HERPES VIRUS INFECTION OF PIGEONS.

BY

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THESIS

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SUMMARY.

The thesis reports the isolation of a herpesvirus from two outbreaks of disease among racing pigeons in the West of Scotland. In both outbreaks the infection seemed to be enzootic and was probably maintained by healthy adult carriers. Thus, although each flock was self-contained, the disease recurred annually in both lofts over several years and only involved birds of five to six weeks of age. Clinical signs included serous conjunctivitis and rhinitis, weakness and general malaise. Some birds were dyspnoeic, probably due to the presence of small diphtheritic patches on the mucous membrane of the larynx. The most constant post-mortem finding was focal hepatic necrosis, but renal necrosis was also noted in some birds. Basophilic or slightly eosinophilic intranuclear inclusions were found in parenchymal cells adjacent to the necrotic foci and were also noted occasionally in epithelial cells adjacent to exudate-coated ulcers in the pharynx and larynx.

Examinations for the ornithosis agent proved negative, but small whitish-cream pocks were produced on the chorio-allantoic membranes of ten-day embryonated hens' eggs inoculated with tissue suspensions from six birds and with a throat swab from a seventh. Embryos became sluggish and often died about the fifth day.

The pocks produced by all isolates generally attained

a maximum diameter of 1 mm. on the fourth day, but dendritic extensions were sometimes present. Intranuclear inclusions, typical o those produced by herpesviruses, were prominent in ectodermal cells on the second day, but diminished in number thereafter and, by the fifth day, were almost entirely absent. Foci of necrosis in the embryonic liver could often be appreciated macroscopically by the fourth day. Intranuclear inclusions were present in hepatic cells adjacent to those foci. Hepatic necrosis was a regular finding in embryos inoculated by the yolk-sac route and also sometimes occurred in embryos infected amniotically, but the virus could not be propagated by allantoic inoculation.

All isolates were cytopathogenic in whole chick-embryo cultures, the cytopathogenic effect (C.P.E.) being characterized by the appearance of foci of round, refractive cells, some of which were considerably swollen. With concentrated inocula, the C.P.E. appeared within 24 hours and the monolayer was completely destroyed by the fourth or fifth day with release of virus into the culture-fluids. The agent was cytopathogenic in cultures of chick-embryo kidney, chick-embryo liver, chicken kidney and pigeon kidney but did not multiply in HeLa cells, in Strain L cells or in primary cultures of dog or calf kidney. The C.P.E. was essentially the same in all avian cultures employed. In contrast to the above findings, a strain of infectious laryngo-tracheitis (I.L.T.) virus was found to produce small syncytia in the epithelial component of whole-embryo cultures

but no C.P.E. in fibroblasts. Intermediate in its effects was the P-5 strain of pigeon I.N.I. virus isolated by Smadel et al. in 1945. This strain, kindly supplied to the writer, produced small syncytia in the epithelial component of whole-embryo cultures and rounding of the fibroblasts.

All isolates produced plaques in whole-embryo cultures maintained under an agar overlay. These appeared on the second or third day and by the fourth day, varied in diameter from 0.5 mm. to 2 mm. though the largest did not increase in size thereafter. Although "small-plaque" variants were found to be present, much of the variation in plaque-diameter was shown to be non-genetic in character. A linear relationship was found to exist between plaque-count and virus input and evidence was obtained to indicate that the distribution of plaques follows the Poisson equation. Replicate assays of stock virus gave approximately the same titre in cultures of different batches. The ratio of plaque-forming to pock-forming units was found to be around 1.5 to 1. Plaques were also formed under methylcellulose the relationship between plaque-count and relative virus concentration again being linear. The plating-efficiency under methylcellulose was approximately double that under agar. About 50 per cent of the virus attached to monolayers of whole-embryo cells in 95 minutes but adsorption was not complete before the end of the sixth hour.

The DNA nature of the virus was demonstrated by inhibition

with bromo- and iodo-deoxyuridines. By means of the negative staining technique of electron microscopy, the virion was shown to be typical of that of the herpesvirus group. The virus was ether-sensitive and was destroyed by exposure to a pH of 4.0. It was quickly destroyed at 56°C. but was relatively stable at 4°C. Antiserum prepared in rabbits against one of the author's isolates was found to neutralize the American P-5 strain as well as three strains of virus from the United Kingdom. The virus appeared to be serologically distinct from that of I.L.T.

CONTENTS

PART 2.

GROWTH AND ASSAY OF VIRUS IN EMBRYONATED EGGS.

PART 3.

GROWTH AND ASSAY OF VIRUS IN TISSUE CULTURE.

Page.

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PREFACE.

The purpose of this thesis is threefold: (1) to report the isolation of a virus, hitherto unrecognized in the United Kingdom, from racing pigeons with a disease which, both in clinical signs and in post-mortem features, bore a quite striking resemblance to ornithosis, (2) to describe the means by which the virus may be propagated in the laboratory and its infectivity measured and (3) to demonstrate that the agent belongs to the herpesvirus group.

The virus was discovered during an investigation into the aetiology of two outbreaks of disease in which the affected birds were dull, anorectic and disinclined to move and sometimes showed small diphtheria-like lesions in the mouth or a slight serous discharge from the eyes and nares. Dyspnoea occasionally occurred as a result of partial occlusion of the larynx by a diphtheritic membrane. At autopsy, however, hepatitis was generally found to be the main lesion, though it varied very considerably in severity. In both lofts, the disease recurred annually and principally affected birds of five to six weeks of age.

To those in medical or veterinary virological laboratories who occasionally examine pigeons in an attempt to trace possible sources of human psittacosis infection, the following communication may not be without relevance.

HISTORICAL INTRODUCTION.

In 1943, an unusual outbreak of ornithosis was described in pigeons in New York State (Smadel et al.). Sick birds presented signs of enteritis and, in some instances, of respiratory involvement. Six of them were subjected to detailed pathological and microbiological examination. The abnormalities encountered at autopsy varied considerably. In two of the birds, flecks of fibrin were found on the peritoneum and the liver was enlarged, but quite the most striking abnormality was focal pancreatitis with opaque, chalky areas which resembled the lesions of fat necrosis of the human pancreas. In a further case, the liver and spleen were enlarged and mottled, the lungs were congested and oedematous and the peritoneal cavity contained sero-gelatinoid exudate which in places adhered to the viscera.

Histopathological examination revealed that the liver of all six birds contained foci which consisted of large mononuclear cells and lymphocytes and which occurred mainly in the periportal connective tissue although, in some places, they also extended into the parenchyma where they replaced hepatic cord cells that had undergone necrobiosis. Focal necrosis was present in the liver of each of the birds, the foci being distributed randomly within the lobules. In five of the birds, the spleen also showed

areas of cellular destruction which varied in both size and number. Vascular congestion, haemorrhage, endothelial hyperplasia and diffuse infiltrations of large mononuclear cells were also recorded. Pancreatic necrosis was found only in the two cases which showed gross lesions of the organ and, in one of them, the lesions appeared to be recent since the cellular outlines were still discernible and leucocytes were quite absent. In the other instance, areas of complete destruction alternated with those of necrobiotic change and mononuclear as well as polymorphonuclear leucocytes were present in the older foci.

Elementary bodies, closely similar to those seen in sections of tissue from birds infected with the agent of psittacosis, were found in the liver of five of the pigeons, in the spleen of six and in the pancreas from the two cases which showed necrosis of that organ. Both parenchymatous cells and mononuclear leucocytes were therein involved.

An unusual feature of the disease was the presence of large, eosinophilic, intranuclear inclusions of the herpetic type in one or more organs of all six cases. In the liver of one of the birds, they were so numerous that, in some fields, practically every intact cell contained one. Most of them occurred in parenchymatous cells but occasionally they were to be found in the epithelial cells lining the small bile ducts and, rarely, they were apparent in large mononuclear leucocytes. In the spleen, inclusions were

present in the cells of the pulp and in those of the Malpighian bodies, while in the pancreas they were visible more often in the islets of Langerhans than in the acinar cells.

Primary intracerebral inoculation into mice of tissue suspensions from five of the birds produced an illness that generally terminated fatally. After a few intracerebral passages in mice, the agent responsible for that disease became so exalted in virulence that the inoculated mice died in from two to four days. In smears made from the meninges, elementary bodies were demonstrable by Machiavello's method of staining. Although intraperitoneal inoculation of infective material failed to produce death in mice, typical L.C.L. bodies were to be found in the peritoneal exudate.

The tissue-suspensions which proved infectious for mice also produced pocks on the chorio-allantoic membranes of 10-day-old embryonated eggs. On the second or third day after inoculation, the lesions were visible as tiny dew-drop structures and, by the fifth day, had become larger and opaque. They contained many cells rich in elementary bodies. Preparations from the liver and spleen of the remaining bird also gave rise to similar chorio-allantoic changes but were incapable of establishing disease in mice. Moreover, although elementary bodies were to be found in the latter pocks, egg-passage material proved to be non-pathogenic for mice.

Histologically, the membranes showed focal areas of ectodermal hyperplasia together with infiltration of polymorphonuclear and mononuclear leucocytes into the underlying mesoderm. Large intranuclear inclusions of the herpetic type were present in the ectodermal cells of all membranes infected with either pigeon or egg-passage material but were difficult to find in membranes inoculated with infective mouse tissue.

Two mutually-exclusive hypotheses were put forward to account for those findings, namely: (a) certain strains of the ornithosis agent are characterized by their ability to produce intranuclear inclusions in infected tissue under appropriate conditions or (b) the disease was caused by a mixture of two agents.

In 1945, a similar outbreak was described in American army pigeons (Smadel et al.). During the spring of 1942, the birds concerned had been apparently normal but early in the summer they were joined by 200 others from a neighbouring state. A number of the newcomers were found to be sick and several were dead on arrival. Illness continued among the new pigeons but did not appear in the original stock until a month later when infection spread rapidly throughout the flock. Many of the infected birds recovered but some 300 succumbed during the summer and autumn. The epizootic diminished in intensity in November and, by the end of the year, the number of birds showing disease of any kind had fallen to from

15 to 20. Quarantine was then lifted and an extensive interchange of pigeons with other contingents took place. Over a period of several months, most of the trained birds were sent to other units and replaced by several thousand adolescent or young adult birds from British sources. The incidence of infection continued at a low rate throughout the first eight months of 1943, during which period, as a rule, one or two dozen birds were involved at any one time. By then the disease was limited almost entirely to young birds from six weeks to three months of age. In August, 1943, the Virus Division of the United States Army was asked to investigate the nature of the disease which had become enzootic in the flock. Eleven sick pigeons were submitted for full investigation.

Clinical signs included conjunctivitis, dyspnoea, diarrhoea, dehydration and emaciation but so considerable was the variation that a characteristic clinical picture was not definable. The post-mortem appearances were equally varied and did not include any feature common to all cases. The sole point of special interest was the presence of focal necrosis in the pancreas of one bird.

Isolation of virus was attempted from the liver and spleen of all eleven cases. The tissue suspensions were inoculated onto the chorio-allantoic membrane of embryonated eggs and into mice by the intracerebral route. Histopathological studies of

liver, spleen, pancreas, pectoral muscle and kidney were also made.

The results of the mouse inoculation tests showed that four of the birds had become infected by the ornithosis agent. Tissue suspensions from two of those four cases produced pocks on the chorio-allantoic membrane, as did similar suspensions from a fifth bird, shown to be free of ornithosis. Histopathological examination of the membranes revealed the presence of intranuclear inclusions within the lesions. Suspensions from the other two ornithosis cases did not evoke formation of pocks. Conversely, the pock-forming agent was non-pathogenic for mice by the intracerebral route. It was concluded, therefore, that two micro-organisms were present in the flock and the one responsible for the pocks was a hitherto unrecognized virus. The latter was called the pigeon intranuclear inclusion (I.N.I.) virus.

The I.N.I. virus appeared to be more closely associated with lesions than did the ornithosis agent. Thus, hepatic necrosis was found only in the three cases from which the virus had been isolated. Respiratory abnormalities were not present in either of the cases of pure ornithosis infection but one of the cases of mixed infection showed conjunctivitis and dyspnoea. The latter was the main clinical sign in the bird which was solely infected with the virus and, at post-mortem examination, tracheitis was found to be the principal lesion. Intranuclear inclusions of the herpetic type were present in the liver of one and in the liver,

spleen and pancreas of two of the cases from which the virus had been isolated.

In addition to the large yellowish pocks on the chorio-allantoic membranes of 10-day embryonated hens' eggs, the I.N.I. virus produced yellow-grey areas of focal necrosis in the liver of the embryo. Intraperitoneal inoculation into pigeons did not evoke any sign of disease but, after intracerebral administration, two birds died and a third became ill. Attempts to produce disease in rabbits and guinea-pigs were unsuccessful.

Two years later an outbreak of ornithosis in pigeons was described in Berkshire, England (Hughes, 1947). The flock was a self-contained one founded on stock imported from America. Affected birds did not manifest any definitive clinical signs but, at post-mortem examination, focal necrosis was conspicuous in the liver and spleen and intranuclear inclusion bodies were to be found at the edges of those lesions. The I.N.I. virus was believed to be present in the flock along with the ornithosis agent but attempts to isolate it were not made.

Further reports of I.N.I. virus infection did not appear until 1966 when Marthedal and Jylling described a disease of pigeons which was subsequently shown to be caused by the I.N.I. virus (Jylling 1967). The condition was first recognized in 1950 and accounted for three per cent of all cases of pigeon disease

investigated by the Royal Veterinary and Agricultural College, Copenhagen, during the succeeding 15 years. Initially, the infection seemed to be limited to the mouth, pharynx and oesophagus, but it was later found to involve the liver and other internal organs. The disease was generally seen in birds of from one to six months of age and seldom occurred in older pigeons. The clinical manifestations varied considerably in severity, some birds merely showing coryza-like signs while, in other instances, dyspnoea and snoring respiratory noises were pronounced. Diarrhoea and loss of condition also occurred. Mortality ranged from an insignificant level in some flocks to above 50 per cent in others, the average amounting to between 10 and 15 per cent of the total number of birds hatched within one season. The duration of clinical signs varied from several days to several weeks.

Examination of the oral cavity and pharynx revealed the presence of greyish-yellow mucoid exudate but the changes considered most characteristic of the disease occurred in the oesophagus, where greyish-yellow or greyish-green patches of diphtheroid nature were generally present and often involved large areas of the mucosa. Similar but smaller lesions were recorded in the mouth and pharynx of a high proportion of cases. Small grey foci - pin-head in size - were sometimes noted in the liver while tracheitis as well as enteritis was often observed.

The histopathology of the above disease was described

by Jylling (1967). The earliest lesion in the oesophagus was a ballooning and hydropic degeneration of cells in the stratum Malpighii, intranuclear inclusions sometimes being recognizable therein. Such necrosis resulted in the formation of ulcers which often became covered by fibrinous and purulent exudates as secondary bacterial infection was established. The liver contained numerous foci of necrosis of eosinophilic character with vascular congestion and haemorrhage also conspicuous. Intranuclear inclusions were present in both von Kupffer cells and in hepatic parenchyma though they were most numerous in areas immediately adjacent to the necrotic foci. A catarrhal, croupous tracheitis, in which focal necrosis involved primarily the glandular parts of the organ, was also observed and typical inclusion bodies were to be detected in association with that lesion. In the lungs, the pathological picture was characterized by an interstitial pneumonia in which proliferation of alveolar epithelium was accompanied by infiltration of lymphocytes into the interstitium. Numerous foci of necrosis were present in the spleen while hyperplasia of the reticular cells was noted in localised areas. Degenerative changes, together with intranuclear inclusions, were to be found in the epithelial cells of the renal tubules and lymphocytic infiltration was to be seen also.

In addition to their presence in the tissues already mentioned, inclusion bodies were described as occurring in alveolar

and bronchial epithelium as well as in cells of unspecifed type in the spleen and pancreas. They were of the Cowdry Type A pattern, eosinophilic in nature and surrounded by a clear "halo". The nucleolus was generally pushed aside against the nuclear membrane to which beads of chromatin were sometimes attached. Rarely, inclusions were found in multinucleated giant cells in the liver and lung. Attempts to demonstrate the presence of the agent of ornithosis by means of the complement-fixation test were all negative, but virus was isolated in embryonated eggs inoculated with a suspension of liver from an unspecified number of cases. Pocks appeared on the chorio-allantoic membrane and focal necrosis was to be found in the liver and spleen of the embryo. Microscopic examination of those lesions revealed the presence of intranuclear inclusions identical with those observed in the pigeon tissues.

Lehner et al. (1967) described a similar disease in pigeons of from six weeks to four months of age. Out of a total of 295 birds bred on the premises, 31 died. Clinical abnormalities were not detected in all of those cases but some birds were inactive and anorectic for approximately one week prior to death. Post-mortem and histopathological examinations were carried out on 14 cases, in eight of which the morbid process was found to be characterized by focal necrosis in the liver, spleen and pancreas accompanied by intranuclear inclusions.

The liver lesions varied from multiple, discrete, small

foci of eosinophilic cellular debris to quite massive areas of necrosis, usually haemorrhagic. Parenchymal cells at the periphery of the necrotic foci as well as the occasional intact cell within the lesion contained eosinophilic intranuclear inclusions. In some cells, the inclusion body seemed to fill the entire nucleus but, in the majority of them, there was a clear 'halo' between the inclusion and the nuclear membrane. Four of the birds had focal necrosis of the pancreas and one had similar lesions in the spleen. As in the liver, the necrotic foci were associated with eosinophilic intranuclear inclusions. Although the presence of the latter structures indicated that the disease was caused by a virus, attempts to isolate the agent were not made.

Concurrent with the studies of Jylling (1967) and Lehner et al. (1967) were two investigations into a similar disease prevalent in the United Kingdom. Dr. J. B. McFerran of the Veterinary Research Division of the Northern Ireland Ministry of Agriculture isolated a virus from the pharynx of a pigeon with diphtheroid lesions (personal communication). The agent, which gave rise to intranuclear inclusions and pocks on the chorio-allantoic membrane of 10-day embryonated eggs, was supplied to the writer who designated it Strain B-1 and carried out further examination of it, the results of which will be described in succeeding pages of this thesis. Part of the author's own investigations, which form the bulk of the thesis, have already been briefly reported (Cornwell et al., 1967).

PART 1. AETIOLOGY AND PATHOLOGY.

SECTION I: NATURAL DISEASE.

(1) INTRODUCTION.

Two outbreaks of disease were studied, one in Renfrewshire (birds of the 'H' series) and the other in Dunbartonshire (birds of the 'M' series). Both occurred during the summer of 1964, chiefly involved pigeons of from one to three months of age and lasted for about two months. In each loft, birds manifested general malaise, dullness and inappetence, were reluctant to move and tended to perch huddled together. The solitary clinical sign common to the majority of cases was a mild serous conjunctivitis though, in both flocks, a minority of affected birds possessed small diphtheroid patches in the mouth or pharynx.

In both lofts, the disease seemed to be enzootic, recurring annually in the young stock. In the first flock, the original source of the infection was unknown but in the second, it was traced back to the accommodation of a stray pigeon. On the day after arrival, the latter was found to have diarrhoea and was immediately killed but, within two weeks, coryza-like signs developed among the stock and affected birds of all ages. Since then the disease has continued to appear in young offspring bred on the premises as well as in birds of any age that were brought in. Because of the latter aftermath, the owner eventually

abandoned the practice of buying in birds for breeding. It is probable, therefore, that a reservoir of infection is maintained by the presence of adult carriers. Possibly, maternal antibody may be conveyed to the young to afford defence for some weeks but, by the time that birds are four to five weeks old, such protection may have so waned that infection acquired from adult carriers is beyond restraint.

(2) MATERIALS AND METHODS.

(a) Pathology.
(b) Bacteriology.
(c) Virology.
(d) Examination for the agent of ornithosis.

Altogether, investigation was made of:

(A) Four carcases of birds which died after serious illness (Nos. M-1 M-2, M-3 and M-4).

(B) One living bird with severe dyspnoea (No. H-1).

(C) Four carcases (Nos. M-5, M-6, M-7 and M-8) of birds which had been killed by the owner because they seemed unwell though the clinical details were not recorded.

(D) Seven living birds with mild serous conjunctivitis or rhinitis associated with slight malaise (Nos. H-2, H-3, H-4, H-5, H-6, H-7 and H-8).

(E) Throat swabs from two birds showing small amounts of clear oculo-nasal discharge and slight hyperaemia of the pharynx

(Nos. HS-1 and HS-2).

(F) Certain tissues, fixed in formol saline, from a bird (No. C-1) from Cambridgeshire which had a marked conjunctivitis as well as indications of systemic upset.

(a) Pathology. Complete autopsy was conducted in all appropriate cases, and, for the purposes of histopathological study, portions of larynx, trachea, lung, heart, liver, spleen, oesophagus, pancreas, small intestine, brain and kidney were fixed in corrosive-formol and embedded in paraffin wax. All subsequent sections were stained by haematoxylin and eosin.

(b) Bacteriology. Bacteriological examination was carried out on freshly killed birds. Blood-agar and MacConkey plates prepared from the trachea, lungs, heart-blood, liver, spleen and intestine were incubated aerobically and anaerobically, as well as under conditions of increased tension of carbon-dioxide.

Examination was made also for the presence of Mycoplasmata, for which purpose plates of Edward's medium and tubes of sloppy agar were inoculated with material from the trachea and lungs and incubated for five days at 37°C. Thereafter, the plates were examined for the presence of colonies characteristic of Mycoplasmata and the contents of the tubes transferred to further plates of Edward's medium, which were incubated for five days and examined as before.

(c) Virology. In the earlier part of the work, virological investigation was limited to examination of suspensions prepared from the lungs and trachea but, in some cases, hepatic suspensions also were investigated. Later a composite suspension was prepared from the trachea, lungs, liver and spleen of each case.

Tissues were macerated in a mortar and pestle with the aid of sterile sand. Sterile normal saline solution was added to yield a 10 per cent suspension which was then admixed with penicillin and streptomycin to a final concentration of 200 units and 200 µg/ml., respectively. Each suspension was inoculated onto the dropped chorio-allantoic membrane of two 10-day embryonated eggs in an amount of 0.2 ml. per egg. The technique used was that described by Burnet and Faris (1942). The inoculated eggs were incubated for four days at 39.5° C., and the membranes were then examined. In all cases, further passage was effected by use of a 10 per cent suspension of the membranes from the first passage. If the membranes of the second passage were normal, the result was recorded as negative but, if any suggestion of abnormality was present, further passages were made until the result was indisputable.

Throat-swabs were obtained by direct application to the palate of the bird whereby as much mucus was removed from the region as possible. The swab was then placed into a vacuum flask, packed with "dry-ice", and transported to the laboratory where it

was immediately transferred to the barrel of a 10 ml. syringe along with 1 ml. of sterile normal saline solution that contained the usual concentration of antibiotics. The saline solution was then repeatedly expelled from and aspirated into the syringe as pressure was applied to the cotton-wool by the piston of the syringe. In that way, the swab was thoroughly washed with the saline, which latter was then used for the inoculation of the dropped chorio-allantoic membrane of two 10-day embryonated eggs, to an amount of 0.3 ml. per egg. Those membranes found to have lesions were fixed in corrosive formol for 24 hours, for which purpose each membrane was coiled round a small glass rod to which it was held by means of fine thread. When the preparation had been embedded in paraffin-wax, the glass rod was withdrawn and thereafter sections were cut and stained by haematoxylin and eosin.

(d) Examination for the agent of ornithosis. A 10 per cent suspension of trachea, lung, liver and spleen was prepared in sterile normal saline solution containing 200 µg. of streptomycin per ml. The technique used for demonstration of the presence of the agent was a modification of that described by Grist and McLean (1964). Six young adult mice were inoculated with the suspension, of which 0.05 ml. was given intracerebrally and 0.5 ml. intraperitoneally. Ten days afterwards, the mice were sacrificed and the brain and spleen removed to provide smears which were then stained by the Jenner-Giemsa method and by Macchiavello's technique and examined

for the presence of elementary bodies. At the same time, a 10 per cent suspension was prepared from the above organs and inoculated into a further six mice by the intracerebral and intraperitoneal routes. After a further 10 days, the brain and spleen were removed and smears examined in the manner just described. A negative diagnosis was recorded if evidence of infection did not emerge after the second passage.

(3) RESULTS.

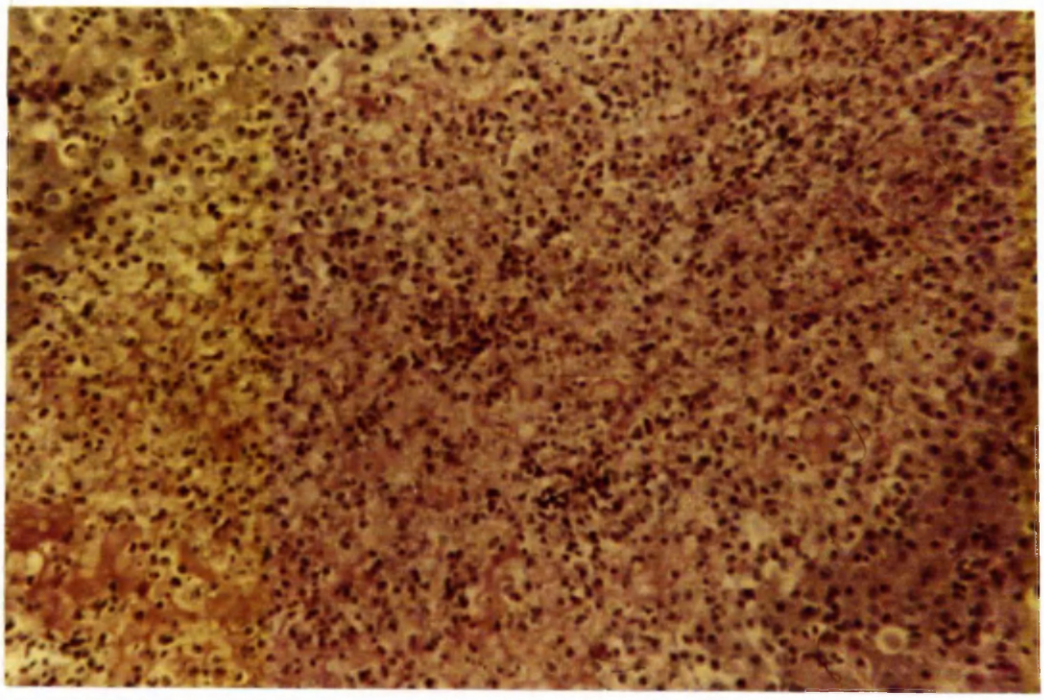
(a) Pathology.
(b) Virology.
(c) Other microbiological examinations.

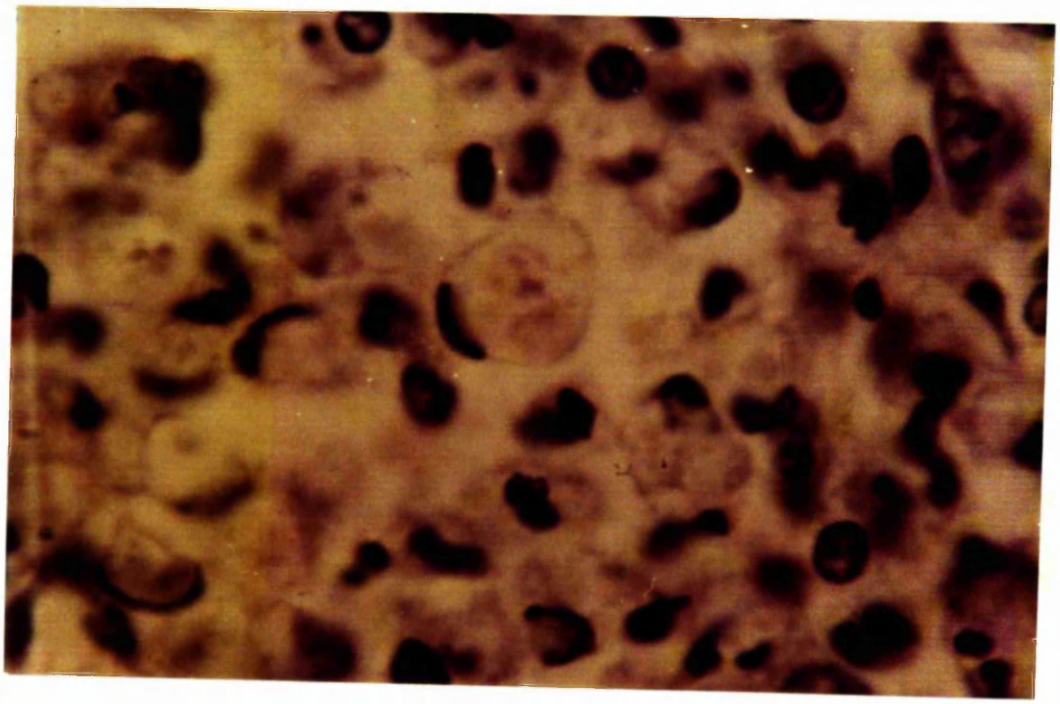
Evidence of virus infection was found in a total of nine cases, two isolations being made from birds of the H-series and five from those of the M-series, with, in two cases (Nos. C-1 and M-2), a diagnosis that rested entirely on the results of histopathological examination. The following account describes the findings in those nine cases.

(a) Pathology. The situations principally affected were the liver, the kidneys and the upper respiratory tract but case No. M-4 presented a severe peritonitis that involved almost the whole of the abdominal cavity and that was attended by adhesions between the liver, the air-sacs and the abdominal wall.

Figure 1. Advanced hepatic necrosis. x 150.

Figure 2. Early hepatic necrosis with crescentic fragments of nuclear membrane. x 1,500.



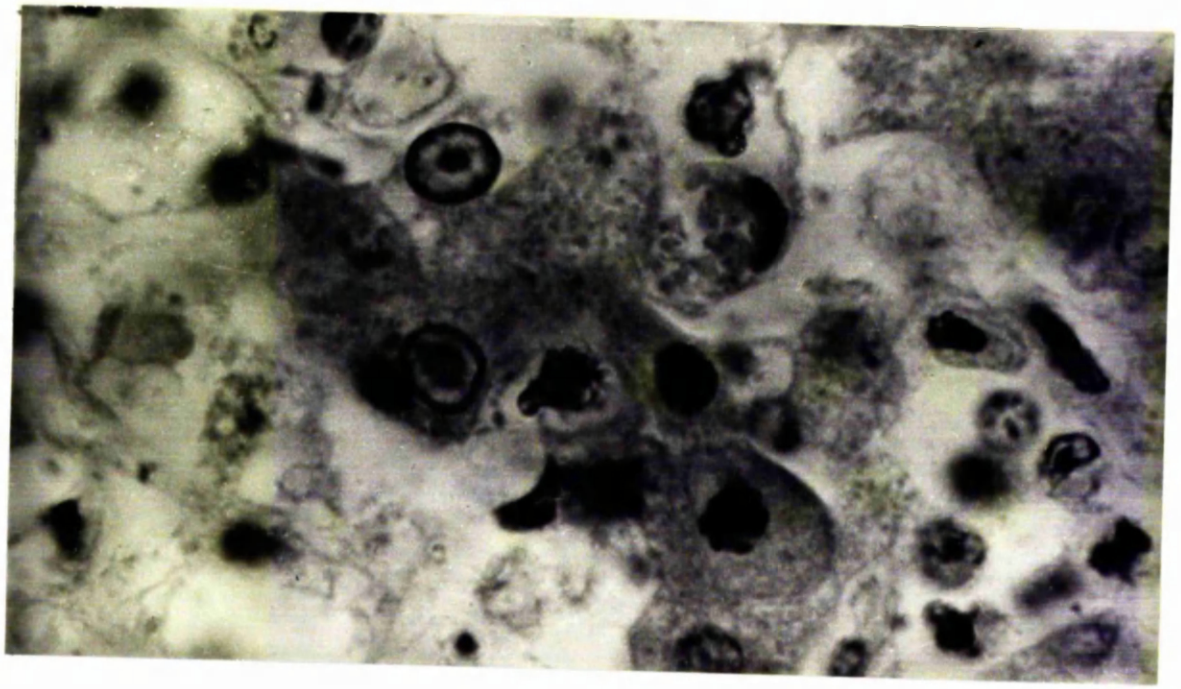


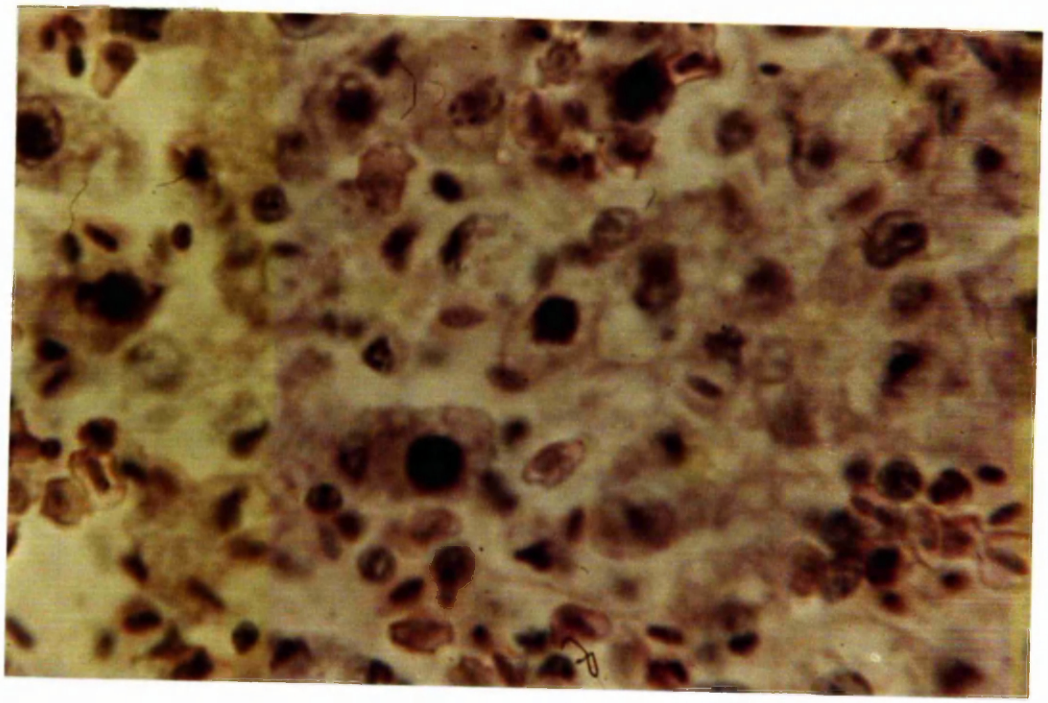
The most common lesion, present in six birds, was a focal necrotizing hepatitis, the severity of which varied considerably from case to case. The foci found in pigeon No. M-4 were scattered throughout the organ and were generally circular in outline, up to 1 cm. in diameter, yellow in colour and soft and dry in consistence. Histopathological examination revealed that they were composed mainly of homogenous eosinophilic material (Figure 1), although the outlines of bile-ducts and of major blood vessels were sometimes discernible amidst the mass of necrotic tissue. Also present were small foci of necrosis that contained conspicuous bicorn fragments of nuclear membranes (Figure 2). Other degenerating nuclei showed what was probably an earlier stage of the same process, namely, accumulation of chromatin at one sector, and almost total loss of staining affinity of the remainder of the nuclear membrane. Heterophil leucocytes and macrophages were present at the margin of lesions, the former being sometimes numerous. The bile-ducts contained much cell debris while the capsule of the liver was covered with fibrin containing many heterophil corpuscles as well as smaller numbers of macrophages.

Intranuclear inclusion bodies were to be found in hepatic cells widely throughout the organ but were most numerous at the periphery of the smaller necrotic foci. In staining reaction, they varied considerably, some being pale pink, others a dull purple-red, and a few almost as basophilic as the nuclear membrane (Figure 3). In most of the affected cells, the cytoplasm

Figure 3. Basophilic and eosinophilic intranuclear inclusions in liver. x 1,500.

Figure 4. Large basophilic inclusions occupying the whole of hepatic nuclei. x 1,200.





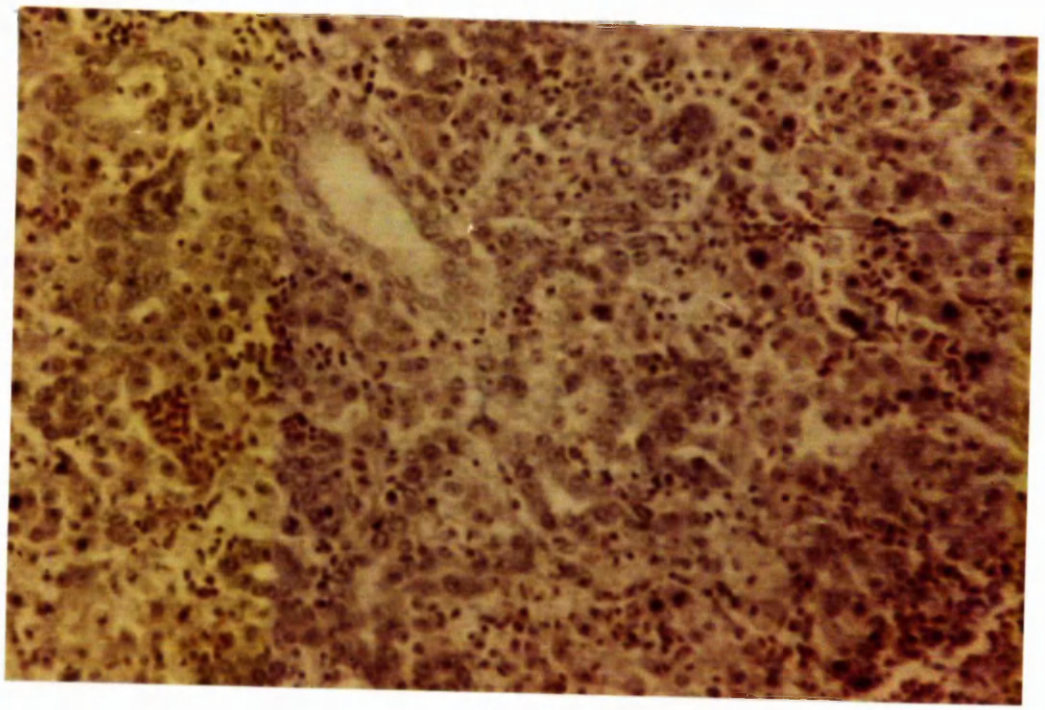
appeared normal and the nucleus remained vesicular with the nucleolus pushed against the nuclear membrane where there was also some accumulation of chromatin. Occasionally the nuclei were shrunken and exhibited marked margination of chromatin, while the cytoplasm was intensely basophilic. In those cases, the inclusion body occupied almost the whole of the nucleus when it was surrounded by a narrow or incomplete 'halo'.

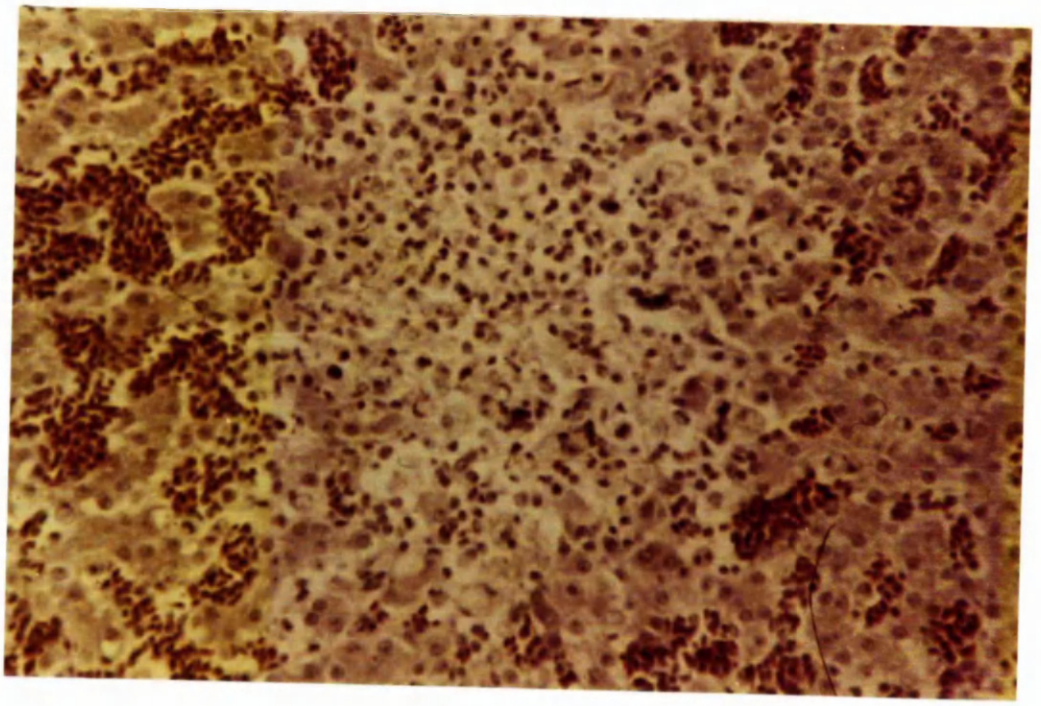
The liver of case No. M-2 was also extensively involved, though the distribution of lesions was quite different. Small semi-confluent foci of necrosis - each generally affecting a mere half dozen cells - were scattered throughout the entire organ. There, too, crescentic nuclear fragments were conspicuous while very large numbers of intranuclear inclusions were present throughout the whole of the surviving parenchyma. Generally, the inclusions were darkly basophilic and only the smallest ones were stained a purple colour. The largest inclusions were impossible to distinguish from the nuclear membrane and the nucleus then appeared as a dense basophilic, cronated mass that projected into the cytoplasm at many points (Figure 4). Most inclusion bodies, however, were of smooth outline and were separated from the nuclear membrane by a distinct 'halo'.

A conspicuous feature associated with the destruction of hepatic parenchyma was hyperplasia of the epithelium of the

Figure 5. Hyperplasia of bile-duct epithelium. x 150.

Figure 6. Focal hepatic necrosis. x 150.





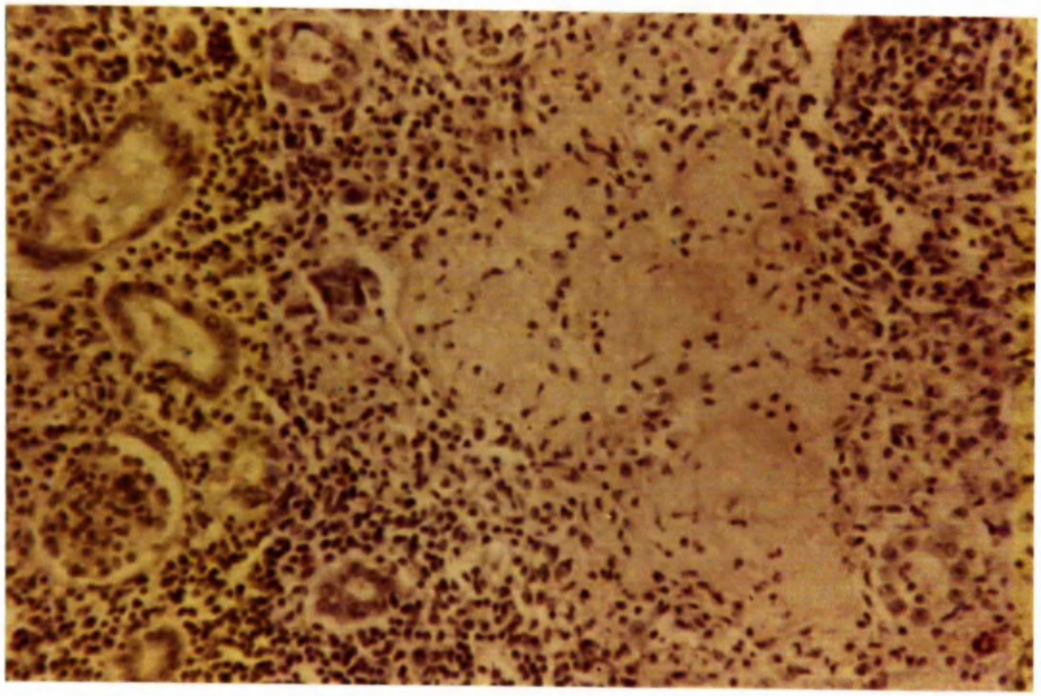
bile-ducts (Figure 5). In some places, the newly formed ducts were patent but, elsewhere, solid masses of epithelial cells were present. Although the sinusoids were sometimes markedly congested, leucocytic activity was limited to the accumulation of small numbers of heterophil leucocytes at the margin of necrotic foci.

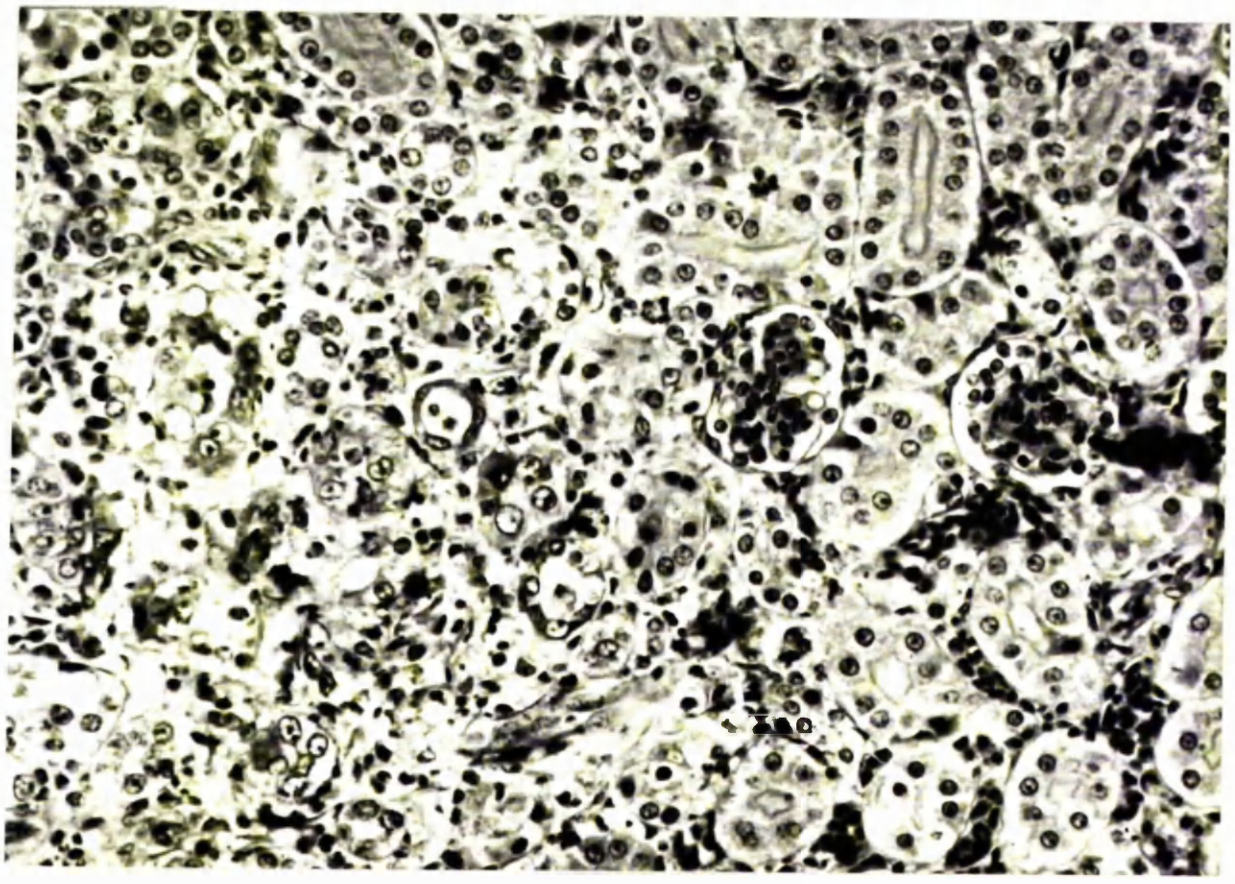
Focal hepatic necrosis was found in pigeons Nos. C-1, M-1, M-3 and M-7 but not in any of these instances were the changes comparable in severity with those of the two preceding cases. In bird No. C-1, such lesions were numerous but small and had not advanced to the amorphous stage (Figure 6). In pigeon No. M-3, affected foci varied in size and structure from homogenous eosinophilic areas that involved several lobules to small intralobular foci consisting of cells in various stages of disintegration. In case No. M-1, the greater part of a lobule was occasionally involved and there were small circumjacent foci present surrounding it but, as a rule, the lesions were small and widely spaced. The foci in bird No. M-7 were likewise minute and scattered. Degenerative changes found in the lesions of all four birds were similar to those already described. Intranuclear inclusions were present in the liver of each bird but were rare in case No. M-7.

