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Susceptibility of the LLC-MK_2 line of monkey kidney cells to human enteroviruses

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The isolation and identification of human enteroviruses in tissue culture is often a laborious and expensive procedure. In many laboratories the tests are made in primary or secondary monkey kidney cultures despite the disadvantages associated with the use of these tissues. Adequate supplies may be difficult to obtain when required and the maintenance of a monkey colony is expensive. A further disadvantage of primary monkey kidney cultures is the not infrequent presence of adventitious agents (Tobin, 1960). The use of a continuous line of cells eliminates these difficulties. If it is to be a satisfactory substitute, a continuous cell line should be at least as susceptible to the virus under investigation as primary cultures.

This report contains the results of experiments planned to determine whether the continuous line of LLC-MK_2 cells (Hull, Cherry & Johnson, 1956; Hull, Cherry & Tritch, 1962) would be a suitable replacement for primary rhesus monkey kidney cultures for the isolation and identification of enteroviruses. The relative susceptibility of LLC-MK_2 and primary monkey kidney cells to prototype enteroviruses before and after passage in LLC-MK_2 is shown. This is followed by the number of isolations recorded when faecal extracts were inoculated into both types of tissue. Finally, the two tissues are compared when used for the identification of enteroviruses by neutralization with specific antisera.

MATERIALS AND METHODS

Virus strains

The prototype ECHO, Coxsackie B and poliovirus strains used are listed in Table 1. With the exception of ECHO 21, which was grown in human amnion cultures, all the strains were propagated in primary rhesus monkey kidney tissue. When extensive cytopathic effect (CPE) was present the cultures were frozen and thawed, and centrifuged at about 100 *g* to remove the cell debris. The supernatant was stored at $-30°$ C. until required. These supernatant fluids were passaged twice in LLC-MK_2 cells before titrations for comparative infectivity were made. Faecal extracts from which cytopathic agents had previously been isolated were removed from storage and re-examined in LLC-MK_2 and primary

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monkey kidney tissues. Cultures, in which no CPE was present after 10 days, were regarded as negative only after further passage had failed to reveal a cytopathic agent.

Tissue cultures

Primary monkey kidney. Kidneys from freshly killed rhesus monkeys were trypsinized by a method based on that described by Rappaport (1956). Cultures were made in tubes ($4 \times \frac{1}{2}$ in.) incubated at 36–37° C. in stationary racks. Growth medium consisted of Hanks's balanced salt solution containing 0·5 % lactalbumin hydrolysate, 2 % bovine serum, 0·05 % sodium bicarbonate and antibiotics. Maintenance fluid was medium 199 containing 0·22% sodium bicarbonate and antibiotics.

LLC-MK$_2$ cells. The continuous line of LLC-MK$_2$ cells in the 285th passage was received from Dr R. N. Hull, Lilly Research Laboratories, Indianapolis. All the cultures used in this investigation were made from cells between the 298th and 328th passage. Stock cultures were grown in flat bottles on medium 199 containing 1 % horse serum, 0·15 % sodium bicarbonate and antibiotics. Subcultures were made when the cell sheet was confluent. The cells were removed from the glass by a 0·02 % solution of Sequestric acid (EDTA) and incubation at 37° C. for 15–30 min. followed by agitation. The cell suspension was centrifuged slowly and the deposit resuspended in growth medium. Each tube received 1 ml. of cell suspension containing approximately 10^5 cells. Incubation was at 36–37° C. in stationary racks. Before the cultures were inoculated, the growth medium was replaced by medium 199 containing 0·22 % sodium bicarbonate and antibiotics. Cultures were used within 4 days of preparation.

Virus titrations

Serial 0·5 log dilutions of virus suspension were prepared in maintenance medium. At each dilution 0·1 ml. volumes were transferred to four culture tubes containing 1 ml. of maintenance medium. Cultures were incubated at 36–37°C. in stationary racks and examined at intervals for CPE. Final readings were recorded after 7 days when any culture showing distinct CPE was considered to be positive for infective virus. The method of Reed & Muench (1938) was used to estimate 50 % infectivity end-points which are recorded as the log tissue culture infective dose per 0·1 ml. (log TCD 50/0·1 ml.). Logarithms are expressed to the base 10.

Virus identification

Viruses were identified in neutralization tests with specific antisera. The method employed was that described by Hambling, Davis & Macrae (1963) in which composite antiserum pools were used. Agents were typed in both LLC-MK$_2$ and primary rhesus monkey kidney cultures whenever the tissues supported sufficient virus growth to produce at least 1000 TCD 50/0·1 ml. If either tissue yielded insufficient virus, at least three passages were made before the tissue was rejected as unsuitable for use in identification of the agent.

RESULTS

Virus suspensions which had been propagated in primary rhesus monkey kidney tissue (pMK) were passaged twice in LLC-MK$_2$ cultures. Infectivity titrations of this second passage material, and also of the virus suspension before passage in LLC-MK$_2$ cells were made in both pMK and LLC-MK$_2$ cultures. Distinct cytopathic effect in LLC-MK$_2$ cultures was produced by thirty-four of the thirty-

Table 1. *Comparison of infectivity titrations of enteroviruses in primary rhesus monkey kidney (pMK) and LLC-MK$_2$ cultures before and after two passages in LLC-MK$_2$ cells*

		Titration of virus grown in pMK†		Titration of virus after two passages in LLC-MK$_2$†	
Virus*	Strain	In pMK	In LLC-MK$_2$	In pMK	In LLC-MK$_2$
ECHO 1	Farouk	6·8	6·2	6·9	6·2
ECHO 2	Cornelis	6·2	6·0	6·3	5·8
ECHO 3	Morrisey	5·6	5·8	5·4	5·9
ECHO 4	Pesascek	4·4	2·8	4·8	3·3
ECHO 5	Noyce	6·5	6·1	7·2	7·0
ECHO 6	D'Amori	6·8	6·1	6·9	6·3
ECHO 7	Wallace	5·8	5·8	6·8	6·9
ECHO 9	Hill	6·9	6·0	7·1	6·9
ECHO 11	Gregory	6·8	5·8	7·0	6·5
ECHO 12	Travis	6·7	6·1	6·3	7·0
ECHO 13	11-4D	4·2	2·9	5·3	4·8
ECHO 14	Tow	4·5	3·3	5·3	5·3
ECHO 15	Charleston	5·0	4·3	4·6	4·8
ECHO 17	CHHE-29	5·3	3·3	6·1	3·8
ECHO 19	Burke	7·3	7·3	7·0	6·5
ECHO 20	JV-1	5·5	4·9	6·1	5·9
ECHO 22	Harris	5·2	1·9	5·1	2·3
ECHO 24	DeCamp	4·7	3·6	6·3	5·2
ECHO 25	JV-4	5·7	3·8	6·2	4·6
ECHO 26	Coronel	5·3	4·7	6·8	6·1
ECHO 27	Bacon	5·7	3·0	7·1	4·3
ECHO 29	JV-10	7·0	5·3	7·5	5·7
ECHO 30	Bastianni	6·1	5·0	6·0	5·3
ECHO 31	Caldwell	5·4	1·8	5·2	2·2
ECHO 32	PR 10	5·3	4·5	6·8	5·1
Coxsackie B1	P.O.	5·8	6·2	6·2	6·4
Coxsackie B2	Ohio (Red)	5·0	4·4	7·0	7·0
Coxsackie B3	Nancy	6·2	6·3	6·9	7·0
Coxsackie B4	J.V.B.	5·5	5·8	6·5	6·3
Coxsackie B5	Faulkner	5·8	5·8	7·0	6·5
Coxsackie B6	Schmitt	5·3	6·0	6·8	7·2
Poliovirus type 1	Mahoney	6·1	5·8	7·0	6·8
Poliovirus type 2	YSK	5·8	6·2	6·2	7·0
Poliovirus type 3	Saukett	6·3	5·4	7·2	5·8

* ECHO types 16 (Harrington), 18 (Metcalf), 21 (Farina) and 23 (Williamson) did not cause distinct CPE in LLC-MK$_2$ cells.

† Expressed as log TCD50/0·1 ml.

eight enteroviruses examined; only ECHO types 16, 18, 21 and 23 failed to show this effect.

The Coxsackie B and polioviruses readily caused CPE in LLC-MK_2 cells and with the possible exception of poliovirus type 3, showed no great difference in titre when titrated in both tissues (Table 1). The poliovirus type 3 (Saukett) both before and after passage in LLC-MK_2 cells, showed higher titres in pMK than in LLC-MK_2 cultures. Further investigations are necessary to determine whether this is a real difference and due to the LLC-MK_2 cells being less susceptible than the pMK to this virus strain.

Table 2. *Isolation and identification of enteroviruses from faecal extracts in primary rhesus monkey kidney (pMK) and LLC-MK_2 cultures*

Isolates		No. isolated and identified in		
Virus and type represented	No.	pMK and LLC-MK_2	pMK only	LLC-MK_2 only
Poliovirus (types 1, 2 or 3)	11	11	—	—
Coxsackie B (types 2, 3, 4 or 5)	15	15	—	—
ECHO (types 1, 3, 6, 7, 9, 11, 13, 14, 16, 17 or 21)	57	23	33	1
Totals	83	49	33	1

Variation in infectivity titres was obtained when the ECHO viruses, propagated in pMK, were titrated in the two tissues (Table 1). Seven viruses (ECHO types 4, 17, 22, 25, 27, 29 and 31) had infective virus concentrations of at least 1·5 log greater in pMK than in LLC-MK_2 cells and this difference persisted after the agents had been passaged twice in LLC-MK_2 cultures.

Enteroviruses were isolated from eighty-three faecal extracts (Table 2). This number comprised fifty-seven members of the ECHO group (types 1, 3, 6, 7, 9, 11, 13, 14, 16, 17 or 21), fifteen Coxsackie B viruses (types 2, 3, 4 or 5) and eleven polioviruses (types 1, 2, or 3). All Coxsackie B and polioviruses were isolated in both LLC-MK_2 and pMK cultures. In contrast, only twenty-three of the fifty-seven ECHO viruses were isolated in both tissues and thirty-three caused CPE only in pMK. All the agents were typed by neutralization tests in the tissue in which they were isolated. Those which caused CPE in the two tissues were identified in both; in no instance did the results differ.

The time for distinct CPE to appear in the tissues inoculated with faecal extracts varied. In general, the ECHO viruses took longer to produce CPE in LLC-MK_2 cells than in pMK, whereas the Coxsackie B and polioviruses usually caused CPE in both tissues at about the same time.

DISCUSSION

In 1955 the continuous line of LLC-MK_2 cells was derived from a pool of cells prepared by trypsinization of kidneys from six rhesus monkeys (Hull *et al.* 1962). It has advantages over primary monkey kidney cultures in being free from

adventitious viruses, readily available, and economical in serum requirements as it is grown on a medium containing only 1 % horse serum. If it could be shown to be as susceptible to viruses as primary cell cultures it should provide an excellent replacement for them.

In this investigation thirty-eight prototype strains of human enteroviruses and eighty-three agents isolated from faecal extracts have been examined in LLC-MK$_2$ and primary rhesus monkey kidney cultures. The results indicate that for some laboratory investigations involving Coxsackie B and polioviruses LLC-MK$_2$ cells could be an adequate substitute for the primary cultures. For example, it may prove useful as the tissue culture system in tests for the estimation of neutralizing antibodies to the Coxsackie B viruses.

The prototype strains of ECHO types 16, 18, 21 and 23 failed to produce CPE in LLC-MK$_2$ cells. If other strains of these types behave similarly, this lack of CPE may serve as a preliminary indication that an unknown agent belongs to one of these four types. The LLC-MK$_2$ cell line was susceptible to the remaining prototype ECHO viruses, but suspensions of types 4, 17, 22 and 31, despite two passages, only contained low concentrations of virus when titrated in LLC-MK$_2$ cultures.

It is possible that the susceptibility of a continuous line of cells may change after subculture. In this study extensive experiments to investigate this were not made. However, the suspensions of three viruses which had been titrated in 298th or 299th passage cells were stored at $-30°$ C. and re-titrated at a later date in 321st passage cells. No significant differences in the titres were observed.

The faecal extracts examined were from specimens sent to the laboratory for virus isolation so they contained a high percentage of the agents which were prevalent at that time. This was especially noticeable in the ECHO group where there was a predominance of ECHO type 6 and to a lesser extent ECHO type 9 viruses. As many of the ECHO type 6 viruses caused CPE in pMK but not in LLC-MK$_2$ cultures, the isolation rate of ECHO viruses in LLC-MK$_2$, when ECHO type 6 is not prevalent, may well be higher than that shown in Table 2. However, as the extracts tested were from the actual specimens which the laboratory had been requested to examine, it seemed justifiable to use this material to evaluate the use of LLC-MK$_2$ cells for virus isolations.

Prime strains of ECHO type 6 virus are recognized (Melnick, 1957) and variation in the susceptibility of cultures to ECHO type 6 viruses has been reported. Pal, McQuillin & Gardner (1963) described the isolation of eighty-nine ECHO type 6 viruses in Hep 2 cells of which only thirty-seven were also isolated in primary cynomolgos monkey kidney cultures. Hsiung (1962) found that the prototype (D'Amori) ECHO type 6 virus failed to cause CPE in Hep 2 cells. During the time faecal specimens were collected, prime strains of ECHO type 6 virus were present in the population so it is possible that the ECHO type 6 viruses which were isolated in pMK but not in LLC-MK$_2$ cells fall into this category, whereas the few which cause CPE in both tissues resembled the prototype strain. This would be supported by the susceptibility of LLC-MK$_2$ cells to the prototype strain as shown in Table 1.

The identification of forty-nine enteroviruses by neutralization tests in both

pMK and LLC-MK_2 cultures showed complete agreement, so it appears that the continuous line would be of value for this purpose. It is satisfactory for the isolation of Coxsackie B and polioviruses. The variation in susceptibility to the ECHO viruses makes it advisable to use the LLC-MK_2 line in conjunction with other culture systems for maximum isolation rates.

SUMMARY

The relative susceptibility of the LLC-MK_2 cell line and primary rhesus monkey kidney cultures to thirty-eight prototype strains of human enteroviruses is described; of these strains only ECHO types 16, 18, 21 and 23 failed to cause CPE in the continuous cell line. The efficiency of the two tissues for the isolation of enteroviruses from faecal extracts is compared. The results show that the LLC-MK_2 cell line is very satisfactory for the isolation of Coxsackie B and polioviruses, but not so useful for the isolation of ECHO viruses. The identification of enteroviruses by neutralization tests in LLC-MK_2 cells is successful.

We wish to thank Dr R. N. Hull (Lilly Research Laboratories, Indianapolis) who supplied us with the LLC-MK_2 line of cells, and also the following members of the Public Health Laboratory Service; Dr A. S. Beare, Dr Joan R. Davies, Dr L. A. Hatch, Dr Hélène J. Mair, Dr G. B. Bruce White and Dr A. E. Wright who made available to us the specimens for virus isolation.

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Evolutionary changes in myxoma virus in Britain

An examination of 222 naturally occurring strains obtained from 80 counties during the period October–November 1962

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INTRODUCTION

Myxomatosis evolved in association with *Sylvilagus* in the Americas, but since 1950 it has become established as an enzootic–epizootic disease in the populations of European wild rabbits (*Oryctolagus cuniculus*) of three continental areas, Europe, Australia and Chile (Fenner & Ratcliffe, 1965). The disease was established in the wild rabbits of Australia in 1950, and in those of Europe in 1952. The first outbreak in Britain occurred at Edenbridge in Kent, in September 1953 (Armour & Thompson, 1955), and the virus (England/Kent/10–53/1 of Fenner & Marshall, 1957) was shown to have the same high virulence as the Lausanne strain used to initiate the European outbreak in France 15 months earlier.

In Australia the virus employed was the highly virulent standard laboratory strain of myxoma virus, originally recovered in Brazil by Moses (1911) and passaged for many years in laboratory rabbits. Not only was it used on a substantial scale in 1950, but each year since then the same virus (or a closely related derivative of it) has been used on a large scale in mass inoculation campaigns (Fenner & Ratcliffe, 1965). Annual examinations of naturally occurring strains of virus, obtained from many localities in Australia for the first 9 years of the epizootic (1951 until 1958–59), revealed the early occurrence in many parts of Australia, and the subsequent dominance throughout the continent, of less virulent strains (Fenner & Marshall, 1957; Marshall & Fenner, 1960).

Nowhere in Europe was deliberate inoculation of wild rabbits with highly virulent virus encouraged, and in Britain deliberate spread of the disease was made illegal in 1954. The continued occurrence of myxomatosis in Britain may be ascribed to the spread of the virus introduced into Kent in 1953. The occasional occurrence of attenuated strains (case mortality rates of 90% or less) was

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recognized in 1955 (Hudson & Mansi, 1955; Fenner & Marshall, 1955; Jacotot, Vallée & Virat, 1955), and two attenuated strains were obtained from Sussex in September and October 1954 (Fenner & Marshall, 1957). Strains recovered from the counties of Brecknockshire and Durham in 1961 were found by Chapple & Bowen (1963) to be very similar in symptomatology and virulence to the common Australian strains of the late 1950s.

However, no attempt had been made in Europe to determine the overall position as far as virus virulence or the genetic resistance of the rabbit were concerned. The former, in particular, was a matter of considerable interest, since not only was a different virus used to initiate the European outbreak, but in England the disease was transmitted by the rabbit flea and not to any extent by mosquitoes (Andrewes, Thompson & Mansi, 1959), and since inoculation of virulent virus was illegal the situation was not complicated by re-introductions.

This paper reports the results of a study of the virulence of 222 strains of myxoma virus obtained from 80 of the 85 counties of Britain during October–November 1962.

MATERIALS AND METHODS

Capture of rabbits and collection of infected tissues

Wild rabbits, killed when obviously suffering from myxomatosis, were collected by field officers of the Ministry of Agriculture, Fisheries and Food and the Department of Agriculture and Fisheries for Scotland. They were caught by a variety of methods: shooting, snares, by dog or ferret, by hand, or killed with a stick. As soon as they were caught they were placed in insect-proof linen bags and immediately dispatched to the Infestation Control Laboratory at Worplesdon. Here they were processed by one of us (P. J. C.), who weighed and sexed the rabbits, collected ectoparasites, and obtained specimens of skin lesion material, blood clot, and lung. A fragment of skin lesion material was placed in 50% glycerol-saline in a plastic tube, and stored at 4° C. until enough tubes had accumulated for dispatch by air to Canberra. Here it was processed as described below, and the resulting virus suspension was eventually tested for virulence by a standard procedure. Other portions of the skin lesion, lung and blood clot were tested for antigens and antibodies by gel diffusion tests; a procedure which had been used for some years by the Central Veterinary Laboratory, Weybridge, for the routine diagnosis of myxomatosis (Mansi & Thomas, 1958; Chapple, Bowen & Lewis, 1963).

Collection and identification of ectoparasites

All fleas and ticks were removed by combing the carcass with a fine comb. They were identified, counted and sexed by Dr A. R. Mead-Briggs and Mr R. J. C. Page at Worplesdon. Lice and mites were obtained by removing a tuft of hair, and mites were identified by Dr A. M. Hughes, Royal Free Hospital School of Medicine, London.

Gel diffusion tests

Slightly different procedures were adopted with material obtained from wild rabbits in England, and from infected laboratory rabbits in Australia. For the

former Oxoid Ionagar No. 2 (1·5 %) buffered to pH 7·2 in 0·01 M sodium barbitone–hydrochloric acid, was used. Preparation of plates, distribution of tissues and antisera in the wells, and the method of recording the results have been described by Chapple *et al.* (1963).

Sera obtained from laboratory rabbits on the eleventh or twelfth day after inoculation were tested for the presence of antibody against an antigen preparation made from skin lesion material obtained from a rabbit infected with the standard laboratory strain, and for the presence of antigen against antiserum from a rabbit which had recovered after infection with the same strain (Woodroofe & Fenner, 1965). Such antisera and antigen give precipitin lines indistinguishable from those obtained with Lausanne virus (Fenner, 1965). These tests were carried out with 0·75 % agar, buffered to pH 8·9; the cups being 4 mm. apart. They were read after 7 days incubation at 35° C. in a humidified incubator.

Preparation of virus for virulence tests

General experience (see Burnet, 1955), and our own recent investigations, have shown that, if stored or aged suspensions which contain a few viable particles and much non-infective virus are used for inoculating rabbits, they suffer a milder disease than if they are inoculated with a diluted, but freshly prepared, suspension of the same virus. This is not due to a genetic change or selection in the virus, as Burnet once suggested, but probably to a combined effect of interferon and antibody response (Fenner & Woodroofe, unpublished results).

As specimens were unavoidably kept at environmental temperatures for several days between their collection in Britain and receipt in Australia, the skin lesion material was passed once in domestic rabbits before the virus was tested for virulence. A slice of the surface of the 7-day-old skin lesion was removed, ground with sand, suspended in diluent, and stored at −60° C. A sample was thawed and assayed on the chorioallantoic membrane before use. The pock count was usually about 10^6 p.f.u. per ml.

The test for virulence

Although expensive in the numbers of rabbits used, and in space, there is no satisfactory alternative to the inoculation of rabbits for the assay of the virulence of myxoma virus for *Oryctolagus cuniculus* (Fenner & Ratcliffe, 1965). In the present series we followed the procedure outlined by Fenner & Marshall (1957). Groups of six laboratory rabbits (young adult New Zealand whites, purchased from a commercial breeder) were inoculated in the flank with 10 ID 50 (approximately 5 pock-forming units, Fenner & McIntyre, 1956) of the virus which had been passed once in domestic rabbits, as described above. High or low environmental temperatures exert a pronounced effect on the course and outcome of myxomatosis, especially with strains of reduced virulence (Marshall, 1959). To avoid this the animal rooms were heated in winter and air-conditioned in summer so that the temperature was maintained at 70° F. ± 2°.

The inoculated rabbits were observed daily, and carefully examined at weekly intervals, and the signs of myxomatosis were recorded. The primary lesion was classified as protuberant, raised or flat. The severity of the disease was assessed,

and the survival times were recorded. The mean survival time is the best index of the virulence of a strain of myxoma virus (Fenner & Marshall, 1957). Samples of virus were allocated to one of six virulence grades according to mean survival time, as shown in Table 1.

Plaque morphology

Myxoma virus produces well-defined plaques on rabbit embryo fibroblasts and rabbit kidney cells, and characteristic differences in plaque morphology have been observed with material obtained from different parts of the world (Woodroofe & Fenner, 1965). Virus obtained from the first passage skin slice was examined on monolayers of rabbit embryo fibroblasts. Methods of producing the monolayers have been described in detail elsewhere (Woodroofe & Fenner, 1965). Plaques were examined after 7 days incubation at 35° C., neutral red in agar having been added on the sixth day.

RESULTS

The complete data relating to each virus strain, and the wild rabbit from which it was recovered, are available on request. Various aspects of these data are analysed below.

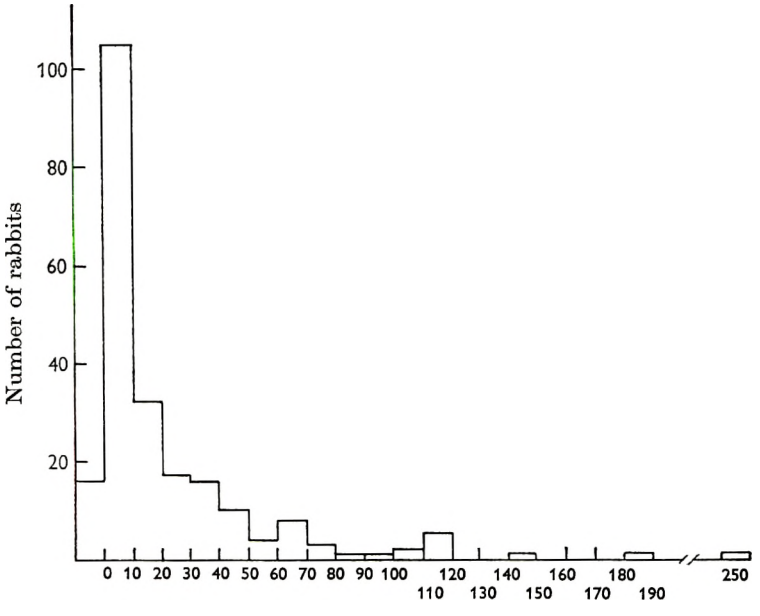

Fig. 1. Histogram showing the frequency distribution of numbers of fleas (*S. cuniculi*) per wild rabbit.

Ectoparasites found on infected wild rabbits

Only 16 (7·2 %) of the rabbit carcasses were free of rabbit fleas (*Spilopsyllus cuniculi*). The number of fleas upon each rabbit varied greatly, as shown in the histogram (Fig. 1). Female fleas were usually more common than males (in 68 % of the flea-bearing rabbits), the numbers being equal in 8 %. Scattered straggler fleas were found, sixteen rabbits being infected with thirty-six specimens of ten

different species of flea. Details of the occurrence and distribution of fleas on these rabbits are published elsewhere (Mead-Briggs, 1964*b*; Mead-Briggs & Page, 1964).

Ticks (usually nymphs of *Ixodes ricinus*) were found on fourteen rabbits, more commonly on those from Scotland. The only other ticks found were single specimens of *I. trianguliceps* and *I. hexagonus*.

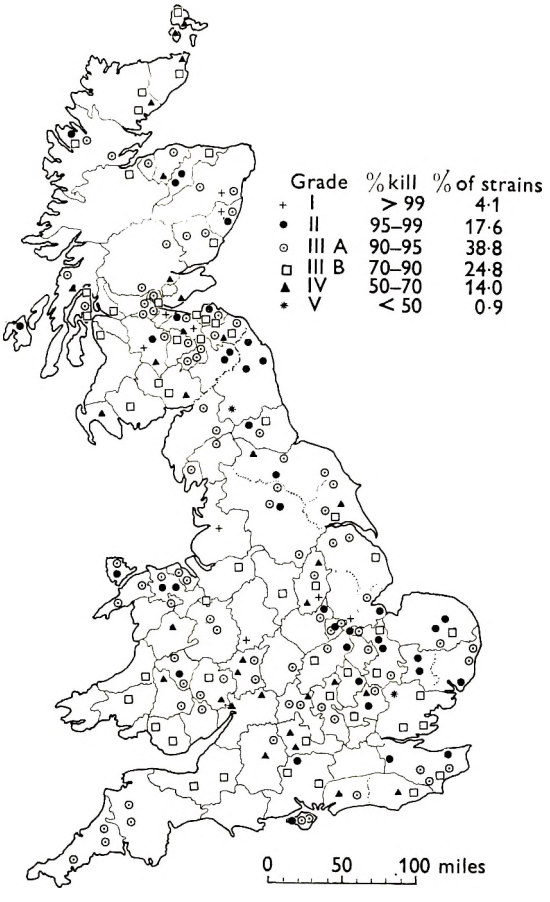

Fig. 2. Map of Britain showing county boundaries, the distribution of samples of myxoma virus collected in October–November, 1962, and their virulence as determined by rabbit inoculation tests.

Classification of virulence by rabbit inoculation tests

The detailed results of the standard inoculation tests are shown in Fig. 2, which shows the virulence and source of each strain of virus examined. The percentages of samples in each virulence grade are shown in Table 1, which also shows the relevant figures for Australia 9 years (1958–59) and thirteen years (1963–64) after the introduction of the virus into that continent. Details of both the Australian groups will be given in a forthcoming paper (Fenner & Woodroofe; to be published). In Britain, as in Australia, there are now to be found a variety of strains of myxoma virus which differ greatly in their virulence for *Oryctolagus cuniculus*.

Symptomatology of rabbits infected with British field strains

The symptomatology of myxomatosis of European rabbits produced by the Lausanne strain of myxoma virus, which was used to initiate the European epizootics, and early English derivatives of this, has been described at length (Fenner & Marshall, 1957). Like all recently recovered South American strains (Fenner, 1965) but unlike the 'standard laboratory strain', which was used in Australia, and its less virulent derivatives, the European viruses produced a florid disease with very large protuberant skin lesions. The strains France/Loiret/4–55/1 and England/Nottingham/4–55/1 (attenuated), which were designated prototype European strains for virulence grades IV and V (Fenner & Marshall, 1957), were associated with these protuberant skin lesions, although they developed this character much more slowly than the virulent strain. However, it was noted that the strain England/Sussex/10–54/1, which was of grade IIIA virulence, produced relatively flat skin lesions. Chapple & Bowen (1963) noted the same characteristics in two attenuated (grade IIIB) strains recovered in England in 1961.

Table 1. *Comparison of the virulence of naturally occurring strains of myxoma virus in Australia and Great Britain, at the time of the first epizootics and at various periods after this. Figures represent the percentages allocated to the virulence grades shown*

		Virulence grade						
		I	II	IIIA	IIIB	IV	V	
		Mean survival time (days)						
		⩽13	14–16	17–22	23–28	29–50	—	
		Case mortality rate (%)						
Country	Year	>99	95–99	90–95	70–90	50–70	<50	Sample size
Australia	1950–51	100	—	—	—	—	—	—
	1958–59*	0	24·6	29·2	26·1	14·0	6·1	130
	1963–64†	0	0	26·4	42·6	25·6	5·4	129
Great Britain	1953	100	—	—	—	—	—	—
	1962	4·1	17·6	38·8	24·8	14·0	0·9	222

* Data from Marshall & Fenner (1960), revised by Fenner & Woodroofe (unpublished).
† Data from Fenner & Woodroofe (unpublished).

In the present survey regular observations were made of the nature of the primary and secondary skin lesions in all inoculated rabbits, and all gradations were found between protuberant (like the European prototype strains of Fenner & Marshall (1957)) and flat (like the Australian prototype strains). In many cases the lesions were intermediate between these extremes, and have been designated raised. The type of response of the six inoculated rabbits was usually uniform and the nature of the primary lesion associated with each strain examined was classified as protuberant, raised or flat. In Table 2 the strains are grouped in virulence grades according to the clinical characters of the primary lesion.

There has clearly been a great change in the type of disease produced as well as in its lethality. Whereas only one of the strains tested from Europe, from collections made in the first three years after release of the virus, did not produce a

protuberant lesion, whatever its virulence, in 1962 only 20 % of all strains produced protuberant skin lesions. The majority were classified as flat, although the lesions were often more prominent than those associated with Australian strains, and a substantial proportion of strains produced large raised but not protuberant primary lesions. There was no absolute correlation between type of lesion and virulence, but most of the protuberant skin lesions were associated with more virulent strains (grades I, II and IIIA). However, more of the highly virulent strains were associated with flat skin lesions than with any other type, in striking contrast to the situation in 1953–55, when most strains recovered were of grade I virulence, and all of these were associated with protuberant lesions.

Table 2. *The relation between virulence, as judged by mean survival time, and symptomatology, as determined by the prominence of the primary lesion produced at the site of inoculation. Figures are percentages of* 222 *strains for the* 1962 *data, and the absolute numbers for* 1953–55 *data**

Type of primary lesion		Totals	Virulence grade I	II	IIIA	IIIB	IV	V
Protuberant	1953–55	17	13	0	1	0	2	1
	1962	20·4	0·4	8·4	8·9	2·7	0	0
Raised	1953–55	0						
	1962	34·7	0·9	5·3	10·7	11·6	5·8	0·4
Flat	1953–55	1	0	0	1	0	0	0
	1962	44·9	3·1	4·4	19·1	9·8	7·6	0·9

* Data for 1953–55 strains from Fenner & Marshall (1957).

The type of disease represented by the strain England/Nottingham/4–55/1 (attenuated), i.e. grade V virulence associated with very prominent lumps in the skin, was not found in the present survey, nor did any of the viruses of grade IV virulence produce this type of syndrome.

Samples from each county were deliberately obtained from different areas and different outbreaks of myxomatosis. Not enough were examined from any single county to give a detailed picture, but from the map (Fig. 2) it is apparent that strains of all types were recovered widely over the island. The only strains classified as grade V virulence came from Essex and Northumberland, and strains classed as grade I were collected in Staffordshire, Lincolnshire, Nottinghamshire, Lancashire and from Aberdeenshire, Lanarkshire, Kincardineshire, Midlothian and West Lothian in Scotland. There was a comparable general distribution of the three symptom types, flat, raised and protuberant, throughout Britain.

Gel-diffusion tests for antigen and antibody

Blood and tissues of the wild rabbits forwarded for study were tested for antigens and antibody by the gel-diffusion test, following the routine described by Mansi & Thomas (1958). In these acute cases which furnished material for virulence tests antigen was always found in the local lesion. Serum which exuded from the blood clot sometimes contained antigen, sometimes antibody, and sometimes neither. In

Table 3 the results of this test on the wild rabbits which provided the virus samples are arranged according to the results of the rabbit inoculation (virulence) tests. In this material there is clearly no indication that the results of the gel-diffusion test could have been used to predict the virulence of the virus recovered from the sample; a result which confirms the conclusions drawn by Chapple *et al.* (1963) on more limited material.

Table 3. *The results of gel diffusion precipitin tests for myxoma antigen and antibody in the blood clot of wild rabbits from which virus samples were obtained grouped according to the virulence grade of the viruses recovered from the rabbits. Figures are percentages of the tests on material from* 220 *rabbits*

Gel diffusion tests with blood showed:	Totals	Virulence grade I	II	IIIA	IIIB	IV	V
Antigen only	32·0	3·1	5·4	11·7	8·6	3·1	0
Antibody only	54·5	0·9	7·7	22·5	12·6	10·4	0·5
Neither antigen nor antibody	13·5	0	4·5	4·5	3·6	0·5	0·5

Table 4. *Correlation between the survival times and presence of antibody and/or antigen in the serum of rabbits* 11 *or* 12 *days after they were infected with* 10 *ID*50 *of some sixty European field strains. Figures express percentages of the* 332 *rabbits tested*

Serological findings	Week of disease during which rabbit died: Second	Third	Fourth	Fifth	Survivors	Total
Antigen only	9·3	38·3	8·4	3·0	0·3	59·3
Antibody only	0	1·5	3·0	0·3	0·9	5·7
Antigen and antibody	1·8	6·0	4·0	1·2	1·2	14·2
Neither antigen nor antibody	0·3	4·8	6·0	4·2	5·4	20·8

Such wild rabbits were obviously at different stages of the disease when captured. In order to obtain more uniform material for gel-diffusion tests use was made of 332 laboratory rabbits inoculated with some sixty strains of virus, for the virulence tests. Eleven or twelve days after inoculation samples of blood were taken from the ear vein, and the sera thus obtained were tested for the presence of both antigen and antibody, and the results recorded in Table 4. The results have been set out according to the survival time of the individual rabbit from which the sample was obtained. At this stage of the disease (11–12 days after infection) nearly three-quarters of the rabbits showed circulating antigen, whereas only 20 % had antibody in their serum, an equal proportion showing neither. In almost all rabbits infected with viruses of grade I or grade II virulence (i.e. those which died late in the second or early in the third week) only antigen was found in the serum, whereas in those which ultimately recovered antigen alone was found in only one of the twenty-six animals, neither antigen nor antibody being found in eighteen.

Production of pocks on the chorioallantoic membrane and plaques on rabbit embryo fibroblast monolayers

The doses used for rabbit inoculations were calculated from the results of pock counts of suspensions of infected skin. Although no particular attention was paid to pock size and morphology any deviation from the appearance usually associated with virulent Brazilian myxoma virus and its derivatives as great as that of either neuromyxoma or Nottingham attenuated (Fenner & Marshall, 1957) would have been noticed. Nothing of this sort was recorded.

All tissue suspensions were also assayed on rabbit embryo fibroblasts, on which all strains of myxoma virus produce well-defined plaques with rather irregular edges (Woodroofe & Fenner, 1965). In all cases the plaque assay was the same as the pock count or slightly higher. Although there was a good deal of variability in the size of plaques on single plates four different types of plaque could be distinguished; large, medium and small clear plaques, all of which had irregular edges, and medium-sized hazy plaques. There was no correlation between plaque type and virulence.

DISCUSSION

This survey shows that in Britain, as in Australia, a variety of strains of myxoma virus now circulate naturally in the wild rabbit population. These differ from one another in several properties: their virulence (lethality) for *Oryctolagus*, the type of lesion they produce in this host, and the type of plaque they produce on rabbit cell monolayers, to list three that have been examined.

Geographically, strains of differing virulence and lesion type are scattered widely throughout Britain, even among these samples, which were collected over a restricted period of time.

The situation in Britain, in regard to myxomatosis, differs in three important respects from that in Australia. There was probably only one introduction of virus, that which started the Bough Beech epizootic in 1953. This strain was indistinguishable from Lausanne, being highly lethal (grade I) and associated with protuberant skin lesions. In Australia the standard laboratory strain produced less prominent lesions, and it has been introduced annually on a large scale during mass inoculation campaigns. The major vector in Britain is the rabbit flea, and not the mosquito, as in Australia.

In spite of these epidemiological differences, moderately attenuated strains of virus are now almost as common in Britain as they were in Australia in 1958–59, although during the last 5 years the level of virulence in Australia has dropped even further, for no grade I or grade II strains have so far been recovered in the 1963–64 tests (Table 1). There are two important contrasts between the situations in Britain and Australia, 9 years after the introductions; strains of grade I virulence were still to be found in Britain, and strains of grade V virulence were less common, in spite of the early appearance of such viruses (exemplified by England/Nottingham/4–55/1 (attenuated)). It has been predicted (Fenner & Marshall, 1957; Andrewes *et al.* 1959) that attenuated viruses would be strongly selected against, if fleas are the major vectors and only leave rabbits when they

die. To some extent this may have happened, for highly virulent strains have persisted in a way not found in Australia, in spite of their annual reintroduction there. However, recent work has shown that the rabbit flea is much less sedentary than was previously believed (Mead-Briggs, 1964*a*; Rothschild, unpublished observations) although not as mobile as mosquitoes, which may help to explain the common occurrence of moderately attenuated strains found in the present survey.

Flea transmission occurs throughout the year, and myxomatosis in Britain does not show the same pronounced seasonal incidences as it does in Australia. During the relatively cold British winter moderately attenuated strains would often cause lethal infections (Marshall, 1959), which could be expected to promote their spread by leading to a dissemination of infected fleas. But the emergence of attenuated strains cannot be explained thus, and must be due to factors in the host–virus balance of which we have little understanding.

The virus introduced into Britain in 1953 was indistinguishable from that introduced into France in June 1952, and produced a disease characterized by very florid symptoms and protuberant skin lesions. In October 1954 a strain of virus was recovered in Sussex which produced flatter skin lesions, not unlike those found in rabbits inoculated with the standard laboratory strain and Australian field derivatives of this. This type of lesion has now become somewhat more common than the original disease with protuberant lesions. In neither of the original situations was the type of lesion correlated with virulence, for all Australian strains produce flat primary skin lesions and the early European strains, which produced protuberant skin lesions, ranged in virulence from grade I to grade V. The data recorded in Table 2 show that in the material examined in this survey there is no correlation between type of lesion and lethality.

Elsewhere it has been shown (Woodroofe & Fenner, 1965) that a plaque mutant of the Lausanne virus obtained from a stock preparation of that virus produced a disease of slightly lower virulence (grade II instead of grade I), which was characterized by raised, and not protuberant, skin lesions. The capacity to produce particular types of lesion, like the virulence, may be controlled by many genes; and because of covariation even single mutational steps may lead to changes in both characters.

