

CANINE DISTEMPER—IMMUNISATION WITH AVIANISED VIRUS.

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INTRODUCTION.

Canine distemper occurs in most parts of the world. The disease is not confined to dogs and a number of animals of various families are known to be susceptible. These are the ferret, mink, weasel, ermine and marten (*Mustelidae*), the raccoon (*Procyonidae*) and the wolf, fox and jackal (*Canidae*).

It has long been known that canine distemper is a highly contagious condition with protean manifestations but very little was known about the aetiology until the classical work of Laidlaw and Dunkin proved conclusively that true distemper was the result of infection with a specific virus.

Most early investigators incriminated bacteria. However, Carré (1905) claimed that he was able to reproduce the disease with bacteria-free filtrates of pericardial and other body fluids. His observations were confirmed by the experiments of Lignieres (1906).

Ferry (1911) was unable to confirm Carré's claims. He realised that bacteria were usually secondary invaders but believed that *Bacillus bronchicanis* (- *Bacillus bronchisepticus* - *Brucella bronchiseptica*) was the primary cause, and made the position more confused when he showed that this organism could be passed through filters regarded as bacteria-proof by most workers at that time (Dunkin 1930). M'Gowan (1911) and Torry and Rahe (1913) held similar views. Vaccines prepared from this organism were used with seemingly encouraging results (Ferry 1912, Torry and Rahe 1913). However, Hardenburgh (1926) proved that they conferred no protection against distemper.

On the other hand Puntoni (1923, 1924) in Italy showed that it was possible to passage the causal agent of distemper by intracerebral injection of dogs, and that the brains of these animals were apparently free of bacteria. Vaccine prepared from formalised brain suspensions gave favourable results. Unfortunately his work was hampered by difficulty in obtaining susceptible dogs. Later Labailly (1927) who accepted Carré's view on the aetiology of distemper showed in a brief report that vaccine might be made from formalised infected dog spleen. Kantorowicz (1933) used brain emulsions collected during the second period of the febrile reaction in dogs. Although he claimed good results his work could not be confirmed.

In England the ravages of distemper had become so serious that in 1923 the Field Distemper Fund was established with the object of investigating this disease under rigorously controlled conditions. Dr. Laidlaw and Major Dunkin were appointed to carry out the research work.

At the commencement of their investigations Laidlaw and Dunkin (1926) stated they were prepared to accept either view as to the aetiology of dog distemper. It soon transpired from their experiments that Carré was correct. Although they were unable to reproduce his results on the filtration of the "body fluids" they did show that under suitable conditions infective filtrates of spleen could be prepared. Moreover, bacteriological examinations of infected ferret and dog tissues indicated that a bacterium was not the cause.

The conclusions of Laidlaw and Dunkin were later challenged by Schlingman (1932, 1933) who could not reproduce their filtration results and who believed *B. bronchisepticus* to be the cause of natural cases of distemper. However, in spite of his findings Alexander (1933) stated that it must be accepted as proved that distemper is the result of infection with a specific filterable virus.

Of inestimable value were the observations of Dunkin and Laidlaw (1926) on the uniform susceptibility of ferrets which can easily be bred in large numbers. In a detailed account they described the symptoms in ferrets infected with dog distemper. The duration of the incubation period was found to be remarkably constant: after exposure it was ten days, while after artificial infection it was usually eight or nine days but occasionally as long as twelve. The eyes became watery and the eyelids swollen; a watery discharge from the nose was commonly seen. The skin of the chin, pads and abdomen was flushed. Twenty-four hours later the discharge from the eyes became purulent and soon the eyelids were glued together. Vesicles appeared on the chin; these eventually burst and became purulent. As the disease progressed listlessness and inappetance developed. Death usually resulted on the fifth day after the appearance of symptoms. Nervous symptoms were seen in about seven per cent of cases. The mortality rate was over 90 per cent.

During the course of their experiments, Laidlaw and Dunkin (1927) found that inactivated distemper virus was capable of conferring immunity to ferrets. Of the various methods tried, inactivation of spleen suspensions with formalin was considered most suitable. Work by subsequent investigators has shown that other issues of infected ferrets may be superior to spleen (Watson and Heath 1942); while Perdrau and Todd (1933) and Dempsey and Meyer (1934) found that photodynamic inactivation of the virus in methylene blue might be advantageous. Immunological differences were observed between various strains of distemper virus when ferrets immunised with inactivated preparations were challenged with strains from different sources; these differences disappeared when immunity was consolidated by the administration of active virus of the same strain (Dunkin and Laidlaw 1927). Most subsequent investigators agree that the immunity conferred by the injection of live virus into ferrets is effective against challenge by other strains. However, Slanetz and Smetana (1937) isolated a strain of virus from a natural outbreak of what appeared to be typical distemper in a colony of ferrets which had no apparent immunological relationship to canine distemper.

Unfortunately vaccines prepared from formalised infected ferret spleen gave most disappointing results when tested in dogs. Similarly prepared vaccine made from dog tissue was better but for the production of persistent immunity a subsequent administration of live virus was required. Dogs treated in this way were found to be immune to all virus strains tested and it appeared that the central nervous system shared in the general immunity (Laidlaw and Dunkin 1927). Extensive field trials with this method (Method No. I) were then conducted with the aid of private practitioners under carefully controlled conditions. Reports by Dunkin (1929) and Comerford (1929) indicated that there were apparently few severe reactions following immunisation and that a solid immunity resulted.

Once it had been established that a satisfactory method of immunisation was at hand, preparation and distribution on a commercial scale was undertaken at the Wellcome Research Laboratories by Major Dalling, who from time to time introduced certain modifications. He (Dalling 1929) found that 99 per cent of inoculated puppies showed no ill effects or only transient listlessness and diarrhoea; the other one per cent of puppies became seriously sick or died.

Dalling (1929) then reported on over 12,000 animals treated by "vaccine and virus". Where severe reactions had been noted he believed the majority were due to natural infection with distemper or other infections which simulated distemper. Abscess-formation was said to be rare but Bowden (1932) was rather

concerned about the number and severity of abscesses produced. A number of cases of "fits or hysteria" were reported following vaccination but it was believed these were not associated with the immunisation process. Of the 12,000 vaccinated dogs there were fifty cases of possible failures. Dunkin and Laidlaw (1931) concluded from the answers to a questionnaire that 98.6 per cent of inoculated hounds developed a solid and durable immunity.

During their early work Laidlaw and Dunkin (1927) were of the opinion that the immunity in distemper was cellular. Later they found that under appropriate conditions potent antiserum could be prepared (Laidlaw and Dunkin 1931) which conferred passive immunity. This finding led to the introduction of the serum-virus method of immunisation (Method No. 2). Preliminary work indicated that the immunity engendered was satisfactory and there was the added advantage that animals would be immune from the time of vaccination. The method has been used with success but Dalling (1932) found that a disturbing number of dogs failed to develop an immunity.

It was found that dogs or ferrets that received virulent virus in conjunction with either formalised vaccine or hyperimmune serum were infective for other susceptible animals.

With either method of vaccination the development of durable immunity was dependent upon the injection of virulent virus of known potency. In their trials Laidlaw and Dunkin used freshly prepared suspensions of virus under controlled conditions (O'Brien and Dalling 1932). When issued on a commercial scale the limitations of these preparations became obvious, and a number of failures resulted which O'Brien and Dalling (1932) concluded were due to administration of inactive virus. Fortunately a method of drying virus suspensions was evolved which largely overcame this difficulty. It was claimed that these preparations would maintain viability for some weeks at ordinary temperatures (Dalling 1932).

On the basis of the work of Laidlaw and Dunkin vaccines against distemper were prepared commercially in many parts of the world and were undoubtedly of considerable value. The generally excellent results obtained have led investigators to suspect that some cases of apparent failure in immunity might be due to disease entities which on clinical grounds were confused with distemper (Hinz 1930). The subsequent discovery of such diseases of the dog as infectious canine hepatitis and toxoplasmosis might thus be considered direct results of the work of Laidlaw and Dunkin.

Working on different lines Green (1939, 1945) noticed during a study on a number of strains of distemper virus obtained from natural cases in a variety of animals that there were marked differences in virulence for the various species of animals. He also observed that a strain of distemper virus was apparently so altered as a result of prolonged serial passage in a single species that its virulence was decreased for other susceptible but unrelated species. Thus he found that after 50 passages in the ferret a dog strain of distemper virus was so modified that no sign of disease could be detected when it was injected into foxes. He suggested that such a modified strain be termed "distemperoid" virus, and claimed (Green 1939) that more than 50,000 silver foxes had been successfully vaccinated with this strain of virus.

In a trial, dogs of various breeds ranging from three weeks to eight months or more of age were inoculated with distemperoid virus. No disturbing reactions were seen but many were definitely sick for a day or two. Close observation showed that most inoculated animals had a slight febrile reaction at about the

seventh day and again about the fourteenth: a solid and durable immunity appeared to result. However, when the virus was injected into puppies which were in poor condition some died and it was obvious that this vaccine could be used only for healthy animals (Green and Swale 1939). Further field trials by Green, Carlson and Swale (1940) confirmed these findings; in addition they claimed that the infection set up in dogs was not contagious. Furthermore they stated that dried preparations retained viability for at least three months but the conditions of storage were not stated.

Commercial issue of this vaccine was undertaken and because of the excellent keeping qualities claimed, was also used in many parts of the world.

