EXPERIMENTAL STUDIES OF THE PATHOGENESIS OF THE VIRUSES OF VACCINIA AND LOUPING-ILL WITH PARTICULAR REFERENCE TO THE MECHANISM OF ANTIVIRAL IMMUNITY.

by

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INTRODUCTION.

In the past much attention has been devoted to the properties of antiviral sera but only recently has an attempt been made to determine precisely how they act. In 1928, Andrewes performed experiments with the vaccinia virus, and came to the conclusion that there was no union of antigen and antibody in neutral mixtures *in vitro*, but in a later communication (1930) he reported that firm antigen-antibody union took place though extremely slowly. As no attempt has been made to repeat and develop his work, the experiments recorded in this thesis were undertaken to throw some further light on the problem.

The discovery of Alston and Gibson (1931) in this laboratory that Louping-ill is transmissible to small inexpensive animals, *viz.* mice, has greatly facilitated work on the pathogenesis and immunity reactions of a neurotropic virus. Moreover it has been possible to compare the mechanism of antiviral immunity to this virus with that to the vaccinia virus and thus to obtain additional data towards the solution of the question at issue.

In this thesis a review of the literature on the subject is given and then the details of the investi-:gations made. Some data regarding the pathogenesis of both the viruses studied are also included.

REVIEW OF LITERATURE ON THE EXPERIMENTAL PATHOGENESIS OF THE VIRUS OF VACCINIA.

Most mammals and birds are susceptible to vaccinia inoculated cutaneously, the calf and rabbit are those most frequently employed. When rabbits are inoculated cutaneously the inoculation lightly done gives a satisfactory vesiculation, while if the trauma be deeper, vesiculation may not occur, and only a leathery crust may form. Inoculation of the cornea causes a keratitis with minute elevations on the surface, which, with the recognition of Guarneiri bodies, is used diagnostically (Blaxall, 1930).

Gordon (1925) showed that when vaccinia virus was introduced into the nasal mucosa, after an incubation-period of 6-8 days, a condition of acute nasal catarrh developed accompanied by a muco-purulent discharge which at the onset contained the virus in abundance.

Though dermal inoculation with the vaccinia virus derived from cutaneous lesions did not lead to generalisation this could be effected experimentally in several ways. Calmette and Guerin (1901) showed that after intravenous injection of cutaneous vaccinia of low virulence, a general eruption might result, especially on areas of skin previously shaved and irritated. This was by no means a constant reaction, however, and frequently no generalisation occurred.

Douglas/

Douglas, Smith and Price (1929) injecting full doses of neuro-vaccine and neuro-testicular vaccine (derived from rabbits) by intravenous and other routes, found typical vesiculation on the skin and marginal mucous membranes, and also described macroscopic lesions in most of the viscera. Intratesticular injection produced immunity and also set set up "sympathetic" vaccinial lesions in the other testicle, if that were injured. Repeated testicular passage could increase the virulence so that the virus applied to the skin produced not only a cutaneous eruption but generalisa-:tion (Ohtawara, 1922) or injected intracerebrally set up a fatal encephalitis (Levaditi, Harvier and Nicolau, 1921; Marie, 1920).

Histology of Exanthem.

Ledingham (1927) came to the conclusion that the reticulo-endothelium was primarily and dominantly involved, and that this involvement might or might not be accompanied by secondary disturbance of adjacent epiblast. He characterised the lesion as of the nature of an acute infective granuloma. He found that the response was of the same nature, when inoculation was made into meso-blastic tissue like the spleen as into the epiblast, and considered that Levaditi and Nicolau's view (1923) that vaccinia manifested a specialised and definite affinity for epiblast/

epiblast was not justified.

Acute Disseminated Encephalo-myelitis.

Clinically signs of encephalitis or paraplegia have been observed within a fortnight of vaccination in a certain proportion of cases in England, Holland and other countries (Greenfield, 1929, 1930). Histologically the disease was characterised by peri-vascular zones of demyelination, accurately centred on a vessel and following it throughout its course. Not infrequently zones of demyelination were found around the margins of the cord, or on the lip of the ventral median fissure, and those were quite indepen-:dent of the arrangement of the vessels. Similar zones might surround the ependyma of the lateral ventricles, and seemed often to be associated with the marginal zones of the cord. In addition to the characteristic demyelination of peri-vascular zones there was usually some degree of infiltration of the adventitial lymphatics with cells of the lymphocyte class (Westphal, 1872; McIntosh and Blaxall, 1928).

The role of vaccinia virus in the causation of the above-mentioned disease was the subject of investi-:gation by various workers. Hurst and Fairbrother (1930) suggested that intradermal inoculation of sheep-lymph or neuro-vaccine was, rarely, if ever, followed by encephalitis. On intracerebral inocula-:tion/

:tion of virulent material, no difficulty attended the transmission of vaccinial encephalitis. They concluded that the essential lesion was a fibrinous, haemorrhagic and poly-morphonuclear meningitis, and such alteration as might be present in the underlying structures was of secondary importance; they were seen only when meningitis was most severe and were no doubt due, at any rate in part, to disturbances in nutrition consequent upon that, and upon compression resulting from the intense local oedema. They thought it highly improbable that the virus of vaccinia played a direct part in the causation of post-vaccinial encephalitis.

McIntosh and Scarff (1930) suggested that the above-mentioned workers used an abnormal strain of the vaccinia virus in producing vaccinial encephalitis. They concluded from their work that virulent strains of vaccinia could produce in rabbits a definite meningo-encephalitis after intracerebral, intravenous and intradermal inoculations; the lesions produced were strictly comparable with the visceral lesions in rabbits, and with those of post-vaccinial and post-variolar encephalitis in man.

Paschen bodies.

Paschen (1906) described the bodies which bear his/

his name as being small, round, coccus-like in form and about 0.2μ in diameter. They were Gram-negative, and were not stained by Sudan III or osmic acid. They were resistent to 2% potash, 2% acetic acid, alcohol, ether and chloroform. They were seen in variolous lymph, child lymph, inoculated rabbit cornea and in other vaccinial material, e.g. neuro-virus (Blaxall, 1930).

Huntemüller (1914) and others claimed that such forms could not be differentiated from albumin or colloidal granules. On the other hand, these bodies, with which might be associated Weigert's granules, Mann's Z granules, Prowazek's elementary bodies, have been said to constitute some form of aetiological agent. (Blaxall, 1930).

Ledingham (1931) was successful in securing pure suspensions of the elementary bodies associated with vaccinia, and such suspensions were agglutinated by the sera of rabbits after recovery from vaccinia, but not by sera from normal animals. In 1932, Ledingham reported that in immune rabbits, the development in the serum of agglutinins for Paschen bodies ran a course differing in no essential particular from that characteristic of ordinary bacterial infections. Eagles and Ledingham (1932) showed that by high speed centrifugalisation it was possible to deplete Berkefeld filtrates/

filtrates of vaccinia virus almost entirely of their virus content and to recover the great bulk of the virus in deposits consisting in the main of Paschen bodies; such deposits after repeated washings in saline retained their potency, while the saline mentruum in which the bodies were suspended was entirely free from virus. In the light of these experiments the conclusion was reached from all the evidence then available that the causal agent of vaccinia is the Paschen-body.

Serology of vaccinia.

It was clearly established that the production of immunity in vaccinial affections was attended with the formation of specific antibodies. The most important of these was a "viricidal" antibody, and subsidiary were a precipitating or agglutinating antibody and a complement-fixing antibody (Gordon,1925).

Viricidal antibody.

This antibody was shown to be closely related to the production of immunity, it was found accompanying the onset of immunity or shortly after; it increased in strength as immunity advanced, but there was no absolute parallelism, since in vaccinated animals it frequently showed decline and might entirely disappear while immunity was still present (Beclere, Chambon, Menard/

Menard, 1899; Henseval and Convent, 1912). It would seem that immunity was increased by a revaccination, though that be confined to an immediate reaction (Sato, 1921; Fugii, 1922), a view at variance with Gins's expressed opinion that a rise in the immunity level could only follow a proliferation of the virus as evidenced by vesiculation (Gins, 1916). Antibody might have sufficient power to inactivate its own originating virus in vitro but be incapable of pro-:ducing the same effect in vivo, since scales con-:taining active virus might still remain attached to the body, although the patient was quite convalescent and fully immune (Winkler, 1925; Sobernheim, 1927).

Wilson Smith (1929) showed that the blood of an infected rabbit might contain at the same time active vaccinia virus (associated with the leucocytes) and antibodies (in the plasma). That finding also suggested that antibodies might be unable to interact with virus which was intracellular. Olitsky and Long (1929) and Olitsky, Rhoads and Long (1929) published evidence showing that from the organs of vaccinated rabbits and the cords of monkeys which had recovered from poliomyelitis, the viruses, though not demonstrable by ordinary methods of subinoculation might be revealed by cataphoresis of the tissue. It was also known, that the salivary gland virus of Cole/

Cole and Kuttner could be readily recovered from immune guinea-pigs (Cole and Kuttner, 1926).

Precipitating antibody.

Wilson Smith (1932) summarised his work as follows:

(1) Extracts of testicular vaccine virus were obtained which gave a specific precipitating reaction with immune sera. The precipitating substance withstood repeated boiling.

(2) Chemical tests showed that both protein and carbohydrate radicle were present in the precipitating extracts.

(3) Filtration experiments indicated that the precipi-:tating substance consisted of particles with an approximate size of 0.004μ. These particles probably could not be the product of the autolysis of the virus, the resulting debris would have consisted of particles of all shapes and sizes.

(4) The heat-stable extract possessed no antigenic power.

(5) The study of serum changes during hyper-immunisa-:tion afforded evidence that precipitating antibodies were probably identical with virus neutralising antibodies.

(6) Allergic skin reactions were elicited by inoculation of the extracts into previously vaccinated human subjects/

subjects, but not in those who had never been vaccinated.

Complement-fixing antibody.

Gordon (1925) adopting careful quantitative procedure with antivaccinial serum succeeded in detecting vaccinia virus up to a dilution of 1 in 2000 and with variolous material to 1 in 1800, and considered it to be a valuable diagnostic measure. He was also successful in eliciting specific flocculation with the viruses of vaccinia and variola against an antivaccinial serum.

Further references to previous studies on antivaccinial immunity will be made in a later section.

REVIEW OF LITERATURE ON THE EXPERIMENTAL PATHOGENESIS OF THE VIRUS OF LOUPING-ILL.

The disease which is variously known as Louping-ill, Trembling or Thwarter-ill, had been recognised for the last hundred and fifty years as one of the serious scourges of sheep flocks. In its occurrence, it seems to be peculiar to Scotland and the north of England (Greig, 1932).

It was early supposed that the British tick, Ixodes Ricinus probably played some part in the production of Louping-ill. This supposition was supported by strong circumstantial evidence in that the disease appeared in its greatest incidence during spring and early summer, when ticks were most active, and it was always found in areas which were tick infested, whereas it did not occur in other and adjoining areas which were tick free (Greig, 1932).

In 1930, Pool, Brownlee and Wilson successfully and regularly transmitted Louping-ill to sheep and pigs by the intracerebral inoculation of material from the brain and spinal cord of affected sheep. All attempts to demonstrate the presence of visible micro-organisms in the infective material failed. No recognisable organism was found in the blood. It was an evident possibility that the infective agent would be found to represent a filterable virus. This view

was further supported by the histo-pathological findings, the nature of the lesions in the meningo-encephalo-myelitis being suggestive of those caused by a neuro-tropic virus (Greig, Brownlee, Wilson and Gordon, 1931).

Pool, Brownlee and Wilson (1930) failed in their attempts to filter the virus through various types of filter candles.

In 1931, Greig, Brownlee, Wilson and Gordon developed this work. In the course of this study, a Berkefeld filtrate prepared by Professor T.J. Mackie proved effective for the first time in producing typical Louping-ill in a sheep injected intracerebrally. Bacteria-free filtrates from Berkefeld N and W and Chamberland L_2 and L_3 candles produced the typical experimental disease. The Chamberland L_5 candle yielded an inactive filtrate on all occasions.

Attempts to infect laboratory animals with Louping-ill.

Alston and Gibson (1931) found that the mouse was susceptible to Louping-ill on intracerebral inoculation. Greig, Brownlee, Wilson and Gordon (1931) confirmed this but failed to infect mice by subcutaneous and intravenous injections in a limited number of cases. A subcutaneous injection of the virus followed by an intracerebral injection of saline 24 hours later produced the disease in 2 out of 6 mice.

Attempts/

Attempts to infect rabbits and guinea-pigs failed. One out of ten rats showed weakness and lack of co-ordination of movements of limbs on the twenty-fifth day, but its brain failed to produce the disease in mice on intra-cerebral injection, but a sheep on similar inoculation succumbed to the typical disease.

Alston and Gibson (1931) transmitted the typical infection to uninoculated mice placed in the same cage with those suffering from the disease.

Webster and Fite (1933) succeeded in transmitting Louping-ill to mice by intranasal insufflation, but these mice were brother to sister inbred for at least 10 generations.

Elford and Galloway (1933) were able to infect mice by intradermal, intranasal and intramuscular routes, without cerebral trauma. Infection could be produced by the application of the virus to the depilated and scarified skin. Attempts to transmit the infection to uninoculated mice placed in the same cage with diseased ones succeeded only in one instance. The success was attributed to the fact, that the healthy mouse ingested the brain of one dead of the disease.

Weston Hurst (1931) successfully transmitted the infection to monkeys by intracerebral inoculation of a 5 per cent emulsion of Louping-ill mouse brain. Single/

Single attempts to infect monkeys by intramuscular or intrasciatic injections failed.

Greig, Brownlee, Wilson and Gordon (1931) failed to produce Louping-ill in sheep by administering the virus by subcutaneous, intradermal and intranasal routes. A thermal reaction was, however, produced. Gordon, Brownlee, Wilson and Macleod (1932) stated that on intracerebral or subcutaneous inoculation virus was present in the blood during the course of the disease, appearing on the day on which the temperature rose and persisting until the occurrence of rapid defervescence before death, at which no virus could be detected. In some experiments, virus in low concentration was shown to be present in the blood to the time of death of the sheep.

On recovery after inoculations other than intra-:cerebral, the sheep were solidly immune for a con-:siderable time against a severe intracerebral infec-:tion (Greig, Brownlee, Wilson and Gordon, 1931).

Role of ticks in Louping-ill.

Macleod and Gordon (1932) found that ticks, which had engorged on louping-ill sheep, were capable of producing Louping-ill within twelve days after moulting. Since ticks after moulting required about eight or nine days to harden before they would attach themselves to a host, it followed that they were infective/

infective as soon as they were able to attach themselves. The virus might survive in the body of the tick for a period of at least 24 days. It appeared, that in the case of larvae at least, Louping-ill virus ingested by this stage could survive a moult, and could be demonstrated in the bodies of the resulting nymphs.

Gordon, Brownlee, Wilson and Macleod (1932) found that ticks collected from Louping-ill pastures were capable of producing a temperature reaction, when allowed to feed on normal sheep. By means of immunity tests this reaction was proved to be the manifestation of a condition other than that of Louping-ill and was called "Tick-borne-fever".

As the natural infection in sheep was due to an "intradermal injection" of the virus by the infected ticks, and there was considerable difficulty in producing Louping-ill experimentally, unless the infective material was introduced directly into the central nervous system, the part played by Tick-borne-fever in the causation of natural infection was investigated by Macleod and Gordon (1932).

In the infestation experiments, in which Tick-borne-fever was induced prior to, or simultaneously with, exposure to Louping-ill infection, Louping-ill developed in the experimental sheep. Infection of central nervous system occurred only in those sheep which/

which were undergoing a concurrent infection of Tick-borne-fever, and it was possible that the presence of this disease facilitated the invasion of the central nervous system by the Louping-ill virus. It was shown, however, that sheep could become infected with, and even die from Louping-ill infection by tick bite, although Tick-borne-fever be not present.

Louping-ill in man.

Rivers and Schwentker (1933) reported that certain workers who had been in close contact with louping-ill virus developed what seemed to be influenza. In each instance this illness in the patient was followed by apparent health lasting a few days which in turn was followed by a definite encephalitis. The spinal fluids were sterile and showed a mono-nuclear pleo-:cytosis and an increased amount of globulin. Attempts to demonstrate the virus in the blood and spinal fluid of two of them were made without success. Five out of fifteen possessed sera that definitely neutralised the virus of Louping-ill.

Cultivation of the virus.

Rivers and Ward (1933) claimed to have cultivated the Louping-ill virus in a medium consisting of minced chicken embryo and monkey serum diluted with nine volumes of tyrode solution. Elford and Galloway (1933) estimated/

estimated the size of the virus to be 15-20 mμ by the method of ultra-filtration analysis using carefully graded collodion membranes.

Effect of chemical and physical agents on the virus.

Gladney and Hurst (1931) made the following observations:

There was no significant difference in the keeping properties of Berkefeld filtrates of the Louping-ill brain emulsions kept under aerobic and anaerobic conditions. Filtrates might retain virulence for as long as fourteen days at +4°C, for 4 days at room-temperature and for only one day at 37°C. The virulence of brain kept frozen at -10°C remained unaltered over a period of at least 60 days; in the same time in the cold room definite loss of virulence occurred. The virus survived but very few days (three days in one case, eight days in the other) at room-temperature. The results at 37°C were invalida-:ted on account of the drying and shrivelling of the brain. That the virus did survive for limited periods at 37°C was shown in further experiments in which brain was kept in a fluid medium. In broth or tyrode solution, virulence was maintained for 48 hours at 37°C, in 50% glycerol for 24 hours. Mouse brains stored in 50% glycerol were active for 110 days when direct emulsion was used. Clear evidence of the ability/

ability of the virus to diffuse into liquid media was obtained.

Histo-pathology.

Brownlee and Wilson (1932) found lesions of meningo-encephalo-myelitis in all natural and experi-:mental cases of Louping-ill in sheep. In the pig, the nervous tissue showed very intense cellular infil-:tration and relatively little nerve-cell destruction. In the mouse, the principal lesion was the necrosis of the large nerve cells of the medulla and cord.

Findlay (1932) gave the following histo-patholo-:gical findings in the monkey: The most constant lesions were in the cerebellar cortex. Many of the granular cells were definitely pyknotic, while in two cases there were petechial haemorrhages in this region. The most striking lesion, however, was the extensive degeneration and in many cases the almost total dis-:appearance of the Purkinje cells, with only now and then a recognisable survivor, without any proliferative changes in the neighbouring micro-glial cells. Of the few remaining Purkinje cells scarcely any were normal, pyknosis, vacuolation and distortion being very common. In other parts of the brain and cord the lesions were of varying intensity. Meningeal infiltration which was restricted to a few mononuclear cells, was always very slight. The lesions consisted of slight peri-vascular/

vascular cuffing, microglial proliferation, an increase in the number of invading cells and occasional degenera-:tion of the neurones with neuronophagia. Acute necrosis of the anterior horn cells was rarely seen, though there was a considerable degeneration and chromatolysis. No inclusion-bodies were found in the nerve-cells of the monkeys.

Findlay (1932) stated that intra-cytoplasmic inclusion-bodies in the neurones of mice were present. They were most numerous in the neurones of pons and mid-brain, rather less frequent in the cord and Purkinje cells and rather rare in the cerebral cortex, where they were always small. They varied in size from 1 to 10μ and were usually somewhat oval in shape when small; kidney-shaped or elongated when larger. The size and number of the bodies found within the same cell showed considerable variation. No definite internal structure was seen within the bodies.

REVIEW OF LITERATURE ON THE MECHANISM OF ANTIVIRAL IMMUNITY.

It is a well established fact, that the filter-:able viruses are capable of giving rise to specific "neutralising" antibodies, but the nature and mode of action of these antibodies still remains somewhat obscure. How far the virus-neutralising effect of the anti-viral serum observed *in vivo* is to be attributed to phagocytosis of the opsonized virus, or how far a direct viricidal or inactivating action of serum may be involved is not definitely known.

Sobernheim (1925) believed that the virus was directly killed by the antiserum. Gordon (1925) spoke of the antibody as a "lysin" because in one experiment fresh guinea-pig complement increased the action of the antiserum, but he could not confirm this. Schultz (1928) concluded that there was no evidence to indicate that complement played a major rôle in the inactivation of a virus. Also, heated serum was found to give similar results to unheated serum.

Andrewes (1928) showed that preliminary incuba-:tion of serum-virus mixtures was not necessary in the case of vaccinia virus in order to demonstrate neutralisation after injection into the skin. He suggested/

suggested that the immune serum acted directly on the tissues producing a local "passive immunity", which in turn was responsible for the neutralisation of the virus.

Fairbrother (1932) supported this view, and showed that immune serum administered 15 minutes after intradermal injection of the virus in exactly the same area neutralised the virus. In contrast to the intra-:dermal route of injection, the activity of the virus was not easily modified by the immune serum when the mixture was injected into the brain: an appreciable alteration in the infectivity of the virus was only shown when the virus suitably diluted remained in contact in vitro with the immune serum for at least 4 hours. Zinsser and Tang (1926) stated that herpes virus and serum must be incubated together before intracerebral inoculation in rabbits, if antibodies were to be demonstrated.

On the other hand Schultz, Gebhardt and Bulloch (1931) working with poliomyelitis virus found that a concentrated immune serum added to an appropriate quantity of virus might serve to render such a virus suspension innocuous immediately after the serum was added. This suggested that the anti-viral serum did not act directly on the virus, but "changed the sus-:ceptibility of the tissues of the host in some indirect manner". With dilution of the immune serum it/

it became necessary to prolong its contact with the virus in vitro showing clearly that immune serum did act directly on the virus.

The same phenomenon was observed with bacterio-:phage. Schultz, Quigley and Bulloch (1929) found, that whereas a serum dilution of 1/128 served to inactivate a given bacteriophage preparation in 30 minutes, a serum dilution of 1/4098 required as long as 8 days to render the same bacteriophage preparation completely inactive.

Antibody absorption.

Experiments were devised by Andrewes (1928) to show whether antibody absorption occurred during inactivation of the virus. The conclusion was that absorbed sera were quite as active as unabsorbed sera. Later Andrewes (1930) found that firm antigen-antibody union took place extremely slowly.

Friedberger and Eisler (1907) reported in the case of the rabies virus successful antibody absorption by means of virulent brain material. They relied, however, upon centrifugalisation to get rid of the excess virus from serum-virus mixtures, and failed to test the absorbed sera for virus content.

Wilson Smith (1930) working with vaccinia recalled the necessity of using enormous doses of bacteria for agglutinin absorption, and decided to increase as much as/

as possible the absorbing dose of the virus, whilst at the same time reducing the antibody content of the serum, by dilution, and greatly prolonging the contact of the virus and antibody. The antibody absorption was shown clearly in that experiment. It might have been due, however, to necrotic cell-products in the vaccinial tissue rather than to the virus itself. A strain of herpes virus behaving more or less like vaccinia in its pathogenesis and antibody production was available. It was possible to carry out cross-absorption tests on the lines of the well-known cross-absorption test used in the analysis of antibacterial immunity. The result showed that each virus absorbed its homologous antibodies from a mixed serum. In two experiments, virus was inactivated by immersion in a water-bath at 58°C to 60°C for one hour. In one experiment the killed virus failed to absorb any antibodies, but in the other a small amount of absorp-:tion occurred. Heated virus therefore differed from heated bacterial suspensions, which in general are found to absorb antibodies readily.

Recovery of virus from neutral serum-virus mixtures.

An attempt was made by Andrewes (1928) to recover the vaccinia virus from an overneutralised serum-virus mixture by adsorbing the virus on to Kaolin, this was successful. The difficulty arose, however, that the antibody/

antibody was also adsorbed on to Kaolin. The same result was obtained by precipitating the virus along with the euglobulins from an overneutralised serum virus mixture. Dilution worked consistently in reactivating those serum-virus mixtures, in which the source of the virus was a centrifuged suspension of vaccinial testis. But when a mixture of diffusate from the same source and the antiserum was kept at room-temperature for 24 hours, dilution failed to reactivate the virus. It must be borne in mind, that the virus in the diffusate might have become inactive on being exposed to room-temperature for 24 hours; as no adequate control had been put up to test the potency of the virus, no definite conclusion could be drawn from the experiment. In 1930, Andrewes found that after long contact of virus and immune serum, virus was progressively less and less easy to recover. But, some reactivation was possible even after 4 days at room-temperature, or 24 hours at 37°C. He con-:cluded that firm antigen-antibody union took place extremely slowly.

Todd (1928) found that a mixture of fowl-plague virus with the corresponding immune serum, so prepared as to be just non-virulent, when injected intramuscul-:arly into a fowl was rendered virulent by simple dilution with saline. If fresh normal fowl serum was used/

used as the diluent in place of saline the phenomenon did not occur. The result in these experiments was the same whether the undiluted serum and the virus were mixed and kept for 2 hours at 37°C, and then diluted, or the serum and virus were separately diluted before mixing. When the mixture of immune serum and virus was kept at 28°C for 24 hours, and then diluted with ten times its volume of saline, and injected intramuscularly, the dilution phenomenon was no longer observed. As the virus might have died out during the long exposure to this temperature, the lack of an adequate control to test the potency of the virus invalidated the experiment. Todd (1928) pointed out, that the serum-virus mixture, which was neutral on intra-muscular injection was virulent on intravenous injection, although the fowl is not more susceptible to an intravenous injection of the virus alone than to an equivalent intramuscular injection. A mixture which contained any considerable excess of immune serum did not become virulent on dilution.

These observations were not in harmony with the findings of Andrewes (1928, 1930) in connection with the vaccinia virus. It must be borne in mind, that Andrewes was working within a very narrow range of virus dilutions in serum-virus mixtures; and the so-called over-neutralised serum-virus mixtures might not/

not have contained a sufficient excess of immune serum to render them comparable to those of Todd.

Bedson (1928) found with herpes virus that reac-:tivation by dilution was no longer possible when virus and serum had been in contact for 4 hours at room-temperature.

