THE ANTIGENIC RELATIONSHIPS OF INFLUENZA A VIRUSES AND THE INCIDENCE IN ANIMALS OF ANTI INFLUENZA A ANTIBODIES A STUDY BY IMMUNODIFFUSION IN CELLULOSE ACETATE

by

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ABSTRACT

Immunoprecipitin reactions in cellulose acetate were examined by studying the immunological relationships among viruses within the Myxovirus group, which included: influenza A/PR8, A/FM1/Can/53, A2/Can/57, A2/Hong Kong /1/68, A/Equi 1, A/Swine, A/Duck, influenza B/Can/5/66, and parainfluenza Sendai. Homologous and cross immunoprecipitin reactions were carried out by diffusing virus particles and virus soluble antigens against specific anti-influenza antisera from convalescent and hyperimmune rabbits, and from hyperimmune roosters. In homologous reactions, a distinct virus-specific precipitin band pattern was demonstrated for each virus strain.

During these studies, certain aspects of immunoprecipitin reactions were clarified:

1. Virus-specific immunoprecipitin reactions were identified and differentiated from host (CE)-specific ones in the majority of immunoprecipitin reactions by comparison and by cross reactions with a host (CE) -specific antigen antibody system analogous to the virus system. Antiviral and antihost convalescent rabbit sera, and hyperimmune rabbit antisera absorbed with N-CAM did not possess detectable antihost antibodies against N-ALF or against N-CAM soluble antigens, but hyperimmune rooster sera did so against N-CAM soluble antigen, but not against N-ALF. All immunoprecipitin reactions between virus antigens and antisera devoid of antihost antibody, were considered to be virus-specific, even though purified viruses contained detectable host contaminants.

2. SDS and DOC caused non-specific precipitation of serum components and of virus components which could be confused with immuno-

precipitin reactions. Nonidet P40 caused a characteristic non-specific precipitation, which was distinct from immunoprecipitin reactions. It was used to disrupt virus particles, in preference to the other surfactants.

3. Non-specific inhibitors present in normal and immune sera, weakly precipitated some influenza virus components in virus particles. Inhibitors were tentatively identified in immunoprecipitin reactions on the basis of susceptibility to V. cholerae filtrate RDE and to periodate. Strongly precipitated components such as the major influenza A type-specific component were precipitated by antibody, demonstrated to be a 7 S gamma-globulin.

The major type-specific component was identified in all influenza A strains studied and was distinct from that of influenza B or parainfluenza Sendai. It was antigenically identical in virus particles and in virus soluble antigens, and appeared to be the same as the type-specific component identified by Schild and Pereira (1969) as the RNP. However, evidence suggests that the major type-specific component may not be the RNP, but may be the major or membrane protein ('M') recently isolated and described (Kilbourne et al., 1972)

A second group of influenza A type-specific components, less strongly precipitated than the major one, appeared to be antigenically the same in virus particle and soluble antigens, and could be the type-specific RNP complex.

A third component found only in virus soluble antigen may be a non-structural one.

The major influenza A type-specific component demonstrated strain heterogeneity in immunoprecipitin cross reactions between influenza A virus particle and soluble antigens, and respective immune sera. This heterogeneity cannot be easily resolved by the type-specific complement fixation test.

Similar strain heterogeneity was also demonstrated by the less strongly precipitated type-specific components in virus soluble antigens.

The identity of secondary virus antigens, which included all but the primary or major type-specific one, was not established by immunoprecipitin tests. However, relationships among influenza A strains revealed by cross reactions among secondary antigens agreed with established ones, and disclosed others not previously demonstrated. The nature of these relationships is discussed. A comparison is made with relationships revealed by HAI tests.

A survey of 15 species of normal animal, avian, and human sera by immunoprecipitin tests in cellulose acetate using virus particle antigens, revealed an extremely high incidence of precipitins against a broad range of influenza A virus strains; the most prevelant being against A2/Hong Kong/1/68 and A/PR8.

Treatment of the normal sera by trypsin-heat-periodate, $NaIO_4$, V. cholerae filtrate RDE, or kaolin, eliminated the precipitins in the majority of sera, suggesting that they were non-specific inhibitors. Complementary HAI tests of the treated sera confirmed the presence of inhibitors. However, in a number of sera, these treatments did not eliminate, or only altered, anti-influenza precipitins, and their identity as antibody or inhibitor could not be established unequivocally by the criterion of sensitivity to the treatments. Similarly, residual HAI components remaining in treated sera could not be unequivocally identified, but, the persistence of both precipitins and HAI activity in treated sera strongly suggested the presence of antibody.

Precipitation of the influenza A major type-specific component in virus soluble antigens by human 7 S gamma-globulin was demonstrated to

be virus-specific and antibody initiated. It was established as a reference reaction to identify similar immunoprecipitin reactions occurring between virus soluble antigens and normal or immune sera.

Thus, precipitins in normal animal sera were identified as anti-influenza antibody by demonstrating that the precipitin band formed between normal sera and influenza A type-specific antigen (SA) linked in identity with the type-specific reference reaction. Continuation of the linkage to a reaction occurring between type-specific antigen and known influenza antiserum, such as convalescent phase human, gave further proof that the precipitin in the normal sera was antibody. The demonstration that influenza A type- and/or strain-specific complement fixing antibodies were present, was additional evidence that normal sera contained anti-influenza A antibodies.

Using the criteria evolved, it became clear that the type-specific precipitating antibodies against A2/Hong Kong/1/68 and against A/PR8 soluble antigens were found in only a few sera of five species of animals: cat, dog, rabbit, goat, and chipmunk. Complement fixing type- and/or strain-specific antibodies against A2/Hong Kong/1/68 were found in six species: cat, dog, rabbit, fowl, sheep, and cottontail rabbit.

The epidemiological implications of the results from the animal serum survey are discussed.

The effects of the different inhibitor inactivation treatments on precipitins and hemagglutinin inhibitors in the sera of different species are presented.

These investigations have revealed that the immunoprecipitin test in cellulose acetate is a valuable complement to existing serological methods for immunological studies of influenza viruses.

CONCLUSIONS

1. Established antigenic relationships among influenza A viruses were confirmed by immunoprecipitin tests in cellulose acetate, and new relationships were revealed (Fig. 8). The influenza A major type-specific component was demonstrated to be antigenically heterogeneous. (Part I, pp 171-216)

2. Because of its strength and heterogeneity in immunoprecipitin reactions, the influenza A major type-specific component is thought to be the membrane protein ('M') of the virus rather than the ribonucleoprotein. (Part I, pp 243-248)

3. Sodium dodecyl sulfate and sodium deoxycholate should not be used to disrupt virus particles for diffusion in cellulose acetate, because non-specific precipitation of serum and virus components by these surfactants interferes with immunoprecipitin reactions. (Part I pp 144-148)

4. Non-specific inhibitors in both normal animal sera, and in the sera of animals immunized against influenza, weakly precipitate at least one influenza A virus component. Precipitins which are eliminated by V. cholerae filtrate RDE, sodium metaperiodate, or trypsin-heat-periodate are tentatively identified as inhibitors. The influenza A major type-specific component is not precipitated by inhibitors, but by a 7 S gamma globulin. (Part I, pp 134-143; Part II, 345-350, 359-361)

5. In both immunoprecipitin and hemagglutination inhibition tests, the treatment required to eliminate non-specific inhibitors of influenza A viruses from normal sera varies according to the animal species and virus subtype. (Part II, pp 338-350)

6. Only a few individual sera from fourteen animal species examined in the Ottawa area possessed precipitins which could be identified as anti-influenza antibodies. Therefore, a reservoir of human influenza virus does not exist in these animals. (Part II, pp 370-380, 385-390)

7. The natural transmission of influenza viruses between animal and man must be demonstrated in order to establish that a reservoir for human influenza exists in animals. Surveys such as the one reported in this thesis are required to indicate which animal species could be potential reservoirs.

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C, D, 7S γ-G and A/PR8 soluble antigen 504

ABBREVIATIONS

The following abbreviations are commonly used throughout the text.

CE	chick embryo
ALF	chick embryo allantoic fluid
N-ALF	normal chick embryo allantoic fluid
V/ALF	virus infected allantoic fluid
CAM	chick embryo chorioallantoic membranes
N-CAM	normal chick embryo chorioallantoic membranes
SA	soluble antigen, extracted from chorioallantoic membranes
V/SA	soluble antigen extracted from virus infected chorioallantoic membranes
N-CAM/SA	soluble antigen extracted from normal chorioallantoic membranes
V/C	virus concentrate suspension prepared from virus infected allantoic fluid by differential centrifugation
V/C_1	ten-fold virus concentrate suspension
V/C_2	one hundred-fold virus concentrate suspension
V/C_2'	two hundred-fold virus concentrate suspension
V/SDG	virus concentrate purified by sucrose density gradient centrifugation
V/KTDG	virus concentrate purified by potassium tartrate density gradient centrifugation
$N-ALF/C_2$	normal allantoic fluid concentrated one hundred fold by differential centrifugation
N-ALF/SDG	normal allantoic fluid concentrate purified by sucrose density gradient centrifugation
SNF	supernatant fluid

NS	normal serum
As	antiserum
R	rabbit
F	rooster or hen
IN	intranasal
IM	intramuscular
RAs-IN	convalescent rabbit serum
RAs-IN/V	convalescent serum of rabbit intranasally infected with virus
RAs-IN/N-ALF	'convalescent' serum of rabbit intranasally exposed to normal allantoic fluid
hRAs	hyperimmune rabbit serum
hRAs/V	hyperimmune serum of rabbit parenterally immunized with virus
hRAs/V abs	as above, but absorbed with normal chorioallantoic membranes
hRAs/N-ALF	hyperimmune serum of rabbit parenterally immunized with normal allantoic fluid
hRAs/N-ALF abs	as above, but absorbed with normal chorioallantoic membranes
hFAs	hyperimmune rooster serum
hFAs/V	hyperimmune serum of rooster parenterally immunized with virus
hFAs/N-ALF	hyperimmune serum of rooster parenterally immunized with normal allantoic fluid

RBC	red blood cells or erythrocytes
n-saline	physiological saline (0.85% NaCl in distilled water w/v)
PBS	phosphate buffered saline
STE buffer	saline - tris - EDTA buffer (Pons and Hirst, 1968)
Tris	Tris(hydroxymethyl)aminomethane
EDTA	ethylenediamine tetraacetic acid

DOC	sodium deoxycholate
NP 40	Nonidet P40
SDS	sodium dodecyl sulfate
THP	trypsin - heat - periodate (serum treatment for inhibitors)
RDE	receptor destroying enzyme as V. cholerae filtrate (serum treatment for inhibitors)
$NaIO_4$	sodium metaperiodate (serum treatment for inhibitors)
IDD	micro immunodouble diffusion in cellulose acetate
HA	hemagglutinin
HA test	hemagglutination test
HAU	hemagglutinating units with respect to the hemagglutination test
HAI	hemagglutination inhibition test
CF	complement fixing
CFT	complement fixation test
EID_{50}	egg infecting dose of virus, 50% endpoint
EM	electron microscope
PAGE	polyacrylamide gel electrophoresis

INTRODUCTION

During investigations of immunological aspects of influenza A/PR8 infection, Johnson and Westwood (1965) using a newly-developed, highly sensitive technique of micro-immunodoublediffusion in cellulose acetate (Johnson et al., 1964), observed that normal sera of a variety of domestic animals contained precipitins which reacted with strain-specific V antigens of diffusing influenza A/PR8 whole virus particles. Further investigations led to the conclusion that the precipitating component was antibody because it identified with a corresponding component in convalescent rabbit serum (Johnson and Westwood, 1968), and it was found to be present in the gamma-globulin fraction of serum (Johnson and Westwood, 1969).

The animal species tested, rabbit, guinea pig, goat, and sheep, are not known to be susceptible to natural influenza virus infection, but the presence of precipitating antibodies to a virus absent from the human population since 1947 implied unsuspected infection of these animal species, and a wider distribution of Myxovirus among domestic animals than had been revealed by standard serological testing.

The present work investigates the implication of these findings with regard to the epidemiology and possible animal reservoir of Myxovirus infections. The study was divided into two parts.

1. Examination of the immunodiffusion reaction in cellulose acetate by studying the immunological relationships of viruses within the Myxovirus group, and comparing them with those revealed by standard serological testing. The study was confined mainly to the influenza A group.

2. By this immunodiffusion technique, normal animal sera were surveyed to determine the incidence of reactivity to selected Myxovirus antigens.

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PART I

LITERATURE REVIEW

Interrelationships of Influenza Virus Antigens

Influenza viruses are a group of myxoviruses causing the respiratory disease influenza, or related manifestations, which may occur as major epidemics in humans and a host of animal and avian species.

Classification

Influenza A viruses, along with types B and C, were included with Newcastle disease virus (NDV), fowl plague virus (FPV), and mumps virus in the original group designated myxovirus by Andrewes, Bang, and Burnet (1955). They described myxoviruses as a moderately sized (60 - 200 nm diameter) group of animal viruses, having specific affinity for certain mucins, a sensitivity to inactivation by ether, and the ability to adsorb to, and to agglutinate, erythrocytes of fowl and other vertebrates by attachment to mucoprotein receptors, with subsequent spontaneous elution from those sites by means of an enzyme-like action (virus-associated neuraminidase). By electron microscopy, pleomorphic, but usually spherical particles were observed, whose lipoprotein envelope, radiating regularly arranged projections, enclosed the nucleocapsid possessing helical symmetry (Horne _et al_., 1960; Hoyle _et al_., 1961), which consisted of ribonucleoprotein (RNP) (Hoyle, 1952; Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956; Waterson, 1962, Cruickshank, 1964).

These viruses were subdivided into two groups on the basis of differences in biological and morphological characteristics (Andrewes and Worthington, 1959; Waterson, 1962; Andrewes, 1964; Waterson and Almeida, 1966): subgroup I, the influenza group, consisting of influenza types A (human and animal strains), B, and C; subgroup II, the Newcastle disease virus group (NDV), consisting of NDV, mumps, and parainfluenza viruses. A group resembling myxoviruses morphologically, were designated pseudomyxoviruses, and includes measles, rinderpest, distemper, and some bovine parainfluenza 3's. Melnick and McCombs (1966) and Melnick (1971) have designated the influenza group as orthomyxovirus; and in the group designated paramyxovirus, they have included the NDV group, the medipest group (measles, distemper, rinderpest), and simian paramyxoviruses. Major differentiating properties of these groups are found in Table 1 (Melnick, 1971). The comparative properties of the influenza and NDV groups have been extensively reviewed (Hoyle, 1968; Robinson and Duesberg, 1968; Scholtissek et al., 1969; Wilner, 1969; Blair and Duesberg, 1970; Kingsbury, 1970; Barry and Mahy, 1970), and will not be detailed here.

Influenza viruses, notably type A, are the objects of this study, and will therefore be the main viruses discussed.

The influenza group is divided into types A, B, and C, the main criterion of differentiation being the type-specific internal ribonucleoprotein (RNP) antigens (WHO Expert Committee on Influenza, 1953, 1959), also known as the 'S' (Hoyle, 1952; Lief and Henle, 1956), or

TABLE 1. Current classification of animal RNA viruses (from Melnick, 1971)

Nucleic acid core	RNA								
Capsid symmetry	Cubic		Helical			Uknown			
Virion: naked or enveloped	Naked		Enveloped			Enveloped			
Site of capsid assembly	Cytoplasm		Cytoplasm			Cytoplasm			
Site of nucleocapsid envelopment			Surface Membrane			Surface Membrane		Intracytoplasmic Membrane	
Reaction to ether treamtnet	Resistant		Sensitive			Sensitive		Sensitive	
Number of capsomeres	32	92						32	
Diameter of helix (nm)			6-9	18	18				
Diameter of virion (nm)	18-30	54-75	80-120	100-300	60x225	About 100	110-130	35-40	80-160
Molecular wt. of nucleic acid in virion (x10^6)	2	10	2-5	8	3-4	10-12	?	2	?
Virus group	Picorna-Virus	Reovirus (Diplorna-Virus)	Ortho-Myxovirus	Paramyxo-Virus	Rhabdo Virus	Oncorna Virus (Leukovirus)	Arena-Virus	Toga Virus	Corona Virus

'Gebundenes' (G) antigen (Schaffer, 1957; Rott and Schafer, 1964), which was detected by the type-specific complement fixation test (CF) (Hoyle, 1948; Lief and Henle, 1959). It was demonstrated in NDV by immunodiffusion in agar gel (Rott and Schafer, 1964). Influenza type A infects human, animal and avian species, whereas types B and C infect only humans (McQueen et al., 1968; Pereira, 1969).

Apostolov and Flewett (1969) suggest that influenza C may need to be separated taxonomically from types A and B, because the diameter of its internal RNP helix is larger (9nm) than that of type A (6nm), and because of other morphological and biological differences not in keeping with those of influenza A and B.

Each influenza virus type was further subdivided into subtypes or strains on the basis of species of origin, and the predominant, strain-specific envelope or V antigen complex, detected serologically by the hemagglutination inhibition test (HAI), or by the strain-specific complement fixation test (WHO Expert Committee on Influenza 1953, 1959). The strains were also designated by geographical origin, strain number, and year of isolation.

Type A viruses demonstrated the most variation, forming a complex group of subtypes infecting man (A0, A1, A2), animals (swine, equine 1, equine 2), and birds (FPV, N, tern, duck, turkey, etc) (see Review, Part II for description of these), whereas there were only a few B strains and one, or possibly three, type C strains (Hoyle, 1968; Pereira, 1969). All were fully described by Andrewes (1964). Tables conveniently categorizing

influenza viruses in this way have been compiled (Rhodes and Van Rooyen, 1962; Hoyle, 1968).

The most recent classification of influenza viruses was proposed by the WHO Committee on Influenza Virus Nomenclature (1971). It retains as much as possible of the old system of classification described above, but incorporates information which has recently come to light. In addition to the species of origin, and the type-specific RNP antigen (A, B, or C), two virus-coded surface antigens, formerly associated with the V antigen, are considered separately, the hemagglutinin and the neuraminidase, the latter recently isolated and characterized (reviewed by Webster and Laver, 1971; Kilbourne et al., 1972). These surface antigens are morphologically (Drzeniek et al., 1968; Laver and Valentine, 1969; Webster and Darlington, 1969) and immunologically (Seto and Rott, 1966; Webster and Laver, 1967) distinct from each other, and undergo independent antigenic variation (Laver and Kilbourne, 1966; Kilbourne, 1968 ; Webster et al., 1968; Easterday et al., 1969). The neuraminidase antigens are detected and differentiated by the neuraminidase inhibition test (NAI).(Webster and Pereira, 1968). Immunoprecipitin tests in agar gels are considered to be complementary alternatives to the standard complement fixation test (Beard, 1970; Schild and Pereira, 1969), hemagglutination inhibition (HAI) (Schild, 1970), and neuraminidase inhibition (NAI) (Schild and Pereira, 1969; Schild and Newman, 1969) tests. The use of monospecific antisera against isolated ribonucleprotein, hemagglutinin, and neuraminidase antigens derived from appropriate reference strains is recommended.

Strain-specific antigenic characteristics of reference influenza A viruses are divided into eight neuraminidase sybtypes and fifteen hemagglutinin subtypes distributed amongst strains originating from four host species: human, swine, equine, and avian (Table 2 from WHO Committee on Influenza Virus Nomenclature 1971). Hemagglutinins and neuraminidases of human strains may be similar to, or identical with, those of strains from non-human hosts. This table will be referred to as the discussion proceeds.

The relationships illustrated in Table 2 depend upon immunologically demonstrating three distinct characteristics for each strain in this group, ribonucleoprotein (RNP), hemagglutinin (HA), and neuraminidase (NA).

Influenza viruses are morphologically distinguishable from other enveloped RNA viruses (Horne et al., 1960; Hoyle et al., 1961), type C can be distinguished from types A and B (Apostolov and Flewett, 1969), but influenza virus subtypes or strains can not.

However, influenza strains can be characterized, and relationships amongst them determined, by examination of antigenic similarities and variations. This involves an analysis of the structure and biological function of viral components as isolated subunits, and as the integrated virus particle, which may be accomplished visually (by EM) chemically, and immunologically. Many recent publications excellently review the voluminous amount of literature related to this subject (for example, Duesberg and Robinson, 1967; Robinson and Duesberg, 1968; Hoyle, 1968; Pereira, 1969; Scholtissek et al., 1969; Blair and Duesberg, 1970;

TABLE 2. Antigenic characteristics of reference influenza A viruses*
(from WHO Committee on Influenza Virus Nomenclature, 1971)

Neuraminidase Subtypes	Hemagglutinin subtypes														
	HO	H1	H2	H3	Hsw1	Heq1	Heq2	Hav1	Hav2	Hav3	Hav4	Hav5	Hav6	Hav7	Hav8
N1	1	2			5										
N2			3	4									14		
Neq1						6		8	10						
Neq2							7							15	
Nav1										11	12				
Nav2												13			
Nav3								9							
Nav4															16

*Previous designations:

1 = A0/PR/8/34
2 = A1/FM/1/47
3 = A2/Singapore/1/57
4 = A2/Hong Kong/1/68
5 = A/swine/Wisconsin/15/30
6 = A/equine-1/Prague/56
7 = A/equine-2/Miami/63
8 = A/FPV/Dutch/27
9 = A/turkey/England/63
10 = A/chicken/Germany N/49
11 = A/duck/England/56
12 = A/duck/Czech./56
13 = A/tern/South Africa/61
14 = A/turkey/Mass./65
15 = A/duck/Ukraine/1/63
16 = A/turkey/Ontario/6118/68

Kingsbury, 1970; Webster and Laver, 1971; Shatkin, 1971). Two comprehensive publications resulted from a recent symposium on the current state of knowledge of the molecular and cellular biology of influenza and other RNA viruses (Barry and Mahy, 1970), and from a workshop on influenza virus polypeptides and antigens (Kilbourne et al., 1972). The authors in the latter publication have suggested designations for, and summarized properties of, polypeptides comprising influenza viruses, resolved by fractionation of disrupted, purified virus particles (Table 3). Further reference to this table will be made.

Morphology

Intact influenza virus particles have a diameter of 80-120 nm, demonstrated by electron microscopy of shadowed and negatively stained preparations (Waterson, 1962). Recent examination by freeze-drying and freeze-etching reveal the particles to be more uniform than generally believed, and to be icosahedral in shape, with an average diameter of approximately 100 nm (Nermut and Frank, 1971). Filamentous forms occur (Waterson, 1962), usually in freshly isolated human strains, suggesting the possibility that this morphology is the more usual form occurring in natural infections. The surface properties of filamentous forms do not appear to differ from those of spherical ones (Webster and Laver, 1971).

Chemical Composition

The chemical composition of influenza viruses has been estimated as follows: protein, 70-74% (Hoyle, 1968; Blair and Duesberg, 1970), and

TABLE 3. Proposed designations of influenza-virus polypeptides (from Kilbourne *et al.*, 1972)

Designation	Approximate mol wt (daltons)	Function	Remarks
P_1 P_2	81,000-94,000	?	Internal, nonglycosylated proteins present in small amounts - possibly the virion polymerase(s).
HA	75,000-80,000	Hemagglutinin	Glycoprotein. May be cleaved to two smaller polypeptides, HA_1 and HA_2, which are held together in the virion by disulfide bonds. Two HA molecules (or HA_1+HA_2 complexes) form the hemagglutinin spike.
NP	53,000-60,000	Nucleocapsid subunit	......
NA	55,000-70,000*	Neuraminidase	Glycoprotein. The neuraminidase may consist of two polypeptides in some strains, and NA_1 and NA_2 can then be used as designations.
HA_1	50,000-60,000	Hemagglutinin	See remarks on HA
HA_2	23,000-30,000	Hemagglutinin	See remarks on HA
M	21,000-27,000	Major or "membrane" protein of viral envelope	Associated with the inner surface of the lipid layer of the envelope and thought by some to be the major structural protein of the viral membrane.
NS	∿ 25,000	?	A nonstructural protein that is synthesized in the cytoplasm and migrates to the nucleus.

*Active enzyme may be a tetramer of 200,000-240,000 molecular weight.

for FPV, 60-70% (Zilling et al., 1955); lipids, 18.5-20% for influenza A and B (Frommhagen et al., 1959; Blough and Merlie, 1970), 24% for FPV (Klenk et al., 1972), and for influenza A and B consist of phospholipid 10-11%, cholesterol 6.5-7.7%, glycolipids 0.4-0.6%, glycerides 0.8%, free fatty acids 1%, whereas for FPV respective percentages are 12.5, 4.6, 2.4, 1.2, and 0.07 for cholesterol esters; non-nucleic acid carbohydrate 5-7% (Frisch-Niggemeyer and Hoyle, 1956; Frommhagen et al., 1959); ribonucleic acid 0.7-1% for influenza A and B (Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956; Frommhagen et al., 1959); and 1.8% for FPV Zillig et al., 1955).

Derivation of Influenza Virus Components

The disruption of purified influenza virus particles into subunits by various agents, and subsequent fractionation and isolation of these by different methods, has facilitated the study of the structure (morphologic and antigenic), chemistry, and function of the isolated virus components, and has helped to elucidate the integrated virus particle structure in relation to its functions biologically and immunologically.

Virus Disruption The actions disrupting agents have, on influenza viruses, have been extensively investigated; and many have been compiled by Hoyle (1968), Biddle and Kendal (1971), and have been described in the reviews of influenza and myxoviruses referred to above.

Disrupting agents vary in action from mild degradation, in which one or several surface antigens are removed from the intact particle,

to complete disintegration of the virus particle into its basic subunits. A number of agents affecting surface components of the virus, yielding subunits which may or may not be biologically active, are: ether (Hoyle, 1952, 1968; Lief and Henle, 1956; Paucker et al., 1959; Davenport et al., 1960), and urea (Dorman, 1964), release the internal component, the S or complement-fixing antigen from the surface antigens, the hemagglutinin and neuraminidase, with little or no loss of their activites; trypsin releases most of the neuraminidase, but does not disrupt the virus particles (Mayron and Rafelson, 1960; Noll et al., 1962); pyridine (Biddle and Kendal, 1971) selectively inactivates the hemagglutinin leaving other activities and the basic integrity of the virus unaffected; Tween 20, a non-ionic detergent, at high pH, efficiently liberates biologically active hemagglutinin and neuraminidase subunits (Webster and Darlington, 1969); pronase selectively destroys the hemagglutinin, leaving the neuraminidase intact (Biddle, 1968); caseinase C (Reginster, 1966), and the proteases subtilin (Biddle, 1968) and nagarse (Kendal et al., 1968, 1969), act similarly, stripping off the spiky layer, decreasing infectivity, and hemagglutinin but not neuraminidase activity, nagarse in addition, disrupting the internal component; chymostrypsin removes all viral spikes and reduces or eliminates associated infectivity, hemagglutinin, and neuraminidase activities, leaving a smooth particle (much like that left by nagarse) devoid of all glycoproteins, containing only carbohydrate-free polypeptides enclosed in a lipid layer and a protein membrane (Schulze, 1970, 1972); bromelain has essentially the same effect as chymotrypsin, but

does not remove the smallest glycoprotein (Compans et al., 1970; Klenk et al., 1972); phospholipase C inactivates influenza virus (Simpson and Hauser, 1966), removes ^{32}P from virus particles (Mizutani and Mizutani, 1964), and removes 70% of phospholipid from chymotrypsin stripped particles (Schulze, 1970); Nonidet P40, a nonionic detergent, liberates almost all the viral RNP in a single 38 S peak by removing the phospholipid layer enclosing it, and destroys a great deal of the hemagglutinin (90%) and neuraminidase (80%) activities of A/WSN but not of B/Lee, leaving the RNA and three carbohydrate-free polypeptides (Pons et al., 1969; Schulze, 1970, 1972; Schulze et al., 1970); Triton N 101, an anionic detergent, releases neuraminidase, a monovalent hemagglutinin substance, and the internal S-antigen (Corbel and Rondle, 1970; Corbel et al., 1970).

Agents completely disrupting the virus particle into its subunits are the anionic detergents, sodium deoxycholate (DOC) and sodium dodecyl sulfate (SDS), the latter the most effectively. The biological activities of liberated hemagglutinin and neuraminidase subunits may be destroyed in some virus strains (Laver, 1963, 1964; Laver and Valentine, 1969; Schild and Pereira, 1969).

Viruses disrupted by SDS (Easterday et al., 1969; Schild and Pereira, 1969; Schild and Newman, 1969; Schild, 1970; Schild et al., 1970; Kilbourne et al., 1971), DOC (Hana and Hoyle, 1966; Styk and Hana, 1966; Schild et al., 1969; Styk et al., 1970a, 1971a), Nonidet P 40 (Schild and Pereira, 1969; Schild, 1970), and Triton N 101 (Corbel and

Rondle, 1970) have been used to study the antigenic structure of the virus by immunodiffusion in agar gels, which is pertinent to the purpose of the current investigation, that is, the examination of influenza A relationships revealed by immunodiffusion in cellulose acetate.

Fractionation of disrupted virus particles by electrophoresis in cellulose acetate (Laver, 1963, 1964; Laver and Webster, 1966, 1968; Laver and Valentine, 1969; Skehel and Schild, 1971, Webster and Laver, 1972); or in agarose (Hjerten et al., 1970), by density gradient centrifugation in sucrose, tartrate, or in cesium chloride (Davies and Barry, 1966; Duesberg and Robinson, 1967; Pons, 1967; Barry and Davies, 1968; Kingsbury and Webster, 1969; Webster and Darlington, 1969; Kingsbury, 1970; Duesberg, 1970, Skehel, 1971 a; Laver, 1971), by column chromatography (Eckert, 1969; Webster, 1970a ; Lazarowitz et al., 1971); and by polyacrylamide gel electrophoresis (Duesberg and Robinson, 1967; Dimmock and Watson, 1969; Pons et al., 1969; Taylor et al., 1969; Haslam et al., 1970 a, b; Compans et al., 1970; Schulze, 1970; Webster, 1970a Lazarowitz et al., 1971; Kilbourne et al., 1971; Skehel and Schild, 1971; Klenk et al., 1972), have demonstrated that influenza viruses are composed of a limited number of components or subunits.

Resolution of Components The number of components resolved varied with the disruption and fractionation procedures used, and the purpose, whether to isolate specific components such as the hemagglutinin (Laver, 1964, 1971, Webster and Laver, 1972), the neuraminidase (Laver, 1963, 1964; Seto et al., 1966 ; Webster and Laver, 1967), or the RNA or

RNP segments (Pons and Hirst, 1968a,b; Duesberg, 1968; Pons et al., 1969; Duesberg, 1970), or, to resolve disrupted influenza virus particles into the maximum number of basic subunits, as did many of the investigators quoted above.

Influenza viruses had been separated into two components (by ether disruption, and adsorption to, and elution from erythrocytes), the S (internal complement-fixing or ribonucleoprotein), and the V (envelope, hemagglutinating) antigens (Hoyle, 1952; Lief and Henle, 1956). As techniques of resolution were refined, an increasing number of polypeptides were detected in virus particles: three (Laver, 1964; Drzeniek et al., 1966b; Duesberg and Robinson, 1967; Taylor et al., 1969; Holland and Kiehn, 1970), four to five (Pons and Hirst, 1969; Content and Duesberg, 1970), and seven, if minor components were included (Dimmock and Watson, 1969; Joss et al., 1969). By immunodiffusion in agar gel, Dimmock (1969) demonstrated seven antigen-antibody precipitin lines which could be attributed to components of the influenza virus particle. This fact was noted by Compans et al. (1970).

Recently, by polyacrylamide gel electrophoresis under reduced conditions, several laboratories have clearly demonstrated seven, and in some cases, eight distinct polypeptides in influenza A strains, the number depending on the strain examined, and the host in which it was grown: WSN in MDBK cells, rhesus MK cells, and in BHK21-F line of baby hamster cells (Compans et al., 1970); WSN in MDBK cells and in chick embryo fibroblasts (CEF) (Schulze, 1970); BEL/42, and recombinants X-31

(A2/Hong Kong/68 - A/PR8, Kilbourne, 1969), and X-7F1 (AO/NWS - A2/RI/5+, Laver and Kilbourne, 1966) in eggs (Skehel and Schild, 1971) (eight polypeptides were resolved from the latter virus). Seven polypeptides were found in each of twelve other strains including those of avian, equine, human, and swine origin (Skehel and Schild, 1971). In addition, the seven structural proteins of WSN virion were identified in association with the plasma membranes of WSN infected cells, BHK21-F, MDBK, and HeLa-S3;and a nonstructural protein, associated with the infected cell nucleus, and slightly smaller than the smallest structural protein (Lazarowitz et al., 1971). Six different polypeptide species were found in fowl plague virus propagated in CEF, compared to seven found in WSN propagated in these cells, and in rhesus MK cells, FPV lacking the largest glycoprotein (protein 2 of the WSN strain) (Klenk et al., 1972). The total molecular weight of virion polypeptides estimated by the above groups ranged from 296,000 to 420,000 daltons.

The polypeptides resolved, which were demonstrated to consist of three to four glycoproteins comprising the spiky layer, possessing hemagglutinating and neuraminidase activities of the virion, and three to four carbohydrate-free proteins of the core enclosed in a lipid bilayer (Kilbourne et al., 1972) were characterized;the hemagglutinin, neuraminidase, and ribonucleoprotein complex had been isolated and characterized previously. The polypeptides known to constitute influenza viruses have been compiled and designated (Table 3, from Kilbourne et al., 1972).

The current state of knowledge regarding influenza virus polypeptides will be considered, particularly their antigenic functions

as revealed by immunodiffusion, and how they influence the antigenic relationships amongst influenza strains.

Ribonucleoprotein

The internal component of influenza virus, known as the nucleocapsid, the ribonucleoprotein (RNP), the S or complement-fixing antigent (NP in Table 3), is enclosed in a double-walled sac containing carbohydrate-free protein overlaid by a layer of lipid, which is surrounded by a layer of spikes containing the hemagglutinin and the neuraminidase (Schulze, 1970, 1972; Apostolov and Flewett, 1969; Compans and Dimmock, 1969; Compans *et al*., 1970; White *et al*., 1970). Treatment of virions with ether (Hoyle, 1952) or with detergents (Duesberg, 1969; Kingsbury and Webster, 1969; Pons *et al*., 1969; Schulze, 1970, 1972; Skehel, 1971 a; Pons, 1971) releases the ribonucleoprotein, which is described by Pons *et al*., (1969), as composed of a single-stranded RNA molecule in association with protein in what appears to be a helical configuration, the protein acting as a structural backbone for specifically ordered RNA fragments; or by Schulze (1972), as a copolymer made up of viral RNA and a chain of molecules of viral protein (VP II) in a continuous strand, which is turned back on itself to form twisted, double-stranded structures. The structural symmetry is still a matter of controversy (Duesberg, 1970).

Influenza RNAs in the ribonucleoprotein, unlike parainfluenza RNA, are hydrolyzed by ribonuclease (Schafer and Wrecker, 1958; Duesberg, 1969; Kingsbury and Webster, 1969; Pons *et al*., 1969), and can be

displaced by polyvinyl sulfate (Pons et al., 1969; Goldstein and Pons, 1970), which suggests that the RNAs are not completely surrounded by capsid proteins (Kingsbury and Webster, 1969), but rather that the RNA is an outer structural component of the nucleoprotein which can function in protecting the core against proteolytic enzymes (Schafer and Wrecker, 1958; Duesberg, 1969).

The RNA contained in the influenza virion is of a single type, designated parental or virion-type RNA (Scholtissek and Becht, 1971; Pons, 1971). Because of genetic evidence, the RNA of influenza virus had been postulated to exist in several pieces (Burnet, 1956; Hirst, 1962; Cruickshank, 1964), and had been so observed under differing conditions (Schafer, 1963; Agrawal and Bruening, 1966; Nayak and Baluda, 1967; Barry and Davies, 1968; and others). Polyacrylamide gel electrophoresis resolved purified virus RNA into five distinct pieces, with sedimentation coefficients of 9-18 S, and molecular weights ranging from 2.7×10^5 to 7×10^5 daltons (Duesberg, 1968; Pons and Hirst, 1968a). Corresponding RNA segments were found in virus infected cells (Duesberg, 1968; Pons and Hirst, 1968 b). Recently influenza A RNA has been resolved in polyacrylamide gels into six electrophoretic zones of three size classes, with molecular weights ranging from 3×10^5 to 1.0×10^6 (Skehel, 1971 b; Content and Duesberg, 1971). Possibly more than six RNA pieces are present (Young and Content, 1971). Corresponding electrophoretic zones were resolved in virus-specific, double-stranded RNA from infected cells by Content and Duesberg (1971), who also demonstrated that some distinct RNA components of influenza virus have different base sequences,

and presumably different genetic information. End group studies on the virus RNA by Young and Content (1971) have revealed the presence of 5'-terminal nucleoside di- or tri-phosphates, indicating that the segmented genomes are individually synthesized and subsequently assembled to form the viral genome (Shatkin, 1971). The total molecular weight of the genome has been estimated at $2x10^6$ to $3.9x10^6$ daltons (Ada and Perry, 1954; Frisch-Niggemeyer, 1956; Pons and Hirst, 1968a; Li and Seto, 1971; Skehel, 1971 b), and constitutes about 15% of the nucleoprotein complex (Duesberg, 1969).

Purified RNA demonstrates no infectivity (Duesberg, 1970), which may be a result of destruction of RNA polymerases within the virion during purification, or due to its fragmented nature in the purified state (Hirst and Pons, 1972). However, Pons (1971) had demonstrated that all intracellular negative-stranded RNA was in the form of an RNP and suggested that negative-stranded RNA conatining RNP is involved in replication rather than free RNA.

The nuclocapsid or nucleoprotein (RNP) of the influenza virus has been estimated to have a molecular weight of $5.3x10^4$ to $6.5x10^4$ daltons; a sedimentation coefficient of 4 S when dissociated from RNA; and constitutes about 90% of the isolated ribonucleoprotein and 17-30% of the virion protein (Compans et al., 1970; Schulze, 1970; Skehel and Schild, 1971; Klenk et al., 1972). It is particularly rich in arginine (Schulze et al., 1970; Becht, 1971; Klenk et al., 1972), and contains only one demonstrable species of protein (Laver, 1964; Duesberg, 1969; Skehel and Schild, 1971; Pons, 1972).

The nucleoprotein has been resolved into multiple components comparable with, and corresponding to, the number of viral RNAs (Duesberg, 1969; Pons et al., 1969). Sedimentation coefficients of separated components varied, which was attributed by Pons (1971) to differences in the technique of nucleoprotein extraction. Ether or DOC extracted nucleoprotein yielded three components, 68 S, 56 S, 48 S (Paucker et al., 1959) or 70 S, 60 S, 50 S (Duesberg, 1969), or two components, 64 S and 56 S (Kingsbury, 1970; Kingsbury and Webster, 1969) on sucrose density gradients, whereas Nonidet P40 extracted nucleoprotein yielded slightly different values, 48 S, 40 S, 34 S, which on glycerol gradient had sedimented into a component ranging in sedimentation coefficients from 27-44 S, peaking at 38 S (Pons, 1971). The sizes of viral RNA are reflected in the sizes of viral nucleocapsids, for example, 18 S RNA is contained in the 64 S RNP component, and 15 S and 9 S RNA are contained in the 56 S RNP component (Kingsbury, 1970; Kingsbury and Webster, 1969; Duesberg, 1970).

Influenza RNP in virus infected cells has been isolated (Pons, 1971; Krug, 1971), and consists of 90% positive and 10% negative-stranded RNA, little or none of the negative-stranded RNA being found in mature virions (Nayak and Baluda, 1968; Pons, 1971). Biological activity of isolated RNP had not yet been achieved (Content and Duesberg, 1971), but the demonstration by Hirst and Pons (1972) that the RNP of wild-type virus (WSN) was genetically competent in rescuing temperature sensitive mutants, while the viral RNA demonstrated no such capacity, and the finding, that the viral RNP-peptide is associated with polysomes, has

prompted Pons (1972) to suggest, that the RNP of influenza virus plays a much more significant role in replication and translation than was previously thought, a fact which had been suggested by Schafer and Wrecker (1958), and by Kingsbury and Webster (1969).

RNP - Antigenically Homogeneous or Heterogeneous? Immunologically, the internal, soluble (S), or ribonucleoprotein (RNP) antigen has formed the basis for differentiating influenza viruses by the type-specific complement fixation (CF) test into types A, B, and C. The S antigens, derived from virus infected chorioallantoic membranes, or from ether disrupted viral particles of both human and animal type A influenza strains, have been considered to be serologically indistinguishable, as no antigenic differences could be demonstrated among S antigens to homotypic strains by complement fixation tests (Lief et al., 1958; WHO Expert Committee on Influenza, 1959; Lief, 1963). However, Davenport et al. (1960), applying a sensitive complement fixation test (Fulton and Dumbell, 1948) in chessboard experiments, using S antigen prepared from ether disrupted influenza A strains, were able to demonstrate that small, quantitative differences could be detected between the internal S antigens of human strains and those of swine and fowl plague virus.

Schild and Pereira (1969), by immunodiffusion in agar gel, characterized the type-specific ribonucleoprotein of various influenza A strains, human A0, A1, A2, and FPV. The RNP was shown to be composed of a single antigenic component, identical in all influenza A strains

studied, because the precipitation, by anti-MEL-S antiserum, of various detergent (SDS, NP 40, etc.) treated influenza A viruses, revealed a single, well-defined precipitin line, which was continuous for all type A strains, whereas cross reactions between influenza A and B strains were not detected.

In a similar manner, using SDS disrupted virus concentrates, Schild and Newman (1969) demonstrated that the influenza A type-specific RNP was a single component in influenza A2/Hong Kong/68 and was identical to that of A/PR8, A2/England/64, and various avian influenza A strains. The specificity of influenza A RNP and its immunological homogeneity in immunoprecipitin reactions has been subsequently demonstrated by others: Easterday et al. (1969); Beard (1970); Schild (1970); Samadieh and Bankowski (1971 a) (the latter discussed in Literature Review Part II); Corbel and Rondle (1970); Hjerten et al. (1970); Skehel and Schild, (1971); Krug, (1971). The characterization of influenza A RNP by the immunoprecipitin test is now accepted as a complementary alternative to the complement fixation test (WHO Committee on Influenza Virus Nomenclature, 1971).

However, the homogeneity of influenza A RNP has been questioned by a number of investigators in Czechoslovakia (Hana and Hoyle, 1966; Styk and Hana, 1966, 1968; Styk et al., 1968, 1970 a, b; 1971a), who have demonstrated, by immunoprecipitin reactions, that although influenza A RNP is type-specific, it is immunologically heterogeneous. This has been revealed by micro immuno-

doublediffusion in agarose, employing techniques of Wadsworth (1957), Crowle, (1958), and Krause and Raunio, (1967). Purified and concentrated S-antigen preparations of a wide variety of influenza A strains were diffused against anti-S antigen guinea pig hyperimmune sera, and, DOC disrupted purified influenza A strains were diffused against human convalescent and animal immune sera (rabbit, horse, pig, mouse, etc.). The formation of numerous precipitin lines (2-9) which were RNP specific, suggested that multiple antigenic determinants could exist in the nucleoprotein component of influenza virus. The heterogeneous precipitin pattern between animal sera and RNP antigens revealed antigenic differences between the RNP antigens of different influenza A strains. This supported the evidence presented by Davenport et al. (1960). In relation to the above findings, Duesberg (1969) has stated that the antigenic heterogeneity of DOC treated influenza viruses in the Ouchterlony (1948) diffusion test may be explained by the fact that the RNA and RNP of influenza A exists in several distinct pieces. This statement and the demonstration by others that the ribonucleoprotein and associated RNA is segmented in viral particles and in virus infected cells (discussed above) supports the suggestion by Davenport et al. (1960), that, if the nucleoprotein directs infected cells to produce progeny virus with hemagglutinin that is serologically unique, it might be expected that the parent internal S antigens would be different, and therefore possibly serologically distinguishable under different conditions.

Chemical evidence revealed that differences existed in the RNP of influenza A virus strains. Chromatographic maps of the tryptic

peptides from ribonucleoproteins of various influenza A strains revealed that small differences occurred in the amino acid sequences of A/MEL and A/BEL (Laver, 1964); that amino acid sequences of recombinant X-7 (A/NWS + A2/RI/5^{+}, Kilbourne et al., 1967) and parent A/NWS were identical, but both differed slightly from that of the other parent A2/RI/5^{-} (Laver and Kilbourne, 1966); and that amino acid sequences of a Swine and an Asian A2 strain differed slightly (Laver, 1969, Webster and Laver, 1971). Some soubt exists as to whether the proteins analyzed by chromatography were ribonucleoprotein, or possibly that of another internal protein present in larger amounts in the virus (Webster and Laver, 1971), which could be the one associated with the inner protein membrane described by Schulze (1972) (M' in Table 3); this presumption and whether the differences observed in amino acid sequences are related to antigenic differences, have yet to be determined.

Further immunological evidence supporting the antigenic heterogenity of influenza A RNP or S antigen will be presented in this thesis.

Genetic Interactions of Influenza Viruses The genome of influenza virus has been demonstrated biophysically to exist in subgenomic fragments, possibly reflected immunologically (discussed above), which could account for its unique and unusual genetic behaviour, distinguishing it from parainfluenza and other viruses containing single-stranded nucleic acids (Hirst, 1962; Scholtissek et al,, 1969; Simpson and Hirst, 1968; Duesberg, 1970; Blair and Duesberg, 1970); it resembles,

in this respect, reovirus, which contains segmented double-stranded RNA (Shatkin, 1971). The subgenomic structural units of influenza are organized into functional segments, affording an excellent mechanism for reassortment of genetic characters (Kingsbury, 1970; Shatkin, 1971), evidenced when the following genetic interactions, peculiar to influenza viruses, occur: 1) high frequency recombination between two active viruses, much higher than predicted for the size of genome present (Hirst, 1962; Kilbourne, 1963; Kilbourne et al., 1967; Simpson and Hirst, 1968; Webster, 1970b ; Webster et al., 1971); 2) cross reactivation or marker rescue between active virus and virus partially inactivated by UV (Simpson and Hirst, 1961; Tumova and Pereira, 1965; McCahon and Schild, 1971), or by heat to produce temperature sensitive (ts) mutants (Simpson and Hirst, 1968; Mackenzie, 1970; Hirst and Pons, 1972); 3) multiplicity reactivation between two viruses inactivated by UV (Barry, 1961) or chemically (Scholtissek et al., 1969); 4) von Magnus virus, which is non-infectious, produced by cells infected at high multiplicity (von Magnus, 1954), resulting from defective RNA biosynthesis (Kingsbury and Webster, 1969; Nayak, 1969, 1972), yielding an 'incomplete' virus, deficient in the heaviest piece of genome segment containing 18 S RNA and its corresponding nucleoprotein, but demonstrating an increase in the amount of the small heterogeneous RNA (Duesberg, 1968; Kingsbury and Webster, 1969; Pons and Hirst, 1969; Choppin and Pons, 1970).

By such genetic interactions between influenza A strains, it has been possible to produce recombinants or antigenic hybrids in which the hemagglutinin is derived from one parent and the neuraminidase from

another antigenically different parent (Laver and Kilbourne, 1966; Sugiura and Kilbourne, 1966; Kilbourne et al., 1967; Kilbourne, 1968 ; Easterday et al., 1969; Schulman and Kilbourne, 1969a). By genetic manipulation, the distribution of major surface antigens in progeny virus can be controlled, and thus "tailor made" hybrids can be produced (Kilbourne, 1969; Schild et al., 1970; Webster, 1970b).

Recombinants have furthered the study of the immune response to each antigen, thus avoiding steric interference from antibody to the other antigen (Schulman and Kilbourne, 1969; Webster, 1970b).

Segregation of surface antigens in recombinant strains has permitted the isolation of pure hemagglutinin and pure neuraminidase for biochemical studies (Laver and Kilbourne, 1966; Laver and Webster, 1968, 1972; Laver, 1971), and for antigenic studies to prepare monospecific antisera in animals (Webster and Laver, 1967; Kilbourne et al., 1968b; Webster and Pereira, 1968; Maeno and Kilbourne, 1970; Schild, 1970). The antisera have been used for immunological characterization of hemagglutinin and neuraminidase in HAI, NAI, CF, immunoprecipitin, plaque-size reducing (PSR), plaque inhibition (PI), and cross protection tests (Laver and Kilbourne, 1966; Brown and Laver, 1968; Schulman and Kilbourne, 1969a; Schulman et al., 1968; Webster et al., 1968; Easterday et al., 1969; Schild and Pereira, 1969; Schild, 1970; Kilbourne et al., 1971; Webster and Laver, 1972).

In addition, recombinants are useful in studies on the probable origin of new influenza strains after mixed infections in animals or

birds (Webster, 1970b ; Webster et al., 1971; Webster and Campbell, 1972; refer to Review Part II).

By using immunoprecipitin tests it was possible to clearly demonstrate that in genetic interactions between antigenically different influenza A strains, antigenic exchange of neuraminidase, hemagglutinin, and RNP had taken place to produce hybrids or recombinants with antigenic specificities derived from both parents (Schild and Pereira, 1969; Schild et al., 1969; Schild and Newman, 1969; Easterday et al., 1969; Schild et al., 1970; Kilbourne et al., 1971; Styk et al., 1971a).

Membrane or 'M' Protein

The smallest structural protein of influenza virus, molecular weight 20,000 to 27,000 daltons (Table 3, M polypeptide) is carbohydrate-free, arginine-rich, and is the major protein of the virus by total mass and number of molecules, the virus particle containing seven times as many molecules of this polypeptide as of the nucleoprotein (Compans et al., 1970; Schulze, 1970, 1972; Taylor et al., 1970; White et al., 1970; Lazarowitz et al., 1971; Skehel and Schild, 1971; Klenk et al., 1972). A distinct protein is revealed by polypeptide mapping (Lazarowitz et al., 1971), and this may be the internal protein mapped by Laver (1964, 1969) and Laver and Kilbourne (1966), which differed slightly in its amino acid sequences in different influenza A strains (Webster and Laver, 1971). This protein is located in subviral particles from which the spiky layer has been stripped and the lipid layer removed,

forming a continuous membrane enclosing the nucleocapsid, the membrane being resistant to proteolysis (Compans et al., 1970; Haslam et al., 1970 b; Schulze, 1970, 1972; White et al., 1970; Skehel, 1971 a; Stanley and Haslam, 1971; Klenk et al., 1972). Evidence from EM observations, X-ray diffraction studies, and its resistance to proteolytic digestion, suggests that the inner protein membrane provides major structural support of the viral envelope (Harrison, 1972). The existence of this small structural polypeptide had been demonstrated, and was observed in the electron microscope by Compans and Dimmock, 1969; Kendal et al., 1969; Joss et al., 1969; Taylor et al., 1969; Holland and Kiehn, 1970; Nermut, 1970).

Taylor et al. (1969, 1970) demonstrated that after being synthesized in the cytoplasm, the smallest structural polypeptide migrated more rapidly and mainly into the nucleolus, while the slower moving nucleoprotein migrated into the nucleoplasm. However, Lazarowitz et al. (1971) suggested that this protein is not found in the nucleus, but, with six other structural proteins, is associated with the plasma membrane, and proposed that the protein observed by Taylor et al. (op cit) was of similar size (25,000 daltons), but was a virus specific non-structural protein (NS in Table 3).

Four internal proteins of several different A strains examined by Skehel and Schild (1971), showed identical migrations in polyacrylamide gels, suggesting that, in terms of size, these proteins were similar amongst influenza A viruses. Preliminary evidence indicates

that the smallest structural protein (the membrane protein) may also be antigenically similar in different type A strains. Nucleocapsid proteins of all influenza A strains are related, and so may be the membrane proteins (Skehel and Schild, 1972).

Schulze (1970) has raised questions regarding the function of this protein as an antigen: 1) Whether it functions as a group-specific or strain-specific antigen, 2) whether antisera, specific for this protein, can neutralize infective virus.

If this protein does function as an antigen, and if it is antigenically similar in influenza A strains, then a type-specific antiserum, like that against ribonucloprotein or S antigen, could precipitate the membrane protein in any given strain of influenza A virus, which raises the question as to what polypeptide is being detected in type-specific complement fixation and immunoprecipitin tests when S antigen is being used?

Virion Polymerase(s)

The largest carbohydrate-free protein resolved by polyacrylamide gel electrophoresis of influenza particles has a molecular weight of 81,000 to 94,000 daltons (Table 3, P1, P2); is associated with the viral internal proteins, the membrane and ribonucloprotein, when the spike layer is removed by proteolytic enzymes (e.g. chymotrypsin, pronase, bromelain), remaining with the RNP through the early stages of purification; and is present in only small amounts, 12-52 molecules per virion,

about 0.5-1.8% of the virus protein (Compans et al., 1970; Schulze, 1970, 1972; Skehel and Schild, 1971; Klenk et al., 1972). Schulze (1972) has suggested that it may be the RNA-dependent RNA polymerase isolated by Chow and Simpson (1971) which was found in association with virions and with stripped particles of a number of influenza A strains (human, avian, and porcine) and a B strain. There seems to be general agreement that this is the most likely function for this protein (Kilbourne et al., 1972). What antigenic role, if any, is played by this protein is not clear.

Baltimore (1971) has proposed the following concept of virion nucleic acid polymerases:

"whenever the first function performed by virion nucleic acid after its introduction into the cell is the transfer of its information to another nucleic acid, the enzyme responsible for this transfer is likely to be found in the virion".

He has noted that wherever virion polymerases exist, the nucleic acid is non-infectious (e.g. reovirus, vesicular stomatitis virus), and where the nucleic acid is infectious (e.g. picornavirus) virion polymerases do not exist or at least could not serve an obligate role. This may account for the non-infectivity of influenza virus RNA because RNA polymerases have been demonstrated in the influenza particle and have been induced in influenza infected cells (Ho and Walters, 1966 Skehel and Burke, 1969; Mahy, 1970).

In addition to the above mentioned publications, further information on influenza virus RNA polymerase may be found in Scholtissek and Rott (1969); Scholtissek (1969, 1970); Brammer et al., (1970); Kilbourne et al., (1972).

Nonstructural Protein

In addition to the seven structural proteins of influenza virus, a virus-specific nonstructural protein, molecular weight∿25,000 daltons (Table 3, NS), distinct by peptide mapping, was found in the nuclei of influenza virus infected cells (Lazarowitz et al., 1971), and was not found in uninfected cells, The authors equated it to one of the three nonstructural proteins found by Dimmock and Watson (1969) in influenza virus-infected chick cells using virus-specific mouse antiserum, and suggested that the nonstructural antigen demonstrated in the nucleolus by Dimmock (1969), and the small protein detected in the nucleolus by Taylor et al. (1969, 1970) was the nonstructural protein. In this thesis, evidence of a virus-specific nonstructural protein, detected by immunodiffusion, in influenza virus soluble antigens prepared from infected chorioallantoic membranes, is presented, and may bear some relationship to the nonstructural antigen described above.

Envelope Antigens

The strain specificity of influenza virus is determined by the external virus-specific or V antigen, which consists of two antigenically distinct, virus-coded, surface antigens, the hemagglutinin and the neuraminidase. These antigens are not covalently linked (Laver, 1963; Laver and Kilbourne, 1966; Rott et al., 1970), and their synthesis is controlled by separate genetic loci (Noll et al., 1962; Laver, 1963; Seto et al., 1966; Laver and Kilbourne, 1966; Schulman and Kilbourne, 1969a). Carbohydrate, which is apparently covalently attached to the viral proteins, has been referred to as host-cell antigen (Haukenes et al., 1965, 1966; Laver and Webster, 1966).

There are two distinct types of spikes on the virion surface; by morphology and by biological activity, one type has been related to the hemagglutinin, and the other to the neuraminidase (Laver and Valentine, 1969; Webster and Darlington, 1969). Excellent reviews discussing these antigens have described their separation and isolation from the intact virion, and the subsequent characterization of the structure and function of each component (Hoyle, 1968; Pereira, 1969; Rott et al., 1970; Webster and Laver, 1971; Kilbourne et al., 1972). The latter review has comprehensively summarized current knowledge about the surface antigens, but exact references have not been cited, therefore, highlights of structure and function will be presented in relation to these components as antigenic substances, which could be detected by immunodiffusion.

Selective degradation of intact virions with such agents as chymotrypsin, pronase, or bromelain, removes the spiky layer leaving subviral particles essentially devoid of infectivity, hemagglutinin, and neuraminidase activities, thereby associating these functions of the virions with the spikes (see Virus Disruption). The shape of the particle appears to be maintained by the spike layer because its removal caused distortion of the particles (Schulze, 1972). Fractionation of intact virus and spike material by polyacrylamide gel electrophoresis and by density gradient centrifugation in guanidine hydrochloride, both under reduced conditions, revealed that the spikes were composed of three to four different glycoproteins, which were subunits of hemagglutinin,

and neuraminidase (Compans et al., 1970; Haslam et al., 1970 a, b; Schulze, 1970; Laver, 1971; Skehel and Schild, 1971; Klenk et al., 1972).

Hemagglutinin

The hemagglutinin spike is the functional molecule (a monomer in SDS which adsorbs to, but does not agglutinate erythrocytes) (Webster, 1970a; Laver, 1971); molecular weight 150,000 daltons; sedimentation coefficient 7-8 S; and consists of a rod 40 Å wide and 140 Å long, which tends to aggregate into rosettes in the absence of SDS or other dispersing agent, agglutinating erythrocytes in the aggregated state (Eckert, 1967; Laver and Valentine, 1969; Webster and Darlington, 1969; Rott et al., 1970; Webster, 1970a; Laver, 1971). It is composed of two hemagglutinin dimers (Table 3, HA, mol. wt. 75,000 to 85,000 daltons), each of which can be dissociated under reduced conditions in SDS or in guanidine hydrochloride into two subunits, HA1 (Table 3, mol. wt. 50,000 to 60,000 daltons), and HA2 (Table 3, mol. wt. 23,000 to 30,000 daltons), which in the dimer are held together by covalent linkages or by disulfide bonds, the latter linkage predominating in the intact virion (Compans et al., 1970; Haslam et al., 1970 a; Webster, 1970a; Schulze, 1970, 1972; Laver, 1971; Skehel and Schild, 1971; Stanley and Haslam, 1971; Klenk et al., 1972). Laver (1971) had demonstrated that in egg-grown virus (BEL), the carbohydrate content of HA1 was 20% and that of HA2 5%; HA1 had nine times as much proline as HA2; and that some differences occurred between them in amino acid composition, as revealed by peptide maps of each subunit. Recent polypeptide mapping experiments by Laver and

Webster (1972), have revealed that striking differences occur in amino acid sequences of both HA1 and HA2 prepared from various A2 strains isolated between 1968 and 1971. Great differences occurred between polypeptide chains of HA subunits from "old" A2 strains isolated in 1968 (pre-Hong Kong) and those from three Hong Kong strains (1968-1971), the polypeptide maps of the "old" viruses bearing no resemblance to those of the Hong Kong strains. Smaller variations within each group were also obvious, particularly in the "old" A2 group, but occurred only in the HA1 subunit of the Hong Kong group.

HA1 is more exposed than HA2, which is suggested by evidence from iodination experiments (Stanley and Haslam, 1971), and the fact that HA2 is the last glycoprotein to be removed from the virion by proteolysis, remaining with the lipid layer after bromelain treatment (Compans et al., 1970; Schulze, 1970, 1972). Klenk et al. (1972), selectively precipitating spike carbohydrate and lipid bound carbohydrate with phytagglutinins, demonstrated that two layers of carbohydrate are present in the viral envelope, one in spikes covalently linked to protein, and the other in lipid layers covalently linked to lipid. White (1972) has proposed that the hemagglutinin spike consists of two molecules of HA1 held together by hydrogen bonds and ionic interactions forming the inner part of the spike, whereas two molecules of HA2 extend from the tip of the spike into the lipid layer, each one held in place by disulfide linkage to HA1. A similar structure has been proposed by Laver, (1971), Schulze (1972).

Both cleaved and uncleaved hemagglutinin subunits have been observed at the plasma membrane after synthesis in the cytoplasm, and both can be incorporated into the virion (Compans et al., 1970; White et al., 1970; Taylor et al., 1970; Lazarowitz et al., 1971). Although the cleaved subunits are consistently observed in all influenza strains the presence of uncleaved hemagglutinin subunit varies in different virus strains, and seems to depend upon the ability of the host cell, in which the virus was grown, to cleave this molecule. It is absent from WSN, AO/BEL, and FPV strains grown in eggs (Haslam et al., 1970 a; Laver, 1971; Lazarowitz et al., 1971; Stanley and Haslam, 1971; Klenk et al., 1972), but is present in WSN virus grown in other cells such as CEF, MDBK, Rhesus MK, or BHK 21-F cells (Compans et al., 1970; Schulze, 1970; Klenk et al., 1972), and in AO/BEL grown in primary embryonic calf kidney cells or in HeLa cells (White et al., 1970; Stanley and Haslam, 1971; Gandhi et al., 1972).

Besides these host dependent variations, spike glycoproteins also show strain dependent differences, such as differences in molecular weights of corresponding glycoproteins in FPV and in WSN, as well as differences in susceptibility to bromelain treatment, which suggests that these constituents might be the molecular basis for strain-specific antigenicity of influenza virus (Klenk et al., 1972).

Laver and Webster (1968) had demonstrated differences in the amino acid sequences of isolated hemagglutinin subunits from wild-type strains (Swine and AO/BEL) and from their antigenic mutants. The mutants,

which differed in HAI and neutralization tests from wild strains, had been obtained by serial passage of wild strains in the presence of homologous antibody of low avidity. The experiments, mimicing antigenic drift (Burnet, 1955) suggested that antigenic variation among influenza virus was related to changes in the amino acid sequence in their hemagglutinin subunits, but it was not known whether changes occurred in the antigenic determinants of the viral protein. In addition to differences between polypeptide maps of Swine and AO/BEL, many similarities were also noted. The recent experiments of Laver and Webster (1972) with a group of naturally occurring A2 variants isolated 1968 to 1972 (discussed above), confirmed that small changes in amino acid sequences occurred in closely related strains (among "old" A2 group or among Hong Kong group), which is strongly suggestive of antigenic drift; and, they have demonstrated that the antigenic shift of the hemagglutinin antigen between "old" 1968 and Hong Kong strains (Coleman et al., 1968, Dowdle et al., 1969; Schulman and Kilbourne, 1969 a) was accompanied by major changes in the polypeptide composition of both HA1 and HA2 subunits. These results were confirmed immunologically, using monospecific antisera to hemagglutinins from A2 strains isolated 1957-1971, in HAI and immunoprecipitin tests. In homologous immunoprecipitin reactions, "old" A2 strains and Hong Kong strains each gave a single precipitin line, but no cross reactions occurred between the two groups, demonstrating that antigenic differences existed in the hemagglutinins (Webster and Laver, 1972).

Therefore, two, or possibly three hemagglutinin subunits could be antigenically active in influenza viruses, depending on the strain and propagating host. Opinions differ as to which subunits contribute to the antigenic site of the hemagglutinin. Kilbourne (1972) suggests that HA1 and HA2 might be under separate genetic control, which precludes the presence of uncleaved precursor hemagglutinin suggested above. The independent changes in polypeptide maps of HA1 and HA2 demonstrated by Laver and Webster (1972) tend to support Kilbourne (1972). Experiments using plaque neutralization tests with recombinants X-12 and X-29, demonstrated that although both have identical parentage (A0/NWS hemagglutinin and A2/RI/5$^+$ neuraminidase), X-12 hemagglutinin seems to be wholly derived from A0/NWS, whereas that of X-29 seems to be biparentally derived, as it has predominantly A0 antigenic activity, but A2/RI/5$^+$ resistance to inactivation of hemagglutinin activity by trypsin which implies separate genetic control of these two characteristics (Kilbourne, 1972). White (1972) suggests that HA1 and HA2 both contribute to the antigenic site of the hemagglutinin. Eckert (1966, 1969, 1972) agrees that only a single kind of glycoprotein (mol. wt. 80,000 daltons) forms the hemagglutinin antigen, and is reducible into the equivalent of HA1 (40,000 daltons). It is non-hemagglutinating, but capable of blocking HAI antibodies, and inducing hemagglutination inhibition and neutralization antibodies. Hobson (1966) also demonstrated that a non-hemagglutinating extract of degraded A/WS (obtained by DOC treatment and ether extraction) could block HAI antibodies in a highly strain-specific reaction (strain-specific serum blocking activity, or SSB), and could induce the production

of hemagglutination inhibition antibodies of a spectrum greater than expected. Hobson's results were confirmed by Corbel and Rondle (1970) using Triton N 101 degraded viruses. They believed that SSB in degraded viruses was one of the components precipitated by specific antiserum in immunodiffusion in agar gel as has been demonstrated by Eckert (1972).

The hemagglutinins of various influenza A strains and recombinants were characterized by immunodiffusion in agar gel (Schild et al, 1969; Schild, 1970; Schild et al., 1970). A0/BEL hemagglutinin, isolated by electrophoresis in cellulose acetate (Laver, 1963), was diffused against homologous monospecific rabbit antiserum (Schild, 1970). The hemagglutinin was precipitated as two or three closely associated lines which coalesced into a single, well-defined precipitin line when the same serum precipitated the hemagglutinin in SDS disrupted A0/BEL virus. The A0/BEL specific reaction was verified by comparing it with that obtained in diffusions of anti-hemagglutinin serum against A0-A2 recombinants known to contain the hemagglutinin from the A0 parent and the neuraminidase from the A2 parent, for example, X-7 (A0/NWS - A2/RI/5^{+}) (Kilbourne et al., 1967), and an A0/BEL - A2/Singapore/1/57 recombinant (Schild et al., 1970). The A0 hemagglutinin in the latter recombinant and in its parent had been determined serologically by HAI tests, and by its failure to be inhibited by γ inhibitor or to agglutinate horse erythrocytes. In homologous immunoprecipitin reactions between disrupted A0/BEL particles and corresponding serum, Schild (1970) differentiated the hemagglutinin from the neuraminidase- and RNP-specific precipitin lines. With monospecific anti-hemagglutinin serum, the hemagglutinins

of A0/PR8, A0/NWS, A/FM1, and A1/Neth/1/56 were demonstrated to be partially related to that of A0/BEL, a relationship not obvious by conventional HAI tests, but suggested by the ACU photometric test of Drescher (1967), antibody absorption tests of Jensen et al. (1956), and by cross-protection tests in mice (Schulman and Kilbourne, 1965). The hemagglutinins of several A2 strains (isolated 1957-1968, and including Hong Kong/68), Swine, and FPV showed no relationship with that of A0/BEL in immunodiffusion (Schild, 1970), nor did those of Duck/Germany/68 or Turkey/Mass/65 (Schild et al., 1969), supporting the corresponding results achieved by Paniker using HAI tests (1968).

Kilbourne et al. (1971), using essentially the same procedures as Schild (1970), demonstrated that the hemagglutinin of recombinant X-31 (Kilbourne, 1969) and parent A2/Hong Kong/68 contained no A0/BEL hemagglutinin, but that the other parent, A0/PR8 did, resolving as a single line when precipitated by A0/BEL anti-hemagglutinin serum.

Styk et al.(1970a, 1971a),in an exhaustive series of immunodiffusion experiments in agarose, circumvented the use of isolated hemagglutinin subunit and homologous monospecific antiserum. Influenza A recombinants, containing the hemagglutinin and neuraminidase of different subtypes, and their respective parent strains, all DOC disrupted, were diffused in various template patterns against a number of convalescent and immunized human and animal sera. By this method, the hemagglutinin specific reaction, common to both the recombinant and parent virus, were identified in the complex precipitin patterns resolved. Hemagglutinin

specific reactions were identified in the following recombinants and hemagglutinin originating parent: X-15 and A/Equi/1/56, R4 and FPV, X-31 and A2/Hong Kong/68.

Although this system is useful, the interpretation of results was not as clear-cut and reliable as those obtained using a combination of the monospecific hemagglutinin reaction and recombinants employed by Schild (1970).

Schild (1970) advocated the use of the immunoprecipitin test using the monospecific hemagglutinin reaction, in addition to the HAI test, because it is not subject to difficulties encountered in interpreting the latter test, such as differences in avidity of test virus for antibody, differences in avidity of anti-hemagglutinin antibodies (Webster, 1968), or in susceptibility to normal serum inhibitors, although this last point is debatable (see Inhibitor Review, Part II).

The immunoprecipitin test is now considered a complementary alternative to the HAI test in the detection of hemagglutinin. Monospecific anti-hemagglutinin antiserum is recommended in both test systems to eliminate steric hindrance of hemagglutination by neuraminidase antibody, which has been demonstrated to occur in reactions using intact virus, resulting in misleading interpretations of HAI cross reactions (Kilbourne, 1968; Kilbourne et al., 1968b; Webster et al., 1968; Schulman and Kilbourne, 1969a; Webster and Laver, 1971; 1972). In the latter publication, Webster and Laver, agreeing with Schulman and Kilbourne (1969a), have clearly demonstrated that low-level cross

reactions among "old" A2 strains and Hong Kong srains observed by others (Fazekas, 1969a, 1970; Dowdle et al., 1969), were spurious and due to antibody against the neuraminidase, which was a common antigen to the two groups of viruses. They re-emphasized the use of monospecific anti-hemagglutinin antiserum for meaningful serological analysis of antigenic variation in influenza viruses.

The observations by Styk et al. (1970a, 1971a) and by Schild (1970), that the hemagglutinin was precipitated as two or three lines is in accord with the finding that the hemagglutinin subunit exists as the HA dimer, and/or HA1 and HA2 subunits (discussed above). However, in other cases, the hemagglutinin is precipitated as only one line (Kilbourne et al., 1971; Webster and Laver, 1972), which suggests the presence of only one hemagglutinin antigen. Schild (1970) stated that, whereas the immunoprecipitin test reflected the immunological specificity of the whole hemagglutinin subunit, the HAI test detected the presence of antibody reacting with only a portion of it, that part responsible for attachment to erythrocytes.

At the present time, fifteen hemagglutinin subtypes have been identified by these tests (WHO Committee on Influenza Virus Nomenclature, 1971). They are distributed amongst viruses originating from human,swine, equine, and avian hosts. It will be of interest to determine the manner in which they evolved.

The use of isolated HA dimer, HA1 and HA2 subunits in immuno-precipitin experiments should clarify which polypeptides are actively precipitated by antibodies in immune sera, and by which specific antibody

type, and should determine more exactly, in conjunction with other immunological methods, the immunological relationships existing amongst the hemagglutinins of influenza A strains. Also resolved could be the polypeptides eliciting antibodies for virus neutralization and protection from infection (Hoyle, 1968; Pereira, 1969; Webster and Laver, 1971), and plaque inhibition (Jahiel and Kilbourne, 1966; Schulman and Kilbourne, 1969a). The exact biochemical analysis of the hemagglutinin of each influenza A strain, already in progress (Laver and Webster, 1972), will finally elucidate such relationships, and perhaps indicate the mode of their genetic reassortment by mutation or recombination (Laver and Webster, 1972; Webster and Laver, 1972) to produce new influenza variants.

Neuraminidase

The active neuraminidase of A2/RI/5$^+$ isolated from SDS disrupted X-7F1 (Laver and Kilbourne, 1966), appears as an oblong structure 85 Å by 50 Å, connected at midpoint by a 100 Å long fibre to a knob approximately 40 Å in diameter. Subunits reaggregate into structures resembling heads of seeding dandelions when SDS is removed (Laver and Valentine, 1969). Similar enzymically active structures of slightly different dimensions were observed in pronase treated A2/Singapore/1/57 by Drzeniek et al. (1968).

Depending upon the virus strain and method of isolation, by detergent or by proteolytic anzymes, the sedimentation coefficient of active neuraminidases varies from 8 to 10 S, which suggests a molecular weight of 200,000 to 300,000 daltons (Noll et al., 1962; Laver, 1963; Wilson and Rafelson, 1963; Drzeniek et al., 1966b; Seto et al., 1966;

Kendal et al., 1968; Laver and Valentine, 1969; Webster and Darlington, 1969; Drzeniek, 1970; Haslam et al., 1970 b; Bucher, 1972). The enzyme constitutes 3 to 17% of the total viral protein, the percentage varying with the virus strain (Noll et al., 1962; Laver, 1963; Kendal et al., 1968; Webster et al., 1968; Schulze, 1970; Skehel and Schild, 1971; Klenk et al., 1972). The size of the neuraminidase indicates it is an aggregate of several polypeptides (Webster, 1970a; Haslam et al., 1970 b; White, 1972; Bucher, 1972).

The neuraminidase subunit (Table 3, NA) has been isolated from disrupted virus particles by fractionation methods similar to those used for hemagglutination subunits (discussed above), often in the same experiment. The number of enzyme polypeptides resolved varied with the virus strain and technique used: one neuraminidase subunit, molecular weight 55,000 to 72,000 daltons was resolved from WSN, an A2 strain, X-31 (A2/Hong Kong/68 - A/PR8), and A0/BEL (Compans et al., 1970; Schulze, 1970; Laver, 1971; Skehel and Schild, 1971); two subunits, molecular weights 45,000 to 80,000 daltons were resolved from X-7, X-7F1, B/Lee, FPV (Webster, 1970; Haslam, et al., 1970 b; Skehel and Schild, 1971; Bucher et al., 1972; Klenk et al., 1972); and an even smaller basic neuraminidase subunit, molecular weight 27,000, has been isolated (Bucher et al., 1972).

Bucher (1972) has proposed that the active form of neuraminidase is a tetramer, molecular weight 240,000, composed of two chains with a molecular weight of 56,000 and two with a molecular weight of 66,000; whereas White (1972), from the data of Haslam et al. (1970 b) has

suggested that two disulfide linked molecules of 63,000 molecular weight polypeptide linked noncovalently to one molecule of 56,000 molecular weight polypeptide to form the active enzyme.

The antigenic neuraminidase subtypes of influenza A viruses, which number eight (Table 2, from WHO Committee on Influenza Nomenclature, 1971), reflect the variation apparent in influenza neuraminidase subunits and in their organization into the active enzyme molecule. These differences are correspondingly reflected in the activity of enzymes (Michaelis constant, K_m) in different influenza strains measured in kinetic experiments, and which appear to be strain-specific (Kendal and Madeley, 1969; 1970; Schild et al., 1970).

A marked difference in the neuraminidase activity between strains has been noted by others, for example, A0/NWS has about 1% of the enzyme activity of A2/Japan (Meier-Ewert and Dimmock, 1970), and recombinant X-7F1 has more than twice the activity of X-7 (Jahiel and Kilbourne, 1966, Webster et al., 1968).

The structure of the neuraminidase, as it exists on the virion, has not yet been determined, but, like the hemagglutinin, its base is thought to be attached by hydrophobic bonds to the lipid bilayer of the virus (Laver and Valentine, 1969; Compans and Dimmock, 1969; Compans et al., 1970; Schulze, 1972; Klenk et al., 1972). Rather than being randomly distributed on the virus surface, it may be present in discrete enclaves amongst the hemagglutinin spikes (Compans et al., 1969; Apostolov et al., 1970; Madeley et al., 1971); when virus particles were flocculated

by specific antineuraminidase antiserum, surface spikes, not obscured by antibody, were clearly seen on the virus, but a mixture of enzyme and hemagglutinin antibodies obscured almost the whole virus surface (Kendal and Madeley, 1970).

The enzyme, like hemagglutinin, is thought to be synthesized in the cytoplasm of infected cells, and is found at the plasma membrane with other structural proteins of the virus (Taylor et al., 1970; Lazarowitz et al., 1971). In mammalian cell lines such as calf kidney, Hep 2, HeLa, and others, it is predominantly membrane bound (Kendal and Apostolov, 1970; Kendal et al., 1971). Carbohydrate incorporated into this glycoprotein is probably of host origin (Haukenes et al., 1966; Laver and Webster, 1966; Taylor et al., 1970; Becht et al., 1972; Klenk et al., 1972).

The enzymic action of neuraminidase has been defined by Gottschalk (1966) as "the hydrolytic cleavage of the glycosidic linkage joining the keto group of neuraminic acid to D-galactose or D-galactosamine and possibly to other sugars". This action, on the terminal sialic acid of glycoprotein cell receptors for influenza virus hemagglutinin, causes virus particles to elute. Neuraminidase is believed to aid the release of mature influenza virions from receptors at the cell surface, because it had been demonstrated that specific antineuraminidase antibodies greatly reduced or inhibited virus release, and reduced plaque size (Drzeniek et al., 1966b; Jahiel and Kilbourne, 1966; Seto and Rott, 1966; Webster and Laver, 1967; Brown and Laver, 1968; Webster et al., 1968; Kilbourne et al., 1968b; Compans et al., 1969; Seto and Chang, 1969), but

did not exert a direct effect on viral maturation (Compans *et al.*, 1969). Electron microscopy studies by Klenk *et al.* (1970) detected an absence of sialic acid at areas of cell surface where virus maturation occurred, which suggested that the neuraminidase could also play a role in the assembly of the virus particle. Becht *et al.*, (1971) proposed that the enzymatic function of neuraminidase is not essential for virus release, as mature virions were released in the presence of monovalent antibody which inhibited the enzymatic activity of FPV, but bivalent antibodies inhibited virus release. They suggested that neuraminidase could mediate a critical step in virus assenbly at the cell membrane.

Kendal and Madeley (1970) suggested that the flocculation of purified influenza virus by specific antineuraminidase antibodies indicated that the release of virus particles may be inhibited by antibodies to the enzyme aggregating virions to the infected cells, as had been observed by Compans *et al.* (1969). A similar phenomenon in infected cells (Seto and Chang, 1969), and a reduction in plaque size but not of plaque numbers in cell cultures by antibody to neuraminidase (Jahiel and Kilbourne, 1966) was thought to be due to specific inhibition of the enzyme site. The mechanism of viral release has yet to be resolved.

Although they do not prevent the infection of susceptible cells (Webster and Laver, 1967), the presence of antineuraminidase antibodies after infection or immunization, may offer a degree of protection from further influenza infection, complementing the protection offered by antibodies to the hemagglutinin. This has been demonstrated by cross-protection tests in mice (Schulman *et al.*, 1968; Schulman and Kilbourne,

1969a), and in chickens (Allan et al., 1971), provided that the challenge virus possessed homologous neuraminidase. It has been implied in humans in the following way: the convalescent serum of influenza patients showed a marked increase in antineuraminidase antibody titre from the acute phase (Kilbourne et al., 1968a) suggesting that residual antibody to neuraminidase, like that to hemagglutinin, persists in human sera following infection; therefore, a factor in the limited spread of A2/Hong Kong/68 was attributed to the fact that its neuraminidase, antigenically indistinguishable from that of previously circulating A2 strains, was inhibited by antibodies to the previous A2 neuraminidase present in the human population, but its antigenically distinct hemagglutinin was not (Coleman et al., 1968; Schulman, 1969; Schulman and Kilbourne, 1969 a, b; Allan et al., 1971). In the pandemic of 1957-1958, a major change had occurred in both neuraminidase and hemagglutinin antigens (Paniker, 1968), which would account for its widespread occurrence and severity. Therefore, antihemagglutinin antibody offers resistance to the initiation of infection, whereas antineuraminidase antibody impedes transmission of infection by immune animals (Schulman, 1969), and possibly by humans. Thus, antibodies to both antigens may be present in the sera of animals exposed to influenza viruses.

Antibodies to neuraminidase are detected serologically by the strain-specific complement fixation (CF) test and by the neuraminidase inhibition (NAI) test (Webster, et al., 1968; reviewed by Webster and Laver, 1971). The NAI test has been used as the basis for differentiating influenza neuraminidases into sybtypes (Coleman et al., 1968; Dowdle et al., 1969; Paniker, 1968; Webster and Pereira, 1968; Tumova and Easterday, 1969; Kendal and Madeley, 1969; Madeley et al., 1971), which at present number eight (see Table 2 from WHO Committee on Influenza Virus

Nomenclature, 1971).

When intact virus is used as a source of neuraminidase in NAI tests, neuraminidase activity may be sterically hindered by antihemagglutinating antibody (Laver and Kilbourne, 1966; Schulman and Kilbourne, 1969a; Easterday et al., 1969; Webster and Laver, 1971). This can be avoided by treating the intact virus with pronase, which selectively destroys the hemagglutinin, but not neuraminidase activity (Easterday et al., 1969); or by use of a monospecific antineuraminidase antiserum prepared against isolated neuraminidase (Webster and Laver, 1967). The use of monospecific antineuraminidase antiserum for division of neuraminidase antigens into subtypes is recommended in the WHO Memorandum on influenza virus nomenclature (WHO Committee on Influenza Nomenclature, 1971).

Characterization of neuraminidase by immunoprecipitin reactions has been accomplished using purified recombinant strains and parent viruses, isolated neuraminidase subunits, and monospecific antineuraminidase antisera.

By immunoelectrophoresis in cellulose acetate (Laver, 1963), Schild and Pereira (1969) demonstrated that the isolated neuraminidase of A2/Eng/66 and of recombinant FPV-A2 (R4) (Easterday et al., 1969) was precipitated as a single line by monospecific anti-A2/57 neuraminidase antiserum. On immunodiffusion in agar gel, using SDS disrupted viruses, the monospecific antiserum precipitated the neuraminidases of a number of A2 strains, and those of recombinants containing A2 enzyme, R4 and

X-7Fl, as two separate lines in close proximity, but not the neuraminidases of A0, A1, or B strains. The enzymes of recombinants and enzyme-originating parent A2/Singapore/1/57, were shown to be the same, because, when precipitated, they linked in reactions of identity. Neuraminidase specific precipitin lines were clearly differentiated from RNP-specific lines in similar diffusions against mixtures of the two monospecific antisera, anti-V antiserum, and human convalescent antisera. It is possible that the two neuraminidase specific lines resolved could be the two subunits of neuraminidase demonstrated to exist in some strains of influenza, including X/7Fl (discussed above) used in these experiments; or the single neuraminidase subunit could be precipitated by two different antibody types, 19 S and 7 S (Schild and Pereira, 1969).

By the same general method, using monospecific antineuraminidase antiserum (to A2/Singapore/1/57 and to A2/RI/5$^+$), the neuraminidases of a number of recombinants and respective parent viruses have been identified in immunoprecipitin reactions, usually precipitated as a single, well-defined line. Antigenic relationships amongst influenza neuraminidases revealed in this way are:

a) The neuraminidases of A2 strains, Singapore/1/57, RI/5$^+$, England/64, /66, and Hong Kong/68 are closely related, but are distinct from those of A0/BEL and A/FM1, which are related (Schild and Newman, 1969; Schild et al., 1969; Easterday et al., 1969) A2/HK/68 enzyme differs from that of A0/PR8 (Kilbourne et al., 1971). The isolated neuraminidase of A2/Singapore/1/57 and that of the SDS disrupted virus

particle differ slightly antigenically (Easterday et al., 1969).

b) A2/Singapore/1/57 neuraminidase and those of avian A strains Turkey/Mass/65 and Turkey/Wisc/66 are all related, whereas A2/Hong Kong/68 enzyme is related to those of Turkey/Wisc/66 and to Duck/Germany, but neither human A2 enzyme is similar to any other avian influenza A strains including FPV (Schild et al., 1969; Schild and Newman, 1969). A2/RI/5^{+} and Turkey/Mass/65 enzymes are related, but not FPV (Easterday et al., 1969). Duck/Germany/68 enzyme is similar to that of A0 and A1 (Schild et al., 1969).

c) Influenza A neuraminidases are antigenically distinct from those of influenza B (Easterday et al., 1969), confirming the results of NAI tests (Paniker, 1968).

These results of immunoprecipitin tests generally agreed with those obtained from parallel NAI tests, except that the immunoprecipitin test did not, which the NAI test did, detect slight antigenic differences between enzymes of A2 strains, notably between A2/57 and A2/Hong Kong/68, which was strong evidence of antigenic drift (Schild and Newman, 1969; Paniker, 1968; Webster and Laver, 1972).

Schild et al. (1970) demonstrated that the neuraminidase in their A0/BEL-A2/Singapore/1/57 recombinant was antigenically identical to the A2 parent in immunoprecipitin tests, and that its enzyme activity had similar K_m values, but that it differed from the parent enzyme in its electrophorectic mobility, and instability in the presence of detergents (SDS). Therefore, immunologically and kinetically, the enzyme reflected

the A2 parentage, but instability in the presence of detergent reflected its A0 parentage. Immunoprecipitin tests did not reveal these differences, but had the isolated neuraminidases been diffused along with disrupted particles, a difference may have been apparent.

In the same series of immunoprecipitin experiments as described for RNP and hemagglutinin, Styk et al. (1970a,1971a) identified the neuraminidase specific reaction in the following recombinants and enzyme-originating parents: X-15 and A2/RI/5+, R4 and A2/Singapore/1/57, X-31 and A2/Hong Kong/68, AOE(HK) and A2/Hong Kong/68. Using swine field sera, a dissimilarity was demonstrated between A2/57 and A2/Hong Kong/68 neuraminidases, agreeing with the serological observations of Paniker (1968) and Schild and Newman (1969), but using human convalescent sera, antigenic differences were not observed between the A2/57 enzyme in X-15 and A2/Hong Kong/68 enzyme. Neuraminidase was often precipitated as two distinct lines in these experiments, as had been observed by Schild and Pereira (1969) and it was suggested that different antigenic sites were involved, either on the same subunit, or in separate subunits.

Comparing the results of Styk et al. (op cit) and those of Schild and his group (discussed above), it is obvious that the use of monospecific antineuraminidase antisera is essential, and isolated neuraminidase subunits advantageous, to clearly identify the neuraminidase specific reaction in immunoprecipitin tests.

The immunoprecipitin test has been accepted as a complementary test to NAI in the subtyping of influenza neuraminidases (WHO Committee on Influenza Virus Nomenclature, 1971), and the use of monospecific antiserum is recommended. The test compares favourably with the NAI test

and will, no doubt, be used to advantage to define the immunological structure of neuraminidase.

Host-Derived Components

Reviews by Pereira (1969) and Webster and Laver (1971) describe the discovery and characterization of host-derived antigen associated with the influenza virion.

The host-cell antigen, an integral part of the virus particle acquired during virus maturation, is a mucopolysaccharide, covalently linked to the coat proteins (hemagglutinin and neuraminidase), and is not associated with the internal antigen of the virus (Smith et al., 1955; Harboe, 1963a, b; Haukenes et al., 1965, 1966; Drzeniek et al., 1966a Laver and Webster, 1966). The latter authors found it to be a heat-stable, low molecular weight (15,000 daltons) carbohydrate, comprising less than 5% of the dry weight of the virus, and detected only in virus grown in the allantois of chick embryo, or of turkey embryo (Harboe, 1963a), but not in that of duck embryo, nor in mouse lungs or various cell culture systems (Webster and Laver, 1971).

Antibody to the host antigen inhibited viral hemagglutinin, which could be applied in blocking tests to detect host antigen (Harboe, 1963a, b; Laver and Webster, 1966) along with other immunological methods such as complement fixation (Smith et al., 1955), immunodiffusion and immunoelectrophoresis (Howe et al., 1967; Haukenes and Gitay, 1968; Lee et al., 1969). In immunodiffusion, the host antigen was precipitated as a double line, which could have been due to specific precipitation with

both 7 S and 19 S antibody in the specific rabbit antiserum against which it was diffused (Haukenes and Gitay, 1968; Lee et al., 1969). Although the basic structure of host antigen is similar to that of blood group substances, it does not possess blood group ABO reactivity, and it does not cross react with type 14 pneumococcal polysaccharide (Lee et al., 1969).

Fractionation of influenza viruses by polyacrylamide gel electrophoresis revealed that the three or four glycoproteins resolved were associated with hemagglutinin and neuraminidase spikes (discussed above). The electrophoretic mobilities of these glycoproteins varied with the type of host cell in which a given strain of virus was grown, whereas those of nonglycosylated polypeptides did not, which suggested that the carbohydrate moiety of the glycoproteins seem to be specified by the host, whereas the protein moiety was specified by the virus (Compans et al., 1970; Haslam et al., 1970 a). Additional evidence for the host origin of spike glycoprotein carbohydrate has been provided using the specific property of the phytagglutinin, concanavalin A, which flocculates cell-associated carbohydrate antigens, and which was found to flocculate intact, but not stripped viral particles (Becht et al., 1972; Klenk et al., 1972).

Blood group A antigen is incorporated into viruses grown in embryonated eggs; blood group B antigen into those grown in monkey kidney cells; Forssman and mononucleosis antigens are incorporated by viruses grown in both systems, but none of these glycolipid antigens could be

detected in duck embryo grown virus. However, using phytagglutinins, specific cell-derived carbohydrates were detected in duck embryo cells and in duck grown influenza virus (Rott et al., 1970).

A phytagglutinin from Dolichos biflorus, reacting specifically with the terminal sugar of blood group A glycolipid, has been used to demonstrate (by flocculation) that glycolipids are associated only with spikeless particles and not with the intact virion, indicating these are bound to the lipid layer (Klenk et al., 1972).

Lipids of influenza virions are believed to be of host origin (Kates et al., 1962; Tiffany and Blough, 1969). Klenk et al. (1972) found that the lipid pattern of FPV is characterized by a high content of cholesterol, sphingomyelin, and glycolipid, which are typical features of many enveloped viruses, and indicate that the viral lipids are derived from the plasma membrane of the host cell (Klenk and Choppin, 1969). The lipid layer lying beneath the spikes of the virion is thought to be in the form of a bimolecular leaflet (Klenk et al., 1972, Kilbourne et al., 1972).

Influenza virus contains little or no host protein (Laver and Webster, 1966), less than 1% (Holland and Kiehn, 1970). Prelabeling experiments demonstrated that, at the plasma membrane, from which the virus was budding, host membrane proteins were completely replaced by virus envelope proteins (Holland and Kiehn, 1970), and pulsechase experiments demonstrated that as they budded through the cell membrane, no host components were incorporated into the virions (Etchison et al.,

1971). The reproducibility of seven or eight polypeptides resolved by polyacrylamide gel electrophoresis of purified influenza viruses grown in various hosts, and the consistent results obtained in the analysis of fourteen influenza virus strains has reinforced the conclusion that only virus-coded protein is present in the influenza virion (Compans et al., 1970; Skehel and Schild, 1971).

Influenza Virus Structure

Each influenza component has been described above. The components integrate to form the virus particle. The model for the structure of the virus particle, based on current evidence discussed in part in this review, was presented by Compans during the Influenza Workshop (Kilbourne et al., 1972) as:

> "The hemagglutinin and neuraminidase are glycoproteins which form the surface projections or spikes on the virion; the bases of these spikes are attached, possibly by hydrophobic bonds, to the lipid layer of the virus. However, the spikes do not penetrate through the lipid bilayer. Beneath this lipid, and in association with its inner surface, the smallest viral protein forms a continuous layer which encloses the viral nucleocapsid. The nucleocapsid is composed of a single species of protein subunit and, when isolated from the virion, the nucleocapsid is found in pieces which contain segments of RNA of various sizes. Somewhere in the interior of the virion, one or two other proteins are located. These are the largest viral proteins and they are not glycosylated."

Cross Reactions of Influenza A Viruses

Antigenic relationships amongst influenza viruses have been based on three virus components, the RNP, the hemagglutinin, and neuraminidase (see previous discussion), and have been reviewed by Jensen (1957 a), and by Pereira (1969). The other structural components of the virus, recently identified (see previous discussion), have not yet been implicated in these relationships. The antigenic role they play has to be established.

In order to determine antigenic relationships, cross reactions amongst influenza viruses have been carried out by numerous investigators using the variety of immunological methods mentioned throughout this review: HAI, NAI, CF (type- and strain-specific), virus neutralization, cross-protection, plaque inhibition (Pereira et al., 1966; Coleman et al., 1968; Paniker, 1968; Tumova and Pereira, 1968; Webster and Pereira, 1968; Dowdle et al., 1969; Drescher and Weidauer, 1969; Fazekas, 1969 a, b; Kendal and Madeley, 1969; Schulman and Kilbourne, 1969 a, b; Tumova and Easterday, 1969; Madeley et al., 1971). Antigens employed in most tests were usually intact viruses or ether extracted S and V antigens, the latter containing both hemagglutinin and neuraminidase. These antigens were used to prepare antisera which were multispecific or broadly reactive.

The interpretation of hemagglutinin and neuraminidase cross reactions using such antigens and antisera was difficult because as discussed earlier (see Hemagglutinin, Neuraminidase), steric inhibition was encountered by antineuraminidase antibodies interfering in HAI tests, and

antihemagglutinin antibodies interfering in NAI tests, thus producing a misleading picture of antigenic relationships (Schulman and Kilbourne, 1969 a; Webster, 1970 c; Webster and Laver, 1971, 1972).

To overcome this problem, recombinants containing segregated hemagglutinin and neuraminidase antigens were used (see RNP-Antigenically Homogeneous or Heterogeneous?), viruses were pronase treated to eliminate the hemagglutinin antigen, leaving the neuraminidase intact (Easterday et al., 1969), and most important, monospecific antiserum prepared from isolated hemagglutinin and neuraminidase subunits were applied in the analysis of these influenza antigens by serological tests, including the immunoprecipitin test (Webster and Pereira, 1968; Dowdle et al., 1969; Schild and Pereira, 1969; Schild and Newman, 1969; Schild, 1970; Webster and Laver, 1972; Kilbourne et al., 1971). These and other experiments have been discussed under Ribonucleoprotein, Hemagglutinin, and Neuraminidase.

In this way the distinct hemagglutinin and neuraminidase antigens of influenza A viruses were characterized and are compiled in Table 2 (WHO Committee on Influenza Virus Nomenclature, 1971)., and will be referred to in the discussion following.

The ribonucleoprotein or internal antigen of all influenza A strains is thought to be distinct, and to be the same for all strains, but there may be interstrain differences (see discussion under Ribonucleoprotein).

The hemagglutinin subtypes, fifteen of which have been identified, are distinct in each of four human strains, A0, A1, A2, and

A2/Hong Kong/68 (Coleman et al., 1968; Paniker, 1968; Dowdle et al., 1969; Fazekas, 1969 b; Pereira, 1969; Schulman and Kilbourne, 1969 a, b; Schild, 1970; Schild et al., 1970; Kilbourne et al., 1971; Webster and Laver, 1972); in the swine strain (Coleman et al., 1968, McQueen et al., 1969; Paniker, 1968; Pereira, 1969; Meier-Ewert et al., 1970; Schild, 1970); in each of the two equine strains, 1 and 2, (Sovinova et al., 1958; Waddell et al., 1963; Pereira et al., 1966; Coleman et al., 1968; McQueen et al., 1969; Tumova and Pereira, 1968; Tumova and Easterday, 1969); and in all avian strains except FPV and Turkey/England/63, which contain the same hemagglutinin subtype (Pereira et al., 1966, 1967 b; Tumova and Pereira, 1968; Easterday et al., 1969; Tumova and Easterday, 1969; Schild et al., 1969; Madeley et al., 1971).

In addition, certain interspecies hemagglutinin relationships have been revealed. By the immunoprecipitin test, it has been demonstrated that the hemagglutinins of A0 and A1 strains are similar but not identical, and both are distinct from A2, A2/Hong Kong, Swine, and FPV strains (Schild, 1970).

At present eight neuraminidase subtypes have been identified, fewer than hemagglutinin subtypes (WHO Committee on Influenza Virus Nomenclature, 1971; Kendal and Madeley, 1969). There is more interstrain sharing of neuraminidase than of hemagglutinin, among human strains, among avian strains, between human and swine strains, between human and avian strains, and between equine and avian strains Table 2 (from WHO Committee on Influenza Virus Nomenclature, 1971). The relationships of neuraminidase antigens revealed by immunoprecipitin tests using mono-specific antisera have been described in the section on Neuraminidase.

A common, distinct neuraminidase subtype is found in human A0 and A1 strains, and in swine strains (Coleman *et al.*, 1968; Paniker, 1968; Pereira, 1969; Dowdle *et al.*, 1969; Easterday *et al.*, 1969; Schild and Newman, 1969; Tumova and Easterday, 1969; Schild *et al.*, 1970; WHO Committee on Influenza Virus Nomenclature, 1971; Kilbourne *et al.*, 1971); and A0/BEL neuraminidase is similar to that of A/Duck/Germany/68 (Schild *et al.*, 1969).

Distinct from this enzyme is that of A2 strains (1957-present Hong Kong strains) which is essentially the same within the subtype, but has drifted slightly, so that the neuraminidase of early A2 strains differ from that of later and Hong Kong strains (Coleman *et al.*, 1968; Paniker, 1968; Dowdle *et al.*, 1969; Schild and Newman, 1969; Schild *et al.*, 1969; Schulman and Kilbourne, 1969 a, b).

As already mentioned (see Neuraminidase), Turkey/Mass/65 and /Wisc/66 neuraminidases are similar to that of A2/Sing/57, the latter avian enzyme and that of Duck/Germany/68 are similar to A2/Hong Kong/68 (Pereira *et al.*, 1967a; Tumova and Pereira, 1968; Webster and Pereira, 1968; Easterday *et al.*, 1969; Schild and Newman, 1969; Schild *et al.*, 1969).

The neuraminidase of equine 1 is distinct from that of equine 2; equine 1 enzyme is similar that of FPV and Virus N; and equine 2 enzyme is similar to that of A2/Hong Kong/68 and to that of Duck/Ukraine (both strains) (Coleman *et al.*, 1968; Tumova and Pereira, 1968; Schild and Newman, 1969; Schulman and Kilbourne, 1969 b; Tumova and Easterday, 1969).

Avian influenza viruses are composed of eight neuraminidase subtypes (Table 2) (Pereira et al., 1967 b; WHO Committee on Influenza Virus Nomenclature, 1971), although Madeley et al. (1971) were able to separate only five types. The relationships of avian and other strains have been discussed.

The above summary of relationships amongst the surface antigens of influenza A viruses demonstrates the complexity of this group. The analysis of isolated antigens using monospecific antisera has been firmly established, and antigenic relationships amongst hemagglutinin and amongst neuraminidase antigens have been determined in many cases as outlined in the WHO Memorandum (WHO Committee on Influenza Virus Nomenclature, 1971). Further investigations will gradually reveal the antigenic pattern which exists amongst these viruses and possibly indicate the manner in which they have evolved. The influence that other structural antigens have on these relationships will be of extreme interest.

The immunoprecipitin test has been invaluable in clearly demonstrating certain relationships, and although it is more broadly reacting than HAI, NAI, and CF (strain-specific) tests, it complements these standard serological methods.

Corbel and Rondle (1970) applied immunoprecipitin tests in checkerboard cross reactions of human and swine strains, but this method has not been applied in checkerboard cross reactions of human animal and avian strains. Therefore, an attempt was made to do so in this thesis.

OBJECTIVES OF PRESENT WORK

As apparent from the literature review, immunodiffusion in agar gel has been widely applied in the immunological analysis of influenza A virus antigens. Immunodiffusion in cellulose acetate has been demonstrated to be a more sensitive technique with greater resolution of antigen and antibody precipitin reactions than demonstrated in agar gel.

In the present investigation, the immunodiffusion reaction in cellulose acetate was studied because certain aspects remained questionable. The technique could not successfully determine the presence of influenza A antibodies in normal animal sera until a clearer interpretation could be made of precipitin bands that were the result of interactions between virus and other antigens, and serum precipitins. The following approach was used.

