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The serological response of sheep to infection with louping-ill virus

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INTRODUCTION

With the discovery of the virus aetiology of louping ill in sheep by Pool, Brownlee & Wilson, in 1930, some of the fundamental characteristics of the virus in relation to the disease in sheep, its transmission, and the identification of the tick vector, *Ixodes ricinus* were soon elucidated (Pool *et al.*, 1930; Alston & Gibson, 1931; Hurst, 1931; Brownlee & Wilson, 1932; McLeod & Gordon, 1932; Gordon, Brownlee, Wilson & McLeod, 1932).

A number of distinct yet related diseases occurring over a wide geographical area from the far east of the Soviet Union to Western Russia were shown in 1937 to be tick-borne virus diseases (Smorodintseff, 1958) and the viruses involved were closely related to the virus of louping ill (Casals & Webster, 1943, 1944). Likewise, a virus isolated from cases of meningoencephalitis in man in Czechoslovakia in 1948, and now generally termed Central European tick-borne encephalitis was shown by Edward to be closely related to the Russian spring–summer encephalitis (R.S.S.E.) and louping-ill viruses (Edward, 1950).

All these diseases are now grouped together under the term Russian spring–summer complex and with louping ill constitute subgroup of the arthropod-borne group B viruses, according to the classification of Casals & Brown (1954).

Certain improvements in the design and technique of complement fixation tests (Fulton & Dumbell, 1949; Mayer, Osler, Bier & Heidelberger, 1946) and the development of haemagglutination inhibition tests with some members of the arbor viruses (Clarke & Casals, 1958) have made possible the examination of material on a larger scale than has readily been accomplished up to now. The ability to perform wider epidemiological surveys, the possibility of offering a diagnostic aid to those concerned with louping ill in sheep and the chance of finding evidence of human infection as suggested by the findings of Likar & Dane (1958) in Ireland, seemed to make a wider investigation in this area worth while.

With these points in view we have carried out a series of investigations in this area; we report here the results of a serological investigation on a small experimental flock of fifteen sheep maintained over a period of 11 months. This was carried out as a preliminary exercise to find the limits of the different techniques employed by us. The fifteen sheep were part of a larger flock maintained for the period at the North of Scotland College of Agriculture at Aberdeen.

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MATERIAL AND METHODS

Virus

The limb strain of louping ill obtained from the Wellcome Research Laboratories was used throughout.

Mice

Mice were all of the TO strain, the foundation stock supplied by the National Institute for Medical Research.

Antigens

3–5 day-old mice were inoculated intracerebrally with approximately 0·02 ml. of a 1:50 dilution of infected mouseling brain suspension. Mice were inoculated in batches of fifty or more, and on the 5th day after inoculation, by which time the mouselings were dead or dying, the brain tissue was harvested, pooled and stored in weighed bottles at -20° C. Brain pools were made as was convenient and the brains stored 1–4 weeks prior to antigen extraction. The preparation of the antigen for complement fixation followed very closely the method described by Williams (1958). A 20% brain emulsion in deionized water was allowed to stand 18 hr. at 4° C. The emulsion was transferred to Petri plates and dried over calcium chloride in a desiccator, which after evacuation was placed in the refrigerator until drying was complete. The dried tissue was then collected and extracted with a weight/volume amount of benzol, nine times that of the equivalent weight of wet brain. Three benzol extractions of 30 min. each were made on the magnetic stirrer and the tissue recovered by centrifuging. After the last extraction, the material was placed in the desiccator in the cold for about 1–2 hr. to remove the residual benzol. The dried benzol extracted brain was finally suspended in 0·9% saline, one part of the original brain weight to nine parts of saline. The suspensions were kept overnight at 4° C. and centrifuged the following morning. The supernatant represented the final antigen. It was distributed in 5 ml. bottles and stored at -20° C. Under these conditions the antigen maintained its titre for about 2 months. Complement-fixing titres of the antigen so prepared varied from 1:4 to 1:16. The antigen prepared as above for complement fixation could also be used for haemagglutination inhibition tests, the dilution representing one haemagglutinating unit being in the region 1:16 to 1:64.

