TERN VIRUS

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by

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CONTENTS.

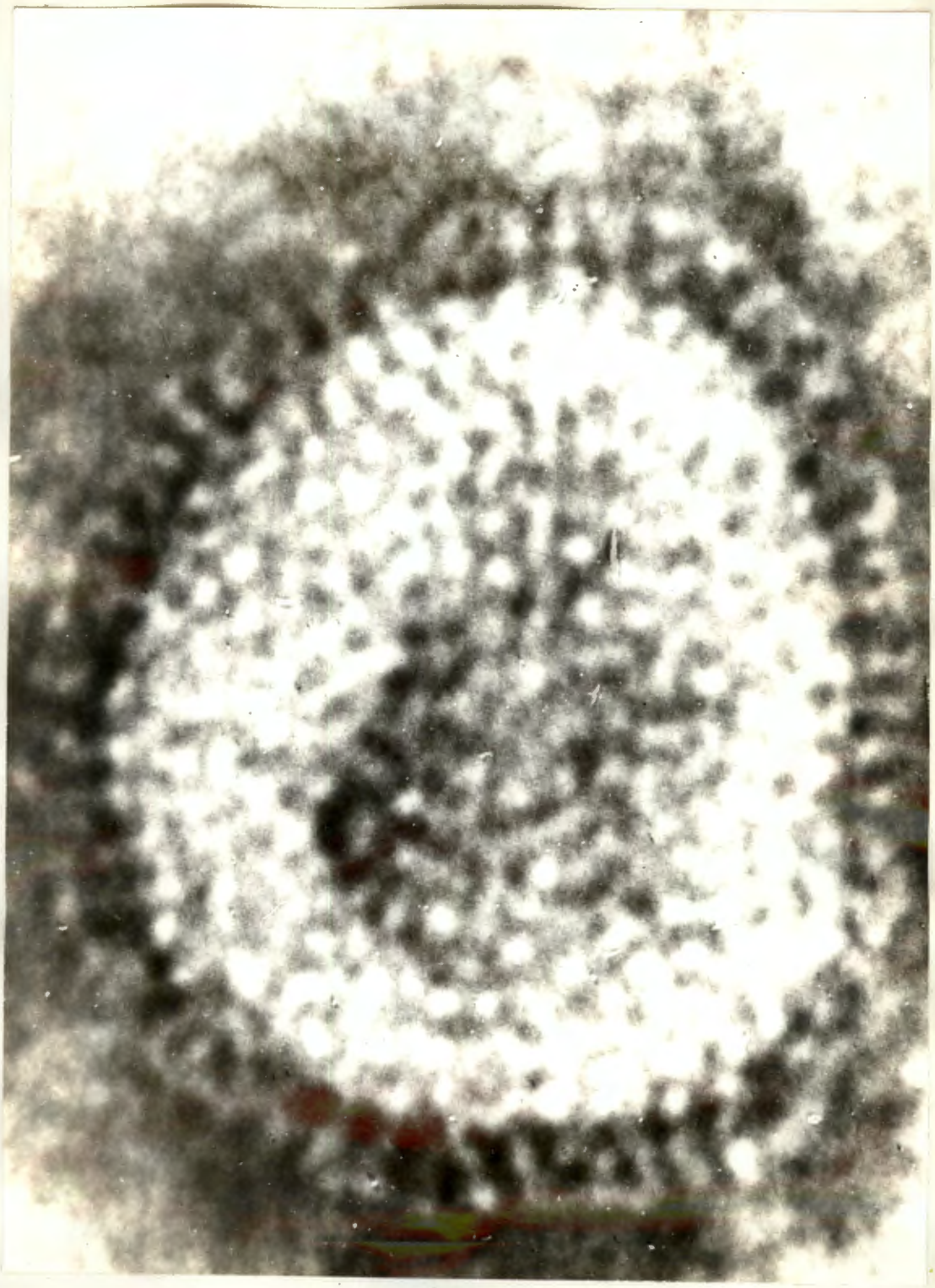


Fig. 1. Electron micrograph of a Tern virus particle.
Negative staining technique.
Magnification: x 1,000,000.

SUMMARY.

An epizootic affecting Common Terns occurred along the Cape Coast in April and May 1961. Afflicted birds were examined and the causative agent was isolated and named Tern Virus.

The properties, antigenic composition and morphology of Tern Virus were determined; it was classified as an Avian strain of Myxovirus influenzae A.

Tern Virus strain-specific antigen was found to be distinct from all other Myxovirus influenzae A strains investigated with the single exception of Chicken/Scotland/1959 Virus to which it was closely related. The pathogenesis of the disease produced in chickens by Tern Virus was investigated and compared with that produced by Chicken Virus. The latter virus was responsible for an outbreak of infection with a high mortality affecting chickens in Scotland in 1959.

The fact that the Common Tern migrates between Europe and South Africa raised interesting epidemiological problems in trying to connect the two outbreaks.

Electron microscopical study of Tern Virus resulted in a number of original contributions to knowledge of the structure of Influenza Viruses.

SECTION I: INTRODUCTION

Common Terns (Sterna hirundo) along the shores of the Republic of South Africa were struck by an epizootic during the months of April and May 1961.

The causal agent was isolated and named Tern Virus. It was subsequently characterised and classified within the Myxovirus group as an avian strain of Influenza type A (see Tables 16 a and b, pages 77 and 77a.)

THE COMMON TERN - GEWONE SEESWAVEL - STERNA HIRUNDO

Although a certain amount of knowledge has been accumulated on the Common Tern and related species of birds, the available information is by no means complete. The following data have been derived from several sources (Witherby, 1941; McLachlan and Liversidge, 1957; Voous, 1960; Serventy and Whittel, 1962).

Description and Distinguishing Features of the Common Tern

The Common Tern is a slenderly built bird 13-14 inches long with tapering bill and forked tail. Adults have been found to weigh between 100 and 175 g. The bill measures 35-39 mm. from its tip to the edge of the feathers. The tarsus is 19-21 mm. and each wing is 255-288 mm. in length. The depth of the tail fork is 64-95 mm. The feet are webbed, and the irises dark brown.

Non-breeding Plumage. (See fig. 2, page 2). In the non-breeding season, i.e. during their stay in the Republic, the back is pale grey with a dark, almost black mark on the front of the wing in the shoulder region. The forehead and the front of the crown are white but the latter may have dark streaks. The back of the crown and nape of the neck are black-brown. The back of the neck and also the underparts are white. The bill

Fig 2/.....

Fig. 2. Sterna hirundo - The Common Tern. About half life-size, depicted in non-breeding plumage as it is usually seen in the Republic of South Africa. The characteristic stance adopted is depicted by the bird in the background.

has more black and less red than in the breeding season, while the legs are usually red throughout the winter.

Breeding Plumage. In the breeding season the main differences are a scarlet bill (the shade of red is distinctive), usually with a black tip. The legs are red. The head is jet-black down to the level of the orbit. The back of the neck is blue-grey and the underparts are off-white.

Distinction from Other Terns. It is necessary to examine the Common Tern at close quarters to distinguish it from the Arctic Tern (Stern macrura) in particular, and also from the Roseate Term (Sterna dougallii). The main differentiating features of the Arctic Tern are that it has extremely short legs with a tarsus 15-17 mm. in length and that the bill is usually shorter (30-33 mm.) and blood-red in the breeding season, at which time the legs are also red. In the non-breeding season the bill and the legs are black. The Roseate Tern is distinguished from the above two species by the long outer tail feathers which are white, and in addition by the pink tinge of the underparts in the breeding season. The bill is long and slender.

Diet.

The diet of the Common Tern, as indicated from an examination of the stomach contents, includes fish (constituting 50% of the diet), crustaceae (30% of the diet), molluscs (12%), and insects and worms, the latter even being obtained from nearby cultivated fields.

Migratory and Breeding Habits

The Common Tern is a migratory sea-bird which breeds in the temperate regions of Europe (including the British Isles), Asia and eastern North America. Occasional breeding places are found in the tropics, probably due to some birds failing to return to the temperate

breeding/.....

breeding grounds.

Common Terns arrive in Europe for breeding from the second week of April onwards, and the eggs are usually laid at the end of May or beginning of June. The birds frequent the inshore waters along low-lying coasts and usually breed in colonies on the coast on shingle banks, sand dunes, salt marshes and rocky islets. However in Scotland, Ireland and on the Continent, though not in England, they may breed inland on rivers, lakes and lochs. The breeding place is not infrequently shared with other terns, including the Arctic Tern which has similar migratory habits but whose breeding grounds extend further north, as its name suggests, into the Arctic regions. The nest is a hollow in rock or scratched out in sand, shingle or grass. The young begin to fly four weeks after the hatching and the breeding grounds are deserted in July or August, but the birds seem to wander before actual migration occurs.

After the breeding season, the birds migrate southwards to America (including South America), South Africa, and also to Australia. Birds from Western Europe (Sweden), as well as from eastern Siberia reach Australia. At the start of migration, terns are frequently found on inland waters as well as at the coast in Britain. Terns ringed in Britain have been recovered from France, Portugal, Spain, Senegal and the Gold Coast. Terns migrate from the Mediterranean down the west coast of Africa to the Cape of Good Hope. In the Republic of South Africa they are very rarely found inland, and have not been found on the east coast further north than Natal. Terns ringed in Finland, Denmark and Germany have been found in the Republic. The Common Tern usually remains in the Republic from October to February, but some birds remain throughout the year. They roost especially along estuaries, on sandbanks and in harbours on boats.

CIRCUMSTANCES/.....

CIRCUMSTANCES OF THE EPIZOOTIC AFFECTING COMMON TERNS IN APRIL AND MAY 1961

Description of the Epizootic. The circumstances of the epizootic have been described by Rowan (1962) in detail, and the salient features only are given below.

Documentation of the epizootic was best in the region of the Cape Peninsula. In 1961 many Common Terns delayed their departure for the North until April and May. Several dead terns were reported in the second and third weeks of April in the region of the Cape Peninsula, but the onset of the epizootic was explosive in the third and fourth weeks of April and either spread rapidly, or was multifocal in origin along the thousand-mile coastline stretching from Port Elizabeth to Lambert's Bay (see Fig. 3, page 6).

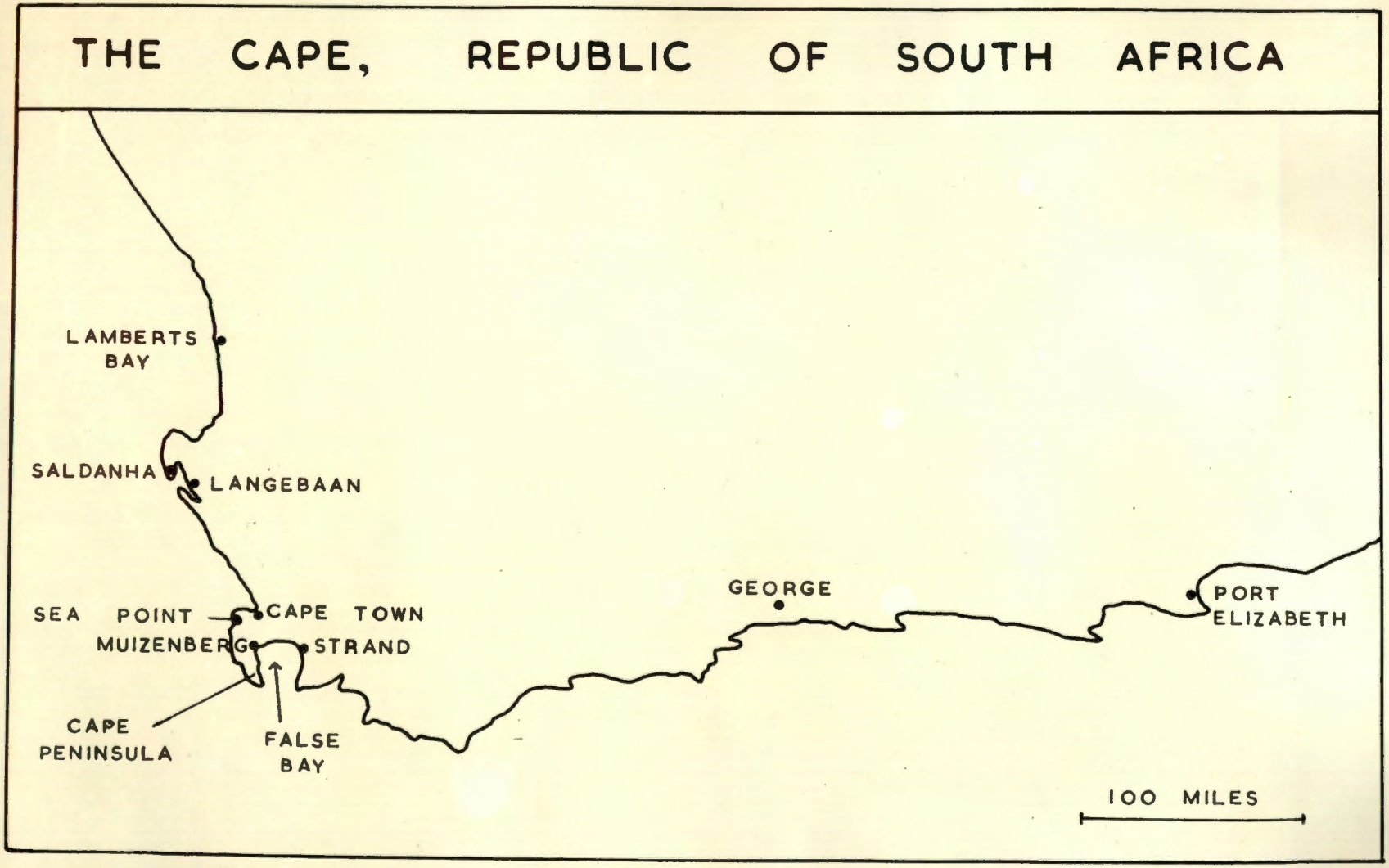
A four-mile stretch of False Bay beach between the Strand and the Eerste River mouth was covered from the 23rd to 26th of April. At the start of the investigation the population of Terns was estimated at 1200-1500, and consisted of Common and Arctic Terns (these two species can only be distinguished on close examination) with an occasional Sandwich Tern (Sterna Sandvicensis). During the four-day period 469 dead Common Terns were found. Sick birds were reluctant to fly, some could no longer fly at all, and the affected birds produced a copious fluid excrement.

No dead Arctic Terns or other birds which mingled with the Common Terns were found. Scavenging Black-backed Gulls (Larus dominicanis) and Cape Ravens (Corvis albicollis) fed on the dead birds but were not themselves overtly affected.

During the end of April and the beginning of May, 473 dead terns were found on the beach at Strandfontein near Muizenberg. Dead terns were also reported during the period of the epizootic at Sea Point, Langebaan, Saldanha Bay, Lambert's Bay, George and the Port

Elizabeth/.....

FIG. 3



PLACES WHERE DEAD COMMON TERNS WERE FOUND DURING THE EPIZOOTIC OF 1961.

Elizabeth area.

No more deaths were noted after the second week in May, by which time most of the recovered or unaffected birds had left, presumably on their migration northwards.

Thirteen of the sixteen ringed Common Terns that were found had been ringed in Finland, Heligoland, Sweden, Russia or Holland. Eight of the thirteen were first-year birds, three were second-year birds, one was fourth- and one a fifth-year bird.

The striking features of the epizootic were the explosive nature of the outbreak, the host specificity in affecting only Common Terns, and the high mortality as evidenced by the finding of 1300 dead birds in four small areas alone.

Weather conditions. It is possible, as will be discussed later, that the weather may have played a role in the onset of the epizootic. At this juncture it may be noted that just prior to and during the epizootic, grossly abnormal weather conditions were recorded. These included heavy rainfall and the appearance of "red tide" in Cape Peninsula waters. The latter is a phenomenon that is little understood, but it is a red colouration of the sea due to excessive multiplication of a dinoflagellate; associated with this or perhaps due to it, whether directly or indirectly, various marine animals may die in large numbers.

SECTION II: ISOLATION AND IDENTIFICATION OF THE CAUSATIVE VIRUS

A: MATERIALS AND METHODS

VIRUS STRAINS EMPLOYED

The myxovirus strains used in this work are listed below. The name used in the text is given, followed by the full designation and source.

1. Tern virus ..... Myxovirus influenzae A avian strain/ Cape Town/1961; UCT and CSIR Virus Research Unit.
2. Chicken virus ..... Myxovirus influenzae A avian strain/ Scotland/1959; Dr. J.E. Wilson, Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Eskgrove, Lasswade, Midlothian; via Onderstepoort Veterinary Laboratory, S.A.
3. Influenza A_2 ..... Myxovirus influenzae A_2 hominis/Cape Town/ 1961; UCT and CSIR Virus Research Unit.
4. Influenza B ..... Myxovirus influenzae B Lee; World Influenza Centre, N.I.M.R., London.
5. Influenza C ..... Myxovirus influenzae C/JJ/1950; Elva Minuse, School of Public Health, University of Michigan; via Professor J. Gear, Polio Research Foundation, Johannesburg.
6. NDV ..... Newcastle disease virus, strain Komarov; Onderstepoort Veterinary Laboratory, S.A.

IMMUNE/.....

IMMUNE SERA EMPLOYED

The following immune sera were prepared against the viruses indicated:-

1. Tern Virus: "Anti-nucleoprotein" ("Anti-NP") and Anti-haemagglutinin (anti-V) guinea-pig immune sera; convalescent ferret and chicken immune sera.

2. Chicken virus: "Anti-NP" and anti-V guinea-pig immune sera; convalescent chicken immune sera.

3. Influenza A_2: "Anti-NP" and anti-V guinea-pig immune sera.

4. Influenza B: "Anti-NP" and anti-V guinea-pig immune sera.

5. Influenza C: "Anti-NP" guinea-pig immune sera; convalescent ferret immune serum.

Details of other immune sera employed and the source from which they were obtained were as follows:-

1. Anti-Fowl plague virus strain Brescia Lot 28/May 1957.

2. Anti-Fowl plague virus strain Alexandrien Lot 30/June 1957.

3. Anti-Chicken virus strain 'N' Lot 31/June 1957.

US Department of Agriculture - Plum Island Animal Disease Laboratory

4. Anti-/.....

4. Anti-Chicken virus/Scotland/1959 chicken serum	Dr J.E. Wilson, via Onderstepoort Veterinary Laboratory.
5. Anti-NDV hyperimmune chicken serum Lot of 21/11/1957	Onderstepoort Veterinary Laboratory South Africa.
6. Anti-NDV hyperimmune chicken serum Lot of 14/11/1960	
7. Human convalescent mumps serum 1960	UCT and CSIR Virus Research Unit
8. Human convalescent mumps serum 1961	
9. Anti-Influenza A_2/CT/57 immune ferret serum	
10. Anti-Influenza A_2/Asia/57 hyperimmune rabbit serum	N.V. Philips-Duphar Amsterdam, Holland
11. Anti-Influenza B/Johannesburg/33/58 hyperimmune rabbit serum	
12. Anti-Influenza A/Philadelphia/53 strain-specific guinea-pig hyperimmune serum	Microbiological Associates Incorporated, USA - through courtesy of Dr. C. Chany, Hopital St Vincent de Paul, Paris.
13. Anti-Influenza A/FM_1/47 strain-specific guinea-pig hyperimmune serum	

14. Anti-/.....

14. Anti-Influenza A/PR8/34
strain-specific guinea-pig
hyperimmune serum

15. Anti-mumps virus
strain-specific guinea-pig
hyperimmune serum

16. Anti-Parainfluenza 1
strain-specific guinea-pig
hyperimmune serum

17. Anti-Parainfluenza 2
strain-specific guinea-pig
hyperimmune serum

18. Anti-Parainfluenza 3
strain-specific guinea-pig
hyperimmune serum

19. Anti-Simian myxovirus (SV5)
hyperimmune rabbit serum

Microbiological Associates
Incorporated, USA -
through courtesy of
Dr. C. Chany,
Hopital St Vincent de Paul,
Paris.

20. Anti-Simian myxovirus (SV5)
(Hull's prototype strain)
hyperimmune rabbit serum 1960

Burroughs Wellcome
via Dr. M.S. Pereira
Central Public Health
Laboratory
Collindale Ave., London.

SEA-BIRDS/.....

SEA-BIRDS INVESTIGATED

During the epizootic three affected Common Terns were submitted for virus studies. Two of these were received on the 27th April from the Percy Fitzpatrick Institute of African Ornithology where they had been stored in the deep freeze for several days after collection. The third bird was caught in extremis by Professor C.J. Uys on the 11th May when it was sacrificed and autopsied.

In addition, studies were carried out on 13 sea-birds, most of them terns, collected in 1961 after the epizootic had ended and on a further 3 Common Terns received in 1963 (see Table 4, page 45,46).

AUTOPSY PROCEDURE

Live birds were exsanguinated by means of cardiac puncture, but if the birds were already dead on receipt, blood clot was obtained after autopsy from the opened heart.

The bird was pinned out on a board. The ventral skin was reflected and the liver, heart, lungs and kidneys removed in that sequence. The skin was then reflected over the cranium which was opened and the brain removed. Separate sterile instruments were used for the removal of each organ; alternatively the instruments were sterilised by flaming and cooled by plunging into sterile saline between the handling of different organs. Care was taken as far as possible to avoid contact of one organ with another. This was best achieved by harvesting the organs in the order stated, but some contact was practically unavoidable, except in the case of the brain. Consequently, each organ except the brain was washed in three 20 ml. lots of sterile saline before portions were removed for viral studies. The remaining portion of each organ was fixed in 10% formol-saline for histological examination which was carried out by Professor C.J. Uys.

PREPARATION/.....

PREPARATION OF TISSUE EMULSIONS FOR VIRAL STUDIES

The serum was separated from the blood taken from the live birds and stored at -20°C.

The blood clot and the portion of each organ was carefully weighed and a 10% emulsion (w/v) made in KB maintenance medium without added serum (see Table 1, page 17) using a pestle and mortar and ground glass. After two cycles of freezing and thawing, the emulsions were spun at 10,000 r.p.m. for 10 minutes in the high speed head of an MSE refrigerated centrifuge and the supernatant fluids were retained. The latter were cultured aerobically on blood agar and were satisfactory for viral culture if no growth was obtained after 48 hours at 37°C. If not used immediately the fluids were stored in dry ice and/or at -20°C in a deepfreeze cabinet.

THE USE OF EMBRYONATED EGGS

Leghorn-Australorpe-cross eggs from the same commercial source were used throughout this work.

The Inoculation of Eggs

The methods used involved a number of modifications of standard procedures.

Embryonated eggs were used on their 10th to 12th day of incubation for inoculation by the allantoic or amniotic routes. Each egg was transilluminated to check on viability, to delineate the air-sac and to mark the position of the embryo and its overlying allantoic cavity.

For Allantoic Inoculation The shell over the air sac was painted with tincture of iodine. Using a sharp-pointed instrument (basically a

weighted/.....

weighted steel nail) a small hole was pierced through the shell and attached shell membrane into the air sac close to its margin, and the area again painted with iodine. Either a finely drawn-out Pasteur pipette, or, if accurate inocula were required, a tuberculin syringe with a suitable needle was used to introduce the inoculum into the allantoic cavity by traversing the air sac and piercing the reflected portion of the shell membrane and the underlying chorio-allantoic membrane. The hole in the shell was then sealed with adhesive tape.

The Amniotic Sac was approached by using a dental grinding disc to make a hole 1 cm. in diameter over the air sac. A minimal quantity of sterile liquid paraffin was used to clarify the reflected portion of the shell membrane so that damage to blood vessels could be avoided while piercing the shell and chorio-allantoic membranes with fine, pointed forceps in order to pick up the amniotic sac. The latter was pulled up into the air sac and the inoculum was introduced into it with a finely drawn-out Pasteur pipette or a tuberculin syringe and needle. Thereafter the amniotic membrane was allowed to drop back into position and the hole in the shell sealed with adhesive tape.

For Chorio-allantoic membrane(CAM) inoculation eggs on their 12th or 13th day of incubation (with well developed membranes) were selected. Under transillumination the air sac was delineated and a line 1-2 mm. long made immediately over an area of the CAM which was free of large blood vessels. The appropriate areas of shell were cleaned with ether. A hole was pierced into the air sac through the overlying shell and its membrane. With a dental burr the shell was gently and carefully drilled away over the linear mark to reveal the underlying shell membrane. The resultant dust was blown away, the egg placed in a horizontal position and the exposed shell membrane covered with a drop of pre-warmed (37°C) sterile saline. The shell membrane fibres were split transvere to the long axis of the egg with a dissecting needle, care being taken to avoid any damage to the underlying CAM. The sterile saline penetrated along

the/.....

the plane of cleavage of the CAM and the shell membrane, thus aiding the weight of the egg contents to obliterate the true air sac while pulling down the CAM and creating a false air sac between it and the shell membrane. If necessary the process of dropping the CAM could be initiated or completed by means of gentle suction with a rubber teat over the hole pierced into the true air sac. The eggs were then returned to the incubator for 1-4 hours and again transilluminated before use to exclude eggs in which haemorrhage had occurred.

The volume of the inoculum was 0.1 ml. per egg, irrespective of the route employed.

Harvesting of Eggs

Prior to harvesting, the eggs were chilled to minimise bleeding. Those eggs inoculated via the allantoic or amniotic routes were opened by cutting along the circumference of the air sac with curved scissors. The allantoic fluid was harvested with a Pasteur pipette, followed by the amniotic fluid. If necessary, the remaining contents of the egg could be tipped out into a sterile petri dish and the required tissues harvested. Inoculated CAM's were harvested by cutting round the edge of the false air sac and detaching the CAM from the removed portion of shell.

THE USE OF WHITE MICE

The laboratory strain of white mice used was a highly inbred strain ex the Onderstepoort Veterinary Laboratory. Suckling mice less than 24 hours old were inoculated intracerebrally in 0.02 ml. amounts, and the brains subsequently harvested and emulsified according to standard procedures for further passage.

THE/.....

THE PREPARATION AND USE OF TISSUE CULTURES (TC)

The materials and methods employed in the in vitro culture of tissue cells are now basically standard (Parker, 1961).

STRAIN KB MALIGNANT HUMAN EPITHELIAL CELL LINE.

This cell line was originally established by Eagle (1955) from an epidermoid carcinoma of the floor of the mouth and tongue.

Media and Solutions.

The composition of the solutions used is set out in Table 1, page 17.

KB cells were incubated at 37°C and maintained by serial sub-culture on glass. The cells were loosened from the glass and dispersed by means of a solution containing trypsin and versene, then washed and resuspended at a suitable concentration (usually 0.1 ml. of packed cells per 100 ml. of medium) in growth medium for seeding into further flasks for stock or into tubes. The tube cultures were sloped and kept stationary until the cells were attached to the glass, and then rotated in a roller-drum at 15 r.p.h. When an almost confluent sheet had formed, the growth medium was replaced with maintenance medium and the tubes were ready for use. The media were standard media based on Hanks' balanced salt solution (table 1).

TABLE 1/.....

TABLE 1. MEDIA AND SOLUTIONS FOR KB CELL CULTURE.

	KB GROWTH MEDIUM	KB MAINTENANCE MEDIUM	PHOSPHATE BUFFERED SALINE	TRYPSIN BUFFER	TRYPSIN-VERSENE SOLUTION
	g.	g.	g.	g.	g.
NaCl	8.0	8.0	8.0	8.0	8.0
KCl	0.4	0.4	0.2	0.4	0.4
$MgSO_4.7H_2O$	0.2	0.2			
$CaCl_2.2H_2O$	0.19	0.19	0.1		
$Na_2HPO_4.2H_2O$	0.076	0.076	1.44	0.076	
KH_2PO_4	0.06	0.06	0.2	0.06	
Glucose	1.0	1.0		1.0	1.0
Phenol red	0.02	0.02			0.02
$MgCl_2.6H_2O$			0.1		
Lactalbumin hydrolysate	5.0	5.0			
$NaHCO_3$	0.35	1.35			0.58
Trypsin					0.5
Disodium versenate					0.2
Calf serum	100 ml.				
Fowl serum		100 ml.			
Distilled water added to	1000 ml.	1000 ml.	1000 ml.	1000 ml.	1000 ml.

Sodium benzyl penicillin 100 i.u./ml., streptomycin sulphate 100 i.u./ml., neomycin sulphate 100 i.u./-l. were incorporated into the solutions and media. Sometimes aseptically prepared mycostatin was added to the filtered media, 25 i.u./ml.
Sterilization was by filtration, employing positive pressure in the case of solutions containing bicarbonate.

Preservation of KB Cells

A suspension of 0.1-0.2 ml. of packed cells in 1 ml. of growth medium with glycerol added to 20% concentration was sealed in a glass ampoule, which was wrapped in cotton wool and placed in a small tin container in a dry ice cabinet. When needed, an ampoule was thawed rapidly at 37°C, its contents diluted in 30-40 ml. of growth medium and seeded into a 20 oz. flat bottle.

MONKEY KIDNEY EPITHELIAL CELL CULTURES

Roller tube cultures were prepared by standard methods from trypsinised Vervet Monkey (Cercopithecus aethiops pygerythrus) kidney cells. The media were identical with those used for KB cells except that 5% calf serum was used in the growth medium, and 0.5% fowl serum in the maintenance medium.

CHICK EMBRYO FIBROBLAST CULTURES (CETC)

These were prepared with slight modifications of the method described by Porterfield (1960), and based on the technique of Dulbecco (1952).

Solutions and Media (see Tables 2 and 3, pages 19 and 20).

Tris (tris-(hydroxymethyl)-amino-methane): A stock 0.05M solution pH 7.2-7.6 was prepared by dissolving 2.42 g. of Tris in about 100 ml. of salt solution (Gey's solution A 18 vols + solution B 1 volume, see tables 2 and 3), adding 76.8 ml. of 0.2M HCl and making up to 400 ml. with more salt solution.