Homologous and cross reactions of selected influenza A strains, influenza B, and parainfl. Sendai were examined by immunodiffusion in cellulose acetate. Antigens used were 1) virus concentrates, which were semi-purified, and purified by sucrose density gradient (both concentrates were undisrupted and chemically disrupted), 2) virus soluble antigens. Antisera used were 1) convalescent sera of intranasally infected rabbits, 2) hyperimmune rabbit sera, and 3) hyperimmune rooster sera.

In the course of homologous and cross reaction studies, a number of aspects needed clarification:

1. The differentiation of virus-specific from host-specific precipitin reactions.
2. The differentiation of precipitin reactions caused by antibody from those caused by inhibitor.
3. The effect, on homologous and cross immunodiffusion reactions, of using virus concentrates disrupted by sonication and various surface active agents, sodium dodecyl sulfate, sodium deoxycholate, and Nonidet P40.

In order to define virus components precipitated in immunodiffusion reactions using whole virus concentrates, certain disrupted viruses were successfully fractionated by polyacrylamide gel electrophoresis (PAGE). The separated components were isolated and used in immunodiffusion reactions in an attempt to identify corresponding components of whole virus concentrates.

Immunodiffusion cross reactions were compared with hemagglutination inhibition cross reactions as a standard serological control.

Biological characteristics of influenza viruses purified by sucrose density gradient and potassium tartrate density gradient were described.

MATERIALS AND METHODS

Viruses

Ten strains of Myxoviruses were used. These included eight strains of Influenza A, one of influenza B and parainfluenza Sendai. A list of these, their source and relevant passage history are found in Table 4 .

Preparation of Virus Antigens

Seed Virus

Seed virus was prepared from each strain by several 24-hour passages through the allantoic sac of 10-11 day old embryonated hens' eggs at 35.5°C until maximum infectivity and hemagglutinating titre were reached. Care was taken to cultivate each virus type on different days to avoid any cross contamination. Stock seed virus was aliquoted in 1 ml volumes in vials and stored at -80°C. Fresh seed was prepared in this way again in 1968 and used as stock for the bulk of virus produced.

TABLE 4. Viruses, Source and History.

VIRUS	SOURCE	DATA
Infl. A0/PR/8	Stock strain, Dept. Microbiology and Immunology, University of Ottawa (originally from Laboratory of Hygiene, Canada Dept. National Health and Welfare).	ALF seed (22/11/65) stored at -80°C.
Infl. A1/FM/1/Can/53	Reference strain 10/53, Ottawa Civic Hospital	Lyophilized ALF (10/53)
Infl. A2/Can/57	Dr. C.A. Mitchell, Dept. Microbiology and Immunology, University of Ottawa (originally from Laboratory of Hygiene, Canada Dept. National Health and Welfare).	ALF (12/10/65)
Infl. A2/Hong Kong/1/68	WHO Influenza Centre, London, U.K. via Laboratory of Hygiene, Canada Dept. National Health and Welfare.	MK_2, AL_1, AM_1, AL_3 ALF
Infl. A/Equi 1/Praha/56	Dr. I. McPherson, Toronto, Canada.	ALF seed (8/7/63)
Infl. A/Swine/Shope 15 /30	Dr. C.A. Mitchell, Dept. Microbiology and Immunology, University of Ottawa.	ALF (18/12/65) stored at -80°C.
Infl. A/Duck/Can/53	Dr. C.A. Mitchell, University of Ottawa from Animal Disease Research Institute, Canada Dept. Agriculture (Mitchell et al, 1967).	ALF stored at -80°C
Infl. A2/Ottawa/68	Isolation from patient 1/68, Ottawa Civic Hospital	ALF (1/68)
Parainfl. Sendai	Dr. C.A. Mitchell, University of Ottawa from Laboratory of Hygiene, Canada Dept. National Health and Welfare.	ALF (12/65) stored at -80°C
Infl. B/Can/5/66	Dr. J.R. Polley, Laboratory of Hygiene, Canada Dept. National Health and Welfare.	ALF_6 lyophilized stored at -20°C.
Infl. A/Melbourne	Laboratory of Hygiene, Canada Dept. National Health and Welfare.	ALF_3 lyophilized stored at -20°C.

Allantoic Fluid (ALF)

For each virus type, large batches of infected chick allantoic fluid were prepared in the following manner. Tenfold virus dilutions were made in *n-saline containing 1000 units of Penicillin G and 1000 μgm of Streptomycin sulfate per ml. Ten to eleven-day hens' embryonated eggs were each inoculated by the allantoic route with 0.1 ml of a 10^{-5} to 10^{-6} dilution of virus seed. These were incubated at 35.5°C for 48 hours in a humidified egg incubator, chilled overnight at 4°C, and harvested for allantoic fluid (ALF) and chorioallantoic membranes (CAM). Eggs infected with infl.A/Swine were incubated for 36 hours to reduce the high egg mortality (up to 50%). This resulted in lower virus yield per egg but increased the total yield of a large harvest. Infected ALF was held at 4°C for up to 72 hours to precipitate phosphates and urates, then clarified by centrifugation at 3000 rpm for 30 min. at room temperature to remove cell debris, precipitated phosphates, etc. In the latter half of the work this speed was increased to 4000 rpm for 30 min. at 10°C to improve the efficiency of clarification. Hemagglutinating (HA) titre was determined by macro method in tubes and perspex trays and later changed to a micro technique (Sever, 1962) using Takatsy loops (see below).

Samples of clarified, infected ALF (V/ALF) were dispensed in vials and stored at -80°C to be used for serological tests and intranasal immunization of rabbits. The bulk was concentrated by differential centrifugation.

Normal, uninfected allantoic fluid (N-ALF) was prepared and used by procedures similar to those employed for infected ALF.

(*0.85% NaCl in distilled water - w/v)

Chorioallantoic Membranes (CAM)

Both normal and viral infected chorioallantoic membranes were washed in sterile n-saline, dispensed in vials, and initially stored at -80°C, then at -20°C (limited storage space).

Soluble antigens (SA) were prepared from these (see below).

Normal chorioallantoic membranes (N-CAM) were used for absorption of CE -specific antibodies in anti-viral hyperimmune rabbit sera.

Concentration of Viruses

Virus concentrate (V/C) was prepared by two cycles of differential centrifugation of clarified, infected allantoic fluid using a Beckman Spinco Model L2 preparative ultracentrifuge for high speed concentration and an International Centrifuge Model PR6 for low speed clarification.

In concentration cycle 1, ALF was ultracentrifuged at high speed, the pellet resuspended in n-saline or saline-tris-EDTA (STE) buffer (Pons, Hirst, 1968a) at 1/10 the original volume and clarified at low speed. The supernatant decanted was designated V/C_1, ten-fold concentrated infected ALF. Concentration cycle 2 reduced V/C_1 to 1/10 volume by the same procedure to yield V/C_2, 100-fold concentrated infected ALF.

Originally a 21 fixed angle rotor was used at 18,000 rpm for 90 min. at 10°C. for concentration followed by clarification at 3000 rpm for 30 min. at 25°C. More efficient concentration with higher virus yield was achieved by increases of speed and time to 19,000 rpm for 3 hours at 10°C, and 4000 rpm for 30 min. at 10°C. by low speed centrifugation. A fixed angle 50 rotor at 45,000 rpm for 60 min., or a SW41 rotor at 38,000

rpm for 50-60 min. was used for smaller volumes in concentration cycle 2. Infl. A2/Can/57, A2/Hong Kong/1/68, A/Equi 1, and Sendai required 200 times concentration to yield a satisfactory antigen (V/C_2') for immunodiffusion. (See Appendix 1, Table 1 for complete details of centrifugation.)

V/C_1 was inoculated to nutrient broth, blood agar plate, thioglycollate broth, cooked meat broth, and incubated at 37°C for 2-5 days to check for bacterial contamination. Contaminated concentrates were filtered by Millipore filter and further reconcentrated, because filtering caused a drop in virus concentration. Only 3 batches of 66 virus and N-ALF concentrates required filtration.

All virus concentrates were dispensed in small volumes and stored at -80°C. Samples of supernatant fluid held from each ultracentrifugation cycle were called SNF_1, SNF_2, and stored at -80°C.

Hemagglutination titrations were done on concentrates and supernatant fluid, by the same method as allantoic fluids (Table 5). (See Appendix 1, Table 2 for complete details).

Virus concentrates V/C_2 and V/C_2' were used for immunodiffusion experiments, immunization of animals, and were further purified by sucrose and potassium tartrate density gradient centrifugation.

Virus Purification by Density Gradient Centrifugation

<u>Sucrose</u> Virus concentrates (C_2 and C_2') were purified by rate-zonal centrifugation on linear sucrose gradients following the principles and methods described by Martin and Ames (1961), Anderson (1966),

Anderson et al. (1966), Brakke (1967), Laver and Webster (1966), Laver (1969); and by equilibrium density-gradient centrifugation (isopycnic) in potassium tartrate described by McCrae et al. (1961), Taylor et al. (1969), and Griffith (1969, personal communication).

A Beckman Spinco L2 preparative ultracentrifuge was used for all density gradient work.

All sucrose density gradient centrifugations were done in a Spinco SW 25.2 rotor head with three tube capacity, using 60 ml cellulose nitrate tubes. Linear sucrose gradients of 32 ml were prepared by carefully layering on top of each other, 4 ml volumes of 40, 35, 30, 25, 20, 15, 10, and 5% sucrose (ribonuclease - free) in STE buffer, pH 7.4 (0.1M·NaCl, 0.001 M EDTA in 0.05 M Tris-HCl) (Appendix 2, p. 465). These were equilibrated overnight at 4°C. Virus concentrate was sonicated for 30 sec., to dispel aggregates, with a Blackstone Sonicator using the 1/8" probe tip, ice-cooled, or the 25 ml cup tip, water-cooled. One ml of sonicated virus was immediately layered on top of each gradient and overlayed with light liquid paraffin (Saybolt viscosity 125/135) to within 1/8" of the top of the tube. In later experiments 4 ml. of 2% sucrose was layered on the virus before adding oil to counteract adsorption of virus particles to oil. The prechilled rotor was immediately loaded, put into the precooled centrifuge, and the run began when the temperature reached 4°C. Speeds and times of centrifugation varied from early to late experiments and depended on the virus. Estimates of conditions necessary for centrifugation of viruses on sucrose density gradients were calculated according to McEwen's tables (1967). The overall range was from 12,000 rpm (17,400 g) x 40 minutes for infl. A/Duck, to 15,000 rpm

(27,200 g) x 75 minutes for infl. A2/HK/1/68. Data is compiled in Table 12 . Each virus was processed in triplicate and many of the runs were duplicated.

The relative position of visible virus bands formed in the gradient was determined at the end of each run by measuring from the gradient top in centimeters. This indicated band movement during centrifugation. If banding was successful and visible, the gradients were photographed using an Ashai Pentax camera and Ilford FP4 film.

Fractions were collected in vials by puncturing the bottom of the tube with a 19 gauge hypodermic needle with the opening a few mm above the pellet. GE Silicone Seal was used to seal around the hole to prevent leakage. One, 2, and 4 ml fractions were collected and drops were also counted. Fractions were assayed for hemagglutinin with 0.5% fowl RBC (1 drop fraction: 4 drops n-saline: 5 drops RBC) In some experiments UV absorption was measured at 280 nm on a Beckman Model DB Spectrophotometer. Opalescent fractions containing banded virus were obvious by visual examination of these in a diffuse light slit in a darkened room. These correlated well with HA and UV absorbance. Positive fractions were pooled, dialyzed in boiled visking tubing against STE buffer pH 7.4 (1 vol. fraction: 50 vol. buffer) overnight at 4°C with one change of buffer. Dialyzed fractions were centrifuged in fixed angle 21, 50, or SW 41 rotor heads (speeds and times are found in Table 12). The virus pellet was resuspended in 0.6 ml STE buffer pH 7.4 per 1 ml of original virus concentrate (Laver, 1969, states 65% recovery of virus by density gradient centrifugation). Half the volume of buffer was used in resuspending pellets if the original virus concentrate was of low

concentration. The resuspended pellet was centrifuged at 4000 rpm for 30 min. to remove clear, yellow, egg-like material, and the final sucrose density-gradient purified virus suspension (V/SDG) was opalescent. Each purified virus was checked by:

1. Micro HA titration using 0.5% fowl RBC (Sever, 1962).
2. Protein estimation (Lowry et al., 1951).
3. Infectivity titration using 10-day hens' embryonated eggs to determine EID_{50} (calculated according to Reed and Muench, 1938).
4. Electron microscopy using negative staining with phosphotungstic acid neutralized (Brenner and Horne, 1959).
5. Micro immunodiffusion in cellulose acetate for homologous reactions with specific antisera: RAs-IN, hRAs (abs), hFAs; and with CE-specific antisera: hRAs/N-ALF, hFAs/N-ALF.

Details of these methods are described in Materials and Methods following.

Linear gradient formation was checked by centrifuging in parallel, a gradient to which one ml of buffer had been added, and a gradient to which one ml of virus had been added. One ml fractions were collected, and the refractive index of each one measured on a Bausch and Lomb Abbe 3L Refractometer (kindly lent by Dr. V.N. Iyer, Biology Dept., Carleton University). These values were plotted on a graph with the UV absorbance values of virus fractions so that the virus banding could be correlated with sucrose concentration.

Banding of virus was compared with banding of proteins of known

molecular weight: bovine serum albumin Fraction V, 2x crystallized horse haemoglobin, trypsin. One percent solutions of these were put on separate gradients and centrifuged in parallel with virus on a gradient. One ml fractions were collected, UV absorbance was read on a Beckman Spectrophotometer at 280 nm, and these values were plotted with the virus readings.

Potassium Tartrate   Sucrose density gradient purified viruses were further purified by equilibrium density gradient centrifugation in potassium tartrate. A Spinco SW 65 rotor head was used initially, but almost all experiments were done using a SW 41 rotor head to which the discussion below refers.

Following the same method of preparation as with sucrose gradients, 11.5 ml 20-40% gradients of potassium tartrate in STE buffer pH 7.4 were made by layering 2.3 ml of 40, 35, 30, 25, 20% solutions on top of each other;these were equilibrated overnight, and overlayed with 0.3 ml of virus sample;or more satisfactorily 11.4 ml 20-45% gradients were used with 0.2 ml of virus suspension. Light paraffin oil was used to fill tubes to within 1/8 inch of the top. Precooling of rotor and centrifuge enabled centrifuging to be carried out at 4°C. All SW 41 centrifugation was at 38,000 rpm, 174,000 g, 180 min., 4°C (details in Table 12). Each virus sample was processed at least in duplicate. If visible, the virus band position in the gradient was measured from the top in centimeters and the gradient photographed.

Fractions of 0.9 ml were collected by the same method as sucrose density gradients, that is, by puncturing the tube bottom with a No. 19 gauge hypodermic needle. Fractions were assayed for HA, UV absorbance at 280 nm, and checked by eye in a slit of diffuse light. Positive fractions were pooled, dialysed at 4°C overnight against STE buffer pH 7.4, and were centrifuged in an SW 41 rotor (38,000 rpm, 174,000 g, 60 min., 10°C). Pellets were

resuspended in 2/3 or 1/2 the volume of the original sample put on the gradient, labelled virus/potassium tartrate density gradient (V/KTDG), and stored at -80°C in vials. Each purified virus was checked by the same tests as V/SDG, i.e. by micro HA titration, mg Protein/ml, EID_{50}, electron microscopy, and by microimmunodiffusion in cellulose acetate for homologous precipitin reactions with specific antisera.

All successfully purified viruses were used for immunodiffusion reactions and in polyacrylamide gel electrophoresis.

Soluble Antigen Preparation (SA)

Soluble antigens were prepared from virus infected and normal chorioallantoic membranes of 12 day old chick embryos. The general method recommended in WHO technical Report Series, 1959 was followed, and a modification of the method outlined by Beard (1970). Each SA was prepared separately, care being taken to prevent any cross contamination.

CAM were subjected to three freeze-thaw cycles at -20°C and RT respectively. A Servall Omnimix Model MM1 (Lourdes Instrument, kindly lent by the Dept. of Biochemistry, University of Ottawa), was used to homogenize membranes at 14,000 rpm, 1½ - 2 min. in a 50 ml. cup immersed in an ice bath to prevent any alteration of components by the heat generated. The suspension was clarified at 3000 rpm for 30 min. at 10°C on an IEC Model PR-6. The top three-quarters of the supernatant was decanted and labelled virus/soluble antigen/ crude (V/SA Cr). Normal egg soluble antigen was labelled N-CAM/SA. A portion of each one was further ultracentrifuged to attempt to remove all whole virus particles. An SW41 rotor was used in a Beckman Spinco Model L2 preparative ultracentrifuge

at 38,000 rpm, 174,000 x g, 50 min. at 10°C. The top two-thirds of the supernatant fluid was dec nted without stirring, and this material was labelled virus/soluble antigen/ clarified (V/SA). Normal egg soluble antigens were prepared by the same method and labelled N-CAM/SA.

An HA micro titration (Sever, 1962) was performed in duplicate on each type of SA produced, both crude and clarified. Any V/SA showing an HA titre of greater than ½ was absorbed using 0.1 ml packed fowl erythrocytes to 3 ml SA for one hour at 4°C. This material was centrifuged at 1500 rpm 25 min. to remove debris and the decanted SNF was labelled V/SA abs. HA titration was repeated and if HA titre was ≤1:2, the majority of whole virus particles and hemagglutinin were considered to have been removed. V/SA abs. were dispensed in vials and stored at -20°C.

The Canadian Communicable Disease Centre, Canada Dept. of National Health and Welfare kindly typed each V/SA and N-CAM/SA by the Type Complement Fixation Test ("S"), and determined its titre using the following antisera: G.pig/A2/HK/1/68 (Lot 22), G.pig/B/Can/5/67 (Lot 24), Hamster/Para D Sendai (Table 28).

All SA were used in immunodiffusion tests in homologous and cross reactions with rabbit, rooster, and other antisera.

Hemagglutination Titration (HA)

Hemagglutination titrations were performed initially by macro-technique using Kahn tubes and perspex trays, but the majority of titrations were performed by the microtechnique outlined by Sever (1962).

Macrotechnique The method outlined by Hsiung (1964) was used.

Physiological or n-saline (0.85%, pH 7.2) was used as virus diluent. Fowl RBC at 0.5% were suspended in PBS pH 7.4 from 10% suspension in Alsever's buffer. Starting with a 10^{-1} or 10^{-2} initial dilution of virus, 0.4 ml volumes of doubling dilutions were made up to 1/5120 or 1/51,200. An equal volume of 0.5% fowl RBC was added, the tray agitated, and incubated one hour at room temperature, or occasionally overnight at $4^{o}C$. Four red blood cell controls were set up using 0.4 ml n-saline: 0.4 ml 0.5% fowl RBC. Hemagglutination was read at a 50% end point. Titres were expressed as the reciprocal of the dilution and as hemagglutinating units per ml (HAU/ml).

<u>Microtechnique</u> Takatsy's method as outlined by Sever (1962) was used with the microtitrator system supplied by Linbro, consisting of 96 hole disposable solid plastic trays, 0.025 dropping pipettes, Takatsy loops 0.05 and 0.025 ml. All Takatsy loops were calibrated, the loop delivery volume was checked and only those with an error of $\leq$ 2% were used. Virus diluent used was n-saline pH 7.2 or PBS pH 7.4. Fowl RBC at 0.5% were suspended in PBS. Initial virus dilutions depended on virus form: 10^{-1} for infected ALF, SNF's and suspected low titre suspensions; 10^{-2} for V/C_1, V/C_2, V/C_2', V/SDG. Using 0.05 ml. volumes, doubling dilutions of virus were made to 1/5120 or 1/51,200 (depending on initial dilution). An equal volume of 0.5% fowl RBC was added with the dropper, trays agitated, and incubated for 30 min. at room temperature. Four RBC controls consisted of 0.05 ml n-saline or PBS: 0.05 ml fowl RBC. Endpoints were read as 50% hemagglutination and titres were expressed as reciprocal of the dilution and as hemagglutinating units per ml (HAU/ml).

Results of haemagglutination titrations are to be found in Tables 5, 28 and in Table 2 Appendix 1.

Protein Estimations

Protein estimations of virus and normal allantoic fluid suspensions were made using the procedures of Lowry et al. (1951).

General Method of Protein Estimation The method of Lowry was exactly followed using the recommended volumes of 0.2 ml protein sample, 1 ml reagent C, 0.1 ml reagent E. All measurements were done with measuring pipettes, volumetric pipettes, and calibrated micropipettes (Calbiochem). Test tubes were especially cleaned. Equivalent conditions were adhered to each time protein estimations were made. Samples were immediately mixed by agitation on a rotomixer after addition of each reagent. Time of reactions were kept constant, 13-15 minutes after addition of Reagent C, and 45-60 minutes after addition of Reagent E. Readings were made on a Beckman Spectrophotometer DB Model in matched Coleman cuvettes at 700 nm, the optimum wavelength determined, as 500 nm was found to be too low for the system used.

Preparation of Standard Curves Lab-Trol (Dade) was used as a protein standard in preference to bovine serum albumin to prepare standard curves for protein estimations. Two standard curves were calibrated: one with n-saline pH 7.2 as diluent (Fig. 1); the other with saline-Tris-EDTA buffer (STE of Pons and Hirst, 1968) pH 7.4 as diluent (Fig. 2). Using the appropriate buffer to prepare each working standard, the initial stock solution of 70 mg/ml was diluted 100-fold so that the working standard

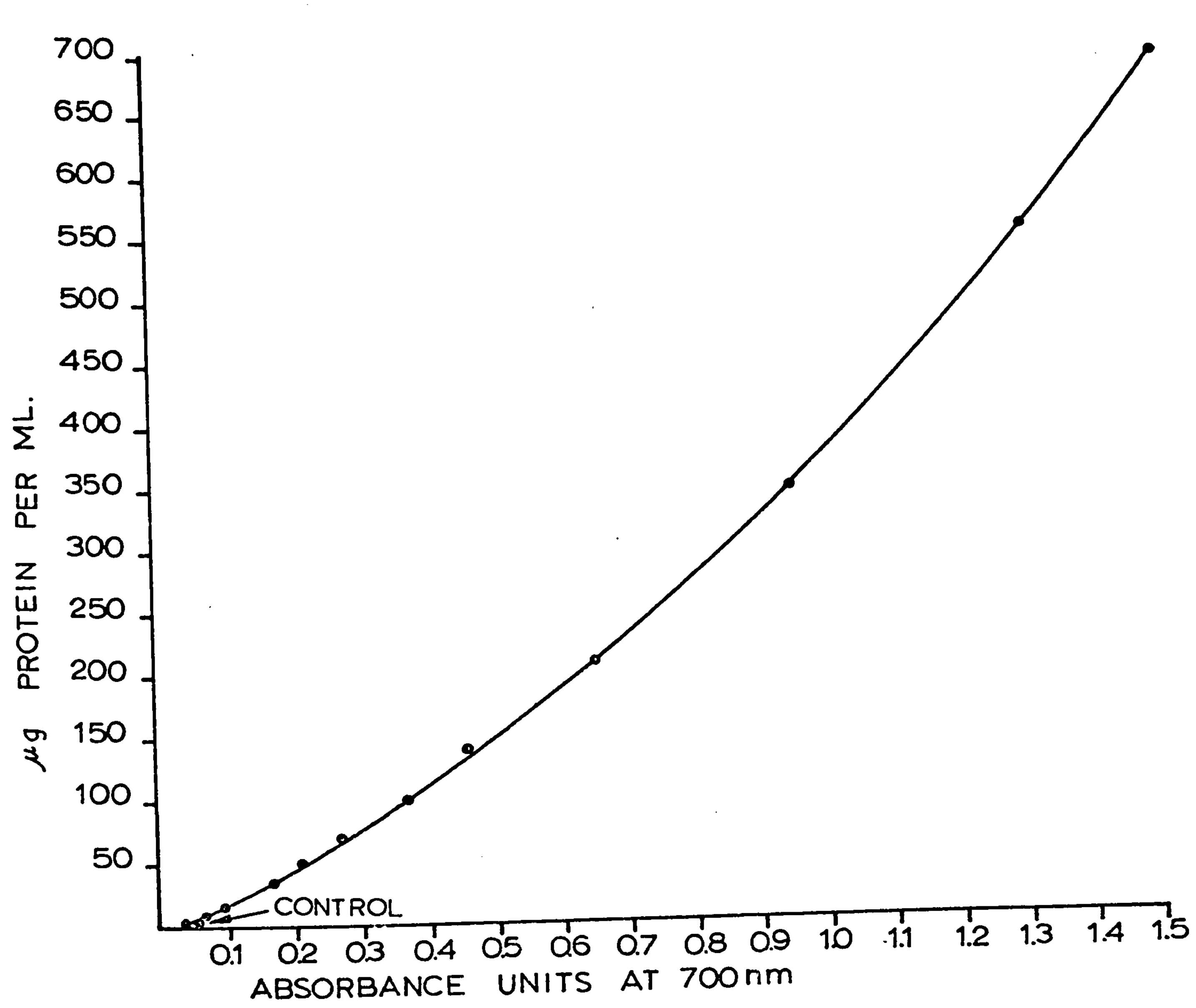


Figure 1. Standard curve for protein estimations. Lab-Trol (Dade) protein standard in n-saline, pH 7.2 (Lowry et al., 1951)

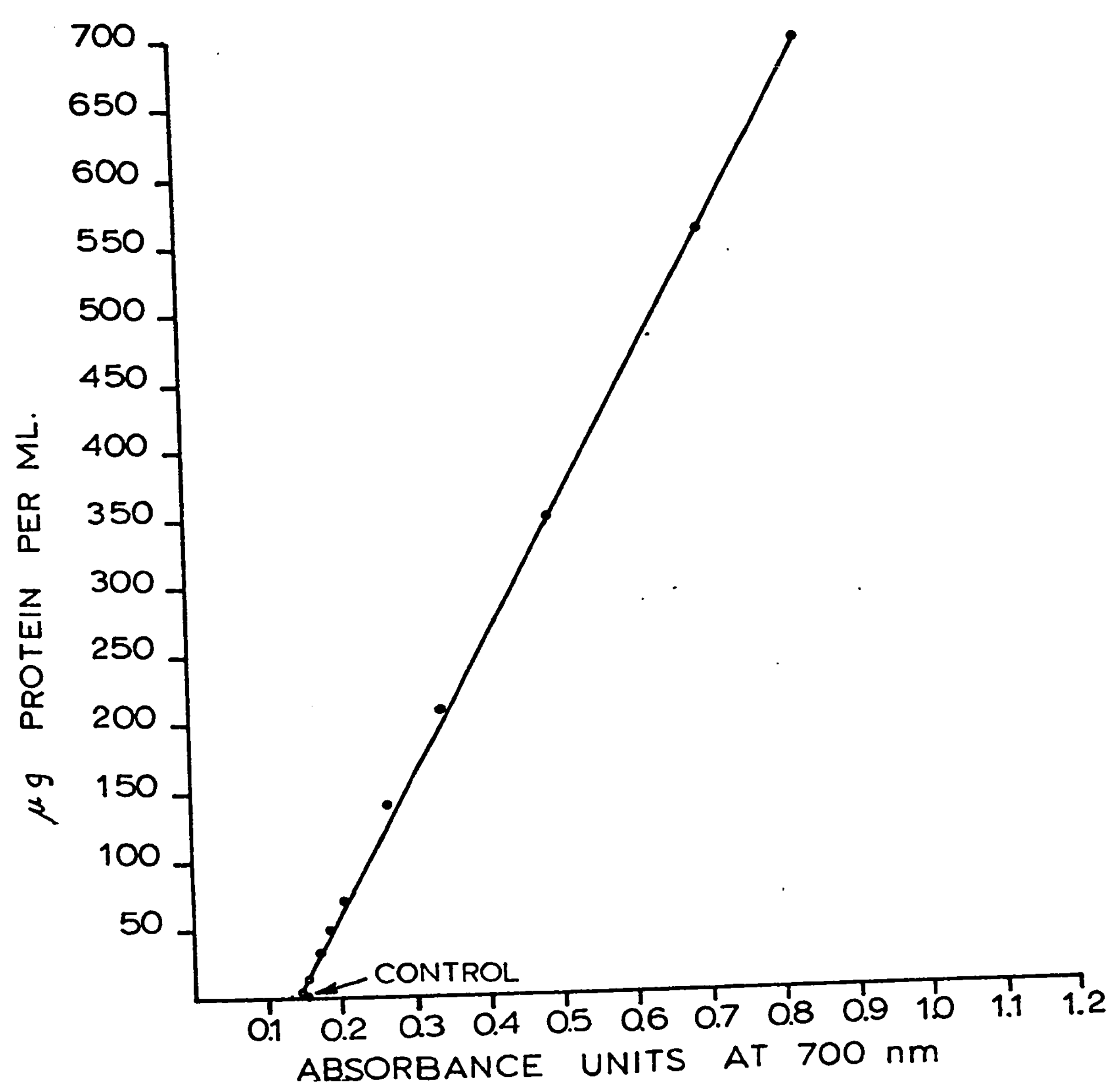


Figure 2. Standard curve for protein estimations. Lab-Trol (Dade) protein standard in STE buffer, pH 7.4 (Lowry *et al.*, 1951)

contained 700 µg Lab-Trol/ml. This material was stored at -20°C in new, cleaned, screw-capped vials.

Eleven values of standard were used to calibrate each curve: 700, 560, 350, 210, 140, 100, 70, 50, 35, 14, 7 µg/ml. Each curve was determined at least three times at three samples per value per determination so that each point on the curve is the mean of at least nine readings. In the majority of virus protein estimations, the bottom half of each curve was used.

<u>Virus and Allantoic Fluid Protein Estimations</u> Protein estimations were made on normal chick allantoic fluid (N-ALF), its concentrate (N-ALF/C_2), its sucrose density gradient purified preparation (N-ALF/SDG), and on each virus strain produced as the following preparations: infected allantoic fluid (V/ALF), concentrate (V/C_2, V/C_2'), sucrose density gradient purified virus (V/SDG), tartrate gradient purified virus (V/KTDG). Using fresh working standard Lab-Trol for reference values @ 700, 350, and 70 µg/ml for each set of protein estimations, at least three estimations were made on each virus suspension and N-ALF using the method outlined above. The dilution of test sample was adjusted so that the final readings would fall in the bottom half of the appropriate standard curve, the diluent being the same as that used in suspending the virus and the protein standard. If reference readings deviated slightly from the standard curve, a suitable correction was made and all readings adjusted accordingly. Values for protein concentrations of test samples were read off the appropriate standard curve directly. The n-saline curve was used for allantoic fluid and concentrate suspensions (if virus was suspended in n-saline), and the

STE curve for STE-suspended V/C_2, V/SDG, and V/KTDG. The mean of the three values was taken as the concentration of protein in the sample expressed as mg Protein/ml.

Results of protein estimations are to be found in Tables 5, 28 and in Appendix 1 , Table 4 .

Infectivity Titrations

Infectivity titrations in embryonated eggs, to determine EID_{50}, were carried out on the following preparations of all virus strains: infected allantoic fluid (V/ALF), concentrate (V/C_2, V/C_2'), sucrose density gradient purified (V/SDG), potassium tartrate gradient purified (V/KTDG).

Five ml volumes of tenfold dilutions of each virus preparation (usual range 10^{-2} - 10^{-13}) were made aseptically in sterile n-saline containing 1000 units Penicillin G per ml and 1000 g Streptomycin sulphate per ml. When quantity of material was limited eg. V/C_2, V/SDG, V/KTDG, the initial dilution from the original sample was made in 1 ml. The allantoic sac of each of four 10 - 11 day embryonated hens' eggs was inoculated with 0.1 ml of each virus dilution, the site wax sealed, the eggs incubated at 35.5°C for 48 hours, and then chilled at 4°C overnight. Each inoculation site was swabbed with metaphen in 70% ethyl alcohol prior to inoculation, and syringe and needles were changed for each dilution.

Each egg was tested separately for the presence of virus by spot hemagglutination test in perspex trays: 1 vol. virus/ALF to 4 vol. n-saline to 5 vol. 0.5% fowl RBC in PBS. After agitation, the trays were allowed to incubate for 1 hour at room temperature. During hot spells in the summer, incubation was overnight at 4°C. Positive hemagglutination

reactions were read to the 50% end point and recorded.

HA testing in microtrays (Linbro) was attempted using 1 vol ALF: 2 vol n-saline: 3 vol 0.5% fowl RBC in PBS but these were not as easily read as the macro test and were abandoned in its favour.

The EID_{50} of each virus preparation was calculated by the method of Reed and Muench (1938) with the interpretative aid of Kalter (1963), and the final results recorded as EID_{50} per ml, expressed as the power of the $\log_{10}$.

The results of infectivity titrations are summarized in Tables 5, 28 and are detailed in Table 3 , Appendix 1 .

Electron Microscopy

The following virus preparations: V/C_2, V/C_2', V/SDG, V/KTDG, and virus fractions from SDS polyacrylamide gel electrophoresis (PAGE) were examined by electron microscopy.

Formvar-coated 400 mesh copper grids stabilized with carbon were used for mounting all virus specimens examined.

All specimens were negatively stained using 2% phosphotungstic acid neutralized (pH 7.0) with 1 N NaOH or KOH. A drop of virus suspension was placed on a grid, washed 1 - 3 times with distilled water and the excess fluid drawn off with filter paper, then stained with PTA for 30 sec., excess stain drawn off, and the grid examined in the electron microscope when dry.

In addition, polyacrylamide gel fractions were stained by the following methods (from Pons, et al., 1969):

The preparation applied to a grid was fixed with 2.5% gluteraldehyde pH 7.0, washed three times with 0.1 M Phosphate buffer pH 7.0, prior to staining with 2% PTA (NaOH was used to neutralize PTA rather than KOH recommended by Brenner and Horne, 1959).

The preparation on a grid was washed with 0.1 M ammonium acetate and stained with 1% aqueous uranyl acetate.

All stained grids were examined by a Phillips EM 300 electron microscope. Micrographs were made at varying magnifications using 35 mm Kodak EM film, developed with D19 developer, and printed on high contrast paper, Agfa BEH 1.

Preparation of Specific Antisera

Rabbit (R) Immunization

White male rabbits of approximately 2 kg. weight were obtained at intervals from the University of Ottawa Medical Faculty Animal House. Each one was ear tattooed with a code number, A1, A2, etc. and put into a Horsfall unit. A normal blood sample was taken from the ear, the extracted serum dispensed into vials, labelled normal rabbit serum (NRS - A1, etc.), and stored at -20°C. Each serum was tested by micro-immunodouble diffusion on cellulose acetate (IDD) against all viruses involved in this study and by hemagglutination inhibition (HAI) against the homologous immunizing virus.

Each rabbit was intranasally infected (IN) with one virus type by dropping 0.25 ml infected allantoic fluid into each nostril with a

spraying action. Infection by each virus type was done on different days in a monkey Horsfall unit which was disinfected thoroughly after each animal. Each rabbit was kept in isolation until the end of incubation (1 - 2 months) when it was again ear bled, the convalescent serum labelled (RAs-IN/V), and stored at -20°C. Individual sera were tested by HAI and IDD.

Rabbits were then removed to individual cages in a common room and immunization was continued parenterally by the intramuscular route (IM). Each dose of the vaccine, administered at two week intervals, consisted of 1 ml virus concentrate (V/C_2) at 10^{-1} in n-saline,mixed with an equal volume of Freund's Complete Adjuvant (Difco). When test bleeding revealed a maximum HAI titre had been reached and a satisfactory IDD pattern obtained, animals were bled by cardiac puncture, the hyperimmune serum dispensed in vials, labelled hRAs/V, and stored at -20°C. The best of each group of sera were pooled (usually two) in equal volumes and used as specific antiserum of the immunizing virus (Table 8). Only half of the 44 animals attempted yielded reasonable antiserum. The immunizing schedule and relevant data are presented in Tables 7 and 8.

Normal allantoic fluid (N-ALF) and its concentrate ($N\text{-}ALF/C_2$) were used to immunize rabbits by the same procedure outlined above but these were done at a time when no other animals were being immunized. Serum from rabbits to which N-ALF had been applied intranasally was labelled RAs-IN/N-ALF (convalescent). Hyperimmune serum was labelled hRAs/N-ALF (Tables 7 and 8).

Rooster (F) Immunization

Two batches of white pullet roosters were immunized parenterally, as facilities for individual isolation of birds were not available. The first batch came from a poulty farm supplying the embryonated hens' eggs

and were numbered F1 - F8. The second batch came from the Animal Disease Research Institute, Canada Dept. Agriculture, from the normal animal section and had had no contact with any humans except one caretaker. These were numberedF 9 - F17. Normal serum was obtained from cardiac puncture blood, dispensed, numbered and labelled NFS/R1 etc., then stored at -20°C.

Birds were kept individually in cages in a common room. One ml of vaccine consisting of virus concentrate (V/C_2) at 10^{-1} in n-saline and an equal volume of Freund's Complete Adjuvant (Difco) was injected intramuscularly at two week intervals until test bleeding showed a reasonable HAI titre and immunodiffusion pattern. Birds were then bled by cardiac puncture, serum extracted and dispensed in vials, labelled hyperimmune fowl serum (hFAs/V), and stored at -20°C. Rooster sera were used individually and not pooled because often only one bird was successfully immunized against a virus type. The immunizing schedule and relevant data are presented in Table 9.

A control bird was immunized with normal allantoic fluid concentrate ($N\text{-}ALF/C_2$) by the same procedure as virus immunization. It was kept in a separate room away from virus immunized birds. Hyperimmune serum obtained from cardiac puncture blood was labelled hFAs/N-ALF and stored at -20°C (Table 9).

<u>N-CAM Absorption of Serum</u>

Each Hyperimmune rabbit serum pool was absorbed with N-CAM to attempt to remove host (CE)-specific antibodies produced when animals were immunized parenterally with egg grown virus concentrate.

The original method used was suggested by Dr. R. Siboo (personal communication) and was later modified.

Frozen normal chorioallantoic membranes (N-CAM) were thawed, washed with an equal volume of saline, and packed at 5000 rpm for 30 min. at 10°C. The supernatant was decanted. Hyperimmune rabbit serum was added in the proportion of 1 vol serum to 10 vol. packed N-CAM, thoroughly mixed, held at 37°C for 1 hour with frequent shaking, then overnight at 4°C. The mixture was centrifuged at 5000 rpm for 30 min., the SNF decanted, measured, clarified at 4000 rpm for 30 min. to remove debris. This supernatant was labelled hyperimmune rabbit serum/virus, /N-ALF, N-CAM absorbed (hRAs/V abs, hRAs/N-ALF abs).

Other proportions of serum to packed N-CAM were tried eg. 1:5, and 1:7. The latter one was optimum if membranes and serum were shaken in a 37°C. water bath for one hour and put on a wrist action shaker overnight at 4°C.

Using the same methods of absorption as above, two other forms of N-CAM were tried:

1. Acetone dried membranes in the proportion 3 vol dried membrane to 1 vol serum. Absorbed serum was labelled hRAs/V abs D.

2. Lyophilized membranes in the proportion 0.125 gm. membrane to 1 vol. serum plus one vol. PBS buffer (pH 7.3). Absorbed serum was labelled hRAs/V abs Ly.

All absorbed sera were dispensed in vials, labelled, and stored at -20°C.

Immunodiffusion in cellulose acetate was used to detect the efficiency of absorption of host (CE)-specific antibodies in hyperimmune rabbit serum absorbed by chick N-CAM.

Various combinations of CE-specific and virus-specific reactions were integrated to evaluate if host (CE)-specific antibodies had been removed from absorbed hyperimmune rabbit sera. The reagents used included: N-ALF C_2 and C_2' and homologous rabbit antisera; N-CAM soluble antigen; virus C_2 and C_2' and homologous convalescent hyperimmune rabbit antisera. Representative template patterns used are to be seen in Plate 4.

The CF titre of each absorbed serum pool was determined by microtitration using influenza A2/Hong Kong/1/68 type-specific antigen. CF titres are to be found in Table 8. P. Phipps, Virus Laboratory, Ottawa Civic Hospital, kindly performed the tests.

Immunological Methods

Micro Immunodoublediffusion in Cellulose Acetate (IDD)

The technique of immunodiffusion used, followed the princples and interpretations of Crowle (1961) and Ouchterlony (1968). The specific adaptation of micro immunodoublediffusion (IDD) in cellulose acetate by Johnson *et al*. (1964) formed the basic technique of all immunodiffusions

carried out in this study. All references to immunodiffusion or immunoprecipitin tests in the experimental work relate to this technique.

General Procedure After experimentation with several buffers, the two selected for use in the majority of immunodiffusion experiments were Tris no. 4 (0.2 M Tris, 0.1 M NaCl, 0.1% sodium azide, pH 7.4, ion conc. 0.15 (determined by Solu Bridge) (Burstone, 1962)) for reactions with all animal sera; and Tris no. 6 (0.05 M Tris, 2.0 M NaCl, 0.1% sodium azide, pH 7.4 (adapted from Gallagher and Voss, 1969b) for reactions with fowl sera. (See Appendix 2 for formulae).

Cellulose acetate strips (S and S Cellulose Acetate Strips for Electrophoresis, Consolidated Laboratories) 2.4 x 5 cm were soaked in one of the above buffers and mounted wet on a glass slide. A perspex template, perfectly smooth, was carefully slipped to a central position, care being taken to eliminate all air bubbles and to avoid scratching the paper. Excess buffer was blotted off and stainless steel chromatography clips (calibrated by manometer to exert a pressure of 1.5 cm) were used to hold the template in place as illustrated in Fig. 3 .

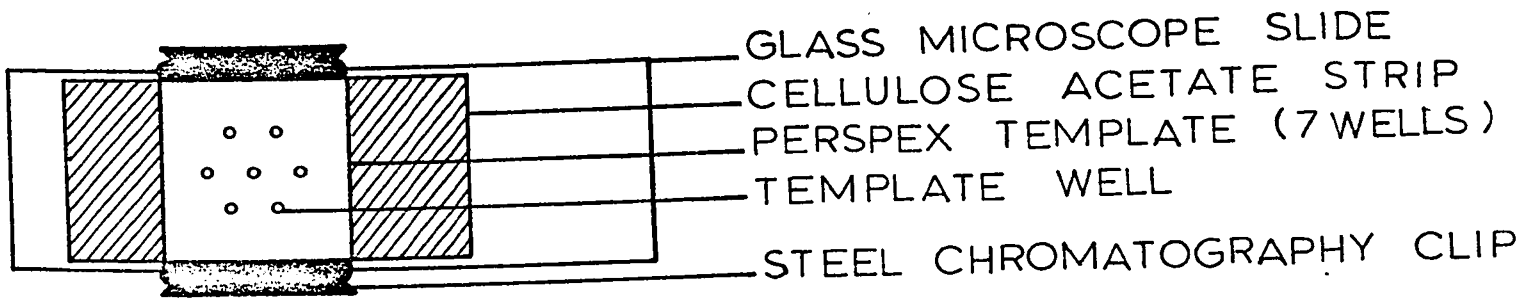


Fig.3 Assembly for immunodiffusion reactions in cellulose acetate.

These were then placed individually in moist chambers (small square Petri dishes containing filter paper saturated with 1% aqueous sodium azide) which were placed in a large moist airtight plastic container and allowed to equilibrate for a few hours at the incubation temperature used for all immunodiffusions, 22.5°C.

Three sizes of perspex templates were used containing 7, 13, and 25 wells, typical examples of which are found in Plates 16C, 15B, and 16A respectively, and in Fig. 4.

Materials to be diffused, that is, antigens and antisera, were applied to template wells by finely drawn out Pasteur pipettes, each well requiring 0.025 ml to be filled. After 44-48 hours incubation in moist chambers at 22.5°C, the templates were removed. The cellulose acetate strips containing immunoprecipitin reactions were then rinsed with cold water, and washed in several changes of 0.85% NaCl for at least 4 hours at room temperature if stained by thiazine red R, but required at least 12 hours wash at 8°C if stained by coomassie blue. Saline washes were followed by rinsing in several changes of distilled water.

Immunoprecipitin reactions were stained in 0.1% thiazine red R in 1% acetic acid (Crowle, 1961), followed by decolorization twice in 5% glacial acetic acid, and a rinse in distilled water. They were then blotted and dried at room temperature.

Coomassie blue, a much more sensitive protein stain (Fazekas de St. Groth et al., 1963), used by Webster (1970 a) to stain influenza polypeptides in polyacrylamide gels, was adapted to stain immunodiffusion reactions in cellulose acetate. However, the decolorizer suggested by Webster curled the cellulose acetate, and a different decolorizing

technique was developed. The following procedure gave excellent results. Saline washed, and distilled water rinsed precipitin reactions were placed in coomassie blue stain (0.05% in 45.5% methanol and 0.1% acetic acid, (Webster, 1970a) in a glass tray so that no overlapping occurred. After one hour of undisturbed staining, the strips were removed and decolorized in at least 3 changes of decolorizer (100 ml methanol: 200 ml distilled water: 17.5 ml glacial acetic acid) until the background was white, but the stained precipitate not overdecolorized. The strips were then soaked in distilled water for at least 30 minutes to rehydrate them, blotted by placing on blotting paper and dried.

All dried strips were cleared in Whitmore oil no. 120 (Consolidated Laboratory), mounted on glass slides under coverslips and sealed with clear nail enamel after trimming and removing excess oil.

Using the slides as negatives in an enlarger, the precipitin reactions were recorded photographically on Agfa BEH 1 paper for thiazine red stained reactions, and on Kodak Rapid Pan paper for reactions stained with coomassie blue. An orange filter enhanced recording of blue reactions on Rapid Pan paper.

The majority of stained slides were examined by a Zeiss Stereographic microscope (kindly lent by the Geology Dept. of this University). Coomassie blue stained reactions were very clear and more easily seen than those stained with thiazine red.

Special Stains were used in an attempt to detect specific components of viruses precipitated in immunodiffusion reactions. Vapours from iodine crystals were used to detect lipoproteins; methyl-green-pyronin

Y to detect RNA (Brachet, 1953, Kurnick, 1955); and acridine orange to detect RNA (Mayor and Hill, 1961, Mayor 1962, Bradley, 1965). Details of procedures are in the Appendix 2 . Difficulty was encountered with all stains because the solvents used attacked the cellulose acetate causing it to curl.

Equivalence Immunoprecipitin Reactions

Equivalences for the immunoprecipitin reactions of each virus with its homologous hyperimmune rooster (hFas/V), and rabbit serum N-CAM absorbed (hRAs/V abs) and unabsorbed (hRAs/V), were establish in two ways:

1. Antigen concentration constant (V) and antisera (As) varying from undiluted to 1:32 dilution.
2. Antigen concentration varying from undiluted to 1:8 with antiserum concentration constant.

The following template patterns were set up for the reactions:

As 1:1 | As 1:2 | As 1:4 | As 1:8 | As 1:16 | As 1:32 | V constant (centre)

hRAs/V | hRAs/N-ALF | hRAs/V abs | hRAs/V | hRAs/N-ALF | hRAs/V abs | V 1:1-1:8 (centre)

Hyperimmune rabbit serum to normal allantoic fluid (hRAs/N-ALF) was included to differentiate host (CE)-specific and virus-specific reactions.

Virus concentrate antigens were found to react optimally at full strength in precipitin reactions. Hyperimmune antiviral rabbit serum, N-CAM absorbed, already at 1:2 - 1:3 dilution, had the greatest spectrum of precipitin bands at full strength. Hyperimmune rooster serum also produced the best results at full strength. However, adjustments of antigen and antibody concentrations were made as the work progressed to keep precipitin reactions at equivalence.

Soluble antigens were found to react optimally at 1:2 dilution in most cases.

Hemagglutination Inhibition (HAI)

The basiç microtechnique outlined by Sever (1962), and detailed procedure described in "Microtiter Manual" (Cooke Engineering Co.) were followed in performing HAI tests. Recommendations from a study of the standardization of viral hemagglutination inhibition tests (Hierholzer et al., 1969) was used as a guide of materials and procedures chosen.

Most equipment was supplied by Linbro: 0.025 and 0.05 ml platinum Takatsy loops, 'U' rigid styrene plates, and 0.025 ml droppers (and from Cooke Engineering Co.).

Diluent used throughout was phosphate buffered saline (PBS) pH7.3 (Appendix 2). Fowl erythrocytes were used at 0.5% in PBS (determined by hematocrit).

Antigen was virus infected allantoic fluid (V/ALF) suitably diluted to contain 4 HAU/0.025 ml, calculated from its HA titer (see HA Titrations above). A preliminary virus HA unit check was done: to each

of 4, 2, 1, ½, 0 HAU of virus in 0.025 ml was added 0.025 ml PBS, 0.05 ml 0.5% fowl RBC (simulating the relative proportions used in HAI test). Incubation was at room temperature 30 min. The end point, read as 50% hemagglutination, represented 1 hemagglutinating unit (HAU) of virus. Red blood cell controls consisting of 0.05 ml PBS and 0.05 ml 0.5% fowl RBC were always included.

For the HAI test, doubling dilutions of 0.025 ml volumes of treated sera were made in the appropriate dilution range. 4 HAU virus in 0.025 ml was added to each serum dilution, the mixture agitated, incubated at room temperature 30 min., and 0.05 ml 0.5% fowl RBC added to each cup. The trays were again agitated and a 50% endpoint was read after 30 min. incubation at room temperature. RBC controls were always included. A serum control of each serum tested was included in the same dilution range and consisted of 0.025 ml serum dilution, 0.025 ml PBS, 0.05 ml 0.5% fowl RBC. HA Unit controls accompanied all HAI tests.

Treatment of Sera for Inactivation of Inhibitors of Influenza Virus Hemagglutination and Precipitation

Trypsin-Heat-Periodate (THP) The methods of Davenport and Minuse (1964), and Casals (1967) were followed with slight modifications. One volume of serum was mixed with a half volume of trypsin (Bacto 250 at 8 mg/ml), the mixture heated at 58°C - 60°C for 30 min., and 1.5 volumes of M/90 potassium periodate added. After 15 min. at room temperature, 1.5 volumes of 1% glycerol in PBS were added to stop the action of periodate. After 15 min. at room temperature, a half volume of PBS was added to ajust the serum dilution to 1:5.

Sodium Periodate ($NaIO_4$) The conditions laid down by Levinson et al. (1969) were strictly adhered to. To one volume of undiluted serum were added 3 volumes M/90 sodium meta-periodate (Sigma) in 0.15 M NaCl at pH 4.5 (adjusted with 0.2 M acetate buffer) and the mixture held at 4°C for 15 min. in the dark. One volume 10% glucose in 0.15 M NaCl was added to stop the reaction and further incubated for 15 min. at 4°C. The treated serum at 1:5 dilution was now ready for testing.

Receptor-Destroying Enzyme (RDE) *V. cholerae* filtrate (Sigma) was titrated with infl.A/Melbourne to determine its neuraminidase potency (French and Ada, 1959) and was found to contain 1000 units neuraminidase per ml. Using a modification of the method recommended by Hierholzer et al. (1969), 4 volumes of RDE were added to 1 volume of serum, the mixture held at 37°C for 16 hr., then heated at 58°C x 1 hr. to destroy residual enzyme. Different strengths of RDE were used in serum treatments: 200, 500, and 1000 units per ml (Coleman and Dowdle, 1969), depending on the serum and conditions of the experiment. Most normal animal sera were treated with RDE at 500 units per ml. Final serum dilutions were at 1:5.

Kaolin, see part II, same heading.

Disruption of Virus Antigens

Virus antigens V/C_2, V/C_2', V/SDG, and V/KTDG of all virus strains used in this study, were disrupted by various methods for use in immunodiffusion cross reaction experiments of influenza viruses, for

fractionation of the viruses by polyacrylamide gel electrophoresis (PAGE), and for fractionation of Duck C_2 by column chromatography using Sephadex G-200. A description of each method used follows.

Sonication

A Blackstone High Intensity Ultrasonicator was used to sonicate influenza A Duck C_2. The 1½" water-cooled cup was sterilized by isopropyl alcohol followed by UV light for 45 min. A 4 ml sample of the virus was disrupted at a 60 power setting for 1, 2, 3, 5, 10, 15, and 20 min, 0.2 ml samples removed at each time interval. Each sample was diffused with homologous convalescent rabbit serum (RAs-IN/Duck) and hyperimmune rabbit serum absorbed by acetone dried N-CAM (hRAs/Duck abs D) to determine the effect of sonication on the immunodiffusion pattern of whole virus particles.

Sodium Dodecyl Sulfate (SDS)

V/C_2, V/C_2', V/SDG, and V/KTDG were disrupted at room temperature by addition of a 10% aqueous solution of highly purified and recrystallized SDS (BDH sodium lauryl sulfate) to a final concentration of 1% in the virus suspension (Laver, 1963). The clarified suspensions were used for immunodiffusion experiments and for fractionation by polyacrylamide gel electrophoresis. Duck C_2 was disrupted in the same way for column chromatography.

Sodium Deoxycholate (DOC)

V/C_2, V/C_2' were disrupted by adding 10% aqueous DOC (Fisher)

to a final concentration of 1% in the virus suspension (Laver, 1963), and holding at 37°C for 1 hour before use. These preparations were used for immunodiffusion experiments only.

Sodium Dodecyl Sulfate (SDS) - β-mercaptoethanol - Heat

V/C_2, V/C_2', and V/SDG were disrupted by adding 10% aqueous SDS to a final concentration of 1%, holding for 15 min at room temperature, then adding β-mercaptoethanol (SIGMA) to a final concentration of 1%, and immersing the virus suspension in boiling water at 100°C for 1 minute (Maizel, 1969). Viruses disrupted in this way were used for immunodiffusion cross reaction experiments and for fractionation by polyacrylamide gel electrophoresis.

Nonidet P40 (NP 40)

This was a gift of the Shell Oil Company of Canada. V/C_2, V/C_2' and V/SDG were disrupted by adding 10% aqueous NP 40 to a final concentration of 1% in the virus suspension, and held at room temperature about 1 hour before used (Schild and Pereira, 1969, Pons et al., 1969). These suspensions were used for immunodiffusion homologous and cross reaction experiments.

Virus Fractionation Procedures

An attempt was made to fractionate viruses into various antigenic components, to identify them, and to use them in immunodiffusion experiments as known antigenic components and thus detect specific anti-

bodies in animal sera, both "normal" and immune. Two techniques of fractionation used are described below.

Column Chromatography using Sephadex G-200

A Pharmacia column 2.5 x 45 cm was packed at $8^{o}C$ with Sephadex G-200 in 0.01 M Tris HCl, 0.01 M NaCl, 0.02% sodium azide buffer (adapted from Duesberg and Robinson (1965) according to directions in Pharmacia Bulletin and under the guidance of Dr. R. Siboo. 1.5 ml of SDS treated infl. A/Duck (see under Virus Disruption, SDS above) was applied to the column and samples collected in 1.5 ml aliquots with a Gilson fraction collector. Protein was determined in each sample by UV absorption at 277 nm in a Coleman Hitachi Model 101 spectrophotometer. The amount of protein recorded was almost negligable and it was obvious that the virus was still in the column. However samples within 2 mini peaks, peak 1, peak 2, were pooled separately. To elute virus adhering to the column, the pH of the buffer was changed to 7.9 and 0.001 M EDTA added. Two ml samples were collected, checked for protein by UV absorption as before, and pooled from the one peak formed peak 3. All pooled samples were dialyzed and concentrated to 1/20 the volume in S and S collodion membranes.

The dialyzed material from each peak was tested by hemagglutination titrations and by diffusion in cellulose acetate against homologous convalescent and hyperimmune rabbit sera, and anti-host (CE) hyperimmune rabbit serum. Peak 3 material was also checked for infectivity in ovo, and by complement fixation using a type A group (A2/Hong-Kong) guinea pig antiserum (this latter test kindly performed by Lab of Hygiene, Canada Dept. National Health and Welfare).

The fractionation procedure was not particularly succesful as technical difficulties were encountered with the fraction collector, and the recovery of virus material was very inefficient.

Polyacrylamide Gel Electrophoresis (PAGE)

The general principles and procedures described by Ornstein (1964) Davis (1964), Loening (1967), and Maizel (1969) were followed.

Virus concentrates, which were semi-purified (C_2, C_2'), and purified (by sucrose density gradient - SDG), were disrupted by two methods: 1) SDS-β-mercaptoethanol - heat and 2) SDS.

Disrupted viruses were fractionated by polyacrylamide gel electrophoresis following the neutral SDS method described by Maizel (1969). All reagents were prepared as described, and stored as recommended (see Appendix 2 for reagent formulae). Five percent acrylamide gels were prepared by mixing the following reagents at room temperature: 1.67 ml acrylamide-bis acrylamide (30:0.8); 1.0 ml sodium phosphate (1 M); 0.1 ml sodium dodecyl sulfate (10% aqueous w/v); 0.005 ml TEMED - N, N, N', N'-tetramethylethylenediamine; 7.1 ml distilled water; 0.1 ml ammonium persulfate (10% aqueous w/v). Glass tubes 7.5 x 0.8 cm were carefully filled to within one cm of the top, overlaid with distilled water, and the gels photopolymerized in a fluorescent light for 45 minutes. Distilled water at the top of the gels was then replaced by electrophoresis buffer, 0.01 M sodium phosphate containing 0.1% SDS. The tubes containing gels were positioned into a Buchler Disc Electrophoresis apparatus (1000 ml), the lower and upper reservoirs were filled with buffer and the gels pre-electrophoresed at 5 mA/tube (using a Buchler Power Pack) for 4-7 hours

to remove persulphate (Fantes and Furminger, 1967). At the end of this time, gel tubes were removed from the apparatus and the buffer emptied from the tops of the gels and from the upper reservoir.

Virus samples, brought to 1% SDS, 0.01 M phosphate, 1% (v/v) β-mercaptoethanol, and 10% sucrose, were applied to the top of the gels in 0.1 ml volumes (and 0.15 ml), carefully overlaid with 2% sucrose, and the tubes gently filled with buffer to avoid mixing. In one set of experiments, β-mercaptoethanol and heat were omitted from the above treatment. Duplicate samples were always run. Bovine serum albumin Fraction V (0.1%) was treated in exactly the same way as virus samples, and included as a standard in each run. The protein load per tube was not more than 0.15 mg. N-ALF was treated in the same way.

Tubes with gels and virus samples were carefully replaced in the apparatus, the buffer levels appropriately adjusted, and the samples were stacked for 20-25 minutes at 6 mA/tube. Electrophoresis was carried out at 10 mA/tube for 2 hours. At the end of electrophoresis, tubes were taken out and the gels carefully rimmed and removed. Gels were stained for 18 hours in 0.05% coomassie blue in 45.5% methanol and 0.1% acetic acid, and were decolorized in several changes of 45.5% methanol, 9.1% acetic acid (Webster, 1970). Stained gels were stored in the dark, in glass tubes containing 7% acetic acid.

Records of fractionated viruses were made by drawings and by photography using a Polaroid Industrial Camera (by kind permission of the Biochemistry Department, Univ. of Ottawa), or by an Ashai Pentax camera, with orange filter, and Panatomic X film.

Each purified virus strain, disrupted by SDS, was fractionated by this electrophoresis method. Five samples per strain were processed.One gel was stained by coomassie blue; the other four were stained for one minute by 8-Anilino-1-naphthalenesulfonic acid (ANA) magnesium salt (0.003% in 0.1 M potassium phosphate buffer, pH 6.8), and viewed under an ultraviolet light (Fisher, 1970; Hartman and Udenfriend, 1969). Protein in bands fluoresced under the UV light when stained by this compound, and was not denatured by it. Fluorescent protein bands situated in the same relative position in each gel were cut out with a scalpel, placed in a new vial, and labelled Virus/Fraction 1, V/Fr 2, etc. Identical fractions of the same virus strain were pooled. 0.05 ml phosphate buffer (0.01 M) was added per gel fraction and the gels broken up, but not crushed, with a glass rod. Vials were put on a shaker for 18-24 hours at 4°C. Eluted fractions of each virus strain were diffused in conjunction with the original disrupted virus sample against homologous antisera: convalescent rabbit, hyperimmune rabbit (abs), and hyperimmune rooster. N-ALF was included as a host control antigen. The following 25-well template pattern was used (S = antiserum, V = disrupted virus, F1, F2, etc. = Fraction 1, Fraction 2 etc.).

	S1		S1		S1		S1		S1		S1		S1		S1	
N-ALF		V		F1		F2		F3		F4		F5		F6		V
	S2		S2		S2		S2		S2		S2		S2		S2	

Diffusions were carried out and stained by coomassie blue as described previously.

Each fraction was tested for only one biological activity, hemagglutination. To one drop (0.025 ml) of fraction was added 2 drops of n-saline, and 3 drops of 0.5% fowl RBC. Incubation in microtrays was 30 minutes at room temperature, and fractions were examined for hemagglutination.

Negatively stained preparations of each fraction were examined by electron microscopy.

Identification of the Type-Specific Reaction in Immunodiffusion

Soluble antigens of each virus studied, influenza A/PR8, FM1/Can/53, A2/Can/57, A2/Hong Kong/1/68, Equi 1,Swine, Duck, influenza B/Can/5/66, and parainfl. Sendai, were diffused with each type of homologous antiserum, convalescent rabbit, hyperimmune rabbit (abs) and hyperimmune rooster to establish the identity of the type-specific reaction. These reactions were run independently and in conjunction with virus concentrates containing whole virus particles and respective antigens. Virus concentrates were undisrupted and NP 40 disrupted.

Two template patterns were used:

1. Seven well templates for homologous reactions in which immune sera (peripherally located) were diffused with soluble antigens, and virus concentrates, the latter antigen undisrupted and NP 40 disrupted (Plate 11 is a representative pattern).

2. Twenty-five well templates were used to establish the identity of the type-specific reaction of all influenza A strains, influenza B, and parainfl. Sendai. Each immune serum was diffused with all virus soluble antigens (Plate 12 is a representative pattern).

Immunoprecipitin reactions were carried out at equivalence. Virus soluble antigens had the same relative order of value established by complement fixation tests, except influenza B/Can/5/66 (Table 28), which was low. Virus concentrate values varied (Table 5), but they were used at full strength in homologous reactions with immune sera to maintain equivalence of the reactions. Interpretations were made on a qualitative basis only.

Immunodiffusion Cross Reactions of Influenza A Viruses

Virus concentrates, semi-purified (C_2 and C_2') and sucrose density gradient purified (SDG), of influenza A/PR8, A/FM1/Can/53, A2/Can/57, A2/HK/1/68, A/Equi 1, A/Swine, A/Duck, B/Can/5/66, and parainfl. Sendai were diffused in cellulose acetate with their corresponding sera of three sorts: convalescent rabbit, hyperimmune rabbit N-CAM absorbed, hyperimmune rooster. A checkerboard pattern was followed which enabled the comparison of homologous and cross reactions.

Virus concentrates were used undisrupted, or disrupted in the following ways: 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate (DOC), 1% Nonidet P40 (NP 40), SDS-β-mercaptoethanol-heat. Each experiment

was done using either undisrupted virus, or viruses disrupted by one method only.

Equivalence of the homologous reaction was maintained in cross reactions.

Three general template patterns were used:

1. 25-well template to determine the general pattern of cross reactivity.
2. 13-well template to determine the general pattern of cross reactivity and to compare different relative positions of antigen and antibody in the template.
3. 7-well template to examine the precipitin reactions of related viruses (revealed by the other two templates) more closely.

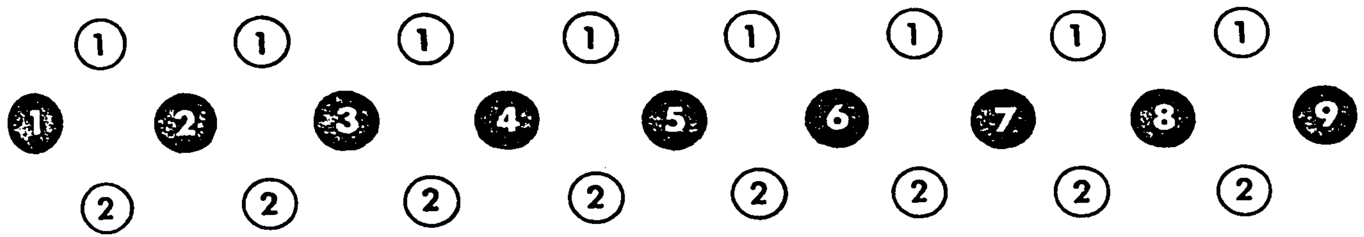
The template patterns used are schematically presented below (Fig. 4).

Each cross reaction slide was analyzed by stereomicroscopy and recorded photographically.

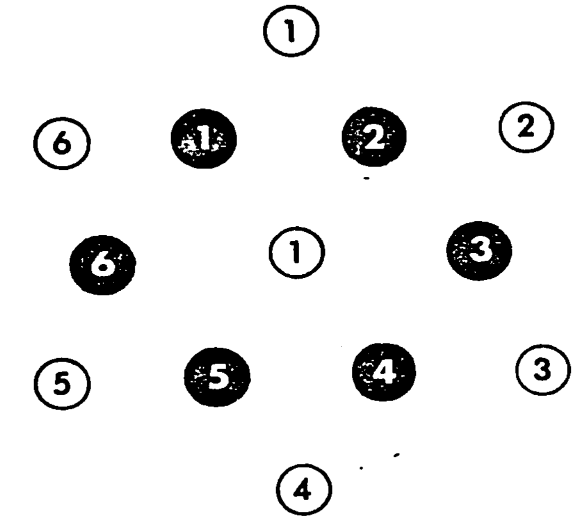
Hemagglutination Inhibition (HAI) Cross Reactions

Using the HAI technique described, hemagglutination inhibition cross reactions of all influenza A strains, PR8, FM1/Can/53, A2/Can/57, A2/Hong Kong/1/68, Equi 1, Swine, Duck, influenza B/Can/5/66, and parainfl.

25 WELL TEMPLATE



13 WELL TEMPLATE



7 WELL TEMPLATES

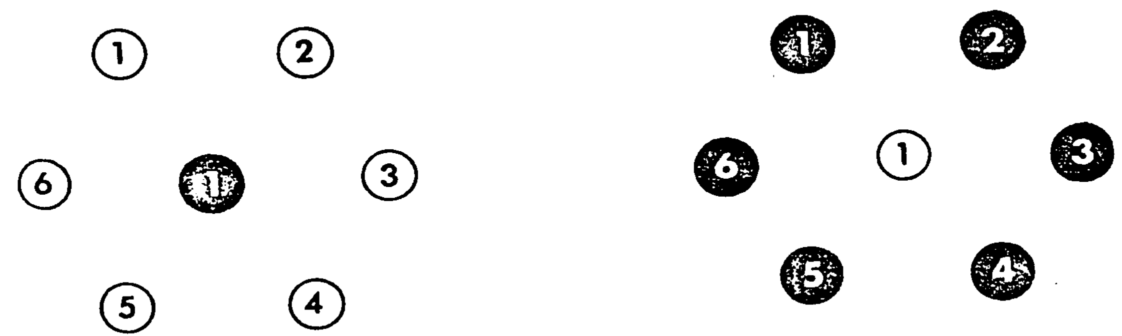
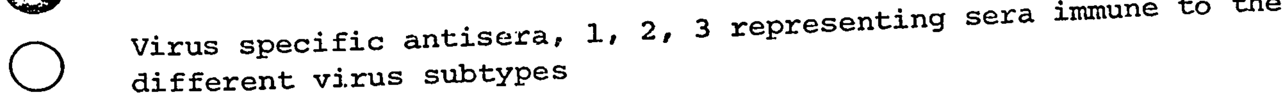


Figure 4. Schematic representation of immunodiffusion template patterns used in influenza virus cross reactions

Virus antigens, 1, 2, 3, etc. representing different subtypes


Virus specific antisera, 1, 2, 3 representing sera immune to the different virus subtypes

Sendai were carried out in a checkerboard pattern, with the following corresponding immune sera: convalescent rabbit,hyperimmune rabbit, and hyperimmune rooster. As a control, anti-N-ALF sera were also included in the checkerboard. All sera were THP, RDE, or $NaIO_4$ treated. Four HA units of virus antigens from infected allantoic fluid were used.

RESULTS

Antigens

Detailed biological characteristics (hemagglutinin activity, infectivity, and protein content) of influenza A strains, influenza B, para-influenza Sendai, and host (chick embryo or CE) antigens are compiled in Appendix 1, Tables 2, 3, 4. The biological characteristics of virus antigens produced are somewhat diverse within each virus strain, and a great variation existed between strains. With refinements in techniques, the biological qualities of each virus strain produced improved, and virus antigens most suitable for immunodiffusion experiments and for fractionation were selected (Table 5). Their biological activity ratios (EID_{50} : Protein, mg/ml; EID_{50} : HAU/ml) are found in Table 6.

Allantoic fluid and virus concentrate (C_2, C_2') antigens will be discussed first, followed by a detailed discussion of density gradient purified viruses.

Virus Infected Allantoic Fluids

Influenza A/PR8 and Duck infected allantoic fluids had the highest HA activity (10^4 HAU/ml), A2/Can/57 and Equi 1 had lower activity (10^3 HAU/ml), the other influenza A strains and B ranging between. Parainfluenza Sendai had very low HA activity (10^2 HAU/ml) (Table 5).

LEGEND - TABLE 5

HAU/ml	:	hemagglutinin units per ml using 0.05 ml virus volumes, 50% end point
$log_{10}EID_{50}/ml$	:	Reed and Muench (1938)
Protein mg/ml	:	determined by method of Lowry *et al.* (1951)
ALF	:	infected allantoic fluid
C_2	:	virus concentrated 100 x, *virus concentrated 200 x
SDG	:	Sucrose gradient purified virus
KTDG	:	Potassium tartrate gradient purified virus
NIL	:	no activity
ND	:	not done

TABLE 5. Hemagglutinin (HAU/ml), infectivity ($log_{10}EID_{50}$/ml), and protein content (mg/ml) in different preparations of influenza A strains, influenza B, parainfluenza Sendai, and N-ALF

Influenza Virus	Batch No.	HAU/ml				$log_{10}EID_{50}$/ml				PROTEIN mg/ml			
		ALF	C_2	SDG	KTDG	ALF	C_2	SDG	KTDG	ALF	C_2	SDG	KTDG
A/PR8	9	$1.28X10^4$	$1.02X10^6$	$1.02X10^6$	$1.02X10^5$	10	11.5	11	7.5	0.96	1.88	2.33	0.23
$A/FM_1/Can/53$	5	$6.4 X10^3$	$5.12X10^5$	$1.6 X10^4$	NIL	7.8	11	8.7		0.96	0.48	0.15	<0.05
A2/Can/57	6	$1.6 X10^3$	$*2.56X10^5$	$1.6 X10^4$	ND	8.5	*8.8	5		1.16	*0.78	0.51	ND
	7	$6.4 X10^3$	$*1.02X10^6$	$4.8 X10^4$	NIL	9	*9.5	7.5	4	1.56	*1.23	0.27	<0.01
A2/HK/1/68	3	$3.2 X10^3$	$*2.56X10^5$	$1.02X10^5$	NIL	8.5	*8.7	8	NIL	2.08	*0.91	<0.01	NIL
	4	$3.2 X10^3$	$*1.02X10^6$	$2.05X10^5$	NIL	7.6	*8.5	7.5	NIL	1.98	*1.44	0.17	<0.01
A/Equi 1	4,5,6	$1.4 X10^3$	$*1.6 X10^4$	$4 X10^3$	ND	7	*8	4.9		1.58	*0.44	0.2	ND
	7	$3.2 X10^3$	$*5.12X10^5$	$3.2 X10^4$	NIL	8.8	*9.8	9.5	4.2	1.44	*0.9	<0.01	<0.01
A/Swine	7	$6.4 X10^3$	$2.56X10^5$	$5.12X10^5$	$2.56X10^4$	9.3	10.3	8.7	6.3	1.14	0.83	0.47	0.23
A/Duck	3	$3.2 X10^3$	$1.28X10^5$	$6.4 X10^4$	$3.2 X10^4$	9.3	9.3	10.2	4.7	1.74	1.5	1.75	ND
	4	$6.4 X10^3$	$5.12X10^5$	$2.56X10^5$	$1.02X10^5$	10.6	10.3	10.3	3.3	1.52	2.05	0.9	0.17
	5	$1.28X10^4$	$2.05X10^6$	$1.02X10^6$	ND	9.5	9	9.5		1.46	0.75	1.0	
B/Can/5/66	1	$3.2 X10^3$	$1.28X10^5$	$1.28X10^4$	NIL	5.5	5.3	3.5	NIL	1.55	1.5	0.35	<0.03
Parainfl. Sendai	3	$8 X10^2$	$*2.56X10^5$	NIL	ND	8.5	*9.2	5.0	ND	0.74	*0.63		ND
N-ALF	2	NIL	NIL	NIL	ND	ND	ND	ND	ND	1.20	0.05	0.21	ND
	3	NIL	NIL			ND	ND			0.84	0.16		

LEGEND - TABLE 6

ALF	:	virus infected allantoic fluid
C_2	:	virus concentrated 100x; * 200x
SDG	:	sucrose density gradient purified virus
KTDG	:	potassium tartrate density gradient purified virus
EID_{50}/ml	:	infectivity in ovo per ml (Reed and Muench, 1938)
HAU/ml	:	hemagglutinating units per ml
Protein, mg/ml	:	Lowry _et al._ (1951)
Blank Spaces	:	one of values too low to detect, therefore ratio is meaningless and not recorded
N/A	:	not applicable

TABLE 6. Biological activity ratios of influenza A strains, influenza B, and parainfluenza Sendai

Influenza Virus	Batch No.	EID_{50} : Protein, mg/ml ALF	C_2	SDG	KTDG	EID_{50} : HAU/ml ALF	C_2	SDG	KTDG
A/PR8	9	$1.0X10^{10}$	$2.7X10^{11}$	$4.3X10^{10}$	$2.2X10^{8}$	$7.8X10^{5}$	$4.0X10^{5}$	$9.7X10^{4}$	$4.9X10^{2}$
A/FM 1/Can/53	5	$8.8X10^{7}$	$2.1X10^{11}$	$4.7X10^{9}$		$1.3X10^{4}$	$1.9X10^{5}$	$4.4X10^{4}$	
A2/Can/57	6	$*4.3X10^{8}$	$1.0X10^{9}$	$2.0X10^{5}$		$3.2X10^{5}$	$3.4X10^{3}$	6.3	
	7	$*6.4X10^{8}$	$4.1X10^{9}$	$1.9X10^{8}$		$1.6X10^{5}$	$4.9X10^{3}$	$1.1X10^{3}$	
A2/HK/1/68	3	$*2.4X10^{8}$	$1.1X10^{8}$			$1.6X10^{5}$	$2.7X10^{3}$	$9.7X10^{2}$	
	4	$*3.0X10^{7}$	$3.5X10^{9}$	$3.0X10^{8}$		$1.9X10^{4}$	$4.9X10^{3}$	$2.5X10^{2}$	
A/Equi 1	4,5,6	$*6.3X10^{6}$	$2.3X10^{8}$	$4.5X10^{5}$		$4.8X10^{3}$	$6.3X10^{3}$	$2.3X10^{5}$	
	7	$*6.1X10^{8}$	$8.8X10^{9}$			$2.5X10^{5}$	$1.7X10^{4}$	$1.6X10^{5}$	
A/Swine	7	$3.0X10^{8}$	$3.6X10^{10}$	$1.5X10^{9}$	$1.3X10^{7}$	$4.8X10^{5}$	$1.2X10^{5}$	$1.4X10^{3}$	$1.2X10^{2}$
A/Duck	3	$1.7X10^{9}$	$2.0X10^{9}$	$1.1X10^{10}$		$9.3X10^{5}$	$2.3X10^{4}$	$3.2X10^{5}$	2.2
	4	$4.0X10^{10}$	$1.5X10^{10}$	$3.3X10^{10}$	$1.8X10^{4}$	$9.6X10^{6}$	$6.0X10^{4}$	$1.2X10^{5}$	0.03
	5	$3.5X10^{9}$	$1.3X10^{9}$	$5.0X10^{9}$		$3.9X10^{5}$	$4.9X10^{2}$	$4.9X10^{3}$	
B/Can/5/66	1	$3.2X10^{5}$	$2.0X10^{5}$	$1.5X10^{4}$		$1.6X10^{2}$	2.3	0.4	
Parainfl. Sendai	3	$*7.0X10^{9}$	$8.0X10^{9}$	N/A		$6.5X10^{5}$	$7.8X10^{3}$	N/A	

Excluding influenza B ($log_{10}EID_{50}$=5.5), there was a 3½ log variation of infectivity among the viruses, Duck with the highest ($log_{10}EID_{50}$=10.6), and a pooled batch of Equi the lowest ($log_{10}EID_{50}$=7). The protein content showed a two-fold variation, PR8 infected ALF with the lowest (0.96 mg/ml), and A2/HK/1/68 with the highest (2.08 mg/ml) values. The variation in protein content between two batches of normal ALF (0.84 and 1.20 mg/ml) suggested that the batch of eggs used did influence the protein content of virus infected ALF. Biological activity ratios were highest with PR8, Duck, and Sendai infected ALF, followed by the A2 viruses, then Equi, Swine, and FM 1; influenza B had the lowest ratios (Table 6).

Infected allantoic fluids were used as a source of virus antigen for hemagglutination inhibition reactions throughout this study.

Virus Concentrates (C_2, C_2')

Concentration and partial purification of viruses from infected allantoic fluid by differential centrifugation yielded virus concentrates of varying biological activity. The A2 viruses, Equi, and Sendai required 200-fold concentration of infected ALF to produce satisfactory antigens for immunodiffusion (asterisked in Tables 5, 6, Appendix 1, Tables 3, 4, and underlined in Appendix 1, Table 2), all other virus strains required 100-fold concentration. The actual virus concentration achieved, as reflected by the values of biological characteristics, did not always correspond to the concentration factor of 100 or 200 times.

The most successful virus concentrate antigens for immunodiffusion, judged by the number and strength of precipitin bands formed in reactions

with homologous antisera (Tables 7, 8, 9, 14), were influenza A/PR8, Duck, A2/HK/1/68, followed by Swine, FM 1, B/Can/5/66, Sendai, and A2/Can/57. The weakest was A/Equi 1. The antigenic qualities of these virus concentrates were not always reflected by their biological activities.

The highest HA activity was demonstrated by PR8, Duck, and A2 viruses (10^5-10^6 HAU/ml), all others were grouped in a lower general level (10^5 HAU/ml), except an Equi pool which had the lowest activity (10^4 HAU/ml (Table 5). Excluding influenza B virus concentrate, the infectivities demonstrated a 3½ log variation, PR8 and FM 1 with the highest ($\log_{10}EID_{50}$ = 11.5 and 11 respectively), and a pooled batch of Equi with the lowest ($\log_{10}EID_{50}$ = 8) infectivity values. A greater than four-fold variation in protein content was demonstrated, Duck and PR8 with the highest (2.05 and 1.88 mg/ml respectively) and FM 1 and a pooled batch of Equi the lowest (0.48 and 0.44 mg/ml respectively), the other virus concentrates ranging between. Contaminating CE material could form as much as 1/3 of a virus concentrate (e.g. FM 1 or Equi pool) suggested by the values obtained with N-ALF concentrates. The evaluation of protein content is discussed below, in Density Gradient Purification of Viruses (p. 164).

Concentration of viruses generally increased, by at least a log, the biological activity ratios (EID_{50} : Protein, mg/ml) of all viruses but Duck, B, and Sendai (Table 6). The increase was often below expected values but this was attributed to lower actual concentrations attained than the concentration factor of 100 or 200 times, and to loss of activity in handling. However, some degree of virus purification was achieved at this stage with all viruses. EID_{50} : HAU/ml ratios generally dropped in

most cases, except those of FM 1 and an Equi pool, which demonstrated a slight rise. This ratio reflected the relative lability of virus biological activity, the infectivity, which is high with A2 viruses, Duck, B, and Sendai; and lower with the other A virus strains; PR8, FM 1, and Swine seemed the most stable.

Although a detailed study by electron microscopy of the viruses in this investigation was not attempted, negatively stained concentrates of each virus strain were examined by EM. All influenza viruses exhibited typical size, structure, and pleomorphism (Duck C_2 in Plate 10 A is a typical example), and differences amongst them were not obvious, except that A2/HK/1/68 preparations contained a higher proportion of filaments (Plate 10 B). Parainfluenza Sendai was characteristically larger, and contained typical RNP structures (Plate 10 C).

Antisera and Homologous Immune Reactions

Rabbit Immune Sera

Convalescent Intranasal infection of rabbits with influenza A strains, influenza B, and parainfl. Sendai yielded small or undetectable antibody responses (Table 7). Each strain of virus infected allantoic fluid used for infection was the maximum concentration obtained on serial passage in eggs. There was an eight-fold difference in HAU/ml content amongst the infecting virus strains and the infectivity, $\log_{10}EID_{50}$ varied from 7.8 to 8.9 for all strains but infl. B whose value was 5.5 (Appendix 1, Table 3, refer to earliest batch number of each strain).

LEGEND - TABLES 7, 8, and 9

[a]Vaccine HAU	:	Number of hemagglutinating units contained in intranasal dose of 0.25 ml virus infected ALF in 0.25 ml n-saline.
[b]HAI Titre	:	Homologous hemagglutination inhibition titre expressed as the reciprocal of the dilution. Sera RDE (*V. cholerae* filtrate) treated. See Tables 22 and 23 for homologous HAI titres of $NaIO_4$ treated sera. Blank spaces indicate HAI titre <10
[c]CF Titre	:	Complement fixation titre expressed as the reciprocal of the dilution using type-specific influenza A2/HK/1/68 soluble antigen. Only sera titrated are recorded.
[d]IDD Bands	:	Number of precipitin bands yielded in immunodiffusion with homologous virus concentrate (C_2, C_2') or N-ALF concentrate (C_2'). Blank spaces indicate no precipitation.
[e]Vaccine HAU	:	Number of hemagglutinating units contained in each parenteral dose (IM) of vaccine consisting of 0.5 ml virus or N-ALF concentrate (C_2, C_2') at 10^{-1} in 0.5 ml Freund's Complete Adjuvant.
[f]Protein mg/ml	:	estimated by method of Lowry *et al*. (1951).
[g]Serum Pools	:	Each antiviral serum pool consisted of equal volumes of listed individual sera. These were absorbed by N-CAM for use in immunodiffusion.
Asterisked Sera,	*	in Tables 7 and 9, were those used for immunodiffusion experiments chosen from two or more available.
ND	:	Not done.

TABLE 7. Rabbit immunization : Convalescent sera (RAs-IN)

Immunizing Antigen	Animal No.	Vaccine [a]HAU	Incubation Weeks	[b]HAI Titre	[c]CF Titre	[d]IDD Bands
Infl. A/PR8	A1	$1.6x10^3$	3.5			
	*A3	$1.6x10^3$	3.5	10	<5	1
	A4	$1.6x10^3$	3.5			
	A6	$1.6x10^3$	3.5			
Infl.A/FM1/Can/43	*A23	$8x10^2$	8.5	10	<5	2
	A24	$8x10^2$	8.5			1
Infl. A2/Can/57	*A29	$1x10^2$	8.5	20	<5	2
	A30	$1x10^2$	8.5			
Infl. A2/KH/1/68	*A46	$8x10^2$	7	20	10	2
	*A47	$8x10^2$	7	10	8	2
Infl. A/Equi 1	*A19	$2x10^2$	17		<5	1-2
	A26	$2x10^2$	8.5			
Infl. A/Swine	A7	$8x10^2$	3.5			1
	*A13	$8x10^2$	3.5	10	<5	1
Infl. A/Duck	*A27	$3.2x10^3$	9	20	20	2
	A28	$3.2x10^3$	9	10		2
Infl. B/Can/5/66	*A43	$8x10^2$	4	10		2
	A44	$8x10^2$	4			2
Parainfl. Sendai	A31	$2x10^2$	4			
	*A32	$2x10^2$	4	20		1-2
		[f]Protein mg/ml				
N-ALF	*A40	1.20	4		<5	
	A42	1.20	4			

TABLE 8 Rabbit immunization: Hyperimmune sera (hRAs), individual and pooled (N-CAM absorbed).

Immunizing Antigen	Animal No.	Vaccine [e]HAU	No. of Doses	[b]HAI Titre	[d]IDD Bands	[g]Serum Pools N-CAM abs. IDD Bands	CF Titre
Infl. A/PR8	A1	1.6×10^3	12	5120	7	5-7	80
	A3	1.6×10^3	12	10,240	10		
	A4	1.6×10^3	12	2560	7		
	A6	1.6×10^3	12	5120	7		
Infl. A/FM 1/Can/53	A23	8×10^2	11	1280	8	4	40
	A24	8×10^2	11	2560	6		
Infl. A2/Can/57	A29	2×10^2	11	10,240	6	3-4	40
	A30	2×10^2	11	5120	4		
Infl. A2/HK/1/68	A46	5.1×10^4	4	5120	6-7	3-4	40
	A47	5.1×10^4	4	2560	10-11		>128 (unabs.)
Infl. A/Equi 1	A19	4×10^2	11	320	2	2	10
	A26	4×10^2	11	320	2		
Infl. A/Swine	A7	1.6×10^3	11	5120	8	4-5	20
	A13	1.6×10^3	11	1280	7		
Infl. A/Duck	A27	1.6×10^3	11	1280	10	7-8	320
	A28	1.6×10^3	11	1280	7		
Infl. B/Can/5/66	A43	6.4×10^3	5	1280	7-8	3-4	ND
	A44	6.4×10^3	5	1920	11		
Parainfl. Sendai	A31	2×10^2	10	160	3	4	ND
	A32	2×10^2	10	320	5		
		[f]Protein mg/ml					
N-ALF	A40	0.16	4	<10	3	none	ND
	A42	0.16	4	<10	2		

TABLE 9. Rooster immunization : Hyperimmune sera (hFAs)

Immunizing Antigen	Animal No.	Vaccine [e]HAU	No. of doses	HAI [b]Titre	IDD [d]Bands
Infl. A/PR8	R1	$1.6X10^3$	4	40,960	4-5
	*R9	$1.6X10^3$	4	10,240	4
Infl. A/FM 1/Can/53	R3	$1.6X10^3$	4	60	2
	*R10	$1.6X10^3$	6	240	3
Infl. A2/Can/57	*R4	$4X10^2$	4	640	2-3
	R11	$1.6X10^3$	7	240	2
Infl. A2/HK/1/68	R17	$6.4X10^3$	4	640	4
Infl. A/Equi 1	R12	$8X10^2$	6	10,240	2-3
Infl. A/Swine	R7	$1.6X10^3$	4	1280	3
	*R13	$1.6X10^3$	5	2560	3-4
Infl. A/Duck	R14	$1.6X10^3$	6	320	5-6
Parainfl. Sendai	R15	$4X10^2$	6	640	4
		[f]Protein mg/ml			
N-ALF	R6	0.16	4	<10	none

HAI titres of RDE (V. Cholerae filtrate) treated sera were 1/10 and 1/20. The HAI titre obtained depended more on time of incubation than the dose of the infecting virus. One to two precipitin bands were formed by all sera with HAI titres when they were diffused with homologous virus concentrate (C_2, C_2'). Four convalescent sera (A/FM 1, Equi, Swine, B) lacking HAI titres, formed one to two precipitin bands in homologous immunodiffusion reactions.

Hyperimmune Continued immunization of each rabbit parenterally (IM), with the same virus strain as a concentrate vaccine in Freund's Complete Adjuvant, produced a marked increase in HAI antibody response to most viruses except A/Equi and Sendai. All sera were V. cholerae RDE treated (Table 8). The low HAI antibody response to the latter viruses may have been due to the low hemagglutinin content of the immunizing vaccines. However, A2/Can/57 with equivalent low HA content induced a very high HAI antibody response tantamount with that of Rabbit A3 immunized by A/PR8 with eight times as much hemagglutinin. This suggests that the antibody response is influenced by the immunizing virus strain and the individual animal. The levels of HAI antibody in rabbits differed in most cases; HAI antibody levels in rabbits immunized by A/Equi and by Sendai were similar, probably a coincidence. The number of immunizing doses listed in Table 8 are the total administered at 2 week intervals. The high number of doses (10 - 12) in many cases were a result of an unpredicted interruption in the immunizing schedule which had to be repeated.

Each hyperimmune serum, in homologous immunodiffusion reactions with virus concentrates (C_2, C_2') formed precipitin bands, which varied

in number between animals immunized with the same virus strain, and with different virus strains. Antisera of rabbits immunized by PR8, A2/HK/1/68, Duck, and B/Can/5/66 exhibited the greatest number of precipitins (10-11), those immunized by Equi and Sendai the lowest (2-5), the others ranging between (Table 8).

The precipitin bands formed were virus-and host-specific (see Differentiation of Host-and Virus-Specific Immunoprecipitin Reactions). Pooled huperimmune sera of each virus strain were absorbed by N-CAM to remove host (CE)-specific precipitins (resulting in a 1:2 - 1:3 dilution of serum). In homologous reactions with virus concentrates, the absorbed sera formed fewer bands, but all those formed were virus-specific (Tables 8, 14). A discussion of this is to be found in Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions; examples of absorption of anti-Duck and anti-FM 1 hyperimmune rabbit sera are illustrated in Plate 4 A, B.

The correlation between HAI titres of hyperimmune rabbit sera and number and strength of precipitin bands formed in homologous reactions, was irregular; high HAI titre anti-PR8 and anti-Swine antisera formed many bands (5-7 and 4-5 respectively), lower HAI titre anti-Duck antiserum formed as many or more (7-8), but low HAI titre anti-Equi antiserum formed few (2) (Table 8).

Homologous Immunoprecipitin Reactions The immunization of each rabbit produced antisera, which, in homologous immunodiffusion reactions with virus concentrate (C_2, C_2'), formed precipitin bands that increased in strength and number as the immunization progressed. A precipitin

pattern unique to each animal developed. Two examples are demonstrated in Plate 1 A, B.

Rabbit A47 contained a weak A2/HK/1/68 precipitin (see Final Selection of Normal Animal Sera Containing Influenza A Antibodies). The same component (identified by precipitin band linkage, (Plate 1 A, arrow 1) progressively increased in convalescent serum,and,as immunization continued, reached maximum strength in the hyperimmune serum. The precipitin band formed was virus-specific because it did not form part of the reaction between host-specific antiserum (hRAs/N-ALF u) and the virus, but it linked in identity with the virus-specific reaction formed between convalescent serum and the antigen (see Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions). The number of precipitin bands formed increased from 2 by convalescent serum, to 10 by hyperimmune serum. A host (CE)-specific precipitin resulting from parenteral immunization (arrow 2) was identified by its precipitin band linkage with a band formed between host-specific serum and the virus.

A similar pattern was developed when Rabbit A13 was immunized with influenza A/Swine (Plate 1 B). An increase in the number and strength of precipitin bands was noted as the animal was progressively immunized, from one band in convalescent serum to 7 in hyperimmune serum. A strong virus-specific immunoprecipitin reaction of the virus with convalescent serum (which contained only virus-specific antibody, arrow 1) increased in a reaction with hyperimmune serum. The specificity of the other precipitin bands formed was not determined as a host-specific antiserum control was not included. A precipitin in normal serum identified with one in hyperimmune serum (by precipitin band linkage, arrow 2), but was not present in convalescent serum.

Rooster Immune Sera

Hyperimmune Parenteral immunization of roosters with influenza A strains, and parainfl. Sendai was less successful than immunization of rabbits (Tables 9, 14). Although the hemagglutinin content of immunizing antigens was generally greater and more even, the HAI antibody response was generally lower and of greater range than that of the rabbits. Because the first group of birds immunized (R1 - R7) responded poorly and were debilitated on caging, a second group of birds (R9 - R17) was immunized.

Immunization with A/PR8 and Equi induced the highest HAI titres (all sera were V. cholerae filtrate treated);moderate titres were induced by Swine;and the lowest titres by FM 1 and A2/Can/57. Intraperitoneal immunization could have increased the antibody response (Greenham and Harber, 1971), and will be applied in future.

Similar to the rabbits, the HAI antibody response did not always reflect the strength of the vaccine administered. The HAU content of PR8, FM 1, A2/Can/57, Swine, and Duck vaccines were the same ($1.6x10^3$), but the HAI antibody responses varied in titre from 1/40,960 - 1/320 (Table 9).

The homologous precipitin responses of hyperimmune rooster sera were less varied than those of rabbit antisera, resembling more closely the virus-specific precipitin responses of N-CAM absorbed sera. The immuno-precipitin reactions of hyperimmune rooster sera with virus concentrate antigens in immunodiffusion were considered to be virus-specific (see Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions). Again, the vaccine dosage was not reflected in the number of precipitins produced by each serum.

A correlation between HAI titres of each antiserum and the

number of precipitin bands they formed in homologlus reactions with virus concentrates was not consistent.

The varied individual antibody response was influenced by the strain of immunizing virus and by the individual bird.

<u>Homologous Immunoprecipitin Reactions</u> of virus concentrates with parenterally immunized roosters were somewhat weaker on the whole than those of rabbits. As each bird was progressively immunized, precipitin bands yielded by the serum, increased in strength and number, producing a characteristic immunoprecipitin pattern with the immunizing virus. Two examples are demonstrated in Plate 1 C, D.

Normal rooster serum (NFS) R1 did not contain any PR8 precipitins (Plate 1C). As the bird was immunized, the number of precipitin bands formed between the serum and the virus concentrate increased from 2 in early stages (FAs-IM) to 4-5 in hyperimmune serum (hFAs). The early precipitins formed increased in strength in hyperimmune serum (indicated by precipitin band linkage, arrowed).

The precipitin response of NFS R4 immunized by A2/Can/57 was not so marked as that of R1 (Plate 1 D), Normal serum contained a component which, with A2/Can/57 virus concentrate, formed a precipitin band cutting the one formed between the virus and hyperimmune serum in non-identity (arrow 1), but linked in identity with another faint one (arrow 2). The component in normal serum was very suggestive of an inhibitor, but could also have been an antibody (see Part II); this will have to be determined. Precipitins resulting from immunization were detected only in hyperimmune sera and numbered two.

Using the above mentioned rabbit and rooster antisera, homologous immune reactions detected by hemagglutination inhibition and immunoprecipitin tests, were established, and helped to form the basis upon which the cross reactions between viruses were interpreted.

Stereomicroscopy of Immunodiffusion Reactions in Cellulose Acetate

During the examination of immunodiffusion reactions in cellulose acetate by the stereomicroscope, it was observed that the immunoprecipitin reactions did not lie in one horizontal plane, but were distributed at varying levels beneath the surface of the acetate strip. The effect was a three-dimensional one, which was most surprising because the descriptions of immunoprecipitin reactions in the literature imply that the reactions lie in one horizontal plane.

It was also observed that the immunoprecipitin reactions were not "lines" as they are commonly referred to, but resembled ribbons or bands, which are inclined at varying angles from the vertical. Because of this, immunoprecipitin reactions are referred to in this thesis as "bands", not as "lines".

As a simple example, the immunoprecipitin reactions resulting from the diffusion of A/Duck purified concentrate and soluble antigen against homologous immune sera (RAs-IN, hRAs abs, hFAs) and against anti-host immune sera (hRAs/N-ALF, hFAs/N-ALF) (Plate 9C) is schematically drawn in Figure 5.

The strongest band (arrows 2) lies at the highest level, and crosses over a weaker band (arrows 1). Beneath both these bands, lies a third band (arrow 3). Near the well containing hFAs/Duck, is a broad diffuse band (arrow 4, stippled in Fig. 5), which slopes towards, and lies

under, bands 1 and 2.

Similar observations with the stereoscopic microscope were used in interpreting all immunodiffusion reactions in this thesis. From these, it is suggested that antigen and antiserum components diffuse along different paths in the cellulose acetate, in three dimensions rather than in two. Each class of immunoglobulin (IgG, IgA, IgM), because of its size, could diffuse at a different level from the other. Similarly, each antigen, because of its size, could also diffuse at different levels. Thereby the number of potential interactions which can take place betweeen antigen and antibody molecules is greater than if diffusion occurred only at one level.

When the reacting serum and antigen components are characterized by immunodiffusion in cellulose acetate, it will be possible to make a more definite interpretation of this phenomenon.

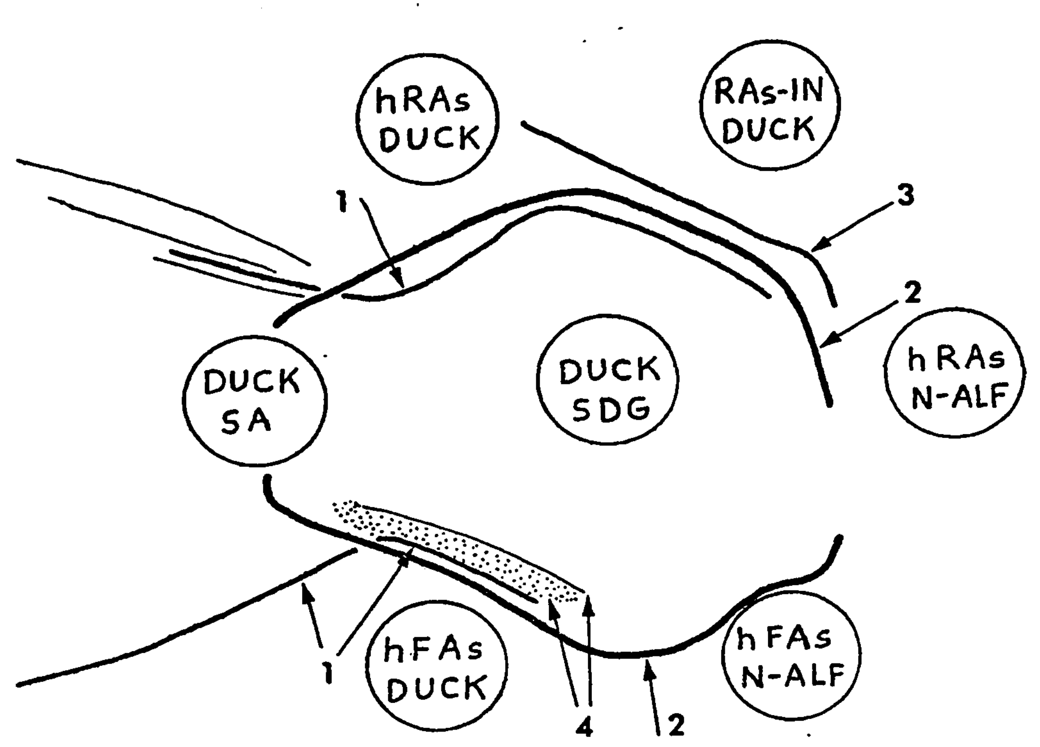


Figure 5. Schematic diagram of the homologous immunoprecipitin reation of A/Duck as viewed by stereomicroscope

(See Appendix 3, p 472 and Plate 9 for abbreviations)

Immunodiffusion Control Experiment

Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions

Animals were immunized with virus concentrate (V/C_2 and V/C_2') and not with density gradient purified virus, therefore the antiserum produced contained not only antibodies to virus, but also to associated host material, that is, to chick embryo (CE). Because both virus-specific and host (CE)-specific antibodies reacted with their homologous antigens to form precipitin bands in immunodiffusion reactions, a control system was used to differentiate them. Two testing systems were carried out:

I. Paralleling the pattern of virus immunization of animals, an immune host (CE)-specific system was developed. Normal chick allantoic fluid (N-ALF) was inserted intranasally, to produce 'convalescent' rabbit serum (RAs-IN/N-ALF), and N-ALF/C_2 parenterally (IM), to produce hyperimmune rabbit serum (hRAs/N-ALF), and hyperimmune rooster serum (hFAs/N-ALF) directed against chick embryo (CE) antigens, corresponding to those in the appropriate infected preparations. Using the antisera produced and the antigens N-ALF/C_2,/C_2', and N-CAM/SA, the antibody response of these animals to host (CE) material could now be determined by precipitin patterns developed in immunodiffusion. These precipitin patterns were notated host (CE)-specific. Host (CE) control experiments were done

independently, parallel to, and in conjunction with virus-specific immunodiffusion reactions, always under the same conditions.