Normal mouseling brain antigen for specificity studies was prepared in the same fashion.

Antisera

Sheep antiserum was prepared in hoggs selected from louping ill-free flocks, by injection of formolized and active virus. Similarly, antiserum was prepared in mice, guinea-pigs and rabbits. These sera were stored at -20° C. without preservative.

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Complement fixation tests

All serum was stored at $-20°$ C. prior to testing. Sheep serum was inactivated at 62° C. for 20 min. Sometimes it was necessary to repeat this heat treatment with strongly anticomplementary specimens.

The tests were carried out in Perspex trays in the usual way after the preliminary titration of antigen, standard serum, haemolysin and complement. A calcium–magnesium veronal buffer was used as diluent and dilutions of the serum under test made from 1:1 to 1:64. Complement ($2\frac{1}{2}$ HD50) was added, followed by appropriately diluted antigen. Overnight fixation at 4° C. followed. Next morning the trays were removed from the refrigerator and left on the bench for 30 min. when the haemolytic system was added and incubation at 37° C. for 30 min. carried out. Readings were made after the trays had stood on the bench for 2 hr. The necessary controls for antigen, serum, complement and cells were included with each batch of tests.

Haemagglutination inhibition tests (H.A.I.)

A good deal of information has been obtained over the past decade of the conditions that are necessary for haemagglutination with different members of the arbor virus group as regards pH range, erythrocyte species and non-specific inhibition (Sabin, 1951; Casals & Brown, 1954; Clarke & Casals, 1955; Porterfield, 1957; Clarke & Casals, 1958). The technique that was finally adopted is described here and discussed later.

Goose cells obtained from the wing vein of a bird anaesthetized with chloroform–ether mixture were taken into Alsever's solution and washed three times in saline. They were made up to a concentration of 0·2% in a phosphate buffer made up from stock solutions of 0·15 molar sodium chloride with 0·2 molar disodium hydrogen phosphate (Na_2HPO_4) and 0·15 molar sodium chloride with 0·2 molar sodium dihydrogen phosphate (NaH_2PO_4) adjusted to produce the desired pH when mixed with an equal volume of bovalbumin borate saline at pH 9·0 (Clarke & Casals, 1958). The antigen was diluted from 1:2 to 1:64 in bovalbumin borate saline solution pH 9·0 and the optimum dilution of antigen for the test determined by noting the highest dilution at which haemagglutination occurred. The volumes used were 0·1 ml. of appropriately diluted antigen, 0·1 ml. of bovalbumin borate saline (to compensate for the serum in the test proper) held for 2 hr. at 4° C. and 0·2 ml. of goose cells in diluent at pH 6·2, found to be the optimum pH, then added. Readings were made after the plates had stood 2 hr. at room temperature. When the antigen titre had been determined the H.A.I. test was carried out with four units of antigen. These tests were carried out on Perspex trays with 0·1 ml. of serum under test and 0·1 ml. of appropriately diluted antigen added, the serum antigen mixtures held 2 hr. at 4° C., the cells added, the readings taken 2 hr. later, the plates having stood those 2 hr. at room temperature. This reading was found to be unchanged 24 hr. later. Cell and antigen controls were included.

Sheep serum contains natural agglutinins to goose cells. These can be removed readily by preliminary absorption at room temperature (one drop of packed goose

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cells to 1 ml. of serum). Non-specific inhibitors of haemagglutination also occur. These can be absorbed with bentonite. One part of unheated serum was added to nine parts of 1 % bentonite in borate saline pH 9·0, the mixture kept at room temperature for 20 min. with shaking at 5 min. intervals, centrifuged, and the supernatant taken as 1/10 dilution of serum. This is a modification of the original method (Clarke & Casals, 1955).