Multiple cream-coloured foci - approximately 2 mm. in diameter - were present in the renal cortex of cases Nos. M-1 and M-3. Histopathological examination showed those lesions to be

Figure 7. Advanced renal necrosis. x 150.

Figure 8. Early renal necrosis. x 330.



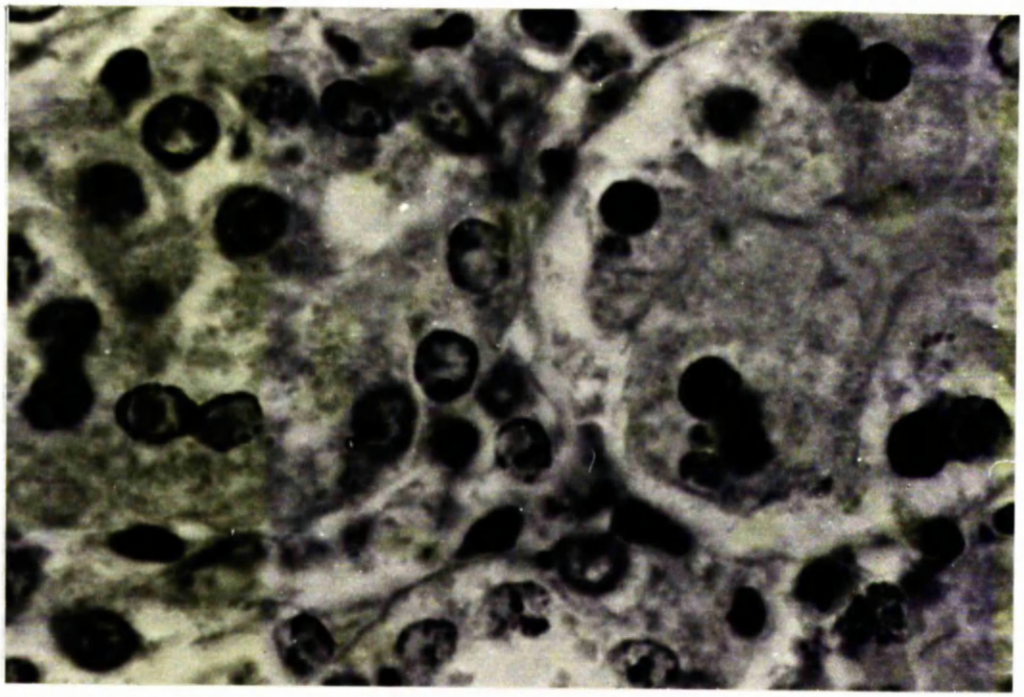


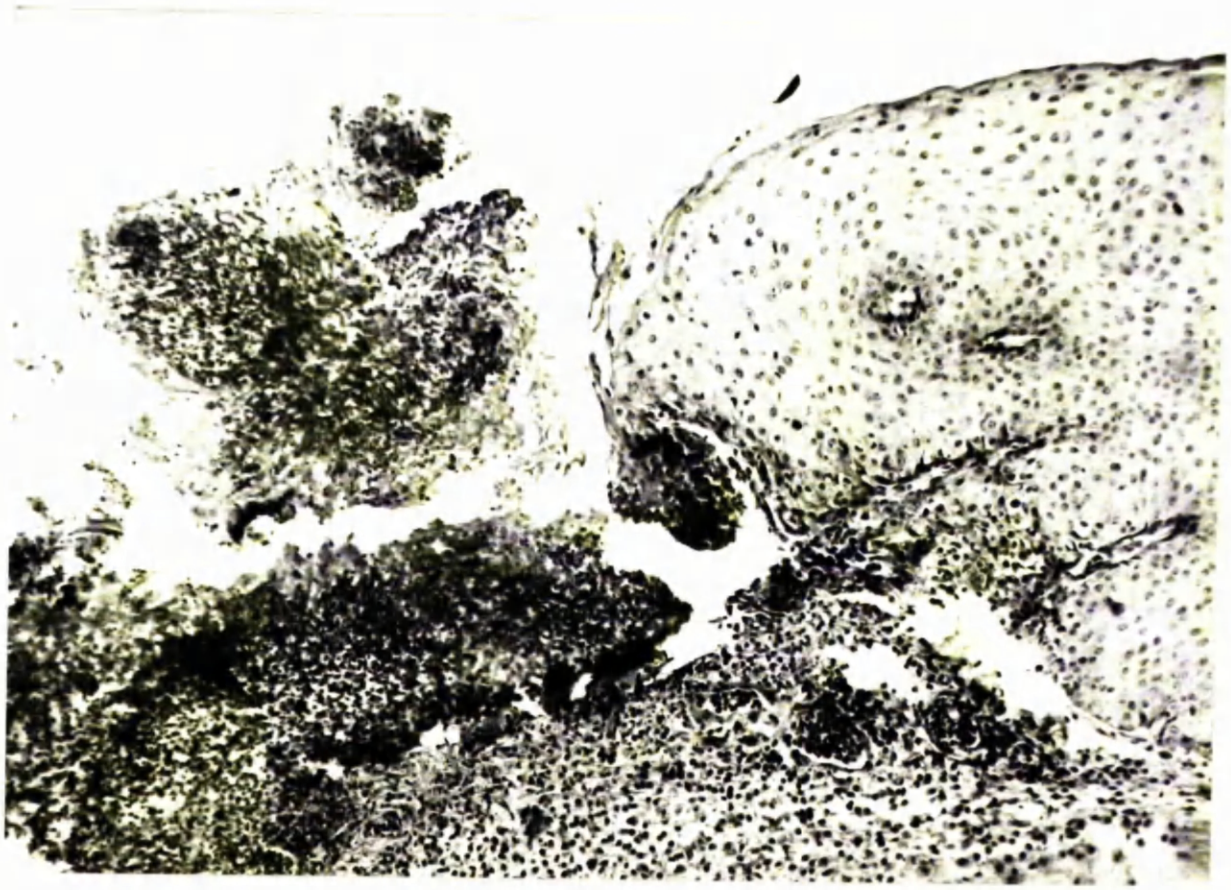
areas of tubular necrosis in which complete disintegration of the renal structure was accompanied by haemorrhage from necrotic blood-vessels as well as by infiltration of lymphocytes and of smaller numbers of heterophil leucocytes (Figure 7). The inflammatory cells were concentrated mainly at the borders of the necrotic foci where, also, surviving tubules were found to contain casts of cell-debris. Fibroblasts also were present at the margin of some lesions, a finding probably indicative of the onset of organization. The presence of smaller foci consisting of degenerating renal cells, macrophages and lymphocytes was also revealed by histopathological examination (Figure 8). Intranuclear inclusions, similar to those already described, were found in tubules at the margin of necrotic foci (Figure 9). Although the number of nephrons so affected was small, inclusions were often present in most of the cells therein.

Although excessive lacrimation was a conspicuous clinical feature in most of the birds and mild serous rhinitis was also sometimes present, overt lesions were found in the respiratory system of only five cases. The outstanding clinical sign in pigeon No. H-1 was severe dyspnoea. The bird stood crouched with the neck extended and the beak open. Respiration was forced and was accompanied by stertorious gasping. At post-mortem examination,

Figure 9. Intranuclear inclusions in renal tubules. x 1,500.

Figure 10. Laryngeal ulcer. x 150.



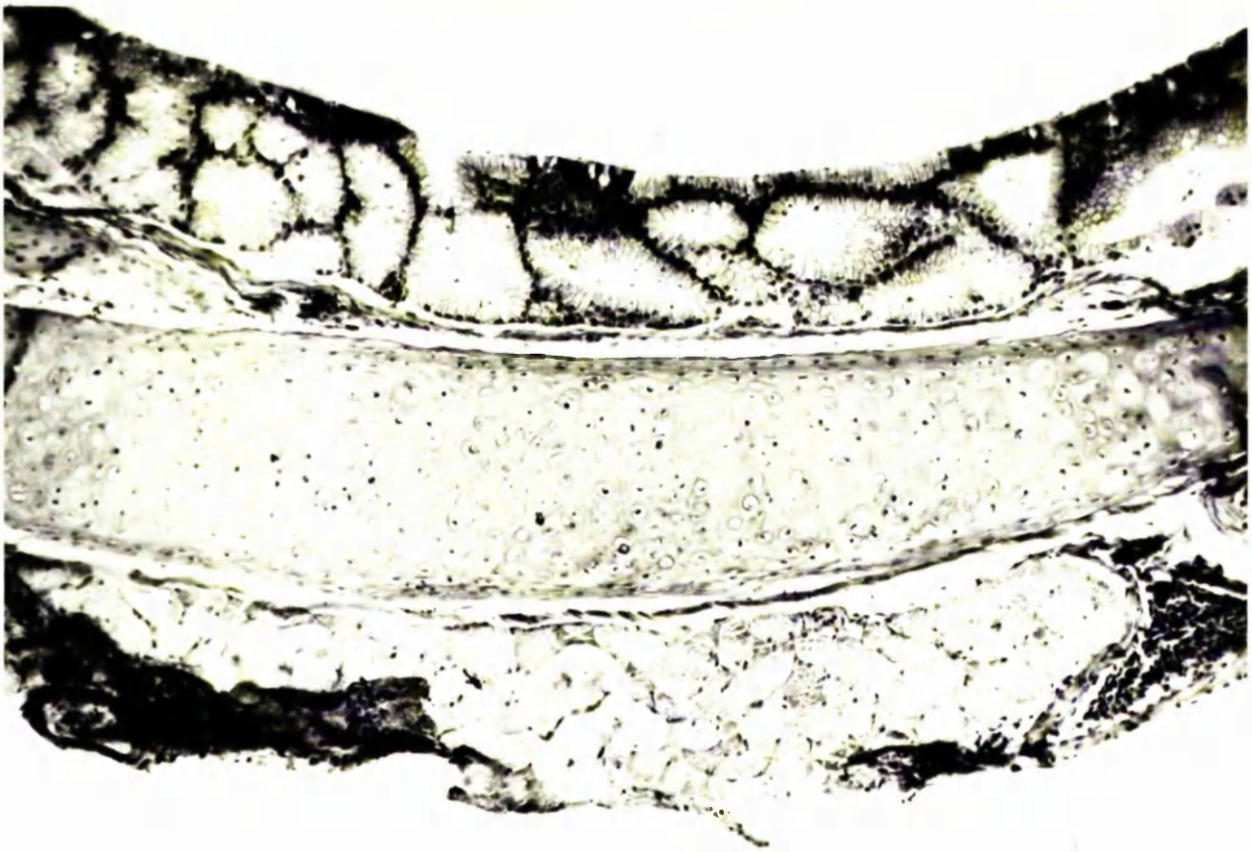


the mucosa of the larynx was found to be coated by a diphtheritic membrane which caused partial occlusion of the lumen of the organ (Figure 10). Small ulcers coated with adherent yellow-brown caseous material occurred also in the pharynx and larynx of cases Nos. M-2 and M-3. Histopathological examination of the lesions in those three birds revealed complete destruction of the epithelium together with vascular engorgement and mononuclear infiltration of the subjacent connective-tissue. The surface of the ulcer was covered with exudate containing cell debris and small numbers of macrophages. In case No. M-3, faintly eosinophilic intranuclear inclusions were discovered in epidermal cells at the edge of the lesion, particularly those of the basal layers.

A moderately severe tracheitis was present in case No. M-1 (Figure 11), though inclusion bodies were not detectable. In places, loss of cilia was associated with slight epithelial hyperplasia, while heterophil leucocytes were visible between individual epithelial cells as well as within the lumen of the tube. The mucus-glands were almost totally destroyed and the greatly thickened mucosa contained accumulations of macrophages and lymphocytes. Similar changes were found in the bronchi of case No. C-1.

(b) Virology. The results of virological examinations were as follows:

Figure 11. Upper: normal pigeon trachea. Lower: tracheitis, case No. M-1. Both x 150.



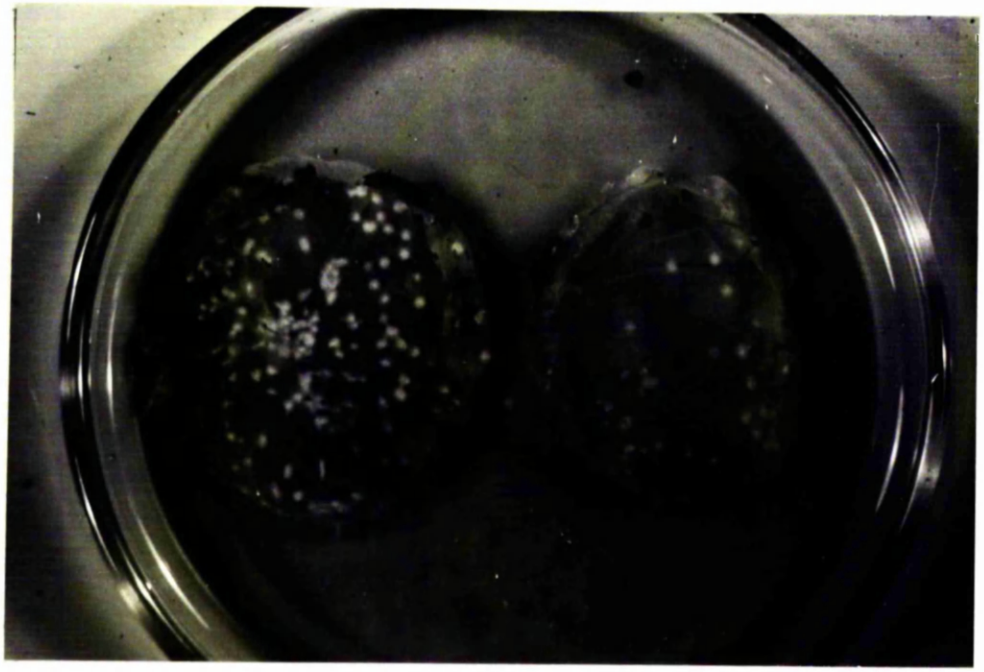
Case number.	Virus isolated from:
H-1	Composite of larynx, trachea and lungs.
HS-2	Throat swab.
M-1	Composite of trachea and lungs.
M-3, M-4, M-7, M-8	Composite of trachea, lungs, liver and spleen.

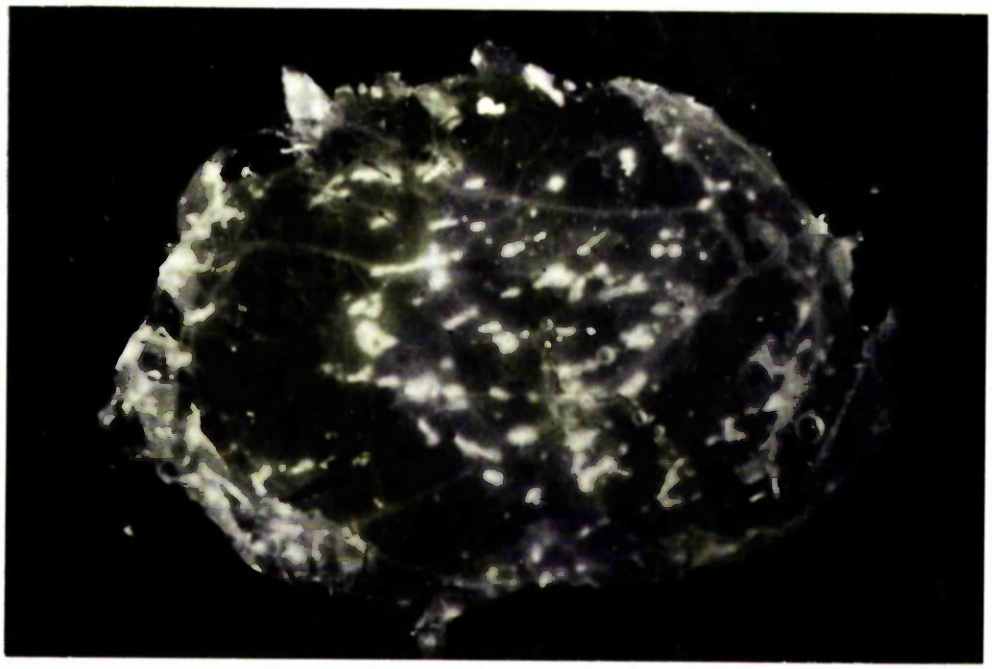
Despite the presence of large numbers of inclusions in the liver of case No. M-2, all attempts at the isolation of virus from that bird proved unsuccessful, both via embryonated eggs and by way of two young pigeons. The latter were inoculated intra-peritoneally with the pooled tissue suspension but neither inclusions nor lesions were found when the creatures were sacrificed six days later.

Numerous pocks appeared on chorio-allantoic membranes inoculated with the tissue-suspensions from cases Nos. M-1, M-3, M-4 and M-8 as well as with the extract from the throat swab taken from case No. HS-2. A central area of thickening and opacity developed in the case of membranes that received suspensions from cases Nos. H-1 and M-7 but, when a second serial passage was effected, discrete pocks were produced. Embryonic death often occurred about the fifth day after infection and yellow foci of

Figure 12. Pocks on chorio-allantoic membrane. x 1.

Figure 13. Pocks on chorio-allantoic membrane. x 2.
Note the linear form of some of the lesions.





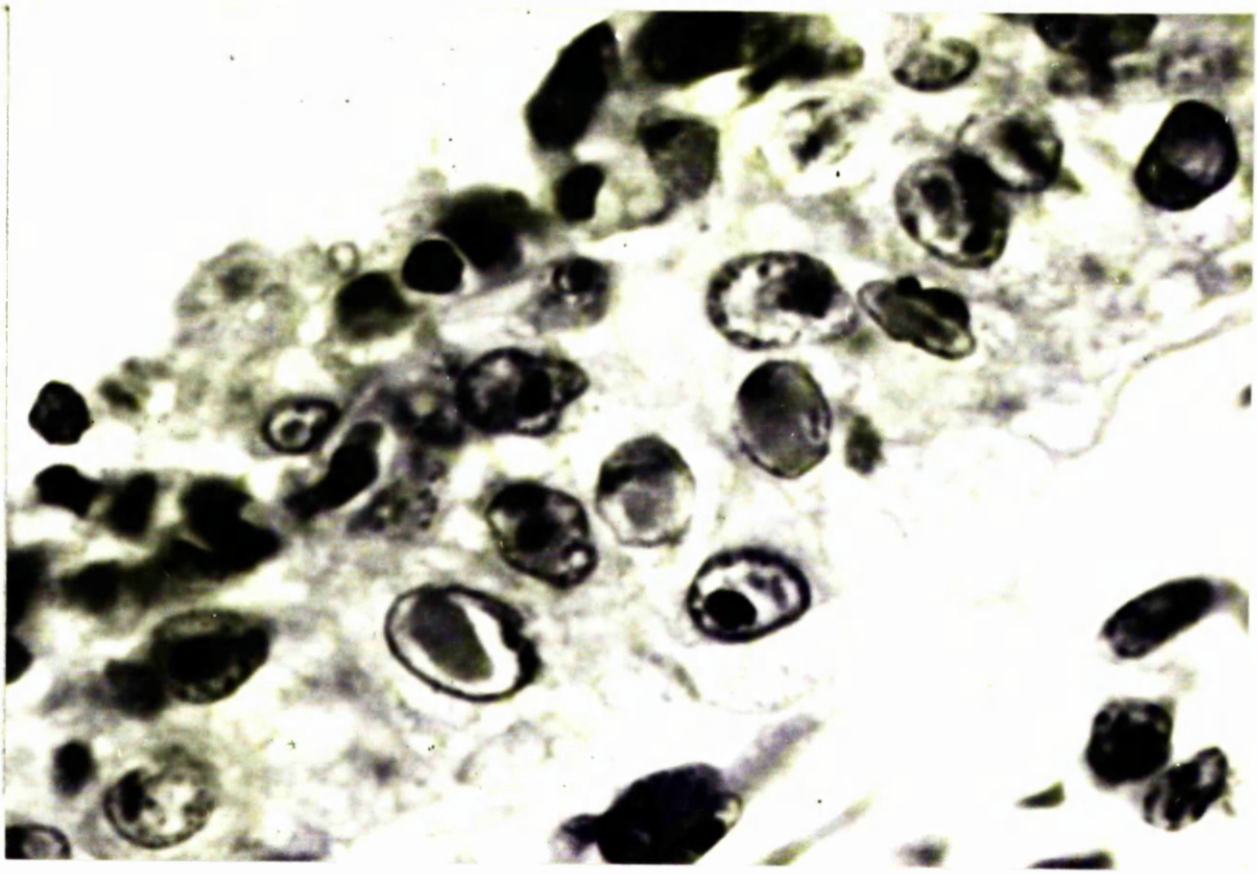
necrosis were perceptible in the liver of sluggish or of dead embryos.

The pocks varied in morphology from discrete circular yellowish-white foci, that reached a size of about 2 mm. by the fourth day, to oval or linear lesions which often coalesced to produce yellowish areas of comma-like or irregular shape (Figures 12 and 13). They were distinctly elevated above the surface of the membrane and a central crater was discernible in many of them.

In membranes fixed and stained on the fourth day after infection, small numbers of weakly eosinophilic inclusions were noted in epithelial cells at the periphery of lesions and were similar in appearance to those illustrated by Smadel et al. (1945) in their description of the pigeon I.N.I. virus. Nevertheless, although the number of pocks increased on passage, the inclusions were never numerous in four or five-day old lesions and were sometimes discernible only after prolonged search. They stained pale pink or dull purple but were never brightly eosinophilic (Figure 14). Marginal accumulation of chromatin was noticeable only in nuclei which contained purple-coloured inclusions. Basophilic intranuclear inclusion bodies were also to be found in parenchymatous cells at the periphery of necrotic foci in the embryonic liver.

(c) Other microbiological examinations. Examinations for mycoplasmata and for the agent of ornithosis proved negative in

Figure 14. Intranuclear inclusions in the chorio-allantoic membrane. x 2,000.



all the seventeen birds studied. Cultural examination occasionally yielded a few colonies of various bacteria, e.g. E. coli, a non-haemolytic Lancefield-negative streptococcus and a small non-motile Gram-negative cocco-bacillus which failed to grow on MacConkey's medium, did not split urea and did not ferment glucose or lactose, but there were not any uniform bacteriological findings.

(4) DISCUSSION.

Virus was isolated from seven out of a total of 18 cases. Typical inclusion bodies were encountered in the tissues of only four of the birds (Nos. M-1, M-3, M-4 and M-7) from which the virus was recovered. Since all strains produced similar inclusions in the chorio-allantoic membrane of embryonated eggs, it seemed probable that the virus responsible for the inclusions in vivo was the same as that grown in embryonated eggs.

In the more severe cases, virus was discovered in organs which showed obvious lesions or to which outstanding clinical signs were related. Thus, case No. M-1 was severely dyspnoeic and virus was isolated from a "pool" of larynx, lung and trachea. Cases Nos. M-2, M-3 and M-4 had a moderate to severe hepatitis and inclusion bodies were present in the liver of each of them. Inclusions were also to be observed in the kidneys of cases Nos. M-1 and M-3 in association with foci of necrosis. It is difficult to avoid the conclusion that the virus was responsible for those lesions.

All attempts at the isolation of the agent of ornithosis were unsuccessful. Thus, none of the mice inoculated intracerebrally with tissue suspensions from the diseased pigeons developed signs of illness and, when they were sacrificed ten days afterwards and smears of brain were examined, elementary bodies were not found. Similarly, signs of illness did not attend passage and preparations of brain were again negative. By contrast, Smadel et al. (1943) found that primary intracerebral inoculation of the agent of ornithosis produced illness that generally terminated fatally and, after a few passages, virulence was so exalted that inoculated mice died in from two to four days. Moreover, the same workers demonstrated elementary bodies in the tissues of the diseased pigeons as well as in those of the inoculated mice whereas, in the present study, elementary bodies proved to be absent from both species of animals. That any false negative result obtained is, therefore, unlikely.

The clinical signs and post-mortem features exhibited by affected birds closely resembled those described for ornithosis by Coles (1940), Meyer et al. (1942), van Vloten (1954), Davis (1955), Fritzsche et al. (1956) and Jansen (1959). Thus, in both diseases, conjunctivitis and respiratory sounds have been manifest clinically and hepatitis found at autopsy so that it is impossible to differentiate the two infections on the grounds of either clinical manifestations or morbid anatomy. The statement by Coles

(1940) and by Jansen (1959) that, whenever a pigeon shows evidence of conjunctivitis, a causal association with the agent of ornithosis should at once be suspected, must now be extended to include the I.N.I. virus as well. Although differential diagnosis may be effected by microbiological or by serological examination, confirmation by histopathological procedure is essential since birds may carry the agent of ornithosis and not show any lesion other than slight splenomegaly (Meyer, 1965).

Certain results of the present investigation were closely similar to those obtained by Smadel et al. (1945). Thus, in the one case of pure intranuclear virus infection described by the latter workers, dyspnoea and diphtheroid lesions of the pharynx were apparent at clinical examination and tracheitis and focal hepatic necrosis were conspicuous at autopsy. Conjunctivitis, dyspnoea, fibrinous peritonitis and hepatic necrosis were found in one of the two cases of dual infection (I.N.I. virus and ornithosis) described by the same authors, and fibrinous peritonitis and hepatic and renal necrosis were present in the other. Each of those abnormalities was noted in at least one bird of the present series. Other similarities lay in the morphology and staining reaction of the inclusions, in the non-pathogenicity for mice of the American and British strains of virus and in the ability of each strain to produce pocks on the chorio-allantoic membrane of embryonated eggs as well as yellowish foci of necrosis in the liver of the embryo.

The only lesion described by Smadel et al., which was not observed in the present investigation was focal pancreatic necrosis.

The disease described by the writer also resembles the conditions reported by Marthedal and Jylling (1966), Jylling (1967) and Lehner et al. (1967). The Danish workers, however, found oesophageal diphtheritic membranes in a high proportion of their cases, a finding unconfirmed by other authors. In the present investigation, diphtheroid lesions were to be observed in the larynx of three cases (M-1, M-2 and M-3) and in the pharynx of two birds (M-2 and M-3), inclusions being associated with the necrotic epithelium in one of them. Jylling (1967) likewise noted the occurrence of inclusions in early lesions of the oesophagus and McFerran (loc. cit.) isolated the B-1 strain from pharyngeal diphtheritic membranes. It is probable, therefore, that the I.N.I. virus is capable of producing diphtheria-like changes in the stratified squamous epithelium of the larynx and upper alimentary system. Prior to the investigations of the above workers, the causation of such lesions was commonly ascribed to Trichomonas gallinae or to pigeon-pox virus. Lesbouyries (1935), who examined a total of 7,226 pigeons and found diphtheritic membranes in no less than 2,534 of them, believed that Trichomonas was responsible for most, if not all, disease of that type. That the protozoan may be associated with such lesions in the absence of the I.N.I. virus has been established by the writer (unpublished observations) but,

clearly, the converse is equally true since, in the present investigation, examination of smears for the presence of motile trichomonads was negative.

Although serological comparison of all the strains of I.N.I. virus so far isolated has not yet been made, it is likely that the infection described in the United States, the British Isles and Denmark is caused by the same virus or by several closely-related viruses. The condition may, therefore, be defined as a specific columbine disease which varies both in severity and in the distribution of lesions but which, in gravest form, is characterized by necrosis of the liver, kidneys, pancreas and spleen, by conjunctivitis, by diphtheroid lesions of the pharyngo-laryngeal location and, possibly, by inflammatory changes in other parts of the respiratory system. The isolation of virus from, or the demonstration of typical inclusion bodies in, pigeons obtained from Cambridgeshire, Northern Ireland and two counties in the West of Scotland suggests that infection is widely distributed in the United Kingdom.

Finally, mention must be made of other avian viruses productive of eosinophilic intranuclear inclusions and pocks on the chorio-allantoic membrane of embryonated eggs and concerned with disease of the respiratory system or liver. The most important is the virus of infectious laryngo-tracheitis (I.L.T.) which primarily attacks the respiratory system of the domestic fowl and pheasant.

Beach (1931), however, found small amounts of the infective agent to occur in the liver and spleen of affected birds while Thorp and Graham (1934) described the presence of microscopic lesions in the liver and kidneys. Helmboldt and Frazier (1963) described intranuclear inclusions in the liver of chickens which were suffering from laryngo-tracheitis. By use of the negative-staining technique of electron microscopy as described by Brenner and Horne (1959), Cruickshank et al. (1963) and Watrach et al. (1963) disclosed that, morphologically, the virus was closely similar to that of herpes simplex.

Andrewes (1964) has suggested that, although confirmation has yet to be received from electron microscopy, various other avian viruses may belong to the herpesvirus group since they produce pocks in embryonated eggs and intranuclear inclusions similar to those of I.L.T. While one of those agents was isolated by French (Andrewes, 1964) from the blood of a seemingly normal pied cormorant (Phalacrocorax melanoleucos), the others have been obtained from cases of hepatitis in various avian species. Pachoco (1930, 1931) described a disease of parrots which was clinically similar to psittacosis. Necrotic foci were found in the liver and spleen of infected birds. Rivers and Schwentker (1932) later showed the virus in question to be productive of eosinophilic intranuclear inclusions. A disease with a similar pathological appearance was noted by the present writer (unpublished observations) in recently-imported parakeets in

the West of Scotland. Green and Shillinger (1936) reported a fatal disease in wild horned owls (Bubo virginianus) which was also characterized by the presence of focal hepatic necrosis and eosinophilic intranuclear inclusions. More recently, Burtscher (1965) studied a similar condition of owls which he termed hepatosplenitis infectiosa strigorum (H.S.I.S.). Virus was isolated from three cases and intranuclear inclusions were found in association with necrotic areas in the liver and spleen of a further eleven birds. Diphtheroid lesions or foci of necrosis were also observed in the mouth, pharynx, oesophagus, glandular stomach, intestines, larynx, lungs and kidneys but inclusions were not detected at any of those sites.

The inter-relationship of the pigeon I.N.I. virus with the above-mentioned agents is not clear. Beach (1931) inoculated I.L.T. virus intratracheally into nine pigeons but was unable to produce any signs of disease. Similar failure to demonstrate pathogenicity on the part of that virus for the pigeon was experienced by Brandly and Bushnell (1934) and Seddon and Hart (1936). French (Andrewes, 1964) failed to infect chicks, pigeons or parrots with his cormorant virus. At first glance, therefore, it would seem that each of those viruses has a narrow host range, which view is supported by the finding of Green and Shillinger (1936) that their owl virus was transmissible to a screech owl (Otus) but not to a barred owl (Strix). On the other hand, since the immune status of

the experimental birds was not investigated prior to experimental infection, it may well be that the non-appearance of lesions in another species was due to immunity resulting from previous infection.

As Andrewes (1964) has remarked, the ability of the pigeon I.N.I. virus to produce pocks in embryonated eggs and intranuclear inclusions typical of the herpesvirus group suggests that its provisional incorporation within that group may not be inappropriate, but information concerning its morphology and nucleic acid composition is essential before its place is ultimately determinable. Of more immediate import, however, is proof of the pathogenicity of the virus for the pigeon together with an account of the laboratory methods available for its growth and assay.

SECTION II: EXPERIMENTAL DISEASE.

(1) INTRODUCTION.

In order to fulfill the third of Koch's Postulates, resort was had to intraperitoneal inoculation of the virus into young pigeons and day-old chicks. Thereafter, the fluorescent antibody technique was employed to determine whether, or not, the distribution of virus antigen corresponded with that of any lesions forthcoming in the experimental pigeons.

A supplementary experiment involved direct introduction of virus into the larynx of pigeons with the object of proving that it was capable of evoking necrosis and ulceration of stratified epithelium and other lesions in the respiratory system.

(2) MATERIALS AND METHODS.

Nineteen pigeons, about eight weeks of age, were inoculated intraperitoneally with approximately 10^5 P.F.U. of the M-3 strain of virus. The latter had been thrice passaged in embryonated eggs and five times in cell cultures prepared from whole chick-embryos (see Part Three). Eight more pigeons from the same source were kept as controls. Additionally, six White Leghorn chicks were inoculated intraperitoneally with a similar dose of virus within twelve hours of hatching, while four more from the same batch of eggs were maintained as controls.

Inoculated pigeons and chicks were killed by cervical dislocation at three, four, six and seven days after inoculation. Control birds were sacrificed on each occasion. Portions of tissue taken from all pigeons were fixed in mercuric chloride-formol and paraffin-wax sections, cut at six microns, were stained by haemalum and eosin.

Immunofluorescence studies were carried out on the pigeon tissues by means of hyperimmune serum to the M-3 strain of I.N.I. virus prepared in a rabbit by the method to be described in Part Three. The prepared serum served to provide the first layer of the indirect method of the fluorescent antibody technique (Coons et al., 1942) while the second layer consisted of goat anti-rabbit globulin conjugated with fluorescein isothiocyanate (Difco). Before use, the conjugate was adsorbed for two hours at 4°C. with 100 mg. of pigeon liver tissue powder per 1 ml. of conjugate.

Small portions of liver and pancreas were placed on the sides of Pyrex test-tubes, rapidly frozen in a dry-ice alcohol mixture at -70° C. and stored at -30° C. for later use. The blocks were removed from the tubes when required, placed on chucks in a Pearse cryostat and cut at -20° C. into sections 3-5μ. thick. The sections were fixed in acetone for 15 minutes and exposed to the antiserum for 30 minutes in a moist chamber at room temperature. They were then washed in phosphate-buffered saline (P.B.S., pH 7.1) for ten minutes and, finally, washed again in P.B.S. The stained sections were then mounted in phosphate-buffered saline and

examined under a Reichert "Zetopan" fluorescence microscope equipped with exciter filter UGI/1.5 mm., barrier filter GG9/1 mm. and a darkground condenser. Control sections comprised (a) sections of tissues from uninfected pigeons stained as above, and (b) infected tissues exposed to normal rabbit serum in place of the hyper-immune serum.

For fluorescence photomicrography, a "High-Speed Ektachrome" (160ASA) colour film was used. Black and white prints were obtained from the colour transparencies.

In the supplementary experiment, five feral pigeons, of unknown age and immune status, were inoculated by the intra-laryngeal route with approximately 10^3 P.F.U. of strain HS-2. The latter had been thrice passaged in embryonated eggs and eight times in tissue-culture. All five birds were sacrificed on the sixth day along with ten control birds obtained from the same source. The larynx, trachea, lungs and liver were fixed, stained with haemalum and eosin and examined as previously described.

(3) RESULTS.

The main post-mortem changes were found in the pancreas, liver and mesentery and consisted of discrete white focal lesions located in the pancreas and liver together with a deposit of fibrin on the peritoneal surfaces.

TABLE 1

RESULTS OF INTRAPERITONEAL INOCULATION

Pigeon No.	Day Examined	Pancreatic Necrosis	Hepatic Necrosis	Peritonitis	Inclusions		Virus Antigen	
					Pancreas	Liver	Pancreas	Liver
1	3	+	+	+	-	+	-	N.E.
2	3	+	-	+	+++	-	+++	+
3	3	+	-	+	-	-	N.E.	N.E.
4	3	-	-	-	-	-	N.E.	N.E.
5	4	-	-	+	-	-	N.E.	N.E.
6	4	+	-	++	+	+	-	-
7	4	+	-	+	-	-	-	-
8	4	-	-	+	-	-	N.E.	N.E.
9	4	+++	-	++	+++	-	+++	+
10	6	++	-	++	+++	-	+++	+
11	6	+	-	+	-	-	N.E.	N.E.
12	6	+	-	+	-	-	N.E.	N.E.
13	6	+++	-	+++	+	+	++	+
14	6	+	-	+	+	-	+	+
15	7	-	-	+	-	-	N.E.	N.E.
16	7	+	-	+	-	+	N.E.	N.E.
17	7	+	-	++	-	-	N.E.	N.E.
18	7	+	+	++	-	+	N.E.	N.E.
19	7	+++	+	+++	+	+	N.E.	N.E.

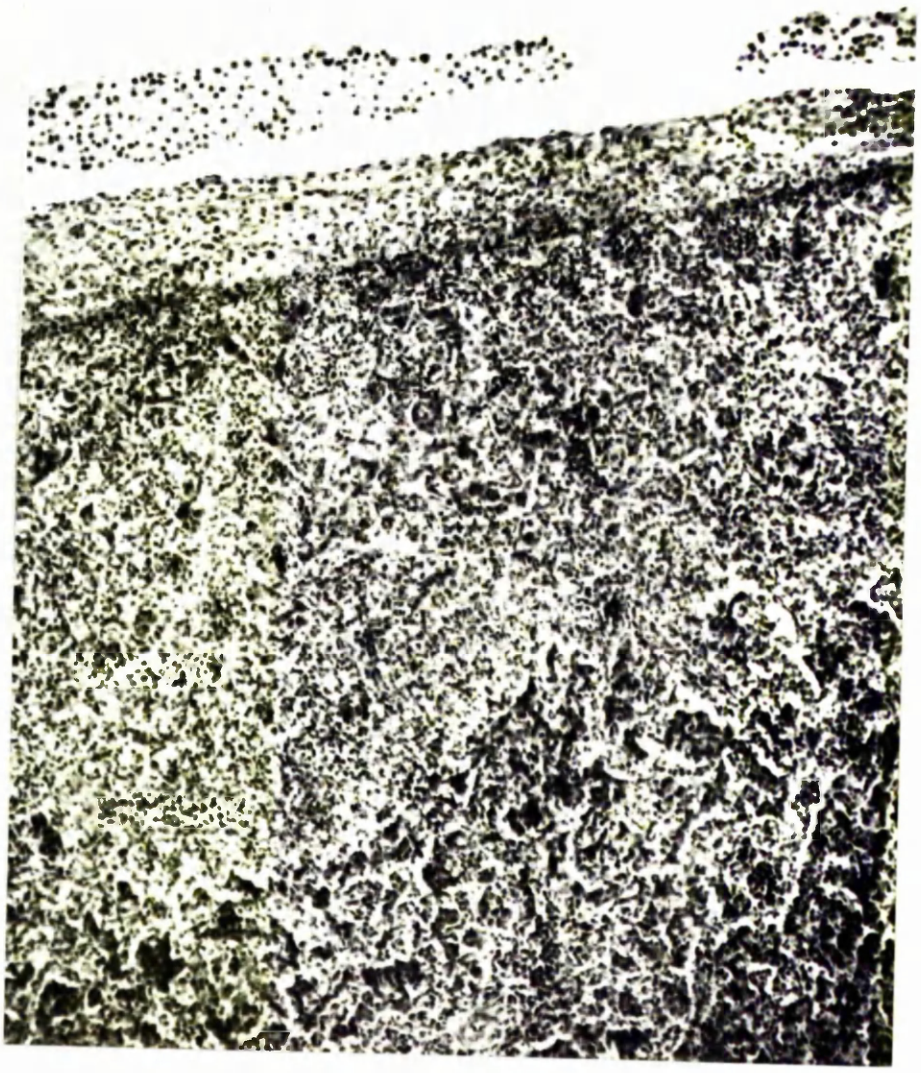
N.E. = Not Examined.

Histological abnormalities are summarized in Table 1, from which it may be seen that the commonest lesion was an acute pancreatitis. The latter varied from slight mononuclear infiltration to areas of frank necrosis associated with marked accumulation of neutrophils and macrophages (Figure 15). These lesions were especially prominent in the sub-capsular region of the organ. Large eosinophilic intranuclear inclusions were found in acinar cells adjacent to the areas of necrosis (Figure 16). Evidence of pancreatitis was sometimes found in pigeons which did not manifest any lesions at autopsy.

Hepatic lesions, consisting of focal necrosis, were present in only three birds. In those animals, and in three other cases as well, intranuclear inclusions were encountered in the parenchymal cells.

Peritoneal changes were characterized by the presence of a fibrinous exudate with numerous mononuclear and heterophilic leucocytes. Lymphoid hyperplasia was evident both in the spleen and the intestine but significant alterations were not detectable in the other organs of the body. By means of immunofluorescence, specific viral antigen was demonstrated in many of the nuclei of pancreatic acinar cells and also in those of a few hepatic cells. Antigen was located either on the nuclear margin or uniformly throughout the nucleus (Figure 17), but was not to be found in any of the control birds.

Figure 15. Pancreatic necrosis and peritonitis. x 75.



Gross and histopathological abnormalities were not present in any of the inoculated or control chicks.

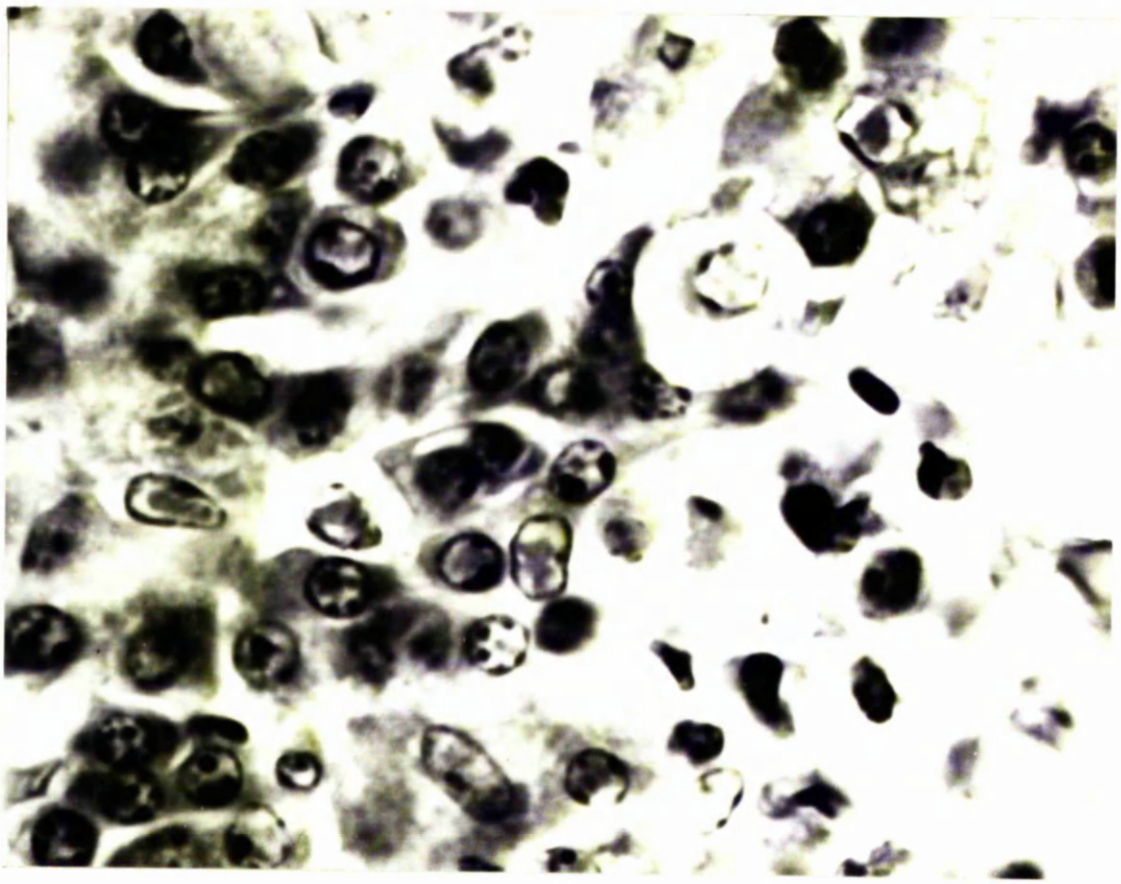
Multiple microscopic foci of epithelial necrosis were detectable in the larynx of two of the five pigeons killed six days after intralaryngeal inoculation of the virus. Some foci extended from the stratum germinativum into the underlying connective-tissue. Both eosinophilic and basophilic intranuclear inclusions were evident throughout all layers of the epithelium (Figure 18). Intranuclear inclusions were to be found in the liver of one of these birds but not in the lungs where, however, perivascular accumulation of macrophages and lymphocytes was prominent. One large vessel showed a necrotic vasculitis, with invasion of the tunica media by macrophages, and perivascular "cuffing" was sometimes conspicuous even around the smaller arterioles. Similar changes, again occurring in the absence of inclusions, were found in the lungs of one of the three inoculated birds which did not develop laryngeal lesions. The respiratory system of all ten control birds remained normal.

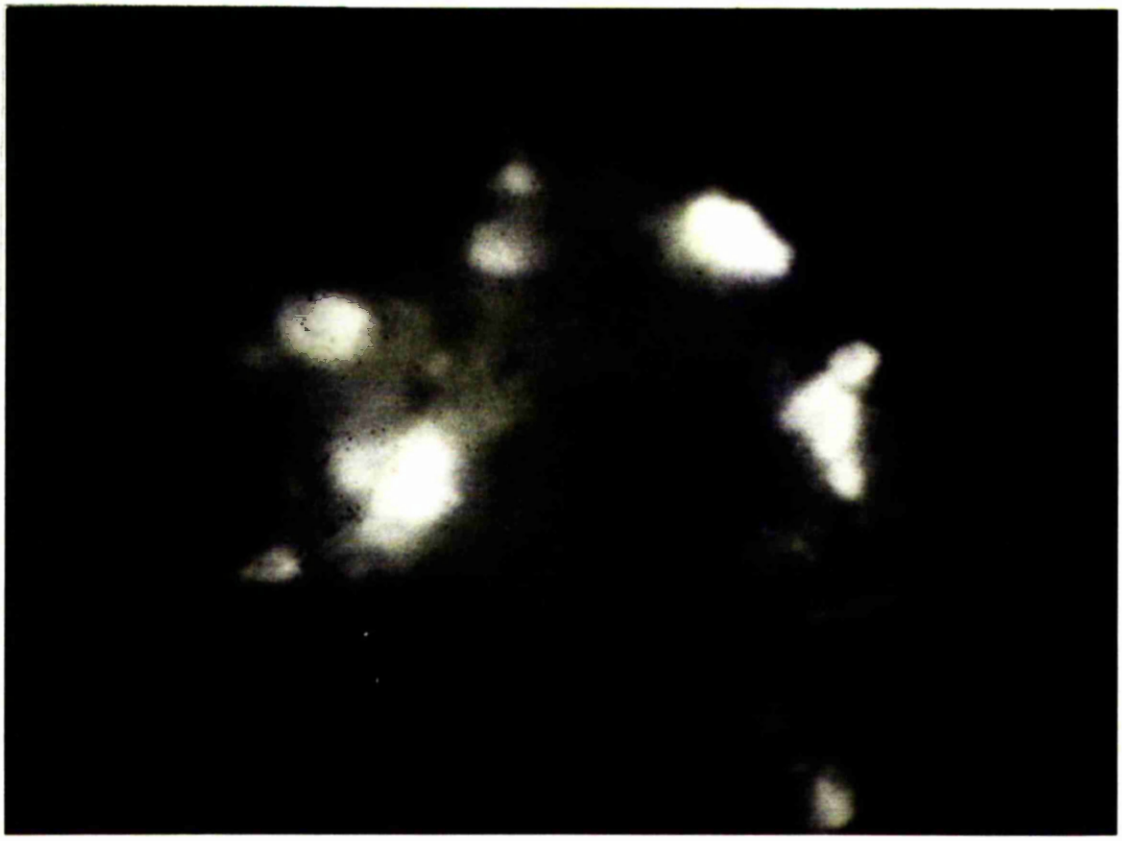
(4) DISCUSSION.

The results presented proved that the M-3 strain of virus was pathogenic for pigeons after administration by the intraperitoneal and intralaryngeal routes. The main features of the experimental disease were pancreatitis, peritonitis and, in some cases, hepatic necrosis. Eosinophilic intranuclear inclusion bodies

Figure 16. Intranuclear inclusions in the pancreas. x 1,500.

Figure 17. Viral antigen in the pancreas. x 1,500.



