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Colicine production as an epidemiological marker of *Shigella sonnei*

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INTRODUCTION

For at least 20 years Sonne's dysentery bacillus has been responsible for most cases of diarrhoea in which a causal agent has been incriminated. Several attempts have been made to establish a method of differentiating strains of this organism, which, among the Shigellae, is unique in being a serological entity; biochemical reactions (Bojlén, 1934; Tee, 1952) allow only subdivision into strains which do or do not ferment xylose. The value of such a marker is further reduced since only a minority of strains ferment this substrate.

Bacteriophage typing, introduced in Sweden (Hammarström, 1947; 1949), has been assessed in Britain by Mayr-Harting (1952) and Tee (1955); although at least thirteen types could thus be identified evidence of type instability *in vivo* discouraged the further use of this method for epidemiological purposes.

Davies (1954) reported that the determination of sensitivity of *Shigella sonnei* to sulphonamides may yield useful information regarding the spread of infection; similarly, she noted that occasional strains were exacting in regard to tryptophan or histidine with the synthetic medium employed for sensitivity testing. Such information may be useful on a short term basis but it is of restricted application, since in many areas the majority of Sonne strains are now resistant to members of the sulphonamide group.

In 1958, Abbott & Shannon investigated the susceptibility of *S. sonnei* to colicines produced by strains of *Escherichia coli*; the variation in sensitivity to the active coli strains of Sonne cultures which were epidemiologically related led these authors to conclude that 'it was unlikely, therefore, that the sensitivity patterns of Sonne strains to colicines could be used as a basis for a typing method'. Conversely, they noted that epidemiologically significant types could be established if colicine production by the Sonne strains was used as a marker; thus colicine types of *S. sonnei* are identified by the patterns of inhibition produced on selected indicator or passive strains of other Shigellae.

Subsequently Abbott & Graham (1961) detailed the type incidence of 1247 strains of *S. sonnei* in England (mostly from Manchester) and extended the number of recognizable types from 7 to 15; Barrow & Ellis (1962) recorded the type incidence of 896 strains in the Bradford area and added further evidence of the reliability of colicine typing as an epidemiological marker.

The present paper describes modifications to the technique used by previous workers and records the validity of the method as an epidemiological tool.

MATERIALS AND METHODS

Strains. Indicator strains and colicine-type strains of *S. sonnei* were obtained from Dr J. D. Abbott and have been maintained on Dorset's egg medium at 4° C. with subculture every 3–4 months. Strains of *S. sonnei* to be typed were acquired from Ruchill Hospital, Glasgow, during 1959–60 and from Leeds since 1960, as well as strains from our own diagnostic laboratories since 1959; all strains were checked biochemically using composite media (Gillies, 1956) and by agglutination tests with specific antiserum before being tested for colicine production and determination of sensitivity to sulphonamides, tetracyclines, chloramphenicol, streptomycin, 'Humatin' and 'Colomycin' using the disc diffusion technique.

Media. Infusion broth was used to culture the indicator strains once weekly or as often as required and was prepared according to Cruickshank (1960, p. 190).

Tryptone Soya Agar (T.S.A.) (Oxoid) was prepared according to the maker's instructions and various concentrations of horse blood added (T.S.B.A.).

Dorset's egg medium was prepared according to Cruickshank (1960, p. 214).

RESULTS

Modifications of the typing technique

Abbott & Shannon's technique requires 3 days preliminary incubation of the strain to be typed (producer strain) and thereafter 5 hr. processing before the indicator (passive) strains are applied. Several preliminary experiments were undertaken with the aim of reducing these times and otherwise streamlining the technique so that it would remain accurate but become more acceptable for 'routine' use. The results of these experiments are summarized in the following paragraphs and detailed protocols are available on request.

(1) The producer strain can be removed from the medium before exposure to $CHCl_3$ and this is done with a microscope slide; the end of the slide used to clear the growth is sheathed with a layer of sellotape. This allows complete removal of the macroscopic growth very cleanly and without requiring the removal of a portion of the medium; any danger of laboratory infection from this manoeuvre is reduced by jettisoning the material on to cellulose wadding soaked with $CHCl_3$.

(2) Effective sterilization of the surface of the medium before applying the indicator strains requires not more than 15 min. exposure to $CHCl_3$ vapour.

(3) Residual traces of $CHCl_3$ are removed by only a few minutes exposure to the natural atmosphere, whether the $CHCl_3$ has acted as a vapour or in liquid contact with the medium ($CHCl_3$ is not adsorbed by solid media).

(4) There is no significant difference in size of zone of inhibition of the indicator strains with various blood concentrations or depths of medium; these facts were established by varying the concentration of blood ($2\frac{1}{2}$, 5, 10 %) and also the depth of the medium by using small and large Petri dishes (diameters internally of $3\frac{1}{2}$ in. and 4 in. respectively) and in each case using 10, 15 and 20 ml. volumes of medium so that six different depths of medium were investigated. At this time all plates were still poured in two layers as recommended by Abbott & Shannon (1958), the basal layer of plain T.S.A. and the top layer incorporating the

concentrations of blood as indicated—the medium volumes in any one plate were equally divided between the layers.

(5) No advantage was found in layered plates except perhaps the saving in blood but this is offset since for routine use 5% blood is used in place of 10% as used by Abbott & Shannon.

Thus the procedure which has been in use for more than 4 years can be summarized as follows: the Sonne strain to be typed is inoculated in a diametric streak on a T.S.B.A. plate (15 ml. of medium regardless of whether small or large size plates are in use); incubation is continued for 24 hr. at 35–36° C. Thereafter the macroscopic growth is removed with a sellotape-sheathed slide, 3–5 ml. of $CHCl_3$ is placed in the lid of the Petri dish and the medium containing portion replaced for 15 min.; the plate is then opened and the residual $CHCl_3$ decanted into a beaker and retrieved for further use by filtering through filter paper.

The medium is exposed to the air for a few minutes and the fifteen indicator strains applied at right-angles to the original line of growth; eight strains are placed on the left and the other seven on the right half of the plate. This number of strains can be accommodated without difficulty even on a small size Petri dish and the resultant zones of inhibition, although only half the width of those obtained by full application across the plate, are easily noted (Pl. 1, figs. 1, 2).

Finally 8 or more hours incubation at 37° C. allows growth of the indicator strains and recognition of patterns of inhibition.

Validity of the technique

More than 5000 strains have been typed by the modified method described; the first 480 strains were examined in parallel by Abbott & Shannon's method and gave identical results. Occasional batches of strains have been thus examined in duplicate over the last 3 years and no discrepancy in type identification noted.

The reliability of colicine typing as an epidemiological marker has been assessed by two indices; first, by noting the constancy of type excreted by any one individual in serial isolations during clinical illness and convalescence (Table 1).

In addition to the 521 individuals who showed uniformity of colicine type of *S. sonnei* on two or more occasions, there were twenty-eight who showed variation in the type excreted.

The second index of reliability was that employed by Abbott & Shannon, namely the uniformity of type in any one epidemic; an epidemic is defined as two or more isolations of *S. sonnei* within a 4-week period from different persons in a family or institution. The epidemics in which there was uniformity of colicine type are summarized in Table 2.

Of these 534 epidemics 472 were in private households and sixty-two in residential or day-care institutions (the majority being children's nurseries); additionally there were twenty-three epidemics where more than one colicine type was encountered.

In Table 3 is given the type distribution for Edinburgh cases between 1959 and 1961; only the first isolation from any individual case or any epidemic is included and instances of mixed type infection have been excluded.

