergent was removed.

As our goal was the study of the immunogenicity of NA, we decided to use the X-7(H1N2) strain and disruption with 1% SDS-10% Triton X-100 as it had been shown that NA from this strain could be rather easily purified by affinity chromatography with high specific activity (Bucher, 1977). In addition, more than 98% of (^{3}H) Triton X-100 could be removed by one week dialysis (Markovitz and Bucher, unpublished).

Figure 6.1. Specific Activity of Neuraminidase Following Detergent Solubilization and Dialysis. The X-31B influenza A recombinant virus was diluted to a concentration of 1mg/ml and brought to a concentration of 1%, 0.5%, or 0.15% SDS or 1% or 0.5% CTAB. After fifteen minutes stirring at room temperature, the samples were brought to (1). 10% Triton X-100, or (2). 10% Tween 80, or (3). 20% Tween 80. Samples were dialyzed for 24 hours at $4^{o}C$ against pH 5 absorbing buffer containing 0.1% of the non-ionic detergent used for solubilization. The dialysates were then assayed for NA activity and protein as described in text.

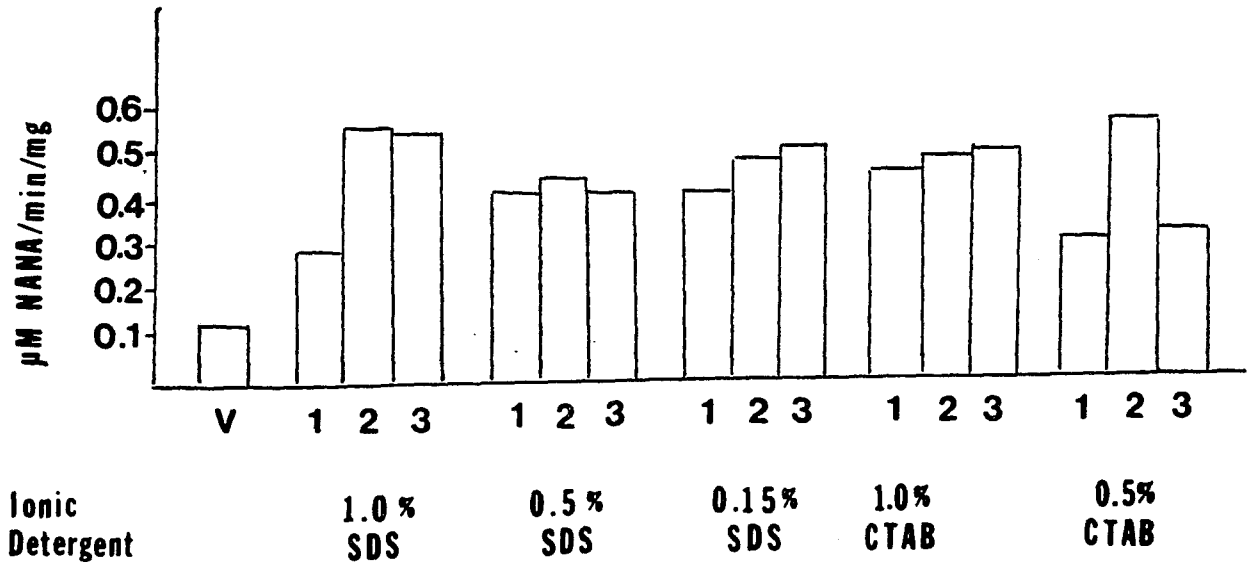

µM NANA/min/mg
0.6
0.5
0.4
0.3
0.2
0.1
V
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3
Ionic Detergent
1.0 % SDS
0.5 % SDS
0.15% SDS
1.0% CTAB
0.5% CTAB

Figure 6.2. Protein Distribution During Neuraminidase Isolation by Affinity Chromatography. Concentrated virus(V) was disrupted with (1). 0.17% SDS-10% Tween 80, (2). 1% SDS-10% Tween 80, or (3). 1% CTAB-10% Tween 80. Disrupted virus samples were dialyzed, and solubilized viral protein (SUP) was separated from unsolubilized (P) by centrifugation. Supernatants were applied to the affinity column as described in text and viral proteins that did not absorb to the column were pooled(P.P.), as were the enzyme fractions eluting at pH 9 (Enz). Total protein recovered (T.P.R.) represents the sum of the protein seen in the protein and enzyme pools. Protein was assayed by the method of Lowry with 0.25% SDS added to allow reading in the presence of Triton X-100 or Tween 80.

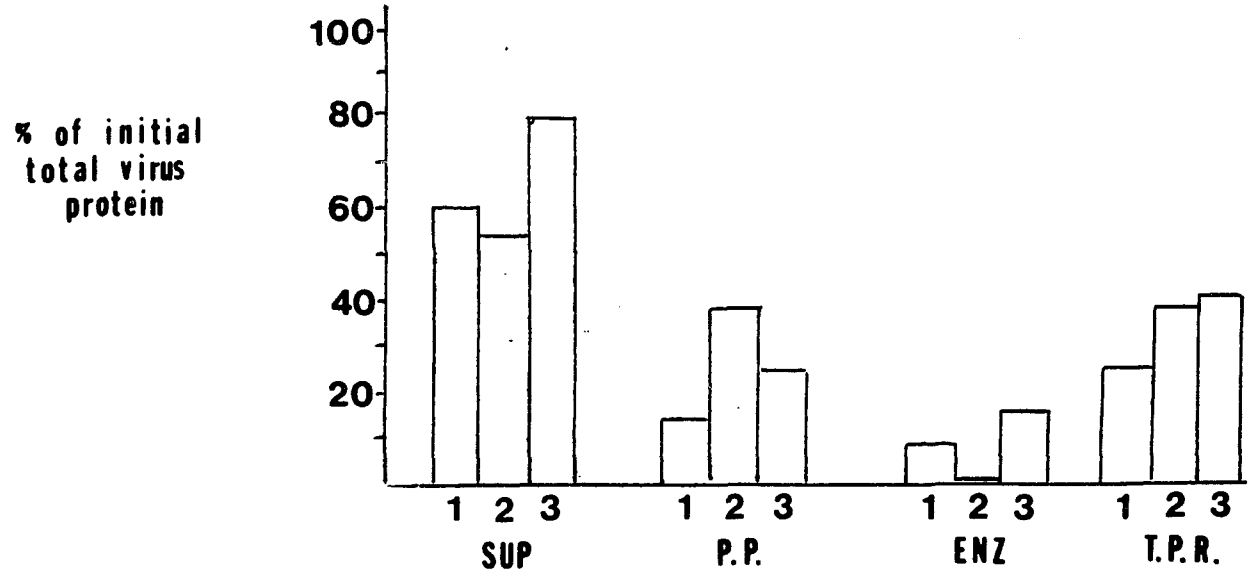
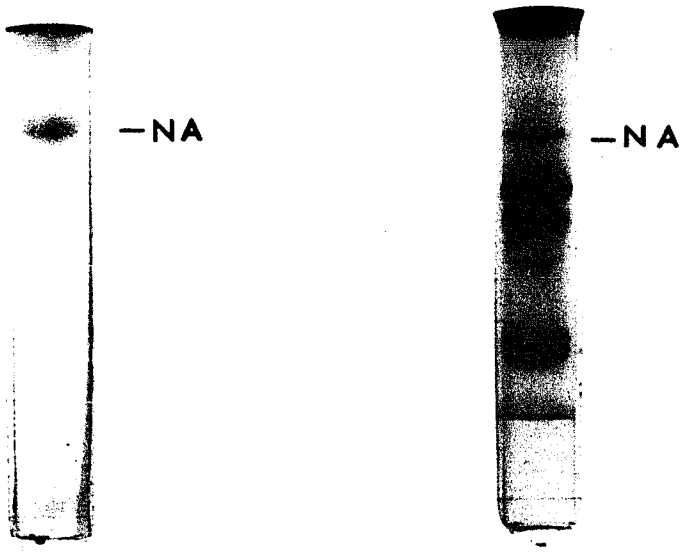

% of initial total virus protein
100
80
60
40
20
1 2 3
SUP
1 2 3
P.P.
1 2 3
ENZ
1 2 3
T.P.R.

Figure 6.3. SDS Polyacrylamide electrophoresis gels of neuraminidase purified from influenza A recombinant X-31B (H3N2). SDS PAGE of total virus protein (right) and of neuraminidase purified by affinity chromatography using 0.17% SDS and 10% Tween 80 (left).



Purified Neuraminidase

Whole Virus Protein

Isolation of neuraminidase from Influenza recombinant X-31 B(H_3N_2) using 0.17% SDS and 10% Tween 80

Figure 6.4. Specific Activity of Neuraminidase during Affinity Chromatography Varies with Detergent Treatment. A). X-31B was disrupted with the indicated concentration of SDS or CTAB and then solubilized with 10% Tween 80 and dialyzed for two days against pH 5 absorbing buffer. Non-solubilized virus was pelleted and the supernatant was applied to the affinity column as described in the text. B). X-7 was disrupted with 1% SDS-10% Triton X-100 and neuraminidase was purified by affinity chromatography as above. As the specific activity of X-7 is about two-fold higher than that of X-31B, the specific activity scale for X-7 was adjusted so that specific activities of the two viruses appear of the same magnitude. V, virus; S, supernatant; P, pellet after centrifugation; E, enzyme pool; PP, protein pool.

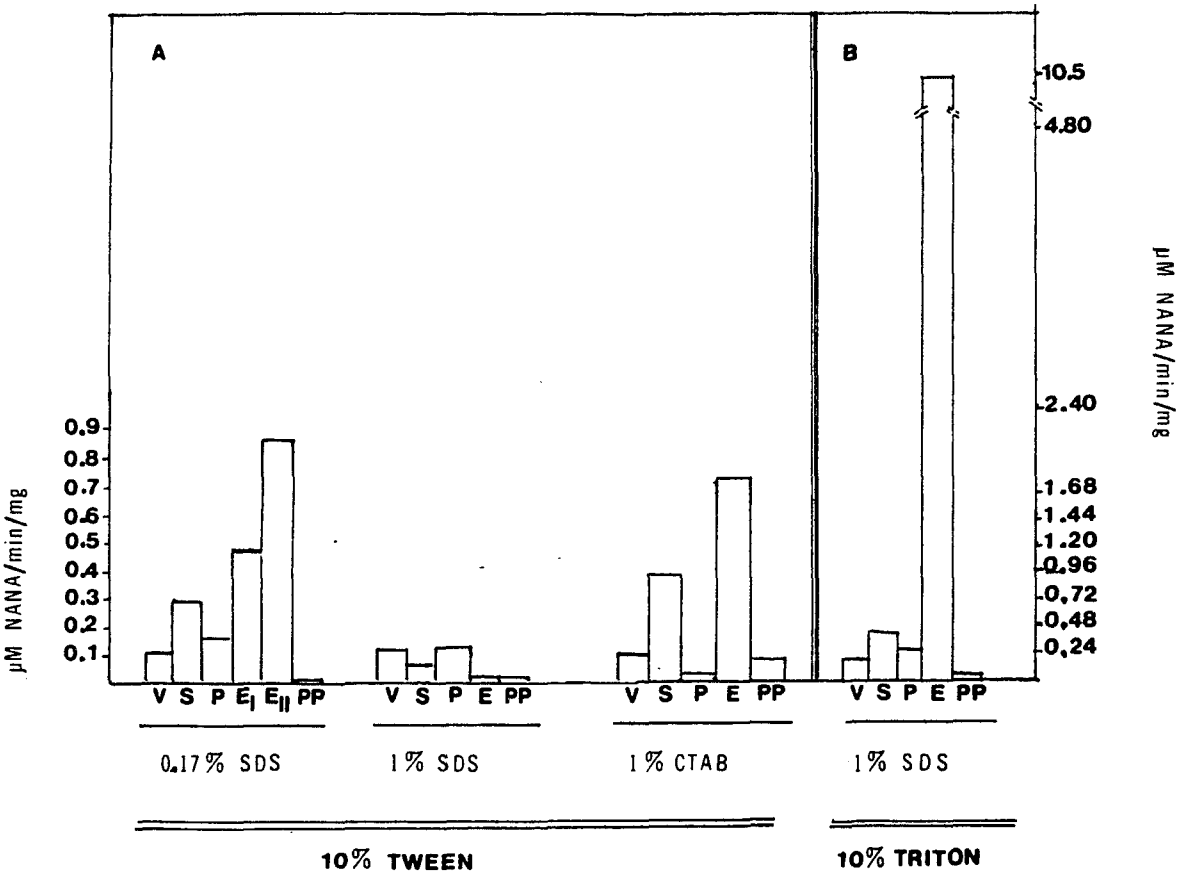
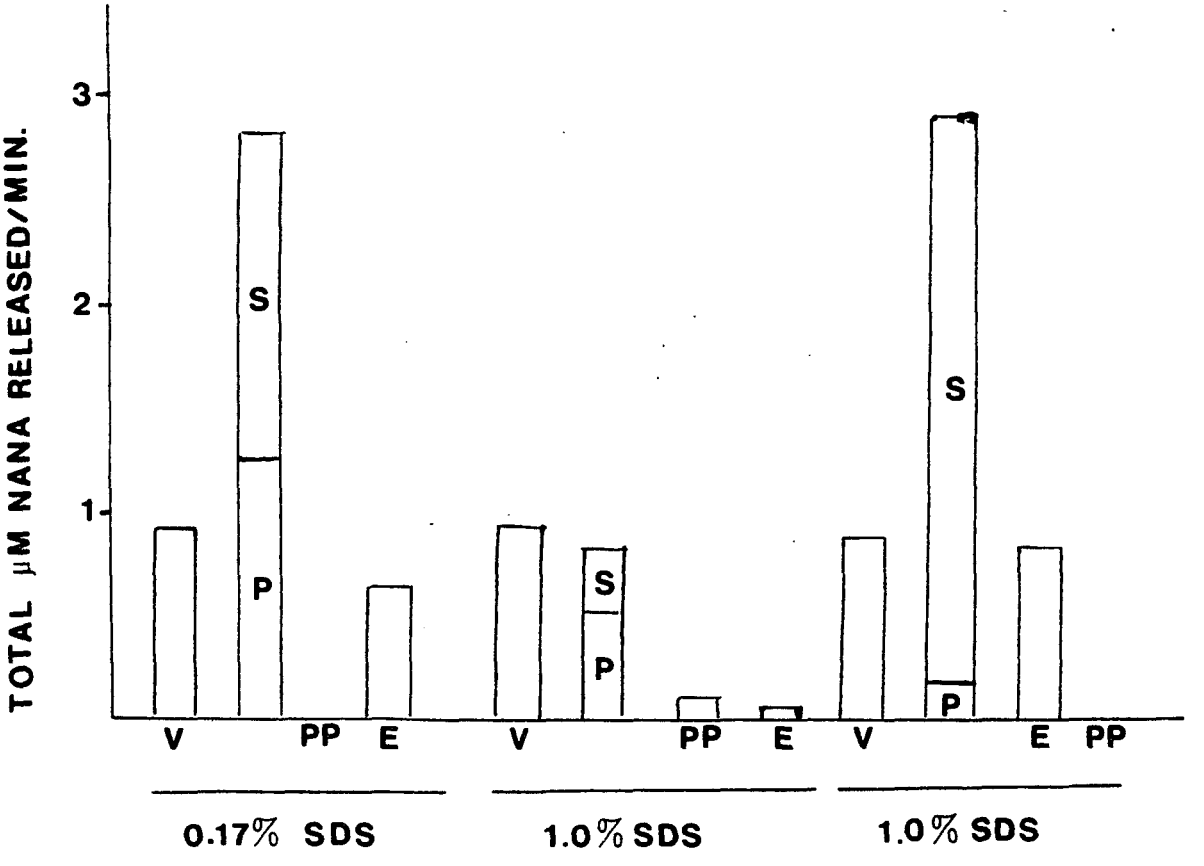

A
B
µM NANA/min/mg
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10.5
4.80
2.40
1.68
1.44
1.20
0.96
0.72
0.48
0.24
µM NANA/min/mg
V S P E$_I$ E$_{II}$ PP
V S P E PP
V S P E PP
V S P E PP
0.17% SDS
1% SDS
1% CTAB
1% SDS
10% TWEEN
10% TRITON

Figure 6.5 Total Neuraminidase Activity at Various Steps in Affinity Chromatographic Purification of Neuraminidase. Virus (X-31B) was disrupted with 0.17% SDS-10% Tween 80, 1% SDS-SDS-Tween 80, or 1% CTAB-10% Tween 80. Disrupted virus was dialyzed and solubilized viral protein, supernatant (S) was separated from unsolubilized, pellet(P) by centrifugation. Viral proteins that did not absorb to the column were collected in the protein pool(PP); material eluting in the enzyme pool was pooled (Enz). Total NA activity was determined by multiplying the volume in milliliters by the activity(μM/min/ml).


TOTAL µM NANA RELEASED/MIN.
3
2
1
S
P
S
P
S
P
V
PP
E
V
PP
E
V
E
PP
0.17% SDS
1.0% SDS
1.0% SDS

CHAPTER 7. STUDIES ON THE IMMUNOGENICITY OF NEURAMINIDASE

INTRODUCTION

It was our goal to investigate the immunologic potential of a single purified viral protein and in doing so to develop criteria for immunogenicity. Briefly we presented the neuraminidase antigen in equivalent amounts as monomer (unaggregated), in aggregated form, incorporated into liposomes, incorporated into liposomes with sonication, or in whole virus. These forms were characterized by enzyme activity, electron microscopy, and immunogenicity.

Morein and colleagues (1978) have shown that the glycoproteins of SFV are effective immunogens if aggregated or incorporated into lecithin vesicles. Both glycoproteins of the SFV were used, eliminating the need for extensive purification.

Oxford and associates (1981) used partially purified HA in aggregates and liposomes and reported that liposomally associated HA was a good immunogen but that aggregated HA was a poor immunogen. However, their HA(aggregated) was a mixture of monomer and aggregated HA.

Neuraminidase appeared an ideal candidate for a study correlating immunogenicity with conformation as enzyme activity provides an extremely sensitive index to conformation. A further advantage to use of neuraminidase in such a study is the potential usefulness of a purified neuraminidase vaccine for the NA-specific vaccine (see Chapter 3).

MATERIALS AND METHODS

Preparation of the virus. The X-7(H1N2) influenza A strain is a recombinant of the NWS(H1N1) and RI/5+(H2N2) strain: the neuraminidase of X-7 has been well characterized (Chapter 3). Virus was grown in the allantoic sac of embryonated eggs and purified as described in Appendix III. Virus used for purification of NA was from a pool of X-7 virus prepared at various time intervals over a two year period and stored at -20^{o}C.

Previous experience had shown that the X-7 neuraminidase is stable under these conditions and can be isolated in pure form.

Purification of the neuraminidase. Affinity chromatographic isolation of neuraminidase was as described in Appendix I. Viral preparations were adjusted to a protein concentration of 1mg/ml as determined by the Lowry assay -- throughout, stock buffer, pH 7, 0.05M sodium acetate, 0.002M $CaCl_2$, is used, unless otherwise stated. Viral preparations were disrupted with 1% SDS and mixed at 37^{o}C for 15 minutes. Triton X-100 was added to a final concentration of 10% and the preparations were dialyzed against pH 5 absorbing buffer (0.05M sodium acetate, 0.002M $CaCl_2$, 0.1% Triton X-100) for 48 hours. NA was isolated by affinity chromatography using the procedure of Cuatrecasas and Illiano (1971) as modified by Bucher (1977).

All fractions were assayed for protein and neuraminidase activity and purity of fractions was assayed by SDS polyacrylamide gel electrophoresis (PAGE), as described (Bucher and Kilbourne, 1972). Fractions that showed no contaminating protein by PAGE were pooled and concentrated by ultrafiltration in an Amicon model 52 and then dialyzed against stock buffer for 2 days.

Antigen preparation. Whole virus was diluted to 0.4mg/ml and dialyzed against stock buffer for one week. Monomer NA was prepared by dialysis of purified NA at a concentration of 0.04mg/ml against 2mM $CaCl_2$. Using (3H) Triton X-100 this laboratory has determined that this procedure removed more than 98% of Triton X-100 (Markovitz and Bucher, unpublished). Liposomal NA was prepared by mixing purified NA at a final concentration of 0.4mg/ml with the lipid mixture in a NA:lipid weight ratio of 1:100. The lipid mixture consisted of lecithin, stearylamine and cholesterol in a 7:2:1 weight ratio (lipids were obtained from Grand Island Biologicals). Liposomes were dialyzed against 2mM $CaCl_2$ for one week. Sonicated liposomes were prepared by 15 minutes sonication at room temperature . Due to instability of the liposomal NA activity, all NA liposomes were freshly prepared for secondary immunization.

All dialysis buffers were changed daily. Following dialysis, antigen solutions were diluted stepwise with dialysis buffer until doses were in final volumes of 0.25ml.

Electron Microscopy. Within two days of primary immunization neuraminidase preparations in all forms were appropriately diluted and mounted on carbon coated Formvar grids by application of a drop of sample to the grid for 1 - 2 minutes.

Staining was with 1% uranyl acetate for one minute. Grids were photographed the day of preparation. During the fourth week the stability of preparations was checked by electron microscopy.

Density Gradient Centrifugation. Neuraminidase liposomes were prepared as described above and the preparation brought to 45% (weight/volume) sucrose by addition of 85% sucrose. This solution was layered over the 60% sucrose cushion of a 5 - 30% sucrose gradient. Centrifugation was at 25,000 rpm for 18 hours at 5°C in a Beckman SW 41 rotor. Fractions (0.6ml) were collected and absorbance at 260nm was monitored. Neuraminidase activity was monitored immediately using 1µl volumes with a 30 minute incubation at 37°C. Fractions were dialyzed to remove sucrose and reassayed for enzymatic activity.

Immunization Protocol. A total of nine 3-4 month old rabbits were used to test each form of antigen -- three at each dose level. Animals were immunized on day 0 (primary immunization) and boosted (secondary immunization) on day 40 with the same dose. All immunizations were intravenous (no adjuvant was used). Bleedings were taken on days 0, 10, 28, 40, and 47.

Determination of antibody titers. The Neuraminidase Inhibition (NI) and Hemagglutination Inhibition (HI) assays were performed on all sera as described in Appendix II and III.

The NI titer is expressed as the inverse of the titer at which 50% inhibition of control level activity was observed. Actual titers were derived by plotting % activity against dilution on semilog paper and fitting the line by eye.

HI assays were performed in microtiter plates at 4°C. In all cases HI was performed in duplicate using both X-7(H1N2) and NWS(H1N1) strains: the HA of X-7 is derived from NWS: the NA is antigenically distinct.

RESULTS

Purification. As assayed by SDS PAGE, neuraminidase preparations used for antigen preparation appeared pure (Fig. 7.1). Heavy loading of gels revealed a band which co-migrated with nucleoprotein (NP) under non-reducing and reducing conditions, and did not stain for carbohydrate with periodic acid-Schiffs reagent; nucleoprotein is non-glycosylated and reduction insensitive. We assume that any hemagglutinin contamination would constitute considerably less than 5-10% of the total protein in the preparations.

Specific Activity. Table 7.1 gives the specific activities of the various antigen forms. The specific activity of intact virus was calculated on the assumption that neuraminidase constituted 10% of X-7 viral protein (Bucher and Kilbourne, 1972). As can be seen, specific activity was characteristic of antigen form and varied up to ten fold. The liposomal preparation was the most active, followed by monomer, viral and then aggregated neuraminidase forms.

When the neuraminidase assay is performed in the presence of Triton X-100 there is a three-fold increase in the activity of liposomal NA and a two-fold increase in the activities of the other forms. The three-fold increase in liposomal activity could reflect release of NA trapped in the interior lamellae of multi-lamellar liposomes.

Stability. Table 7.1 also shows the stability of these preparations (stored at 4°C) over the course of the experiment. The two fold increase in monomer activity may reflect instability of this form: this laboratory has observed an increase in NA activity of X-7 stored at 4°C. The activity of intact virus decreased by half and that of liposomes decreased four-fold. Liposomal activity did not decrease below that of aggregated. Because of the decrease in liposomal NA activity and because the NA liposomes appeared to degenerate with time

as revealed by electron microscopy, fresh NA liposomes were prepared and used for secondary immunization.

Electron Micrographs. Monomer NA (Fig. 7.2a) existed predominantly in the monomer form although in some fields small aggregates of NA were observed (not shown). Monomers were aligned head up so that the doughnut form of the tetrameric head (~80Å x 80Å) was clearly visible. No NA stalks or tails were visualized. No HA trimers were identified.