Schultz, Gebhardt and Bulloch (1931) working with the poliomyelitis virus were able to reactivate a neutral serum-virus mixture by addition of a suitable volume of physiological saline. They concluded that the inactivation of poliomyelitis virus by immune serum resembled more closely a toxin-antitoxin reaction rather than a bactericidal type of action. But there was no need to postulate a union between the virus and antibody; they had also indicated that the non-infectivity of the serum-virus mixture might have been due to the action of the antibody on the tissues of the host, which in turn acted on the virus.

Craigie and Tulloch (1931) found from their experiments that when acri-flavine was injected intra-:dermally in different concentrations and the vaccinia virus was administered in exactly the same spot 4 hours later, inhibition of cutaneous reactions occurred in those areas, which had been treated by the higher concentrations of acri-flavine, although such concen-:trations/

:concentrations did not kill the virus per se. They suggested that Indian ink, acriflavine, quinine, hydrochloride and possibly certain immune sera either prevented the virus injected intracutaneously from gaining access to the cells or allowed the cells to deal effectively with it.

Bedson (1928) found that collodion particles were capable of adsorbing both herpes virus and herpes antibody, but the particles which had first adsorbed the antibody showed an increased avidity for the virus. Washed colon bacilli could be used in place of collodion particles. This experiment suggested that antigen-antibody union did occur in vitro. It was also recognised that neutralising antibody and complement-fixing antibody were not necessarily identical. The positive results with the complement-fixation reaction (vide infra) supported the view that virus and anti-:body did unite in vitro.

Complement-fixation and Flocculation.

Gordon (1925) obtained specific complement-fixation and flocculation with the viruses of vaccinia and variola. Ciuca (1929) produced evidence to show that the complement-fixation reaction could be obtained with the virus of foot and mouth disease. Takaki, Bonis and Koref (1926) applied this reaction in the identification of the viruses of herpes, rabies and

Japanese/

Japanese encephalitis. Bedson and Bland (1929) obtained specific complement-fixation with the viruses of herpes and vaccinia and hyper-immune sera.

The successes reported were called in question by Schultz and his co-workers (1928) on the ground of the non-specificity of the reactions. Burgess, Craigie and Tulloch (1929) and Craigie and Tulloch (1931) obtained specific flocculation in mixtures of extracts of variola crusts and anti-vaccinial serum, and applied the reaction in the practical diagnosis of variola. The test was applied to over 200 specimens of material with an experimental error of less than 1.5%. It was also definitely shown that the flocculation which occurred when antivaccinial serum was brought in contact with extracts of vaccinia or variola material was not due to adventitious antibodies reacting with secondarily infecting bacteria that might be present on the skin. Further, the serological identity of variola major and variola minor with both dermal vaccinia and generalised neuro-vaccinia was established.

E. St. G. Gilmore (1931) made the following observations in the tests with vaccinia culture virus and rabbit serum. The reagents were free from bac-:teria, and the antigen and antiserum were obtained from the same species, the results demonstrated beyond question/

question the specific union *in vitro* of vaccinia virus and a corresponding antibody. The complement-fixa-:tion property of the serum of rabbits was demons-:trable as early as 10 days after infection, persisted for at least nine months. (Olitsky and Long in 1929 recovered by cataphoresis vaccinia virus from the tissues of rabbits as long as 133 days after dermal infection.) The amount of complement-fixing anti-:body did not depend upon the extent of infection. Immune rabbit serum was unsatisfactory for the complement-fixing experiments with viruses, because of its anti-complementary nature.

Bedson (1932) found that the washed elementary bodies of psittacosis derived from mouse spleen were agglutinated specifically by an anti-psittacosis serum, and fixed complement in its presence, but did not react in either way with an antiserum for mouse spleen.

Craigie (1932) stated that two serologically active fractions might be obtained from extracts of vaccinial tissue derived from rabbits, *viz.* lapine, (a) a Seitz-filterable and precipitable substance and, (b) elementary bodies. Both fractions were floccula-:ted specifically by the same antibody of antivaccinial serum; they did not react with normal rabbit sera. The flocculation reaction as performed with crude suspensions/

suspensions, involved both a precipitin and agglu-:tinating reaction, and was referable to the same antigen in two different states. A thrice washed suspension of elementary bodies exhibited a virus content similar to that of the extract from which they were obtained. These results warranted the conclusion that the Seitz-filterable flocculable substance was a specific product of the elementary bodies of vaccinia, and supported the view of Paschen, Ledingham and others, that these bodies were the virus of vaccinia.

Findlay (1931) concluded from his work that in vaccinia, the greater part of the immune body appeared to be associated with the euglobulin fraction, part however with the pseudoglobulin fraction. Ledingham, Morgan and Petrie (1931) observed that the anti-:vaccinial body was found to be associated with the euglobulin as well as the pseudoglobulin fraction of the serum, but not with the albumin. The euglobulin fraction contained the antibody in highest concentra-:tion per gramme of protein, but its absolute amount fell below that present in the pseudoglobulin fraction. On the other hand Marrack (1934) pointed out that bacterial antibodies were not as a rule found to be confined to any particular fraction of the globulins. The distribution of antibodies in the fractions separated/

separated by ammonium sulphate was most thoroughly studied in the case of diphtheria antitoxin, which was not confined to any one fraction (Banzhaf and Gibson, 1907; Barr and Glenny, 1931; Barr, Glenny and Pope, 1931; Barr, 1932).

The fact that *in vitro* union of virus-antigen and antiviral body was usually weak was held to differentiate the antiviral body from other immune substances such as bacteriolysins or bactericidins. On the other hand, it is well-known that mixtures of bacteria and their corresponding antisera in which there is little evidence of *in vitro* bacteriolysis may behave as neutral mixtures in the living body, where opsonic and phagocytic processes came into play (Gye and Ledingham, 1930) .

Douglas and Smith (1930) working with vaccinia suggested that some of the phagocytic cells of an immune rabbit might be actively concerned in the increased resistance of such animals.

EXPERIMENTAL STUDIES OF THE PATHOGENESIS OF THE VIRUS OF VACCINIA WITH PARTICULAR REFERENCE TO THE MECHANISM OF ANTIVIRAL IMMUNITY.

Technique of neutralisation experiments with immune serum.

Rabbits weighing 2000-3000 grammes were used throughout the experiments. In the beginning, they were depilated extensively on the back a few hours before the experiment, but as some of them were found dead next morning, this method was abandoned and thereafter depilation was done the day before and the rabbit kept warm.

Two specimens of the virus were used: one was a commercial calf lymph (Jenner Institute), the other was supplied by courtesy of Captain S.R. Douglas, National Institute for Medical Research. In each case the virus was passed at frequent intervals through rabbit brains, and the resulting neuro-virus was used in the experiments. It was preserved in 50% glycerol in saline in the ice-chest. A 10% emulsion in saline was made for each experiment and was first centrifuged for 5 minutes at 2000 r.p.m. Decimal dilutions of the supernatant fluid were then made up to 10^{-6}. 0.2 c.c. of each of these were mixed/

mixed with an equal volume of undiluted immune serum (vide infra) and the mixtures were then injected in 0.2 c.c. amounts in the depilated area, generally in four rows; while the dilutions of virus only were injected in the fifth row as a control. The animal was kept under observation for at least 6 days, and the diameters of the resulting vaccinial lesions were then measured and recorded.

Following an intradermal injection of virus alone in a rabbit, the small swelling produced disappeared within a few hours, and the site of injection then appeared normal for a varying period depending on the concentration of the virus used. In general the greater the concentration of virus used, the earlier the subsequent reaction appeared. Using undiluted virus, a papule appeared at the site of injection two days later, and this gradually increased in size reaching a maximum on the sixth or seventh day. When approaching its maximum size, the central area of the papule became first congested and later bluish-purple in colour. The tissue underlying the papule was markedly indurated, and central necrosis left the surface of the papule flat or slightly umbilicated. The papule then commenced to retrogress slowly, and the induration gradually passed off but scarring persisted in the site of a large reaction for many months/

months. With low concentrations of virus the reaction was essentially of the same kind, but it did not make its appearance until 6-7th day following injection, and the area of induration was much smaller. When determining the size of a reaction, maximum diameters attained by the area of induration were noted. Occasionally instead of a single papule being formed, there were several small discrete papules. These reactions were few in number, and were recorded in the tables as "papules". Occasion-:ally erythema without induration followed injection, and this was also recorded in the tables.

Two samples of immune serum were used (1) obtained by giving six intradermal injections of 0.2 c.c. of the decimal dilutions of neuro-virus for the titration of the virus, and three weeks later drawing the blood by cardiac puncture. The serum so obtained was pooled and stored in the ice-chest without pre-:servative. (2) Hyper-immune serum: some of the rabbits treated as in (1) were given in addition intravenous injections of 1/50 dilution of the super-:natant fluid from centrifugalised neuro-virus emulsion starting three weeks after the intradermal titration of the virus. The injections were given weekly for at least three months, the dosage ranging from 4-10 c.c. The rabbits were then bled, the serum pooled and stored/

stored in the ice-chest.

The following symbols were used in the tabulation of results.

V	=	virus
I-S	=	immune serum
N-S	=	normal serum
V+I-S	=	mixture of virus and immune serum
---	=	absence of any reaction
X mm	=	diameter of the area of induration in mm

Experiments on neutralisation of the virus by immune sera.

Whenever the hyperimmune serum is not mentioned specifically, the ordinary immune serum (1) was used in the experiments.

In the first place, the thermostability of the virus neutralising antibody of the antivaccinial serum was tested. A sample of immune serum was divided into five portions. These were heated in the water-bath at different temperatures as follows.

(1) 55°C for 30 minutes.

(2) 55°C for 60 minutes.

(3) 60°C for 30 minutes.

(4) 60°C for 60 minutes.

Equal volumes of the undiluted serum and decimal dilutions of the virus were mixed in sterile tubes, and injected intradermally into rabbits immediately after mixing. The results on the seventh day were as follows//

follows.

Injected immediately after mixing.

Dilution of virus.	V	V+I-S	V+heated I-S			
			(55°-30m)	(55°-60m)	(60°-30m)	(60°-60m)
10^{-5}	2mm	---	---	---	---	---
10^{-4}	13mm	---	---	---	---	---
10^{-3}	19mm	---	---	---	---	---
10^{-2}	21mm	---	---	---	---	---
10^{-1}	17mm	erythema	1mm	---	---	2mm
Undil.	22mm	1mm	2mm	2mm	3mm	4mm

There was no appreciable decrease in potency after heating the serum to 55°C-60°C for 30 minutes or at 55°C for 60 minutes, but there was a slight de-:crease after 60 minutes at 60°C.

In a further experiment, the results were as shown below.

Dilution/

Dilution of virus	V	V+I-S	V+heated I-S		
			(55°-60m)	(63°-30m)	(63°-60m)
10^{-6}	---	---	---	---	---
10^{-5}	---	---	---	---	---
10^{-4}	14mm	---	---	---	---
10^{-3}	15mm	---	---	---	---
10^{-2}	17mm	---	---	---	8mm
10^{-1}	24mm	---	---	---	14mm
Undil.	---	---	---	12mm	17mm

The previous observations were confirmed. In addition, it showed that heating the serum at 63°C for 30 minutes was more effective in lowering potency than heating for 60 minutes at 55°C. But when heating was continued at the higher temperature of 63°C for the longer period of 60 minutes, the potency of serum was not completely destroyed.

Action of complement on heated immune serum and virus mixtures.

The action of fresh guinea-pig complement on virus and heated immune serum mixtures was tested. A control was included by adding to virus and heated immune serum mixtures the same amount of guinea-pig serum heated at 56°C for 30 minutes to inactivate the complement.

Dilution/

Dilution of virus.	V	V+I-S	V+heated I-S 55°C-30 min	V+heated I-S 55°C-30 min	
				-fresh guinea-pig serum	-heated guinea-pig serum
10^{-6}	---	---	---	---	---
10^{-5}	---	---	---	---	---
10^{-4}	---	---	---	---	---
10^{-3}	11 mm	---	---	---	6 mm
10^{-2}	14 mm	---	---	---	8 mm
10^{-1}	16 mm	---	---	7 mm	12 mm

This experiment showed clearly that compared with heated guinea-pig serum, the fresh serum definitely enhanced the action of heated immune serum. The fallacy was, that any direct action of the guinea-pig serum per se on the virus was not taken into considera-:tion. In the next experiment, this was investigated. The antivaccinial serum was diluted 1/50 before mixing with the virus emulsion.

Dilution of virus.	V+Saline	V+Compl.	V+I-S	V+heated I-S +complement.	V+I-S +heated guinea-pig serum.
10^{-6}	7 mm	---	---	---	---
10^{-5}	13 mm	7 mm	---	---	---
10^{-4}	17 mm	17 mm	---	---	papules
10^{-3}	24 mm	20 mm	7 mm	7 mm	21 mm
10^{-2}	27 mm	19 mm	13 mm	17 mm	19 mm
10^{-1}	18 mm	17 mm	14 mm	20 mm	22 mm
Undil.	---	---	16 mm	24 mm	17 mm

The conclusion was drawn that the complement although it slightly enhanced the action of the heated immune serum acted by itself on the virus. Thus no true complementing action could be demonstrated.

Local passive immunity.

In the previous experiments, the immune serum and virus were mixed, and after a few seconds were tested in vivo for neutralisation. The possibility of neutralisation effect being due to a tissue reaction mediated by the antiserum was considered. This was investigated in the following experiments.

A 10^{-1} dilution of a 10% emulsion of neuro-virus was injected intradermally into several areas in 0.2 c.c. amounts, and 0.2 c.c. undiluted antivaccinial serum was injected exactly into each area from 5-30 minutes later. In another series of injections, the serum was first given and the virus was super-imposed from 5-30 minutes later.

Dilution of virus.	V	Time I-S given.	Time V 10^{-1} given.
10^{-4}	16 mm	I-S 5 min after V ---	V 10^{-1} 5 min after I-S ---
10^{-3}	20 mm	10 " " " ---	10 " " " ---
10^{-2}	22 mm	15 " " " ---	15 " " " ---
10^{-1}	25 mm	20 " " " ---	20 " " " ---
Undil.	32 mm	25 " " "erythema	30 " " " ---
		30 " " slight induration.	

The/

The results showed, that immune serum introduced within twenty minutes of the virus intradermally in exactly the same spot neutralised the virus. Con-:versely serum introduced 30 minutes before the virus in the same spot afforded complete protection.

In the following experiment, the same points were investigated but in addition the effect of variation in dilution of virus and increase in the period of time between injection of virus and serum were also considered.

Time I-S given.	Virus emulsion strength		
	Undil.	10^{-1}	10^{-2}
15 min after V	---	---	---
20 " " "	---	---	---
25 " " "	4 mm	erythema	---
30 " " "	6 mm	"	---
35 " " "	4 mm	"	---
40 " " "	12 mm	"	---
Control V alone	22 mm	21 mm	18 mm

Neutralisation of the virus in the skin could be effected without any in vitro contact of the serum and virus. The serum could neutralise the virus, if administered before the virus was established in the tissues.

Recovery/

Recovery of virus from neutral serum-virus mixtures.

Attempts were made to ascertain whether firm antigen-antibody union could be demonstrated in neutral serum-virus mixtures. The experiments were based on the work of Andrewes (1928).

Dilution experiments.

Decimal dilutions of the virus were mixed with an equal amount of undiluted immune rabbit serum and a few seconds later the mixture was diluted 10^{-1} in saline, and injected intradermally. The resulting reactions on the 7th day are given in the following table.

Dilution of virus.	V	V + I-S	(V+I-S) diluted 10^{-1}
10^{-6}	7 mm	---	---
10^{-5}	10 mm	---	---
10^{-4}	12 mm	---	---
10^{-3}	16 mm	---	12 mm
10^{-2}	22 mm	21 mm	15 mm
10^{-1}	16 mm	23 mm	9 mm
Undil.	16 mm	24 mm	14 mm

These indicated that the neutral serum-virus mixture (containing virus dilution 10^{-3}) which was just balanced, could be dissociated by 10^{-1} dilution in saline. The titre of the virus was reduced to 10^{-4} from/

from 10^{-3} by such dilution, but as the control with virus alone was positive in 10^{-6} dilution, the absence of reactivation could not be due to loss of potency of the virus. Firm antigen-antibody union had occurred in mixtures containing smaller amounts of the virus, i.e. 10^{-4} - 10^{-5}, and a relative excess of immune serum.

N.B. From the table, it is seen that the last positive reaction due to undiluted serum-virus mixture was that in which the dilution of the virus was 10^{-2}. All mixtures containing lower concentra-:tions of the virus gave negative reactions. For the purpose of investigation of the dilution phenomenon, the mixture containing the highest concentration of the virus, viz. 10^{-3}, which gave a negative reaction was considered as "just-balanced".

The above experiment was twice repeated with corresponding results.

As previously stated, Todd (1928) in studying the dilution phenomenon in the case of the virus of fowl-plague, could not reactivate a neutral serum-virus mixture by dilution in normal fowl-serum, but only by dilution in saline. Andrewes (1928) working with vaccinia virus found no difference in the results of dilution experiments by using either serum or saline. In the following experiment, the serum-virus mixtures were/

were diluted with nine volumes of saline and normal rabbit serum respectively.

Dilution of virus.	V	V+I-S	(V+I-S) diluted 10^{-1} in	
			N-S	saline
10^{-5}	18 mm	---	---	---
10^{-4}	22 mm	---	---	---
10^{-3}	38 mm	---	---	4 mm
10^{-2}	42 mm	---	---	18 mm
10^{-1}	38 mm	6 mm	12 mm	24 mm
Undil.	26 mm	17 mm	27 mm	28 mm

The neutral serum-virus mixture containing 10^{-2} and 10^{-3} dilutions of the virus could be definitely reactivated by dilution in saline, but not the one containing 10^{-4} dilution. The absence of reactivation in the last could not be due to the incidental dilution of the virus to 10^{-5}, as the control with virus only was positive in that dilution. On the other hand, there was a relative excess of immune serum in the aforesaid mixture. The serum-virus mixtures containing higher concentrations of the virus were partially neutralised by the immune serum, and only slight reactivation was observed by 10^{-1} dilution in normal rabbit serum.

In another experiment dilution in saline yielded dissociation/

dissociation but no so dilution in normal serum.

Dilution of virus.	V	V+I-S	(V+I-S) diluted 10^{-1}	
			N-S	Saline
10^{-5}	12 mm	---	---	---
10^{-4}	14 mm	---	---	---
10^{-3}	17 mm	---	---	---
10^{-2}	19 mm	---	---	6 mm
10^{-1}	32 mm	---	---	12 mm
Undil.	35 mm	17 mm	12 mm	17 mm

The absence of reactivation of the virus from neutral serum-virus mixtures by dilution in normal rabbit serum might have been due to the presence of natural antibodies. Some of these being thermolabile, the normal rabbit serum was heated at 55°C for 30 minutes in an attempt to reduce or inactivate the same. And in another experiment, the 10^{-1} dilutions were made respectively in saline, unheated normal rabbit serum, and heated normal rabbit serum.

Dilution of virus.	V	V+I-S	(V+I-S) diluted 10^{-1} in		
			Saline	N-S	heated N-S
10^{-6}	papules	---	---	---	---
10^{-5}	7 mm	---	---	---	---
10^{-4}	16 mm	---	---	---	---
10^{-3}	17 mm	---	---	---	---
10^{-2}	19 mm	---	---	3 mm	---
10^{-1}	20 mm	---	11 mm	4 mm	6 mm
Undil.	20 mm	7 mm	3 mm	3 mm	2 mm

In/

In this case, the serum-virus mixtures which were more or less balanced were reactivated by dilution not only in saline, but also in unheated normal rabbit serum, and normal rabbit serum heated at 55°C for 30 minutes.

The variable results obtained by diluting neutral serum-virus mixtures in normal rabbit sera from different animals were probably due to differences in the natural antibody content of those sera.

The experiment was repeated with the following result:

Dilution of virus.	V	V+I-S	(V+I-S) diluted 10^{-1} in		
			Saline	N-S	heated N-S
10^{-6}	3 mm	---	---	---	---
10^{-5}	4 mm	---	---	---	---
10^{-4}	11 mm	---	---	---	---
10^{-3}	17 mm	---	---	---	---
10^{-2}	22 mm	---	---	---	10 mm
10^{-1}	25 mm	---	---	---	papules
Undil.	27 mm	---	---	---	14 mm

In this experiment, the reactivation of the virus occurred only in the dilutions made in normal heated serum. The absence of dissociation of the neutral serum-virus mixture by dilution in unheated normal serum was probably due to the high natural antibody content of the serum, which caused masking of the virus/

virus. The cause of failure of reactivation of the virus from the neutral serum-virus mixture by dilution in saline was not quite clear. Andrewes (1928) pointed out that the virus survived better in normal serum than saline, low concentrations of the virus might have been inactivated in saline and failed to produce a cutaneous reaction.

In all these experiments, the contact of the serum and virus was only for a few seconds. The effect of incubation of serum-virus mixtures for 24 hours at 37°C before attempted dissociation by dilution was next investigated. The degree of neutralisation of the serum-virus mixtures, where the contact was for a few seconds was also compared with that observed in mixtures, which had been incu-:bated for 24 hours at 37°C. Dissociation was tested for by 10^{-1} dilution in saline as in the previous observations.

Dilution of virus.	V+Saline (24 hours	V+N-S - 37°C)	V+I-S (0)	V+I-S (24hrs-37°C)	V+I-S 24hrs-37°C and diluted in saline
10^{-3}	31 mm	18 mm	---	---	---
10^{-2}	32 mm	19 mm	---	---	6 mm
10^{-1}	28 mm	22 mm	---	5 mm	17 mm
Undil.		27 mm	papules	11 mm	16 mm

The/

The mixture containing 10^{-2} dilution of the virus was just balanced, and could be reactivated by dilution. When the serum-virus mixtures were incubated before injection, the results were similar to those obtained when the contact was just for a few seconds. Normal rabbit serum had slight inactivating effect on the virus, the reactions with V + Saline being more marked than those given by V + N-S.

Over-neutralised serum-virus mixtures.

In the next series of experiments, the dilution-phenomenon was investigated in regard to mixtures of virus and antiserum, in which definite over-neutrali-:sation of the maximum concentration of virus was produced by the use of hyperimmune serum. It has been suggested by Andrewes (1928) that the dilution phenomenon depends on the fact that diluted virus establishes itself rapidly in the tissues while the protective action of diluted serum takes much longer to develop. An adequate control was provided by diluting the serum beforehand and mixing with the decimal dilutions of the virus to demonstrate the potency of the diluted serum.

Dilution/

Dilution of virus.	V	V+I-S	(V+I-S) diluted in saline to		
			10^{-1}	10^{-2}	10^{-3}
10^{-6}	---	---	---	---	---
10^{-5}	6 mm	---	---	---	---
10^{-4}	7 mm	---	---	---	---
10^{-3}	10 mm	---	---	---	---
10^{-2}	9 mm	---	---	---	---
10^{-1}	11 mm	---	---	---	---
Undil.	18 mm	---	---	---	---

Control with diluted serum

Dilution of virus.	V	V+ diluted I-S		
		I-S 10^{-1}	I-S 10^{-2}	I-S 10^{-3}
10^{-6}	5 mm	---	---	---
10^{-5}	12 mm	---	---	3 mm
10^{-4}	21 mm	---	5 mm	7 mm
10^{-3}	21 mm	---	9 mm	11 mm
10^{-2}	19 mm	---	11 mm	9 mm
10^{-1}	17 mm	---	10 mm	11 mm
Undil.	23 mm	11 mm	11 mm	13 mm

In previous experiments, the failure to reactivate a neutral serum-virus mixture by 10^{-1} dilution in saline might have been due to the immune serum not being/

being put out of action by dilution. In this case, even 10^{-3} dilution of the mixture failed to reactivate the virus. In the control, the immune serum was diluted 10^{-1} - 10^{-3} before mixing with the virus dilutions, and showed a low neutralising titre in 10^{-2} and 10^{-3} dilutions. Firm antigen-antibody union had taken place immediately after mixing, when by the use of a hyperimmune serum, the virus was over-neutralised.

In view of the possibility that qualitative differences between hyperimmune and ordinary immune serum were responsible for the variation in the results of the dilution-phenomenon, the following experiment was devised. Hyperimmune serum was diluted 10^{-1} before testing for antigen-antibody union.

Dilution of virus.	V	V+I-S	(V+I-S)diluted with saline		Control
			10^{-1}	10^{-2}	V+I-S diluted 10^{-2}
10^{-6}	3 mm	---	---	---	---
10^{-5}	14 mm	---	---	---	---
10^{-4}	20 mm	---	---	---	3 mm
10^{-3}	21 mm	---	---	---	11 mm
10^{-2}	22 mm	---	---	---	12 mm
10^{-1}	20 mm	---	12 mm	4 mm	9 mm

The neutral serum-virus mixture, which contained the/

the greatest relative proportion of virus was reac-:tivated on dilution. Reactivation by dilution of diluted hyperimmune serum and virus mixture was shown to correspond with the same phenomenon in ordinary immune-serum virus mixture. Hyperimmune serum appeared to differ from ordinary immune serum only in the degree of neutralising potency.

Concentration of virus by Chamberland L_2 filter-powder.

An attempt was made to concentrate the virus by adsorption to Chamberland L_2 filter powder. 50-60 mgms of filter powder were added to 2 c.c. of a neuro-virus emulsion, which was frequently shaken. After 3 hours contact at room-temperature, the emulsion was centrifuged at 2000 r.p.m. for 10 minutes. Decimal dilutions of the deposit were made and injected intra-:dermally into rabbits, the dilutions of virus only were injected into the same rabbit as a control.

The virus in the filter powder was positive up to 10^{-12}. The control was tested up to 10^{-6} dilution and was positive.

On repetition of the same experiment, the contact of the filter-powder and virus emulsion was for one hour only. The intradermal titration of the powder gave positive results up to 10^{-12}, while the virus alone was positive up to 10^{-9} dilution and not in higher/

higher dilutions. The virus was adsorbed and definitely concentrated by the filter-powder.

In the following experiments, an attempt was made to recover the virus from neutral serum-virus mixtures by adsorption to the filter powder.

Recovery of virus from serum-virus mixtures by powdered filter.

Technique.