Neutralization tests

The serum neutralization test was carried out in mice about 21 days old and the interpretation recommended by Rivers (1948) applied where neutralization indices 0–9 are presumed negative, 10–49 doubtful, and 50 or more, positive.

Because of the number of laboratory accidents that have occurred in the past with louping-ill virus (Rivers & Schwentker, 1934; Edward, 1948), use was made as far as possible of protection hoods during manipulation. The tests were, however, performed on the open bench. The work was carried out in a small laboratory adjacent to the Animal House and separate from the main laboratory building.

EXPERIMENTAL RESULTS

A difficulty in C.F. tests with sheep serum was the strong anticomplementary effect of most samples. Heating at 62° C. for 20 min. in the waterbath was usually effective in eliminating this anticomplementary effect, although on occasion this heat treatment had to be repeated. Diluted serum could often be cleared of anticomplementary activity at temperatures somewhat lower than 62° C., but dilution was of limited use because some of the C.F. titres were low, e.g. 1:2 to 1:4. Although destruction of antibodies is possible at temperatures above 60° C. this point was not pursued, since with the heat treatment employed C.F. antibodies could be detected early in the course of immunization of animals which had shown no antibody prior to immunization.

The development of the H.A.I. test followed closely the method of Clarke & Casals (1958) with the exception of the antigen preparation which has already been described. The optimum pH was 6·2 as shown in Table 1. All batches prepared were active in the range pH 6·2–6·6.

Time and temperature were varied in an attempt to enhance the H.A.I. titres, e.g. by allowing the serum antigen mixture to remain overnight at 4° C., but no difference was detected whether the serum antigen mixture was held 2 or 24 hr. at 4° C. Furthermore, after the addition of erythrocytes the end titres obtained were identical whether the test proceeded at room temperature or at 37° C.

As with other haemagglutination inhibition tests the problems of non-specific inhibitors loom large. Serum from non-immunized sheep had H.A.I. titres which varied between 1:20 to 1:640. In other arbor virus infections and with human serum, Clarke & Casals (1958) described the removal of non-specific inhibitors by treatment of serum with acetone or kaolin, and Clarke & Casals (1955) described a method using bentonite. These methods were compared on a number of samples from immunized and non-immunized sheep when it was found that non-specific

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inhibitor was successfully removed by all three methods. Bentonite extraction was adopted, bentonite or kaolin extraction being simpler than the acetone method.

In the techniques used the time of contact between the bentonite and serum was not critical, although the concentration of bentonite is said to be so in treating human serum (Salminen, 1960).

Table 1. *Determination of optimum* pH *for H.A.I. tests*

pH	Antigen dilution in bovalbumin borate saline 1/2	1/4	1/8	1/16	1/32	1/64	Erythrocyte controls
5·75	1	1	1	0	0	0	0
6·0	3	3	2	1	1	0	0
6·2	4	4	4	4	3	2	0
6·4	4	4	4	3	2	1	0
6·6	1	1	2	0	0	0	0
6·8	0	0	0	0	0	0	0

The pH is that resulting from the mixing of the erythrocyte adjusting diluent and bovalbumin borate saline.

4, Maximum haemagglutination; 3, 2, 1, degrees of haemagglutination; 0, complete haemagglutination inhibition.

Table 2. *Removal of non-specific inhibitors of haemagglutination by absorption with* 1 % *bentonite*

	Exposure time to 1 % Bentonite (min.)	Reciprocal of dilution of serum 10	20	40	80	160	320	640	1280	2560
Immune serum	Nil	0	0	0	0	0	0	0	2	4
	10	0	0	0	0	0	2	4	4	4
	20	0	0	0	0	0	2	4	4	4
	30	0	0	0	0	0	2	4	4	4
	60	0	0	0	0	0	2	4	4	4
Non-immune serum	Nil	0	0	0	0	0	4	4	4	4
	10	4	4	4	4	4	4	4	4	4
	20	4	4	4	4	4	4	4	4	4
	30	4	4	4	4	4	4	4	4	4
	60	4	4	4	4	4	4	4	4	4

4, Maximum haemagglutination; 3, 2, 1, degrees of haemagglutination; 0, complete haemagglutination inhibition.