SUMMARY

Samples of lesions containing viable myxoma virus were collected from 222 infected wild rabbits captured in 80 counties of Britain during October–November 1962. They were dispatched by air to Australia and passaged once in domestic rabbits before being tested for their virulence by the intradermal inoculation of groups of six laboratory rabbits with small doses of virus.

The results showed that a wide range of viruses of differing properties now coexist in Britain. Their virulence ranges from very high (99% lethal) to low (about 50%), but the majority of strains fall into the grade III virulence group, with estimated case mortality rates varying between 70 and 95%. The viruses also produce disease of varying symptomatology, skin lesions being very prominent, raised or flat. There was no obvious association between lesion type and virulence.

Tests made on the wild rabbits from which the samples were obtained, and on

inoculated domestic rabbits, showed that virulence could not be predicted by gel-diffusion tests.

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An aetiological study of respiratory infection in children, Edinburgh City Hospital, 1961–1963

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The aetiology of acute respiratory infection in young children is still not fully understood and numerous combined clinical and laboratory investigations have been carried out in an attempt to elucidate this problem (Morrison *et al.* 1957; Gardner *et al.* 1960; Holland, Tanner, Pereira & Taylor, 1960; Garrow & Taylor, 1962; Tobin, 1963; Clarke *et al.* 1964). No such study had been carried out in Edinburgh and a fact-finding survey was therefore planned to provide a picture of the bacterial and viral flora occurring in children admitted to hospital with respiratory infection. In this survey, no effort was made to examine a control group of children but it was hoped that by investigating *all* cases of respiratory illness certain aetiological patterns might be found to be associated with certain syndromes. Although the sera were tested for antibodies to respiratory syncytial virus no particular attempt was made to isolate this virus; optimum conditions for isolation of the common cold viruses were likewise not provided. The part played by these viruses may be considerable and must not be overlooked when examining the present results.

MATERIALS AND METHODS

The investigations were carried out during two winter periods; (1) from 1 October 1961 to 30 April 1962; (2) from 1 November 1962 to 31 May 1963. The procedures adopted during the first period are described below; alterations to the protocol made during the second period are listed separately.

Clinical cases and procedures

All children 12 years of age and under admitted to Edinburgh City Hospital with acute respiratory infection were included in the survey. All patients were examined clinically and classified into the following disease categories:

(*a*) upper respiratory tract infection (URTI) which included all coryza-like illness and conjunctivitis, pharyngitis and stomatitis;

(*b*) bronchitis, which included croup;

(*c*) pneumonia which was confirmed radiologically;

(*d*) whooping cough, which was a clinical diagnosis and included complicating bronchitis or pneumonia.

On admission two cough swabs were taken by passing the swab through the

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mouth to the posterior pharyngeal area; one of the swabs was sent to the laboratory for bacteriological examination while the other was immersed immediately in 3 ml. transport medium for virus isolation. A specimen of venous blood and a faecal specimen were also collected for virus studies and in cases of whooping cough a pernasal swab was examined bacteriologically. Ten to fourteen days after onset of the illness a second blood specimen was taken for investigation of virus antibodies.

Bacteriological procedures

The cough swabs were serum-coated according to the method of Rubbo & Benjamin (1951). In all cases the cough swab was cultured on blood agar and on heated blood agar and a direct smear was Gram-stained. The pernasal swab was inoculated on to Bordet–Gengou medium. Sera from cases of whooping cough were examined for antibodies to *Bordetella pertussis* by the tube method (Evans & Maitland, 1939).

Virological procedures

Specimens for virus isolation were transported to the laboratory without delay and stored at $-70°$ C. until cell cultures were available; the transport medium consisted of 2 % inactivated horse serum in Hanks's solution and contained penicillin (100 units per ml.) and streptomycin (100 μg./ml.). Serum samples were stored at $-25°$ C. All attempts to isolate virus from throat swabs and faecal specimens were made in secondary cultures of monkey kidney cells and in Hep-2 cells. The maintenance medium for all tissue cultures was medium 199 with 2 % calf serum and in all cases penicillin and streptomycin were included in the medium. Tubes were inoculated with 0·2 ml. of the specimen and incubated in roller drums at 35° C.; the cells were examined microscopically for cytopathic effect every 2 days for 18 days. Haemadsorption tests using 0·4 % human group 'O' erythrocytes were carried out on all monkey kidney cultures inoculated with throat swabs 10 days after inoculation. Throat swab specimens were also inoculated into the amniotic cavity of 11-day chick embryos; the eggs were incubated for 2 days at 35° C. and the amniotic fluids tested for haemagglutination. A further passage was then carried out.

Complement fixation tests were carried out according to the technique described by Bradstreet & Taylor (1962). The sera were screened at a dilution of 1/16 against the following antigens: influenza A, B and C, Sendai, para-influenza 1, 2, 3, adenovirus, mumps S and V and respiratory syncytial (RS) virus. All complement fixing reagents except RS were obtained from the Standards Laboratory for Serological Reagents, Central Public Health Laboratory, Colindale. The RS antigen was prepared in monkey kidney cells grown on serum-free medium from the Randall strain of virus previously passaged in Hep-2 cells.

Neutralization tests were carried out on patients' serum against any virus isolated from that patient. The virus, diluted to contain 100 TCD 50 per 0·2 ml., was mixed with 0·25 ml. amounts of twofold dilutions of serum. The mixtures were allowed to stand for an hour at room temperature and then 0·2 ml. amounts were inoculated into each of two tubes of the appropriate tissue culture. The neutralizing

titre was taken as the highest dilution showing inhibition of the virus when complete degeneration of the cells was evident in the tubes inoculated with virus alone.

Identification of virus strains was carried out by neutralization tests using the following antisera: poliovirus 1, 2, 3, coxsackie B 1–6, A 7, A 9, echo 1–10, 12, 14, 15, 16, 18, 19, 20, 22, 25, 26, adenovirus 1–11, 14, 15, 16, para-influenza 1, 2, 3, herpes simplex. Unidentified viruses were inoculated into litters of unweaned mice to aid identification; the mice were inoculated by the intracerebral and intraperitoneal route and examined for 21 days for signs of paralysis.

Alterations during winter 1962–63

The following changes were made to the above procedures:

(1) the investigations were limited to children of 6 years or under because of the poor isolation rate in those over 6 during the first year;

(2) a nasal swab was examined bacteriologically in an attempt to improve the isolation rate of pneumococci;

(3) the monkey kidney cells were maintained on serum-free medium, i.e. 7·5 % liver digest (Burroughs Wellcome) in Earle's solution (Smith, 1961);

(4) throat swabs were not inoculated into chick embryos as it was considered that most influenza infections would be detected in monkey kidney cells and by the complement fixation test.

RESULTS

During 1961–62, 131 children were included in the survey; of these, 73 % were admitted to hospital during the 3 months October to December. Over the winter of 1962–63, 133 patients were examined; these cases were distributed more evenly throughout the period of study. Approximately a quarter of the patients occurred in each of the four age groups, 0–1 years, 1–2 years, 2–3 years, and over 3 years. Because of the exclusion of children over 6 years of age in the second winter and because 50 % of the children in the oldest age group in the first winter were over 6 it is evident that there were more younger cases in 1962–63.

Distribution of disease categories

The distribution of disease categories throughout the periods surveyed is shown in Fig. 1. During the first winter each disease category was quite evenly distributed throughout the whole winter; 37·4 % of the cases were in the URTI group and 29·8 % in the pneumonia group, while bronchitis and whooping cough were responsible for 16·0 and 16·8 %, respectively. In the second winter pneumonia occurred mainly during the early part of the winter and whooping cough during the latter half. In contrast to the previous year only 29·3 % of cases were in the URTI group; 32·3 % were in the pneumonia group; and bronchitis and whooping cough were again least encountered and were responsible for 18·1 and 20·3 % of the cases. The disease categories were distributed evenly throughout the age groups.

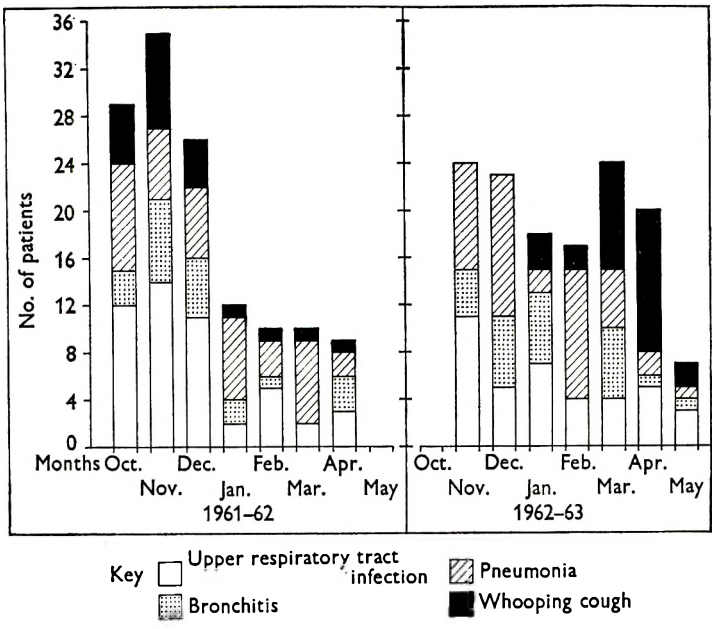

Fig. 1. The distribution of disease categories thoughout the two periods of study.

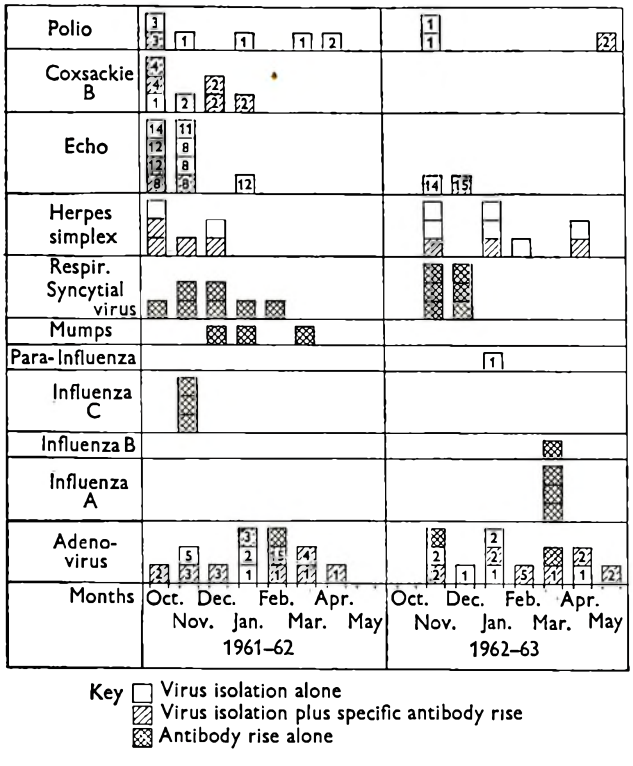

Fig. 2. The seasonal incidence of virus throughout the two periods of study. Numbers indicate type of virus isolated.

Incidence of virus

The viruses encountered over the two winters are shown in Fig. 2. The percentage incidence of virus during the two periods of study was 41·2 and 28·6 %, respectively. This percentage includes viral isolation with or without antibody rise as well as a fourfold or greater rise in antibody *without* isolation of the virus. Excluded from these figures are twenty-three unidentified viruses isolated over both winters; twenty-two of these were isolated from faecal specimens alone and twenty-one grew only in Hep-2 cells; none was pathogenic for unweaned mice. The general pattern of virus incidence was similar in both periods apart from a difference in the number of enterovirus isolations; these formed 40·7 % of the total viruses in 1961–62, but only 13·2 % in 1962–63; this reduction was due to the complete absence of coxsackie B viruses and a decrease in the number of echo viruses in the second winter. There was no evidence of influenza C or of adenovirus types 3, 4, or 15 in 1962–63. The enteroviruses, apart from poliovirus, were encountered early in the winter in both surveys; influenza A and influenza C infections were also demonstrated within a relatively short period of time as was RS in the second winter. The adenoviruses were more evenly distributed throughout the survey period.

More than one virus was isolated from a patient in eleven cases as follows: adenovirus with mumps, influenza B, influenza C and para-influenza virus respectively in four cases, RS with echo, herpes simplex and adenovirus respectively in three cases, herpes simplex with influenza C and an unidentified virus in two cases; echo 14 and coxsackie B1 occurred together once and two apparently different unidentified viruses were isolated from the same patient in one case.

Relation of virus incidence to disease category

The incidence of virus within the disease categories is shown in Fig. 3. The percentage of patients in each group with evidence of virus can be seen in the first part of Table 1.

Of twenty-three adenovirus isolations ten were from patients in the URTI group, all of whom showed an antibody rise to the virus isolated; one of these cases had associated splenomegaly. Two patients in this group showed a rise in antibody but no virus was isolated. Two cases of pharyngoconjunctival fever were diagnosed and were associated with the isolation of adenovirus type 3; both patients were under 2 years old. The other types encountered in the URTI group were types 1, 2, 4 and 15. Half the patients with URTI had associated diarrhoea. There were three adenovirus isolations (types 1 and 2) in the bronchitis group, which included one patient with croup, but no antibody response occurred with these isolations. Adenovirus types 1, 2, and 5 were isolated from children with pneumonia and from those with whooping cough. The two patients in the whooping cough group who produced antibody rises both had bronchitis; the three whooping cough cases who did not show an antibody rise to the adenoviruses isolated had pneumonia as a complication. Of the twenty-six cases with evidence of adenovirus infection or carriage, 70 % were under 2 years of age.

The three cases of influenza A which occurred in March 1963 were associated in time with eleven adult admissions of influenza and were distributed throughout the age groups. Only one case of para-influenza virus infection was diagnosed virologically; it occurred in a child aged one year with uncomplicated croup.

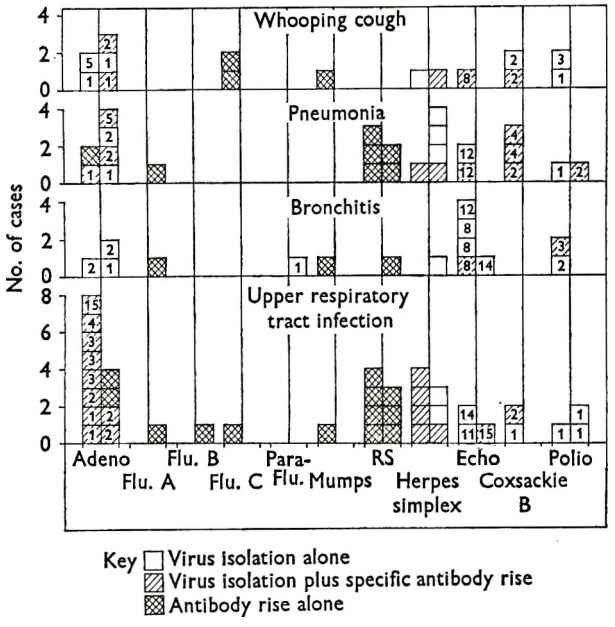

Fig. 3. The incidence of virus within the defined disease categories. 1961–62, left side of column. 1962–63, right side of column. Numbers indicate type of virus isolated. Total patients in each disease category: URTI 89; bronchitis 45; pneumonia 82; whooping cough 49.

Table 1. *The relation between virus isolation and serological response*

	URTI		Bronchitis		Pneumonia		Whooping cough	
	1961–62	1962–63	1961–62	1962–63	1961–62	1962–63	1961–62	1962–63
Percentage patients in each disease category with evidence of virus*	47	39	38	29	30	28	52	14
Percentage patients in each disease category with antibody rise	38·8	18	14·3	8·3	25·7	16·3	22·7	11
Ratio of antibody rise to virus isolation†	13/15	4/6	2/5	0/0	6/7	4/6	2/7	3/4

* Including virus isolation with or without antibody rise as well as a fourfold or greater rise in antibody *without* isolation of virus. Unidentified viruses are excluded.

† Excluding those patients from whom serum was not available and those with antibody rise with no virus isolation.

Thirteen cases of respiratory syncytial (RS) virus infection were diagnosed serologically. Seven of these had mild URTI, five had pneumonia and one had bronchitis. All the cases except one were over the age of 1 year and half of them were over the age of two.

Herpes simplex was isolated from fifteen patients. Of these, three cases were diagnosed as aphthous stomatitis, one as bilateral conjunctivitis and one as a case of pneumonia which also had facial herpes; these five cases had an associated antibody rise to herpes simplex. The remaining ten cases which were distributed throughout the disease categories showed no clinical evidence of herpes simplex infection although three of them had an associated antibody rise.

Three of the four isolations of echovirus type 8 were made from children admitted from the same day nursery with clinical bacillary dysentery; their ages were 14 months, 2 years and 3 years. All of these children had associated bronchitis and no bacterial pathogens were isolated from their faeces; only one of the children, however, showed an antibody rise. The remaining echo 8 was isolated from a child of 15 months with whooping cough and bronchitis and was accompanied by a rise in antibody. A specific antibody rise was also detected in a case of pneumonia from whom echovirus type 12 was isolated and in a case of URTI with cough from whom echovirus type 15 was isolated. The remaining five echovirus isolations, which included types 11, 12 and 14, were not accompanied by a rise in antibody and occurred throughout the disease categories, showing no special features.

Coxsackie virus type B 2 was isolated from four children, three of whom showed a serological rise. Of these three, one child had a mild aseptic meningitis with URTI, a second had pneumonia with rash and diarrhoea and the third had whooping cough pneumonia. The remaining type 2 virus was isolated from a patient with whooping cough bronchitis. Two B 4 viruses were isolated in the pneumonia group; only one of these produced an antibody rise. One type 1 virus was isolated from a child with uncomplicated URTI; there was no antibody rise.

In only two of the nine cases from whom poliovirus was isolated was an antibody rise detected. One of these occurred in a case of 'croup' from whom poliovirus type 3 was isolated and the other in a pneumonia case with associated diarrhoea. Of the isolations occurring without antibody rise only in one case was a definite history of recent vaccination obtained. This child had bronchitis with diarrhoea. No central nervous system complications occurred in any of the cases with poliovirus isolations.

No special clinical features were associated with the isolation of the unidentified viruses.

Antibody response

Over both winters the percentage of patients with a fourfold or greater antibody response was 28·2 and 17·3 %, respectively, giving an average response of 23 %. In Table 1 the relation between virus isolation and serological response can be seen; the relationship is expressed as a ratio of fourfold antibody rise to virus isolation. From the table it is evident that the antibody response was best in the URTI and pneumonia groups and was variable in the whooping cough and

bronchitis groups. The antibody response in the different age groups was also expressed as a ratio of antibody rise to virus isolation and the response was found to be similar throughout the age groups.

During the course of the survey it was noted that when the same virus was isolated from both throat swab and faeces a fourfold or greater rise in antibody to that virus was always associated. In contrast to this only 32 % of viruses isolated from the throat alone and 17 % of the viruses isolated from faeces alone gave a fourfold or greater antibody rise.

Table 2. *The isolation of potential bacterial pathogens within the disease categories*

	URTI		Bronchitis		Pneumonia		Whooping cough		Total
	1961–62	1962–63	1961–62	1962–63	1961–62	1962–63	1961–62	1962–63	
Staphylococcus pyogenes	15	5	5	9	6	13	4	4	61
Pneumococcus	0	5	0	1	0	3	0	4	13
Bordetella pertussis	—	—	—	—	—	—	2	0	2
Haemophilus influenzae	2	0	1	0	1	0	1	0	5
Escherichia coli	5	3	0	3	3	1	3	1	19
Monilia	0	1	0	0	0	1	1	0	3
Pneumococcus plus other bacterial pathogens	0	4	0	2	0	1	0	1	8
Other double bacterial pathogen isolations	0	2	0	0	0	0	0	1	3
Total patients with pathogenic bacteria	22	20	6	15	10	19	11	11	114
Total patients	49	39	21	24	39	43	22	27	264
Percentage with pathogenic bacteria	45	51	29	63	26	44	50	41	43

Bacteriological findings

Potential bacterial pathogens were isolated from 37 % of patients in 1961–62 and from 49 % in 1962–63. These findings are summarized in Table 2, where it may be seen that *Escherichia coli* was included as a potential pathogen. During the first winter, isolations were highest in the whooping cough and URTI groups; isolations in 1962–63 were highest in the URTI and bronchitis groups.

The commonest potential bacterial pathogen isolated was *Staphylococcus pyogenes*. No pneumococci were isolated in 1961–62 but the examination of a nasal swab during the second winter gave isolations of pneumococci in 15 % of cases. *Bordetella pertussis* was isolated twice in 1961–62 but not at all in 1962–63. Agglutinating antibodies to *B. pertussis* were investigated in twenty-one patients during 1961–62 and in nineteen patients during 1962–63. Of the forty patients, eight had

no detectable antibody at 1/30, twenty-five had stable titres of 1/30 or 1/60, four had stable titres of 1/120 or higher, two patients had a fourfold antibody rise and one had a fourfold fall in titre. Seven of the children tested had been immunized; five had stable titres at 1/30, two had stable titres at 1/60. Of the forty-nine cases of whooping cough examined during the survey only nine (18%) had been immunized.

There was no significant difference in the isolation rate of bacterial pathogens between those patients who had received antimicrobial drugs before admission and those who had not. It is worthy of note that half the children from whom *Esch. coli* was isolated had *not* had previous chemotherapy.

Summary of laboratory findings

The combined bacteriological and virological results obtained during the survey are summarized in Table 3. Approximately one third (36%) of the cases had no known aetiology and a further 6% had viruses alone without any serological rise, indicating doubtful aetiology. Serological evidence of virus infection with or without bacteria was shown in 22%, and 29% had bacterial pathogens alone.

Table 3. *Incidence of bacterial and viral infections during the survey*

	B	VI	VS	B+VI	B+VS	Nil	Total
1961–62	32	11	26	7	10	45	131
1962–63	45	6	12	9	11	50	133
Total both winters	77	17	38	16	21	95	264
Percentage both winters	29·2	6·4	14·4	6·0	8·0	36·0	—

B = Bacterial pathogens alone.
VI = Virus isolation alone.
VS = Serological evidence of virus with or without virus isolation.

DISCUSSION

The proportion of respiratory illnesses associated with known viral agents has been found to be high in infancy and childhood (Chanock & Johnson, 1961) and the figures presented in this paper are comparable with those from other surveys (Gardner *et al.* 1960; Hilleman *et al.* 1962; Sterner & Tunevall, 1962). The aetiological structure of respiratory illness in a 'non-epidemic' period is also known to be complex (Sterner & Tunevall, 1962); this is borne out here by the fact that, with the exception of the possible association between upper respiratory tract infection and adenoviruses, no specific virus or virus group was associated with any particular disease category.

Antibody studies are essential to differentiate between virus carriage and virus infection; infection is defined here as a fourfold or greater rise in antibody whether or not virus has been isolated. We have probably underestimated the proportion of virus isolations which were infections in the true sense. The relatively low figure of 23% may have been due to the fact that insufficient time was allowed between acute and convalescent serum specimens for antibody rise to take place or that convalescent specimens could not be obtained, and in some cases a long period of illness before admission to hospital may have made it impossible to

demonstrate an antibody rise. The latter probably accounts for the apparently poor viral antibody response in the whooping cough group; that most of these cases had had experience with *B. pertussis* is borne out by the agglutination studies, in which 32 out of 40 (80 %) cases were found to possess antibodies, whereas only a very small proportion (18 %) had been artificially immunized with this antigen. The viral infections which did occur in this condition were probably super-infections although we cannot exclude the possible aetiological role of viruses in clinical whooping cough (Goodpasture, Auerbach, Swanson & Cotter, 1939; Chany *et al.* 1958; Farber & Vawter, 1961; Olsen, Miller & Hanshaw, 1964).

The endemic types of adenovirus, types 1, 2 and 5, formed the bulk of our adenovirus isolations, whereas members of the so-called pathogenic group of adeno-viruses, types 3, 4, 7 and 14, occurred in our series only during the first winter. Two of the three cases of adenovirus type 3 infection showed the characteristic syndrome of pharyngo-conjunctival fever, an association which is well documented (Bell *et al.* 1955; Kjellén, Zetterberg & Svedmyr, 1957; Huebner, Rowe & Chanock, 1958). The single isolation of adenovirus type 15 was found in association with upper respiratory tract infection. An antibody rise accompanied 60 % of our adenovirus isolations. These were therefore considered to be infections; of these 67 % occurred with upper respiratory tract syndromes and 20 % in pneumonia. We conclude that although 10 % of our total population had evidence of adeno-virus carriage 7 % actually had virus infection (see Fig. 3); the majority of the viruses were of the endemic type and were probably the cause of respiratory disease predominantly of the upper respiratory tract.

The myxovirus group, which includes the influenza and para-influenza viruses, mumps and possibly respiratory syncytial virus, are all regarded as pathogenic, and evidence of myxovirus infection was found in 9 % of our population, this being the highest infection rate for the groups of virus encountered. Influenza A infection occurred during a localized outbreak in March 1963 when eleven adult cases were diagnosed in our hospital. All the influenza infections encountered occurred throughout the disease categories, two cases of influenza C occurring in the whooping cough group. The single para-influenza infection was associated with croup; this association is now well established (Parrott *et al.* 1962). Two of our three mumps infections were cases of clinical mumps and were included in the survey on account of associated respiratory infection. One of these carried adeno-virus type 2 while the remaining mumps infection was subclinical.

Respiratory syncytial virus has been established as a major cause of respiratory illness, especially of lower respiratory tract infection in infants and children (Chanock & Finberg, 1957; Forbes, Bennett & Gray, 1961; Chanock *et al.* 1962; Hilleman *et al.* 1962). Our apparently low figure of 5 % is probably an under-estimate because of the insensitivity of our serological technique, in which we used only one unit of antigen. Furthermore, no particular attempt was made to isolate the virus. In a recent study of an outbreak of acute bronchiolitis in Sunderland only 50 % gave unequivocal serological evidence of infection with RS virus, pre-sumably because of the inability of the younger children to produce detectable antibodies (Crone, Heycock, Noble & Patton, 1964).

Herpes simplex virus was found in 6 % of our cases and half of these had virus infections. Four of these cases showed a characteristic illness and one other case had facial herpes; it is unlikely that the remaining ten isolations were aetiologically concerned with the associated respiratory illness.

Enteroviruses were found in 10 % of our population and formed the largest viral group encountered. It has been shown by many workers (Walton & Melnick, 1953; Moffet & Cramblett, 1962; Hilleman, 1963) that, during a period of increased prevalence of coxsackie B virus, a variable proportion of minor illness (3–14 %), not infrequently mild respiratory illness, is due to this virus group. Coxsackie B viruses occurred in 7 (3 %) of our cases; five of these represented infection and occurred throughout the disease categories. Certain types of echoviruses, notably types 11, 20 and 28, have been established as causing respiratory illness, usually of a mild type. Of these types only one type 11 was encountered, associated with upper respiratory tract infection: there was no antibody response. The three cases of enteric–respiratory disease from a children's nursery with echo 8 isolations compare with a similar outbreak described by Rosen, Johnson, Huebner & Bell in 1958. The remaining echovirus types in our series, 12, 14 and 15, have not previously been incriminated in respiratory tract disease. Echoviruses were isolated from 11 (4 %) patients, but in only four of these was there a rise in antibody. We conclude that, although coxsackie B virus, when present, may account for a small proportion of respiratory illness, the other enteroviruses did not seem to play an important role in our population. Only one enterovirus, a coxsackie B 2 virus, was associated with clinical involvement of the central nervous system.

Bacterial pathogens were isolated from half of our total cases but no great emphasis can be put on the presence of pathogens in the throat (Rabe, 1948). It is of interest that, in accordance with the findings of Masters, Brumfitt, Mendez & Likar (1958), the isolation of pneumococci was increased in the second year when nasal swabs were examined. The low isolation rate of *B. pertussis* in whooping cough may be related to the long duration of illness before admission to hospital.

Although bacteria or viruses could be implicated in approximately two-thirds of the cases in this survey, the remaining patients gave negative results. This again emphasizes the need for further investigations in this field, bearing in mind the possible significance of such virus groups as the rhinoviruses. It seems inevitable, however, that the aetiology of a proportion of cases will always be unknown because of the poorer antibody response in the youngest age group; it is therefore important that more should be known of the pathogenesis of the virus groups so that the significance of their presence in disease may be better understood even in the absence of antibody response.

SUMMARY

The findings are described of a combined clinical, bacteriological and virological study which included all children admitted to the City Hospital, Edinburgh, with acute respiratory infection and whooping cough during the winters 1961–62 and 1962–63. During the first winter 131 cases aged 0–12 years and in the second winter 133 aged 0–6 years were examined. The respiratory illnesses were divisible

into upper respiratory tract infection, bronchitis, pneumonia, and whooping cough; many of the cases of whooping cough had respiratory complications with bronchitis or pneumonia.

Paired sera, a throat swab and a faecal specimen were taken from each child and investigated virologically. Over both winters the highest total virus isolation rate was found in the group suffering from upper respiratory disease. Approximately two-thirds of the total number of patients from whom virus was isolated and from whom both acute and convalescent sera were available gave a serological response to the homologous virus; the highest proportion of these patients occurred in the pneumonia and URTI groups. The groups of viruses associated with a fourfold or greater rise in antibodies occurred in the following proportions of the cases: myxovirus 9 %; adenovirus 7 %; enterovirus 4 %; herpes simplex 3 %.

Bacterial pathogens were isolated from 37 % of patients in 1961–62 and from 49 % in 1962–63, *Staph. pyogenes* being the most common pathogen. Isolation of pneumococci was facilitated during the second year by the examination of a nasal swab. Pre-admission chemotherapy did not significantly alter the bacterial isolation rates. Agglutination studies were carried out on forty clinical cases of whooping cough admitted during the two winters and thirty-two showed significant stable titres to *Bordetella pertussis*; only 9 (18 %) of these cases gave a history of prophylactic immunization.

A third of the patients had neither bacterial nor viral pathogens.

The findings in this survey illustrate the need for further intensive virological and bacteriological studies of acute respiratory infections in early childhood.

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Estimating titres and their approximate standard errors in complement fixation tests

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INTRODUCTION

In the complement fixation test indirect measures are obtained of an antigen-antibody reaction. Each test comprises a three-dimensional array of reaction mixtures, the three variables being antigen, antiserum and complement. The different levels of each variable are equally spaced on a logarithmic scale, except that one of the levels is the zero level, with none of the variable present. For a full account of the test the reader is referred to Fulton (1958), who describes the calculation of antigen and antiserum maxima titres but notes that, to judge the significance of a difference between two titres, it is usual to set arbitrary conservative limits to the estimates in view of the difficulty of obtaining their variances. The main purpose of this paper is to give formulae whereby these variances may be approximately calculated in a relatively simple fashion, and to give an appropriate method for the comparison of the titres of two antisera (or antigens) when they react homologously.

METHOD AND RESULTS

(1) *The amount of complement fixed*

The amount of complement fixed at any particular level of antigen and antiserum is measured indirectly by the amount required so that enough is left over, after fixation, to cause 50 % haemolysis in a standard indicator system of sensitized red blood cells. Let the true amount required be ζ. Then Fulton (1958) suggests the following method to obtain an estimate z of ζ. If we look at the reaction mixtures corresponding to the different levels of complement, we find, for an appropriate indicator system, that the transition from no lysis to complete lysis is so rapid that only one of two effects can be observed. Either the reaction mixtures can be divided into two sets, one set appearing to have all the indicator cells lysed and the other no indicator cells lysed; or else just one reaction mixture shows partial lysis of the indicator cells. If the former, we take z to be the geometric mean of the levels of complement at the transition point; if the latter, we take z to be the level of complement that shows partial lysis.

Let the spacing between successive levels of complement be h when logarithms to base 10 are taken, i.e. if c is the constant dilution factor $h = \log_{10} c$. Let $y = \log_{10} z$ and $\eta = \log_{10} \zeta$. Thus any estimate y of η is either one of the log levels of

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complement used or an average of two consecutive log levels of complement used. Suppose that of a large number of estimates obtained in this way θ_1 is the proportion that are log levels of complement used, i.e. that are determined by a partial lysis. Then, in any interval h that can give rise to an estimate, a range $\theta_1 h$ would give rise to an estimate of this type. Thus it is reasonable to assume that if a log level of complement used falls in the interval $(\eta - \theta_1 h/2,\ \eta + \theta_1 h/2)$ then that log level will be taken as the estimate y. We can therefore consider such an estimate y to be a random variable uniformly distributed on the interval $(\eta - \theta_1 h/2,\ \eta + \theta_1 h/2)$, with the result that it is an unbiased estimate of η and has a variance of $\theta_1^2 h^2/12$. Similarly any estimate y that is the average of two log complement levels used can be considered to be a random variable uniformly distributed on the interval $(\eta - (1-\theta_1)\frac{1}{2}h,\ \eta + (1-\theta_1)\frac{1}{2}h)$, with the result that it is again an unbiased estimate of η but in this case the variance of the estimate is $(1-\theta_1)^2 h^2/12$. Expressing the variance of z by var (z) we have

$$\text{var}\ (z) \doteqdot z^2\ \text{var}\ (\log_e z) = (z \log_e 10)^2\ \text{var}\ (\log_{10} z) = (z \log_e 10)^2\ \text{var}\ (y).$$

Thus

$$\text{var}\ (z) \doteqdot (zth \log_e 10)^2/12, \tag{1}$$

where
$$t = \left\{ \begin{array}{l} \theta_1 \text{ if } z \text{ is a level of complement used,} \\ 1-\theta_1 \text{ if } z \text{ is a geometric mean of two} \\ \qquad \text{levels of complement used.} \end{array} \right\} \tag{2}$$

Now with some indicator systems the transition from no lysis to complete lysis is not so rapid, and the reaction mixtures can be classified into more states than the three simple ones of 'no lysis,' 'partial lysis' and 'complete lysis'. In such cases the variances of the estimates z, and so also the variance of the final estimate of the titre, can be materially reduced by making use of the extra information available. To illustrate this we shall consider in some detail the situation in which there are altogether five recognizable states, i.e. those that are judged as being '0 % lysis', '25 % lysis', '50 % lysis', '75 % lysis' and '100 % lysis'.

Let the five states, in sequence of increasing lysis, be scored 0, 1, 2, 3 and 4. Then for a given indicator system and a given value of h there are three possible cases.

Case 1. h is so large that the two levels of complement on either side of η can never be scored 1 and 3. Then one of the four following effects may be observed:

(i) Just one reaction mixture is scored 2; y is taken to be the level of complement at which this occurs.

(ii) Two consecutive reaction mixtures are scored 0 and 3; y is taken to be a weighted average of the two corresponding complement levels.

(iii) Two consecutive reaction mixtures are scored 1 and 4; y is taken to be a weighted average of the two corresponding complement levels.

(iv) Two consecutive reaction mixtures are scored 0 and 4; y is taken to be the simple average of the corresponding complement levels.

Case 2. h is so small that the two levels of complement on either side of η can never be scored 0 and 3 or 1 and 4 (nor, *a fortiori*, 0 and 4). Then only two effects are possible, and y will be taken to be either a level of complement scored 2 or the

average of two levels scored 1 and 3. This case is thus equivalent to the situation in which only three states are recognizable, and this has been dealt with above.

Case 3. h is intermediate in size between the two extremes of cases 1 and 2. In this case the two levels of complement on either side of η can never be scored 0 and 4. There are four possible effects, given by (i), (ii) and (iii) under case 1 above and (iv) Two consecutive reaction mixtures are scored 1 and 3; y is taken to be the simple average of the corresponding complement levels.

The weights used to obtain the weighted averages in cases 1 and 3 are so chosen that the estimates y are unbiased, and for this purpose it is necessary to know the expected proportion θ_1 of estimates of type (i) and the expected proportion θ_2 of estimates of type (ii). It is reasonable to assume that θ_2 is also the proportion of type (iii), and so θ_2 should be taken to be half the proportion of a large number of estimates of either types (ii) or (iii). Then the proportion of type (iv) is $1-\theta_1-2\theta_2$. Let y_0, y_1, y_3 and y_4 be log complement levels scored 0, 1, 3 and 4, respectively. An estimate of type (ii) is then $wy_0+(1-w)y_3$, for a suitably chosen w, and, since $y_0 = y_3-h$, this estimate is equal to y_3-wh. Now by an argument analogous to that used previously y_3 can be considered to be a random variable uniformly distributed on the interval $(\eta+\theta_1 h/2, \eta+\theta_1 h/2+\theta_2 h)$, and so for the estimate to be unbiased w must be so chosen that

$$\int_{\eta+\theta_1 h/2}^{\eta+\theta_1 h/2+\theta_2 h} [(y_3-wh)/\theta_2 h]\,dy_3 = \eta,$$

the solution to which is $w = (\theta_1+\theta_2)/2$. The variance of this estimate is the same as that of y_3, i.e. $\theta_2^2 h^2/12$. In a similar manner it is found that an estimate of type (iii) is $(1-w)y_1+wy_4$, with the same value of w as before, and the variance of this estimate is $\theta_2^2 h^2/12$.

By the same argument estimates of type (i) and (iv) are also unbiased, and their variances are respectively $\theta_1^2 h^2/12$ and $(1-\theta_1-2\theta_2)^2 h^2/12$. In each case z is taken to be the antilog of y, and so the variances of these estimates z are again given by (1), but where now

$$t = \left\{ \begin{array}{l} \theta_1 \text{ for estimates of type (i),} \\ \theta_2 \text{ for estimates of types (ii) or (iii),} \\ 1-\theta_1-2\theta_2 \text{ for estimates of type (iv).} \end{array} \right\} \qquad (3)$$

A value of z can be found for each combination of levels of antigen and antiserum used in the test. In particular, let k be the value found when both antigen and antiserum are at the zero level. Then, provided the effect is additive, $z-k$ estimates the amount of complement fixed at any given level of antigen and antiserum; it also estimates the amount of complement fixed by the antigen-antibody complex formed at that level provided there are no anticomplementary or procomplementary effects.

These effects, however, may be present. Now for any particular estimate z let u be the corresponding estimate at the same level of antiserum, but at the zero level of antigen, and let v be the corresponding estimate at the same level of antigen but

at the zero level of antiserum. Then, provided the various effects are additive, the amount of complement fixed by the antigen-antibody complex is given by

$$f = z - u - v + k. \tag{4}$$

This reduces to $z-v$ if the antiserum shows no anticomplementary or pro-complementary effects ($u = k$), to $z-u$ if the antigen shows no such effects ($v = k$), and to $z-k$ if neither antiserum nor antigen show such effects ($u = k = v$). The variances of u, v and k are all given approximately by (1), with u, v and k respectively replacing z, and so the variance of (4) is given approximately by a sum of such expressions.

(2) *Antiserum (antigen) maxima titre*

In many systems the antiserum (or antigen) maxima line can be determined by only a few points. We shall here consider in detail the case where only two points are used to define the maxima line, and obtain the appropriate variance for the estimate of the antiserum (or antigen) titre. Only the antiserum maxima line need be discussed, as analogous results are obtained for the antigen maxima line by interchanging the words 'antigen' and 'antiserum', and the definitions of u and v, in what follows.