Decimal dilutions of neuro-virus were mixed with equal amounts of undiluted antivaccinial rabbit serum, and a few seconds later 20-30 mgms of filter powder were added to each mixture. The contact was for 15 minutes at room-temperature (20°C), the tubes being frequently shaken. The suspensions were then centri-:fuged for 10 minutes at 2000 r.p.m.; the supernatant fluid was poured off and saline was added to restore the original volume (1 c.c.). The tubes were again shaken to ensure a uniform suspension, of which 0.2 c.c. was injected intradermally in the depilated skin of rabbits.

The dissociation of the serum-virus mixture by adsorbing the virus with the filter powder was investi-:gated in the following experiment, the results of which are shown.

Dilution/

Dilution of virus.	V	V+I-S	(V+I-S) in filter powder.
10^{-6}	12 mm	---	---
10^{-5}	15 mm	---	---
10^{-4}	22 mm	---	---
10^{-3}	27 mm	---	11 mm
10^{-2}	28 mm	---	papules
10^{-1}	25 mm	papules	24 mm
Undil.	26 mm	"	32 mm

Firm antigen-antibody union had occurred in those serum-virus mixtures which contained a relative excess of immune serum, or in other words a relative low concentration of virus. Dissociation occurred in mixtures in which the proportion of virus and serum were more nearly balanced.

The experiment was repeated, but in this case no virus could be recovered from neutral serum virus mixtures.

Dilution of virus.	V	V+I-S	(V+I-S) in filter powder.
10^{-6}	---	---	---
10^{-5}	---	---	---
10^{-4}	6 mm	---	---
10^{-3}	13 mm	---	---
10^{-2}	15 mm	---	---
10^{-1}	17 mm	7 mm	9 mm
Undil.	19 mm	12 mm	12 mm

As the contact of the immune serum and virus had been only for a few seconds in previous experiments, the effect of incubation for 24 hours at 37°C on the serum-virus mixtures before adsorption was investi-:gated. The immune serum and the virus dilutions were kept separately under the same conditions and mixed immediately before treatment with the powdered filter. A control was included to test the potency of the virus alone, kept at the same temperature for the same period.. The results are shown below.

Dilution of virus.	V (24 hrs-37°C)	V+I-S (0)	(V+I-S) (0) in filter	(V+I-S) (24 hrs-37°C) in filter
10^{-4}	---	---	---	---
10^{-3}	---	---	---	---
10^{-2}	18 mm	---	---	---
10^{-1}	25 mm	---	9 mm	9 mm
Undil.	15 mm	erythema	20 mm	20 mm

The incubation of the serum-virus mixtures at 37°C for 24 hours did not improve the antigen-antibody union. The filter powder produced dissociation in mixtures, which were more or less balanced.

In another experiment, the fallacy of adsorbing both the virus and antibody from the mixtures thereby masking/

masking the virus was obviated by the following control. The virus and immune serum were separately treated with the powdered filter and the deposits were mixed and injected.

Dilution of virus.	V	V in filter.	V+I-S	(V+I-S) in filter.	V in filter+ I-S in filter.
10^{-6}	---	---	---	---	---
10^{-5}	---	---	---	---	---
10^{-4}	---	---	---	---	---
10^{-3}	13 mm	4 mm	---	---	---
10^{-2}	14 mm	11 mm	---	---	3 mm
10^{-1}	16 mm	12 mm	---	---	5 mm

The filter powders separately treated by the virus dilutions and immune serum, when mixed and injected intradermally gave reactions up to 10^{-2} dilution, while the control with virus only was positive in 10^{-3} dilution. The indication was, that both antibody and virus were adsorbed by a powdered filter. When the virus dilutions were first mixed with the immune serum and then treated by the filter, no active virus was recovered from neutral serum-virus mixtures. This could not be due to adsorption of the antibody masking the virus, as the control showed that the filter powder treated by the antiserum did not adsorb/

adsorb sufficient antibody to neutralise the virus to the same extent as the antiserum. The only satis-:factory explanation was, that firm antigen-antibody union had occurred in neutral mixtures.

The experiment was repeated with the following result.

Dilution in virus.	V	V in filter.	V+I-S	(V+I-S) in filter.	V in filter + I-S in filter.
10^{-6}	7 mm	5 mm	---	---	---
10^{-5}	15 mm	8 mm	---	---	---
10^{-4}	21 mm	12 mm	---	---	---
10^{-3}	22 mm	19 mm	---	---	4 mm
10^{-2}	25 mm	20 mm	---	2 mm	6 mm
10^{-1}	18 mm	24 mm	---	4 mm	10 mm

Those serum virus mixtures, which were just balanced or contained a relatively slight excess of immune serum could be dissociated.

On repetition, the following result was obtained.

Dilution of virus.	V	V in filter.	V+I-S	(V+I-S) in filter.	V in filter + I-S in filter.
10^{-6}	6 mm	---	---	---	---
10^{-5}	12 mm	4 mm	---	---	3 mm
10^{-4}	13 mm	6 mm	---	---	4 mm
10^{-3}	14 mm	7 mm	---	---	5 mm
10^{-2}	18 mm	18 mm	4 mm	2 mm	5 mm
10^{-1}	19 mm	22 mm	9 mm	11 mm	6 mm

The/

The virus could not be recovered from the neutral serum-virus mixtures. The filter powder was shown to adsorb most of the virus and some of the antibody.

Concentration of virus by blood clot.

An attempt was made to adsorb the virus by rabbit blood, which was allowed to clot. A few drops of normal rabbit blood, taken directly from the ear-vein were added to a neuro-virus emulsion, and the blood was allowed to clot and retract. The clot was emulsified in a few drops of saline, and decimal dilutions were prepared. On intradermal injection of the same, positive results were obtained up to 10^{-11} dilution; while the control with virus emulsion was positive up to 10^{-8} dilution.

On repetition, it was confirmed that the virus could be concentrated by the addition of rabbit blood, which was allowed to clot.

In the following experiments, the neutral serum-virus mixtures were dissociated by adsorbing the virus with rabbit blood clot.

Dissociation of neutral serum-virus mixtures by blood clot.

Technique.

Decimal dilutions of virus in saline were mixed with an equal volume of antivaccinial serum. A few drops/

drops of blood were allowed to drop from the ear-vein of a normal rabbit into the mixture. When clotting and retraction had taken place, the mixtures were centrifugalised at 2000 r.p.m. for ten minutes and the supernatant fluid poured off. Normal saline was added to the clot to make up the original volume, and a uniform suspension made, of which 0.2 c.c. was in-:jected intradermally.

In one experiment, the serum-virus mixtures were incubated at 37°C for 24 hours, the serum and virus dilutions were also kept separately under the same conditions, and were mixed immediately before being subjected to dissociation by rabbit blood clot. The idea was to test the effect of incubation on antigen-antibody union, and to compare the results with un-:incubated mixtures.

Dilution of virus.	V	V+I-S (24 hrs-37°C)	(V+I-S) (24hrs-37°C) in clot.	(V+I-S) (0) in blood-clot.
10^{-5}	---	---	---	---
10^{-4}	---	---	---	---
10^{-3}	16 mm	---	---	---
10^{-2}	17 mm	---	---	9 mm
10^{-1}	15 mm	---	---	5 mm
Undil.	17 mm	8 mm	3 mm	19 mm

Virus/

Virus could be recovered to some extent from the neutral serum-virus mixtures when the contact was for a few seconds, but after 24 hours contact at 37°C, this could not be effected.

The experiment was repeated, but a control was included to obviate the fallacy of adsorbing the virus on the blood-clot and thus masking the virus.

Dilution of virus.	V	V in clot	(V+I-S)	(V+I-S) in clot	V in clot + I-S in clot
10^{-6}	3 mm	---	---	---	---
10^{-5}	11 mm	---	---	---	---
10^{-4}	16 mm	---	---	---	---
10^{-3}	17 mm	---	---	---	---
10^{-2}	19 mm	19 mm	---	---	---
10^{-1}	22 mm	26 mm	---	---	---

No virus could be recovered from neutral serum-virus mixtures. It was also shown that a considerable amount of antibody was adsorbed by the blood-clot, hence the result was not valid. It also showed, that the blood clot method was not very satisfactory for the demonstration of dissociation.

Recovery of the virus from neutral serum-virus mixtures by precipitating euglobulins.

Technique.

The neuro-virus emulsion under investigation was diluted with nine volumes of distilled water, carbon-

di/

di-oxide gas was passed through the mixture for at least three minutes till a fine precipitate was visible. The precipitate was thrown down by centrifugalisation at 2000 r.p.m. for ten minutes. The supernatant fluid was poured off, and the deposit was taken up in saline and brought to the original volume (1 c.c.). Serial dilutions were then made, and 0.2 c.c. of each was injected intradermally.

The possibility of a firm antigen-antibody union taking place after incubation of the serum-virus mixtures for 24 hours at 37°C was investigated and compared with the serum-virus union in the unincubated mixtures. Decimal dilutions of neuro-virus emulsion were kept in contact with equal volumes of undiluted immune rabbit serum for 24 hours at 37°C. In another batch, the serum and virus were kept separately at 37°C for the same period, and were mixed immediately before dissociation, which was tested for by precipitating the virus in the euglobulins.

Dilution of virus.	V (24hrs-37°C)	V+I-S (0)	euglob. from (V+I-S) (0)	euglob. from (V+I-S) (24hrs-37°C)
10^{-4}	---	---	---	---
10^{-3}	---	---	---	---
10^{-2}	22 mm	---	4 mm	4 mm
10^{-1}	20 mm	---	5 mm	8 mm
Undil.	papules	---	10 mm	9 mm

The/

The antibody content of the antiserum was probably comparatively low,, and excess of immune serum was not present in the neutral serum-virus mixtures, hence virus was recovered to some extent in the euglobulins from all the neutral serum-virus mixtures. Incuba-:tion of the serum-virus mixtures for 24 hours at 37°C did not improve the antigen-antibody union as compared with a few seconds contact.

In another experiment, a control was included to find out if the antibody was associated with the euglobulins.

Dilution of virus.	V	V+I-S	euglob.from (V+I-S)	V+ euglob. from I-S
10^{-6}	---	---	---	---
10^{-5}	4 mm	---	---	---
10^{-4}	12 mm	---	---	---
10^{-3}	16 mm	---	---	3 mm
10^{-2}	20 mm	---	---	4 mm
10^{-1}	22 mm	---	---	11 mm

When undiluted immune serum and virus dilutions were mixed, and an attempt was made to recover the virus by precipitating the euglobulins, no virus was recovered from neutral serum-virus mixtures. This was either due to inactivation of the virus or pre-:cipitation of a considerable amount of antibody in the euglobulins along with the virus, which led to masking/

masking of the virus. In the control, the antibody content of the euglobulins of the antiserum was tested by titrating it against virus dilutions. Positive results up to 10^{-3} dilution of the virus were obtained, while the virus by itself was positive up to 10^{-5} dilution. It followed therefore, that the non-recovery of the virus in the euglobulins from neutral serum-virus mixtures was not due to neutra-:lisation of the virus by concomitant antibody. The only satisfactory explanation was the inactivation of the virus.

The experiment was repeated with the following result.

Dilution of virus.	V	V+I-S	euglob.from (V+I-S)	V+euglob.from I-S
10^{-6}	3 mm	---	---	---
10^{-5}	5 mm	---	---	---
10^{-4}	8 mm	---	---	---
10^{-3}	11 mm	---	---	---
10^{-2}	12 mm	---	5 mm	---
10^{-1}	17 mm	6 mm	12 mm	6 mm

Dissociation of the virus was demonstrable in the just balanced serum-virus mixture. When the immune serum and virus dilutions were mixed and then disso-:ciated/

:ciated by precipitating the euglobulins, positive reactions up to 10^{-2} dilution of the virus were obtained on testing in vivo. But when the euglo-:bulins from the antibody were mixed with virus dilutions, positive results only up to 10^{-1} dilution of the virus were demonstrable. The antibody content of both the series of mixtures being the same, the suggestion was, that more of the virus than anti-:body was precipitated in the euglobulins.

In another experiment, the control consisted in mixing the euglobulins both from the virus emulsion and the antiserum instead of using the serum-globulins only as in the previous experiments.

Dilution of virus.	V	euglob. from V.	V+I-S	euglob. from (V+I-S)	Control V euglob. + I-S euglob.
10^{-6}	---	---	---	---	---
10^{-5}	6 mm	3 mm	---	---	---
10^{-4}	9 mm	6 mm	---	---	---
10^{-3}	13 mm	11 mm	---	---	4 mm
10^{-2}	16 mm	12 mm	---	---	6 mm
10^{-1}	21 mm	24 mm	---	11 mm	9 mm
Undil.	24 mm	29 mm	6 mm	7 mm	

This experiment confirmed the previous findings, the virus was recovered from those neutral serum-virus mixtures/

mixtures, which contained the least relative proportion of antibody.

Had the absence of the virus from neutral serum-virus mixtures containing 10^{-3} to 10^{-2} dilutions of the virus been due to an excessive amount of antibody being precipitated in the euglobulins thereby neutralising the virus, the control would have given negative results. But the control being positive, inactivation of the virus must have occurred in over-neutralised serum-virus mixtures.

Neutralisation experiments by routes other than intradermal.

In the experiments conducted so far the serum-virus mixtures were injected intradermally for the demonstration of neutralisation. Andrewes (1928) showed that a mixture, which was found to be neutral on intradermal injection was active when administered intracerebrally or intratesticularly. He failed to demonstrate neutralisation of the virus by the antiserum, when the mixture was injected into the brain.

Fairbrother (1932) came to the same conclusion, but he was able to demonstrate neutralisation of the virus by the antivaccinial serum, when the virus suitably diluted remained in contact with the antiserum for at least four hours at room-temperature.

He/

He used 1000 skin-doses of the virus for this purpose, and did not attempt seriously to show, if smaller ammounts of the virus were more susceptible to the antiserum.

In the succeeding experiments, minimal amounts of the virus were used, and their effects observed. Hyper-immune serum was used throughout in an attempt to demonstrate neutralisation, when mixtures were injected into the brain, anterior chamber of eye and the cornea.

Intracerebral and intra-ocular administration.

Technique.

The hair of the scalp were clipped and tincture of iodine was applied. The rabbit was fully anaesthe-:tised, and the middle point of the line joining the two outer canthi was the site of injection. With the half blade of the scissors, a hole was bored and 0.5-0.8 c.c. of the emulsion under investigation was in-:jected deeply into the brain substance. The rabbits were kept under observation for at least 14 days. The symptoms of virus activity were anorexia, loss of weight, and marked asthenia with ataxia, tremor and paresis in a certain number of cases.

Intra-ocular injection.

The rabbit was fully anaesthetised, an intra-:dermal/

:dermal needle was introduced at the corneo-scleral junction into the anterior chamber, and a few drops of aqueous humor were allowed to flow out; then the syringe was attached in position, and 0.2 c.c. of the material under investigation injected intraocularly. After an incubation-period of 4-5 days, the symptoms of infection were congestion, muco-purulent discharge, ground-glass appearance of the cornea with pannus-formation in certain cases.

Minimal amounts of the virus required to produce encephalitis and irido-cyclitis.

An emulsion of neuro-virus, which was positive up to 10^{-6} dilution on intradermal titration, was inoculated intracerebrally in 10^{-5} and 10^{-6} dilutions in adult rabbits. The animals were kept under obser-:vation for 3 weeks, but no untoward symptoms were demonstrable.

An attempt was next made to produce an irido-cyclitis by intra-ocular injections of 10^{-3} - 10^{-6} dilutions of the same virus emulsion, but no reaction occurred. In later experiments, 10^{-2} - 10^{-1} dilutions of the virus gave positive results on intracerebral and intra-ocular injections.

In short, 10^{-3} to 10^{-4} skin-doses were required to produce encephalitis or irido-cyclitis on injection into adult rabbits.

IMMUNITY/

IMMUNITY REACTIONS.

An attempt was made to demonstrate neutralisation of the virus by the hyper-immune serum after injection of the serum-virus mixture into the brain, anterior chamber and cornea. The effect of incubation on serum-virus union was investigated, and compared with unincubated mixtures. Decimal dilutions of neuro-virus emulsion were mixed with 0.5 c.c. of undiluted hyper-immune antivaccinial serum in equal volumes and (a) immediately after were diluted 10^{-1} in Locke's fluid for the demonstration of dissociation, (b) were incubated at room-temperature for 18 hours, and then diluted as in (a). The mixtures were injected intra-:cerebrally, intra-ocularly and intra-corneally into adult rabbits. The mixture and the control were injected into the same rabbit as far as possible.

(a)

Contact for few seconds at room-temperature.

Dilution of virus.	V+N-S	V+I-S	(V+I-S) dil.10^{-1}	V+diluted I-S 10^{-1}
1/20	I-C + I-O +			
1/10		I-C + I-O +		I-C + I-O +
Undil.		I-C - Cornea -	I-C +	I-C + Cornea -

I-C = Intra-cerebral.
I-O = Intra-ocular.
\+ = denotes active infection.
\- = denotes absence of any reaction.

Thus/

Thus virus when mixed with an equal amount of immune serum, and injected intracerebrally immediately after was found to have been neutralised, but the neutral serum-virus mixture was reactivated on dilution. It was also found that a serum-virus mixture, which was neutral on injection into the cornea, was able to produce a positive result on intracerebral injection into the same rabbit. The varying resistance of rabbits to intracerebral injec-:tion of the virus was noted. Thus in one animal the intracerebral injection of (Undil. V+I-S) produced no effect, the other injected with (V 10^{-1} + I-S) developed typical encephalitis.

(b)

Contact for 18 hours at room-temperature.

Dilution of virus.	V+N-S	V+I-S	(V+I-S) diluted 10^{-1}	(V+diluted I-S 10^{-1})
1/20	I-C ±			
1/10	I-C + I-C +	I-C – I-C +	I-C –	I-C + Cornea –
Undil.		I-C – I-C +	I-C –	I-C + I-C +

Definite neutralisation of the virus by immune serum after intracerebral inoculation was demonstrable, but the serum-virus mixture, which was found to be neutral/

neutral on intracerebral inoculation, produced a definite reaction when injected into the anterior chamber, Similarly the mixture which was found to be neutral on intra-corneal injection, was active on intracerebral injection.

The varying results obtained after injection of the serum-virus mixture into the brain and cornea were probably due to a comparative rapid dispersion of the constituents in the former site. The serum-virus mixture which was negative on intracerebral injection and positive intra-ocularly was probably neutralised by the brain tissue, but aqueous humor could not effectively deal with it. Moreover a mild reaction in the anterior chamber could be easily observed, but the same did not obtain in the brain.

The dilution phenomenon in the neutral serum-virus mixtures could not be elicited due to the deterioration of the virus at room-temperature in 18 hours.

In the next experiment, decimal dilutions of the neuro-virus were mixed with equal volumes of undiluted immune serum and incubated at 37°C for 24-48 hours, and then injected intracerebrally and intra-ocularly. The virus was active on intradermal titration in a dilution of 10^{-6}.

Route/

Route of inoculation ..... Intracerebral.

Dilution of virus.	V + Saline (24 hrs - 37°C)	V + I-S (24 hrs - 37°C)
10^{-3}	---	---
10^{-2}	+	---
10^{-1}		---

Route of inoculation ..... Intra-ocular.

Dilution of virus.	V+ Saline (48 hrs - 37°C)	V + I-S (48 hrs - 37°C)
10^{-1}	++	---
Undil.	+++	+

At least ten lethal doses of the virus were neutralised by an equal volume of undiluted immune serum after 24 hours incubation at 37°C. After 48 hours incubation at 37°C neutralisation of the virus by the immune serum was demonstrable by intra-ocular injection.

The previous experiment revealed that neutralisa-:tion of the virus by the antiserum was very difficult to demonstrate after intra-ocular injection of the serum-virus mixture. In the following experiment, the serum-virus mixtures were injected intra-ocularly after varying periods of incubation, the control with V + Saline being injected into the opposite eye. The result/

result was as under.

Dilution of virus.	V+Saline (0)	V+I-S (0)	V+Saline (24 hrs-37°C)	V+I-S	V+Saline (48 hrs - 37°C)	V+I -S
10^{-3}			---	---		
10^{-2}			---	---		
10^{-1}	+++	+	+++	+	---	---
Undil.					+++	---

Unheated virus, which was positive in the skin in 10^{-6} dilution produced an irido-cyclitis and keratitis in 10^{-1} dilution (after 24 hours incubation at 37°C), but not in smaller concentrations. The immune serum neutralised the virus to some extent immediately on mixing, complete neutralisation occurred after 48 hours incubation at 37°C. The virus deteriorated at least by 10^{-1} in this time.

Dissociation of the neutral serum-virus mixtures.

An attempt was again made to demonstrate antigen-antibody union after intracerebral injection. The neuro-virus dilutions and undiluted immune serum were mixed in equal volumes and (a) diluted 10^{-1} in saline and injected intracerebrally (b) incubated at 37°C for 24 hours and again diluted as before.

Injected/

Injected immediately after mixing:

Dilution of virus.	V + Saline	V+I-S	(V+I-S) diluted 10^{-1}
10^{-1}	---	---	---
Undil.		---	+

Incubated at 37°C for 24 hours.

Dilution of virus.	V + Saline	V+I-S	(V+I-S) diluted 10^{-1}
10^{-1}	---	---	---
Undil.	+	---	+

Immune serum could neutralise the virus immediately on mixing but the neutral serum-virus mixture could be reactivated after 10^{-1} dilution in saline, although the contact was prolonged for 24 hours at 37°C. The controls injected with 10^{-1} dilution of virus with an equal volume of saline failed to react showing thereby the varying response of rabbits to intracerebral injec-:tion of neuro-virus.

The surviving rabbits were re-injected intra-:cerebrally with undiluted neuro-virus on the 14th day, all died of encephalitis within 6 days.

The experiment was repeated. The serum-virus mixtures were injected immediately after mixing both intracerebrally/

intracerebrally and intra-ocularly. Dissociation was tested for by dilution with 4 volumes of saline.

Dilution of virus.	V+N-S	V+I-S	(V+I-S) dil.1/5	V + dil. I-S 1/2
1/20	I-O +			
1/10	I-C + I-O +	I-C - I-O +		
1/5	I-O +			
Undil.	I-C + I-O +	I-C - I-O +	I-C + I-O +	I-C +

Firm antigen-antibody union could not be demonstrated in serum-virus mixtures after intracerebral injection. The control showed that antiserum diluted even 1/2 before mixing with undiluted virus failed to neutralise it. The neutral serum-virus mixtures were in all probability more or less balanced, the virus being inhibited therein. Had a more potent serum been available, firm antigen-antibody union might have been demonstrated in overneutralised mixtures. When a similar neurotropic virus, viz. louping-ill, was over-neutralised by means of a potent antiserum, inactivation of the virus was demonstrable. This would be fully described in a later section.

Absorption experiments.

An attempt was made to demonstrate antibody absorption in serum-virus mixtures containing an excess of the antigen.

Technique.

A 10% emulsion of bacteria-free neuro-virus was incubated with an equal amount of immune serum for 18 hours at 37°C. Then it was centrifuged at 2000 r.p.m. for 15 minutes. A small amount of the supernatant fluid was kept to see if excess of virus had been used for absorbing the antibodies, the rest was filtered through a Seitz E.K. filter to remove the virus. (Non-filterability of the virus through a Seitz E.K. filter will be described in a later section). The filtrate was labelled "absorbed serum". It's antibody content was tested, it was found to be as active as the immune serum.

Dilution of virus.	Serum-virus supernatant.	Seitz filtrate.	V	V+I-S	V+absorbed serum.
10^{-3}	---	---	---	---	---
10^{-2}	---	---	14 mm	---	---
10^{-1}	---	---	19 mm	---	---
Undil.	8 mm	---	25 mm	---	---

The result also showed that an excess of virus had been used to absorb the antibodies, and the Seitz filtrate was free from virus.

In/

In another experiment, the absorbing dose of the neuro-virus was markedly increased, and the immune serum was diluted to reduce its antibody content. Absorption was allowed to take place over a long period at 37°C. The neuro-virus and antiserum, which had proved bacteria-free by anaerobic and aerobic cultures were used exclusively. 10 c.c. of 10^{-1} and 10^{-2} dilutions of antivaccinial serum were used to emulsify 6 gms of vaccinial rabbit brain. 1 c.c. of ether was added to counteract bacterial contamination. Rubber-stoppers sealed with paraffin were used to prevent evaporation of the ether. The emulsion was incubated for 3 days at 37°C. The tubes were vigorously shaken once every day to ensure thorough mixing of the contents. After 3 days, the emulsion was freed from ether by keeping it exposed to air for some time. It was then centrifuged for 30 minutes at 2500 r.p.m. The supernatant fluid was pipetted off and spun twice for 30 minutes at the same speed. The supernatant fluid was then heated at 55°C for 45 minutes to destroy the virus. This absorbed serum was tested for the presence of antibodies by titration against the virus. The control immune serum was kept under identical conditions.

Dilution/

Dilution of virus.	V	V+dil. 10^{-1}	I-S V+dil. I-S 10^{-2}	V+absorbed serum 10^{-1}	V+absorbed serum 10^{-2}
10^{-6}	4 mm	---	---	---	---
10^{-5}	6 mm	---	---	---	---
10^{-4}	8 mm	---	---	---	---
10^{-3}	9 mm	---	---	---	3 mm
10^{-2}	12 mm	---	5 mm	---	6 mm
10^{-1}	14 mm	3 mm	6 mm	4 mm	11 mm

Partial antibody absorption from the 10^{-2} dilution of the serum had apparently occurred.

The experiment was repeated, and the technique employed was the same as before, except that the virus was removed by Seitz E.K. filter instead of heat from the absorbed serum.

Dilution of virus.	V	V+diluted I-S 10^{-1}	V+diluted I-S 10^{-2}	V+absorbed serum 10^{-1}	Seitz filtrate
10^{-6}	---	---	---	---	
10^{-5}	9 mm	---	---	---	
10^{-4}	15 mm	---	---	---	
10^{-3}	19 mm	---	---	7 mm	
10^{-2}	24 mm	---	11 mm	11 mm	
10^{-1}	26 mm	---	15 mm	31 mm	---
Undil.					---

Partial/

Partial antibody absorption from 10^{-1} dilution of absorbed serum was demonstrable.