Details of the breakdown of this distribution seasonally and in different wards of Edinburgh will be incorporated in a later publication, but it should be noted that even in the brief period of 3 years there was evidence of the originally predominant Type 7 strain being ousted by other types and particularly that for brief periods untypable strains accounted for almost all isolations.

Table 1. *Serial isolations from* 521 *excretors of* Shigella sonnei *who showed uniformity of colicine type**

Colicine Type	No. of repeat isolations (excluding original) 1	2	3	4	5	6	7	Total no. of strains
7	227	68	17	7	4	.	2	476
u/t	29	18	8	5	3	.	.	124
4	28	8	8	1	.	.	.	72
6	16	3	1	1	.	.	.	29
14	7	4	2	.	.	.	.	21
2	28	3	.	.	.	.	.	34
11	9	4	.	.	.	.	.	17
1*a*	3	1	.	.	.	.	.	5
3*a*	1	1	.	.	.	.	.	3
8	2	.	.	.	.	.	.	2
9	1	.	.	.	.	.	.	1
3	1	.	.	.	.	.	.	1
No. of persons	352	110	36	14	7	.	2	785

* 28 (5·37 %) other individuals showed variation in the type excreted.
u/t = untypable strains.

Table 2. 534 *epidemics of* Shigella sonnei *in which there was uniformity of colicine type**

Colicine Type	No. of persons in epidemic 2	3	4	5	6	7	8	> 8†	No. of Epidemics	No. of People
7	146	78	31	15	9	3	5	6‡	293	1006
u/t	48	22	15	6	2	.	2	10§	105	533
2	26	15	8	1	.	.	1	$2^{15,22}$	53	179
4	26	4	3	4	1	1	.	$2^{10,15}$	41	134
11	6	2	2	2	.	.	1	.	13	44
6	3	.	3	.	1	.	.	1^{22}	8	46
1*a*	4	.	2	.	.	.	.	1^{14}	7	30
14	2	.	1	1	.	.	.	1^{31}	5	44
3	3	1	.	1	.	.	.	.	5	14
8	1	1	.	.	.	.	.	.	2	5
3*a*	2	.	.	.	.	.	.	.	2	4
Total	267	123	65	30	13	4	9	23	534	2039

* In twenty-three (4·31%) other epidemics there was lack of uniformity of type.
u/t = untypable strains.
† Superscribed figures indicate the number of cases in such epidemics.
‡ 13, 16, 19, 21, 37 and 60 cases.
§ 9, 10, 12, 15, 15, 23, 24, 35, 43 and 67 cases.

Table 3. *Frequency distribution of colicine types of* Shigella sonnei *Edinburgh* 1959–61*

Colicine Type	No. of strains	As percentage of All strains	As percentage of Typable strains
7	620	58·7	63·9
2	127	12·02	13·1
4	91	8·62	9·4
u/t	86	8·14	—
11	51	4·83	5·3
6	38	3·59	3·9
14	12	1·14	1·2
3	10	0·95	1·0
8	9	0·85	0·9
3*a*	6	0·57	0·6
1*a*	4	0·38	0·4
9	1	0·09	0·1
12	1	0·09	0·1
Total	1056	.	.

* Only the first strain isolated from any individual or epidemic is included; instances of mixed type infection are excluded.

Table 4. *Patterns of inhibition of* Shigella sonnei *on indicator strains*

Indicator strain no.	Colicine type 1*a*	1*b*	2	3	3a	4	5	6	7	8	9	10	11	12	13	14
1	+	+	−	+	+	+	+	−	−	+	+	+	−	+	−	+
2	+	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+
3	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	+
4	−	−	−	−	−	v	v	−	−	−	−	−	−	−	−	v
5	−	−	−	+	+	+	+	−	−	+	v	+	−	+	−	+
6	+	+	−	+	+	−	+	+	−	−	+	+	−	+	+	+
7	+	+	−	+	+	−	+	+	−	−	+	+	−	+	+	+
8	−	−	−	+	+	+	+	−	−	+	+	+	−	+	−	+
9	−	+	+	+	+	+	+	+	−	−	+	+	−	+	+	+
10	+	+	−	+	−	−	+	−	−	−	−	+	−	−	−	−
11	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+
12	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+
13	−	−	−	+	+	+	+	−	−	+	+	+	−	+	−	+
14	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
15	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+

The indicator strain numbers correspond respectively with: *S. sonnei* 2, 56, 17, 2M, 38, 56/56, 56/98, R 1, R 6; *S. schmitzi* M. 19 (NCTC 8218); *S. sonnei* 2/7, 2/64, 2/15, R 5; and *Bact. coli* Row.

\+ = Inhibition of an indicator strain; v = variable reaction; − = no inhibition of an indicator strain.

Identity of Type 14 *strains*

The pattern of inhibition given by Sonne strains of this hitherto undescribed type is given in Table 4 along with the characteristic patterns for the types previously described by Abbott and his colleagues.

Although Type 14 strains have occurred infrequently the evidence for its identity and epidemiological significance is clear cut; strains of this type appeared in June

and July 1959 in a Borders village with four household epidemics and several isolated cases occurring and was the only type isolated in the area.

Its next appearance was in February 1960 in a private school in a small village 20 miles north and west of the earlier episode; in the school outbreak with thirty-one excretors of Type 14 *S. sonnei* it was noted that the only symptomless excretor was one of the domestic staff who had suffered clinical illness with this type when living in the Borders village the previous year. One further case occurred in May 1960 in Edinburgh which could not be related epidemiologically to any of the previous excretors.

It will be seen from Table 4 that Type 14 differs from Types 3 and 3*a* in the variability of its activity against indicator strain no. 4 and in lacking activity against indicator strain no. 14; it differs further from Type 3 strains by lacking inhibitory activity on indicator strain no. 10.

DISCUSSION

Colicine typing of *Shigella sonnei* can be performed more rapidly and economically and without loss of accuracy by the modified technique described; type identification can be made within 36 hr. of isolation using only one plate.

Considering the reliability of colicine typing for epidemiological purposes it should be noted that Abbott & Shannon give no detail regarding the first index used in the present investigation other than that 'repeated isolations from the same patient were tested on a number of occasions, with identical results'.

The results given in Table 1 show that 521 (94·9 %) of 549 individuals showed uniformity of colicine type on two or more occasions. In fourteen of the twenty-eight cases with lack of uniformity in the type of *S. sonnei* excreted serially, only one colony was tested on each occasion; the results are shown abstractly where *X* and *Y* represent different colicine types:

	Isolation	
No. of cases	1st 2nd 3rd	
13	$X \rightarrow Y \rightarrow Y$	(replacement)
1	$X \rightarrow Y \rightarrow X$	(replacement with reversion)

In the remaining fourteen patients, multiple colonies (3–15) were tested from each isolation and eight patients showed excretion similar to the thirteen in the above group, i.e. on second or subsequent isolations there was apparent replacement of one type by another. In the six other cases the patterns were as outlined:

	Isolation			
No. of cases	1st	2nd	3rd	
3	*X* and *Y*	→ *X* or *Y*	→ *X* or *Y*	(elimination)
3	*X*	→ *X* and *Y*	→ *X* and *Y* or *X* or *Y*	(acquisition: with maintenance, with reversion, with replacement)

This latter situation was also seen on two occasions in household epidemics each involving two siblings of school age in whom a second type was acquired and with one pair of children was maintained along with the original type over five more isolations; in the second pair the freshly acquired strain was maintained for two further isolations when the original type could not be discovered in spite of multiple colonies being examined from each diagnostic plate.