Aggregated NA (Fig. 7.2b) existed in both the aggregated and unaggregated forms. About half of aggregated NA existed as monomer. Aggregates ranged in size up to ~350Å and had the appearance of solid balls. The NA stalks and hydrophobic tails were not visible.

A typical field of NA liposomes in shown in Fig. 7.2d. Several large smooth surfaced vesicles can be seen. The fields of NA liposomes were characteristically studded with electron-lucent, "minisomes": these are not seen in fields of plain liposomes (Fig. 7.2c).

Sucrose Density Gradient Centrifugation. Analysis of neuraminidase liposomes by upwards flotation on a 10-30% sucrose gradient with a 60% sucrose cushion, indicated that neuraminidase was associated with liposomes of heterogeneous densities (Fig.

7.3). Association of neuraminidase with lipid is demonstrated by its ability to float under the conditions of centrifugation.

Immunogenicity studies. Following primary immunization with all antigen forms, NI titers were low or not detectable (Table 7.2). Secondary immunization revealed that all antigen forms had primed at the highest dose level (Table 7.2 and Fig. 7.4). The response to monomer was lowest and was very spotty (one animal responding well, one, poorly, and one not al all (Fig. 7.4), confirming our original observation on the variable immunogenicity of purified NA preparations that were not aggregated. The secondary NI response to aggregated NA was the highest: four-fold greater than to monomer ($P < 0.01$) and two-fold greater than than that elicited by intact virus. At the median dose level, no response was made to monomer NA and titers to viral, aggregated and liposomal NA were low and indistinguishable from each other.

The results confirm that monomer NA is a poor antigen and indicate that aggregated and liposomal NA are capable of priming to a NA response equal to or better than that of intact virus. The equivalence of the aggregated and liposomal forms of NA indicate that association with lipid per se did not improve the antibody response to the NA antigen.

DISCUSSION

The primary purpose of this experiment was to determine if a purified glycoprotein could be manipulated in such a way as to result in a highly immunogenic product which is potentially suitable for vaccine purposes. We were able to demonstrate that the purified NA molecule of influenza A was as immunogenic as the NA in whole virus if it were aggregated or incorporated into liposomes.

In the experiments presented in this chapter, we show that immunogenicity correlates better with aggregation than with activity: aggregated NA being the immunological equivalent of liposomal NA which had a nine-fold higher specific activity. This is in accordance with the findings of Helenius' laboratory (Morein et al., 1978; Balcarova et al., 1981) in studies of SFV glycoprotein vaccines, where they found no advantage of liposomal associated protein over aggregated protein.

Sonication of NA liposomes (data not shown) reduced immunogenicity; only one rabbit of three receiving 10µg of sonicated liposomal NA had a measureable NI. Sonication dramatically altered the morphology of the liposomes: following sonication the spheroid liposomes were seen to possess multi-

ple myelin type forms projecting from the surface. Sonication also reduced NA activity about one-third and led to earlier loss of NA activity(Table 7.1). Oxford and associates(1981) have reported that HA liposomes formed by sonication were more immunogenic than aggregated HA. They did not compare immunogenicity of purified HA samples with that of HA in whole virus. The disparity between Oxford's results and those obtained by Helenius' laboratory could well be due to either the difference in lipids and/or the variation between proteins, i.e. lipids could serve to enhance the immunogenicity of HA but not serve that function for other proteins. It may also be that HA is stable to sonication while NA is not. Moreover, the lipid-protein associations formed by sonication may be quite different from those formed when liposomes are made by dialysis. When liposomes are formed by dialysis (Helenius et al., 1977) the protein spikes appeared oriented outward as they are in the virion. In contrast liposomes formed by sonication in Oxford's laboratory showed the protein oriented both externally and internally. The dialysis method of liposomal formation may provide for a more orderly transition of the protein back into the lipid bilayer. It remains to be seen if the lipid-protein interactions formed by both methods are equivalent.

It would appear that aggregation is not the sole determinant of immunogenicity; the electrophoretically purified NA preparations of Laver and Valentine (1969) contain aggregated NA but are reported to be poorly immunogenic (Webster and Laver, 1967). In this regard it is of interest that our aggregated NA protein (which was immunogenic) has a very different appearance from that of electrophoretically purified NA. Our aggregates appear very similar to SFV aggregated spike protein (Simons et al., 1978). It is possible that isolation by SDS electrophoresis induces permanent conformational changes in the NA molecule. We have observed that denatured (enzymatically inactive) tetrameric NA purified by SDS chromatography is still capable of interacting with lipid (Chapter 9), indicating that denaturation does not affect the ability of NA to maintain conformation of that portion of the molecule which is capable of hydrophobic interactions.

Several interesting conformational dependent differences in neuraminidase were noted. As mentioned there were large differences in the specific activities of different forms: especially dramatic was the nine-fold difference in the specific activities of aggregated and liposomal neuraminidase. Differences were also observed by electron microscopy. Monomer NA was shown to be predominantly in the unaggregated state with a head size (80 x80Å) and appearance very similar

to that reported by others(Laver and Valentine, 1969; Wrigley et al., 1973).

It was difficult to characterize the appearance of NA in liposomal preparations. NA did not appear as the expected slender stalk, but appeared to aggregate in the lipid (Fig. 7.2d). The different appearance of NA aggregates and NA minisomes, as well as the dramatically different specific activities of the two forms indicated that the association of NA with liposomes seen on sucrose density gradient centrifugation was not due to electrostatic interaction between NA aggregates and lipid but represented actual incorporation of NA into lipid.

Oxford and colleagues (1981) have reported that non-ionic detergent purified NA appeared to be only superficially associated with liposomes: in their experiments lipid-protein interaction was effected by sonication. It may be that formation of liposomes by dialysis resulted in different lipid-protein interaction than is seen with the sonication method. In our experience sonicated NA liposomes lost immunogenicity even though specific activity only decreased by a third, indicating that some conformational change had occured.

In analyzing the predicted amino acid sequence from the known nucleotide sequences of N1 and N2 neuraminidases (Field

et al., 1981; Blok and Air, 1980a,b) it is noted that the neuraminidase shows a highly conserved sequence of twelve amino acids at the amino terminal: six hydrophilic amino acids followed by a hydrophobic stretch which is predicted to provide lipid anchorage. In light of what is known of the structure of other membrane glycoproteins these authors predict that NA would be a transmembrane protein with the amino terminal stretch of hydrophilic amino acids appearing on the cytoplasmic side of the membrane where they may serve some recognition function.

It may be that our failure to visualize spikes on NA liposomes is a result of the fact that NA is deeply embedded in lipid. Such a conformation would be consistent with the hypothesis that neuraminidase is a transmembrane protein, i.e. the stalk-tail length of 100Å reported by Wrigley and Laver and Valentine would allow 70Å for traversing the lipid bilayer and 30Å of stalk (and 40Å of head) extending from the lipid surface. More detailed studies are needed as at present it is difficult to say with confidence what conformation the NA actually assumes in the virion, as length measurements are made of the viral fringe i.e. viewed in profile, and from this angle it is difficult to distinguish NA from the far more numerous HA spikes.

SUMMARY

One promising approach to vaccines against influenza and other enveloped viruses is the use of purified viral glycoprotein vaccines (Neurath and Rubin, 1971; Crumpacker, 1980). Glycoprotein vaccines have thus far shown themselves to be of low reactogenicity; they have the advantage of immunizing to only the antigens capable of inducing relevant antibodies; and in the case of viruses which have the potential to be latent, purified protein vaccines appear to be a safer immunogen than whole virus vaccines.

The major obstacle to the use of purified influenza A viral glycoprotein vaccines has been their relatively poor immunogenicity (Neurath and Rubin, 1971). We have shown the NA of influenza to be as effective an antigen in aggregated or liposomal form as in intact virus, whereas in monomer form it was very poorly immunogenic.

As there is evidence that the immune response to NA in primed humans is dampened by the presence of the HA antigen (Chapter 3), it is possible that the immunogenicity of aggregated NA in humans may be even greater than is indicated by the NI response in rabbits.

As there is no immunological advantage to liposomes over aggregated NA, and as immunogenicity did not correlate with enzymatic activity but with the state of aggregation it is concluded that aggregation, whether into protein or lipid micelles, is a better predictor of immunogenicity than enzyme activity. This is not to say that enzyme activity is not needed, or that a change in the NA activity of an antigen form was not an indication of change in conformation (as seen with sonicated liposomes).

As aggregated NA was as immunogenic as liposomal NA and because of the instability of liposomal NA, it would appear that aggregated NA is the better candidate for a purified NA vaccine.

Figure 7.1. SDS polyacrylamide gel of purified neuraminidase. Affinity purified neuraminidase was concentrated and then dialyzed to reduce Triton X-100 concentration to about 3%. 20µg by the Lowry assay was loaded on a 10% SDS PAGE gel and run at 3ma/gel tube till the dye front had migrated to the end of the tube. Under these conditions NA migrates with an approximate molecular weight of 240,000 daltons(Bucher and Kilbourne, 1972).

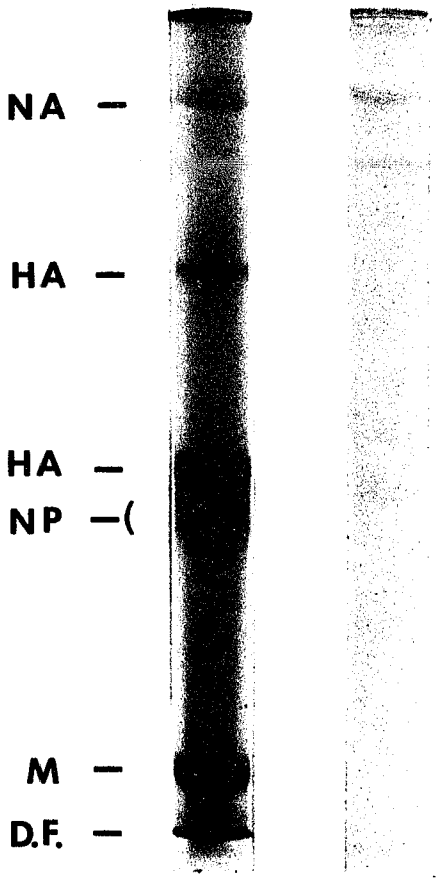

NA
HA
HA
NP
M
D.F.

Figure 7.2a. Monomer form of the neuraminidase. Purified NA was brought to 0.1% Triton X-100 concentration by one week dialysis against 0.1% Triton X-100, 2mM $CaCl_2$. Samples were mounted on carbon coated Formvar grids and stained with 1% uranyl acetate as described in text. NA heads measure ~80 x 80Å. Bar represents 500Å. Final magnification, 148,000. Electron micrograph by Dr. J. Schwartz.

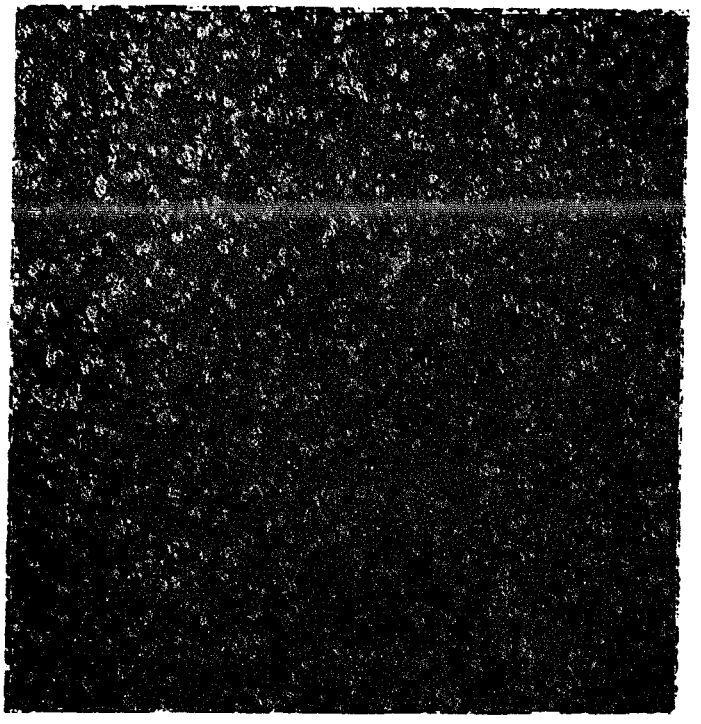


Figure 7.2b. Aggregated Form of Neuraminidase. Purified NA was dialyzed against 2mM $CaCl_2$ for one week, at the end of which time Triton X-100 levels were below detection. This procedure aggregated about half of the the NA molecules. This field showed a higher percentage in aggregated form. Aggregates varied in size up to ~350Å. Bar represents 1000Å. Final magnification, 165,000. Electron micrograph by Dr. J. Schwartz.

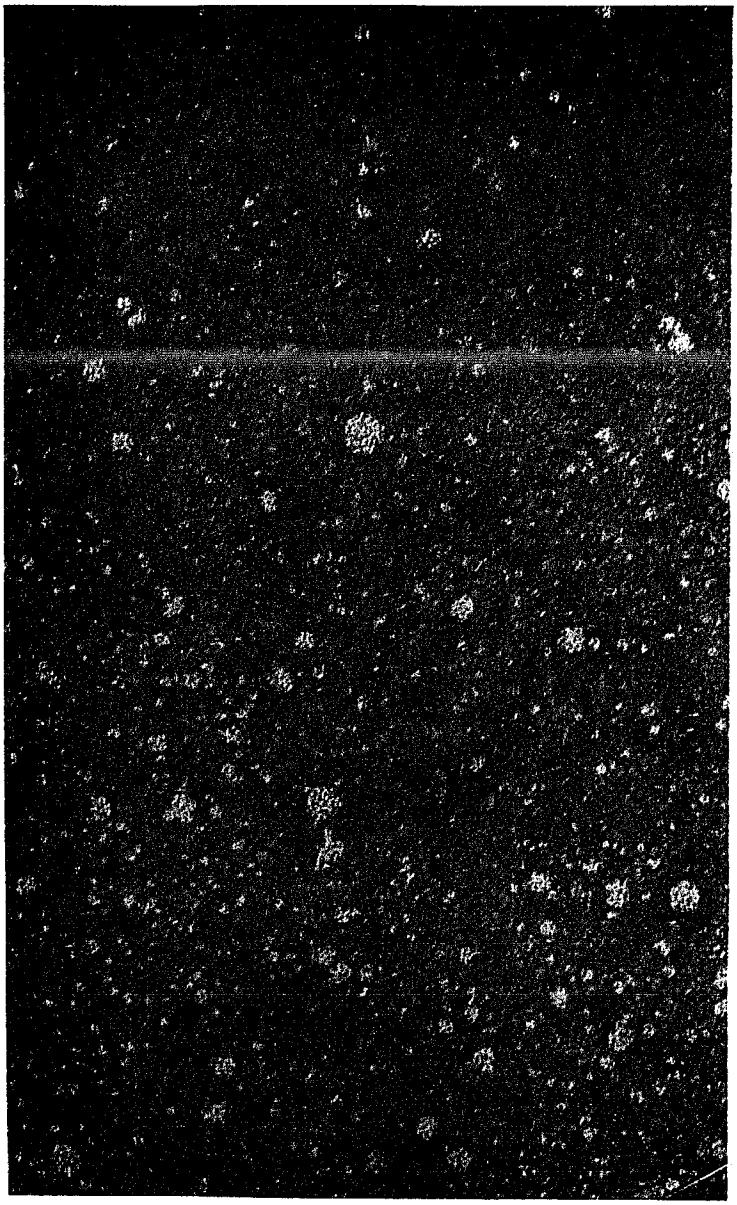


Figure 7.2c. Plain Liposomes. Liposomes were dialyzed against 2mM $CaCl_2$ for one week. The pleomorphism and aggregation appear to be calcium ion effects on the membrane. Bar represents 1000Å. Final magnification, 158,000.

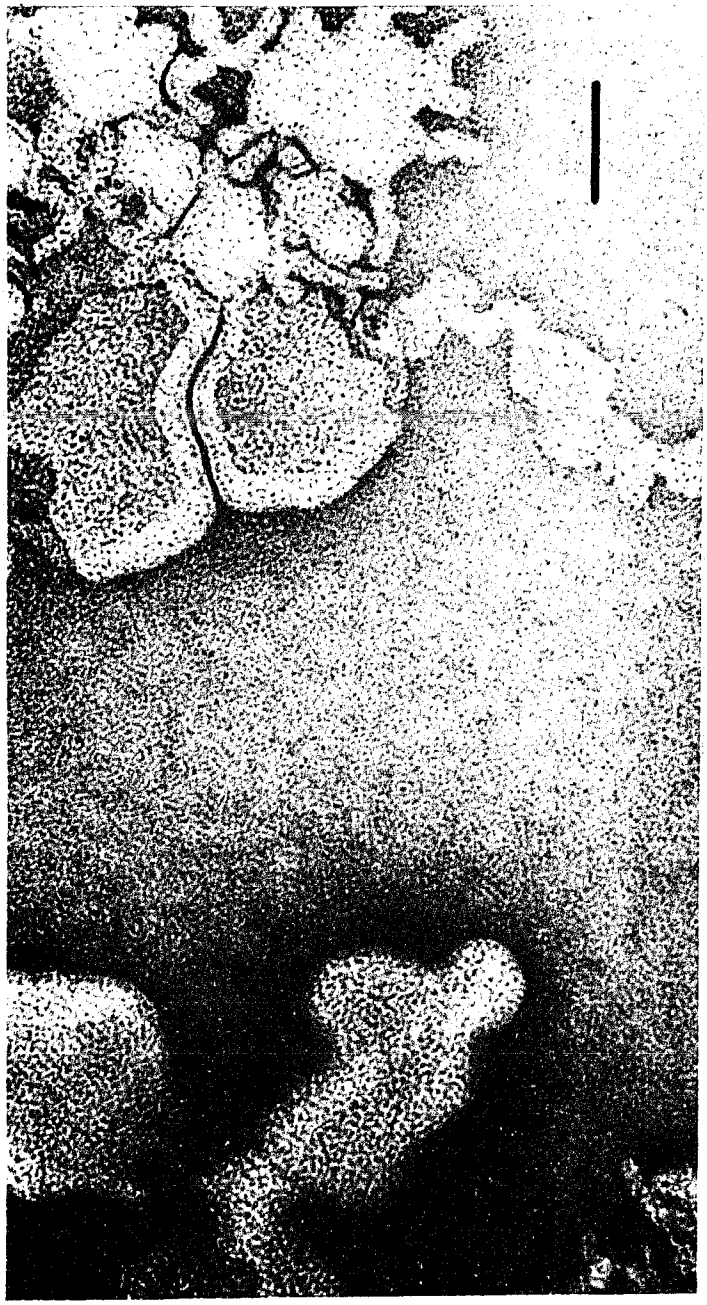


Figure 7.2d. Neuraminidase Liposomes of 1.07gms/ml density. Note the presence of "minisomes", small vesicles that range in size up to 600 Å, and larger vesicles. Particles approximately the size of NA heads can be seen aggregated on the surface of the vesicles (arrows). Bar represents 1000Å. Final magnification, 158,000.

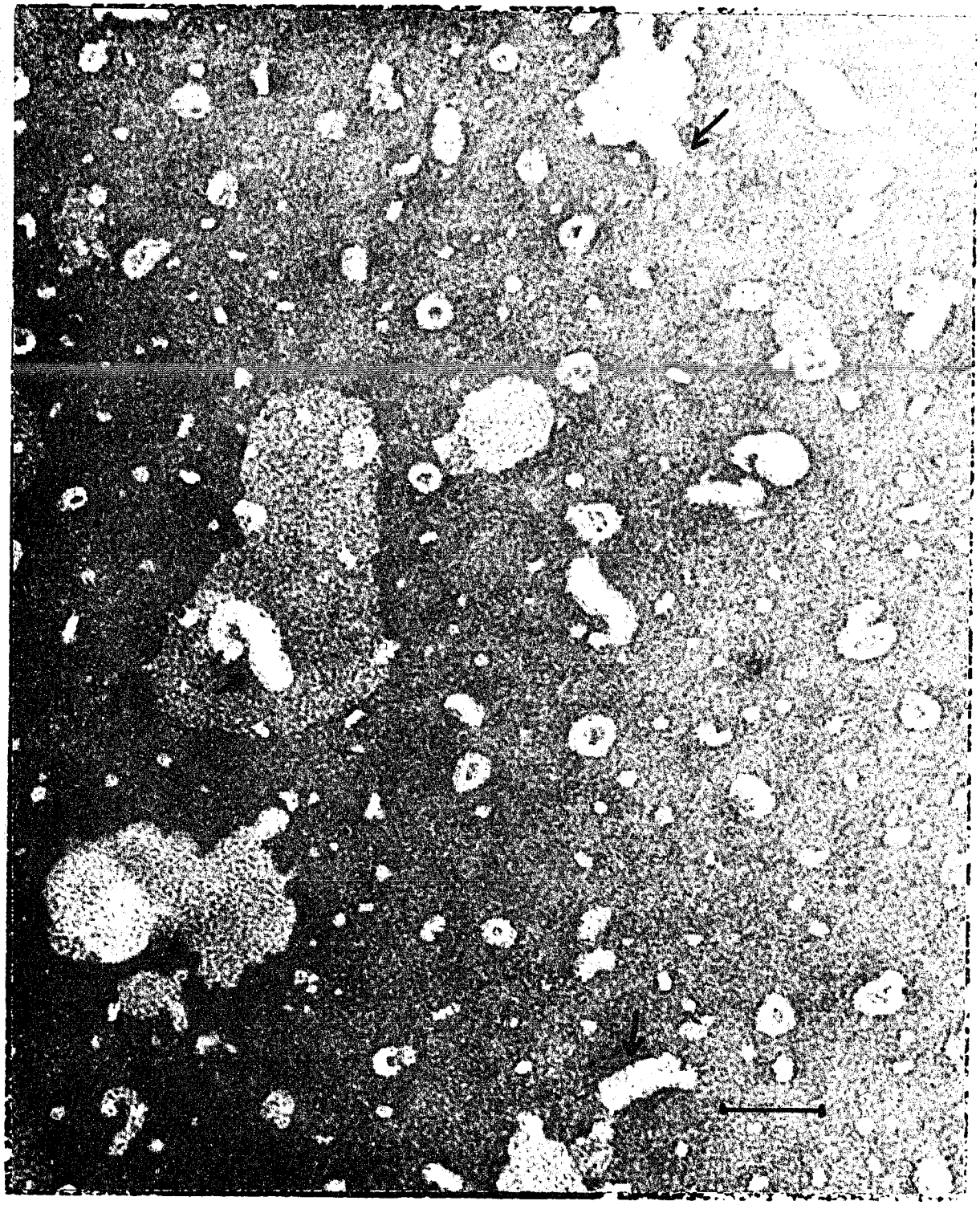


Figure 7.3. Flotation of NA Liposomes on Sucrose Density Gradient. NA lipsomes or plain liposomes in 45% sucrose solution were layered over the 60% cushion of a 10-30% sucrose gradient made with 2mM $CaCl_2$, and centrifuged at 25,000 rpm for 18 hours at 5^oC in a Beckman SW 41 rotor. 0.6ml fractions were collected and absorbance at 260nm was monitored. NA activity was assayed on dialyzed samples as described in materials and methods. The upwards flotation of NA with lipid indicated association with lipid. NA activity was associated with lipid (A_{260nm}) of density 1.18-1.03gm/ml. Plain liposomes -----; NA liposomes ———; NA activity •--•--•; density ———.