Fulton & Almeida (1962) have shown that the antiserum maxima line can be defined either as the linear relation between the maximum value of f at any one level of antiserum and the concentration of antiserum at that level, or as the linear relation between the logarithm of the maximum value of f and the logarithm of the antiserum concentration. The latter definition is preferable, but in many practical situations the two definitions are equivalent. In view of this, and since the assumption of a linear relation between the logarithms leads to a computationally much more cumbersome expression for the variance of the antiserum maxima titre, the former definition will be used here. The antiserum maxima titre is then taken from this line to be that antiserum concentration at which f is equal to k (i.e. k is taken to be the unit of measurement).

Suppose we have two points (f_1, x_1) and (f_2, x_2) on the antiserum maxima line, where corresponding to (4) we have

$$f_1 = z_1 - u_1 - v_1 + k \quad \text{and} \quad f_2 = z_2 - u_2 - v_2 + k.$$

Then the antiserum maxima titre is estimated by

$$x_t = G/F, \tag{5}$$

where

$$F = f_1 - f_2 \tag{6}$$

and

$$G = (f_1 - k)\, x_2 - (f_2 - k)\, x_1 \tag{7}$$

The variance of (5) can be approximately determined as follows. We have (provided, as would always occur in practice, F^2 is large relative to its variance)

$$s_x^2 \doteq s_G^2/F^2 - 2Gs_{FG}/F^3 + G^2 s_F^2/F^4, \tag{8}$$

where

s_x^2 = variance of x_t,
s_F^2 = variance of F,
s_G^2 = variance of G,
s_{FG} = covariance of F and G.

These variances and covariance depend upon whether anticomplementary or

procomplementary effects are shown by the antiserum alone ($u \neq k = v$), by the antigen alone ($u = k \neq v$), by both ($u \neq k \neq v$), or by neither ($u = k = v$). For this reason it is convenient to define three quantities, S_1, S_2 and S_3, in four different ways according to which of these conditions holds, and then it will be possible to express s_x^2 in terms of S_1, S_2 and S_3. Let

$$\text{if } u \neq k = v: \left.\begin{aligned} S_1 &= (z_1 t)^2 + (u_1 t)^2, \\ S_2 &= (z_2 t)^2 + (u_2 t)^2, \\ S_3 &= (kt)^2; \end{aligned}\right\} \tag{9}$$

$$\text{if } u = k \neq v: \left.\begin{aligned} S_1 &= (z_1 t)^2 + (v_1 t)^2, \\ S_2 &= (z_2 t)^2 + (v_2 t)^2, \\ S_3 &= (kt)^2; \end{aligned}\right\} \tag{10}$$

$$\text{if } u \neq k \neq v: \left.\begin{aligned} S_1 &= (z_1 t)^2 + (u_1 t)^2 + (v_1 t)^2, \\ S_2 &= (z_2 t)^2 + (u_2 t)^2 + (v_2 t)^2, \\ S_3 &= 0; \end{aligned}\right\} \tag{11}$$

$$\text{if } u = k = v: \left.\begin{aligned} S_1 &= (z_1 t)^2, \\ S_2 &= (z_2 t)^2, \\ S_3 &= 4(kt)^2; \end{aligned}\right\} \tag{12}$$

where in each product t is given by (2) or (3) and is determined by the type of estimate with which it is multiplied. Then using (1) it follows that

$$\begin{aligned} s_F^2 &= (h \log_e 10)^2 (S_1 + S_2)/12, \\ s_G^2 &= (h \log_e 10)^2 (S_1 x_2^2 + S_2 x_1^2 + S_3 [x_2 - x_1]^2)/12, \\ s_{FG} &= (h \log_e 10)^2 (S_1 x_2 + S_2 x_1)/12. \end{aligned}$$

Finally, substituting into (8) these expressions, (5) and $(\log_e 10)^2/12 = 0{\cdot}442$, we obtain for the variance of x_l

$$s_x^2 \doteq 0{\cdot}442 h^2 [(x_2 - x_l)^2 S_1 + (x_1 - x_l)^2 S_2 + (x_2 - x_1)^2 S_3]/F^2.$$

The standard error of the antiserum maxima titre is therefore taken to be approximately,

$$0{\cdot}665 h [(x_2 - x_l)^2 S_1 + (x_1 - x_l)^2 S_2 + (x_2 - x_1)^2 S_3]^{\frac{1}{2}}/F. \tag{13}$$

(3) *Comparing two antiserum (antigen) maxima titres*

One of the main purposes of estimating an antiserum maxima titre and its standard error is to compare it with another such estimate. If the two antiserum preparations that are being compared contain entirely different antibodies, or at any rate antibodies that do not react in a homologous manner, then the interpretation of such a comparison is hazardous. If, on the other hand, the one preparation is a simple concentration of the other, or else the two antisera react homologously (so that one can be considered to be a concentration of the other), then a comparison of their maxima titres is the same as a comparison of their average titres, and this is essentially the estimation of the concentration of the one relative to that of the other. On a logarithmic scale the relative concentration (i.e. multiplicative factor) is given by a log difference (i.e. additive quantity), and we shall proceed to give a method of estimating this and its standard error. As before, it is not necessary to go into the comparison of two antigens, since this is completely analogous.

There are standard procedures for estimating a relative concentration when some known single-valued function of the response involved bears a linear relationship to some known single-valued function of the concentration (Finney, 1952). But in a complement fixation test, in which procomplementary or anti-complementary effects may be present, the functional form of the relation between the response (i.e. amount of complement fixed) and the concentration of antiserum is in general unknown; and furthermore the response may not be strictly monotonic, in which case no transformation can lead to a linear relationship. It is reasonable, however, to assume that over a limited range the response curve can be well represented by a parabola, and a full account of a method for estimating relative concentration in such a situation is presented elsewhere (Elston, 1965). An outline of the computational details, adapted to the special case of estimating the relative concentration of two antisera by the complement fixation test, will be given here.

For computational simplicity we wish all the responses that are measured in a given test to have approximately the same variance; this will be so for the estimates y on a logarithmic scale. It will not be possible to use the estimates obtained at the zero level of antiserum, and it is preferable not to use the estimates obtained at the zero level of antigen. Suppose that for each of the antiserum preparations a test has been carried out at d non-zero levels of antiserum and r non-zero levels of antigen, so that for each preparation we have dr estimates y. If, for one of these preparations, p_j is the proportion of these dr estimates of the jth type, the weighted average variance of y for that preparation is

cases 1 and 3: $$[p_1\theta_1^2+(p_2+p_3)\,\theta_2^2+p_4(1-\theta_1-2\theta_2)^2]\,h^2/12. \tag{14}$$

case 2 (and only three states recognizable):

$$[p_1\theta_1^2+(1-p_1)\,(1-\theta_1)^2]\,h^2/12. \tag{15}$$

In (15) p_1 is the proportion of estimates y scored 2 (or determined by a partial lysis if only three states are recognizable).

Denote the two antiserum preparations A and B, and suppose we wish to determine the concentration of B relative to A. Define the orthogonal polynomial coefficients

$$x_{1i} = \begin{cases} i-\frac{1}{2}(d+1) & \text{if } d \text{ is odd,} \\ 2i-(d+1) & \text{if } d \text{ is even} \end{cases} \tag{16}$$

and $$x_{2i} = x_{1i}^2-\sum_i x_{1i}^2/d \quad (i = 1, 2, \ldots, d). \tag{17}$$

Let $$C = r\sum_i x_{1i}^2 \quad \text{and} \quad D = r\sum_i x_{2i}^2. \tag{18}$$

Let the sum of the r estimates y at the ith level of antiserum be $y_{i.}$ for preparation A and $y'_{i.}$ for preparation B (the first level is the most dilute and the dth level the most concentrated). The following sums of squares are calculated to check the validity of the method of estimation:

$$L = (\sum_i x_{1i}y_{i.})^2/\mathrm{C}, \quad \text{with 1 degree of freedom,}$$

$$Q = (\sum_i x_{2i}y_{i.})^2/\mathrm{D}, \quad \text{with 1 degree of freedom,}$$

and $$R = \sum_i y_{i.}{}^2/r-(\sum_i y_{i.})^2/dr-L-Q, \quad \text{with } d-3 \text{ degrees of freedom.}$$

L, Q and R are divided by the weighted average variance of y for A and compared with the tabulated values of the χ^2-distribution with the degrees of freedom indicated. L and/or Q should be significantly large, but R, which is a measure of the deviation from a parabolic response curve, should not be significantly large. Similarly, three quantities L, Q and R are calculated for preparation B, using $y'_{i.}$ instead of $y_{i.}$, and these are divided by the weighted average variance of y for B; as before L and/or Q should be significant, but R should be not significant. Let the sum of the two weighted average variances of y for A and B be s^2. L, Q and R are now calculated using $y'_{i.}+y_{i.}$ instead of $y_{i.}$, divided by s^2 and referred to the same χ^2-distributions; as before L and/or Q should be significant and R should not be significant. Finally, these three sums of squares are computed using $y'_{i.}-y_{i.}$ instead of $y_{i.}$, divided by s^2, and again referred to the same χ^2-distributions; in this case neither Q nor R should be significantly large—if Q is significant this may indicate that the two preparations are not reacting homologously.

Provided the above checks do not indicate any invalidity of the method, the estimate of log relative concentration is obtained by calculating, in sequence:

$$\begin{aligned} a &= \sum_i (y'_{i.}-y_{i.})/dr, & b &= \sum_i x_{1i}\,(y'_{i.}-y_{i.})/C, \\ b_1 &= \sum_i x_{1i}\,(y'_{i.}+y_{i.})/C, & b_2 &= \sum_i x_{2i}\,(y'_{i.}+y_{i.})/D, \\ m_1 &= 2a/b_1, & m_2 &= b/b_2, \\ W_1 &= (m_1^2/C+4/dr)/b_1^2, & W_2 &= (m_2^2/D+1/C)/b_2^2 \end{aligned} \tag{19}$$

$$m = (W_1 m_2 + W_2 m_1)/(W_1+W_2). \tag{20}$$

At this point m is substituted for m_1 and m_2 in (19) to obtain new values of W_1 and W_2, which are then used in (20) to recalculate m; this process is repeated until m becomes stable. Then we calculate

$$s_m^2 = s^2\, W_1 W_2/(W_1+W_2), \tag{21}$$

using the last values of W_1 and W_2.

Now the r levels of antigen used for A must be the same as the r levels used for B, but the d levels of antiserum used need not be the same for both preparations; it is essential, however, that the d levels of antiserum used for A should be a constant multiple of the d levels used for B. Let the logarithm of this constant multiple be k, so that if the concentration of A at the ith level is z_i, and that of B is z'_i, $k = \log_{10}(z_i/z'_i)$ and is the same for all i. Then the concentration of B relative to A, in $\log_{10}$ units, is estimated by

$$\begin{cases} mh+k, \text{ with standard error } s_m h, \text{ if } d \text{ is odd}, & (22) \\ \frac{1}{2}mh+k, \text{ with standard error } \frac{1}{2}s_m h, \text{ if } d \text{ is even}. & (23) \end{cases}$$

(4) *Examples*

Table 1 (sample A) summarizes the results of a complement fixation test in which the antigen is human albumin and the antibody is made in rabbits. The successive concentrations used for all three variables were arranged to be 0·2 $\log_{10}$ units apart. The reaction mixtures were classified into five states, and so there are four different types of estimates. From about 200 such estimates obtained in

different tests, all using the same indicator system as in this example and with $h = 0{\cdot}2$, the proportions of the different types were found to be $\theta_1 = 0{\cdot}23$ and $\theta_2 = 0{\cdot}24$. The estimates in the table were therefore obtained using $w = 0{\cdot}47/2$.

Table 1. *Estimates, z, of amount of complement required for 50 % haemolysis in indicator system—sample A*

Antiserum concentration	Antigen concentration					
	0·0251	0·0158	0·0100	0·0063	0·0040	0
0·0251	0·178	0·158	0·112	0·100	0·079	0·020
0·0158	0·141	0·141(iii)	0·141	0·100	0·079	0·025(i)
0·0100	0·112	0·112	0·112	0·089	0·079	0·032
0·0063	0·089	0·089(iii)	0·089	0·071	0·063	0·040(i)
0	0·032	0·032(iv)	0·032	0·040	0·045	0·050

(i), (iii), (iv), type of estimate.

The last column of Table 1 gives the values of u, the last row the values of v; k is given in the lower right corner, i.e. $k = 0{\cdot}050$. From this table it is found that the largest values of f at each of the four antisera concentrations are:

$$f = 0{\cdot}176 \quad \text{at} \quad x = 0{\cdot}0251,$$
$$f = 0{\cdot}134 \quad \text{at} \quad x = 0{\cdot}0158,$$
$$f = 0{\cdot}098 \quad \text{at} \quad x = 0{\cdot}0100,$$
$$f = 0{\cdot}067 \quad \text{at} \quad x = 0{\cdot}0063.$$

However, inspection of the first line of Table 1 shows that at antiserum concentration 0·0251 a maximum has not necessarily been reached, and so it is best to ignore the point ($f = 0{\cdot}176$, $x = 0{\cdot}0251$). The other three points lie approximately on a straight line, and the two points out of these three that best determine the antiserum maxima line are the two extreme points ($f_1 = 0{\cdot}134$, $x_1 = 0{\cdot}0158$) and ($f_2 = 0{\cdot}067$, $x_2 = 0{\cdot}0063$). We shall use these two points to determine an estimate of the antiserum maxima titre and its standard error.

Using (6), (7) and (5) we obtain

$$F = 0{\cdot}134 - 0{\cdot}067 = 0{\cdot}067,$$
$$G = (0{\cdot}134 - 0{\cdot}050)\,(0{\cdot}0063) - (0{\cdot}067 - 0{\cdot}050)\,(0{\cdot}0158) = 0{\cdot}0002606$$

and $$x_t = 0{\cdot}0002606/0{\cdot}067 = 0{\cdot}00389 \text{ or } 1:257.$$

It is evident that both antiserum and antigen show procomplementary effects ($u \neq k \neq v$), and so to obtain the standard error of this estimate we use (11) to define S_1, S_2 and S_3. Thus

$$S_1 = (0{\cdot}141 \times 0{\cdot}24)^2 + (0{\cdot}025 \times 0{\cdot}23)^2 + (0{\cdot}032 \times 0{\cdot}29)^2 = 0{\cdot}001264,$$
$$S_2 = (0{\cdot}089 \times 0{\cdot}24)^2 + (0{\cdot}040 \times 0{\cdot}23)^2 + (0{\cdot}032 \times 0{\cdot}29)^2 = 0{\cdot}000627,$$
$$S_3 = 0.$$

Therefore, using (13), the approximate standard error is

$$0{\cdot}665 \times 0{\cdot}2 \times [(0{\cdot}0024)^2 \times 0{\cdot}001264 + (0{\cdot}0119)^2 \times 0{\cdot}000627]^{\frac{1}{2}}/0{\cdot}067 = 0{\cdot}00062.$$

From this we obtain an approximate 95 % confidence interval for x_t as $0{\cdot}00389 \pm 2 \times 0{\cdot}0062$, i.e. 0·00265 to 0·00513.

Table 2 (sample B) summarizes the results of a similar complement fixation test, the only differences from sample A being that the antiserum is different, but believed to act as a concentration of the antiserum in sample A, and that different levels of antiserum are used. If we estimate the antiserum maxima titre in the same way as for sample A we find $x_t = -0{\cdot}00009$ with a standard error of 0·00036. Thus this estimate of the titre is not significantly different from zero, in accordance with the fact that the true titre cannot be negative. This is a case in which it would be distinctly preferable to define the antiserum maxima line as the linear relation

Table 2. *Estimates, z, of amount of complement required for* 50 % *haemolysis in indicator system—sample B*

Antiserum concentration	Antigen concentration					
	0·0251	0·0158	0·0100	0·0063	0·0040	0
0·0100	0·251	0·224	0·141	0·100	0·063	0·020
0·0063	0·200	0·200$^{(iv)}$	0·141	0·112	0·071	0·020$^{(iv)}$
0·0040	0·141	0·141	0·141	0·112	0·079	0·020
0·0025	0·112	0·112$^{(ii)}$	0·112	0·089	0·079	0·020$^{(iv)}$
0	0·032	0·032$^{(iv)}$	0·032	0·040	0·045	0·050

$^{(ii)}$, $^{(iv)}$, type of estimate.

between the logarithm of the maximum value of f and the logarithm of the antiserum concentration, for then estimates of the titre cannot be negative. However, if our main purpose is to estimate the antiserum concentration of sample B relative to that of sample A, rather than to estimate the individual titres, the method given in §3 should be used; this method will now be illustrated.

Logarithms of the estimates z in Tables 1 and 2 are given, multiplied by minus one, in Table 3; multiplication of all the y-values by minus one has no effect on the results, and so is done to avoid unnecessary minus signs. The type of each estimate is indicated, and the proportions of the types in the two samples are:

$$\begin{aligned} \text{sample A:} \quad p_1 &= 3/20 = 0{\cdot}15 \\ p_2+p_3 &= 14/20 = 0{\cdot}70 \\ p_4 &= 3/20 = 0{\cdot}15 \\ \text{sample B:} \quad p_1 &= 3/20 = 0{\cdot}15 \\ p_2+p_3 &= 13/20 = 0{\cdot}65 \\ p_4 &= 4/20 = 0{\cdot}20 \end{aligned}$$

Taking $\theta_1 = 0{\cdot}23$, $\theta_2 = 0{\cdot}24$ and $h = 0{\cdot}2$ as before, (14) gives the weighted average variance to be 0·000203 for sample A and 0·000207 for sample B. These two values and their sum are entered in the last row of Table 4.

For these data $d = 4$ and $r = 5$, and so from (16), (17) and (18):

$$\begin{aligned} &x_{11} = -3, \quad x_{12} = -1, \quad x_{13} = 1, \quad x_{14} = 3, \\ &x_{21} = 4, \quad x_{22} = -4, \quad x_{23} = -4, \quad x_{24} = 4, \\ &C = 100 \quad \text{and} \quad D = 320. \end{aligned}$$

Each of the four sets of sums of squares L, Q and R in Table 4 is then computed from these values and the appropriate set of sums or differences given in the last

two columns of Table 3. For example, the last column of Table 4 is obtained as follows:

$$L = [(-3)(-0{\cdot}50)+(-1)(-0{\cdot}40)+(1)(-0{\cdot}30)+(3)(-0{\cdot}15)]^2/100 = 0{\cdot}0132,$$
$$Q = [(4)(-0{\cdot}50)+(-4)(-0{\cdot}40)+(-4)(-0{\cdot}30)+(4)(-0{\cdot}15)]^2/320 = 0{\cdot}0001,$$
$$R = [(-0{\cdot}50)^2+(-0{\cdot}40)^2+(-0{\cdot}30)^2+(-0{\cdot}15)^2]/5-[-0{\cdot}50-0{\cdot}40-0{\cdot}30-0{\cdot}15]^2/20-L-Q = 0{\cdot}0001.$$

Table 3. *Negative logarithms of estimates, z, in Tables* 1 *and* 2 *(non-zero levels of antigen and antiserum), together with required sums and differences*

Anti-serum level, i.	Sample A					Total y_i.	$y'_i.+y_i.$
4	$0{\cdot}75^{(ii)}$	$0{\cdot}80^{(i)}$	$0{\cdot}85^{(iii)}$	$0{\cdot}95^{(ii)}$	$1{\cdot}10^{(iv)}$	4·45	8·75
3	$0{\cdot}85^{(iii)}$	$0{\cdot}85^{(iii)}$	$0{\cdot}85^{(iii)}$	$1{\cdot}00^{(i)}$	$1{\cdot}10^{(iv)}$	4·65	9·00
2	$0{\cdot}95^{(ii)}$	$0{\cdot}95^{(ii)}$	$0{\cdot}95^{(ii)}$	$1{\cdot}05^{(iii)}$	$1{\cdot}10^{(iv)}$	5·00	9·60
1	$1{\cdot}05^{(iii)}$	$1{\cdot}05^{(iii)}$	$1{\cdot}05^{(iii)}$	$1{\cdot}15^{(ii)}$	$1{\cdot}20^{(i)}$	5·50	10·50
	Sample B					Total $y'_i.$	$y'_i.-y_i.$
4	$0{\cdot}60^{(i)}$	$0{\cdot}65^{(iii)}$	$0{\cdot}85^{(iii)}$	$1{\cdot}00^{(i)}$	$1{\cdot}20^{(i)}$	4·30	−0·15
3	$0{\cdot}70^{(iv)}$	$0{\cdot}70^{(iv)}$	$0{\cdot}85^{(iii)}$	$0{\cdot}95^{(ii)}$	$1{\cdot}15^{(ii)}$	4·35	−0·30
2	$0{\cdot}85^{(iii)}$	$0{\cdot}85^{(iii)}$	$0{\cdot}85^{(iii)}$	$0{\cdot}95^{(ii)}$	$1{\cdot}10^{(iv)}$	4·60	−0·40
1	$0{\cdot}95^{(ii)}$	$0{\cdot}95^{(ii)}$	$0{\cdot}95^{(ii)}$	$1{\cdot}05^{(iii)}$	$1{\cdot}10^{(iv)}$	5·00	−0·50

$^{(i)}$, $^{(ii)}$, $^{(iii)}$, $^{(iv)}$, type of estimate.

Table 4. *Sums of squares and χ^2-values for checking the validity of the method*

Component	D.F.	$y_i.$ S.S.	$y_i.$ χ^2	$y'_i.$ S.S.	$y'_i.$ χ^2	$y'_i.+y_i.$ S.S.	$y'_i.+y_i.$ χ^2	$y'_i.-y'_i.$ S.S.	$y'_i.-y'_i.$ χ^2
L	1	0·1221	601	0·0552	267	0·3422	835	0·0132	32
Q	1	0·0045	22	0·0061	29	0·0211	51	0·0001	< 1
R	1	0·0000	< 1	0·0001	< 1	0·0001	< 1	0·0001	< 1
Weighted average variance		0·000203		0·000207		0·000410			

(A computational check is afforded by noting that for each component the sum of the sums of squares in the first two columns should be half the sum in the last two columns.) The χ^2-values in Table 4 are obtained by dividing each corresponding sum of squares by the variance at the bottom of its column. We see that L is significantly large in all four columns, Q is significant in all columns but the last and R is never significant. There is thus no reason to doubt the validity of the method, and so we can proceed to estimate the relative concentration.

From the last column of Table 3 we find

$$a = -0{\cdot}0675, \quad b = 0{\cdot}0115,$$
$$b_1 = -0{\cdot}0585, \quad b_2 = 0{\cdot}008125,$$

and so $m_1 = 2{\cdot}308$ and $m_2 = 1{\cdot}415$.

Substituting these values into (19) we obtain

$$W_1 = 74{\cdot}0 \quad \text{and} \quad W_2 = 246{\cdot}3,$$

so that from (20) $m = 2{\cdot}10$. Substituting this value for m_1 and m_2 in (19) we obtain

$$W_1 = 71{\cdot}3 \quad \text{and} \quad W_2 = 360{\cdot}2,$$

so that using these new values in (20) $m = 2{\cdot}16$. Finally, if this value is substituted for m_1 and m_2 in (19) we obtain

$$W_1 = 72{\cdot}1 \quad \text{and} \quad W_2 = 372{\cdot}3$$

and m remains 2·16. Then using (21) we calculate

$$s_m^2 = (0{\cdot}000410)\ (72{\cdot}1)\ (372{\cdot}3)/444{\cdot}4 = 0{\cdot}0248.$$

Inspecting Tables 1 and 2 we see that each concentration of antiserum for sample A is 2·51 times the corresponding concentration for sample B. Thus $k = \log_{10} 2{\cdot}51 = 0{\cdot}4$ (i.e. two levels that were arranged to be 0·2 $\log_{10}$ units apart), and from (23) the concentration of B relative to A, in $\log_{10}$ units, is estimated by

$$(2{\cdot}16)\ (0{\cdot}2)/2 + 0{\cdot}4 = 0{\cdot}616$$

with standard error $[\sqrt{(0{\cdot}0248)}]\ (0{\cdot}2)/2 = 0{\cdot}0157$. It follows that an approximate 95 % confidence interval for the log concentration of B relative to A is given by $0{\cdot}616 \pm 0{\cdot}031$, and taking antilogs the 95 % confidence interval for the concentration of B relative to A is 3·85–4·44.

DISCUSSION

In the examples only two points were used to estimate the absolute antiserum maxima titre and its standard error. It is not suggested that the other points on the maxima line should be ignored. All the points should be used to determine the titre, fitting a line either by eye or by least squares (in this latter case it would be possible to take account of the different variances and covariances among the points, but this would hardly be worth while). It is, however, suggested that for computational simplicity only the two most extreme points on the line be used to obtain an approximate standard error for the estimate. If all the points were uncorrelated and supplied an equal amount of information about the titre, then using just two out of n points would result in estimating $\sqrt{\frac{1}{2}n}$ times the required standard error. But since the most extreme points contain most of the information, and since the points are all correlated, the estimate that is obtained is less than $\sqrt{\frac{1}{2}n}$ times too big; it can therefore be regarded as a conservative estimate of the true standard error. Thus if, for sample A, a regression line is fitted to the three points by unweighted least squares, the antiserum maxima titre is found to be 0·00358. This estimate is probably better than the estimate 0·00389 previously obtained, and it is reasonable to assume that 0·00062 is a conservative estimate of its standard error; since three points are available, we may expect this standard error to be less than $\sqrt{\frac{3}{2}} = 1{\cdot}2$ times too big. Thus a conservative 95 % confidence interval is given by $0{\cdot}00358 \pm 2 \times 0{\cdot}00062$, i.e. 0·00234 (about 1:430) to 0·00482 (about 1:210).

Provided it is valid the method given in §3 should always be used to compare two titres, in preference to the simpler method of estimating the absolute titres separately and comparing these estimates, as it uses much more of the information available. Checking the validity of the method is an essential part, requiring little extra computation (it should be noted that b, b_1 and b_2 can be calculated at the same time as some of the sums of squares are calculated). Occasionally both components L and Q may be non-significant for $y_{i.}$ and/or $y'_{i.}$. In such a case the antigen contours should be plotted and a set of antigen concentrations that give similarly shaped contours (the same set for both A and B) used to estimate m and s^2_m. It may be possible to pick out another set of antigen concentrations and so obtain a second pair of values, say m' and s'^2_m. (For each set of antigen concentrations L and/or Q must be significant for both $y_{i.}$ and $y'_{i.}$.) Then instead of m take the weighted average $(ms'^2_m + m's^2_m)/(s^2_m + s'^2_m)$ with standard error $s_m s'_m/\sqrt{(s^2_m + s'^2_m)}$.

In certain cases the calculation of m can be simplified. If W_1 is very large compared with W_2, m is virtually equal to m_2 and $s^2_m = s^2 W_2$; while if W_2 is very large compared with W_1, m is virtually equal to m_1 and $s^2_m = s^2 W_1$. However if, as in the example, W_1 and W_2 are of the same order of magnitude, m must be obtained by an iterative process.

Finally, it should be noted that the method given for comparing two titres gives the most precise result when all d quantities $y'_{i.} - y_{i.}$ are near zero. This can to a large extent be arranged, as in the example, by having different antiserum concentrations of the two preparations, i.e. by appropriately choosing k.

SUMMARY

A method is given for obtaining the approximate standard error of the antiserum (or antigen) maxima titre in the complement fixation test when the maxima line is determined by only two points; the same formulae may be used to obtain a conservative estimate of the standard error when more than two points are available. If the degree of lysis in each reaction mixture can be scored more finely than into the three simple states of 'no lysis', 'partial lysis' and 'complete lysis', then more accurate estimates can be obtained. A method is also given for estimating the concentration of one antiserum (or antigen) relative to that of another when they react homologously.

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Infection of sheep and monkeys with Langat virus: cross-protection against other viruses of the Russian spring-summer complex

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Langat virus, a member of the Russian spring–summer (RSS) complex of group B arboviruses, was isolated from *Ixodes granulatus* ticks found on forest rats near Kuala Lumpur, Malaya (Smith, 1956). It was shown to be distinct from the other members of the complex by Clarke (1962). Natural infections with Langat virus appear to be rare and no overt disease has been observed: of 386 Malayan human sera (including 215 from forest people) only two (of the latter) were found to have neutralizing antibody. In thirty-two patients with malignant disease, infection with Langat virus (TP-21-7th mouse passage) usually caused only a mild febrile illness although evidence of encephalitis was found in two (Webb, Wetherly-Mein, Smith & McMahon, unpublished). Price, Lee, Gunkel & O'Leary (1961) showed that one strain of Langat virus (TP-21-9) which had been passaged in chick embryo tissue cultures did not produce encephalitis when inoculated intracerebrally into rhesus monkeys and that when three live viruses (yellow fever, West Nile and Langat (TP-21-9)) were given in series there was subsequently good protection against a wide range of group B arboviruses. Price *et al.* (1963*a*) reported that 70% of the plaque-purified clones prepared from the 12–20th chick embryo tissue culture passages of Langat virus showed increased virulence for mice inoculated intraperitoneally and caused more lesions of the central nervous system in rhesus monkeys inoculated intracerebrally than the parent 8th mouse passage. There was no comparable increase in virulence following passage in hamster kidney cultures. Shah *et al.* (1962) studied Langat virus in mice and guinea-pigs and demonstrated a good measure of protection against some viruses of the RSS complex. Smorodintsev (1963) has carried out preliminary trials in man with live Langat virus as a vaccine against Russian spring–summer encephalitis.

The present experiments were designed to investigate the possibility of using Langat virus as a live vaccine against louping ill in sheep and against the RSS complex of viruses in man.

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MATERIAL AND METHODS

Viruses

(i) The 6th and 9th unweaned mouse passages of the TP-21 strain of Langat virus.

(ii) The Absettarov strain of Central European tick-borne encephalitis virus at an unknown mouse passage level obtained from the Walter Reed Army Institute of Research, Washington, D.C.

(iii) The W 377 CD IV strain of Kyasanur Forest disease virus at the 8th mouse passage obtained from Dr J. S. Porterfield ex Dr M. Theiler.

(iv) The Moredun strain of louping ill virus (LI).

Neutralization tests

Qualitative tests were done in 3 to 4-week-old mice by the method of Smith & Westgarth (1957). Quantitative tests were similar to the qualitative tests described by Smith *et al.* (1964), except that sera were mixed with equal volumes of four 10-fold dilutions of virus. The log neutralizing index was the difference between the log LD 50 of the virus after incubation with test sera and the log LD 50 of the virus after incubation with antibody-free serum. The end-points were calculated by the method of Reed & Muench (1938).

Haemagglutinin-inhibition (HI) tests

These were done at room temperature using 8–16 units of antigens prepared from unweaned mouse brain by high-speed centrifugation (Sabin, 1956; Smith *et al.* 1964).

RESULTS

A. Laboratory experiments in sheep

Results of infection

Subcutaneous (SC) inoculation

Forty-five adult sheep and seven lambs aged 1–7 days were given $10^{1\cdot6}$–$10^{9\cdot6}$ mouse IC LD 50 of 9th passage Langat virus. None showed evidence of clinical disease during the following 3–4 weeks and none of ten adults and two lambs tested showed detectable viraemia 1–5, 7 or 9 days after infection; seven were also tested on the 11th day with negative results. No detectable neutralizing antibody was found 21 days after infection (Table 1).

Intracerebral (IC) injection

Ten adult sheep and four lambs were injected IC with $10^{2\cdot3}$–$10^{8\cdot0}$ mouse IC LD 50 of 9th passage virus. Apart from two traumatic deaths among the adults and one among the lambs, no clinical disease was seen during the following 3–4 weeks. Adult sheep which received $10^{6\cdot0}$–$10^{8\cdot0}$ mouse IC LD 50 developed significant levels of neutralizing antibody to Langat virus but not to louping ill virus. The one given $10^{2\cdot3}$ mouse IC LD 50 failed to do so. Of the lambs, one

showed a probable antibody response to Langat virus, but two others given 10-fold more virus showed no response (Table 1). All the lambs were 1 day old at the time of infection.

Protection against louping ill virus

A number of sheep vaccinated either SC or IC with doses of Langat virus ranging from $10^{1\cdot6}$ to $10^{6\cdot0}$ mouse IC LD 50 were challenged with louping ill virus either by the method of Edward (1947) or by direct IC inoculation. Eight out of twelve sheep vaccinated SC with at least $10^{2\cdot3}$ mouse IC LD 50 of Langat virus survived SC challenge with louping ill virus followed three days later by 1 ml. of 2 % starch IC.

Table 1. *Antibody responses in sheep following infection with Langat virus and subsequent challenge with louping ill virus*

Langat virus infection			Neutralizing indices			
			21 days after infection with Langat		21 days after challenge with LI	
Route	Age of animal	log mouse IC LD 50	Langat	LI	Langat	LI
SC	Adults	1·6–9·6	< 1·0* (25)	< 1·0* (9)	1·4–1·6† (5)	2·0–2·6† (5)
	Lambs	2·0–6·0	< 1·0* (4)	—	1·0–2·2† (4)	1·7–3·6† (4)
IC	Adults	2·3	< 1·0 (1)	—	—	—
		6·0	1·6* (5)	0·7* (5)	2·6* (3)	2·2* (3)
		8·0	2·0* (2)	0·3* (2)	2·0* (2)	—
	Lambs	6·5	1·2 (1)	—	—	—
		7·5	< 1·0* (2)	—	—	—

( ) Number of sera examined.
* Pooled sera.
† Range of neutralizing indices for individual sera.

Table 2. *Sheep vaccinated with Langat virus and challenged with louping ill virus*

Langat infection		Louping ill challenge		
Route	Dose (log mouse IC LD 50)	Method	Survivors	
SC	1·6	LI SC plus starch IC	0/5	
	2·3		3/3	8/12 (67 %)
	4·6		2/6	
	5·9		3/3	
IC	2·3		1/1	4/4 (100 %)
	6·0		3/3	
	Nil		1/7	(14 %)
SC	5·9	LI virus IC	0/2	
IC	6·0		0/1	
	Nil		0/1	

All the sheep vaccinated by the IC route survived a similar challenge, but no sheep, whether vaccinated SC or IC, withstood IC challenge with louping ill virus (Table 2). Table 1 shows that after challenge, all surviving animals developed

louping ill neutralizing antibody and that the levels of Langat antibody in the same specimens were not significantly different regardless of their level before challenge.

B. FIELD EXPERIMENTS IN SHEEP

As the viruses of Langat and louping ill are closely related serologically and the former is not known to cause naturally occurring disease in either man or sheep, it was felt that it might be used as a potential live virus vaccine. Since it is transmitted only by ticks, the infected sheep used in the field trial were kept on tick-free land for a month after inoculation. Viraemia has not been detected in sheep, and it was considered, therefore, that there was no risk of infecting ticks.

Vaccination

Fifty yearling sheep (hoggs) were obtained in March 1961, from a tick-free area and moved to the West of Scotland College of Agriculture at Auchincruive, near Ayr. After preliminary blood sampling, twenty-five were inoculated SC with $10^{3\cdot0}$ mouse IC LD 50 of the 6th mouse passage Langat virus and the remainder kept as controls. They were retained there on tick-free pasture for 1 month, then, at the end of April, were placed in equal numbers of vaccinated and control hoggs on three hirsels (areas of hill sheep farms confined naturally by burns, ravines, etc.) on Camlarg, Dalcairnie and Knockgray farms in an endemic louping ill area in Ayrshire (see map, Smith *et al.* 1964).

Results of field exposure

Deaths from louping ill

Only those deaths confirmed by virus isolation from the central nervous system were accepted. The development of neutralizing and HI antibody to louping ill was taken as evidence of infection.

Louping ill killed one of six vaccinated hoggs at Camlarg. No cause of death was found in the seventh hogg. All of five non-vaccinated hoggs on the same hirsel died of louping ill; a sixth had a *Pasteurella* septicaemia and the seventh was lost. At Dalcairnie, two of the four vaccinated hoggs infected with louping ill and all three infected in the non-vaccinated group died of louping ill. Thus, among the vaccinated hoggs which became infected on both hirsels there were 3/10 deaths from louping ill compared with 8/8 in the infected non-vaccinated hoggs (Table 3). As there was no evidence of infection at Knockgray up to the end of June, the hoggs there were transferred to Camlarg where, although 93% (13/14) became infected, no evidence of disease was observed.

Antibody responses

None of the sheep had antibody to either virus before vaccination. Twenty-one days after Langat infection eight of twenty-five had Langat neutralizing antibody and three HI antibody. After 13 days exposure on the Camlarg hirsel, antibody responses showed that the majority had been infected with louping ill. All the vaccinated animals had either or both HI and neutralizing antibody to both louping ill and Langat; all except one of the control animals developed similar

antibodies. Those animals which survived retained louping ill neutralizing antibody for the 4 months to the end of the experiment. HI antibody titres, however, fell markedly; the mean Langat titre fell from 1/320 in May, to 1/40 in June and to 1/10 in September, while the corresponding mean louping ill titres were 1/320, 1/80 and 1/20. On the Dalcairnie hirsel no change in antibody was detected after 8–14 days exposure. The majority of the louping ill infections and deaths occurred

Table 3. *Field trial: infection rates and death rates from louping ill*

		Vaccinated		Non-vaccinated		
Hirsel	Number per group exposed	Infected	Deaths from LI among infected	Infected	Deaths from LI among infected	Total infection rates
Camlarg	7	7	1/6*	7	5/5†	14/14 (100 %)
Dalcairnie	11	4	2/4	3	3/3	7/22 (32 %)
Total	18	11	3/10	10	8/8	21/36
%	—	—	30	—	100	58
Knockgray	7	0	0	0	0	0/14 (0 %)
Camlarg July/Sept.	7	6	0/6	7	0/7	13/14 (93 %)

* One died of another cause.

† One died of Pasteurella septicaemia and one was lost.

between the May and June bleedings and only two of the animals infected survived to demonstrate HI and neutralizing antibody in June and September. On the Knockgray hirsel no antibody changes attributable to louping ill infection had occurred by the end of June. After transfer to Camlarg all the control and all except one of the vaccinated animals developed louping ill HI and neutralizing antibody.

The infection rate on the Camlarg hirsel was 100 % in May and 93 % in July–September. On Dalcairnie it was 32 % in May–June.

C. Experiments in monkeys

Results of infection

Intracerebral

Two rhesus monkeys were inoculated IC with $10^{7.0}$ mouse IC LD 50 of 6th passage Langat virus. No fever (apart from the first 48 hr.) or other symptoms were observed during 40 days observation. No virus was isolated from their blood on the 7th day or from their brains on the 40th day. Their serology is shown in Table 4.

At autopsy monkey A was found to have extensive generalized tuberculosis. The brains of these monkeys were fixed by perfusion *in situ* and examined by Dr V. Udall of the Wellcome Research Laboratories. He reported that in monkey A there was no evidence of a virus infection in the central nervous system and in monkey B the only lesion observed was a small area of neuronal damage and neuronophagia in the embolic nucleus of the cerebellum. The significance of this finding is not clear.

Subcutaneous

A cynomolgus monkey (C) was given $10^{6\cdot0}$ mouse IC LD 50 Langat virus SC. Viraemia was detected in low titre on the 2nd and 4th days by inoculation of unweaned mice but not subsequently up to the 21st day when tested on alternate days. The monkey had no fever or other symptoms. The serology is shown in Table 4.