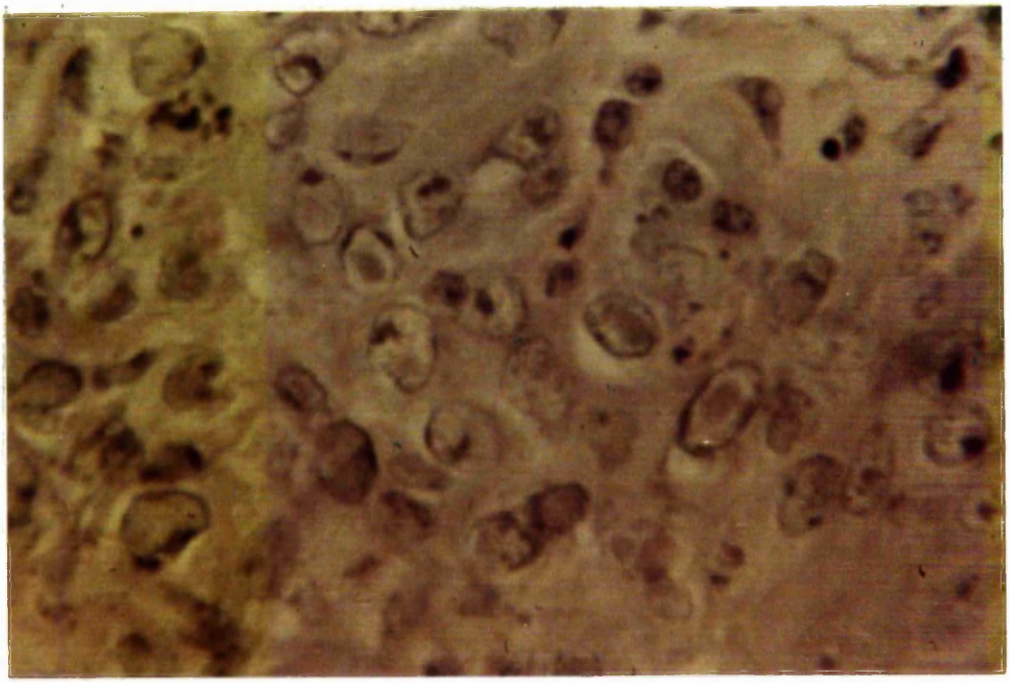


and specific viral antigen were demonstrated in, and adjacent to, those necrotic areas, findings which the writer considered to be of greater significance than would have been attachable to re-isolation of the virus from the experimentally infected birds.

Pancreatic necrosis was not observed in the spontaneous disease already described but was a prominent feature in the experimental disease now under report, as it had been in several of the natural cases described by Smadel et al. (1945) and by Lehner et al. (1967). Although pancreatitis has been recorded in laboratory animals experimentally infected with the virus of foot-and-mouth disease (Platt, 1956:1958) as well as occasionally in natural disease (Venezuelan equine encephalitis, Kissling et al. 1956), the occurrence of the lesion is uncommon. The prominence of lesions on the surface of the pancreas in the present investigation suggested direct penetration of the organ by virus from the abdominal cavity. The view that a similar sequence of events occurred in mice inoculated intraperitoneally with the virus of herpes simplex has already been advanced by Beswick (1958).

Intranuclear inclusions were numerous in parts of the epitholium adjacent to the laryngeal lesions present in two out of the five pigeons employed in the supplementary experiment. That the lesions were produced by the pigeon I.N.I. virus thus seems certain. Their microscopic size suggested that they were of recent

Figure 18. Intranuclear inclusions in the larynx. x 1,200.



origin. Moreover, in view of the total absence of similar lesions in ten control pigeons obtained from the same source, they probably resulted from the intralaryngeal introduction of virus six days previously. If that surmise is correct, it may be concluded that the virus is capable of establishing lesions after topical application as well as after direct inoculation into tissues. Similarly, the presence of intranuclear inclusions in the liver of one of these experimentally infected birds indicates that dissemination of virus may follow infection of respiratory or alimentary epithelium.

PART 1: SUMMARY AND CONCLUSIONS.

Evidence of I.N.I. virus infection was found in nine pigeons suffering from an illness clinically similar to ornithosis. The virus was isolated from seven of the birds by way of the chorio-allantoic membrane of embryonated hens' eggs, where it produced small, yellowish-white, crateriform pocks. Histopathological examination of those lesions and of foci of necrosis in the embryonic liver revealed the presence of intranuclear inclusion bodies identical to those seen in tissues of the affected birds. Inclusions were found in the liver of two further cases from which the virus was not isolated. All attempts at the demonstration of the agent of ornithosis were unsuccessful.

The main changes found in the naturally-infected pigeons were hepatic and renal necrosis, peritonitis and diphtheroid foci in the pharynx and larynx. Experimentally, the virus produced pancreatic as well as hepatic necrosis and peritonitis when administered intraperitoneally, and necrosis of the stratified squamous epithelium of the larynx in birds infected by the intralaryngeal route. By means of immunofluorescence, virus antigen was revealed in necrotic foci within the pancreas of experimentally-infected pigeons, a finding which clearly demonstrates the pathogenicity of the I.N.I. virus for that species. By contrast, the agent failed to produce lesions in day-old chicks. That it may play a part in disease of the domestic fowl, therefore, seems highly unlikely.

PART 2. GROWTH AND ASSAY OF VIRUS IN EMBRYONATED EGGS.

(1) INTRODUCTION.

The paucity of inclusions in chorio-allantoic membranes harvested four days after inoculation suggested that higher yields of virus might be obtainable at an earlier stage of the infection. Accordingly, serial studies on the development of the pocks were undertaken. At the same time, inoculation via the amnion, the allantois and the yolk-sac was investigated in an effort to establish the most effective route of inoculation and the optimal time for harvesting.

The investigation also provided the means for comparison of the pocks produced by the pigeon I.N.I. virus with those formed by the viruses of pigeon-pox and avian infectious laryngo-tracheitis (I.L.T.) since it was felt that the morphology of the lesions might prove a useful feature in the differentiation of the three viruses.

A further aim was to determine whether or not virus could be accurately titrated by means of an enumeration assay based on the number of pocks formed on the chorio-allantoic membrane.

(2) MATERIALS AND METHODS.

All the fertile eggs used in the investigation were obtained from a single flock of White Leghorn hens.

(a) Methods of Inoculation. The chorio-allantoic membranes of 12-day embryonated eggs were inoculated with the H-1, HS-2, M-3, M-4, P-5 and B-1 strains of pigeon I.N.I. virus.

The P-5 strain was obtained by courtesy of Dr. E. B. Jackson, formerly of Dr. Smadel's team, and was contained in material consisting of a freeze-dried suspension of infected chorio-allantoic membranes from the 64th egg-passage, which had been held at -20°C. for 13 years. The B-1 strain was isolated and supplied by Dr. J. B. McFerran of the Veterinary Research Division of the Northern Ireland Ministry of Agriculture and has already been mentioned in Part One.

For comparative purposes, eggs of the same age were inoculated with the 102nd. egg-passage of the B.E.E. strain of avian I.L.T. virus, kindly supplied by Dr. F. T. W. Jordan of the University of Liverpool Veterinary School as well as with a tissue-culture adapted strain of pigeon-pox virus provided by Mr. W. Baxendale of the Wellcome Research Laboratories.

The method used for the inoculation of the chorio-allantoic membrane was that recommended by Westwood et al. (1957) since those workers claimed that that technique produced the minimum of damage to the embryonic and extra-embryonic tissues, and hence provided greater uniformity of results. The main features of the method may be summarized as follows:-

(1) The eggs are used on the twelfth day of incubation.

(2) One incision is made in the shell for the purpose of dropping the membrane and a second for introducing the inoculum.

(3) The membranes are dropped with the aid of McIlvaine's citric acid / di-sodium phosphate buffered distilled water, 0.004 M., pH 7.2, (Clarke, 1928), heated to 50° C.

(4) After the membrane has been dropped, eggs showing signs of haemorrhage are discarded and the remainder replaced in the incubator at 37° C. for one to four hours prior to inoculation.

(5) The virus is suspended in McIlvaine's buffer at refrigerator temperature and without the addition of serum.

Amniotic inoculation of 14- to 15-day embryonated eggs was carried out with pigeon I.N.I. strains HS-2, M-3, M-4 and M-7, the method used being that described by Busby et al. (1964).

The allantoic cavity of 10-day eggs and the yolk-sac of seven-day embryos were inoculated with strain M-3, again according to the methods advocated by Busby et al. (1964).

In all cases, the virus subjected to study had been passaged through chick embryo tissue-culture as described in Part Three, the number of passages varying from three to fifteen.

(2) Examination of inoculated eggs. The embryos together with the associated membranes were removed from infected eggs at daily intervals from the first to the fourth day following inoculation by the chorio-allantoic route and were fixed in mercuric-chloride-

formol for one day. Representative blocks were embedded in paraffin-wax and sections were stained with haemalum and eosin as described in Part One.

Two chorio-allantoic membranes, inoculated with sufficient of the HS-2 strain of virus to produce confluent pocks and of uniform size and appearance as well as devoid of non-specific lesions, were harvested at daily intervals from the first to the fourth day after inoculation and stored at -40° C. for one to two weeks. The virus content of the membranes was then titrated by the plaque-assay method described in Part Three.

Similar procedures were carried out in eggs inoculated with the M-3 strain via the amnion, the allantois and the yolk-sac, the details being as follows:-

Route of inoculation.	Tissues for histopathology.	Tissues for virus titration.
Amniotic	Embryo	Amniotic fluid.
Allantoic	Chorio-allantoic membrane.	Allantoic fluid.
Yolk-sac	Embryo	Yolk-sac wall.

In the case of eggs inoculated by the amniotic and allantoic routes, preliminary experiments were designed to determine the effect of the virus on the embryo and the content of virus in the fluids was not ascertained. In later studies, histopathological examinations were accompanied by titrations of virus. Enumeration

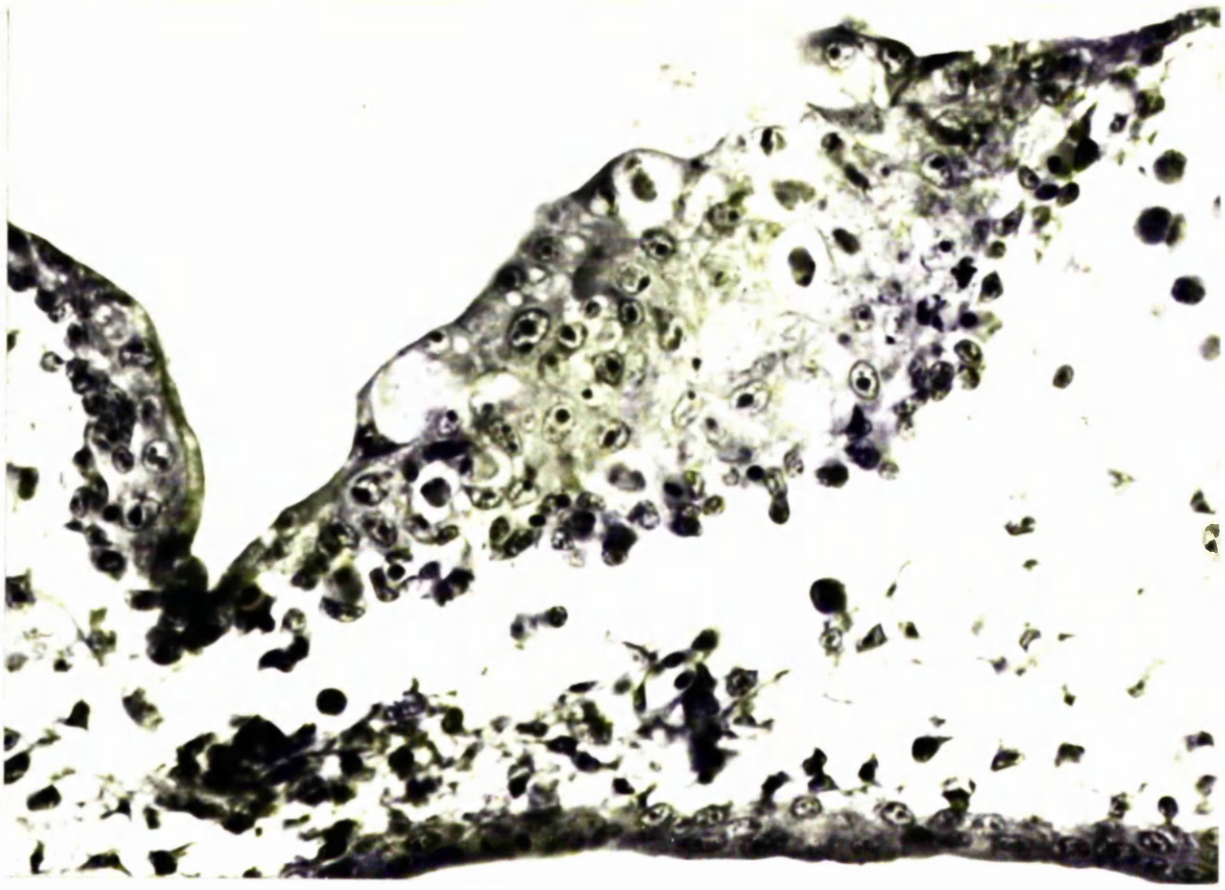
assays, based on the number of pocks produced, were set up with fourfold and 0.5 $\log_{10}$ dilutions of strains H-1 and B-1. The counts were carried out on the third day of incubation in order to minimise error resulting from the appearance of secondary foci.

(3) RESULTS.

All strains of pigeon I.N.I. virus produced identical pocks. Minute white foci appeared on the first and second day and rapidly increased in size and number until the fourth day, by which time they appeared as small, raised, whitish-cream pocks (0.5-1 mm. in diameter), often with a central crater, or as linear extensions of larger cream-coloured plaques which probably resulted from coalescence of smaller lesions. Those changes were accompanied by thickening and opacity of the uninfected portions of the membrane. By the third day the embryos had generally become sluggish, and death often supervened on the fourth day.

Pocks also appeared two days after infection with I.L.T. and pigeon-pox viruses. Those produced by the former agent reached a size of 6-8 mm. by the fourth day, when they occurred as large, circular, flat, greyish ulcers that were sharply demarcated from the surrounding uninfected tissue. The pigeon-pox lesions, on the other hand, were distinctly elevated above the surface of the membrane and were not ulcerated. They possessed opaque white centres which extended outwards through various shades of whitish-grey to

Figure 19. Intranuclear inclusions and ectodermal hyperplasia. x 530.

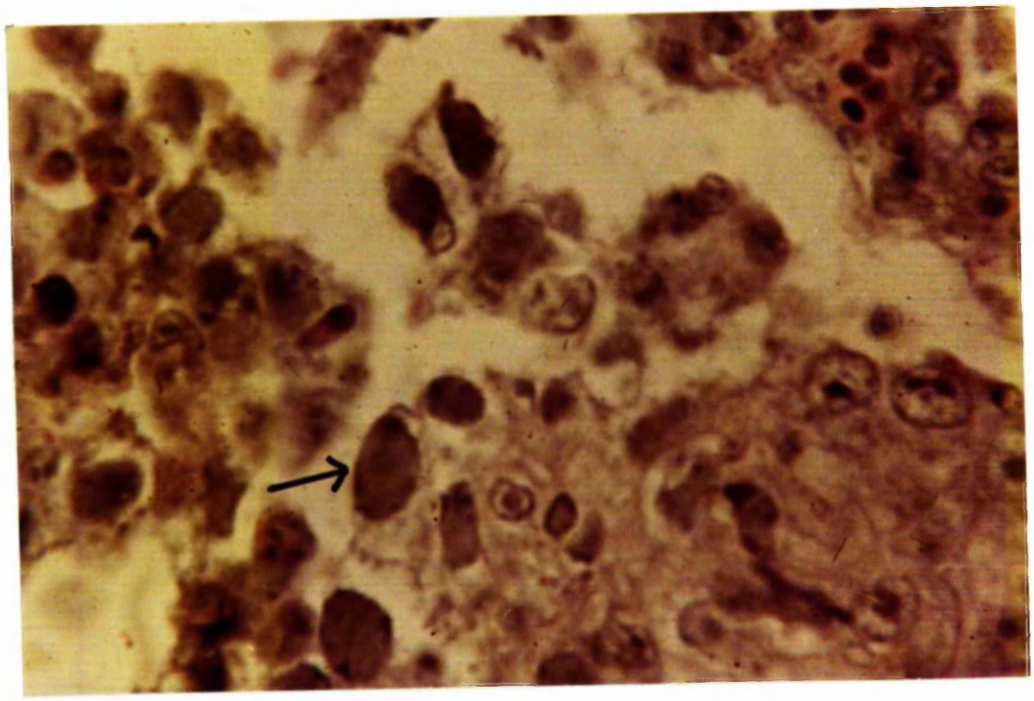


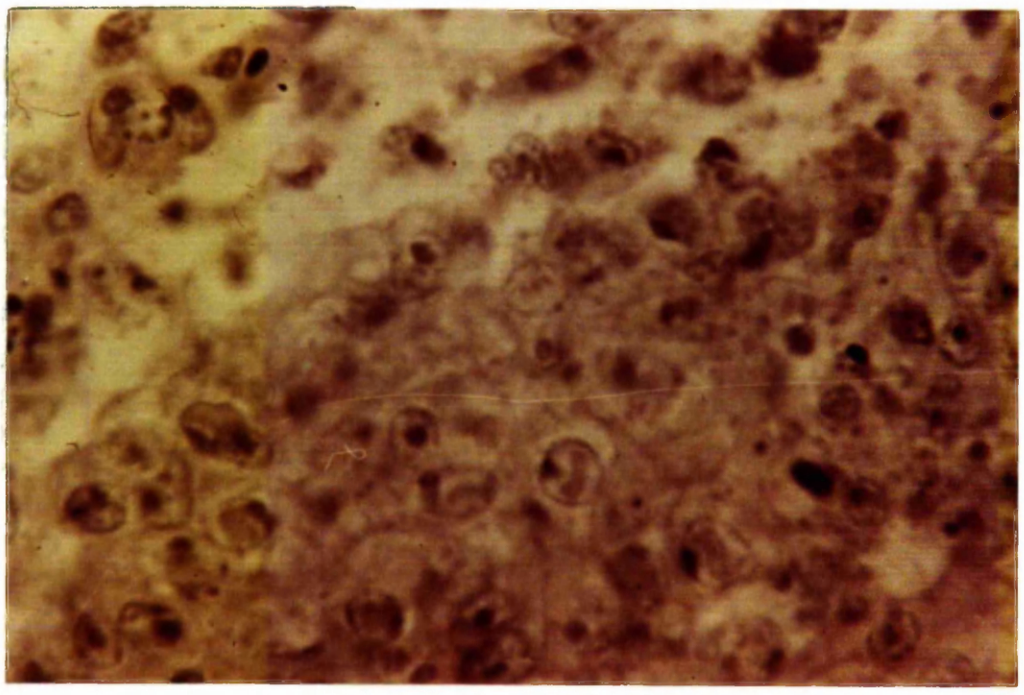
merge with the normal tissue. They were circular in shape and fairly uniform in size, approximately 2-3 mm. in diameter by the fourth day. Embryonic deaths were not observed in eggs infected with either virus.

Histopathological examination of chorio-allantoic membranes, infected 24 hours earlier with the pigeon I.N.I. virus, revealed foci of ectodermal proliferation in which intranuclear inclusions were prominent in affected epithelium, which ranged from six to eight cells in depth (Figure 19). Most of the inclusions were of basophilic type and occupied the whole of the nucleus, the chromatin being gathered into small clumps at the nuclear membrane (Figure 20). Other inclusions were stained reddish-purple and were surrounded by distinct 'halos', the nucleolus generally being located on the nuclear membrane (Figure 21). Such lesions were unaccompanied by necrosis but, below some of them, there was a zone of similar thickness consisting of macrophages, fibroblasts and heterophil leucocytes. Inclusion bodies were not to be found in those areas and hyperaemia was not evident, though heterophil leucocytes were sometimes diffusely scattered in the mesenchyme near the lesions. The endoderm showed slight hyperplasia in areas immediately below the ectodermal foci where the cells were elongated with the long axis at right angles to the surface; in most instances, these hyperplastic zones were from two to four cells in thickness. Inclusions were never detectable in endodermal cells.

Figure 20. Basophilic intranuclear inclusions occupying the whole of the nucleus. x 1,200.

Figure 21. Eosinophilic Type A inclusions. x 1,200.



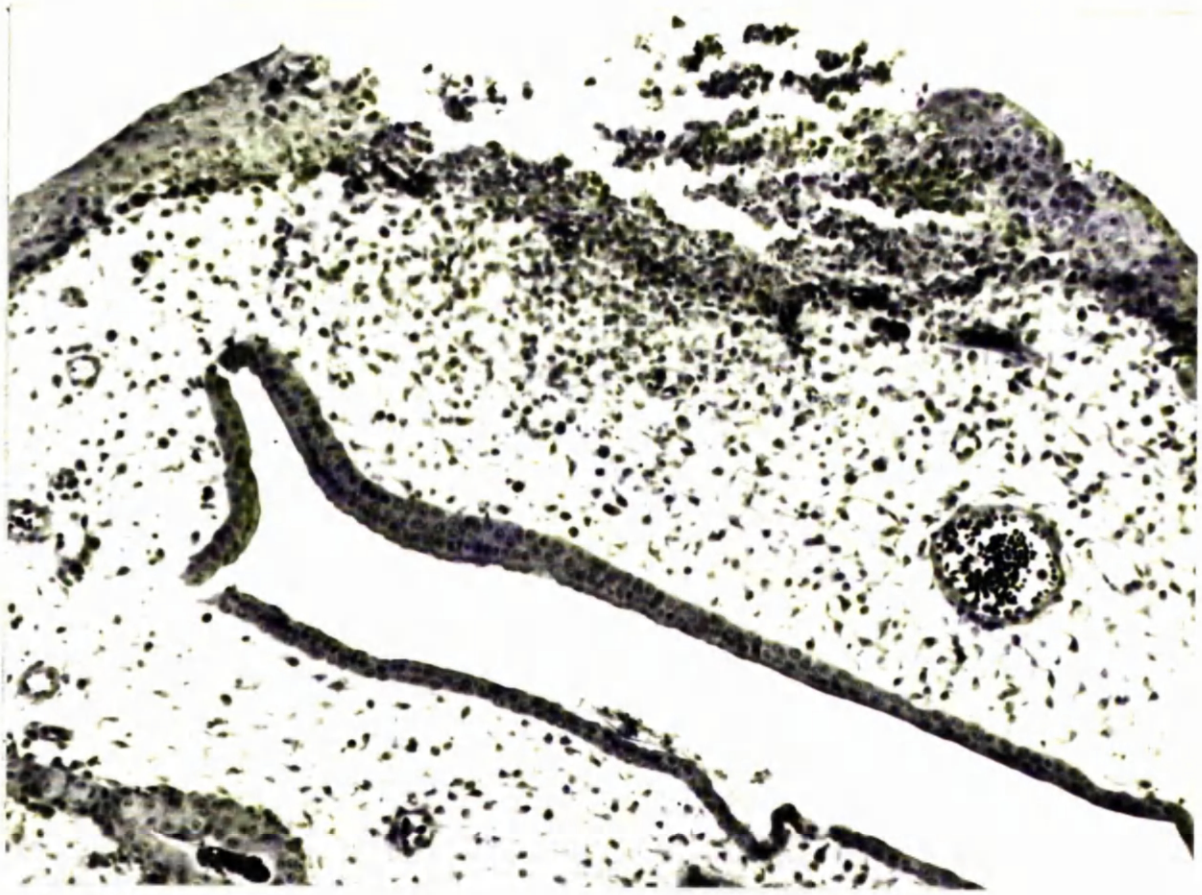


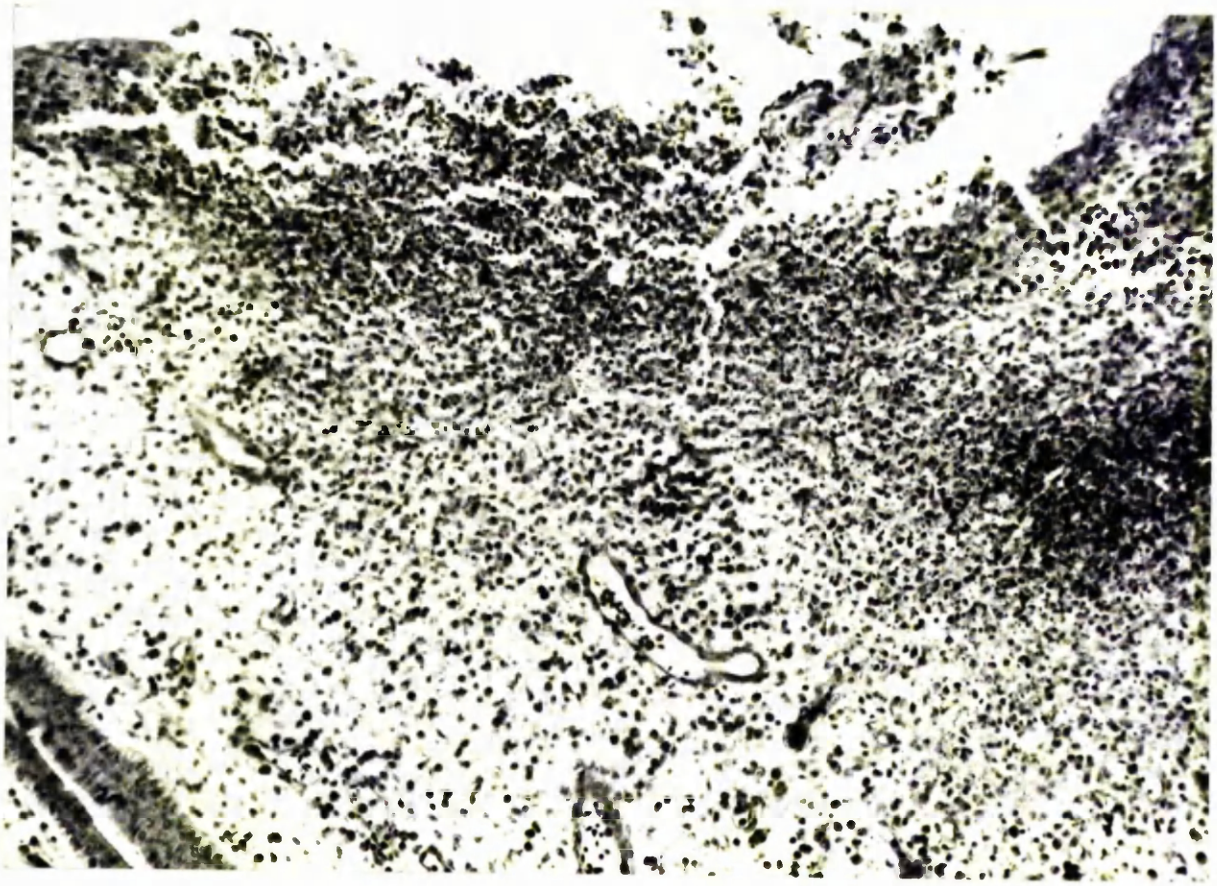
Similar changes were present 48 hours after infection and were accompanied by small foci of necrosis at the centre of most of the pocks as well as by some degree of separation of the hyperplastic ectoderm from the underlying mesenchyme (Figure 22). Inclusion bodies were numerous. By the 72nd. hour, pocks of three different stages of development were recognizable. In the most advanced form, there was complete loss of ectoderm over large areas and the necrosis had extended into the mesenchyme where the inflammatory zone containing heterophil leucocytes and macrophages merged imperceptibly with erythrocytes from damaged blood-vessels and debris derived from necrotic mesenchymal cells and leucocytes. Intranuclear inclusions were present at all levels of the mesenchyme, the eosinophilic type with 'halos' being the most prominent. The mesoderm was congested and the endoderm hyperplastic. The latter occasionally reached approximately 40 cells in thickness but, in most instances, was limited to a depth of four to eight cells; inclusions were absent.

In less mature pocks, both proliferative and necrotic changes were present, the lesion consisting of a central crater surrounded by a zone of hyperplastic ectoderm. Inclusions, largely of the eosinophilic type, were present in the epithelial cells bordering the ulcer and necrosis often extended into the superficial part of the mesoderm where it gradually blended with a highly cellular portion consisting of macrophages, heterophil

Figure 22. Necrosis of ectoderm at centre of pock. Infiltration of leucocytes into the mesoderm. x 150.

Figure 23. Necrosis extending into mesoderm and blending with cellular infiltration. Note absence of capillary "line". x 150.





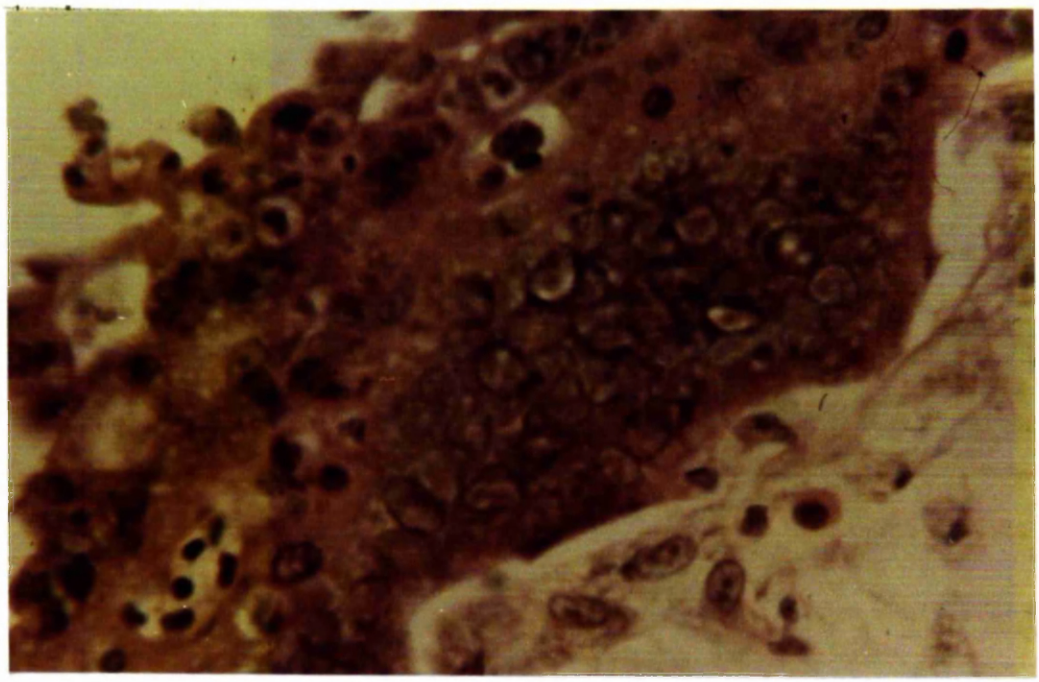
leucocytes and mesenchymal cells (Figure 23). The remainder of the mesoderm was congested and the endoderm attained three or four cells in thickness. The most immature pocks were similar to those seen during the 24-48 hour period; the ectoderm was hyperplastic and contained inclusions, mostly of the basophilic type, while minor degrees of vacuolation and necrosis were present at the surface.

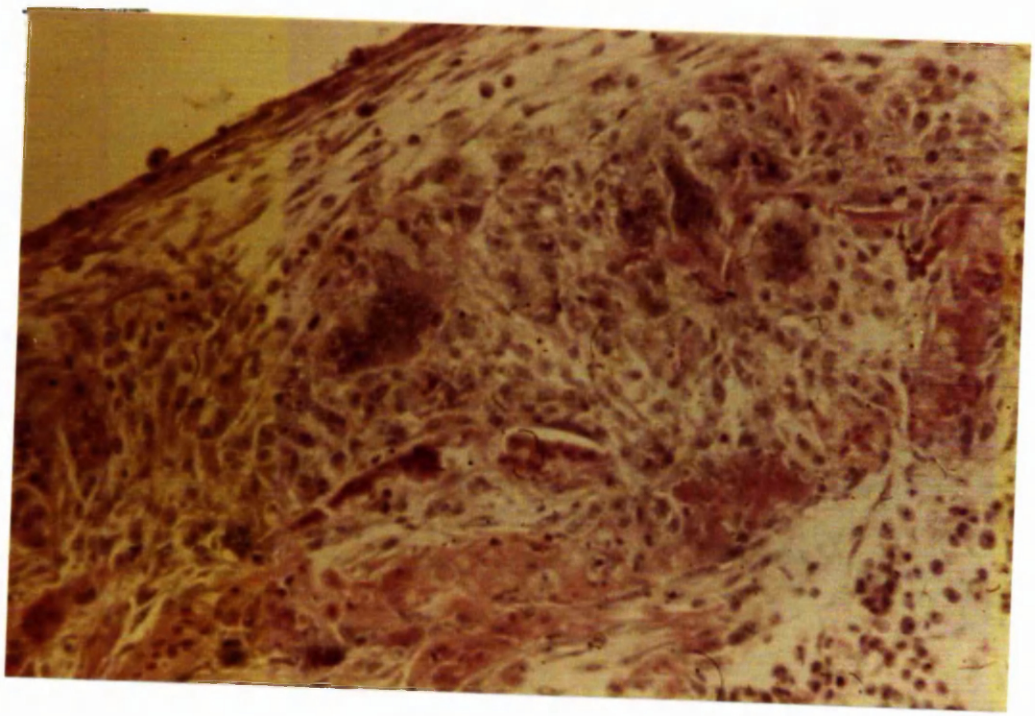
By 96 hours, the great majority of the lesions showed extensive ectodermal necrosis and inclusions were less numerous. The necrotizing process continued until, at 120-144 hours, the membrane had been reduced to a hyperplastic endoderm, a mesenchyme heavily infiltrated with leucocytes and small remnants of ectoderm. At that stage, inclusion bodies were to be found only with difficulty and were almost entirely of the eosinophilic type, well demarcated from the nuclear membrane by a clear 'halo'.

A few, small, multinucleated syncytia, containing inclusions and accompanied by slight proliferative and degenerative changes, were present in the ectoderm of chorio-allantoic membranes inoculated 24 hours earlier with I.L.T. virus but, by the 48th hour, necrosis and inflammation were advanced. At the latter time, there was little evidence of ectodermal hyperplasia. Indeed, in some places, the epithelium had entirely disappeared and, in others, it had been reduced to a mass of necrotic tissue containing macrophages, numerous heterophil leucocytes and a few epithelial cells. In less

Figure 24. Syncytium in ectoderm of chorio-allantoic membrane infected with I.L.T. virus. x 1,200.

Figure 25. Chorio-allantoic membrane infected with I.L.T. virus. Note fibroblasts at surface and remnants of syncytia below. x 150.





advanced lesions, the ectoderm was still largely intact but showed vesiculation and infiltration by heterophil leucocytes. Multinucleated cells, in which all nuclei contained inclusions, were also sometimes present in those parts of the epithelium (Figure 24). All inclusions in each syncytium were generally of the same type, either the large basophilic pattern occupying the whole nucleus or the smaller, discrete, eosinophilic form.

Reactive changes in the mesenchyme underlying the ulcerated or necrotic ectoderm occurred as early as the 48th hour and were a conspicuous feature of I.L.T. infection. A layer of fibroblasts, occupying up to approximately two-fifths of the depth of the mesoderm, lay immediately below the necrotic epithelial mass, the outermost cells being orientated parallel with the surface (Figure 25). The fibroblastic tissue was separated from the remainder of the mesoderm by a well-defined stratum which consisted of budding capillaries, fibroblasts and macrophages (Figure 26). In some areas, large numbers of fibroblasts, arranged perpendicularly to the surface, were seen to extend upwards from the capillary loops. Apart from the presence of small numbers of widely scattered heterophil leucocytes, the portion of the mesoderm below the capillaries appeared normal. The endoderm was slightly hyperplastic and varied from two to ten cells in depth. Inclusions were not detectable in any tissue other than ectoderm. Similar changes were present in chorio-allantoic membranes inoculated three to five days

Figure 26. Chorio-allantoic membrane infected with I.L.T. virus. Note complete destruction of the ectoderm and demarcation of normal mesoderm by a well-defined capillary "line". x 110.

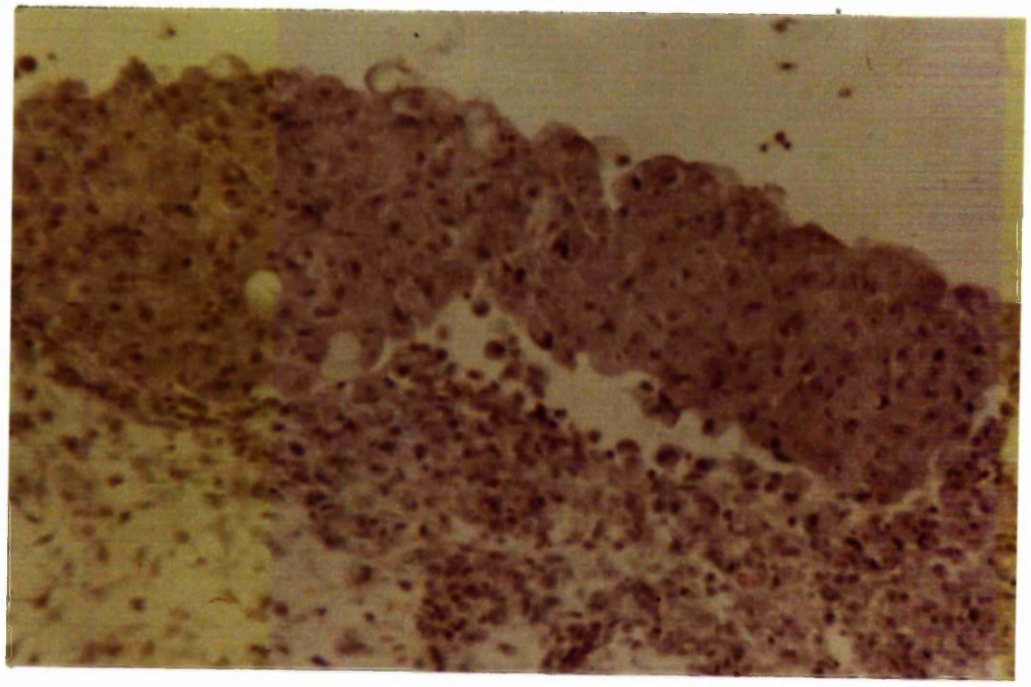
previously with I.L.T. virus but, as destruction of ectoderm advanced, the number of inclusions diminished.

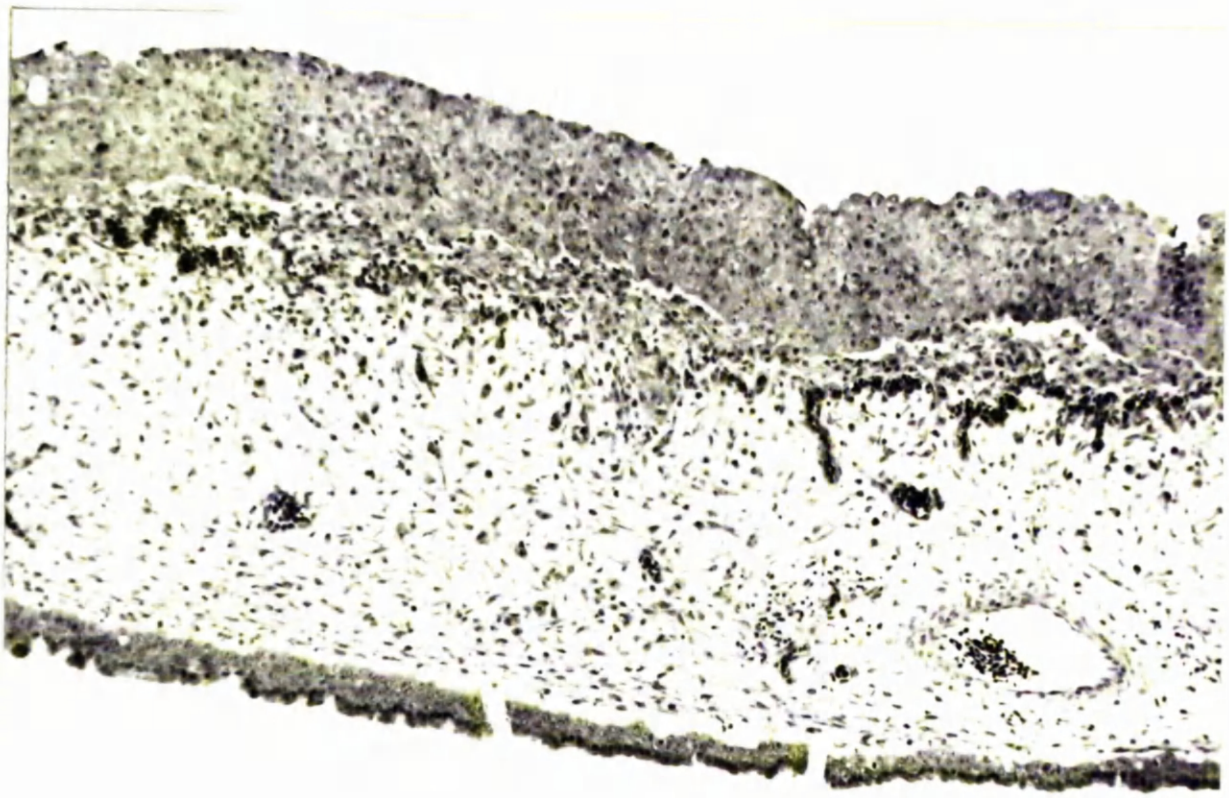
Ectodermal hyperplasia was the outstanding feature of chorio-allantoic membranes inoculated with pigeon-pox virus and, by the 72nd. hour after infection, degenerative changes had also appeared. At that time, the ectoderm was up to 20 cells in thickness and many of those elements exhibited 'balloon' degeneration whereby the surface of the epithelium became undulate (Figure 27). Numerous swollen, round, epithelial cells were partly or wholly detached from the remainder of the ectoderm which, in places, was itself separated from the underlying mesenchyme. In some areas, the outermost portion of the mesoderm was separated from the remainder by a line of budding capillaries and fibroblasts (Figure 28), though that feature was less well-developed than it was in membranes infected with I.L.T. virus. The endoderm was slightly hyperplastic and varied from three to five cells in depth.

Embryonic deaths were noted only in eggs infected with the pigeon I.N.I. virus and never occurred before the fourth day. Histopathological examination of embryos likewise revealed lesions only in those eggs which had been inoculated with that agent. Changes were limited to the liver and spleen which usually contained several minute, cream-coloured foci that, microscopically, closely resembled the necrotic lesions to be seen in the organs of pigeons

Figure 27. Chorio-allantoic membrane infected with pigeon-pox virus. Hyperplastic ectoderm with undulate outline caused by "ballooning" of cells. x 150.

Figure 28. Chorio-allantoic membrane infected with pigeon-pox virus. Note capillary "line". x 110.





suffering from natural I.N.I. infection. In hepatic cells toward the periphery of the lesions, basophilic and eosinophilic inclusion bodies were present and were generally demarcated by clear 'halos'.

The titres of pigeon I.N.I. virus (strain HS-2), obtained from chorio-allantoic membranes inoculated with sufficient of the agent to produce semi-confluent lesions by the fourth day, were as follows:

Day One	6.4×10^3	P.F.U. per membrane.
Day Two	6.7×10^5	P.F.U. do. "
Day Three	1.9×10^5	do. "
Day Four	1.0×10^5	do. "

The results of amniotic inoculations were inconclusive. Preliminary work with four strains of pigeon I.N.I. virus (M-3, M-4, M-8, HS-2) suggested that the method might be useful, in so much as each strain of virus produced hepatic necrosis but in only one out of three eggs inoculated. Typical inclusions were encountered in localized areas within the tracheal epithelium of one of these embryos but, in the other three instances, they were discoverable only in association with the foci of hepatic necrosis. When, however, the experiment was repeated with 12 eggs inoculated with 10^4 P.F.U. of the M-3 strain of virus, hepatic necrosis was not to be found in any of the embryos, and amniotic fluid harvested on the third and fourth days proved devoid of infective virus.

Allantoic inoculation of the M-3 strain of I.N.I virus also led to varying results. Specific abnormalities were not detected in the chorio-allantoic membrane though proliferation of the endoderm was occasionally noted. Out of nine infected eggs, only one embryo showed hepatic necrosis on the fourth day and the titre of virus in the allantoic fluid was but 5×10^1 P.F.U. per ml. The pooled allantoic fluid from the remaining eight inoculated eggs contained < 10 P.F.U. of virus on the fourth day.

Foci of necrosis appeared in the liver of embryos on the sixth day following inoculation of the yolk-sac with strain M-3 and minute pocks were present on the chorio-allantoic membrane on the seventh day. Lesions were not detected in the wall of the yolk-sac though small numbers of intranuclear inclusions were recognizable therein from the fourth day onwards. Titration of virus present in the latter structure yielded the following results:-

Day Three	4.7×10^3	P.F.U. per yolk-sac.
Day Five	1.0×10^4	do.
Day Seven	2.1×10^5	do.

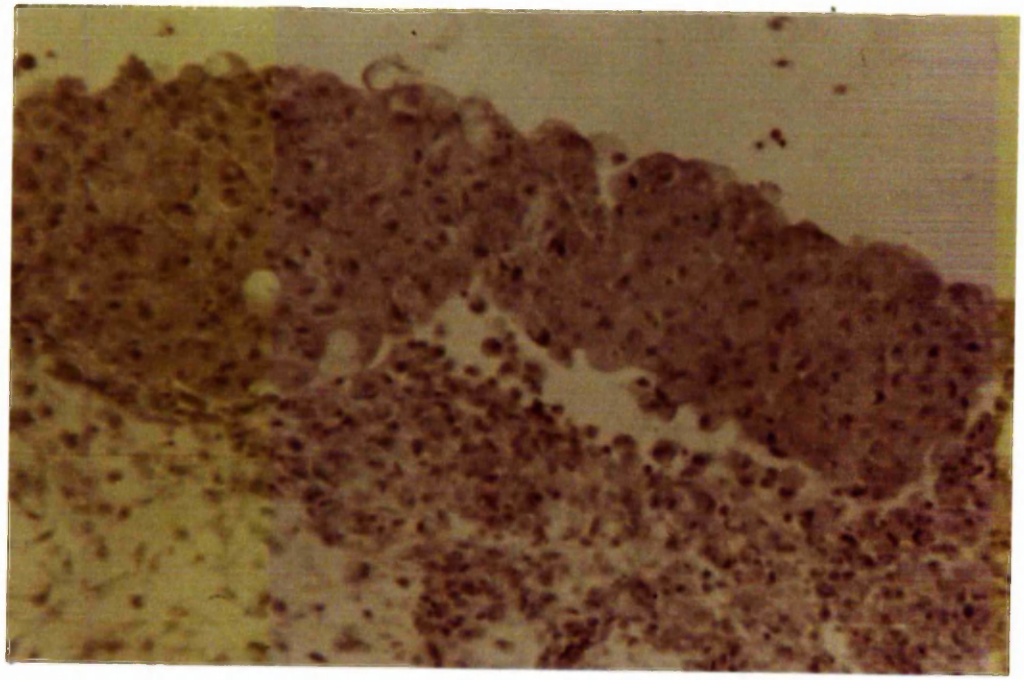
When attempts were made to establish an assay based on the relationship between the pock counts and the relative concentrations of virus employed, it was found that very considerable variation existed from egg to egg in the number of lesions produced by a single dilution of virus. Two examples may be cited:-

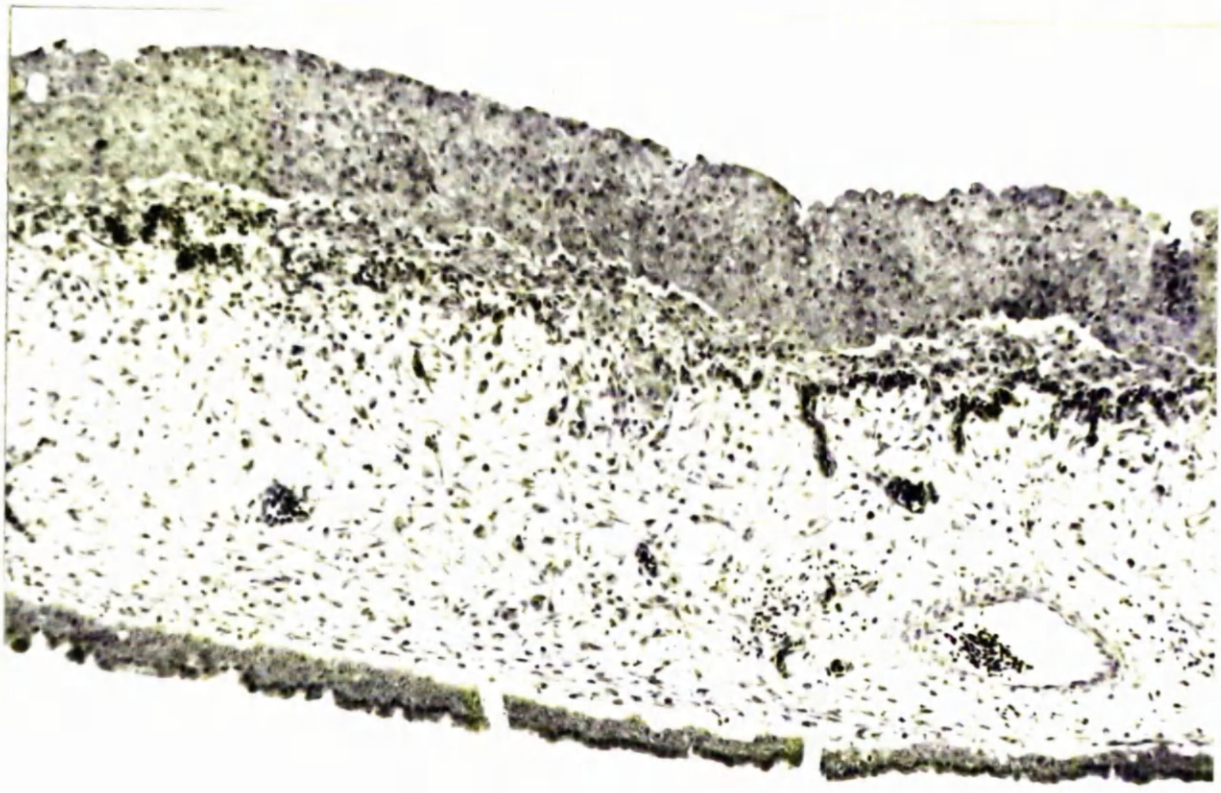
Virus dilution.	No. of eggs.	Pock-counts.	Mean pock-counts.	Coefficient of variation.
$10^{-1.5}$	11	35, 7, 54, 29, 10, 5, 28, 17, 19, 15, 5.	20.4	72.4%
10^{-2}	10	4, 0, 2, 2, 11, 3, 0, 22, 3, 5.	5.2	128.4%

The standard deviation was sometimes too great to support the validity of the mean and, for that reason, it was impossible to determine whether or not a linear relationship existed between the mean pock-count and the relative virus concentration.

