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ISOLATION AND CHARACTERISATION OF ADENOVIRUSES AND REOVIRUSES OF DOMESTIC HENS IN NEW ZEALAND

A thesis presented in partial fulfilment of the
requirements for the degree of
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ABSTRACT

This investigation was undertaken to determine whether or not adenoviruses or reoviruses could be recovered from domestic hens in New Zealand. Using cultures derived from neonatal chicken kidney tissue 30 cytopathic agents were recovered from domestic hens.

Electron microscope examination following staining by sodium phosphotungstate showed that 25 of these agents had the morphology of adenoviruses and the remaining 5 agents were morphologically indistinguishable from reoviruses.

Some selected strains of adenoviruses and reoviruses were subjected to physico-chemical tests and it was found that both groups of agents were unaffected by chloroform and the replication of adenovirus strains, but not the reovirus, was inhibited by IDU. These results provide confirmatory evidence for the identification of these agents as avian adenoviruses and reoviruses. The avian adenoviruses like the analogous mammalian agents were found to have diminished thermostability in the presence of 1.0M Ca^{++}.

Cross-neutralisation tests showed that four serologically distinct adenovirus strains were recovered and 16 of the remaining adenovirus isolates were assigned to one or other of the four serotypes. All 5 reoviruses were assigned to one serological type on the basis of neutralisation tests.

A preliminary serum survey for neutralising antibody to the 4 adenovirus serotypes showed that antibody to one or more of these viruses was present in a high proportion of flocks of domestic fowl in the Manawatu district of New Zealand and antibody to two of the serotypes was particularly common.

This work represents the first report of the recovery of avian adenovirus and reovirus from domestic hens in New Zealand. However their clinical and pathological significance remain to be investigated.

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INTRODUCTION

Virology is becoming an increasingly important field of study as far as domestic hens are concerned. With the advent of more intensive production methods, requiring a high population density of birds, an ideal situation is created for the survival and spread of viruses. The pathological and economic importance of avian viruses, however varies from country to country so to place the present study in perspective, the groups of viruses known to infect domestic hens are listed in Table 1. This table also lists the diseases known to be caused by these agents; how the viruses are commonly propagated in the laboratory, and whether or not they are believed to be present in New Zealand.

At the initiation of the present work it was clear that the study of viruses of the domestic hen population of New Zealand had to be limited for practical reasons to one, or at the most to a few virus groups. The choice of the groups to be included in this study was partially governed by the fact that inoculation of embryonated eggs was the only technique fully exploited for the study of viruses of domestic hens in New Zealand, although cell culture techniques are necessary to isolate some agents. However, since cell cultures are capable of propagating a relatively large range of viruses (see Table 1) it was decided to further limit the present study to non cell-associated agents giving a readily identifiable cytopathic effect in cell cultures, and to concentrate on agents infecting the alimentary and respiratory tracts. Since the virus causing infectious laryngotracheitis (ILT) can be studied by egg inoculation techniques, it also was excluded from the present study.

The above considerations, in conjunction with the knowledge that adenovirus and reovirus infections appear to be widespread in domestic hen populations of other countries, led us to concentrate on determining whether or not adenoviruses and reoviruses were present in New Zealand, and, if present, to make at least a preliminary assessment of their prevalence.

Several groups of workers in overseas countries have published data dealing with the isolation and characterization of adenoviruses and reoviruses of domestic hens; this work is reviewed below. A consideration of the relationship of these two virus groups to diseases of the domestic hen is included in the final discussion.

Table 1: Viruses known to infect domestic hens, including the diseases they cause, how the agents are commonly propagated, and whether of not they are present in New Zealand.

Virus group	Disease	Grown in	Present in New Zealand
Myxovirus	Fowl plague	eggs	no [a]
Paramyxovirus	Newcastle disease	eggs, cell cultures	no [a]
Coronavirus	Infectious bronchitis	eggs	yes [b]
Poxvirus	Fowlpox	eggs	yes [b]
Enterovirus	Avian encephalomyelitis	eggs	yes [b]
Herpesvirus	Infectious laryngotracheitis	eggs, cell cultures	yes [b]
Herpesvirus	Marek's disease	cell cultures	yes [b]
Leukovirus	Sarcomas, leukoses	cell cultures	yes
Reovirus	Uncertain	cell cultures	yes [c]
Adenovirus	Inclusion body hepatitis, others uncertain	cell cultures	yes [c]
Parvovirus	Unknown	cell cultures	no [d]

a. this applies with certainty only to strains of classical virulence

b. McCausland, 1972

c. established in this study

d. has not yet been detected in New Zealand, but no systematic investigation has been made.

Isolation and characterization of avian adenoviruses

In retrospect it now seems clear that the first agent related (or identical) to adenoviruses of domestic hens was isolated in 1949 in the United States by Olson (1950) from bobwhite quail. The flock of birds from which this agent was recovered had a respiratory disease described as bronchitis, which caused up to 80% mortality. In 1956 another agent was isolated from bobwhite quail, from a farm reporting up to 70% losses due to a respiratory disease, and was found (Du Bose et al., 1958) to be serologically related to the agent isolated by Olson (1950). When inoculated into embryonated eggs, both these "quail bronchitis" viruses caused diminished growth of the embryo ("dwarfing"), or embryo death.

Yates and Fry (1957) isolated a number of agents from domestic hens. The agents could be serially passaged in embryonated eggs and killed the embryos. Since there was no apparent disease associated with the source of any of the agents, these agents were designated as strains of "chicken embryo lethal orphan" (CELO) virus, "orphan" being the term which by tradition was applied to any agent which did not cause any apparent pathological condition in its host in natural conditions.

Yates and Fry (1957) also found that CELO virus was serologically related to the quail bronchitis virus isolated by Olsen (1950), but serologically distinct from the viruses of Newcastle disease, infectious bronchitis, laryngotracheitis, fowlpox and avian encephalomyelitis.

Burke et al. (1959a) recovered agents from cloacal swabs of both diseased and healthy domestic hens. The agents gave cytopathic effects in chick kidney (CK) cell cultures, and were called avian enteric cytopathogenic viruses. In a further study of a prototype strain of virus (designated EV89) selected from their isolates, Burke and co-workers (1959b) reported a serological relationship (by neutralisation tests) between their prototype EV89 and avian encephalomyelitis virus, and incorrectly concluded (see below) that EV89 could be the avian encephalomyelitis virus.

In 1961 Taylor and Calnek (1962) tested a number of cloacal swabs from domestic hens for the presence of cytopathic agents, using chick embryo kidney cell cultures. They recovered agents giving two different kinds of cytopathic effect (CPE): one was characterized by rounding and

increased refractility of the cells, and the other was characterized by the formation of syncytia. Serological classification by neutralisation tests of all the agents resulted in the establishment of a large number (15) of ill defined groups, in which all the agents giving the syncytial type of CPE were assigned to one group, and one of the other groups was found to be antigenically indistinguishable from CELO virus.

In 1962 Sarma and Pomeroy (1962) reported isolations of viruses from caecal tonsils of chickens. They referred to their isolates as "enteroviruses", and two of them (A66 and A82) were studied in some detail, but the conclusion drawn was simply that the two isolates were "strains of the same virus, and differed from other viruses of poultry animals and man".

The first suggestion that any cytopathic agent which was isolated from domestic hens might be grouped with the adenoviruses, was made by Burmester et al. (1960), who investigated some of the properties of a virus originally thought to be a causative agent of avian lymphomatosis. The virus was found to resemble the human adenoviruses in morphology, chemical characteristics, site of replication in the cell, and heat stability; thus it was suggested that this cytopathogenic agent be referred to as Gallus adeno-like (GAL) virus.

In 1962, after a number of strains of cytopathic agents given the designation "GAL" virus were recovered, Sharpless (1962) investigated the serological relationships of these GAL virus isolates, and on the basis of cross-neutralisation tests, divided them into four serological groups which were called GAL 1 to 4. However this serological grouping was later (see below) shown to be incorrect.

Davies and Englert (1961) studied the morphology of GAL virus by negative contrast electron microscopy. They reported that the virus particles had an average diameter of 86 nm, and each particle had a total of 162 capsomeres, whereas human adenoviruses were slightly smaller (60-80 nm) and had 252 capsomeres per particle. Nevertheless they suggested that their results supported the conclusion that GAL virus should be grouped with the adenoviruses.

At the same time, MacPherson et al. (1961) also studied the fine structure of GAL virus by negative contrast electron microscopy, and

their interpretation was that like mammalian adenoviruses, GAL virus had 252 capsomeres, but they suggested that these capsomeres resembled the capsomeres of herpesvirus and polyoma virus rather than those of mammalian adenoviruses, because the capsomeres of GAL virus were "hollow and elongated".

In spite of the apparent differences between GAL virus and the mammalian adenoviruses, both groups of workers (Davies and Englert, 1961; MacPherson et al., 1960) inclined to the view that GAL virus should be assigned to the adenovirus group. This conclusion however, was not generally accepted at that time, not only because of the confusing morphological findings, but also because it was generally assumed that all adenoviruses had a common antigen which could be detected by gel precipitation and complement fixation tests (Ginsberg, 1962). No common antigen however was detected between GAL virus and the mammalian adenoviruses.

The first detailed study of CELO virus was carried out by Petek et al. (1963), who found CELO virus shared many biological and physico-chemical properties with the mammalian adenoviruses. They also concluded from electron microscope observations of thin sections that CELO virus most likely did not belong to the RNA-containing enteric group of viruses although it was well known that CELO virus was frequently found in the alimentary tract of birds.

Further isolations of viruses from the alimentary tract of domestic hens were reported by Khanna (1964) in Hungary. All isolates were recovered from cloacal swabs, and the agents were called avian enteroviruses - a term applied earlier by Sarma and Pomeroy (1962), but which was probably only intended to indicate that these agents were isolated from the alimentary tract of domestic hens.

Khanna divided his isolates into five serological types, and the type most frequently isolated was serologically identical to avian enteric cytopathogenic virus strain EV89 (Burke et al., 1959b) mentioned previously (see above).

Meanwhile studies of the morphology of CELO virus using negative contrast electron microscopy showed this agent to have 252 capsomeres and a diameter of 73 nm (Dutta and Pomeroy, 1963). This finding added

further weight to the conclusion that CELO, like GAL virus, should be grouped with the mammalian adenoviruses. It should be noted that up to this stage apparently, nobody had determined if there was a serological relationship between CELO virus and any of the strains of GAL virus, even though it had been suggested that both could be classified as adenoviruses.

Japanese workers in 1963 (Kawamura and Tsubahara, 1963) found a partial antigenic relationship between CELO and a strain of GAL (not indicated) by agar gel precipitation tests, but this work, like much of the other work done in Japan on avian viruses, was to go apparently unnoticed for a number of years.

Probably the main reason why investigators, apart from the Japanese, were reluctant to group these avian adeno-like viruses with the mammalian adenoviruses was the absence of a common antigen. Apart from this, properties such as size, morphology, heat stability in the presence of various cations, chloroform sensitivity and site of virus replication, all favoured the conclusion that GAL, CELO and related agents should be regarded as avian adenoviruses.

In 1968 in the United States Burke et al. (1968) reported a comprehensive study of the properties of a number of agents isolated from domestic hens but not yet assigned to a virus group. These agents included CELO, the 4 strains of GAL virus, "enteric virus" EV89, and a latent virus recovered from spontaneously degenerating chicken kidney cell cultures. All displayed properties typical of adenoviruses (Table 2) and following the publication of this data it has been increasingly accepted that avian adenoviruses constitute a legitimate virus group and the requirement of Ginsberg (1962) that adenoviruses, by definition, have a common antigen with standard mammalian strains has been dropped (Wildy 1971).

It should be pointed out, however, that avian adenoviruses have an antigen in common (Burke et al., 1968) although this antigen is serologically unrelated to that found in the mammalian agent.

Clarification of avian adenovirus serology.

Initially there was some confusion over the serological relationship of the adenoviruses of domestic hens in the United States. Some of the results of early serological classification have been discussed

Table 2: Comparison of properties of avian adeno-like viruses and mammalian adenoviruses.

	Mammalian	Avian
Nucleicacid type	DNA	DNA
Ether/chloroform stability	stable	stable
Stability at pH 3	stable	stable
Size	60-80 nm	70-85 nm
Morphology	icosahedral	icosahedral [a]
Number of capsomeres	252	252 [a]
Stabilized by monovalent but not divalent cations at $50^{o}C$	yes	yes
Intranuclear inclusion bodies in infected cells	present	present

a. for CELO and a strain of GAL only - none of the other avian strains examined.

briefly above, but it was not until 1968 that Burke et al. (1968) showed that most of the strains of avian adenovirus that had been isolated and studied in the United States were serologically related to either CELO or GAL 1, by neutralisation tests. It also became apparent from this work that the strains most commonly isolated in the United States, as well as in Hungary (Khanna, 1964), belonged to the "CELO serotype".

However, as early as 1964 in Japan 111 strains of avian adenoviruses had been recovered from domestic hens (Kawamura et al., 1964) and neutralisation tests divided these strains into 8 distinct serological types. Again, of these 8 serotypes, the most frequently isolated (38.8%) was the CELO serotype (designated "Ote" by the Japanese). At 9.9%, GAL 1 (SR48) was the fourth most frequently isolated of the 8 serotypes. Complement fixation and agar gel precipitation tests revealed an antigen common to all the 8 serotypes.

In a comprehensive investigation of the serological relationships of avian adenoviruses recovered from domestic hens, McFerran et al. (1972) in Northern Ireland, confirmed the serological results of the Japanese workers. Furthermore, McFerran et al. (1972) recovered 167 avian adenoviruses from domestic hens in Northern Ireland and showed that they could be divided into 7 serological types. Six of these serotypes were closely related to the Japanese strains, but the remaining strain was readily distinguishable in cross-neutralisation tests from the Japanese prototypes. They therefore concluded that at least 9 serotypes of avian adenovirus had been established, viz. GAL 1, CELO and 7 others.

Unfortunately, no rational numbering system has yet been accepted for avian adenovirus serotypes; however Table 3 shows the present designations and synonyms for the nine established serotypes of avian adenoviruses.

Table 3: Avian adenovirus serotypes: designation of prototype strains in the United States, Japan and Northern Ireland.

Designation in:		
United States [a]	Japan [b]	Northern Ireland [c]
CELO	Ote	112
GAL1	SR-48	685
	KR-5	506
	TR-22	340
	YR-36	
	SR-49	75
	TR-59	58
	CR-119	
		764

a. Burke *et al*. (1968)

b. Kawamura *et al*. (1964)

c. McFerran *et al*. (1972)

Reoviruses of domestic hens

Taylor and Calnek (1962) reported the isolation of five cytopathic agents from cloacal swabs taken from domestic hens. These agents gave a syncytial type of CPE in chicken embryo kidney (CEK) cell cultures. All 5 isolates were assigned to one serological type by neutralisation tests, and antiserum to the "prototype" strain did not neutralise infectious bronchitis virus or ILT virus, nor did it inhibit haemagglutination by Newcastle disease virus. Although no further tests were carried out, it now seems likely, on the basis of CPE, that these "enteric cytopathic" agents were reoviruses.