Chick/.....

TABLE 2. MEDIA USED FOR CHICK EMBRYO FIBROBLAST CULTURES

STOCK FOR GEY'S BALANCED SALT SOLUTION

STOCK SOLUTION A (may be made up 10x concentrated and diluted for use)		STOCK SOLUTION B		STOCK SOLUTION C	
	g.		g.		g.
NaCl	7.0	$MgCl_2.6H_2O$	4.2	$NaHCO_3$	22.5
KCl	0.37	$MgSO_4.7H_2O$	1.4		
$Na_2HPO_4.12H_2O$	0.301	$CaCl_2$	3.4		
KH_2PO_4	0.0237				
Glucose	1.0				
Distilled water added to 1000 ml.		Distilled water added to 1000 ml.		Distilled water added to 1000 ml. (adjust to pH 7.9 by bubbling CO_2 through).	

Sodium benzyl penicillin 100 i.u./ml. and streptomycin sulphate 100 i.u./ml. were incorporated into the solutions.

Sterilization was by filtration, with positive pressure filtration in the case of solutions containing bicarbonate.

TABLE 3. MEDIA AND SOLUTIONS FOR CHICK EMBRYO FIBROBLAST CULTURES

	STANDARD GEY'S BALANCED SALT SOLUTION	TRIS GEY'S SOLUTION	TRIS GROWTH MEDIUM	TRIS OVERLAY MEDIUM	TRIS STAINING MEDIUM
Stock soln A	90%	90%	90%)))) 89%	20% of 10x concentrated	
Stock Soln B	5%	5%	5%))	10%	Tris overlay 50%
Stock Soln C	5%		1%))		
0.05M tris,pH 7.6		5%	4%)	10%	
0.5% lactalbumin hydrolysate (in distilled water)			5%	10%	
Calf serum			5%	10%	
Chick embryo extract			1%	5%	
Distilled water				35%	45%
Neutral red 0.4% in distilled water					5%

Sodium benzyl penicillin 100 i.u./ml. and streptomycin sulphate 100 i.u./ml. were incorporated into the solutions and media.

Sterilization was by filtration, with positive pressure filtration in the case of solutions containing bicarbonate.

Chick Embryo Extract. The head, limbs and viscera were removed from 10-12 day-old chick embryos and homogenised in 1 ml. of Standard Gey's solution (see Tables 2 and 3) per embryo. The homogenate was centrifuged at 2,000 r.p.m. for 30 minutes in the cold (MSE centrifuge) and the supernatant fluid stored at -20°C. This extract was thawed and centrifuged at 2,000 r.p.m. for 10 minutes before use.

Trypsin Solution. A stock 5% solution was prepared by dissolving 5 g. of Difco trypsin in trypsin buffer (see Table 1, page 17) warmed to 37°C and stirred continuously. After approximately 30 minutes the solution was filtered rapidly through a Seitz pad (for sterilization), dispensed in 5 ml. amounts and stored at -20°C.

For use, a 0.25% trypsin solution was prepared by adding 5 ml. of stock solution to 95 ml. of trypsin buffer containing 0.1% of $NaHCO_3$, and penicillin 100 i.u./ml. and streptomycin 100i.u./ml.

Other Solutions and Media. The composition of other solutions and of the media is given in Tables 2 and 3, pages 19 and 20.

The Preparation of Chick Embryo Fibroblast Cultures

Chick embryos, 10-12 days old, were harvested aseptically and the head, limbs and abdominal viscera removed. The embryos were washed in two changes of Tris Gey's solution (see Table 3), minced with scissors and transferred to a trypsinizing flask containing 8 ml. of 0.25% trypsin per embryo. The tissues were trypsinized under continuous agitation by means of a magnetic stirrer for 30 minutes at 37°C, and then filtered through gauze to remove undigested fragments. The cells were next washed twice in Tris growth medium (see Table 3) and resuspended in 2 ml. of growth medium per embryo. The concentration of cells was determined by counting in a haemocytometer and the suspension diluted with growth

medium/.....

medium to give a concentration of 5 x 10^6 cells / ml. Finally this suspension was filtered through a coarse sintered glass filter to remove cell clumps before seeding into petri dishes or tubes.

Petri Dish Cultures of Chick Embryo Fibroblasts. Petri dishes, approximately 70 mm. in diameter were seeded with 5 ml. of cell suspension and placed level in an incubator at 37°C. After overnight incubation cell sheets had formed and the cultures were ready for use. For inoculation the growth medium was removed and 0.5 ml. of inoculum (virus diluted in growth medium or growth medium alone in the case of controls) was pipetted onto each cell sheet. The virus was allowed to adsorb at room temperature (18-23°C) for one hour with occasional rocking to ensure even distribution of virus over the cell sheet. After the adsorption period the fluid was removed and each cell sheet covered with 6 ml. of agar overlay prepared at the moment of using by mixing equal quantities of 1.2% thrice-washed oxoid agar in distilled water and overlay medium, both at 44°C. After a period of incubation at 37°C in a humidified incubator the cell sheets were stained with 4-5 ml. of Tris staining medium (see Table 3) containing neutral red, and examined after a further four hours incubation at 37°C for the presence of plaques.

Roller Tube cultures of Chick Embryo Fibroblasts. Roller tubes (with or without coverslips) were seeded with 1 ml. of filtered cell suspension diluted to contain 0.1 ml. of packed cells per 100 ml. of growth medium (approximately 2.5 x 10^5 cells per ml.) and sloped overnight at 37°C. The growth medium was renewed and the tubes, which were then incubated at 37°C in a revolving drum (15 r.p.h.) were ready for use either immediately or up to 1-2 weeks later. The volume of inoculum employed was 0.1 ml. per tube containing 0.9 ml. of medium.

CRITERIA OF VIRUS INFECTION OF TISSUE CULTURES

In roller tube tissue cultures evidence of virus infection was

one/.....

one or more of the following:-

1. a cytopathic effect (CPE),

2. a positive haemagglutination (HA) test on the fluid,

3. a positive haemadsorption test.

In the case of petri dish cultures, virus infection was detected by the formation of plaques.

VIRUS INFECTIVITY TITRATIONS

Serial tenfold dilutions of the virus suspensions were made and inoculated in 0.1 ml. amounts into four or more eggs per dilution via the allantoic route. Alternatively tissue culture tubes were employed in similar numbers, each tube containing 0.9 ml. of medium. Eggs were examined 48-72 hours after inoculation for death of the embryo and/or a positive HA test on the embryonic fluids. Tissue culture tubes were examined for CPE daily for 7-14 days until the end-point was reached.

Calculation of the 50% infective dose end point (ID_{50}) was made according to the method of Reed and Muench (1938) and the virus content of the titrated suspension expressed either as $\log_{10}$ ID_{50} per ml., or ID_{50} per ml. In the case of 10% tissue emulsions the virus content was expressed per gram of tissue.

The result of titrations on tissue culture plates were expressed as $\log_{10}$ plaque-forming-units (pfu) per ml.

FIXATION AND STAINING (HAEMATOXYLIN AND EOSIN) OF TISSUE CULTURES GROWN ON COVERSLIPS

The cytopathic effect of virus infection was studied by fixing

and/.....

and staining infected cells grown on coverslips in roller tubes and noting the changes produced as compared to uninfected controls. The cells were washed well in two changes of phosphate buffered saline (PBS - see Table 1, page 17), and fixed by one of the two methods below:

either a) in absolute alcohol for 15-20 minutes and then stored in 70% alcohol for subsequent staining

or b) Bouin's fluid, which was composed as follows:

Saturated aqueous solution of picric acid	15 parts
Formalin (40% aqueous formaldehyde)	5 parts
Glacial acetic acid	1 part

After 30-60 minutes the fixative was replaced with absolute alcohol for 10 minutes, and this in turn by 70% alcohol in which the coverslips were stored to await staining with haematoxylin (Harris's) and aqueous eosin (or eosin/phloxin in the proportion of 7 parts to 3 parts) following standard procedures.

ACRIDINE ORANGE STAINING AND RIBONUCLEASE DIGESTION OF VIRUS-INFECTED CELLS

The reagents used were:

Acetate Buffer: 0.2 M pH 4.7.

Acridine Orange (Gurr 07183) : Stock 0.5% solution in acetate buffer; diluted 1/100 in acetate buffer for use.

Phosphate Buffer: 0.2 M in 0.14 M NaCl, pH 7.3.

Ribonuclease: Seravac chromatographed crystalline ribonuclease - 0.01% solution in phosphate buffer.

Chick/.....

Chick embryo tissue cultures grown on coverslips were inoculated with approximately 1000 $TCID_{50}$ of virus and harvested when 25-50% of the cells showed cytopathic effect. They were rinsed thoroughly in PBS, fixed in absolute alcohol for 15 minutes and stored in 70% alcohol at 4°C for several hours until employed. Before use, the coverslips were washed free of alcohol in distilled water; some were digested in 0.1% ribonuclease for 15 minutes at 18°C and others were placed in phosphate buffer alone as controls. The coverslips were then washed in acetate buffer, stained in Acridine orange for 30 minutes and differentiated and mounted in acetate buffer using nail varnish to cover the edges of the coverslips. Uninfected controls were also always prepared with each experiment.

The stained preparations were examined with the Zeiss fluorescence microscope equipment using an Osram HBO 200 maximum pressure mercury vapour lamp, a BG 12 exciter filter and an OG 5 barrier filter. The former transmitted light of wave-length 3,500-5,200 Å, while the latter transmitted light of wave-length greater than 5,200 Å.

HAEMAGGLUTINATION (HA) AND HAEMAGGLUTINATION - INHIBITION (HI) TESTS

Preparation of Washed Red Cell Suspensions:

Chickens or guinea-pigs were bled by cardiac puncture; for certain tests blood from other species and humans was obtained by cardiac- or venipuncture. Five to ten ml. of blood was drawn off and transferred to 50-100 ml. of sterile 3.8% sodium citrate in saline (0.85% NaCl - w/v). The cells were washed in three changes of approximately 10 volumes of PBS and resuspended in PBS containing penicillin 100 i.u./ml. and streptomycin 100 i.u./ml. to give a 10% (v/v) stock suspension which was renewed weekly. For use, a 0.6% suspension in saline was prepared from the stock solution, and tested to

exclude/.....

exclude auto-agglutination.

Virus Titration by the Haemagglutination Test

Standard perspex plates were employed. Serial doubling dilutions of the material to be tested were made in 0.5 ml. amounts. Sterile saline or PBS was used as the diluent. Red cells, 0.5 ml. of an 0.6% suspension, were added to each dilution, and to a control cup containing 0.5 ml. of diluent. The results were read after 1 hour at room temperature, but in certain experiments tests were also carried out at 4°C and/or 37°C. The highest dilution of material giving complete agglutination was assumed to contain one haemagglutinating unit (HAU) per 0.5 ml. If this dilution was e.g. 1/160, the original material contained 320 HAU per ml.

Haemagglutination-Inhibition (HI) Test

The haemagglutination-inhibition test, based on the fact that viral haemagglutination was specifically inhibited by specific immune sera, was used to identify viruses by means of known immune sera, and to test sera for antibody content to known viruses. All sera were heat-inactivated (56°C for 30 minutes). The virus suspension was titrated in 0.5 ml. amounts and an aliquot diluted to contain 4 HAU per 0.25 ml. The procedure was to add 4 HAU of virus contained in 0.25 ml. to an equal volume of each of a series of doubling dilutions of serum, followed by 0.5 ml. of chicken red cell suspension. A parallel test on pre-immune or normal serum was carried out, and the standard controls set up, i.e. serum control to exclude the presence of naturally-occuring agglutinins, cell control and control titration of the 4 HAU dilution of antigen used in the test.

The HI antibody titre of a serum was expressed as the reciprocal of the highest dilution which caused complete inhibition of agglutination

by/.....

by 4 HAU of antigen. This did not take into account the further dilution of the serum occasioned by the addition of the other reagents in the test.

Non-Specific Inhibitors

The effect of non-specific inhibitors of viral haemagglutination in serum was eliminated where necessary by one of the following standard procedures (WHO Report, 1959):-

1) Treatment of the sera with standardised crude cholera filtrate obtained commercially in the lyophilised state from N.V. Phillips-Duphar, Amsterdam, Holland. In HI tests with these treated sera 2% sodium citrate in saline was used as the diluent.

2) treatment of the sera with freshly prepared M/90 potassium periodate.

The dilution factors resulting from these treatments were taken into account when interpreting the HI test.

Haemadsorption and Haemadsorption-Inhibition Test

These are based on the "adsorption-agglutination" test of Vogel and Shelekov (1957).

The haemadsorption test was used as an indicator of viral infection of tissue culture cells with resultant production of haemagglutinin. The roller tube tissue cultures were rinsed out well with three changes of PBS, and 1 ml. of an 0.6% suspension of washed red cells (guinea-pig or fowl) was introduced and the tubes sloped in the refrigerator or at room temperature. After 15 minutes the tubes were

gently/.....

gently rinsed out with PBS, 1 ml. of tissue culture fluid introduced and the tubes examined microscopically for adsorption of red blood cells to infected tissue culture cells.

Uninoculated control tubes were similarly treated.

NEUTRALISATION TEST

The neutralisation test was used either to identify an unknown virus with known immune sera, or to test a serum for antibodies to a known virus.

A standard procedure, using a constant amount of virus was followed. An aliquot of a previously titrated virus suspension was diluted to contain an estimated 200 ID_{50} per 0.1 ml. for the culture system employed. A suitable volume of this dilution was mixed with an equal volume of each dilution of the serum (previously heated at 56°C for 30 minutes) to be tested, and allowed to stand in the dark at room temperature (18-23°C) for 60-90 minutes. Each serum-virus mixture was then inoculated in 0.1 ml. amounts into three or more chick embryo tissue culture tubes (in one test embryonated eggs were used). The neutralisation test was considered positive if the effects of virus infection were either prevented or significantly delayed i.e. by at least 48 hours, as compared to controls set up with known normal serum.

The criteria of infection in embryonated eggs was death of the embryo and/or a positive HA test on the embryonic fluids and in CETC tubes the criterion used was CPE and/or a positive haemadsorption test.

The neutralising antibody titre of a serum was expressed as the reciprocal of the highest dilution giving a positive result in at least 50% of the inoculated cultures, and did not take into account the further dilution due to the other reagents in the test.

COMPLEMENT/.....

COMPLEMENT FIXATION TESTS

PREPARATION OF ANTIGENS FOR USE IN COMPLEMENT FIXATION TESTS

PREPARATION OF THE TYPE-SPECIFIC NUCLEOPROTEIN (NP) ANTIGEN AND THE STRAIN-SPECIFIC (V) ANTIGEN OR HAEMAGGLUTININ

The procedure followed for the preparation of the above two antigens was similar to the standard procedure followed for influenza viruses (WHO report, 1959) based on the methods of Hoyle (1952) modified by Lief and Henle (1956 a and b) and Fabiyi et alia (1958). This involved the preparation of a partially purified and concentrated suspension of virus which was fractionated with ether and the two antigens contained in the aqueous phase were then separated by adsorption of the haemagglutinin to red cells followed by elution into buffer.

In some experiments, formalin-treated red cells were employed in order to avoid the haemolysis which occured when fresh cells were used. The cells were prepared according to the method of Cox and Pirtle (1956) but were washed free of formalin before use.

Preparation of Partially Purified and Concentrated Virus Suspensions

Seed virus was diluted in PBS and 0.1 amounts of a 10^{-3} dilution were inoculated into the allantoic cavity of embryonated eggs in their 10th-12th day of incubation. After further incubation for approximately 36 hours at 37°C, the eggs were chilled and the allantoic and amniotic fluids were harvested into a container standing in an ice bath. The pooled fluids were clarified by centrifuging in the cold at 1500 r.p.m. for 5 minutes in an MSE refrigerated centrifuge, and replaced in a container in an ice bath. Washed fowl red cells were added to a final concentration of 1-2% and virus was allowed to adsorb to them over a

period/.....

period of 1-2 hours with occasional agitation.

Next, the agglutinated cells were sedimented at 500 r.p.m. for 5 minutes, the supernatant fluid decanted, and the cells washed three times in about 20 volumes of ice-cold saline. The cells were always triturated with a fine Pasteur pipette to ensure thorough washing. Finally, the agglutinated cells were resuspended in a volume of PBS 1/20th of that of the original infected fluid and placed in a water bath at 37°C for 90 minutes, with occasional agitation, to allow the virus to elute from the red cells. The latter were then sedimented, the supernatant being saved and the cells washed in a further volume of warm PBS. The two volumes of PBS were combined to constitute the first eluate, which represented an approximate tenfold concentration of virus.

A second cycle of adsorption and elution was carried out on the first eluate using the same volume of red cells as in the first cycle, thus effecting a further tenfold concentration of virus. The second eluate constituted the suspension of partially purified and concentrated virus.

Influenza C was an exception and could not be concentrated satisfactorily and conveniently by adsorption and elution. Instead the clarified infected fluids were concentrated in the Spinco preparative centrifuge rotor 21 by centrifuging for 70-90 minutes at 19,000 r.p.m. The pellets were resuspended in PBS and clarified by low-speed centrifugation.

Ether Fractionation of Whole Virus

The virus suspension, prepared as above, was treated with an equal volume of freshly distilled di-ethyl ether at room temperature (18-23°C) for 90-120 minutes with continuous agitation by means of a magnetic stirrer. After low-speed centrifugation (2000 r.p.m. for 5 minutes) the aqueous phase, consisting of a mixture of the haemagglutinin

and/.....

and nucleoprotein, was transferred to a wide-mouthed container and placed at 37°C overnight to remove residual ether. The suspension was spun in a Spinco preparative centrifuge at 33,000 r.p.m. for 1 hour in an attempt to remove unsplit live or partially split virus. The supernatant fluid was then chilled in an ice bath and packed, washed guinea-pig cells were added until the haemagglutinin was exhaustively adsorbed so that when the cells were sedimented the supernatant fluid constituted the nucleoprotein fraction. The red cells were washed thoroughly 6 times in about 10 volumes of ice-cold PBS, resuspended in a small volume of PBS and elution of the haemagglutinin allowed to occur at 37°C for 1-2 hours. After centrifugation, the SNF was removed and pooled with an additional amount of warm PBS which was used to wash the red cells. This pool constituted the haemagglutinin fraction.

The fractions were used as antigens in the complement fixation tests, and to produce immune sera in guinea-pigs. In the latter case the fractions were checked to exclude residual live virus by two consecutive allantoic passages in eggs.

The exception was influenza C in which the haemagglutinin could not be effectively separated from its soluble antigen by adsorption and elution. The ether-treated purified virus was therefore dialysed overnight in the cold against distilled water, and the haemagglutinin-associated precipitate spun out by low-speed centrifugation and the supernatant fluid finally absorbed exhaustively with washed fowl cells. This supernatant, i.e. the soluble antigen fraction, was retained. In another preparation, the haemagglutinin and soluble antigen were not separated, and the mixture was designated influenza C (V + NP).

PREPARATION/.....

PREPARATION OF IMMUNE SERA FOR USE IN COMPLEMENT FIXATION TESTS

PREPARATION OF ANTI-HAEMAGGLUTININ (ANTI-V) SERA

The method of Fabiyi et alia (1958) was used. Guinea-pigs were injected intraperitoneally with 1.0-2.0 ml. amounts of purified haemagglutinin containing more than 2560 HAU per ml. (titrated with guinea-pig red cells). Three injections were given with an interval of one week between the first and second, and of three weeks between the second and third. The guinea-pigs were bled by cardiac puncture 7 days after the last dose, the sera were separated, inactivated at 56°C for 30 minutes, absorbed for 5-10 minutes at room temperature with washed sheep red cells, and stored frozen at -20°C.

PREPARATION OF "ANTI-NUCLEOPROTEIN" (ANTI-NP) SERA

The method used was that of Lief et alia (1958 a), but because the same strain of virus was used for the intranasal inoculation as in the preparation of nucleoprotein for the booster inoculation, the immune sera were expected to possess good titres of both anti-nucleoprotein and anti-haemagglutinin antibodies. Guinea-pigs were inoculated under light ether anaesthesia with freshly harvested allantoic fluid, diluted, if necessary, to contain approximately 10^6 EID_{50} of virus per 0.1 ml. This amount was introduced into each nostril. After an interval of 5-8 weeks, each animal received an intraperitoneal inoculation of 1.0-2.0 ml. homologous nucleoprotein antigen prepared by ether fractionation as described above. The animals were bled 7 days later by cardiac puncture and the sera heat-inactivated, absorbed with sheep red cells and stored at -20°C.

TESTS/.....

TESTS FOR THE PURITY OF THE HAEMAGGLUTININ AND NUCLEOPROTEIN ANTIGENS AND THEIR RESPECTIVE GUINEA-PIG IMMUNE SERA

Because preliminary experiments showed that Tern virus nucleoprotein was antigenically similar to that of Influenza A_2, but that their haemagglutinins were antigenically distinct, Influenza A_2 nucleoprotein and "anti-nucleoprotein" serum could be used to test the purity of the Tern virus material, and vice versa (Lief et alia, 1958a; Fabiyi et alia, 1958).

To this end, Tern virus haemagglutinin was considered to be free of its own nucleoprotein by the inability to demonstrate complement fixation with Influenza A_2 "anti-nucleoprotein" serum, while anti-haemagglutinin serum was shown to be free of anti-nucleoprotein antibodies by the inability to demonstrate complement fixation with influenza A_2 nucleoprotein. The nucleoprotein preparations could then be shown to be free of haemagglutinin by testing with homologous anti-haemagglutinin serum which had been shown to be free of anti-nucleoprotein antibodies.

A similar procedure was followed for the Chicken virus.

As expected, the "anti-nucleoprotein" sera showed high titres of homologous anti-haemagglutinin as well as anti-nucleoprotein antibodies (Lief et alia, 1958 a).

PREPARATION OF OTHER IMMUNE SERA

Ferret Immune Serum

A sample of pre-inoculation serum was obtained by cardiac puncture. Later, the ferrets were anaesthetised with ether and approximately 10^6 EID_{50} of virus introduced into each nostril. The animals were bled 3-4 weeks later, rested for two weeks, again inoculated intranasally and then exsanguinated 7-10 days later. The sera were separated, heated at 56°C for 30 minutes and stored at -20°C.

Fowl/.....

Fowl Immune Serum

Fowls (Leghorn-Australorpe cross) surviving experimental infection (by the intranasal and conjunctival sac routes) with Tern or Chicken virus were given one or more challenge inoculations and bled by cardiac puncture. The serum was heat-inactivated and stored at -20°C.

Rabbit Immune Serum

After preliminary bleeding a mature rabbit was immunised with allantoic fluid infected with Tern virus according to the following schedule:

Day 1. Intramuscular inoculation (IMI) of 0.5 ml. of allantoic fluid to which formalin had been added to a concentration of 0.1% and allowed to act at room temperature for 24 hours.

Day 4. IMI and intraperitoneal inoculation (IPI) of 0.5 ml. formolised allantoic fluid

Day 12. IPI 0.75 ml. and intravenous inoculation (IVI) 0.25 ml. allantoic fluid.

Day 37. IPI 1.5 ml. and IVI 0.5 ml. allantoic fluid.

Day 44. Bled and serum separated, heat-inactivated and stored at -20°C.

A control rabbit was inoculated with normal allantoic fluid in an identical manner.

REAGENTS/.....

REAGENTS FOR THE COMPLEMENT FIXATION TEST

Diluent

The "Veronal" buffer of Mayer et alia (1946) was used throughout. The stock solution contained:-

NaCl	85.0 g.
5-5 diethylbarbituric acid	5.75 g.
Sodium 5-5 diethylbarbiturate	3.75 g.
$CaCl_2$	0.28 g.
$MgCl_2.6H_2O$	1.68 g.
Glass distilled water to	2,000 ml.

The acid was dissolved in approximately 500 ml. of hot glass distilled water and the NaCl and Sodium barbiturate added. The volume was made up to 2,000 ml. and the $CaCl_2$ and $MgCl_2.6H_2O$ added. Sterilisation was by autoclaving at 15 lbs/square inch for twenty minutes. The pH was 7.2 and the stock was stored in the refrigerator. For use each day an appropriate amount of this stock was diluted 1/5 in glass distilled water, and gelatin added to a concentration of 0.1%.

Sheep Red Cells

Blood was collected aseptically from the jugular vein of a sheep into two volumes of a sterile mixture of equal amounts of 3.8% sodium citrate in saline and Alsever's solution as modified by Bukantz et alia (1946).

The latter solution had the following composition:

Dextrose	20.5 g.
Sodium citrate	8.00 g.
Sodium chloride	4.20 g.
Citric acid (to adjust pH to 6.1)	0.55 g.

Distilled/.....

Distilled water ..... to 1000 ml.

The cells were stored in the refrigerator and remained suitable for use for several months. They were allowed to age for one week before use. An appropriate quantity of cells was washed thrice in 4-5 volumes of diluent. A 2% (v/v) suspension was made in diluent under standard conditions (Kolmer et alia, 1951), and the haemoglobin concentration of this suspension was measured with the MRC photometer.

For use in complement fixation tests a 2% suspension of washed cells was prepared as follows:-

the haemoglobin concentration of an initial suspension of washed cells was estimated and the suspension then diluted to give the haemoglobin concentration indicative of a 2% suspension.

Fresh suspensions were prepared every 2 days.

Haemolysin

The anti-sheep haemolysin was prepared in rabbits and titrated according to standard methods (Kolmer et alia, 1951). It was diluted with an equal volume of neutral glycerin and stored in the refrigerator. A stock solution of 1/100 was prepared by diluting 2 ml. of the haemolysin-glycerin mixture with 94 ml. of normal saline plus 4 ml. of 5% phenol in saline. The stock solution was also stored in the refrigerator, and for use an aliquot was diluted in "Veronal" buffer to contain 8 minimal haemolytic doses (MHD) per unit volume.

Complement

Pooled guinea-pig serum was used as the source of complement and was preserved either by distributing suitable aliquots in ampoules and storing in dry ice, or by the method of Richardson (1941) as

described/.....

described below:-

Solution A	Boric acid (H_3BO_3)	1.55 g.
	Saturated NaCl to	100 ml.
Solution B	Sorbitol ($C_6H_{14}O_6$. $1/2H_2O$)	9.55 g.
	Sodium azide (NaN_3)	0.81 g.
	Saturated NaCl to	100 ml.
Solution C	Sodium azide	0.81 g.
	Saturated NaCl to	100 ml.

To each 8 ml. of guinea-pig serum 1 ml. of solution A and 0.5 ml. each of solutions B and C were added. The preserved complement was stored in the refrigerator and diluted 1/8 with distilled water before titration, giving an initial dilution of 1/10 of complement.

PROCEDURE FOR THE COMPLEMENT FIXATION TEST

The small volume method in Wasserman tubes (7.5 x 1 cm.) was also used, but the micromethod employing perspex plates was found perfectly satisfactory provided careful attention was paid to all the details of technique as described below. This method was also more economical and convenient.

MICROMETHOD

This method was carried out on the standard perspex plates used for HA tests and a "40 dropper pipette" (0.025 ml. per drop) was employed. The exact volume delivered by the pipette was not important as long as the same pipette was used for a given batch of tests and thoroughly washed out with diluent between reagents.

To avoid a fall in the titre of the complement dilution while distributing the reagents, it was essential to keep all reagents chilled

in/.....

in an ice bath and to pre-chill the perspex plates. The latter were taken out of the refrigerator when needed for the distribution of a particular reagent, and returned immediately until needed for the distribution of the next reagent. From the time of pre-chilling until the reading of the test, each plate was kept covered with a well-fitting sheet of plastic (1.5 mm. in thickness) except while reagents were added and while the plate was warming to room temperature before the addition of sensitised cells.

Complement Titration

Chemically preserved complement was used and was titrated under the identical conditions in which the test was carried out. From the starting dilution of 1/10, a series of dilutions (1/70, 1/80 ..... 1/160) was made in Wasserman tubes and four drops of each dilution were transferred to a row of cups in a (pre-chilled) perspex plate. Four drops of diluent were added to each cup and the plate covered and placed overnight for 16-18 hours in the refrigerator. The next morning the plate was left uncovered to warm up to room temperature whilst sheep cells were being sensitised for 15 minutes at room temperature by mixing equal volumes of a 2% washed sheep cell suspension and appropriately diluted haemolysin. Four drops of sensitised cell suspension were then added to each of the cups and the reagents mixed by tapping the plate which was then covered and incubated at 37°C for 90 minutes. The plates were not stacked but placed on separate slatted shelves to allow access of air all around them. It was important that the contents of the plates be mixed after 20 minutes and again 10 minutes later. This was best carried out by gently tapping the plates.

One minimal haemolytic dose (MHD) was contained in the highest dilution of complement showing complete haemolysis as judged by reading with an inverted microscope. Two MHD were used in the test. All the

tests proper were two-dimensional so that it was not necessary to titrate complement in the presence of antigen.

The chemically preserved complement was found to maintain its titre for at least two months.

The Test Proper

The detailed procedure was similar to that described above for the titration of complement. Apart from screening tests all the tests were two-dimensional.

The reagents in each cup were added in the following order and the amounts used were as shown or half these quantities:

Serum ..... 2 drops (0.05 ml.)
Antigen ..... 2 drops
Complement ..... 4 drops (2 MHD)
Incubation at 4°C for 16-18 hours
Incubation at room temperature for 15 minutes

Sensitised cell suspension ..... 4 drops
Incubation at 37°C for 90 minutes

The standard controls were set up:

Antigen controls:
Each antigen dilution was set up with diluent instead of immune serum.

Serum controls:
Each serum dilution was set up with diluent instead of antigen.

Cell and diluent controls:

a) Equivalent to grade 4 reading - cup finally contained 8 drops of diluent and 4 drops of sensitised cells.

b) Equivalent to grade 2 reading - cup finally contained 10 drops of diluent and 2 drops of sensitised cells.