Another experiment brought out the contrast between immune and absorbed serum more clearly.

Dilution of virus.	V	V+diluted I-S 10^{-2}	V+diluted I-S 10^{-1}	V+absorbed serum 10^{-2}	V+absorbed serum 10^{-1}
10^{-6}	---	---	---	---	---
10^{-5}	---	---	---	---	---
10^{-4}	7 mm	---	---	---	---
10^{-3}	12 mm	---	---	5 mm	4 mm
10^{-2}	14 mm	---	---	15 mm	9 mm
10^{-1}	17 mm	11 mm	4 mm	17 mm	11 mm

The previous results were confirmed as shown in the protocol.

Antibody absorption by killed virus was next attempted. Two vaccinial brains were emulsified in 20 c.c. of 10^{-2} dilution of immune serum. The emulsion was divided into two portions, of which one was immed-:iately heated at 56°C for 45 minutes to kill the virus. Both portions were incubated for 5 days at 37°C. After 5 days, the tubes were opened, the ether was allowed to evaporate and the emulsions were centri-:fuged as described before. The supernatant fluid, which was obtained from the emulsion containing the live/

live virus was heated at 56°C for 45 minutes to destroy the virus. The serum, which had been treated by dead virus was not heated a second time. The results of titration of the antibody content of absorbed sera are shown below.

Dilution of virus.	V	V+diluted I-S 10^{-2}	V+serum absorbed with live virus 10^{-2}	V+serum absorbed with heated virus 10^{-2}
10^{-6}	3 mm	---	4 mm	2 mm
10^{-5}	11 mm	2 mm	7 mm	9 mm
10^{-4}	25 mm	6 mm	papules	17 mm
10^{-3}	32 mm	14 mm	16 mm	29 mm
10^{-2}	34 mm	24 mm	22 mm	28 mm
10^{-1}	12 mm	28 mm	21 mm	24 mm
Undil.	19 mm	22 mm	22 mm	14 mm

Partial antibody absorption by killed and live virus was demonstrable but the results with live virus were better.

The experiment was repeated. Incubation of the serum-virus emulsion was reduced to 22.5 hours at 37°C (instead of 5 days).

Dilution/

Dilution of virus.	V	V+ diluted I-S 10^{-2}	V+ serum absorbed with live virus 10^{-2}	V+ serum absorbed with dead virus 10^{-2}
10^{-6}	12 mm	---	---	---
10^{-5}	13 mm	---	---	---
10^{-4}	13 mm	---	---	---
10^{-3}	18 mm	---	4 mm	---
10^{-2}	21 mm	12 mm	papules	4 mm
10^{-1}	22 mm	16 mm	13 mm	5 mm
Undil.		20 mm	14 mm	11 mm

Killed virus failed to absorb the antibodies, but the live virus produced some degree of absorption.

The antibody absorption as shown in the previous experiments might have been due to non-specific factors. In the following experiment, normal rabbit brain was emulsified in 10^{-1} dilution of immune serum to act as a further control.

Dilution/

Dilution of virus.	V	V+I-S	V+ serum absorbed with normal brain	V+ serum absorbed with heated virus	V+ serum absorbed with live virus.
10^{-6}	---	---	---	---	---
10^{-5}	5 mm	---	---	---	---
10^{-4}	3 mm	---	---	---	2 mm
10^{-3}	4 mm	---	---	6 mm	7 mm
10^{-2}	4 mm	---	17 mm	18 mm	14 mm
10^{-1}	12 mm	---	19 mm	26 mm	16 mm
Undil.		papules	22 mm	28 mm	17 mm

Rabbit brain containing live virus absorbed neutralising antibodies from immune serum to a greater extent than did brain containing heat killed virus, but the latter was more effective than normal brain. Partial antibody absorption by normal brain could only be due to non-specific factors, as the immune serum and normal brain were obtained from homologous animals, viz. rabbits.

Antibody absorption by normal brain was again tested. The immune serum was diluted 1/50 beforehand, and incubation of serum-virus emulsion was for 3 days at 37°C.

Dilution/

Dilution of virus.	V	V + I-S	V+serum absorbed with normal brain
10^{-6}	5 mm	---	---
10^{-5}	papules	---	---
10^{-4}	papules	---	---
10^{-3}	13 mm	13 mm	13 mm
10^{-2}	16 mm	16 mm	16 mm
10^{-1}	16 mm	16 mm	19 mm

Antibody absorption by normal brain was not demonstrable in this instance.

Role of leucocytes in the immunity mechanism.

Douglas and Smith (1930) found that whole blood from rabbits immunised against vaccinia produced a better neutralisation of the virus than the separated serum. Washed blood cells from the same source, when compared with normal cells, definitely enhanced the action of the serum.

Fairbrother (1933) suggested that leucocytes from immune rabbits in the presence of antiserum were more potent in their action on the virus, than normal leucocytes; but in his comparative tests he used an amount of "immune" leucocytes which was three times that of the normal cells.

In the following experiments, the action of the leucocyte/

leucocyte fraction of blood from normal and immune rabbits was investigated. It should be noted that the leucocyte fraction contained a certain admixture of platelets.

Method of preparation of leucocyte suspensions.

As immune animals rabbits were used, which 7-21 days previously had been employed for intradermal titrations of the vaccinia virus and serum-virus mixtures (vide supra). The hair of the chest was clipped as close as possible, and tincture of iodine was applied. 10 c.c. of equal volumes of a 5% suspension of aleuronat and sodium nucleinate were injected intra-pleurally on each side under ether anaesthesia. 20-24 hours later, the rabbit was bled by cardiac puncture, and the blood so obtained was mixed with a solution of 10% sodium citrate and 5% glucose (nine volumes of blood to one volume of glucose-citrate). The exudate from the pleural cavity was mixed with an equal volume of a solution containing 2% citrate and 1% glucose. The blood and pleural exudate were spun for 20 minutes at 2500-3000 r.p.m. The supernatant fluid was pipetted off, and the leucocytic cream was removed. At first the leucocytes from the blood and pleural exudate were mixed, but later they were kept separate. The

leucocytes were taken up in 7-10 c.c. of 1% citrate solution, thoroughly shaken and respun for 7-10 minutes at 2000-3000 r.p.m. In this manner they were washed at least three times. The total leucocyte counts were made and it was found that animals varied considerably in their yield of leucocytes. Rabbits rendered immune to vaccinia virus invariably gave better yields than normal rabbits in both blood and pleural exudates. In any one experiment the leuco-:cyte suspensions from normal and immune rabbits were numerically equalised by dilution.

Effect of leucocytes on the vaccinia virus.

The action on the virus of "immune" leucocytes per se, and through the mediation of the immune serum was tested. The immune serum was diluted 1/50 beforehand. Decimal dilutions of neuro-virus were mixed with equal volumes of (a) suspensions of leucocytes from normal rabbits, (b) suspensions of leucocytes from immune rabbits, (c) 1/50 dilution of antivaccinial serum, (d) a mixture of leycocyte suspension and diluted immune serum. The concentra-:tion of virus in those mixtures, which were to be compared with each other, was equalised by the addition of a solution of 1% sodium citrate. After varying periods of incubation, 0.2 c.c. of the mixtures were injected/

injected intradermally.

Experiment No.1.

Incubated for 18 hours at 37°C.

Dilution of virus.	V	V+I-S+ citrate	V+I-S+immune leucocytes	V+immune leuco-:cytes + citrate
10^{-4}	---	---	---	---
10^{-3}	11 mm	---	---	---
10^{-2}	13 mm	---	---	---
10^{-1}	17 mm	16 mm	14 mm	papules
Undil.		17 mm	18 mm	21 mm

The "immune" leucocytes definitely inactivated the virus per se, but had no definite action on the immune serum-virus mixtures.

Experiment No.2.

The behaviour of leucocytes from normal and immune rabbits was compared. The total leucocyte count was equalised in all the suspensions to 2000 per c.mm. The mixtures were (a) injected intra-:dermally immediately after mixing, (b) incubated for 2 hours at 37°C before testing in vivo.

(a)/

(a)

Injected immediately after mixing.

Dilution of virus.	V	V+N-Leuco.	V+Imm. Leuco.	V+Imm.Leuco. + citrate	V+I-S + citrate
10^{-6}	9 mm				
10^{-5}	22 mm				
10^{-4}	22 mm				
10^{-3}	19 mm	17 mm	11 mm	papules	13 mm
10^{-2}	23 mm	18 mm	12 mm	4 mm	21 mm
10^{-1}	23 mm	21 mm	18 mm	12 mm	22 mm
Undil.		20 mm	17 mm	22 mm	23 mm

N-Leuco. = Normal leucocytes
Imm.Leuco. = Immune leucocytes

(b)

Time of contact (2 hours at 37°C).

Dilution of virus.	V	V+N-Leuco.	V+Imm. Leuco.	V+N-Leuco. + I-S	V+Imm.Leuco. + I-S
10^{-6}	---				
10^{-5}	---				
10^{-4}	---				
10^{-3}	16 mm	11 mm	---	---	---
10^{-2}	19 mm	16 mm	---	---	---
10^{-1}	16 mm	18 mm	12 mm	4 mm	---
Undil.		31 mm	21 mm	17 mm	9 mm

"Immune" leucocytes were effective in neutralising

the/

the virus to some extent immediately on mixing, but the action was more marked after incubation: they also definitely enhanced the action of immune serum on the virus. In experiment No.1 the variable result was probably due to the fact, that leucocytes had clumped together, and settled at the bottom during 18 hours incubation at 37°C; and so were not in close contact with the serum-virus mixtures.

Normal leucocytes had no action on the virus per se even after incubation.

Experiment No.3.

The previous experiment was repeated with the following results. The total leucocyte count was 63,000 per c.mm.

Injected immediately after mixing.

Dilution of virus.	V	V+N-Leuco. +citrate	V+Imm.Leuco. + citrate	V+N-Leuco. + I-S	V+Imm.Leuco. + I-S
10^{-6}	---				
10^{-5}	4 mm	papules	---	---	---
10^{-4}	15 mm	16 mm	---	13 mm	3 mm
10^{-3}	16 mm	16 mm	13 mm	16 mm	7 mm
10^{-2}	18 mm	17 mm	18 mm	16 mm	8 mm
10^{-1}	21 mm	17 mm	18 mm	15 mm	9 mm
Undil.		24 mm	19 mm	15 mm	12 mm

Time/

Time of contact (2 hours - 37°C).

Dilution of virus.	V	V+N-Leuco. + citrate	V+Imm.Leuco. + citrate	V+N-Leuco. + I-S	V+Imm.Leuco. + I-S
10^{-6}	---				
10^{-5}	21 mm	---	---	---	---
10^{-4}	24 mm	18 mm	---	18 mm	---
10^{-3}	28 mm	18 mm	4 mm	22 mm	---
10^{-2}	36 mm	24 mm	18 mm	24 mm	9 mm
10^{-1}	37 mm	23 mm	22 mm	28 mm	15 mm
Undil.		24 mm	18 mm	26 mm	12 mm

The previous observations were confirmed.

Experiment No.4.

The previous experiments were repeated, only "immune" leucocytes were used this time instead of normal and "immune" both.

Injected immediately after mixing.

Dilution of virus.	V	V+Leuco. +citrate	V + Leuco. + I-S	V + I-S +citrate
10^{-5}	---	---	---	---
10^{-4}	---	---	---	---
10^{-3}	papules	papules	---	---
10^{-2}	16 mm	9 mm	---	---
10^{-1}	17 mm	11 mm	---	---
Undil.		12 mm	17 mm	---

Time/

Time of contact (2 hours at 37°C).

Dilution of virus.	V	V+Leuco. +citrate	V+ Leuco. + I-S	V+ I-S + citrate
10^{-6}	papules			
10^{-5}	papules			
10^{-4}	13 mm	12 mm	---	---
10^{-3}	18 mm	12 mm	---	---
10^{-2}	24 mm	17 mm	9 mm	---
10^{-1}	39 mm	18 mm	14 mm	14 mm
Undil.		19 mm	18 mm	19 mm

The "immune" leucocytes had no effect on the virus, they rather protected it from the action of the antiserum. They behaved like normal leucocytes. Probably the rabbit in this case was not highly immune. In the previous experiments, the leucocytes from the blood and pleural exudate had been pooled. The action of the immune leucocytes on the virus per se might have been due to the antibodies absorbed from the circulation, which might not have been removed even after three washings with large volumes of glucose-citrate solution. As the antibody content of the pleural exudate was in all probability very low, the leucocytes from the pleural exudate were kept separate from those of the blood, and were tested separately in the following experiment.

Experiment/

Experiment No.5.

(a)

Contact 2 hours at 37°C.

Dilution of virus.	V	V+ N-Leuco.	V+Imm.Leuco. (Blood)	V+Imm.Leuco. (Pleura)
10^{-5}	---			
10^{-4}	---	7 mm	---	---
10^{-3}	9 mm	12 mm	---	---
10^{-2}	17 mm	12 mm	---	---
10^{-1}	19 mm	22 mm	10 mm	14 mm
Undil.		20 mm	11 mm	14 mm

(b)

Contact for 20 hours at room-temperature.

Dilution of virus.	V	V+N-Leuco.	V + Imm.Leuco. (Blood)	V+Imm.Leuco. (Pleura)
10^{-3}	---	21 mm	10 mm	10 mm
10^{-2}	28 mm	25 mm	17 mm	15 mm
10^{-1}	31 mm	26 mm	21 mm	20 mm
Undil.		29 mm	22 mm	23 mm

Normal leucocytes had a protective effect on the virus. "Immune" leucocytes both from blood and pleura definitely inactivated the virus. 20 hours incubation at 18°-22°C did not give such good results as two hours at 37°C.

In/

In the experiments so far recorded, the leucocyte-virus mixtures were injected intradermally. The effect of "immune" leucocytes on the inactivation of the virus after intracerebral injection into young growing rabbits was next investigated.

Experiment No.6.

Contact for 18 hours at room-temperature.

Dilution of virus.	V + Saline	V+Imm.Leuco.	V + I-S
1/16	+	−	
1/8		+	+
1/4		+	+
1/2	+	+	+

Minimal concentrations of the virus were inacti-:vated by "immune" leucocytes.

Summary of previous observations.

The "immune" leucocytes definitely inactivated the virus per se after the leucocyte-virus mixtures were injected intradermally or intracerebrally. The only exception was Experiment No.4, in which they behaved more or less like normal leucocytes, and protected the virus from the action of the antiserum, probably the rabbit from which they were derived had not developed a high grade of immunity.

Normal/

Normal leucocytes either did not diminish the potency of the virus as in Experiments Nos.2 and 3 or had a protective effect on the virus (No.5).

"Immune" leucocytes were effective immediately on mixing, but the action was better marked when the mixtures were incubated for two hours at 37°C (Experiments Nos.1,2, and 3). In Experiment No.5 20 hours incubation at room-temperature did not give such good results as 2 hours at 37°C.

"Immune" leucocytes definitely enhanced the action of immune serum-virus mixtures (Nos.2 and 3), but had no definite action on immune serum-virus mixtures in No.1. In the previous experiments the maximum time of incubation was 2 hours, while in this experiment (No.1) the incubation was for 18 hours at 37°C, and the leucocytes had clumped together, and settled at the bottom, and so were not in close contact with serum-virus mixtures.

Effect of spleen emulsion on the virus.

The effect of a spleen emulsion from normal and immune rabbits on the virus was next tested. The spleen was emulsified in saline and freed from blood by at least three washings in saline. Equal volumes of spleen emulsion and decimal dilutions of the virus were mixed and incubated for 2 hours at 37°C and then injected/

injected intradermally. The action of immune and normal leucocytes obtained from the blood and pleural exudates of the same rabbits was again investigated at the same time. The result was as under.

Dilution of virus.	V	V+N-Leuco.	V+Imm.Leuco. (Blood)	V+Imm. Leuco. (Pleura)	V+N spl.	V+Imm. spleen
10^{-6}	---	---	---	---	---	---
10^{-5}	---	---	---	---	---	---
10^{-4}	---	papules	---	---	papules	10 mm
10^{-3}	---	12 mm	papules	---	3 mm	12 mm
10^{-2}	18 mm	15 mm	11 mm	---	15 mm	17 mm
10^{-1}	19 mm	15 mm	17 mm	20 mm	16 mm	17 mm
Undil.		18 mm	18 mm	22 mm	17 mm	18 mm

The effect of leucocytes on the virus was con-:firmed, but the normal spleen acted much better on the virus than immune spleen. This might have been due to the persistence of the virus in the spleen of the immune animal.

In the next experiment, the spleen emulsions were first heated at 56°C for 45 minutes and then tested for their inactivating effect on the virus.

Incubated/

Incubated for 2 hours at 37°C.

Dilution of virus.	V	V+N spleen	V+Imm. spleen	V+heated Imm. spleen.	V+heated normal spleen
10^{-6}	---	---	---	---	---
10^{-5}	---	---	11 mm	7 mm	---
10^{-4}	---	---	11 mm	8 mm	---
10^{-3}	16 mm	---	16 mm	9 mm	6 mm
10^{-2}	22 mm	---	18 mm	17 mm	16 mm
10^{-1}	27 mm	24 mm	18 mm	19 mm	17 mm
Undil.		26 mm	20 mm	20 mm	18 mm

The heated normal spleen inactivated the virus to a certain extent per se. The heated immune spleen on the other hand enhanced the action of the virus on the tissues.

Active immunisation of rabbits.

An attempt was made to demonstrate active immunity in rabbits to an intracerebral injection after intra-:dermal administration of the virus. A single series of seven injections of decimal dilutions of the virus ranging from undiluted to 10^{-6} were given in 0.2 c.c. amounts to a number of rabbits. At 24 hour intervals the rabbits received an intracerebral injection of undiluted neuro-virus. Immunity was not present on the second, third, fourth and fifth day. On the sixth/

sixth day immunity was demonstrable to this extent, that the animal showed symptoms of encephalitis but recovered. On the seventh day solid immunity was demonstrable.

Protection experiment with hyper-immune antivaccinial serum.

An attempt was made to induce passive immunity in rabbits against an intracerebral injection of the neuro-virus. Hyper-immune antivaccinial serum was injected intravenously into three rabbits. One received 1 c.c. of serum, the other 2 c.c. and the third 4 c.c. On simultaneous administration of the undiluted virus by the intracerebral route, typical encephalitis developed in all of them in 5 days. The serum had no influence on the course of the disease.

Filterability of the vaccinia virus.

An attempt was made to filter the vaccinia virus through a Seitz E.K. filter. A 10% emulsion of neuro-virus in saline was centrifuged at 2500 r.p.m. for 15 minutes. The supernatant fluid was filtered through a Seitz E.K. filter at a negative pressure of 50 c.c. of mercury. The filtrate was injected intratesticularly in 0.5 c.c. amount into a rabbit, the unfiltered emulsion being injected into another as/

as a control. After 3 days, haemorrhagic orchitis and inflammation of tunica vaginales resulted in the control, while the filtrate did not produce any reaction. Three more attempts were made on the same lines to filter the virus through a Seitz E.K. filter with negative results. In two further experiments, the virus brain was emulsified in normal rabbit serum, and the Seitz filtrate was tested by intradermal injection with negative results, while the control with the unfiltered emulsion was positive.

When the same neuro-virus emulsion was filtered through (1) British Berkefeld candle, (2) L_2 Chamber-:land candle, the filtrate produced no positive results on intradermal injection, though positive controls were obtained.

The neuro-virus emulsion was next treated with an equal amount of ether. The slightly opalescent fraction (vide infra) was tested for its virus content by intradermal injections, and was found to be positive, but the Berkefeld and Chamberland L_2 filtrates prepared from the same gave negative results. The virus in short, could not pass, Seitz E.K., British Berkefeld and Chamberland L_2 candles.

Action of ether on neuro-virus.

When ether was added to an equal volume of neuro-virus/

virus emulsion, and thoroughly mixed by frequent shaking, an almost water-clear fraction separated at the bottom of the tube. On centrifugalisation, three layers were clearly discernible. A semi-fatty layer intervened between an ether soluble fraction on top, and an ether insoluble slightly opalescent fraction at the bottom. The bottom layer was removed and tested for its virus content. After 3 hours contact of ether with the virus emulsion, the potency of the virus was reduced by 10^{-1}. After 24 hours contact of the two at room-temperature the potency of the virus was reduced by 10^{-2} (from 10^{-6} to 10^{-4})

Pathogenicity of encephalitic strain of vaccinia virus on animals.

In the following experiments, the effect on the brain of administration of neuro-virus by various routes to animals was investigated.

(a) Rabbits.

By intratesticular injection.

Neuro-virus was administered intratesticularly in one c.c. amount. After three days, the rabbit had a definite orchitis, but in addition showed general symptoms. It lost weight, did not take its food and died on the sixth day.

This observation was confirmed in 3 further experiments.

By/

* 0·5 cc of the undiluted neuro-VIRUS emulsion was injected intradermally in 14 different areas amounting in all to 7 ccs.

By intradermal injection.

7 c.c. of an undiluted emulsion of neuro-virus were administered intradermally.* There was a definite local lesion, but in addition the animal lost progressively in weight amounting to 18 oz. in three weeks, then it began to recover. No symptoms of encephalitis appeared.

By intravenous injection.

4 c.c. of vaccinial brain diffusate (prepared by leaving a small piece of brain in broth for three days at room-temperature) were administered intravenously. The animal definitely lost weight, and was off food. On the eighth day, it looked definitely ill, showed a gasping respiration and had a coarse lateral tremor. It was then killed. Lesions of generalised vaccinia were present in lungs, liver, spleen and intestine. The same observation was confirmed several times. No signs of encephalitis appeared. Thus, on admini-:stration of an encephalitic strain of vaccinia virus, viz. neuro-virus, by intratesticular, intradermal and intravenous routes, symptoms of encephalitis failed to appear.

The effect of traumatising the brain in conjunc-:tion with an intravenous injection of the virus was next investigated.

Cerebral/

Cerebral trauma in conjunction with virus administration intravenously.

Vaccinia brain emulsion was administered intravenously in 2 c.c. amounts and Locke's fluid was injected intracerebrally at the same time. On the sixth day, the rabbit had symptoms of typical vaccinial encephalitis.

On another occasion, a rabbit was given 10 c.c. of neuro-virus emulsion intravenously, and 1 c.c. Locke's fluid intracerebrally. It died on the tenth day with definite clinical manifestations of encephalitis.

The effect of administering minimal doses of the virus intravenously in conjunction with cerebral trauma was next investigated. 1 c.c. saline was injected intracerebrally into all the rabbits shown in the table.

Amount intravenously.	Result.
0.1 c.c.	+ dead ninth day
0.5 c.c.	negative
1.0 c.c.	+ recovered
4.0 c.c.	+ dead third day

The result was not uniform due to the varying susceptibility of the rabbits, but it confirmed the previous/

* An attempt was made to inject maximal amounts of the neuro-virus emulsion consistent with safety; from 0·025 c.c of the undiluted emulsion up to 0·1 cc of a 10^{-3} dilution being used, the dosage varied with the size of mice, smaller mice receiving relatively less than large.

previous finding.

(b) Mice.

In an attempt to transmit vaccinial encephalitis to mice, neuro-virus emulsion was injected intracere-:brally up to 10^{-3} dilution.* Those receiving 10^{-3} and 10^{-2} dilutions survived, while others receiving 10^{-1} dilution were ill and slightly ataxic, and were found dead in 4-7 days.

In the next experiment, only the undiluted emulsion injected intracerebrally produced symptoms of weakness and ataxia leading to death in 1 out of 5 mice.

When the neuro-virus was administered intra-:peritoneally with simultaneous cerebral trauma to 3 mice, one had ataxia, displayed clonic movements and died on the sixth day; others were negative. The controls injected with the virus intraperitoneally per se showed no symptoms.

Concentration of virus in brain and spleen tissue of rabbits following an intracerebral injection.

The behaviour of neuro-virus during the incubation period following an intracerebral injection was investi-:gated. Four rabbits were injected intracerebrally with the undiluted neuro-virus, and were killed at 24 hour intervals. The presence of the virus in brain and spleen suspensions was titrated intradermally.

Dilution/

Dilution of virus.	24 hours		48 hours		72 hours		96 hours clinically +	
	Brain	Spleen	Brain	Spleen	Brain	Spleen	Brain	Spleen
10^{-6}	---	---	6 mm	---	5 mm	---	6 mm	---
10^{-5}	---	---	7 mm	---	6 mm	---	11 mm	---
10^{-4}	---	---	7 mm	---	6 mm	---	13 mm	---
10^{-3}	---	---	11 mm	---	8 mm	---	14 mm	---
10^{-2}	---	---	17 mm	---	9 mm	---	13 mm	4 mm
10^{-1}	6 mm	---	17 mm	---	15 mm	8 mm	14 mm	5 mm
Undil.	12 mm	---	22 mm	---	17 mm	9 mm	16 mm	11 mm

The virus multiplied locally in the brain and was recovered from the spleen only when the concentration of the virus in the brain had risen very high. The concentration of the virus in the spleen then rose indicating either a local multiplication of the virus in the spleen or a continued inflow from the brain into the circulation. The virus multiplied at least 10^{-4} times in 48 hours, the concentration remaining more or less constant on subsequent titrations. Clinical symptoms of encephalitis were not manifest after 48 hours, although the concentration of the virus in the brain was nearly the same as after 96 hours. When a high concentration of the virus was present in the brain for 48 hours, then clinical manifestations supervened.

Distribution/

Distribution of the virus in blood.

The association of the virus with the cellular constituents of blood was next investigated. 10 c.c. of a neuro-virus emulsion were administered intra-:venously to a rabbit. After one hour, the rabbit was bled from the marginal ear vein into 2% citrate solution, and the blood was fractionated. The leucocyte and red-cell fraction were then injected intradermally. The virus was found to be associated with the leucocyte fraction. The red cells failed to give any reaction.

