QUANTITATIVE STUDIES ON THE VIRUS OF LOUPING ILL
IN SHEEP AND TICK

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by

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INTRODUCTION

Severe lamb losses in south-west Ayrshire during 1960 prompted a revival of interest in the epidemiology of louping ill (Smith, McMahon, O'Reilly, Wilson and Robertson, 1964). In Argyllshire an intensification of the field problem had already been noticed in 1959. The Veterinary Investigation Officer, Oban, Mr. C.C. Bannatyne, obtained the co-operation of the Moredun Institute of the Animal Diseases Research Association in investigating the outbreaks in Argyll. Those particularly concerned were Mr. J.G. Brotherston, Deputy Director of the Institute, Dr. R.M. Barlow of the Experimental Pathology department and Mr. J. Campbell of the Microbiology department.

The events in Argyll presented disturbing features in that what had traditionally been regarded as a disease of hoggs (Pool, Brownlee and Wilson, 1930) was now being encountered in alarming outbreaks among sheep of all ages, ranging from lambs less than three weeks old to cast hill ewes at six years of age. This altered pattern indicated the need for epidemiological study.

Dr. J.A. Campbell of the Sub-department Parasitology, Department of Zoology, University of Edinburgh, suggested to the author that the examination of certain facets of the vector-virus relationship would form a convenient and useful adjunct to the Moredun investigations. This would also be a logical extension of Dr. Campbell's own work (1948) on the life cycle of the vector Ixodes ricinus, L. Concurrently further work on the tick life cycle was to be implemented in Dr. Campbell's department by Mr./
Mr. D.H. Kemp.

The author was employed in October, 1963, on a three year grant made by the Agricultural Research Council to Dr. Campbell. By kind permission of the Director of Moredun Institute, Dr. J.T. Stamp, the author was granted excellent facilities in Mr. Brotherston's department to pursue studies on the tick-virus relationship under the joint supervision of Dr. Campbell and Mr. Brotherston.

The programme of research originally proposed, comprised three sections:

1. An examination of methods for the isolation of virus from ticks.

2. The application of such a method to a survey or incidence of infection in field tick populations in the area of special interest in Argyllshire.

3. The laboratory study of the vector-virus relationship, with reference to such aspects as the threshold level of viraemia for virus uptake by the tick, the multiplication of virus in the tick and the possibility of transovarial passage of the agent.

Under the first item of the programme the use of cell cultures was tested as an alternative to mouse inoculation for the isolation of virus from ticks. The tests soon reached a stage where it was logical to proceed with the survey of incidence of infection in/
in questing ticks. Levels of tick infestation encountered on sheep pastures at numerous sites in Argyllshire were low and it became evident that one person would be unable to collect sufficient material for extensive survey of infection in questing tick populations. It was decided to concentrate instead on laboratory study of the vector-virus relationship.

The inadequacy of extant information on the course of viraemia and immunity in the acute phase of infection in the sheep precluded an immediate experimental approach to section three. Studies on viraemia and immune response had to be undertaken as a preliminary to tick-feeding experiments. The opportunity was taken to make detailed observations on the clinical features of the disease in sheep. This information not only facilitated the designing of tick experiments, but also allows some deductions to be made about the role of the sheep as a donor of virus for the tick.

In the discussion of the project, the findings in sheep and tick are examined separately and are then related to each other in an attempt to assess their epidemiological significance.
I. \textbf{THE ISOLATION OF LOUPING ILL VIRUS FROM TIKES.}

The recovery of virus from the vector is integral to the study of a vector-virus relationship and the examination of methods for carrying out such isolations formed an obvious starting point for this programme. Mouse inoculation is the definitive procedure. The intention was to examine whether an alternative would offer advantage in sensitivity, convenience, labour or time required.

The cell culture technique has widespread application in virology and this was extended to the direct recovery of arbovirus from the vector, for use in population surveys of certain mosquito-borne viruses (Welsh, Neff and Lennette, 1958a,b; Diercks and Hammon, 1959). The technique had to accommodate the toxicity of arthropod tissue to the mammalian cell monolayers on first passage.

With the object of evaluating the use of cell culture for the isolation of loping ill virus from ticks the investigation covered in sequence:- the preparation of tick pool suspensions and elimination of contaminant micro-organisms, the estimation of toxicity of the tick inocula for the cell monolayers, the examination of the effect of the tick material on the cytopathogenicity of mouse brain virus, and the experimental recovery of virus from infected ticks. Comparative information on the relative efficiency of cell culture and mouse inoculation was then accumulated in the later course of the programme.
Arboviruses of the B group are poor cytopathic agents (Libiková, 1962; Henderson and Taylor, 1960), but titration of louping ill virus by its cytopathogenic effect had been reported (Oker-Blom, 1956, 1959; von Zeipel and Svedmyr, 1958; Williams, 1958a). The pig kidney method of Williams, although subject to the disadvantages of primary cell culture, appeared most promising for the present purposes and, furthermore, was already in use at Moredun Institute.

MATERIALS AND METHODS

Viruses. The reference strain of louping ill virus, currently designated LI 31, had been maintained at Moredun Institute for over thirty years by an unrecorded number of serial intracerebral passages in sheep interrupted by prolonged periods of storage in dried or frozen brain. It was originally isolated from a field case of the disease in a sheep. The virus was used throughout the study in lyophilised form in its third and fourth mouse passages from sheep brain 180.

LI 68 is a recent field isolate from the brain of a four-year-old Scots Blackface tup which had died on Blarcreen Farm, North Connel, Argyllshire, in October 1962. It, likewise, was used in third and fourth passage lyophilised mouse brain form.

Each freeze drying ampoule was prepared with 0.25 ml of 10% mouse brain antigen in inactivated horse serum and was sealed under vacuum. Reconstitution in 2.25 ml cell culture medium/
medium or phosphate buffered serum—saline was considered to yield a $10^{-2}$ dilution of whole mouse brain.

**Mice.** The Moredun colony of Schneider Swiss albino mice was established in 1942 from stock supplied by the Medical Research Council. It is a conventional closed colony of heterogenetic mice maintained by random breeding. The mice are free from the major murine diseases including ectromelia and salmonellosis. Apart from a minor outbreak, in two adjacent cages, of a diarrhoea associated with a Paracolon bacterium, no trouble was experienced with intercurrent disease in the mice used in the study.

For routine purposes three-week-old mice were injected by the intracerebral route under light ether anaesthesia. The volume of inoculum was 0.03 ml and it was administered by 0.25 ml tuberculin syringe with $\frac{1}{2}$" 26 B.W.G. needle. The technique for one-day-old suckling mice was to pass the needle forward under the skin along the back to deliver 0.01 ml into the cranium, and then to deposit a further 0.02 ml subcutaneously as the needle was withdrawn.

Deaths during the first four days after inoculation were discounted as resulting from traumatic, toxic or contaminant causes, and the result of the test in such instances was scored from the remainder of the group of mice. Louping ill mortalities occurred typically between the fifth and fourteenth days, with the majority of mice given high doses of virus dying at the beginning of this period./
period. Observation was discontinued after twenty-one days.

Cells. Pig kidney cells were prepared by trypsin-dispersion and grown out in Roux flasks following the technique used by Williams (1958a,b), except that the maintenance medium of 0.5% lactalbumen hydrolysate, 10% pooled calf serum and antibiotics, was made up in Hank's instead of Earle's saline. After incubation for seven days the cultures were ready for trypsin-verene harvesting and seeding into 'Pyrex' tubes (16 x 150 mm) containing 'flying' coverslips (c.6 x 22 mm), at the rate of 3 x 10^5 cells per tube, in 1.0 ml of medium. The medium was usually replaced with 1.5 ml fresh medium on the day after seeding. Monolayering was usually complete after a further twenty-four hours at 37°C.

Line cells studied included:— a HeLa line obtained from the Virology Unit of Dr. R.H.A. Swain of the Department of Bacteriology and Virology, University of Edinburgh, in 1963; a 'Stice' line of pig kidney epithelium, presumably tracing back to Dr. E. Stice of the Cutter Laboratories, Berkeley, California, and supplied in 1963 by Dr. G. Fraser, Department of Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh; a baby hamster kidney (BHK 21) line received from Dr. I. Macpherson of the Department of Virology, University of Glasgow, in 1963, and 'PK 15' pig kidney epithelial cells, which came from the American Type Culture Collection Cell Repository, Rockland, Maryland, U.S.A., in 1965, with a history of derivation from clone '15' of a line established by Dr. E. Stice in 1955.
The BHK 21 were maintained in Roux flask or baby-bottle cultures with Paul's modification (1960) of Eagle's (1955) medium, plus 10% calf serum, while all the other line cells were grown in Eagle's (1959) medium with 10% calf serum. Weekly sub-culturing of the stock cultures followed trypsin-versene breakdown in all but the HeLa cultures, for which 0.02% versene was used.

Ticks. For the initial toxicity experiments, unengorged *Ixodes ricinus* L. nymphs were obtained from Mr. D.H. Kemp of the Zoology Department, University of Edinburgh. They had been reared from engorged females collected at random from Argyllshire sheep during 1963, by Mr. C.C. Bannatyne, Veterinary Investigation Officer, Oban. The larvae derived from these females had been fed on hedgehogs in the laboratory. Further tests were done with adults taken in various degrees of engorgement from sheep and cattle in Argyll by the author.

For the trial recovery of virus from tick, replete and partially engorged imaginal ticks, removed from field cases of louping ill were supplied by Mr. Bannatyne.

Finally, opportunity was taken to compare the sensitivity of the cell culture and mouse inoculation methods by utilising the ticks infected in the tick-feeding experiments described in Section III.

**Tick pool suspensions.** Tick pools were weighed to 0.0001 g. and maintenance medium added to the nearest 0.1 ml for the initial dilution. Trituration was achieved variously by pestle and mortar, ground glass tissue grinder or motor-driven teflon plunger. The suspensions were subjected to two cycles of rapid freezing and thawing to release virus from tissue. Freezing was accomplished by immersing the tubes of suspension in alcohol/
alcohol with dry ice at $-70^\circ\text{C}$ and thawing was done in a $37^\circ\text{C}$ water bath. After centrifugation (3,000 g, for 20 minutes) the supernatants were filtered in the initial experiments through 'Oxoid' membranes in modified Hemming's filters. Sometimes preclarification was necessary and the supernatants were then first filtered through 'Ford Sterimats' previously rinsed with copious sterile saline to reduce toxicity. Filtration was replaced by high speed centrifugation (12,000 g, for 30-60 minutes) in most of the later work.

All operations were carried out in the cold (0 to $4^\circ\text{C}$) as far as possible.

Titrations. Strict aseptic virological technique was applied throughout the cell culture work. Manipulations were carried out under a perspex hood fitted with bunsen burner. Dilutions of virus and tick suspension in medium were prepared in the customary manner using a fresh sterile pipette for each serial transfer.

Suspensions of virus antigen or tick pools in course of virus assay were made up and introduced to the cell cultures in medium which had been cooled to $4^\circ\text{C}$. The remainder of each original tick pool was stored for future reference at $-20^\circ\text{C}$.

Toxicity of tick suspensions was initially titrated at $\log_{10}$ intervals, and in later experiments at $\log_{2}$ intervals between 1 in 50 and 1 in 6,400 of tick material per ml. Virus titrations were done in $\log_{10}$ interval dilutions up to $10^{-9}$ of the reconstituted mouse brain antigen. The volume of inoculum in both instances/*

* Average pore diameter 0.5 μ.
instances was 1.0 ml. When tick suspension and virus were to be inoculated together, each was made up at double the final concentration required. The two were then added in equal amounts before use.

After adsorption for one hour at $37^\circ C$ the inocula were replaced by 1.5 ml of fresh medium. In the later stages of the work tick suspensions were left in contact with the monolayers for twenty-four hours or even for the full five-day observation period.

Three to ten (or even twenty) tubes per dilution of the material under titration, plus an equal number of controls, were used on different occasions. The monolayers were observed daily by low power microscopy and the medium changed when excess acidity became apparent, usually on the third day.

A proportion of the coverslips was fixed, stained and mounted on slides on each of the five days. The cells were stained with either Giemsa or haematoxylin and eosin. The degree of degeneration of monolayers was classified on an arbitrary scale 0 to 4+ in relation to controls and 2+ degeneration was considered indicative of virus cytopathogenesis or tick toxicity.

Tick pools under assay for toxicity were also inoculated intracerebrally into three to five 3-week-old mice at the lowest dilution. This monitored the possibility of a tick virus, rather than toxicity, causing monolayer degeneration. As a further check, material from degenerated monolayers was serially passaged.
Five or six 3-week-old mice per dilution were inoculated in parallel with the cell cultures where tick-pools were undergoing assay for virus. The culture fluids at each dilution were pooled on the fifth day and inoculated into a further three to five mice. Thus each of these tick pools was titrated in terms of mouse lethal doses \( (LD_{50}) \), cytopathic doses \( (CPD_{50}) \), and cell or "tissue" culture infective doses \( (TCID_{50}) \), of virus.

The fifty per cent end-points were estimated by the moving average probability method of Thompson (1947).

**Neutralisation tests.** Tick pools being examined for virus were concurrently screened with hyperimmune sheep serum. Test cultures were inoculated with final concentrations of 1:200 tick suspension plus 1:50 immune serum in medium, after the mixture had stood for one hour at 22°C. Controls on each test occasion consisted of dilutions of LI 31 virus, approximating to 100 and 1,000 \( LD_{50} \) per 1.0 ml, likewise treated with immune serum and inoculated into 5 replicate cultures, the lower virus dose being tested also with non-positive sheep serum. Pooled culture fluids of each test group were inoculated into 3 to 5 mice on the fifth day.

Three isolates from ticks were compared with LI 31 virus in cross-neutralisation tests. Log\(_{10}\) dilutions between $-2^*$ and $-3$ of the viruses in serum-saline were added in equal volume to 0.02 ml/

* No end-point was determined for the titre of this serum since it neutralised 100 metabolic inhibition doses of virus at the highest dilution tested, namely, 1:6,250 \( (P_{40}) \).
ml amounts of inactivated (56°C for 30 minutes) test and control sheep sera. The mixtures were stood at 22°C for one hour and then transferred to an ice bath. Each dilution of virus with control or test serum was inoculated intracerebrally into 5 mice. The mice were observed for 21 days and 50% end-points of the titrations were calculated by the method of Thompson (1947). The log neutralising index of a test serum was calculated as the difference between the negative log LD₅₀ titres of virus with control and test serum. Interpretation followed convention; an index of 1.7 or over was taken to indicate positive neutralisation while indices between 1.0 and 1.7 were considered doubtful (Hammon and Work, 1964).

RESULTS.

Ground glass tissue grinders proved most suitable for the preparation of suspensions of small numbers of ticks, but these varied individually in the efficiency with which they macerated the unfed immature stadia. Filtration of the suspensions was hampered by frequent clogging of the membranes in spite of preclarification of the material by centrifugation and passage through pad filters. Once regular access to a high-speed centrifuge was acquired, filtration was discontinued. High-speed centrifugation had the additional advantage of reducing fluid loss in the processing of the suspensions so that small tick pools could be handled. Thus it was found practicable to use as few as 25 or 50 flat nymphs in 1.7 ml of diluent to prepare a 1:200 or a 1:400 concentration of tick tissue in the fluid.

Contamination in batches of pig kidney cultures inoculated with tick suspensions was common at first and a yeast-like organism was most often observed. Little difficulty was encountered after the change to high-speed centrifugation even although nystatin was omitted from the culture medium.
Toxic degeneration of monolayers became evident within 24 hours following the introduction of high concentrations of tick tissue, with granularity of the cells seen by low-power microscopy. Individual cells became rounded and the medium contained detached debris. The monolayer stripped easily if the tube was shaken. This effect was evident with inocula consisting of 1.0 ml of tick suspension at a dilution of 1:100 or lower, but not at 1:200. This distinction was sharp and regular, with 3+ to 4+ degeneration (complete stripping) of monolayers at 1:100 of the tick tissue in the medium, but with no visible damage to cells at 1:200 (Figure 1a).

On occasion it appeared as though tick tissue was beneficial to cell growth at dilutions which were not actually toxic. Monolayers inoculated with 1:200 or higher dilutions of the tick material appeared to be denser than control cultures when the coverslips were stained and mounted, as though the tick material had contributed nutrients to the maintenance medium.

The dividing line between toxic and non-toxic concentrations of tick tissue was constant for eggs and any of the tick instars, irrespective of the state of engorgement, and was not altered by the length of time for which tick material was left in contact with the monolayers before the medium was changed. The toxicity did not persist in subcultures.
Fig. 1. The use of monolayer cell cultures for the isolation of virus from tick tissue suspensions. Coveralip cell cultures are shown stained and mounted on microscope slides.

a) The toxicity of tick tissue to pig kidney monolayers is evident at a concentration of 1:100 of tick tissue in growth medium but not at a concentration of 1:200 or less.

b) The cytopathogenic effect produced by mouse-brain virus is evident up to the highest dilution tested here, namely, $10^{-7}$.

c) The cytopathogenic effect produced by mouse-brain virus is not inhibited by the inclusion of tick tissue at a concentration of 1:200 in the medium.

d) Mouse-brain virus titrated by its cytopathogenic effect on HeLa cell monolayers. Degeneration is present in cultures inoculated with virus up to a dilution of $10^{-6}$ and in two out of five cultures inoculated at a dilution of $10^{-7}$.

e) Following tick passage the ability of mouse-brain virus to produce a cytopathogenic effect in pig kidney cell monolayers is reduced. Partial degeneration of monolayers is evident in cultures inoculated with virus up to a dilution of $10^{-5}$ only.

f) The original mouse-brain virus produces degeneration of pig kidney monolayers up to a dilution of $10^{-7}$. 
subcultures.

The cytopathogenicity of mouse-brain virus was not affected by non-toxic levels of tick-tissue (Figures 1b and 1c). Various combinations of virus and tick material concentrations were tested. Results with the laboratory virus, LI 31, and the recent field isolate, LI 68, of ovine origin, were similar.

It remained to test the method for actual recovery of virus from infected ticks. For this purpose it was convenient to use material taken from field cases of louping ill, since tick-feeding experiments were not as yet under way at the laboratory. Partially engorged females taken from eleven suspected cases of the disease in Argyllshire were obtained through Mr. Bannatyne. Virus was recovered from tick pools in three instances.

One pool of ten partially engorged females was taken from a hogg which was found to have clinical symptoms of louping ill on 1st April, 1964. The animal was killed in extremis two days later but virus was not recovered from the brain, although histopathological lesions of encephalitis were reported. This sheep was one of a number from Blarcreen Farm, North Connel, which were being over-wintered on Glencruitten Golf Course, Oban. The pool suspension killed mice at a concentration of 1:50 of tick tissue in culture medium. Pig kidney monolayers inoculated at a 1:200 dilution of the material did not show microscopic evidence of cytopathogenesis, but the staining of the coverslips appeared to be less intense than that of control cultures when compared macroscopically. The /
material was transferred serially in cell culture for five passes without an apparent increase in cytopathogenicity. At each pass aliquots of the culture fluid regularly killed mice, with symptoms typical of louping ill. Incorporation of reference hyperimmune louping ill antiserum in the culture medium at a final concentration of 1:50 resulted in failure of the subinoculations to kill mice. Cultures inoculated at a $10^{-3}$ dilution of the original tick pool were not found to contain virus on mouse subinoculation.

The isolate was designated LI 2/7, Glencruitten, 1964. The 2/7 is the pool serial number and refers to the seventh pool of ticks obtained on the second field trip to Argyll in 1964.

The other virus recoveries were made from tick pools numbered B/11 and B/13, being the eleventh and thirteenth suspensions prepared from material forwarded to the laboratory by Mr. Bannatyne. Tick pool B/11 consisted of five females taken off a lamb on Glenforsa Estate, Mull, and B/13 was a pool of six females from a sheep on Barguillean Farm, Taynuilt. Both animals were suspected of having louping ill in May, 1964. The suspensions of tick tissue failed to kill mice at the initial concentration of 1:40 in culture medium. Monolayers inoculated with the suspensions at a dilution of 1:200 appeared to stain less intensely than control cultures. No other signs of cytopathogenesis were evident in three serial culture passages. Aliquots of culture fluid from the third passage of both pools killed three-week-old mice, but failed to do so in the instances of parallel cultures grown in the presence of a 1:50/
1:50 concentration of hyperimmune louping ill antiserum. Cultures inoculated with a $10^{-3}$ dilution of the original tick suspensions failed to yield virus, even on passage.

After three passages in cell culture and one in mice, each of the three isolates was further inoculated into mice for the purpose of preparing lyophilised virus stock for future study. Full neutralisation tests were not convenient at this juncture, but the screening tests applied during cell culture passage left little doubt about the identity of the agents isolated. The three viruses were subsequently used in the sheep studies reported below (Section III) in which the serum antibody responses of the sheep were determined by using the laboratory virus strain, LI 31, as diagnostic antigen. Antibodies were demonstrated at high titre.

Cross-neutralisation tests in mice between the three tick viruses and LI 31 were done with sera taken from the sheep on the seventh day after inoculation, although such early sera could not be expected to have high titres. The choice was, nevertheless, deliberate, since arbovirus antisera become widely cross-reactive after hyperimmunisation and are most specific early in the course of a primary infection (Porterfield 1962a). The control serum was taken from sheep 1007 seven days after it had been inoculated with non-infective lyophilised mouse brain. The test with LI 31 serum was done first and the three other sera were then tested simultaneously. The log neutralising indices are shown in table 1. Some of the figures reflect no significant neutralisation/
TABLE 1.

Cross-neutralisation between LI, 31, 2/7, B/13 and B/11 viruses.

Antisera:

<table>
<thead>
<tr>
<th>Viruses</th>
<th>31</th>
<th>2/7</th>
<th>B/13</th>
<th>B/11</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>3.2*</td>
<td>1.9</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>2/7</td>
<td>0.2</td>
<td>1.4</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>B/13</td>
<td>2.0</td>
<td>2.0</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>B/11</td>
<td>1.8</td>
<td>2.0</td>
<td>1.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* $\log_{10}$ neutralising index.
neutralisation; they lie in the doubtful range between 1.0 and 1.7.
The difference, however, between the three logs of homologous virus
which the LI 31 antiserum neutralised and the 0.2 logs of LI 2/7
which it neutralised, is striking. In general the LI 2/7 virus was
least neutralised by heterologous sera but LI 2/7 antiserum neutral-
ised the other viruses. The information is too limited for firm
conclusions to be made, but it did suggest the need for further
comparison of the strains. A full antigenic study lay outside of
the scope of this project, however, and was not pursued beyond a
preliminary examination of the suitability of the Moredun mice for
use in protection tests. The mice were resistant to large doses of
virus administered peripherally without previous immunisation, but they
could not be protected against intracerebral challenge with homologous
virus even by five intraperitoneal injections of live homologous
virus. Peripheral administration of virus followed by 2% starch
injected intracerebrally also regularly killed the immunised mice.
The introduction of mouse strains bred for use with encephalitis
viruses was prohibited owing to the lack of quarantine facilities at
the Institute at the time of this work.

While louping ill virus had been recovered directly from
ticks in pig kidney cultures, the method relied on mouse
subinoculation to demonstrate virus. Haemadsorption was tried as
an alternative to cytopathogenesis as an indicator of virus recovery.
The method of Buckley (1959) using arbovirus haemagglutination
buffers (Clarke and Casals 1958), as well as the original Shekolov,
Vogel and Chi (1958) haemadsorption technique was attempted.
attempted. Erythrocytes from sheep, rabbits, guinea pigs and geese were all used without success.

Several line cells were tested for susceptibility to louping ill cytopathogenesis, although it was unlikely that they would prove more sensitive as indicators of primary isolation than the pig kidney cultures. All of the cells listed under 'materials' showed some evidence of virus activity at the lower dilutions tested, but HeLa cells appeared to be most affected (Figures 2a and b). Titration of unadapted laboratory strain virus was possible on HeLa monolayers by adjusting the seeding rate to 200,000 cells per tube in 1 ml of medium. Heavier seeding resulted in overgrowth of the cells masking cytopathogenesis, while lighter seeding resulted in failure to monolayer even in control cultures. Monolayers inoculated with the lowest dilution \(10^{-1}\) of mouse brain virus appeared to be less degenerated than higher dilutions (Figure 1d). The titration on HeLa cells shown in the photograph was carried out in parallel on six mice per virus dilution. On the basis of microscopic scoring of the partial cytopathogenesis of the monolayers the fifty per cent end-point of virus activity \(\text{CPD}_{50}\) may be calculated by the method of Thompson (1947) as follows:

\[
\text{Log Dose} | \text{CPE} | p | \text{Moving average} / p
\]

\[
\text{Log Dose} | \text{CPE} | p | \text{Moving average} / p
\]
Fig. 2. The cytopathogenic effect produced by stock mouse-brain virus in HeLa cell monolayers. a) Control culture at six days showing excessive growth of cells. b) Infected culture at six days showing rounding and stripping of cells.
Since the inoculum volume was 1.0 ml, the original material contained $10^{6.9}$ CPD$_{50}$ per 1.0 ml. In the mouse titration all the animals inoculated up to the $10^{-4}$ dilution of virus died, four out of six died at $10^{-5}$ and only one died at $10^{-6}$. The virus content of the original material calculated in the same way from the mouse/
mouse figures, therefore, is $10^{5.3}$ LD$_{50}$ per 0.03 ml. Correcting this figure for the inoculum volume (i.e. multiplying by $\frac{1.00}{0.03}$) the virus content becomes $10^{6.8}$ LD$_{50}$ per 1.0 ml, which is comparable to the figure for the cell cultures.

The calculation is included here as an example of the application of Thompson's method as used on all titrations in this programme. The 95% fiducial limits were not calculated for each of the numerous titrations involved in the project since it was assumed from similar work in the laboratory that these limits would fall within one log$_{10}$ dilution of the estimated 50% end-points in most instances.

Unlike the laboratory virus, the culture-passaged tick isolates failed to produce degeneration of line cell monolayers. As in pig kidney cultures, the presence of these viruses had to be demonstrated by mouse subinoculation.

Comparison of the sensitivity of pig kidney monolayers and mice for the recovery of virus from ticks was carried out with material from the tick-feeding experiments described below (section III). Ticks were infected by feeding them on sheep inoculated with the laboratory strain of virus.

Twenty-one pools of infected ticks were titrated in mice and on pig kidney monolayers. It was found that the ability of the laboratory virus to produce degeneration of cell cultures was markedly reduced following tick passage. Consequently, the fluids/
fluids derived from cultures inoculated at each dilution in a
titration were injected into a further group of mice after pooling
on the fifth day. In 17 of the 21 titrations the results of the
subinoculations were clear cut: all the mice within a group either
died or survived. This was taken to indicate the difference between
infected and non-infected cultures on the assumption that any virus
which was recovered in the cultures became amplified to a level at
which it regularly killed mice. In the four exceptions single mouse
deaths occurred and these may be assumed to be due to causes other
than louping ill.

Hypothetical 50% end-points for the infection of the cell
cultures (TCID\textsubscript{50}) could be set by the Thompson (1947) moving average
method at 0.5 log\textsubscript{10} higher than the maximum dilutions from which
culture fluids killed mice. It was found that the TCID\textsubscript{50} titres of
the tick pools were at an average of 1.3 log\textsubscript{10} dilution higher than
the corresponding direct mouse titration ID\textsubscript{50} titres, with a range
from 0.6 to 1.8 log\textsubscript{10} difference in the two levels. Since this
difference in titres corresponds closely to the difference in inoculum
volumes, the cell cultures and mice were effectively of equal
sensitivity to infection with virus from ticks.