Complement control:

A control titration of the dilution of complement used was carried out to verify that it contained 2 MHD per 4 drops.

Reading of Results

Each cup of the test was read and the degree of complement fixation graded according to the size of the cell button, the cell-diluent controls serving as comparison:-

No lysis	= grade 4 complement fixation
Complete lysis	= grade 0
Grades 3, 2, 1 and trace (tr)	= intermediate grades

The complement fixing titre of the antigen or immune serum was defined as the highest dilution which gave grade 4 or 3 fixation with the highest dilution of immune serum or antigen respectively. This dilution, as determined by homologous titrations, contained one complement fixing unit of the antigen or antibody respectively (see Tables 8, 9 and 10 on pages 58, 59 and 60).

The serial serum dilutions used started at 1/8 or lower, and the initial dilution contained a minimum of 8 units of antibody. The series of antigen dilutions included the original undiluted suspension which contained 8-64 units of antigen.

SMALL/.....

SMALL VOLUME METHOD

This was performed in Wasserman tubes and reagents were distributed with 1.0 ml. graduated pipettes.

Complement titration

To 0.2 ml. of each of a series of dilutions was added 0.2 ml. of diluent. The tubes were placed in a waterbath at 37°C for 15 minutes during which time sheep cells were sensitised as described above. 0.2 ml. of sensitised cell suspension was added to each of the tubes which were replaced in the waterbath at 37°C for 30 minutes. The highest dilution showing complete haemolysis contained one minimal haemolytic dose (MHD) of complement. Two MHD were used in the test.

Test Proper

Racks and tubes were chilled and the reagents were added as follows:-

serum ..... 0.1 ml.
antigen .....0.1 ml.
2 MHD complement dilution ..... 0.2 ml.

After incubation for 16-18 hours in the refrigerator the racks were placed for 15 minutes in a waterbath at 37°C. Then 0.2 ml. of sensitised cell suspension was added to each tube and the racks were replaced in the waterbath for a further 30 minutes.

The controls and grading of results were as described above.

RECIPROCAL CROSS-COMPLEMENT FIXATION TESTS

All reciprocal cross-complement fixation tests were two-dimensional and were carried out at the same session.

PREPARATION/.....

PREPARATION OF STOCK VIRUS SUSPENSIONS. METHOD OF PASSAGE.

The virus strains were not "purified" e.g. by means of a series of passes at limit dilutions, in order to avoid the possibility of selecting mutant populations.

Accordingly, all the virus strains,except for Influenza C, were propagated by inoculating 10^3 to 10^5 EID_{50} of the respective strains into the allantoic cavity of 3 or more eggs and subsequently harvesting and pooling the infected allantoic fluids, which were then designated according to the number of serial allantoic passes as Allantoic 1, or 2, etc.

In the case of Influenza C the amniotic route of inoculation was employed for propagation and the harvested infected amniotic fluids were designated as Influenza C Amniotic 1, or 2, etc.

A reference stock of each virus strain was prepared from freshly harvested virus infected allantoic, or, in the case of Influenza C, amniotic fluids, which were lyophilised and stored at -20°C. An ampoule of lyophilised virus was reconstituted at appropriate intervals and subcultured in eggs to prepare a working stock of virus suspension which was distributed in ampoules and stored frozen at -20°C.

The virus infected fluids usually contained between 10^7 and 10^9 EID_{50} per ml.

Those virus strains adapted to CETC were designated according to their chronological passage history e.g. Tern virus (Allantoic 9, CETC 11) indicated 9 consecutive allantoic passes followed by the 11th consecutive pass in CETC.

PASSAGE HISTORY OF THE VIRUS STRAINS EMPLOYED

Tern virus was used at the 3rd to 11th allantoic passage level except for experiments in CETC where Tern virus (Allantoic pass 9, CETC

pass/.....

pass 11) was employed.

Chicken virus was originally isolated in eggs, and made available to the Onderstepoort Veterinary Laboratory by Dr. J.E. Wilson. At Onderstepoort it was given one pass in eggs, the infected allantoic fluids of which were lyophilised and a sample made available to our laboratory.

Chicken virus was used at the same passage levels in eggs in this laboratory as Tern virus. In CETC experiments Chicken virus (allantoic pass 9, CETC pass 2) was employed.

Influenza A_2 allantoic pass 3 and 4 were employed.

Influenza Lee B, a long-established laboratory strain, was used at the 3rd or 4th allantoic passage level in this laboratory.

Influenza C/JJ/1950 was also an established laboratory strain, and amniotic pass 3 and 4 were employed.

NDV strain Komarov was an established laboratory strain. Allantoic pass 3 and 4 in this laboratory were used, except for CETC experiments where NDV (allantoic pass 3, CETC pass 2) was employed.

PRECAUTIONS AGAINST LABORATORY CROSS-INFECTION

Particularly stringent precautions were observed to avoid any danger of a laboratory 'pick-up'. In addition to the usual precautions, the programme was planned to avoid handling different viruses on the same day. Occasionally, when this was not possible, separate cubicles and usually different workers were used for each virus.

B: RESULTS

ISOLATION OF TERN VIRUS

DATA ON SEA-BIRDS INVESTIGATED

Data on 19 sea-birds investigated during and subsequent to the epizootic of 1961 are presented in Table 4, pages 45 and 46. Most of the birds were terns, either Sterna hirundo (Common Terns), Sterna macrura (Arctic Terns), or Sterna bergii (Swift Terns), but two Cape Cormorants (Phalacrocorax capensis) and a Hartlaub's Gull (Larus hartlaubii) were included in the group. Birds 1, 2, and 3, all Common Terns, were the only birds collected at the time of the epizootic for virus studies.

Investigation of Birds 1, 2 and 3

Two pools were prepared from the organ emulsions of each bird by mixing equal portions of the emulsions of brain and liver for one pool and heart, lung and kidney for the other. The pools were inoculated in 0.1 ml. amounts into embryonated eggs via the allantoic route, into both KB and monkey kidney tissue culture roller tubes, and into newborn mice intracerebrally.

Bird 1:

Inoculated eggs. In the case of Bird 1, the embryos of the inoculated eggs died within 48 hours and presented a rather striking appearance; their external surfaces were congested and showed punctate and frequently larger focal haemorrhages. The allantoic fluids harvested from these eggs agglutinated chicken red cells and contained 80 HAU per ml. while the allantoic fluids of uninoculated eggs of the same batch did not agglutinate chicken cells. No bacteria were detected in the fluids on examination of a

smear/.....

TABLE 4. (Contd. on next page)

DATA ON SEABIRDS INVESTIGATED

BIRD NO.	1 *	2 *	3 *	4	5	6	7	8	9	10
SPECIES	S.hirundo	S.hirundo	S.hirundo	S.bergii	S.macrura	S.hirundo	S.bergii	S.macrura	S.hirundo	S.hirundo
SOURCE	<---------- CAPE PENINSULAR REGION ---------->									
RECEIVED	27.4.61	27.4.61.	11.5.61.	1.6.61.	8.6.61.	8.6.61.	1.6.61.	14.6.61.	4.7.61.	4.7.61.
CONDITION	Dead 2 days		Ill	Well	Shot 8 days before		Well	Dead 2 days	Shot 2 days before	
AUTOPSY	27.4.61.	27.4.61.	11.5.61.	2.6.61.	8.6.61.	8.6.61.	15.6.61.	14.6.61.	4.7.61.	4.7.61.
STORAGE BEFORE TITRATION	72 days at -20°C	78 days at -20°C	5 days at -70°C	Titrated stat.	36 days at -20°C	41 days at -20°C	34 days at -20°C	51 days at -20°C	31 days at -20°C	39 days at -20°C
TERN VIRUS CONTENT OF: ***										
HEART	4.3	1.9	trace	-	-	-	-	-	-	-
LUNG	5.1	3.3	trace	-	-	-	-	-	-	-
LIVER	3.7	trace	trace	-	-	-	-	-	-	-
KIDNEY	3.5	1.7	trace	-	-	-	-	-	-	-
BRAIN	1.7	-	-	-	-	-	-	-	-	-
BLOOD	5.1	3.7	-	NT	NT	NT	-	-	-	-
SERUM HI ANTIBODY TITRE	NT	NT	< 5	< 5	NT	NT	< 5	NT	20	20

* = The only birds received during the epizootic. - = No virus isolated after 2 passes in eggs.

*** = Virus content expressed as the logarithm$_{10}$ EID$_{50}$ per gram of tissue. NT = Not tested.

TABLE 4. (Continued) DATA ON SEABIRDS INVESTIGATED.

BIRD NO.	11	12 **	13 **	14	15	16	17	18	19 **
SPECIES	S.macrura	S.bergii	S.bergii	S.hirundo	S.hirundo	S.hirundo	P.capensis	P.capensis	L.hart-laubii
SOURCE	<-- CAPE PENINSULAR REGION ->			<---------- PORT ELIZABETH REGION ---------->					Cape Town
RECEIVED	4.7.61.	1.6.61.	1.6.61.	19.4.63.	6.8.63.	26.9.63	29.6.61.	2.7.61.	6.9.61.
CONDITION	Shot 2 days	Well	Well	<-------- Dead approx. 24 hours -------->				Well	Alive
AUTOPSY	4.7.61.	30.8.61.	6.11.61.	19.4.63.	6.8.63	26.9.63	29.6.61.	2.7.61.	14.9.61.
STORAGE BEFORE TITRATION	46 days at -20°C	1 day at -20°C	NT	Titrated stat.	2 days at -20°C	5 days at -20°C	2 days at -20°C	17 days at -20°C	1 day at -20°C
TERN VIRUS CONTENT OF:									
HEART	-	-	NT	NT	-	-	-	-	3.75
LUNG	-	-	NT	-	-	-	-	-	3.5
LIVER	-	-	NT	-	-	-	-	-	5.25
KIDNEY	-	-	NT	NT	-	-	-	-	4.75
BRAIN	-	-	NT	-	-	NT	-	-	-
BLOOD	NT	-	NT	NT	-	-	NT	NT	NT
SERUM HI ANTIBODY TITRE	< 5	< 5 (Pre) 160(post)	< 5(pre) 80(Post)	NT	NT	NT	NT	NT	5

** = Birds inoculated experimentally with live Tern virus.

Pre = Pre-inoculation serum sample.
Post = Post-inoculation serum sample.

smear stained by Gram's method, nor could bacteria be cultured. These facts suggested that the agent isolated might be a myxovirus.

Inoculated Tissue Cultures. The KB tubes were subinoculted after 9 days into fresh KB tubes, and seven days thereafter CPE was noted. A second subinoculation resulted in CPE in 6 days, and the culture fluid contained 1280 HAU per ml. in contrast to the fluid of control uninoculated tubes which showed no haemagglutinating activity. Culture in monkey kidney cells for a total of 21 days yielded negative results and the inoculated newborn mice suffered no ill effects during the 14-day period of observation.

Birds 2 and 3:

A similar agent was isolated from Birds 2 and 3 in eggs, but not in KB or in monkey kidney tissue culture nor in newborn mice.

CULTURE SYSTEM OF CHOICE

The embryonated egg thus proved to be a sensitive and convenient culture system and was used for the major part of this work. It was found that infection of the embryonated egg with Tern virus could be achieved by all the routes of inoculation tried, and that virus could be recovered from the allantoic fluid, the amniotic fluid, the embryonic membranes, the emulsified embryo and the yolk sac in approximately equal concentrations. The allantoic route of inoculation was chosen as the most suitable and the allantoic and amniotic fluids as the most suitable sources of virus. These fluids usually contained between $10^{7.5}$ and 10^{9} EID_{50} of Tern virus per ml. End-points were sharp in titrations of Tern virus in eggs, and the results could be read within 48-72 hours.

PROTOTYPE/.....

PROTOTYPE STRAIN OF TERN VIRUS

The agent isolated in eggs from the heart-lung-kidney pool of Bird 1 was employed as the prototype strain throughout this work. It was named Tern virus. Immune serum prepared in rabbits against the prototype strain of Tern virus had the same HI antibody titre when tested against the homologous strain and the strains isolated from the heart-lung-kidney pool of Bird 2 and the lung of Bird 3, indicating that the same virus had infected all three Common Terns obtained during the epizootic.

EVIDENCE THAT TERN VIRUS WAS A MYXOVIRUS

Evidence that Tern virus was a myxovirus was in the first instance obtained from the following experiments:-

1a. Adsorption and Elution from Erythrocytes

An aliquot of allantoic fluid from allantoic pass 2 containing 640 HAU of Tern virus per ml. was absorbed overnight at 4°C with washed fowl cells added to a concentration of 1.5%. The agglutinated cells were washed thrice in 20 volumes of cold saline, then resuspended in a volume of saline equal to that of the original aliquot and stood at room temperature ($\pm$ 20°C) for 30 minutes with occasional mixing. After light centrifugation a sample of the supernatant fluid was removed; it contained no haemagglutinating activity. The sedimented cells were immediately dispersed and transferred to a waterbath at 37°C for 30 minutes and thereafter centrifuged. The supernatant fluid was then found to contain 640 HAU per ml.

This property of adsorption and elution was repeatedly demonstrated in the present study by the standard technique employed for purifying Tern virus, but the recovery, though adequate, was not so complete with the larger quantities and the shorter adsorption and elution times employed.

1b. Preparation/.....

1b. Preparation of Indicator Virus

A second aliquot of allantoic fluid was treated in a similar manner but was first heated at 56°C for 30 minutes. This treatment did not destroy the haemagglutinating ability of the virus, but it could no longer elute from the red cells even after 150 minutes incubation at 37°C.

2. HA Titration of Tern and Influenza A_2 Virus Suspensions using Erythrocytes previously incubated with Tern Virus.

Washed fowl erythrocytes were made up to an 0.6% suspension in infected allantoic fluid containing 640 HAU of Tern virus per ml. and incubated at 37°C for 3 hours. Control cells were incubated in normal allantoic fluid. The "treated" and control cells were centrifuged and washed thrice in saline in which they were then made up to 0.6% suspensions which were used for the HA titration of a Tern and an Influenza-A_2 virus suspension.

The Tern virus suspension contained 640 HAU per ml. with control erythrocytes and less than 10 HAU with the "treated" cells. The Influenza-A_2 suspension contained 160 HAU with control cells while only a trace of haemagglutination was present at a dilution of 1/10 using "treated" cells.

The lower titres of the two virus suspensions with "treated" cells were interpreted as an indication of the similarity of the enzymes of the two viruses.

VIROLOGICAL INVESTIGATION OF THE SEA-BIRDS

The results of virus studies carried out on 19 sea-birds are presented in Table 4, pages 45 and 46.

The virus content of each organ was titrated in eggs employing

the/.....

the allantoic route of inoculation and using 5 eggs per dilution except for Bird 19 where 4 eggs per dilution were inoculated. The serum, where available, was tested for HI antibodies against Tern virus.

Tern virus was only isolated from Birds 1, 2 and 3, which were received during the epizootic, and from Bird 19 which was inoculated experimentally.

Birds 1 and 2 were received dead, having been picked up ill and then sacrificed. Bird 3 was received alive but ill and sat huddled up with ruffled feathers and was unwilling to move when disturbed. It was sacrificed on receipt.

It is noteworthy that the virus content of some of the organs of Tern 1 was moderately high despite the storage of the emulsions at -20°C for 72 days. Less virus was found in the tissues of Tern 2 and only traces in those of Tern 3 in the serum of which no HI antibody to Tern virus was detected.

In the post-epizootic period the only evidence of natural infection with Tern virus was the presence of specific HI and neutralising antibodies in the serum of each of two Common Terns (Birds 9 and 10) shot in July 1961. No HI antibodies were detected in the serum of an Arctic Tern (Bird 11) shot at the same time.

Birds 12, 13 and 19 were inoculated experimentally. Birds 12 and 13, both Swift Terns, remained well after inoculation with live Tern virus. Bird 12 was inoculated intramuscularly (IM) with approximately 10^6 EID_{50} and this was repeated 9 days later. It died after a further interval of 40 days as a result of cardiac puncture. No virus was isolated from any of its organs, nor were lesions detected histologically in them. Its serum however had an HI antibody titre of 160 against Tern virus. Bird 13 had a series of 4 IM inoculations of approximately 10^6 EID_{50} at intervals of 17, 17 and 22 days and died 19 days later as a result of cardiac puncture. Virus isolation was not attempted, but no lesions were

found/.....

found on histological examination of the organs. The HI antibody titre of the serum was 80 against Tern virus.

No HI antibodies were detected in pre-inoculation samples of serum of the two above birds.

Bird 19, a Hartlaub's Gull, showed no HI antibodies in a pre-inoculation sample of serum, and died on the third day after IM inoculation of approximately 10^6 EID_{50} of Tern virus. Virus was present in moderately high amounts in the tissues, but histological examination of the latter proved negative, possibly because the bird died before sufficient time had elapsed for lesions to develop. This point will be discussed in section III.

HISTOLOGICAL EXAMINATION OF THE SEA-BIRDS

The histology was reported on as follows by Professor C.Uys (1963):

Birds 1, 2 and 3 (Common Terns):

"Except for bird 3, the organs other than the brain proved to be extremely autolytic and thus valueless for histological examination. Although autolytic changes were present in the brain as well, the changes there could still be evaluated.

Evidence of encephalitis was present in all three birds. The most characteristic lesion was focal infiltration of the brain substance by mononuclear cells, associated loss of neurones and focal proliferation of glial cells. These cellular nodes appeared to occur around a central capillary-sized vessel and were distributed sparingly throughout the cerebrum and cerebellum. A more diffusely distributed perivascular cuffing of lymphoid cells and slight increased cellularity of the meninges were also noted.

The/.....

The liver, lungs, kidneys and heart of Bird 3 were normal. The spleen showed slight increase in reticular cells around the sinusoids, but otherwise was not remarkable.

Hartlaub's Gull:

Eye, heart, brain, kidney, liver and lung showed no change of note.

Other birds, including experimentally inoculated Swift Terns:

Brain, myocardium, kidney, lung, liver, spleen and eye showed no change of note."

SEROLOGICAL CLASSIFICATION OF TERN VIRUS

HAEMAGGLUTINATION-INHIBITION TESTS WITH TERN VIRUS AGAINST NORMAL ANIMAL SERA

This experiment was carried out to determine if normal sera might have a non-specific inhibitory effect on Tern virus haemagglutination. No inhibition was detected in any of the following normal sera which were tested at an initial dilution of 1/10:- Sera of 3 ferrets, 5 rabbits, 4 Swift Terns, pooled and individual guinea-pig sera, pooled and individual fowl sera, pooled human serum, calf and horse serum, pooled rat and pooled mouse serum. Serum from a fowl undergoing artificial immunisation against Tern virus was included in the test and gave inhibition up to a dilution of 1/40, while the pre-immune serum gave no inhibition of haemagglutination. All except the rat and mouse sera were previously heated at 56°C for 30 minutes in order to remove heat-labile inhibitors.

HAEMAGGLUTINATION-/.....

HAEMAGGLUTINATION-INHIBITION TESTS WITH TERN VIRUS AND MYXOVIRUS STRAIN SPECIFIC IMMUNE SERA

Results of HI tests with various specific immune sera are presented in Table 5, page 53. The corresponding specific antigens were available for some of the immune sera used, in which case the homologous HI test was also carried out. These sera were treated with receptor-destroying enzyme to eliminate non-specific inhibition. Only Chicken virus had demonstrable antigenic sharing with Tern virus, to which it was closely related.

TABLE 5. HI TESTS WITH TERN VIRUS ANTIGEN AND MYXOVIRUS STRAIN-SPECIFIC IMMUNE SERA.

STRAIN-SPECIFIC IMMUNE SERUM	SOURCE OF SERUM	HI ANTIBODY TITRE AGAINST 4HAU OF TERN VIRUS	HOMOLOGOUS HI ANTIBODY TITRE
Tern virus/SA/1961	rabbit	160	160
Chicken virus/Scot./ 1959	chicken	160	640
Fowl plague virus 1. strain Brescia	-	< 4	-
2. strain Alexandrien	-	< 4	-
Chicken virus N	-	< 4	-
NDV, 2 lots	chickens	< 5	160
Influenza			
-A/PR8/1934	guinea-pig	< 10	-
-A/FM1/1947	guinea-pig	<10	-
-A/Phil/1953	guinea-pig	<10	-
-A_2/Asia/1957	rabbit	< 6	192
-A_2/CT/1957	ferret	< 5	160
-B/Jo'burg/1958	rabbit	< 6	768
-C/JJ/1950	ferret	< 6	1280
Mumps- 2 sera	human	< 6	-
Mumps	guinea-pig	<10	-
Para-influenza 1	guinea-pig	<10	-
" 2	guinea-pig	<10	-
" 3	guinea-pig	<10	-
Simian virus 5	rabbit	<10	-

- = information not available.

TERN VIRUS NEUTRALISATION TESTS IN EGGS

Neutralisation tests were performed in eggs with Tern virus (allantoic 3) against homologous, Chicken virus and NDV immune sera and several control sera as set out in Table 6. The test was positive for homologous and Chicken virus immune serum but negative for the others.

TABLE 6. NEUTRALISATION TESTS WITH TERN VIRUS (200 EID_{50} OF ALLANTOIC PASS 3) IN EMBRYONATED EGGS

Strain specific and control sera	Source	Dilution	Result	Homologous HI titre of Serum
Tern/SA/1961	rabbit	1/5	+	160
Chicken/Scot./1959	chicken	1/6	+	640
NDV (Komarov)	chicken	1/6	-	160
Control	chicken	1/5	-	
Control	rabbit	1/5	-	
Control	guinea-pig	1/5	-	
Control	ferret	1/5	-	

+ = neutralisation - = no neutralisation

CROSS-NEUTRALISATION TESTS IN CETC TUBES

The results of neutralisation tests in CETC tubes using Tern, Chicken and Newcastle disease viruses and a series of strain-specific immune sera are recorded in Table 7, page 55. Approximately 100 $TCID_{50}$ of Tern virus (allantoic 9, CETC 11), of Chicken virus (allantoic 9, CETC 2) and of NDV (allantoic 3, CETC 2) were used in the tests.

TABLE 7/.....

TABLE 7. CROSS-NEUTRALISATION TESTS IN CHICK EMBRYO TISSUE CULTURE TUBES

Strain-specific immune serum	Source	Dilution used	Results			Homologous HI antibody titre
			Tern virus	Chicken virus	NDV	
Tern/SA/1961	rabbit	1/5	+	+	-	160
Chicken/Scot./ 1959	chicken	1/10	+	+	-	640
Influenza A_2/Asia/1957	rabbit	1/6	-	-	-	192
Influenza A_2/CT/1961	guinea-pig	1/5	-	-	-	40
NDV (Komarov)	chicken	1/5	-	-	+	160
Fowl plague 1. strain Brescia	data not available	1/5	-	-	-	
Fowl plague 2. strain Alexandrien	data not available	1/5	-	-	-	
Chicken virus N	data not available	1/5	-	-	-	
Influenza B/Jo'burg/1958	rabbit	1/6	-	-	-	
Influenza C/JJ/1950	ferret	1/5	-	-	-	
Mumps (2 sera)	human	1/5	-	-	-	
SV5	rabbit	1/5	-	-	-	

+ = neutralization - = no neutralization.

SEROLOGICAL CROSS-TESTS WITH CONVALESCENT CHICKEN SERA

The results of cross-neutralisation and cros-haemagglutination-inhibition tests with immune chicken sera showing the close reciprocal antigenic relationship between Tern and Chicken viruses are presented in Section III dealing with the experimental infection of chickens (see Table 21, page 102.

COMPLEMENT FIXATION (CF) TESTS

Six lots of haemagglutinin and nucleoprotein were prepared from Tern virus, four from Chicken virus and five from Influenza A_2 virus. All of these antigens proved to be "pure" on testing, thus demonstrating the adequacy of the methods employed in their preparation. Three lots of Influenza B (V) and (NP) antigens were prepared, and from Influenza C two lots of nucleoprotein and one of haemagglutinin plus nucleoprotein.

"Anti-nucleoprotein" sera were prepared for each of the above strains, and anti-haemagglutinin sera for all except Influenza C in lieu of which the "anti-nucleoprotein" sera were used.

Cross-complement fixation tests were done with the reagents of each virus strain and those of each of the other 4 strains above, and repeated on two or more occasions using different batches of antigens and different sera. The results of these tests indicated that the nucleo-proteins of Tern, Chicken and Influenza A_2 viruses were closely related, and that the haemagglutinins of Tern and Chicken viruses were closely related to each other but not at all to that of Influenza A_2. There was no antigenic relationship between Influenza B and C viruses nor between these two viruses and Tern, Chicken or Influenza A_2 viruses.

Reciprocal/.....

Reciprocal Complement Fixation Tests with the reagents of Tern, Chicken and Influenza A_2 Viruses

Representative examples of the results obtained in the cross-complement fixation tests with the NP antigens of these three viruses and the V antigens of Tern and Chicken viruses are presented in Tables 8, 9 and 10 on pages 58, 59 and 60.

Although the antibody content understandably varied between sera, any given serum reacted at least as well with homologous antigen as with similar amounts of the heterologous antigens.

Table 8 presents the results obtained in two-dimensional cross-CF tests with the three type-specific antigens (NP). The NP antigens of Tern and Chicken viruses showed very similar results. Influenza A_2 NP antigen reacted with its own serum to a slightly higher titre than did the heterologous antigens, and it reacted with heterologous sera to about a two-fold lower titre than their respective homologous antigens.

Tables 9 and 10 show the results of cross-tests with the strain specific (V) antigens of Tern virus and Chicken virus. Similar results were obtained when purified haemagglutinin or whole virus was used as the V antigen; it was regularly found that the Tern virus anti-V sera reacted to practically the same titre with Tern virus as with the Chicken virus V antigen, but that the Chicken virus anti-V sera reacted to a two- to four-fold higher titre with homologous than with Tern virus V antigen.

TITRATION/.....

TABLE 8. CROSS-COMPLEMENT FIXATION TESTS WITH THE NUCLEOPROTEIN (NP) ANTIGENS AND "ANTINUCLEOPROTEIN SERA" OF TERN, CHICKEN AND INFLUENZA A_2 VIRUSES.

	INFLUENZA A_2 VIRUS "ANTI-NP SERUM"								TERN VIRUS "ANTI-NP SERUM"								CHICKEN VIRUS "ANTI-NP" SERUM								Antigen Controls
	1/8	16	32	64	128	256	512	1024	1/8	16	32	64	128	256	512	1024	1/8	16	32	64	128	256	512	1024	
undil	4	4	4	4	4	4	4	.	4	4	4	4	4	.	.	.	4	4	4	4	tr	.	.	.	.
1/2	4	4	4	4	4	4	4	.	4	4	4	4	4	.	.	.	4	4	4	4	tr	.	.	.	.
Influenza 4	4	4	4	4	4	4	4	.	4	4	4	4	4	.	.	.	4	4	4	4	1	.	.	.	.
A_2 8	4	4	4	4	4	4	4	tr	4	4	4	4	4	.	.	.	4	4	4	4	3	.	.	.	.
Virus NP 16	4	4	4	4	4	4	3*	tr	4	4	4	4	3	.	.	.	1	3	4	4	2	.	.	.	.
32	tr	1	2	3	2	tr	.	.	tr	tr	tr	tr	.	.	.	.	.	.	tr	.	.	.	.	.	.
UNDIL	4	4	4	4	4	.	.	.	4	4	4	4	4	4	4	3	4	4	4	4	2	.	.	.	.
1/2	4	4	4	4	4	.	.	.	4	4	4	4	4	4	3	3	4	4	4	4	2	.	.	.	.
4	4	4	4	4	4	4	.	.	4	4	4	4	4	3	3	2	4	4	4	4	3	.	.	.	.
Tern 8	4	4	4	4	4	4	2	.	4	4	4	4	4	3	3	.	4	4	4	4	4	.	.	.	.
Virus NP 16	4	4	4	4	4	4	3	.	4	4	4	4	4	3	1	.	4	4	4	4	4	1	.	.	.
32	4	4	4	4	4	4	3	.	4	4	4	4	4	3*	1	.	4	4	4	4	4	3	.	.	.
64	.	tr	1	1	1	.	.	.	2	3	3	3	2	2	1	.	.	tr	2	2	2	.	.	.	.
undil	4	4	4	4	4	4	tr	.	4	4	4	4	tr	.	.	.	4	4	4	4	4	4	.	.	.
1/2	4	4	4	4	4	4	tr	.	4	4	4	4	4	1	.	.	4	4	4	4	4	3	.	.	.
4	4	4	4	4	4	4	tr	.	4	4	4	4	4	3	.	.	4	4	4	4	3	tr	.	.	.
Chicken 8	4	4	4	4	4	4	2	.	4	4	4	4	4	3	.	.	4	4	4	4	3	tr	.	.	.
Virus NP 16	4	4	4	4	4	4	3	.	4	4	4	4	4	3	tr	.	4	4	4	4	3	tr	.	.	.
32	4	4	4	4	4	4	3	.	4	4	4	4	4	3	tr	.	4	4	4	4	3*	tr	.	.	.
64	tr	tr	tr	tr	tr	.	.	.	tr	tr	tr	tr	tr	.	.	.	.	.	.	.	.	.	.	.	.
Serum Controls	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

* Indicates the dilution of serum or antigen containing one unit of complement fixing activity.
. = no detectable complement fixation. 4 = maximal complement fixation.
1, 2, 3 = intermediate grades of complement fixation.

TABLE 9. CROSS-COMPLEMENT FIXATION TESTS WITH THE STRAIN-SPECIFIC ANTIGENS AND IMMUNE SERA OF TERN AND CHICKEN VIRUSES.