In the next experiment, 6 c.c. of the neuro-virus emulsion were administered intravenously, and after two hours, the rabbit was bled from the ear into glucose-citrate solution (2% sodium citrate and 1% glucose). The blood was fractionated, and serial dilutions in 1% citrate of each fraction were made and injected intradermally into a second rabbit. 1 c.c. Locke's fluid was injected intracerebrally at the same time. No signs of encephalitis supervened, but the skin reactions were as follows.

Dilution/

Dilution of virus.	Virus.	Leucocytes	Platelets	Red-cells	Plasma
10^{-6}	4 mm	---	---	---	---
10^{-5}	15 mm	---	---	---	---
10^{-4}	22 mm	---	---	---	---
10^{-3}	26 mm	---	2 mm	---	---
10^{-2}	28 mm	---	3 mm	---	---
10^{-1}	34 mm	12 mm	4 mm	2 mm	---

In a further experiment, 6 c.c. of the virus emulsion were injected intravenously, the rabbit was bled after 30 minutes and 4 hours respectively.

Dilution of virus.	Virus	Leucocytes (30 minutes in circulation)	Platelets (30 minutes in circulation)	Red-cells (30 minutes in circulation)	Leucocytes (4 hours)
10^{-6}	6 mm	---	---	---	---
10^{-5}	7 mm	---	---	---	---
10^{-4}	13 mm	---	---	---	---
10^{-3}	14 mm	---	papules	---	---
10^{-2}	15 mm	---	6 mm	---	---
10^{-1}	13 mm	8 mm	9 mm	---	7 mm

The virus, when present in blood was associated with the leucocyte fraction, but more so with the platelets.

In the previous experiments, the virus was recovered/

recovered from blood in low concentration only; most of it was inactivated by the normal defence mechanism of the body.

In the following experiments, the effect of injecting the virus into hyper-immune rabbits was investigated.

5 c.c. of the neuro-virus emulsion were injected intravenously into a hyper-immune rabbit. After 15 minutes, the blood was withdrawn and fractionated. The virus could not be recovered in that case.

In another experiment, 6 c.c. of the virus emulsion was in immune rabbit circulation for 30 minutes and 4 hours respectively.

Dilution of virus.	Virus	30 minutes		4 hours	
		Platelets	Leucocytes	Platelets	Leucocytes
10^{-6}	6 mm	---	---	---	---
10^{-5}	12 mm	---	---	---	---
10^{-4}	17 mm	---	---	---	---
10^{-3}	22 mm	---	---	---	---
10^{-2}	22 mm	---	---	---	---
10^{-1}	26 mm	2 mm disappeared 5th day	---	---	---

The virus had mainly disappeared from the circu-:lation in 30 minutes and was not recoverable after 4 hours.

EXPERIMENTAL STUDIES OF THE PATHOGENESIS OF THE VIRUS OF LOUPING-ILL WITH PARTICULAR REFERENCE TO THE MECHANISM OF ANTI-VIRAL IMMUNITY.

Technique.

The louping-ill virus derived from the brain of an infected sheep was subjected to passage in mice by intracerebral inoculation. Thereafter the brains of infected mice were used as the source of the virus. All mice showing the typical symptoms of louping-ill were killed when moribund. The brain was removed aseptically, and sterility tests made in phosphate-broth and cooked-meat medium. The brain, on being found sterile was stored in 50 per cent glycerol in the ice-chest. When required, it was freed from glycerol by washing in saline, weighed and thoroughly ground up in a sterile mortar, and then Locke's fluid was gradually added to produce a 5 per cent uniform suspension. The suspension was centrifuged at 2000 r.p.m. for a period of 5 minutes. The supernatant fluid was pipetted off, and used for intracerebral injection.

For inoculation in mice, the skin of the scalp was depilated at first, but depilation was later abandoned. The animal was fully anaesthetised with ether. The site of inoculation was cleansed with spirit/

spirit, the point of inoculation being mid-way between the root of the ear and the middle line of the skull. A guarded intradermal needle, which allowed penetration to ¼ of an inch was used, the amount of inoculum being about 0.04 c.c. Since the maximum period of incubation in louping-ill in mice has been described as 23 days (Greig, Brownlee, Wilson and Gordon, 1931) the animals were observed for that period at least, the maximum time being 6 weeks.

Typical louping-ill infection in mice.

Unless the animal showed definite paralysis of one or more limbs, it was not regarded as a case of louping-ill. Hyperaesthesia, ataxia, paresis and tremor alone were not diagnostic of the disease, and could be ascribed to various bacterial infections.

In those mice, which showed definite clinical symptoms of louping-ill the inoculation was regarded as yielding a positive result (+) in those with no clinical manifestations but surviving throughout the observation period, the result was stated as negative (---); those animals found dead without having shown symptoms of louping-ill were labelled (D). In all mice showing symptoms not attributable to louping-ill, the result was not recorded.

Louping-ill in rabbits.

An attempt was made to transmit louping-ill to rabbits/

rabbits by an intracerebral injection of infected mouse brain, but without success even on repetition.

Louping-ill in lambs.

3 c.c. of infected mouse-brain emulsion were injected intracerebrally into two lambs. Both succumbed to the disease, their brains were removed aseptically, and dessicated, and then preserved in the refrigerator.

Louping-ill in Macacus rhesus.

One c.c. of infected mouse-brain emulsion was injected intracerebrally into a monkey. No symptoms were observed for 6 days. During this time the temperature ranged between 101.2°F and 102.2°F. On the seventh day, there was a febrile reaction, the temperature being 104.4°F, and the monkey was not as lively as before. It became quieter, and was dis-:inclined to move. Then marked ataxia and a coarse lateral tremor involving the entire body appeared. The temperature showed a diphasic reaction, and on the fourth day after the appearance of the symptoms, the animal was unable to sit up. It lay quietly and maintained its position obstinately. When forced to move, marked tremor and ataxia were still apparent. The tremor became infrequent, paresis more marked, and the temperature dropped to subnormal. Following lumbar/

lumbar puncture the animal recovered some power and was able to move about a little but remained definitely ataxic. When the head was displaced from the resting position, it rocked vigorously and came into violent contact with the sides of the cage, slight nystagmus was present. The temperature rose to normal, but later again showed a diphasic curve. · At this stage there was marked wasting of muscles, tremor was not demonstrable, paresis increased appreciably, moderate ataxia was present, and the animal was unable to feed itself. Muscular weakness increased, and became extreme. There was difficulty in taking solids; the muscles of mastication acted very feebly. Finally the animal became helpless and moribund. It was killed on the 18th day after the inoculation.

The presence of louping-ill virus in the cerebro-spinal fluid was investigated. Lumbar puncture was done on the 7th day after onset of symptoms. The cerebro-spinal fluid was diluted 1/4 before intra-:cerebral injection into three mice, which did not show any symptoms. The conclusion was, that the virus was not present in the cerebro-spinal fluid at that stage.

Mode of infection in mice.

An attempt was made to infect mice by routes other than intracerebral. The minimal amount of the virus/

virus from mouse-brain emulsion required to produce infection was also investigated. In the case of the intracerebral injections, one mouse received 0.04 c.c. of the emulsion, the second only the small amount of virus taken up on a sterile needle which had been dipped into the brain emulsion.

Mode of infection	Dose of brain-emulsion	Number of mice	Result.
Intracerebral	minimal	2	+ +
Intracerebral	0.04 c.c.	2	+ +
Subcutaneous.	0.5 c.c.	2	---
Intraperitoneal.	0.5 c.c.	2	---

\+ = Clinical manifestation of louping-ill

\--- = Survived without showing any symptoms

Thus minimal amounts of the virus introduced into the brain were sufficient to produce infection, while 0.5 c.c. injected intraperitoneally or subcutaneously were ineffective.

The effect of traumatising the brain in conjunc-:tion with intraperitoneal or subcutaneous injection of the virus was investigated. The results of a series of observations are shown below.

Route/

Route of injection.	Dose brain-emulsion.	Additional treatment	Number of mice.	Result.
Intraperitoneal	0.5 c.c.	Cerebral trauma (simultaneous)	3	+ 10 days + 11 days ---
Intracerebral	0.04 c.c.	Nil.	3	+ 6 days + 6 days + 8 days
Intraperitoneal	0.5 c.c.	Cerebral trauma (simultaneous)	2	+ 11 days + 12 days
Intracerebral	0.04 c.c.	Nil.	2	+ 9 days + 9 days
Subcutaneous	0.5 c.c.	Cerebral trauma simultaneous seventh day	2	+ 6 days + 16 days

It will be noted that intraperitoneal and sub-:cutaneous injections of the virus in association with an intracerebral trauma were effective in producing louping-ill; but the incubation period was longer than after intracerebral injection and the successes were not uniform.

The experiment was repeated later. A number of mice were inoculated intraperitoneally with 0.2 c.c. of brain emulsion. One mouse was subjected to cerebral trauma 24 hours after inoculation, another 48 hours later and so on, the last mouse being trauma-:tised after an interval of 8 days. The results were irregular; mice subjected to the trauma on 1st, 2nd and/

and 4th day yielded positive results, those traumatised on the 3rd, 5th, 8th and 9th day survived.

The surviving mice were tested for immunity 43 days later by intracerebral injection. All succumbed to the typical infection, there being no immunity to the undiluted virus at this period.

As the virus was becoming more virulent on being subjected to passage in mouse-brain, attempts were made again to infect mice by routes other than intra-:cerebral. The results of a series of observations are as follows.

Route of injection.	Dose brain-emulsion.	Additional treatment	Number of mice.	Result.
Subcutaneous	0.5 c.c.	Coal-gas exposure	2	+8 days ---
Intracerebral	0.04 c.c.	Nil.	3	+5 days +5 days +7 days
Intraperitoneal	0.2 c.c.	Nil.	4	+15 days --- --- ---
Subcutaneous	0.2 c.c.	Nil.	3	+14 days --- ---
Intranasal insufflation	0.5 c.c.	Nil.	1	+13 days
Intracerebral	0.04 c.c.	Nil.	2	+ 7 days + 7 days
Intraocular	0.02 c.c.	Nil.	1	+ 8 days

The/

The conclusion drawn was that subcutaneous and intraperitoneal injections of virus exalted in virulence by passage could produce louping-ill per se in a certain proportion of mice after a long incuba-:tion period.

Intraocular injection and intranasal insufflation were also effective.

The experiment was repeated with the following result.

Mode of administration.	Dose brain-emulsion.	Number of mice.	Result.
Intracerebral	0.04 c.c.	3	+ 6th day + 6th day + 6th day
Intraperitoneal with spinal injury 48 hrs later.	0.2 c.c.	3	+ 8 days +10 days ---
Intraperitoneal		14	12 + 7-12 days 2 ---
Right intraocular	0.02 c.c.	3	+ 7th day + 7th day + 9th day
Right Retroocular	0.04 c.c.	3	--- --- ---
Intracerebral	0.04 c.c.	3	+ 5th day + 6th day
Intranasal	0.5 c.c.	4	+ 9th day + 9th day D* 9th day D 9th day
Per Os	0.5 c.c.	2	Louping-ill?

* D = found dead (vide p.103)

It/

It was shown in this way that louping-ill could be transmitted to mice by intraperitoneal, intra-:ocular, and intranasal routes. Retro-ocular injections failed to reproduce the disease. The result of administering the virus by mouth was inconclusive. One out of the two mice was ataxic on the 12th day, its brain was submitted to passage in four mice. One became comatose on the 16th day, the second died on the 21st day, others remained normal.

Those mice, which survived an intraperitoneal injection of the virus were reinjected intracerebrally with undiluted emulsion 32 days later; no immunity was demonstrable and all succumbed to the typical infection.

An attempt was again made to reproduce the infection in mice by administration of the virus per os in four mice. One developed ataxia and paresis on the 11th day; others showed no symptoms. The controls injected intracerebrally died on the 6th day showing typical symptoms of the disease.

The virus which had become highly virulent by passage was used later to infect mice per os. Three out of 9 mice were successfully infected after an incubation period of 13-15 days.

In another experiment a Berkefeld filtrate of the brain/

brain emulsion when administered intracerebrally caused infection on the 7th day, while intranasal insufflation was effective in 8-14 days in 3 out of 4 mice.

In a further experiment louping-ill mouse brain emulsion was injected intracerebrally, and the mice were killed after 24 and 48 hours respectively. Their brains were emulsified and administered to mice by intracerebral and intraperitoneal routes. The brain of the mouse injected 24 hours previously gave a positive result on intracerebral administration, and a negative by the intraperitoneal route. The brain of the mouse injected 48 hours previously yielded positive results by both routes. This experiment suggested that the virus gained in potency when it was allowed to multiply in the brain for 48 hours instead of 24 hours. A highly virulent strain could cause infection when injected intraperitoneally, otherwise an intracerebral injection was essential to reproduce the disease.

Effect of physical and chemical agencies on the virus.

The effect of heat on the potency of the virus was investigated. The brain emulsion was divided into two parts. One was heated at 57°C in the water-bath for 30 minutes, the other was kept in the ice-chest to act as a control. Two mice were then injected intracerebrally/

intracerebrally to test for the presence of live virus.

Number of mice.	Heated virus 57°C - 30 min.	Control.
2	---	+
	---	+

The virus was apparently killed by exposure to 57°C for 30 minutes, while the control was positive.

The experiment was repeated with the following result.

Dilution of virus.	Heated virus.			Control.
	55°C-5 min.	55°C-15 min.	55°C-30 min.	
10^{-3}				+
10^{-2}	--- D			+ +
10^{-1}	--- +	--- +		+ +
Undil.	+ +	+ +	--- ---	+ +

Note: In this and subsequent tables the number of mice tested is indicated by the number of symbols indicating the results of the experiment.

The brain emulsion was positive in at least a 10^{-3} dilution. After 5 minutes exposure to 55°C the virus was active in a 10^{-1} dilution. The same result/

result was given by exposure to 55°C for 15 minutes. It was destroyed at 55°C in 30 minutes.

The virus was next exposed to a temperature of 37°C for 24 hours.

Number of mice.	Heated virus (24 hours - 37°C)		
	1st experiment.	2nd experiment.	3rd experiment.
2	+	+	+
	+	+	+

Louping-ill mouse brain emulsion in Locke's fluid was active after being exposed to 37°C for 24 hours. The same result was obtained in three experiments.

Next the effect of keeping the virus emulsion in Locke's fluid for 48 hours at 37°C was investigated.

Dilution of virus.	Heated virus 48 hrs - 37°C	Control.
10^{-3}		+
10^{-2}		+
		+
10^{-1}		+
		+
Undil.	---	+
	---	+

The virus became inactive in Locke's fluid in 48 hours at 37°C, while the control from the ice-chest was positive/

positive in at least a 10^{-3} dilution.

Effect of ether on the virus.

The effect of ether on the brain emulsion was investigated.

The emulsion was left in contact with an equal amount of ether at room-temperature for two hours. During this time the mixture was shaken vigorously several times, then it was centrifuged at 2000 r.p.m. for 5 minutes. The mixture separated out into three fractions - an opalescent fraction at the bottom, a semi-fatty layer intervening with an ether-soluble fraction on top. The opalescent fraction was tested for the presence of the virus.

The virus could not be recovered, while the control was positive.

In the next experiment, ether was allowed to act on the emulsion for one hour and it was found that one hour's contact with ether inactivated the virus.

In a later experiment, the virus emulsion was divided into four parts. Ether was allowed to act on the emulsions for 15, 30 and 60 minutes respectively; the fourth part served as a control.

Number of mice.	Virus treated with ether			Control.
	15 min.	30 min.	60 min.	
2	--- ---	--- ---	--- ---	+ +

15/

15 minutes contact with ether inactivated the virus.

Effect of normal sheep serum on the virus.

It was incidentally noticed during an experiment that when the virus was kept in Locke's fluid for 4 hours at room-temperature, its maximum effective titre was 10^{-2}, but if it was in contact with an equal amount of normal sheep serum, which had been heated at 56°C for 30 minutes, its titre after 4 hours was definitely higher, 10^{-3}.

Dilution of virus.	Virus in Locke 4 hrs - room temp.	V in Locke sheep serum 4 hrs - room temp.
10^{-3}	---	+
10^{-2}	+	+
10^{-1}	D*	+
Undil.	+ +	D

*D = found dead.

The effect of normal sheep serum on the toxic action of Locke's fluid on the virus was further investigated. The virus emulsion in Locke's fluid was subdivided into two parts. One was left in contact with an equal volume of normal sheep serum, the other was kept as a control.

Dilution/

Dilution of virus.	4 hours at room-temperature.	
	Virus in Locke	V in Locke Normal serum
10^{-2}	---	+
10^{-1}	+	

The previous observation was confirmed.

In another experiment, it was found, that while after 24 hours contact at room-temperature, Virus + undiluted normal sheep serum acted in a titre of 10^{-3}, the virus in Locke's fluid was active only when undiluted.

In a further experiment, brain was ground up and divided into two portions, one was emulsified in Locke's fluid, the other in normal sheep serum. Both portions were incubated at 37°C for 24 hours, and then injected intracerebrally into mice.

Dilution of virus.	Virus in Locke	Virus in sheep serum
10^{-1}	---	---

		+
Undil.	+	
	+	

These observations showed clearly that virus survived better in normal sheep serum than in Locke's fluid.

Effect/

Effect of physical agencies on the virus.

The resistance of the virus to glycerol, dessication & Locke's fluid at various tempera-tures was investigated as follows.

V in 50% glycerol at +4°C		Dessicated Lamb brain at -10°C		V in Locke's fluid	
4 months	+	4.5 months	+	7 days room	+
	+		+		+
	+		+		
4 months	+	6 months	+	9 days room	---
17 days	---	9 days	+		---
5 months	---	8 months	+	18 days +4°C	+
	---	10 days	+		+
	---		+		+
5 months	---	15 months	+		
18 days	---	22 days	+		
	---		---		

The virus survived for at least 4 months 17 days, when preserved in 50% glycerol (made up with Locke's fluid) in the ice-chest. When dessicated, and kept in the refrigerator, it survived for at least 15 months and 22 days. In Locke's fluid, it survived for 7 days at room-temperature and for at least 18 days in the ice-chest.

Passage through filters.

The virus could be consistently filtered through a British Berkefeld candle.

An attempt was made to filter it through a Chamberland/

Chamberland L_5 candle. A Berkefeld filtrate of the virus emulsion was prepared and about 15 c.c. of the same were passed through a Chamberland L_5 candle at a negative pressure of 50 c.c. of mercury. The filtrates were then injected intracerebrally into mice.

Filtrate		Control
Chamberland L_5	Berkefeld	Unfiltered emulsion
---	+	+
---	+	+

The Chamberland L_5 filtrate was inactive, while the Berkefeld filtrate gave positive results.

Detection of virus in tissues of mice at various intervals during the incubation-period.

An attempt was made to determine the site of multiplication of the virus following an intracerebral injection.

Louping-ill mouse brain emulsion was injected intracerebrally into mice. They were killed at 24 hour intervals, and their brains were emulsified and injected intracerebrally into other mice to determine the presence of the virus. The virus was not demon-:strable in the brain after 24 hours, the result was doubtful after 48 hours. After 72 hours, the virus

was/

was present in the brain when tested in 10^{-1} dilution.

The experiment was repeated.

Number of mice.	brain 24 hr.	brain 48 hr.	brain 72 hr.
2	+ +	+ D	+ +
Number of mice.	spleen 24 hr.	spleen 48 hr.	spleen 72 hr.
2	--- ---	--- D	+ D

The virus was consistently recovered from the brain after 24, 48 and 72 hours. From the spleens of the same mice, the virus could not be recovered after 24 and 48 hours, but was demonstrable after 72 hours.

The incubation period when subinoculations were made from the brain after 48 hours was 8-9 days, after 72 hours 5-6 days.

In a further experiment it was difficult to demonstrate the virus in the brain 24 hours after the infection. After 48 hours the virus was demonstrable in 10^{-1} dilution and the spleen from the same mouse yielded a similar titre. After 72 hours, it was demonstrable in the same concentration. After 96 hours, it was demonstrable in 10^{-2} dilutions of brain and spleen emulsions and at this stage manifestations of/

of louping-ill began to appear.

	A. Brains.				
Dilution of virus.	24 hour	48 hour	72 hour	96 hour	120 hour
10^{-2}				+	+
10^{-1}		+	+	+ +	+
Undil.	+ ---	+	+ +		
B. Spleens.					
Dilutions of virus.	24 hour	48 hour	72 hour	96 hour	120 hour
10^{-2}				+	+
10^{-1}		+ +	+ +	+	
Undil.	--- ---	+	+	+	+

The experiment was repeated later on. It con-:firmed the previous findings, and showed in addition, that the virus appeared and multiplied in the blood after 48 hours, but it was not demonstrable in the circulation after 72 hours.

A/

A. Brain.

Dilution of virus.	24 hour	48 hour	72 hour	96 hour
10^{-2}			+ D	+
10^{-1}		D ---	---	D
Undil.	+ +	+ +	+	

B. Spleen.

Dilution of virus.	24 hour	48 hour	72 hour	96 hour
10^{-2}			--- D	+
10^{-1}		D +	+ D	D
Undil.	--- D	+ D	D	

C. Blood.

Dilution of virus.	24 hour	48 hour	72 hour	96 hour
10^{-2}			D ---	
10^{-1}		+ +	--- ---	---
1/2	--- ---	+	D	

Thus/

Thus it was shown that on intracerebral injection the virus could be recovered with difficulty from the brain, but not from spleen, suggesting a local multi-:plication. After 48 hours, it could be recovered from brain, blood and spleen. 72 hours later it was not demonstrable even in low dilutions in blood, but was present in brain and spleen in high concentration. Manifestations of louping-ill appeared after the virus had multiplied in the brain to a high level.

Concentration of virus by addition of normal rabbit blood.

A few drops of normal rabbit blood were allowed to drop directly from the ear-vein into the brain emulsion. When clotting and retraction had taken place, the emulsion was centrifuged at about 2000 r.p.m. for 10 minutes. The supernatant fluid was pipetted off, and the clot was taken up in a little Locke's fluid to ensure a uniform suspension. Ten-fold dilutions were prepared, and the concentration of the virus in the clot was determined by intra-:cerebral injection into mice, the control being injected with the emulsion alone. The virus adsorbed by the blood-clot gave positive results up to at least 10^{-6} dilution, while the emulsion alone reacted up to 10^{-3} dilution only. The virus was thus concentrated at least 10^{-3} times in the clot.

The/

The possibility of concentration of the virus by defibrinated blood was next investigated. The virus alone was ineffective in a 1/100 dilution, but mixed with defibrinated rabbit blood and injected immediately after, the M.L.D. was 1/200. When the emulsion and defibrinated blood were in contact for 20 hours in the ice-chest, the M.L.D. was again 1/200. No evidence of definite concentration of the virus by defibrinated blood was forthcoming.

Relation of the virus to the blood-constituents.

The distribution of the Louping-ill virus in blood was determined. Louping-ill mouse-brain emulsion was left in contact with nine volumes of citrated sheep blood for two hours at room-temperature; then the blood was fractionated into leucocytes, red-cells and plasma. The various fractions were diluted ten-fold in 1% sodium citrate, and injected intracerebrally into mice. The virus was recovered from all the fractions but more so from leucocytes.

In another experiment, the emulsion was left in contact with sheep platelets and leucocytes for 20 hours in the ice-chest. Normal sheep blood was mixed in an equal volume with a solution containing 2% sodium citrate and 1% glucose. It was centrifuged for 7 minutes at 2500-3000 r.p.m. The supernatant fluid/

fluid was respun at the same speed for 20 minutes, and the deposit consisting of platelets was suspended in glucose-citrate solution; the leucocytes being obtained from the deposit in the first tube. On intracerebral injection of the virus-platelet emulsion, the virus was found to be concentrated ten times, the leucocytes gave the same titre as the brain emulsion.

Dilution of virus.	V emulsion.	V Leucocytes	V Platelets
10^{-5}	---	---	---
10^{-4}	---	---	+
10^{-3}	+	+	+
10^{-2}	+		
10^{-1}	+		
Undil.	+		

In the next experiment, the emulsion was in contact with the various constituents of sheep blood for 18 hours at room-temperature. The results were irregular. The red-cells in one case produced the typical infection in 10^{-3} dilution, while the platelets gave a positive in 10^{-6} dilution. In a further experiment, the contact of the emulsion with the various constituents of sheep blood was for 3 hours at room-temperature. The mixture was shaken for 30 minutes/

minutes during that time, then it was centrifuged for 20 minutes at 2000 r.p.m. and the deposits were diluted and injected intracerebrally.

Dilution of virus.	Red-cells.	Leucocytes.	Platelets.
10^{-7}		--- D	D D
10^{-6}		+ +	+ +
10^{-5}	--- ---	D +	+ +
10^{-4}	+ +	+ +	+ D
10^{-3}	+ D	+ +	+ D
10^{-2}	+ +		
10^{-1}	+ +		

When present in blood, the virus was mainly associated with the platelet and leucocyte fraction.

Experiments on concentration of virus by
(a) precipitation of euglobulins, and
(b) adsorption on Berkefeld filter powder.

A 10% centrifugalised emulsion of mouse-brain was diluted with nine volumes of distilled water and carbon-di-oxide was passed through it for 3 minutes. The resulting precipitate was thrown down by centri-
:fugalisation/

:fugalisation and tested for the presence of the virus. The control was positive, but no virus was demonstrable in the euglobulins.

A powdered British Berkefeld filter was sterilised. The powder was added to the Berkefeld filtrate of the infected mouse brain, and left in contact for 15 minutes, being shaken vigourously several times. It was then centrifuged, the deposit serially diluted and injected intracerebrally, but no concentration of the virus resulted, the material being only as active as the Berkefeld filtrate, viz. in 10^{-1} dilution.

Immunisation of animals against Louping-ill.

(A) Sheep.

A sheep was immunised with the virus by sub-:cutaneous injections of a 20% emulsion of brains from infected mice. At first injections were given on alternate days for one month, later bi-weekly for one week, and finally weekly for 3 weeks. The dose of the emulsion was increased from 4 to 40 c.c. The sheep was bled after two months and the serum was stored in the ice-chest without a preservative.

(B) Pig.