Kawamura et al. (1965) reported the isolation of 77 agents, 75 of which were from the alimentary tract and the remaining two were from the trachea of domestic hens. All 77 agents gave a syncytial type of CPE in CK cell cultures and they were assigned to 5 distinct serological types by cross-neutralisation tests.

Each prototype strain was examined with respect to a number of biological properties. These properties are listed in Table 4 and are compared with the corresponding properties of the mammalian reoviruses as described by Wilner (1964).

On the basis of the overall similarity of the avian agents to the mammalian reoviruses Kawamura et al. (1965) proposed that these agents should be classified as avian reoviruses.

In 1965, in the United States, Dutta and Pomeroy (1967) isolated an agent from chicks with severe cloacal pasting. Negative contrast electron microscopy revealed the double layered nature of the virus particles, consisting of an inner core and an outer shell, like mammalian reoviruses. The average diameter of virus particles was reported to be 60 nm which is significantly less than the generally accepted figure (70-80 nm) and the number of capsomeres has not been determined. However, because of morphological similarities of this avian virus to mammalian reoviruses and some other common properties such as ether resistance and thermostability, it was suggested (Dutta and Pomeroy 1965) that their agent should be considered as an avian reovirus.

An investigation of the nature of the "Crawley agent", originally isolated by Fahey and Crawley (1954) from the respiratory tract of chickens with chronic respiratory disease, was undertaken many years later by Petek et al. (1967). The agent was found to be stable to a pH of 3.0; its growth was not inhibited by IDU; and it caused the formation of cytoplasmic eosinophilic inclusions in infected CK cells.

Table 4: Comparison of some properties of avian reoviruses and mammalian reoviruses.

	Mammalian reoviruses [a]	Avian reoviruses [b]
Nucleic acid type, determined by sensitivity to IDU	RNA	RNA
Ether/chloroform stability	stable	stable
Stability at pH 3.0	stable	stable
Overall particle diameter	70-77 nm	70-82 nm
Morphology	icosahedral	icosahedral
Presence of inner core and outer shell	present	present
Number of capsomeres	92	92 [c]
Cytoplasmic inclusions in infected cells	present	present

a. Wilner (1964)

b. Kawamura *et al*. (1965)

c. not unequivocally established for either mammalian or avian strains at that time

Electron microscopic examination of thin sections of cells infected with the "Crawley" agent revealed particles with a structure similar to that of human reoviruses. Differences between the "Crawley" agent and mammalian reoviruses in the type of CPE produced and in haemagglutinating properties were noted, but since the more fundamental properties of the "Crawley" virus were shared with mammalian reoviruses, Petek et al. (1967) suggested that the "Crawley" virus be grouped with the reoviruses. In retrospect, therefore, it may be concluded that Fahey and Crawley (1954) were the first to isolate avian reoviruses.

In 1969 three agents were recovered (Deshmukh and Pomeroy, 1969a) from 7 to 10 day old chicks. These chicks, like those from which Dutta and Pomeroy (1967) isolated a reovirus, also showed cloacal pasting. Further investigation of the properties of the three agents was undertaken (Deshmukh and Pomeroy, 1969b; Déshmukh, Sayed and Pomeroy, 1969), and these workers reported that the three viruses displayed the following properties:

(a) They contained RNA, as determined by IDU and BDU sensitivity, and RNase and DNase treatment. [a]

(b) They did not exhibit diminished heat stability in the presence of Mg^{++} (unlike mammalian adenoviruses).

(c) The particle diameter determined by ultrafiltration was 50 to 100 nm.

(d) They were stable in the presence of chloroform or ether.

(e) They were related to human reoviruses 1, 2 and 3 as shown by haemagglutination inhibition, complement fixation, serum-neutralisation and agar gel precipitin tests. [b]

a. Note: the authors claimed that the infectivity of virus preparations was diminished by exposure to RNase, but was unaffected by DNase. They apparently assumed that the virus could be penetrated by these enzymes which consequently could digest their nucleic acid.

b. Note: these results have not been confirmed, and are not generally accepted.

On the basis of the morphology and physico-chemical properties of these three agents, it was suggested that they be classified as reoviruses, and, in spite of the claimed serological relationships with the mammalian reoviruses, they were regarded as "avian" reoviruses.

In 1971 (McFerran et al., 1971) reported the isolation of reoviruses from domestic hens in Northern Ireland. These agents were identified as reoviruses by negative contrast electron-microscopy, and by this stage the structure of reoviruses was sufficiently well established and distinctive to allow identification of the agents by morphological criteria.

As can be seen from the above synopsis of the literature, reoviruses of domestic hens have not been investigated in detail. However, it is clear that they have frequently been recovered from domestic hens, usually from the alimentary tract. The most comprehensive study was made by Japanese investigators (Kawamura et al., 1965), and as a result of their work it is now accepted that there are 5 distinct serological types of avian reovirus. Unfortunately these serotypes have not yet been compared serologically with most of the reoviruses isolated in other countries.

MATERIALS AND METHODS

1. Chick Kidney (CK) Cell Cultures

Kidneys were removed aseptically from three 0 to 2 day old chicks and chopped finely with scissors into Earles based washing solution (EWS, see Appendix). The chopped kidneys were washed twice in EWS, shaken vigorously to facilitate fragmentation and dispersal of the tissue, and transferred into 20 ml of 0.1% trypsin solution (see Appendix). The suspended tissue fragments were then further dispersed by stirring at 37°C using a magnetic stirrer and external magnet. Dispersal of the cells was completed after 30 minutes.

The cells were then centrifuged at 80g for approximately 5 minutes in graduated conical glass centrifuge tubes and resuspended in 10 ml of growth medium (HTC, see Appendix). The resuspended cells were centrifuged at 80 g for exactly 5 minutes, the supernatant fluid was removed, and the packed cell volume (PCV) was noted. The cells were then suspended in 500 times their volume of fresh HTC medium saturated with CO_2.

The cell concentration was also estimated using a haemocytometer, although this procedure was only used as an additional check on cell concentration.

The cell suspension was dispensed in aliquots using a Cornwall pipette, which was sterilized by autoclaving at 5 p.s.i. for 5 minutes. The volume of cell suspension added to each type of culture container was as follows:

tubes (16 x 125 mm screw cap culture tubes)	0.7 ml
glass petri dishes (45 mm diameter)	5 ml
bottles (160 ml screw cap milk dilution bottles)	15 ml

Tubes were placed in a roller drum, which was placed on a roller apparatus, left undisturbed at 37°C for 24-48 hours, and then rolled. Petri dish cultures were incubated in an atmosphere of 5% CO_2 in air. Bottles were incubated at 37°C.

Monolayer cultures formed about two days after seeding, and at this stage HTC medium was replaced by maintenance medium

saturated with CO_2 (EaM, see Appendix), using a Cornwall pipette, and the following volumes:

tubes	1.5 ml
petri dishes	5 ml
bottles	20 ml

Monolayer cultures persisted intact for 7 to 8 days without a further change of the medium.

2. Virus Isolation

(a) Post mortem specimens

The following tissues or specimens taken from healthy and diseased birds, were used for virus isolation:

	abbreviation used
lung	L
trachea	T
intestinal contents	IC
caecal contents	CC
caecal tonsil	CT
spleen	S
bursa of Fabricius	B
kidney	K
pancreas	P
liver	LV
heart	H
oviduct	O

All material was ground with acid-washed sand using a pestle and mortar, to which a small amount (1-2 ml) of standard diluent (SD, see Appendix) was added. The diluent contained 1000 units/ml of penicillin and 1000 ug/ml of streptomycin. SD was then added to the ground material to give an approximately 10% suspension.

The suspended tissue was centrifuged at 4000 g for 20 minutes at 4°C. The supernatant fluid was then either inoculated into cell cultures immediately, or stored at -40°C (the lowest available temperature) until culture tubes were available.

(b) Swabs from live birds

Cloacal and tracheal swabs (CS and TS respectively) were taken from healthy and diseased birds. The swab ends were broken off into bijou bottles containing SD medium as used for post mortem tissue. The bottles were shaken vigorously, the swabs removed, and the resulting suspensions treated as described for P.M. specimens.

(c) Inoculation of specimens into cell cultures

With all types of samples, 0.2 ml of the final suspension was inoculated into duplicate cell culture tubes, the tubes were rolled at 37°C, and examined for cytopathic effects (CPE) daily for seven days. If no CPE appeared a further "blind" passage of 0.5 ml was made. After incubating for a further seven days the original sample was regarded as negative if no CPE was observed. When a CPE was observed, the supernatant was passaged into several fresh culture tubes to prepare a small pool of virus. This pool was stored at -40°C until required for typing or further characterization.

3. <u>Virus identification by electron microscopy</u>

When a virus was isolated, it was given a further passage into a culture tube, which was incubated, with rolling, until most of the monolayer had degenerated. Any fragments of monolayer still adhering to the glass were scraped off, and the cell debris was sedimented by centrifuging at 500 g for 10 minutes. The supernatant fluid was then completely removed with a Pasteur pipette, leaving a pellet of cell debris, to which 1 to 3 drops of deionized water were added. Note: the volume added was adjusted according to the amount of sediment. The suspension was then frozen and thawed to lyse any intact cells. A drop of the lysed cell suspension was placed on a slide, 2 carbon coated EM grids were placed carbon side down on top of the drop, removed after a few minutes, and blotted by touching only the edge of the grids. The grids were then air dried, floated carbon side down on a drop of 5% sodium phosphotungstate (pH 6.5) for a few seconds, removed, blotted gently at the edge, and allowed to dry.

Grids were examined in a Philips EM 200 electron microscope; photographs of particles were taken and printed so as to give a final magnification of 200,000. Agents were identified on the basis of their size and structure.

4. Production and standardisation of reagents for serological typing of virus isolates

(a) Production of antisera to isolates

An isolate was "biologically purified" by passaging three times at limit dilution. The isolate was inoculated into several culture tubes which were incubated at 37°C until about 50% of the cells had degenerated. Medium plus cell debris was then harvested and pooled. Aliquots of the pool were dispensed into bijou bottles and stored at -40°C. This material was thawed when required, and inoculated into a rabbit according to the following immunization schedule:

days	material inoculated	site of inoculation
0	1 ml of 1:1 mixture of Freund's complete adjuvant and virus suspension	intramuscular (both hind legs)
7	1 ml virus suspension	intramuscular (all four legs)
14	1 ml virus suspension	intravenous (ear)

Rabbits were bled 10 days after the final inoculation, and serum was stored in 2 ml aliquots at -20°C.

(b) Virus standardisation for neutralisation tests

Virus was inoculated into several cell culture tubes and incubated at 37°C. When about 20% of the cells showed a CPE the supernatant was removed, pooled and stored at -40°C in 1 ml aliquots. For quantal titration of the virus pools serial tenfold dilutions of virus (usually from 10^{-1} to 10^{-7}) were made, and 0.5 ml of each dilution was inoculated into each of 4 culture tubes, rolled at 37°C, and the cultures were examined for the next 6 days for the appearance of CPE. Titres were calculated using the method of Kaerber (1931).

(c) Antiserum titration

Antiserum to be titrated was thawed, inactivated by heating at 56°C for 30 minutes, then diluted in SD in serial two-fold

steps from 1 in 10 to 1 in 40,960. The volume used was 0.6 ml. An equal volume of homologous virus was added to each tube in the dilution series. This virus was previously diluted to give an estimated titre of 200 $TCID_{50}$/0.5 ml: thus each tube in the dilution series then contained an estimated virus titre of 100 $TCID_{50}$/0.5 ml, and the antiserum had final dilutions ranging from 1 in 20 to 1 in 81,920.

The virus-serum mixtures were then incubated at 37°C for one hour, then two 0.5 ml aliquots of each dilution were inoculated into duplicate culture tubes, which were then rolled at 37°C and examined for the appearance of CPE for the next 6 days. Serum titres were calculated using the method of Kaerber (1931). As a control, the virus suspension diluted to give an estimated titre of 200 $TCID_{50}$/0.5 ml was further diluted by a factor of two with inactivated pre-immune rabbit serum (arbitrarily diluted 1:1000), and incubated for one hour at 37°C. Tenfold dilutions of the mixture were made, and inoculated into culture tubes as in 4(b) to check that the actual virus titre agreed with the calculated value.

Provided this control titration showed a titre of 30-300 $TCID_{50}$/0.5 ml, the corresponding antiserum titre was regarded as valid.

5. Serological typing of virus isolates and selection of prototype strains

Virus isolates were assigned to a group by negative contrast electron microscopy, and isolates within each group, i.e. adenoviruses and reoviruses, were further assigned to serological types using neutralisation tests described below.

Antiserum was initially prepared to one "purified" isolate from each group, titred as described above (4(c)) and diluted in SD to a concentration corresponding to 20 times the endpoint titre as estimated against the homologous virus. This dilution of antiserum was mixed with an equal volume of a 10^{-1} and a 10^{-3} dilution of each untyped isolate of the same virus group. The serum-virus mixtures and controls (in which the serum was replaced with SD) were incubated at 37°C for one hour, and two 0.5 ml aliquots from each of the two mixtures and two controls were inoculated into each of two CK culture tubes and rolled at 37°C. The cultures were examined daily and the results recorded when one or other of the control tubes showed a clearly defined CPE.

An isolate was regarded as being of the same serological type as the prototype strain when an unequivocal delay was detected in the time of appearance of CPE in the presence of the antiserum when compared with the control cultures.

Following this typing procedure, one untyped isolate was randomly selected, passaged three times at limit dilution, and used to immunize a rabbit. This new antiserum was titred using the homologous virus, and used to type the remaining isolates. This procedure was repeated until all the isolates were assigned to serological types. The strains of virus used to produce antisera for typing were regarded as prototype strains.

6. Cross-neutralisation tests with prototype strains

After the selection of prototype strains, each antiserum was titrated against all of the other virus prototypes as described below:

1. Serial two-fold dilutions of each antiserum were made, ranging from 1:10 to 1:40,960
2. All sera were inactivated by heating at 56°C for 30 minutes.
3. To each tube in one of the dilution series an equal quantity of the appropriate virus was added. This virus was previously diluted to give a final calculated titre of 100 $TCID_{50}$/0.5 ml when mixed with the antiserum.
4. All virus-antiserum mixtures were incubated at 37°C for one hour, then duplicate CK culture tubes were inoculated with 0.5 ml of each of the dilutions and rolled at 37°C.
5. The tubes were examined daily, and any CPE was recorded. Endpoints were calculated provided the control titrations of the respective viruses contained 30-300 $TCID_{50}$/0.5 ml.

Flow diagram of procedures for typing viruses and cross-neutralisation tests

Virus isolation:
arbitrarily designated type A (etc)

↓

Virus "purification" by three passages at limit dilution

↓

Production of virus pools

↓

Pool 1 (of type A)
Contained cell debris and supernatant. Aliquots stored at -40°C for rabbit inoculation

↓

Antiserum production

Pool 2 (of type A)
Supernatant fluid only. Aliquots stored at -40°C

↓

Titrate virus pool

→ Standardize antiserum ←

↓

Test for ability to neutralise new isolates

↓

Isolate not neutralised: called type B, and new anti-serum to type B prepared.