(4) DISCUSSION.

When chorio-allantoic membranes were inoculated with sufficient pigeon I.N.I. virus to produce confluent lesions by the fourth day, the number of inclusions reached a maximal level on the second and third days after infection. Thereafter, the number gradually declined as progressively less ectoderm remained to support further cycles of virus multiplication. Those findings explained the paucity of inclusions in membranes removed on the fifth day (to which experience reference was made in Part One) and suggested that the optimal time for harvesting might be on the second or third day. That hypothesis was confirmed by the results of virus titration which showed that the greatest yield of virus (6.7×10^5 P.F.U.) was obtainable from membranes procured on the second day and that thereafter the concentration of virus diminished.





Following inoculation into the amniotic and allantoic sacs, the virus seemed to be capable of invading the embryo and establishing a focal hepatitis though only in a small proportion of eggs. The trifling amounts of virus present in the allantoic fluid and the absence of inclusions in the endodermal cells both suggest that the allantois is highly resistant to the virus and that, therefore, attempts to infect the embryo by the allantoic route are unlikely to succeed. The solitary successful endeavour may be explained by contamination of the ectoderm by the needle of the syringe, though it is possible that the endoderm may be partially susceptible in a small proportion of eggs. Considerable fluctuation in the yield of I.L.T. virus released into the allantoic fluid was reported by Beveridge and Burnet (1946). The reason for the variable results obtained by amniotic inoculation is difficult to account for satisfactorily. The finding of inclusions in the trachea of one embryo suggests that infection occurs directly from the amniotic fluid, rather than by accidental contamination of the chorion in the act of inoculation. If that surmise be correct, the apparent resistance of some eggs may be explained by the resistance of the respiratory epithelium to the virus. Burnet and Foley (1941) found that I.L.T. virus grew in the respiratory system of some embryos but not in that of others and similar variation in the susceptibility of epithelium may have obtained in the present investigation. The only general conclusion to be drawn is that neither allantoic nor

amniotic inoculation offers a satisfactory method for the propagation of the virus.

The results of the investigation under report differ slightly from those of Jylling (1967). The latter author stated that inoculation by the allantoic route produced the same changes as were seen following infection of the chorio-allantoic membrane and described focal necrosis of the lungs, kidneys, gizzard, intestine, and heart as well as plaques in the skin, none of which findings were observed by the present writer. The difference in the distribution of the necrotic foci is probably unimportant since Jylling recorded that the embryos usually died in from six to nine days after inoculation whereas, in the work under report, embryos were seldom examined after the fifth day. Further difficulty attends comparison of the pocks produced by the strains isolated in the two countries for Jylling gave but the barest of details on the histopathology of the lesions. However, it would seem that, in both instances, a characteristic feature of the pocks was the presence of necrosis and inflammatory changes in the mesoderm.

The changes produced by the pigeon I.N.I. virus are strikingly similar to those caused by Pacheco's parrot virus. Thus, Rivers and Schwentker (1932) reported that the embryos of eggs inoculated with the latter agent died in three to five days and that small white foci of necrosis were visible in the liver, spleen and

kidneys. Histological examination revealed proliferation and necrosis of the epithelium of the chorion together with infiltration of the mesoderm with leucocytes. Intranuclear inclusions were present in the ectodermal cells of the pocks, in some of the leucocytes in the underlying mesoderm and in foci of necrosis found in the viscera of the embryo.

The morphology of the pocks incited on the chorio-allantoic membrane by the three viruses employed in the present investigation served to provide a ready means of distinguishing between them. The lesions produced by I.L.T. virus were much larger and appeared as sharply demarcated, grey ulcers. Those associated with the two columbine viruses were cream-coloured and distinctly elevated but, in the case of pox infection, were distinguished by the absence of craters and of elongated foci. In respect of the embryo, abnormalities were observed only in eggs inoculated with the pigeon I.N.I. virus.

Histopathologically, the pocks associated with the three viruses also differed widely. The two intranuclear agents produced early necrosis but, whereas in the case of the agent of I.L.T. the extent of that process was confined to the ectoderm and was bounded by granulation-tissue, the foci of destruction caused by the pigeon I.N.I. virus were not so demarcated but extended into the deeper parts of the mesoderm. Thus, inclusions were absent from the

mesoderm in the former case but were numerous in the latter. Pigeon-pox virus gave rise to foci characterized by massive ectodermal hyperplasia, the proliferative changes being succeeded by 'balloon' degeneration which seemed to occur simultaneously throughout the affected parts of the epithelium. Granulation-tissue was present immediately beneath the ectoderm but was much less conspicuous than that observed in lesions of I.L.T. Multinucleated cells were found in the membranes infected by I.L.T. virus but not in those inoculated with either of the other two agents. Both intranuclear viruses produced identical inclusions which varied from basophilic to eosinophilic in type.

The gross and microscopic changes produced by I.L.T. and by pigeon-pox viruses were closely similar to those described by other workers. Thus, Burnet (1934) reported that the pocks formed by I.L.T. virus appeared to primarily represent the response of the ectodermal epithelium to the presence of virus, with the development of secondary changes in other layers. Initial hyperplasia was succeeded by necrosis so that, by the fourth day, the ectoderm had largely disappeared and had been replaced by oedematous inflammatory tissue. Burnet (1938) and Glover (1939) emphasized the proliferative nature of the pocks caused by pigeon-pox virus; although the ectoderm was often five to six times the normal thickness, abnormalities were not found in the embryos, most of which hatched normally.

The ease with which the pigeon I.N.I. virus could be propagated in embryonated eggs suggested that an enumeration assay, based on the number of pocks found on infected chorio-allantoic membranes, might prove a useful and statistically-acceptable method of titration. When, however, the matter was investigated, it was found that the coefficient of variation for the pock-counts produced by a given virus dilution was very large, generally over 60 per cent. That finding is in accordance with the results obtained by some authors, studying certain other viruses. Thus, Fenner and McIntyre (1956), working with the rabbit myxoma virus, accepted a wide coefficient of variation (up to 110 per cent) as an intrinsic defect of the pock-assay. Armitage (1957) carried out a detailed statistical analysis of the results, both published and unpublished, obtained by other workers and concluded that, in all cases, the variance in the pock-count produced by a given virus dilution was considerably greater than that expected if the distribution of virus particles was of the Poisson form. Westwood et al. (1957), however, found that variation in the number of pocks produced by vaccinia virus could be reduced to a level approaching that expected from the Poisson equation by an improved method of inoculation. In the hands of the present author, however, the latter technique did not seem to encourage uniformity in the number of pocks formed by the pigeon I.N.I. virus. The variation experienced was not due to aggregation of virus particles since, as will be related in Part Three, the same virus

dilutions produced plaques in cell-culture which were distributed between culture-vessels according to the Poisson equation. Possibly related to the high variance pertaining to assays carried out on the chorio-allantoic membrane was the observation that some of the lesions were of linear or comma-shaped appearance, a phenomenon which suggested that they might have been derived from more than one virus particle. It was, therefore, concluded that, although the embryonated egg was satisfactory for primary isolation and propagation of the virus, the precision of the pock-assay was insufficient to allow proper titration of the virus.

(5) SUMMARY AND CONCLUSIONS.

Inoculation of the chorio-allantoic membrane was found to be a satisfactory method of propagating the pigeon I.N.I. virus, and optimal yields were obtained on the second day. Smaller amounts of virus were procurable by infection of the yolk-sac but other routes of inoculation proved unreliable. The morphology and histopathology of the pocks allowed a sharp distinction to be made between the pigeon I.N.I. virus and the agents of I.L.T. and pigeon-pox. The precision of the pock-assay was found to be too low to allow proper titration of the virus.

PART 3. GROWTH AND ASSAY OF VIRUS IN TISSUE-CULTURE.

INTRODUCTION.

Although the embryonated egg had been found to provide a satisfactory medium for the isolation of the agent from diseased birds, the inherent superiority of tissue-culture for more refined studies, together with its greater convenience, led to early investigation of its suitability for study of the virus.

The preliminary aim of the present part of the thesis was to find a system of cell-culture in which the pigeon I.N.I. virus might propagate with the production of an easily-recognizable cytopathogenic effect (C.P.E.). If that end were realized, the following studies were intended to ensue:-

1) Comparison of the cytopathogenicity of the author's strains with that of others encountered in the British Isles and in the United States of America.
2) Investigation into the range of cells susceptible to the virus in order to determine the comparative sensitivity of certain types of avian cell and to ascertain whether, or not, the virus was able to multiply in representative mammalian cells.
3) With special reference to taxonomy and differential diagnosis, a comparison of the C.P.E. of the columbine virus with that of avian viruses relevant to the work under report, i.e., I.L.T. virus by virtue of its ability to produce intranuclear inclusions,

and pigeon-pox virus because of its possible association with laryngeal pseudo-diphtheritic membranes.

The foregoing studies, considered in Section One, infra, were hoped not only to assist differential diagnosis and define a method most suited to propagation of the virus but also to lead to the development of a procedure of assay of the virus, a necessary pre-requisite for further investigation. Section Two is concerned with the establishment of such a procedure and with the conditions which govern its use.

SECTION I: GROWTH OF THE VIRUS.

(1) MATERIALS AND METHODS.

(a) Culture vessels.
(b) Whole-chick-embryo monolayers.
(c) Chick-embryo kidney cultures.
(d) Chick-embryo liver cultures.
(e) Chicken kidney cultures.
(f) Pigeon kidney cultures.
(g) Mammalian cell cultures.
(h) Media.
(i) Virus strains.
(j) Cytological studies.
(k) Infectivity titrations.

(a) Culture vessels. Cells were grown in 4-oz. flat medicine bottles for preparation of virus stocks, in Leighton tubes with cover-slips for cytological studies, and in roller tubes in stationary racks for titration of virus.

(b) Whole-chick-embryo monolayers. Cultures were prepared from 10/12-day-old eggs by a method based upon the technique of Dulbecco (1952) as modified by Postlethwaite (1960). After removal of the embryos, the heads were discarded and the remaining portions finely minced. The tissue fragments were then thoroughly washed with P.B.S., free of calcium and magnesium ions, and transferred to a flask of warm trypsin solution which was placed on a magnetic stirrer in an incubator at 37° C. After ten minutes, the crude cell suspension was

discarded and replaced by fresh pre-heated trypsin solution. After 45 minutes more at 37°C., the cell suspension was collected and placed in a refrigerator. Further trypsin solution added to the tissue fragments was collected after 30 minutes incubation. The two cell suspensions were pooled and centrifuged at 800 r.p.m. for ten minutes. The sediment was washed once with maintenance medium and then resuspended in growth medium. A viable cell count was performed by means of 0.1 per cent trypan blue and the cell suspension diluted to provide the required concentration of cells. Tubes were seeded with 1×10^6 cells in 1 ml. of growth medium and bottles with 1.8×10^7 cells in 12 ml. of medium.

(c) Chick-embryo kidney cultures. The kidneys were removed from 19-day embryos and, after mincing, were washed thoroughly with P.B.S. Following a final wash in trypsin, the tissue fragments were suspended in a flask of trypsin solution which had been warmed to 37°C. The flask was then immersed in a water-bath at the same temperature and the fragments kept in motion by agitation. The resultant cell suspension was collected at the end of 5-10 minutes and placed in the refrigerator. Fresh heated trypsin solution was then added to the fragments and the process repeated. Normally, three runs of 5-10 minutes each were sufficient to break down the tissue and release the cells. The first suspension collected was discarded if it contained a high proportion of erythrocytes. Otherwise, all cell suspensions were pooled and centrifuged at 500 r.p.m. for five minutes. The cells

were resuspended in maintenance medium, sedimented again at the same speed and finally diluted in growth medium. A viable cell count was then performed, and the cell concentration adjusted to provide 10^6 cells per 0.5 ml. 1 ml. amounts were then dispensed into Leighton tubes containing coverslips.

(d) Chick-embryo liver cultures. Such preparations were made by a modification of the method described by Kaeberle, Drake and Hanson (1961). The livers were removed from 17-day chick embryos and trypsinized by the same method as that employed in the preparation of embryonic kidney cultures. The cell suspensions were pooled and centrifuged at 500 r.p.m. for five minutes. The cells were then washed once in maintenance medium, sedimented again in a graduated tube and resuspended in an amount of growth medium equal to 100 times the packed cell volume. The cell suspension was distributed into Leighton tubes, to a volume of 1 ml. per tube.

(e) Chicken kidney cultures. The method employed was that described by Churchill (1965). Kidneys from a four-week-old chicken were finely minced and the fragments washed three times with P.B.S. Twenty ml. of trypsin solution, preheated to 37° C., were added to the tissue and disaggregation allowed to proceed for five minutes at the same temperature. The solution was then removed from the flask, mixed with 1 ml. of calf serum and placed in a refrigerator. The process was repeated five or six times. After centrifugation of the

cell suspensions at 500 r.p.m. for five minutes, the sedimented cells were resuspended in growth medium and a viable cell count carried out. The concentration was then adjusted to provide 4 x 10^5 cells per ml. Tubes were inoculated with 1 ml. and bottles with 12 ml. of the suspension.

(f) Pigeon kidney cultures. Kidneys were removed from young birds and cultures prepared by the method used for the chicken kidneys.

(g) Mammalian cell cultures. Primary cultures of dog and calf kidney cells were prepared according to the method described by Cornwell et al. (1965). HeLa and Strain L cells, routinely maintained in the department, were also examined for their ability to support growth of the virus.

(h) Media. For chick-embryo kidney and whole-chick-embryo cultures, growth medium consisted of Hank's balanced salt solution (B.S.S.) supplemented with 0.25 per cent lactalbumin hydrolysate and 10 per cent inactivated calf serum. Eagle's basal medium with 10 per cent tryptose phosphate broth, five per cent chick-embryo extract and 30 per cent inactivated calf serum was used for the cultivation of chick-embryo liver cells, while the same medium minus chick-embryo extract and with only 10 per cent of calf serum sufficed for establishment of chicken kidney and pigeon kidney monolayers.

Hank's B.S.S. with 0.25 per cent lactalbumin hydrolysate was employed for the maintenance of cultures after inoculation.

For disaggregation of tissues, 0.25 per cent trypsin (1 : 250, "Difco", certified) in phosphate-buffered saline (P.B.S.), free of calcium and magnesium ions, was used. All embryos were obtained from White Leghorn eggs of the same strain.

(i) Virus strains. Third- or fourth-egg passages of strains H-1, HS-2, M-3, M-4, M-8 and B-1, and the 66th egg passage of Smadel's P-5 strain were selected for inoculation into tissue-culture. In each case, the chorio-allantoic membranes from the last egg passage were macerated in a Griffith's tube with maintenance medium to produce a 20 per cent suspension. After clarification by centrifugation, 3 ml. amounts of each suspension were inoculated onto monolayers of whole-chick-embryo cells grown in bottles. After three hours at 37° C., the inoculum was removed, the cells washed twice with P.B.S. and fresh maintenance medium added. All cultures were incubated at 37° C.

From the second or third passage onwards, the virus was harvested when the C.P.E. involved approximately one-third to one-half of the monolayer. The cells were scraped from the glass surface, resuspended in their own maintenance medium and macerated in a Griffith's tube. A 1 in 10 dilution of that fluid was employed for passage. Occasionally transfers were made with infected culture

fluids only. Adsorption time varied from two and one half to four hours.

Virus from the eleventh passage of strain M-3 in whole-chick-embryo monolayers was transferred to cultures of chicken kidney, chick-embryo-kidney and chick-embryo liver. Virus from the fourth passage of strain M-4 was inoculated into cultures of pigeon-kidney and chick-embryo liver. Monolayers of pigeon-kidney were also infected with the fourth passage of Smadel's strain P-5.

All mammalian cultures employed in the investigation were inoculated with the eleventh passage of strain M-3 and thereafter examined daily for a period of one to two weeks. In each case, an inoculated culture was harvested on the third day and macerated in a Griffith's tube. The resultant suspension, clarified by centrifugation, was then transferred to cultures of whole-chick-embryo cells which were maintained under observation for seven days for the development of a typical cytopathogenic effect.

The 102nd egg passage of the BEE strain of I.L.T. virus, kindly supplied by Dr. F. T. W. Jordan of the University of Liverpool Veterinary School, was inoculated into cultures of whole-chick-embryo cells and thence directly into monolayers of chick-embryo kidney, chick-embryo liver, chicken kidney and pigeon kidney.

For comparative purposes, cultures of whole-chick-embryo

cells were also inoculated with the Burroughs-Wellcome strain of pigeon-pox virus, which is commercially available for the protection of poultry against fowl-pox.

(j) Cytological studies. Coverslip cultures from Leighton tubes were washed in physiological saline, fixed in mercuric chloride-formol for three hours and stained by haematoxylin and eosin.

(k) Infectivity titrations. Ten-fold dilutions of virus were prepared in Hank's B.S.S. Each dilution was inoculated into a minimum of six roller-tube cultures, 0.5 ml. per tube. 50 per cent end-points were calculated according to the method of Kärber (1931).

(2) RESULTS.

(a) Growth of the virus in whole-chick-embryo cells and comparison of the cytopathogenic effect produced by different strains.
(b) Comparison of the growth and cytopathogenicity of the virus in different types of cell-culture.
(c) Comparative studies with I.L.T. and pigeon-pox viruses.

(a) Growth of the virus in whole-chick-embryo cells and comparison of the cytopathogenic effect produced by different strains.

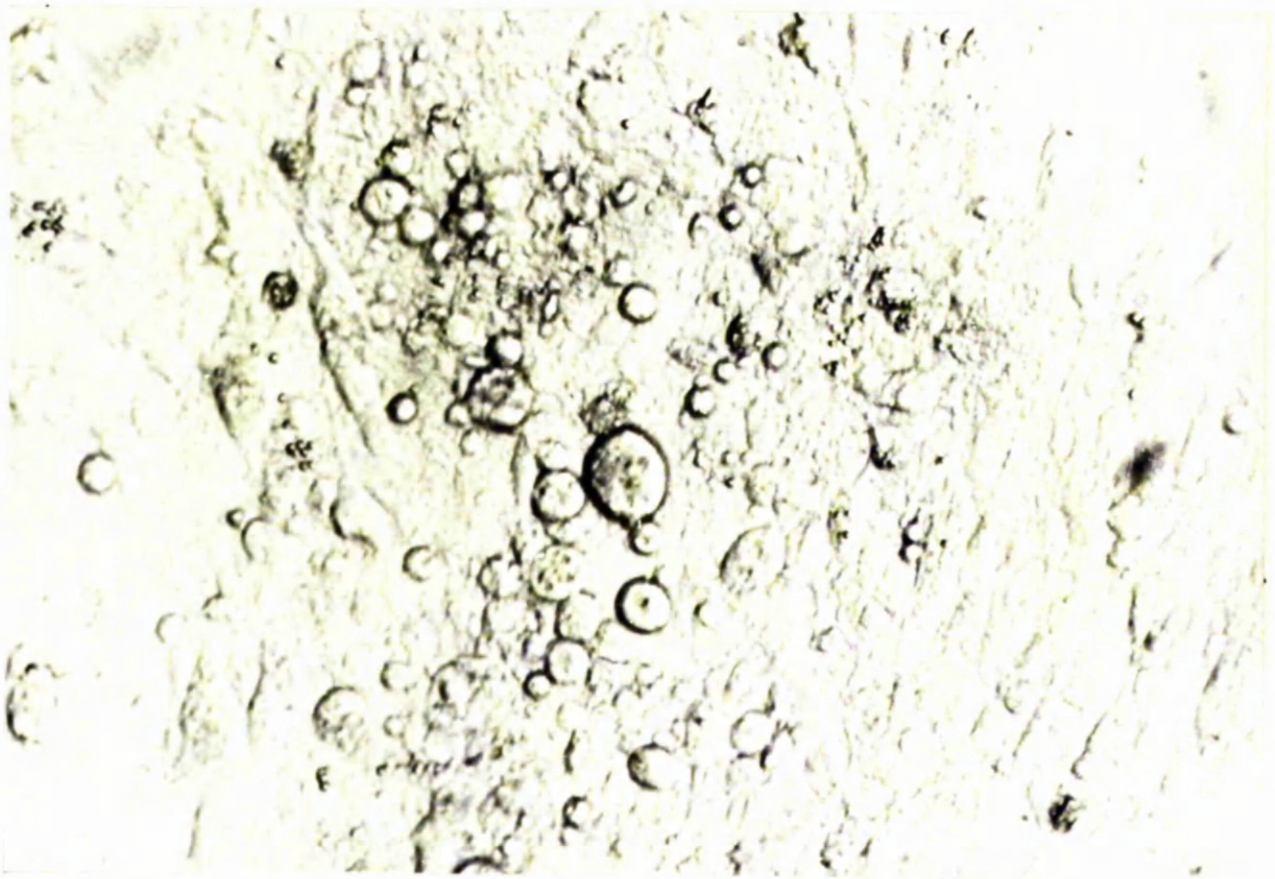
All strains isolated by the author grew well in whole-chick-embryo cultures and gave rise to the same highly characteristic focal cytopathogenic effect.

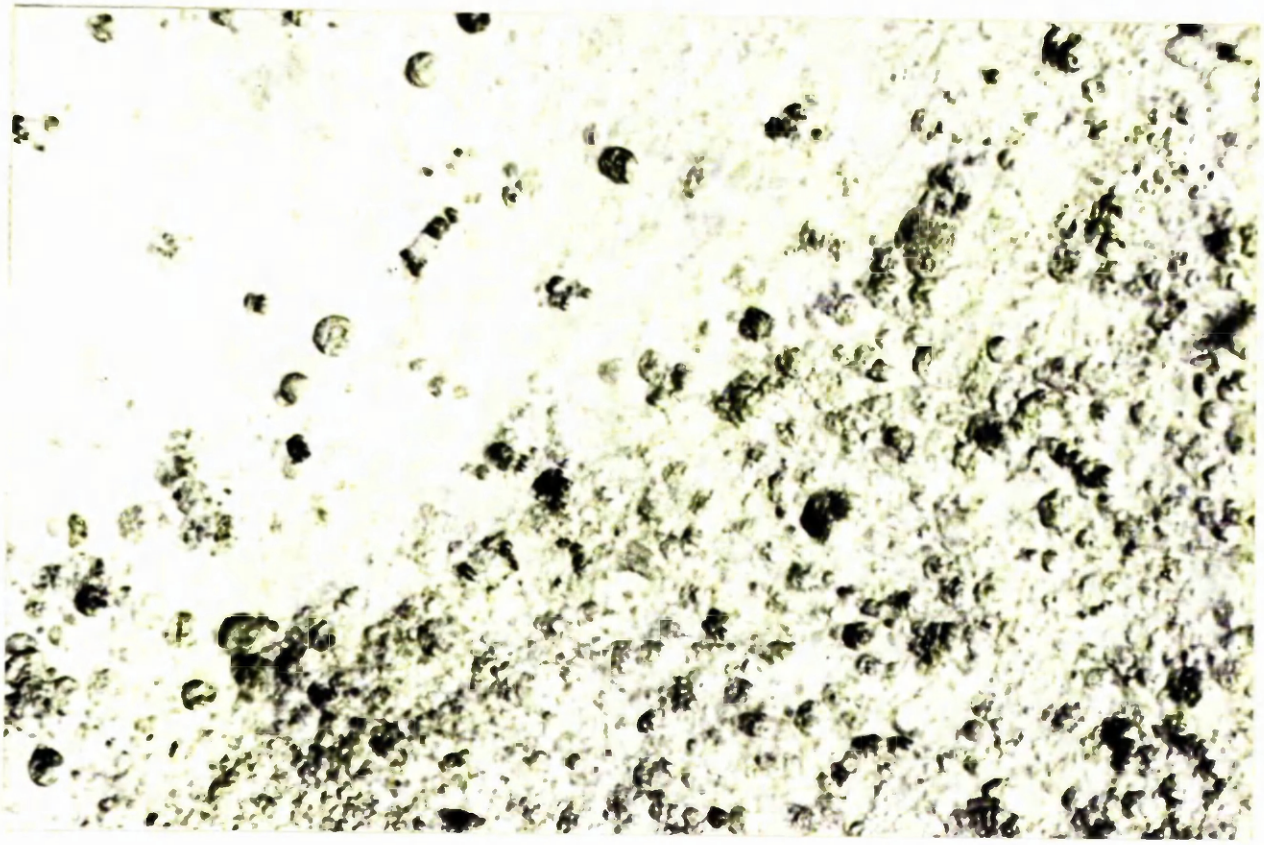
The earliest change to be seen in monolayers inoculated with suspensions of infected chorio-allantoic membranes was the appearance, on the third or fourth day after inoculation, of a few small clusters of round, refractile cells. When fresh monolayers were infected with suspensions prepared from those cultures, a focal cytopathogenic effect developed on the second day. The foci enlarged and increased in number so that, by the fourth day, many had reached a size of 1 mm. in diameter and appeared as circular, opaque, greyish-white plaques when viewed by oblique illumination. In subsequent passages initiated with undiluted inocula, the cytopathogenic effect was visible within 24 - 48 hours and involved the whole of the monolayer by the third or fourth day but, when high (10^{-4} or 10^{-5}) dilutions of virus were employed, the changes remained focal in character.

The cytopathogenic effect was characterized by the occurrence of clusters of round, refractile cells (Figure 29), the latter varying considerably in size, the largest having a diameter of approximately two to three times that of the smallest. As each focus enlarged, it gradually lost its compact cluster-like morphology. The refractile cells then formed the outwardly-expanding rim of the lesion, the internal portion consisting mainly of shrunken cells and granular debris (Figure 30). With further enlargement, a clear space sometimes developed in the centre of the plaque but the granular debris, which gave the affected area an amber hue, usually remained

Figure 29. Early focal cytopathogenic effect in whole-chick-embryo cultures. x 320.

Figure 30. Late focus consisting mainly of granular debris. x 320.





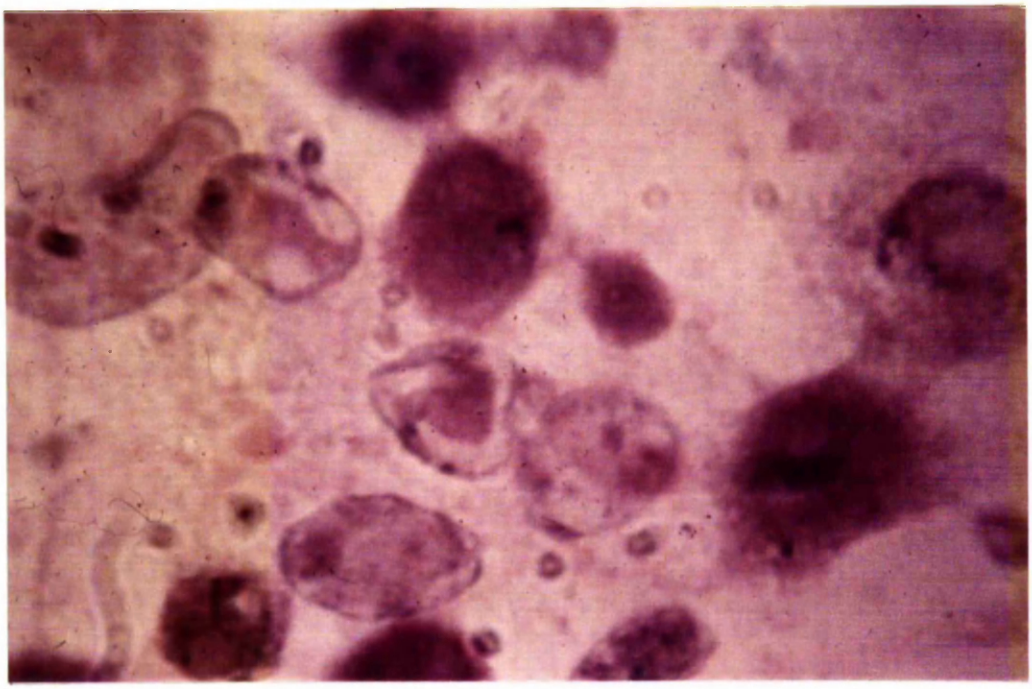
adherent to the glass for several days. In one instance, an infected culture, in which the cytopathogenic effect had appeared on the second day, was kept under observation for 14 days without macroscopically-visible spaces being formed. Microscopical examination of that specimen revealed the presence of a few fibroblasts, intermingled with a small number of granular round cells and a large amount of amorphous debris.

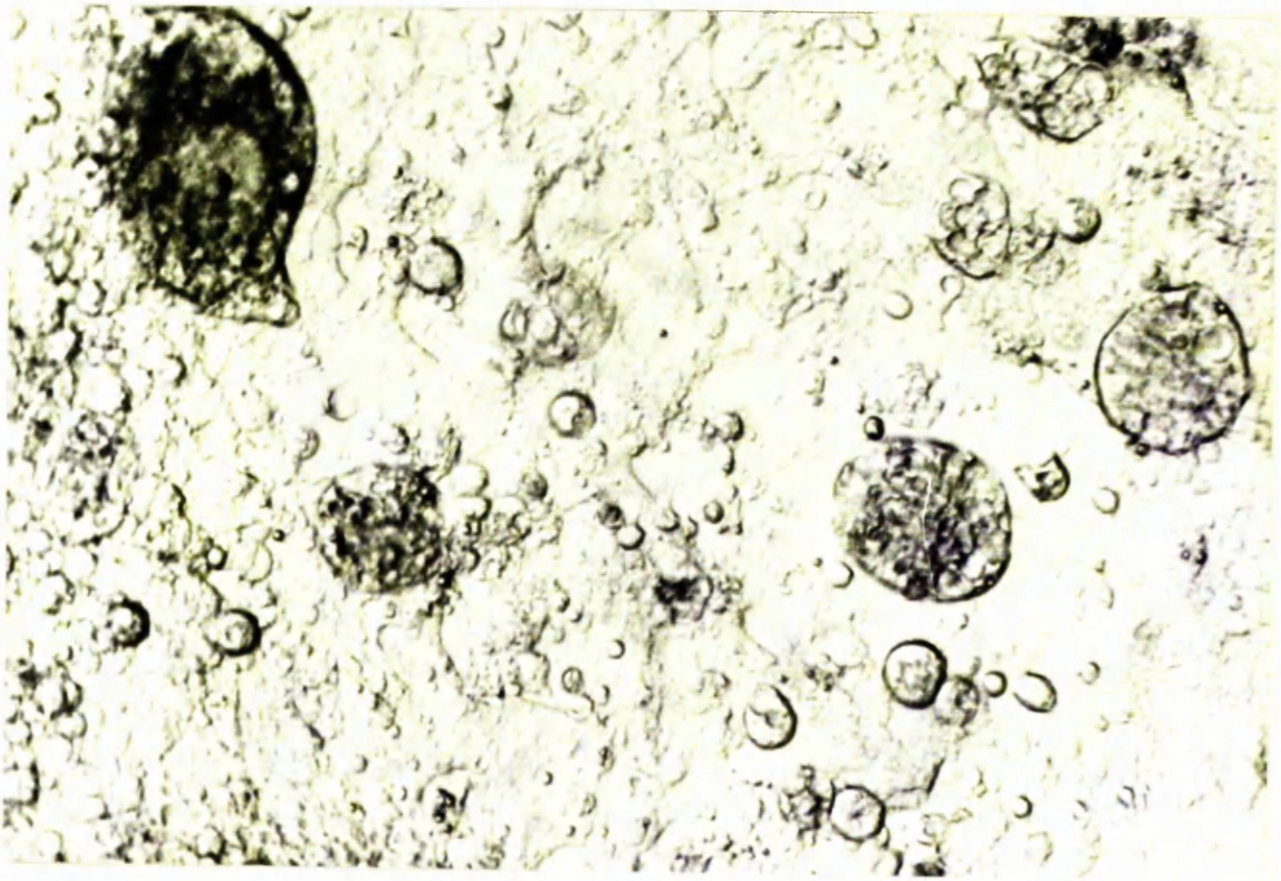
Observation of developing foci showed that the following cytological events took place. The earliest change was swelling of the infected fibroblast whereby the latter became navicular and refractile. Retraction of the cytoplasm at one or both poles rendered the cell pyriform or leaf-like. Very fine strands of cytoplasm were sometimes seen to protrude from the poles of affected cells. As the process continued, the cell became entirely spherical and appeared bright, homogenous and refractile. Later, however, the latter condition was lost as the cell became increasingly shrunken and granular.

In stained preparations, intranuclear inclusions were to be noted in cells of normal shape (Figure 31) as well as in some rounded ones and were of the same two types visible in embryonated eggs, namely, the basophilic or amphophilic mass occupying the whole of the nucleus and the weakly eosinophilic Cowdry Type A inclusion, surrounded by a clear 'halo', the latter form generally being in the

Figure 31. Intranuclear inclusions in infected whole-chick-embryo cultures. x 3,000.

Figure 32. Syncytia in whole-chick-embryo cultures infected with strain P-5. x 320.





majority. In most of the round cells, particularly the smaller ones, the nuclei were pyknotic and inclusion bodies were not distinguishable. Many of the cells seemed to be binucleate and some of the large round ones noted in unstained cultures were found to possess three or four nuclei. Others contained a single nucleus partially divided into two lobes by a transverse restriction. In such nuclei, division of the inclusion body was evident on rare occasions.

Islands of epithelial cells were often conspicuous in whole-embryo cultures but they seldom became involved in the cytopathic process until foci were well established among the fibroblasts. Once infected, however, they manifested the same cytological changes as did the latter cells.

The infectivity titres measured between the second and the fourth days generally ranged from 10^4 to 10^6 $TCID_{50}$ / ml. Virus seemed to be readily released from the cells and transfer was easily accomplishable with medium from infected cultures. For example, culture-medium collected on the second day from the third passage of strain M-4 contained $10^{4.9}$ $TCID_{50}$ of virus per ml. compared with $10^{5.6}$ $TCID_{50}$ in the case of cells.

In order to identify the cytopathogenic agents with the virus strains isolated in eggs, medium from infected cultures was inoculated onto the chorio-allantoic membranes of embryonated eggs. Different passage levels of the various strains were examined in

that way, (e.g. first, third, sixth and ninth of strain H-1 and third, sixth and eleventh of strain M-3) and, in all cases, typical pocks appeared on the membranes and hepatic necrosis was found in the embryo. Histological examination of two-day-old pocks and of liver lesions revealed the presence of numerous characteristic inclusions. Suspensions of normal chorio-allantoic membranes and of whole-chick-embryo cultures failed to produce any cytopathogenic effect in tissue culture.

McFerran's B-1 strain and Smadel's P-5 strain both produced changes identical with those described above. The latter virus, however, gave rise to an additional effect which clearly distinguished it from strains isolated in the British Isles. Large, irregularly-shaped areas of cytoplasm were sometimes present near the centre of a focus and moderate numbers of extremely large, round cells were conspicuous at the margins of the foci, while others were to be found floating in the medium. In unstained monolayers, the detached cells were seen to consist of a spherical, granular body embedded in a clear refractile mass of cytoplasm. In stained preparations, the large spherical cells proved to be small syncytia containing 12 to 15 nuclei, within which latter basophilic or eosinophilic inclusions were present. The irregularly-shaped areas of cytoplasm were also found to be small syncytia but with pyknotic nuclei. Whether those structures had been derived from fibroblasts or from epithelial cells was not always easy to establish but, in

some cases, they were obviously of latter origin (Figure 32). Culture medium collected on the second day from the fourth passage contained $10^{4.7}$ $TCID_{50}$ of virus per ml., compared with $10^{5.9}$ $TCID_{50}$ per ml. in the case of cells. As with the other isolates, the cytopathogenic agent produced typical pocks and inclusion bodies in embryonated eggs.

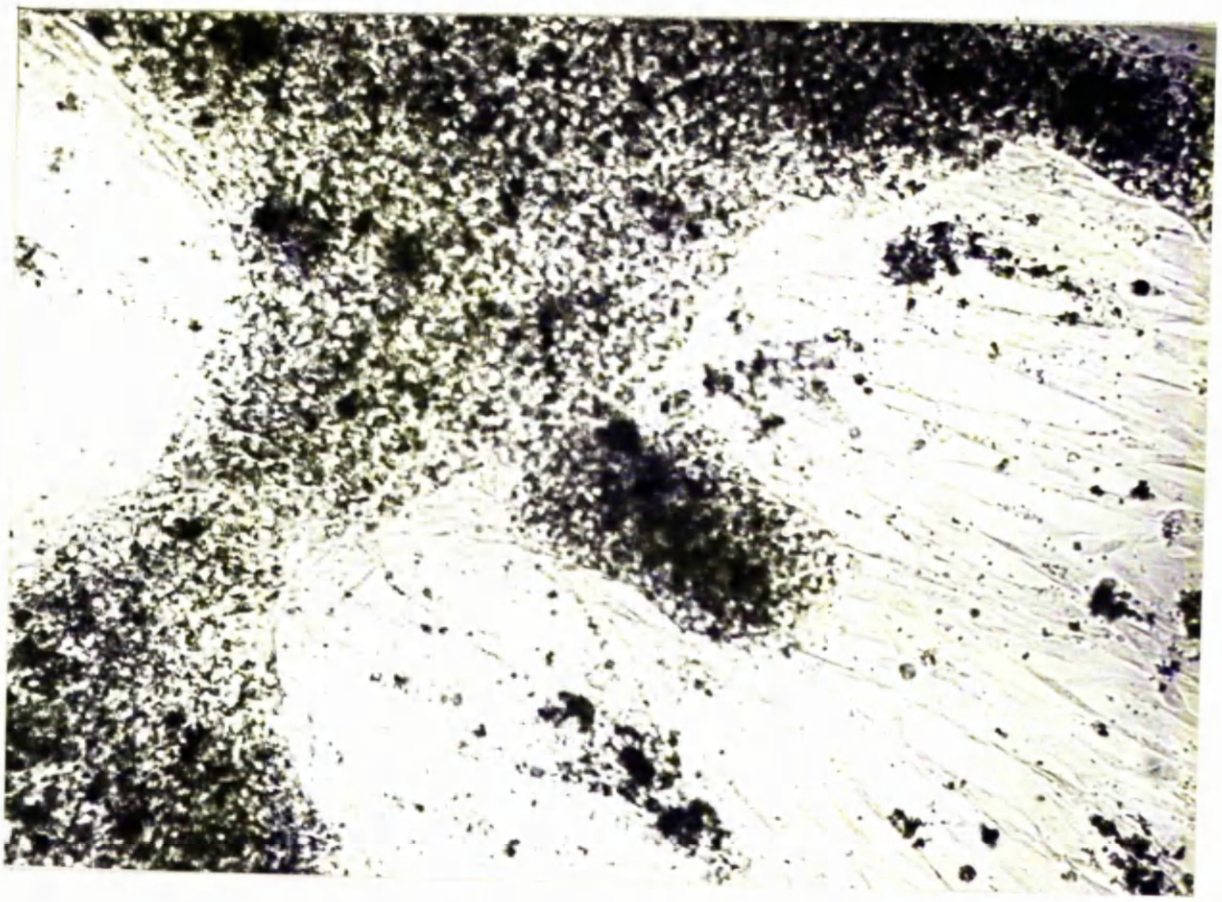
(b) Comparison of the growth and cytopathogenicity of the virus in different types of cell-culture.

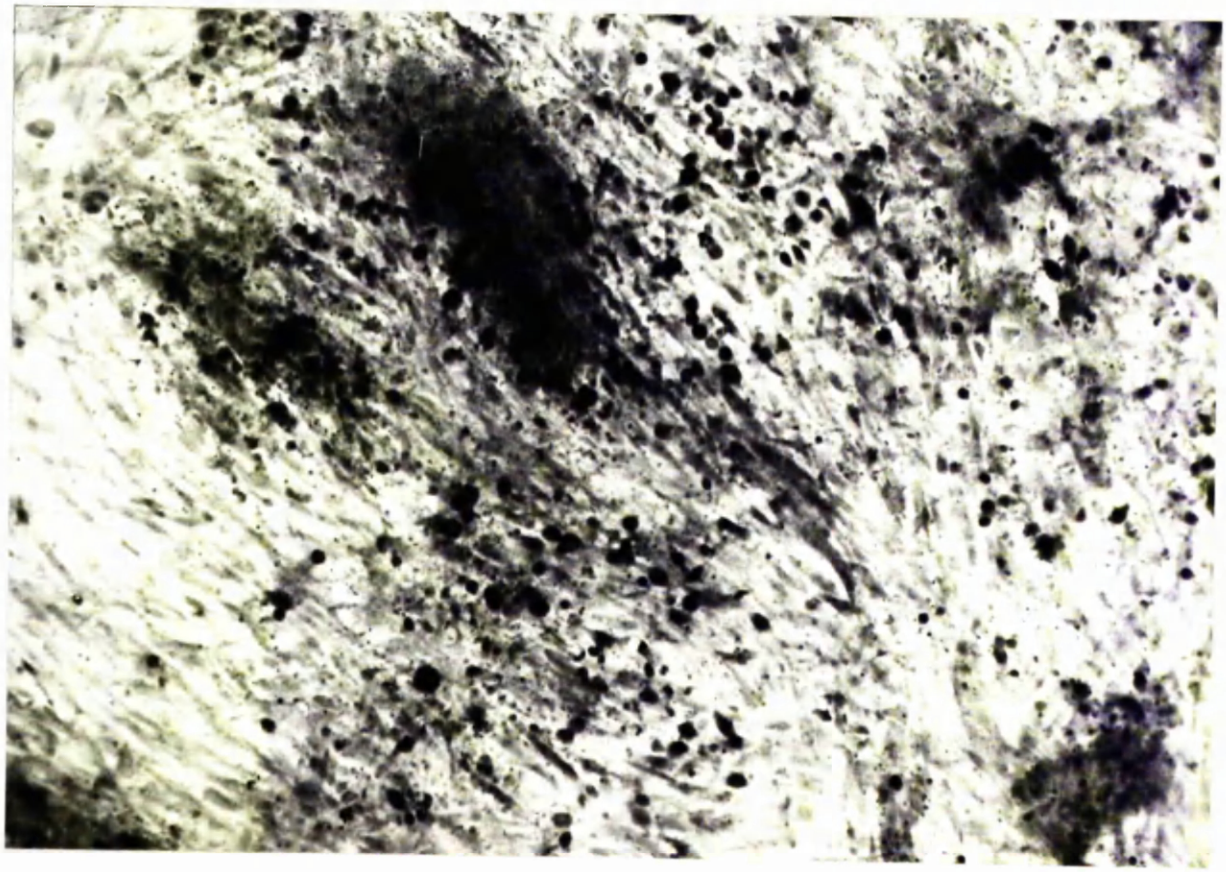
The virus was found to be cytopathogenic in all of the avian cultures examined but did not grow in any of the mammalian cells employed in the investigation. The different types of avian cell seemed to vary in susceptibility to the virus but the cytopathology was essentially the same in all.

Normal cultures of chick-embryo liver consisted of large islands of epithelial cells separated from each other by a network of fibroblasts. The former spread slowly over the glass surface and were numerically predominant during the first two days but, by the fourth day, a vigorous growth of fibroblasts halted further extension (Figure 33). Within 24 hours of the inoculation of virus, a characteristic rounding of epithelial cells was observable and a few fibroblasts were also involved. In stained preparations, moderate numbers of typical, eosinophilic Cowdry Type A inclusions were to be found in epithelial cells of normal shape,

Figure 33. Normal chick-embryo liver culture, unstained. x 150.

Figure 34. Infected chick-embryo liver culture, stained. x 150.





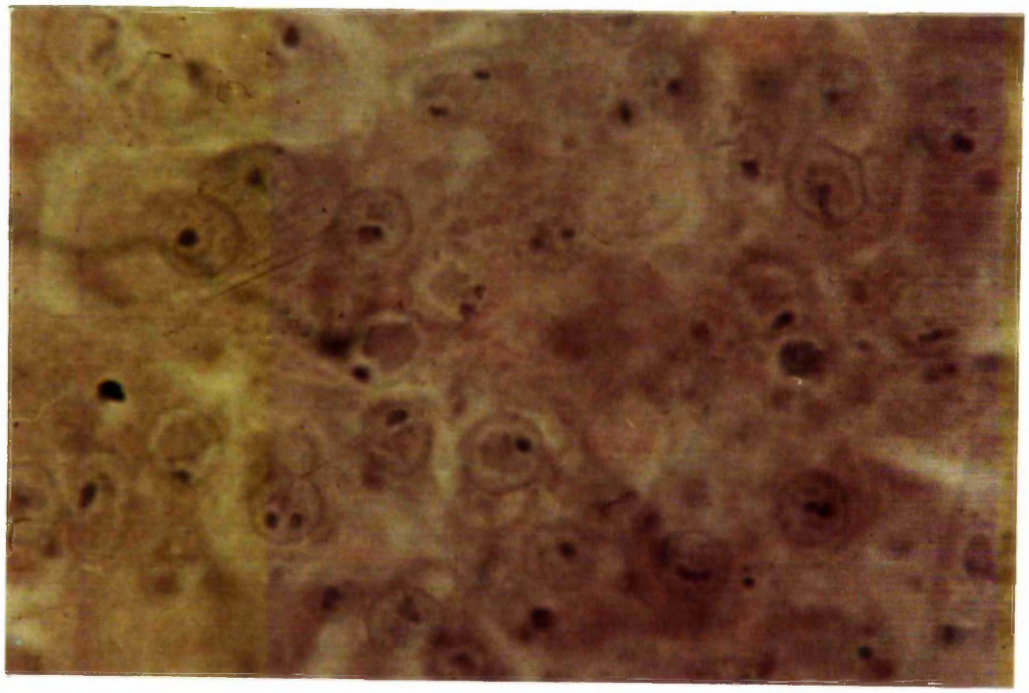
(Figure 35). The cytoplasm of the rounded epithelial cells was intensely hyperchromatic and the nuclei were shrunken and pyknotic or, in some cases, reduced to basophilic fragments. Typical inclusion bodies were not to be seen in those cells but were present in swollen fibroblasts.