2. Virus antigens V/C_2, V/C_2', V/SDG, V/SA, and antiviral sera RAs-IN, hRAs/V (abs), hFAs/V, were tested with the above mentioned host (CE)-specific antigens and antisera in immunodiffusion reactions, to detect the presence of host (CE)-specific components, and to determine their relationship to virus-specific precipitin reactions.

The comparison of host (CE)-specific and virus precipitin patterns by immunodiffusion helped to elucidate virus-specific precipitin reactions within the limits of sensitivity of immunodiffusion in cellulose acetate.

The results of the discussion following are summarized in Table 10.

Rabbit Antisera 'Convalescent' sera of rabbits, intranasally exposed to chick N-ALF (RAs-IN/N-ALF), or to live virus as infected allanotic fluid (RAs-IN/V), on diffusion with N-ALF C_2 or with N-CAM/SA, did not form visible precipitin bands, suggesting they did not contain detectable host (CE)-specific antibodies. The lack of precipitin reactions is demonstrated between 1) N-ALF/C_2 (centre row) and convalescent rabbit sera to various influenza A strains, B, and parainfl. Sendai (top row) in Plate 2A, 2) N-CAM/SA (centre row) and the same group of convalescent rabbit sera as in (1) (bottom row) in Plate 2B. Precipitin reactions were absent on immunodiffusion of RAs-IN/N-ALF against virus concentrates or soluble antigens, reaffirming the conclusion, that, intranasal exposure of rabbits to host (CE) material (N-ALF) did

LEGEND - TABLE 10

RAs-IN/N-ALF	:	Convalescent serum of rabbit intranasally exposed to chick normal allantoic fluid (N-ALF)
hRAs/N-ALF	:	Hyperimmune serum of rabbit parenterally immunized with N-ALF
hRAs/N-ALF abs	:	Hyperimmune serum of rabbit parenterally immunized with N-ALF, absorbed with N-CAM, and contains residual N-CAM components
hFAs/N-ALF	:	Hyperimmune serum of rooster parenterally immunized with N-ALF
RAs-IN/Virus	:	Convalescent sera of rabbits intranasally infected with virus infected allantoic fluid
hRAs/Virus	:	Hyperimmune sera of rabbits parenterally immunized with virus concentrate (V/C_2)
hRAs/Virus abs	:	Hyperimmune rabbit sera as above, but absorbed with N-CAM, and contain residual N-CAM components
hFAs/Virus	:	Hyperimmune sera of roosters parenterally immunized with virus concentrate (V/C_2)
Virus/C_2	:	Virus concentrate
Virus/SA	:	Virus soluble antigen

Only homologous precipitin reactions are considered with viruses

Numbers in parentheses under + indicate number of host (CE)-specific precipitin bands

- : absence of precipitin bands

ND : not done

TABLE 10 Immunodiffusion host (CE)-specific control reactions

Antiserum	Antigens					
	N-ALF/C_2	N-CAM/SA	hRAs/N-ALF abs	Virus/C_2	Virus/SA	hRAs/Virus abs
RAs-IN/N-ALF	-	-	-	- +[c]	-	-
hRAs/N-ALF	+ (2-4)	+ (3-5)	+ (2)	+ (1-2)	ND	+ (2-3)
hRAs/N-ALF abs	-	-	-	- +[d]	-	ND
hFAs/N-ALF	-	- + (1)[a]	(±)[b]	-	- + (1)[e]	ND
RAs-IN/Virus	-	-	-	+	+	-
hRAs/Virus	+ (≥1)	ND	+	+	ND	+ (≥2)
hRAs/Virus abs	-	-	ND	+	+	-
hFAs/Virus	-	-	+ (1)	+	+	+ (1-3)[f]

[a] Faint precipitin reaction recorded <u>once</u> when hFAs/N-ALF located next to strongly reacting hRAs/N-ALF.

[b] One precipitin band produced only with absorbed hRAs/N-ALF, not with unabsorbed, therefore, not an interspecies reaction.

[c] Reaction shown to be RDE sensitive inhibitor reacting with influenza A2/Hong Kong/1/68 and not antibody.

[d] Faint reaction shown to be RDE sensitive inhibitor reacting with influenza A2/Hong Kong/1/68 and not antibody.

[e] Faint precipitin band produced on 25-well template, occasionally on 7-well. No reaction with Duck/SA, B/SA, Sendai/SA.

[f] hFAs/Virus versus residual N-CAM in hRAs/Virus abs: all strains but A2/Can/57, A2/HK/1/68, and A/Equi produce 1-3 bands.

not stimulate the production of perceivable host (CE)-specific serum antibodies, whereas intranasal infection of rabbits with live viruses induced the production of virus-specific antibodies. However, a precipitin band was formed between RAs-IN/N-ALF and influenza A2/Hong Kong/1/68 C_2' (Plate 3A), which disappeared when the serum was RDE treated suggesting that RDE sensitive A2/Hong Kong/1/68 inhibitor, commonly found in rabbits, was responsible for the reaction (see Part II, Inhibitor Review, and Results, Immunodiffusion and Inhibitors). The precipitin, barely detectable in the normal and hyperimmune (N-ALF) sera of this rabbit, disappeared on RDE treatment. On the basis of the evidence presented, precipitin bands produced by immunodiffusion between RAs-IN/V and virus antigens were considered to be virus-specific.

Hyperimmune rabbit serum to normal allantoic fluid (hRAs/N-ALF) contained antibodies to 3 components in N-ALF/C_2 and up to 5 components in N-CAM/SA (Plate 3B, C). Of these, two components were common to both CE substances, as demonstrated by precipitin band linkage (Plate 3B, arrows 1 and 2).

All detectable host (CE)-specific antibodies were removed when hRAs/N-ALF was absorbed with N-CAM, as illustrated by the absence of reaction between absorbed serum and N-ALF/C_2 and N-CAM/SA (Plate 3B, C). However, at least three residual normal chorioallantoic membrane antigen components were left in absorbed serum, two of which reacted with antibodies in unabsorbed serum (Plate 3B, arrows 1' and 2'). These two were identified as N-CAM components by precipitin band linkage with those produced between N-CAM/SA and hRAs/N-ALF (arrows 1 and 2). A precipitin reaction occurred between V/C_2 or V/SA (for example with Duck C_2 in

Plate 4 A, E-LINK arrow) and unabsorbed hRAs/N-ALF, but not between these antigens and absorbed hRAs/N-ALF. The absence of precipitates between hRAs/N-ALF (abs) (top row) and soluble antigens of influenza A strains, B, and parainfl. Sendai (centre row) is demonstrated in Plate 2 C. These observations further strengthened the conclusion that adequately absorbed serum did not contain host (CE)-specific antibodies detectable by immunoprecipitin reactions.

Similarly, hyperimmune antivirus rabbit sera (hRAs/V) contained precipitating antibodies which were virus-specific and CE-specific (Plate 4 A, B); representative reactions of these are arrowed (V arrow, E arrow respectively). Upon adequate absorption of hRAs/V with chick N-CAM (wet or lyophilized), detectable host (CE)-specific antibodies were removed, leaving only virus specific antibodies. Typical reactions of unabsorbed and absorbed sera are illustrated by precipitin reactions with influenza A/FM1 (Plate 4B) and influenza A/Duck (Plate 4A). Note that precipitin bands produced by absorbed serum do not link with host (CE)-specific reactions, but those produced by unabsorbed serum do so. Precipitin bands formed by absorbed serum link with those formed by convalescent rabbit serum (V-LINK arrow), concluded from the discussion above to be virus-specific, and identifying the absorbed serum bands as virus-specific. All adequately absorbed hRAs/V sera used in this study did not contain detectable host (CE)-specific antibodies in immunodiffusion reactions with N-ALF/C_2 (Plate 2A) and N-CAM/SA (Plate 2B). In addition, each absorbed serum contained up to 7 or more residual N-CAM components which reacted with host (CE)-specific antibodies in unabsorbed serum (Plates 4A,B , arrowed E). Within the limits of sensitivity of immuno-

diffusion in cellulose acetate, precipitin reactions between adequately N-CAM absorbed hyperimmune antivirus rabbit sera (hRAs/V abs) and virus antigens were considered to be virus-specific.

Adequate absorption of sera was obtained with wet or lyophilized N-CAM, but not with acetone-dried N-CAM.

Rooster Antisera Roosters were chosen to be immunized with chick embryo (CE) grown virus because it was hoped that by containing the immune system within the species, antibodies produced would be virus-specific, and few, if any, host (CE)-specific. As with rabbit antivirus serum, it was important to identify virus-specific precipitin bands in the immunodiffusion reaction, and to identify, if not eliminate, host (CE)-specific ones.

The hyperimmune serum produced by parenteral immunization with N-ALF/C_2 (hFAs/N-ALF) was checked in numerous immunodiffusion experiments for the presence of egg-specific antibodies using N-ALF/C_2 and N-CAM/SA as host antigens. Precipitin reactions were not observed with either antigens (Plate 3B). Switching of relative positions of antigen and antiserum did not alter the negative reaction. CE-grown virus concentrates did not react with hFAs/N-ALF, but did react in homologous and cross hFAs/V reactions. But, host (CE)-specific antibody in hFAs/N-ALF was detected occasionally: once in a very faint reaction with N-CAM/SA (arrowed, Plate 3C), next to the strong response of host (CE)-specific hyperimmune rabbit serum; and when positioned next to hRAs/N-ALF (abs), which contained residual N-CAM components. It never occurred when hFAs/N-ALF was situated next to unabsorbed hRAs/N-ALF, indicating that it was not an interspecies reaction.

Immunoprecipitin reactions did not occur on immunodiffusion of virus concentrates against hFAs/N-ALF. However, when virus soluble antigens of influenza A strains, B, and parainfl. Sendai (centre row) were diffused with hFAs/N-ALF (bottom row), a faint precipitin band appeared, linking each virus strain except Duck/SA, B/SA, and Sendai/SA (Plate 2C). The common component in each virus type was presumed to be CE material as the lack of reaction with Duck/SA ruled out the possibility of virus-specificity.

There was complete absence of precipitin reactions on immunodiffusion of hyperimmune antivirus rooster sera (hFAs/V) against N-CAM/SA.

In experiments of viruses versus homologous hyperimmune rooster and absorbed rabbit sera, one to three precipitin bands were produced between the sera when they were located next to each other. All but A2/Can/57, A2/HK /1/68, A/Equi 1 exhibited this reaction. This suggested that the A2 and Equi 1 viruses did not carry as much host (CE) material in the concentrate form as other virus types in this study, hence immunized roosters had a lower host (CE)-specific antibody level, and thus reaction with residual N-CAM components in absorbed hRAs was absent.

Immunization of roosters with CE grown virus did not guarantee absence of anti-host antibody. If CE grown virus concentrates (V/C_2) were used in immunodiffusion reactions with hyperimmune antivirus rooster sera (hFAs/V), precipitin reactions produced were considered to be virus-specific, but if the soluble antigen form of virus was used (V/SA), faint CE-specific reactions could occur and careful interpretation of results was required to determine which reactions were virus-specific.

Because viruses purified by density gradient (V/SDG) were shown to contain host (CE) material, though less than semi-purified virus concentrates (V/C_2) (see Density Gradient Purification of Viruses and N-ALF, and Virus Fractionation by Polyacrylamide Gel Electrophoresis), the above discussion of differentiation of virus-specific from host-specific immunodiffusion reactions can be applied and interpreted in the same way as with semi-purified virus concentrates (V/C_2).

Differentiation of Antibody- from Inhibitor-Specific Immunoprecipitin Reactions

When normal animal sera were examined for the presence of influenza antibodies by immunodiffusion in cellulose acetate, it was observed that many precipitin reactions with virus concentrates were due to inhibitors, components of animal sera, which, much like antibodies, react with the envelope antigens of influenza viruses (Part II, Effect of Inhibitor Inactivation Treatments on Precipitins). Inhibitors rise in titre during disease and on immunization of animals with influenza viruses (Part II, Inhibitor Review, Summary and Discussion). Therefore, the immune sera of rabbits and roosters specifically immunized in this study could contain, in addition to specific antibodies, inhibitors of the α, β, or γ type which precipitate influenza viruses. Virus precipitation by the latter two inhibitors would be most commonly encountered, as α inhibitors form unstable precipitates with live influenza viruses, which dissolve on continued incubation (Svedmyr, 1949 a, b); both are conditions of immunodiffusion experiments. The presence of inhibitors could confuse the interpretation of immunoprecipitin reactions between animal antisera (antibodies) and virus antigens occurring as homologous and cross reactions.

Preliminary Experiment A preliminary experiment was carried out to determine whether the immune sera of rabbits and roosters (infected and/or immunized with influenza A/PR8, FM 1/Can/53, A2/Can/57, A2/Hong Kong/1/68, A/Equi 1, Swine, Duck, influenza B/Can/5/66, parainfl. Sendai,

and N-ALF) contained inhibitors which, along with true antibodies, precipitated virus antigens in homologous immunodiffusion reactions.

Sera were treated with V. cholerae filtrate RDE plus heat (58° Cx1 hr) which eliminated α and β inhibitors, or with $NaIO_4$ which eliminated α and γ inhibitors. The final dilution of sera after treatment was 1:5, therefore a control serum at 1:5 dilution was included for comparison.

Each serum, native, diluted 1:5, and treated with V. cholerae RDE or with $NaIO_4$, was diffused with its homologous virus antigen as concentrate (V/C_2 or $/C_2$'), and as soluble antigen (V/SA). The latter antigen was included because reactions of inhibitors with soluble antigens have not been encountered and are not believed to occur. Typical template patterns are demonstrated in Plate 5A,B,C .

In diffusion with virus antigens, the precipitin bands formed at 1:5 serum dilution were used as a base; any precipitin bands eliminated by V. cholerae RDE and /or by $NaIO_4$ treatment could have been inhibitor initiated; all remaining bands were considered to be antibody initiated.

Observations Because a specific analysis was considered to be premature at this stage, only preliminary observations were made. Analysis of precipitin reactions between antisera treated in various ways and virus concentrates (V/C_2, including C_2') or soluble antigens (V/SA) (Table 11, Plate 5) revealed the following:

Undiluted Sera : A greater than usual number of precipitin bands were formed in immunodiffusion between virus concentrate antigens and the following sera: convalescent rabbit

LEGEND TABLE 11

1:5	:	1:5 dilution of antiserum in PBS
RDE	:	V. cholerae filtrate RDE treated antiserum
$NaIO_4$	:	$NaIO_4$ treated antiserum
RAs-IN	:	convalescent serum of rabbit intranasally infected with virus or intranasally immunized with N-ALF
hRAs abs	:	hyperimmune serum of rabbit parenterally immunized or N-ALF and absorbed with N-CAM
hFAs	:	hyperimmune serum of rooster parenterally immunized with virus or N-ALF
C_2	:	virus concentrate antigens or N-ALF concentrate antigen
SA	:	virus soluble antigens or N-CAM soluble antigen
Blank spaces	:	negative reaction recorded by absence of precipitin bands
ND	:	not done

TABLE 11. Effect of inhibitor inactivation treatments of immune animal sera on homologous immunodiffusion reactions.

Influenza Antiserum	No. Precipitin Bands Formed with Homologous C_2				with Homologous SA			
	Native	1:5	RDE	$NaIO_4$	Native	1:5	RDE	$NaIO_4$
A/PR8:								
RAs-IN								
hRAs abs	5-6	3-4	2-3	1-3	2	2	2	2
hFAs	4-5	3-4	3-4	3-4	2	2	2	2
A/FM 1/Can/53:								
RAs-IN	1				1			1
hRAs abs	4	2	1	1	2	1	1	1
hFAs (1:2)	3	1	1	1	1	1	1	
A2/Can/57:								
RAs-IN	1				1			
hRAs abs	4-5	4	2	2	1	1	1	1
hFAs	5	3	3	2	3	3	4	3
A2/Hong Kong/1/68:								
RAs-IN	3	2	1	1	1			
hRAs abs	4	3	2	2	1	1	1	1
hFAs	4	3	2	2	1	1	1	1
A/Equi 1:								
RAs-IN	2				1			
hRAs abs	1	1	1		1			
hFAs	3	2	3	2	3	3	3	4
A/Swine:								
RAs-IN	1	1	1					
hRAs abs	4	3	3	2	3	1	1	1
hFAs	3	2	2	3	2	2	2	2
A/Duck:								
RAs-IN	4	3	2	2-3	3	2		2
hRAs abs	9	4	5	3	4	4	2	2
hFAs	6	4	4	2	3	2	2	3
B/Can/5/66:								
RAs-IN	3-4	1	1	1	1	1	1	2
hRAs abs	ND	2	3		1	1	1	1
P. Sendai:								
RAs-IN	2	2	1	2				
hRAs abs	5	1	2	2	1			
hFAs	5	3	3	2	1	1	1	1
N-ALF:								
RAs-IN								
hRAs abs								
hFAs			1		1		1	

against A2/HK/1/68, A/Duck, and B/Can/5/66; hyperimmune rabbit (abs) against A/Duck and Sendai; hyperimmune rooster against A2/Can/57 and Sendai. All extra bands formed were weak ones. Reactions with convalescent sera were, on the whole, weak, as was the reaction with Equi hyperimmune rabbit serum (abs).

1:5 Dilution

and V/C_2

Elimination or reduction in the number of weaker precipitin bands was noted in almost all sera except Swine and Sendai convalescent rabbit, and Equi hyperimmune rabbit (abs). Two major bands, one, identified as type -specific (Plate 5A,B,C arrow 1) and the other suspected of being neuraminidase-specific (because it resembled the neuraminidase-specific reaction described by Schild and Pereira, 1969) (Plate 5A,B,C arrow 2), were usually not affected. However, dilution of Sendai hyperimmune rabbit serum (abs) resulted in the absence of one major band, and dilution of FM 1 hyperimmune rooster serum eliminated the suspected neuraminidase-specific band (Plate 5C).

and V/SA

Dilution of most hyperimmune rabbit sera (abs) and rooster sera did not eliminate the major type-specific band formed (Plate 5A,B,C , arrow 1), nor most of the minor ones; however, the weaker bands formed by FM 1 and Swine hyperimmune rabbit sera (abs), and by

Duck hyperimmune rooster serum were eliminated. The only reaction formed with Equi 1 and with Sendai hyperimmune rabbit sera (abs) vanished. Precipitin bands were eliminated or reduced in reactions involving the following convalescent rabbit sera: FM 1, A2/Can /57, A2/HK/1/68 (plate 5B), and Equi 1.

The effect of inhibitor inactivation treatments could not be determined on sera whose reactions were eliminated by 1:5 dilution

RDE

and V/C_2 : Precipitins forming two major bands with V/C_2 were not eliminated, but a variation occurred in the number of weaker bands formed (Plate 5A,B,C). A reduction in numbers was noted in all three types of A2/HK/1/68 sera (Plate 5B), in the convalescent rabbit serum against Duck and Sendai, and in hyperimmune rabbit sera (abs) against PR8, FM1/Can/53, and A2/Can/57. An increase in number occurred in the hyperimmune rabbit sera (abs) against Duck, B/Can/5/66, and Sendai, and in Equi hyperimmune rooster serum. The number of precipitin bands formed by all other sera remained unchanged. for example, hyperimmune rooster serum against PR8 (Plate 5A).

and V/SA The major type-specific band was not affected (Plate 5A,B,C arrow 1), but minor ones were altered in a few instances: for example, RDE treatment eliminated the reactivity of Duck convalescent rabbit serum and

reduced the number of bands formed by the hyperimmune serum, but increased the number of bands formed with A2/Can/57 hyperimmune rooster serum

$NaIO_4$

and V/C_2

The two major precipitin bands formed with V/C_2 were not affected in most instances (Plate 5A, B, C, arrows 1 and 2), but a variation occurred in the minor ones formed. The type-specific band (Plate 5C, arrow 1) was not formed by treated FM1 hyperimmune rooster serum; and all precipitin reactivity of influenza B and Equi hyperimmune rabbit sera, and of Swine convalescent serum was eliminated. After $NaIO_4$ treatment, fewer minor bands were formed by all three types of A2/HK/1/68 sera (Plate 5B), and by the hyperimmune rabbit sera (abs) against PR8, FM1, A2/Can/57, Swine, Duck, and by the hyperimmune rooster serum against A2/Can/57, Duck, and Sendai, but not against PR8 (Plate 5A). In two cases, one extra band was formed, by Sendai hyperimmune rabbit serum (abs), and by Swine hyperimmune rooster serum.

and V/SA

The component forming the major type-specific band survived in the majority of cases (Plate 5A, B, arrow 1). It was eliminated in reactions with FM1 hyperimmune rooster serum (Plate 5C). Minor precipitin band formation was altered in several cases: fewer bands

were formed in reactions with Duck hyperimmune rabbit serum (abs); an increase in the number of bands formed was observed in reactions with B convalescent rabbit serum, and with Equi and Duck hyperimmune rooster serum. All precipitin reactions with the other $NaIO_4$ treated sera remained unchanged.

Interpretation Most hyperimmune rabbit and rooster sera and many convalescent rabbit sera used in this study possessed weakly precipitating components, which were probably non-specific inhibitors because: they precipitated virus particle concentrate components which contained envelope antigens, and they were eliminated by inhibitor inactivation treatments. Stronger serum components which precipitated two of the major antigens, the type-specific (Plate 5 A,B, arrow 1), and possibly neuraminidase (Plate 5 A,B, arrow 2), usually survived these treatments, and were considered to be antibody. Supporting this observation is the demonstration that the serum component precipitating the type-specific antigen is a 7 S gamma-globulin (see Part II, Results, Identification of Influenza Antibodies in Immune Preparations, and Plate 26).

The reactions of immune sera with virus soluble antigens were not generally affected by inhibitor inactivation treatments. This fact supports the evidence that type-specific antigen is precipitated only by antibody (7 S gamma-globulin) and not by inhibitor.

In instances when the stronger serum components precipitating two of the antigens described above were eliminated, the precipitates they formed with virus concentrate or virus soluble antigen, before

being treated for inhibitors, were usually weak; for example, the reactions formed by FM 1 hyperimmune rooster serum (Plate 5C) and Equi hyperimmune rabbit serum (abs). Inhibitor inactivation treatments are known to attack serum antibody (Part II, Inhibitor Review, pp.285;& 350). Therefore, the inability of treated sera to precipitate influenza virus concentrate or soluble antigens, known to be precipitated by antibodies, suggested that these treatments lowered the active antibody to a level not detectable in immunodiffusion reactions. This is proof that influenza virus antibodies can be adversely affected by V. cholerae filtrate RDE and $NaIO_4$.

Evidence of the alteration of antibody by V. Cholerae filtrate RDE and by $NaIO_4$ were the observations that, in immunodiffusion, sera treated by the former substance formed split precipitin bands with suspected viral neuraminidase (as occured with Duck hyperimmune rooster serum), and sera treated by the latter substance could form extra precipitin bands with viral soluble antigen (as observed with Equi and Duck hyperimmune rooster serum). These observations suggested that an additional component in the serum, presumably antibody, had been activated which could precipitate influenza virus antigen. The identity of the activated component has not been established. In reactions with virus concentrates, extra precipitin bands produced by treated sera could be due to activation of different inhibitor types by the treatments as described by Levinson et al. (1969) (Part II, Inhibitor Review).

This preliminary experiment demonstrated that homologous immunoprecipitin reactions of virus soluble antigens with the antisera of animals immunized with influenza A strains, B, or parainfl. Sendai, were antibody-initiated, especially the type-specific reaction, and could be interpreted

as such in homologous and cross reactions.

However, homologous immunoprecipitin reactions between the above sera and virus concentrates were a mixture of antibody- and inhibitor-initiated reactions. Antibody was responsible for the strong precipitation of two of the major antigens, the major type-specific and the suspected neuraminidase. Although the serum precipitins weakly reacting with other components of virus concentrates have been generally grouped together as antibody or inhibitor, the precipitin reactions they formed with viral components have not as yet been differentiated. Further detailed studies are required to do this (see Part II, Summary and Discussion). Therefore, immunoprecipitin reactions, either homologous or cross, involving virus concentrate antigens should be interpreted with the above facts in mind.

Disruption of Virus Antigens for Immunodiffusion

Influenza virus particles in agar gel immunodiffusion reactions are disrupted to facilitate the diffusion of viral antigens through the agar. Surface active agents such as sodium dodecyl sulfate (SDS) or sodium deoxycholate (DOC) are very commonly used (Hana and Hoyle, 1966; Easterday et al., 1969; Schild and Pereira, 1969; Schild, 1970; Styk et al., 1970a,1971a).

Various disrupting methods were applied in this study to increase the number of antigens detected in homologous and cross precipitin reactions of influenza virus concentrates (semi-purified - C_2 or sucrose density gradient purified - SDG), with corresponding immune sera.

Sonication Disruption of influenza A/Duck concentrate by sonication for up to 20 minutes did not appreciably improve or expand the immuno-precipitin pattern produced with homologous immune sera compared to that of the untreated concentrate. Sonication for ½ - 1 minute was useful for dispersing virus aggregates and was applied in the preparation of all virus concentrates.

Non-Specific Immunodiffusion Reactions Caused by Chemical or Surface Active Agents Influenza viruses disrupted by SDS, DOC, Nonidet P40 (NP 40), and SDS-β-mercaptoethanol-heat were used in immunodiffusion cross reactions with immune sera to broaden the antigenic spectrum being investigated.

The occurrence of a great deal more cross-reactivity than expected, much of it due to fuzzy precipitin bands as well as sharp ones, suggested that part of the precipitation was non-specific. Plate 6 A illustrates a typical precipitin pattern formed by DOC disrupted influenza A, B, and Sendai viruses (centre row) diffusing with hyperimmune rabbit sera (abs) against A/Swine (top row), and against parainfl. Sendai (bottom row). Sendai antiserum normally precipitated only its homologous virus antigens, and not those of any influenza A or B strains. However, in this experiment, it precipitated all of them, suggesting that the precipitates formed were of non-specific nature. Similar phenomena were noted in most cross reactions using viruses disrupted with the above-mentioned reagents.

Therefore, in order to detect whether non-specific precipitation was occurring in these reactions, the following control experiments were carried out using template patterns identical to those employed for virus cross reactions (Plate 14, Fig. 4).

Replacing the disrupted viruses, each disrupting agent alone, in the same diluent, concentration, volume, and template position as used with the viruses, was diffused with each immune serum employed in cross reaction studies. Plate 6 B illustrates non-specific precipitation of convalescent rabbit sera (bottom row), and hyperimmune rabbit sera (abs) (top row) by 1% DOC (centre row). SDS (1%) caused similar non-specific precipitation. The precipitation of hyperimmune rooster sera by 1% SDS (Plate 6C), or by 1% DOC was even more pronounced. SDS-β-mercaptoethanol-heat treatment of sera caused the greatest amount of non-specific precipitation in all antisera tested.

Characteristic rings around the central well containing NP 40 (1%) were formed by precipitated serum components, when the surfactant was diffused against convalescent rabbit sera (Plate 7A bottom row), hyperimmune rabbit sera (abs) (Plate 7A top row), and with hyperimmune rooster sera. Non-specific precipitin rings were also formed when virus concentrates (C_2 and SDG), disrupted by 1% NP 40 (Plate 7B centre row), were diffused against phosphate buffered saline (PBS) alone, which replaced antiserum in the template (top and bottom rows). The well defined precipitin ring formation was easy to locate in immunodiffusion reactions between NP 40 disrupted influenza viruses and corresponding immune sera (Plate 11B, NS).

Viruses, disrupted by SDS, DOC, or SDS-β- mercaptoethanol-heat, were placed in central wells and diffused against PBS which replaced immune sera in the peripheral wells (Plate 7C). Non-specific precipitation of components in both C_2 and SDG concentrates was not so great as that observed with antisera.

In cellulose acetate immunodiffusion, non-specific precipitation of serum and viral components by SDS, DOC, SDS-β-mercaptoethanol-heat, and to a lesser extent by NP 40, made the interpretation of immuno-precipitin reactions involving disrupted virus very difficult. The non-specific precipitin bands observed in the control experiments could be confused with, or obscure, those resulting from precipitation of antigen by antibody. In addition, components, which would otherwise enter into the immune reaction, could be precipitated. Their elimination by non-specific precipitation would result in an incomplete representation of

the possible immune reaction. Palmer et al. (1971) have demonstrated that SDS precipitates human 7S immunoglobulin in a single line in agar gel. Its removal by SDS in an immunodiffusion reaction with disrupted virus, could decrease the sensitivity of the reaction. In our experiments, antiserum components precipitated by each disrupting agent differed because the precipitin patterns resulting from the action of each agent were not identical. The same can be said about the precipitation of viral components, although the precipitin patterns produced by each agent were not so diverse.

Viruses disrupted with certain reagents can be useful in carefully monitored immunodiffusion reactions, with cautious interpretation of results. Observing the limitations outlined above, NP 40 disrupted virus concentrates, in conjunction with undisrupted virus, were utilized in 1) homologous immunodiffusion reactions identifying the type-specific component (Plate 11 A, B), and 2) in the study of influenza A cross reactions. The use of SDS, DOC, and SDS-β-mercaptoethanol-heat disruption of viruses was abandoned because the non-specific precipitation could not be so easily recognized as it was ith NP 40.

The results of these control experiments in cellulose acetate are in agreement with the observations of similar non-specific precipitation in agar gel by Corbel and Rondle (1970), Palmer et al. (1971), and in cellulose acetate by MacDonald (1971). Corbel and Rondle have recommended using the non-ionic detergent Triton N101 to disrupt influenza viruses because it did not give non-specific precipitation with antisera or other antigens in immunodiffusion in agar gel. This and other disrupting agents

will have to be investigated for future application to immunodiffusion experiments in cellulose acetate.

The control experiments above point out the necessity for extremely strict controls to be applied to all reagents used in immunodiffusion experiments, and for the cautious interpretation of results.

Density Gradient Purification of Viruses and N-ALF

Purification of viruses by density gradient procedures is standard in most virus research laboratories. The procedures followed were ones most commonly seen in the literature. Sucrose density gradient purification of influenza viruses is well established. A comparison of behaviour of representative members of influenza A viruses on sucrose and potassium tartrate gradients, and subsequent biological activity was undertaken.

Each virus purification in sucrose and potassium tartrate is discussed separately on the basis of five criteria: HAU/ml, EID_{50} in ovo, mg protein per ml, immunoprecipitin reaction with homologous antisera, and electron microscopy. The results are then summarized and compared.

Discussion of Individual Viruses

Influenza A/PR8 An easily manipulated virus, PR8 in a sucrose density gradient (5-40%), forms a uniform, reproducible, tight band, one-third to one-half way down the gradient, depending on the "g" force (Table 12, Figs. 6, 7 a, Plate 8). Biological activity was maintained the best of any virus studied (Tables 5, 6, 12). In immunodiffusion, the immunoprecipitin reaction was strong and clear with homologous immune sera, RAs-IN, hRAs (abs), hFAs, forming 1, 3, and 4 precipitin bands

LEGEND - TABLE 12

All centrifugation performed with Beckman Spinco Model L_2 preparative ultracentrifuge

SDG : sucrose density gradient 4.8 cm height

KTDG : potassium tartrate density gradient 8 cm height

[a]Measured from top of gradient to bottom of visible band

[b]Extent of band given. Low position due to virus aggregates

HAU/ml : haemagglutinin units per ml using 0.05 ml virus volumes, 50% end point

Protein mg/ml: determined by method of Lowry _et al_. (1951)

$\log_{10}EID_{50}$: in ovo, calculated according to Reed and Muench (1938)

C_2, C_2' : virus concentrated 100 x, 200 x respectively

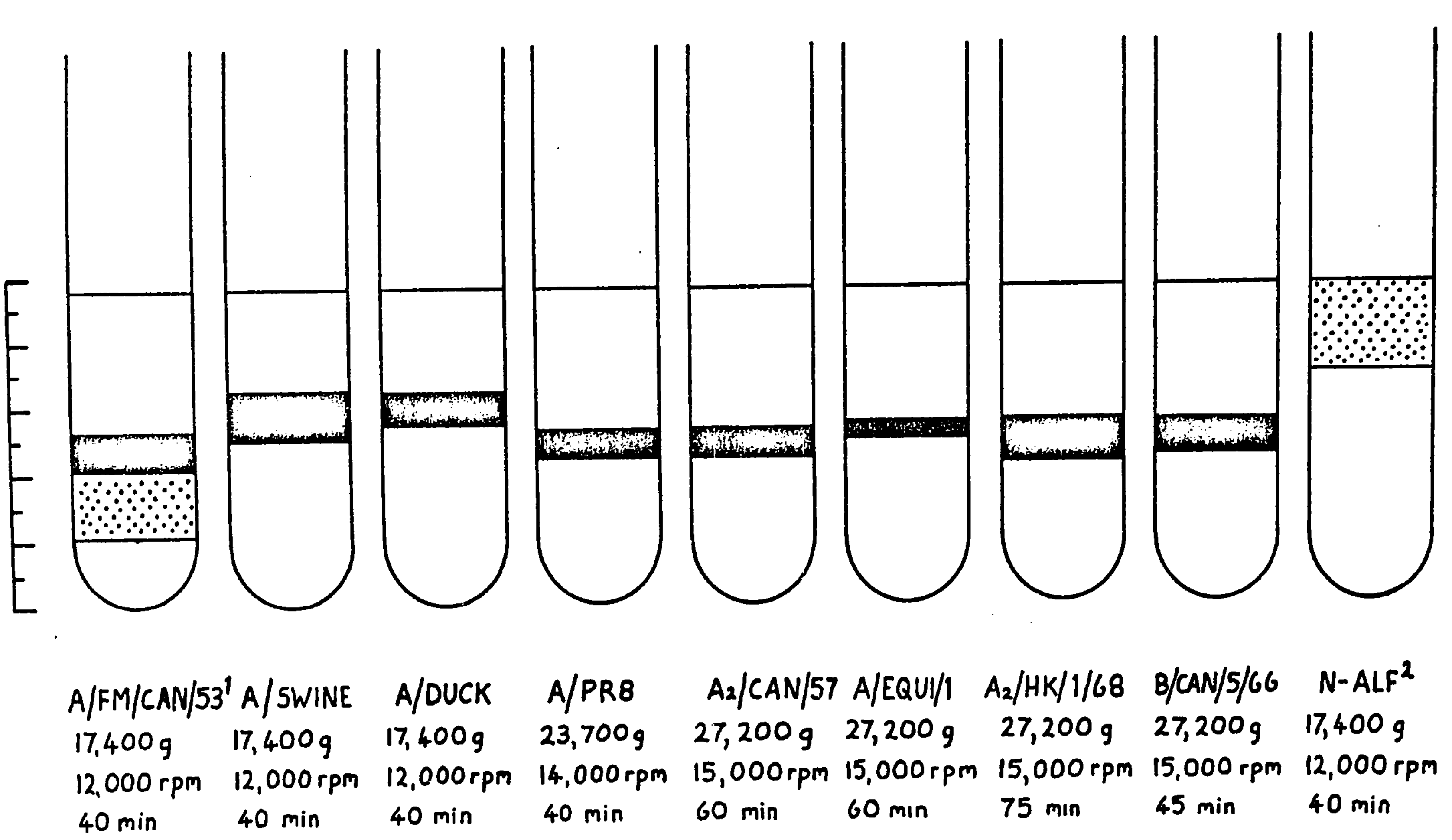
"g" : centrifugal force values are rounded off to nearest hundred

Blank spaces : not done

TABLE 12. Density gradient centrifugation of influenza viruses, parainfluenza Sendai, and N-ALF

Material C_2 and C_2'	Batch No.	No. of Samples	Gradient	SW Rotor	rpm	Time min.	"g"	Temp °C	Band Position cm. from Top[a]	Purified Virus Characteristics: HAU/ml	Purified Virus Characteristics: Protein mg/ml	Purified Virus Characteristics: $\log_{10} EID_{50}$
Infl.A/PR8	7	3	SDG	25.2	12,000	40	17,400	4	1.7	5.2×10^5		11.2
	9	3	SDG	25.2	14,000	40	23,700	4	2.5	1.02×10^6	2.33	11
	9	2	KTDG	41	38,000	180	174,000	4	6.2	1.02×10^5	0.23	7.5
Infl.A/FM1/Can/53	5	5	SDG	25.2	12,000	40	17,400	4	2.7	1.6×10^4	0.15	8.7
	5	2	KTDG	41	38,000	180	174,000	4	4.7	NIL	<0.05	4.3
	4	3	KTDG	65	45,000	135	144,000	4				
Infl.A2/Can/57	6	3	SDG	25.2	15,000	40	27,200	4	2.5	1.6×10^4	0.51	5
	7	3	SDG	25.2	15,000	60	27,200	4	2.5	4.8×10^4	0.27	7.5
	7	2	KTDG	41	38,000	180	174,000	4		NIL	<0.05	4
Infl.A2/HK/1/68	3	3	SDG	25.2	15,000	60	27,200	5	2.6	1.02×10^5		8
	4	3	SDG	25.2	15,000	75	27,200	4	2.6	2.05×10^5	0.17	7.5
	3	2	KTDG	41	38,000	180	174,000	4		NIL	<0.05	4.7
Infl.A/Equi 1	4-6	6	SDG	25.2	15,000	40	27,200	4	0.9-2.6[b]	4×10^3	0.2	4.9
	7	3	SDG	25.2	15,000	60	27,200	4	2.2	3.2×10^4		9.5
	7	2	KTDG	41	38,000	180	174,000	4		4×10^2	<0.05	4.2
Infl.A/Swine	7	6	SDG	25.2	12,000	40	17,400	4	2.2	5.12×10^5	0.47	8.7
	7	3	KTDG	41	38,000	180	174,000	4	7.8	2.56×10^4	0.23	6.3
Infl.A/Duck	3	3	SDG	25.2	12,000	40	17,400	4	2.0	6.4×10^4	1.75	10.2
	4	4	SDG	25.2	12,000	40	17,400	4	2.0	2.6×10^5	0.9	10.3
	5	6	SDG	25.2	15,000	40	27,200	4	2.5	1.02×10^6	1.0	9.5
	3	3	KTDG	65	45,000	180	144,000	4	3.9	3.2×10^4		4.7
	4	3	KTDG	41	38,000	180	174,000	4	7.0	1.02×10^5	0.17	3.3
Infl.B/Can/5/66	1	3	SDG	25.2	15,000	45	27,200	4	2.5	1.3×10^4	0.35	3.5
	1	2	KTDG	41	38,000	180	174,000	4		NIL	<0.05	NIL
P.Sendai	3	3	SDG	25.2	15,000	45	27,200	4	1.1	NIL		5.0
N-ALF	Pool	2	SDG	25.2	12,000	40	17,400	4		NIL	0.21	NIL

Figure 6. Relative positions of visible bands formed by influenza viruses and N-ALF in sucrose density gradients of 32 ml volume. Spinco head SW 25.2, $4^{o}C$.



[1]Stippled band represents diffuse area in gradient possessing some activity

[2]Stippled band is protein detected by UV absorption at 280 nm

cm scale represents gradient height, which is 4.8 cm.

Legend Figure 7.

Sedimentation profiles of influenza strains a) A/PR8 b) A2/Hong Kong/ 1/68 c) A/Duck and of N-ALF, after centrifugation in linear sucrose gradients (10-40%) in Spinco head SW 25.2. Bovine serum albumin (1%) standard centrifuged in parallel with A/PR8 and A2/Hong Kong/1/68. One ml volumes of each sample placed on each gradient. Linearity of gradients is demonstrated.

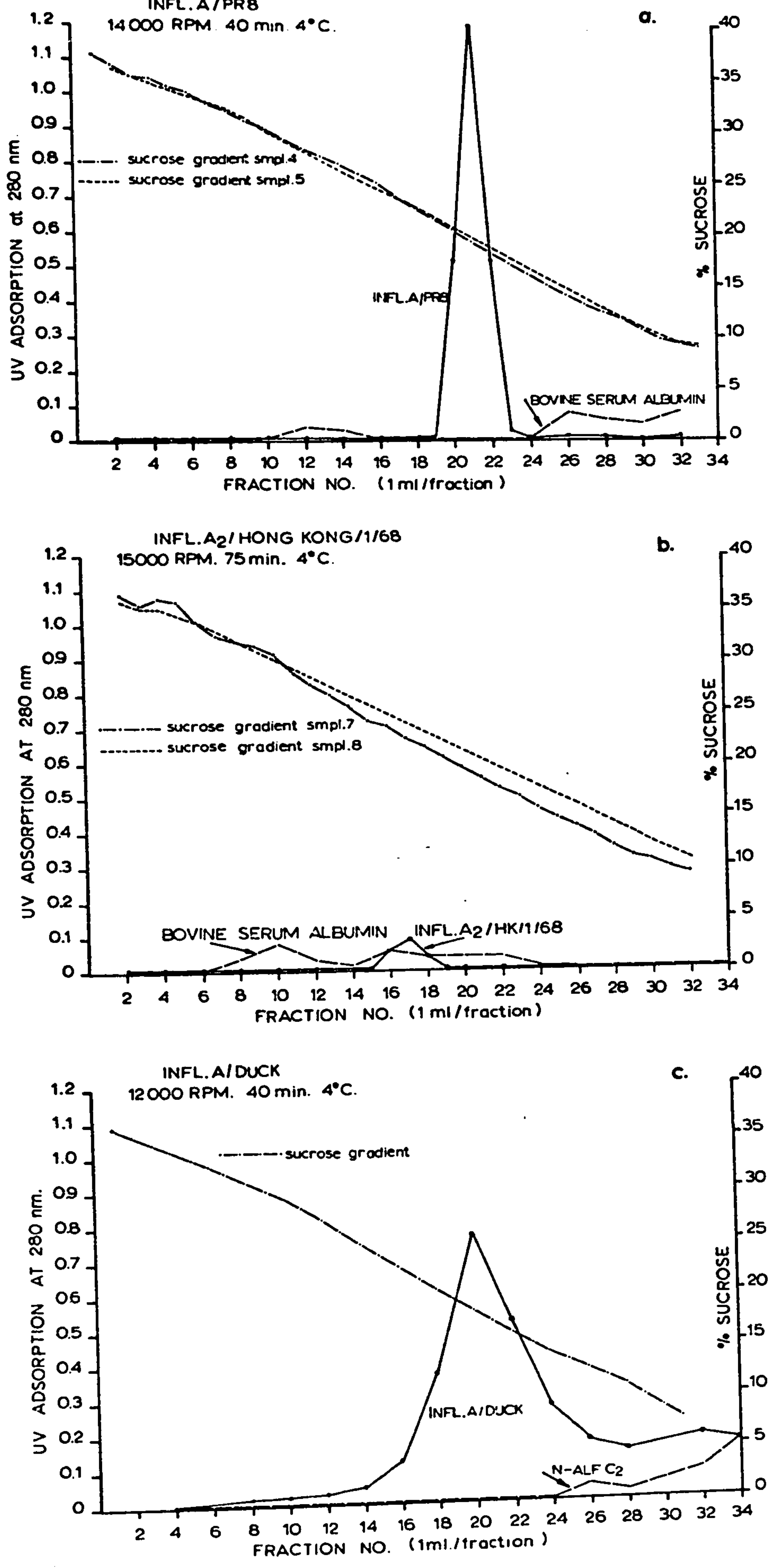


Figure 7. Sedimentation profiles of influenza A strains in linear sucrose gradients

respectively (Plate 9 A). An antigenic component, common to the virus and its respective soluble antigen in reactions with homologous hyperimmune rabbit and rooster sera, could be the type specific component (arrowed 1). When diffused with anti-host (CE) hyperimmune rabbit serum, purified virus (SDG) was shown to contain at least one detectable host (CE)-specific component compared to two demonstrated in semi-purified preparations (C_2). Polyacrylamide gel electrophoresis of SDS disrupted virus revealed host (CE) material in both preparations (Plates 19 and 20).

The second purification cycle in potassium tartrate gradient was not so successful. A band formed near the bottom of the gradient (Table 12), but virus biological activity was lowered (Tables 5, 6, 12), and no detectable precipitation by homologous immune sera was recorded in immunodiffusion.

Electron microscopy of negatively stained sucrose gradient purified virus revealed typical influenza virus with little extraneous material (Plate 10 E), whereas tartrate purified virus appeared to have atypical surface projections (Plate 10 G).

Influenza A/FM 1/Can/53 FM 1 purification by sucrose density gradient yielded purified virus with reduced biological activity compared to semi-purified preparations (Tables 5, 6, 12). Two bands were formed, a dense upper and a diffuse lower one (Fig. 6, Plate 8). Hemagglutinating activity of the diffuse fraction was six times that of the dense one, but infectivity, in ovo, of the latter was two logs greater (Appendix 1, Tables 2 and 3). Material from both bands formed similar precipitin band patterns in immunodiffusion, precipitated weakly by homologous RAs-IN, and faintly by homologous hRAs (abs). Host component, detected

in immunodiffusion with host (CE)-specific hyperimmune rabbit serum, was not completely removed by this purification cycle. Electron micrographs were poor, but typical virus was seen in both fractions.

Tartrate gradient centrifugation produced sufficient virus to be precipitated by homologous convalescent and hyperimmune (abs) rabbit sera in immunodiffusion, but the only biological activity expressed was low level infectivity ($\log_{10}EID_{50} = 4$) (Tables 5, 6, 12). Virus "ghosts" were seen by electron microscopy of negatively stained preparations.

Influenza A2/Can/57 Banding, about half way down a sucrose gradient, was finally attained at 27,200 g (15,000 rpm) for 60 min and was relatively tight, but was not so dense as that of other influenza A strains (Table 12, Fig. 6, Plate 8). Biological activity was reduced in the purified virus, especially infectivity (Tables 5, 6, 12). In immunodiffusion with homologous antisera, the only immunoprecipitin reaction which occurred was a single band formed with convalescent rabbit serum. The virus preparation contained host material which was precipitated by host (CE)-specific hyperimmune rabbit serum in immunodiffusion. Typical virus was seen by electron microscopy of negatively stained preparations.

The only activity demonstrated by potassium tartrate purified virus was low infectivity ($\log_{10}EID_{50} = 4$) (Tables 5, 6, 12).

Influenza A2/Hong Kong/1/68 Banding of this virus was achieved with difficulty. Although a band formed in 60 min. at 27,200 g (15,000 rpm), 75 min. was required for it to consolidate into a tight, but not very dense band half way down the gradient (Table 12, Figs. 6 and 7 b, Plate 8).

Biological acitivity of the purified virus dropped slightly compared with that of semi-purified virus, but surprisingly it was one of the hardier influenza A viruses with which to work (Tables 5, 6, 12). The purified virus did not diffuse easily in cellulose acetate immunodiffusion. Two precipitin bands were formed when virus was diffused with homologous antisera (Plate 9 B). The virus contained an antigenic component identical to one found in its soluble antigen, because precipitin bands formed by these, with homologous hyperimmune rabbit serum (abs) linked (arrowed), suggesting the component could be the type-specific one. Host (CE)-specific material was still contained in the virus preparation. It was detected when diffusion of purified virus with anti-N-ALF hRAs yielded a precipitin band; and when SDS disrupted virus was fractionated by polyacrylamide gel electrophoresis (Plate 20).

Typical pleomorphic influenza virus morphology, including filaments were seen by electron microscopy of negatively stained preparations of purified virus (Plate 10 D).

The virus was not successfully purified in potassium tartrate gradients because the duration of centrifugation was not long enough for the virus to band. Using the data obtained in sucrose density gradients, a relative adjustment of time could be made and the virus successfully banded. A few typical viruses were seen by electron microscopy.

<u>Influenza A/Equi 1</u> Tight banding of Equi virus was achieved at 27,200 g (15,000 rpm) in 60 min in a sucrose density gradient; and the light band formed was half way down the gradient (Table 12, Fig. 6). Biological activity dropped when the virus was purified, and only

infectivity was maintained (Tables 5, 6, 12). In immunodiffusion with homologous antisera, one linking precipitin band formed with normal rabbit (A19), convalescent rabbit, and hyperimmune rooster serum, signifying that the same antigen was reacting with antibody in each serum. Host (CE)-specific component, revealed when precipitated by anti N-ALF hyperimmune rabbit serum in immunodiffusion, was present. Little virus, but with typical morphology, was seen in negatively stained virus preparations.

Tartrate purification yielded virus with an EID_{50} of 10^4, but no other demonstrable biological activity (Tables 5, 6, 12). It resembled influenza A2/Can/57 in this respect. Both viruses required longer centrifugation for successful banding. Virus was not seen by electron microscopy in negatively stained preparations.

Influenza A/Swine An easily formed, reproducible, tight band was formed by Swine half way down the sucrose gradient (Table 12, Fig. 6). Although infectivity dropped by 1½ logs, HA activity was maintained, with some lowering of the biological activity ratio (Tables 5, 6, 12). In immunoprecipitin reactions with homologous RAs-IN, hRAs (abs), and hFAs, two antigenic components were precipitated by the rabbit, and three by the rooster serum (Plate 9 D). One of the latter components was also found in Swine soluble antigen, and was identified as the same in both virus preparations by precipitin band linkage (arrowed), suggesting it could be the type-specific component. The purified virus contained host (CE)-specific component because it was precipitated by anti-host hyperimmune rabbit serum, and hyperimmune rooster serum in immunoprecipitin tests.

In negatively stained preparations, Swine virus appeared typical, and the preparation was devoid of extraneous material when compared with semi-purified preparations by electron microscopy (Plate 10 F).

Virus activity dropped after a potassium tartrate purification cycle, but not to the same extent as other viruses (Tables 5, 6, 12). By electron microscopy, the morphology of the virus appeared altered especially the envelope projections.

Influenza A/Duck Duck influenza virus was easily banded by sucrose density gradient centrifugation, forming tight bands about one-third to one-half way down the gradient (Table 12, Figs. 6, 7 c, Plate 8). The biological activity of the virus was maintained throughout the purification process (Tables 5, 6, and 12). Precipitin reactions in immunodiffusion occurred with all homologous immune sera (RAs-IN, hRAs abs, hFAs) and normal rabbit serum A27. One antigenic component was detected by homologous absorbed hyperimmune rabbit serum when virus antigen was peripherally located. When the antigen was located centrally, two or three precipitin bands formed. The antigenic component of one of these was the same as a component in Duck soluble antigen, which was demonstrated by precipitin band linkage (arrowed 1, Plate 9 C). This could be the type-specific component. Host (CE) material was not completely removed by this purification cycle since in immunodiffusion, CE component was detected by rabbit host (CE)-specific immune serum, and by polyacrylamide gel electrophoresis of SDS disrupted virus (Plate 20).

Potassium tartrate density gradient was a further step in purification, but even though a purer virus preparation was obtained,

there was drastic loss of biological activity, especially an infectivity drop of 6-7 logs (Tables 5, 6, and 12). A faint precipitin reaction with homologous hyperimmune rabbit serum (abs) was detectable in immunodiffusion.

On electron microscopy, negatively stained sucrose gradient purified virus looked typical with less extraneous material than Duck C_2 (Plate 10 A), but potassium tartrate purified virus appeared distorted, especially the envelope projections.

Influenza B/Can/5/66 Influenza B virus banded easily in about the same position in sucrose gradients as influenza A viruses under the same conditions (Table 12, Fig. 6, Plate 8). Biological activity dropped compared to the semi-purified preparation (C_2), especially infectivity (Tables 5, 6, 12). A freezer breakdown may have partly contributed to the infectivity drop, because this virus seemed particularly susceptible to elevated storage temperatures. The purified virus was not precipitated by homologous antiserum in immunodiffusion.

Electron microscopy of negatively stained preparation revealed typical influenza virus morphology.

Tartrate gradient purification was not successfully completed.

Parainfluenza Sendai For reasons unknown, the virus was not successfully banded in sucrose density gradients. The sample migrated only one-quarter of the way down the gradient, and the fraction collected had an EID_{50} of 10^5, but no detectable hemagglutinin or protein (Tables 5, 6, 12). A precipitin band did form with homologus convalescent rabbit

serum in immunodiffusion. Sheared off virus particles were seen by electron microscopy of negatively stained preparations but semi-purified particles had characteristic morphology (Plate 10 C). Altered conditions are required to purify this virus successfully.

Potassium tartrate purification was not attempted.

N-ALF Visible banding of N-ALF was not seen, but a small peak was detected near the top of the gradient by UV absorption at 280 nm. This was collected, concentrated, and labelled N-ALF/SDG' (Figs. 6, 7c , Plate 8). Duck virus, run in parallel, banded distinctly. N-ALF fractions paralleling those of Duck were collected to determine the host (CE) material present in the same part of the gradient as the Duck virus band. This material, concentrated, was labelled N-ALF/SDG. Only protein content was detectable (Tables 5, 12). Host (CE)-specific antigenic components were present in both N-ALF/SDG and N-ALF/SDG', because on immunodiffusion with homologous hyperimmune rabbit serum, one precipitin band was formed, and two precipitin bands with hRAs/Duck. On polyacrylamide gel electrophoresis, SDS-disrupted N-ALF/SDG resolved into two faint bands about half way down the gel (Plate 20 A).

Indefinite, scattered debris was seen by electron microscopy of negatively stained material.

A potassium tartrate purification cycle was not attempted, but would be valuable in order to determine if host components are present in potassium tartrate purified viruses.

Comparison

All influenza A viruses and influenza B formed bands on sucrose

density gradient centrifugation and thus underwent a stage 1 purification cycle. The viruses fell into two groups with regard to conditions necessary for sharp band formation (Table 13).

Group I banded relatively easily requiring 17,400 g for 40 min. The order of listing within the group (Table 13) is from highest to lowest rate of sedimentation, that is, the greatest to the least distance the band migrated in the gradient under the given conditions: infl. A/FM1 with the greatest, and PR8 with the shortest distance of migration. With an increase in centrifugal force, both infl. A/PR8 (23,700 g for 40 min.) and infl. A/Duck (27,200 g for 40 min.) moved a greater distance, comparable to that moved by the viruses in Group II. These latter viruses required the higher g of 27,200 for a longer time, 60 to 75 min., to form sharp bands. Thus the rate of sedimentation of Group II is relatively slower than Group I, which suggests they are behaving as lighter particles.

The behaviour of influenza A viruses in sucrose density gradients with respect to rate of sedimentation is interesting in that the faster sedimenting members of Group I are A0, A1, and serologically related types, all originating in epidemics before the A2/Asian era (pre 1957), and the slower sedimenting members of Group II are A2/Asian, A2/Hong Kong and A/Equi 1 originating in 1956 and later.

Infl. A2/HK/1/68 was the most difficult virus to band, forming a diffuse band in 60 min. at 27,200 g (bracketed in Table 13), but requiring 75 min. to form a sharp one. The rate of band migration and formation was slower, yet the distance of migration was greater than other viruses of the group. Possibly, although the morphology of infl.

TABLE 13. Relative positions of bands formed by influenza viruses in sucrose density gradients (32 ml volume, Spinco head SW 25.2, 4°C).

Influenza Virus	Distance of Band Migration from Gradient Meniscus in cm				
	17,400 g (12,000 rpm)	23,700 g (14,000 rpm)	27,200 g (15,000 rpm)		
	40 min.	40 min	45 min.	60 min.	75 min.
Group I					
A/FM1/Can/53	2.6				
A/Swine	2.2				
A/Duck	2.0		2.5 (40 min.)		
A/PR8	1.7	2.5			
Group II					
A2/Can/57				2.5	
A/Equi 1				2.2	
A2/HK/1/68				(2.6)	2.6
B/Can/5/66			2.5		

A2/HK/1/68 is similar to other influenza A viruses, the higher proportion of filaments to spherical particles may be a physical factor influencing band formation.

Influenza B is included for comparison of type B with type A viruses and is found to be similar in general behaviour to Group I viruses in sucrose density gradient centrifugation.

Only certain influenza A viruses purified by sucrose density gradient centrifugation maintained their biological activity at the same level as before purification (Tables 5, 6, 12).

Infectivity of infl. A/PR8, A2/HK/1/68, Duck, and Equi 1 was retained or dropped less than a log, but all other virus types suffered a greater than one log drop.

The number of hemagglutinin units per ml remained much the same in infl. A/PR8, Swine, and Duck, but dropped by a log or more in the other viruses.

Protein concentrations increased in infl. A/PR8 and Duck suspensions, but the other organisms showed decreases especially infl. A/FM1, A2/HK/1/68, Equi 1, and infl. B. In one batch of Equi virus and one of Hong Kong, the protein was not detectable in spite of reasonable infectivity titre and haemagglutinin content. The reason for these low readings is unknown but could be due to interference of sucrose or buffer (STE), but these were compensated for, by dialysis, to remove sucrose, and, preparation of a standard curve for protein estimation using STE buffer to suspend the standard protein used.

The Lowry _et al._ (1951) method is used extensively in protein

estimations of viruses and other substances as it is an easy and convenient procedure to use. It is not always universally applicable to all viruses in all states and Lowry says, "the amount of color will vary with different proteins" and, "color is not strictly proportional to the concentration". Lab-trol, the standard used for the preparation of two protein estimation standard curves, is a different protein to that found in influenza viruses. At the infected chick allantoic fluid and virus concentrate (V/C_2) level, the protein being measured is virus plus CE material, the latter giving a certain base to the measurement of protein content and thus yielding more consistent results. Sucrose gradient purified viruses contain minimal CE protein and mainly virus protein. Influenza viruses vary within the group and a certain inconsistency of response to the Folin-Ciocalteu reagent is to be expected. The final color developed in the reaction depends on: biuret reaction of protein with copper ion in alkalai; and reduction of the phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophane present in the treated protein (Layne, 1957). The Lowry method is used extensively for protein estimations of influenza and other viruses on the assumption that the tryptophane and tyrosine content does not vary markedly amongst the virus types. In such a variable group as influenza A viruses, this assumption may not hold with the result that inconsistency in protein values may be partly due to a variation in the amino acid composition of these viruses which has as yet not been completely determined. The Lowry method of protein estimation was used in this study and the results interpreted with these facts in mind.

Immunoprecipitin reactions of sucrose gradient purified virus with homologous virus-specific antisera in immunodiffusion (Plate 9), were not

so strong as those of virus concentrate (V/C_2). The number of antigenic components detected as precipitin bands varied with the serum used (Table 14).

When compared, homologous convalescent rabbit sera reacted with the same antigenic component in purified virus as in virus concentrate, indicating that the purification process had not altered the precipitin relationship of virus and convalescent antiserum. Exceptions to this were an extra antigenic component detected as a precipitin band in Swine and Duck viruses (Table 14).

Fewer precipitating antigenic components were detected in purified virus than in semi-purified concentrate (C_2, C_2') by absorbed hyperimmune rabbit serum, for example, Duck virus precipitin bands were reduced from 7 or 8 to 2 components (Table 14). This suggested that purified virus suspension was more homogeneous antigenically, containing antigens of the same order as whole virus particles, and did not contain the heterogeneous mixture of virus and virus products found in semi-purified virus concentrate.

Immunoprecipitin reactions with rooster hyperimmune sera varied, remaining unchanged in number with purified Equi and Swine viruses, reduced in number with PR8, A2/HK/1/68, and Duck, and non-existant with FM1 and A2/Can/57 (Table 14).

Parainfl. Sendai, though listed, is not discussed here as it was not considered to be successfully purified.

The number of host (CE)-specific components detected by immunoprecipitin reactions with host (CE)-specific rabbit antiserum was reduced

TABLE 14. Comparison of number of precipitin bands formed by semi-purified and sucrose density gradient purified Myxoviruses in homologous immunoprecipitin tests

Virus	No. Precipitin Bands Formed with Homologous Antisera on Immunodiffusion					
	Antigen as C_2 or C_2'			Antigen SDG purified		
	RAs-IN	hRAs(abs)	hFAs	RAs-IN	hRAs(abs)	hFAs
Infl.A/PR8	1	5-7	4	1	3	3
Infl.A/FM1/Can/53	1-2	4	3	1	2	–
Infl.A2/Can/57	1	3-4	2	1	1	–
Infl.A2/HK/1/68	2	3	4	2	2	1
Infl.A/Equi 1	1	2	2	1	–	2
Infl.A/Swine	1	4-5	3-4	2	2	3
Infl.A/Duck	2	7-8	5	3	2	3
Infl.B/Can/5/66	1-2	3-4	2	–	–	ND
Parainfl. Sendai	1-2	4	4	1	–	–
N-ALF	–	–	–	–	–	–
		$hRAs_3$			$hRAs_1$	

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C_2 : virus concentrated 100x

C_2' : virus concentrated 200x

SDG purified : virus sucrose density gradient purified

RAs-IN : convalescent rabbit serum

hRAs (abs) : hyperimmune rabbit serum, absorbed with N-CAM

hRAs : hyperimmune rabbit serum

hFAs : hyperimmune rooster serum

ND : not done

– : no reaction

in most viruses from two in the semi-purified concentrate preparations to one in the purified preparations, the exceptions being Swine (three reduced to one), and A2/Can/57 (one in both). The host components in purified virus did not visibly react with host (CE)-specific/'convalescent' rabbit serum, /N-CAM absorbed hyperimmune rabbit serum, nor with /hyperimmune rooster serum. Therefore precipitin reactions between purified viruses and their homologous antisera, containing only virus-specific precipitins, were considered to be virus-specific. Paralleling this, in precipitin reactions with host (CE)-specific rabbit antiserum three CE antigen components in N-ALF C_2 were reduced to one in sucrose gradient purified N-ALF.

Sucrose density gradient purification of viruses was most successful with infl. A/Duck, and PR8, moderately successful with infl. A/Swine, A2/HK/1/68, Equi, and FM1, and least successful with A2/Can/57 and B/Can/5/66. Success was not obtained with parainfl. Sendai. With the knowledge gained from working with these viruses and enough time spent on each one, most difficulties encountered could be surmounted and all purified satisfactorily.

The virus product obtained on sucrose gradient purification was not completely pure, but for the purpose of this study i.e. immunoprecipitin reactions of virus and virus-specific antisera, the antigen(s) obtained were considered to be viral in nature so long as strict controls were adhered to, and host (CE)-specific reactions eliminated or identified.

Potassium tartrate gradient purification was not successful enough for virus products to be utilized in this study. Not enough time was

available to work out the conditions required for satisfactory purification of so many viruses. Some success was had with infl. A/PR8, Duck, and Swine, but in all of these, biological activity, especially infectivity, dropped sharply, and the morphology of the virus was considerably altered, especially envelope projections. Potassium tartrate does cause precipitation of some viruses which may have been a contributing factor in the lack of success with some of the viruses. However, experience was gained in manipulation of gradients and viruses. In conjunction with sucrose density gradient purification procedures, it is felt that most viruses could be purified in a second cycle to produce an almost pure virus suspension which could be used to further this study.

Linearity of Gradients

The linearity of sucrose gradients formed was fairly even under all conditions of centrifugation used, and the reproducibility with each set of fixed conditions was within 3% (Fig. 7 a, b, c). Hand-pipetted equilibrated gradients were probably not quite so accurate as those made by a gradient mixer. Gradients must have been fairly even and uniform as banding of viruses was reproducible in triplicate within each run, and under the same cnditions, each run was reproducible.

Sucrose Density Gradient Centrifugation of Standard Proteins

A preliminary start was made to qualitatively compare the relative sedimentation rates of virus and proteins of known molecular weights, e.g. bovine serum albumin, horse hemoglobin, and trypsin. Bovine serum albumin (BSA), mol. wt. 68,000 (Weber and Osborn, 1969) migrated well in a 5 - 40 % sucrose gradient (Fig. 7 a, b). At 17,400 g for 40 min.

it formed two peaks, one just over half way down the gradient, and the other in the top third, with infl. A/PR8 positioned between, about one-third down the gradient. At 27,200 g for 75 min., the bottom BSA peak was about two-thirds down the gradient, and the upper peak coincided with the peak of infl. A2/HK/1/68, about half way down the gradient. This supported the finding that infl. A2/HK/1/68 tended to behave as a lighter particle than infl. A/PR8 in sucrose density gradient centrifugation. However, Kendal (1968) quoted by Apostolov et al. (1970), found that the buoyant densities of A2/Singapore/57 and A0/PR8/34 in a linear sucrose gradient (12-60%) were very similar (1.200 and 1.210 gm/cm^3 for the A2 and 1.205 gm/CM^3 for the A0 virus). Why Kendal's results differed from the ones presented here is not known.

Horse haemoglobin (Laver and Webster, 1966) and trypsin were not successfully used as standards.

Identification of the Type-Specific Reaction in Immunodiffusion

The type-specific reaction was clearly identified in homologous immunodiffusion reactions of all influenza viruses (A/PR8, A/FM 1/Can/53, A2/Can/57, A2/Hong Kong/1/68, A/Equi 1, A/Swine, A/Duck, and B/Can/5/66); it was not so clearly defined in reactions of parainfl. Sendai. The influenza A type-specific reactions of individual strains were interpreted with the following facts in mind: 1) all virus soluble antigens except infl. B were of the same relative order of CF value (Table 28), 2) influenza A hyperimmune rabbit sera (abs) demonstrated some variation of CF titre (Table 8), 3) of the convalescent sera, only those infected by A2/Hong Kong/1/68 and Duck recorded CF titres (Table 7), 4) the CF titres of hyperimmune rooster sera were not determined, and 5) the variations in HAI titres of all homologous antisera reflected differences in their overall reactivity (Tables 7, 8, and 9). Virus concentrates used in homologous reactions varied in biological values as well (Table 5). However, all homologous immunoprecipitin reactions were carried out at equivalence.

The type-specific reactions of each virus-antiserum system were represented by many photographs, therefore only a few examples representative of the more strongly reacting viruses will be discussed. Details of these and of other relevant virus reactions are included in Table 15.

Homologous Reactions The major precipitin band formed between Duck soluble antigen (SA) and homologous immune sera was the type-specific reaction (Plate 11 A, arrow 1), which linked in identity with one of the

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RAs-IN : Convalescent serum of rabbit infected with virus

hRAs : Hyperimmune serum of rabbit parenterally immunized with virus, absorbed with N-CAM

hFAs : Hyperimmune serum of rooster parenterally immunized with virus

Major RNP : Strong precipitin band formed by antiserum with virus concentrate (C_2) and with soluble antigen (SA) which links across in identity

Minor RNP : Weaker precipitin band formed by antiserum with virus concentrate and with soluble antigen which links across in identity

Only SA Component : Precipitin band formed by antiserum with virus SA only

+ : in homologous reaction, one precipitin band formed; $+_2$ two precipitin bands formed; *± band present only in reactions combined with NP 40 disrupted virus concentrate

+ : in cross reaction, one or more precipitin bands formed, one of which links in reactions of all influenza A strains; **± weak A/FM1 convalescent serum forms precipitin bands with A strains which do not link across

\- : no reaction

Blank spaces : Serum too weak to form visible precipitin reaction with virus antigens

ND : Not done

TABLE 15. Influenza and parainfluenza type-specific components revealed by immunoprecipitin reactions.

Influenza Antiserum		Homologous Reactions: Major RNP	Minor RNP	Only SA Component	Cross Reactions: Influenza Type A	Type B	Parainfl. Sendai
A/PR8	RAs-IN	+					
	hRAs	+	+	+	+	−	−
	hFAs	+	+	+	+	−	−
A/FM1/Can /53	RAs-IN	+	−	−	**±	−	−
	hRAs	+	−	+	+	−	−
	hFAs	+	+	+	+	−	−
A2/Can/57	RAs-IN	+	−	+	+	−	−
	hRAs	+	−	+	+	−	−
	hFAs	+	−	+	+	−	−
A2/HK/1 /68	RAs-IN	+	−	−	+	−	−
	hRAs	+	+	−	+	−	−
	hFAs	+	+	−	+	−	−
A/Equi 1	RAs-IN	+	−	+	+	−	−
	hRAs	+	+	−	+	−	−
	hFAs	+	+	−	+	−	−
A/Swine	RAs-IN	+					
	hRAs	+	+	+	+	−	−
	hFAs	+	*±	−	+	−	−
A/Duck	RAs-IN	+	−	+	+	−	−
	hRAs	+	$+_2$	+	+	−	−
	hFAs	+	−	$+_2$	+	−	−
B/Can/5 /66	RAs-IN	+	−	+	−	+	−
	hRAs	+	−	+	−	+	−
	hFAs	ND	ND	ND	−	+	−
Parainfl. Sendai	RAs-IN				−	−	+
	hRAs	+	−	+	−	−	+
	hFAs	+	−	+			

major reactions occurring between virus concentrate antigens and immune sera (Table 15, Major RNP). The serum component precipitating the major type-specific antigen has been demonstrated to be a 7S gamma-globulin (presumably antibody) (Part II, Identification of Influenza Antibodies in Immune Preparations; Plates 25 and 26). The antibody precipitating the major type-specific component was the same in all sera because the precipitin band formed by soluble antigen and virus concentrate, with convalescent, and with absorbed hyperimmune rabbit sera, linked in identity (Plate 11A, arrow 1, top); that of hyperimmune rabbit serum (abs) and hyperimmune rooster serum is also identical (Plate 11A, arrow 1, bottom); and those of convalescent rabbit sera and hyperimmune rooster sera had also been shown to be identical by the same type of linkage.

Whether the whole virus concentrate was undisrupted (Plate 11 A), or disrupted with Nonidet P40 (NP 40) (Plate 11 B), the type-specific reaction (arrow 1) remained the same as described above. NP 40 releases nearly all RNP from influenza virus particles in a single 38 S peak (Pons et al., 1969), and on immunodiffusion in agar gel, gives strong, clearly defined precipitin lines for the type-specific ribonucleoprotein antigen, and for neuraminidase, but much fainter lines for suspected hemagglutinin (Schild and Pereira, 1969). The second strong precipitin band, present only in reactions between Duck influenza virus concentrate, unidsrupted or NP 40 disrupted, and all homologous immune sera (Plate 11 A, B, arrow 2) was similar to the neuraminidase specific reaction described by Schild and Pereira (1969). The hemagglutinin reaction could not be clearly identified.

Other precipitin bands were formed by precipitation of components in Duck SA by precipitins in each of the homologous immune sera, (Plate 11 A, B). Of three additional bands formed with hyperimmune rabbit serum (abs),

one was the result of precipitation of a component present only in soluble antigen (arrow 3) (Table 15, Only SA Component), and was also found in reactions with convalescent serum; two, which linked to precipitin bands formed between virus concentrate and the serum, were the result of precipitation of components present in both soluble antigen and in the virus itself (Plate 11 A, arrows 5, 6, Table 15, Minor RNP). All are considered to be virus-specific reactions (see Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions). One of the additional precipitin bands formed between soluble antigen and hyperimmune rooster serum was host (CE)-specific, because it linked in identity with a host-specific reaction between hyperimmune rooster serum and residual N-CAM components in absorbed hyperimmune rabbit serum (arrow 4) (refer to Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions).

Cross Reactions In order to confirm the identity of the type-specific antigen, the type-specificity of influenza A, B, and parainfl. Sendai antigen had to be demonstrated. Soluble antigens of all influenza A strains used, influenza B, and parainfl. Sendai (Plate 12 A, centre row) were diffused against Duck hyperimmune rabbit serum (abs) (top row), and against Duck convalescent rabbit serum (bottom row). A major precipitin band linking across all influenza A soluble antigens was formed by both types of serum, but reactions with B or Sendai SA did not occur. A similar linking precipitin band was formed by PR8 (top row) and FM 1 (bottom row) hyperimmune rooster sera which precipitated all influenza A soluble antigens in a linking band, but did not precipitate B or Sendai SA (Plate 12 B). These results were confirmed in 7-well template patterns. The influenza A type-specificity of these reactions was clearly demonstrated,

and it identified the precipitated components as the type-specific antigen. Moreover, following the characterization of type-specific ribonucleoprotein (RNP) in immunoprecipitin reactions by Hana and Hoyle (1966), Schild and Pereira (1969), Beard (1970), and Styk et al. (1970a), this antigen appears to be the RNP (see Summary and Discussion for further comments). The influenza A type-specific component was also demonstrated in immunodiffusion reactions of A2/Hong Kong/1/68 (Part II, Identification of Influenza A Type-Specific Antigen by Immunodiffusion, Plate 24).

The type-specific B reaction was demonstrated by the isolated homologous reactions formed between B soluble antigen with convalescent and hyperimmune (abs) rabbit sera, and complete lack of reaction with various influenza A, and Sendai soluble antigens (Plate 12 C, extreme left). Similar results were obtained using 7-well template patterns. The type-specific reaction of parainfluenza Sendai was similarly demonstrated.

In addition to the main reaction weaker precipitin bands were found in many of these cross reactions but their significance has yet to be established. They could be precipitated components which are related to RNP, or they could be minor RNP antigens. The identity of one of these precipitin bands formed by rooster hyperimmune sera has been established as a host-specific reaction to host components in the soluble antigen mentioned above.

Following both patterns described above, the type-specific reaction was established for each kind of antiserum immune to each of the other influenza A strains used, influenza B, and parainfl. Sendai (Table 15). Excepted were convalescent rabbit sera to A/PR8, A/Swine, and parainfl. Sendai which, independently, were too weak to form visible precipitates

with other than homologous virus antigens. In homologous reactions, more than one component in virus soluble antigens were precipitated by the majority of antisera. All sera precipitated the major type-specific component present in both soluble antigen and in virus concentrate. Lesser or minor RNP components present in both SA and C_2 were variably precipitated by antisera to all influenza viruses except A2/Can/57, B/Can/5/66, and parainfl. Sendai. Many antisera precipitated components present in virus soluble antigen only. In rabbit sera, this could be interpreted as virus-related host component not immunologically identifiable in the structure of the virus; this interpretation could also apply to rooster sera except those immune to A/PR8, A/FM 1, and A/Duck, where the component was normal host, because it was demonstrated to be the same as residual N-CAM components found in absorbed hyperimmune rabbit sera.

The above experiments established the identity of the type-specific reaction of each virus studied, which was applied to the interpretation of immunodiffusion cross reactions between viruses, as soluble antigens and as concentrates, and to the detection of antibodies to these components in normal animal sera.

Immunoprecipitin Cross Reactions of Influenza A Soluble Antigens

Preliminary immunodiffusion studies of the immunoprecipitin cross reactions with soluble antigens of influenza A viruses (7 strains), influenza B, and parainfl. Sendai, using corresponding immune sera of three sorts: convalescent rabbit, hyperimmune rabbit (abs), and hyperimmune rooster, have revealed the following.

In many cases, a complex precipitin band pattern was formed, which indicated that more than one component in virus soluble antigen was being precipitated by specific antibodies occurring in the different sorts of antisera (Table 16, Plates 12 A, B, 13 A, B, C). The main linking precipitin band formed with each anti-influenza A immune serum, by precipitation of soluble antigens of all influenza A strains used, but not with B or Sendai, was the major type-specific reaction, and has been described in the preceding section.

In most cross reactions between the soluble antigens and immune sera used in this study, this influenza A type-specific band was continuous and uniform, which indicated that one type of antibody in the immune sera was precipitating a component common to all influenza A soluble antigens, that is, the influenza A type-specific antigen (Plates 12A,B upper reaction, 24B). The antibody had been demonstrated to be a 7 S gamma-globulin (See Part II, Identification of Influenza Antibodies in Immune Preparations, Plates 25 and 26).

However, in reactions with certain antisera, the continuity and

LEGEND - TABLE 16

RAs-IN : convalescent serum of rabbit infected with virus

hRAs abs : hyperimmune serum of rabbits parenterally immunized with virus, absorbed with N-CAM

hFAs : hyperimmune serum of rooster parenterally immunized with virus

Influenza Soluble Antigens (SA): PR8 (A/PR8), FM1 (A/FM1/Can/53), A2 (A2/Can/57), HK (A2/HK/1/68), Equi (A/Equi 1), SW (A/Swine), Duck (A/Duck), B (B/Can/5/66); and Sendai (parainfl. Sendai)

\+ : Major type-specific immunoprecipitin band linking across all influenza A reactions, or isolated influenza B type-specific reaction, or isolated parainfl. type-specific reaction

⊕ : Isolated immunoprecipitin band which does not link

Number following +, e.g. +2, indicates number of precipitin bands formed in addition to major type-specific band

*+ : indicates break in the continuity (e.g. barb formation, band splitting) of type-specific band linkage

w : very weak type-specific precipitin band formation

\- : no reaction

Blank Spaces : Complete lack of reaction due to weak antiserum used

TABLE 16. Immunoprecipitin cross reactions of influenza A, B, and parainfluenza Sendai soluble antigens, preliminary results

Antisera	Soluble Antigen: Influenza A							Infl.	Parainfl.
	PR8	FM1	A2	HK	Equi	SW	Duck	B	Sendai
Ras-IN									
A/PR8	+	+	+	+	+	−	−	−	−
A/FM1/Can/53	⊕	⊕	−	−	⊕	⊕	⊕	−	−
A2/Can/57	+	+1	+	+1	+	+	+	−	−
A2/HK/1/68	+	+	+	+	+	+	+	−	−
A/Equi 1	+2	+2	+3	+1	+3	+1	+1	−	−
A/Swine									
A/Duck	+	+	+1	+	+2	+	+1	−	−
B/Can/5/66	−	−	−	−	−	−	−	+	−
Sendai									
hRAs abs									
A/PR8	+1-2	+2	*+2	*+1	*+2	*+2	*+2	−	−
A/FM1/Can/53	+2	+1	+	+1	+	+1	+1	−	−
A2/Can/57	+1	+1	+2	+1	+	+	+	−	−
A2/HK/1/68	+1	+1	+1	+1	+1	+1	+1	−	−
A/Equi 1	+	*+	+1	+	+1	+	+	−	−
A/Swine	+1	+1	+	+1	+1	+2	+1	−	−
A/Duck	+3	+3	+2	+2	+3	+3	+3	−	−
B/Can/5/66	−	−	−	−	−	−	−	+	−
Sendai	−	−	−	−	−	−	−	−	+
hFAs									
A/PR8	+1	+1	+1	+1	+3	+2	+2	−	⊕
A/FM1/Can/53	+3	+	+	+	+1	*+1	*+1	−	−
A2/Can/57	+2	+	+1	+1	+2	*+	*+	−	⊕
A2/HK/1/68	+$_w$1	+1	+1	+2	+1	+$_w$1	+1	−	−
A/Equi 1	*+3	+1	+1	+1	+1	+	+	−	−
A/Swine	+1	+$_w$1	+$_w$1	*+	+$_w$1	+2	+2	⊕	⊕
A/Duck	+$_w$1	+$_w$1	+2	+2	+2	+2	*+1	−	−
Sendai	−	−	−	−	−	−	−	−	+

the uniformity of the precipitin band formed was not wholly maintained (Table 16 , asterisked). The formation of barbs (Plates 12B arrowed, 13 A arrowed 1, 13C arrowed), splitting of the band into two, and other similar phenomena, suggested that the type-specific antibody in each immune serum was not uniformly specific for all strains of influenza A type-specific component.

The following examples are cited.

The A type-specific precipitin band formed between various influenza A soluble antigens and A2/Can/57 hyperimmune rooster serum (Plate 13 B) was beset with splits and barbs which suggested that type-specific components in at least two of the soluble antigens, Swine and Duck, only partially resembled the others. However, some continuity of the reaction was maintained. When the positions of the serum and soluble antigens were reversed and a seven well template pattern used (Plate 13 A), the precipitin band formed with Duck SA again only partially identified by linkage (evidenced by barb formation, arrows 1) with the uniform band formed by the other influenza A SA. The barb formation observed at the junction of Duck and Equi bands was seen in both reactions (Plate 13 A, B, arrowed) and implied some difference in the type-specific component of each virus. Barb formation was also noted in reactions with FM1 hyperimmune rooster serum (Plate 12 B, arrowed), and with PR8 hyperimmune rabbit serum (abs) (Plate 13 C, arrowed). The uniqueness of the Duck virus type-specific component and its corresponding antibody, was apparent in reactions with Duck hyperimmune rooster serum (Table 16), wherein the single type-specific precipitin band formed with all other influenza A soluble antigens split into two at the homologous reaction.

The type-specific component of influenza A Swine also

differed somewhat from the type-specific components of other influenza A strains (Table 16) when precipitated by PR8 hyperimmune rabbit serum (Plate 13 C arrowed), by hyperimmune rooster sera to FM 1 (Plate 12 B, arrowed), and to A2/Can/57 (Plate 13 B arrowed). The reaction with A2/HK/1/68 hyperimmune rooster serum was very weak (Table 16). In reciprocal reactions of these soluble antigens with Swine hyperimmune rooster serum, A2/HK/1/68 SA partially identified by precipitin band linkage, as evidenced by barb formation; and, FM 1, A2/Can/57, and Equi SA are all very weakly precipitated by anti-Swine antibody. Here again was a demonstration of a type-specific component and its specific antibody which differed from those of other influenza A virus strains.

When influenza A soluble antigens are diffused with PR8 hyperimmune rabbit serum (abs) (Plate 13 C), the continuity of the main type-specific precipitin band formed was interrupted by splits and barbs (arrowed) similar to those described above, and was especially evident with A2/HK/1/68 and Equi SA (arrowed) implying a discrepancy between PR8 SA and these two soluble antigens, although this was demonstrated only by reciprocal reactions with Equi hyperimmune rooster serum (Table 16). Further detailed studies will be necessary to establish the significance of the immunological differences observed in influenza A major type-specific antigens.

In addition to the main type-specific band, other minor bands were variably formed in cross reactions between immune sera and influenza A soluble antigens (Table 16, minor band occurrence indicated by numbers following + signs). These have been described and their specificity indicated in homologous reactions discussed above (Table 15). Minor

precipitin bands have been demonstrated to originate by the precipitation of three general types of components: 1) virus-specific, common to both virus particle and soluble antigen; 2) virus-specific, found only in soluble antigen: 3) host-specific, resulting from precipitation of N-CAM soluble antigen components by hyperimmune rooster sera. The components were not related to the viral hemagglutinin, nor to the neuraminidase (see discussion in preceding section), and those with viral specificity were thought to be antigens which could be associated with the internal components of the virus, in particular, the RNP. On the other hand, they could be the same components precipitated by another type of antibody, for example IgM. Only further detailed investigation will determine their nature and that of the immunoprecipitin reaction.

Use could be made of such minor components to detemine relationships among viruses. Rooster serum, hyperimmune to A2/Can/57, precipitated a common component in the soluble antigens of PR8, A2/Can/57, A2/HK/1/68, Equi, Duck, and FM 1 to form the A type-specific precipitin band (Plate 13 A, arrow 2). A second linking precipitin band (arrow 3) was formed with the soluble antigens of two serologically related viruses, A2/Can/57, and A2/HK/1/68, and with Equi, indicating that they possessed a common component. The band was not formed with the other three serologically unrelated viruses. This observation confirmed the established relationship of A2/Can/57 with A2/HK/1/68, and demonstrated that both of these were related to Equi by a secondary soluble antigen in addition to the type-specific antigen.

An attempt was not made at this stage to analyze the intricacies of the cross reactions among the influenza A virus soluble antigens and

their related sera. The results are presented (Table 16) only to point out that the influenza A soluble antigens, as revealed by immunoprecipitin reactions in cellulose acetate, are not simple and straight-forward as suggested by the type-specific complement fixation test, but comprise a group of related substances which, when identified, might contribute information which could help to clarify the involved genetics of this group of viruses.

For such a study, it would be necessary to perform parallel and more detailed experiments using the type-specific component(s) prepared from whole virus particles, and to compare, and possibly equate, the results obtained with those presented here.

The analysis of these soluble antigen immunoprecipitin patterns has been valuable for interpreting similar reactions with virus concentrate. Using the results obtained above, it was possible to identify the major type-specific immunoprecipitin reactions in cross reactions between virus concentrates and corresponding immune sera. However, in soluble antigen cross reactions, further analysis is required to establish the identity and significance of other minor immunoprecipitin reactions related to the major type-specific component.

Immunoprecipitin Cross Reactions of Influenza A Virus Concentrates

Cross reactions were interpreted only qualitatively because virus strains exhibited different values in biological characteristics (Tables 5, 6), and immune sera varied in potency, as revealed by serological testing (Tables 7, 8, 9). However, homologous immunodiffusion reactions, on the whole, were maintained at equivalence.

The initial screening of immunoprecipitin cross reactions occurring amongst influenza A/PR8, A/FM1/Can/53, A2/Can/57, A2/Hong Kong /1/68, A/Equi 1, A/Swine, A/Duck ,influenza B/Can/5/66, and parainfluenza Sendai, was done in 25-well templates by diffusing semi-purified virus concentrates (C_2 and C_2') against each corresponding immune serum of three sorts: convalescent rabbit, hyperimmune rabbit (abs), and hyperimmune rooster. The antiviral (precipitating) spectrum of each antiserum against each virus strain, and the converse relationship was examined in a checkerboard pattern (Table 17). From this data certain virus relationships were implied (Table 18), and these served as the basis for more detailed studies using 7-well templates (Tables 19, 20).

Initial Screening Cross Reactions

Major Type-Specific Immunoprecipitin Reactions Only the influenza A major type-specific reaction was well-defined and easily identified (Table 17, notation +) because, a) it occurred when each influenza A virus strain was diffused against the following anti-influenza A

LEGEND - TABLE 17

RAs-IN : convalescent serum of rabbit infected with virus

hRAs abs : hyperimmune serum of rabbits parenterally immunized with virus, absorbed with N-CAM

hFAs : hyperimmune serum of rooster parenterally immunized with virus

Influenza virus concentrate antigens C_2, C_2' (semi-purified): PR8 (A/PR8), FM1 (A/FM1/Can/53), A2 (A2/Can/57), HK (A2/HK/1/68), Equi (A/Equi 1), SW (A/Swine, Duck (A/Duck), B (B/Can/5/66); and Sendai (parainfl. Sendai)

\+ : Major type-specific precipitin band linking across all influenza A reactions, or isolated influenza B type-specific reaction

⊕ : Isolated precipitin band which does not link

Number following +, e.g. +2, indicates number of precipitin bands formed in addition to major type-specific band

*+ : indicates break in the continuity (e.g. barb formation, band splitting) of the major type-specific band linkage

w : very weak major type-specific precipitin band formation

\- : no reaction

TABLE 17 Immunoprecipitin cross reactions of influenza A, B, and parainfluenza Sendai virus concentrate antigens. Initial screening.

Antisera	Concentrate Antigen C_2, C_2'								
	Influenza A							Infl.	Parainfl.
RAs-IN	PR8	FM1	A2	HK	Equi	Swine	Duck	B	Sendai
A/PR8	⊕	–	–	–	–	–	–	–	–
A/FM1/Can/53	⊕	⊕	–	–	–	⊕	⊕	–	–
A2/Can/57	*+	+	+	+	+	+	+	–	–
A2/HK/1/68	+	+	+	+1	+	+	+	–	–
A/Equi 1	–	–	–	–	⊕	–	–	–	–
A/Swine	–	–	–	–	–	⊕	–	–	–
A/Duck	*+	*+	+	*+	+	+	*+1	–	–
B/Can/5/66	–	–	–	–	–	–	–	⊕	–
Sendai	–	–	–	–	–	–	–	–	–
hRAs abs									
A/PR8	*+5	+2	+1	+	*+2	*+3	+2	–	–
A/FM1/Can/53	*+2	+3	*+1	+	+	+2	+1	–	–
A2/Can/57	+1	*+1	+3	*+3	+1	*+2	*+1	1	–
A2/HK/1/68	*+	+1	+	+2	+	+1	*+1	–	–
A/Equi 1	+	+1	+1	+1	+1	+1	*+	–	–
A/Swine	+2	+2	$+_w$	$+_w^1$	$+_w$	*+4	+3	–	–
A/Duck	+3	+3	+1	+1	+1	+4	+10	–	–
B/Can/5/66	–	–	–	–	–	–	–	3	–
Sendai	–	–	–	–	–	–	–	–	4
hFAs									
A/PR8	+3	+1	+	+1	+1	+1	+1	–	1
A/FM1/Can/53	+1	+2	+1	+1	+1	+2	+1	–	–
A2/Can/57	+1	+1	+3	+1	+	+1	+1	–	–
A2/HK/1/68	+1	+	+	+4	$+_w$	–	+	–	–
A/Equi 1	*+1	*+1	+3	*+1	+1	*+1	*+1	1	1
A/Swine	+1	*+	+	+1	+	*+3	*+1	1	1
A/Duck	+2	+2	+1	+2	+	+2	*+5	–	1
B/Can/5/66	–	–	–	ND	–	–	–	2	–
Sendai	–	–	–	–	–	–	–	–	4

immune sera: i) sufficiently potent convalescent rabbit sera (anti- A2 /Can/57, -A2/HK/1/68, -A/Duck), ii) all hyperimmune rabbit sera (abs), and iii) all hyperimmune rooster sera, with one exception, anti-A2/HK /1/68 serum did not precipitate A/Swine C_2; b) the A type-specific precipitin bands so formed, linked in identity, indicating that a similar component in each influenza A virus strain was being precipitated by an identical or similar antibody in each anti-influenza A serum; c) the A type-specific reaction did not occur with influenza B or parainfluenza Sendai antigens or antisera, and these yielded only homologous type-specific reactions. These observations agreed with, and confirmed the results obtained in cross reactions, using viral soluble antigens (Table 16). They are also in accord with the identification of the type-specific reaction by Schild and Pereira (1969), which they believe is the RNP.

Cross reactions with influenza A virus concentrates have strengthened the evidence revealed by similar cross reactions using soluble antigens, that the major type-specific component was not immunologically identical in all influenza A virus strains. Barbs and splits occured in the type-specific precipitin band and its linkages, when the type-specific component in one virus was similar, but not identical, to the type-specific component of the virus homologous to the precipitating antibody. The type-specific reactions of viruses demonstrating this phenomenon are asterisked (*+) in Table 17. The incidence was higher than that demonstrated by soluble antigens and the reason for this was not known. Further studies using isolated major type-specific component of each virus strain are required to confirm and relate the above observations.

Examples of the type-specific reactions described above are illustrated in Plates 14-17, and are discussed below.

Discrete homologous immunoprecipitin reactions occured when parainfluenza Sendai (Plate 14 A, extreme right) and influenza B (extreme left) were diffused with their respective hyperimmune rabbit sera (abs). Anti-Sendai (bottom row) and anti-B (top row) rabbit sera did not precipitate any influenza A antigens (centre row). The discreteness of the homologous influenza B reaction was further demonstrated in Plate 15 C. Influenza B virus was not precipitated by anti-Swine hyperimmune rabbit serum (abs), and influenza A virus antigens (inner ring of wells) were not precipitated by anti-B hyperimmune rabbit serum (centre well). Which precipitin band represented the B type-specific reaction was not known, but the one arrowed (arrow 2) was suspected. It occured whether the serum was positioned peripherally or centrally with respect to the virus antigen, and bore no relation whatsoever to the A type-specific band (arrows 1), which linked homologous and cross influenza A reactions in a star-like pattern. Similarly, anti-influenza B convalescent rabbit serum (Plate 15 A, bottom row) precipitated only the homologous virus antigen (extreme left), which was not precipitated by anti-A/Duck convalescent serum (top row). The latter antiserum precipitated only influenza A antigens (centre row) to form a linking precipitin band considered to be the influenza A major type-specific reaction. The discrete specificity of the parainfluenza Sendai reaction was determined in the same way.

Anti-A/PR8 hyperimmune rabbit serum (abs) (Plate 14 B, top row) precipitated only influenza A virus antigens (centre row). The major

type-specific linking precipitin band (arrows 1) was not uniform and continuous, but a barb (arrow 2), and a splitting of the band (arrows 3) appeared, implying that the type-specific component being precipitated in PR8, Equi, and Swine virus was not identical to that in the other influenza A viruses. The strong reaction which swept out on each side illustrated one of the drawbacks of using this template pattern. However, the reaction was influenza A specific, confirmed by other diffusion experiments (e.g. Plate 15 C).

The A type-specific linking band (arrow 1) which resulted when anti-A/FM 1 hyperimmune rabbit serum (Plate 14 B, bottom row) precipitated all influenza A antigens was more even and consistent, but barbs at the PR8-FM 1 linkage area (arrow 4), and at the FM 1-A2/Can/57 linkage area (arrow 5), implied that immunological differences occurred between the major type-specific component of PR8 and FM 1, and the major type-specific component of FM 1 and A2/Can/57 respectively.

Similarly, the major linking influenza A type-specific reaction was demonstrated in diffusions with anti-PR8 hyperimmune rooster serum (Plate 14 C, top and bottom rows, arrows 1); with anti-FM 1 hyperimmune rooster serum (Plate 14 D, bottom row, arrows 1); and with the following hyperimmune rabbit sera (abs), anti-Swine (Plate 16 A, top and bottom rows, arrows 1), anti-A2/Can/57 (Plate 17 A, top row, arrows 1), and anti-A2/HK/1/68 (Plate 17 A, bottom row, arrows 1). Plate 16 A demonstrated a swept out reaction which occasionally occurred when using 25-well templates, but the type-specific precipitin band could be traced across the template.

Using the template pattern in Plate 15 B, and diffusing six influenza A viruses (PR8, FM 1/Can/53, A2/Can/57, A2/HK/1/68, Swine, and Duck - inner ring of wells) with the homologous hyperimmune rabbit sera (abs) (outer ring of wells), a combination of homologous and cross immunoprecipitin reactions occurred. The resulting star pattern which the linking influenza A major type-specific band formed (arrows 1) demonstrated that a similar major type-specific component in each virus was being precipitated by a similar antibody in each antiserum in both homologous and cross reactions. Some irregularity did occur in the type-specific reaction which indicated that all major type-specific components were not immunologically identical (arrows 2, 3, 4). When positions of antiserum and antigen were reversed, a single linking precipitin band (arrow 5) occurred between anti-FM 1 hyperimmune rabbit serum (centre) and the above mentioned influenza A viruses, which was considered to be the major type-specific reaction. This template pattern has demonstrated that in this immunodiffusion system, the broadest precipitin band pattern was achieved when the antisera were positioned peripherally, and the antigen centrally.

By using the above described immunodiffusion patterns, the major influenza A type-specific reaction was identified in homologous and cross reactions between influenza A viruses and convalescent rabbit sera, hyperimmune rabbit sera (abs), and hyperimmune rooster sera.

<u>Secondary Immunoprecipitin Reactions</u> In addition to the major type-specific immunoprecipitin reaction, other precipitin bands, some major, some minor, occurred in homologous and cross reactions. These will

be referred to as secondary immunoprecipitin reactions (Table 17, Plates 14-18. In homologous reactions, a few of these were well-defined and discrete, for example, those occurring between A/PR8 and its hyperimmune rabbit serum (abs) (Plate 14 B, top row); or its homologous hyperimmune rooster serum (Plate 14 C); or between A/Swine and its homologous hyperimmune rabbit serum (Plate 16 A). Secondary precipitin bands formed in homologous reactions were much weaker, reflecting less potent reagents, for example, those occurring between A/FM 1 and its homologous hyperimmune rabbit serum (Plate 14 B, bottom row), or its homologous hyperimmune rooster serum (Plate 14 D, bottom row).

Most secondary precipitin bands yielded by influenza A cross reactions were of moderate or weak strength, as demonstrated by diffusion of influenza A viruses and rabbit sera hyperimmune to A/PR8 (Plate 14 B, top row), and to A/FM 1 (Plate 14 B, bottom row), or by rooster sera hyperimmune to each of these viruses (Plate 14 C and 14 D respectively). Cross reactions between influenza A viruses and convalescent rabbit sera yielded few, if any, secondary precipitin bands as demonstrated by the serum of a rabbit convalescent to A/Duck (Plate 15 A, top row). Some variation in the number of bands formed occurred between experiments, therefore the figures in Table 17 represented the maximum number observed in several experiments.

Attempts were made to characterize the precipitated viral components by immunodiffusion using specific antiserum and viral components isolated by column chromatography (Sephadex G-200), or by polyacrylamide gel electrophoresis of SDS disrupted viruses. The non-specific precipitation by SDS of serum and viral components in immunodiffusion

hampered interpretation of the reactions (see Disruption of Virus Antigens for Immunodiffusion, and Virus Fractionation by Polyacrylamide Gel Electrophoresis), and viral components were thus not characterized in this way.

Therefore, the number of secondary bands occurring in each homologous and cross reaction were counted and recorded (Table 17); and the discreteness of the band(s), or linkage(s) with related band(s) of homologous and cross reactions were noted.

The following examples illustrate linking and independent bands formed in influenza A virus cross reactions. Swine and Duck influenza viruses shared an antigen which was precipitated by anti-FM 1 hyperimmune rabbit serum (abs) to form a linking band (Plate 14 B, arrow 6), whereas an independent PR8 antigen was precipitated by the same serum (arrow 7); A2/Can/57 and FM 1 viruses shared an antigenic component precipitated as a linking band by anti-FM 1 hyperimmune rooster serum (Plate 14 D, arrow 2), while an independent component was also precipitated in the homologous reaction (arrow 3), and in a reaction with Swine virus (arrow 4) . An independent precipitin band formed between Duck virus and its homologous convalescent rabbit serum Plate 15 A indicated the component precipitated was unique to the Duck influenza A virus.

The analysis and compilation of secondary precipitin reactions in this way contributed towards determining the relationships between influenza A viruses.

<u>Influenza A Virus Relationships</u> The initial screening of influenza A virus cross reactions revealed that:

1) all influenza A viruses possessed an immunologically similar,

though not always identical major type-specific component, which was demonstrated reciprocally in all immunodiffusion cross reactions with hyperimmune rabbit and rooster sera except one, and with sufficiently potent convalescent rabbit sera (Table 17). The component was unique to influenza A viruses and was not related to influenza B or parainfluenza Sendai type-specific components respectively.

2) Certain influenza A viruses were more closely related immunologically than others because they were shown, by precipitin band linkages of identity in immunodiffusion reactions to share one or more antigenic components in addition to the major type-specific component. Some viruses formed independent precipitin bands in homologous and cross reactions, in addition to linking bands, or in their absence. Independent precipitin bands resulting from cross reactions suggested that there was some association between antigens of the precipitated virus and the virus homologous to the precipitating antiserum. However, it could have been the reflection of a viral component which bore no such relationship.

Therefore, a diminishing order of relationships amongst influenza A viruses was tentatively suggested by demonstrating a) the presence of shared antigens, b) the occurrence of independent and possibly related antigens in association with (a), or separately. Those viruses which cross reacted reciprocally were considered to be the most closely related. The results of these experiments are summarized in Table 18. Viruses are

LEGEND - TABLE 18

hRAs/V abs : hyperimmune serum of rabbits parenterally immunized by virus, absorbed with N-CAM

hFAs/V : hyperimmune serum of rooster parenterally immunized by virus

Influenza viruses : PR8 (A/PR8), FM1 (FM1/Can/53), A2 (A2/Can/57) HK (A2/Hong Kong/1/68), Equi (A/Equi 1), SW (A/Swine), Duck (A/Duck), B (B/Can/5/66)

and Sendai (parainfluenza Sendai)

The major influenza A type-specific immunoprecipitin reaction (band) is formed by cross reactions between all influenza A viruses listed, and corresponding hyperimmune sera (exception: A/Swine not precipitated by hFAs/HK). Influenza B and parainfluenza Sendai each form homologous type-specific reactions.