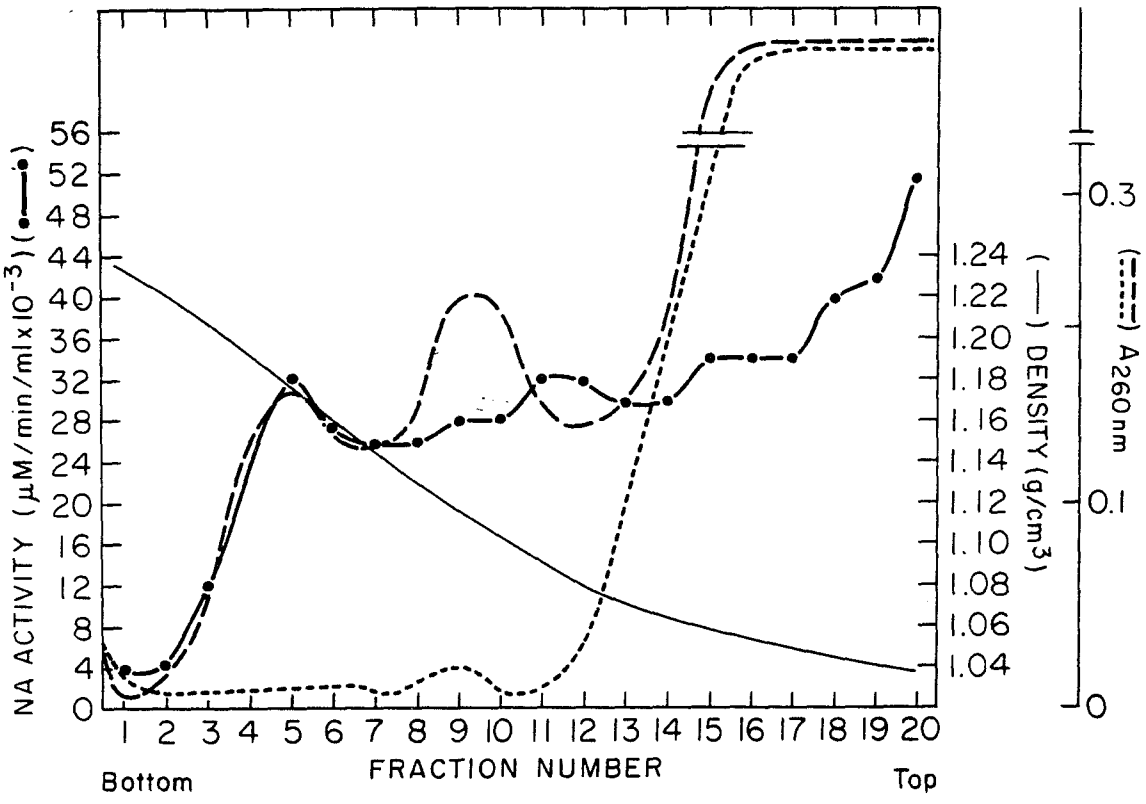

NA ACTIVITY (μM/min/ml x 10⁻³) (•—•)
56
52
48
44
40
36
32
28
24
20
16
12
8
4
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Bottom
FRACTION NUMBER
Top
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1.10
1.08
1.06
1.04
(—) DENSITY (g/cm³)
0.3
0.1
0
(- - -) A260nm

Figure 7.4. Final Day NI Titers. Three animals were immunized twice with 10, 1 or 0.1 µg of NA in intact virus, monomer, aggregated or liposomal form as described in materials and methods. Animals were bled seven days after secondary immunization and NI titers were calculated as described in the text. No response to the 0.1µg levels were seen. At the 10µg NA dose level aggregated NA was significantly more immunogenic ($P < 0.1$) than monomer. The NI response to aggregated NA is two-fold higher than the NI response to whole virus but this is not statistically significant(there is a great variation in individual rabbit response and the number of rabbits in each group is small).

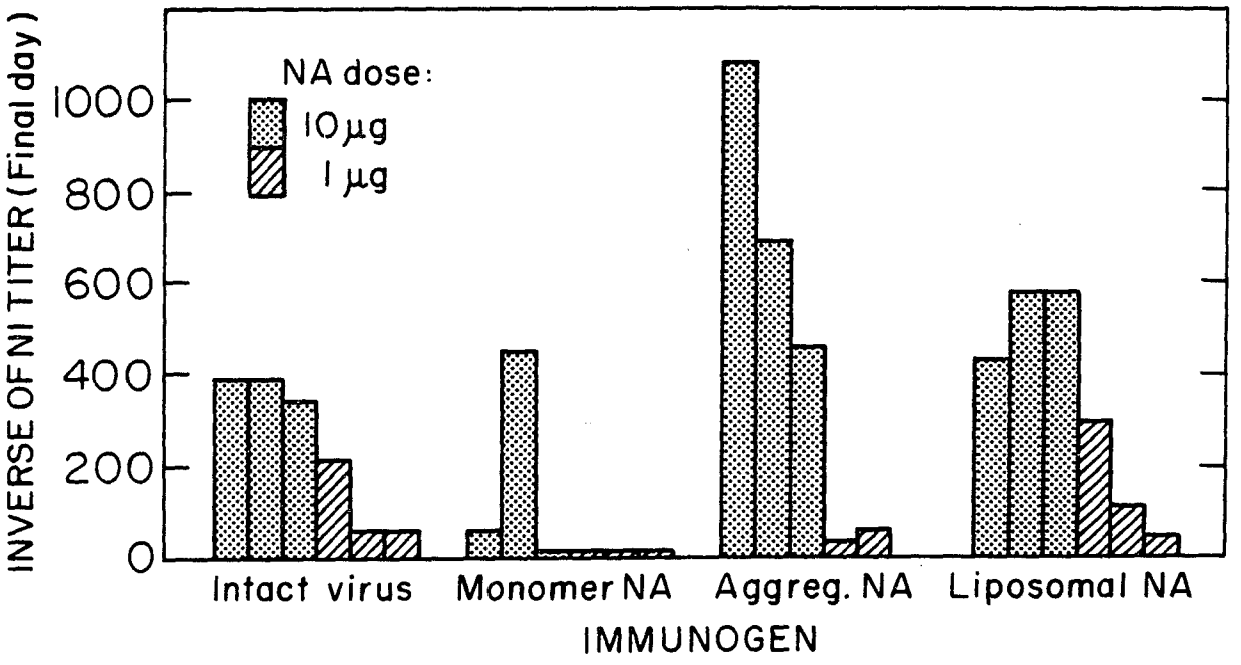

INVERSE OF NI TITER (Final day)
1000
800
600
400
200
0
NA dose:
10 µg
1 µg
Intact virus
Monomer NA
Aggreg. NA
Liposomal NA
IMMUNOGEN

Figure 7.5. Correlation of Enzyme Activity with Immunogenicity. The mean final NI titer following secondary immunization of animals receiving 10μg NA doses was divided by the specific activity on day 0.

NI Response per Enzyme Unit

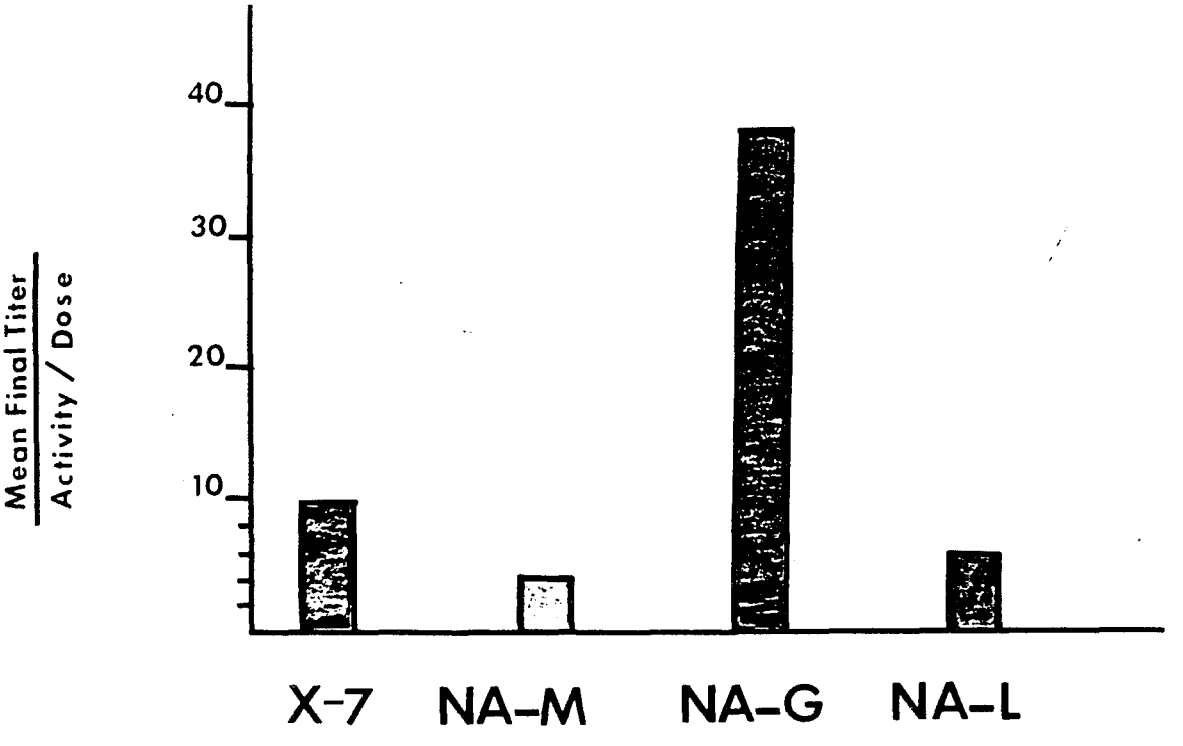


Table 7.1. Enzymatic Stability of NA Samples Over the Course of the Experiment. Enzyme activity is expressed as specific activity(μM/min/mg). The specific activity of intact virus is calculated on the basis that NA constitutes 10% of viral protein, i.e. the specific activity of virus on day 0 was 0.2 μM/min/mg of total protein. Samples were stored at $4^{o}C$ and assayed on days indicated.

a). Assay was performed in the presence of 0.1% Triton X-100. Number represents average fold increase in specific activity done in duplicate of three assays on three different days.

b). Specific activity of freshly prepared liposomal NA used for secondary immunization.

TABLE 7.1

Immunogen	Day				Triton X-100
	0	8	19	36	Enhancement Factor
Intact Virus(X-7)	2.0	0.9	0.9	0.9	2
Monomer NA	4.0	5.3	4.5	7.9	1
Aggregated NA	1.0	1.3	1.2	1.2	2
Liposomal NA	9.5	2.75	2.5	2.9	3
				(12.5)b	
Liposomal NA sonicated	N.D.	3.0	0.5	N.D.	N.D.
			(7.03)b		

Table 7.2. Average NI Responses to Secondary Immunizations. Secondary NI titers represent the average of the three animals at each dose level.

Table 7.2

Immunogen	dose (μg)	2° NI Response 47d
Intact Virus (X-7)	100	380
	10	117
	1	-
Momomer NA	10	175
	1	-
	0.1	-
Aggregated NA	10	757
	1	34
	0.1	-
Liposomal NA	10	538
	1	158
	0.1	-

CHAPTER 8. INCORPORATION OF M PROTEIN INTO LIPOSOMES

INTRODUCTION

THE M (matrix or membrane) protein is a major protein constituent of three groups of enveloped viruses: orthomyxoviruses (influenza viruses); paramyxoviruses; and rhabdoviruses (Fenner, 1970). These groups of viruses are defined as enveloped because the external glycoprotein antigens are situated in a lipid bilayer. In the case of influenza virus, M protein appears to form an electron-dense layer inside the lipid bilayer (Compans and Dimmock, 1969; Bachi et al., 1969; Choppin and Compans, 1975). Although the M protein is the most abundant protein in the virus particle, relatively little is known about its function, perhaps in part due to its hydrophobic properties (Gregoriades, 1973).

SDS gel chromatography permits the isolation of milligram quantities of M protein (Bucher, 1975; Bucher et al., 1976). Since the influenza virion is an enveloped virus, we examined the interaction of M protein with artificial lipid bilayers, or liposomes. An artificial system of M protein and lipid should provide an interesting model for studying the function of M protein in the virion and its possible interaction with the glycoprotein spikes and/or the ribonucleoprotein of influenza virus.

METHODS

Preparation of M protein. M protein was purified from a commercial lot of X-38 (H7N2) virus which had been formalin treated for vaccine use. The vaccine was the gift of Lederle Laboratories, Pearl River, N.Y.. M protein was isolated by SDS gel chromatography on Bio Gel A-5m (Bio Rad) columns following disruption of the virus preparation with 10% SDS. As previously described, M protein eluted from the A-5m column under non-reducing conditions following the major peak of hemagglutinin and nucleoprotein, with a molecular weight of

25,000 daltons (Bucher et al., 1976). SDS polyacrylamide gel electrophoresis was performed as previously described (Bucher and Kilbourne, 1972).

Removal of SDS from M protein. SDS was removed by continuous flushing of the M protein preparation while the protein was maintained in a large volume to promote formation of the monomer form of SDS (Reynolds and Tanford, 1970a). Approximately 10mg of M protein was diluted to 200 ml with distilled water in an Amicon model 202 ultrafiltration unit equipped with PM-10 membranes. The unit was connected to an RG12 reservoir (Amicon) containing 4 liters of distilled water. The entire 4 liters of distilled water were flushed through the 200ml of dilute M protein solution and the M protein sample concentrated to 10ml. The M protein sample was shown to be free of SDS by the absence of hemolysis of an equivalent volume of a 0.5% solution of erythrocytes in microtiter plates; this indicates a concentration below 0.00045% SDS, the lower limit of detection in this test.

Formation of liposomes containing M protein. The appropriate phospholipid mixtures were mixed with M protein and solubilized by the use of octylglucoside (Sigma) in a ratio of 1 mg protein : 5 mg total lipids : 25 mg octylglucoside (Baron and Thompson, 1975; Helenius et al., 1977). All lipids employed

were products of Grand Island Biological Company. Liposomes were prepared with ovolecithin alone or with a mixture of lipids to provide a net negative or a net positive charge. The liposomes containing a net negative charge were prepared in the molar ratio of lecithin:dicetyl phosphate:cholesterol of 9:2:1: the liposomes containing a net positive charge were prepared in the same molar ratio with stearylamine substituted for dicetylphosphate. Liposomes formed on dialysis of the octylglucoside from the preparation versus 0.002M $CaCl_2$ at $4^{o}C$ for 48 hours: the dialysis medium was replaced and dialysis continued for an additional two days.

Sucrose Gradient Centrifugation. Following formation of the liposomes by dialysis, the liposomes were fractionated by centrifugation or flotation on sucrose gradients. The liposome mixture (1 ml) was either overlaid on a 10-30% sucrose gradient with a 1.5 ml sucrose cushion of 60% or placed in a layer of 40% sucrose between the gradient and the 60% sucrose cushion and the liposomes separated by flotation. Centrifugation was performed in an SW 27.1 rotor at 120,000g for 18 hours. Fractions were collected from the gradients and assayed for protein by the Lowry assay (Lowry et al., 1951); one drop of 10% SDS was added to each assay to facilitate solubilization of the liposomal preparation. Density of fractions was determined by refractive index measurements. Liposomal preparations were dialyzed versus distilled water to remove sucrose.

Electron Microscopy. The liposomal suspension was placed on the surface of carbon coated formvar grids and stained with a 1% aqueous solution of uranyl acetate. Freeze fracture was performed by a standard technique. A drop (~1 ul) of liposomal pellet in distilled water was frozen on 3 mm Balzars gold discs in Freon-22 cooled to -150^{o}C in liquid nitrogen. Cryoprotective agents were not used. Discs were mounted on the specimen stage of a Balzars BAF-301 unit. The fracture procedure was carried out at -130^{o}C and at a pressure of 110^{-6} torr. The fractured surface was shadowed with platinum/carbon. The replicas were cleaned with sodium hypochlorite for at least 6 hours, rinsed twice in distilled water, then mounted on electron microscopic grids. Specimens were examined in a JEM-100B electron microscope at an instrument magnification of 30,000.

RESULTS

A large proportion of the liposomes formed in the presence of M protein migrated to a density of 1.22 gm/ml with a

smaller proportion of these liposomes having a light density of 1.04gm/ml(Fig. 8.1). Liposomes with no added protein had a density of 1.03 gm/ml (Fig. 8.1). Heavy density liposomes (1.20-1.22 gm/ml) formed in the presence of M protein for all mixtures of lipids employed; net negative charge, net positive charge or neutral charge (lecithin only). M protein was shown to be present in the heavy density liposomes by SDS PAGE (Fig. 8.2).

Liposomes containing M protein with a net negative charge were chosen for additional studies since they appeared to be more stable than positively charged or neutral liposomes. After storage at $4^{o}C$ for a one week period, liposomes with a net positive or neutral charge showed considerable loss of the discrete heavy fraction on sucrose gradient centrifugation with formation of heterogeneous lighter components (data not shown). The M-liposomes with a net negative charge maintained the same distribution on sucrose gradient centrifugation even after a one week period of storage at $4^{o}C$.

Negative staining and examination by electron microscopy showed no difference in the external apppearance of liposomes containing M protein to those without M protein (Fig. 8.3). Specimens prepared by freeze-fracture for electron microscopy showed dramatic difference in the liposomes with and without

M. Liposomes with M protein were frequently multilammelar with no specific surface features (Fig. 8.4). Liposomes containing M protein were distinct mulberry-like structures which appeared to be unilamellar (Fig. 8.4). The M liposomes are studded with 100 Å particles: allowing 10 - 20 Å for shadowing (Baskins, 1977) the actual particle size is estimated to be 80 - 90 Å.

One field (area = 0.1 um^2) was sampled from each of 13 M liposomes in figure 8.4 to determine the particle density of these liposomes. The median particle density was 3000/um^2 with a mean particle density of 2885/um^2. The range in particle density was 1600 - 3600/um^2.

DISCUSSION

M protein purified from X-38 virus vaccine readily associated with lipid vesicles. The M protein integrated into the lipid bilayer during formation of liposomes by dialysis regardless of the net charge on the lipid vesicles.

Liposomes formed in the presence of M protein had two discrete densities, either a heavy density of 1.22gm/ml which contained M protein or a light density of 1.04 which contained no detectable M protein. A continuum of M liposomes of various densities was not seen, indicating that the formation of M liposomes involved cooperative interaction, i.e. condensation or coalescence of the M protein with the lipid bilayer and with itself.

The size (80 - 90 Å) and distribution (3000/um^2) of the particles seen in M liposomes is similar to that found for intramembranous particles of other biological membranes examined by electron microscopy following freeze fracture preparation. Segrest and associates (1974) found 80Å particles at a density up to 4000/um^2 in preparations of the membrane penetrating peptide of the MN glycoprotein in phospholipid vesicles. Freeze etch preparations of erythrocyte membranes show 75Å particles at a concentration of 4200- 4900 particles/um^2 (Tillack et al., 1972). Vesicles of fragmented sarcoplasmic reticulum prepared by freeze fracture show 80Å intramembranous particles at a density of 3800 - 3900/um^2(Baskin, 1977).

The liposomes formed in the presence of M protein appeared unilamellar; multilamellar structures were not seen on electron microscopic examination of M protein liposomes

prepared by freeze fracture. Multilamellar liposomes were frequently seen in the absence of M protein in addition to smaller vesicles which appeared unilamellar. The incorporation of M protein into the lipid bilayer appears to alter liposomal structure.

CONCLUSIONS

Self assembly has been amply demonstrated in the case of non-enveloped viruses with helical or icosahedral symmetry (Kaper, 1968). Reconstitution of solubilized membranes with recovery of biological activity has been demonstrated for a paramyxovirus, HVJ or Sendai virus (Hosaka and Shimizu, 1972). Glycoprotein "spikes" of influenza virus have been shown to insert into artificial membranes (Almeida et al., 1975: Huang et al., 1979: Oxford et al., 1981). Co-formation of M protein liposomes with the glycoprotein spikes will provide a model system for the study of the assembly of enveloped viruses (see Chapter 9).

Assembly of the virion in the host cell may be dependent on the ability of M protein to form domains on the inner sur-

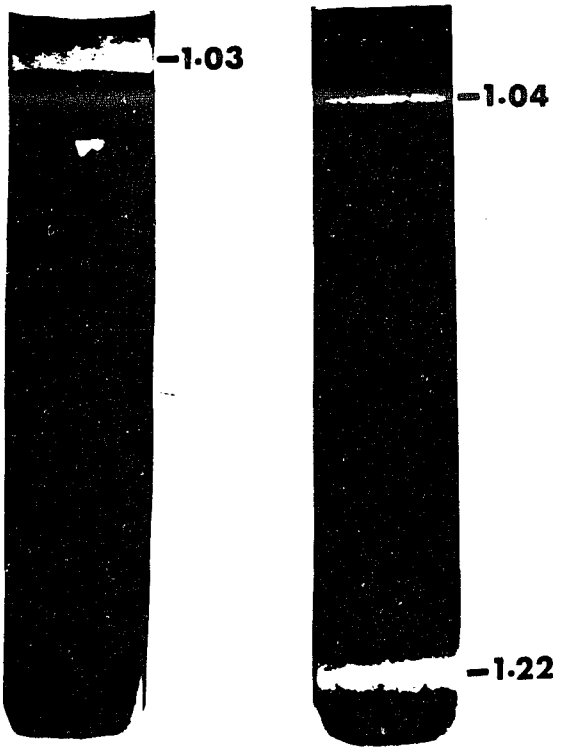
face of the cell membrane (Choppin and Compans, 1975). We have shown the considerable affinity of M protein for interaction with itself and the lipid bilayer, which indicates the possibility that maturation of influenza virus involves formation of M protein domains in infected cell membranes as has been observed by Bachi (1980) for Sendai virus.

M protein domains in the membrane of infected cells could result in concentration of glycoprotein spikes in these areas. The M protein domains in turn may then interact with ribonucleoprotein complexes causing the "budding" and maturation of viral particles. It is entirely possible that the conformation of M protein in the presence of the other viral proteins will be quite different than seen in M liposomes.

Studies of inhibitors which will interact with M protein and prevent its interaction with itself and with other viral proteins or the lipid bilayer in model liposomal systems could be of considerable assistance in the design of antiviral pharmacologic agents effective against influenza and other enveloped viruses.

Work presented in this chapter was done in collaboration with I.G. Kharitonenkov, J.A. Zakomirdin, V.B. Grigoriev, S.M. Klimenko and published by Bucher et al., in 1980.

Figure 8.1. Sucrose gradient centrifugation of a). liposomes without M protein added and b). liposomes formed in the presence of M protein. Note heavy band with a density of 1.22gm/ml in the presence of M protein on gradient (b). Light density bands had densities of 1.03 gm/ml on gradient (a) and 1.04 gm/ml on gradient (b).



M-liposomes

Figure 8.2. SDS polyacrylamide gel electrophoresis of M protein preparation (gel a) and M protein liposomes (gel b) isolated from the sucrose density gradient with a density of 1.22 gm/ml (see Figure 8.1). Approximately 10 μg of protein was applied to the gel.

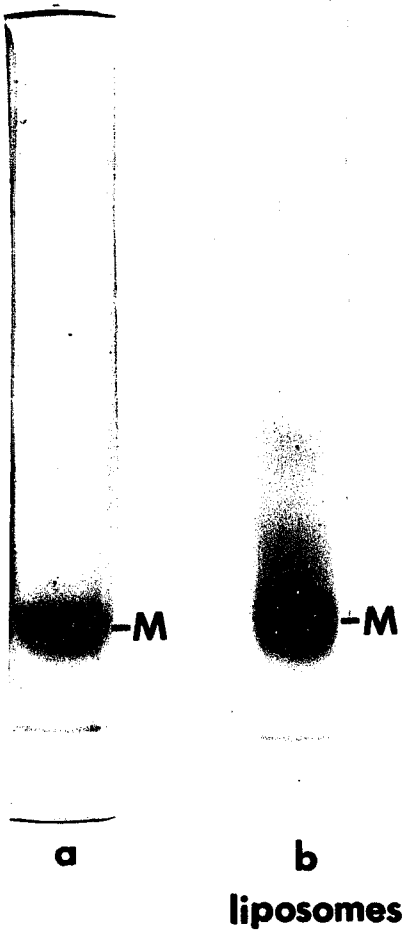

M
M
a
b
liposomes

Figure 8.3. Electron micrographs of liposomes following negative staining. Upper portion of figure (a), liposomes without M protein; (b) liposomes formed in the presence of M protein and migrating to a density of 1.22 gm/ml on sucrose gradient centrifugation (see Figure 8.1). Final magnification is 60,000. Electron micrograph by V.B. Grigoriev.