5 c.c. of a 10% emulsion of brain from infected mice were administered subcutaneously to a pig. The animal was watched for any adverse symptoms for a week, and then further injections of the same emulsion/

emulsion were given on alternate days for two weeks, and later weekly, the dose being increased gradually from 6-55 c.c. The blood was withdrawn at intervals and the serum tested for antibodies, (vide infra). The animal was bled after 5 months, and the serum was stored in the ice-chest without preservatives.

The antiserum from the pig was more potent in neutralising properties than the sera from rabbits or sheep. Hence the pig serum was mostly used in neutralisation experiments.

Neutralisation experiments.

In performing such experiments, decimal dilutions in Locke's fluid of the supernatant fluid from a 10% centrifugalised mouse-brain emulsion were mixed with equal volumes of the serum under investigation (0.2 c.c. each) in sterile tubes, and the mixtures incu-:bated for varying periods, then injected intracere-:brally into mice. In the control, normal sheep serum was added in equal volume to the virus dilutions in Locke's fluid, as it was previously shown that the virus survived better in a mixture of normal sheep serum and Locke's solution than in Locke's fluid alone.

Determination of neutralising power of immune sheep serum.

In the following experiment, the immune sheep serum was tested for the presence of virus-neutralising antibodies/

antibodies, and the thermostability of such anti-:bodies was also investigated. The technique outlined in the previous paragraph was used, the period of incubation of the serum-virus mixtures being fixed at 4 hours at 37°C. The results were as follows.

Dilution of virus.	V + N-S	V + I-S	V + heated I-S (55°C - 30 min.)
10^{-4}	--- ---		
10^{-3}	+ ---	--- ---	--- ---
10^{-2}	+ +	--- --- ---	--- ---
10^{-1}	+ +	+ --- ---	+ ---
Undil.		† D	+ ---

V = Virus
N-S = Normal serum
I-S = Immune serum
V+I-S = Mixture of virus and immune serum
--- = Survived (negative)
† = Symptoms of Louping-ill

At least ten minimum lethal doses of the virus could be neutralised by the immune serum after 4 hours incubation at 37°C, and it was also found that the immune serum was thermostable, i.e. its neutralising power was not reduced by heating to 55°C for 30 minutes.

These/

These observations were confirmed in two similar experiments.

Neutralising power of heated sheep serum.

The effect of heating the immune serum to 55°C for 60 minutes and to 58°C - 62°C for 60 minutes was then investigated.

Time of contact 4 hrs at 37°C.

Dilution of virus.	V + N-S	V + heated I-S (55°C - 60 min.)	V + heated I-S (58°C-62°C for 60 min.)
10^{-3}	+	---	---
	+	---	---
	+	---	D
10^{-2}	+	+	+
	+	---	+
	+		+
10^{-1}	+	+	+
	+	+	+
	+		D
Undil.	+	+	+
	+	+	+
	+	+	+

It was concluded that the neutralising antibody was still present after the serum had been heated for 60 minutes at 55°C, but at a temperature of 58°C to 62°C, the potency of the serum was definitely reduced.

Similar results were obtained on repetition.

Time and temperature factors in neutralisation.

The/

The effect of temperature on the neutralisation of the virus by the immune serum was investigated. Serum-virus mixtures were kept at room-temperature (18-22°C) and at 37°C, (the time of contact being in all cases 4 hours) and then injected intracerebrally into mice.

Dilution of virus.	V + N-S (4 hrs-37°C)	V + I-S (4 hrs-37°C)	V + N-S (4hrs-room.)	V + I-S (4hrs-room.)
10^{-4}	+ ---	--- ---	D	--- ---
10^{-3}	+ +	--- ---	+	--- ---
10^{-2}	+ D	--- ---	+	+ +
10^{-1}	+ D	+ +	+	+ +
Undil.	+ +	+ D	+	+ +

The higher temperature accelerated neutralisation of the virus. Approximately 100 M.L.D. at least of the virus were neutralised at 37°C, while only 10 M.L.D. were neutralised at room-temperature (18°C-22°C).

In the next experiment, the effect of variation of both time and temperature during the contact of virus and immune serum was investigated. Mixtures of antibody and virus were kept at room-temperature (22°C) for one hour, while another series of the same were in contact/

contact in the incubator at 37°C for 30 minutes. The result of intracerebral injection into mice was as follows.

Dilution of virus.	Number of mice.	V + I-S (30 min-37°C)	V + I-S (60 min-room.)
10^{-3}	2	--- ---	--- ---
10^{-2}	2	+ D	--- ---
10^{-1}	2	+ +	D D
Undil.	1	+	+

Thus, better neutralisation occurred at room-temperature in one hour than at 37°C in 30 minutes.

In a further experiment, the contact of immune sheep serum and the virus dilutions was for 4 hours at 37°C and 26 hours at room-temperature.

Dilution of virus.	Number of mice.	V + I-S (26hrs-room. 18-22°C)	V + I-S (4hrs-37°C)
10^{-3}	2	+ D	+ ---
10^{-2}	2	+ +	--- ---
10^{-1}	2	+ D	+ ---
Undil.	1	+	---

The/

The contact of immune-serum and virus at 37°C for 4 hours gave better neutralisation than at room-temperature for 26 hours.

In previous experiments, the serum-virus mixtures were always incubated for the demonstration of neutra-:lisation. Whether incubation was necessary for this purpose was investigated in this experiment.

Dilution of virus.	V + N-S (4hrs-37°C)	V + I-S (0)	V + I-S (4hrs-37°C)
10^{-3}	+	---	---
	+	---	---
10^{-2}	D	+	---
	D	+	---
10^{-1}	+	+	+
	+	+	+
Undil.	+	D	+
		D	---

High dilutions of the virus could be rendered inactive immediately on mixing, but on incubation, the neutralisation was much better.

In the next experiment, the incubation of the serum-virus mixture for 4 hours at 37°C produced better neutralisation than 30 minutes at 37°C, or injection immediately after mixing. 0.5 c.c. of the immune serum injected intraperitoneally gave no protection against a simultaneous intracerebral injection of the minimum lethal dose of the virus.

Dilution/

Dilution of virus.	V+I-S (O)	V + I-S (30min-37°C)	V + I-S (4hrs-37°C)	V I-cerebral I-S I-periton.
10^{-3}			--- ---	
10^{-2}	+ D	+ D	--- ---	+ D
10^{-1}			+ D	

That incubation of the serum-virus mixture was not necessary to demonstrate neutralisation of low concentrations of the virus was confirmed in a further experiment.

The effect of incubation on the neutralisation of the virus by the antiserum was clearly shown in the following experiment with a sample of serum obtained from a rabbit immunised against Louping-ill.

Injected immediately after mixing.

Dilution of virus.	V+ N-S sheep	V + I-S rabbit
10^{-4}	--- ---	---
10^{-3}	--- ---	--- ---
10^{-2}	+ +	--- --- ---
10^{-1}	+ +	+ +

Injected/

Injected after 4 hours contact at 37°C.

Dilution of virus.	V + N-S sheep	V + N-S rabbit	V + I-S rabbit
10^{-6}	---	---	---
	---	D	D
10^{-5}	---	---	---
	---	---	---

10^{-4}	---	+	---
	---	+	---
		+	---
10^{-3}	+	+	---
	+	+	---
		---	---
10^{-2}		+	---
		+	
		+	
10^{-1}		+	
		+	+
		+	+

The immune rabbit serum neutralised the virus immediately on mixing but the result was 10^{2} times better, when the serum-virus mixtures were incubated for 4 hours at 37°C.

With the immune pig serum, neutralisation of the virus was demonstrable immediately on mixing and even when the serum was diluted 10^{-1} neutralisation still occurred.

Serum-virus mixture injected immediately after mixing.

Dilution/

Dilution of virus.	V + N-S sheep.	V + diluted I-S 10^{-1} pig.
10^{-4}	--- D	--- ---
10^{-3}	+ +	--- ---
10^{-2}	+ +	+
10^{-1}	+ +	+ +
Undil.		+ + +

Injected immediately after mixing, and after two hours at room-temperature.

Dilution of virus.	V + N-S (0)	V + I-S (0)	V + I-S (2 hrs-22°C)
10^{-3}	+ +	--- --- ---	--- --- ---
10^{-2}	+ D	--- --- +	--- --- ---
10^{-1}	+ +		

The latter result showed better neutralisation after incubation.

In those experiments, in which the virus and immune serum were injected intracerebrally immediately on/

on mixing, the in vitro contact was for a few seconds only.

The possibility of neutralisation of the virus by the immune serum without any in vitro contact was next investigated. The immune serum was injected intracerebrally, and after a few seconds, the virus was injected in exactly the same spot, control mice receiving the same dose of virus emulsion alone.

Dilution of virus.	V only intracerebrally.	I-S intracerebrally, then V intracerebrally.
10^{-5}	D	---
	D	---
10^{-4}	+	---
	---	---
10^{-3}	+	+
	+	---

The experiment was repeated with a less potent virus and the same immune serum with the following result.

Dilution of virus.	V only intracerebrally.	I-S intracerebrally, then V intracerebrally.
10^{-3}	---	---

10^{-2}	+	---
	---	---
10^{-1}	+	---
	+	+

Definite/

Definite protection was afforded by the immune serum injected intracerebrally a few seconds before the virus. The suggestion was that the antibody changed the susceptibility of the tissues, which were able to inactivate the virus to some extent.

Action of complement on serum-virus mixtures.

In previous experiments, it was shown that the heated immune serum (55°C-30 min.) was as active as the unheated. As the neutralisation of the virus was demonstrated after the injection of the serum-virus mixture into the tissues of the host, the action of complement in vivo could not be excluded.

In the following experiments, the action of complement (in fresh guinea-pig serum) on the heated serum and virus mixture was investigated. As the guinea-pig serum might act on the virus per se, this was first determined.

Three volumes of fresh guinea-pig serum were added to one volume of the virus dilution; normal sheep serum was used in the control in place of the guinea-pig serum.

Dilution of virus.	V + N-S sheep.	V + N-S guinea-pig.
10^{-3}	+	---
10^{-2}	+	---
	+	+
10^{-1}	+	+
	+	+
Undil.	+	+
	+	+

Normal guinea-pig serum was thus found to inactivate the virus to a certain extent.

The experiment was repeated with a different guinea-pig serum and the same strain of virus.

Dilution of virus.	V + N-S sheep.	V + N-S guinea-pig.
10^{-4}	+	

10^{-3}	+	---
	+	---
10^{-2}	+	---
10^{-1}	+	

The previous observation was confirmed.

Equal volumes of the virus dilutions and the immune sheep serum were mixed with double the volume of fresh guinea-pig serum. In the control, the guinea-pig serum was used after heating it at 56°C for 30 minutes to inactivate the complement.

Dilution of virus.	V+N-S sheep	V+N-S guinea-pig	V+I-S +heated guinea-pig serum	V+heated I+S +fresh guinea-pig serum
10^{-3}	+	---	---	
	+	---	---	
10^{-2}	+	+	+	---
	+	---	---	---
10^{-1}	+	+	+	---
	+	+	+	---
Undil.	+	+	+	D

Although/

Although the action of the heated immune serum on the virus was definitely enhanced by the normal guinea-pig serum, this could be explained by the direct action of this serum on the virus.

The action on the virus of the tissues of a refractory animal (rabbit).

Louping-ill could not be transmitted to rabbits (vide p.103). Of the many possible explanations for this failure, one was the possible presence in the animal's blood of a natural protective antibody. The effect on the virus of normal rabbit blood and serum was therefore investigated.

In the first experiment, the mixtures of virus and serum were injected intracerebrally after incuba-:tion for 4 hours at 37°C.

Dilution of virus.	V+N-S sheep (4hrs-37°C)	V+N-rabbit serum (4hrs-37°C)	V+N-rabbit blood (4hrs-37°C)
10^{-4}	+ +		
10^{-3}	+ +	+ +	D
10^{-2}	+ +	+ +	+
10^{-1}	+ +	+	+ +
Undil.		+ D	+ +

No definite action on the virus of normal rabbit serum/

serum or blood was demonstrable.

The experiment was repeated:: normal rabbit blood was incubated with the virus dilutions for 2 hours at 37°C.

Dilution of virus.	V + N-S sheep (2hrs-37°C)	V + N-rabbit blood (citrated) (2hrs-37°C)
10^{-3}	+ + +	+ +
10^{-2}	+ + +	+ + +
10^{-1}	+ +	+ + +
Undil.		+ + +

The previous observation was confirmed.

The following experiments were devised to test the clearing rate of virus following intravenous injection from the blood of a refractory animal.

10 c.c. brain emulsion containing the virus were injected intravenously into a rabbit. After two hours, it was bled, and the blood was fractionated. No virus could be recovered from 10^{-2} dilutions of the leucocytes and platelets, although the control with virus emulsion only was positive in 10^{-4} dilution.

In/

* The exact dilution of the VIRUS after introduction into the circulation could not be determined. 6 cc of the mouse brain emulsion were injected intravenously, the emulsion was most probably diluted from 1/10 - 1/20 in the blood stream. It was not considered necessary to provide an adequate control, the dilutions of the. The control set forth in the left hand column of the table could not be exactly correlated to the dilutions of the Virus in the blood; the dilution of the virus in the blood stream has not been taken into account in tabulating the results. The table has to be interpreted bearing the limitations in mind.

In the next experiment, 10 c.c. of the mouse-brain emulsion were injected intravenously into a rabbit, blood was withdrawn from the marginal ear-vein after 30, 60 and 120 minutes respectively. The virus was recovered in 10^{-1} of whole blood up to 60 minutes, but was not demonstrable in the 120 minute sample. *

In a further experiment, 6 c.c. of the mouse-brain emulsion were injected intravenously, the rabbit was bled from the ear-vein after 60, 90 and 120 minutes respectively, and the presence of virus in the whole blood dilutions tested for by intra-:cerebral injection into mice as follows.

Dilution of virus.	V	60 minutes	90 minutes	120 minutes
10^{-4}	+			

10^{-3}	---	+		
	---	---		
10^{-2}	+	+		
	---	+		
		+		
10^{-1}	+	+		+
	+	+		---

1/2		+	+	+
		+	+	+
				+
Undil.	+			

The/

The virus was recovered from all the samples taken up to 120 minutes after injection, but it was difficult to recover at that time as shown by the single positive result among three mice injected with a 10^{-1} dilution of whole blood.

In a further experiment, the virus could not be recovered after 60 minutes in the rabbit circulation, although the control with the virus emulsion was positive in at least a 10^{-3} dilution.

In refractory animals the virus was rapidly removed from the peripheral blood, although the speed with which this occurred differed markedly in differ-:ent animals.

In the next experiment the result of incubation of the virus dilutions with citrated normal rabbit blood was compared with that of immune blood and serum. As the rabbit blood was diluted 1/2 by the addition of an equal volume of 2 per cent citrate, the immune serum was correspondingly diluted.

Incubated for 4 hours at 37°C

Dilution/

Dilution of virus.	V + N-blood	V + I-blood	V + I-S
10^{-3}	+ +	--- ---	---
10^{-2}	+ +	+ ---	---
10^{-1}	+ +	--- ---	---
1/2	+ +	+ ---	--- ---

The immune blood and serum both definitely neutralised the virus.

Protection experiments with immune sheep serum.

0.2 c.c. of immune sheep serum was injected intraperitoneally in a number of mice; after twenty-four hours decimal dilutions of the virus were injected intracerebrally. The result was as follows.

Dilution of virus.	Result.
10^{-3}	+
10^{-2}	+ + +
10^{-1}	+ + +
Undil.	+ + +

No/

No protection was afforded by intraperitoneal administration of immune serum against intracerebral injection of the virus even in high dilutions.

Recovery of virus from neutral serum-virus mixtures.

An attempt was made to recover the virus from neutral serum-virus mixtures by dilution of the same in Locke's fluid.

Equal volumes of decimal dilutions of the mouse-brain emulsion were mixed with undiluted immune rabbit serum, and after 4 hours incubation at 37°C, the mixtures were diluted 10^{-1} and 10^{-2} in Locke's fluid and injected intracerebrally into mice. A control was provided by mixing the virus dilutions with an equal amount of undiluted normal sheep serum, and keeping the mixtures at the same temperature for the same period. On intracerebral injection of the same, the potency of the virus could be determined.

Contact for 4 hours at 37°C.

Dilution of virus.	V + N-S sheep	V + I-S rabbit	(V+I-S) diluted in Locke's fluid	
			10^{-1}	10^{-2}
10^{-4}	+	---		
	+	---		
10^{-3}	+	---		
	+	---		
10^{-2}	D	---	---	

10^{-1}	+	+	+	+
		---	D	+
Undil.		+		
		+	+	+
				+

The/

The serum-virus mixture containing 10^{-2} dilution of the virus was found to be neutral, and could not be reactivated by 10^{-1} dilution in Locke's fluid. By dilution, the virus in the aforesaid mixture was diluted to 10^{-3} from 10^{-2}, but as the virus in the control was positive in 10^{-4} dilution, the absence of reactivation of the virus could not be due to dilution beyond the reacting concentration of the virus. The mixture containing the higher concentration of the virus, viz. 10^{-1} was partially neutralised, and could be reactivated on dilution.

In the next experiment, immune sheep serum was used for neutralisation of the virus.

Contact for 4 hours at 37°C.

Dilution of virus.	V + N-S sheep.	V + I-S sheep.	(V + I-S) diluted with Locke's fluid.		
			10^{-1}	10^{-2}	10^{-3}
10^{-3}	+	---			

10^{-2}	+	---	---		
	+				
10^{-1}	+	+	+	---	
	+	---	+	---	
Undil.	+	+	+	---	+
	+	+		---	

The neutral serum-virus mixture containing 10^{-2} dilution of the virus could not be reactivated by 10^{-1} dilution in Locke's fluid, while the attempt with/

with the partially neutralised mixture containing 10^{-1} dilution of the virus was successful.

The same experiment was repeated with immune pig serum.

Contact for 4 hours at 37°C.

Dilution of virus.	V + N-S sheep.	V + I-S pig.	(V + I-S) diluted in Locke's fluid.	
			10^{-1}	10^{-2}
10^{-4}	--- ---			
10^{-3}	--- D	--- ---		
10^{-2}	+ +	---	--- ---	
10^{-1}	+ +	--- ---	---	--- ---
Undil.		--- ---		--- ---

Firm antigen-antibody union had taken place in the neutral mixtures, and attempts at reactivation by dilution failed.

As the dilution-phenomenon depends on diluting the neutral serum-virus mixture to such an extent, that the diluted virus is still effective, while the immune serum is diluted beyond the range of effective-:ness, in the next experiment a control was included to ascertain whether the concentration of immune serum had been sufficient to render it inactive.

The/

The immune pig serum was diluted 10^{-1} beforehand, and then mixed with decimal dilutions of the virus. The serum-virus mixtures were diluted 10^{-1} immediately after mixing. As the final dilution of the serum was 10^{-2} in the mixtures, a control was included by diluting the serum 10^{-2} before mixing with virus dilutions.

Injected intracerebrally immediately after mixing.

Dilution of virus.	V + N-S sheep.	V + I-S pig.	(V + I-S) dil. 10^{-1}	V + diluted I-S 10^{-2}
10^{-4}	---			
10^{-3}	+	---	---	---
	+	---		---
10^{-2}	+	+	---	+
	+	---	---	---
10^{-1}	+	+	---	+
	+	+	---	+
Undil.		+	+	+
		+	+	---

The serum-virus mixture containing 10^{-2} dilution of the virus was partially neutralised. On dilution, the virus was completely inactivated. This was due to neutralisation by the immune-serum in the diluted mixture, as the control with 10^{-3} dilution of virus + N-S was positive.

Adsorption/

Adsorption of the virus to L_5 filter powder.

Decimal dilutions of the mouse-brain emulsion were mixed with an equal volume of the undiluted serum under investigation. 20-30 mgms of Chamberland L_5 filter powder were then added, and left in contact for 15 minutes at room-temperature, the mixture being shaken several times during this period. The mixture was then centrifuged at 2000 r.p.m. for 10 minutes, the supernatant fluid poured off, and Locke's fluid was added to the filter powder to restore the original volume (1 c.c.). The result of intracerebral injection of the filter powder emulsion into mice was as follows.

Dilution of virus.	V + N-S sheep.	(V + N-S) in filter.
10^{-3}	---	---
10^{-2}	---	---
	---	---
10^{-1}	+	+
	+	+
Undil.		+
		+

It was possible to adsorb the virus from the normal serum-virus mixtures by the powdered filter.

Recovery of virus from neutral serum-virus mixtures by filter powder.

In/

In the following experiments, an attempt was made to recover the virus from apparently neutral serum-virus mixtures by adsorption by the filter powder.

Undiluted immune pig serum was mixed in equal volumes with decimal dilutions of the virus, part of the mixture was treated immediately after with filter powder as in the previous experiment. As a control, mice were injected with the same mixtures without treatment, and the virulence of the virus was indica-:ted by injecting filter powder treated by virus and normal sheep serum mixtures. The result was as follows.

Dilution of virus.	V + N-S in filter.	V + I-S	(V + I-S) in filter.
10^{-4}	+ ---		
10^{-3}	+ +	--- ---	--- ---
10^{-2}	+ D	--- ---	+ ---
10^{-1}	+ D	+ D	+ +
Undil.		+ +	+ +

The neutral serum-virus mixture containing 10^{-2} dilution of the virus was partially reactivated. Serum-virus mixtures containing smaller concentrations of/

of the virus could not be reactivated at all showing that firm antigen-antibody union had probably occurred in mixtures containing an excess of immune serum.

In the next experiment, the serum-virus mixtures were incubated for 4 hours at 37°C before treatment with the filter powder. In the same experiment, the capacity of filter powder to adsorb the antibody was investigated by separately treating the virus dilu-:tions and the immune serum with filter powder, removing the supernatant layers by centrifugation, mixing the deposits and immediately injecting intra-:cerebrally.

Dilution of virus.	V + N-S (4 hours	V+I-S - 37°C)	V + I-S in filter.	V in filter+ I-S in filter. (0)
10^{-6}	+	---	+	---
	D	---	+	---

10^{-5}	+	---	+	---
	D	---	---	---
10^{-4}	+	---	+	---
	+	---	---	---

10^{-3}	+	---		---
	+		+	---
		D	+	

10^{-2}	+	---	---	---
	+	---	---	---
10^{-1}		+		+
		+		+

(0) = injected immediately after mixing.

Partial/

Partial dissociation of the virus occurred in nearly all the neutral serum-virus mixtures. The virus used was highly potent and acted up to the highest dilution tested. There was therefore the possibility that in no mixture was there sufficient excess of the immune serum to overneutralise the virus, and hence dissociation occurred. The suspen-:sion in Locke's fluid of the filter powder, which was first treated by the immune serum before mixing with the virus dilutions proved more potent than the antiserum in neutralising the virus. The reactiva-:tion by filter powder of the virus in immune serum virus mixtures showed that relatively much more of the virus than antibody was adsorbed by the powder.

In the following experiments, it was again shown, that when the virus dilutions were mixed with the undiluted immune serum and then treated with the filter powder, the virus could be reactivated to some extent. But filter powder, which was first treated by the immune serum, and then mixed with the powder treated by the virus dilutions, was very potent in neutralising the virus.

Dilution/

Dilution of virus.	V + N-S (4 hrs	V + I-S - 37°C)	(V + I-S) in filter. (4 hrs-37°C)	V in filter + I-S in filter. (0)
10^{-3}	--- ---	--- ---	--- ---	---
10^{-2}		--- ---	---	---
10^{-1}		D	+ ---	--- ---
Undil.		--- D	+ ---	

(0) = Contact few seconds.

The experiment was repeated.

Dilution of virus.	V+N-S (4 hrs-37°C)	V+I-S	(V+I-S) (4 hrs-37°C) in filter.	(V+N-S) (0) in filter	(V+I-S) (0) in filter	V in filter+ I-S in filter (0)
10^{-3}	---	--- ---	--- ---	--- ---	---	--- ---
10^{-2}	+ +	--- ---	--- ---	---	---	---
10^{-1}	+ +	--- ---	--- ---	+ ---	---	--- ---
Undil.		---	+ ---	+ +	+ ---	

The neutral serum-virus mixtures could be partially reactivated by the filter powder. But the filter powder first treated by the immune serum had marked avidity for the virus.

Reactivation/

Reactivation of virus from neutral serum-virus mixtures by blood-clot.

It had been shown previously that Louping-ill virus could be concentrated by the rabbit blood clot. In the following experiments, decimal dilutions of the mouse-brain emulsion were mixed with undiluted serum, and after varying periods of incubation the mixtures were treated with a few drops of normal rabbit blood taken directly from the ear-vein. The blood was allowed to clot and retract, the mixture was then centrifuged at 2000 r.p.m. for 10 minutes. The supernatant fluid was poured off, and the deposit was taken up in Locke's fluid, which was brought to the original volume. After breaking up the clot as finely as possible, the resulting emulsion was injected intracerebrally into mice.

In the following experiment, decimal dilutions of mouse-brain emulsion were mixed with equal volumes of normal and immune sheep sera. The mixtures were immediately after dissociated by the rabbit blood clot. The result was as under.

Dilution/

Dilution of virus.	V + N-S	blood + clot from V + N-S	blood-clot from V + I-S
10^{-3}	---	---	---
10^{-2}	+ D	+ +	--- ---
10^{-1}	+ D	+ ---	--- ---
Undil.	+ D	+ +	+ ---

The virus was partially dissociated in the mixture containing the undiluted virus and immune serum, and not in those containing smaller concentra-:tions of the virus.

It had been shown before, that there was better neutralisation of the virus by immune serum after incubation of the serum-virus mixtures for 4 hours at 37°C than when the contact was for a few seconds only. In the following experiment immune serum virus mixtures were dissociated (a) a few seconds after mixing, (b) after 4 hours incubation at 37°C.