It is not surprising that an individual may harbour more than one colicine type at any one time when Sonne dysentery is so ubiquitous; it is accepted that a person may excrete two separate and distinct types of salmonella and instances of combined excretion of a shigella and salmonella occur in diagnostic laboratories. Such findings do not invalidate serological typing techniques and at present there is no evidence that the presence of two colicine types of *S. sonnei* in one individual is associated with type instability *in vivo* which would of course invalidate the technique for epidemiological purposes; there are, on the other hand, incidents which lend weight to *in vivo* stability and show that excretion of two types is a real occurrence; two children are known to have excreted Type 4 *S. sonnei* more or less regularly for more than 3 years without any change of type (neither child has ever had a recognizable attack of dysentery). Another child participated in two epidemics in March and July 1959 and on the first occasion she and the other cases were excreting Type 2 strains, whereas on the second occasion all of the cases were of Type 7 except this child who was excreting Types 2 and 7 and continued to do so for five further examinations over 3 weeks.

Further evidence of such occurrences must be sought by follow-up studies so that *in vivo* instability of colicine production can be conclusively eliminated. With regard to *in vitro* instability, there is no evidence of change in colicine production either qualitatively or quantitatively when cultures have been checked at 6- to 12-month intervals over the last 4 years.

The second index of reliability revealed, in the present study, that 534 (95·9 %) of the 557 epidemics were homogeneous as regards colicine type. Regarding the twenty-three epidemics which were exceptional in showing lack of uniformity of colicine type it is noteworthy that eight were in households and fifteen in institutions, whereas uniformity was seen in 472 households and sixty-two institutional outbreaks; this high proportion of mixed type epidemics in institutions is statistically significant. One institutional outbreak of mixed types has been summarized above and in the few other instances when epidemiological data were available it was apparent that mixed type epidemics were real and most unlikely to be due to type instability *in vivo*; in all such mixed epidemics the majority of cases were of one type and frequently only one patient deviated from the common type.

The incidence of colicine types in Edinburgh during 1959–61 differs appreciably from that reported in Manchester and Bradford over approximately the same period; this is particularly obvious in regard to untypable strains with an incidence of 8·1, 16·9 and 43 % respectively in these cities. In Manchester Types 1*a* and 7 account for 60 % of typable strains, and in Bradford Types 2 and 4 for 83 %; in Edinburgh, however, 63·9 % of typable strains are Type 7 and types 2 and 4 account respectively for 13·1 and 9·4 % whilst Type 1*a* has rarely been encountered.

Type 6 strains amounted to 3·9 % of typable strains in Edinburgh but this type is notable for its rarity in the other two centres.

Abbott & Graham considered that on the basis of epidemiological evidence Types 1*a*, 1*b*, 2, 4 and 7 could be regarded as distinct types; we have insufficient experience of either of the Type 1 strains but can confirm their view on the other three types and in addition consider that Types 6, 11 and the new Type 14 are distinctive from evidence gathered during this investigation.

These differences in type distribution in the three centres should excite renewed interest in Sonne dysentery and it is hoped that other workers will report their experience with this recently acquired epidemiological tool; further inter-regional differences and annual variations in type incidence within certain regions have been studied and along with other epidemiological data are being prepared for publication.

SUMMARY

1. Modifications of the technique of colicine typing are reported which allow more rapid and economical identification of *Shigella sonnei* for epidemiological purposes without loss of accuracy.

2. The technique is epidemiologically valid as judged by the constancy of type in repeated isolations from individuals and the uniformity of type in epidemics; it is considered that the few instances of lack of uniformity in these indices are probably examples of true mixed type infection. There is no evidence of type instability *in vitro*.

3. Type incidence differs significantly from that reported in two other centres; a new colicine type (Type 14) is described which brings the total of recognizable types to 16. It is considered that in addition to the five types which were previously thought distinctive, three other Types, 6, 11 and 14, can now be so regarded.

4. There is a need for continued studies to resolve the few discrepancies associated with infection by more than one type either in the individual or in an epidemic; it is hoped that colicine typing will reawaken interest in Sonne dysentery.

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Fig. 1

Fig. 2

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EXPLANATION OF PLATE 1

Fig. 1. Pattern of inhibition given by *S. sonnei* colicine type 7.

Fig. 2. Pattern of inhibition given by *S. sonnei* colicine type 3.

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Intradermal cholera vaccination

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INTRODUCTION

Immunization by the intradermal route against the enteric group of diseases and against tetanus has been shown to be particularly free from post-inoculation reactions (Barr, Sayers & Stamm, 1958; Noble, 1963). The employment of this method of inoculation during the past 4 years has led to a considerable saving of manpower in Army personnel.

Cholera vaccine is still administered by the subcutaneous route with a significant number of both local and general reactions.

Bearing in mind the successful reduction in number and severity of reactions experienced in enteric and tetanus vaccination, it was decided to investigate the possibility of producing an intradermal cholera vaccine. This article is an account of the experiments carried out to compare and contrast the biological effects produced by cholera vaccine administered by both the subcutaneous and intradermal routes.

Investigating the inoculation of cholera vaccine by the intradermal route, Panja & Das (1947) expressed the opinion that this method appeared to be superior to inoculation by the subcutaneous route. These authors also stressed the economy of materials which resulted, and the negligible reactions which followed injection of cholera vaccine by the intradermal route.

Singer, Wei & Hoa (1948) reported better antibody formation after intradermal vaccination than after inoculation by the subcutaneous route in human volunteers. These authors, however, reported abscess formation at the site of inoculation in a few cases following the administration of a third dose of *Vibrio cholerae* vaccine by the intradermal route. The intradermal inoculations consisted of 1500×10^6 heat-killed *V. cholerae* organisms administered in a dose of 0·2 ml. As 0·1 ml. is now used as the standard intradermal dose it is likely that ulceration was caused by the volume rather than the contents of the inoculations.

All virulent strains of *V. cholerae* possess a common O antigen. By agglutinin–absorption techniques subsidiary O antigenic components can be demonstrated. Gardner & Venkatraman (1935) classify *V. cholerae* strains into three subtypes, designated according to the standard strains, Inaba, Ogawa and Hikojima. Inaba and Ogawa each possess distinct subsidiary O antigens. Hikojima possesses both these subsidiary O antigens. All strains of *V. cholerae* and some other non-pathogenic vibrios possess a common H antigen (Pollitzer, 1959).

Cholera vaccine prepared at the David Bruce Laboratories, for administration

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to Service personnel, is made in accordance with the recommendations laid down by World Health Organisation (1959) and contains *V. cholerae* strains Inaba and Ogawa. The vaccine consists of a heat-killed and phenol-preserved suspension of 4000 million Inaba and 4000 million Ogawa organisms per ml. in 0·5 % phenol-saline.

Vella (1963), in his experiments with the El Tor vibrio, showed that the mouse strain C/57/Black was suitable for experimental studies with *V. cholerae*. As he had established a readily available supply of this strain of mice to these laboratories, it was decided to use C/57/Black mice in these studies.

MATERIALS AND METHODS

Preliminary animal virulence tests

These tests were performed by challenging mice and guinea-pigs intraperitoneally with various strains of *V. cholerae* in order to assess their suitability as challenge organisms, and to establish the lethal dosage for 100 % of animals inoculated (LD 100).

(*a*) *Mice.* Groups of mice (C/57/Black—ten mice, of average weight 22 g., in each group) were inoculated by the intraperitoneal route with graded doses of challenge organism contained in 0·5 ml. of isotonic saline. The mice were observed for 72 hr. and the numbers dead, at the end of that time, were recorded. Mucin was not used. The challenge organisms used were National Collection of Type Cultures' strains Inaba (7258, 7260 and 8039) and Ogawa (5596 and 8040).