Nine out of ten sheep serum samples contained agglutinins to goose cells to an average titre of 1:20. These were readily removed by absorption with goose cells; the agglutinins were not affected by bentonite absorption.

Examination of sera

Six animals, five of them from a louping ill-free flock (nos. 1–5) and the sixth from an infected flock were maintained in a louping ill-free area and serologically examined at monthly intervals between November 1959 and September 1960.

They were inoculated in August 1960 with 1·0 ml. of an infected mouse brain

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suspension diluted 1:100 in saline (approximately 10^6 mouse LD50 units). The September bleedings were carried out 28 days later. The results month by month are shown in Table 3.

Table 3. *Serum titres (C.F. and H.A.I.) of six sheep at monthly intervals over 11 months and their response to live virus inoculation*

Animal no.	Test	Nov.	Dec.	Jan.	Feb.	March	Apr.	May	June	July	Aug.*	Sept
1	C.F.	0	0	0	0	0	0	16	0	0	0	64
	H.A.I.	0	0	0	0	0	0	0	0	0	0	320
2	C.F.	0	0	4	0	0	0	2	0	0	0	0
	H.A.I.	0	0	0	0	0	0	0	0	0	0	0
3	C.F.	0	0	0	0	0	0	16	0	0	0	16
	H.A.I.	0	0	0	0	0	0	0	0	0	0	80
4	C.F.	0	4	4	0	0	0	2	0	0	0	32
	H.A.I.	0	0	0	0	0	0	0	0	0	0	160
5	C.F.	0	0	0	0	0	0	2	0	0	0	32
	H.A.I.	0	0	0	0	0	0	0	0	0	0	320
6	C.F.	64	32	8	16	8	8	16	8	16	16	32
	H.A.I.	20	40	40	40	40	80	20	40	40	80	160

* All animals inoculated August. Titres expressed in reciprocals. In H.A.I. test 0 = < 1/10.

Animals 1–5, free from infection remained over-all serologically negative during the observation period, apart from an unexplained rise in the C.F. titre during May—a rise that was also noted in another group of animals and was confirmed on repeating, but which was not paralleled by a coincidental rise in the H.A.I. titre. Uninfected animals remained serologically negative by the C.F. and H.A.I. tests. At least four of the five animals were immunologically competent as judged by the serological response following virus inoculation in August. For comparison the fluctuation in the monthly serum titres obtained from sheep number 6 from an infected flock which was serologically positive at the beginning of the experiment is included in Table 3. Here the C.F. titre fluctuated around 1:16 and the H.A.I. titre around 1 : 40. No marked increase in titre in either test was observed after inoculation in August.

A further ten sheep from a louping ill-free flock were observed over a similar period during which they were subject to a series of inoculations with vaccine and infective virus. It was particularly desired to see how animals would respond serologically to heavy and persistent infection after initially receiving commercial vaccine.

The results are shown in Table 4.

Blood samples examined on 20 November 1959 at the beginning of the experiment from each of the ten sheep showed an absence of detectable C.F. or H.A.I. antibodies. After the series of inoculations administered in November (Table 4) and the subsequent bleedings 4 weeks later (18 December 1959) C.F. antibodies were present to a titre of about 1:16 and H.A.I. antibodies had risen from zero

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to 1:320. Four weeks later saw some decline in these titres but thereafter the H.A.I. antibody levels, in contrast to the C.F. antibodies, were maintained during the remaining 8 months of the experiment.