Viruses are horizontally listed in relative order of relationship to the virus in column 1. The order is based on the number of precipitin bands formed in addition to the major type-specific one. Many of these are minor and their identity has yet to be established. Viruses considered to be most closely related are those which cross interchangeably and which share antigenic components as demonstrated by linking precipitin bands.

[a]Cross :Virus Antigens Precipitated : Antigens of virus listed are precipitated by the homologous antiserum of the virus indicated in column 1 (Influenza Virus)

[b]Reciprocal Cross : Virus Antigen Precipitins : Precipitins in antisera listed, precipitate antigens of the virus indicated in column 1

[c]Cross and Reciprocal Cross : Both above described immunoprecipitin reactions occur. Homologous antiserum of the virus in column 1 precipitates the listed virus antigens, and the homologous antisera of these viruses reciprocally precipitate the virus indicated in column 1

Viruses in brackets, e.g. (PR8), form only the major type-specific precipitin band

TABLE 18 Relationships of influenza A viruses revealed by immunoprecipitin cross reactions. Initial screening using 25-well templates.

A. Undisrupted virus concentrates (C_2, C_2') and hRAs/V (abs)

Influenza Virus	[c]Cross and Reciprocal Cross	[a]Cross: Virus Antigens Precipitated	[b]Reciprocal Cross: Virus Antigen Precipitins
A/PR8	PR8 Swine Duck FM1 A2 (HK)	Equi	
A/FM1/Can/53	FM1 Swine PR8 A2 Duck		HK Equi
A2/Can/57	A2 Equi Duck PR8 FM1	HK Swine	
A2/HK/1/68	HK Swine Duck (PR8)	FM1	A2 Equi
A/Equi 1	Equi A2	HK FM1 Swine	Duck PR8
A/Swine	Swine Duck PR8 FM1 HK		A2 Equi
A/Duck	Duck Swine PR8 FM1 A2 HK	Equi	
B/Can/5/66	B		
Sendai	Sendai		

B. Undisrupted virus concentrates (C_2, C_2') and hFAs/V.

Influenza Virus	[c]Cross and Reciprocal Cross	[a]Cross: Virus Antigens Precipitated	[b]Reciprocal Cross: Virus Antigen Precipitins
A/PR8	PR8 FM1 Swine Duck Equi		A2 HK
A/FM1/Can/53	FM1 PR8 A2 Equi Duck	Swine	HK
A2/Can/57	A2 Duck FM1	Swine, PR8 HK	Equi
A2/HK/1/68	HK	PR8	FM1 A2 Duck Equi Swine
A/Equi 1	Equi PR8 FM1	A2 HK Swine Duck	
A/Swine	Swine Duck PR8	HK	FM1 A2 Equi
A/Duck	Duck Swine PR8 FM1 A2	HK	Equi
B/Can/5/66	B		
Sendai	Sendai		

listed (horizontally) in tentative order of diminishing relationships to the virus strain in the lefthand column according to the scheme just described; Part A using hyperimmune rabbit serum (abs); Part B using hyperimmune rooster serum. Cross reactions with convalescent rabbit sera revealed mainly the major type-specific reaction, and were therefore not included.

A slightly different order of influenza A virus relationships was revealed by cross reactions using each serum system. However, both systems demonstrated that, in addition to the major type-specific component, each influenza A virus strain shared, or was associated with, at least one additional antigen of another strain. Certain viruses interrelated consistently:- PR8, Swine, Duck, and FM1/Can/53. A2/Can/57 bore some relationship to this group, especially to FM1, but appeared to be more closely related to Equi 1. Equi shared very few antigens with the first mentioned group but this may have been influenced by the low potency of antigen and antiserum used. The connection between A2/Hong Kong/1/68 and the other influenza A viruses was fairly remote.

The general observations above were used as a basis for detailed studies of influenza A virus cross reactions by immunodiffusion in 7-well templates. The listings in Table 18 and the data in Table 17 were used as a basis, to guide the juxtapositioning of viruses and immune sera, so that the relationships amongst the viruses could be best revealed.

Preliminary Detailed Studies

A closer, more detailed examination of the interrelationships of

influenza A virus strains, as revealed by immunodiffusion cross reactions in 7-well templates, was initiated. Both undisrupted semi-purified virus concentrates (C_2, C_2') and Nonidet P40 disrupted semi-purified and sucrose density gradient purified (SDG) concentrates were used. Only reactions with hyperimmune rabbit sera (abs) were considered here because many of the diffusions using hyperimmune rooster sera were unsatisfactory. The results reported at this stage were considered to be preliminary, but they did contribute some useful information (Tables 19, 20, Plates 16, 17, 18).

Juxtapositions of Antigens and Antisera The relative positioning of antigens and antisera in cross reactions influenced the number of precipitin bands yielded, that is, the viral components precipitated. More were formed when the antigen was located centrally and the antisera peripherally, than in the converse arrangement. For example, more precipitin bands were formed when centrally located FM1 C_2 was diffused against hyperimmune rabbit sera (abs) to FM1/Can/53, PR8, Duck, Swine, Equi 1, A2/Can/57 (Plate 18 B), than when the FM1 antiserum was central, and the antigens were peripheral (Plate 18 A).

Each arrangement yielded slightly different information. The first arrangement demonstrated the capacity of different antisera (peripherally located) to precipitate the antigens of one virus strain (centrally located) (Plates 18 B, C, D, 16 C, D), whereas the latter arrangement demonstrated the capacity of the centrally located antiserum to precipitate antigens of different A strains which were peripherally located (Plates 18 A, 16 B).

Shared and independent antigens were thus revealed by the two arrangements. For example, the major A type-specific component of influenza A virus strains was precipitated by anti-influenza A antisera to form a precipitin band linking all around (Plates 18 A, B, 16 B, arrows 1); a secondary linking precipitin band was formed when various anti-influenza A sera (Swine, Duck, PR8, FM 1) precipitated the same FM1 antigen (Plate 18 B, arrows 2), implying that the homologous viruses of these sera shared this antigen; and an independent band was formed by a homologous FM1 reaction (Plate 18 A, arrow 2), by a cross reaction between anti-Swine hyperimmune rabbit serum (abs) and FM1 C_2 (Plate 18 B, arrow 3), and between anti-Duck serum and FM1 C_2 (arrow 4). The independent reactions suggested that these antigens were unique to each virus precipitated and to the homologous virus of the precipitating antiserum.

The arrangement most used in this study was the one in which a centrally located antigen was diffused against peripherally located antisera.

Use of Undisrupted and NP40 Disrupted Virus Concentrates in Cross Reactions The use of undisrupted and NP40 disrupted semi-purified and purified virus concentrates was compared in cross reactions (Table 19, Plate 18). Disrupting viruses with NP 40 had variable effects on reactivity compared to that of undisrupted viruses.

In homologous reactions, NP 40 disruption of virus concentrates (C_2, C_2') increased the number of components precipitated by antisera in A2/Can/57 and Equi 1 C_2'; and in A2/Hong Kong/1/68 SDG compared to the number obtained with undisrupted virus C_2; but did not affect, or lowered,

LEGEND - TABLE 19

Same notations as in Table 17, with the following additions:

SDG	:	influenza A virus concentrate purified by sucrose density gradient
NP 40 disrupted:		virus concentrates disrupted by 1% Nonidet P40
ND	:	not done

TABLE 19 Immunoprecipitin cross reactions of influenza A virus concentrates, undisrupted and Nonidet P40 disrupted. Preliminary detail studies.

Influenza A Antigen	Antiserum hRAs/Influenza A (abs)						
	PR8	FM1	A2	HK	Equi	Swine	Duck
C_2, C_2' (Undisrupted)							
PR8	+3	+1	+1	+	+	+2	+3
FM1/Can/53	+1	+2-3	+2	+	+	+3	+3
A2/Can/57	+1	+1	+1	+	+1	+1	+1
A2/HK/1/68	+	+	+1	+1	+1	$+_w$	+
Equi 1	+	+	+1	+	+	+	+
Swine	+1	+	+	+	+	+4	+1
Duck	+2	+1	+1	+	+	+2	+5
C_2, C_2' (NP 40 Disrupted)							
PR8	+1	+	ND	+	$+_w$	+	+
FM1/Can/53	+	+1	+	ND	+	+1	+2
A2/Can/57	+1	+2	+3	+	+2	+1	+1
A2/HK/1/68	+2	ND	+1	+1	+1	+	+1
Equi 1	+	+	+3	+	+1	+	+
Swine	+	+	ND	+	+	+	+
Duck	+	+	ND	+	+	+	±4
SDG (NP 40 Disrupted)							
PR8	+2	+1	+	+	+2	+2	+1
FM1/Can/53	+1	+2	+	+	ND	+	+
A2/Can/57	ND	ND	ND	ND	ND	ND	ND
A2/HK/1/68	+	-	+1	+2	+	+	-
Equi 1	ND	ND	ND	ND	ND	ND	ND
Swine	+1	+	+	+1	+	+3	+1
Duck	+1	+1	+	+	+	+	+4

LEGEND - TABLE 20

Same notations as in Table 18, with the following additions:

Virus Concentrate (SDG): sucrose density gradient purified virus concentrate

Virus horizontally listed PR8 FM1, etc. : forms secondary precipitin band(s) in cross reactions, which link in reactions of identity with those of homologous virus reactions

Virus horizontally listed and dot underscored e.g. PR8 FM1 : forms secondary precipitin band(s) in cross reactions, independent of homologous virus reaction

Virus horizontally listed and underlined e.g. PR8 FM1 : forms secondary precipitin band(s) which link in identity with homologous reaction band(s), and forms band(s) independent of homologous reaction

Viruses in brackets, e.g. (HK Equi), form only the major type-specific precipitin band.

Independent components were classified in the same way as shared components with respect to cross and/or reciprocal cross reactions. Influenza A virus strains demonstrating shared and independent components in mutual cross reactions were considered to be the most closely related.

LEGEND - TABLE 20 (contd)

To illustrate the interpretation of Table 20, the relationship between influenza A/PR8 and other influenza A strains (Part B) is described:

Under "Cross and Reciprocal Cross", anti-PR8 hyperimmune rabbit serum (abs) precipitated secondary components, both shared and independent, of the homologous virus, of Swine and Duck, The homologous antisera of the latter two viruses reciprocally precipitated PR8 virus components (PR8 Swine Duck) (See Table 19 for data). An independent FM1 component was precipitated by anti-PR8 serum, and anti-FM1 serum precipitated an independent PR8 component (FM1). Under "Cross", anti-PR8 serum precipitated two A2/HK/1/68 components, one shared and one independent (HK), but precipitated only a shared A2/Can/57 component (A2). Under "Reciprocal Cross", anti-Equi serum precipitated two PR8 components, one shared and one independent (Equi). To illustrate one further designation in the table (refer to Part A, same virus, A/PR8); anti-PR8 serum precipitated only the major type-specific component of A2/HK/1/68 and of Equi 1, and the homologous sera of these viruses precipitated only the major type-specific component of PR8 [(HK Equi)] .

TABLE 20 Relationships of influenza A viruses revealed by immunoprecipitin cross reactions. Preliminary detail studies using 7-well templates.

A. Undisrupted virus concentrates (C_2, C_2') and hRAs/V (abs).

Influenza Virus	[c]Cross and Reciprocal Cross	[a]Cross: Virus Antigens Precipitated	[b]Reciprocal Cross: Virus Antigen Precipitins
A/PR8	PR8 Duck Swine FM1 A2 (HK Equi)		
A/FM1/Can/53	FM1 Duck A2 PR8 (HK Equi)		Swine
A2/Can/57	A2 FM1 PR8 Equi Duck	HK	Swine
A2/HK/1/68	HK (PR8 FM1 Duck Swine)		A2 Equi
A/Equi 1	(Equi) A2 (PR8 FM1 Duck Swine)	HK	
A/Swine	Swine Duck PR8 (HK Equi)	FM1 A2	
A/Duck	Duck PR8 FM1 Swine A2 (HK Equi)		

B. Nonidet P40 disrupted virus concentrates (C_2, C_2', SDG) and hRAs/V (abs)

Influenza Virus	[c]Cross and Reciprocal Cross	[a]Cross Virus Antigens Precipitated	[b]Reciprocal Cross: Virus Antigen Precipitins
A/PR8	PR8 Swine Duck FM1	HK A2	Equi
A/FM1/Can/53	FM1 Duck PR8 (Equi)	A2	Swine (HK)
A2/Can/57	A2 Equi	HK	FM1 PR8 Swine Duck
A2/HK/1/68	HK	Swine (FM1)	PR8 A2 Equi Duck
A/Equi 1	Equi A2 (FM1 Swine Duck)	PR8 HK	
A/Swine	Swine PR8 (Equi)	FM1 A2	HK Duck
A/Duck	Duck FM1 PR8 (Equi)	A2 HK Swine	

the number of components precipitated in the other virus preparations (Table 19). In cross reactions, greater cross reactivity (exhibited as precipitin bands) was observed when using NP 40 disrupted A2/Can/57, A2/HK/1/68, and Equi C_2' and Swine SDG, than when using undisrupted viruses, but equal, or less reactivity, occurred using the other virus preparations (Table 19). For example, when various influenza A antisera were diffused with undisrupted FM1 C_2 (Plate 18 B), more secondary cross reactions occurred than in diffusions with NP 40 disrupted FM1 C_2 (Plate 18 C) or FM1 SDG (Plate 18 D). The homologous reaction was not much affected. The major influenza A type-specific component was usually precipitated as a band linking all around (Plate 18 B, C, D, arrows 1); its position in relation to secondary components was altered in reactions with NP 40 disrupted FM1 C_2 (compare Plates 18 B and C, arrows 1).

Non-specific precipitation, by NP 40, of serum proteins and viral components (Plates 16 - 18, label NS in all reactions using NP 40 disrupted virus antigens), could have depleted potentially reactive antibody and/or virus components which would have accounted for the more restricted reactions observed. However, the desired increase in precipitin band spectrum was attained with the other viruses discussed above. Therefore, it was advantageous to use and compare immunodiffusion cross reactions using both undisrupted virus C_2 and NP 40 disrupted virus C_2 and SDG.

<u>Analysis of Immunoprecipitin Cross Reactions</u> Influenza A virus relationships listed in Table 20 were inferred from the data compiled in Table 19, by the analysis of immunodiffusion cross reactions, typified by those found in Plates 16 B, C, D, 17 B, C, D, 18 A-D. A few examples are described to demonstrate the basis for Tables 19 and 20.

Initial screening of influenza A/Swine in cross reactions (Tables 17, 18, Plate 16 A) suggested that it was more closely related to certain influenza A strains - Duck, PR8, FM1 - than to others. Detailed cross reactions confirmed the previous observations that influenza A Swine shared the A major type-specific component with all other influenza A strains examined (Plate 16 B, C, D, arrows 1, Table 19). Anti-Swine hyperimmune rabbit serum (abs) precipitated the major type-specific components of various A strains, forming precipitin bands which linked in identity (Plate 16 B, arrow 1) (cross reaction), and the antisera of these viruses precipitated Swine virus as undisrupted C_2, or as NP 40 disrupted C_2 and SDG (Plate 16 C, D, respectively, arrow 1) (reciprocal cross reaction). One other virus component (secondary) was precipitated in undisrupted PR8 and Duck C_2 by anti-Swine rabbit serum (abs) (Plate 16 B, arrows 2); in a reciprocal reaction, a secondary component in disrupted Swine SDG was precipitated by hyperimmune rabbit sera (abs) against PR8 and A2/HK/1/68, which linked with the homologous reaction (Plate 16 D, arrows 2). In another experiment, anti-Duck rabbit serum also precipitated a secondary Swine component. Two independent precipitin bands adjacent to the antiserum well were formed in Swine homologous reactions (Plate 16 C, D). The results of several such experiments were thus analyzed and recorded in Table 19.

Influenza A2/HK/1/68, in initial screening cross reactions, was demonstrated to be related to influenza A virus strains by sharing the major influenza A type-specific component, and at least one other secondary antigen (Tables 17, 18, Plate 17 A, bottom row). Detailed studies confirmed the presence of the common type-specific component when undisrupted C_2 (Table 19) and NP 40 disrupted C_2 were diffused with various

anti-influenza A hyperimmune rabbit sera (abs) (Plate 17 B, arrows 1), but the reaction was much weaker when disrupted, less potent purified virus was used (Plate 17 C, arrows 1). The reaction was very weak between the virus and hyperimmune rabbit sera to PR8 and Swine, and was absent with the anti-FM1 serum. This observation suggested that the major type-specific component of Equi 1 and A2/Can/57 was probably more closely related to that of A2/HK/1/68 than was the major type-specific component of the other three viruses - PR8, FM1, Swine. Anti-PR8 hyperimmune rabbit serum (abs) precipitated two secondary A2/HK/1/68 components, forming one precipitin band (Plate 17 B, arrow 2) which linked with the one formed by precipitation of the same component by antiserum against Equi and against the homologous virus; and forming a second precipitin band (Plate 17 B, arrow 3) which linked with the band of the same component precipitated by anti-Duck antiserum. This demonstration of antigen sharing implied that closer relationships existed amongst these strains than others. Anti-A2/Can/57 rabbit sera precipitated a closely related homologous A2/HK/1/68 component (Plate 17 B, arrow 4). Secondary components of disrupted purified A2/HK/1/68 virus were precipitated faintly and were difficult to interpret (Plate 17 C).

A close relationship between A2/Can/57 and Equi 1, suggested by initial screening (Tables 17, 18) was confirmed in detailed studies in which various anti-influenza A hyperimmune rabbit sera (abs) were diffused with NP 40 disrupted A2/Can/57 C_2' (Plate 17 D). The major influenza A type-specific component was precipitated by all anti-A sera to form a clearly-defined, linking precipitin band (arrows 1), which implied that the type-specific component was shared by each influenza A

strain examined - A2/Can/57, Equi, A2/HK/1/68, Swine, FM1, PR8. Two of three secondary components precipitated in the homologous reaction were also precipitated by anti-Equi serum, and the bands thus formed, linked (arrows 2, 3), indicating a sharing of antigens by the two virus strains. The component arrowed 3 was also shared by PR8, FM1, and A2/HK/1/68, because the antisera against these viruses weakly precipitated it to continue the linking precipitin band. An independent A2/Can/57 component precipitated by anti-Swine serum, which lay adjacent to the serum well, implied the sharing of a unique antigen between these two influenza A strains.

Immunological differences observed amongst the major type-specific component of influenza A strains in cross reactions using soluble antigens and in initial screening cross reactions were not as evident in detailed studies. They were notably lacking when NP 40 disrupted virus antigens were used, except in those instances where the major type-specific cross reaction was much weaker than the homologous one.

Other experiments in addition to those described revealed additional information which was incorporated into Tables 19 and 20. In this way immunodiffusion cross reactions, carried out with all influenza A strains studied, were analyzed to determine what relationships existed amongst them as revealed by this method.

<u>Influenza A Virus Interrelationships</u> The limited immunoprecipitin cross reactions carried out (Table 19) inferred a certain relative order of relationships amongst influenza A strains which are listed in Table 20. Interpretations given below must be regarded with some caution because they

are based on reactions with single or double sera.

The results yielded by cross reactions using undisrupted semi-purified virus concentrates (C_2 and C_2') (Table 20 A) were not in complete agreement with those obtained using NP 40 disrupted semi-purified and purified (SDG) virus concentrates (Table 20 B). However, they were complementary and were therefore interpreted collectively. All influenza A viruses were demonstrated to possess the influenza A type-specific component by cross and reciprocal cross reactions. Continuing the usage in the section on Initial Screening Cross Reactions, this component is referred to as the primary or major type-specific component, and all other components as secondary.

Virus strains sharing secondary components were regarded as more closely related than those which did not. At this stage of knowledge in the investigation, the closeness of the relationship between virus strains was considered to vary directly with the number of shared secondary components. The demonstration of mutual or reciprocal cross reactions suggested a closer association between virus strains than one way cross reactions. Thus homologous antibody to one strain, precipitating a common component in a heterologous strain, indicated an association existed between the strains.

In addition to shared antigens, precipitation of independent components in cross reactions were also considered in determining relationships amongst influenza A strains. The precipitation of a component in a heterologous strain by the homologous antibody to form an independent or discrete reaction, implied an association of some sort between the two strains. The demonstration of such interactions reciprocally, strengthened the relationship between them.

When an independent component was precipitated in homologous reactions, its presence contributed towards, and reflected the uniqueness of the virus character.

The significance of various cross reactions and reciprocal reactions is weighted by the criteria given in Table 20, which indicates interstrain relationships. The strains are horizontally listed in order of diminishing relationship to the virus strain in the left hand column.

The interrelationships among influenza A strains tabulated in Table 20 are schematically summarized in Figure 8. The arrows represent the precipitating activity of the antiserum and point towards the heterologous virus whose antigens are precipitated.

Influenza A strains demonstrating the greatest number of interrelationships are: Duck and PR8; A2/Can/57 and Equi 1. Anti-Duck antiserum precipitated three PR8 components (shared and independent), whereas only two Duck components (shared and independent) were precipitated by anti-PR8 serum, implying that the anti-Duck serum had a greater capacity to precipitate PR8 antigens than the anti-PR8 serum had to precipitate Duck antigens. Three Equi components were precipitated by anti-A2/Can/57 serum, and two A2 components by anti-Equi serum, which suggested that anti-A2/Can /57 serum had the greater precipitating capacity.

Because an equal number of shared and/or independent components were mutually precipitated in cross reactions, relationships between certain viruses were balanced, and the closeness of the association varied directly with the number of components precipitated. Such

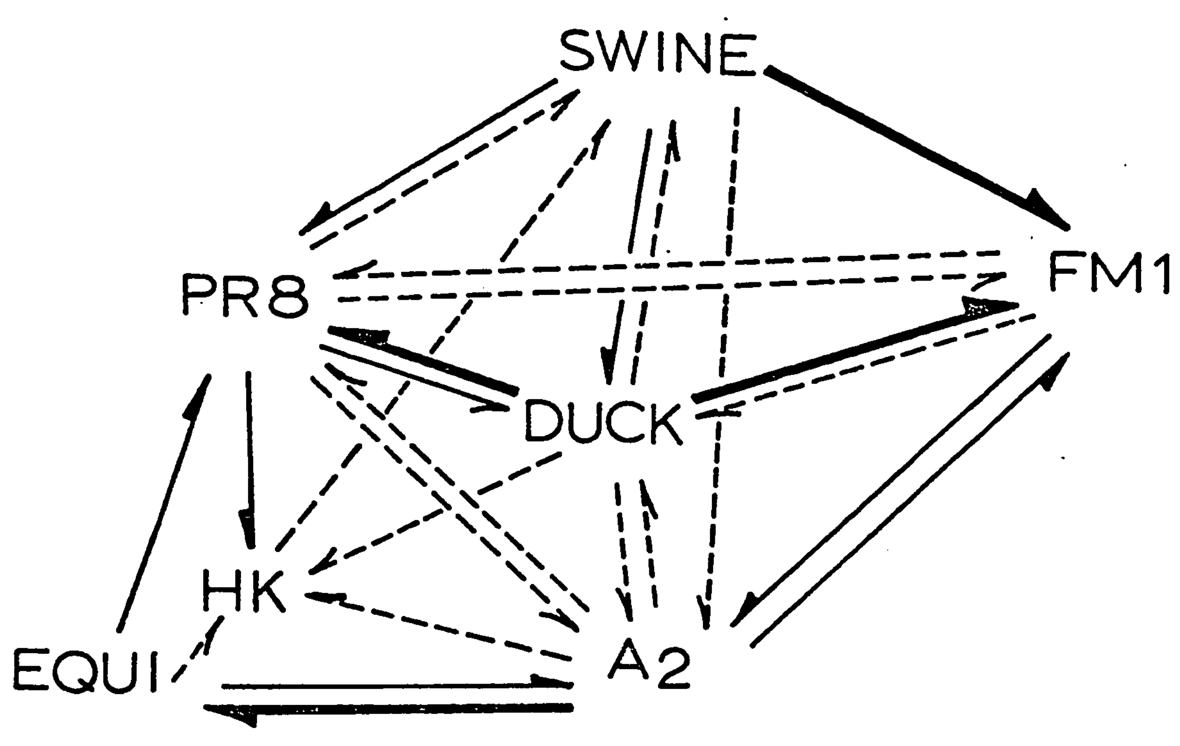
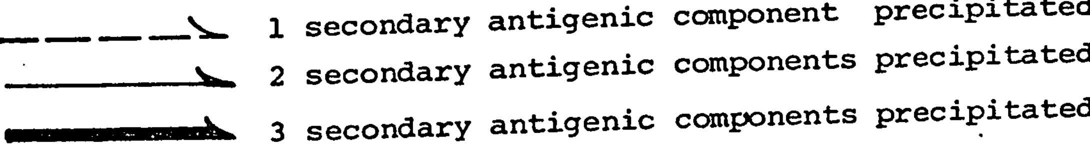


Figure 8. Interrelationships of influenza A virus strains revealed by secondary immunoprecipitin reactions.

Arrows represent the precipitating capacity of the specific antiserum and point towards the heterologous virus whose antigens are precipitated



(Lengths of arrows not significant)

viruses are listed in the following diminishing order of relationship: A2/Can/57 and FM1/Can/53 (2shared antigens); PR8 and FM1, PR8 and A2/Can/57 (one shared antigen); A2/Can/57 and Duck (one independent antigen).

All other relations were not balanced. When the homologous antiserum of one virus, precipitated more components of a second virus than were precipitated in the reciprocal reaction, the first antiserum demonstrated the greater precipitating capacity. When the reaction occurred in only one direction, and not reciprocally, the relationship was more distant. Viruses demonstrating such uneven associations, in decreasing order of relationship are (numbers of virus components precipitated in parentheses): Duck and FM1/Can/53 (3 FM1: 1 Duck); Swine and Duck (2Duck: 1 Swine); Swine and PR8 (2 PR8: 1 Swine); Swine and FM1/Can/53 (3 FM1); PR8 and A2/HK/1/68 (2 A2/HK/1/68); Equi 1 and PR8 (2 PR8) A2/HK/1/68 and A2/Can/57, Equi 1, Duck (1 HK); A2/HK/1/68 and Swine (1 Swine); Swine and A2/Can/57 (1 A2). Therefore, anti-Duck serum had greater precipitating capacity than anti-FM1 serum, and anti-Swine serum had a greater capacity than anti-Duck serum, and so on to the end of the list. Table 20 indicates whether the components were shared and/ or independent.

The relationships of each influenza A strain with all the others are summarized as follows (Fig. 8);

<u>A/PR8</u> had reciprocal relations with all strains except Equi 1 and A2/HK/1/68. Duck was closest, followed by Swine, FM1, and A2/Can/57, in that order. A2/HK/1/68 and Equi 1 were more distant, because

precipitation of A2/HK/68 components by anti-PR8 serum was not reciprocated, nor was precipitation of PR8 components by anti-Equi serum.

A/Duck was more closely related to PR8 than to FM1 or Swine and more distantly to A2/Can/57 or A2/HK/1/68; the connection with Equi 1 was only through the primary type-specific component.

A2/Can/57 had some association with all strains, the closest being with Equi 1, then with FM1 followed by PR8 and Duck. The one-way reactions of anti-A2/Can/57 serum precipitating A2/HK/1/68, and of anti-Swine serum precipitating A2/Can/57, suggested more distant associations.

A/Equi 1 was associated with only three strains: most intimately with A2/Can/57, and more remotely with PR8 and A2/HK/1/68. The connection with the other A strains was only through the primary type-specific component.

A/Swine had reciprocal relations with only Duck and PR8. A close association with FM1/Can/53, indicated by anti-Swine serum precipitating three FM1 components, was not reflected in converse reactions. A one-way reaction with anti-A2/HK/1/68 serum suggested a distant association.

A/FM1/Can/53 relationships were more restricted. Reciprocal ones occurred with three strains, the closest with A2/Can/57, followed by Duck and PR8 in that order. Anti-FM1 serum did not precipitate Swine components despite the strong converse precipitation by anti-

Swine serum, demonstrating an uneven association. Only the primary type-specific component was shared with Equi 1 and A2/HK/1/68.

A2/Hong Kong/1/68 was remotely associated with all other strains because all reactions were unidirectional. Swine was the only strain affected by anti-A2/HK/1/68 serum; whereas A2/HK/1/68 components were precipitated by the antisera against PR8, A2/Can/57, Equi 1, Duck, in order of diminishing reactivity. Only the primary type-specific component was shared with FM1/Can/53.

It is of note that PR8 (antigens and specific antiserum) entered into the greatest number of cross reactions - 16. The reactivity exhibited by the other strains was: A2/Can/57 - 15, Duck - 15, FM1/Can/53 - 13, Swine - 11, Equi 1 - 8; and the least reactive was A2/HK/1/68 - 6.

Possibly related to the above, is the observation that secondary cross reactions occurred by PR8 and A2/Can/57 with all other strains; by Duck and Swine with all strains except Equi; by FM1/Can/53 with all strains except Equi and A2/HK/1/68; by A2/HK/1/68 with all strains except FM1/Can /53; and Equi 1 only cross reacted with A2/Can/57, PR8, and A2/HK/1/68.

Therefore, the most highly cross reactive strain was PR8, and the least was A2/HK/1/68, the other strains ranging in activity between these two.

The results of detailed studies generally confirmed and elaborated those obtained in the initial screening cross reactions. On the basis of secondary reactions, PR8, Duck, and Swine had close, consistent inter-relationships, and FM1/Can/53 to a slightly lesser degree. A2/Can/57 was

related to this group especially to FM1, but was most closely connected to Equi 1. In addition to A2/Can/57, Equi was related to only PR8 and A2/HK/1/68. The relationships between A2/HK/1/68 and all other strains was weak.

Each individual strain was characterized by one or more antigens which were immunologically unique.

The next stage in this investigation will be to establish the identity of precipitin band components involved in homologous and cross reactions. It should be possible to identify the antigens being precipitated, and to establish which ones are unique to individual strains, and which are common to two or more strains.

The influenza virus (WSN) has been shown to be composed of at least seven proteins of different sizes, three to four glycoproteins, and three to four carbohydrate-free proteins (Compans et al., 1970; Schulze, 1970, 1972; Skehel and Schild, 1971 Klenk et al., 1972). Each of these could be precipitated by a specific antibody to yield seven precipitin bands in immunodiffusion. In this investigation, one of these has been identified as the primary or major type-specific component which could be the RNP (see Discussion following), therefore the other immunoprecipitin reactions detected could be due to precipitation by antibody of the three to four glycoproteins which include neuraminidase and hemagglutinin, and the other two to three carbohydrate-free virion proteins, Whether these are precipitated as monomers or as polymerized components is not known. In addition, up to two minor components, related to the virus, and associated with the primary or major type-specific component originating

from soluble antigen extracts, have also been detected in this investigation. The influenza virus components they represent have yet to be determined.

Not all precipitation of the virus antigens described was by specific antibody. Envelope associated antigens such as the hemagglutinin could be precipitated by influenza inhibitors of the β-or γ-type, shown to be present in hyperimmune and convalescent sera. Therefore this factor will also have to be considered when identifying the origin of precipitin bands.

A true interpretation of immunological interrelationships amongst influenza A strains, as revealed by immunodiffusion, can be made only when the antigenic and serum components forming each precipitin band are accurately defined by using isolated and purified reagents: virus antigens, serum antibodies (specific gamma-globulins), and serum inhibitors.

The investigation to this stage has merely pointed out, that, on the basis of immunoprecipitin reactions using virus particle concentrates, virus soluble antigens, and convalescent and hyperimmune sera, certain relationships have been shown to exist among influenza A strains. Having established that these relationships exist, intensive and detailed studies as described above will be required to determine the exact nature of influenza A virus interrelationships.

Hemagglutination Inhibition Cross Reactions of Influenza A Virus Strains

Hemagglutination inhibition (HAI) cross reactions were carried out on all immune sera used in immunodiffusion cross reactions, which included convalescent rabbit, hyperimmune rabbit, and hyperimmune rooster against influenza A/PR8, A/FM1/Can/53, A2/Can/57, A2/Hong Kong/1/68, A/Equi 1, A/Swine A/Duck, influenza B/Can/.5/66, parainfl. Sendai, and N-ALF. Corresponding virus strains as infected ALF were used as antigens; N-ALF was not used (Tables 21-23).

It was difficult to correlate HAI and immunodiffusion results because the HAI test measures the presence of antibodies to only one component, the hemagglutinin, whereas the immunodiffusion test detects the presence of antibodies to hemagglutinin, and to at least five other viral components including the RNP (Compans et al., 1970; Schulze, 1970; Skehel, Schild, 1971). Because the sera used in HAI tests were multi-specific, the steric effects of neuraminidase-specific reactions also came into play (Kilbourne, 1968) making comparative interpretations difficult. The presence of non-specific inhibitors of influenza viruses also interfered with the interpretation of both tests (see Inhibitor Review, Part II). Because the hemagglutinin-specific immunoprecipitin reaction was not identified in immunodiffusion reactions, it was not possible to directly compare the cross reactivity amongst influenza A strains detected by HAI and immunodiffusion tests. However, some general observations were made, but a detailed analysis was not undertaken.

Legend TABLES 21-23

Hemagglutination Inhibition (HAI) titres expressed as reciprocal of the dilution. Micro method using 0.025 ml volumes and 4 HAU/0.025ml virus antigen

Serum treatments (in parentheses following serum type):

THP : trypsin, heat, potassium periodate (M/90)

RDE : RDE (*V. cholerae* filtrate) @ 500 units /ml

$NaIO_4$: $NaIO_4$ (M/90)

In Table 26, serum treatment is designated after each virus strain because it was determined by the strain used as antigen (e.g. PR8 (RDE))

Blank Spaces: HAI titre < 10

In Table 22, hRAs ($NaIO_4$), all blank spaces signify not done.

ND : not done

TABLE 21. Hemagglutination inhibition cross reactions of influenza A, B, and parainfl. Sendai. Convalescent rabbit sera (RAs-IN).

Antisera	Virus Antigen (ALF)								
	Influenza A							Infl. B	Parainfl. Sendai
RAs-IN	PR8 (RDE)	FM1 (RDE)	A2 ($NaIO_4$)	HK (RDE)	Equi ($NaIO_4$)	Swine (RDE)	Duck (RDE)	(RDE)	(RDE)
A/PR8	10								
A/FM1/Can/53		10							
A2/Can/57			20		20				
A2/HK/1/68				20					
A/Equi 1									
A/Swine						10			
A/Duck							20		
B/Can/5/66								10	30
Sendai			40		30				20
N-ALF									

TABLE 22. Hemagglutination inhibition cross reactions of influenza A, B, and parainfl. Sendai. Hyperimmune rabbit sera (hRAs).

Antisera	Virus Antigen (ALF)								
	Influenza A							Infl.	Parainfl.
hRAs (THP)	PR8	FM1	A2	HK	Equi	Swine	Duck	B	Sendai
A/PR8	15 360	40	40	1280	20	80		ND	
A/FM1/Can/53	80	1280	120	320	120	60		ND	80
A2/Can/57	10		5120	480	2560	60		ND	10
A2/HK/1/68	40	20	80	10 240	80	20		ND	10
A/Equi 1	40	40	240	10	640	15		ND	
A/Swine	80	60	120	320	80	5120		ND	10
A/Duck	10		40	320	80		1280	ND	
B/Can/5/66	80	20	240	640	60	40		ND	20
Sendai	40	40	120	640	60	20		ND	480
N-ALF	40		160	320	160			ND	
hRAs (RDE)									
A/PR8	10 240	160	80	30	40	640			
A/FM1/Can/53	40	1280	160	40	40	40			
A2/Can/57	10		5120	60	1280				
A2/HK/1/68	120		160	5120	40				
A/Equi 1			480	40	320				
A/Swine	40	60	60	40	40	5120			
A/Duck			60	20	20		1280		
B/Can/5/66	20		60	20	30			1280	40
Sendai	10		60	40	40				240
N-ALF	20		20	10	20				

TABLE 22 (Cont'd) Hemagglutination inhibition cross reactions of influenza A, B, and parainfl. Sendai. Hyperimmune rabbit sera (hRAs).

Antisera	Virus Antigen (ALF)								
	Influenza A							Infl.	Parainfl.
hRAs ($NaIO_4$)	PR8	FM1	A2	HK	Equi	Swine	Duck	B	Sendai
A/PR8			30	40	20				
A/FM1/Can/53			40	20	40				
A2/Can/57			2560	40	1280				
A2/HK/1/68			40	2560	20				
A/Equi 1			120	40	480				
A/Swine			40	20	30				
A/Duck			20	20	10				
B/Can/5/66			20	20	40				
Sendai			30	20	30				
N-ALF			20	20	15				

TABLE 23. Hemagglutination inhibition cross reactions of influenza A, B and parainfl. Sendai. Hyperimmune rooster sera (hFAs).

Antisera	Virus Antigen (ALF)							
	Influenza A							Parainfl.
hFAs (THP)	PR8	FM1	A2	HK	Equi	Swine	Duck	Sendai
A/PR8	10 240	120		80		10		
A/FM1/Can/53		160						
A2/Can/57		10	1280	320	1280			
A2/HK/1/68	ND	ND	ND	ND	ND	ND	ND	ND
A/Equi 1			960	640	10 240			
A/Swine	10			240	15	5120		
A/Duck		20		160	10		320	15
Sendai				160	60			640
N-ALF	10			20				
hFAs (RDE)								
A/PR8	10 240	60						
A/FM1/Can/53		240						
A2/Can/57			640	10	1280			
A2/HK/1/68			30	640	20			
A/Equi 1			2560	80	10 240			
A/Swine						2560		
A/Duck							320	
Sendai								640

Effect of Inhibitor Inactivation Treatments on Immune Sera

Hyperimmune Rabbit Sera The complete elimination of non-specific inhibitors from hyperimmune rabbit sera by each of the treatments used, trypsin-heat-periodate, RDE (as *V. cholerae* filtrate), or $NaIO_4$, was not successful (Table 22).

THP eliminated inhibitors against Duck virus. THP did not eliminate inhibitor activity against almost all other strains in the majority of sera.

RDE lowered the general level of inhibitor activity against most virus strains in many sera, but did not eliminate it completely; it was effective against Duck, Swine, and FM 1 inhibitors; in some cases, the activity increased after RDE treatment. However, RDE was generally the most effective treatment used for rabbit serum inhibitors against all virus strains except A2/Can/57 and A/Equi 1. It lowered the homologous HAI titres of anti-A2/HK/1/68, anti-Equi, and anti-Sendai sera, compared to those recorded after THP treatment.

$NaIO_4$ very effectively lowered inhibitor activity against A2/Can/57 and A/Equi 1 in most rabbit sera. Its action against A2/HK/1/68 inhibitors was comparable to that of RDE treatment. Homologous HAI titres of anti-A2/Can/57 and A2/HK/1/68 sera were lower than after THP or RDE treatment.

Convalescent Rabbit Sera The treatments which most effectively eliminated inhibitors from hyperimmune rabbit sera, determined by the virus strains used as antigen, were applied to convalescent rabbit sera: $NaIO_4$ for A2/Can/57 and Equi inhibitors and RDE for inhibitors to all

other strains. Inhibitor activity, after appropriate treatment, was almost eliminated from the serum of these animals which had not been parenterally immunized (Table 21).

Hyperimmune Rooster Sera RDE treatment of hyperimmune rooster sera successfully eliminated inhibitory activity against all virus strains in the majority of sera. THP treatment was not as effective (Table 23). The homologous HAI titre of anti-Duck serum was lower after RDE treatment than after THP treatment.

Cross Reactions and Comparisons

Convalescent Rabbit Sera Low titre (1/10-1/20) homologous reactions were recorded by all convalescent sera except anti-Equi 1 (HAI- 1/10) (Table 21). Cross reactivity amongst the different convalescent sera was minimal: anti-A2/Can/57 inhibited Equi HA to low titre (1/20). The inhibition of A2/Can/57 and Equi HA by anti-Sendai serum, and Sendai HA by anti-B serum was a puzzle; but could have been due to inhibitors not affected by $NaIO_4$ treatment in the first instance, or by RDE in the second instance, as similar HAI titres were recorded in the hyperimmune sera of the same animals after the same treatments (Table 22). These cross reactions were not confirmed by immunodiffusion cross reactions (Table 17).

Hyperimmune Rabbit Sera Only RDE and $NaIO_4$ treated sera are described (Table 22). Please refer to $NaIO_4$ treated sera for HAI titres recorded against A2/Can/57, A2/HK/1/68, and A/Equi 1, and to RDE treated sera for HAI titres recorded against all other strains.

Homologous HAI titres were demonstrated by all hyperimmune sera, anti-PR8 with the highest (1/10,240), and anti-Equi (1/320) and anti-Sendai (1/240) with the lowest.

It was difficult to determine which cross reacting HAI titres were due to antibody and which to inhibitor, therefore, it was decided that all antibody activity was > 1/60, and all inhibitor activity was < 1/60, a very arbitrary value. On this basis, the following HAI cross reactions occurred: anti-PR8 serum inhibited FM 1 and Swine HA; anti-A2/Can/57 serum inhibited Equi 1 HA and the converse; anti-A2/HK/1/68 serum inhibited PR8 HA.

Secondary antigen cross reactions revealed by immunodiffusion (Tables 17-20, Fig. 8) agreed with the PR8-FM 1-Swine association, and the A2/Can/57-Equi 1, but did not confirm the A2/HK/1/68-PR8 association, only a reciprocal one.

Hyperimmune Rooster Sera RDE treated sera only are considered (Table 23). The range of homologous HAI titres was broad, anti-PR8 and anti-Equi the highest (1/10,240), and Duck (1/320) and FM 1 (1/240) the lowest.

Cross reactivity revealed by HAI tests were: a reciprocal A2/Can/57-Equi 1 reaction; and FM1 HA inhibited by anti-PR8 serum.

Immunodiffusion experiments (Tables 17 and 18) confirmed the PR8-FM1 association, but revealed only the precipitation of A2/Can/57 by anti-Equi 1 serum.

These comparisons were very tenuous because direct evidence was not available to correlate influenza A strain relationships revealed by HAI and immunodiffusion reactions, although some general agreement did occur. When the hemagglutinin-specific immunoprecipitin reaction

is identified in cellulose acetate immunodiffusion reactions, including differentiation of antibody from inhibitor initiated precipitations, it can be applied to determining hemagglutinin associations amongst influenza A strains by immunodiffusion which are possibly more complex than revealed by serological testing (Schild, 1970; Schulze, 1972). These, then, can be compared with hemagglutination inhibition cross reactions revealed by standard serological procedures.

Virus Fractionation

Column Chromatography

Column chromatography, using Sephadex G-200, of SDS disrupted Duck influenza virus was not successful because, virus components remained in the column until 0.001 M EDTA was added to the buffer, and the pH was raised from 7.3 to 7.9. The recovery of protein was poor. When dialyzed and concentrated, only one fraction pool yielded a precipitin band when diffused with homolgous convalescent rabbit serum, and not with hyperimmune serum. The precipitin band could have been due to non-specific precipitation by SDS which was not entirely removed from the fraction by dialysis. Neither infectivity in ovo nor hemagglutinin activity could be demonstrated.

Greater success was achieved with polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE)

Semi-purified Virus Concentrates (C_2, C_2') Electrophoresis in polyacrylamide gels of semi-purified concentrates of influenza A strains (PR8, FM1/Can/53, A2/Can/57, A2/HK/1/68, Equi 1, Swine, Duck), influenza B/Can/5/66, parainfl. Sendai, and N-ALF, disrupted by SDS - β-mercaptoethanol - heat, yielded varying gel patterns. Examples are illustrated in Plate 19.

N-ALF C_2' separated into 21 protein components on electrophoresis. Host (CE) proteins were visible in the gel patterns of all virus concentrates, indicating the amount of host material present in semi-purified viruses. Other influenza A strains possessed varying degrees of host material. This confirmed the presence of host-specific components in C_2 and C_2' preparations revealed by immunodiffusion reactions with anti-viral, and anti-N-ALF hyperimmune rabbit serum, not absorbed with N-CAM.

When influenza A PR8 and Duck C_2 were fractionated (Plate 19), similar polyacrylamide gel patterns of protein components were revealed, which differed from those of influenza B and parainfl. Sendai; the gel patterns of the latter two viruses also differed from each other. Because virus preparations used were only semi-purified further comments can not be made.

Purified Virus Concentrates (SDG) Sucrose density gradient purification (SDG) of viruses and N-ALF concentrates drastically reduced, but did not completely eliminate, host-specific material. This was revealed by electrophoresis of SDS - β-mercaptoethanol - heat disrupted purified virus concentrates (Plate 20 A, not all bands are visible in photograph). Purified N-ALF contained only very weak protein component(s)(marked by arrow in Plate 20 A). This confirmed the immunological detection of host-specific material in purified virus concentrates when they were diffused with anti-N-ALF (anti-host)-specific

hyperimmune rabbit serum (see Density Gradient Purification of Viruses and N-ALF).

Visible separation of viral components was achieved with A/PR8, A/FM1/Can/53, A/Swine, and A/Duck; it was less apparent with A/Equi 1, and B/Can/5/66; and was almost imperceptible with A2/Can/57, A2/HK/1/68, and parainfl. Sendai (μg protein per sample was: 180, 11, 34, 75, <10, 26, 38, <10, <10 respectively). Gel pattern drawings of each fractionated virus strain demonstrated more components than were detected photographically. Protein bands representing separated components were indicated by numbers to the left of each gel; in those gels containing virus, these represented viral components, whereas in the other two they were related to host (CE) material, or to the bovine serum albumin standard.

Each of the separated components could have been a potential antigen which could have been precipitated by a specific homologous antibody to form a precipitin band on immunodiffusion. Therefore, in homologous reactions, the number of precipitin bands formed with PR8 and Swine could be 8, 7 with Duck, 6 with FM1/Can/53 and so on. This does not take into account the state in which separated components exist, as monomers or polymerized; very likely the latter, because the separations in these experiments were not carried out under reducing conditions (Webster, 1970 a , c).

<u>Isolation and Identification of Separated Viral Components</u>

Each influenza A virus strain, purified and SDS disrupted, was fractionated in polyacrylamide gels. One gel representing each strain was stained with coomassie blue (Plate 20 B). The other four were stained

with ANA magnesium salt. In coomassie blue stained gels, detectable components of five virus strains were separated (Plate 20 B, numbered to the left of each gel): 7 components in PR8, 9 in FM1, 12 in A2/HK/1/68, 11 in Swine, 8 in Duck; not so obvious, but still detectable were 4 components in A2/Can/57, and 4 in Equi 1; none were apparent in N-ALF. Bands labelled "H" were thought to be host (CE) material, because they were similar in appearance and position to host material detected in semi-purified virus, and in N-ALF concentrates (Plate 19). A2/HK/1/68 components no. 1 and 2 could have possibly been host material but this was debatable. Because disruption and electrophoresis were not carried out under reduced conditions, the components separated were not necessarily in a monomeric state, but were probably in a polymerized one, or a combination of both.

The separated polypeptides of each influenza A strain yielded slightly different gel patterns which were influenced by variation in : 1) the migration rates of protein 2) relative positions in gels with respect to the other components, 3) the number of polypeptides separated. Only a few general features are pointed out because a detailed analysis can only be made when virus preparations are free of extraneous host material, and viral components are separated by electrophoresis under reducing conditions.

PR8, FM1/Can/53, and Swine gel patterns were very similar, but differed from the individual patterns of Duck and A2/HK/1/68. Although very weak, A2/Can/57 and Equi 1 patterns resembled each other. The

similarities amongst influenza A strains demonstrated in this way were in general agreement with those revealed by immunodiffusion cross reactions (see Immunodiffusion Cross Reactions of Influenza A Viruses, Table 20, Fig. 8), which were a function of interactions amongst related viral antigenic components and their specific antibodies.

Certain polypeptides (presumably viral) in all influenza A strains appeared in the same relative gel position, and therefore could have been the same component:

	PR8	FM1	A2/Can/57	HK	Equi	Swine	Duck
Components:	PR8/4	FM1/4	A2/Can/57/3	HK/6	Equi/3	Swine/5	Duck/3
"	5	" 5	" " 4	" 7	" 4	" 6	" 4
"	6-7	" 7-9	" absent	" 9-10	" absent	" 1-11	" 7-8

The bottom component(s) in each gel appeared to be similarly positioned in relation to the others but had slightly different rates of migration. Protein bands, which strongly fluoresceed under UV light after ANA magnesium salt staining, were cut out as indicated in Plate 20B (cut fractions are marked [4 and include numbered components(s), those of A2/Can/57 and Equi are omitted), and eluted. Immunodiffusion of eluted fractions with homologous immune sera yielded precipitin bands, but sufficient residual SDS was present in the polyacrylamide gel fractions, to cause non-specific precipitation of serum proteins and viral components. It was not possible to differentiate precipitin bands due to virus-antibody interactions from non-specific precipitation (see Disruption of Virus Antigens for Immunodiffusion). The removal of SDS by dialysis or KCl precipitation was not feasible because the eluted fraction volumes were so small.

The detection of hemagglutinin in fractions was complicated by the fact that SDS inactivates the hemagglutinin of certain influenza A strains, for example NWS, and that it also lyses red blood cells. However, hemagglutination was exhibited by the following fractions : PR8/5, Swine/5-6, Duck/3-4, which suggests that these could have been associated with the hemagglutinin of these viruses.

Electron microscopy of eluted fractions did not yield any usable information and the failure to do so was attributed to lack of experience with this method on the part of the investigator.

Although separation of viral components in polyacrylamide gels by electrophoresis was accomplished in over half of seven influenza A strains examined (PR8, FM1/Can/53, A2/HK/1/68, Swine, and Duck) which were disrupted either by SDS or by SDS - β -mercaptoethanol-heat, the ultimate goal of these experiments was not achieved, that is, the separated and isolated viral polypeptides were not characterized by immunodiffusion reactions nor by electron microscopy.

However, the general method could be successfully applied provided that viruses could be disrupted by, and fractionated in, the presence of a compound which did not precipitate serum proteins or viral components non-specifically. Alternatively, isolated components could be utilized for the immunization of suitable animals to produce monospecific sera for use in immunodiffusion reactions to immunologically characterize virus components. Valuable experience in the use of polyacrylamide gel electrophoresis has been gained by these experiments.

SUMMARY AND DISCUSSION

The most significant results of the present investigation can be summarized and discussed as follows.

Before meaningful interpretations of influenza A homologous and cross reactions in immunoprecipitin tests could be made, it was necessary to establish 1) the virus specificity of immunoprecipitin reactions, 2) that the immunoprecipitin reaction was caused by antibody and not by inhibitor, 3) the identity of the virus components being precipitated.

The progressive immunization of rabbits and roosters by chick embryo (CE)-grown influenza and parainfluenza viruses induced the formation of an increasing number of serum precipitins which, when diffused with homologous semi-purified virus particles, precipitated up to thirteen components, some of which were virus-specific, and others host (CE)-specific. The serum precipitins are mainly antibodies, and are referred to as such throughout the following discussion, but they may also include non-specific inhibitors, which have not yet been unequivocally differentiated in immunoprecipitin reactions.

The virus-specific and host (CE)-specific components were differentiated in the majority of immunoprecipitin reactions by comparison and by cross reactions with an analogous host-specific antigen anti-body system.

The host system revealed that rabbits intranasally exposed to host material (N-ALF) did not form detectable anti-host antibodies;

whereas the same rabbits parenterally hyperimmunized by host material (N-ALF $/C_2$) formed anti-host antibodies which precipitated a number of components in N-ALF/C_2 and in N-CAM/SA, a few of which were related.

Absorption of the hyperimmune rabbit sera with N-CAM removed all detectable anti-host antibody, and was complete because residual N-CAM material was left in the absorbed serum. This residual N-CAM material was precipitated by homologous antibodies in unabsorbed serum.

The sera of roosters hyperimmunized in the same way as rabbits, did not precipitate N-ALF components, but unexpectedly formed anti-host antibodies which precipitated a component in N-CAM/SA, a host component in many virus soluble antigens, and a component in the residual N-CAM material in hyperimmune rabbit serum absorbed with N-CAM. Rabbit and rooster antisera immune to N-CAM/SA will have to be included in these experiments to complete the study of the antihost spectrum in these sera.

The normal sera of these animals did not precipitate any host components. Therefore, antihost antibody formed was the result of antigenic stimulation by host material.

The convalescent sera of rabbits intranasally infected with CE-grown viruses possessed only virus-specific precipitating antibodies; host-specific precipitating antibodies were not detected.

Absorption with N-CAM of hyperimmune sera of rabbits parenterally immunized with CE-grown viruses, removed all detectable host-specific antibodies, as demonstrated by the analogous absorption in the host system. The absorbed antiviral hyperimmune serum contained only

virus-specific antibodies. The removal of host-specific flocculating or precipitating antibodies in this way had been demonstrated previously by Belyavin (1955), and recently by Corbel and Rondle (1970).

Semi-purified virus particles contained a number of host components as contaminants (cf Smith et al., 1955), which were reduced, but not completely eliminated on further virus purification in a sucrose density gradient. Host component(s) in these virus preparations were demonstrated in immunoprecipitin tests by diffusion with antihost rabbit serum; and in polyacrylamide gels, when semi-purified and purified virus particles were fractionated in parallel with corresponding N-ALF preparations. Although approximately 21 components were separated by polyacrylamide gel electrophoresis of semi-purified N-ALF, and two in purified N-ALF, only 5 in the former and one in the latter were significantly antigenic, because this was the maximum number precipitated by antihost hyperimmune rabbit serum. The reason for this is not known. Dimmock and Watson (1969) observed no host specific polypeptide peaks in the region of virus polypeptides, when virus sample, to which infected tissue culture fluid had been added, was fractionated in polyacrylamide gels. The use of concentrated tissue culture fluid might have revealed the presence of contaminating host material as was demonstrated above.

Anti-viral convalescent and hyperimmune (N-CAM absorbed) rabbit sera, containing solely virus specific antibodies, precipitated only virus components when diffused against the following virus preparations: semi-purified and purified particles, which were both undisrupted and Nonidet P40 disrupted, and virus soluble antigens.

Hyperimmune sera of roosters, parenterally immunized by CE-grown viruses, appeared to possess only virus-specific antibodies because when diffused against concentrated virus particles, only virus components were precipitated. However, the weak precipitation of a host component in some virus soluble antigens, or of residual N-CAM material in absorbed hyperimmune rabbit sera against all strains but A2/Can/57, A2/HK/1/68, and Equi 1, and against N-ALF, suggested the presence of antibody to the host material present in soluble antigens.

The demonstration that roosters produce intraspecies antihost antibodies, limits their use for production of virus-specific antisera by immunization with CE-grown virus. In immunoprecipitin reactions with virus particles, all components precipitated were considered virus-specific, but in reactions with virus soluble antigens, only careful monitoring enabled valid interpretation of virus-specific reactions to be made. To ensure antiviral specificity of such antisera, host-specific antibodies will have to be absorbed out in future.

By homologous immunoprecipitin tests, a distinct virus-specific precipitin band pattern was demonstrated for each virus strain, when each virus preparation (semi-purified and purified, both undisrupted and Nonidet P40 disrupted) was diffused against each type of homologous antiserum, convalescent rabbit, hyperimmune rabbit (N-CAM absorbed) and rooster. The number of immunoprecipitin bands yielded for each strain-specific reaction varied with the antigen and antiserum used (Tables 7, 8, 9, 14, Plates 1, 4, 9, 11). Generally fewer bands formed with

convalescent than with hyperimmune sera because the former reflected the primary immune response, and the latter the secondary response with a broader antibody spectrum (see Webster and Laver, 1971). Immunoprecipitin patterns formed when antiviral hyperimmune rabbit sera (abs) and hyperimmune rooster sera precipitated components of the same virus preparation, differed slightly, and reflected the differences in the antibody spectrum of each animal species (see Grey, 1969; Gallagher and Voss, 1969).

Purification of semi-purified virus particles in linear sucrose gradients removed many of the degraded virus components (and much contaminating host material) resulting in fewer virus components being precipitated by hyperimmune sera, but approximately the same number by convalescent sera (Table 14).

Disruption of virus particles by agents such as SDS, DOC, or Nonidet P40 is necessary to liberate virus components for diffusion through agar in immunoprecipitin tests (see Part I, Review, pp. 15-16), but has not proven necessary for virus particle diffusion through cellulose acetate; although viruses were disrupted by these agents to ensure that the maximum number of virus antigens were being detected in virus homologous and cross reactions. These agents are known to cause some non-specific precipitation of antigens and sera in agar (Corbel and Rondle, 1970; Palmer *et al.*, 1971). In cellulose acetate, the use of SDS and DOC disrupted virus was precluded because these agents caused marked non-specific precipitation of serum components and some precipitation of virus material in such a way that the immunoprecipitin patterns could

not be interpreted accurately. Non-specific precipitation of serum components in cellulose acetate was also described by MacDonald (1971). Nonidet P40 disrupted viruses were used, with reservations, because the non-specific precipitation of sera and virus antigens does occur, but as distinct rings around the central well of templates, usually discrete from the antigen-antibody precipitin pattern (Plate 11). The depletion of virus antigen and antiserum components by such non-specific precipitation raises the question as to whether the resulting immunoprecipitin patterns represent the complete picture of virus antigen-antibody reactivity in the reagents used. This point needs further investigation to evaluate the use of disrupting agents in immunoprecipitin tests.

Nonidet P40, gentler in action than SDS, strips spikes (hemagglutinin and neuraminidase) and the underlying lipid layer from influenza virus (WSN), leaving behind a subviral particle consisting of RNP enclosed in a protein membrane (Schulze, 1970, 1972), and on continuing action liberates the RNP as a single 38 S component (Pons et al., 1969; Schulze et al., 1970). Thus Nonidet P40 disrupted viruses were included in homologous and cross immunoprecipitin reactions among influenza A viruses to achieve a clearer separation of spike and internal antigen components. However, when viruses were disrupted, the resolution of precipitated virus components was not markedly better than when virus particles were undisrupted; and, an increase in the number of components precipitated occurred only with disrupted semi-purified A2, Hong Kong, and Equi, and with purified Swine, but not with the other strains studied (Table 19).

Ideally a disrupting agent should only disrupt the virus particle and should not cause non-specific precipitation of virus and serum components. Triton N 101 has been recommended by Corbel and Rondle (1970) because, although it is an efficient virus disrupting agent, it causes minimal non-specific precipitation in immunoprecipitin reactions. The application of this agent for disruption of viruses to be used in cellulose acetate immunoprecipitin tests has to be investigated.

Non-specific inhibitors present in the normal sera of a number of animal and avian species, and in the sera of immune animals, precipitated or flocculated influenza virus particles (see Part II, Inhibitor Review, and Screening of Normal Sera for Anti-Influenza Activity). In the immunoprecipitin reactions of this study, precipitating inhibitors were identified on the basis of their susceptibility to inactivation by RDE (as V. cholerae filtrate) or by $NaIO_4$ (see Part II, Antibody Detection in Sera Demonstrating Anti-Influenza Activity).

Virus components in homologus virus particles were weakly precipitated by non-specific inhibitors in the antisera of rabbits and roosters, convalescent or hyperimmune to each influenza A and B strain studied, or to parainfl. Sendai.

Virus components related to the major type-specific component of virus particle antigens, or of virus soluble antigens, or those related to suspected neuraminidase, which were usually precipitated as strong bands, were not precipitated by inhibitors. This is because the strong bands formed by precipitation of these components with native immune sera were not appreciably altered when the sera were treated

by RDE or $NaIO_4$. It is demonstrated in Part II that influenza type-specific component is precipitated by 7S γ-G, a serum fraction with which non-specific inhibitors are not associated. Therefore, when the treatment of serum causes these bands to be altered or eliminated, it is the antibody in the serum which is attacked.

Presumably, the components that were weakly precipitated were associated with the hemagglutinin subunit, because influenza inhibitors are known to interfere with the hemagglutinating capacity of influenza viruses, and with virus infectivity; both of these activities are neutralized by antibody against the hemagglutinin with which the inhibitor is known to compete (see Part II, Inhibitor Review). Therefore, the weak precipitin bands that were eliminated when the sera were inactivated are very likely precipitated hemagglutinin. This fact indirectly identifies the hemagglutinin specific reaction in immuno-precipitin tests, and it would be a useful test for hemagglutinin antigen.

As observed for normal sera, discussed in Part II, treatment of serum by RDE or $NaIO_4$ to inactivate precipitating inhibitors in immune sera, had the following effects: 1) susceptible inhibitors were reduced or eliminated, 2) resistant inhibitors were not affected, 3) certain inhibitors were activated by one or the other treatment (cf Levinson et al., 1969), 4) antibody was attacked in some instances.

Therefore, inhibitor initiated reactions are usually weak when immune sera of animals are diffused against virus concentrates, and are most likely associated with the viral hemagglutinin. Inhibitors have not been found to precipitate components associated with the major type-specific antigen of the virus, nor with the neuraminidase. As

discussed in Part II (p. 388), specific inhibitors must be isolated, and their identity in immunoprecipitin reactions established in order to clearly differentiate them from precipitating antibody.

The identity of virus-specific and host-specific reactions was thus established in immunoprecipitin tests for this study, and it was determined that the majority of virus components were precipitated by antibody, but that the hemagglutinin could be precipitated by antibody and by non-specific inhibitor. The next step was establishing the identity of virus components being precipitated.

The major type-specific component, thought to be the RNP, was identified in all influenza A strains studied, in influenza B, but was not clearly established in parainfl. Sendai. Each type specific component was distinct from that of other types, and cross reactions between them did not occur regardless of whether the type-specificity was expressed as virus antigen or as antivirus antibody (Table 15, Cross Reactions, Table 16). This finding is in agreement with the characterization of RNP by immunoprecipitin tests in agar gel by Schild and Pereira (1969), Schild and Newman (1969), Easterday _et al_. (1969), and Beard (1970).

The homologous type-specific reaction for each virus strain as virus particle antigen (undisrupted or Nonidet P40 disrupted) and as virus soluble antigen was established in diffusions against each homologous convalescent and hyperimmune serum (Table 15, Homologous Reactions, Tables 16, 17, 19). More than one component in virus particle

and in soluble antigens were precipitated by the majority of antisera. The major type-specific component was strongly precipitated from virus particle and virus soluble antigens by all antisera. The component was identical in both types of virus preparations. It was often the only component precipitated by convalescent antisera possessing primary response antibody. A second group of type-specific components, the lesser or minor RNP, which were the same in both antigen preparations, were variably precipitated by all anti-influenza A hyperimmune sera (rabbit and rooster), but not by that against A2/Can/57. They were not precipitated by anti-influenza B or anti-Sendai, nor by any convalescent sera. Both major and minor type-specific components were structural because they were shown to be present in virus particles and in virus soluble antigens. They were not host components because they were precipitated in virus particle and in virus soluble antigens by homologous hyperimmune rabbit (abs) and rooster anti-sera, furthermore, antihost antibodies are not present in absorbed hyperimmune rabbit serum.

A third component was precipitated from only virus soluble antigens, and not from virus particle antigens by many antisera against all strains except A2/Hong Kong/1/68; two such components were precipitated in Duck soluble antigen (Table 15). This component could be regarded as non-structural, similar to those detected in immunodiffusion studies by Dimmock (1969), and subsequently identified by others (Dimmock and Watson, 1969; Taylor et al., 1969, 1970; White et al., 1970; Lazarowitz et al., 1971; see Part I, Review, p33 and Table 3 from Kilbourne et al., 1972). This assumption is made because the component is found only in extracts from virus infected cells, and antibody against it is

present in convalescent as well as in hyperimmune sera (both immunized by live influenza viruses). It is not host component because it is precipitated by rabbit antisera which do not contain antihost antibody against the host material in virus particles or in virus soluble antigens. Why the component is absent from the A2/HK/68 reaction is not known at present.

Classically, the influenza type-specific component is thought to be the RNP (Schild and Pereira, 1969; see Part I, Review, p. 23). However, upon examination of the evidence from recent investigations (Kilbourne et al., 1972; Webster and Laver, 1971; see Part I, Review, Membrane or 'M' Protein), some doubt is raised as to the identity of the type-specific component. The major structural component detected in type-specific immunoprecipitin reactions may not be the RNP, but could be the smallest structural protein, which is the major protein ('M' in Table 3, from Kilbourne et al., 1972) in total mass and number of molecules of the influenza virus, and which forms the membrane enclosing the internal antigen or RNP (Compans et al., 1970; Schulze, 1970, 1972; Kilbourne et al., 1972; see Part I, Review, p. 29). The minor structural component in type-specific immunoprecipitin reactions could be the RNP. The main evidence for this is:

1. There are seven times as many molecules of 'M' protein as of nucleoprotein in the influenza virus particle (see Part I, Review, p. 29), and the 'M' protein is external to, and therefore a more readily exposed antigen, than the nucleoprotein. Therefore, the antibody

specific component which occurs in all homologous and cross immunoprecipitin reactions of influenza A strains and influenza B, could be a reflection of the antigenic activity by the dominant 'M' protein; whereas the weak precipitation of the minor type-specific component in many immunoprecipitin reactions could reflect the antigenic activity of the less abundant and less exposed RNP component. Both polypeptides are present in virus particles and in extracts from infected cells (as demonstrated by Lazarowitz _et al_., 1971;and Pons, 1971;see Part I, Review, p. 22, 30), and therefore could be the antigenic components described above.

2. Nonidet P40 strips spikes and lipid from the influenza virus leaving a subviral particle consisting of 'M' protein and RNP (demonstrated by Schulze, 1970, 1972). Immunoprecipitin reactions using Nonidet P40 disrupted virus particles resulted in the same major and minor type-specific reactions as described for undisrupted particles. Subviral particles as described by Schulze (_op cit_) have been seen by EM examination of negatively stained virus soluble antigen preparations used in this study, which have not been reported because of the tentative nature of the results.

3. The heterogeneity of the major type-specific component in immunoprecipitin cross reactions among influenza A subtypes, does not agree with the homogeneous character of the RNP described by Schild and Pereira (1969)

Evidence from other sources is:

1. S antigen, prepared from virus infected cell extracts, or from ether disrupted (WHO Expert Committee on Influenza, 1953, 1957), or from DOC disrupted (Schild and Pereira, 1969) viruses, is used to prepare anti-S serum for use in type-specific complement fixation tests and to

identify the RNP in immunoprecipitin tests. The S antigen contains internal antigen which consists of RNP and very likely the 'M' protein. Therefore, the type specificity of influenza viruses may be mainly determined by the abundant 'M' protein rather than by the RNP as has been believed.

2. The demonstration that the type-specific reaction consists of multiple components when purified S antigen and DOC disrupted viruses were diffused against anti-S serum (Styk and co-workers, see Part I, Review, p. 24), tends to lend support to the proposal that more than one antigen is involved in the type-specific reaction which could include the 'M' protein.

3. Laver has suggested that polypeptide maps of tryptic digests thought to be RNP, could possibly be the 'M' protein instead (Webster and Laver, 1971).

4. Schulze (1970) has raised the question of the function of 'M' protein as antigen and whether it functions as a group-specific or a strain-specific antigen, and, in addition, whether antisera specific for this protein can neutralize infectious influenza virus.

5. Skehel and Schild (1971, 1972) have demonstrated that the internal proteins of several influenza A strains are of similar size, and are antigenically related, and suggest that the 'M' protein may also be antigenically similar in different type A strains. The evidence presented in this study tends to support this suggestion.

The isolation of the 'M' protein, and its antigenic characterization by complement fixation and by immunoprecipitin tests using monospecific antisera, should determine whether or not the type-specificity of influenza viruses is dependent upon it, or upon the RNP, or upon the combined effect of these two antigens.

Attempts to directly identify other antigens of influenza viruses by fractionation of purified particles in polyacrylamide gel electrophoresis yielded good separation of virus components, but when they were extracted and diffused against specific antisera, non-specific precipitation by residual SDS made interpretation of immunoprecipitin patterns impossible and this approach had to be modified. Isolated viral components can be used to prepare monospecific antisera by which various antigens can be detected, and this method, used by others (Schild and Pereira, 1969; Schild and Newman, 1969; Schild, 1970; Schild et al., 1970), will be applied in future investigations.

Immunoprecipitin cross reactions between soluble antigens of influenza A viruses and their respective convalescent and hyperimmune sera revealed that, although the major type-specific reaction was strictly confined to influenza type A viruses, it was not completely homogeneous for all influenza A strains. Minor type-specific components were also variably precipitated from different strains (Table 16). The type specificity, but strain heterogeneity of the major component was also apparent when the antigens used were virus particles, but in these reactions, the minor type-specific components were not distinguishable

from the other precipitated virus antigens. Only a detailed study would enable them to be resolved.

The heterogeneity of the main type-specific reaction among influenza A strains was suggested by the following observation. Although the major type-specific component was precipitated as a single band linking all influenza A strains, with certain strains, barbs and splits occurred in the continuity of the band. This indicated partial rather than complete identity between the virus component homologous to the precipitating antibody, and the component of other strains (see Crowle, 1961). For example, differences between homologous and cross precipitation of A2/Can/57, Duck and Swine type-specific components by anti-A2/Can/57, antibody were demonstrated to occur, suggesting that the antibody was not as specific for Duck and Swine type-specific components as for that of the homologous antigen. In addition, a minor type-specific virus component common to A2/Can/57, A2/HK/1/68, and Equi 1, did not occur in Duck or Swine, nor in PR8 or FM1. Other similar examples are found in the text and in Table 16.

The evidence presented in this study suggests that the major type-specific component could be the 'M' protein rather than the RNP, because of the abundance of 'M' protein and its accessibility as an antigen. 'M' protein is thought to be similar amongst influenza A strains, but could demonstrate antigenic differences. Laver has demonstrated that some differences do occur in the amino acid sequences of what he now suspects to be the 'M' protein of different influenza A strains, but did not determine if these differences were reflected

antigenically (Webster and Laver, 1971). The results of immunoprecipitin cross reactions between the major type-specific components described in this thesis strongly suggest that antigenic differences do occur among influenza A strains, and that they may be due to variation in the 'M' protein. This can only be finally proven by investigating immunoprecipitin cross reactions between the isolated 'M' protein from different influenza A strains using monospecific antisera, and comparing these with the above results.

The minor type-specific virus components precipitated, which show considerable variation from strain to strain (Table 16), could be the RNP component(s). The RNP is known to exist in distinct pieces (three have been isolated), in both the virus particle and in infected cells (see Part I Review, p. 20). Therefore, the weak precipitation of multiple type-specific components of the less abundant antigen demonstrated in this investigation, and the precipitation of multiple type-specific components by others (Styk and co-workers, see Part I, Review, p. 24) could be a reflection of these pieces, a suggestion put forth by Duesberg (1969), which could easily vary in specificity from strain to strain. Davenport et al. (1960) proposed that strain variation in the type-specific component could occur because slight differences were observed to occur in the S antigen between certain influenza A strains in complement fixation tests. The expression of such variation would result in the heterogeneity thought to exist in the internal antigen of influenza A strains by the above mentioned authors.

The heterogeneity may be due to a combination of variation in the 'M' protein and in the RNP component(s) of influenza A strains. Only further detailed work with the isolated components described, using monospecific antisera, will reveal the influence of these components, in addition to that of hemagglutinin and neuraminidase, on the antigenic variation among influenza A strains.

Cross reactions among influenza A viruses were examined by checkerboard immunoprecipitin tests. Semi-purified and purified virus particles, both undisrupted and Nonidet P40 disrupted, were diffused against respective convalescent and hyperimmune (abs) rabbit antisera, and against hyperimmune rooster antisera. The following observations were made.

All influenza A strains possessed an immunologically similar, though not always identical, major type-specific component (generally believed to be the RNP, see the discussion immediately preceeding). This was demonstrated reciprocally in the majority of immunoprecipitin cross reactions with hyperimmune sera, but only with sufficiently potent convalescent sera. The component was not related to that of either influenza B or parainfl. Sendai (Table 15). The results achieved by cross reactions among virus soluble antigens were thus confirmed, and were in general agreement with similar observations of others (for example, Schild and Pereira, 1969; Schild and Newman, 1969; Easterday et al., 1969; see Review p.58).

The pattern of relationships among influenza A strains revealed by the hyperimmune rabbit serum system differed slightly from that revealed by the hyperimmune rooster serum system. Generally, in addition to the major type-specific component, other virus antigens (referred to as secondary) were variably shared, indicating that certain strains were more closely related than others. (Tables 17-20). The specificity of a strain depended upon the possèssion of one or more independent components which were not shared by other strains. This was demonstrated by each virus strain examined. The secondary antigens include hemagglutinin and neuraminidase, which form the basis for establishing the strain specificity of influenza A viruses (Table 2 from WHO Committee on Influenza Virus Nomenclature., 1971). Secondary antigens could also include at least one of the other recently isolated influenza virus polypeptides (Compans et al., 1970; Schulze, 1970; Skehel and Schild, 1971; Klenk et al., 1972) whose antigenic role has not been established. It is not known exactly which antigen, if any, in addition to hemagglutinin and neuraminidase, may be responsible for the inter-relationships revealed by immunoprecipitin cross reactions using virus particles and related components reported here, because only the identity of the type-specific component was established, but not that of the other secondary components. Because relationships between strains beyond those established for hemagglutinin and neuraminidase antigens were apparent, the presence of at least one other antigen was thought to be possible.

Therefore, the identity of secondary antigens remaining unknown, associations between different strains could only be determined on the basis of their immunoprecipitin activity with respective antisera, and

the relationships inferred were judged by the manner and degree by which the following conditions were fulfilled.

The most closely related strains were those sharing a common secondary component which was demonstrated by reciprocal reactions between antigens and specific antisera. Strains were considered not so closely related if the shared component was precipitated in oneway reactions and not reciprocally. A distant association between strains occurred when antibody against one precipitated the antigen of another independently, indicating that some common factor, reflected by the mutually precipitating antibody, was present in the two strains (Tables 18 and 20); the relationship was considered closer if the precipitation was conversely reciprocated.

The relationships amongst strains established in this way are schematically summarized in Figure 8 (data from Tables 17-20).

Briefly, general observations on the immunoprecipitin activity among the secondary antigens of influenza A strains are:

1. PR8, Duck, and Swine had close consistent interrelationships which extended to FM1 to a lesser degree. These results agreed in part with relationships known to exist among these strains. The neuraminidase antigens of PR8, FM1 and Swine are known to be the same, as is the hemagglutinin of PR8 and FM1, but not that of Swine (Table 2 from WHO Committee on Influenza Virus Nomenclature, 1971). HAI tests did not conclusively confirm the established hemagglutinin relationships (Tables 22 and 23). Duck hemagglutinin bears no significant relationship to

any other A strains (Mitchell et al., 1967; Tumova and Easterday, 1969), which was confirmed in HAI tests (Tables 22 and 23). Information about its neuraminidase is not available. Corbel and Rondle (1970) had similar results in agar gel diffusions between Swine and PR8, PR8 and FM1, but not between Swine and FM1.

2. A2/Can/57 was most closely related to Equi 1, less closely to FM1, more distantly to PR8, Duck, and Swine, and remotely to A2/HK/68. The first relationship was confirmed in HAI tests (Tables 22 and 23), but not the others. The only agreement with established relationships is that A2/57 and A2/HK/68 neuraminidases are similar (cf Coleman et al., 1968; Dowdle et al., 1969; Schild and Newman, 1969; Table 2 from WHO Committee on Influenza Virus Nomenclature, 1971; see Review, p. 51), but this is palely reflected in the immunoprecipitin results achieved here.

3. Except for the strong relationship with A2/Can/57, and very weak ones with PR8 and A2/HK/68, Equi 1 shared only the type-specific component with the other A strains tested. This may be due in part to the lack of potency of the antigen and antiserum employed. None of the above relationships have been reported elsewhere (see Review, p. 54).

4. The weakest interrelationships with all other strains was demonstrated by A2/HK/68. This was not due to lack of potency of antigen or antiserum. Like Equi 1, the virus appeared remote from the other strains, and the established relationship with A2/Can/57 neuraminidase was weakly reflected in the immunoprecipitin results reported here.

A suggestive cross reaction observed in HAI tests could have been due to steric hindrance by the common neuraminidase antibody (Tables 22, 23).

5. Strains demonstrating the greatest number of interactions in immunoprecipitin tests, which suggests they are closely related are: Duck and PR8; A2/Can/57 and Equi; A2/Can/57 and FM1; Duck and FM1. What antigens are reflected in these reactions are not known except for the A2/Can/57 - Equi 1 hemagglutinin relationship (Table 22).

6. Antisera against Duck, A2/Can/57, and Swine, appear to have greater precipitating capacity than antisera against the other strains. This is not because of extra potency (Table 22), but may reflect the presence of more broadly reactive antibodies. Such broad reactivity may be due in part, to less specificity on the part of the antibodies (see Edelman, 1970), which is reflected in its avidity for different antigens, which may be "junior" or "senior" (Fazekas, 1969 a, b). "Senior" antigens are likely to stimulate more broadly cross-reactive antibodies, for example A2/Can/57, whereas "junior" antigens will induce narrower responses, for example Equi 1.

7. The most highly cross reactive strain was PR8 and the least was A2/HK/1/68, the other strains ranging in activity between these two, The potency of PR8 antigens and antisera may be in part responsible for this, but those of A2/HK/68 were not that much weaker.

The relationships revealed in immunoprecipitin cross reactions in cellulose acetate agree in part with ones established by standard serological tests and by the immunoprecipitin test in agar gel, but

extend beyond them. Fazekas (1969 a, b) has been able to demonstrate that some of these relationships could exist. Whether the additional relationships demonstrated in the cross reactions of this study reflect the detection of additional antigens active in immunoprecipitin tests which are not detected by other tests, or whether interfering substances such as non-specific inhibitors are causing confusing precipitations has not yet been fully determined.

As has been advocated throughout this thesis, only a detailed study, in which each precipitated component and each active precipitin are clearly identified, will reveal the true nature of antigenic relationships among influenza A strains.

Although certain aspects of the immunoprecipitin test in cellulose acetate have been clarified, and additional information gained about influenza virus antigens, the objectives of this part of the study have been only partially fulfilled, and further detailed work must be continued.

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PART II

LITERATURE REVIEW

Influenza in Animals

Where is the reservoir of human influenza virus? Does it lie in animal and/or avian species, or is man his own reservoir?

Over the past 50 years a relationship between human and animal influenza viruses has gradually been revealed but has not been completely and clearly defined. Because animals and birds are naturally infected by influenza viruses belonging to the same antigenic group as those infecting man, and respond to experimental infection with human viruses, the part they play in the epidemiology of human influenza has raised much speculation. It has been suggested that virus variants causing human pandemics may originate in animals (Andrews, 1959; Fenner, 1968; Hoyle, 1968; McQueen *et al.*, 1968) but this has not been unequivocally proven. Pereira (1969) has postulated that new strains of influenza may arise by mutation or by genetic recombination. However, the mechanism of antigenic variation and the origin of new influenza virus subtypes is still poorly understood, as is the epidemiology of influenza.

Many problems are still to be resolved as has been pointed out in numerous articles and excellent reviews, the most recent including those by McQueen *et al.* (1968), Hoyle (1968), Pereira (1969), Webster and Laver (1971), and Steele (1971). The World Health Organization Conference

on Hong Kong Influenza held in the U.S. in 1969 , brought to light many observations and theories concerning the relationship of human and animal influenza viruses, and pointed out that much work was necessary to fill in existing gaps of knowledge.

Natural Infections

Influenza infections occurring in animal and avian species are only of the A type. Humans are infected not only by influenza type A, but also by types B and C (McQueen *et al.*, 1968, Pereira, 1969). Influenza A virus infections are widespread and are known to occur as epizootics in animals and birds. They have been established as the etiologic agents by their isolation from infected animals and birds.

Swine influenza Infections in swine were first noted in 1918 in North America (Koen, 1919), and were thought to have arisen from, and be related to, the human influenza pandemic of 1918-1919 which had originated several months earlier (McBryde, 1927). Shope (1931, 1932) isolated and identified the etiologic agent as a filterable virus which, in a synergistic action with *H. influenzae suis*, gave rise to the clinical disease. McQueen *et al.* (1968) excellently reviewed Shope's work over the next 24 years, in which he defined the total epizootiology of influenza in swine, implicating swine lungworm and the earthworm as intermediate hosts, and the need for meterological stress to activate masked viruses to active infection in perpetuating the swine flu cycle. Hoyle and Fairbrother (1937) demonstrated that human and swine viruses have a common internal antigen (S), thus classifying swine influenza as a type A influenza (Andrewes, 1964).

The disease is endemic in the Mid West U.S. (Shope, 1964). No evidence of swine flu has occured in the Eastern U.S. since 1941, although inapparent infection may be present, as Nakamura et al. (1969) have detected swine influenza antibodies (HAI) in serum from swine native to New York. Swine-like influenza infections have been reported in Russia, Poland, the Balkans, Germany, Scandanavia (Shope, 1964) and in Czechoslovakia (Blaskovic et al., 1970a).

Swine influenza isolates from pigs have remained antigenically stable, showing minor antigenic drift in hemagglutinin and neuraminidase, but no major antigenic shift (Jensen and Petersen, 1957; Meier-Ewert et al., 1970) from the original isolated strain SW/Shope 15. A recent isolation from pigs in Taiwan, during an influenza epidemic in humans by A2/Hong Kong/68 (Kundin, 1970), has revealed a new strain A/Swine/Taiwan/70, antigenically identical to human A2/Hong Kong/68 virus (Beare et al., 1971), and apparently unrelated to the "classical" swine strain Shope S15 or A/Swine/Man/67, or A/Swine/Wisc/66. The authors stated that this was the first instance of an influenza A virus of animal origin to be shown to have an antigenic composition identical with that of a human strain. In this instance, interspecies transfer was suspected of having taken place from man to swine.

However, Barb et al. (1962) had earlier reported the isolation of a PR8-like influenza virus from pigs with acute respiratory illness. The identity of the virus as PR8 was confirmed by serum absorption and mouse pathogenicity tests. A2-related and PR8-related viruses were also reported to have been isolated from pigs (Romvary et al., 1962).

Equine influenza Influenza epizootics in horses have been described since the 12th century (Steele, 1960), and recorded in 1688 in England and Ireland[1](Short, 1749), in Edinburgh in 1732 (Gibson, 1754; Thompson, 1852) and in North America in 1872 (Judson, 1874a,b). The etiologic agent of such an epizootic in horses in Czechoslovakia was established as A/Equi 1/Prague/56 (Sovinova et al, 1958; Tumova and Fiserova-Sovinova, 1959), and Equi 1-related virus was reported by others to cause epidemics in the U.K. (Beveridge et al., 1965; Rose, 1966), in India (Feldman, 1965; [1]Singh, 1965), in the U.S. (Bryans, 1964), and in Canada (Ditchfield et al., 1965). In 1963, Waddell et al., isolated a new subtype of equine influenza virus, A/Equi 2/Miami/1/63 which subsequently spread to Canada (Marois et al., 1963), to South America (Somma et al., 1963), to the U.K. (Beveridge et al., 1965; Rose, 1965), to France [1](Sohier, 1965), and to Switzerland (Beveridge, 1965, et al.).

Both subtypes of equine influenza virus are still prevelant in horse populations and have remained antigenically stable (McQueen et al., 1968).

Avian influenza Influenza-like viruses, including fowl plague, which naturally infect avian species are considered to be Myxoviruses (Andrewes and Worthington, 1959) of the influenza A type (Pereira et al., 1965a, 1967). A filterable virus was thought to cause infections of fowl in Italy in the late 19th century (Stubbs, 1951), which, when isolated from similar infections later was identified as fowl plague (FPV), an influenza A type virus (Schafer, 1955, 1957). It is thought to be endemic in North Africa (Wells, 1963). An FPV-related

[1]cited by McQueen et al., 1968

strain, A/Turkey/Eng/63, infects turkeys (Wells, 1963), and may attack other avian species, ducks, geese, and pigeons. Wild birds such as sparrows, may be infected (Gerlack and Michalka, 1926), and an infection in terns (Rowan, 1962) by A/Tern/South Africa/61 has been related to A/Chicken/Scotland/59 causing a fowl plague infection in chickens (Becker, 1963, 1966). A virus related to, but not identical with FPV, is virus N (Dinter, 1949), a type A influenza (Rott and Schafer, 1960) infecting chickens.

Lang et al. (1965) first reported a disease of turkeys in Wilmot Township, Ont., caused by an influenza A, designated A/Turkey/Canada/63 (Lang and Willis, 1966). Subsequent outbreaks have been recorded in the U.S. and in Canada caused by antigenically different strains: T/Wisconsin/66 (Pereira et al., 1966; Smithies et al., 1969a), T/Mass/3740/65, T/Ont/7732/66 (Lang et al., 1968; Narayan et al., 1969, 1970), T/Wisconsin/68 (Smithies et al., 1969b). The behaviour of influenza virus in turkeys may be lethal or hardly infectious (Steele, 1971).

Epornitics have occurred in ducks, and several strains of virus have been isolated: Duck/Czech/56 (Koppel et al., 1964), Duck/Eng/56 (Simmins and Asplin, 1956; Roberts, 1964), Duck/Canada/53 (Walker and Bannister, 1953, 1953; Mitchell et al., 1967), Duck/Eng/62 (Roberts, 1964). Influenza A infections in domestic ducks have also been reported from Russia, Italy, Germany, and Yugoslavia (Hwang et al., 1970).

Avian influenza A viruses and 16 antigenically related, but not identical, strains of influenza A viruses isolated from quail, ducks, turkeys, pheasants, and chickens on poultry farms near Pavia in Northern

Italy were divided into 6 groups on the basis of reciprocal HAI tests using post-infection avian sera (Pereira et al., 1966; Pereira, 1969): 1) FPV, T/Eng/53 2) Virus N, Quail/Italy/65, Pheasant/Italy/65 3) Duck/Eng/56 4) Duck/Czech/56, Duck/Eng/62 5) T/Can/63, T/Mass/65, T/Wisc/66 6) Chicken/Scot/59, Tern/S.Africa/61. The most recent classification of avian influenza viruses has added two additional types, A/Duck/Ukraine/63 and A/Turkey/Ontario/6118/68 (WHO Committee on Influenza Virus Nomenclature, 1971).

Influenza infections in migratory waterfowl, other than the one reported in terns (Rowan, 1962), had not been noted in the 10 to 15 years prior to 1968 (Easterday et al., 1968).

Influenza in other animals Dogs may transiently carry almost any human virus (Clapper, 1970), and a few Myxoviruses have been isolated, canine distemper, mumps, several strains of parainfluenza virus, but as yet, not influenza virus (Binn et al., 1968; Binn, 1970; Appel and Percy, 1970; Rosenberg et al., 1971). Topciu et al. (1966b) did not isolate any influenza viruses from the lung specimens of over 100 dogs examined.

Meenan et al. (1962), in a survey of domestic animals in Ireland, were unable to isolate influenza viruses from cattle, sheep, poultry, horses, dogs, or swine. These results and negative serological evidence led to the conclusion that a reservoir of A2/Asia/57 viruses did not exist in domestic animals, nor had the virus spread to them during a human epidemic in 1961. Barb et al. (1962), however, reported the isolation of Shope virus from diseased cattle and sheep.

Confirmed influenza virus isolation resulting from natural infection in other animal species have not been reported. Little evidence

has been available on the occurrence of influenza virus infection in wild animals because of the difficulty of isolating influenza virus from them (Hoyle, 1968). But a continuing investigation of a wide host range, including wild animal species, may reveal the existence of other strains of influenza viruses causing natural infections, as recommended by Pereira (1969), Kaplan (1969), and others.

Experimental Infections

Many animals have been experimentally infected with influenza A type viruses of animal and human origin, and occasionally with influenza B. Such infections, mainly by the respiratory route, have demonstrated that different strains of influenza viruses have the capacity, under appropriate controlled conditions, to infect a wide variety of host species, animals, birds, and man, with the variable production of disease and immune response. The potential wide host range of these viruses is relevant to the study of influenza epidemiology; there is the possibility that interspecies transfer of viruses may occur between potential lower animal reservoirs and humans, giving rise to new virus subtypes, and subsequent disease.

Experimental infection of animals in the laboratory and in the field has made possible the study of a) the development of disease in infected animals with the possible recovery of the infecting virus, b) the immune response in the animal induced by the infecting virus, c) antigenic relationships among the viruses as revealed by immunological reactions, d) the experimental epidemiology of influenza; and the maintenance of the virus by serial passage in a suitable animal host.

Hoyle (1968) and McQueen *et al.* (1968) have reviewed experi-

mental infection of animals with animal viruses. This discussion will be confined to relevant observations of experimental infections in animals pertaining to this study.

Many species of laboratory animals have been successfully infected with influenza virus strains. The ferret was the first one successfully infected, developing clinical disease and/or immune response on infections with influenza A/WS, Swine (Smith et al., 1933;,Shope, 1934), PR8 (Haff et al., 1966a, b), Equi 1, Duck/Eng/56 (Andrewes and Worthington, 1959). Pinto et al. (1969) were able to demonstrate that measured manifestations of infections with influenza viruses (clinical disease, serum antibody, etc.) differed quantitatively with the strain of virus used and the inoculum concentration. There was a progressive decrease in such manifestations from A/PR8 to A2 to B to A1 to respiratory syncytial virus.

Laboratory mice, a common influenza host, were first readily infected by human A strains and by A/Swine (Andrewes et al., 1934; Shope, 1935). Stuart-Harris (1939) adapted human WS influenza intracerebrally in mice to produce a neurotropic strain, NWS. In studying the experimental epidemiology of influenza, Schulman (1968) used mice as an animal model to investigate the transmission of influenza virus infection. Mice varied in their ability to transmit the disease, the transmission varied with environmental conditions, and viruses varied in their ability to be transmitted, the A2 or Asian viruses more readily transmitted than strains of other subtypes. The latter finding could be related to the great spread of A2 in the 1957 pandemic.

Rabbits are apparently insusceptible to infections with human influenza viruses such as A/PR8 (Francis and Magill, 1935, Hyde, 1942). But Warburton stated (1969) that if rabbits were held at low temperatures during intranasal infection with influenza virus, and during subsequent incubation, they could develop clinical disease and die.

Adult fowl are resistant to infection by human strains of influenza viruses (Tang, 1938). Samadieh and Bankowski (1971b) found that starlings were not susceptible nor responsive to infections with avian influenza viruses; but in experiments with turkeys and turkey influenza they found that influenza viruses may be egg transmitted.

Dogs, intranasally infected with a pre-1958 human influenza strain, shed virus, and demonstrated seroconversion (Ado and Titova, 1959). Todd and Cohen (1968) recovered virus up to 5 days post infection from throat and nasopharyngeal secretions of dogs intranasally infected with A2 and B virus strains. Virus replication was thus indicated. However, there was no evidence of transmission from infected to contact dogs. Animals shedding virus had complement fixing S and V serum antibodies, which in 2 animals persisted for 14 months. The persisting antibody may have been a reflection of persisting virus thus inferring a carrier state in healthy dogs of a human type influenza. The evidence was not strong enough to incriminate dogs as a secondary host of human influenza virus, but merely suggested a potential role for dogs in this respect. Therefore continued monitoring of canine populations was recommended in order to detect any natural infections should they occur.

Influenza infection of cats has been recorded (Lozovaya, 1942). Paniker and Nair (1970) infected cats with A2/Hong Kong/68 by intranasal

inoculation, by contact with an infected cat, and with a human influenza patient. Clinical illness was not apparent, but virus was shed from the throat for one week post infection. All infected animals developed HAI antibodies. Reinfection with the same virus 2 months later produced no shedding of virus, but stimulated a significant increase in HAI antibody titre. Traditionally, cats live in close contact with humans, thus the infection of a cat by a human case of influenza suggests that cats may be potential vectors in the transmission of human influenza virus. They recommended serum surveys of cats to reveal the presence of antibodies to influenza viruses.

Little is known about anthroponoses and influenza virus, but a number of Myxoviruses have been found to produce infection in non-human primates. Kalter et al. (1969) infected Kenya baboons (Papio sp.) with an A2/Hong Kong/68-related virus. Few clinical symptoms were noted but the animals shed virus, transmitting it to contact animals which in turn shed virus. Seroconversion was demonstrated in all infected animals. The experiment demonstrated yet another species susceptible to human influenza virus infection. Kalter and Heberling (1971) have recently reviewed the incidence of influenza infections in primates.

The speculation that there was an association between influenza epidemics in man and epidemics in horses (in McQueen et al., 1968); the finding that sera from persons born before 1900 contained antibodies to A Equi 2-related viruses (Voth and Feldman, 1963; Minuse et al., 1965; Schild and Stuart-Harris, 1965; Masurel and Mulder, 1966; Davenport et al., 1967), and to A2/Asia/57 and the A2/Hong Kong/68 variant (Masurel, 1969a; Fukumi, 1969; Marine et al., 1969, Marine and Workman, 1969); and that

antigenic similarities existed between A Equi 2 strains and the newest human variant (Coleman et al., 1968; Kasel et al., 1969; McQueen et al., 1969; Masurel, 1969b) suggested that recycling of viruses as proposed by Jensen and Francis (1953) could have occurred. These facts prompted the experimental infection of horses in order to investigate the potential role of this animal species as a source of influenza virus capable of infecting man.

Ponies and horses have been experimentally infected with A2/Hong Kong/68 via the respiratory route by Todd et al. (1969), Blaskovic et al. (1969 a, b), and Kasel and Couch (1969). Febrile illness developed in the latter group of horses only, but all infected animals shed virus for up to 5-7 days post infection. Virus was not successfully transmitted to contact animals. Many infected animals responded with rises in serum antibody levels, but these varied with the conditions of each experimental group.

Todd et al., (op. cit) found that all infected ponies registered significant rises in neutralising and CF titres to A2/Hong Kong/68 and to A/Equi 2, the latter neutralising titres were higher than those of the infecting virus. One pony developed a significant A/Equi 1 antibody rise. There were no antibodies to an earlier A2 strain which shared a neuraminidase with A2/Hong Kong/68. Most antibody responses were of secondary nature indicating a previous encounter with a Hong Kong strain. A close relationship between A2/Hong Kong/68 and A/Equi 2 was demonstrated.

Kasel and Couch (op cit) found only a few infected ponies produced neutralising antibodies to the infecting virus but no hetero-

logous antibody response to A/Equi 2 was detected. When challanged with A/Equi 2, the infection by A2/Hong Kong/68 afforded little protection and some animals developed a febrile response.

Horses infected by Blaskovic et al. (op cit) produced HAI antibody only to A2/Hong/Kong/68 and to A/Equi 1. Intravenous challange with A/Equi 2 virus caused an increase in HAIantibodies to A2/Hong Kong but not to A/Equi 2. Another group of horses infected intranasally by A/Equi 2 developed active disease, shed virus, and produced low titre HAI antibodies to A/Equi 1 and A2/Hong Kong/68. Homologous antibody was produced only by an intramuscular booster dose. Challange with A2/Hong Kong/68 induced a titre rise in only Hong Kong HAI antibodies.

Although the results of all these experiments are not consistent, infection of horses by A2/Hong Kong/68 virus was demonstrated, accompanied by homologous antibody production, and in some cases heterologous antibody to A/Equi 2, revealing the antigenic relationship of these two viruses. The reciprocal response was not present in A/Equi 2 infected horses as only antibody to A2/Hong Kong was demonstrated in the primary response. However, McQueen et al. (1969) did detect significant CF and HAI titre rises to A/Equi 1 strains, A2/Asian strains, A2/Hong Kong/68, and A/Equi 2 in horses infected by the latter strain.

Kasel and Couch (op cit), and Todd et al. (op cit) concluded that their experiments did not offer any proof that horses were active reservoirs of virus or sources of new antigenic variants for man. Kasel's group did argue that the appearance of antigenically related viruses in man suggested an exchange could occasionally occur between them in nature.

Styk et al. (1970b) continued the study of Blaskovic's group described above. Horse sera were examined by immunodouble diffusion (ID) in agarose gel using DOC disrupted viruses and nucleoprotein (NP or S-, g-) antigens. There was some correlation between HAI and ID results. However, especially in early sera, ID proved more sensitive than HAI and revealed a broader antibody response in several horse sera. Unique and shared antigenic components were shown to exist amongst A/Equi 1, A/Equi 2, and A2/Hong Kong/68 viruses. Some difficulties were encountered with this technique which affected the results: equivalence of reactions was not always obtained, and there was a lack in potency of some antigens, especially Equi 1 and Equi 2 NP. The authors chose to ignore the presence of precipitating serum inhibitors, and did not always clearly differentiate host-specific and virus-specific reactions. Immunodiffusion was considered to offer valuable complementary information to HAI tests in studies on the antibody response after infection or immunization of animals with influenza virus.

An influenza B infection of ponies was accomplished by Kasel et al. (1968). Eight out of twelve ponies developed fever, shed virus, and showed significant rises in the titre of serum neutralizing antibodies. Natural infections of animals by influenza B have not been reported, although serum antibodies to influenza B have been detected (Ditchfield et al., 1965; Brontiki et al., 1965; Topciu et al., 1966a; Blaskovic et al., 1970a). The demonstration that animals are susceptible to infections with influenza B raises the question of the position of influenza B in the epidemiology of influenza.

The results of epidemiological, serological, and antigenic

studies have yielded circumstantial evidence relating the virus that caused the swine epizootic in 1918 to the virus that caused the human pandemic of 1918-1919. However, no proof exists that one caused the other (McQueen et al., 1968; Pereira, 1969; Steele, 1971).

Attempts have been made to demonstrate that such an interspecies transfer was feasible. Shope and Francis (1936) showed that swine infected by human A/PR8 virus under natural conditions developed a mild swine flu-like disease. Transmission by contact to other swine succeeded in only one case. Swine with a previous history of swine flu were immune to PR8 infection, but those infected with PR8 were not immune to challange with swine influenza. Human FM 1 strains may cause a mild disease in swine, and virus is recoverable from the lungs (Gulrajani, 1951). Infection of young piglets with a human A2/Asian isolate produced only virus shedding and homologous HAI antibodies (Wallace and Kissling, 1959). Isolated virus, on intranasal passage to a second group of pigs was also shed and the pigs developed only HAI but not complement fixing antibodies. Because the Asian strain had not established itself in swine in the U.S., the conclusions were that pigs did not play a significant role in the Asian epidemic in that country.

The evidence presented is not very convincing that transfer from man to swine can occur and persist.

Blaskovic et al. (1970 a, b, c) studied the epidemiology of swine influenza by introducing Czech/57 swine influenza virus into a disease-free pig farm. They demonstrated the following. Susceptible weanlings lacking specific antibody could become infected intranasally

and develop some clinical symptoms. Actively infected animals shed virus up to 3 months or more, harboring virus in lung tissue in the presence of humoral antibodies, and could infect susceptible pigs. All infected pigs developed significant rises in HAI and VN (and some in CF) titres which persisted for up to 1 year and 1½ years respectively. HAI antibodies were not produced against PR8, FM1, A2/Sing/57, or A2/Hong Kong/68 (inhibitor resistant variant). Neither lungworm nor meteorological stress seemed to be necessary to perpetuate the infection as demonstrated by Shope (1941ab,1943ab). Virus was not transmitted in ovo or through the placenta in off-spring, but protective antibodies were passed to piglets through the colostrum protecting them from infection for up to 30 days. These finding have added valuable information regarding influenza infection and transmission in swine. The use of a virus different from the classical strain has evolved a different idea of its transmission in pigs. The strain of the virus seems all important in epidemiological studies of this sort.