		TERN VIRUS ANTI-V SERUM							CHICKEN VIRUS ANTI-V SERUM							Antigen Controls
		1/8	16	32	64	128	256	512	1/8	16	32	64	128	256	512	
	undil	4	4	4	4	4	3	.	4	4	4	4	1	.	.	.
	1/2	4	4	4	4	4	4	1	4	4	4	4	2	.	.	.
	4	4	4	4	4	4	4	2	4	4	4	4	2	.	.	.
Tern	8	4	4	4	4	4	4	1	4	4	4	4	2	.	.	.
Virus	16	4	4	4	4	3*	2	.	4	4	4	2	1	.	.	.
Haemagglutinin	32	.	tr	tr	tr	tr	.	.	1	2	tr	tr	.	.	.	.
	64	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	128	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	256	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	undil	4	4	4	4	4	4	tr	4	4	4	4	4	3	.	.
	1/2	4	4	4	4	4	4	1	4	4	4	4	4	4	.	.
	4	4	4	4	4	4	4	1	4	4	4	4	4	4	1	.
Chicken	8	1	2	4	4	3	tr	.	4	4	4	4	3*	2	1	.
Virus	16	.	.	.	.	.	.	.	4	4	4	2	1	tr	.	.
Haemagglutinin	32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	64	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	128	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	256	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Serum Controls	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

* Indicates the dilution of serum or antigen containing 1 unit of complement fixing activity.

TABLE 10 CROSS-COMPLEMENT FIXATION TESTS WITH THE STRAIN-SPECIFIC ANTIGENS AND IMMUNE SERA OF TERN AND CHICKEN VIRUSES.

		TERN VIRUS ANTI-V SERUM								CHICKEN VIRUS ANTI-V SERUM								Antigen Controls
		1/8	16	32	64	128	256	512	1024	1/8	16	32	64	128	256	512	1024	
Whole Tern Virus Antigen	undil	4	4	4	4	3	1	.	.	4	4	4	4	2	2	2	.	.
	1/2	4	4	4	4	4	3	2	.	4	4	4	4	3	2	.	.	.
	4	4	4	4	4	4	4	3	.	4	4	4	4	4	tr	.	.	.
	8	4	4	4	4	4	4	4	.	4	4	4	4	4	2	.	.	.
	16	4	4	4	4	4	4	4	1	4	4	4	4	4	4	.	.	.
	32	4	4	4	4	4	4	3	2	4	4	4	4	4	4	.	.	.
	64	4	4	4	4	4	4	3*	tr	4	4	4	4	4	3	.	.	.
	128	4	4	4	3	tr	.	.	.	4	4	4	4	4	2	.	.	.
	256	.	.	.	.	.	.	.	.	2	2	2	1	.	.	.	.	.
Whole Chicken Virus Antigen	undil	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	4
	1/2	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3
	4	4	4	4	4	4	4	4	1	4	4	4	4	4	4	3	1	.
	8	4	4	4	4	4	4	4	2	4	4	4	4	4	4	3	tr	.
	16	4	4	4	4	4	4	4	3	4	4	4	4	4	4	3	1	.
	32	4	4	4	4	4	4	4	3	4	4	4	4	4	4	3	1	.
	64	4	4	4	4	4	4	3	1	4	4	4	4	4	4	3	1	.
	128	4	4	4	4	3	2	1	.	4	4	4	4	4	4	3*	1	.
	256	.	.	.	1	2	2	.	.	4	4	4	4	1	1	.	.	.
Serum Controls		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

* Indicates the dilution of serum or antigen containing 1 unit of complement fixing activity.

TITRATION OF HOMOLOGOUS HAEMAGGLUTINATION-INHIBITING, COMPLEMENT FIXING AND NEUTRALISING ANTIBODIES IN STRAIN-SPECIFIC GUINEA-PIG IMMUNE SERA

Tern virus and Chicken virus strain-specific (anti-V) guinea-pig immune sera were tested with the homologous antigen to determine whether the purified haemagglutinin (V) had produced anti-V antibodies demonstrable by the HI and neutralisation tests as well as the CF test.

The results (see Table 11, page 62) showed that good titres of antibodies were demonstrable by all three serological tests.

THE SPECTRUM OF RED CELLS AGGLUTINATED BY TERN AND CHICKEN VIRUSES

Haemagglutination titrations of whole virus suspensions (freshly harvested infected allantoic fluids) were carried out with human, animal and avian 0.6% washed red cell suspensions.

The cells were collected not more than three days before use. A series of dilutions of each virus was made in bulk in saline and aliquots then distributed into the required numbers of rows of cups in perspex plates.

The HA spectrum of each virus was tested at 4°C, at room temperature (18-22°C) and at 37°C.

Readings which were made at 90 minutes and at 16 hours are recorded in Tables 12, 13 and 14, Pages 62a, 63 and 64. The plates at 37°C were read a third time at 20 hours.

HA Spectrum at 4°C (Table 12, page 62a)

After 16 hours at 4°C the haemagglutination titres with the various cell suspensions remained unchanged for both viruses as compared with the readings taken at 90 minutes.

TABLE 11. TITRATION OF HOMOLOGOUS HAEMAGGLUTINATION-INHIBITING, COMPLEMENT FIXING, AND NEUTRALISING ANTIBODIES IN STRAIN-SPECIFIC GUINEA-PIG IMMUNE SERA.

TERN VIRUS ANTIGEN			
HOMOLOGOUS IMMUNE SERUM (guinea-pig)	ANTIBODY TITRES		
	HI	CF	N
1	80	128	2560
2	40	128	1280
3	160	256	2560
CONTROL SERUM	< 5	< 8	< 5

CHICKEN VIRUS ANTIGEN			
HOMOLOGOUS IMMUNE SERUM (guinea-pig)	ANTIBODY TITRES		
	HI	CF	N
1	160	256	5120
2	20	128	320
3	160	512	5120
CONTROL SERUM	< 5	< 9	< 5

HI = Haemagglutination-inhibiting antibody titre using 4HAU of virus.

CF = Complement fixing antibody titre with 1 unit of V antigen.

N = Neutralising antibody titre using approx. 100 $TCID_{50}$ of Tern virus (Allantoic 9, CETC 11) and of Chicken virus (Allantoic 9, CETC 2).

TABLE 12.

HAEMAGGLUTINATION SPECTRUM OF TERN AND CHICKEN VIRUSES AT 4°C.

Read at 1½ hours

CELLS	TERN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120	CHICKEN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120
Fowl	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+−	.	.	.
Monkey	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+−	.	.	.
Goose	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	+−	.	.	.
Goat	+	+	+	+	+	+	+	.	.	.	+	+	+	+	+	+	.	.	.	.
Horse	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	.	.	.	.
Human	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	+−	.	.	.
G.Pig	+	+	+	+	+	+	+	+−	.	.	+	+	+	+	+	+	.	.	.	.
Mouse	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	.	.	.	.
Sheep	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	.	.	.	.
Rabbit	+	+	+	+	+	+	+	+	+	+−	+	+	+	+	+	+	+	.	.	.

Read after 16 hours

CELLS	TERN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120	CHICKEN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120
Fowl	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+−	.	.	.
Monkey	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+−	.	.	.
Goose	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	+−	.	.	.
Goat	+	+	+	+	+	+	+	.	.	.	+	+	+	+	+	.	.	.	.	.
Horse	+	+	+	+	+	+	+	+−	.	.	+	+	+	+	+	+	.	.	.	.
Human	+	+	+	+	+	+	+	+−	.	.	+	+	+	+	+	+	+−	.	.	.
G.Pig	+	+	+	+	+	+	+	+−	.	.	+	+	+	+	+	+	.	.	.	.
Mouse	+	+	+	+	+	+	+−	.	.	.	+	+	+	+	+	+	.	.	.	.
Sheep	+	+	+	+	+	+	+	.	.	.	+	+	+	+	+	.	.	.	.	.
Rabbit	+	+	+	+	+	+	+	+	+	+−	+	+	+	+	+	+	+	.	.	.

+ = HA

0 = elution after previous HA

. = no HA.

TABLE 13. HAEMAGGLUTINATION SPECTRUM OF TERN AND CHICKEN VIRUSES AT 18-22°C.

Read at 1½ hours

CELLS	TERN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120	CHICKEN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120
Fowl	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	.	.	.	.
Monkey	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	.	.	.	.
Goose	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	.	.	.
Goat	+	+	+	+	+	+	+	.	.	.	+	+	+	+	+	+−	.	.	.	.
Horse	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	+−	.	.	.
Human	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	+	.	.	.
G.Pig	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+−	.	.	.
Mouse	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+	.	.	.
Sheep	+	+	+	+	+	+	+	+−	.	.	+	+	+	+	+	+−	.	.	.	.
Rabbit	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+	.	.	.

+ = HA

0 = elution after previous HA

. = no HA

Read after 16 hours

Fowl	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	.	.	.	.
Monkey	+	+	+	+	+	+	+	+−	.	.	0	0	0	0	0	+−	.	.	.	.
Goose	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	.	.	.
Goat	+−	+	+	+	+	+	+−	.	.	.	0	0	0	0	0	0	.	.	.	.
Horse	+	+	+	+	+	+	+	+	+−	.	+	+	+	+	+	+	+−	.	.	.
Human	0	0	+	+	+	+	+	+	+−	.	0	0	0	0	0	+−	.	.	.	.
G.Pig	0	0	+	+	+	+	+	+−	.	.	+−	+−	+−	+−	+−	+−	.	.	.	.
Mouse	+	+	+	+	+	+	+	+−	.	.	+	+	+	+	+	+	+−	.	.	.
Sheep	0	0	+	+	+	+	+	.	.	.	0	0	0	0	0	0	.	.	.	.
Rabbit	+	+	+	+	+	+	+	+	.	.	+	+	+	+	+	+	+	.	.	.

TABLE 14.

HAEMAGGLUTINATION SPECTRUM OF TERN AND CHICKEN VIRUSES AT 37°C.

Read at 1½ hours

CELLS	TERN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120	CHICKEN VIRUS 1/10	20	40	80	160	320	640	1280	2560	5120
Fowl	+	+	+	+	+	+	+	+-	.	.	+	+	+	+	+	+	.	.	.	.
Monkey	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.
Goose	+	+	+	+	+	+	+	+	+	+-	+	+	+	+	+	+	+	.	.	.
Goat	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Horse	+	+	+	+	+	+	+	+	+-	.	+	+	+	+	+	+	.	.	.	.
Human	+	+	+	+	+	+	+	+	+-	.	0	0	0	+	+	+	+-	.	.	.
G.Pig	+	+	+	+	+	+	+	+	.	.	+-	+	+	+	+	+	.	.	.	.
Mouse	+	+	+	+	+	+	+	+	+-	.	+	+	+	+	+	+	+	.	.	.
Sheep	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Rabbit	+	+	+	+	+	+	+	+-	.	.	0	0	+	+	+	+	.	.	.	.

+ = HA

0 = elution after previous HA

. = no HA

Read after 16 hours

Fowl	+-	+	+	+	+	+	+	+-	.	.	+-	+	+	+	+	+	.	.	.	.
Monkey	0	0	0	0	0	+-	+-	+-	.	.	.	.	.	.	.	.	.	.	.	.
Goose	+	+	+	+	+	+	+	+	+	.	+	+	+	+	+	+	+	.	.	.
Goat	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.
Horse	0	0	0	0	0	0	0	0	0	.	0	0	0	0	0	0	.	.	.	.
Human	0	0	0	0	0	0	+-	+	.	.	0	0	0	0	0	0	0	.	.	.
G.Pig	0	0	0	0	0	0	0	0	.	.	0	0	0	0	0	0	.	.	.	.
Mouse	0	0	0	0	0	0	0	0	0	.	0	0	0	0	0	0	0	.	.	.
Sheep	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.
Rabbit	0	0	0	0	0	0	0	0	.	.	0	0	0	0	0	0	.	.	.	.

HA Spectrum at Room Temperature (Table 13, page 63)

After 90 minutes at room temperature the readings were the same as at 4°C. However after an interval of 16 hours at room temperature Tern virus had begun to elute from human, guinea-pig and sheep cells. By this time the Chicken virus had eluted completely or almost completely from monkey, goat, human and sheep cells and commenced elution from guinea-pig cells.

HA Spectrum at 37°C (Table 14, page 64)

After 90 minutes at 37°C the spectrum of Tern virus was the same as after 90 minutes at 4°C and room temperature, while the Chicken virus showed no agglutination of monkey, goat or sheep cells and elution of human and rabbit cells had already started. The spectrum after 16 hours at 37°C showed that both viruses still agglutinated fowl and goose cells, and Tern virus still showed some agglutination of monkey and human cells. The result read at 20 hours was practically identical for the two viruses, but the rate of reaching this point had been more rapid with the Chicken virus. At this time a trace of agglutination was still present in the higher dilutions with fowl cells. Goose cells were the only cells from which no elution had occurred.

It was also established that Tern virus agglutinated Swift Tern and Common Tern erythrocytes which were obtained from some of the captured birds.

TEMPERATURE STABILITY OF THE HAEMAGGLUTININ AND INFECTIVITY OF TERN AND CHICKEN VIRUSES

Tern virus- and Chicken virus-infected allantoic fluids of allantoic pass 11 and 12 respectively were tested. In each case equal volumes of allantoic fluid from 5 eggs were pooled and distributed in

ampoules/.....

ampoules in 1.0 ml. amounts. These were immersed in a 56°C waterbath and batches of ampoules removed at predetermined intervals to be cooled rapidly in an ice-bath in which control unheated ampoules had been placed at the start of the experiment. The contents of each ampoule were titrated by the haemagglutination method using 0.5% fowl cells, and residual live virus titrated in the allantoic cavity of embryonated eggs. The results are recorded in Table 15, page 67. It will be seen that the haemagglutinins of both viruses showed a four-fold drop in titre after 20 minutes and then remained relatively stable even after 150 minutes at 56°C. The infectivity of Tern virus was not destroyed after 60 minutes at 56°C while the infectivity of the Chicken virus was destroyed after 20 minutes at 56°C.

THE ADAPTATION OF TERN VIRUS AND CHICKEN VIRUS TO GROWTH IN TISSUE CULTURE

Both Tern and Chicken virus were readily adapted to growth in CE tissue cultures in which both produced similar cytopathic effects. The infected cell fluids usually contained approximately $10^{6.5}$ $CETCID_{50}$ per ml. of the respective viruses. The endpoints of titrations were usually reached in about 7 days.

Tern virus was also adapted to growth in KB cells in which it produced CPE. The infected cell fluids usually contained about $10^{6.5}$ $KBTCID_{50}$ per ml.

Cytopathic Effect of Tern Virus and Chicken Virus in Chicken Embryo Tissue Cultures as revealed by Staining with Haematoxylin and Eosin

Both viruses produced similar cytopathic effects. The initial changes were produced in the nucleoli which enlarged and became eosinophilic in contrast to their usual basophilic staining. They developed into irregular, eosinophilic inclusions, one or more per nucleus, and enlarged to fill the nucleus in which scattered fragments of chromatin remained.

Finally/.....

TABLE 15. TEMPERATURE STABILITY OF THE HAEMAGGLUTININ AND THE INFECTIVITY OF TERN AND CHICKEN VIRUSES.

TERN VIRUS

Time Interval in minutes at 56°C	HA Titration 1/5	10	20	40	80	160	320	640	1280	2560	Infectivity
0	+	+	+	+	+	+	+	+/-	.	.	+
10	+	+	+	+	+	+	+/-	.	.	.	+
20	+	+	+	+	+	+/-	.	.	.	.	+
30	+	+	+	+	+	+/-	.	.	.	.	+
60	+	+	+	+	+	+/-	.	.	.	.	+
90	+	+	+	+	+	+/-	.	.	.	.	
120	+	+	+	+	+	+/-	.	.	.	.	
150	+	+	+	+	+	+	+/-	.	.	.	

CHICKEN VIRUS

Time Interval in minutes at 56°C	HA Titration 1/5	10	20	40	80	160	320	640	1280	2560	Infectivity
0	+	+	+	+	+	+	.	.	.	.	+
10	+	+	+	+	+	.	.	.	.	.	+
20	+	+	+	+	.	.	.	.	.	.	-
30	+	+	+	+	.	.	.	.	.	.	-
60	+	+	+	+/-	.	.	.	.	.	.	-
90	+	+	+	+/-	.	.	.	.	.	.	
120	+	+	+	+/-	.	.	.	.	.	.	
150	+	+	+	+/-	.	.	.	.	.	.	

+ = haemagglutination or virus still infective.

. = no haemagglutination.

- = virus inactivated.

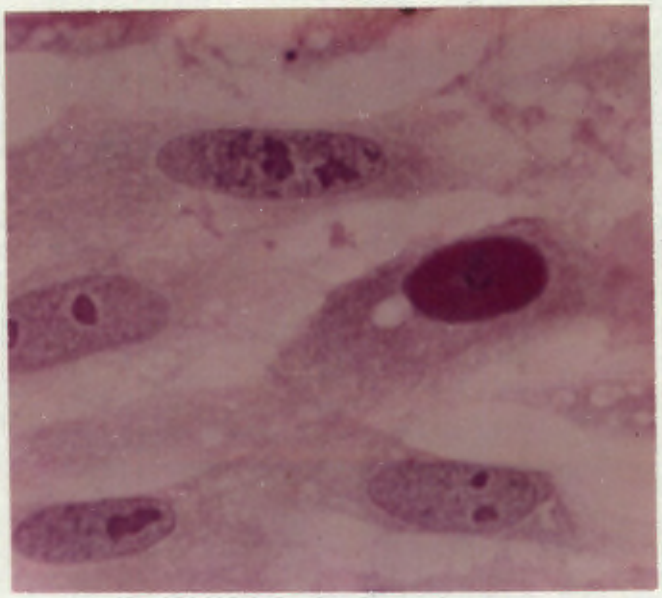


Fig. 4. Chick embryo fibroblast culture infected with Tern virus. There is one fully developed eosinophilic intranuclear inclusion occupying the whole of a nucleus. There is a normal-looking nucleus at the bottom right of the field. The remaining nuclei show early nucleolar changes. Haematoxylin and Eosin. Magnification: x 1,200.

Finally the nucleus and the rest of the cell became fragmented. No specific changes were noted in the cytoplasm of affected cells. No syncytia were produced.

In stationary tube cultures the viruses produced plaques, and spread to the rest of the cell sheet was delayed compared to roller tube cultures where the whole of the cell sheet was soon uniformly affected and where CPE reached a maximum within about 24 hours of commencing.

Some of the stages in intranuclear inclusion formation together with apparently normal nuclei for comparison are illustrated in Fig. 4, page 68.

Acridine Orange Staining and Ribonuclease Digestion of Chick Embryo Tissue Cultures Infected with Tern or Chicken Virus

The intranuclear inclusions corresponding to those seen in preparations stained with haematoxylin and eosin showed the characteristic fluorescence associated with ribonucleic acid (Armstrong and Niven, 1957). In preparations first digested with ribonuclease and then stained with Acridine Orange only a slight green fluorescence remained, indicating the ribonucleic-acid content of the intranuclear inclusions. See Figs. 5a, b and c, pages 70, 71 and 72.

"Auto-Inhibition" by Tern Virus.

When titrating Tern virus-infected allantoic fluid on CETC plates the largest number of plaques was usually obtained with inocula diluted 10^{-3} or 10^{-4}, and the number of plaques tailed off with more highly diluted inocula. However there was also a progressive decrease in the number of plaques formed by the more concentrated inocula, and few plaques were formed by undiluted allantoic fluid. See Fig. 6, page 74.

This phenomenon of "auto-inhibition" was also noted in KB tissue cultures. Although the strain isolated in KB cells from Bird 1

gave/.....

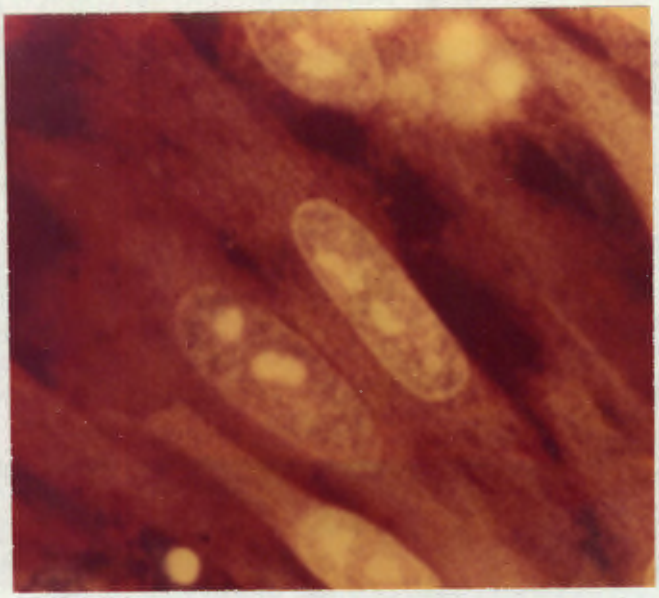


Fig 5A. Normal chick embryo fibroblasts stained with Acridine Orange (AO). Magnification: x 1,800.

Fig 5B. Chick embryo fibroblasts infected with Tern virus. The intranuclear inclusion shows the characteristic fluorescence of ribonucleic acid (RNA). AO. Magnification: x 2,700.

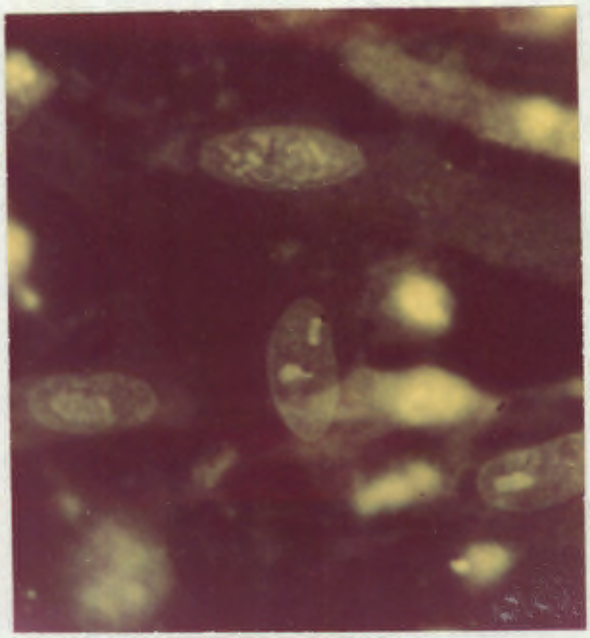


Fig 5C. Chick embryo fibroblasts infected with Tern virus. Digested with ribonuclease before staining with AO. The intranuclear inclusions, top centre and left centre, show a pale green fluorescence and not the characteristic fluorescence of RNA seen in Figure (B). Magnification: x 1,200.

gave 1280 HAU per ml. at the third pass, further subculture was not successful. Undiluted fluids had been used as inocula in the previous passes. Subsequently Tern virus was adapted to KB cells and easily propagated by serial subculture of infected cell fluids, which contained about $10^{6.5}$ $KBTCID_{50}$ per ml., provided that dilute inocula of about 1000 $KBTCID_{50}$ were employed. The use of undiluted inocula for serial subcultures resulted in "auto-inhibition" and loss of the strain.

Fig 6/.....

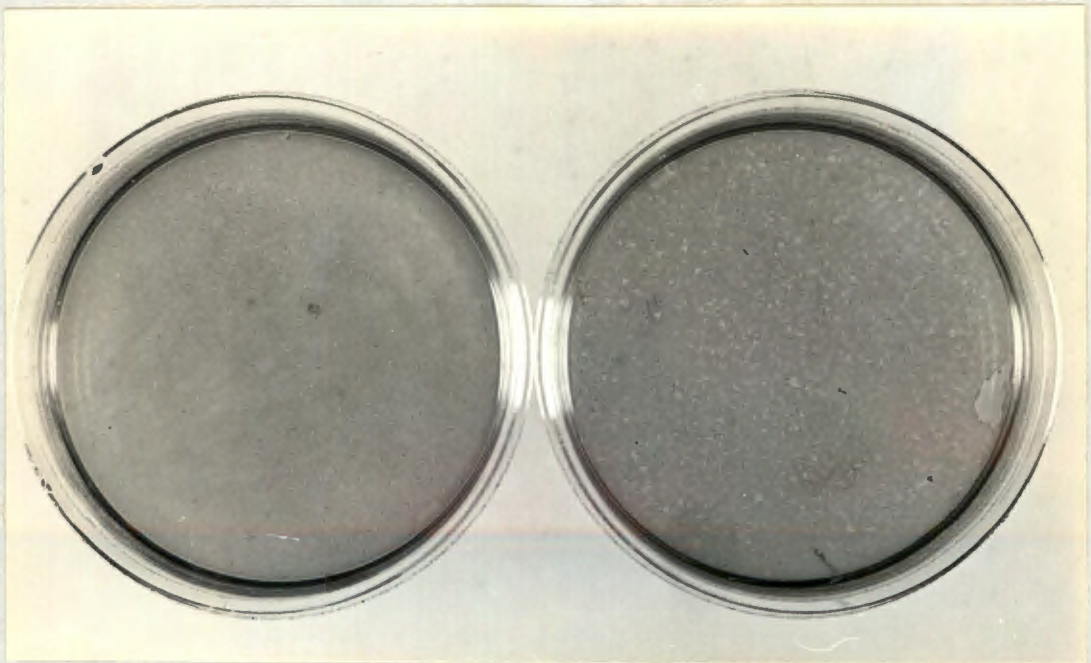


Fig. 6. Chick embryo fibroblast Petri dish cultures. The culture on the left was inoculated with a 10^{-1} dilution and the one on the right with a 10^{-4} dilution of Tern virus (infected allantoic fluid). There were less plaques with the more concentrated inoculum. The petri dishes were 70 mm. in diameter.

C: DISCUSSION

THE MYXOVIRUSES

Myxovirus is the name originally suggested by Andrewes et alia (1955) for a group of viruses having similar properties, particularly an affinity for mucoproteins such as are found on the surface of red cells. The ability of myxoviruses to agglutinate red blood cells was first discovered by Hirst (1941) and McClelland and Hare (1941). These authors showed that the influenza virus particles adsorbed to red cells and caused agglutination and that this agglutination could be specifically inhibited by homologous immune serum. Hirst (1942b) established that the haemagglutination titre could be used as an index of the concentration of a given virus suspension and that the level of antibodies as determined by the haemagglutination-inhibition test could be used as an index of the level of neutralising antibodies.

Hirst (1942a) also pointed out the enzymatic nature of the adsorption of influenza virus to and its elution from red cells. Francis and Salk (1942) described the method of purifying and concentrating influenza virus by the adsorption-elution procedure. The enzyme has since been identified as sialidase (Seto et alia 1959, 1961).

Hirst (1941) initially drew attention to the occurrence in human and animal sera of non-specific inhibitors of viral haemagglutination and showed that these inhibitors could be destroyed by prolonged exposure to active virus which remained unaltered in the process (Hirst 1942b). Francis (1947) confirmed the latter observation and noted that inactivation of the enzyme of influenza virus by heat treatment destroyed the ability of the virus to elute from red cells and rendered the viral haemagglutination markedly sensitive to inhibition by non-specific inhibitors.

Later, Burnet et alia (1946), Burnet and Stone (1947), and Stone (1948) prepared an

enzyme/.....

enzyme which was named receptor destroying enzyme (RDE) from the filtrate of Vibrio cholerae and showed that its action was similar to that of viral enzyme. RDE could consequently be used to eliminate non-specific inhibitors in serum. Despite this, non-specific inhibitors still cause difficulty in HI tests so that some workers prefer the complement fixation test which gives comparable results and is unaffected by non-specific inhibitors (Fulton and Dumbell, 1949; Henle et alia, 1958).

These basic observations have now been amply confirmed and apply to all myxoviruses with a few known exceptions which will be discussed below.

THE SUB-DIVISION OF MYXOVIRUSES INTO TWO GROUPS

Currently recognized myxoviruses are listed in Table 16 a and b together with references to appropriate works on their antigenic composition and ultrastructure. The binomial system of nomenclature for the Myxoviruses (see Andrewes, 1964) has not been fully developed for all the myxovirus strains, particularly for those of sub-group II. Andrewes and Worthington (1959) pointed out that there were physical and biological differences between influenza viruses (types A, B and C) and other myxoviruses and suggested the subdivision of the myxoviruses into two groups on this basis. The morphology of the myxoviruses has since been elucidated in fine detail following on the work of Horne et alia (1960) and Hoyle et alia (1961), and it is apparent that the two groups are distinguishable also on structural grounds. The biological and morphological characters of the two sub-groups have been discussed by Waterson (1962). Waterson and Hobson (1962) suggested the broadening of the second sub-group of myxoviruses to include the Measles-Rinderpest-Distemper (MRD) trio and Respiratory Syncytial Virus. These four viruses resemble the other members of the group in certain characteristics

particularly/.....

TABLE 16a. A CLASSIFICATION OF MYXOVIRUSES.

SUB-GROUP 1 - THE INFLUENZA VIRUSES.			
Host	Virus	Serology[3]	Structure[3]
HUMAN	M. influenzae A hominis	77,55,64,	128,62,67,40
	M. influenzae B	133,78,81	128
	M. influenzae C	35	128
AVIAN	M. influenzae A avian strain		
CHICKEN	Strain 1. Classical Fowl Plague	90	62,40,131
	2. Virus N	41	131
	3.a) Scotland/1959	16,90	14
COMMON TERN	b) Tern/SA/1961	16,90	89,14
DUCK	4. Anatis/Britain/1956	90	128
	5. Anatis/Britain/1962	90	128
	6. Anatis/Czech/1956	90	128
SWINE	M. influenzae A suis		
	Strain 1. Shope	108,53,97	128
	2. Cambridge	97	
	(M.influenzae A hominis PR8)[2]	97	
	(M.influenzae A_2 hominis)	97	
HORSE	M.influenzae A equi Praha/1956	116	128
	(M.influenzae A_2 hominis)	116	
COW	(M.influenzae A suis. Shope Strain)	97	
SHEEP	An as yet unclassified strain	97	
	(M.influenzae A_2 hominis)	97	

[1]Adapted from Andrewes (1964).