Tick-passaged laboratory virus thus behaved in the same way as
the original mouse-brain stock as regards relative infectivity to
cell cultures and mice, but there was a marked difference in
cytopathogenicity. Mouse-brain virus was cytopathogenic even at the
minimal infective level for the pig kidney cultures. Virus from
ticks produced a partial cytopathogenic effect only when inoculation/
inoculation rates were well in excess of the minimum infective level. For example, figure 1c shows that tick pool 20 caused only slight degeneration of pig kidney monolayers at dilutions of $10^{-3}$ to $10^{-5}$. The culture fluids of this tick pool, which had a titre by direct mouse titration of $10^6$ ID$_{50}$ per 0.03 ml, killed five out of five mice up to the $10^{-7}$ level. The lyophilised mouse-brain stock of the same virus, LI 31, produced degeneration of monolayers up to the $10^{-7}$ level (figure 1f). Culture fluids from this titration also killed mice up to the $10^{-7}$ level, and the direct mouse titre was $10^6.3$ ID$_{50}$ per 0.03 ml.

Although the comparative titrations indicate that mice and cell cultures are equally sensitive to infection, in practice primary isolation of virus from field-collected ticks proved to be more successful with cell cultures than with mice. Two of the three isolations from field ticks reported above, were made in cell cultures but not in mice in the first instance. Similarly, three out of four viruses isolated in the survey of incidence of infection in wild tick populations (section II), were recovered in mice only after passage of the tick suspensions in pig kidney cultures. The fourth virus was recovered by direct mouse inoculation but not in pig kidney cultures.

Nevertheless, both three-week-old mice and pig kidney cultures appear to be inferior to one-day-old mice as critical means of testing for virus. In three instances in the course of the tick experiments (section III) virus was recovered from tick suspension in one-day-old mice where virus had not been demonstrated by either direct inoculation of three-week-old mice or by mouse subinoculation/
subinoculation following pig kidney culture passage.

In summary - Suitable suspensions of tick tissue for the inoculation of cell cultures were prepared by means of ground glass tissue grinders. Contaminant micro-organisms were eliminated from the suspensions by centrifuging them at 12,000 G. for 30-60 minutes. The suspensions were toxic to cell culture monolayers at a concentration of 1:100 of tick tissue in growth medium, but not at a concentration of 1:200 or less. The division between toxic and non-toxic concentrations of tick tissue was constant for eggs and any of the tick instars, irrespective of the state of engorgement, and was not altered by the length of time for which tick material was left in contact with the monolayers before the medium was changed. The toxicity did not persist in subcultures.

Non-toxic concentrations of tick tissue appeared to be beneficial to cell growth, as though tick material contributed nutrients to the medium. At such concentrations tick tissue did not affect the cytopathogenicity of mouse-brain virus: stock mouse-brain viruses could be titrated by their cytopathogenicity for pig kidney and HeLa cell cultures irrespective of whether or not tick tissue suspensions were included in the growth medium.

The use of cell cultures for the isolation of virus from ticks was tested with ticks removed from field cases of louping ill. Three-week-old mice were inoculated along with the pig kidney cultures for comparison. The same procedure was adopted during the survey of incidence of infection in wild tick populations (section II). A total of seven virus isolations were made. Five/
Five of the viruses were isolated in cell culture but not in mice. One isolation was made in mice but not in cell culture.

Primary isolates from ticks produced little evidence of cytopathogenesis in cell cultures; the presence of the viruses was indicated by the fact that infected cultures stained less intensely than control cultures. Virus isolation was confirmed by subinoculating culture fluids into mice. A limited number of serial passages of new isolates in cell cultures failed to exalt cytopathogenicity. The presence of the viruses in cell culture could not be demonstrated by haemadsorption.

Isolates were identified by a neutralisation screen test. The three initial tick isolates were used in a full cross-neutralisation test with the laboratory strain of virus. There were quantitative antigenic differences between the viruses.

Although new isolates were obtained from ticks more readily in cell cultures than in mice, comparative titrations with 21 pools of ticks infected with the laboratory strain of virus (section III) indicated that mice and pig kidney cultures are equally sensitive to infection. The cytopathogenicity of the laboratory virus, however, was reduced following tick passage. As with new isolates, mouse subinoculation had to be used to demonstrate the presence of the tick-passaged laboratory virus in cell cultures.

Both cell cultures and three-week-old mice appear to be inferior to one-day-old mice as critical means of testing for virus. In three instances in the course of the tick experiments (section III)/
(section III) virus was recovered from tick suspension in one-day-old mice where virus had not been demonstrated in three-week-old mice or in cell cultures.
II. SURVEY OF INFECTION IN WILD TICK POPULATIONS.

Louping ill is widely distributed in Argyllshire. The disease was confirmed by the Moredun workers in field specimens which Mr. Bannatyne obtained from numerous localities in the county. Three farms, with which good liaison had been established in the course of the investigations, were chosen for particular attention in the projected survey of infection in tick populations (Figure 3).

One of the selected farms, Barguillean, Taynuilt, consists of 3,000 acres of rough hill grazing with heather, and a further 1,000 acres of marginal wood and scrub. The altitude ranges from about 300 to 1,500 feet above sea level. Blarcreen, North Connel, is a similar, large property with three hill hirsels rising steeply from the sea loch, Etive. The lower slopes are heavily wooded and bracken is prominent in the vegetation. The third site, Glenshellach, Oban, is a small-holding consisting of a steep, treeless hillside and a few low-lying fields with patches of rushes where drainage is poor. This last farm is unrepresentative of hill-farming in that sheep and store-cattle are frequently bought in. The other two farms are stocked with bound flocks of Scots Blackface sheep.

The incidence of the disease in livestock is heaviest in the April-June period each year, with a second peak during September-November. With the exception of January, however, cases have been recorded in every month of the year, although rarely in/
Fig. 3. Sites from which confirmed cases of louping ill were recorded in Argyllshire, 1961 to 1965. Farms selected for survey of incidence of virus in questing ticks are shown at A (Glenshellach), B (Barguillean) and C (Elarcreen).
in December. Field trips in Argyll for tick-collecting were planned to coincide with the spring and autumn peaks of tick activity.

**MATERIALS and METHODS.**

**Tick collection and handling.** White blankets used for collecting questing ticks were prepared by fixing a 3" x 1" plank along the hem at one end to act as a weighted leading-edge. The blankets were then dragged over ground-layer vegetation by string attached to the ends of the plank. Terry towelling was later substituted for blanket, on the recommendation of Mr. Kemp, and was found to improve results.

The blankets were examined after every 100 to 200 paces walked. The under-surface was exposed in sections by rolling the blanket up on to the plank and the ticks were collected by suction apparatus (pooter) directly into 3" x 1" flat-bottomed tubes. Most of the ticks collected were nymphs but occasional batches of larvae were encountered. The few adults seen were pulled off the blanket by hand and put into 1/2" x 1 1/2" flat-bottomed tubes. Labels were included in all tubes and after they had been securely stoppered with cotton wool they were put into large, wide-mouth jars with screw tops. The jars contained a 2" layer of plaster of Paris on the bottom which was kept moist with water to maintain humidity at saturation point. The 3" x 1" tubes were also used separately with their own plaster layers.

Flat ticks not immediately processed for virus examination at /
the laboratory were found to survive well when stored at 4°C in the refrigerator.

Partially engorged ticks taken from sheep consisted almost entirely of adults. These were put into cotton-wool stoppered \(\frac{1}{2}\)" x 1\(\frac{1}{2}\)" tubes in the field and transported in plaster-jars. At the laboratory they were stored at \(-20\)°C until examined for virus.

**Tick pool examination.** Twenty-five out of every 200 field-collected nymphs were examined under a stereoscopic binocular to check the species.

The preparation and centrifugation of tick suspensions were as previously described.

Field-collected, flat nymphs were lighter than the laboratory-reared material used in the cell-culture toxicity tests; 0.0125 - 0.0234 g as opposed to an average of 0.0340 g per 100 nymphs. It was convenient, nevertheless, to continue to use 50 nymphs with 3.4 ml of culture medium to prepare suspensions. This allowed three pig kidney cultures and three mice to be inoculated with the original suspension after centrifugation. The common regimen for preparing arthropod suspensions for virus examination is, apparently, to use 50 individuals per 2 - 4 ml of diluent (Work, 1964).

The partially engorged females were used in pools of 5 individuals and weighed to make up suspensions of known dilution so that five cultures could be inoculated with 1:200 of tick tissue in /
in medium. Three mice were inoculated at the same time with the initial dilution of the suspensions, in some instances as low as 1:16.6 of tick tissue in medium.

The pooled culture fluids of each tick suspension were inoculated into three further mice on the fifth day, when the coverslips were stained and mounted. The culture fluids and, where possible, the original material were stored at −20°C for the 21 day mouse observation period.

RESULTS

A total of four weeks was spent in Argyllshire in four visits during the period from March to June, 1964. The results obtained by blanket dragging during this period were very poor. Apart from the three selected farms, numerous sites between Benderloch in the north and Lochgilphead in the south were tested. Blanketing was also attempted on the island of Mull. Different altitudes were tried and various types of vegetation were covered. Only on Blarcreen were sufficient questing ticks obtained to examine for virus, but more time was spent on this site than on the others. The total of 359 nymphs from Blarcreen were used as seven pools of 50 individuals each and no virus was recovered from them.

Infestation on sheep and cattle was noticed to be moderate or slight on the farms visited. About 20 partially engorged females on a sheep was usual. Small numbers of ticks were taken/
taken from animals for use in examining the toxicity of tick tissue to cell cultures as previously described. A few cases of louping ill were encountered and ticks were taken from these to test the use of cell culture for virus recovery as mentioned earlier (Section I).

The field trips were too expensive and time-consuming in relation to the results achieved and no further visits were made to Argyll in 1964. In 1965 three trips were made in the early part of the year, the last visit being for two weeks at the end of April. The results were again unsatisfactory and only by concentrating on Blarcrean for most of the last two-week visit were 925 nymphs collected. The ticks were collected on pastures where the ground-cover was three inches high at most. Adjacent rough grazing and bracken yielded far fewer ticks. Many of the nymphs were taken from points lying below the spring tide flood level of the sea loch.

The 925 nymphs were examined for virus as 18 pools of 50 individuals each. One of these pools, serial number 5/66, caused symptoms typical of louping ill in one mouse out of three, seven days after inoculation. The mouse was killed and brain material tested in three-week-old mice without causing death. The material was also tested in two litters of suckling mice and virus was recovered which subsequently killed three-week-old mice. In the instance of this isolate, virus was not recovered from subinoculation of culture fluid either in three-week-old or/
or in suckling mice.

A number of partially engorged female ticks were removed from sheep on Glenshellach and on Achnacone Farm, Glencoe, during the 1965 trips when blanketing proved fruitless. From Achnacone, 115 females were examined for virus as 23 pools of 5 ticks each and 155 females from Glenshellach formed 31 pools. Direct mouse inoculation with these 54 pools did not yield virus but three recoveries were made from the subinoculation of culture fluids. One mouse which died after being inoculated with culture fluid of pool 5/38, from Achnacone, was putrified by the time it was seen. Virus was then recovered by using the culture fluid of this pool, stored at -20°C, to inoculate two litters of suckling mice. The other two recoveries were from pools 5/25 and 5/45 from Glenshellach and Achnacone respectively. The culture fluids of these two pools killed three-week-old mice.

The four isolates were subjected to a neutralisation screen-test and stored at -20°C for future reference. For the test, 10^{-2} dilutions of infective mouse brain were added in equal volume to dilutions of a louping ill hyperimmune serum and a non-positive sheep serum respectively, to give final concentrations of 1:50 of the sera. At the same time two dilutions of LI 31 virus, containing 10^1 and 10^3 mouse ic LD_{50} per 0.03 ml, were treated in like manner. After standing one hour at bench temperature (22°C) the mixtures were each inoculated into 5 mice. The immune serum neutralised all four /
four isolates. The survey was abandoned after April, 1965, in order to concentrate attention on the more profitable laboratory studies. During May the blanketing activities were switched to Perthshire to collect material for use in the tick feeding experiments.

In summary - Tick recoveries from blanketing in Argyllshire were very poor. In the spring of 1964 a total of 359 nymphs were collected on one farm, Blarcreen, and these were tested for virus as seven pools, without positive result. Insufficient questing ticks for virus examination were collected at other sites.

During the spring of 1965, recoveries on Blarcreen amounted to 925 nymphs. These were tested in 18 pools and one virus isolation was recorded.

Another virus recovery was made from one of 31 pools of five partially engorged females (155 ticks) taken from sheep on Glenshellach in 1965. Twenty-three similar pools comprising 115 partially engorged females were taken from sheep on Achnacone, Glencoe, and these yielded two virus isolates.

The survey was abandoned after April, 1965, in order to pursue the laboratory studies.
III. EXPERIMENTAL STUDIES OF VIRUS IN HOST AND VECTOR

The recovery of arbovirus or antibody from vertebrates in an epidemiological survey will indicate that certain species are meeting infection but does not prove that they are playing a role in the circulation of the virus in the biocoenose. Critical evidence is obtained by determining the threshold level of virus in blood required for infection of the vector and then ascertaining to what extent infections in the particular vertebrate species are characterised by a viraemia of sufficient intensity and duration.

The concept of threshold levels of viraemia for arthropod infection was established in the classic mosquito studies with the North American encephalitides (Chamberlain, Sikes, Nelson and Sudia, 1954). A number of difficulties arise when this concept is applied to tick-borne infections. Ticks feed over a period of days and therefore, unlike mosquitoes, they experience a changing pattern of virus and antibody content in the blood meal. Moreover, the fate of the ingested blood is not constant throughout the period of feeding. Blood is utilised for growth in the initial slow phase of engorgement but up to three-quarters of the intake occurs by simple rapid expansion of the tick during the last twelve hours of feeding (Sutton and Arthur, 1962). In contrast to the forty-eight hours or so in which mosquitoes complete digestion, most of this blood may remain undigested for weeks while metamorphosis proceeds. Another obvious difference from mosquitoes is that three instars of the tick are parasitic. The sensitivity of/
of these to virus infection may vary.

The main purpose of this investigation was to determine the threshold for infection of the tick *Ixodes ricinus* L. with the louping ill virus. It was considered important to take cognisance of the points raised above in designing the tick feeding experiments and hence two preliminary obstacles had to be overcome. These concerned the choice of tick material and the inadequacy of information on viraemia and immunity in the early phase of infection in the sheep.

This tick is known to be difficult to maintain in culture and laboratory-bred nymphs and adults are erratic in their feeding rates. Thus the decision was made to use ticks which were collected as active questers in the field. The rearing of a suitable population of culture ticks would, in any event, have taken up too much of the time available. By immunising the virus-donor sheep to tick-borne fever it was hoped to avoid complication by introducing this disease with wild ticks. The possible presence of louping ill in such ticks will be discussed below.

The information required on experimental infections in sheep concerned the duration and intensity of viraemia as well as the appearance and rise of antibody levels. This was needed because it was planned to attach batches of ticks in such a manner as to ensure that the separate groups were experiencing differences in composition of the blood meal. It was logical to take the opportunity to extend the monitoring of experimental infections/
infections to a detailed clinical study of the disease in sheep.

MATERIALS AND METHODS

Viruses. The choice of virus for the clinical monitoring experiments was determined by the requirement of the Moredun workers for serological study of a number of field strains. The original three tick isolates (LI 2/7, LI B/11 and LI B/13) and the laboratory strain (LI 31) were included so that ten strains in all were involved. The particulars of LI 68 appear in Section I.

LI 174 had been isolated from a grouse brain received in July, 1963, from Speyside where there had been a history of deaths in young birds. It was used in lyophilised second mouse passage form.

LI 188 and LI 189 were isolates from ewe hoggs which had died in a severe outbreak of louping ill in October, 1963, on Barguilllean Farm, Taynuilt, Argyllshire. They likewise were used in second mouse passage form.

LI 226 was recovered from the brain of a 6-year-old ewe which died on Barguilllean Farm in April, 1964. This animal had undergone repeated vaccination. The virus was in its fourth mouse passage form.

LI 274 came from the brain of a hogg found dead on Glenomore Farm, Oban, Argyllshire in June, 1964, and also followed second mouse passage when used.

It was intended that each sheep should receive about $10^6$ mouse IC LD$_{50}$ of virus but there had been no opportunity to do full/
full titrations of the lyophilised field strains before use. On the basis of experience with the laboratory virus it was decided that a standard $3 \text{ ml of } 10^{-2}$ dilution of each virus would be administered to the sheep, and that mouse titration of the actual dose would be carried out simultaneously.

The virus suspensions in phosphate buffered serum–saline were injected subcutaneously in the short-haired area just posterior to the axilla of the sheep.

**Sheep.** The particulars of the sheep are listed in table 2. They were all from tick-free sources and were kept permanently housed at the Institute on a diet of concentrate nuts with hay and water *ad lib.* With the exception of two Scots Blackface cross-breds all sheep were either Cheviots or Cheviot cross-breds. Two animals were aged but the remainder were one or two years old when used. During experiments the sheep were housed in loose boxes.

Clinical monitoring was commenced up to three days before infection or infestation and was continued for fourteen days after, or beyond when symptoms persisted.

Temperatures were recorded at 9 a.m. and again between 4 and 5 p.m. each day. Blood was taken each morning, with due aseptic precaution, from the jugular vein. Large bore serum needles were used and the bleeding was done with as little excitement as possible so as not to distort the leucocyte picture. Three separate samples were taken on each occasion for the purposes of/
### TABLE 2.
**PARTICULARS OF EXPERIMENTAL SHEEP**

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of titrating viraemia, obtaining serum and making leucocyte
counts. The sheep were weighed on a portable machine every
second or third day.

Full post-mortem examination of animals which succumbed in
the course of experiments was avoided since this would have exposed
other workers to the hazard of louping ill infection. Autopsy was
limited to the removal of brain and cervical cord for
histopathological and virological examination, and to a superficial
inspection of the thoracic and abdominal viscera in situ. Samples
of lung, spleen, bone marrow and prescapular lymph node were taken
for histopathology in some instances.

Viraemia. Blood was collected in Bijou bottles which had been
prepared with 0.2 ml of sterile acid-citrate-dextrose (ACD)
(Clarke & Casals, 1958) anticoagulant. The dilution of blood, by
this volume of ACD, was disregarded. The amount of ACD was
sufficient to delay clotting while log<sub>10</sub> dilutions of blood were
made up in 10 per cent inactivated horse serum in phosphate-buffered
saline, pH 7.4. The serum-saline was held at 4°C in quantities
suitable for immediate use each morning.

At first the numbers of mice available were limited and only
three mice could be used per dilution of blood but later, in the
tick experiments, it became possible to use five. Until
experience had shown on which days the peak titres of viraemia
could be expected the mice were so allocated that the end-points
of virus level could not be determined in a few instances. Where/
Where the titration of sample tick pools had indicated the unlikelihood of transmission of louping ill only the lower dilutions of blood were tested.

The fifty per cent end-points were estimated by the moving average probability method of Thompson (1947).

**Blood cell counts.** Blood was collected in Bijou bottles prepared with approximately 8 mg of dry sequestic acid (versene) as anticoagulant, so that no dilution was involved. Counts were always done on the day of bleeding; usually shortly after the blood had been taken in the morning.

For the total leucocyte count, the appropriate blood dilution (1:20) was made in 2 per cent acetic acid solution in a 'strict' grade haemocytometer pipette. Five large squares were counted in a Fuchs-Rosenthal haemocytometer and the concentration of white cells per cubic millimetre calculated. Where large variation in the totals for each square indicated uneven filling of the chamber counts were repeated. The total counts were found to be of good reproducibility.

Straight-edged blood smears were made for the differential counts. These were fixed and stained by McNeal's tetrachrome stain (George T. Gurr & Co.). Counts were made along the edges by battlement traverse of the microscope field. Three hundred cells were usually classified and the results recorded to the nearest 1 per cent of each cell type. During leucopaenia it was sometimes possible to classify only 100 cells per slide and/
Immature cell types were not classed separately. The distinction made between monocytes and atypical lymphocytes was arbitrary but an effort was made to be consistent. The nuclei of monocytes were often distinctly lobular and were always more textured than those of lymphocytes. The cytoplasm of monocytes tended to show very slight focal metachromasia.

No consistent monitoring of erythrocytes had been intended in the louping ill sheep but a few examinations were made as a casual check. The red blood cell status of two tick-borne fever sheep was followed more thoroughly.

Few erythrocyte counts were made before this technique was abandoned as being too laborious in view of the relative imprecision of the information gained. Packed cell volumes were determined by M.S.E. capillary tube haematocrit. Haemoglobin concentrations were estimated by the acid-haematin conversion method (Gradwohl, 1956). The equivalent haemoglobin levels for the photometrically measured optical densities were read from a table in use in the clinical biochemistry unit at the Institute.

**Tick-borne fever counts.** A surveillance was kept for tick-borne fever bodies whilst carrying out the differential white cell counts. The choice of McNeal's stain had been made partly for this reason. Where infection was evident, 100 polymorphs were examined and the number parasitised was recorded.
Sera. Blood for serum was collected in sterile, dry McCartney bottles. For reasons explained later it was found desirable to adhere to a strict pattern for serum handling. (p.45) The blood was allowed to coagulate at bench temperature for thirty minutes after the bleeding and the clots were loosened from the glass. The bottles were then placed in the refrigerator at 4°C for three hours while shrinkage of the clots occurred. After centrifugation 0.5 ml amounts of serum were sealed into sterile ampoules. These were labelled and immediately stored in the deep freezer at -20°C, to be thawed only once before use. As a routine, fifteen ampoules were prepared daily from the blood of each sheep on experiment.

Neutralisation tests. Cell culture metabolic inhibition neutralisation tests were based on the technique of Kaariainen and Halonen (1961) and Kaariainen (1965).

In this test virus activity is manifested by its suppression of cell metabolism. Phenol red in the culture medium turns yellow with the acidity produced by growing cells. Virus infection inhibits growth and prevents the indicator change. Neutralisation of virus by specific antiserum results in normal cell growth and colour change. Cells are seeded at a rate which will ensure that control cultures attain end-point acidity between the fifth and seventh days. Virus inhibition of cell metabolism is delayed and hence too high an initial concentration of cells can impair the test by making the medium acid before cell damage occurs.
The origin and maintenance of the HeLa cell stock cultures has been detailed in Section I (page 7). Cells harvested in 0.02% versene were suspended in a medium of critical composition, as determined by Kaariainen (1965). The medium consisted of Eagle's minimal essential amino acids (1959) in Hank's salt solution with 0.1% glucose and 0.05% sodium bicarbonate, plus 5% inactivated calf serum. The high levels of antibiotics, namely, 400 units penicillin, 400 mcg streptomycin and 50 mcg nystatin per ml, which had been incorporated to permit the test to be carried out in disposable plastic cell culture panels, were retained to avoid buffering alterations.

Preliminary HeLa cell titrations were done with 0.2 ml volumes of doubling dilutions of cell suspension containing 160,000 to 5,000 cells, together with 0.4 ml of medium. Two types of plastic panel tested proved unsuitable in that they supported HeLa cell growth only in concentrations so great that acidity of the medium supervened in one or two days. Growth of HeLa cells was improved by replacement of the liquid paraffin used to seal the culture wells by adhesive film. The cells grew better however, in rubber-stoppered tubes (16 x 150 mm). Twenty thousand cells in tube culture sufficed to produce a colour change corresponding to a final pH of 6.6 in six or seven days at 37°C, and this regimen was adopted for further use.

Virus made up in log_10 dilutions was titrated by adding 0.2 ml amounts to 0.2 ml of cell suspension and 0.2 ml of medium in each/
each of five tubes per dilution. Simultaneous comparative
titrations were made by intracerebral mouse-inoculation.

Ampoules of sera stored at -20°C were thawed and inactivated
at 56°C for 30 minutes, before dilutions in five-fold steps between
l in 10 and l in 6,250 were prepared in medium. Each serum
dilution was then added to an equal volume (usually 2 ml) of a
suspension containing one hundred metabolic inhibition doses
(MID50) of virus per 0.2 ml. After allowing neutralisation to
proceed for one hour at room temperature (22°C), 0.4 ml of serum-
virus mixture was added to 0.2 ml of cell suspension in each of
five tubes per serum dilution.

A labile serum factor could not be discerned in the range
of dilutions used since the titre of fresh sheep serum remained
unaltered after inactivation. Nevertheless, it was regular
practice to inactivate all sera before testing.

Titrations of virus-and cell-controls were done along with
each test. Daily serum samples from individual sheep taken
before inoculation and on each of the first fourteen days of
looping ill infection were tested simultaneously, so that on
each test occasion negative and positive sera of different titres
were included.

Results were recorded on the sixth or seventh day of
incubation when control cell cultures were at pH 6.6. The
difference between virus-positive (pH 7.4) and virus non-positive
tubes (pH 6.6) was obvious, but in instances of doubt visual/
visual comparison of the cultures with 'Capillator' tubes (B.D.H.) was resorted to. A colour corresponding to pH 6.8 or under was then arbitrarily taken to indicate positive cell growth or virus-neutralisation, while pH 7.0 or over was taken to signify virus growth.

The fifty per cent end-points of virus and serum titres were estimated by the moving average probability method of Thompson (1947).

Examples of cultures with neutralisation are shown in figure 4.

**Haemagglutination-inhibition tests.** The haemagglutination-inhibition tests were based on the standard arbovirus techniques of Clarke and Casals (1958).

**Antigen.** The test was not in use at the laboratory at the outset of this work and the author first prepared antigen by the alkaline extraction method. The titre of this antigen expressed as the reciprocal of the end-point dilution of 10% mouse brain material, was 64, i.e. 1 in 640 of the original mouse brain, at an optimal test pH of 6.4.

Subsequently, Mr. J. Campbell used acetone-ether extraction to prepare antigen of much greater potency. Titres of 256, 512, and on one occasion even 1024, were attained. By the definitive sucrose-acetone method, the author also produced antigens with titres of 256 and 512. These titres are considerably higher than any previously reported for louping ill./
Fig. 4. Metabolic inhibition neutralisation test with sera taken from immune sheep 1CO3 on fourth and fifth days after tick infestation. Colour change of growth medium from red to yellow indicates that neutralising activity was present in both sera to a dilution of 1:50. The photograph fails to convey accurately the colour changes actually observed.

Fig. 5. Haemagglutination-inhibition test of sera taken during the first fourteen days of infection in a sheep. Antibodies appeared on the sixth day and reached the highest titre tested here, 1:2,560, on the tenth day.
The sucrose-acetone extraction differed from the original Clarke and Casals method in some details. Three-week-old mice sufficed in place of suckling mice and these were inoculated intracerebrally with 0.03 ml of a $10^{-2}$ dilution of lyophilised LI 31 antigen. The brains of the mice were harvested when the individuals were obviously ill and were stored at $-20^\circ C$ for subsequent use.

A 20% brain homogenate in 8.5% sucrose solution was prepared with a motor-driven teflon plunger and care was taken to add it dropwise to briskly-stirred cold acetone for the first extraction. After the third change of acetone drying was completed by high vacuum pump. The fine pink powder was reconstituted in sufficient borate-buffered saline (BS), pH 9.0, to make up the original homogenate volume, in place of the 0.4 volume of physiological saline recommended for antigens to be used in complement-fixation tests. This suspension is the haemagglutinating antigen in subsequent references.

The antigen was deproteinised in the cold by the standard protamine sulphate precipitation method and then centrifuged for one hour at 10,000 rpm. At this point the supernatant often showed only partial haemagglutination at low dilutions. Contrary to the suggestion of Clarke and Casals it was found that antigen of eventual good quality need not show any evidence of activity before protamine treatment.

Further/
Further precipitation occurred if the antigen was diluted in BS and stored at 4°C. Thus over the course of a week or two each antigen was subjected to several cycles of high-speed centrifugation until good haemagglutination patterns with a stable end-point were obtained. The fluoro-carbon alternative to the protamine treatment was found to be less satisfactory.

Antigen was stored at 4°C in dilutions of 1 in 8 or 1 in 16 in BS. A limit to the storage period was not determined, but a sample of the original alkaline extract antigen showed only a two-fold drop in titre after two years at 4°C.

Sera. Preliminary comparisons were made between bentonite- and kaolin-absorption and acetone extraction for the removal of non-specific haemagglutinin inhibitors from test sera. Positive and negative sera of humans, sheep and mice were treated by all three methods, both with and without prior inactivation. The 1% bentonite absorption method of Williams and Thorburn (1961) was found to give results closest to the reference acetone extraction procedure. Nevertheless, the acetone technique was chosen for use throughout this programme.