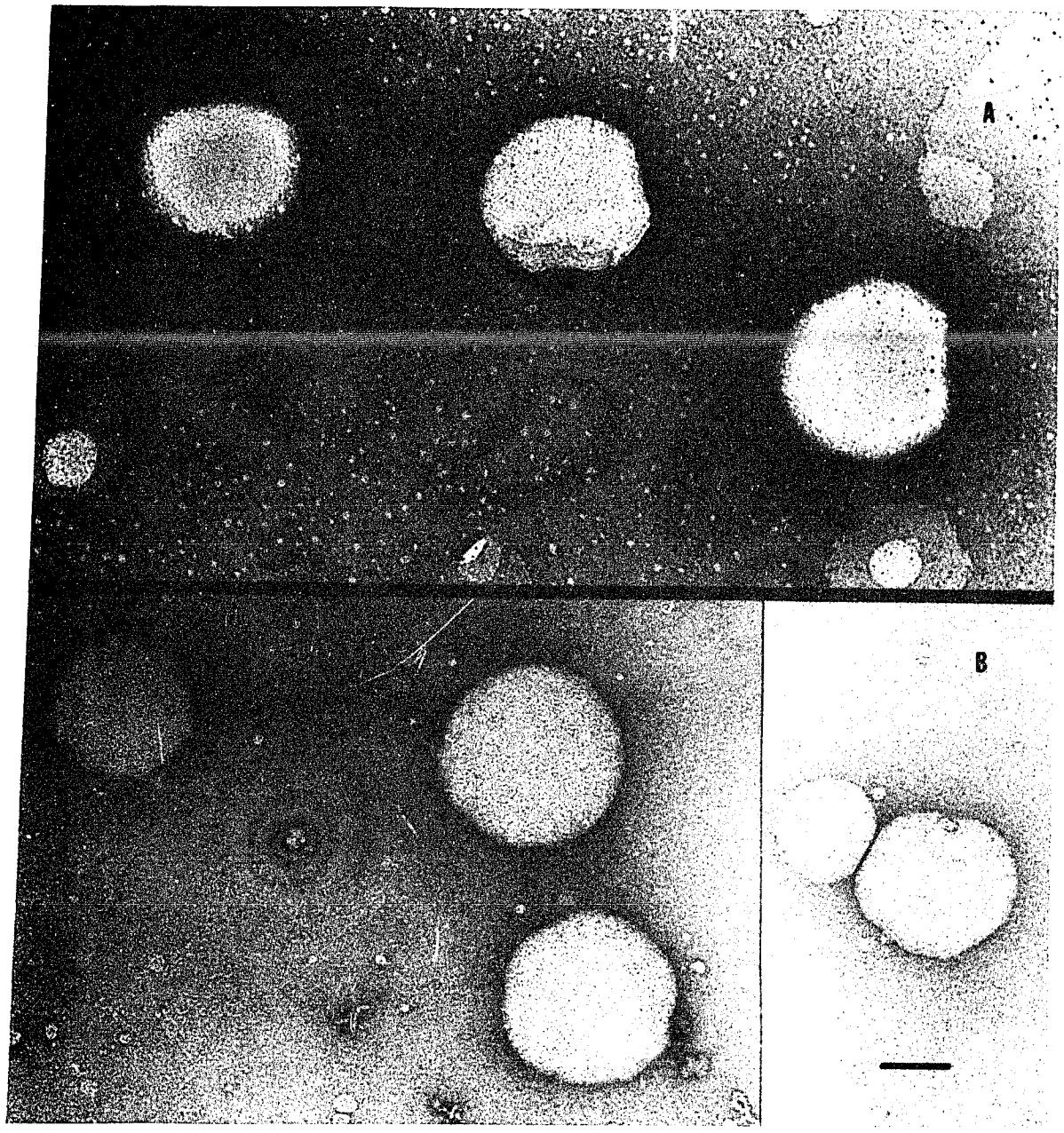
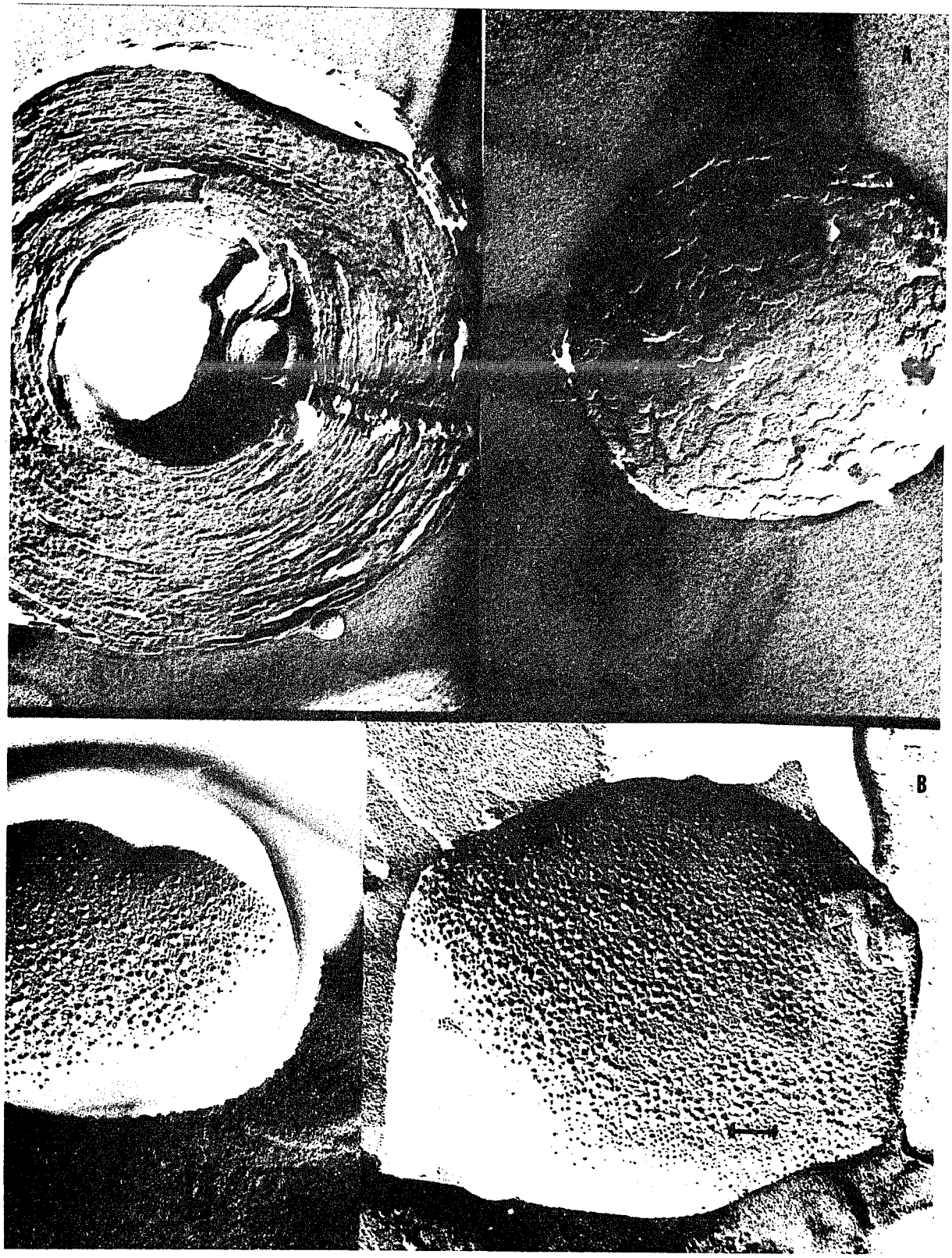

A
B

Figure 8.4. Electron micrographs of specimens prepared by freeze fracture (see Methods). Liposomes formed in the absence of M protein are shown in the figure (a). Liposomes formed in the presence of M protein having a density of 1.22gm/ml are shown in the lower figure (b). Final magnification is 60,000 for multilamellar vesicles in upper part of (a); 90,000 for vesicles shown in lower half of (a) and in (b). Electron micrograph by V.B. Grigoriev.


A
B

CHAPTER 9. INTERACTION OF NEURAMINIDASE AND M PROTEIN

INTRODUCTION

The M protein of type A influenza virus has been visualized as forming a capsid-like structure internal and proximal to the virus membrane(Compans and Choppin, 1973). As reviewed in Chapter 4, the M protein is a major structural protein of the virus constituting about 40% of the total viral protein. M protein's other functions are not clearly understood -- that it is multifunctional is indicated by the following: production of M protein is the rate-limiting step in virus replication; M is associated with virus yield, amantadine sensitivity and the ability of a strain to become cold-adapted (Chapter 4). These facts suggest a structural role for M protein and its probable central role in virus assembly and perhaps also in dis-assembly (in the initiation of the replicative cycle).

This laboratory has demonstrated that purified M protein of influenza A incorporates into the lipid bilayer to form a discrete population of liposomes with a density of 1.22gm/ml and a morphology similar to that reported for other intramembranous particles. We have also seen that M protein is highly aggregated in aqueous suspension (data not shown). The affinity of M protein for a lipid environment, and its tendency to self-aggregate both in lipid and aqueous solutions suggests that incorporation of newly synthesized M protein into patches in the membranes of infected cells is feasible.

Bachi (1980) recently demonstrated that Sendai virus infected cells display viral protein domains in the cell membrane. These domains are formed by intramembranous particles arranged in a crystalline pattern; both nucleocapsids and Sendai-specific glycoproteins are seen associated with these domains. Freeze etch micrographs show the presence of intramembranous particles during and for a short period following budding. With maturity the intramembranous particles disappear and the virions take on the typical myxovirus morphology: the M protein appears as a capsid-like structure beneath the virion lipid bilayer. Bachi has suggested that the intramembranous particles seen in viral domains of infected cell membranes is M protein and that it appears to be serving a recruitment purpose.

A possible model for virus assembly is as follows: M protein incorporates into the cellular membrane via its affinity for lipid and its tendency to self-aggregate. M protein domains recruit viral glycoproteins by specifically interacting with these, and the M-glycoprotein domain is recognized by nucleocapsids. To explain how M could be extruded from the lipid bilayer between assembly and maturation it must be postulated that the virion morphology is altered, either as a function of changed interactions among the viral proteins or indirectly by a change in the lipid environment of released virions.

In this paper we report experiments aimed at elucidating the interaction of M protein with the neuraminidase of influenza virus.

MATERIALS AND METHODS

Preparation of protein. M protein was purified by SDS chromatography from a batch of X-38 (H7N2) virus prepared for vaccine use (a gift of Lederle Laboratories, Pearl River N.Y.) or

from a batch of X-53a (H1N1) virus prepared for vaccine use(a gift of the Canadian government) which was β propriolactone inactivated. M was purified as described in Chapter 8.

Neuraminidase was isolated by affinity chromatography from the X-7 (H1N2) recombinant as described in Chapter 8. Neuraminidase from such preparations is highly immunogenic if aggregated (by removal of Triton X-100) or incorporated into liposomes (Chapter 7).

Highly purified, denatured neuraminidase (designated NA_{TSS}) was purified from affinity chromatography enriched preparations by the following procedures.

NA enriched fractions from the enzyme pool of affinity chromatography were pooled and concentrated to 1 - 2 mg/ml protein concentration by the Lowry assay in an Amicon model 52, using a Diaflo XM-50 ultra-filtration membrane. Three ml of this preparation were applied to a 2.5 x 90 cm Bio-gel A-5m column (Bio-rad) previously equilibrated with TSTR buffer pH 7.4, 0.02M Tris-HCl, 0.1% Triton X-100, 0.05% sodium azide). All fractions were collected with a LKB 2000 fraction collector in 1 ml volumes and the A_{260nm} was monitored with an LKB Uvicord I. NA rich TSTR fractions were identified by enzyme activity and PAGE gels, and pooled and concentrated as before using a Diaflo PM-10 membrane. Concentrated fractions were

brought to 5% SDS (weight/vol.), heated in a 56^{o}C water bath for 30 minutes and 2 - 3 ml samples were applied to a Bio-gel A-5m column previously equilibrated with TSS buffer (pH 7.4, 0.02M Tris-HCl, 0.1% SDS, 0.05% sodium azide). One ml fractions were collected as above. NA fractions were identified by SDS PAGE gels (there is no detectable NA activity at this point), pooled and concentrated to 2 - 3 ml by ultrafiltration using PM-10 membranes and dialyzed against daily changes of distilled water for five days. All but the last change of water contained 0.05% sodium azide. In this laboratory this procedure has been shown to reduce SDS to below detectable levels.

Preparation of Liposomes. Liposomes were formed by dialysis following solubilization of the lipids or lipid-protein mixture with octylglucoside. Control liposomes were prepared after solubilizing the mixture of lecithin:dicetyl phosphate: cholesterol (molar ratio 9:2:1) in 2% octylglucoside. Liposomes containing viral proteins were formed by the addition of the purified protein(s) to the same ratio of lipids as used for control liposomes. The protein:lipid ratios (by weight) were: M protein liposomes, 1:5 (0.8mg:4mg); neuraminidase liposomes, 1:25 (0.16mg:4mg); and neuraminidase-M-protein liposomes, 1:5:25 (0.16mg:0.8mg:4mg). Liposomal preparations were dialyzed against 2mM $CaCl_2$ for one week to remove detergent.

Sucrose Gradient Centrifugation. Liposomes were characterized by sucrose gradient centrifugation as described in Chapter 9.

Electron Microscopy. Neuraminidase-M-liposomes and M liposomes were mounted on carbon coated Formvar grids by the application of a drop of sample to the grid for one minute, and stained for one minute with 1% phosphotungstic acid or 1% uranyl acetate.

RESULTS

Sucrose Density Gradient Centrifugation.

Control liposomes (those containing no added protein) showed multiple forms which banded in an area of light density (1.04 - 1.07 g/ml) as shown in Fig. 9.1. These multiple bands may represent liposomes containing varying numbers of lamellae in the liposome.

M protein liposomes formed two major liposomal peaks on centrifugation confirming our earlier observations with M protein liposomes (Fig. 9.2). There was a fraction rich in M

protein, of heavy density, and a second fraction of light density with little or no detectable protein. The density of the heavy band (1.15gm/ml) is lighter than the 1.22 gm/ml found in our earlier studies. M protein liposomes formed with X-53a M protein had a density of 1.17gm/ml (data not shown).

The protein found at the top of the gradient is not M protein but appears to be a contaminant of the egg lecithin batch: the protein co-migrates with ovalbumin on 10% SDS PAGE gels (data not shown).

Neuraminidase liposomes. When liposomes were formed in the presence of affinity purified neuraminidase, NA activity was found associated with the light (1.07 - 1.03) density liposomal bands (fig. 9.3). Association of neuraminidase with lipid did not significantly alter the densities of the liposomal fractions, unlike the case with M protein liposomes where the M protein served to greatly increase the density of the M protein liposomes. A substantial portion of the neuraminidase aggregated and sedimented to the bottom of the gradient. No substantial amount of lipid was associated with this fraction as judged from lack of turbidity at 260nm.

Neuraminidase-M protein liposomes.

The presence of M protein greatly enhanced the incorporation of neuraminidase into the lipid bilayer (Fig. 9.4).

Three times as much neuraminidase activity is associated with liposomes in the presence of M protein as compared to that found in the absence of M protein (Fig. 9.3); the same quantity of neuraminidase from the same batch had been added during formation of liposomes in both cases. Nearly two-thirds of the neuraminidase activity associates with the heavy density M protein containing liposomes. A slight increase in density is detected, from 1.15gm/ml as seen in Fig. 9.2 for M protein liposomes to 1.17 gm/ml, an increase of 0.02gm/ml. The amount of neuraminidase activity associated with the light density liposomes is similar to that seen for NA liposomes (Fig. 9.3).

NA_{TSS} liposomes.

Because denatured NA is believed to non-immunogenic, we were interested in characterizing the interaction of NA_{TSS} (which was denatured and had been purified for sequencing) with lipid and with M protein to determine if we could detect clues as to the nature of the change in conformation. As can be seen in Fig. 9.5, NA_{TSS} incorporated into negative liposomes as evidenced by the fact that no protein was seen at densities greater than 1.12 gm/ml. What is interesting is that under our conditions of liposome formation, denatured NA appears to be more efficiently incorporated into lipid than active neuraminidase: no protein appears to have sedimented to the bottom of the tube as in Fig. 9.3.

NA_{TSS}-M protein liposomes.

When M protein was present with NA_{TSS} during liposomal formation, two protein containing populations of liposomes were formed (Fig. 9.6), a light fraction with density ranging from 1.10 - 1.04gm/ml and a dense population with a density of 1.17, which is the density of X-53a M liposomes. As there is no NA activity on this gradient it was not possible to determine if there was NA in the heavy density liposomes. A lack in shift in density for the M protein liposomes suggests there was not. It is interesting that significantly more protein than can be accounted for by NA_{TSS}, remains with the lighter density NA_{TSS} in the lighter density liposomes. It may be that interaction with NA_{TSS} somehow prevented M from self-aggregating into heavy density liposomes. This experiment suggests that while NA_{TSS} was capable of interacting with lipid it did not seem capable of interacting with M protein in the same cooperative manner that was seen with active neuraminidase.

Electron Microscopy. M liposomes in this experiment were formed in the presence of $CaCl_2$ since neuraminidase has a calcium requirement (Chapter 3). In the presence of calcium, M liposomes are pleomorphic (Fig. 9.7), and display the same tendency to aggregate that is seen with plain liposomes (Fig.

7.2c). Liposomes containing neuraminidase and M protein are shown in Fig. 9.8b and d. They do not differ significantly in appearance from plain liposomes. (Fig. 9.8a and c).

DISCUSSION

M liposomes formed with M protein from X-38 (H7N1) vaccine and used for the experiments reported in Chapter 8 showed a density of 1.22 gm/ml. In subsequent experiments M protein liposomes derived from X-38 showed a density of 1.15gm/ml. Liposomes made from the X53a (H1N1) vaccine showed a density of 1.17gm/ml. It is probable that the variation in density seen in M protein liposomes at different times is both strain dependent, virus treatment dependent, and dependent on the lipid batch used for liposomal formation. There may be other variables such as temperature during formation and centrifugation that also affect the density.

The results show that although density may vary, in all cases M protein liposomes formed a discrete heavy density liposomal population as well as a protein free, light density population.

That the method of protein purification is of importance in predicting protein behavior is illustrated by a comparison of the characteristics of affinity purified neuraminidase and NA_{TSS}. The enzymatically inactive highly purified NA_{TSS} appeared to interact more efficiently with lipid than enzymatically active neuraminidase. During centrifugation a substantial portion of affinity purified neuraminidase did not interact with lipid but remained as aggregates and precipitated to the bottom of the tube, whereas it appears that all NA_{TSS} interacted with lipid to form light density NA_{TSS} liposomes.

When M protein was added during liposomal formation, affinity purified neuraminidase was more efficiently incorporated into lipid and appears to have formed liposomes containing both proteins. NA_{TSS}, however, does not appear to have interacted with M protein to form heavy density liposomes; indeed it appears that in the presence of NA_{TSS} some M protein failed to be incorporated into heavy density liposomes. An interesting possibility is that NA_{TSS} interaction with M protein or with lipid somehow hindered the co-operative process by which M protein is incorporated into lipid.

Incorporation of NA into liposomes. When NA liposomes were formed with positively charged lipids at an NA:lipid ratio of 1:100 all neuraminidase was incorporated into lipid

(Fig. 7.3). When neuraminidase was incorporated into negatively charged liposomes at an NA:lipid ratio of 1:25 not all NA was incorporated into lipid. Bucher and Schwartz had previously observed (unpublished) that NA appeared to be more efficiently incorporated into positively charged liposomes. The experiments performed here do not substantiate or disqualify this observation as the protein to lipid ratios as well as lipid charge and detergents are different in this experiment than in that outlined in Chapter 7. In this experiment negatively charged liposomes were used, as M protein liposomes of positive charge are unstable (Chapter 8).

CONCLUSIONS

In the absence of M protein, neuraminidase appeared in liposomes of light density and migrated as non-lipid associated NA aggregates. When neuraminidase and M protein were present during liposomal formation, all neuraminidase, as assayed by activity, was associated with lipid: some NA activity was associated with liposomes of light density, but most of the NA activity appeared in a discrete liposomal peak of

density 1.17. The presence of M protein enhanced neuraminidase activity considerably. Comparison of Fig. 9.3, neuraminidase liposomes, with Fig. 9.4, neuraminidase-M protein liposomes shows that more than twice as much activity was present on the latter gradient.

The interaction of M and NA to form a discrete liposomal population, the enhancement of NA incorporation in lipid in the presence of M protein, and the high specific activity of the NA in the neuraminidase-M protein liposomes are all indicative of specific interaction of the two proteins.

The enhanced incorporation of neuraminidase into liposomes in the presence of M protein suggests two possibilities for the mechanism of formation of neuraminidase-M protein liposomes. Neuraminidase may directly interact with M protein through its hydrophobic tail resulting in incorporation of the enzyme into liposomes containing M protein, or it may be that M protein alters the lipid bilayer in such a way that the lipid is more conducive to insertion of the neuraminidase.

We have previously shown the considerable affinity of M protein for interaction with itself and the lipid bilayer (Chapter 8), and the ability of neuraminidase to interact with lipid (Chapter 7). We have now demonstrated the interaction of neuraminidase and M protein in co-formation of liposomes.

Interaction of M protein with the lipid bilayer could alter membrane structure during viral infection of the host cell. This interaction could result in concentration of glycoprotein spikes in an area of "condensed" M protein-lipid domains (Choppin and Compans, 1975; Robertson et al. 1979; Bachi, 1980). The M protein in turn may then interact with ribonucleoprotein complexes causing the "budding" of viral particles (Robertson et al. 1979; Bachi, 1980).

The ability to construct virosomes containing one or more of the viral components may also help us to understand the mechanisms by which these proteins interact with each other and with the lipid bilayer and should provide a valuable tool for exploring many facets of virus cell interaction: viral maturation, viral penetration (Huang et al., 1979; Rott, 1980; Oxford et al., 1981) and immune conformation and processing. They may also prove a useful in vitro system for development of effective anti-viral agents.

Figure 9.1. Control liposomes. Turbidity of the liposomal fractions on the gradient was monitored by absorbance at 260nm(-----). Density was measured by refractive index(_____).

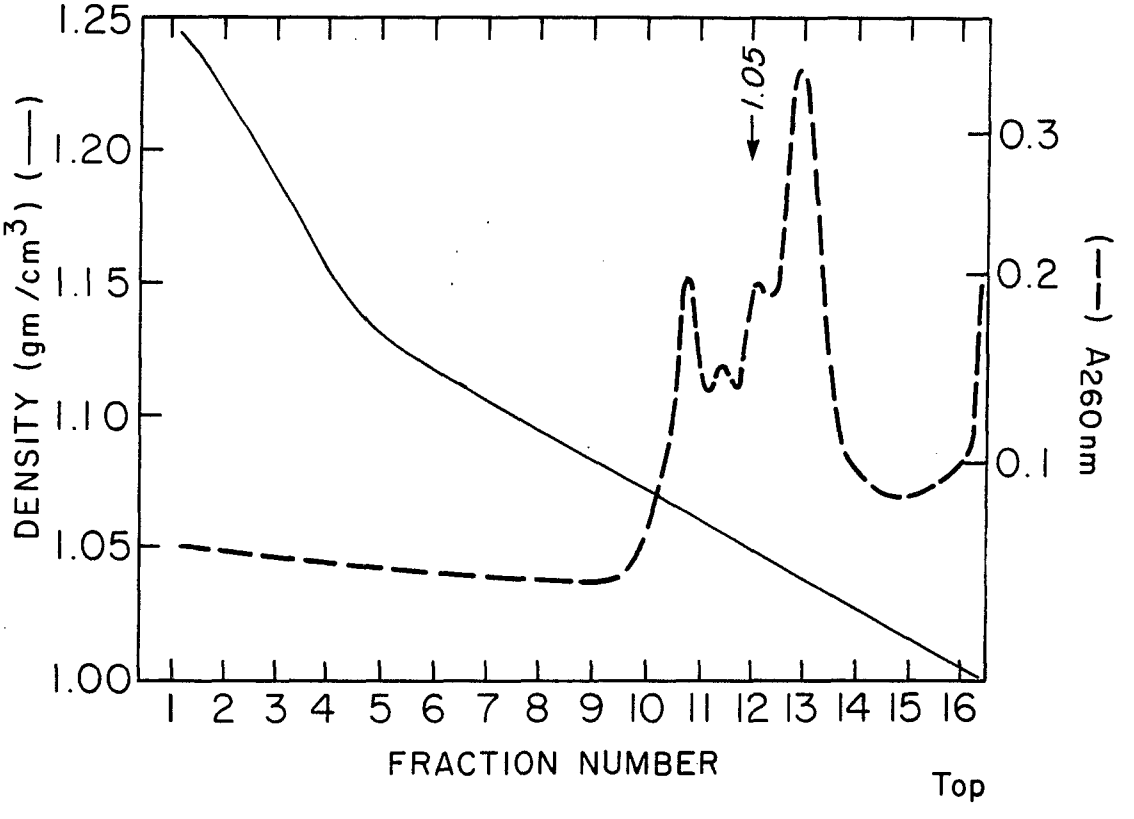

1.25
1.20
1.15
1.10
1.05
1.00
DENSITY (gm /cm3) (—)
1.05
0.3
0.2
0.1
(---) A260nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
FRACTION NUMBER
Top

Figure 9.2. M protein liposomes. Protein concentration is indicated by the shaded area (-o-o-o); turbidity by absorbance at 260nm (- - -). M protein is associated with the liposomal fraction of 1.15gm/ml.