In the United States of America reports on its use were favourable, provided healthy animals only were inoculated (Stader and Slaughenhaupt 1942, Jonas 1947, Glenny 1947, Edgett, Groth, La France, Schroeder and Zipp, 1948). However, reports from other countries varied considerably. Yutuo (1948) in the Philippines claimed good results but in England (Anon. 1947) it was considered that the available evidence did not favour the method and that, in any case, the virus was not always viable when it arrived. It was also stated that serial passage of many English strains through a large number of ferrets had not resulted in modification similar to that reported by Green. In the Union of South Africa the consensus was that the use of this vaccine was not entirely safe (Council S.A.V.M.A. 1948).

From Sweden it was reported that the immunising power of distemperoid virus was excellent but that there were many severe reactions (Bodin 1947, and Christiansen 1946). In France, Goret and Yvore (1947) stated that vaccine imported from America was used with success but when the strain was passed through locally reared ferrets for the preparation of vaccine there was apparently a reversion of virulence for the dog.

During the war years it was found in France that because of numerous technical difficulties the recognised methods of vaccine production could not be employed and a vaccine was devised in which distemper virus was absorbed on aluminium hydroxide and then dried by the method of Mudd and Flosdorff (Goret 1946). It was claimed that the virus was then avirulent but immunogenic. In practice, this vaccine proved unsatisfactory since about 15 per cent of failures ensued. However, it was pointed out that during this period an apparently virulent variant of distemper virus had become prevalent. When the serum-virus method was again resorted to, failures continued to appear. The impression gained was that whatever method of immunisation was employed very little immunity resulted against this virus variant. Similar disturbing observations were made at about the same time in other parts of the world (Jonas 1947; and Edgett *et al.* 1948 in the United States, MacIntyre 1949 in England, and Ullrich 1950 in Germany). Because the majority of these cases showed central nervous system involvement, a great deal of attention has been paid in recent years to the causes of canine encephalitis. (It should be noted that in veterinary literature the term "encephalitis" is usually used in its clinical sense).

That the central nervous system might be involved in cases of distemper has long been known. Blaine (1817) mentions "convulsive fits" and Ferry (1911) encountered an outbreak of what he considered distemper where the animals showed "simply a slight cough and convulsions". Laidlaw and Dunkin (1926) observed that nervous symptoms may be met with at two different stages of distemper in dogs; either early in the second febrile reaction or after three to four weeks of continuous illness. The early cases were accepted as manifestations of distemper

but they considered it an open question whether the late manifestations were really due to distemper or to some other cause (Laidlaw and Dunkin 1927). At the time Pugh (1927) had no doubt that these cases of "so-called nervous distemper" were indeed distemper but he stated that the causal organism was apparently neurotropic and that previous attacks of catarrhal distemper conferred no immunity to the encephalitic form. Later Perdrau and Pugh (1930) made the important observation that in some cases of canine encephalitis, demyelination could be demonstrated. They believed that demyelination was a sequel to infection with distemper virus rather than a direct result. On the other hand, Hurst, Cooke and Melvin (1943) believed that "nervous distemper" of dogs in which demyelination could be demonstrated was caused by the virus of distemper itself.

Pugh (1936, 1937) was apparently greatly concerned about the diagnosis of distemper and described what he thought was a distinct disease entity in dogs—"dry distemper" or "infectious brown mouth"—which occurred in animals of any age and had no apparent relationship to natural or artificial immunity to distemper. From one outbreak only was it found possible to isolate a virus in ferrets. Pugh (*loc. cit.*) suggested that this entity was the cause of nervous distemper. However, it would seem from his description that he might have been dealing with a variety of conditions including perhaps leptospirosis.

Cordy (1942) felt that the evidence obtained in his experiments justified the conclusion that there existed a highly neurotropic immunologically distinct strain of distemper virus.

So increasingly prominent did cases of canine encephalitis become that even such experienced practitioners as Kirk (1948) considered that a new disease of dogs might have made its appearance, and mooted a possible relationship between this disease and human poliomyelitis. From only one of his cases could a virus be transmitted to a ferret. The incubation period was 20 days.

MacIntyre, Trevan and Montgomerie (1948) working at the Wellcome Research Laboratories became greatly interested and described what they considered five distinct neuropathological entities among their cases of clinical encephalitis.

In their first group they included cases from which a virus infectious for the ferret could be demonstrated in brain emulsions. They suggested two sub-divisions in this group.

In the first were placed those cases in which the brain lesions were essentially nerve cell destruction. The responsible virus was believed to be the true distemper virus of Laidlaw and Dunkin. While in the second sub-division they included those in which the lesions observed were demyelination and inflammatory encephalitis. Once it had been pointed out by Margret Scheitlin that encephalitis was often accompanied by hyperkeratosis of the foot-pad ("hard-pad") this clinical manifestation was frequently encountered. Dogs of all ages were liable to develop this form of the disease. When artificial transmission with "hard-pad" disease virus was attempted only mild reactions resulted. Only slight evidence of cross immunity could be found between these and true distemper strains. Ferrets inoculated with emulsions from infected dogs usually reacted after an incubation period of about 23 days; occasionally this period was as short as ten days and at times as long as 32. Recoveries in ferrets were not uncommon. It was concluded that although the virus of "hard-pad" disease and that of classical distemper were not entirely unrelated, their differences were sufficient for them to be accepted as separate entities.

In the second group were included cases in which demyelination and inflammatory encephalitis were observed but from which no virus infectious for the ferret could be demonstrated. In one case transmission to a dog was accomplished.

The cases in the third group showed inflammatory encephalitis but transmission experiments were unsuccessful. All were in old dogs and in the opinion of Innes (1950) it would seem this condition corresponds with Cordy's "old dog encephalitis".

In the fourth group was placed toxoplasmosis. [Toxoplasmosis has been observed in the brains of dogs in many parts of the world and Fankhauser (1951) found that hardening of the foot-pads may accompany this form of encephalitis.]

Miscellaneous conditions such as nutritional deficiency and helminthiasis were included in the fifth group.

For the sake of completeness other virus diseases can be added to their list. Infectious canine hepatitis (Rubath) which has been shown to be identical with Green's fox encephalitis (Siedentropf and Carlson 1949) may be responsible for nervous symptoms (Frei 1950). Mention might also be made of the virus of lymphocytic choriomeningitis (Green 1942); whether or not this virus is capable of causing disease symptoms in dogs is unknown. Schlotthauer (1941) found indirect evidence that dogs might be infected naturally with the virus of equine encephalitis. It has also been reported that dogs may be infected with a virus of Aujeszky (Boecker 1939). In conclusion it should be stated that the differential diagnosis between several viral and protozoal diseases and leptospirosis may be most difficult.

Support for the views of MacIntyre *et al.* (1948) came from Innes (1950) and from Verlinde (1948) in Holland who had previously isolated a virus (virus B) which he claimed was immunologically distinct from classical distemper, and which he now thought was none other than the virus of "hard-pad" disease. Pugh (1948) believed that "hard-pad" disease had existed for some considerable time but, although he had seen hard-pads before he had not realised their significance; he then considered that the conditions which he had described earlier were manifestations of this disease. However, from the discussion which followed his paper it appeared that many practitioners had great difficulty in determining what should be considered "hard-pad" disease and what distemper.

On the other hand Weipers (1948) stated that he believed "hard-pad" disease and distemper were closely related. He did not consider hygiene and nutrition were in any way responsible for the development of central nervous symptoms but rather it was the difference in the way in which experimental and field dogs were kept. In this connection it might be added that Flashman (1950) in Australia believed fatigue was of major importance.

In an interesting series of observations on the variations in response to the injection of active virus following the use of formalised vaccine Bateman (1949) found that 79.5 per cent of a group of greyhound puppies could be classed as non-reactors. An epidemic occurred among these animals of what he considered ordinary distemper but which was diagnosed as "hard-pad" disease by the investigators at the Wellcome Research Laboratories. Bateman noticed that not one of the dogs which had been classed as a reactor to vaccination was affected while 95 per cent of the non-reactor group developed the disease. He then had grave doubts as to the existence of a "hard-pad" disease virus as a separate disease entity and believed that immunisation had been inefficient. Similar observations

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were made by Flashman (1950) who considered that a dog solidly immune to distemper would also be immune to "hard-pad" disease. Greer (1949) concluded that there was an inverse ratio between the degree of reaction following vaccination (Laidlaw and Dunkin Method No. I) and susceptibility to "hard-pad" disease.

In the United States of America it is generally held that there is very little, if any, difference between the two viruses (Koprowski, Jervis, James, Burkhart and Poppensiek 1950, and Cabasso 1952).

Steel and Whitem (1950) in Australia were careful to point out the markedly divergent forms distemper might assume and stated that although the literature indicated that three distinct viruses existed, namely distemper, hard-pad and contagious hepatitis virus they were unable to differentiate the diseases clinically. Furthermore, they stated that the criteria of MacIntyre *et al.* (*loc. cit.*) for the differentiation of true distemper and hard-pad virus were insufficient. One of the difficulties they stressed in any work of this nature was the uncertainty of the purity of any one strain of virus. They believed the virus of hard-pad was primarily viscerotropic. However, Mansi (1951) isolated in ferrets a virus from a case of canine encephalitis which appeared to be markedly neurotropic; no immunological differences between this virus and those of distemper, hard-pad or Green's distemperoid virus could be shown.

Goret (1950) stated that he had frequently observed differences in virulence among various strains of distemper virus, some of which could not readily be established in ferrets. His explanation for the numerous failures in immunity was that strains might become so modified in their pathogenic characteristics, that they caused infections with atypical symptoms and that some were able to overwhelm protection conferred by laboratory strains. Mansi (1948) had earlier expressed somewhat similar views.

