STUDIES IN THE EXPERIMENTAL PATHOLOGY OF LOUPING-ILL ENCEPHALITIS

by

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SUMMARY

Aspects of the pathogenesis and neuropathology of louping-ill encephalitis have been investigated in rodents and sheep.

In animals inoculated intraperitoneally virus was detected in the circulation before being isolated from nervous tissue. There was, however, no indication as to the means by which virus particles passed from blood to brain. Replication of virus within the central nervous system appeared to be confined to the cytoplasm of nerve cells. In infant hamster cerebellum virions were found within abnormal cytoplasmic membranes in both Purkinje cells and granule cells. Neurons containing these membranes were depleted of granular endoplasmic reticulum and showed loss of Nissl substance. Chromatolysis was seen also in ventral horn cells of moribund sheep but, although viral antigen was demonstrated in the majority of such cells by immunofluorescence, virus particles were not found on ultrastructural examination. Necrosis of ventral horn cells in sheep was accompanied by diminution in acid phosphatase activity and fragmentation of the Golgi apparatus.

Onset of symptoms preceded obvious neuronal loss in 75 per cent. of C57 black mice inoculated intraperitoneally. However in most other experiments nerve cell necrosis could be correlated with neurological dysfunction. In infant hamsters and moribund sheep the distribution patterns of neuronal damage and cells containing virus particles and viral antigen were similar. It is concluded that the basic cause of louping-ill is damage of nerve cells by the virus.

Neuronal damage in moribund sheep was most apparent in the vestibular nuclei, the motor nuclei, the Purkinje cells of the cerebellum and the

ventral horns of the spinal cord. The telencephalon was relatively unaffected. This distribution of lesions was seen in animals that were inoculated intracerebrally or subcutaneuosly, and in natural cases. Neuropathological changes were more generalized in mice inoculated intracerebrally, and were not obviously affected either by the strain of host or the presence of concurrent lesions of scrapie. Severe neuron necrosis was seen in infant hamsters and rats, but not in those more than 14 days old at inoculation. In sheep, however, the severity of lesions was similar in all age groups. No general principle can be formulated to explain the distribution of virus infected nerve cells in animals with louping-ill.

Virus was detected in nervous tissue before inflammatory changes were apparent. Severe neuron necrosis was seen in all moribund sheep, and inflammatory changes were most marked in those with the longest incubation times. There were slight generalized inflammatory lesions, which were either perivascular or associated with effete neurons, in all surviving sheep and in subclinically affected hamsters and rats. Inflammation is essentially a secondary phenomenon, which occurs after nerve cells are infected with virus.

The perivascular cuff was studied intensively in moribund sheep. Histologically most constituents of the cuff appeared to be of the lymphoid type, with a few classical plasma cells and monocytes. The majority were shown to contain globulin in their cytoplasm, when stained with a fluorescent conjugate prepared against sheep IgG. Electron microscopy showed that most of the perivascular inflammatory cells were plasmacytes. Infiltrating cells were also seen to be of the plasma cell type and were observed to enter

nervous tissue without causing any obvious damage to either endothelial cells or their basement membranes. Thus perivascular cuffing in sheep with louping-ill results mainly from migration of circulating plasma cells, or plasma cell precursors, into the central nervous system and is a specific response to the presence of viral antigen in the tissues. The inflammatory reaction is a protective mechanism, and is not responsible for the development of symptoms. In this respect it is unlike the histologically similar delayed allergic reaction.

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INTRODUCTION

Louping-ill virus is classified serologically as a member of the tick-borne encephalitis (T.B.E.) complex of the group B arboviruses (Andrewes and Pereira, 1967). It is a well known cause of fatal encephalomyelitis in sheep and cattle in nature (Pool, Brownlee and Wilson, 1930; Dunn, 1952) and is the only arbovirus yet isolated in the United Kingdom.

A feature common to all of the naturally occurring arbovirus encephalitides of animals and man is that only a proportion of an infected population develop clinical neurological disease. The nervous symptoms may range from slight meningitis to fatal encephalitis. This differential susceptibility of apparently similar hosts is a major problem in our understanding of arbovirus diseases. The subject matter of this thesis is concerned with relating factors affecting susceptibility to louping-ill infection with the nature and pathogenesis of lesions in the central nervous system (C.N.S.).

There are two basic experimental approaches that can be used to investigate the pathogenesis of an infectious process with a variable clinical outcome. Firstly it may be possible to manipulate experimental factors, such as the route of inoculation or the age of the host, so that the consequence of infection becomes predictable. Animals can then be killed for examination at intervals throughout the incubation period, with a reasonable certainty that they would have developed a particular symptom if allowed to survive. This serial killing approach is most applicable to studies in rodents, where large numbers of genetically defined hosts of specific pathogen free (S.P.F.) status are readily available. It is the only feasible method for investigating the histopathogenesis of lesions in nervous tissue.

The alternative is to take sequential samples from individual animals, from the day of inoculation to the time of development of a particular symptom. This technique is suitable for studies of viraemia and the immune response, but obviously only the final pathology can be determined. Relatively large animals, such as sheep, are extremely useful for these serial sampling studies. Considerable volumes of blood can be taken regularly, and there is sufficient nervous tissue for examination by a range of histological techniques requiring different fixation procedures.

These two approaches are complementary. In the serial killing experiment humoral factors and histopathogenesis are investigated in populations of animals selected to give a uniform response. The infectious process in individuals developing a range of symptoms can be studied by the serial sampling technique.

Serial killing studies in rodents have formed the basis of most experimental investigations of the pathogenesis of arbovirus encephalitis (Albrecht, 1968; Johnson and Mims, 1968). The majority of these viruses were examined because they were known human pathogens. Comparative studies were obviously impossible in the medically important host and monkeys are too expensive and rare for large scale experiments.

Louping-ill is thus of considerable general interest as a model for investigating the pathogenesis of arbovirus encephalitis. Comparative experimental studies are possible both in rodents and the normal host, and the pattern of clinical susceptibility in naturally infected sheep is typical of the arboviruses (Gordon, Brownlee, Wilson and MacLeod, 1962). It is also of specific medical interest, for louping-ill virus is well

known as a cause of human laboratory infection (Rivers and Schwentker, 1934; Edward, 1948; Cooper, Green and Fresh, 1964; Webb, Connolly, Kane, O'Reilly and Simpson, 1968), and there is tentative evidence that the disease occurs in man in nature (Likar and Dane, 1958; Williams and Thorburn, 1962; Schonell, Brotherston, Burnett, Campbell, Coghlan, Moffat, Norval and Sutherland, 1966). Again the clinical pattern in man is characteristic of arbovirus infections in general.

Arboviruses are spread in nature by arthropods in which, by definition, they also replicate (Andrewes and Pereira, 1967). The natural vector for louping-ill virus is the sheep tick Ixodes ricinus (MacLeod and Gordon, 1932), though the disease has also been transmitted experimentally by Rhipicephalus appendiculatus (Alexander and Neitz, 1933, 1935). A somewhat speculative account can be given of the sequence of events in the passage of virus from an infected tick to the C.N.S. of a susceptible mammalian host. Following tick bite, which may be regarded as equivalent to intradermal inoculation, the virus is carried by the lymphatics to the regional lymph-node where it may replicate (Malkova, 1968). It then enters the blood via the thoracic duct (Malkova, 1960, a) and further cycles of replication occur in a number of visceral sites, such as the spleen and other lymph nodes (Pool et al, 1930; Malkova, 1960, c; Gresikova, Albrecht and Ernek, 1961). The release of this virus into the blood gives rise to a continuing plasma viraemia (Malkova, 1967) which finally terminates at about the time of formation of specific antibody (Malkova, 1960, b; Swanepoël, 1968). The virus probably enters nervous tissue after multiplying in, or passing through, the endothelial cells of small blood vessels throughout

the C.N.S. (Johnson and Mims, 1968; Albrecht, 1968). An alternative hypothesis, whereby virus is excreted onto the olfactory mucosa and enters the brain via the olfactory bulb, was proposed by Burnet and Lush (1938). There is no evidence that arboviruses enter the C.N.S. by centripetal spread along peripheral nerves (Johnson and Mims, 1968), though this has been demonstrated for other virus groups e.g. the herpesviruses (Johnson, 1964; Yamomoto, Otani and Shiraki, 1968).

The fate of viruses once they have entered the C.N.S. and the means by which they cause disease and death are not well understood (Smith, 1969). There is little comprehensive information on the interaction of louping-ill virus with the mammalian C.N.S., for most previous workers with louping-ill were concerned with other aspects of the disease process. Even so many of the papers published before 1967, when the present work was commenced, contain some information of limited relevance. These are mentioned here rather briefly; any specific points of interest will be considered in the discussion.

The histopathogenesis of C.N.S. changes was investigated in pigs showing a range of symptoms by Dow and McFerran (1964). Gresikova et al (1961) made a small scale pathogenesis study of clinical and subclinical infection in sheep inoculated with virulent and attenuated strains of virus. The development of neuropathological changes in monkeys with louping-ill was studied by Galloway and Perdrau (1935). Subclinical infection in the rat (Burnet, 1936, a; Miles, 1951) and the fatal disease in the mouse (Fite and Webster, 1934; Burnet and Lush, 1938) were investigated in serial studies of virus titres in nervous tissue.

Histological lesions of non-suppurative encephalitis have been described in clinical louping-ill produced by inoculating the virus into a number of species; sheep (Brownlee and Wilson, 1932; Wilson, 1945; Williams, 1958, b); cattle (Dunn, 1952); pigs (Brownlee and Wilson, 1932); monkeys (Hurst, 1931; Findlay, 1932); mice (Hurst, 1931, 1950; Brownlee and Wilson, 1932) and voles (Findlay and Elton, 1933).

There is relatively little published information on the nature of louping-ill encephalitis in mammalian hosts showing differing susceptibility to experimental infection.

MATERIALS AND METHODS

Experimental Animals

The mice, hamsters and rats are defined with respect to age and weight at inoculation in Table 1. With the exception of the hamsters they were derived from S.P.F. colonies. The C57 black and C57 brown mice were the only inbred strains used. All rodents were weaned at 20 or 21 days post partum and were from litters containing at least 3 offspring.

The sheep and lambs were females or wethers, and were either born on the Moredun Institute farms or purchased from other tick-free environments. The majority (58 animals) were Scottish Blackfaces (B.F.), with some B.F. crosses (103, 104, 105, 106, 108, 111, 3K42, 3K43, 3K44, 3K45, 3K46), Cheviot crosses (3K39, 3K40, 3K41, 3K49) and Cheviots (641, 643). None of the 75 animals had serum haemagglutination inhibiting (H.I.) antibody to louping-ill virus when inoculated at 3 days, 6 months or 9 months of age.

Inocula

The Moredun strain of sheep louping-ill virus (LI.31) was used in all experiments with rodents. The passage history of LI.31 has been described by Swanepoël (1968). Stock virus was stored at -20°C, as freeze-dried 0.25 ml. aliquots of a 10 per cent. suspension of fourth or fifth passage mouse brain (LI.31.M4 or LI.31.M5) in 10 per cent. inactivated horse serum saline (H.S.S.). This freeze-dried virus was re-constituted for use by suspending the contents of 2 ampoules in 4.5 ml. of cold H.S.S. and centrifuging at 1200 g, for 15 minutes at 4°C. The supernate was then diluted to the required concentration in cold H.S.S.. Pooled contents from 2 ampoules were used in order to minimize variations in virus titres between inocula (Williams, 1958, b). A similar preparation was made from normal

TABLE 1

AGE AND WEIGHT OF REPRESENTATIVE GROUPS
OF RODENTS AT TIME OF INOCULATION

Animal	Group	Age	Weight in grams (mean ± S.E.)
C57 black mice	weaned	21 days	7.2 ± 0.2
	adult	3 months	28.9 ± 0.5
C57 brown mice	weaned	21 days	11.9 ± 0.9
	adult	3 months	25.7 ± 0.8
Porton mice	weaned	21 days	18.3 ± 0.5
	adult	3 months	42.1 ± 0.7
White mice	weaned	21 days	13.3 ± 0.3
	adult	3 months	40.9 ± 0.8
Syrian hamsters	infant	4 days	4.5 ± 0.1
	large suckling	14 days	14.1 ± 0.5
	newly weaned	22 days	30.5 ± 0.7
	young adult	6 to 8 weeks	86.3 ± 1.0
	aged	aged *	126.2 ± 3.2
Porton rats	infant	4 days	7.9 ± 0.3
	newly weaned	20 days	37.6 ± 1.1
	young adult	40 days	108.0 ± 3.5

* = Cast breeding females

mouse brain and used as a control.

The sheep and lambs were inoculated with one of two recent isolates (SB.526 or SB.527) of louping-ill virus. These were originally recovered by injecting mice intracerebrally with brain suspensions from moribund sheep, from separate louping-ill outbreaks in the West of Scotland (Brotherston, Boyce and Bannatyne, unpublished data).

Inoculation Techniques

Animals were inoculated by the intracerebral (i.c.), intraperitoneal (i.p.) or subcutaneous (s.c.) routes. The term "peripheral" is used in a general sense to describe parenteral administration by other than the i.c. route.

Depending on their size the rodents were given an i.c. dose of from 0.02 to 0.06 ml., or a peripheral dose of from 0.2 to 0.6 ml.. The inocula were given i.c. into the left cerebral hemisphere, s.c. along the back or i.p. in the ventral midline.

The older sheep were given either 1.0 ml. i.c. or 10.0 ml. s.c., and the lambs were similarly inoculated with 0.5 ml. or 5.0 ml.. Inocula were given i.c. into the left cerebral hemisphere, or s.c. in the medial aspect of the thigh of the right hind leg.

Titration of Virus Inocula

Virus inocula were titrated by i.c. inoculation into weaned white mice. Serial ten-fold dilutions of centrifuged supernates were made in cold H.S.S.. These were held on melting ice, for a maximum interval of 1 hour, until groups of 5 or 6 mice were injected i.c. with each dilution. The mice were observed daily for symptoms of louping-ill, as described by Hurst (1931)

and Alston and Gibson (1931). Clinically affected animals were killed and the observations were terminated on the fourteenth day, when the survivors were also destroyed. The amount of virus in the inoculum was calculated by the method of Reed and Muench (1938) and expressed as the total number of mouse infectious doses given.

Virology

The virus isolation and titration results reported in this thesis are all from studies in rodents. Animals were killed by decapitation, and blood was collected into sterile, weighed bottles. Samples of nervous tissue were removed aseptically into similar bottles. These specimens were made to 10 per cent. (w/v) suspensions in cold H.S.S., the blood by pipetting and the tissue by maceration in Griffith's tubes. Each suspension was then divided into 2 aliquots and these duplicate samples were stored at -20°C, for a maximum interval of one month, or at -70°C for longer periods.

One aliquot was thawed rapidly, centrifuged at 1200 g for 15 minutes at 4°C and the undiluted supernate was tested in mice for presence of virus. The second aliquot of positive samples was then titrated as described previously (Titration of Inocula). Virus titres (Reed and Muench, 1938) were expressed as the reciprocal of $\log_{10} ID_{50}/0.03$ ml. of 10 per cent. suspension. If virus was detected at a level that was too low to calculate as a titre it was said to be present in trace (T) amounts.

Histology

Both rodents and sheep were killed by decapitation and exsanguination. Brains were fixed routinely in 10 per cent. formol saline for at least 10 days, for rodents, or 1 month, for sheep. The rodent brains were bisected

longitudinally. Sagittal blocks of sheep tissue were cut from the olfactory bulbs, the cerebrum at the frontal pole and the level of the optic chiasma, the thalamus, midbrain, pons (2 blocks), cerebellum, medulla (3 blocks) and spinal cord (C2, C7, T9 and L4), and post-fixed in saturated mercuric chloride. All blocks were dehydrated through a graded alcohol-toluene series and embedded in paraffin with a melting point of 56°C.

Paraffin sections (6 μ) were stained routinely with haematoxylin and eosin (H. & E.). The acridine orange stain was used at pH 4.0 for demonstrating nucleic acids (Mayor and Diwan, 1961) in mouse brains that had been fixed in Carnoy's fluid (48 hours at room temperature) and embedded in paraffin. Other stains used on paraffin sections were periodic acid-Schiff (P.A.S.), luxol fast blue (Pearse, 1955), methyl green pyronin, phospho-tungstic acid haematoxylin, Sudan black B, Best's carmine and toluidine blue. These techniques are described in Culling (1963) and Pearse (1968).

A few suckling hamster brains were also fixed in calcium formol and frozen sections were examined for presence of lipids and myelin by the OTAN (Adams, 1959), Sudan IV and Smith Quigley techniques.

Lesion Scoring

Semiquantitative estimates of lesion severity were made on paraffin sections stained with H. & E.. Following routine histological examination all sections were randomised and read blind. The severity of histological changes was assessed on the neuropathological lesion grading scale of Nathanson, Goldblatt, Thind, Davis and Price (1965). A grade of 1 was given for a few inflammatory foci, 2 for numerous inflammatory foci, 3 for very severe inflammation and/or necrosis of at least 30 per cent. of neurons,

and 4 for necrosis of at least 90 per cent. of neurons. The term "necrosis" was used to describe irreversible neuronal damage, as illustrated in the classical study of Bodian (1948).

Each rodent half brain was graded (by examining several interrupted serial sections mounted on the one slide) in the 5 broad anatomical regions shown in Fig. 1. The randomised series of slides was examined 3 times. In some experiments a total value was calculated for each half brain by summing the 15 readings made (3 replicates on 5 regions). This total was then divided by 3 to give an average called the "encephalitis score". When both halves of the brain were examined the 2 encephalitis scores were added and the average taken. The maximum possible encephalitis score was thus 20 (e.g. Table 7 in results). In other experiments the lesion grades for each region were combined for identical groups of animals, calculated as percentages and expressed in the results as horizontal histograms (e.g. Fig. 10).

In blocks of sheep tissue the severity of lesions in various anatomical sites (listed in Fig. 44 in the results) was graded on a single examination of sections mounted on 3 separate slides (representing intervals of 60 μ through the block). Thus for each bilaterally symmetrical component, e.g. the red nucleus, there were ideally 6 estimates of lesion severity (2 readings from each of 3 slides) and there were more numerous readings for areas extending over several blocks e.g. the reticular formation. These readings were either averaged for individual animals (e.g. Table 15) or combined, for groups of similar animals. The relative proportions of different lesion grades in these combined readings were calculated as percentages and

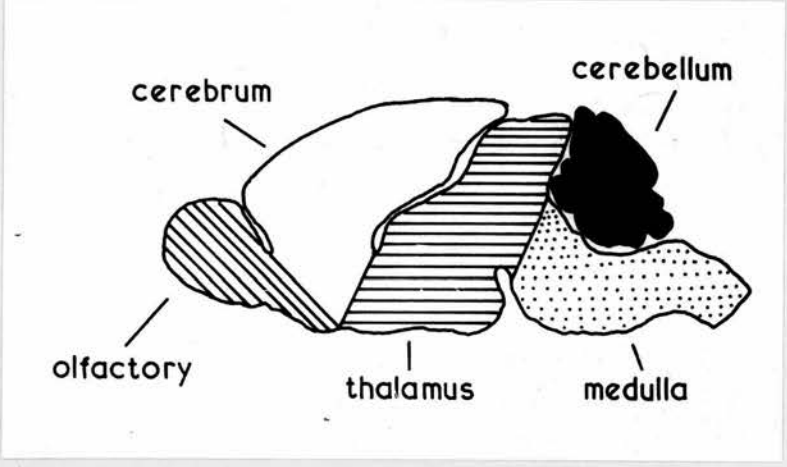


Fig. 1 Encephalitis grading sites in rodent half brains.

expressed as horizontal histograms (e.g. Fig. 44).

Anatomical features in the brainstem of sheep were identified with the aid of the atlases of Palmer (1958) and Richard (1967). The lobes of the cerebrum and cerebellum were identified according to the respective nomenclatures of Ranson (1935) and Innes and Saunders (1962).

Enzyme Histochemistry

The cervical spinal cords were removed within 5 minutes of sheep being killed and small blocks were fixed for 48 hours in cold neutral calcium formol. Frozen sections (12 μ) were stained to demonstrate either acid phosphatase, by the Gomori lead nitrate method (Pearse, 1968), or thiamine pyrophosphatase (Novikoff and Goldfischer, 1961). The acid phosphatase technique is effectively a marker for lysosomes, whereas thiamine pyrophosphatase activity is localized in the Golgi apparatus.

Immunofluorescence

A number of attempts were made to develop a specific immunofluorescent test that was sufficiently sensitive to detect louping-ill infected cells in cryostat sections. Conjugates of immune globulin with fluorescein isothiocyanate (F.I.T.C.) were prepared from ascitic fluid of hyperimmunized mice (Brandt, Buescher and Hetrick, 1967), and sera of hyperimmunized rabbits and guinea pigs. The indirect test (Nairn, 1962) was also tried, overlaying sections with immune sheep serum followed by conjugated anti-sheep globulin. None of these proved very successful, an experience shared in similar systems by Johnson (1964) and Henderson, Peacock and Randles (1967).

This problem was overcome when Mr. H. W. Reid produced a serum with a

very high titre ($\frac{1}{250,000}$) to louping-ill virus, by hyperimmunizing a sheep with the methanol precipitated adjuvant vaccine developed by Brotherston and Boyce (1969). The globulin fraction was precipitated with saturated ammonium sulphate and conjugated with F.I.T.C. by Mr. A. C. Gardiner, using the dialysis method of Clarke and Shepard (1963). This conjugated globulin was diluted 1:1 in phosphate buffered saline (P.B.S.) and absorbed once with mouse liver powder and once with sheep liver powder (Nairn, 1962). The conjugate was then dispensed into ampoules and stored at -70°C, following clarification by centrifuging (18,000 g for 30 minutes at 4°C) and filtering through a millipore membrane. Thawed aliquots were used after a further 1:7 dilution in P.B.S..

A mono-specific antiserum to the IgG (7S) class of sheep globulin was produced by Mr. H. W. Reid and Mr. A. McL. Dawson. The IgG component of sheep serum was isolated by methods similar to those described by Aalund, Osebold and Murphy (1965) and Penhale and Christie (1969). A rabbit was then hyperimmunized with sheep IgG incorporated in Freund's incomplete adjuvant. This rabbit anti-sheep IgG globulin was conjugated with F.I.T.C. and processed, stored and diluted as described above, with the exception that guinea pig liver powder was used for both absorptions.

Fresh nervous tissue for examination was taken into small polythene bags which were sealed, quenched in a eutectic mixture (dry ice and petroleum ether, -70°C) and stored at -20°C. Blocks were trimmed with a cold scalpel blade and mounted, with a couple of drops of 15 per cent. ovalbumin, on cryostat chucks. Sections (8 μ) were cut at -18°C and lifted off the knife with cleaned glass slides kept at room temperature. These were held

at -18°C in the cryostat cabinet until processed, usually within 30 minutes.

Sections were removed from the cryostat, fan-dried for 10 minutes at room temperature, fixed for 10 minutes in cold acetone (4°C) and dried again for a further 10 minutes. They were then overlaid with the conjugate and incubated in a humid chamber for 30 minutes at 37°C. This was followed by 3 ten minute washings in P.B.S. (at 37°C on a rotator) and one 5 minute washing in distilled water. They were then mounted in glycerol (buffered to pH 8.0) and examined the same day using a "Reichert Zetopan" microscope fitted with a HBO 200 mercury vapour lamp, a UG1/2.5 exciter filter, a GC9/1 barrier filter, a glycerol immersion dark field condenser and a glycerol immersion X60 objective. Photomicrographs were taken on Kodak Tri-X film, giving exposures of about 2 minutes.

Slices of mouse brain were also embedded in paraffin at low temperature, by the technique of Albrecht, Mrenova and Karelova (1966). The tissue slices (1.5 to 2.0 mm. thick) were fixed in 3 changes (20, 40 and 60 minutes) of cold (4°C) Carnoy's fluid and cleared in three 30 minute changes of chloroform, the first 2 at 4°C and the third at room temperature. Impregnation with paraffin was done at 54°C (3 changes of 20 minutes through wax with melting points of 39°C, 45°C and 50°C) and blocks were stored at 4°C. Sections (6 μ) were cut at room temperature, floated onto water at 40°C and spread on slides without any adhesive. After drying for 30 minutes at 37°C, they were passed through xylene and an alcohol series to water, each change lasting for 1 to 2 minutes. They were then stained with conjugate as described above.

Minor modifications were made, because of inadequate embedding, when

this technique was used with sheep tissue. The blocks were fixed in cold Carnoy's fluid for 24 hours and were passed through absolute alcohol before embedding, to improve dehydration.

The specificity of the anti-louping-ill and anti-IgG conjugates was established both by staining control sections from normal animals and inhibiting fluorescence by the use of a blocking test (Nairn, 1962). Sections from positive cases were first overlaid with unconjugated specific immune serum. After 30 minutes this was washed off and the sections were stained with the comparable conjugate. Greater than 80 per cent. inhibition of fluorescence was observed with both conjugates, in all the experimental systems used.

Pre-treatment of unfixed cryostat sections, by immersion for 2 hours in citrate buffer at pH 3.3 (Gajl-Peczalska, Fish, Meuwissen, Frommel and Good, 1969), did not affect subsequent staining of cell-associated IgG. However when anti-IgG-stained sections were immersed in this eluting solution all fluorescence was removed, though staining could still be detected in similar preparations held in P.B.S. at pH 7.4. Thus the intracellular antigen stained (IgG) was closely associated with the cells, and could not be eluted by a treatment sufficient to dissociate the normal antigen-antibody staining reactions.

In conjugate stained sections the number of cells showing strong apple-green fluorescence in a particular anatomical site were estimated subjectively and graded from + to ++++. The very approximate equivalents of these readings are; + = 5 to 25 per cent.; ++ = 26 to 50 per cent.; +++ = 51 to 75 per cent.; ++++ = 76 to 100 per cent.. At least 3 sections, on 3

separate slides, were examined at random from each block. These 3 readings were averaged to give the final value quoted in the results.

Electron Microscopy

The lumbar spinal cords of sheep were perfused with 3 per cent. glutaraldehyde in sodium cacodylate buffer at pH 7.4 (Mercer and Birbeck, 1966). A 500 ml. bottle of fixative was connected to a 14 swg. needle, via a blood transfusion drip and a flexible polythene tube. As soon as the sheep had been killed the abdominal cavity was opened and the needle was inserted and tied, pointing towards the heart, in the caudal end of the dorsal aorta. Following section of the posterior vena cava the drip was started at a flow rate of about 20 ml. per minute (approximately 3 drops per second). Both common iliac arteries were clamped and the aorta was also occluded immediately caudal to the left renal artery. The perfusion was maintained until 1.0 litre of fixative had been used. The perfused tissue, which could be identified by its hard yellow consistency, was then removed into cacodylate buffered glutaraldehyde.

Blocks of ventral horn (1 mm. cubes) were cut and immersed for a further 2 hours in glutaraldehyde. They were then washed in buffer, post-fixed in 1 per cent. osmium tetroxide, washed again in buffer, dehydrated through an alcohol series, cleared in epoxy propane and embedded in "Araldite" (Glauert and Glauert, 1958).

Sections were cut with glass knives on an LKB Ultrotome 1. Areas for ultrastructural examination were selected on optical microscopy of 1 μ Giemsa stained thick sections of the whole block face. The blocks were trimmed to the selected area and ultrathin sections were cut and stained

with lead citrate. All of the section cutting, and most of the tissue processing, was done by Mr. E. W. Gray.

The ultrathin sections were examined on a Siemens Elmiskop 1 electron microscope. The instrument was largely operated by Mr. W. Smith, who also photographed the fields selected by the author for more detailed examination.

Suckling hamsters were decapitated and 0.1 ml. of glutaraldehyde was immediately injected through the skull in the region of the cerebellum. The cerebellum was removed into a drop of fixative and cut into 1 mm. cubes with razor blades. These cubes were fixed in glutaraldehyde for 2 hours, and then processed and examined as described above.

RESULTS

I. STUDIES IN MICE

(1) The Susceptibility of C57 Black Mice to Inoculation by Different Routes

The intention was to establish an experimental system whereby parenteral inoculation with virus would regularly produce fatal infection. It was thought that the C57 black strain of mice might be a suitable host, for Pogodina and Savinov (1964) have shown that they are highly susceptible to i.p. and s.c. inoculation with a number of other T.B.E. viruses.

A total of 252 three to 4 week old C57 black mice were inoculated by the s.c., i.p. or i.c. routes with ten-fold dilutions of the same re-constituted aliquot of LI.31.M4. Twelve animals were used for each dilution for each route. The results of this titration give a measure of the susceptibility of these mice to louping-ill (Table 2). The only certain way of producing the disease was by i.c. injection of virus, which caused a 100 per cent. morbidity with a predictable incubation period. The most consistent pattern of susceptibility to peripheral inoculation was found in mice inoculated i.p. with the 10^{-2} dilution of virus; 11 of 12 first showed signs of severe neurological disease, as described by Hurst (1931) and Alston and Gibson (1931), at 8 days after inoculation. The remaining clinically normal mouse survived to day 21. This route and dose were thus used in all the pathogenesis studies described in section (2).

(2) Pathogenesis Studies in I.P. Inoculated C57 Black Mice

These serial killing experiments were done to investigate the sequential development of virus titres and histological changes in the brains of animals that could reasonably be expected, from the results obtained in section (1), to develop neurological symptoms following

TABLE 2

TITRATION OF LI.31.M4 IN C57 BLACK MICE BY VARIOUS ROUTES

Route	Virus titre ($log_{10}ID_{50}$/0.03ml.)	Incubation time (days, mean ± S.E.)	
s.c.	3.5	11.04 ± 0.31	) $p < 0.01$
i.p.	4.6	9.70 ± 0.26	) $p < 0.001$
i.c.	7.2	6.76 ± 0.27	

TABLE 3

VIRUS TITRE IN C.N.S. AND BLOOD OF C57 BLACK MICE SAMPLED SEQUENTIALLY * AFTER I.P. INOCULATION WITH LI.31.M4

	Days after inoculation							
	1	2	3	4	5	6	7	8
C.N.S.	T	T	0	3.8	3.6	2.6	5.3	6.2
Blood	0.5	0.2	T	T	T	T	0	0

* = Pools of brain and spinal cord, and blood from 3 mice were used for each titration.

T = Trace

0 = No virus isolated

peripheral inoculation with louping-ill virus.

The virological and histological results can be considered separately, as individual animals were examined by one or other of these techniques. It is convenient to precede the presentation of this data by a general account of the groups of animals inoculated, and the efficacy of the inoculum in producing clinical neurological disease.

The inocula were 10^{-2} dilutions of re-constituted LI.31.M4. Five were prepared and, from titrations in white mice, it was calculated that each C57 black mouse was inoculated i.p. with between 1.5×10^5 and 5.0×10^5 infectious doses of virus. There were 70, 20, 7, 24 and 48 C57 black mice in the respective groups. Animals were killed at random, for both virological and histological examination from the first and last groups and for virological examination alone from the other 3 groups.

A total of 61 mice, including some from each group, were allowed to survive for the full incubation period. Clinical louping-ill was seen in 88.5 per cent. of these on the eighth day after inoculation. This incidence of symptoms was considered acceptable for serial killing studies. The presence of an occasional negative sample in the pools from 3 animals that were examined virologically (see below) would have caused a negligible drop in titre (0.2). Two of the 7 mice without symptoms on day 8 remained unaffected until day 21, the other 5 were killed on day 8 for histological examination.

Virology The time of appearance of virus and the magnitude of titres in the C.N.S. were determined by titrating pools of 3 brains and spinal cords and bloods each day for 8 days. Virus was detected in the C.N.S. from

day 4 (Table 3), though clinical signs were not seen until day 8. The small amounts of virus isolated from nervous tissue on days 1 and 2 were attributed to presence of viraemic blood.

The distribution of virus in nervous tissue was determined by titrating pooled regions of C.N.S. (Fig. 2) from 3 mice from days 4 to 8. Virus was present in all regions from the fifth day, but was detected in only 2 regions on day 4 (Fig. 3). In order to ascertain if these represented preferential sites for early virus detection in the C.N.S. the experiment was repeated for day 4, but on this occasion no virus was isolated from any segment.

It was thought that a more accurate estimate of the time of first detection of virus in the brain might be obtained by titrating at intervals on the fourth day. Thus, pools of 3 brains and bloods were examined at 3 hourly intervals from 90 to 102 hours. Individual samples were also taken from 3 mice at 96 hours. There was no consistent pattern in the results from the brain pools, the titres being; 90 hours 0.7, 93 hours $\geqslant$2.4, 96 hours $\geqslant$2.5, 99 hours 0.1 and 102 hours 1.4. Virus was not isolated from the blood at 90 hours, though it was present in trace amounts in the other 4 samples. Only one of the brains titrated individually at 96 hours contained any virus, at a titre of $\geqslant$2.4.

Burnet and Lush (1938) inoculated 6 to 8 week old mice i.p. with louping-ill virus and found that, in a high proportion, virus was present in the olfactory bulbs before it could be detected in the rest of the brain. This did not appear to be the case on days 4 to 8 of these experiments. An investigation was made of the possible presence of virus in different

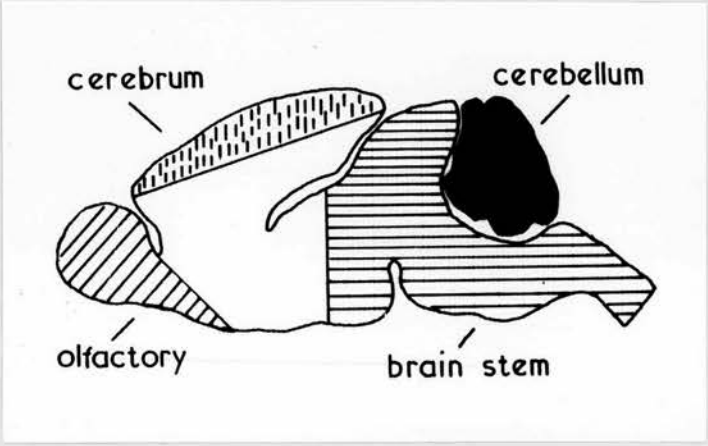


Fig. 2 Regions of the brains of i.p. inoculated C57 black mice that were sampled for virus isolation and titration.

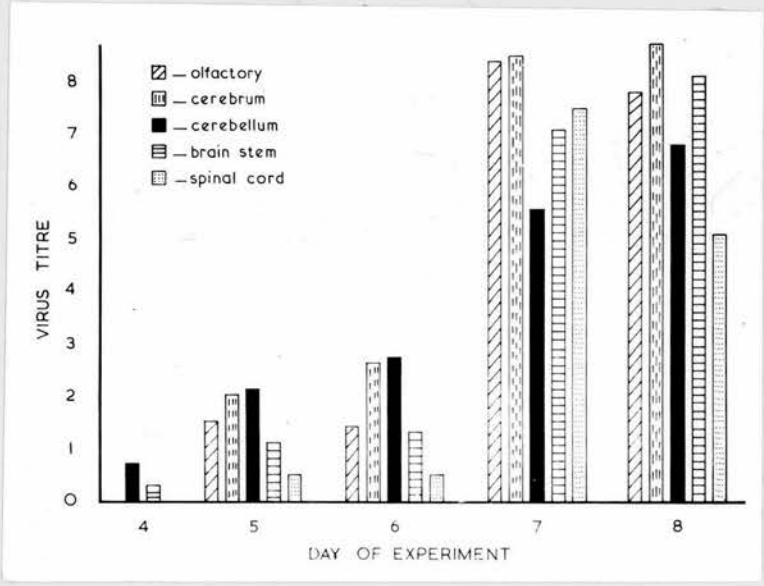


Fig. 3 Virus titre in pools of spinal cords and various regions of the brains (Fig. 2) from 3 C57 black mice that were inoculated i.p. with LI.31.M4. The virus was not isolated from the blood.

segments from the brains of individual mice from days 1 to 4. Groups of 4 mice were examined each day and blood and the olfactory, cerebral and cerebellar segments were taken separately for virus isolation without titration. Virus was only found in nervous tissue from 2 of the mice from day 4; in all 3 brain segments of one and the olfactory and cerebral segments of the other. Viraemia was demonstrated in 12 of the 16 mice, with no particular pattern of occurrence.

Thus it appeared that there was no preferential site of virus entry into the C.N.S. or of virus replication in nervous tissue.

Histology A series of formol fixed brains were taken from 23 clinically normal mice (3 from days 1 to 7 and 2 from day 8) and 5 mice with marked neurological symptoms on day 8. Histological changes were seen in H. & E. stained paraffin sections from only 4 of them, 2 from each at days 7 and 8. The most severely affected brain showed small perivascular inflammatory foci of lymphocytes in the thalamus, cerebral cortex and corpus callosum, and some neuron necrosis in the cerebral cortex and the granular layer of the cerebellum. The other 3 had slight inflammatory changes but neuron necrosis was not observed. The distribution of Nissl substance, as seen in paraffin sections stained with methyl green pyronin, was normal in all but obviously necrotic neurons.

As the observation that brains from 3 of 5 clinically affected mice were histologically normal required confirmation, another series was examined. These were fixed in Carnoy's fluid and were taken from 12 clinically normal mice (3 on each of days 5 to 8) and 14 with nervous symptoms on days 7 and 8. Five control mice were also examined on day 8. The only lesions found in

H. & E. stained paraffin sections were in the brains of 5 mice from days 7 and 8, including one that was clinically normal. The most severe changes are illustrated in Figs. 4 and 5. Fluorescence microscopy of acridine orange stained paraffin sections revealed no differences in distribution of nucleic acids in the brains of infected and control mice.

Immunofluorescence A highly specific fluorescent anti-louping-ill conjugate (p. 11) later became available (about 18 months after the preceding experiments were performed), and it was thought that this might be suitable for determining the distribution of virus infected cells in the brain.

Twenty-six C57 black mice were each inoculated i.p. with 6.7×10^4 infectious doses of LI.31.M4, and 4 other mice were injected with control material. Three virus infected mice were killed each day and the controls were killed on days 2, 4, 6 and 8. One half of the brain was quenched for cryostat sectioning and the other half was fixed in cold Carnoy's fluid and embedded in paraffin at low temperature.

The only clinically affected animals were seen on day 8, when 2 of the 5 virus infected animals remaining were found dead, 2 had neurological symptoms and 1 was normal. Specific viral immunofluorescence was not detected in cryostat sections from any animal killed before day 6, 1 clinically normal animal killed on each of days 7 and 8, or from the controls. There were no histological changes in H. & E. stained paraffin sections from any of these mice.

In cryostat sections fluorescent neurons were found in all regions of the brain (Table 4). This fluorescence was confined to neuronal cytoplasm (Fig. 6). A similar distribution was observed in the cold Carnoy paraffin

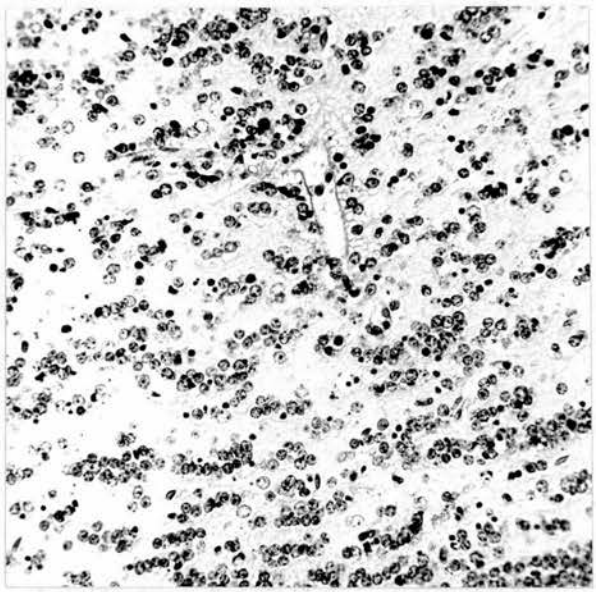


Fig. 4 Infiltrating lymphocytes and necrotic cells in the olfactory bulb of a clinically normal C57 black mouse that was inoculated i.p. with LI.31.M4 and killed 7 days later. H. & E. X240.