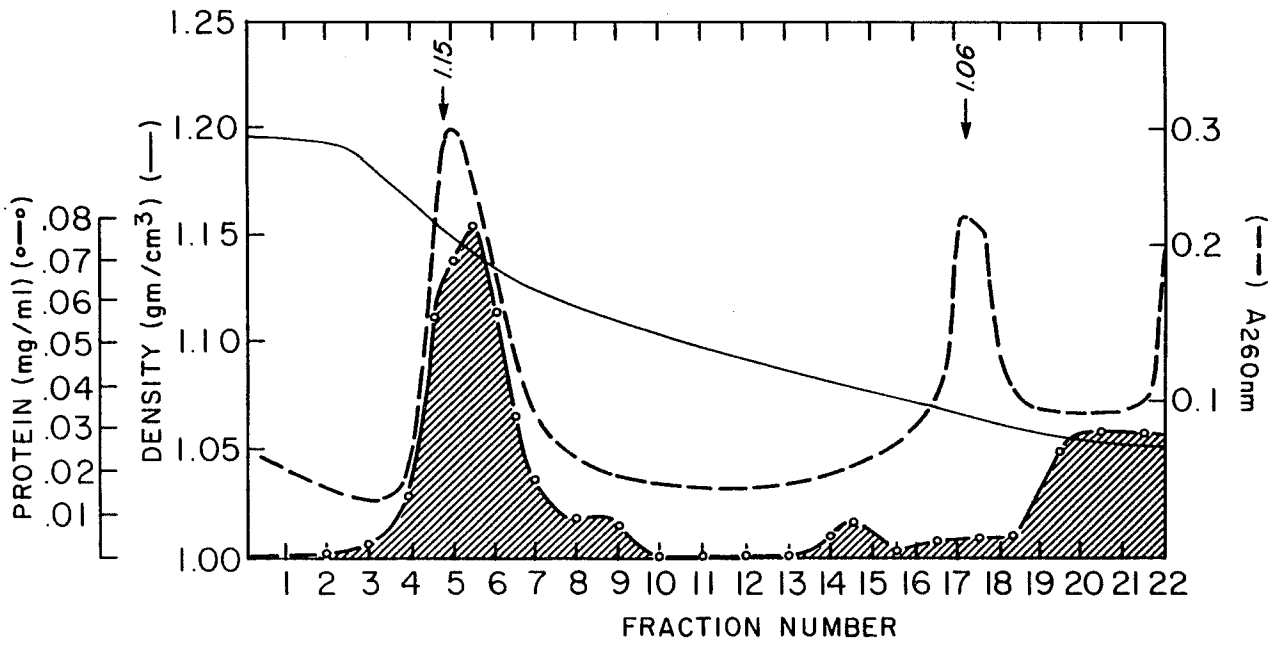

1.25
1.20
1.15
1.10
1.05
1.00
DENSITY (gm/cm³) (—)
1.15
1.06
0.3
0.2
0.1
(– – –) A260nm
.08
.07
.06
.05
.04
.03
.02
.01
PROTEIN (mg/ml) (o—o)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
FRACTION NUMBER

Figure 9.3 Neuraminidase liposomes. Neuraminidase activity is represented by the stippled area; turbidity by absorbance at 260nm (- - -). Neuraminidase activity is associated with low density liposomal fractions (1.03 to 1.07gm/ml). A significant portion of the neuraminidase activity sediments under these conditions.

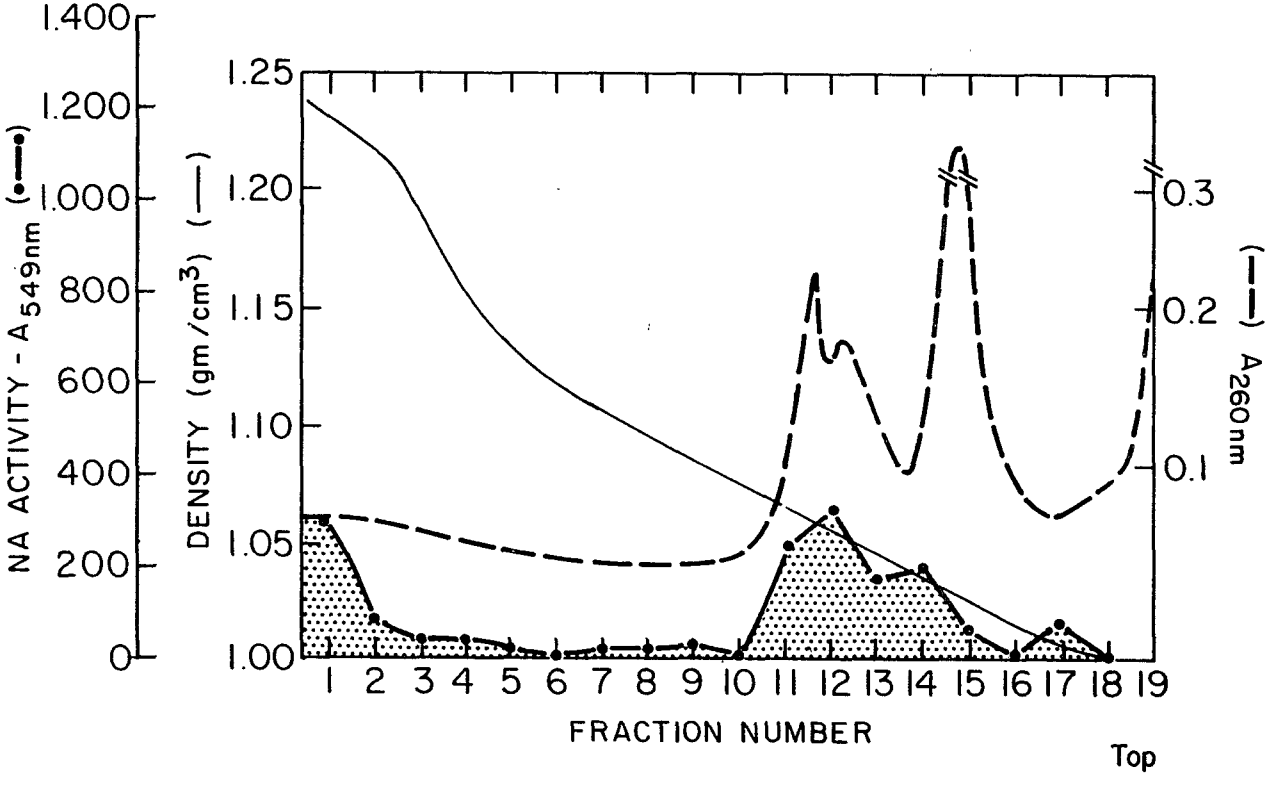

1.400
1.200
1.000
800
600
400
200
0
NA ACTIVITY - A549nm (•—•)
1.25
1.20
1.15
1.10
1.05
1.00
DENSITY (gm/cm3) (—)
0.3
0.2
0.1
(—) A260nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
FRACTION NUMBER
Top

Figure 9.4. Neuraminidase-M protein liposomes. Neuraminidase activity is represented by the stippled area; turbidity by absorbance at 260nm (- - -). A substantial portion of neuraminidase activity is associated with the heavy density liposomal fraction containing M protein (1.17gm/ml).

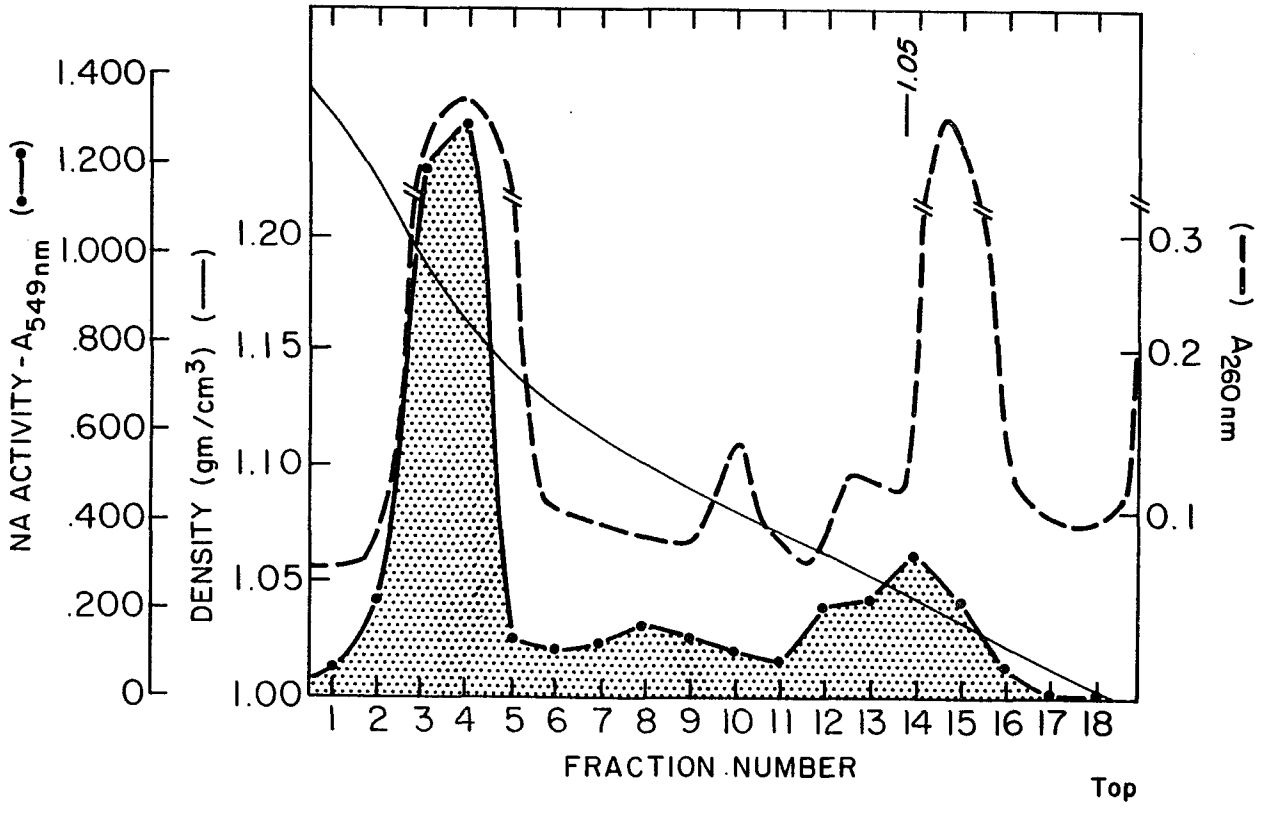

NA ACTIVITY - A_{549nm} (—•—)
1.400
1.200
1.000
.800
.600
.400
.200
0
DENSITY (gm/cm³) (—)
1.20
1.15
1.10
1.05
1.00
(– – –) A_{260nm}
0.3
0.2
0.1
1.05
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
FRACTION NUMBER
Top

Figure 9.5. NA_{TSS} liposomes. NA_{TSS} is highly purified, enzymatically inactive neuraminidase and is represented by protein(--o--o--o) as NA_{TSS} showed no activity even on 18 hour incubation; turbidity by absorbance at 260nm (- - -). NA_{TSS} is seen associated with liposomes of heterogeneous densities (1.12 ml to 1.03gm/ml).

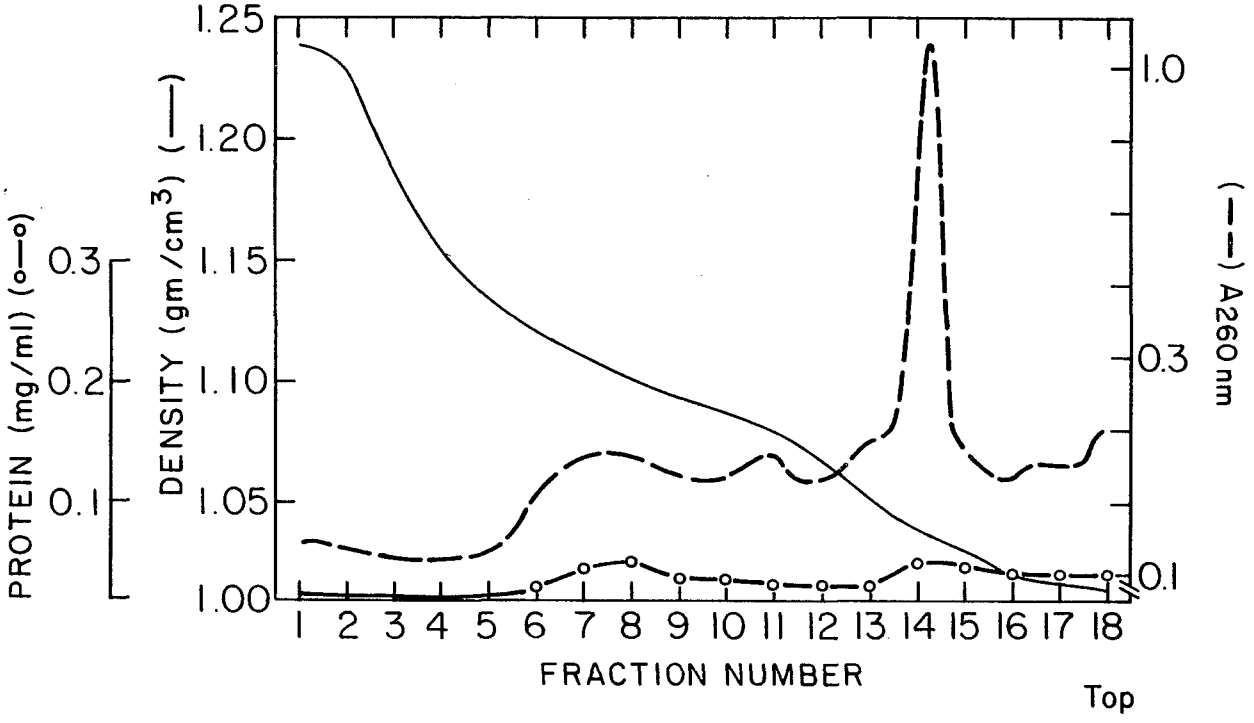

PROTEIN (mg/ml) (o—o)
0.3
0.2
0.1
DENSITY (gm/cm3) (—)
1.25
1.20
1.15
1.10
1.05
1.00
1.0
0.3
0.1
(– – –) A260 nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
FRACTION NUMBER
Top

Figure 9.6. NA_{TSS}-M protein liposomes. Protein (NA_{TSS} and M) is represented by --o--o--o); turbidity by absorbance at 260nm (- - -). A discrete liposomal population with a density of 1.17 is seen: this is identical to that seen with X-53a M protein liposomes, in indicating that there is no contribution to density of M protein liposomes made by NA_{TSS} as there was for neuraminidase(Fig. 9.4). Considerably more protein remained in the light NA_{TSS}-M protein lipsomal population than appeared in either the NA_{TSS} liposomes or M liposomes indicating that under these conditions some M protein was not incorporated into heavy density liposomes.

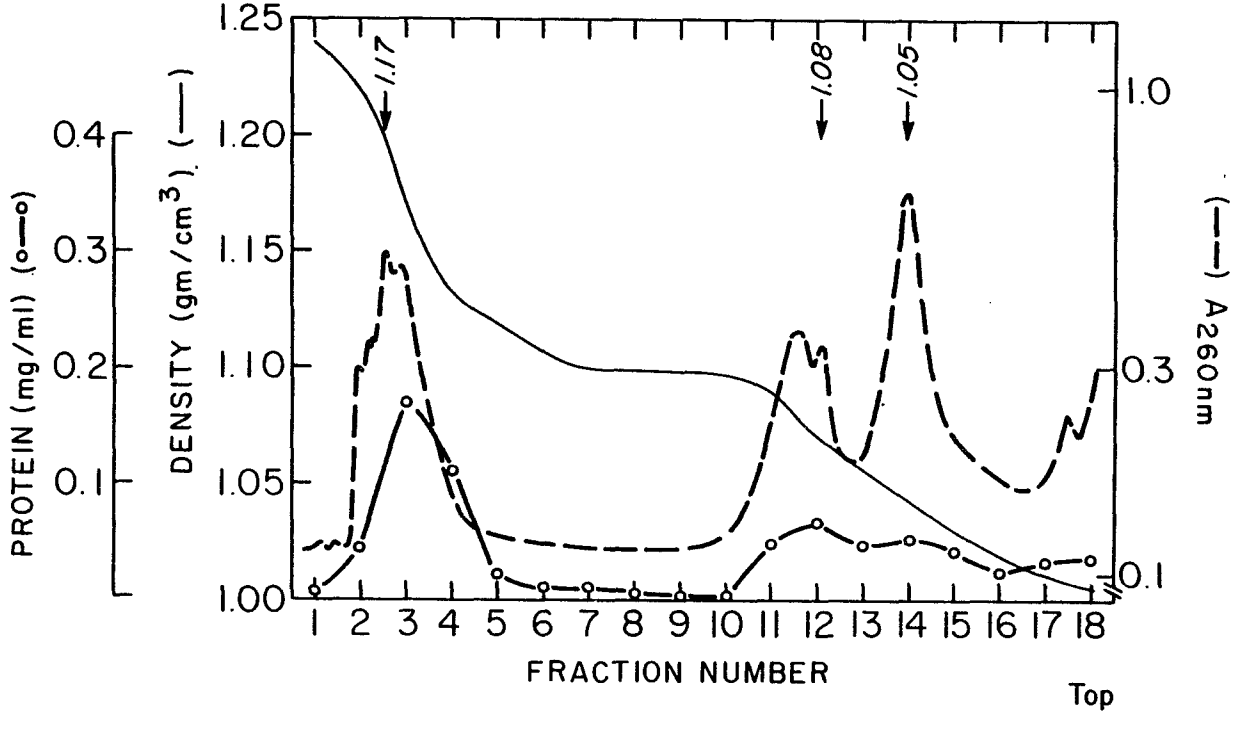

PROTEIN (mg/ml) (o—o)
DENSITY (gm/cm³) (—)
(—) A260nm
1.17
1.08
1.05
0.4
0.3
0.2
0.1
1.25
1.20
1.15
1.10
1.05
1.00
1.0
0.3
0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
FRACTION NUMBER
Top

Figure 9.7. Electron Micrograph of M(X-53a) protein liposomes (d 1.17 gm/ml) maintained in the presence of 2mM $CaCl_2$. Note the pleomorphism and similarity to plain liposomes formed under the same conditions (Fig. 7.2c). Bar represents 1000Å. Final magnification 158,000.

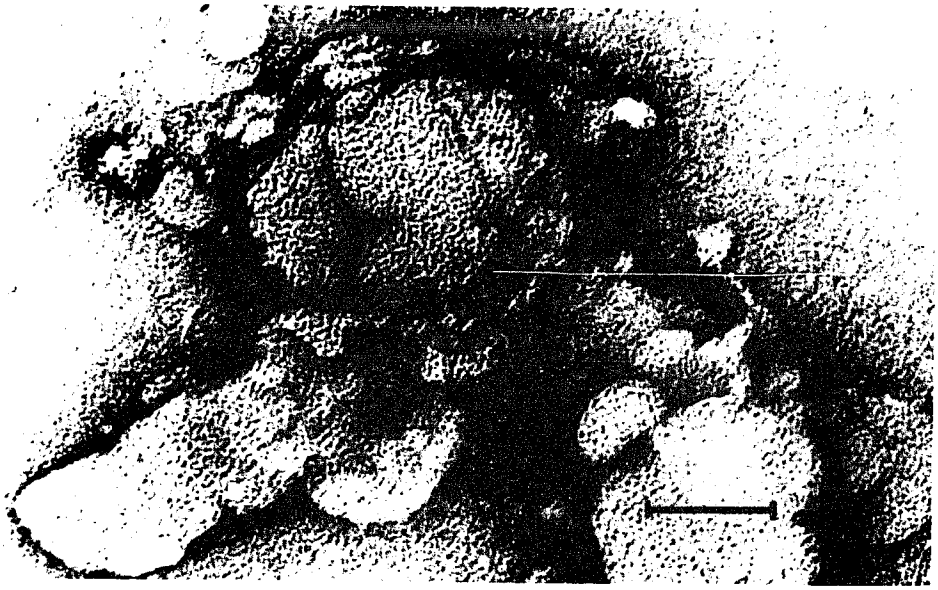
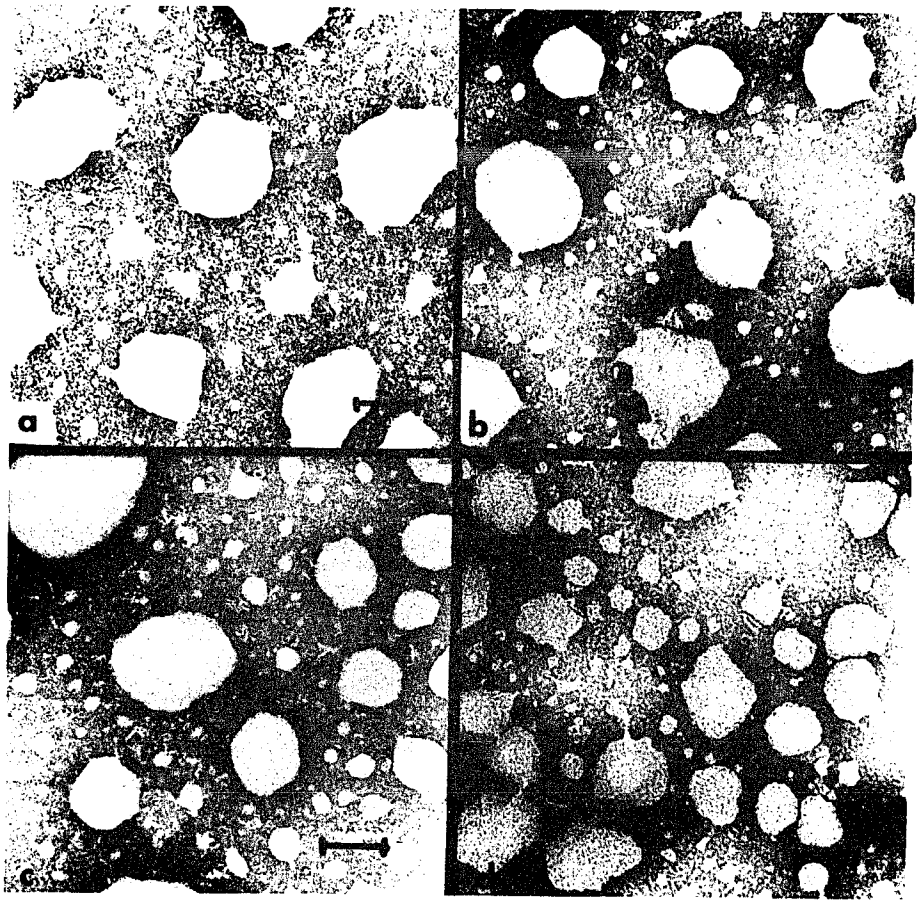


Figure 9.8. Neuraminidse-M protein liposomes. a). Plain liposomes (density 1.03) stained with PTA. b). Neuraminidase -M protein liposomes stained with PTA: note that these do not differ significantly from plain liposomes, except for the fact that minisomes have a more clearly defined shape. c). Plain liposomes stained with uranyl acetate. d). neuraminidase-M protein liposomes stained with uranyl acetate. Note that there does not appear to be any stain related difference in the morphology of the liposomes. Bar represents 1000Å. Final magnification, 75,000.


a
b
c

CHAPTER 10. INCORPORATION OF HEMAGGLUTININ AND THE HA_2 POLYPEPTIDE INTO LIPOSOMES

Encouraged by what we felt was a mounting body of evidence of cooperative interaction of influenza membrane associated proteins with one another and the implications that this information held for self-assembly, we undertook to study the interaction of the hemagglutinin and the HA_2 polypeptide of hemagglutinin with liposomes and with M protein and liposomes.

MATERIALS AND METHODS

Preparation of Proteins

M protein was isolated from X53a (H1N1) vaccine strain as described in Chapter 8. Hemagglutinin was obtained by lowering the Triton X-100 concentrations of HA rich fractions of the NA affinity chromatography protein pool when freshly pre-

pared X-7 had been used for isolation of NA. Triton X-100 levels were 3 - 4% -- slightly higher than those present in NA samples used to prepare NA liposomes.