[2]Bracketed strains are those which appear primarily to infect other species

[3]The numbers indicate references listed in the bibliography.

TABLE 16b. A CLASSIFICATION OF MYXOVIRUSES.

SUB-GROUP II - THE NDV - PARA-INFLUENZA SUB-GROUP			
Host	Virus	Serology[3]	Structure[3]
HUMAN	M.para-influenzae 1	29,35	61,62
	" 2	35	
	" 3	29,35	129
	" 4	72	
	" 5(SA,DA viruses)	103(SA virus) 68(SA,DA,SV5) 28(SA,DA)	
	M.parotitidis	35	61,62
	Measles	130	130,126
	Respiratory syncytial virus	30,127	127
CHICKEN	Newcastle Disease Virus	35	98,61,62
	Yucaipa virus	13	42
OTHER AVIAN SPECIES	NDV	19,20 104,134	
SWINE	African Swine fever		125
	(M.para-influenzae 1-Sendai)[2]	10	
CATTLE	Rinderpest	130	92
	(M.para-influenzae 3)	1	
DOG	Distemper	130	37
MOUSE	M.para-influenzae 1(Sendai)	34	
	Peromyscus virus	88	
MONKEY	MINIA (Measles)	100	
	Simian para-influenza viruses		
	1 SV5 (M.para-influenzae 5)	69,28,68	32,33
	2 SV41	6	
CHIMPANZEE	Respiratory Syncytial virus	87	

[1]Adapted from Andrewes (1964).

[2]Bracketed strains are those which appear primarily to infect other species.

[3]The numbers indicate references listed in the bibliography.

particularly in morphology, though differing in other respects (Table 17, page 79). None of the four viruses has neuraminidase activity, and only Measles virus agglutinates red cells. Their classification as myxoviruses implies attaching a greater importance to morphology than to haemagglutination as a taxonomic criterion.

Almeida et alia (1961) tentatively suggested the inclusion of Rabies virus in the NDV - para-influenza sub-group of myxoviruses on the basis of morphology.

THE INFLUENZA SUB-GROUP OF MYXOVIRUSES

Antigenic composition of the Influenza Viruses

The existence in the influenza virus particle of two kinds of complement fixing antigens is now well established as a result of serological, biochemical and morphological observations. The nucleoprotein (or S or soluble or internal) antigen is type specific i.e. similar for all strains of type A influenza as opposed to a different antigen common to all strains of type B influenza (Lennette and Horsfall, 1941; Henle and Wiener, 1944; Wiener et alia, 1946; Hoyle, 1945; Lief et alia, 1958a; Lief et alia, 1958b; Cook et alia, 1959) and yet another common to influenza C strains (Sigel et alia, 1949; Lief et alia, 1958b; Cook et alia, 1959).

The haemagglutinin constitutes the strain specific (or V or external) antigen and differs from the nucleoprotein antigen but may include some components which cross-react with other strains within the same type, (Henle and Wiener, 1944; Wiener et alia, 1946; Hoyle, 1945; Friedewald, 1943 and 1944; Fulton and Dumbell, 1949; Fabiyi, 1958).

The nucleoprotein antigen is enclosed within the intact virus particle, but is also found free in infected tissues and tissue fluids (Hoyle, 1945, 1950 and 1952; Frisch-Niggemeyer and Hoyle, 1956; Lief and Henle, 1945a and b; Schafer, 1957). The nucleoprotein and haemagglutinin

may/.....

TABLE 17. COMPARISON OF THE MEASLES-RINDERPEST-DISTEMPER AND RESPIRATORY SYNCYTIAL VIRUSES WITH THE INFLUENZA AND THE NDV - PARA-INFLUENZA SUBGROUPS OF MYXOVIRUSES.

(Waterson and Hobson, 1962).

	NDV particle structure	Syncytia and eosinophilic cytoplasmic inclusions in tissue culture	Haemagglutination	Neuraminidase activity	Intranuclear inclusions in tissue culture
Influenza group	-	-	+	+	- *
NDV - para-influenza group	+	+	+	+	-
Respiratory syncytial virus	+	+	-	-	-
Measles	+	+	+	-	+
Distemper	+	+	-	-	+
Rinderpest	+	+	-	-	+

+ = virus possesses the indicated property.

- = virus lacks the indicated property.

* = Tern and Chicken viruses both produce intranuclear inclusions.

may be prepared from intact virus particles by fractionating the virus with ether and separating the haemagglutinin from the nucleoprotein by adsorption to and subsequent elution from red cells (Lief et alia, 1958a; Fabiyi et alia, 1958; Lief and Henle, 1956a; Hoyle, 1952; Ada, 1957; Horne et alia, 1960; Hoyle et alia, 1961; Becker, 1963; Schafer, 1957).

Both the haemagglutinating and enzymatic activities are associated with the haemagglutinin (Hoyle, 1952; Lief and Henle, 1956a; Schafer, 1959; Davenport et alia, 1960) which has the same antigenic specificity as the intact viral particle in complement fixation tests (Fabiyi et alia, 1958; Davenport et alia, 1960), haemagglutination-inhibition tests (Davenport et alia, 1960) and precipitation experiments (Schafer, 1957). The haemagglutinin represents the surface layer of the intact particle, as will be discussed under the section dealing with morphology, and appears to contain the essential antigen needed to confer protection by vaccination as has been shown for Fowl Plague virus (Schafer, 1957). The latter view is supported by the demonstration of neutralising antibodies to Tern and Chicken viruses in the serum of guinea-pigs immunised with purified homologous haemagglutinin (Table 11 a and b, page 62).

Pure nucleoprotein and haemagglutinin are useful in the type and strain specific serodiagnosis of influenzal disease (Henle et alia, 1958), and in the preparation of type and strain specific sera (Lief et alia, 1958a; Fabiyi et alia, 1958). Specific sera are useful in the rapid identification of newly isolated strains as to type (Lief et alia, 1958b; Sigel et alia, 1949), and in the determination of the antigenic patterns of strains (Fabiyi et alia, 1958; Lief et alia, 1958b; Pereira, 1963).

Species Specificity of Influenza A Strains

It is well known that human strains of influenza A viruses differ in antigenic(V antigen) composition, and while there are also different

influenza/.....

influenza B strains, the influenza C strains isolated have proved antigenically homogeneous.

All the influenza strains that have so far been isolated from vertebrates other than humans were A strains. The Shope strain of Swine influenza has been isolated from swine and cattle, the PR8 strain from swine as well as humans, the Influenza A_2 from swine and sheep as well as humans (Romvary et alia, 1962). Influenza A_2 has also been isolated from horses (Tůmova and Sovinova, 1959). Horse influenza strains have so far not been isolated from other species.

Amongst the avian strains only, the closely related Tern and Chicken viruses were isolated from different species.

NDV - PARA-INFLUENZA SUBGROUP OF MYXOVIRUSES

Antigenic Composition

The viruses of this subgroup have structural components similar to the influenza subgroup but are antigenically separate from the influenza viruses. They do not possess a single common antigen; each virus is antigenically distinct although (with the known exception of Respiratory Syncytial virus - Pereira, 1964) sharing antigens with one or two or more other viruses in the subgroup (Johnson et alia, 1960; Chanock et alia, 1961; Hsiung et alia, 1962; Andrewes, 1964). Para-influenza virus 1 and Sendai virus share antigens to such an extent that they appear to represent subvarieties of the same strain (Andrewes et alia, 1959).

Para-influenza 5 includes the SV5 virus isolated from monkey kidney tissue cultures (Hull et alia, 1956), the SA virus of human origin isolated from throat washings during common cold studies (Schultz and Habel, 1959), and the DA virus isolated at autopsy from the blood of a case of infective hepatitis (Hsiung et alia, 1962). The latter authors

established that the three viruses were antigenically indistinguishable.

The inclusion of Measles virus in the NDV - para-influenza group was suggested by Waterson et alia (1961), of Rinderpest virus by Plowright et alia (1962) and of Distemper virus by Cruickshank et alia (1962). The question of the inclusion of these three viruses and Respiratory Syncytial virus in this subgroup has been discussed above.

MINIA virus, isolated from uninoculated monkey kidney tissue cultures, is identical with measles virus (Ruckle, 1957).

Yucaipa virus was isolated from the laryngo-tracheal exudate of chickens in California, and characterised as a myxovirus with similar properties to NDV although it was antigenically distinct (Dinter et alia, 1964).

Andrewes (1964) suggests that the following viruses probably belong in the NDV - Para-influenza subgroup of myxoviruses; African swine fever virus, Peromyscus virus which was isolated from white-footed mice by Morris et alia (1963); and also another simian virus, SV41.

Species Specificity of the Viruses of the NDV - Para-influenza Subgroup of Myxoviruses

The host range of some of the viruses in this subgroup overlap. Para-influenza I infects humans (Chanock et alia, 1958), and Para-influenza I strain Sendai causes a latent infection in laboratory mice (Chu and Chu, 1956, quoted by Andrewes,1964). As discussed above, both Para-influenza 5 and Measles virus infect man and monkey. Respiratory Syncytial virus infects man (Chanock et alia, 1957) and the chimpanzee (Morris et alia, 1956). Para-influenza 3 causes disease in man (Chanock et alia 1958) and also infects cattle (Abinanti et alia, 1960).

Newcastle disease is usually regarded as a disease of chickens, but NDV may infect other avian species. NDV was responsible for an

outbreak/.....

outbreak in Grey Parrots (Psittacus erithacus) in Kenya in 1955 (Scott and Winmill, 1960). The virus has also been isolated from sea-birds in Great Britain: from a gannet (Wilson, 1950) and from shags and cormorants (Blaxland, 1951). Laboratory infection of humans with NDV may manifest itself as a conjunctivitis, an influenza-like illness, or an illness resembling abortive poliomyelitis (Howitt, et alia 1956).

It appears that guinea-pigs may suffer natural infections with the myxoviruses which usually infect man (Cook et alia, 1959).

CLASSIFICATION OF TERN VIRUS

Tern virus can unequivocally be classified as an avian strain of Myxovirus influenzae A. Its haemagglutinating properties were similar to those of the influenza viruses, and serological studies showed that it had the same type-specific nucleoprotein antigens as the Influenza A_2 strain with which it was compared.

The haemagglutinin of Tern virus was found to be antigenically distinct from that of other influenza A strains investigated with the single exception of Chicken virus which had an antigenically closely related haemagglutinin. This relationship was confirmed by Pereira (1963) who also found no antigenic sharing of Tern virus haemagglutinin with Fowl Plague virus nor with two British strains and one Czechoslovakian strain of Duck influenza virus.

When grown in tissue cultures Tern virus produced intranuclear inclusions which contained RNA as shown by ribonuclease digestion and acridine orange staining. This suggested that as has been shown in the case of Fowl Plague virus by Breitenfeld and Schafer (1957), Tern virus nucleic acid was ribonucleic acid and that the site of formation of viral nucleic acid was in the nucleus. These points would require confirmation by fluorescent antibody studies.

Furthermore, Tern virus had the same structure as the influenza viruses, as will be discussed in the section on the morphology

of Tern virus.

TERN VIRUS AS THE CAUSAL AGENT OF THE TERN EPIZOOTIC

The reasons for regarding Tern virus as the causal agent of the epizootic are:

Firstly, Tern virus was isolated from all three diseased Common Terns (Birds 1, 2 and 3 in Table 4, page 45) collected during the epizootic and the same virus was isolated from a further two diseased Common Terns examined at the Onderstepoort Veterinary Laboratories (Alexander, 1961). In Birds 1 and 2 in Table 4 the virus content of the organs was relatively high considering the long interval of storage at -20°C before titration. Bird 3 was probably sacrificed at an early stage of infection, before the virus had had sufficient time to establish itself. In addition, serum was obtained from each of two Common Terns collected shortly after the epizootic and both sera contained HI and neutralising antibodies to Tern virus, indicating that they had been infected with Tern virus.

It seems reasonable to conclude that at the time of the epizootic infection with Tern virus was widespread among the population of Common Terns. It is interesting that two Swift Terns were clinically unaffected by experimental inoculation with live Tern virus which did, however, stimulate antibody formation.

Secondly, as will be described in a later chapter, domestic chickens could regularly be infected with Tern virus and developed an acute illness similar to that noted in Terns (Uys, 1963). Unfortunately, it was not possible to capture live Common Terns and none were available for experimental inoculation. All three diseased Common Terns showed a meningo-encephalitis histologically similar to that seen in the experimentally infected chickens.

INFLUENZA/.....

INFLUENZA VIRUS INTRA-STRAIN VARIATION

Tern virus and Chicken virus were originally isolated from different hosts. The process of adaptation of an influenza virus to a new host might result in the selection of a mutant population differing from the original strain in antigenic and/or other properties (Hilleman, 1954).

Hirst (1947) isolated the same strain of Influenza A virus from the throat washings of two patients by egg inoculation. These isolates were antigenically different from, although related to, the viruses isolated from the same washings by ferret inoculation and subsequent mouse passage. Not only did the ferret-mouse-adapted strains differ from the egg-adapted strains, but they also differed from each other indicating that antigenic changes had occurred during the ferret-mouse passage.

Fabiyi et alia (1958) noted antigenic change in an egg-adapted PR8 strain of influenza after it had been adapted to mice.

Sugg (1949) showed that antigenic change of an egg-adapted strain of influenza A occurred after it was adapted to mice. By contrast, he also showed that influenza A may undergo changes in virulence without any apparent change in antigenic pattern.

Wang (1948) and Anderson and Burnet (1947) reported observations on egg lines of influenza A which were adapted to mice without any resulting change in antigenic structure.

The use of the egg (an avian system) in preference to other culture systems for comparative experiments with avian viruses seemed advisable from the point of view of avoiding the selection of mutants (Jensen et alia, 1957).

It was decided not to purify the viruses used in this work, e.g. by means of a series of passes at limit dilutions, in order to avoid the

possible/.....

possible selection of mutants. Instead, the viruses (pooled infected embryonic fluids) were passed using inocula containing approximately 10^4 or 10^5 EID_{50}.

RELATIONSHIP OF TERN VIRUS TO CHICKEN VIRUS

Antigenic analysis showed that Tern virus and Chicken virus were very similar in antigenic make-up. The cytopathic effect of both viruses was the same in chick embryo tissue culture, with the production of intranuclear inclusions containing ribonucleic acid. Electron microscopy revealed a similar structure for both viruses. In the experiments to determine the spectrum of red cells agglutinated, the viruses apparently differed only in their rate of elution from agglutinated cells. The temperature stability of the two viral haemagglutinins was similar, although the infectivity of Tern virus was more heat-stable than that of Chicken virus.

Although the two viruses produced a similar disease in experimentally infected chickens there were distinguishing features which are described in a later section. This point was confirmed in an independent study by Wilson (1962) who also investigated the host range of Tern virus and of his Chicken virus and found that both produced fatal infection in chickens and quails, that Tern virus but not the Chicken virus affected turkeys, and that neither affected duck,pigeons, rooks, jackdaws, Hooded and Carrion Crows.

Particularly if cogniscance is taken of the fact that Tern virus and Chicken virus were originally isolated from different hosts, it may be concluded that they are variants of the same strain. This conclusion has an important bearing on the discussion of the epizootiology of Tern virus infection in a later chapter.

THE/.....

THE SERUM ANTIBODY CONTENT OF GUINEA-PIGS IMMUNISED WITH PURIFIED HAEMAGGLUTININ.

Guinea-pigs immunised with the purified haemagglutinin of Tern or Chicken virus developed HI and neutralising antibodies to whole homologous virus as well as strain specific complement fixing antibodies in their sera. The results of experiments illustrating these points are recorded in Tables 11 a and b on page 62. The titres of neutralising antibodies in the guinea-pig sera compared favourably with the titres attained in the sera of chickens convalescing from Tern or Chicken virus infection (see Table 21 a and b, page 102).

THE PHENOMENON OF AUTO-INHIBITION

The auto-inhibition noted with Tern virus has been observed with other myxoviruses. Chany (1961) studied the phenomenon with Para-influenza 3 virus grown in KB cells, and Cantell (1961) reported the study of the same phenomenon with Mumps virus. The evidence in both instances suggested that the inhibition was mediated by an interferon-like substance produced by the host cells.

SECTION III. EXPERIMENTAL INFECTION OF CHICKENS WITH TERN AND CHICKEN VIRUSES.

MATERIALS AND METHODS

The only feasible way of catching live healthy terns in sufficient numbers would have been at their breeding grounds in Europe and so it was decided to study the pathogenesis of Tern virus infection in chickens instead, and to compare the resulting disease with that caused in Chickens by the Chicken virus.

Crossed Leghorn-Australorpe chickens from a commercial source were used for all the experiments. The materials and methods used were as described in the previous section unless otherwise stated. Infectivity titrations were carried out in the allantoic cavity of eggs using 6 eggs per dilution.

EXPERIMENTAL TERN VIRUS INFECTION OF CHICKENS

SUSCEPTIBILITY OF CHICKENS TO DIFFERENT ROUTES OF INOCULATION WITH TERN VIRUS

Intramuscular Route

Freshly harvested Allantoic pass 8 of Tern virus was titrated in 3-day-old chickens which were kept at an environmental temperature of 27-31°C. A series of tenfold dilutions from 10^{-1} to 10^{-9} was prepared and each dilution inoculated in 0.1 ml. amounts into the thigh muscles of 10 chickens. Four "running-in" uninoculated controls were placed with each group of 10 chickens in 24 x 18 x 9 inch cages.

Ten control chickens were inoculated with diluent only and

their/.....

their cage kept at least three feet away from the other cages.

A parallel titration of the same dilutions was carried out in eggs.

Other Routes

One group of 3-day-old chickens (environmental temperature 27-31°C) was presented overnight with infected drinking water containing 10^6 EID_{50} of Tern virus per ml.

Groups of 3-week-old chickens were inoculated with approximately 10^5 EID_{50} of Tern virus intranasally or into the conjunctival sac. Environmental temperature was maintained at between 20°C and 22°C.

Virological and histological investigations were carried out on 4 chickens infected by the intramuscular route, 3 infected via their drinking water, 2 by intranasal and 2 by conjunctival sac inoculation.

THE PATHOGENESIS OF TERN VIRUS INFECTION OF CHICKENS

Two experiments were carried out on six-week-old chickens with their environmental temperature kept at 20-22°C. The aims were to establish the signs and course of the disease, to study the development of both gross and microscopic lesions in the tissues, and to determine the distribution of virus in the tissues and excreta.

Experiment I

Twenty-four chickens were inoculated with approximately 10^5 EID_{50} of freshly harvested Tern virus which was in its eighth Allantoic passage. Half the inoculum of 0.1 ml. was introduced into the right conjunctival sac and half into the right nostril.

Six control chickens were inoculated with normal allantoic

fluid/.....

fluid and kept in the same room at a distance of at least three feet from the other chickens.

Three or more of the 24 chickens were autopsied daily for six days and the two convalescent survivors on day 10. One control chicken was autopsied with each of the above batches except on day 4.

Emulsions were prepared from the following tissues and stored at -20°C : breast muscle, liver, spleen, lungs, kidneys, heart and brain. In addition to the above tissues which were collected in the stated order, portions of comb, soft palate, skin and small intestine and also both eyeballs with lids were taken for histological examination.

Experiment 2

Twenty-eight six-week-old chickens were inoculated with Tern virus in the same way as in the first experiment. On the fifth day after inoculation six moribund infected chickens were sacrificed, and on the twelfth day another. Two of three surviving birds were sacrificed on the 19th day, and the third survivor on the 25th day. One control bird was sacrificed on the 4th and another on the 25th day.

The blood, heart, spleen, lungs, and brain of each chicken was harvested in that order for viral studies. A portion of each organ, and the proventriculus and ventriculus were taken for histological examination.

The tissue emulsions were ampouled and stored in a dry ice cabinet and their virus content titrated within two weeks of preparation during which time the virus is known to maintain its titre.

THE PATHOGENESIS OF CHICKEN VIRUS INFECTION OF CHICKENS

Thirty six-week-old chickens were inoculated with freshly harvested Allantoic pass 9 of Chicken virus. The inoculum of 0.1 ml. contained 10^6 EID_{50} and the routes of inoculation were the same as those

used/.....

used in the two experiments with Tern virus.

The chickens were distributed in 24 x 18 x 9 inch cages, six to a cage, with one uninoculated "running in" control per cage.

Six chickens were inoculated with Tern virus, also at the 9th allantoic passage level, and placed in a cage on their own.

Three control uninoculated chickens were kept in the same room, and one was sacrificed on the third day of the experiment.

All the cages were kept on the same level and at least three feet from one another.

All surviving chickens were bled at the termination of the experiment on day 25.

At autopsy blood and heart, lungs and brain were collected for virus investigations. The following specimens were collected for histological examination: heart, lungs, brain, liver, spleen, kidney, skin, comb, small gut and proventriculus.

Tissue emulsions were stored in a dry ice cabinet and titrated for virus content within two weeks of preparation, inoculating four eggs per dilution.

RESULTS/.....

RESULTS

INFECTION OF CHICKENS WITH TERN VIRUS

SUSCEPTIBILITY OF CHICKENS TO DIFFERENT ROUTES OF INOCULATION WITH TERN VIRUS

Intramuscular Route

The titrated Tern virus suspension contained $10^{7.6}$ LD_{50}/ml. for 3-day-old chickens and $10^{8.3}$ EID_{50}/ml.

Most of the affected chickens died within three days and all within seven days of inoculation.

The uninoculated "running-in" controls remained well during the 15-day period of the experiment.

Other Routes

Seven out of ten birds died within 5 days of being presented with infected drinking water.

Five out of eight chickens inoculated intranasally died, all within five days. Use of the conjunctival-sac route resulted in seven out of eight birds dying, six within six days and the seventh on the 13th day.

The course of the illness appeared to be similar, whichever route of inoculation was employed.

THE PATHOGENESIS OF TERN VIRUS INFECTION IN CHICKENS.

Course of the Disease.

The incubation period was 3-5 days.

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The earliest signs of illness were listlessness, ruffling of the feathers and retraction of the head, but the affected chicken looked normal when roused to strut around. Some birds had ventral soiling due to loose stools. Within several hours of signs of illness the affected chickens developed a "dozing-off" appearance with drooping head and wings, and sometimes preferred to squat. At this time the palpebral conjunctivae were congested and on the conjunctival surfaces of the lower eyelids towards the inner canthus little pin-head sized, white nodules of lymphoid tissue stood out prominently. Some birds continued to eat and drink though ill. Most affected birds died within about 48 hours. They developed one or more of the following signs:- paresis, tremors, muscle spasms and convulsions. Terminally they became stuporose and often lay in bizarre spastic attitudes, sometimes exhibiting slow spastic or convulsive movements. Some birds remained in this state for about 24 hours before death. The combs were congested and changed from red to blue. In chickens where the illness ran a protracted course, necrotic lesions developed on the combs and sometimes on the eyelids, and healed slowly in birds that recovered.

There were no signs of respiratory difficulty in any of the chickens, nor was there any discharge of secretions from the nasopharynx.

The occasional chicken appeared to be refractory to infection, and some showed only mild symptoms of late onset followed by recovery.

Of the 28 chickens inoculated in experiment 2 most died within one week, but three did not die till the 11th or 12th day, and another three survived. One of the latter remained persistently well throughout, and the other two were recovering after showing signs of the disease.

The controls remained healthy throughout.

Autopsy Findings

At autopsy there were no prominent lesions. The earliest

change/.....

change noted was a reactive spleen and some fatty change of the liver. At a later stage of the disease the myocardium was pale and flabby.

Virological Investigations

Experiment 1.

The results of the first experiment are given in Table 18. The virus content of the emulsions of a given tissue of all the chickens were titrated at the same time or in two batches within four days of each other. All the titrations were completed within four months, during which time the emulsions were stored at -20°C.

In chickens sacrificed one day after inoculation the only organ in which virus could be detected was the kidney. A transient viraemia associated with widespread distribution of virus in the tissues occurred early in the course of the disease. Thereafter virus was detected in the brain, heart and lung almost invariably, and not infrequently in the breast muscle. A low titre of antibodies was demonstrated in the serum of one of the three birds autopsied on the 5th day, but virus was still present in the tissues of this chicken. No virus was detected in the tissues of the two convalescent chickens sacrificed on the 10th day and both had antibodies in their serum.

Chicken No. 9 apparently escaped infection: no virus was detected in any of the tissues examined, no antibodies were detected in the serum, and no lesions were found histologically.

Experiment 2.

The results of Experiment 2 are presented in Table 19.

The shorter and colder storage conditions of the tissues in Experiment 2 resulted in the demonstration of higher concentrations of

virus/.....

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TABLE 18. PATHOGENESIS OF TERN VIRUS INFECTION OF CHICKENS.

EXPERIMENT 1. RESULTS OF VIROLOGICAL INVESTIGATIONS

	INOCULATED CHICKENS																							CONTROL CHICKENS					
Day of Experiment	1			2			3			4				5							6		10		1	2	3	5	6	10
Chicken Number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6
Virus content of:*																														
Blood	-	-	-	2.2	2.2	-	2.0	-	-	-	-	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Brain	-	-	-	3.0	1.8	1.8	2.8	2.2	-	1.8	2.8	2.8	6.4	5.3	4.0	5.0	6.0	5.8	4.8	5.0	3.3	4.8	-	-	-	-	-	-	-	-
Heart	-	-	-	1.8	1.8	-	3.5	1.8	-	-	<3.5	<3.5	5.0	<3.5	<3.5	4.0	4.0	<3.5	<3.5	4.0	<3.5	<3.5	-	-	-	-	-	-	-	-
Lung	-	-	-	2.8	2.8	2.4	4.2	1.8	-	2.5	4.8	5.3	6.0	3.8	3.8	2.3	3.7	2.3	2.2	1.6	-	1.8	-	-	-	-	-	-	-	-
Liver	-	-	-	1.8	-	-	1.8	-	-	-	-	2.3	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	2.6	2.0	-	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kidney	2.0	-	1.8	-	-	-	3.2	-	-	-	-	-	2.8	-	-	1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Muscle	-	-	-	2.2	-	-	1.8	-	-	-	-	4.0	1.8	1.8	-	1.8	3.0	-	-	-	-	-	-	-	-	-	-	-	-	-
Serum HI antibody Titre	-	-	-	-	-	-	-	-	-	-	-	NT	NT	-	NT	NT	-	NT	NT	20	NT	NT	80	40	-	-	-	-	-	-

* Virus content expressed as the $\log_{10}$ EID_{50} per g. of tissue.

- = no virus or no antibodies detected.

NT = not tested.

TABLE 19. PATHOGENESIS OF TERN VIRUS INFECTION OF CHICKENS.

EXPERIMENT 2. RESULTS OF VIROLOGICAL INVESTIGATIONS OF INFECTED CHICKENS.

Day of experiment:	5						12	19		25
Inoculated Chicken number:	1	2	3	4	5	6	7	8	9	10
Virus content of:-*										
Blood	-	-	-	-	-	-	-	-	-	-
Brain	6.0	7.5	7.7	6.3	7.5	7.7	-	-	-	-
Heart	5.2	5.7	5.7	4.0	4.8	5.5	-	-	-	-
Lung	2.0	3.5	4.3	4.2	4.7	5.8	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-	-
Palatal swab	NT	+	+	-	+	+	-	-	-	-
Cloacal swab	NT	-	+	+	-	-	-	-	-	-
Serum HI antibody titre:	-	-	-	-	5	-	NT	40	-	40

* Virus content expressed as the logarithm $_{10}$ EID_{50} per gram of tissue.

- = no virus detected or no antibodies detected.

+ = virus present.

NT = not tested.

virus in them than in the corresponding tissues in Experiment 1. Tern virus was detected in all six moribund chickens sacrificed on the fifth day. The titre was high in the brain, less so in the heart and lungs, and virus was isolated from the palatal swabs and/or cloacal swabs of all the five chickens so tested. No virus was detected in the blood nor in the spleen. Only one of the six chickens had demonstrable HI antibodies in the serum. No virus was isolated from the moribund chicken sacrificed on the 12th day, nor from the two convalescent chickens (Nos 8 and 10) sacrificed on the 19th and 25th day after infection and whose sera contained antibodies. Chicken 9 was apparently refractory to infection: it remained well throughout the experiment, no virus was detected in its tissues, no antibodies were detected in the serum and no histological lesions were found in its tissues.

The control chickens of both experiments yielded no evidence of infection with Tern virus.

THE PATHOGENESIS OF CHICKEN VIRUS INFECTION IN CHICKENS

Course of the Disease

The first signs of illness in chickens inoculated with Chicken virus were listlessness and apathy, and death usually followed rapidly. Twenty-one of the thirty inoculated chickens died on the third or fourth day after inoculation, and of these the majority were found dead in the morning, without having shown noticeable signs of illness the previous evening. However, four chickens survived for 2, 3 or 4 days after the first sign of illness and two recovered. Three apparently escaped infection completely; they remained well throughout the experiment and did not develop specific antibodies. All chickens which showed signs of illness did so within five days of inoculation. One "running-in" control

became/.....

became ill on the 7th day after the start of the experiment, and died within 36 hours. It had gross lesions of the eyelids as well as all the other signs noted in the inoculated chickens. Two of the six cage companions had died on the third and two on the fifth day, while the remaining two recovered. In contrast with Tern virus infection, no signs of irritation of the nervous system, e.g. spasms, tremors or head retraction, were noted in any of the birds.

On external examination the striking feature was congestion of and haemorrhage into the tissues, a distinctive feature which was not seen in chickens infected with Tern virus. This was particularly evident in the skin around the joints, and on the conjunctival surfaces of the lower lids near the inner canthus. The combs were congested and cyanosed and developed ulcerating lesions in birds in which the illness ran a protracted course.