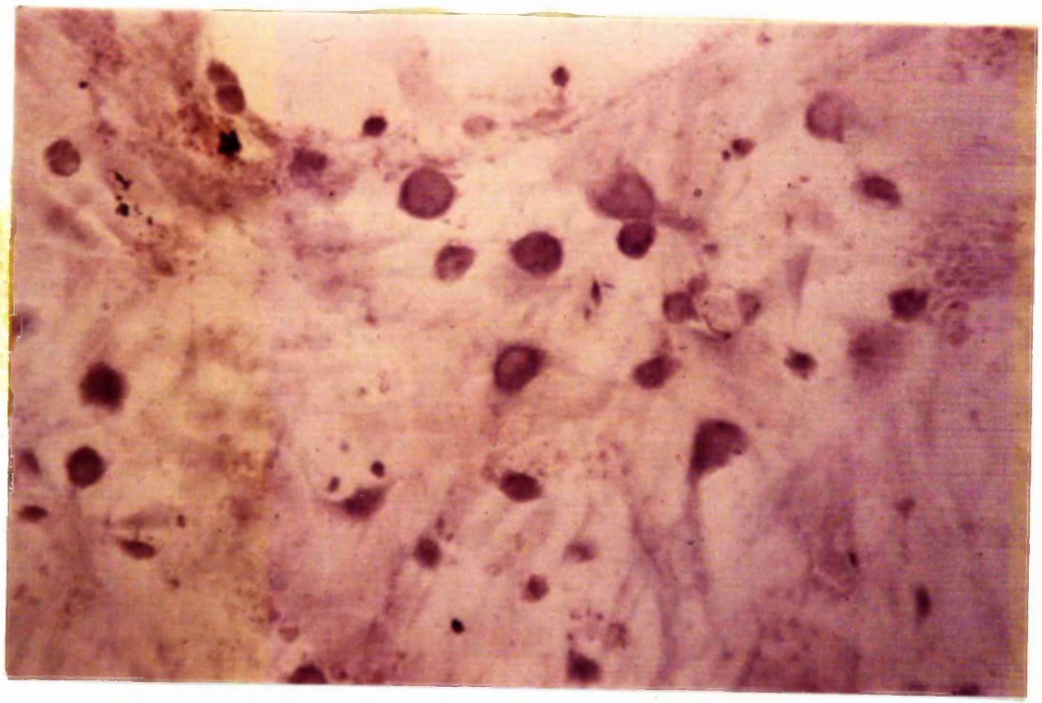
By 48 hours after inoculation, little remained of the epithelial islands. In some places, epithelial cells of normal shape, with classical Type A inclusions, were still distinguishable but were generally embedded in a mass of eosinophilic cell-debris along with small numbers of round cells. Many of the islands had largely disappeared and rounding of fibroblasts was now prominent (Figure 34). Examination carried out between the third and sixth days revealed widespread involvement of fibroblasts and, rarely, traces of epithelial islands. The general appearance was very similar to that seen during the later stage of infection in whole-embryo cultures when the highly granular cell-sheet, with much cell debris still adherent to the glass, exhibited few clear spaces.

In chick-embryo kidney cultures, the cytopathogenic effect was characterized by the appearance of round, refractile cells concentrated round the edges of the cell-sheet and distributed singly or in small clusters elsewhere (Figure 36). Many of those cells corresponded in size with the large round cells seen in infected whole-embryo cultures. Intranuclear inclusions were to be noted in

Figure 35. Intranuclear inclusions in infected chick-embryo liver culture. x 1,500.

Figure 36. Cytopathogenic effect in chick-embryo kidney culture. x 1,200.





cells of normal shape but were not observable in the round cells, the nuclei of which were pyknotic. Although small foci of such cells regularly appeared within 24 hours of inoculation, fewer secondary foci seemed to be produced in these monolayers than occurred in whole-embryo cultures, the rate of progression of the cytopathogenic effect being noticeably slower.

Strain M-4 showed the same cytopathogenicity in pigeon kidney cultures as it did in chicken kidney cultures but appeared to spread more quickly in the former. In both types of monolayer, a characteristic rounding of cells was observable within 24 hours of infection. As in the case of whole-embryo cultures, the round cells varied considerably in size but a higher proportion were of very large type. Many occurred singly but, occasionally, they were present in a cluster reminiscent of a bunch of grapes. By the third day, small circular plaques with clear centres were distinguishable with the naked-eye. Microscopical examination at that time revealed the presence of clumps of swollen, refractile cells surrounding the clear spaces. By the ninth day, virtually all the cells of the monolayer had become infected and were detached from the glass.

Examination of stained cultures showed that the round cells generally contained one or two nuclei, rarely three or four. There was not any relationship between the size of a cell and the number of nuclei which it contained. Thus, some of the largest cells

contained only one nucleus whereas some of the smaller ones possessed two. Intranuclear inclusions were sometimes visible and occasionally were clearly demarcated by a wide 'halo' but, more often, closely impinged on the nuclear membrane. In most cases, the nucleolus was pushed aside against the latter but sometimes was embedded within the inclusion. Margination of chromatin, sometimes occurring evenly and, at others, irregularly along the nuclear membrane, was a constant feature while small, round, shrunken nuclei as well as those of clavate and other irregular form, were often to be seen. Rather granular, eosinophilic, intranuclear inclusions were found in cells of normal shape.

In pigeon kidney cultures, strain P-5 produced a cytopathogenic effect similar to that induced by strain M-4, but, possibly due to prolonged passage in embryonated eggs, the rate of spread through the monolayer was noticeably slower.

Parallel titration of strain M-3 carried out in whole-embryo cultures, chick-embryo-liver cultures and chicken kidney cultures by means of the method of plaque-assay described in Section Two yielded the following results:

	Whole-chick-embryo cells.	Chick-embryo liver cells.	Chicken kidney cells.
Infectivity titre. F.F.U./ml. of virus.	$10^{5.2}$	$10^{4.5}$	$10^{2.4}$

Monolayers of HeLa cells, strain L cells and primary

renal cultures derived from the calf and the dog remained morphologically normal after inoculation and attempts to extract infective virus were unsuccessful.

(c) Comparative studies with I.L.T. and pigeon-pox viruses.

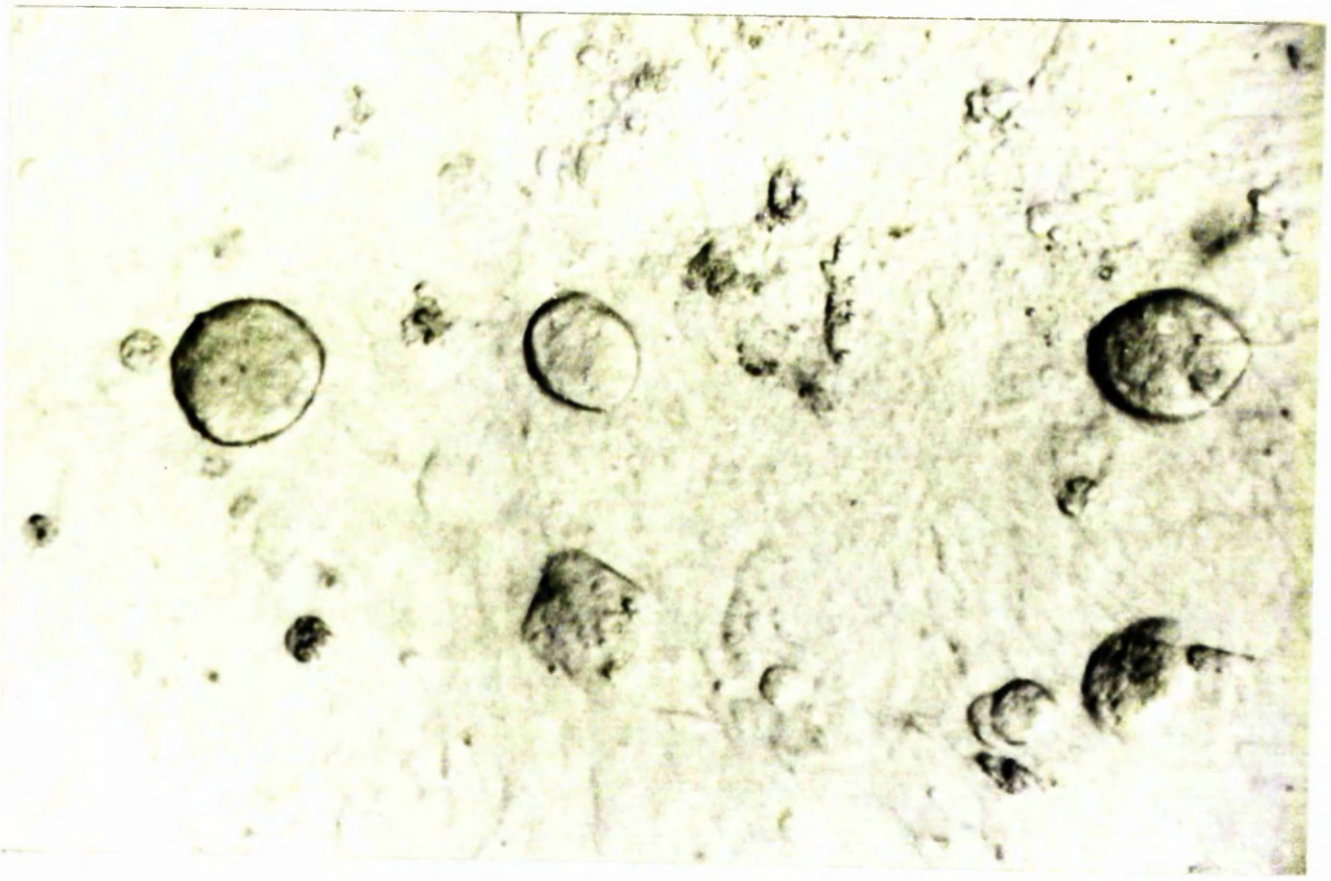
Both above viruses produced a cytopathogenic effect which was readily distinguishable from that caused by the columbine agent.

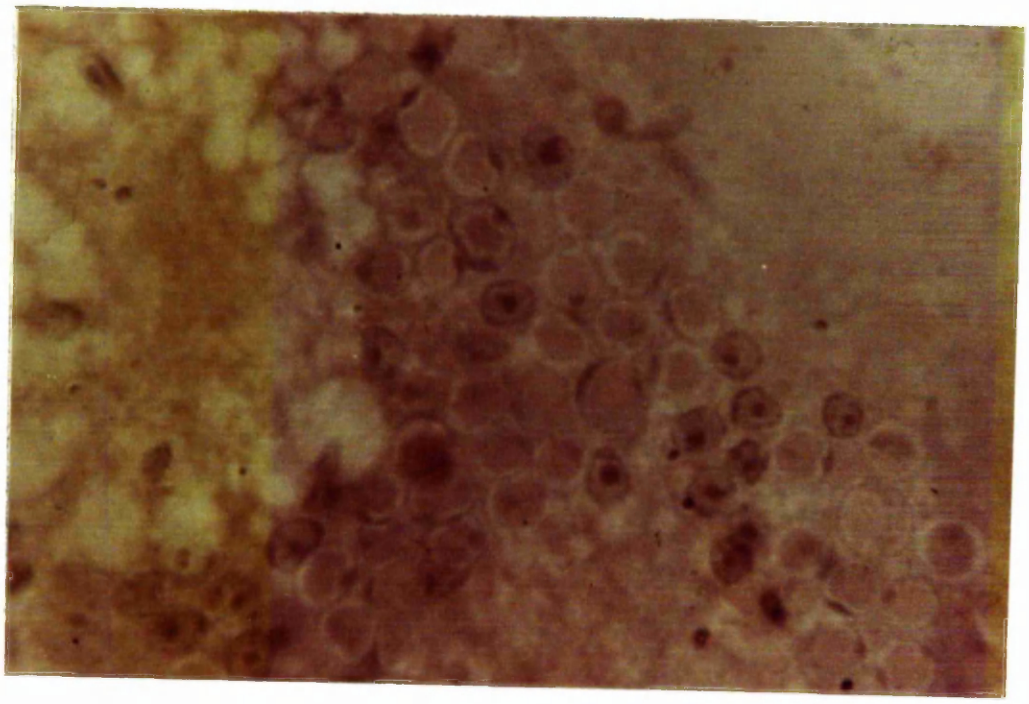
In preliminary work with I.L.T. virus, a cytopathogenic effect was observed in whole-chick-embryo cultures inoculated 24 hours earlier with a 1 in 50 dilution of macerated chorio-allantoic membranes which had shown confluent pocks. The characteristic feature was the development, within small epithelial islands, of multinucleated syncytia which, in unstained monolayers, were distinguishable by a highly refractile cell membrane of uniform circular, or ovoid, contour (Figure 37). In some cases, the nuclei were seen to be arranged in a ring or horse-shoe pattern or huddled together. By the second day, detached syncytia were manifest floating in the culture fluid.

Intranuclear inclusions were found in stained monolayers and varied considerably in size and in tinctorial reaction. Some were of classical, eosinophilic Cowdry Type A pattern; others were amphophilic or basophilic and occupied almost the whole of the nucleus without any surrounding halo. The inclusions of individual

Figure 37. Syncytia in whole-chick-embryo culture infected with I.L.T. virus. x 150.

Figure 38. Syncytia in chick-embryo liver culture infected with I.L.T. virus. x 1,200.





syncytia were generally of the same type although wide differences, ranging from highly eosinophilic Type A to small, densely basophilic structures, were sometimes recorded. Each syncytium possessed from 12 to 40 nuclei, depending on the size of the epithelial island. By the fourth or fifth day after infection, most of the syncytia had disappeared, though a few were sometimes to be found as late as the eighth day.

Despite the formation of syncytia in epitholial cell islands, a cytopathogenic effect was never to be observed in fibroblasts. Thus, on the sixth day, cultures consisted of dense sheets of apparently normal fibroblasts. On passage, the same changes appeared in the epithelial islands but a C.P.E. in fibroblasts was never appreciable.

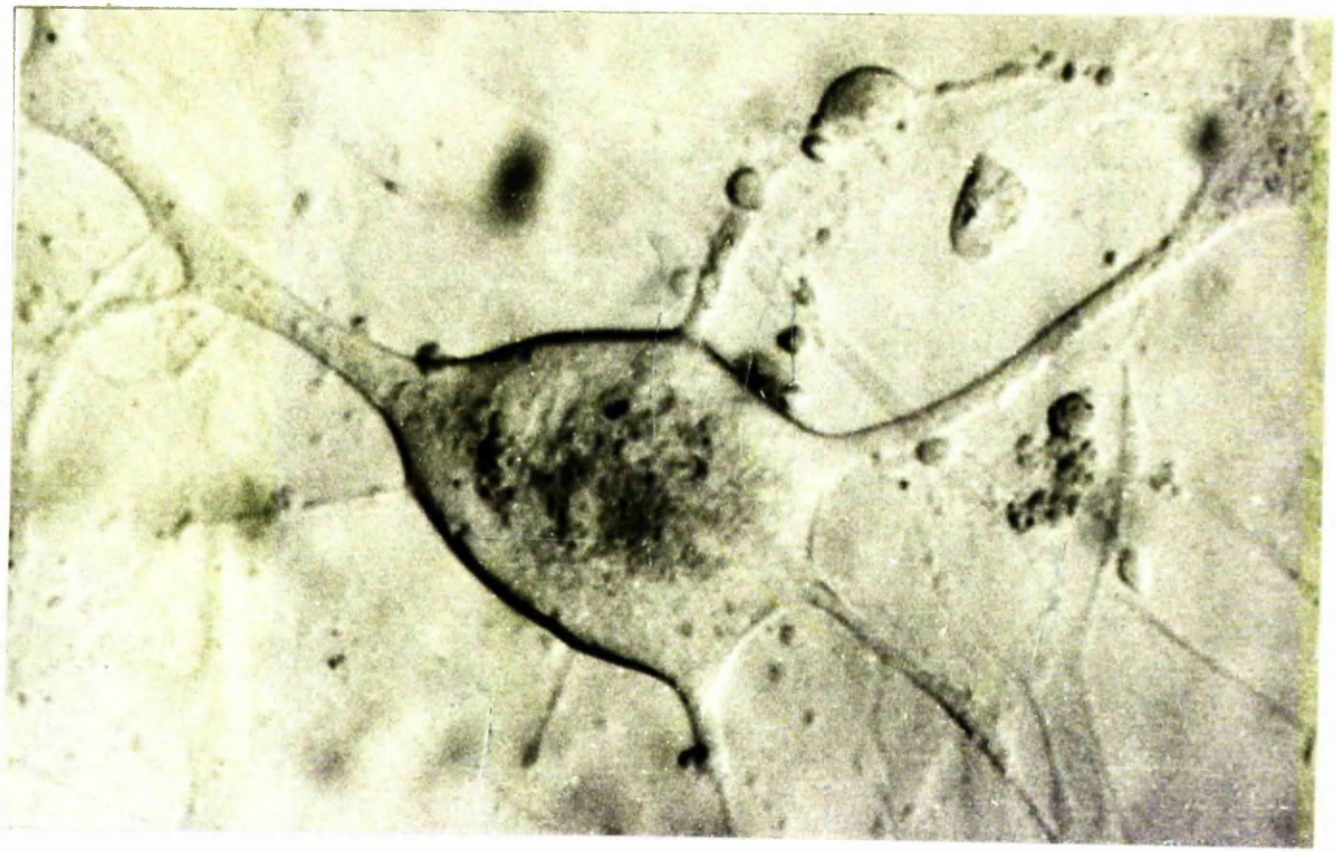
I.L.T. virus harvested on the third day of its first passage in whole-chick-embryo cultures was employed for infection of monolayers of chick-embryo liver. Multinucleated syncytia appeared in the epithelial islands on the day after infection and were of two main patterns. The first was generally highly vacuolated, had an indistinct border and contained up to approximately 100 nuclei, the majority of which contained eosinophilic inclusions (Figure 38). The second syncytial type was smaller with about 20 nuclei, often arranged in a ring, a more hyperchromatic cytoplasm, less vacuolation, a distinct membrane and a rounded or ovoid contour.

On the second day, most of the syncytia appeared degenerate, the nuclei being shrunken and pyknotic and inclusions difficult to see. By the third day, all that remained of the epithelial islands was a shrunken eosinophilic mass, devoid of all morphological detail. The place of the syncytia was occupied by fibroblasts and, by the fifth day, the cultures consisted of dense sheets of normal fibroblasts with occasional syncytial remnants. A cytopathogenic effect was not observable in the fibroblasts at any time.

Large multinucleated syncytia were formed in chicken kidney cultures infected with I.L.T. virus. They appeared within 24-48 hours of inoculation and differed from those observed in chick-embryo liver cultures in that they were stellate, with numerous elongated branches which imparted a lace-like appearance to affected parts of the monolayer. By the fourth day, many large spaces with ragged edges had formed while, during the succeeding two or three days, most of the remaining cells were destroyed.

In chick-embryo kidney cultures infected with I.L.T. virus, typical syncytia were again produced but the cytopathogenic effect differed from that noted in the foregoing cultures in that it spread much more slowly. A few stellate syncytia (Figure 39) were to be seen on the second day but, even by the fifth day, little further advancement had occurred. A cytopathogenic effect was not

Figure 39. Stellate syncytium in chick-embryo kidney culture infected with I.L.T. virus. x 150.



observed in pigeon-kidney cultures inoculated with I.L.T. virus.

Pigeon-pox virus gave rise to swelling and rounding of cells in whole-embryo cultures but the cytopathogenic effect was readily distinguished from that caused by I.N.I. virus by the much smaller number of cells involved at any one time. Infection spread slowly through the monolayer and spaces were formed as a result of cell-death but, even as late as the eighth day, approximately 50 per cent of the cells looked normal. The large, swollen cells, so characteristic of the cytopathogenic effect caused by I.N.I. virus, were never observed. Infected cells were shrunken and did not occur in well-defined clusters and neither intranuclear nor cytoplasmic inclusions were detectable in stained monolayers. The nuclei of the round cells were generally reduced to basophilic fragments.

(3) DISCUSSION.

All strains of pigeon I.N.I. virus isolated in eggs were cytopathogenic in tissue-culture. In each case, the cytopathogenic agent was capable of producing lesions in embryonated eggs, even after 11 passages in cell culture. Conversely, a 20 per cent suspension of chorio-allantoic membranes from uninoculated eggs was non-cytopathogenic for whole-embryo tissue cultures and extracts of normal monolayers did not give rise to pocks. Inclusion bodies of similar appearance were found in infected cultures, infected eggs and in the organs removed from diseased pigeons. Finally, the cyto-

pathogenic agent produced typical changes in sheets of pigeon cells, whereas I.L.T. virus had not any such effect. Those findings strongly suggest that the agent grown in cell-culture was derived from that present in the original pigeon tissues.

All strains of virus isolated in the British Isles produced the same focal cytopathogenic effect, characterized by swelling and rounding of cells. Smadel's P-5 strain, however, caused the formation of multinucleated syncytia in the epithelial islands of whole-embryo cultures in addition to eliciting the former change in individual fibroblasts. That phenomenon, however, must not be assumed to signify fundamental taxonomic difference between the isolates. Both types of effect are typical of the herpesvirus group. The viruses of bovine malignant catarrh (Plowright et al. 1960), of bovine mammillitis (Martin et al. 1966), and of I.L.T. (Churchill, 1965) are capable of producing large syncytia (containing 20-100 nuclei) whereas cytomegaloviruses (Smith, 1959) and canine herpes virus (Cornwell et al., 1966) cause rounding of individual cells. Moreover, a clear-cut dividing line cannot be drawn between those members of the group which provoke one effect and those which give rise to the other. Both types of change may be seen in monolayers infected with herpes simplex virus (Crandell, 1959), herpes B virus (Reissig and Melnick, 1955), and pseudorabies viruses (Tokumaru, 1957). Differences in cytopathology may be dependent upon factors such as the existence of variants, the amount of virus present in the

inoculum or the passage-history of the virus. Thus, a stable variant of herpes simplex virus, characterized by its increased capacity to produce syncytia, was separated from its parent strain by Hoggan and Roizman (1959a) while Tokumaru (1957) described a gradual increase in capability of the pseudorabies virus to produce syncytia during the course of serial propagation in monkey-kidney cells. Accordingly, in the present investigation, the capacity of strain P-5 to form syncytia possibly arose as a result of selection of a variant during prolonged passage in the chorio-allantoic membrane.

The requirements of the virus were not restricted to one particular type of avian cell. Growth occurred in fibroblasts and in different types of epithelial cell derived from the domestic fowl or the chick-embryo as well as in epithelial cells procured from the natural host. The cytopathology of the infection was essentially the same in all those cultures, the differences being quantitative rather than qualitative. The cells found to be most sensitive to the virus were fibroblasts derived from the whole-chick-embryo. Hepatic epithelial cells from the chick-embryo were almost as susceptible but chicken kidney cells were much more resistant. Thus, when titrations were carried out in parallel in the three different types of culture, the virus suspension employed was found to have a titre of $10^{5.2}$ P.F.U. / ml. in whole-embryo cells, $10^{4.5}$ P.F.U. / ml. in monolayers of chick-embryo liver but only $10^{2.4}$ P.F.U. / ml. in chicken kidney cells.

The cytopathogenicity of I.L.T. virus contrasted markedly with that of the columbine agent. With the exception of strain P-5, the latter never produced the large syncytia so characteristic of I.L.T. infection. Conversely, I.L.T. virus was non-pathogenic for fibroblasts. Those differences were most marked in cultures of chick-embryo liver which contained large numbers of both epithelial cells and fibroblasts. The pigeon virus produced an early rounding of epithelial cells and fibroblasts and caused extensive damage to both cellular components within two days. Infection with I.L.T. virus, on the other hand, rapidly resulted in the formation of massive epithelial syncytia which quickly degenerated but the fibroblasts remained normal and continued to grow until they occupied the spaces left by detached syncytia. The ability of I.L.T. virus to produce syncytia in chick-embryo kidney and in chicken kidney cultures has already been reported by Watrach and Hanson (1963) and by Churchill (1965), respectively. The specificity of I.L.T. virus in its requirement for epithelial cells may, possibly, be correlated with its inability to infect the mesoderm of the chorio-allantoic membrane. Churchill (1965) commented on the unsuitability of fibroblasts for the growth of I.L.T. virus but the present investigation showed that whole-embryo cultures may be employed for the propagation of the virus provided that they contain moderate numbers of epithelial islands.

The cytopathogenic effect of the pigeon I.N.I. virus was found to be very similar to that produced by the cytomegaloviruses.

Thus, Smith (1956), employing cultures of human fibroblasts for the propagation of the salivary-gland virus of man, described the development of small, circular foci in which the cells were enlarged, rounded or ovoid and more refractile than normal. The foci slowly increased in size and number, enlargement of a focus being followed by degeneration at the centre. The degenerating cells became granular and disintegrated, leaving masses of dense refractile granules. Similar results attended the investigations of Smith (1954), Hartley et al. (1957), Black et al. (1963) and Hartley and Done (1963) into cultivation of salivary-gland viruses from the mouse, the guinea-pig, the African green monkey and the sheep, respectively. However, although the pigeon I.N.I. virus resembles the cytomegaloviruses in its cytopathogenicity for fibroblasts, it differs from them in its ability to multiply in epithelial cells. Lack of cytopathogenicity for cells of the latter type is a recognized feature of salivary-gland viruses (Plummer, 1967), though inclusions have been noted by Rowe et al. (1956) and Weller et al. (1957) in epithelial cells lying adjacent to infected fibroblasts.

The cytopathogenicity of the pigeon I.N.I. virus also bears some resemblance to that of varicella virus. The latter produces foci of swollen, rounded, refractile cells in epithelial as well as in fibroblastic cultures, though syncytia may also develop, particularly in monolayers of epithelial cells (Weller, 1964). The micro-epidemiology of varicella infection, however, differs from

that found in cultures infected with the columbine agent in that infective virus particles are released from the cell only in the most minute quantities (Weller, 1964). In the present investigation, the virus was found to be released into the culture medium in moderately large amounts. Thus, a titre of $10^{4.9}$ $TCID_{50}$ / ml. was found in the fluid harvested from cultures infected with the third passage of strain M-4.

In conclusion, the columbine agent may be said to behave in cell-culture in a manner consistent with the idea that it is a herpes virus but not in such a way as to suggest that it has a special relationship with any single member of the herpesvirus group.

(4) SUMMARY AND CONCLUSIONS.

All strains of pigeon I.N.I. virus isolated in the British Isles produced the same focal cytopathogenic effect in a variety of avian cultures. Whole-chick-embryo cultures, consisting largely of fibroblasts, appeared to be much more susceptible than monolayers of renal epithelium but were only slightly more sensitive than cultures of hepatic epithelial cells. Smadel's P-5 strain produced small syncytia in the epithelial component of whole-embryo cultures and caused rounding of individual fibroblasts, a pattern of behaviour exhibited also by varicella virus. Unlike the latter, however, all strains of the columbine agent were released into the culture fluids in infective form. The virus of I.L.T. was readily distinguishable

since it formed large syncytia in epithelial cells but failed to grow in fibroblasts. Pigeon-pox virus was recognizable by reason of its lower cytopathogenicity and by the absence of intranuclear inclusions.

SECTION TWO: THE PLAQUE ASSAY.

(1) INTRODUCTION.

Since the cytopathogenic effect was found to be of focal distribution after monolayers had been infected with high dilutions of pigeon I.N.I. virus and maintained under liquid media, plaques produced under an agar overlay seemed to afford a convenient method for the assay of the infectivity of virus suspensions.

The first plaque assay in animal virology was introduced by Dulbecco (1952) and the method has since been applied to the study of a wide range of mammalian and avian viruses. The general aspects of the technique and the various factors essential to its use have been reviewed in detail by Cooper (1961a) and need not here be discussed.

The advantage of the plaque assay over the end-point dilution method is largely one of economy. At the 50 per cent end-point, an average of two cultures are required to detect one infective particle. Half the cultures do not receive any but, purely as a result of chance, some of the others receive more than one infective unit. From the Poissonian distribution of particles, it has been calculated that the infected monolayers acquire, on average, 1.38 particles each. Thus, taking non-infected as well as infected cultures into consideration, the average number of particles which

each one receives is 0.69. One hundred cultures, therefore, are necessary for the detection of 69 infectious units of virus, if each is inoculated with an average of 1 $TCID_{50}$. Since 69 plaques are accurately countable in one overlay culture, the latter may be regarded as statistically equivalent to 100 tube cultures, inoculated with limiting dilutions and maintained under liquid media. By those arguments, Dulbecco and Vogt (1954) calculated that one monkey kidney would yield 15-30 Petri dish cultures, statistically equivalent to 1,200-2,400 roller tubes. Thus, to attain similar exactitude, the plaque method is much more economical in both cells and labour.

The formation of plaques under an overlay of solid medium is also of importance in studies on viral genetics, since a clone of virus is thereby obtainable from a single plaque. Moreover, since plaques of particular appearance may be associated with certain other genetic markers, plaque morphology has been used as a criterion in the characterization and differentiation of a number of viruses. Thus, Bohl et al. (1960) showed that five serologically-distinct porcine enteroviruses caused the formation of four different types of plaques in monolayers of pig-kidney cells.

Because of the above advantages, investigations were carried out in order to establish a satisfactory system of plaque-assay for the pigeon I.N.I. virus.

(2) MATERIALS AND METHODS.

(a) Petri dishes.
(b) Cell system.
(c) Infection of cultures.
(d) Agar overlay.
(e) Methylcellulose overlay.
(f) Detection of plaques.
(g) Measurement of plaque size.

(a) Petri dishes. 50 mm. glass-plates (Anumbra) and 50 mm. plastic dishes (Falcon, Sterilin and Esco, Grade AA) were employed.

(b) Cell system. Whole-chick-embryo cultures were prepared from 10-day embryonated eggs as has been described in the previous section. Seven million cells, suspended in 5 ml. of growth medium, were pipetted into each Petri dish. Monolayers were complete within 24 hours and were used without further delay. Monolayers of chick-embryo liver and chick-embryo kidney, prepared as recorded in the preceding section, were also employed.

(c) Infection of cultures. After removal of the growth medium, the cells were washed once with P.B.S. prior to inoculation. Virus dilutions were made up in Hank's B.S.S. The volume of inoculum added to each plate was 0.5 ml. To promote attachment of virus, plates were incubated at 37°C. for four hours in an atmosphere of five per cent carbon dioxide.

(d) Agar overlay. Following adsorption of virus, the inoculum was

removed and the cells washed once with P.B.S. The overlay was prepared by mixing 1.8 per cent agar (Difco-Bacto) in deionised water with an equal volume of double-strength Earle's B.S.S. containing 0.25 per cent lactalbumin hydrolysate and five per cent inactivated calf serum. The volume of overlay added to each plate was 4 ml.

(e) Methylcellulose overlay. The method used for the preparation of the overlay was that described by Rapp et al. (1959), though the methylcellulose was not washed with alcohol prior to use. One gram of the compound was suspended in 40 ml. of boiling water, autoclaved and cooled to 40° C. An equal volume of double-strength Eagle's medium, containing the usual antibiotics, and 20 ml. of horse serum were then added. The resultant suspension was employed in precisely the same way as the agar overlay.

(f) Detection of plaques. Plates were incubated at 37° C. in an atmosphere of five per cent carbon dioxide in air for four days. Initially, 1 ml. of overlay, containing a 1 in 20,000 dilution of neutral red, was added to each plate on the third day. Although that procedure generally proved satisfactory, toxic effects sometimes occurred and results were thus nullified. The method was, therefore, abandoned and replaced by a conventional technique of fixation and staining, as follows: 3 ml. of mercuric-formol were added to each plate on the fourth day. After 5-24 hours, the fixative was decanted and the agar separated from the sides of the dish by means of a fine

needle, following which the overlay was removable in one piece without damage to the cell-sheet. The monolayers were then washed in methyl alcohol and stained by Leishman's method.

(g) Measurement of plaque size. A plastic block, with aperture just wide enough to allow insertion of a Petri dish, was secured to the metal plate located immediately behind the lens casing of a Kershaw 'Daylight' strip projector, the slide-holder first being removed. In that way, stained monolayers were projected onto a screen which consisted of several large pieces of graph paper, which were divided into square inches, each sub-divided into small squares 1/10 ins. in length. A Petri dish with a 1 cm. square marked out of the cell-sheet was then inserted into the projector and the position of the latter adjusted until the image of the 1 cm. line on the dish occupied eight inches on the screen. The diameters of the plaques, one at right angles to the other, were then recorded in terms of the number of small squares covered. The real dimensions of the plaque were then calculated, on the basis of eight small squares on the screen being equivalent to 1 mm. Miscalculation of one small square introduced an error of only 0.125 mm. in the final calculation of the plaque diameter.

(3) EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT ONE: Production of Plaques under Agar.

The aim of this experiment was to determine whether plaques were producible under an agar overlay and, if so, to study their morphology, size and rate of development.

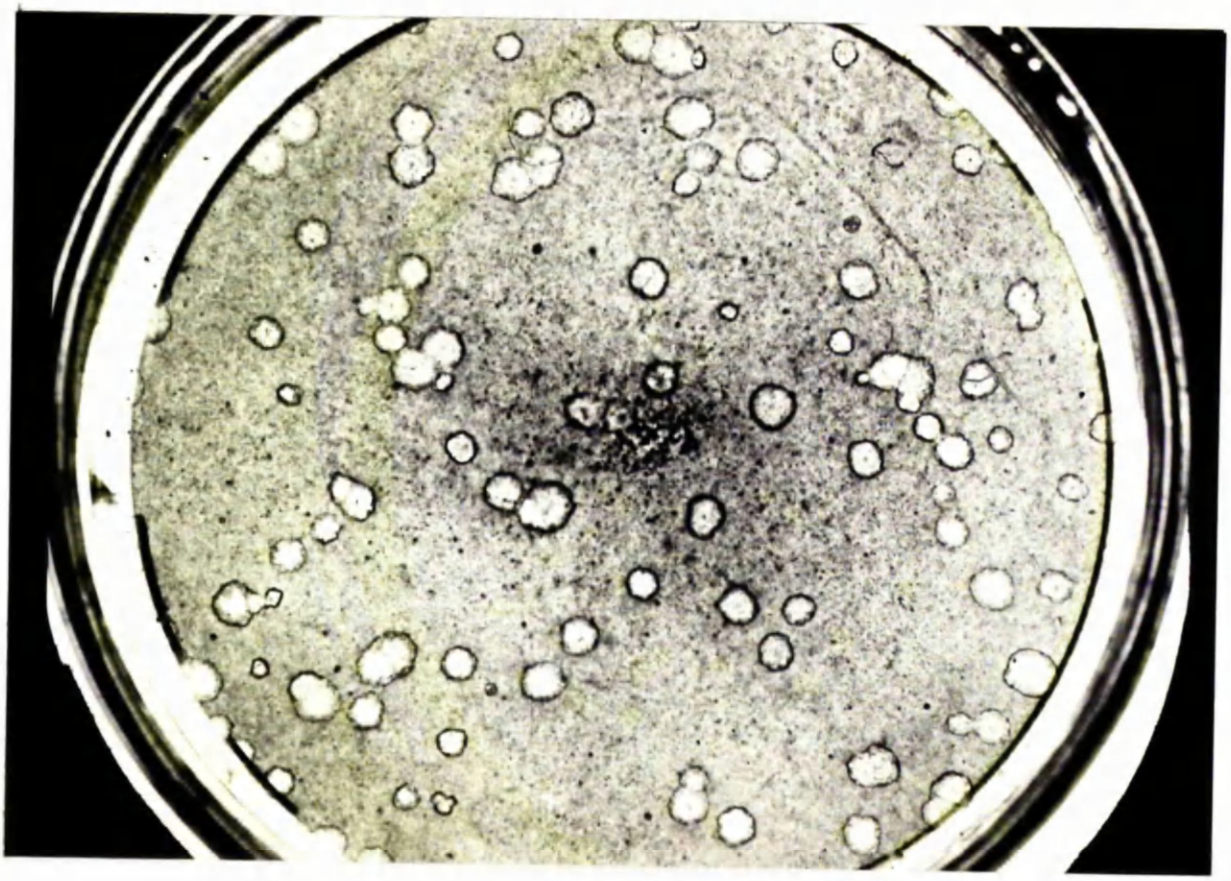
For the above purposes, whole-embryo cultures were inoculated with ten-fold dilutions of strains of tissue-culture-adapted pigeon I.N.I. virus, Nos. H-1, HS-2, M-3, M-4 and P-5, while monolayers of chick-embryo liver and chick-embryo kidney cells were infected with dilutions of strain M-3. Overlays of agar were added and the plates incubated as already described.

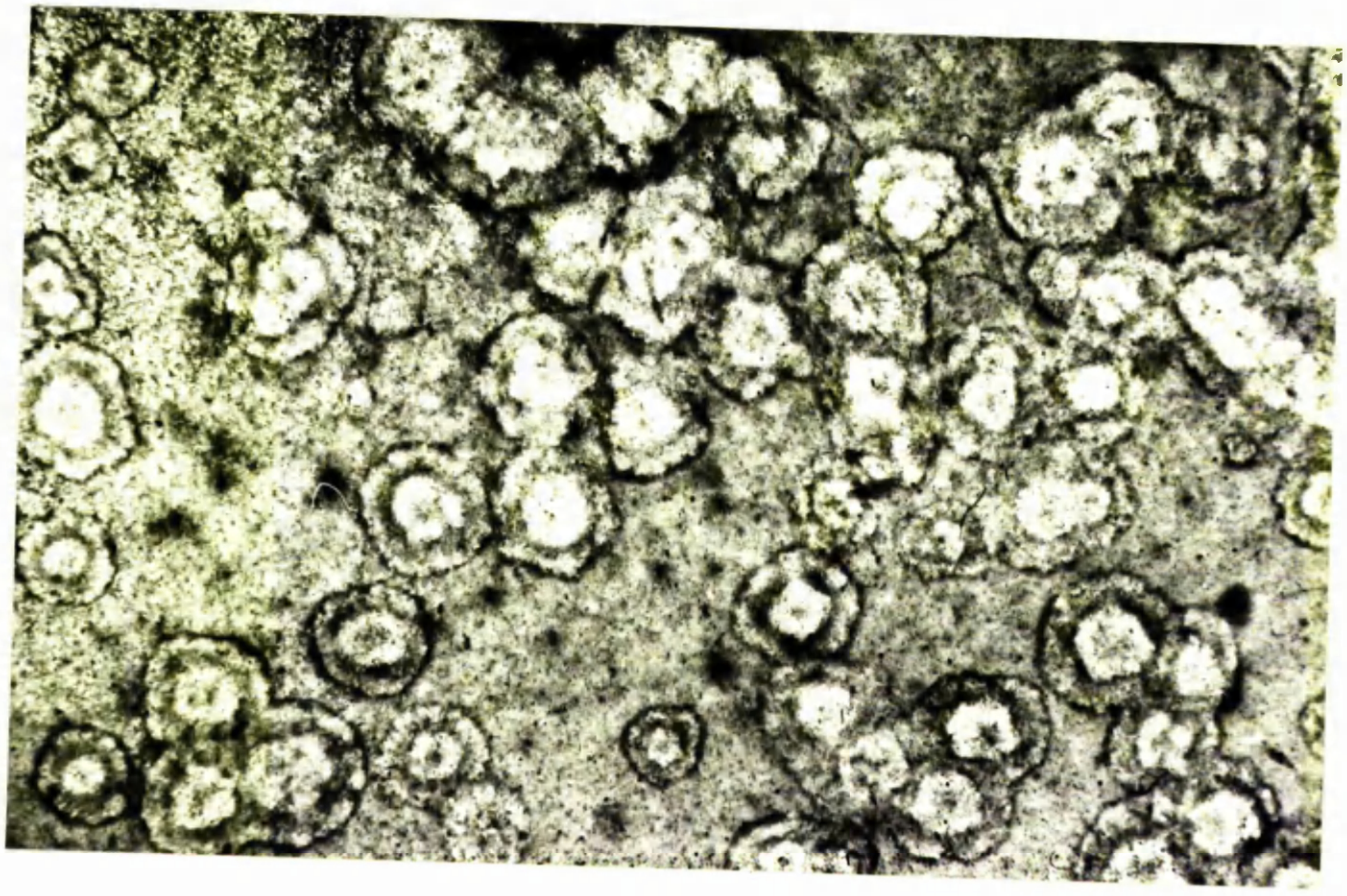
Macroscopic plaques generally appeared in whole-embryo cultures on the third day but were rarely appreciable on the second day. The larger ones were distinguishable without the aid of neutral red or other stain and appeared as greyish, opaque, circular foci when viewed with oblique illumination against a dark back-ground.

The plaques were circular or slightly ovoid in shape and, prior to the fifth day, were always sharply demarcated by a narrow band of round or swollen cells which stained very deeply with both neutral red and Leishman's stain (Figure 40). In some instances, the interior was entirely clear so that the plaques had a "punched-out" appearance but, usually, cell debris remained and was distributed

Figure 40. Plaque-formation under agar. x 2.4.

Figure 41. Plaques of "target-board" appearance. x 10.





in a variety of ways. Thus, in the smaller plaques, the debris, comprised of cells at various stages of dissolution and stained pale lilac with Leishman's stain, occupied the whole of the area within a deeply-coloured peripheral ring. In larger plaques, the centre was often clear but the largest ones occasionally resembled a target-board, with a central core of debris surrounded by a clear zone which was encircled by another band of debris that was enclosed by a hyperchromatic border (Figure 41).

Plaques produced under agar in cultures of chick-embryo liver and chick-embryo kidney conformed in size and morphology with those observed in monolayers of whole-embryo cells though, owing to the strong affinity of the hepatic epithelial cells for Leishman's stain, the hyperchromatic border was less conspicuous in the liver cultures. A small number of plaques had clear centres with consequent "punched-out" appearance but the great majority contained debris which was loosely scattered in the central area but more compactly disposed immediately inside the hyperchromatic rim.

The principal disadvantage of cultures of embryonic liver over those of the whole-embryo was that a much smaller number of cultures was obtained from the same quantity of eggs. A second major drawback was that two types of cell were always present, the proportion of epithelial cells sometimes varying considerably among different batches of cultures. The islands of fibroblasts stained

very weakly and, although easily differentiable from the larger plaques, were sometimes to be confused with the smaller ones unless a colony microscope was used. Since cultures of chick-embryo kidney were not producible in bulk and, as reported in Section One, seemed less susceptible than monolayers of whole-embryo cells, the decision was made to standardise the latter type of culture for all subsequent work.

When it became clear that all the strains of virus employed in the investigation gave rise to plaques, a further 30 plates of whole-embryo cultures were each inoculated with approximately 50 P.F.U. of strain M-3 and overlaid with agar in order to study the rate of growth of the plaques. The plates were examined on the third, fourth and fifth days of incubation, ten being dealt with per day. The number of plaques observed was recorded and their size on the third and fourth days then measured, for which purpose one plate was stained on each occasion.

The plaque-counts on successive days are shown in Table 2. The number of plaques counted on the fourth day was about 50 per cent higher than that recorded on the preceding day, i.e., on the third day, about one-third of the plaques were of microscopic size but, by the following day, they had enlarged sufficiently to be seen with the naked eye. On the fifth day, however, a diffuse non-specific degeneration, a phenomenon occasionally encountered in

TABLE 2

PLAQUE COUNT PER DAY

Strain M-3, twelfth passage in cell-culture

Day	Number of plates	Plaque counts	Mean	Variance	Standard deviation	Coefficient of variation
3	10	26, 28, 41, 24, 30, 29, 25, 30, 30, 36	29.9	26.55	5.15	17.23%
4	10	37, 61, 42, 59, 47, 48, 33, 37, 48, 41	45.4	85.58	9.25	20.38%
5	10	40, 33, 36, 31*, 29*, 35*, 30*, 30*, 36*, 39	33.9	-	-	-

* plates showed a considerable loss in staining affinity.

N.B. Among the 20 plates examined on the third and fourth days, in only five do the individual counts differ from the mean by more than the standard deviation and none exceeds or falls short of the mean by a value greater than twice the standard deviation so that both means are statistically acceptable. When the "t" test is applied, the difference between the two means is found to be highly significant ("t" = 4.63, P = < .001).

cultures maintained under agar for more than four or five days and resulting in loss of contrast between the plaques and the lightly-staining degenerate monolayer, prevented the making of an accurate count.

The sizes reached by the plaques on the third and fourth days are shown in Table 3. The largest plaque observable on the third day was 1.25 mm. in diameter, the smallest 0.5 mm. and the overall average diameter 0.84 mm. On the fourth day, approximately 38 per cent of the plaques were larger than the maximum recorded on the previous day, the largest reaching 2 mm. in diameter, or four times the width of the smallest. The mean overall diameter was 1.11 mm.

In size and rate of growth, the plaques described closely resemble those produced by herpes simplex virus. Thus, Kaplan (1957) found that the plaques formed by the latter virus in rabbit kidney cultures appeared on the second day but did not reach maximal size or number until the fourth or sixth day. By the fourth day, most of the herpes plaques had attained a diameter of 1-2 mm. but, unlike the plaques produced by poliovirus, did not continue to increase in size thereafter. Not all herpesviruses, however, behave in that manner. Thus, Kaplan and Vatter (1959) reported that pseudorabies virus formed much larger plaques, 5-10 mm., in rabbit kidney cells by the third day.

TABLE 3

PLAQUE SIZE PER DAY

Strain M-3, twelfth passage in cell-culture

Plaque diameter in mm.	Number of plaques Day 3	Day 4
0.500	5	3
0.625	2	4
0.750	3	5
0.875	2	5
1.000	4	3
1.125	3	2
1.250	1	3
1.375	0	6
1.500	0	3
1.625	0	2
1.750	0	2
1.875	0	1
2.000	0	1
Total	20	40
Mean plaque diameter in mm.	0.84	1.11

The largest number of plaques allowable for assay purposes in a single Petri dish has been calculated by Dulbecco and Vogt (1954) from the average plaque size and the area of the culture-vessel. The maximum number of non-overlapping circles (N) which can be accommodated on the surface of a disc of area A is given by the equation:

$$N = \frac{A}{0.866d^2}$$

where d is the root mean square of the diameter of the circles. If that formula is applied to the results of the present investigation where d = 1.16 mm. and the diameter of the Petri plate is 50 mm., N is equivalent to 1685. As the amount of virus (V) added to a plate is increased, the proportion of plaques formed from two or more infected cells in the same vicinity also rises, so that the higher the value of V, the greater will be the difference between the number of plaques initiated and the number of observable plaques (P). The quantity P is given by the equation:

$$P = N(1 - e^{-\frac{V}{N}}) \qquad (i)$$

where e is the base of natural logarithms and N and V are as previously defined.

From equation (i), $$P = N - \frac{N}{e^{\left(\frac{V}{N}\right)}} \qquad (ii)$$

If d = 1.16, then N = 1685, as previously calculated. If 300 plaque-forming units (P.F.U.) of virus are used to infect a 50 mm. plate,

$$\text{then } P = 1685 - \frac{1685}{2.71828^{\left(\frac{300}{1685}\right)}}$$

$$= 1685 - \frac{1685}{2.71828^{(0.178)}}$$

$$= 1685 - \frac{1685}{1.195}$$

$$= 1685 - 1410$$

$$= 275.$$

Thus, 300 P.F.U. of virus would give a count of 275 plaques, the error due to overlapping being $\frac{25}{300}$ or 8.33 per cent. Similarly, if 200 P.F.U. were employed, the number of plaques observed would be 189, an error of five per cent, and if 100 P.F.U. were utilised, the count would be 97, and the error three per cent. From those figures, it may be concluded that, if the results of an assay are read on the fourth day, the time at which the mean plaque diameter is around 1.1 mm., and if the error due to overlapping is to be kept to five per cent, the maximal plaque count in a 50 mm. plate should not exceed approximately 200.

EXPERIMENT TWO: Attempted Isolation of Virus from Plaques and from Agar.

Certain criteria, reviewed by Cooper (1961a), are essential to the establishment of a statistically-valid assay-system:-

(1) Monolayers should remain normal in the absence of virus.

(2) The virus must be isolable from a plaque in far higher concentration than from distant areas.

(3) A linear relationship must exist between the plaque count and the amount of virus used to infect the culture.

(4) Plaques should be allocated among cultures of one batch according to the Poisson distribution, and the coefficient of variation of duplicate assays should approximate to the theoretical value expected from the total number of plaques counted.

(5) Plaque formation should be inhibited by low concentrations of specific antiserum but not by normal serum.

(6) Repeated assays of a virus stock should give the same titre with cultures of different batches.

The aim of the present and of succeeding experiments was to demonstrate that, when the pigeon I.N.I. virus is titrated by means of a plaque-assay, the above criteria are fulfilled.

Early experiments indicated that plaques were not produced in the absence of virus. In order to confirm the converse, attempts were made in the present experiment to isolate infective virus from a number of plaques of varying size. By means of a Pasteur pipette,

a cylinder, taken from the agar above a plaque according to the method described by Dulbecco and Vogt (1954), was expressed into 2 ml. of Hank's B.S.S. and the pipette washed with the same solution. The fluid was frozen to -40° C., thawed out and inoculated into a tube-culture which was kept under observation for the appearance of the specific cytopathogenic effect. Provided that the plaques examined were larger than approximately 1 mm. in diameter, most attempts at the recovery of virus from them were successful, but difficulty was often experienced in the isolation of virus from the smaller plaques, presumably because of the lesser amount of virus present therein together with the problem of aligning the pipette correctly over such a small area.