(*b*) *Guinea-pigs.* Animals of the Dunkin–Hartley strain, weighing 250–300 g., were injected intraperitoneally with graded doses of *V. cholerae* Inaba (NCTC 7260) suspended in 1·0 ml. physiological saline. Mucin was not used. The number dying in 72 hr. was recorded.

Active mouse protection tests

This group of experiments was devised to compare the protection afforded to mice by the administration of *V. cholerae* vaccine by both the subcutaneous and intradermal routes. Dosage and time interval between inoculations and between immunization and challenge were varied, as were the strains used for preparation of the vaccines and subsequent challenge.

In each experiment two immunizing inoculations only were given; subcutaneous injections were given in a standard dose volume of 0·5 ml. introduced into the loose areolar tissue of the 'scruff' of the neck; intradermal injections were administered in constant dose volume of 0·05 ml. introduced into the shaved flank of the animals.

Challenge doses were suspended in sterile isotonic saline and administered in a constant dose volume of 0·5 ml. injected intraperitoneally. Mucin was not used.

(*a*) Two groups of mice (C/57/Black strain—forty mice in each group) were inoculated with current issue David Bruce Laboratories (D.B.L.) cholera vaccine batch no. 398, one group subcutaneously and the other by the intradermal route. Seven days later a second immunizing dose was given. As the vaccine contained 8000 million organisms per ml., the group injected with 0·5 ml. subcutaneously received 4000 million organisms per mouse at each inoculation, whereas those

inoculated with 0·05 ml. intradermally received 400 million organisms per mouse at each inoculation. Ten days after the second immunizing dose all mice were challenged with 1 LD 100 (1000×10^6 organisms) *V. cholerae* strain Inaba (NCTC 7260), together with twenty unimmunized control mice. The mice were observed for 72 hr. and survivors recorded.

(*b*) The previous experiment was repeated with twenty mice in each group and a challenge dose of 2 LD 100 (2000×10^6 organisms).

(*c*) A further experiment was performed, with ten mice in each group, in which each vaccine was so diluted as to give an immunizing dose of 100 million organisms by both inoculation routes. The interval between the first and second inoculation and between the second inoculation and the 1 LD 100 challenge dose was 14 days.

(*d*) Two vaccines were prepared, both containing 4000×10^6 organisms per ml. of *V. cholerae* strain Ogawa (NCTC 8040) in 0·5 % phenol-saline. One contained in addition Inaba (NCTC 7260) of 4000×10^6 organisms per ml. whilst the other contained Inaba (NCTC 8039) of the same strength. Both vaccines therefore contained 8000×10^6 organisms per ml. Two groups of forty-five mice (C/57/Black Porton) were immunized with these vaccines by two intradermal injections of 0·05 ml. at an interval of 7 days. Seven days later, the mice were challenged in groups of 15 by the intraperitoneal route, in doses of 0·5 ml., with three strains of Inaba (NCTC 8039, 7258 and 7260), 900×10^6 organisms in each dose. Unimmunized control challenges were set up in parallel.

Active guinea-pig protection test

Animals of the Dunkin–Hartley strain of guinea-pigs, weighing between 250 and 350 g., received two immunizing doses of D.B.L. issue *V. cholerae* vaccine batch no. 398 containing 8000 million organisms per ml., at an interval of 5 days. Five animals received 0·1 ml. and 0·2 ml. injected by the intradermal technique, and five animals received 0·5 ml. and 1·0 ml. by the subcutaneous route.

Seven days after the second immunizing dose these ten animals, together with five unprotected ones as control, received a challenge of live *V. cholerae* (NCTC 7260) intraperitoneally, 5000 million organisms suspended in 1·0 ml. isotonic saline. The animals were observed and the survivors at 72 hr. noted.

Agglutination response in rabbits

This experiment was devised to compare the antibody response in rabbits after immunization with cholera vaccine given subcutaneously and intradermally.

Six rabbits (Porton coloured) were divided into three groups (A, B and C) of two animals in each group.

Two vaccines were prepared containing equal amounts of *V. cholerae* strains Inaba and Ogawa (NCTC 7260 and 8040, respectively) to strengths of 20,000 million and 8000 million organisms per ml.

All rabbits were bled to establish a base-line titre of agglutination. No agglutinins to several *V. cholerae* suspensions were detected. Group A rabbits received two inoculations of 0·1 ml. of the 20,000 organisms per ml. vaccine intradermally, with an interval of 16 days. They were bled 7 days after the second injection. Eight

days after the second injection a further dose of 0·1 ml. of the same vaccine was given intradermally. After a further 7 days they were bled again.

Rabbits of groups B and C were given two inoculations of 0·5 ml. of the 8000 million organisms per ml. vaccine subcutaneously, with an interval of 16 days between each injection. Seven days after the second injection they were bled. On the following day group B received a third dose of 0·5 ml. of the 8000 million organisms per ml. vaccine by the subcutaneous route and group C received an intradermal dose of 0·1 ml. of the vaccine of 20,000 million organisms per ml. Seven days after these inoculations both groups B and C were bled again.

Agglutinations were performed on all sera by the Felix & Bensted (1954) technique using an antigenic suspension of equal parts of *V. cholerae* strains Inaba and Ogawa. The titres were expressed as the reciprocal of the lowest dilution of serum in the last tube showing granularity of deposit visible to the naked eye.

Agglutination response in human volunteers

Eighteen human volunteers, from the staff of the David Bruce Laboratories, were found to have no agglutinin titres to several *V. cholerae* suspensions.

These volunteers fell into two groups: group A who had received no previous experience of cholera vaccination, and group B who had previously been given various cholera injections, but none within the previous 12 months.

Group A was subdivided into two further subgroups of five volunteers each and group B was divided into two groups of four volunteers in each.

Half of group A received two subcutaneous injections of D.B.L. current issue vaccine batch no. 400, in doses of 0·5 and 1·0 ml. at an interval of 10 days. The other half received two intradermal inoculations, of 0·1 ml. on both occasions, of the same vaccine, at the same interval.

The two subgroups of group B were inoculated in a similar manner except that they received one dose only of 0·1 ml. intradermally or 0·5 ml. subcutaneously.

All volunteers were bled at intervals of 4, 8 and 14 weeks after their last inoculation, and the agglutination titres of their sera determined.

Passive mouse protection tests

Rabbit immune serum was obtained by inoculating Porton coloured rabbits, either intradermally or subcutaneously, with three injections of current issue D.B.L. cholera vaccine batch no. 400, at intervals of 7 days. Intradermal inoculations were of 0·1 ml. volume and subcutaneous inoculations of 0·5 ml.

Seven days after the third inoculation the rabbits were bled and the agglutination titres determined.

The titres of agglutination produced by intradermal and subcutaneous inoculation were 1:320 and 1:160 respectively.

Two groups of twenty mice (Ajax albino), and one group of ten mice, were inoculated subcutaneously with immune serum from rabbits inoculated either intradermally or subcutaneously, and with normal rabbit serum, respectively. Twenty-four hours later all mice were challenged intraperitoneally with *V. cholerae* strain Inaba (NCTC 7260), in a saline suspension of 1500 million organisms

contained in a dose of 0·5 ml. Unprotected mice were challenged in parallel. All mice were observed and survivors at 72 hr. noted.

A further passive mouse protection test using sera obtained from human volunteers was carried out.