HA_2 was isolated by SDS gel chromatography as previously described (Bucher et al., 1976) and labelled by bringing the purified HA_2 sample to a concentration of 6M urea and 0.01M dithiothreitol and incubating at 37°C for two hours in sealed tubes. After cooling to room temperature, samples were alkylated with 0.025mCi of ^{14}C iodoacetic acid (New England Nuclear) in 0.050ml for five minutes followed by addition of 0.04M iodoacetic acid. Samples were dialyzed against two changes of 20% acetic acid to remove unreacted iodoacetic acid and then two changes of distilled water. HA_2 prepared by such techniques is highly immunogenic in rabbits (two ten microgram doses given I.V. on days 0 and 40, produce an ELISA titer of 16,000 (Khan and Bucher, personal communication), indicating that it is aggregated.

Liposomes. Negative liposomes were formed as described in Chapters 9 and 10 using an HA:lipid ratio of 3:17; an HA:M:lipid ratio of 3:3.5:17; an HA_2:lipid ratio of 1:17; and an HA_2:M:lipid ratio of 1:3.5:17. Ratios were arrived at by attempting to approximate the ratio of the proteins to each other in the virion.

Sucrose Gradient Centrifugation. Liposomal samples were layered over a 10 - 30% sucrose gradient with a 1-2ml cushion of 60% sucrose and centrifugation was at 33,000 rpm for 20 hours at 10^{o}C in a Beckman SW 41 rotor. For details of the procedure see Chapter 8.

RESULTS

We attempted to form HA liposomes using HA enriched fractions that eluted in the void volume of affinity chromatography. As seen in Fig. 10.1 we formed a dense HA liposomal peak at 1.17gm/ml but about half the protein on the gradient stayed with the light density liposomes. When HA and M protein were present during liposomal formation heavy density liposomes (d. 1.22 gm/ml) were formed. The density of HA-M protein liposomes was significantly greater than that of either HA liposomes or M(X-53a) liposomes, both of which had densities of 1.17 gm/ml indicating the presence of both proteins in this liposomal peak. On this gradient as on the HA liposomal gradient much protein remained associated with light density liposomes.

When we attempted to determine HA titers for these fractions we discovered that there was lysis across the gradient. Even the heavy density HA liposomes and HA-M liposomes were lytic at a 1:64 dilution indicating a Triton X-100 concentration of 0.03%. No hemagglutination was observed. The implications of this will be discussed later.

When HA_2 is present during liposomal formation all HA_2, as indicated by radioactivity, associated with liposomes of 1.24 gm/ml density (Fig. 10.3).

When both HA_2 and M were present during liposomal formation all of HA_2 and almost all M protein (indicated by protein) was present in liposomes of 1.23 gm/ml density and no protein was associated with the light density liposomes. The density of HA_2-M protein liposomes is essentially the same as that seen for HA_2 liposomes but is greater than that routinely seen with M(X-53a) liposomes (d. 1.17 gm/ml), indicating that both M and HA_2 are present in the same liposomes.

DISCUSSION

On both HA and HA-M protein liposomal gradients much protein remained associated with the light density liposomes. The presence of relatively high levels of Triton X-100 associated with even the heavy density liposomes on both gradients, made it impossible to draw firm conclusions from this data. However the HA liposomal experiments did demonstrate that HA incorporated into lipid as reported by others (Huang et al., 1979; Oxford et al., 1981). The heavier density of the HA-M protein liposomes suggested that there was cooperative interaction between the HA and M proteins as was observed for NA-M protein liposomes.

The difficulty in removing Triton X-100 from HA indicated that another detergent should be used for experiments involving HA. Even when HA samples in Triton X-100 were solubilized with 5% octyl glucoside and centrifuged at 35,000 rpm in a Beckman SW 41 rotor for 20 hours on a 10 - 30% sucrose gradient with 60% sucrose cushion, we failed to dissociate any meaningful amount of HA from the light density Triton X-100 peak. Using Triton X-100 to solubilize the envelope glycoproteins of Semliki Forest Virus Simons and colleagues (1978) reported that some Triton remained associated with the aggregated glycoprotein spikes.

The formation of dense HA_2 liposomes (d. 1.24 gm/ml) and light non-protein containing liposomes when HA_2 was present during liposomal formation suggested that there is cooperativity in the incorporation of HA_2 into lipid.

The density of HA_2-M protein liposomes(d. 1.23gm/ml) was similar to that of HA_2 liposomes. The presence of both proteins in the same liposomes is indicated by the fact that no liposomal peak was observed at the characteristic density of M protein liposomes (d. 1.17gm/ml). Once again this suggested cooperative interactions between the two proteins.

Recent evidence (Dourmashkin, personal communication), indicates that M liposomes of heavy density are solid balls of lipid and protein in which the multilamellar structure characteristic of liposomes formed by dialysis is lost. It will be most interesting to look at heavy density liposomes containing M and other proteins to determine if the lipid organization is also disrupted in these structures. Use of liposomes containing more than one protein and characterization of the immune response to these should shed light on how the conformation and processing of proteins varies with context.

Figure 11.1 HA liposomes: sucrose density gradient centrifugation. Negative liposomes were formed by mixing detergent solubilized HA with detergent solubilized lipid mixture in a 3:17 ratio and dialysis for one week as described in the text. Protein--o--o--o; lipid (A_{260nm}) -- --- -- , density _____ .

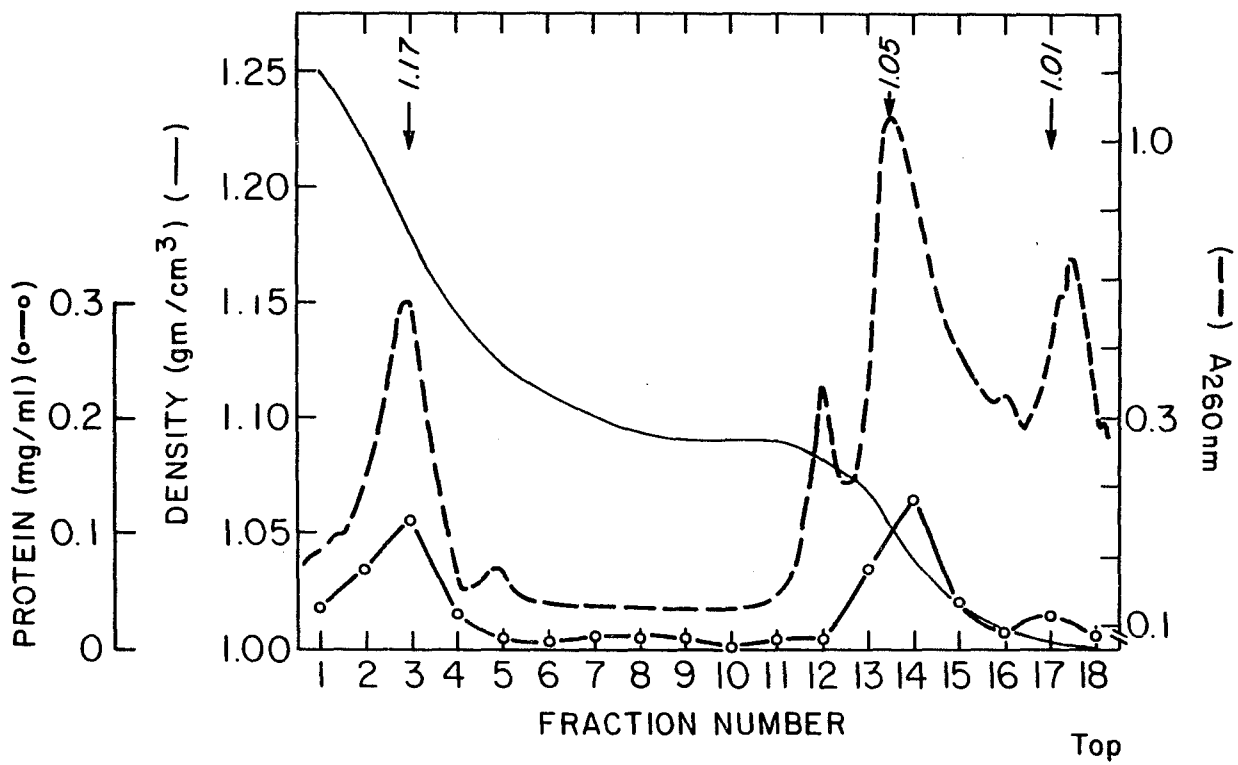

1.17
1.05
1.01
PROTEIN (mg/ml) (o—o)
DENSITY (gm/cm³) (—)
(---) A260nm
0.3
0.2
0.1
0
1.25
1.20
1.15
1.10
1.05
1.00
1.0
0.3
0.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
FRACTION NUMBER
Top

Figure 11.2 HA-M protein liposomes: sucrose density gradient centrifugation. Negative liposomes were formed by mixing detergent solubilized HA with M protein and lipid in a HA:M:lipid ratio of 3:3.5:17 and dialysis for one week as described in text. Protein, o--o--o; lipid, A_{260nm}, -- -- --; density _____.

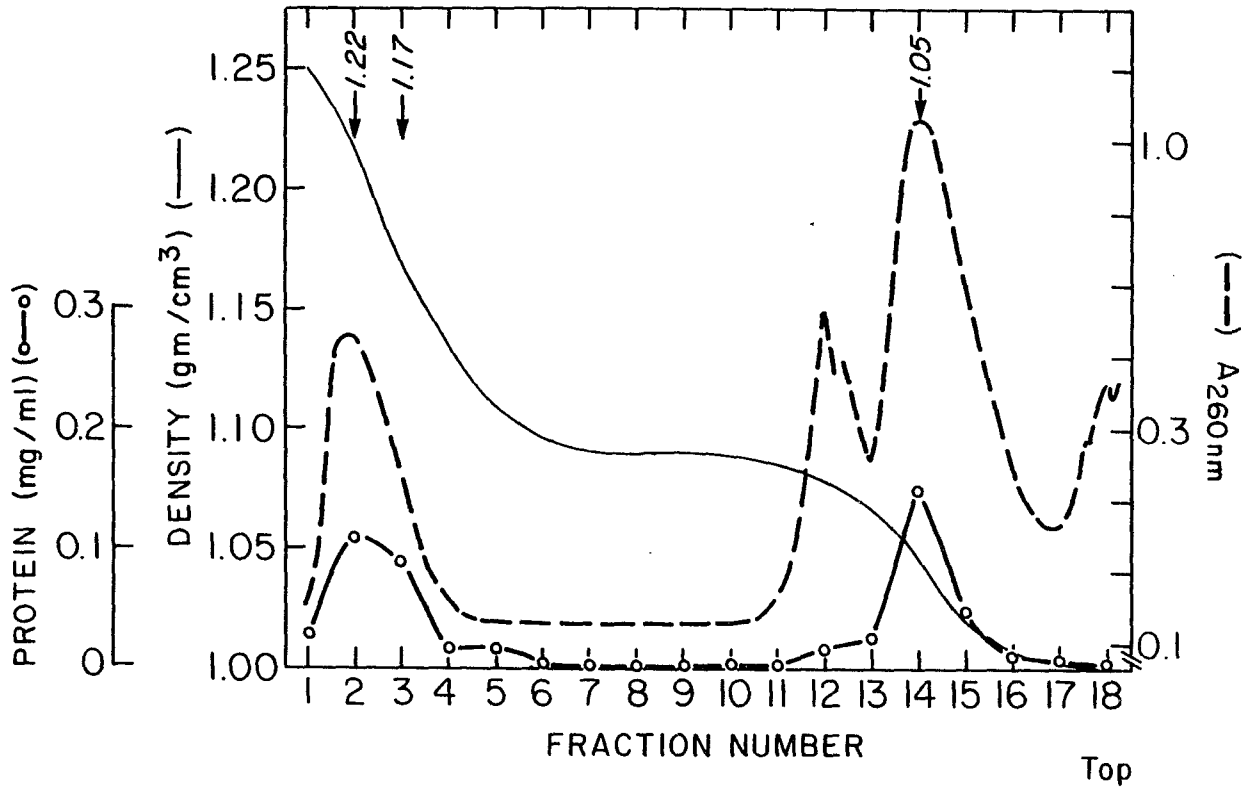

PROTEIN (mg/ml) (o—o)
0.3
0.2
0.1
0
DENSITY (gm/cm³) (——)
1.25
1.20
1.15
1.10
1.05
1.00
1.22
1.17
1.05
1.0
0.3
0.1
(— —) A260nm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
FRACTION NUMBER
Top

Figure 11.3. HA_2 liposomes: sucrose density gradient centrifugation. Negative liposomes were formed by mixing purified ^{14}C labelled HA_2 with lipid in a HA_2:lipid ratio of 1:17 and dialysis for one week, as described in text. Protein o--o--o; HA_2, x--x--x; lipid, A_{260nm}, -- -- --; density _____.

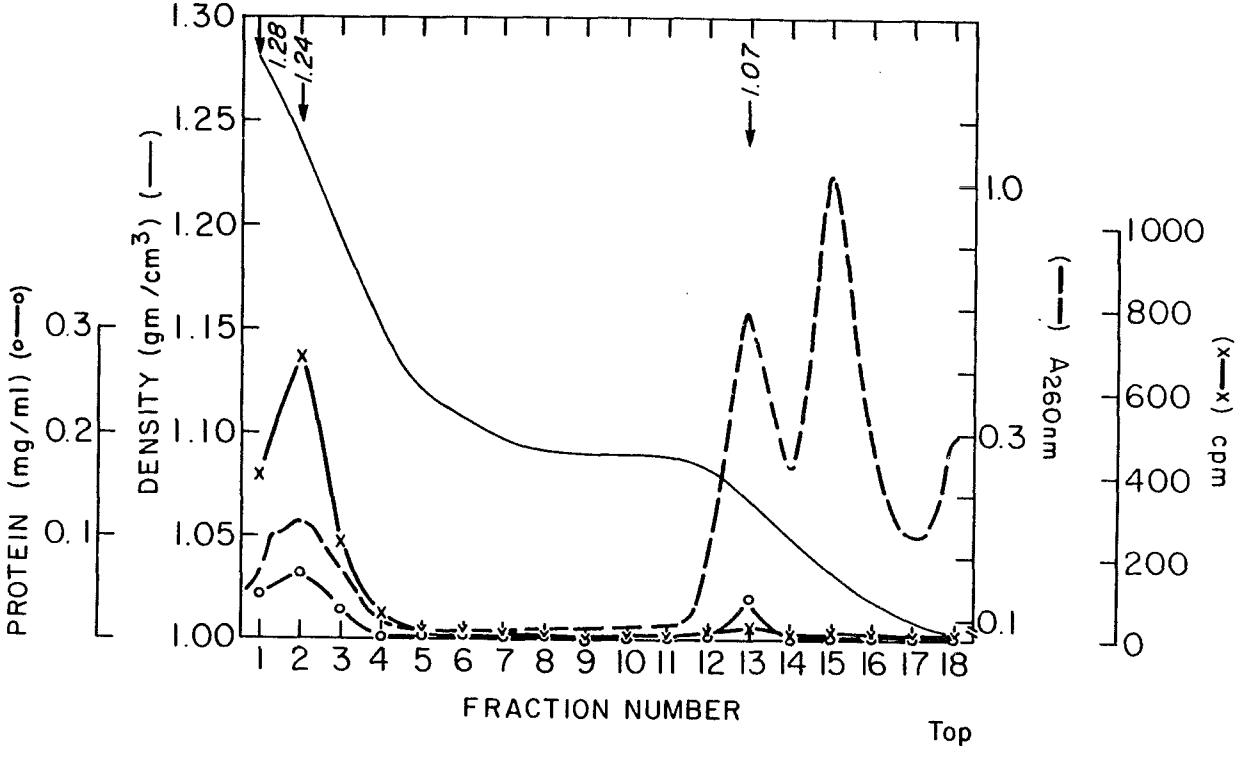
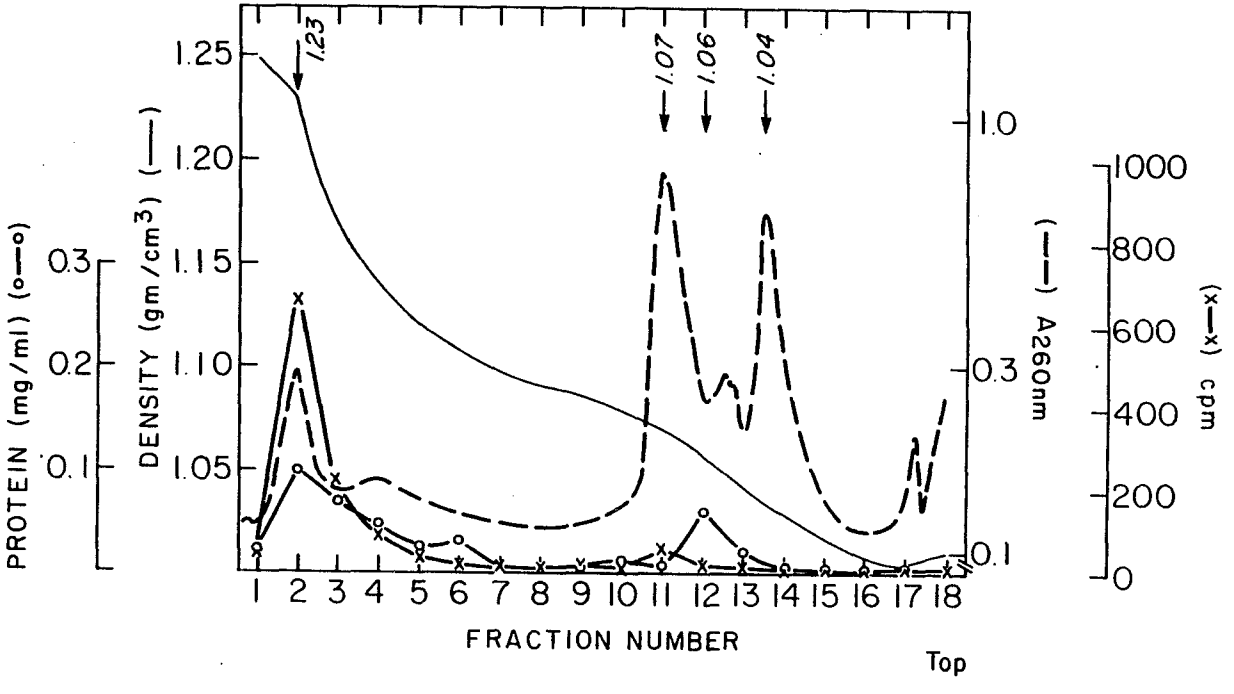

1.30
1.25
1.20
1.15
1.10
1.05
1.00
DENSITY (gm /cm3) (—)
1.28
1.24
1.07
0.3
0.2
0.1
PROTEIN (mg/ml) (o—o)
1.0
0.3
0.1
(—) A260nm
1000
800
600
400
200
0
(x—x) cpm
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
FRACTION NUMBER
Top

Figure 11.4 HA_2-M protein liposomes: sucrose density gradient centrifugation. Negative liposomes were formed by mixing HA_2:M:lipid in a ratio of 1:3.5:17 and dialysis for one week as described in text. Protein (o--o--o), HA_2(cpm) x--x--x, lipid(A_{260nm}) -- -- --, density, _____.


PROTEIN (mg/ml) (o—o)
DENSITY (gm/cm³) (—)
(—) A260nm
(x—x) cpm
1.23
1.07
1.06
1.04
FRACTION NUMBER
Top

CHAPTER 11. SUMMARY

The work presented in this thesis had its starting point in our interest in the use of purified neuraminidase of type A influenza virus as an immunogen in the neuraminidase-specific vaccine (Kilbourne et al., 1972). It was our hope that a careful study correlating immunogenicity with conformation would yield information that would be of general use in the design of purified protein vaccines.

Since the inception of our studies, parallel studies with Semliki Forest virus from the laboratories of Helenius, Simons, Morein and others (Morein et al., 1978; Balcarova et al., 1981) and the hemagglutinin of influenza A (Oxford et al., 1981) have been reported. We believe our studies to be unique in two ways: firstly, the enzyme activity of neuraminidase allowed us to monitor otherwise undetectable changes in conformation; secondly, we have compared the response of the purified antigen to that of the antigen in the intact virus and shown it to be an equal or superior immunogen. We have shown that the purified neuraminidase of type A influenza

virus is as immunogenic as that in intact virus if it were aggregated or incorporated into positively charged liposomes, and that the monomer form was only poorly immunogenic.

We found that neuraminidase activity did not correlate with immunogenicity: aggregated neuraminidase had nine-fold lower specific activity than the immunologically equivalent liposomal neuraminidase.

Several interesting conformationally dependent differences were noted. Specific activity of neuraminidase in different forms varied widely and appeared to be characteristic of that conformation. By electron microscopy differences were also noted. Our aggregated neuraminidase had a dramatically different appearance from that seen with SDS electrophoresis purified NA which is reported to be poorly immunogenic (Webster and Laver, 1967; Laver and Valentine, 1969). Our aggregates appeared very similar to those observed with the glycoprotein spikes of SFV: these were isolated by methods very similar to those used by us and were also highly immunogenic.

Neuraminidase in liposomes appeared to be aggregated on the surface of large liposomes or in smaller vesicles which we termed "NA-minisomes". When liposomes were sonicated we found that specific activity dropped one third but that the immune response was similar to that observed with monomer neuramini-

dase. It may be that sonication dissociates neuraminidase from lipid or disaggregates protein micelles.

An enhanced immune response to HA incorporated into negatively charged liposomes has been observed (Oxford et al., 1981). Bucher and Schwartz (unpublished), in preliminary experiments had shown that neuraminidase did not associate well with negative liposomes. Indeed when we formed negative liposomes in the presence of neuraminidase a substantial portion of the protein was shown to migrate on sucrose density gradient centrifugation as non-lipid associated neuraminidase aggregates, unless M protein were present. The immunogenicity of negatively charged neuraminidase liposomes should be investigated to determine if these liposomes enhance the immunogenicity of neuraminidase as was reported for hemagglutinin.

Although in our system, liposomes turned out to offer no immunologic advantage over protein micelles our studies of liposomes made us appreciative of the profound alterations in protein conformation when it is lipid associated as opposed to when it is not. We were also tantalized by the possibilities liposomes offered for studying viral morphology.

The discovery that M protein could insert into the lipid bilayer of liposomes in what appeared to be a cooperative process, suggested that the influenza virus could assemble by the

formation of M protein domains on the cytoplasmic surface of the lipid membrane of infected cells (Choppin and Compans, 1975), and that this domain might serve to recruit other viral proteins. As the first step in investigating this hypothesis we decided to see if we could detect evidence for specific protein-protein interaction between M protein and the influenza glycoproteins.

We have been able to show specific neuraminidase-M protein interaction during the formation of liposomes by dialysis. (We did not investigate the association of M protein with neuraminidase aggregates, though this informtion would be most interesting.) The evidence for specific neurminidase-M protein interaction was as follows:

1. The presence of M protein facilitated the incorporation of neuraminidase into lipid. During the formation of negatively charged neuraminidase liposomes not all neuraminidase was incorporated into lipid, while in the neuraminidase-M protein liposomal preparation all neuraminidase was associated with lipid.

2. In the presence of M protein a substantial portion of neuraminidase was incorporated into liposomes of heavy density. This was a discrete liposomal population which showed evidence of neuraminidase and M protein existing in the same liposomes.

3. Further evidence of neuraminidase-M protein interaction was the two to three-fold higher enzyme activity seen on the neurminidase-M protein liposomal gradient, than on the gradient containing neuraminidase liposomes.

Unfortunately, we were not able to use electron microscopic methods for extensive study of the morphology of neuraminidase-M protein liposomes. It would be most interesting to see if M protein remains an intramembranous protein in the presence of neuraminidase, and to observe the conformation of neuraminidase in negative liposomes to determine if M-dependent conformational changes are detectable.

Studies were begun on elucidating the interaction of hemagglutinin with liposomes. Hemagglutinin prepared from the protein pool of neuraminidase affinity chromatography did not prove an ideal source; Triton X-100 levels could not be lowered sufficiently. An interesting possibility is that hemagglutinin bound hydrophobic moieties more tenaciously than did neuraminidase. However, we were able to demonstrate that Triton X-100 solubilized hemagglutinin was incorporated into heavy density, negatively charged liposomes. The presence of two lipid populations, one with light density and a low protein to lipid ratio and one with heavy density with a high

protein to lipid ratio, suggested that we were seeing cooperative interaction during incorporation of hemagglutinin into lipid (even in the presence of low Triton X-100 concentrations). Electron micrographs of hemagglutinin liposomes by Oxford et al. (1981) showed the presence of both protein rich and protein poor liposomes (these were not separated by sucrose gradient centrifugation).

In the presence of hemagglutinin and M protein, we formed a discrete population of heavy density hemagglutinin-M protein liposomes, which were substantially more dense than either the M protein or hemagglutinin liposomes, indicating that this population contained both proteins, presumably in the same liposomes although this was not directly demonstrated.