Because of the numerous obvious difficulties attendant on the preparation of the vaccines which have been mentioned there has been a continuous search for means other than inoculation of carnivores for the propagation of distemper virus. Green (1945) stated that in his hands the cat, rabbit, mouse, rat and guinea-pig proved refractory. Mansi (1948) made the claim that he could infect the white mouse but it was pointed out by Montgomerie (1948) that the possibility that Mansi was dealing with the virus of L.C.M. had not been eliminated.

Goret, Martin, Joubert and Pouvoir (1950) and Martin, Goret, Joubert and Toucas (1950) have recently stated that strains of distemper can be adapted to growth in the rabbit. Dedié and Klapötke (1951) successfully cultivated distemper virus in tissue culture. It is, as yet, too early to know the significance of these claims.

The outstanding value of the developing chick embryo for the propagation of certain viruses is well known and has the added advantage that in many instances continued serial passage has resulted in marked and permanent decrease in virulence for the natural host [Goodpasture (1940)].

Mitscherlich (1938) found that the virus of distemper could survive six days on the chorio-allantoic membrane of eggs but sub-inoculations were unsuccessful. Plummer (1939) in two attempts maintained the virus for five and six passages but failed to carry the strains further.

At Onderstepoort it was considered that the possibility of cultivating distemper virus in eggs warranted re-examination. The successful serial propagation of Green's strain of distemper (Haig 1948, 1949), its attenuation and use for mass immunisation of dogs form the subject of this thesis.

EXPERIMENTAL OBSERVATIONS.

I. METHODS AND MATERIALS.

*A. Strains of Distemper Virus employed.**(1) Green's Distemperoid Strain.*

A vial labelled "Canine Distemper Vaccine, Ferret Origin, Green Method" was obtained which carried the serial number 57AX4. The contents of the ampoule were reconstituted in 2.0 ml. normal saline solution and 1.0 ml. of this was inoculated intraperitoneally into each of two ferrets. On the fourth day after injection one of the ferrets showed a marked febrile reaction and on the following morning this animal was killed and its spleen removed. This was suspended in about 40 ml. broth with the aid of a Waring blender. The supernatant fluid obtained by centrifugation at about 3,000 r.p.m. for 30 minutes in a Clay Adams angle-head centrifuge was used to seed the chorio-allantoic (C.A.) membranes of developing chick embryos. All precautions possible with the facilities available were taken to ensure bacteriological sterility of this and other similar material that was handled. Antibiotics were not added as they were not available at the time.

(a) Onderstepoort strain.—The variant obtained by continued serial passage of Green's distemperoid virus in eggs was termed the "Onderstepoort" strain.

(b) EF strain.—Material prepared from the spleen of a ferret that reacted to injection of C.A. membranes from the fourth egg transfer of Green's distemperoid virus was stored in sealed glass ampoules in a dry-ice cabinet and was named the EF strain. This strain has regularly proved fatal for ferrets in which characteristic symptoms developed after an incubation period of about eight days. It was used for homologous challenge of ferrets immunised with the Onderstepoort strain.

(2) Collie Strain.

A Collie pup that had been inoculated with Onderstepoort virus (egg-passage 135) about seven days previously was presented at the laboratory when it was critically sick; it showed marked nervous symptoms, greatly thickened foot-pads and slight discharge from the eyes.

Blood drawn from this dog into citrate was injected subcutaneously into two ferrets. After an incubation period of 13 days both animals showed characteristic symptoms of distemper. On the following day they were killed and suspensions of their spleens were stored in a dry-ice cabinet.

The dog's condition became progressively worse and about one week later it was killed and portions of its brain, spleen, liver and foot-pads were collected for biological and histological studies.

At autopsy the brain was very moist in appearance and histological examination revealed a patchy demyelination with some gliosis especially of the cerebellum and medulla. The foot-pads showed a marked hyperkeratosis. Slight degenerative changes and signs of venous stasis were observed in the liver.

Two ferrets inoculated with 1.0 ml. each of a 10 per cent brain suspension developed characteristic distemper symptoms; one after nine days and the other after eleven days. Both were killed when very sick and suspensions of their spleens were stored in a dry-ice cabinet.

Another two ferrets inoculated at the same time with 10 per cent suspension of the dog's spleen reacted with similar symptoms after an incubation period of 16 days. Suspensions of their spleen were stored at -76° C.

(3) *Ridge Strain.*

A Ridgeback pup of about five months of age showed marked nervous symptoms and slight catarrhal discharge from the eyes; its foot-pads appeared normal. This animal had received Onderstepoort virus (egg-passage 142) about two weeks previously.

Blood was collected in citrate from this dog and 1.0 ml. was injected into each of two ferrets. One developed characteristic distemper symptoms after 17 days but the other remained healthy until it was killed 30 days later.

After three days the dog was killed, *in extremis*. At autopsy the brain was moist in appearance. Histologically there was evidence of perivascular infiltration in the cerebrum and mid-brain; focal areas of glia proliferation in the cerebrum, hippocampus, midbrain and cerebellum; and focal demyelination especially in the mid-brain and cerebellum. No inclusion bodies were observed. The liver showed degenerative changes and signs of venous stasis.

Two ferrets received 1.0 ml. of a 10 per cent suspension of the dog's brain subcutaneously, while another 0.25 ml. intracerebrally. The former two showed characteristic distemper symptoms ten and eleven days later; they were killed on the 14th day and their spleens were stored in a dry-ice cabinet. The third ferret showed similar symptoms on the 14th day and died seven days later.

(4) *Ruby Strain.*

A Dobermann pup approximately five months of age was very sick and showed nervous symptoms; its foot-pads appeared normal. Nine days previously this animal had received Onderstepoort virus (egg-passage 148). It was killed and at autopsy the brain was moist.

Histological examination revealed an acute encephalomyelitis with perivascular infiltration, focal demyelination and glia proliferation of the brain-stem and cerebellum. A few eosinophilic intra-nuclear inclusions were encountered.

Four ferrets were inoculated with freshly prepared 10 per cent brain emulsion in broth. Two which received 0.25 ml. of ten per cent brain emulsion intracerebrally failed to react within 30 days. However, one of the two treated with 1.0 ml. subcutaneously developed typical clinical symptoms of distemper after an incubation period of 13 days. Spleen material from this animal was subinoculated by the subcutaneous route into a ferret which showed characteristic symptoms nine days later; this ferret was killed and spleen suspension was stored in a dry-ice cabinet.

(5) *English Strain.*

A strain of distemper virus was obtained from England through the courtesy of Dr. I. Galloway of Pirbright. It was injected into ferrets and their spleens were stored in a dry-ice cabinet.

The above-mentioned virus strains were all found to be potent for periods of at least six months when stored at -76° C.

B. *Source of Eggs.*

The eggs used were obtained from a flock of B.W.D.-free* White Leghorns kept at Onderstepoort. After a preliminary incubation of eight days in a Jamesway forced draft incubator at 37.5° C. those containing vigorous embryos were selected. Unless otherwise stated egg inoculations were made onto the chorio-allantoic membrane by the method of Alexander (1938) and re-incubation was at 35° C. With slight modifications the methods in general use at the laboratory which have been described by Alexander (1947) for the handling of eggs and virus suspensions were employed. Injections by the yolk-sac, allantoic or amniotic route were made by standard methods.

C. *Source of Ferrets.*

Ferrets (*Mustela eversmanni furo* L.) came from the colony at Onderstepoort which was started in 1937 with animals introduced from England. The colony is situated about 500 yards from the laboratory. Reasonable but not meticulous precautions are taken to prevent accidental infection of the stock and at no time has a disease resembling distemper been observed. They are fed an adequate ration of meat, milk and maize porridge.

When drafted into experiments they were from four to twelve months old; no selection on sex or colour was made. In most of this work only one experiment was conducted at a time. When an experiment was completed the survivors were killed. After disinfection the cages and rooms were left vacant for at least seven days. On those infrequent occasions when more than one strain was being investigated simultaneously the ferrets were housed in separate buildings under the care of separate attendants.

To differentiate the various animals in an experiment they were marked with dyes and in addition when large numbers were used, they were ear-tagged with numbered chicken wing-bands. These were found very satisfactory and only occasionally did they become dislodged.

It has been stated (Laidlaw and Dunkin, 1926) that observations on thermal reactions in ferrets are unreliable since struggling usually induces a temperature elevation of about 2° F. Ferrets bred at Onderstepoort are reasonably docile. Provided their temperatures were taken by one person who grasped the tail of the animal with one hand and allowed the ferret to grip the mesh on the top of the cage while the thermometer was inserted, it is believed that reasonably accurate readings were obtained.

Owing to the necessity for conserving the supply of available ferrets, usually a single animal only was employed for each test. However, when batches of vaccine were titrated two were inoculated with each dilution. The dose of the inoculum was 1.0 ml. administered either subcutaneously or intraperitoneally.

D. *Source of Dogs.*

Considerable difficulty was experienced in obtaining susceptible dogs for experimental purposes, so that the amount of work which could be carried out on these animals at the laboratory was unfortunately very limited. However, a few litters of puppies with no history of previous illness were obtained and isolated as soon as they could be weaned. No definite information concerning the susceptibility of the puppies nor that of their dams could be obtained. They were fed an adequate ration.

* Bacillary white diarrhoea.

Most of the preliminary work on the safety and efficacy of the Onderstepoort virus for dogs was done in conjunction with private practitioners in Pretoria. Once it had been shown that this strain could safely be used further trials were conducted at the South African Police Dog Depot at Quaggaspoort, where distemper had been rife for many years.