Table 4. *Serum titres (C.F. and H.A.I.) of ten sheep examined at monthly intervals over 11 months inoculated with vaccine and infective virus*

Animal no.	Test	Nov.*	Dec.	Jan.	Feb.†	Mar.‡	Apr.	May	June	July	Aug.§	Sept.
7	C.F.	0	4	0	0	0	0	0	0	0	0	0
	H.A.I.	0	640	40	40	40	80	80	40	20	20	20
10	C.F.	0	16	8	4	8	0	16	0	8	8	32
	H.A.I.	0	.	80	80	.	20	80	40	80	80	160
12	C.F.	0	4	0	0	0	0	8	0	0	0	8
	H.A.I.	0	40	0	0	0	0	0	0	0	0	0
13	C.F.	0	16	2	0	0	0	16	0	0	0	16
	H.A.I.	0	.	80	20	10	0	0	0	0	0	0
17	C.F.	0	0	16	8	2	0	8	4	4	8	16
	H.A.I.	0	640	40	40	80	80	80	60	20	20	20
19	C.F.	0	8	8	8	8	0	16	4	4	8	16
	H.A.I.	0	160	40	40	160	160	80	80	80	80	160
21	C.F.	0	16	8	0	0	0	32	0	0	8	.
	H.A.I.	0	80	320	10	160	320	320	160	80	20	80
23	C.F.	0	16	16	4	0	0	8	0	0	0	32
	H.A.I.	0	160	160	80	80	80	80	80	80	80	80
25	C.F.	0	16	8	4	4	0	8	4	4	8	.
	H.A.I.	0	320	160	80	160	320	160	160	160	80	160
W 19	C.F.	0	16	4	0	0	0	0	0	0	0	0
	H.A.I.	0	20	10	0	0	0	0	0	0	0	0

* 2·0 ml. commercial vaccine 29 November 1959; 0·75 ml. infective virus 3 December 1959; 1·0 ml. infective virus 8 December 1959; 2·0 ml. infective virus 15 December 1959.
† 2·0 ml. infective virus 19 February 1960.
‡ 2·0 ml. infective virus 24 March 1960.
§ 2·0 ml. infective virus 21 August 1960.
In H.A.I. tests 0 = < 1/10.
1 ml. infective virus = 10^6 mouse LD 50 units (approximately).

Further inoculations in February, March and August with serum examinations carried out 4 weeks after each inoculation showed a failure of any sustained serological response to these repeat infections by either test until September when the C.F. titres rose erratically.

After the initial serological response these animals maintained an appreciable H.A.I. titre but showed a poor response to subsequent infections. There was no consistent pattern in the C.F. antibody response in the face of repeat infections.

During the course of the experiment, lambs were born, fourteen of which survived, nine from the non-immunized group and five from the immunized. The lambs reflected the antibody status of the mother, the nine from the first group lacked C.F., H.A.I. and neutralizing antibodies at birth, and when inoculated at about three months old with a single dose of infective virus they produced measurable C.F., H.A.I. and neutralizing antibody titres (B 1 in Fig. 1).

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The lambs from the immunized group of ewes had initially H.A.I. and neutralizing antibodies but were without C.F. antibodies like their dams. These antibodies showed a slow decline over the first 3 months of life and the rate of disappearance was unaffected by repeated inoculation of infective virus (R1 in Fig. 1).

In addition to the results reported above we examined on behalf of veterinary officers in this area forty samples of serum from sheep suffering from louping ill. In these, while the C.F. test varied between nil and 1:32 the H.A.I. titre was 1:40 or greater in every instance. Because of the sharp rise in the H.A.I. titre after inoculation of infective virus (Tables 3 and 4) and the constantly elevated level we found in clinical cases, we tested the serological response of three louping ill-free sheep, two of which were inoculated with formolized sheep brain vaccine