Table 4. *Homologous antibody responses following Langat virus infection in monkeys*

Route	Intracerebral				Subcutaneous		Oral	
Monkey	A		B		C		D	
Day	HI*	NI†	HI	NI	HI	NI	HI	NI
0	< 10	0·0	< 10	0·0	< 10	0·0	< 10	0·0
4	—	—	—	—	< 10	—	< 10	—
7	< 10	—	< 10	—	—	—	—	—
11	—	—	—	—	40	—	20	—
30	—	—	—	—	—	> 1·8	20	> 1·8
40	20	2·4	80	2·5	—	—	—	—

* HI = Reciprocal of highest dilution causing complete haemagglutinin-inhibition.
† NI = Neutralizing index.

Oral

A cynomolgus monkey (D) was given $10^{8\cdot0}$ mouse IC LD 50 of Langat virus orally. Viraemia was detected on the 2nd, 4th and 7th days but not subsequently up to 21 days. The monkey had no fever or other symptoms. The serology is shown in Table 4.

Protection against related viruses

Central European tick-borne encephalitis

Monkeys C and D together with two controls (E and F) were given $10^{5\cdot0}$ mouse IC LD 50 of the Absettarov strain SC. Three days later 1 ml. of 2% starch was given IC. No symptoms or fever were detected in controls or in C and D. They were killed 22 days after challenge, the brains were fixed by perfusion *in situ*, and were examined histologically by Dr V. Udall, who reported as follows:

Subcutaneously vaccinated monkey (C). *Brain.* No evidence of encephalitis or of starch inoculation.

Orally vaccinated monkey (D). *Brain.* No evidence of virus infection but there is a small zone of inflammation probably due to the injection of starch.

Control monkeys (E). *Brain.* There are many small foci of infiltration and cuffing throughout the cerebral hemispheres. In the cerebellum there is patchy loss of the granular layer and of some Purkinje cells. The fastigial nucleus is damaged. *Cord.* There is infiltration and some chromatolysis of the anterior grey matter.

(F). *Brain.* There is evidence of injection trauma in the forebrain and of meningoencephalitis affecting numerous sites. In general, there is infiltration and cuffing with less effect on the neurones, except in the cerebellar cortex, fastigial nucleus and anterior horn cells of the cord. The inflammatory reaction can be seen

in the anterior commissure, and in the rhinencephelon inferior to it, the thalamus, subthalamic nucleus, substantia nigra and nuclei pontis. There is no evidence of disturbed function in any other tissue.

Thus although symptoms were not produced, there was marked encephalomyelitis in the control monkeys but not in the monkeys previously infected with Langat virus. Twenty-two days after challenge with Absetterov virus, monkey C had shown only a twofold rise in HI antibody to Langat virus, monkey D, a fourfold rise. In contrast, the control monkeys showed increases from < 1/10 to 1/160 and > 1/1280.

Kyasanur Forest Disease (K.F.D.)

A similar experiment (summarized in Table 5) was made with K.F.D. virus. The vaccinated monkeys showed no symptoms after K.F.D. infection but the controls were dull and had no appetite from the 4th to the 9th day. Viraemia was observed in the control but not in the vaccinated monkeys. The brains were perfused on the 28th day after challenge and histological examination by Dr V. Udall showed no evidence of encephalomyelitis in the vaccinated monkeys or in one of the controls. The other control had diffuse encephalitis involving the frontal, parietal and hippocampal areas of the cerebral cortex and the dentate nucleus.

Table 5. *Vaccination of monkeys with Langat virus and challenge with Kyasanur Forest disease virus*

	Vaccination (Langat)		Challenge (K.F.D.)		
Route	Dose (log mouse IC LD 50)	Viraemia (days)	Dose (log mouse IC LD 50)	Viraemia (days)	Histology encephalitis
Oral	5·8	2–6	6·4	Nil	–
SC	3·8	1–6	6·4	Nil	–
Control	Nil	–	6·4	1–8	+
Control	Nil	–	6·4	1–8	–

DISCUSSION

Langat virus is clearly of very low pathogenicity for sheep; it causes no illness even by intracerebral inoculation, does not cause detectable viraemia and antibody responses are absent or minimal. In contrast, monkeys can be infected by the intracerebral, subcutaneous and oral routes without clinical or histopathological disease but with marked viraemia and strong antibody responses. The 6th mouse passage appears less virulent for monkeys than the 8th which was studied by Price *et al.* (1963*a*). In view of the known susceptibility of arboviruses to acid, the oral infection presumably took place in the mouth, pharynx or oesophagus.

One third of the hoggs vaccinated with Langat virus developed either neutralizing or HI antibody to it before leaving Auchincruive. Infection with louping ill virus stimulated formation of both types of antibody to Langat virus. However, there was no significant difference in the HI titre to either Langat or louping ill

viruses whether the animals had been vaccinated or not. Langat HI titres tended to fall more rapidly than louping ill titres following louping ill infection: Langat titres fell an average of eightfold in the first 6 weeks compared with fourfold for louping ill titres. In the following three months both fell a further fourfold.

Because of the poor antibody responses in sheep, Langat virus makes a relatively poor vaccine in them against RSS complex of viruses. On the other hand it is an effective vaccine in monkeys and probably in man. The virus causes viraemia and good antibody responses in patients with malignant disease and although encephalitis has occurred in these abnormal individuals, they were specially at risk because of malignant deposits in the central nervous system (Webb *et al.* unpublished).

Smith *et al.* (1964) discussed the control of louping ill and the outstanding problems in the pathogenesis of the encephalitis. One of these problems, the influences of age and nutrition on the risk of encephalitis, is posed by the hoggs exposed at Knockgray. None of them showed evidence of infection while exposed there but 93 % of them were infected in July–September at Camlarg. None showed evidence of disease although among similar hoggs exposed at Camlarg from April to June, 73 % of those infected died of louping ill.

Of sheep vaccinated subcutaneously with at least $10^{2\cdot3}$ mouse IC LD 50 of Langat virus and challenged with louping ill virus by the method described by Edward (1947) 67 % survived; this is about the same as the survival rate in laboratory sheep vaccinated with formalinized sheep brain vaccine and challenged in the same way (O'Reilly & White, unpublished). Among sheep infected with louping ill in the field trial 30 % of the Langat-vaccinated hoggs and 100 % of the non-vaccinated controls died of louping ill. Although this difference is significant, the protection is not so much better than the formalinized sheep brain vaccine as to justify the use of a live virus vaccine.

Langat virus infection protects monkeys against encephalitis and reduces or eliminates viraemia in them due to other viruses in the RSS group. This confirms work by Price *et al.* (1963*b*) who followed us in using Edward's method of challenge employing peripheral inoculation followed by starch intracerebrally. The possible use of Langat virus as a vaccine in man shows promise, either alone against the RSS complex of viruses (Smorodintsev, 1963), or in combination with other group B arboviruses against the whole group (Price *et al.* 1961).

SUMMARY

1. The susceptibility of sheep to infection with Langat virus has been studied. No viraemia or symptoms were detected in sheep inoculated either subcutaneously or intracerebrally.

2. Only those sheep inoculated intracerebrally with $10^{6\cdot0}$–$10^{8\cdot0}$ mouse IC LD 50 of virus developed significant quantities of neutralizing antibody.

3. Two-thirds of sheep vaccinated with varying doses of Langat virus withstood subcutaneous challenge of louping-ill virus followed by intracerebral starch. All the intracerebrally vaccinated sheep survived this form of challenge but no sheep,

whether vaccinated subcutaneously or intracerebrally, withstood intracerebral challenge of louping ill.

4. In a field trial, three of ten hoggs vaccinated with Langat virus and exposed to natural louping ill infection at Camlarg and Dalcairnie died of the disease compared with all eight of the non-vaccinated hoggs. At Knockgray, there was no louping ill infection, but 93 % of the hoggs from this hirsel developed louping ill antibody after transfer to Camlarg.

5. Monkeys infected intracerebrally, subcutaneously or orally with Langat virus showed a low titre viraemia without clinical symptoms or histological changes in the brain and developed high titres of antibody. Vaccinated monkeys challenged with either Central European tick-borne encephalitis or Kyasanur Forest disease viruses remained healthy compared with control monkeys which showed evidence of disease.

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Salmonellae in cattle and their feedingstuffs, and the relation to human infection

*A Report of the Joint Working Party of the Veterinary Laboratory Services of the Ministry of Agriculture, Fisheries and Food, and the Public Health Laboratory Service**

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INTRODUCTION

The majority of food poisoning outbreaks in man that are due to the salmonella group of organisms follow the consumption of food directly or indirectly associated with infection of some animal. The chief animal reservoirs of *Salmonella* are fowls, pigs and cattle. Fowls and pigs have been regarded as forming a more important source of human infection than cattle (Wilson & Miles, 1955). The number of different serotypes that have been isolated from cattle is less than the number isolated from fowls and pigs. There has, therefore, been a tendency to minimize the part played by cattle in the spread of salmonella infection to man.

Abattoir surveys in Yorkshire and South Wales (McDonagh & Smith, 1958; Harvey & Phillips, 1961) have shown a relatively close relationship between *S. typhi-murium* infection in animals and man over corresponding time periods; approximately 77 % of the typable strains isolated from human infections in South Wales belonged to phage-types found in the abattoir environment or in local farm animals. It would, therefore, be reasonable to assume that many salmonella incidents in man are caused by the consumption of meat or meat products derived from native animals.

As will be shown later, *S. typhi-murium* is the second most common type isolated from cattle, and it is also the major cause of human infections. The development of

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a scheme for phage-typing of strains of *S. typhi-murium* (Felix & Callow, 1943; Callow, 1959), has allowed accurate comparisons to be made between human and animal infection. It was observed by Anderson (1960), that 97 % of animal strains of *S. typhi-murium* submitted for typing during a particular period of 1958, came from chickens and calves. The phage-type distribution in fowls was quite different from that in cattle, but when the groups were combined the distribution of common types had general similarities to that in human beings. This suggested that fowls and calves, or their products, might be the source of most human infections with *S. typhi-murium* in England and Wales. Other salmonellae also might originate from this source.

Table 1. *Areas served by veterinary investigation laboratories on the working party*

Veterinary investigation centre	Areas served	Veterinary investigation centre	Areas served
Bristol	Dorset Gloucestershire Somerset Wiltshire	Liverpool	Cheshire Lancashire
		Norwich	Norfolk East Suffolk
Cardiff	Carmarthenshire Glamorgan Monmouthshire Pembrokeshire	Penrith	Cumberland Westmorland
		Reading	Berkshire Buckinghamshire Oxfordshire Northamptonshire
Exeter (Starcross)	Cornwall Devon		
Leeds	East Riding Yorkshire West Riding Yorkshire		

In 1959, a joint working party of the Public Health Laboratory Service and the Veterinary Laboratory Services was set up to study salmonella infection in cattle and calves and in their feedingstuffs. The problem of the spread of salmonella infection from cattle to man was also investigated when opportunity offered. The areas served by the Veterinary Investigation Laboratories on the working party are shown in Table 1.

The husbandry and movement of cattle has a direct effect on the epidemiology of cattle salmonellosis, and a review of the situation is therefore given.

THE CATTLE POPULATION OF ENGLAND AND WALES

The total number of calves and cattle in England and Wales is approximately 8,800,000 (Agricultural Statistics, 1962).

A clear-cut division into beef and dairy cattle is impossible because many herds serve a dual purpose. Thus about three-quarters of home produced beef originates from dairy herds and consists either of 'cow beef' or, of greater importance, of beef produced from the bull calves and surplus heifer calves from dairy herds.

In March 1961 (Agricultural Statistics, 1962), there were about 3,850,000 cows and heifers in milk or in calf, of which 84 % formed part of the national dairy herd and 16 % formed part of the national beef herd. A preponderance of dairy cows is found, with few exceptions, in most areas of England and Wales.

The cattle population includes some 2,200,000 bull calves and steers being reared for beef, and about 2,700,000 young females. The latter include both beef animals and dairy replacements.

The annual calf crop amounts to about 3,500,000 calves of which 500,000 form replacements for the national dairy herd, about 2,000,000 are reared for beef production and 1,000,000 are slaughtered as calves. The number of calves born monthly is roughly 300,000 but there is some seasonal variation.

The distribution and movement of dairy cattle

The counties with the largest number of dairy cows are, in order, Somerset, Devon, Cheshire, Lancashire, the West Riding of Yorkshire, Shropshire and Staffordshire, all of which have more than 100,000 head. In Wales the chief dairying counties are Carmarthenshire and Pembrokeshire with approximately 80,000 and 51,000 respectively.

In the past there has been much short-term movement of adult dairy cows as herd replacements, but movement of this kind has now decreased.

The distribution and movement of beef cattle

The distribution of *adult* beef cattle shows some overlapping with that of the national dairy herd, the counties with the highest populations being Northumberland, Devon, the Lindsey division of Lincolnshire, Herefordshire, Cornwall and Shropshire. All these counties have more than 20,000 beef cows. In Wales, Montgomery, Radnor and Brecon each have 15,000 to 16,000 head.

Concentrations of *young* cattle being reared and fattened for beef are found in the Midlands, Yorkshire and East Anglia.

In contrast to the limited degree of movement shown by dairy cattle, the movement of calves and store cattle is an outstanding feature of the beef industry. Until recently store cattle commonly changed hands several times before slaughter, but the present trend is to reduce the number of changes to one or two. The main streams of movement are as follows.

Calves and store cattle

Large numbers of surplus calves from the dairy herds move from the west and south-west into East Anglia, the East Midlands and some other eastern counties for rearing and feeding for beef.

Considerable numbers of calves are retained in the south-west and west for fattening in the valleys of Devon, Somerset and Cornwall. Calves that are bought privately, or by the better class of calf-dealer, may be taken direct to their new premises without passing through a market. Others are exposed for sale in one or more markets—each occasion providing opportunities for cross-infection, chilling and dietary abuse. Because of these risks direct sales between calf breeders and calf rearers are becoming more common.

Considerable numbers of weaned calves move each autumn from the uplands of southern Scotland and northern England, from Wales and from south-west England to the arable areas of the Midlands and East Anglia where they are fattened in

yards to light weight beef over winter or finished on grass the following summer. Each spring, yearlings and older cattle are sent to the Midlands and Southern grazing areas, although the high price of these older stores is now tending to restrict this trade.

Imported fat and store cattle

In the decade 1951–60, live cattle imported into Great Britain averaged 604,000 annually, all from Northern Ireland and Eire except for about 0·1 % from the Channel Islands and the Isle of Man. During this period the average number of live cattle imported annually from Northern Ireland was approximately 267,000 (fat cattle 177,000; store cattle 90,000) and from Eire approximately 336,000 (fat cattle 86,000; store cattle 250,000).

Whatever their origin, fat cattle move again when they are sold for beef. This generally involves exposure in a market followed by a journey—often of considerable length—to the slaughter point. These movements provide opportunities for cross-infection.

THE MEAT TRADE

Home killed meat

About 3,300,000 cattle and 1,000,000 calves are slaughtered annually in the United Kingdom. Of the cattle slaughtered, approximately 2,500,000 are beef or

Table 2. *Estimated beef and veal supplies in the United Kingdom* 1960

(Thousand tons)

Home produced or imported	Country	Beef fresh and salted	Chilled beef	Frozen beef quarters, sides, carcasses, and bones	Veal	Total supplies
Home produced	United Kingdom	800·6	—	—	19·3	819·9
Imports	Irish Republic	13·8	0·1	1·0	—	14·9
	Australia	—	1·7	61·8	1·1	64·6
	Bechuanaland	—	—	6·2	—	6·2
	New Zealand	—	1·5	17·3	1·8	20·6
	Rhodesia and Nyasaland	—	1·3	3·7	—	5·0
	Other Commonwealth countries	—	—	0·1	—	0·1
	Argentina	—	181·8	22·2	—	204·0
	Brazil	—	0·2	—	—	0·2
	Netherlands	—	—	—	2·6	2·6
	Uruguay	—	20·6	12·0	—	32·6
	United States	—	—	0·3	—	0·3
	Other foreign countries	—	1·1	1·9	—	3·0
Total		13·8	208·3	126·5	5·5	354·1

Total home produced	819·9 (70)
Total imports	354·1 (30)
Total supplies	1174·0 (on the bone 1125) (boneless 49)

Figures in parentheses are percentages as integers.

* Modified from Tables 25 and 26, *Meat* (1962). Commonwealth Economic Committee. London: H.M.S.O.

dual purpose cattle or the progeny of dairy cows reared for beef, 500,000 are discarded dairy cows and rather more than 250,000 are imported as fat cattle ready for slaughter.

The calves slaughtered are almost entirely from dairy or dual purpose herds. The majority are killed within a few days after birth as 'bobby' calves; the remainder are slaughtered at about 3 months of age as veal.

Imported meat

Table 2 shows the countries of origin of our imports of beef and veal and compares these imports with our home-produced supplies.

Table 3. *Salmonella serotypes identified from bovine sources in the United Kingdom* 1956–61

Serotype	No. of cultures identified	Serotype	No. of cultures identified
S. abaetetuba	1	*S. llandoff*	1
S. agama	27	*S. london*	1
S. anatum	5	*S. manhattan*	3
S. bareilly	1	*S. menston*	3
S. binza	1	*S. newport*	2
S. bovis morbificans	2	*S. niloese*	2
S. braenderup	2	*S. oranienburg*	3
S. brancaster	4	*S. poona*	1
S. bredeney	4	*S. pullorum*	6
S. bury	1	*S. reading*	6
S. california	1	*S. richmond*	1
S. cholerae-suis	2	*S. ruiru*	1
S. cubana	1	*S. saint-paul*	7
S. derby	1	*S. senftenberg*	2
S. dublin	1375	*S. simsbury*	1
S. emek	7	*S. stanley*	1
S. enteritidis	21	*S. stanleyville*	1
S. gallinarum	2	*S. tennessee*	6
S. give	4	*S. thompson*	9
S. heidelberg	29	*S. typhi-murium*	560
S. kinshasa	1	*S. vejle*	5
S. liverpool	2		

Of the 2116 cultures listed above, 276 were derived from the Salmonella Reference Laboratory, Colindale, and 1840 were derived from the Weybridge Laboratory, having been isolated either there, or at Veterinary Investigation Centres. Of these 1840 cultures, 1147 (62%) originated from calves and 693 (38%) from older cattle.

Salmonella serotypes isolated from bovine sources

This information was derived from the records of the Central Veterinary Laboratory, Weybridge, and the Salmonella Reference Laboratory of the Public Health Laboratory Service, Colindale. The period covered is 1956–61. The results are given in Table 3.

Certain serotypes are relatively host specific; *S. cholerae-suis*, which is common in pigs, was isolated only twice from bovine sources, and *S. gallinarum* and *S.*

pullorum, common in fowls, only eight times. The specificity of *S. dublin* for bovine animals is shown by the great number of isolations compared with other serotypes. *S. typhi-murium* is not host-specific, but was the second most common serotype, 560 having been isolated in a total of 2116. Because of the lack of host-specificity of *S. typhi-murium*, a wide search may be necessary for the source of infection. The presence of certain other serotypes (Walker, 1957; Report, 1959*a*; Report 1961; Harvey & Price, 1962), suggests that animal feedingstuffs may be implicated.

The dominance of *S. dublin* and *S. typhi-murium* in Table 3 is noteworthy. *S. dublin*, although the commonest cause of salmonellosis in cattle in England and Wales, plays little part in human infection. *S. typhi-murium*, in contrast, causes by far the greatest number of infections in man. It was, for example, responsible for 73% of the incidents of salmonella infection in man in the period 1954–59 (Report, 1960).

Table 4. *Seasonal incidence of salmonella infection in calves and older cattle*

	Calves				Older cattle			
Month	*S. dublin*	*S. typhi-murium*	Other sero-types	Total	*S. dublin*	*S. typhi-murium*	Other sero-types	Total
Jan.	43 (6)	31 (9)	4 (11)	78 (7)	18 (3)	22 (15)	1 (5)	41 (6)
Feb.	55 (7)	21 (6)	0	76 (7)	11 (2)	8 (6)	0	19 (3)
Mar.	52 (7)	24 (7)	5 (14)	81 (7)	31 (6)	14 (10)	1 (5)	46 (7)
Apr.	41 (6)	11 (3)	2 (5)	54 (5)	25 (5)	1 (1)	2 (9)	28 (4)
May	40 (5)	9 (2)	2 (5)	51 (4)	38 (7)	5 (3)	4 (18)	47 (7)
June	36 (5)	12 (3)	1 (3)	49 (4)	47 (9)	2 (1)	1 (5)	50 (7)
July	37 (5)	18 (5)	0	55 (5)	56 (11)	11 (8)	1 (5)	68 (10)
Aug.	42 (6)	18 (5)	3 (8)	63 (6)	54 (10)	9 (6)	0	63 (9)
Sept.	65 (9)	43 (12)	7 (19)	115 (10)	62 (12)	13 (9)	2 (9)	77 (11)
Oct.	113 (15)	60 (17)	4 (11)	177 (15)	59 (11)	27 (19)	3 (14)	89 (13)
Nov.	119 (16)	62 (17)	4 (11)	185 (16)	67 (13)	12 (8)	4 (18)	83 (12)
Dec.	103 (14)	52 (14)	5 (14)	160 (14)	52 (10)	20 (14)	3 (14)	75 (11)
Total	746	361	37	1144	520	144	22	686

Figures in parentheses are percentages as integers.

In calves the incidence of both *S. dublin* and *S. typhi-murium* infection is highest in the autumn and winter. In older cattle, *S. dublin* infection shows a similar seasonal incidence, but the pattern of *S. typhi-murium* infection is not quite so clear cut. Rearrangement of the incidence on a quarterly basis, however, does show a more definite seasonal variation (see Table 5).

SEASONAL INCIDENCE OF SALMONELLA INFECTION IN CATTLE AND CALVES

The seasonal incidence derived from the records of the Central Veterinary Laboratory, Weybridge, is given in Table 4. The figures were collected over the period 1958–61, and refer mainly to incidents. An incident comprises either a sporadic case, or a group of epidemiologically related cases.

The pattern of incidence in Table 4 is a general one for England and Wales as a whole and may obscure local variations.

Table 5 relates the incidence of calvings to the incidence of salmonella infection in calves and cattle. In this table the average number of calves born during the

period 1958–61 in different quarters of the agricultural year is shown. It has not been possible to obtain figures for each separate month, or to arrange the data in terms of a calendar year. The seasonal incidence of infection in Table 4 has, therefore, been re-arranged in terms of the quarters of the agricultural year.

In cattle and in calves, the peak period of infection is September–November for *S. dublin* and *S. typhi-murium*. In cattle *S. dublin* is low in December–February, but *S. typhi-murium* infection is high in the same period. A similar raised incidence in December–February is seen in *S. typhi-murium* calf infections. The September–November quarter is that during which the maximum number of calvings takes place. It is probable that a high incidence of calvings in certain quarters is related to an increased spread of salmonella infection.

Table 5. *Relation of incidence of calvings to incidence of bovine salmonella infection*

Agricultural year quarter	Average no. of calvings 1958–61	Incidents *S. dublin*		Incidents *S. typhi-murium*		Incidents All serotypes	
		Calves	Older cattle	Calves	Older cattle	Calves	Older cattle
Dec.–Feb.	823,000 (26)	201 (27)	81 (16)	104 (29)	50 (35)	314 (27)	135 (20)
Mar.–May	795,000 (25)	133 (18)	94 (18)	44 (12)	20 (14)	186 (16)	121 (18)
June–Aug.	580,000 (18)	115 (15)	157 (30)	48 (13)	22 (15)	167 (14)	181 (26)
Sept.–Nov.	976,000 (31)	297 (40)	188 (36)	165 (46)	52 (36)	477 (42)	249 (36)

Figures in parentheses are percentages as integers.

MATERIALS AND METHODS

Materials examined

(*a*) Faeces from cattle, calves and other animals.
(*b*) Faecal pats from grassland on infected farms.
(*c*) Swabs from walls of loose-boxes on infected farms.
(*d*) Necropsy specimens from cattle, calves and other animals.
(*e*) Milk.
(*e*) Animal feedingstuffs.
(*g*) Moore's swabs (Moore, 1948) from drains of farms, abattoirs and retail shops.
(*h*) Faeces from human beings.

Bacteriological methods

Little alteration was made in the technique normally used by both services for salmonella isolation. In general, selenite F broth or tetrathionate broth was used as enrichment medium. Deoxycholate–citrate agar was employed as the main plating medium. Some laboratories also used Wilson and Blair's medium and brilliant green neutral red taurocholate lactose agar. Enrichment media were incubated at 37° C. and were subcultured at 24 hr. Plating media were incubated at 37° C. and were examined after 24–48 hr. incubation.

There were modifications of this general routine depending on personal preference.

RESULTS

The results of the investigations of the laboratories constituting the working party are given in a general fashion in Tables 6–8.

Table 6 shows the three main classes of serotype isolated from bovine salmonella incidents. The total cattle population in each area is included in this table and the percentage of cattle under one year old. *S. dublin* was isolated from 68 % of the incidents, *S. typhi-murium* from 27 % and other serotypes from 6 %.

Table 6. *Bovine salmonella incidents at veterinary investigation centres* 1959–61

Veterinary investigation centre	Total cattle in area served	Percentage of total cattle under 1 year	Incidents			
			S. dublin	*S. typhi-murium*	Other serotypes	Total
Bristol	1,008,771	19	56 (59)	34 (36)	5 (5)	95
Cardiff	478,583	22	178 (89)	13 (7)	9 (5)	200
Exeter*	804,401	23	30 (68)	11 (25)	3 (7)	44
Leeds	525,220	26	73 (72)	23 (23)	5 (5)	101
Liverpool	579,659	18	37 (73)	7 (14)	7 (14)	51
Norwich	274,258	30	125 (57)	82 (38)	11 (5)	218
Penrith	427,940	25	3 (33)	6 (67)	0	9
Reading	473,069	23	78 (55)	55 (39)	8 (6)	141
Total			580 (68)	231 (27)	48 (6)	859

Figures in parentheses are percentages as integers.

* Starcross.

Table 7 gives the distribution of phage-types of *S. typhi-murium* isolated from 155 of the 231 incidents recorded in Table 6. Untypable strains have been excluded. The total number of phage-types isolated was 28. The types most frequently encountered were 1 var. 5 (U 9) and 1 a (2) which together represent 42 % of the isolations in the period studied. Isolations of *S. typhi-murium* from animal feedingstuffs are shown and the commonest phage-types found in man are marked with an asterisk. The isolations from feedingstuffs were made from material distributed during 1959–61, although a few of the actual isolations were made in 1962.

Ten incidents of bovine infection were studied in detail by members of the working party in order to determine if spread had occurred. Table 8 shows the local and general distribution of infection in these incidents.

Salmonella infection and calf movements

Calf infection on a dealer's premises

Calves entering a dealer's premises in Norfolk were surveyed by the Norwich Veterinary Investigation Centre. The calves were purchased in the West Country. Most were bought at markets, but some were collected direct from their home

farms. They were brought to Norfolk in the dealer's own lorries, and rectal swabs were taken from them within an hour or so of their arrival at his premises.

By the end of December 1961, eighty-three batches of calves comprising 4258 animals had been examined. Salmonellae were isolated from twenty (0·5 %) of the calves. Of the eighty-three batches, eleven (13 %) were infected. Five serotypes were found. The results are given in Table 9.

Table 7. *Bovine* Salmonella typhi-murium *incidents at veterinary investigation centres* 1959–61, *untypable strains excluded*

Phage-type	A	B	C	D	E	F	G	H	Total	Animal feeding stuff isolations 1959–61
1 (1)*	—	1	1	—	—	8	—	1	11 (7·1)	—
1 var. 2 (U41)	—	—	—	—	—	3	—	1	4 (2·6)	—
1 var. 5 (U9)*	7	1	—	1	—	22	1	7	39 (25·2)	—
1a (2)*	8	—	—	1	—	12	—	5	26 (16·8)	5
1a (U57)*	—	1	1	1	5	2	2	—	12 (7·7)	2
1a var. 1 (3an)*	—	—	—	—	—	6	—	3	9 (5·8)	—
1a var. 2 (3aer)	—	1	2	—	—	—	—	1	4 (2·6)	—
1b (4)	—	—	—	—	—	—	—	—	—	1
1b (4a)	—	—	—	—	—	—	—	—	—	1
2 (9)	—	—	1	—	—	—	—	—	1 (0·6)	—
2 (10)	—	—	—	—	—	—	—	1	1 (0·6)	1
2 (12)*	2	2	—	—	—	10	—	1	15 (9·7)	2
2 (12a)*	—	—	—	—	—	—	—	—	—	1
2a (13)	—	—	1	—	—	—	—	—	1 (0·6)	—
2b (17)	—	—	—	1	—	—	—	—	1 (0·6	—
2b (18)	1	—	—	—	—	—	—	—	1 (0·6)	—
2b (29)	—	—	—	1	1	—	—	—	2 (1·3)	—
2c (14)*	—	—	—	—	—	3	—	5	8 (5·2)	—
2c (15a)	—	—	—	—	—	1	—	—	1 (0·6)	—
2d (20a)	—	1	—	—	—	2	—	1	4 (2·6)	—
4 (8)*	—	—	—	—	—	2	—	—	2 (1·3)	—
22	—	—	—	—	—	—	—	—	—	1
28	1	—	—	—	—	—	—	—	1 (0·6)	—
32	—	—	1	1	—	—	—	—	2 (1·3)	1
U15	—	—	—	—	—	1	—	—	1 (0·6)	—
U20	—	—	—	—	—	—	—	—	—	1
U29	—	—	—	—	—	1	—	—	1 (0·6)	—
U38	—	1	—	—	—	—	—	—	1 (0·6)	—
U39	1	—	—	—	—	—	—	—	1 (0·6)	—
U59	—	—	—	—	—	2	—	—	2 (1·3)	—
U72	1	—	—	—	—	—	—	—	1 (0·6)	—
U107	2	—	—	—	—	—	—	—	2 (1·3)	—
U117	1	—	—	—	—	—	—	—	1 (0·6)	—

A = Bristol
B = Cardiff
C = Exeter (Starcross)
D = Leeds
E = Liverpool
F = Norwich
G = Penrith
H = Reading

Phage-typing column. Figures in parentheses refer to the new typing scheme (Callow, 1959). All type designations preceded by letter U are provisional.

Total column. Figures in parentheses are percentages.

* Common phage-types in man. Phage-type 2c (14) usually associated with fowls. Phage-type 4 (8) frequently associated with ducks.

Most owners treated the infected calves when told the result of the examination. It was noted that at this stage none of the calves showed signs of clinical salmonellosis.

This survey confirmed beyond doubt that dealers' calves were introducing a

Table 8. *Distribution of salmonella infection in ten incidents*

Incident and description	Infection in man	Infection of other animals and animal feedingstuffs, etc.
1. *S. typhi-murium* 1 a (2). Calf infection	2 infections in children	Infection in contact calves and older store cattle
2. *S. typhi-murium* 1 a (2). and *S. dublin*. Calf infection	1 infection in child	*S. dublin* infection of dog
3. *S. typhi-murium* 1 a (U 57). Calf infection	2 infections on farm; 6 infections in village receiving milk from farm	
4. *S. dublin*. Calf infection		*S. dublin* infection of dog
5. *S. typhi-murium* 1 (1). Bovine infection	1 infection of milk handler on farm	
*6. *S. typhi-murium* 2 (12). Bovine infection	122 infections in county	*S. typhi-murium* 2 (12) isolated by Moore's swabs from 2 abattoirs. 1 pig and 1 rat at abattoir infected. Origin of pig traced to local farm. Same phage-type isolated from 13 butchers' shops by drain swabs
7. *S. typhi-murium* 1 var. 5 (U 9). Calf infection	3/8 infections on farm 1 infection from direct contact with calves	
8. *S. typhi-murium* 1 a var. 2 (3 aer). Calf infection	Infection of farm workers or their families	Symptomless infection of chickens
*9. *S. heidelberg*. Udder of cow and milk infected	3/3 symptomless infections of farm workers. 74 infections in community from milk	*S. heidelberg* isolated from rectal swab of calf fed on milk from herd. *S. heidelberg* was also isolated from English meat and bone meal samples at factory supplying cattle cake to farm. *S. heidelberg* was *not* isolated from the cattle cake
10. *S. typhi-murium* 2 (12). Bovine infection	1 infection on farm	Geese and ducks on farm infected. Other farms receiving turkey poults from original farm experienced outbreaks due to *S. typhi-murium* 2 (12). Same type was isolated from 2/2 opened bags of poultry food fed to affected poults on original farm

* For full description of these incidents see (incident 6) Harvey, Price, Bate & Allen (1963); (incident 9) Knox *et al.* (1963), Davies & Venn (1962).

variety of salmonella serotypes into East Anglia. As far as could be ascertained, each infected calf came from a different farm.

Investigations were made by the Bristol Veterinary Investigation Centre on the home farms of three of the calves, with negative results.

Table 9. *Serotypes isolated in a survey of a dealer's calves*

Serotype	No. of strains isolated
S. agama	8
S. cubana	1
S. dublin	4
S. saint-paul	1
S. typhi-murium, 1 var. 2 (U41)	3
S. typhi-murium, 1a (2)	1
S. typhi-murium, U29	1
S. typhi-murium, untypable	1

Table 10. *Farm outbreaks caused by* S. typhi-murium *in East Anglia*

Outbreak no.	Phage-type
1	1a (2)
2	1a (2)
3	1a (2)
4	1a (2)
5	1a (2)
	1 var. 5 (U9)
6	1 (1)
7	4 (8)

Farm outbreaks, East Anglia

A series of outbreaks in calves was also studied by the Norwich Veterinary Investigation Centre. In this investigation the starting point was the occurrence of an outbreak on a farm. Seven outbreaks were investigated. The infecting serotype was *S. typhi-murium*. The phage-types isolated are given in Table 10.

In outbreaks 1–5, the clinically affected batches of calves had all been purchased from a dealer near Wrexham, Denbighshire. Their common origin, the common phage-types of the strains, and the fact that some of the affected batches had been delivered direct to the purchasers, without passing through local markets, showed convincingly that the infection had been brought into East Anglia with the calves. It seems probable that cross-infection occurred either at the dealer's premises or during transit.

Farm outbreaks, East Yorkshire

Similar observations in the East Yorkshire area (Leeds Veterinary Investigation Centre; Hull Public Health Laboratory) were made during 1959 and 1960. Here it was found that 18/19 incidents of salmonella infection in calves followed within 2 weeks of the arrival of calves on the farms. These calves were purchased locally and from Lancashire, Hertfordshire, Cheshire and South Wales. It is of interest

that the calves responsible for two incidents due to *S. typhi-murium* 1a (U57) came from South Wales. This phage-type was responsible for some bovine infection in Glamorgan in 1958.

Contact with other animals in transit

Contact with other farm animals in the course of calf movement may be responsible for some infection. An outbreak caused by *S. heidelberg* occurred on a farm receiving calves from the Chichester area. These calves arrived at irregular intervals over a period of 3 months from one dealer. During investigation of the source of the outbreak, *S. heidelberg* was isolated from pig faeces collected at the market through which these calves passed. Pigs were taken to the market in lorries. Calves were taken from the market in the same lorries and cross infection may have taken place in these vehicles.

Persistence of infection on farm premises

The Norwich Veterinary Investigation Centre demonstrated the survival of *S. dublin* for up to 36 weeks in pats of infected faeces on rough grassland, and for at least 11 months on the walls of a loose-box soiled by a carrier cow. A group of young calves housed in the loose-box at the end of this period developed infection with *S. dublin* within 2–4 days.

It is known that salmonellae can exist a very long time in faeces and on pasture. For instance, Henning (1939) showed *S. dublin* to survive for at least 1069 days in artificially dried bovine faeces. Josland (1951) in Australia demonstrated that *S. typhi-murium* could be recovered from shaded pastures over periods ranging from 14 to 22 weeks. Watts & Wall (1952), using earth contaminated with infected sheep faeces, found that samples were still positive for *S. typhi-murium* after 200 days. Mair & Ross (1960), showed the survival of *S. typhi-murium* in garden soil in this country for at least 251 days.

Table 11. *Salmonellae in animal feedingstuffs in relation to bovine salmonellosis*

	Raw materials	Protein conc. meals	Meals/ mashes	Pelleted foods	Bovine sources
Total samples	2801	893	1955	1459	—
Samples positive	587	94	64	8	2116
S. dublin	2 (0·3)	0	0	0	1375 (64·9)
S. typhi-murium	37 (6·3)	5 (5·3)	0	1 (12·5)	560 (26·5)
Other serotypes	548 (93·4)	89 (94·7)	64 (100)	7 (87·5)	181 (8·6)

Figures in parentheses are percentages of number of positive samples.

Persistence of infection in a locality

The development of phage-typing of *S. typhi-murium* has enabled this to be observed more effectively than hitherto.

In Monmouthshire, incidents occurred due to *S. typhi-murium*, phage-type

2 (12), on farms in the same area in 1960 and 1961. Pigs were involved in the first incident and a heifer and poultry in the second. Persistence of infection in a locality has also been recorded by Anderson, Galbraith & Taylor (1961). The infections observed were caused by *S. typhi-murium*, phage-type 20a.

DISCUSSION

Table 11 presents the relationship between salmonellae isolated from animal feedingstuffs and salmonellae isolated from bovine sources. Both home produced and imported animal feedingstuffs are included. This table is derived from data in Table 3 and from sources already published (Walker, 1957; Report, 1959*a*; Report, 1961; Harvey & Price, 1962). The data as presented in Table 11 are not in the form in which they were originally published.

The contrast between serotypes commonly found in feedingstuffs and those found in bovine infections is marked. The most important types found in bovine salmonellosis are *S. dublin* and *S. typhi-murium*. These two serotypes are rarely isolated from animal feedingstuffs. Dutch and American workers have made somewhat similar observations in their own countries (van der Schaaf, van Zijl & Haagens, 1962; Edwards, 1962). This evidence does not suggest that animal feedingstuffs are an important cause of bovine infection due to *S. dublin* and *S. typhi-murium*.

When, however, the phage-types of *S. typhi-murium* found in animal feedingstuffs and in bovines are compared, some similarity of type emerges. This may be fortuitous and may indicate that materials derived from native animals have been used for the manufacture of the meat and bone meal present in these feedingstuffs. Seven out of the ten phage-types listed in the last column of Table 7 have been found in bovine infection in the United Kingdom (Anderson, E. S., Personal Communication). These phage-types are 1a (2); 1a (U57); 2 (10); 2 (12); 2 (12a); 22; and 32, although types 2 (12a) and 22 were not encountered in cattle during the present survey period, 1959–61.

In Table 7, nine phage-types are marked with an asterisk as being frequent causes of human disease (Anderson, E. S., Personal Communication). In 1961, these types were together responsible for 70 % of human infection with *S. typhi-murium* in England and Wales. Their order of frequency in that year was as follows:

2c (14) 1a (2) 1 var. 5 (U9) 1a var. 1 (3an) 2 (12a)
1 (1) 4 (8) 1a (U57) 2 (12)

It is pointed out in a footnote to Table 7 that types 2c (14) and 4 (8) are commonly associated with fowls and ducks respectively, and these avian sources are probably responsible for a considerable proportion of infections caused by these two types. In 1961, types 2c (14) and 4 (8) caused jointly some 23 % of human infections.