↓

Cross-neutralisation tests, using antisera to types A, B etc.

Isolate neutralised: i.e. it is type A

7. Further characterization of virus isolates: Physico-Chemical tests

(a) Chloroform sensitivity

This was determined by the method of Feldman and Wang (1961). Cell-free virus suspension was mixed with 5% of its volume of chloroform and shaken at room temperature for 10 minutes, centrifuged at 100 g for 10 minutes to sediment the chloroform, and the supernatant was titrated as previously described.

A preparation of the same virus suspension without chloroform was also shaken and centrifuged, and titrated as a control.

(b) IDU sensitivity

Presumptive evidence for nucleic acid type was obtained by investigating the effect of 5-iodo 2 deoxyuridine (IDU) on virus replication, as described by Salzman (1960).

After the formation of monolayers in culture tubes, HTC medium was replaced with maintenance medium which contained no serum. This assured the depletion of the supply of free thymidine. After 24 hours, the maintenance medium was removed, and 1000 $TCID_{50}$ of virus was inoculated into each tube, and at the same time fresh maintenance medium containing 1% calf serum plus the following amounts of IDU and thymidine was added:

2 tubes	MM only (controls)
2 tubes	MM plus 10^{-4}M IDU (35 ug/ml)
2 tubes	MM plus 10^{-4}M IDU plus 2 x 10^{-4}M thymidine (50µg/ml)

The cultures were incubated with rolling at 37°C until the controls showed an unequivocal CPE. All cultures were then frozen and thawed and the extent of virus replication was determined by quantal titration.

(c) Effect of cations on heat stability

The effect of Na^{+}, Mg^{++} and Ca^{++} on the heat stability of the avian adenoviruses and reoviruses was determined as described by Wallis, Yang and Melnick (1962).

The following solutions were prepared in deionized distilled water and sterilized by autoclaving:

NaCl	4M
$MgCl_2$	2M
$CaCl_2$	2M

Equal volumes of virus and the appropriate solution, or sterile water in the case of the controls, were mixed in ampoules which were then sealed, and completely submerged for one hour at 50°C. The ampoules were then opened and the contents assayed by quantal titration.

For one virus preparation, the titrations of the following mixtures were made:

virus in distilled water (no heating): initial control (IC)
virus in distilled water (heated 50°C one hour): final control (FC)
virus in 2M Na^{+}
virus in 1M Mg^{++} — all heated at 50°C for one hour
virus in 1M Ca^{++}

8. Screening tests for neutralising antibodies

Sera to be examined for the presence of neutralising antibodies to each of the adenovirus serotypes isolated in this study were diluted 1:6 in SD and inactivated by heating at 56°C for 30 minutes. To 0.6 ml of each diluted serum an equal volume of virus suspension was added. The virus added was previously diluted in SD to give a final calculated titre of 100 $TCID_{50}$/0.5 ml.

The virus-serum mixtures were then incubated at 37°C for one hour, and two 0.5 ml aliquots of each mixture were inoculated into duplicate CK culture tubes, which were rolled at 37°C and examined daily for the appearance of CPE. At the same time, a control titration of the virus suspension was made, and the results of the serum neutralisation tests were regarded as satisfactory provided the controls indicated that the virus titre was within the range of 30-300 $TCID_{50}$/0.5 ml.

This procedure was repeated for each of the adenovirus isolates characterized in this study.

A number of positive sera were also titrated, as described in "Materials and Methods 4(c)" except that the final serum dilutions tested ranged from 1:20 to 1:640, instead of 1:20 to 1:81,920.

9. Staining CK monolayers

CK monolayers were grown on coverslips in petri dishes, which were incubated at 37°C in an atmosphere of 5% CO_2 in air. Cultures were inoculated with virus at the time of replacing growth medium (HTC) with maintenance medium (EaM). The monolayers were examined daily for the appearance of CPE; when a CPE was clearly visible the coverslips were processed by the following method:

(1) The maintenance medium was removed from the petri dish, and the cells were washed with PBS and fixed with either methanol or 10% formol saline for 5 minutes.

(2) The fixative was removed and the cultures were washed again with PBS. The coverslips were placed in Khan tubes and stained for 24 hours with Giemsa's stain diluted 1:75 in tap water.

(3) The stained preparations were rinsed in tap water and dehydrated by washing twice with acetone; then a 1:1 mixture of acetone and xylene, followed by two washes in xylene. The coverslips were mounted in Depex.

RESULTS

1. Growth of chick kidney (CK) cell cultures

One factor essential for the production of satisfactory cell monolayers in tubes which were not individually "gassed", was that the growth medium (HTC) and maintenance medium (EaM) should both be saturated with CO_2 before use (Clarke, 1973). When HTC medium not fully saturated with CO_2 was used to make up the seeding suspension, monolayers did not develop; and when EaM medium for maintenance was not saturated with CO_2 before use the cells rounded up, and the monolayers degenerated within 1-3 days.

However, the major problem to be overcome in the production of good cell monolayers, which could be maintained for a long enough period of time to propagate viruses, was to produce an initial seeding suspension of the right concentration. Since the methods described in the literature (see Discussion) for the preparation of CK cell cultures vary in many details, it was necessary to standardize on a convenient and reproducible technique suitable for the available equipment.

In the first attempt, CK monolayers were produced in tubes seeded at the following dilutions of packed cell volume (PCV) in growth medium:

1:50
1:100
1:200
1:300

In all cases, complete monolayers formed within two days, but they were uneven, due to the high density of the "cell clumps" (see Plates 1 to 3). Furthermore, cells continued to divide after the growth medium had been replaced with maintenance medium, with the result that monolayers quickly became multi-layered and, as a result, became detached from the glass surface.

After initiating a number of cultures, using dilutions in the range of 1:400 to 1:700 it was found that a 1:500 dilution of packed cells in growth medium gave the most satisfactory results, in terms of the proportion of tubes (about 95%) with satisfactory monolayers. At a dilution of 1:600 monolayers took longer to develop, and in 10-15% of the tubes monolayers did not form at all.

Throughout the duration of the experimental work, the concentration of the seeding suspensions was checked by counting cell clumps in a haemocytometer. Provided the time of trypsinization and the time and speed of centrifugation were not varied, a 1:500 dilution of packed cells in growth medium gave counts of 13,000-15,000 clumps per ml. It was concluded that seeding cell suspensions of the right concentration could consistently and more conveniently be prepared without the use of a haemocytometer.

Plates 1, 2 and 3 show the appearance of the cell clumps or "tubules", as well as single (kidney) cells and erythrocytes in the haemocytometer. Although outgrowths did occur from the single cells, development of monolayers appeared to be due mainly to the growth of cells from the periphery of cell clumps.

2. Virus isolation and identification by electron microscopy

Specimens to be screened for the presence of cytopathic agents were inoculated into culture tubes which were rolled at 37°C and examined for the next 6 days for cytopathic effects (CPE). Where no CPE were observed, supernatant fluid was given a further "blind" passage, and if no CPE were observed after a further 6 days, specimens were considered negative and discarded.

From a total of 150 post mortem samples taken from the Department of Veterinary Science at Massey University, and 44 swabs taken from live birds in commercial flocks in the Manawatu district, 27 cytopathic agents were recovered. Of these, 65% gave a CPE after initial inoculation of the sample into culture tubes, and the remaining 35% gave a CPE which was first detected following the "blind" passage. Three distinct types of CPE were observed:

(a) cells rounded up and became slightly more refractory than uninfected cells (Plate 4)

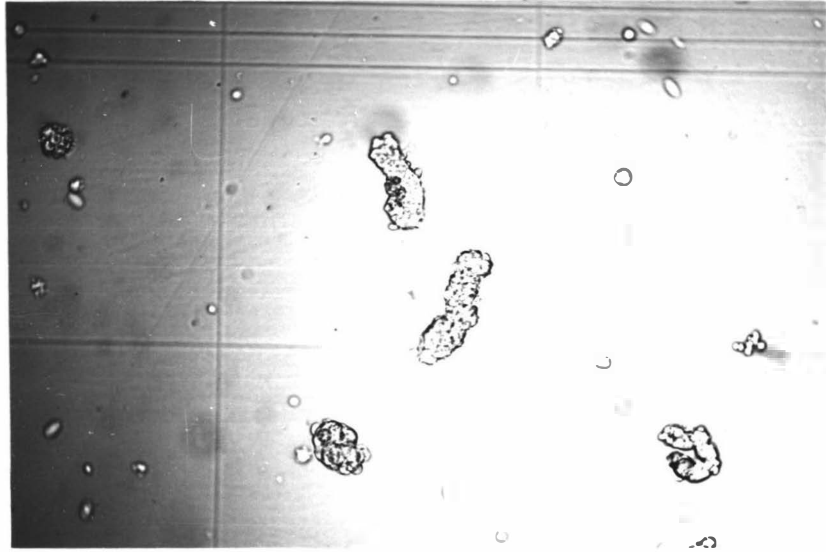
(b) cells became vacuolated, then after a further 24-48 hours they started to round up as in (a) (Plate 5).

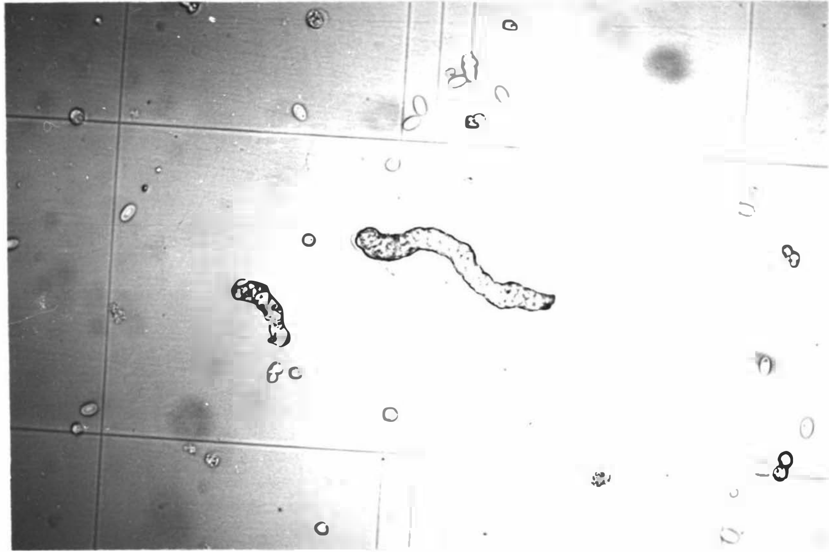
(c) cells became slightly vacuolated and developed a granular appearance. They then fused to form large irregular syncytia (Plate 6), which after a further 24-48 hours degenerated to leave cobweb-like processes attached to the glass surface.

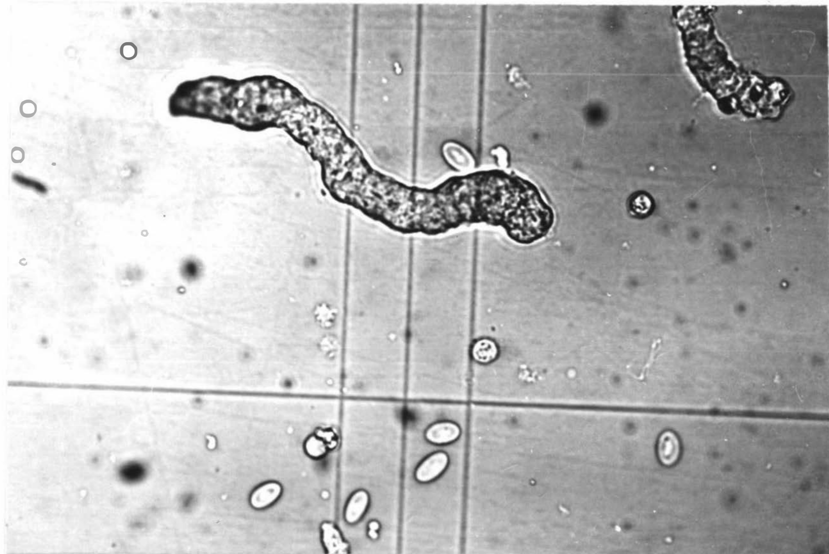
Plates 1, 2 and 3.

Kidney tissue, from day old chicks, dispersed by trypsin. The dispersed tissue was centrifuged and the pellet resuspended in 500 times its volume of growth medium. The resuspension is viewed in a haemocytometer and consists mainly of fragments of kidney tubules; erythrocytes and a few individual kidney cells. The kidney cells may be distinguished from erythrocytes because they appear granular and spherical.

This suspension corresponds to the concentration of tissue used to seed tubes, petri dishes or bottles for the production of monolayer cultures.







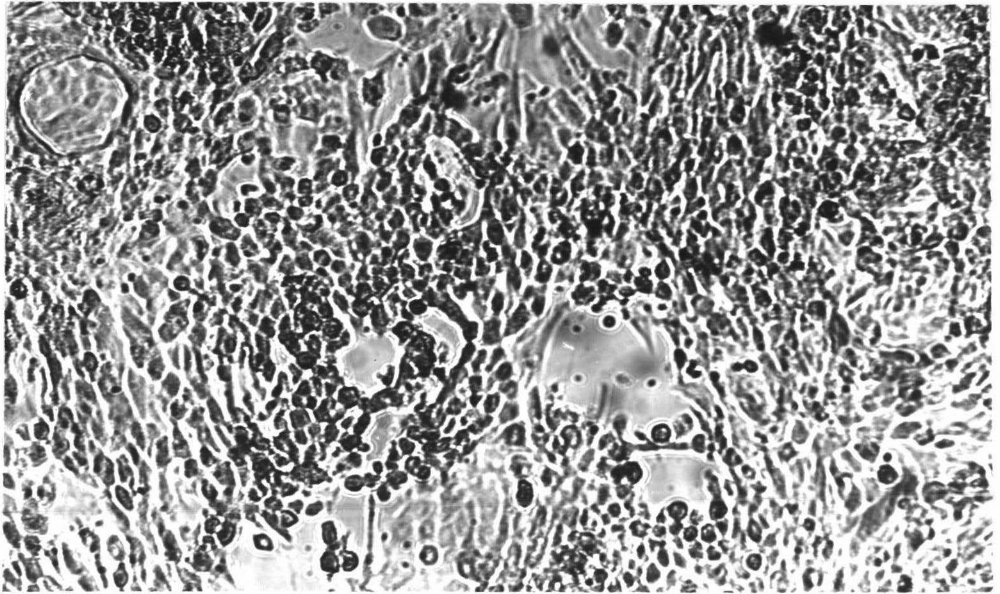


Plate 4. Chick kidney cell culture showing foci of cell rounding following inoculation with avian adenovirus type A. CPE caused by types B and C are indistinguishable from that illustrated. Unstained preparation x 100.