Autopsy Findings

At autopsy there was oedema of the tissues of the head, the spleen was reactive and there was congestion of and haemorrhages into the mucous membrane of the proventriculus and marked congestion of the cloacal mucous membrane.

One dead chicken, one moribund and one sick chicken (chickens 1, 2 and 3 in table 20, page 99) were harvested on the third day after inoculation, and three moribund birds on the fourth day. A convalescent chicken was sacrificed on the 14th day.

Chickens Inoculated with Tern virus

Three moribund chickens, two of which became ill on day 4 and one on day 5, were autopsied on the sixth day. Two chickens which became ill on days 6 and 7 recovered after a protracted illness; one of them

was/.....

TABLE 20. PATHOGENESIS OF CHICKEN VIRUS INFECTION OF CHICKENS

RESULTS OF VIROLOGICAL INVESTIGATIONS.

	Chickens inoculated with Chicken virus							Sick Contact Control	Normal Control	Chickens inoculated with Tern Virus.			
Day of experiment:	3			4			14	9	3	6			14
Chicken number	1	2	3	4	5	6	7			1	2	3	4
Virus content of:-*													
Blood	NT	3.0	3.25	3.25	3.0	4.0	-	> 3.5	-	-	-	-	-
Brain	4.25	5.0	1.75	4.0	3.0	3.25	-	3.5	-	>4.5	>4.5	>4.5	-
Heart	6.0	4.25	1.75	4.5	3.0	3.25	-	3.75	-	3,75	3.25	3.0	-
Lung	7.25	>4.5	4.0	7.5	4.5	>4.5	-	> 4.5	-	3.5	-	>4.5	-
Serum HI antibody titre	NT	-	-	-	-	-	160	NT	-	-	-	-	40

* Virus content expressed as the logarithm $_{10}$ EID_{50} per gram of tissue.

- = no virus detected or no antibodies detected.

NT = not tested.

was sacrificed on day 14. The sixth and last chicken apparently escaped infection altogether.

In these chickens the features of the disease and the autopsy findings were similar to those in experiments 1 and 2.

Control Uninoculated Chickens

These showed no evidence of virus infection apart from the one "running-in" control already mentioned.

Virological Investigations

The results of virological studies are presented in table 20, page 99.

Good concentrations of virus were present in the blood and tissues of the moribund chickens inoculated with Chicken virus, and no HI antibodies were present in the sera. No virus was detected in the convalescent chicken's tissues, but the serum contained HI antibodies to a dilution of 1/160.

The chickens inoculated with Tern virus showed results in keeping with those of the previous experiments.

CROSS-CHALLENGE INOCULATION OF CHICKENS CONVALESCENT FROM TERN OR CHICKEN VIRUS INFECTION

A chicken recovering from infection with Chicken virus, and another from infection with Tern virus were challenged with 10^6 EID_{50} of freshly harvested homologous virus inoculated approximately 4 months after and by the same routes as the initial dose. Both remained well and were unaffected by rechallenge with heterologous virus two weeks later.

CROSS/.....

CROSS HAEMAGGLUTINATION-INHIBITION AND NEUTRALISATION TESTS WITH TERN AND CHICKEN VIRUSES AND THE RESPECTIVE CONVALESCENT CHICKEN SERA.

The sera of two chickens convalescent from Tern virus infection, of two convalescent from Chicken virus infection and of one control chicken were tested against both Tern and Chicken viruses. A single series of doubling dilutions starting at 1/5 was prepared from each serum and aliquots of these distributed to give the required number of sets of dilutions for the cross-tests.

For the HI tests, freshly harvested virus suspensions (Allantoic pass 9) were used as antigens.

The neutralisation tests were carried out in CETC using approximately 100 $TCID_{50}$ of Tern virus (allantoic 9, CETC 11) and 50 $TCID_{50}$ of Chicken virus (allantoic 9, CETC 2).

The results are presented in Tables 21 a and b, page 102, and illustrate the close reciprocal antigenic relationship between the two viruses.

HISTOLOGICAL FINDINGS IN CHICKENS INFECTED WITH TERN OR CHICKEN VIRUS.

Histological studies were undertaken by Professor C.J. Uys, of the Department of Pathology, UCT. He made the following points:

1. "The distribution and character of the lesions produced by Tern virus were similar no matter which route of inoculation was employed." This is in contrast to NDV where the route of inoculation can affect the tissue tropism of a given strain (Jungherr and Markham, 1962; Brandly, 1959; Sinha et alia, 1957).

2. "Tern virus produced a distinctive pattern of pathological changes in infected chickens as compared with the pattern produced by Chicken virus (Uys and Becker, 1964) and with the reported findings in Newcastle Disease and Fowl Plague (Jungherr et alia, 1946)".

DISCUSSION/.....

TABLE 21. CROSS HAEMAGGLUTINATION-INHIBITION AND NEUTRALISATION TESTS WITH TERN AND CHICKEN VIRUSES AND THE RESPECTIVE CONVALESCENT CHICKEN SERA.

(a) CROSS HI TESTS

	SERUM HI ANTIBODY TITRE AGAINST	
	TERN VIRUS	CHICKEN VIRUS
Serum 1	80	40
2	80	20
3	80	160
4	320	320
5	<5	<5

(b) CROSS NEUTRALISATION TESTS

	SERUM NEUTRALISING ANTIBODY TITRE AGAINST	
	TERN VIRUS ($100\ TCID_{50}$)	CHICKEN VIRUS ($50\ TCID_{50}$)
Serum 1	320	320
2	1280	320
3	160	2560
4	1280	5120
5	<5	<5

Sera 1 and 2 were from chickens convalescent from Tern virus infection.

Sera 3 and 4 were from chickens convalescent from Chicken virus infection.

Serum 5 was from a normal control chicken.

DISCUSSION.

Tern virus produced in chickens an acute disease with a high mortality similar to that caused in Common Terns at the time of the epizootic (Uys, 1963). Chicken virus also produced an acute disease with a high mortality in chickens similar to the disease seen in the outbreak in chickens from which this virus was originally isolated (Wilson, 1962). Chickens infected with Tern virus could be readily distinguished from those infected with the Chicken virus because the difference in morbid anatomical appearance was sufficiently striking (see page 97). This difference was confirmed by the independent observations of Wilson (1962) who described the morbid anatomical picture of Chicken virus disease as that of classic Fowl Plague. As stated earlier, the histopathological pattern of the two virus diseases was also different.

A viraemia was no longer present in chickens dying of Tern virus infection, but it was a constant feature in the case of infection with Chicken virus and the fact that virus may still be isolated from the tissues of sick birds despite the presence of antibodies in their sera has also been noted in Newcastle Disease (Sinha et alia 1952). The close antigenic relationship between Tern and Chicken viruses is illustrated by the results of the cross-protection tests in chickens, and of the cross-HI and -neutralization tests with the sera of convalescent chickens (see Table 21, page 102).

An interesting feature was the presence of significant amounts of Tern virus in the lungs of affected chickens in the absence of a viraemia and in the absence of histological lesions recognisable by the methods used. This phenomenon is not unknown, for instance it has been noted in cases of disseminated herpesvirus infection in humans (Becker et alia, 1963) and it is well known that viruses may multiply in tissue culture without producing CPE.

It may be pertinent to recall that the Hartlaub's Gull (Bird

19, Table 4, page 46) died on the third day after inoculation with live Tern virus, presumably due to infection with Tern virus. Although the virus was recovered from its tissues, no histological lesions were detected in them.

The low incidence of disease in contact chickens (in the case of both Tern and Chicken viruses) was in marked contrast to the ease with which contacts become infected in Newcastle Disease (Andrewes and Allison, 1961; Kaschula, 1961).

It was interesting that some chickens seemed to escape infection altogether, but no evidence of a carrier state or of latent infection was found in these studies in chickens.

The complexity of factors that may be involved in the transmission of avian virus infections is illustrated by the studies of Andrewes and Allison (1961) on NDV infection in chickens. In addition it has been shown that the environmental temperature can determine the tissue tropism in chickens of a given strain of NDV. (Brandly, 1959; Sinha et alia, 1957).

It is clear that the transfer of epidemiological information from experimentally infected chickens in the laboratory to naturally infected terns in the field could not be assumed to be valid, and so the observations were limited mainly to possible routes of infection of and dissemination from chickens.

Infection could be established via the conjunctival sac, the nares, infected drinking water and by parenteral inoculation. The source of infecting virus could be the nasopharyngeal secretions, cloacal excretions, external lesions e.g. of the eyelids, exposed internal tissues or blood. The role of ectoparasites is an unknown factor.

EPIZOOTIOLOGY OF TERN VIRUS INFECTION

A thorough search of the literature has failed to reveal a report of any other epizootic in terns. However, Pollard (1947) in carrying out a survey on the sera of sea-shore birds in the USA found psittacosis CF antibodies in 36% of the sera including the sera of three Common Terns.

Epizootics in other species of wild birds, including sea-birds, have been reported, but few of these were properly investigated and most of the reports are not very informative. A few exceptions, where unusually favourable circumstances prevailed, provide elegant examples of epidemiological study. One of these concerns a viral infection of Manx Shearwaters (Puffinus puffinus) on islands off the West coast of Britain (Dane, 1948; Dane et alia, 1953). The causative agent was thought to be a new virus but not a myxovirus (Stoker et alia, 1953). A second example is the infection of Fulmar Petrels (Fulmaris glacialis) in the Faroe Islands with Psittacosis virus (Miles and Shrivastav, 1951).

Of particular interest is the investigation of Blaxland (1951) into the possible role of sea-birds in the spread of NDV amongst domestic poultry in Scotland, neighbouring off-shore islands and Ireland. NDV was isolated from the bone marrow emulsions of 6 out of 26 shags (Phalacrocorax aristotelis). Antibodies to NDV were found in 18 of 54 blood samples of shags and cormorants (P. carbo). Blaxland considered sea-birds as a possible means of spread of NDV because of the local habit of shooting shags for the table and throwing the offal to the poultry. Wilson (1950) isolated NDV from a gannet (Sula bassana) caught on the Orkney islands.

No report of avian influenza in sea-birds was found.

A/.....

A discussion of the epizootiology of Tern virus infection is necessarily based largely on speculation. The first possibility which may be considered is that the terns contracted the infection locally from another vertebrate. The explosive and widespread nature of the outbreak along a 1000-mile stretch of coast implies that this vertebrate would have been mingled with the terns over the area concerned and that the virus would have been widely distributed in that species without producing the dramatic effects seen in terns. The fact that Swift Terns experimentally inoculated with Tern virus (Birds 12 and 13, Table 4, page 46) remained well, serves to illustrate the possible variation in susceptibility of different avian species.

Alternatively if the infection were not multifocal in origin a very rapid spread among terns from the original focus would have to be assumed.

The circumstances of the epizootic might be better explained if the virus was present in a latent form in the terns, poised to produce overt widespread infection when the right ecological conditions presented themselves. These conditions may have been created by the very abnormal weather which prevailed just prior to the tern epizootic. A prolonged masked or latent virus infection which may become overt under particular precipitating circumstances is well known. Apart from man, where the recurrent herpesvirus 'feverblister' is a classic example, prolonged latency is a common feature of ornithosis (Pollard, 1947), and NDV has been recovered from apparently normal chickens which were sacrificed two months after exposure to the virus (Brandly et alia, 1946).

Ticks, mites and lice are known to harbour NDV for several days but are not known to transmit infection (Stubbs, 1959; Brandly et alia, 1946).

Little is known about the epizootiology of avian influenza A virus strains. The role of ectoparasites is an unknown factor. No virus

with/.....

with strain-specific antigens related to Tern virus has been isolated in South Africa before or since the tern epizootic.

An intriguing fact is the outbreak in chickens in Scotland caused by Chicken virus about 18 months before the outbreak of the tern epizootic (Wilson, 1962). This virus which is so closely related to Tern virus, is the only other known virus strain which shares haemagglutinating antigens with Tern virus. This, coupled with the fact that terns migrate between Europe and South Africa tempts further speculation to try and link up the two outbreaks.

A direct link might be postulated, as contact between terns and chickens is possible. Terns breed in Scotland along inland waters as well as along the coast, and are known to feed in cultivated fields. Even so, it would still be necessary to postulate an initially inapparent infection within the population of Common Terns.

An indirect link might have involved an intermediary host or hosts, or it might be that the two outbreaks had a common source of infection. The differences in the properties of the two isolated strains of virus could be readily explained by variation occasioned by passage in different hosts (as discussed in the previous section). The occurrence of mass mortality in Kittiwakes (Rissa tridactyla) and Fulmars (Fulmaris glacialis) from February to August, 1959 (Joensen, 1959) off the coasts of Britain and Scandinavia may be pertinent. The aetiology of this outbreak was unfortunately not established but, if the same virus was involved in both cases, infection might have been transmitted to the chickens in Scotland by sea-birds particularly if the stormy weather which prevailed at that time and forced fishing vessels to take shelter drove sea-birds a little inland to do likewise. In fact large numbers of sea-birds (Herring Gulls-Larus argentatus) were noted at the time on the farm where the outbreak in chickens occurred. Terns may likewise

have/.....

have contracted the infection from other sea-birds, either in South Africa if these were also migrants, or in the breeding grounds in Europe. In the latter event the infection would initially have been latent in view of the fact that the tern epizootic occurred after the terns had been in South Africa for several months.

SECTION IV. ELECTRON MICROSCOPY OF TERN VIRUS AND CHICKEN VIRUS

INTRODUCTION

THE STRUCTURE OF THE MYXOVIRUSES

Since the introduction of the negative staining technique to supplement the existing techniques of shadow casting and ultrathin sectioning in electron microscopy, it has been possible to elucidate the ultrastructure of many viruses in fine detail. Consequently, the morphology of viruses has assumed greater taxonomic importance.

Although all the myxoviruses have a characteristic basic structure, the influenza viruses types A, B and C (subgroup I, Table 16 a, page 77) are structurally distinguishable from other Myxoviruses (Sub group II, Table 16 b, Page 77a). The morphological characters of each subgroup were originally reported by Horne et alia (1960), and Hoyle et alia (1961), and have now been amply confirmed as a result of electron microscopical studies on practically every one of the currently known myxoviruses.

The brief summary given below of the morphological characters of the two subgroups is based on the original observations of Horne et alia (1960) and Hoyle et alia (1961) and is represented diagrammatically in Fig. 7, page 124. References to morphological studies on specific viruses are given in tables 16 a and b.

Structure of Influenza Viruses

The structure of the Influenza viruses is typified by the structure of Tern virus which will be described in detail later in this section. Briefly, the influenza virion measures between 800 and 1200Å in

diameter/.....

diameter, spontaneous disruption of the particle is uncommon, the surface bears prominent radially orientated 'rods' of haemagglutinin, and there is an internal nucleocapsid which is a slender double helix measuring about 90-100Å in diameter (this latter structure has not yet been demonstrated in influenza C virus).

Structure of Myxoviruses Other than Influenza Viruses

The virion is larger than that of the Influenza viruses, disrupts easily, the surface projections are less prominent and the helical nucleocapsid has a diameter of 170-180Å and a characteristic herringbone appearance.

In come of the viruses in this subgroup the nucleocapsid appears to be a double helix, which is, however, a coarser structure than that of the Influenza viruses.

A detailed study of the morphology of Tern virus was undertaken and resulted in several original contributions to knowledge on the structure of myxoviruses.

Chicken virus was also examined in the electron microscope.

MATERIALS AND METHODS

TERN VIRUS

The details of the methods used to prepare the purified whole virus and subunit (nucleoprotein and haemagglutinin) suspensions have been described in section two. The slight modifications of these methods employed in the preparation of suspensions for electron microscopy are given below.

Preparation/.....

Preparation of Purified Virus Suspensions

Seed virus was allantoic fluid from the fifth, sixth, or seventh allantoic passage, and was diluted 10^{-4}, 10^{-5}, or 10^{-6} for inoculation into the allantoic cavity of embryonated eggs. The eggs were then incubated for 24-36 hours at 37°C after which the embryonic fluids were harvested and the virus purified and concentrated by two cycles of adsorption to and elution from chicken erythrocytes. The final suspension contained about 10,000 haemagglutinating units per ml. using 0.5% fowl cells.

Portions of the suspension were dialysed overnight at 4°C against distilled water or 1% ammonium acetate. In the former case a virus-associated precipitate regularly formed.

Controls were similarly prepared from eggs inoculated with diluent.

Preparation of Virus Subunit (nucleoprotein and haemagglutinin) Suspensions

The virus was propagated, purified, and concentrated approximately tenfold by two cycles of adsorption to and elution from chicken erythrocytes. An aliquot of the suspension was set aside and the rest was then treated with a half volume of freshly distilled ethyl ether by shaking for several seconds, allowing it to stand at room temperature (19°C) for one hour, shaking again and allowing it to stand for a further hour. After low-speed centrifugation the aqueous phase was transferred to a wide-mouthed container and placed at 37°C overnight to remove residual ether. This was followed by exhaustive adsorption of haemagglutinin to washed guinea-pig erythrocytes leaving the nucleoprotein in the supernatant fluid after centrifuging down the cells. The latter were washed four times in cold PBS and elution allowed at 37°C for 1-2 hours, the eluate

constituting/.....

constituting the haemagglutinin fraction which usually contained about 100,000 HAU per ml.

The nucleoprotein and haemagglutinin fractions, and the aliquot of virus suspension which was not treated with ether were spun in a Spinco model L preparative centrifuge at 37,000 r.p.m. for 6 hours. The nucleoprotein formed a minute translucent pellet that was easily resuspended in either distilled water or 1% ammonium acetate. The haemagglutinin did not form a compact pellet and was thus collected with the bottom 0.5 ml. of fluid in the centrifuge tubes. This suspension was dialysed overnight at 4°C against 1% ammonium acetate or distilled water, in the latter case forming a haemagglutinin-associated precipitate. The pellet of whole virus was resuspended in distilled water or 1% ammonium acetate.

Controls were prepared in the same way from non-infected allantoic fluids.

Mounting of the Suspensions for Electron Microscopy

Negative Staining method. The method developed by Brenner and Horne (1959) was used for examination of the virus and subunit suspensions of which several batches were prepared. A portion of each suspension was mixed with an equal quantity of 2% phosphotungstic acid which was neutralised to pH 7.0 with N-NaOH or N-KOH. The next step was to place a small drop of mixture onto a carbon-coated copper grid and immediately aspirate it, leaving behind a thin film which dried almost instantly.

Metal Shadowing. A small drop of whole virus suspension was placed on a grid and immediately aspirated. The grid was shadowed with palladium at an angle of 35°.

Preparation/.....

Preparation of Ultrathin sections of virus particles. The virus was concentrated and purified by two cycles of adsorption to and elution from chicken erythrocytes and then dialysed against distilled water overnight at 4°C. The virus-associated precipitate was fixed in 1% osmium tetroxide in Veronal buffer (pH 7.2) for two hours at 4°C. After dehydration in acetone and embedding in methacrylate (4/5 n-butyl and 1/5 methyl methacrylate), sections were cut in a Philips ultramicrotome and mounted on carbon-coated copper grids.

The sections were then stained by floating the inverted grids for 2-5 hours on 0.5% uranyl acetate adjusted to pH 6.0.

Electron Microscope

The grids were examined in a Siemens electron microscope at 80Kv at verified magnifications of 19,250 and 40,000, using double condensor illumination.

Measurements

All measurements were made on positive prints using a micro-comparator calibrated in microns.

Printing of Electronmicrographs

Enlarged positive prints were made directly from the negative plates. The extent of magnification is limited by the degree of resolution obtained. However a higher resolution and consequently a higher useful magnification was obtained by interposing an intermediate step which took advantage of the greater sensitivity of the negative as opposed to the print emulsion. This step involved making intermediate negatives from the electronmicrograph or a low magnification print thereof, enlarging

the/.....

the field of interest in the process, and then preparing positive prints from these intermediate negatives.

CHICKEN VIRUS

Suspensions of whole virus, of haemagglutinin and of nucleoprotein were prepared as described for Tern virus and examined by the negative staining technique.

RESULTS

TERN VIRUS

NEGATIVELY STAINED PREPARATIONS

Intact Virus Particles (Viria)

Intact Tern virus particles showed considerable pleomorphism. Filamentous forms were easily found, showing an external structure similar to that of the more frequent rounded forms (Figs 8 and 9). The overall diameter of apparently intact rounded particles varied between 850 and 1550Å, usually lying in the upper half of this range. Irregularly-shaped smaller or larger particles were seen. Filamentous forms varied greately in length and diameter. The surface of the virion was formed by prominent, radially orientated rod-like projections of haemagglutinin. These 'rods' projected free for approximately 120Å, were 50-60Å in diameter and had a centre to centre distance of about 65Å.

The'rods' of haemagglutinin were well seen in profile around the circumference of the virus particle and end-on over the presenting

surface/.....

surface. The latter point is well illustrated in Figure 10 by the large particle which was apparently flattened out. Most of the 'rods' of haemagglutinin appeared to be surrounded by six neighbours but it would be difficult to determine the precise method of packing because of the relative mobility of their free ends. Hexagonal packing was noted on the surface of filamentous forms of Influenza C by Waterson et alia (1962).

Haemagglutinin

After ether fractionation of the whole virus the haemagglutinin consisted of star-like balls of radially arranged 'rods' (Figures 12, 13, 14, 15). This was also the case after spontaneous disintegration of the virus particle (Figures 20, 23, 25). These 'rods' corresponded to those forming the surface of the intact virus particle and appeared to be linked to one another via the subunits on their central ends. The full length of the 'rods' measured approximately 190Å.

By taking a through-focus series of micrographs it was shown that the 'rods' had a central core of greater electron density (Figures 14, 15). This appeared to be due to the entry of phosphotungstate into a central canal indicating that the haemagglutinin was tube-like.

The diameter of the balls lay between 420 and 500Å. The width of the tube-like projections measured 50-60Å, and their internal diameter measured approximately 12Å.

Nucleocapsid (Nucleoprotein)

The helical structure of the nucleocapsid i.e. the nucleoprotein, was demonstrated. It was seen in the form of a double helix (Figure 16,

17A and B) which consisted of two spiral strands with the same directional sense and intertwined. In the portion of helix in Figure 16 at A each spiral strand had a pitch of about 160Å. In ether-fractionated preparations the helix was seen in lengths of about 1000 to 2000Å and its overall diameter usually measured between 118 and 130Å depending upon the degree of teasing apart of the two strands of the double helix. Each strand appeared to consist of roughly spherical subunits about 50Å in diameter.

It was noted that there were two forms of double helix differing in that the one was the mirror image of the other i.e. they were enantimorphous. This meant that the two forms twisted in opposite directional senses (Figure 17A and B). Examples of both forms were easily found in the same electron micrograph, but the proportion of each was difficult to assess accurately.

Numbers of ring-like structures were seen with overall diameters similar to that of the helix and with central holes which usually measured 35-40Å across. Each was interpreted as a single turn of a spiral strand viewed end-on, and could be compared to a spring washer. These 'washers' were roughly pentagonal in outline (18A, B), the bossing at each of the five angles being due to a subunit. Because the subunits in any given 'washer' are part of a spiral they may be seen with varying degrees of sharpness depending on their relation to the plane of focus.

Spontaneously Disrupting Virus Particles

Particles undergoing spontaneous disruption were not frequently found, but a study of them provided valuable information on the structure of Tern virus.

It was clear that the 'tubes' of haemagglutinin were linked to

one/.....

one another via their central ends to form the viral envelope. Empty envelopes or portions of envelopes, rather resembling strings of Chinese crackers, were seen (Figure 19). An empty shell was seen in the process of breaking down into rosettes of haemagglutinin (Figure 20). These rosettes were interpreted as rolled up fragments of viral envelope. This point was well illustrated by the occasional fragments which were larger than usual as in Figure 13 at A. The length of the 'tubes' of haemagglutinin as measured in empty envelopes and in the rosettes of haemagglutinin was about 190Å compared with approximately 120Å in the intact virus particle. This seeming discrepancy is explained by the fact that in the intact virus particle the true limits of the central ends of the 'tubes' were not apparent; consequently the measurement of 120Å represented only the length of the free-standing portions of the 'tubes' of haemagglutinin.

Viruses were seen with an apparently whorled arrangement of the nucleocapsid; in Figure 21 the nucleocapsid of the virus particle is seen protruding through a small break in the external haemagglutinating layer. In Figure 22 the nucleocapsid appears to be orientated in parallel. These two appearances lend support to the suggestion of an orderly arrangement of the nucleocapsid, possibly in a spiral about a central axis of the viral particle. This would then present a whorled appearance when viewed along the axis, and a parallel arrangement when viewed at right angles to the axis (Hoyle et alia, 1961).

In some partially disrupted virus particles a structure was clearly seen (Fig. 23) which has been described as a membrane (thought to be derived from host-cell membrane) in the case of other myxoviruses (Horne et alia, 1960; Hoyle et alia, 1961).

The large irregular disrupting virus in Fig. 25 showed a number of interesting features. In places around the edge of the particle the

haemagglutinin was stripped off (A) and balls of haemagglutinin (B) were present in the neighbourhood of the virus. The 'washer' (C) was interpreted as a piece of helical strand of nucleocapsid seen end-on. In addition the virus had a 'membrane'. Closer examination of this membrane suggested that it may have a helical structure, in which case it could be interpreted as a layer of nucleocapsid lying adjacent and internal to the envelope of haemagglutinin. Support for this suggestion came from the examination of the small particle (D) which showed a 'membrane' protruding from the virus in the form of a double helix.

The diameters of the 'membrane' of the large virus and the 'washer' were similar (125-130Å) and corresponded with that of the nucleocapsid prepared by ether fractionation. The membrane of the particle at D measured somewhat less, about 110Å.

At this stage it is necessary to note the possible variation in the diameters of both the nucleocapsid and the 'membrane' under different circumstances. The nucleocapsid in Fig. 21 measured about 80Å and that in Fig. 22 about 82Å in diameter, compared to between 118 and 130Å or sometimes more in ether-treated material. It should be noted that the 'tubes' of haemagglutinin in each of these micrographs corresponded in size and spacing with each other and with those in other preparations. The latter statement also applied to the micrographs which showed 'membranes'. The 'membrane' in Fig. 23 measured approximately 83Å. In virus particles fixed with formalin prior to examination in the electron microscope, a 'membrane' could also be seen, as in Fig. 24 where it measured about 86Å. At the point indicated in Fig. 24 the 'membrane' appeared to be a double helix, a fragment of which had apparently been displaced so that it presented itself end-on. The resulting ring-like structure was similar to those seen in the preparations of nucleocapsid. The 'tubes' of haemagglutinin, which were less distinct

in/.....

in the formalin-fixed preparations, corresponded in size and spacing with those in preparations that were not fixed with formalin.

Ultrathin Sections

In ultrathin sections the pleomorphism of the virus particles was again evident (Fig. 26). The rounded and filamentous forms appeared to have the same structure. The viria showed an outer coat which usually had an inner zone of lesser electron density. In some particles the outer coat could be seen to consist of radially arranged striations which would correspond to the haemagglutinin of particles examined by the negative staining technique. Just internal to the outer coat was a well-defined 'membrane' approximately 77Å wide which would correspond to the 'membrane' seen in particles examined by the phosphotungstate method (Fig. 23, 24, 25). Careful examination of this 'membrane' (Figs. 27A and B) revealed that it was a double helix. In addition it was again noted that there were two forms of double helix: the one seen in Fig. 27A twisted in the opposite direction to that in Fig. 27B.

In some particles the plane of section and of focus was such that the double helix simulated a 'double membrane'. Similar 'double membranes' were seen in virus suspensions examined by the negative staining technique (Figs. 24, 25) and the nucleocapsid also sometimes assumed this appearance as in Figs. 21 and 22.

Some of the particles showed what has been interpreted as an "internal body" in the case of other influenza viruses (Morgan et alia, 1956).

The appearance of the particles was very similar to that described for influenza virus in ultra-thin sections of infected tissues (Morgan et alia, 1956) and in ultrathin sections of pellets of influenza virus (Birch-Andersen and Paucker, 1959), but a higher resolution was

obtained, and revealed that the structure called a 'membrane' in the above two papers was a double helix. Valentine and Isaacs (1957 a and b) examined several myxovirus strains electron microscopically. They mounted virus particles on grids and digested them enzymatically before fixing in osmium tetroxide and staining with phosphotungstic acid. Their electron micrographs appeared to show that the nucleocapsid was arranged in a ring-like fashion around the circumference of the virus particle. The arrangement of the nucleocapsid was comparable to that seen in ultrathin sections of Tern virus.

METAL-SHADOWED VIRUS PARTICLES

In metallised preparations (Fig. 11) the viria cast shadows which indicated their three-dimensional form. The projections of haemagglutinin could be distinguished on the circumference of the virus while over its presenting surface the metal tended to bridge the gaps between the ends of the haemagglutinin to form a delicate lacework pattern.

CONTROLS

The preparations made from uninfected eggs were subjected to identical manipulations and were devoid of any of the above features.

CHICKEN VIRUS

Suspensions of viria, of haemagglutinin, and of nucleocapsid were examined by the negative staining technique. Their morphology showed no differences from that of the corresponding Tern virus preparations.

A/.....

A group of Chicken virus particles is seen in Fig. 28, and the purified nucleocapsid of Chicken virus in Fig. 29.

DISCUSSION.

Morphology of Tern virus

The morphology of Tern virus was similar to that described for other Influenza A virus strains (Horne et alia, 1960), but on the basis of observations made in this study it was suggested that Tern virus had the following morphology.

The viral particles were pleomorphic. Filamentous forms were easily found but rounded forms predominated. The overall diameter of apparently intact rounded particles usually measured between 850 and 1550Å. A filamentous particle measuring 1500 mu long by 90 mu wide was seen, but filamentous forms were usually shorter and often narrower.