It is interesting to note that inactivation appeared to alter results in these trial extractions. For this reason, and because previous experience with repeated freezing and thawing of serum stored in bulk had shown that the titres did not remain stable, sera were stored at -20°C in small samples to be thawed only once before use.
After thawing 0.2 ml amounts of the sera were extracted in the prescribed manner and then the reconstituted 1 in 10 dilutions in BS were absorbed with packed goose erythrocytes to remove non-specific haemagglutinins. The centrifuged supernatants were then ready for use in the test at a starting dilution of 1 in 10.

**Erythrocytes.** Two domestic white ganders were bled alternately for the cells used in these tests. The preparation of the erythrocyte stock suspensions in dextrose-gelatin-veronal buffer was as described by Clarke and Casals, but they were standardised by spectrophotometer at a slightly higher optical density than recommended (nearer to 5.0 than 4.5 at a wavelength of 490 nm). This improved the haemagglutination patterns and was necessary in order to bring the settling time of the test down to the optimal one to two hour period.

**Tests.** The tests were done in the standard W.H.O. pattern agglutination trays in the manner described by Clarke and Casals. As with neutralisation tests, the sera of an individual sheep taken before inoculation and on each of the first fourteen days following louping ill infection, were tested together. Standard positive and negative sera were included on each test occasion. Doubling dilutions of 0.2 ml volumes of each serum between 1 in 10 and 1 in 2560 were prepared in fresh 0.4% bovine albumen borate-saline (BA-BS), pH 9.0, in separate columns of wells. Sera with titres higher than 2560 were taken to end-point dilution in subsidiary tests. It was found to be sufficient to rinse out pipettes three times with BS between serial/
serial transfers in making up the dilutions, but a clean, short pasteuer pipette was used with the automatic pipetting syringe for each separate serum. An extra well at the 1 in 10 serum dilution served as control for the presence of non-specific haemagglutinating activity in sera. To these serum controls, 0.2 ml of BA-BS was added, while 4-8 units of haemagglutinating antigen in 0.2 ml BA-BS were distributed into the test serum wells. Finally, 0.4 ml amounts of BA-BS were added to enough wells to serve as erythrocyte controls and to allow control titration of the antigen over the range from stock-concentration to beyond the known end-point. After 0.4 ml stock antigen had been added to one empty well and to one of those with 0.4 ml BA-BS the trays were held overnight at 4°C to allow serum-antigen fixation.

On the following morning a 1 in 24 dilution of the stock goose erythrocytes was prepared in fresh, pre-cooled phosphate buffer. This buffer was so constituted that it would adjust the final pH to 6.4, when added in equal volume to the test reagents. The dilutions for the antigen titration were then completed and 0.4 ml volumes of the erythrocyte suspension added to all wells. Trays were shaken gently to ensure mixing of reagents, and they were then left for two hours to settle at room temperature (22°C) before the tests were read.

Test patterns ranging from complete haemagglutination to discrete button formation were classified on the arbitrary scale 0 to 4+. In cases of doubt the partial haemagglutination pattern 2+ was taken as an end-point for antigen activity.
activity. Conversely, in the serum tests, the 3+ pattern was considered to be the inhibition end-point. As a rule, serum end-points were abrupt and distinct. The results were highly reproducible.

The complete test of the sera of one sheep is shown in figure 5.

**Ticks.** *Ixodes ricinus* L. nymphs and a few adults were collected by blanket dragging on Acharn Farm, Killin, Perthshire, during May 1965. The farm was selected for its reported freedom from louping ill in recent years and because blanketing yields there were heavier than those obtained in Argyllshire. Larvae were derived from females which Mr. Kemp had in culture.

A few exotic tick species also in culture at the Department of Zoology of the University were included in the experiments to see whether any of these would prove easier to maintain as laboratory models than *I. ricinus* for use in studying such aspects as transovarial passage of virus. These comprised *Rhipicephalus appendiculatus* Neumann, originally from Kenya, *R. bursa* Canestrini and Fanzago, obtained from Hamburg, Germany, *Hyalomma anatolicum anatolicum* Koch, from Iran and *Dermacentor andersoni* Stiles, from Alberta, Canada.

**Tick feeding experiments.** Three sheep, numbers 1009, 1010 and 1028, were each injected intravenously with 1 ml of blood from sheep 4355, one of Dr. Foggie's current tick-borne fever reactors. The temperature responses were recorded and the presence of tick-borne fever bodies confirmed in bloodsmears on the one occasion/
occasion of examination.

Two months later the three sheep were each injected subcutaneously with $10^6.7$ mouse LD$_{50}$ of LI 31 virus for use as virus-donor hosts in the tick infection experiments. The overall design of the experiments is shown in figures 6 and 7.

Three separate groups of both larvae and nymphs of *I. ricinus* were fed on each of the sheep. On the basis of the information gained in the clinical studies the attachments were so arranged that the first groups would complete engorgement just before viraemia became demonstrable, while the second groups would be replete when viraemia was at the peak but before the appearance of antibodies. The third groups would feed from the peak of viraemia into the phase during which diminishing amounts of virus would be present with increasing levels of antibody.

In addition a few *I. ricinus* adults and some *H. anatolicum anatolicum* nymphs were fed on sheep 1028. *D. andersoni* nymphs and *R. bursa* adults were attached to sheep 1010, while *R. appendiculatus* nymphs were placed on sheep 1008.

The virus and antibody levels of the sheep were monitored during the tick feeding. Sample pools of both the engorged ticks and of the succeeding instars were assayed for virus content. The remaining ticks were then tested for transmission to susceptible sheep and again assayed for virus in the engorged state./
Fig. 6. Schematic design of the tick-feeding experiments, showing the feeding periods of the various tick groups on sheep 1C06 and 1C28 and the subsequent examination of the ticks.
Fig. 7. Schematic design of the tick-feeding experiments, showing the feeding periods of the various tick groups on sheep 1010 and the subsequent examination of the ticks. Column headings as in figure 6.
Details of the tick feeding and sheep transmissions will be discussed below.

Subsidiary tick infection experiments were carried out by placing laboratory-bred larvae on sheep 9049 and 1003 during their reactions to tick transmissions. These larvae were tested for virus content before and after moulting.

Tick feeding and handling procedures. Ear bags were made of white cotton material. They were in the form of a sleeve open at both ends but shaped to fit firmly round the base of the ear. They were flared to a wide mouth at the free end. This allowed the open end to be reflected back to expose the ear. Four stout tapes were sewn on at the corners of the base so that the bags could be tied down firmly by passing the tapes around the throat.

Before applying the bag, the hair at the base of the ear was clipped short. A piece of three inch wide elastic adhesive tape was folded lengthwise so as to expose adhesive surface on both sides. This was then applied around the base of the ear to adhere to the skin medially while the bag was fitted over the outer sticky surface. After the bag had been tied in place narrow non-elastic adhesive tape was passed round the outside of the base of the bag to seal it. Tension on the base was adjusted to avoid swelling of the ears. Severe swelling did arise in two instances but this appeared to be from pyogenic infection associated with the tick feeding.

Immature ticks were introduced into the ear bag either in/
in short lengths of pasteur pipette sealed by cotton wool stoppers or in the 1\(\frac{1}{2}\)" x 1\(\frac{1}{4}\)" flat-bottomed glass tubes with cotton wool stoppers. The stoppers were withdrawn and the glass containers were left in the bags for twelve hours while the ticks became attached. Adult ticks or even immature ticks were also added by merely brushing them from their tubes into the bag. After the ticks had been placed in the bag a stout elastic band was wound tightly around to close the open end. (Figure 8.)

All three tick instars and several species could be attached to one ear at a time and subsequently sorted out again on recovery. By using the ears alternately, several batches of the same instar of one species could be kept separate on a single host.

The bags were opened and the ticks examined every twelve hours after the first two days since detached ticks died from dehydration if left in the ear bags for longer periods. To recover the ticks the sheep were immobilised in metal cradles with large sheets of white paper spread on the floor beneath their heads to facilitate recovery of ticks accidentally dropped. As the bags were reflected (Figure 9) the replete ticks were brushed into a large plastic funnel with a stopper in its spout. Material from each ear bag was transferred into a labelled container for cleaning and sorting. On each occasion the elastic band was replaced by a new one.

The engorged ticks were accompanied by copious tick faeces and dried blood as well as wool fluff. The faeces and blood/
Fig. 8. Ear bags affixed to sheep.

Fig. 9. Ear bag reflected to show nymphs nearing repletion.
blood dissolved readily in cold water and the ticks were then recovered and dried on muslin in a Büchner filter. They were placed in clean 1½" x 1¼" tubes at the rate of a hundred larvae, twenty nymphs or one female per tube. Data were pencilled on strips of plastic tracing material and enclosed in each tube. The details on the labels were duplicated in tick feeding record books. These included the serial number of the tubes, description of contents, date of recovery and host number. The cotton wool stoppered tubes were then placed in moist plaster-base jars and kept in a 22°C constant temperature room with eighteen hour artificial daylight periodicity. Ticks to be assayed for virus content in the engorged state were transferred directly to -20°C for use within a fortnight.

At the end of each tick feeding experiment the bedding in the loose boxes was incinerated and the impervious concrete walls and floors were cleaned with strong caustic solution.

Six weeks after detachment the majority of ticks had moulted and they were transferred into clean tubes. The records were brought up to date to take account of mortalities and ticks which had failed to moult. One month later the ticks were again put into clean tubes and then moved to a cold room at 10°C for six weeks before the next sorting. Thereafter they were kept at 22°C as the transmission attempts proceeded over the next few months.

**Tick pool virus assays.** Six pools of 50 individuals each of the field-collected nymphs were tested for virus before the tick/
tick feeding experiments. They were tested by mouse inoculation at 1:200 dilution of tick suspension in medium and by the cell culture with mouse subinoculation method.

The preparation of tick-pool suspensions has been described above (Section I). It was found convenient to use the following numbers of ticks for individual pools: 100 engorged larvae, 25 engorged nymphs, 100 flat nymphs, 25 flat imaginal ticks and single engorged females. All pools were weighed to 0.0001 g. for preparation of the suspensions at known concentrations. Batches of eggs and unfed larvae were weighed uncounted.

After high speed centrifugation the tick pool suspensions were inoculated into pig kidney cultures in a log_{10} titration of each pool up to a 10^{-9} dilution in medium as described in Section I. At the same time 3 to 5 three-week-old mice were inoculated with the tick material at the original dilution which varied between 1:16.6 and 1:200. A sample of each tick suspension was also made up in a 1:200 concentration in medium incorporating a final concentration of 1:50 of a standard louping ill antiserum. These were inoculated after standing one hour at bench temperature (22°C) into 5 pig kidney cultures. This served as a screening test to prove that louping ill was in fact being handled. Pools of culture fluids representing each dilution were inoculated into 3 to 5 mice on the fifth day.

Where specific deaths occurred a full mouse-titration was carried out on the original material stored at -20°C. Where no/
no deaths occurred the original tick pools were passaged in two litters of day-old suckling mice.

Mouse brains harvested from the titrations were subsequently used as antigen in full metabolic inhibition neutralisation tests to identify the virus being measured in the experiments.

RESULTS

A. SHEEP

The clinical data included here comprise the experimentally-induced infections from all stages of the project and are presented together to facilitate comparisons. They include observations on uncomplicated louping ill infections with the various strains of virus and dual infections with louping ill and tick-borne fever.

The results are presented graphically in Figures 10 to 43 but the full protocols have been deposited at Moredun Institute. A selection of the information is also presented in tables 3 to 9. These include titrations of virus dosages administered to sheep, one example of the daily records of experimental animals, the results of haemagglutination-inhibition tests on all the sheep sera and the serum neutralisation titres for those sheep which were tested. The titres of viraemia in subcutaneously infected or challenged sheep are also listed. Since only one of the tick-infested sheep (9C49) developed a demonstrable viraemia the/
<table>
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<tr>
<th>Sheep Number</th>
<th>Virus Strain</th>
<th>Mouse deaths at log(_{10}) dilutions tested</th>
<th>LD(_{50}) titres per 0.05 ml dilution</th>
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<tr>
<td>1003</td>
<td>31</td>
<td>5/5   5/5   5/5   4/5   4/5   4/5   0/5   0/5   0/5</td>
<td>10(^6) 6 LD(_{50})</td>
</tr>
<tr>
<td>1006</td>
<td>6/6</td>
<td>6/6   6/6   6/6   6/6   6/6   6/6   0/6   0/6   0/6</td>
<td>10(^5) 6 &quot;</td>
</tr>
<tr>
<td>1005</td>
<td>6/6</td>
<td>6/6   6/6   6/6   6/6   6/6   6/6   0/6   0/6   0/6</td>
<td>10(^6) 7 &quot;</td>
</tr>
<tr>
<td>1008) 1010) 1028)</td>
<td>31</td>
<td>-     3/5  3/5  3/5  3/5  3/5  3/5  3/5  3/5  3/5</td>
<td>10(^6) 7 &quot;</td>
</tr>
<tr>
<td>4B55</td>
<td>5/5</td>
<td>5/5   5/5   5/5   5/5   5/5   5/5   5/5   5/5   5/5</td>
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<tr>
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<tr>
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<td>6/6</td>
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<tr>
<td>1014</td>
<td>189</td>
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<tr>
<td>1025 8/13</td>
<td>5/5</td>
<td>5/5   5/5   5/5   5/5   5/5   5/5   5/5   5/5   5/5</td>
<td>10(^5) 7 &quot;</td>
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<tr>
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<td>274</td>
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<td>9B99</td>
<td>8/11</td>
<td>-     5/5  5/5  5/5  5/5  5/5  5/5  5/5  5/5</td>
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<td>5016) 5017) 5022) 5023) 5029) 8070) 8071)</td>
<td>31</td>
<td>5/5   5/5   5/5   5/5   5/5   5/5   5/5   5/5   5/5</td>
<td>10(^6) 3 &quot;</td>
</tr>
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</table>
TABLE 4.

DAILY RECORD OF SHEEP 9B99, INOCULATED $10^{6.2}LD_{50}$ LI B/11, 7/6/65.

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<tr>
<th>DATE</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<td>3</td>
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<td>9</td>
<td>10</td>
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<th>TEMPERATURE a.m.</th>
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<th>2.6</th>
<th>5.1</th>
<th>5.0</th>
<th>6.0</th>
<th>5.4</th>
<th>4.1</th>
<th>6.2</th>
<th>6.2</th>
<th>5.7</th>
<th>3.7</th>
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<tbody>
<tr>
<td>(°F - 100)p.m.</td>
<td>3.6</td>
<td>2.5</td>
<td>6.3</td>
<td>6.9</td>
<td>7.1</td>
<td>-</td>
<td>-</td>
<td>4.4</td>
<td>5.6</td>
<td>3.8</td>
<td>Died</td>
</tr>
</tbody>
</table>

| WEIGHT lbs.  | 145 | 147 | 145 | -   | 141 | 138 | -   | 137 | -   | 132 | -   |

| LEUCOCYTES, $10^9$/mm$^3$ | 8.4 | 9.8 | 5.2 | 3.5 | 2.8 | 2.2 | 1.6 | 2.3 | 3.9 | 2.8 | 6.3 |

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<th>DIFFERENTIAL COUNTS %</th>
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<th>60</th>
<th>43</th>
<th>43</th>
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<th>48</th>
<th>61</th>
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<th>60</th>
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<td>5</td>
<td>1</td>
<td>2</td>
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<tr>
<td></td>
<td>B</td>
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<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

L = lymphocytes
M = monocytes
B = basophils
N = neutrophils
E = eosinophils.
## TABLE 5.

**SHEEP HAEMAGGLUTINATION INHIBITION TITRES**

| Sheep number | Days after inoculation/infestation: | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|--------------|-----------------------------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|
| 1C03a*      |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80 | 640 | Died |    |
| 1C03b*      |                                   | 20| 20| 40| 160| 320| 640| 640| 1280| 1280| 2560| 2560|    |    |    |    |
| 1C05        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80 | 80 | 80 | 40 |
| 1C06        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 40 |
| 1C07        |                                   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 1C08        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20| 40 | 80 | 80 | 80 | 320| 640 | Died |
| 1C10        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20| 40 | 40 | 40 | 160| 640 | 640 | 640 |
| 1C14        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 20| 40 | 160| 320| 640| 640 | 320 | 320 | 320 |
| 1C25        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 20| 40 | 40 | 160| 640| 1280| 1280|    |    |
| 1C28        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| 1C32        |                                   | 0 | 0 | 0 | 0 | 10| 0 | 10| 160| 160| 320| 320| 320| 160| 160 | 160 |
| 4B55a       |                                   | 0 | 0 | 0 | 0 | 10| 20| 40 | 160| 320| 320| 320| 320| 80 | 2560| 640 |
| 4B55b       |                                   | 0 | 0 | 0 | 0 | 10| 20| 40 | 160| 320| 320| 320| 320| 80 | 1280| 640 |
| 5C16a       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 160| 160| 160| 160| 160 | 160 | 640 |
| 5C16b       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |    |    |    |    |    |    |    |
| 5C17a       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 640| 640| 640| 640| 640 | 640 | 640 |
| 5C17b       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 640| 640| 640| 640| 640 | 640 | 640 |
| 5C19        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 640| 640| 640| 640| 640 | 640 | 640 |
| 5C20        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10| 20| 80| 320| 1280| 2560| 1280 | 1280 |
| 5C21        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10| 40 | 80| 160| 160 | 160 | 160 | 160 |
| 5C22        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80 |
| 5C22b       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80 |
| 5C23a       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5C23b       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5C25        |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5C28a       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5C28b       |                                   | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 640 |

---

5096/
### TABLE 5. Continued

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>Days after inoculation/infestation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>5C96</td>
<td>0</td>
</tr>
<tr>
<td>5C97</td>
<td>0</td>
</tr>
<tr>
<td>5C98</td>
<td>0</td>
</tr>
<tr>
<td>6C00</td>
<td>0</td>
</tr>
<tr>
<td>8C22</td>
<td>Negative throughout. Killed subsequently for other reasons.</td>
</tr>
<tr>
<td>8C70a</td>
<td>Negative throughout</td>
</tr>
<tr>
<td>8C70b</td>
<td>0</td>
</tr>
<tr>
<td>8C71a</td>
<td>Negative throughout</td>
</tr>
<tr>
<td>8C71b</td>
<td>0</td>
</tr>
<tr>
<td>9C49</td>
<td>0</td>
</tr>
<tr>
<td>9B99</td>
<td>0</td>
</tr>
</tbody>
</table>

*a and b refers to first and second occasion on which sheep monitored.*
TABLE 6.
NEUTRALISING ANTIBODY TITRES OF EXPERIMENTAL SHEEP.

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>Days after infection or infestation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 3 4 5 6 7 8 9 10 11 12 13 14</td>
</tr>
</tbody>
</table>

(a) Sheep used as virus-donors for ticks:

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>0 3 4 5 6 7 8 9 10 11 12 13 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1010</td>
<td>- - - - 1.35* 2.05 2.75 3.8+ 3.8 3.8 3.8 3.8 3.8 3.8</td>
</tr>
<tr>
<td>1028</td>
<td>- - - - 2.2 2.75 3.3 3.45 Dead - - - -</td>
</tr>
<tr>
<td>1008</td>
<td>- - - - 1.35 2.05 2.75 3.45 Dead - - - -</td>
</tr>
</tbody>
</table>

(b) Immune sheep infected by ticks:

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>0 3 4 5 6 7 8 9 10 11 12 13 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1003</td>
<td>2.05 2.05 2.05 2.05 2.75 3.3 3.8 3.8 3.8 3.8 3.8 3.8</td>
</tr>
<tr>
<td>4355</td>
<td>2.05 2.05 2.33 2.75 3.3 3.8 3.8 3.8 3.8 3.8 3.8 3.8</td>
</tr>
</tbody>
</table>

(c) Susceptible sheep infected by ticks:

<table>
<thead>
<tr>
<th>Sheep number</th>
<th>0 3 4 5 6 7 8 9 10 11 12 13 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>5019</td>
<td>- - - - 1.35 2.05 2.75 3.45 3.45 3.45 3.45 3.45 3.45</td>
</tr>
<tr>
<td>9049</td>
<td>- - - - 2.05 2.75 3.6 3.8 3.8 3.8 3.8 3.8 3.8</td>
</tr>
</tbody>
</table>

* 0.2 ml of serum neutralises 100 MID\textsubscript{50} of virus to a dilution of $10^{-1.35}$

+ Sera not tested beyond 1 : 6,250 i.e. $10^{-3.8}$
TABLE 7.

VIRAEMIA TITRES OF SUBCUTANEOUSLY INFECTED SHEEP

<table>
<thead>
<tr>
<th>Sheep Number</th>
<th>Days after infection:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>(a) Sheep infected with field virus strains:</td>
<td></td>
</tr>
<tr>
<td>1032</td>
<td>0.8</td>
</tr>
<tr>
<td>1014</td>
<td>-</td>
</tr>
<tr>
<td>5096</td>
<td>TR</td>
</tr>
<tr>
<td>5097</td>
<td>-</td>
</tr>
<tr>
<td>5021</td>
<td>-</td>
</tr>
<tr>
<td>5020</td>
<td>0.2</td>
</tr>
<tr>
<td>6000</td>
<td>-</td>
</tr>
<tr>
<td>9899</td>
<td>-</td>
</tr>
<tr>
<td>1025</td>
<td>-</td>
</tr>
<tr>
<td>5098</td>
<td>0.8</td>
</tr>
<tr>
<td>(b) Sheep infected with laboratory strain virus:</td>
<td></td>
</tr>
<tr>
<td>1003</td>
<td>0.8</td>
</tr>
<tr>
<td>1006</td>
<td>-</td>
</tr>
<tr>
<td>1005</td>
<td>-</td>
</tr>
<tr>
<td>4B55</td>
<td>-</td>
</tr>
<tr>
<td>1010</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 7  Continued

<table>
<thead>
<tr>
<th>Sheep Number</th>
<th>Days after infection:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1028</td>
<td></td>
</tr>
<tr>
<td>1008</td>
<td></td>
</tr>
</tbody>
</table>

* $10^{0.8}$ mouse ic $LD_{50}$ of virus per 0.03 ml blood

+ TR = trace; whole blood kills less than 50% of mice inoculated.
**TABLE 8.**

**VIRAEMIA TITRATIONS OF SHEEP 9C49 AFTER TICK INFESTATION.**

<table>
<thead>
<tr>
<th>Blood log_{10} dilutions</th>
<th>Days after infestation:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14</td>
</tr>
<tr>
<td>-7</td>
<td>- - - - 0/5 0/5 0/5 - - - - - -</td>
</tr>
<tr>
<td>-6</td>
<td>- - - - 0/4 0/5 0/5 - - - - - -</td>
</tr>
<tr>
<td>-5</td>
<td>- - - 0/5 0/5 0/5 0/5 0/5 - - - - -</td>
</tr>
<tr>
<td>-4</td>
<td>- - - 0/5 0/4 0/5 0/4 0/5 - - - - -</td>
</tr>
<tr>
<td>-3</td>
<td>- - 0/5 0/5 0/5 0/5 0/5 0/5 0/5 - - - - -</td>
</tr>
<tr>
<td>-2</td>
<td>- - 0/5 0/5 0/3 0/5 0/5 0/5 0/5 - - - - -</td>
</tr>
<tr>
<td>-1</td>
<td>1* /5 0/5 1/5 1/5 0/5 0/5 0/5 0/5 0/4 0/5 - - - -</td>
</tr>
<tr>
<td>0</td>
<td>1* /5 0/5 2/5 3/5 1/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
</tbody>
</table>

**LD_{50}**

<table>
<thead>
<tr>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>TR</td>
</tr>
</tbody>
</table>

* Non-specific deaths; in this case killed because of injuries sustained in fighting.
**TABLE 9.**

**VIRAEMIA TITRES OF SHEEP CHALLENGED AFTER TICK EXPERIMENTS.**

<table>
<thead>
<tr>
<th>Sheep Number</th>
<th>Days after infection:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5016</td>
<td>-</td>
</tr>
<tr>
<td>5017</td>
<td>TR</td>
</tr>
<tr>
<td>5022</td>
<td>-</td>
</tr>
<tr>
<td>5023</td>
<td>-</td>
</tr>
<tr>
<td>5028</td>
<td>-</td>
</tr>
<tr>
<td>8070</td>
<td>TR</td>
</tr>
<tr>
<td>8071</td>
<td>-</td>
</tr>
</tbody>
</table>
the protocols of virus monitoring in this animal are given fully as an example of the way in which the mice were allocated on the various days to cover the expected viraemic patterns.

The sheep are placed in five categories for discussion below but it will be apparent that consecutive infections in some of the individuals fall into separate categories. Although the contexts in which tick transmission infections arose will become evident later it is useful to outline the preceding sequence of events at this point.

A pilot investigation was carried out in the Autumn of 1964 on three sheep inoculated subcutaneously in succession to establish a routine for the monitoring of infection in the future experiments. One of these sheep (1032) received a field strain of virus, while the other two (1003 and 1006) were given the reference strain, L131. It was decided from the findings that a thorough clinical study would be practicable only on groups not exceeding 3 or 4 animals. Thus during the Summer of 1965 groups of three and four sheep were infected at fortnightly intervals, nine sheep receiving the field strains, and one the L1 31 virus.

Three further sheep which received L1 31 virus were those used in feeding and infecting the ticks (1008, 1010 and 1028). All three developed intercurrent tick-borne fever in spite of having received the immunising dose of tick-borne fever blood two months previously. The question now arose as to whether the sheep/
sheep were experiencing a recrudescence of the earlier tick-borne fever precipitated by the looping ill or whether the field-collected nymphs had introduced a tick-borne fever to which cross-immunity was incomplete. To check the first of these possibilities the tick-borne fever donor sheep (4B55), whose fever reaction had preceded the others by only a few days, was obtained from Dr. Foggie and given a similar subcutaneous inoculation of LI 31 virus, (p.72).

By courtesy of Dr. Foggie uncomplicated tick-borne fever reactions were monitored for comparative purposes in three sheep (7C93, 8050 and 6129) which he was infecting and challenging.

The remainder of the infections described concern the transmission attempts with the experimental ticks and the subsequent challenge of those sheep which failed to develop looping ill from the infestation.

Figure 10 is a key to the information furnished in the diagrams of the sheep reactions. In addition it should be noted that the areas enclosed by the temperature curves have been shaded above an arbitrary base-line of pyrexia 105°F (40.55°C).

One animal, 1C07, was given 3 ml of a 10⁻² dilution of lyophilised ordinary mouse brain as a control (figure 11). It will be noticed that the total leucocyte, lymphocyte and neutrophil counts are plotted together, while the eosinophils and monocytes are placed below with the abscissal scale expanded ten times. Neutrophils and eosinophils are plotted with broken lines.
Table 10. Key to abbreviations used in figures 11 to 46.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kg</td>
<td>Body weight</td>
</tr>
<tr>
<td>N</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>L</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>M</td>
<td>Monocytes</td>
</tr>
<tr>
<td>E</td>
<td>Eosinophils</td>
</tr>
<tr>
<td>W/W</td>
<td>White blood cells</td>
</tr>
<tr>
<td>RBC, Hb</td>
<td>Red blood cells, hemoglobin</td>
</tr>
<tr>
<td>PCR, WBC</td>
<td>Peripheral blood cell</td>
</tr>
<tr>
<td>HPL</td>
<td>Hemolysin production</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotide triphosphate</td>
</tr>
<tr>
<td>ID₅₀, ID₅₀</td>
<td>Dose of infection, dilution</td>
</tr>
<tr>
<td>PRD</td>
<td>Plaque reduction</td>
</tr>
</tbody>
</table>

Note: The table contains abbreviations and their definitions, and the text provides instructions for interpreting the abbreviations used in figures.
Fig. 11. Response of sheep 1007 to subcutaneous injection with 3 ml of a $10^{-2}$ dilution of non-infective mouse-brain.
lines. Basophils have been disregarded. This pattern is adhered to throughout the diagrams. After the injection of normal mouse brain, sheep 1007 showed transient rises of neutrophils and lymphocytes in sequence followed by a delayed eosinophil response.