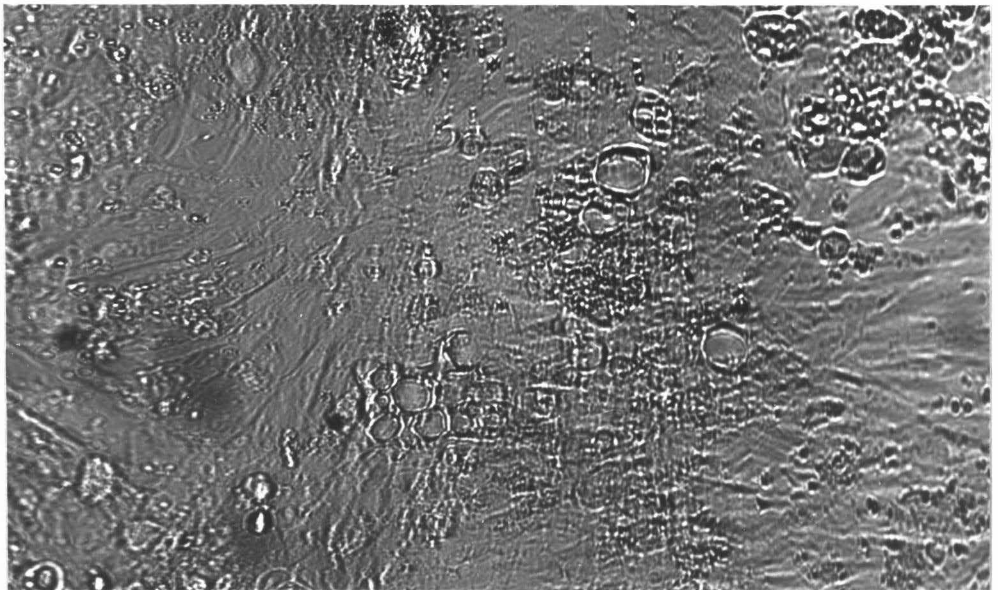


Plate 5. Chick kidney cell culture showing foci of vacuolated cells, following inoculation with avian adenovirus type D. Unstained preparation x 100.

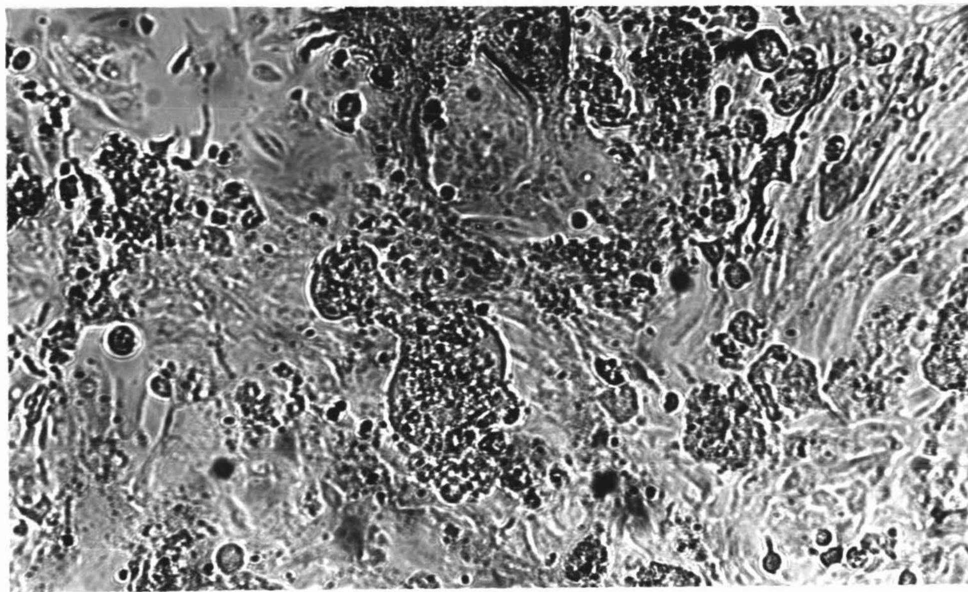


Plate 6. Chick kidney cell culture showing the formation of a syncytium (dumbell shaped object in the centre of the micrograph) following inoculation with avian reovirus. Smaller degenerating cells are also seen scattered throughout the monolayer. Unstained preparation x 100.

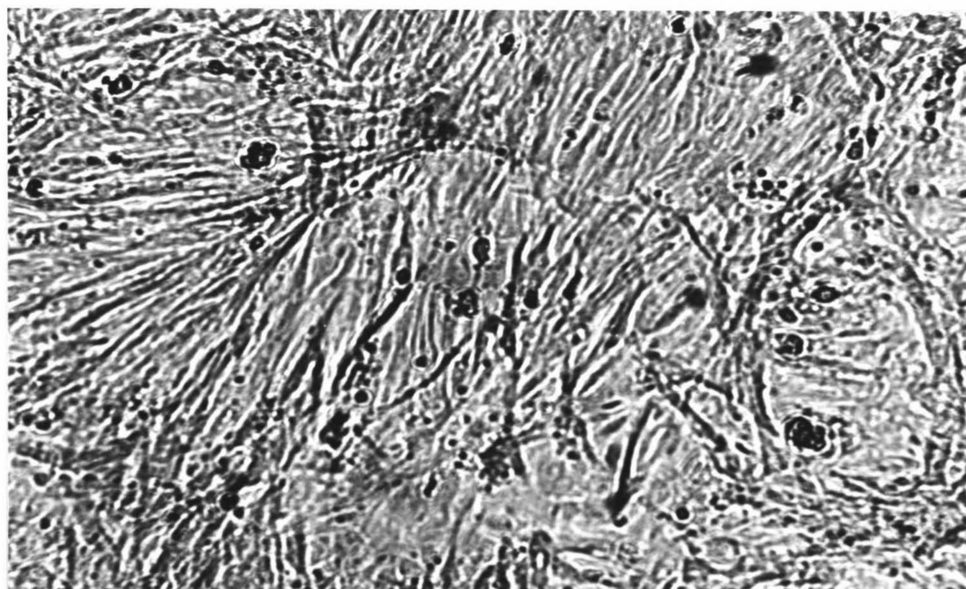


Plate 6a. Uninoculated chick kidney cell culture. Compare with plates 4, 5 and 6. Unstained preparation x 100.

All agents were examined by negative-contrast electron microscopy. Plate 7 is the best electron micrograph obtained of particles with adenovirus-like morphology and size. The particle represented in Plate 7 is clearly hexagonal in outline, has a diameter of about 80 nm, and triangular faces may be discerned. Such agents are indistinguishable in morphology from the mammalian adenoviruses as described by Horne (1962).

Plate 8 is more typical of what was usually seen in the electron microscope using the diagnostic method described for negative staining. Note that most of the particles have been penetrated by the stain and some particles are partly broken; however, such structures are still distinguishable from agents other than those of the adenovirus group. Agents having this adenovirus morphology gave rise to a CPE in CK cell cultures of the types described in (a) or (b) above.

The second, and only other, type of virus particle isolated in cell cultures during this study is illustrated in Plate 9. This particle has an inner core and an outer shell structure. The average diameter of the core was found to be 46 nm, and the average overall diameter of the particles was 72 nm.

Reported measurements of human reoviruses (types 1, 2 and 3) vary from 60 to 77 nm for the overall diameter and 32 to 46 nm for the core diameter (Loh et al. 1965), so the agents isolated in this study having this morphology and size were considered to be members of the avian reovirus group.

All agents with reovirus morphology gave rise to the syncytial type of CPE (described in (c) above, and see Plate 6) in CK cell cultures.

Table 5 lists the viruses isolated in this study, and includes the source of each isolate, the disease (if any) of the bird from which the material was taken, and the group to which the virus belongs, as determined by negative contrast electron microscopy.

Taking into account those agents, samples 19, 23 and 24, which ultimately (see results of IDU sensitivity tests) were found to be mixtures, a total of 25 adenoviruses and 5 reoviruses were recovered from the 194 specimens screened. However, in many cases more than one specimen was taken from a bird.

194 specimens (or parts of specimens - see Table 5) were taken from a total of 95 diseased and healthy birds. Twenty-eight percent of these birds were found to be infected by either an adenovirus, a reovirus, or by both types of virus.

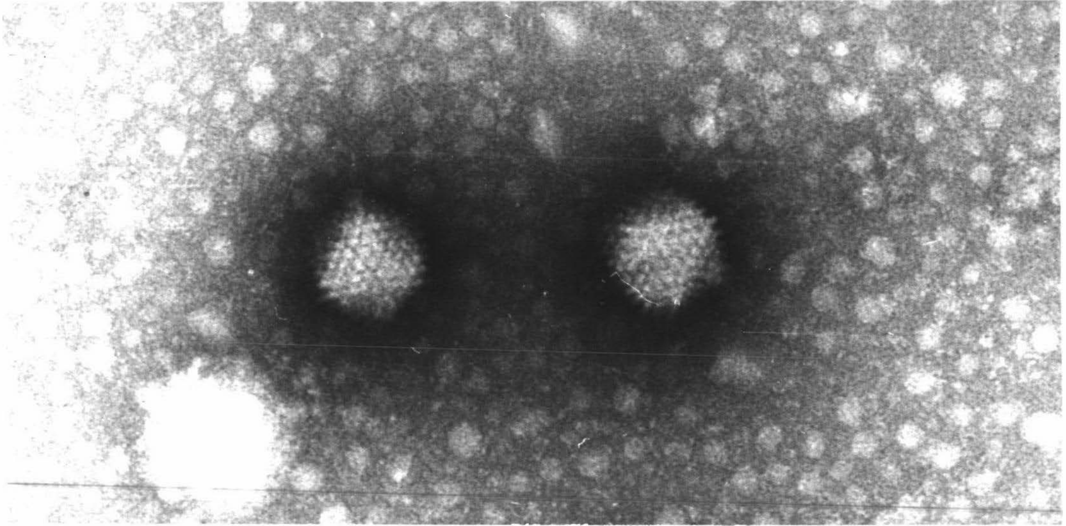


Plate 7. Avian adenovirus type A negatively stained with sodium phosphotungstate. The two particles shown are not penetrated by stain, and are apparently stained on one surface only. Some of the triangular faces can be discerned. x 200,000.

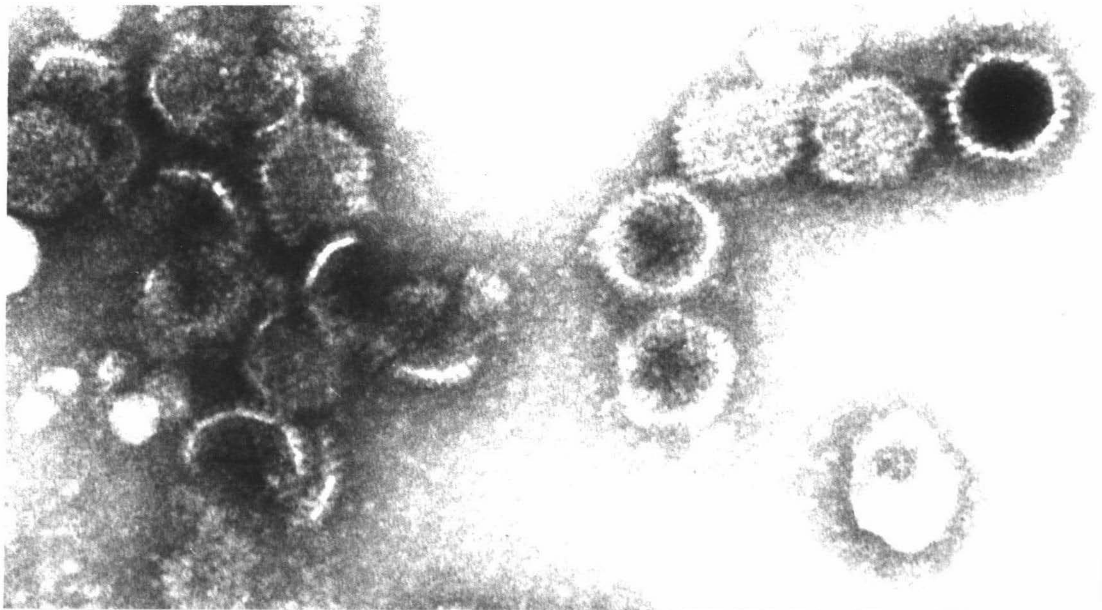


Plate 8. Avian adenovirus type B negatively stained with sodium phosphotungstate. This micrograph is typical of the types of structures seen when impure preparations of avian adenoviruses are examined routinely for identification purposes. Many particles are partly broken, and partly penetrated by stain. One particle (top right) is fully penetrated by stain, and individual capsomeres are seen in profile. Note the absence of an inner shell. x 200,000

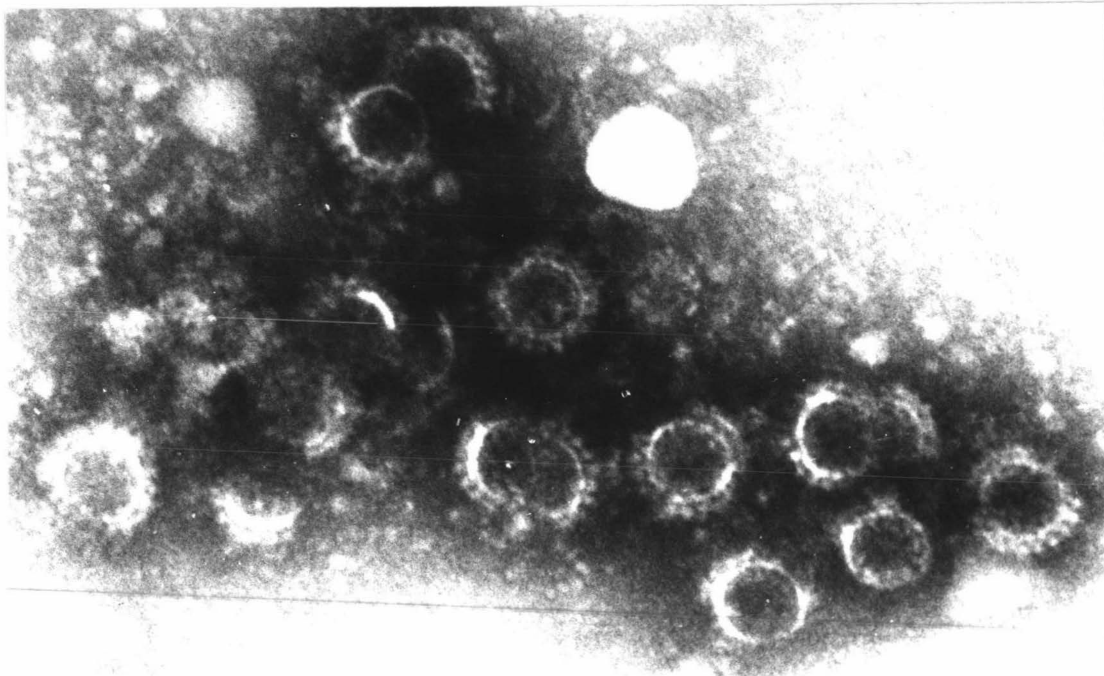


Plate 9. Avian reovirus negatively stained with sodium phosphotungstate. All particles are penetrated by stain. The particles are spherical in outline, and have an inner layer of capsomeres which appears as a narrow unstained ring. x 200,000.