Dilution/

Dilution of virus.	clot from (V + N-S) (0)	clot from (V + I-S) (0)	clot from (V + I-S) (4hrs-37°C)	V + I-S (4hrs-37°C)
10^{-6}	---	---		

10^{-5}	---	---		
	---	---		
10^{-4}	+	---		

10^{-3}	+	---	---	---
	+		---	

10^{-2}	+	+	---	---
	+	+	---	---

10^{-1}	+		+	+
	+	+	+	+
		---	---	
Undil.			+	+
			+	+

When the serum-virus mixtures were subjected to dissociation after a few seconds contact, the mixture containing 10^{-2} dilution of the virus was definitely reactivated. But after 4 hours incubation of the serum-virus mixture, no dissociation of the neutral serum-virus mixtures was demonstrable; firm antigen-antibody union had taken place.

Antibody absorption experiments.

An attempt was made to demonstrate antibody absorption/

absorption by the virus in serum-virus mixtures. The principle was to use an excess of virus and to allow it to act on the immune serum for a long period at 37°C. Three bacteria-free Louping-ill mouse brains (0.9 grammes approx.) were emulsified in 3-4 c.c. of immune serum. Rubber stoppers sealed with paraffin were used to prevent bacterial contamination. The time of incubation varied between 18-48 hours in several experiments. After incubation, the emulsion was centrifuged at 2500 r.p.m. for 15 minutes. The supernatant fluid was pipetted off and respun for the same period, then it was heated at 55°C for 30 minutes to free it from virus. It was labelled "absorbed serum" and was titrated against the virus to determine its antibody content. A control was always included by keeping the immune serum under identical conditions of temperature, and then comparing its titre with the absorbed serum.

In the following experiment, 3 bacteria-free mouse brains were emulsified in 3 c.c. of immune pig serum. The emulsion was divided into two portions, which were separately incubated for 48 hours at 37°C. The absorbed serum was tested for the presence of antibodies. Bacterial growth occurred in one of the emulsions. The result of intracerebral injection into mice was as follows.

Dilution/

Dilution of virus.	V + N-S (4hrs-37°C)	V + absorbed serum (4hrs-37°C)	V + absorbed serum contaminated	V + I-S
10^{-4}	---	---	---	---
	---	---		---
10^{-3}	---	---	---	---
	---	---	---	---
10^{-2}	+	---	---	---
	---	---		
10^{-1}	+	---		---
	+	---		---
Undil.				

No antibody absorption was demonstrable.

The experiment was repeated. 4 mouse brains were emulsified in 1/20 dilution of immune pig serum, the incubation being for 48 hours at 37°C.

Dilution of virus.	V + N-S (0)	V + I-S (0)	V + absorbed serum (0)
10^{-3}	---	---	---
	---	---	
10^{-2}	+	---	
	+	---	---
10^{-1}	+	+	+
	+	+	+
Undil.		+	
		+	

The absorbed serum was as active as the immune serum/

serum, no antibody absorption was demonstrable.

In a further experiment, immune sheep serum was substituted for the immune pig serum. 3 mouse brains were emulsified in 1.5 c.c. of serum, the incubation being for 18 hours at 37°C.

Dilution of virus.	V + N-S (4hrs-37°C)	V + I-S (4hrs-37°C)	V + absorbed serum (4hrs-37°C)
10^{-3}	---		
10^{-2}	+	---	---
	+	---	
10^{-1}	+	---	+
	+		D
Undil.	+	D	+
			+

Partial antibody absorption was demonstrable.

In the next experiment, the antibody content of the immune pig serum was reduced by diluting it to 10^{-2} before it was emulsified in mouse-brain emulsion. The result of titrating the antibody content of the absorbed serum was as under.

Dilution/

Dilution of virus.	V + N-S (0)	V + I-S (0)	V + absorbed serum (0)
10^{-3}	--- ---	--- ---	--- --- ---
10^{-2}	+ +	--- ---	+ --- ---
10^{-1}	+ +	+ +	+ +
Undil.		+ +	+ +

Partial antibody absorption was demonstrable.

Antibody absorption occurred only from those sera, whose antibody content was low.

In the following experiment, a highly virulent strain of virus was used for antibody absorption. The serum-virus emulsion was divided into two parts. One was treated by 10% ether to inactivate the virus, then both were incubated for 48 hours at 37°C.

Dilution of virus.	V + N-S (4hrs-37°C)	V + serum absorbed etherised virus (4hrs-37°C)	V + absorbed serum (4hrs-37°C)	V + I-S
10^{-3}	--- D	---	---	--- ---
10^{-2}	+ +	--- ---	--- ---	---
10^{-1}	+ +	--- ---	+ ---	--- ---
Undil.		--- D	+ ---	--- ---

Partial/

Partial antibody absorption was demonstrable from the absorbed serum, which had been treated with live virus, the immune serum treated by etherised virus, was as potent as the unabsorbed.

Complement-fixation and flocculation reactions.

Complement-fixation test.

The supernatant fluid from a 5% centrifugalised Louping-ill mouse brain emulsion was used as the antigen, normal mouse brain being used as a control. The haemolytic system was prepared by adding 6 M.H.D. of the immune body to a 3% emulsion of thrice washed sheep corpuscles. 0.25 c.c. of the antigen and 0.1 c.c. of the immune serum were used in the tests. The usual antigen, serum and complement dose controls were included.

	M.H.D. of complement fixed.	
	Normal brain.	Louping-ill brain.
Immune sheep serum	8	8
Immune rabbit serum	0	2
Immune pig serum	0	3

Of the three sera under examination, the titre of the neutralising antibody was previously found to be highest in the immune pig serum, which also showed the definite presence of the complement-fixing antibody.

Flocculation test.

5%/

5% emulsions of normal and Louping-ill mouse brain emulsions were mixed with an equal volume of ether, and frequently shaken. After two hours contact at room-temperature, the almost water clear fraction from the bottom was used as the antigen. Immune sera were heated for 30 minutes at 55°C, and 0.5 c.c. of two-fold dilutions were mixed with equal volumes of the antigen. After four days incubation at 55°C, definite flocculation occurred with immune pig serum up to a serum-dilution of 1/32. The immune sheep serum gave a negative result, the immune rabbit serum gave a positive reaction up to 1/8 dilution. The floccules examined under the microscope consisted of an aggregation of minute round granules.

✱

In carrying out the flocculation reaction with Louping-ill mouse brain emulsion and immune pig serum, three sets of controls were provided - (a) antigen control, (b) immune pig serum control - serial two-fold dilutions of the serum, (c) immune sheep serum - two-fold dilutions mixed with an equal amount of antigen.

As immune sheep serum containing definite neutralising antibodies gave a negative flocculation reaction, this test was not considered of great practical value and was not pursued any further.

DISCUSSION.

The problem of the biological nature of viruses has given rise to a great deal of controversy. If viruses could be cultivated on inanimate food media, they might justifiably be regarded as living organisms, but it is doubtful whether this conclusion can be legitimately drawn from the data so far available. Eagles and McClean (1931) claimed to have cultivated the vaccinia virus in a cell-free medium. Maitland, Laing and Lyth (1932) failed to confirm this. The general failure to cultivate viruses *in vitro* apart from living tissue has supported the hypothesis that they are not living entities but catalysts or ferments capable after entering the susceptible cells of so affecting its metabolism that more catalyst of the same kind is produced, which then proceeds to "infect" more widely. This theory, however, is completely out of accord with what we know of the action of ordinary ferments, which exhibit no powers of reproduction when in contact with specific substances.

Although viruses vary in size, each species maintains a constant order of size irrespective of the environment in which it is multiplying. The capacity to adapt themselves to a changed environment in highly characteristic of living things and is shown strikingly by some viruses. The first example of this is found in/

in the adaptation of smallpox virus in calves. When smallpox virus has been passed through a sufficient number of calves in succession, or even through rabbits only, it becomes so modified that even after repeated passages in man, it does not return to its original condition. Similar phenomena have been observed in the adaptation of herpes virus to brain tissue, of contagious epithelioma of fowls to pigeons, and of the mosaic disease of tobacco to cucumbers. It is difficult to reconcile such facts with any conception of viruses as inanimate principles.

In certain respects the pathogenesis of virus diseases appears to differ to a greater or less degree from bacterial infections. The tendency to selective localisation appears to be more definite, and it has become customary to use such terms as dermotropic or neurotropic to define the special but by no means exclusive affinities of a given species or strain. Another peculiarity of the viruses is their tendency to produce characteristic "inclusion bodies" in certain cells. It seems possible that this habbit of functioning as intracellular parasites has an important bearing on anti-viral immunity.

Of the ultimate mechanism by which viruses produce their harmful effects we as yet know little or nothing. We do not, for instance, know whether any of them produce soluble toxins with a characteristic biological/

biological action, as in the case of certain bacteria. Wilson Smith (1932) and Craigie (1932) isolated a specific soluble substance derived from the vaccinia virus, which shares with the bacterial haptens the capacity to yield specific precipitation *in vitro*, although it is unable to stimulate antibody production *in vivo*.

If the mechanism of anti-viral immunity could be shown to be that of anti-bacterial immunity, the supposed relationship of viruses to the bacteria would be strengthened. There was a tendency amongst earlier workers to differentiate somewhat sharply between immunity to viruses on the one hand and to bacterial infections on the other. Almost all experiments on the transmission of virus infection and on immunity reactions *in vivo* and *in vitro*, have of necessity been carried out with tissue extracts or with other crude material containing host products as well as virus. Under these conditions the qualitative or quantitative analysis of such reactions is rendered very difficult, and for this reason knowledge of the mechanism of anti-viral immunity had tended to lag behind that of anti-bacterial immunity.

Before discussing the mechanism of anti-viral immunity in the light of the present work and its similarity to or difference from that of anti-bacterial immunity/

immunity, some reference may be made to mechanisms involved in the latter state.

Anti-bacterial sera may be subdivided into two groups according to the mechanism by which they bring about the death of the homologous organisms. The serum principles involved may be either (a) an anti-:body which along with complement leads to the death of corresponding bacteria with or without lysis- bactericidal action, or (b) an antibody which sensitises bacteria to phagocytosis without the necessary inter-:vention of complement. In the study of the mechanism of anti-viral immunity, it is impossible at present to test any direct in vitro viricidal action of an anti-:serum, and the same applies to the investigation of any effect analogous to bacteriotropic action. Pfeiffer and Issaef (1894) and Pfeiffer (1895) found that on injection of an immune serum along with the corresponding organism into a normal animal, bacterio-:lysis could be observed within a few minutes. Bezzola (1909) found that lysis as a rule occurred more readily in the peritoneal cavity than when the test was made in vitro. On the other hand when toxin and antitoxin are brought together in vitro, a definite period of time elapses before neutralisation of toxin is complete. Morgenroth (1907) showed that in the case of diphtheria toxin this is considerable - about twenty-four hours.

The/

The determination of the period of time required for the inactivation of virus *in vitro* by the corresponding serum would be of great interest. If inactivation were shown to occur within a few minutes, then a close analogy could be drawn with bactericidal action.

Morgenroth (1907) found that a neutral mixture of diphtheria toxin and antitoxin could be broken up by dilute hydrochloric acid, and the two constituents recovered, this union being thus reversible. Ramon (1923) showed that salt-free toxin-antitoxin floccules dissociate upon the addition of 1 in 1000 to 1 in 1,500 acetic acid. Upon heating for an hour at 58°C, toxin is destroyed and anti-toxin remains. Otto and Sachs (1906) dissociated toxin-antitoxin by dilution, but pointed out that when the mixture was allowed to stand for twenty-four hours, so that combination is complete, the phenomenon no longer occurred. If virus could be recovered from a neutral serum-virus mixture, then obviously its behaviour would resemble that of a toxin, which is not destroyed in a neutral toxin-antitoxin mixture and can be recovered by various means.

Camus (1908), Sato (1921) and Soberheim (1925) in connection with vaccinia virus were unable to immunise animals by injection of immune serum-virus mixtures, which/

which suggested the destruction of the virus-antigen. Gordon (1925) supported this view: that the antiserum directly kills the virus *in vitro*, because in one of his experiments, complement enhanced the action of heated serum on the virus, but he could not confirm his first result. Andrewes (1928) showed that vaccinia virus could be recovered from an apparently neutral serum-virus mixture and this led to the idea that neutralisation of the virus by an antiserum resembles a toxin-antitoxin union. This view cannot be accepted, because recovery of the virus from the immune serum-virus mixture, which had not been shown to be neutral has no significance. Although the serum-virus mixture was found to be neutral on injec-:tion into a test animal, no antigen-antibody union might have taken place *in vitro*. In 1930, in care-:fully controlled experiments, Andrewes observed that with prolonged contact virus was more and more difficult to recover, but some dissociation was possible even after several days. He did not test the action of high concentrations of antiserum on minimal amounts of the virus, and the results encountered might have been very different, as will be shown in succeeding pages.

Anti-bacterial immune substances may also be demonstrable by their effects in producing clumping or/

or agglutination of corresponding bacteria or by producing a precipitate with a filtrate of culture. Precipitation however also results from the interaction of toxin and antitoxin. If the precipitated floccules could be shown to consist of aggregations of specific elementary bodies, then the viruses would be comparable to bacteria in relation to this phenomenon. This has been achieved by Ledingham (1931) and Bedson (1932) in connection with vaccinia and psittacosis respectively.

The complement-fixation reaction is characteristic of antigen-antibody reactions generally, and its study in relation to anti-viral immunity has not yielded data of special significance as regards the question at issue: and the same applies to the phenomenon of antibody absorption by the particular antigen.

In the study of the mechanism of anti-viral immunity, the following questions arise:
(1) Whether an antigen-antibody union has occurred in vitro. It would not be possible, however to determine, whether the virus was killed in vitro, or only sensitised to the destructive action of the phagocytes or other cells of the test animal.
(2) The time taken in inactivation: whether inactivation of the virus occurs rapidly like the lysis of bacteria or more slowly like the neutralisation of toxin/

toxin.

(3) Recovery of virus from neutral serum-virus mixtures.

In the present work, the problem of the state of the virus in neutral serum-virus mixtures was re-investigated. Dissociation of the neutral serum-virus mixtures was effected by (1) dilution, (2) adsorption by powdered Chamberland L_2 filter and blood-clot, (3) precipitation of euglobulins. It must be borne in mind that only a partial separation of virus and antibody was effected by any of the above-mentioned methods.

Dilution phenomenon.

Bedson (1928) gave the following rationale of the phenomenon: when a mixture of virus and antiserum just neutral as tested in vivo, in which the virus concentration is considerable, is diluted, the highest effective dilution of the serum is rapidly exceeded while the diluted virus is still effective; and in this manner, it may be possible to determine the state of the virus in an apparently neutralised mixture and so ascertain whether any interaction between virus and antibody is taking place in the test tube. At first sight this conception might appear unreasonable because dilution of a balanced mixture of virus and antibody/

antibody reduces both these factors in the same pro-:portion. But the rate at which antigen and antibody unite depends on their concentration so that by diluting a balanced mixture of virus and antiserum the time required for their interaction is prolonged. When a balanced mixture of virus and antibody is diluted and injected into a test animal, the diluted antiserum would act on the diluted virus in the tissues. The time of their union having been con-:siderably prolonged, the virus would establish itself in the tissues before the antigen-antibody union occurred. A negative reaction would only result when the time required for the union of virus and antibody was less than that required for the virus to infect the tissues. Thus by the simple procedure of dilution and consequent prolongation of the time required for the serum to act, active virus may be unmasked in an apparently neutralised mixture.

The sera used by Andrewes varied considerably in potency, a fairly potent undiluted sample inactiva-:ted from 100-1000 m.i.d. of virus. In experiments with neutral serum-virus mixtures, he had to work within a very narrow range of virus dilutions. In the experiments recorded in this thesis, ordinary immune serum was obtained from several rabbits. The titre of the various fractions varied to some extent, but/

but 10^3 - 10^5 skin doses of the virus were usually neutralised. Those serum-virus mixtures which were more or less balanced could be regularly reactivated by a 10^{-1} dilution in saline. On the other hand neutral serum-virus mixtures containing an excess of immune serum or minimal concentrations of the virus showed a stable antigen-antibody union. The anti-:serum used by Andrewes had a low antibody content, and it was not practicable for him to attempt dilution of over-neutralised mixtures of serum and virus, as such mixtures already contained a low concentration of virus and further dilution would have rendered it inactive. It is therefore obvious that he was working with only just balanced mixtures, which have been shown to be readily reactivated by dilution.

In working with fowl-plague virus, Todd (1928) was able to reactivate neutral serum-virus mixtures by dilution in saline, but not by dilution in normal fowl-serum. But Andrewes (1928) in the case of vaccinia virus successfully reactivated both by dilution in saline and in normal rabbit serum. Normal rabbit sera were used in four dilution experi-:ments of the present work (vide page 43). Reactivation of the virus definitely occurred in one experiment; only a trace in the second and none at all in the other two. The failure of dissociation by/

by dilution was probably due to a high natural pro-:tective action of the sera used. Although the natural immune property of the sera was not thoroughly investigated, the varying response of rabbits to vaccinia infection which is well-known was constantly observed. In one experiment, normal rabbit serum was heated at 55°C for 30 minutes in an attempt to inactivate or reduce the natural neutralising anti-:bodies, and dilution in this heated normal serum reactivated the virus, but not dilution in the unheated normal serum. The varying results of the work of Todd and Andrewes may possibly be explained by the varying natural protective action of the sera used.

Craigie and Tulloch (1931) found that a floccu-:lating antiserum might inhibit the dermal infectivity of vaccinia exposed thereto <u>in vitro</u>, but that the infective agent was not killed by such exposure, for by testicular inoculation, the virus was found to be still active. They did not explain the discrepancy between their results and those of Andrewes. In the experiments recorded in this thesis, it was shown that hyperimmune serum neutralised the virus almost immediately on mixing, the antigen-antibody union being stable, as the neutral serum-virus mixtures could/

could not be dissociated even on 10^{-3} dilution in saline. When the hyperimmune serum was diluted 10^{-1} before use in neutralisation experiments, it was found that balanced mixtures could be dissociated by dilution in saline. The serum-virus mixtures con-:taining diluted hyperimmune serum behaved like those containing ordinary immune serum, the only difference between the two seemed to be a question of the amount of antibody being greater in hyperimmune serum. On hyperimmune serum being previously diluted to 10^{-2} and then mixed with the virus dilutions, neutralisa-:tion of the virus was very unsatisfactory; but when hyperimmune serum was mixed with the virus dilutions and then the mixture diluted 10^{-2}, reactiva-:tion of the virus failed to occur. A few seconds contact of undiluted hyperimmune serum with the virus was enough to produce a stable union.

Andrewes (1928) found that a neutral mixture of vaccinia virus and immune serum could be reactivated by 1/125 dilution in saline, but not by 1/5 or 1/25 dilution. While Todd (1928) working with fowl-plague virus found that a serum-virus mixture containing an excess of immune serum could not be reactivated on dilution. Todd, however, did not test high dilutions of the overneutralised mixtures. In the present work, when/

when the neutral serum-virus mixture was diluted even up to 10^{-3}, no reactivation occurred, although the control with the 10^{-3} dilution of virus alone was positive.

Craigie and Tulloch (1931) suggested that the efficacy of Indian ink, acriflavine, quinine hydro-:chloride, and possibly certain immune sera to modify the lesions resulting from inoculation of vaccinia virus into the skin was solely an *in vivo* effect. Their presence might either prevent the virus from gaining access to the cells or allow the cells to deal effectively with it. Andrewes (1928) in connection with vaccinia postulated a local passive immunity of the tissues produced by the antiserum, which was responsible for the neutralisation of the virus in the skin of rabbits. It is shown in the present work (*vide* page 39), that antivaccinial serum introduced within twenty minutes of the undilu-:ted neuro-virus in exactly the same area neutralised the virus; also serum introduced 30 minutes before the virus in the same area afforded complete protection. It was further demonstrated that diluted virus given intradermally was completely neutralised by the serum introduced in the same spot even 40 minutes later. But it was also observed that when undiluted virus and immune serum were mixed and after a few seconds the mixture was diluted 10^{-2} and injected intradermally, no/

no lesion appeared. But when 10^{-2} dilution of the virus was mixed with 10^{-2} dilution of immune serum and tested in vivo, there was a definite reaction. The concentration of the constituents of the mixture was the same in both intradermal tests, but the results were markedly different. The capacity of the immune serum to modify the vaccinial lesion could not solely be an in vivo effect, as a definite in vitro inactivation of the virus was demonstrable. This was confirmed by experiments carried out with filter powder and euglobulins.

Reactivation of neutral serum-virus mixtures by (1) Chamberland L_2 filter powder and (2) blood-clot.

Andrewes (1928) devised[1] a method to adsorb the virus from neutral serum-virus mixtures by Chamberland L_2 filter powder, but he discarded it, because he found that the antibody was also adsorbed. Carefully controlled experiments (vide page 54) showed that although both the virus and antibody were adsorbed, much more of the virus than the antibody was present in the filter powder. This method was a valuable adjunct to dilution method in reactivating neutral serum-virus mixtures. The previous observations were confirmed that only just balanced mixtures were dissociable. The result was the same, when the blood-clot method was employed, but as blood-clot tends/

tends to adsorb larger proportions of antibody from neutral mixtures, this method was not so satisfactory.

Reactivation of neutral serum-virus mixtures by precipitating euglobulins.

Virus could be recovered in the euglobulins obtained from more or less balanced mixtures. The controls showed that a considerable amount of antibody was also associated with the euglobulins, but carefully controlled experiments (vide page 62) revealed that much more of the virus than antibody was precipitated in the euglobulins. The conclusion of Andrewes (1928) that this was the best method of reactivating the virus from neutral serum-virus mixtures could not be borne out. Findlay (1931) and Ledingham, Morgan and Petrie (1931) have also found that a considerable amount of vaccinial antibody is associated with the euglobulins of the antivaccinial serum.

Craigie and Tulloch (1931) found that immune serum-virus mixtures could not be dissociated by precipitation of euglobulins. The mixtures could not be dissociated by precipitation of euglobulins. The failure might have been due to a concomitant high antibody content of the euglobulins, which masked the virus on intradermal injection.

Intracerebral/

Intracerebral and intra-ocular investigation.

All the points so far raised have been in relation to the intradermal titration of serum-virus mixtures.

Andrewes (1928) showed that a serum-virus mixture, which was found to be neutral on intradermal injection was active when administered intracerebrally or intra-testicularly. Fairbrother (1932) succeeded in demonstrating neutralisation of the virus after the serum-virus mixture was injected intracerebrally. He found that the incubation of serum-virus mixtures was necessary for the demonstration of neutralisation.

An attempt was made (vide page 65) to test the action of hyperimmune serum on minimal concentrations of the virus. It was interesting to note that 10^3 - 10^4 skin doses of the virus were required to produce encephalitis or irido-cyclitis in adult rabbits, smaller doses having no visible effect. Undiluted virus when mixed with an equal amount of hyperimmune serum and injected intracerebrally immediately after was found to be neutral, but the neutral serum-virus mixture could be reactivated on dilution. Higher concentrations of the virus were neutralised by the immune serum, when the serum-virus mixture was incubated showing therby that the serum was acting directly on the virus. The varying response of tissues/

tissues to infection by the same serum-virus mixture was noted. A serum-virus mixture, which was found to be neutral on intra-corneal injection was active on intracerebral injection. This anomaly could be explained on the ground that there was extremely slow diffusion of the mixture in the cornea allowing pro-:lomged contact of the serum and virus in vivo, while there was rapid diffusion of the constituents in the brain. A serum-virus mixture which was found to be neutral on intracerebral injection produced a definite reaction on intra-ocular inoculation. It was probably due to the varying resistance of the tissues to the same infection, the brain was capable of neutralising infective material which the aqueous humor was unable to deal with.

A stable antigen-antibody union could not be demonstrated after the mixture was injected into the brain. This was to be explained on the ground that intracerebral injection required a high concen-:tration of virus for a positive result, and therefore a correspondingly high concentration of immune serum was necessary to produce overneutralisation. Such a serum was not to hand, hence the serum-virus mixtures used were more or less balanced, and reactiva-:tion of virus on dilution consequently occurred.

Andrewes/

Andrewes (1930) averred that there was evidence of slow union of vaccinia virus with the antibody *in vitro*, but the neutralisation *in vivo* occurred in a few minutes. Thus there was a radical difference between serum-virus neutralisation in the living animal and the test tube. The present work indicated that there was a close parallelism between neutralisa-:tion *in vivo* and *in vitro*.

The data so far given indicated that a direct union of virus and antibody may occur under suitable conditions *in vitro*. This view is further strengthened by the results of antibody absorption tests, in that partial antibody absorption from serum-virus mixtures was clearly demonstrated (*vide* page 75).

Virus and excess of immune serum, left in contact for a few seconds, failed to give a positive reaction on intradermal injection; the same mixture was found to be neutral on intracerebral inoculation, but on dilution reactivation occurred and positive reactions to intracerebral injection resulted.

Heated immune serum was found to be as active as unheated serum, and there was no evidence to indicate that complement acted on the serum-virus mixture *in vitro*. The experiment reported by Gordon (1925) in which complement was found to assist serum-virus union was not adequately controlled in that the action of/

of the complement containing serum or virus *per se* was not determined over a sufficiently wide range of virus dilutions. In these experiments the *in vivo* action on the serum-virus mixtures of the complement could not be excluded. Since complement was not necessary for serum-virus neutralisation to occur, and dissociation by dilution etc. was possible, the union of immune serum and virus resembled toxin-antitoxin fixation. On the other hand specific flocculation (Craigie and Tulloch, 1931) and comple-:ment-fixation reactions (Gilmore, 1931) could be demonstrated which implied definite *in vitro* union of virus and antibody.

It must be borne in mind that immune sera seem to kill bacteria only with the co-operation of leucocytes or of complement.

Ledingham (1931) has put forward evidence to show that vaccinia virus is probably identical with the Paschen bodies. The virus only passes the coarse filters with difficulty and so there seems no doubt that it is much larger than the molecules of bacterial toxin. Vaccinia virus is thus intermediate in size between bacteria and toxins, hence it may have its own peculiar mechanism in dealing with antisera.