In order to determine whether or not the virus was capable of diffusing through the agar, cylinders were removed therefrom at distances of two, four and six mm. from the nearest plaque and examined for the presence of virus. A small portion of agar was also removed from the surface of the overlay (depth = 4 mm.) immediately above a plaque and tested in the same manner. In all those instances, however, recovery of virus was unsuccessful. It may be concluded, therefore, that the present experiment not only served to prove that plaques were produced by the virus but also, by showing that the virus was unable to diffuse through the agar, demonstrated the effectiveness of the latter in preventing the appearance of secondary plaques.

EXPERIMENT THREE: Linearity of the Relationship between Plaque-Count and Virus Concentration.

The demonstration of a linear relationship between the amount of virus employed and the number of plaques produced is essential for the establishment of a plaque-assay since it offers experimental support for the hypothesis that one particle is sufficient for the infection of a cell and therefore provides a valid method for the computation of infectivity. Thus, Dulbecco and Vogt (1954) showed that the number of plaques formed by poliovirus in monkey-kidney cells was directly proportional to the quantity of virus in the inoculum and that the same relationship would not obtain if more than one particle was necessary for the creation of a plaque.

The object of the present experiment was to determine whether, or not, the number of plaques produced by the pigeon I.N.I. virus was proportional to the virus concentration employed. Initially, plates of whole-embryo cells were infected with ten-fold dilutions of strain H-1 (sixth passage in cell culture). Thereafter, two-fold dilutions, ranging from approximately 100 P.F.U. to 1 P.F.U., were employed. Similar experiments were conducted with strain M-3 (eleventh passage in cell culture).

The results of the first titration, in which ten-fold dilutions of strain H-1 were used, are shown in Table 4. As far as the range of dilutions allows, the figures indicate that the dose-

TABLE 4

TITRATION OF STRAIN H-1 BY MEANS OF TEN-FOLD DILUTIONS

Virus dilution	Number of plates	Number of plaques	Mean	Variance	Standard deviation	Coefficient of variation
10^{-1}	4	All confluent	—	—	—	—
10^{-2}	4	Uncountable	—	—	—	—
10^{-3}	4	104, 109, 129, 131	118	189	13.75	11.65%
10^{-4}	4	9, 7, 12, 13	10.3	7.66	2.77	27.77%

TABLE 5

TITRATION OF STRAIN H-1 BY MEANS OF TWO AND FOUR-FOLD DILUTIONS

Relative virus concentration	Number of plates	Number of plaques	Mean	Variance	Standard deviation	Coefficient of variation
8x	2	144, 125	134.5	—	—	—
4x	3	65, 60, 61	62	7.0	2.65	4.27%
2x	5	25, 25, 27, 30, 35	28.5	17.81	4.22	14.81%
1x	5	10, 14, 15, 17, 18	14.8	9.75	3.12	20.8%

response relationship is linear. The results of titrations employing two-fold dilutions of virus are recorded in Tables 5 and 6. The linearity of the relationship is illustrated in Figure 42 which has been constructed from the data supplied in the latter two tables. Table 7 shows the numbers of plaques produced by two-fold dilutions of strain M-3. Again, the dose-response relationship is approximately linear over the range of infectivity employed. The mean plaque - counts recorded in Tables 5-7 are statistically-valid since approximately 70 per cent of the individual counts differ from them by less than the standard deviation and only three (2.6 per cent) deviate by more than twice the latter value.

Thus, in four titrations, a linear relationship between plaque-count and virus input was obtained. That finding, confirmed by subsequent experience in assays based on two or more dilutions, demonstrates the validity of the system for the measurement of infectivity.

TABLE 6

TITRATION OF STRAIN H-1 BY MEANS OF TWO-FOLD DILUTIONS

Relative virus concentration	Number of plates	Number of plaques	Mean	Variance	Standard deviation	Coefficient of variation
16x	9	63, 48, 39, 51, 48, 48, 66, 51, 41	50.56	79.32	8.96	17.64%
8x	12	24, 25, 28, 26, 20, 30, 18, 20, 29, 23, 22, 19	23.66	16.24	4.03	17.03%
4x	13	13, 12, 11, 9, 16, 15, 19, 11, 9, 6, 7, 11, 8	11.3	13.39	3.66	32.4%
2x	12	6, 2, 7, 3, 5, 8, 5, 3, 6, 7, 16, 8	6.33	13.15	3.63	57.26%
1x	12	1, 4, 1, 4, 1, 2, 2, 0, 2, 2, 4, 1	2.0	1.82	1.35	67.4%

LINEARITY of DOSE-RESPONSE RELATIONSHIP

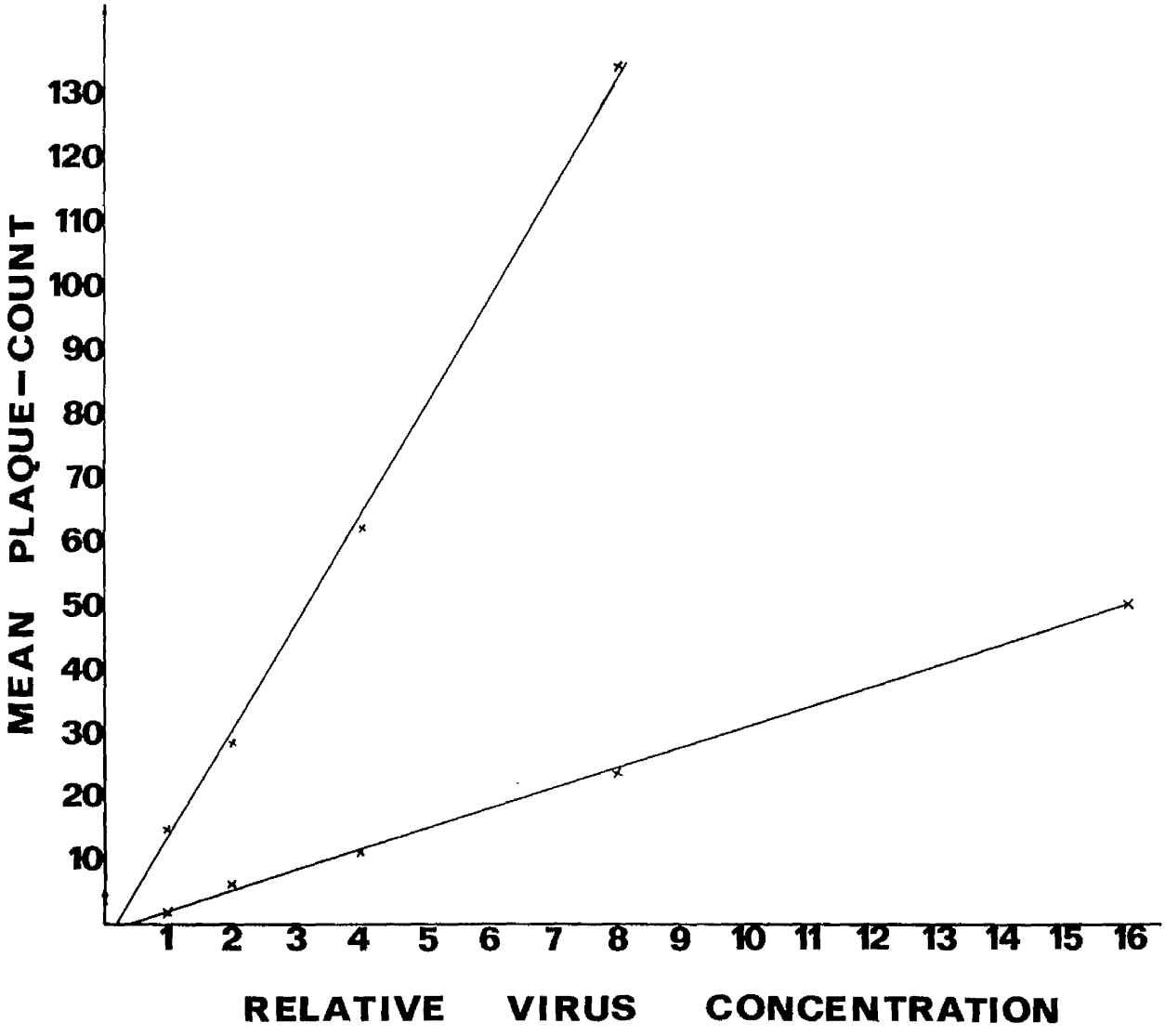


TABLE 7

TITRATION OF STRAIN M-3 BY MEANS OF TWO-FOLD DILUTIONS

Eleventh passage in cell-culture

Relative virus concentration	Number of plates	Number of plaques	Mean	Variance	Standard deviation	Coefficient of variation
16x	7	47, 58, 57, 44, 44, 38, 36	46.3	73.0	8.54	18.45%
8x	10	21, 26, 23, 25, 26, 17, 27, 33, 25, 20	24.3	19.44	4.41	18.37%
4x	10	13, 18, 14, 16, 9, 14, 16, 11, 12, 10	13.3	8.23	2.87	21.57%
2x	10	6, 10, 4, 8, 12, 6, 5, 5, 6, 8	7.0	6.22	2.49	35.63%
1x	7	5, 3, 1, 2, 1, 3, 3	2.6	1.95	1.40	43.35%

EXPERIMENT FOUR: The Poisson Distribution of Plaques.

The experiment under report was intended to reveal the degree to which the allocation of plaques between Petri dishes conformed with that expected from the Poisson distribution. The work comprised a study of the relationship between the actual and the theoretical distribution of plaques when (a) a large number of plates was inoculated with limiting dilutions of virus and (b) when the quantity of cultures employed was restricted to the amount most likely to be used routinely in the laboratory, i.e. between four and 12 per virus dilution. Thus, it was hoped that the favourable statistical conditions provided in the former part of the investigation would demonstrate the frequency-distribution of plaques to be of the Poisson form, while the latter portion would show that, when the results of a significant number of titrations are analysed, most of the coefficients of variation found to obtain broadly correspond with those expected from theory.

In order to obtain a curve of the frequency-distribution of plaques, 50 plates were inoculated with a limiting dilution of strain H-1, the mean concentration of virus being approximately 5 P.F.U. per plate. The results are shown in Table 8 and may be analysed in the following manner. If 100 plates are inoculated with an average of m P.F.U. of virus, the number of plates (Pr) expected to bear x plaques is given by the Poisson distribution as follows:-

TABLE 8

FREQUENCY-DISTRIBUTION OF PLAQUES PER PLATE

Strain H-1

Number of plates with		0	plaques	=	0
"	"	1	"	=	2
"	"	2	"	=	4
"	"	3	"	=	8*
"	"	4	"	=	8*
"	"	5	"	=	9*
"	"	6	"	=	8*
"	"	7	"	=	5*
"	"	8	"	=	2
"	"	9	"	=	3
"	"	10	"	=	2
"	"	11	"	=	0
"	"	12	"	=	0

* Plates with counts within one standard deviation on either side of mean.

Mean plaque count	=	4.94
Variance	=	4.96
Standard deviation	=	2.23
Coefficient of variation	=	44.53%

$$Pr = \frac{e^{-m} m^x}{x!} \times 100 \qquad \text{or} \qquad Pr = \frac{m^x}{e^m x!} \times 100$$

Substituting the figures recorded in Table 8, where the mean plaque count in 50 plates is 4.94, the number of plates expected to have four plaques would be :-

$$\frac{4.94^4}{2.71828^{(4.94)} \times 4!} \times 100 = \frac{4.94^4}{139.7 \times 24} \times 100 = 17.75.$$

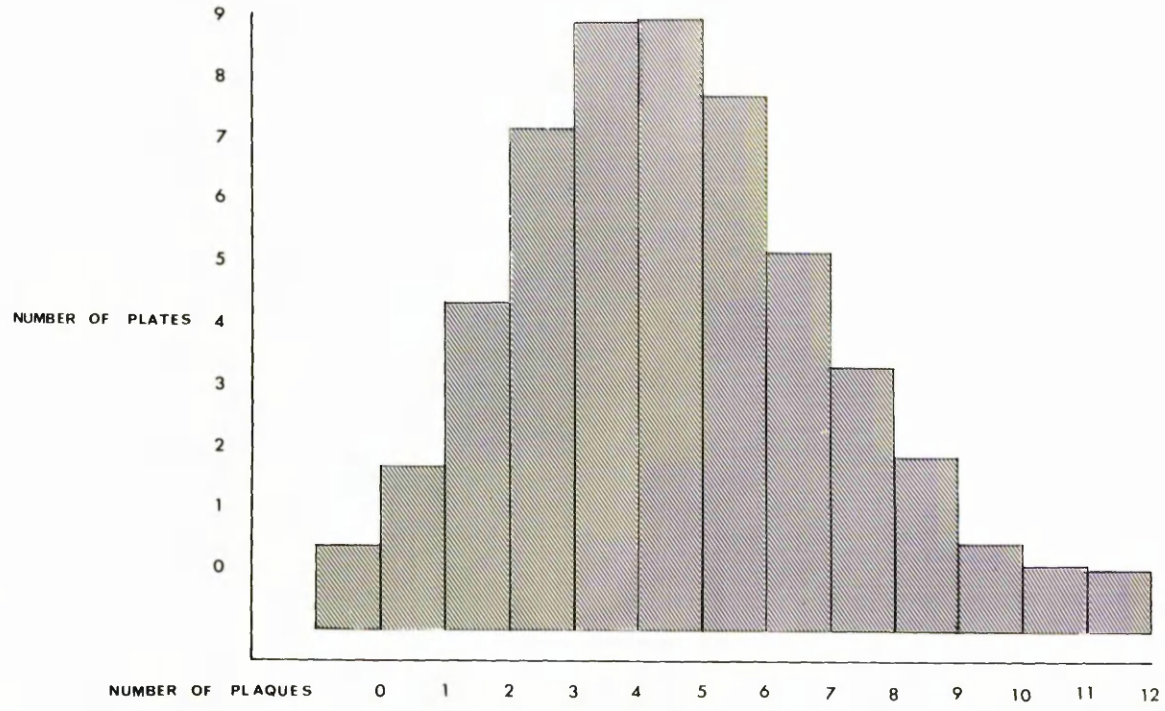
The number of plates expected to have 0, 1, 2, 3, etc. plaques may be similarly calculated and, in that way, a histogram (Figure 43) obtained. When the actual distribution of plaques is compared with the theoretical allocation, it is found that the former approximates to the latter, i.e., the allocation of plaques between Petri plates follows the Poisson equation.

The coefficient of variation is given by the expression $\frac{\sqrt{V}}{m} \times 100$ where V is the variance and m the mean. As Isaacs (1957) has shown, when distribution of individuals follows the Poisson form, the variance is equivalent to the mean. The expected coefficient of variation in a Poisson distribution, therefore, is given by the expression $\frac{\sqrt{m}}{m} \times 100$. Thus, from any mean, it is possible to calculate the coefficient of variation to be expected if the allocation of plaques was Poissonian. In that way, a graph relating the theoretical coefficient to the mean plaque count may be drawn.

In the second part of the experiment, the actual coefficients

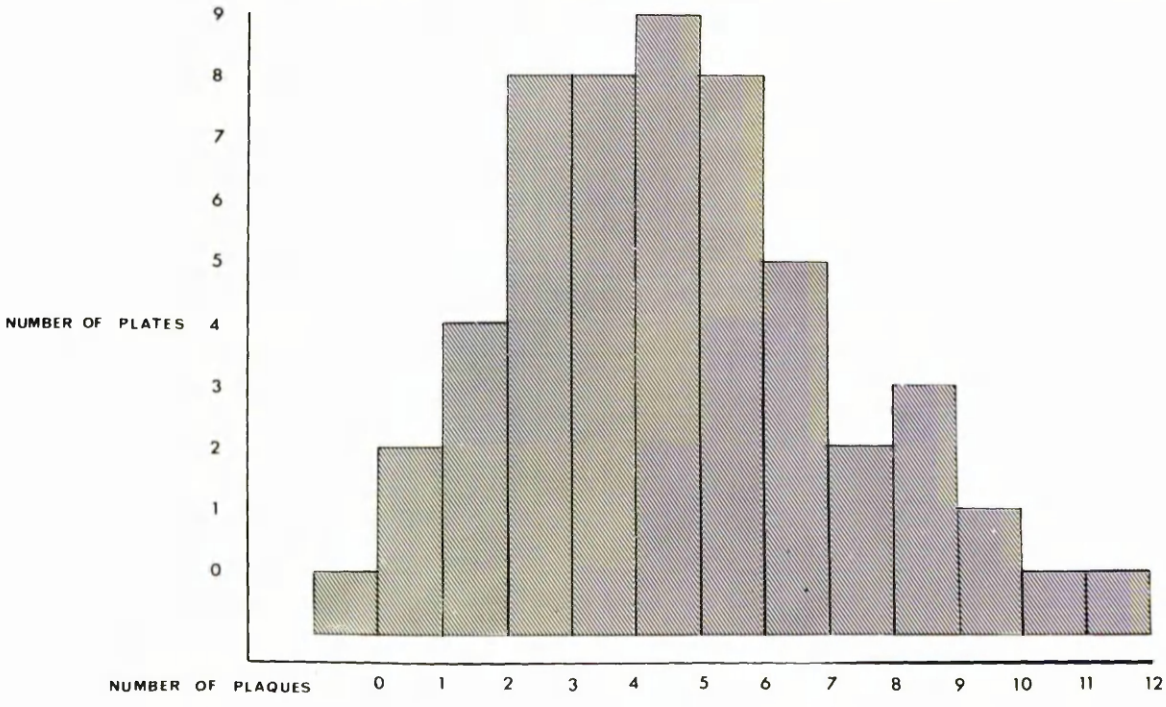
THEORETICAL DISTRIBUTION OF PLAQUES IN 50 PLATES.

MEAN PLAQUE COUNT 4.94

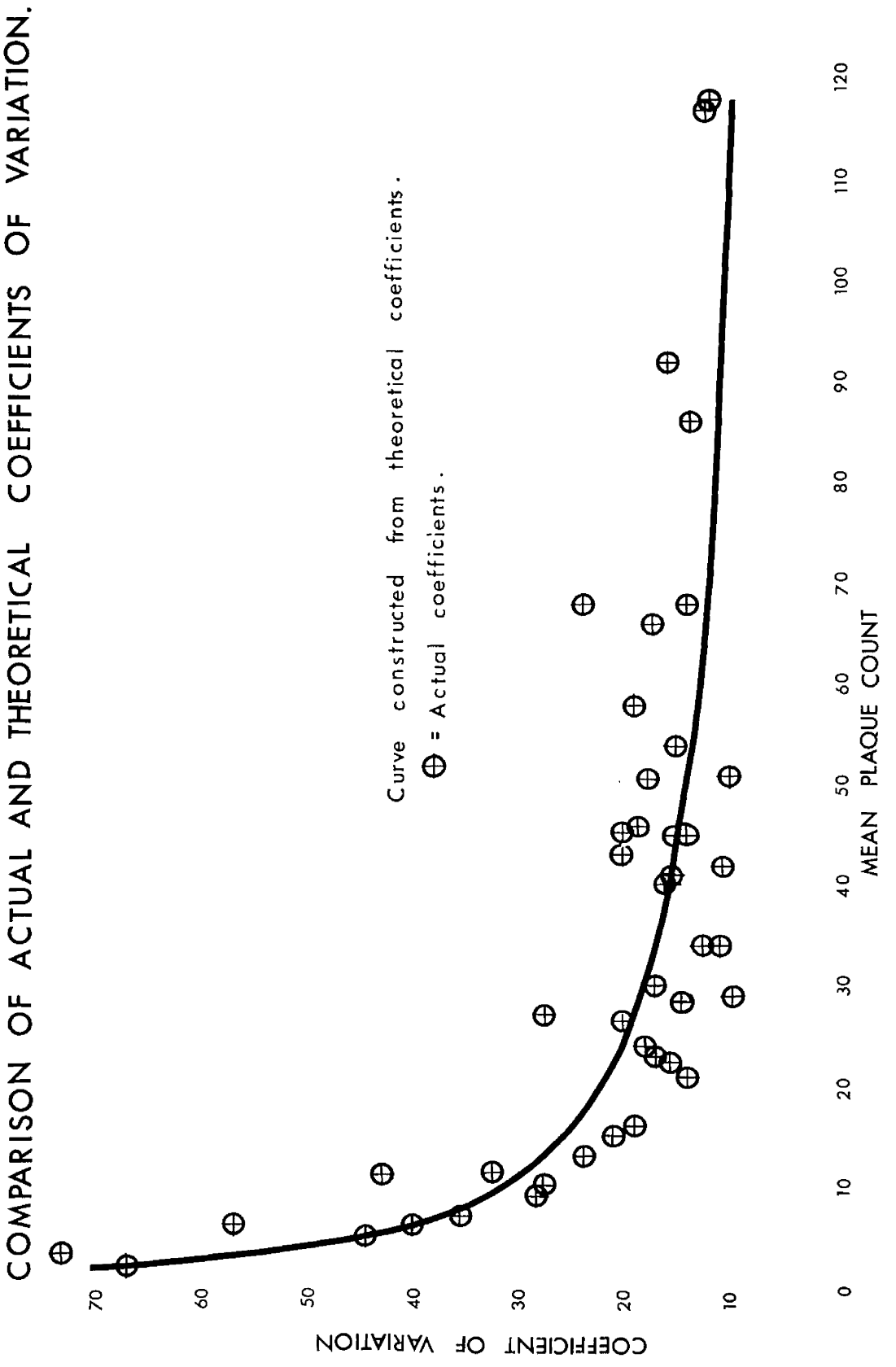


ACTUAL DISTRIBUTION OF PLAQUES IN 50 PLATES.

MEAN PLAQUE COUNT 4.94



of variation were calculated from the individual plaque-counts produced by 43 different dilutions of virus (the average number of plates used for each one being 8.5) and plotted on a graph (Figure 44) constructed from the theoretical values as described above. Approximately 19 per cent of the results shown in that figure fall on the curve drawn from the theoretical coefficients, while 23 per cent fall short by a factor of less than 0.25 and another 14 per cent exceed by a factor of less than 1.25. When plates were infected with more than about 40 P.F.U. of virus, the actual coefficient of variation tended to surpass the theoretical whereas, with smaller amounts of virus, it was generally lower. In view of the small number of Petri dishes sometimes employed, the degree of correspondence is satisfactory. Had the number of cultures supporting each mean been greater, the actual coefficients are likely to have approached the theoretical more closely. Thus, as has been shown, when 50 plates were infected (Table 8), the mean and the variance were almost identical (4.94 and 4.96 respectively), the coefficient of variation thus corresponding to the theoretical value expected from the Poisson distribution.


COMPARISON OF ACTUAL AND THEORETICAL COEFFICIENTS OF VARIATION.
Curve constructed from theoretical coefficients.
⊕ = Actual coefficients.
COEFFICIENT OF VARIATION
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
110
120
MEAN PLAQUE COUNT

EXPERIMENT FIVE: Inhibition of Plaque-formation by Specific Antiserum.

As a further test of the specificity of the plaques, attempts were made to determine whether, or not, their formation could be prevented by incubation of the virus with homologous antiserum prior to inoculation of the cultures.

Antiserum was produced in a rabbit by the following proceedure. After 20 ml. of blood had been collected to serve as a control in subsequent neutralization tests, the animal was inoculated intravencously with 1 ml. of a suspension of the M-3 strain of virus, prepared as follows. Virus was released from the cells by treatment in an M.S.E. ultrasonic disintegrator and was found to have an infectivity titre of 2×10^5 P.F.U. per ml. After clarification by centrifugation, the suspension was distributed into ampoules which were stored at -40°C. Intravenous inoculation of 1 ml. was repeated at approximately weekly intervals for six weeks. Ten days after the final injection, 20 ml. of blood were removed and the serum separated.

To determine whether, or not, anti-cell antibody was present in the serum, plate-cultures of whole-chick-embryo cells were inoculated with pre-inoculation serum and others with post-inoculation serum, the volume in both cases being 0.3 ml. per plate. After three hours incubation at 37°C., the serum was removed and the cells washed six times with P.B.S. The cultures were then challenged with ten-fold dilutions of the M-3 strain of virus and, after the lapse

of four hours to permit attachment of virus, preparations were covered with agar overlay in the manner already described. The plaques were counted after four days of incubation and the numbers compared. The results were as follows:

	Pre-inoculation serum.		Post-inoculation serum.	
Virus dilution.	Number of plates.	Plaque counts.	Number of plates.	Plaque counts.
10^{-3}	5	25, 60, 64, 56, 68.	3	40, 41, 51.
10^{-4}	5	7, 6, 7, 7, 2.	4	3, 5, 5, 4.
Titre calculated from both dilutions.	1.1×10^5 P.F.U./ml.		8.6×10^4 P.F.U./ml.	

In view of the possibility that small traces of antiviral antibody remained despite washing of the cells, the difference in titre is small. It may be concluded, therefore, that the serum did not have any significantly deleterious effect on the capacity of cells to adsorb virus or to produce a new generation of fully-infective particles.

Neutralization tests were then carried out with the M-3 strain of virus. The sera were inactivated at 56°C. for 30 minutes prior to use. Ten-fold dilutions of virus strain M-3 were mixed with equal volumes of a 1 in 10 dilution of post-inoculation serum according to the "constant serum - variable virus" method. The same procedure was followed with the pre-immunisation serum. The

constituents of the mixtures were allowed to react for one hour at room temperature (23°C.) followed by 18 hours at 4°C. Each mixture was then inoculated into a number of plate-cultures of whole-chick-embryo cells, 0.5 ml. per plate. Details of attachment time, agar overlay, etc., were as already described but the cells were washed twice with P.B.S. after removal of the inoculum. Results were as follows:

	Virus and pre-inoculation serum.		Virus and post-inoculation serum.	
Virus dilution.	Number of plates.	Plaque counts.	Number of plates.	Plaque counts.
10^{-1}	3	All confluent.	2	40, 27.
10^{-2}	4	All N.P.C.	4	2, 2, 2, 2.
10^{-3}	3	24, 34, 29.	5	0, 0, 0, 0, 0.
10^{-4}	4	4, 6, 4, 4.	4	0, 0, 0, 0.
Titre from two dilutions P.F.U./0.5 ml.		3.8×10^4		2.45×10^2
Titre in $\log_{10}$ P.F.U./0.5 ml.		4.57.		2.38.

The neutralization index, the difference between the two titres, was therefore 2.19 in respect of the 1 in 10 dilution of post-inoculation serum.

The above findings showed that antiserum to the pigeon I.N.I. virus, free of demonstrable anti-cell antibody, was producible in the rabbit. The results also satisfied the fifth of the six

criteria mentioned previously inasmuch as plaques did not appear when the virus was incubated with homologous antiserum prior to inoculation but did develop when the virus was mixed with serum taken from the animal before immunization.

EXPERIMENT SIX; Reproducibility of Results.

When experiments are being conducted which necessitate the titration of a virus suspension at intervals of a week or more, and hence involve the use of cultures derived from different individuals or groups of individuals, it is clearly necessary to know that such monolayers are uniform in susceptibility. The aim of the present experiment was, therefore, to compare the results obtained when stocks of pigeon I.N.I. virus were titrated in cultures of different batches.

Replicate titrations of strains H-1 and M-3 were carried out in cultures of different lots and, for comparative purposes, in ones of the same batch. In all cases, dilutions of stock virus were made up afresh for each titration. The infectivity titres obtained are recorded in Table 9, from which it may be seen that, if any variation existed in the susceptibility of cultures of different groups, the corresponding divergence in titre is unlikely to exceed that arising from errors of dilution.

TABLE 9

REPLICATE TITRATIONS

(1) Titration of virus in cultures of different batches

Virus strain	Titre from first assay P.F.U. per ml.	Titre from second assay P.F.U. per ml.
H-1	1.28×10^5	1.36×10^5
M-3	1.87×10^5	1.61×10^5
M-3	1.72×10^5	1.84×10^5

(2) Titration of virus in cultures of the same batch

Virus strain	First assay P.F.U. per ml.	Second assay P.F.U. per ml.	Third assay P.F.U. per ml.
M-3	2.68×10^5	2.44×10^5	2.03×10^5
M-3	8.24×10^4	1.10×10^5	1.06×10^5

EXPERIMENT SEVEN: Plaque-formation under Methylcellulose.

The experiments already reported showed that, when agar was employed as the overlay, the plaque-assay method provided a convenient and statistically-valid means of titrating virus. The only disadvantage in the technique was that non-specific degeneration of cells sometimes occurred. The present experiment was, therefore, intended to reveal whether, or not, equivalent or better results were obtainable when methylcellulose was used in place of agar.

Preliminary studies, carried out with strains B-1 and M-3, showed that macroscopic plaques generally appeared on the second day and continued to increase in size until, at least, the seventh day when many had reached a diameter of 4 mm. As well as enlarging more rapidly than those formed under agar, they also differed in possessing a distinctly tesselated border. Non-infected cultures maintained under methylcellulose remained free of non-specific degeneration for at least a week.

To determine whether the dose-response relationship was linear when methylcellulose was employed in place of agar, plates of whole-embryo cells were infected with two-fold dilutions of strain M-3 and overlaid with the former material. The infected cultures were incubated for four days and the plaques then counted. The results are shown in Table 10, from which it will be seen that the mean plaque-counts were approximately proportional to the

TABLE 10

TITRATION OF STRAIN M-3 BY MEANS OF METHYLCELLULOSE

Relative virus concentration	Number of plates	Number of plaques	Mean	Variance	Standard deviation	Coefficient of variation
64x	8	All N.P.C.	-	-	-	-
32x	7	167, 133, 125, 137, 134, 134, 123	136.1	211.5	14.54	10.68%
16x	8	63, 60, 74, 77, 67, 69, 59, 67	67.0	40.29	6.35	9.47%
8x	7	30, 26, 21, 28, 23, 37, 27	27.4	27.00	5.20	18.94%
4x	8	19, 12, 19, 29, 23, 22, 17, 19	20.0	24.29	4.93	24.64%
2x	8	7, 9, 5, 12, 10, 10, 10, 5	8.5	6.41	2.53	29.79%
1x	8	5, 5, 5, 1, 2, 3, 4, 2	3.4	2.55	1.60	47.34%

N.P.C. = Not possible to count.

quantities of virus in the inocula and that the coefficients of variation corresponded well with those expected from the Poisson distribution.

The above findings indicated that a plaque-assay system, based on the use of methylcellulose, could provide a convenient and statistically-valid means of titrating the pigeon I.N.I. virus. Nevertheless, due to fear that vibration might sometimes allow localized dissemination of virus through the liquid overlay, the author considered that it would be safer to adopt the agar overlay for all future investigations. A non-linear dose-response relationship has been obtained with methylcellulose by Rapp et al. (1959) who found that the number of foci produced by measles virus in Hep-2 cells rose more rapidly with increase in virus concentration than was to be expected from the single-particle hypothesis.

EXPERIMENT EIGHT: Relative Sensitivity of Different Methods of Assay.

The purpose of the experiment under report was to compare the relative sensitivity of the following methods of titration:-

(1) Plaque-assay with agar overlay.

(2) Plaque-assay with overlay of methylcellulose.

(3) Quantal (50 per cent end-point) method performed with tube-cultures.

(4) Pock-assay.

For comparison of the plating efficiency under the two different types of overlay, four plates of whole-embryo cells were infected with a 10^{-3} dilution of strain M-3 and, after removal of the inoculum four hours later, were covered with methylcellulose. Four further plates, infected with the same dilution of virus in identical manner, were overlaid with agar. The number of plaques present after four days of incubation were as follows:-

Overlay,	Number of plates.	Plaque count.	Mean plaque count.
Agar	4	27, 29, 29, 33.	29.5
Methylcellulose	4	61, 72, 50, 63.	61.5

Those results suggest that, possibly due to unfavourable micro-environmental conditions obtaining under a solid overlay, some of the infected cells are unable to initiate the formation of plaques when covered by agar.

For comparison of the TCID50, as determined by means of

tube-cultures, with the P.F.U., ten-fold dilutions of a stock of strain M-3 (ranging from 10^{-4} to 10^{-8}) were inoculated into tubes of whole-embryo cells, nine tubes being used for each dilution and the volume of inoculum being 0.5 ml. per tube. Dilutions of 10^{-5} and 10^{-6} were employed for the infection of cells grown in plates, five of the latter being used for each dilution, the inoculum again being 0.5 ml. An attachment time of four hours was allowed for both tubes and plates. After removal of the inoculum, both types of culture were washed twice with P.B.S. before the addition of maintenance medium and agar overlay, respectively. From the third to the sixth day, all tubes were examined every 24 hours and the numbers of those showing C.P.E. were recorded. In the case of the plaque assay, however, the counts were made on the fifth day.

The time at which the C.P.E. was first detected in inoculated tubes is shown in Table 11, while the plaque-counts produced by the same dilutions of virus are given in Table 12. It is highly probable that all infected tubes contained foci by the fourth day; those which were positive on the fifth or sixth days, but seemingly negative earlier, may have contained a single focus on the fourth day which escaped detection at that time. Tubes found to be positive six days after inoculation with dilutions of 10^{-7} and 10^{-8} contained one focus only. The titre per 0.5 ml. was $10^{7.167}$ $TCID_{50}$ whereas that given by the plaque assay was 4.57×10^{6} P.F.U., the ratio between the two being 3.21 to 1. Since the plating-

TABLE 11

TITRATION OF VIRUS BY 50% END-POINT METHOD

Virus dilution	Time of appearance of C.P.E.			
	Day 3 Tube number 1 2 3 4 5 6 7 8 9	Day 4 Tube number 1 2 3 4 5 6 7 8 9	Day 5 Tube number 1 2 3 4 5 6 7 8 9	Day 6 Tube number 1 2 3 4 5 6 7 8 9
10^{-4}	+ + + + + + + + +	N.E.	N.E.	N.E.
10^{-5}	- - + - - - - - -	+ + + + + + + + +	N.E.	N.E.
10^{-6}	- - - - - - - - -	- + + - + - - - +	- + + + + + + - +	+ + + + + + + + +
10^{-7}	N.E.	- - - - - - - - -	- + - - - - - - -	- + + - + + + - -
10^{-8}	N.E.	N.E.	- - - - - - - - -	- - + - - - - - -

Infected tubes inoculated with 10^{-7} and 10^{-8} dilutions of virus contained one focus of C.P.E. only.

TITRE = $10^{7.167}$ T.C.I.D.$_{50}$/0.5 ml.

N.E. = Not Examined.

TABLE 12

TITRATION OF SAME VIRUS BY PLAQUE METHOD

Virus dilution	Number of plates	Number of plaques	Mean plaque count
10^{-5}	4	60, 34, 65, 39	49.5
10^{-6}	5	7, 4, 4, 3, 3	4.2

TITRE = 4.57×10^{6} P.F.U./0.5 ml.

efficiency in tube-cultures may differ from that in plates, it is impossible to ascertain the extent to which the observed ratio between the two units of infectivity depended upon the inability of infected cells to produce plaques under agar. For statistical reasons alone, titres expressed in $TCID_{50}$ should be 1.44 times higher than those given in P.F.U. since, as was stated in the introduction to the present section, at the 50 per cent end-point, the average number of particles per culture (non-infected included) is 0.69, i.e. 1 $TCID_{50}$ is equivalent to 0.69 P.F.U.

To compare the plaque-forming unit with the pock-forming unit, the dropped chorio-allantoic membranes of 10-day embryonated eggs were inoculated with ten-fold dilutions of strain M-3, prepared from the eleventh passage in cell-culture, the method of inoculation being that of Beveridge and Burnet (1946). Petri plates of whole-embryo cells were inoculated with a single dilution of virus suspension. The same volume of inoculum (0.5 ml.) was used for eggs and plates. The infected cultures were not washed before the addition of the overlay but in other respects were handled as before. In order to lessen the risk of counting secondary pocks, three days of incubation were allowed for both types of assay, after which time the plaques were counted, the membranes removed from the eggs and the number of pocks determined.

The results of the latter investigation are recorded in

TABLE 13

COMPARISON OF PLAQUE-FORMING AND POCK-FORMING UNITS

Virus dilution	Number of eggs	Pock counts	Mean	Number of plates	Plaque counts	Mean
1×10^{-2}	3	180, 115, 91	129	-	-	-
1×10^{-3}	5	6, 8, 10, 15, 12	10	-	-	-
2×10^{-3}	-	-	-	10	9, 6, 9, 8, 10, 8, 6, 8, 8, 9	8.1
1×10^{-4}	7	2, 2, 0, 0, 0, 1, 0	0.71	-	-	-

Table 13. Comparison of the number of pocks produced by three ten-fold dilutions of virus reveals a slight deviation from linearity, but the titre of virus, as measured by the pock-counting method, clearly was of the order of 2×10^4 Po.F.U. per ml. The titre calculated from the plaque-assay was 3.24×10^4 P.F.U. per ml., or approximately 1.5 times greater than that determinable by the pock-counting method. Had the results been read on the fourth day, the number of plaques would have been higher but it is possible that the quantity of pocks might also have been greater.

It may be concluded, therefore, that calculation of the 50 per cent end-point in tube-cultures and plaque-formation under methylcellulose each provides a slightly more sensitive means of titration than does the standard plaque-assay method adopted by the author. As well as sometimes causing degeneration of the monolayer, the agar overlay seemingly interfered with the ability of some infected cells to initiate plaques. Similar results have occasionally been reported by other workers. Levine and Sharpless (1959), for example, found that the titre of GAL virus, as measured by the plaque-assay, was only 10-50 per cent of that determined by the quantal method. Nevertheless, despite these drawbacks, the agar overlay was deemed to provide the most acceptable method of assay in the present investigation since, in addition to being more reliable than methyl-cellulose as a means of eliminating diffusion of virus, it prevented sloughing of the monolayer, an event which occurred not infrequently

in cultures maintained under liquid media. The pock-assay was subject to greater variation than either type of plaque-assay and was inferior to both in its sensitivity to the virus.

EXPERIMENT NINE: Rate of Attachment of Virus.

In previous experiments, the time allowed for the attachment of virus was around four hours, which period was considered ample for adsorption of most, if not all, of the virus particles. To determine the optimal attachment-time and, thereby, establish a standard procedure for future assays, it was deemed essential to estimate the adsorption-rate of the virus. Four experiments, each with a different concentration of strain M-3, were, therefore, carried out to ascertain the amount of virus which would attach to monolayers of cells in a given time.

In the first experiment, the intention was to employ approximately 200 P.F.U. of virus per plate in order to produce statistically-optimal numbers of plaques in those Petri dishes incubated for minimal periods prior to removal of the inoculum. Plates of whole-embryo cells were inoculated with the virus as rapidly as possible and subjected to incubation at 37° C., under guidance by an automatic timer. After five, 15, 30, 60 and 90 minutes and two, three and four hours of incubation, five to seven plates were taken out of the incubator and drained of fluid. To reduce error from the short delay between inoculation and the commencement of incubation, the plates removed after five minutes were the last infected, those taken out after 15 minutes were the penultimate inoculated, and so on. Following withdrawal of the fluid, the cells were washed twice

with P.B.S. before being overlaid with agar and incubated for four days.

As may be seen from Table 14, the mean plaque-count produced by an attachment-time of four hours was approximately 250 instead of the expected 200. Loss of plaques due to overlapping, therefore, almost certainly occurred. The numbers of plaques likely to have obtained in the absence of overlapping are recorded in Table 14, the adjustments being made according to the method described in Experiment One on the assumption that the mean plaque-diameter was around 1.1 mm. Initially, the plaque-count increased very rapidly but, after about 15 minutes, the rate of growth slowed down and then proceeded at a uniform velocity until the end of two to two and one half hours. Thereafter, growth decreased during each successive hour though, even at the end of the fourth hour, it was continuing slowly.

When the experiment was repeated with only approximately 30 P.F.U. of virus per plate, similar results were obtained (Table 15). During the second hour of incubation, the proportion of virus attaching to the cells (i.e. the fraction of that present at the start of the second hour) was closely similar to that adsorbing during the first hour, a finding which suggests that the rate of attachment was exponential. Figure 45, constructed from the data given in Tables 14 and 15, implies that the rate of attachment is independent of virus-concentration though, since the plaque-counts

TABLE 14

ATTACHMENT RATE WITH HIGH TITRE INOCULUM

Time mins.	Number of plates	Plaque counts	Mean	Mean corrected for overlapping	Percentage of four-hour counts
5	6	29, 40, 32, 37, 35, 37	35.0	35	13
15	7	49, 53, 40, 42, 46, 48, 46	46.3	47	17.15
30	7	81, 82, 74, 78, 73, 79, 55	74.6	77	28.5
60	7	88, 97, 85, 81, 101, 89, 97	91.1	94	34.8
90	7	143, 140, 153, 126, 151, 131	136.9	142	52.6
120	5	190, 167, 177, 174, 184	178.5	188	69.6
180	7	238, 208, 205, 230, 253, 195, 200	218.4	233	86.3
240	6	230, 252, 267, 279, 238, 253	249.8	270	100

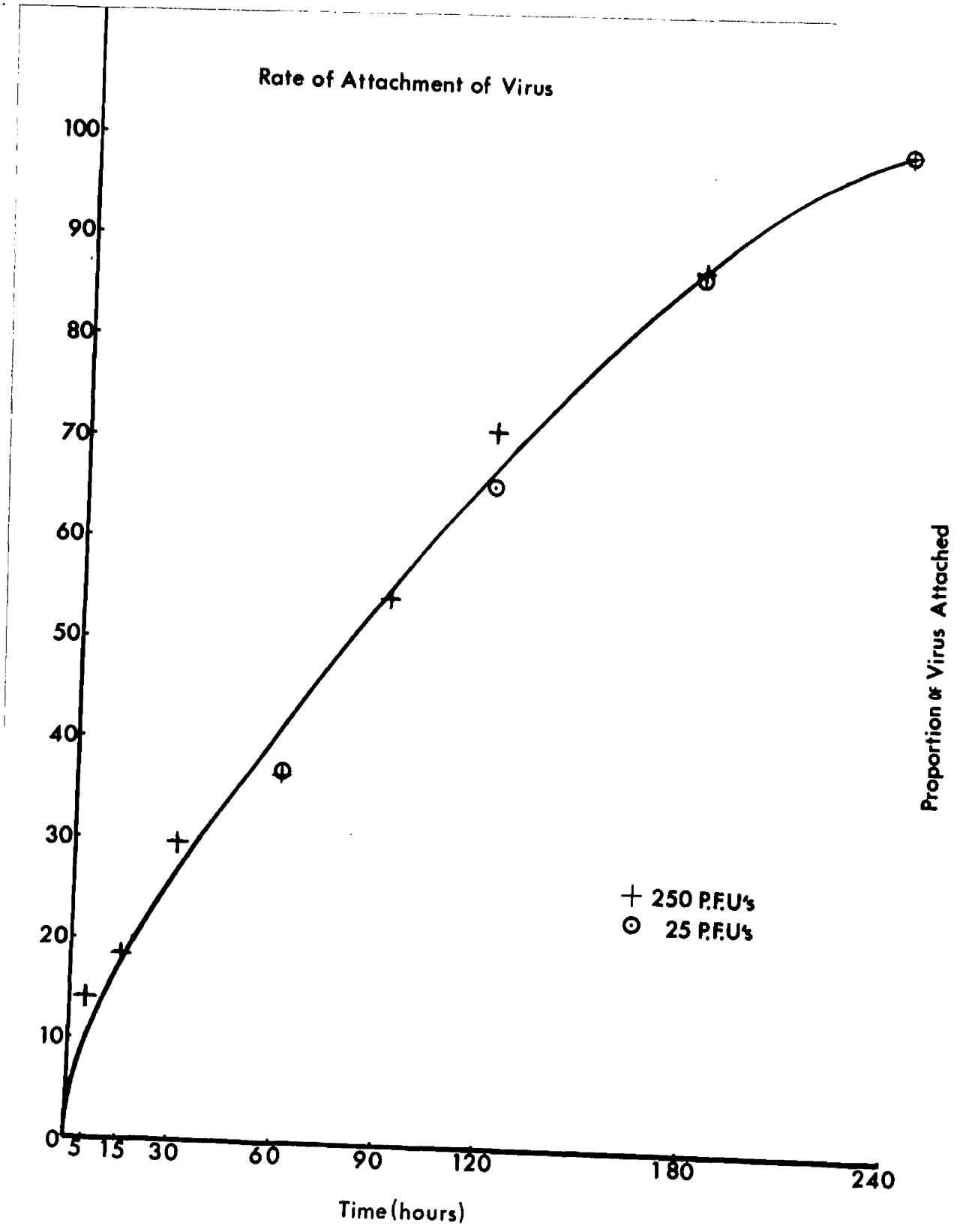
TABLE 15

ATTACHMENT RATE WITH LOW TITRE INOCULUM

Time (hours)	Number of plates	Plaque counts	Mean	Percentage of four-hour counts
1	9	10, 8, 15, 8, 10, 8, 8, 6, 8	9.0	36.9
2	10	17, 12, 17, 17, 13, 18, 16, 12, 22, 15	15.9	65.2
3	9	19, 18, 25, 22, 18, 22, 20, 18, 26	20.9	85.6
4	5	25, 23, 30, 21, 23	24.4	100

are expressed not as absolute values but as percentages of those recorded after an adsorption-time of only four hours, the apparent similarity between the rates of attachment for the two different virus-concentrations may not be entirely valid. When the experiment was repeated on two further occasions, with measurements made at two, three, four, five and six hours, it was found that, in the first of those investigations, the plaque-counts produced by attachment-times of four and five hours were 82 and 92 per cent of the total count effected by an exposure of six hours, while in the second, the figures were 89 and 97 per cent respectively.

Kaplan (1957) has shown that the attachment-rate of herpes simplex virus to rabbit-kidney cells is independent of virus concentration, the curve of the attachment rate for 25 P.F.U. coinciding with that for 100 P.F.U. The results of the present study point to a similar conclusion in respect of pigeon I.N.I. virus, The curve of virus adsorption obtained in the present investigation is similar in shape to those published by Kaplan (1957) for herpes simplex virus in rabbit-kidney cultures, by Youngner (1956) for vaccinia and poliomyelitis viruses in monkey-kidney cultures and by Rubin et al. (1957) for Newcastle disease virus in chick embryonic lung cultures. Kaplan, for example, found that 50 per cent of herpes simplex virus attached within 90 minutes, while Youngner reported that an adsorption period of at least six hours was required for complete attachment of vaccinia virus. In the present investigation, an


Rate of Attachment of Virus
100
90
80
70
60
50
40
30
20
10
0
5
15
30
60
90
120
180
240
Time (hours)
Proportion of Virus Attached
+ 250 P.F.U's
⊙ 25 P.F.U's

almost analogous rate was found to obtain. Thus, 50 per cent of pigeon I.N.I. virus required approximately 95 minutes for adsorption, but attachment of the remainder was incomplete until, at least, the end of the sixth hour. Hence, an adsorption time of not less than six hours is essential when accurate measurement of infectivity is required, e.g. in the determination of the particle:infectivity ratio or in experiments involving comparison between infectivity and other properties of the virus. When the infectivity of two suspensions is under contrast, an attachment period as short as four hours should suffice.

EXPERIMENT TEN: Effect of Washing the Monolayer before and after Inoculation.

In the previous experiments, cultures were washed once, or twice, with P.B.S. immediately before inoculation and once after removal of the inoculum. The object of the present investigation was to determine the extent to which those procedures influenced the plaque-count.

A total of 23 plate-cultures of whole-embryo cells were employed. After the growth medium had been removed, six cultures were washed twice with P.B.S. and then infected with approximately 60 P.F.U. of strain M-3. The remaining 17 cultures were inoculated with the same quantity of virus without prior washing. After incubation at 37° C. for four hours, the inoculum was withdrawn from all of the plates. Six of the seventeen cultures mentioned above were then washed twice with P.B.S. before they were covered with agar and the remainder were similarly overlaid without preliminary treatment with P.B.S. After four days incubation, the number of plaques was counted and the results compared.