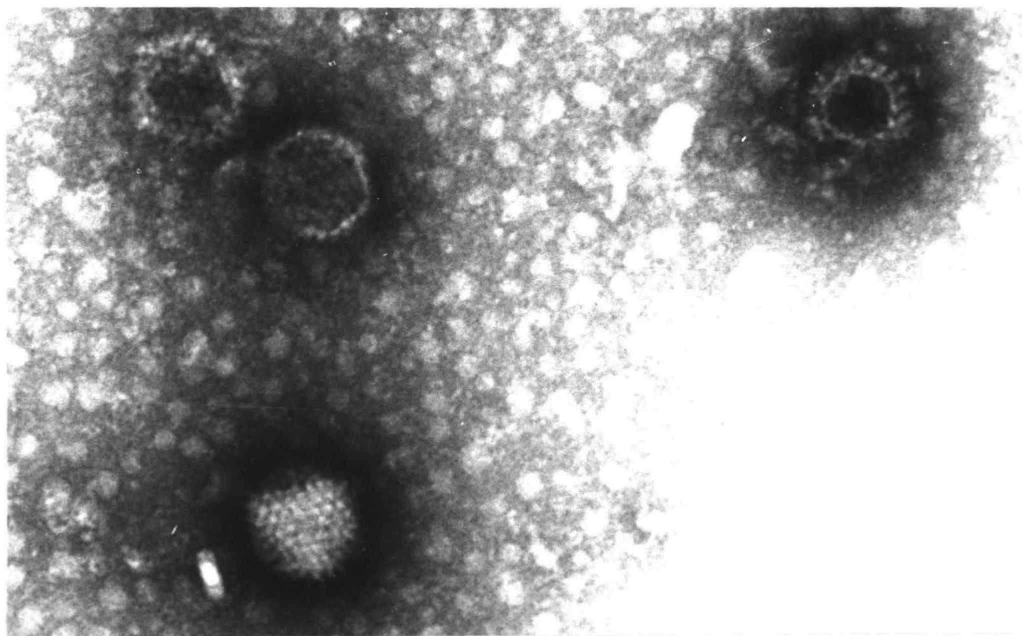


Plate 10. Mixture of adenovirus and reovirus negatively stained with sodium phosphotungstate. One penetrated reovirus particle is present (top right), and it can be distinguished from the adenovirus particles (left, top and bottom) because of the presence of an inner shell. x 200,000.

Table 5

This table lists the virus group to which each isolate was assigned; the specimens from which it was recovered and the pathology, if any, of the birds from which the specimens were taken. The association, if any, between the observed lesions and the virus isolated remains to be established. Note that isolates were occasionally recovered from healthy birds and some were recovered from birds with lesions caused by non-viral agents.

a. See Materials and Methods, sections 2(a) and (b) for abbreviations. Where more than one type of material is listed under "source", this means that all types listed were ground and pooled before testing.

b. Sample 24 was found by electron microscopy to contain a mixture of particles with adenovirus and reovirus morphology (Plate 10). Only particles with reovirus morphology were initially detected in negatively stained preparations from samples 19 and 23; however these samples were later found to contain a mixture of adenoviruses and reoviruses (see Results, IDU sensitivity: reoviruses).

Table 5: Viruses recovered from domestic hens using CK cell cultures.

Isolate number	Source [a]	Pathology	Virus group
1	CT, CC, IC	Acute form of Mareks disease, and heterakis irarus	adenovirus
2, 3	CS	Laying hen, slight respiratory signs	"
4	CT, CC, IC	Dead bird. Congestive heart failure	reovirus
5	"	Endoparasite infections (histomoniasis, capillaria)	adenovirus
6	"	Staphylococcosis (arthritis, hepatitis, septicaemia)	"
7 to 11	CS	None	"
12	CT, CC, IC, T	Staphylococcosis (arthritis, hepatitis, septicaemia)	"
13	B	" " "	"
14 to 16	CT, CC, IC, T	Infectious bronchitis (respiratory and kidney)	"
17	"	Necrotic enteritis	"
18	CT, CC, IC	None	"
19	"	"Pullet disease"	reovirus and adenovirus
20	"	" "	reovirus
21	"	Necrotic enteritis	adenovirus
22	CS	None	"
23	"	"	reovirus and adenovirus
24 [b]	"	"	" "
25	K	"	adenovirus
26	CT, CC, IC	"	"
27	CC	"Sudden drop in egg production"	"

3. Serological classification of viruses recovered from New Zealand domestic hens

(a) Adenovirus typing

Preliminary serotyping divided 20 of the 25 adenoviruses isolated into 4 different serological types. The remaining 5 adenovirus isolates do not belong to serotypes A. B, C or D, and have not yet been studied in cross-neutralisation tests.

Table 6 shows the distribution of the 20 isolates within the 4 serological types, and also gives the relative frequency of isolation of each adenovirus serotype.

Having divided the adenoviruses into 4 groups by the preliminary typing, cross-neutralisation tests were undertaken to confirm that the serological "prototype" strains are distinct.

The results of these cross-neutralisation tests, using the 4 adenovirus prototypes (A, B, C and D) and their antisera, are shown in Table 7. The lowest antiserum dilution used in the cross-neutralisation tests was 1:40. Concentrations greater than this were toxic for the cell cultures because of the presence of anti-chick cell antibodies (see Materials and Methods).

No cross-neutralisation was observed between the 4 types. Hence these tests confirmed the result of preliminary typing which indicates that these 4 isolates were serologically distinguishable.

All of the adenovirus isolates belonging to types A, B and C gave a CPE of the rounding up type (Plate 4) described above, while type D adenovirus (of which isolate 17 was the only member) gave CPE of the vacuolating type (Plate 5). One of the untyped adenovirus strains (isolate 9) also gave rise to the vacuolating type of CPE. Typing tests showed that this strain did not belong to the A, B, C or D serotypes but it has not yet been investigated by cross-neutralisation tests.

(b) Typing of reovirus isolates

Isolate 23 was arbitrarily selected as the prototype reovirus strain, and used to produce an antiserum. This antiserum had a relatively low neutralisation titre of 160, possibly because the concentration of antigen used in the rabbit inoculum was low: thus when titrated in CK cells, reoviruses grew to a titre of only $10^{3.5}$ - 10^4 $TCID_{50}/0.5$ ml, whereas the adenoviruses invariably grew to titres of 10^6 - 10^7 $TCID_{50}/0.5$ ml, and in their case, sera with high antibody titres were consistently produced (see Table 7).

Table 6: Results of typing adenovirus isolates.

The relative frequency of recovery of each type and the provisional designation of each serotype is also shown.

Isolate No.[a]	Provisional designation of serotype	Other isolates of the same serological type	Relative frequency of isolation of serotype
1	A	none	5%
5	B	3, 7, 8, 15, 16, 21	35%
6	C	12, 13, 14, 19, 22 to 27	55%
17	D	none	5%

a. isolate numbers as in Table 5.

An adenovirus isolate was picked at random, purified by passaging three times at limit dilution, and used to prepare an antiserum. This antiserum was tested for its ability to neutralise the remaining adenovirus isolates. Of those not neutralised, one was picked at random, and the above process repeated until 20 adenovirus isolates were assigned to four serological types.

Table 7: Cross-neutralisation tests between prototype strains of avian adenovirus.

	Antiserum titre			
Virus	Anti A	Anti B	Anti C	Anti D
A	5,120	- [a]	-	-
B	-	70,000	-	-
C	-	-	40,960	-
D	-	-	-	10,240

a. indicates serum titre of less than 40

Each virus prototype was added to a twofold dilution series of each antiserum, incubated for one hour at 37°C and then each dilution was inoculated into CK culture tubes. Inoculated tubes were incubated at 37°C and examined for CPE for 6 days. Serum titres were then calculated using the method of Kaerber (1931).

The antiserum to reovirus 23 (diluted 1:80) nevertheless neutralised the other four reovirus isolates; thus all 5 reovirus isolates were assigned to one serological group. Consequently no cross-neutralisation tests between reovirus isolates were performed.

4. Physico-Chemical tests

(a) Chloroform sensitivity

Avian adenoviruses and reoviruses were titrated before and after exposure to chloroform in standard conditions (see Materials and Methods). A poxvirus was included as a positive control. The results are shown in Table 8. Titres indicate that the infectivity of avian adenoviruses and reoviruses was unaffected by chloroform treatment, whereas the poxvirus (pigeon pox, obtained from TVL, Wellington) was sensitive to chloroform. Stability to chloroform is a known property of the avian adenoviruses (McFerran et al. 1972) and reoviruses (Deshmukh and Pomeroy, 1969b).

(b) IDU sensitivity: adenoviruses

Replication of all four prototype strains of adenovirus was compared in the presence and absence of 5-iodo 2-deoxyuridine (IDU). As a control, the ability of thymidine to reverse any inhibition caused by IDU was also tested.

The results are recorded in Table 9. In all cases the yield of infectious virus in the presence of IDU was less than 1% of the control value (in the absence of IDU), and the presence of thymidine completely reversed the inhibition.

This finding is consistent with the conclusion that the four prototype strains (A, B, C and D) are DNA viruses.

(c) IDU sensitivity: reoviruses

Isolate 23 (see Table 5), selected to be the first reovirus prototype, was "purified" by passaging three times at limit dilution, and used to prepare an antiserum for serological typing. While the antiserum was being prepared, the purified agent was tested for its sensitivity to IDU, and was found to be sensitive. This initial result was inconsistent with the conclusion (based on morphology) that it was a reovirus; consequently, the "purified" agent was re-examined by negative contrast electron microscopy, and found to have adenovirus morphology. Since it was known that

Table 8: Stability of avian adenoviruses and reoviruses in the presence of chloroform. An avian poxvirus was included as a positive control.

	Adenovirus (Type A)	Reovirus	Pigeon Poxvirus
Untreated	3.25 [a]	3.5	3.0
Chloroform treated	3.25	3.5	< 0.5

a. titres are expressed as $\log_{10}$ $TCID_{50}$/0.5 ml.

A mixture of cell culture supernatant fluid containing virus, plus 5% of its volume in chloroform, was shaken at room temperature for 10 minutes, centrifuged at 100 g for 10 minutes to sediment the chloroform, and the supernatant was subsequently titrated to assay the surviving virus.

Table 9: Replication of avian adenoviruses in the presence of IDU.

	Adenovirus serotype			
	A	B	C	D
Controls	4.75 [a]	6.5	5.0	4.0
10^{-4}M IDU	2.5	2.5	2.25	1.75
10^{-4}M IDU plus 2 x 10^{-4} thymidine	5.0	6.5	5.0	4.0

a. titres expressed as $\log_{10}$ $TCID_{50}$/0.5 ml

Virus was allowed to replicate in CK culture tubes containing no IDU (controls), IDU or IDU plus thymidine, until the controls showed extensive CPE. All cultures were then frozen and thawed, and the virus titre was assayed by quantal titration.

one other specimen had originally contained a mixture of adenovirus and reovirus (number 24, from electron microscopy - see Plate 10), it seemed likely that the specimen from which the prototype reovirus was isolated and "purified" (i.e. isolate 23) also contained a mixture of two agents, and that during the course of passaging at limit dilution, the reovirus had been diluted out, because, as noted before, reoviruses do not replicate to as high a titre in CK cells as the adenoviruses. It was also found that during the course of purification of an agent from sample 23 the syncytial type of CPE initially observed (at the screening stage) gave way to the rounding up type of CPE, but it was initially assumed that this change in CPE could be due to a process of adaptation of the reovirus to growth in CK cells. However, in retrospect, it seems likely that this phenomenon was actually due to the loss of the reovirus from a mixture of adenovirus and reovirus in the original sample.

To recover the reoviruses free of contaminating adenovirus, first passage material (stored at $-40^{o}C$) from the five specimens known to contain reoviruses was further passaged up to five times in the presence of $10^{-4}M$ IDU. The remaining transmissible agents were observed by negative contrast electron microscopy. In all five "purified" preparations, only particles with typical reovirus morphology were then detected.

The prototype (23) was then retested for IDU sensitivity, using the same method as for the avian adenoviruses. The results are shown in Table 10. The replication of the virus was not inhibited in the presence of IDU. This result indicates that this prototype strain is an RNA virus, which is consistent with the conclusion (based on morphology) that, following purification, it is a reovirus.

(d) Heat stability of avian adenoviruses and reoviruses in the presence of various cations

The stability of two adenovirus prototypes (B and C) and the reovirus prototype to heat ($50^{o}C$ for one hour) in the presence of Na^{+}, Ca^{++} and Mg^{++} was tested by the method described. The results of these tests are shown in Table 11.

Table 10: Replication of the prototype strain of avian reovirus in the presence of IDU.

	Virus titre
Controls (no IDU)	4.0 [a]
10^{-4} M IDU	4.25
10^{-4}M IDU plus 2 x 10^{-4} thymidine	4.25

a. virus titre expressed as $\log_{10}$ $TCID_{50}$/0.5 ml.

Virus was allowed to replicate in CK culture tubes containing no IDU (controls), IDU and IDU plus thymidine until the controls showed an extensive CPE. All cultures were then frozen and thawed, and the virus titre was assayed by quantal titration.

Table 11: The effect of cations on the heat stability of avian adenoviruses and reoviruses.

	Adenovirus		Reovirus
	Type B	Type C	(prototype strain)
IC [b]	6.5 [a]	6.0	3.5
FC [c]	6.75	6.0	2.75
2.0M Na^{+} [d]	6.5	6.0	2.75
1.0M Ca^{++} [d]	2.0	1.0	2.5
1.0M Mg^{++} [d]	6.5	6.0	2.75

a. titres expressed as $logs_{10}$ $TCID_{50}$/0.5 ml

b. IC = initial control; virus titrated without heating

c. FC = final control: virus titrated after heating at 50°C for one hour

d. virus was titrated after heating for one hour at 50°C in the presence of the ions indicated.

With adenovirus prototypes B and C the results indicate that:

a. There is no drop of titre following heating without the addition of cations so both B and C prototypes are stable at $50^{\circ}C$ for one hour.

b. The addition of either Na^{+} or Mg^{++} caused no change in titre as compared with the controls (IC or FC), i.e. both prototypes are stable to heating in the presence of Mg^{++} or Na^{+}.

c. In the presence of 1.0M Ca^{++}, the surviving fraction of both prototypes following heating, was less than 0.01%; i.e. both B and C were markedly destabilized by heating for one hour at $50^{\circ}C$ in the presence of 1.0M Ca^{++}.

Kawamura et al. (1964) also found avian adenoviruses to be destabilized by heating in the presence of Ca^{++}, but not Mg^{++}; whereas other investigators (Yadav et al., 1974; McFerran et al., 1972) reported that avian adenoviruses were destabilized at $50^{\circ}C$ by both Ca^{++} and Mg^{++} (see Discussion).