The HA_2 polypeptide of hemagglutinin was incorporated into liposomes to form very heavy density liposomes (d. 1.28gm/ml). Oxford and collegues (1981) have shown that bromelain derived hemagglutinin did not associate with liposomes. We have confirmed that it is the hydrophobic HA_2 portion of the hemagglutinin molecule that is responsible for attachment of hemagglutinin to lipid.

In the presence of HA_2 and M all protein was incorporated into a discrete population of very heavy density liposomes, suggesting that HA_2 and M were selectively interacting.

The term "cooperative interaction" is used by us to describe the following phenomenon. When positively charged NA liposomes were formed, sucrose gradient centrifugation showed them to be a heterogeneous population with a density range of 1.18 - 1.03gm/ml. In this case incorporation of neuraminidase into liposomes would appear to have occurred by random association of neuraminidase with lipid, even though liposomally associated neuraminidase in small aggregations was observed by electron microscopy. However, when we observed the formation of two very distinct liposomal populations, one with heavy density, one with light, we were led to speculate that during the process of protein associating with lipid there was a greater probability of protein associating with a lipid micelle that already contained protein than with micelle containing no protein, and that this interaction is due to protein-protein interaction. We apply the term "cooperative interaction" to describe this phenomenon and would suggest that it is the ability of influenza virus glycoproteins to selectively interact with each other in a lipid environment that may be the process by which virus assembles on the surface of influenza virus infected cells. Confirmation of the selective interaction of influenza virus glycoproteins will have to be obtained using liposomal systems that contain both cellular membrane associated proteins as well as viral proteins.

Most of the steps in influenza virus replication in host cells utilize host cellular machinery and hence agents that affect these steps in viral replication also affect the host. The indication that influenza virus may self-assemble at the membrane of infected cells is of potential interest in the development of antiviral agents. It indicates that there may be a step in viral replication that could be interfered with, with minimal damage to the host. Liposomal systems may prove a useful tool in the designing and testing of such agents.

APPENDIX I. PURIFICATION OF NEURAMINIDASE BY AFFINITY CHROMATOGRAPHY

(Cuatrecasas and Illiano, 1971; Bucher, 1977)

Preparation of the Neuraminidase Affinity Column. (Bucher laboratory procedure, unpublished)

1. Wash 200ml of Sepharose 4B with 2 liters of borate buffer (0.2M boric acid, 0.15M NaCl, adjusted to pH 8.0 with NaOH). Centrifuge at 1000rpm for 10 minutes Repeat two additional times.

2. Suspend Sepharose in 400ml of borate buffer in a 2 liter flask and add CNBr (16 gm in 320 ml of distilled water, adjusted to pH 11 with 6M NaOH -- do this in hood). Stir for 20 minutes maintaining mixture at pH 10.9 by addition of 1M NaOH.

3. Terminate reaction by rapid washing of CNBr Sepharose in Buchner funnel with 1 liter of cold distilled water and then 500 - 1000 ml of cold borate buffer.

4. Place activated Sepharose 4B in 200 ml borate buffer with 4 gm of glycyl-glycyl-tyrosine and stir overnight at $4^{o}C$.

5. Place washed, activated Sepharose in column and wash with 8 liters of 0.1N NaCl.

6. Wash column with 400 ml of pH 8.9, 0.5M sodium bicarbonate.

7. Remove sepharose from column and dissolve in 200ml, of 0.5M sodium bicarbonate (pH 8.9), in beaker.

8. Dissolve 540 mg AMPOX (preparation given immediately following) in 200 ml ice cold 0.4N HCl by sonication. Then add 500 mg sodium nitrite in 2.2ml cold distilled water over a one minute period. Wait 5 minutes and then add the prepared agarose.

9. Adjust pH to 8.8 with 1.0N NaOH and stir 8 hours at room temperature.

10. Pour in column and wash with 6 liters of 0.1N NaCl.

PREPARATION OF P-AMINOPHENYLOXAMIC ACID (AMPOX)

(Chemische Berichte, 1903, 36:413). Bucher laboratory procedure, unpublished.

1. Dissolve 5 gm p-phenylenediamine and 20 gm oxalic acid in 500 ml hot distilled water. Cover beaker and use stirring rod and maintain at a low boil for 3 - 4 hours (color changes from orange to deep pink).

2. Allow to cool: a very heavy AMPOX precipitate forms.

3. Centrifuge for 20 minutes at 8,000 rpm and discard supernatant. Wash precipitate. in 500 ml ice cold distilled water. Centrifuge as before and save precipitate.

4. Redissolve precipitate in 15 ml, 4N NaOH.

5. Filter, save filtrate. Reprecipitate AMPOX with 4N H_2SO_4.

6. Wash precipitate with 100 - 200 ml ice cold distilled water. Centrifuge and save precipitate.

7. Dry in drying oven with no heat.

AFFINITY COLUMN CHROMATOGRAPHY.

(Bucher, 1977)

COLUMN PREPARATION

1. Gently stir prepared column material in 3 volumes of 0.1M pH 9.1, 0.1M sodium bicarbonate, 0.1% Triton X-100(TX-100).

2. Allow column material to settle and decant supernatant.

3. Pour column material into column and wash with 3 bed volumes of bicarbonate buffer.

4. Wash column with adsorbing buffer,(pH 5, 0.05M sodium acetate, 1mM $CaCl_2$ and 0.1mM EDTA, 0.1% TX-100) until eluant is pH 5. Shake column and wash with another 2 - 3 volumes of absorbing buffer.

PREPARATION OF VIRUS

1. Dilute virus to 1mg/ml (Lowry assay) with pH7, 0.05M sodium acetate, 2mM $CaCl_2$.

2. Incubate viral protein with 1% SDS at 37°C for 15 minutes Use syringe with large gauge needle to facilitate disruption (for N1 strains disrupt at room temp.).

3. Centrifuge at 20,000rpm in Beckman JA 21 for one hour. Solubilized NA is in the supernatant.

APPLICATION OF SAMPLE TO COLUMN

1. Apply 1 column volume of sample to column. Stop the column for 15 minutes Repeat these two steps until all of sample is applied to column.

2.Wash column with 3 - 4 volumes of absorbing buffer

ELUTION OF NA FROM COLUMN

1. Elute NA by switching to pH 9.1, bicarbonate buffer.

2. Collect samples in 1 - 2 ml aliquots and as soon as possible adjust pH of aliquots to pH 6 or 7 with 1N HCl.

DETERMINATION OF PURITY

1. Assay all column fractions and starter virus preparations for NA activity (use 25µl).

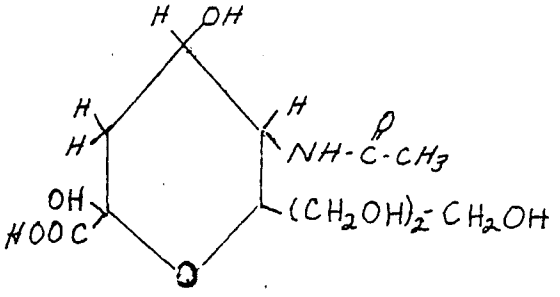
2. Run 50µl aliquots of the following on 10% page gels virus; supernatant, pellet; protein pool; enzyme pool fractions.

HANDLING OF THE NEURAMINIDASE.

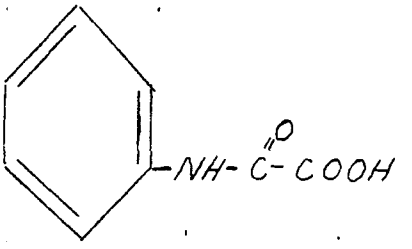
1. Pool and concentrate enzyme pool fractions that appear pure by PAGE. NA can be stored in the Revco at -70^{o}C for at least one year with no noticeable loss of activity.

2. Neuraminidase can be dialyzed before storing if this is desirable. Sodium azide can be added if sample is not intended for biological use.

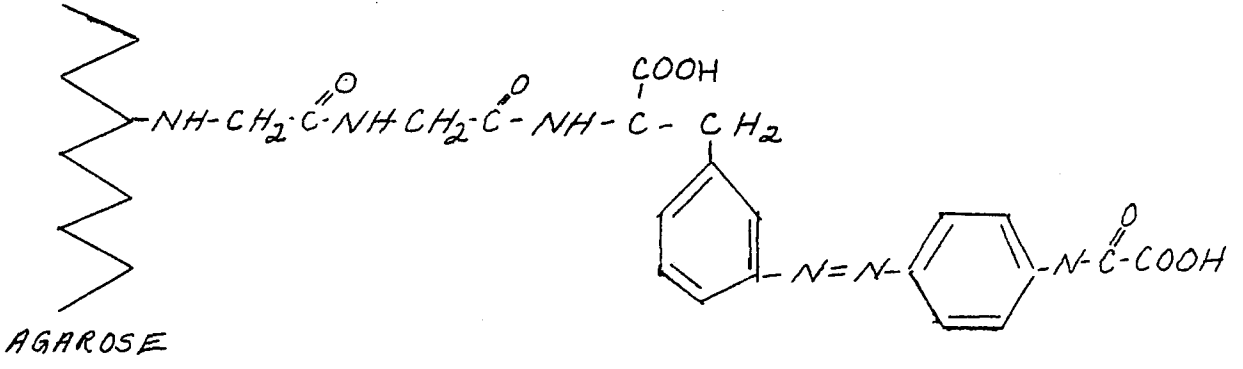
Figure A. 1. Chemical Structures for Neuraminidase Affinity Column.



N-acetyl neuraminic acid



N-(p-aminophenyl)oxamic acid



Affinity Column. The diazonium derivative of N-(p-aminophenyl)oxamic acid is coupled via an azo linkage to agarose with a glycl-glycl-tyrosine leash (from Cuatrecasas and Illiano, 1971).

APPENDIX II. NEURAMINIDASE ASSAYS

THE NEURAMINIDASE ASSAY

(Warren, 1959, 1963; Aminoff, 1959, 1961)

(This laboratory procedure adapted from the laboratory procedure of Barbara Pokorny by Bucher, unpublished)

1. 10 to 50µl of sample to be tested is brought to 150µl with pH6, 0.05M sodium acetate, 2mM $CaCl_2$.

2. To this add 50µl of fetuin (protein concentration -- 25mg/ml).

3. Incubate at 37°C for 15 minutes (longer times may be used).

4. Cool and add 50µl of periodate solution (0.2M sodium metaperiodate in 62% phosphoric acid) and incubate at room temperature for 20 minutes.

5. Add 0.5ml of arsenite reagent(10% sodium arsenite in 0.5M sodium sulphate with 0.3% sulphuric acid), and shake until the brown color disappears.

6. Add 1.5ml of TBA solution (0.6M 2-thiobarbituric acid in 0.5M sodium sulphate) and shake the tubes. Place in boiling water bath for 15 minutes.

7. Add 2ml of Warrenoff Reagent (5% HCl in butanol) and vortex. Clarify by centrifugation at 1000 rpm for 5 minutes.

8. Color is extracted into the acid butanol(top) phase and this solution is read at 549mu.

9. The number of μM/min/ml is calculated from the equation

$$\mu M/min/ml = O.D._{549} \times 0.034 \times \text{time in } min^{-1} \times 1000/\#\mu l \text{ used}$$

Periodate cleaves free neuraminic acid to yield β formyl pyruvic acid (carbon atoms 1 - 4). Excess periodate is reduced by arsenite. β-formyl pyruvic acid reacts with 2-thiobarbituric acid to yield a red chromophore with an aborbance peak at 550mu.

THE NI ASSAY

(Aymard-Henry et al., 1973)

(This laboratory procedure adapted from the laboratory procedure of Barbara Pokorny by Bucher, unpublished)

1. A standard virus preparation is titered so that in the NA assay, 50µl of virus on 16 hour incubation at 37°C with 50µl of fetuin yields an $O.D._{549}$ of between 0.400 and 0.800.

2. Serum is heat inactivated at 56°C for 30 minutes to destroy serum neuraminidase activity. Serum is diluted in four-fold steps using calcium acetate buffer.

3. 50µl of an appropriate dilution of virus and 50µl of the serum dilutions (1:16 - 1:1028 are standardly used) are mixed and allowed to stand at room temperature for 30 minutes. The solutions are brought to 0.2ml with calcium acetate buffer and 50µl of fetuin are added. Controls containing only virus or only serum are run. All samples are assayed in duplicate or tri-

plicate. Samples are incubated at 37°C for 16 hours and then taken through the standard NA assay and read at 549mu.

4. Calculation of the NI titer. NA activity is expressed as a percentage, i.e. NA activity of a given dilution of sera/virus control. The % activity is plotted on semilog paper, % activity vs dilution, and a best-fit line is drawn. The inverse of the dilution at which 50% inhibition is expected is used as the NI titer. (Aymard-Henry et al., 1973).

APPENDIX III. ASSAYS FOR HEMAGLUTINATION

THE HEMAGGLUTINATION TEST

(Sever, 1962)

(This laboratory procedure adapted from the laboratory procedure of Barbara Pokorny by Bucher, unpublished)

1. Add 25µl of 1x PBS to all wells of a microtiter plate except well #1.

2. Add 25µl of virus solution to be tested to wells #1 and #2.

3. Dilute out starting with well #2 and continuing through well #24.

4. Add 25µl of 0.5% human type "O" red blood cells to all wells.

5. Refrigerate for one hour. Viral neuraminidase can cause virus to elute from red blood cells but it is not active at $4^{o}C$

6. Score HA. Hemagglutination appears as a fine pink sediment on the bottom of the well. Where there is no hemagglutination, a small compact red button is seen on the bottom of the plate. An HA pattern in well 1 represents an HA titer of 2 (the inverse of the virus dilution), well 2, a titer of 4; well 3, a titer of 8, etc.

DETERMINATION OF HI TITER

(Hierholzer and Sugg, 1969)

(This laboratory procedure adapted from the laboratory procedure of Barbara Pokorny by Bucher, unpublished)

RDE treat antisera (a.s.) to remove sialic acid residues on serum proteins which could interfere with the HA reaction.

1. To 1 part antisera add 4 parts RDE working solution. Stock solution is 5mg of commercial RDE (receptor destroying enzyme of Vibrio cholerae) in 5ml distilled water. Working solution is prepared by diluting stock 1:10 with calcium saline immediately before use. Incubate overnight at 37°C.

2. Add 3 parts 2.5% sodium citrate and incubate overnight at 56°C for 30 minutes. This inactivates serum neuraminidase.

3. Add two parts 1x PBS. Serum is now at 1:10 dilution.

TITER VIRUS

1. Dilute virus 1:10, 1:100, 1:500, etc. Add 25µl of each dilution to wells 1 and 2 of microtiter plate.

2. Add 25µl of PBS-alb (1x PBS, 0.1% albumen) to wells two though 8 and dilute out starting at well 2.

3. Add 25µl PBS-alb to all wells.

4. Add 50µl 0.25% human type "O" red blood cells to all wells.

5. Refrigerate for 1 - 2 hours.

6. Select a starting dilution of virus giving 2 - 3 wells of hemagglutination. Confirm this before doing HI. N.B. As ANAb may interfere with the HA especially at high titers use both the virus to which the antisera has been made and a virus carrying an irrelevant NA.

DETERMINATION OF HI TITER.

1. Add 25µl of PBS-alb to all wells of a microtiter plate except well 2.

2. Add 25µl of RDE treated antisera To wells 1,2 and 3. and dilute out from well 3.

3. Add 25µl of virus dilution indicated by HA titering in all wells but well 1.

4. Incubate at room temperature for one hour.

5. Add 50µl of 0.25% red blood cells (in PBS-alb) to all wells and refrigerate for 1 - 2 hours.

6. Score HI in the following way. First well should not show HA as it received no virus. If there is no inhibition HA should be seen in all other wells. HI will appear as a pattern like that in well 1. If this occurs in well 2 the HI titer is 40; in well 3, 80 etc.

APPENDIX IV. GROWTH AND PURIFICATION OF INFLUENZA A VIRUS

(This laboratory procedure adapted from the laboratory procedure of Barbara Pokorny by Bucher, unpublished)

1. Seed virus (in allantoic fluid) is diluted to 10^{-4} with sterile PBS, 1% penicillin and streptomycin.

2. Candle ten day eggs and mark with pencil where injection should be made to hit allantoic cavity and avoid major blood vessels, tap this spot with diamond pencil. Make small opening at the top of the egg.

3. Inject each egg with 0.1ml of seed virus dilution. Seal up injection site with warm liquid paraffin.

4. Incubate at $37^{o}C$ for 24 - 40 hours.

5. Harvest allantoic fluid.

6. Filter crude allantoic fluid through cheese cloth to remove large debris.

7. Spin at 10,000 rpm for 10 minutes to pellet out red blood cells and other cellular debris. Discard pellet.

8. Pellet virus from allantoic fluid by spinning at 20,000 rpm in Beckman JA 20 for 1 hour. Discard supernatant and resuspend virus in pH 7 PBS or pH 7 calcium acetate buffer.

9. Layer over a discontinuous sucrose gradient (in a 40ml cellulose acetate centrifuge tube, place 6ml of 60% and over this 6ml of 30% sucrose diluted in appropriate buffer). spin in SW 27 rotor at 25,000 rpm for 1 hour at $4^{o}C$.

10. Collect the dense band that forms at the 30 - 60% interface. it has a density of about 1.22gm/ml. The lighter band is also collected it has lower infectivity than the more dense band but can be used for priming runs for affinity chromatography.

11. Dilute out heavy and light bands 1:5 in appropriate buffer and spin at 20,000rpm as before to pellet virus. Resuspend virus in appropriate buffer.

12. Characterize the virus from both bands by HAU, NA, and Lowry. Heavy band protein content should equal a harvest of about 0.1mg/egg used. There should be about 25% of this amount in light band fraction. Do not reclaim virus if heavily contaminated with yolk.

13. Use immediately or quick freeze and store at -70°C. Check HA and NA activity when defrosting.

APPENDIX V. CLASSIFICATION OF INFLUENZA A VIRUSES (W.H.O., 1980)

Current subtype designation	Previous subtype designation*
H1	H0, H1, Hsw1
H2	H2
H3	H3, Heq2, Hav7
H4	Hav4
H5	Hav5
H6	Hav6
H7	Heq1, Hav1
H8	Hav8
H9	Hav9
H10	Hav2
H11	Hav3
H12	Hav10
N1	N1
N2	N2
N3	Nav2, Nav3
N4	Nav4
N5	Nav5
N6	Nav1
N7	Neq1
N8	Neq2
N9	Nav6

* sw, swine; eq, equine; av, avian; no species designation, human

BACKGROUND ON INFLUENZA A VIRUS STRAINS USED IN THESE STUDIES.

(All seed virus were the gift of Dr. E.D. Kilbourne)

X-31B (H3N2). A recombinant of A/Aichi/2/68 (H3N2) and A/PR/8/34 (H1N1). Used as vaccine strain.

X-7 (H1N2). A recombinant of RI/5+(H2N2) and NWS (H1N1).

X-53a (H1N1). A recombinant of A/New Jersey/11/76 (H1N1) and A/PR/8/34 (H1N1). This virus was from outdated vaccine and was the gift of the Canadian Bureau of Biologics.

X-38 (H7N2). A recombinant resulting from the triparental cross of A/Eng/42/72 (MRC-11)(H3N2), A/PR/8/34 (H1N1)(Ann Arbor variant), and A/equine/Prague/1/56 (H7N7). This virus was from outdated vaccine and was the gift of Lederle Laboratories.

BIBLIOGRAPHY

Ada, G.L., Yap, K.L.(1979). J. gen. Virol. 42:541.

Allan, W.H., Madeley, C.R., Kendal, A.P.(1971). J. gen. Virol. 12:79.

Allen, A.K., Skehel, J.J., Yuferov, V. (1977). J. gen. Virol. 37:625.

Almeida, J.D., Edwards, D.C., Brand, C.M., Heath, T.D.(1975). Lancet 2:899.

Almeida, J.D., Hoyle, L. (1971). Micron. 3:306.

Aminoff, D. (1959). Virology 7:355.

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

Aminoff, D., Bruegge, W.F.V., Bell, W.C., Sarpolis, K., Williams, R. (1977). Proc. Nat. Acad. Sci. (USA) 74;1521.

Ashwell, G., Morell, A.G. (1974). Adv. Enzymol. 41:99.

Aurora, D.J.S. (1979). Canadian Med. Assoc. J. 121:1575.

Aymard-Henry, M., Coleman, M.T., Dowdle, W.R., Laver, W.G., Schild, G.C. Webster, R.G. (1973) Bull. W.H.O. 48:199.

Bachi, T. (1980). Virology 106:41.

Bachi, T., Gerhard, W., Lindenmann, J., Muhlethaler, K. (1969). J. Virol. 4:769.

Balcarova, J., Helenius, A., Simons, K. (1981). J. gen. Virol. 53:85.

Bangham, A.D. (1978). In "Liposomes and their Uses in Biology and Medicine" (D. Papahadjopoulos, ed.). Annals N.Y. Acad. of Sci. 308:2

Bangham, A.D., Horne, R.W. (1964). J. Mol. Biol. 8:660.

Bangham, A.D., Standish, A.M., Watkin, J.C., Weissman, G. (1967). Protoplasm 63:183.

Baron, C., Thompson, T.E. (1975). Biochim. Biophys. Acta 382:276.

Barry, D.W., Staton, E., Maynor, R.E. (1974). Infect. Immun. 10:1329.

Baskin, R.J. (1977). In "Membrane Proteins and Their Interactions with Lipids". (R.A. Capaldi, ed.) pp 151-188. Marcel Dekker, Inc. N.Y., N.Y. pp. 151-188.

Bekesi, J.G., Arneault, G. St., Walter, L., Holland, J.G. (1972). J. Natl. Canc. Inst. 49:107.

Beutner, K.R., Rizzone, C., DeMelto, S., Ogra, P.L. (1976). Develop. biol. Standard. 33:162.

Beutner, K.R., Chow, T., Rubi, E., Strussenberg, J., Clement, J., Ogra, P.L. (1979). J. Infect. Dis. 140:844.

Beveridge, W.I.B. (1977). "Influenza: the Last Great Plague." Prodest, Neal Watson Academic Publications, Inc., N.Y.

Biddison, W.E., Doherty, P.C., Webster, R.G. (1977). J. Exp. Med. 146:690.

Blix, G., Gottschalk, A., Klenk, E. (1957). Nature 179:1088.

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

Blok, J., Air, G.M. (1980). In "Structure and Variation in Influenza Virus" (G. Laver and G.M. Air, eds.) pp 135-146. Elsevier/North-Holland, N.Y.

Blok, J., Air, G.M. (1980). Virology 107:50.

Boschman T.A.C., Jacobs, J. (1965). Biochem. Z. 342:532.

Both, G.W., Sleigh, M.J., Bender, V.J., Moss, B.A. (1980). In "Structure and Variation in Influenza Virus (G.W. Laver and G.M. Air, eds), pp 81-89. Elsevier/North -Holland, Amsterdam.

Braciale, T.J. (1977). J. Exp. Med. 146:673.

Bucher, D.J. (1975). In "The Megative Strand Viruses" (R. Barry and B. Mahy, eds) pp 133-144. Academic Press, N.Y.

Bucher, D.J. (1977). Biochim. et Biophys. Acta 482:393.

Bucher, D.J., Kharitonenkov, I.G., Zakomirdin, J.A., Grigoriev, V.B., Klimenko, S.M., Davis, J.F. (1980). J. Virol. 36:586.

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

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

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

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

Burnet, F.M. (1955). "Principles of Animal Virology", 1st ed. p. 380. Academic Press, N.Y.

Chapman, D., Peck, W.E., Quinn, P.J. (1978). In "Liposomes and their Uses in Biology and Medicine" (P. Papahadjopoulos, ed.) Annals N.Y. Acad. Sci 308:67.

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

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

Compans, R.W., Choppin, P.W. (1973). In "Ultrastructure of Animal Viruses and Bacteriophages: An Atlas" (J.A. Dalton and F. Hagnenau, eds.) p. 213. Academic Press, N.Y.

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

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

Compans, R.W., Nakamura, K., Roth, M.G., Holloway, W.L., Kemp, M.C. (1980). In "Structure and Variation in Influenza Virus" (G.W. Laver and G.M. Air, eds.) pp 223-232. Elsevier/North-Holland, N.Y.

Couch, R.B., Kasel, J.A., Gerin, J.L., Schulman, J.L., Kilbourne, E.D. (1974). J. Infect. Dis. 129:411.

Cretescu, L., Beare, A.S., Schild, G.C. (1978). Infect. Immun. 22:322.

Crumpacker, C. S. II (1980). Rev. Infect. Dis. 2:78.