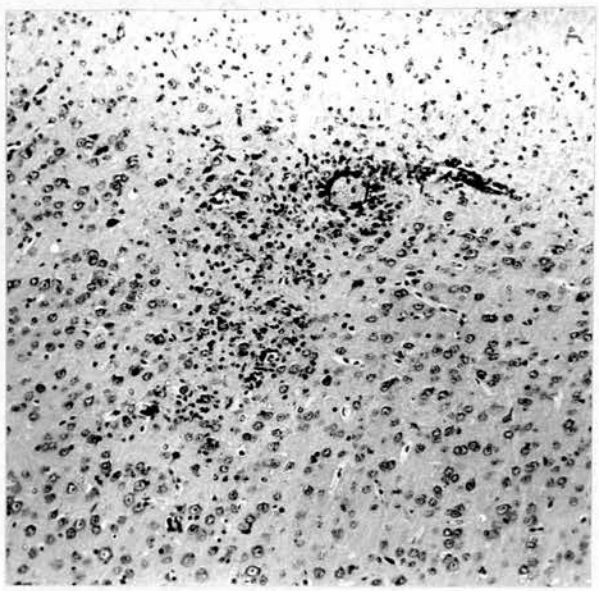


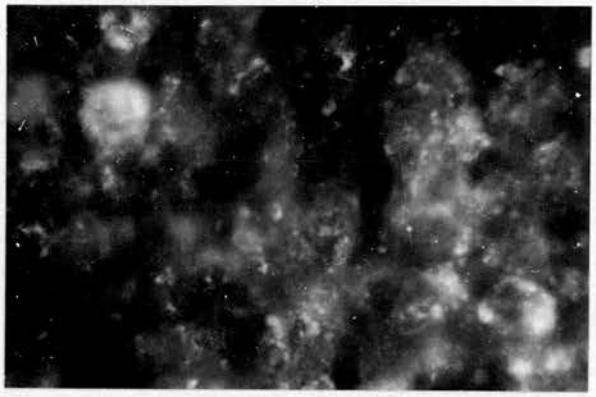
Fig. 5 Inflammation and neuron necrosis in the cerebral cortex of a clinically affected C57 black mouse that was inoculated i.p. with LI.31.M4 and killed 8 days later. H. & E. X120.

TABLE 4

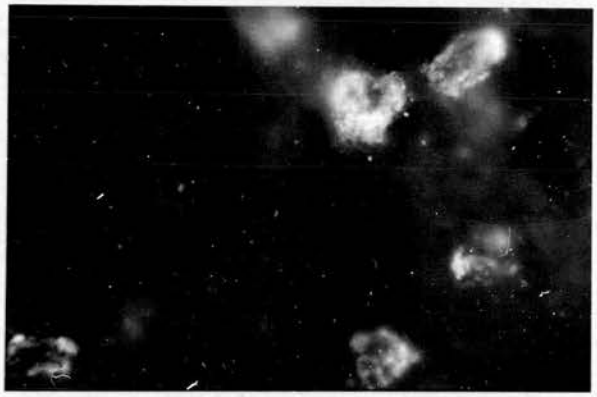
THE DISTRIBUTION OF IMMUNOFLUORESCENCE AND HISTOLOGICAL LESIONS IN THE BRAINS OF C57 BLACK MICE INOCULATED I.P. WITH LI.31.M4

Days after inoculation	Animal	Immunofluorescence* in cryostat sections					Histological* lesions in paraffin sections				
		Olfactory bulb	Cerebrum	Cerebellum	Thalamus	Medulla	Olfactory bulb	Cerebrum	Cerebellum	Thalamus	Medulla
6	A	++	+	-	-	-	0	0	0	0	0
	B	+	+	-	-	-	0	0	0	0	0
	C	-	+	-	+	++	0	0	0	0	0
7	A	++	++	+	+	+	1	2	1	2	1
	B	+	+	-	+	++	0	0	0	1	1
8	A	+	+	+	+	+	0	0	0	0	0
	B	+	++	+	+	+	0	0	0	0	0

* Details of the immunofluorescence and histological grading systems are given on pages 9 and 14.



(a)



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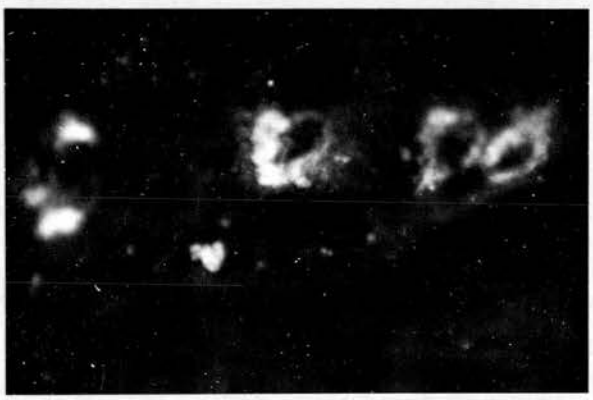
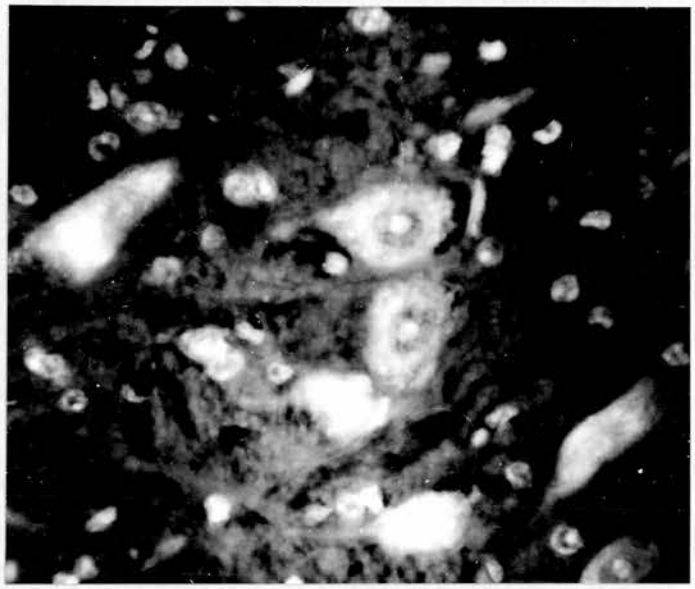
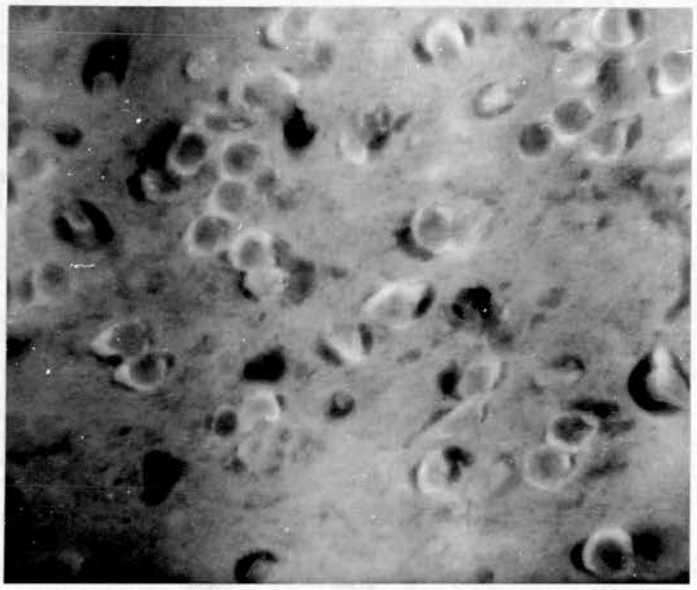


(c)

Fig. 6 Fluorescing cells in the anterior part of the rhinencephalon (a), the cerebral cortex (b) and the medulla (c) of a clinically normal C57 black mouse that was killed at 7 days after i.p. inoculation with LI.31.M4. Cryostat section, anti-louping-ill conjugate X720.



(a)



(b)

Fig. 7 Fluorescing neurons in the dentate nucleus (a) and cerebral cortex (b) in a cold-Carnoy paraffin section from the case illustrated in Fig. 6. Anti-louping-ill conjugate X720.

sections (Fig. 7) from positive cases (Table 4), though there was a general diminution in the intensity of staining and some blocks cut rather badly due to imperfect embedding. There was no correlation between the distributions of fluorescent neurons and the minimal inflammatory reaction (Table 4).

Comment The most interesting feature of louping-ill in i.p. inoculated C57 black mice is that there were histological lesions in the brains of only 6 of the 26 clinically affected animals examined, even though there were high concentrations of virus and many infected cells in nervous tissue. Neuropathological changes were also detected in 5 of 8 clinically normal mice that were killed on day 7. These would probably have soon developed symptoms. Thus, in this experimental system, there is little correlation between clinical disease and histological lesions in the brain.

(3) The Severity and Distribution of Neuropathological Changes in Different Strains of Mice that were Inoculated I.C.

The low incidence of neuropathological changes in the brains of clinically affected i.p. inoculated C57 black mice was rather surprising, in view of the severe lesions described in other strains of mice that had been given louping-ill by the i.c., intranasal or intramuscular routes (Hurst, 1931, 1950; Fite and Webster, 1934; MacKenzie, Wilson and Dennis, 1968). This difference could be a function either of the strain of host or the route of inoculation.

Six 3 week old C57 black mice were inoculated i.c., each with 5.0×10^2 infectious doses of LI.31.M4. Five were clinically affected on day 7 and one had already died. All 5 showed marked neuropathological changes, which were very severe in one animal. Both inflammation and neuron necrosis

(Figs. 8 and 9) were more evident in these mice than in i.p. inoculated animals with lesions (section (2)).

Thus the route of inoculation was important in determining the severity of neuropathological changes in clinically affected animals. This did not, however, eliminate the possibility that the strain of mouse might also have an effect. Comparisons between strains of mice could only be made in i.c. inoculated animals, for this is the only known way (p. 17) of producing a standard incubation time and a 100 per cent. incidence of neurological disease.

These comparative studies were done in two age groups of mice, because Hurst (1950) has described differences in the distribution and severity of lesions in mice of different ages. Groups of 60 C57 black and C57 brown inbred mice and Swiss white and Porton random bred mice were produced at the same time. Thirty of each strain were inoculated i.c. with a 10^{-4} dilution of LI.31.M5 at either 3 weeks or 3 months of age. The amount given was calculated as 1.4×10^3 virus doses for each 3 week old mouse and 3.2×10^3 virus doses for each 3 month old mouse.

All animals had neurological symptoms, or were found dead, at 6 days after inoculation. Those that were clinically affected were killed, and the patterns of lesion distribution were determined in 15 formol fixed brains from each strain at each age, by the semiquantitative lesion grading technique described on page 9.

The distribution and severity of histological changes in the various groups is shown in Fig. 10. The cerebellum was the most affected area in the 3 week old inbreds and all the 3 month old mice, whereas the most severe

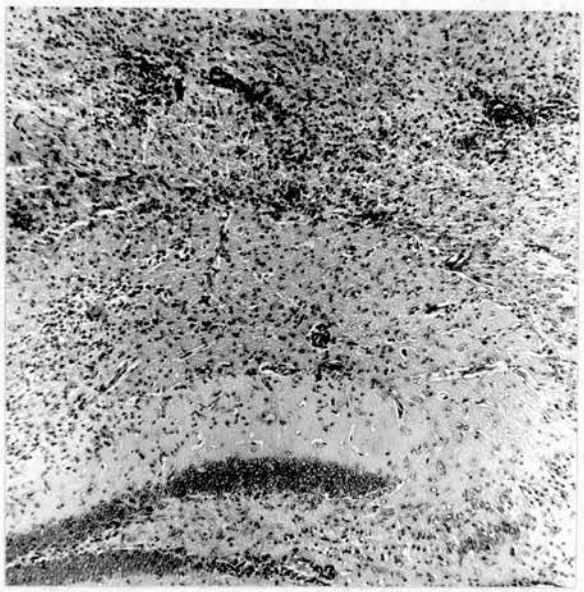


Fig. 8 Inflammation and necrosis in the cerebrum and hippocampus of a clinically affected C57 black mouse that was inoculated i.c. with LI.31.M4 and killed 7 days later. H. & E. X60.

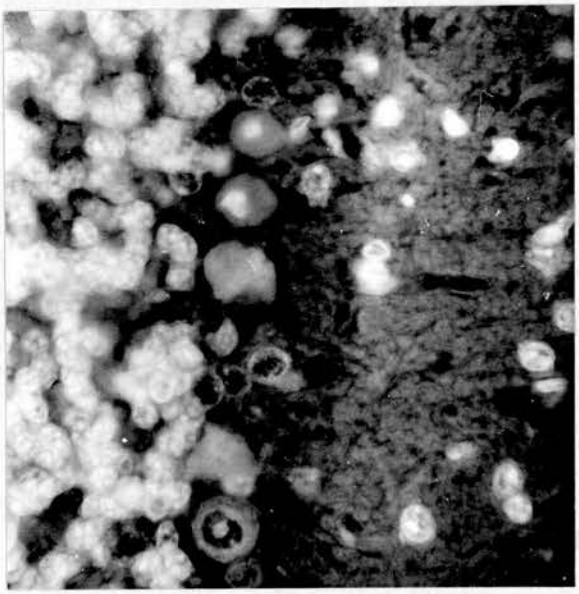


Fig. 9 Fluorescence photomicrograph of a row of necrotic Purkinje cells in the cerebellar cortex of a clinically affected C57 black mouse that was inoculated i.c. with LI.31.M4 and killed 7 days later. Acridine orange X1200.

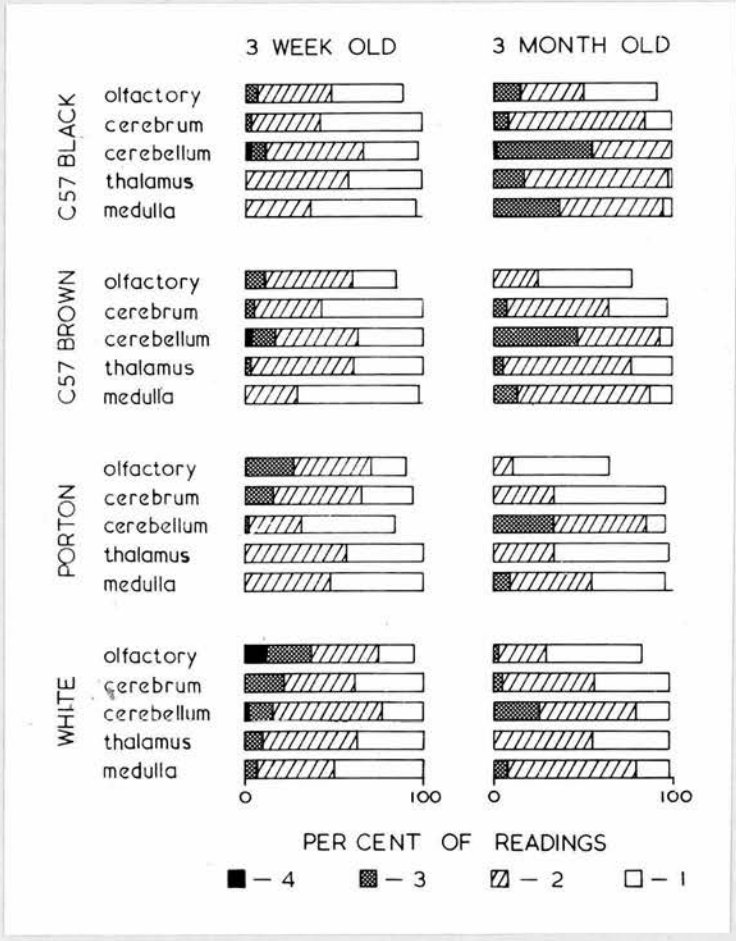


Fig. 10 The distribution of neuropathological changes in 4 strains of mice that were inoculated i.c. with LI.31.M5 at 3 weeks or 3 months of age and were killed, when clinically affected, at 6 days after inoculation. Each of the 8 groups of histograms represents results from 15 mice. The lesion grading system is described on pages 9 and 10.

lesions in the 3 week old random breds were in the olfactory bulbs. In all strains the damage to the medulla was more marked in the older animals. The Swiss white mice were the most severely affected in the younger groups, whereas in older animals there were slightly more severe lesions in the C57 black mice.

In general, though there were differences in lesion severity with age for each mouse strain, the strains themselves did not show characteristic patterns of pathology.

(4) The Nature of Neuropathological Changes in C57 Black Mice with Scrapie that were Inoculated I.C. with Louping-ill Virus

Both louping-ill and scrapie are naturally occurring neurological diseases of sheep in the United Kingdom. Louping-ill is an acute infection, whereas scrapie is an extremely chronic condition. Thus, as louping-ill is known to occur in quite old sheep (R. M. Barlow, pers. comm.), it is not inconceivable that scrapie affected sheep might contract louping-ill. Such a dual infection might produce qualitatively different pathological changes in the brain from those normally associated with either disease. This possibility can be investigated experimentally in C57 black mice, which are susceptible to both scrapie and louping-ill.

When C57 black mice are inoculated i.c. with the ME.7 scrapie agent (Zlotnik and Rennie, 1963) lesions of status spongiosus and neuronal vacuolation can be detected in the brain from about 12 weeks after inoculation (Fraser and Dickinson, 1968). These changes become progressively more widespread, until the mice are severely debilitated by about 23 weeks after inoculation.

Two groups of 12 C57 black mice were each inoculated i.c. with 3.2×10^3 infectious doses of LI.31.M5, at 20 weeks after i.c. injection with either

a 10^{-1} dilution of the ME.7/6 scrapie agent or control material. Ten other scrapie mice were injected i.c. with control material at the same time that the louping-ill virus was given. The virus inoculated animals all had clinical louping-ill, or were found dead, 6 days later and 10 brains from each group were taken into formol saline. The 10 control scrapie mice were killed at the same time. One half of each brain was blocked longitudinally and the severity of inflammation and neuron necrosis in H. & E. stained paraffin sections was graded as described on page 9. The other half of the brain was blocked transversely (Mould, Dawson, Slater and Zlotnik, 1967) and the severity of scrapie lesions was determined (Fraser and Dickinson, 1968) by an experienced observer, Mr. J. C. Rennie.

The distribution of the louping-ill encephalitis grades is shown in Fig. 11. The only obvious difference was that the medulla was more severely affected in control mice that had been inoculated with virus than it was in comparable scrapie mice. It is unlikely that this was due to suppression of louping-ill encephalitis by the scrapie disease process, for lesions caused by mouse passaged scrapie agents in mice tend to be relatively less severe in the medulla than they are in the telencephalon (Zlotnik and Rennie, 1963). The overall louping-ill encephalitis scores (p. 10) were unaffected by the presence of concurrent scrapie (Table 5), but it appeared the scrapie lesion scores were depressed in animals with louping-ill. This result merits further investigation, but it is outside the scope of this thesis.

The histological picture in mice with both diseases (Fig. 12) was of the inflammation and neuron necrosis seen in louping-ill (Fig. 13) superimposed on the status spongiosus and neuronal vacuolation of scrapie (Fig. 14).

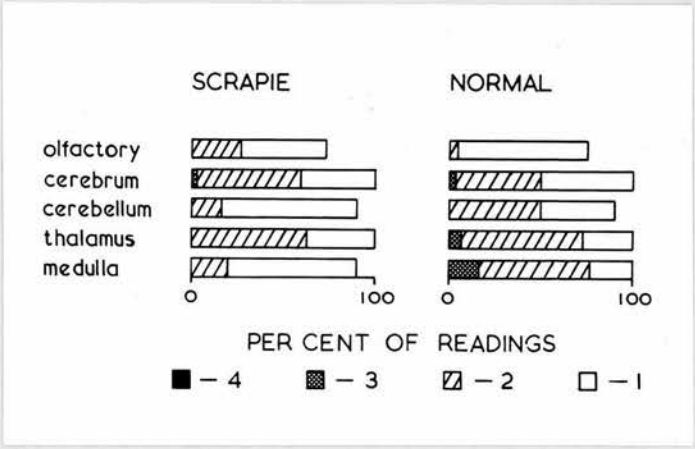


Fig. 11 The distribution of louping-ill encephalitis grades in clinically affected C57 black mice that had been injected i.c. with LI.31.M5 six days previously, at 20 weeks after i.c. inoculation with ME.7/6 scrapie agent or control material.

TABLE 5

THE SEVERITY OF LESIONS IN SCRAPIE AND NORMAL MICE THAT WERE INOCULATED I.C. WITH LI.31.M5 OR CONTROL MATERIAL

Week of i.c. inoculation		louping-ill encephalitis scores[x] (mean ± S.E.)	scrapie lesion scores*
0	20[+]		
Control	LI.31.M5	7.7 ± 0.4	1.3 ± 0.4
scrapie	LI.31.M5	6.4 ± 0.5	9.2 ± 0.7
scrapie	control	0.1 ± 0.1	14.6 ± 0.8

There were 10 mice in each group.

\+ = All animals were killed 6 days later.

x = This term is defined on page 10.

* = These values were calculated by summing semiquantitative estimates of lesion severity in various areas of the brain (Fraser and Dickinson, 1968). The readings were made by Mr. J. C. Rennie.

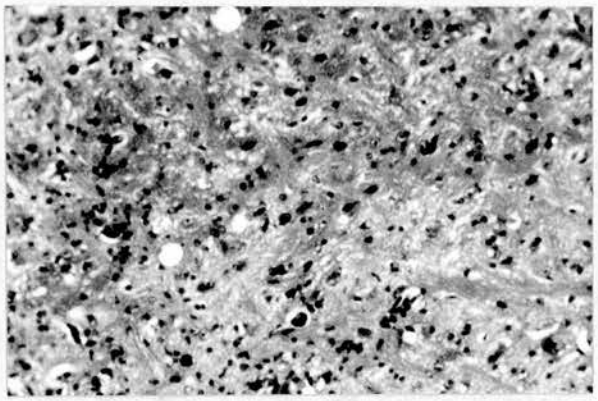


Fig. 12

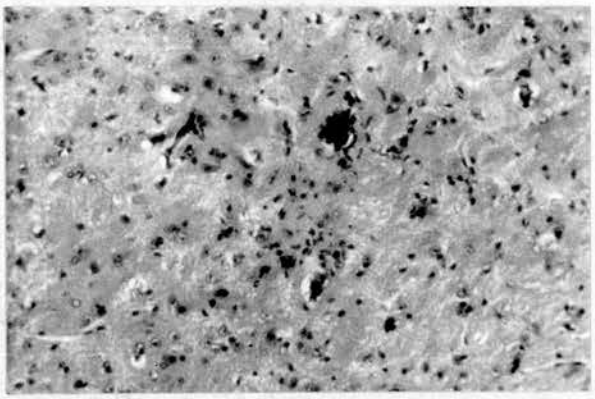


Fig. 13

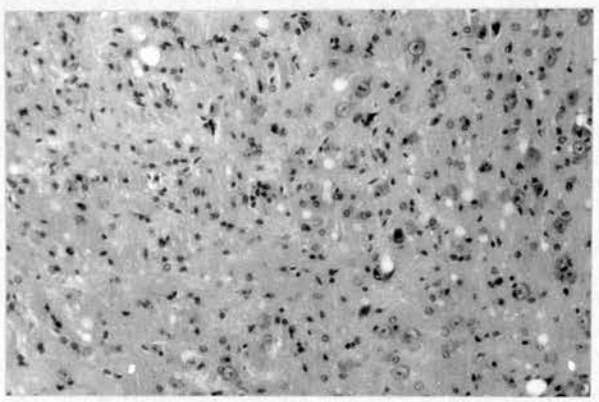


Fig. 14

Figs. 12, 13 and 14 The thalamus of C57 black mice that were inoculated i.c. with scrapie agent (12 and 14) or control material (13) at 20 weeks before i.c. inoculation with LI.31.M5 (12 and 13) or control material (14). H. & E. X80.

II. STUDIES IN SYRIAN HAMSTERS

(1) The Effect of Route of Inoculation and Age on Susceptibility and Neuropathological Changes

There were considerable differences in the severity of neuropathological changes in moribund C57 black mice, depending on whether the virus was given i.p. or i.c.. Comparative studies of the relationships between route of inoculation, clinical susceptibility and neuropathological lesions were limited by the lack of another host species known to be regularly susceptible to peripheral exposure to louping-ill virus. The weaned Syrian hamster develops a fatal disease following i.c. inoculation with louping-ill virus (Pogodina and Savinov, 1964), but an asymptomatic infection results when the virus is given s.c. (Pogodina, 1964). It was thought that this resistance to peripheral inoculation might not be present in younger hamsters, for suckling mice are generally much more susceptible than adults to arbovirus infections (Lennette and Koprowski, 1944).

Eighty hamsters (15 litters), aged from 3 to 25 days (Fig. 15) and weighing between 3.2 g and 48.4 g, were used. A 10^{-2} dilution of re-constituted LI.31.M5 was inoculated i.c. into one-third of the animals in each litter, i.p. into another third and control material was injected i.p. or i.c. into the remainder. Infected hamsters were given approximately 2.4×10^4 mouse infectious doses i.c., or 10 times that amount i.p.. Brains for histological examination were taken from 28 clinically affected animals and 18 clinically normal animals, including 16 controls.

In all i.c. inoculated hamsters incubation periods were very uniform (5 to 7 days) (Fig. 16), clinical signs were severe and weight gains were

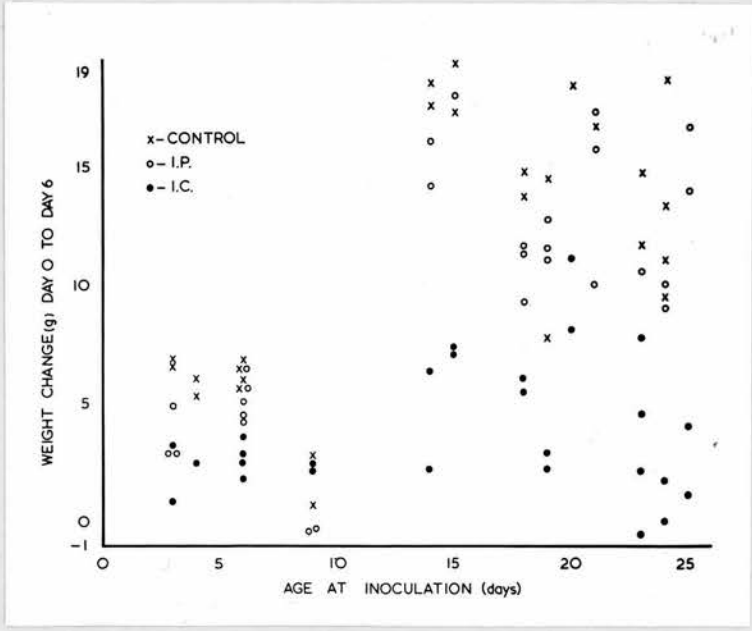


Fig. 15 Body weight change from day of i.p. or i.c. inoculation with LI.31.M5 or control material (day 0) to day 6, in hamsters of various ages.

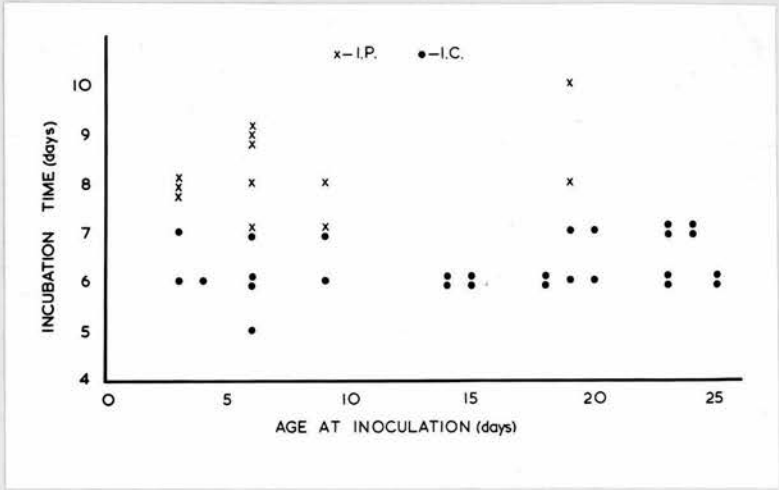


Fig. 16 Incubation times to detection of neurological symptoms in hamsters which were inoculated i.p. or i.c. with LI.31.M5 at different ages. The 2 inoculated i.p. when 19 days old showed slight symptoms, from which they subsequently recovered. The remainder were found dead (9 animals) or killed with severe clinical signs (28 animals).

considerably lower than in controls (Fig. 15). Incubation times were generally longer, however, in clinically affected animals that were given virus i.p. (Fig. 16). Fatal symptoms and depressed weight gains (Fig. 15) were only seen in those less than 10 days old at i.p. inoculation.

All clinically affected hamsters had lesions of encephalitis (Fig. 17) and the severity of neuropathological changes was not greatly influenced by the route of inoculation. In animals exposed by both routes the severe neuronal necrosis seen in infants gave way, with increasing age (Fig. 17), to a predominantly inflammatory lesion. This gradation was only apparent in hamsters less than 14 days old at inoculation. The controls were histologically normal.

Thus the nature of the neuropathological lesions in clinically affected Syrian hamsters is considerably affected by the age of the host but, compared with the C57 black mouse, is relatively independent of the route of inoculation.

(2) Pathogenesis Studies in Hamsters that were Inoculated I.P. at Different Ages

The pattern of increasing resistance with age provided an experimental model for investigating the nature and development of neuropathological changes in animals with a range of symptoms resulting from i.p. inoculation with virus.

Five groups of hamsters were inoculated i.p. with control material or a 10^{-2} dilution of re-constituted LI.31.M5, as shown in Table 6. The control and virus inoculated animals were from the same litters and were housed together. Infected hamsters were killed serially, as shown in

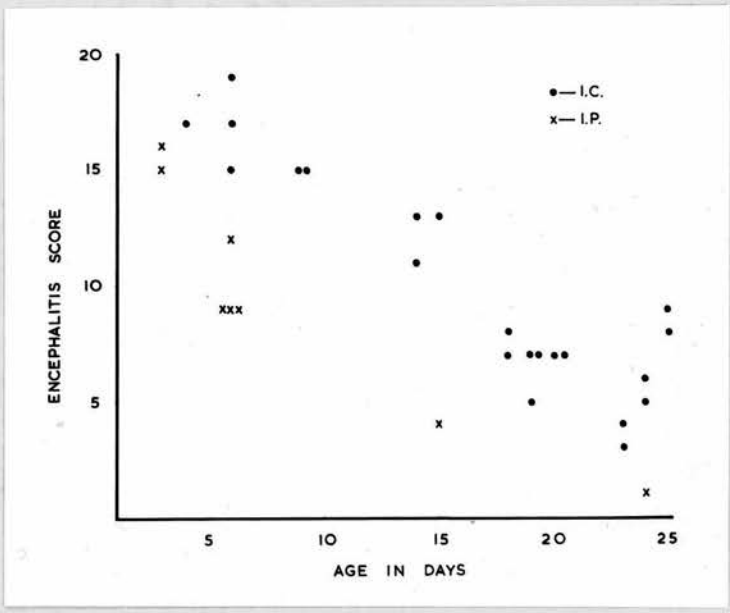


Fig. 17 Encephalitis scores (p. 10) in hamsters inoculated i.p. or i.c. with LI.31.M5 at different ages. The 2 inoculated i.p. when 15 and 24 days old were clinically normal throughout. The remainder had symptoms of neurological disease.

TABLE 6

DETAILS OF I.P. INOCULATION OF HAMSTERS OF DIFFERENT AGES WITH LOUPING-ILL VIRUS OR CONTROL MATERIAL

Group	Age at inoculation	Number inoculated		Number examined*	
		Virus†	Control	Virus	Control
Infant	4 days	26	10	18	10
Large suckling	14 days	33	9	26	6
Newly weaned	22 days	34	4	33	4
Young adult	6 to 8 weeks	22	4	22	4
Aged	♀	6	2	6	2

* = Five inocula were titrated in mice and it was calculated that each hamster was given between 1.6×10^5 and 3.9×10^5 infectious doses of LI.31.M5.

† = Excess animals were inoculated to allow for unforeseen deaths; 4 infant hamster brains were used to provide formol-fixed frozen sections for lipid histochemistry.

♀ = These animals were cast breeding females.

Tables 7, 8 and 9 and Fig. 18, and the controls were sacrificed at comparable times. In the 3 younger groups a sample of blood and one half of the brain was processed for virus titration and the other half of the brain was examined histologically. The brains of young adult and aged hamsters were taken for histological examination alone.

Two of the infant hamsters were found dead on day 7 and the remainder were moribund on day 8. One large suckling hamster had neurological symptoms on day 12, but there were no symptoms in any of the other animals listed in Table 6.

The titres of virus in samples of blood and brain from the younger hamsters are given in Tables 7 to 9. Viraemia was detected fairly consistently for 5 to 6 days in the large suckling and newly weaned animals. However infant hamsters had generally higher levels of viraemia which persisted until death on day 8. Brain titres were also high in the infants, whereas the smaller amounts detected in nervous tissue from newly weaned hamsters tended to disappear by about day 11. The virus was also isolated from blood and brain of one clinically normal control infant killed on day 6. This may have resulted from cross infection when litter mates were inoculated with virus, for some inoculum often seeps out along the needle track in these very small rodents. No isolations were made from any of the other 16 controls examined.

The earliest pathological change apparent in the infant hamsters was slight focal vasculitis in the cerebral cortex of one animal killed on day 4 (Table 7). Foci of cellular necrosis were apparent from day 5 and became progressively more widespread until death on day 8. The changes in the

TABLE 7

ENCEPHALITIS IN INFANT HAMSTERS WHICH WERE
4 DAYS OLD AT I.P. INOCULATION WITH LI.31.M5.

Day after inoculation		1	2	3	4	5	6	7	8
Virus titre in blood	Hamster 1	1.7	3.4	3.0	2.4	1.5	2.2	2.2	2.5
	Hamster 2	2.8	4.1	3.5	$\geqslant$ 4.2	1.4	2.5	2.5	2.4
Virus titre in brain	Hamster 1	0	2.7	5.2	6.2	6.8	6.6	6.2	5.6
	Hamster 2	0	2.9	4.2	5.3	5.8	5.8	6.2	6.4
Encephalitis score	Hamster 1	0	0	0	0	8	13	15	13
	Hamster 2	0	0	0	1	8	12	14	14

0 = No virus isolated, or lesions detected.

The term "encephalitis score" is defined on page 10.

TABLE 8

ENCEPHALITIS IN LARGE SUCKLING HAMSTERS WHICH WERE 14 DAYS OLD AT I.P. INOCULATION WITH LI.31.M5.

Day after inoculation		1	2	3	4	5	6	7	8	9	10	12	14	22
Virus titre in blood	Hamster 1	T	1.4	0.9	T	1.0	1.2	0	0	0	0	NT	NT	NT
	Hamster 2	1.5	1.6	0.9	0.5	0	0.5	0	0	0	0	NT	NT	NT
Virus titre in brain	Hamster 1	0	1.5	1.5	2.6	3.0	3.5	3.0	1.2	1.5	1.2	NT	NT	NT
	Hamster 2	0	0.2	2.0	2.5	2.5	3.0	1.8	2.0	1.5	2.4	NT	NT	NT
Encephalitis score	Hamster 1	0	0	0	2	1	3	7	8	6	4	7	9	3
	Hamster 2	0	0	0	0	0	6	7	8	7	4	5	12	5

0 = No virus isolated or lesions detected.

T = Trace.

NT = Not tested.

TABLE 9

ENCEPHALITIS IN NEWLY WEANED HAMSTERS WHICH WERE 22 DAYS OLD AT I.P. INOCULATION WITH LI.31.M5.

Day after inoculation		1	2	3	4	5	6	7	8	9	10	11
Virus titre in blood	Hamster 1	2.1	2.3	1.2	0.2	T	0	0	0	0	0	0
	Hamster 2	1.6	1.6	1.0	0.5	3.2	0	0	0	0	0	0
	Hamster 3	0.6	0.6	2.0	0.5	0.6	1.4	0	0	0	0	0
Virus titre in brain	Hamster 1	0	0	0	2.5	0.5	1.5	2.0	1.8	0.2	1.2	T
	Hamster 2	T	1.5	1.0	2.2	T	1.2	1.5	1.5	1.2	T	0
	Hamster 3	0	0	0	1.8	2.8	2.4	T	1.0	0	T	0
Encephalitis score	Hamster 1	0	0	0	2	0	4	2	4	3	5	5
	Hamster 2	0	0	0	1	0	2	5	7	5	6	5
	Hamster 3	0	0	0	0	1	0	3	3	5	8	4

0 = No virus isolated, or lesions detected.

T = Trace.

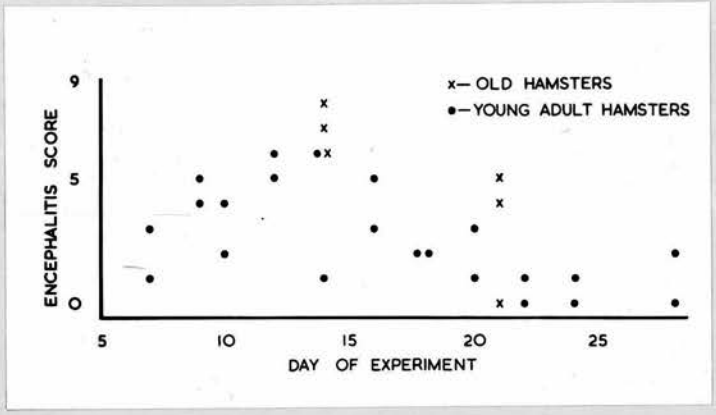


Fig. 18 **Encephalitis scores (p. 10) in young adult (6 to 8 week old) and old ("aged" in text) hamsters which were inoculated i.p. with LI.31.M5 and were clinically normal when killed.**

cerebellar cortex were particularly severe (Fig. 19) and myelin was absent from the cerebellar folia, though present in the comparative controls. Granules of P.A.S. positive material were found in foam cells and in clumps of cellular debris in necrotic areas (Fig. 20). This material was not metachromatic, did not stain for glycogen or lipid and was only weakly eosinophilic. Its chemical nature is obscure. Cellular necrosis was widespread in clinically affected animals, especially in the cerebral cortex (Fig. 21) and the colliculi. The olfactory bulbs and the medulla tended to be less severely damaged. Seven of the 8 controls were histologically normal, and there were slight lesions in the 1 animal from which virus was isolated.

Inflammation was, however, the predominant pathological change in the brains of all the older hamsters (Tables 8 and 9, Fig. 18). Slight inflammation was first detected at from 4 to 7 days after inoculation and, with the exception of one large suckling hamster with neurological symptoms on day 14 (Table 8), there was little progression in severity of lesions. The characteristic changes were of microglial proliferation and perivascular infiltration with round cells (Figs. 22 and 23). Neuron necrosis and P.A.S. positive material were, if present, associated with perivascular foci. There was no preferential distribution of inflammation throughout the C.N.S., and the lesions tended to resolve with time (Fig. 18). The 16 controls examined were histologically normal.