The results of heating the prototype strain of reovirus showed the following:

a. A drop in titre occurred after heating the control preparation at $50^{\circ}C$ for one hour. This indicated that the reovirus strain, although it could not be called heat-labile, when compared for example with herpesviruses (Wallis et al. 1962), is less heat stable than the avian adenoviruses tested in this study.

b. The heat stability of the virus was not significantly affected by the addition of Ca^{++}, Mg^{++} or Na^{+}.

5. Screening sera from domestic hens for neutralizing antibody to avian adenoviruses.

Three sera from each of 16 flocks of domestic hens housed in the southern half of the North Island were screened for the presence of neutralising antibodies to each of the four avian adenovirus serotypes (A, B, C and D). The sera were screened at a dilution of 1:12.

Results of these tests are recorded in Table 12. All sera except those in flock 1 (see Table 12) had antibodies to at least two of the adenovirus serotypes. It is concluded that adenovirus infections occur frequently among New Zealand domestic hens.

Table 12: Results of screening sera from domestic hens for the presence of neutralising antibody to avian adenoviruses, Types A, B, C and D.

	Virus used in neutralisation test			
Flock No.	Type A	Type B	Type C	Type D
1	[a] 0/3 [b]	0/3	0/3	0/3
2	1/3	3/3	3/3	3/3
3	0/3	3/3	3/3	3/3
4	0/3	3/3	3/3	2/3
5	0/3	0/3	3/3	3/3
6	3/3	3/3	3/3	3/3
7	1/3	3/3	3/3	3/3
8	1/3	2/3	3/3	3/3
9	1/3	3/3	3/3	3/3
10	0/3	1/3	3/3	3/3
11	1/3	3/3	3/3	3/3
12	0/3	3/3	3/3	3/3
13	1/3	3/3	3/3	3/3
14	0/3	3/3	3/3	3/3
15	1/3	3/3	3/3	3/3
16	0/3	0/3	3/3	3/3

a. number of sera which were positive

b. number of sera tested

An indication of the prevalence of infection by a particular serotype of a virus may be given by:

(a) The proportion of sera with antibodies to that serotype
(b) The relative frequency of isolation of that virus serotype from random specimens.

Table 13 gives a comparison of the proportion of sera positive for each of the 4 adenovirus serotypes with the relative frequency of isolation of each serotype. Type C was the adenovirus serotype most frequently isolated in this study, and it was found that a high proportion of flocks had antibody to this type. However, an equal number of flocks had sera positive for type D adenovirus, which was one of the least frequently isolated serotypes. Possible reasons for this "anomaly" are considered in the Discussion.

One serum from each of 13 of the 16 flocks from which sera were taken and screened for neutralising antibodies to all 4 adenovirus serotypes, was titrated to find the titre of antibody to type C avian adenovirus. The distribution of these titres is recorded in Figure 1. The geometric mean serum titre was calculated to be 160.

6. Staining of CK cell cultures

Satisfactory staining of infected and uninfected CK monolayers was obtained, using coverslip preparations which were fixed for 5 minutes in either methanol or 10% formol saline. The fixed preparations were stained for 24 hours with Giemsa's stain diluted 1:75 in tap water.

Plate 11 is a micrograph of Giemsa stained uninfected CK cells fixed with 10% formol saline.

Plates 12 and 13 are micrographs of Giemsa stained adenovirus infected CK cells. Nuclei of infected cells are enlarged, and contain inclusion bodies. All four serotypes of avian adenovirus isolated in this study gave rise to eosinophilic intranuclear inclusion bodies in CK cells.

When reovirus was inoculated into coverslip preparations of CK cell cultures and incubated at $37^{\circ}C$ in petri dishes, syncytia did not develop to the same extent as was observed in rolled culture tubes. However, relatively small syncytia were detected when the preparations

were stained (Plates 14, 15) and typically contained 5-10 nuclei. Nuclei of syncytia appeared to be enlarged, but otherwise they looked normal. Cytoplasmic eosinophilic inclusions were occasionally seen in these syncytia.

Table 13: The proportion of sera positive for each of the 4 adenovirus serotypes compared with the relative frequency of isolation of each serotype.

	Avian adenovirus serotype			
	A	B	C	D
Percentage of flocks with one or more sera positive (three tested)	50%	81%	94%	94%
Relative frequency of isolation of each adenovirus serotype	5%	35%	55%	5%

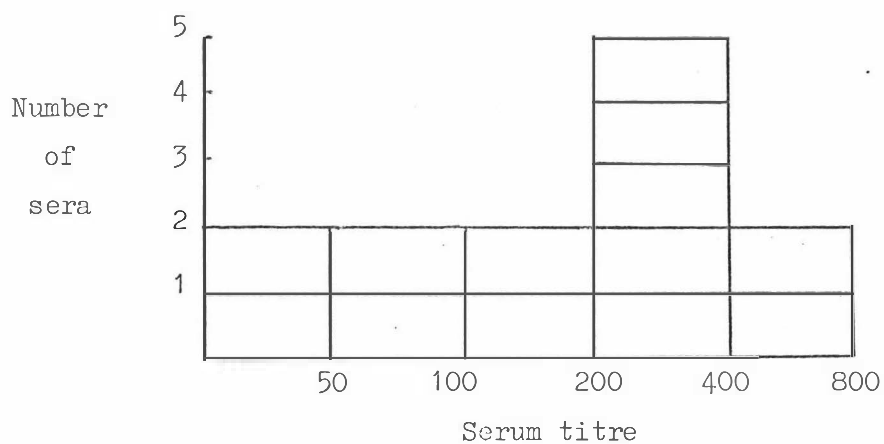


Figure 1. Distribution of the titres of neutralising antibody to avian adenovirus type C in a small sample of randomly selected positive sera.

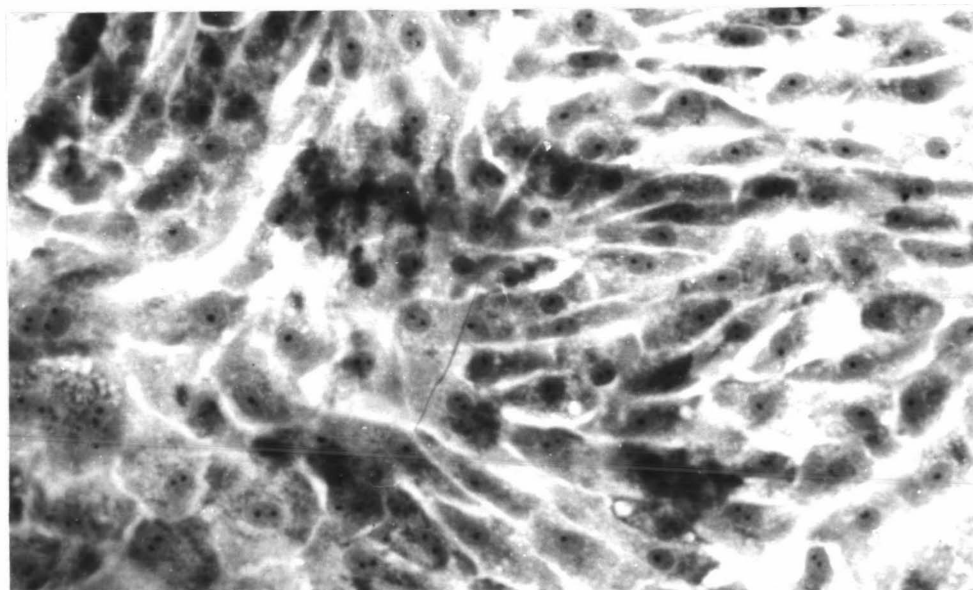


Plate 11. A typical area of a chick kidney cell monolayer. Giemsa's stain. x 200.

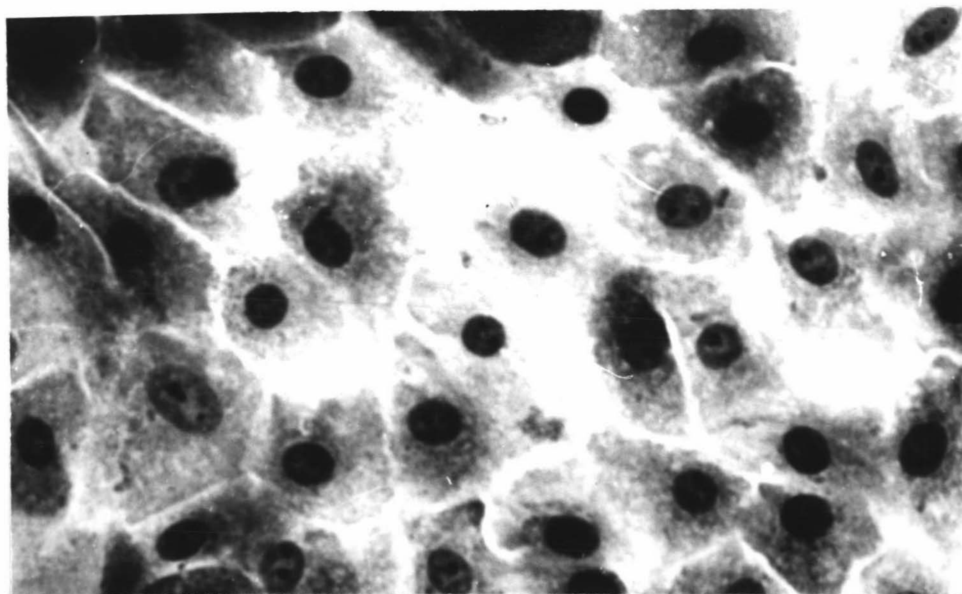
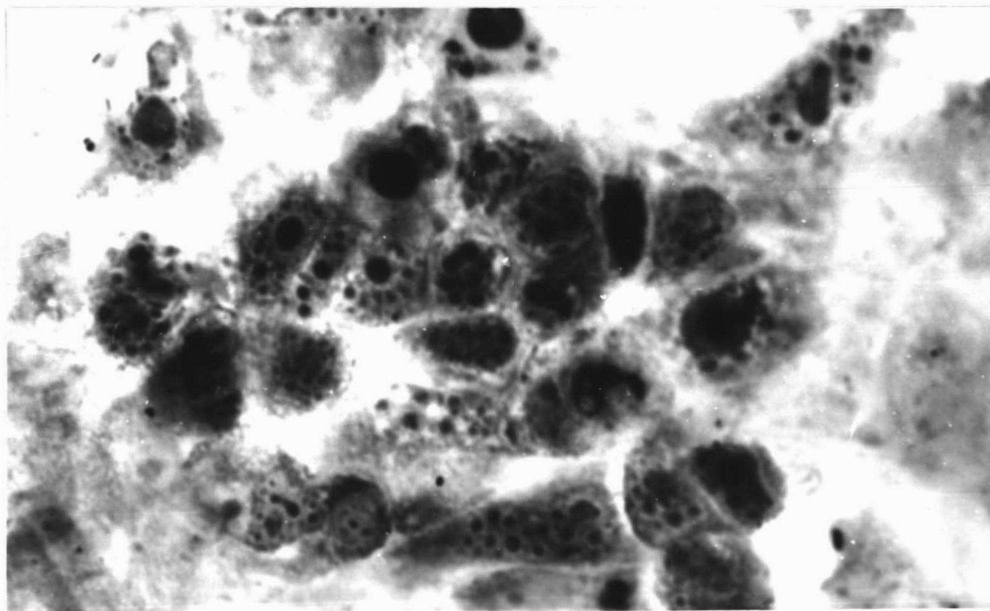
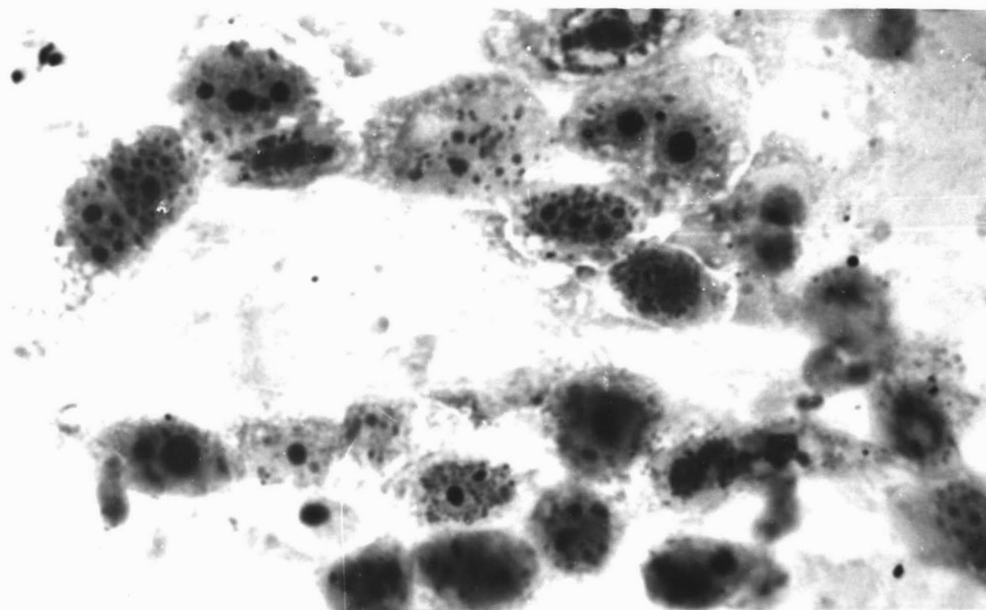


Plate 11a. Part of an island of epithelial cells in a culture of chick kidney cells. Most cultured chick kidney cells were more fibroblastic in morphology (Plate 11). Giemsa's stain. x 300.



Plates 12 and 13. Chick kidney cell cultures inoculated with avian adenovirus type A. Infected nuclei contain one or several eosinophilic inclusions of different size. Giemsa's stain. x 300.



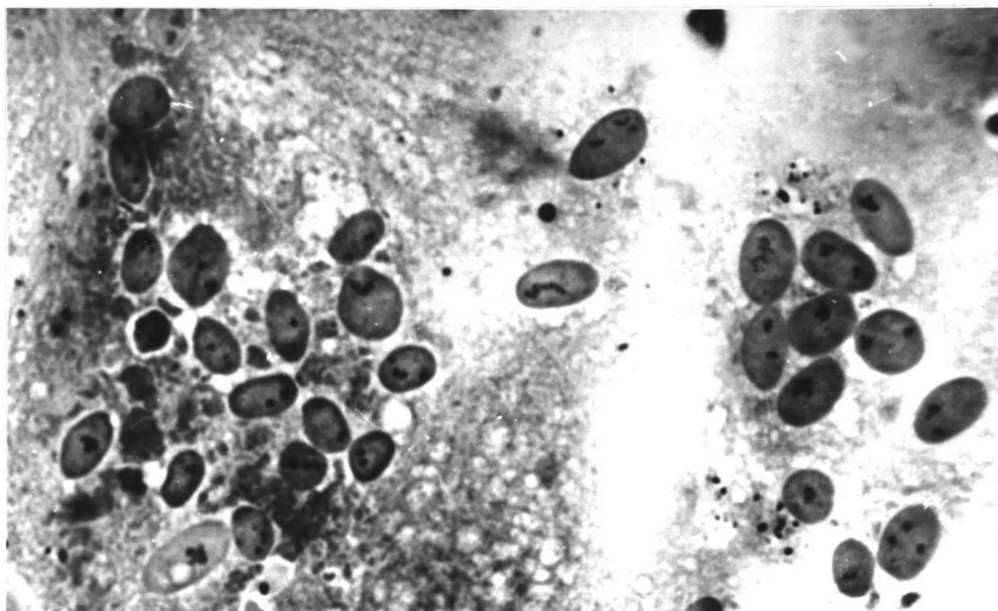


Plate 14. Chick kidney cell culture inoculated with avian reovirus. Two relatively large syncytia are present. Giemsa's stain. x 300.