Cuatrecasas, P., Illiano, G. (1971). Biochem. Biophys. Res. Commun. 44:178.

Currie, G.A., Bagshawe, K.D. (1968). Br. J. Cancer 22:588.

Davenport, F.M. (1961). Amer. Rev. Resp. Dis. 83:146.

Davenport, F.M. (1973). Med. J. Aust. Spec. Suppl. 1:33.

Davis, J.F., Bucher, D.J. (1981). Fourth Intl. Negative Strand Virus Conf.(R. Compans, and D.H.L. Bishop, eds.) pp. 203-208. Elsevier/North-Holland, N.Y.

Davis, B.D., Dulbecco, R., Eisen, H.N., Ginsberg, H.S., Wood, W.B., McCarty, M. (1973). "Microbiology" second edition, pp. 1307-1328. Harper and Row, Hagerstown, Md.

Desselberger, U. (1977). Arch. Virol. 53:335.

Desselberger, U., Zamecnik, P., Palese, P. (1980). In "Structure and Variation in Influenza Virus" (G.W. Laver and G.M. Air, eds.) pp. 169-179. Elsevier/North-Holland, Amsterdam.

Dimmock, N.J. (1971). J. gen. Virol. 13:481.

Dopheide, T.A., Ward, C.W. (1980). In "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp. 21-26. Elsevier/North-Holland, Amsterdam.

Dourmashkin, R.R., Tyrrell, D.A.J. (1974). J. gen. Virol. 24:129

Dowdle, W.R., Mostow, S.R., Coleman, M.T., Kay, H., Schoenbaum, S.C. (1973). Postgrad. Med. J. 49:159.

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

Drzeniek, R. (1972). Current Top. Microbiol. Immunol. 59:35.

Drzeniek, R. (1973). Histochem. J. 5:271.

Drzeniek, R., Seto, J.T., Rott, R. (1966). Biochim. Biophys. Acta 128:547.

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

Edmond, J.D., Johnson, R.G., Kidd, D., Rylance, H.J., Sommerville, R.G. (1966). Brit. J. Pharmacol. 27:415.

Fazekas de St. Groth, S. (1962). Advan. Virus Res. 9:1.

Fazekas de St. Groth, S. (1963). Ann. N.Y. Acad. Sci. 103:674.

Fenner, F. (1970). "The Biology of Animal Viruses", Vol.I. Academic Press, Inc., N.Y.

Fields, S. Winter, G., Brownlee, G.C. (1981). Nature 290:213.

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

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

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

Gesner, B.M., Ginsberg, V. (1964). Proc. Nat. Acad. Sci. (USA) 52:750.

Gething, M.J. Bye, J., Skehel, J., Waterfield, M. (1980). In "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp. 1-10. Elsevier/North-Holland, Amsterdam.

Gottschalk, A., Drzeniek, R. (1972). In "The Glycoproteins", (A. Gottschalk, ed.) pp. 381-402. Elsevier/North-Holland, Amsterdam.

Gottschalk, A. (1957). Biochim. Biophys. Acta 23:645.

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

Gregoriades, A. (1973). Virology 54:369.

Gregoriades, A. (1980). J. Virology 36:470.

Greenberg, S.B. Couch, R.B., Kasel, J.A. (1974). Am. J. Epidemiol. 100:209.

Groome, N.P., Belyavian, G., Lansdell, A., Ashford, D. (1977). Biochim. Biophys. Acta 495:54.

Gulik-Krzywicki, T. (1975). Biochim. Biophys. Acta 415:1.

Hackett, C.J., Askonas, B.A., Webster, R.G., van Wyke K. (1980). J. gen. Virol. 47:497.

Han, T. (1973). Clin. Exp. Immunol. 13:165.

Haukenes, G., Harboe, A., Mortensson-Egmund, K. (1966). Acta Pathol. Microbiol. Scand. 66:510.

Hay, A.J., Kennedy, N.C.T., Skehel, J.J., Appleyard, G. (1979). J. gen. Virol. 42:189.

Helenius, A., Simons, K. (1975). Biochim. Biophys. Acta 415:29.

Helenius, A., Fries, E., Kartenbeck, J. (1977). J. Cell Biol.

Helenius, A., Kartenbeck, J., Simons, K., Fries, E. (1980). J. Cell Biol. 84:404. 75:866.

Hennessy, A.V., Minuse, E., Davenport, F.M. (1972). J. Immunol. 109:213.

Hierholzer, J.C., Sugg, M.T. (1969). Appl. Microbiol. 18:816.

Hill, M.W. (1978). In "Liposomes and their Uses in Biology and Medicine (P. Papahadjopoulos, ed.) p. 101-111. Ann. N.Y. Acad. Med , N.Y.

Hirst, G.K. (1941). Science 94:22.

Hirst, G.K. (1942a). J. Exp. Med. 75:47.

Hirst, G.K. (1942b). J. Exp. Med. 76:195.

Hosaka, Y., Shimizu, Y.K. (1972). Virology 49:640.

Hoskins, T.W., Davies, J.R., Smith, A.J., Miller, C.L. Allchin, A. (1979). Lancet 1:33.

Huang, R.T.C., Rott, R., Klenk, H.-D. (1981). Virology 110:243.

Huang, R.T., Wahn, K., Klenk, H.-D., Rott, R. (1979). Virology 97:212.

Inglis, S.C., Barrett, T., Brown, C.M., Almond, J.W. (1979). Proc. Nat. Acad. Sci.(USA) 76:3790.

Jackson, G.G. (1976). In "Influenza Virus-Vaccines-Strategy", (P. Selby ed.) Academic Press, N.Y.

Jahiel, R.I., Kilbourne, E.D. (1966). J. Bacteriol. 92:1521.

Kaper, J.M. (1968). In "The Molecular Basis of Virology" (H. Frankel-Conrat, ed.) pp. 1-133. Reinhold Book Corp. N.Y.

Kedar, E., Ortiz, L.M., Fahey, J.L. (1973). J. Immunol. 112:26.

Kendal, A.P., Cox, N.J., Murphy, B.R., Spring, S.B., Maasab, H.F. (1977b). J. gen. Virol. 37:145.

Kendal, A.P., Eckert, E.A. (1972). Biochim. Biophys. Acta 185:163.

Kendal, A.P., Galphin, J.C., Palmer, E.L. (1977c). Virology 76:186

Kendal, A.P., Kiley, M.P. (1973). J. Virol. 12:1482.

Kendal, A.P., Kiley, M.P. (1975). In "The Negative Strand Viruses" (B.Mahy, and R. Barry, eds.) pp 145-160. Academic Press, N.Y.

Kendal, A.P., Minuse, E., Maasab, H.F., Hennessey, A.V., Davenport, F.M.(1973). Am. J. Epidemiol. 98:96.

Kendal, A.P., Noble, G.R., Dowdle, W.R. (1977a). J. Infect. Dis. 136:S415.

Kilbourne, E.D. (1963). In "Preventive Medicine and Public Health" (W.G. Smillie, ed.) p. 192. Macmillan, N.Y.

Kilbourne, E.D. (1975). In Influenza Viruses and Influenza" (E.D. Kilbourne, ed.) pp 483-538. Academic Press, N.Y.

Kilbourne, E.D. (1976). J. Infect. Dis. 134:384.

Kilbourne, E.D., Chanock, R.M., Choppin, P.W., Davenport, F.M., Fox, J.P., Gregg, M.B., Jackson, G.G., Parkman, P.D. (1974). J. Infect. Dis. 129:750.

Kilbourne, E.D., Laver, W.G., Schulman, J.L., Webster, R.G. (1968). J. Virol. 2:281.

Kilbourne E.D., Schulman, J.L., Couch, R.B. (1972). In In "Proceedings of the 2nd International Congress for Virology (J.L. Melnick, ed) p. 121. Karger, Basel.

Kim, H.W., Arrobio, J.O., Brandt, C.D., Parrott, R.H., Murphy, B. R., Richman, D.D., Chanock, R.M. (1976). Pediatr. Res. 10:238.

Klenk, H.-D. (1980). In "Structure and Variation in Influenza Virus" (G.W. Laver and G.M. Air, eds) p. 213-222. Elsevier/North- Holland, Amsterdam.

Klenk, H.D., Compans, R.W., Choppin, P.W. (1970). Virology 42:1158.

Klenk, H.-D., Rott, R., Becht,H. (1972). Virology 47;579.

Klenk, H.D., Rott, R. Orlich, M. (1977). J. gen. Virol. 36:151.

Knop, J., Sedlacek, H.H., Seiler, F.R. (1978). Immunology 34:181.

Lake, P., Mitchison, N.A.(1976). Cold Spring Harbor Symp. Quant. Biol. 41:589.

Lamb, R.A., Choppin, P.W. (1979). Proc. Nat. Acad. Sci. (USA) 76:4908.

Laver, W.G. (1963). Virology 20:251.

Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W., Ward, C.W., Dopheide, T.A.A. (1979a). Virology 98:226.

Laver, W.G., Baker, N. (1972). J. gen. Virol. 17:61.

Laver, W.G., Gerhard, W., Webster, R.G., Frankel, M.E., Air, G.M. (1979b). Proc. Nat. Acad. Sci.(USA) 76:1425.

Laver, W.G., Kilbourne, E.D. (1966). Virology 30:493.

Laver, W.G., Valentine, R.C. (1969). Virology 38:105.

Laver, W.G., Webster, R.G. (1966). Virology 30:104..

Lazarowitz, S.G., Choppin, P.W. (1975). Virology 48:440.

Lazdins, I., Haslim, E.A., White, D.O. (1972). Virology 49:758.

Leavitt, J., Mayner, R., Phelan, M., Leavitt, A., Ennis, F. A. (1979). In "Structure and Variation in Influenza Virus" (G.W. Laver, ed.) Elsevier/North-Holland, Amsterdam.

Lee, A. (1968). Proc. Soc. Exp. Biol. Med. 128:891.

le Maire, M., Miller, J.V., Tanford, C. (1976). Biochem. 15:2336.

Lenard, J., Wong, C.Y., Compans, R.W. (1974). Biochim. Biophys. Acta 332:341.

Liacopoulos, P., Ben-Efrain, S. (1975). Prog. Allergy 18:97.

Liebovitz, A., Coultrip, R.L., Kilbourne, E.D., Legters, L.J., Smith, C.D., Chin, J., Schulman, J.L. (1971). J. Infect. Dis. 124:481.

Lohmeyer, J., Klenk, H.-D. (1979). Virology 93:134.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). J. Biol. Chem. 193:265.

Lubeck, M.D., Schulman, J.L., Palese, P. (1978). J. of Virol. 28:710.

Maasab, H.F. (1969). J. Immunol. 102:728.

Mackenzie, J.S., Mackenzie, S., Lloyd, J., Dent, V. (1975). J. Hyg. Canb. 75:425.

Makino, S., Reynolds, J.A., Tanford, C. (1973). J. Biol. Chem. 248:4926.

Manesis, E.K., Cameron, C.H., Gregoriadis, G. (1979). FEBS Letters 102:107.

Mayron, L.W., Robert, B., Winzler, R.J. (1961). Arch. Biochem. Biophys. 92:475.

Meindl, P., Tuppy, H. (1966a). Monatsh. Chem. 97:990.

Meindl, P., Tuppy, H. (1966b). Monatsh. Chem. 97, 1628.

Meindl, P., Bodo, G., Lindner, J., Palese, P. (1974). Virology 58:457.

Milstein, C., Brownlee, G.G., Harrison, T.M., Mathews, M.B. (1972). Nature New Biol. 239:117.

Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., Fiers, W., Threlfall, G., Barber, C., Carey, N., Emtage, S. (1980). Cell 19:683.

Min Jou, W., Verhoeyan, M., Devos, R., Saman, E., Huylebroeck, D., Rompuy, L.V., Fang, R., Fiers, W. (1980). In "Structure and Variation in Influenza Virus" (W.G. Laver and G. M. Air, eds.) p.63-68. Elsevier/North-Holland, Amsterdam.

Morbidity and Mortality Weekly Report (1977). 26:193.

Morbidity and Mortality Weekly Report (1978). 27#6.

Morbidity and Mortality Weekly Report (1978). 27#16.

Morein, B., Helenius, A., Simons, K., Pettersson, R., Kaariainen, L. (1978). Nature 276:715.

Moss, B.A., Underwood, R.A., Bender, V.J., Whittaker, R.G. (1980). In "Structure and Variation in Influenza Viruses" G.W. Laver and G.M. Air, eds.) p. 329-338. Elsevier/North-Holland, Amsterdam.

Mostow, S.R., Schild, G.C., Dowdle, W.R., Wood, R.J. (1975). J. Clin. Microbiol. 2:531.

Murphy, B.R., Kasel, J.A., Chanock, R.M. (1972). N. Engl. J. Med. 286:1329.

Murphy, B.R., Markoff, L.J., Chanock, R.M., Douglas, R.G., Bitts, R.F., Cate, T.R., Couch, R.B. (1977). Dev. Biol. Standard. 39:47.

Murphy, B.R., Richman, D.D., Spring, S.B., Chanock, R.M. (1976). Postgrad. Med. J. 52:381.

McCauley, J., Skehel, J., Elder, K., Gething, M.-J., Smith, A., Waterfield, M. (1980). In "Structure and Variation in Influenza Virus" (G. Laver and G.M. Air, eds.) pp 97-104. Elsevier/North-Holland, N.Y.

Nakajima, S., Suigura, A. (1980). Virology 101:450.

Neurath, A.R., Rubin, B.A. (1971). In "Monographs in Virology" Vol 4. (J.L. Melnick, ed.) pp.1-87. S. Karger, Basel.

Noll, H., Aoyagi, T., Orlando, J. (1962). Virology 18:154.

Ogra, P.L., Chow, T., Beutner, K.R., Rubi, E., Strussenberg,J., DeMello, S., Rizzone, C. (1977). J. Infect. Dis. 135:499.

Osborn, J.E. (1977). "Influenza in America 1918-1976", p. 89. Prodist, N.Y.

Oxford, J.S., Hockley, D.J., Heath, T.D., Patterson, S. (1981). J. gen. Virol. 52:329.

Palese, P., Bucher, D., Kilbourne, E.D. (1973). Appl. Microbiol. 25:195.

Palese, P., Compans, R.W. (1975). J. gen. Virol. 33:159.

Palese, P., Ritchey, M.B., Schulman, J.L. (1977). Virology 76: 114.

Palese, P., Schulman, J.L., Bodo, G., Meindl, P. (1974). Virology 59:490.

Palese, P., Schulman, J.L. (1976). Proc. Nat. Acad. Sci.(USA) 73:2142.

Palese, P., Schulman, J.L. (1976). J. Virol. 17:876.

Palese, P., Tobita, K., Ueda, M., Compans, R.W. (1974). Virology 61:397.

Paniker, C.K.J. (1968). J. gen. Virol. 2:385.

Papahadjopoulos, D., Portis, A., Pangborn, W. (1978). In "Liposomes and their Uses in Biology and Medicine" (D. Papahadjopoulos, ed.). Ann. N.Y. Acad. Sci. 308:50.

Parkman, P.D., Galasso, G.J., Fox, F.H., Jr., Noble, G.R. (1976). J. Infect. Dis. 134:100.

Pons, M.W. (1975). In "Influenza Viruses and Influenza" (E.D. Kilbourne, ed.) pp 145-170. Academic Press, N.Y.

Porter, A.G., Barber, C., Carey, N.H., Hallewell, R.A., Threllfall, G., Emtage, J.S. (1979). Nature 282:471.

Rafelson, M.E. Jr. (1963). Exposes a Biochim. Med. 24:121.

Rafelson, M.E., Schneir, M., Wilson, V.W. (1963a). Arch. Biocem, Biophys. 103:424.

Rafelson, M.E., Schneir, M., Wilson, V.W. (1963b). In "The Amino Sugars" Vol. II (R.W. Jeanolz and E.A. Balags, eds.) p. 171-179. Academic Press, N.Y.

Reginister, M., Rentier, B. Dierickx, L. (1975/76). International Virology 4:344.

Reginister, M., Joassin, L., Fontaine-Delcambe, P. (1979). J. gen. Virol. 45;283.

Reynolds, J.A., Tanford, C. (1970a). J. Biol. Chem. 245:5161.

Reynolds, J.A., Tanford, C. (1970b). Proc. Nat. Acad. Sci.(USA) 66:1002.

Richardson, C.D., Scheid, A., Choppin, P.W. (1980). Virology 105:205.

Richman, D.D., Murphy, B.R., Belshe, R.B., Rusten, H.M., Chanock, R.M., Blacklow, N.R., Parrino, T.A., Rose, F.B., Levine, M.M., Caplan, E. (1977). J. Infect. Dis. 136:256.

Rifkin, D.B., Compans, R.W., Reich, E. (1972). J. Biol. Chem. 247:6432.

Ritchey, M.B., Palese, P. (1976). Virology 72:410.

Ritchey, M.B., Palese, P., Schulman, J.L. (1976). J. Virol. 20:307.

Robertson, B.H., Bhown, A.S., Compans, R.W., Bennet, J.C. (1979). J. Virol. 30:759.

Robinson, N.C., Tanford, C. (1975). Biochem. 14:369.

Rott, R. (1980). In "Structure and Variation in Influenza Virus" (G.W. Laver and G.M. Air, eds.) pp 201-211. Elsevier/North- Holland, Amsterdam.

Rott, R., Becht, H., Orlick, M. (1974). J. gen. Virol. 22:35.

Rott, R., Drzeniek, R., Frank, H. (1970). In "The Biology of Large RNA Viruses" (R.D. Barry and B.W.J. Mahy, eds.) pp. 75-85. Academic Press, N.Y.

Russell, S.M., Liew, F.Y. (1979). Nature 280:147.

Sanford, H.B. (1967). Transplantation 5:1273.

Schild, G.C., Dowdle, W.R. (1975). In "The Influenza Virus and Influenza" (E.D. Kilbourne, ed.) pp 316-372. Academic Press, N.Y.

Schulman, J.L., Palese, P. (1977). J. Virol. 24:170.

Schild, G.C., Pereira, H.G. (1969). J. gen. Virol. 4:355.

Schulman, J.L. (1969). Bull. W.H.O. 41:647.

Schulman, J.L. (1975). In "The Influenza Virus and Influenza" (E.D. Kilbourne, ed.) pp 373-394. Academic Press, N.Y.

Schulman, J.L., Khakpour, M., Kilbourne, E.D. (1968). J. Virol. 2:778.

Schulman, J.L., Kilbourne, E.D. (1965). J. Bacteriol. 89:170.

Schulman, J.L., Kilbourne, E.D. (1969). Proc. Nat. Acad. Sci.(USA) 63:326.

Schulman, J.L., Palese, P. (1975). Virology 63:98.

Schulman, J.L., Palese, P. (1978). In "The Negative Strand Viruses and the Host Cell" (B.W.J. Mahy and R.D. Barry, eds.). pp 667-674. Academic Press, N.Y.

Schulze, I.T. (1970). Virology 42:890.

Schulze, I.T. (1972). Virology 47:181.

Schulze, I.T. (1973). Advan. Virus Res. 18:1.

Schulze, I.T. (1975). In "The Influenza Virus and Influenza" (E.D. Kilbourne, ed.) pp 53-83. Academic Press, N.Y.

Schwarz, R.T., Schmidt, M.F.G., Anwer, U., Klenk, H.-D. (1977). J. Virol. 23:217.

Sedlacek, H.H., Meesman, H., Seiler, F.R. (1974). Int. J. Cancer 15:409.

Sedmak, J.J., Grossberg, S.E. (1973). Virology 56:658.

Segrest, J.P., Gulik-Krzywicki, T., Sardet, C. (1974). Proc. Nat. Acad. Sci.(USA) 71:3294.

Shope, R.E. (1931). J. Exp. Med. 54:373.

Simons, K., Helenius, A., Garoff, H. (1973). J. Mol. Biol. 80:119.

Simons, R.L., Rios, A., Ray, P.K. (1971). J. Nat. Cancer Inst. 47:1087.

Skehel, J.J. (1971). Virology 45: 275.

Skehel, J.J., Hay, A.J., Armstrong, J.A. (1977). J. gen. Virol. 38:97.

Skelly, J., Howard, C.R., Zuckerman, A.J. (1981). Nature 290:51.

Skehel, J.J., Schild, G.C. (1971). Virology 44:396.

Skehel, J.J., Waterfield, M.D. (1975). Proc. Nat. Acad. Sci (USA) 72:93

Skelly, J., Howard, C.R., Zuckerman, A.J. (1981) Nature 290:51.

Sleigh, M.J., Both, G.W., Brownlee, G.G., Bender, V.G., Moss, B.A. (1980). In "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp. 69-78. Elsevier/ North-Holland, Amsterdam.

Smith, W., Andrews, C.H., Laidlaw, P.P. (1933). Lancet 2:66.

Staihelin, L.A. (1968). J. Ultrastructure Res. 22:326.

Stanley, P., Haslam, E.A. (1971). Virology 46:764.

Sugiura, A. (1975). In " The Influenza Virus and Influenza" (E.D. Kilbourne, ed.)pp 171-214. Academic Press, N.Y.

Takatsu, K., Ishazaka, D.(1976). J. Immun. 116:1257.

Tanford, C. (1974). J. Phys. Chem. 78:2469.

Tanford, D., Reynolds, J.A. (1976). Biochim. Biophys. Acta 457:133.

Taussig, M.J. (1976). In "The Antigens" Vol IV. (M. Sela, ed.) pp 333-368. Academic Press, N.Y.

Taylor, R.M. (1949). Am. J. Pub. Hlth. 39:171.

Threlfall, G., Barber, C., Carey, N., Emtage, S. (1980). In "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp. 51-61. Elsevier/North- Holland, Amsterdam.

Tillack, T.W., Scott, R.E., Marchesi, V.T. (1972). J. Exp. Med. 135:1209.

Tyrrell, D.A.J., Smith, J.W.G. (1979). Brit. Med. Bull. 35:77.

Verhoeyen, M., Fang, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E., Fiers, W. (1980). Nature 286:771.

Voller, A., Bidwell, D.E., Bartlett, A. (1976). Bull. W.H.O. 53:55.

Vonka, V., Zavedova, H., Bruj, J., Skovil, V., Janout, V., Uvizl, M., Kotikova, J. (1977). Develop. biol. Standard 39:337.

Walop, J.N., Boschman, T.A.C., Jacobs, J. (1960). Biochim. Biophys. Acta 44:185.

Ward, C.W., Dopheide, T.A. (1980). Aust. J. Biol Sci. 33:449.

Ward, C.W., Dopheide, T.A. (1980). In Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp. 27-38. Elsevier/North-Holland, Amsterdam.

Warren, L. (1959). J. Biol. Chem. 234:1971.

Warren, L. (1963). In "Method in Enzymology," Vol. 6 (S.P. Colowick and N.O. Kaplan, eds.) pp. 463-473. Academic Press, N.Y.

Waterfield, M.D., Espelie, K., Ebler, K., Skehel, J.J. (1979). Brit. Med. Bull. 35:57.

Webster, R.G. (1970). Virology 40:643.

Webster, R.G., Campbell, C.H., Granoff, A. (1971). Virology 44:317.

Webster, R.G., Campbell, C.H., Granoff, A. (1973). Virology 51:149.

Webster, R.G., Hinshaw, V. (1977). Infect. Immun. 17:561.

Webster, R.G., Kendal, A.P., Gerhard, W. (1979). Virology 96:258.

Webster, R.G., Laver, W.G. (1967). J. Immunol. 99:49.

W.H.O. Memorandum, (1980). Bull. W.H.O. 58(4):585.

Weiss, L., Mayhew, E., Ulrich, K. (1966). Lab. Invest. 15:1304.

Wiley, D.D., Wilson, I.A., Skehel, J.J. (1981). Nature 289:373.

Wilson, V.W., Rafelson, M.E. (1967). Biochim. Biophys. Acta 146:160.

Wilson, J.A., Skehel, J.J., Wiley, D.C. (1981).

Winter, G., Fields, S. (1980). Nucleic Acids Res. 8:1965.

Wrigley, N.G. (1979). Brit. Med. Bull. 35:35.

Wrigley, N.G., Skehel, J.J., Charlewoood, P.A., Brand, C.M. (1973). Virology 51:525.

Yamane, N., Arikawa, J., Odagiri, T., Sukeno, N., Ishida, N. (1978). Jpn. J. Med. Sci. Biol. 31:431.

Yewdell, J.W., Frank, E., Gerhard, W. (1981). J. Immunol. 126:1814.

Young, J., Palese, P. (1979). Proc. Nat. Acad. Sci.(USA) 76:6547.