Sheep inoculated with field strains of virus. The ten sheep grouped here comprise eight of which each received different field viruses and two (1032 and 6000) which were both given LI 68. The virus doses varied between $10^{4.5}$ and $10^{6.2}$ mouse ic ID$_{50}$. The haemagglutination-inhibition titres only are cited as an index of the antibody response in these sheep.

The reactions of the sheep fall into two obvious classes: those which were fatal (sheep 5C98, 1C25, 9B99 and 6000) and those which were not (5C20, 5C21, 5C96, 5C97, 1C14 and 1C32).

Three of the fatal infections in sheep 5C98, 1C25 and 9B99 (Figures 12, 13 and 14) had features in common which distinguished them from the reaction of sheep 6000 (Figure 15).

All three had a disphasic fever starting on the second day after inoculation. The remission of temperature occurred on the sixth day and the second febrile phase lasted a further three days. Virus became demonstrable in the blood on the first or second day after inoculation and reached maximum concentration on the third to fifth days. The titre dropped on the fifth or sixth days and virus was not demonstrable thereafter. In two of the sheep the/
Fig. 12. Response of sheep 5098 to subcutaneous infection with $10^{5.4}$ LD$_{50}$ of LI 2/7 virus.
Fig. 13. Response of sheep IC25 to subcutaneous infection with \(10^{5.7} ID_{50}\) of LI B/13 virus.
Fig. 14. Response of sheep 9B99 to subcutaneous infection with $10^{6.2}$ LD\textsubscript{50} of LI B/11 virus.
the end-points for the peak titres were not determined but viraemia in all three sheep exceeded $10^4$ mouse ic LD$_{50}$ of virus per 0.03 ml blood. At the highest level of viraemia in sheep 5C98 there was $10^7.5$ mouse ic LD$_{50}$ of virus per 0.03 ml of blood.

Haemagglutination-inhibition antibody became measurable on the fourth or fifth days of infection while high titres of virus were still present in the blood. Very high antibody titres were attained in these sheep and a levelling off of the immune response occurred eight or nine days after infection. The antibody level went into decline immediately prior to death in sheep 5C98.

A small initial rise in the leucocyte total was associated in two of the three sheep with the neutrophils rather than the lymphocytes. On the second day the leucocyte totals declined sharply chiefly as a result of a decrease in lymphocytes. When maximum leucopaenia was reached, on the fifth to seventh days, the neutrophils had also fallen. In two of the sheep the cell totals fell to below 25% of the original count. Counts recovered slightly after this point and two of the sheep (1C25 and 9B99) had a terminal neutrophilia. Monocyte and eosinophil levels appeared to be depressed during the reactions.

Apart from panting and a clear, watery nasal discharge the sheep showed no symptoms during the first phase of fever and they continued to eat well. Specific symptoms appeared only by the/
the seventh (9B99) or eighth days after inoculation when viraemia had passed and antibody was already present in high titres.

The sheep became hyperaesthetic at first and reacted wildly to any visual or auditory stimulus. They had a 'wild' stare giving the impression of intense awareness of activity in their vicinity. A nervous licking of the lips, rolling of the eyes and holding of the ears at odd angles was also noticeable. Slight trembling of the head and forequarters could be discerned and sheep 9B99 had dextral torticollis. Sheep 1C25 had a slight diarrhoea. All three animals had dirty, crusted discharge around the nostrils.

On day nine trembling became more noticeable and involuntary jerking and stamping of the limbs developed. Sheep 5C98 could still walk normally but the other two were ataxic with weak, swaying hindquarters and a characteristic high-stepping action more noticeable in the forelimbs. All three sheep became easily excited and the two ataxic ones would run off at an angle, stumble and struggle wildly to get up. Sheep 9B99 frothed slightly at the nostrils and mouth and had bouts of gasping dyspnoea. When undisturbed the sheep were depressed. Their heads were held low with eyes closed and they ground their teeth. Anorexia was slight and sheep 1C25 was observed to feed up to day nine, nevertheless, a rapid loss of weight was evident in all sheep. The loins and flanks became hollowed and the faces became thin with the disappearance of the fat in the supraorbital fossae/
foseae especially noticeable.

All three sheep were recumbent on the tenth day but 5098 could raise itself with difficulty and walk a few paces with the back arched and the hind legs tucked under the body. It would assume a sejant posture with the weak hindquarters resting on the ground. It died overnight and was found on the eleventh day with froth at the nostrils. Sheep 9B99 was prostrate with its head resting on the floor but could walk if lifted and supported. It later developed convulsive bouts of galloping motions with the limbs and it died suddenly in the early afternoon of the tenth day. It too had been frothing at the mouth and nostrils. Sheep 1025 was lying outstretched on the tenth morning with occasional convulsive galloping motions of the limbs. It appeared to be in extremis and was killed.

Hydrothorax was found at post-mortem in 9B99 and 5098. The lungs were congested and oedematous with copious fluid and froth in lung tissue and the lumina of bronchi and trachea. Narrow bands of collapse were evident on the ventral margins of the lungs. Those lymph glands which were inspected - bronchial, mediastinal, mesenteric and prescapular - appeared swollen and moist in all three sheep. The spleens were atrophied, with wrinkled capsules and narrow margins. The malphigian corpuscles were not evident on cut surfaces.

Half brains and the cervical portions of the spinal cord were submitted to the Experimental Pathology department of the/
the Institute for histopathological examination and the remaining halves of the brains were tested for virus by mouse inoculation. The histopathological lesions were confirmed to be those found in louping ill and were reported merely as "non-suppurative encephalitis". Virus was not recovered from the brains of these sheep.

The monophasic febrile reaction in sheep 6000 (Figure 15) became apparent only on the ninth day and had passed off on the twelfth day. Virus in the blood was demonstrable, in low titre, only on the third day. Haemagglutination-inhibition antibody could be detected on the fourth day the titre becoming 1:80 by the sixth day. It remained at this comparatively low level throughout the course of the disease. Both lymphocytes and neutrophils showed a transient rise initially. Thereafter the neutrophils remained constant while the lymphocytes fluctuated but showed an overall decrease so that the two cell types were present terminally in almost equal proportion.

Sheep 6000 was panting on day nine and appeared to be weak on the following two days. On the thirteenth day the animal was lying down but was able to rise and walk. It was depressed and could not be frightened or chased easily. Brachyplegia had set in on the fourteenth day and the sheep could still propel itself forward on its sternum by use of the hind limbs. The head was held erect and there appeared to be none of the disorientation encountered in the three sheep above. The animal was comatose/
Fig. 15. Response of sheep 6000 to subcutaneous infection with $10^{5.0} \text{LD}_{50}$ of LI 68 virus.
Comatose on the fifteenth morning and was killed. No macroscopic lesions were noted at post-mortem. The histopathology was reported as "very severe non-purulent encephalitis". Virus was not recovered from the brain.

Nervous symptoms were marked in one of the non-fatal infections (sheep 5C20) but were discerned with certainty in only one other (5C21). Both of these sheep had a biphasic fever (Figures 16 and 17). The peak levels of virus in the blood were below $10^{3.0}$ LD$_{50}$ per 0.03 ml. Antibodies appeared on the fifth and sixth days and rose to a maximum of 1:2560 on the ninth day in sheep 5C20, while the highest titre in sheep 5C21, 1:640, was recorded on the fourteenth day. Leucopaenia was severe in sheep 5C20 and followed the pattern seen in the acutely fatal infections, with a transient neutrophilia followed by a relative neutrophilia as the lymphocyte level went into sharp decline. Neutrophil counts fell in turn. Maximum leucopaenia occurred on the sixth day. Thereafter the lymphocyte level was more immediately buoyant than the neutrophil level, but the recovery was not sustained and neutrophils exceeded lymphocytes in the fifth week. The degree of leucopaenia in sheep 5C21 was less pronounced but the same pattern occurred of an initial lymphocyte decrease followed by neutrophil decrease. After maximal leucopaenia on the seventh day the lymphocytes returned to a level just below the initial count. Neutrophilia developed on the eleventh day and persisted to the fourteenth day but had subsided at the examination a week later.
Fig. 16. Response of sheep 5C20 to subcutaneous infection with $10^{5.5}$ LD$_{50}$ of LI 274 virus. The viraemic titres are inadvertently plotted one day too early. The curve should be read one day to the right.
Fig. 17. Response of sheep 5C21 to subcutaneous infection with $10^{4.8} \text{LD}_{50}$ of LI 188 virus.
Symptoms appeared in both sheep on the twelfth day, just following the second peak of fever. Sheep 5C20 held its head to one side and moved its ears independently at odd angles while rolling its eyes. It had a 'stupid' expression as though perplexed and would attempt to climb up the wall or on to other sheep. The movements were slow and deliberate and not frenzied. A slight posterior ataxia was evidence. The animal improved over the course of a week. Sheep 5C21 was excitable on day twelve and had very fine tremors of the head and neck. It recovered over the next three days.

Of the remainder, sheep 5C96, 5C97 and 1C32 (Figures 18, 19 and 22) had diphasic fever while 1C14 (Figure 20) had a monophasic reaction. Viraemia was demonstrable in all these sheep within the first five days, but only in sheep 5C96 did the titre exceed 2.0 logs of virus per 0.03 ml, although end-points were not determined in sheep 1C32. Haemagglutination-inhibition antibody appeared between days four and six. In only 5C97 was a titre of 1:640 exceeded, and then only on the tenth day. The other sheep reached maximum levels between the eighth and eleventh days after inoculation. Although there were initial transient rises of neutrophils and lymphocytes in sheep 5C96 and 5C97 respectively, neutrophil counts became slightly depressed in both sheep and their lymphocytes fluctuated irregularly. The tendency to suppression of eosinophil and, to a lesser extent, monocyte levels during the reaction was evident in these sheep as in the others/
Fig. 18. Response of sheep 5096 to subcutaneous infection with $10^{4.7} \text{LD}_{50}$ of LI 174 virus.
Fig. 19. Response of sheep 5097 to subcutaneous infection with $10^{5.1}$ LD$_{50}$ of LI 226 virus.
Fig. 20. Response of sheep LC14 to subcutaneous infection with $10^{4.5} \text{LD}_{50}$ of LI 189 virus.
Fig. 21. Response of sheep 1C06 to subcutaneous infection with $10^{5.6}$ LD$_{50}$ of LI 31 virus.

Fig. 22. Response of sheep 1C32 to subcutaneous infection with $10^{5.0}$ LD$_{50}$ of LI 68 virus.
others but eosinophilia was noted after some weeks. White cell counts altered gradually in sheep $1.4$ repeating in effect the pattern seen previously, namely, a lowered lymphocyte level followed by a reduction in neutrophils. Apart from panting during pyrexia, the only symptom observed in these four sheep was an apparent excitability after subsidence of the second phase of fever and this occurred only in 5C96 and 5C97. But these two sheep had been particularly wild when taken on to experiment and it is impossible to be certain about the specificity of this symptom. Some weeks later these two sheep were still troublesome to catch and handle.

As shown in the figures, ten to twenty per cent weight less was common even in mild infections.

**In summary** - Of the ten sheep infected subcutaneously with $10^{4.5}$ to $10^{6.2}$ mouse i.c. $\text{LD}_{50}$ of recent isolates of louping ill virus, four died or had to be killed and two others developed specific symptoms from which they recovered. The fatal cases were infected with $10^{5.0}$ to $10^{6.2}$ $\text{LD}_{50}$ of virus, nevertheless, three of the six survivors also received at least $10^{5.0}$ $\text{LD}_{50}$ of virus.

Two sheep developed monophasic fevers, one on the ninth day, a fatal case, and the other on the seventh day, a non-fatal case, while all the rest had diphaphic fevers. In these last the initial rise in temperature occurred two or three days after inoculation. The first phase ended on day six or seven and the second phase ended between the ninth and twelfth days. Viraemia became demonstrable one to three days after infection and was not found after the sixth day. The duration of viraemia in individual/
individual sheep ranged between one and five days and maximum titres occurred on days three, four or five. Haemagglutination-inhibition activity also appeared during the first febrile stage, on days four to six, when high levels of virus were still circulating in some sheep. These antibodies attained peak levels between the eighth and tenth days but in two sheep this happened on the sixth and fourteenth days. Severe leucopaenia involving both the major cell types was seen in some of the infections and tended to be associated with the more intense viraemias. Characteristic behavioural symptoms, when they occurred, developed during or after the second febrile stage when viraemia was no longer demonstrable and antibodies had already attained maximum titres. Ten to twenty per cent of body-weight loss during the acute phase of the disease was common even in the absence of behavioural symptoms.

Three sheep which had acute, fatal infections received viruses isolated from ticks as opposed to vertebrate isolates given to the remainder of the sheep. The infection in these three was characterised by intense viraemia exceeding $10^{4.0}$ LD$_{50}$ per 0.03 ml at maximum, severe leucopaenia and high antibody levels. Symptoms included excitability, cerebellar ataxia, convulsive fits and paraplegia. Deaths all occurred on the tenth day and asphyxiation from fulminating lung oedema was the probable immediate cause of death in two of the animals.

One sheep had a subacute, fatal infection which appeared to be exceptional in some respects. A low titre of virus was /
was demonstrated in the blood on only one day, the antibody response was feeble and leucopaenia was slight. The animal became weak during a monophasic fever and remained slightly depressed as a flaccid brachyplegia developed. It was killed while comatose and in extremis on the fifteenth day.

Two sheep had a mild form of the disease and recovered from symptoms. Viraemia in these two sheep did not exceed $10^{3.0} \text{LD}_{50}$ of virus per 0.03 ml. One animal had a strong immune response as well as a marked leucopaenia and showed signs of disorientation and ataxia over the course of a week. The other sheep had a weak immune response, a slight leucopaenia followed by a neutrophilia and showed excitability and fine muscular tremors over the period of a few days.

The remaining four sheep had comparatively low viraemic titres, and their antibody levels were intermediate. Leucopaenia was not very obvious but the basic trends seen in the other reactors could also be discerned in these sheep. These infections were atypical in the sense that behavioural symptoms could not be detected with certainty.

Sheep inoculated with the laboratory strain of virus. Three of the seven sheep grouped here were those which served as tick-hosts during loping ill infection. They developed intercurrent tick-borne fever in spite of having had an immunising infection two months previously. The tick-borne fever infective-blood donor was itself given loping ill virus after three months and this/
this infection is included here. The remaining three sheep underwent uncomplicated infection with the laboratory strain virus. The virus doses inoculated into the seven sheep varied between $10^{5.3}$ and $10^{6.7}$ LD$_{50}$.

The temperature responses of sheep 1008, 1028 and 1010 to the preliminary tick-borne fever infections are shown in Figures 23, 24 and 25. Bloodsmears were made on the first day of pyrexia and parasitaemia was confirmed in all three sheep. The infective cell counts indicated heavy infections and no further blood observations were deemed necessary. The tick-borne fever in sheep 1008 had a protracted course, but all three sheep appeared to be well at the start of the tick experiments. The first tick attachments were made three days before the louping ill inoculations. Tick-borne fever bodies were noticed in the blood smear of sheep 1008 on the day of louping ill infection (Figure 23), and they appeared in the polymorphs of the two other sheep on the second day after infection. The bodies remained demonstrable for periods of four to seven days in the three sheep and maximum counts varied from 34 to 44 infected polymorphs per 100 counted.

Fever started on the day after the louping ill injection in all three sheep (Figures 23, 24 and 25), and virus became demonstrable in the blood on the second or third day. Viraemia lasted six or seven days and the maximum levels recorded on the fourth and fifth days after subcutaneous infection were $10^{2.5}$ LD$_{50}$ of virus per 0.03 ml in sheep 1010, $10^{4.5}$ LD$_{50}$ in 1028 and $10^{7.5}$ LD$_{50}$ in 1008. Haemagglutination-inhibition antibodies appeared/
Fig. 23. a) Response of sheep 1C08 to intravenous infection with 1 ml of tick-borne fever blood. b) Response of 1C08 to subcutaneous infection with $10^6.7$ LD$_{50}$ of UI 31 virus and infestation with ticks.
Fig. 24. a) Response of sheep 1C28 to intravenous infection with 1 ml of tick-borne fever blood. b) Response of 1C28 to subcutaneous infection with $10^{6.7} \text{LD}_{50}$ of L131 virus and infestation with ticks.
Fig. 25. a) Response of sheep 1C10 to intravenous infection with 1 ml of tick-borne fever blood.  b) Response of 1C10 to subcutaneous infection with $10^{6.7}$ ID$_{50}$ of LI 31 virus and infestation with ticks.
appeared on the sixth day after infection and reached the highest titre of 1:640 on the ninth or tenth days. Neutralising antibody preceded haemagglutination-inhibition by one day in sheep 1C10 but in the other two sheep the two antibody activities became detectable simultaneously. High levels of virus were still circulating when the antibodies appeared. The end-point for neutralising activity in the serum of sheep 1C10 after the seventh day lay beyond the highest dilution of serum tested, namely, 1:6,250. After slight initial rises in both neutrophils and lymphocytes in two of the sheep, the cell counts dropped steeply in all three animals to maximum leucopenia on the fifth and sixth days. At the lowest point there were only six hundred leucocytes per cubic millimetre of blood in sheep 1C08. Cell counts recovered up to day 14 in sheep 1C10 but again fluctuated after that day.

All three sheep began panting on the second day after inoculation and as fever was sustained they remained constantly distressed until the pathognomonic symptoms appeared. By the sixth day the clear watery nasal discharge seen with the onset of fever became dirty and crusted. The sheep had obviously lost weight and were weak, lying down to rest for much of the time. They were also depressed and stood with their heads down, resting against the wall while they gnawed their teeth.

On the eighth day sheep 1C28 was decidedly ataxic with characteristic high-stepping gait and weak, swaying hindquarters. That afternoon it had convulsive fits and would attempt to climb/
climb the walls if lifted. It was very weak and would support itself by leaning its head against the wall while the legs were moved constantly in an involuntary, trampling motion. It also had a catarrhal diarrhoea. On this same day, the eighth, sheep 1C08 was slightly excitable and had a wild staring expression while 1C10 remained dull and depressed.

On the ninth day paraplegia became marked in sheep 1C28 and it would remain in sejant posture unless disturbed. Locomotion was very poor. The animal could not regain its legs from a recumbent position but would struggle and thrash about in wild galloping movements. Trembling of the head and involuntary leg jerks were seen when the animal was not struggling. It became prostrate later that day and died in the early evening. Sheep 1C08 was ataxic on the ninth day, with similar swaying hindquarters and high-stepping gait. Its forequarters also appeared to be very weak and it lay down most of the time with its head held low. It died overnight.

Sheep 1C10 did not become ataxic until the tenth day and then the high-stepping action and weak hindquarters were not very noticeable. On the following day, however, the sheep developed severe muscular trembles which became progressively worse over the succeeding days. The animal lay on its sternum but could rise. It walked with difficulty, the back arched and the hind limbs tucked under the belly as it shuffled along. The impression was gained that the essential lesion was one of muscular weakness rather/
rather than nervous derangement. The body tremble and stilted locomotion seemed to stem from the intense muscular effort required to maintain posture. It was expected that the animal would die but by the sixteenth day it was somewhat stronger. Four weeks after infection the gait was still a little unsteady but after six weeks the sheep appeared well.

At post mortem sheep 1C28 was found to have hydrothorax and fibrinous adhesions of the lungs to the parietal pleura. The lungs were congested and oedematous. There was froth in the bronchi. Lymph glands were congested and swollen. The histopathology of the prescapular lymph node was reported as intense vascular congestion and a lack of lymphoid activity with no lymphoid follicles to be discerned in the cortex. The brain histopathology was reported as non-suppurative encephalitis. Virus was not recovered from the brain of this sheep.

Sheep 1C08 was found to have hydrothorax plus lung congestion and oedema together with a few superficial patches of collapse. The mucous membranes of the bronchi were congested and the lumina frothy. Lymph glands were swollen and congested. The spleen was normal in size but malphigian corpuscles were not evident on cut surfaces. There was a Corynebacterium pyogenes abscess at the injection site. The histopathology was reported as intense congestion of alveolar capillaries and interlobular oedema in the lungs. The spleen showed autolysis. A specimen of bone marrow from the sternum did not appear to be abnormal in/
in composition but peculiar 'chromatin' condensations on the outer surface of immature neutrophils and myelocytes were described. The prescapular lymph gland showed congestion and a lack of lymphoid follicular activity. The brain and cord were reported to have lesions of non-suppurative encephalitis. Virus was recovered from the brain of this animal.

None of the remaining four sheep developed significant symptoms. At most, panting was noticed during pyrexia. Weight loss was recorded in one animal (1005) and was suspected but not proven in the two sheep infected in the pilot investigation (1003 and 1006).

Monophasic febrile reactions in sheep 1006 (Figure 21), sheep 1003 (part of Figure 33) and 4B55 (Figure 26) corresponded to the usual first fever of diphasic reactions in that they were concurrent to viraemia and the appearance of antibody. The hyperthermia in sheep 4B55 was only just perceptible. The monophasic reaction in sheep 1005 (Figure 27), on the other hand, was delayed until antibodies had already circulated at maximum level. Demonstrable viraemia in 1006, 1003 and 4B55 occurred over two to four of the initial five days after infection and maximum titres were lower than $10^{3.0}$ LD$_{50}$ of virus per 0.03 ml. No virus was demonstrable in the blood of 1005 at all. Haemagglutination-inhibition antibody appeared in all sheep three to five days after the injection of virus and peak titres were obtained by days seven to twelve. The lowest maximum titre (1:160) was recorded in the/
the sheep without demonstrable viraemia. White cell counts were made in only two of the four sheep. In sheep 1C14 fluctuating excursions of the lymphocyte level were followed by a decrease in neutrophils. No definite trend occurred in the lymphocyte and neutrophil counts of sheep 4B55, which was still apparently slightly neutropaenic following its earlier tick-borne fever infection. This sheep, however, did show an eosinophilia, starting towards the end of the fourteen day observation period. Despite the earlier tick-borne fever infection, no tick-borne fever bodies were seen in the blood of 4B55 throughout the louping ill infection.

In summary - The LI31 strain of louping ill virus was inoculated subcutaneously into a total of seven sheep. The doses varied from $10^{5.3}$ to $10^{6.7}$ mouse ic LD$_{50}$.

Three sheep which were infected with the louping ill virus and then used as tick-hosts, developed intercurrent tick-borne fever in spite of an earlier immunising infection with this latter disease. Two of these dual infections were characterised by sustained fever, intense louping ill viraemia of seven days duration and severe leucopaenia. Maximum virus levels in the blood were $10^{7.5}$ and $10^{4.5}$ LD$_{50}$ per 0.03 ml respectively. Neutralising and haemagglutination-inhibition antibodies appeared on the sixth day after inoculation while high levels of virus still circulated. The disease ran an acute, fatal course with cerebellar ataxia and paraplegia more prominent in one animal. The deaths occurred on the ninth day and both sheep were found to have lung oedema at/
at post-mortem. Virus was recovered from the brain of only one, but both had histopathological lesions. The third animal had a sustained fever in which the diphasic pattern could be discerned. Virus was demonstrated in the blood for a period of six days; the maximum level was $10^{2.5}$ LD$_{50}$ per 0.03 ml. Neutralising antibodies appeared on the fifth day and by the eighth day the titre exceeded the maximum dilution tested, namely 1:6,250. Haemagglutination-inhibition was detected one day later than neutralising activity and the maximum titre of 1:640 was attained by the tenth day. Leucopaenia was severe during the first nine days but cell counts recovered over the following five days. This animal developed severe symptoms aptly described by the alternative name for louping ill disease, namely, 'trembles'. It was expected to die but recovered fully over the course of six weeks.

Four sheep which had uncomplicated infections with LI 31 virus did not exhibit specific symptoms of the disease. These infections could be grouped in two ways. All four sheep had monophasic fevers but one of them was exceptional in exhibiting only marginal hyperthermia and no respiratory distress or loss of weight, in spite of a tick-borne fever infection three months previously. On the other hand, this sheep, and two of the three which had definite pyrexia, developed moderate levels of viraemia (below $10^{3.0}$ LD$_{50}$ of virus per 0.03 ml) while the third pyrexic animal had no demonstrable viraemia. The antibody response was poorest in the non-viraemic animal.

Sheep infected with tick-borne fever. Uncomplicated infections of/
of tick-borne fever were monitored by courtesy of Dr. Foggie, for comparison with the dual infections encountered in the three tick-infested sheep.

Sheep 8050 (Figure 28) and 7C93 (Figure 29) developed intense pyrexia of short duration four days after the initial intravenous injection of tick-borne fever blood. Well defined leucopaenia was coincidental to the fever and was maximal eight and ten days after infection in the two sheep respectively. The leucopaenia followed the pattern seen in uncomplicated louping ill infections, with an early lymphocyte drop being followed shortly by neutropaenia. The lymphocyte counts recovered steadily after the lowest point so that the leucocyte total count was restored to its initial level by the third week. Neutropaenia was more prolonged and even by the fourth week the neutrophil levels were not quite restored. A surge of the eosinophil counts in both sheep corresponded with the recovery of the total cell counts at three weeks. Infected polymorphs were seen in the bloodsmears for five and six day periods at the time of fever and onset of leucopaenia. A maximum count of sixty-six per cent infected polymorphs was recorded in both animals. Anaemia was not seen. Both sheep were weak and listless for about three weeks and, by appearances, must have lost a good deal of weight.

Neither sheep developed pyrexia when subjected to a similar uncomplicated tick-borne fever infection seven months later. Low levels of infected polymorphs were, however, observed in the bloodsmears of sheep 7C93 on three consecutive days. No trends could be deduced from the white cell count fluctuations on this /
Fig. 26. Response of sheep 4B55 to subcutaneous infection with $10^{5.3} \text{LD}_{50}$ of LL 31 virus.
Fig. 27. Response of sheep 1005 to subcutaneous infection with $10^{6.7}$ LD$_{50}$ of LI 31 virus.
Fig. 29.  a) Response of sheep 7093 to intravenous infection with 1 ml of tick-borne fever blood.  b) Response of 7093 to intravenous challenge with 1 ml of tick-borne fever blood.
Fig. 28. a) Response of sheep 8C50 to intravenous infection with 1 ml of tick-borne fever blood. b) Response of 8C50 to intravenous challenge with 1 ml of tick-borne fever blood.
this occasion, but the lymphocytes did decrease in 7093 on the fourteenth day. Cell counts were discontinued after this day.

A susceptible sheep, number 6129, was inoculated along with the above two sheep on the second occasion to check the infectivity and virulence of the challenge dose. Fever began on the fourth day in this animal (Figure 30) and gradually subsided over the next ten days. Leucopaenia was moderately severe. The lymphocyte count fell with the onset of pyrexia and recovered slightly over the fourteen day observation period. Neutrophil counts fell towards the end of this period. An eosinophilia, stemming from an earlier injection of non-infective blood, disappeared rapidly at the beginning of the fever. Tick-borne fever parasitised cells were seen in bloodsmears over a nine day period, and the proportion of infected polymorphs exceeded fifty per cent for six of the nine days. Sheep 6129 was an aged animal and did not appear to be unduly discomfited by the infection.

*In summary* - Leucopaenia involving both major cell types was found to be a feature of primary tick-borne fever infection resulting from the intravenous injection of infective blood in a total of three sheep. All three became hyperthermic and tick-borne fever bodies could be seen in their bloodsmears for at least a five day period. The maximum count of infected polymorphs exceeded sixty per cent in each of the sheep. The disease in the two younger animals was characterised by listlessness and weakness for three weeks after pyrexia and by an apparent loss of weight. The/
Fig. 30. Response of sheep 6129 to intravenous infection with 1 ml of tick-borne fever blood.
The older sheep did not become ill apart from experiencing the febrile reaction.