As will be seen from Table 16, the mean plaque-count was highest in the group of plates which had been washed before inoculation and was lowest in the batch similarly treated after removal of the inoculum. That result accords with theory since washing of the monolayer prior to inoculation removes dead or

TABLE 16

THE EFFECT OF WASHING THE MONOLAYER BEFORE AND AFTER INFECTION

Procedure	Number of plates	Plaque counts	Mean
No washes	11	47, 59, 62, 64, 75, 81, 57, 57, 52, 58, 60	61.1
Two washes prior to inoculation	6	61, 67, 77, 63, 61, 84	68.8
Two washes after removal of inoculum	6	67, 63, 52, 50, 50, 61	57.2

unattached cells to which the virus might otherwise adsorb, while washing of the cells after withdrawal of the inoculum may loosen virus affixed to the cell-membrane. The former procedure, therefore, is likely to be associated with a high plaque-count while the latter treatment may lead to a lower count than that encountered with unwashed plates. Sellars and Stewart (1959), for example, found that washing culture-vessels before inoculation doubled the plating-efficiency of foot-and-mouth disease virus.

When the "t" test was applied to the results of the present experiment, the effect of washing was not clearly substantiated. Thus, when the plaque-counts from plates washed before inoculation were compared with those from unwashed vessels, "t" was found to be 1.578 and P greater than 0.10 but less than 0.20, a figure of minor statistical significance. Similarly, comparison of the counts from dishes washed after removal of the inoculum with those from unwashed plates revealed "t" to be 0.858 and P greater than 0.20 but less than 0.50, i.e. a difference again statistically indefinite.

From the above results, slight increase of plaque-count may occur if a plate is washed before inoculation but similar treatment after removal of the inoculum has little, if any, influence on the count. Hence, in future investigations, washing was restricted to determination of the absolute titre of virus present in a suspension and was excluded from comparative tests of infectivity.

EXPERIMENT ELEVEN: Variation in the Size of Plaques produced by a Viral Clone.

When micro-environmental conditions are kept constant, plaque morphology associated with a particular strain of virus generally remains uniform. A clear exception to the genetic stability of the plaque-characters specific to a given strain of virus, however, is the wide variation of size sometimes experienced, which tendency is not alterable by the employment of selected viral clones. Such non-genetic variation has been described by Dulbecco and Vogt (1954), Hsiung and Melnick (1957) and Cooper (1961b) in relation to various enteroviruses and Buthala (1960) and Macpherson (1960) apropos of GAL virus.

In Experiment One of the present investigation, plaques measured on the fourth day of incubation were found to be from 0.5 mm. to 2.0 mm. in diameter. Such variation may have been due to the existence of variants of diverse rate of growth or may have been of the non-genetic type mentioned above. In quest of the latter possibility, the current experiment was designed to study variation in plaque-size produced by a single clone of virus.

Plate-cultures of whole-embryo cells were inoculated with limiting dilutions of strain M-3. After incubation at 37°C. for four hours, the inoculum was removed, the cells washed three times with P.B.S. and agar overlay added. In one plate, a single plaque,

approximately 0.8 mm. in diameter, appeared on the third day and from it virus was extracted as described in Experiment One and inoculated into a tube-culture. In the latter, a typically focal C.P.E. was evident on the second day of incubation and the new virus stock so procured was designated "Clone 1A". The process of cloning was then repeated but, on this occasion, all plates inoculated with limiting dilutions of clone "1A" were found to produce two or more plaques. Accordingly, "Clone 1B" was prepared from a vessel with two plaques, the one selected for isolation being approximately 2 cm. distant from the other. A number of plate-cultures were then inoculated with 50 P.F.U. of "Clone 1B". At daily intervals from the second to the seventh day after infection, one culture was removed from the incubator, fixed and stained by Leishman's method. The dimensions of the plaques were then measured as previously described.

Plaque sizes between the second and seventh days are recorded in Table 17 which shows that, whereas minimal and maximal dimensions varied little after the third day, the mean diameter did not approach its maximum until the fifth day. It may, therefore, be concluded that a small number of plaques attained maximum size on the fourth day but that enlargement did not continue beyond the fifth day. The mean diameters encountered on the third and fourth days were slightly greater than those recorded with uncloned virus in Experiment One, while the standard deviation was lower e.g. 0.27 mm.

TABLE 17

SIZE OF PLAQUES PRODUCED BY " CLONE 1B "

Day	Number of plaques measured	Plaque dimensions in mm. Minimum	Maximum	Mean
2	24	0.50	0.875	0.69
3	46	0.50	1.50	0.94
4	52	0.75	2.00	1.25
5	33	0.875	2.00	1.40
6	26	0.75	1.875	1.42
7	27	0.875	2.375	1.45

against 0.41 mm. on the fourth day.

The results of the present experiment clearly demonstrate the existence of considerable non-genetic variation in plaque-diameter. Comparison with those recorded in Experiment One also suggests that part of the variation in plaque-size experienced with uncloned virus may be genetic in origin. The mean diameter of plaques produced by cloned virus slightly exceeded that associated with uncloned stock, e.g. 1.25 mm. against 1.11 mm. on the fourth day. When the "t" test is applied to the figures, the difference is found to be of moderate statistical significance ("t" = 1.91, $P => 0.05$, $P =< 0.10$). An even more important distinction in the behaviour of the cloned and of the uncloned virus lies in the pattern of the frequency-distribution for the various sizes of plaque seen at any given time. In the present experiment, it was found that, when the plaque-diameters measured on the fourth day were plotted against the proportion of plaques of each particular size, the curve obtained had a single peak, i.e. it indicated that the relationship between number and size followed a normal distribution. In Experiment One, however, the corresponding graph had two peaks, which characteristic suggested that the population of virus particles consisted of two types, one of fast growth and the other of slow development.

EXPERIMENT TWELVE: The Genetic Origin of Variation in Plaque-diameter.

The object of the present experiment was to determine the existence, or otherwise, of a variant productive of plaques significantly smaller than those formed by other genotypes.

Eight clones of virus (strain B-1) were prepared by the methods described in Experiment Two. In all instances, the parent plaque selected was less than 0.8 mm. in diameter and the virus isolated from it was recloned at least once prior to study. Plaques were measured by means of a microscope fitted with an ocular micrometer, which was calibrated by use of a stage-micrometer of graticular length = 2 mm. Plate-cultures of whole-embryo cells were infected with approximately 150 P.F.U. of each clone and the diameter of plaques measured along two axes, one at right angles to the other. To avoid bias, the study was made of the first 50-75 plaques observed in each plate.

Out of eight clones examined, seven produced plaques of approximately the same size as those formed by uncloned virus (the dimensions of which were recorded in Experiment One). The eighth ("Clone X") gave rise to foci which were significantly smaller. The following account compares the plaques produced by "Clone X" with those formed by "Clone Z", which latter was representative of the seven clones referred to above.

Both variants were re-cloned twice. Each produced macroscopic foci under liquid medium when first isolated from the respective parent plaque but, whereas those formed by "Clone Z" were easily recognizable with the naked eye on the third day, those induced by "Clone X" were barely discernible, even on the fourth day (Figure 46). The same result obtained (1) when virus from those cultures provoked the formation of plaques under an agar overlay, (2) when virus isolated from the latter foci was made to infect other cultures maintained under a liquid medium, and (3) when virus from the latter cultures provided further plaques under agar, the dimensions of which are recorded in Table 18.

The sizes of the plaques produced by "Clone Z" were closely similar to those of the foci established by uncloned virus, the mean diameter of each being 0.88 mm. and 0.84 mm. respectively, on the third day, and 1.12 and 1.11 mm. respectively, on the fourth day. Corresponding figures for "Clone X" were 0.52 mm. and 0.73 mm. Similarly, the largest plaques present on the fourth day were 2.08 mm. for "Clone Z", 2.00 mm. for uncloned virus and only 1.01 mm. for "Clone X". The differences between the plaque-diameters associated with each clone is highly significant statistically. Thus, when the dimensions of the foci present on the third day are subordinated to the formula for the standard error of the difference between two means ($\sqrt{\frac{\sigma x^2}{N_x} + \frac{\sigma z^2}{N_z}}$ where σ is the standard error of the mean and N the number of measurements), the standard error of the difference

Figure 46. Plaque-formation by clones Z (upper) and X (lower). x 3.

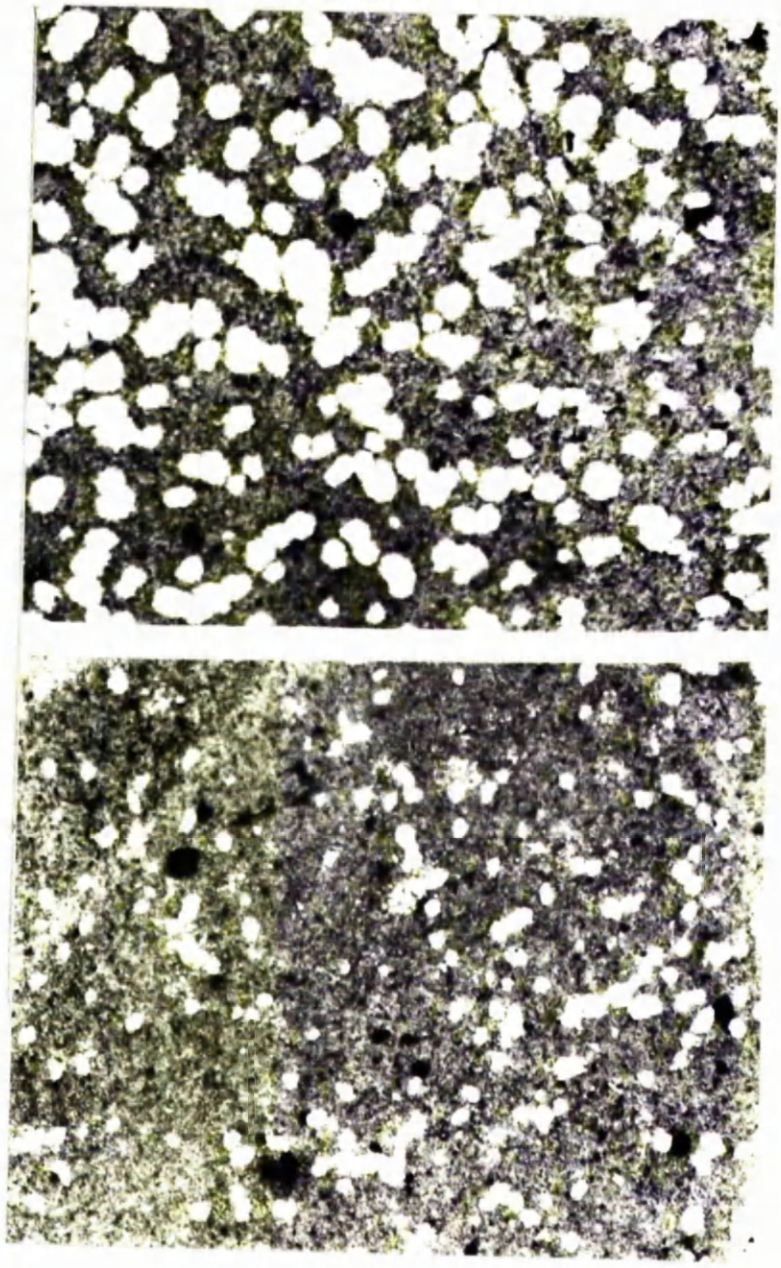


TABLE 18

SIZES OF PLAQUES. CLONES X AND Z

		Clone X	Clone Z
Day 3	Number of plaques measured	50	50
	Minimum diameter	0.25 mm.	0.35 mm.
	Mean diameter	0.52 mm.	0.88 mm.
	Maximum diameter	0.75 mm.	1.45 mm.
Day 4	Number of plaques measured	65	75
	Minimum diameter	0.37 mm.	0.37 mm.
	Mean diameter	0.73 mm.	1.13 mm.
	Maximum diameter	1.00 mm.	2.13 mm.

is found to have a value of 0.1939 divisions of the micrometer-scale. The observed difference, again in divisions of that scale, between the two means was 11.46, i.e. 59.1 times greater than the maximum difference resulting from chance. Analysis of like figures obtained on the fourth day also denoted that the dissimilarity in dimensions was highly significant. The foregoing results, therefore, indicate that variation in the size of the plaques produced by uncloned virus is partly due to genetic differences among virus particles.

(4) SUMMARY AND CONCLUSIONS.

All strains of the virus produced small, round plaques in cultures of whole-chick-embryo as well as in monolayers of chick-embryo liver and chick-embryo kidney cells. About two-thirds of the foci were clearly visible to the naked eye on the third day. The remainder became apparent on the following day, which was the optimal time for counting, when plaque-size ranged from 0.5 mm. to 2.00 mm. Enlargement generally terminated about the fifth day. "Small-plaque" variants existed but much of the variation in size was non-genetic in origin.

A linear relationship between the plaque-count and the virus input was established. Evidence that the distribution of plaques follows the Poisson equation was obtained by inoculating 50 plates with a single virus dilution and by comparing the actual coefficients of variation with the theoretical ones in 43 different "one-dilution" titrations. The results of the latter study suggested that, when eight or nine plates were used for each dilution, the coefficient of variation approached the level to be expected from the Poisson distribution. Replicate assays of stock-virus gave approximately the same titre in cultures of different batches. Plaque-formation was inhibited by a specific antiserum prepared in a rabbit but not by normal rabbit serum. Those results indicate that plaque-assay provides a statistically-valid method for the computation

of virus infectivity. In its sensitivity to virus, the system was superior to that based on the pock-count but was inferior to the 50 per cent end-point method performed by means of tube-cultures.

When methylcellulose was employed in place of agar, the growth of plaques occurred more rapidly and continued for a longer period of time. A linear relationship existed between the plaque-count and the concentration of virus. The coefficients of variation for the plaque-counts produced by different dilutions of virus were similar to those to be expected from the Poisson distribution. Plating-efficiency was approximately double that of plaque-assay with agar.

Approximately 95 minutes were required for half of the amount of virus present in an inoculum to affix to cells but attachment of the remainder was not complete until, at least, the end of the sixth hour. A standard adsorption time of four hours allowed about 85 per cent of inoculated virus to enter the cells. Investigations into the effect of washing the cell-sheet before infection and previous to addition of the overlay proved inconclusive but slight increase of the plaque-count may ensue when unattached cells and pieces of cellular debris have been removed from the culture-vessel prior to inoculation.

PART 4. CHARACTERIZATION OF THE VIRUS.

(1) INTRODUCTION.

The formation of Cowdry Type A intranuclear inclusions and the production of a focal cytopathogenic effect similar to that caused by cytomegaloviruses suggested an affinity on the part of the pigeon I.N.I. virus with the herpesvirus group. The work about to be described was designed to elucidate those properties of the virus which would allow its unequivocal incorporation within that group.

The system of classification adopted in the present investigation is that originally proposed by Lwoff, Horne and Tournier (1962) and later recommended by the International Subcommittee on Virus Nomenclature (1963). According to that scheme, taxonomy should rest on:

(a) The chemical nature of the nucleic acid,
(b) The symmetry of the nucleocapsid.
(c) The presence or absence of an envelope.
(d) In the case of viruses with cubic symmetry, the number of capsomeres.

Additional characters deemed distinctive of the virion have been recommended for classification and are used for that purpose in the present investigation. They include the sensitivity of the virus to ether and its stability or lability under acidic conditions, both of which phenomena are genetically stable (Hamparian, Hilleman and Ketler, 1963).

In addition to providing data of taxonomic importance, the investigations about to be described were intended to supply information on the thermo-resistance of the virus and on its serological relationship with I.L.T. virus. The opportunity was also taken to determine whether, or not, the virus was endowed with haemagglutinating capacity, though it was recognized that the latter characteristic is not a feature of the herpesvirus group.

(2) ANTIGENIC RELATIONSHIP OF STRAINS.

(1) INTRODUCTION.

The information already presented revealed very close similarities in the properties of the various isolates. All formed the typical Cowdry Type A intranuclear inclusion and gave rise to identical lesions in embryonated eggs. Moreover, all produced the same, highly-characteristic type of cytopathogenic effect in cell-culture. Those findings strongly suggested that each of the strains belonged to one viral genus, though it was appreciated that some antigenic differences might exist.

At the commencement of the present part of the investigation, the writer realized that a detailed study of more than one strain of virus was not feasible. At the same time, it was felt that it might prove advantageous, purely on grounds of temporary convenience, to be in a position to carry out examinations with strains other than that appointed as the "type-strain". The author therefore considered that an essential pre-requisite to the pursuit of taxonomic studies would be to demonstrate a serological relationship among those strains of virus which he might wish to employ. As far as he is aware, such a relationship exists only among viruses which possess the same fundamental structure. Thus, in studies of the physical and chemical structure of the virion, it is possible to substitute one strain of virus for another if evidence of their mutual serological

identity has already been obtained.

When two viruses have a soluble antigen in common, as in the case of the G antigen of the influenza A viruses or the NP antigen of the pox viruses, an antiserum prepared against one of them may give a positive reaction with the other in complement-fixation or precipitation tests but a negative result in the neutralization test. For that reason, the latter serological procedure provides the most sensitive index of antigenic similarity. The primary aim of the present experiment was, therefore, to determine whether, or not, an antiserum capable of neutralizing the "type-strain", M-3, had a similar effect on the HS-2, B-1 and P-5 strains of virus. Confirmatory evidence was sought in fluorescent antibody tests carried out in cell-culture.

(2) MATERIALS AND METHODS.

Initial efforts to produce an antiserum in cockerels having failed, an attempt was made to produce antiserum in a rabbit, which animal was presumed to be free of antibody to the agent as well as suitable for intravenous inoculation and easy recovery of blood. The methods employed to that end and the satisfactory degree of neutralization observed with the homologous virus (strain M-3) were described in Part Three, Experiment Five. Cross-neutralization tests were then carried out with the P-5, HS-2 and B-1 strains of virus. In each case, ten-fold dilutions of virus were mixed with a

1 in 10 dilution of rabbit immune serum and incubated at 37°C. for one hour followed by exposure at 4°C. for 18 hours before inoculation into plates of whole-chick-embryo cells. Controls consisted of the same dilutions of virus incubated with a 1 in 10 dilution of normal rabbit serum. Details of the attachment time and plaque-assay were as reported in Part Three.

For fluorescent antibody studies, coverslip cultures of whole-embryo cells were infected with approximately 10^5 P.F.U. of each of the four strains of virus. 24 hours after inoculation, the coverslips were removed from the Leighton tubes, fixed in acetone at 4°C. for 10 minutes, and divided into two lots, one of which was treated with immune rabbit serum and the other with normal serum, both of which had been adsorbed overnight with a tissue-powder prepared from a fowl-liver. Uninfected monolayers were also stained with each of the sera. After 30 minutes in a moist chamber at 37°C., the coverslips were washed thoroughly in P.B.S., stained with goat anti-rabbit globulin conjugated with fluorescein isothiocyanate (Difco Laboratories, Detroit, Michigan) and examined by means of a Reichert " Zetopan " fluorescence microscope, as described in Part One for the examination of tissue-sections from experimentally-infected birds.

(3) RESULTS.

As reported in Part Three, Experiment Five, a 1 in 10

dilution of the rabbit M-3 antiserum had a neutralization index of 2.19 against the homologous strain. Against the other strains, the neutralization indices were as follows:

P-5	2.01
B-1	1.94
HS-2	1.38.

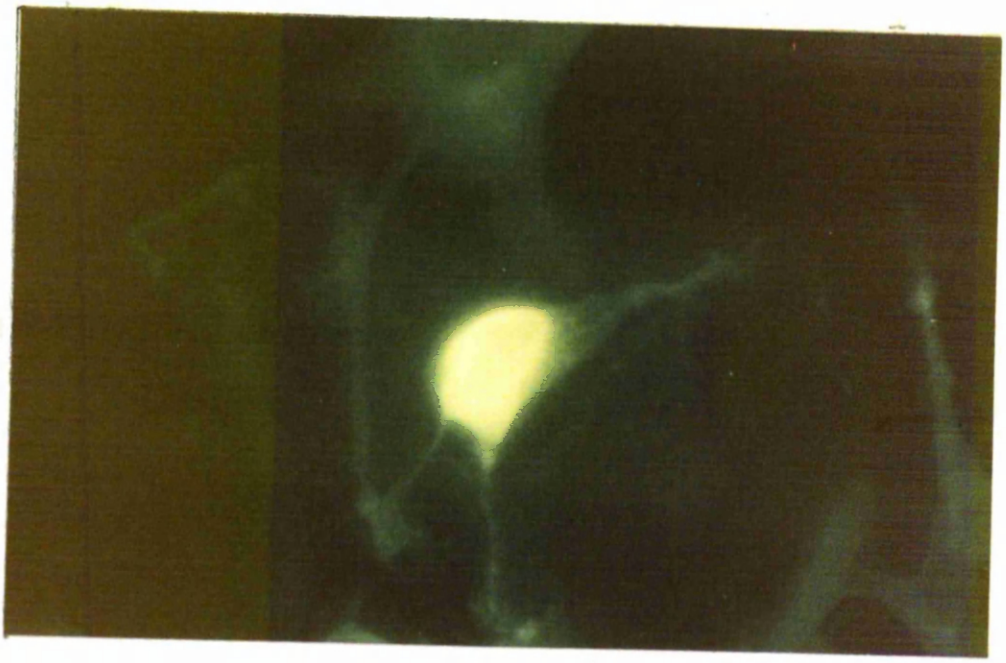
By the use of M-3 antiserum in the indirect fluorescent antibody technique, viral antigen was detected in the nuclei of cells infected with heterologous isolates (P-5, B-1 and HS-2) as well as with the homologous strain. Fluorescence was of two main types though intermediate stages were often to be found. In the first, the staining reaction was intense throughout the whole of the nucleus (Figure 47) but, sometimes, the nuclear membrane was appreciable by reason of its greater brilliance. In the second, fluorescence was restricted to the nuclear membrane (Figure 48). Both patterns of viral distribution were found in all infected cultures but fluorescence was not manifest in infected cultures stained with normal serum or in uninfected cultures treated with immune serum.

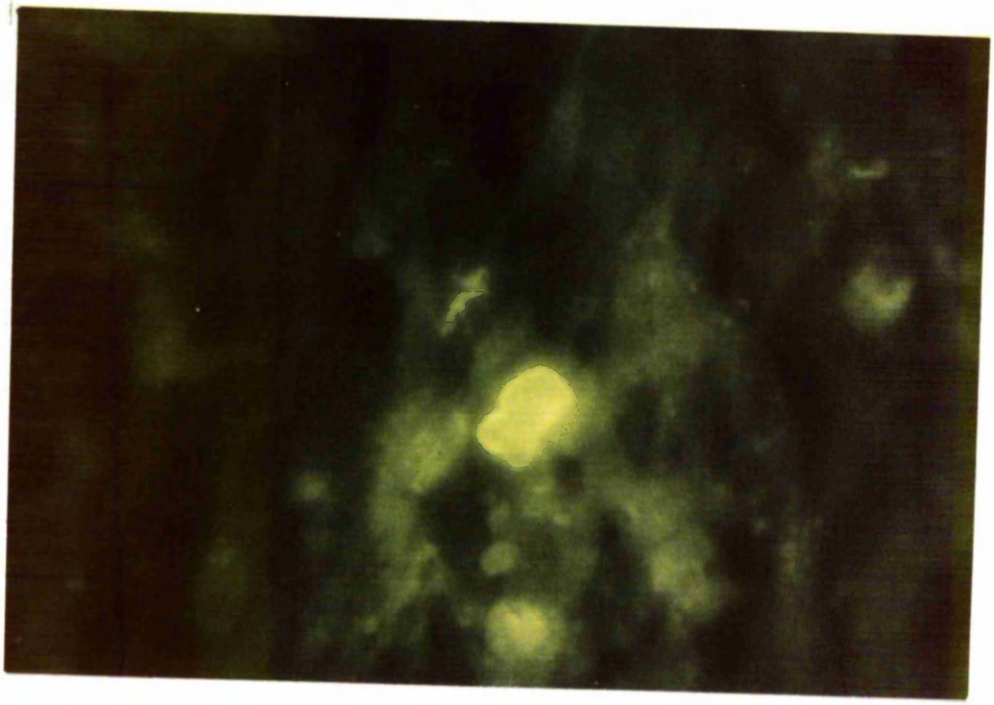
(4) DISCUSSION.

M-3 antiserum in a 1 in 10 dilution was found to have moderate neutralizing activity against the P-5 and B-1 strains as well as against the homologous virus, the neutralization indices being 2.01, 1.94, and 2.19, respectively. Although reciprocal cross-

Figure 47. Virus antigen stained by fluorescent-antibody in nucleus of fibroblast *in vitro*. x 2,000.

Figure 48. Virus antigen concentrated mainly at nuclear membrane. x 2,000.





neutralization tests with P-5, B-1 and HS-2 antisera were not carried out, the author considers that the results fulfil the main purpose of the investigation and confirm that the M-3, P-5 and B-1 strains are very closely related. Those agents may, therefore, be substituted for each other in studies pertaining to the type of nucleic acid, particle morphology, ether sensitivity and resistance to acid on the part of the virus.

The results indicated that M-3 antiserum had a noticeably lower neutralizing index against the HS-2 strain than against the other strains, but differences in the degree of antigenic relationship exhibited by the four strains are not properly assessable by the methods used in the present investigation. In order to demonstrate fine antigenic differences between, for example, the P-5 and M-3 strains, it would be necessary to compare either the rate of neutralization of each strain with homologous and heterologous antiserum or the neutralization slope constructed from the serum titres obtained from a number of " constant virus-variable serum" tests carried out with different concentrations of virus. The former method was employed by McBride (1959) to differentiate between minor "within-type" variants of poliomyelitis virus. Carmichael (1962) used both procedures to distinguish two strains of canine hepatitis (ICH) virus. Antiserum neutralized the homologous virus more quickly than it did the heterologous one and the neutralization slopes of the two strains with homologous antisera were quite different, 0.4 and 0.7. Experiments

of that type are clearly required if the question of minor antigenic differences among strains of pigeon I.N.I. virus is to be resolved.

(3) NUCLEIC ACID COMPOSITION.

(1) INTRODUCTION.

Since Cooper (1961c) proposed that viruses be divided into two main groups, the riboviruses and the deoxyviruses, various techniques have been employed to determine the type of nucleic acid forming the core of the virus. They include (a) digestion of virus preparations with ribonuclease and deoxyribonuclease (McAllister et al., 1963), (b) treatment of infected tissue-cultures with halogen derivatives of deoxyuridine (Lam and Atherton, 1963), (c) electron microscopy of virus particles stained with uranyl acetate and lead hydroxide (Smith and Melnick, 1962) which reveal DNA and RNA respectively and (d) various histochemical methods (McAllister et al., loc. cit., Mayor and Diwan, 1961), such as the Feulgen method for DNA and acridine orange for RNA. On account of their simplicity, the second and fourth of those methods were chosen for the purpose of the investigation under report.

Inhibition of the growth of herpes simplex virus in HeLa cells by means of 5-fluoro-2'-deoxyuridine (FUDR) was first demonstrated by Newton and Tamm (1959). Salzman (1960) demonstrated that the same compound prevented the growth of vaccinia virus, but it was soon recognized that the inhibitory effect did not extend to the replication of RNA viruses. Herrmann (1961), for example, reported that, although 5-iodo-2'-deoxyuridine (IUDR) and 5-bromo-2'-deoxy-

uridine (BUDR) suppressed plaque formation by vaccinia and herpes viruses, they were without effect on Newcastle disease and West Nile viruses, both of which contain RNA. More recently, however, Bader (1964) found that BUDR inhibited the multiplication of Rous sarcoma virus which, seemingly, requires the participation of DNA at an early stage in the growth cycle. Although similar results were obtained by Thormar (1965) for Visna virus, it is generally agreed that the halogenated deoxyuridines do not adversely influence the multiplication of RNA viruses (Appleyard, 1967). It may, therefore, be safely concluded that inhibition by those compounds is strongly suggestive of the presence of DNA in the virus core. That contention is especially true when the inhibitory effect is weighed in conjunction with other similarities between the agent under examination and a virus known to contain DNA.

Although the Cowdry Type A inclusion body is a recognized feature of herpesvirus infection, it represents merely a terminal stage in the infective process. Crouse et al. (1950) emphasized that the earliest inclusions produced by herpes simplex virus in chorio-allantoic membranes differed considerably from those of the classical type A in that they were basophilic, Feulgen-positive and completely filled the nucleus. As the pocks developed, the inclusions became eosinophilic and Feulgen-negative and were separated from the nuclear membrane by a clear 'halo'. A similar sequence of events was described by Watrach and Hanson (1963) in studies on the

cytopathology of I.L.T. virus in chick-embryo-kidney tissue-culture. Between 12 and 36 hours after infection, the nuclei of infected cells were filled to capacity with basophilic DNA-containing material which was indistinguishable from the nuclear membrane. During the succeeding 36 hours, the inclusions gradually became separated from the nuclear membrane by a clear zone and there was concurrent loss of basophilia as well as a reduction in the intensity of the Feulgen reaction. By the 72nd. hour, many of the inclusions were eosinophilic and Feulgen-negative.

The above changes may signify fundamental processes common to the growth of all herpesviruses. Early increase of nuclear DNA, as revealed by the Feulgen reaction, is most likely to be due to the replication of viral DNA. Conversely, lack of staining affinity for acridine orange implies the absence of RNA synthesis. The association of Feulgen-positive, acridine orange-negative, nuclear material with basophilic inclusions occupying the entire nucleus, followed by the appearance of Feulgen-negative type A inclusions, may be regarded as strong evidence of herpesvirus multiplication.

(2) MATERIALS AND METHODS.

IUDR and BUDR were obtained from the Sigma Chemical Company and thymidine from British Drug Houses, Ltd. Difficulty was experienced in dissolving the deoxyuridines but satisfactory results were eventually obtained when the compound was added to a small volume

of medium, preheated to 37°C., and then thoroughly agitated in an MSE ultrasonic disintegrator.

The effect of IUDR and BUDR on the multiplication of the virus was investigated in five experiments, the details being as follows:

Experiment 1. To test the inhibitory efficacy of deoxyuridine on a known DNA virus, cultures of chick-embryo-liver cells were infected with I.L.T. virus and, after they had been washed in P.B.S. following removal of the inoculum, were divided into three groups, namely:

1. Those in which 20 µg. IUDR/ml. was incorporated into the maintenance medium.
2. Those in which the maintenance medium contained 20 µg. IUDR/ml. + 200 µg. thymidine/ml.
3. Those covered by maintenance medium only.

The media were removed daily and replaced with fresh solutions of appropriate type. Those recovered at 48 hours after infection were assayed in whole-embryo cultures for their virus content.

Experiment 2. The procedure of Experiment 1 was repeated with the M-3 strain of pigeon I.N.I. virus, but with the quantities of IUDR and thymidine increased to 50 µg/ml. and 500 µg/ml. respectively and an amount of virus equal to 1.8×10^3 P.F.U. per plate-culture. At 24, 48 and 72 hours after infection, the cellular fraction of the cultures was harvested and the virus content titrated by the plaque-

assay method.

Experiment 3. In this investigation, three parallel plaque-assays of strain M-3 were carried out, the agar in the first containing 50 µg. IUDR/ml., that in the second incorporating 50 µg. IUDR/ml. and 500 µg. thymidine/ml. and that in the third being without supplement.

Experiment 4. Cultures of whole-embryo cells were each infected with 1.8×10^4 P.F.U. of strain M-3 and, after removal of the inoculum, were washed thoroughly in P.B.S. before the addition of normal maintenance medium. The latter was removed for virus assay 24 hours later and replaced by IUDR in a concentration of 20 µg./ml. and IUDR plus thymidine in concentrations of 20 µg./ml. and 200 µg./ml., respectively. Again, control cultures were maintained in medium without any supplement. At the 48th hour, the fluids were withdrawn for virus assay.

Experiment 5. The inhibitory effect of BUDR was studied in cultures infected with the HS-2 strain of virus and maintained in the presence of 30 µg. BUDR/ml. Those preparations were examined daily for the presence of CPE and harvested on the fourth day for assay of virus content (cells and fluids together). Untreated infected cultures were similarly investigated but reversal of inhibition by use of thymidine was not attempted.

For cytochemical studies coverslip-cultures of whole-embryo cells were infected with the M-3 strain of virus. At 6, 22 and 30 hours after inoculation, media were removed and the monolayers washed six times with P.B.S. preparatory to fixation in corrosive-formol for 18-36 hours. The cultures were divided into two groups, one of which was treated with DNAase. Both lots were then stained by the fluorescent reagent technique of Cutting and Vassar (1951) and examined under a Reichert ' Zetopan ' fluorescence microscope. The same method was employed for the study of chorio-allantoic membranes fixed two days after infection with the M-3 strain of virus.

(3) RESULTS.

Typical syncytia were present on the second day in chick-embryo-liver cultures infected with I.L.T. virus. The changes were progressive and, by the fourth day, had involved the whole of the epithelial component of the monolayers. Identical alterations, proceeding at the same rate, occurred in infected cultures maintained in the presence of IUDR and thymidine. By contrast, both infected and uninfected cultures treated with IUDR remained morphologically normal over the four-day period of observation. The quantities of virus present in the culture fluids harvested on the second day were as follows:

Content of medium	Titre of virus TCID50/0.5 ml.
Virus alone	$10^{2.75}$
IUDR	0
IUDR + thymidine	$10^{2.25}$

Numerous characteristic foci were present on the second day in whole-embryo cultures infected with 1.8×10^3 P.F.U. of the M-3 strain of pigeon I.N.I. virus. The changes were progressive and the entire monolayer became involved by the fifth day. Identical results were obtained with infected cultures maintained in the presence of IUDR and thymidine, but monolayers treated with IUDR alone remained morphologically normal. The quantity of virus present in the cells on the first, second and third days are recorded in Table 19 and illustrated graphically in Figure 49.

Incorporation of IUDR in the agar overlay reduced the number of mature plaques by over 200 times (Table 20) but several hundred minute, punctiform foci were present in the 10^{-1} dilution and from 10 to 30 occurred in the 10^{-2} dilution. The minute size (less than 0.4 mm.) of the foci made gross counting difficult since they were generally impossible to differentiate from cell clumps. Those examined under the microscope were seen to consist of rounded or swollen hyperchromatic cells and cell debris and appeared to represent immature plaques. They were entirely absent in cultures inoculated with higher dilutions of the virus.

Numerous small foci were present 24 hours after inoculation of cultures with 1.8×10^4 P.F.U. of virus. The addition of IUDR at that time did not prevent extension of the CPE to virtually the whole of the monolayer by the 48th hour. The amount of virus present in the

TABLE 19

EFFECT OF IUDR ON YIELD OF VIRUS (EXPERIMENT 2)

Time (hours)	Titre of virus in $\log_{10}$ P.F.U./ml.		
	Virus control	Virus + IUDR	Virus + IUDR + Thymidine
24	4.01	2.13	3.46
48	6.10	1.72	4.97
72	5.41	1.38	4.47

Inhibition Of Viral Multiplication By Use Of IUDR

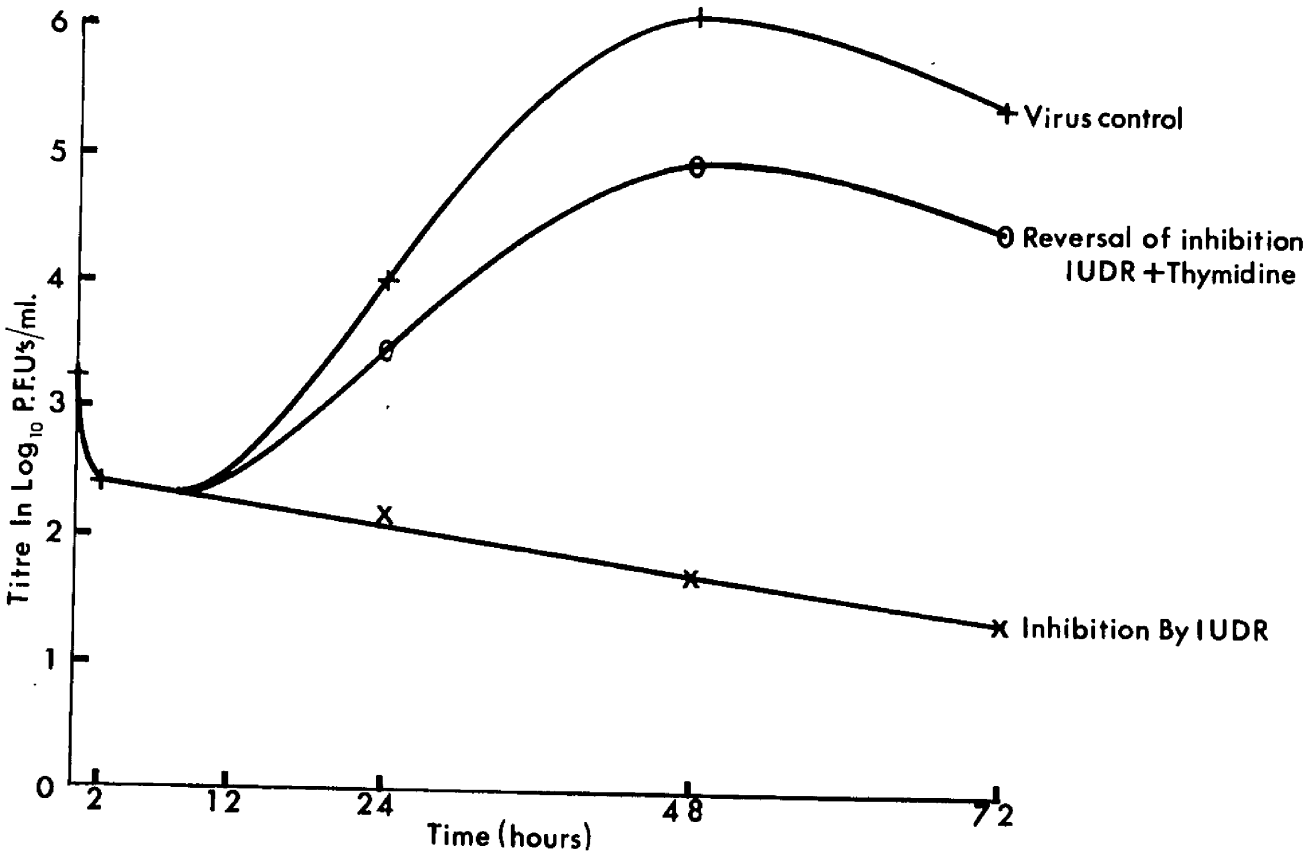


TABLE 20

EFFECT OF IUDR ON THE PLAQUE-COUNT

Content of overlay	Virus dilution	Number of plates	Plaque-counts	Mean plaque-count
No addition (Virus control)	10^{-4}	5	0, 1, 3, 3, 2	1.8
IUDR	10^{-1}	5	4, 7, 7, 7, 8	6.6
	10^{-2}	5	0, 1, 2, 1, 0	0.8
	10^{-3}	5	0, 0, 0, 0, 0	0
IUDR + thymidine	10^{-3}	5	28, 29, 25, 25, 19	25.2
	10^{-4}	5	2, 2, 2, 3, 1	2.0

culture-fluids was, however, considerably reduced by the procedure, as may be seen from Table 21.

In cultures infected with strain HS-2 and maintained in the presence of BUDR, a CPE appeared on the second day but consisted merely of a few widely-scattered, round, refractile cells. The latter increased in number during the succeeding two days but, in most cases, remained surrounded by normal fibroblasts. Multicellular foci of infection were few in number and were comprised only of a small knot of cells. In contrast, many foci were present on the third day in infected (control) cultures and an extensive, confluent CPE was manifest on the fourth day. Cultures maintained under BUDR and harvested on the fourth day contained 2.6×10^1 P.F.U. of virus per ml. whereas in control cultures sampled at the same time, 4.0×10^3 P.F.U. of virus per ml. were found to occur.

Infected coverslip cultures stained by the fluorescent Feulgen method contained varying numbers of cells with brightly fluorescent golden-yellow nuclei. They were only rarely to be seen in cultures fixed as early as six hours after infection but were numerous in those examined at 22 and 30 hours. The whole of the nucleus was involved and discrete inclusion bodies were not detectable. As the foci developed, the Feulgen-positive nuclei tended to occur around the expanding margins and many of the remaining cells contained dull green intranuclear inclusions, clearly separated from the nuclear

TABLE 21

EFFECT OF IUDR ON YIELD OF VIRUS (EXPERIMENT 4)

Time (hours)	Titre of virus in $\log_{10}$ P.F.U./ml.		
	Virus control	Virus + IUDR	Virus + IUDR + Thymidine
24	4.92	5.04	5.02
48	4.90	2.92	4.54

membrane. Examination of further infected cultures stained by haematoxylin and eosin suggested that the fluorescent golden nuclei revealed by the Feulgen method corresponded with those which were almost entirely occupied by basophilic material. Conversely, the non-fluorescent, green, intranuclear inclusions appeared to be identical with those stained by eosin. The same impression was gained from a study of serial sections of infected chorio-allantoic membrane. Thus, where swollen basophilic nuclei were numerous, large fluorescent Feulgen-positive nuclei also occurred while, in those areas where clusters of eosinophilic inclusions were present, groups of Feulgen-negative inclusion bodies were also to be found.

In infected cell-cultures treated with DNAase, Feulgen-positive material was not encountered.

(4) DISCUSSION.

The concentrations of IUDR employed in the present investigation did not produce any microscopical evidence of cellular damage and coverslip-cultures stained with haematoxylin and eosin remained normal in appearance. Buthala (1964) reported that gross cellular, nuclear or mitochondrial alterations were not to be noted in rabbit kidney cultures when IUDR was used in concentrations below 100 µg./ml. and that 'cytotoxicity was not acute below 1000 µg./ml.' In experiments with I.L.T. and herpes simplex viruses growing in chick-embryo kidney cultures, Tannock (1965) applied IUDR in

concentrations of up to 1000 µg./ml. but did not describe any evidence of toxicity. Indeed, after treatment with IUDR at levels of 100 µg./ml. and 1000 µg./ml. those cultures still seemed able to support the growth of a mutant of herpes simplex virus, resistant to the action of IUDR. On the other hand, Perecchino and Orfei (1965) reported that, whereas the use of 100 µg. of IUDR per ml. led to vacuolation, together with increased granularity and some nuclear changes in bovine embryonic kidney cultures, 50 µg. per ml. was non-toxic. The concentrations of IUDR employed in the investigation under report were probably not of an order sufficient to derange the metabolism of resting cells. In the absence of toxicity and in view of the finding (Buthala, 1964) that 100 µg./ ml. of IUDR did not completely arrest multiplication of pseudorabies and herpes simplex viruses, the levels of inhibition attained in the present investigation furnish prima facie evidence that the inhibitory action of the compounds was selectively directed against replication of the virus under examination.

The investigations under report showed that IUDR in concentrations of 20 and 50 µg/ml. prevented the formation not only of pigeon I.N.I. virus but also that of I.L.T. virus. Since the inhibitory effect of IUDR on the growth of the latter microbe has already been demonstrated (Tannock, 1965), it seems reasonable to conclude that the columbine agent should be classed as a deoxyvirus. In Experiment 2, the incorporation of 50 µg. of IUDR into each ml. of

medium served to reduce the maximal titre of pigeon I.N.I. virus from 6.10 $\log_{10}$ P.F.U. to 1.72 $\log_{10}$ P.F.U. per culture, a decrease comparable with that obtained by Tannock (1965) in respect of I.L.T. virus. The addition of thymidine counteracted the effect of IUDR to the extent of allowing the maximal titre of virus to reach 4.97 $\log_{10}$ P.F.U. per culture, i.e. 7.3 per cent of that produced in untreated cultures. While that figure is low, corresponding measurements made at other times in the multi-step growth cycle, such as 28 per cent at 24 hours and 11.5 per cent at 72 hours, bear comparison with the 15 per cent recorded by Roizman et al. (1963). Moreover, the quantity of virus present in cells maintained under IUDR plus thymidine and harvested at the end of 48 hours was 1782 times greater than that contained in cells kept under IUDR alone. The efficacy of thymidine in reversing the effect of IUDR indicates that the inhibition brought about by the latter substance resulted from its ability to substitute for thymidine and was not attributable to genetic alteration of the cell. Interestingly enough, under an agar overlay, thymidine seemed to entirely abolish the inhibitory effect (Experiment 3).

Although the number of typical, mature plaques was reduced by incorporation of IUDR into the agar overlay (Experiment 3), numerous foci barely visible to the naked eye were present in the 10^{-1} dilution and microscopic examination showed them to be plaque-like in appearance. The view that they represented plaques was reinforced by the finding of identical foci, to about one-tenth the

number, in the 10^{-2} dilution. Nevertheless, even if allowance is made for those foci, it is clear that the concentration of IUDR employed reduced the plaque-count as well as the mean plaque-diameter. Persechino and Orfei (1965), working with infectious bovine rhinotracheitis (I.B.R.) virus in bovine embryonic kidney cultures, reported that, whereas the use of 50 µg. of IUDR per ml. completely suppressed plaque formation, the application of the compound in concentrations of 10 and 20 µg./ml. allowed a small number of undersized plaques to develop. Buthala (1964) recorded that complete suppression of plaque-formation by herpes simplex virus occurred under agar containing as little as 1 µg. of IUDR per ml. and that, when smaller amounts were employed, plaques of reduced size developed, the mean diameter diminishing in proportion with increase in drug concentration. While the results of the present investigation are qualitatively similar to those of the above workers, the degree of inhibition achieved was considerably less.

The development of a small number of plaques as large as 1 mm. in diameter in plates infected with the 10^{-1} dilution is difficult to interpret. The possibility that they arose from selection of a mutant virus resistant to IUDR seems unlikely because such did not seem to occur under liquid media. Thus, in Experiment 2, the titre of infective virus in treated cultures declined continuously over the three-day period of examination. That only a very small proportion of cells were rendered incapable of forming new virus also

appears improbable, if only because in Experiment 2, the CPE was completely prevented by the compound. Moreover, in Experiment 5, BUDR seemed to hinder extension of the CPE from the initially infected cells. Round (presumably infected) cells occurred singly or in very small clusters but typical expanding foci never developed. A similar observation was made by Rawls et al. (1964) in studies of the inhibition of varicella.

The cytochemical results correspond with those of Crouse et al. (1950) for herpes simplex and of Watrach and Hanson (1963) for I.L.T. Basophilic intranuclear inclusions, occupying the whole of the nucleus, were prominent in situations and at times when there was a marked increase of DNA throughout the nucleus. Conversely, eosinophilic type A inclusions were generally Feulgen-negative, though intermediate stages were sometimes found. The round cells seen in infected tissue-cultures rarely contained demonstrable DNA.

(4) MORPHOLOGY.

(1) INTRODUCTION.

The information already presented on the nucleic acid composition, the inclusion body development and the character of the CPE strongly suggested that the pigeon I.N.I. virus might belong to the herpesvirus group. In order, therefore, to provide further information on the agent's place in viral taxonomy, studies of the morphology of the virion were undertaken.

By use of the negative staining technique of electron microscopy (Brenner and Horne, 1959), Wildy et al. (1960) showed the virus of herpes simplex to consist of a polyhedral core, a capsid of icosahedral shape composed of 162 hollow, elongated, polygonal capsomeres, and an envelope of varying size and shape surrounding the nucleocapsid. Following the work of Wildy et al. (1960), a number of viruses of human and animal origin have been assigned to the herpesvirus group on the basis of their very close structural similarity with herpes simplex virus, those most pertinent to the present investigation being the virus of avian I.L.T. (Watrach et al. 1963; Cruickshank et al. 1963) and the human cytomegalovirus (Smith and Rasmussen, 1963).

(2) MATERIALS AND METHODS.

The M-3 strains of pigeon I.N.I. virus was grown in

whole-embryo cultures and harvested on the second day after infection. Cultures were frozen and thawed once to remove the cells from the walls of the container and 500 ml. of the pooled cell suspension were homogenized for two minutes in an MSE ultrasonic disintegrator at an amperage of 1.5. After clarification of the crude suspension by centrifugation at 3500 r.p.m. for 30 minutes, the virus was concentrated by rotation at 20,000 r.p.m. for 30 minutes in a Spinco L1 preparative ultracentrifuge. All but one of the pellets were resuspended in an equal volume of distilled water and a loopful of the suspension transferred to a carbon-coated copper grid which was then inverted over a drop of 2 per cent phosphotungstic acid adjusted to a pH of 6.0 with normal potassium hydroxide. After approximately five seconds, the grid was transferred to a Siemens Elmiskop electron microscope and examined at instrumental magnifications of 40,000 with double condenser illumination. For estimation of the particle/infectivity ratio, the other pellet was resuspended in 2 ml. of Hank's B.S.S. and 0.1 ml. of the preparation mixed with 0.1 ml. of a suspension of latex containing 1.2×10^9 spheres per ml., 0.1 ml. of a 2 per cent solution of phosphotungstic acid and one drop of 0.1 per cent bovine serum albumen. The mixture was examined in the electron microscope as described above and the number of virus particles accompanying 60 latex spheres was counted. The infectivity of the remainder of the virus suspension was measured by the plaque-assay method.