Two pairs of human volunteers were given issue D.B.L. cholera vaccine batch no. 400 in two inoculations of either 0·5 and 1·0 ml. subcutaneously or 0·1 ml. intradermally, at an interval of 10 days. Fourteen days later the volunteers were bled and the agglutination titres of their sera determined.

The titres of the individuals inoculated by the subcutaneous route were 1:640 and 1:320 whilst those injected intradermally were 1:320 and 1:160.

The sera were pooled and filtered and gave final titres of 1:320 and 1:160 respectively. Serum obtained from non-immunes tested at the same time showed no agglutination in the lowest dilution (1:10).

Two groups of twenty C/57/Black mice were inoculated with the immune sera and one group of ten mice received normal human serum.

The protective inoculation consisted of 0·5 ml. of serum administered subcutaneously, 24 hr. before challenge.

All mice were challenged with *V. cholerae* Inaba (NCTC 7260) live suspensions in a dose of 0·5 ml. given intraperitoneally, and containing 1500 million organisms suspended in isotonic saline.

Intradermal skin tests

This group of experiments was designed to study the effect of cholera vaccine and its components when inoculated intradermally into the skin of guinea-pigs.

Cholera vaccines consisting of Inaba and Ogawa strains (NCTC 7260 and 8040 respectively) mixed in equal proportions were used. The vaccines were heat-killed, preserved with 0·5 % phenol-saline, and made up to strengths of 64,000, 32,000, 16,000 and 8,000 million organisms per ml. Controls included isotonic saline, 0·5 % phenol-saline, and stock TABT intradermal vaccine (D.B.L. batch no. 31).

Guinea-pigs were inoculated intradermally on the shaved abdomen with 0·1 ml. of each of these preparations. The inoculation site and any skin lesions produced were observed and measurements taken daily.

The effect of repeated intradermal inoculations was studied in both guinea-pigs and rabbits by injecting those animals with doses of 0·1 ml. intradermally at weekly intervals for 9 successive weeks. The vaccine used was D.B.L. issue cholera vaccine batch no. 395. The injections were all placed into a 1 in. diameter shaved area of abdominal skin but not into the exact site of previous inoculations. The inoculation sites were examined daily for evidence of ulceration, and the presence or absence of indurated nodules assessed by palpation. Daily records of these observations were kept.

RESULTS

Animal virulence tests

These tests (see Tables 1 and 2) show that the strains used were lethal for mice and guinea-pigs, conform to the standards of virulence laid down by W.H.O. (1959), and were suitable as challenge organisms in active and passive protection tests.

The *V. cholerae* Inaba strains were more virulent for mice (C/57/Black) than the Ogawa strains. The virulence of *V. cholerae* strains was found to vary slightly from one experiment to the next and a preliminary virulence test was performed with each experiment in order to adjust challenge doses to an accurate LD 100.

Table 1. *Number of mice killed by graded challenge doses of various strains of* Vibrio cholerae

(Expt. 1. Virulence test. *V. cholerae*. C/57/Black mice. Av. wt. 22 g.)

Organisms used	Challenge dose live organisms in millions						
	250	500	1000	1500	2000	2500	3000
Inaba (7258)	1	4/7	9	10	9	—	—
Inaba (7260)	0	5	6	9	10	10	—
Inaba (8039)	0	5	10	10	—	—	—
Ogawa (5596)	0	1	4	8	7	10	10
Ogawa (8040)	1	0	6	—	10	—	—

Challenge dose given in a standard volume of 0·5 ml. saline intraperitoneally. Mice: C/57/Black—10 mice in each group unless otherwise stated. Controls: no deaths occurred with dead organisms inoculated at the same time, in the highest dose used for live challenge.

Table 2. *Number of guinea-pigs killed by graded doses of* Vibrio cholerae, *Inaba type* (NCTC 7260)

(Expt. 2. Virulence test. *V. cholerae*. Av. wt. 250–300 g.)

Challenge Inaba 7260—millions of organisms	2000	3000	4000
Deaths at 72 hr.	1/3	2/3	3/3

Numerators represent animals killed. Denominators represent number of animals challenged. Challenge given in standard dose of 1·0 ml. intraperitoneally.

Active mouse protection tests

V. cholerae vaccine, inoculated by both the subcutaneous and intradermal routes, gave almost complete and equal protection against an LD 100 challenge of live *V. cholerae* organisms. Table 3, Expt. 3 (*a*), shows the results obtained. As the same vaccine, containing 8000 million organisms per ml., was used for inoculation by both routes, the organism content of the intradermal injections (400 million organisms in 0·05 ml.) was only 1/10 of the strength of the subcutaneous inoculation (4000 million in 0·5 ml.) yet, in this experiment, showed equal protection.

Expt. 3 (*b*), Table 3, showed that about half the mice were still protected when the lethal dose was doubled to 2 LD 100, irrespective of the route of inoculation.

Expt. 3 (*c*), Table 3, demonstrated that the organism content of the inoculum could be lowered to only 100 million organisms, injected by either route, and still give absolute and equal protection to an LD 100 challenge of live organisms.

Expt. 3 (*d*), Table 4, shows that protection with *V. cholerae* vaccine by the intradermal route in mice (C/57/Black) was almost complete, and independent of the Inaba strains used both in the preparation of the vaccine and as challenge organism. Inaba strains were selected as being the most virulent strains of *V. cholerae* available in these laboratories at the time.

Table 3. *A comparison of the number of mice surviving a challenge of* 1 *LD*100 *and* 2 *LD*100 *Inaba organisms* (*NCTC* 7260) *after two inoculations of cholera vaccine administered by the subcutaneous and intradermal routes*

(Active mouse protection test. C/57/Black mice. Av. wt. 20–30 g.)

		Subcutaneous 0·5 ml. × 2 at 7 days	Intradermal 0·05 ml. × 2 at 7 days	Nil (control group)
	Protection ...			
	No. orgs × 10^6 ...	4000	400	Nil
Expt. 3 (*a*)	Challenge at 10 days—no. orgs. × 10^6	1000	1000	1000
	Survivors at 72 hr.	39/40	38/40	2/20
	Increased challenge dose			
Expt. 3 (*b*)	Challenge at 10 days—no. orgs. × 10^6	2000	2000	2000
	Survivors at 72 hr.	10/20	6/20	0/20
	Decreased protection dose—longer interval			
	Protection ...	0·5 ml × 2 at 14 days	0·05 ml × 2 at 14 days	Nil (control group)
	No. orgs. × 10^6 ...	100	100	
Expt. 3 (*c*)	Challenge at 14 days—no. orgs. × 10^6	1000	1000	1000
	Survivors at 72 hr.	10/10	8/10	0/10

Numerators represent survivors at 72 hr. Denominators represent number of animals challenged. Challenge organism Inaba (NCTC 7260) was given in a standard dose of 0·5 ml. intraperitoneally.

Table 4. *Active mouse protection test comparing two strains of* Vibrio cholerae *Inaba incorporated in the vaccine. Vaccine administered intradermally. Challenge—three strains of* V. cholerae *Inaba.*

(Expt. 3 (*d*). Active mouse protection test. C/57/Black mice. Av. wt. 18–25 g.)