E. Freeze-Drying Process.

Freshly harvested C.A. membranes were mixed with an equal volume of diluent and were macerated in a Waring blender. In early work the diluent used was distilled water. Later this was replaced by a mixture composed of 2 per cent peptone, 10 per cent lactose in M/25 phosphate buffer at pH 7.4. The supernatant fluid obtained by light centrifugation was then freeze-dried in 0.5 ml. amounts in an Edwards model 3PS centrifugal freeze-drier. The interval between harvesting and the commencement of the drying-cycle was approximately one hour.

At the beginning of this work the ampoules were sealed under air but it was found that the virus deteriorated rapidly at ambient temperatures. Therefore the ampoules were subsequently sealed under dry nitrogen and stored at -15° C. The viability of the virus was then found to be more stable (refer to experiments on the keeping quality of the virus).

II. PROPAGATION OF GREEN'S DISTEMPEROID VIRUS (ONDERSTEPOORT VIRUS) IN CHICK EMBRYOS.

Eighteen eggs containing eight-day old embryos were seeded on the C.A. membrane with 0.2 ml. each of supernatant fluid obtained by centrifugation of ferret spleen emulsion infected with Green's distemperoid virus. The eggs were then re-incubated at 35° C.

On the following day it was found that 15 of the embryos had died and examination of smears revealed the presence of numerous bacteria. The remaining three embryos were alive on the fourth day after inoculation when they were opened and the C.A. membranes examined. The only apparent deviation from normal was slight oedema of the C.A. membrane at the site of inoculation. The membranes were harvested and after emulsification in the type of mincer tube described by Alexander (1947) a small amount of broth was added. After light centrifugation the supernatant fluid was transferred to the C.A. membranes of other eggs. None of these eggs died. When their membranes were harvested four days later they were used to inoculate two separate groups of eggs. In this way serial transfers were made at four- or five-day intervals. At each passage sterility tests were made in ordinary broth and smears were made from many of the eggs in which the embryos died. Only occasionally were bacteria found; that line of passage was abandoned and the duplicate group was used to start two separate lines. In this way a total of over 200 serial passages was made. At various levels of passage undiluted macerated membranes were stored in sealed glass ampoules in a dry-ice cabinet for future reference.

All subinoculations were made within two hours of harvesting infected membranes. Until about the eighth transfer no marked macroscopic changes were noticed in the C.A. membranes but from then on it was found that many were considerably thickened, and showed numerous small light-grey areas which tended to coalesce. With continued passage the appearance of these lesions became more uniform and regularly involved not only the site of inoculation but also a portion of the membrane immediately below the true air-space.

In early passages only occasional embryos died during the period of observation but by the 130th transfer a large number died after three or four days of incubation when the inoculum was used in concentrations not greater than 1/10 to 1/40. The cause of these deaths was not determined.

A. Influence of Temperature of Incubation on Multiplication of Virus.

Alexander (1947) has stressed the importance of the temperature of incubation on the multiplication of virus in developing embryos and showed that in the case of bluetongue virus the optimum temperature was considerably lower than those routinely used by most workers.

When the Onderstepoort strain of distemper virus had been taken 32 serial passages at 35° C. a series of experiments was made to determine at what temperature of incubation multiplication in the C.A. membrane was most satisfactory. Three separate lines of serial passage were initiated in which the temperature of incubation of infected eggs was 32° C., 35° C. and 37° C. In each case transfers were made at four- or five-day intervals with 1 in 10 dilutions of infected membranes. Twelve eggs were used for each transfer.

At different levels of egg-passages the viral activity of the membranes was measured by titration in ferrets. These titrations made with serial decimal dilutions of infected membranes are listed in Table 1.

TABLE 1.
Influence of Temperature of Incubation on Multiplication of Distemper Virus in Embryonated Eggs.

Temperature of Incubation.	Number of Passages.*	Time of Incubation.	Infectivity.	
32° C.	3	Days. 5	10 ⁻³	
	13	4	10 ⁻³	
	21	4	10 ⁻³	
	27	4	10 ⁻⁴	
	48	}	2	10 ⁻²
			4	10 ⁻²
35° C.	9	2	10 ⁻³⁺	
			14	10 ⁻⁵⁺
	25	4	10 ⁻³	
	22	2	10 ⁻⁴	
	51	4	10 ⁻⁴⁺	
			10 ⁻³	
	52	}	6	10 ⁻³
			8	10 ⁻⁴
37° C.	3	5	10 ⁻⁵	
	12	4	10 ⁻³	
	22	}	3	10 ⁻⁵⁺
			6	10 ⁻⁵
	45	}	2	10 ⁻⁴⁺
			4	10 ⁻⁴⁺
	7	}	9	10 ⁻⁵
			4	10 ⁻⁴
54	4	10 ⁻⁴		

NOTE.—10⁻³⁺ = end point not determined.

* Strain had previously been passaged 32 serial passages at 35° C.

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(1) *Propagation at 32° C.*

In the series propagated at 32° C. a gradual decrease in the macroscopic changes in infected C.A. membranes become apparent, and by the 30th passage little indication of cellular proliferation could be seen; the membranes were soft, translucent and greatly distended with clear fluid.

(2) *Propagation at 35° C.*

In the series propagated at 35° C. the C.A. membranes were uniformly thickened. Numerous coalescent grey areas were seen at the site of inoculation and to some extent in the part of the membrane under the true air-space.

(3) *Propagation at 37° C.*

It was noticed that the C.A. membranes in the series propagated at 37° C. did not show such uniformity in their appearance as those incubated at 35° C.; in most of the membranes the lesions were considerably more marked but in a few very little evidence of change could be seen. Some difficulty was experienced in maintaining the series passaged at this temperature as many early deaths were encountered among the embryos.

Conclusions.

From these experiments it was apparent that over the range of incubation temperatures investigated 37° C. was most favourable for the multiplication of the Onderstepoort virus in the C.A. membranes of eight-day old embryos. However, considerable difficulty was experienced in maintaining the strain in serial passage at this temperature of incubation as there were many early deaths among the embryos. It should be emphasised that transfers were made with freshly harvested material. Eventually those lines passaged at 32° C. and 37° C. were abandoned and serial passage of the strain was continued at an incubation temperature of 35° C. only.

B. Propagation of Virus in Tissues other than the Chorio-Allantoic Membrane.

It is known that different viruses multiply more readily in certain tissues of chick embryos, and that the rate of attenuation which might occur with serial passage may vary when propagation is encouraged in different tissues. Thus Jenkins and Shope (1946) found that only after rinderpest virus was adapted to propagation in the C.A. membrane could the strain be passaged by the yolksac route; attenuation then proceeded more rapidly.

A preliminary experiment made when the Onderstepoort strain was in its 16th transfer showed that in eggs inoculated by the C.A. membrane route, virus was present in considerable quantities in tissues other than the C.A. membrane. Titrations made by the inoculation of ferrets with serial ten-fold dilutions of embryos, extra-embryonic fluid and C.A. membranes harvested after four days' incubation gave end points of 10^{-3} in each instance.

Once it had been determined that the virus could be cultivated in the C.A. membranes of developing embryos a series of experiments was undertaken in an attempt to increase the yield of virus and hasten attenuation. Infected membranes from the routine passages were used to initiate the experiments described below; incubation was at 35° C.

(1) Injection by the Yolk-Sac Route.

Six eight-day old embryos were inoculated by the yolk-sac route with a 1:10 suspension of C.A. membranes from the routine 25th passage. Five days later all were alive when the embryos and portions of the yolk-sacs were removed and used to inoculate the yolk-sacs of other eggs. After five serial passages had been made in this way 1.0 ml. of mixed embryo and yolk-sac emulsion was administered intraperitoneally to a ferret. It showed no apparent reaction and was fully susceptible to challenge with undiluted C.A. membrane material from the current routine passage.

This experiment was repeated with negative results.

(2) Injection by the Allantoic Route.

A 1:10 suspension of infected C.A. membranes from the 40th routine passage was inoculated into the allantoic cavities of twelve eight-day old embryos; at five-day intervals subinoculations of allantoic fluid were made. One of two ferrets which received 1.0 ml. allantoic fluid from the fifth transfer showed characteristic symptoms of distemper. The other showed no apparent reaction. A third ferret inoculated with material from the 15th allantoic transfer failed to react and the series was abandoned.

(3) Injection by the Amniotic Route.

In a similar series of transfers made by the amniotic route with inoculum from the 90th C.A. membrane passage, no virus could be demonstrated by subinoculation of a ferret with amniotic fluid from the fifth passage.

Conclusions.

Under the conditions of these experiments it was not found possible to propagate the strain serially other than in the C.A. membrane.

C. Effect of the Concentration of the Inoculum on Multiplication.

It has been stated that the multiplication of influenza virus in embryonated eggs may occur to a higher degree when dilute inoculum is used than when the inoculum is injected in concentrated form (Horsfall 1948). On numerous occasions when titrations of Onderstepoort virus were made in eggs it was found that extensive lesions could be expected only in those C.A. membranes that received material rich in virus. Examples are to be found in Table 8. Early deaths, however, were then troublesome and it was found that some judgment had to be used in determining the most satisfactory concentration of inoculum. When extensive lesions were present in the membranes used for the preparation of the suspensions it was found advisable to inject a dilution of 1:20 or 1:40.