During 1959–61, the majority of animal strains of type 2 (12a) were isolated from pigs, although more recent work has shown it to be commonest in bovines. The remaining six phage-types, 1a (2), 1 var. 5 (U9), 1a var. 1 (3an), 1 (1), 1a (U57) and 2 (12), are primarily of bovine origin. These types were responsible, in 1961, for 41 % of human infections, a high figure for a single animal source. This is

all the more striking when it is pointed out that the total number of phage-types of *S. typhi-murium* isolated in 1961 was about 80. All the above information is derived from Anderson (in the press).

Finally, when animal feedingstuff isolations of *S. typhi-murium* are compared simultaneously with bovine and human isolations in Table 7, it is noted that phage-types 1a (2), 1a (U57) and 2 (12) are commonly found both in cattle and in man.

Two local comparisons of cattle and human strains of *S. typhi-murium* were also made. In Yorkshire, before 1959, phage-type 1 var. 5 (U9) had not been isolated. In 1959, however, this type was found both in cattle and in man and since then has become the dominant type in human infections. In South Wales, comparison of the phage-types of *S. typhi-murium* isolated from local animals and from local infections in man has been routine practice since 1958 (Harvey & Phillips, 1961; Harvey *et al.* 1963). From 1958 to 1961, phage-types 1a var. 1 (3an), 1a (U57), 1 (1) and 2 (12) have been isolated from local cattle in this sequence. The same phage-types have been isolated over the same period and *in the same sequence* from human infections in Glamorgan. The human infections invariably occurred within a few weeks of the bovine isolations. The association is naturally emphasized by the coincidence in location and timing.

Further work is clearly required into the epidemiological significance of animal feeds as a source of salmonellosis. Although, as yet, there is little evidence to incriminate them as a major cause of bovine salmonellosis, their importance should not be entirely disregarded as a potential starting point of a chain of infection with man as the ultimate host (Report, 1961; Niven, 1962).

The spread of bovine salmonellosis, particularly infections caused by *S. dublin* and *S. typhi-murium*, may be attributed largely to the movement of cattle and calves. The movement of calves is probably the most important factor.

The seasonal incidence of infection is shown in Tables 4 and 5. It is evident that with both *S. dublin* and *S. typhi-murium* the peak incidence occurs in the last quarter of the year. It is suggested that the concentration of calf infections in this quarter is connected with the sale and movement of calves through markets and dealers' premises to calf rearing areas and abattoirs and with overcrowding of a susceptible population.

The conditions under which calves travel encourage the spread of disease, as animals are herded together in temporary accommodation and lorries on their way to their ultimate destination. Stress factors such as travel weariness, dietary changes and chilling on the journey are also thought to increase susceptibility to infection.

The seasonal incidence of *S. typhi-murium* infection in bovines is different from that in man, as warm atmospheric temperatures leading to bacterial multiplication in food is a major factor in determining the human peak incidence in the third quarter of the year. This factor is of little importance in bovine infection.

In Table 6 the frequency of infection due to *S. dublin* and *S. typhi-murium* is apparent, as is the high relative incidence of *S. dublin* infection in South Wales. This is in keeping with previous surveys (Field, 1948, 1949*a*, *b*). Gibson (1961), commented on the fact that *S. dublin* is endemic in adult and yearling cattle in

South Wales whereas it is rarely found in adult cattle in East Anglia. There is a marked contrast between these two regions, because South Wales is a breeding area in which the effect of calf movement is little felt, and East Anglia is a rearing area where the movement of calves is probably of major importance. The distinction between breeding and rearing areas is not quite so clear cut in the other regions.

Table 8, which gives details of the spread of salmonella infection to different animal species in ten of the incidents studied by members of the Working Party, places the limited problem of bovine salmonellosis in a wider context. The discovery, in two of the episodes in Table 8, that poultry were infected is in keeping with the observations of previous authors (Stenert, 1938; Sellers & Sinclair, 1953), who found poultry infections in mixed farms where cattle salmonellosis was discovered. This finding is possibly of importance. Müller (1957) stated that in Denmark *S. typhi-murium* infection of calves is often traced to poultry. It is possible that some bovine salmonellosis in this country may originate from the same source.

The farm dogs from which salmonellae were isolated in Incidents 2 and 4 (Table 8), were probably examples of secondary infection. Dogs may play some part in the persistence of infection on a farm or in the transfer of salmonellae to young children in close contact with them.

Cross-infection of calves during transport has been discussed. The observations on the effect of moving young animals from breeding to rearing areas also suggest strongly that infection is conveyed to the rearing areas by this means. Gibson (1961) considered that *S. dublin* was introduced into East Anglia by calves bought from areas such as those in South Wales (Field, 1948, 1949*a*, *b*), where the serotype causes endemic infection in cattle. It would appear that the transfer of calves from Wales has introduced particular phage-types of *S. typhi-murium* into Yorkshire and East Anglia.

The length of time during which infection persists in a herd after termination of an outbreak of clinical disease depends on a number of factors including the following:

(*a*) The duration of the active carrier state in recovered clinical cases and in symptomless excreters.

(*b*) The establishment of infection in other animals in contact with the infected cattle.

(*c*) The survival of organisms shed by infected animals.

Adult and yearling cattle recovering from clinical salmonellosis due to *S. dublin* usually excrete the organisms constantly in the faeces for years, possibly for life. Subclinical infection can also result in an active carrier state of similar duration. The term 'active carrier' denotes an apparently healthy animal which excretes salmonellae in its faeces; a 'latent carrier' is an animal from whose organs salmonellae can be isolated although they are not found in the faeces during a limited number of examinations. It is thought that various stress factors, such as intercurrent disease, can cause latent carriers to become active carriers, or to develop clinical signs of salmonellosis. The carrier state that occurs in adult cattle

after infection with *S. typhi-murium* probably does not last as long as that induced by *S. dublin* (Report, 1959*b*).

In contrast to what occurs in adult cattle, calves that recover clinically from either *S. dublin* or *S. typhi-murium* infection almost invariably cease to excrete the organism, although it is possible that latent carriage may persist in some of these animals (Gibson, 1958). Exceptionally, active carriage may continue (Field, 1948).

As adult cattle may be active carriers of *S. dublin* for long periods and may give rise to gross contamination of pastures and buildings, the question of the disease establishing itself in other domestic or wild animals would seem to be of secondary importance in maintaining infection. Infection of farm rats (Field, 1948; Gibson, 1958, 1961) and sheep (Shearer, 1957; Watson, 1960), may be considered as possible causes of prolonging *S. dublin* incidents on a farm, but observations on farm rats in contact with infected livestock do not suggest that rodents form permanent reservoirs of *S. dublin*. Cross-infection of other animal species seems to be more important with *S. typhi-murium* because of the wide host range of this serotype compared with *S. dublin*.

Table 12. *Holding time in abattoir lairages in England and Wales*

Maximum holding time (days)	Calves	Older Cattle
1	117	106
2	10	47
3	2	31
4	4	24
5	0	8
6	0	6
7	0	15
Over 7	0	4

McDonagh & Smith (1958) produced evidence to show that the shortening of the holding time in Bradford abattoir controlled cross-infection in animals and was followed by a reduction in human salmonellosis in the area. The importance of cross-infection in lairages has also been demonstrated by Galton, Smith, McElrath & Hardy (1954) and Anderson, Galbraith & Taylor (1961).

Information on the range of holding times in a series of abattoirs was collected by members of the Working Party. This information is given in Table 12. It is obvious that the range is wide and that holding times could profitably be shortened by extension of legislation.

On farms, cross-infection can be reduced by preventing indiscriminate intermingling of cattle and other animals (Dolman, 1957).

Conclusions

When we consider the control of salmonellosis, many difficulties are encountered. Several lines of attack on the problem do exist, that on the animal-to-animal cycle probably being the most important (Dolman, 1957; Newell, 1959). Methods of practical control of infection can be classified under three main headings:

(1) *Prevention of infection of cattle on farms*

(*a*) The freeing of animal feedingstuffs from salmonellae.

Although such materials are apparently unimportant in causing *S. dublin* infection, they carry other salmonella serotypes which may be responsible through cattle for human disease. Since Report (1961) appeared the contamination of animal feedingstuffs with salmonellae has been greatly reduced by selection of raw materials. Bone meal is not used by compounders as a source of minerals in animal foods on account of the risk of its containing anthrax spores, and steamed bone flour has been, to a great extent, replaced by purely inorganic phosphates, which are free from micro-organisms (McCoy, J., Personal Communication). The identification of Angola sun-dried fish meal as the most heavily contaminated raw material of animal foods led to its disappearance. The introduction of new and improved processing plants for the production of meat meal and meat and bone meal from native animal wastes has reduced the salmonella content of materials to probably the irreducible minimum. In addition to the reduction in contamination of raw materials, the process of pelleting is known to diminish 10-fold the salmonella content of meals and mashes produced from contaminated raw materials. It must be appreciated that even the sterilization of all animal foods used in the United Kingdom could not prevent infection of man from the serotypes of salmonellae established in native flocks and herds. These are responsible for the majority of human infections.

(*b*) The maintenance of a high standard of cleanliness in farm buildings.

(*c*) It has been shown that the purchase of infected calves from other farms and markets is important in the introduction of salmonellosis into clean herds. It is difficult to see how this source can be controlled.

(2) *Prevention of spread of infection from animal to animal in transit*

(*a*) Implementation of the recently introduced Statutory Instrument (S.I. 1963) requiring improved conditions of transport for young calves.

(*b*) Control of housing of animals during transport. Special attention should be paid to calf housing with the object of reducing stress factors.

(*c*) Encouragement of direct sales to eliminate intermediate housing.

(3) *Prevention of spread from animals to man*

(*a*) Reduction of holding time for live animals en route for the abattoir.

(*b*) Improvement of abattoir hygiene in order to avoid contamination of flesh by infected material such as faeces.

(*c*) Improved hygiene during transport of carcasses from abattoirs.

(*d*) Improvement of hygiene in butchers' shops in order to minimize cross-contamination of meat.

(*e*) Increased use of refrigeration in shops and houses in order to prevent multiplication of salmonellae in the final products.

SUMMARY

1. The incidence of salmonellosis in cattle in England and Wales has been investigated.

2. *S. dublin* and *S. typhi-murium* were much the commonest serotypes encountered.

3. The frequency of both serotypes was highest in the last quarter of the year, which is the period of maximum calvings. The high incidence of calf infection in this quarter is probably related to calf sales and movement.

4. In the previous investigations (Reports, 1959*a*, 1961), these serotypes were isolated only infrequently from the raw materials of animal feedingstuffs and from complete animal foods.

5. The distribution of phage-types of *S. typhi-murium* in cattle was compared with that in man and in animal feedingstuffs. Six phage-types were common both in cattle and in man. These types were: 1a (2), 1 var. 5 (U9), 1a var. 1 (3an), 1 (1), 1a (U57) and 2 (12). Three of the types found in feedingstuffs were common in both human and bovine infections. These types were: 1a (2), 1a (U57) and 2 (12).

6. In a study of ten incidents of salmonellosis in cattle, it was shown that the same phage-type or serotype was commonly found in man in other farm animals and occasionally in feedingstuffs.

7. It is clear that salmonellosis is spread into different regions of the country by calf movement.

8. The importance is endorsed of conditions of travel and lairage, and of the holding time at abattoirs, in the promotion of cross-infection with salmonellae.

9. Measures are outlined for the reduction of salmonellosis in cattle and of its transfer to man.

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The influence of mineral carriers on the simultaneous active and passive immunization of guinea-pigs against tetanus

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INTRODUCTION

There is considerable interest in the use of adsorbed tetanus toxoid given concurrently or at close intervals with prophylactic horse tetanus antitoxin as a means of conferring immediate protection against tetanus, whilst promoting potential active immunity at the same time.

Simultaneous administration of antitoxin with fluid tetanus toxoid was found to inhibit the normal response to toxoid in humans (Barr & Sachs, 1955). This was also shown in rabbits by Suri & Rubbo (1961) and in guinea-pigs by Smith (1964) who showed that in similar tests with adsorbed preparations interference was much less.

Adsorbed preparations of tetanus toxoid in simultaneous active and passive immunization against tetanus have been used with satisfactory results by several workers in the field (Gold & Bachers, 1943; Ericcson, 1948; Tasman & Huygen, 1962; Eckmann, 1959; Smith *et al.* 1963).

The experiments recorded in this paper were carried out to investigate further the differences betweeen adsorbed and fluid preparations of tetanus toxoid in simultaneous immunization procedure.

MATERIALS AND METHODS

Tetanus antitoxin (horse)

A single preparation of pepsin refined horse globulin (Harms, 1948) was used in all experiments. The high-potency material was suitably diluted to contain 150 units of antitoxin in 1·0 ml.

Tetanus toxoid

Two preparations of concentrated tetanus toxoid were used to provide adsorbed toxoids of suitable strength. The first preparation was used in those experiments recorded in Tables 1–7 and the second preparation for those in Tables 8–14. No comparison has been drawn between these two series of results. The adsorbed materials were prepared by adding calculated amounts of concentrated toxoid (*ca.* 1000 Lf/ml.) to suitably diluted and buffered adsorbent. The material was left for 7 days at room temperature with occasional shaking for adsorption to take place.

* Dr Fulthorpe died while this paper was in the press. Requests for reprints should be addressed to The Secretary, Department of Immunology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent.

Toxoid estimation by flocculation

Constant volumes of a suitable dilution of toxoid were pipetted into a series of $\frac{3}{8} \times 3$ in. glass tubes. A working standard antitoxin (23 units/ml.) was added in volumes differing by 10%. The tubes were incubated at 50° C., with one-third length of the tubes immersed to allow for convection, and inspected at intervals. Estimation of the toxoid content of the preparation was based on the principle that the mixture which flocculated most rapidly contained an equal number of Lf doses of toxoid and provisional *in vitro* units of antibody.

Toxoid estimation by the in vivo *total combining power test (TCP)*

Volumes of toxoid, suitably diluted to contain about 1 unit equivalent of toxoid and differing by 20%, were measured into a series of tubes and a constant volume of antitoxin containing 2 units was added, the mixtures were gently shaken and allowed to stand for 1 hr. Constant volumes of a test toxin equivalent to 1 unit of standard antitoxin were then added and mixed and allowed to stand for $\frac{1}{2}$ hr. The whole mixture (restricted to a reasonable total volume) was then injected into mice. The mice were observed twice daily and the end-point of the *in vivo* test was based on the deaths of the test animal on the fourth day or definite symptoms of tetanus on the third day. If an animal was dead on the second or third day and the next animal in the series was alive, the end point was arrived at by interpolation. Several estimates were made on each preparation and the value allotted was the arithmetic mean of individual results. It has been observed that with a satisfactory toxoid the dose of toxoid equivalent to 1 unit, as determined by this method, was considerably lower than the Lf dose (Fulthorpe & Thomson, 1960).

Antitoxin titrations

The titrations of antitoxin in guinea-pig sera were carried out in mice by the technique of Glenny & Stevens (1938).

RESULTS

Simultaneous immunization with fluid toxoid

Guinea-pigs given two doses each of 2·25 Lf of fluid tetanus toxoid, at 28 days interval, were bled 10 days after the second dose of toxoid, and the antitoxin titres of individual animals estimated by *in vivo* titration in mice. The group was found to have responded well, with a geometric mean titre of 28·2 units/ml. (Table 1). When similar groups were also given 150 units of horse tetanus antitoxin (pepsin refined globulin) by the intraperitoneal route at different periods of time in relation to the first dose of toxoid, the antigenic responses were grossly impaired and the difference between these results and that with toxoid alone were significant ($P = < 0{\cdot}05$). There was no significant difference however, between results when antitoxin was given from 4 hr. before to 4 days after the toxoid ($P = > 0{\cdot}05$); in a considerable proportion of the guinea-pigs in these groups no detectable secondary response was observed. When doses of 20–50 Lf of toxoid were given concurrently with antitoxin very little improvement in response occurred.

Table 1. *The effect of horse tetanus antitoxin (pepsin refined globulin) 150 units, on the antigenic responses of guinea-pigs to two doses of 2·25 Lf of* fluid *tetanus toxoid given subcutaneously at 28 days interval*

(The horse antitoxin given intraperitoneally at different times in relation to the first dose of toxoid. Guinea-pigs bled 10 days after second dose of toxoid.)

Horse tetanus antitoxin	No. of guinea-pigs with antitoxin titres (units/ml.)															Geometric means
	< 0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·5–1·0	1·0–2·0	2·0–5·0	5·0–10·0	10–20	20–50	50–100	> 100	Total	
Not given	—	—	—	—	—	—	—	—	—	1	1	6	1	1	10	28·2
150 units 4 hr. before toxoid	2	5	1	—	2	—	—	—	—	—	—	—	—	—	10	< 0·020
150 units concurrently with toxoid	4	3	1	—	1	—	—	—	—	—	—	—	—	—	9	< 0·016
150 units 4 hr. after toxoid	5	2	2	—	1	—	—	—	—	—	—	—	—	—	10	< 0·015
150 units 24 hr. after toxoid	7	—	—	1	2	—	—	—	—	—	—	—	—	—	10	< 0·020
150 units 4 days after toxoid	2	2	2	1	1	—	1	—	—	—	—	—	—	—	9	< 0·037

Simultaneous immunization with adsorbed toxoid

A similar experiment was carried out using 2·25 Lf of tetanus toxoid adsorbed on aluminium hydroxide (Table 2). The antigenic response to the toxoid alone (G.M. 38·7 units/ml.) was of the same order as that obtained with fluid toxoid. When passively administered antitoxin was given as before there was a reduction in the response to the toxoid. The differences in response were, however, not

Table 2. *The effect of horse tetanus antitoxin (pepsin refined globulin)* 150 *units, on the antigenic responses of guinea-pigs to two doses of* 2·25 Lf *of* adsorbed *tetanus toxoid given subcutaneously at* 28 *days interval*

(The horse antitoxin given intraperitoneally at different times in relation to the first dose of toxoid. Guinea-pigs bled 10 days after second dose of toxoid.)

	No. of guinea-pigs with antitoxin titres (units/ml.)								
Horse tetanus antitoxin	1·0–2·0	2·0–5·0	5·0–10·0	10–20	20–50	50–100	>100	Total	Geometric means
Not given		2			8	6	1	17	38·7
150 units 4 hr. before toxoid		2	1	3	1	3		10	15·3
150 units concurrently with toxoid		1	1	6	2	5		15	21·2
150 units 4 hr. after toxoid	3	1	3	4	2	1	1	15	9·9
150 units 24 hr. after toxoid		1	1	5	7	1		15	16·3
150 units 4 days after toxoid			1	2	5	5	1	14	33·1

significant between those results where the antitoxin was given 4 hr. before, concurrently with, 24 hr. or 4 days after the first dose of toxoid when compared with the response to toxoid alone. The response observed when the antitoxin was given 4 hr. after the first dose of toxoid was, however, significantly different from that obtained with toxoid alone ($P = < 0{\cdot}05$). There is a suggestion in these results that there is an optimum time for interference by passive antitoxin in the simultaneous immunization method. The immunizing efficiency of adsorbed toxoid in the presence of passive antitoxin was thus found to be satisfactory and remarkably different from that observed when fluid toxoid was used.

When the dose of antitoxin given concurrently with 2·25 Lf of aluminium hydroxide adsorbed tetanus toxoid was increased progressively (Table 3), it was found that there was no statistically significant reduction in response with 300, 600 or 1200 units of antitoxin but that a significant reduction in response occurred when 2400 units was given ($P = < 0{\cdot}05$).

The route of injection of the concurrently administered antitoxin did not significantly affect the responses to toxoid except where the intravenous route was used (Table 4). It must be assumed that the greater effectiveness of antitoxin administered in this way was due to rapid transfer to the site of toxoid injection.

Table 3. *The effect of increasing doses of horse tetanus antitoxin (pepsin refined globulin) on the antigenic responses of guinea-pigs to two doses of* 2·25 Lf *of* adsorbed *tetanus toxoid given subcutaneously at* 28 *days interval*

(The horse antitoxin given intraperitoneally at the same time as the first dose of toxoid. Guinea-pigs bled 10 days after the second dose of toxoid.)

	No. of guinea pigs with antitoxin titres (units ml.)								
Horse tetanus	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	> 100	Total	Geometric means
Not given	—	2	—	—	8	6	1	17	38·7
150 units	—	1	1	6	2	5	—	15	21·2
300 units	—	1	2	1	4	2	—	10	19·5
600 units	—	—	1	3	4	2	—	10	23·2
1200 units	—	1	1	3	3	1	1	10	18·4
2400 units	3	—	3	1	2	1	—	10	7·7

Table 4. *The effect of giving* 150 *units of horse tetanus antitoxin (pepsin refined globulin) on the antigenic responses of guinea-pigs to two doses of* 2·25 Lf *of* adsorbed *tetanus toxoid given subcutaneously at* 28 *days interval.*

(The horse antitoxin given by different routes at the same time as the first dose of toxoid. Guinea-pigs bled 10 days after the second dose of toxoid.)

	No. of guinea-pigs with antitoxin titres (units/ml.)										
150 units of antitoxin given	0·1–0·2	0·2–0·5	0·5–1·0	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	Total	Geometric means
Intraperitoneal	—	—	—	—	1	1	6	2	5	15	21·2
Subcutaneous	—	—	—	—	2	1	4	1	2	10	13·6
Intramuscular	—	—	—	1	—	2	3	3	1	10	13·5
Intravenous	1	—	—	3	—	1	2	2	1	10	5·1

Elimination of passive antitoxin

In order to investigate the rise of actively produced antitoxin in actively and passively immunized animals it was first necessary to investigate the rate of turn-out of passively administered horse tetanus antitoxin in normal and serum-sensitive guinea-pigs. Groups of animals (250–350 g.) were injected with 150 units of antitoxin by the intraperitoneal route and separate groups were bled at intervals and the antitoxin titres estimated (Table 5). After 24 hr. the antitoxin titres ranged from 3·3 to 4·5 units/ml., after 7 days approximately one-tenth of this (0·42–0·47) and after 14 days most animals in the group had 0·02–0·05 units/ml. At 21 days there was no measurable antitoxin (< 0·01 units/ml.). In serum-sensitive guinea-pigs, i.e. animals which had received 150 units of antitoxin 3 months previously, the rate of loss of a further dose of antitoxin was much accelerated; at 7 days several members of the group had no measurable antitoxin and the titres in the remainder varied from 0·02 to 0·5 units/ml., and at 14 days there was no measureable antitoxin in that group. These observations indicate the scavenging effect of anti-horse antibodies and the considerable degree of variation between different individuals in the effectiveness of this process.

Table 5. *Antitoxin titres (units/ml.) of separate groups of guinea-pigs* (250–350 g.) *at different times following a single intraperitoneal injection of* 150 *units of horse tetanus antitoxin (pepsin refined globulin)*

(A) In normal guinea pigs.
(B) In serum sensitised guinea-pigs (450–650 g.).

(A)

Guinea-pig no.	Group 1 24 hr. later	Group 2 7 days later	Group 3 14 days later	Group 4 21 days later
1	4·0	0·42	0·01–0·02	< 0·01
2	4·0	0·45	0·02–0·05	< 0·01
3	3·6	0·45	0·02–0·05	< 0·01
4	4·5	0·42	0·02–0·05	< 0·01
5	4·0	0·45	0·02–0·05	< 0·01
6	4·0	0·45	0·02–0·05	< 0·01
7	4·5	0·45	0·02–0·05	< 0·01
8	4·0	0·45	0·05	< 0·01
9	3·3	0·47	0·01	< 0·01
10	4·0	0·42	0·01	< 0·01

(B)

	Group 5 7 days later	Group 6 14 days later
1	< 0·01	< 0·01
2	0·1–0·2	< 0·01
3	0·2–0·5	< 0·01
4	< 0·01	< 0·01
5	0·1–0·2	< 0·01
6	0·2–0·5	< 0·01
7	0·02–0·05	< 0·01
8	< 0·01	< 0·01
9	< 0·01	< 0·01

Rate of development of actively produced antitoxin

Two further groups of guinea-pigs (Table 6A and B) were immunized with two doses of 2·25 Lf of fluid toxoid at 42 days interval. One of these groups was in addition given 150 units of antitoxin intraperitoneally at the same time as the first dose of toxoid. All animals were bled at 14, 21, 28 and 42 days and the antitoxin titres following the primary response were estimated. A further titration was made at 52 days to estimate the value for the secondary response. At 14 days it was found that in the group given toxoid only, eight out of ten animals had measurable antitoxin and that active production of antitoxin had begun. In the group given antitoxin concurrently with the first dose of toxoid the values obtained were of the same order as those found when antitoxin alone was given (Table 5A).

Table 6. *Antitoxin titres (units/ml.) of two groups of guinea-pigs*

((A) Given 2·25 Lf of *fluid* tetanus toxoid subcutaneously, and (B) given 2·25 Lf of *fluid* tetanus toxoid subcutaneously and 150 units of tetanus antitoxin (pepsin refined globulin) intraperitoneally at the same time. All animals bled 14, 21, 28 and 42 days later, a further 2·25 Lf of toxoid given on the 42nd day and the animals again bled 10 days later.)

Guinea-pig no.	Day 14	Day 21	Day 28	Day 42	Secondary response Day 52
			(A)		
1	< 0·01	0·01–0·02	0·01–0·02	0·2–0·5	5–10
2	0·02–0·05	0·1	0·5–1·0	1–2	50–100
3	0·01	0·02–0·05	0·5–1·0	1·0	50–100
4	0·01–0·02	0·02–0·05	0·5–1·0	0·5–1·0	20–50
5	0·01–0·02	†	†	†	†
6	< 0·01	< 0·01	< 0·01	0·01–0·02	5–10
7	0·01–0·02	0·02	0·01–0·02	0·1	10
8	0·02–0·05	0·02–0·05	0·02	0·02–0·05	20–50
9	0·02–0·05	0·02–0·05	0·5–1·0	1–2	50–100
10	0·02–0·05	0·02	0·5	1–2	50–100
G.M.	< 0·016	< 0·026	< 0·15	0·31	28·6
			(B)		
1	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
2	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
3	0·02–0·05	< 0·01	< 0·01	< 0·01	0·5
4	0·02	< 0·01	< 0·01	< 0·01	0·01
5	0·02–0·05	†	†	†	†
6	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
7	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
8	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
9	0·02–0·05	< 0·01	< 0·01	< 0·01	1–2
10	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
11	0·05	< 0·01	< 0·01	< 0·01	< 0·01
12	0·01–0·02	< 0·01	< 0·01	< 0·01	0·01–0·02
13	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
14	0·05	< 0·01	< 0·01	< 0·01	< 0·01
15	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
G.M.	0·03	< 0·01	< 0·01	< 0·01	< 0·02

† Animal died.

At 21, 28 and 42 days the group given fluid toxoid alone showed a slow rise in titre with some animals responding much better than others. The final titres for

this group after a second dose of toxoid varied from 5 to 100 units/ml., the highest values being given by the animals in which antitoxin appeared at an early stage and increased most rapidly in titre after the first injection of toxoid. Where antitoxin was given concurrently with the first dose of toxoid, passive antitoxin was, as expected, not present at 21 days and no circulating antitoxin was found and, after the second dose of toxoid, only four animals of a total of fourteen gave a measurable secondary response.

Further groups of guinea-pigs were treated with aluminium hydroxide adsorbed tetanus toxoid (2·25 Lf) with and without concurrently administered antitoxin, and combined immunization was carried out in a similar fashion with serum sensi-

Table 7. *Antitoxin titres (units/ml.) of three groups of guinea-pigs*

((A) Given 2·25 Lf of tetanus toxoid *adsorbed* on aluminium hydroxide, subcutaneously, (B) 2·25 Lf of *adsorbed* toxoid subcutaneously and 150 units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the same time, and (C) horse serum sensitized guinea-pigs treated with both toxoid and antitoxin. All animals bled 14, 21, 28 and 42 days later, a further 2·25 Lf of *adsorbed* toxoid given on the 42nd day and the animals bled 10 days later.)

Guinea-pig no.	Day 14	Day 21	Day 28	Day 42	Secondary response Day 52	
			(A)			
1	0·01	1·0	2–5	2–5	100	
2	0·01	0·1	0·2	0·2–0·5	20–50	
3	0·02–0·05	2·0	5·0	5–10	100–200	
4	0·01	1–2	1–2	2–5	50–100	
5	0·02–0·05	1·0	2–5	5–10	10–20	
6	0·01	0·2	1·0	1·0	10–20	
7	0·01–0·02	2·0	2·0	5–10	20–50	
9	0·01–0·02	1·0	1·0	†	†	
10	0·01–0·02	1·0	2·0	2–5	100–200	
G.M.	0·014	0·78	1·6	2·8	47·9	
			(B)			
13	0·02	< 0·01	< 0·01	< 0·01	10–20	
16	0·02–0·05	< 0·01	< 0·01	< 0·01	5–10	
8	0·02–0·05	< 0·01	< 0·01	0·01–0·02	10–20	G.M.
4	0·02–0·05	< 0·01	< 0·01	0·02–0·05	10–20	11·9
1	0·02	< 0·01	< 0·01	0·05–0·1	10	
12	0·02–0·05	< 0·01	< 0·01	0·01	10–20	
19	0·02	< 0·01	0·02	0·1–0·2	20–50	
18	0·01–0·02	< 0·01	0·05–0·1	0·2	20–50	
2	0·02–0·05	0·01–0·02	0·5–1·0	0·5–1·0	10–20	G.M.
9	0·01	0·01–0·02	0·02–0·05	0·02–0·05	10–20	27·5
7	0·05	0·05	0·2–0·5	0·2–0·5	10–20	
10	0·02–0·05	0·02	0·5–1·0	0·5–1·0	20–50	
3	0·01–0·02	0·05–0·1	0·2–0·5	1–2	50–100	
6	0·02	0·05	0·2–0·5	1–2	20–50	
14	0·01–0·02	0·1–0·2	0·5	1–2	50	G.M.
20	0·01–0·02	1·0	1·0	2·0	10–20	28·1
17	0·05	1–2	2·0	2·0	20–50	
15	0·05	2·0	2–5	2–5	50	
G.M.	0·025	< 0·038	< 0·11	< 0·23	20·9	

† Animal died.

Table 7 (*cont.*)

(C)

1	0·01–0·02	0·2–0·5	1–2	2–5	20–50
2	< 0·01	0·01–0·02	0·1	1–2	10
3	0·01–0·02	0·1–0·2	1–2	2–5	20–50
4	< 0·01	0·1–0·2	0·5	†	†
5	0·01–0·02	0·2	1·0	2–5	20–50
6	< 0·01	0·1–0·2	0·2–0·5	0·5–1·0	2–5
7	0·02–0·05	0·02	0·1–0·2	1·0	20
8	< 0·01	0·2–0·5	1–2	†	†
9	< 0·01	0·1–0·2	1·0	2–5	20
10	< 0·01	0·05	1·0	†	†
11	0·01	0·01	0·2–0·5	1·0	2–5
12	0·01	0·1	0·5	2·0	5–10
13	< 0·01	0·02	0·5	1–2	20–50
14	0·01–0·02	2–5	10	10–20	50–100
15	< 0·01	0·02	1–2	2–5	20–50
16	0·02–0·05	0·01–0·02	0·01	0·2–0·5	1–2
G.M.	< 0·011	0·078	0·55	1·9	14·1

tive animals. These groups were bled at intervals as before (Table 7A, B and C). The group given adsorbed toxoid only showed active antitoxin production at 14 days of a similar order to that found in Table 6A with fluid toxoid alone. Thereafter the rise in titre with adsorbed toxoid was more rapid and although the final titres after the second dose of toxoid were not proportionately as great, this could well be due to a flattening of the dose response curve when approaching the optimum response for this dose and time interval. When 150 units of antitoxin was given with the first dose of adsorbed toxoid (Table 7B), the antitoxin titres at 14 days were of the same order as those found with antitoxin alone. At 21 days there was evidence of active antitoxin production in ten out of eighteen animals; the titres at this time were, however, considerably lower than those found where no horse tetanus antitoxin had been given, and the subsequent rise in titre was much slower. Even at 42 days two animals had failed to produce measurable antitoxin, while others had responded well, and the picture was one of considerable individual variation between animals. All members of this group responded well to a second dose of toxoid, and arrangement of the results according to the primary response indicates that interference with the primary response did in fact have a slightly depressing effect on the secondary response.

Serum-sensitive guinea-pigs were immunized with adsorbed toxoid and given 150 units of antitoxin at the same time as the first dose of toxoid. At 14 days it was found that eight out of sixteen animals had no detectable antitoxin presumably as a result of rapid removal of the passively acquired antitoxin. The remainder of animals in the group had a low level of circulating antitoxin. It was hardly possible that this was horse globulin since when antitoxin alone was given to serum-sensitive animals (Table 5B) it was found that after 14 days no detectable antitoxin remained. It must therefore be assumed that active antibody production had begun in these animals, in spite of the concurrent administration of heterologous antitoxin. Moreover, when the results in Table 7B and C are compared it can be seen

Table 8. *Antitoxin titres (units/ml.) of groups of guinea-pigs given two doses of* 2·25 Lf *of tetanus toxoid, with different aluminium hydroxide content, subcutaneously at* 42 *days interval and* 150 *units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the time of the first dose of toxoid. All animals bled* 10 *days after the second dose of toxoid*

Aluminium hydroxide (μg./Lf)	No. of guinea-pigs with antitoxin titres (units/ml.)													Geometric means
	< 0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·5–1·0	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	Total	
71·7 (M/27)	—	—	—	—	—	—	—	—	1	2	3	5	4	15	22·4
33·0	—	—	—	—	1	—	—	1	1	1	2	7	—	13	10·2
16·5	—	—	1	—	1	—	—	1	1	2	3	—	3	12	5·1
8·2	5	3	1	2	—	—	1	1	—	—	1	—	1	15	< 0·078
3·8	11	1	—	—	—	1	—	1	—	—	—	—	—	14	< 0·018
1·6	11	1	—	—	—	1	—	—	—	—	—	1	—	14	< 0·022
Nil	11	1	—	2	—	—	—	1	—	—	—	—	—	15	< 0·013

Table 9. *Antitoxin titres (units/ml.) of groups of guinea-pigs given two doses of* 2·25 Lf *of tetanus toxoid with different aluminium phosphate content, subcutaneously at* 42 *days interval and* 150 *units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the same time as the first dose of toxoid. All animals bled* 10 *days after the second dose of toxoid*

Aluminium phosphate μg./Lf	No. of guinea-pigs with antitoxin titres (units./ml.)													Geometric means
	< 0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·2–1·0	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	Total	
239 (M/10)	—	—	—	—	—	—	—	—	1	2	2	7	3	15	20·4
119	—	—	—	—	—	—	—	—	—	4	4	4	3	15	19·0
55·1	—	—	—	—	—	—	—	—	1	1	7	4	2	15	17·8
27·5	—	2	1	1	3	1	—	3	3	1	—	—	—	15	0·31
13·7	2	4	2	—	1	—	—	4	2	—	—	—	—	15	< 0·11
6·4	9	3	—	1	2	—	—	—	—	—	—	—	—	15	< 0·016
Nil	10	3	1	—	1	—	—	—	—	—	—	—	—	15	< 0·013

Table 10. *Antitoxin titres (units/ml.) of groups of guinea-pigs given two doses of* 2·25 Lf *of tetanus toxoid, with different calcium phosphate content, subcutaneously at* 42 *days interval with* 150 *units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the same time as the first dose of toxoid. All animals bled* 10 *days after the second dose of toxoid*

Calcium phosphate (μg./Lf)	No. of guinea-pigs with antitoxin titres (units/ml.)											Geometric means
	< 0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·5–1·0	1·0–2·0	2·0–5·0	5·0–10	10–20	Total	
316 (M/20)	7	2	—	—	1	1	—	1	2	1	—	15	< 0·06
158	5	1	—	—	2	—	—	3	1	1	2	15	< 0·11
79	4	4	—	—	—	2	1	—	3	1	—	15	< 0·09
39·5	9	1	1	—	1	—	—	2	—	1	—	15	< 0·011
19·8	11	1	1	—	1	—	—	—	1	—	—	15	< 0·010
9·9	12	1	1	—	—	—	—	1	—	—	—	15	< 0·010
Nil	12	1	2	—	—	—	—	—	—	—	—	15	< 0·010

that there were no non-responders at 21 days in Table 7C, indicating that production of antitoxin had begun earlier. The titres attained at this time were not however so high as those found in Table 7A where no heterologous antitoxin had been given; the rate of increase of titre from 21 to 42 days was more rapid however, and at 42 days the titres were not significantly different from those found in Table 7A. The final titres in serum-sensitive guinea-pigs although not significantly different from those found with unsensitized animals were significantly lower than the results where heterologous antitoxin had not been given ($P = < 0{\cdot}05$).

Influence of concentration of adsorbents

The influence of different adsorbents at different concentrations on the antigenic efficiency of tetanus toxoids in the presence of heterologous antitoxin was investigated (Tables 8–10). Tetanus toxoid (approximately 50 Lf/ml.) was adsorbed into (*a*) aluminium hydroxide in concentrations from 71·6 μg./Lf (M/27) down to 1·6 μg./Lf; (*b*) aluminium phosphate 239 μg./Lf (M/10) down to 6·4 μg./Lf, and (*c*) calcium phosphate 316 μg./Lf (M/10) down to 9·9 μg./Lf. Groups of guinea-pigs were given two doses of 2·25 Lf of each of these materials at 42 days interval; 150 units of heterologous antitoxin was given intraperitoneally at the same time as the first dose of toxoid. Antitoxin titres were estimated from bleedings taken 10 days after the second dose of toxoid.

It was quite evident from the results obtained that the efficiency of the preparations depended on the concentration of the adsorbent. With the aluminium salts at concentrations customarily used in human prophylactics the antigenic response obtained was very satisfactory. Calcium phosphate, although an excellent adsorbent for proteins, was singularly ineffective. Although at the highest level of concentration there were a number of animals in the calcium phosphate group giving reasonable responses, there were many non-responders and the values were widely scattered. There was however a gradation of response which indicated that some effect had been produced. The aluminium salts produced results which demonstrated that the adsorbent content of the toxoid preparation was critical and that amounts below a certain level were quite inadequate. This critical concentration appeared to be slightly lower for the hydroxide than for the phosphate.

Adsorption characteristics of different adsorbents

The adsorption characteristics of different mineral carriers in relation to tetanus toxoid were investigated (Table 11). Tetanus toxoid of approximately 50 Lf/ml. was adsorbed into different concentrations of aluminium hydroxide, aluminium phosphate and calcium phosphate. The unadsorbed toxoid remaining in the supernatant fluid from such material was titrated by flocculation after 1 week and after 9 months. The concentrations of carrier used were of a similar order, the highest concentration of aluminium hydroxide being 71·7 μg./Lf (M/27) and subsequent reductions in concentration at about 50 % differences were made down to 1·6 μg./Lf. Aluminium phosphate was used at an initial concentration of 239 μg./Lf (M/10) and calcium phosphate at an initial concentration of 314 μg./Lf (M/20). After

adsorption for one week it was found that the most effective adsorbent on a basis of the molar concentration of the different preparations was calcium phosphate (complete adsorption at M/80): this material took up further toxoid on standing at 0–4° C. for 9 months. On this basis aluminium hydroxide was nearly as effective (complete adsorption at M/58), and there was only slight improvement in the position after 9 months. Aluminium phosphate was relatively poor as an adsorbent and complete adsorption was only effected at M/10, hardly any improvement taking place with this material after 9 months.