The external surface of the virion was formed by the haemagglutinin which consisted of radially arranged tube-like projections approximately 120Å long and 50-60Å wide, and spaced to give a centre to centre distance of about 65Å. These tubes had an inner diameter of approximately 12Å, and were linked together via their central ends thus forming an envelope within which lay the nucleocapsid. The true length of the tubes of haemagglutinin, approximately 190Å, was evident when measured in empty envelopes or in the rosettes of haemagglutinin which could be regarded as rolled-up fragments of virus envelope.

The nucleocapsid consisted of a double helix of which there were two forms, one winding in the opposite directional sense to the other. Gardner (1963) discusses some of the many spirals that occur in nature, and gives examples of spirals which twist in one or other of the

two/.....

two directional senses. An interesting example is an animal with spiral horns, which are enantiomorphous. The double helix was a slender structure measuring about 90Å in diameter unless it was teased out e.g. in ether-fractionated preparations where measurements of 130Å or more were recorded. The spiral strands of the double helix appeared to consist of subunits measuring about 50Å in diameter. Each turn of a strand apparently consisted of five or six of these subunits.

The nucleocapsid lay close up against the internal surface of the haemagglutinin envelope and sometimes simulated a membrane. It appeared that the nucleocapsid might be wound in an orderly spiral manner about a central axis of the virus particle as has been suggested for other influenza viruses (Hoyle et alia, 1961; Waterson et alia, 1962). If this were so, and if the nucleocapsid were thrown into contrast by entry of phosphotungstate into a particle viewed along its axis, the helix would be seen side-on as a more or less continuous 'membrane'. If the particle were slightly tilted, the 'membrane' would appear wider and less prominent, or it might be irregular due to collapse or folding of the virus particle. If viewed at right angles to the particle axis the helix would be seen at the periphery of the virus as a series of cross-sections forming at most an indistinct 'membrane'.

Conclusive evidence that the 'membrane' was a double helix was provided by the ultrathin sections of whole virus particles.

Contributions of the Study of Tern Virus to Knowledge of the Structure of Influenza Viruses

The morphology of Influenza A viruses has now been widely studied and human, animal and avian strains have the same structure (Waterson et alia, 1962). These authors also reported that the viria of two strains each of influenza B and C which they examined were

morphologically/.....

morphologically the same as those of influenza A.

It therefore seems probable that the new observations made on Tern virus will apply to other influenza virus strains. These observations were:

1. That the rod-like projections of haemagglutinin were hollow i.e. tube-like.
2. That the viral envelope was formed by the tube-like projections of haemagglutinin which were linked together via their central ends.
3. That there was no viral membrane but that the appearance of a membrane might be simulated by the double helix of nucleocapsid.
4. That the nucleocapsid was enantimorphous.

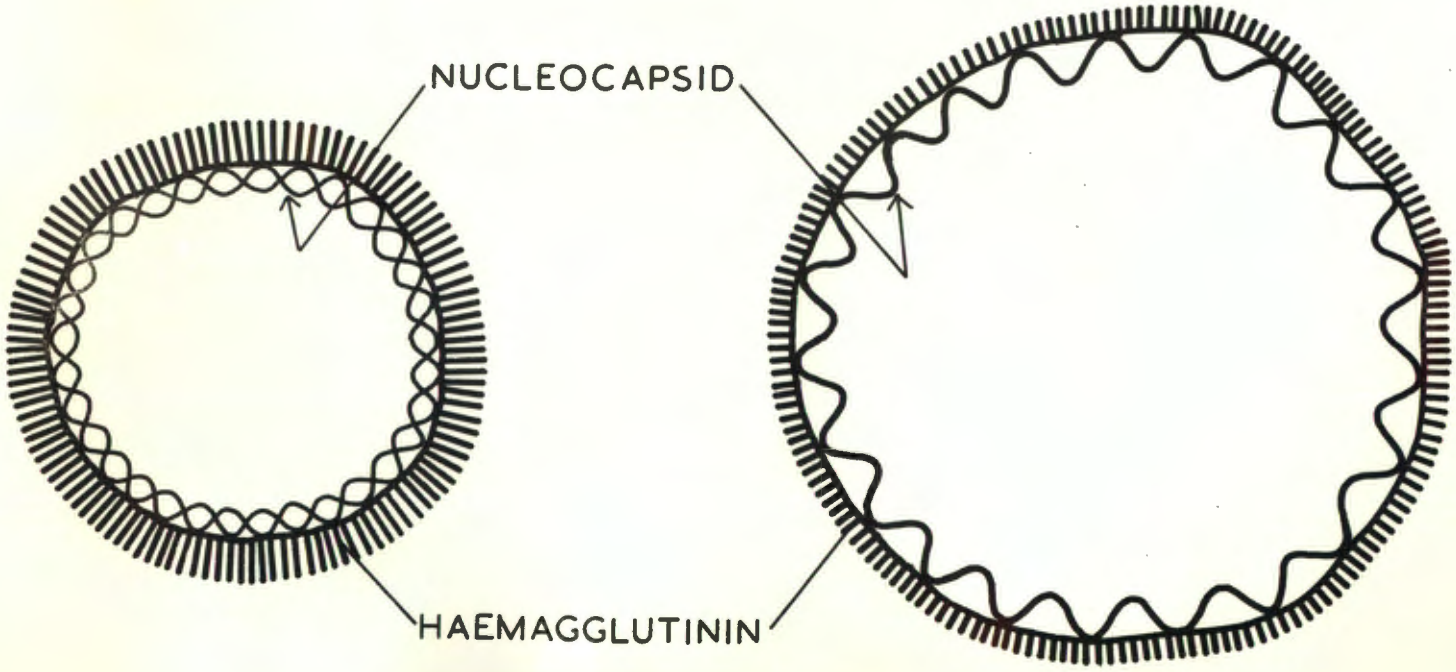
The last observation raised the question as to whether each particle contains only one or the other of the two forms of nucleocapsid, or some of each. The former case would be compatible with the nucleocapsid consisting of one long length of double helix, while the second possibility would presumably require multiple lengths of helix.

FIG. 7

THE STRUCTURE OF MYXOVIRUSES

INFLUENZA
SUB-GROUP

NDV – PARAINFLUENZA
SUB-GROUP



The linear scales in the figures which follow represent 1000Å.

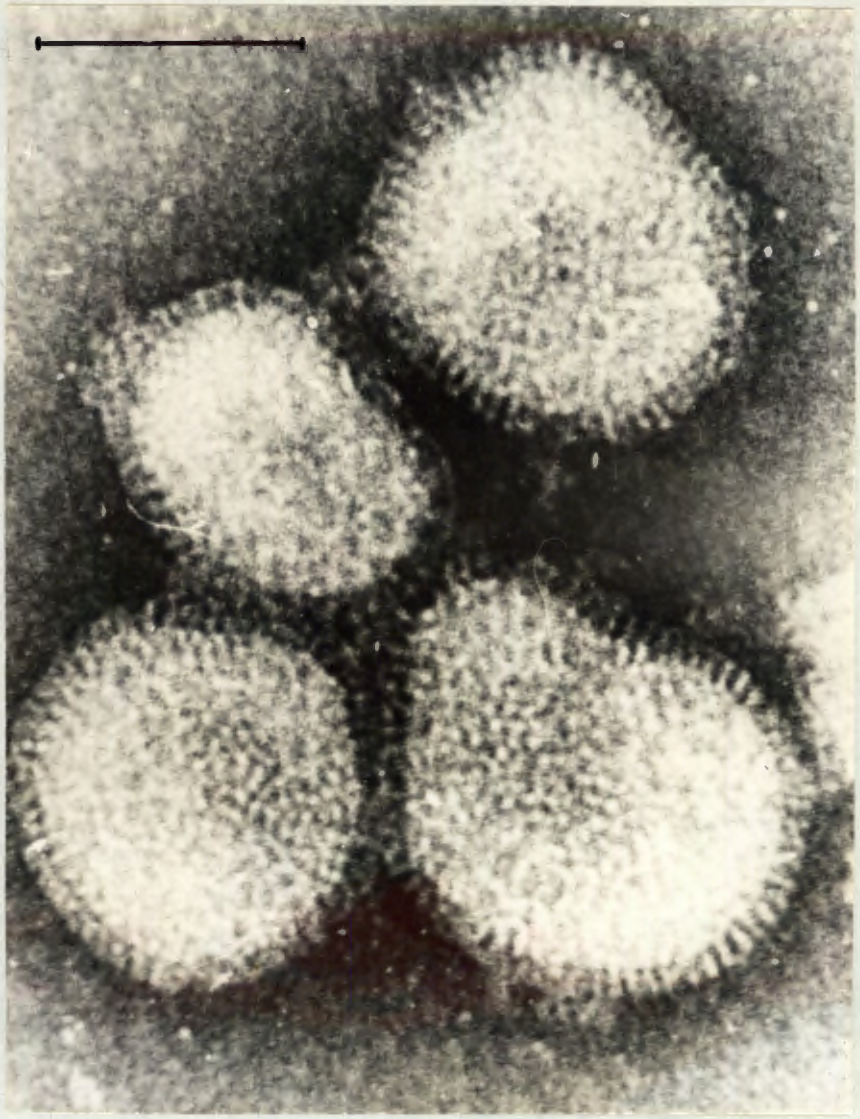


Fig. 8. Electron micrograph of particles of Tern virus. The viral surface is formed by radially arranged rod-like projections of haemagglutinin which are seen in profile around the circumference and end-on over the surface of the particles. Negative staining technique. Magnification: x 346,500.

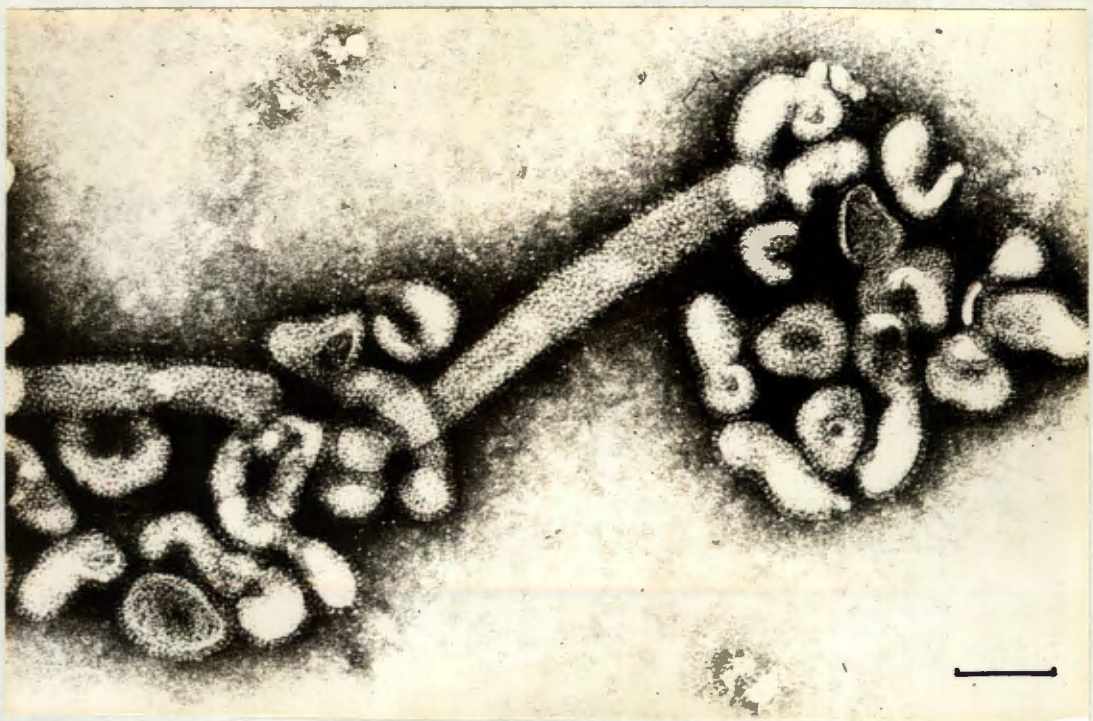


Fig. 9. Particles of Tern virus. The pleomorphism of the particles is apparent. Magnification: x 124,500.

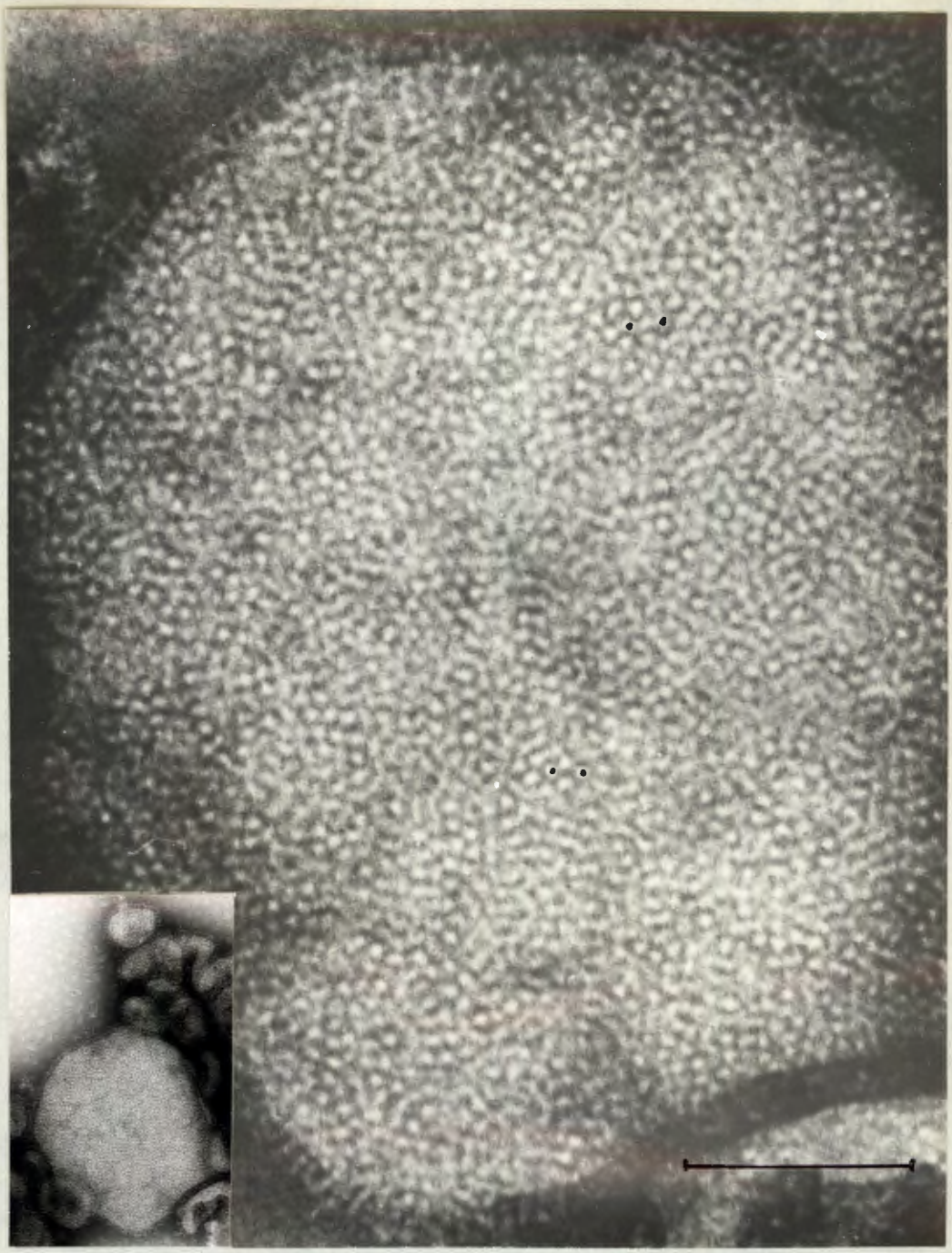


Fig. 10. Tern virus particle. Enlargement of the apparently flattened particle shown in the inset. The projections of haemagglutinin seen end-on appear to show hexagonal packing (marked area). Magnification: x 330,000; inset, x 57,750.

Fig. 11. Metal-shadowed Tern virus particles. The rod-like projections of haemagglutinin can be seen at the circumferences of the virus particles (arrow). Over the presenting surfaces of the particles the metal has tended to bridge the gaps between the free ends of the rod-like projections of haemagglutinin to form a lacework pattern. Shadowed with palladium at an angle of 35°. Magnification: x 96,250.

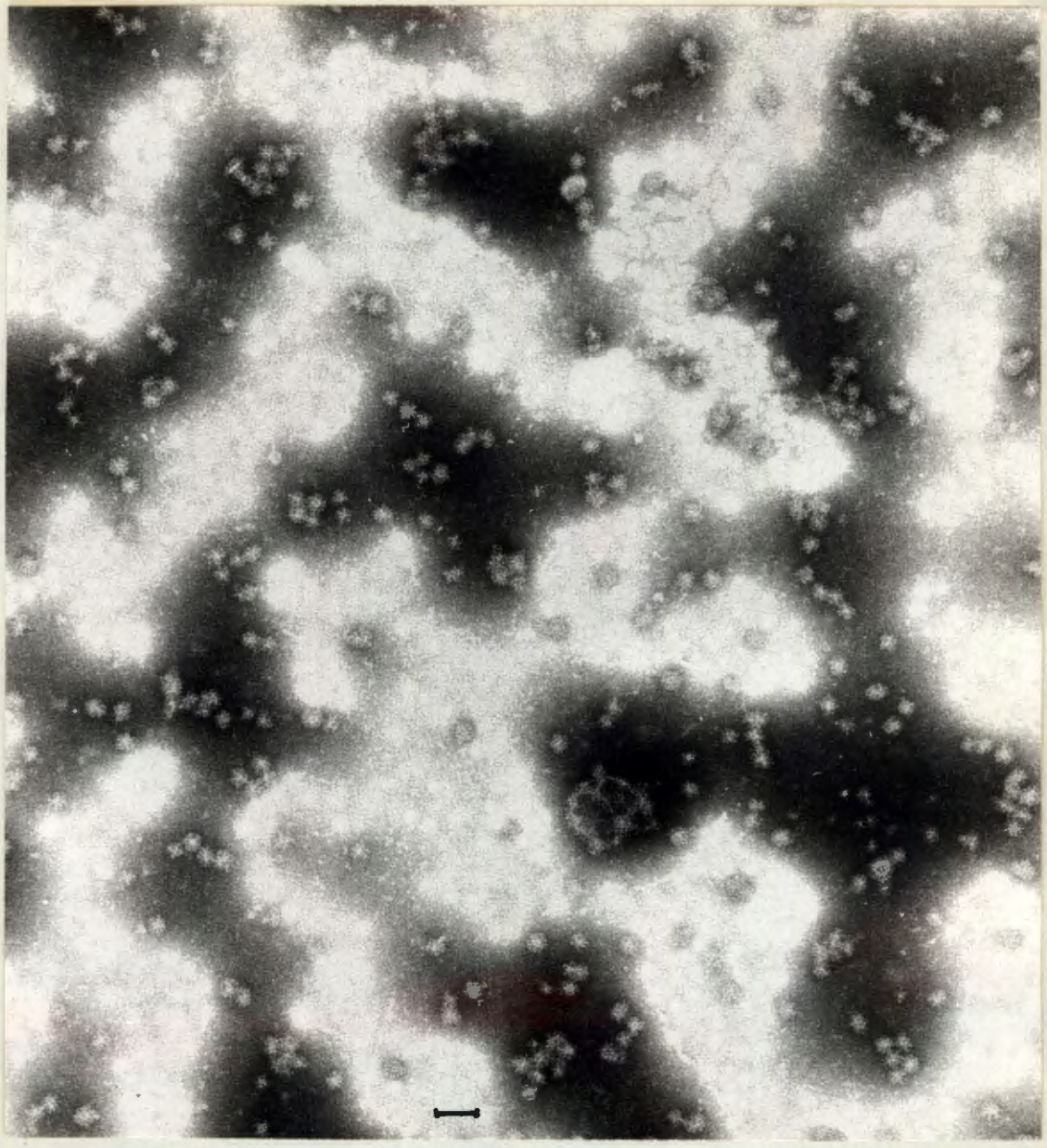


Fig. 12. The haemagglutinating component (haemagglutinin) of ether-fractionated Tern virus. It consists of star-like balls of radially arranged rods corresponding to those which form the external covering (envelope) of intact virus particles. Negative staining technique. Magnification: x 57,750.

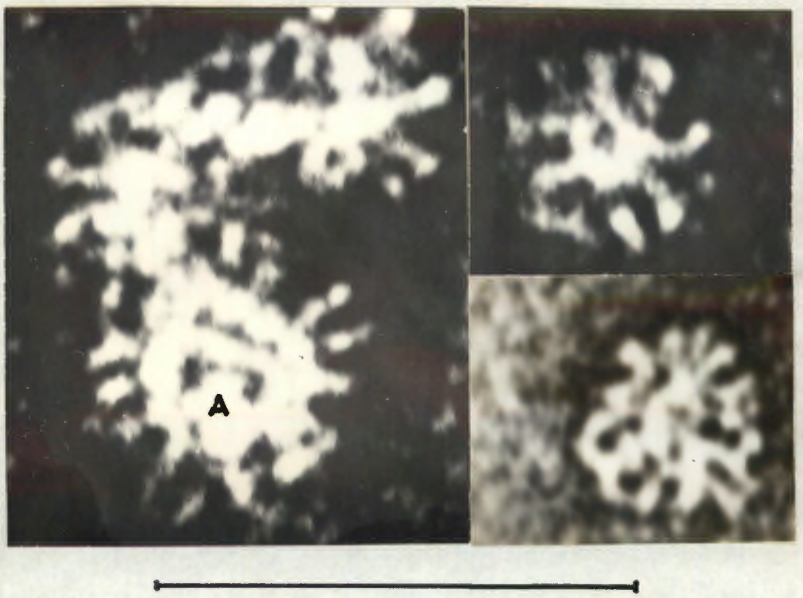


Fig. 13. Tern virus haemagglutinin. The balls of haemagglutinin may be regarded as rolled up fragments of viral envelope, well illustrated by the ball at A. Magnification: x 637,500.

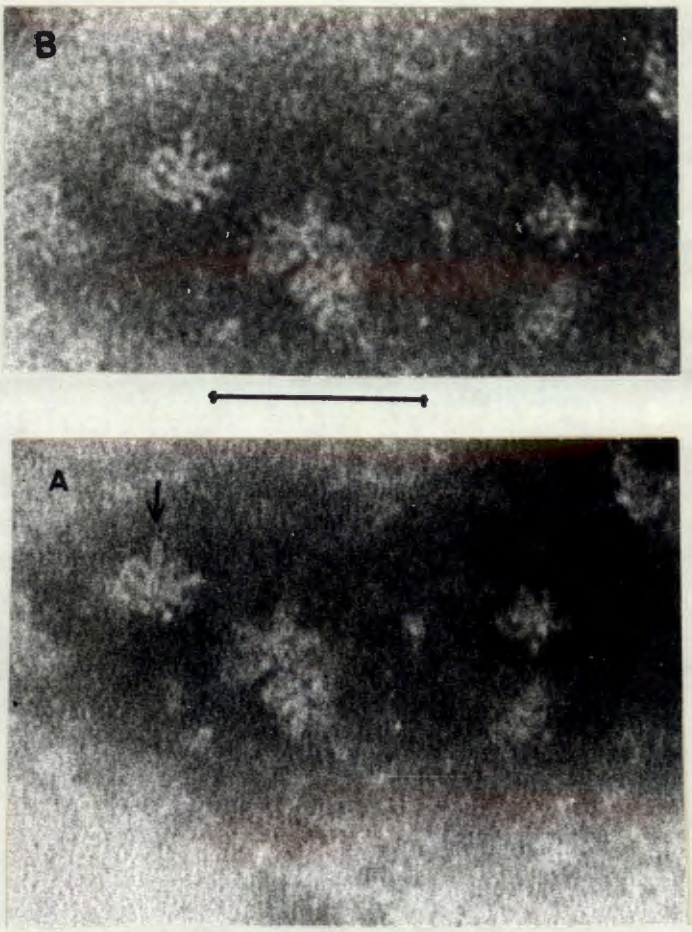


Fig. 14 A and B. Tern virus haemagglutinin. Two of a through-focus series of micrographs illustrating how the critical plane of focus in A reveals the hollow tube-like nature of the radiating rods of haemagglutinin. This is not evident in the different plane of focus in B.
Magnification: x 280,000.

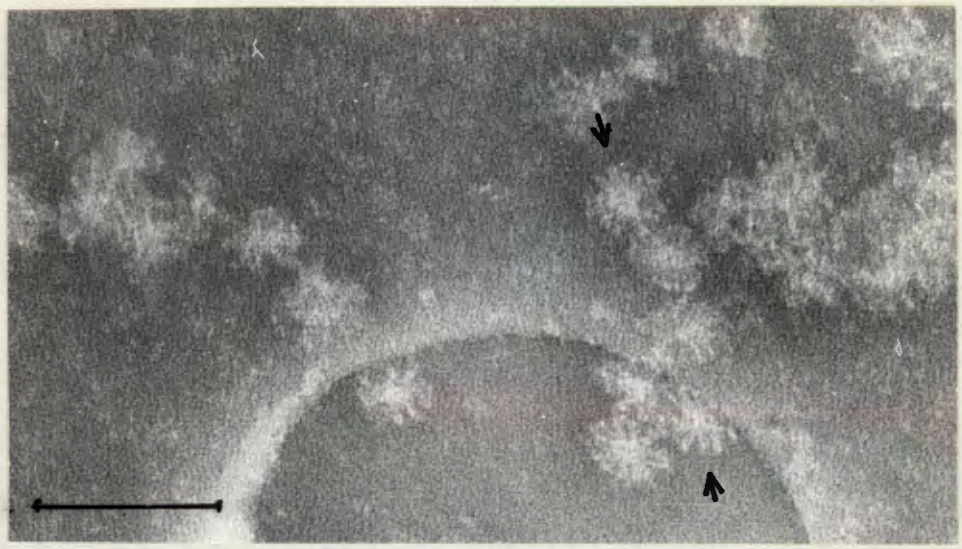


Fig. 15. Tern virus haemagglutinin. One of a through-focus series of micrographs showing the balls of haemagglutinin. Phosphotungstate appears to have entered the radiating rods indicating that they are tube-like. Magnification: x 240,000.

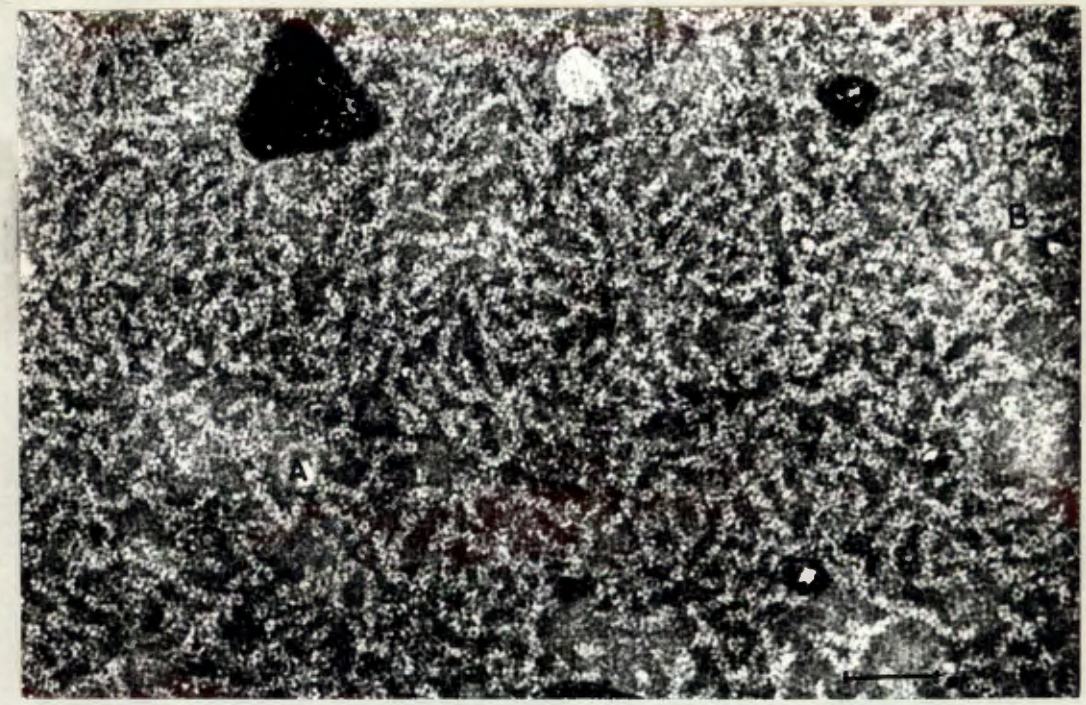


Fig. 16. Purified nucleocapsid of ether-fractionated Tern virus. The nucleocapsid consists of a double helix, well seen at A and B, occurring in lengths of 1000-2000 Å in ether-fractionated preparations. Some ring-like structures are seen and interpreted as end-on views of broken-off portions of one of the helical strands of the nucleocapsid e.g. at C. Negative staining technique. Magnification: x 120,000.