Styk et al. (1971 b) intranasally infected piglets with A2/ Hong Kong/68 and with A/Swine/Taiwan/70 (Kundin, 1970). Although virus was nasally shed up to seven days post-infection, very little response was apparent: no disease symptoms, no viraemia, and little or no antibody to hemagglutinin or neuraminidase was detected. Antibody to S antigen was detected by immunoprecipitin tests. A single contact pig may have been infected.

Steele (1971) has attributed the persistance of swine influenza virus to the yearly turnover of pig population, whereas the immunity of long-lived humans has caused human strains of virus to fade.

Although natural infections of humans from an animal reservoir or vector have not been proven, there is a recent report of an isolation of a Fowl Plague-like virus from a patient suffering with infectious hepatitis (DeLay _et al_., 1967; Campbell _et al_., 1970). This is most unusual and offers unpleasant possibilities of a human vector of FPV, and a potential source of new human viruses by recombination between FPV and human influenza A viruses, which has already been accomplished experimentally (Tumova and Pereira, 1965; Easterday _et al_., 1969).

Humans have been experimentally infected with an animal influenza strain, A/Equi 2 (Kasel _et al_., 1965, Alford _et al_., 1967; Kasel and Couch, 1969). Some developed an influenza-like febrile illness shedding virus up to 6 days post infection. The majority of individuals developed significant serum neutralising antibodies to Equi 2, and a portion of these developed heterologous antibodies to human A2/Asia (Kasel _et al_., 1965; Alford _et al_., 1967), and others to A2/Hong Kong/68 (Kasel and Couch, 1969). The latter authors found that heterologous antibody developed by Equi 2 infected humans protected the humans from intranasal challange by A2/Hong Kong/68, the degree of protection depending on the magnitude of the initial heterologous response. Kasel and Couch cautioned about the interpretation of surveillance data based on serological surveys alone, as these could not be a reliable indicator of interspecies spread during outbreaks of influenza in either species. However, the occasional exchange of antigenically related viruses in nature was thought to be possible.

Blaskovic _et al_. (1970a) were not able to demonstrate virus infection of man during the epidemiological studies of swine influenza

discussed above. The slight rise in the serum antibodies of some individuals exposed to the virus was not considered to be significant.

Experimental influenza infections of various squirrels, minks, lemmings, and field voles have been reported (Hoyle, 1968).

The above discussion has pointed out that animals can be infected with human strains of influenza viruses. The antibody response of such infection may be of heterologous nature, which influences the interpretation of results of serological surveillances of animal sera. As yet, definite proof implicating animals as a source of human infection is lacking, but a potential has been demonstrated which warrants continuing investigations into this field.

Recombination of Influenza Viruses

It has been proposed that new antigenic strains capable of causing pandemic influenza in man have arisen from recombinations of human and animal or avian species (Pereira, 1969). Webster and Laver (1971) have recently reviewed this subject and describe the following circumstantial evidence supporting this theory.

Only type A influenza viruses cause both human pandemics and infections in animals and birds. Influenza A viruses have a high recombination frequency which may be because they appear to have a segmented genome. Antigenic hybrids of various combinations of human, animal, and avian influenza viruses have been produced in the laboratory. Influenza viruses isolated from natural infections of birds possess one of the surface antigens (neuraminidase) which is immunologically indistinguishable from that of some human influenza strains. They

suggest that the recombination of influenza viruses demonstrated in the laboratory may be the mechanism by which new strains arise.

Webster *et al.* (1971) demonstrated the production of influenza antigenic hybrids *in vivo*. The new viruses were virulent for host animals causing either a severe fatal disease or a mild respiratory infection. Mixed infections of turkeys with FPV and turkey influenza virus yielded a high rate of antigenic hybrids containing FPV hemagglutinin and turkey influenza neuraminidase; reciprocal hybrids were also produced. Similar mixed infections of pigs with FPV and swine influenza yielded antigenic hybrids with FPV hemagglutinin and swine influenza neuraminidase, and their reciprocal hybrids.

Webster and Campbell (1972) have demonstrated, using chickens and a turkey-FPV recombinant, that selection of new influenza viruses can occur *in vivo* , yielding genetically stable hybrids, which can cause severe or fatal infection in natural hosts, in this case, chickens. They suggest that "new" influenza viruses of man could arise by a similar mechanism.

Thus genetic interaction between mammalian and avian viruses is possible in both a mammal and a bird, between avian viruses in a bird, and genetic recombination can take place *in vivo*, which strengthens the theory that human pandemic viruses could originate from recombinations between viruses in lower animals. However, until a recombination is demonstrated which yields viruses capable of producing transmittable disease in man, the evidence for this source of pandemic influenza strains is still circumstantial.

Serological Surveillance of Influenza Infections

The detection of antibodies to influenza viruses in animal, avian, and human sera by serological tests such as HAI CF, VN, etc. have been used to determine if infection has occurred in a species, which is of significance in elucidating the epidemiology of influenza.

Kaplan and Payne (1959) reported on the first large serological survey of animals, organized by the World Health Organization in 1957, following the observations that the new human influenza variant A/Asia/57, causing a human pandemic, was reported to be associated with an epizootic of pigs in China. This occurrence was likened to a previous human pandemic-swine eipizootic association which had occurred in 1918 and in which the etiologic agents were thought to be similar if not identical, namely the type A swine influenza virus. The isolation of other type A influenza viruses from natural infections in horses, fowl, and ducks added to the speculation that lower animals could possibly serve as a reservoir for new variants of human influenza viruses.

Swine and horse sera were surveyed for the presence of antibodies to Asian, equine, and swine influenza in order to determine if these animals were reservoirs and a possible source of the new human influenza variant which had suddenly appeared.

Twenty countries participated in carrying out complement fixation and hemagglutination inhibition tests on horse sera, and HAI tests on swine sera, using as antigens newly isolated human A2 strains, and the natural influenza strain of each species. Standard procedures recommended by the WHO Expert Committee on Influenza (1953, 1959) were

followed. The CF test was to detect type A specific antibodies of recent infections, and the HA would cover recent and remote strain-specific reactions. Much trouble was experienced with non-specific inhibitors because the recommended V. Cholerae filtrate and periodate treatments did not seem to eliminate them effectively, especially from horse serum, which made interpretation of results difficult. However, the survey revealed that 1) the A2 strain can cause a natural infection in horses, 2) equine influenza caused by A-Equi or a closely related strain is present in many countries, 3) the A2 strain can cause natural infection in swine, 4) A/swine strain infection in pigs, long endemic in the U.S.A. was present in at least two European countries, West Germany, and Czechoslovakia. These results were added to the accumulated knowledge concerning the source of human influenza which was being intensively studied. The origin of the new strain was not apparent from these studies.

A survey carried out in pigs by Akao et al. (1960) in Japan showed a very low incidence of antibodies to swine influenza and none to the human A2 strain. The authors concluded that swine did not play a part in the epidemiology of the new variant in Japan.

Similar surveys have been carried out before and since, with much the same objectives. A few of these will be discussed.

As mentioned above, the identity of the virus causing the human pandemic of 1918-1919 was circumstantially established as a swine influenza virus by the detection serologically, of the presence of specific antibodies in human sera.

Antibodies to human influenza viruses have been serologically detected in swine in a similar manner, and have implied infection of swine by human influenza viruses.

In 1937, in the Eastern U.S., antibodies to human influenza viruses found in the sera of herds of swine neutralized the virus isolated from a current influenza epidemic occurring in the same institution, but would not neutralize swine influenza virus (Stokes et al., 1937; Shope, 1938). The serological evidence implied that the pigs were infected with the human virus, which is evidence of interspecies transfer of influenza virus from man to swine, similar to that suspected of occurring in 1918.

A similar situation was revealed in Hungary by Barb et al. (1962), who found that post-epizootic swine sera contained HAI antibodies reacting specifically with virus isolated from pigs during the epizootic which had been identified as A/PR8 by serum absorption and mouse pathogenicity tests.

The most recent and most convincing suggestion of inter-species transfer was detected by Kundin (1970), in an investigation to determine the role of pigs in human influenza infection. During an influenza surveillance study of apparently healthy pigs in Taiwan in 1969, a steady increase in the number of sera with HAI titres ($\geq$ 1:40) to A2/Hong Kong/68 was noted from June/69 (1%) to February/70 (38%), and an increase in the incidence of virus isolations. A human epidemic caused by A2/Hong Kong/68 occurred December/69 to January/70. Virus isolated from the pigs was antigenically identical to human A2/Hong Kong /68, and was shown to infect humans (Beare et al., 1971, see discussion

above). Preliminary indications at that time were that the infection appeared in man first.

The information yielded by serological studies, is often an indication of a trend of infection in an animal population, indicating the most likely place to look for possible virus isolates, and whether such viruses have been present in the population.

In Czechoslovakia, a comparative study of infection of horses by A/Equi 2 and A2/Hong Kong/68 required non-immune horses (Blaskovic et al., 1969a). Periodate treated sera of over 800 horses demonstrated a low incidence of HAI antibodies to A/Equi 1 and a complete lack of HAI antibodies to A/Equi 2 which correlated with low incidence of infection by Equi 1 and the absence of an eipzootic and infection by Equi 2 in the few years before the survey. Non-specific inhibitor to A2/Hong Kong/68 found in untreated sera (all positive with inhibitor sensitive variant - IS, and a few positive with inhibitor resistant variant - IR) was almost completely eliminated by periodate treatment, only a few sera reacting with the IS variant. The authors considered the residual HAI activity to be due to nonspecific inhibitors but stated a more thorough study would be needed to determine whether they should be considered antibodies.

Ditchfield et al. (1965) reported a high incidence of antibodies to influenza B in a serological survey of horses in the Toronto area. Antibodies to influenza A and B type viruses have also been reported in cattle and sheep (Macpherson et al., 1963; Brontiki et al., 1965), and the latter authors have also reported influenza A and B type antibodies in the sera of dog, fox, wolf, hare and duck.

Cats live in close contact with humans. Paniker and Nair (1970) were successful in infecting cats with A2/Hong Kong/68 influenza and therefore conducted a survey of 28 cat sera to detect antibodies to the virus. Six out of 28 trypsin-heat-periodate treated sera had HAI titres ≥ 1:80, but it was not established whether these represented specific antibody or non-specific inhibitors.

In a survey of domestic animals in Ireland, 13/45 dog and 4/20 cat sera were found to inhibit hemagglutination by A2/Asia/57 inhibitor sensitive but not by inhibitor resistant strains. Therefore, all HAI was considered due to non-specific inhibitor because antibody, if present, would have inhibited hemagglutination by the inhibitor resistant strain (Meenan et al., 1962).

Topciu et al. (1966b) investigated the presence of influenza viruses in dogs because of their close proximity to man thus forming a potential reservoir of influenza viruses. A high incidence (70-88%) of high titre (1:3500) influenza hemagglutinin inhibitors to two strains of B type virus, a low incidence (4-1%) of low titre inhibitors to PR8, FM1, and A2 strains, and no inhibitors to B/Lee or A swine strains was found. Later work (Topciu et al., 1967) revealed that the inhibitor of influenza B in normal dog serum was distinct from known influenza inhibitors (alpha, beta, and gamma) as it resisted all usual inhibitor inactivation methods. He was not able to identify it as specific or non-specific in nature.

When over 700 dog sera from various sources were examined serologically by Lundegren et al. (1969), low HAI titres to an A2 strain were found in 6% of the sera (RDE treated). A group of these retained

HAI activity after treatment with either periodate, trypsin-periodate, or CO_2, but the reacting component was not referred to as antibody, perhaps because of Topciu's findings (1967). Complement fixing antibodies were demonstrated in the sera, 8% with type A, 6% with type B, and 1% with type C. There was no correlation between HAI and type A CF positive results. The authors stated that the results reflected the dogs had been exposed to influenza virus in the few months prior to sampling and any infections had been of subclinical nature.

It is apparent that the presence of non-specific inhibitors of influenza virus hemagglutination in animal sera interferes with the determination of influenza antibody content of sera, and makes the interpretation of data difficult in relation to epidemiological and other studies. Attempts have been made to overcome this problem.

Nakamura and Easterday (1967) attempted to establish an effective method for differentiating specific antibody to influenza viruses from non-specific inhibitors occurring in animal and avian sera reported to contain antibodies to human influenza viruses. These were differentiated on the basis of the type of antibody response to challange with virus.

Animals containing only non-specific inhibitor were shown by these authors to produce a primary antibody response when exposed to influenza virus, whereas those with antibody from previous antigenic exposure produced a secondary antibody response following challange with the same virus antigen as had been first experienced. On this basis, sheep and cattle examined in the field produced only primary responses,

suggesting the non-specific nature of inhibitor substances in the sera. Animals with previous influenza infections, eg. swine and turkeys, produced secondary antibody responses. Further studies such as this would give more meaning to the evaluation of the role of animals in the epidemiology of influenza.

Migratory birds have been suggested as vectors in the dissemination of influenza viruses (Wells, 1963; Becker, 1966; Pereira et al, 1967b), therefore Easterday et al., (1968) examined 50 sera of 9 species of migratory waterfowl in North America for the presence of influenza antibodies to 16 different avian virus strains isolated in North America or related to these. Serological methods included HAI, CF, and VN (eggs). Two of 9 species examined, Canada goose and snow goose, in good correlation between serological tests, were found to contain antibodies reacting with 5 strains of Turkey influenza viruses and with Duck/Eng/66. In HAI tests sera cross-reacted with several antigens. The Canada geese were represented by 3 populations. These serological observations indicated in what species circulating virus could be isolated and identified, as data to determine the mode of dissemination of avian viruses among wild and domestic bird population is lacking.

Asplin (1970) did not find HAI antibodies to Duck/Eng/56 and /62 in 8 species of wildfowl in England including mallard, pintail, teal, tufted, pochard, shoveller, coot, and moorhen.

Continuing the examination of wild bird sera for influenza antibodies, Dasen and Laver (1970) found that 10% of over 300 shearwater (Puffinus pacificus) living on a small uninhabited island on the NE coast

of Australia inhibited the neuraminidase of A2/Asian/57 virus, some to high titre, and inhibited the neuraminidase of A2/Hong Kong/68 to a lesser degree, but did not inhibit the enzyme of AO/Bel at all. The inhibiting substance in serum was assumed to be avian IgG (7S) because in a sucrose density gradient, it had a sedimentation coefficient of 6S. Absorption experiments further confirmed the identity of A2/Asian/57 anti-neuraminidase. Antibody to type-specific A internal antigen was not detected, suggesting the birds were infected long before by an influenza virus possessing neuraminidase identical to that of A2/Asia/57.

Ten percent of one group of the Shearwaters contained antibody to type-specific A antigen (RNP), which was revealed by immunodiffusion in agar gel, suggesting a more recent influenza epidemic had occurred in this group. They were in contact with a species of migratory birds coming across Asia, Europe, and North America (Kilbourne et al., 1972). Immunodiffusion and neuraminidase inhibition test results did not correlate. Sera of 100 chickens in New South Wales were all negative for the above antigens.

The demonstration of antibodies related to human influenza viruses in these wild birds supported the idea that human influenza epidemics may originate from avian or animal reservors and the authors suggested that the isolation of as many avian and animal influenza strains as possible would be expedient in the epidemiology of influenza virus infections.

During the screening of 90 animal sera from the London Zoo for non-specific inhibitors of human influenza virus hemagglutination (Shortridge et al., 1970), three sera, two Asiatic black bears and one

orangutan, after neuraminidase and metaperiodate treatment, exhibited an inactivation pattern more like specific antibody than inhibitor. The presence of antibody to A2/Sing/57 was confirmed by complement fixation and virus neutralization (eggs). Complement fixation and hemagglutination inhibition activity of one Asiatic black bear serum were shown to be associated with a 7.2 S component obtained by sucrose density gradient centrifugation of the serum. The complement fixing activity was exclusively associated with an IgG.

Except for contact infection in a baboon (Kalter et al., 1969, see above), the unique finding of an antibody to a human influenza virus in a wild species of animal suggested a human source of infection (possibly at the zoo), and added yet another species to the potential group of animal reservoirs of human influenza virus, this one originating from the same general area as the last two major variants of human influenza.

An important immunological contribution of the papers by Dasen and Laver (1970) and by Shortridge et al. (1970) was the definite confirmation that the anti-influenza substance in the sera of birds and animals was a 7 S component suggestive of antibody rather than a non-specific inhibitor.

Inhibitors of Influenza Virus Activity

The usefulness and reliability of standard immunological methods involving the detection and measurement of influenza viruses and their specific antibodies is plagued by the presence of inhibitors, often

referred to as "non-specific", in animal sera and secretions. These inhibitors by antibody-like reactions, hinder the interpretation of hemagglutination inhibition tests, virus neutralisation tests, and precipitation tests, especially in immunodiffusion, but do not pose a problem with complement fixation tests.

Viral inhibitors in serum do not confine their activity to influenza and other Myxoviruses, but are also complicating factors with many other virus groups e.g. adeno-, arbo-, picodna-, reo-, rubella, vaccinia and others (Allen et al., 1958; Tauraso et al., 1971).

The inhibitors of influenza viruses have been widely investigated and discussed, the subject referred to in publications including the immunological aspects of influenza (Chu, 1951; Burnet and Stanley, 1959; Cohen et al., 1963; Gottschalk, 1966 a, b; Hoyle, 1968 ;Krizanova and Rathova, 1969, are a few examples).

The following is a discussion of influenza virus inhibitors relevant to this study, that is, those concerned with hemagglutination inhibition and precipitation in immunodiffusion.

There are three known types of influenza inhibitors associated with immunological reactions: α or Francis, heat-stable (Francis, 1947); β or Chu, heat-labile (Hirst, 1942a, Smith et al., 1951; Chu, 1951) and γ, found in high titres in horse and guinea pig serum and active against Asian influenza viruses (Shimojo et al., 1958; Cohen and Belyavin, 1959, etc.).

The initial independent discovery of Hirst (1941) and McClelland and Hare (1941) that chicken erythrocytes were agglutinated by live

influenza virus (B/Lee) in allantoic fluid and that specific antibody inhibited virus hemagglutination, was applied to detect and measure the virus present in infected materials, and to determine the antibody content of sera. However, normal serum of rabbits and ferrets was found to inhibit virus hemagglutination (Hirst, 1942a,1943). A component in normal rabbit serum inhibiting A BEL D hemagglutination was destroyed on heating the serum 15 - 20 min. at 62°C, while the inhibiting activity of immune rabbit serum of the same virus was relatively thermostable (McCrea, 1946). The stability of antibody to heating at 62°C for 30 min. differentiated it from labile inhibitor (Anderson, 1959). Burnet and McCrea (1946) found that a heat-stable component in normal ferret sera inhibited hemagglutination of BEL D strains and neutralized infection of chick embryos and mice by this virus.

α Inhibitors

Francis (1947) discovered that the normal sera of humans, rabbits, and ferrets and their corresponding B/Lee immune sera inhibited the active, and to a higher titre, the indicator state of influenza B/Lee. This inhibitor became known as Francis, or α inhibitor (Smith and Westwood, 1950). Indicator virus is active virus heated at 56°C for 30 min., which loses its infectivity and power to elute from red cells but retains its hemagglutination capacity (Hirst, 1942 a, b; Briody, 1948; Stone, 1949a,b). It is very sensitive to inhibition of hemagglutination by inhibitor and has lost its power to react with antibody (Francis, 1947). Francis inhibitor was identified by McCrea (1948) as a component of the heat stable (up to 100°C) mucoprotein fraction of human and rabbit sera.

These mucoprotein α inhibitors of influenza A and B strains were found in the sera, tissues, and secretions of many animal species: in the normal sera of ferrets (Burnet and McCrea, 1946); normal human sera and tissue extracts, and normal rabbit and guinea pig sera (Friedenwald et al., 1947; Smith and Westwood, 1949); normal rabbit sera (Smith et al., 1951); normal ferret, fowl, rabbit, and guinea pig sera (Sampaio, 1952); and in many others. The presence of α inhibitors was revealed in other biological fluids, the two most powerful inhibiting mucoproteins being ovine submaxillary gland (McCrea, 1948, thesis) and the urinary mucoprotein of Tamm and Horsfall (1950, 1952). Others occurred in saliva (Francis and Minuse, 1948); tears (Anderson, 1948); ovarian cyst fluid (Burnet, 1948a); ovomucin (Gottschalk and Lind, 1949); normal allantoic fluid of chick embryo (Svedmyr, 1949a); bronchial secretions (Curtain et al., 1953b); human meconium (Curtain et al., 1953 a, b); glycoproteins isolated from human erythrocytes (Kathan et al., 1961; Kathan and Winzler, 1963); edible bird's nest (Howe et al., 1961; Biddle and Belyavin, 1963).

The α inhibitor of the above mentioned mucoproteins actively inhibited hemagglutination of indicator viruses (Stone, 1949b) of influenza A strains such as WSE, PR8, BEL, Swine, and especially influenza B strains, such as Lee and Rob, but had diminished or no activity against the same strains of living viruses. Chu (1951) stated that active virus formed a transient combination with the inhibitor and subsequently destroyed it. Alpha inhibitors have been shown to react with indicator A and B strains (Cohen et al., 1963). Mucoprotein inhibitors react with viruses in a receptor gradient, that is, an inhibitor treated by an active virus

will no longer inhibit the indicator virus of the same strain, but will inhibit viruses further down the gradient (Stone, 1949b). Not all mucoprotein inhibitors react with all viruses, some have more restricted action. However, some powerful inhibitors such as urinary mucoprotein inhibit both active and indicator MEL and WSE (Burnet, 1952).

In both sera and mucoproteins, α inhibitors have not been shown to neutralise infectivity of influenza viruses (Chu, 1951; Cohen et al., 1963; Cohen and Dorman, 1965).

Alpha inhibitors in normal rabbit and human sera were inactivated by the receptor-destroying enzyme (RDE) of V. cholerae filtrate (isolated by Burnet et al., 1946 ; Burnet and Stone 1947), and by living influenza viruses (Anderson, 1948). Anderson also noted that specific antibody titer was diminished by RDE treatment in a small proportion of human immune sera. Purified RDE was subsequently found to destroy α inhibitors in normal ferret sera (Isaacs and Bozzo, 1951) and in normal rabbit, ferret, guinea pig, and fowl sera (Sampaio, 1952).

Proteolytic agents such as trypsin inactivated α inhibitor in normal animal sera: rabbit and human (McCrea, 1948); ferret, fowl, guinea pig, and rabbit (Samapio and Isaacs, 1953).

Dilute periodate, as potassium or sodium salt, rapidly inactivated the inhibitor at room temperature in normal rabbit, human and other sera (McCrea, 1948; Burnet and Lind, 1954; Jensen, 1957a).

Kaolin adsorption reduced the inhibitory titer but was less effective than heat (Samapio, 1952).

The α inhibitors in other mucoprotein substances such as ovarian cyst fluid (Burnet, 1948 a) and ovomucin (Gottschalk and Lind, 1949) were also inactivated by dilute periodate solutions (0.01 M), by the action of RDE, and by the enzymic action of live influenza viruses, the strain B/Lee the most active of those tested. However, Burnet (1948 b) demonstrated that such mucoids, virtually inactive against live A strains, could be converted into powerful inhibitors of those strains both *in vitro* and *in vivo*, by treatment with extremely small amounts of periodate.

An investigation of the chemistry of mucoproteins initiated by Gottschalk, Blix, Klenk, and others gave an insight into the mechanisms involved in the inhibition of influenza virus hemagglutination by mucoproteins. This is excellently presented by Gottschalk (1959, 1966 a, b), and only a very brief reference will be made here.

The observations that: Francis inhibitor in normal serum strongly inhibited indicator viruses (Francis, 1947); the inhibitory component was a mucoprotein (McCrea, 1948) and was susceptible to RDE (Anderson, 1948); human red cells contain carbohydrate material which inhibited virus hemagglutination (de Burgh *et al*., 1948); a potent inhibitor in ovomucin was destroyed by treatment with active influenza virus, RDE, or periodate (Gottschalk and Lind, 1949) led the latter authors to suggest that certain soluble glycoproteins inhibited hemagglutination by competing with red cell receptors for the influenza virus particle and that a common structural feature, a carbohydrate, served as a substrate for both enzyme and RDE. The loss of biological activity of these mucoid inhibitors by treatment with RDE or living virus was accompanied

by the release of a low molecular weight component identified by Klenk _et al_. (1955) to be a sialic acid, the group name for various types of acetylated neuraminic acids (N-acetylneuraminic acid, N-glycolylneuraminic acid, N, O-diacetylneuraminic acid, etc., after the nomenclature of Blix _et al_. (1957)). Gottschalk (1957 a, b) identified neuraminidase as the enzyme present in RDE, and the enzyme embedded in the influenza virus surface, which inactivated the inhibitor by a hydrolytic cleavage of the sialic acid linkage. He stated that all virus hemagglutination inhibitors were glycoproteins containing sialic acid, the consituent split off by neuraminidase. He defined glycoproteins as conjugated proteins, containing as prosthetic group, one or more heterosaccharides with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the polypeptide chain. (Gottschalk, 1962).

Live influenza virus neuraminidase destroyed virus receptors at the red cell surface rendering them inagglutinable (Hirst, 1942 b), and both RDE and live virus neuraminidase destroyed virus receptors at erythrocyte and host cell surface (Burnet _et al_., 1946) with the release of sialic acid (Klenk _et al_, 1955). Gottschalk (1966 a, b) stated that sialic acid is an important factor in attaching virus to receptor in the erythrocyte surface and to the soluble inhibitory glycoprotein. Therefore, the inhibition of influenza virus hemagglutination by a glycoprotein is the outcome of competition for the virus particle between the glycoprotein receptors embedded in the red cell surfaces and the soluble inhibitory glycoprotein. To be a potent inhibitor of viral hemagglutination, the glycoprotein must have a high affinity for the indicator virus. The inhibitory potency is determined by a combination of the number of sialic

acid residues available per glycoprotein molecule, and the complementariness of the contacting areas on the surface of the virus and glycoprotein (Fazekas de St. Groth and Gottschalk, 1963).

The electrophoretic mobility of α inhibitors has been investigated. Sialic acid is responsible for the high electrophoretic mobility of sialoglycoproteins (Gottschalk, 1966 a). The electrophoretic mobility is reduced when sialic acid is split off by neuraminidase (Ada and Stone, 1950). Tyrrell (1954), by starch zone electrophoresis, demonstrated that α inhibitor migrated with α globulins even in immune serum and was quite distinct from β inhibitor and from antibody. Alpha inhibitor of human sera was elevated during the acute phase of various infectious diseases. In rabbit and human serum, α inhibitor contained at least two components of different electrophoretic mobility and biological specificity: the first migrated with α_2-macroglobulins and inhibited hemagglutination of heated B/Lee and to a lesser extent heated PR8; the second minor one moved more rapidly than the first and inhibited both viruses to the same degree. Harboe _et al._, (1958) showed α inhibitor to be electrophoretically heterogeneous, the activity present in several components of rabbit serum. Cohen and Belyavin (1961) fractionated normal rabbit serum by precipitation, and on paper electrophoresis found alpha inhibitor activity in at least three fractions, the euglobulin, pseudoglobulin, and albumin. Fractionation studies on human sera by Biddle and Shortridge (1967) demonstrated the neuraminidase-sensitive α inhibitor to be an α_2-macroglobulin, similar to the 18 S compound described in horse serum (Biddle _et al._, 1965). Human serum had been shown to contain primarily α inhibitor (Hilleman and Werner 1953; Levinson _et al._, 1969). Horse serum also contains α inhibitor

especially atypical serum which contains little, if any γ inhibitor (Cohen et al., 1963, Cohen et al., 1965). A discussion of α inhibitor in horse serum is included below in the section on γ inhibitors.

Alpha inhibitors have a wide range of sedimentation coefficients e.g. 200 S in normal allantoic fluid (Svedmyr, 1949 a); 31-37 S in ovomucin (Sharp et al., 1951); 29.5 S in human urine (Perlmann et al., 1952, Tamm et al., 1955); 8.5 S in ovine submaxillary glands (Gottschalk and Simmonds, 1960; Gottschalk and McKenzie, 1961); 2.16 S in human erythrocytes (Kathan et al., 1961). Details of these with molecular weights are to be found in Gottschalk (1966 a).

β Inhibitors

Heat-labile inhibitors in serum were first noted by Hirst (1942a) and McCrea (1946). Ginsberg and Horsfall (1949) described a heat -labile component in human, rabbit, guinea pig, and mouse sera which inhibited hemagglutination and neutralized infectivity of NDV, influenza A and B, and mumps viruses. Because it was not removed by absorption with a heterologous antigen-antibody complex, and addition of sufficient virus did not alter complement activity, the thermolabile component was not hemolytic complement. Smith and Westwood (1949), in addition to α inhibitors, described similar thermolabile inhibitors of live influenza hemagglutination in sera of mice, rabbits, guinea pigs, and human sera. All authors noted that the inhibitory titres of thermolabile components were lower than those of heat-stable Francis inhibitors.

Based on detailed investigations of normal mouse serum, Chu (1951) presented the following description of heat-labile "Chu" inhibitor.

It inhibits hemagglutination of all egg-grown A-prime strains isolated since 1946, equivalent titers achieved with live or heated virus. Mouse adapted strains, and those egg-passaged in the presence of normal mouse serum , were resistant to the inhibitor. Therefore, the inhibitor was more active against unadapted (mouse) than mouse-adapted virus.

Like the thermolabile component in guinea pig serum described by Ginsberg and Horsfall (1949), Chu inhibitor required the presence of calcium ions for activity, formed a stable combination with the virus, and was not destroyed by active virus. Chu suggested that the removal of inhibition is due to specific adsorption by unadapted (mouse) virus and is not adsorbed by mouse-adapted virus whose charged surface configuration prevents effective union.

Kaolin effectively adsorbed inhibitor but also drastically lowered antibody in immune sera. The inhibitor was not inactivated by 0.05 M sodium periodate. Crude cholera filtrate destroyed the inhibitor of hemagglutination and the neutralising factor but semi-purified RDE did not. Neither RDE nor crystalline trypsin affected inhibitor, but an unidentified agent present in both crude enzyme preparations did. Sampaio and Isaacs (1953) suggested that a trypsin-like enzyme known to be present in crude V. cholerae extracts was the component destroying β inhibitor in rabbit serum. These authors also showed that crystalline trypsin was highly active in destroying Chu inhibitors in normal ferret, fowl, rabbit, guinea pig, and mouse sera with no observable significant effect on specific antibody.

The inhibiting and neutralising factors were considered by

Chu (1951) to be closely related, if not identical, because of similar patterns of anti-viral activity and susceptibility to inactivation.

Chu (1951) also investigated inhibitors in other animal species and found that unadapted (mouse) A-prime strains were inhibited to higher titers than adapted strains. Human, hamster, and cotton rat sera inhibited hemagglutination to low titers (<1/10 - 1/20), and mouse, ferret, guinea pig, and rabbit sera to higher titers (1/60 - 1/140).

Smith et al., (1951) described an inhibitor in normal rabbit sera more active against living A-prime strains isolated in 1951 than against standard reference strains. Neither RDE nor RDE followed by heating (56°C for 30 min.) destroyed it. They proposed the name β inhibitor to distinguish it from heat-stable α (Francis) inhibitor, also present in normal rabbit serum.

Sampaio (1952), comparing α and β inhibitors in six different animal species, confirmed Chu's findings and noted a variation in the types of inhibitor present in these sera. Mouse serum contained only β inhibitor, ferret and fowl sera mainly α, guinea pig and rabbit both, and hamster neither. Brans et al. (1953) equated heat labile and Chu or β inhibitors. The presence of a thermolabile component in ox serum which inhibited hemagglutination and neutralised infectivity of A-prime and B influenza was reported by Briody et al. (1955). Henry and Younger (1957) noted that a thermolabile substance in rabbit, guinea pig, and calf serum neutralized the cytopathogenic effects of A-prime strains in monkey kidney cell cultures. It did not affect B or mouse adapted A strains and was believed to be a β inhibitor. They recommended heat

inactivation of all sera used in tissue culture systems. Wagner (1955) felt that Chu inhibitor resembled the properdin system because treatment of mouse serum with zymosan, known to inactivate properdin, completely inactivated hemagglutination inhibition of unadapted (mouse) A strains.

Tyrrell (1954), by starch-zone electrophoresis of normal sera of mice, guinea pigs, rabbits and normal beef plasma, and of immune rabbit and human sera, separated α and β inhibitory activities from that of antibody. Alpha inhibitors were present in α-globulins, β inhibitors in slow-moving β-globulins, and all antibody activity was confined to γ-globulins. Cohen and Belyavin (1961), separated inhibitory components in normal rabbit serum by fractional precipitation methods with subsequent paper electrophoresis of the separated fractions. Beta inhibitory activity was found mainly in the pseudoglobulin fraction. They found it difficult to relate their results to those of McCrea (1946) and Tyrrell (1954) because of the varying and scanty criteria employed to recognize hemagglutination inhibitors in general, and β inhibitor in particular.

In a study of inhibitors of bovine sera active against polio, polyoma, and PR8 viruses, Ackerman and Dinka (1965) found all such activity present in the macroglobulin fractions in which early or primary antibodies are usually found, and not in the 7 S gamma-globulin fraction. The inhibitory activity was destroyed by β-mercaptoethanol which is known to inactivate the immunoglobulin IgM. Because no A-prime strains were used to detect β inhibitory activity, it is difficult to evaluate this study.

Krizanova and Sokol (1966) isolated purified β inhibitor from bovine serum by chromatography on silica gel columns, followed by

fractionation with DEAE cellulose, the final purified product achieved by complexing-dissociation methods involving A-prime virus, the presence of calcium ions, and EDTA. The purified β inhibitor had a sedimentation constant of about 4S; a buoyant density in cesium chloride of 1.35 gm/cm^3; and HAI activity of 16,000 to 25,000 units per mg protein; formed one or two precipitation zones on immunodiffusion in agar gel; and on paper electrophoresis migrated in the region of fast-moving serum gamma-globulins or β_2 -globulins. This β inhibitor was not in the same globulin fraction as the one isolated by Hana et al., (1963) on Sephadex G-200 whose activity was associated with the macroglobulin peak. The inhibitor was shown not to be a lipoprotein as had been suggested by Polyak et al. (1959).

The actual chemical structure of β inhibitor has not been described. Unlike α inhibitor it is not inactivated by KIO_4 or purified RDE, thus suggesting that it doesn't depend on a polysaccharide prosthetic group like sialic acid for its activity (Cohen and Belyavin, 1961). Ackermann and Dinka (1965) noted that although characterisation of α inhibitor is well-defined, the origin and nature of β are not. The definition of the chemical structure of β inhibitors may help to reveal the manner in which it acts in the same way as it has with α and γ inhibitors, thus defining its anti-viral activity in relation to that of antibody.

γ Inhibitors

When a new antigenic strain of influenza virus, the Asian or A_2, appeared in 1957, a hemagglutination inhibitor that was active almost

exclusively against this strain, living or heated, was found in high titres in normal horse and guinea pig serum; at lower titres in normal rabbit and ferret sera (Shimojo _et al._, 1958; Cohen and Belyavin, 1959; Takatsy and Barb, 1959; Volakova _et al._, 1959); but not in human serum (Cohen and Dorman, 1965). This inhibitor differed markedly from the known α- and β - type inhibitors and became the prototype of a new class of inhibitors termed the γ-type (Shimojo _et al._, 1958, Choppin and Tamm, 1959a; Cohen and Belyavin, 1959, 1961).

The inhibitor actively inhibited hemagglutination and neutralisation of inhibitor-sensitive but not inhibitor-insensitive Asian strains (Choppin and Tamm, 1959b,1960 a, b; Cleeland and McKee, 1958). It was inactive against heated B/Lee and a live A prime strain (Cohen and Belyavin, 1959). Like other potent inhibitors of hemagglutination by living viruses which neutralized the infectivity of corresponding inhibitor sensitive strains (Burnet and McCrea, 1946; Smith and Westwood, 1949; Chu, 1951), the gamma inhibitor in horse serum neutralised infectivity of A_2 viruses in eggs (Cohen and Belyavin, 1959); and when administered intranasally shortly before or after infection of mice with A_2, protected them from death (Cohen, 1960, Link _et al._, 1964).

The inhibitor was heat stable (Volakova and Jandasek, 1959; Cohen and Belyavin, 1961) but heating at 70°C for 1 hour increased its HAI activity against sensitive A_2 strains 3-50 fold (Cohen _et al._, 1963).

Initially γ-inhibitor was found relatively resistant to the action of RDE neuraminidase (Cohen and Belyavin, 1959), but in later experiments it was shown that treatment with neuraminidase released 40%

of its sialic acid (Cohen et al., 1963). Trypsin partially destroyed it (Cohen and Belyavin, 1959, James and Fiset, 1959). Kaolin adsorption removed a large proportion of inhibitory activity (Spence, 1960). Oxidation by M/90 periodate as sodium or potassium salts completely destroyed the activity against A_2 strains (Jensen 1957a, Choppin and Tamm, 1959a, James and Fiset, 1959; Cohen et al., 1963).

Cohen et al. (1963) suggested that because γ inhibitors are inactivated by periodate, they were mucoproteins. They differed from periodate- and neuraminidase-sensitive α inhibitors present in about 10% of horse sera lacking γ inhibitors (therefore termed atypical), because neuraminidase did not completely release the bound sialic acid and did not affect their inhibitory powers. The susceptibility of both types of inhibitors to periodate and/or RDE confirmed these were not antibodies. The chemical identity of γ inhibitors in horse serum was revealed in a series of investigations involving its isolation, purification, and characterization.

Attempts had been made to separate active γ inhibitor from horse serum, but the severe precipitation and separation methods, including dialysis against distilled water, revealed inhibitory activity in several isolated components, with a wide variation in sedimentation coefficients and electrophoretic mobilities (Krizanova-Lauckova et al., 1961; Boretti et al., 1964; Krizanova and Lesko, 1964), rather than a single substance or group of substances. Cohen et al. (1965) found the inhibitory activity exclusively in the α-globulin fraction of horse serum when relatively mild methods of fractionation were used. Biddle et al. (1965) separated inhibitor from horse serum by centrifugation, electrophoresis, and

chromatography under mild conditions of ionic strength and pH. The purified and concentrated γ inhibitor with high HAI activity was mainly found in a single fast-sedimenting component (extrapolated S20,w = 18.0) with the electrophoretic mobility of an α_2-macroglobulin. The slow moving components isolated by earlier workers represented breakdown products of naturally occurring 18 S material. Pepper (1968a, b) continued the investigation, improving isolation and purification methods. He found, by the hydrolysis of horse serum and its sub-fractions by dilute acid, alkali, V. cholerae and influenza virus neuraminidases, that the sialic acids present are a mixture of N-acetyl- and N-glycolylneuraminic acids together with the 4-O-acetylated derivatives of these acids.

The inhibitory activity of the component in horse sera active against influenza A_2 viruses was found to be determined by a 4-O-acetyl-N-acetylneuraminic acid substituted α_2-macroglobulin. Observations based on both sedimentation and molecular sieve chromatography indicated that γ inhibitor could exist in 2 different molecular sizes which are interconvertible: a) in fresh normal serum its sedimentation coefficient is 12 S and molecular weight 0.5×10^6; b) under mildest purification procedures it forms a dimer and therefore as fractionated α_2-macroglobulin has a sedimentation coefficient of 18.1 S and molecular weight of 1.0×10^6. The purified component forms less than 1/5 of the total α_2-macroglobulin; its chemical composition of 85% protein and 15% carbohydrate was similar to that of α_2-macroglobulins in other species; and it has a capacity for linear polymerisation, behaving as a highly asymmetric, semi-stiff rod in solution.

Levinson <u>et al</u>. (1969) studied the effect of influenza virus strains, A2 and a type B isolated in 1965, on horse serum inhibitor treated with combinations of mild alkali, $NaIO_4$, and neuraminidase, to determine the important prosthetic sialic acid groups active in inhibition.

Sodium periodate treatment of the inhibitor, under conditions designed to reduce non-Malapradian oxidations to a minimum (Neuberger, and Marshall, 1966; Schneir and Rafelson, 1966), inactivated inhibitory action against A2 viruses by oxidation of the straight chain cis-hydroxyl groups of 4-O-acetyl NANA (Fig. 9).

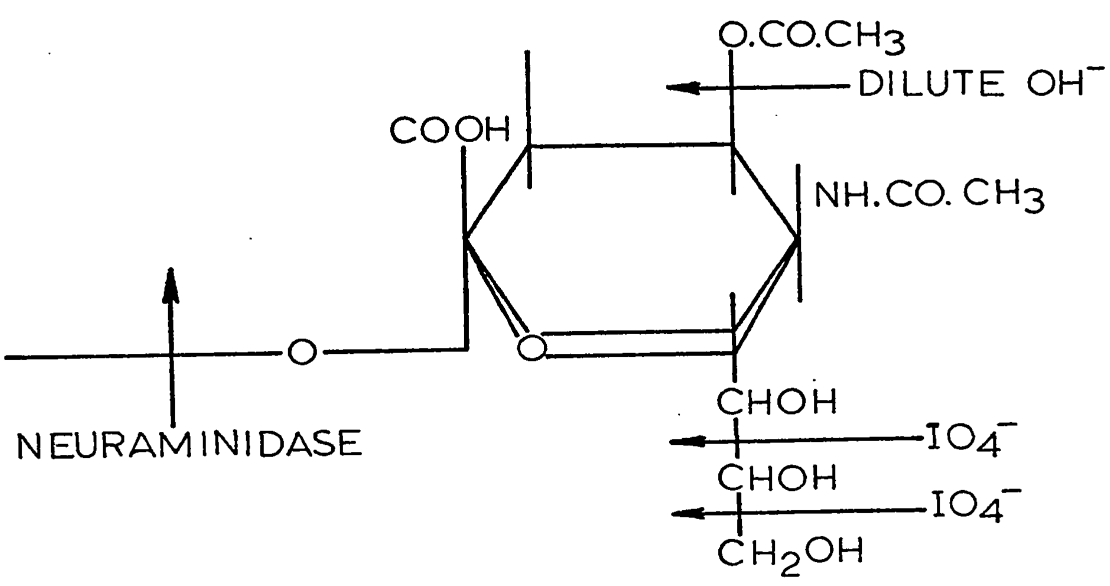

Fig. 9 Sites of action of $NaIO_4$, neuraminidase, and dilute alkali on the 4-O-acetyl-N-acetylneuraminic acid (from Levinson, Pepper, Belyavin, 1969).

However, this greatly enhanced the activity for B virus serum inhibitor as the new prosthetic group formed was a more effective inhibitor of B hemagglutination, that is a better fit was created for the receptor at the virus surface.

The A2 virus inhibitor was resistant to neuraminidase, but treating with 0.1 N NaOH removed O-acetyl groups (Fig. 9), altering the sialic acid prosthetic groups of the γ inhibitor to NANA, and thus converted it to an α inhibitor, which was neuraminidase sensitive (Pepper, 1968a, Levinson _et al._, 1969). The NANA became an active receptor site for both A2 and B strains; and it was susceptible to periodate enhancement of HAI activity against B viruses.

The results of the above experiments explained and confirmed the earlier observations of Choppin and Tamm (1960 b) that a single inhibitory substance can cause different types of inhibition depending on which virus is used to measure the activity. They found that a mucoprotein fraction of rabbit serum separated by electrophoresis behaved as a neuraminidase sensitive α inhibitor against heated Lee virus and as a characteristic γ inhibitor against A2 virus particles $RI/5^+$. Urinary mucoprotein, a powerful inhibitor (α) of heated Lee virus, also inhibited both heated and living A2 virus.

The experiments of Pepper and Levinson described above, with others, emphasised the importance of the configuration of the prosthetic sialic acid group of glycoprotein inhibitors in relation to the configuration of the receptor at the virus surface, that is, to be effective as an inhibitor of influenza virus hemagglutination, the

sialic acids must exactly fit into a site on the virus. Because the same type of sialic acid mediates both hemagglutination inhibition and neuraminidase resistance, only one type of binding site is necessary on the virus surface to explain the action of inhibition and its inactivation (Pepper, 1968 a; Levinson et al., 1969). These authors added a fourth criterion to the three criteria stated by Gottschalk (1966a) which glycoproteins must fulfill to be effective inhibitors of influenza hemagglutination: the structure of neuraminic acid.

These experiments also showed how to establish the identity of an inhibitor and thus suggest the most effective treatment for its inactivation.

Comparison

Choppin and Tamm (1960b, 1964) pointed out that a single inhibiting substance can cause different types of inhibition depending on which virus is used to measure the activity. Thus the same mucoprotein fraction of rabbit serum separated by electrophoresis, may behave as a neuraminidase sensitive α inhibitor against heated Lee virus and may behave as a characteristic γ inhibitor against sensitive A2 virus. They prefer to refer to serum inhibitors as horse serum mucoproteins, human serum mucoproteins, etc., and to identify such features as electrophoretic mobility and relate these to their biological activities.

The major inhibitory component in horse serum was shown to be an 18 S α_2-macroglobulin (Biddle et al., 1965). An antiserum prepared against an isolated human α_2-macroglobulin cross-reacted with partially purified α_2-macroglobulins (possessing influenza virus inhibitory

activity) of horse, monkey, ox, goat, and sheep sera (James and Stanworth, 1964; James, 1965). Biddle and Shortridge (1967), by gel diffusion and immunoelectrophoresis, confirmed the presence of an antigenically common α_2-macroglobulin in the sera of horse, human, monkey, bovine, calf, and ferret. In addition, they were able to show that neuraminidase sensitive 18 S α_2-macroglobulin in human serum which inhibited heated B virus was antigenically similar to a periodate sensitive 18 S α_2-macroglobulin in horse serum which inhibited sensitive A2 virus. Although both of these are glycoproteins containing sialic acids, and are physically and chemically similar, they express different biological activities. A common antigenic determinant is shared by the α_2-macroglobulins of different species and this property is shared by influenza virus inhibitors which occur in α_2-macroglobulin fractions of the sera. The reason for differences in biological expression of these has not yet been elucidated. However, as discussed above, Pepper (1968 a) and Levinson _et al_. (1969) were able to relate the inhibitory action of horse serum inhibitors to their chemical structure. This work could be expanded and applied to explain the action of inhibitors in other animal sera.

From the above discussion of the inhibitors of influenza viruses, it is apparent that the inhibitor expression of an animal serum may be due to a) the presence of more than one inhibitor type, or b) different activities by the same molecular structure, and these depend upon the virus strain. This is an important factor to consider when treating different species of animal sera for inactivation of these inhibitors.

Ananthanarayan and Paniker (1960) compared the effect different methods of inactivation had on normal animal serum inhibitors and their

hemagglutination inhibition of five prototype influenza strains: B, AO, A1, A2, and Swine. Nine species of sera were used: guinea pig, horse, monkey, dog, cow, rabbit, sheep, fowl. Sera were inactivated by five standard treatments commonly used in laboratories: heat, RDE, KIO4, trypsin-heat-periodate (Alabama method), trypsin. No one technique was applicable for all types of sera against all strains. The inhibitors differed qualitatively and quantitatively in different animal species as revealed by their varied response to destruction. The treatment with broadest application was found to be trypsin-heat-periodate (Alabama method).

Inhibitors of Influenza A2/Hong Kong/1/68

The appearance of influenza A2/Hong Kong in 1968 created additional problems with inhibitors. The inhibitory pattern of this virus was not the same as that of previous A2 viruses. They were highly sensitive to inhibitors present in horse, guinea pig and human sera, and in the latter were sensitive to RDE but not to KIO_4 (Fedova *et al.*, 1969). Terzin and Vujikov (1969) found that KIO_4 inconsistency inactivated inhibitors of A2/Hong Kong within the same species, and in some cases increased activity.

Coleman and Dowdle (1969), also found that A2/Hong Kong varied widely in its sensitivity to inhibitors in animal sera. Because a consistent inhibitory pattern could not be established, an effective treatment applicable to all species of serum could not be determined. Species of sera tested were listed in order of inhibitory activity from least to greatest: monkey, goat, chicken, human, rabbit, ferret, guinea-pig, horse. The effect of various inactivation treatments of these sera

on their inhibitory capacity were summarized in the following table.

TABLE 24: The effect of treatments on inhibitors of A2/Hong Kong influenza virus haemagglutinins in normal sera of 8 animal species (from Coleman and Dowdle, 1969)

Species	No. of Sera	Treatment[a]						
		None	Heat	Trypsin (T)	Periodate (P)	Heat T-P	RDE	Kaolin
Monkey	3							
Goat	3							
Chicken	7		16					
Man	8	23	25	21	17	11		
Ferret	8	38	37	13			16	
Rabbit	10	50	31	11	22	92		
Guinea-pig	14	93	618	546	91	118	288	
Horse	7	525	525	589			172	40

[a]The inhibition titre is the reciprocal of the highest serum dilution which inhibited 4 HA units of antigen. Values shown are reciprocals of geometric means of serum titres against influenza A2/Hong Kong/1/68, 8/68, 16/68, 50/68, A2/Aichi/2/68, and A2/Aichi/2/68, and A2/Georgia/25/69. No entry indicates a geometric mean titre less <1:10.

The expression of inhibitor varied with the serum species, in horse serum a γ-type inhibitor was active, in another experiment an α-type was active. Although it had some characteristics of β inhibitor

in some sera e.g. in goat and chicken sera it was sensitive to trypsin and RDE, and was adsorbed by kaolin, it was not affected by these treatments in other sera. Coleman and Dowdle (op cit) concluded that hemagglutinin inhibitors in animal sera are complex and unpredictable, and that empirical findings must be considered in the interpretation of HAI results.

Serum inhibitors of hemagglutination by A2/Hong Kong/68 present in human, guinea pig, rabbit, and rat sera were not reliably eliminated by M/90 KIO_4, or *V. cholerae* RDE, but were susceptible to 1% trypsin (Jandasek *et al.*, 1969). In addition, the high degree of thermostability suggested to the authors that it was similar to a gamma inhibitor.

De Sousa and Bal (1971) found that serum inhibitors of hemagglutination by A2/Hong Kong and later strains detected in normal bovine serum were very resistant to sodium metaperiodate, whereas those of A2/Asia strains were extremely sensitive. On this basis, the inhibitors of A2/Hong Kong and later strains in normal bovine serum were likened to β type inhibitor which was more like A0 and A1 inhibitors than the predominantly γ type A2/Asia inhibitor. However, the A2/Hong Kong inhibitor was extremely thermostable at 56°C and irregularly or moderately thermolabile at 65°C, which does not conform with the thermolabile character of β inhibitor. Inhibitors to pre-1968 A2 and to post-1968 A2 strains found in lower titres in horse, guinea pig, rabbit, rat, fowl, and foetal calf sera, were all sensitive to periodate.

As with the inhibitors of other influenza viruses, until the chemical nature of inhibitors of A2/Hong Kong is established and the mode of action determined, the empirical approach will continue to be

used in treatments chosen for their inactivation.

Hoyle (1968) has conveniently tabulated the inhibitor distribution in various species of animal sera (Table 25).

TABLE 25: Distribution of inhibitors to egg adapted viruses in animal sera (from Hoyle, 1968, modified)

Francis inhibitor		Chu inhibitor		γ inhibitor	
Ferret	+ + +	Rabbit	+ +	Horse	+ + +
Fowl	+ +	Guinea pig	+ +	Rabbit	+ +
Guinea pig	+ +	Ox	+ +	Guinea pig	+ +
Human	+ +	Sheep	+ +	Pig	+ +
Mouse	+	Human	(+ +)	Human	−
Cat	−	Mouse	+ +		
Hamster	−	Ferret	+ +		
Rat	−	Cotton rat	+ +		
		Fowl	−		
		Hamster	+ −		
		Rat	−		

Human sera does not contain β inhibitor (Belyavin, 1956) and thus it has been put in parenthesis in the table.

Precipitation and Flocculation by Inhibitors

The agglutination of influenza viruses by immune serum was first reported by Magill and Francis (1938). Very little investigation of this phenomenon was made.

Svedmyr (1948, 1949 a, b, c) in a study of reactions between influenza A/PR8 and normal chick allantoic fluid found that allantoic fluid inhibited hemagglutination of the virus. When mixed at equivalent proportions, active concentrated PR8 and purified inhibitor from allantoic fluid formed an unstable precipitate which dissolved when the temperature was raised to 35°C. The precipitation- dissolution phenomenon could be repeated on the addition of fresh normal allantoic fluid and a quantitative release of hemagglutinin accompanied dissolution.

PR8 inactivated by heat and purified inhibitor formed a stable precipitate that did not dissolve on incubation at 35°C for 48 hours. This suggested that a virus enzyme was responsible for the dissolution phenomenon seen with active virus.

The inhibitor was destroyed by active but not by heated virus, and by low concentrations of periodate. Periodate caused partial dissolution of stable precipitate formed between heated virus and inhibitor, and released about 2/3 of the hemagglutinin. Crystalline trypsin and ficin did not destroy the capacity to precipitate heated virus, but reduced the inhibitory capacity.

Specific antibody in periodate treated immune ferret sera, if added first, prevented union of virus and inhibitor, but if added to the virus after the inhibitor, did not affect the stable precipitate formed. This revealed the competition between antibody and inhibitor for receptor sites on the virus surface. From the description, the inhibitor appeared to be an α-type.

Belyavin (1955) observed that highly purified strains of AO, Al,

and B were flocculated by specific immune rabbit sera in a typical precipitin-like reaction requiring reagents to be at equivalence and demonstrating zone inhibition when antigen or antibody were in excess. Like Svedmyr (1948, 1949 a, b, c), he observed the reaction could be blocked by inhibitory components present in virus-infected allantoic fluids. A thermolabile component in normal sera of rabbits, guinea pigs, humans, mice, fowl, and horse flocculated PR8 and B viruses, but the strongest reactions were with A-prime strains (Belyavin, 1956). The component resembled β inhibitor (Chu, 1951) because it was thermolabile, resisted the action of crude and purified RDE, but was inactivated by crystalline trypsin. However, Belyavin discounted this identity because he had found human serum to contain no β inhibitor. Because RDE inactivation of the inhibitor was done with only human and rabbit sera, and the trypsin treatment with human sera, it is not possible to apply the results obtained with these to all normal sera tested. The normal sera of the animals mentioned above flocculated PR8 in a pattern which was similar to that produced by PR8 and egg white saline, thus suggesting that an α-type inhibitor, sensitive to RDE, was the active flocculating component (Belyavin, 1957).

Horse serum inhibitor was shown to flocculate sensitive A2 strains (Biddle and Stevenson, 1966). Treatment of the serum with M/90 periodate completely destroyed the flocculating ability, suggesting that the flocculation was due to gamma inhibitor present in the horse serum. The HAI titre was reduced 60 - 80 fold. Other viruses were examined but required 21 hours for flocculation rather than the 4 required by sensitive A2 viruses. The viruses tested formed a flocculation sensitivity gradient: A2 (sensitive) = PR8 > ASH (A1) > A2 (resistant) > Lee.

The precipitation and flocculation discussed above were done in tubes.

Styk and Hana (1966) by agar-gel immunodiffusion, demonstrated that ether-treated purified influenza A1 was precipitated by purified inhibitor (prepared on Sephadex G-200) to produce one distinct and one faint line. Precipitate was not formed between whole virus and inhibitor. In spite of the fact that the evidence presented shows the strong band formed between disrupted virus and inhibitor linking in identity with an equally strong band formed between disrupted virus and specific immune rabbit serum, presumably an antigen-antibody reaction, the authors considered the first reaction to be due to inhibitor defined by them as a $beta_2$-macroglobulin (Hana and Styk, 1962). However, their discussion ends with this statement, "It is possible that there was another substance present in the preparation of inhibitor which reacted with ether split A1 virus, the precipitin line being thus not formed by inhibitor". In the discussion of inhibitor above, Krizanova and Sokol (1966) doubt that the component isolated by Styk and Hana (op cit) was inhibitor.

The application of the above observations to our use of immunodiffusion in cellulose acetate was important in order to determine whether the precipitating components in normal sera of animals were antibodies or inhibitors.

To summarize the diverse data relevant to influenza virus inhibitors, Table 26 has been compiled from Chu (1951), Cohen et al. (1963), Cohen and Dorman (1965) with additions from the references discussed above.

It is apparent that inhibitors of influenza viruses are a group

TABLE 26 : Characteristics of influenza virus inhibitors

Characteristics	Inhibitor Type		
	α	β	γ
Heat stability @ 100°C	+	-	+
Effect of heat (70°C x 1 hr) on inhibitory activity	increased	inactivated	increased
Inhibition of : living virus	-	+	+
indicator virus	+	+	+
Type of combination with active virus	transient	stable	
Destruction by living virus neuraminidase	destroyed	not destroyed	partial destruction
Virus strains inhibited (main ones)	AO, A1, A_2, B	AO, A1	A_2 (sensitive)
Prototype virus used to identify inhibitor	indicator B	A1	A_2 (sensitive)
Inhibition of unadapted (mouse) and mouse adapted virus strains	both	mainly unadapted	
Neutralization of infectivity	-	+	+
Inactivation by:			
Crude V. Cholerae Filtrate	+	+	Partial
RDE (Neuraminidase)	+	-	partial (40%)
Trypsin	+	+	±
Periodate	+	-	+
Adsorbed by Kaolin	-	+	±
Chemical identity	Glycoprotein (mucoprotein)	Protein	Glycoprotein (mucoprotein)
Active component necessary for inhibitory activity	sialic acid	not defined	sialic acid
Electrophoretic mobility	α-Globulin α_2-macroglobulin	β-Globulin	α_2-Macroglobulin
Sedimentation coefficient of purified inhibitor	wide range N-ALF 200 S ovomucin 31-37S horse serum 3.66S	4S	18S
Flocculation or precipitation of viruses	+stable with indicator -unstable with living	+	+
Distribution-major sources	Sera, Secretions and Tissues of most species	Bovine, Mouse, Rabbit Ferret Sera	Horse, Guinea pig, Rabbit, Ferret Sera

of substances whose chemical structure and biological activity have been well defined in some cases, but poorly or not at all in others. In only a few instances have the structure and biological activity been related. Before a full understanding of inhibitors in animal sera and tissues is achieved, it will be necessary to clearly define each one chemically, and to relate the structure to biological activity. When inhibitors are clearly identified in this way, it will be possible to completely differentiate them from antibodies, or show a relationship with them, then, to entirely eliminate them by appropriate treatment, and finally to establish the part they play in the immunological response of animals to infection.

Detection of Influenza Serum Antibodies by Immunodiffusion

The survey of normal animal sera for the presence of influenza A antibodies was carried out using the immunodiffusion technique in cellulose acetate, therefore some reference to the application of the immunoprecipitin test in the detection of influenza antibodies in animal sera is considered to be relevant.

Immunodiffusion has been used to demonstrate various antigens of influenza viruses, neuraminidase (Schild and Pereira, 1969), hemagglutinin (Schild, 1970), ribonucleoprotein (Hana and Hoyle, 1966; Schild and Pereira, 1969), and the complexity of the antigenic components of influenza viruses (Styk and Hana, 1966, Corbel and Rondle, 1970; Styk et al., 1970a; Johnson and Westwood, 1971).

However, immunodiffusion has rarely, and only recently been

applied to serological surveys of animal sera for virus antibodies. Johnson and Westwood (1968), using a microtechnique in cellulose acetate and whole virus antigen, detected precipitins of influenza A/PR8 in the normal sera of rabbits, guinea pigs, rooster, a goat, a sheep, and identified them as immunoglobulins (1969).

Papadoulos et al. (1970) used the Ouchterlony method of immunodiffusion in agar gel to identify arboviruses and to detect specific antibody to these viruses in sentinel rabbits and squirrels.

An agar gel precipitin test (AGP) was developed by Beard (1970) to identify type-specific antibody in avian and mammalian sera using type-specific ribonucleoprotein(RNP) or soluble (S) antigens prepared from chick chorioallantoic membranes individually infected with human, horse, swine, and avian influenza A viruses, and a human influenza B virus.

The type specificity of various influenza strains designated A, and one B, by complement fixation, and the type-specificity of corresponding complement fixing antibodies in convalescent and hyperimmune sera was confirmed by precipitin reactions of identity in the AGP test. A common type-specific A antigen was present in the following viruses: PR8, A2/Jap/57, Equi 1, Swine/Shope/S15, various turkey influenza strains, various duck influenza strains, quail influenza, and FPV. These reactions remained distinct from, and confirmed a type-specific B reaction.

The sensitivity of CF and immunodiffusion tests were comparable and good correlation was noted in examination of human and equine acute and convalescent sera.

The AGP test was incorporated as a complementary method to

standard serological methods. Because of its broad specificity, it was valuable in detecting recent influenza infection in animals requiring antigen from only one virus strain, whereas the strain-specific HAI test required antigens from a multitude of strains to achieve the same spectrum of detection.

The method was also applied to examine sera from naturally occurring field outbreaks of influenza in turkeys (Beard, 1970).

Samadieh and Bankowski (1971 a), applied the same principles and a similar type of immunodiffusion test, but used the internal antigen of ether disrupted influenza virus as type-specific RNP antigen. They confirmed the presence of an identical ribonucleoprotein (S antigen) in avian (various turkey strains) and human (NWS) influenza A viruses.

Good correlation was demonstrated between type-specific CF and immunodiffusion tests but poor correlation between HAI and immunodiffusion tests, suggesting that HAI antibodies differ from precipitin antibodies.

OBJECTIVES OF PRESENT WORK

The above review reveals that a vast amount of knowledge has been accumulated regarding influenza virus, its antigenic structure, and the immune response it induces in mammals and birds. The incidence and spread amongst human, animal, and avian populations, that is the epidemiology of influenza, has been well documented but only partially defined. Most hypotheses fall short of a complete explanation because convincing supporting experimental evidence is lacking. The origin or reservoir of new variants causing human pandemics and animal epizootics is not known. Do they arise from a lower animal reservoir, from a mutation, or recombination between animal and human viruses, or from a spontaneous mutation in humans? Continuing investigations into this area have been recommended by most workers in the field such as Hoyle (1968), Pereira (1969), Easterday (1969), Webster and Laver (1970), Steele (1971), and many others. Another curious question is why the last two human pandemics have both originated from a common area, eastern Asia?

In order to continue the search for a possible animal reservoir, a highly sensitive serological method, micro-immunodiffusion in cellulose acetate (Johnson *et al.*, 1964) has been applied in a serum survey of "normal" animals to detect the presence of "antibodies" (precipitins) to whole virus antigens of selected strains of influenza A and parainfluenza

Sendai. The immunodiffusion survey was paralleled by HAI testing of selected sera. An investigation was made of the effect non-specific inhibitor inactivation treatments had on the reactivity of selected normal sera with influenza A virus antigens in immunodiffusion and HAI reactions. An attempt was made to establish the identity of influenza A virus precipitating antibodies in normal animal sera and to detect these in a selected group of animal species.

MATERIALS AND METHODS

Normal Animal Serum Samples

Over six hundred 'normal' mammalian and avian sera, as well as a small proportion of human sera, were collected between May, 1966 and June, 1970 in Ottawa and nearby localities in Ontario and Quebec (Table 27). The sera from fifteen different species fell into four animal categories:

1. Pets: cat (NCS), dog (NDS), rabbit (NRS)
2. Domestic: bovine (NBS), equine (NES), fowl and rooster (NFS), goat (NGS), sheep (NSS).
3. Wild: chipmunk (NC_HS), cottontail rabbit (NC_RS), ground hog (NG_HS), mink (NM_KS), snowshoe hare (NS_HS), squirrel (NS_QS).
4. Human: NHS

All sera except those of wild animals were collected or received as freshly clotted blood. This was held at 37°C for 1 hr., overnight at 4°C, and the clear serum decanted after two centrifugation cycles at 2500 rpm for 25 min. at 10°C. Rooster and fowl sera were held 1 hr. at 37°C, then 1 hr. at 4°C before centrifuging at 3500 rpm for 30 min.

TABLE 27 : Details of Normal Sera

Normal Serum	No. of Samples	Lab Code Number	Date Received	Source
Cat (NCS)	53	OU 1-3	5/70	Animal house, Ottawa Univ., Med.
		H1-50	6/70	Humane Society, Ottawa
Dog (NDS)	109	OU 1-10	3-4/70	Animal house, Ottawa Univ., Med.
		22-81		
		H1-89	6/70	Humane Society, Ottawa
Rabbit (NRS)	106	A1-A20	4-6/66	Animal house, Ottawa Univ., Med.
		A21-A32	10/66	" " " " "
		A34-A39	5-6/68	" " " " "
		A40-A42	8/68	" " " " "
		A43-A44	10/68	" " " " "
		A45-A47	12/69	" " " " "
		101-112	1/70	Farm near Kars, Ontario
		113-162	6/70	Farm near Orleans, Ontario
Bovine (NBS)	100	1-54	1/70	Abbatoir, Kars, Ont.
		55-100	7/70	" " "
Equine ((NES)	5	1-5	1/70	Farm near Kars, Ont.
Fowl and Rooster (NFS)	67	R1-R8(rst)	8-9/68	Poultry farm, Kemptville, Ont.
		R9-R17	10/69	Canada Dept. Agriculture, ASRI, normal animal farm, Hull, Que.
		101-151	1/70	Poultry abbatoir, Kars, Ont.
Goat (NGS)	7	1-4	1968	Dr. Mitchell, Dept. Microbiol. &Immunol., Ottawa Univ., Med.
		5-7	1/70	Farm near Kars, Ont.
Sheep (NSS)	75	1-75	1/70	Farms near Smiths Falls, and Kars, Ont.
Chipmunk	6	1-6	1969-1970	Dr. J.A. McKiel, Zoonosis Laboratory, CCDC, Canada Dept. National Health and Welfare. (Specimens were from Area H, Richmond to Smiths Falls, Ont.
Cottontail rabbit (NC_RS)	13	1-13	"	"
Ground hog (NG_HS)	25	1-25	"	"
Mink (NM_KS)	1	1	"	"
Snowshoe hare (NS_HS)	42	1-42	"	"
Squirrel (NS_QS)	4	1-4	"	"
Human	23	Names (initials)	1/70 6/70	Members, Dept. Microbiol. and Immunol., Ottawa Univ., Medicine.

to obtain maximum recovery of serum. The decanted serum was clarified at 2500 rpm for 25 min. All sera were dispensed in vials, labelled with animal designation and lab code number (e.g. NCS 5), then immediately stored at $-20^{o}C$.

Antigens

The following strains of viruses were included: influenza A/PR8, A/FM1/Can/53, A2/Can/57, A2/Hong Kong/1/68, A/Equi 1/Praha/56, A/Swine, A/Duck, B/Can/5/66, parainfluenza Sendai; and N-ALF.

Details of the source, methods of preparation, and evaluation of biological characteristics (HAU/ml, EID_{50}, mg protein/ml, etc.) of antigens used in the serum survey are to be found in Part I.

Antigens for immunodiffusion were virus particles from infected chick allantoic fluid concentrated 100x (V/C_2) and 200x (V/C_2') by differential centrifugation; and soluble antigens (V/SA) made from extracts of infected chick chorioallantoic membranes. Table 28 lists immunodiffusion antigens with their biological characteristics.

Antigens for hemagglutination inhibition (HAI) tests were virus infected allantoic fluids. A list of these and their biological characteristics is to be found in Table 5, Part I.

Screening of Normal Sera for Anti-Influenza Activity

Immunodiffusion (IDD)

Normal animal sera were examined by immunoprecipitin test

LEGEND - TABLE 28

C_2, C_2'	:	100x and 200x concentrated virus from infected allantoic fluid
$C_{2/5}$	:	500x concentrated tissue culture fluid (vaccine)
HAU/ml	:	hemagglutinating units per ml.
$\log_{10}$ $EID_{50/ml}$	:	infectivity per ml in eggs, expressed as power of $\log_{10}$.
Protein mg/ml	:	estimated by Lowry et al. method (1953)
CF titre	:	complement fixation titre expressed as reciprocal of dilution

*TCD_{50} of original tissue culture fluid obtained from Connaught Laboratories, Toronto.

ND : Not done

\- : No reaction

TABLE 28. Biological characteristics of virus antigens used with normal animal sera in immunodiffusion reactions.

Virus	Antigen Form	HAU/ml	$\log_{10}$ EID_{50}/ml	Protein mg/ml	Antigen	HAU/ml	CF Titre
Infl.A/PR8	C_2	10^4-10^5	8-11	1.7-2.0	SA	< 20	128
Infl.A/FM_1/Can/53	C_2	10^4	8-11	0.6	SA	< 20	128
Infl.A2/Can/57	C_2'	10^4	9	0.24	SA	< 20	256
Infl.A2/HK/1/68	C_2'	10^5-10^6	8-9.5	0.5-1.5	SA	< 20	128
Infl.A/Equi 1	C_2'	10^4	8-10	0.44	SA	< 40	512
Infl.A/Swine	C_2	10^4	10	0.6	SA	< 20	128
Infl.A/Duck	C_2	10^4-10^6	9-10	0.75-2.0	SA	< 20	256
Infl.A/Ottawa/68	C_2	10^3	ND	0.32	ND	ND	ND
Infl.B/Can/5/66	C_2	10^5	5	1.5	SA	< 20	16
Parainfl. Sendai	C_2'	10^3-10^5	9	0.5-0.6	SA	< 20	> 64
Canine distemper (CDV)	$C_{2/5}$	-	*5	0.1	ND	ND	ND
N-ALF	C_2	-	-	0.16	SA	-	-

reactions in cellulose acetate (prodedure described in Part I) for the presence of precipitins to the following viruses: infl. A/PR8, FM 1 /Can/53, A2/Can/57, A2/HK/1/68, Equi 1, Swine, Duck, infl.B/Can/5/66, parainfluenza Sendai; and to N-ALF. Wild animal sera were tested with only infl. A/PR8 and A2/HK/1/68 because small volumes of sera were available.

Antigens used were virus concentrates described above.

The template pattern used to initially screen all six hundred sera was:

	Normal Serum NS1		NS2	
NS6		Virus Antigen		NS3
	NS5		NS4	

In the first half of the survey, both native and heated (58 -60°C x 1 hr) sera were examined in adjacent wells following the above template pattern. Only native sera were examined in the latter part of the survey. Necessary adjustments in concentrations of reactants were made to keep precipitin reactions at equivalence (See Part I Equivalence Immunoprecipitin Reactions). All reactions were carried out in duplicate slides with further repetitions if necessity warranted.

Most immunodiffusion reactions in the initial screening were stained by Thiazine red R (Crowle, 1961, Appendix 2), examined by binocular stereographic microscope (Zeiss)(kindly lent by Dept. of

Geology, Univ. of Ottawa) and all positive, doubtful, and some negative reactions were photographed. A more sensitive stain, Coomassie blue (see Part I, Materials and Methods) was used to stain faint reactions.

To ensure that the precipitates formed were a result of specific virus-precipitin reactions, random samples of serum types were tested by immunodiffusion in the following ways:

1. Serum sample and N-ALF concentrate were diffused to detect any host (CE)-specific serum components.
2. Serum samples, virus antigens, and buffer were individually diffused in separate reactions to detect any non-specific precipitation.

Hemagglutination Inhibition (HAI)

Because the immunodiffusion survey of normal sera revealed the highest incidence of positive reactions with infl. A/PR8 and A2/HK/1/68, all sera were examined for the presence of HAI antibodies to these viruses (see Part I for HAI test procedure). Sera precipitating other virus strains on immunodiffusion were examined for HAI activity with these other viruses e.g. infl. A/Swine Duck, Equi, etc. All HAI tests were carried out in duplicate and repeated as necessity warranted.

Originally A2/HK/1/68 was not included in the serum survey, therefore the serum treatment chosen to inactivate inhibitors of virus hemagglutination (α, β, and γ-type) was trypsin-heat-periodate (THP) (Davenport and Minuse, 1964, Casals, 1967). However, with the inclusion of infl. A2/HK/1/68, THP treatment proved to be inadequate for removal

of inhibitors to this virus. Therefore a proportion of sera (1/3) demonstrating precipitating and HAI activity were subject to additional types of treatment for inactivation of inhibitors: RDE as crude V. cholerae filtrate, Kaolin, $NaIO_4$ (Tables 5 A to I, Appendix 1). The details of THP, RDE, and $NaIO_4$ treatments are to be found in Part I (Materials and Methods), Kaolin treatment details follow.

The effects of various inhibitor inactivation treatments of sera on their hemagglutination inhibition activity were examined.

Serum Treatments for Inhibitor Inactivation

Heat Human sera were inactivated at 56°C for 30 min. Animal sera required higher inactivation temperatures. However, it was found that temperatures above 60°C for 30 min. often coagulated the small volumes of sera available, therefore most animal sera were inactivated at 58-60°C for 30 min.

Trypsin-Heat-Periodate (THP), RDE, $NaIO_4$ (see Part I, Materials and Methods)

Kaolin A modification of the method outlined by Casals (1967) was used. 25gm acid washed kaolin was added with constant stirring to 100 ml borate-saline pH9.0 and the final pH adjusted to 7.2 (Coleman and Dowdle, 1969). Five volumes of 25% kaolin suspension were added to 1 volume of serum, the mixture held at room temperature for 20 min. with occasional shaking, then centrifuged at 2500 rpm for 30 min. The supernatant serum was at a dilution of 1:6.

Removal of Natural Agglutinins Some sera contained natural

agglutinins for fowl erythrocytes used in HAI tests. They were removed by adsorbing 5 ml serum with 0.1 ml packed fowl erythrocytes for 30 - 60 min. at 4°C. Red cells were removed by centrifuging at 1500 rpm for 20 min. and the adsorbed supernatant serum was decanted (Casals, 1967).

Antibody Detection in Sera Demonstrating Anti-Influenza Activity

Selection of Sera

Over 50 normal sera demonstrating strong influenza A precipitating activity, and HAI activity not eliminated by inhibitor inactivation treatments, were selected for scrutiny to determine whether the reaction components were antibody or inhibitor. Some negative controls were also included.

Virus Antigens

The initial screening of sera revealed the highest incidence of precipitation and hemagglutination inhibition of infl. A2/HK/1/68 and PR8, therefore, these were the main strains of antigens used. Appropriate other influenza A strains (FM 1/Can/53, A2/Can/57, Equi 1, Swine Duck) were included in tests with sera precipitating more than one influenza A virus strain. Viruses were used as concentrates and as soluble antigens (described above).

Immodiffusion in Cellulose Acetate (IDD)

Selected sera that were native, at 1:5 dilution, and inhibitor treated, were examined by immunodiffusion to determine the effect of

inhibitor inactivation treatments on their immunoprecipitin reactions with influenza A viruses. Because the final dilution of treated sera was at 1:5, a dilution control serum at 1:5 in PBS was included.

Influenza A virus precipitating components in normal animal sera were compared and equated with serum gamma-globulins and immune sera, both containing antibody to influenza A viruses. These were:

1. Gamma-globulins of human, especially 7S gamma-globulin, and animal origin whose immunoglobulin specificity and anti-virus spectrum had been determined (see below and Table 30).
2. Sera of animals immunized with influenza A virus strains homologous with antigen used in immunodiffusion reactions.
3. Paired human normal-convalescent and acute-convalescent sera showing significant rises in HAI and CF titers, and in some cases proven virus isolation of infl. A2/HK/1/68.

Details of these specific immune substances follow.

Gamma-Globulin Preparations

Human Three types of commercial human gamma-globulin preparations were used:

Immune serum globulin U.S.P. (Human Gamma-Globulin) (Lederle, lot 221-561) in undiluted liquid form and at 1;2 in sterile PBS.

Gamma Globulin (Human) Fraction II, 98% pure (by electrophoresis), lyophilized (Schwarz Mann), was reconstituted in sterile PBS at the following concentrations: 10, 15, 20, 30, 40, 50 mg/ml.

7S Gamma Globulin (Human), electrophoretically purified, lyophilized (Schwarz Mann), was reconstituted in sterile PBS at the following concentrations: 5, 10, 20, 30, 40, 50 mg/ml.
All were stored at $-20^{o}C$.

The specific gamma-globulin constitution of each one was determined by immunodiffusion in cellulose acetate using the following specific anti-human gamma-globulin sera (Hyland):

Goat anti-human IgA α chain specific
" " " IgM μ " "
" " " IgG γ " "
" " " IgG H and L chain specific

Equivalence reactions were established by varying the concentrations of reactants in immunodiffusion.

Animal The following lyophilized commercial preparations of animal globulins (Schwarz Mann) were used:

Gamma-globulin (bovine) Fraction II, 100% pure (by electrophoresis)
" " (canine), 98% pure (by electrophoresis)
" " (equine) Fraction II, 98% pure (by electrophoresis)
" " (rabbit), 98% pure (by electrophoresis)
" " (sheep)

These were all reconstituted in sterile PBS at the following concentrations: 10, 20, 30, 40, 50 mg/ml, and stored at $-20^{o}C$.

Gamma-globulin specificity of each gamma-globulin type was determined by immunodiffusion using available specific anti-animal gamma-globulin sera:

Rabbit anti-canine gamma-globulin (Hyland)

Rabbit anti-equine " " "

Goat anti-rabbit " " "

and:

Goat anti-bovine serum globulin (Microbiological Associates)

In addition, the relationship of equine gamma-globulin to specific human gamma-globulins was examined using goat anti-human IgA, IgG, and IgM sera (Hyland).

Serological Determinations Using microtitration techniques (Sever, 1962), each human and animal gamma-globulin was checked for HAI activity against infl. A/PR8, and A2/HK/1/68, and for complement fixing antibodies to S and V antigens of infl.A2/HK/1/68. All human gamma-globulin preparations were tested by immunodiffusion in cellulose acetate for precipitating activity with all influenza A strains used in this study, influenza B, parainfluenza Sendai, and N-ALF as concentrate antigen (C_2 - 100x conc., C_2' - 200x conc.), and as soluble antigen (SA). Animal gamma-globulin preparations were tested against these influenza A strains and N-ALF.

Sera of Specifically Immunized Animals

Rabbit sera, intranasally infected convalescent (RAs-IN/V) and hyperimmune, N-CAM absorbed (hRAs/V abs), and hyperimmune rooster sera (hFas/V) to infl. A/PR8, FM 1/Can/53, A2/Can/57, A2/HK/1/68, Equi 1, Swine, Duck, and infl. B/Can/5/66, parainfluenza Sendai, and N-ALF were used as examples of specific immune sera with which to compare normal

animal sera in virus precipitin reactions. Details of the preparation and characterization of these are given in Part I (Materials and Methods); treatment for inhibitors in Part I (Materials and Methods) and above. Determination of CF titres to infl. A2/HK/1/68 S and V antigens is outlined below.

<u>Normal (NHS), Acute (HAs/A) and Convalescent (HAs/C) Human Sera</u>

Six paired human sera together with their CF titres were kindly supplied by J. Mingle of this department. Sera were obtained in January and February, 1970, from patients during the acute phase of illness and 2 weeks later. These were lab coded VP 39/A and VP39/C etc. respectively. Infl. A2/HK/68 virus was isolated from three of the patients. About a month after their normal serum samples had been taken, two members of this department contracted influenza, D.K. in January, 1970, and N. Mc D. in August, 1970, and serum samples were taken from each one two weeks after recovery.

All were dispensed in vials, labelled with initials and stored at -20°C.

The antiviral spectrum of paired sera was determined by the following methods:

1. Micro HAI tests of RDE treated sera for HAI antibodies to infl. A/PR8, Duck, and A2/HK/1/68.
2. Micro CF tests of heated (56°C x 30 min.) sera for CF antibodies of infl. A2/HK/1/68 S and V antigens, and infl. B/Can/5/66 S antigens.

3. Immunodiffusion of native sera for precipitating activity with all viruses used in this study, i.e. influenza A strains, influenza B, parainfl. Sendai, and N-ALF.

Foetal Calf Serum (FCS)

Foetal calf serum containing β-globulins was included in immunodiffusion experiments as a potential β inhibitor control because it should contain no IgM or IgG. Since these experiments were carried out, Schultz et al. (1971) have identified IgM and IgG in a few foetal calf sera as early as 145 days gestation, and in a greater number of older foetuses. Therefore it is possible that the foetal calf serum used in these experiments may also have contained these Ig classes.

Foetal calf serum (GIBCO) was treated in the following manner: left native undiluted, diluted 1:5, heated (58°C x 1 hr.) RDE treated, and $NaIO_4$ treated.

The following antiviral activities were determined: HAI with infl. A2/HK/1/68 and PR8 using all serum forms in microtitrations; CF with S and V antigens of infl. A2/HK/1/68 using heat inactivated serum in microtitrations; immunoprecipitin reactions of all influenza A viruses employed in this study by all forms of FCS.

Complement Fixation (CF)

Micro complement fixation (CF) tests using infl./A2/HK/1/68 S and V antigens were kindly performed by the Virus Laboratory, Ottawa Civic Hospital (P. Phipps) on the following: selected normal animal sera, animal gamma-globulin preparations, human gamma-globulin preparations,

foetal calf serum, sera of rabbit and roosters immunized with infl. A2 /HK/1/68, and paired human sera. Fowl and rooster sera required indirect CF testing.

Animal sera were inactivated at 58°C for 30 min. and human sera at 56°C for 30 min. Usual dilution range was 1/1 - 1/16 but these were extended when necessary. All sera were coded and included with analyses of hospital routine specimens.

RESULTS

Screening of Normal Sera for Anti-Influenza Activity

Immunodiffusion (IDD)

Following the suggestion that infl.A/PR8 was precipitated by specific antibodies in normal animal sera (Johnson and Westwood, 1968, 1969), immunodiffusion in cellulose acetate was applied to determine the incidence of precipitins against influenza A strains in a wide range of normal animal sera (Figs 10 to 13).

Serum components precipitating influenza A viruses are not necessarily antibody but may also be influenza inhibitors of the α-type (Svedmyr, 1949 a, b, c; Belyavin, 1957), β-type (Styk and Hana, 1966), or the γ-type (Biddle and Stevenson, 1966). Thus the immunoprecipitin reactions of these components with influenza viruses were not distinguishable by the simple immunodiffusion patterns used in the initial part of this survey. Additionally parallel HAI tests on THP treated sera (discussed below) revealed the presence of a high incidence of hemagglutination inhibition of two strains of influenza, A2/HK/1/68 and PR8, suggesting that the precipitating components detected in the normal sera were not solely antibody. Nevertheless, the screening of sera by immunodiffusion was useful in that it enabled precipitin positives to be selected and further examined for the presence of antibodies.

Legend Figures 10-14

"Normal" serum type is named as EQUINE, GOAT, RABBIT, etc. Number in parenthesis following serum type is numbers of sera examined.
Bar width is proportional to numbers tested.
NIL is absence of detectable precipitins. (Figs. 10 to 13); or absence of detectable hemagglutination inhibition (Fig. 14).
THP treated - trypsin-heat-periodate treated. (Fig. 14)

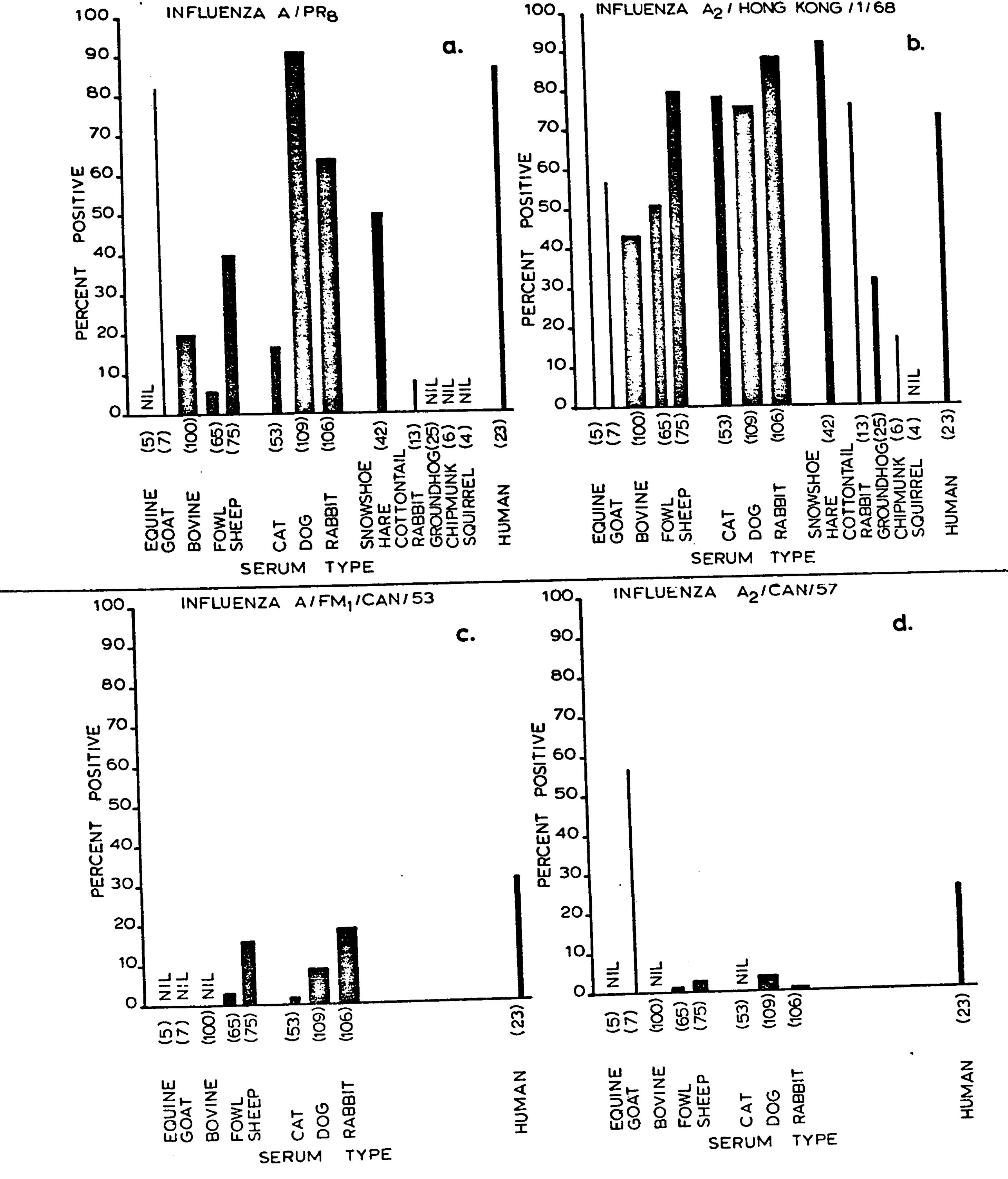

Figure 10. Immunodiffusion of normal animal sera. Incidence of precipitins against human influenza A strains

INFLUENZA A/SWINE

a.

PERCENT POSITIVE

100 90 80 70 60 50 40 30 20 10 0

NIL NIL

(5) (7) (100) (65) (75) (53) (109) (106) (23)

EQUINE GOAT BOVINE FOWL SHEEP CAT DOG RABBIT HUMAN

SERUM TYPE

INFLUENZA A/DUCK

b.

PERCENT POSITIVE

100 90 80 70 60 50 40 30 20 10 0

NIL

(5) (7) (100) (65) (75) (53) (109) (106) (23)

EQUINE GOAT BOVINE FOWL SHEEP CAT DOG RABBIT HUMAN

SERUM TYPE

INFLUENZA A/EQUI 1

c.

PERCENT POSITIVE

100 90 80 70 60 50 40 30 20 10 0

NIL NIL NIL

(5) (7) (100) (65) (75) (53) (109) (106) (23)

EQUINE GOAT BOVINE FOWL SHEEP CAT DOG RABBIT HUMAN

SERUM TYPE

PARAINFLUENZA SENDAI

d.

PERCENT POSITIVE

100 90 80 70 60 50 40 30 20 10 0

NIL NIL NIL NIL NIL

(5) (7) (100) (65) (75) (53) (109) (106) (23)

EQUINE GOAT BOVINE FOWL SHEEP CAT DOG RABBIT HUMAN

SERUM TYPE

Figure 11. Immunodiffusion of normal animal sera. Incidence of precipitins against animal influenza A strains and parainfluenza Sendai

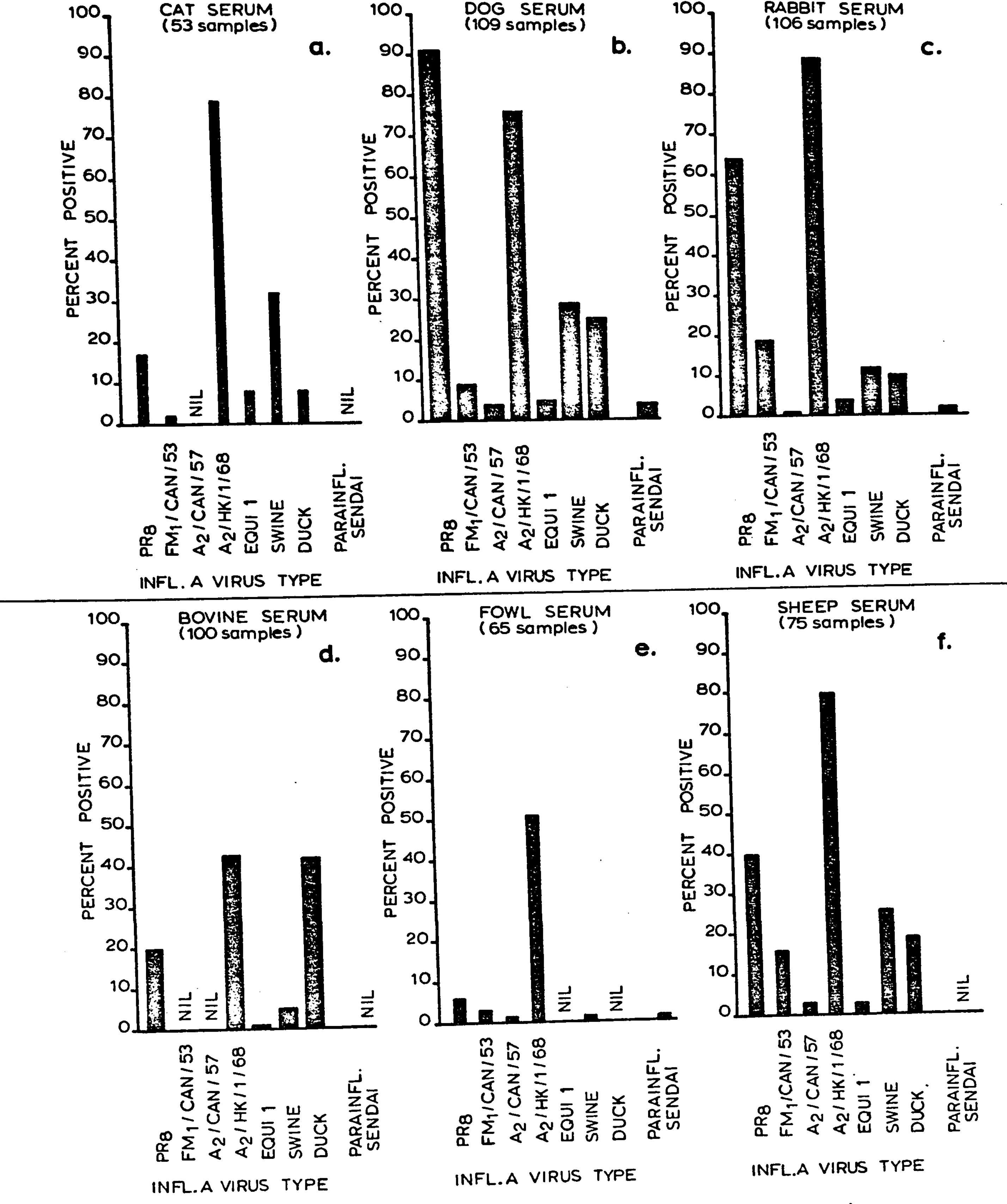

Figure 12. Immunodiffusion of normal animal sera. Influenza A virus precipitin spectrum

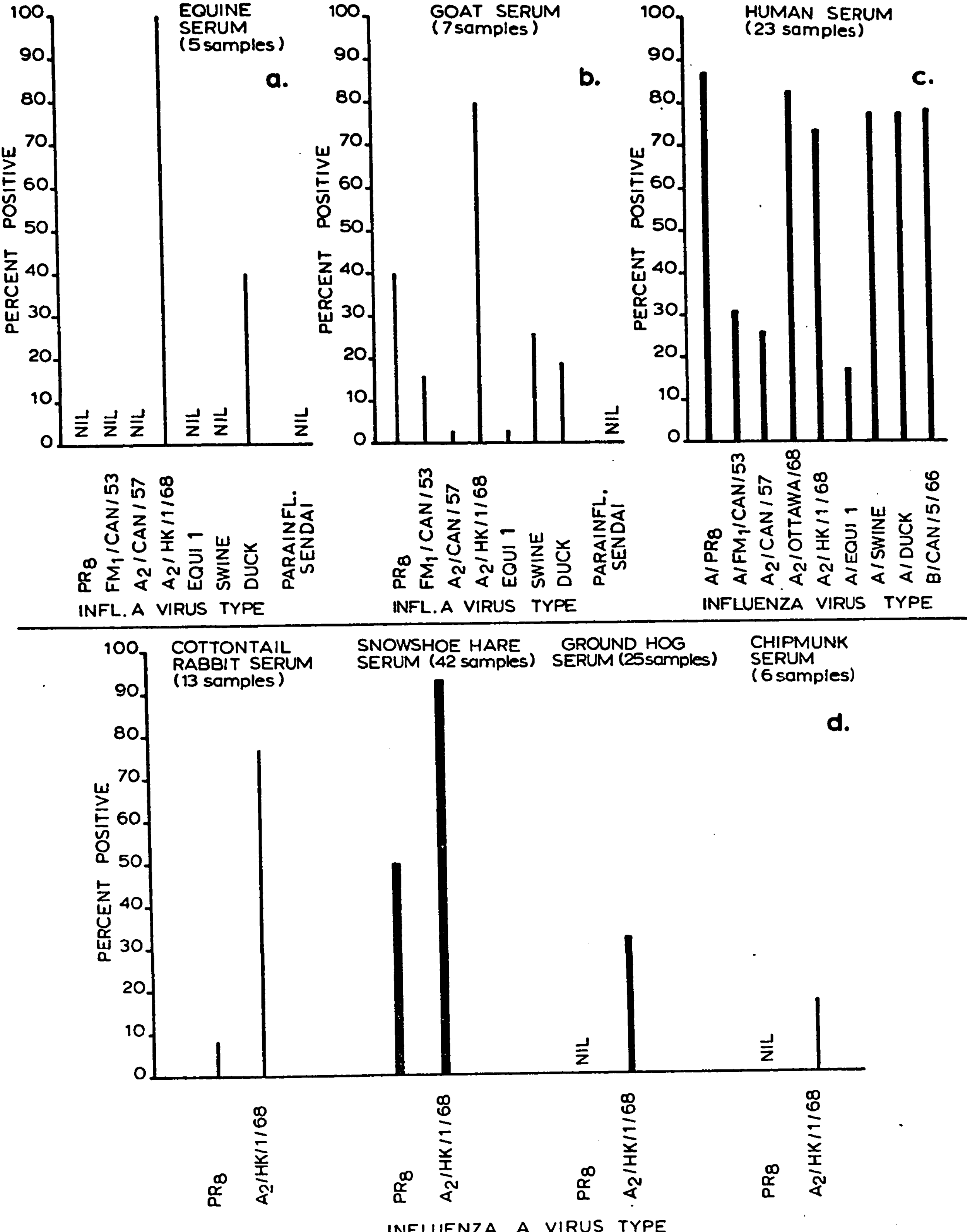

Figure 13. Immunodiffusion of normal animal sera. Influenza A virus precipitin spectrum

Bands formed by immunoprecipitin reactions between normal sera and influenza viruses ranged in appearance from intense and well-defined, to faint and fuzzy. Four examples of immunoprecipitin reactions (chosen from over 2000 slides) of normal sera (outer wells) with influenza A virus concentrates (centre wells) A2/HK/1/68 (Plate 21 A, B), PR8 (Plate 21 C), Duck (Plate 21 D) include both positively and negatively reacting sera. Some sera formed more than one precipitin band with whole virus antigen e.g. NCS H27 with A2/HK/68 in Plate 21 B. A number of sera precipitated more than one influenza A virus strain. Reactions of virus with different sera often linked in identity indicating a similar component reacting in each serum, e.g. in reactions with A2/HK/1/68, normal rabbit sera (outer wells) form linking precipitin bands (arrows, Plate 21 A). Components in the same relative position and same plane, although not linking because of a sweeping out of the reaction, are also considered to be common e.g. normal sheep sera form similar bands which do not link in reactions of identity (arrowed, Plate 21 D). Heating of dog sera (NDS 8 Ht outer well) at 58°C x 30 min. did not affect its precipitating ability with infl.A/PR8 (centre well, Plate 21 C). Note that bovine serum in the same reaction did not precipitate the virus. However, heating of animal sera gave such inconsistent results that it was abandoned half way through the survey. Control experiments (discussed under Materials and Methods, Immunodiffusion Screening), confirmed that these precipitates resulted from specific reactions between serum precipitins and virus antigen, and were not host (CE)-specific nor nonspecific.

Reactions similar to those described above formed the basis of the analysis of normal sera for precipitins of influenza A viruses. Many

of the fifteen species of normal sera examined displayed a wide range of precipitating activity with a few or all virus strains i.e. infl.A/PR8, FM1/Can/53, A2/Can/57, A2/HK/1/68, Equi 1, Swine Duck, parainfluenza Sendai (Figs. 10 to 13).

The sera of most species examined, except squirrel, precipitated infl.A2/HK/1/68 (Figs. 10 b, 12, 13), with a high incidence of positive reactions: 93% in snowshoe hare, 89% in rabbit, and over 70% in dog, cat, sheep, cottontail rabbit, and human. All five horses were positive. (Note that the number of samples of each serum species varied considerably, from 4 squirrels to 109 dogs). Rabbit sera collected in 1966 and 1967 (Table 27) had an extremely high incidence of precipitins to A2/HK/1/68, 28 out of 30, but rooster sera collected in 1968 had a low incidence, 2 out of 8 birds.

Precipitins against infl. A/PR8 (Figs. 10 a, 12, 13) were not as prevelant as those against A2/HK/1/68. Five species demonstrated an incidence of 40% or greater e.g. dog 91%, goat 86%, rabbit 64%, snowshoe hare 50% and sheep 40%.

The incidence of normal serum precipitins against infl.A/Duck (10-40%) (Figs. 11 b, 12, 13) and Swine (10-30%) (Figs. 11 a, 12, 13) was lower and fewer species of sera reacted, e.g. pets, a few domestic, and human.

The percentage and number of species of reacting sera dropped sharply in reactions with infl. A/FM1 (Figs. 10 C, 12, 13) A2/Can/57 (Figs. 10 d, 12, 13), Equi 1 (Figs. 11 c, 12, 13), and almost no activity was recorded with parainfluenza Sendai (Figs. 11 d, 12, 13). The low results

recorded with Equi may be due in part to the weak antigen used.