III. ANIMAL INOCULATION OF THE ONDERSTEEPOORT VIRUS.

(1) *Observations on Ferrets.*

Freshly prepared C.A. membrane suspensions from the third and from a number of subsequent serial egg-passages were injected into ferrets, firstly to demonstrate the presence of virus and secondly to follow the progress of any attenuation that might result. The results of these observations are listed in Table 2.

TABLE 2.

Reactions observed in Ferrets Inoculated with C.A. Membrane Emulsions from Different Serial Egg-passages.

Egg Passage.	Dilution.	Nature of Reaction in Ferrets.	Result.
3.....	Undiluted.....	C.S. (7).....	K.E. (10).
4.....	Undiluted.....	C.S. (7).....	K. (8).
5.....	Undiluted.....	Peracute.....	D. (10).
9.....	Undiluted.....	C.S. (7).....	K. (10).
	10 ⁻¹	C.S. (9).....	K.E. (12).
	10 ⁻²	C.S. (7).....	K.E. (17).
14.....	Undiluted.....	C.S. (6).....	D. (8).
	10 ⁻²	Peracute.....	D. (5).
	10 ⁻⁴	C.S. (7).....	K. (12).
	10 ⁻⁵	Peracute.....	D. (6).
	10 ⁻⁶	N.R.....	—
25.....	10 ⁻²	C.S. (4).....	K. (6).
	10 ⁻³	C.S. (5).....	K. (6).
	10 ⁻⁴	N.R.....	—
	10 ⁻⁵	N.R.....	—
	10 ⁻⁵	C.S. (6).....	K.E. (18).
54.....	10 ⁻³	C.S. (8).....	K. (10).
	10 ⁻⁴	C.S. (8).....	D. (21).
	10 ⁻⁴	C.S. (14).....	Recovered.
	10 ⁻⁵	C.S. (8).....	Recovered.
92.....	Undiluted.....	C.S. (7).....	K.E. (18).
97.....	Undiluted.....	No apparent reaction.....	Immune.
110.....	Undiluted.....	No apparent reaction.....	Immune.
	10 ⁻¹	No apparent reaction.....	Immune.
	10 ⁻²	No apparent reaction.....	Immune.
	10 ⁻³	No apparent reaction.....	Immune.
	10 ⁻⁵	No apparent reaction.....	Not immune.
158.....	10 ⁻¹	No apparent reaction.....	Not immune.
	10 ⁻²	No apparent reaction.....	Not immune.
	10 ⁻³	No apparent reaction.....	Not immune.
	10 ⁻⁴	No apparent reaction.....	Not immune.
	10 ⁻⁵	No apparent reaction.....	Not immune.

NOTE.—D. (5) = Died on fifth day after injection.
 C.S. (7) = Characteristic symptoms on seventh day.
 K.E. (10) = Killed in extremis on tenth day.
 N.R. = No apparent reaction.

From Table 2 it can be seen that the incubation period varied from four to fourteen days when the appearance of visible symptoms was regarded as the criterion of infection.

Examples of the peracute form of the disease in ferrets were encountered among those animals inoculated with material from the first 14 egg passages of the strain. Until the 54th transfer typical reactions were the rule, but from then onward it was noticed that the reaction produced was rather less severe and more

prolonged and although most ferrets had to be destroyed when very sick, a few did recover. When material from the 110th or higher levels of passage were inoculated into ferrets it was found that only an occasional animal died. The others showed no visible signs of illness and were immune to challenge.

Ferrets infected with third egg-passage material were found to be contagious by co-habitation with controls. This property of the virus disappeared with continued passage possibly before the fourteenth generation and certainly by the 25th.

(2) *Observations on Dogs.*

When it became apparent that it was possible to propagate Green's distemperoid virus in chick embryos it was considered that since this strain was in commercial use as an immunising agent for dogs, egg-propagated virus could safely be used for this purpose. At the time no facilities whatever were available at the laboratory for either obtaining or breeding known distemper-free dogs. It was, therefore, necessary to obtain the assistance of colleagues to infect what dogs they were able to obtain for this purpose and to keep what records they could.

The supernatant fluid obtained from freshly harvested C.A. membranes was used either undiluted or diluted 1 in 10 in saline. Each dog received 1.0 ml. subcutaneously on the day of preparation. The results of these experiments are summarised in Table 3.

TABLE 3.

Reactions Observed in Dogs Inoculated with Infected C.A. Membranes from Different Levels of Egg-passage.

Egg Passage.	Breed.	Age.	Reaction.
6.....	Dachshund.....	2 months.....	No reaction.
	Dachshund.....	2 months.....	No reaction.
	Dachshund.....	2 months.....	No reaction.
	Dachshund.....	2 months.....	No reaction.
11.....	Ridgeback.....	6 months.....	No reaction.
	Mastiff.....	5 months.....	No reaction.
15.....	Irish Setter.....	5 months.....	Severe reaction complicated with biliary fever; recovered.
	Pointer.....	2 months.....	Severe reaction; died.
16.....	Mastiff.....	12 months.....	Severe reaction; recovered.
	Fox-terrier.....	3 years.....	Mild reaction.
	Dobermann.....	10 months.....	No reaction.
	Mastiff.....	8 months.....	No reaction.

Sixth Passage.—Four Dachshund cross-bred pups about two months old, which had no history of previous illness, received material from the sixth egg-passage.

They showed no apparent reaction.

Eleventh Passage.—A six-month old Ridgeback and a five-month old Mastiff cross-bred pup were inoculated with material from the eleventh passage.

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No apparent reaction was observed.

Fifteenth Passage.—Emulsion from the fifteenth passage was injected into an Irish Setter five months old, and a Pointer, two months old. The former showed a temperature elevation of 104° F. on the fourth day after injection; on the sixth day its temperature was 105·8° F. The temperature gradually returned to normal but from the eighth day after injection eye lesions developed and on the eleventh day cutaneous pustules appeared on the groins. The animal recovered in spite of a concurrent attack of biliary fever diagnosed and treated on the 15th day. The other dog had a temperature of 103° F. on the sixth day following injection; the temperature remained more or less at this level until the twelfth day when the animal collapsed and died. Cutaneous pustules were first seen on the seventh day and eye lesions on the ninth. Shortly before death marked nervous symptoms were evident.

Sixteenth Passage.—A one year-old Mastiff, a three-year old Fox-terrier, a ten-month old Dobermann and an eight-month old Mastiff were inoculated with material from the 16th egg transfer.

The Fox-terrier was seen to be slightly sick from the seventh to the ninth day, when it recovered. The year-old Mastiff, however, was very sick from the fifth to the ninth day; it would not eat and vomited frequently. Recovery was rapid. The other two dogs showed no apparent reaction.

These results showed that the strain at the passage levels tested could not be used with any degree of safety for the immunisation of dogs, and observations on reactions in this animal were discontinued until the strain was in its 130th transfer. At this level, ferret inoculations indicated that a marked decrease in virulence for the ferret had occurred with continued passage in eggs. Preliminary experiments indicated that a similar decrease in virulence for the dog had indeed occurred. Issues of fluid preparations of virus made from current serial passages from the 130th to 190th were issued on a gradually increasing scale to local private practitioners, and to the South African Police Dog Depot at Quaggaspoort for use on dogs older than ten weeks. Approximately 1,000 doses were issued.

When it was apparent that the strain could safely be used to immunise dogs in the field and when it had been determined that reasonably stable freeze-dried preparations could be made, vaccine was issued on a commercial scale in September 1950. During the last three years some 40,000 doses of vaccine were used.

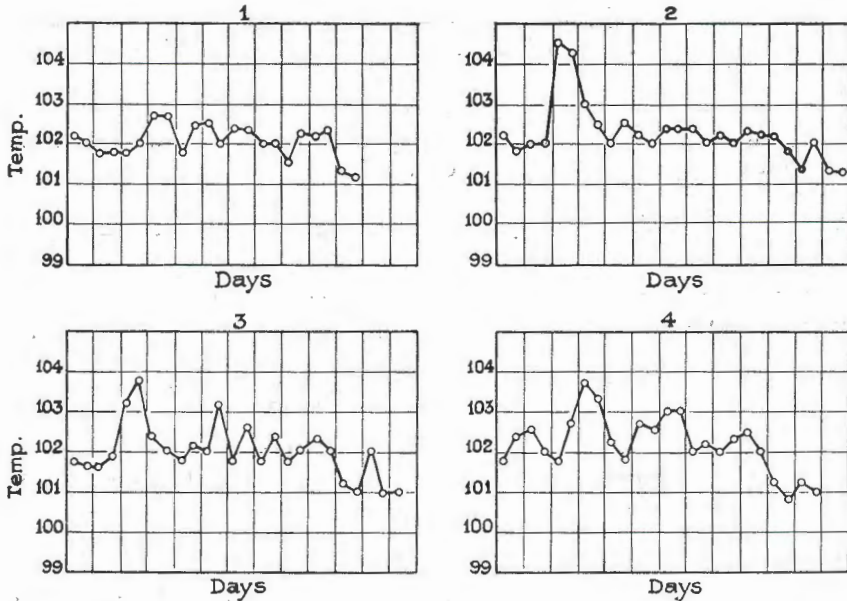
In the majority of instances no visible reactions to inoculating of attenuated virus were noticed, but it was found that there was a slight febrile reaction from about the fourth to the eighth day after injection. In the subjoined Figure 1 examples of temperature curves are shown.

Occasionally reports of dogs being listless during or after the febrile reaction were also received.

During early issues of vaccine for field use, when considerable attention was paid to reactions by private practitioners, a number of cases (about ten) of encephalitis, usually one to 14 days after injection were reported and several of

these cases were brought to the laboratory. Three cases were examined in detail: the isolated viruses were referred to above as the Collie, Ridge and Ruby strains.