Protected 0·05 ml. intradermally × 2 at 7 days.	Ogawa plus 7260 vaccine			Ogawa plus 8039 vaccine		
At 7 days challenged Inaba 900 × 10^6 in 0·5 ml. intraperitoneally	7258	7260	8039	7258	7260	8039
Survivors at 72 hr.	15/15	15/15	12/15	14/15	15/15	12/14
Control virulence test						
Inaba strain	7258		7260		8039	
Dose in 0·5 ml. intraperitoneally × 10^6	450	900	450	900	450	900
Survivors at 72 hr.	5/10	1/10	7/10	0/10	0/10	0/10
Plate count viability	67 %		60 %		47 %	

Numerators represent the number of animals to survive. Denominators represent the number of animals inoculated.

Active guinea-pig protection tests

Table 5 demonstrates that equal and good protection was afforded to guinea-pigs when vaccinated by either the intradermal route or the subcutaneous route with *V. cholerae* vaccine, and challenged with live virulent cholera organisms.

Table 5. *The numbers of guinea-pigs protected by* Vibrio cholerae *vaccine when challenged by a lethal dose of live* Vibrio cholerae

(Expt 4. Guinea pig active protection test. Av. wt. 250–300 g.)

Protected D.B.L. cholera vaccine. Batch no. 398, 2 doses at 5 days	Intradermal, 0·1 and 0·2 ml.	Subcutaneous, 0·5 and 1·0 ml.	Nil (control group)
Challenge Inaba 7260 orgs. $\times 10^6$ intraperitoneally in 1·0 ml.	5000	5000	5000
Survivors at 72 hr.	5/5	5/5	1/5
Plate count viability	49%		

Numerators represent number of animals to survive. Denominators represent the number of animals inoculated.

Table 6. *Agglutinin titres produced in rabbits after two and three doses of cholera vaccine given either intradermally or subcutaneously.*

(The antigen suspension contained both Inaba and Ogawa strains mixed in equal quantities.) (Expt. 5).

Rabbits			
Group	No.	Titre after two inoculations	Titre after three inoculations
A	R 6	80	320
	R 27	160	320
B	R 40	320	320
	R 48	160	320
C	R 5	640	320
	R 22	160	640

Group A received 3 doses intradermally (20,000 $\times 10^6$ orgs. per ml.). B received 3 doses subcutaneously (8000 $\times 10^6$ orgs. per ml.). C received 2 doses subcutaneously and 1 dose intradermally. Titres expressed as reciprocal of the lowest dilution of serum in the test tube showing granularity of deposit visible to the naked eye.

Agglutinin response in rabbits

Agglutinin titres in previously unprotected rabbits, inoculated with two or three doses of cholera vaccine, were comparable, independent of whether the animals were inoculated by the subcutaneous or intradermal routes. Table 6 shows the responses obtained.

Agglutinin responses in human volunteers

No material difference could be detected in the agglutinin response in human volunteers when they were inoculated with issue D.B.L. cholera vaccine by either the subcutaneous or intradermal routes (Table 7). Previously unprotected

volunteers showed good agglutinin responses, and others requiring booster dosage, after at least 1 year's lapse since previous inoculation, showed an equally good response by either vaccination route. The actual agglutinin titres obtained were very much the same as those obtained with a similar vaccine, by Singer *et al.* (1948).

Table 7. *Agglutinin response in human volunteers to D.B.L.* Vibrio cholerae *vaccine, batch no.* 400, *administered by either the intradermal or subcutaneous route*

Expt. 6 Volunteers	Route	No.	Weeks after second inoculation or booster dose 4	8	14
Group A. No previous cholera inoculations	Subcutaneous 0·5 and 1·0 ml., interval 10 days	5	20	80	80
		6	160	80	40
		7	160	160	160
		17	40	80	160
		18	80	160	320
	Intradermal 0·1 ml. × 2, interval 10 days	2	320	160	80
		3	160	80	40
		4	40	80	40
		15	20	20	20
		16	20	40	20
Group B. Various previous cholera inoculations. None during the past 12 months	Subcutaneous 0·5 ml. once only	11	80	160	80
		12	20	80	40
		13	160	320	80
		14	320	160	160
	Intradermal 0·1 ml. once only	1	320	40	40
		8	40	160	160
		9	20	20	20
		10	160	160	160

Titres expressed as the reciprocal of the lowest dilution of serum in the test tube showing granularity of deposit to the naked eye.

Passive mouse protection tests

This experiment demonstrates that about half the mice passively protected with immune rabbit serum survive a challenge of an LD 100 dose of live *V. cholerae*, as compared with no survivors in the control group receiving serum from unimmunized rabbits. No difference could be demonstrated in the protection afforded by sera obtained from rabbits immunized by either the subcutaneous or intradermal routes.

A temporary shortage in the supply of C/57/Black mice necessitated the use of Ajax (Porton) albino mice in the experiment described. The result was confirmed later in C/57/Black mice. The inclusion of the white mice experiment also illustrates that strains other than C/57/Black are equally effective in this type of cholera experiment. Table 8 shows the results obtained.

Table 9 shows the results with sera from human volunteers, and it is seen that the passive protection of C/57/Black mice is identical with that afforded by immune rabbit serum described above. Again the fact emerges that no material difference could be demonstrated between immunization by either the intradermal or subcutaneous routes.

Table 8. *Passive mouse protection test using sera from rabbits inoculated with* Vibrio cholerae *vaccine by the intradermal and subcutaneous route*

(Expt. 7. Passive protection test. Ajax white mice. Av. wt. 25 g.)

Protection 0·5 ml. subcutaneous	Serum: Rabbit 1 (intradermal titre 1:320)	Serum: Rabbit 2 (subcutaneous titre 1:160)	Serum: Neutral serum titre 0	Unprotected (control group)
Challenge Inaba 7260 0·5 ml. intraperitoneally orgs. × 10^6	1500	1500	1500	1500
Survivors at 72 hr.	9/20	11/20	0/10	1/10

Numerators represent survivors. Denominators represent number of animals inoculated.

Table 9. *Passive mouse protection test using serum from human volunteers inoculated with* Vibrio cholerae *vaccine by the subcutaneous and intradermal routes*

(Expt. 8. Passive protection test. C/57/Black mice. Av. wt. 22 g.)

Protection 0·5 ml. subcutaneous	Serum: Pool I, subcutaneous, titre 1:320	Serum: Pool II, intradermal, titre 1:160	Serum: Neutral serum, titre 0
Challenge Inaba 7260 0·5 ml. intraperitoneally orgs. × 10^6	1500	1500	1500
Survivors at 72 hr.	11/20	12/20	0/20

Numerators represent survivors. Denominators represent the number of animals inoculated.

Intradermal skin tests

(*a*) Cholera vaccine, inoculated intradermally, in doses of 0·1 ml. of vaccines containing 64,000 and 32,000 million organisms per ml. produced severe persistent reactions in the shaved abdominal skin of guinea-pigs. Firm, indurated nodules of average diameter 12 and 8 mm. respectively were produced. For the first few days the nodules were accompanied by a narrow band of erythema. In 10 days the nodules produced by the larger dose were about half their original size whereas those of the lower dose resolved in 5 days.

Guinea-pigs inoculated with vaccine containing 16,000 and 8000 million organisms per ml. showed no reactions at all. The reaction at the inoculation site measured less than 5 mm. 8 hr. after injection, and was not detectable 18 hr. later.

The TABT vaccine given intradermally produced no reactions, and was indistinguishable from the two lower cholera vaccine doses.

The phenol-saline (0·5 %) and isotonic saline produced no reaction, and were absorbed in 4 hr.

(*b*) Repeated intradermal inoculations in doses of 0·1 ml. of an 8000 million organisms per ml. cholera vaccine, at weekly intervals for 9 weeks into a limited

area of both rabbit and guinea-pig skins, failed to produce ulceration due to local tissue hypersensitivity. No reactions of any type occurred, except that early in the series it was noted that the inoculum was completely absorbed in approximately 8 hr., whereas after the sixth injection the inoculum tended to require a little longer time to absorb, which never exceeded 24 hr.