Table 11. *Degree of adsorption of tetanus toxoid (approximately* 50 Lf/ml.) *by different concentrations of* (a) *Aluminium hydroxide*, (b) *Aluminium phosphate*, (c) *Calcium phosphate*

Aluminium hydroxide (μg./Lf)	Toxoid content of supernatant fluid (Lf/ml.) After 1 week	After 9 months
71·7 (M/27)	< 2 (< 1)*	< 2
33·0	< 2 (< 1)	< 2
16·5	23 (25)	8·7
8·2	45 (67)	39
3·8	52·5 (91)	52·5
1·6	52·5 (91)	52·5
Nil	52·5 (91)	52·5
Aluminium phosphate (μg./Lf)		
239 (M/10)	< 2 (< 1)	< 2
119	10·8 (18)	6
55	25 (50)	26
27·5	39 (67)	41
13·7	47·5 (77)	47·5
6·4	49 (83)	49
Nil	49 (83)	49
Calcium phosphate (μg./Lf)		
314 (M/20)	< 2 (< 1)	< 2
157	< 2 (< 1)	< 2
78	< 2 (1·7)	< 2
39	10·3 (14)	< 2
19·5	32 (42)	25
9·7	46 (61)	41
Nil	51 (91)	51

* Figures in parentheses represent toxoid content of supernatant fluid estimated by total combining power method in mice (unit-equivalents/ml.).

It was demonstrated by Holt (1950) that elution of diphtheria toxoid from mineral carrier occurred in normal human serum. This characteristic was investigated (Table 12). Mineral precipitates from fully adsorbed toxoid were resuspended in an equivalent volume of normal horse serum and incubated at 37° C. for 2 and 6 hr. The supernatants from these materials were then tested by the

Table 12. *Elution of tetanus toxoid from totally adsorbed preparations by incubation of precipitates with an equivalent volume of normal horse serum at* 37° *C.*

Total combining power (TCP) estimation of eluates (unit-equivalents/ml.)

Time of elution	Aluminium hydroxide adsorbed	Aluminium phosphate adsorbed	Calcium phosphate adsorbed
2 hr.	5·0 (5·5 %)	27·7 (33·3 %)	16·7 (18·3 %)
6 hr.	4·5 (5·0 %)	26·3 (31·6 %)	12·5 (13·7 %)
TCP of unadsorbed control	91	83	91

Table 13. *Percentage reduction in antitoxin combining power (unit-equivalents/ml.) of tetanus toxoid adsorbed on to different quantities of three different adsorbents*

Aluminium hydroxide			Aluminium phosphate			Calcium phosphate		
Adsorbent content (μg./Lf)	Combining power unit-equivalents/ml.	Reductions (%)	Adsorbent content (μg./Lf)	Combining power unit-equivalents/ml.	Reductions (%)	Adsorbent content (μg./Lf.	Combining power unit-equivalents/ml.	Reductions (%)
71·7 (M/27)	50	45·1	239/(M/10)	83	Nil	314 (M/20)	59	35·2
33·0	53	42·2	119	83	Nil	157	59	35·2
16·5	56	37·7	55	80	3·6	78	59	35·2
8·2	77	14·4	27·5	80	3·6	39	59	35·2
3·8	83	7·7	13·7	83	Nil	19·5	67	26·4
1·6	91	Nil	6·4	83	Nil	9·7	83	8·8
Nil	91	—	Nil	83	—	Nil	91	—

total combining power (TCP) method for any toxoid which had been eluted. Only 5 % of the total toxoid adsorbed was eluted from aluminium hydroxide precipitates by this method, whereas 31–35 % of the toxoid was eluted from the aluminium phosphate carrier and 13–18 % was eluted from the calcium phosphate carrier.

The ability of antitoxin to combine with toxoid adsorbed on to mineral carriers was investigated by TCP tests with adsorbed materials (Table 13). Toxoid adsorbed on to aluminium hydroxide did not combine so well with antitoxin, and at the highest level of concentration of adsorbent, this resulted in a 45 % reduction in combining power. With aluminium phosphate-adsorbed toxoid, however, there was no significant reduction in combining power for antitoxin. With calcium phosphate-adsorbed toxoid there was some reduction in combining power which, as with the aluminium hydroxide-adsorbed material, reached an optimum point consistent with almost complete adsorption as judged by adsorption experiments shown in Table 11. In the circumstances the failure of aluminium phosphate to interfere with antitoxin combination was inexplicable.

Table 14. *Combining power of antitoxin in the presence of different mineral carriers at the calculated concentration used in the total combining power test for toxoid*

Apparent antitoxin value (units/ml.) in the presence of:			
No carrier	Aluminium hydroxide	Aluminium phosphate	Calcium phosphate
42	42	42	45

The power of adsorbents to interfere with toxin–antitoxin titrations was investigated to ensure that the results obtained with toxoid were not due to adsorption of toxin in the second part of the total combining power test (Table 14). In this laboratory standard antitoxin was titrated in the presence of such quantities of adsorbent as would be used in the total combining power test, if adsorbed toxoid were under test. By this method there was no evidence of interference with the results using the aluminium compounds, but a slight increase of 7 % in the apparent antitoxin value where calcium phosphate adsorbent was added to the mixture. This would have the effect of lessening the percentage reduction in combining power with calcium phosphate at the highest level of adsorbent concentration in Table 13.

The observations on the characteristics of different adsorbents when taken in conjunction with the results of the antigenicity tests with adsorbed toxoids in the presence of heterologous antitoxin, indicated that the efficiency of toxoids adsorbed on aluminium compounds could hardly be due to the physical fact of adsorption alone. This suggests that the very different efficacy of the two adsorbents might be associated with some specific difference in action of calcium and aluminium ions on tissue cells.

Histological observations

Guinea-pigs were injected subcutaneously with 1·0 ml. amounts of suspensions of (*a*) aluminium phosphate, and (*b*) calcium phosphate. Small volumes (0·2 ml.)

of suspensions of approximately ten times the strength used in antigenicity tests at the highest level of concentration of mineral carrier, were used to exaggerate any effects produced. An equal number of animals was treated with the same materials to which 10 Lf of tetanus toxoid had been added. Direct comparison between the effects of the two minerals was made by injecting both mineral carriers into the same animal in each experiment. Different individual animals were killed after 1, 2, 3, 5 and 9 days, and the lesions excised and examined histologically.

Histological appearances showed both qualitative and quantitative differences in the tissue reactions between aluminium and calcium phosphate treated animals, both with and without toxoid, over the nine-day observation period. As the differences in reaction were more easily seen when the salts alone were injected, the features of these will be described first.

After one day both salts were detected in the lymphatics. Polymorphonuclear leukocytes were seen around the inoculum, but the degree of cellular response showed a marked difference between the two salts, that for calcium being profound whilst this was not so for aluminium. Macrophages were also present and both salts were detected in them.

After 3 days in the aluminium-treated animals, in addition to an increase in the cellular response, many cells of a different type first appeared in or near the lymphatics. These cells had a pale-staining cytoplasm with a well-defined, medium-sized nucleus; the chromatin was vesiculated. They were phagocytic as shown by the presence of aluminium in the cytoplasm. The blood vessels did not show any leukocytosis.

During the rest of the observation period, this special type of cell increased in numbers and appeared to be attracted to the aluminium, forming zones around it. These cells were not present except in aluminium treated animals.

The calcium salt appeared to be more noxious and induced a more severe cellular reaction, active phagocytosis was seen to be present, but the cell types involved did not include the particular type observed in aluminium treated animals. Giant cells were in evidence at 5 and 9 days and calcium was detected in them, but the majority of the mineral was extracellular. The development of fibrous tissue was considerable only in the calcium treated animals. At 9 days at the site of injection, the calcium salts were much less in evidence than the aluminium salts.

The addition of toxoid to both mineral carriers had the effect of increasing the polymorphonuclear response. The large pale-staining cells were still in evidence in those animals receiving aluminium salt, but the presence of an increased polymorphonuclear response confused the picture, making the differences between the effects of the different forms of treatment more difficult to distinguish.

DISCUSSION

It is well known that the presence of heterologous antibodies interferes with the primary response to fluid tetanus toxoid (Ramon & Laffaile, 1925; Cooke & Jones, 1943; Barr & Sachs, 1955). These observations were confirmed in animals by Suri & Rubbo (1961) who also observed that the degree of interference depended on the

ratio of toxoid to antitoxin, and that postponement of the toxoid injection for 7 days improved the response to it. The degree of interference with the response to fluid toxoid in guinea-pigs has been amply demonstrated in this paper in Table 1. It was, however, surprising to find that passive antitoxin was capable of severe interference when given up to 4 days after the first dose of fluid toxoid. This observation has been made previously by Uhr & Baumann (1961), with diphtheria toxoid and antitoxin.

It is reasonable to assume that when antitoxin was present at the time of injection of fluid toxoid, it caused interference by combining with and rendering unrecognizable the determinant antigenic groups. Antigen-antibody mixtures, especially when antibody is in excess, are poorly antigenic. However, it would be expected that when antitoxin was injected 4 days after the first dose of toxoid, antigen would have already been taken up by the receptor cells. It was therefore surprising that interference should still be evident at that stage.

It has been shown that many phagocytic cells such as the Kupffer cells of the liver which are not responsible for antibody production will take up antigens (White, 1963). Moreover, antigen taken up by many different cell types disappears in a few days (Coons, Leduc & Kaplan, 1951). The work of Gowans, McGregor, Cowan & Ford (1962) demonstrated that the primary antigenic response depends on the presence of small lymphocytes. This suggests either that antigen is passed from phagocytic cells to the small lymphocytes or that some processed material is handed on in this way, or that only antigen taken up by small lymphocytes is responsible for the initiation of the immune response and that phagocytosis serves some other purpose. There is a strong possibility that antibody-producing plasma cells are derived from small lymphocytes following stimulation by an antigen, and that this development process is essential to the production of specific antibody (McGregor & Gowans, 1963). In that case phagocytic cells may act merely as a holding depot, capable of transforming additional cells of the lymphocyte series. It is possible that antigen may be transferred directly from phagocytes to immunologically competent cells (White, 1963).

Buxton & Allen (1963) demonstrated in chickens that leucocytes sensitized *in vivo* with *Salmonella gallinarum* polysaccharide were sensitive to antisera to that organism. They attributed the cytotoxic effect to an anaphylactic type of reaction. It is possible that destruction of cells of the lymphatic series, from which antibody-producing cells are probably derived (Gowans *et al.* 1962) could be responsible for the failure to respond when toxoid was followed by antitoxin 4 days later.

When simultaneous active and passive immunization was carried out with aluminium hydroxide-adsorbed toxoid the antitoxin responses were of quite a different order. Although some interference with the responses was evident it was of a low degree where an adequate amount of mineral carrier was used. This difference did not seem to be a simple quantitative effect, for if the dose of fluid toxoid was increased to 20 or 50 Lf, with 150 units of antitoxin, no great improvement in active response was achieved.

It is well known that foreign particles such as insoluble aluminium salts are readily taken up by phagocytic cells, but it does not follow that soluble antigens

are so readily taken up, although Robineaux & Pinet (1960) demonstrated the uptake of fluorescein-labelled human serum protein by chicken macrophages. However, Glenny & Pope (1925) showed that, although fluid diphtheria toxoid injected intravenously into rabbits failed to immunize, if the toxoid was partly neutralized when antigen-antibody aggregates would be formed an immune response was obtained, and this applied to both heterologous and homologous antitoxins. Moreover, Glenny, Buttle & Stevens (1931) found that fluid toxoid was rapidly eliminated from the body, and they found some evidence that it was lost rapidly from the site of subcutaneous injection whereas adsorbed toxoid was not. These observations strongly suggest that mineral adsorbents produce an enhanced effect by promoting phagocytosis and acting as a long-term reservoir of antigen, protected from interference by antibody.

It can be seen in Tables 8 and 9 that the response to adsorbed tetanus toxoid by the simultaneous immunization procedure was directly related to the concentration of aluminium adsorbent and that on an equimolar basis the responses to the two preparations were very similar. However, in Table 10 the results when calcium phosphate was used as an adsorbent were very unsatisfactory, although there was some evidence of an increased effect at the highest levels of concentration of adsorbent.

Investigation of the adsorption characteristics of these three preparations gave no clue to the considerable differences in results. When the three different adsorbents were compared (Table 11) on a molecular basis, the calcium phosphate was the best and the aluminium phosphate preparation the least effective, and there was some slight improvement with time. The relative capacity of such adsorbed materials to cling to the adsorbent when inactivated in the presence of normal serum, which has been regarded as of significance in antigenicity (Holt, 1950), was investigated (Table 12). It was found that aluminium hydroxide-adsorbed toxoid was the most stable under these conditions and aluminium phosphate-adsorbed toxoid the least. There was then no correlation with immunizing capacity in the simultaneous immunization procedure.

Further, observations on the ability of the adsorbents to cover the antibody combining sites of the toxoid (Table 13) showed a similar lack of correlation with immunizing capacity.

White, Coons & Connolly (1955*a*) investigated the morphological changes in rabbits and guinea-pigs following the injection of alum precipitated diphtheria toxoid. They concluded that the improved antigenicity of alum precipitated toxoid was due to the development of an alum granuloma in which antigen was retained and released over a prolonged period, and that the development locally of antibody producing plasma cells contributed to the improved performance. Observations showed that the zone of macrophages built up around the alum deposit contained numerous alum particles. Further observations by White *et al.* (1955*b*) demonstrated a marked increase in the proliferation of macrophages both locally and in regional lymph nodes when the wax fraction of *Mycobacterium tuberculosis* was injected in a water-in-oil emulsion, and this was associated with plasma cell proliferation in the regional nodes; this material increased antibody titres to

antigens incorporated in the mixture. Humphrey (1963) has demonstrated that mycobacteria in oily emulsions cause a rise in γ-globulin in guinea-pigs without any increase in any known antibody. Moreover, the antitoxin response to diphtheria toxoid can be enhanced by a delayed hypersensitivity response to purified protein derivative at the site of injection of toxoid (Humphrey & Turk, 1963). These observations suggest that adjuvants have a double effect in that there is increased production of macrophages in which White *et al.* (1955*a*) found no evidence of antibody production, and proliferation of antibody producing plasma cells.

In the present experiments it was found that aluminium phosphate alone produced the typical macrophage reaction in tissue and calcium phosphate did not. The relative inefficiency of calcium phosphate-adsorbed toxoid as an antigen in the presence of passive antitoxin could be due simply to its poor qualities as an adjuvant, but very large doses of fluid toxoid do not produce satisfactory immunity when passive antitoxin is present. This suggests that some special condition may be operative. Since a feature of the alum granuloma is rapid phagocytosis it is possible that toxoid is protected from interference by antitoxin when it is incorporated within the intact cell. In addition localization of antigen at the site of injection rather than the rapid dispersion of a fluid antigen would expose the material to relatively less antitoxin.

SUMMARY

Guinea-pigs given two doses of 2·25 Lf of fluid tetanus toxoid at 28 days interval had very satisfactory antitoxin titres 10 days after the second dose of toxoid (G.M. 28·2 units/ml.). Similar groups of animals given 150 units of horse tetanus antitoxin simultaneously with the first dose of toxoid responded very badly (G.M. $< 0{\cdot}016$).

Interference by passive antitoxin occurred even when the antitoxin was given as late as 4 days after the first dose of toxoid.

Interference by passively administered antitoxin was minimal when aluminium hydroxide-adsorbed toxoid was used. It was necessary to increase the dose of antitoxin from 150 to 2400 units before significant interference occurred.

The route of administration of antitoxin did not significantly affect the results except when the antitoxin was given intravenously.

When guinea-pigs were immunized and bled at regular intervals it was found that with both fluid and aluminium hydroxide-adsorbed preparations, titratable antitoxin was present on the 14th day. The increase in titre thereafter was more rapid with the adsorbed preparation, but after a second dose of toxoid there was no significant difference in titre.

Passively administered antitoxin virtually abolished the active response to fluid toxoid, but with aluminium hydroxide-adsorbed preparations the primary response was not abolished but reduced and delayed and there was much individual variation.

Horse serum-sensitive guinea-pigs given adsorbed toxoid with simultaneous passive horse antitoxin gave a better primary response to the toxoid than did unsensitive animals.

The effectiveness of adsorbed tetanus toxoid in the simultaneous immunization procedure was directly related to the concentration of aluminium hydroxide or phosphate used: this concentration was critical and amounts below a certain level were ineffective. Calcium phosphate used as an adsorbent was unsatisfactory in this way, although it was an excellent adsorbent.

Investigation of the adsorbent characteristics of aluminium hydroxide and phosphate and of calcium phosphate, showed that the calcium salt on a molar basis was the most effective and that aluminium phosphate was the least effective.

Elution of toxoid from centrifuged precipitates of the three types of adsorbent showed that only 5% of toxoid was removed from the aluminium hydroxide, 13–18% from the calcium phosphate and 31–33% from the aluminium phosphate preparation when incubated with normal serum at 37° C.

Aluminium hydroxide adsorption *in vitro* interfered with the ability of antitoxin to combine with toxoid and to a lesser extent calcium phosphate had the same effect; aluminium phosphate, however, did not appear to interfere at all in this way

Histological observations on the tissue response to aluminium phosphate and calcium phosphate indicated that the typical alum granuloma produced by aluminium phosphate was not produced by the calcium salt.

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Studies on the pathogenesis of rinderpest in experimental cattle

III. Proliferation of an attenuated strain in various tissues following subcutaneous inoculation

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The pathogenesis of rinderpest, following experimental infection of cattle with the virulent RGK/1 strain of virus, has been the subject of two recent investigations (Liess & Plowright, 1964; Plowright, 1964). Further experiments have now been undertaken to provide comparative data on infection with a culture-attenuated strain of virus which is widely used as a vaccine for cattle in East and West Africa. It was hoped that the information obtained would be useful in explaining its lack of pathogenicity and high immunogenicity (Plowright & Ferris, 1962*b*). There was also a need to determine how long vaccine virus persisted in the tissues of inoculated cattle, so that meat from potentially infected animals would not be exported to countries free from the disease (see Provost, 1960).

The procedure adopted was to kill experimental animals at daily intervals after administration of vaccine and titrate infective virus in selected tissues.

MATERIALS AND METHODS

Virus

Serial propagation of the virulent Kabete 'O' strain of rinderpest virus in primary calf kidney (BK) cells selected a virus population which, after seventy passages, could be inoculated into cattle without producing pyrexia or other clinical signs (Plowright & Ferris, 1962*b*). This attenuated strain produced a good neutralizing antibody response and is currently used for vaccine production in its 91st to 100th BK passages. For the present experiments virus of the 95th passage was used; it was freeze-dried in glass-sealed ampoules which were stored at $-20°$ C. and constituted a routine production batch of vaccine.

For every cattle inoculation 2 ampoules were each reconstituted in 1 ml. of sterile deionized water and the contents pooled. A twofold dilution was then prepared in tissue culture growth medium (Plowright & Ferris, 1959) and each animal received 2 ml. subcutaneously, on the left-hand side of the neck. The inoculation was made immediately above and in front of the left prescapular (posterior superficial cervical) lymph node, where the point of injection was marked by trimming off a small amount of hair.

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On five occasions during the course of the experiments, portions of the inoculum were titrated in BK cells by the method of Plowright & Ferris (1959, modified 1962*b*). All the results fell within the range $10^{4.0}$ to $10^{4.6}$ TCD 50 per ml. of reconstituted vaccine. It has been shown, for this strain of virus, that one TCD 50 is equivalent to one cattle ID 50 (Plowright & Ferris, 1962*b*); each experimental animal therefore received between 10,000 and 40,000 cattle ID 50 or about 100 times the normal field dose of vaccine.

Experimental cattle

The animals used were grade steers or heifers, the majority being Red Poll or Ayrshire crosses. They were aged between 1 and 3 years, and their sera contained no rinderpest-neutralizing antibody (Plowright & Ferris, 1961). All cattle were segregated after inoculation but they were not housed and had continual access to grass and water. Temperatures were recorded daily before 8.30 a.m., all inoculations being performed before this time. Animals were killed at daily intervals after inoculation; in all instances this was completed before 9 a.m.

Collection of tissues

The technique employed was essentially that described by Plowright (1964). *Blood*, for the recovery of virus and serum, was always collected in the manner detailed in the same paper, as were also portions of the following tissues, viz.: mucosae of the *base of the tongue*, *dorsal turbinate bone*, *abomasum* (*pylorus*), *ileum*, *caecum* and *colon;* one large, or several small *haemolymph nodes* taken from adipose tissue of the left prescapular groove; *spleen*, *bone marrow* and *palatal tonsil; pharyngeal*, *left prescapular*, *right prescapular* and *mesenteric* (jejuno-ileal) *lymph nodes; liver*, *kidney*, *lung*, *myocardium* and *brain*.

Two additional tissues were obtained at autopsy. First, a portion of *muscle* and *subcutaneous tissue* was removed from the neck immediately below the point at which virus was inoculated. Secondly, in seventeen instances, the total lymphoid tissue comprising a *Peyer's patch* was collected; the intestinal contents were first removed from the mucosa by liberal washing with tap water and the tissue of the patch was then dissected clear of the underlying muscle layers.

Subsequent treatment of tissues

The methods used in the preparation of 10-fold dilution series, from the blood, bone marrow and each solid tissue, followed in detail those described by Plowright (1964). In the early stages, dilutions covering the range 10^{-2} to 10^{-5} were prepared from each tissue; concentrations greater than 10^{-2} (w/v) could not be used without inducing cytotoxic changes in the monolayers inoculated. As the experiment progressed it became apparent that a large number of tissues did not support virus multiplication (see Results) and for these the dilution series was first reduced to 10^{-2} and 10^{-3}; later only a 10^{-2} suspension was tested, while finally, in the case of the last three animals employed (nos. 9712, 9716 and 9737), these tissues were excluded.

Where virus proliferation was detected the dilution range extended to a mini-

mum of 10^{-4} and a maximum of 10^{-7}. In a few instances titres were higher than expected and the end-point was not determined.

Viraemia was detected by preparing leucocyte fractions from 20 ml. samples of blood with EDTA and inoculating these into five BK cultures, as already described (Plowright & Ferris, 1962*a*). In addition a 10^{-1} dilution in culture maintenance medium was prepared from blood-EDTA mixtures and inoculated into five additional BK cultures in a dose of 2 ml. All dilutions of solid tissues were inoculated, in the same dosage, into five BK cultures.

Preparation and maintenance of cell cultures

All virus titrations were carried out in 6- to 9-day-old cultures of BK cells in tubes of 160 × 15 mm. dimensions. For convenience, suspensions of trypsin-dispersed BK cells in growth medium were held at 4° C. for up to 2 days before seeding. The handling of the monolayers before and after inoculation was as described by Plowright (1964). Using the antibiotics described by that author bacterial and fungal contamination were only encountered occasionally and never in serious proportions.

The cultures were examined by low-power microscopy on the 5th, 7th and 9th days after inoculation, final readings being made on the 9th day. Titres were calculated by the method of Thompson (1947) and expressed as $\log_{10}$ TCD 50/g. of solid tissue.

The level of viraemia was never sufficiently high to be expressed as a 50 % end-point; virus was only detectable in the leucocyte concentrates and the results were expressed as the number of positive tubes out of five inoculated (Tables 1–3).

In the case of solid tissues, where only one tube inoculated with a 10^{-2} dilution was positive the result was expressed as a 'trace'. Where two or more were infected it was assumed that all the tubes receiving a hypothetical 10^{-1} dilution would have been positive, and the result was given as a 50 % end-point.

Serum neutralization tests

Quantitative neutralization tests were performed on sera collected from every animal at the time of its destruction. All sera were tested simultaneously against the same stock of virus, yielding $10^{2.6}$ TCD 50 per tube (Plowright & Ferris, 1961).

RESULTS

Clinical signs in cattle

No pyrexial response was recorded and no visible abnormalities were detected in any of the animals while alive. Mild engorgement of carcass lymph nodes was occasionally found at autopsy but otherwise no significant lesions were observed.

Virus proliferation

According to the results, the tissues divided themselves into two clear-cut categories—those in which virus proliferation could be detected, at least occasionally, and those which failed to support virus multiplication at any time. The latter

group included ten tissues, comprising six mucosae and four parenchymatous organs, viz.: the mucosae of the nasal cavity (turbinate bone), base of the tongue, abomasum, ileum, caecum and colon; liver, kidney, myocardium and brain.

Virus multiplication was demonstrated in twelve of the twenty-two tissues

Table 1. *The titre of virus in various tissues of cattle killed on days 1–4 after inoculation*

Time after infection ...	1 day		2 days		3 days		4 days	
Animal no. ...	9385	9531	9379	9535	9607	9873	9603	9727
Left prescapular lymph node	0·0	0·0	0·0	0·0	0·0	0·0	Tr.	0·0
Right prescapular lymph node	0·0	0·0	0·0	0·0	0·0	0·0	Tr.	0·0
Pharyngeal lymph node	0·0	0·0	0·0	0·0	0·0	0·0	2·6	Tr.
Mesenteric lymph node	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Peyer's patch	N.T.	0·0	N.T.	N.T.	N.T.	0·0	N.T.	0·0
Tonsil	0·0	0·0	0·0	0·0	0·0	0·0	⩾ 3·2	0·0
Blood*	0/5	0/5	0/5	0/5	0/5	0/5	2/5	0/5
Spleen	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Haemolymph node	0·0	0·0	0·0	0·0	0·0	0·0	⩾ 3·2	1·6
Bone marrow	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Muscle and subcutaneous tissue	0·0	0·0	0·0	0·0	0·0	0·0	⩾ 3·2	0·0
Lung	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0

* Result expressed as number of tubes positive out of five inoculated with each leucocyte fraction.

Table 2. *The titre of virus in various tissues of cattle killed on days 5–7 after inoculation*

Time after infection ...	5 days			6 days			7 days		
Animal no. ...	9612	9626	9737	9600	9645	9670	9611	9632	9712
Left prescapular lymph node	2·6	⩾ 4·2	4·0	2·8	2·4	2·4	4·0	0·0	2·4
Right prescapular lymph node	0·0	Tr.	3·2	3·2	3·2	3·2	3·6	⩾ 3·4	⩾ 5·0
Pharyngeal lymph node	2·5	2·8	3·6	2·8	3·4	4·8	⩾ 4·2	3·4	4·4
Mesenteric lymph node	3·0	2·2	2·0	1·6	2·8	0·0	⩾ 4·0	4·6	4·4
Peyer's patch	0·0	N.T.	Tr.	N.T.	3·4	3·2	N.T.	3·8	4·8
Tonsil	2·4	2·6	3·8	3·6	3·8	3·7	3·8	3·0	4·6
Blood*	0/5	1/5	1/5	1/5	2/5	0/5	1/5	2/5	5/5
Spleen	1·7	2·2	1·6	3·0	1·8	3·0	1·8	1·6	3·4
Haemolymph node	Tr.	⩾ 3·2	4·0	3·4	3·8	4·2	5·4	4·6	5·4
Bone marrow	0·0	0·0	0·0	1·6	0·0	0·0	0·0	0·0	1·8
Muscle and subcutaneous tissue	0·0	0·0	0·0	2·2	⩾ 3·2	0·0	0·0	0·0	0·0
Lung	0·0	Tr.	0·0	0·0	0·0	0·0	0·0	0·0	1·8

* Result expressed as number of tubes positive out of five inoculated with each leucocyte fraction.

tested, viz.: muscle and subcutaneous tissue at the site of inoculation; left and right prescapular, pharyngeal and mesenteric lymph nodes, Peyer's patches, tonsil, spleen and haemolymph node; blood, bone marrow and lung.

Virological events could be roughly divided into three phases, i.e. those of virus eclipse, multiplication and decline.

Table 3. *The titre of virus in various tissues of cattle killed on days* 8–10 *after inoculation*

Time after infection ...	8 days				9 days		10 days	
Animal no. ...	9622	9683	9716	9732	9618	9714	9729	9733
Left prescapular lymph node	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Right prescapular lymph node	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Pharyngeal lymph node	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Mesenteric lymph node	0·0	2·8	4·0	0·0	1·8	0·0	0·0	0·0
Peyer's patch	0·0	Tr.	0·0	0·0	$\geqslant$ 5·0	0·0	0·0	0·0
Tonsil	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Blood*	0/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Spleen	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Haemolymph node	0·0	Tr.	0·0	0·0	3·0	0·0	0·0	0·0
Bone marrow	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Muscle and subcutaenous tissue	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
Lung	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0

* Result expressed as number of tubes positive out of five inoculated with each leucocyte fraction.

The eclipse phase

After the inoculations a period of 3 days elapsed during which no virus could be demonstrated in any tissue. This phase ended on day 4, when two animals were killed. In the first (no. 9603), trace amounts of virus were found in both prescapular lymph nodes but more substantial amounts were found in the pharyngeal lymph node ($10^{2\cdot6}$ TCD 50/g.), tonsil ($\geqslant 10^{3\cdot2}$ TCD 50/g.) and prescapular haemolymph node ($\geqslant 10^{3\cdot2}$ TCD 50/g.). A low grade viraemia was present (2/5 tubes infected with a leucocyte fraction) and virus was also detected in the muscle and subcutaneous tissue ($\geqslant 10^{3\cdot2}$ TCD 50/g.).

The second animal killed on day 4 (no. 9727) had a trace of virus in a pharyngeal lymph node and small quantities in a haemolymph node ($10^{1\cdot6}$ TCD 50/g.). No virus could be detected in the blood, prescapular lymph nodes or tissue removed from the site of inoculation.

The phases of virus multiplication and decline in various tissues

Left prescapular lymph node. On day 5 a peak mean titre of $\geqslant 10^{3\cdot6}$ TCD 50/g. was recorded in this structure (Fig. 1). Thereafter the quantity of virus declined, so that on day 7 the mean was $10^{2\cdot1}$ TCD 50/g. (Fig. 1). No virus could be recovered from this node in any of the 8 animals killed on the 8th day or later (Table 3).

Right prescapular lymph node. On day 5 three animals were tested and the mean titre was $10^{1\cdot1}$ TCD50/g. (Fig. 1). By day 6 the titres had risen considerably (Table 2), whilst the peak mean value of $\geqslant 10^{4\cdot0}$ TCD50/g. was reached on day 7. This was followed by an abrupt fall in infectivity, as none could be demonstrated in this node on day 8 or on either of the two succeeding days.

Pharyngeal lymph node. The mean titre rose steadily from $10^{1\cdot3}$ TCD50/g. on day 4 to a peak of $10^{4\cdot0}$ TCD50/g. on day 7, identical with that obtained in the right prescapular node. As in the prescapular nodes, no virus could be recovered on day 8.

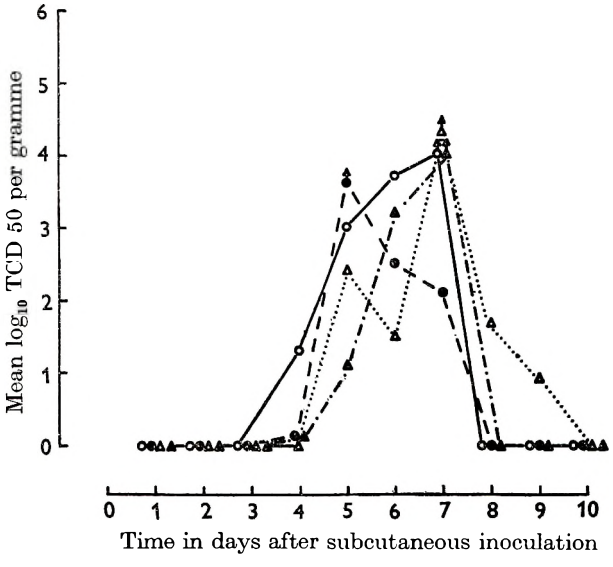

Fig. 1. Proliferation of culture-attenuated rinderpest virus, strain RBOK, in the lymph nodes of cattle. ○——○, Pharyngeal L/N; ● – – ●, left prescapular L/N; △·····△, mesenteric L/N; ▲–·–·–▲, right prescapular L/N.

Mesenteric lymph node. The results for this tissue were more irregular than those for the other lymph nodes studied and virus persisted for a longer time (Fig. 1). Virus multiplication was not detected until day 5 after inoculation, when virus was recovered from all three animals tested (see Table 2 and Fig. 1). On day 6 one of three cattle (no. 9670) had no detectable infectivity, whereas 24 hr. later all animals tested yielded relatively high concentrations of virus; the mean titre on the 7th day was $10^{4\cdot3}$ TCD50/g., one of the highest recorded during these experiments. On day 8, two cattle, nos. 9732 and 9622, showed no virus in mesenteric lymph nodes, whereas two others had moderate amounts. On day 9, low-titre virus was still present in one of two animals but finally, on the 10th day, neither of the two cattle killed had any demonstrable infectivity in this node.

Palatal tonsil. The tonsil behaved in a very similar manner to the pharyngeal lymph node, virus appearing in one of two animals on the 4th day and disappearing by the 8th (Fig. 2).

Peyer's patches. Virus in this tissue was not present in appreciable quantities until day 6, although a trace was recorded in one of two animals (no. 9737) tested

on day 5. The mean titre rose rapidly to a peak of $10^{4\cdot3}$ TCD 50/g. on day 7. Of four animals tested on day 8 only one (no. 9683) yielded virus and this was in trace amount. However, on the 9th day one animal, no. 9618, showed high-titre virus in a Peyer's patch ($\geqslant 10^{5\cdot0}$ TCD 50/g.) and accounted for the sharp second peak in the mean line (Fig. 2). No virus was recovered from either of the two animals tested on the 10th day post-inoculation.

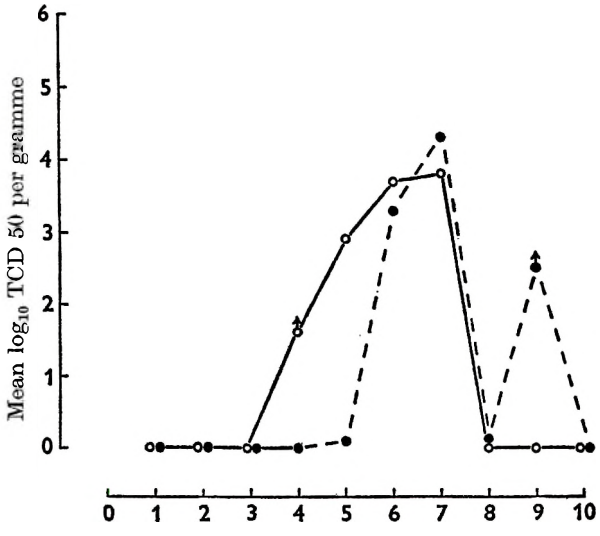

Fig. 2. Proliferation of culture-attenuated rinderpest virus, strain RBOK, in the lympho-epithelial tissues of cattle. ○—○, Tonsil; ● – – ●, Peyer's patch.

Spleen. No virus was detected in this organ until day 5, and the peak mean titre of $10^{2\cdot6}$ TCD 50/g. was recorded on day 6 (Fig. 3). A rapid fall ensued and no virus was recovered from the spleens of four animals killed on the 8th day. Mean titres were the lowest recorded for any of the tissues that regularly supported virus proliferation.

Prescapular haemolymph nodes. Virus was demonstrable in the haemolymph nodes of both animals tested on day 4 (Table 1), the mean titre on this day being $10^{2\cdot4}$ TCD 50/g. On day 5 substantial amounts of virus were found in cattle nos. 9626 and 9737 but only a trace was found in no. 9612 (Table 2). Thereafter the mean titre rose steadily, reaching a peak of $10^{5\cdot1}$ TCD 50/g. on day 7, the highest figure recorded for any tissue (Fig. 3). Only a trace of virus was recorded from one of the four animals tested on the 8th day, but 24 hr. later one of two animals (no. 9618) still yielded $10^{3\cdot0}$ TCD 50/g. (Table 3). No virus was detected in this tissue on day 10.

Blood. As observed in the section on Materials and Methods, the amount of virus in the blood was never sufficiently great to be expressed as a 50 % end-point. Hence, in preparing Figure 3, one positive tube was arbitrarily taken to represent 0·1 $\log_{10}$ units. Virus was first found in the blood on day 4 (no. 9603, Table 1). A low-grade viraemia was also detected in eight of the thirteen animals killed on days

5–8 inclusive. Virus in the blood probably reached a maximum on the 7th day (Tables 2 and 3).

Bone marrow. Virus was recovered from the bone marrow on only two occasions, the titre being low in each instance (no. 9600 with $10^{1.6}$ TCD 50/g. on day 6; no. 9712 with a titre of $10^{1.8}$ TCD 50/g. on day 7).

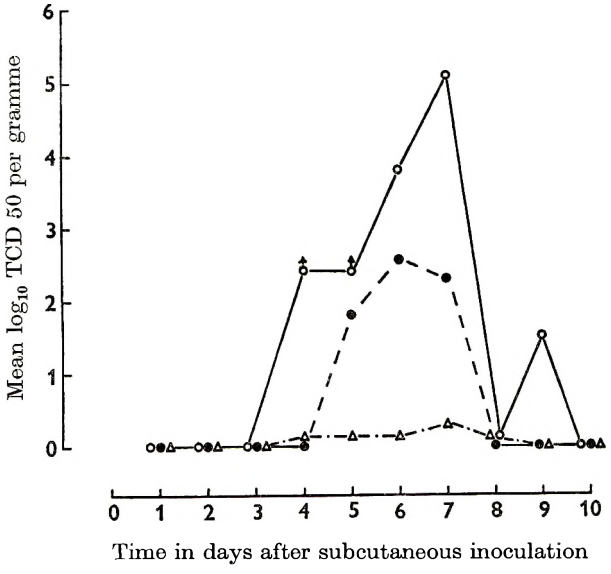

Fig. 3. Proliferation of culture-attenuated rinderpest virus, strain RBOK, in the lymphopoietic tissues and blood of cattle. ○—○, Prescapular HL/N; ● – – ●, spleen; △—·—△, blood.

Lung. Virus was isolated from the lungs of two cattle only, no. 9626 (day 5) and no. 9712 (day 7); in both cases the amount of virus was very small (see Table 2).

Muscle and subcutaneous tissue at site of inoculation. Infectivity was demonstrated in these tissues on three occasions, once on day 4 (no. 9603, $10^{3.2}$ TCD 50/g.) and twice on day 6 (no. 9600, $10^{2.2}$ TCD 50/g.; no. 9645, $\geqslant 10^{3.2}$ TCD 50/g.).

Development of neutralizing antibody

As shown in Table 4, no animal produced neutralizing antibody before day 7. At this time one ox (no. 9712) of three tested, showed a trace of antibody although large quantities of virus were present in many of its tissues. On the 8th day, three of four animals tested had neutralizing antibody in their sera and two of these (nos. 9622 and 9732) were devoid of infectious virus. No. 9683 still had virus in four sites, in spite of the presence of low-titre antibody. One of two animals (no. 9618) killed on the 9th day, had no detectable antibody and virus was still present in three tissues (Table 3); both cattle tested on the 10th day had circulating antibody.