FIG. I.—*Febrile Reactions in Puppies Inoculated with Onderstepoort Virus, Egg-passage 150.*



In spite of extensive trials it was in no case found possible to propagate these viruses in eggs. As a result of histological examination of material from these cases and studies made on the viruses isolated, it was believed that the Onderstepoort virus could not be held responsible. Rather it seemed that the animals had been inoculated during the incubation period of natural infection.

In spite of numerous requests to private practitioners for reports on the safety and efficacy of this vaccine in the field, none other than verbal statements were obtained. However, from the information received, it appeared that the vaccine could be used with safety on dogs older than ten weeks. The immunity conferred was apparently durable and solid. Isolated reports of failures in immunity were received. These occurred in dogs varying from eighteen months to two years of age; most showed encephalitis of undetermined aetiology.

At the South African Police Dog Depot, Quaggaspoort, distemper had for many years been rife and had greatly interfered with breeding and training. During the last three years it has become customary to vaccinate all animals at the age of ten weeks with Onderstepoort virus. No case of distemper has been diagnosed at this station during that time.

IV. IDENTIFICATION OF THE VIRUS PROPAGATED IN EMBRYONATED EGGS.

The result obtained from ferret and dog inoculations left little doubt that it was the virus of distemper which had been adapted to growth in eggs. Nevertheless, to verify this assumption ampoules of canine anti-distemper serum

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supplied by Burroughs Wellcome and Co. and labelled Q1946N with an expiry date of 1 May, 1949, were obtained. The serum contained 0.35 per cent cresol as preservative. A preliminary neutralisation test in which serum and virus were mixed *in vitro* gave inconclusive results and it was assumed that the virus had been inactivated by the preservative contained in the serum. In another experiment injections of 5.0 ml. of serum and 1.0 ml. of undiluted C.A. membrane emulsion (57th egg-passage) were made subcutaneously at two different sites of a ferret; a second ferret received the same amount of membrane emulsion only.

The first ferret showed no apparent reaction and was subsequently immune to challenge with homologous virus. The other ferret reacted with characteristic symptoms on the eighth day and died on the 21st.

Intranasal instillation of supernatant fluid from the 16th serial egg-passage of the strain into mice failed to produce lesions by the time they were killed and examined twelve days later. Subinoculations of the lung suspensions into other mice were negative.

A study was made of the possible adaptation of Hirst's chicken red cell agglutination phenomenon to the virus contained in C.A. membrane suspensions or in allantoic fluid. The results were negative. However, a report from Moscow (Vladimirov, 1949) stated that such agglutination is possible under certain conditions of experimentation.

Onderstepoort virus was sent to Doctors Cabasso and Cox of Lederle Laboratories, Pearl River, New York, who compared the behaviour of this strain with that of the Lederle strain which they had propagated in eggs (Cabasso and Cox, 1949).

Conclusion.—It was concluded that the virus which had been adapted to propagation in developing eggs was that of distemper.

V. IMMUNITY TESTS IN FERRETS AND DOGS VACCINATED WITH ONDERSTEPSOORT VIRUS.

Because a great deal of controversy has raged concerning the relationship between true distemper virus and virus obtained from cases of "hard-pad" disease or canine encephalitis, the immunity conferred by the Onderstepoort strain of distemper to challenge with a variety of strains of virus was examined.

(1) *Tests in Ferrets.*

Three ferrets were inoculated subcutaneously with 1.0 ml. of freshly harvested C.A. membrane suspension infected with the Onderstepoort strain (190th egg-passage). Four weeks later two of these ferrets and two controls received 1.0 ml.

of spleen suspension infected with the Collie strain (isolated from the blood) which had been stored in a dry-ice cabinet. The third vaccinated ferret and two other controls were inoculated with Ridge strain (isolated from the brain). The results of this experiment are shown in Table 4.

TABLE 4.
Immunity Tests in Ferrets Vaccinated with Onderstepoort Virus.

Inoculum.	Immunity Test. with Strain.	Result.
Onderstepoort virus.....	Collie.....	I.
Onderstepoort virus.....	Collie.....	I.
Control.....	Collie.....	NI.
Control.....	Collie.....	NI.
Onderstepoort virus.....	Ridge.....	I.
Control.....	Ridge.....	NI.
Control.....	Ridge.....	NI.

I. — Showed no apparent reaction.
NI. = Reacted and died.

In a similar experiment 0.5 ml. of freshly harvested membrane suspension (egg-passage 196) was injected into each of ten ferrets. One died about three weeks later of an undiagnosed disease. Twenty-seven days later the remaining animals, together with eight controls were tested for immunity with four strains. The results of this experiment are shown in Table 5.

TABLE 5.
Immunity Tests in Ferrets Vaccinated with Onderstepoort Virus.

Inoculum.	Immunity Test with Strain.	Result.
Onderstepoort virus.....	Collie.....	I.
Onderstepoort virus.....	Collie.....	I.
Onderstepoort virus.....	Collie.....	I.
Onderstepoort virus.....	Collie.....	I.
Onderstepoort virus.....	Collie.....	I.
Control.....	Collie.....	NI.
Control.....	Collie.....	NI.
Onderstepoort virus.....	Ridge.....	I.
Onderstepoort virus.....	Ridge.....	I.
Control.....	Ridge.....	NI.
Control.....	Ridge.....	NI.
Onderstepoort virus.....	Ruby.....	I.
Onderstepoort virus.....	Ruby.....	I.
Control.....	Ruby.....	NI.
Control.....	Ruby.....	NI.
Onderstepoort virus.....	E.F.....	NI.*
Control.....	E.F.....	NI.
Control.....	E.F.....	NI.

Note.—* It is believed that this ferret was missed at the time of inoculation with Onderstepoort virus; it will be seen from Tables 6 and 7 that complete cross immunity exists between the Onderstepoort and E. F. virus strains.

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From Tables 4 and 5 it is seen that complete immunity was conferred by Onderstepoort virus to challenge with a variety of strains obtained from cases of canine encephalitis.

(2) Tests in Dogs.

Five nondescript pups of about three months of age which were not known to have had any illness were inoculated with 1.0 ml. of undiluted C.A. membranes from the 133rd egg-passage. They showed no apparent reaction.

Five weeks later these animals, together with an uninoculated litter mate, received 1.0 ml. of 10 per cent suspension in broth of freshly harvested ferret spleen infected with the English strain of distemper.

The five vaccinated animals showed no apparent reaction while the control showed a slight temperature elevation on the third day and again from the fifth to the thirteenth days. The animal was listless for two days but showed no other signs of illness. (See Fig. 2.)

In another experiment three Great Dane pups, about four months old, were inoculated with dried vaccine (egg-passage 190). Four weeks later these animals and two controls of unknown breed but of approximately the same age received subcutaneously 1.0 ml. of a freshly prepared 10 per cent spleen suspension infected with Collie (brain) in its second ferret passage.

The Great Danes showed no apparent reaction. One of the controls was seen to be sick on the eighth day; its condition improved but on the 16th day it was again listless and its condition then gradually became worse until it died four days later. Mild eye lesions and diarrhoea were observed, and shortly before death the right front leg was very painful.

Two ferrets were subinoculated with blood drawn from this dog on the day before it died. Both reacted with characteristic symptoms of distemper after an incubation period of 16 and 18 days; one died two days later, the other was killed when very sick.

The second control dog was sick on the 14th day after injection, and was listless for about another eight days, when it recovered. Blood was drawn on the 21st day after injection and inoculated into two ferrets. One showed a characteristic reaction eleven days later but the other ferret was normal when killed after 21 days.

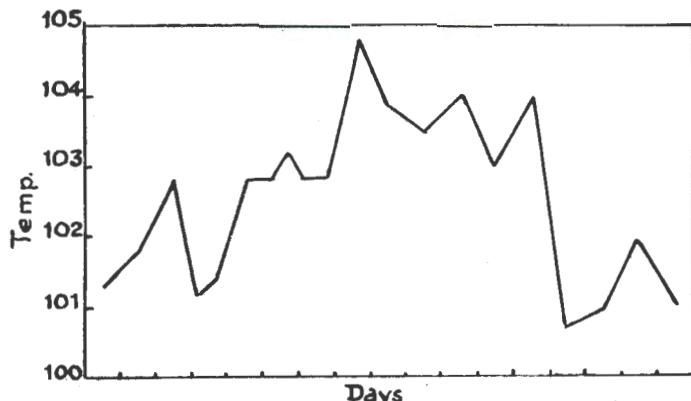


Fig. 2.—Febrile reaction in control dog inoculated with English strain of distemper.

Conclusions.

The immunity conferred to ferrets and dogs by the Onderstepoort virus was solid when challenged by a variety of strains, including some isolated from cases of canine encephalitis.

VI. KEEPING QUALITIES OF THE ONDERSTEPSPOORT VIRUS.

(1) *Viability in the Fluid State.*

Four sealed glass ampoules containing 2.0 ml. each of undiluted supernatant fluid, obtained from freshly harvested and macerated C.A. membranes (egg-passage 143) were placed in an incubator at 32° C. Titrations in ferrets of viral activity were made of the suspension before incubation and after 24 and 48 hours incubation. Four weeks later the immunity of these ferrets was challenged with homologous E.F. strain.

The results of this experiment are shown in Table 6.

TABLE 6.
Keeping Quality of Suspension of Onderstepoort Virus.