The two younger sheep did not develop pyrexia when challenged with the same tick-borne fever strain seven months later. The only sign of infection on this occasion was a low level of infected polymorphs noted in the bloodsmears of one of the two animals for a three day period.

**Sheep used in tick transmission experiments.** The thirteen sheep here were all used in attempted transmissions with the later instars of the ticks fed on the viraemic sheep 1C08, 1C10 and 1C28 above. The results fall into three main groups, namely, four sheep which became infected with both louping ill and tick-borne fever, five sheep which became infected with tick-borne fever only and four sheep which acquired neither infection.

The infected *I. ricinus* ticks derived from preceding instars which fed on viraemic sheep 1C08 and 1C28 were fed together on individual sheep in the transmission attempts and were responsible for the four dual louping ill and tick-borne fever infections. The ticks are not of concern at present, but it should be noted here that these ticks were attached in two groups at a five day interval on each of the four test sheep. In this way it is possible to establish that the first group of ticks transmitted louping ill before the second group fed in each instance. For example, while no demonstrable viraemia occurred in sheep 5C19 (Figure 31), louping ill neutralising antibody appeared in the
the serum after the attachment of nymphs derived from larvae which fed on sheep 1C28 but before the attachment of larvae derived from nymphs which fed on sheep 1C08. The onset of pyrexia preceded the feeding of the second group of ticks in this instance, and the appearance of tick-borne fever bodies in bloodsmears coincided with the appearance of the louping ill antibody. In one of the three remaining sheep viraemia was demonstrable before the attachment of the second group of ticks, while in the two others a rise of antibodies occurred before the second attachment of ticks. It should also be recorded here that clean laboratory-bred larvae were attached along with the second group of infective ticks on two of the sheep (9C49 and 1C03).

Two of the four dual transmissions of louping ill and tick-borne fever were to wholly susceptible sheep, numbers 5C19 and 9C49. The other two animals, 1C03 and 4B55, had previously been infected with louping ill and 4B55 had experienced tick-borne fever as well. None of these four animals died following the dual infections from tick infection.

There were slight quantitative differences in the reactions of the two susceptible sheep. The temperature rose four days after the initial infestation in 5C19 (Figure 31) and remained high for ten days, although there was a pattern of several peaks within this period. In sheep 9C49 (Figure 32) the temperature showed irregular fluctuations between the second and sixteenth days after infestation, but was significantly elevated for only the eighth, ninth and tenth days. No viraemia was demonstrated in 5C19, and/
Fig. 31. Response of sheep 5C19 to infestation with nymphs derived from sheep 1C28 to 1C08.
Fig. 32. Response of sheep 9C49 to infestation with adult ticks derived from sheep 1008 to 1028.
and only a very low level of virus was found over three days in the blood of 9C49. Haemagglutination-inhibition antibody was detected on the sixth day in both animals and was accompanied by neutralizing antibody in 9C49. In 5C19 the neutralizing antibody had appeared one day earlier. The antibodies reached their peak levels by the ninth and eleventh days and titres were highest in 9C49 in which viraemia had been demonstrable. The tick-borne fever parasitaemia was more intense and prolonged in sheep 5C19, in inverse relation to the louping ill virus and antibody levels in the two animals. The infected polymorph counts were recorded over a thirteen day period following the fifth day in 5C19 and for six days after the eighth day in 9C49. Maximum levels of infected polymorphs exceeded 60 per cent in both instances. Moderate leucopenia developed in both animals. Lymphocyte levels fell early but fluctuated. Neutrophil counts tended to decrease about a fortnight after the first infestation. It is interesting to observe on figure 32 that the initial fall in lymphocytes in sheep 9C49 came as soon as the second day after infestation when the fluctuation of temperature began and one day before viraemia became demonstrable.

Both sheep became weak during the infection. On the twelfth day 5C19 would go down to rest, and exhibited a slight nervousness if disturbed. On the thirteenth day it seemed unsteady on its legs. It remained depressed for the following three days. Both animals were much improved at the end of three/
three weeks. They appeared brighter and were eating well. These sheep appeared to be distressed during the fever to a degree not seen in experimentally induced fevers of either louping ill or tick-borne fever. This feature was common to all the tick-borne fever cases arising from tick-transmission, whether accompanied by louping ill or not. These sheep would stand panting with their heads down, their ears hung horizontally. They had a wide-eyed, alarmed expression, but were not excitable.

The temperature curves of the two immune sheep bear a resemblance to those of the last two sheep. Sheep 1003 (Figure 33b) had a few peaks of fever between the sixth and fifteenth days after the first infestation, while 4B55 (Figure 34) had a single short period of pyrexia after the third day. Viraemia was not demonstrated in either sheep, but antibody levels were boosted by the fifth day. Tick-borne fever infected polymorphs were found in the bloodsmears of 4B55 on the fifth to ninth days and the highest count exceeded sixty per cent infected cells. In sheep 1003 parasitaemia was seen for nine days starting one week after infestation, and the highest count was 80 per cent infected polymorphs. There was moderately severe leucopaenia in both animals one week after infestation. Up to this day it was chiefly the lymphocytes that were affected but thereafter a neutropaenia was seen in both animals. The low level of neutrophils in sheep 1003 between the seventh and twenty-first days is especially striking, and appreciable recovery was noticed only in the fifth week. The restoration of lymphocyte counts was also delayed in /
Fig. 33. a) Response of sheep 1C03 to subcutaneous infection with $10^{6.6}$ LD$_{50}$ of LI 31 virus. b) Response of 1C03 to infestation with adult ticks derived from sheep 1C28 and 1C08.
Fig. 34. Response of sheep 4B55 to infestation with nymphs derived from sheep 1008 and 1028.
in these two sheep.

Sheep 1003 became markedly distressed at the start of fever seven days after infestation. It was panting with its head held down and its ears lying flat. There was a crusted nasal discharge. It remained in an apparent state of depression until the sixteenth day when it was noticed to be slightly nervous. On this day there was a tendency to stamp its feet occasionally, apparently involuntarily. On the following day there was a vacant stare in its eyes and a twitching of the head and neck muscles. By the nineteenth day the sheep was steady, looked brighter and was eating well. The other sheep, 4B55, also behaved in a somewhat unexpected way for an immune animal, although it did not exhibit nervous symptoms. It started panting on the fourth day when the temperature rose and on the seventh and eighth days it was recumbent and showed great reluctance to rise. On the ninth day it suddenly recovered.

Tick-borne fever infection was confirmed in the bloodsmears of five further sheep which became pyretic following tick infestation. No louping ill viraemia or serum antibody was demonstrated in this group of animals. Counts of the tick-borne fever infected polymorphs were not recorded for two of them, 5C16 and 8C70, since leucocytes were in any case only monitored on a few key days in this pair of animals, but the presence of the agent was confirmed during the fevers.
fevers.

Sheep 5C25 started panting on the fifth day when an intense temperature reaction began (highest temperature 108°F) (\textsuperscript{35}). The following day it seemed to be slightly excitable and exhibited the nervous licking movements sometimes seen a little later on in the course of louping ill infections. By the seventh day the animal was depressed and had lost its appetite, but it was eating again and appeared calm for the following three days. On the morning of the eleventh day the sheep was found lying down and it could only stand and walk if supported. There were no nervous symptoms associated with the weakness which took the form of a general lack of tonus in the musculature. The pattern of the temperature curve was suggestive of the classical diphaseic reaction of louping ill, but it also resembles the thermal response of sheep 5C17 (Figure 36a), which had uncomplicated tick-borne fever, and which was on experiment at the same time with similar ticks. It was too early to consider the mouse viraemia tests non-positive, and serum tests had not yet been done, so that the possibility of louping ill could not be discounted. Tick-borne fever bodies were first seen in the bloodsmears of 5C25 on the sixth day and were still present at a fairly high level on the eleventh day, although the maximum count of infected polymorphs recorded was only 52 per cent. A marked leucopaenia coincided with pyrexia and involved only lymphocytes on the eleventh day when the sheep were slaughtered but there had been a transient fall of neutrophils.
Fig. 35. Response of sheep 5C25 to infestation with nymphs derived from sheep 1C10.
neutrophils. It was decided to kill the animal for post-mortem and histopathological examination.

In the event, no louping ill viraemia or serum antibody was found over the entire eleven day period. No histopathological lesions were seen in the central nervous system. No virus was recovered from brain material, spleen or prescapular lymph gland. The latter two organs were swollen at post-mortem, but malphigian corpuscles could not be recognised in the spleen. The histopathology of these organs was curious. The lymphoid follicles were active in the lymph node, but large numbers of macrophages, may in mitosis, were present in the cortex and medullary sinuses. Single or multiple eosinophilic intranuclear inclusion bodies were described in the macrophages. In the spleen the malphigian bodies were sparse and ill defined, and again there was a predominance of macrophages, many with the intranuclear inclusions.

Protracted fever occurred in two of the remaining sheep which had uncomplicated tick-borne fever, namely, 5C17 and 5C16 (Figures 36a and 38a). The other two sheep, 5C28 and 8C70 (Figures 37a and 39a), had fleeting thermal responses.

In the two in which full counts were made (5C17 and 5C28, Figures 36a and 37a), the tick-borne fever bodies were seen in bloodsmears over ten and six day periods. The maximum counts of/
Fig. 36. a) Response of sheep 5C17 to infestation with nymphs derived from sheep 1C10. b) Response of 5C17 to subcutaneous challenge with $10^{6.3} \text{LD}_{50}$ of LI 31 virus.
Fig. 37. a) Response of sheep 5C28 to infestation with adult ticks derived from sheep 1C10. b) Response of 5C28 to subcutaneous challenge with $10^{6.3} \text{LD}_{50}$ of LI 31 virus.
Fig. 38.  a) Response of sheep 5C16 to infestation with adult ticks derived from sheep 1C28.  b) Response of 5C16 to subcutaneous challenge with $10^{6.3}$ ID$_{50}$ of LL 31 virus.
Fig. 39. a) Response of sheep 8G70 to infestation with nymphs derived from sheep 1G08, 1G10 and 1G28. b) Response of 8G70 to subcutaneous challenge with $10^{6.3}$ LD$_{50}$ of LI 31 virus.
of infected polymorphs exceeded 50 per cent in both animals. A transient neutrophilia followed infestation in sheep 5026. Leucopenia then developed in this animal as in 5017, with a fall in lymphocytes preceding a decrease in neutrophils. The few cell counts made in 5016 (Figure 38a) suggest a less severe cell response of the same pattern. A neutrophilia on the fourth day in sheep 8070 (Figure 39a) may have been associated with pyogenic infection of the ear associated with tick-bite. The swelling of the ear receded rapidly after an intra-muscular injection of penicillin and streptomycin.

All four sheep appeared very distressed during pyrexia and the two with the longer periods of fever, 5017 and 5016, became dull and somewhat lethargic a week after infestation. They recovered swiftly after fever subsided.

The final group of four sheep were those which failed to develop either tick-borne fever or louping ill following infestation. Three of these, 8022, 5022 and 8071, were infested with the succeeding instars of the exotic tick species which had fed on the virus-donor hosts. The three were taken on to experiment simultaneously and all three had diarrhoea during the first two weeks of the observation period, but this was thought to be associated with some extraneous cause. They were under observation for just over three weeks and failed to develop pyrexia.

Sheep 8022 was slightly lame in the off-hind limb before tick infestation. This lameness was reported to be due to joint ill/
ill which the animal had experienced as a lamb. Shortly after infestation its temperature wavered in a manner which suggests a mild response to the tick infestation. (Figure 40). The white cell counts were extremely high and fluctuated markedly over the three weeks of observation. It seems likely that the leucocytosis was allied to a chronic joint ill infection, as the lameness became worse soon after the tick attachment and some weeks later the hock was too painful to take the weight of the animal. The temporal coincidence of tick feeding and exacerbation of joint-ill symptoms is striking. There was no evidence of louping ill or tick-borne fever transmission and it was decided to kill the animal rather than keep it in prolonged discomfort for louping ill challenge.

Sheep 5C22 did not manifest pyrexia or other symptoms during the feeding of adult D. andersoni ticks, but there was a transient rise of white cells of both main types centering on the twelfth day after infestation (Figure 41a). There was no evidence from blood and serum observations of disease transmission. Subsequent to this infestation, and before the sheep was challenged with louping ill, it was used as host to a number of field-collected I. ricinus adult ticks. The ticks were required for a separate purpose and the reaction of the sheep, being of no concern, was not monitored at first. It was noticed on the sixth day, however, that the sheep was panting. On the ninth day the sheep showed severe nervous symptoms. The animal held its head at an angle with the ears hanging down and rolled its eyes. There was a/
Fig. 40. Response of sheep 8C22 to infestation with *H. anatolicum* adults derived from sheep 1C28. Sheep 8C22 had a history of joint ill as a lamb. At the time of experiment it had a slight residual lameness which was exacerbated following tick infestation. The leucocytosis is probably associated with chronic infection.
Fig. 41. a) Response of sheep 5C22 to infestation with D. andersoni adults derived from sheep 1010. b) Response of 5C22 to infestation with field-collected I. ricinus adults and, subsequently, to subcutaneous challenge with \(10^6.3\) LD\(_{50}\) of LI 31 virus.
a slight trembling of the head and neck. The hindquarters were weak and swayed as the sheep walked, sometimes so much that the animal fell down. In short, the symptoms were characteristic of an acute case of louping ill. Furthermore, one ear was swollen and had small pustules at the sites of tick-bite. For this reason an intramuscular injection of penicillin and streptomycin was administered. The temperature was elevated at this point, and tick-borne fever bodies were seen in bloodsmears (Figure 41b). There was also a severe leucopaenia.

The animal recovered strength over the next three days, and by the sixteenth day after infestation it appeared thin but otherwise normal. The lymphocyte count had been restored, but neutropenia continued. No louping ill antibody could be demonstrated in serum samples taken on the ninth to sixteenth days. On the sixteenth day the animal was included with others being challenged by subcutaneous louping ill infection.

Apart from the diarrhoea mentioned above, sheep 3071 (Figure 42a) did not exhibit any symptoms during tick infestation, and in sheep 5023 (Figure 43a) there was only a transient neutrophilia after tick attachment.

In summary - Thirteen sheep were infested with the later instars of ticks fed on viraemic sheep 1008, 1010 and 1028.

Four sheep became infected with both louping ill and tick-
Fig. 42.  a) Response of sheep 8C71 to infestation with *R. bursa* larvae derived from females which fed on sheep 1C10.  b) Response of 8C71 to subcutaneous challenge with $10^{6.3} ID_{50}$ of II 31 virus.
Fig. 43. a) Response of sheep 5G23 to infestation with adult ticks derived from sheep 1G10. b) Response of 5G23 to subcutaneous challenge with $10^{6.3} \text{ ID}_{50}$ of LI 31 virus.
tick-borne fever, but none of these developed severe symptoms or died. Two were susceptible to both diseases before infestation, while two had recovered from subcutaneous infections of louping ill. One of the latter had a previous infection with tick-borne fever by needle as well. Louping ill viraemia, of a very low intensity and short duration, was recorded in only one of the susceptible sheep. But, louping ill infection was evidenced in all four animals either by the appearance of antibodies or by the boosting of pre-existing antibody levels on the fifth and sixth days after infestation. All four sheep appeared distressed during pyrexia and transient nervous symptoms were seen in one susceptible and one immune animal. The other immune animal became temporarily recumbent, without nervous symptoms, in spite of having experienced both diseases before. The severity of tick-borne fever parasitaemia in this animal was beyond what could be expected in a re-infection with this agent alone. In general, the clinical effects of the dual infection following tick infestation seemed more severe in the two immune sheep than in the susceptible ones.

Tick-borne fever infection was confirmed in the bloodsmears of five further sheep which became pyretic following the tick infestations, but no louping ill viraemia or serum antibody was demonstrated in this group. One of these animals was killed on the eleventh day after infestation when it was too weak to stand unsupported. No evidence of louping ill was obtained after death from histopathological or virological examinations. The four/
four others recovered from non-specific symptoms associated with pyrexia.

A final group of four sheep failed to develop pyrexia during infestation and they were monitored in the daily routine manner for over three weeks without evidence of louping ill or tick-borne fever. One of these sheep was used subsequently to feed field-collected, adult *I. ricinus* ticks and developed behavioural symptoms characteristic of acute louping ill. It recovered without serological evidence of this disease, but tick-borne fever bodies were seen in bloodsmears.

**Sheep challenged after the tick transmission experiments.** The sheep which had failed to show evidence of louping ill in the tick transmission experiments were challenged simultaneously with LI 31 virus. An inoculum of $10^{6.3}$ mouse ic LD$_{50}$ was administered subcutaneously.

Full clinical monitoring in the routine manner was not practicable for seven sheep and the number of observations was therefore reduced. White cells were counted on four key days only. Serum for antibody tests was taken on the day of inoculation and on the seventh and fourteenth days afterwards. Viraemia was titrated to maximum dilutions of $10^{-2}$ on the second and fifth days and up to $10^{-3}$ on the third and fourth days. Whole blood only was tested in mice on the other days of the fourteen day /
The features of the challenge infections in the seven sheep are shown individually in Figures 36b - 39b and 41b - 45b inclusive. All the sheep had diphasic fevers but the febrile response of 8C71 (Figure 42b) was weak. Moderate leucopaenia was recorded in all the animals. Viraemia was intense in three sheep, numbers 8C70, 8C71 and 5C23 (Figures 39b, 42b and 43b) but the end-points of their maximum titres were not determined. An intermediate viraemia in 5C16 (Figure 38b) exceeded $10^{2.0}$ LD$_{50}$ of virus per 0.03 ml on one day only. The remaining three sheep, 5C17, 5C28 and 5C22 (Figures 36b, 37b and 41b), circulated low levels of virus. Haemagglutination-inhibition antibody was not detected in the initial serum samples but appeared in the subsequent sera of all seven animals. Titres were higher than 1:640 only in the two sheep which survived intense viraemias, namely, 8C71 and 5C23 (Figures 42b and 43b).

Nervous symptoms were seen in four of the animals. Two of these, 5C17 and 5C28 (Figures 36b and 37b), which had mild viraemias, exhibited only transient excitability associated with the second stage of fever. Two sheep with more intense viraemias, 8C70 and 5C23 (Figures 39b and 43b), became ataxic after similar excitability. Sheep 5C23 had the characteristic high-stepping gait on the eighth, ninth and tenth days and tended to roll its eyes and ears at odd angles. The ataxia disappeared after the tenth day and the animal gradually recovered strength. Sheep 8C70 had torticollis on the/
the ninth day and would circle to the left and stumble. On the
tenth day it was recumbent and went into convulsions of galloping
and paddling movements. When raised it would charge wildly to the
left or circle and fall. It was killed in extremis later on the
same day. At post-mortem the lymph glands were enlarged and moist.
The spleen was atrophied and the malphigian corpuscles were indistinct.
The lungs appeared to be normal. Histopathological lesions of
non-suppurative encephalitis were reported and virus was recovered
from brain material.

In summary — Seven sheep which had failed to show evidence of
louping ill in tick transmission experiments were challenged by
subcutaneous inoculation of $10^{6.3} L_{D50}$ of LI 31 virus.

All seven sheep developed diphasic fevers. Viraemia was
intense in three of them, with maximum titres exceeding $10^{3.0} L_{D50}$
per 0.03 ml. An intermediate viraemia in one animal exceeded
$10^{2.0} L_{D50}$ per 0.03 ml at the highest point and the remaining
three sheep circulated low levels of virus. Haemagglutination-
inhibition antibody, not demonstrable in sera taken before
inoculation, appeared in the seven and fourteen day sera of all the
animals. Titres exceeded 1:640 only in two animals which had
intense viraemias.

Two sheep with low grade viraemias exhibited transient
excitability associated with the second fever. Two animals with
intense viraemias became ataxic after similar excitability. One/
One of these recovered after walking in the characteristic high-stepping manner for three days. The other animal had torticollis and severe cerebellar ataxia with convulsive fits. Histopathological lesions of encephalitis were reported and virus was recovered from the brain.

It is concluded that all seven animals were immunologically competent and susceptible to louping ill when challenged. They had not, therefore, experienced louping ill infection in the course of the tick transmission experiments.
B. Ticks.

Sheep 1C08, 1C10 and 1C28 were used as virus donors for studying infection of the tick. The maximum titres of viraemia attained in these sheep were $10^{2.5}$ LD$_{50}$ per 0.03 ml in 1C10, $10^{4.5}$ LD$_{50}$ per 0.03 ml in 1C28 and $10^{7.5}$ LD$_{50}$ per 0.03 ml in 1C08. Each animal underwent a tick-borne fever reaction during the tick feeding experiment despite the fact that all three had been subjected to an immunising infection. The dual infections are described above.

The bulk of the tick material fed on the sheep consisted of I. ricinus L. larvae and nymphs and the total recoveries of these are listed in table 10. Most of the available ticks were placed on the animals to feed during the period of patent viraemia so that the earlier groups of ticks fed on each sheep contained relatively smaller numbers of individuals. This disparity was exaggerated by the loss of ticks in the early groups through desiccation when collections were made at 24 hourly intervals. The recoveries improved remarkably after a change to 12 hourly intervals of collection and 93.5% of 200 nymphs were recovered fully engorged and alive in one instance. The feeding times of the exotic species and of the few I.ricinus adults were protracted and death supervened in sheep 1C08 and 1C28 before many of these ticks were replete.

At the first sorting and cleaning six weeks after detachment it was found that as many as 97 live nymphs had hatched from 100 engorged larvae per tube. Moulting was well in excess of 70% in most instances, but there was complete mortality with fungal growth/
TABLE 10.

RECOVERIES OF ENGORGED I. RICINUS TICKS FROM SHEEP 1C08, 1C28 AND 1C10

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Nymphal tick groups:</th>
<th>Larval tick groups:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>1C08</td>
<td>48/100</td>
<td>111/180 (61.7%)</td>
</tr>
<tr>
<td>1C28</td>
<td>103/e.150</td>
<td>141/180 (78.3%)</td>
</tr>
<tr>
<td>1C10</td>
<td>31/e.150</td>
<td>157/180 (87.2%)</td>
</tr>
</tbody>
</table>
growth in a few tubes. One month later the losses had not been heavy and between 40% and 70% of the original numbers of ticks in the various tubes were still alive. The ticks were then transferred from the 22°C constant temperature room to 10°C for six weeks and during this time the mortalities were slight. At the end of this period, four months after detachment, the post-moult virus assays of the ticks were done and it was the intention that transmission attempts should follow immediately. Most of the transmission experiments had to be delayed for two months, however, owing to the pressure of work on other aspects of the project and considerable tick losses occurred in the interim. This might have been avoided if the material had again been sorted and transferred into clean tubes.

Figures 44–46 show the feeding periods of the various groups of ticks in relation to the course of the dual infections in the hosts and include a schematic representation of the subsequent investigation of the ticks. For example, in figure 44 it is shown that the first group of I. ricinus L. nymphs fed on sheep 1008 consisted of 100 individuals placed on the animal three days prior to virus inoculation. Thirty-seven of the nymphs were recovered in a fully engorged and viable state on the day of inoculation. A further eleven were recovered on the following morning, making a total of 48 ticks which were fed successfully. Twenty-five of the engorged nymphs were pooled and tested for virus content. Tick pool number 1 was found not to contain virus.
Fig. 44. Feeding periods of the various tick groups on sheep. 1088
hers in relation to the course of the dual louping ill and tick-
borne fever infection of the host. Subsequent examination of the
ticks is shown schematically.
ticks on all three sheep. The larvae and nymphs in these groups fed before demonstrable viraemia occurred and they failed to gain infection.

The second group of nymphs on sheep LCO8 consisted of 180 individuals placed on the animal on the day of virus inoculation to feed during early viraemia. On the third morning after inoculation 61 of these nymphs were recovered in a fully engorged and viable state. A further 21 nymphs were replete by that evening and recoveries of 13 and 16 ticks on the fourth day complete the figures for this group. Thus 111 (61.7%) of the original 180 were fed successfully. Twenty-five of the engorged nymphs were pooled (serial number 2) and were found not to contain virus. Twenty-five adult ticks which had moulted from nymphs of both the first and second groups fed on sheep LCO8 constituted tick pool 33. This, too, was virus non-positive and the remaining adult ticks of these two groups failed to transmit loup ing ill to sheep 5623. Virus was not recovered from individual engorged female ticks after the transmission attempt.

The third group of nymphs, attached during patent viraemia, was arbitrarily divided into two sets representing shorter and longer engorgement periods and tick pools 3 and 4 were tested separately for virus. Both were positive at the same titre, $10^{5.1}$ ID$_{50}$ per 0.03 ml. The rest of the diagram follows the same pattern. Details of the infective titres of the tick pools and of the virus and antibody content of host blood are shown in the schematic table 11.
### TABLE 11.

THE INFECTION OF *I. ricinus* TICKS FED ON SHEEP 1C08 IN RELATION TO BLOOD MEAL COMPOSITION.

<table>
<thead>
<tr>
<th>Days after inoculation:</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viraemia, log LD&lt;sub&gt;50&lt;/sub&gt;/0.03 ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>6.8</td>
<td>7.5</td>
<td>2.75</td>
<td>2.5</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>HI titre dilution reciprocal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>640</td>
<td></td>
</tr>
<tr>
<td>HI titre neg. log dilution</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.35</td>
<td>2.05</td>
<td>2.75</td>
<td>3.45</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days on which nymphs fed</th>
<th>Grp. 2</th>
<th>Grp. 68.2%</th>
<th>Grp. 31.8%</th>
<th>Grp. 76.7%</th>
<th>Grp. 23.3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days on which larvae fed</td>
<td>Grp. 2</td>
<td>Grp. 68.2%</td>
<td>Grp. 31.8%</td>
<td>Grp. 76.7%</td>
<td>Grp. 23.3%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Titre post-transmission titres.</th>
<th>9649</th>
<th>1003</th>
<th>4B55</th>
<th>5019</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg. log post-transmission titres.</td>
<td>5.1</td>
<td>5.7</td>
<td>5.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*Infective ticks pooled with comparable group from sheep 1C28*

ND = Virus assay not done.

- = Non-positive result
In brief, only the third group of larvae and of nymphs which fed on sheep 1C08, i.e. those which had been attached during maximal viraemia, were found to be infected. Within these virus-positive groups, pools of individuals which had fed rapidly showed virus titres similar to those of pools of ticks which had taken longer to feed (table 11). The second group of nymphs, which fed during early viraemia, did not acquire infection. Most of the nymphs in this group, however, were replete before peak viraemia. Only five larvae which had fed to repletion during early viraemia were recovered alive and these were not examined for virus.

After the moult, the succeeding instars of the infected larvae and nymphs from sheep 1C08 had slightly higher virus titres than the engorged ticks from which they were derived, but the increases were within the limits of error of virus titration (table 11).

Figures 45 and 46 show that the *I. ricinus* ticks were similarly handled in relation to sheep 1C28 and 1C10. Certain differences from sheep 1C08 are immediately evident in the results of these animals. On sheep 1C28, infection took place in the group of nymphs which fed during early viraemia as well as in the larvae and nymphs which fed during maximal viraemia. Only four larvae which had fed during early viraemia were recovered alive and these were not tested for virus. Within the virus-positive groups from sheep 1C28, pools of individuals which fed rapidly had higher virus titres than pools of ticks which took longer to feed (table 12).

In two instances the virus titres recorded after the moult in/
Fig. 45. Feeding periods of the various tick groups on sheep 1028 shown in relation to the course of the dual louping-ill and tick-borne fever infection of the host. Subsequent examination of the ticks is shown schematically. Column headings as in figure 44.
TABLE 12.

THE INFECTION OF I. RICINUS TICKS FED ON SHEEP 1C28 IN RELATION TO BLOOD MEAL COMPOSITION.