The antiviral spectrum of each serum species (Figs. 12, 13) included many of the viruses tested. It was complete in dog, rabbit, and human; it included all influenza A strains but not parainfluenza Sendai in sheep and goat; the other species did not precipitate all influenza A strains and precipitated parainfluenza Sendai variably. Equine serum had the narrowest anti-viral spectrum, precipitating only A2/HK/1/68 and Duck. Of the wild animals, cottontail rabbit and snowshoe hare precipitated both test antigens, PR8 and A2/HK/1/68,while ground hog and chipmunk precipitated only the latter. Squirrels demonstrated no precipitins. Human sera from members of this department, had a high incidence of precipitins with a broad anti-viral spectrum. It is interesting to note that precipitins against PR8, A2/HK/1/68, Duck, Swine, A2/Ottawa/68, infl. B were of the same high level, while those against FM1, A2/Can/57, and Equi were low.

The influenza precipitins found in such high incidence in dog sera could have been a reflection of the distantly related canine distemper virus (CDV) (Andrewes, 1964) which commonly infects dogs, and against which many dogs are immunized. However, on diffusion with dog sera, the influenza A viruses formed precipitin bands which remained quite discrete from those formed with CDV indicating that the antigenic components involved were different. Therefore, the influenza precipitins detected in the survey were not related to those of canine distemper virus precipitins.

The general conclusion is that a high percentage of the sera

of most animal species examined possessed a wide range of precipitins against influenza A viruses, but whether the serum precipitins were antibody or inhibitor was not revealed.

Hemagglutination Inhibition (HAI) and Effect of Inhibitor Inactivation

In conjunction with the immunodiffusion survey, HAI tests performed on THP treated sera revealed that 13 animal species demonstrated an extremely high incidence of HAI activity with A2/HK/1/68; and that 11 of these demonstrated a lower incidence with PR8, the other 2 species showing no activity (Fig. 14). The HAI activity of all animal species with the other A strains of this study was almost non-existant, occasional low titres (1/5-1/20) being recorded e.g. 1 dog with Equi, 1 dog with Swine, 1 bovine, 1 goat, 2 fowl with A2/Can/57. As all sera of the wild animal group, and 75% or more of cat, dog, rabbit, and bovine sera demonstrated HAI activity with A2/HK/1/68, it became obvious that most of this activity, and possibly that against PR8, was due to inhibitor. Therefore the HAI activity of these sera had to be re-examined after treatment by other inhibitor inactivation methods.

Over 200 sera demonstrating influenza hemagglutination inhibition and strong influenza precipitation on immunodiffusion were selected and treated with RDE; sera whose HAI titre was not eliminated by THP or by RDE were further treated with kaolin and $NaIO_4$, and the HAI activity of all of these determined. Listed in Tables 5 A-I, Appendix 1, are the selected sera, the inhibitor inactivation treatments to which they were subjected,and the resulting HAI titres with infl.A2/HK/1/68, PR8, and other A strains used in this study.

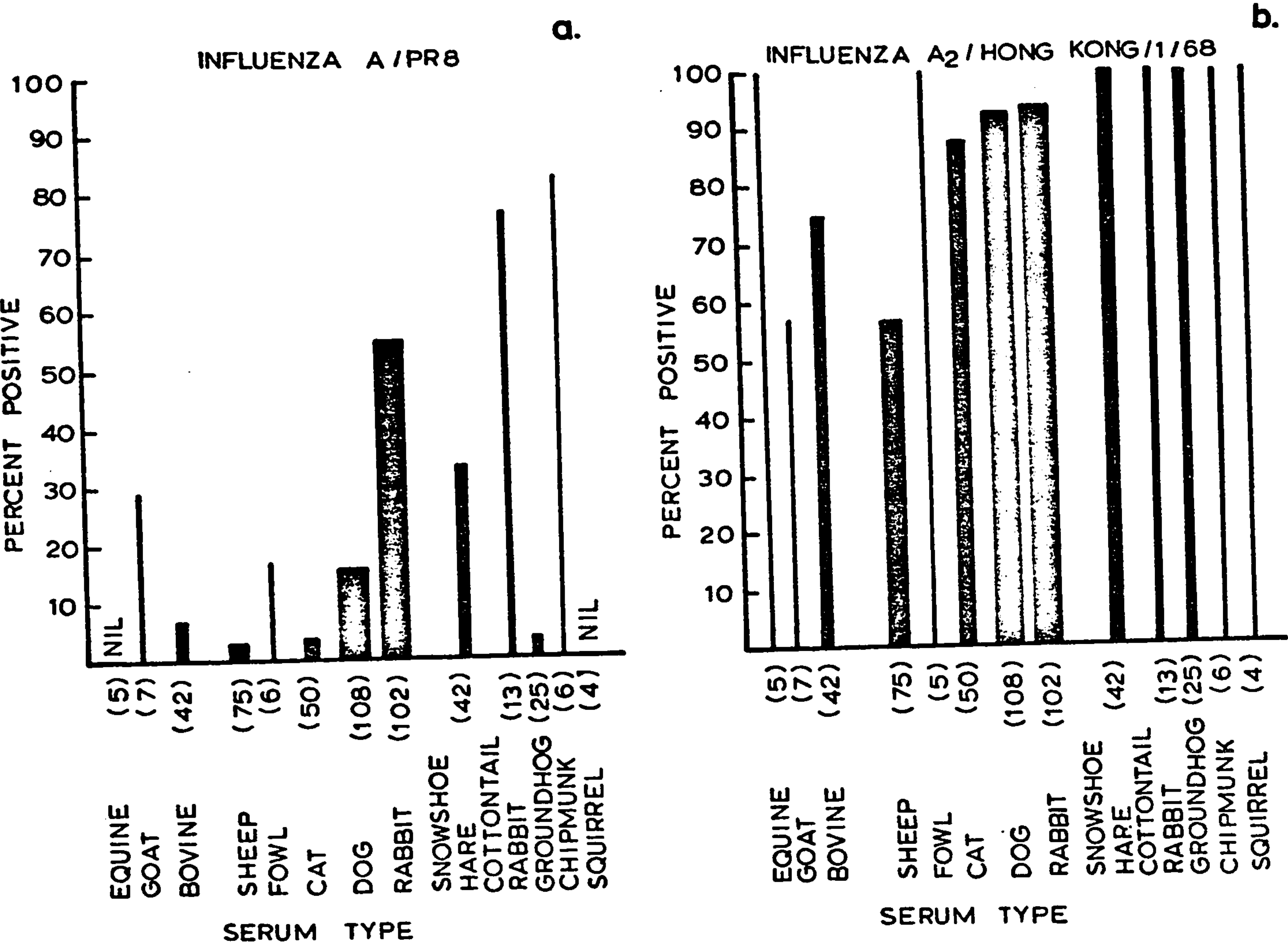

Figure 14. Hemagglutination inhibition tests of normal animal sera (THP treated)

a) Incidence of HAI activity against influenza A/PR8
b) Incidence of HAI activity against influenza A2/Hong Kong/1/68

TABLE 29: Effect of inhibitor treatments on HAI activity of selected normal sera

Normal Serum Species	Number of Sera tested	Infl. A2/HK/1/68 No. Sera with HAI titre After treatment with: THP	RDE	Kaolin	$NaIO_4$	HAI titre range after treatment with THP	all others	Infl. A/PR8 Number of sera tested	No. sera with HAI titre after treatment THP	RDE	$NaIO_4$	HAI titre range After treatment with: THP	all others
Bovine (NBS)	6	4	4	0	ND	20	5	6	2	ND	ND	5	
Cat (NCS)	27	24	6	12/12	6/6	20-160	5-60	27	1	ND	0/6	5	<4
Dog (NDS)	29	27	8	9/10	4/8	10-160	10-80	27	7	ND	0/4	5-40	<4
Equine (NES)	5	5	5	5	2/3	40-480	10-20	5	0	ND	0/3	<4	<4
Fowl and Rooster (NFS)	19	5/5	10	ND	1/2	20-80	5-40	19	1/6	1	0/2	10	15
Goat (NGS)	9	6	4/7	1/2	ND	5-20	5-20	7	2	ND	ND	30,40	
Rabbit (NRS)	26	25	25	19/20	6/6	160-5120	5-96	26	16	1/25	0/4	5-20	10
Sheep (NSS)	26	16	23	ND	5/11	5-40	5-40	26	1	ND	ND	5	<4
Chipmunk (NC_HS)	6	6	4	ND	ND	10-40	5-10	6	5	ND	ND	5-10	
Cottontail Rabbit (NC_RS)	13	13	5	2/3	1/1	15-160	5-20	13	10	ND	ND	5-10	
Ground Hog (NG_HS)	13	13	1	1/1	ND	20-160	10	13	0	ND	ND	<4	
Mink (NM_KS)	1	1	0	ND	ND	40	<4	1	1	ND	ND	10	
Squirrel (NS_QS)	4	4	0	ND	ND	10-40	<4	4	0	ND	ND	<4	
Snowshoe Hare (NS_HS)	24	24	2	3/6	1/1	10-160	5-12	24	5	ND	ND	5-10	

HAI titres expressed as reciprocal of dilution

ND: Not done

Table 29 summarizes the effect of the other inhibitor inactivation treatments compared with that of THP on the HAI activity of the selected sera and can be interpreted as follows:

A2/HK/1/68

THP did not effectively inactivate inhibitor in most animal sera examined.

RDE as crude V. cholerae filtrate plus heating at 58°C x 1 hr., most effectively removed HAI activity against A2/HK/1/68 in squirrel, ground hog, snowshoe hare, dog, cat. Low level residual HAI titre was left in bovine, fowl, rabbit, and cottontail rabbit sera. RDE had little or no effect on HAI activity in goat,horse, sheep, and chipmunk sera.

Kaolin Removed all HAI activity from bovine sera, but left low level residual HAI titres in all other species treated.

$NaIO_4$ when used, gave much the same result as THP but residual HAI titres were lower, the relative values similar to those left after RDE treatment.

A/PR8

THP eliminated all or most inhibitors of PR8 hemagglutination in equine, ground hog, squirrel, cat, sheep, snowshoe hare, fowl and rooster sera. Some residual HAI activity was left in bovine, dog, goat sera. THP had little or no effect on PR8 inhibitors in rabbit, chipmunk, cottontail rabbit, and mink sera.

RDE was more effective in rabbit sera than THP, but less effective in sheep sera.

Kaolin - use not required

$NaIO_4$ when used, eliminated all PR8 inhibitory activity in cat, dog, horse, rooster, and rabbit sera.

Additionally, not included in Table 29 but in Tables 5 A-I, Appendix 1:

Other infl. A strains (used in this study)

THP effectively removed all HAI activity in most serum species in reactions with infl.A/Duck, Swine, and FM1.

RDE and Kaolin - use not required.

$NaIO_4$ completely removed all horse serum inhibitors against, infl.A/Equi 1, and goat serum inhibitors against infl.A2/Can/57.

From the above summary, Table 29, and Tables 5 A-I, Appendix 1, the following observations can be made.

THP did not affect inhibition of A2/Hong Kong/1/68 HA in most animal sera.

RDE eliminated the major part of A2/HK/1/68 activity in the species: cat, dog, fowl, snowshoe hare, cottontail rabbit, ground hog, and squirrel. Therefore, the high incidences of HAI activity with A2/HK/1/68 in these species were due to the presence of inhibitor, sensitive to RDE (as V. cholerae filtrate) but not to THP. Any residual

HAI activity left reflected either an inhibitor or antibody.

It could not be established whether A2/HK/1/68 antibody, inhibitor, or both were the active components in bovine, equine, rabbit, sheep, and chipmunk sera, as neither THP nor RDE eliminated HAI activity. Kaolin, a harsh treatment, did eliminate activity in bovine sera but was ineffective in the other species of sera examined. $NaIO_4$ eliminated HAI activity in some dog and sheep sera but not in others. The activity eliminated was due to $NaIO_4$-sensitive inhibitor, the residual HAI activity was not. The identity of serum components responsible for residual HAI activity of all these species could not be determined.

Similarly, with infl. A/PR8, THP effectively reduced HAI activity in most species of sera examined except rabbit, cottontail rabbit, and chipmunk. The HAI activity in rabbit sera was due to inhibitor because it was almost entirely eliminated by RDE treatment; only one animal in 26 retained low level residual HAI titre. Unfortunately the limited quantities of wild animal sera available did not allow examination of cottontail rabbit, chipmunk, or snowshoe hare sera for PR8 HAI activity after RDE treatment, but it is suspected that the results would be similar to those obtained with rabbit sera. $NaIO_4$, when used in the few sera available, eliminated HAI activity in a cat serum (NCS 11) resisting THP treatment, and in a rabbit serum (NRS 160) resisting both THP and RDE treatment. Because the supply of individual sera was exhausted, this treatment was not always completed to the extent desirable.

It was concluded that almost all HAI activity against PR8 in normal animal sera examined was due to inhibitors which were susceptible to one

or more of the inactivation treatments used. In a few sera, HAI activity survived these treatments but the component responsible could not be identified as being antibody or inhibitor.

The HAI activity left after all inactivation treatments could be due to : a) a residual inhibitor not affected by any treatment used; b) antibody. Residual inhibitor could have been present because not all inhibitor was eliminated, or, because more than one inhibitor type was present. A combination of two or more inactivation treatments could have been effective, but this was not done. Thus, as the various treatments were conducted separately rather than together, it is most likely that not all types of inhibitor present in sera were eliminated by individual treatments.

Therefore, it was not possible to establish unequivocally by hemagglutination inhibition tests, whether inhibitor or antibody was responsible for HAI activity against A2/HK/1/68 or PR8 persisting after different inhibitor inactivation treatments. HAI tests detect antibody to only one antigenic component of influenza A viruses, the hemagglutinin, but immunodiffusion detects precipitating antibody to at least three known antigenic components of influenza A viruses, hemagglutinin, neuraminidase, and type-specific or RNP antigen. Therefore sera with persisting residual HAI activity which also precipitated the same virus or viruses on immunodiffusion were further investigated to determine the nature of the serum components exhibiting anti-influenza activity (asterisked in Table 5 A-I, Appendix 1).

Antibody Detection in Sera Demonstrating Anti-Influenza Activity

Effect of Inhibitor Inactivation Treatments on Precipitins

The main objective of this investigation was to determine the significance of precipitating antibodies detected in animal sera against influenza viruses, especially A2/HK/1/68 and PR8. Therefore, the prime concern was the detection and elimination of precipitating inhibitors by various inactivation treatments, rather than identification of specific inhibitor types.

The first stage in the precedure was to describe the effect of inhibitor inactivation treatments on all precipitating activity of normal animal sera. As shown below, considerable variation was demonstrated in the susceptibility of individual sera within a species to inhibitor inactivation treatments, and the range of susceptibility was influenced by the strain of virus. Because of this, a pattern could not be established and tabulated, thus only general observations are discussed.

The final dilution of sera treated for inhibitors was at 1:5, and at this dilution, many sera lost detectable precipitating activity. Therefore, it was not possible to assess the effects of inhibitor inactivation treatments on the precipitating capacity of such sera.

RDE, THP, $NaIO_4$, and Kaolin variably affected precipitin activities with influenza viruses, ranging from little or no effect through to complete elimination of the activity. THP treated sera were altered and produced fuzzy immunoprecipitin reactions. RDE had the same effect but occasionally also changed the position of precipitin bands suggesting an alteration in the normal serum component. Kaolin, when it did not eliminate the reaction, caused no qualitative alteration.

The following is a more detailed and specific summary of these effects.

A2/HK/1/68

Dilution of 1:5 caused some bovine, cat, and a chipmunk serum to lose precipitating activity.

RDE as crude *V. cholerae* filtrate completely removed precipitin components from fowl and goat sera. It varied in its action on precipitins in cat, dog, equine, rabbit, cottontail rabbit, and snowshoe hare sera by reducing, fuzzing, or completely eliminating any precipitation between native serum and the virus. The component in bovine sera and in some equine sera was not affected.

THP removed precipitins in bovine sera and varied in its effect on the precipitating activity of cat, dog, horse, fowl, rabbit, cottontail rabbit, and snowshoe hare sera by reducing, fuzzing or completely eliminating precipitation of the virus demonstrated by native serum.

$NaIO_4$ when used, completely eliminated all precipitating activity in fowl and rabbit sera, and fuzzed or eliminated precipitation by cat, dog, rabbit, and cottontail rabbit sera. The action on equine and snowshoe hare sera varied.

A/PR8

Dilution of 1:5 caused a few dog and bovine sera to lose detectable precipitating activity.

RDE as crude *V. cholerae* filtrate eliminated precipitins in snowshoe hare but not in goat sera. In bovine, cat, dog, equine, and rabbit sera, RDE caused a fuzzy precipitate to appear where none had been formed between native serum and the virus.

THP removed precipitins from rabbit and snowshoe hare sera but didn't affect those in goat sera.

$NaIO_4$ when used, inactivated precipitins in rabbit sera.

Kaolin when used, eliminated the precipitating capacity of snowshoe hare sera and lowered the activity of goat sera.

Other Influenza A Virus Strains

Dilution of 1:5 removed detectable precipitation of influenza A/Duck and A/Swine by a few cat dog, goat, and rabbit sera.

RDE as *V. cholerae* filtrate inactivated A/Duck precipitins in cat sera, but not in equine sera. A/FM1 precipitins were not eliminated in dog sera.

THP fuzzed precipitates formed between A/Duck and cat sera but didn't affect those formed between A/FM1 and dog sera.

$NaIO_4$ eliminated precipitins of influenza A/FM1 in dog sera and of A/Duck in equine sera.

Kaolin had no effect on the precipitation of influenza A/Duck by a cat serum.

The following examples of immunodiffusion reactions illustrate some of the effects of inhibitors described above.

Plate 22A shows an immunoprecipitin reaction between centrally located A2/HK/1/68 C_2' and peripherally located normal dog serum (NDS) H62 diluted 1:5, THP treated, and RDE treated. Of two precipitin bands formed between diluted serum and virus, only the inner one is relatively unaffected by 1:5 dilution and serum treatments, but some reduction in the precipitate is seen with RDE treated serum. In a similar reaction between A2/HK/1/68 and normal fowl serum (NFS) 115 (Plate 22B), the precipitin band formed is not affected nor altered by RDE treatment shown to remove HA inhibitors from fowl sera. However, in Plate 22C, the immunoprecipitin reaction formed between normal rabbit serum (NRS) A46 and A2/HK/1/68 C_2' is fuzzed when serum is THP or RDE treated. Normal cat serum (NCS) H27 in Plate 22D gradually has its precipitin activity diminished on dilution

of 1:5, on treatment with THP, with $NaIO_4$, and the precipitin band formed between RDE treated serum and the virus is just barely visible (arrowed), yet in another reaction (Plate 23B) the effect of RDE is not so marked (arrowed). In all plates, linkage of identity confirms that the same component in each serum is precipitating the virus despite inhibitor treatments.

Due to the small sampling and the variation in susceptibility to inhibitor inactivation treatments within a species discussed above, it was difficult to establish, for each species of serum examined, a precipitating inhibitor pattern on the basis of susceptibility to inactivation by various serum treatments. This could have been a simple way to distinguish inhibitor from antibody in immunoprecipitin reactions because α inhibitors are inactivated by RDE, and by THP; β inhibitor by V. cholerae filtrate, heat, trypsin, and kaolin; γ inhibitor by periodate as $NaIO_4$ or in THP (Table 26, Inhibitor Review). Antibody is not completely destroyed by any of these treatments, thus any precipitating activity remaining could be due to antibody.

In the sera of a few species, all inhibitor activity was completely eliminated by specific serum treatments, and any other activity was presumably due to antibody. For example, RDE treatment of goat and fowl sera, and THP treatment of bovine sera consistently inactivated A2/HK/1/68 inhibitors while RDE and THP treatment of snowshoe hare sera eliminated PR8 inhibitors. However, in the majority of sera the following questions arose; 1) if the precipitating activity of a serum was not

affected by an inactivation treatment, was it due to antibody or to resistant inhibitor? 2) if the precipitating activity was not eliminated but merely altered or reduced, was the residual activity due to resistant inhibitor or to an antibody affected by the treatment? Two further complications were: 1) the fact that virus particles contain more than one antigen which may be precipitated by more than one type of antibody (Pike, 1967), and by more than one type of inhibitor (Svedmyr, 1949 a; Belyavin, 1955, 1956, 1957; Hana and Styk, 1966; Biddle and Stevenson, 1966); 2) that serum treatments may attack antibody, RDE (Anderson, 1948), periodate (Andersen *et al.*, 1966; Cohen and Dorman, 1965), and Kaolin (Chu, 1951; Coleman and Dowdle, 1969) have all been shown to do so.

Therefore, on the basis of susceptibility to inhibitor inactivation treatments, it was not possible by immunodiffusion alone to definitely establish the identity of precipitin components in normal sera as inhibitor or antibody. However, the presence of antibody was strongly suggested if:

a. The precipitating component was not completely eliminated by the inactivation treatments used, that is, by RDE as *V. cholerae* filtrate, by THP, by $NaIO_4$. Kaolin was not always considered because it is known to adsorb immunoglobulins.

b. The precipitin band formed on immunodiffusion by the serum component and virus concentrate remained the same in the serum despite inactivation treatments.

c. The precipitin bands, formed with the virus by native sera and sera inactivated in various ways, all linked in identity.

d. The precipitin bands described in a, b, and c linked with reactions between the virus and immune preparations known to contain influenza antibody (discussed below).

A few sera retained the capacity to precipitate influenza virus concentrates despite all serum treatments described (Table 32, + signs), and thus fulfilled the requirements a, b, c, and d for the suggested presence of precipitating antibody. These included normal sera precipitating influenza A2/HK/1/68: cat - NCS H27 (Plate 22D 23B), dog - NDS H62 (Plate 22A), fowl - NFS 115 (Plate 22B), and with some doubt, rabbit - NRS A46 (Plate 22C), and NRS A47; and sera precipitating influenza A/PR8: goat - NGS 2 (Plate 23A).

Several sera fulfilled requirement d, and a, b, and c only partially, because inhibitor treatments of sera did alter their precipitin pattern, but did not eliminate it completely (marked P in Table 32). A number of sera fulfilled only requirement d (marked D in Table 32). The precipitins in P and D sera could be considered as antibody provided that the precipitins in the immune preparations, with which they identified, were all antibodies.

The second stage in the procedure to identify precipitating antibody in normal sera was to include in immunodiffusion reactions, not only native and treated sera, but also anti-sera and immunoglobulin

preparations known to contain influenza antibody as a result of infection or immunization; in particular, sera of specifically immunized roosters and rabbits, especially convalescent rabbit sera, human convalescent sera, human gamma-globulin preparations of known immunoglobulin constitution, and animal gamma-globulins. It was important to demonstrate that the precipitating component was a 7S gamma-globulin, IgG, because inhibitors of influenza viruses are not found in this fraction of serum proteins. Inhibitors of influenza viruses, α-type and γ -type, are 18S α_2-macro-globulins (Biddle et al., 1965, Biddle and Shortridge, 1967) and could be confused in immunoprecipitin reactions with 19S IgM. Because IgA may occur as 7S to 17S depending on the degree of polymerization (Fudenberg, 1965), and possesses agglutinating powers (Pike, 1967), it could also be confused in its polymerized form with these inhibitors. β inhibitor is more easily distinguishable because it exists in a 4S state (Krizanova and Sokol, 1966).

To clearly identify precipitating antibody in immune sera and in immunoglobulins, the major type-specific antigen was used because it is known to be precipitated only by antibody.

Identification of Influenza A Type-Specific Antigen by Immunodiffusion

One of the influenza virus components precipitated by normal sera has been identified as the type-specific antigen (see Part I, Identification of the Type-Specific Reaction in Immunodiffusion for a full discussion). Immunodiffusion of soluble antigens made from extracts of

infected chorioallantoic membranes with rabbit and rooster immune sera has demonstrated the type-specific antigen of influenza A/PR8, FM1/Can/53, A2/Can/57, A2/HK/1/68, Equi 1, Swine, Duck, and of influenza B, and parainfluenza Sendai. Table 28 contains the biological characteristics of these soluble antigens (SA).

A single example is cited describing the type-specific component in influenza A2/HK/1/68, thus relating the work in Part I to normal sera examined in Part II. Plate 24A illustrates a homologous A2/HK/1/68 reaction of viral antigen as virus concentrate and as soluble antigen with convalescent and hyperimmune rabbit sera. Several precipitin bands are formed between the virus concentrate (centre) and immune sera (top and bottom). The outermost band (arrowed) links in a reaction of identity with a band formed between SA and both immune sera indicating a common component. This was thought to be the type-specific component. To establish the type-specific antigen identity, influenza A type specificity had to be demonstrated. When the soluble antigens of all influenza A strains used in this study, influenza B, and parainfluenza Sendai were diffused against hyperimmune (top) and convalescent (bottom) rabbit anti-A2/HK/1/68 antisera, a precipitin band linking across all influenza A soluble antigens was formed by both types of sera (Plate 24B). No reactions occurred with influenza B or parainfluenza Sendai. The converse reaction in which sera were varied, and virus was constant gave the same result. All reactions described were virus-specific (refer to Part I, Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions).

This clearly demonstrated the influenza A type-specificity of the immunoprecipitin reaction and identified the precipitated antigen as being influenza A type-specific antigen which is believed to be the ribonucleoprotein (Schild and Pereira, 1969). These results are in agreement with those found in Part I (Identification of the Type-Specific Reaction in Immunodiffusion) and with the immunoprecipitin characterization of the type-specific component as RNP by Hana and Hoyle (1966, 1968); Schild and Pereira (1969); Beard (1970); Styk *et al.*, 1970 a).

The type-specific antigen is precipitated only by influenza A antibody, but not by inhibitors, and was therefore used to identify precipitating antibody in normal and immune preparations described below.

Identification of Influenza Antibodies in Immune Preparations

Gamma-Globulins The specific constitution of human gamma-globulins was determined by immunoprecipitin tests. Commercial preparations of human gamma-globulins, 7S gamma-globulin, Schwarz Mann (7S γ -G), gamma-globulin, Schwarz Mann (γ -G/SM), and gamma-globulin Lederle (γ -G/L) diffused against specific anti-human immunoglobulin goat antisera, anti-IgA, anti- IgG, and anti-IgM, yielded the following results (Plate 25):

7S γ -G was found to contain IgG and IgA, but no detectable IgM (Plate 25 A, B). Anti-IgG serum precipitated one component, IgG, in 7S γ -G (Plate 25 A, B, arrow 1), whereas anti-IgA serum precipitated three components, probably IgA of various molecular weights (Plate 25 A, arrows 2). The absence of reaction between anti-IgM and 7S γ -G (Plate 25 B)

indicated that detectable IgM was not present in the 7S γ -G preparation. In some instances, anti-IgG precipitins did not diffuse readily and precipitated IgG in 7S γ -G and in γ -G/SM close to the antiserum well (Plate 25 A, B, arrow 3).

γ -G/SM contained IgA, IgG, and IgM in order of diminishing concentration (Plate 25 A, B, C). Anti-IgA serum precipitated two components, presumably IgA of differing molecular weights, in γ -G/SM (Plate 25 A, C, arrows 4). By precipitin band linkage, both components were demonstrated to be similar to two IgA components in 7S γ -G (Plate 25 A, arrows 4). Anti-IgG serum precipitated one component, IgG, in γ -G/SM (Plate 25 C, arrow 5), whereas anti-IgM serum precipitated two components, suggesting two forms of IgM present in γ -G/SM (Plate 25 B, arrows 10), which was not detected in another experiment (Plate 25 C).

γ -G/L contained IgA, IgM, and IgG in order of diminishing concentration (Plate 25 A, B). Anti-IgA serum precipitated two components, IgA of differing molecular weights, in γ -G/L (Plate 25 A, arrows 7), and anti-IgM precipitated two components in γ -G/L, possibly different forms of IgM (Plate 25 B, arrows 6). Anti-IgG weakly precipitated one component, IgG (Plates 25 A, B, arrow 8), which, in the latter reaction was precipitated as a split band.

Precipitin bands formed between specific anti-human immunoglobulin goat antiserum, for example, between anti-IgG and anti-IgM (Plate 25B, arrow 9) were due to excess IgG remaining after absorption of anti-IgG antibodies in anti-IgM serum to yield specific anti-IgM antiserum. This a common commercial practice. Similar reactions were also noted with anti-IgA serum.

The identity of canine, equine, and rabbit gamma-globulins was confirmed by immunodiffusion with the respective homologous anti-gamma-globulin serum. Canine and equine gamma-globulins contained one reacting component and rabbit three. Equine gamma-globulin was shown to contain components equivalent to the human immunoglobulin classes IgG, IgA, IgM. Bovine gamma-globulin consists of at least seven serum globulin components.

Table 30 summarizes human and animal gamma-globulin HAI and CF reactions with influenza A2/HK/1/68 and PR8; and immunoprecipitin reactions with all influenza A strains used in the immunodiffusion study.

Each preparation of human gamma-globulin, RDE treated, inhibited hemagglutination by the test viruses, but to differing titres, a result of the different batches and concentrations employed. RDE treatment of human gammaglobulins was selected because in human serum it eliminates inhibitors of A2/HK/1/68 and PR8 hemagglutination. Therefore, the active HAI component present in these preparations was assumed to be antibody. Of animal gamma-globulins, only equine reacted. When treated with RDE and with $NaIO_4$ it inhibited A/Equi 1. The active HAI component in this preparation was considered to be antibody.

All human and animal gamma-globulins except bovine were anti-complementary.

Immunoprecipitin reactions of gamma-globulins with influenza A antigens paralleled their HAI activity. Only equine (horse) gamma-globulins possessed activity. Untreated, RDE treated, and $NaIO_4$ treated

LEGEND - TABLE 30

Serum treatments for HAI and IDD tests:

1:5 : this dilution of serum in PBS

RDE : receptor destroying enzyme as crude V. cholerae filtrate at 500 units/ml.

$NaIO_4$: M/90 sodium metaperiodate

S : type-specific complement fixing antigen

V : strain-specific complement fixing antigen

ac : anti-complementary

All HAI and CF titres are expressed as reciprocal of dilution

C_2 and C_2' : virus concentrates 100x and 200x concentrated respectively

SA : virus soluble antigen

- HAI : titre $< 1:4$

- CFT : titre $< 1:1$

- IDD : no detectable precipitin bands formed

+ IDD : one precipitin band formed, +2 : 2 precipitin bands formed, etc.

Blank spaces: reactions not done.

TABLE 30. Influenza A hemagglutination inhibition (HAI), complement fixation (CF), and immunoprecipitin (IDD) characteristics of human and animal gamma-globulins, and foetal calf serum.

Immune Preparation	Serum Treatment for HAI	HAI Infl. A HK	HAI Infl. A PR8	CF Infl. A2/HK/68 S	CF Infl. A2/HK/68 V	Serum Treatment for IDD	IDD Infl. A C_2 and C_2' HK	PR8	Duck	Swine	FM1	A2	Equi	Infl A SA
Human gamma-globulin, (Lederle) (γ-G/L)	RDE	120	160	ac	ac	Native	$+_{2-3}$	$+_{2-3}$	$+_{2}$	+	+	+	+	+
						1:5	$+_{2-3}$	$+_{3}$	$+_{2}$	-	-			All A
						RDE	$+_{2-3}$	$+_{2}$	$+_{2}$	-	-	+		strains
Human gamma-globulin, (Schwarz, Mann) (γ-G/SM) (20-30mg/ml)	RDE	10	40	ac	ac	Native	$+_{3}$	$+_{4}$	+	$+_{2}$	$+_{3}$	$+_{2-3}$	$+_{2-3}$	+
						1:5	$+_{2}$	$+_{3}$			+			All A
						RDE	$+_{2}$	$+_{2}$	$+_{2}$	$+_{2}$				strains
Human 7S gamma-globulin (7S γ-G) (40mg/ml)	RDE			ND	ND	Native	$+_{2}$	+	$+_{1}$	$+_{2}$	$+_{2}$	$+_{2}$	+	+ All A strains
Bovine gamma-globulin (20mg/ml)	RDE	-	-	1	1	1:5	-	-	-					
	$NaIO_4$	-				RDE	-	-	-					
Canine gamma-globulin (20mg/ml.)	RDE	-		ac	ac	1:5	-	-	-					
						RDE	-	-	-					
Equine gamma-globulin (20mg/ml)	RDE	10	-	ac	ac	1:5	$+_{2}$		+					+
	$NaIO_4$	15	-			RDE	$+_{2}$		+					All A
		(Equi 1-10)				$NaIO_4$	$+_{2}$	-	+				-	strains
Rabbit gamma-globulin (20mg/ml)	RDE	-	-	ac	ac	1:5	-	-						
						RDE	-	-						
Sheep gamma-globulin (20mg/ml)	RDE	-	-	ac	ac	1:5	-	-		-				
						RDE	-	-		-				
Foetal calf serum	Native	12	4			1:5	+	-	-	-	-			
	58^oCx 1 hr	4	-	-	-	58^oCx 1hr	+							
	RDE	-	-			RDE	+							
	$NaIO_4$	40												

equine (horse) gamma-globulin precipitated A2/HK/1/68 and Duck virus concentrates and soluble antigens. The precipitating component was most likely antibody because the precipitin band formed with the virus was not affected by inactivation treatments of the preparation and a linkage of identity was obvious between bands formed by untreated and treated preparations (outermost band, Plate 23 C).

Each type of human gamma-globulin in immunodiffusion tests precipitated all influenza A strains used in this study, as well as influenza B, but not parainfluenza. Sendai (Table 30). RDE treatment of these preparations did not alter the immunodiffusion pattern formed with any of the viruses except PR8, where RDE eliminated one band in γ -G/SM. All precipitin reactions with influenza virus concentrates and with soluble antigens were shown to be virus-specific because none of the gamma-globulins precipitated normal allantoic fluid concentrate (N-ALF/C_2), nor normal chorioallantoic membrane soluble antigen (see Part I, differentiation of Host- and Virus-Specific Immunoprecipitin Reactions).

The influenza A precipitating spectrum of human 7S gamma-globulin (7S γ-G) and gamma-globulin/Schwarz Mann (γ-G/SM or γ-G) is illustrated in Plate 26 A, B, C. 7S γ-G (top wells), and γ-G (bottom wells) form precipitin bands with virus concentrates (centre wells) (Plate 26A), and form a linking band with all influenza A soluble antigens (Plate 26 B, C). The linking band in Plate 26 B, C was shown to be an influenza type-specific reaction (see above) associated with type-specific antigen, which may be the RNP (Schild and Pereira, 1969; Beard, 1970). A linking band, not so

obvious, is also formed by both gamma-globulins with all virus concentrates in Plate 26 A, and has been shown in other experiments to be the type-specific reaction (see Part I, Identification of the Type-Specific Reaction in Immunodiffusion).

The influenza A type-specific band formed between influenza A soluble antigens (V/SA) and 7S γ -G (Plate 26 B) results from the precipitation of the type-specific component in V/SA by the IgG component in 7S γ -G. The type-specific component present in V/SA is not precipitated by IgA because when it is precipitated by 7S γ -G, the band formed cuts an IgA-specific reaction in non-identify (Plate 26 B, arrow 1). A similar situation is demonstrated when antigens are virus concentrates (Plate 26 A, arrow 1). In an analogous experiment, the type-specific component was not precipitated by IgM because 7S γ -G does not contain detectable IgM (Plate 26 C, arrow 1, Plate 25 B). Therefore, it is concluded that the type-specific component in influenza A virus soluble antigens, and in virus concentrate antigens, is precipitated by an IgG class of antibody, not by an inhibitor.

A similar situation has been demonstrated with γ -G/SM (γ -G). It forms an influenza A type-specific band linking across all A soluble antigens which cuts an IgA-specific reaction (in non-identity) (Plate 26 A, B, arrow 2), and does not identify with an IgM specific band in Plate 26 C, arrow 2. In Plate 26 B, a suggestion of linkage with an IgG-specific band is indicated by arrow 3. Therefore, it may be concluded that the component in γ -G/SM which precipitates type-specific antigen is also an IgG class of antibody, not an inhibitor.

The main precipitin band formed between influenza A virus soluble antigens and human 7S γ -G is the influenza A type-specific band. It is also the dominant band in reactions between soluble antigens and γ -G/SM.

Therefore, the influenza A specific precipitin band formed as a result of precipitation of type-specific antigen (presumably RNP) by 7S γ -G was used to identify type-specific antibody in normal animal sera, in immunized animals, and in human convalescent sera.

γ -G/L by immunodiffusion with anti-IgM μ chain serum was found to contain IgM (19S) which could easily be confused with 18S α_2-macroglobulin inhibitors (α - and γ -type) in immunodiffusion reactions with virus concentrates. The components in γ -G/L which precipitated virus concentrates could not be considered as only antibody. However, in immunodiffusion reactions with A2/HK/1/68 concentrate, two components common to γ -G/L (Native and RDE treated) and γ -G/SM (native and RDE treated) precipitated the viruses. Each gamma-globulin preparation formed a linking, stronger, outer band, and a linking, weaker, inner band. A similar component resembling the one forming the strong precipitin band described above was demonstrated in immunoprecipitin reactions with PR8, FM1, A2/Can/57, and Duck influenza virus concentrates. The component forming the strong band resembled the component precipitating the type-specific antigen identified above, but the one forming the weak band has not as yet been proven to be an antibody, although this is suggested by its resistance to RDE treatment in reactions with A2/HK/1/68, PR8, and Duck strains.

A component in equine (horse) gamma-globulin (native, RDE treated, and $NaIO_4$ treated), also identified with the component forming the strong, outer precipitin band described above, in reactions with A2/HK/1/68 and Duck virus concentrates. Its identification as antibody was confirmed by its resistance to inactivation treatments (outer band, Plate 23 C).

Immune Rabbit and Rooster Sera Tables 7, 8, 9, Part I, describe the HAI and immunodiffusion characteristics of convalescent rabbit (RAs-IN/V), hyperimmune rabbit, N-CAM absorbed (hRAs/V abs), and hyperimmune rooster sera (hFAs/V) against all influenza viruses used in this study, and special reference is made to influenza A2/HK/1/68 and A/PR8 which were the principal strains used in the analysis of normal animal sera. Significant rises in HAI titre occurred as the animals were progressively immunized, accompanied by a definite expansion of the immunoprecipitin pattern formed with influenza virus concentrates (Plate 1). The complement fixing titre of RAs-IN/A2/HK/1/68 was 1/8 with type-specific S antigen in both rabbits, and rose to greater than 1/128 in hRAs/A2/HK/1/68. The demonstration of an immunological response on the part of animals to antigenic stimulation by influenza viruses must be accepted as being due to the production of specific antibodies. Immunoprecipitin reactions were shown to be virus-specific (Part I, Differentiation of Host- and Virus-Specific Immunoprecipitin Reactions). Excepted were certain host-specific reactions occurring between hyperimmune rooster sera and virus and host soluble antigens.

At this point, immunoprecipitin reactions in immune sera are assumed to be due to induced antibody, although inhibitor may also play a part (see Part I, Differentiation of Antibody-from Inhibitor-Specific Immunoprecipitin Reactions).

The immune sera of specifically immunized animals were used in immunodiffusion reactions between normal sera and influenza viruses e.g. A2/HK/1/68 and PR8, to help identify precipitating components in normal sera as influenza A virus-specific. They could be used to definitely indicate the presence of antibody only in the following instance: if in immunodiffusion reactions with a specific virus antigen (C_2 and SA), the precipitin bands formed by the immune serum and human 7S γ -G linked in identity. In Plate 28 A, A2/HK/1/68 soluble antigen (centre) is precipitated by homologous hyperimmune rabbit serum (N-CAM absorbed) (periphery) and by human 7S γ -G; the precipitin bands formed link in identity indicating a common component present in both immune preparations, which has been identified as antibody in 7S γ -G (see above). A similar A2/HK/1/68 precipitin has been demonstrated in homologous hyperimmune rooster serum (Plate 28B), and a PR8 precipitin (Plate 28 C, D).

The pattern of precipitin band linkage formed with γ -G/SM is similar to that demonstrated with 7S γ -G in precipitin reactions with soluble antigens.

In immunodiffusion reactions with virus concentrates, the precipitin bands formed by animal sera link with those formed by human γ -G/SM and γ -G/L. For example (Plate 22 A), precipitins in homologous

hyperimmune and convalescent rabbit sera (bottom outer wells) form two precipitin bands with A2/HK/1/68 C_2' (centre) - which are the same as formed by γ -G/L and the virus. In Plate 22 B, RDE treated hyperimmune rooster serum (outer well) forms a precipitin band with A2/HK/1/68 C_2' (centre) and is the same as that formed by RDE treated γ -G/L. Because not all influenza precipitating components found in these two human gamma-globulin preparations have been established as antibody (discussed above), the same interpretation must be applied to similar components found in the sera of immunized animals.

The titre of α inhibitor rises during the acute phase of various infectious diseases including influenza (Tyrrell, 1954), and multiple immunization of rabbits with virus antigens A2 and PR8 causes a rise in titres of thermostable serum inhibitors (Rovanova and Kosyakov, 1967) demonstrating HAI and virus neutralization activity.

It is therefore possible that precipitating components, even in the sera of immunized animals, may be a combination of antibody and inhibitor, which cannot be entirely distinguished in immunoprecipitin reactions with virus concentrates. Their presence, however, is strongly suggestive of antibody.

Thus, the sera of immunized animals can help to determine the presence of precipitating antibody in normal animal sera, by utilizing the precipitation of the type-specific component in virus soluble and concentrate antigens, demonstrated to be precipitated by antibody in 7S γ -G (see Identification of Influenza Antibodies in Immune Preparations), and

in immune sera (see Differentiation of Antibody- from Inhibitor-Specific Immunoprecipitin Reactions).

Human Convalescent Sera On examination of paired human sera from influenza patients, antibodies to A2/HK/1/68 in convalescent sera were revealed by significant rises in HAI and in CF titres; the diagnosis of influenza was confirmed in some cases by isolation of A2/HK/1/68 virus from the patients (Table 31). The difference in precipitation of influenza A virus concentrates (Plate 27, centre rows) by acute (top rows) and convalescent (bottom rows) sera is illustrated in Plate 27 A and B. In both patients, A2/HK/1/68 virus was isolated. An immune response to influenza A strains is evident in the convalescent sera by an increase in the number and intensity of precipitin bands formed. Note that there is no change in the precipitin response to influenza B in either patient. Influenza A and B reactions remain discrete and cut each other in non-identity (Plate 27 A and B, arrowed). Similar immunoprecipitin patterns were demonstrated by the other VP sera listed in Table 31.

In Plate 27 C, the difference in precipitation of influenza A strains by "normal" (top row) and by convalescent (bottom row) human serum (DK) is very clear, illustrating the increase in precipitin response after influenza infection, which agrees with serological findings (Table 31). A less marked response was demonstrated by NM convalescent serum (not illustrated).

The type-specific reaction linking all influenza A strains is distinguishable when acute and normal sera are diffused against virus

LEGEND - TABLE 31

Human sera: RDE (500 u/ml) treated for HAI tests
inactivated 30 min. at 56°C for CF Tests

*NM and DK "A" sera were normal having been taken about a month before illness set in

A : acute phase serum

C : convalescent phase serum

A/S : type-specific complement fixing antigen influenza A2/HK/1/68

A/V/HK : strain-specific complement fixing antigen influenza A2/HK/1/68

B/S : type-specific complement fixing antigen influenza B/Can/5/66

- : negative results, that is titres less than 1/5 (HAI), less than 1/4 (CF), and no virus isolation

ND : not done

TABLE 31 Characteristics of normal, acute, and convalescent human sera in relation to influenza infection as determined by hemagglutination inhibition (HAI), complement fixation (CF) tests, and virus isolation.

Human Serum No.	Date taken	HAI						CFT						Virus isolation
		Infl.A2HK68		Infl.A/PR8		Infl.A/Duck		A/S		A/V/HK		B/S		
		A	C	A	C	A	C	A	C	A	C	A	C	
VP-39	1-2-70	-	80	10	10	-	-	-	128	8	128	-	-	Infl.A2/HK/1/68
VP-41	1-2-70	-	40	-	-	-	-	-	128	-	128	-	-	-
VP-54	1-2-70	-	40	20	30	-	-	8	32	16	256	-	-	-
VP-66	1-2-70	-	160	-	-	-	-	16	32	8	218	8	8	Infl.A2/HK/1/68
VP-68	1-2-70	-	160	-	-	-	-	-	16	32	32	8	8	Infl.A2/HK/1/68
VP-73	1-2-70	-	80+	60	60	-	-	8	256	16	1024	16	16	-
*NM	7-8-70	-	-	-	-	-	-	4	4	-	16	ND	ND	ND
*DK	1-2-70	-	60	-	-	-	-	-	>16	-	>64	ND	ND	ND

concentrates (Plate 27 A, B, C. top), indicating the presence of antibody to the type-specific component. An increase in the type-specific antibody is obvious in convalescent sera, because the influenza A type-specific reactions are more pronounced when convalescent sera are diffused against the same virus concentrates (Plate 27 A, B, C, bottom).

The precipitating type-specific antibody in convalescent human sera was used in conjunction with human 7S gamma-globulin (7S γ -G) to identify similar antibodies in normal animal sera (Plate 28). The type-specific precipitin in convalescent serum VP 73 or VP 68 was shown to be the same anti-influenza A antibody as present in 7S γ -G, because when serum and gamma-globulin were simultaneously diffused against A2/HK/1/68 SA (Plate 28 A, B), or against A/PR8 SA (Plate 28 C, D), the precipitin bands formed linked in identity. A precipitin in convalescent serum forms another inner band which is independent of the one formed by 7S γ -G (Plate 28 B, C, D). Its nature is not known.

The recall of influenza antibodies in human sera against all influenza A strains, human, animal, and avian, after an infection by a human strain, has been clearly demonstrated by the immunoprecipitin test, and is a further illustration of the phenomenon termed Original Antigenic Sin by Francis (1953), discussed by Webster and Laver (1971).

Foetal Calf Serum Foetal calf serum precipitated only A2/HK/1/68 in immunodiffusion reactions (Table 30). It could not be used as an indicator of inhibitor precipitin because it retained its precipitating activity even after treatments which should eliminate this inhibitor,

that is, heating at 58°C x 1 hour, and RDE (V. cholerae filtrate) treatment, and because it has been shown to contain immunoglobulins (Schultz et al., 1971). A typical immunoprecipitin reaction with A2/HK/1/68 (centre) is shown in Plate 22 B, where the precipitin band formed with the virus is not related to any precipitin bands formed by all other sera and immune preparations. The only exception was found in normal sheep serum which yielded a precipitin band with A2/HK/1/68, which linked with one produced by foetal calf serum.

In view of the behaviour of its HAI activity with respect to inactivation treatments (Table 30), the identity of the reacting component in foetal calf serum remained uncertain.

Complement Fixation Tests Normal sera fixing complement in reactions with influenza A2/HK/1/68 S and V antigens are listed in Table 32, and in Tables 5 A - I, Appendix 1. Most CF titres were very low except for that of Dog H36.

Complement was fixed in reactions with both S and V antigens by sera of : Cat H27, Dog OU1, Rabbits A5, A19, 160, and Sheep 62. These sera contained influenza A type-specific antibodies and demonstrated A2/Hong Kong/1/68 strain specificity. Complement fixation with V antigens only was demonstrated by the sera of: Dogs H36, H48, H86, Rabbits A47, 127, 160, and Cottontail rabbits 395, 435. The A2/Hong Kong/1/68 strain specific antibodies of these sera could be related to the hemagglutinin (Lief and Henle, 1959) or the neuraminidase (Webster and Laver, 1971) of that virus.

These sera were considered to contain antibody to influenza A viruses because inhibitor has not yet been shown to fix complement in reactions with virus antigens.

Of 19 fowl sera examined, complement was fixed in reactions with S and V antigens by 16 sera, with S antigen only by one serum, and with V antigen only by one serum; one serum was negative. Because of such an overwhelming positive response, these results suggest that non-specific factors were involved. Because the immunoprecipitin type-specific reactions were also doubtful, these results will not be interpreted until they are further clarified.

Complement fixing antibodies appear to be a result of recent infection, and are relatively short-lived, so that only animals having a recent infection would be positive (Casals, 1967). Hence, although the presence of complement fixing antibodies should indicate recent infection, their absence would not negate the possibility of an earlier infection.

Final Selection of Normal Animal Sera Containing Influenza Antibodies

Normal animal sera, scrutinized by the procedures discussed above, and compared with immune preparations containing influenza antibody, were judged for influenza antibody content on the following basis (Table 32):

1. The presence of influenza type A antibodies was established if:

   a. The immunoprecipitin reaction between normal animal serum component and influenza antigen linked

LEGEND - TABLE 32

HAI : Microhemagglutination titres expressed as reciprocal of dilution

Serum treatments for inactivation of inhibitors : V. cholerae filtrate RDE; THP (Trypsin-heat-periodate); $NaIO_4$ (sodium metaperiodate); Kaolin (adsorption)

CF : Microcomplement fixation tests expressed as reciprocal of dilution. A2/Hong Kong/1/68 S and V antigens.

C_2, C_2' : virus concentrated 100x and 200x respectively

SA : virus soluble antigen prepared from extract of infected CAM.

\+ : confirmed precipitin antibody

P : precipitin reactions suggest presence of antibody (see Results and Summary for discussion)

D : precipitin reactions doubtfully suggest presence of antibody (see Results and Summary for discussion)

\- : negative reaction

Blank spaces : test not done because of lack of serum or not considered necessary.

TABLE 32. Comparison of hemagglutination inhibition, complement fixation, and immunoprecipitin reactions of "normal" animal sera

Normal animal serum species	Serum No.	HAI Infl.A2/HK/1/68 RDE	HAI Infl.A2/HK/1/68 THP	HAI Infl.A2/HK/1/68 $NaIO_4$	HAI Infl.A2/HK/1/68 Kaolin	HAI Infl. A/PR8 RDE	HAI Infl. A/PR8 THP	HAI Infl. A/PR8 $NaIO_4$	CF Infl.A2/HK/1/68 S	CF Infl.A2/HK/1/68 V	Immunoprecipitin: Antibody to virus C_2,C_2' A2/HK/68	Immunoprecipitin: Antibody to virus C_2,C_2' PR8	Immunoprecipitin: Antibody to virus C_2,C_2' Other Infl.A	Immunoprecipitin: Antibody to virus SA A2/HK/68	Immunoprecipitin: Antibody to virus SA PR8
Bovine (NBS)	24	10	10		-		5		-	-	-	-	-/Duck		
	82	5	20		-		-		-	-	-	-			
Cat (NCS)	H19	10	10	10	12		-	-	-	1	D			-	-
	H27	60	30	40			-	-	1	2	+		P/Duck	+	+
	H37	-	80	5-10	12		-	-	-	-	-		-/Equi		
Dog (NDS)	OU1	-	-	-			-		1	8	-	-		-	-
	OU22	-	20	-	6		-	-	NS	NS	-	-	-/Duck		
	H36	80		40			-		-	20				+	P
	H48	20	160	20	12		10 (Swine 10)		-	4	P	-	-/Swine	P	-
	H52	-	120		-						-		-/FM1		
	H62	-	20	-	18		-	-	NS	NS	+	-	-/Duck	-	-
	H68	-	160	-	12		-	-	-	-	D	-	-/FM1		
	H86	-	20	10			-	-	NS	8	-	-		P	-
Equine (NES)	1	480	320		192		-	-	-	-	D				
	3	160	120	-	192		-	-	NS	NS	-	-			
	4	160	320	20	72		-	-	-	-	P	-	-/Duck	-	-
	5	320	80	10	96		-	-	-	-	-	-			
Fowl and Rooster (NFS)	R2	20		-		-		-	indirect	CF 16	P				
	115	20		10		-		-	16	-	+			P	-
Goat (NGS)	1	20	20		48		30		-	-	-	D	-/A2/Can/57	+	
	2	10	-		-		40		-	-	-	+	P/Duck		

TABLE 32. (Cont'd) Comparison of hemagglutination inhibition, complement fixation, and immunoprecipitin reaction of "normal" animal sera

Normal animal serum species	Serum No.	HAI Infl.A2/HK/1/68 RDE	HAI Infl.A2/HK/1/68 THP	HAI Infl.A2/HK/1/68 $NaIO_4$	HAI Infl.A2/HK/1/68 Kaolin	HAI Infl. A/PR8 RDE	HAI Infl. A/PR8 THP	HAI Infl. A/PR8 $NaIO_4$	CF Infl.A2/HK/1/68 S	CF Infl.A2/HK/1/68 V	Immunoprecipitin: Antibody to virus C_2,C_2' A2/HK/68	Immunoprecipitin: Antibody to virus C_2,C_2' PR8	Immunoprecipitin: Antibody to virus C_2,C_2' Other Infl.A	Immunoprecipitin: Antibody to virus SA A2/HK/68	Immunoprecipitin: Antibody to virus SA PR8
Rabbit (NRS)	A5	10	1280	40	24	-	20	-	1	4	D	-		-	-
	A19	20	3840	40	96	-	-		2	4	D	-		-	-
	A29	10	5120		24	-	20		-	-	D				
	A46	20	640		24	-	10		10		P				
	A47	5	120	40	24	-	-	-	-	2	D			-	P
	127	20	160	20	36	-	-		-	2	D				
	157	20	640	40	24	-	-	-	-	-	P			-	-
	160	20	160	20	24	10	10	-	2	2	D	D		P	+
Sheep (NSS)	62	15	30	-	-		-		1	8	D	-		-	-
	75	10	-		-		-				-	-			
Cottontail Rabbit (NC_RS)	395	-	20	60			10		-	2	D			-	-
	442	-	60		12		10				-				
Snowshoe hare (NS_HS)	352	-	160				-		-	-	-	-			
	359	10	160	20	-		-		-	-	-	-			
Chipmunk (NC_HS)	575	10	40				10		-	-	D	-		P	+

in a reaction of identity with the influenza A type-specific reaction formed between the type-specific antigen and human 7S gamma-globulin (7S γ -G, IgG).

b. The type-specific precipitin band in a) linked in a reaction of identity with immunoprecipitin reactions of convalescent and immunized animals, and with those given by human influenza convalescent serum.

c. The normal serum was shown to fix complement in reactions with an influenza A type-specific (S) and/or strain-specific (V) antigen.

2. The presence of influenza A antibodies was suggested, but not fully established if:

a. HAI activity remained despite RDE, THP, or $NaIO_4$ treatments of sera for inhibitor inactivation. Kaolin was not always considered because it is known to remove immunoglobulins containing antibody.

b. Immunoprecipitin reactions between influenza virus concentrates and normal sera remained the same despite the inhibitor inactivation treatments listed in 2a

c. The precipitin bands formed with the virus in 2b linked in a reaction of identity with those formed between virus and;

i. The sera of convalescent and specifically immunized animals.

ii. Human and animal gamma-globulin preparations known to contain influenza antibody, especially 7S gamma-globulin.

The following normal animal sera completely fulfilling the conditions set down in 1a and 1b, and completely or partially fulfilling those set down in 1c and in 2, are considered to contain antibody to influenza A viruses.

Normal cat serum

NCS H27 contained antibodies to influenza A type-specific antigens, possibly A2/HK/1/68, because it completely fulfilled all the above conditions. In reactions with A2/HK/1/68, NCS H27 formed a precipitin band on immunodiffusion with soluble antigen which linked in identity with one formed between soluble antigen and human convalescent serum (VP 73C), human 7S gamma-globulin, and absorbed hyperimmune rabbit serum to A2/HK/1/68 (Plate 28 A); the normal serum contained low titre complement fixing antibodies to S and V antigens (Table 32); it retained precipitating activity of virus concentrate, despite inhibitor inactivation treatments, and the reaction linked with that of human gamma-globulin (Plates 22 D and 23 B); HAI activity was not eliminated by inhibitor inactivation treatments (Table 32).

Additionally, in an immunodiffusion reaction with PR8 soluble antigen, similar to that described with A2/HK/1/68, a precipitin band was formed, with corresponding linkages,

which suggested the presence of a cross reacting influenza A type-specific antibody, because no other evidence indicated the presence of antibody to influenza A/PR8. A faint reaction with influenza A/Duck C_2 was also observed, which is even more suggestive that the reaction was a type-specific one.

Normal dog serum

NDS H 36 possessed antibodies to influenza A type-specific antigen, possibly A2/HK/1/68 because it fulfilled all conditions in 1 and partially fulfilled those in 2. On immunodiffusion with A2/HK/1/68 soluble antigen (Plate 28 B) it formed 2 precipitin bands which linked in identity with 2 formed by human convalescent serum (VP 73 C). The precipitin in the outer band identified with a precipitin in human 7S gamma-globulin, in anti-A2/HK/1/68 hyperimmune rooster serum, and also in anti-A2/HK/1/68 hyperimmune rabbit serum (absorbed) in another similar reaction. NDS H36 reacting with A2/HK/1/68 V antigen fixed complement (Table 32). Precipitation of virus concentrates and HAI activity were not eliminated after treatment with RDE and $NaIO_4$ (Table 32).

NDS H 36 also formed a precipitin band with PR8 soluble antigen which linked with precipitin bands formed by soluble antigen and human convalescent serum, human

7S gamma-globulin, and homologous hyperimmune fowl serum. The reaction may be an expression of type-specific cross reactivity between soluble antigens of A2/HK/1/68 and PR8.

Normal goat serum

NGS 1 contained antibody to influenza A type-specific antigen. Conditions 1a and 1b were met in reactions with A2/HK/1/68 soluble antigen. A strong precipitin band formed with the soluble antigen linked with the common one formed by human convalescent serum (VP 73C), human 7S gamma-globulin, and homologous absorbed hyperimmune rabbit serum as well as with horse gamma-globulin. None of the inhibitor inactivation treatments carried out eliminated HAI activity with A2/HK/1/68 (Table 32). The small amount of serum curtailed further investigations.

Normal rabbit serum

NRS 160 possessed antibody to influenza A type-specific antigen. All of condition 1 was met and condition 2 partially. In reactions with influenza A/PR8, the outer of the two precipitin bands formed with soluble antigen (Plate 28 D) linked in identity with the common one formed between soluble antigen and human convalescent serum, human 7S gamma-globulin, and homologous hyperimmune fowl serum.

The precipitin in NRS 160 forming the inner band identified with a similar component in human convalescent serum precipitating the soluble antigen. Complement was fixed to a low titre in reactions with A2/HK/1/68 S and V antigens (Table 32). Inactivation of the serum did not affect precipitins in NRS 160, but $NaIO_4$ eliminated low-level HAI activity.

A precipitation of influenza A2/HK/1/68 soluble antigen was faintly suggested which identified with immune preparations (P in Table 32), and may be a reflection of type-specific cross reactivity with PR8. HAI activity was not eliminated by inactivation treatments (Table 32); and only $NaIO_4$ eliminated precipitating activity with virus concentrate.

Normal chipmunk serum

NC_HS 575 demonstrated the presence of influenza A type-specific antibody only in immunodiffusion reactions. In Plate 28 C, of two precipitin bands formed with PR8 soluble antigen, the inner one linked in identity with human convalescent serum (VP 68 C) signifying a common component; but the outer band (situated behind rather than in front of the well because of an unequivalent reaction) shared a common precipitin with human convalescent serum, human 7S gamma-globulin, and PR8 hyper-

immune fowl serum. The small sample did not allow a thorough investigation of HAI activity (Table 32), but low titre PR8 and A2/Hong Kong/68 HAI was recorded.

A suggestion of a precipitin reaction with A2/HK/1/68 soluble antigen identifying with those of immune preparations, may be due to a type-specific cross reaction with PR8.

The presence of influenza antibody was suggested but not established in certain sera which only partially fulfilled conditions in 1 and 2 . These are:

Normal dog serum

NDS H48 did not completely fulfill all conditions set down in (1) but partially met them, and those in (2). Complement was fixed to low titre in reactions with A2/HK/1/68 V antigen (Table 32), and there was a suggestion of a reaction of identity with the common precipitin band formed between A2/HK/1/68 soluble antigen and human convalescent sera, human 7S gamma-globulin, and homologous hyperimmune rabbit serum (absorbed), but not with homologous hyperimmune fowl serum (latter reaction in Plate 28 B). The precipitating antibodies of fowl and dog sera appeared to differ. NDS H48 retained precipitation of virus concentrate and HAI activity despite inhibitor inactivation treatments. Therefore, NDS H48 could contain type-specific influenza A antibody, possibly against A2/HK/1/68.

Normal Fowl serum

Difficulty was experience in interpreting normal fowl serum immunodiffusion reactions because fowl serum has been shown to precipitate host (CE) components in N-CAM and in virus soluble antigens. NFS 115 precipitin was not clearly demonstrated to be the same as homologous hyper-immune rooster serum precipitin, or that of 7S gamma-globulin, or of human convalescent serum. It demonstrated complement fixing antibodies in an indirect CF test with A2/HK/1/68 V antigen. Thus, it could contain antibody to influenza A type-specific antigen. A similar phenomenon was demonstrated by the sera of 6 other fowl. (Table 32, Plate 22B).

SUMMARY AND DISCUSSION

The survey of 15 species of normal animal, avian, and human sera by micro immunoprecipitin tests in cellulose acetate, using whole virus antigen, has revealed an extremely high incidence of precipitins against a broad range of influenza A virus strains (Figs. 10-13). The most prevelant precipitins were those active against A2/Hong Kong/1/68, occurring in all species of normal sera examined except squirrel. These against A/PR8 were next in order of incidence. The occurrence and distribution of precipitins against other influenza A strains was not so great; in order of diminishing occurrence they are Duck and Swine, FM1, Equi, and A2/Can/57. Precipitins against parainfluenza Sendai, of the lowest incidence, were found only in dog, rabbit, and fowl sera. Recently, a number of parainfluenza isolations from dogs have been reported (Binn et al., 1968; Appel and Percy, 1970; Rosenberg et al., 1971), which substantiates the presence of anti-Sendai precipitins in dog sera.

A broad anti-influenza A spectrum was exhibited by the sera of certain species. Precipitins in dog, rabbit, human, sheep, and goat sera affected all seven A strains used in this study, whereas those in cat, bovine, and fowl sera affected only a few, and equine sera only two strains. Precipitins against A2/Hong Kong/1/68 and PR8 were detected in the sera of wild animals such as cottontail rabbits and snowshoe

hares; only A2/Hong Kong/68 precipitins in groundhog and chipmunk; and neither precipitin in squirrel.

Certain individual animal sera precipitated more than one virus strain, occasionally all seven strains; human sera often demonstrated the latter capacity.

These findings suggested that influenza A infections were widely distributed among animal species, which would support the theory that animals could serve as a reservoir for influenza A viruses.

Complementary HAI tests performed on the sera after THP treatment (Fig. 14) revealed an extremely high incidence and widespread distribution of anti-influenza activity, particularly against A2/Hong Kong/68 and A/PR8. The incidence was as high as 100 per cent of the sera of some species, which suggested that the HAI activity observed could have been due to predominantly non-specific inhibitors rather than to specific antibodies.

Correlation between HAI and immunoprecipitin test results were inconsistent, and the considerable variation observed depended upon the animal species and the virus strain used. The lack of correlation between the tests may be due to the fact that antibodies against different influenza antigens were being detected by each test system.

Various inhibitor inactivation treatments of sera (THP, $NaIO_4$, V. cholerae RDE, Kaolin), which demonstrated HAI and precipitating activity, demonstrated that much of the HAI activity against A2/Hong Kong/68 and most, but not all of that against PR8, was due to non-specific inhibitors. However, in a number of sera from five animal

species, the residual HAI activity remaining after different treatments (Table 32) could not be established as due to antibody or to resistant inhibitor. The persistence of the residual HAI activity suggested that the active HAI component left was antibody. Low level HAI titres (1/20-1/40) are usually considered to be due to non-specific inhibitors; however, similar titres recorded in convalescent rabbit sera after influenza infections (Tables 7 and 21) were presumably due to antibody.

Anti-influenza precipitins in the normal sera examined could not be readily differentiated in immunoprecipitin tests as being specific antibody or non-specific inhibitor on the basis of sensitivity to inhibitor inactivation treatments as was demonstrated in HAI tests. The sensitivity of precipitins to these treatments varied greatly, even in individual sera within a species, particularly those against A2/Hong Kong/68.

The effects of THP, $NaIO_4$, and RDE treatments on serum precipitins in immunodiffusion reactions with virus concentrates ranged from causing no change, through fuzzing of the immunoprecipitin reaction, to its complete elimination (Plates 22 and 23). Those precipitins, which were consistently eliminated within a species, were considered to be non-specific inhibitors. The other precipitins, which were so affected by the treatments as to cause a fuzzy precipitation of the antigen to occur, or a change in the relative position of the band (noted with RDE treated sera), could be either 1) inhibitor, whose character had been altered by the treatments (see Levinson et al., 1969), or 2) altered antibody, which is known to be attacked by these treatments (see Part II, Review, Inhibitors of Influenza Viruses). The glycoproteins attached to

the heavy chain of immunoglobulins (Grey, 1969; Edelman et al., 1969; Edelman, 1970; Niedermeier et al., 1971; Shimizu et al., 1971) could have been attacked by periodate or RDE in the same way as glycoprotein inhibitors are (see Part II, Review, Inhibitors). Thus the function of the immunoglobulin could have been affected, which was demonstrated in the immunoprecipitin reactions described above by atypical precipitation of the virus antigen.

Precipitins present in a few sera were strongly suspected of being antibodies because 1) they were not eliminated by any of the treatments for inhibitor inactivation, and 2) in diffusions with virus concentrates, they continued to form precipitates which linked in reactions of identity with those formed when virus was diffused against anti-influenza immune preparations such as human gamma-globulins, and homologous convalescent and huperimmune animal sera (+ and P in Table 32).

The precipitins in a number of other sera were doubtfully considered to be antibody (D in Table 32), when, on diffusion with virus antigen, the precipitin band yielded linked with the one formed between the virus and the immune preparations. However, the immune preparations used were known to contain inhibitors as well as antibody (see Tyrrell, 1954; Rovanova and Kosyakov, 1967). In Part I, animal sera immune to influenza had been demonstrated, by immunodiffusion reactions with virus particle antigens, to contain precipitating inhibitors in addition to precipitating antibodies (Differentiation of Antibody-from Inhibitor-Specific

Immunoprecipitin Reactions). Only the influenza A major type-specific antigen was demonstrated to be precipitated solely by antibody in diffusions with virus concentrate and virus soluble antigens.

Following this, the immunoprecipitin reaction in cellulose acetate between the influenza A major type-specific antigen (as soluble antigen) and human 7 S gamma-globulin was established as virus-specific, and caused by antibody (IgG). It was used as a reference reaction to identify immunoprecipitin reactions due to antibody that occurred when influenza A major type-specific antigen (as SA) was diffused against a) known anti-influenza immune sera, such as human influenza convalescent phase, convalescent and hyperimmune rabbit and rooster, and b) normal animal sera. The identification of the precipitating antibody as an IgG class of immunoglobulin further defined similar animal, and avian sera described by Schild and Pereira (1969), Beard (1970), and Samadieh and Bankowski (1971a).

Precipitins in normal animal sera were identified as anti-influenza antibody by the demonstration that the immunoprecipitin reaction between normal animal sera and influenza A type-specific antigen (as SA) linked in identity with the type-specific reference reaction, and with a similar reaction occurring between type-specific antigen and known influenza antiserum (Plate 28). Antibody against influenza A type-specific antigen was thus identified in the sera of a cat (H27), a dog (H36), a goat (1), a rabbit (160), and a chipmunk (575), and was tentatively identified in the sera of a second dog (H48), a fowl (115), and

both rabbits discussed above, correlated with the presence of low titre strain- or type-specific complement fixing antibodies (Table 32). Only the latter antibodies were detected in a few other normal sera, fowl (R2), rabbits (A5, A19), Sheep (8), and cottontail rabbit (395). These correlations are similar to those observed between immunoprecipitin tests in agar gel and CF tests on acute and convalescent horse and human sera (Beard, 1970; Samadieh and Bankowski, 1971a).

The results obtained from the strain-specific CF test, HAI tests, and immunoprecipitin tests (Table 32) suggested that anti-A2/Hong Kong/68 antibody was most likely present in the cat (H27) and both dog (H36, H48) sera; whereas the very strong type-specific immunoprecipitin reaction with PR8 soluble antigen suggested the presence of anti-PR8 antibody in the chipmunk (575) and both rabbit (160, A47) sera.

Type-specific precipitating antibodies against influenza A viruses were demonstrated in only a few sera of five species of animals; cat, dog, rabbit, goat, and chipmunk. Complement fixing type- and strain-specific antibodies of low titre were found in six species: cat, dog, rabbit, fowl, sheep, and cottontail rabbit.

The relatively low numbers of sera found to contain anti-influenza A antibody within each species was perhaps due to the following factors:

The method by which sera, most likely to contain anti-bodies, were selected from the vast numbers precipitating influenza virus concentrates; the screening techniques used

eliminated all sera but those with a definite possibility of containing antibody. The detection of only IgG type-specific precipitating antibodies by immunodiffusion had less chance of detecting positive sera than complement fixation which involves both IgG and IgM antibodies (see Pike, 1967). Antibodies against the influenza type-specific component are relatively short-lived and indicate recent infection only (Lief and Cohen, 1966; Beard, 1970). Sera were sampled randomly, at no specific time of the year, thus recent infections could have been missed.

Type-specific antibodies resulting from recent infections by influenza A viruses have been detected in the sera of 'normal' animals by a very sensitive immunodoublediffusion test in cellulose acetate. In spite of its limitations, this test system could be easily applied to detecting recent infections caused not only by influenza A strains, but by B or C strains, and possibly by parainfluenza viruses. A similar test system in agar gel had been recommended for a similar purpose by Beard (1970) and Samadieh and Bankowski (1971 a). Beard (1970) had applied it to examine the sera of turkeys infected by influenza virus.

The basic method used to identify antibodies against type-specific antigens could also be applied to the identification of antibodies against hemagglutinin, neuraminidase, and other influenza antigens recently identified (see Kilbourne et al., 1972, and Part II, Review). Hemagglutinin and neuraminidase have been isolated (by Laver, 1963, 1964); Wilson and Rafelson, 1963; Seto et al., 1966; Drzeniek et al., 1966b;

Laver and Valentine, 1969; Webster and Darlington, 1969), and characterized by immunoprecipitin tests in agar gel, using monospecific sera and recombinant strains (by Schild and Pereira, 1969; Schild and Newman, 1969). A reference reaction with a known immunoglobulin, 7S or IgG, which is independent of precipitating inhibitors, must be established, and used to identify the same reaction in immune preparations and in 'normal' animal sera. The immunoprecipitin reactions of influenza envelope antigens with other immunoglobulins such as IgM and IgA must be differentiated from those initiated by nonspecific inhibitors. By an extension of the technique, using purified inhibitors from 'normal' animal sera such as γ in horse serum (see Biddle et al., 1965;Pepper, 1968, a,b), and β in bovine serum (see Krizanova and Sokol, 1966), the immunodiffusion reactions due to known inhibitors could be established and distinguished from those initiated by specific antibody.

Thus, if immunoprecipitin reactions using whole virus concentrates can be specifically defined as suggested, both recent and long-standing antibodies to at least three influenza antigens could be identified simultaneously in one test. A sensitive and broadly reacting test like this would greatly increase the probability of detecting antibodies to influenza viruses in a survey such as this one, and would be a valuable complement to existing serological methods.

The results of this survey demonstrate that, in the Ottawa area, antibodies of recent influenza A infections, possibly by A2/Hong Kong/68 and PR8, existed in at least eight animal species, most of which have some

human contact. The significance of antibody in the wild animal sera is not known. Antibody to A_2/Hong Kong/68 is not surprising in sera collected after 1968 because the virus had been circulating in the human population since that time, and has been shown experimentally, to be capable of infecting animals (see Review, Influenza in Animals). But the presence of CF type- and strain-specific A2/Hong Kong/68 antibodies in the sera of two rabbits, A5 and A19, collected in 1966 leads to the speculation that this virus could have been circulating in this species of animal two years before it emerged in the human population. High titres of A2/Hong Kong/68 inhibitor (1:1280-1:5120) were found in the sera of these and other rabbits collected in 1966. Whether this also was an indication of the presence of the virus is not known but it is highly suggestive as the titre of inhibitors in rabbits has been shown to rise on immunization with PR8 and A2 viruses (Rovanova and Kosyakov, 1967). Antibodies to PR8 had been demonstrated in animal sera before (Johnson and Westwood, 1969). Their presence is a puzzle as PR8 virus has been out of human circulation since 1946, but the antibody expression could be a reflection of a cross reaction with a related virus such as A/Swine, which has been demonstrated to occur (Part I, Immunodiffusion Cross Reactions of Influenza A Virus Concentrates).

The scattered occurrence of these antibodies suggests random or chance infection of the animals and does not implicate any one species as a possible reservoir except perhaps rabbit in relation to early demonstration of A /Hong Kong/68 antibodies. The possibility of multiple reservoirs is indicated by the broad host range of influenza A viruses revealed by this

study and in numerous others (see Review, Influenza in Animals). However, the fact that animals in close contact with man can be infected with influenza viruses indicates they could serve as vectors, or a possible site for recombination to take place between influenza A strains with the production of new antigenic hybrids as has recently been demonstrated experimentally in vivo (Webster et al., 1971; Webster and Campbell, 1972). This may well be the source of new sybtypes pathogenic for humans and for animals. However, until influenza viruses are isolated from naturally occurring infections in animals which can be related to human infections, definite proof of animal involvement in the influenza cycle of man will remain lacking.

Very few sera, less than 2%, of over 600 examined, contained antibodies to influenza virus, but many of them had an extremely high incidence of high titre A2/Hong Kong/68 hemagglutination inhibitor, and a lower incidence and titre of PR8 and other influenza A inhibitors.

The expression of A2/Hong Kong/68 hemagglutination inhibitor in the sera of 15 species of animals varied with the species. This agrees with similar observations of Coleman and Dowdle (1969), and is analogous with those of Anantharayan and Paniker (1960), who, using a variety of animal sera and pre-1968 influenza A strains, found that inhibitors differed qualitatively and quantitatively in different animal species. The current investigation revealed, that in seven species of animal sera (cat, dog, fowl, snowshoe hare, cottontail rabbit, groundhog, and squirrel), A2/Hong Kong/68 inhibitors resistant to THP and $NaIO_4$, and

sensitive to V. cholerae RDE, resembled β inhibitor, yet were thermostable, which was not characteristic of a β -type inhibitor. The thermostability of these inhibitors had been noted by others (Coleman and Dowdle, 1969; Fedova et al., 1969; Jandasek et al., 1969; De Sousa and Bal, 1971). The inhibitor in bovine sera resisted all inactivations except koalin treatment, which was not characteristic of β inhibitor (see Part II, Review). The periodate insensitivity of Hong Kong inhibitor in bovine serum has prompted De Sousa and Bal (1971) to relate it to a β -type. The current study revealed that the A2/Hong Kong/68 HAI activity exhibited in the sera of other animal species examined, was only partially or not at all affected by these treatments, and could not be attributed with certainty to either antibody or inhibitor.

In immunoprecipitin tests, a definite pattern of inhibitor inactivation for all species examined could not be determined, but those precipitins eliminated consistently within a species were thought to be inhibitors. Thus, A2/Hong Kong precipitins were eliminated by V. cholerae RDE in fowl and goat sera, by THP in bovine sera, and by $NaIO_4$ in fowl and rabbit sera. PR8 precipitins in snowshoe hare sera were inactivated by V. cholerae RDE, THP, $NaIO_4$, and in rabbit sera by THP and $NaIO_4$. Inactivation of precipitins did not parallel inactivation of hemagglutination inhibitors except in fowl and snowshoe hare sera.

The identity of A2/Hong Kong/68 hemagglutination inhibitors has not been resolved by the above investigations (Coleman and Dowdle, 1969; Jandasek et al., 1969; De Sousa and Bal, 1971), but useful treatments to eliminate them from some animal species have been indicated. The

application of these treatments to eliminate precipitating inhibitors of influenza viruses from the sera of certain animal species has been demonstrated in the present study.

Rabbit sera collected in 1966 contained strain-specific CF antibodies against A2/Hong Kong/68 and high titres of A2/Hong Kong/68 hemagglutination inhibitor, which was not found in sera collected later. The relationship between these was not clear and was not encountered again in this survey. The sera of certain animal species possessed consistently high titres of A2/Hong Kong/68 inhibitor: domestic and wild rabbits, hares, horses, cats and dogs. The high incidence in dog sera was in marked contrast to the low incidence of influenza A inhibitors found by Topciu (1966b). Inhibitor titres are known to increase in infection and on immunization (Tyrrel, 1954; Rovanova and Kosyakov, 1967), but their significance in the immune response has not been revealed.

Like antibodies, serum inhibitors interfere with influenza A virus activities. They inhibit hemagglutination by the virus, neutralize its infectivity in ovo, in tissue culture, and in mice, and precipitate its antigenic components (see Review - Inhibitors). Neutralization is a protective characteristic, and the other antiviral activities a reflection of this quality in vivo.

Antibodies are immunoglobulins and have restricted antigenic specificity. Inhibitors have less specificity with respect to antigens and are either β-globulins (β inhibitor), or glycoproteins, α_2-macroglobulins (α and γ inhibitors). Glycoprotein inhibitors depend on a

sialic acid prosthetic group for their activity in combining with viruses (Gottschalk, 1966a; Pepper, 1968a, b; Levinson et al., 1969). They are chemical analogues of sialic acid-containing glycoproteins which form an integral part of the red cell membrane (Gottschalk, 1966a: Branton, 1971[1]; Bretscher, 1971), and other tissue cells (Hirst, 1943). Glycoproteins (glycopeptides) are an integral part of immunoglobulin molecules, and are mainly attached to the constant portion of the heavy chain (Edelman et al., 1969; Davie and Osterland, 1971). They do contain sialic acid (Edelman and Gall, 1969; Niedermeier et al., 1971). Although carbohydrate units do not appear to have a definite role in specific reactions between antibody and antigens (Frommel et al, 1971; Shimizu et al, 1971), the latter authors suggest that the carbohydrate may facilitate non-specific binding of IgM antibodies to the glycolipoprotein membrane surfaces of red cells and similar substrates for 19 S antibodies.

Is there a connection between glycoprotein inhibitors and the glycoproteins of immunoglobulins, both of which demonstrate anti-influenza activity? The increase in sialic acid levels in human sera as a result of infections, malignancies, and autoimmune diseases, and in dogs with canine distemper (Engen, 1971), could be accounted for by the primary response antibody IgM (Burnet, 1970), which contains 10-12% carbohydrate (Edelman and Gall, 1969; Spiegelberg et al., 1970). However, it could be possible that as a result of infection with, in this case, influenza viruses, glycoprotein inhibitors are the precursor components produced in an immune response, offering some protection against the infecting virus,

[1]Paper presented at Can. NRC Symposium "Biomembranes" (1971).

until the primary antibody IgM is mobilized, and gradually decline on the production of IgG. Do the inhibitors bear any relation to the carbohydrate moiety of immunoglobulins? Could they be intimately involved in activation of gamma-globulins to the active antibody molecules? An investigation into this question could throw some light on the significance of serum inhibitors against influenza and other viruses.

Serum surveys such as this one continue the recommended surveillance of animal populations for influenza virus infections (see Review, Influenza in Animals), and the information gained by these studies may be added to general knowledge regarding the epidemiology of influenza, contributing towards resolving the mechanism by which influenza viruses originate and spread in human and animal populations.

The critical evaluation of the epidemiological study in this thesis in Part II, depended upon charactrization and definition of the immunoprecipitin reactions in cellulose acetate revealed by the investigations undertaken in Part I. These included:

1. The differentiation of virus-specific from host-sepcific immunoprecipitin reactions.

2. The differentiation of virus antigen precipitation caused by antibody from that caused by inhibitor, particularly apparent in the case of the influenza A major type-specific antigen. This fact was reinforced in the second part by demonstrating that the major type-specific component was precipitated by a human 7S gamma globulin. Therefore, the precipitins in normal sera precipitating this component were thus proven to be anti-influenza antibody and not non-specific inhibitor.

3. The comparison of virus specific immunoprecipitin reactions

formed when virus antigens were diffused against normal sera with those formed when virus antigens were diffused against the sera of animals immune to influenza was a valuable aid in establishing the identity of anti-influenza antibodies in normal sera. A similar comparison of HAI reactions was also helpful.

Although the investigations in Part I were carried further than originally intended, they fully complemented and were essential for valid interpretations of the epidemiological studies to be made, and must be continued.

CONCLUSIONS

1. Established antigenic relationships among influenza A viruses were confirmed by immunoprecipitin tests in cellulose acetate, and new relationships were revealed (Fig. 8). The influenza A major type-specific component was demonstrated to be antigenically heterogeneous. (Part I, pp 171-216)

2. Because of its strength and heterogeneity in immunoprecipitin reactions, the influenza A major type-specific component is thought to be the membrane protein ('M') of the virus rather than the ribonucleoprotein. (Part I, pp 243-248)

3. Sodium dodecyl sulfate and sodium deoxycholate should not be used to disrupt virus particles for diffusion in cellulose acetate, because non-specific precipitation of serum and virus components by these surfactants interferes with immunoprecipitin reactions. (Part I pp 144-148)

4. Non-specific inhibitors in both normal animal sera, and in the sera of animals immunized against influenza, weakly precipitate at least one influenza A virus component. Precipitins which are eliminated by <u>V. cholerae</u> filtrate RDE, sodium metaperiodate, or trypsin-heat-periodate are tentatively identified as inhibitors. The influenza A major type-specific component is not precipitated by inhibitors, but by a 7 S gamma globulin. (Part I, pp 134-143; Part II, 345-350, 359-361)

5. In both immunoprecipitin and hemagglutination inhibition tests, the treatment required to eliminate non-specific inhibitors of influenza A viruses from normal sera varies according to the animal species and virus subtype. (Part II, pp 338-350)

6. Only a few individual sera from fourteen animal species examined in the Ottawa area possessed precipitins which could be identified as anti-influenza antibodies. Therefore, a reservoir of human influenza virus does not exist in these animals. (Part II, pp 370-380, 385-390)
7. The natural transmission of influenza viruses between animal and man must be demonstrated in order to establish that a reservoir for human influenza exists in animals. Surveys such as the one reported in this thesis are required to indicate which animal species could be potential reservoirs.

SUGGESTIONS FOR FUTURE WORK

Suggestions for future work have been indicated in the discussion of each phase of this study. The most pertinent suggestions are:

1. To continue the characterization of components entering into immunoprecipitin reactions in cellulose acetate immunodiffusion. The components to be studied would include: a) antigens originating from influenza A viruses and from host material, and b) serum precipitins, which may be specific anti-influenza antibodies or non-specific inhibitors of influenza viruses. The inclusion of recombinant influenza A strains with segregated antigenic components such as hemagglutinin and neuraminidase will be a necessity.

   a. Antigens Pure antigenic components from each virus strain should be isolated by disruption of purified whole virus particles and subsequent fractionation. Disrupting agents employed should have mild, but efficient action, and not cause non-specific precipitation of either virus or serum components in immunoprecipitin tests, and include such agents as Triton N 101 or bromelain. Disrupted virus particles could be fractionated by : electrophoresis in polyacrylamide gels or in cellulose acetate, by isoelectric focusing, or by centrifugation in density gradients (for example, sucrose, guanidine hydrochloride, dextran sulfate).

   Each isolated component should be characterized

   i. Biologically, for hemagglutinin activity, neuraminidase activity, infectivity, and protein content.

   ii. Morphologically, by electron microscopy.

   iii. Immunologically, by HAI, NAI, CF (type- and strain-specific),

VN, and immunoprecipitin tests, using monospecific reference sera (recommended by the WHO Committee on Influenza Virus Nomenclature, 1971). The immunological characterization of those influenza A antigens for which the biological activity has not been established will depend on immunoprecipitin tests.

Isolated host antigen components can be identified in immunoprecipitin tests by similar methods used for identifying virus components.

b. Antisera Monospecific antisera should be prepared against each isolated virus component and used to characterize the component in immunoprecipitin and other serological tests (see iii. above). Fractionation of monospecific sera is necessary to isolate precipitins to determine to which class of immunoglobulin (IgG, IgA, IgM), or to which type of inhibitor (α, β, or γ), each belongs. The demonstration of precipitating activity in the IgG antibody fraction is most important. Fractionation of normal animal sera precipitating influenza A antigens, and of anti-influenza antisera, should be carried out for the same purpose.

The interactions of isolated viral antigen components and isolated serum precipitins in immunoprecipitin tests will make it possible to identify each reacting component, antigen and precipitin, and thus to establish reference immunoprecipitin reactions which can be used to identify these components in: 1) virus particle and soluble antigens; and 2) normal and immune human, animal, and avian sera.

2. When reference reactions characterizing each antigen and serum precipitin component are established, the following may be resolved:

   a. Unequivocal differentiation of host- and virus-specific components precipitated in immunoprecipitin tests by serum precipitins.

b. The identity of the major type-specific component precipitated in immunoprecipitin tests. Is the component 'M' protein, or the RNP complex? The identity of the same component in complement fixation tests.

c. The identity of the minor type-specific components precipitated in immunoprecipitin tests. Are the components the RNP complex, or other influenza virus antigens? The use of autoradiography would be advantageous in both (b) and (c).

d. The identity of other antigenic components such as the hemagglutinin and neuraminidase in immunoprecipitin reactions using virus particle antigens.

e. The nature of the specificity of the major and minor type-specific antigens of influenza A viruses. Immunoprecipitin cross reactions between isolated 'M' protein of various influenza A strains using monospecific sera, should be examined and compared with the results of the present study to determine if the type-specific antigens are also strain-specific. A similar comparison should also be carried out with isolated RNP components.

f. The identity of specific influenza A antigens that cross react in immunoprecipitin tests. This will clearly reveal the antigenic relationships among influenza A viruses.

g. Which precipitins in 'normal' and in immune animal sera are specific anti-influenza antibodies and which are non-specific inhibitors of influenza virus antigens.

h. The identity of virus components precipitated by normal serum precipitins. This may indicate the most efficient antigen(s) for detecting anti-influenza antibodies in serum surveys employing the immunoprecipitin test.

3. To extend the survey for antibodies to influenza A viruses in the sera of 'normal'animals, using virus particle antigens in conjunction with the reference reactions described above. A larger sampling of the animal species already examined should be continued and new species, both domestic and wild, added. Strains of influenza A viruses, such as A/Equi 2 and A/Turkey, should be included in the antigenic spectrum.
4. To determine if a relationship exists between serum glycoprotein inhibitors and the glycoprotein moiety of immunoglobulins. The effect that inhibitor inactivation treatments have on immunoglobulin glycoproteins could be included.

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A P P E N D I X   I

APPENDIX TABLE 1 Differential centrifugation of virus concentrates

Virus	Batch No.	C_1					C_2				
		Rotor	RPM	"g"	Min	^oC	Rotor	RPM	"g"	Min	^oC
Infl. A/PR8	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	2	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	4	21	18,000	32,600	90	10	21	18,000	32,600	110	10
	5	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	6	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	7	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	8	21	19,000	36,300	120	10	21	19,000	36,300	120	10
	9	21	18,900	35,900	150	10	21	19,000	36,300	150	10
	10	21	19,000	36,300	180	10	21	19,000	36,300	180	10
Infl. A/FM1/Can/53	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	2	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	3	21	18,000	32,600	90	10	21	18,000	32,600	110	10
	4	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	5	21	19,000	36,300	120	10	21	19,000	36,300	150	10
	6	21	19,000	36,300	180	10	21	19,000	36,300	180	10
Infl. A2/Can/57	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	4	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	5	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	6	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	7	21	19,000	36,300	180	10	50	45,000	122,200	60	10
	4-5 $\frac{C2}{2}$										

TABLE 1 Continued

Virus	Batch No.	C_1					C_2				
		Rotor	RPM	"g"	Min	°C	Rotor	RPM	"g"	Min	°C
Infl. A/Equi 1	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	2	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	3	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	4	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	5	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	6	21	19,000	36,300	120	10	21	19,000	36,300	120	10
	7	21	19,000	36,300	180	10	50	45,000	122,200	75	10
	4+5 $\frac{C_2}{2}$						21	19,000	36,300	120	10
Infl. A2/HK/1/68	1	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	2	21	18,900	35,900	120	10	21	18,900	35,900	120	10
	3	21	19,000	36,300	180	10	21	19,000	36,300	180	10
	4	21	19,000	36,300	180	10	21	19,000	36,300	210	10
	5	21	19,000	36,300	180	10	SW41	38,000	174,000	60	10
	6	21	19,000	36,300	180	10	SW41	38,000	174,000	50	10
	7	21	19,000	36,300	180	10	50	45,000	122,200	60	10
	8	21	19,000	36,300	180	10	SW41	38,000	174,000	60	10
	9										
	10										
	11	21	19,000	36,300	180	10	SW41	38,000	174,000	60	10
	12	21	19,000	36,300	180	10	SW41	38,000	174,000	50	10
Infl. A2/Ottawa/68	1	21	18,000		120	10	21	18,000		110	10

TABLE 1 Continued

Virus	Batch No.	C_1					C_2				
		Rotor	RPM	"g"	Min	°C	Rotor	RPM	"g"	Min	°C
Infl. A/Swine	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	2	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	3	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	4	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	5	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	6	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	7	21	19,000	36,300	120	10	21	19,000	36,300	120	10
	8	21	19,000	36,300	180	10	21	19,000	36,300	180	10
Infl. A/Duck	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	2	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	3	21	19,000	36,300	120	10	21	19,000	36,300	120	10
	4	21	19,000	36,300	180	10	21	19,000	36,300	120	10
	5	21	19,000	36,300	180	10	21	19,000	36,300	180	10
Infl. B/Can/5/66	1	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	2	21	19,000	36,300	180	10	21	19,000	36,300	180	10
Parainfl. Sendai	1	21	18,000	32,600	90	10	21	18,000	32,600	90	10
	2	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	3	21	19,000	36,300	210	10	21	19,000	36,300	210	10
	4	21	19,000	36,300	180	10	50	45,000	122,200	60'	10

TABLE 1 Continued

Virus	Batch No.	C_1					C_2				
		Rotor	RPM	"g"	Min	°C	Rotor	RPM	"g"	Min	°C
Canine Distemper Virus (Rerun)	1	21	19,000	36,300	180	10	SW41	38,000	174,000	45'	10
		21	19,000	36,300	16 hr	10	SW41	38,000	174,000	60'	10
Antigen											
N-ALF	1	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	2	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	3	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	4	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	5	21	18,000	32,600	110	10	21	18,000	32,600	110	10
	6	21	19,000	36,300	180	10	21	19,000	36,300	180	10

"g" centrifugal force is rounded off to nearest hundred.

C_1 virus concentrated 10 x by differential centrifugation

C_2 " " 100x " " " (A2/Can/57, A2/Hong Kong/1/68, A/Equi 1, and N-ALF batches were concentrated 200x, underlined in Appendix Table 2)

APPENDIX TABLE 2 LEGEND

* HAU determined by microtitration (Sever, 1962), all others by macro method in tubes or WHO perspex plates.

ALF : virus infected allantoic fluid or normal allantoic fluid (N-ALF).

C_1 : virus concentrated 10x

SNF_1 : supernatant fluid from C_1

C_2 : virus concentrated 100x; underlined figures are C_2', virus concentrated 200x

SNF_2 : supernatant fluid from C_2

SDG : virus purified by sucrose density gradient centrifugation

SDG/SNF: supernatant fluid from SDG

KTDG/SNF: virus purified by potassium tartrate density gradient centrifugation following SDG purification

APPENDIX TABLE 2 Hemagglutination Units (HAU) per ml of influenza A strains, influenza B, parainfluenza Sendai, and N-ALF preparations

Virus	Batch No.	HAU/ml								
		ALF	C_1	SNF_1	C_2	SNF_2	SDG	SDG/SNF	KTDG	KTDG SNF
Infl. A/PR8	1	$6.4x10^3$	$8x10^3$	$4x10^2$	$6.4x10^4$	$4x10^2$				
	4	$6.4x10^3$	$1.6x10^4$	*$1.28x10^3$	$3.2x10^4$	ND				
		*$1.28x10^4$								
	5	$1.28x10^4$	$4x10^3$	$9.6x10^3$						
	6	$1.6x10^3$	$8x10^3$	$2x10^2$	$3.2x10^4$	$1x10^2$				
		*$6.4x10^3$								
	7	$6.4x10^3$	$3.2x10^4$	$2x10^2$	$3.2x10^4$	$2x10^2$	*$5.12x10^5$	*$8x10^2$		
		*$1.28x10^4$			*$1.28x10^5$					
	8	*$2.56x10^4$	*$6.4x10^5$	*$1.6x10^3$	*$2.48x10^6$	*$3.2x10^3$				
	9	*$1.28x10^4$	*$3.2x10^4$	*$8x10^2$	*$1.024x10^6$	*$1.6x10^3$	*$1.024x10^6$	*$4x10^2$	*$1.024x10^5$	*$2x10^2$
	10	*$2.56x10^4$	*$5.12x10^5$	*$1.6x10^3$	*$2.04x10^5$	$8x10^2$	*$5.12x10^4$			
	11	*$1.28x10^4$								
Infl. A/FM1/Can/53	1	$4x10^2$			$1.6x10^4$					
	3	$3.2x10^3$		$2x10^2$		*$2x10^2$				
	4	$8x10^2$	$8x10^3$	$2x10^2$	*$3.2x10^4$	$2x10^2$	*$3.2x10^4$	*2x10		
	5	*$6.4x10^3$	*$2x10^3$	*$1.6x10^3$	*$5.12x10^5$	*$8x10^2$	*$1.6x10^4$	*$2x10^4$	*Nil	* 2
	6	*$6.4x10^3$	*$4x10^2$	*$6.4x10^4$	*$2x10^1$	*$1.28x10^5$				
	4-6 Pool (milipore filtered)				*$3.2x10^4$		*$4x10^3$	* $2x10^2$		

TABLE 2 Continued

Virus	Batch No.	HAU/ml								
		ALF	C_1	SNF_1	C_2	SNF_2	SDG	SDG/SNF	KTDG	KTDG/SNF
Infl. A2/Can/57	1	$4x10^2$	2.5x10	8x10	$4x10^3$	$2x10^2$				
	4	$1.6x10^3$	$1.6x10^4$	*10^2	$8x10^3$	5x10				
	5	$1x10^2$	$1x10^3$	2.5x10	$8x10^3$	5x10				
	6	$1.6x10^3$	*$1.6x10^4$	$1x10^2$	*$2.56x10^5$	*$4x10^2$	*$1.6x10^4$	* $2x10^2$		
	7	*$6.4x10^3$	*$3.2x10^4$	*$8x10^2$	*$1.024x10^6$	*$1.6x10^3$	*$4.8x10^4$	*$8x10^2$	*Nil	*Nil
	4-5 Pool				*$1.6x10^4$	$1x10^2$				
Infl. A2/HK/1/68	1	*$1.6x10^3$	*$3.2x10^4$	$2x10^2$	*$6.4x10^4$	*$2.0x10^2$				
	2	*$3.2x10^3$	*$4x10^3$	*$4x10^2$	*$3.2x10^4$	*$1.6x10^3$	* $2x10^2$	*$8x10^2$		
	3	*$3.2x10^3$	*$1.6x10^4$	*$2.0x10^2$	*$2.56x10^5$	*$4x10^2$	*$1.024x10^5$	*$4x10^2$		
	4	*$3.2x10^3$	*$3.2x10^4$	*$2x10^2$	*$1.024x10^6$	*$2x10^2$	*$2.048x10^5$	* $2x10^2$	*Nil	*Nil
	5	*$3.2x10^3$								
	6	*$3.2x10^3$	*$1.6x10^4$	*$4x10^2$	*$1.28x10^5$	*$4x10^2$				
	11	*$6.4x10^3$	*$5.12x10^4$	*$4x10^2$	*$1.024x10^6$	*$4x10^2$				
	12	*$3.2x10^3$	*$1.28x10^4$	ND	*$1.28x10^5$	ND				
	7	*$3.2x10^3$								
	8	*$1.6x10^3$								
	9	*$3.2x10^3$								
	10	*$6.4x10^3$								

TABLE 2 Continued

Virus	Batch No.	HAU/ml								
		ALF	C_1	SNF_1	C_2	SNF_2	SDG	SOG-SNF	KTDG	KTDG/SNF
Infl. A/Equi 1	1	8x10^{2}								
	2	4x10^{2}	1x10^{3}	4x10^{2}	8x10^{3}	8x10^{2}				
	3	4x10^{2}	1x10^{3}	5x10	4x10^{3}	1x10^{2}				
	4	3.2x10^{3}		*1.6x10^{2})	pooled	*3.2x10^{2}				
	5	8x10^{2}	*1.28x10^{4}	*6.4x10^{2})		*1.6x10^{2}				
	4-5 Pool				*1.6x10^{4}	*1.6x10^{2} (SNF3)	*4x10^{3}			
	6	2x10^{2}	1x10^{3}	5x10	8x10^{3}	5x10				
	7	*3.2x10^{3}	*3.2x10^{4}	*4x10^{2}	*5.12x10^{5}	*1.6x10^{3}	*3.2x10^{4}	* 2x10^{2}	*Nil	*Nil
Infl. A/Swine	1	3.2x10^{3}	4x10^{3}		3.2x10^{4}	5x10				
	3	3.2x10^{3}	1.6x10^{4}	8x10^{2}	3.2x10^{5}	3.2x10^{3}				
	5	1.6x10^{3}	4.0x10^{3}	1x10^{2}	3.2x10^{4}	2x10^{2}				
	6	8x10^{2}	2x10^{3}	5x10	3.2x10^{4}	5x10				
	7	*6.4x10^{3}	*6.4x10^{4}	*4x10^{2}	*2.56x10^{5}	*4x10^{2}	*5.12x10^{5}	8x10^{2}	*2.56x10^{4}	*2x10^{2}
	8	*2.56x10^{4}			*5.12x10^{5}					
Infl. A/Duck	1	1.28x10^{4}		2x10^{2}	6.4x10^{4}	2x10^{2}				
	2	1.6x10^{3}	4x10^{3}	4x10^{2}	3.2x10^{4}	2x10^{2}				
	3	3.2x10^{3}	8x10^{2}	4x10^{2}	1.28x10^{5}	1x10^{2}	*6.4x10^{4}	* 2x10^{2}	*3.2x10^{4}	* 2x10^{2}
	4	*6.4x10^{3}	*3.2x10^{4}	*4x10^{2}	*5.12x10^{5}	*2x10^{2}	*2.56x10^{5}	*1.6x10^{3}	*1.024x10^{5}	*4x10^{2}
	5	*1.28.10^{4}	*5.12x10^{5}	*4x10^{2}	*2.048x10^{6}	*8x10^{2}	*1.024x10^{6}	*1.6x10^{3}		

TABLE 2 Continued

Virus	Batch No.	HAU/ml								
		ALF	C_1	SNF_1	C_2	SNF_2	SDG	SOG-SNF	KTDG	KTDG/SNF
Infl. B/Can/5/66	1	$3.2x10^3$	$1.28x10^4$	$2x10^2$	*$1.28x10^5$	*$2x10^2$	*$1.28x10^4$	*$8x10^2$	*Nil	*Nil
	2	*$6.4x10^3$		*$1.6x10^2$	*$3.84x10^5$					
Infl. A/Ottawa/68	1	5x10	$2x10^3$	$2x10^2$	$8x10^3$	5x10				
Parainfl. Sendai	1	$8x10^2$	*$1.6x10^2$	* $2x10^2$	$4x10^3$	* $2x10^2$				
	2	$2x10^2$	$1x10^3$	5x10	*$8x10^3$	5x10	*Nil	*Nil	ND	ND
	3	$8x10^2$	*$3.2x10^4$	* $2x10^2$	*$2.56x10^5$	* $2x10^2$				
	4	*$1.28x10^4$	*$1.28x10^4$	*4x10	*$2.56x10^5$	* $2x10^2$				
Infl. A/Melbourne	1	*$8.19x10^4$								
N-ALF	1	-	-	-	-	-	*Nil	*Nil		
	2	* -	* -	* -	* -	* -				
	3	* -	* -	* -	* -	* -				
	4	* -	* -	* -	* -	* -				
	5	* -	* -	* -	* -	* -				

APPENDIX TABLE 3 Infectivity (EID_{50}) per ml of influenza A strains, influenza B, and parainfluenza Sendai preparations

Virus	Batch No.	ALF	$\log_{10}$ EID_{50}/ml C_2	SDG	KTDG
Infl. A/PR8	5	8.2			
	6	9.0			
	7	7.7	7.8	11.2	
	8		10.2		
	9	10.0	11.5	11.0	7.5
Infl. A/FM1/Can/53	5	7.8	11.0	[a]6.7 8.7	4.3
	6	9.8			
	[b]4 & 6		8.0		
Infl. A2/Can/57	6	8.5	*8.8	5.0	4.0
	7	9.0	*9.5	7.5	
Infl. A2/HK/1/68	1	8.0	*8.7	8.0	Nil
	3	8.5	*9.5	7.5	Nil
	4	7.6			
Infl. A/Equi 1	1	8.9	*8.0	4.9	
	4-6	7.0	*9.8	9.5	4.2
	7	8.8			
Infl. A/Swine	4	8.0			
	6	8.8	10.3	8.7	6.3
	7	9.3			
Infl. A/Duck	2	8.2	9.3	9.2	4.7
	3	9.3	10.3	10.3	3.3
	4	10.6	9.0	9.5	
	5	9.5			
Infl. B/Can/5/66	1	5.5	5.3	3.5	Nil
Parainfl. Sendai	3	8.5	*8.2	5.0	
CDV		TCD50=5			

Legend

EID_{50} : infectivity titre of virus in ovo determined by Reed and Muench (1938)

ALF : virus infected allantoic fluid

C_2 : virus concentrated 100x; *virus concentrated 200x

SDG : sucrose density gradient purified concentrates

KTDG : potassium tartrate density gradient purified concentrates

a 6.7 is value of diffuse band, 8.7 is value of dense band

b Pooled batches were millipore filtered

APPENDIX TABLE 4 LEGEND

Protein determinations done according to Lowry *et al.* (1951)

ALF : virus infected allantoic fluid, or normal ALF

C_2 : virus or N-ALF concentrated 100x; *200x; **500x

SDG : virus or N-ALF sucrose density gradient purified and concentrated

KTDG : virus or N-ALF potassium tartrate density gradient purified and concentrated

[a] 0.03 is value of diffuse band, 0.15 of the dense band

[b] Pooled batches were millipore filtered

APPENDIX TABLE 4 Protein content (mg/ml) of influenza A strains, influenza B, parainfluenza Sendai, CDV, and N-ALF preparations

Material	Batch No.	mg Protein/ml (Lowry, 1951)			
		ALF	C_2	SDG	KTDG
Infl. A/PR8	7	0.94	1.7		
	8		1.45		
	9	0.96	1.88	2.33	0.23
	10		2.0		
Infl. A/FM1/Can/53	3		0.34		
	4	0.90	0.63	[a] 0.03, 0.15	0.03
	5	0.96	0.48		
	6		1.44		
	[b] 4 & 6		0.33		
Infl. A2/Can/57	4		0.23		
	4&5		*0.24		
	6	1.16	*0.78	0.51	0.01
	7	1.56	*1.23	0.27	
Infl. A2/Ottawa/68	1		0.32		
Infl. A2/HK/1/68	1		0.22		
	3	2.08	*0.91	0.01	0.01
	4	1.98	*1.44	0.17	
Infl. A/Equi 1	3		0.12	0.2	
	4-6		*0.44		
	5	1.58	*0.9	0.01	0.01
	7	1.44			
Infl. A/Swine	6	0.93	0.83	0.47	0.23
	7	1.14			
Infl. A/Duck	2		0.95	1.75	
	3	1.74	1.5	0.9	0.17
	4	1.52	2.05	1.0	
	5	1.46	0.75		
Infl. B/Can/5/66	1	1.55	1.5	0.35	0.03
Parainfl. Sendai	1		0.05		
	2		*0.49	0.01	
	3	0.74	*0.63		
	4		*4.7		
CDV	1		**0.05		
N-ALF	2	1.20	0.05	0.21	
	3	0.84	0.16		
	4		0.16		

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APPENDIX TABLES 5 A - I. Serological testing of normal sera by hemagglutination inhibition (HAI), complement fixation (CF), and immunodiffusion (IDD).