(*c*) During the course of the experiments in this paper some hundreds of intradermal inoculations, usually in pairs at an interval of 7–10 days, have been administered to mice, guinea-pigs, rabbits and human volunteers, and no reactions, except where provoked in (*a*) above, have been detected, and at no time has ulceration occurred at the site of injection.

DISCUSSION

The important methods available in the laboratory for assessing the immunizing properties of a vaccine may be classified as follows: (1) active immunization experiments in laboratory animals; (2) serological tests to determine the presence of agglutinating antibodies in the serum of actively immunized animals and man; (3) passive protection tests with the serum of vaccinated animals and man. Each of the three methods has its advocate. Burrows, Mather, Elliott & Havens (1947) stressed the value of active immunization tests, but formed an unfavourable opinion of passive mouse protection tests in assessing cholera vaccines. Griffitts (1944), on the other hand, strongly recommended passive mouse protection tests for assaying the results of cholera vaccination. Panja & Das (1947) and Singer *et al.* (1948) not only confirmed the value of active protection tests, but found excellent agglutination responses with cholera vaccine administered by both the subcutaneous and intradermal routes.

In the foregoing experiments, all three methods have been used to compare the immunizing properties of cholera vaccine injected both intradermally and subcutaneously. It should be noted that, except where otherwise specifically stated, the cholera vaccines contained a fixed organism content, and consequently mice injected intradermally with 0·05 ml. received one-tenth of the immunizing dose compared to those injected subcutaneously with 0·5 ml. Similarly, human volunteers injected with 0·1 ml. intradermally received one-fifth of the subcutaneous immunizing dose. No material difference in the protection afforded by cholera vaccine could be demonstrated between the two routes of administration. Active and passive mouse protection tests, active guinea-pig protection tests, and the agglutination responses in humans and rabbits, all gave excellent and parallel results independent of the inoculation route.

Miles (1958) studying the toxicity of intracutaneous TAB vaccine concluded that provided the dose of TAB bacilli was about half the minimum dose inducing necrosis at the centre of the injection site, it could be as safely injected in a 0·05 ml. volume as in a volume of 0·5 ml. or greater. He further stated that, in spite of some pronounced differences in structure, there is *prima facie* evidence that, so far as intradermal inoculations are concerned, human skin reacts in a similar way to the skin of guinea-pigs and rabbits.

In the experiments described above doses of cholera vaccine as large as

6400 million organisms (0·1 ml. of $64{,}000 \times 10^6$ organisms per ml.) intradermally inoculated in rabbits failed to cause ulceration, although unacceptable indurated nodules did occur. Smaller doses produced no such unacceptable reactions. Doses of 800 million organisms (0·1 ml. of 8000×10^6 organisms per ml.) in human volunteers produced no reactions of any kind.

Singer *et al.* (1948), whilst finding better antibody production with cholera vaccine administered by the intradermal route than by subcutaneous injection, rejected the former method as they experienced some cases of ulceration at the injection site when giving a third injection. This was not our experience. Doses of 0·1 ml. of a vaccine containing 8000 million cholera organisms per ml., injected intradermally at weekly intervals for 9 weeks into the skin of both rabbits and guinea-pigs, failed to produce ulceration.

Panja & Das (1947), whilst noting the economy of materials and negligible reactions resulting from the intradermal inoculation of cholera vaccine, considered the method impracticable in mass inoculation programmes. Combined enteric and tetanus prophylactic by intradermal inoculation has been in use in the Army for 4 years (Noble, 1963). During this time large numbers of intradermal inoculations have been given by Service medical officers without any technical difficulties being experienced.

It is the experience of the author, and a generally accepted fact among Service medical officers, that cholera vaccine administered by the subcutaneous route produces both local and general reactions in about 10% of patients inoculated. No reactions have occurred when cholera vaccine was administered by the intradermal route either to animals or to a small number of human volunteers.

Barr *et al.* (1958) demonstrated a dramatic fall in the incidence of reactions to combined enteric–tetanus prophylactic. The use of the intradermal route of inoculation for this prophylactic in the Army since 1959 had led to a considerable saving of manpower, due to the decrease in numbers and severity of reactions (Noble, 1963). As cholera vaccine, administered by the intradermal route, gives excellent protection, equal to that afforded by subcutaneous vaccination, it is suggested that reactions to cholera vaccine would be greatly reduced if the intradermal route of inoculation were adopted. Furthermore, in order to reduce the number of inoculations required by individuals proceeding overseas, it should be possible to combine intradermal TABT prophylactic with intradermal cholera vaccine, and still retain the freedom from undesirable inoculation reactions, shown separately by the two preparations.

SUMMARY

The protection afforded by cholera vaccine administered by the intradermal route, and demonstrated by active and passive mouse protection tests, active guinea-pig protection tests, and agglutination titres, is excellent and equal to that given by subcutaneous inoculation.

It is suggested that cholera vaccine administered by the intradermal route would greatly reduce the incidence of reactions which occur when the vaccine is given subcutaneously.

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A new enrichment medium for salmonellae*

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The so-called enrichment media are of great importance in the isolation of salmonellae from faeces and other material. Two of them, namely the tetrathionate broth according to Mueller–Kauffmann (Kauffmann, 1930–31) and the selenite F broth according to Leifson (1936), are most frequently used. The first appears to be superior in the isolation of *Salmonella typhi*, the second in the isolation of other salmonella types.

Some of the coliform organisms, especially *Proteus* species, *Pseudomonas aeruginosa* and *Aerobacter aerogenes*, are not always inhibited in the enrichment media mentioned. As a consequence of this, these media sometimes give negative results. For this reason the attempts to develop a new enrichment medium for the isolation of salmonella types appeared justified to the present author. As a result of a laborious investigation a medium was developed which proved, in experiments with pure cultures of enteric bacteria, to be very selective for salmonellae. The medium was then tested in routine diagnosis and compared with other standard media used in this laboratory. The purpose of this paper is to report the results obtained.

MATERIALS AND METHODS

Media. The new enrichment medium (called MS = magnesium chloride–selenite medium) is composed of nutrient broth Difco + 0·2 % yeast extract Difco + 0·05 % sodium hydrogen selenite ($NaHSeO_3$) Merck + 3·4 % $MgCl_2$.

To make 300 ml. of the medium, put 600 mg. of yeast extract and 150 mg. of sodium hydrogen selenite in a sterile bottle, and add 274·5 ml. of sterile nutrient broth. When these are dissolved, adjust to pH 7·5, first with 2–3 drops of 10 N-NaOH and then with N-NaOH, using an electric pH-meter. Then add 25·5 ml. of 40 % $MgCl_2$ solution. The medium is sterilized by standing in boiling water for 10 min., and is distributed aseptically in 10 ml. amounts in sterile test tubes. The final pH of the medium is about 6·8. The medium should be kept at 4° C. until use.

The tetrathionate broth was prepared according to the formula given by Kauffmann (1941).

The Wilson–Blair agar and the SS agar were prepared according to the Yugoslav *Manual of the Microbiological Methods* (1953).