<table>
<thead>
<tr>
<th>Days on which nymphs fed</th>
<th>Days after inoculation:</th>
<th>Tick pool virus assays:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 4 5 6 7 8 9</td>
<td>Sheep number</td>
</tr>
<tr>
<td>Viraemia, log 50/0.03 ml</td>
<td>- - 0.2 3.5 4.5 4.5 1.2 1.5 0.2 -</td>
<td>Neg. log post-transmission</td>
</tr>
<tr>
<td>HI titre dilution reciprocal</td>
<td>- - - - - - - 40 160 640 640</td>
<td></td>
</tr>
<tr>
<td>NI titre neg. log dilution</td>
<td>- - - - - - - 2.2 2.2 3.3 3.3</td>
<td></td>
</tr>
<tr>
<td>Grp 2 55.1%</td>
<td>X X X X X</td>
<td>4.7 4.7 1003 + 1.7, 1.8, 2.5, 3.7, 4.2</td>
</tr>
<tr>
<td>Grp 3 44.9%</td>
<td>X X X X X</td>
<td>4.3 3.3 9049 + 4.3, 5.2</td>
</tr>
<tr>
<td>Days on which larvae fed</td>
<td>Grp 3 11.8%</td>
<td>X X X X X</td>
</tr>
</tbody>
</table>

* Infective ticks pooled with comparable groups from sheep 1C08.
in ticks from sheep 1C28 differed significantly from the pre-moult titres. Adult ticks showed an increase in titre over the rapid feeding nymphs from which they were derived while slow feeding larvae showed a decrease in titre after the moult (table 12).

Sheep 1C10 produced quite different results from the other two sheep on account of its much lower viraemia. Virus was recovered from newly engorged ticks solely in the instance of larvae attached during maximal viraemia. This was detectable only in one-day-old mouse passage. After the moult, however, virus was detected in the ticks of this group to a titre of $10^{2.1}$ LD$_{50}$ per $0.03$ ml in three-week-old mice (table 13). Although virus was not detected immediately following engorgement in any of the other tick groups fed on sheep 1C10, traces of virus were found after the moult in adults derived from the nymphs attached during maximal viraemia (one-day-old mouse passage).

The *I. ricinus* ticks from all three sheep were tested for louping ill transmission two months after the post-moult virus assays were performed. The relationship of the transmission tests to the host-origin of the tick material can best be seen from the experimental design, which is shown graphically in figures 6 and 7. Certain tick groups were used together on individual sheep in the transmission tests, while in one instance a single tick group was split on to two susceptible animals. The reasons for these combinations will be explained here.

In planning the tests it was originally intended that/
Fig. 46. Feeding periods of the various tick groups on sheep 1010 shown in relation to the course of the dual louping ill and tick-borne fever infection of the host. Subsequent examination of the ticks is shown schematically. Column headings as in figure 44.
TABLE 13.

THE INFECTIO Nit THE RICINUS TICKS FED ON SHEEP 1610 IN RELATION TO BLOOD MEAL COMPOSITION

<table>
<thead>
<tr>
<th>Days after inoculation:</th>
<th>Tick pool virus assays:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viraemia, log LD&lt;sub&gt;50&lt;/sub&gt;/0.03 ml</td>
</tr>
<tr>
<td></td>
<td>0 1 2 3 4 5 6 7 8 9</td>
</tr>
<tr>
<td></td>
<td>- - 1.8 2.5 2.2 0.8 1.5 TR 3.8</td>
</tr>
<tr>
<td></td>
<td>- - - - - - 20 40 40 160</td>
</tr>
<tr>
<td></td>
<td>- - - - - - 1.35 2.05 2.75 3.8* 3.8</td>
</tr>
<tr>
<td>Days on which nymphs fed</td>
<td>X X X X X</td>
</tr>
<tr>
<td>Days on which larvae fed</td>
<td>X X X X X</td>
</tr>
<tr>
<td></td>
<td>X X X X X</td>
</tr>
</tbody>
</table>

TR = trace amount of virus, demonstrated by suckling mouse passage
ND = Virus assay not done
- = Non-positive result
* = End-point of neutralising activity beyond highest dilution tested, i.e. 1:6,250 or 10<sup>3.8</sup>.
that comparable groups of infected ticks should be fed on immune and susceptible sheep in parallel, to check the effect of antibody on virus already present in the tick. Because of the losses which had been sustained, the groups of infected ticks were too small to split for this purpose. It was then decided to attempt a compromise by using the infected ticks from 1C08 and 1C28 together.

By placing half the surviving adults of the infected group from 1C08 on the susceptible sheep 9C49 five days before similar ticks from 1C28, it was hoped to show that the 1C08 ticks were transmissive before the second attachment occurred. The order of attaching the remaining halves of the tick groups from the two donor sheep was reversed on an immune sheep, 1C03, so that ticks from 1C28 could be proven transmissive before those from 1C08 fed. Comparison of the titres of the ticks after feeding would indicate the effects of susceptible and immune hosts on virus in the tick.

The same principle was adopted for feeding infected nymphs on susceptible sheep 5C19 and on immune sheep 4B55. The pattern of successive feedings reversed that employed for the adults: in this instance the ticks from 1C08 preceded those from 1C28 on the immune animal and followed them on the susceptible.

The evidence presented above in the sheep results does, indeed, confirm transmission of both lousing ill and tick-borne fever by ticks from both donor hosts.
Nymphs derived from the larvae fed on sheep 1C10, which had the least intense viraemia, were divided on to two susceptible sheep, 5C25 and 5C17, in order to increase the chances of transmission. The evidence for the transmission of tick-borne fever only in both instances is presented in the sheep results. Sheep 5C25 was killed when it became recumbent, but no proof of louping ill infection was obtained.

The larvae which had fed before viraemia on all three donor sheep were tested together because so few were recovered from any one animal. The virus assays (pools 22, 48 and 78) were non-positive for louping ill, and tick-borne fever only was transmitted to sheep 8C70.

The remaining transmission tests concern the adult ticks derived from sheep 1C10 and the adults of the nymphs which fed before viraemia on sheep 1C28. These were placed on susceptible sheep 5C28 and 5C16 respectively, and tick-borne fever only was recorded in the animals.

The tick transmissions are summarised in table 14 which shows the actual numbers of ticks which fed to repletion in the tests. In brief, louping ill was transmitted only by nymphs and adult \textit{I. ricinus} ticks which had fed as larvae and nymphs during demonstrable viraemia on either sheep 1C08 or 1C28. These transmissions involved four sheep and in each tick-borne fever was recorded as well. The remainder of the \textit{I. ricinus} ticks transmitted only tick-borne fever in five instances, while one/
**TABLE 14.**

**SUMMARY OF THE TICK TRANSMISSIONS SHOWING THE NUMBERS OF TICKS FED TO REPLETION IN THE TESTS.**

<table>
<thead>
<tr>
<th>Test sheep</th>
<th>Nymphs</th>
<th>Females</th>
<th>Exotic ticks</th>
<th>Original host</th>
<th>Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L.I.</td>
</tr>
<tr>
<td>1003</td>
<td>-</td>
<td>3/10</td>
<td>-</td>
<td>1628</td>
<td>+</td>
</tr>
<tr>
<td>1004</td>
<td>-</td>
<td>3/12</td>
<td>-</td>
<td>1608</td>
<td>*?</td>
</tr>
<tr>
<td>4B55</td>
<td>5/15</td>
<td>-</td>
<td>-</td>
<td>1608</td>
<td>+</td>
</tr>
<tr>
<td>4B56</td>
<td>17/27</td>
<td>-</td>
<td>-</td>
<td>1628</td>
<td>?</td>
</tr>
<tr>
<td>5016</td>
<td>-</td>
<td>9/18</td>
<td>-</td>
<td>1628</td>
<td>-</td>
</tr>
<tr>
<td>5017</td>
<td>17/33</td>
<td>-</td>
<td>-</td>
<td>1610</td>
<td>-</td>
</tr>
<tr>
<td>5019</td>
<td>15/27</td>
<td>-</td>
<td>-</td>
<td>1628</td>
<td>+</td>
</tr>
<tr>
<td>5020</td>
<td>10/15</td>
<td>-</td>
<td>-</td>
<td>1608</td>
<td>?</td>
</tr>
<tr>
<td>5022</td>
<td>-</td>
<td>-</td>
<td>37/9 females</td>
<td>1610</td>
<td>-</td>
</tr>
<tr>
<td>5023</td>
<td>8/20</td>
<td>-</td>
<td>-</td>
<td>1608</td>
<td>-</td>
</tr>
<tr>
<td>5024</td>
<td>24/33</td>
<td>-</td>
<td>-</td>
<td>1610</td>
<td>-</td>
</tr>
<tr>
<td>5025</td>
<td>-</td>
<td>10/31</td>
<td>-</td>
<td>1610</td>
<td>-</td>
</tr>
<tr>
<td>5026</td>
<td>-</td>
<td>4/9 females</td>
<td>1628</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5027</td>
<td>8/15</td>
<td>-</td>
<td>1608, 1610, 1628</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5028</td>
<td>-</td>
<td>254/7 nymphs</td>
<td>1610</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5029</td>
<td>-</td>
<td>6/12</td>
<td>-</td>
<td>1608</td>
<td>+</td>
</tr>
<tr>
<td>5030</td>
<td>-</td>
<td>3/10</td>
<td>-</td>
<td>1628</td>
<td>?</td>
</tr>
</tbody>
</table>

*?? indicates transmission not proven as previously attached ticks had already transmitted agent.*
one infestation was not followed by disease. The investigation was concerned primarily with louping ill and the criteria for tick-borne fever in the sheep were restricted to the occurrence of a temperature reaction plus/or the demonstration of infected polymorphs in bloodsmears. The non-reactor was not challenged with tick-borne fever, nor was blood taken for subinoculation into susceptible animals.

The results of the transmission tests are thus in accordance with the evidence from the tick-pool assays; the louping ill infection in sheep 1C1O differed fundamentally from that in the other two virus-donor hosts as regards the infection of immature I. ricinus ticks.

Virus was recovered subsequent to the transmission tests only from the ticks which had engorged on the four animals in which louping ill transmission had occurred. In each instance the ticks, nymphs and females derived from sheep 1C08 and 1C28, showed a fall in titre following engorgement (tables 11 and 12). The nymphs were tested immediately after engorgement but the females were held for three months to oviposit. Consequently, some of the females were in a moribund state when tested and this may account for the low and varied titres recorded in these ticks. Nevertheless, there was no significant difference in titre between nymphs and females which had fed on susceptible sheep and those which had fed on immune animals. Thus there is no evidence of a deleterious effect on virus in the tick following engorgement on immune as opposed to susceptible hosts.
When it became clear that the intensity of viraemia in none of the original three hosts had been below an absolute threshold for the infection of ticks, it was decided to carry out subsidiary tests. Attachments of laboratory-bred larvae were made on sheep 9C49 and 1C03 on the fifth day after these sheep had been infested in the transmission tests with ticks known to contain virus. The course of the infections in these two animals are reported above with the sheep results.

Sheep 9C49 had a trace amount of virus in the blood on the day of attachment of the larvae, i.e. whole blood killed only one out of 5 three-week-old mice, and on the following day antibodies became detectable. The 701 larvae which fed on this animal took between 4 and 6 days to become replete and virus was not recovered from the engorged ticks or from the succeeding instar. Viraemia was not demonstrable in sheep 1C03 which was immune, and the pre-existing level of antibodies was already boosted by the time that the larvae attached. A total of 841 larvae fed on this sheep, and again virus was not found either before or after the moult.

Ticks other than *Ixodes ricinus* larvae and nymphs were not systematically studied and the tests on them were opportunistic. Nine of fourteen *I. ricinus* females placed on sheep 1C28 detached themselves from the ear which had been removed after death. These ticks were only partially engorged but five remained alive and each of these produced a small batch of eggs. The spent females were tested for virus three months after detachment. Three were/
were positive. The titres of two were $10^{2.3}$ and $10^{3.3}$ ID$_{50}$ per 0.03 ml and the third suspension killed only one three-week-old mouse at the lowest dilution tested, namely, $10^{-2}$. One month later the few eggs which they had produced and the small number of larvae which had hatched were pooled and tested for virus. Tick pool 47 failed to kill three-week-old mice at the lowest dilution, $10^{-2}$, but virus was isolated in one-day-old mouse passage. This was subsequently neutralised by louping ill antiserum in a metabolic inhibition test. As far as is known this is the first account of transovarian passage of the agent. Virus could not be isolated by the same method from the eggs of eleven females which had oviposited after transmitting louping ill, even although the infective titres of some of these eleven females exceeded those of the three females above (table 12).

No result was obtained with *R. appendiculatus* since virus assay was prevented by the death of the ticks. This was probably due to a fault in environmental conditions although all 27 engorged nymphs moulsted to adults before death. The *D. andersoni* nymphs were fed on sheep 1C10 which had the least intense viraemia. Tick pool 39 comprising 25 of the derived adults was virus non-positive and the remaining adults failed to transmit louping ill to test sheep 5C22. In view of the results obtained with *I. ricinus* ticks on sheep 1C10, the failure to infect *D. andersoni* nymphs is not surprising. Five *R. bursa* adults were also fed on 1C10 but neither their derived larvae nor the spent females contained virus. The larvae failed to transmit louping ill to sheep 3C71. Larvae-nymphs of a/
a two host strain of *H. anatolicum anatolicum* which fed on sheep 1C28 were tested only after the adult moult and were found to contain $10^{3.1}$ mouse ic ID$_{50}$ of virus per 0.03 ml. These ticks were tested for louping ill transmission on susceptible sheep 8C22. There was no transmission, but only four out of nine females and their attendant males attached and fed to repletion.

In summary - Three sheep were used as virus donors for studying the uptake of louping ill infection by ticks. The maximum titres of viraemia were $10^{2.5}$ ID$_{50}$ per 0.03 ml in sheep 1C10, $10^{4.5}$ ID$_{50}$ per 0.03 ml in 1C28 and $10^{7.5}$ ID$_{50}$ per 0.03 ml in 1C08.

The bulk of the tick material consisted of *I. ricinus* larvae and nymphs. Three groups of larvae and three groups of nymphs were fed on each sheep. These were attached in consecutive order so that the first groups fed before demonstrable louping ill viraemia occurred and the second groups were replete at the peak of viraemia before antibody became detectable. The third tick groups fed from the peak of viraemia into the phase during which antibody replaced virus in the blood.

Larvae and nymphs which fed before demonstrable viraemia occurred, failed to gain louping ill infection.

Of the larvae and nymphs which fed on sheep 1C08, only the third groups, i.e. those groups which fed during maximal viraemia, contained virus when tested in the engorged state. This sheep had the most intense viraemia but the majority of the nymphs in the/
the second group, which fed during early viraemia, were replete before the maximum levels of virus were circulated. Only five larvae which had fed during early viraemia were recovered alive and these were not examined for virus. Within the infected groups, pools of individuals which had fed rapidly showed virus titres similar to those of pools of ticks which had taken longer to feed.

After the moult, the succeeding instars of the infected larvae and nymphs from sheep 1028 had slightly higher virus titres than the engorged ticks from which they were derived, but the increases were within the limits of error of virus titration (table 11).

Both groups of nymphs which fed during viraemia on sheep 1028 contained virus when tested in the engorged state. The larvae which fed during maximal viraemia on this sheep also contained virus. Four larvae which had fed during early viraemia were not examined for virus. Within the virus-positive groups of engorged ticks from sheep 1028, pools of individuals which fed rapidly had higher virus titres than pools of ticks which took longer to feed.

In two instances the virus titres recorded after the moult in ticks from sheep 1028 differed significantly from the pre-moult titres. Adult ticks showed an increase in titre over the rapid feeding nymphs from which they were derived while slow feeding larvae showed a decrease in titre after the moult (table 12).
The infected ticks from sheep 1C28 and 1C08 were used together in transmission tests. The nymphs were placed on two sheep, one susceptible to louping ill and one immune. The adult ticks were likewise attached to an immune and to a susceptible sheep.

Nymphs from sheep 1C28 were placed on the susceptible test animal five days before the nymphs from 1C08 were attached. In this way it was possible to ascertain that the nymphs from 1C28 transmitted louping ill before those from 1C08 fed. The order of attachment was reversed on the immune animal to confirm transmission by the ticks from sheep 1C08. The same principle was applied in attaching the adult ticks to an immune and a susceptible sheep.

It was thus shown that both the nymphs and the adult ticks derived from sheep 1C28 transmitted louping ill. Likewise, both the nymphs and the adult ticks from sheep 1C08 transmitted louping ill. Comparison of the infective titres of the ticks, determined after the transmission tests, indicated that feeding on an immune as opposed to a susceptible host had no apparent effect on virus in the tick.

Of the ticks derived from the third sheep, 1C10, which had the least intense viraemia, only the larvae which fed during maximal viraemia yielded virus when tested in the engorged state. The virus content of these larvae was demonstrable in suckling mice only.
After the moult to nymphs, the virus titre of the ticks in this group was $10^{2.1}$ LD$_{50}$ per 0.03 ml. Seventeen of these nymphs fed to repletion on one susceptible sheep and twenty-four nymphs fed on another animal. There was no transmission of louping ill and virus was not recovered from the engorged ticks after the transmission tests.

Adult ticks derived from the nymphs which fed during maximal viraemia on sheep 1C10 were found to contain virus after the moult, but this was demonstrable in suckling mice only. The adults failed to transmit louping ill to a susceptible sheep and virus was not detected in the engorged ticks after the attempted transmission.

It was concluded that the viraemias in sheep 1C08 and 1C28 had been sufficiently intense to bring about the infection of *I. ricinus* larvae and nymphs. The viraemia in sheep 1C10 differed fundamentally; it was marginal for the infection of the tick.

Two further tests were made with larvae. Sheep 1C03 and 9C49, which became infected as a result of tick infestation in the transmission tests, were used as virus-donor hosts. Sheep 1C03 was immune to louping ill and it did not develop a demonstrable viraemia. Sheep 9C49 had a low level of virus in the blood on the day that the larvae were attached. Whole blood killed only one out of five mice inoculated.

Neither of the two sheep was able to infect larvae. Virus was not isolated from the engorged ticks or from the succeeding instar.
Ticks other than *I. ricinus* larvae and nymphs were not studied systematically. A few *I. ricinus* adults were placed on sheep 1C28, but the animal succumbed to encephalitis before the female ticks were replete. Three months after detachment three out of five partially engorged females contained virus, the highest titre being $10^{3.3} \text{LD}_{50}$ per 0.03 ml. One month later virus was isolated in suckling mice from the pooled eggs and larvae of these females. This is apparently the first record of transovarial passage of louping ill virus in the tick.

A limited number of observations were made with exotic tick species. The *R. appendiculatus* ticks which fed on sheep 1C08 all died before they could be tested for virus content. The *D. andersoni* nymphs fed on sheep 1C10, which had the least intense viraemia, failed to become infected. Five *R. bursa* females which fed on sheep 1C10 also failed to gain infection. Adult *H. anatolicum anatolicum* ticks derived from larvae-nymphs which fed on sheep 1C28, contained virus at a titre of $10^{3.1} \text{LD}_{50}$ per 0.03 ml. These adults failed to transmit louping ill to a susceptible sheep, but only four females fed to repletion in the transmission test.
DISCUSSION.

The prevailing theory of louping ill pathogenesis was already implicit in the conclusions of MacLeod and Gordon (1932). It is given recent statement by Smith et al (1964).

These authors describe louping ill as a biphasic illness in which the first phase, characterised by mild symptoms, corresponds to the visceral multiplication of virus. They continue:— "The virus may or may not succeed in reaching the cells of the central nervous system: if it does then the second phase of [sic] encephalitis follows: namely clinically recognizable louping ill."

It is central to the theory that infection follows a sequential course from systemic to nervous; the transition depending upon virus overwhelming the "blood-brain barrier". Since the sequence is not always completed, factors mediating penetration of the barrier assume key significance in the theory. MacLeod and Gordon (1932) considered that, in nature, concurrent tick-borne fever played the major role in facilitating virus invasion of the central nervous system. Smith et al (1964) list age, nutritional state, concurrent disease and stress as factors affecting the development of encephalitis, but they assert that the process is basically a quantitative one:— "The encephalitic phase ... depends on the degree and duration of viraemia ..."
The present experimental observations appear to be in agreement with the theoretical model of the disease in one respect only, namely, the tendency for severe encephalitis to be associated with intense viraemia. Of the twenty-four sheep infected subcutaneously in the study, the highest levels of virus were circulated in six of the seven which sustained fatal encephalitis.

Nevertheless, the symptoms of encephalitis in these six sheep were discernible within three to six days after maximum viraemia. So short an interval throws suspicion on the assumption that brain infection is initiated at the peak of viraemia. The objection may be raised that the influence of concurrent tick-borne fever on the course of louping ill invalidates argument based on sheep 1C06 and 1C28, but in three of the animals with uncomplicated infections the nervous symptoms supervened only four days after peak viraemia. The entry of virus into the nervous system in these instances must have occurred well in advance of the peak of viraemia since in both sheep and mice massive infection by the direct intracerebral route requires a minimum period of five days before nervous symptoms are manifest. Moreover, it has been shown after intranasal infection that the presence of louping ill virus in the brain precedes the appearance of lesions and nervous symptoms by several days (Fite and Webster, 1934; Burnet and Lush, 1938).

The inference is, therefore, that virus was still in relatively low concentration in the blood at the stage when brain infection took place in the six sheep. In the seventh case of fatal encephalitis/
encephalitis there can be no doubt that virus reached the central nervous system independently of intense viraemia. This sheep, 6C00, had a viraemia which was detectable on one day only, and then at nearly the lowest maximum level recorded in any animal.

Among the seventeen sheep which survived subcutaneous infection, eight exhibited symptoms indicative of encephalitis. The symptoms were pronounced in four animals and in three of these the viraemias were of more than average intensity for non-fatal infections. In the other four sheep encephalitis was evidenced by transient nervous excitability only and viraemia was of a moderate or low intensity.

Tick transmission of virus to four animals resulted in demonstrable viraemia of a low degree in only one of two susceptible sheep. The failure of two immune sheep to circulate virus was to be expected. Mild nervous symptoms were observed in one each of the susceptible and immune animals, in the absence of demonstrable viraemia. It is perhaps inadmissible to draw inferences on the relationship of brain infection to the level of viraemia here as loping ill was accompanied by tick-borne fever in all four instances.

Three lines of evidence thus emerge which argue against the contention that intense viraemia is the crucial factor determining infection of the nervous system. These are: the outright exception, in sheep 6C00, to the general regression of the severity of nervous symptoms on the intensity of viraemia; the discrepant timing of maximum viraemia in relation to the probable infection of the brain in acutely fatal encephalitis and the occurrence of mild/
mild encephalitis in association with low intensity viraemia. It follows, therefore, that the link observed between the level of viraemia and the severity of nervous symptoms is not a direct causal one but a reflection of the separate dependence of viraemia and encephalitis upon a third factor which, in effect, constitutes the susceptibility of the host to the agent.

According to Smith et al. (1964) the viraemia is controlled by antibody production: "... the degree and duration of viraemia ... is largely determined by the rate of development of circulating antibody."

In the present study the higher levels of antibody response were seen to follow the more intense viraemias with remarkable regularity among the 24 subcutaneously infected sheep. The two primary infections resulting from tick infestation, in sheep 5C19 and 9C49, conformed to this pattern, too, in that the greater degree of immune response took place in 9C49 in which viraemia had been demonstrable. Clearly then, failure to circulate high levels of virus cannot be ascribed to the suppression of viraemia by strong antibody response.

Furthermore, the rate of antibody development was not accelerated in animals with low intensity viraemia. Antibodies became detectable between the third and sixth days after infection and usually attained maximum titres between the eighth and twelfth days; the actual variations within these periods not being significantly correlated with the amount of virus circulated.
Low intensity viraemia thus did not result from an early immune response blocking maximal viraemia.

The duration of a viraemia tended to increase directly with the maximum titre, the result being that the more intense viraemias extended further into the period during which antibody was present in the blood. In these instances there was a decline in the level of virus in circulation once antibody became detectable: viraemia nevertheless remained demonstrable for a number of days after the onset of the immune response, as for example in sheep 1C28 (figure 45). The impression here is that while antibody reduced the infectivity of the agent in the blood, virus continued to be released into the circulation. The in vitro interaction of coincident antibody and virus in the blood was minimised during the tests for viraemia by using pre-cooled diluents and by avoiding delay in inoculating the mice.

These observations on the relationship of antibody to viraemia are based in the main on the haemagglutination inhibition response, but apply equally well to neutralising activity where this was tested. Apart from the seven sheep in which neutralising antibody response was monitored for the purposes of this project, a number of random tests on the sera of the remaining sheep showed good accordance between the degree of immune response as measured by haemagglutination inhibition and as measured by in vitro neutralisation of virus.
There is no published evidence for an inverse relationship of viraemia to antibody response. Early investigations of viraemia in louping ill usually comprised random or non-quantitative tests (Pool et al., 1930; Greig et al., 1931; Gordon et al., 1932a; MacLeod and Gordon, 1932) and were not linked to antibody studies. A recent study of viraemia (Pogodina, 1964) also omitted reference to serum immunity. Conversely, investigations of antibody response (Williams, 1958b; Williams and Thorburn, 1961; Smith et al., 1964) contain no information on viraemia. Indeed, the present project appears to be the first in which these two features of acute louping ill infection have been studied together. Moreover, the rise of antibodies has not previously been monitored in the first two weeks of infection.

It is of diagnostic significance that antibody levels were in the maximal range by the time that behavioural symptoms of the disease appeared in experimental sheep, including those animals infected by tick transmission. This presumably represents the stage at which the clinical disease would first be suspected in the field. On present evidence, a serum sample taken at this point for diagnostic purposes would show similar or higher antibody levels than convalescent serum taken two weeks later.

The present findings suggest a model of pathogenesis in which peripherally introduced virus frequently invades the central nervous system. Entry into the nervous system occurs early in the infection and is independent of the level of viraemia ultimately/
ultimately achieved. In those instances where maximal virus multiplication follows, the result is both intense viraemia and overt encephalitis. On occasion, encephalitis may occur without a high degree of virus circulation. A strong immune response is associated with intense viraemia and to this extent severe encephalitis is frequently accompanied by high antibody levels. The encephalitis may terminate in death or, as in asymptomatic brain infection, in recovery with subsequent immunity.

This interpretation of the disease is in keeping with recent opinion on virus pathogenesis (Bang and Luttrell, 1961) in two important respects: it does not automatically equate brain infection with encephalitis and it allows that there may be a pathogenic element to the immune response of the host. * Furthermore, no vasculo-meningeal barrier to virus is recognised. The mechanisms which determine the course of an infection are unknown, but external or pathogen-related and internal or host-related components can be identified.

The experiments with field strains of louping ill afford an indication that virus-related factors affect pathogenesis. Of three sheep infected with strains isolated from ticks, all sustained acutely fatal encephalitis. Among the seven sheep inoculated with strains of virus isolated from vertebrates only one, 6000, developed severe encephalitis. Although this encephalitis, too, was fatal, the subacute course of the infection was distinct from that induced by the viruses of tick origin. The disparity between the results/

* Dr. Gordon Smith has drawn the attention of the author to the publication of Webb and Smith (1966) in which the probable role the immune response in the pathogenesis of encephalitis is discussed.
results in the two groups suggests that the tick-derived viruses were more virulent than vertebrate-derived viruses when tested at similar low mouse-passage level. Clear examples of variation in pathogenicity of louping ill strains have been recorded by Grešíková, Albrecht and Ernek (1961) and by Pogodina et al. (1964)

The occurrence of fatal encephalitis in sheep 6C00, which circulated less virus than most animals in the study, indicates that the multiplication of virus and the manifestation of disease are separately related to host susceptibility. Sheep 1C32, which was infected with a similar dose of the same strain of virus, had a more intense viraemia than 6C00 without developing nervous symptoms.

The results of the experimental infections with the laboratory strain of virus show that the host is subject to predisposition to the disease. There were three severe infections, two of them fatal, among the seven sheep in this group. These animals, 1C08, 1C10 and 1C28, underwent an immunising infection with tick-borne fever two months prior to infestation with ticks and inoculation with virus. They experienced a second bout of tick-borne fever at the time of the louping ill infection. The severe clinical reactions in these three animals contrast strongly with the mild disease which the laboratory virus produced in the four infections uncomplicated by tick attachment and, hence, tick-borne fever.