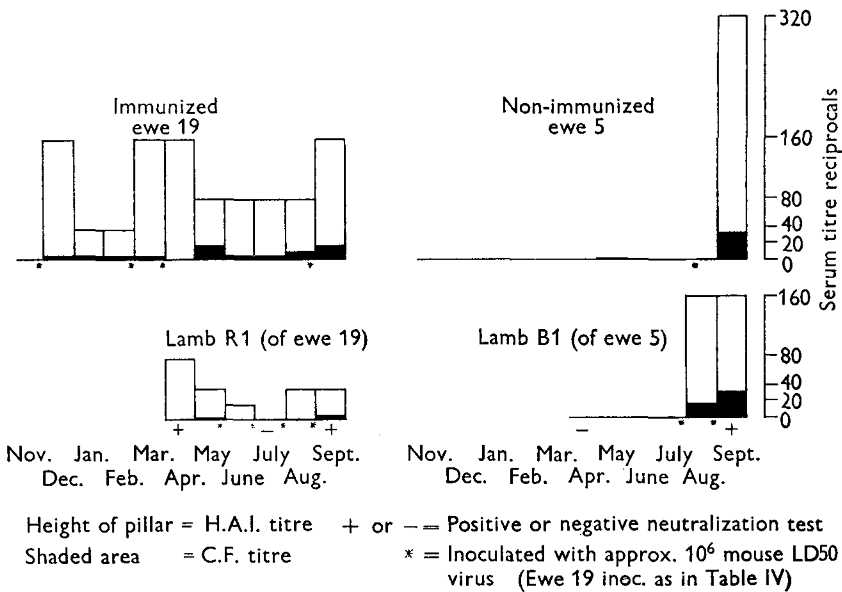

Fig. 1. Serum titres, C.F., H.A.I. and neutralizing, at monthly intervals, of representative lambs from immunized and non-immunized dams.

Table 5. *Response in sheep to inoculation with formolized and infective brain suspensions*

Animal	Inoculation	Test	Pre-inoculation	Period after inoculation 21 days	45 days	88 days
1	Formolized	C.F.	0	16	4	0
	Sheep brain	H.A.I.	0	0	0	0
2	Formolized	C.F.	0	8	4	0
	Sheep brain	H.A.I.	0	0	0	0
3	Infective	C.F.	0	16	4	0
	Mouse brain	H.A.I.	0	160	160	40

Titres expressed in reciprocals. In H.A.I. tests 0 = $<1/10$. Animals 1 and 2 were inoculated with 2 ml. of commercial (sheep brain) vaccine. Animal 3 was inoculated with approximately 10^6 mouse LD 50 units of infective mouse brain suspension.

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and the other infective virus in mouse brain suspension. Although all three animals developed C.F. antibodies, only the sheep inoculated with infective virus developed H.A.I. antibody (Table 5). This was of some interest, and since we have confirmed this finding in small-scale experiments using mice and guinea-pigs inoculated with formolized and infective mouse brain suspension respectively, may be of more fundamental significance. If it can be confirmed on a larger series it may have a practical value as an aid to the serological differentiation between vaccination and infection.

DISCUSSION

Williams (1958) reporting on the C.F. test for louping ill in sheep found that antibody appeared 2–6 weeks after experimental infection, persisted less than 21 weeks in almost half the sheep he examined, and 6 weeks only in one case. In contrast he found that neutralization titres appeared 1–4 weeks after infection and were stable over a long period. Due to delayed appearance, borderline titres and rapid decline and disappearance of C.F. antibody in a high proportion of recovered cases, only 50 % of field cases positive to the neutralization test were also positive to the complement fixation test. Our results confirm the transience of complement-fixing antibodies in sheep serum and underline the limitations of the C.F. test in sheep.

The stability of neutralizing antibodies makes the mouse neutralization test of more value for field survey work and it has been successfully used for this purpose by Dunn (1960) in the examination of deer serum, but the test has many disadvantages especially in the large numbers of mice required, all of which have to be inoculated intracerebrally. The development of an *in vitro* neutralization test as described by Porterfield (1960) might be of advantage in this respect.