Time stored at 32° C.	Concentration of Suspension Injected into Ferrets.							
	10 ⁻¹		10 ⁻²		10 ⁻³		10 ⁻⁴	
	A.	B.	A.	B.	A.	B.	A.	B.
0 hours.....	—	—	I	I	I	I	I	I
24 hours.....	I	I	I	I	NI	NI	—	—
48 hours.....	NI	NI	—	—	—	—	—	—

NOTE.— I = Immune to homologous challenge.
NI = Not immune.

From Table 6 it is seen that the freshly prepared suspension was active in a dilution of at least 10⁻⁴. After 24 hours activity could be demonstrated in dilution of 10⁻² but after 48 hours 10⁻¹ dilution failed to confer immunity. From these results it must be concluded that the potency of the Onderstepoort virus is unstable when held at 32° C. for 24 to 48 hours.

(2) *Viability in the Freeze-Dried State.*

During the course of the preparation of a large number of freeze-dried batches of vaccine minor modifications were introduced from time to time in an attempt to improve the potency and keeping quality of the virus. A number of experiments were made to determine the keeping qualities of the vaccine maintained at different temperatures.

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Serial ten-fold dilutions in broth of reconstituted dried preparations (egg-passage 193) were made and were injected into ferrets. Two animals were injected with each dilution. Four weeks later these animals, together with an adequate number of controls, were challenged with the E.F. strain.

The results of some of these experiments are set out in Table 7.

TABLE 7.
Keeping Qualities of Freeze-dried Onderstepoort Virus Preparations.

Batch No.	Vehicle.	Sealed Under.	Stored At.	Time in Days.	Dilution Injected into Ferrets.							
					10 ⁻¹		10 ⁻²		10 ⁻³		10 ⁻⁴	
					A.	B.	A.	B.	A.	B.	A.	B.
2	Aq. dist....	Air.....	-15°C. 37°C.	81	I	I	I	I	I	I	—	—
				7	NI	NI	NI	NI	NI	NI	—	—
23	Aq. dist....	Nitrogen...	-15°C. 37°C.	8	—	—	I	I	I	I	—	—
				7	I	NI	I	NI	I	NI	—	—
37	Aq. dist....	Nitrogen...	-15°C. 37°C.	16	I	I	I	I	I	I	NI	NI
				7	I	I	I	NI	NI	NI	—	—
93	Lactosepeptone buffer	Nitrogen...	-15°C. 37°C.	17	—	—	—	I	I	I	I	I
				7	—	—	I	I	I	I	I	I

NOTE.— I = Immune to homologous challenge.
NI = Not immune.

From Table 7 it can be seen that when ampoules were sealed under air complete inactivation of the virus resulted after exposure at 37° C. for seven days. In addition it was observed that a change in colour had occurred in these preparations. When the tubes were sealed under nitrogen the rate of inactivation was considerably retarded. No explanation can be offered for the irregular results obtained when Batch 23 was held seven days at 37° C.

The substitution of lactose and peptone in buffered distilled water for plain distilled water for vaccine preparation improved the keeping qualities of the virus. This was observed both during the drying cycle as well as when such dried preparations were held at 37° C.

VII. TITRATION OF ONDERSTEPSOORT VIRUS IN EMBRYONATED EGGS.

The limited supply of ferrets and accommodation did not permit the titration of each batch of vaccine. Attempts were therefore made to determine the potency of virus by titration in eggs.

Serial ten-fold dilutions of a mixture of infected C.A. membranes in buffered lactose-peptone solution, were made before and after freeze-drying. Each dilution was injected into six eggs. The membranes were examined for lesions, seven days later. The dried material was again titrated in eggs after storage at 37° C. for one week. The results of this experiment are shown in Table 8.

TABLE 8.
Titration of Onderstepoort Virus in Eggs, before and after Freeze-drying.

Dilution.	Before Freeze-Drying.	After Freeze-Drying.	After Storage of Dried Material for 7 Days at 37°C.
10 ⁻¹	+ + + +	+ + + +	+
10 ⁻²	+ + +	+ + +	+ +
10 ⁻³	+ +	+ +	+
10 ⁻⁴	?	+	+
10 ⁻⁵	—	?	—

NOTE.—+ + + + = well marked lesions of the C.A. membrane.
? = doubtful lesions.
— = no lesions seen.

Considerable difficulty was experienced in deciding whether or not the scanty lesions produced by dilute suspensions of Onderstepoort virus in egg were specific but it was believed that reasonably reliable estimates of viral activity could be made in this way.

This experiment also confirmed previous findings that suspensions made in buffered lactose-peptone solution showed very little, if any decrease, in activity after freeze-drying. However, after storage of dried material at 37° C. for one week, it was found that although the end-point of activity was apparently unaltered, the inoculation of 1:10 dilutions did not produce well marked lesions and it was believed that there was some degree of interference from inactive virus.

VIII. CORRELATION OF TITRES OBTAINED BY TITRATION IN EGGS, FERRETS AND DOGS.

Once it had been found that titrations of the Onderstepoort strain could be made in eggs, an experiment was made to correlate the titre obtained in this way with ability to confer immunity to both ferrets and dogs.

Serial ten-fold dilutions were made in broth of freeze-dried vaccine (Batch 135). Six eggs, two ferrets and one dog, were inoculated with each dilution. Seven days later the C.A. membranes of the eggs were examined for lesions and after three weeks the immunity of the dogs and ferrets was challenged with Collie virus.

The results of this experiment are shown in Table 9.

TABLE 9.

Correlation of Infectivity of Onderstepoort Virus for Eggs and its Immunising Potency for Ferrets and Dogs.

	Infectivity for Eggs.	Immunising Potency for.		Neutralisation Tests on Dog Sera.	
		Ferrets.	Dogs.	Before Immunisation.	After Immunisation.
10 ⁻¹	+++	I; I	I	Negative	Positive
10 ⁻²	++	I; I	I	Negative	Positive
10 ⁻³	+	NI; NI	NI	Negative	Negative
10 ⁻⁴	+	NI; NI	NI	Negative	Negative
10 ⁻⁵	Doubtful	NI; NI	NI	Negative	Negative

NOTE.—+ + + = well marked lesions of the C.A. membrane.

+ = scanty lesions confirmed by passage.

I = immune to challenge.

NI = not immune to challenge.

From Table 9 it will be seen that the vaccine was infective for eggs in a dilution of at least 10⁻⁴. On the other hand protection was conferred to ferrets and dogs at a dilution not greater than 10⁻². The nature of the reactions observed in the dogs is shown in Fig. 3

Usually, rather higher titres were obtained where vaccine batches were titrated in ferrets. It was thought that this might be due to the use of a heterologous strain of distemper for challenge. However, serum neutralisation tests made after the method of Cabasso (1952) on sera of the dogs, collected both before immunisation and before challenge, gave similar results. The sera were used undiluted. The virus used for these tests was the homologous E.F. strain.

Conclusions.—Although considerable difficulty was experienced in determining end-points of titrations made in eggs, it was apparent that suspensions which were capable of producing well defined lesions in eggs, would confer immunity to dogs and ferrets.

DISCUSSION.

From a perusal of the literature, it was apparent that considerable progress had been made in the evolution of satisfactory methods of immunising dogs against distemper, chiefly as a result of the work of Laidlaw and Dunkin (*loc. cit.*) and Green (*loc. cit.*). Nevertheless, there were certain disadvantages inherent to each method that had been advocated.

Vaccines dependent on inactivated virus for their immunising properties, gave only transient protection. The Laidlaw and Dunkin Method No. 1 took advantage of this transient immunity to render harmless the administration of virulent virus for the consolidation of immunity. There was unfortunately no way of determining what the response to the formalised vaccine had been. In dogs where it had apparently been poor, severe mishaps resulted when virulent virus was injected.

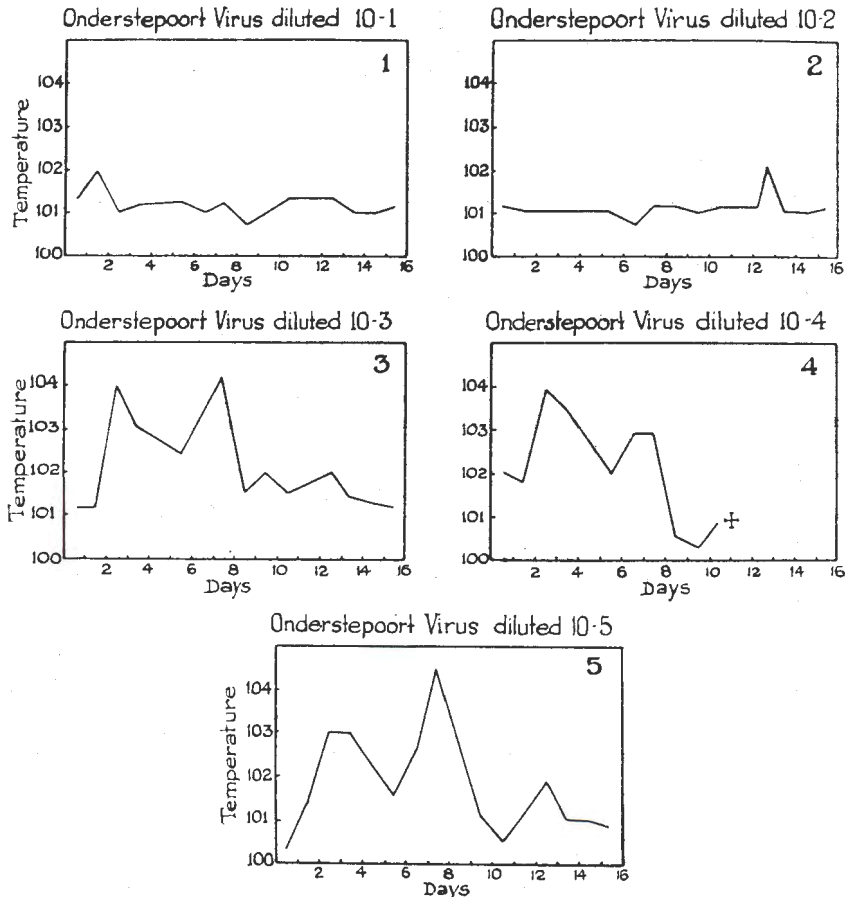


Fig. 3.—Febrile reactions shown by puppies previously inoculated with various concentrations of Onderstepoort virus when challenged three weeks later with the Collie strain.