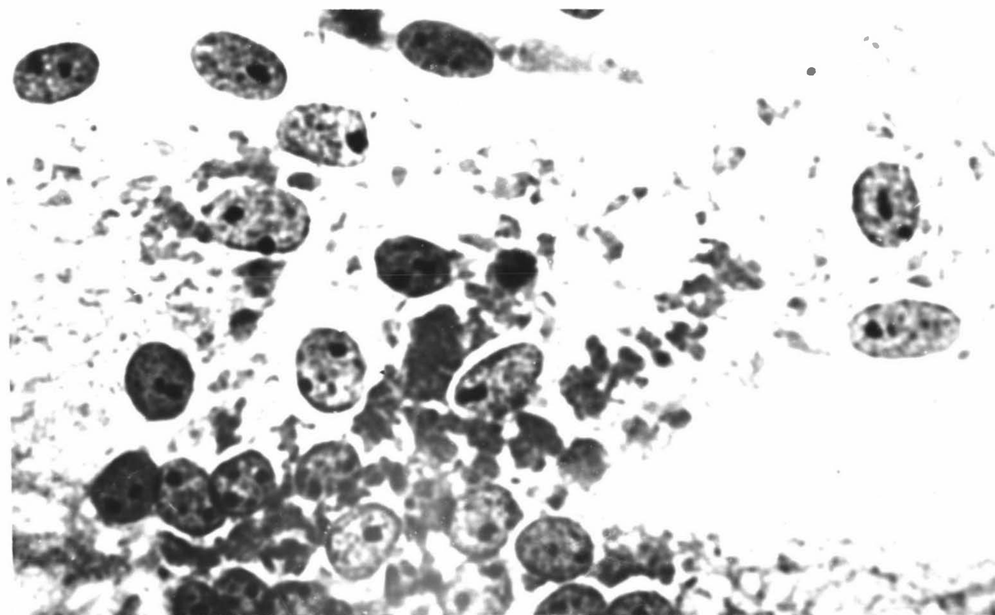


Plate 15. Chick kidney cell culture inoculated with avian reovirus. Note the presence of cytoplasmic inclusion bodies in the syncytium. Giemsa's stain. x 400.

DISCUSSION

1. Production of chick kidney cell cultures.

At the initiation of this study it was clear that it was necessary to produce chick kidney cell cultures free of contaminating viruses, and in particular, free of cytopathic agents. Other workers confronted with the same problem have used kidneys from birds of varying ages, viz. chick embryos near the point of hatch (18 - 21 days), neonatal chicks (0 - 4 days old), and young birds 2 - 10 weeks old.

The obvious advantage of using birds 2 - 10 weeks old is that they have bigger kidneys, so that the same amount of kidney tissue can be obtained from fewer birds. However, the major disadvantage of using older birds is that the risk of contamination of the kidney by a cytopathic agent is high, unless the birds are maintained in isolation until they are used. No facilities were available for this purpose during the course of this investigation. However, in one instance, because of a lack of neonatal chicks, kidneys were obtained from a 6 week old bird (which had not been kept in isolation), and the cultures prepared from this kidney tissue were found to degenerate spontaneously, and the cytopathic agent was found to be an adenovirus (isolate 25).

Thus two alternatives remained, viz. the use of either chick embryos near the point of hatch, or neonatal chicks. Initially kidneys were taken from 19 - 21 day old chick embryos for the preparation of cell cultures. Since cytopathic agents are not normally egg transmitted, the risk of contamination of cultures prepared from embryonic kidney was low. However, it was noted that the kidneys of a 1 - 2 day old chick were much larger, and easier to remove, than those of a chick at the point of hatch. For this reason, and also because it was relatively easy to keep chicks in isolation for up to four days after hatching, neonatal chicks were used as a source of kidney material for cell cultures for the remainder of the investigation. No spontaneously occuring CPE were observed in any of the cell cultures prepared from kidneys taken from either chick embryos or neonatal chicks so contaminant viruses did not present a problem during the course of this work.

A number of different methods for the preparation of chick kidney cell cultures have been described in the literature; however some of them included what appeared to be unnecessarily complicated operations, such as straining trypsinized kidney tissue through sterile gauze to remove unwanted material (Burke *et al*., 1959a). This particular step was avoided by allowing large tissue fragments (if they were present) to settle out of the suspension following trypsinization, decanting the supernatant into conical glass centrifuge tubes and spinning to sediment the remaining cells and cell clumps to allow removal of the trypsin.

It was noted that there were two critical requirements for the production of satisfactory cell cultures in screw topped tubes with an atmosphere of air:

(a) Both growth medium (HTC) and maintenance medium (EaM) were saturated with CO_2 before use

(b) The seeding chick kidney cell suspension had to be standardized within narrow limits.

Provided the above requirements were met, cell culture monolayers lasted for 6 to 7 days in maintenance medium without a further change of medium. Although this period is not particularly long it was adequate to allow the isolation and assay of many cytopathic agents.

2. Isolation of cytopathic agents

During the process of virus isolation from a large number of clinical specimens, it is obviously necessary to avoid cross contamination during handling. Cross contamination can most easily occur if the medium in inoculated culture tubes is changed during the incubation process, so for that reason medium was not renewed in this study. This allowed the maintenance of inoculated cultures for no longer than 7 days.

Unfortunately many clinical specimens, particularly faeces and faecal swabs, were toxic for cell cultures and in most cases the monolayers were not maintained for a long enough period to allow the development of a recognizable CPE particularly in the presence of

non-specific (toxic) degeneration. This problem was circumvented by giving all apparently negative specimens a "blind" passage following incubation for 7 days or until the monolayers had degenerated. Passaged material was rarely if ever toxic for cell cultures.

The results clearly indicate that it is advantageous to give specimens a "blind" passage, since 35% of all the isolates produced a detectable CPE only after one passage. It could be argued that one blind passage may not be enough to detect a high proportion of cytopathic agents. However, in a similar study undertaken in Northern Ireland it was found (McFerran *et al.*, 1971) that 96% of all isolates were recovered either in the initial culture or after one blind passage. Furthermore, it was noted in the present study that virus which caused a CPE only after blind passage, tended nevertheless, to give a marked CPE after 2 to 3 days' incubation of passaged material, which suggests that much virus replication normally occurred even in the absence of a detectable CPE This in turn suggests that more than one blind passage should in most cases be unnecessary for the recovery of the type of agents which were investigated in this study.

3. Identification of virus isolates by negative contrast electron microscopy.

Agents giving a transmissible CPE were examined in the electron microscope, and recognizable virus particles were detected in all cases, usually at the first attempt. In fact the ease with which virus particles were detected was unexpected, since the technique used involved neither purification nor concentration of the virus.

In retrospect, the ease of visualization of the agents was probably due to the fact that only reoviruses and adenoviruses were recovered, and both of these agents replicate well in cell cultures, have a distinctive morphology, and are large enough to be seen easily.

The routine identification of unpurified viruses in the electron microscope is a well established technique in diagnostic veterinary virology (McFerran *et al.*, 1971a), and depends on variations in the morphology of different virus groups. However, in distinguishing virus groups by this method, absolute criteria, e.g. number of capsomeres in icosahedral viruses, are not applied, since routine

electron micrographs rarely resolve particle morphology in sufficient detail as to be adequate for that purpose. Nevertheless, there are some routinely applicable objective criteria for distinguishing virus groups, including particle, capsid or nucleocapsid diameter, presence or absence of an envelope, and the morphology of individual capsomeres.

It turned out, however, that all of the agents detected in this study consisted of naked capsids which measured between 70 and 85 nm in diameter. The two types of virus particles seen in the electron microscope were nevertheless distinguishable on the basis of morphological characteristics.

The type most frequently seen often had a distinctive hexagonal (or sometimes pentagonal) outline (Plate 8). In some electron micrographs triangular faces could be discerned; and the average particle diameter was 80 nm. This type of particle was morphologically indistinguishable from mammalian adenoviruses, as described by Horne (1962), and was thus classified as an avian adenovirus. Supporting evidence for this conclusion, viz. inclusion body formation and the physico-chemical properties of the virus, are discussed elsewhere.

The second type of virus particle, which was detected less frequently than the adenoviruses, could be distinguished from them by the presence of an inner capsid appearing as a continuous ring 46 nm (mean) in diameter. This ring (Plate 9), was visible only in penetrated particles, but since most virus particles were penetrated by stain, this presented no problem.

Particles of this morphology were indistinguishable from mammalian reoviruses (Loh et al., 1965) and since it has already been established that mammalian reoviruses are morphologically indistinguishable from avian reoviruses (Kawamura et al., 1965; Petek et al., 1967), it is concluded that the isolates recovered in this investigation with the morphology described above represent isolates of avian reoviruses.

Recent work on the structure of "reovirus-like" mammalian viruses has established that these agents, although similar in morphology to mammalian reoviruses, can be divided into two or possibly three different groups on the basis of morphological differences. Thus the name "Diplornaviridae" is now frequently

applied to the family of double-stranded RNA "reovirus-like" agents, although this family has no official status (Wildy, 1971). This group of double-stranded viruses of mammalian origin has been subdivided into reoviruses and orbiviruses by some authors. Jawetz, Melnick and Adelberg (1972) distinguish orbiviruses from reoviruses by the large doughnut-shaped capsomeres (as seen end on) which orbiviruses have, but which are absent from reoviruses.

There is also the possibility of a third group of mammalian agents within the diplornaviruses, viz. agents seen in the faeces of children and neonatal calves suffering from acute diarrhoea. Flewett et al. (1974) stated that "judging from published micrographs, the calf and human viruses also differ in morphology from the orbiviruses". This distinction as yet appears to be based on subjective criteria. Nevertheless, the name "rotavirus" was suggested by Flewett et al. (1974) for this type of particle.

The situation is further confused, since in an earlier report Bishop et al. (1974) described the agents detected in the faeces of children suffering from acute gastroenteritis, and classified them as orbiviruses. However, these agents are morphologically indistinguishable from those later described by Flewett et al. (1974).

Happily, the agents isolated from domestic hens in this investigation, and described as having reovirus morphology, are clearly distinguishable from both orbiviruses and those agents detected in faeces of children and neonatal calves. The major distinguishing feature of the avian agents is the clearly defined inner capsid structure with a mean diameter of 46 nm. Thus when these agents are classified as avian reoviruses in this work, the term is applied in its narrow sense, and does not include orbiviruses or rotaviruses.

4. Serological typing of isolates by neutralisation tests

Norrby (1971) has suggested that the method of choice for the serological classification of adenoviruses is the neutralisation test, and although this suggestion was made in relation to mammalian adenoviruses, it seems to have been generally applied to avian adenoviruses (Kawamura et al., 1964; Burke et al., 1968; McFerran et al., 1972) and this approach was adopted in the present study.

The same approach to the classification of avian reoviruses has also been used (Kawamura _et al._, 1965), and was likewise applied to the typing of those agents isolated in this study which were classified as avian reovirus.

It would have been desirable in this study to have obtained prototype strains of adenoviruses and reoviruses from overseas to prepare antisera, so that the results of serotyping of isolates would be directly comparable with those of overseas workers. However, since the importation into New Zealand of veterinary viruses (or even sera) not already known to be present in the country is rarely allowed, especially when the laboratory concerned has no adequate isolation facilities, it proved necessary to proceed with this study without the standard antisera.

The alternative method is to arbitrarily establish local "prototype" strains of each serotype, which can ultimately be sent overseas for typing with standard sera. This approach has been taken in the present study, but the final typing of the local prototype strains has not yet been accomplished.

The results of cross-neutralisation tests (Table 7) demonstrate that in the present study 4 serotypes of adenovirus have been recovered in New Zealand. Similar studies reported by overseas workers (Kawamura _et al._, 1964; Burke _et al._, 1968; McFerran _et al._, 1972) show that there is a total of 9 avian adenovirus serotypes. Some of these serotypes showed low level one way cross reactions in the neutralisation tests, whereas others did not.

As is evident from Table 7 the 4 prototype strains isolated in New Zealand are serologically distinguishable and exhibit no detectable cross reactions in neutralisation tests.

In the present study 5 agents remained untyped, although they were tested with the 4 prototype antisera, so it seems evident that at least 5 serological types of avian adenovirus have been recovered. In preliminary work (Green, unpublished results) an antiserum prepared to a fifth adenovirus, that was not neutralised by the 4 prototype antisera, neutralised two of the 4 prototype strains at a high level. Thus although the first 4 prototype strains established were clearly distinguishable, the situation must be investigated in more detail before a fifth serotype is unequivocally established.

Serological typing of the reoviruses isolated in this study was done in conditions which were not optimal because a prototype antiserum with a relatively high titre was not obtained. It would have been preferable to have an antiserum with a titre of at least 1000, particularly when cross-neutralisation tests are used to establish new prototype strains.

However, since the prototype antiserum neutralised all 5 isolates as efficiently as it neutralised the homologous virus, it is unlikely that more than one serotype of avian reovirus has been recovered in this study. Hence the problem of performing cross-neutralisation tests with low titre antiserum did not arise.

5. Physico-chemical tests

Physico-chemical tests seem to have been developed initially in relationship to virus classification, but are also used as an aid to the identification of viruses routinely isolated in diagnostic laboratories. Such tests are undoubtedly of value in some circumstances, e.g. viruses which require an envelope for their infectivity can be distinguished from naked viruses by their sensitivity to lipid solvents.

The prototype strains of isolates recovered in this study were tested for their sensitivity to chloroform, and were found to be resistant to it (Table 8). This result is consistent with the conclusion based on electron microscopy that the isolates were either adenoviruses or reoviruses.

Apart from tests for essential lipid, one of the most useful physico-chemical tests as a routine aid to virus identification is to establish the effect of IDU on virus replication. The recognition of RNA viruses which (due to reverse transcriptase) are inhibited by IDU may appear to invalidate the use of IDU as a method of establishing the type of nucleic acid contained in a virus. However, all RNA viruses inhibited by IDU are enveloped, whereas the agents isolated in this study are not enveloped, and there is no known exception among the unenveloped agents to the rule that IDU inhibits DNA but not RNA viruses.

Consequently the agents studied in this investigation could be classified as RNA or DNA viruses on the basis of IDU sensitivity, taking into account the fact that they were unenveloped, as determined by chloroform sensitivity and electron microscopy.