Fatal louping-ill in i.p. inoculated infant hamsters is thus a function of persistent viraemia, high levels of virus in nervous tissue and progressive cellular necrosis in the brain. Whereas the subclinical infection in hamsters

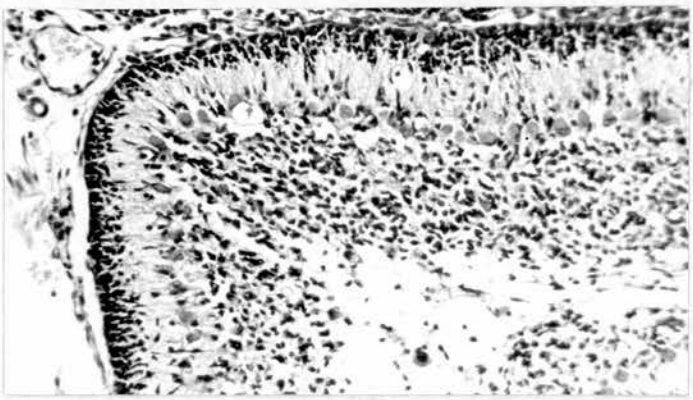


Fig. 19

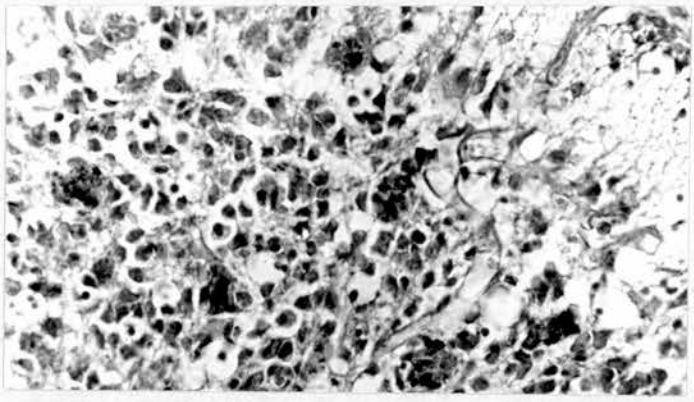


Fig. 20

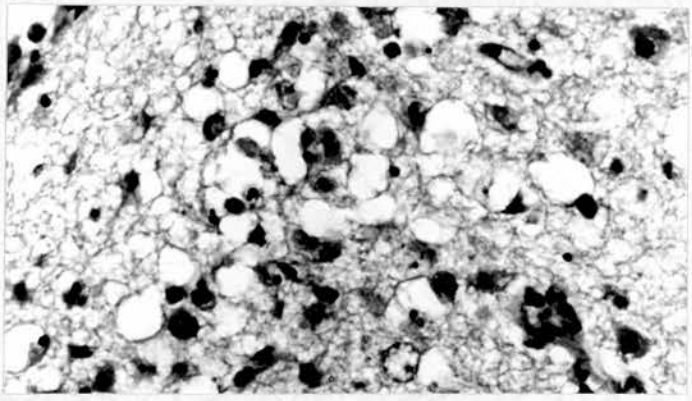


Fig. 21

Figs. 19 to 21 Cellular necrosis in an infant hamster that was moribund at 8 days after i.p. inoculation with LI.31.M5.

Fig. 19 Cerebellum, the nuclei of the Purkinje cells are displaced and shrunken and those of the granule cells are pyknotic. This lesion was given a grade of 4. H. & E. X165.

Fig. 20 Cerebellum, P.A.S. positive material in the Purkinje and granule cell layers. P.A.S. X440.

Fig. 21 Cerebral cortex, cellular necrosis and vacuolation of the ground substance. H. & E. X500.

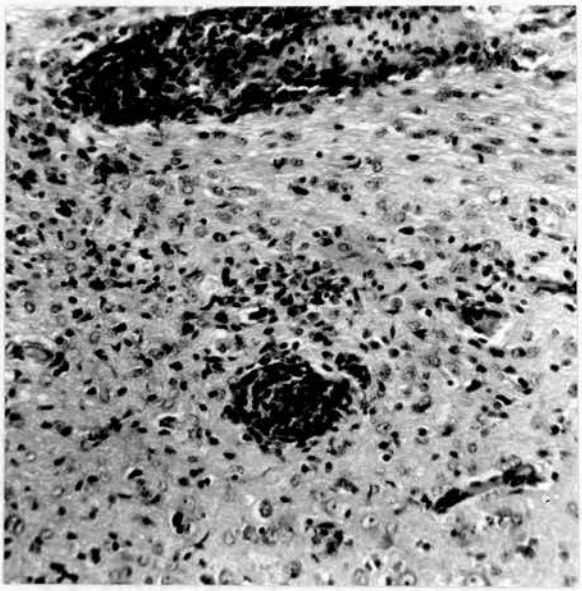


Fig. 22 Inflammatory reaction in the thalamus of a clinically normal large suckling hamster at 14 days after i.p. inoculation with LI.31.M5. This lesion was given a grade of 2. H. & E. X165.

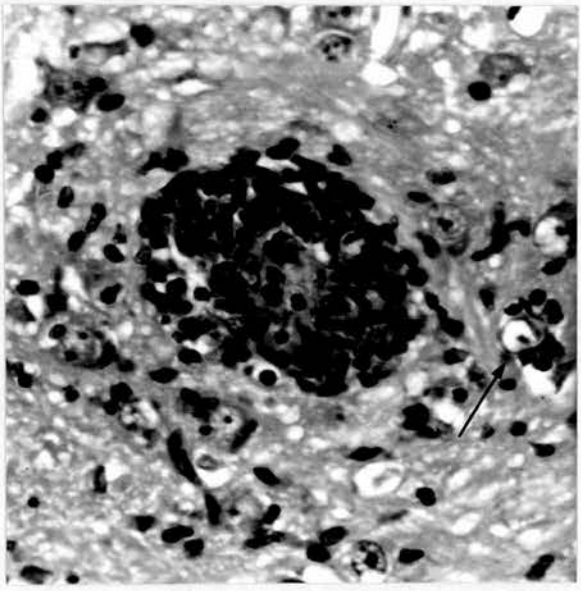


Fig. 23 Neuronal satellitosis (arrow) and an isolated focus of inflammatory cells in the thalamus of a clinically normal newly weaned hamster that was killed at 11 days after i.p. inoculation with LI.31.M5. H. & E. X440.

inoculated i.p. at more than 14 days of age is characterized by low levels of virus in the brain and fairly slight inflammatory changes which tend to resolve with time.

(3) The Effect of Cortisone on Subclinical Infection

The susceptibility of rodents to group B arbovirus infections is generally increased by giving cortisone early in the incubation period (Southam and Babcock, 1951; Imam and Hammon, 1957; Kasova, 1962). It thus seemed possible that the clinical pattern in large suckling hamsters inoculated i.p. with louping-ill virus, and perhaps the severity of neuropathological changes, might be modified by cortisone treatment. The possible effect on lesions in brain has not been studied previously.

A small experiment was done as shown in Table 10. The symptoms in these animals were very acute and 9 were found dead in the morning, though they seemed normal on the previous night. Severe generalized neuropathological changes were seen in moribund cortisone treated animals (Fig. 24). These were predominantly inflammatory in nature with large perivascular cuffs (Fig. 25), diffuse infiltrations of inflammatory cells and some neuron necrosis (Fig. 26). There were only slight lesions in the brains of virus inoculated animals that were given N saline instead of cortisone (Controls in Fig. 24). No neuropathological changes were detected in the cortisone treated hamsters that were inoculated with normal mouse brain, instead of virus.

The neuropathological changes in these cortisone treated large suckling hamsters were comparable in severity to those seen previously in 3 moribund hamsters that were inoculated i.c. at about the same age (Fig. 17), and one large suckling hamster that developed symptoms following i.p. inoculation

TABLE 10

EFFECT OF CORTISONE ON THE SUBCLINICAL DISEASE IN LARGE SUCKLING (14 day old) HAMSTERS*

Simultaneous inocula	Number inoculated	Number with symptoms	Incubation time (days, mean ± S.E.)
Virus[+] and cortisone[x]	21	16	18.1 ± 1.1
Normal mouse brain and cortisone[x]	6	0	---
Virus[+] and N saline	15	0	---

* = All hamsters were given drinking water containing 0.0125 mg. per ml. of aureomycin (Cyanamid of Great Britain Ltd., London). This water was changed daily throughout the experiment.

\+ = 1.2×10^5 mouse infectious doses of LI.31.M5 given i.p..

x = 5.0 mg. of cortisone acetate (Roussel Laboratories Ltd., London) given s.c. in 2.0 ml. of N saline.

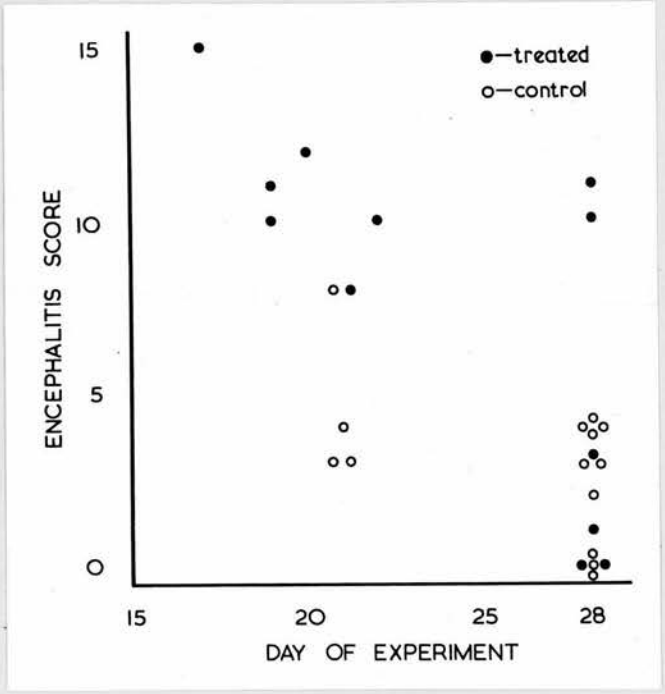


Fig. 24 Encephalitis scores (p. 10) in large suckling hamsters, some of which were treated with cortisone at time of i.p. inoculation with LI.31.M5. The controls (virus and N saline) were clinically normal, as were all animals killed on day 28. The remainder were moribund.

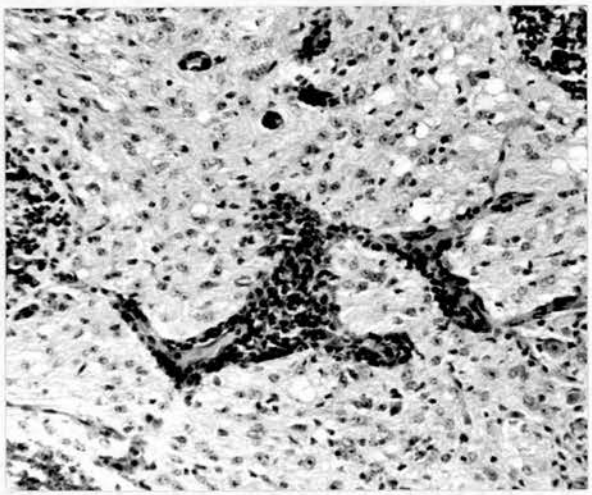


Fig. 25 Perivascular cuffing and diffuse inflammation in the cerebellar white matter of a cortisone treated large suckling hamster that was moribund when killed at 17 days after i.p. inoculation with LI.31.M5. H. & E. X110.

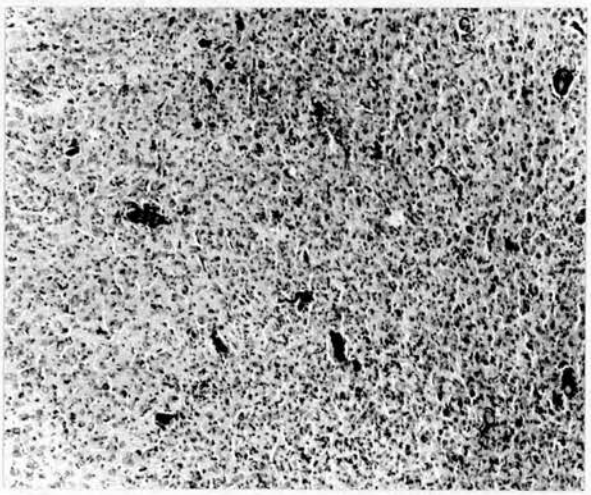


Fig. 26 Disseminated inflammation and neuron necrosis in the cerebral cortex of a cortisone treated large suckling hamster that was clinically normal when killed at 28 days after i.p. inoculation with LI.31.M5. H. & E. X70.

(Table 8). This offers further support to the earlier observation (p. 27) that lesion severity in suckling hamsters is closely related to clinical susceptibility and is not largely a function of the route of inoculation, as it is in weaned C57 black mice (Section I, (2) and (3)).

(4) Titration of the Virus I.C. in Weaned Hamsters

In weaned hamsters, however, lesion severity was not related to either clinical susceptibility or the route of virus inoculation. This is apparent when the encephalitis scores from clinically affected i.c. inoculated animals (Fig. 17) are compared with those from clinically normal animals that were given virus i.p. (Table 9). A possible explanation is that the disease in moribund animals was so acute that there was insufficient time for severe pathological changes to develop. The most obvious way of increasing the incubation time was to decrease the dose of virus given.

Newly weaned hamsters and mice (for comparison with the standard titration system) were inoculated i.c. with ten-fold dilutions of virus (Table 11). The hamsters proved to be almost as susceptible as mice, though their incubation times were longer. The severity of lesions in hamsters was not obviously related to either the incubation time or the dose of virus (Fig. 27). Inflammatory changes were found in all regions of the brain (Fig. 28) and the olfactory bulb (Fig. 29) was the most consistent site for severe lesions.

A further experiment was done in 2 groups of 5 young adult hamsters, which were inoculated i.c. with either a 10^{-2} or a 10^{-5} dilution of re-constituted LI.31.M5 (equivalent to 10^{-0} and 10^{-3} on Table 11). One of those given the lower dose died soon after injection, 1 was clinically

TABLE 11

MORTALITY* IN NEWLY WEANED HAMSTERS
AND MICE INOCULATED I.C. WITH VIRUS

Log_{10} dilution of standard inoculum†	Hamsters		Mice	
	Incidence (groups of 6)	Day p.i. (mean ± S.E.)	Incidence (groups of 5)	Day p.i. (mean ± S.E.)
-4	4	12.8 ± 1.1	5	7.0 ± 0.0
-3	5	14.3 ± 1.6	5	7.0 ± 0.0
-2	6	11.2 ± 0.4	5	6.0 ± 0.0
-1	6	9.8 ± 0.5	5	6.4 ± 0.2
-0	6	8.5 ± 0.5	-	-

* = Including animals which were killed when moribund.

† = The inoculum was a 10^{-2} dilution of re-constituted LI.31.M5.

p.i. = Post inoculation.

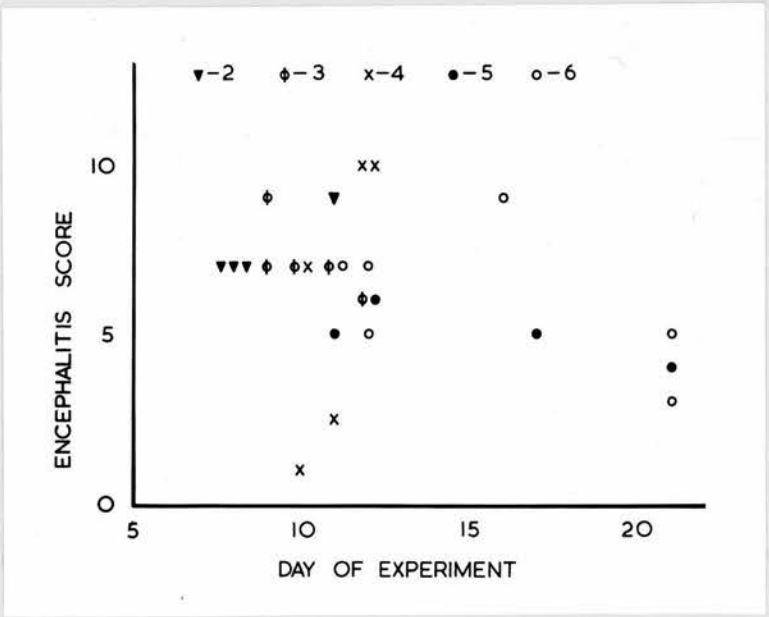


Fig. 27 Encephalitis scores (p. 10) in newly weaned hamsters that were inoculated i.c. with ten-fold dilutions of LI.31.M5. The 3 animals killed on day 21 were clinically normal. The remainder had severe neurological symptoms.

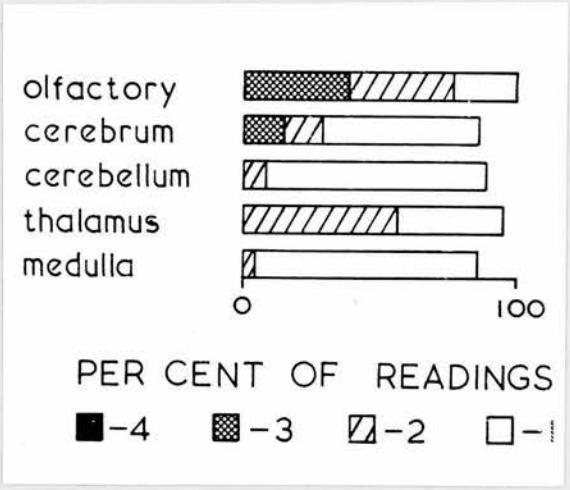


Fig. 28 The distribution of lesion grades (p. 9) in 21 moribund newly weaned hamsters that were inoculated i.c. with LI.31.M5.

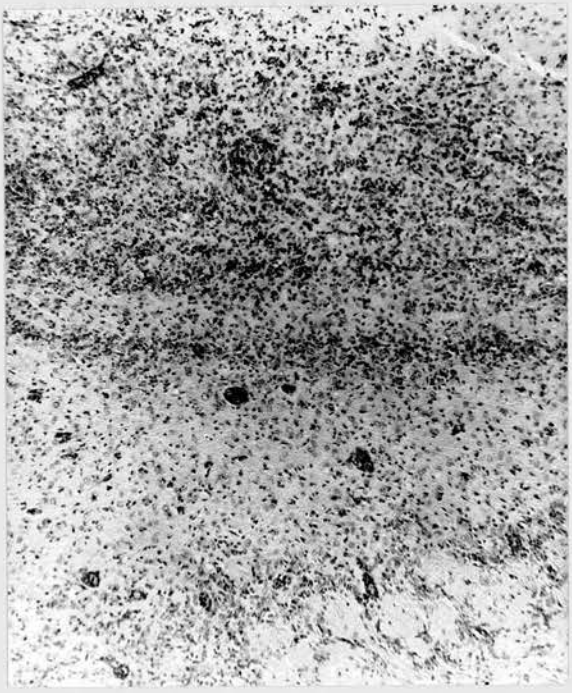


Fig. 29 Inflammation and neuron necrosis in the olfactory bulb of a moribund newly weaned hamster that was killed at 12 days after i.c. inoculation with LI.31.M5. H. & E. X66.

normal until killed on day 21 and the remainder developed symptoms of posterior paresis (Table 12). Four of those given the higher dose were killed with posterior paresis, between days 7 and 11, and 1 survived. The encephalitis scores from clinically affected hamsters ranged between 4 and 7. The symptoms in these young adults were generally less marked than those in the newly weaned animals. Head movements and feeding behaviour were still quite normal in 4 of the 7 young adults that were killed with posterior paresis.

Thus the severity of neuropathological changes in clinically affected weaned hamsters was not altered when the incubation time was increased by giving a smaller dose of virus i.c.. There was slight evidence for a marginal decrease in susceptibility with increasing age at i.c. inoculation. The older hamsters tended to develop long lasting posterior paresis.

(5) Chronic Neurological Symptoms and the Effect of Cortisone

The demonstration of persistent posterior paresis in i.c. inoculated young adult hamsters suggested that this might possibly be a model for studying chronic non-fatal neurological disease. With the exception of rare "tolerant" infections in mice inoculated with Kyasanur Forest disease virus (Price, 1966) there are not, to the author's knowledge, any other instances of chronic C.N.S. symptoms in rodents inoculated with arboviruses. This does occur in other species e.g. louping-ill in man is characterized by non-fatal neurological involvement (Rivers and Schwentker, 1934).

Corticosteroids were recently used by Webb et al (1968, a) to treat such symptoms in laboratory workers that had contracted louping-ill. In an experimental situation early administration of cortisone would probably

TABLE 12

SYMPTOMS AND LESIONS IN 3 YOUNG ADULT HAMSTERS
THAT WERE INOCULATED I.C. WITH LI.31.M5

Paresis		Symptoms on day killed	Encephalitis* score	Lesion grade* in spinal cord		
Day of onset	Duration			Cervical	Thoracic	Lumbar
8	3	moribund, anterior and posterior paresis	4	1	1	1
8	6	" " "	6	1	1	1
8	6	posterior paresis only	2	0	1	1

* These terms are defined on pages 9 and 10.

increase the incidence of fatal louping-ill (p. 30). However giving the drug just before the expected development of symptoms might mimic the chemotherapeutic effect demonstrated by Webb et al (1968, a), and perhaps decrease the degree of neurological involvement.

An experiment was done, as shown in Table 13, to study both the nature of the chronic disease and the effects of cortisone. All animals were also given aureomycin (as described in Table 10) in their drinking water. Female hamsters received one half the dose of cortisone given to males, for they are very susceptible to the toxic effects of this drug (Frenkel and Havenhill, 1963). In a preliminary experiment 4 of 5 females died when given the full 9.0 mg. of cortisone acetate, whereas all 5 males survived. Another 12 hamsters (6 males and 6 females) were inoculated i.c. with control material and then treated with cortisone on the schedules shown in Table 13. One male died, and the other 11 were clinically and histologically normal.

Neurological symptoms were seen in 65 per cent. of the untreated hamsters (Table 13) and the disease progressed to a fatal conclusion in approximately half (55 per cent.) of these cases. The other half developed posterior paresis, from which some animals recovered, commencing on about day 11. Lesions were detected in the spinal cords of the 3 paralysed hamsters examined (Fig. 30) and in 1 of 3 that remained clinically normal throughout (Table 14). There were only minimal inflammatory changes in the brains of these surviving animals (Figs. 31, 32 and 33).

All of the cortisone treatments increased the incidence of both neurological involvement and fatal infection (Table 13). Treatments, either commencing or continuing, from 6 days after inoculation tended to delay both

TABLE 13

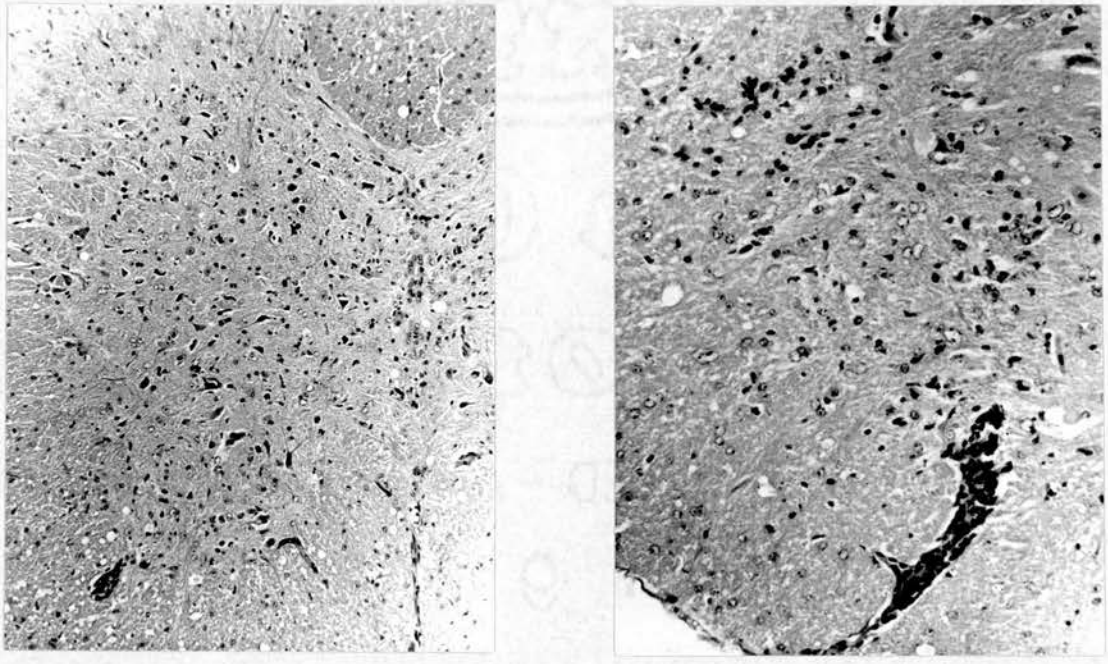
THE EFFECT OF CORTISONE ACETATE ON LOUPING-ILL IN YOUNG ADULT HAMSTERS THAT WERE INOCULATED I.C. WITH LI.31.M5[x]

Animals		Cortisone* administration		Neurological symptoms		Posterior paresis			Mortality	
Sex	No.	Total dose (mg.)	Days given	No.	Day of onset (mean ± S.E.)	No.	Day of onset (mean ± S.E.)	Duration in days (mean ± S.E.)	No.	Day (mean ± S.E.)
Male	10	Nil	Nil	5	11.8 ± 1.2	5	11.8 ± 1.2	5.6 ± 1.7[+]	3	14.3 ± 0.7
	10	9.0	3 to 7	10	14.8 ± 1.0	3	15.0 ± 0.6	2.0 ± 0.6	9	14.8 ± 1.0
	10	9.0	6 to 10	10	16.4 ± 0.9	7	17.7 ± 0.7	1.6 ± 0.3	9	16.2 ± 1.0
Female	10	Nil	Nil	8	11.1 ± 0.4	8	11.1 ± 0.4	7.5 ± 2.2[+]	4	12.5 ± 0.3
	10	4.5	3 & 4	8	11.6 ± 1.5	3	12.1 ± 0.7	2.0 ± 0.0	8	12.0 ± 1.6
	10	4.5	6 & 7	6	15.2 ± 0.7	4	16.0 ± 0.7	2.0 ± 0.4	6	16.0 ± 1.0

x = Each animal was given 1.2×10^2 mouse infectious doses i.c..

* = Treated animals were given 3.0 mg. s.c. on the first day, followed by 1.5 mg. on successive days. The cortisone was prepared as described in Table 10. The untreated animals were given N saline instead.

+ = These times would have been longer except that 6 untreated animals with posterior paresis were killed at 23 days after inoculation, though they appeared normal in every other respect.



(a) (b)

Fig. 30 Inflammation and neuron necrosis in the lumbar spinal cord of an untreated young adult female hamster that had posterior paresis for 13 days and was killed at 23 days after i.c. inoculation with LI.31.M5. These lesions were given grades of 2. (a) H. & E. X110, (b) H. & E. X180.

TABLE 14

THE SEVERITY OF SPINAL CORD LESIONS IN UNTREATED YOUNG ADULT HAMSTERS THAT WERE KILLED 23 DAYS AFTER I.C. INOCULATION WITH LI.31.M5

Sex	Duration of posterior paresis (days)	Encephalitis* score	Lesion grades* in spinal cord		
			Cervical	Thoracic	Lumbar
M	8	1	0	0	2
M	0	3	0	0	0
F	14	5	0	1	2
F	13	4	1	2	2
F	0	2	0	1	1
F	0	2	0	0	0

* These terms are defined on pages 9 and 10.

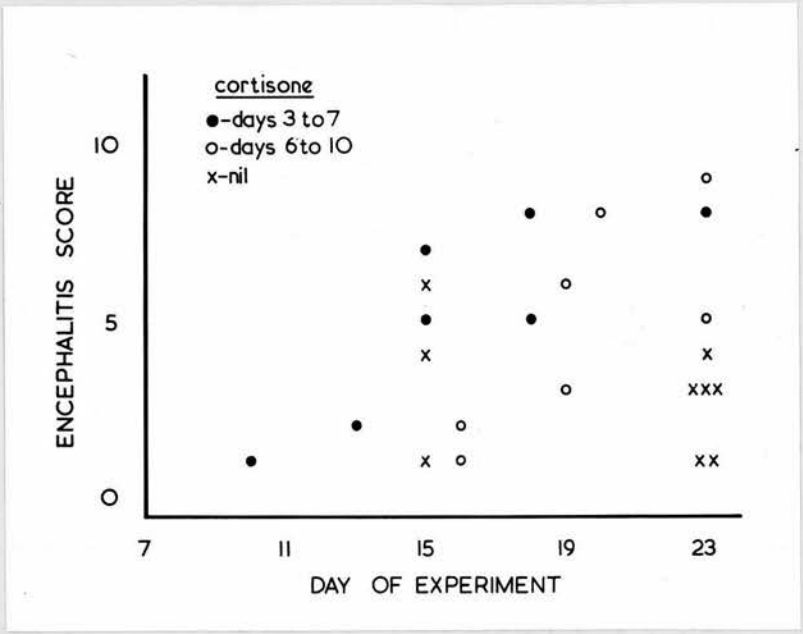


Fig. 31 Encephalitis scores (p. 10) in young adult male hamsters, some of which were given cortisone after i.c. inoculation with LI.31.M5. All of the animals killed before the termination of the experiment on day 23, and one of those from day 23 (with the highest score), were moribund.

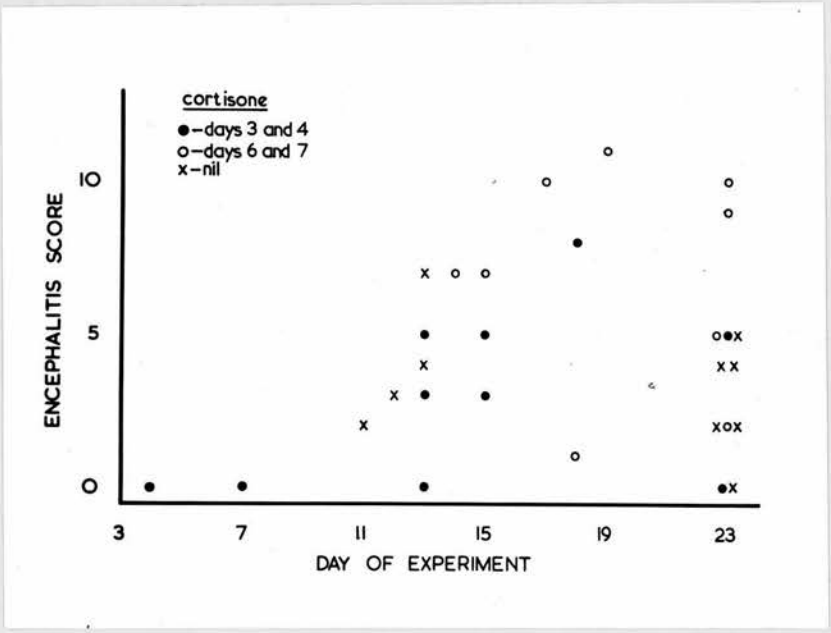


Fig. 32 Encephalitis scores in young adult female hamsters, some of which were given cortisone after i.c. inoculation with LI.31.M5. All of the animals killed before day 23 were moribund.

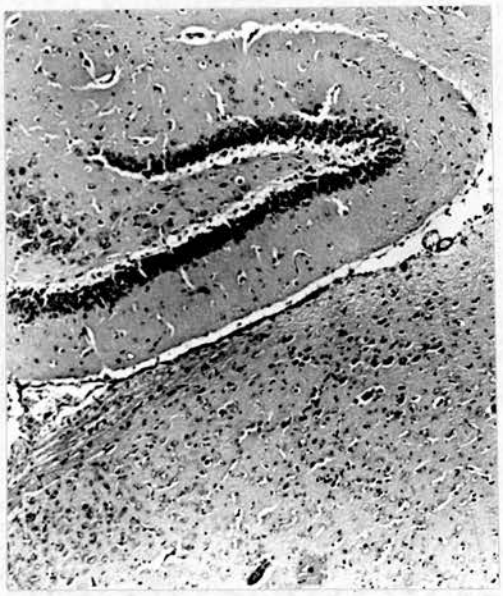


Fig. 33 Minimal inflammation in the thalamus and hippocampus of an untreated young adult male hamster that was killed, when clinically normal, at 23 days after i.c. inoculation with LI.31.M5. H. & E. X60.

Fig. 34 Meningitis, disseminated inflammation and neuron necrosis in the hippocampus; and perivascular cuffs in the thalamus of a young adult male hamster. This animal was inoculated i.c. with LI.31.M5, given cortisone from 6 to 10 days after inoculation and killed, when moribund, at 23 days after inoculation. H. & E. X60.

the onset of symptoms and the time of death. Once clinical signs were apparent the disease was rapidly progressive and posterior paresis, if detected, was only a transient symptom. The neuropathological changes seen in the longest surviving moribund treated animals (Figs. 31, 32 and 34) were among the most severe detected in any i.c. inoculated weaned hamsters (cf. Figs. 17 and 27). Cortisone may have allowed marked lesions to develop by effectively prolonging the incubation period.

Subclinical infection and chronic symptoms can thus occur in young adult hamsters that are inoculated i.c. with a fairly small dose of virus. Chronic posterior paresis is associated with moderate lesions in the lumbar spinal cord. Cortisone treatment may delay the onset of symptoms but increases both the incidence of fatal infection and, if the animals survive long enough, the severity of neuropathological changes.

(6) The Ultrastructure of Cerebellar Neurons in I.P. Inoculated Infant Hamsters

The pathogenesis of acute necrotizing encephalitis in infant hamsters was defined by virus titration techniques and optical microscopy of paraffin sections (Table 7, p. 29). It was thus logical to use this experimental system for an ultrastructural study of the development of pathological changes in the neuron. The feasibility of this approach was demonstrated in a small preliminary technical study (Smith and Doherty, 1969).

Infant (4 day old) hamsters were inoculated i.p. with 1.2×10^5 mouse infectious doses of LI.31.M5, or control material. They were killed serially and their cerebellums were processed for electron microscopy (p. 16). The investigation was restricted to the cerebellum because of the ease of

identifying different cell types in this tissue. At the time of writing blocks have been examined from 1 control from each of days 6 and 8, 1 clinically normal infected hamster from each of days 4 and 6, and 1 moribund hamster from day 8.

A single focus of about 12 neurons containing dark-staining intracytoplasmic bodies was observed on optical microscopy of Giemsa stained 1 μ thick sections from the infected hamster killed on day 4. Adjacent ultrathin sections of this focus were examined in the electron microscope and these dark-staining bodies were found to correspond to large discrete membraneous areas (Fig. 35). These structures were present in the cytoplasm of both Purkinje cells and granule cells and were not detected in the normal neurons (Fig. 36) of control hamsters. The cytoplasm external to the membraneous areas was considerably depleted of normal granular endoplasmic reticulum and consequently appeared rather "watery" (Fig. 35).

These membraneous areas surrounded numerous small electron-dense bodies, which were more apparent at higher power (Fig. 37). The membranes were composed of several layers and were devoid of ribosomes. They are unlike any normal component of neuronal cytoplasm.

There were many more neurons (approximately 50 per cent.) containing membraneous areas in the infected animal killed on day 6. Such cells were morphologically similar to those seen in the previous case. They tended to occur in foci and included both Purkinje cells and granule cells.

However, in the moribund hamster killed on day 8, cells containing these structures also showed many dark-staining shrunken mitochondria (Fig. 38). Cytoplasmic vacuolation and neuronal disintegration were also

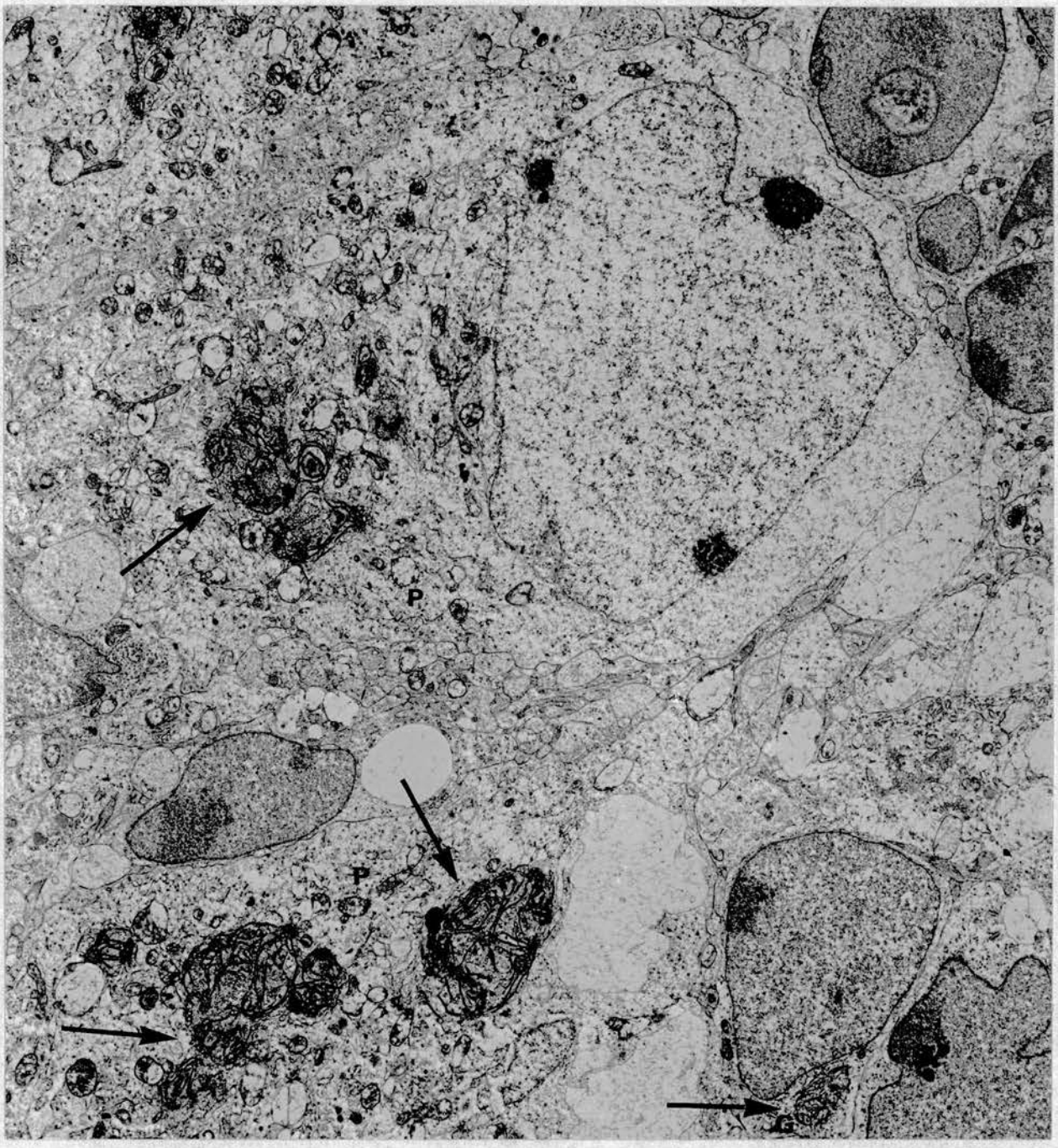


Fig. 35 Dark staining membraneous areas (arrows) in the cytoplasm of Purkinje cells (P) and granule cells (G). The cytoplasm has a rather "watery" appearance and is depleted of normal granular endoplasmic reticulum. Infant hamster killed at 4 days after i.p. inoculation with LI.31.M5. Lead citrate X6,000.