Legend

Inhibitor inactivation treatments of sera for HAI:

RDE: receptor destroying enzyme as crude V. cholerae filtrate (@ 500 units/ml for most sera) and $58^{\circ}C \times 1$ hr.

THP: trypsin, heat ($58-60^{\circ}C \times 1$ hr), M/90 KIO_4

$NaIO_4$: M/90 $NaIO_4$

Kaolin: 25% suspension

S: group complement fixing antigen

V: strain-specific complement fixing antigen

All HAI and CF titres are expressed as reciprocal of dilution

- for HAI: titre of 1:5

- for CF: titre of 1:1

- for IDD: no visible precipitin band formed with virus

+ for IDD: one precipitin band formed, $+_2$:2 precipitin bands formed, etc.

All virus strains listed are influenza A except parainfluenza Sendai and canine distemper virus (CDV).

ac: anti-complementary

na: not applicable because of lack of serum

NS: non-specific reaction

Blank spaces: tests not done

TABLE 5A

Serum Type	Serum No.	HAI A2/HK/1/68 RDE	HAI A2/HK/1/68 THP	HAI A2/HK/1/68 $NaIO_4$	HAI A2/HK/1/68 Kaolin	HAI A/PR8 RDE	HAI A/PR8 THP	HAI A/PR8 $NaIO_4$	HAI Other infl. A $NaIO_4$	CF A2/HK/68 S	CF A2/HK/68 V	IDD PR8	IDD A2HK68	IDD Duck
Equine (NES)	*1	480	320	na	192		-			-	-	-	+	-
	2	40	120	na	48		-			NS	NS	-	+	-
	*3	160	120	-	192		-	-	-Equi	-	-	-	+	-
	*4	160	320	20	72		-	-	-Equi	1	-	-	+	+
	*5	320	80	10	96		-	-	-Equi	-	-	-	+	+
Horse (Equine) -Globulin (20 mg/ml)		10		15		-		-	10 Equi	NS	NS	+	+	+
									THP					
Bovine (NBS)	*24	10	10		-		5		A/Duck-	-	-	+	+	+
	*32	5	15		-		5		"	-	-	$+^{2}$	+	+
	*82	5	20		-		-		"	-	-	-	+	+
	* 2	-					-		"	-	-	-	-	-
	13	5					-		"	-	-	-	+	$+^{2}$
	48	-					-		"	-	-	-	+	+
Bovine γ-Globulin (20 mg/ml)		-		-		-				1	1	-	-	-
Foetal Calf (FCS)		- (Native sera 12) (@ 58°Cx1 hr 4)		40	-	- (Native sera 4) (@ 58°Cx1 hr -)				-	-	-	+	-

TABLE 5B

Serum Type	Serum No.	HAI						CF		IDD					
		A2/HK/1/68				A/PR8		A2/HK/68		PR8	A2HK68	FM1	Equi	Swine	Duck
		RDE	THP	$NaIO_4$	Kaolin	THP	$NaIO_4$	S	V						
Cat (NCS)	1	-	40		12	-				-	+	-	-	-	-
	2	10	160		24	-				-	+	-	-	-	-
	4	5	60		24	-				-	-	-	-	-	+
	*5	-	40		48	-				-	+	-	-	-	-
	H9	-	80			-				-	+	-	-	-	-
	H10	-	40			-				-	+	-	-	-	-
	H11	-	160	10	12	5	-	-	-	+	-	-	-	-	-
	H12	-	40			-				-	-	-	-	-	-
	H13	5	80			-				-	+	-	-	-	-
	*H19	10	10	10	18	-	-	-	1	-	+	-	-	-	-
	H20	-	20		12	-				-	+	-	-	-	-
	H28	5	40			-				-	+	-	-	-	-
	H29	-	40			-				-	-	-	-	-	-
	H30	-	40			-				-	+	-	-	-	-
	H31	-	20			-				-	+	-	-	-	-
	H32	-	20			-				-	+	-	-	-	-
	H33	-	10			-				-	+	-	-	-	-
	H34	-	80		12	-				-	-	-	-	-	-
	*H37	-	80	5-10	12	-	-	-	-	-	+[2]	-	+	-	-
	*H38	-	80	10	12	-	-	-	-	-	+[2]	-	+	-	-
	H39	-	-			-				-	+	-	+	+	-
	H40	-	40			-				-	+	-	+	+	-
	H41	-	-			-				-	+	-	-	+	-
	*H43	-	10	5-10	24	-	-	-	-	+	+[3]	-	-	+	-
	H47	-	160		24	-				+	+	-	-	+	-
	*H27	60	30	40		-	-	1	2	-	+	+	-	-	+
	H35		-			-		-	-	-	-	-	-	-	-

TABLE 5C

Normal Serum Type	Serum No.	HAI							CF		IDD					
		A2/HK/1/68				A/PR8		Other infl. A	A2/HK/68		PR8	A2HK68	FM1	Swine	Duck	Other infl. A etc
		RDE	THP	$NaIO_4$	Kaolin	THP	$NaIO_4$	THP	S	V						
Dog (NDS)	*OU 7	-	40		12	10					+	+	-	-	-	-
	OU 9	-	30		24	5					+	+	-	+	-	-
	*OU 22	-	20	-	6	-	-		NS	NS	+	+	-	-	+	-
	*OU 1	-	-	-		-			1	8	+	+	-	-	+	-
	*OU 86	-	20	10		-			NS	8	+	+	-	+	+	-
	*OU 57	10	10	20		-			-	2	+	+	-	-	-	-
	H35	-	160		6	-					+	+	-	-	-	-
	*H36	80		40	na	-			-	20	+	+[2]	+	+	+	+A2/Can/57
	H37	-	10			-					+	-	-	-	-	-
	H38	5	30			-					+	-	-	-	-	-
	H39	-	60			-					+	+	-	-	-	-
	H40	-	20			5					+	-	-	-	-	-
	H41	-	20			-					+	+	-	-	-	-
	H42	-	20			-					+	+	-	-	-	-
	H43	10	30			-					+	+	-	-	-	-
	H44	-	20			-					+	+	-	-	-	-
	H45	5	80			40		-Swine			+	+	-	+	-	-
	H46	10	80			5		" "			+	+	+	+	-	-
	H47	20	60			-	-	-Swine, Duck			+	+	+	+	+	+A2/Can/57, Equi
	*H48	20	160	20	12	10		-Swine	-	4	+	+	-	+	-	-
	H49	-	40			-		"			+	+	-	+	-	-
	H50	-	120		18	-		"			+	+	-	+	-	-
	H51	-	120		12	-		"			+	+	-	+	-	-
	*H52	-	120		-	-		10 Swine			+	+	-	+	-	+CDV
	H53	-	80			-					+	+	-	+	-	-
	H54	-	120			-					+	+	-	+	-	-
	H55	-	80			10					+	+	-	-	-	-
	*H62	-	20	-	18	-	-		NS	NS	+	+	+	-	+	-
	*H68	-	160	-	12	-	-		-	-	+	+	-	-	-	-
Dog γ-Globulin (20 mg/ml)		-				-			ac	ac	-	-	-	-	-	-

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TABLE 5D

Serum Type	Serum No.	HAI						Indirect CF		IDD			
		A2/HK/1/68			A/PR8			A2/HK/68		PR8	A2HK68	FM1	Other infl. A
		RDE	THP	$NaIO_4$	RDE	THP	$NaIO_4$	S	V				
Fowl (NFS)	104	-			-			-	16	-	-	-	-
	*109	20			-			16	10	-	+[3]	-	-
	123	10			15	10		16	16	-	-	-	-
	128	-			-			-	-	-	+[2]	-	-
	150	10			-			16	16	-	+	-	-
	105	40			-			16	16	-	+	-	-
	*118	20			-			16	16	-	+[2]	-	-
	*115	20		10	-		-	16	-	-	+[2]	-	-
	*R2	20		-	-		-	-	16	-	+	-	-
	*R4	10			-			16	16	-	+	-	+A2/Can/57
	R5	-			-			16	16	+	-	-	-
	R6	-			-			16	16	+	-	-	-
	R7	-			-			16	16	+[2]	+	-	-
	*R8	10	20		-	-		16	16	-	-	-	-
	R10	-			-			16	16	+	-	-	-
	R13	-	15		-	-		16	16	-	-	+	-
	R14	5	80		-	-		16	16	-	-	+	-
	*R15	-	20		-	-		16	16	-	-	-	+Swine
	R17	-	15		-	-		16	16	-	-	-	-

TABLE 5E

Serum Type	Serum No.	HAI							CF		IDD			
		A2/HK/1/68			A/PR8		Duck	A2Can57	A2HK68		PR8	A2HK68	A2Can57	Duck
		RDE	THP	Kaolin	RDE	THP	RDE	$NaIO_4$	S	V				
Goat (NGS)	*1	20	20	48		30	-		-	-	+	+	-	$+_2$
	*2	10	-	-		40	-	-	-	-	$+_2$	+	+	+
	3	-	-			-	-		-	-	+	+	+	+
	4	-	-			-	-	-	-	-	-	-	+	$+_2$
	5	10	5			-	-	-	-	-	+	-	-	$+_2$
	6	10	10			-	-		-	-	+	-	-	+
	*7	10	10			-	-	-	-	-	+	+	+	+
	8	5			-		-	-	-	-				
	9	-			-		-	-	-	-				

TABLE 5F

Normal Serum Type	Serum No.	HAI A2/HK/1/68 RDE	HAI A2/HK/1/68 THP	HAI A2/HK/1/68 $NaIO_4$	HAI Kaolin	HAI A/PR8 RDE	HAI A/PR8 THP	HAI A/PR8 $NaIO_4$	CF A2/HK/1/68 S	CF A2/HK/1/68 V	IDD PR8	IDD A2HK68	IDD Swine	IDD Other infl. A etc.
Rabbit (NRS)	*127	20	160	20	36	-	-		-	2	+	+2	-	-
	133	15	160		18	-	5		-	-	+	+2	-	-
	148	10	320		18	-	5				+	+	-	-
	149	10	320		12	-	-				+	+	-	-
	150	20	320		24	-	10				+	+2	-	-
	152	5	320			-	10				+	+	-	-
	153	10	160			-	5				+	+	-	-
	154	10	160			-	10				+	+	-	+ Sendai
	155	10	160			-	-				+	+2	-	-
	156	5	160			-	5				+	+2	-	-
	*157	20	640	40	24	-	-	-	-	-	+	+2	-	-
	158	5	240			-	5				+	+	-	-
	*159	20	320		12	-	10				+	+2	-	-
	*160	20	160	20	24	10	10	-	2	2	+	+2	-	-
	*A5	10	1280	40	24	-	20	-	1	4	-	+	-	-
	*A19	20	3840	40	96	-	-		2	4	-	+	-	+ Equi, Duck
	A20	10	1920		18	-	-				+	+	-	-
	*A21	5	240		96	-	-				+	+	-	-
	*A29	10	5120		24	-	20		-	-	+	+	-	-
	A30	10	5120		24	-	10				+	+	-	-
	A31	20	3840		36	-	5				+	+	-	-
	A32	10	2560		36	-	-				+	+	-	-
	A45	20	640		48	-	5				-	+	+	-
	*A46	20	640		24	-	10				+	+	+	-
	*A47	5	120	40	24	-	-	-	-	2	+	+	+	-
	A44	-	-		-		-		NS	NS	-	-	-	-
Rabbit γ-Globulin (20 mg/ml)		-	-			-			ac	ac	-	-	-	-

TABLE 5G

Normal Serum Type	Serum No.	HAI: A2/HK/1/68 RDE	HAI: A2/HK/1/68 THP	HAI: A2/HK/1/68 $NaIO_4$	HAI: A2/HK/1/68 Kaolin	HAI: A/PR8 THP	HAI: Other infl. A THP	CF: A2/HK/68 S	CF: A2/HK/68 V	IDD: PR8	IDD: A2HK68	IDD: Swine	IDD: Other infl. A
Sheep (NSS)	4	10	20			-				-	+	-	
	5	10	10			-	-Swine			-	-	+[2]	-
	6	10	5			-				-	-	-	-
	7	-	5			-				-	+	-	-
	8	10	10			-				-	+	-	-
	9	10	-			-				-	+	-	-
	10	10	10			-	-Swine			-	-	+	-
	11	5	-			-				-	-	-	-
	12	20	5		-	-		-	-	-	+	-	-
	*14	20	-		6	-	-Swine			-	+	+	-
	20	40	-		-	5				-	+	-	+ FM1 Equi
	25	10	30			-				+[2]	+	+	-
	26	-	20			-				+[2]	+	+	-
	30	10	10		6	-	-Swine, Duck	-	-	+	+	+	-
	44	10	-			-				-	+	-	-
	45	10	-			-				-	+	-	-
	46	15	20			-				+	+	-	-
	47	10	-			-				+	+	-	-
	48	40	-		-	-				-	+	-	-
	*56	10	40		6	-				+	+[2]	-	-
	61	20	40		6	-	-Duck			+	+	-	+ Duck
	*62	15	30	-	-	-	-Duck	1	8	+	+	-	+[2] Duck
	*63	20	30		-	-				+	+	-	+ Equi
	71	40	40		6	-				+	+	-	-
	*75	10	-		-	-	-Swine, Duck			+	+	+	+ FM1, Duck
	37		-			-		-	-	-	-	-	-
Sheep γ-Globulin (20 mg/ml)		-				-(RDE)		NS	NS	-	-	-	-

TABLE 5H

Normal Serum Type	Serum No.	HAI A2/HK/1/68 RDE	HAI A2/HK/1/68 THP	HAI A2/HK/1/68 $NaIO_4$	HAI A2/HK/1/68 Kaolin	HAI A/PR8 THP	CF A2/HK/1/68 S	CF A2/HK/1/68 V	IDD PR8	IDD A2HK68
Snowshoe Hare (NS_HS)	325	–	60			–			–	+
	326	–	40			–			–	+
	331	–	60			–			–	+
	*333	–	40		–	5			–	+
	334	–	80			–			–	+
	340	–	160		6	–			–	+
	*352	–	160		–	–	–	–	–	+
	353	–	60			–			–	+
	*359	10	160	20	–	–	–	–	–	+
	363	–	160		12	–			–	+
	480	–	20			–			–	+
	491	–	40			10	NS	NS	–	+
	494	–	60			5			–	+
	498	–	20			–			+	+
	510	5	80			10			–	+
	513	–	80			–			+	+
	520	–	80		6	–			–	+
	546	–	20			–			–	+
	548	–	20			–			–	+
	550	–	10			–			–	–
	561	–	10			–			–	+
	595	–	10			–			–	–
	0	–	40			–			–	+
	497		80			10	–	–	+	+
Cottontail Rabbit (NC_RS)	316	–	40			5			–	–
	355	–	15			10			–	+
	*395	–	20	60		10	–	2	–	+
	435	10	20			–	–	2	–	+
	437	–	40			10			–	–
	440	5	160		12	10			–	–
	*442	–	60		12	10			–	+
	443	5	60		–	–			–	+
	508	20	40			10			–	+
	526	–	15			–			+	+
	439	–	40			10			–	+
	396	–	20			5			–	+
	*434	5	40			10			–	+

TABLE 5I

Normal Serum Type	Serum No.	HAI A2/HK/1/68 RDE	HAI A2/HK/1/68 THP	HAI A2/HK/1/68 $NaIO_4$	HAI A2/HK/1/68 Kaolin	HAI A/PR8 THP	CF A2/HK/1/68 S	CF A2/HK/1/68 V	IDD PR8	IDD A2HK68
Groundhog (NG_HS)	284	-	20							+
	401	-	40			-	-	-	-	+
	408	-	20			-			-	+
	412	-	20			-			-	+
	416	-	20			-			-	-
	436	-	20			-			-	-
	447	-	20			-			-	-
	448	-	20			-			-	-
	449	10	160		12	-			-	+
	452	-	60			-			-	+ 2
	471	-	20			-			-	-
	477	-	40			-			-	+
	483	-	80			-			-	+
Chipmunk (NC_HS)	551	5	10			-			-	-
	562	-	20			10			-	-
	570	-	20			10			-	-
	571	10	20			5			-	-
	574	10	20			10			-	-
	*575	10	40			10	-	1	-	+
Squirrel (NS_QS)	397	-	10			-			-	-
	398	-	40			-			-	-
	399	-	20			-			-	-
	563	-	40			-			-	-
Mink (NM_KS)	559	-	40			10			-	+

APPENDIX II

Buffers

<u>Tris No. 4 for Immunodiffusion</u> (modified from Burstone, 1962)

0.2 M Tris, 0.1 M NaCl, 01.% sodium azide, pH 7.4, ion conc. 0.15.

NaCl	5.84 gm
Trizma-HCl (Sigma)	26.44
Trizma Base (Sigma)	3.88
Na Azide	1.0

Add deionized water to 1 litre.

<u>Tris No. 6 for Immunodiffusion</u> (modified from Gallagher and Voss, 1969)

0.05 M Tris, 2.0 M NaCl, 0.1% sodium azide, pH 7.4

NaCl	116.98 gm
Trizma-HCl (Sigma)	6.61
Trizma Base (Sigma)	0.97
Na Azide	1.0

Add deionized water to 1 litre.

<u>Tris-NaCl Buffer for Gel Chromatography</u> (from Duesberg and Robinson, 1965)

0.01 M Tris, 0.1 M NaCl, 0.001 M EDTA, 0.02% sodium azide, pH 7.3

Tris	1.21 gm
NaCl	5.84
EDTA	0.336
Na Azide	0.2

Add deionized water to 1 litre.

<u>STE</u> (from Pons and Hirst, 1968 a)

0.1 M NaCl, 0.001 M EDTA, 0.05 M Tris-HCl, pH 7.2-7.3

NaCl	5.84 gm
Trizma-HCl (Sigma)	13.22
Trizma Base (Sigma)	1.94
EDTA	0.3362

Add deionized water to 1 litre.

Phosphate Buffered Saline (PBS) (R. Siboo, personal communication)

Stock Solutions: 0.4 M Na_2HPO_4 -- 56.78 gm/l H_2O

0.4 M NaH_2PO_4 -- 55.196 gm/l H_2O

To make 0.01 M PBS, pH 7.3:

20 ml 0.4 M Na_2HPO_4

5 ml 0.4 M NaH_2PO_4

26 gm NaCl

Add deionized water to 1 litre.

Physiological Saline or n-saline

8.5 gm NaCl

Add deionized water to 1 litre.

Reagents for Polyacrylamide Gel Electrophoresis

(from Maizel, 1969)

Preparation of Reagents (numbers of reagents as in Maizel)

1. Acrylamide-bis acrylamide (30: 0.8)

Acrylamide (Eastman 5521)	30 gm
N,N'-bis-methylene acrylamide (Eastman 8383)	0.8
Deionized water	100 ml

4. Sodium dodecyl sulfate (10%)

Sodium dodecyl sulfate (BDH)	10.0 gm
Deionized H_2O	100.0 ml

5. TEMED - N,N,N', N' - tetramethylenediamine (Matheson, Coleman and Bell TX 405)

4. Ammonium persulfate (10%)

Ammonium persulfate (Fisher A-682)	1.0 gm
Deionized water	9.0 ml

(make fresh every two weeks)

10. Sodium phosphate (1 M)

Na_2HPO_4	81.0 gm
$NaH_2PO_4 \cdot H_2O$	59.3

Add deionized water to 1 litre.

To prepare resolving gels:

For 10 ml 5% gel, mix the reagents in the following order:

1.67 reagent (1)
1.00 ml reagent (10)
0.10 ml reagent (4)
0.005 ml reagent (5)
7.10 ml deionized water
0.10 ml reagent (6)

For Electrode buffer, mix:

100 ml reagent (10)
10 ml reagent (4)
890 ml deionized water

Stains

Thiazine Red R (Crowle, 1961, Appendix IV. p. 304)

Thiazine red R	0.1 gm
Acetic acid (1%)	100.0 ml

Differentiate in 70% ethanol containing 1% acetic acid.

Methyl-Green-Pyronin and Ribonuclease Method for RNA (modified from Brachet, 1942)

Stain Preparation:

Soln. A	5% aqueous pyronin	17.5 ml
	2% aqueous methyl green (chlorform washed)	10.0
	Deionized water	250.0
Soln. B	M/5 acetate buffer pH 4.8	

To use:

Mix equal volumes of Soln. A and Soln. B in Coplin jar. Mixture keeps about 1 week. Do not use any longer.

Method:

1. Bring sections to water.
2. Stain in methyl-green-pyronin solution 10 min. to 24 hrs.
3. Rinse in deionized water a few seconds.
4. Blot dry.

RNA: stains red.

Methyl-Green-Pyronin Y Method for DNA and RNA (modified from Kurnick, 1955)

Stain Preparation:

1. Make 2% aqueous solution Pyronin Y.
2. Extract with $CHCl_3$, by shaking in separatory funnel until chloroform layer becomes colorless.
3. Make up 2% solution aqueous solution methyl green.
4. Extract with $CHCl_3$ as above until chloroform layer is no longer violet above.

For use:

12.5 ml pyronin Y solution + 7.5 ml methyl green solution + 30 ml deionized water.

Stain Method;

1. Stain for 6 min. in methyl-green-pyronin.
2. Blot with filter paper.
3. Immerse in 2 changes n-butyl alcohol, 5 min. in each.
4. Immerse in Whitmore oil for 5 min.
5. Mount in oil under coverslip.

Results: RNA bright red

DNA clear green

Acridine Orange Staining of Purified Virus (modified from Mayor and Hill, 1961; Mayor, 1962)

1. Immerse cellulose acetate strip in McIlvaine's buffer at pH 4.0 for 10 mon.
2. Stain in 0.01% acridine orange in McIlvaine's buffer at pH 4.0 for 10 min., rinse in fresh buffer for 2-3 min. and mount in buffer, or dry and mount in Whitmore oil.

Results: RNA stains red

ssDNA stains red

dsDNA stains green

Acridine Orange Staining of Purified Virus (modified from Bradley's modification (1965) of Mayor and Hill (1961))

Procedure:

1. Immerse cellulose acetate strips in buffered saline for 1 min.
2. Fix in sulphosalicylic acid (200 g/l deionized water) for 1 min. or do not fix, but go directly to step 3.
3. Stain with acridine orange (1% aqueous) for 5 min. in the following modified McIlvaine's buffer:

	ml
0.1 M Citric acid	6
0.015 M Na_2HPO_4	4
1% acridine orange at pH 3.8	0.1

4. Rinse in buffer used in Step 3 without acridine orange.
5. Transfer to 0.15 M Na_2HPO_4 15 min.
6. Let dry at room temperature on blotting paper, and view under UV lamp (direct viewing is substituted for fluorescent microscope, using 257 nm wavelength UV lamp).

Results:

Note color of virus smears (or bands) on slide. If red, slides placed in 0.1 M citric acid and examined at intervals of 1, 2, and 3 min., color changes are noted.

dsDNA viruses: fluorescent green after phosphate treatment in Step 5 (if band stains green, there is no need for citric acid treatment above.)

ssRNA / ssDNA viruses: stain flame red, after phosphate treatment.

With subsequent citric acid exposure, RNA viruses do not fade over 3 min. period; but DNA viruses fade noticeably and may change to green.

dsRNA viruses may stain like dsDNA viruses.

A P P E N D I X   III

Abbreviations for Plates

The notations listed below are the most commonly encountered in most Plates. Explanation for other notations accompany relevant plates.

Antigens

Virus concentrates, semi-purified (C_2 100x, and C_2' 200x concentrated)

Influenza :

PR8 : A/PR8
FM1 : A/FM1/Can/53
A2 : A2/Can/57
HK : A2/Hong Kong/1/68
EQUI : A/Equi 1
SW : A/Swine
DUCK : A/Duck
B : B/Can/5/66

Parainfluenza :

SEND : Sendai

SDG following virus notation is virus concentrate purified by sucrose density gradient, e.g. PR8/SDG, FM1/SDG, A2/SDG, etc.

SA following virus notation is virus soluble antigen, e.g. PR8/SA, FM1/SA, A2/SA, etc.

N-ALF : normal allantoic fluid semi-purified concentrate (C_2'), i.e. normal host (CE) concentrate

N-CAM/SA : normal host (CE) soluble antigen extracted from normal CE chorioallantoic membranes

Antisera

RAs-IN : convalescent serum of rabbit intranasally infected with virus or exposed to host material

RAs-IM : immune serum of rabbit parenterally (IM) immunized by virus or host material;early stages of immunization

hRAs : hyperimmune serum of rabbit parenterally (IM) immunized with virus or host material, absorbed with N-CAM

hRAs u : hyperimmune serum of parenterally immunized rabbit, as above, but <u>not</u> absorbed with N-CAM

Abbreviations for Plates

Antisera cont'd

FAs-IM : immune serum of rooster parenterally (IM) immunized with virus or host material;early stages of immunization

hFAs : hyperimmune serum of rooster parenterally (IM) immunized with virus or host material

Infecting or immunizing antigen, virus or host respectively, follows each antiserum notation above, indicating specificity of antiserum, e.g. RAs-IN/PR8, hRAs/PR8, hFAs/N-ALF, etc.

PLATE 1

Homologous immunoprecipitin patterns formed by rabbit and rooster immune sera when diffused against virus concentrates.

Antisera in peripheral wells; antigens in central wells

NRS : normal rabbit serum; A47, A13 are code numbers
NFS : normal rooster serum; R1, R4 are animal code numbers

A. As a rabbit is progressively immunized with A2/Hong Kong/1/68, an increase in the immune response is reflected by increase and expansion of the homologous immunoprecipitin reaction. The two precipitin bands formed between virus and convalescent serum increases to ten when the serum becomes hyperimmune. A linking precipitin band (arrow 1) formed by the antigen and sera is barely detectable in normal serum, but increases in strength to a maximum in hyperimmune serum. Virus specificity of the band is determined by a linkage of identity with the virus-specific reaction between the convalescent serum and the antigen; and by no linkage (non-identity) with a host-specific reaction between anti-host antiserum and the virus antigen. Linkage with the host-specific reaction (arrow 2), identifies host-specific reactions in hyperimmune and immune sera, which are due to anti-host precipitins formed when the rabbit was parenterally immunized with egg-grown virus C_2.

B. A similar pattern evolves when a rabbit is immunized with A/Swine. The number of precipitin bands increases from one in convalescent serum to 7 in the hyperimmune serum. The precipitin in convalescent serum forming the band indicated by arrow 1, increases in strength as the animal is further immunized. A precipitin in normal serum identifies, by precipitin band linkage, with one in hyperimmune serum (arrow 2).

C. Serum from early stages (FAs-IM) of PR8 immunization of a rooster forms two precipitin bands with the virus which increase in strength in hyperimmune serum (precipitin band linkage arrowed). In addition, hyperimmune serum contains components forming 2-3 other precipitin bands with the antigen.

D. A component detectable in both normal and hyperimmune serum forms a precipitin band with A2 which links (arrow 2). This band cuts another one formed by hyperimmune serum and the virus (arrow 1), indicating non-identity of reacting components forming each band. Precipitins in hyperimmune serum form 3 precipitin bands with the virus antigen.

(Thiazine red R stain)

PLATE 1

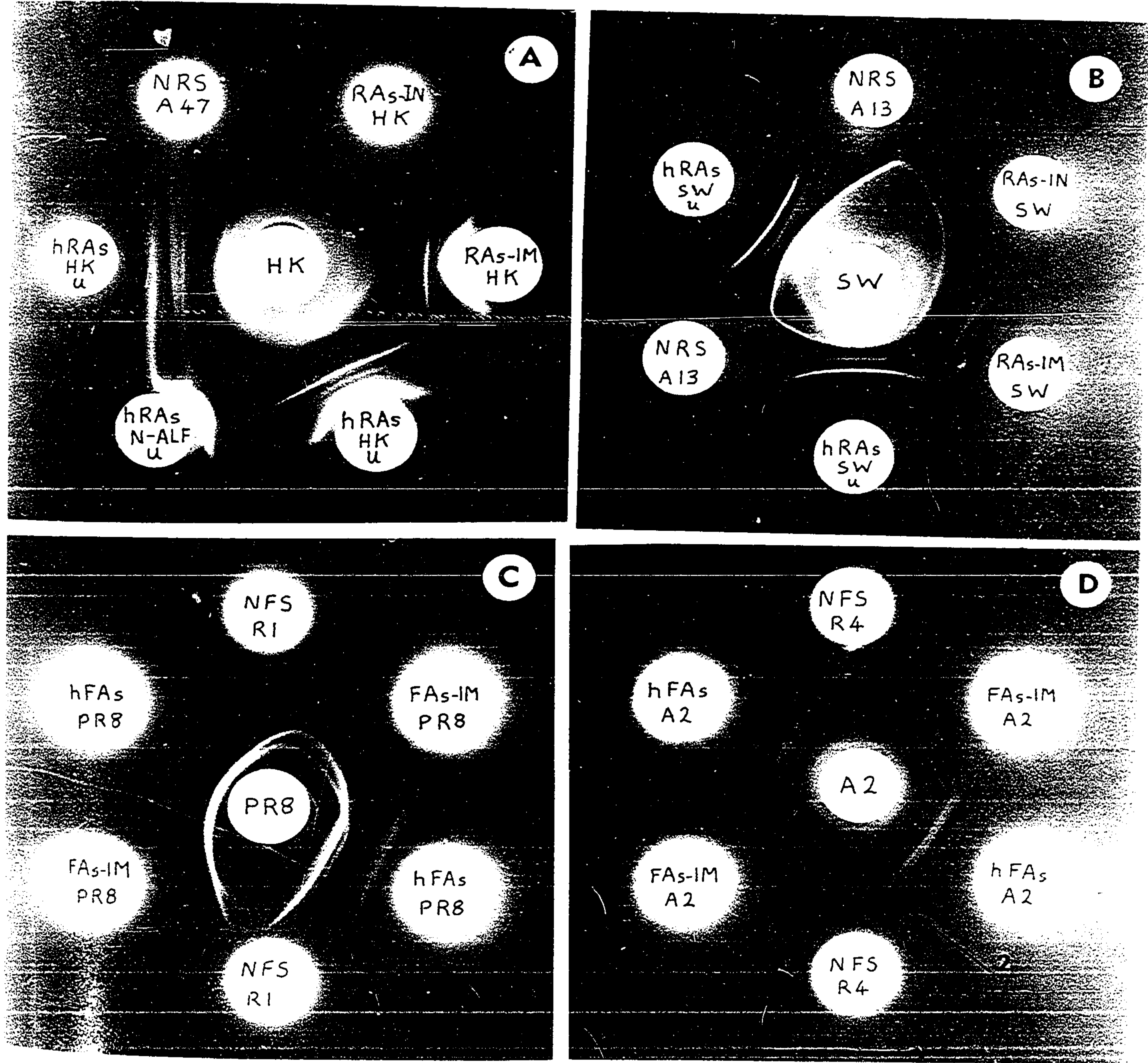


PLATE 2

Control experiments to determine host(CE)-specific and virus-specific immunoprecipitin reactions.

A. Absence of precipitin bands on diffusion of N-ALF C_2 (centre row) with virus infected convalescent rabbit sera (top row and extreme right centre) and virus immunized hyperimmune rabbit sera (absorbed with N-CAM) (bottom row and extreme left centre), indicates absence of host(CE)-specific antibodies demonstrable by use of N-ALF C_2.

B. Absence of precipitin bands on diffusion of N-CAM/SA (centre row) with the same group of rabbit sera as in (A) (RAs-IN/V - bottom row and extreme left centre; hRAs/V - top row and extreme right centre) indicates absence of host(CE)-specific antibodies which is demonstrated by use of N-CAM/SA.

C. Absence of immunoprecipitin reactions between hRAs/N-ALF (N-CAM absorbed) (top row) and various influenza A strains, influenza B, and Sendai soluble antigens (centre row) indicates the absorbed sera do not contain antibodies to host components present in virus soluble antigens. A linking precipitin band formed between hFAs/N-ALF and all virus soluble antigens but Duck, B, and Sendai suggests anti-CE antibody present in the hyperimmune rooster serum. Lack of reaction with Duck/SA rules out the virus specificity of this reaction.

(Coomassie blue stain)

PLATE 2

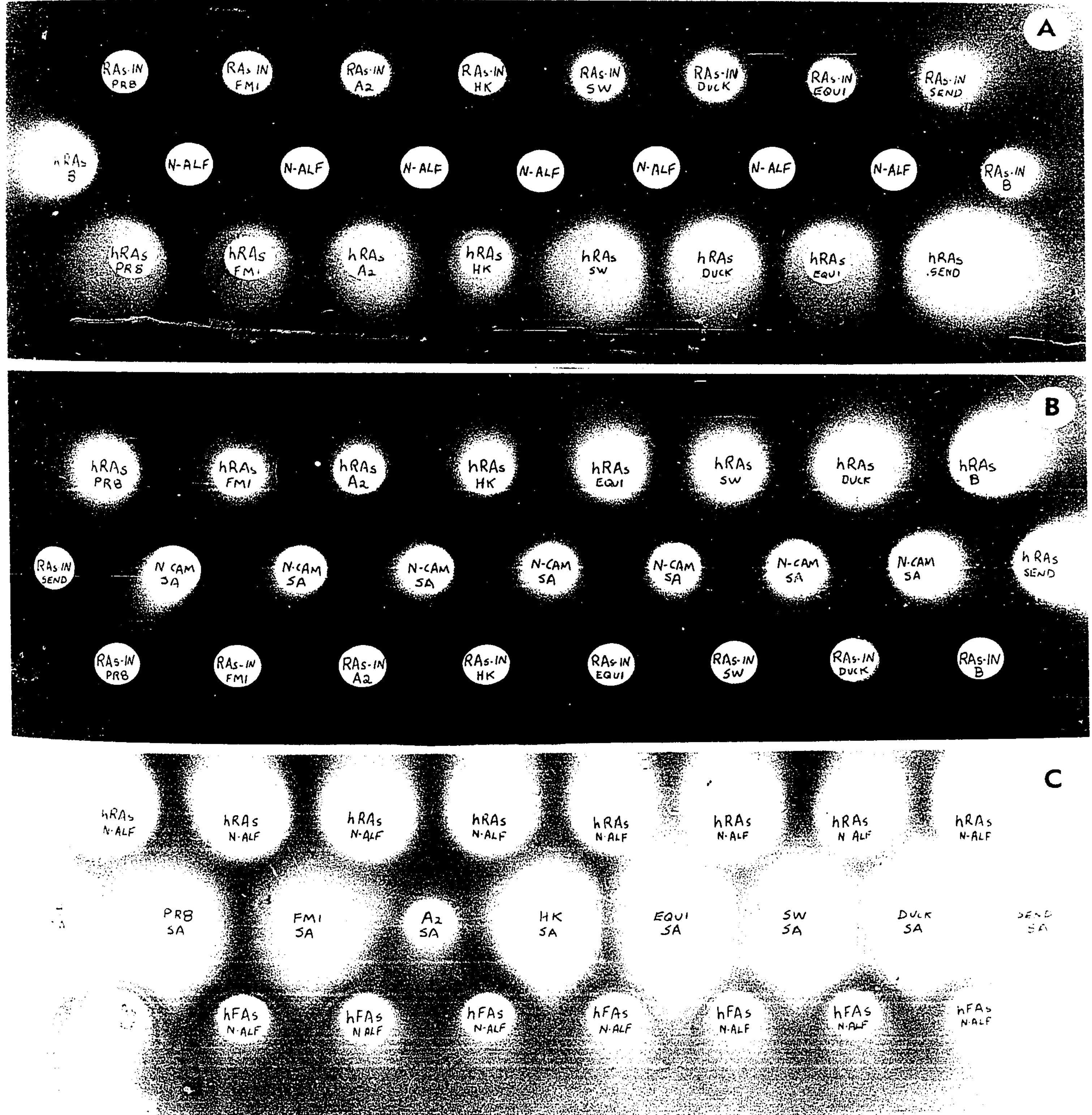


PLATE 3

Determination of host-and-virus-specificity of immunoprecipitin reactions.

1:2, 1:5 : these dilutions of sera in PBS

A. RDE : sera treated with RDE as V. cholerae filtrate

A precipitin band formed between convalescent serum and HK is weaker on dilution of the serum, and vanishes when serum is RDE treated. The same precipitin in hyperimmune serum is very weak, forming a barely visible precipitin band with the antigen which vanishes when the serum is diluted and RDE treated. The RDE susceptibility of the precipitin suggests it is an inhibitor.

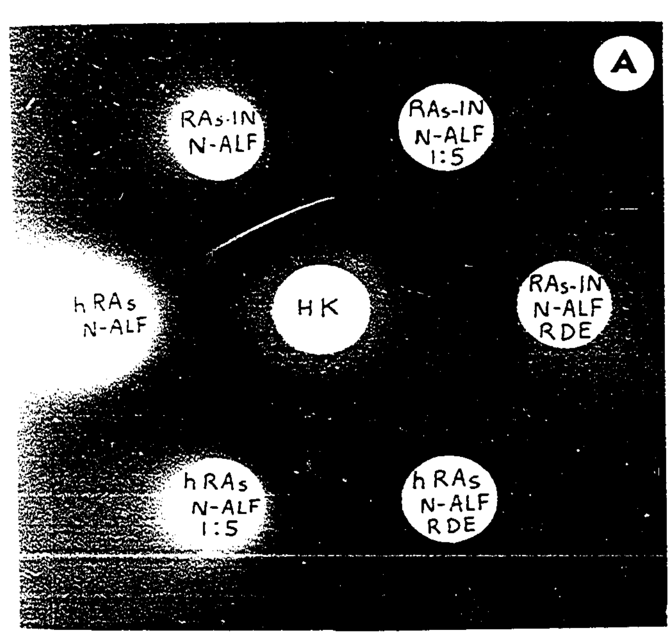
B. Precipitating antibody responses of a rooster and a rabbit serum parenterally immunized with host material (N-ALF C_2) in immunodiffusion reactions with the immunizing antigen and N-CAM/SA; and the effect of N-CAM absorption of hRAs/N-ALF on its precipitin antibody pattern with the CE antigens. Unabsorbed rabbit serum precipitates 3 N-ALF components, and 5 N-CAM components, two of which (arrows 1 and 2) are host components common to N-ALF and N-CAM. Absence of immunoprecipitin reactions between absorbed serum and both host antigens illustrates the successful removal of host-specific antibodies found in unabsorbed serum. Three residual N-CAM components remaining in absorbed serum are precipitated by unabsorbed serum antibodies, two of which (arrows 1' and 2') identify by precipitin band linkage with two components common to N-ALF and N-CAM (arrows 1 and 2).

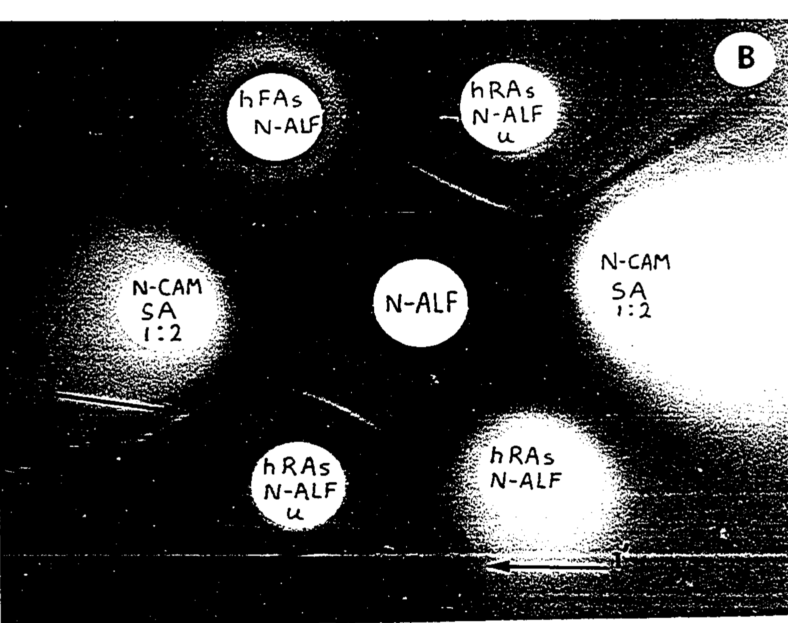
Complete lack of reaction of hFAs/N-ALF with both host antigens illustrates the absence of detectable anti-host antibodies in the rooster serum.

C. Arrow points to faint precipitin band formed between hyperimmune serum of rooster immunized with N-ALF C_2 and soluble N-CAM antigen (photography has not adequately recorded this band which is present in the coomassie blue stained slide), demonstrating that roosters can develop precipitating antibody to host (CE) material, an intra-species phenomenon. This reaction appears occasionally, when the serum is situated next to unabsorbed hRAs/N-ALF.

(Coomassie blue stain)

PLATE 3





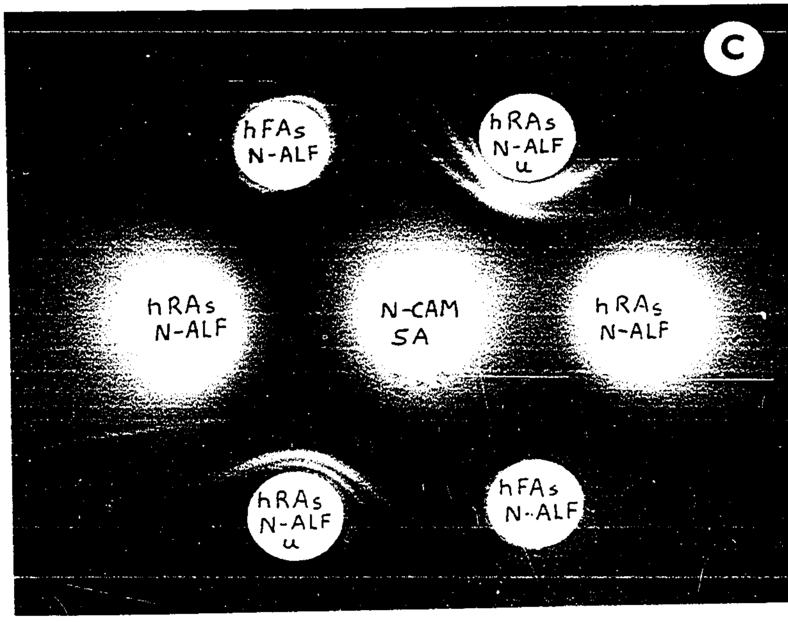


PLATE 4

Determination of host-and-virus-specificity of immunoprecipitin reactions. Absorption by N-CAM, of rabbit sera hyperimmune to A/Duck and A/FM1, and its effect on the precipitating antibody pattern with homologous virus and host antigens.

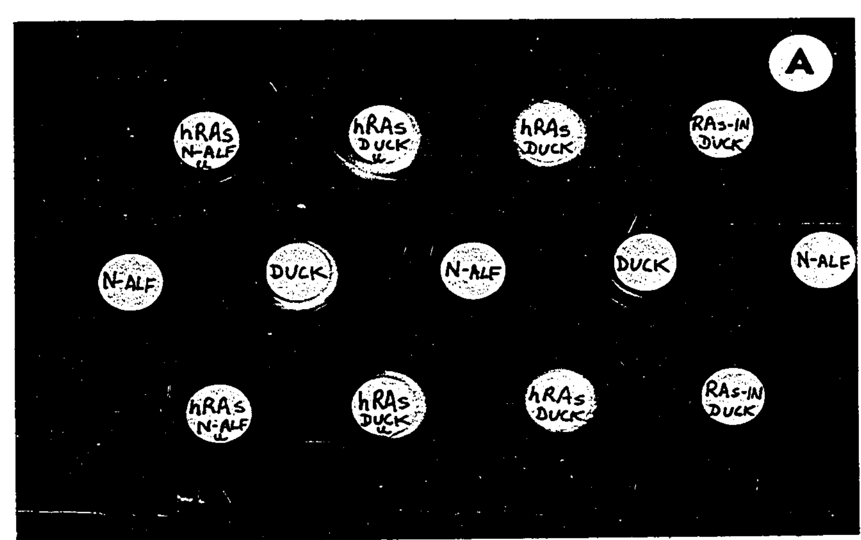
A. Rabbit serum hyperimmunized with Duck C_2, and N-CAM absorbed, forms 4-5 virus-specific precipitin bands with homologous Duck antigen (V arrows), two of which link in identity with two virus-specific precipitin bands formed between the virus antigen and homologous convalescent rabbit serum (V-LINK arrows). Neither serum precipitates N-ALF components and therefore do not contain host-specific antibodies. N-CAM absorption of hRAs/Duck was successful.

In contrast, the same hyperimmune rabbit serum unabsorbed forms 9-10 precipitin bands with homologous Duck antigen. One band is host-specific because it links with a CE-specific reaction formed by unabsorbed hRAs/N-ALF with virus antigen (Duck C_2) and with host antigen (N-ALF C_2) (E-LINK arrows). Host specificity of other of these bands is suggested by direct linkage with host specific reactions or by curving in towards host-specific reactions. Host antibodies in unabsorbed hRAs/Duck precipitate residual N-CAM components in the absorbed serum forming 2 host-specific precipitin bands (E arrows). One of these links with the host-specific reaction described above (E-LINK) suggesting that a common component present in N-CAM, N-ALF, and Duck C_2 is precipitated by homologous antibody which is similar in host immunized and virus immunized rabbits.

B. An experiment similar in principle to that described in (A) but using a different virus system, infl. A/FM1 and its homologous antisera. V arrows signify virus-specific precipitin bands, E arrows, host(CE)-specific bands. V-LINK arrows signify linkage of identity of the only virus specific band formed by homologous antisera with their virus antigen (FM1 C_2). E-LINK arrows signify linkage of identity of one host(CE)-specific band. Neither absorbed hyperimmune serum nor convalescent serum contain host-specific antibody evidenced by lack of reaction with N-ALF C_2, but unabsorbed hyperimmune serum does. At least 6 residual N-CAM components in absorbed serum are precipitated by host-specific antibodies in unabsorbed serum (E arrows). The absorption of host-specific antibodies in hyperimmune rabbit serum by N-CAM was successful.

(Thiazine red R stain)

PLATE 4



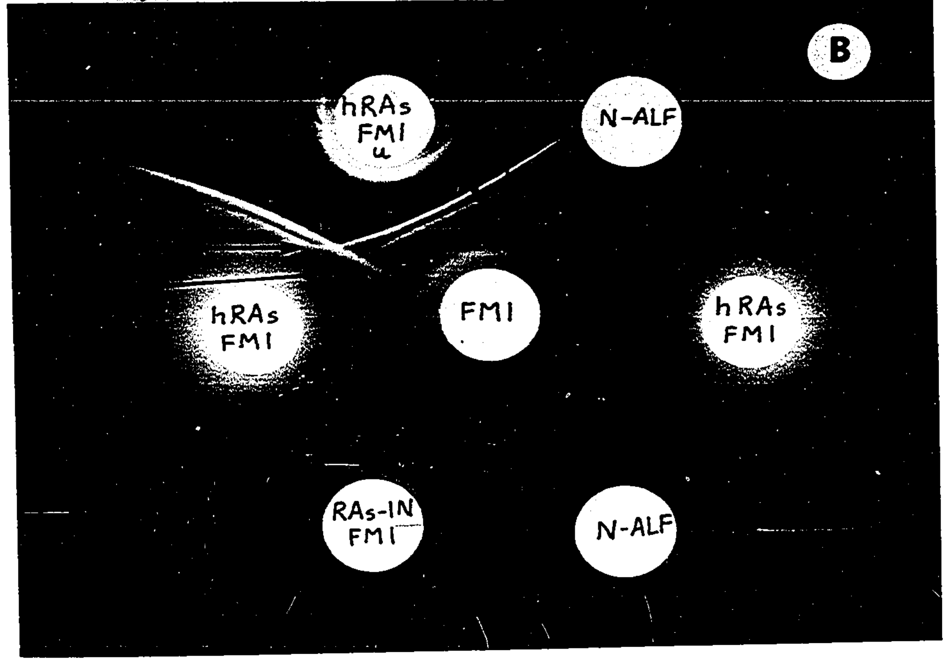


PLATE 5

Effect of serum inhibitor inactivation treatments on homologous immunoprecipitin reactions between animal antisera and virus concentrate or soluble antigens.

Antisera, top and bottom rows; antigens centre rows

1:5, 1:2 :these dilutions of serum in PBS
RDE :V. cholerae filtrate RDE treated sera
$NaIO_4$:$NaIO_4$ treated sera
NS (Plate 5C) :non-specific precipitate resulting from overlapping slides in staining process

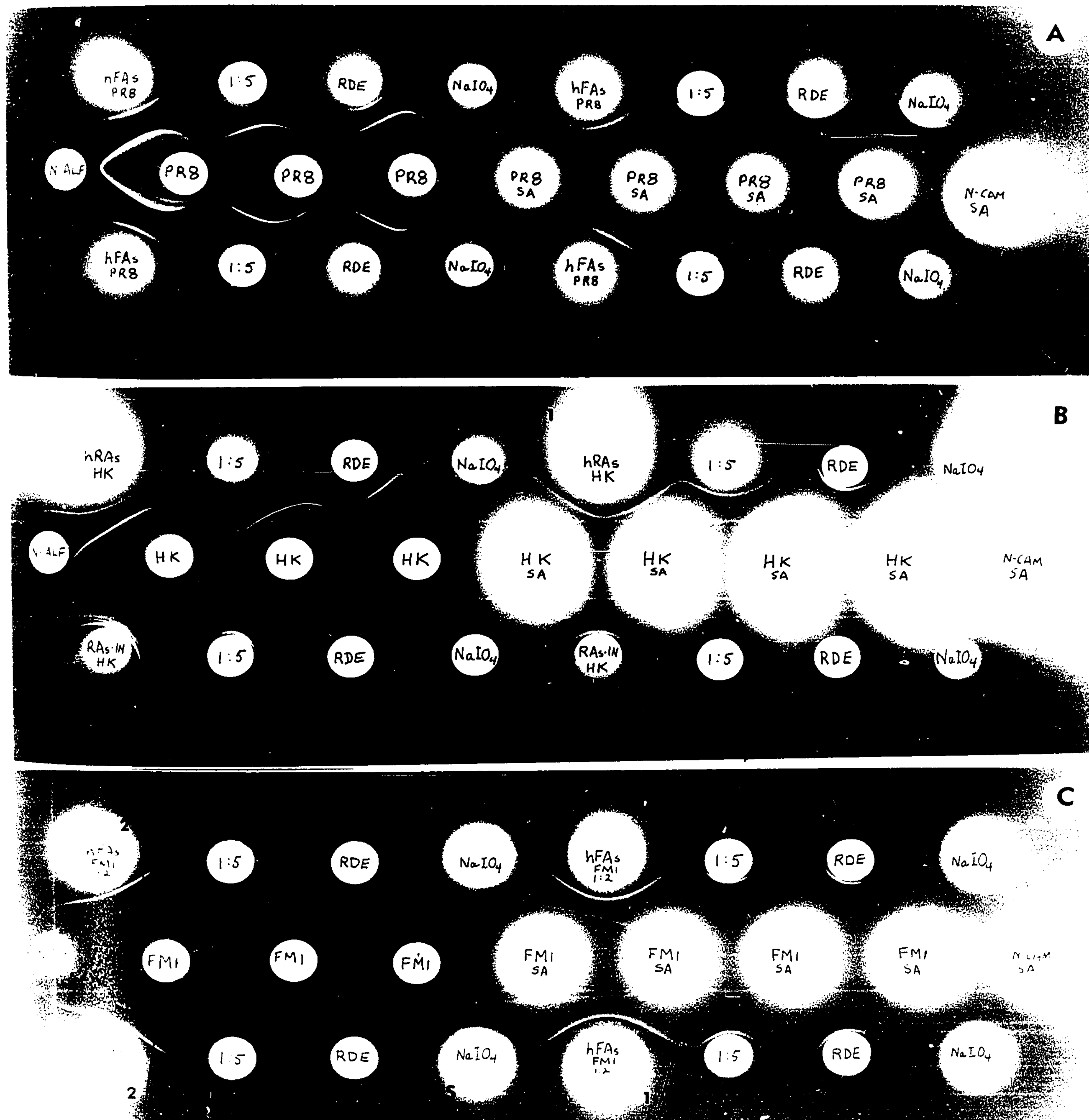
A. Two strong precipitin bands are formed on diffusion of PR8 hyperimmune rooster serum against virus concentrate: a type-specific reaction (arrow 1), and a suspected neuraminidase-specific reaction (arrow 2); a number of weaker bands are also formed. Dilution, RDE, or $NaIO_4$ treatment of serum do not affect the component(s) precipitating each of these antigens, but a reduction is noted in the number of weaker bands formed (refer to Table 11). The same type-specific reaction is also formed with soluble antigen by the same serum component (demonstrated by precipitin band linkage of identity), which is not affected by dilution or the same inactivation treatments. A weaker precipitin band is also unaltered by treatment of sera.

B. The components of A2/HK/1/68 hyperimmune rabbit serum (abs) (top row) forming two (arrows 1, 2) of three strong precipitin bands in reactions with virus concentrate antigens (centre row) are not affected by dilution, RDE, or $NaIO_4$ treatment. Dilution reduces the serum component forming the innermost third band and eliminates it, as well as a very much weaker band. One of the surviving bands (arrow 1) is the type-specific reaction, identified by its linkage to the same reactions formed with homologous soluble antigen, also unaffected by dilution, RDE, or $NaIO_4$ treatment. The other surviving strong precipitin band (arrow 2) is suspected of being neuraminidase-specific. Precipitins in A2/HK/1/68 convalescent rabbit serum (bottom row) form three bands when diffused against homologous virus concentrate, which are reduced to 2 by dilution, and to one by RDE or $NaIO_4$ treatment. Lack of precipitin band linkage between each reaction does not permit identity of the surviving precipitin band with the type-specific reaction formed by convalescent serum and soluble antigen. This band is eliminated when the serum is diluted.

C. Components in FM1 hyperimmune rooster serum (diluted 1:2) form three precipitin bands with FM1 virus concentrate. Only one of these bands (arrow 1) remains when the serum is diluted 1:5 or when it is treated with RDE. The absence of reaction when serum is $NaIO_4$ treated indicates the precipitin was eliminated. A non-specific (NS) streak occurring during staining is not significant. Although the precipitin band linkage is broken at $NaIO_4$ treated serum, what appears to be the same reaction, the type-specific one occurs between the serum and FM1 soluble antigen. It follows the same pattern of inactivation by $NaIO_4$. The other strong precipitin band (arrow 2) is thought to be neuraminidase-specific.

(Coomassie blue stain)

PLATE 5



PLATES 6 and 7

Non-specific precipitation of antisera and viral antigens by surface active agents.

DOC	: sodium deoxycholate, 1% in n-saline
SDS	: sodium dodecyl sulfate, 1% in n-saline
NP 40	: Nonidet P40, 1% in n-saline
PBS	: phosphate buffered saline (pH 7.3)

Suffix DOC following virus notation signifies disruption by 1% of this reagent, e.g. PR8/DOC
(A/PR8/DOC), FM1/DOC (A/FM1/Can/53 /DOC), etc.

Suffix NP 40 following virus notation signifies disruption by 1% of this reagent, e.g.
PR8/NP 40 (A/PR8/NP 40), etc.

A. Precipitation of DOC disrupted influenza A strains, B, and parainfl. Sendai by hyperimmune rabbit sera (abs) against A/Swine (top row), and against parainfl. Sendai (bottom row). hRAs/Sendai normally precipitates only homologous virus antigens as demonstrated (far right). In this experiment all viruses are precipitated into fuzzy bands linking across the template. hRAs/Swine usually precipitates all influenza A strains but not influenza B or Sendai, whereas here, all virus concentrates are precipitated into a fuzzy band linking across the template in addition to other more distinct precipitin bands formed with type A virus.

B. Non-specific precipitation of rabbit sera, convalescent (bottom row) and hyperimmune (abs) (top row) by 1% DOC (centre row). Note similarity of precipitin bands to those found in 6A.

C. Non-specific precipitation of hyperimmune rooster sera (top and bottom rows) by 1% SDS. Both fuzzy and sharp precipitin bands are formed.

PLATE 7

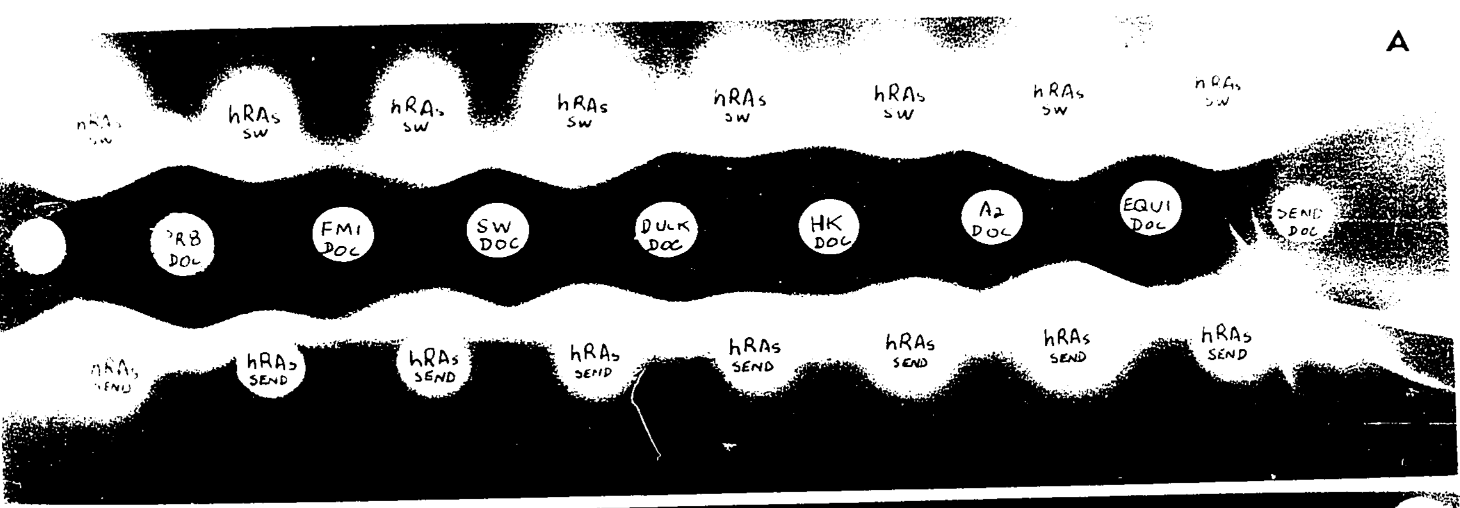
A. Characteristic ring formation by NP 40 precipitated antiviral rabbit sera, convalescent (bottom row), and hyperimmune (abs) (top row).

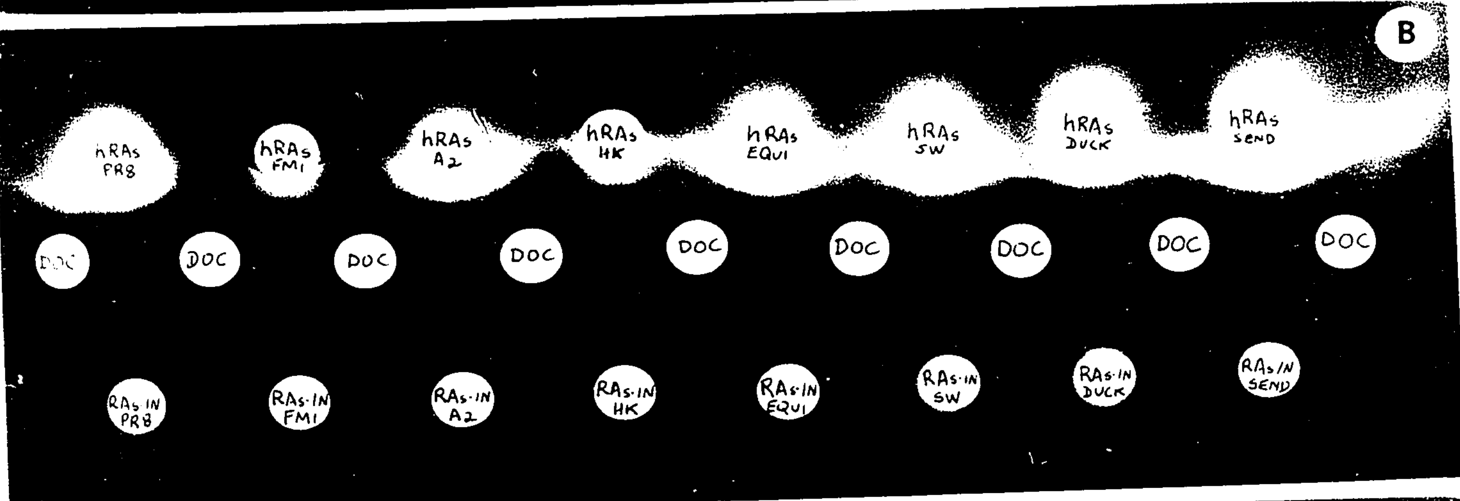
B. Characteristic ring formation by NP 40 precipitated virus concentrates (C_2 and C_2') diffused against PBS.

C. Precipitation of DOC disrupted viruses is much less than that observed with antisera. Not all viruses are non-specifically precipitated, only B, PR8, FM1, Duck, and Sendai.

(Coomassie blue stain)

PLATE 6





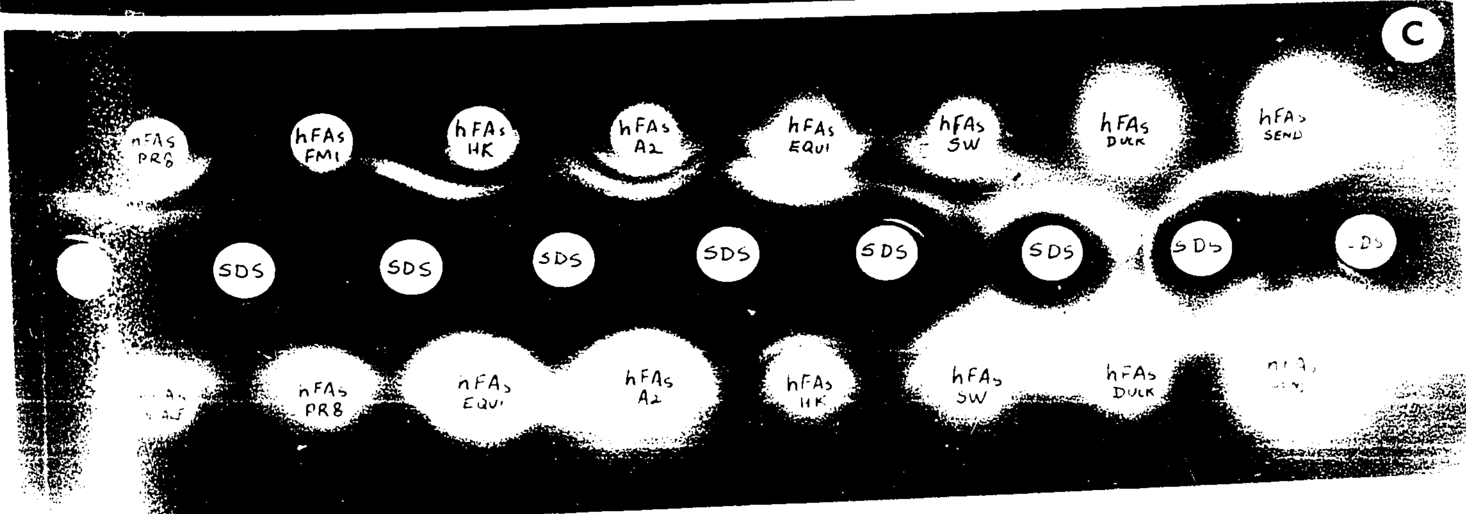


PLATE 7

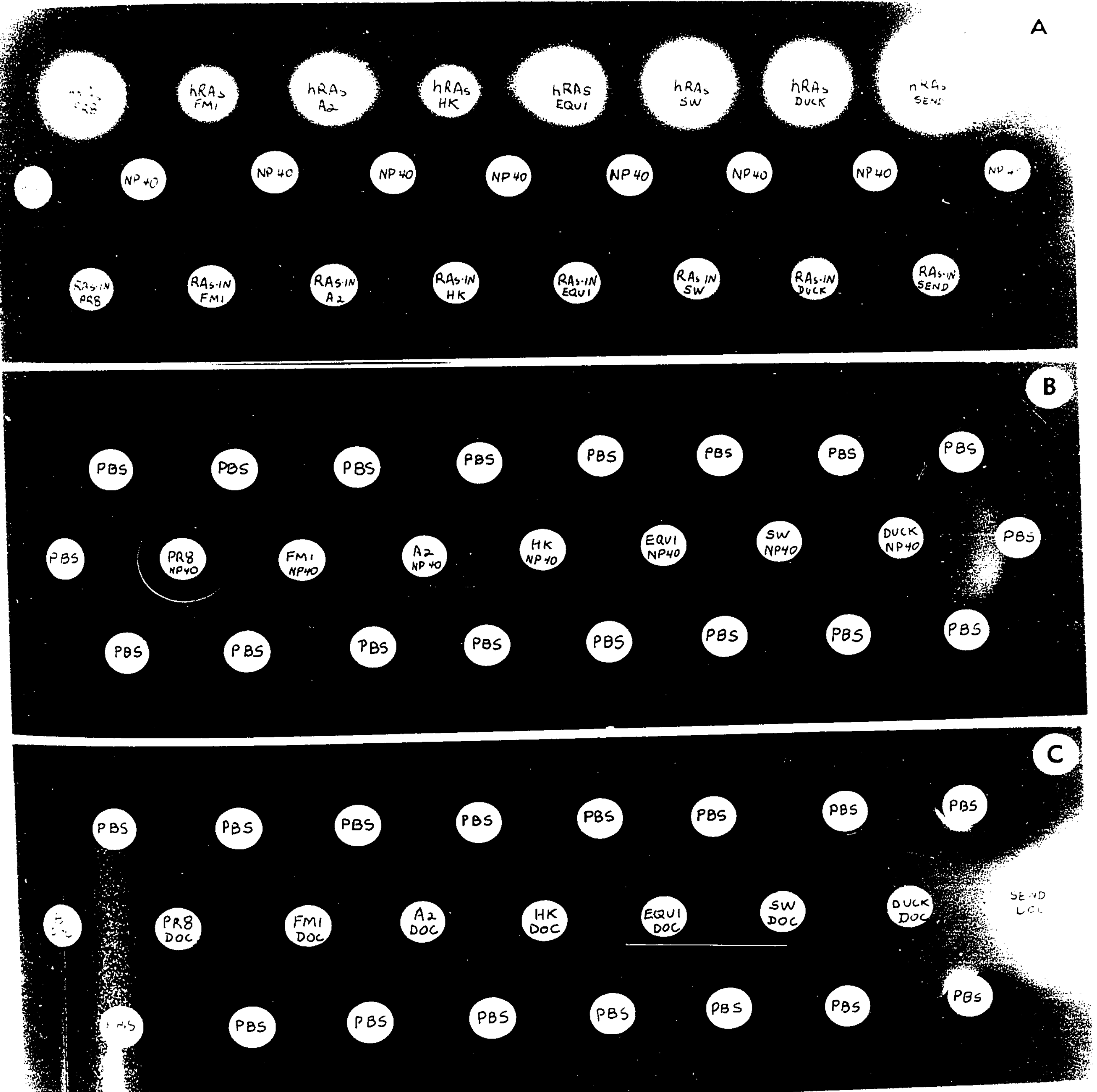


PLATE 8

Banding of influenza viruses in sucrose density gradients. Spinco head SW 25.2 at 4°C.

[1]Diffuse band detected by spectrophotometry

[2]Denser upper band

[3]Diffuse lower band

[4]Diffuse band indicated

PLATE 8

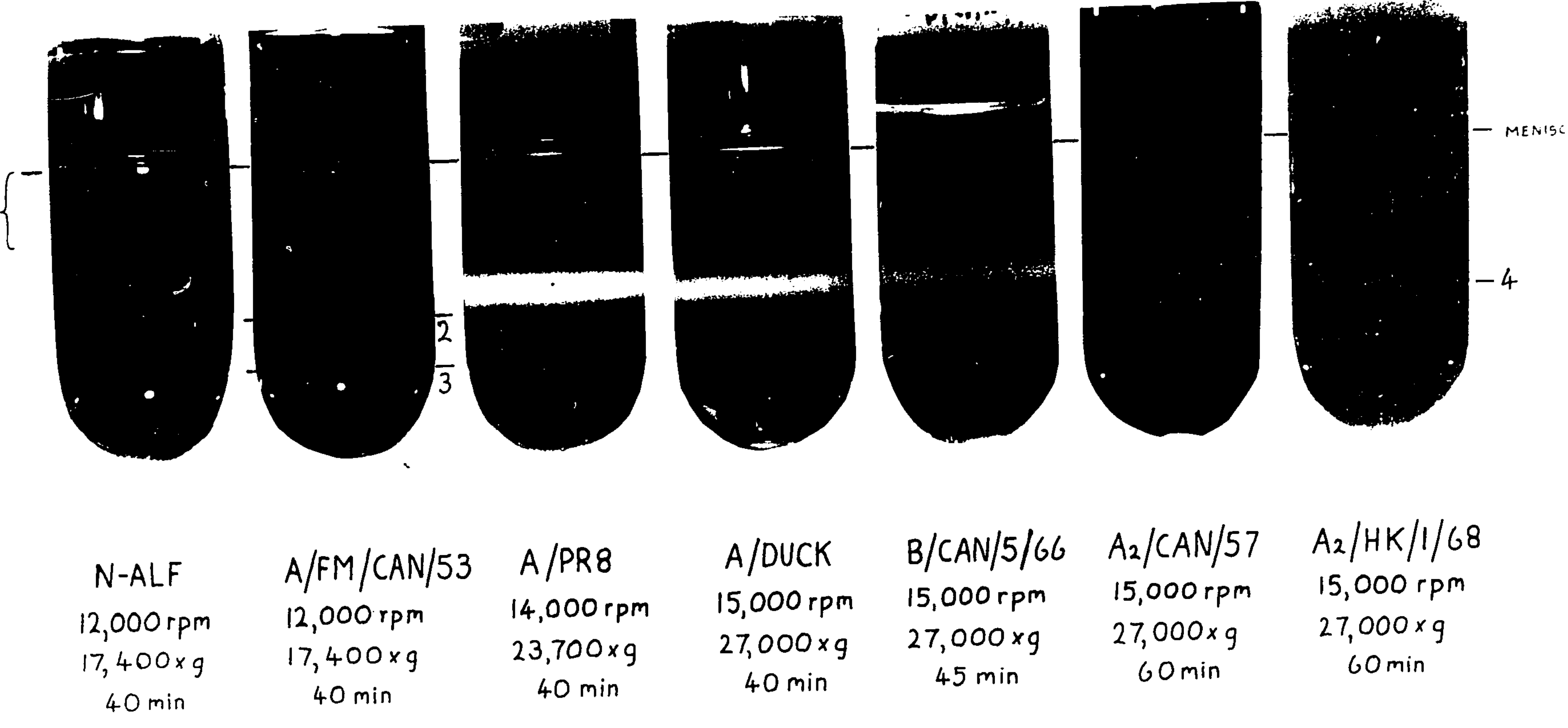


PLATE 9

Homologous immunoprecipitin patterns of sucrose density gradient purified viruses.

Antisera, peripheral wells; antigens, central wells

A. Homologous hyperimmune rabbit and rooster sera each precipitates 3 components of purified PR8 virus, and convalescent serum precipitates one, as indicated by the formation of 3 and 1 precipitin bands respectively. Host-specific hyperimmune rabbit (N-CAM absorbed) and rooster sera do not precipitate the virus. All these precipitin reactions are considered to be virus-specific. Each outermost band of hyperimmune rabbit and rooster serum links with its respective band formed between each of these antisera and PR8 soluble antigen (arrow 1), suggesting that a type-specific component is being precipitated. A common antibody in all three PR8 antisera is precipitating the same virus component as evidenced by a linking precipitin band formed between these sera and the purified virus (arrow 2). An antibody common to both hyperimmune sera is precipitating a third viral component indicated by precipitin band linkage (arrow 3).

B. Influenza A2/Hong Kong/1/68 purified virus does not diffuse well, as illustrated by the "ringing" of a precipitin band around the antigen well (centre). Interpretation of this reaction is difficult. A linking precipitin band (arrowed) suggests that the common component in soluble antigen and purified virus concentrate precipitated by both homologous rabbit antisera, is the type-specific one.

C. Two of three components of purified virus precipitated by homologous convalescent rabbit serum are also precipitated by homologous hyperimmune rabbit serum. The precipitated component forming the innermost band (arrow 1) is type-specific because it links with the type-specific reaction between hRAs/Duck (abs) and Duck soluble antigen (arrow 1). The other component precipitated by all anti-Duck antisera (arrow 2) is also precipitated by host-specific rooster serum which has been demonstrated to lack host and virus antibodies in reactions with virus and host concentrates. The identity of this component is questionable.

D. Homologous hyperimmune rabbit and rooster sera each precipitate two components of purified swine virus. In each case, the outermost precipitin band formed links in identity with the type-specific immunoprecipitin reaction occurring between the respective sera and soluble antigen (arrow 1). A second component of purified virus is precipitated by all anti-swine antisera. Host-specific rooster antiserum, although demonstrated to lack host and virus antibodies (see C above), precipitates a third component of purified virus. It resembles the reaction described with Duck virus above, but does not link in identity with reactions of other antisera.

PLATE 9

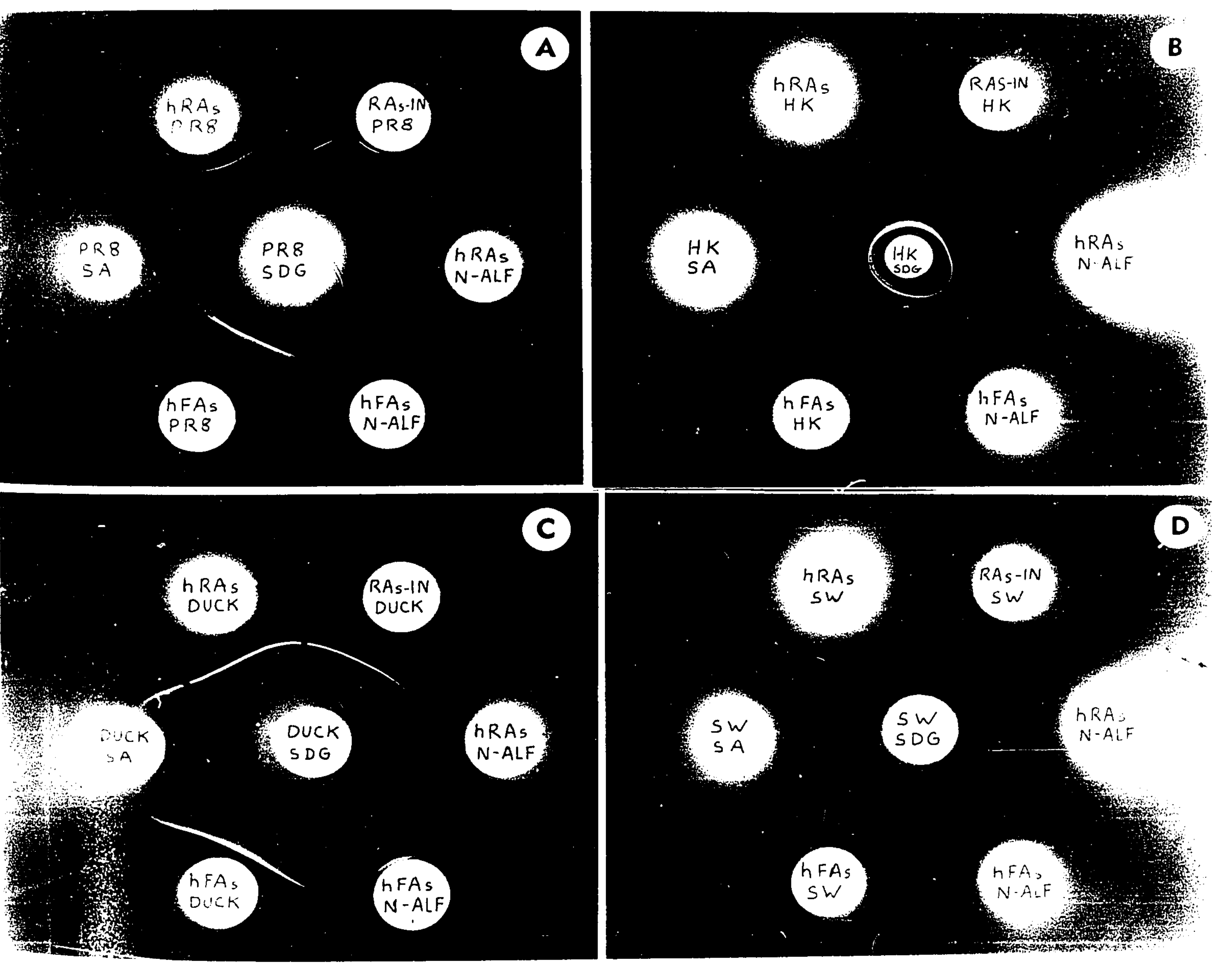


PLATE 10

Electron micrographs of influenza A strains and parainfluenza Sendai (Negatively stained by PTA).
A-D: magnification 83,800 x ; E-G: magnification 84,600 x

A. Influenza A/Duck C_2. Note debris in background.

B. Influenza A2/Hong Kong/1/68 filament in C_2' preparation. Note non-specific debris in background.

C. Prainfluenza Sendai C_2. RNP is characteristically ejected from disrupted virus particle.

D. Influenza A2/Hong Kong/1/68, sucrose density gradient purified preparation. Note pleomorphic forms.

E. Influenza A/PR8, sucrose density gradient purified preparation.

F. Influenza A/Swine, sucrose density gradient purified preparation.

G. Influenza A/PR8, potassium tartrate gradient purified preparation. Virus particles not as clearly stained as those in (E); surface projections are not as clear.

PLATE 10

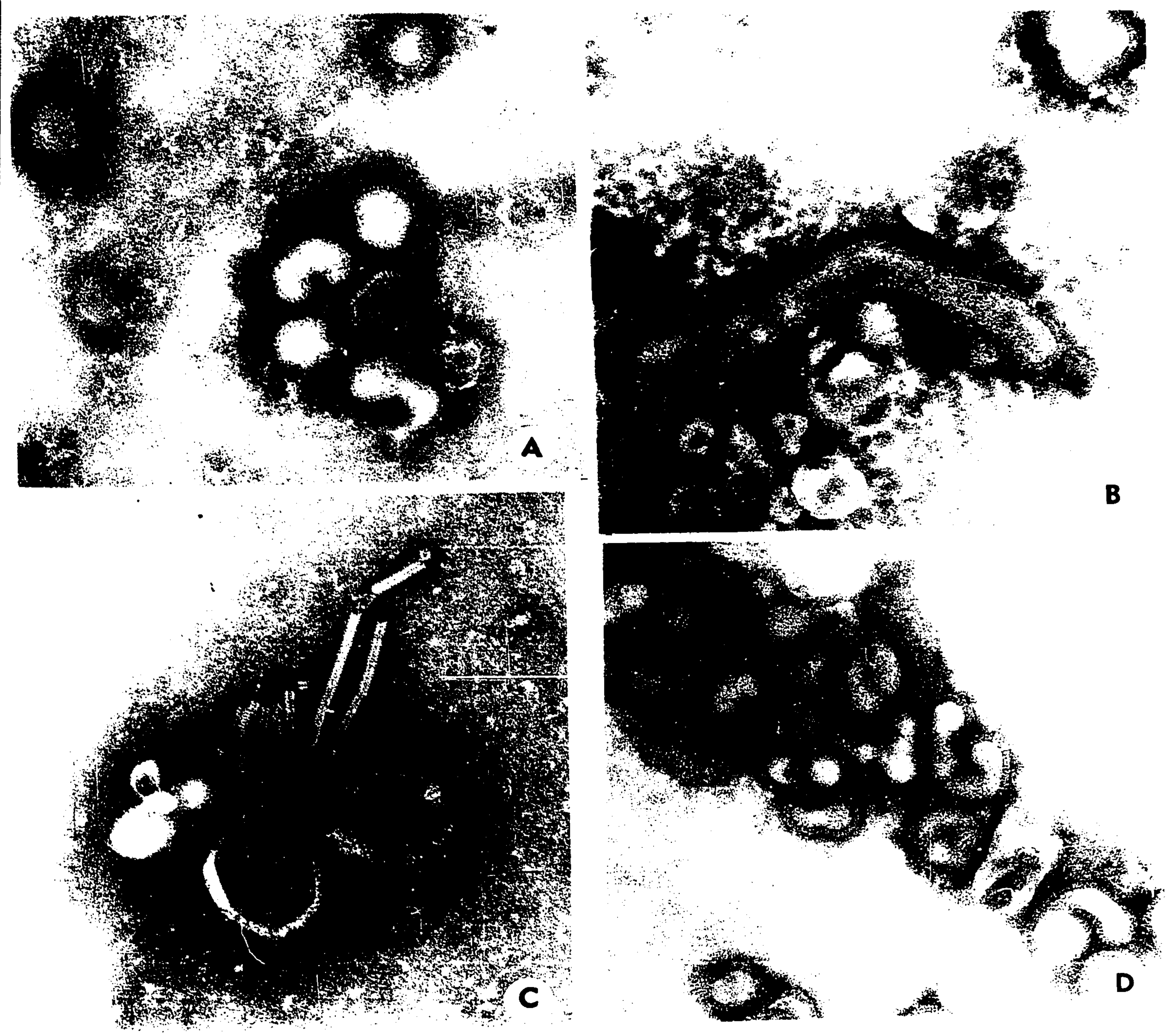


PLATE 10

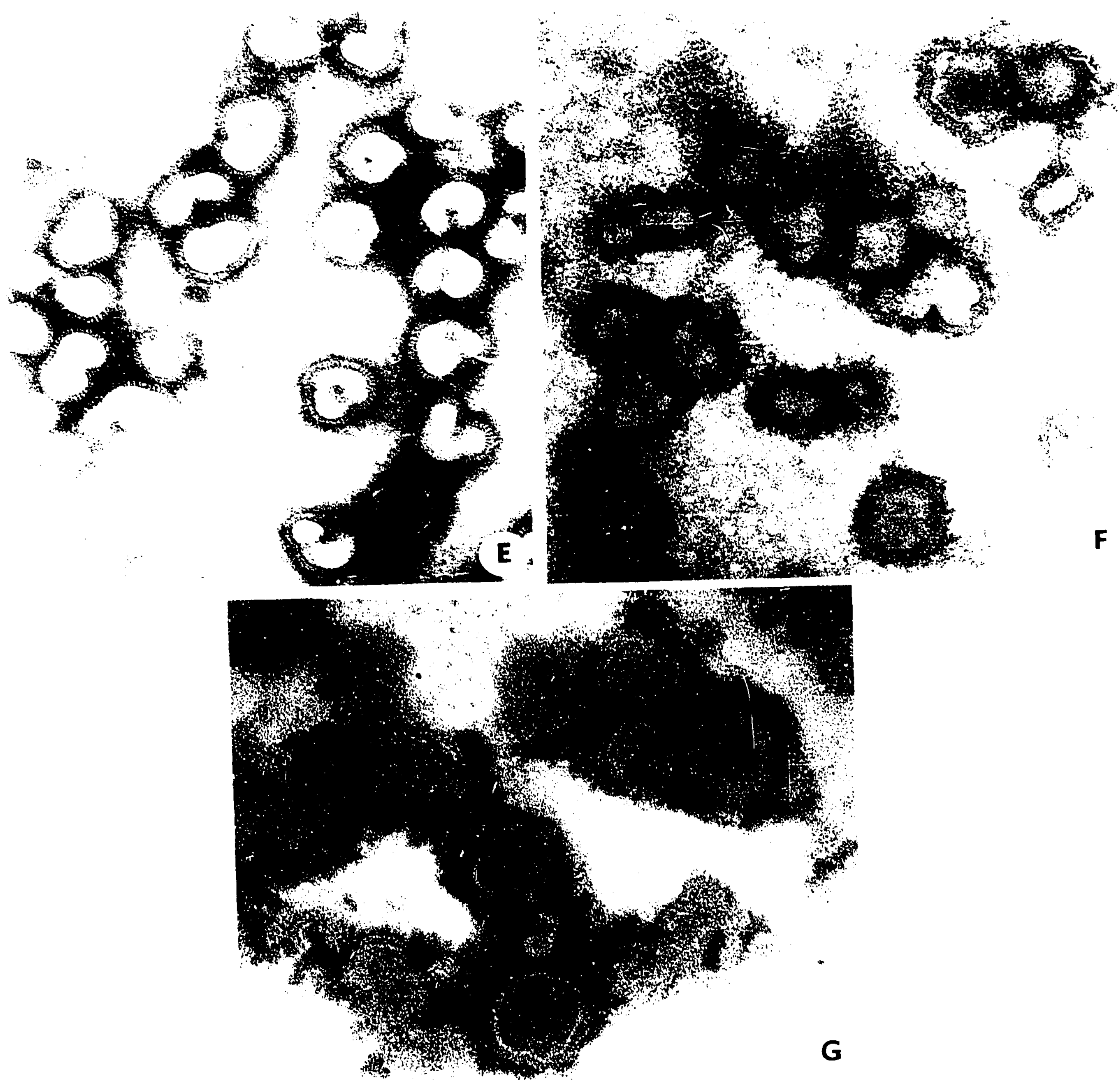


PLATE 11

Identification of the type-specific reaction in homologous immuno-diffusion reactions.

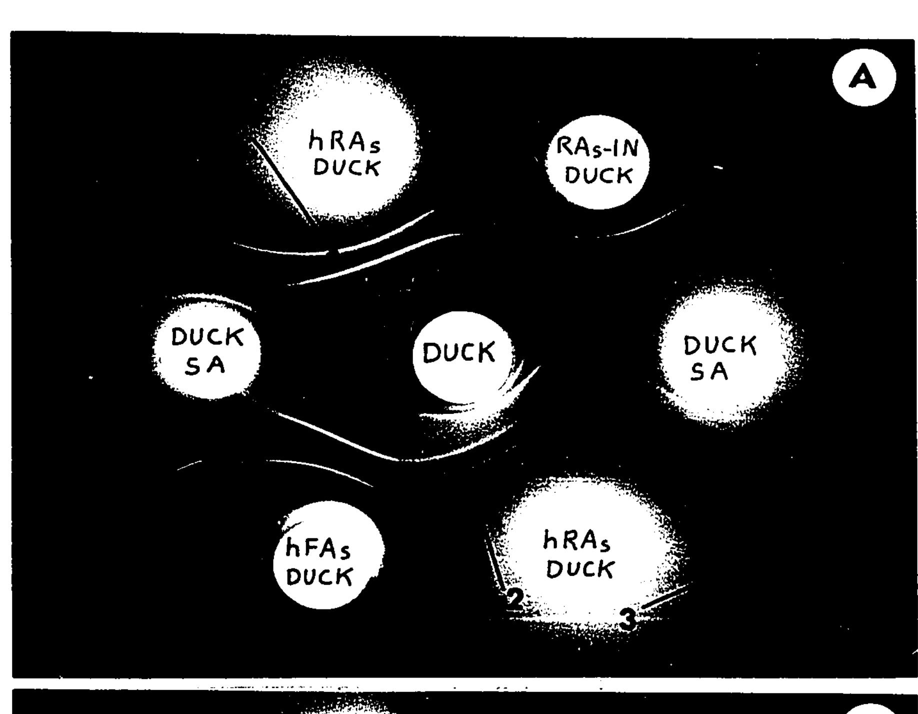
DUCK/NP 40 : influenza A/Duck virus concentrate (C_2), disrupted by 1% Nonidet P40

A. A precipitin band formed by hyperimmune rabbit serum (abs) with SA and with C_2 links, identifying the precipitated component as type-specific antigen, which could be the RNP (arrow 1, top and bottom). This reaction links to one formed by the same antigens with convalescent rabbit serum (arrow 1 top), and with hyper-immune rooster serum (arrow 1 bottom). Two other precipitin bands formed by both SA and C_2 with hyperimmune rabbit serum (abs) (arrows 5, 6), are precipitated components common to virus particles and to soluble antigen. One component found in only soluble antigen is precipitated by hyperimmune rabbit serum (arrow 3), and is a virus-specific reaction. The host specificity of a precipitin band formed between hyperimmune rooster serum and SA (arrow 4) is established by linkage with a precipitin band formed between the serum and residual N-CAM components in absorbed hyperimmune rabbit serum (arrow 4). One other strong precipitin band formed between each immune serum and only virus C_2 (arrow 2) is suspected of being neuraminidase specific.

B. An identical reaction to A above, except that Duck virus concentrate has been disrupted by Nonidet P40 (1%). The above explanation can be applied here. Two precipitin rings (NS) around the central antigen well are formed by non-specific precipitation of antiserum and/or virus concentrate by the detergent.

(Coomassie blue stain)

PLATE 11



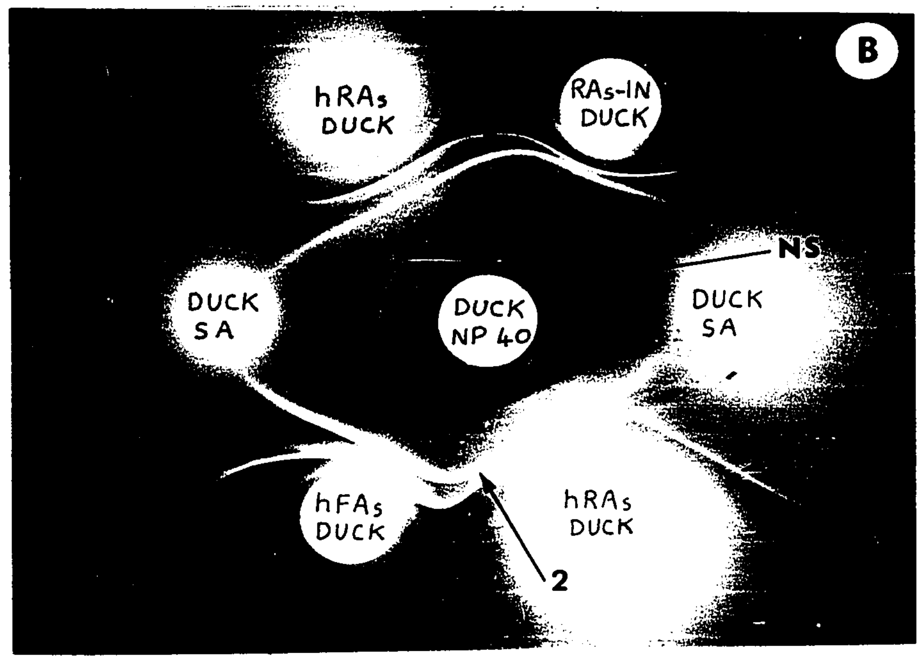


PLATE 12

Identification of the type-specific reaction in immunodiffusion cross reactions.

Antisera, top and bottom rows; antigens, centre row

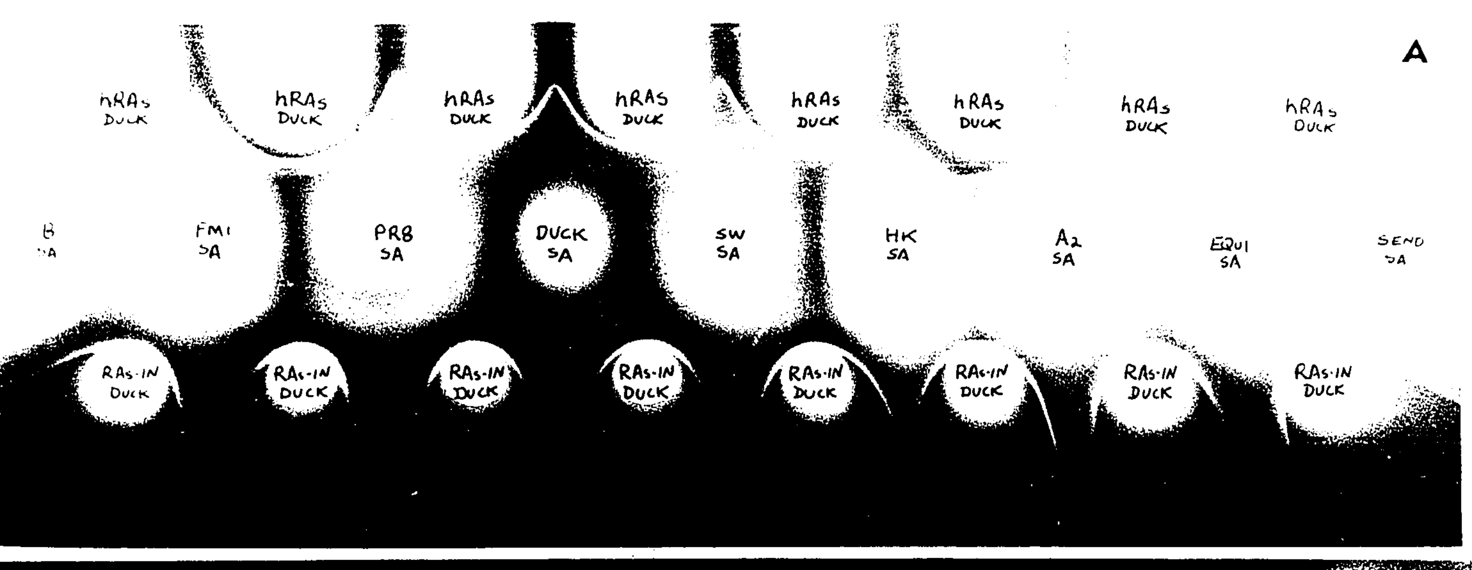
A. The single linking precipitin band formed by anti-Duck hyperimmune rabbit serum (top row) and by anti-Duck convalescent rabbit serum (bottom row) with the soluble antigens of all influenza A strains, but not influenza B or parainfl. Sendai is the major influenza A type-specific reaction. Other minor precipitin bands of type A specificity are also formed.