Thus the three aids to virus identification, viz. electron microscopy, chloroform stability, and IDU sensitivity, all played an important part in reaching the conclusion that all the agents which were isolated from New Zealand domestic hens are either adenoviruses or reoviruses.

Investigations of the effect of cations on the heat stability of some agents have shown that mammalian adenoviruses and reoviruses could be distinguished, since divalent ions (1.0M Mg^{++} and Ca^{++}) increased the thermolability of adenoviruses (Wallis, Yang and Melnick, 1964). These results, however, do not necessarily apply to the avian agents: thus Kawamura et al. (1965) found that Mg^{++} stabilised reoviruses to the effect of heating, but the virus titre was not increased, and in one instance was slightly decreased following heating. As can be seen in Table 11, in the present work it was found that Mg^{++} did not increase the heat stability of the prototype reovirus, which exhibited a similar response to the most sensitive of the strains tested by Kawamura et al. (1965). It is concluded that in the present state of knowledge, heat stability is of less value than some other methods used in the identification of avian reoviruses.

Avian adenoviruses however, are destabilised by 1.0M Ca^{++} (Kawamura et al., 1964; McFerran et al., 1972), as are the mammalian agents (Wallis, Yang and Melnick, 1962), and in the present study, results (Table 11) are consistent with this conclusion. However, the effect of Mg^{++} is variable: Kawamura et al. (1964) found that 1.0M Mg^{++} did not destabilise avian adenoviruses, whereas McFerran et al. (1972) found that Mg^{++} destabilised avian adenoviruses, but to a lesser extent than did Ca^{++}. Our results agree with those of Kawamura et al. (1964), but since different isolates and probably different serotypes were tested by each group of workers, it is likely that the response of avian adenoviruses to heat in the present of Mg^{++} is heterogeneous.

The general agreement that only 0.001 to 0.01% of the infectivity of adenoviruses survives heating at 50°C for an hour in the presence of 1.0M Ca^{++} whereas 10 - 100% of reoviruses survive under the same

conditions means that this technique could be used to isolate a reovirus from a mixture of adenovirus and reovirus.

In this context it is relevant to reiterate that in the present work a "purified reovirus" initially identified as a reovirus by electron microscopy gave an anomalous result in IDU sensitivity tests, which led to a re-examination of the virus in the electron microscope, which in turn led to the conclusion that a mixture of viruses was present in the original specimen, only one of which was seen in the first electron microscopic examination.

6. The prevalence of adenovirus and reovirus infection of domestic hens in New Zealand.

a. Adenovirus

The work reported in this thesis was undertaken primarily to find if avian adenoviruses and reoviruses could be recovered from the domestic hen population in New Zealand. However, since 25 adenoviruses were recovered from 95 birds, most of which did not have pathological lesions which were likely to have been caused by viruses, and could therefore be regarded as almost a random selection of the domestic hen population, it is concluded that adenoviruses are probably widespread in New Zealand.

Immunity to adenoviruses is type specific (Davis et al., 1973) and since at least 4 serotypes have been identified in the present work, it is likely that birds may be repeatedly infected with a series of serologically distinct adenoviruses.

Before discussing the results of serum neutralisation tests, it is relevant to note that non-specific neutralisation of avian adenoviruses by normal chicken sera has not been reported, and repeated testing of sera from SPF birds for their ability to neutralise adenoviruses has given negative results (Clarke, personal communication), so it is concluded that the serological survey detected genuine antibody, even though non-specific neutralisation of some viruses by some sera may occur, e.g. polioviruses are neutralised by bovine sera (McFerran, 1962).

Apart from virus isolation additional evidence that adenovirus infection is common in birds in New Zealand was obtained in a small serological survey, in which the ability of sera from domestic hens to neutralise all 4 adenovirus serotypes was tested. From Table 12 it was concluded that neutralising antibodies to all 4 adenoviruses serotypes were present, and that antibodies to adenovirus serotypes C and D were common.

It was noted that although type D adenovirus was one of the types least frequently isolated, neutralising antibodies to this particular type were found to be very common. The obvious explanation for this is that infection of domestic hens in the southern half of the North Island by this particular adenovirus serotype was common a short time before attempts were made in this investigation to recover avian viruses.

b. Reovirus

Five strains of reovirus were isolated in this investigation. Four of these came from a single flock, and all 5 were assigned to one serological type. Since the total number of isolations was small, it appears that reovirus infections in New Zealand domestic hens are less common than adenovirus infections. It is worth recording that other investigators have also isolated reoviruses from domestic hens less frequently than adenoviruses (McFerran et al., 1971).

However, it must be stressed that any conclusions regarding the prevalence of reovirus based on the present work must be regarded as tentative because the scope of the investigation was very limited, and did not include a serological survey.

7. Clinical and pathological significance of avian adenoviruses and reoviruses.

(a) Adenoviruses

Until about ten years ago, few if any investigations had been made concerning the association of adenovirus infection with disease in the domestic hen. Clemmer (1964) experimentally infected one-day-old chicks with a strain of adenovirus, and noted that "as is commonly the case with human enterovirus infection, overt signs of disease were absent". The term "enterovirus" was applied in the broad sense of the word, meaning a virus recovered from the alimentary tract.

Five years later Berry (1969) reported that experimental infection of laying hens with CELO virus caused a drop in egg production of approximately 10%, which lasted for three weeks. A drop in eggshell quality was also reported in this investigation.

Cook (1972) confirmed the finding of Berry (1969) that the egg production of domestic hens experimentally infected with CELO virus dropped by 10% (for about two weeks), but she found that eggshell quality was not affected.

From the point of view of histopathology, by far the most characteristic lesion known to be caused by an adenovirus is a classical disease of dogs, viz. canine hepatitis. It is of particular interest therefore that there is a disease of broiler chicks which resembles canine hepatitis in that it is characterized by the presence of intranuclear inclusion bodies in the livers of affected birds. The disease was described by Hemboldt and Frazier (1963), and nine years later Pettit and Carlson (1972) called the disease inclusion body hepatitis (IBH), and recovered an unidentified cytopathic agent from the livers of affected chicks. These workers also reported that broilers with IBH suffered up to 7% mortality.

Young et al. (1972) also reported a severe outbreak of IBH in Northern Ireland, affecting a large number of 3 to 5 week old broiler chicks, and resulting in a mortality of up to 5%. An adenovirus, which was found to be the most frequently isolated serotype in Northern Ireland, was recovered from affected birds (Clarke, personal communication).

A year later, Winterfield et al. (1973) experimentally infected three-day-old and four-week-old chicks with an adenovirus they had previously isolated from a domestic hen suffering from IBH, and

produced the disease in the birds, which they had infected by intravenous, intratracheal, eyedrop and subcutaneous inoculation. They were also able to recover the virus from various tissues of these birds, including the liver. In the same investigation, Winterfield <u>et al</u>. (1973) reported that the agent involved was serologically related to CELO virus.

Wells and Harrigan (1974) were also able to produce IBH in seven-day-old chickens, by intravenous and intraperitoneal inoculation with an agent described as having typical adenovirus morphology. Unfortunately, the serological relationship of this agent with other avian adenoviruses was not investigated.

Although it seems fairly certain that adenoviruses can cause IBH in domestic hens, it must be noted that the disease has not yet been produced in birds experimentally infected under field conditions. Further work is necessary to establish the natural conditions under which adenovirus infection will cause IBH in domestic hens.

Apart from canine hepatitis, another well established disease syndrome associated with adenovirus infection is acute respiratory disease (ARD), in military recruits. The critical factor here seems to be the crowding together of young susceptible hosts. This situation may have an epidemiological parallel with the current practice of housing hens in crowded conditions, so it would seem reasonable to look for an association between avian adenoviruses and respiratory disease. Reports in the literature not infrequently mention that the hens from which adenoviruses have been recovered have been suffering from slight respiratory signs, but nobody has concluded that adenoviruses are definitely associated with respiratory disease in domestic hens. For example, McFerran <u>et al</u>. (1971) noted that adenoviruses were recovered with a higher frequency from birds with respiratory disease than from birds without any respiratory signs.

However, more investigation, both in laboratory and field conditions is required in order to establish the role of adenovirus infection in respiratory diseases of the domestic hen.

At present, it may be concluded that although the most likely result of exposure of hens to avian adenoviruses is the establishment

of an inapparent infection, adenoviruses can in some circumstances cause IBH, and probably can also cause drops in egg production, and possibly also cause respiratory signs. The possibility that other pathological conditions may also be caused by adenoviruses cannot be ruled out until these agents have been studied in more detail in field and laboratory conditions in various countries over a considerable period of time.

(b) Reoviruses

As yet no well defined disease has been unequivocally shown to be associated with reovirus infection of domestic hens.

Petek et al. (1967) reported that the "Crawley agent" (an avian reovirus) was originally isolated by Fahey and Crawley (1954) from chickens with chronic respiratory disease. Dutta and Pomeroy (1967), and Deshmukh and Pomeroy (1969a) isolated avian reoviruses from chicks with severe cloacal pasting. However, no success with reproducing either of these signs by experimental inoculation of chicks with avian reoviruses has been reported.

Infectious bursitis, also called Gumboro disease, has been reported to be caused by an avian reovirus (Petek and Mandelli, 1968). However, Lunger and Maddux (1972) reported that the infectious bursal agent (IBA) was probably not a reovirus, and they suggested it might be classified as a picornavirus, in spite of its size (60 nm diameter). More recently, Lukert and Davis (1974) investigated some of the properties of an isolate of IBA, and suggested it should be classified as a diplornavirus.

Obviously further investigation is required before it is established if a reovirus is the causative agent of infectious bursitis.

It has also been suggested that "reovirus could be the causative agent of an ill-defined syndrome called 'pullet disease'." (Andrewes and Pereira, 1972). It was interesting (Results, Table 5) that reoviruses were isolated in this investigation from birds tentatively diagnosed as having "pullet disease". However, since the clinical picture is so ill-defined, no conclusion can be drawn from this.

Aside from possible diseases caused by reovirus infection of domestic hens, it has been suggested that reoviruses could be the cause of infectious enteritis, otherwise known as bluecomb, in turkeys (Dees et al., 1972), and possibly the cause of a disease which resulted in up to 30% mortality in turkey poults but nevertheless was not associated with any gross lesions (Simmons et al., 1972).

However, in these last two cases the picture is just as confused as with reovirus infection of domestic hens, and no extrapolation can be made from one to the other to help clarify the situation.

It is concluded that avian reoviruses have not been shown to be the cause of any well defined disease of domestic hens - a situation which is analogous to that of mammalian reoviruses.

APPENDIX

1. Phosphate buffered saline and mineral salts - PBS (pH 7.5)

Soln. A	NaCl	8.0 gm
	KCl	0.2 gm
	$Na_2HPO_4.12H_2O$	2.9 gm
	KH_2PO_4	0.2 gm
	Dist. H_2O	800 ml
Soln. B	$CaCl_2.6H_2O$	0.19 gm
	Dist. H_2O	100 ml
Soln. C	$MgCl_2.6H_2O$	0.1 gm
	Dist. H_2O	100 ml

1000 ml PBS consists of 800 ml Soln. A, 100 ml Soln. B, 100 ml Soln. C.

2. Trypsin solution (0.1%)

Trypsin	1 gm (1:250 Difco trypsin)
PBS	1000 ml

Final solution is sterilised by filtration through 0.22 μ filters.

3. Earle's based solutions

(a) Earle's stock solution

NaCl	68 gm
KCl	4 gm
$MgSO_4.7H_2O$	2 gm
$NaH_2PO_4.2H_2O$	1.4 gm
Glucose	10 gm
$CaCl_2.6H_2O$	3.94 gm (dissolved separately)
0.4% Phenol red soln.	25 ml
Dist. H_2O up to	1000 ml

(b) Earle's lactalbumin solution - LaE

Stock solution (above)	500 l	Earle's working solution
Dist. H_2O	4500 ml	

To 500 ml of working solution is added 25 gm lactalbumin hydrolysate; the rest of the working solution is dispensed in 360 ml amounts, and all medium is autoclaved, after which 40 ml of the lactalbumin hydrolysate solution is added to each 360 ml amount of Earle's working solution. Final solution is called LaE.

(c) 4.4% bicarbonate buffer

$NaHCO_3$	22 gm
0.4% Phenol red soln.	12.5 ml
Dist. H_2O to	500 ml

Sterilised by autoclaving.

(d) Earle's maintenance medium - EaM
(all components are already sterile, and are added aseptically)

LaE	400 ml
4.4% bicarbonate soln.	20 ml
Foetal calf serum	10 ml
Antibiotics	penicillin and streptomycin, both to final concentrations of 100 units (or μg) per ml.

(e) Earle's based diluent - SD

LaE	400 ml
Foetal calf serum	10 ml
Antibiotics	as for MM

Used for all dilutions of viruses and sera (called standard diluent, SD).

(f) Earle's based washing solution - EWS

LaE	400 ml
Antibiotics	as for MM

Used for washing chick embryo kidney tissue immediately after removal from chick.

4. Hank's solutions

(a) Hank's balanced salt solutions

Soln. A	$NaCl$	160	gm	
	KCl	8	gm	
	$MgSO_4.7H_2O$	2	gm	
	$MgCl_2.6H_2O$	2	gm	(dissolved separately)
	$CaCl_2.6H_2O$	5.5	gm	

Dispensed in 250 ml amounts, autoclaved.

Soln. B	$Na_2HPO_4.2H_2O$	1.2	gm
	KH_2PO_4	1.2	gm
	Glucose	20	gm
	0.4% Phenol red soln.	50	ml
	Dist. H_2O to	1000	ml

Dispensed in 250 ml amounts, autoclaved.

(b) Hank's working solution - LaH

Soln. A	250	ml
Soln. B	250	ml
Dist. H_2O	4500	ml

25 gm lactalbumin hydrolysate added to 500 ml working solution. The rest of the working solution is dispensed in 360 ml amounts, and all medium is autoclaved, after which 40 ml of the lactalbumin hydrolysate solution is added to give each 360 ml amount of Hank's working solution. The final solution is called LaH.

(c) 1.4% bicarbonate buffer

$NaHCO_3$	7	gm
0.4% Phenol red soln.	12.5	ml
Dist. H_2O to	500	ml

Sterilised by autoclaving.

(d) Tryptose phosphate broth

Tryptose	20	gm
Glucose	2	gm
$NaCl$	5	gm
Na_2HPO_4	2.5	gm
Dist. H_2O	1000	ml

Sterilised by autoclaving.

(e) Hank's growth medium - HTC

(all components already sterile, and are added aseptically)

LaH	400 ml
1.4% bicarbonate soln.	20 ml
Calf serum	50 ml
Tryptose phosphate broth	50 ml
Antibiotics	as for MM

5. Giemsa stain

Giemsa's stain (Gurr)	3.8 gm
Glycerin	250 ml
Methanol	250 ml

These are mixed and heated for one hour at 50°C; cooled and filtered (Whatman No. 1).

Immediately before use the concentrated stain is diluted with tap water.

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