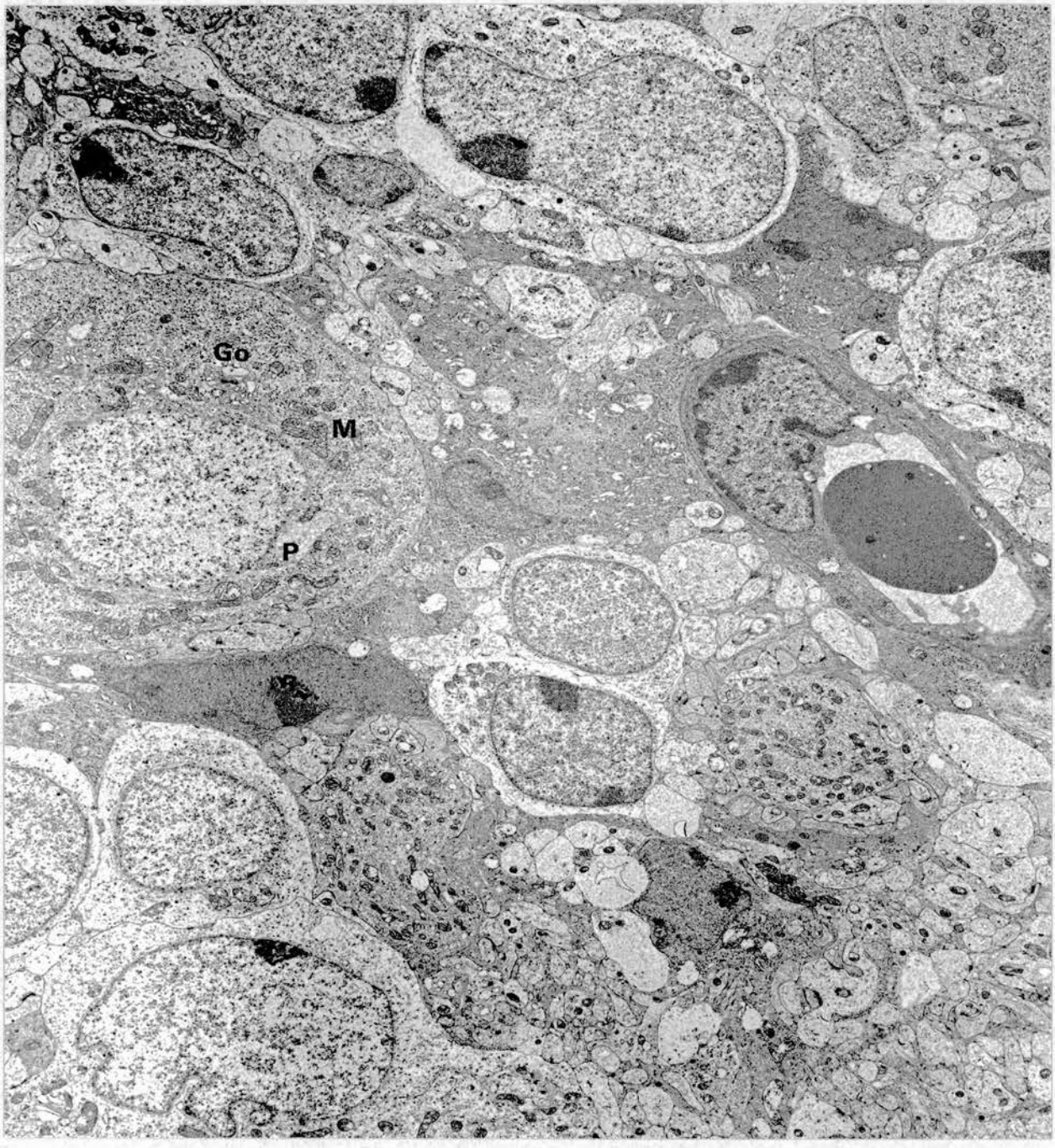


Fig. 36 Normal Purkinje cells (P) with numerous mitochondria (M), normal Golgi apparatus (Go.) and both smooth and granular endoplasmic reticulum. Infant hamster killed at 6 days after i.p. inoculation with control material. Lead citrate X6,000.

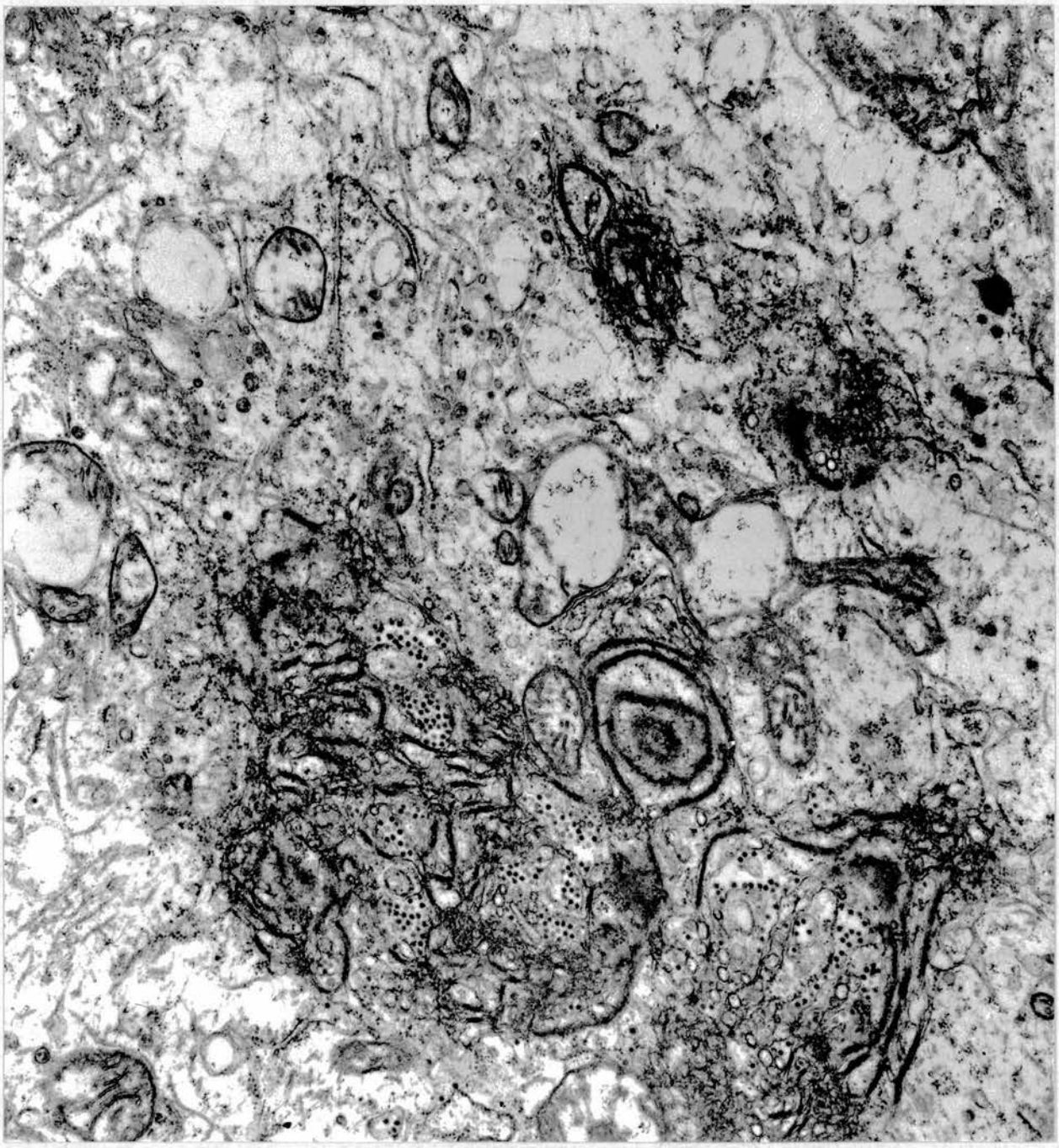


Fig. 37 Membraneous areas containing numerous small electron-dense bodies in the cytoplasm of a Purkinje cell. Infant hamster killed at 4 days after i.p. inoculation with LI.31.M5. Lead citrate X30,000.

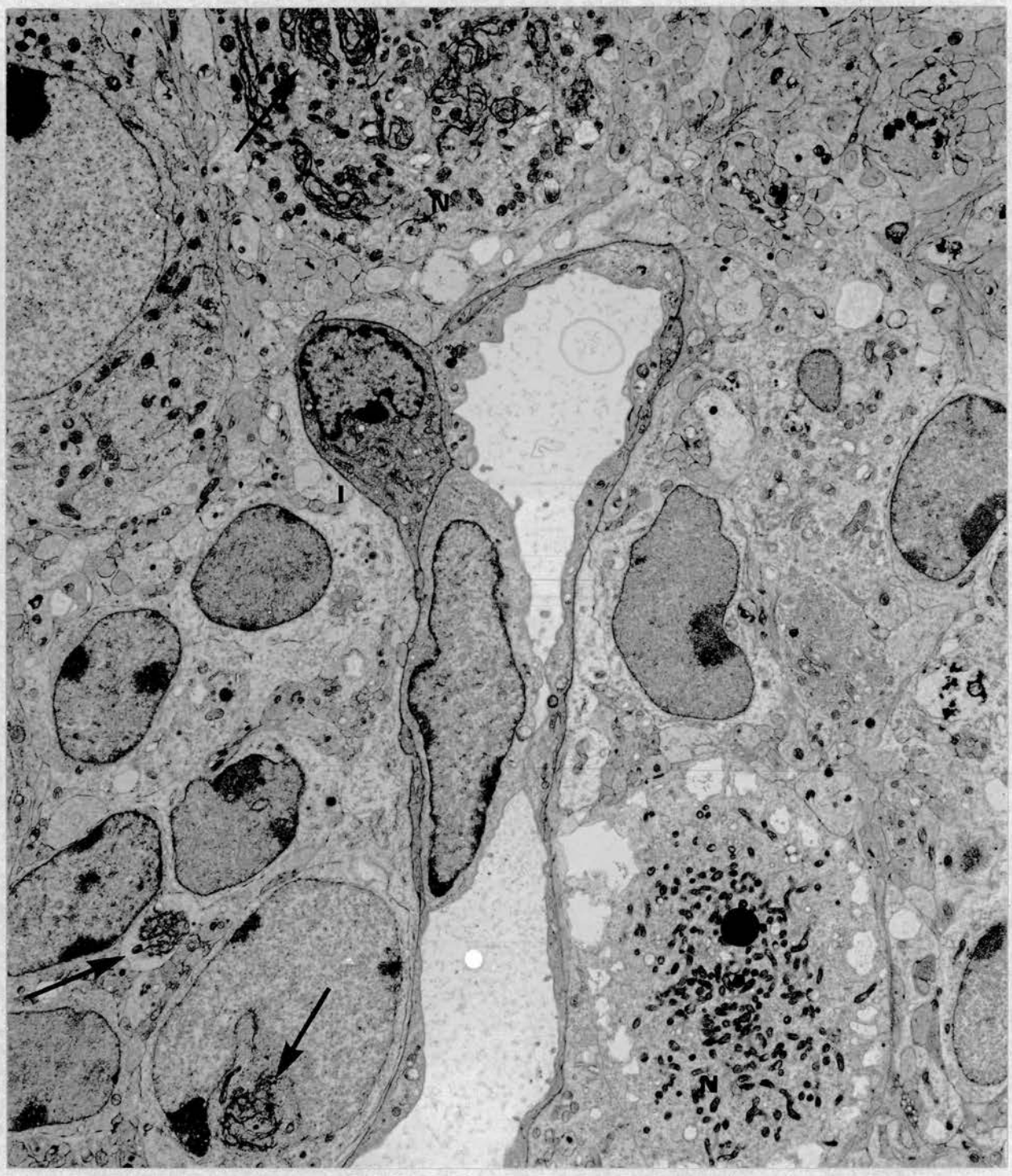


Fig. 38 Neuronal cytoplasm (N) containing numerous dark staining mitochondria and some membraneous areas (arrows), one of which is apparently in a neuronal process. The cell to the right of the blood vessel appears to be in an advanced stage of disintegration. There is also a single inflammatory cell (I), probably a polymorph, outside the endothelium of the vessel. Moribund infant hamster killed 8 days after i.p. inoculation with LI.31.M5. Lead citrate X6,000.

apparent. Another difference was that the abnormal membranes surrounded numerous empty "tubules", as well as some dense bodies (Fig. 39). These "tubules" had been seen previously, but in much smaller amounts. At this higher magnification (Fig. 39) some of the dense bodies can be seen to be surrounded by a less electron-dense "halo". An occasional inflammatory cell (Fig. 38) was seen in this case.

This limited ultrastructural study has, to date, confirmed several observations made earlier in paraffin sections (p. 29). The lesions in infant nervous tissue are initially focal, and the progression of the disease is associated with increasing numbers of abnormal neurons. There is an almost complete absence of any vascular inflammatory reaction in these infant hamsters.

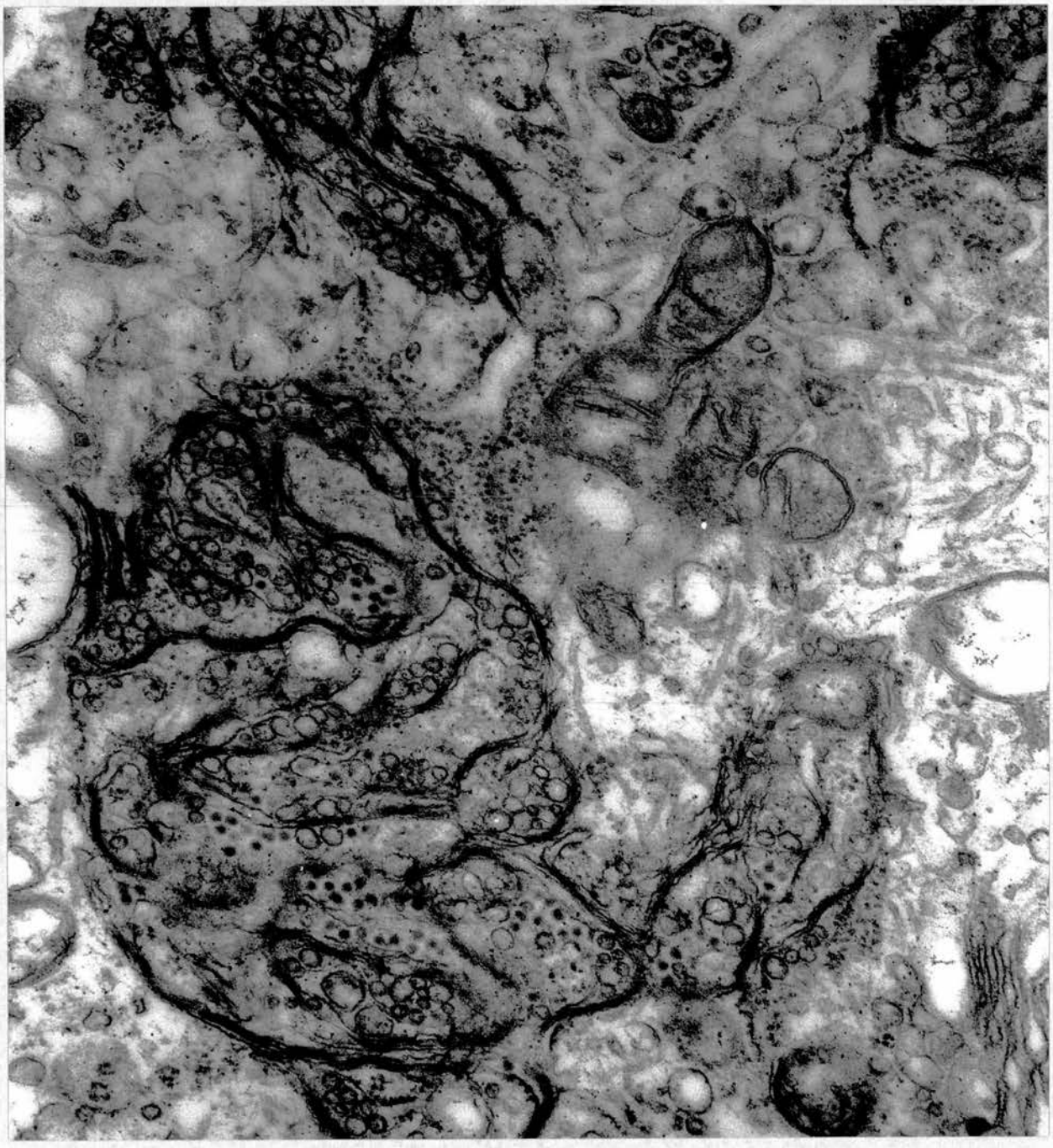


Fig. 39 Membraneous areas containing some dense bodies which appear to be surrounded with a slightly less dense "halo" and numerous empty "tubular" structures. Moribund hamster killed 8 days after i.p. inoculation with LI.31.M5. Lead citrate X45,000.

III. STUDIES IN RATS, GUINEA PIGS AND RABBITS

(1) The Histopathogenesis of Fatal and Subclinical Infection in the Rat

When the virus was inoculated i.p. into newly weaned animals, C57 black mice developed fatal neurological symptoms whereas Syrian hamsters were apparently unaffected. However there were no neuropathological changes in many of the mice, whereas inflammation was detected consistently in the brains of the hamsters. Both species were susceptible to i.c. inoculation, but in this case the neuropathological lesions in the mice were much more severe than those in the hamsters.

The rat is even more resistant than the hamster to experimental infection with the virus. Though infant rats are susceptible to i.c. inoculation (Gresikova et al, 1961), weaned rats do not develop any symptoms following i.c., i.p. or intranasal exposure (Hurst, 1931; Greig, Brownlee, Wilson and Gordon, 1931; Burnet, 1936, a; Miles, 1951). The histopathology of louping-ill encephalitis in the rat has not been described, but it would be interesting to compare it with that in the hamster.

Random bred rats of the Porton strain were inoculated at different ages with a 10^{-2} dilution of re-constituted LI.31.M5, and killed sequentially as shown in Fig. 40. Eleven other rats (corresponding to the groups in Fig. 40) were injected with control material and remained clinically and histologically normal.

The only symptoms seen were in 6 moribund infants remaining on day 7 (4 of these were not examined histologically). Lesions of cellular necrosis were very apparent in infant rat brain by day 5 (Fig. 40) and became progressively more widespread (Fig. 41) with time. Slight inflammation was

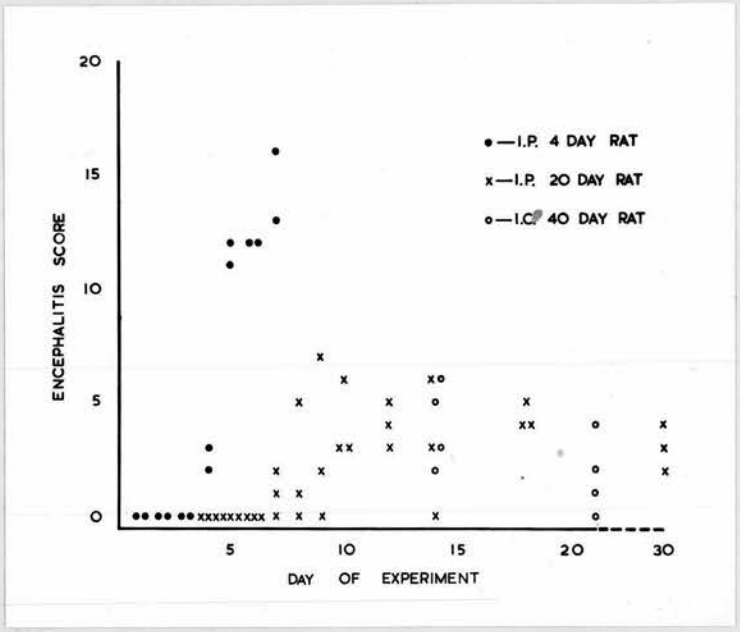


Fig. 40 Encephalitis scores (p. 10) in infant (4 day), newly weaned (20 day) and young adult (40 day) rats that were inoculated i.p. or i.c. with LI.31.M5. The only symptoms were in the infants that were killed on day 7.

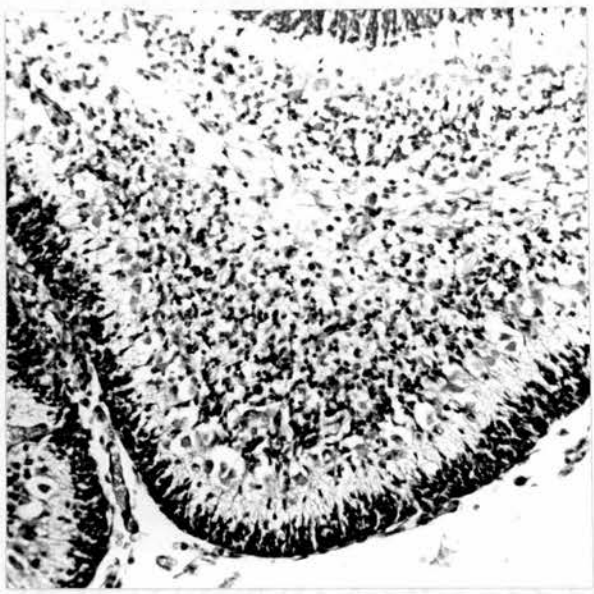


Fig. 41 Cellular necrosis in all layers of the cerebellum of an infant rat that was moribund at 7 days after i.p. inoculation with LI.31.M5. H. & E. X165.

Fig. 42 Inflammation associated with the injection site in a young adult rat that was clinically normal when killed at 14 days after i.c. inoculation with LI.31.M5. H. & E. X165.

found fairly consistently from day 7 in the i.p. inoculated newly weaned rats (Fig. 40), and similar changes were seen in young adults that had been given virus i.c.. These lesions were randomly distributed throughout the C.N.S. and tended to resolve with time.

There was a rather bizarre inflammatory reaction in the region of the i.c. injection site in 4 of the young adults (Fig. 42). The discrete nature of this focus of gliosis and vasculitis might indicate that the infectious process in nervous tissue was largely localized to this area. Lesions of this type were not seen in 3 i.c. inoculated controls and have not been observed in any other species.

Thus, following i.c. inoculation, the resistant weaned rat may show slightly different neuropathological lesions from those seen in the susceptible weaned hamster. However the pathology of louping-ill encephalitis in i.p. inoculated rats is very similar to that seen in the hamster. The disease is, if anything, slightly more acute in infant rats than in infant hamsters.

(2) Histological Examination of I.C. Inoculated Guinea Pigs and Rabbits

Both guinea pigs and rabbits are known to be resistant to i.c. inoculation with louping-ill virus (Hurst, 1931; Greig et al, 1931; Casals and Webster, 1944; Pogodina, 1964), but neuropathological examination of these species has not been reported.

Three 4 day old guinea pigs and three 4 day old rabbits were injected i.c. with a 10^{-2} dilution of re-constituted LI.31.M5. Another guinea pig and rabbit were given control material. All animals remained clinically normal and were killed for histological examination on day 21. Very slight

inflammatory lesions, giving encephalitis scores of 2, were found in brain sections from 2 infected guinea pigs. No neuropathological changes were detected in any of the other 6 animals.

Guinea pigs are very mature at birth when compared with mice, hamsters and rats, all of which were at least susceptible as infants. Even so guinea pigs are not completely resistant to louping-ill, for they develop significant levels of viraemia following both i.c. and s.c. challenge (Pogodina, 1964). The rabbit is, however, relatively immature at birth, and the lack of any discernable response in i.c. inoculated infants indicates that it is the most resistant of the species studied.

IV. STUDIES IN SHEEP

(1) The Susceptibility of Sheep and Lambs to Inoculation by Different Routes and the Distribution and Severity of Neuropathological Changes

The route of inoculation was important in determining the severity of neuropathological changes in clinically affected weaned C57 black mice. Severe lesions were seen in i.c. inoculated mice, but no histological changes were detected in 75 per cent. of those given virus i.p.. However the fairly slight lesions found in subclinically affected i.p. inoculated weaned hamsters were similar to those in moribund hamsters that had been injected i.c.. Infant hamsters and rats developed a fatal necrotizing encephalitis following i.p. inoculation, whereas newly weaned animals showed no symptoms and developed only slight inflammatory changes. The relationships between age, route of inoculation, susceptibility and lesion severity vary considerably between rodent species and no general principles can be promulgated. The interaction between these variables in the sheep has not been previously investigated.

Sixteen 9 month old sheep and 8 three day old lambs were inoculated i.c. or s.c. with one of 2 recent isolates (SB.526 or SB.527) of louping-ill virus. The 6 groups of 4 animals used and the identification numbers of individuals are given in Fig. 43. Sheep inoculated i.c. received 9.5×10^5 mouse infectious doses of SB.527 or 1.9×10^6 mouse infectious doses of SB.526. The lambs were given 2.5×10^6 mouse infectious doses of SB.526 i.c.. Inocula administered s.c. contained 10 times these amounts of virus. Another 4 sheep and 4 lambs were injected i.c. or s.c. with control material. The virus infected animals were killed when moribund or at 20 days after

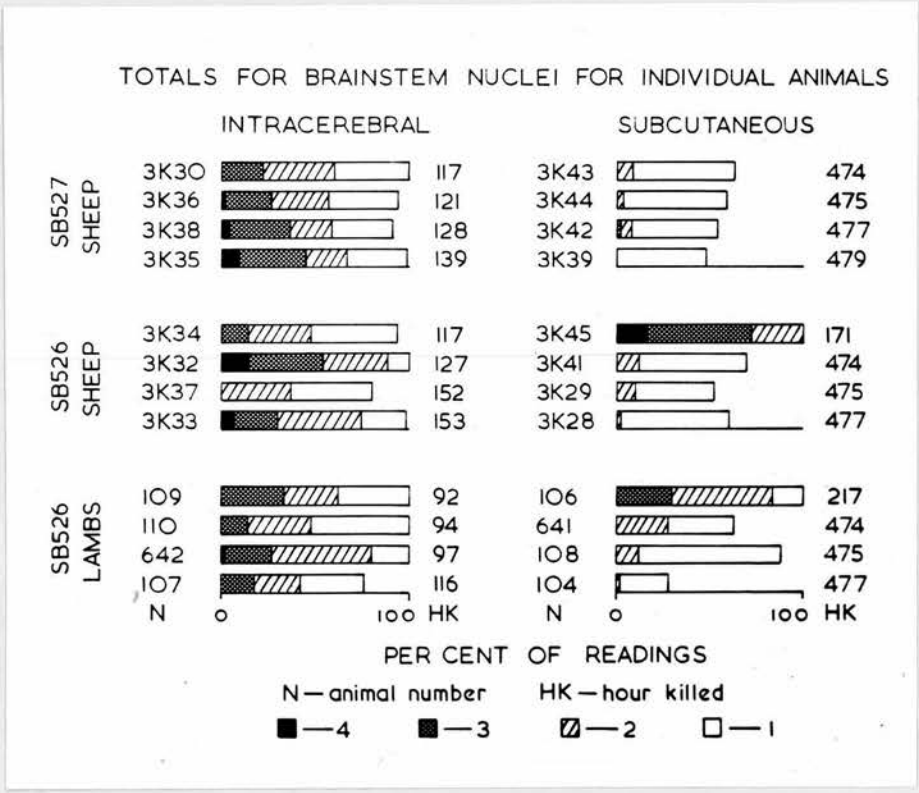


Fig. 43 The severity of encephalitis in individual sheep and lambs that had been inoculated i.c. or s.c. with SB.526 or SB.527. These values were calculated by summing the lesion grades for all of the brainstem nuclei identified in Fig. 44. The lesion grading system is explained on pages 9 and 10.

inoculation, and the controls were killed at comparable times.

There were no symptoms in any of the 8 controls and the disease was subclinical in 6 of the animals that were inoculated s.c. with virus. Non-fatal neurological symptoms were seen in 4 other s.c. inoculated animals. Sheep 3K41 developed a permanent non-progressive posterior paresis from day 12 and 3K28 was slightly ataxic from days 8 to 10, as was 3K43 on days 11 and 12. Lamb 641 was ataxic from days 10 to 15.

The remaining sheep (3K45) and lamb (106) that were inoculated s.c. and all 12 animals that were given virus i.c. were killed when moribund. The incubation times are given in Fig. 43. Typically there was a 4 to 6 day incubation period, then a progression of symptoms from slight ataxia to complete flaccid paralysis over a 6 to 18 hour interval. There was a significant difference in the incubation times ($p<02$) of sheep and lambs inoculated i.c. with SB.526, but there was no difference ($p<50$) between sheep given SB.526 or SB.527 i.c..

Lesions were found in the brains of all animals that had been inoculated with virus, including the 10 surviving to day 20. The severity of neuropathological changes in individuals is compared in Fig. 43; these values were estimated by summing the lesion grades (p. 9) for all the brain-stem nuclei (listed in Fig. 44) of each animal. The most severe damage was seen in the sheep (3K45) that succumbed to s.c. inoculation and there were marked changes in the other 13 animals that developed fatal neurological symptoms.

The distribution and severity of neuropathological changes in i.c. inoculated animals are given in Fig. 44. Slight mechanical damage was

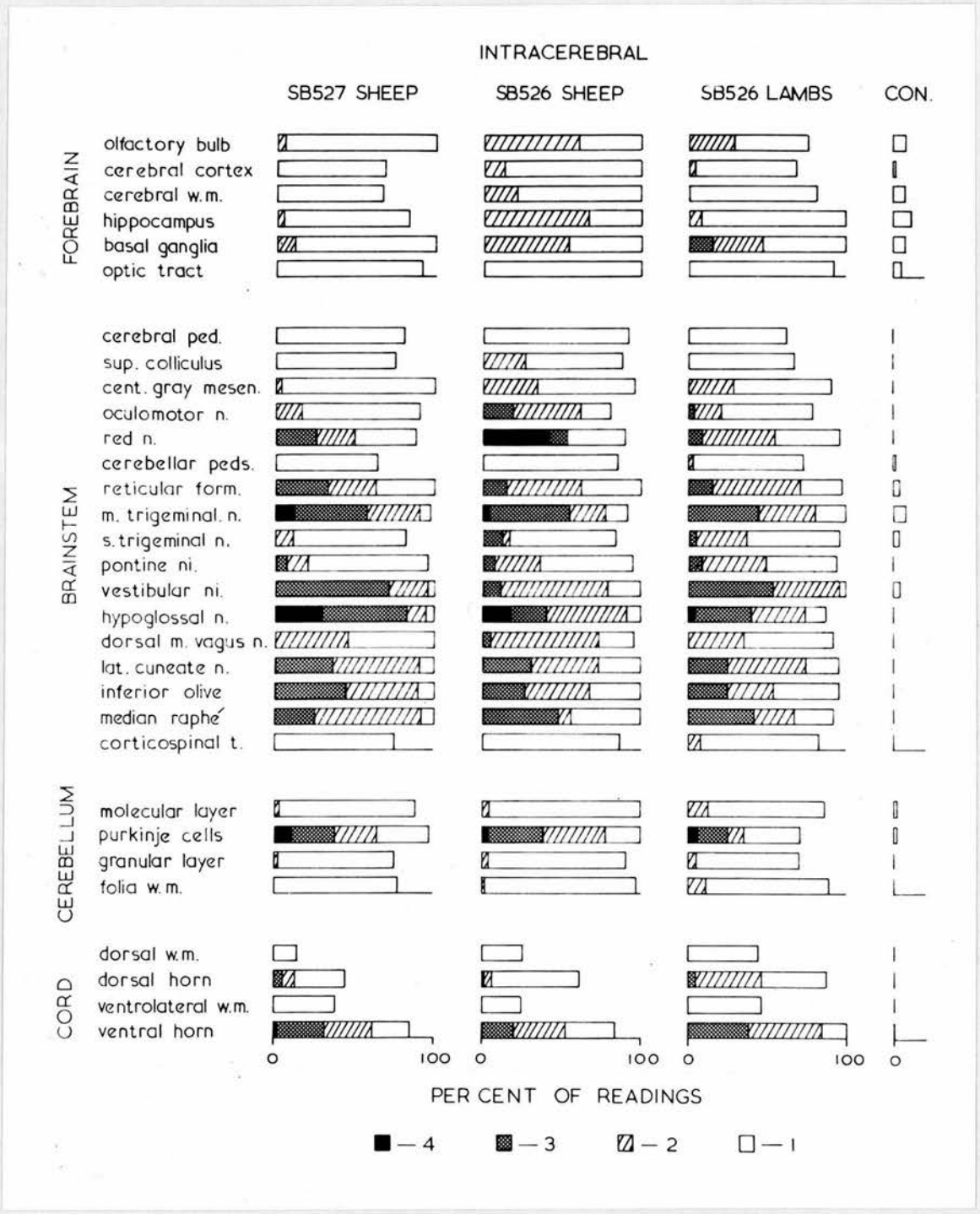


Fig. 44 The distribution and severity of lesions in groups of 4 i.c. inoculated sheep and lambs. The lesion grading system is explained on pages 9 and 10.

w.m. = white matter
ped.(s) = peduncle(s)
m. = motor
n.(i) = nucleus(ei)
mesen. = mesencephalon
s. = sensory

found to be associated with the injection site in the forebrain, as is apparent in the readings from the controls. The distribution and severity of lesions was essentially similar in all 3 groups of virus inoculated animals. Changes in white matter were relatively slight and the most severely damaged sites were the motor nuclei in the brainstem, the vestibular nuclei, the Purkinje cells of the cerebellum and the ventral horns of the spinal cord. Typical severe inflammatory and neuronal lesions in the red nucleus are illustrated in Figs. 45 and 46.

The pattern of lesion distribution in the brainstems of i.c. inoculated animals was comparable to that observed in the 2 (3K45 and 106) that succumbed to s.c. inoculation (susceptibles, in Fig. 47). The inflammatory reaction in these 2 cases was more severe and diffuse (Figs. 48 to 51) than that observed in any other animal. Isolated inflammatory foci were seen throughout the brains and spinal cords of all virus inoculated sheep and lambs that survived to day 20 (Fig. 47). Glial foci in these survivors were often associated with necrotic neurons (Figs. 52 and 53).

The cerebrum was the only major region of the brain where pathological changes in white matter (Fig. 54) were as marked as those in gray matter (Fig. 55). This was apparent in animals inoculated by either route, and the lesions were essentially inflammatory in all sites.

Estimates of lesion severity in the ventral horn at various levels of the spinal cord are given in Fig. 56. In i.c. inoculated animals there was a tendency for the cervical cord to be more severely damaged than the more caudal areas. This was also apparent in sheep inoculated s.c. with SB.526. The ventral horn lesions in animals surviving to day 20 were more marked in

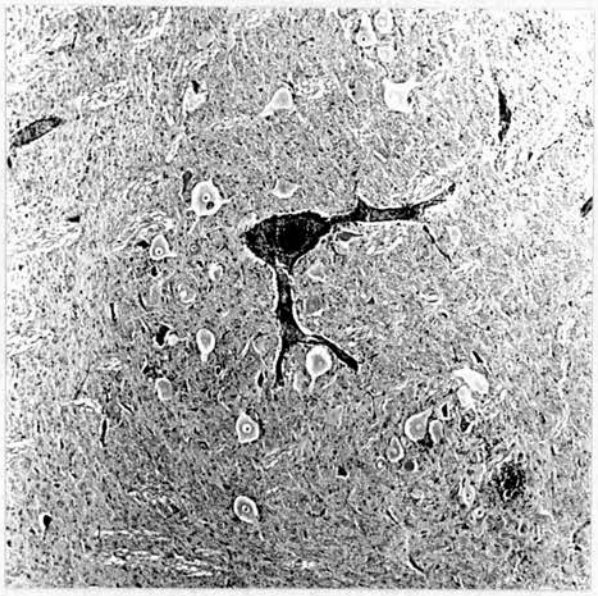


Fig. 45 Neuron necrosis and inflammation in the red nucleus of lamb 642. H. & E. X48.

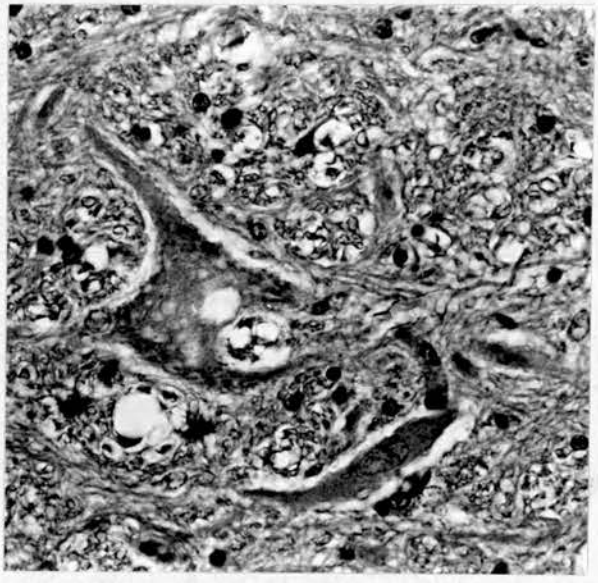


Fig. 46 Necrotic neuron with fragmented nucleus and "foamy" cytoplasm in the red nucleus of sheep 3K33. H. & E. X440.

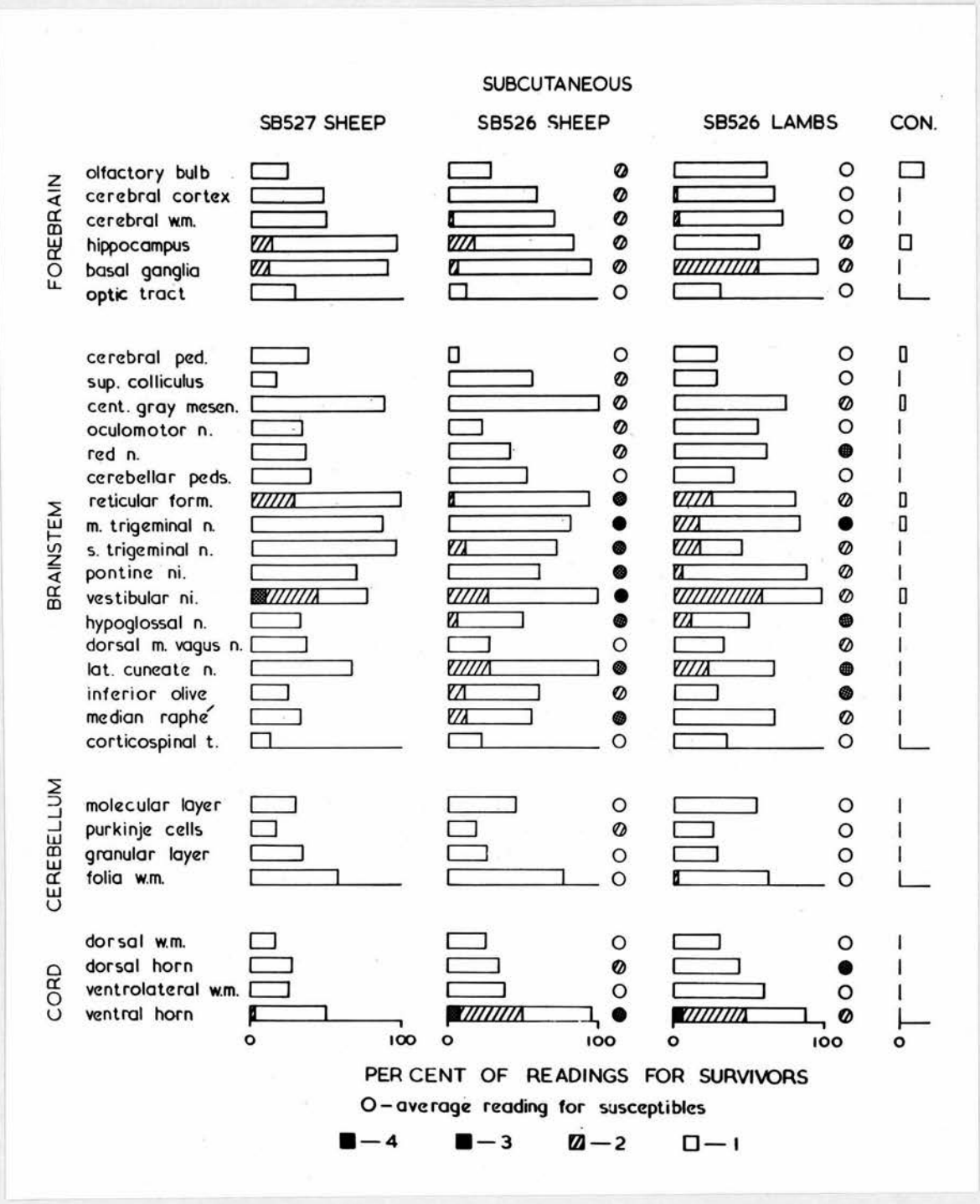


Fig. 47 The distribution and severity of lesions in groups of 4 s.c. inoculated sheep and lambs. The method of expressing these results is explained on page 10.