(3) RESULTS.

Numerous virus particles with, or without, an envelope were observed, those of naked type being in the majority. Although the preparation contained a considerable amount of cell debris, the characteristic appearance of the virions rendered them easily recognizable. Detailed examinations were made from photographs of fourteen particles taken at random.

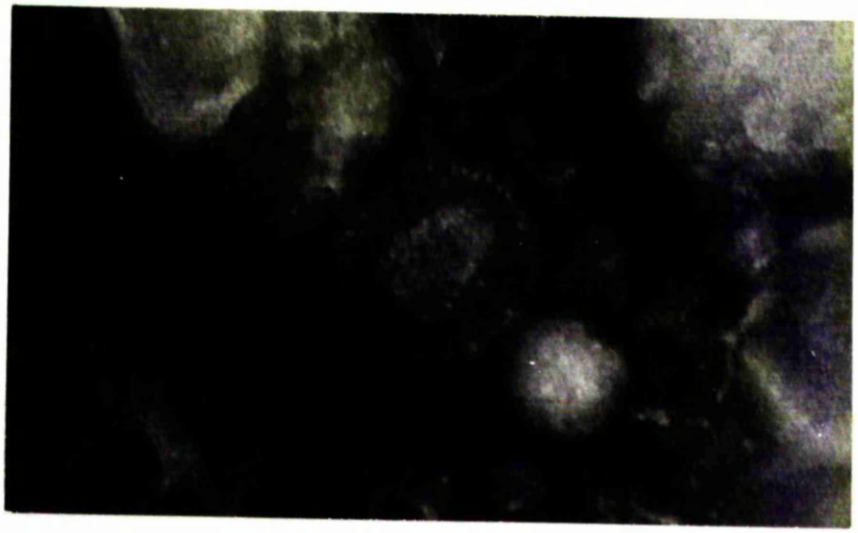
Three main structural components were appreciated, the core, the capsid and the envelope. The envelope was present in only two of the 14 particles photographed. One virion (Figure 50) was devoid of a core i.e. "empty" and three (Figure 51) were partially filled with phosphotungstic acid, i.e. "semi-empty".

The region normally occupied by the core was clearly demarcated by the capsid in the case of the particle entirely filled with the phosphotungstate (Figure 50). It was hexagonal and measured 750 Å between opposite sides and 875 Å between opposite angles. Identical values were obtained for two of the "semi-empty" particles but, in the instance of the third, the distance between opposite sides was 825 Å.

In most cases, the capsid appeared hexagonal, each edge measuring 475 Å ± 25 Å. It had an overall diameter of 1000 Å ± 50 Å and consisted of elongated, hollow and regularly-disposed capsomeres. The diameter of the latter lay between 75 and 100 Å and the axial hole

Figure 50. Empty virion. x 240,000.

Figure 51. Semi-empty virion. x 240,000.



between 25 Å and 40 Å. At one border of the capsid, the elongated form of the capsomeres was sometimes more readily appreciable and their length was found to be between 100 Å and 125 Å. Many of them appeared to be angular, a feature indicative of polygonal form. They were separated from each other by a distance of approximately 25 Å.

The capsomeres were arranged in a highly ordered pattern. When orientation was favourable, equilateral triangular facets were clearly recognizable on the surface of the capsid (Figure 52), a feature indicative of three-fold symmetry. In the latter figure, the capsid is viewed along an axis of two-fold symmetry, so that, whereas the two facets stand out prominently with their capsomeres only diverging slightly from that axis, other facets are sharply inclined to the plane of view and, thus, quickly disappear from sight. It was, therefore, possible to count the number of capsomeres around the periphery of each facet. The total - 12 - indicated that there were five capsomeres along each edge, i.e. shared with the adjoining facet. The central area of each facet contains three capsomeres. In Figure 53, a capsomere is to be seen enclosed by five others. Those features indicated that the capsid possesses 5:3:2 symmetry and takes the form of an icosahedron. From the formula, applicable to capsids of that type of symmetry, $10(n-1)^2 + 2$, where n represents the number of capsomeres along each edge, the total number of capsomeres in the particle is found to be 162.

Figure 52. Complete nucleocapsid. x 400,000.

Figure 53. Particle showing axis of five-fold symmetry.
x 400,000.

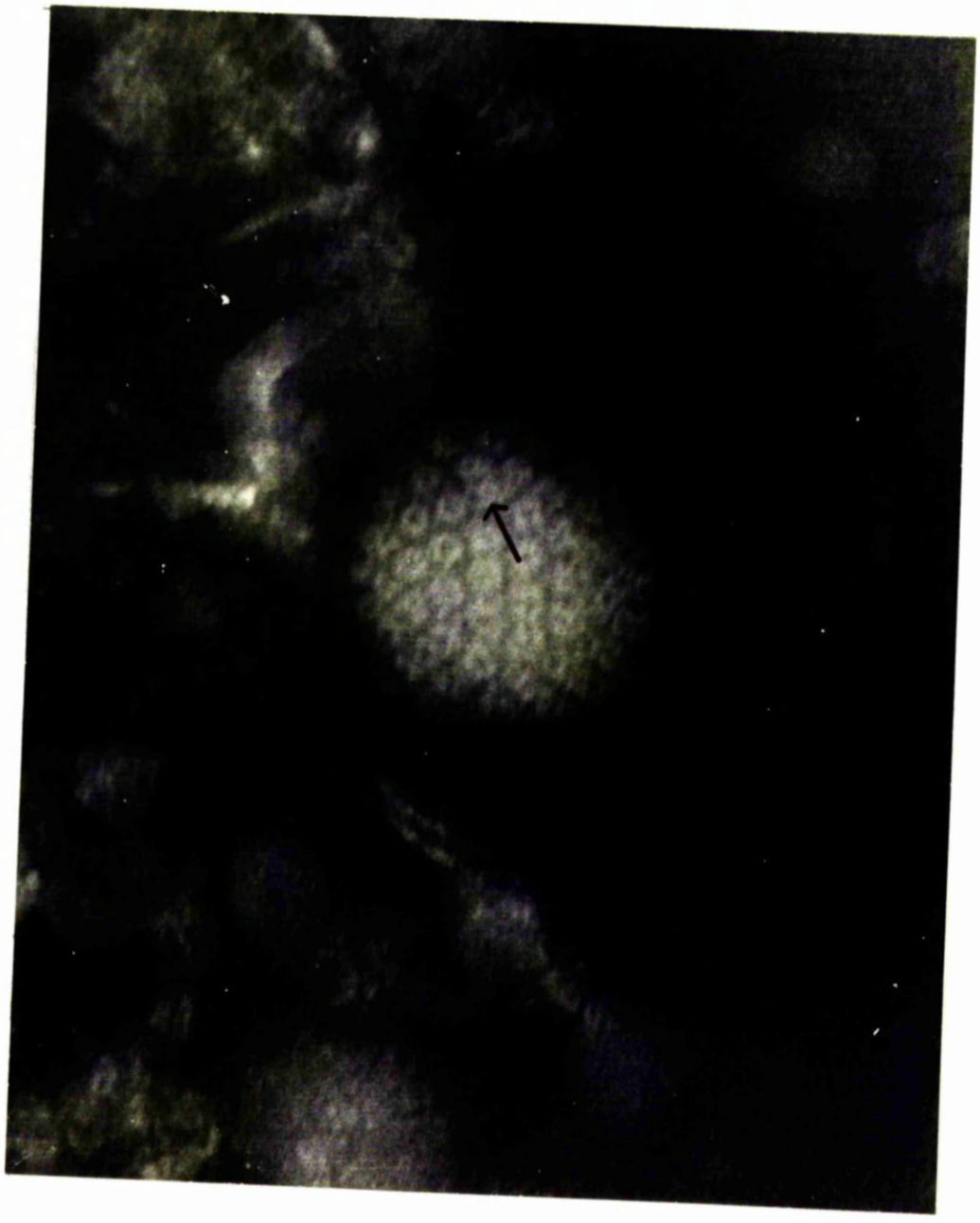
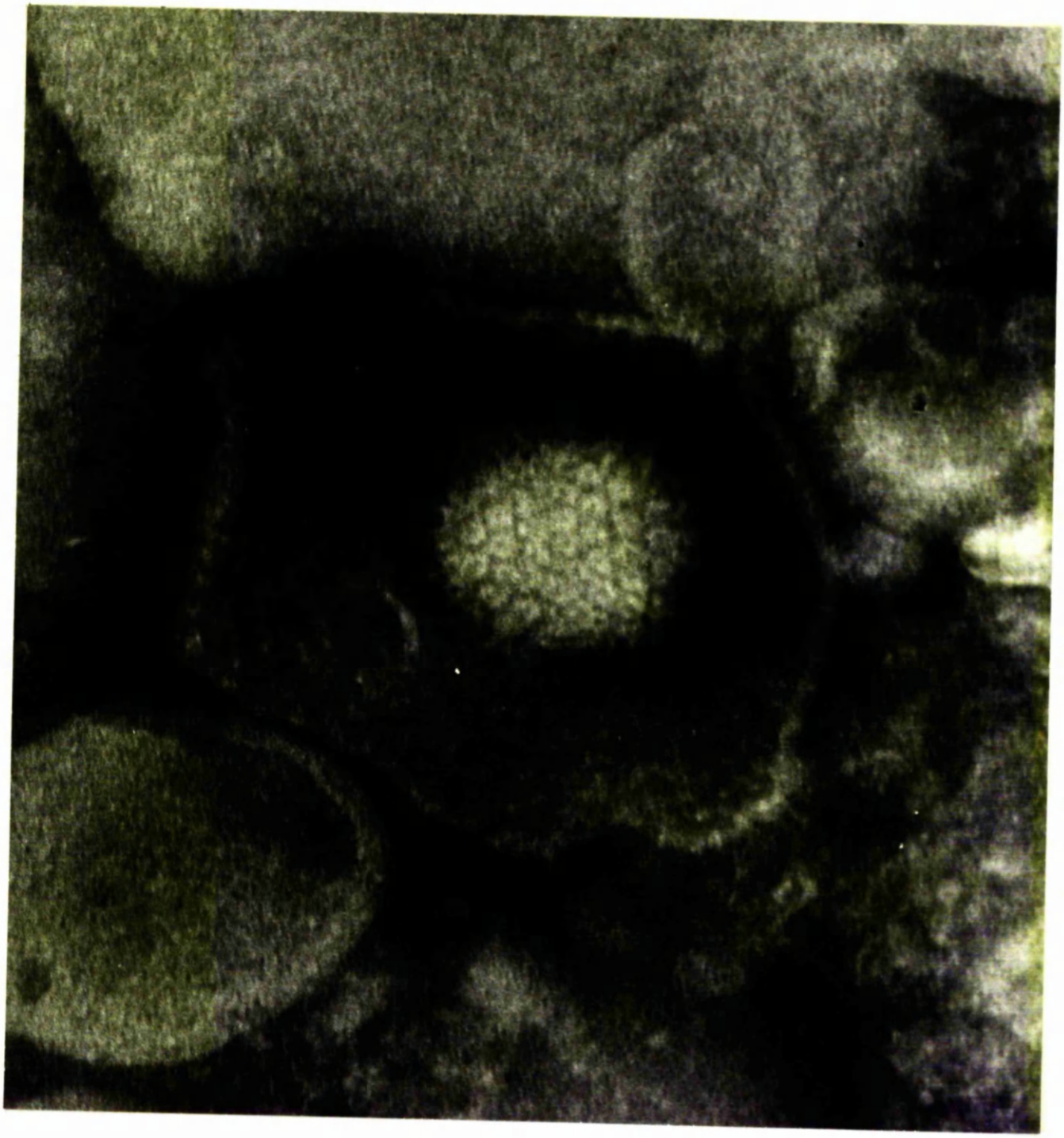


Figure 54. Entire virion. x 400,000.



Since only two photographs were taken of enveloped particles, it is impossible to give a full description of the size and structure of the envelope. In one particle, the envelope was "ballooned" at one pole and disrupted at the other. Smooth in outline and seemingly homogeneous in structure, it varied from 40Å to 225Å in thickness and had a minumum diameter of 2800Å. That of the second particle (Figure 54) was more regular in shape, appeared heterogeneous in structure with occasional fine projections, varied in thickness from 50Å to 150Å and had a maximum diameter of 2300Å.

The number of particles present in 1 ml. of the virus suspension prepared for estimation of the particle/infectivity ratio was approximately 1×10^{10}. A considerable amount of cell debris was present whereby some of the virus particles may have been obscured. It is unlikely, however, that the true number of virions present exceeded the recorded number by a factor of more than two. The infectivity titre of the same suspension was 9.9×10^{6} P.F.U. / ml., so that the particle/infectivity ratio was not less than 1000:1 and, probably, not greater than 2000:1.

(4) DISCUSSION.

The particles revealed by electron microscopy were present in large numbers and were of highly characteristic appearance, entirely consistent with that of virions. The capsid was made up of equilateral triangular facets, a feature indicative of a regular polyhedron.

More precisely, the hexagonal profile of the capsid and the existence of points of five-fold symmetry showed it to be icosahedral. Where orientation was favourable, a group of three capsomeres was seen to occupy the centre of each facet. It was apparent, therefore, that five capsomeres were present along the edge shared by adjoining facets, and that the total number of capsomeres in the virion was 162 - the number characteristic of the herpesvirus group. Other characters denoting membership of that group were the presence of envelopes in a minority of particles, the hollow and elongated form of the capsomeres and the average diameter of the capsid, 1000 ± 50Å as compared with 1050 ± 10Å for herpes simplex virus (Wildy, et al., 1960) and 1075 ± 25Å for I.L.T. virus (Watrach et al., 1963).

Other virus-like particles were not observed in the preparation. Thus, although homogenates of non-infected tissue cultures were not examined, the demonstration of large numbers of typical herpesvirus particles in highly infective suspensions of a virus, which almost certainly contained DNA and which was known to be ether-sensitive (see later) and capable of producing characteristic herpetic inclusions, may be regarded as evidence sufficient to identify the particles with that virus.

The aim of the present investigation was to confirm that the pigeon I.N.I. virus belonged to the herpesvirus group rather than to provide a definitive description of the fine structures of

the virion, the number of particles photographed being insufficient for the latter purpose. Nevertheless, within the limitations imposed by the small number of particles measured, the dimensions recorded for the various viral components are closely similar to those of the corresponding constituents in other members of the group, as may be seen from Table 22. Dubiety does exist, however, as to the morphology of the envelope. Wildy et al., (1960) found that periodic protuberances, about 80-100Å in length and spaced at intervals of about 50Å, were present on the surface of the envelope of herpes simplex virus, while Watrach et al., (1963) described a series of similar but smaller projections in I.L.T. virus. In Figure 54 of the present investigation, fine spikes appear to be present on the surface of the envelope but the clarity of the photograph does not allow a more precise description of the structures to be made.

Due to the presence of cell-debris which may have obscured some virions, the particle counts were not precise. Nevertheless, judging from the relative distribution of cell-debris and clearly visible virus particles, it seemed unlikely that the count underestimated the true value by a factor of more than three. Probably also, the infectivity titre was greater than that recorded since, as was related in Part 3, Section 2, the sensitivity of the standard plaque-assay is inferior to that of other methods of titration in cell-culture. It may be concluded, therefore, that the particle:

TABLE 22

COMPARATIVE DIMENSIONS OF THE STRUCTURAL COMPONENTS OF SOME HERPESVIRUSES

Virus	Pigeon ENI	Herpes simplex	I.L.T.	Human cyto-megalovirus
Reference	(1)	(2)	(3)	(4)
Diameter of core	775 ± 25	775 ± 5	800 ± 20	770
" " nucleocapsid	1000 ± 50	1050 ± 10	1075 ± 25	1130
" " capsomeres	75 - 100	95 ± 1	95	-
Length " "	100 - 125	120 - 125	105	-
Diameter of axial hole	25 - 40	40	35 - 40	40
Distance apart of capsomeres	25	25 - 30	30	-
Thickness of envelope	40 - 225	40 - 100	40 - 100	-
Diameter over "	2300 - 2800	1450 - 2050	1950 - 2500	1800 - 3000

All measurements in Å units.

References:
(1) Present investigation
(2) Wildy *et al.* (1960)
(3) Watrech *et al.* (1963)
(4) Smith and Rasmussen (1963).

infectivity ratio lay around 3 $\log_{10}$ units; it was almost certainly much greater than 2 $\log_{10}$ and considerably less than 4 $\log_{10}$ units. As such, it contrasts sharply with that obtained by Smith and Rasmussen (1963) for human cytomegalovirus (6-8 $\log_{10}$ units). In the latter case, the high ratio correlated with the total absence of particles with complete cores whereas, in the present investigation, virions with complete cores proved to be in the majority. Smith (1964) found that envelopes are essential to the infectivity of herpes simplex virus but, although the proportion of enveloped particles was not high in the work under report, it was not sufficiently low to account for the fairly high particle : infectivity ratio.

(5) RESISTANCE.

(1) INTRODUCTION.

Studies on the resistance of the pigeon I.N.I. virus to chemical and physical agents were designed firstly, to provide further information of taxonomic interest and, secondly, to furnish particulars of value in the storage and handling of the virus in the laboratory.

The significance of ether-sensitivity and of acid-lability in the taxonomy of viruses has already been emphasized. Hamparian et al. (1963) reported that herpes simplex and varicella viruses as well as the human cytomegalovirus were inactivated at pH 3.0 and were sensitive to ether. Similar results have been obtained with other herpesviruses, e.g., that of infectious bovine rhinotracheitis (Griffin et al., 1958) and that of feline rhinotracheitis (Miller and Crandell, 1962). It is apparent, however, that thermo-resistance varies considerably within the group. Thus, Kaplan and Vatter (1959) reported that, whereas 28 per cent of the infectivity of a suspension of pseudorabies virus remained after exposure to 44°C. for five hours, only 0.014 per cent of herpes simplex virus survived such treatment.

(2) MATERIALS AND METHODS.

The method used for the study of ether-sensitivity was

that described by Andrewes and Horstmann (1949). 0.1 ml. of pure anaesthetic ether (May and Baker, London), free from demonstrable peroxides and aldehydes, was mixed with 0.5 ml. of the H-1 strain of virus. The mixture was placed in a screw-cap bottle, bound with adhesive tape to reduce evaporation and the vessel then held at 4°C. for 24 hours along with another bottle containing virus suspension only. At the end of that period, the ether-treated virus suspension was poured into an open Petri dish and left at room temperature for 10 minutes to allow evaporation of the ether. Titrations of treated and control suspensions were then carried out in whole-embryo cultures in the manner previously reported.

Tests of acid-lability were carried out by the methods described by Tyrrell and Chanock (1963). The pH values obtained in the present investigation, however, proved somewhat different from those recorded by the above authors. Thus, when 9 parts of Eagle's medium without sodium bicarbonate were mixed with 1 part of virus suspension (strain M-3), the resultant pH was 4.0 instead of 3.0 as expected. Similarly, when one part of 0.1 M. sodium citrate - citric acid buffer at pH=4.0 was mixed with an equal part of the same virus suspension, the pH obtained was 4.8 instead of 5.0. All pH values were measured in an M.S.E. pH meter and were checked with pH indicator papers (Whatman - B.D.H.). Controls consisted of the following mixtures:-

(a) 9 parts Eagle's medium with bicarbonate + 1 part virus; final

pH=7.6.

(b) 1 part 0.5M phosphate buffer + 1 part virus; final pH=7.2.

All mixtures were held at room temperature for three hours and then titrated in the usual way.

The rate of thermal inactivation was measured under the following conditions:-

Temperature.	Time.	Strain.
56°C.	5 minutes	M-3
50°C.	30 minutes	HS-2
37°C.	1, 2, 3, 4, 5, 15 and 23 hours	B-1
4°C.	208 days	HS-2
-40°C.	10 days	HS-2
-40°C.	21 days	B-1
-40°C.	25 days	M-3

Stocks of virus employed for those studies were grown in whole-embryo cells maintained in Hank's B.S.S. containing 0.25 per cent lactalbumin hydrolysate but without any serum. The agent was released from the cells by maceration in a Griffith's tube and the resultant suspension was clarified by centrifugation at 1500 r.p.m. for ten minutes and then dispensed into ampoules. All samples were titrated by means of the plaque-assay method immediately after the respective period of treatment.

To determine the effect of slow freezing and thawing,

strain B-1 was subjected to three cycles of that treatment. The virus suspension was divided into four parts, one of which was titrated immediately and the remainder was placed in a deep-freeze cabinet at -40°C. Approximately 30 minutes was allowed for the temperature of the three samples to reach -40°C., whereupon they were removed and thawed out at room temperature. One was then titrated and the other two returned to cold storage. The process was repeated until the last sample had been frozen and thawed three times.

Finally, in order to determine whether, or not, ultrasonic treatment of cultures would release virus from infected cells without simultaneously inactivating much of the virus already present in the culture fluid, the following procedure was carried out. A suspension of cells, infected with strain B-1 and containing a large amount of extracellular virus, was divided into five portions, one of which was titrated immediately, and the remainder after exposure to ultrasonic waves in an M.S.E. ultrasonic disintegrator, registering a maximum current of 1.5 amps., for periods of 30 seconds, one minute, two minutes and three minutes duration, respectively. The tubes containing the cell suspensions were immersed in ice to dissipate the heat generated by the ultrasonic waves and so leave the virus unimpaired.

(3) RESULTS.

The virus was found to be highly sensitive to ether, infectivity falling from 5.8×10^5 P.F.U./ml. to 5.5×10^1 P.F.U./ml. in 24 hours at 4° C.

The effects of three hours exposure to acid at 4° C. were as follows:-

Suspension	Titre in P.F.U./ml.			
	pH 4.8	pH 7.6	pH 4.0	pH 7.2
1	2.72×10^4	4.18×10^4	-	-
2	-	-	1.2×10^1	2.01×10^5

The rates of thermal inactivation are shown in Tables 23 and 24. Virus was rapidly destroyed at 56° C. but had a half-life of approximately 15 hours at 37° C. It was preserved well at 4° C. but varying results were obtained at -40° C. Two cycles of slow freezing and thawing had little effect on infectivity, as the following figures demonstrate.

	Initial titre P.F.U./ml.	Residual titre P.F.U./ml.
One cycle	3.2×10^5	8.0×10^4
Two cycles	"	2.04×10^5
Three cycles	"	5.65×10^4

When suspensions of infected cells were exposed to ultra-

TABLE 23

THERMAL INACTIVATION

Temperature	Virus strain	Initial titre P.F.U./ml.	Final titre P.F.U./ml.	Time
56°C.	M-3	4.66×10^3	1.50×10^1	5 minutes
50°C.	HS-2	3.16×10^4	9.65×10^3	30 minutes
37°C.	B-1	4.95×10^3	1.68×10^3	23 hours
4°C.	HS-2	5.16×10^4	1.30×10^3	208 days
-40°C.	HS-2	4.06×10^4	3.16×10^4	10 days
"	M-3	1.52×10^5	2.40×10^4	25 days
"	B-1	4.00×10^5	9.90×10^3	21 days

TABLE 24

THERMAL INACTIVATION AT 57°C.

Time (hours)	Titre of virus P.F.U./ml.
0	4.95×10^5
1	4.50×10^5
2	3.77×10^5
3	3.50×10^5
4	3.60×10^5
5	3.57×10^5
15	3.45×10^5
23	1.60×10^5

sonic waves, the cells were rapidly broken up and intracellular virus released. After approximately half a minute of such treatment, however, the rate of inactivation of extracellular virus exceeded the rate of release and the titre fell. The details were as follows:

Duration of treatment (minutes).	Titre in P.F.U./ml.
0	1.2×10^5
½	8.3×10^5
1	3.6×10^5
2	2.4×10^5
3	6.6×10^4

(4) DISCUSSION.

The experiments reported above showed that the virus was ether-sensitive and acid-labile and thus provided confirmation of its membership of the herpesvirus group. Infectivity was only slightly impaired at pH=4.8, but was almost totally destroyed at pH=4.0. The relationship between pH and the rate of inactivation of herpesviruses has not been often mentioned in the available literature but the virus seems to be rapidly destroyed at a pH of 4.0 and fairly stable at pH=6.0. Griffin et al. (1958), for example, found that the titre of infectious bovine rhinotracheitis (I.B.R.) virus fell from $10^{6.5}$ to $10^{1.5}$ in ten days at pH=4.4 and from $10^{6.5}$ to $10^{3.5}$ in 15 days at pH=5.0 but remained at the original level after 25 days at pH=6.0. The results of the present investigation suggest

that the pigeon I.N.I. virus is less sensitive than I.B.R. virus to pH=5.0 but that both agents are equally labile at pH=4.0.

The work under report suggested that the virus was amongst the most thermostable of the herpesvirus group. That observation may not be entirely valid inasmuch as the virus suspensions employed were undiluted tissue-culture preparations which, though clarified by centrifugation and free of serum, almost certainly contained host-cell material which may have exerted a protective effect on the virus. The reason for the procedure adopted in the present investigation was the need to obtain information on the stability of the virus under the conditions in which it was grown, harvested and preserved in the laboratory. Nevertheless, since similar methods have been employed by other workers e.g., Griffin et al. (1958), comment on the thermostability of the pigeon virus relative to that of other members of the herpes group is not without value.

At 56°C. the titre fell from 4.66×10^3 P.F.U./ml. to 1.5×10^1 P.F.U./ml. in five minutes, a rate of destruction comparable with that which Griffin et al. (1958) found to obtain with I.B.R. virus, in which instance the titre dropped from $10^{6.5}$ $TCID_{50}$ to $10^{3.5}$ $TCID_{50}$ in three minutes, the proportion of virus inactivated being 99.7 per cent compared with 99.9 per cent in the present investigation. At 50°C. the columbine virus was very much more stable than at 56°C., 30.6 per cent surviving an exposure of 30 minutes

duration. Comparison of the thermoresistance of the virus with that of other herpesviruses at 50°C. is difficult, since few figures have been published, but Hull and Nash (1960) stated that 30 minutes exposure at that temperature " did not kill " herpes B virus.

At 37°C. the titre fell by 23.8 per cent during the second hour but remained almost static for, at least, the next three hours. After 15 hours, it had been reduced to 49 per cent of that originally present. The half-life at 37°C. would therefore, seem to be around 15 hours. By comparison, the half-life of certain other herpesviruses recorded in the literature is as follows:

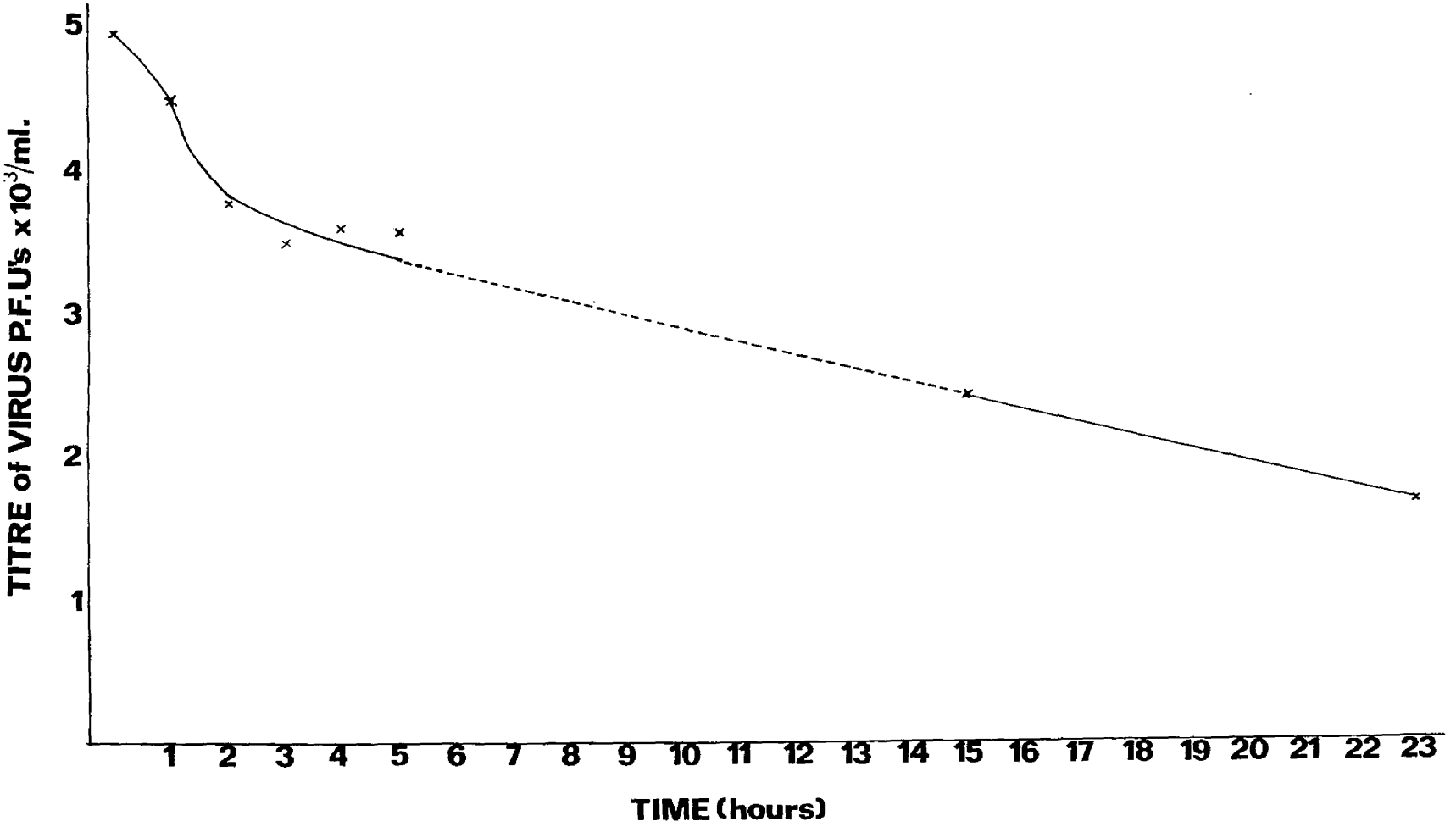
I.B.R.	37°C.	10 hours	Stevens and Groman (1963)
Canine herpes virus	36°C.	5 "	Carmichael et al. (1965)
Herpes simplex virus	37°C.	3 "	Farnham and Newton (1959)
" " "	"	1½ "	Scott et al. (1961)
Human cytomegalovirus	"	1 hour	Krugman and Goodheart (1964)
Pseudorabies	44°C.	2½ hours	Kaplan and Vatter (1959).

The information available, therefore, would indicate that the pigeon virus is amongst the most heat-resistant of the herpesviruses yet encountered.

The shape of the curve of inactivation at 37°C. differs from that reported for other herpesviruses. Farnham and Newton (1959) found that the infectivity of herpes simplex virus was destroyed

according to a first-order reaction whereas Kaplan (1957) as well as Hoggan and Roizman (1959b) found a pronounced shoulder in the curve. In the present investigation, the rate of inactivation was appreciably greater during the first two hours than at subsequent times (Figure 55). The reason for the early decrease in titre is unknown but it was possibly due to the elimination of highly-thermolabile variants or particles already partially damaged.

Weller and Hanshaw (1962) reported that pools of human cytomegalovirus stored in sealed ampoules in dry-ice varied in stability. The present investigation revealed that stability of the columbine virus at -40° C. likewise varied considerably but, since each measurement was made with a different strain of virus, the possibility that the variations were attributable to strain differences cannot be ignored. At -40° C. only 2.5 per cent of the infectivity of strain B-1 survived for 21 days whereas up to 15 per cent of the infectivity of strain M-3 remained after 25 days. The apparent instability of strain B-1 at -40° C. is not correlative with its resistance at 37° C. At 4° C., the viability of the virus was well maintained and storage at that temperature thus appears to be a more satisfactory method of preservation than does maintenance at -40° C. A somewhat similar result was reported by Stoker and Ross (1958) who found that the HFEM strain of herpes simplex virus remained stable at 4° C. for one month whereas storage at -20° C. reduced its infectivity by more than ten times inside two weeks. By contrast, Plummer


RATE of INACTIVATION at 37°c
TITRE of VIRUS P.F.U's x 10^3/ml.
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
TIME (hours)

and Lewis (1965) showed that the human cytomegalovirus was very much less resistant at 4°C. than at 22°C.

Although Krech and Lewis (1954) reported that samples of herpes B virus frozen at -20°C. and -70°C. lost approximately 99 per cent of their infectivity after one cycle of freezing and thawing and Rowe et al. (1956) and Weller et al. (1957) recorded similar findings in respect of the human cytomegalovirus, the results of the present investigation indicated that instability at -40°C. was not due simply to that treatment. Thus, one exposure to slow freezing and thawing reduced the titre by only four times while a second cycle increased the infectivity to 63.8 per cent of the original. That rise was presumed to be due to disruption of infected cells with resultant release of virus. After a third cycle, the titre fell to 17.6 per cent of that present at the commencement of the experiment, a much smaller reduction than that found to occur in virus suspensions stored at -40°C.

Although titration of the infectivity of virus suspensions before, and after, lyophilization was not carried out, the efficacy of that method of preservation was well demonstrated by the discovery reported in Part One that lyophilized virus of strain P-5 remained viable after storage at -20°C. for thirteen years.

The present investigation showed that the pigeon I.N.I. virus was inactivated by ultrasonic waves and that such disruption

of infected cells must not be prolonged if virus so released is to remain viable. Similar results were obtained by Kaplan (1957) in respect of herpes simplex virus. Provided that the rate of inactivation of cell-associated virus is minimal, i.e. very low compared with the rate of release, the optimal time for treatment is likely to depend upon the ratio of intracellular to extracellular virus. Where the proportion of the latter is already high, the amount of virus which can be released from the cells is restricted and inactivation of free virus becomes the critical factor. Conversely, when the proportion of cell-associated virus is very high, ultrasonic disruption may be considerably protracted before the amount of inactivated virus comes to exceed the quantity released.

(6) HAEMAGGLUTINATION.

(1) INTRODUCTION.

The failure of herpesviruses to agglutinate red cells in the standard haemagglutination test has long been recognized (Andrewes, 1964; Kaplan, 1966). A number of reports from Eastern Europe, however, refer to the occurrence of haemagglutination under certain conditions. Thus, Shubladze et al. (1960) asserted that some strains of herpes simplex virus agglutinated goose erythrocytes provided that the pH lay between 5.2 and 6.8, while Semerdjiev (1962) described agglutination of horse and guinea-pig red cells by the virus of equine rhinopneumonitis, particularly after treatment with formalin. Tokumaru and Scott (1964), however, tested 40 strains of herpes simplex virus for their ability to agglutinate goose cells but failed to find any evidence of that activity. McCollum et al. (1956) found that horse erythrocytes were agglutinated by extracts of hamster tissues infected with the virus of equine rhinopneumonitis. Such haemagglutination was not prevented by the serum of convalescent horses but was inhibited by the serum of horses hyperimmunized with infected hamster tissue.

As far as the writer is aware, the foregoing claims have not been confirmed by other workers and the inability of a herpesvirus to haemagglutinate continues to be regarded as a characteristic, if negative, feature of the group. The present

investigation had the aim of determining whether, or not, haem-agglutination was to be achieved with the pigeon I.N.I. virus.

(2) MATERIALS AND METHODS.

Tests were carried out with the B-1, M-3 and H-1 strains of virus, the infectivity titres of which lay between 5 and 6 $\log_{10}$ P.F.U./ml. Two-fold dilutions of viral suspensions were made up with P.B.S. in perspex haemagglutination plates, 0.3 ml. per cup. 1 per cent suspensions of pigeon, goose, fowl, guinea-pig, rat, rabbit and horse red blood cells were prepared in P.B.S., and 0.3 ml. amounts dispensed into the cups containing diluted virus. The pH levels of the mixtures were measured by means of litmus papers (Whatman - BDH) and were found to be between 6.7 and 7.0. Each test was done in triplicate so that one plate was incubated at 37°C., one placed in a refrigerator at 4°C. and the third maintained at room temperature. All plates were examined after 30, 60 and 90 minutes.

The experiment was repeated with goose red cells suspended in phosphate buffer of pH=5.5. The final pH, according to the indicator paper, was approximately 6.0.

(3) RESULTS.

Haemagglutination was not observed with any of the cells or at any of the temperatures employed. Auto-agglutination of rat erythrocytes occurred when they were mixed with virus dilutions of

1 in 16, or above.

(4) DISCUSSION.

The work of Semerdjiev (1962) on equine rhinopneumonitis virus and that of Shubladze et al. (1960) on agglutination of cells by herpes simplex virus had suggested that haemagglutination might be achieved with the pigeon I.N.I. virus if columbine or goose cells were employed. The results, however, showed otherwise. Hence, the only conclusion to be drawn is that the inability of the virus to haemagglutinate accords with membership of the herpesvirus group.

(7) ANTIGENIC RELATIONSHIP WITH ILT VIRUS.

(1) INTRODUCTION.

The objects of the work about to be reported were to discover:

(a) If antiserum to the M-3 strain of virus had any neutralizing activity against three strains of I.L.T. virus and

(b) If antiserum prepared against two strains of I.L.T. virus had any similar capacity against the M-3 strain of pigeon I.N.I. virus.

Experiments were carried out in collaboration with Mr. W. Baxendale of the Wellcome Research Laboratories.

(2) MATERIALS AND METHODS.

Cross-neutralization tests were performed with the M-3 isolate and with three strains of I.L.T. virus. In all cases, the "variable virus - constant serum" method was employed.

In the first series of tests, a 1 in 10 dilution of rabbit antiserum prepared against strain M-3 was mixed with ten-fold dilutions of the B.E.E. strain of I.L.T. virus. After incubation for three-quarters of an hour at 37° C. followed by a similar period of exposure at room temperature, the mixtures were inoculated into tubes of whole-chick-embryo cells, four tubes per dilution of virus. The same procedure was repeated twice, but with 1 in 10 dilutions of normal

calf-serum and antiserum against the B.E.E. strain of I.L.T. virus in place of M-3 antiserum. The B.E.E. antiserum was kindly supplied by Dr. F. T. W. Jordan of the University of Liverpool Veterinary School. The criterion employed in the assessment of the presence or absence of infective virus in virus-serum mixtures was the development of typical, multinucleated syncytia in epithelial islands of the cultures.

The neutralizing capacity of the B.E.E. antiserum against the M-3 strain of pigeon I.N.I. virus was then examined. Ten-fold dilutions of virus were mixed with a 1 in 10 dilution of the antiserum. The time allowed for neutralization was the same as in the tests with I.L.T. virus but the mixtures were inoculated into plates of whole-embryo cells and surviving virus titrated by means of the standard plaque-assay. The test was repeated with a 1 in 10 dilution of normal calf-serum in place of the I.L.T. antiserum.

At the Wellcome Research Laboratories, several young hens were inoculated with the M-3 strain of virus and, along with un-inoculated controls, were challenged three weeks later with a virulent strain of I.L.T. virus. Attempts were also made to produce antibody to the columbine agent by intratracheal injection of ten pullets followed by intravenous inoculation of four of them three and six weeks later. When plaque-inhibition tests were carried out in cultures of chick-embryo liver cells, it was found that serum from three of the hens given multiple inoculations of virus contained

antibody, the neutralization index in each case being slightly greater than 1.4. Plaque-inhibition tests were then performed with one of the M-3 antisera, an antiserum against I.L.T. virus (I.L.T.-2 strain), M-3 virus and two strains of I.L.T. virus (I.L.T.-1 and I.L.T.-2). The agents were mixed and incubated as already described but cultures of chick-embryo liver cells were employed so that both viruses could be titrated by the plaque-assay method. Baxendale (1966) reported that higher plaque-counts were obtained with I.L.T. virus in those cells than in cultures of chicken kidney.

(3) RESULTS.

Cross-neutralization tests with M-3 antiserum and the B.E.E. strain of I.L.T. virus failed to demonstrate any antigenic similarity between the two viruses. The titre of virus in the test employing a 1 in 10 dilution of I.L.T. antiserum was $10^{0.5}$ $TCID_{50}$/0.5 ml. while the corresponding figures for virus incubated with a 1 in 10 dilution of normal calf-serum and with a like dilution of M-3 antiserum were both $10^{4.25}$ $TCID_{50}$/0.5 ml. Work with M-3 virus and B.E.E. antiserum showed that there was little, if any, neutralization of the former by the latter, the titre of virus incubated with the antiserum being 2.7×10^4 P.F.U./0.5 ml. of virus-serum mixture compared with 6.5×10^4 P.F.U./0.5 ml. for virus incubated with normal calf serum.

Birds inoculated with strain M-3 at the Wellcome Research

Laboratories and challenged with I.L.T. virus three weeks later developed clinical signs of I.L.T. which were of severity akin to those shown by the controls. Three of the four birds given multiple inoculations of strain M-3 developed a low level of antibody to the virus and samples of sera possessed neutralization indices of slightly greater than 1.4. Normal fowl sera did not have any neutralizing activity. One of the three sera was then used in a cross-neutralization test, with results as follows:

Neutralization Indices.

Antiserum.	Virus.		
	M-3	ILT-1	ILT-2
M-3	1.2	0.4	0.6
ILT-2	0.9	2.0	2.0

(4) DISCUSSION.

The findings were indicative of slight cross-reaction between the M-3 strain of pigeon I.N.I. virus and the Wellcome I.L.T.-2 virus since the neutralization indices of I.L.T.-2 antiserum were 0.9 against the columbine agent and 2.0 against the homologous virus. The degree of cross-neutralization exhibited by the fowl M-3 antiserum is difficult to assess because, although it had a very low neutralization index against I.L.T.-2 virus (0.6), that against the homologous strain was only 1.2. Since the neutralization index of normal fowl serum was 0 in respect of all three viruses, the above

figures may be regarded as evidence of a minor antigenic relationship. On the other hand, they are not such as to invalidate precise distinctions.

The cross-neutralization test with the B.E.E. strain of I.L.T. virus provided more definitive results. A 1 in 10 dilution of B.E.E. antiserum had the high neutralization index of 3.75 against the homologous virus but one of only 0.38 against strain M-3. Similarly, M-3 antiserum had an index of 0 against the B.E.E. strain compared with that of 2.19 against the homologous virus. Clearly then, the two viruses were antigenically unrelated.

Strain M-3 seemed to be of low immunogenicity to the domestic fowl. Only three out of four birds given three inoculations of virus developed neutralizing antibody and the titre in all cases was low. Moreover, six birds in receipt of a single intratracheal injection failed to produce antibody, possibly because virus was unable to replicate. The antibody levels of the inoculated birds subsequently challenged with I.L.T. virus were not measured but, from the other results, it seems unlikely that the one inoculation of strain M-3 was sufficient to have stimulated the production of antibody. Whether or not birds which develop antibody after multiple inoculations of the virus are rendered resistant to challenge with I.L.T. virus has yet to be determined. As is well-known, puppies vaccinated with measles virus become resistant to the antigenically

related canine distemper virus (Slater and Murdock , 1963). Present evidence, however, does not denote an association, antigenic and otherwise, between the two avian herpes viruses comparable with that which obtains between the viruses of measles and canine distemper. Even if that relationship did exist, the usefulness of the columbine agent as a heterotypic vaccine against I.L.T. would be severely limited by the need to administer it by multiple inoculations.

It must be emphasized that the experiments recorded above were conducted with only one strain of pigeon I.N.I. virus. Although M-3 antiserum has already been shown to neutralize the B-1, P-5 and HS-2 strains of columbine agent, it is possible that antiserum against, for example, strain B-1 might not neutralize strain M-3. The present investigation does not, therefore, purport to have explored the antigenic relationship of I.L.T. virus with the pigeon I.N.I. virus but only that of the former with one strain of the latter.

(8) SUMMARY AND CONCLUSIONS.

Inhibition of viral multiplication by deoxyuridine and demonstration by Feulgen staining of an early increase in the amount of DNA in the nuclei of infected cells indicated that the nucleic acid of the pigeon I.N.I. virus existed in the form of DNA. The nucleocapsid was icosahedral in shape, with a mean diameter of 100 mμ., possessed 162 hollow, elongated capsomeres and was sometimes enveloped. Infectivity was destroyed at a pH of 4.0 and by treatment with ether. Those results clearly show that the agent belongs to the herpesvirus group. Neutralization tests indicated that the strains examined by the author were closely related antigenically but the agent was serologically distinguishable from I.L.T. virus. All attempts to demonstrate haemagglutination were unsuccessful.

FINAL DISCUSSION.

The handbook on cage-birds published by the British Veterinary Association states that insufficient is known about Smadel's I.N.I. virus to justify its inclusion in the list of agents affecting pigeons in the United Kingdom. The present contribution, however, has revealed the existence of a closely related virus in various parts of the country and has clearly demonstrated its pathogenicity for the pigeon. Laboratory methods suitable for its growth and assay have been presented and proof of its place within the herpesvirus group has been established.

The incidence of infection is as yet unknown and future work will be required to determine whether the disease is prevalent in feral as well as in domesticated pigeons. There is, however, some circumstantial evidence to suggest that the virus may be widely disseminated in flocks of the latter type. In a personal communication to the author, Mr. R. H. Axworthy of the Standard Laboratory (Poultry Diagnostic Laboratory), Reading, stated that he had observed a disease of similar clinical and pathological character on many occasions. Examination for the agent of ornithosis was carried out several times but with negative results in each case. According to the handbook referred to above, outbreaks of disease of unknown aetiology but distinguished by the appearance of coryza-like clinical signs have occurred in widely-separated parts of the country during

the past decade. The condition, to which various names have been applied, e.g. "eye-cold", "roup" and "coryza", affects pigeons of up to one year of age and is seemingly contagious. Once recovery has taken place, birds are seldom affected again. Enquiries made by the author in Renfrewshire and Dunbartonshire elicited the information that "eye-cold" is a common disease of young birds which sometimes has a high mortality and which may be associated with the presence of small, yellowish-brown excrescences in the mouth and throat. Since those features were characteristic of the disease described in this thesis, the two conditions are possibly synonymous. However, as the agent of ornithosis can be isolated from the ocular discharge of pigeons with conjunctivitis (Monreal, 1958) and the protozoan Trichomonas gallinae is known to be productive of diphtheritic membranes, further investigation is obviously required before the relative importance of the three organisms in the aetiology of pigeon disease can be properly assessed.

Apart from those basic physical and chemical characters which denote membership of the herpesvirus group, the three most definitive features of the pigeon I.N.I. virus were its affinity for fibroblasts, its release from the infected cell in vitro and the relatively high degree of thermal resistance which it exhibited. In respect of the first of those properties, it resembled the cytomegaloviruses though, since it grew well in epithelial cells of hepatic origin and, to a lesser extent, in those derived from renal

cortex, the similarity was limited. The agent was akin to most members of the herpesvirus group, e.g. herpes simplex virus and murine cytomegalovirus, in being released into the culture-fluid in large amounts and thus differed from the causal organism of herpes zoster, the herpesvirus isolated by Churchill (1957) from cases of Marek's disease and the human cytomegalovirus, all of which may be successfully passaged only by transfer of infected cells.

The columbine agent seemed to differ fundamentally from I.L.T. virus in its ability to grow in fibroblasts and in its greater invasiveness both in vivo and in ovo. In tissue-culture and in the chorio-allantoic membrane, I.L.T. virus remained restricted to epithelial cells. Limitation of the latter agent to the respiratory system of the domestic fowl may, therefore, be correlative with its inability to multiply in cells of mesodermal origin. If that surmise is correct, those other avian viruses productive of hepatic necrosis and intranuclear inclusions may be more closely related to the columbine agent than to I.L.T. virus. In the absence of information on the cytopathogenicity of those agents, speculation is premature but it is conceivable that future work will allow the division of the avian herpesviruses into two sub-groups, one comprising I.L.T. virus and the other consisting of agents capable of growth in cells of diverse origin.

In order to seek out those aspects of pigeon I.N.I. virus

infection which could be exploited to greatest advantage in future work, the present investigation was intended to cover the subject on as broad a basis as possible. For that reason, pursuit or elaboration of certain findings was not possible in the time available. The task of future work will be not only to fill the gaps left by the present contribution, particularly with regard to the incidence of infection and the serological relationship between strains, but also to extend the depth of the investigation by comparison of the base ratios of the viral DNA with those of other herpesviruses and by examination of viral clones for reduced pathogenicity.

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