It is necessary, however, to consider the possibility that field strains of virus were introduced along with the ticks in the above experiments.
In view of the low incidence of infection encountered in questing ticks, the coincidental transmission of virus to three sheep was unlikely. The probability that field viruses were introduced is further reduced by the fact that relatively small groups of field ticks, 100 to 150 nymphs, were attached to the sheep before the massive doses of laboratory viruses were administered. Virus was not recovered from three hundred of the experimental ticks tested before the attachments were made. None of the tick groups which engorged before demonstrable viraemia occurred were subsequently found to contain virus.

The duration of the interval between the subcutaneous inoculation of virus and the onset of demonstrable viraemia or antibody response in each of the three sheep, was in the same range as the corresponding periods in all other subcutaneously infected sheep in the study. The equivalent intervals between the attachment of the first groups of ticks and the onset of demonstrable viraemia or immunity, however, were of greater length than the corresponding incubation periods observed later in tick transmissions.

It is concluded, therefore, that the infections in the three sheep used to feed ticks, were not complicated with field strains of virus.

There is little doubt that the concurrent tick-borne fever infections, on the other hand, represent transmission by the field ticks rather than recrudescence of the earlier immunising infections brought about by louping ill virus inoculation. Since pyrexia in/
in the three sheep followed virus inoculation more rapidly than was usual in subcutaneous louping ill infection, the initial pyretic responses appear to relate to the tick attachments made three days prior to virus administration and, indeed, the tick-borne fever agent was already detectable in the bloodsmear of one animal, 1CO8, at the time of the virus inoculation. The complication with tick-borne fever was foreseen because the incidence of the infection in field tick populations is known to be high (MacLeod, 1936; McEwen, 1947). The immunising infections were carried out as a preliminary to the tick-feeding experiments in the hope that the complication with tick-borne fever would thus be avoided.

Although the concurrent tick-borne fever infections appeared to heighten the pathogenicity of the laboratory strain of virus, the converse is also true: louping ill infection aggravated tick-borne fever. The parasitaemias recorded in the three sheep were of greater intensity than may be expected following challenge with a second strain of tick-borne fever in the absence of louping ill infection (Fogbie, personal communication). Certainly, it was found that a second infection with the same strain of tick-borne fever, uncomplicated by louping ill, was very mild in sheep 7C95 and 8C50 which Dr. Fogbie had on experiment (figures 28 and 29).

Further experience with louping ill and tick-borne fever during the project indicates that the interaction of these diseases is probably more complex than the synergistic dual infections in sheep 1CO8, 1C10 and 1C28 would suggest.
Sheep 4B55 was the tick-borne fever reactor from which infective blood was obtained for the immunising infections of sheep 1C08, 1C10 and 1C28. Shortly after the tick-feeding experiments sheep 4B55 was itself infected with louping ill virus without tick attachments being made. The animal underwent a mild louping ill reaction but it failed to manifest a recrudescence of tick-borne fever infection. At this juncture sheep 4B55 was ostensibly immune to both louping ill and tick-borne fever.

The animal was then subjected to simultaneous infection with louping ill and tick-borne fever by tick infestation. On this occasion it had a marked tick-borne fever parasitaemia. There was no demonstrable louping ill viraemia but the serum antibody levels were boosted. Sheep 4B55 did not manifest nervous symptoms characteristic of louping ill during the dual infection, but it was distressed to the extent that it lay down to rest and was unwilling to rise. It appears that the second louping ill infection facilitated a relatively severe second infection with tick-borne fever. There is no certain indication that the second tick-borne fever infection in turn affected louping ill infection.

Sheep 1C03 also experienced simultaneous infection with louping ill and tick-borne fever as a result of tick transmission. This sheep underwent subcutaneous infection with louping ill virus prior to the tick infestation, but it had had no previous experience of tick-borne fever. Following tick infestation the animal developed a moderately severe tick-borne fever parasitaemia, but it did not/
not circulate detectable levels of louping ill virus. Despite its ostensible immunity to louping ill, sheep 1C03 exhibited transient nervous symptoms. It is possible that here the tick-borne fever infection lowered the resistance of the animal to louping ill.

Two more sheep, 5C19 and 9C49, sustained dual infection with louping ill and tick-borne fever following tick transmission. These two animals were susceptible to both diseases before tick infestation. Both developed moderately severe tick-borne fever parasitaemias, but louping ill viraemia of low intensity was detected in sheep 9C49 only. Both animals showed signs of distress during pyrexia, but transient nervous symptoms were seen in 5C19 only. There is no clear evidence that louping ill and tick-borne fever infections were synergistic in these two animals, except that the slight nervous symptoms in sheep 5C19 were exhibited in the absence of demonstrable viraemia.

Out of the total of seven dual infections reported here, in three instances only can it be said with any certainty that concurrent tick-borne fever aggravated louping ill infection. The infections in these three animals, 1C08, 1C10 and 1C28, are not strictly comparable to the remaining four dual infections in that massive doses of louping ill virus were injected subcutaneously at the time of tick-borne fever infection. The other dual infections resulted solely from tick transmission. Furthermore, sheep 1C08, 1C10 and 1C28 had undergone tick-borne fever infection only two/
two months prior to the dual infection. Although two of the other animals which sustained the dual infection, 4B55 and 1C03, had also experienced earlier infection with tick-borne fever and/or louping ill, this had been eight and fifteen months before the dual infection.

The evidence that louping ill aggravates tick-borne fever infection, on the other hand, is complicated by uncertainty about the virulence of tick-borne fever itself. It was originally described as a mild disease of sheep (Gordon et al, 1932b), but under some circumstances it has been found to be a quite severe disease (Harbour, 1945; Jamieson, 1947, 1950; Littlejohn, 1950; Stamp and Watt, 1950).

Four of the seven dual infections described above were in animals 1C08, 1C10, 1C28 and 4B55 which had previously experienced tick-borne fever. In none of the animals was immunity to tick-borne fever apparent and it must be concluded that louping ill infection facilitated tick-borne fever infection in these animals. The remaining dual infections were in sheep 5C19, 9C49 and 1C03 which had not previously experienced tick-borne fever. The maximum levels of parasitaemia attained in these three sheep exceeded the highest levels recorded in five sheep with uncomplicated tick-borne fever following tick infestation. Nevertheless, one of the animals with uncomplicated tick-borne fever, 5C25, had to be killed when it became recumbent.
From the present findings it can be inferred that where animals meet simultaneous challenge with the two diseases by natural transmission, louping ill infection will facilitate breakdown of an immunity to tick-borne fever. If tick-borne fever is being met for the first time, then louping ill can aggravate the infection. It is less certain that tick-borne fever will weaken an immunity to louping ill or aggravate a primary infection. The impact of tick-borne fever may be conditioned by the age of the animal and by strain differences in the virulence of the agent. McEwen (1947) and Jamieson (1947) found that the disease was mildest in young animals and Foggie (1951) demonstrated that there were differences in virulence between separate ovine strains of tick-borne fever.

The existing experimental data on the effect of tick-borne fever on louping ill infection were analysed by MacLeod (1962). He made a qualitative comparison of uncomplicated louping ill in fifteen sheep with the dual infection in thirteen sheep. The difference in death rate between the two groups does suggest that, contrary to the present conclusion, tick-borne fever aggravates louping ill infection. The disagreement may be related in part to differences in the conditions of experiment, such as variation in the virulence of the tick-borne fever strains used. Clearly, there is room for further examination of the inter-relationship of louping ill and tick-borne fever.
The prevailing opinion is that dual infection is common under field conditions and that tick-borne fever aggravates louping ill (Gordon et al, 1962). The natural incidence of tick-borne fever is high and the challenge rate is such that a vast majority of sheep exposed to ticks undergo infection during the first few weeks of life (MacLeod, 1936; McEwen, 1947). From what is known about natural louping ill challenge rates (Gordon et al, 1962; Smith et al, 1964) it is probable that most of the sheep which become infected encounter the virus later than tick-borne fever. From this it follows that dual infection is modal in a flock only under exceptional circumstances.

The persistently high tick-borne fever challenge rate, however, ensures that louping ill infection is met in face of continuing tick-borne fever challenge. Since the present finding is that louping ill infection induces breakdown of immunity to tick-borne fever, it is, indeed, probable that louping ill is regularly accompanied by tick-borne fever infection. In view of the evidence that a high proportion of the louping ill infections occurring in a bound flock are inapparent (Gordon et al, 1962; Smith et al, 1964), there must be some doubt about the significance of the role of tick-borne fever in aggravating louping ill.

From the present examination of the nature of louping ill infection in the sheep certain aspects of tick feeding and infection are already apparent. It is clear that the infectivity of the animal for the tick is related to the clinical severity of infection.
infection. The severity of the disease tends to increase with the intensity of viraemia. Furthermore, it is obvious that only ticks which become replete in the early stages of viraemia avoid ingesting antibody along with virus. It is also evident that ticks frequently encounter the agent of tick-borne fever together with the virus of louping ill in the blood of sheep.

In evaluating the results of the tick feeding experiments it is immediately apparent that a high proportion of the ticks which fed during viraemia on sheep 1C08 and 1C28 became infected and were able to transmit. Thus five nymphs derived from an original 1,105 larvae in the third group which fed on sheep 1C08 sufficed to transmit louping ill to sheep 4B55 and fifteen nymphs from an original 1,419 larvae on 1C28 transmitted the infection to sheep 5C19 (table 14). A mere three females derived from 328 nymphs which fed on sheep 1C28 and six females from 173 nymphs on 1C08 were sufficient to ensure the transmission of louping ill virus to sheep 1C03 and 9C49. Moreover, the spent females which were tested individually for virus content some three months after the transmissions, were found to be infective in ten out of eleven instances (tables 11 & 12).

By way of contrast, the less intense viraemia in sheep 1C10, served to infect a low proportion of the ticks fed on this animal. Thus 41 nymphs, derived from 1,073 larvae on 1C10, fed to repletion on susceptible sheep 5C17 and 5C25 but failed to transmit the virus (table 14). Since a single infected nymph probably suffices to/
to transmit louping ill it follows that there was, in effect, a total of 41 tests in which transmission did not occur. MacLeod and Gordon (1932) recorded transmission of louping ill to a sheep where only two nymphs became attached. Ten female ticks derived from nymphs on 1C10 also failed to transmit louping ill to a susceptible animal. The only evidence that infection did occur in ticks fed on sheep 1C10 was the isolation of virus from flat nymphs and adults after the moult. Subsequent to the transmission tests, virus was not detected in any of the ticks from 1C10 (table 13).

Analysis of the infective titres determined in the tick suspensions provides further indication of the relative efficiency with which infection took place in the various tick groups. Since the test for virus did not distinguish between ingested infectivity present in gut content and true infection of tick tissue, the infective titres of ticks in the engorged state are less informative than the titres of succeeding instars following the moult. At the time of the post-moult virus assays, four months after tick detachment, all but traces of bloodmeal were utilised or voided. The infective titres of tick pool suspensions at this stage must represent principally, if not exclusively, virus present in tick tissue.

The highest virus titres recorded after the moult were in ticks derived from the third groups of larvae and of nymphs which fed on sheep 1C08 and 1C28. The log 10 LD 50 titres were 5.7, 5.7, 6.1 and 6.1 per 0.03 ml (tables 11 & 12). Within the limits of accuracy of virus titration, these levels must be regarded as identical; a fact/
fact which suggests not only that there was a uniform high rate of infection in these tick pools, but also that there was a measure of uniformity in the titres attained in infected individuals. This finding is consistent with existing evidence that arboviruses, in mosquito (Chamberlain, Corristan and Siikes, 1954) or tick (Varma and Smith, 1962), multiply to an inherently determined titre irrespective of the actual level of virus uptake, provided only that it exceeds the threshold for infection of the arthropod. Virus levels are well maintained in the arthropod and the infective titres of individuals are broadly comparable to each other at any particular interval after the bloodmeal. It follows that significant differences in virus titre between similar pools of flat ticks must represent large differences in the numbers of individuals which are infected.

Consequently, the infection rate in the ticks which fed on sheep 1C10 must have been very low. A single infected nymph, containing virus at the level recorded from sheep 1C08 or 1C28, would have been adequate to yield the titre of $10^{2.1} \text{LD}_{50}$ per 0.03 ml recorded in the pool of 100 individuals derived from sheep 1C10 (table 13). In the adult ticks derived from sheep 1C10, virus was demonstrable at the lowest dilution of tick suspension in suckling mice only. Infectivity at this level suggests residual contamination with ingested virus rather than true infection of tick involving virus multiplication.
It is concluded, therefore, that the levels of viraemia attained in sheep 1C03 and 1C28 were adequate for the infection of the tick, *I. ricinus* L., but that the intensity of the viraemia in sheep 1C10 was marginal for tick infection. The evidence suggests that the intensity of viraemia in sheep 1C10 was actually at the arbitrarily defined threshold level: the level of virus required to infect 1 to 5% of arthropods (Chamberlain, Sikes, Nelson and Sudia, 1954).

For the purpose of arriving at a general statement on louping ill virus uptake by the tick, *I. ricinus*, it is necessary to define the term threshold in relation to ticks. The concept of threshold, the level of virus required to infect 1 to 5% of arthropods, was established with instantaneous feeders, namely, mosquitoes, and hence it involved the need to specify only the level of virus required at the instant of feeding. It was anticipated (p. 33) that other variables would have to be introduced into the concept of threshold on applying it to protracted feeders such as ticks. Ticks experience changing levels of virus during the feed and the problem arises as to which is the more important in determining infection of the tick: the maximum intensity of viraemia or its duration above a certain intensity. Moreover, it is necessary to take account of the potential overriding effect of antibody at various concentrations.

From the study of infection in the sheep it is clear that the duration of viraemia bears a direct relationship to its maximum/
maximum intensity. Similarly, the weight of antibody production is directly related to the maximum intensity of viraemia. Thus, the complexities anticipated in defining threshold conditions are mollified in practice: it is sufficient to define threshold viraemia in terms of its maximum intensity. Provided threshold intensity is attained, the questions of duration and possible interaction of antibody can be disregarded. This does not indicate that the duration of viraemia and the onset of the antibody response are without significance, but merely that the operation of these factors is likely to be consistent in viraemias of given intensity. The duration of viraemia has the ultimate importance that it controls the number of ticks which meet infection. The role of antibodies is discussed below.

From the present findings, therefore, it appears that viraemia with a maximum intensity of $10^{2.5}$ LD$_{50}$ per 0.03 ml, as in sheep 1010, is at the threshold for the infection of the tick, *I. ricinus*, with louping ill virus. The only relevant information which can be traced tends to support this estimate. Dr. Gordon Smith (1965) stated in discussion at a symposium that viraemia, in a hedgehog, with a maximum titre of $10^{1.9}$ LD$_{50}$ per 0.03 ml, was inadequate for the infection of *I. ricinus*. Dumina (1958), working with a Russian tick-borne encephalitis isolate and *I. persulcatus*, concluded that infection of the tick "is an irregular process, occurring most often ... with a virus concentration of $10^3$ or more in ... blood."

The inference made by Dumina, that even a high level of virus/
virus in the blood fails to ensure the infection of all ticks, is in agreement with present experience. Thus, the levels of virus circulated in sheep 1C08 and 1C28 were apparently adequate for the infection of ticks, yet some ticks which fed during viraemia on these two animals failed to become infected. There was no evidence of infection in the second group of nymphs attached to sheep 1C08 (table 11). These nymphs fed during early viraemia and most of them were replete before the maximum levels of virus were circulated. The nymphs may thus have failed to ingest infective doses of virus. It is possible, on the other hand, that virus ingested in the terminal stage of engorgement is less effective in bringing about infection of the tick than is virus taken in with the initial part of the blood-meal which is utilised immediately for cuticle synthesis (Lees, 1952).

Infection did occur in the second group of nymphs on sheep 1C28, but here viraemia became demonstrable one day earlier than in sheep 1C08 (table 12). There was also a tendency for the nymphs on 1C28 to have a more protracted feeding period than the corresponding group on 1C08. The nett effect was to expose the nymphs on 1C28 to a larger dose of virus at an earlier stage of their engorgement than the group on 1C08, although the position was reversed during the terminal stages of engorgement. The difference in virus content of the initial part of the bloodmeal may have been critical and this presumably accounts for the contrasting results obtained in the two groups of nymphs.
The most striking feature about the ticks which fed on sheep 1C28, however, was the tendency for pools of individuals which fed rapidly to have higher virus titres than pools of ticks within the same groups which took longer to feed (table 12). By extension of the argument used above, the differences in virus titre must indicate that infection took place in a lower proportion of slow feeders than rapid feeders. The phenomenon cannot be ascribed to a simple deficiency in virus content of engorged blood since both slow and rapid feeders in the third groups of ticks placed on 1C28 must have become attached and commenced feeding together during the most intense stage of viraemia. \( I. ricinus \) ticks which failed to attach within 24 hours died from desiccation.

The phenomenon must, therefore, be related to the physiology of tick feeding. Theoretically, slow feeding would make it more difficult for the tick to accumulate an infective dose of virus. For infection of the tick to occur the rate of virus intake must exceed the loss of infectivity in engorged blood which is bound to follow prolonged exposure of ticks to the body temperature of the host. Apart from thermal inactivation of virus, the elevated temperature may cause loss of infectivity through facilitating the interaction of virus and antibody in undigested blood. The loss of infectivity of ingested virus during tick attachment can be seen quite clearly in the poor recovery of virus from the engorged ticks from sheep 1C10 (table 13). It appears that there were no infected individuals included among the engorged ticks tested from this animal. In the engorged ticks from the two sheep with the more/
more intense viraemias, 1C08 and 1C28, it must be presumed that multiplication of virus in the tissues of infected individuals masked the loss of infectivity in gut content (tables 11 & 12).

It is notable that there was no significant difference in titre between pools of rapid and slow feeding ticks on sheep 1C08 (table 11). This is not surprising since the extremely high levels of virus recorded in the blood of sheep 1C08, up to $10^{7.5}$ LD$_{50}$ per 0.03 ml, could easily have offset the effects of protracted engorgement by allowing slow feeding ticks to acquire infective doses of virus despite disproportionate loss of ingested infectivity as compared to rapid feeders.

Two tentative conclusions thus emerge for future verification. First, virus ingested during the terminal phase of engorgement is less effective in bringing about tick infection than is virus which is ingested during the initial phase of engorgement. Secondly, infection takes place with greater facility in ticks which feed rapidly than in ticks which are slow feeders.

Although the initial rise of antibodies during viraemia may contribute towards the failure of ticks, particularly slow feeders, to acquire infective doses of virus, once infection of the tick has occurred the ingestion of antibodies appears to be without effect. From the present study it is evident that ticks which feed through the most intense stage of viraemia frequently encounter antibodies at concentrations comparable to those which circulate in immune/
immune animals. Nevertheless, the ingestion of antibodies at such concentration by the slower feeding ticks on sheep 1008, failed to produce lower infective titres in the ticks than were recorded in the rapid feeders which ingested less antibody (table 11).

Furthermore, there was no indication in the limited tests made with infected nymphs and females that feeding on immune hosts had a deleterious effect on virus in the tick. The differences in titre between infected ticks fed on immune hosts and those fed on susceptible hosts are not significant (tables 11 & 12). These findings are consistent with evidence presented by MacLeod (1962) that adult ticks, infected as larvae, are able to transmit loup ing ill after feeding on an immune host as nymphs.

Results obtained with other viruses of the tick-borne encephalitis complex are contradictory. Benda (1958) concluded that ticks infected with the virus of Central European tick-borne encephalitis undergo a reduction in titre after feeding on immune hosts. He added that the infection, nevertheless, persists in the tick irrespective of the immune status of the host. Dumina (1958) concluded that I. persulcatus ticks, infected with a Russian strain of tick-borne encephalitis, fail to lose virus by feeding on immune animals. Similarly, Il'enko (1960) found that the ingestion of antibodies had no effect on a Russian virus in I. ricinus nymphs and adults. Larvae infected as a result of transovarial passage of virus, however, lost their infectivity by feeding on immune hosts. Il'enko was unable to explain this phenomenon.
Accurate assessment of threshold viraemia for the infection of the tick must be based on more exact determination of tick infection rates than was attempted in the present study. This may have been achieved through the exploration of fluorescent antibody methods for the identification of virus in individual ticks. In retrospect, however, it is clear that the high titres of infection which are attained in the tick make it practicable to isolate virus from individual ticks. Moreover, the use of cell cultures together with mouse subinoculation appears to be a suitably sensitive method for testing individual ticks.

Twenty-one pools of experimentally fed ticks were infective to pig kidney monolayers at an average of \( \log_{10} \) dilutions higher than the corresponding ID\(_{50}\) titres in mice. This indicates that pig kidney cultures and mice are, in fact, equally sensitive to infection with the virus since the cell culture dose is 1.5 logs greater than the mouse dose, i.e. an inoculum volume of 1.0 ml as opposed to 0.03 ml. Nevertheless, in practice this difference in the amount of material which can be introduced into cell cultures and mice at similar dilution may significantly alter the chances of virus recovery from low titre material. Five of seven virus strains isolated from field ticks were recovered in cell culture, but not in mice, in the first instance.

The true advantage of cell cultures over mice for the isolation of virus from ticks can be evaluated as follows:
It is assumed that individual nymphs attain virus titres of up to $10^{6.1} \text{LD}_{50}$ per 0.03 ml since this level was recorded in a suspension of 100 ticks which fed as larvae on sheep 1C28 (table 12). A pool of 50 nymphs as used in the survey of wild ticks which includes only one such infected individual, has a 50-fold lower virus content than the above pool. It has a titre of $10^{4.4} \text{LD}_{50}$ per 0.03 ml. The arbitrary preparation of the survey pools of 50 nymphs in 3.4 ml diluent means that on an actual weight/volume basis some suspensions contain only $1:400$ or $10^{-2.6}$ of tick tissue in medium (p. 28). At this dilution the volume of inoculum used in mice, 0.03 ml, contains approximately $10^{1.8}$ or 63 mouse ic LD$_{50}$ of virus; a level which is marginal for virus demonstration in the mouse. The volume of suspension used to inoculate cell cultures on the other hand, contains nearly 2,000 mouse ic LD$_{50}$ at the same dilution, making virus recovery much more certain.

Cell cultures thus offer a useful advantage over three-week-old mice for the isolation of virus from ticks, but it is desirable to eliminate the need for mouse subinoculation to demonstrate virus. The solution may lie in the use of fluorescent antibody to demonstrate the presence of virus in monolayer cultures (Kunz, 1964).

Prior to this study, Libíková, Řeháček and Mayer (1962) compared methods incorporating cell cultures for the isolation of tick-borne encephalitis viruses from tick and mouse tissues. This included recovery of looping ill virus from *I. ricinus* nymphs. Their results, in the form of histograms, indicate the same advantage.
advantage of cell cultures over mice as observed here. The tick-borne encephalitis agents failed to elicit cytopathogenesis in chick embryo fibroblasts but, as in the present study, a method employing cell culture together with mouse subinoculation was adopted for extensive use in screening tick suspensions for virus (Keháček, personal communication).

The failure of tick isolates and tick-passaged laboratory virus to induce cytopathogenesis in pig kidney cultures during the present study, lends support to the notion that arbovirus populations are subjected to alternate types of selection in arthropod and vertebrate hosts (Smith, 1964). Following adaptation of virus through a number of cell culture passages, however, the ability to induce cytopathogenesis may become fixed. Keháček (1962) found that a Central European strain of tick-borne encephalitis virus, adapted through 33 passages in HeLa cell cultures, retained its ability to induce cytopathogenesis following tick passage. The ability of the virus to infect ticks and to undergo transovarial passage in the tick was unimpaired by adaptation to HeLa cells.

The present observations provide little information on the growth cycle of virus in the tick. Comparison of the pre- and post-moult virus titres in each of the tick groups reveals significant changes in three instances only (tables 11, 12 & 13). Adult ticks from sheep 1C28 and nymphs from 1C10 showed increases in titre over the nymphs and larvae from which they were derived. Some of the larvae from sheep 1C28, the slow feeders, showed a/
a decrease in titre after the moult to nymphs. These changes in titre cannot readily be interpreted in terms of a growth cycle of virus in the tick. The figures may represent genuine changes in the virus content of infected individuals, or they may equally well represent differences in the numbers of infected ticks included on each test occasion.

It seems certain that some virus multiplication had already occurred in the ticks by the time that the first virus assays were performed immediately after engorgement. Thus, the infective titre recorded at this stage in the third group of larvae fed on sheep 1C28, $10^{6.1} \text{LD}_{50}$ per 0.03 ml, was significantly higher than the maximum titre recorded in the blood of the host, $10^{4.5} \text{LD}_{50}$ per 0.03 ml (table 12). Moreover, the evidence that there was a loss of infectivity in ingesta during tick attachment, of itself indicates that high infective titres in engorged ticks must be ascribed to the multiplication of virus in tick tissue.

The high infective titres recorded after the moult indicate remarkably efficient maintenance of the virus in tick tissue. Despite the fact that the ticks were held for four months after detachment before the post-moult virus assays were performed, most of the time at $22^\circ \text{C}$, a significant fall in titre was detected only in the slow feeding larvae from sheep 1C28. Two possible mechanisms whereby the infective titres of arboviruses are maintained in arthropods were suggested by Chamberlain, Corristan and Sikes, (1954). These are: exceptional preservation of virus infectivity in/
in arthropod haemolymph and, the occurrence of secondary
multiplication cycles in target organs, such as salivary glands,
following the initial multiplication of virus in gut tissue.

A fuller study of the multiplication of virus in the tick than
was attempted here, should include a greater number of observations
made at close intervals; preferably on a number of individual ticks
on each occasion. In this way, trends in the growth curve of virus
in the tick would emerge clearly.

The isolation of virus from the pooled eggs and larvae of five
females during the present study constitutes the first concrete
evidence of transovarial passage of louping ill infection in the
tick. In an early investigation of louping ill Stockman (1918)
carried death in one lamb and a subinocuable fever in another by
infesting them with larval ticks, but he failed to identify the
causal agent. It seems certain that Stockman encountered both
louping ill and tick-borne fever during the course of his
investigation, but neither disease has since been confirmed to be
transmitted by larvae.

Alexander and Neitz (1935) made an unspecified number of
tries to transmit louping ill with *Rh. appendiculatus* larvae
and MacLeod (1962) performed a total of 18 transmission tests with
*I. ricinus* larvae. MacLeod indicated that only nine of his tests
were valid since the rest involved larvae which probably came from
non-infected females. From comparison with research on Central/
Central European tick-borne encephalitis it is manifest that nine tests are insufficient to eliminate the possibility that transovarial passage of virus occurs in louping ill. Benda (1958) tested the progeny of 62 I. ricinus females infected with Central European virus and recorded transmission of infection to mice in four instances only. Řeháček (1962) examined larvae from 121 infected females and achieved but four isolations.

The evidence produced in the present study indicates merely that the female is capable of passing virus to the egg. This information forms an inadequate basis from which to assess the general significance of transovarial passage of virus in louping ill. Should the incidence of transovarial passage of virus be no greater than the 3.3% or 6.5% recorded with the Central European virus, it will be of low epidemiological significance. Where other sources of virus are eliminated, this rate of reproduction of infection would ensure survival of virus in the short term, but would lead to a sharp decline in the incidence of infection in ticks.

The few exotic tick species were included in the present project merely to determine whether or not the species, which offer advantages over I. ricinus in laboratory culture, could serve as models for I. ricinus in studying infection of the tick. Their prolonged feeding makes them unsuitable for such use.