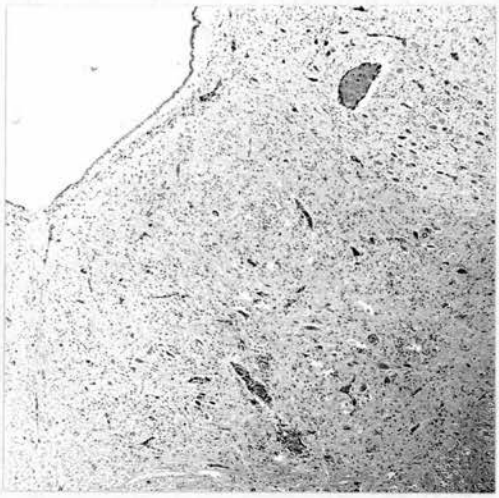


Fig. 48

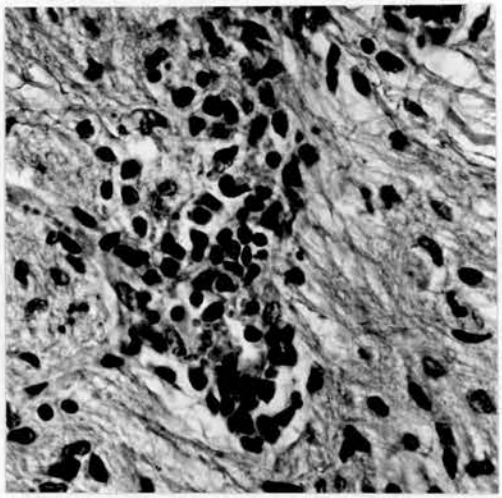


Fig. 49

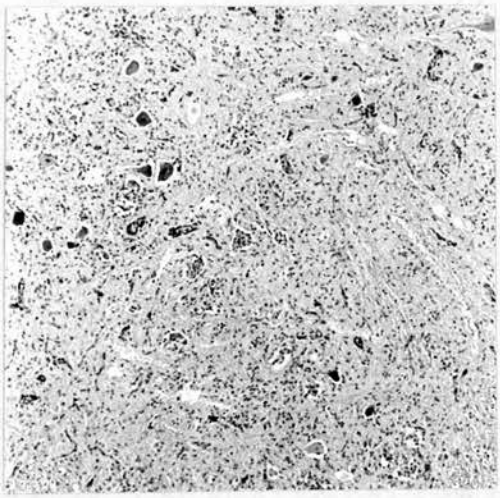


Fig. 50

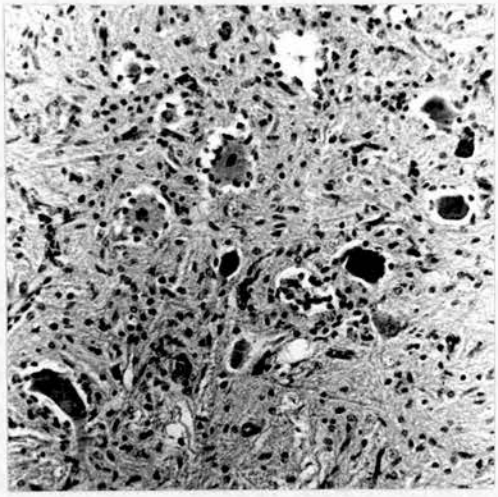


Fig. 51

Figs. 48 to 51 Neuronal and inflammatory lesions in a sheep and lamb that succumbed to s.c. inoculation with SB.526.

Fig. 48 The hypoglossal and dorsal motor vagus nucleus of sheep 3K45. H. & E. X33.

Fig. 49 Perivascular cuffing with mononuclear cells in the medulla of sheep 3K45. H. & E. X440.

Fig. 50 Severe acute lesions in the ventral horn of lamb 106. This lesion was given a grade of 4. H. & E. X48.

Fig. 51 Acute neuron necrosis and neurophagia in the ventral horn of lamb 106. H. & E. X130.

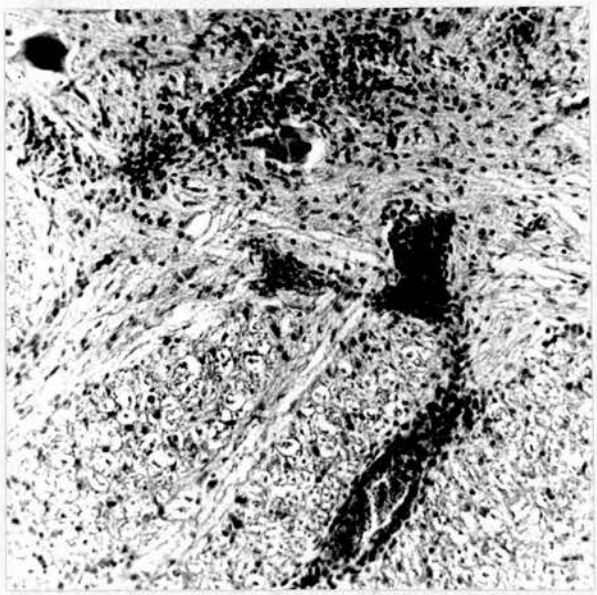


Fig. 52 Inflammation and neurophagia in the ventral horn of lamb 108, which had no neurological symptoms and was killed on day 20. This lesion was given a grade of 2. H. & E. X130.

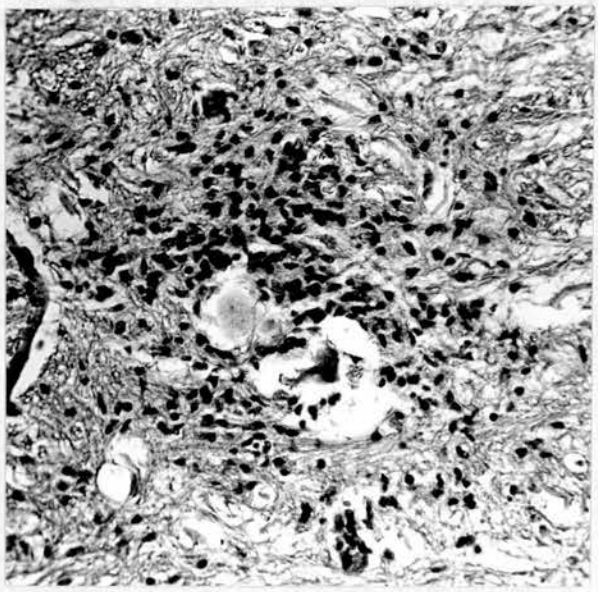


Fig. 53 Inflammation around an effete neuron in the reticular formation of sheep 3K28, which was ataxic from 8 to 10 days after inoculation and was clinically normal when killed on day 20. H. & E. X260.

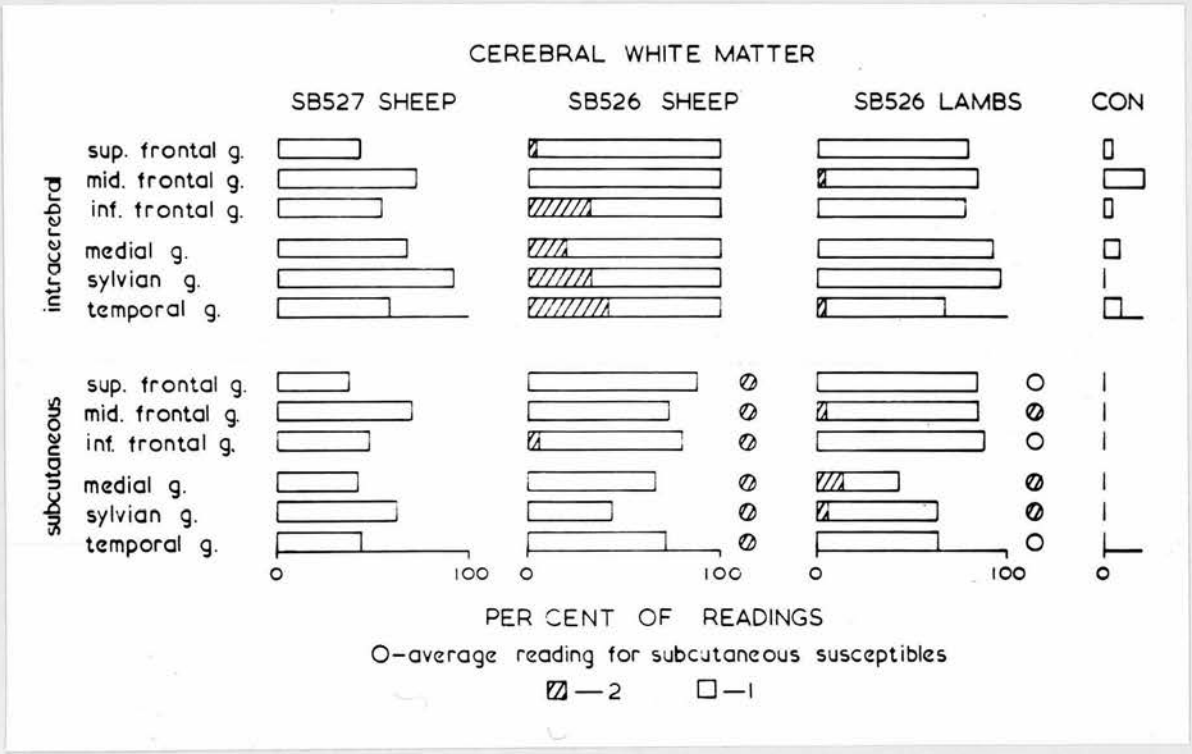


Fig. 54 **The distribution and severity of lesions in cerebral white matter of i.c. and s.c. inoculated sheep and lambs.**

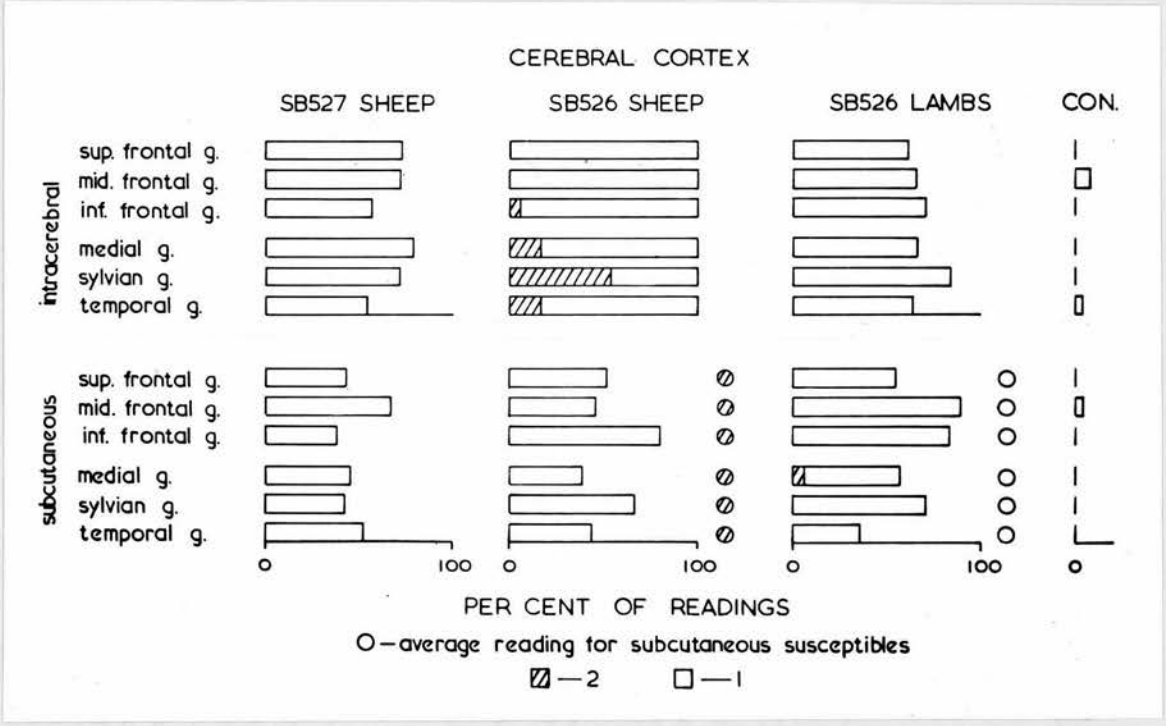


Fig. 55 **The distribution and severity of lesions in the cerebral cortex of i.c. and s.c. inoculated sheep and lambs.**

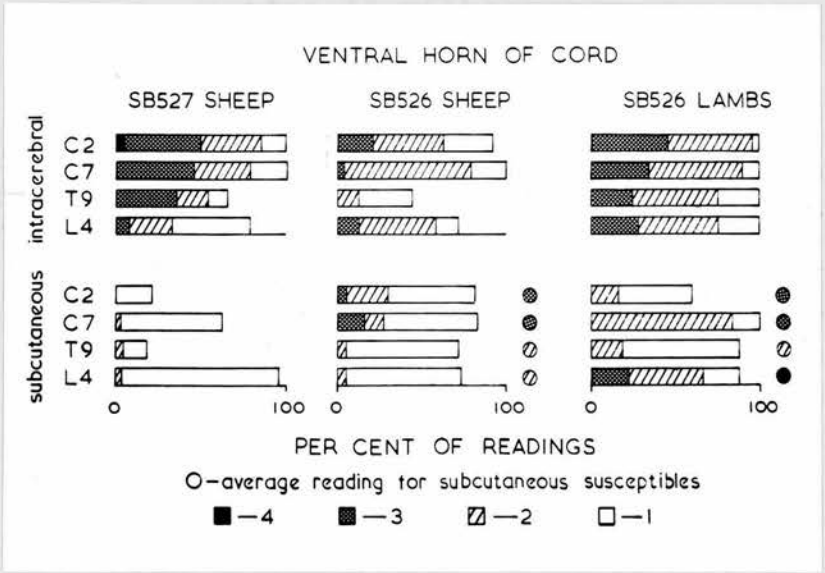


Fig. 56 **The severity of lesions in the ventral horn at different levels of the spinal cord of i.c. and s.c. inoculated sheep and lambs.**

sheep given SB.526 (2 of 3 with symptoms of ataxia or posterior paresis) than in animals inoculated with SB.527 (1 of 4 with symptoms of ataxia).

The Purkinje cells of i.c. inoculated animals were most severely damaged in the ventral parts of the cerebellum (Fig. 57). Only very slight inflammatory lesions were found in the Purkinje cell layers of animals that survived, and in the lamb that succumbed to s.c. inoculation.

Thus susceptibility and the severity of neuropathological changes in the sheep were not obviously affected by the age of the host. Slight generalized histological changes were detected consistently in the C.N.S. of animals that had no neurological symptoms, or only minimal clinical involvement. Marked lesions were found in all moribund animals and the severity of neuronal damage appeared to be largely independent of the route of inoculation, though the inflammatory reaction was more severe in the 2 given virus s.c.. The overall distribution of lesions was essentially similar to that seen in 5 natural cases that were examined (Appendix 1).

(2) Aspects of the Disease in S.C. Inoculated Sheep

In the previous experiment 2 of 8 sheep and lambs inoculated s.c. with the SB.526 virus isolate developed an acute fatal disease, 3 had non-fatal neurological symptoms and the remaining 3 were completely unaffected. There was some indication that clinical susceptibility was related to the rate of production of IgG class H.I. antibody to louping-ill virus (Reid and Doherty, in preparation). This immunological hypothesis could be investigated further by inoculating a fairly large number of sheep s.c. with the SB.526 isolate. Significant groups of animals would reasonably be expected to develop a range of symptoms, from fatal disease to slight fever

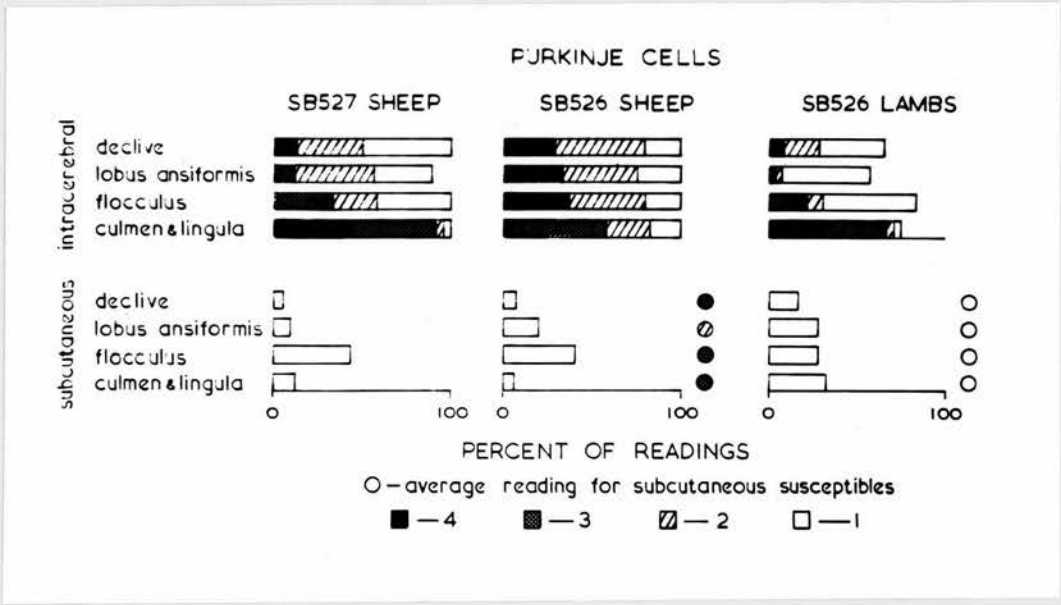


Fig. 57 **The severity of Purkinje cell damage in different lobes of the cerebellum of i.c. and s.c. inoculated sheep and lambs.**

with no obvious neurological involvement, which might correlate with variations in the immune response. Such an experiment would also provide a range of material for neuropathological examination.

Thirty-five 6 month old B.F. sheep were each inoculated s.c. with 7.5×10^5 mouse infectious doses of the SB.526 virus isolate. Fatal symptoms were seen in 23 of these 35 animals (Fig. 58). The high incidence (66 per cent.) may have been related to the daily withdrawal of 30 ml. of blood from each sheep (for virological and serological investigations). In the previous experiment, where there was a 25 per cent. incidence of fatal disease, the usual daily blood sample was 2 ml. and the maximum was 10 ml.. A further 8 sheep that were bled daily (30 ml.) after s.c. inoculation with control material remained clinically normal.

Nervous tissue from moribund, surviving and control animals was processed for examination by a range of techniques. These included, conventional neuropathology, anti-louping-ill and anti-IgG immunofluorescence, acid phosphatase and thiamine pyrophosphatase histochemistry, and electron microscopy. Investigations using enzyme histochemistry and the electron microscope were confined to the spinal cord. This material is still being examined at the time of writing. The limited results presented are from; a randomly selected sample of moribund infected animals from days 7 and 9; the 7 (including 2 controls) whose lumbar spinal cords were perfused for electron microscopy; all of those killed with atypical symptoms; and 2 animals that survived without obvious neurological involvement. There are no results for the sheep found dead on day 28 (Fig. 58), for there was at least a 12 hour delay from death to autopsy.

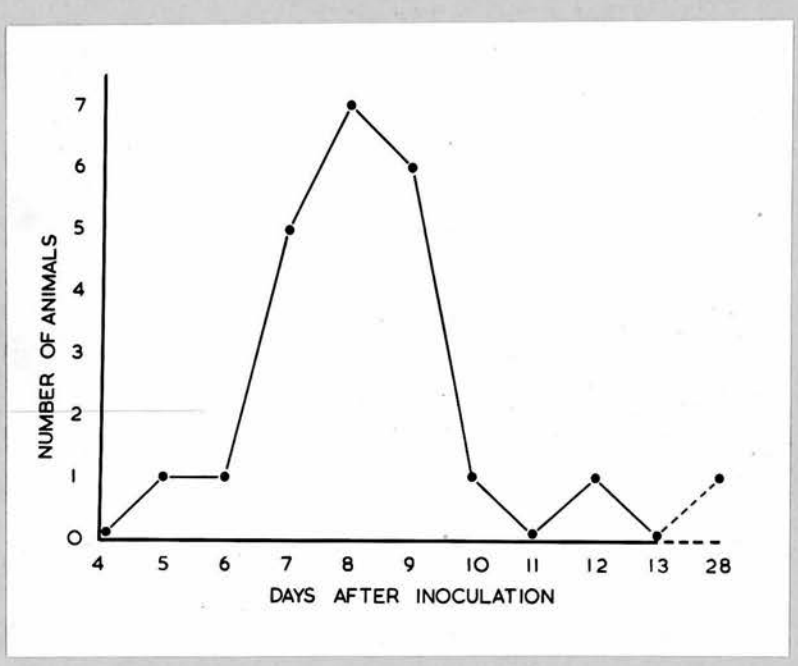


Fig. 58 The incidence of fatal louping-ill in 35 six month old sheep that had been inoculated s.c. with SB.526. In all, 23 of the animals succumbed.

(a) The distribution of anti-louping-ill immunofluorescence and neuropathological changes in sheep with hyper-acute, acute and non-fatal symptoms

The abdominal cavity of the sheep that was moribund on day 5 (Fig. 58) contained copious amounts of sero-sanguineous peritoneal fluid. Pasteurella haemolytica was isolated both from this fluid and the abdominal viscera. There were no obvious neurological symptoms or neuropathological lesions, and fluorescent neurons could not be detected following staining with the anti-louping-ill conjugate. Systemic pasteurellosis was not implicated again, by either bacteriological or morphological criteria, in any subsequent case.

The sheep that died on day 6 (Fig. 58) had no symptoms when last seen at 5 or 6 hours before death. The carcase appeared normal at post mortem and no lesions were detected on neuropathological examination, though a few isolated fluorescent neurons were seen in the pons. This animal had the highest level of viraemia (over 10^8 infectious particles per ml. of blood cf. 10^4 in the previous case, H. W. Reid pers. comm.) found in any of the experimental sheep. In this case louping-ill was apparently a hyper-acute fulminating disease, with death preceding significant C.N.S. involvement.

Fluorescent neurons and neuropathological changes were demonstrated in all 13 moribund sheep examined from days 7, 8, 9 and 12 (Fig. 58). Specific anti-louping-ill fluorescence in cryostat sections could only be definitely identified in neurons and neuronal processes (Fig. 59) and no evidence was found for infection of glia or endothelial cells. The apple-green staining was confined to the neuronal cytoplasm, often in a rather "sponge-like" pattern (Fig. 59, a, b) which corresponded to the "foamy" changes seen previously in some damaged cells (Fig. 46). An earlier stage in the disease

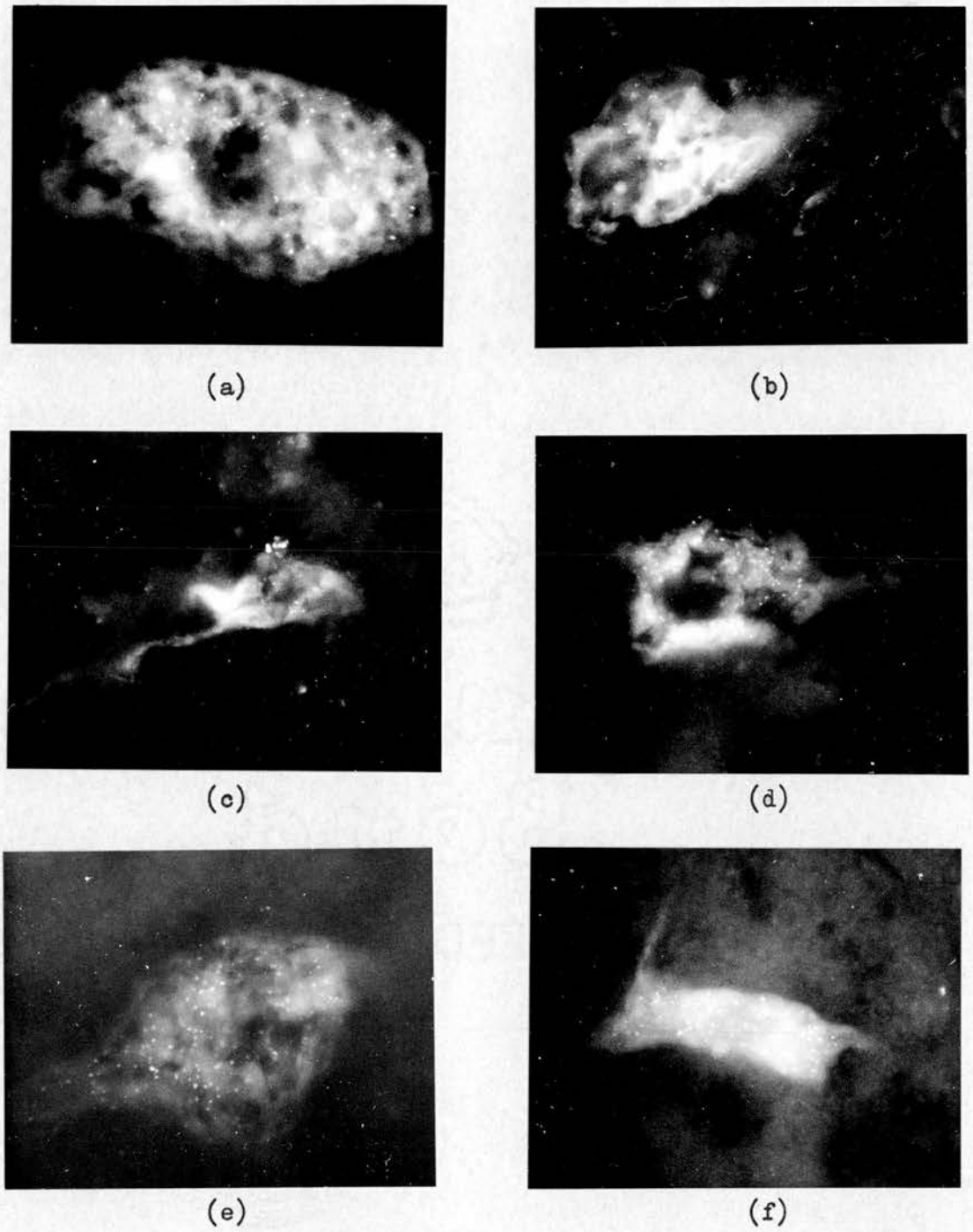


Fig. 59 Fluorescent neurons in the pons of sheep 3N24 which was moribund at 7 days after s.c. inoculation with SB.526. The absence of fluorescence from the nucleus is apparent in (a) and (d), and fluorescence is seen in neuronal processes in (c), (d) and (f). Cryostat sections, anti-louping-ill conjugate X720.

process may be represented by less-intense fluorescence (Fig. 59, e), whereas a few cells with fairly strong dark green homogeneous staining (Fig. 59, f) may be at a late stage of infection.

The distribution of fluorescence in cryostat sections from one half of the brain corresponded fairly well to the pattern of pathological changes in paraffin sections from the other half of the brain (Tables 15, 16 and 17). The most consistent sites for detection of both strongly fluorescent neurons and severe histological lesions were the pons and the ventral horns of the spinal cord. Evidence of widespread neuronal infection was also found, though less consistently, in all cell types in the cerebellar cortex. There were few fluorescent cells and only slight histological changes in the cerebral cortex. The fluorescence that was seen in the cerebrum tended to be focal and may, therefore, have been detected in other cases if more blocks had been examined.

Only a few slightly fluorescent neurons were seen, in the ventral horn, of the animal that was moribund on day 12 (Table 17) and there was no fluorescence in any of the 4 surviving animals killed from days 28 to 30 (Table 18). There were fairly mild neuropathological changes in all 5 cases.

The results of this limited study of the distribution of lesions and anti-louping-ill fluorescence in s.c. inoculated sheep were basically in accord with the semiquantitative neuropathological data recorded previously from other sheep and lambs that were infected by i.c. (Fig. 44), s.c. (Fig. 47) or natural exposure (Appendix 1). Neurons containing viral antigen were mainly found in the ventral horns of the spinal cord, the brainstem and the cerebellar cortex. This distribution was reflected in the pattern of

TABLE 15

THE DISTRIBUTION OF ANTI-LOUPING-ILL IMMUNOFLUORESCENCE AND HISTOLOGICAL CHANGES IN SHEEP MORIBUND AT 7 DAYS AFTER S.C. INOCULATION WITH SB.526

		Immunofluorescence*				Histopathology*			
Animal number		3N12	3N16	3N24	3N04	3N12	3N16	3N24	3N04
Cerebral cortex		++	-	-	-	1	2	1	1
Cerebellar cortex	Purkinje layer	+++	-	++++	+	2	2	2	1
	type II Golgi cells	++	-	+++	-	2	2	2	1
	granular layer	+	-	++	-	1	2	2	1
Pons (all neurons)		++++	+	+++	++++	4	2	2	2
Ventral horn of spinal cord		++++	+	++	+++	4	2	2	2

* The grading systems used in Tables 15 to 18 are explained on pages 9, 10 and 14.

TABLE 16

THE DISTRIBUTION OF ANTI-LOUPING-ILL IMMUNOFLUORESCENCE AND HISTOLOGICAL CHANGES IN SHEEP MORIBUND AT 9 DAYS AFTER S.C. INOCULATION WITH SB.526

		Immunofluorescence				Histopathology			
Animal number		3N17	3N09	3N21	3N34	3N17	3N09	3N21	3N34
Cerebral cortex		-	++	+	-	1	2	1	1
Cerebellar cortex	Purkinje layer	++++	++	-	-	2	3	2	1
	type II Golgi cells	+++	++	-	-	2	3	2	1
	granular layer	++	++	-	-	2	2	1	1
Pons (all neurons)		++	++	++	+	3	2	2	2
Ventral horn of spinal cord		++++	++++	+++	++	4	3	4	3

TABLE 17

THE DISTRIBUTION OF ANTI-LOUPING-ILL IMMUNOFLUORESCENCE AND HISTOLOGICAL CHANGES IN MORIBUND SHEEP WHOSE LUMBAR SPINAL CORDS WERE PERFUSED FOR ULTRASTRUCTURAL EXAMINATION

		Immunofluorescence					Histopathology				
Animal number		3N22	3N38	3N29	3N32	3N31	3N22	3N38	3N29	3N32	3N31
Days after inoculation		7	8	8	8	12	7	8	8	8	12
Cerebral cortex		+	+	-	-	-	1	1	1	1	0
Cerebellar cortex	Purkinje layer	+++	+	-	+	-	3	2	3	2	0
	type II Golgi cells	++	+	-	-	-	3	1	3	0	0
	granular layer	++	+	-	-	-	2	2	2	1	0
Pons (all neurons)		++	NE	++	+++	-	2	3	2	3	1
Ventral horn of spinal cord		++++	NE	++	+++	+	4	4	2	4	3

NE = Not examined

TABLE 18

THE DISTRIBUTION OF ANTI-LOUPING-ILL IMMUNOFLUORESCENCE AND HISTOLOGICAL CHANGES IN SHEEP SURVIVING S.C. INOCULATION WITH SB.526

		Immunofluorescence				Histopathology			
Animal* number		3N02	3N10	3N20	3N44	3N02	3N10	3N20	3N44
Days after inoculation		28	29	30	30	28	29	30	30
Cerebral cortex		-	-	-	-	1	0	1	1
Cerebellar cortex	Purkinje layer	-	-	-	-	0	1	0	0
	type II Golgi cells	-	-	-	-	0	1	0	0
	granular layer	-	-	-	-	0	0	0	0
Pons (all neurons)		-	-	-	-	1	1	1	1
Ventral horn of spinal cord		-	-	-	-	2	1	1	2

* Sheep 3N02 and 3N10 remained clinically normal throughout. Both 3N20 and 3N44 had long standing symptoms of ataxia, debility and apparent muscular weakness.

histological damage.

(b) The pathology of the ventral horn neuron

Marked histological changes and strong fluorescence were detected in ventral horn neurons of the majority of moribund sheep (Tables 15, 16 and 17). Blocks of ventral horn from 5 cases (Table 17) were also examined in the electron microscope. However nothing that might reasonably be considered to be a virus particle (as described in infant mice by Smith and Doherty, 1969) has been seen. Nor has anything been observed that is comparable to the unusual membraneous structures found in infant hamster neurons. Some ventral horn neurons contained abnormal amounts of tubular material (Fig. 60) and many appeared to be depleted of normal granular endoplasmic reticulum (Fig. 61). The Golgi apparatus (Fig. 61) was not readily identified in these damaged cells.

Evidence for loss of the Golgi apparatus in necrotic neurons was also found on optical microscopy of sections stained to demonstrate thiamine pyrophosphatase (T.P.P.). The amount of T.P.P. activity was generally considerably reduced (Fig. 62). Loss of lysosomes, as demonstrated by decreased acid phosphatase (A.C.P.) activity, was also apparent in damaged cells (Fig. 63). Occasionally there was very dark A.C.P. staining in the region of the nucleus (Fig. 63, c).

The necrotic ventral horn neurons of moribund sheep appeared to be depleted of some normal cytoplasmic organelles. The virus could not be readily detected by electron microscopy, even in cases where the presence of viral antigen in most ventral horn neurons was demonstrated in the fluorescence microscope.

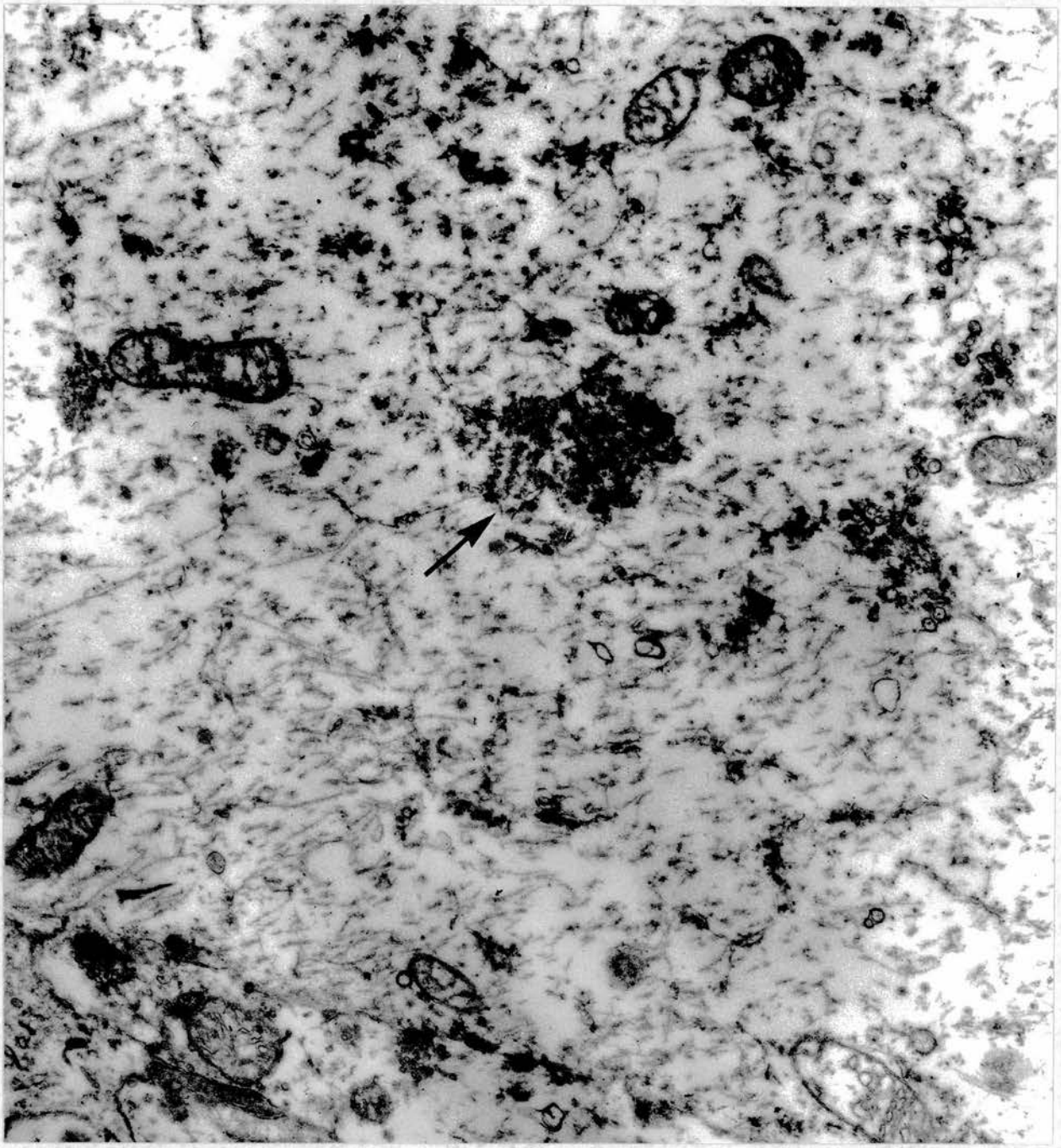


Fig. 60 The cytoplasm of a ventral horn neuron from sheep 3N22. There is an absence of normal granular endoplasmic reticulum, and an accumulation of tubular material (arrow) is apparent. There are no structures that can be definitely identified as virus particles. Lead citrate X30,000.