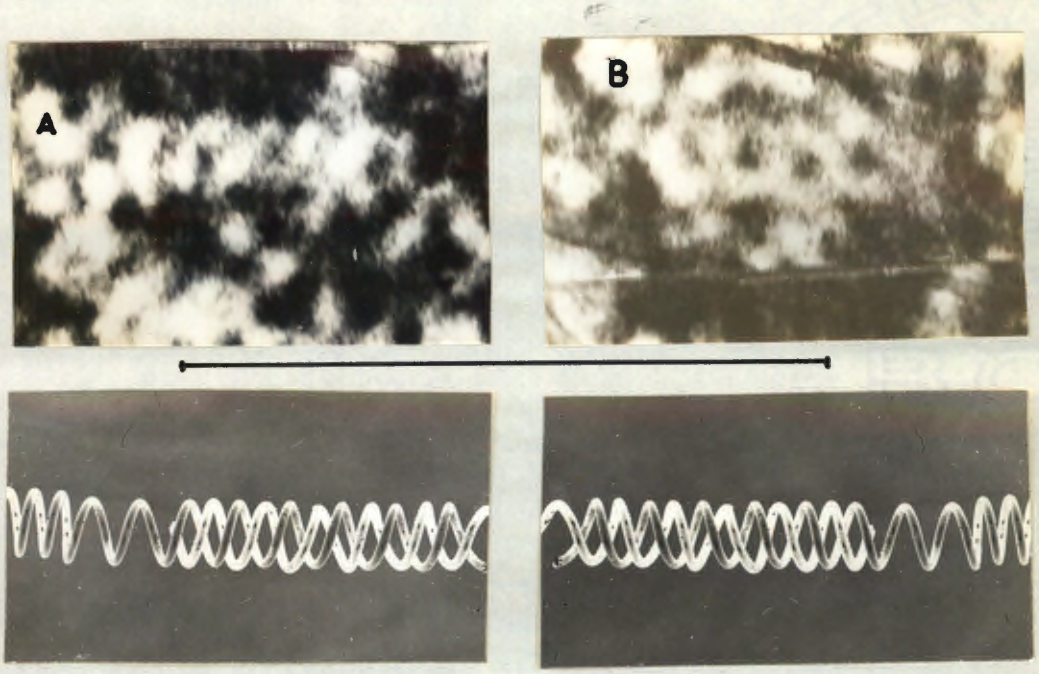


Fig. 17 A, B. Purified nucleocapsid of Tern virus. Enlargements of the two lengths of double helix seen in Fig. 16 at A and B. They are enantiomorphous. The spring models are for comparison. Magnification: x 852,000.

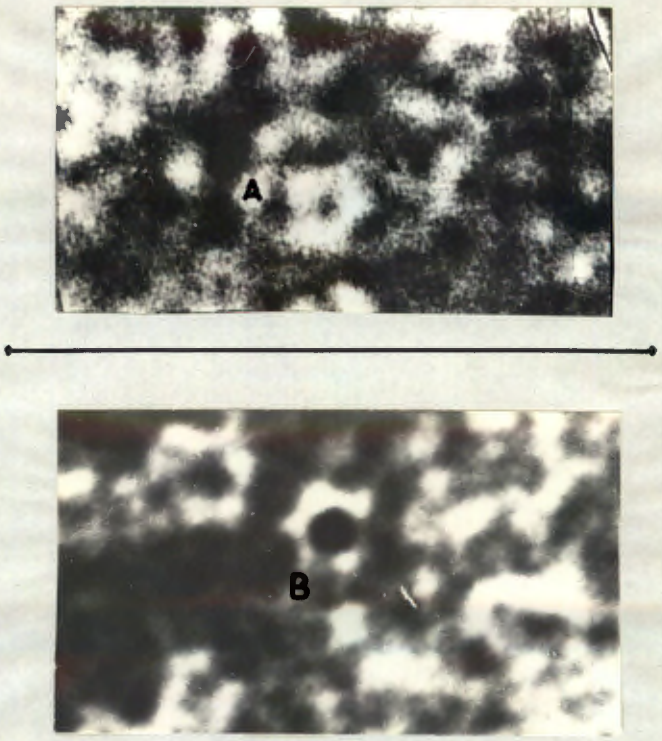


Fig. 18, A, B. Purified nucleocapsid of Tern virus. Enlargement of the ring-like structure seen in Fig. 16 at C (18A), and of a similar structure from another field (18B). Each of these ring-like structures is interpreted as an end-on view of a single turn of a helical strand, and could be compared with a spring washer. The 'washers' are roughly pentagonal in outline, the bossing at each angle being due to a subunit. Magnification: x 852,000.

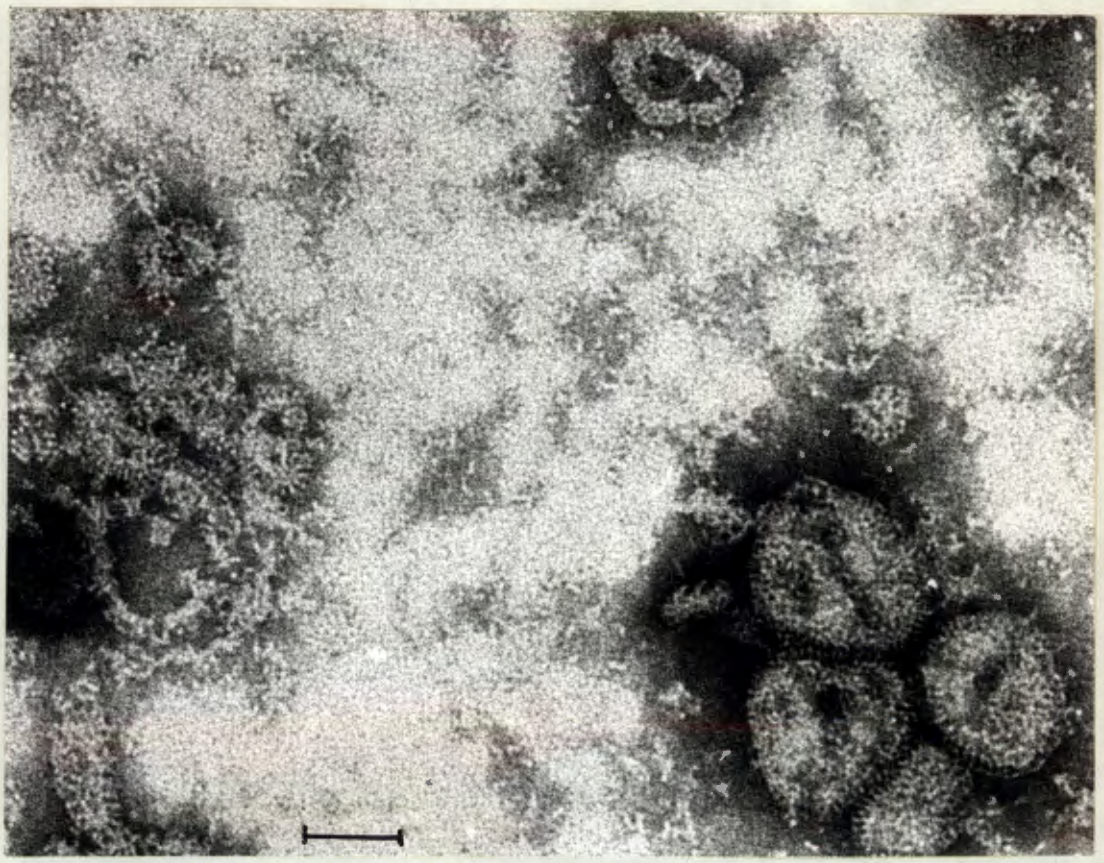


Fig 19. Intact and disrupting Tern virus particles. There are empty shells or viral envelopes formed by the radially arranged rods of haemagglutinin which are linked together via their central ends. Portions of viral envelope are also seen, rather resembling strings of Chinese crackers. None of the shells show a 'membrane'. Magnification: x 134,750.

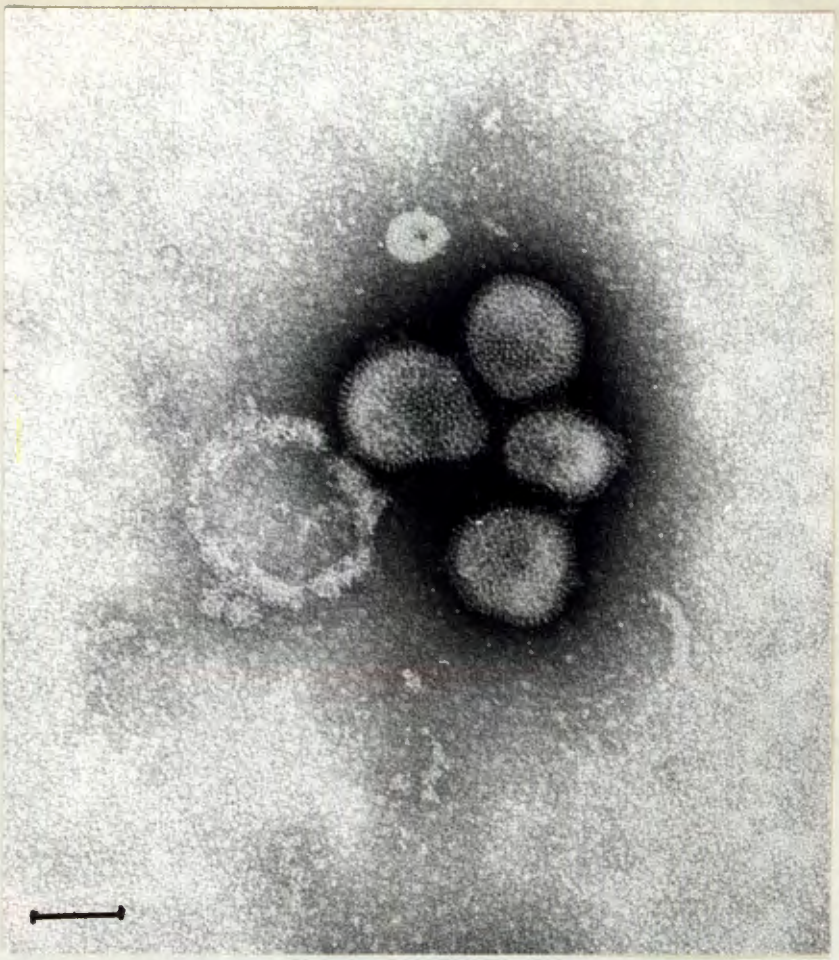


Fig. 20. Adjacent to intact particles of Tern virus are the apparent remains of a disrupted particle. This consists of a shell of haemagglutinin which is itself in the process of breaking up into star-like balls. Magnification: x 115,500.

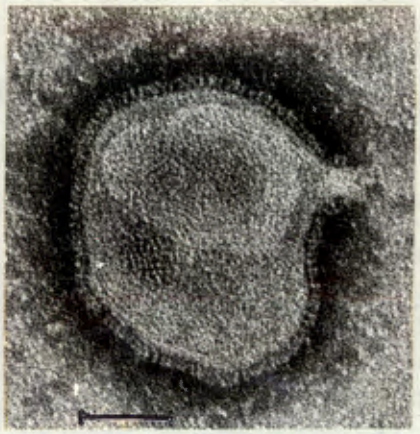


Fig. 21. A disrupting particle of Tern virus. There is an apparently coiled arrangement of the nucleocapsid which has just breached the viral envelope. Magnification: x 115,500.

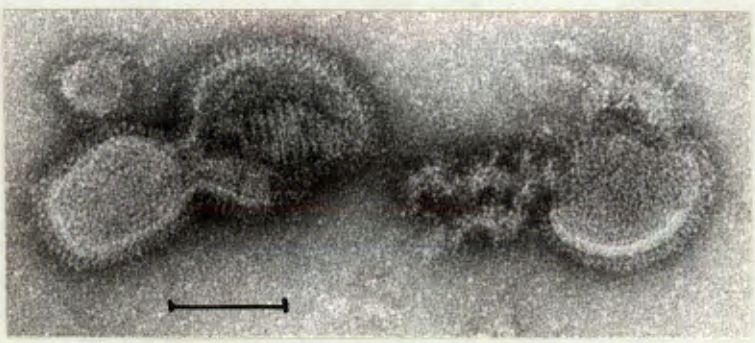


Fig. 22. A disrupting particle of Tern virus. The nucleocapsid is orientated in parallel within the particle. Magnification: x 150,000.

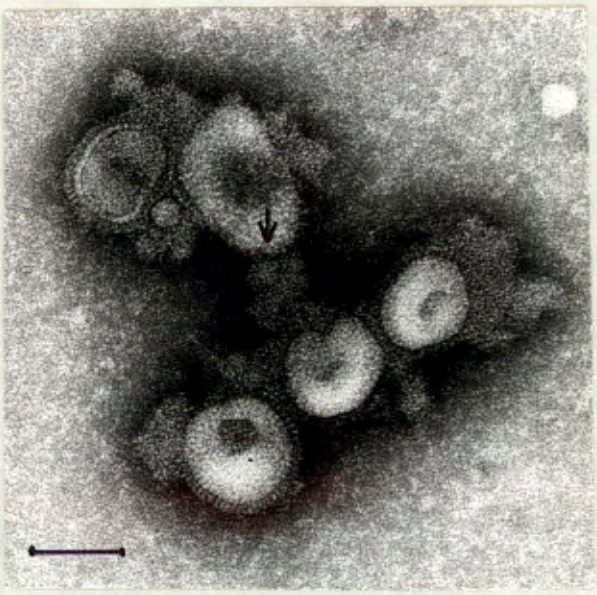


Fig. 23. Particles of Tern virus, one of which shows a well-defined 'membrane'. Balls of haemagglutinin are also present (arrow). Magnification: x 120,000.

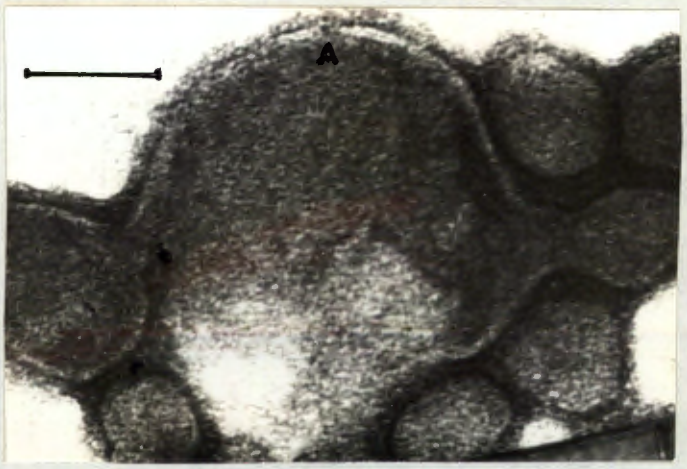


Fig. 24. Tern virus, fixed in formalin and examined with the negative staining technique. The projections of haemagglutinin are less prominent than in preparations not fixed in formalin. Note the membrane at A; it appears to be a double helix, a fragment of which has apparently been displaced and presents itself end-on.
Magnification: x 172,000.

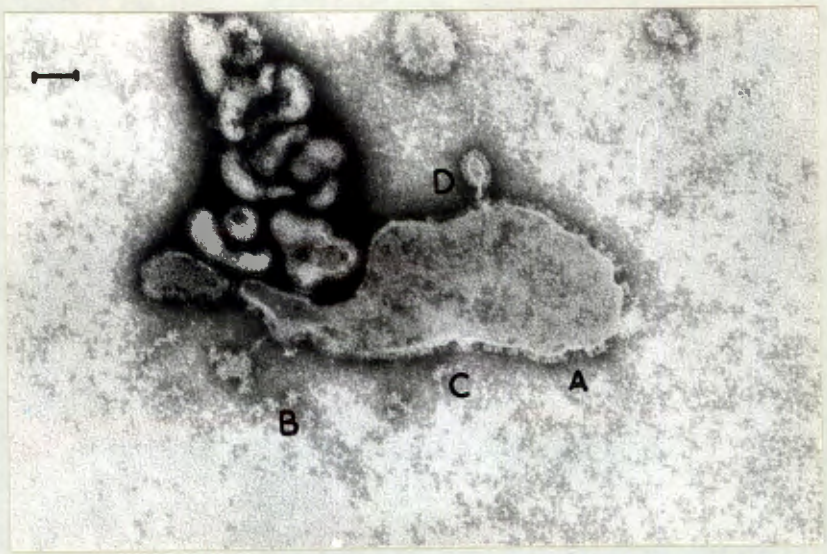


Fig. 25. Tern virus. The large disrupting particle has lost the projections of haemagglutinin over parts of the circumference (A). Star-like balls of haemagglutinin are seen in the neighbourhood (B). The 'washer' (C) is interpreted as a portion of helical component seen end-on. Examination of the 'membrane' of the large particle and of the smaller particle at (D) suggests that the 'membrane' has the structure of a double helix. Magnification: x 57,750.

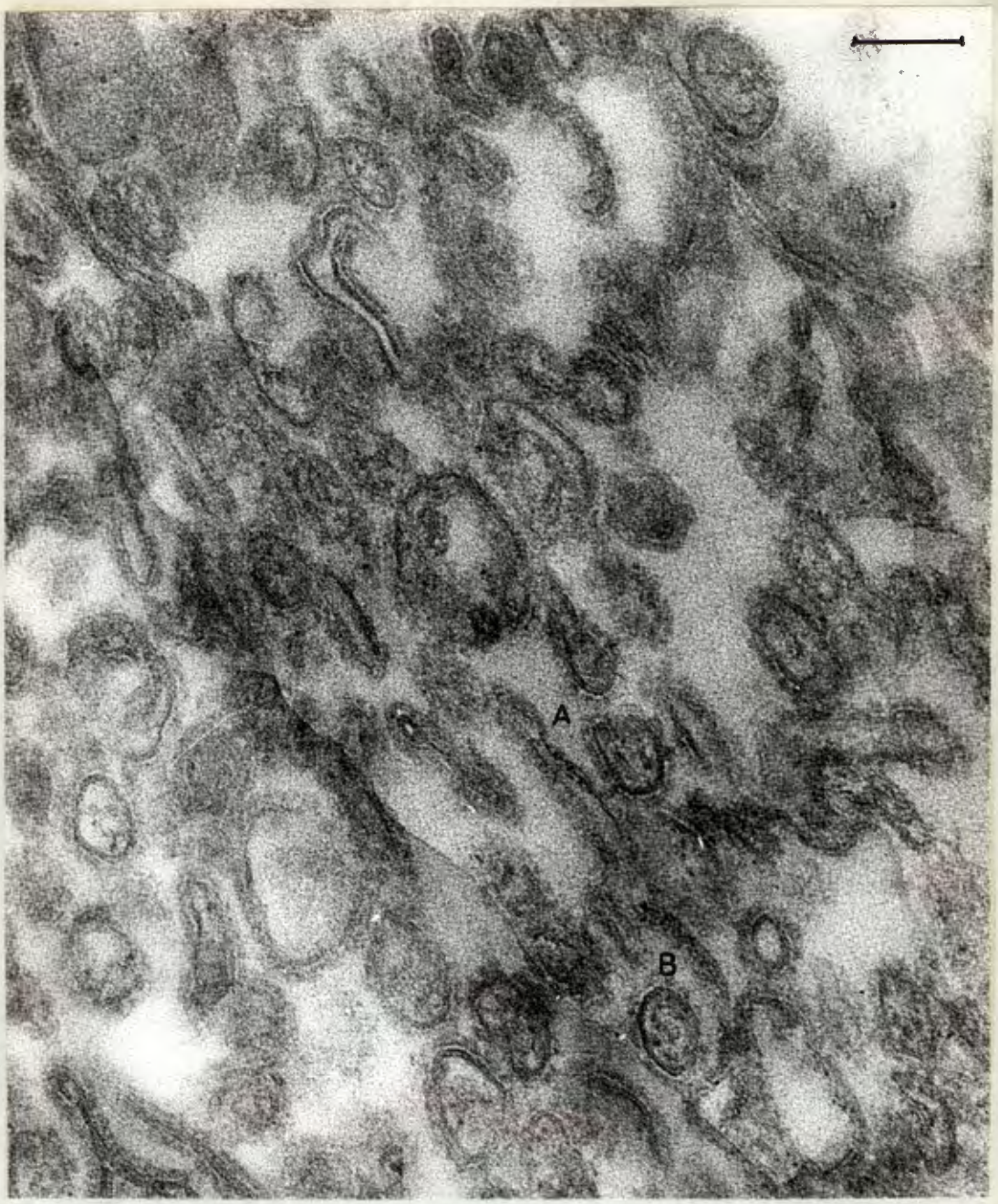


Fig. 26. Tern virus particles embedded in methacrylate. Electron micrograph of an ultrathin section stained with uranyl acetate. The particles show a diffuse outer coat, often with a less dense inner zone. In some particles the outer coat can be seen to consist of radially arranged rods. Adjacent to the inner surface of this outer coat is a well defined 'membrane' which is a double helix - see particles A and B. Magnification: x 154,000.

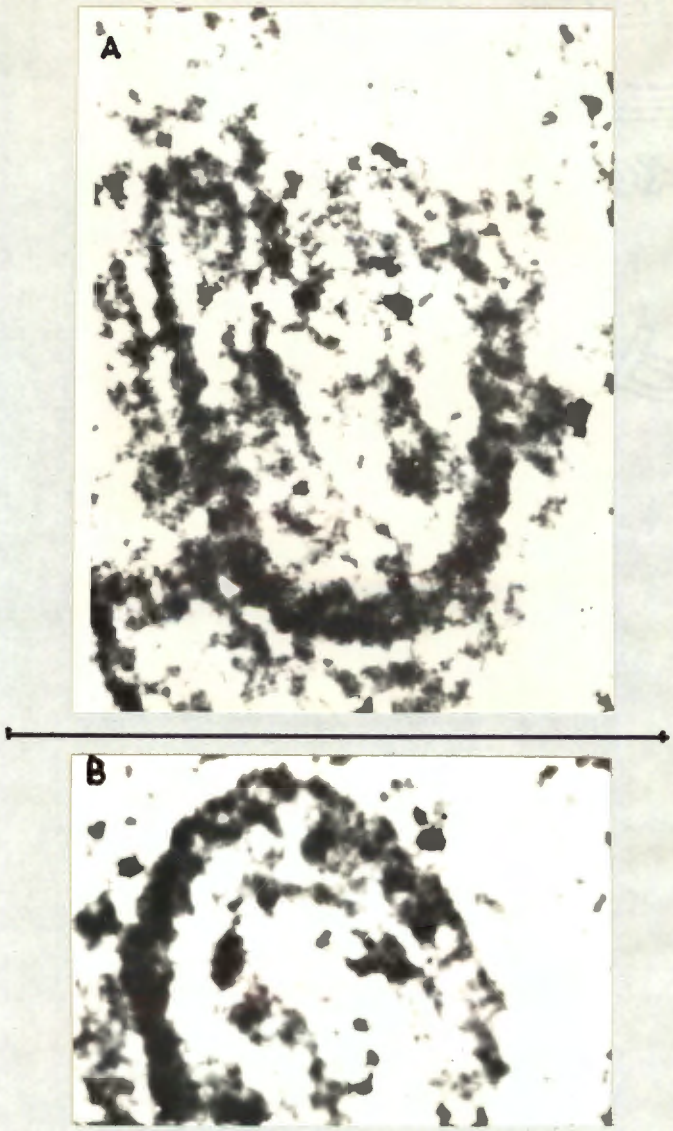


Fig. 27A, B. Ultrathin section of Tern virus. Enlargements of the particles A and B in Fig. 26, illustrating that the 'membranes' are double helixes. Note that the helix in particle A twists in the opposite direction to that at B, providing further evidence that Tern virus nucleocapsid is enantiomorphous. Magnification: x 866,250.

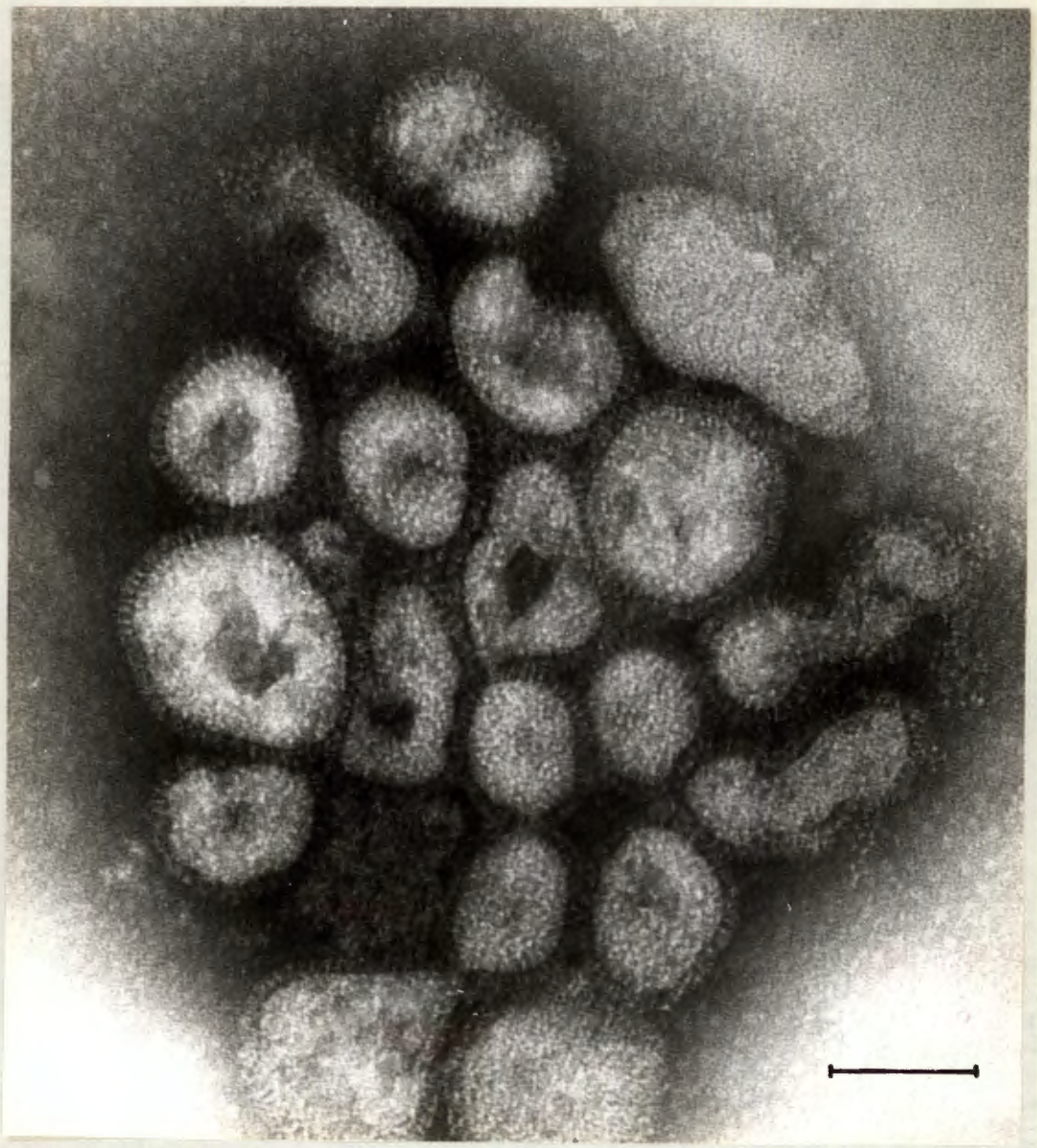


Fig. 28. Chicken virus. Intact virus particles with features similar to those of Tern virus. Magnification: x 192,000.

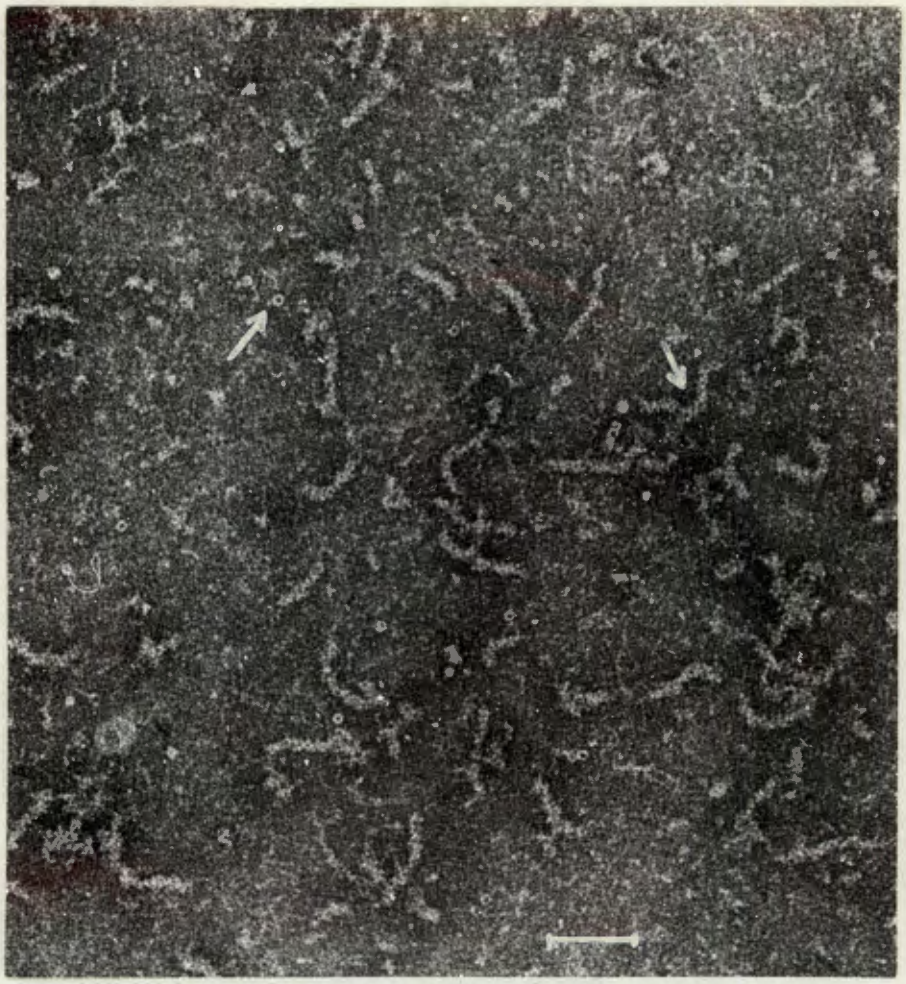


Fig. 29. Chicken virus nucleocapsid prepared by ether fractionation of whole virus. Present are the double helical and 'ring-like' structures seen in Tern virus nucleocapsid preparations. Magnification: x 115,500.

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