Table 4. *Rinderpest neutralizing antibody in the sera of cattle killed at different times after the inoculation of culture-attenuated rinderpest virus*

Animal no.	No. of days after inoculation	Titre of serum*	Animal no.	No. of days after inoculation	Titre of serum*
9385	1	0·0	9611		0·0
9531		0·0	9632	7	0·0
9379	2	0·0	9712		Trace
9535		0·0	9622		0·8
9607	3	0·0	9683	8	Trace
9873		0·0	9716		0·0
9603	4	0·0	9732		0·4
9727		0·0	9618	9	0·0
9612		0·0	9714		1·0
9626	5	0·0	9729	10	0·8
9737		0·0	9733		0·8
9600		0·0			
9645	6	0·0			
9670		0·0			

* Log_{10} SN 50 at time of death. All sera were tested simultaneously against $10^{2\cdot6}$ TCD 50 of virus.

DISCUSSION

The sequence of events following subcutaneous inoculation of culture-attenuated rinderpest virus was not precisely determined. It is probable that part of the inoculum was first transferred by lymphatic vessels to the local lymph node and proliferated there before reaching the blood through the cervical duct. Some evidence for this was seen in the earlier virus peak in the left than in the right prescapular node (5th *v.* 7th day; see Fig. 1) but no animal was examined in which virus was limited to the node draining the site of inoculation. It may be that some inoculated virus passed through the local node directly into the blood stream; this might account, for example, for higher virus titres in tonsil and pharyngeal node of Ox 9603 (4th day) than in the prescapular nodes of the same animals.

Appreciable quantities of virus (*ca.* $10^{2\cdot2}$ to $10^{3\cdot2}$ TCD 50/g.) were recovered, on three occasions, from muscle and subcutaneous tissues at the site of inoculation. The animals involved were killed on the 4th and 6th days after inoculation and, in view of the lability of cultured rinderpest virus (Plowright & Ferris, 1961), it is almost certain that some local proliferation took place. Difficulties in locating the exact site of deposition of the inoculum could easily account for failure to demonstrate local virus growth in the other animals. There were no indications as to which cells in the muscle and subcutaneous tissue supported virus multiplication.

The viraemia produced by attenuated rinderpest virus differed considerably from that produced by virulent strains. Thus, the Kabete 'O' virus, from which our attenuated variant was derived, reappeared in the blood on the day following its subcutaneous inoculation in large dosage; viraemia reached maximum levels on the 4th day after infection and persisted for 13–14 days (MacOwan, 1956). Recent field strains, of low cattle pathogenicity, produced a viraemia detectable on the

2nd to 4th days after subcutaneous inoculation of about 10^4 to 10^5 TCD 50 of virus; peak blood titres of $10^{1\cdot4}$ to $10^{3\cdot0}$ TCD 50/ml. were usually attained on the 6th or 7th days post-infection, the duration of viraemia varying from 3 to 11, with a mean of over 6 days (Plowright, 1963). The highly pathogenic RGK/1 strain was recovered on the first to 3rd days following parenteral inoculation; pyrexia began on the 3rd to 5th days and blood virus reached a mean of $10^{2\cdot3}$ TCD 50/ml. on the 3rd day of fever (Liess & Plowright, 1964), viraemia lasting until the 12th day after infection (Plowright, 1964).

It is immediately evident that the attenuated virus showed a longer 'eclipse' phase in cattle, being demonstrable at the earliest on the 4th day following inoculation. Infectivity was not regularly demonstrable in the blood, except on the 7th day, when it possibly reached a maximum. The duration of viraemia was only 5 days in the group of cattle studied, although figures for individual animals were not obtained. It is clear that some virus must have circulated before the 96th hour, since spread to and growth in the pharyngeal lymph nodes and tonsil had occurred by that time.

After intranasal inoculation of cattle, the RGK/1 strain produced maximal mean titres of $> 10^7$/g. in the cephalic lymph nodes, tonsil and prescapular haemolymph nodes. Somewhat lower but considerable figures were recorded for the spleen, visceral lymph nodes, mucosae of the alimentary tract (tongue, abomasum, caecum, colon and ileum) and lung, whilst a low level of proliferation was detected in the nasal mucosae, bone marrow, liver and kidney (Plowright, 1964). The proliferation of the virulent Kabete 'O' strain of rinderpest virus in different tissues of cattle has not, apparently, been studied systematically but peak titres of about $10^{6\cdot0}$ ID 50/g. have been recorded in the spleen, body lymph nodes and abomasal mucosa (Scott, 1955; MacOwan, 1956). The attenuated Kabete 'O' strain, used in these experiments, reached maximal mean titres which were about 100-fold to 1000-fold lower in various lymph nodes, tonsil, spleen and haemolymph nodes.

Peyer's patches were not, unfortunately, included in the RGK/1 study (Plowright, 1964), but it was evident that a considerable proliferation of the attenuated virus occurred in them and not in the intervening ileal mucosa or in the mucosae of the tongue, abomasum (pylorus), caecum and colon. As lymphoid follicles, either single or aggregrated, and diffuse infiltrations of lymphoid cells are associated with all the alimentary and respiratory mucosae which failed to support a significant growth of attenuated virus, it is of interest to speculate on reasons for the selective localization observed. It may simply be that the mass of susceptible lymphoid cells in mixed lympho-epithelial structures, such as the palatal tonsil and Peyer's patches, is very much greater than that in the mucosa and submucosa of the base of the tongue, abomasum, etc.; virus would not accordingly reach detectable levels in the latter group of sites. This suggestion is difficult to accept, particularly in the case of the tongue mucosae and lung, where many large lymphoid follicles are present and a trace of virus, at least, would have been expected in some animals. It is possible that lymphoid cells in different situations vary in their capacity to support the growth of rinderpest virus or in their accessibility to infection by virus circulating in the blood. Similar factors may

account for the considerably higher titre of virus in the haemolymph nodes than in the spleen (Fig. 3); the great similarity in histological structure of these tissues (Trautman & Fiebiger, 1952) could have led to the expectation that virus titres attained in them would have been comparable.

The infrequent recovery of small quantities of virus from samples of lung (2) and bone marrow (2), suggested that such results may have been due to the trapping in these tissues of virus-carrier leucocytes, rather than active multiplication in local accumulations of lymphoid cells.

Widespread proliferation of the attenuated strain in lymphopoietic tissues can probably be associated with the solid and lasting immunity which it induces in cattle. Recent work has shown that resistance to severe parenteral challenge lasts for at least 3 years, while the high initial titre and slow rate of decline of neutralizing antibody in the serum, support the belief that it is almost certainly lifelong (Plowright, unpublished). So far as the lack of pathogenicity of vaccine virus is concerned it seems reasonable to suppose that this can be associated with its failure to proliferate in the mucosae of the alimentary tract, or in the nasal mucosa and parenchymatous organs. Attenuated virus is, in fact, strictly lymphotropic, whereas virulent strains multiply in the tissues mentioned and produce lesions there which account for the characteristic clinical signs. The absence of attenuated rinderpest virus from surface mucosae and parenchymatous tissues probably resulted in a failure of virus excretion, which would account for the inability of vaccine virus to spread by contact amongst susceptible cattle (Plowright & Ferris, 1962*b*).

It is interesting in this connection to recall comparable findings with virulent and attenuated strains of other viruses. Thus, a mild strain of Newcastle disease virus differed from a highly virulent one only in the rate of its penetration into and proliferation in the central nervous system of chickens (Karzon & Bang, 1951); a mild strain of fowlpox did not multiply to any extent in the liver and bone marrow of inoculated chicks, whereas a virulent strain did so and subsequently produced a secondary viraemia and generalization (Mayr & Wittman, 1957); finally, Roberts (1963) showed that an avirulent strain of ectromelia virus differed from a virulent one in its reduced ability to infect the liver macrophages and hence liver parenchymal cells.

The abrupt decline of attenuated rinderpest virus between days 7 and 8 was undoubtedly associated with the appearance of antibody at that time; it seems improbable that the supply of susceptible cells was anywhere near to exhaustion, especially as the maximal titres registered were so modest. The 7–10 days before antibody became detectable in different animals was comparable to the 9 days recorded in cattle infected intranasally with the virulent RGK/1 strain (Plowright, 1964) and 6–7 days in others given virulent Kabete 'O' virus subcutaneously (Plowright, 1962). The disappearance of virus from all tissues of cattle by the 10th day after inoculation of a large dose of the attenuated strain indicates that, under field conditions, it can probably be assumed with safety that animals given vaccine 2 weeks previously no longer constitute a risk from the point of view of meat exports (see Provost, 1960).

SUMMARY

Twenty-five grade cattle were infected subcutaneously with $10^{4.0}$ to $10^{4.6}$ TCD 50 of a highly-attenuated strain of rinderpest virus which is used as a vaccine. No clinical reaction was observed but the proliferation of virus was studied in twenty-two tissues harvested at daily intervals from the first to the 10th days after inoculation. Serum samples collected at the same times were examined for rinderpest-neutralizing antibody.

There was an 'eclipse' phase of 3 days during which no infectivity could be demonstrated in any tissue. On the 4th day virus had generalized, as shown by its detection in lymphoid tissues which were not associated with the site of inoculation; occasional animals showed evidence of viral proliferation in the local muscle and subcutaneous tissue. A considerable growth of virus, with peak titres between $10^{4.0}$ and $10^{5.0}$ TDC 50/g., was demonstrated in the prescapular, pharyngeal and mesenteric lymph nodes, also in the palatal tonsils and Peyer's patches of the ileum. Highest titres ($10^{5.4}$ TCD 50/g.) were recorded in the prescapular haemolymph nodes, but less virus (up to $10^{3.4}$ TCD 50/g.) appeared in the spleen.

A low-level viraemia was detected in eight of the thirteen cattle killed on the 5th to 8th days inclusive. Minimal quantities of virus were found on two occasions each in the bone marrow and lung. No virus was recovered from the mucosae of the base of the tongue, abomasum, ileum, caecum and colon; liver, heart, kidney and brain tissue also failed to support its multiplication.

Neutralizing antibody was present in all cattle by the 10th day after inoculation, its appearance being associated with the abrupt decline in virus titres, which was usually demonstrable on the 8th day.

The behaviour of the attenuated virus was compared with that of virulent strains, and it was concluded that its lack of pathogenicity was due primarily to its failure to proliferate in the mucosae of the gastro-intestinal and respiratory tracts. Vaccine virus was, in fact, exclusively 'lymphotropic', a characteristic which may account for the solid, lasting immunity it confers and for the considerable antibody response it provokes in inoculated cattle. Inability to spread by contact amongst susceptible cattle may be a result of the absence of virus in mucosae or parenchymatous organs and hence in excretions.

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Two outbreaks of echovirus 14 infection: a possible interference with oral poliovirus vaccine and a probable association with aseptic meningitis

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During 1962 and 1963 we encountered two outbreaks of echovirus 14 infection in northern Japan. In the first, which occurred in Sendai in 1962, it seemed possible that echovirus 14 was interfering with the establishment of infection with Sabin type 1 oral poliovaccine. In the second, which occurred in Aomori in 1963, a small outbreak of aseptic meningitis was observed.

The present communication describes these events and discusses the significance of echovirus 14 infection in man.

MATERIALS AND METHODS

Subjects

In October 1962, babies in an infant institution in Sendai were fed type 1 strain (LSc2ab) of Sabin's oral poliovaccine obtained from the Connaught Medical Laboratories, Canada. Nine babies to whom this vaccine was given were examined. In 1963, fifty nine patients with aseptic meningitis in Aomori City were studied.

Virus isolation

Primary cultures of cynomolgus monkey kidney cells were used. Growth medium for the cell monolayers consisted of Hanks's balanced salt solution containing 0·5% lactalbumin hydrolysate and 5% unheated bovine serum. Maintenance medium was Earle's balanced salt solution containing 0·5% lactalbumin hydrolysate without serum.

Faeces and cerebrospinal fluids (CSF) were kept frozen at $-25°$ C. until tested. Stools for virus isolation were prepared in 10% suspension in the maintenance medium, and centrifuged for 10 min. at 3000 r.p.m. Before infection, fluids of the cell monolayers were replaced by 1·0 ml. of the maintenance medium, and 0·1 ml. of the stool extract or 0·2 ml. of CSF was then inoculated. The cultures were incubated at 35–37° C. in a stationary state and observed for at least 7 days. The fluids of any cultures exhibiting marked cytopathic effect were passaged to fresh cell cultures for confirmation of the viral agents.

Identification of virus

Virus strains were identified by the neutralization test, using antisera to polioviruses 1–3, coxsackie A 9 and B 1-6 viruses, and to echoviruses 1-28. Neutralization of approximately 100 TCD 50 of virus by 20 neutralizing units of antiserum was considered a positive identification.

Neutralizing antibody test

Paired serum specimens from the same person were always tested simultaneously. Sera were heated at 56° C. for 30 min. before testing. Fourfold serial dilutions of the sera were tested against approximately 100 TCD 50 of poliovirus 1, Mahoney strain and of echovirus 14, the current strain, AM-53-63. Serum-virus mixtures were allowed to stand at room temperature for 1 hr. before inoculation into stationary monkey kidney cell culture tubes. Neutralizing-antibody titres were expressed as the reciprocal of the highest serum dilution that completely protected the cells in the presence of approximately 100 TCD 50 of virus.

RESULTS

(1) *Failure of vaccination with Sabin type* 1 *attenuated poliovirus in infants infected with echovirus* 14

Nine babies in the infants' institution in Sendai were chosen to receive Sabin oral poliovaccine as a group of our field trial of the vaccine. On 5 October 1962, faeces and blood specimens were taken before vaccination and examined for enteroviruses and antibody titres. Three days later, 1 ml. of type 1 vaccine virus containing 10^5 or 10^6 TCD 50 was given by mouth. All the babies were under 8 months old at the time of feeding vaccine. Faeces were obtained 10 days and blood specimens 7 weeks after the feeding of vaccine.

Results are shown in Table 1. From the faecal specimens obtained 3 days before feeding vaccine, no virus was recovered in any of the babies tested. From those obtained 10 days after the feeding, however, echovirus 14 was isolated in six of the nine tested. Poliovirus type 1, which probably was the vaccine virus, was isolated only in two babies. No viruses were recovered in one case (no. N-7). Significant rise (fourfold) of polio 1 antibody was observed in only three cases, in spite of the absence of polio-antibodies in all babies before vaccine was given. On the other hand, a significant rise of echovirus 14 antibody was found in eight babies. Seven of them had no detectable antibody against echovirus 14 before feeding vaccine. However, one (no. N-7) showed a higher titre of echovirus 14 antibody in the first serum, suggesting a recent infection with echovirus 14 before poliovaccine was given. Another (no. N-1) showed echovirus 14 antibody to a titre of 1/4 in the first serum. This might have been maternal antibody, as no virus was isolated from the faecal specimen obtained on the day when the blood sample was taken, but echovirus 14 was recovered from the faeces 13 days later. No symptoms due to the alimentary infections were observed in any of the infants.

From the results of virological and serological examinations, it can be postulated

Table 1. *Virus isolation and neutralizing antibody titre in nine infants fed Sabin type* 1 *poliovaccine*

Baby no.	Age (weeks)	TCD50 of fed vaccine (log)	Virus isolation: Sampling time (days)*	Virus isolation: Results	Neutralizing antibody: Bleeding time (days)*	Neutralizing antibody: Titre against Polio 1	Neutralizing antibody: Titre against Echo 14
			−3	Neg.	−3	0†	4
N-1	4	5	10	Echo 14	49	0	16
			−3	Neg.	−3	0	0
N-2	4	5	10	Polio 1	49	0	256
			−3	Neg.	−3	0	0
N-3	4	6	10	Echo 14	49	0	16
			−3	Neg.	−3	0	0
N-4	4	6	10	Echo 14	49	0	256
			−3	Neg.	−3	0	0
N-5	12	5	10	Echo 14	49	0	256
			−3	Neg.	−3	0	0
N-6	13	6	10	Echo 14	49	16	256
			−3	Neg.	−3	0	1024
N-7	13	6	10	Neg.	49	0	256
			−3	Neg.	−3	0	0
N-8	26	5	10	Polio 1	49	16	256
			−3	Neg.	−3	0	0
N-9	27	5	10	Echo 14	49	16	16

* Days from the feeding vaccine.
† Less than 4.

Table 2. *Effectiveness of vaccination with Sabin's attenuated poliovirus* 1 *on infants at the out-patient clinic of National Hospital of Sendai*

Baby no.	Age (weeks after birth)	TCD50 of fed vaccine (log)	Virus isolation: Sampling time (days)*	Virus isolation: Result	Neutralizing antibody against poliovirus 1: Bleeding time (days)*	Neutralizing antibody against poliovirus 1: Titre
			−3	Neg.	−3	0†
K-2	3	5	10	Neg.	49	64
			−3	Neg.	−3	0
K-13	4	5	10	Neg.	49	0
			−3	Neg.	−3	0
K-22	13	5	10	Polio 1	49	64
			−3	Neg.	−3	0
K-23	14	6	10	Polio 1	49	16
			−3	Neg.	−3	0
K-30	24	5	10	Polio 1	49	64
			−3	Neg.	−3	0
K-31	26	6	10	Neg.	49	0

* Days from the feeding vaccine.
† Less than 4.

as follows: case no. N-7 had been infected with echovirus 14 before vaccine was fed and then the virus spread to other infants. The time of infection with echovirus 14 was apparently very close to that of the poliovirus vaccine feeding. Thus, the alimentary infection with echovirus 14 in infants possibly interfered with the establishment of infection with the oral poliovirus, and resulted in a failure of the vaccination in six out of nine babies.

In the same programme of this field trial, thirteen babies in the out-patient clinic of the National Hospital of Sendai were also given the same vaccine in the same manner and were examined by the same procedure. Six of them had no detectable antibody against poliovirus type 1 before vaccination. As shown in Table 2, four out of the six showed a significant rise of antibody after vaccination.

Table 3. *Monthly incidence of aseptic meningitis associated with enteroviruses, Aomori,* 1963

Month	No. of patients tested	No. of patients of positive isolation of following virus type								
		Poliovirus		Coxsackievirus			Echovirus		Un-identified	Total
		1	3	A9	B2	B4	6	14		
January	2	—	—	—	1	—	—	—	—	1
February	1	—	—	—	—	—	—	—	—	0
March	2	—	—	—	1	—	—	—	—	1
April	1	—	—	—	—	—	—	—	—	0
May	4	1	—	—	1	1	—	—	—	3
June	8	—	—	—	1	1	—	—	1	3
July	17	1	2	1	1	—	—	—	4	9
August	10	—	—	—	—	—	—	4	—	4
September	6	—	—	—	—	—	—	2	—	2
October	5	—	—	—	—	—	—	1	—	1
November	2	—	—	—	—	—	—	—	—	0
December	1	—	—	—	—	—	1	—	—	1
Total	59	2	2	1	5	2	1	7	5	25

(2) *An outbreak of aseptic meningitis associated with echovirus* 14

During the longitudinal studies on virus etiology of the involvement of the central nervous system in Aomori district, it was found that echovirus 14 was a main causative agent of aseptic meningitis which occurred in the summer and fall of 1963. Virus isolation was attempted from fifty nine children and infants with aseptic meningitis at the Pediatric Clinic of Aomori Prefectural Central Hospital, Aomori City, during 1963. Results are summarized in Table 3. From the beginning of January to the end of July 1963, poliovirus types 1 and 3, coxsackie A9, B2 and B4 viruses, and unidentified viruses which were probably coxsackieviruses since they caused paralysis in unweaned mice, were isolated from the faecal or cerebrospinal fluid specimens of seventeen patients. On the other hand, during the following 3 months (August–October), only type 14 echovirus was recovered from seven patients, and echovirus 6 was isolated from one patient in December. In one case (AM-53-63 in Table 4) echovirus 14 was recovered from both faeces and CSF.

The results suggested that the main causative virus of aseptic meningitis from August to October 1963 was echovirus 14.

Paired sera from twenty-one patients with aseptic meningitis were examined for neutralizing antibody against echovirus 14. As shown in Table 4, antibody titres against echovirus 14 were detected in all seven cases excreting echovirus 14 and in four of the virus-negative patients. Thus over 50 % of the aseptic meningitis patients found from August to October had been infected with echovirus 14. Although paired sera of fourteen patients with aseptic meningitis found in June and July, from whom no virus was recovered, were examined for neutralizing antibody against echovirus 14, no significant rise of antibody was found in any.

Table 4. *Virological and serological evidence on patients with aseptic meningitis caused by echovirus* 14

Patient no.	Age (year-month)	Onset date	Echovirus 14 isolation			Echovirus 14 Neutralizing antibody	
			Specimen	D.D.*	Results	D.D.*	Titre
AM-36-63	4–6	Aug. 12	Faeces	2	+	2	0†
			CSF	2	–	16	64
AM-37-63	1–10	Aug. 13	Faeces	2	+	2	0
			CSF	2	–	14	256
AM-38-63	2–6	Aug. 16	Faeces	2	+	2	0
			CSF	4	–	36	64
AM-49-63	3–3	Aug. 30	Faeces	2	+	2	0
			CSF	2	–	18	256
AM-41-63	2–6	Aug. 30	Faeces	2	–	4	0
			CSF	2	–	17	16
AM-42-63	6–9	Sept. 11	Faeces	1	–	2	0
			CSF	1	–	15	16
AM-43-63	1–10	Sept. 12	Faeces	2	+	2	0
			CSF	2	–	17	16
AM-45-63	2–6	Sept. 13	Faeces	4	+	4	0
			CSF	4	–	18	16
AM-55-63	2–9	Oct. 3	Faeces	3	–	9	64
			CSF	Not available		29	256
AM-53-63	2–9	Oct. 18	Faeces	2	+	2	0
			CSF	1	+	30	16
AM-52-63	1–6	Nov. 18	Faeces	2	–	1	16
			CSF	2	–	27	64

*Days after onset of illness.
† Less than 4.

These facts strongly suggest that a small epidemic of aseptic meningitis caused by echovirus 14 occurred among children and infants in Aomori in the middle summer and fall of 1963.

Symptoms and signs of most of the patients with echovirus 14 infection were typical of aseptic meningitis due to many other enteroviruses. High fever, nausea,

vomiting, and abnormal findings on examination of CSF, were common in these patients, except in one case (no. AM-55-63 in Table 4) who exhibited transient gait disturbance and a pleocytosis in the CSF but no fever.

DISCUSSION

Isolation of echovirus 14 from patients with aseptic meningitis has been reported from many parts of the world. Especially, isolation of the virus from the CSF confirmed that the virus was associated with asceptic meningitis (Henley, Berger & Hodes, 1958; McLean, McQueen & McNaughton, 1962; Lepow *et al.* 1962; Kelen, Delbin, Lesiak & Labzoffsky, 1963; Miyamoto *et al.* 1964). However, no outbreak of aseptic meningitis caused by this virus has been reported so far. In Japan, the first isolation of echovirus 14 from a case of aseptic meningitis was reported from this laboratory by Miyamoto *et al.* (1964). The virus was recovered from both CSF and faeces of one case which occurred in Sendai in the fall of 1962.

The results described in the present paper suggest that the incidence of aseptic meningitis from August to October 1963 in Aomori was an outbreak, though not on a large scale, due to echovirus 14, because frequent isolation of echovirus 14 from the patients was observed in a limited area and over a short period of time. This is also supported by the fact that echovirus 14 was isolated from the CSF of one patient and by the significant rise of antibody titre in many patients. An epidemic of gastro-enteritis caused by echovirus 14 has been reported by Lépine *et al.* (1960). It was also confirmed that the virus, spread widely in a population, did not cause any detectable illness among infants or children, as reported in the paper by Ingram *et al.* (1962) and in the present paper.

Since 1961, viruses causing aseptic meningitis occurring in Aomori have been studied in this laboratory. A large epidemic associated with Coxsackie B5 virus, and to a lesser extent with Coxsackie A9 virus was observed in 1961, as reported previously by Hinuma *et al.* (1964). In 1962, a prevalent virus was Coxsackie A9 virus, and the incidence pattern of the illness suggested that it was a small outbreak due to the A9 virus (unpublished data). In 1963, as noted in this paper, Coxsackie B2 virus was the principal strain isolated from January to June but echovirus 14 from August to October. Although the study is still in progress, echovirus 4 has been isolated from a fairly large number of patients in the summer of 1964 (unpublished data). Thus, the dominant virus associated with aseptic meningitis in Aomori has apparently changed year by year or even season by season within a year.

Another effect of echovirus 14 infection described in this paper is a possible interference with a vaccine strain of poliovirus 1. Several investigators have reported that naturally occurring infections with enteroviruses may interfere with the intestinal infection with attenuated polioviruses, as cited in the paper by Ingram *et al.* (1962). In contrast to our observations, Ingram *et al.* (1962) reported that echovirus 14, which was prevalent in an infants' institution in Cleveland, U.S.A., appeared to have little suppressive effect upon an attenuated strain of poliovirus 1. Possible interference by the echovirus 14 was suggested in only two

of twenty-five vaccinated babies. In our study poliovirus type 1 was recovered from only two of nine babies who were fed 10^5 or 10^6 TCD50 of type 1 strain of Sabin's poliovaccine, but echovirus 14 was isolated from six babies, 10 days after feeding. Serological examination gave evidence that only three out of nine vaccinated babies showed a rise of poliovirus 1 antibody whereas a significant rise of echovirus 14 antibody was observed in eight babies 7 weeks after the vaccination. This serological evaluation of effectiveness of the vaccine indicates that the vaccination in the infants' institution reported here was less effective than that in the National Hospital of Sendai as shown in Table 2. This failure of vaccination might be caused by the interference with echovirus 14 infection which was evident by virus isolation and serological examination.

In the studies by Ingram *et al.* (1962), antibody response to echovirus 14 was observed in only 12% of the infected infants. In this report, all the serum-negative infants clearly showed the echovirus 14-antibody response. As discussed by Ingram *et al.* (1962) the difference between these two observations may be explained by a variation in antigenicity among echovirus 14 strains distributed in many parts of the world.

SUMMARY

Two events concerned with echovirus 14 infection were reported.

The first, which occurred in Sendai in 1962, was that the intestinal infection with the attenuated poliovirus 1 vaccine was possibly suppressed by naturally occurring infection with echovirus 14. Echovirus 14 was isolated 10 days after the vaccine feeding in six out of nine cases, but poliovirus in only two cases. Six of the vaccinated babies did not exhibit antibody response to the poliovirus 1, while a significant rise of antibody titre against echovirus 14 was seen in as many as eight babies 7 weeks after the vaccination.

The second was a small outbreak of aseptic meningitis due to echovirus 14 in Aomori in 1963. During August to October, echovirus 14 was isolated from the faeces of seven patients with aseptic meningitis. The virus was also isolated from the CSF in one case. The antibody titre against echovirus 14 was detected in all the patients from whom the virus was isolated. In addition, echovirus 14 antibody was detected in four out of fourteen patients with aseptic meningitis from whom no virus was recovered. The onset of serologically positive patients was limited to the months of August to October.

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Staphylococcus aureus strains associated with the hedgehog, *Erinaceus europaeus*

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Early in 1963, Smith & Marples (1964) showed the short eared hedgehog, *Erinaceus europaeus*, to be a natural selector and propagator of penicillin resistant forms of *Staphylococcus aureus*. Until this time, no satisfactory answer to the 'pre-penicillin era' occurrence of penicillin resistant strains had been found. This paper is the full report of the examination of free-living hedgehogs in New Zealand for coagulase positive staphylococci. Hedgehogs appear to constitute a hitherto unrecognized reservoir of penicillin resistant strains of *Staph. aureus*.

MATERIALS AND METHODS

Fifty-nine hedgehogs were collected from urban parks and gardens in various areas of New Zealand. The skin of fifty-six, paws of fifty-seven and anus of eleven were sampled by means of a moistened swab. Nasal samples were obtained from fifty-eight of the animals using a small wire loop which was gently rotated in each nostril.

All swabs and samples were incubated in 10 % salt broth for 12–18 hr. and, after that, each tube of salt broth was inoculated on blood agar. The only swabs which were plated direct, without enrichment in salt broth, were from the skin of six hedgehogs showing obvious skin crusting. All plates were examined after suitable incubation and samples of each colonial type of staphylococci were subcultured on fresh media. After incubation, these subcultures were examined for coagulase production using the tube method.

All coagulase positive strains were phage typed using the international set of twenty-two human phages. Strains typing with phage 81 were listed as phage group I strains. Sensitivity or resistance to disks of penicillin (3 units) was determined. In each case, the Oxford strain of *Staph. aureus* known to be sensitive to 0·03 units of penicillin was included. Fifty of the strains selected at random were examined for resistance to disks of the following antibiotics; chloramphenicol, 20 μg.; streptomycin, 66 units; erythromycin, 20 μg.; and celbenin, 10 μg. The Oxford staphylococcus was used as a control in each experiment. The ability of forty strains to produce β-lysin was determined by stab inoculation on to washed sheep erythrocyte agar plates.

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Table 1. *Phage type and penicillin resistance of* Staphylococcus aureus *strains recovered from hedgehogs*

	Phage type		No.	Penicillin resistance
Phage group I	81	1*	5	5
	52	1*	4	4
	52/52A/80	—	3	3
	52A/81+	1*	2	2
	79+	1*	1	1
	52/52A	—	1	1
	29/52/52A/80	—	1	1
		4	17	17
Phage group II	3A	1*	1	—
	3C	—	1	1
	3A/3B/3C/55/77	—	1	1
		1	3	2
Phage group III	47	2*	8	5
	7/47	5*	5	5
	42E/75	—	3	3
	6/7/42E/47+	—	2	1
	7/47/53/54/75	1*	2	2
	6/47/53/75	1*	1	1
	6/42E/47/54/75	—	1	1
	47/77+	—	1	1
	42E	—	1	1
	6+	—	1	—
		9	25	20
Untypable			12	7
Not typed			6	6

	Phage type		No.	Penicillin resistance
Unclassifiable mixed groups	42E/7/81	—	6	6
	7/79	4*	4	4
	42D/42E/81	—	4	4
	42D/42E/75	—	1	1
	42D/75/77+	3*	3	3
	42D/42E/6/79/81	—	2	2
	42D/47	—	1	1
	42D/54/75/77	1*	1	1
	42D/42E/6/7/47/54/75/77/80/81	—	2	—
	52/42E/7/81	—	1	1
	54/80	—	1	1
	42E/7/77/81	—	2	1
	42E/6/7/47/75/77/79	—	2	1
	42E/6/7/47/75/81	—	1	1
	42E/6/7/47/55/77/81	—	4	3
	42E/6/7/47/77/81	—	1	1
	42E/6/7/47/79/77/81+	—	2	2
	42E/6/47/53/77/79	—	1	1
	42E/6/47/53/75/77/79	—	1	1
	42E/47/77/79+	—	2	2
	29/42D/42E/79/81	—	2	2
	29/42D/42E/7/47/81	—	2	2
	29/42D/42E/6/7/47/79/80/81	—	4	4
	29/42E/6/7/47/53/75/77/79/81	—	1	1
	29/52A/42E/6/7/47/79/80/81	—	2	2
	29/52/52A/47/80	—	3	3
	29/42E/47	—	1	1
	29/47	—	2	1
	29/42D/3A/3C/42E/6/7/47/54/79/81+	—	1	1
	29/52/52A/42D/3A/3C/42E/6/7/47/54/79/80/81	—	1	1
		8	61	55

* Indicate typed At RTD

The sex, weight, and presence of skin parasites *Caparinia tripilis* and *Trichophyton mentagrophytes* var. *erinacei*, was recorded for each animal.

RESULTS

Incidence of Staphylococcus aureus

Fifty (85 %) of the fifty-nine hedgehogs yielded strains of *Staph. aureus*, from either the paws, skin, nasal cavity or anus. The phage types of the strains at RTD and 1000 × RTD are shown in Table 1. Altogether 124 strains were obtained. A summary of the isolations is shown in Table 2. Skin carriage was more frequent than carriage on other sites.

Table 2. *Isolation of* Staphylococcus aureus *from hedgehogs*

Area of animal	No. examined	No. positive	% positive	No. strains
Nasal	58	23	40	27
Skin	56	38	68	44
Paw	57	36	63	45
Anal	11	6	55	8

Table 3. *Phage groups of* Staphylococcus aureus *recovered from hedgehogs in various areas of New Zealand*

Area	Phage group I	II	III	Un-classified	Un-typed	Not typed
Dunedin (Nov. 1962/Mar. 1963)	11	1	1	12	2	3
Dunedin (Nov. 1963/Jan. 1964)	4	1	5	11	3	3
Upper Hutt (Jan. 1963)	—	1	14	18	2	—
Hamilton (Feb. 1964)	1	—	3	18	4	—
Other areas (1962–64)	1	—	2	2	1	—

Phage groups obtained

Phage typing was attempted on 118 of the 124 strains. Seventeen were of phage group I, 3 of phage group II, 25 of phage group III, 61 were typable but unclassifiable into any of the phage groups, and 12 were untypable. At RTD, 96 of the 118 strains were untypable and of these ninety-six, 84 typed at 1000 × RTD.

No predominance of phage groups in any body area was apparent.

Geographical distribution

Shown in Table 3 are the phage groups of staphylococci recovered from hedgehogs collected in various areas of New Zealand. Although ungroupable phage types predominated in each area, phage group I types were relatively common in the early Dunedin groupable strains, while phage group III staphylococci were the main classifiable types recovered from Upper Hutt.

Sex distribution

Male hedgehogs more frequently harboured pathogenic staphylococci than did females. If the number of strains recovered from each sex is compared with the number of body areas sampled, pathogenic staphylococci were recovered from 69·2 % male areas and only 40·0 % female areas. Phage groups were equally spread amongst the two sexes.

Age distribution

All ages of hedgehogs were found infected with staphylococci. Animals were divided into two groups depending on weight: 'young' hedgehogs which weighed less than 500 g. and 'old' hedgehogs which weighed more than 500 g. These represented ages of up to 6 months in the case of 'young 'animals, and between 9 and 18 months for 'old' animals. Few hedgehogs in New Zealand live beyond 18 months (Brockie, 1958). Table 4 reveals that both 'young' and 'old' hedgehogs were colonized by coagulase positive staphylococci to the same extent.

Table 4. *Isolation of* Staphylococcus aureus *from young and old hedgehogs*

	Nasal		Skin		Paw		Anal	
Age	No. examined	No. positive	No. examined	No. positive	No. examined	No. positive	No. examined	No. positive
Young	23	8	23	17	24	15	6	4
Old	26	11	24	17	24	15	5	2

Table 5. *Distribution of mites and* Staphylococcus aureus *on the skin of forty-three hedgehogs*

Normal animals			Slightly scabby animals			Very scabby animals		
No. examined	Staph. aureus	Mites	No. examined	Staph. aureus	Mites	No. examined	Staph. aureus	Mites
17	10	1	14	11	5	12	12	9

Effect of parasitic mites

Hedgehogs in New Zealand are commonly infected with the acarine mite *Caparinia tripilis* (Brockie, 1958). How these parasites influence the skin microflora is difficult to assess. Coagulase positive staphylococci were found on both normal and scabby looking animals. Mites on the other hand appeared to be correlated directly with skin scabbiness and were usually absent from normal animals. These points are shown in Table 5.

Staph. aureus strains could easily be recovered from scabby animals. Direct plating of swabs from the six hedgehogs with obvious skin scaling resulted in almost pure growths of *Staph. aureus*. Enrichment in salt broth was required to recover staphylococci from 'normal' animals. Thus the presence of mites did appear to favour the increased multiplication of staphylococci. Plate 1 is a picture of the snout and ears of a wild hedgehog naturally infected with mites, *T. mentagrophytes* var. *erinacei*, and pathogenic staphylococci.

Effect of fungi

Hedgehogs in New Zealand are also heavily infected with the dermatophyte *T. mentagrophytes* var. *erinacei* (Smith & Marples, 1963). The presence of this fungus in the skin of the animals did not appear to influence the distribution of coagulase positive staphylococci. Of thirty-three hedgehogs suffering from ringworm twenty-seven (82 %) also harboured staphylococci, while twenty-one (81 %) of twenty-six fungus-free animals were colonized by these bacteria.

Antibiotic sensitivities

Of the 124 coagulase positive strains isolated, 107 (86·3 %) were resistant to penicillin G (3 units). All fifty strains examined were sensitive to streptomycin, chloramphenicol, tetracycline, erythromycin and celbenin.

β-lysin production

Of the forty strains examined for β-lysin production, 33 (83 %) were positive.

DISCUSSION

It appears that hedgehogs are very suitable hosts for coagulase positive staphylococci. Unfortunately it is difficult to compare the distribution of staphylococci in hedgehogs with that occurring in other animals, for, apart from man, surveys on the normal animal incidence of pathogenic staphylococci have been largely neglected. Hedgehogs carry coagulase positive staphylococci on the skin much more frequently than is found in man, for these organisms were recovered from the skin of 68 % and paws of 63 % hedgehogs. The corresponding normal human figures are 16 % for skin and 40 % hands (Williams, 1963). Nasal carriage in hedgehogs and man is at approximately the same level (40 %). It would seem that in the hedgehog the skin is the primary site of staphylococcal multiplication.

The growth of dermatophytes in animal skin provides an ideal environment for the colonization and rapid multiplication of *Staph. aureus* (Smith, unpublished). Presumably the high incidence of *T. mentagrophytes* var. *erinacei* in New Zealand hedgehogs is partly responsible for the high incidence of staphylococci on the skin. The acarine mite, *Caparinia tripilis*, may also assist in the establishment of coagulase positive staphylococci by damaging the skin surface. Sections of intact mites revealed the presence of numerous micrococci. These parasites could be responsible for the spread of *Staph. aureus* between individual hedgehogs.

Hedgehog strains of *Staph. aureus* appeared to be relatively susceptible to human staphylophages, only twelve of 118 strains being untypable with a set of twenty-two human phages. However, only 19 % of the strains were typable at RTD, the majority typing satisfactorily only at 1000 × RTD. Cattle and other animal strains do not type satisfactorily with human phages (Marshall, 1964). This worker found only about 60 % of his strains to be typable with human phages. As forty-five of the 106 typable hedgehog strains were groupable into recognized human groups, and as animal strains of staphylococci have been shown to be capable of infecting humans (Moeller, Smith, Shoemaker & Tjalma, 1963), it is

reasonable to assume that hedgehogs may provide a reservoir for human infections. Phage types 52, 52/52A, 80 and 81 or combinations of these types represented 16% of the typable strains. Phage group III made up 24% of the typable strains with types 47 and 7/47 most prominent. While many of the strains were groupable into human phage groups the majority of these were of animal origin, for they produced β-lysin. In general the finding of β-lysin producing strains in routine material is about ten times more likely if the strain originated from animal sources (Elek & Levy, 1950).

The presence of one phage type in hedgehogs did not appear to inhibit the carriage of a second type. This is contrary to the human results reported by Williams (1963), who found that prior infection with one strain excluded the acquisition of other strains. However, the carriage of one phage group in hedgehogs did influence the presence of other groups (see Table 3).

The outstanding feature of hedgehog *Staph. aureus* strains was the high percentage (86·3%) resistant to penicillin and only penicillin. This resistance was due to penicillinase production as all strains were sensitive to celbenin. Penicillin resistance in human pathogenic staphylococci is often coupled with resistance to other antibiotics (Wentworth, 1963). The normal incidence of penicillin-resistant types in the *Staph. aureus* population of man is only about 25% (Rountree & Rheuben, 1956).