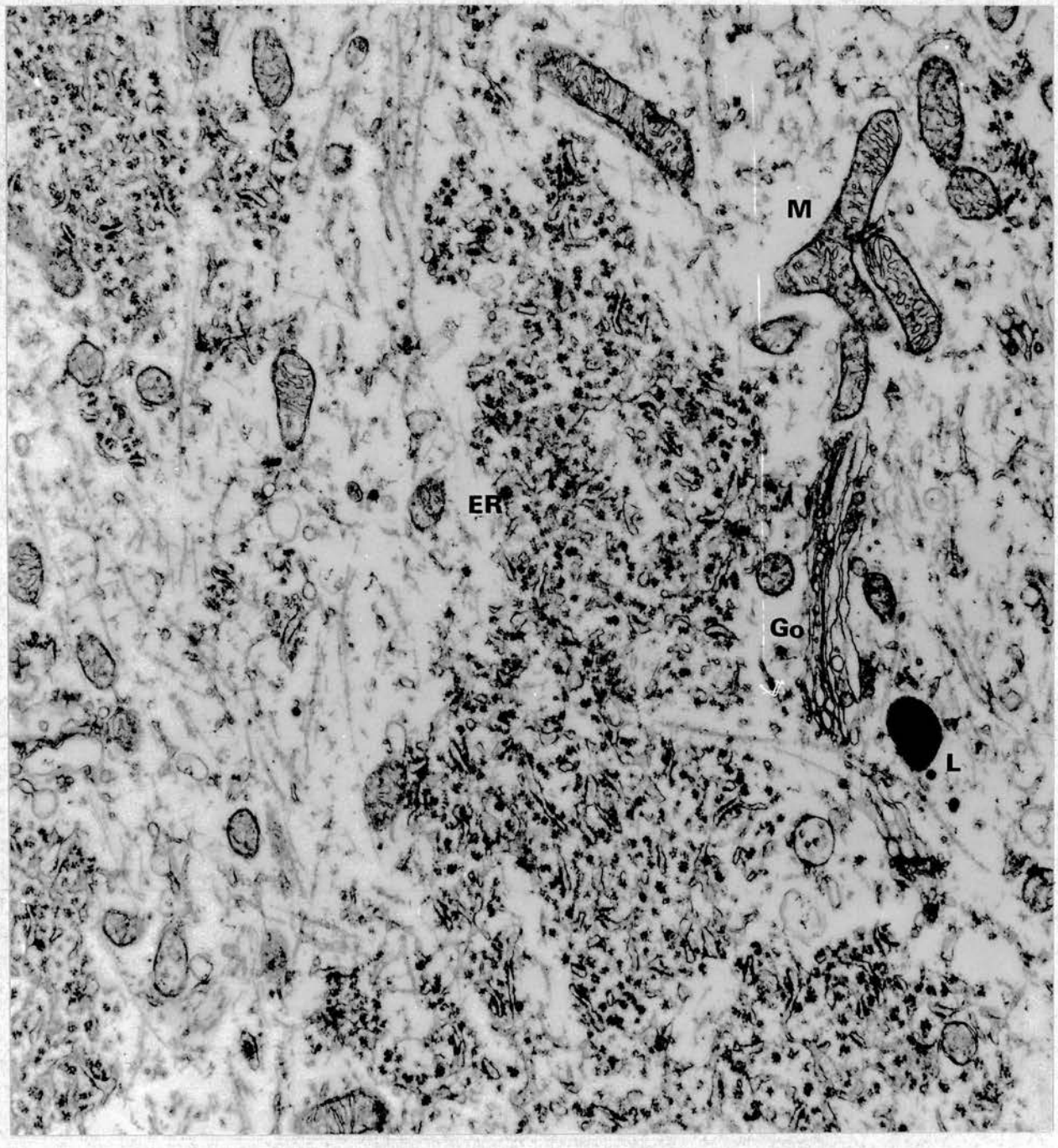
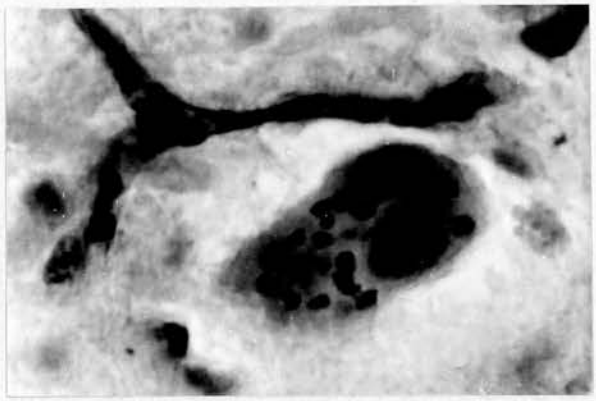
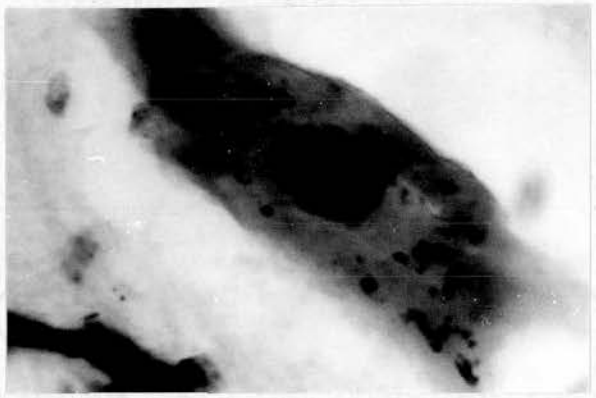


Fig. 61 The cytoplasm of a ventral horn neuron of a normal sheep. A number of structures are apparent including, mitochondria (M), granular endoplasmic reticulum (ER), a lysosome (L) and a Golgi apparatus (Go). Lead citrate 30,000.



(a)



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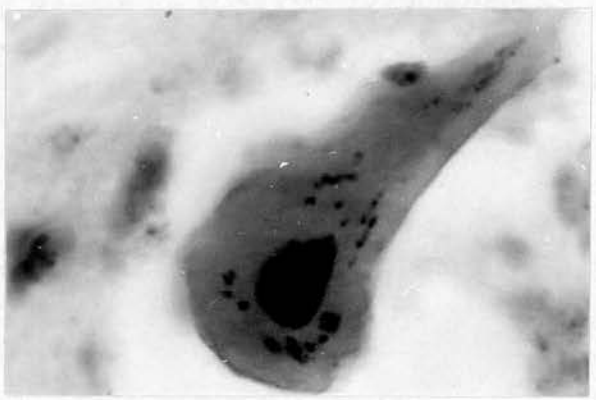
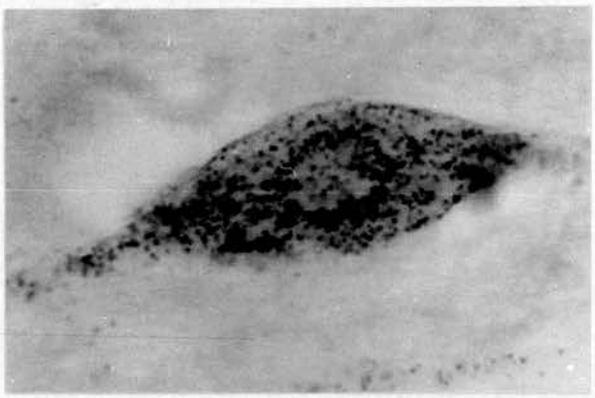
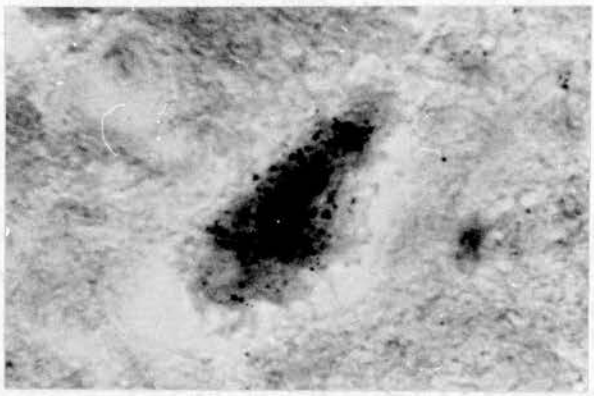


(c)

Fig. 62 Normal Golgi apparatus in a ventral horn neuron of a control sheep (a) and loss and fragmentation of the Golgi apparatus (b) and (c) in sheep 3N21 which was moribund at 7 days after s.c. inoculation with SB.526. Thiamine pyrophosphate X750.



(a)



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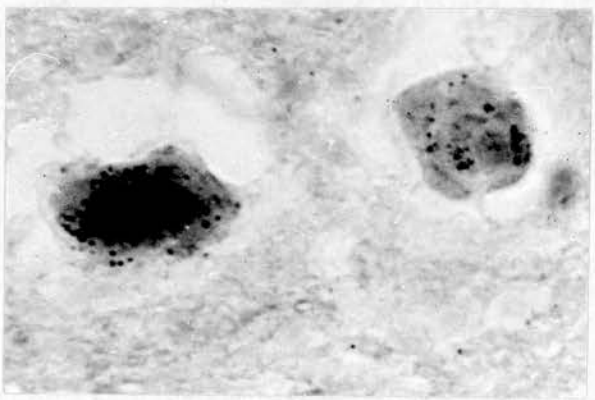


(c)

Fig. 63 Normal distribution of acid phosphatase in the ventral horn neuron of a control sheep (a) and loss of activity in necrotic neurons (b) and (c) from sheep 3N24 which was moribund at 7 days after s.c. inoculation with SB.526. Acid phosphatase X750.

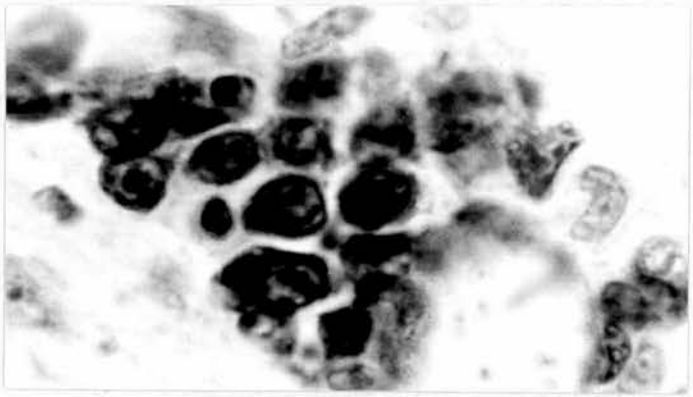
(c) The nature of the perivascular inflammatory reaction

Cells in perivascular cuffs appeared, in methyl green pyronin stained sections, to be predominantly of the mononuclear type (Fig. 64). The characteristic indented nucleus and rather pale cytoplasm of the monocyte was seen in a few cells (Fig. 64, b), but the majority had a more regular nucleus surrounded by cytoplasm with varying degrees of pyroninophilia.

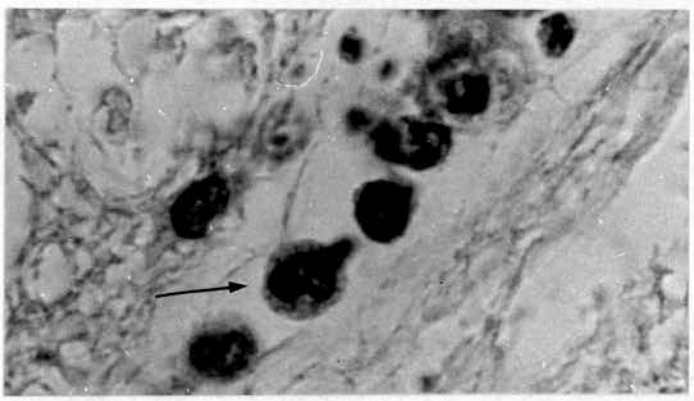
The cytoplasm of many of these cells fluoresced brightly in cold-Carnoy paraffin sections that were stained with the anti-IgG conjugate (Fig. 65). Such fluorescing cells comprised at least 75 per cent. of the constituents of cuffs in most cases (10 of 17) examined, and their incidence was never less than 25 per cent.. The endothelium also tended to fluoresce in both control and infected animals and, in severe acute cases, there was some diffuse staining of nervous tissue. Antigen (IgG) detected in sites other than the cytoplasm of inflammatory cells could be eluted by pre-treatment of unfixed cryostat sections with citrate buffer at pH 3.3 (p. 14).

In Giemsa stained "Araldite" thick sections (Fig. 66) the perivascular inflammatory cells again appeared to be predominantly of the mononuclear type. The nucleus was eccentric in some and most had a more abundant cytoplasm than was seen previously in paraffin sections. Others were probably phagocytes and contained masses of necrotic debris.

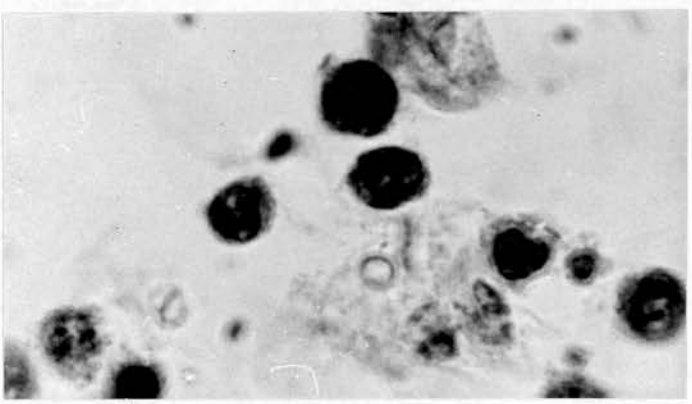
One of these phagocytes is shown, at a greater magnification, in an ultrastructural preparation of an adjacent ultrathin section (Fig. 67). However the majority of the perivascular cells did not appear to contain ingested material. In the cytoplasm of many there were abundant tubular or elongate flattened arrays of granular endoplasmic reticulum, numerous



(a)

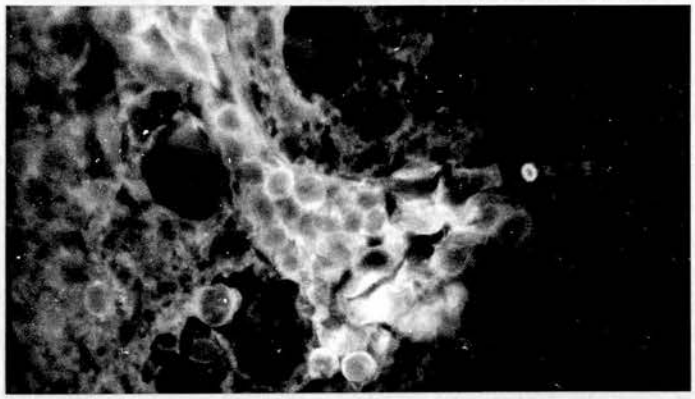


(b)

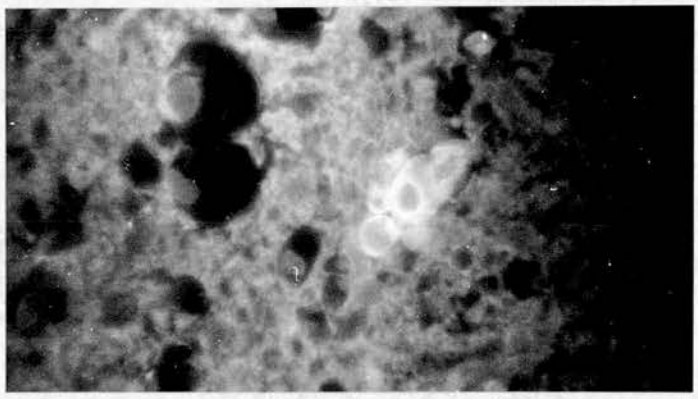


(c)

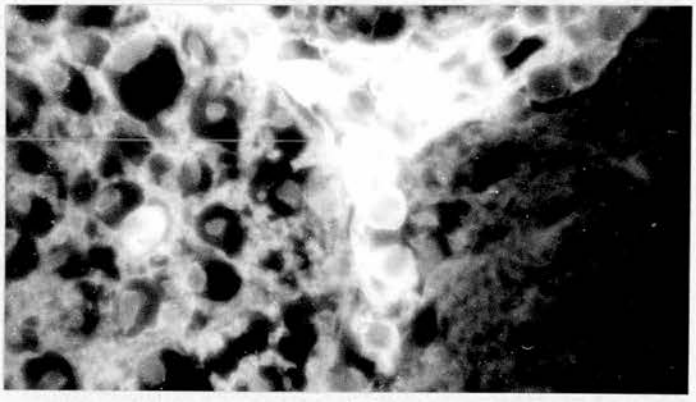
Fig. 64 Inflammatory cells in perivascular cuffs in the spinal cord of sheep 3N22, which was moribund at 7 days after s.c. inoculation with SB.526. The cells are of the mononuclear type and at least one of those shown (arrow) is probably a monocyte. Cold-Carnoy paraffin section X1,200.



(a)



(b)



(c)

Fig. 65 Fluorescing inflammatory cells in perivascular cuffs in the spinal cord of sheep 3N22. The cytoplasm of the majority of inflammatory cells shows strong specific apple-green fluorescence. The small group in (b) are associated with a very small vessel. The bright mass in the middle of (c) is largely due to the blue autofluorescence of red cells in the vessel lumen. Cold-Carnoy paraffin section. Anti-IgG conjugate X720.

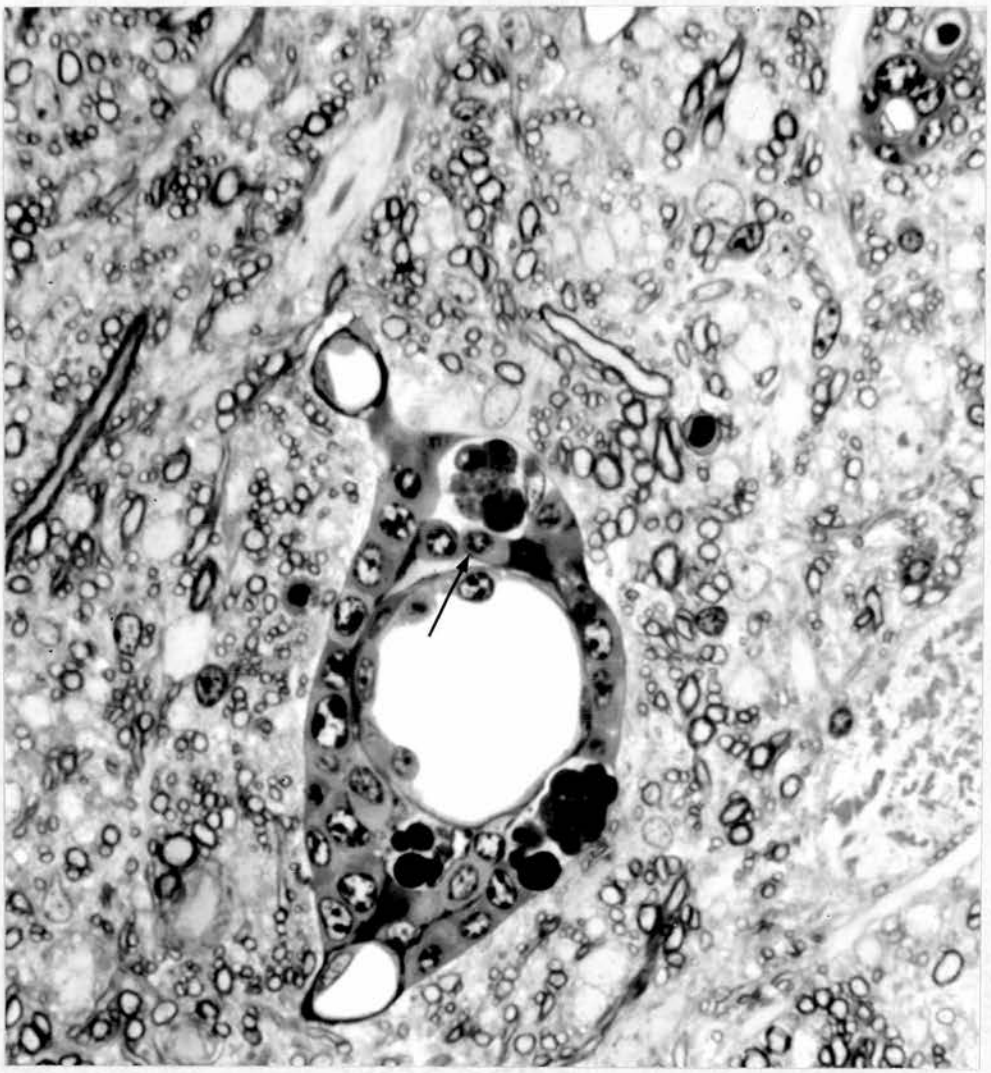


Fig. 66 A large perivascular cuff, surrounding a venule, and a small cuff around a capillary. The infiltrating cells are of the mononuclear type. Some are phagocytes, and in others (arrow) the nucleus is eccentric. "Araldite" 1 μ thick section. Lead citrate X800.

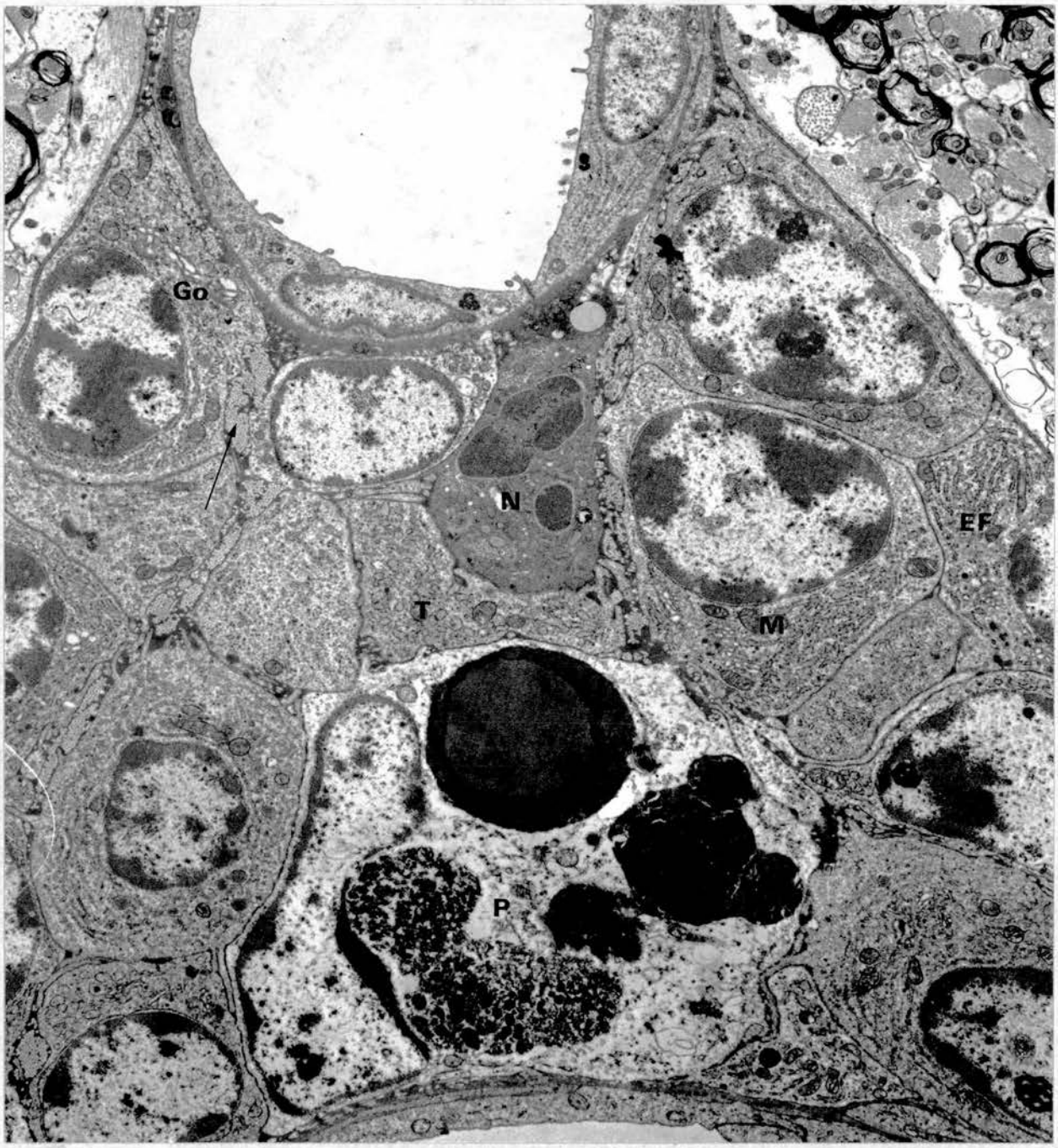


Fig. 67 A perivascular cuff in the ventral horn of sheep 3N38. One of the inflammatory cells is a phagocyte (P) and another (N) may be a neutrophil. The cytoplasm of many of the other cells contains abundant granular endoplasmic reticulum, which seems to be arranged around tubular (T) or elongate flattened (EF) vesicles. Mitochondria (M) and a Golgi apparatus (Go) may also be seen in this type of cell. There is a considerable amount of unstained particulate material (arrow) embedded in an extracellular electron-dense matrix. Lead citrate X9,000.

mitochondria and a Golgi apparatus. Such cells are shown again in Fig. 68 and, at a higher magnification, in Fig. 69.

Similar cells were also seen in the lumen of the venule (Fig. 68) in a poorly perfused area. They apparently leave the circulation either by transport across the cytoplasm of endothelial cells (Fig. 68) or by pushing through at endothelial cell junctions (Fig. 69). Once outside the endothelial barrier the majority seem to remain in the perivascular area, though some may migrate further into the nervous tissue (Fig. 68). Even cells that remain close to the vessel wall cause considerable compression of the surrounding neuropil, and create what is effectively an extracellular perivascular space (Figs. 67 and 69). In normal brain there is no space around vessels of this size (Fig. 70). This extracellular space is largely filled with amorphous electron-dense material in which well defined unstained profiles (Fig. 67) or filaments (Fig. 69) are apparent.

Thus, though some cells in perivascular cuffs are phagocytes, most contain numerous cytoplasmic elongate or tubular vesicles surrounded by granular endoplasmic reticulum. The cytoplasm of the majority of such cells stains brightly with the anti-IgG conjugate, and the antigen (IgG) cannot be readily eluted. The presence of cuffing results in the formation of an abnormal perivascular space.

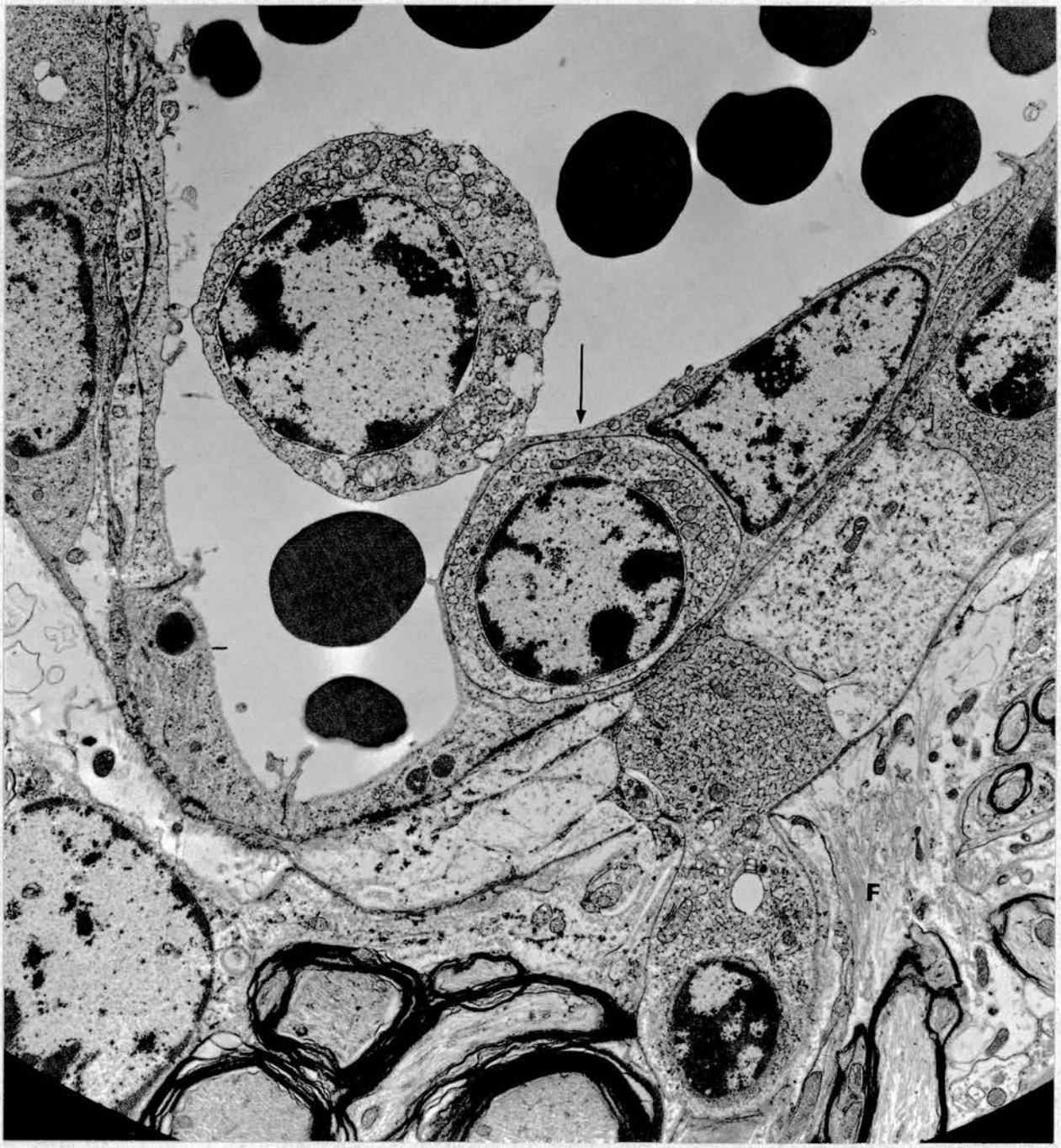


Fig. 68 An inflamed blood vessel in the ventral horn of sheep 3N22. One inflammatory cell is in the lumen of the vessel, another (arrow) appears to be within the cytoplasm of an endothelial cell, while a third seems to be migrating out of the perivascular area by pushing past the foot process of an astrocyte (F). The inflammatory cells contain numerous tubular vesicles surrounded by granular endoplasmic reticulum. Lead citrate X9,000.

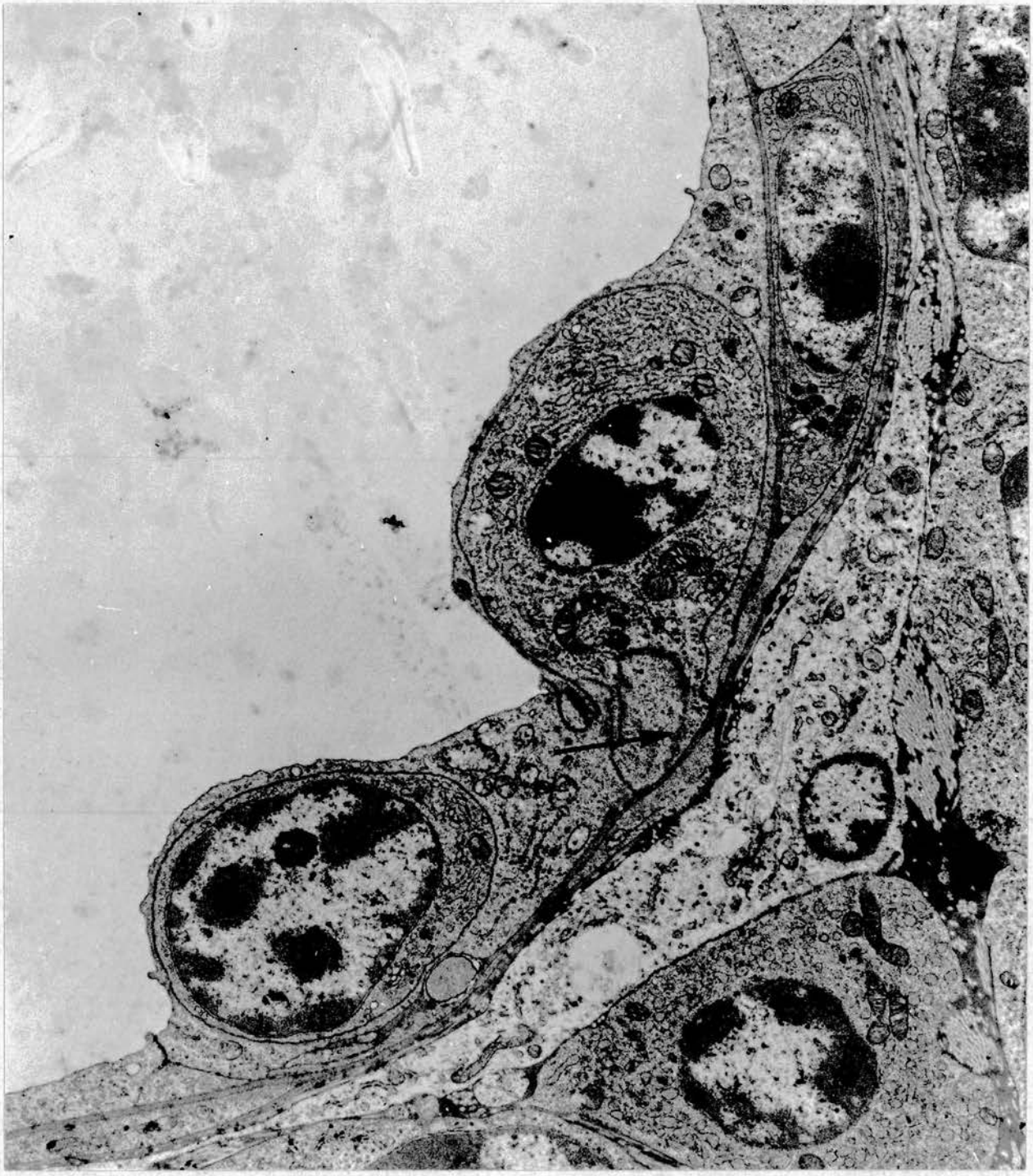


Fig. 69 An inflamed blood vessel in the ventral horn of sheep 3N38. Two inflammatory cells appear to be within the cytoplasm of endothelial cells, though one pole of the larger cell (arrow) may be pushing through at an intercellular junction. Many of the inflammatory cells contain flattened or tubular arrays of granular endoplasmic reticulum. The electron-dense extracellular matrix contains what appear to be unstained filaments. Lead citrate X13,500.

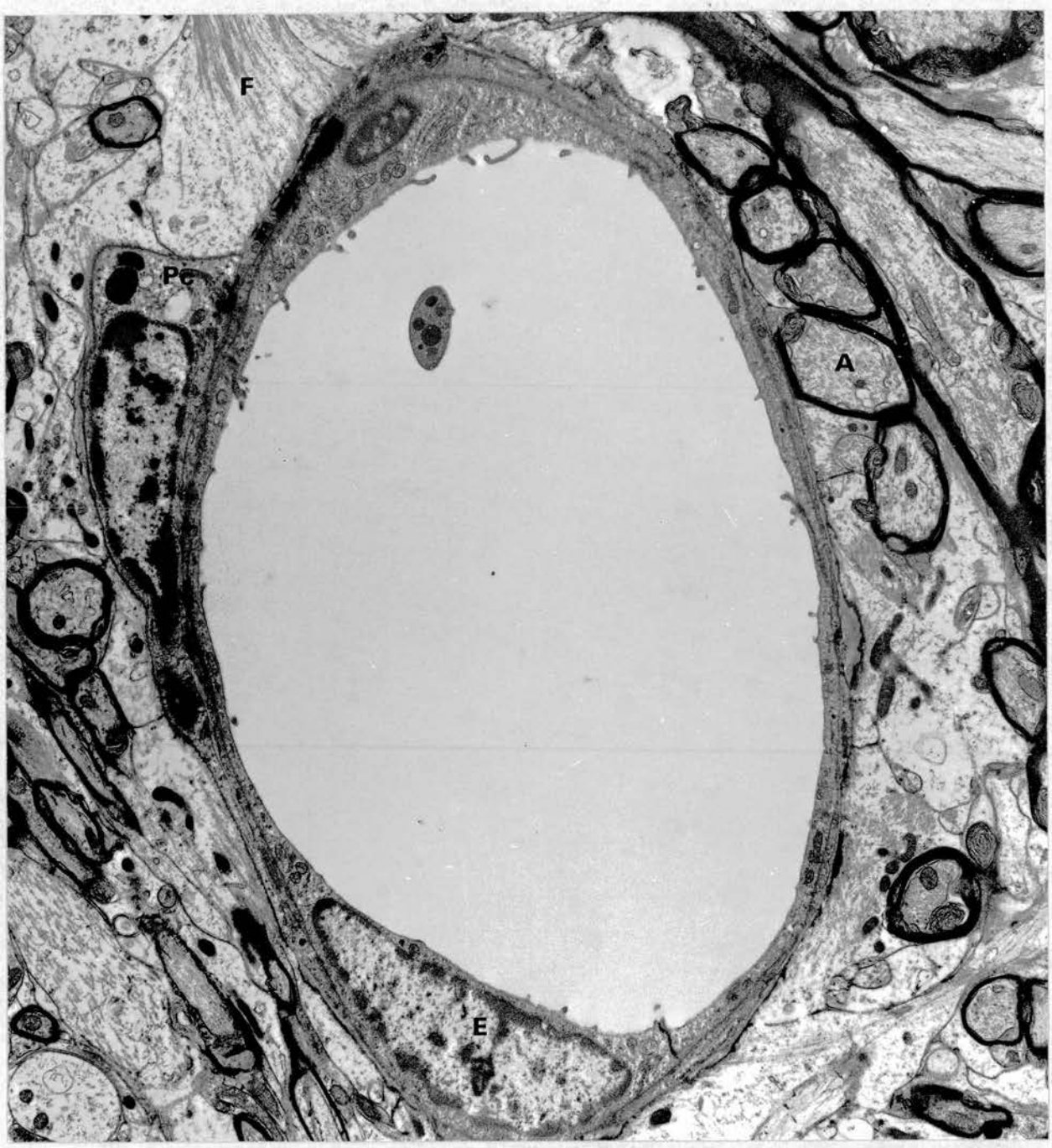


Fig. 70 A small blood vessel in the ventral horn of a control sheep. The perivascular space, seen in other tissues, is almost completely absent and the endothelial cells (E) are surrounded by a pericyte (Pc), foot processes of astrocytes (F) and myelinated axons (A). Lead citrate X9,000.

DISCUSSION

Louping-ill virus was detected in blood of hamsters and mice before being isolated from brain. The duration and intensity of the viraemia may govern the extent of C.N.S. involvement. Higher virus titres were recorded from the blood of susceptible infant hamsters than from resistant large suckling and weaned animals. This would seem to support the idea (Smith, McMahon, O'Reilly, Wilson and Robertson, 1964; Sellers, 1969) that the number of virus particles circulated through the C.N.S. has a direct effect on the degree of subsequent neurological involvement. However in the experiments in sheep and lambs the magnitude of viraemia had no obvious influence on the severity of symptoms (H. W. Reid, pers. comm.), so other factors may be involved.

Viraemia continued until death on day 8 in the infant hamsters but was terminated at about day 6 in lambs and older hamsters, probably by specific circulating antibody (H. W. Reid, pers. comm.; Simon, Slonim and Zavadova, 1966). Infant rodents are unresponsive to antigenic stimuli for the first week or so of post-natal life, whereas the lamb is immunologically competent well before birth (Adinolfi and Wood, 1969). The increased susceptibility of infant hamsters and rats to louping-ill may result from their lack of immunological competence, as was proposed by Richards and Cordy (1967) in similar studies of bluetongue in sheep and mice.

The brains of large suckling hamsters and new-born lambs are at a much later stage of development than that of the infant hamster. A further contributing factor to the severe disease seen in the infants may be that there is increased passage of virus through the immature endothelial barrier. Some substances are known to pass more readily from blood to brain during

times of active growth, but this may be a highly selective phenomenon and cannot be applied as a general principle (Dobbing, 1968). Also the nature of passage may change with age. For instance the enzyme horse-radish peroxidase seems to diffuse through the choroid plexus endothelium of new-born rats, whereas from 5 days of age the substance is transported across the endothelial cells in pinocytic vesicles (Morecki, Zimmerman and Becker, 1969).

There was no definite information on the means by which circulating louping-ill virus passed through the endothelial barrier to enter nervous tissue. Hurst (1936) proposed that virus may "grow through" the endothelial cells of small blood vessels throughout the C.N.S.. However louping-ill virus was not detected in endothelium by either ultrastructural or immuno-fluorescent techniques. There was no histological evidence of vascular damage, and the blood vessels of moribund hamsters and sheep appeared essentially normal in the electron microscope. Endothelial cells are the first cells in brain to be exposed to blood-borne virus. As louping-ill virus causes generalized necrosis in unprotected populations of cells (Burnet, 1936, b; Williams, 1958, a) widespread infection of endothelium could be expected to result in marked endothelial necrosis, that would be apparent by the time symptoms occur. The absence of perivascular haemorrhage suggests that little, if any, virus replication occurs in this site. Necrosis of the endothelium of venules, following replication of influenza virus, resulted in development of severe haemorrhagic encephalopathy (Hook, Luttrell, Slaten and Wagner, 1962).