Virus was isolated at a titre of $10^{3.1} \text{LD}_{50}$ per 0.03 ml from a pool of ten adults of a two-host strain of H. anatolicum anatolicum/
anatolicum which fed as larvae-nymphs on sheep 1C28. Since it is evident from results obtained with I. ricinus that ingested infectivity does not persist at such high titre, it must be accepted that at least a proportion of the hyalommid ticks gained infection and supported multiplication of virus. The remainder of the H. anatolicum anatolicum adults were placed on a susceptible sheep, but they failed to transmit infection. Only four females fed to repletion in the transmission test and these were found not to contain virus after engorgement. No further isolations of virus were made. The remaining exotic ticks either died before they could be tested for virus or else they came from sheep 1C10 in which the viraemia was of marginal intensity even for the infection of I. ricinus ticks. Consequently, the potential role of the exotic ticks as vectors of louping ill remains open.

To the incomplete evidence of infection of H. anatolicum anatolicum ticks can be added the evidence of Alexander and Neitz (1933, 1935) that louping ill is transmitted with facility by Rh. appendiculatus ticks. The fact that the virus infects such zoologically distinct species as an Ixodes, a Rhipicephalus and a Hyalomma, suggests that louping ill can be transmitted by a wide range of ticks. Smith (1964) cites evidence which shows that at least three other tick-borne encephalitis viruses are capable of infecting a range of ticks. He makes the inference that the tick-borne encephalitis viruses in general will readily infect ixodid ticks other than their natural vectors.
In the attempted survey of incidence of virus in questing ticks in Argyllshire, appreciable numbers of nymphs were collected on one farm only, Blarcreem, and the infection rate in these was found to be low. No isolations were made from 359 nymphs in 1964 and only one isolate was obtained from 925 nymphs in 1965.

In addition, six viruses were isolated from partially engorged female ticks removed from sheep elsewhere in Argyllshire. Three initial isolates came from females taken from natural cases of the disease in order to test the cell culture method for recovery of virus from ticks. The remainder comprise two isolates from 115 females from Achnacone farm and one from 155 females from Glenshellach farm. The two isolates from Achnacone could represent material taken from a single viraemic animal.

According to unpublished information, a survey was conducted in Ayrshire during the same period as this study in Argyllshire. On the basis of two virus isolations from some 2,000 nymphs it was estimated that approximately one questing nymph in 600 was infected (Smith, personal communication). The observations in the two counties appear to be broadly in agreement. During the years of study, questing ticks were sparse and the infection rate was low; probably no more than one nymph in 500 to 1,000 was infected. The implications of these findings are discussed below.

Cross-neutralisation between the reference strain of louping ill/
ill virus, LI 31, and the three original tick isolates from Argyllshire was tested in mice with low titre sheep antisera. The sera were deliberately taken early in the course of primary infection in order to emphasise antigenic differences. Arbovirus antisera become progressively less monospecific with the course of infection and hyperimmune sera are widely cross-reactive among related agents (Porterfield, 1962a). The most marked difference appeared to be that between the reference virus, LI 31, and the tick strain LI 2/7 (table 1). In an experiment subsequent to the completion of this project, 12 sheep which had survived peripheral infection with LI 31 virus were challenged subcutaneously in two groups with the LI 31 and LI 2/7 viruses. The immunity conferred by LI 31 virus protected the sheep against clinical response to the challenge with LI 2/7. Hence, the difference between the two agents appears to be of little consequence.

There is no record of significant antigenic differences between loping ill strains being encountered. Begum (1963) and Chan (1963) concluded that there were no immunological variations in the two and three strains which they studied. The last author compared a virus isolated in 1930 with recent isolates from Northern Ireland and Ayrshire. Williams, Thorburn and Ziffo (1963) demonstrated slight quantitative differences in serological comparisons of grouse isolates with a laboratory virus, but they omitted to elaborate on the implications of their findings. Clarke (1964) included three loping ill strains, separated by up to twenty-seven/
twenty-seven years in laboratory passage, in her definitive study of the tick-borne encephalitis complex but she did not find them to be antigenically separable.

The existence of significant antigenic differences between louping ill strains is, in any event, rendered unlikely by the close immunological affinity which exists between all members of the tick-borne encephalitis complex (Casals and Webster, 1943, 1944; Casals, 1944, 1962; Edward, 1950; Pond, Russ and Warren, 1953; Pond and Russ, 1955; Slonim, 1957; Clarke, 1962, 1964; Porterfield, 1962b; Kunz, 1964). In the definitive studies of the complex by Clarke (1962, 1964), the Negishi, louping ill, Central European and Far Eastern tick-borne encephalitis viruses were found to form an especially compact group in which antigenic differences are sometimes clear only unidirectionally in sophisticated antibody-absorption tests.

The implications of the present findings in sheep and tick can best be evaluated by attempting to construct an epidemiological model of the disease.

The potential role of the sheep as a donor of virus for the tick is assessed by determining to what extent natural infection in the sheep is likely to be characterised by viraemia of an intensity greater than threshold. More precisely it is necessary to arrive at an estimate of how frequently, in nature, sheep exhibit viraemia with a maximum intensity which exceeds $10^{2.5} \text{LD}_{50}$ per 0.03 ml.
The sheep experiments were conducted primarily for the purpose of gaining information on the artificial, rather than the natural, disease. Nevertheless, it is unlikely that the principal implications of the present study will alter where there is adherence to the conditions of natural infection. Although the evidence suggests that three tick isolates were more virulent than other recent isolates of vertebrate origin, the essential item is that there was an overall regression of the severity of the disease upon the intensity of viraemia. With the notable exception in sheep 6000 which appears to have been a peculiarly susceptible animal, the correlation between the intensity of viraemia and the severity of disease held good for field viruses following a low number of passages as well as for the laboratory virus with a long history of vertebrate to vertebrate passage.

The tendency was for viraemia of threshold intensity for the tick to be attained only in infections marked by frank disease. Of the eleven subcutaneously infected sheep which showed unmistakable encephalitis or died, nine had viraemias of threshold intensity. Of the remaining thirteen sheep, which manifested transient nervous excitability at most, only two circulated levels of virus in excess of $10^{2.5} \text{LD}_{50}$ per 0.03 ml. Levels of viraemia significantly higher than threshold occurred only among the seven fatal infections.

There is no known reason why this pattern should be altered in natural disease. If anything, the scant available evidence suggests that viraemias may be less intense following tick/
tick transmission than after artificial infection, thus exaggerating the tendency for threshold intensity to be exceeded only with fatal disease. Of the two infections produced in susceptible sheep by tick transmission, one was without demonstrable viraemia and in the other barely detectable levels of virus were circulated.

It would appear, therefore, that the extent to which a sheep population is capable of infecting ticks is related to the incidence of severe disease. In the absence of more precise information it is assumed here that the death rate in a flock serves as an index of ability to infect ticks.

The death rate varies widely with locality and with successive tick seasons. Although fatalities may reach the epidemic proportions seen recently in Ayrshire and in Argyllshire, the vast majority of infections occurring in sheep are inapparent (Gordon et al., 1962; Smith et al., 1964). The most comprehensive information available on louping ill fatalities relates to the field trials of the vaccine in the years 1931 to 1934 (Gordon, 1931a,b; Gordon et al., 1962). The results indicate that approximately 5% of control hoggs and a smaller proportion of lambs succumbed to the disease during spring tick seasons. On individual farms the hogg and lamb mortalities ranged from nil to over 30% (Gordon, 1934b). It was apparently omitted to monitor ewes, but the disease was stated to be rare in animals older than hoggs (Gordon et al., 1962).
It is postulated, therefore, that in a spring season where the incidence of disease is high, some 30% of the hoggs or lambs in a flock are capable, by present definition, of infecting ticks. The infection rate which these sheep produce in ticks depends upon the proportion of the tick population which they feed. Ideally, a hill flock contains, in spring, 4 ewes and 4 lambs to 1 hogg (Milne, 1947). This ratio is accepted here although, in practice, the numbers of hoggs and lambs relative to ewes are probably always lower than the optimal. The ratio applies to each self-contained flock bound to a separate hirsel on a hill farm.

The numbers of female ticks fed per spring season by individual ewes, hoggs and lambs were shown, by Milne (1947), to be in a ratio of 8:6:1. Differences in weight of infestation appeared to be primarily a function of host size, possibly related to area of ground covered in unit time. These factors should operate also for immature ticks. Certainly, Milne found that the number of nymphs fed per season by individual sheep bore a direct relationship to the number of females fed. No exact data are available for larvae, but it is now assumed that all ticks, adult and immature, are fed by individual ewes, hoggs and lambs in a ratio of 8:6:1.

On a flock basis, then, four ewes feed 32 ticks of each instar where four lambs feed 4 ticks and one hogg feeds 6 ticks. Of the total number of ticks fed by a flock, the ewes feed 76.2% i.e. 32 out of every 42 ticks fed. Lambs feed 9.5% of the total and hoggs 14.3%.
Where 30% of the lambs develop sufficiently intense viraemia to infect ticks, 2.9% of all ticks fed by the flock, i.e. 30% of 9.5%, have the potential to become infected. At the height of the tick season sheep carry about one-fifth of the total number of females which they feed over the entire season (Milne, 1947). This must be true also of nymphs since Milne found that the ratio of nymphs to females on sheep remained remarkably constant in a season where their curves of activity were synchronous. It is assumed that one-fifth of larvae, too, feed at peak infestation. Consequently, the greatest number of ticks carried by sheep at one time is equal to one-fifth of the ticks which are fed over the entire season. This applies separately to each instar of tick. Where all 30% of the affected lambs carry the theoretical maximum infestation at the time of their exhibiting viraemia, they produce infection in, at most, 0.57% of the ticks fed by the flock, i.e. one-fifth of 2.9% of ticks. The estimate applies separately to each instar of tick.

In this same model, four-fifths of the tick population are absent from hosts during the period of peak infestation. It is assumed that the feeding of these ticks is evenly spaced over an arbitrary ten week season. It follows that where viraemia in each of the affected lambs fails to coincide with peak infestation, approximately one-tenth of four-fifths of the ticks which the lambs feed over the entire season are exposed to infection. In this instance, viraemia exceeding threshold intensity in 30% of lambs produces infection in 0.23% of the ticks fed by the flock.
flock, i.e. one-tenth of four-fifths of 2.9% of ticks. This estimate has the obvious defect that tick feeding is not evenly spaced over a season. The number of ticks occurring on sheep rises and declines gradually so that an absolute minimum infestation could consist of one tick per animal. Nevertheless, it is unlikely that all of the affected lambs will develop viraemia at the time of least infestation.

The occurrence in 30% of the lambs in a flock of viraemia which exceeds threshold intensity for infection of the tick, therefore, produces infection in 0.23% to 0.57% of the ticks fed by the flock. One tick in every 175 to 435 is infected.

Calculated in the same way, viraemia exceeding threshold intensity in 30% of the hoggs in a flock produces infection in 0.34% to 0.86% of the ticks fed by the flock. Here one tick in every 116 to 294 is infected. Where threshold intensity of viraemia is exceeded in 30% of both lambs and hoggs, between 0.57% and 1.43% of ticks, i.e. between 1 in 70 and 1 in 175, are infected.

In a season where only 5% of lambs develop viraemia which exceeds threshold intensity, 0.038% to 0.095% of the ticks fed by a flock become infected. One tick in every 1,053 to 2,632 is infected. Similarly, 5% of hoggs produce infection in 0.057% to 0.14% of ticks. One tick in every 714 to 1,754 is infected. The occurrence of viraemia in excess of threshold intensity in 5% of both lambs and hoggs, therefore, results in infection of 0.095% to/
to 0.235% of the ticks fed by the flock. One tick in every 4.26 to 1,053 is infected.

In round figures, one tick in every 100 to 500 becomes infected in an exceptional spring season where there is a severe incidence of disease among young sheep. Within this range, the higher rate of infection is unrealistic since it requires that all sheep carry maximum infestations at the time of developing viraemia. More usual levels of disease result in infection of one tick in every 500 to 2,500 fed by the flock. In some seasons, presumably, there is no initiation of infection in ticks.

Where there is an autumn-feeding population of ticks, it is smaller than the spring population. A minor proportion of autumn-feeding ticks in Scotland may become ready to feed in spring and these are incorporated into the spring population. Spring-feeding ticks are less likely to pass into the autumn population (Campbell and Kemp, personal communication). It is improbable, therefore, that there is a significant transfer of infected ticks from one population to the other. Nevertheless, the infection rates in the two populations interact on each other by determining the immune status of the flock.

The factors affecting infection of autumn ticks are complex, but two extreme situations can be recognised. Where peak activity of autumn ticks occurs early in the season, the ticks may encounter a flock in which four out of nine sheep are young susceptible hoggs,
hoggs, no longer protected by maternal antibody. Here the potential of the flock to infect ticks is high. Calculated as before, viraemia exceeding threshold for the infection of ticks in 30% of the young hoggs produces infection in 0.9% to 2.25% of ticks fed by the flock. One tick in every 44 to 111 is infected. Intense viraemia in 5% of the young hoggs produces infection in 0.15% to 0.38% of ticks. One tick in every 258 to 666 is infected. At the other extreme, the peak of tick activity occurs late in the season when surplus hoggs have been sold and others have been removed for away-wintering, so that the available sheep may consist principally of immune ewes. Here the potential of the flock to infect ticks is negligible.

The true role which sheep play in determining the infection rate in the tick population depends upon the extent to which other hosts are involved. Evidence that wild hosts meet infection with louping ill virus has been obtained for the red deer (Dunn, 1960), red grouse (Williams, Thorburn and Ziffo, 1963; Watt, Brotherston and Campbell, 1963), and the common shrew, wood mouse and brown hare (Smith, Varma and McMahon, 1964). Experimental infections have been produced in the field vole (Findlay and Elton, 1953), red grouse (Williams, Thorburn and Ziffo, 1963), wood mouse (Smith, Varma and McMahon, 1964) and the hedgehog (Smith, 1965). In no instance is it established that these species are capable of infecting ticks.
From the definitive study of host relationships of the tick by Milne (1949a,b), it appears that the proportion of the tick population fed by the wild fauna is usually too small for any non-domestic host to be of comparable importance to the sheep. Although wild hosts may have the ability to maintain tick and virus in the absence of sheep, only in seasons of particular abundance can they feed and, hence, infect, a significant number of the ticks on sheep pasture. It is possible, however, that periodic plagues of wild hosts produce surges in the abundance of ticks by ensuring survival of ticks which otherwise fail to find hosts.

Thus although the role of wild hosts remains open, the evidence suggests that the sheep normally plays the dominant part in determining the infection rate in the tick population of hill pasture.

The remaining possible source of infection for the tick is transovarial passage of virus. Where this is assumed to be negligible, the infection rate observed in questing nymphs represents solely the infections produced in larvae. The infection rate in female ticks represents cumulative infections, produced in larvae and nymphs successively. Where the incidence of transovarial passage of virus is appreciable, a significant proportion of questing larvae may carry infection. Here the infection rates in nymphs and females are boosted by transovarially acquired infection.
The models presented above were oversimplified, but the estimates provide a fair indication of the variations in the infection rate of ticks which are likely to follow relatively large fluctuations in the incidence of intense viraemia. Thus, the infection rate observed in the questing nymphs from Blarcreen farm is no greater than may be expected from the estimates made here. Although no figure is available for the incidence of louping ill on Blarcreen in the year prior to the 1965 survey, the losses were slight. Probably less than 5% of young stock plus a few adult sheep could, by present definition, have developed sufficiently intense viraemia to infect ticks. This is consistent with the low infection rate observed in questing nymphs. Since there was a comparatively high incidence of the disease on Blarcreen two and three seasons earlier, it follows that there is little evidence of transovarial passage of virus.

For more exact calculation of tick infection rates than was attempted here, it is necessary to know the average effective duration of viraemia and the daily rate of parasitization of sheep with the various instars of tick. Allowance has to be made for the fact that a proportion of ticks which feed during intense viraemia fails to gain infection. Furthermore, some ticks which feed during viraemia will fail to complete engorgement before the host dies, but this applies particularly to female ticks. Finally, it has to be borne in mind that a proportion of infected ticks may fail to transmit virus.
The rate of challenge which sheep meet, with any incidence of virus in ticks, depends on the average weight of infestation. Provided that the microclimate requirements of the tick are met, the weight of infestation increases with the stocking rate, i.e. with the density of the host population (Milne, 1947). On hill farms in northern England, with a carrying capacity of up to one sheep per acre, the maximum infestations in spring average 100 to 150 female ticks per ewe and each ewe feeds five times this number of females per annum (Milne, 1947). This level of infestation corresponds in particular to Milne’s figures for a farm near Cheviot and the estimate is, therefore, taken to be valid for similar hill farms in the adjacent Border counties of Scotland.

The prevalence of nymphs relative to females must vary from year to year. In a particular season, however, Milne found that sheep fed approximately eight times as many nymphs as females and this ratio is assumed to be usual.

Consequently, it is postulated that on a heavily infested farm in the Border area, where there is a spring peak of tick activity only, each ewe feeds an average of 750 females and 6,000 nymphs per annum. From the ratios determined by Milne (see above) it follows that hoggs on this farm feed approximately 563 females plus 4,500 nymphs and lambs feed 94 females plus 750 nymphs.

No comparable data are available for farms in the Western Highlands of Scotland, but the levels of infestation here are known/
known to be lower than in Northumberland and the Borders area (MacLeod, 1938; Campbell, 1952). The difference is thought to be associated with the fact that farms in the south are generally four times more heavily stocked than Highland farms (Milne, 1949a).

Although the true relationship between the densities of sheep and tick populations is probably quite complex, it is assumed here that the weight of infestation varies directly with the stocking rate. Hence, tick infestation on a Highland farm is taken to be one-fourth as heavy as on the Border farm above. This applies to the spring-feeding population of ticks on the farm. The autumn-feeding population is smaller.

These estimates are consistent with tick burdens observed at various sites in Argyllshire (p. 29).

From calculations made above, it is taken that one questing nymph in every 714 to 1,745 is infected following a spring season during which 5% of hoggs develop sufficiently intense viraemia to infect ticks. At a minimum, the same proportion of females is infected. With this incidence of virus in ticks it follows that all of the hoggs and ewes on the Border farm meet infection. A high proportion, if not all, of the hoggs and ewes on the Highland farm meet infection. On both farms, susceptible animals either die of the infection or else they recover and are immune. Where the same rate of challenge persists the ewe populations consist almost exclusively of immune survivors. Hence, most lambs born of these ewes receive maternal antibody.
Lambing coincides with the spring season of tick activity and since colostral immunity protects against infection for up to three months (Wilson and Gordon, 1948; Williams and Thorburn, 1961; Smith et al, 1964), the lambs are not susceptible to louping ill during their first tick season. Even repeated infection during the period of passive immunity fails to confer active immunity (Wilson and Gordon, 1948; Williams and Thorburn, 1961).

Consequently, the lambs enter their second tick season as fully susceptible hoggs and infection during this season results either in death or in immunity.

On the Border farm, where there is a spring peak of tick activity only, deaths occur when hoggs are one year old. On the Highland farm, with bimodal peaks of tick activity, deaths may occur during the lesser tick season in autumn when hoggs are approximately six months old, or during the main tick season in spring. On both farms, most survivors of the first year of life are immune and there is a sharp decline in mortality with further increase in age. Since deaths here are effectively limited to the one age group, namely, hoggs, losses are likely to be heavy on infrequent occasions only.

Although it can be inferred that louping ill is potentially a disease of hoggs on both types of farm, this pattern of disease is stable on the Border farm only. Here the rate of challenge which sheep meet remains high in seasons where the prevalence of ticks, or the incidence of virus in ticks, falls to a quarter of the levels/
levels indicated above. Following a season during which sheep fail to infect ticks it remains possible for females, infected earlier as larvae, to produce a high incidence of infection in hoggs and ewes. In this way, the high rate of challenge can persist even where hogg mortality, and hence threshold viraemia, fluctuates between nought and 5% per annum.

By contrast, a fall of 1% in the incidence of threshold viraemia in hoggs on the Highland farm produces a 20% decrease in the rate of challenge which hoggs and ewes meet at a constant level of infestation. Thus, following a season during which 2.5% of hoggs attain threshold viraemia, approximately 50% of hoggs and ewes meet infection. Where similar rates of challenge persist the flock contains an increasing number of susceptible ewes and there is a corresponding reduction in colostral immunity among lambs. Consequently, the disease is no longer confined to hoggs and the potential of the flock to infect ticks is increased. Higher tick infection rates accompany disease in sheep of all ages. With a marked increase in the challenge rate, the immune status of the ewe flock is restored and loupimg ill reverts to a disease of hoggs.

It follows that the occurrence of transovarial passage of virus in the tick, or the circulation of virus in wild host and tick, would have little effect on the pattern of disease on the Border farm. If anything, the effect would be to stabilize the high rate of challenge even further. Similarly, surges in the abundance of ticks, following plagues of wild hosts, are unlikely/
unlikely to produce changes in the pattern of disease where the rate of challenge is already high.

On the Highland farm transovarial passage of infection in the tick, or circulation of virus in wild host and tick, would have the effect of ensuring survival of the virus during periods of low challenge. In seasons where wild hosts become particularly numerous, they could probably induce abrupt increases in challenge rate, either by infecting ticks or by promoting an increase in the abundance of ticks. In this way, rodent plagues which follow long periods of low challenge could precipitate severe outbreaks of disease.

Calculation of probable death rates on the two farms is precluded by the unpredictability of factors which govern mortality. Although most natural infections in sheep are in-apparent (Gordon et al., 1962; Smith et al., 1964) the number of susceptible animals which develops fatal disease following infection varies from nought to 100%, depending on climatic conditions and on the nutritional and physiological state of the animals (Edward, 1947; Gordon et al., 1962; O'Reilly, Smith McMahon, Wilson and Robertson, 1965). For this reason epidemiological analysis of louping ill ultimately remains on a stochastic basis.

In general terms it can be stated that where the prevailing rate of challenge is high, louping ill is essentially a disease of hoggs. Here the disease probably kills little more than 5% of hoggs/
hoggs each year, but mortality may exceed 30% on occasion.

Low rates of challenge occur most readily where tick infestation is inherently moderate. In this instance the pattern of disease tends to fluctuate over a period of years to involve hoggs and sheep of all ages alternately, the number of susceptible ewes in the flock being cumulative over periods of low challenge. Following one year of low challenge, only the youngest ewes, the gimmers, and their lambs, are at risk. A death rate of 5 to 30% in this group of animals amounts to an approximate mortality of 1 to 6% of all ewes and lambs in the flock.

Where low rates of challenge persist for a number of years the flock contains a high proportion of susceptible animals. An abrupt increase in challenge rate at this point may, nevertheless, fail to provoke a high incidence of disease. When the sheep are predisposed to encephalitis, however, an abrupt increase in challenge rate produces severe outbreaks of disease involving sheep of all ages. Such outbreaks are likely to occur at widely spaced intervals only; they require that a long period of low challenge is followed by an abrupt increase in challenge rate which coincides with conditions of enhanced susceptibility to encephalitis among sheep.

In brief, the incidence of disease on affected farms falls into two basic patterns; in one pattern the disease is limited almost/
almost exclusively to hoggs and in the other a variable number of
ewes and lambs is affected each year, the losses becoming
particularly severe at intervals of many years only. The first
pattern coincides with the classical form of the disease which
Pool et al (1930) set out to investigate in 1929. The second pattern
presents the features observed in recent outbreaks of the disease in
Argyllshire.

There is some evidence to support the view that the occurrence
of outbreaks of disease involving sheep of all ages is not, in fact,
a recent development. Gordon et al (1962) observed two outbreaks of
disease during the vaccine trials between 1931 and 1934 in which
"the mortality in all ages of sheep was particularly high". These
outbreaks were, however, attributed to the introduction of tick and
virus onto tick-free hill farms through "the mixing of sheep at farm
boundaries". Although this explanation may be correct, it is clear
from lamb deaths recorded on numerous other farms during the vaccine
trials (Gordon, 1934b) that the disease was not strictly limited to
hoggs. A mortality of 38.1% observed among unvaccinated lambs on
one farm in 1933 is comparable to the estimated 50% death rate
reported recently from a farm in Ayrshire (Smith et al, 1964).

Moreover, it should be noted that the problem of disease in
lambs was sufficiently serious to warrant prophylactic use of
antiserum being investigated in large scale field trials between
1938 and 1941 (Wilson and Gordon, 1948). Totals of 20,000 each of/
of test and control lambs were involved. Although the number of deaths recorded among lambs on experiment each year indicates moderate levels of disease only, no figures are supplied for mortality on individual farms.

If, indeed, the incidence of disease in sheep other than hoggs has increased in recent years, then methods used to control the disease may well have contributed to this altered pattern. It is doubtful whether or not a single ixodicide treatment of sheep per tick season has led to an appreciable reduction in levels of infestation over the years. Nevertheless, it follows from the present analysis that the effect of such a reduction in the level of infestation would be to alter a stable pattern of disease in hoggs into an erratic pattern of disease in sheep of all ages. Reduction of tick infestation through pasture improvement would have the same effect.

Vaccination of sheep, too, would ultimately have this effect by reducing the level of virus circulation in the biocenose. Although the vaccine fails to induce production of demonstrable antibodies, the theory is that it sensitizes the animal to louping ill virus. Sensitized animals, by virtue of a rapid immune response, acquire active immunity from natural infection without risk of encephalitis (Gordon 1934a, b; Edward, 1947; Gordon et al., 1962; Smith et al., 1964). The evidence produced in laboratory tests (Edward, 1947) does not allow critical evaluation of the effectiveness of the vaccine, but results obtained on individual farms during the field trials (Gordon/
(Gordon, 1934b) indicate clearly that vaccination reduces mortality.

The deficiency of the vaccine, however, lies in the fact that it relies on high challenge rates to complete immunisation of vaccinated animals, usually hoggs. Where the prevailing rate of challenge is low, hoggs may fail to meet infection in their first tick season following vaccination. There is no evidence that the animals will remain protected against infection in subsequent years: the durability of the protection conferred by the vaccine has not been determined. Even if it is assumed that vaccination protects indefinitely, it remains true that lambs born of vaccinated ewes acquire colostral immunity only in those instances where the ewes have met natural infection. Where low rates of challenge persist for a number of years a high proportion of lambs in the flock fails to gain colostral immunity despite regular use of vaccine.

Under conditions of high challenge rate, where only hoggs manifest the disease, vaccination reduces the incidence of disease in hoggs and, hence, reduces the infection rate in ticks. Where this leads to a reduction in challenge rate, vaccinated animals fail to meet infection and, consequently, they are unable to confer colostral immunity on their lambs. Thus, regular vaccination of hoggs may alter a position where only hoggs are susceptible to the disease into a position where lambs and, possibly, ewes, are susceptible/
susceptible to the disease. Clearly, there is need for a vaccine which induces a strong antibody response, thereby ensuring both immunity in vaccinated animals and colostral protection of lambs.

The principal conclusion to be drawn from the present analysis therefore, remains unaltered where existing methods of controlling the disease are practised: the fluctuating pattern of disease in sheep of all ages which is seen in Argyllshire, arises under conditions of low challenge rate. This conclusion is essentially in agreement with the conclusion reached by Smith et al (1964) in their analysis of the disease problem in Ayrshire.

The experimental findings reported above and the assumptions made in the epidemiological analysis of the disease will undoubtedly require modification as further knowledge becomes available. The present project will, however, fulfill its intended purpose if it serves only to suggest certain points of departure for future research. As an immediate sequel to this programme it would be useful to repeat the threshold experiments with a second strain of virus, preferably with a recent tick isolate. On this occasion threshold viraemia for infection of the tick can be fixed with greater accuracy by determining infection rates through testing of individual ticks. The experiments should include a large number of adult ticks to allow an estimate to be made of the significance of transovarial passage of virus.

The role of the sheep in the circulation of virus can be re-assessed by studying viraemia in animals following simulated natural infection, produced either by tick transmission or by subcutaneous/
subcutaneous infection of small doses of a strain of virus isolated from ticks. Similar laboratory investigation of viraemia in a range of wild hosts would indicate which species merit further attention in ecological studies.

The deterministic approach to the epidemiology of the disease would be taken to its logical conclusion in an integrated field study where monitoring of infection in sheep is accompanied by studies on the ecology of the tick. This would allow assessment of the true quantitative relationships between the incidence of disease, challenge rates, infestation rates and the infection rate of ticks.
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