In staphylococcal strains from man, penicillin resistance appears to favour certain phage types (Parker, 1958). This was not the case with hedgehog strains where antibiotic resistance was spread equally throughout phage groups I, II and III and unclassifiable and untypable strains.

The hedgehog in New Zealand provides a hitherto unrecognized reservoir of penicillin-resistant strains of *Staph. aureus*. Further work is required to determine to what extent these organisms may be transmitted to the human population.

SUMMARY

1. Strains of *Staphylococcus aureus* were obtained from the nostrils of twenty-three of fifty-eight hedgehogs; the skin of thirty-eight of fifty-six hedgehogs; the paws of thirty-six of fifty-seven hedgehogs and the anus of six of eleven hedgehogs.

2. Of 118 strains, 106 (90%) were typable with human staphylophages. Seventeen were phage group I, three phage group II, twenty-five phage group III, sixty-one were typable but unclassifiable into groups, and twelve were untypable.

3. Male hedgehogs were more heavily infected than females, while all ages of hedgehogs appeared equally susceptible to infection.

4. Of the 124 coagulase positive strains obtained, 107 (86·3%) were resistant to penicillin. Resistance to other antibiotics—chloramphenicol, streptomycin, tetracycline, erythromycin, celbenin—was not encountered.

5. Thirty-three (83%) of forty strains produced β-lysin.

6. Mites (*Caparinia tripilis*) and fungi (*Trichophyton mentagrophytes* var. *erinacei*) did not appear to directly influence the carriage of *Staphylococcus aureus* on the hedgehog skin.

J. M. B. SMITH

(*Facing p.* 291)

These investigations were carried out in the Microbiology Department, Medical School, University of Otago, Dunedin, New Zealand. I am indebted to Associate Prof. Mary J. Marples, Microbiology Department, for her continued help and encouragement during these investigations, and to Prof. J. A. R. Miles, Microbiology Department, for providing research facilities. Financial assistance given by the Medical Research Council of New Zealand is gratefully acknowledged. I would also like to thank Mrs Jean Markham for phage typing the staphylococcal strains.

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EXPLANATION OF PLATE

Ears and snout of a hedgehog naturally infected with *T. mentagrophytes* var. *erinacei*, *Caparinia tripilis*, and *Staphylococcus aureus*.

J. Hyg., Camb. (1965), **63**, 293
With 1 *plate*
Printed in Great Britain

Dermatophyte lesions in the hedgehog as a reservoir of penicillin-resistant staphylococci

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The investigations described in this paper were undertaken in an attempt to explain why such a high proportion (86 %) of the strains of *Staphylococcus aureus* carried by hedgehogs were resistant to penicillin (Smith & Marples, 1964). Since the discovery and classification of this antibiotic, several penicillin-like substances have been derived from organisms other than *Penicillium notatum*. These organisms include a number of *Penicillium* and *Aspergillus* species (Peck & Hewitt, 1945). The occurrence of antibiotic substances has been demonstrated in only a limited number of pathogenic fungi. Waksman, Horning & Spencer (1942) noted the production of antibiotics by *Aspergillus fumigatus*. Peck & Hewitt (1945) recorded the production of a penicillin-G-like substance during the *in vitro* growth of a number of dermatophytes, and in particular *Trichophyton mentagrophytes*. Uri, Szathmary & Herpay (1957) succeeded in demonstrating the *in vivo* production in ringworm lesions of an antibiotic biologically resembling penicillin G. The possible ecological implications of this antibiotic production on the microbial flora of a chronic ringworm lesion were not investigated. It is rare that antibiotic production can be shown to have ecological significance in a natural environment (Brian, 1957), although the story of the increasing incidence of antibiotic-resistant staphylococci in such man-made selective environments as the hospital ward has been well documented. Smith & Marples (1964) suggested that chronic mycotic infection of hedgehog skin (*Erinaceus europaeus*) provided an environment in which penicillin-resistant staphylococci had a selective advantage over sensitive strains, and that hedgehog skin is one in which the natural production of an antibiotic is affecting the structure of the biocenose.

This paper embodies the full report of these findings and demonstrates that ringworm lesions may be an important reservoir of penicillin-resistant strains of *Staphylococcus aureus*.

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MATERIALS AND METHODS

In vitro *antibiotic production by dermatophytes*

The ability of the following strains of dermatophytes to produce antibiotic-like substances *in vitro* was determined.

Serial no.	Species	Source
FM 10 (IMI 101, 051)	*Trichophyton mentagrophytes* var. *erinacei*	Hedgehog
FM 16		Hedgehog
FM 22		Hedgehog
Tt	*T. terrestre* (pigmented)	Hedgehog
M. 11	*T. mentagrophytes* var. *granulare*	Mouse
OAM	*T. mentagrophytes* var. *interdigitale*	Human foot
Fi/10	*Epidermophyton floccosum*	Human foot
Fi/140	*Trichophyton rubrum*	Human foot
Fi/146	*Microsporum canis*	Human scalp
TC 11	*Trichophyton concentricum*	Tokelau Is. (human)
TC 17	*T. concentricum*	Tokelau Is. (human)

A small portion (pin head) of a 7-day Sabouraud's dextrose agar culture of each of these eleven dermatophytes was inoculated into 25 ml. Sabouraud's dextrose broth contained in a 50 ml. Erlenmeyer flask. All flasks were shaken in a 30° C. water-bath for 15 days. Each day 0·2 ml. of fluid was removed aseptically from the flasks and examined for antibiotic activity against the Oxford strain of *Staph. aureus*. The method used was that described by Peck & Hewitt (1945).

Any inhibitory substance active against the staphylococcal test strain was examined for inactivation by penicillinase. Sterile Sabouraud's dextrose broth (pH 8·0) was found to have no antagonistic effect on the test strain.

Strains FM 10 and FM 16 were also grown in 25 ml. of 5-strength Sabouraud's dextrose broth and the 'antibiotic' concentrations reached after 5 days agitation at 30° C. recorded. A flask with 25 ml. of Sabouraud's dextrose broth containing 2·5 units/ml. of penicillin G was also shaken in the 30° C. water-bath and the penicillin concentration recorded each day. Any changes in the pH of the fungal filtrates were recorded.

The antibiotic produced by *T. mentagrophytes* var. *erinacei* (FM 10), which gave a zone of inhibition equal to that of 1·1 units of penicillin G per ml. against the Oxford staphylococcus, was also examined for inhibition of the organisms listed in Table 1. In all experiments a control solution of Sabouraud's dextrose broth containing 1·1 units/ml. of penicillin G was run in parallel.

In vivo *antibiotic production by* T. mentagrophytes *var.* erinacei

An attempt was made to determine if the hedgehog strain of *T. mentagrophytes* produced an antibiotic-like substance when growing *in vivo*. Two lines of investigation were adopted.

(i) An attempt to demonstrate, by a plate assay method, antibiotic production by fungus-infected rabbit skin.

(ii) An examination of the organisms, especially *Staph. aureus*, recoverable from

fungus-infected guinea-pig skin to determine if any selection of those showing penicillin resistance had occurred. From previous experiments conducted *in vitro* it had been found that *T. mentagrophytes* var. *erinacei* produced appreciable amounts of an antibiotic substance biologically resembling penicillin G.

Assay of fungus-infected skin

A young male rabbit (600 g.) was experimentally infected with *T. mentagrophytes* var. *erinacei*. A 7-day Sabouraud's dextrose agar culture of the fungus (FM 10) was rubbed into a shaved scarified area of skin on the right hind quarter. When the ringworm had developed for 11 days, fungus-infected skin was removed and placed directly on plates seeded with a lawn of the Oxford strain of *Staphylococcus*. After overnight incubation at 37° C., any zones of inhibition were recorded. Normal skin from the same animal served as a control. Infected skin was also placed on a lawn of the staphylococci to which 6 drops of penicillinase had previously been added.

Investigation of organisms

Four young male guinea-pigs (less than 250 g.) were shaved on the right hind quarter, and the exposed skin scarified with a blunt scalpel. Replica plates were then taken of this shaved area using the method described by Gorill & Penikett (1957).

Swabs were also taken from the shaved area, inoculated into salt broths and incubated at 37° C. for 12–18 hr. Samples from each broth were then streaked on blood agar. After suitable incubation these plates were examined for coagulase-positive staphylococci. Subcultures of representative staphylococcal types from each plate were tested for coagulase production using the tube method.

All *Staph. aureus* strains were phage typed and their penicillin sensitivity was determined in tubes of broth containing penicillin concentrations of 0·037, 0·075, 0·15, 0·312, 0·625, 1·25, 2·5, 5·0, 10·0, 15·0, 20·0, 25·0, and 50·0 μg./ml., incubated overnight at 37° C. With each series of experiments, a control consisting of the Oxford strain of *Staph. aureus* was included.

After the replica plates and swabs had been taken, two of the four guinea-pigs were inoculated with *T. mentagrophytes* var. *erinacei* as described above. At weekly intervals, replica plates and swabs were taken from the two ringworm and two control animals, and examined as just described.

One of the fungus-infected guinea-pigs (and one of the non-infected control pigs) received regular doses of triamcinolone acetonide beginning 4 days after infection, in an attempt to produce a chronic fungal infection as described by Gross, Actor, Jambor & Pagano (1963).

RESULTS

In vitro *antibiotic production*

Of the eleven dermatophyte strains examined, only six showed any sign of antibiotic production during the 15 days of the experiment. These were *T. mentagrophytes* var. *erinacei*, FM 10, FM 16, FM 22; *T. mentagrophytes* var. *granulare*, M 11;

T. mentagrophytes var. *interdigitale*, OAM; and *E. floccosum*, Fi/10. Of these, *T. mentagrophytes* var. *erinacei* strains produced the highest antibiotic yield. The three hedgehog strains (FM 10, FM 16, FM 22) gave uniform results as regards antibiotic production. This reached a maximum after 5 days and was equivalent to 1·1 units/ml. of penicillin G. *T. mentagrophytes* var. *interdigitale* had a maximum yield after 5 days and was equal to 0·2 units/ml. of penicillin G. The maximum reached by *T. mentagrophytes* var. *granulare* occurred after 11 days and was equivalent to 0·3 units/ml. of penicillin. *E. floccosum* appeared to produce two anti-

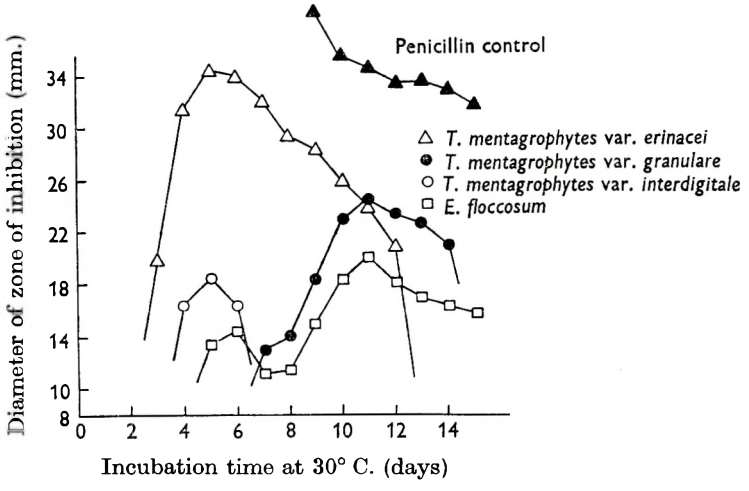

Fig. 1. Production of antibiotics by certain dermatophytes.

biotics, the first reaching a maximum after 6 days and the second after 11 days. Whereas the antibiotic produced by *T. mentagrophytes* strains was inactivated by penicillinase, that produced by *E. floccosum* was not completely neutralized. It appeared that more than one substance inhibitory to the Oxford staphylococcus was being produced by *E. floccosum*. These results are shown in Fig. 1.

If the flasks were not shaken during growth the fungus grew as a surface pellicle, and very little antibiotic was produced as compared with that from shaken flasks. If the flasks were shaken, the growth was microscopically very like that produced *in vivo* by dermatophytes, and it has been termed pseudo-parasitic.

As this paper is concerned with the effect of *T. mentagrophytes* var. *erinacei* on the skin microflora, no attempt was made to investigate fully the antibiotics produced by var. *interdigitale*, var. *granulare* or *E. floccosum*. The following results apply only to var. *erinacei*. The antibiotic factor first appeared in the fungal filtrate after 2 days, the concentration rising sharply to reach a maximum after 5 days. As already stated, this was equivalent to 1·1 units/ml. of penicillin G. This concentration then slowly declined at the same rate as the penicillin G control (see Fig. 1). The antibiotic production was accompanied by a sharp increase in the pH of the filtrate. This rose from the initial 5·6 to 8·3 during the first 4 days, and remained at this level for the remainder of the experiment. Sterile Sabouraud's

dextrose broth, pH 8·0, did not inhibit the staphylococcal test organism. When the fungus was grown in 5-strength broth, the concentration of antibiotic reached after 5 days was equivalent to 7·0 units/ml. of penicillin G.

The antibiotic produced by *T. mentagrophytes* var. *erinacei* was inactivated by penicillinase. The fungal filtrate appeared to have the same effect on the various test organisms as an equal concentration of penicillin G in Sabouraud's dextrose broth pH 8·0. Sensitive and resistant organisms are shown in Table 1.

Biologically therefore, the 'antibiotic substance' produced *in vitro* by *T. mentagrophytes* var. *erinacei* closely resembled penicillin G.

Table 1. *The reaction of various organisms to the antibiotic produced* in vitro *by* T. mentagrophytes *var.* erinacei

Sensitive organisms	Resistant organisms
α-Haemolytic streptococci	*Candida albicans*
Oxford strain of *Staphylococcus aureus*	*Proteus mirabilis*
	Aerobacter aerogenes
Pneumococci	*Escherichia coli*
Sarcina lutea	*Salmonella typhimurium*
	Enterococci
	Penicillin-resistant strain *Staphylococcus aureus*

In vivo *antibiotic production by* T. mentagrophytes *var.* erinacei

Assay of ringworm skin

Skin removed from the var. *erinacei* rabbit lesion inhibited the growth of staphylococci close to the skin. This effect was not shown by normal skin or in the presence of penicillinase. This is shown in Pl. 1.

Examination of organisms

The bacterial counts obtained from the four guinea-pigs by the replica plating technique are shown in Tables 2 and 3. An indication of the species present is also given. Unfortunately the hair had regrown into the uninfected skin after 2 weeks, which made further replica plating from these two animals unreliable. Replicas of the skin and hair were taken and any coagulase-positive staphylococci isolated counted. These isolations are shown in brackets in Table 3. The outstanding feature of the results was the increase of coagulase-positive staphylococci in fungal-infected skin as compared with the non-increase on normal skin. If the number of colonies of *Staph. aureus* is taken as a percentage of the total count both ringworm-infected guinea-pigs present a similar picture. Normal skin is relatively resistant to colonization by pathogenic staphylococci (Williams, 1963). It is probable that isolations obtained from the skin and hair of non-infected guinea-pigs represented transient 'visitors' from the air, and not organisms which were multiplying on the skin.

The low total counts on the fungus-infected animal which received triamcinolone acetonide can probably be attributed to the inhibition of the inflammatory response

in the ringworm lesion by this substance. This lesion was not nearly so scabby and raw as that in the other fungus-infected animal.

The *Staph. aureus* strains isolated from the four guinea-pigs via the salt broth enrichment were all of similar phage type. This strain was lysed by many or all of

Table 2. *Organisms recovered by replica plating from fungal-infected guinea-pig skin*

	Total	Micro-cocci	Gram + rods	α-Haem. streps.	Coag. + staphs
Guinea-pig 1. Ringworm + triamcinolone acetonide					
Original	90	83	7	—	—
Week 1	430	410	20	—	—
Week 2	270	69	17	180	4
Week 3	156	62	2	90	2
Week 4	151	32	1	100	18
Guinea-pig 2. Ringworm only					
Original	110	102	8	—	—
Week 1	420	370	50	—	—
Week 2	500	449	10	20	21
Week 3	500	463	7	10	20
Week 4	500	429	5	—	66

Table 3. *Organisms recovered by replica plating from normal guinea-pig skin*

	Total	Micro-cocci	Gram + rods	α-Haem. streps.	Coag. + staphs
Guinea-pig 3. Control. Triamcinolone acetonide					
Original	126	120	6	—	—
Week 1	30	15	15	—	—
Week 2	—	—	—	—	—
Week 3	—	—	—	—	(1)
Week 4	—	—	—	—	—
Guinea-pig 4. Control					
Original	101	94	7	—	—
Week 1	38	18	20	—	—
Week 2	—	—	—	—	(1)
Week 3	—	—	—	—	(2)
Week 4	—	—	—	—	(1)

the following phages, 6/7/42E/47/53/54/75/77/83A/81. Slight variations were found each week, as shown in Table 4. Also shown in this table is the inhibitory concentration of penicillin for each strain. As shown, the penicillin inhibitory concentration of the staphylococcal strains recovered from the ringworm lesions increased from the initial 5·0 μg./ml. to approximately 20 μg./ml. after 3 weeks. No such increase was apparent with the staphylococci recovered from the normal control animals. Organisms with high penicillin resistance appear to be selected for propagation in *T. mentagrophytes* var. *erinacei* infected guinea-pig skin.

Table 4. *Phage type and inhibitory concentration of penicillin of* Staphylococcus aureus *strains recovered from* Trichophyton mentagrophytes *var.* erinacei *infected and non-infected guinea-pig skin*

The inhibitory concentration of penicillin, in μg./ml., is shown below each phage type.

	Guinea-pig 1. Ringworm + ve. Triamcinolone acetonide	Guinea-pig 2. Ringworm + ve.	Guinea-pig 3. Control. Triamcinolone acetonide	Guinea-pig 4. Control.
Originally	6/7/42E/47/53/54/75/77/83A/81 5·0	—	—	6/7/42E/47/53/54/75/77/83A/81 5·0
Week 1	6/7/42E/47/53/54/75/77/83A/81 5·0	6/7/42E/47/53/54/75/77/83A/81 5·0	6/7/42E/47/53/54/75/83A + 5·0	6/7/42E/47/53/54/75/77/83A + 5·0
Week 2	6/7/42E/47/53/54/75/83A/81 20·0	6/7/42E/47/53/54/75/83A + > 20·0	6/7/42E/47/53/54/75/83A/81 2·5	6/7/42E/47/53/54/75/77/83A + 5·0
Week 3	6/7/42E/47/53/54/75/77/83A + 15·0	6/7/42E/47/53/54/75/77/83A + > 20·0	6/7/42E/47/53/54/75/77/83A + 5·0	6/7/42E/47/53/54/75/77/83A + 5·0
Week 4	6/7/42E/47/53/54/75/83A/ + 20·0	6/7/42E/47/53/54/75/77/83A + > 25·0	6/7/42E/47/53/54/75/83A + 5·0	6/7/42E/47/53/54/75/83A + 2·5
Week 5	6/7/42E/47/53/54/75 + 15·0	6/7/42E/47/53/54/75 + 25·0	6/7/42E/47/53/54/75 + 1·25	6/7/42E/47/53/54/75 + 5·0

DISCUSSION

Skin carrying a pathogenic fungus appears to provide a favourable habitat for coagulase-positive staphylococci (Marples & Bailey, 1957). Guinea-pig skin experimentally infected with *T. mentagrophytes* var. *erinacei* was readily colonized by *Staph. aureus* strains, which appeared to thrive in the ringworm tissue. In wild hedgehogs the skin is the primary site of staphylococcal multiplication (Smith, 1965). The presence of *T. mentagrophytes* var. *erinacei* in the skin of a high proportion of the animals (Smith & Marples, 1963) may be partly responsible for the growth of *Staph. aureus* strains in such an environment. Experiments have been conducted on possible symbiotic relationships between dermatophytes and staphylococci. When they are grown together on solid media, the results depend on which organism is favoured by the cultural conditions (Elek, 1959). Patiala (1947) found fluid cultures of some dermatophytes to exert an inhibitory effect on staphylococci. Elek (1959) considered the clinical significance of such observations to be doubtful. The reverse effect has also received attention. Catanei (1929) and Fabiani (1932) found *T. schoenleini* to be stimulated by staphylococci, while Vanbreuseghem (1948) found that certain staphylococcal strains inhibited this fungus *in vitro*. The present investigations indicate that, in hedgehogs, *T. mentagrophytes* var. *erinacei* and penicillin-resistant strains of staphylococci do live symbiotically on the skin.

In vitro, *T. mentagrophytes* appeared capable of producing an antibiotic biologically resembling penicillin G. Of the three varieties of *T. mentagrophytes* examined, var. *erinacei* was most proficient in producing the antibiotic. This *in vitro* production of penicillin by *T. mentagrophytes* had previously been demonstrated by Peck & Hewitt (1945). These workers found granular strains to be proficient in producing penicillin (var. *erinacei* not being discovered at this time). Whether or not *T. mentagrophytes* var. *erinacei* will prove of value as a commercial source of penicillin is outside the scope of this paper. By simply increasing the concentration of the medium fivefold, 7·0 units of penicillin/ml. could be produced in 5 days. Glaxo Laboratories in England have been able to produce up to 3·0 units/ml. of penicillin from *Trichophyton* strains (other than var. *erinacei*) (Richmond, personal communication).

The penicillin production by var. *erinacei* was accompanied by a sharp increase in the pH of the medium from 5·6 to 8·3. Peck & Hewitt (1945) noted a similar pH rise in the filtrates of their dermatophyte strains. Whereas var. *erinacei* reached its maximum concentration of penicillin and highest pH value after only 5 days growth, Peck & Hewitt found their strains of *T. mentagrophytes* to take nearly 12 days to reach maximum production and pH level. This is similar to the findings with var. *granulare* in the present investigations (see Fig. 1).

Peck & Hewitt (1945) were able to demonstrate antibiotic production in *T. violaceum*, *T. tonsurans* and *E. floccosum* but not in *Microsporum canis*, *M. audouini* or *T. rubrum*. In the present investigations, *M. canis*, *T. rubrum*, *T. concentricum* and *T. terrestre* did not appear capable of producing antibiotics *in vitro*.

T. mentagrophytes and *E. floccosum* have been shown to produce a substance resembling penicillin G when growing *in vivo* (Uri *et al.* 1957). These workers found that skin from ringworm lesions inhibited the growth of penicillin-sensitive organisms (*Bacillus subtilis*), the inhibition being neutralized by penicillinase. Rabbit skin infected with *T. mentagrophytes* var. *erinacei* appeared to have the same effect. A substance active against penicillin-sensitive organisms and neutralized by penicillinase could be demonstrated in fungus-infected skin. Normal skin did not show this effect.

The production of penicillin by var. *erinacei* in guinea-pig skin was well demonstrated by the increase in penicillin resistance of the *Staph. aureus* strain residing in the ringworm. The staphylococci, which were initially inhibited by 5·0 μg./ml. of penicillin G, were found to increase in resistance as the ringworm progressed. After 3 weeks, the inhibition level had risen to above 20 μg./ml. The *in vivo* production of penicillin by var. *erinacei* would not have to be great to account for this increase. Leitner, Sweeney, Martin & Cohen (1963) demonstrated that between 0·6 and 2·0 units/ml. of penicillin had the same effect as 20 units/ml. in triggering off the enzymic action of penicillinase production. Even 0·01–0·02 units/ml. of penicillin were sufficient to induce penicillinase production. It is easy to imagine this concentration being produced in chronic ringworm lesions.

Penicillin-resistant strains of *Staph. aureus* were present before the therapeutic use of penicillin. Until now no satisfactory theory has been advanced to account for this. It appears as if the presence of *T. mentagrophytes* var. *erinacei* in hedgehog skin does provide an environment selective for penicillin-resistant strains of staphylococci. The presence of the fungus is considered the main reason why so high a proportion (86 %) of the strains of *Staph. aureus* carried by hedgehogs are penicillin resistant.

As 90 % of the hedgehog *Staph. aureus* strains were typable with human staphylophages (Smith, 1965) it would appear that these animals provide a reservoir of penicillin-resistant pathogenic staphylococci. We consider that chronic ringworm lesions of other animals and man may also be of importance as reservoirs of penicillin-resistant *Staph. aureus*.

SUMMARY

An antibiotic substance biologically resembling penicillin G was produced by the growth of *Trichophyton mentagrophytes* var. *erinacei*, *T. mentagrophytes* var. *granulare* and *T. mentagrophytes* var. *interdigitale* in Sabouraud's dextrose broth. An antibiotic concentration equivalent to 7 units/ml. penicillin G could be produced by var. *erinacei* when grown in a suitable nutrient medium. *Epidermophyton floccosum* also produced a substance which inhibited the growth of the Oxford staphylococcus. However, this substance was not completely inactivated by penicillinase. No *in vitro* antibiotic production could be demonstrated with *T. rubrum*, *T. concentricum*, *T. terrestre* or *Microsporum canis*.

Penicillin production could be demonstrated in rabbit skin infected with *T. mentagrophytes* var. *erinacei*. Pathogenic staphylococci falling on a var. *erinacei* guinea-

pig lesion increased rapidly in the ringworm tissue. The inhibiting concentration of penicillin G for such staphylococci was shown to increase from 5·0 μg./ml. to above 20·0 μg./ml. as the ringworm progressed.

The presence of *T. mentagrophytes* var. *erinacei* in a high percentage of hedgehogs is considered the main reason why the skin is the primary site of staphylococcal multiplication in hedgehogs and why most of the *Staphylococcus aureus* strains recoverable from these animals are penicillin resistant.

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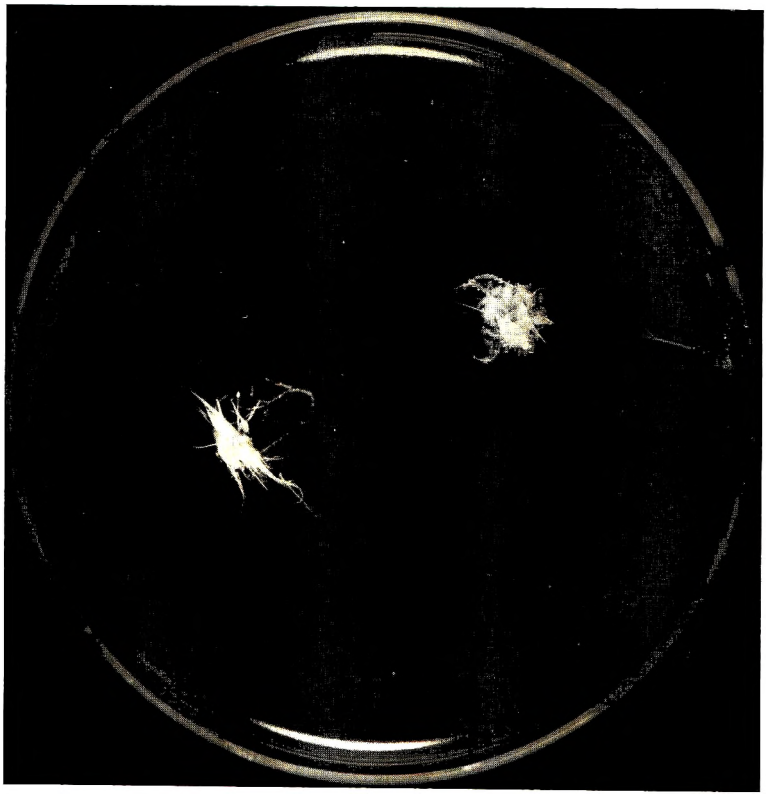

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EXPLANATION OF PLATE

Effect of *T. mentagrophytes* var. *erinacei* infected rabbit skin (right) and normal rabbit skin (left) on the growth of the Oxford strain of *Staphylococcus aureus*.

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Wire mesh screening for the exclusion of houseflies

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INTRODUCTION

Since the introduction of the modern synthetic residual insecticides, with their efficiency and convenience, many older methods of controlling insects have been neglected. The steadily increasing problem of insecticide resistance, however, coupled with growing concern about the possible hazards of toxic residues, have forced us to realize that we may not be able to rely on chemical control measures indefinitely. This is especially true of that versatile insect, the housefly, which has shown itself capable of developing resistance to all the more potent modern insecticides.

Among the older fly control measures, suitable in particular circumstances, is screening, which may be desirable for excluding flies from hospitals, canteens and food manufacturing plants. Moreover, a screen capable of excluding houseflies would also keep out blowflies and wasps (the latter being especially troublesome in jam factories).

Examining the literature, one finds specifications of wire or cloth mesh for the exclusion of mosquitoes; and these are based on actual experiments, usually with anophelines (Davey & Gordon, 1938; Block, 1946). The textbook on the housefly by West (1951) notes that 'A mesh of 14 wires to the inch will exclude houseflies, blowflies and similar species, but it is better to use about an 18-mesh screening in order to exclude smaller insects at the same time'. This may be true in countries plagued with mosquitoes; but, indoors in Britain, these are seldom a problem and it seemed possible that a wider mesh might be adequate to exclude flies, blowflies and wasps. Such wider mesh would allow passage of more light and air and would be cheaper. Accordingly, some simple experiments were undertaken to determine the mesh size required.

WIRE MESH SPECIFICATIONS

The aperture size in wire mesh is dependent on the mesh number (i.e. number of wires per linear inch) and the thickness of the wire. The latter is graded by standard wire gauge (s.w.g.) numbers, which are a set of rather arbitrary figures, ranging from 7/0 (0·5 in.) to 1/0 (0·324 in.) and from 1 (0·3 in.) to 50 (0·001 in.), approximating to a geometrical series. Examples of diameters in the range likely to be used in insect screen gauze are as follows:

s.w.g.	16	18	20	22	24	26	28	30	32	34
d (in.)	0·064	0·048	0·036	0·028	0·022	0·018	0·015	0·012	0·011	0·009
(mm.)	1·63	1·22	0·914	0·711	0·559	0·457	0·376	0·315	0·274	0·234

As a result of the experiments on anopheline mosquitoes, the following recommendations have been made:

16 mesh, 31 s.w.g. (aperture 1·3 mm.). Excludes most mosquitoes.

18 mesh, 33 s.w.g. (aperture 1·15 mm.). Excludes all mosquitoes.

EXPERIMENTS WITH HOUSEFLIES

To determine the gauze dimensions necessary to exclude houseflies, some representative samples were obtained from Messrs N. Greening and Sons Ltd. (whose catalogue gives full details of all relevant dimensions). An extremely simple test method was employed. Batches of twenty to thirty flies were confined in glass jars (7 lb. jam jars) with the mouths covered with the gauze samples under test. Over each mesh, another jar was inverted to catch the flies which escaped. After 5 hr. the flies which escaped (and the remainder) were killed, counted and

Table 1. *Dimensions (mm.) of male flies which did not escape through 7/20 mesh and female flies which did escape. N.B. (a) With proboscis retracted. (b) Including wing base. (c) Pronotum to base of sternopleuron*

	Head		Thorax		Abdomen	
	Width	Depth[a]	Width[b]	Depth[c]	Width	Depth
Males	2·11	1·81	2·15	2·27	2·07	1·48
Females	2·12	1·84	2·35	2·35	2·37	1·91

Table 2. *Dimensions of gauze samples and numbers of flies which escaped through them. (Totals of 50 of each sex confined for 5 hr.)*

Gauze		Aperture length (mm.)		Flies escaped (x/50)	
Mesh	s.w.g.	Theoretical	Measured	Males	Females
7	20	2·71	2·75 ± 0·14	8	23
8	22	2·46	2·49 ± 0·11	4	6
9	24	2·26	2·25 ± 0·06	2	3
10	32	2·26	2·17 ± 0·09	0	0
10	24	1·98	1·77 ± 0·07	0	0
12	31	1·82	1·79 ± 0·10	0	0
14	32	1·54	1·53 ± 0·07	0	0
18	32	1·16	1·16 ± 0·05	—	—

sexed. The experiments were done at 25° C. in a lighted rearing room. The flies used were taken from a laboratory colony maintained at 25° C. The average weight of the males was about 17 mg. and of the females 24 mg. Some of their dimensions were measured by a low power microscope with a graduated eyepiece (Table 1). At the same time, the aperture dimensions of the gauzes were checked and found to depart slightly from their theoretical values (Table 2).

It will be noted that flies escaped from jars covered with gauze of 9 mesh and larger, but none got through 10 mesh or smaller. Curiously enough, more females escaped than males, presumably because their urge to escape was greater. A comparison of the mesh apertures with minimum fly dimensions shows that the

flies had little room to manoeuvre, bearing in mind the necessity of getting through legs and wings. Unlike the mosquito, which has a narrow thorax, the fly cannot take advantage of the extra length of the diagonal, since its thoracic outline is nearly circular.

ADVANTAGES OF WIDER MESH FOR FLY EXCLUSION

It appears that 10 mesh, 32 s.w.g., can be used for fly exclusion with fair confidence; only occasional, undersized flies are likely to get through it. The larger fly mesh will exclude only 20 % of the light as compared with 36 % excluded by a standard mosquito gauze (18 mesh, 32 s.w.g.).

Wider gauze will allow better ventilation, though this is much more difficult to assess than might be expected. Three methods of measurement have been found in the literature. Eckert & Pflüger (1941) measured the pressure drop due to various wire gauze frames in a wind tunnel and thereby worked out various curves relating their characteristic resistance coefficients to wind speed. Lomax (1945) fitted mosquito gauze circles into a pendulum frame and recorded their effects in damping the oscillation of the pendulum. Croton & Crowden (1955) employed an electric fan and a kata-thermometer to determine the degree to which mosquito nets reduced air currents and their cooling effects. This last method seems to give the type of information desired; but it is rather vague and difficult to standardize. On the other hand, the technique of Eckert & Pflüger, though precise, demands rather elaborate equipment. Therefore, some tests with the simple pendulum method were undertaken.

For my tests, I used a stiff wire pendulum, 60 cm. long, suspended from two cotton threads. At the lower end, at right angles to the plane of oscillation, the wire was bent into a 12 cm. diameter circle. The various gauzes were fixed in this ring and the pendulum weight kept constant by addition of lead weights, when necessary. The pendulum was released at an angle of 45° to vertical and allowed to swing in still air. The numbers of oscillations were counted as the amplitude decreased to various angles. Results were based on the number of swings between 15° and 5° to the vertical. Over this range the gauze circle was moving at an average speed of 22 cm./sec. falling to 7·5 cm./sec. At this low speed, where no turbulence occurs, the air behaves as a viscous fluid and it may be assumed that the resistance of the grid is proportional to air velocity.* It can be shown that, under these conditions, the air resistance is proportional to the reciprocal of the number of swings to decrease from one given (small) angle to another. The actual equation (derived on the lines of the well known textbook of Ramsey, 1933) is

$$k = \frac{W \log \theta_1/\theta_2}{ag} \frac{1}{z},$$

where k = air resistance; z = number of swings between angles θ_1 and θ_2; W = weight and a = radius of the pendulum; g = gravity constant.

* This seems a satisfactory approximation for the lower air speeds relevant to ventilation. Eckert & Pflüger (1941) show that the curves relating the 'resistance coefficient' to air speed bend sharply at higher velocities, as turbulence behind the grid wires begins to introduce an additional factor. In the higher range, resistance is related to the square of the air velocity.

Using the data for 10 mesh, 32 s.w.g. gauze given in Table 3, the reciprocal of the number of swings can be plotted against the percentage obstruction of the pendulum (see Fig. 1). There is a good linear relationship, which can be fitted by the equation

$$y = 0{\cdot}001557(x + 8{\cdot}07)$$

(where y = reciprocal of swings; x = % occlusion). When $y = 0$, corresponding to the infinite number of swings expected with no air resistance, $x = -8{\cdot}07$. This value is due to the air resistance of the pendulum frame (apparently equivalent to 8% of the area of the circle).

On this basis, exclusion of air, like that of light, is linearly related to the area of

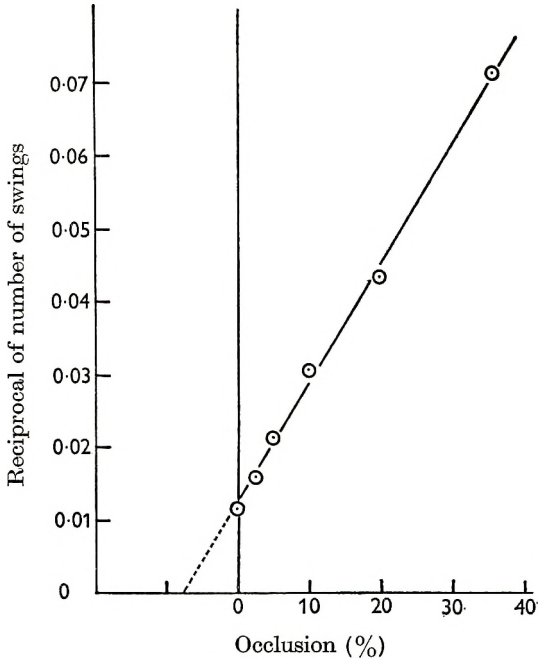

Fig. 1. Damping of a pendulum by air resistance of a circle of wire gauze. Relations between reciprocal of the number of swings between 15° and 5° and the occlusion of the circle by gauze.

Table 3. *Results of tests with the pendulum method of gauze assessment (Lomax, 1945)*

	Pendulum circle		
Nature of obstruction	Obstruction (%)	No. swings 15° to 5°	$\frac{1}{\text{no. swings}}$
Empty	0	86	0·0116
Strip of 10/32 gauze filling $\frac{1}{8}$th circle	2·5	63	0·0159
Strip of 10/32 gauze filling $\frac{1}{4}$ circle	5·0	47	0·0213
Strip of 10/32 gauze filling $\frac{1}{2}$ circle	10·0	32·5	0·0308
Disc of 10/32 gauze filling whole circle	20·0	23	0·0435
Disc of 18/32 gauze filling whole circle	36·0	14	0·0714
Disc of 10/24 gauze filling whole circle	39·0	13·5	0·0741

the obstructing wires. Such a relationship will tend to be distorted as the percentage obstruction increases to an extent that an appreciable proportion of air flows round the pendulum circle, instead of through it. Yet the linear relation appears to hold over the range covered, with wires of the same thickness. In short, exclusion of light and air by a fly gauze would be about half that caused by mosquito gauze.

A final advantage of the wider gauze is that, whatever metal is used, its cost will only be about half that of the finer mosquito gauze. This may be some consideration where a large building is to be proofed.

SUMMARY

Control of houseflies by modern insecticides is becoming unreliable owing to emergence of resistant strains, so that some older measures deserve reconsideration. Exclusion of mosquitoes by gauze has been studied experimentally but not, apparently, of houseflies.

Some simple tests show that a suitable gauze to exclude houseflies (and larger insects) would be 10 mesh 32 s.w.g. with an aperture 2·17 mm. square. This compares with a gauze recommended for excluding mosquitoes, 18 mesh and 32 s.w.g. with an aperture 1·16 mm. square.

The advantages of the wider gauze for flies are admission of more light and air and lower cost. Some simple experiments on the relative air resistance of mosquito and fly gauze were made by a method involving retardation of a pendulum by a gauze circle. The results suggest that the obstruction to ventilation is roughly proportional to the percentage obstruction (as with light) which was 20 % for the fly gauze and 36 % with the mosquito gauze.

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