The haemagglutinating properties of some members of the arbor viruses first discovered by Sabin and his associates (Sabin & Buescher, 1950; Sabin, 1951; Channock & Sabin, 1953) were extensively studied by Casals and his group (Casals & Brown, 1953, 1954; Clarke & Casals, 1955) and the technical difficulties of H.A.I. tests due to non-specific inhibitors largely overcome. In 1953 and 1954, Casals & Brown obtained negative results with R.S.S.E. and louping-ill viruses before the instability of the haemagglutinin from this group was fully appreciated, and later, in 1958 when the techniques for H.A.I. were extensively described by Clarke & Casals, louping ill was not among the group of viruses studied. Salminen (1960) studied the haemagglutinins of the R.S.S.E. group and described the optimum conditions for haemagglutination, the use of erythrocytes from different species and the amounts of non-specific inhibitor in human and animal serum. He concluded that the H.A.I. test was valuable in this group but, due to the presence of infective virus in the antigen, its use on any scale was precluded until a non-infectious antigen could be developed. We have since learned (Gordon Smith, personal communication, 1961) that benzene-extracted antigens are not in general favoured for haemagglutination. The antigen used here was produced originally for complement fixation, and, while found usable for haemagglutination, never yielded high titres. Nevertheless, we found it consistent, reliable and active over

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a reasonable pH range. Simple extracts of mouse brain in alkaline saline, while yielding higher haemagglutination titres, we found less reliable and more exacting in pH requirements, possibly because in their preparation we were not able to employ high-speed centrifugation.

Louping ill is endemic over some areas of North-west Scotland, but within these areas, and bordering on them are others apparently free from the disease, and in some instances the dividing line between such areas appears to be very clear cut. Why this should be so is not clear and may involve complex ecological situations. The possibility of alternative hosts present in the wild fauna providing a reservoir for the infection of domestic animals, while never proved in louping ill, might yet account for the uneven local distribution of the disease.

Where louping ill is endemic it is rare to find adult sheep clinically affected as presumably active immunity is acquired during the first year of life and thereafter reinforced by repeated natural infections. However, Sharman (1960), in his survey of sheep disease in the islands of North and South Uist, did find in this endemic area clinical cases in adult sheep and confirmed that some adults in these areas were not immune. This may merely confirm the patchy distribution of infection in endemic areas, but in this respect it may also be of interest to note the findings in the lambs of the experimental group studied here, where a passively conferred immunity apparently prevented for a time, a response to infective virus. Susceptible adults may be those which received the stimuli of infection while still covered by maternal antibody and failed to respond. Removed for wintering to the low tick-free areas, they may return to the hills the following spring susceptible to the infection. A further study on that transient stage between the passively immune lamb and the actively immune would be of interest. The study of these problems should be furthered by the development and appraisal of serological tests. In the examination of sheep serum for louping-ill antibodies the H.A.I. test promises well and should be useful both for epidemiological and diagnostic purposes.

SUMMARY

1. An H.A.I. test for louping ill in sheep is described and its potentiality investigated alongside a standard C.F. test.

2. Sheep free from infection with, or which have not been vaccinated against, louping ill do not possess serum antibody demonstrable by the C.F., H.A.I. or neutralization techniques as described here.

3. After the administration of commercial vaccine followed by infective virus, C.F., H.A.I. and neutralizing antibodies are detectable. The C.F. antibodies are the least stable and in many instances disappear entirely from the peripheral circulation within 4 months of their appearance; they also appear to be susceptible to fluctuation unassociated with the disease. H.A.I. antibodies elicited under the same circumstances reach a high level early, are maintained for longer periods than C.F. antibodies, and fall to a basic level which tends to be maintained.

4. H.A.I. antibodies do not appear to be elicited in response to inoculation with vaccine only, in contrast to C.F. and neutralizing antibodies which are so elicited. It is suggested in these circumstances, measurement of H.A.I. antibody

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is the most useful of the serological tests available for surveys and for diagnostic purposes in sheep.

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