Vaccinia virus produces lesions in which there is much cellular reaction containing a large number of/

of phagocytes; and experiments performed by Douglas and Smith (1930) suggest that some of the phagocytic cells of rabbits immunised against vaccinia may be actively concerned in the increased resistance of such animals. In the present work (vide page 83) it was noted that there was a radical difference between the leucocytes from normal and immune rabbits. The "immune" leucocytes definitely inactivated the virus per se after the leucocyte-virus mixture was injected intradermally or intracerebrally. (It is possible that this action might have been due to the adsorbed antibodies, which were not removed even after repeated washing). Normal leucocytes either did not diminish the potency of the virus or exerted a protective effect. "Immune" leucocytes definitely enhanced the action of immune serum on the virus in 2 out of 3 experiments, the effect being more marked after incubation. Although no difference has been detected between "immune" and normal leucocytes in immunity studies with bacteria, the very fact that leucocytes acted on the virus seems to differentiate it from toxins and provide an analogy with the bacteria. Also stable antigen-antibody union in overneutralised serum-virus mixtures occurring after a few seconds contact, is strongly in favour of the reaction resembling a bactericidal action rather than a/

a toxin-antitoxin union.

The mechanism of anti-viral immunity in louping-ill.

The problem of inactivation of the virus in neutral serum-virus mixtures in louping-ill had to be investigated on slightly different lines from vaccinia. The presence in any tissue of the virus, on account of its highly neurotropic character, had to be determined by intracerebral injection, whereas vaccinia virus could be tested for by intradermal injections. As was noted before, the results of injection of vaccinia virus and antiserum mixtures into the skin and brain gave slightly different results, firm antigen-antibody union being demonstrable by the first route and not by the latter. It is possible that if a more potent antiserum had been available, the serum-virus mixtures would have been overneutralised and a firm antigen-antibody union might then have been demonstrated after intracerebral inoculation of the same. This was borne out by the results of intracerebral injection of the virus.

It was found, that although on intravenous injection of louping-ill virus into a normal rabbit, the virus was rapidly removed from the circulation, normal rabbit blood or serum had no definite inactiva-:ting effect on the virus in vitro. But neutralising antibodies/

antibodies could be demonstrated in the serum on immunisation of this refractory animal. As regards the time required for neutralisation to occur in immune serum-virus mixtures, Zinsser and Tang (1926) working with the neuro-tropic herpes virus stated that such mixtures must be incubated before intra-:cerebral injection in rabbits. In the present work, however, it was found that a potent antiserum could neutralise minimal concentrations of a similar neuro-:tropic virus, i.e. louping-ill, immediately on mixing. Similarly neutralisation of vaccinia by immune serum occurred immediately on mixing.

Schultz, Gebhardt and Bullock (1931) working with poliomyelitis virus also found that a concentrated immune serum added to an appropriate quantity of virus might serve to render such a virus suspension inno-:cuous immediately after the serum was added. They suggested that the anti-viral serum did not act directly on the virus, but indirectly by diminishing the susceptibility of the tissues of the host. They did not confirm this suggestion by testing the effect of injection of suitable dilutions of the virus into the brain of a monkey which had been previously treated by immune serum. In the present work, when immune serum was injected intracerebrally and louping-ill/

ill virus was administered in the same area a few seconds later, neutralisation of minimal concentra-:tions of the virus was clearly demonstrable. On the other hand, when antisera was allowed to act on the louping-ill virus in vitro and the time of contact and temperature were varied, it was shown that actual neutralisation occurred, the degree of this effect being proportioned to the time of contact and temperature within certain limits.

Heated serum was found to be as potent in neutralising the virus as the unheated serum, and there was no evidence to indicate that complement played a major role in the inactivation of the virus by the antiserum in vitro. But positive complement-fixation and flocculation tests showed that there was a specific antigen-antibody reaction in vitro. Attempts were made to recover the virus from neutral serum-virus mixtures. Those mixtures, which were partially neutralised could be reactivated by dilution in Locke's fluid. Stable union had occurred in completely neutral serum-virus mixtures, and no virus was recovered on dilution of the same. Similarly the results of adsorption of the virus by a powdered Chamberland L_5 filter showed that immune serum-virus mixtures containing an excess of immune serum, or minimal concentrations of the virus could not be reactivated/

reactivated; but virus could be recovered from more or less balanced mixtures. The filter powder which had been treated by the immune serum exhibited a marked avidity for the virus. This observation was comparable to that of Bedson (1928) in connection with collodion particles and herpes virus.

When an attempt was made to recover the virus from neutral serum-virus mixtures by the blood-clot method, the previous observations were confirmed. When an immune serum-virus mixture was incubated, the virus could not be recovered by adsorption in blood-clot, although the attempt was successful, when the serum and virus had been in contact for a few seconds.

The absorption experiments indicated, that a trace of antibody absorption occurred when a great excess of virus was added to a minimal amount of antiserum. Elford and Andrewes (1933) working with bacteriophage found it extremely difficult to demons-:trate antibody absorption, even the balanced mixtures of phage and antiphage serum contained a very large excess of immune serum.

In one experiment, although live virus adsorbed small amounts of antibody, virus inactivated by ether failed to adsorb any. This observation pointed to a difference between the behaviour of this virus and of bacteria/

bacteria in general, since inactivated bacterial emulsions adsorb antibodies to the same extent as living organisms.

Thus recovery of virus from more or less balanced neutral serum-virus mixtures suggested that a certain amount of virus was not actually destroyed but rather inhibited in the presence of antiserum. There was no evidence to indicate that inactivated virus could be recovered by dissociation of an over-neutralised serum-virus mixture. The action of heated immune serum on the virus was not enhanced by the addition of complement to serum-virus mixtures *in vitro*, but it was impossible to exclude the action of complement in the test animal. Further, comple-:ment was fixed by the serum-virus mixtures, only when the serum contained the specific antibody.

The work which has been recorded in this thesis elicits no major or fundamental differences between the mechanisms of anti-viral and anti-bacterial immunity.

DISCUSSION ON THE PATHOGENESIS OF THE VIRUS OF VACCINIA.

An attempt was made by Levaditi and Nicolau (1926) and Walthard (1926) to produce vaccinial encephalitis in rabbits by intradermal administration of the virus, but no symptoms of encephalitis supervened although the virus could be recovered from the brain. When cerebral trauma was inflicted along with intradermal injection of the virus, a greater amount of the virus could be recovered from the brain than after intradermal administration alone.

McIntosh and Scarff (1930) were able to demonstrate microscopic lesions in the central nervous system after intradermal injection of the virus, but did not succeed in producing symptoms of encephalitis.

In the present work, attempts to produce manifestations of encephalitis after administration of the virus by intratesticular, intravenous and intradermal routes failed. Histological lesions of encephalo-myelitis were discernible after intravenous administration of the virus, but outward manifestations did not appear. The administration of the virus by the intravenous route along with cerebral trauma was uniformly successful in producing encephalitis, provided a reasonably large dose of the virus-material was employed.

In/

In another experiment it was found that following intracerebral inoculation, the virus multiplied locally, and when the concentration of the virus had risen to a fairly high level, then signs of encepha-:litis supervened.

Wilson Smith (1929) found that when vaccinia virus was present in blood, it was associated with the leucocyte and platelet fraction, but more so with leucocytes. In the present work it was found that the vaccinia virus was associated more with platelets than leucocytes. Segal and Bacot (1922) made a similar observation with the typhus virus.

DISCUSSION ON THE PATHOGENESIS OF THE VIRUS OF LOUPING-ILL.

For demonstration of the louping-ill virus in any tissue, intracerebral injection of the tissue emulsion into mice was required. The results of administration of the tissue emulsion by routes other than intracerebral were not uniformly successful. The incubation period was shorter when the virus was administered intracerebrally than by other routes. When virus of low virulence was administered sub-:cutaneously or intraperitoneally, symptoms of infection failed to appear, but concomitant cerebral trauma precipitated the characteristic disease. It was found that following intracerebral injection, it was difficult to recover the virus after 24 hours, but later it multiplied locally and was demonstrable in the circulation. When the concentration of the virus rose to a sufficiently high level, then symptoms of encephalo-myelitis supervened. On intravenous injection of the virus into a rabbit, it could be recovered from the whole blood, serum and red-cells respectively, whereas in vaccinia the virus could not be recovered either from whole blood, serum or red-cells, but only from the washed leucocytic fraction. There was a distinct difference between the behaviour of the two viruses in this respect.

SUMMARY AND CONCLUSIONS.

This experimental study of the mechanism of antiviral immunity revealed the following data and conclusions:

(1) When vaccinia virus was mixed with appropriate amount of antiserum and immediately injected into rabbits, inactivation of the virus to a greater or less degree was demonstrable.

(2) The virus could be recovered from neutral serum-virus mixtures, which were more or less balanced.

(3) Those neutral serum-virus mixtures which contained an excess of immune serum or minimal concentrations of the virus could not be dissociated.

(4) Partial antibody absorption was clearly shown, when an excess of virus was in prolonged contact with minimal amounts of antibody.

(5) Firm antigen-antibody union could not be demonstrated after intracerebral injection of neutral serum-virus mixtures. This was considered to be due to lack of excess of immune serum in such mixtures.

(6) In the case of another neurotropic virus, viz. that of louping-ill, it was found that firm antigen-antibody union could be demonstrated in mixtures containing/

containing either an excess of immune serum or low concentrations of the virus.

(7) Neutral serum-virus mixtures could be subdivided into (a) balanced, and (b) overneutralised. Balanced mixtures were reactivable, while definite inactivation of the virus occurred in overneutralised mixtures.

(8) No major differences have been elicited between the mechanism of anti-viral and anti-bacterial immunity.

(9) Attempts to produce manifestations of encepha-:litis after administration of vaccinia virus by routes other than intracerebral failed, but adminis-:tration of the virus by the intravenous route along with cerebral trauma precipitated the characteristic disease. An analogous phenomenon was elicited with a louping-ill virus of low virulence.

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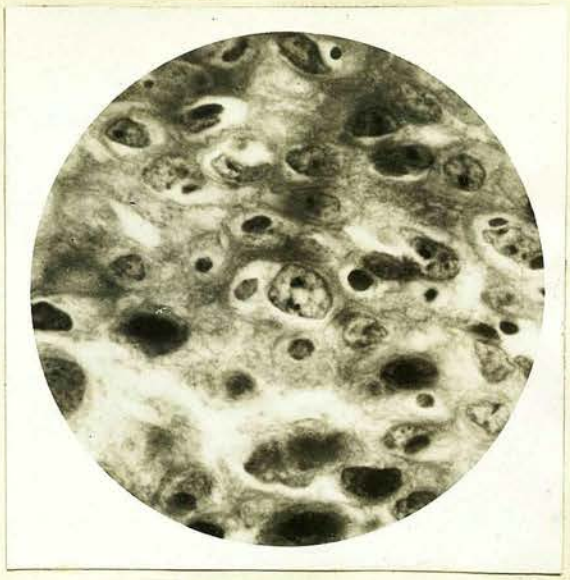

Fig.1. Section of rabbit-cornea showing Guarneiri bodies following intracerebral injection of neuro-virus.

Fig.2. Section of rabbit skin showing Guarneiri bodies following intradermal injection of neuro-virus.

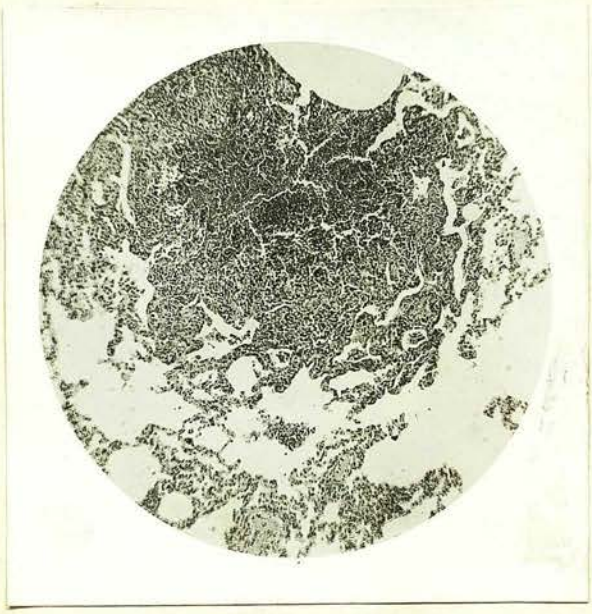

Fig.3. Section rabbit lung showing round-cell focal infil-:tration following intravenous injection of neuro-virus.

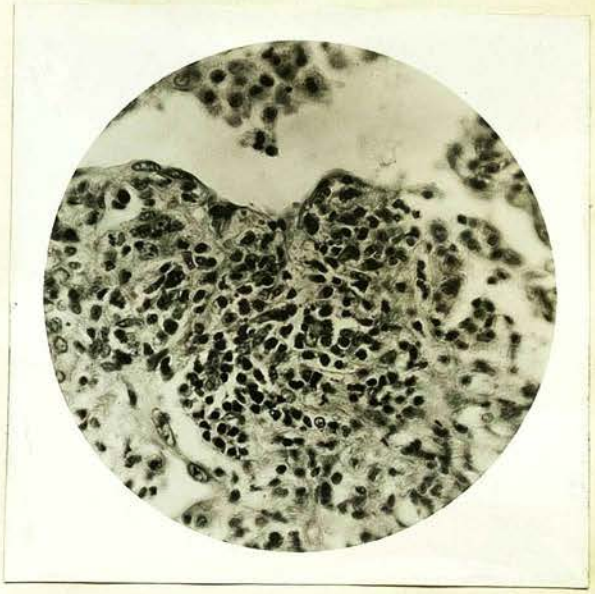

Fig.4. Same as Fig.3, under higher magnification.

Fig.9. Section rabbit cerebrum showing meningeal infiltration following intracerebral injection of neuro-virus.

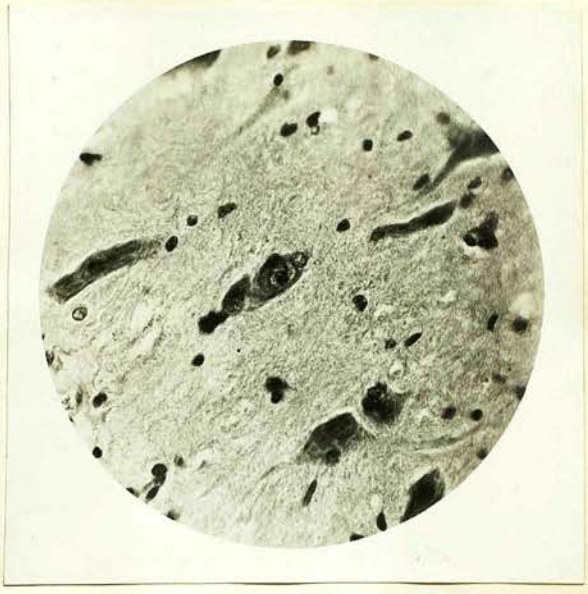

Fig.10. Section rabbit spinal cord showing neuronic degeneration with slight cellular infiltration following intravenous injection of neuro-virus.

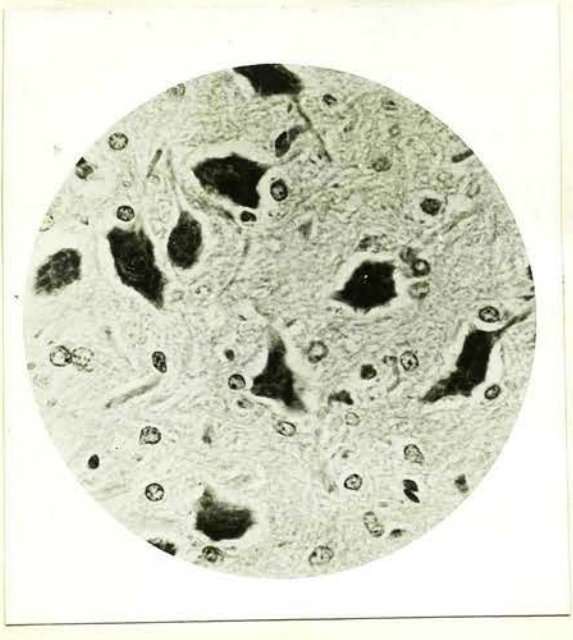

Fig.11. Section rabbit spinal cord-myelitis following intracerebral injection of neuro-virus.

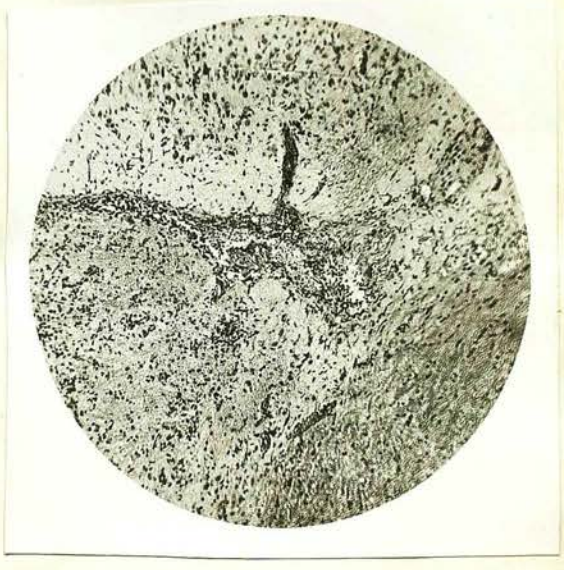

Fig.12. Section mouse-cerebrum showing marked cellular infiltra-:tion with peri-vascular cuffing following intracerebral injection of louping-ill virus.

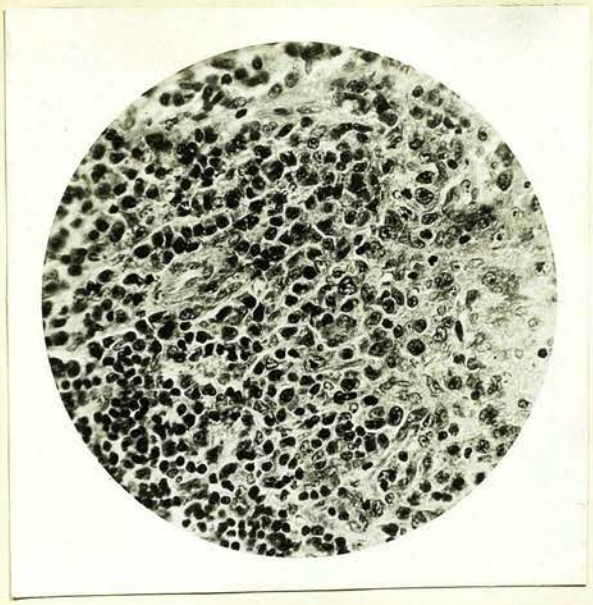

Fig.5. Section rabbit spleen-granuloma-following intravenous injection of neuro-virus.

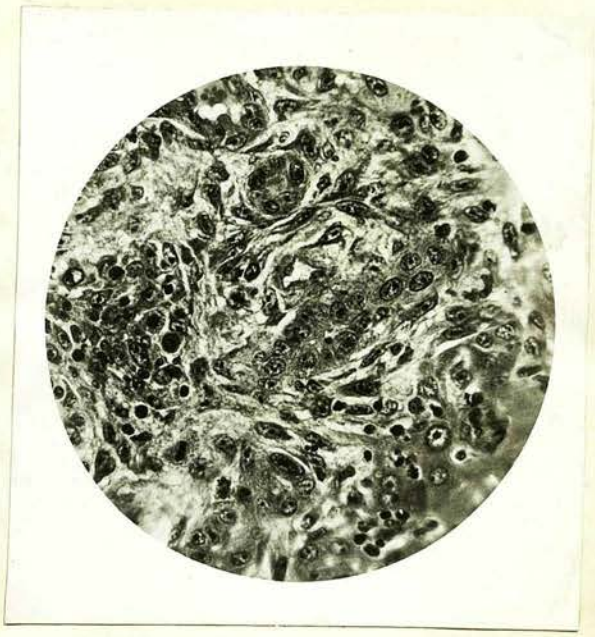

Fig.6. Section rabbit liver showing focal round cell infil-:tration following intravenous injection of neuro-virus.

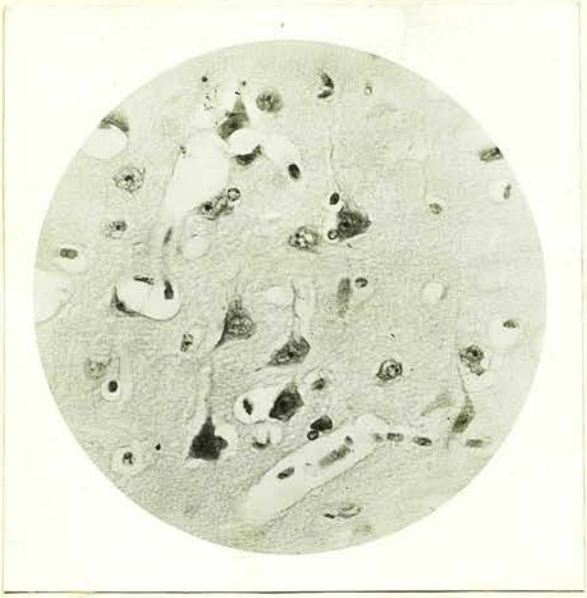

Fig.7. Section rabbit cerebrum showing neuronic degeneration with phagocytosis, i.e. encephalitis following intracerebral injection of neuro-virus.

Fig.8. Same as Fig.7, from a different rabbit.

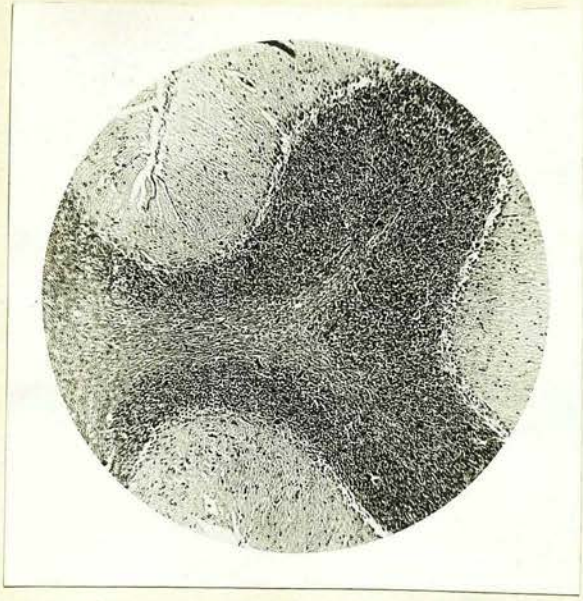

Fig.13. Section cerebellum of monkey showing almost complete disappearance of Purkinje cells following intracerebral injection of louping-ill virus.

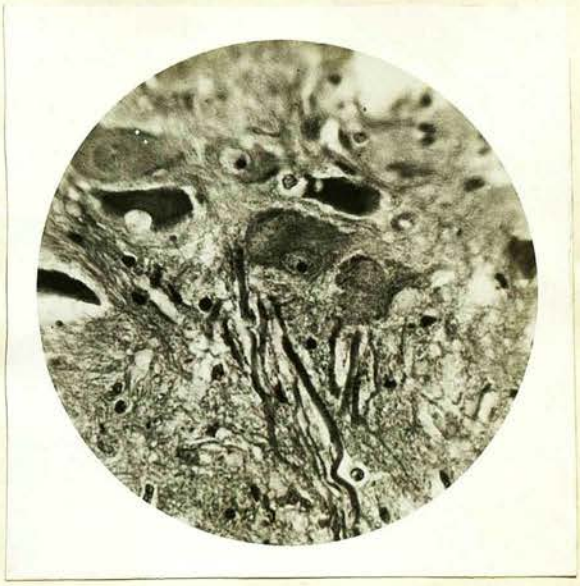

Fig.14. Section of spinal cord of monkey showing neuronic degen-:eration following intracerebral injection of louping-ill virus.

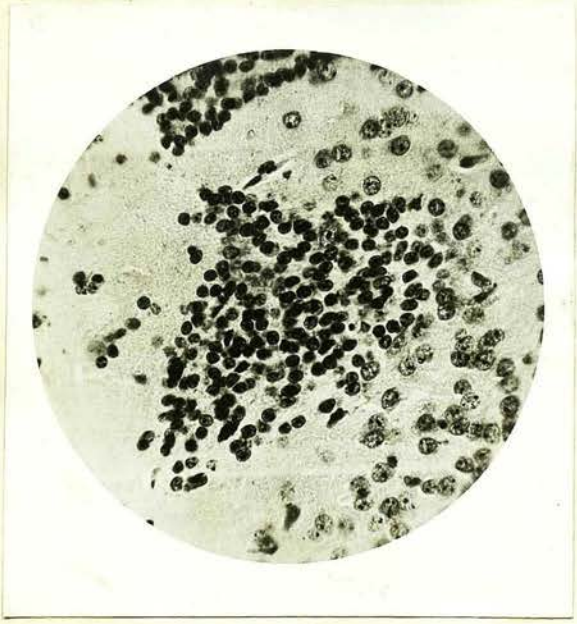

Fig.15. Section cerebrum of mouse showing focal round-cell infiltration following intracerebral injection of louping-ill virus.

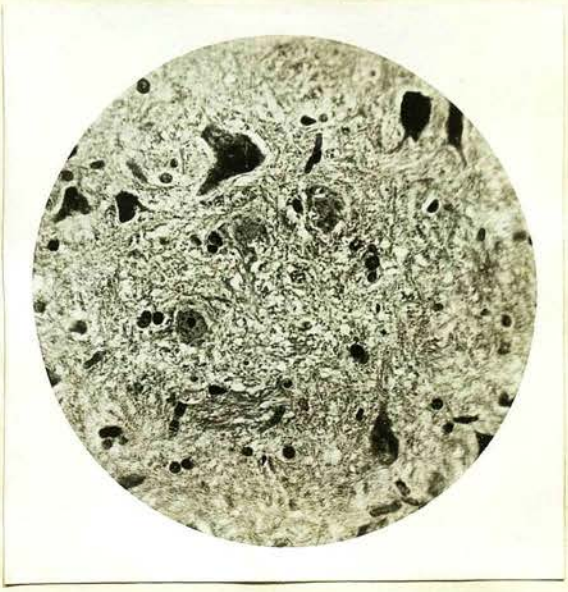

Fig.16. Section cerebrum of mouse showing neuronic degenera-:tion following intracerebral injection of louping-ill virus.