However it is quite possible that endothelial involvement was not observed because only a few cells became infected. These could easily have

been missed both on fluorescence and electron microscopy, for observations were made at magnifications of X600 and X2,000 respectively and only a limited amount of tissue could be examined. The logical sequel of virus entry at a few scattered points is that lesions in nervous tissue would progress from isolated foci. This was not apparent in either immunofluorescent or histological studies of louping-ill in relatively mature animals, though neuronal damage in infant rodents was initially focal. In immunofluorescent studies with related viruses, infected endothelium has been observed in some experiments (Kundin, Liu, Hysell and Hamachige, 1963; Johnson, 1965) but not in others (Mims, 1964; Albrecht, 1962, 1968). Oyanagi, Ikuta and Ross (1969) could find no ultrastructural evidence of growth of Japanese encephalitis (J.E.) virus (a group B arbovirus) in endothelial cells of adult mice.

According to Johnson and Johnson (1968) growth through endothelium of small blood vessels is the only method of arbovirus entry into brain that has been convincingly demonstrated by fluorescent antibody techniques. However immunofluorescence would not be sufficiently sensitive to detect virus particles that were passing across endothelial cells in pinocytic vesicles, without causing any damage. Pinocytic passage through the endothelial barrier is more common with large than with small molecules (Mayerson, Wolfram, Shirley and Wasserman, 1960; Karnovsky, 1967), and has been shown for electron-dense particles that are smaller than louping-ill virus (Pappas and Tennyson, 1962; Brightman, 1965).

A particle of louping-ill virus released on the brain side of the endothelium of a venule or capillary is faced directly with the plasma

membranes of intact cells. The absence of a significant perivascular space (Maynard, Schultz and Pease, 1957) and the nature of the surrounding tissue are well illustrated in Fig. 70 in the results. Astrocytic processes, principally their end feet, constitute about 85 per cent. of these cellular elements (Maynard et al, 1957). Even so there was no ultrastructural or immunofluorescent evidence that louping-ill virus multiplied in astrocytes. Slight generalized astrocytic hypertrophy has been observed in mice from 2 days after i.p. inoculation with louping-ill virus, but the reason for this was not determined (Zlotnik, 1968).

The pericyte, or pericytal microglial cell (Mori and Leblond, 1969), is also contiguous with the endothelial barrier. Louping-ill virus has not been demonstrated in these cells, or in any other glia. Immunofluorescent detection of viral antigen in unspecified glial cells of rodents infected with group B arboviruses has been reported by some (reviewed by Johnson and Mims, 1968) but not all authors (ElDadah and Nathanson, 1967). The related J.E. virus was observed on electron microscopy of microglial cell cytoplasm by Yasuzumi, Tsubo, Sugihara and Nakai (1964) and Yasuzumi and Tsubo (1965, b), but not by Oyanagi et al (1969). Such virus could either have been phagocytosed following release from necrotic cells, or have resulted from replication within microglia. Growth of louping-ill virus in microglia is feasible, for multiplication to a high titre has been shown in in vitro cultures of sheep lung macrophages (J. T. Vantsis, pers. comm.) and many other viruses are known to replicate in fixed tissue macrophages in visceral sites (Mims, 1964).

The endothelial cells may also be in direct contact with, or very close to, the plasma membranes of nerve cells and their processes. According to

Scharrer (1944) no neuron is more than 50 μ from a capillary. All louping-ill virus demonstrated by immunofluorescence or electron microscopy has been confined to the perikaryons, axons or dendrites of neurons. Viral antigen in axons of sheep and mouse neurons was only detected fairly close to the cell body, whence it may have diffused (Droz and Leblond, 1963). Virus was not found in more remote sites, such as the long tracts of the spinal cord.

Direct infection of the axon by extracellular virus would probably be prevented by the presence of a myelin sheath. The small horse-radish peroxidase molecule (M.W. 40,000) is generally not able to penetrate to the peri-axonal space of myelinated nerve fibres, though there may be minimal entry at the nodes of Ranvier (Hirano, Becker and Zimmerman, 1969). The brain of the young lamb is quite well myelinated at time of birth (Barlow, 1969) and the lamb is no more susceptible to louping-ill than the adult sheep. However infant rats and hamsters consistently develop fatal disease following i.p. inoculation with virus, whereas adults remain asymptomatic. In control hamsters evidence of myelination was not detected until 12 days after birth. Consequently the axons of infants would be quite exposed to entry of virus. Once a virus particle had entered the axoplasm it would need to move against the normal direction of axonal flow in order to reach the cell body where synthesis of protein (Droz and Leblond, 1963), and perhaps virus replication, normally occurs. However such a particle might pass down the axon to the cell body of a post-synaptic neuron.

The most useful information concerning virus replication in neuronal cytoplasm was gained from the ultrastructural study of infant hamster cerebellum. In many neurons numerous small electron-dense bodies, some of

which were surrounded by a less dense rim or "halo", were seen enclosed in abnormal membrane profiles. These bodies were probably mature particles, or virions, of louping-ill virus. The electron-dense centre may represent the nucleoid, or ribonucleic acid core (Zemla, Anderleova and Gresikova, 1968), and the halo may be the capsid, or lipoprotein coat (Fenner, 1968). Similar structures have been observed on electron microscopy of nervous tissue from mice and older hamsters with louping-ill (Smith and Doherty, 1969; Zlotnik and Harris, 1970), and many different types of cells infected with other group B arboviruses (Kovac, Kunz and Stockinger, 1961; Abdelwahab, Almeida, Doane and McLean, 1964; Yasuzumi et al, 1964; Yasuzumi and Tsubo, 1965, a and b; Tikhomirova and Karpovich, 1966; Tikhomirova, Karpovich, Reingold, Levkovich and Shestopalova, 1968; Filshie and Rehacek, 1968; Murphy, Harrison, Gary, Whitfield and Forrester, 1968; Oyanagi et al, 1969).

Most of these workers thought that the surrounding membrane profiles were derived from endoplasmic reticulum, though an origin from mitochondria has also been proposed (Zlotnik and Harris, 1970). There were several layers to the membranes seen in infant hamster neurons, which is more like the normal structure of the mitochondrion than that of the endoplasmic reticulum. However virions were not detected in the lumen of either identifiable mitochondria or endoplasmic reticulum.

The Purkinje cells often contained several membraneous profiles, whereas usually only one was seen in the much smaller granule cells. The apparently undamaged nucleus was sometimes displaced, but there was no obvious distension of the plasma membrane. The normal extent of neuronal cytoplasm placed a physical limit on the amount of virus that could be synthesized in the cell.

In conventional histological preparations the Purkinje cells appeared to be severely affected, but the granule cells seemed relatively normal. Optical microscopy of louping-ill infected material thus tended to over-emphasize the degree of damage to the cell with a large cytoplasm.

The cytoplasm of both infant hamster cerebellar neurons and ventral horn cells from moribund sheep was depleted of normal granular endoplasmic reticulum and appeared rather watery. This was the only major similarity, for membrane profiles and virions were readily detected in the former but were not seen at all in the latter. Obviously, because of great differences in size and spacing, it is much more difficult to examine the sheep cells in the electron microscope. However in some cases most ventral horn neurons were shown, by immunofluorescent staining of comparable cryostat sections, to contain large amounts of viral antigen. This discrepancy may simply reflect that virions are much more likely to be present in a large 8 μ cryostat section than in a small ultrathin ($< 1\ \mu$) section. Even so the possible presence of viral antigen that has not been incorporated into virions cannot be discounted. Such antigen might attach fluorescent antibody, but would be unrecognized in electron micrographs.

Necrosis of sheep ventral horn cells was accompanied by diminution of T.P.P. and A.C.P. staining. T.P.P. activity is normally localized in the Golgi apparatus (Novikoff and Goldfischer, 1961). Typical Golgi apparatus could not be demonstrated on ultrastructural examination of damaged nerve cells. Fragmentation of the Golgi apparatus in severely affected neurons of sheep with louping-ill has been described previously by Gresson and Zlotnik (1947), who used the techniques of Aoyama and Da Fano (Baker, 1945).

The normal site of A.C.P. activity is the lysosome (Pearse, 1968). Diminished A.C.P. staining in virus infected cells probably indicates that lysosomal damage has occurred (Allison and Mallucci, 1965). Release of lysosomal hydrolases into cytoplasm may be, at least in part, involved in the development of cellular necrosis (De Duve, 1964). However it is impossible to determine whether the lysosomal changes, indicated by loss of A.C.P. staining, in sheep neurons were a cause or an effect of damage to the cell. In non-cytopathic infection of human amnion cell cultures Lesso and Mayer (1968) found that there was no alteration in the activities of lysosomal hydrolases. Normal A.C.P. activity was also recorded from the brains of mice with severe encephalitis resulting from i.c. inoculation of louping-ill virus (MacKenzie et al, 1968). Thus lack of lysosomal damage is probably not the reason for the absence of obvious neuron necrosis in 75 per cent. of i.p. inoculated C57 black mice.

There were high titres of virus in all regions of the brains of these i.p. inoculated C57 black mice and many neurons contained viral antigen. Generalized dysfunction, as indicated by appearance of neurological symptoms, occurred before structural changes were apparent on conventional histological examination of virus-infected nerve cells. Johnson (1968) has also reported non-cytopathic infection of neurons in mumps virus encephalitis in the hamster. Neuronal damage was minimal in many of the moribund weaned hamsters that had been inoculated i.c.. Again onset of symptoms was probably too acute to allow development of severe lesions, for marked neuropathological changes were seen following prolongation of the incubation period by cortisone treatment. Also neuronal loss, though slight, was apparent in

surviving i.p. inoculated hamsters.

In all the other experiments the nerve cells of clinically affected animals were severely damaged. The distribution of virus observed on electron microscopy of infant hamster cerebellum was similar to the pattern of histological changes in paraffin sections (subject to the reservation mentioned on p. 56). An analogous relationship between occurrence of louping-ill antigen and neuronal damage was observed in moribund sheep. In most experiments the pattern of neuron necrosis in moribund and surviving animals is thus a fairly accurate reflection of the distribution of cells that are, or have been, infected with louping-ill virus. By using the histopathological data rough correlations can be made between the numbers of virus-infected nerve cells and the clinical disease.

Slight scattered neuronal loss was apparent in surviving sheep, whereas there was evidence of massive damage in moribund animals. Similarly there was widespread neuron necrosis in the fatal disease in infant hamsters, but only minimal loss in the older survivors. Therefore the severity of symptoms in louping-ill encephalitis would seem to be directly related to the number of neurons infected with virus. This supports the concept developed by ElDadah and Nathanson (1967) who found, using immunofluorescence, that the severity of clinical disease in rats infected with the West Nile group B arbovirus could be closely correlated with the number of infected neurons in the brain. The basic pathogenic mechanism in these 2 viral encephalitides is evidently direct virus-induced damage to the nerve cell.

The forebrains of sheep with louping-ill were relatively unaffected, compared with the Purkinje cells, the vestibular nuclei, the motor nuclei

and the ventral horns. The objective estimates of neuronal damage in i.c., s.c. or naturally infected animals presented substantially confirm the earlier description of Brownlee and Wilson (1932), who studied the pathology of louping-ill encephalitis in i.c. inoculated sheep and field cases. Though there are well recognized functional relationships (Truex and Carpenter, 1964) between the regions of C.N.S. that were severely damaged it is improbable that the pattern of lesions represents spread of infection along preferential neuronal pathways. This was proposed in cats infected with Newcastle disease virus by Luttrell and Bang (1958), but louping-ill virus did not seem to diffuse very far along axons (p. 54).

A similar distribution of severe neuropathological changes, with relative sparing of the telencephalon, has been demonstrated in monkeys inoculated intrathalamically or intranasally with other T.B.E. and group B arboviruses (Nathanson et al, 1965; Nathanson, Davis, Thind and Price, 1966; Nathanson, Gittelsohn, Thind and Price, 1967; Mayer and Rajcani, 1968). Thus the pattern of lesions in both monkeys and sheep is not obviously affected by the route of administration of virus. Also the lack of damage to the forebrain cannot be explained on a comparative anatomical basis, for the telencephalon of the primate is much better developed than that of the sheep (Ranson, 1935). Nathanson, Stolley and Boolukos (1969) found that in monkeys injected intrathalamically with a group A arbovirus (Eastern equine encephalomyelitis) the lesions were most marked in the cerebrum, whereas the spinal cord was only slightly affected. Nathanson (pers. comm.) has suggested that this may be due to physico-chemical differences between groups A and B of the arboviruses (Andrewes and Pereira, 1967). At present, factors determining the distribution

of virus-infected neurons in arbovirus encephalitis are somewhat obscure.

The needle track was not preferentially associated with neuronal loss attributed to louping-ill virus in any of the animals that were inoculated i.c.. Mims (1960) found that only an insignificant part of an i.c. inoculum remained in the parenchyma of the brain, while the majority passed directly into the cerebrospinal fluid (C.S.F.) and the blood. This may explain why the most marked lesions observed in sheep and adult hamsters were quite remote from the i.c. injection site. Neuropathological changes in i.c. inoculated mice were more generalized, with severe lesions in the cerebrum, olfactory bulb and thalamus as well as in the cerebellum and the medulla. Similar patterns have been determined objectively in mice given other T.B.E. viruses i.c. or s.c. (Denk and Kovac, 1969; Grcevic, Vince and Vesenjak-Hirjan, 1969; Vince and Grcevic, 1969). No general hypothesis can be formulated to relate the i.c. route of challenge with the distribution of neuronal loss.

In the serial killing experiments with i.p. inoculated hamsters and mice relatively high concentrations of virus were recovered from brain before infiltration with inflammatory cells was apparent. The only site of virus replication in C.N.S. that could be demonstrated by morphological techniques was the cytoplasm of the nerve cell (p. 54). The inflammatory response would thus seem to be essentially a secondary phenomenon, occurring after neurons are infected with virus.

The distribution and degree of nerve cell damage and inflammation corresponded in most experiments. The minimal neuronal loss in subclinically affected sheep and hamsters was accompanied by slight generalized inflammatory

changes, which were either mainly perivascular or associated with effete neurons. Similar lesions have been described in the asymptomatic disease in hamsters and monkeys inoculated s.c. with the J.I.R. strain of T.B.E. virus (Simon, Slonim and Zavadova, 1966, 1967), and Porton and Swiss A_2G mice given Langat virus intradermally or i.p. (Seamer and Randles, 1967; Webb, Wight, Platt and Smith, 1968). Varying degrees of inflammation were associated with the consistent marked neuronal damage observed in moribund sheep and lambs. The severity of inflammation was generally related to the incubation time; being least apparent in i.c. inoculated lambs and most obvious in animals succumbing to s.c. exposure. This again indicates that inflammation is a reactive process, which takes time to develop.

The nature of the perivascular inflammatory response was studied intensively in moribund sheep that had been given virus s.c.. Infiltrating cells were predominantly of the mononuclear type, with varying degrees of cytoplasmic pyroninophilia. A few classical plasma cells were seen but, on the basis of conventional optical microscopy, most appeared to be lymphocytes. When these cells were stained with an anti-IgG conjugate the cytoplasm of many showed strong specific fluorescence. This IgG could not be readily eluted and it was apparent that such cells contained globulin. Inflammatory cells with immunoglobulin in their cytoplasm have been demonstrated previously, by a similar technique, in the brain of a child that died from subacute progressive panencephalitis (Ter Meulen and Muller, 1968; Ter Meulen, Enders-Ruckle, Muller and Joppich, 1968).

When such perivascular cuffs were examined in the electron microscope the majority of cells in the infiltrates were found to contain numerous

distended vesicles of granular endoplasmic reticulum, surrounding poorly stained material. This is the characteristic appearance of the secretory cell (Kurosumi, 1961). By inference from the immunofluorescence studies it is apparent that a number of these cells are either synthesizing or carrying globulin. The secreted globulin is probably represented by the poorly stained material enclosed in vesicles. Similar cells in the parenchyma and efferent lymph of sheep lymph nodes given a variety of antigenic stimuli have been identified, first functionally and then by electron microscopy, as plasma cells (Cunningham, Smith and Mercer, 1966; Hall, Morris, Moreno and Bessis, 1967; Smith and Morris, 1970). Thus, by both ultrastructural and immunological criteria, many of the cells in perivascular cuffs of sheep with louping-ill can be considered as members of the plasma cell series (Humphrey and White, 1964). The term "plasma cell" is used in this general sense throughout the remainder of this discussion. Very few of the infiltrated cells were similar to normal sheep lymphocytes in efferent lymph from unstimulated nodes (Hall et al, 1967).

On the basis of ultrastructural examination two slightly different types of plasma cells were recognized. Some had the elongate flattened arrays of granular endoplasmic reticulum and numerous mitochondria that are characteristic of the classical plasma cell (Fitch, Rowley and Coulthard, 1965). The cytoplasm of others contained fewer mitochondria and was largely filled with circular vesicles of granular endoplasmic reticulum. Cells with the second type of morphology were considered to be immature plasma cells by Leduc, Avrameas and Bouteille (1968). This seems unlikely in the present context, however, for the majority of perivascular cells contained

brightly fluorescing globulin in their cytoplasm. Conversely Harris, Hummeler and Harris (1966) thought that such cells were in the final stage of synthesis; the circular vesicles represented discrete globules of antibody, which was only released by dissolution of the cell. Release of globulin by cellular lysis could account for some of the debris observed in perivascular phagocytes in louping-ill.

The moribund sheep that were killed for pathological examination were also investigated by Reid (pers. comm.), who determined the relative proportions of serum and C.S.F. antibodies to several different types of antigen. He concluded that most of the high H.I. antibody titres to louping-ill virus that were detected in C.S.F. originated from sites within nervous tissue, and could not be accounted for on a basis of filtration from serum. This supports earlier observations by Webb et al (1968, a), concerning the source of H.I. and neutralizing antibodies to louping-ill virus in C.S.F. from clinical cases in man. It seems probable that many of the perivascular plasma cells in sheep with louping-ill are producing antibody that is specifically directed against louping-ill virus. The H.I. antibody detected in C.S.F. would be in excess of that reacting with free virus in nervous tissue.

The infiltrating leucocytes demonstrated both in the lumen of venules and in endothelium were morphologically similar to the perivascular plasma cells. The inflammatory reaction in louping-ill encephalitis appears to result mainly from emigration of circulating plasma cells, at least some of which are producing antibody that is specific to viral antigen. Release of such antibody into nervous tissue would, by neutralizing free virus, tend to

prevent spread of infection from cell to cell. Both the limitation of the disease process in subclinical and non-fatal cases in hamsters and sheep and the disappearance of virus from the brains of survivors could be partly a result of local production or release of specific immunoglobulin.

Fatal encephalitis probably occurs when there has been generalized dissemination of virus throughout the C.N.S. prior to cellular emigration. The inflammatory reaction in cases where virus is replicating in many nerve cells may even exacerbate the disease process (Webb and Smith, 1966; Webb, Wight, Wiernik, Platt and Smith, 1968), as large cuffs around small vessels cause considerable compression of nervous tissue and would hinder passage of oxygen and other nutrient materials from blood to brain. Suppression of the inflammatory response was probably a factor in both the delay in onset of symptoms in i.c. inoculated hamsters that were given cortisone, and the successful treatment of cases of louping-ill encephalitis in man with corticosteroids (Webb et al, 1968, a).

Inflammatory changes were not detected in all clinically affected animals. There were no obvious cellular infiltrates in the brains of 75 per cent. of i.p. inoculated C57 black mice with severe neurological symptoms, and only a very few inflammatory cells were seen in nervous tissue from moribund infant rodents. Presence of an inflammatory reaction was certainly not essential for the development of symptoms. A similar conclusion was reached by Hirsch and Murphy (1968), who used anti-thymocyte serum to depress the inflammatory response in mice infected with yellow fever virus and established that infiltration of inflammatory cells into nervous tissue was not of primary importance in the pathogenesis of the fatal disease.

Also Johnson (1968) thought that the inflammatory reaction was a secondary phenomenon, and was of no significance in the development of symptoms in mumps virus encephalitis in the hamster.

Emigrating plasma cells passed through the cytoplasm of endothelial cells without causing any obvious damage to either the endothelial cells or their basement membranes. Lymphocytes pass in this way across the blood vessel walls of normal lymph nodes, whereas in inflamed nodes exit of polymorphs and monocytes causes obvious distension of the junctions between endothelial cells (Marchesi and Gowans, 1964). Infiltration in louping-ill encephalitis thus appears to be a relatively physiological process. Similar passage of lymphocytes through the endothelium has also been observed in rabbit nervous tissue during the early stages of both herpesvirus encephalitis (Baringer and Griffith, 1970) and experimental allergic neuritis (Astrom, Webster and Arnason, 1968). In these conditions perivascular lymphocytes transformed into cells that could be considered, on morphological criteria, as members of the plasma cell series (Humphrey and White, 1964).

The inflammatory response in virus encephalitis has been compared (Webb, 1968; Johnson, 1968) with types I, III and IV of the allergic reactions defined by Coombs and Gell (1968). Experimental allergic neuritis (E.A.N.) is a classical example of the type IV, or delayed hypersensitivity, reaction (Astrom et al, 1968). However the similarity between inflammation in louping-ill and E.A.N. seems only to apply to the initial stage of perivascular cuffing. Symptoms of E.A.N. do not occur until there is massive exudation of monocytes, associated with marked distension of endothelial junctions and leakage of plasma proteins (Lampert, 1969; Schroder and Krucke,

1970). Perivascular cuffs in louping-ill were almost entirely composed of plasma cells, and the relatively few phagocytes seen may have been largely derived from pericytes. The electron-dense matrix observed between infiltrated cells may represent extravasated blood protein, but there was no evidence of the periodicity associated with fibrinogen or fibrin (Pappas, Ross and Thomas, 1958; Porter and Hawn, 1949). Sheep die from the effects of virus-induced nerve cell dysfunction (p. 58) before the inflammatory reaction becomes non-specific and causes secondary damage, such as obvious breakdown of the endothelial barrier and brain oedema.

The course of louping-ill infection in hamsters was profoundly modified by treatment with cortisone acetate. The net result in all experiments was increased mortality, associated with severe neuropathological changes in the animals that survived longest. No detailed explanations can be offered for these results. Cortisone is a potent inhibitor of protein synthesis (Kilbourne, Stewart and Pokorny, 1961). Its pharmacological effects are therefore protean. For instance large doses cause necrosis of cells in the lymph nodes and thymus (Weaver, 1955), and may suppress formation of antibody (Kilbourne, 1956), infectious virus (Hannoun, Fernandes and Macieira-Coelho, 1965) and interferon (Rytel and Kilbourne, 1966). The severe disease seen in hamsters with louping-ill may be generally considered to result from reversal of such inhibition, which is often followed by potentiation, subsequent to withdrawal of treatment. A similar pattern of increased susceptibility associated with marked lesions in brain was observed in mice that were infected with dengue virus (a group B arbovirus) and then given a dose of the immunosuppressive drug "Cyclophosphamide" (Cole and Nathanson, 1968).

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APPENDICES

Appendix 1.

The Distribution and Severity of Lesions in Lambs with Natural Louping-ill

Comparative lesion scoring studies were done on 5 moribund lambs with louping-ill, contracted during a natural outbreak on a property in South East Scotland. All had high H.I. titres to louping-ill virus containing a significant proportion of IgM class antibody. This serological pattern has been associated with the acute disease (Reid and Doherty, in preparation). Louping-ill virus was isolated from the brains of 3 of these animals (H. W. Reid, pers. comm.).

The distribution of neuropathological changes (Fig. 71) was similar to that described in experimental cases (Section IV (1) in results). There was a relative sparing of the telencephalon and the most severe lesions were associated with the motor nuclei, the vestibular nuclei, the Purkinje cells and the ventral horns of the spinal cord.

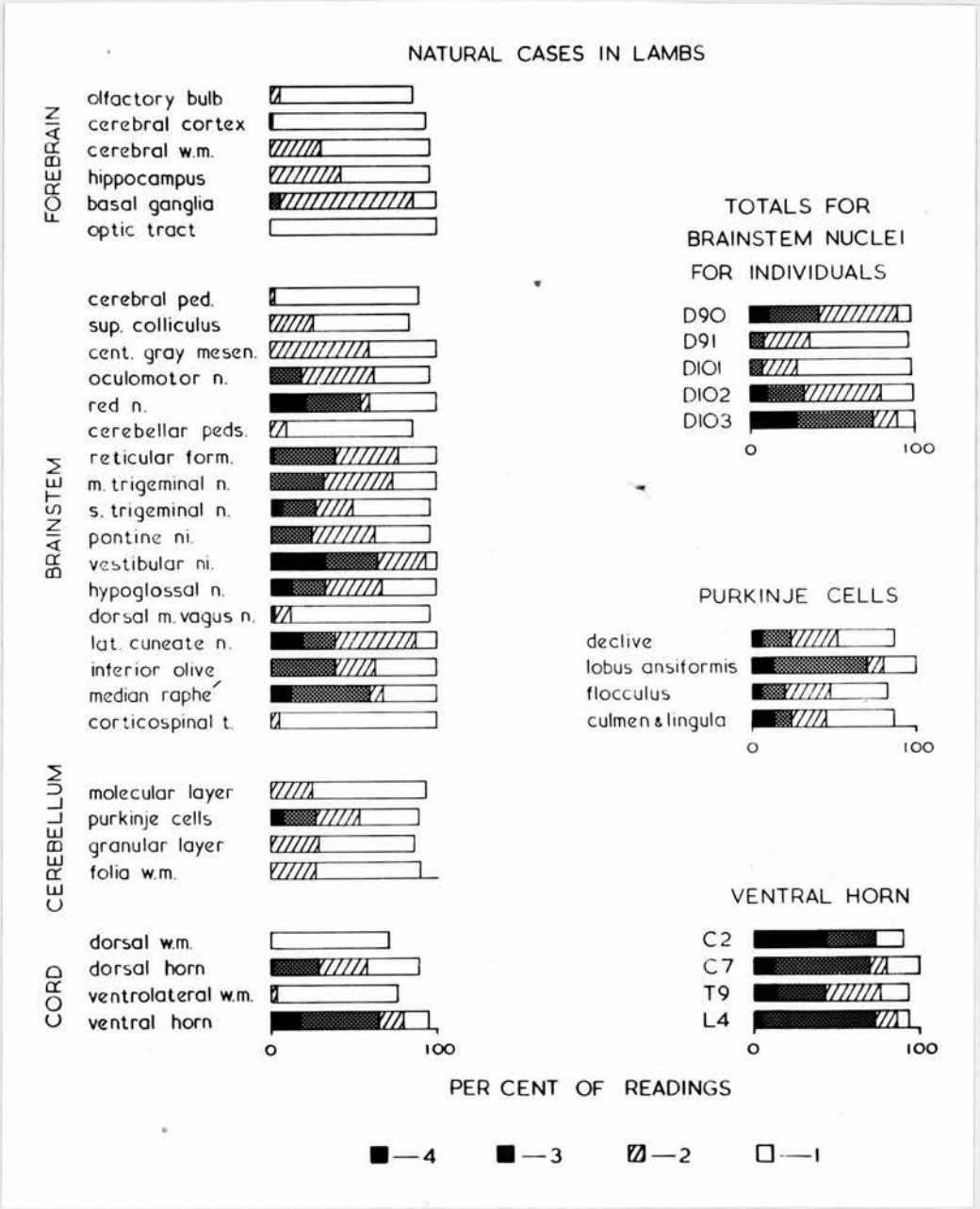

Fig. 71 The distribution and severity of lesions in 5 field cases of louping-ill in lambs. The lesion grading system is explained on page 10.

w.m. = white matter
ped.(s) = peduncle(s)
m. = motor
n.(i) = nucleus(ei)
mesen. = mesencephalon
s. = sensory

DEMONSTRATION OF LOUPING-ILL VIRUS IN ABNORMAL PURKINJE CELLS

BY

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Demonstration of Louping-Ill Virus in Abnormal Purkinje Cells

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SUMMARY. Abnormal Purkinje cells were selected by optical microscopy of cerebellar cortex from sucking mice with louping-ill encephalitis. When these cells were examined in the electron microscope, virus particles were seen in areas of modified endoplasmic reticulum. The capsid consisted of a nucleoid surrounded by a clear space and a dense outer membrane. The diameter of the capsid was approximately 44 nm. and that of the nucleoid 20 nm.

Experimental louping-ill encephalitis in young sucking rodents was characterized by massive cellular necrosis throughout the brain (Doherty, 1969). Damage to neurons could have resulted from intracellular production of virus, which was present at high titre in brain suspensions, or from some other factor, e.g. ischaemia (King, 1940). One way to resolve this question was to demonstrate the virus by ultra-structural examination of individual

neurons which showed damage on examination by the optical microscope. The Purkinje cells were selected because they showed a range of abnormalities and were readily identifiable by the optical and electron microscopes.

MATERIAL AND METHODS

Four-day-old Swiss white mice were inoculated intraperitoneally with 10,000 mouse LD_{50} of the Moredun sheep strain (LI 31) of louping-ill virus in fifth passage mouse brain. Similar mice were inoculated with a control preparation of normal mouse brain.

A total of six infected and six control mice were killed by decapitation on Day four or five after inoculation, when clinical disease became apparent. Three per cent glutaraldehyde (0·1 ml.) was immediately injected through the skull in the region of the cerebellum. The brain was exposed as quickly as possible and small blocks of cerebellum were fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in "Araldite".

Purkinje cells for ultra-structural examination were selected on the optical microscope using 1 μm thick sections which were stained with 1/10 Giemsa at 60°C. The blocks were trimmed to the selected area and ultra-thin sections were cut and then stained with lead citrate or uranyl acetate/lead citrate.

RESULTS

Optical microscopy of thick sections showed abnormal accumulations of blue staining material in Purkinje cells (Fig. 1 inset) of mice with louping-ill. This change was not seen in the controls.

Low-power electron microscopic examination revealed that there was an altered distribution of the endoplasmic reticulum (Fig. 1) in the abnormal cells. At higher power it was seen that the lumen of this modified endoplasmic reticulum contained dense bodies (Fig. 2), some of which were contiguous with the membrane. These bodies had the characteristics of virus particles (Fig. 3) and were not seen in control preparations. Most particles were between 42 and 48 nm. in diameter, a few were as small as 37 nm. and others had diameters up to 55 nm. The capsids were usually spherical and contained a central nucleoid. The nucleoids were approximately 20 nm. in diameter.

DISCUSSION

The morphology of louping-ill virus has not been previously described. The particles seen by us correspond both in their morphology and distribution to published descriptions of other group B arboviruses, namely St. Louis encephalitis virus in mouse neurons (Murphy *et al.*, 1968) and closely related viruses of the tick-borne encephalitis virus complex in cell cultures (Kovac *et al.*, 1961; Abdelwahab *et al.*, 1964; Tikhomirova & Karpovich, 1966; Tikhomirova *et al.*, 1968). Therefore, it seems reasonable to assume that these particles represent louping-ill virus and that the abnormal appearance of the Purkinje cells was associated with virus production. The distribution of complete virus particles in areas of modified endoplasmic reticulum supports the suggestion of Filshie & Rehacek (1968) that the group B arboviruses coat at the internal membranes of the cell, rather than at the plasma membrane.

The size range of louping-ill virus as determined by electron microscopy is considerably greater than that proposed by earlier workers who, from filtration and centrifugation studies, concluded that virus diameters ranged from 15 to 27 nm. (Elford & Galloway, 1933; Olitsky & Casals, 1948).

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—— Karpovich, L. G., Reingold, V. N., Levkovich, E. N. & Shestopalova, N. M. (1968). *Acta Virol.*, **12**, 529.

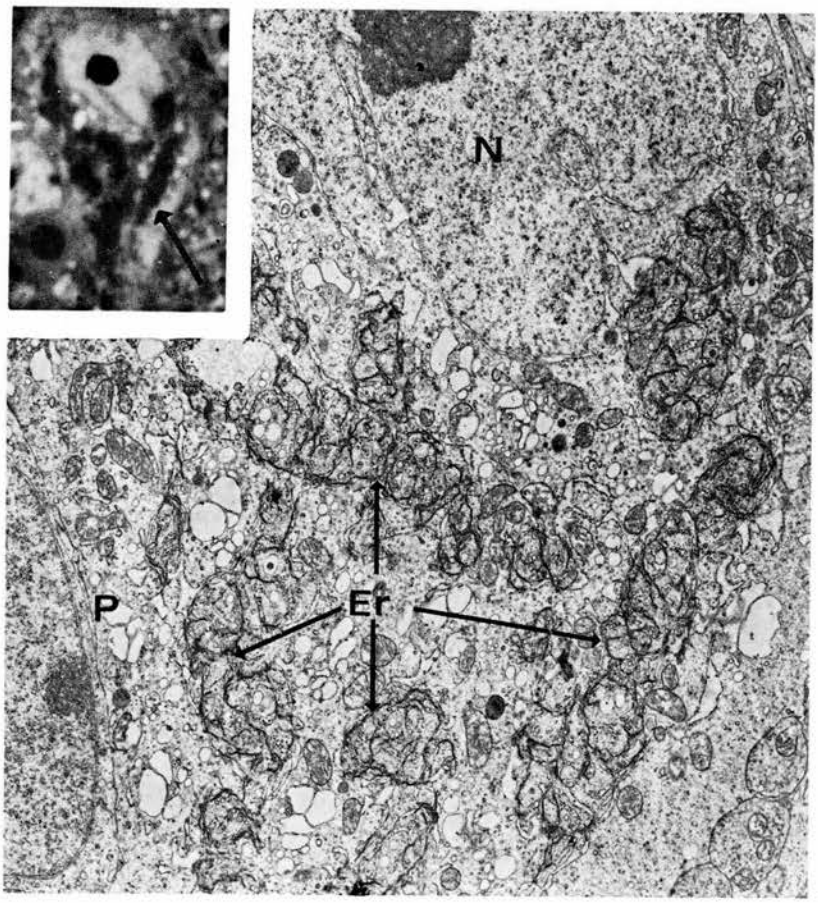

FIG. 1, Inset. Light micrograph of an affected Purkinje cell showing abnormal accumulations (arrow) of blue staining material in the cytoplasm. 1/10 Giemsa × 1,200.
FIG. 1. Electron micrograph of part of the same cell showing areas of modified endoplasmic reticulum (Er), nucleus (N) and plasma membrane (P). Lead citrate × 9,000.

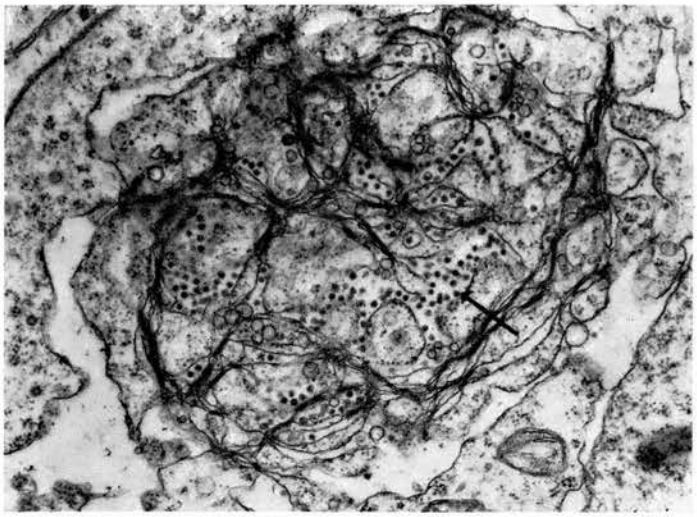

FIG. 2. Virus particles (arrow) in modified endoplasmic reticulum. Lead citrate × 30,000.

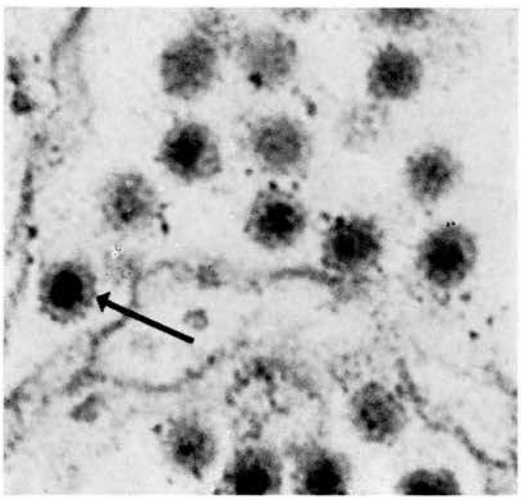

FIG. 3. Virus particles, showing variation in electron density. Capsids can be seen containing a dense nucleoid surrounded by a lighter area enclosed in a membrane (arrow). Uranyl acetate/lead citrate × 200,000.

EFFECT OF AGE ON LOUPING-ILL ENCEPHALITIS IN THE HAMSTER

By

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INTRODUCTION

It has recently been shown that the histopathology of central nervous system lesions caused by bluetongue virus varies considerably with age of the host at inoculation (Richards and Cordy, 1967). The possible effect of age of the host on louping-ill encephalitis has never been investigated experimentally, though there is some evidence from natural cases that such variation does occur (Zlotnik, I. cited by Webb and Smith, 1966). Suckling and adult hamsters develop fatal neurological disease after intracerebral (i.c.) inoculation with louping-ill virus (Gresikova, Albrecht and Ernek, 1961; Pogodina and Savinov, 1964; Zlotnik, 1968). Asymptomatic infection results when the virus is given to adult animals by the subcutaneous (Pogodina, 1964) or the respiratory (Zlotnik, 1968) routes.

It thus seemed likely that the Syrian hamster would be suitable for studying the effects of age on both fatal and subclinical louping-ill.

MATERIALS AND METHODS

Hamsters. The hamsters were from litters containing at least 3 animals and were weaned at 21 days of age. The mean body weights of hamsters were 4·5 g. at 4 days old, 14·1 g. at 14 days old and 30·5 g. at 22 days old.

Inoculation. The LI31 strain of louping-ill virus was in its fifth mouse brain passage and, like the control mouse brain suspension, was stored and re-constituted for use as described subsequently (Doherty, 1969). The resultant 10 per cent. centrifuged brain suspensions were diluted 1:100 to give the inocula used in all of the subsequent experiments. The hamsters were inoculated i.c. with 0·03 ml. or i.p. with 0·3 ml. A number of virus inocula were prepared and 5 of these were titrated i.c. in weaned white mice. The virus titres, expressed as the reciprocal of $\log_{10}ID_{50}/0{\cdot}03$ ml. of 10 per cent. brain suspension, ranged from 6·2 to 6·4. By this criterion hamsters were inoculated i.c. with between $1{\cdot}6 \times 10^4$ and $3{\cdot}9 \times 10^4$ infectious virus particles, or i.p. with 10 times that dose.

Determination of the susceptibility of hamsters to louping-ill. Eighty hamsters (15 litters), aged from 3 to 25 days and weighing between 3·2 and 48·4 g., were used. The virus was inoculated i.c. into one-third of the animals in each litter, i.p. into another third and control material was inoculated i.p. or i.c. into the remainder. They were weighed each day from the fifth day post inoculation (P.I.). Brains were taken for histological examination from 28 clinically affected animals, 2 that were clinically normal, and 16 controls.

Pathogenesis studies. The most interesting feature of the above was the pattern of decreasing susceptibility to i.p. inoculation as the animals grew older. It was thus decided to study the pathogenesis of louping-ill in hamsters that were inoculated at different ages. Three groups were inoculated i.p. as follows; 4 day old, 26 with virus

and 8 controls; 14 day old, 33 with virus and 9 controls; 22 day old, 34 with virus and 4 controls. The control and the infected animals were from the same litters and were housed together.