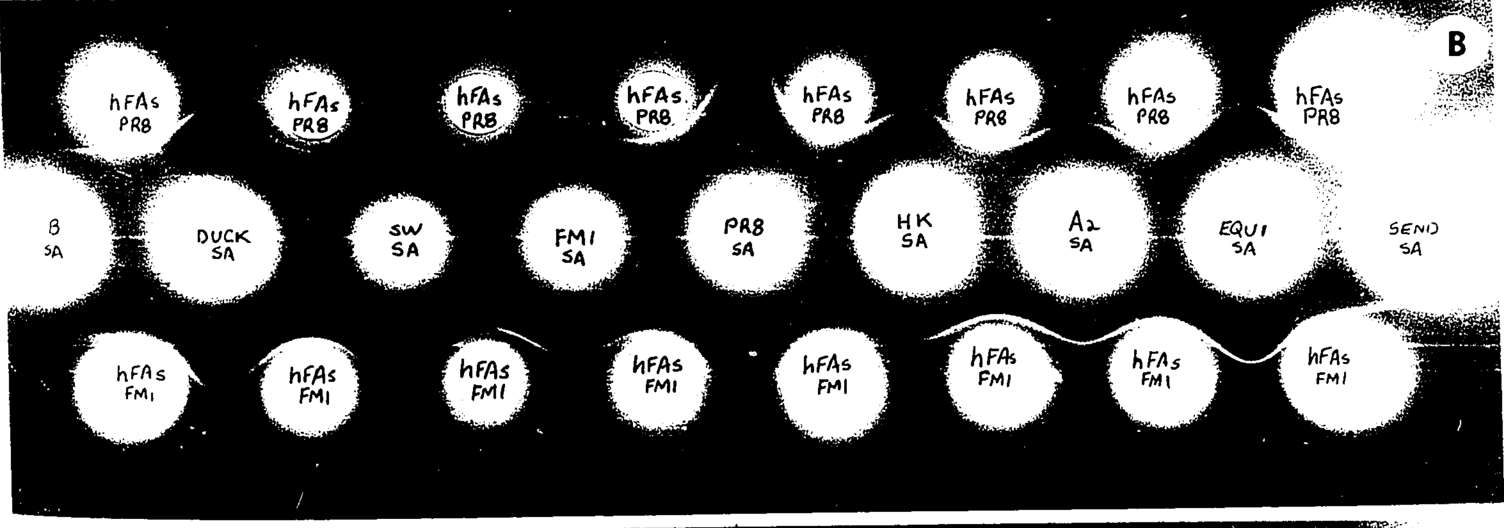
B. The major influenza A type-specific reaction is also demonstrated by hyperimmune rooster sera against PR8 (top row) and against FM1 (bottom row) with the same soluble antigens used in A above. Note lack of precipitation of B and Sendai SA. The type-specific reaction is not completely uniform as demonstrated by a break in the continuity of the precipitin band between the FM1 homologous reaction and the reaction between anti-FM1 antiserum and Swine soluble antigen (arrowed). One of the secondary precipitin bands formed could be a host-specific reaction.

C. The isolated homologous influenza B reaction formed by soluble antigen with hyperimmune rabbit serum (top row left) and with convalescent rabbit serum (bottom row left) is the influenza B type-specific reaction. Note lack of precipitation of influenza A or Sendai SA.

(Coomassie blue stain)

PLATE 12





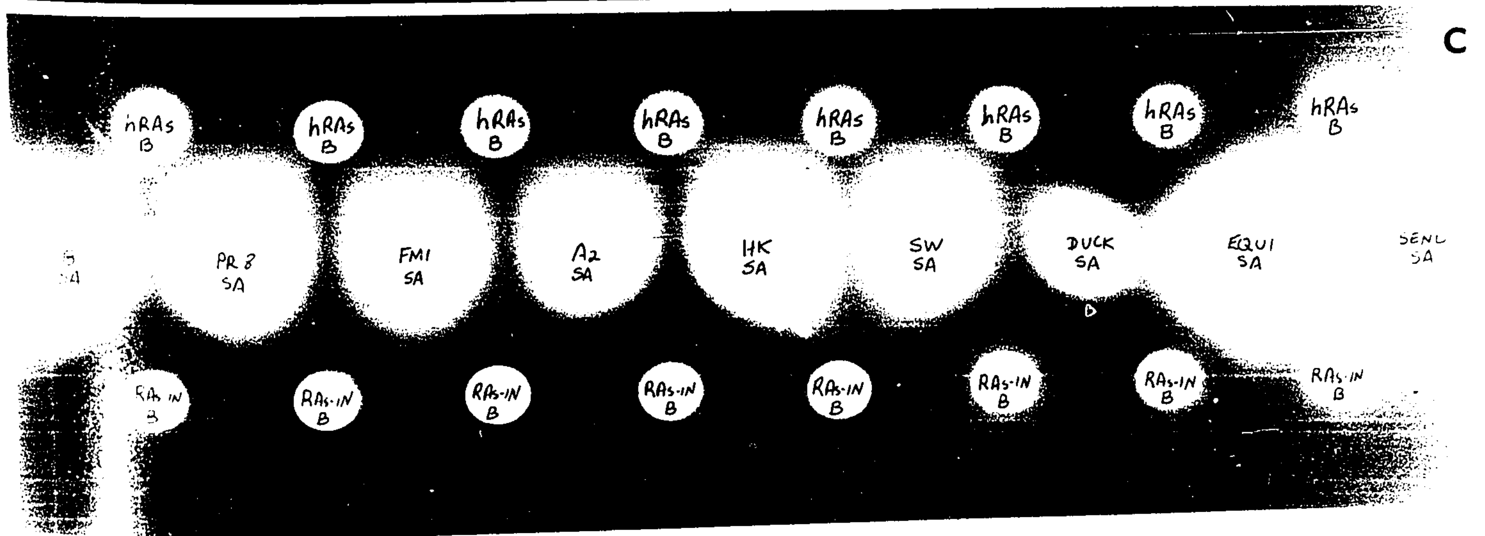


PLATE 13

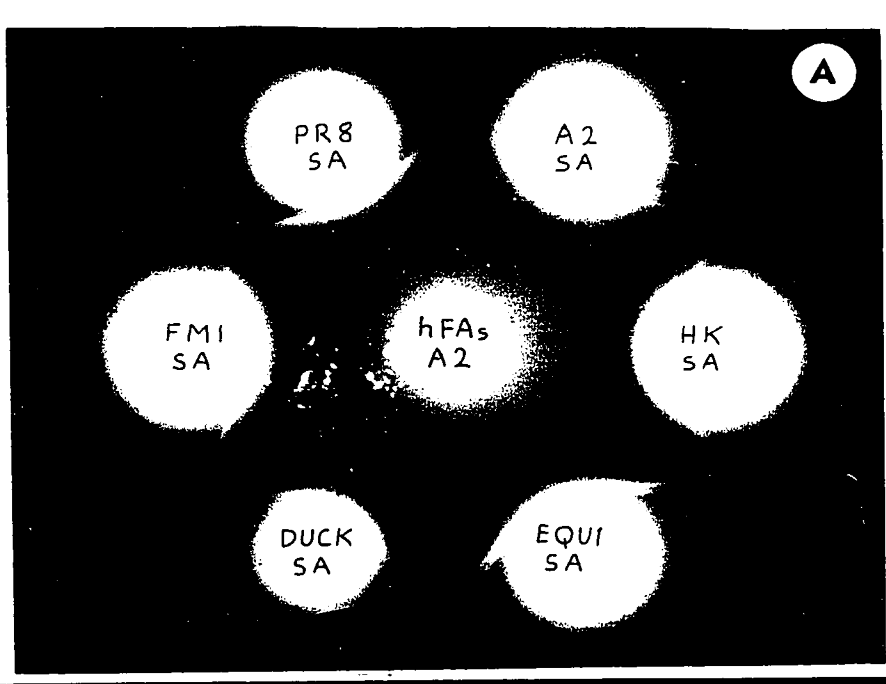
Immunoprecipitin cross reactions of influenza A, B, and parainfluenza Sendai soluble antigens.

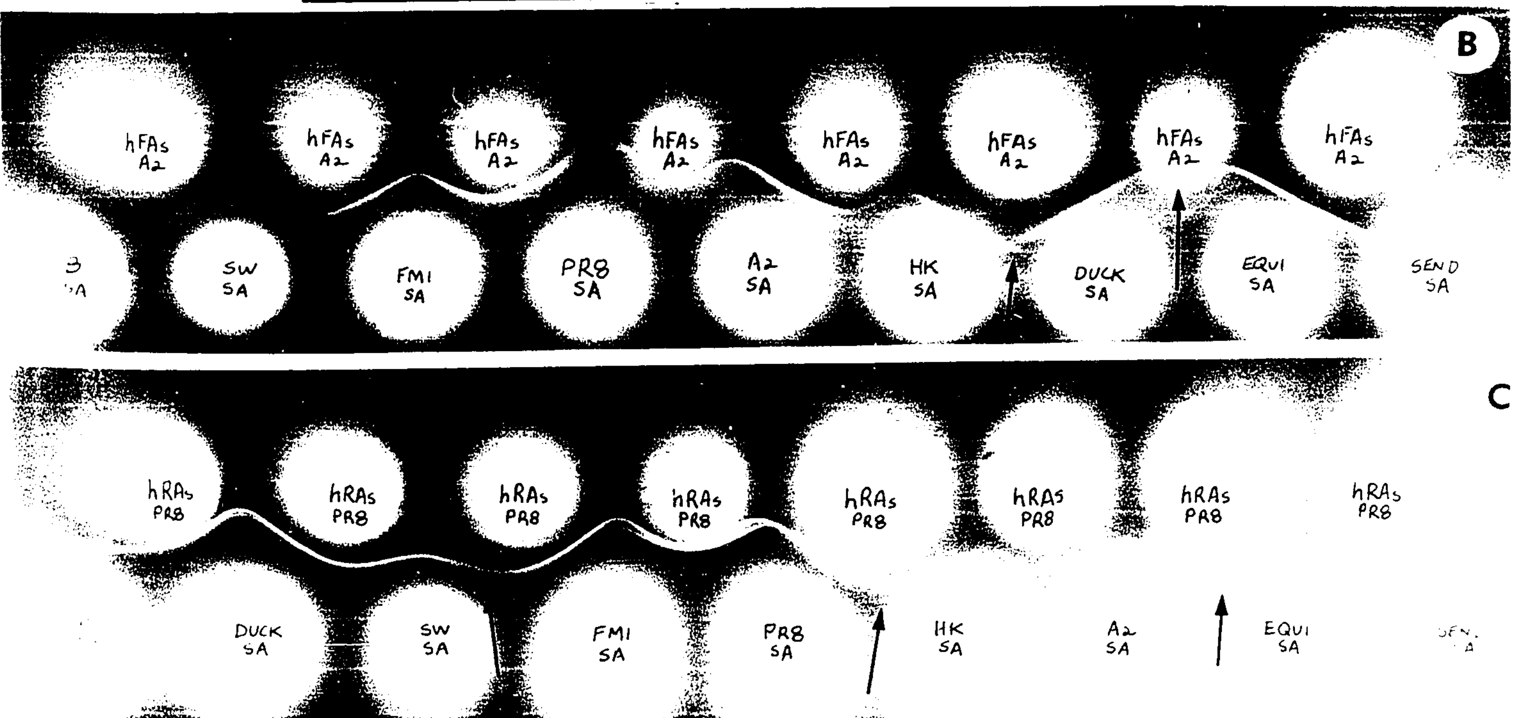
A. A major precipitin band which links all around, is formed between hFAs/A2 and each influenza A soluble antigen (arrow 2). The linkage of identity indicates that a common type-specific component is precipitated by the antiserum. However, the formation of barbs (arrows 1) by the DUCK/SA band indicates only partial identity with the major reaction. This is also demonstrated in Plate 13 B (arrowed). The antiserum precipitates an extra component in the soluble antigens of A2, HK. and Equi, which, because of precipitin band linkages (arrows 3), must be immunologically the same in all three strains.

B. Illustration of the type-specific immunoprecipitin reaction between hFAs/A2 and various influenza A soluble antigens. The major precipitin band formed links across all A type soluble antigens, but barbs indicating partial identity, are evident (arrowed). The type-specific component in SW and DUCK is not immunologically identical to the other five influenza A strains. Minor secondary precipitin bands are also formed. Absence of influenza B and parainfl. Sendai precipitation establishes the type-specific reaction as A.

C. The antibody in hRAs/PR8 (abs) precipitating the major type-specific component in various influenza A soluble antigens, is not uniformly specific for each subtype. Band splitting and barb formation (arrowed) indicate that SW and DUCK type-specific components differ slightly from PR8 and FM1, which differ from HK, A2, and EQUI components. Other minor precipitin bands of type A specificity are also formed. Note lack of reaction with influenza B and parainfl. Sendai.

PLATE 13





PLATES 14-18

Immunoprecipitin cross reactions of influenza A strains using virus concentrates and convalescent and hyperimmune sera.

NP 40: virus concentrates disrupted with 1% Nonidet P40.

PLATE 14

Initial screening of influenza A cross reactions.

A. Note discrete homologous influenza B immunoprecipitin reactions (top, extreme left), and homologous parainfluenza Sendai reactions (bottom, extreme right). There is no cross reaction with influenza A antigens (centre row), by either anti-B (top row), or anti-Sendai (bottom row) hyperimmune rabbit sera (abs).

B. Cross reactions between anti-PR8 hyperimmune rabbit serum (abs) (top row) and influenza A antigens (centre row) yield a major type-specific reaction (arrows 1) which is not continuous and uniform, forming a barb (arrow 2) at the PR8-FM1 linkage, and splits (arrow 3) at the A2/HK/1/68-Equi linkage. Other secondary components are precipitated as independent or linking bands. Cross reactions between anti-FM1 hyperimmune rabbit serum (abs) (bottom row) and influenza A antigens are less complex. The major type-specific reaction (arrows 1) is more uniform, but barbs are formed at the PR8-FM1 linkage (arrow 4) and at the FM1-A2/Can/57 linkage (arrow 5). Other secondary components are precipitated as linking (arrow 6) and independent (arrow 7) bands.

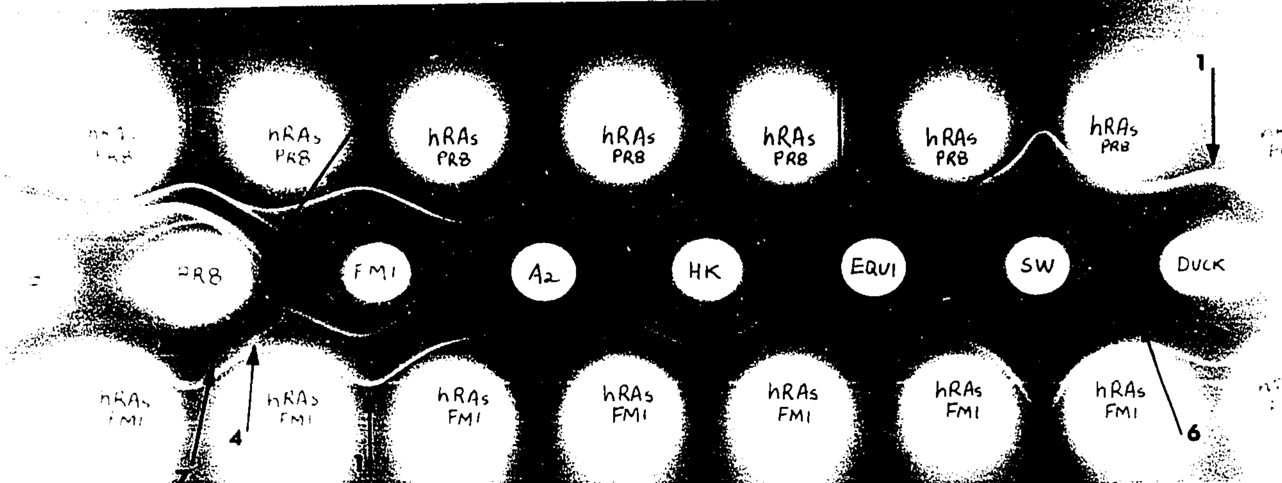
The immunoprecipitin reaction is swept out at each end of the slide, a phenomenon which can occur when using this template pattern. This gives the impression that reactions have occurred between the anti-A sera and B or Sendai antigens, which is not the case, as demonstrated in Plate 16 C and other experiments.

C. Cross reactions between anti-PR8 hyperimmune rooster serum (top and bottom rows) and influenza A antigens (centre row) form the major type-specific band (arrows 1) which is absent in reactions between the antiserum and B or Sendai concentrates. A very strong independent band and several weaker ones are formed by the homologous PR8 reaction.

D. The type-specific band (arrows 1) formed between anti-FM1 hyperimmune rooster serum (bottom row) and influenza A antigens (top row) is a restricted influenza A reaction, which does not extend to B or Sendai. The antiserum precipitates secondary components of two types: one that is shared by two antigens such as FM1 and A2/Can/57 to form a linking band (arrow 2), and independent components seen as discrete bands in the homologous reaction (arrow 3), or in a reaction with Swine concentrate (arrow 4).

(A, B, C Coomassie blue stain; D thiazine red R stain)

hRAs
A2
HK
EQUI
SW
hRAs SEND
hRAs SEND
hRAs SEND
hRAs SEND
hRAs SEND
hRAs SEND
A



hRAs PR8
hRAs PR8
hRAs PR8
hRAs PR8
hRAs PR8
hRAs PR8
1
PR8
FMI
A2
HK
EQUI
SW
DUCK
hRAs FMI
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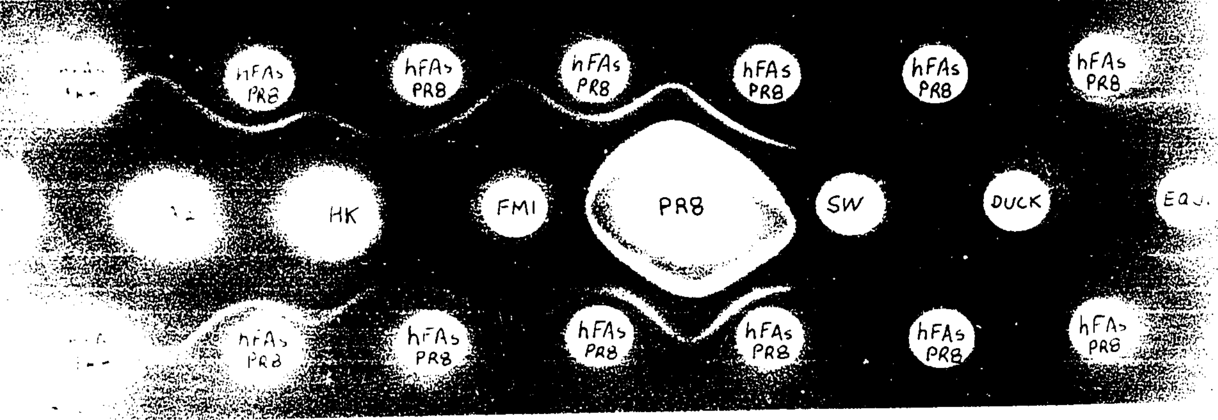
hRAs FMI
hRAs FMI
hRAs FMI
hRAs FMI
hRAs FMI
4
6
B



hFAs PR8
hFAs PR8
hFAs PR8
hFAs PR8
hFAs PR8
hFAs PR8
HK
FMI
PR8
SW
DUCK
hFAs PR8
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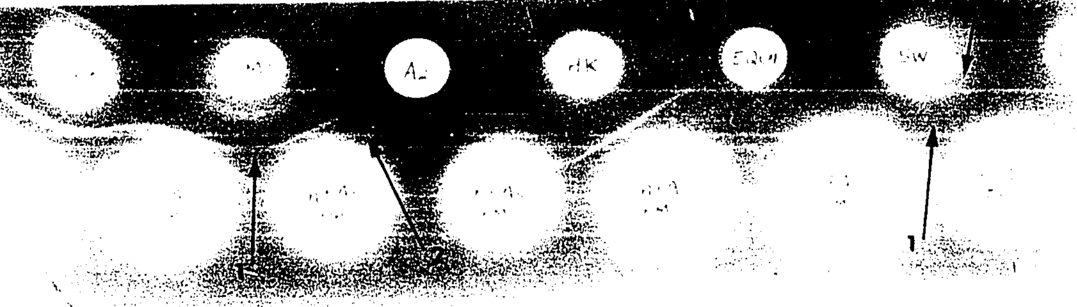
hFAs PR8
hFAs PR8
hFAs PR8
C



A2
HK
SW
1

PLATE 15

Initial screening of influenza A cross reactions using 25- and 13-well template patterns.

A. Anti-Duck convalescent rabbit serum (top row) forms the single linking major type-specific precipitin reaction with all influenza A concentrates, but not with B or Sendai (centre row). A secondary independent antigen is precipitated in the homologous reaction (top right). Note barbs in the type-specific band at the following linkages: PR8-FM1, FM1-A2, A2-HK, Swine-Duck (arrows B). Anti-B convalescent rabbit serum (bottom row) forms only a discrete homologous precipitin band (bottom left), and does not react with any influenza A strains.

B. Immunoprecipitin reaction resulting from different juxtapositions of antigens and antisera are demonstrated.

Each one of six influenza A concentrates (inner ring of wells) is diffused against its homologous hyperimmune rabbit serum (abs) (outer ring of wells), and against anti-FM1 serum (centre well). The linking type-specific precipitin band forms a peripheral star pattern (arrows 1) when the antisera are peripheral, and forms a hexagonal pattern (arrow 5) when the antiserum is central, in relation to the antigen position. Irregularity in the continuity of the peripheral major type-specific band occurs in three places (arrows 2, 3, 4). Note the number of secondary precipitin bands formed in homologous and cross reactions when the antisera are positioned peripherally with respect to the viruses than when an antiserum is positioned centrally.

C. The influenza B homologous reaction remains discrete whether the homologous antiserum is peripheral or central in relation to the antigen. The band arrowed 2 could be the influenza B type-specific reaction. Anti-B hyperimmune rabbit serum (abs) does not precipitate any influenza A strains, either when it is centrally positioned in relation to five influenza A concentrates, or when it is peripherally positioned in relation to A/PR8 C_2. Conversely, influenza B components are not precipitated by anti-Swine serum. The major influenza A type-specific band formed when each influenza A concentrate is diffused against its homologous serum and adjacent other anti-influenza A serum, links all around in a star-like pattern and bears no relation to the influenza B reactions.

(Coomassie blue stain)

PLATE 15

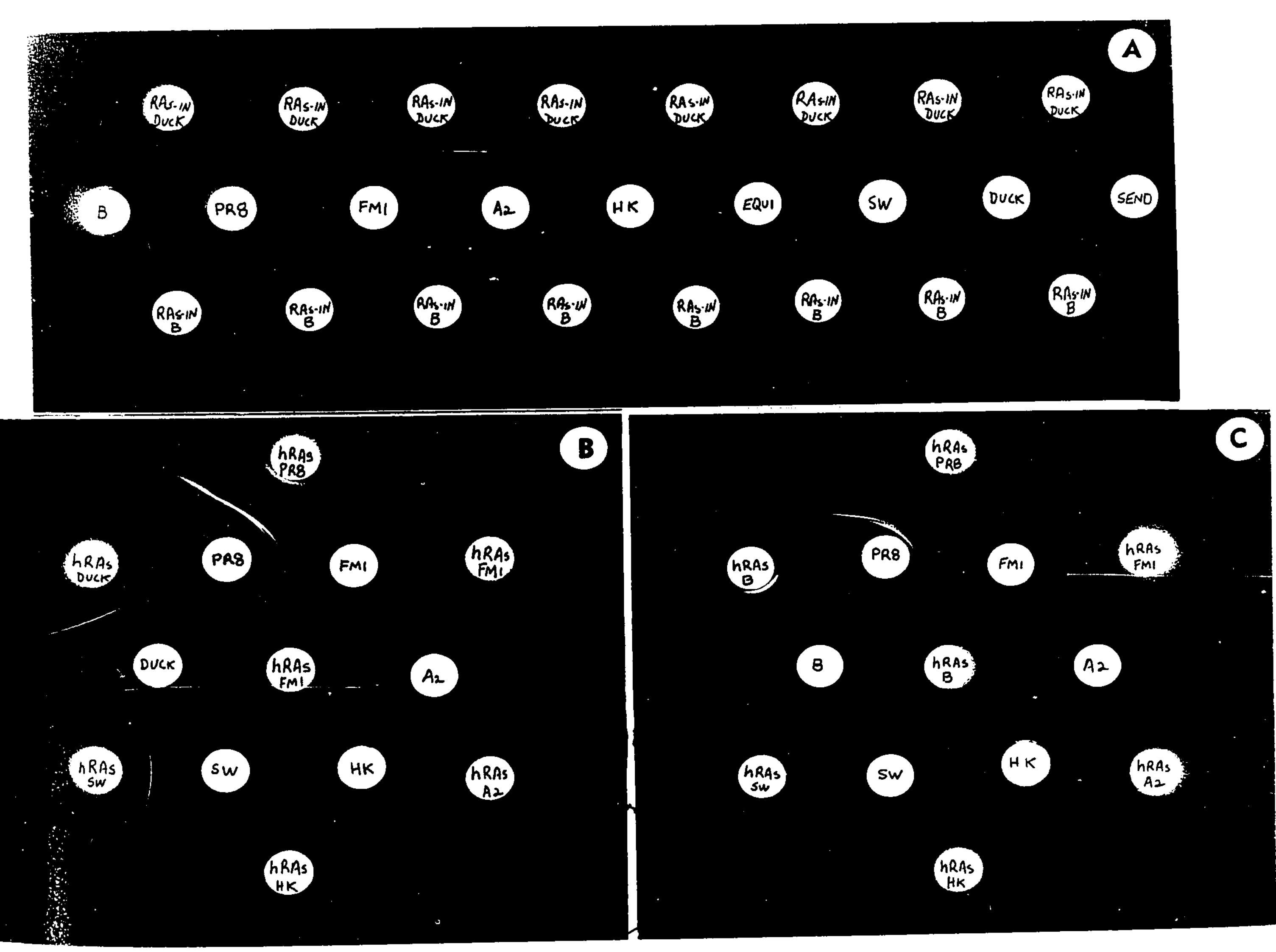


PLATE 16

Initial screening (A) and detailed studies (B-D) of influenza A/Swine cross reactions using semi-purified and purified virus concentrates, undisrupted and Nonidet P40 disrupted, and hyperimmune rabbit sera (abs).

A. Cross reactions between anti-Swine hyperimmune rabbit serum (abs) (top and bottom rows) and influenza A concentrates (centre row), yield the linking major influenza A type-specific reaction (arrows 1), which does not form with influenza B or Sendai. The linkage is best seen in the bottom reaction because the top half of the reaction has "swept out" towards the top of the slide, a phenomenon encountered when using this template pattern. Note the secondary strong independent band formed by the homologous reaction. Other secondary precipitin bands are also formed in homologous and cross reactions and are in the same general area as the type-specific band.

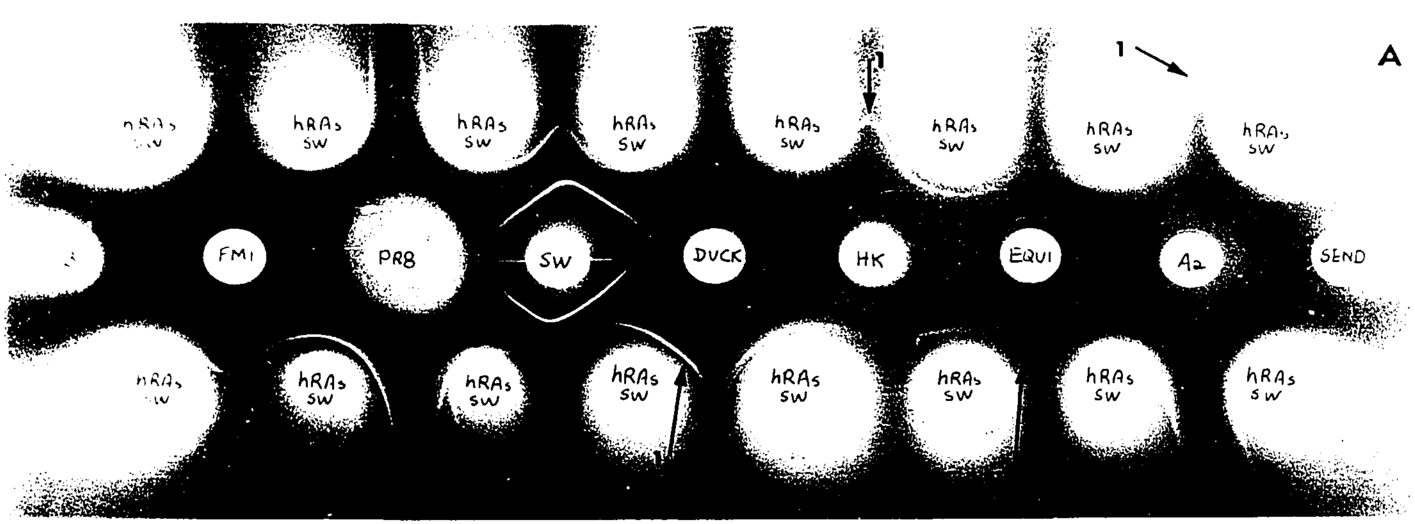
B. Anti-Swine hyperimmune rabbit serum (abs) precipitates the major type-specific component of all influenza A strains, PR8, FM1, A2/Can/57, Equi, Duck and Swine to form a band which links all strains except Swine and Duck, and is very faint at the Swine-Equi linkage (arrows 1). The antiserum precipitates a secondary PR8 and a Duck component (arrows 2). A precipitated independent homologous component lies adjacent to the antigen well.

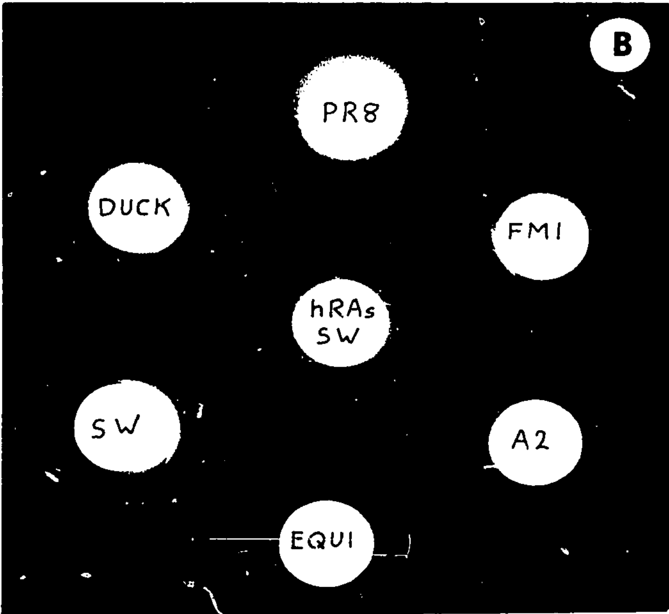
C. The major type-specific component of NP 40 disrupted semi-purified A/Swine (centre well) is precipitated by all anti-influenza A hyperimmune rabbit sera (abs) (peripheral wells) to form a linking precipitin band (arrows 1). In the homologous reaction only, two secondary components are precipitated to from independent bands adjacent to the antiserum well. Non-specific precipitation of serum and/or virus components form a ring around the central antigen well (NS).

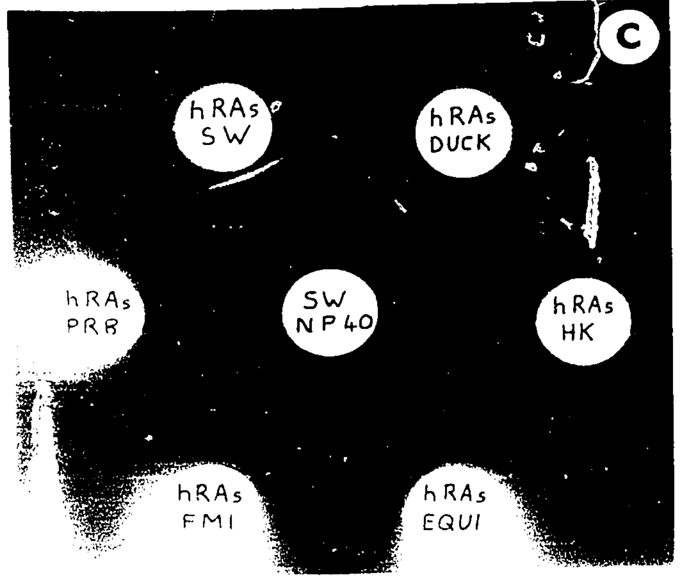
D. Using identical components to (C) above, but substituting NP 40 disrupted purified for semi-purified virus, a slightly different diffusion pattern results. The type-specific component is precipitated by all anti-influenza A sera to form a band which links between each antiserum (arrows 1) except between A2/HK/1/68 and Equi. A secondary Swine component, precipitated by the homologous antiserum, and by anti-PR8 serum, forms bands which link in identity (arrow 2), implying that a common anti-Swine antibody exists in each antiserum. A similar secondary Swine component is precipitated by anti-A2/HK/1/68 serum which could be the same one (arrow 2), but the linkage is obscured, because the band runs under the type-specific one formed by anti-Duck serum. Two independent bands lying adjacent to the homologous antiserum well, are precipitated independent components unique to Swine virus. The non-specific precipitation by NP 40 of antisera and/or antigen components forms a double ring of precipitate around the central antigen well (NS).

(A, C, D, Coomassie blue stain; B, thiazine red R stain)

PLATE 16







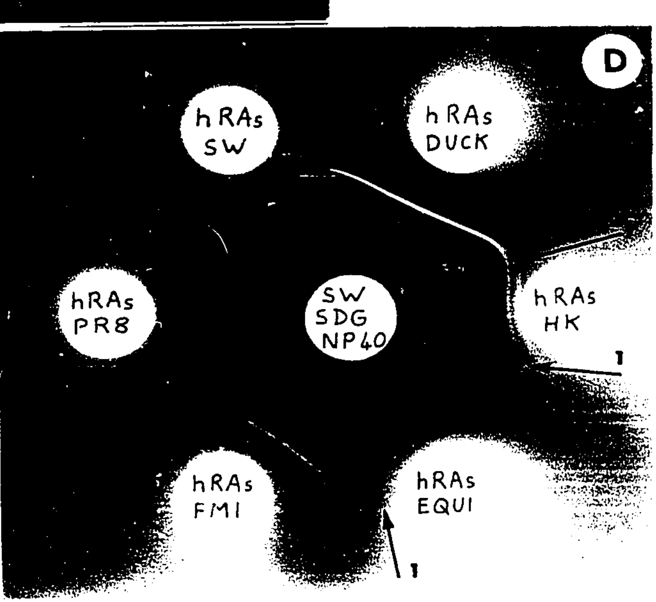


PLATE 17

Initial screening (A) and detailed studies (B-D) of influenza A2/Hong Kong/1/68 and A2/Can/57 cross reactions using Nonidet P40 disrupted semi-purified and purified virus concentrates and hyperimmune rabbit sera (abs).

A. The linking major type-specific influenza A reaction (arrows 1) is clearly formed between anti-A2/Can/57 serum (top row), and influenza A concentrates (centre row), but not with influenza B or Sendai virus. Secondary components of PR8, Swine and Duck are also precipitated by the antiserum.

Similarly the type-specific component of all influenza A strains is precipitated by anti-A2/HK/1/68 hyperimmune rabbit serum (abs) (bottom row) to form a linking precipitin band (arrows 1) which did not extend to B or Sendai virus. A secondary component precipitated by the homologous reaction rings the antigen well. A secondary Duck component is also precipitated.

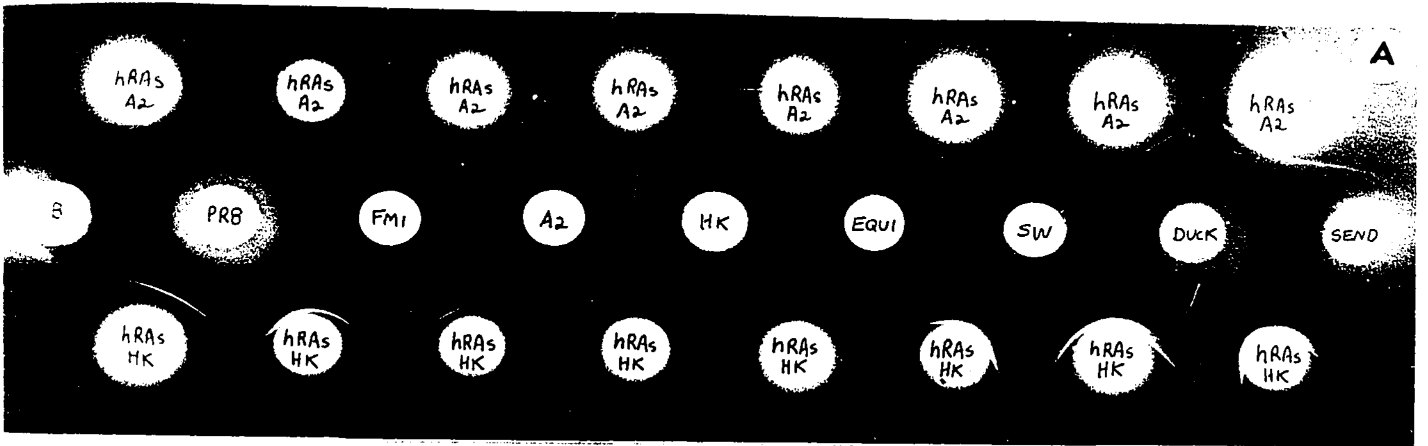
B. When NP 40 disrupted semi-purified A2/HK/1/68 (centre well) is diffused with anti-influenza A sera (peripheral wells), the major type-specific reaction forms a band which clearly links all around. Anti-PR8 serum precipitated two secondary components as bands (arrows 2 and 3). One of these (arrow 2), links with a similar linking precipitin band formed when the HK component was precipitated by anti-Equi serum, and by the homologous antiserum. The other band (arrow 3), links to one formed when the same component was precipitated by anti-Duck serum. Anti-A2/Can/57 serum precipitated a component to form a band (arrow 4) which linked to the homologous reaction. Note typical rings around the antigen well (NS), formed by non-specific precipitation of serum and/or antigen by NP 40.

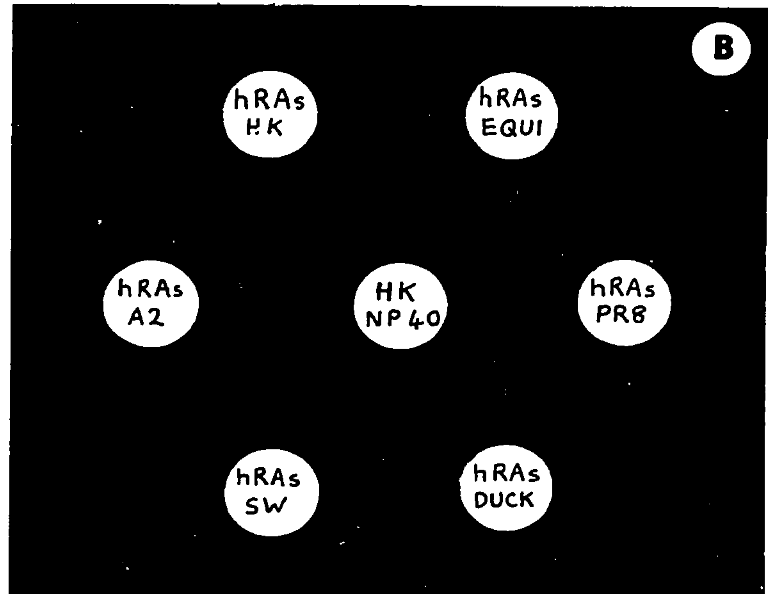
C. A similar reaction to (B), but substituting NP 40 disrupted purified for semi-purified A2/HK/1/68 (centre well), and anti-FM1 serum for anti-Duck serum (lower right). A much weaker precipitin pattern is produced using a weaker antigen. The major type-specific reaction is weak (arrows 1), barely visible when the type-specific component is precipitated by anti-Swine or anti-PR8 serum, and is not visible when precipitated by anti-FM1 serum. An independent component is precipitated by the homologous reaction. Note the non-specific NP 40 precipitation around the central well (NS).

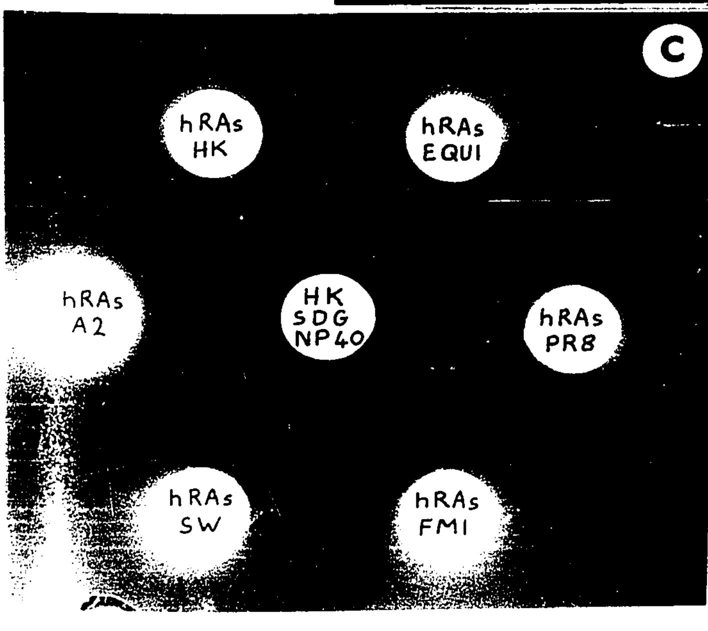
D. The major type-specific component of NP 40 disrupted A2/Can/57 (centre well) is clearly precipitated by all anti-influenza A sera to form a linking precipitin band (arrows 1). Two secondary components precipitated in the homologous reactions form linking bands, which link with those formed by the precipitation of two similar components by anti-Equi serum (arrows 2 and 3). One of these bands (arrow 3) is linked to similar linking ones formed when the same component is precipitated by anti-PR8, anti-FM1, and anti-A2/HK/1/68 sera. In addition, an independent band in the homologous reaction is yielded by the precipitation of a component unique to A2/Can/57. Note characteristic rings around the antigen well resulting from NP 40 non-specific precipitation.

(Coomassie blue stain)

PLATE 17







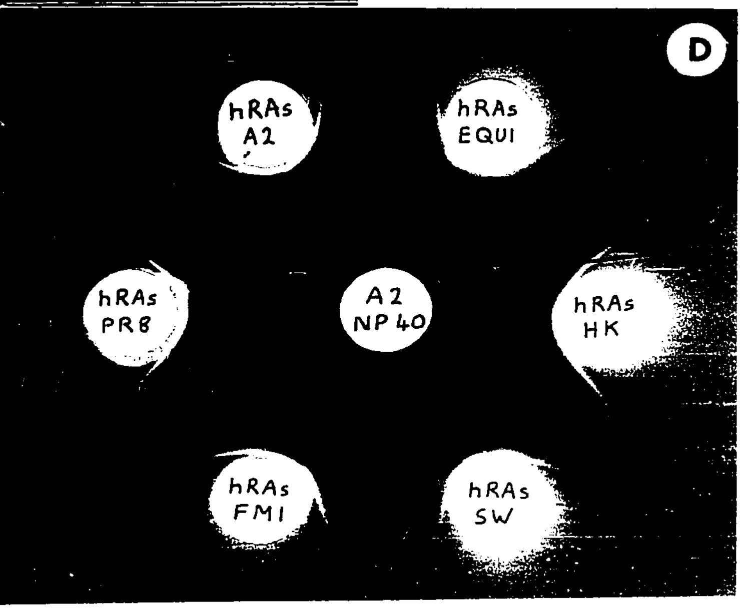


PLATE 18

Immunoprecipitin patterns formed by 1) changing relative positions of virus antigens and antiviral antisera, and 2) using undisrupted and Nonidet P40 disrupted concentrates, semi-purified and purified.

A. When centrally positioned anti-FM1 hyperimmune rabbit serum (abs) is diffused against peripherally positioned influenza A concentrates, the major type-specific component of each strain is precipitated by the serum to form a single linking band (arrows 1). A secondary independent component is precipitated in the homologous reaction (arrow 2). Very weak precipitation of a PR8 component forms a faint band lying just inside the type-specific reaction (arrow 3).

B. More components are precipitated when FM1 concentrate is central, and anti-influenza A sera are peripheral. The FM1 major type-specific component is precipitated by all antisera to form a linking band (arrow 1). Anti-Swine serum precipitates three secondary FM1 components to form bands lying outside the type-specific one: one (arrows 2) is precipitated by anti-Duck, anti-PR8, and anti-FM1 sera to form a linking band; another (arrow 5) is precipitated by anti-Duck serum to form a linking band; and a third (arrow 3) is independent. Anti-Duck serum also precipitates an independent FM1 component (arrow 4).

C. Using the same relative positions of antigen and antisera as in (B), but substituting NP 40 disrupted FM1 for undisrupted C_2 changes the immunoprecipitin pattern slightly. The major type-specific reaction (arrowl) now lies adjacent to the antiserum wells, and the secondary reactions are inside it. Fewer secondary FM1 components are precipitated: two by anti-Duck serum, and one by the homologous antiserum. The characteristic NP 40 non-specific precipitation rings the central well.

D. Using the same relative arrangement of antigen and antisera as in (B) and (C), but substituting NP 40 disrupted purified FM1 concentrate for semi-purified, a pattern resembling (B) rather than (C) is obtained. The major type-specific reaction (arrow 1) is weaker because of the weaker antigen used, but it still forms a linking precipitin band when precipitated by the antisera. Secondary components are precipitated only in the homologous reaction, and form bands which lie adjacent to the antiserum well, and outside the type-specific reaction.

(A, B, thiazine red R stain; CD, coomassie blue stain)

PLATE 18

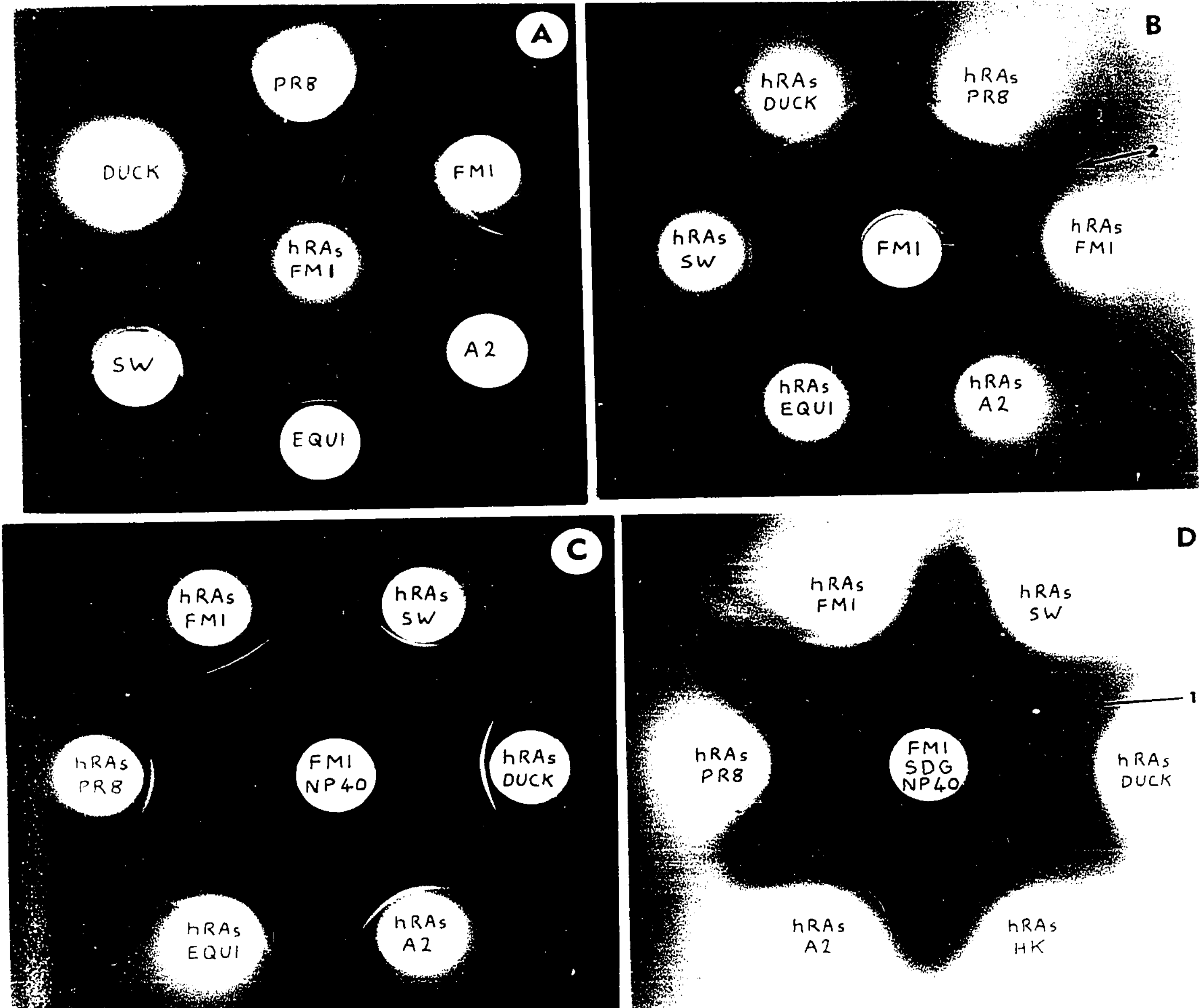


PLATE 19

Polyacrylamide gel electrophoresis of semi-purified influenza A, B, parainfluenza Sendai, and N-ALF concentrates, SDS-β-mercaptoethanol-heat disrupted.

Gel lengths: 6.5 cm
BSA : bovine serum albumin, Fraction V (0.1% w/v aqueous)

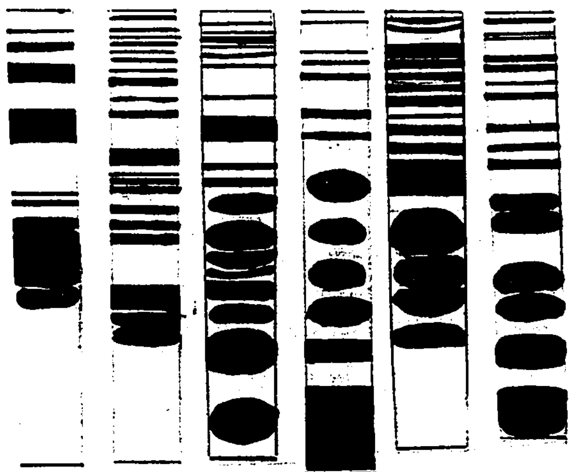
Drawings (top) and Polaroid photographs (bottom) of gels are compared.

See text p 227 for description

Gels are coomassie blue stained.

The direction of migration is toward the anode, from top to bottom.

PLATE 19



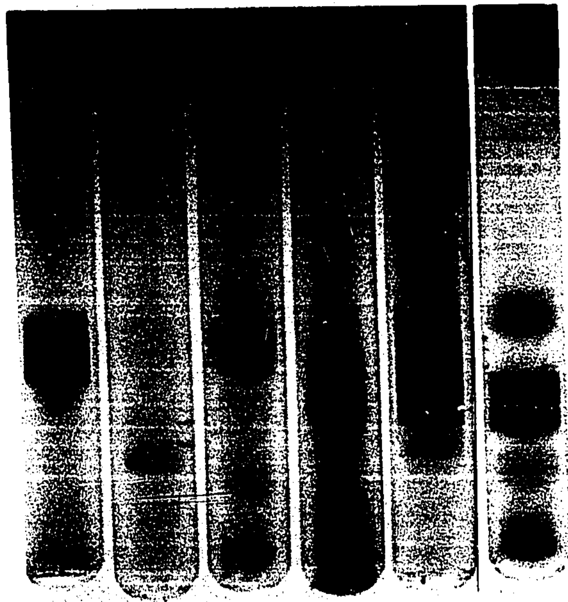


PLATE 20

Polyacrylamide gel electrophoresis of purified (SDG) influenza A strains, influenza B, parainfluenza Sendai, and N-ALF. A. SDS - β-mercaptoethanol-heat disrupted. B. SDS disrupted.

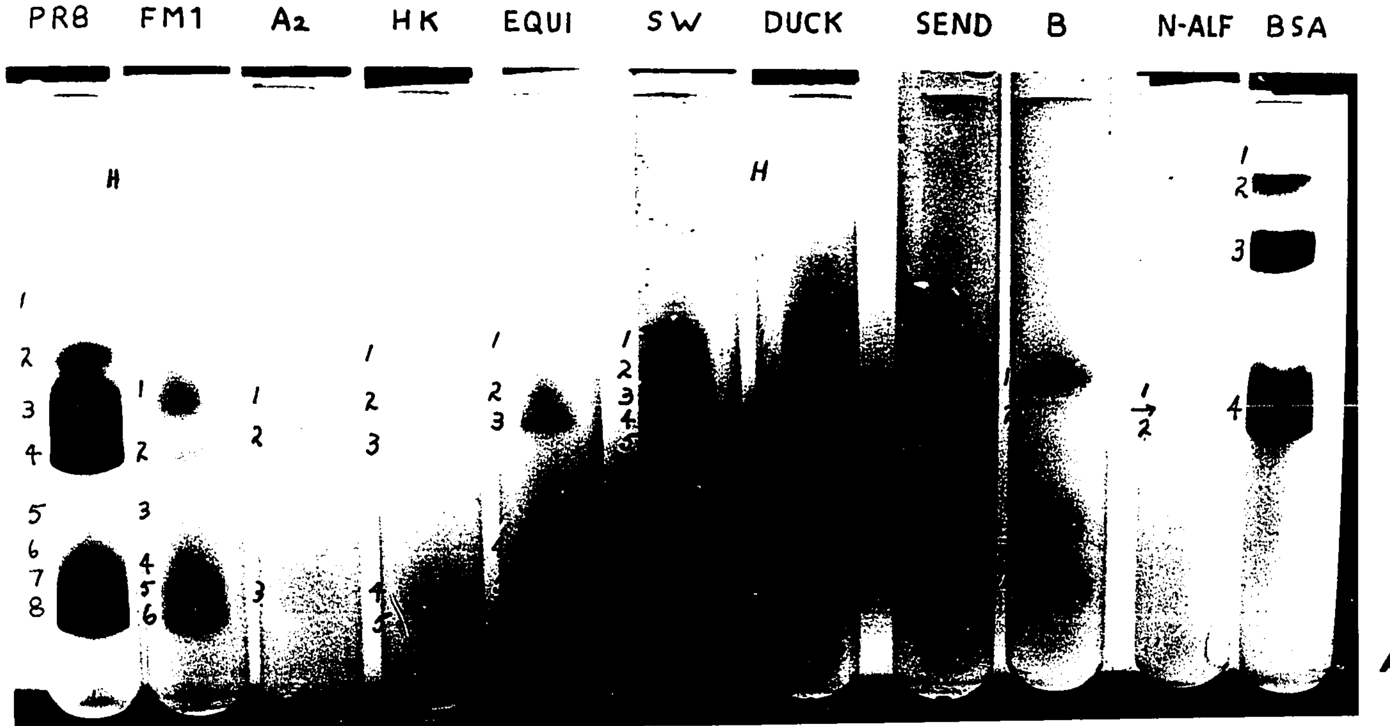
Gel lengths: A.6.8 cm. B. 6.5 cm
BSA : bovine serum albumin, Fraction V (0.1%) in distilled water
H : host (CE) component

Protein bands are numbered on the left from top to bottom of each gel.

Gels are coomassie blue stained.

In (B), fractions cut out of ANA stained gels are marked: 4
The direction of migration is toward the anode, from top to bottom
See text, p 228 for description.

PLATE 20



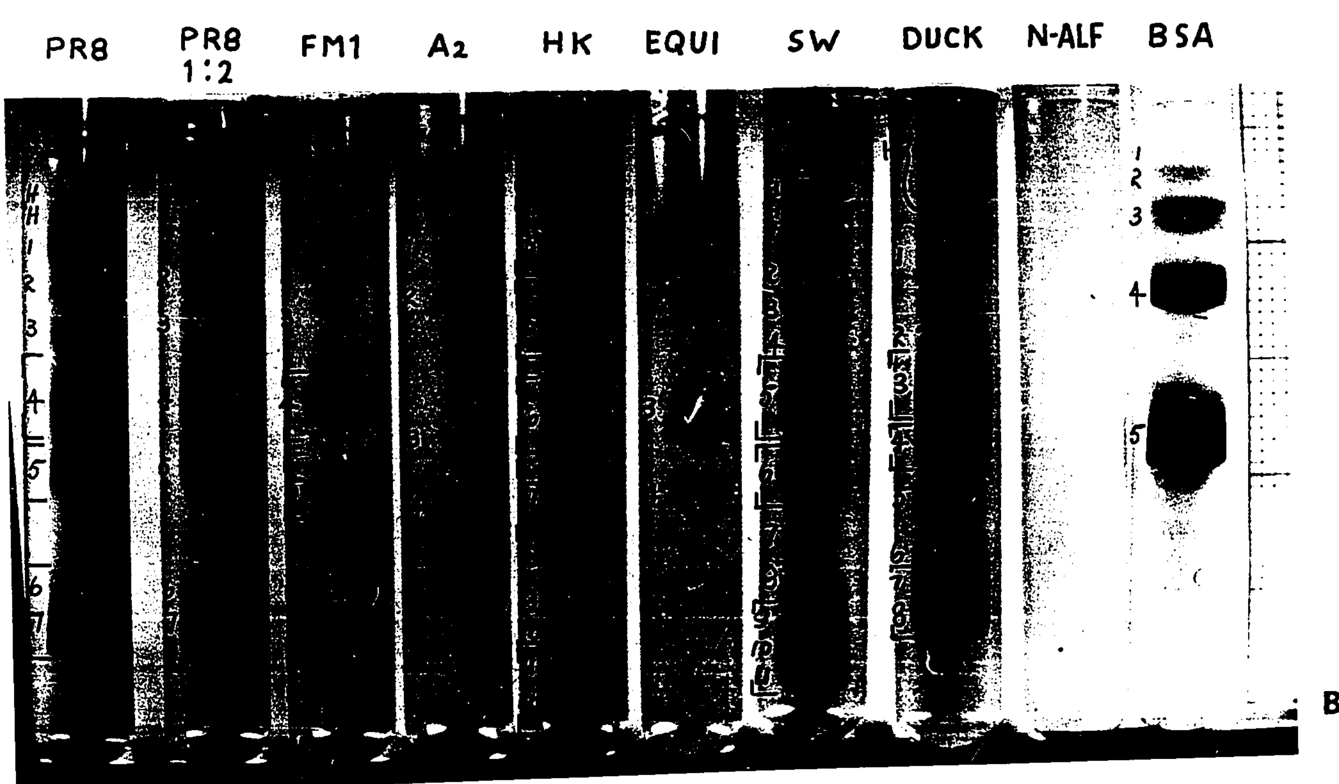


PLATE 21

Typical immunodiffusion reactions demonstrating precipitins against influenza viruses present in normal animal sera.

Normal sera (outer wells); A7, H25, 55, etc. are lab code nos.
NRS : rabbit
NCS : cat
NDS : dog
NBS : bovine
NSS : sheep

Ht : Serum heated at 58 - 60°C x 30 min.

Antigens in centre wells

A. One or more precipitin bands are formed between normal rabbit sera and HK. At least one links all around (arrowed) indicating that a common component in all sera is precipitating the antigen.

B. NCS H27 forms 3 precipitin bands with HK, the innermost one linking in identity with that formed by adjacent NCS H26 and H28.

C. Heating normal dog sera did not affect the precipitin band formed with PR8, nor did heating of bovine serum alter its negative reactivity.

D. Precipitin bands formed between normal sheep sera and DUCK (arrowed) do not link because the reaction sweeps out. However, the precipitating components in the sera are considered to be the same because the bands are in the same relative position and plane.

(A, coomassie blue stain; B, C, D, thiazine red R stain)

PLATE 21

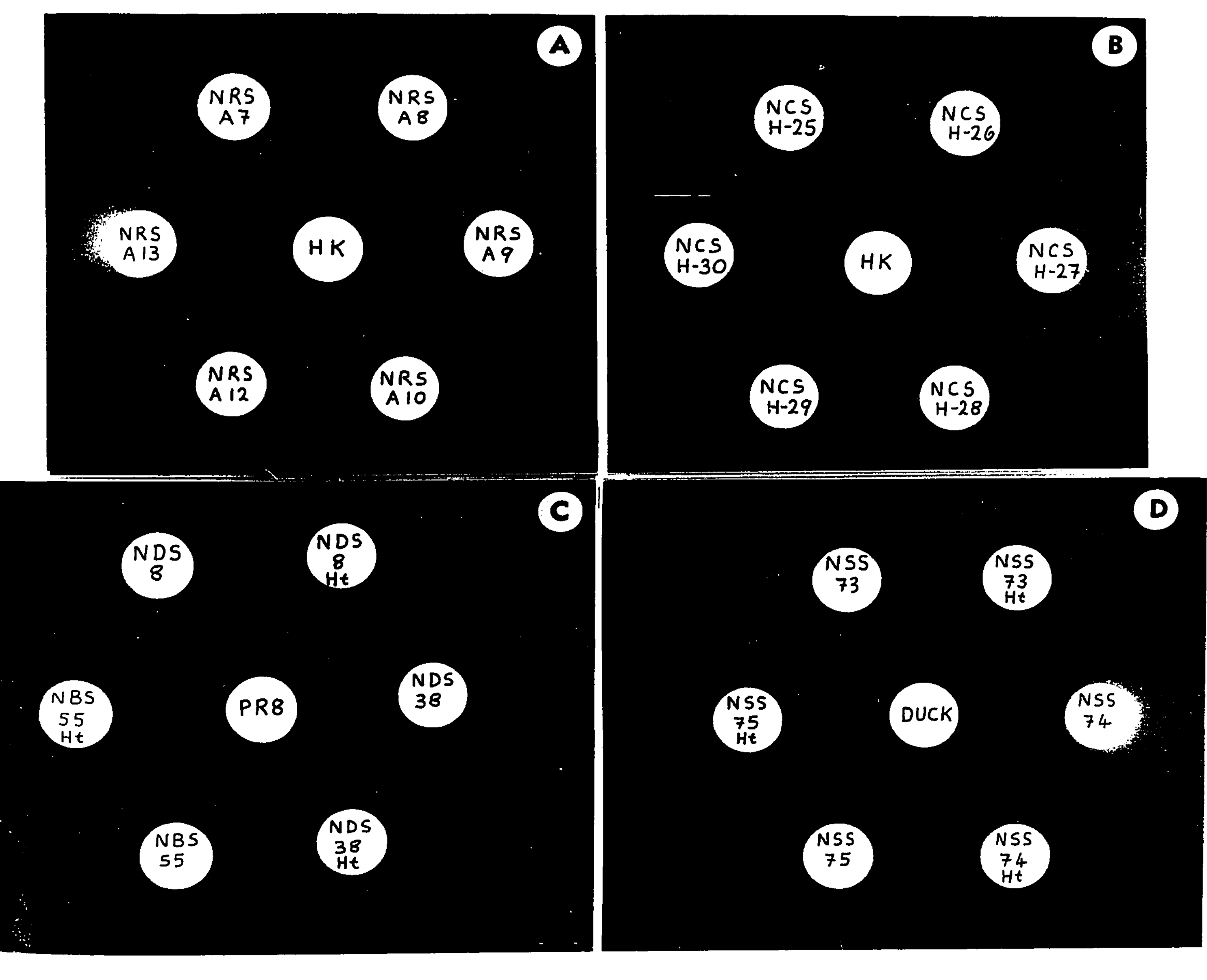


PLATE 22

Comparison of the precipitation of influenza A2/Hong Kong/1/68 concentrate by: 1) normal animal sera, native and treated for inhibitors, 2) homologous immune sera of rabbits and roosters, 3) human gamma-globulin (Lederle) (γ-G/L), and 4) human gamma-globulin (Schwarz-Mann) (γ-G/SM).

1:5 : this dilution of serum in PBS
THP : trypsin-heat-periodate treated serum
RDE : RDE (V. cholerae filtrate) treated serum
$NaIO_4$: sodium metaperiodate treated serum
Kaolin : kaolin adsorbed serum

A. NDS H62 : normal dog serum H62

Two linking precipitin bands formed between A2/Hong Kong/68 concentrate and normal dog, convalescent and hyperimmune rabbit sera, and human gamma-globulin indicates that each serum contains two common precipitating components. Inhibitor treatments of dog sera (THP and RDE) eliminate the outer precipitin band suggesting it could be inhibitor initiated. The inner one is not eliminated which suggests it is antibody initiated.

B. NFS 115 : normal fowl serum 115
FCS : foetal calf serum

A common precipitin band is formed with the antigen by all sera except foetal calf, which forms an independent inner band. RDE treatment does not eliminate the common component found in normal fowl and hyperimmune fowl sera, and in human gamma-globulin. The common precipitin could be antibody to influenza A2/Hong Kong/68.

C. NRS A46 : normal rabbit serum A46

The precipitin band formed by native normal rabbit serum and A2/Hong Kong/68 concentrate is fuzzy when the serum is THP or RDE treated, but is unaffected when the serum is kaolin treated. The precipitin bands of these reactions link amongst themselves and with a precipitin band formed between the antigen and homologous convalescent and hyper-immune rabbit sera. The common precipitin present in all sera is very likely influenza A2/Hong Kong/68 antibody.

D. NCS H27 : normal cat serum H27

Human gamma globulin is diluted 1:5 in PBS to 4 mg/ml. Several precipitin bands are formed by normal cat serum which link with those formed by human gamma-globulin. The outermost one is eliminated by dilution of the cat serum. Only one precipitating component survives all inhibitor inactivation treatments becoming very weak in RDE treated sera as reflected by the faint precipi-tin band produced (arrowed). Two components common to normal cat serum and human gamma-globulin are thereby demonstrated and are very likely antibody to A2/Hong Kong/68.

(A, B, D, coomassie blue stain; C thiazine red R stain)

PLATE 22

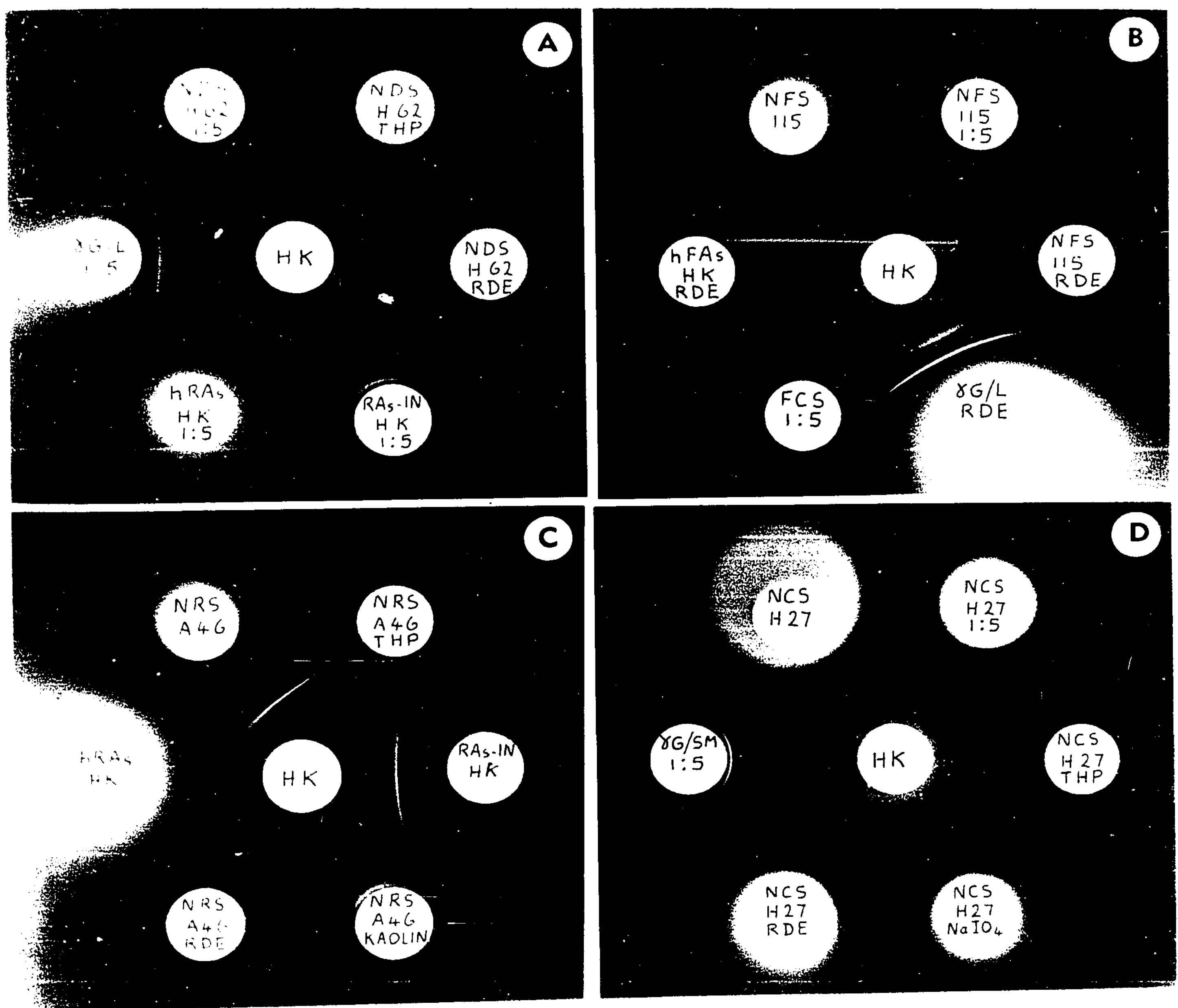


PLATE 23

1:5	: this dilution of serum in PBS
THP	: trypsin-heat-periodate treated serum
RDE	: RDE (V. cholerae filtrate) treated serum
$NaIO_4$	: sodium metaperiodate treated serum
γ-G/L	: human gamma-globulin, Lederle
γ-G/SM	: human gamma-globulin, Schwarz Mann

A. Comparison of the precipitation of influenza A/PR8 concentrate by: 1) normal goat serum, native and treated for inhibitors, 2) homologous convalescent rabbit serum, 3) homologous hyperimmune rabbit serum (abs), and 4) human gamma-globulins

NGS 2 : normal goat serum

Goat serum, diluted or treated for inhibitors, by THP or RDE, precipitates PR8 antigen to form a linking precipitin band. The linkage continues with identical precipitin bands formed when the same antigen is precipitated by gamma-globulin, and by hyperimmune rabbit serum. An identical precipitin exists in all these preparations and may be PR8 antibody. Note that γ -G/L forms two bands which fuse (arrow 1, 2) on linkage with the band formed by diluted goat serum, implying two precipitin components in the gamma-globulin are precipitating the antigen. Two different antibodies may be precipitating the same antigen as γ-G/L contains IgG, IgM, and IgA.

B. Effect of dilution and RDE treatment on immunoprecipitin reactions between A2/Hong Kong/1/68 and normal cat serum, and human gamma-globulin preparations.

NCS H27 : normal cat serum H27

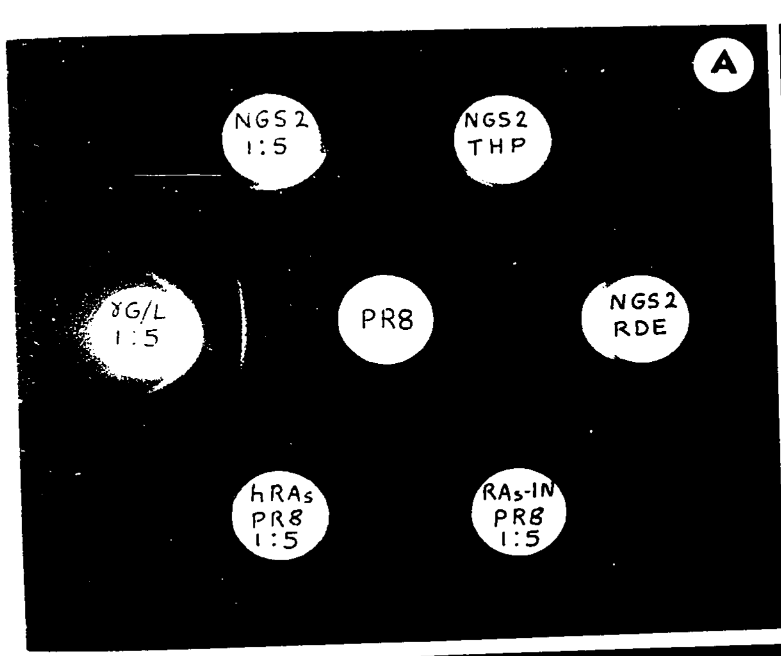
Two precipitin bands are formed with A2/Hong/Kong/68 concentrate by native cat serum and both human gamma-globulins. Linkage of identity confirms that they contain an identical precipitin. RDE treatment of gamma-globulins has not affected the immunoprecipitin pattern found with the antigen. RDE treatment of human serum is recommended for removal of A2/Hong Kong/68 inhibitors, therefore the precipitins must be antibody to A2/Hong Kong/68. Dilution of cat serum has eliminated the outer precipitin band and makes it difficult to assess the effect of RDE treatment. The inner linking band has been weakened by dilution and RDE treatment of the serum (arrowed), but is not eliminated. Both precipitin bands could be caused by antibody and thus all serum and immune preparations contain antibody to A2/Hong Kong/68 antigens.

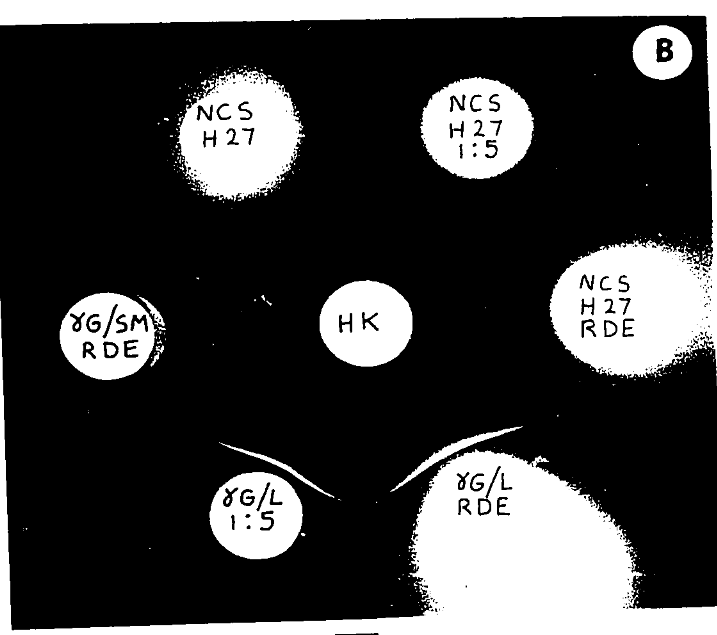
C. NES 3 : normal horse serum
γ/Horse : horse (equine) gamma-globulin

Effect of dilution (1:5), RDE and $NaIO_4$ treatment on immunoprecipitin reactions between A2/Hong Kong/1/68 and horse gamma-globulin. The outermost precipitin band formed on precipitation of HK by horse gamma-globulin is unaffected when the immune preparation is treated with RDE or $NaIO_4$, suggesting that the precipitating component is an antibody. The elimination of a weaker, inner band when the gamma-globulin is $NaIO_4$ treated suggests that the precipitating component in this reaction is an inhibitor sensitive to $NaIO_4$, which also seems to be present in the normal horse serum.

(A, thiazine red R stain; B, C, coomassie blue stain)

PLATE 23





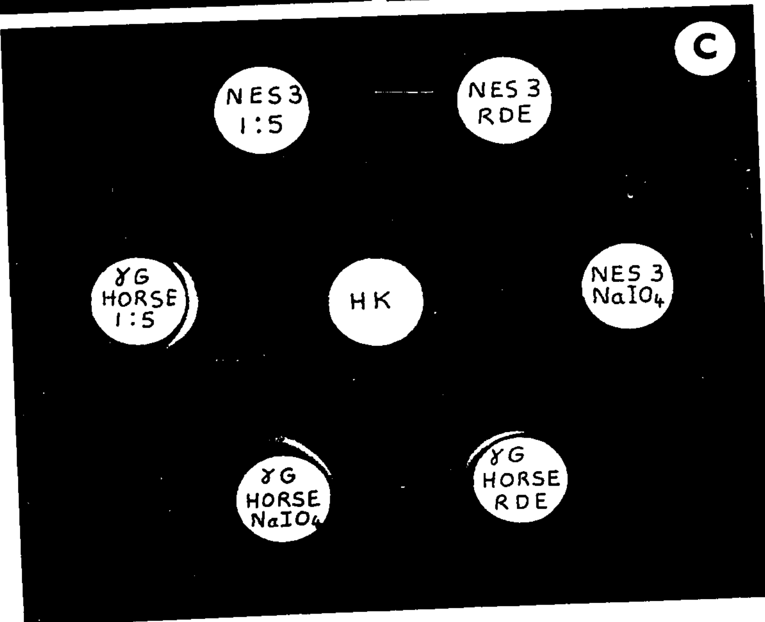


PLATE 24

Identification of the type-specific antigen of influenza A2/HK/1/68 by a comparison of the following immunoprecipitin reactions: homologous immune rabbit sera and whole virus concentrate; homologous immune rabbit sera and soluble antigen.

cr: crude soluble antigen
cl: clarified soluble antigen

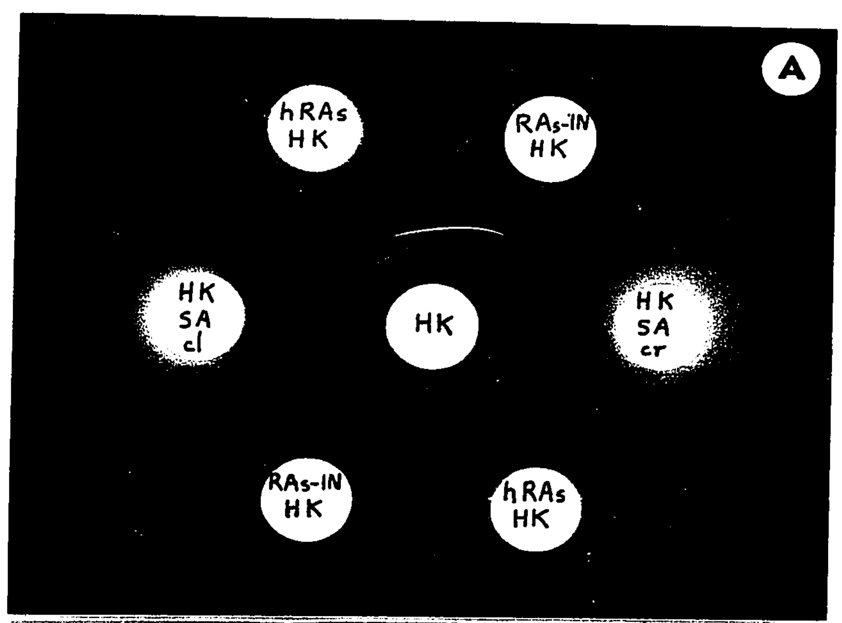
A. The type-specific reaction (arrowed) identifies the major type-specific component in the immunoprecipitin pattern produced when anti-A2/Hong Kong/1/68 antisera are diffused against homologous virus concentrate and virus soluble antigens.

B. Identification, by immunodiffusion, of the major type-specific reaction between influenza A soluble antigens and anti-A2/Hong Kong/1/68 antisera.

A single precipitin band linking all influenza A soluble antigens, but absent in reactions with influenza B and parainfluenza Sendai soluble antigens, is the major influenza A type-specific reaction.

(Coomassie blue stain)

PLATE 24



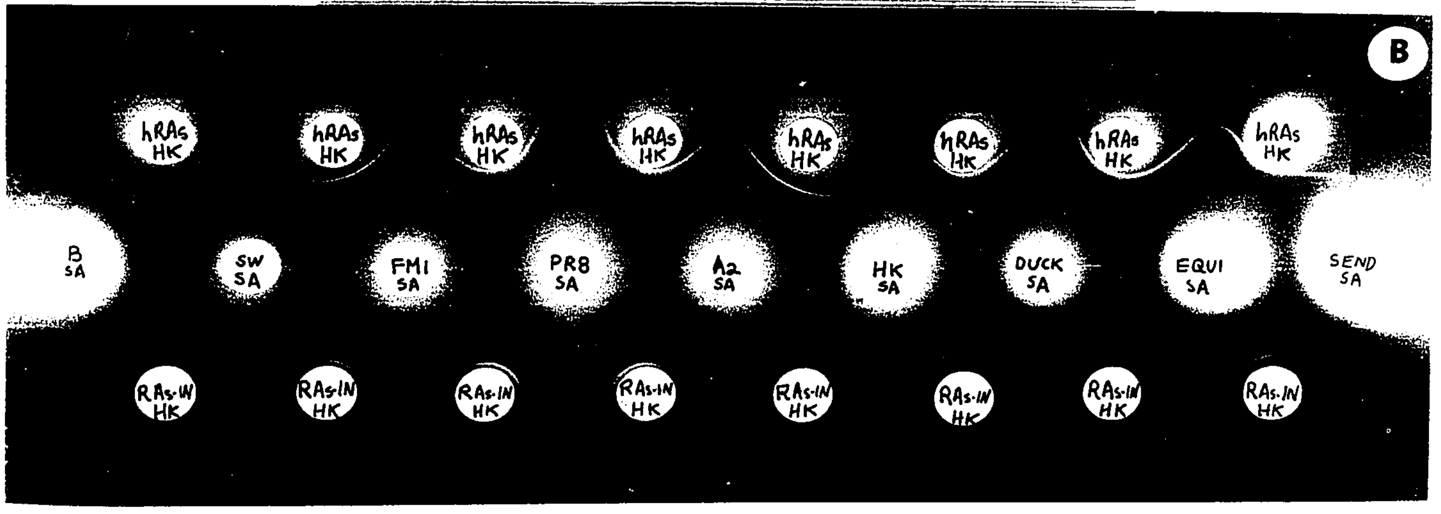


PLATE 25

Determination of immunoglobulin constitution of commercial preparations of human gamma-globulins by immunodiffusion using specific anti-human immunoglobulin goat antisera (Hyland).

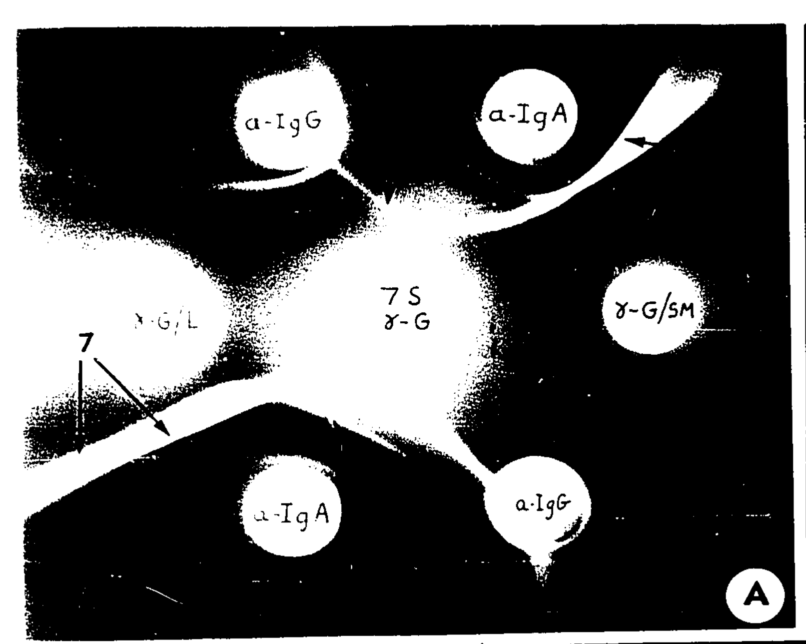
7 S γ-G	: 7 S gamma-globulin (Schwarz Mann) at 30 mg/ml
γ-G/SM	: gamma-globulin (Schwarz Mann) at 30 mg/ml in A, B, at 20 mg/ml in C
γ-G/L	: gamma-globulin (Lederle) at 1:2 dilution
a-IgA	: goat anti-human IgA α chain specific antiserum
a-IgG	: goat anti-human IgG γ chain specific antiserum
a-IgM	: goat anti-human IgM μ chain specific antiserum

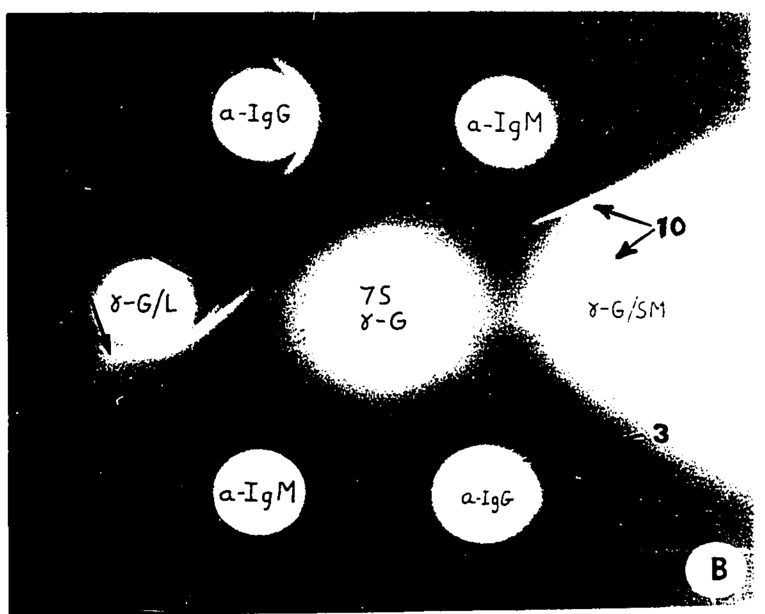
All a-Ig sera marked 1:5 are at this dilution in PBS pH 7.3

See Identification of Influenza Antibodies in Immune Preparations for complete explanation (p.354).

(Coomassie blue stain)

PLATE 25





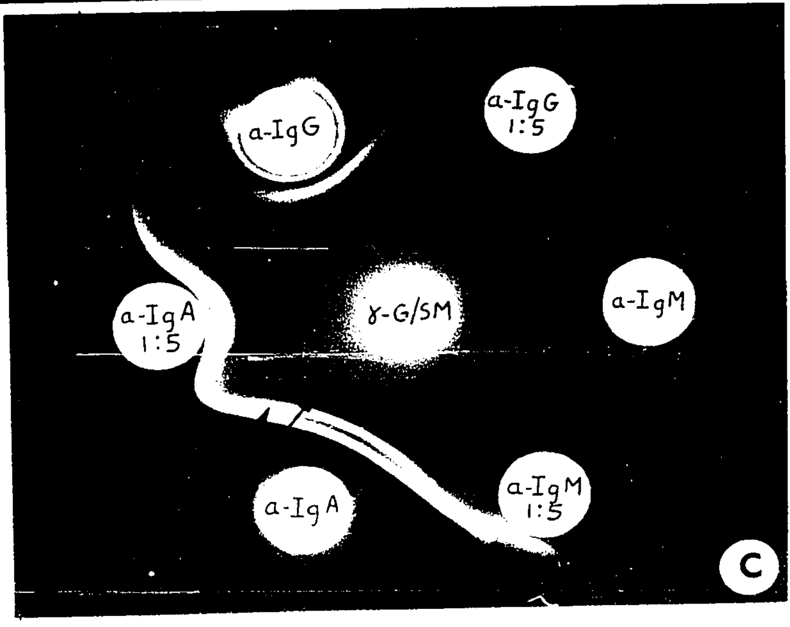


PLATE 26

Determination, by immunodiffusion, of the anti-influenza virus spectrum of human gamma-globulin commercial preparations, and a comparison of these immunoprecipitin reactions with those specific for human IgA, IgG, and IgM.

7 S γ-G : human 7 S gamma-globulin (Schwarz/Mann) at 40 mg/ml
γ-G : human gamma-globulin (Schwarz/Mann) (γ-G/SM) at 30 mg/ml
a-IgA : goat anti-human IgA α chain specific serum (Hyland)
a-IgG : goat anti-human IgG γ chain specific serum (Hyland)
a-IgM : goat anti-human IgM μ chain specific serum (Hyland)

A, B. Arrow 1 : 7 S γ-G virus antigen precipitin band cuts IgA-specific band

Arrow 2 : γ-G virus antigen precipitin band cuts IgA- specific band

B. Arrow 3 : γ-G virus antigen precipitin band has suggestive link with IgG-specific reaction

C. Arrow 1 : 7 S γ-G virus antigen precipitin band has no reaction with anti-human IgM

Arrow 2 : γ-G virus antigen precipitin band remains discrete from IgM-specific band and does not react with anti-human IgM

See text, p.359 for description of reactions of virus antigens diffused against 7 S γ-G and against γ-G (γ-G/SM)

(Coomassie blue stain)

PLATE 26

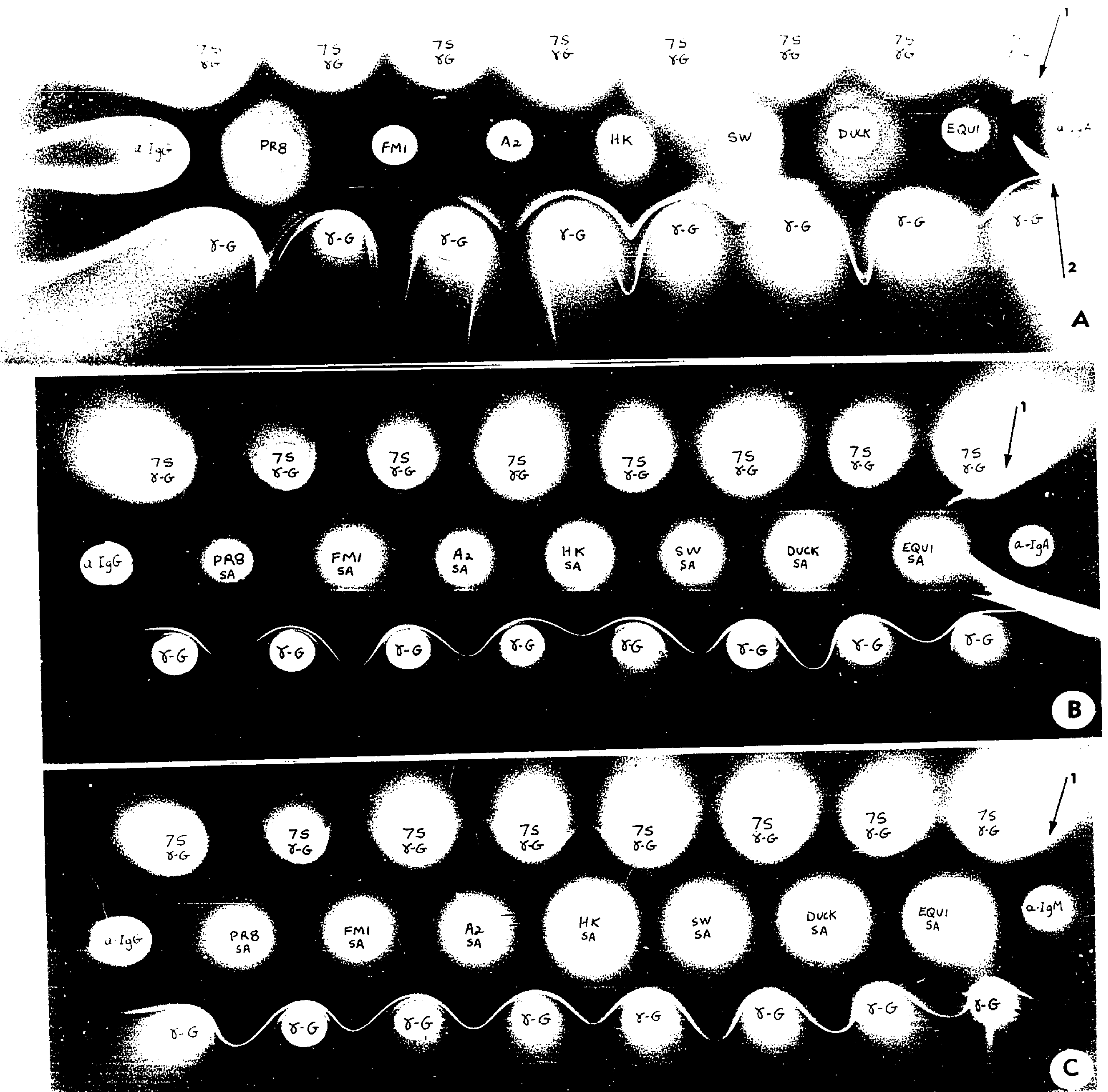


PLATE 27

A comparison, by immunodiffusion, of the anti-influenza spectrum of paired human sera: influenza acute phase with the convalescent phase; or normal serum with influenza convalescent phase serum.

Acute (A) or normal sera (NHS) are in the top rows, and convalescent sera (C) are in the bottom rows. Virus concentrates in centre rows
A2 OTT68 : influenza A/Ottawa/68

A. An increased precipitin response is seen in convalescent serum VP 68C from that of acute phase serum VP 68A. The major influenza A type-specific band links across all influenza A viruses except Equi 1, cutting the influenza B reaction in non-identity (arrowed). A2/HK/1/68 was isolated from the patient (Table 32).

B. Another example of the increase in precipitin response by convalescent serum (VP 66C) from that of acute phase serum (VP 66A) to influenza A strains but not to influenza B. Influenza A & B reactions remain discrete and cut each other in non-identity (arrowed). A2/HK/1/68 was isolated from this patient (Table 32).

C. Marked difference between "normal" human serum (DK/NHS) and convalescent human serum (DK/C) precipitin response to influenza A virus strains, but no change to influenza B or parainfl. Sendai. The major A type-specific component is not easily traced in this reaction.

PLATE 27

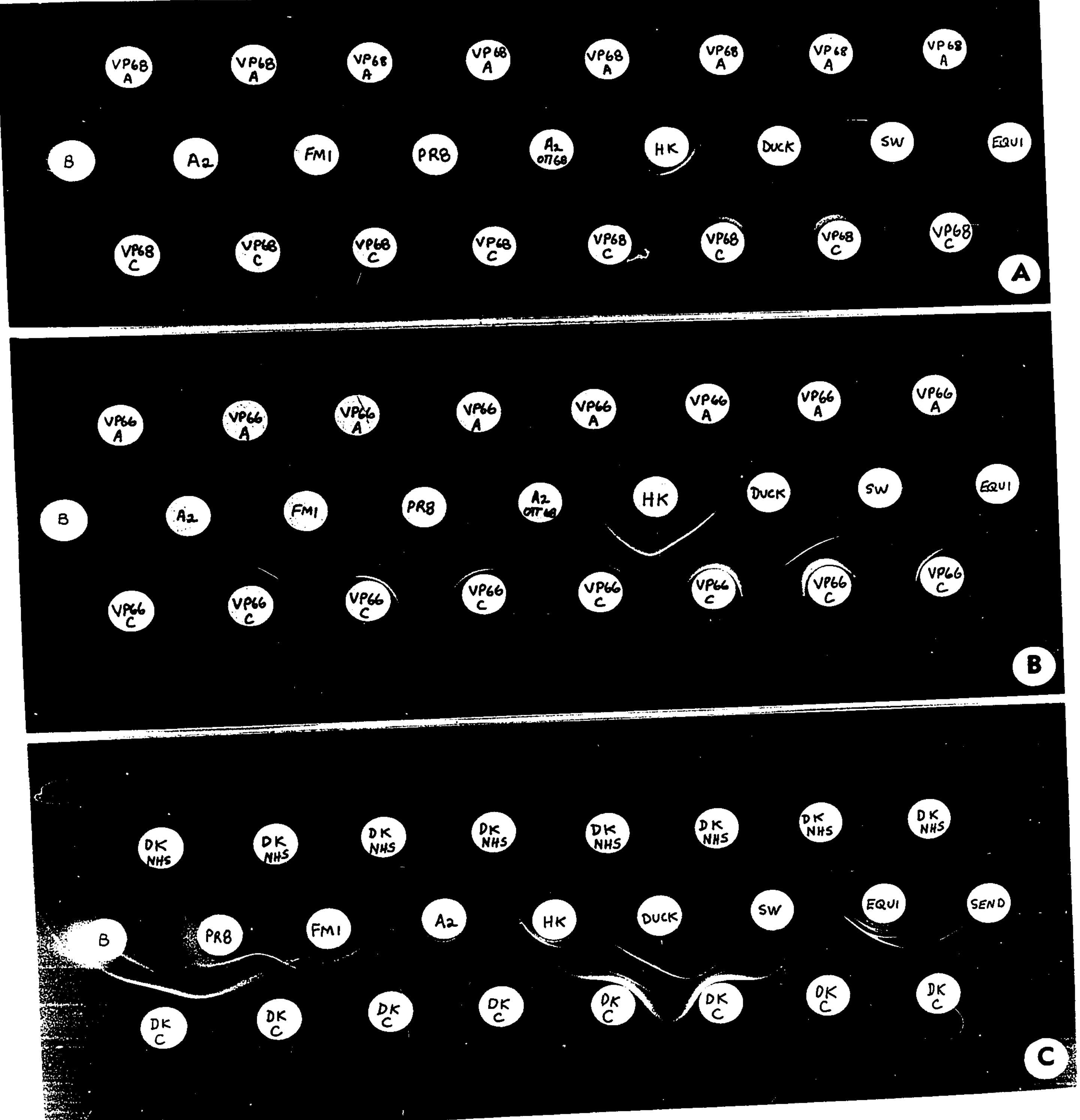


PLATE 28

The establishment of the influenza type-specific virus-antibody reaction in normal animal sera, in human influenza convalescent sera, and in anti-influenza rooster and rabbit antisera, by an immunoprecipitin reaction of identity with a proven influenza A type-specific reaction between:

A, B. 7 S γ-G and A2/Hong Kong/1/68 soluble antigen
C, D. 7 S γ-G and A/PR8 soluble antigen

7 S γ-G : human 7 S gamma-globulin at 50 mg/ml
VP 73C : human convalescent phase influenza serum, VP 73

A. NBS 32 : normal bovine serum no. 32
NCS H19 : normal cat serum; H19, H27 are animal code numbers

The type-specific reaction between 7 S γ-G and HK/SA links with and thus identifies the same reaction between HK/SA and: hRAs/HK, VP 73C, NCS H27.

B. NDS : normal dog serum; H48, H36, OU1 are animal code numbers.
H36 is at 1:5 dilution

The type-specific reaction between 7 S γ-G and HK/SA links with and thus identifies the same reaction between HK/SA and: hFAs/HK, VP 73C, and NDS H36 (outer precipitin band in human convalescent and normal dog sera). The nature of the inner precipitin band formed between HK/SA and human convalescent and normal dog sera is not known.

C. NC_RS 395 : normal cotton tail rabbit serum; 395, 434 are animal code numbers.
NC_HS 575 : normal chipmunk serum 575
VP 68C : human convalescent phase influenza serum VP 68C

The type-specific reaction between 7 S γ-G and PR8/SA links with and thus identifies the same reaction between PR8/SA and: hFAs/PR8, VP 68C (outer band), and NC_HS 575 (outer band - lack of equivalence has pushed this band behind the well). An inner precipitin band formed between antigen and NC_HS 575 links with a similar one formed between antigen and VP 68C. Its nature is unknown.

D. NRS : normal rabbit serum; 127, 157, 160 are animal code numbers.

The type-specific reaction between 7 S γ-G and PR8/SA links with and thus identifies the same reaction between PR8/SA and: hFAs/PR8, VP 73C (outer band), and NRS 160 (outer band - lack of equivalence has pushed this band behind the well). An inner precipitin band between antigen and NRS 160 links with a similar one between antigen and VP 73C.

(Coomassie blue stain)

PLATE 28