In a modification of this method, transient immunity was conferred by the administration of hyper-immune serum while an injection of virulent virus consolidated the immunity (Laidlaw and Dunkin Method No. 2). This method did eliminate many of the accidents at the time of immunisation but it was found that a greater number of animals failed to develop an immunity. With both methods, No. 1 and No. 2, lack of immunity was in all probability usually as a result of the administration of impotent virus. A further disadvantage was that dogs immunised with either method could be contagious to other dogs. The production of formalised virus vaccine, hyper-immune serum and virus was cumbersome, and entailed maintaining large numbers of dogs and ferrets.

Green's method of immunisation with distemperoid virus offered great promise. The need for large numbers of dogs for the preparation of vaccine was eliminated, and it was claimed that dogs while undergoing the process of immunisation were not infective. Vaccine prepared from the distemperoid virus has been used extensively but experience has shown that it is not entirely safe for mass immunisation. Cabasso, Stebbins and Cox (1953) stated that they had difficulty in conceiving how a true modification of the pathogenic characteristic of distemper virus could have occurred as a result of serial passage in a highly susceptible host. Nevertheless it is apparent from the results published by Green (1939) that a progressive decrease in virulence for the fox did accompany serial passage of the strain in ferrets. Modification in virulence for the dog is more difficult to assess. However, it would seem that the great majority of reactions to distemperoid virus were less severe than those reported by Dunkin and Laidlaw (1926). The benign reactions reported by these authors apparently followed the use of ferret-propagated strains of distemper virus which they believed had become relatively avirulent for the dog.

The important disadvantages mentioned above have stimulated investigations on the propagation of distemper virus in hosts other than carnivores, with the hope that a method would be found for the easy production of large amounts of virus, and that attenuation would follow adaptation of the virus to a foreign host. It was found at Onderstepoort that Green's distemperoid virus could readily be propagated in the C.A. membrane of developing chick embryos. At the time it was thought that since Green's distemperoid virus had been used for the immunisation of dogs, egg-propagated virus could safely be employed without further modification. However, early passage material appeared to be more virulent than the parent strain. With serial passage in eggs it was noticed that there was a gradual decrease in virulence for ferrets, so much so that at the 110th egg-passage the strain had been rendered avirulent. In addition transmission by cohabitation no longer occurred after the 25th passage. Cabasso and Cox (1949) stated that the Lederle strain of distemper became attenuated for ferrets between the 24th and 28th egg-passage while a strain isolated from a case of canine encephalitis (Cabasso and Cox, 1952) was markedly modified after the 33rd serial egg-passage. No satisfactory explanation can be given for the difference in the onset of attenuation but it would seem that the prolonged serial passage in ferrets to which Green's distemperoid strain had been subjected made it less amenable to attenuation.

As the Onderstepoort virus from early egg-passages (6th to 16th generation) was rather virulent, tests on dogs were discontinued. Trials were recommenced after the strain had received more than 130 egg-passages. Since it appeared that the degree of attenuation at this and higher levels, was such that it could safely be used, mass immunisation with freeze-dried vaccine was undertaken. Results from the field have been very satisfactory. However, occasional reports of severe reactions within 14 days of vaccination have been received. Thorough investigations on some of these cases showed that the Onderstepoort virus was not responsible, and that the symptoms were due to natural infection prior to vaccination. However, the possibility that an inapparent infection with distemper virus can be provoked by vaccination should be considered.

The time required for the development of immunity in both dogs and ferrets was not determined for periods shorter than 21 days at Onderstepoort. It has been shown by Baker, Leader and Gorham (1952), that ferrets which had received Onderstepoort virus two or more days prior to virulent distemper virus, did not show symptoms of disease. They considered that protection was conferred firstly

by interference and then by active immunity. Their observations were confirmed by Cabasso, Stebbins and Cox (1953) who employed another avianised distemper strain; in addition they showed that a single injection of avianised virus rendered ferrets immune for at least two years. The duration of immunity in dogs vaccinated with avianised virus has not been established. It is possible that they behave in the same way as ferrets but from field reports in South Africa it would seem that immunity may wane; cases of encephalitis have been observed in isolated cases 12 to 18 months after vaccination. For this reason further investigations are considered essential for the determination of the duration and degree of immunity following both natural infection and vaccination.

In the United States avianised distemper virus has been used extensively for the immunisation of dogs and mink against distemper. Cabasso, Burkhart and Leaming (1951) and Brasmer, Whitter and Boley (1953) had most gratifying results with avianised virus while Hartsough and Gorham (1953) stated that some 60,000 mink which had been immunised with the Onderstepoort strain showed no apparent reaction to vaccination; no outbreaks of distemper have occurred among these animals.

In Sweden, Berg (1953) used avianised virus imported from the United States on a limited number of ferrets and dogs. He reported a slight reaction in some of the dogs four to eight days after injection; there was no indication that the animals were contagious and the immunity conferred appeared to be satisfactory.

The results obtained with avianised virus in South Africa have been good. It is unfortunately, not possible to estimate the percentage of failures in immunity that occurred. The majority of failures that were reported showed central nervous system involvement, and were accepted as cases of distemper. It has been pointed out that the differential diagnosis of several canine diseases may offer some difficulty but it is believed that the majority of cases diagnosed clinically as encephalitis are caused by distemper virus. A considerable amount of controversy has arisen as to whether or not all strains of virus isolated from cases of canine encephalitis are the same as the classical distemper virus described by Laidlaw and Dunkin (*loc. cit.*). The terms "hard-pad" disease virus or "para-distemper" virus have been suggested for those obtained from cases in which demyelination occurred.

Although it has been stated that a varying degree of antigenic differences may exist between distemper strains (Laidlaw and Dunkin, *loc. cit.*, Slanetz and Smetana, *loc. cit.*, MacIntyre *et al. loc. cit.*) most investigators have found close similarity (Koprowski *et al. loc. cit.*, Cabasso, *loc. cit.* Mansi, *loc. cit.*, Haig, *loc. cit.*). The length of the incubation period and the severity of the reaction in ferrets of newly isolated strains have also been used for their differentiation. However, these are variable and would appear to depend more on as yet undetermined factors rather than on fixed properties of virus strains. Histological studies have also proved unsatisfactory for differentiation. It is evident that much more experimental work is required for the determination of possible strain differences but at present there seems to be no justification for accepting the view that there are distinct entities. In studies of this nature the state of the host should also be taken into account: the level of nutrition, the presence or absence of concurrent infections such as coccidiosis and helminthiasis, and the degree of physical exertion may have a pronounced influence on the symptomatology of the disease. In addition the possibility should be considered that infection during the period of waning parental or active immunity may influence the course of the disease.

SUMMARY AND CONCLUSIONS.

1. In a brief review of the literature, methods which have been advocated for the immunisation of dogs against distemper and possible reasons for failures in immunity are discussed.

2. The propagation of Green's distemperoid virus in developing chick embryos is described. Conditions found most favourable for multiplication, were injection of eight-day-old embryos by the chorio-allantoic membrane route and incubation at 35° C. An inoculum with a high virus titre was most satisfactory for maintaining the derived Onderstepoort strain in embryonated eggs.

3. Continued serial passage of the Onderstepoort virus in developing chick embryos, resulted in a loss of contagiousness for ferrets by the 25th passage.

4. By the 130th egg-passage the degree of attenuation was such that the Onderstepoort virus could safely be used for the immunisation of both dogs and ferrets.

5. Immunity tests in ferrets and dogs vaccinated with the Onderstepoort virus, showed close immunological similarity between this strain and those obtained from encephalitic forms of distemper.

6. The keeping qualities of the Onderstepoort virus were examined.

(a) Macerated infected chorio-allantoic membranes showed considerable decrease in potency after storage at 32° C. for 24 hours, and possibly complete loss after 48 hours.

(b) Freeze-dried preparations of infected membranes showed very little loss of potency after storage at 37° C. for seven days provided buffered lactose-peptone solution was employed for making suspensions prior to freeze-drying.

(c) Freeze-dried preparations retained potency for at least 81 days when stored at -15° C.

7. Titration of viral activity made in eggs, ferrets and dogs indicated that approximately 500 egg infective doses are required for the immunisation of both ferrets and dogs.

8. Immunisation of dogs with Onderstepoort virus has been undertaken on a large scale and to date approximately 40,000 doses of vaccine have been used in Southern Africa. The results have proved satisfactory.

9. It has become apparent that some artificially or naturally immunised dogs are liable to develop the encephalitic form of distemper. As the pathogenesis of this clinical manifestation is obscure, the necessity for determining the significance of the duration of immunity, the neurotropic affinities of various virus strains, concurrent infections and the general state of health of dogs prior to infection, is stressed.

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