Animals in each group were killed at random for virological and histological examination as follows; 4 day old, 2 infected and 1 control each day for 8 days; 14 day old, 2 infected each day for 10 days and 1 control every second day; 22 day old, 3 infected each day for 11 days and 1 control every third day. A sample of blood and one half of the brain were processed for virological examination and titrated i.c. in weaned white mice. The techniques used were as described subsequently (Doherty, 1969), except that samples were stored at −70°C. for up to 3 months. The other half of the brain was examined histologically. Additional brains for histological examination were taken from 2 infected and 1 control 4 day old hamster at 8 days P.I., and 2 infected 14 day old hamsters on each of 12, 14 and 22 days P.I. The remaining animals in each group were killed at 22 days P.I. and were not examined.

Histology. All brains from animals found dead were discarded as unsuitable for histological examination. Whole or half brains from animals that were killed were fixed in 10 per cent. formol saline. Each half brain was blocked longitudinally (Fig. 1) and 3 paraffin sections (6 μ) were stained with haematoxylin and eosin (H. & E.) and mounted on the same slide. Selected paraffin sections were also stained by the following techniques; periodic acid-Schiff (P.A.S.); Luxol fast blue, for myelin; methyl green pyronin, for plasma cells; Best's carmine, for glycogen; toluidine blue, for metachromasia; and Sudan black, for fat. Formol-fixed frozen sections were cut from the brains of three 4 day old hamsters at 8 days P.I. and stained for lipid by the Sudan IV and OTAN methods.

Lesion scoring system. The H. & E. stained slides were randomised, the 3 sections on each were examined and the severity of lesions in each of the 5 anatomical regions shown in Fig. 1 was graded from 0 to 4. The subjective criteria used were based on those of Nathanson, Goldblatt, Thind, Davis and Price (1965). A grade of 1 was given for a few inflammatory foci, 2 for numerous inflammatory foci, 3 for very severe inflammation and/or necrosis of at least 30 per cent. of neurons, and 4 for necrosis of at least 90 per cent. of neurons. Examples of these grades are given in the results (Figs. 5 and 8). The randomised series of slides was examined 3 times and a total value for each brain was calculated by summing the grades for the 5 regions from each of these readings. This total was divided by 3 to give an average value which was called the encephalitis score. The maximum possible encephalitis score was thus 20.

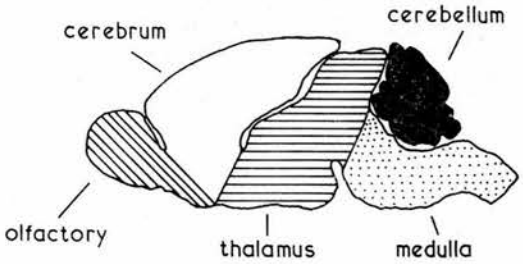

Fig. 1. Encephalitis grading sites.

RESULTS

Susceptibility of Hamsters of Different Ages to Louping-ill

Intracerebral inoculation with virus produced marked signs of neurological disease in all hamsters. The incubation times were very uniform and ranged

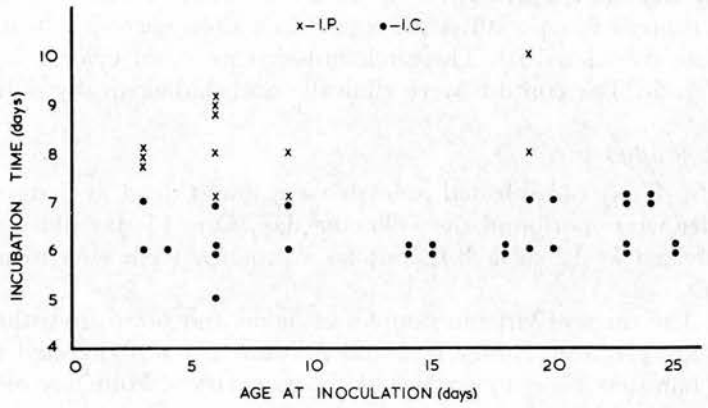

Fig. 2. Incubation time to detection of neurological symptoms in hamsters which were inoculated i.p. or i.c. with virus at different ages. The 2 inoculated i.p. when 19 days old showed slight symptoms, from which they recovered. The remainder were subsequently found dead (9) or killed with severe clinical signs (28).

from 5 to 7 days (Fig. 2). Body weight gain was considerably less than that of the controls over the 6 days P.I. (Fig. 3). When the virus was inoculated i.p., however, severe clinical signs were seen only in animals that were less than 10 days old at inoculation. Mild nervous symptoms were also seen in 2 of the older hamsters. Generally the onset of clinical signs was later than was seen in animals inoculated i.c. (Fig. 2).

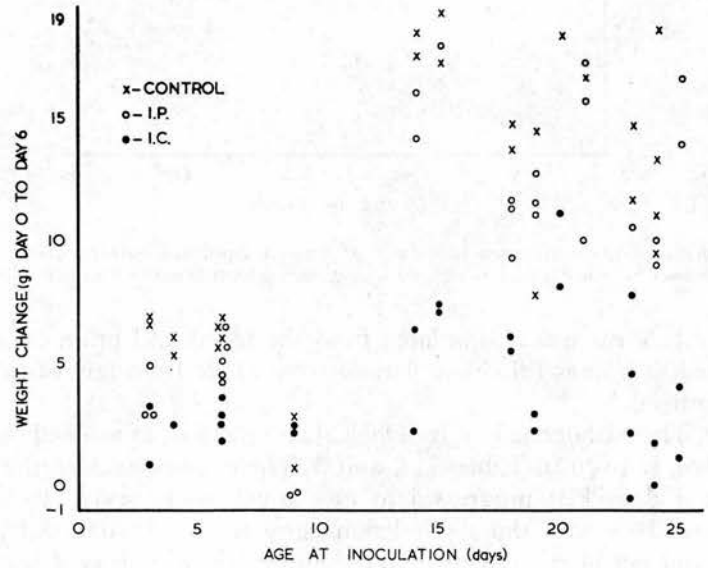

Fig. 3. Body weight change from day i.p. or i.c. inoculation with virus or control (day 0) to day 6, in hamsters of various ages.

As age at inoculation increased up to 14 days the severity of histological encephalitis decreased, especially with regard to neuron necrosis which was more severe in those inoculated i.c. These relationships were not evident in the older hamsters (Fig. 4). The controls were clinically and pathologically normal.

Pathogenesis Studies

Two of the 4 day old infected animals were found dead at 7 days P.I. and the remainder were moribund the following day. One 14 day old hamster was clinically affected at 12 days P.I., but no symptoms were seen in any of the other animals.

Virology. The titres of virus in samples of blood and brain from the 3 groups of hamsters are given in Tables 1, 2 and 3. Viraemia was detected for 5 to 6 days P.I. in hamsters 14 or more days old at inoculation. Four day old hamsters showed viraemia of higher titre, however, which persisted until death at 8 days P.I. Similarly, virus titres in brain were higher in the younger animals. There was a tendency for virus to disappear from the brains of 22 day old hamsters

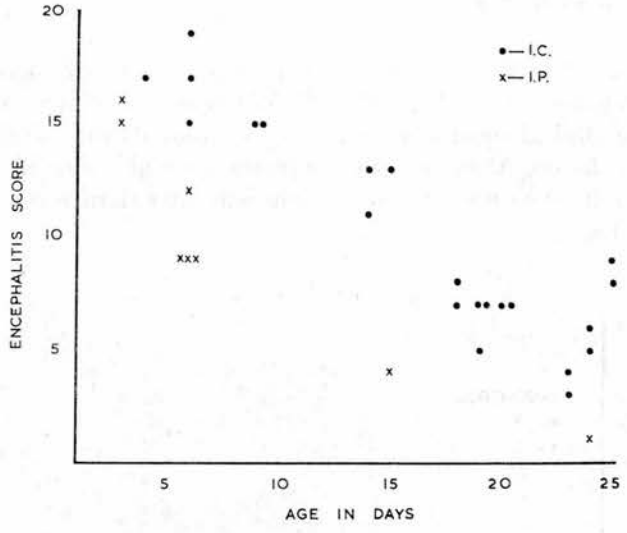

Fig. 4. Encephalitis scores in hamsters inoculated at different ages. The two inoculated i.p. when 15 and 24 days old were clinically normal throughout. The remainder had nervous symptoms.

at 11 days P.I. Virus was also isolated from the blood and brain of one control hamster killed at 6 days P.I. No isolations were made from any of the other 16 controls examined.

Histology. The pathogenesis of histological encephalitis, as assessed by the lesion scoring system, is given in Tables 1, 2 and 3. The changes seen in the 4 day old hamsters at 5 days P.I. progressed to give much more severe lesions on the following day. However, the slight lesions seen in the 14 day old animals at 5 days P.I. did not alter markedly over the next 17 days, except for one killed with neurological disease at 12 days P.I. Slight lesions were also seen consistently in the 22 day old hamsters from 7 to 11 days P.I.

In the 4 day old hamsters the earliest pathological change was slight vasculitis in the cerebral cortex of one animal killed 4 days P.I. Foci of cellular necrosis were apparent from 5 days P.I. and became progressively more widespread. The changes in the cerebellum were particularly severe (Fig. 5); myelin was absent from the cerebellar folia though present in controls of comparable age. Granules of P.A.S. positive material were found in foam cells and in clumps of

TABLE 1
LOUPING-ILL ENCEPHALITIS IN HAMSTERS 4 DAYS OLD AT I.P. INOCULATION

*Day after inoculation**	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Virus titre in blood	1·7 2·8	3·4 4·1	3·0 3·5	2·4 ⩾4·2	1·5 1·4	2·2 2·5	2·2 2·5	2·5 2·4
Virus titre in brain	0 0	2·7 2·9	5·2 4·2	6·2 5·3	6·8 5·8	6·6 5·8	6·2 6·2	5·6 6·4
Encephalitis score	0 0	0 0	0 0	0 1	8 8	13 12	15 14	13 14

* Results in two hamsters on each day.
0 = No virus isolated, or lesions detected.

cellular debris in necrotic areas (Fig. 6). This material was not metachromatic, did not stain for glycogen or lipid and was only weakly eosinophilic. Its chemical nature is obscure. Cellular necrosis was widespread in clinically affected animals, especially in the cerebral cortex (Fig. 7) and the colliculi. The olfactory bulbs and the medulla tended to be less severely damaged.

TABLE 2
LOUPING-ILL ENCEPHALITIS IN HAMSTERS 14 DAYS OLD AT I.P. INOCULATION

*Day after inoculation**	*1*	*2*	*3*	*4*	*5*	*6*	*8*	*7*	*9*	*10*	*12*	*14*	*22*
Virus titre in blood	T 1·5	1·4 1·6	0·9 0·9	T 0·5	1·0 0	1·2 0·5	0 0	0 0	0 0	0 0	NT NT	NT NT	NT NT
Virus titre in brain	0 0	1·5 0·2	1·5 2·0	2·6 2·5	3·0 2·5	3·5 3·0	3·0 1·8	1·2 2·0	1·5 1·5	1·2 2·4	NT NT	NT NT	NT NT
Encephalitis score	0 0	0 0	0 0	2 0	1 0	3 6	7 7	8 8	6 7	4 4	7 5	9 12	3 5

* Results in two hamsters on each day.
0 = No virus isolated, or lesions detected. T = Trace. NT = Not tested.

Inflammation, however, was the predominant pathological change in the brains of the 14 and 22 day old hamsters. There was vasculitis, perivascular infiltration of lymphocytes and microglial proliferation (Fig. 8). Neuron necrosis and P.A.S. positive material, if present, were associated with inflammatory foci. There was no preferential distribution of lesions. With the exception of the one hamster from which virus was isolated, all the controls were histologically normal.

DISCUSSION

The pattern of decreasing severity of histological encephalitis with increasing age after i.c. inoculation was very like that described in suckling rats inoculated i.c. with the West Nile group B arbovirus by El Dadah and Nathanson (1967). However, although the pathological changes were so similar, the clinical syndromes were very different. Hamsters were susceptible to louping-ill given i.c. at all ages, whereas rats did not show significant neurological symptoms when inoculated by this route with West Nile at more than 16 days of age. This is comparable to the observation that there was no difference in the severity of lesions in older clinically affected and clinically normal hamsters inoculated by the i.c. or i.p. routes. Suckling rats became resistant to i.p. inoculation with West Nile virus at about 12 days of age (El Dadah, Nathanson and Sarsitis, 1967), as did hamsters to louping-ill.

TABLE 3
LOUPING-ILL ENCEPHALITIS IN HAMSTERS 22 DAYS OLD AT I.P. INOCULATION

*Day after inoculation**	*1*	*2*	*3*	*4*	*5*	*6*	*8*	*7*	*9*	*10*	*11*
Virus titre in blood	2·1	2·3	1·2	0·2	T	0	0	0	0	0	0
	1·6	1·6	1·0	0·5	3·2	0	0	0	0	0	0
	0·6	0·6	2·0	0·5	0·6	1·4	0	0	0	0	0
Virus titre in brain	0	0	0	2·5	0·5	1·5	2·0	1·8	0·2	1·2	T
	T	1·5	1·0	2·2	T	1·2	1·5	1·5	1·2	T	0
	0	0	0	1·8	2·8	2·4	T	1·0	0	T	0
Encephalitis score	0	0	0	2	0	4	2	4	3	5	5
	0	0	0	1	0	2	5	7	5	6	5
	0	0	0	0	1	0	3	3	5	8	4

* Results in three hamsters on each day.
0 = No virus isolated, or lesions detected. T = Trace.

The inverse relationship between age and susceptibility to louping-ill given i.p. is probably related to the development of immunological competence. The termination of viraemia at about 6 days P.I. in the older hamsters would be associated with the presence of circulating antibody (Slonim, Zavadova and Simon, 1966). This means that the brain was no longer exposed to virus produced in other organs. Antibody may also pass from blood into cerebrospinal fluid (Webb, Connolly, Kane, O'Reilly and Smith, 1968) and thus limit spread of virus in the brain.

In all the i.p. inoculation animals the first histological changes were associated with blood vessels, probably because virus entered the brain substance by the endothelial cells (Johnson and Johnson, 1968). Large molecules may pass more readily from the blood to the brain in times of active growth (Dobbing, 1968), and this may partly explain the increased susceptibility of very young hamsters to louping-ill. Also immature nervous tissue contains no myelin and has a higher water content. The cells are thus in a relatively loose matrix through which small particles, such as arboviruses, might easily diffuse. The progressive increase in size of necrotic area indicates that such a mechanism may operate.

In older animals all significant lesions were close to blood vessels, possibly indicating a change in permeability of the brain substance. It could be argued that isolations of virus from the brains of these animals were due to presence of infected blood cells in vascular inflammatory foci, which remained long after viraemia had ceased. However, Malkova (1967), working with another strain of tick borne encephalitis virus in mice, has demonstrated that most virus in blood is present in the free state and is not cell associated. Thus, it seems more likely that virus did multiply in the brains of these animals, but was localised by the host and finally eliminated (El Dadah and Nathanson, 1967). The mild vascular inflammatory reaction is characteristic of subclinical infections of the brain with arboviruses (Simon, Slonim and Zavadova, 1966; Albrecht, 1968).

Mackenzie, Wilson and Dennis (1968) have also found P.A.S. positive material, similar to that seen in 4 day old hamsters inoculated i.p., in the Purkinje cell layer of weaned mice given louping-ill i.c. Their suggestion that it was probably a tissue breakdown product is in accord with the distribution of areas of necrosis demonstrated here. It is interesting that it survived paraffin embedding.

SUMMARY

Syrian hamsters developed symptoms of neurological disease following intracerebral inoculation with louping-ill virus at from 3 to 25 days of age, or intraperitoneal inoculation at from 3 to 10 days of age. In older animals inoculated intraperitoneally the disease was asymptomatic. The severity of neuron necrosis decreased as the period of inoculation by both routes increased to 14 days.

Four day old hamsters inoculated intraperitoneally showed persistent viraemia and death at 7 or 8 days post inoculation. High titres of virus were found in the brain and there were spreading foci of cellular necrosis associated with P.A.S. positive granules. Older inoculated hamsters generally had a subclinical infection. Lower levels of viraemia were detected for about 6 days and the low titres of virus in the brains of 22 day old hamsters tended to disappear at about 11 days after inoculation. Vascular inflammatory foci of lymphocytes were seen from about 6 days after inoculation.

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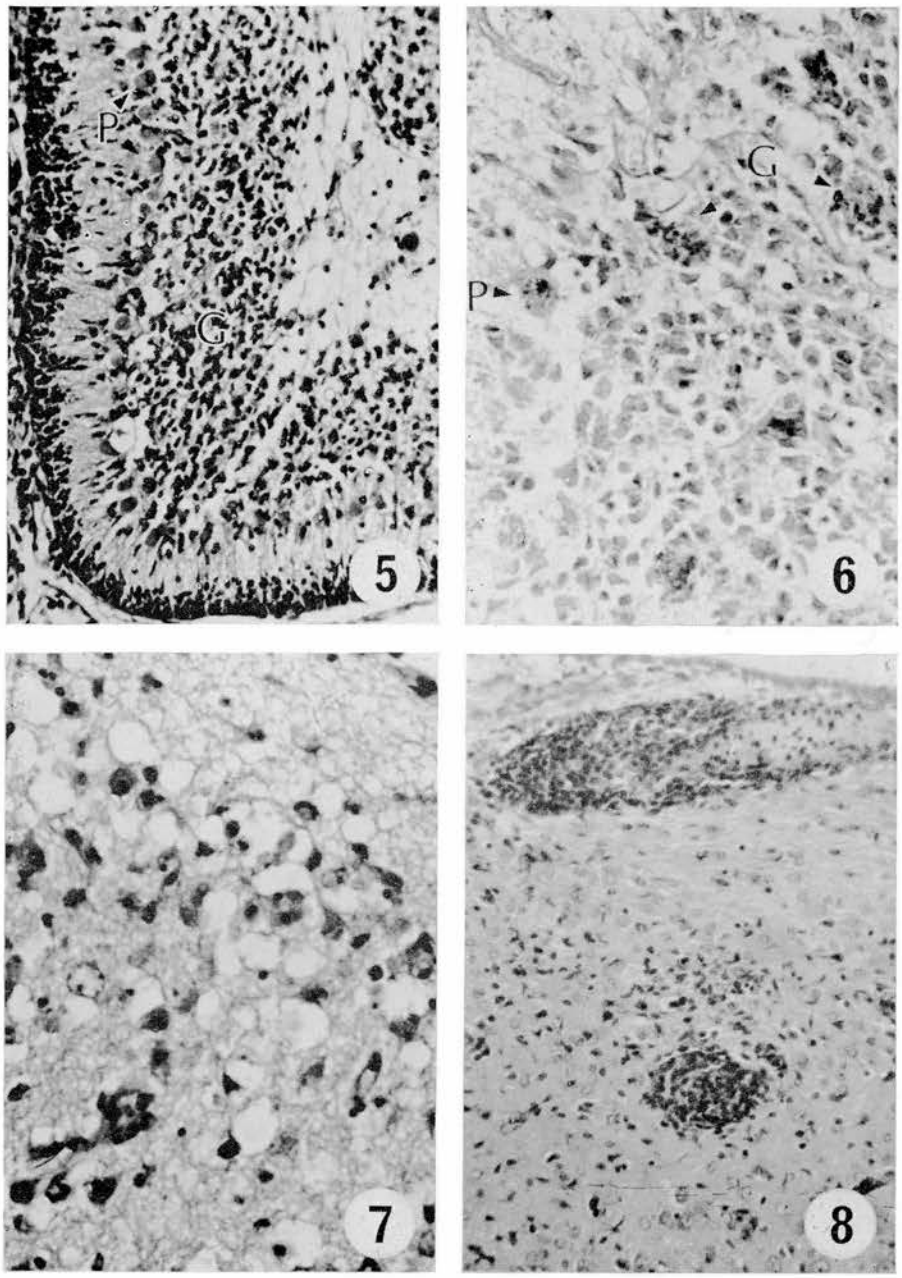

Fig. 5. Cellular necrosis in the cerebellum of a 4-day-old hamster moribund at 8 days P.I. The nuclei of the Purkinje cells (P) are displaced and shrunken and those of the granule cells (G) are pyknotic. This lesion was given a grade of 4. H. & E. × 165.

Fig. 6. P.A.S. positive material in the Purkinje (P) and granule cell (G) layers of the cerebellum shown in Fig. 5. P.A.S. × 440.

Fig. 7. Necrosis and vacuolation of the ground substance in the cerebral cortex (same section as fig. 5). H. & E. × 500.

Fig. 8. Inflammatory reaction in the thalamus of a clinically normal 14-day-old hamster at 14 days P.I. This lesion was given a grade of 2. H. & E. × 175.

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LOUPING-ILL ENCEPHALITIS IN C57 BLACK MICE

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INTRODUCTION

The mode of entry of viruses into the central nervous system (C.N.S.) and the mechanisms whereby they produce disease and death are not well understood (Smith, 1968). Pogodina and Savinov (1964) have shown that C57 black mice are highly susceptible to subcutaneous (s.c.) and intraperitoneal (i.p.) inoculation with a number of group B arboviruses of the tick borne encephalitis complex, to which louping-ill virus belongs (Andrewes and Pereira, 1968). It was thus considered that these mice might be suitable for serial virological and histological studies of the pathogenesis of encephalitis resulting from parenteral inoculation with louping-ill virus.

MATERIALS AND METHODS

Mice. The C57 mice were from a SPF colony, and were inoculated at 3 to 4 weeks of age. A total of 432 mice was used. The mean ($\pm$S.E.) body weight of 30 mice was $12{\cdot}5 \pm 0{\cdot}3$ g.

Inocula. The Moredun strain of sheep louping-ill virus (LI31) was stored at -20°C. as freeze-dried 0·25 ml. aliquots of a 10 per cent. suspension of fourth passage mouse brain in 10 per cent. horse serum-saline. The freeze-dried virus was re-constituted for use by suspending the contents of 2 ampoules in 4·5 ml. of cold serum-saline and centrifuging at 1,200 *g* for 15 minutes at 4°C. Two ampoules were used to minimize variation in virus titre between inocula (Williams, 1958). A similar preparation of normal mouse brain was used as a control.

Titration of the virus inoculum in mice. Ten-fold dilutions of the centrifuged supernatants were made and held on melting ice until inoculated within one hour of preparation. The mice were inoculated with 0·03 ml. by the i.c. route or 0·3 ml. by the i.p. or s.c. routes. Twelve animals were used for each dilution for each route and the survivors were kept for 21 days after inoculation. Virus titres were calculated by the method of Reed and Muench (1938) and expressed as the reciprocal of the $\log_{10}ID_{50}$/0·03 ml. of a 10 per cent. suspension.

General experimental design. In the above titration the most consistent pattern of susceptibility to parental inoculation was found in mice inoculated i.p. with a 10^{-2} dilution of virus; 11 of 12 first showed signs of severe neurological disease (Hurst, 1931; Alston and Gibson, 1931) on day 8. The remaining clinically normal mouse survived to day 21. This route and dose were thus used in all the pathogenesis studies. Five such inocula were prepared for the various experiments and each one was titrated i.c. in weaned Swiss white mice, using 5 for each dilution and keeping the survivors for 14 days after inoculation. It was calculated from these titrations that each black mouse was inoculated i.p. with between $1{\cdot}5 \times 10^5$ and $5{\cdot}0 \times 10^5$ infectious virus doses.

There were 70, 20, 7, 24 and 48 black mice in the respective groups inoculated. Animals were killed for both virological and histological examination from the first

and the last groups and for virological examination from the other 3. The mice in each experiment were numbered and these numbers were randomised. They were then killed in this pre-determined sequence. If an animal was found dead, the next mouse in the series was taken. Some mice from each experiment were allowed to survive for the full incubation period, as a check that each inoculum was producing clinical neurological disease. Five other mice were injected with a similar dose of the control inoculum.

Virology. The purpose of the virological studies was to determine the presence or titre of virus in nervous tissue. Blood samples were also examined so that the contribution of residual viraemic blood to virus recovery from nervous tissue could be assessed. The material was examined from individual mice, or as pooled samples from groups of 3 mice.

Mice were decapitated and blood was collected into sterile, weighed bottles. The brains and spinal cords were removed aseptically into similar bottles. In some experiments the brain was first divided into 4 anatomical segments (Fig. 1). The samples were then made to 10 per cent. (w/v) suspensions in cold serum-saline, the blood by pipetting and the nervous tissue by maceration in Griffiths' tubes, and stored at −20°C. for a maximum interval of one month. Frozen samples were thawed rapidly, centrifuged, diluted and titrated in white mice as described above. If virus was detected at a level that was too low to express as a titre it was said to be present in trace (T) amounts.

Histology. An initial series of brains was fixed in 10 per cent. formol saline. Longitudinal paramedian blocks were taken from one half of each brain and the other half was blocked coronally at 5 levels (Mould, Dawson, Slater and Zlotnik, 1967). Paraffin sections were cut at 6 μ and stained with haematoxylin and eosin (H. & E.) or methyl green pyronin (M.G.P.) for Nissl substance. Thereafter Carnoy's fluid was used for fixation so that the acridine orange (A.O.) stain for nucleic acids could be applied. Longitudinal paraffin sections were cut from all brains and stained with H and E. Selected sections were stained with A.O. at pH 4·0 (Culling, 1963) and examined using a u/v fluorescence microscope.

Histopathology in i.c. inoculated mice. Six mice were inoculated i.c. with a 10^{-4} dilution of virus. The higher dilution of virus was used for this route in order to make the incubation period comparable to that seen in mice inoculated i.p. Brains from clinically affected mice were taken into Carnoy's fluid and processed for histological examination.

RESULTS

Susceptibility of the Mice to Louping-ill

The results of the titration of the virus inoculum by various routes give a measure of the susceptibility of these mice to louping-ill (Table 1).

TABLE 1
TITRATION OF VIRUS INOCULUM IN C57 BLACK MICE BY VARIOUS ROUTES

Route	*Virus titre* ($log_{10}ID_{50}$/0·03 *ml.*)	*Incubation time* (*days, mean* ± *S.E.*)	
I.C.	7·2	6·76 ± 0·27	$P < 0·001$
I.P.	4·6	9·70 ± 0·26	
S.C.	3·5	11·04 ± 0·31	$P < 0·01$

Pathogenesis Studies

On day 8 clinical evidence of louping-ill was seen in 88·5 per cent. of the

61 mice that were kept for the full incubation period including animals from each of the 5 inoculation groups. Two of the 7 mice without clinical symptoms remained unaffected until day 21; the other 5 were killed for histological examination on day 8. No further examinations were made on 34 of the 54 mice with louping-ill, including 13 that were found dead.

Virology. The time of appearance of virus and the magnitude of titres in the C.N.S. were determined by titrating pools of 3 brains and spinal cords, and bloods each day for 8 days. Virus was detected in the C.N.S. from day 4 (Table 2), though clinical signs were not seen until day 8. The small amounts of virus isolated from nervous tissue on days 1 and 2 were attributed to presence of viraemic blood.

TABLE 2
VIRUS TITRE IN C.N.S. AND BLOOD OF SEQUENTIALLY SAMPLED MICE*

	Day of experiment							
	1	*2*	*3*	*4*	*5*	*6*	*8*	*7*
C.N.S.	T	T	O	3·8	3·6	2·6	5·3	6·2
Blood	0·5	0·2	T	T	T	T	0	0

* Pools of brain and spinal cord, and blood from 3 mice were used for each titration.
T = Trace. 0 = No virus isolated.

The regional distribution of virus in nervous tissue was determined by titrating pooled segments of C.N.S. from 3 mice from days 4 to 8. Virus was present in all segments from the fifth day, but was detected in only 2 segments on day 4 (Fig. 2). In order to ascertain if these represented preferential sites for early virus detection in the C.N.S. the experiment was repeated for day 4, but on this occasion no virus was isolated from any segment.

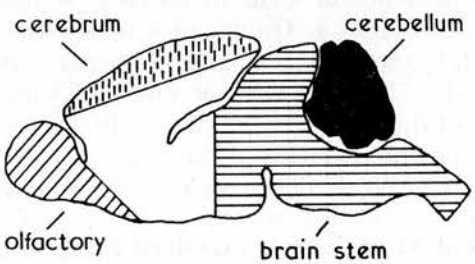

Fig. 1. Segments of the brain sampled for virus isolation and titration.

It was thought that a more accurate estimate of the time of first detection of virus in the brain might be obtained by titrating at intervals on the fourth day. Thus, pools of 3 brains and bloods were examined at 3 hourly intervals from 90 to 102 hours. Individual samples were also taken from 3 mice at 96 hours. There was no consistent pattern in the results from the brain pools, the titres being; 90 hours 0·7, 93 hours $\geqslant$2·4, 96 hours $\geqslant$2·5, 99 hours 0·1 and 102 hours

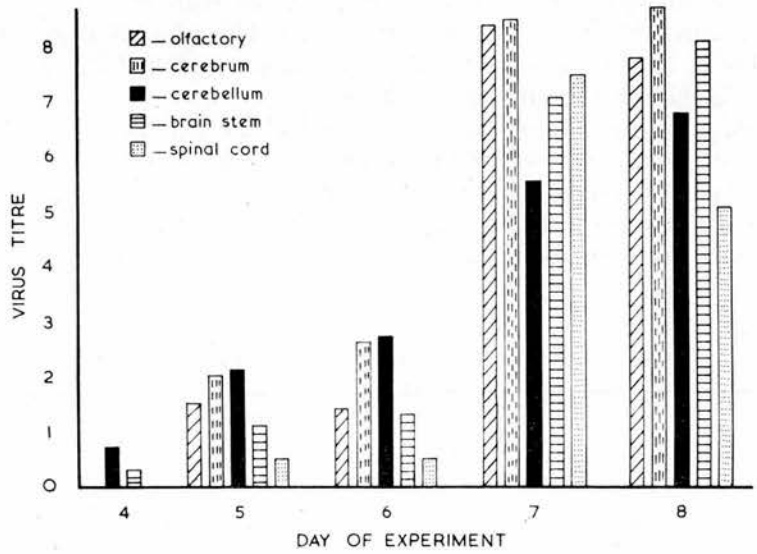

Fig. 2. Virus titre ($\log_{10}ID_{50}$/0·03 ml.) in pools of spinal cord and various segments of the brain (Fig. 1) from 3 mice. The virus was not isolated from the blood.

1·4. Virus was not isolated from the blood at 90 hours though it was present in trace amounts in the other 4 samples. Only one of the brains titrated individually at 96 hours contained any virus, at a titre of $\geqslant$2·4.

Burnet and Lush (1938) inoculated 6 to 8 week old mice i.p. with louping-ill virus and found that, in a high proportion, virus was present in the olfactory bulbs before it could be detected in the rest of the brain. This did not appear to be the case on days 4 to 8 of these experiments. An investigation was thus made of the possible presence of virus in different segments of the brains of individual mice from days 1 to 4. Groups of 4 mice were examined each day, and blood and the olfactory, cerebral and cerebellar segments (Fig. 1) were taken separately for virus isolation without titration. Virus was only found in nervous tissue from 2 of the mice from day 4; in all 3 brain segments of one and in the olfactory and cerebral segments of the other. Viraemia was demonstrated in 12 of the 16 mice, with no particular pattern of occurrence.

Histology. A series of 23 brains was examined from mice showing no clinical symptoms (3 from days 1 to 7 and 2 from day 8) and 5 with marked neurological signs on day 8. Histological changes were seen in only 4 of them, 2 from each of days 7 and 8. The most severely affected brain showed small perivascular and endovascular inflammatory foci of lymphocytes and monocytes in the thalamus, cerebral cortex and corpus callosum, and some neuron necrosis in the cerebral cortex and the granular layer of the cerebellum. The other 3 showed slight inflammatory changes but neuron necrosis was not observed. The distribution of Nissl substance, as seen in the M.G.P. stained sections, was normal in all but necrotic neurons.

As the observation that brains from 3 of 5 clinically affected mice were histologically normal required confirmation, another series was examined. These were taken from 12 mice showing no clinical symptoms (3 on days 5 to 8) and 14 with nervous symptoms on days 7 and 8. Five control mice were also examined on day 8. The only lesions found were in the brains of 5 mice from days 7 and 8, including one that was clinically normal. The most severe changes are illustrated in Figs. 3 and 4. Examination of the A.O. stained sections revealed no differences in distribution of nucleic acids in the brains of infected and control mice.

Histopathology in I.C. Inoculated Mice

It was of interest to determine if brains of mice with clinical neurological disease following i.c. inoculation could also be histologically normal. One of the 6 mice thus inoculated was dead on day 7 and the remainder were clinically affected. There were severe histological changes (Fig. 5) in the brain of one mouse, and moderately severe lesions in the brains of the other 4. Both inflammation and neuron necrosis (Fig. 6) were more marked in these mice than in i.p. inoculated animals.

DISCUSSION

Intraperitoneal inoculation of C57 black mice with high titres of virus has been found to be a reproducible method for producing fatal louping-ill infection. The course of such infection was not completely predictable as virus could not be isolated consistently from the brain at 96 hours, and up to 10 per cent. of mice survived, probably because virus replication did not occur in the C.N.S. It is possible that an occasional negative sample may have been included in brain pools from which virus was isolated at the pre-clinical stages, but this would have only caused a negligible drop in titre (0·2).

The failure to confirm the report of Burnet and Lush (1938) that virus was present in the olfactory bulbs before it was detected in the rest of the brain may be explained by differences in the age (Hurst, 1950) and strains of hosts, and in the titres of the virus inoculated (Seamer and Randles, 1967). In fact, no preferential site of virus entry into the C.N.S. or of virus replication in nervous tissue was found. The presence of virus in the brain for about 4 days before the development of neurological signs is in accord with the results of an earlier study in intranasally inoculated mice (Fite and Webster, 1934).

The most interesting feature of louping-ill infection in the mice inoculated i.p. is that although virus was present at high titre in all regions of the brains of clinically affected animals, there were lesions in the brains of only 6 of the 24 that were examined histologically. Histological changes were also found in the brains of 3 of 6 mice without clinical symptoms killed on day 7, though these would probably have soon developed symptoms. Marked inflammatory lesions were seen, however, in the brains of all of the i.c. inoculated mice with similar neurological signs. The presence of inflammatory cells in nervous tissue did not appear to be important in the development of clinical neurological disease, which seems to be at variance with the suggestions of Webb and Smith (1966). A comparable observation was made by Hirsch and Murphy (1967) who used anti-thymocyte serum to depress inflammation in the brains of mice that had

been inoculated with yellow fever virus, and concluded that the cellular inflamatory response was not of primary importance in the pathogenesis of the neurological disease. There may well have been other morphological changes, such as astrocytosis (Zlotnik, 1968), which were not demonstrated by the techniques used.

The consistent presence of marked lesions in the brains of animals inoculated i.c. is in accord with previous studies in other strains of mice (Hurst, 1931, 1950; Mackenzie, Wilson and Dennis, 1968).

SUMMARY

Weaned C57 black mice developed marked neurological symptoms 8 days after intraperitoneal inoculation with high titres of louping-ill virus. The virus was first detected in nervous tissue on day 4 and replication thereafter proceeded at approximately equivalent rates in different regions of the central nervous system. There was no obvious preferential site of virus entry into the brain. Lesions of encephalitis were found in only 25 per cent. of mice with neurological signs. Histological changes were present in the brains of all i.c. inoculated C57 black mice with symptoms.

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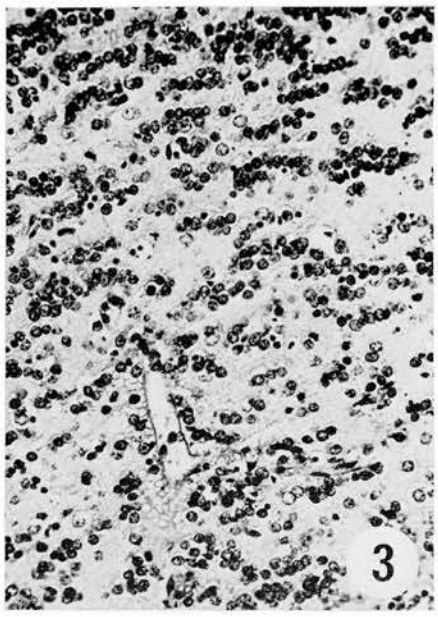

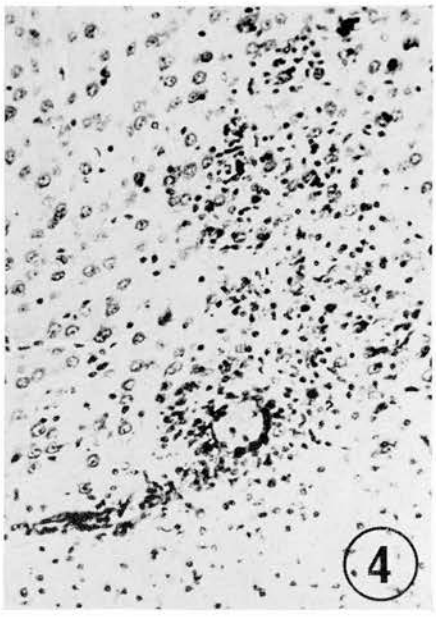

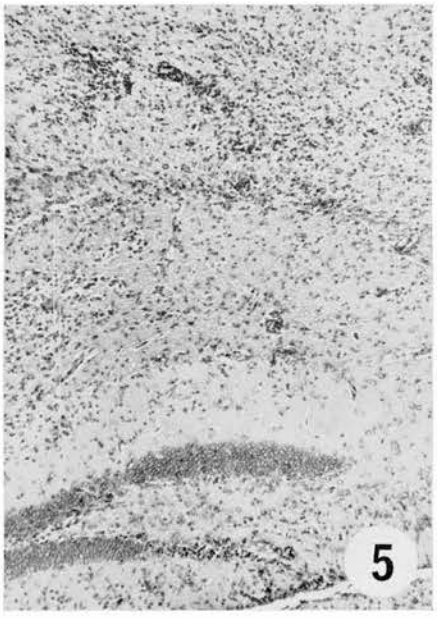

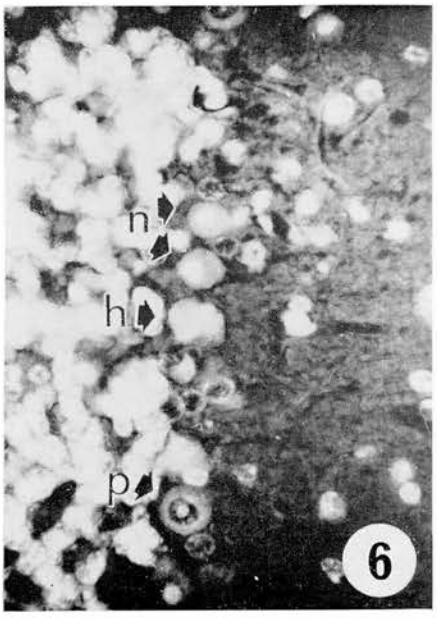

Fig. 3. Necrotic cells in the olfactory bulb of a clinically normal mouse, inoculated i.p. and killed on day 7. H. & E. × 240.

Fig. 4. Perivascular cuff, microglial proliferation and necrotic cells in the cerebral cortex of a clinically affected animal, inoculated i.p. and killed on day 8. H. & E. × 120.

Fig. 5. Inflammation and necrosis in cerebral cortex and hippocampus of a clinically affected mouse, inoculated i.c. and killed on day 7. H. & E. × 60.

Fig. 6. Fluorescence photomicrograph of cerebellar cortex of a clinically affected mouse. inoculated i.c. and killed on day 7. The Purkinje cells are normal (p), undergoing necrosis (h) or necrotic (n). Acridine orange × 1200.

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