A total of 605 specimens of faeces were examined for salmonellae. Each specimen was first seeded on a direct Wilson–Blair and SS agar plate. After that about 1 g. of the faeces was emulsified in 10 ml. of distilled water, and four drops of this suspension were dropped into a tube containing 10 ml. of tetrathionate broth and

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into a tube containing the same amount of the new enrichment medium. After incubation for 24 hr. a large loopful (4 mm. internal diameter) of each of the enrichment media was plated on to Wilson–Blair and SS agar. The plates were incubated overnight and then examined together with the direct plates. Two or three suspected colonies from each plate were selected and sub-cultured on the screening medium (double sugar agar). The inoculated double sugar agars were incubated overnight and then examined for salmonellae according to the standard methods.

RESULTS

From 605 specimens of faeces 95 salmonella strains were isolated. Among them the following salmonella types were identified: 8 strains of *S. typhi*, 41 strains of *S. paratyphi B* (including *S. java*), 27 strains of *S. typhi-murium*, 7 strains of *S. enteritidis*, 6 strains of *S. derby*, and 6 strains of *S. blockley*.

The efficiency of the new enrichment medium in comparison with other media used in the study is shown in Tables 1–3.

Table 1. *Comparison of efficiency of various selective media for the isolation of salmonella types*

(From 605 specimens of faeces, 95 salmonella strains were isolated.)

Name of medium	No. of salmonella strains isolated
Wilson–Blair, direct plate	37
SS agar, direct plate	21
Mueller–Kauffmann medium	82
MS medium	79

Table 2. *Number of salmonella strains isolated on only one of the four selective media*

Name of the medium	No. of salmonella strains isolated
Wilson–Blair, direct plate	0
SS agar, direct plate	1
Mueller–Kauffmann medium	14
MS medium	7

Table 3. *Comparison of the efficiency of various selective media for the isolation of* Salmonella typhi

(The distribution of positive cultures from the eight specimens of faeces from which *S. typhi* was isolated.)

	Patient							
Medium	1	2	3	4	5	6	7	8
Wilson–Blair, direct	+	−	+	+	+	+	+	−
SS agar, direct	+	−	+	+	+	+	+	−
Mueller–Kauffmann	+	−	−	−	+	−	+	−
MS medium	+	+	+	−	+	−	+	+

The new enrichment medium described contains two inhibitory substances, magnesium chloride and sodium hydrogen selenite.

Magnesium chloride was used for the first time for the preparation of an enrichment medium by Rappaport, Konforti & Navon (1956) and Rappaport & Konforti (1959). In an appropriate concentration it is inhibitory for coliform organisms, including *Proteus* species and *Pseudomonas aeruginosa*. The magnesium chloride–malachite green (MM) medium described by Rappaport *et al.* contains magnesium chloride in a 4 % concentration. In our medium 3·4 % of $MgCl_2$ was found to be the optimal concentration.

The second characteristic of our MS medium is that it contains sodium hydrogen selenite in a very low concentration (0·05 %). Such a low concentration was chosen because it was found that higher concentrations may be inhibitory for many salmonella strains. The combined action of 0·05 % sodium hydrogen selenite and 3·4 % $MgCl_2$ makes the medium sufficiently selective.

The instructions for the preparation of the new MS enrichment medium must be strictly followed. The only satisfactory broth found was Difco nutrient broth, and it cannot therefore be replaced by any other nutrient broth or by infusion broth. The same is true for the sodium hydrogen selenite Merck.

The MS medium is very cheap and very easy to prepare. The results obtained indicate that the new medium is an efficient enrichment medium for the isolation of *S. typhi* as well as for the isolation of other salmonella types. From Table 3 it can be seen that the MS medium is in fact superior to the Mueller–Kauffmann medium when dealing with the isolation of *S. typhi*. In the isolation of other salmonella types the MS medium is, however, a little inferior to the Mueller–Kauffmann medium.

From 95 salmonella strains 7 were isolated only on the MS medium. It is clear that this gain would have been much higher if the study had been done in a province in which *S. typhi* is prevalent, as the MS medium compares more favourably with the Mueller–Kauffmann medium in the isolation of *S. typhi* than in the isolation of other salmonella types.

Subcultures from Mueller–Kauffmann and MS medium were made in the present study on Wilson–Blair and SS agar plates. The analysis of the results showed that subcultures on Wilson–Blair medium were more efficient.

SUMMARY

A new enrichment medium for the isolation of salmonella types from faeces is described.

The inhibitory substances for coliform organisms and enterococci in this medium are magnesium chloride and sodium hydrogen selenite.

The new medium was found in routine diagnostic work to be superior to the Mueller–Kauffmann medium in the isolation of *Salmonella typhi* and only a little inferior in the isolation of other salmonella types.

When used in addition to the standard media the new medium increases the number of positive results.

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Disinfection of woollen blanket

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INTRODUCTION

Following the demonstration that conventional laundering does not adequately reduce counts of bacteria from blankets (Steingold, Wood & Finch, 1954; Jerram, 1958) there has been concern that blankets may be important reservoirs of pathogens in hospitals (King Edward's Hospital Fund for London, 1959). Methods have been developed for sterilizing (Humfeld, Elmquist & Kettering, 1937; Gillespie & Alder, 1957; Finch, 1958; Foter, 1960; Stratford, Christie & Dixson, 1960; Caplan & Dickinson, 1961), and for boiling woollen blankets (Cunningham, 1956; Dickinson, Wagg & Fairchild, 1959; Pressley, 1960). Although boiling of blankets on a large scale has been routine practice for several years at hospital laundries in Australia (Cowling, 1959; Standards Association of Australia, 1962), and boiling has the advantage that mycobacteria may be destroyed, it has not yet been universally accepted in other countries. In view of this and the observation that conventional laundering with soap is very ineffective in removing bacteria (Blowers & Wallace, 1955; Frisby, 1957; Ravenholt, Baker, Wysham & Giedt, 1958; Schwabacher, Salsbury & Fincham, 1958; Thomas, Liddell & Carmichael, 1958; Larkin, Bridson, Grieve & Gibson, 1961; Dickinson, Wagg & Carter, 1962) the potentialities of washing with bactericides warrant closer investigation. Bactericides have not proved as effective in laundering trials with new woollen blankets as would be expected from the activities of the bactericides as measured by standard methods; the present paper is concerned with the possibility that this low efficiency of the bactericides may be due to their adsorption by the wool.

MATERIALS

Blanket

All-wool blanket was used; it was undyed and not treated for shrink-resistance. For all tests it was used as squares, area 25 cm.^{2} and weight about 1 g.

Cultures

Cultures of *Staphylococcus aureus* and *Escherichia coli* were used for contamination of blanket. These bacteria were isolated from disease sites, and were cultured in the following medium: (g./l.), concentrated beef extract (Oxo Ltd. London), 10; peptone (Bacto-peptone, Difco, Detroit), 10; yeast extract (Difco), 5; K_2HPO_4, 2; $MgSO_4.7H_2O$, 1; (pH = 7). Cultures (50 ml. in 250 ml. conical flasks) were incubated overnight at 37° C., and were started with 1 ml. inocula from similar, fresh cultures.

Bactericides and detergents

Bactericides used were P.C.M.X. (3,5-dimethyl-4-chlorophenol), C.T.A.B. (cetyl trimethyl ammonium bromide), Hibitane (chlorhexidine diacetate, i.e. bis-(4-chlorophenyl diguanido)-hexane diacetate), and detergents were Alkanate D (sodium dodecylbenzene sulphonate 82 %, sodium sulphate 15 %, water 3 % w/w) and Lissapol N 450 (ethylene oxide condensate compound of octyl cresol, Imperial Chemical Industries).

Antagonists

Antagonist for P.C.M.X. was Tween 80 (Erlandson & Lawrence, 1953), and for C.T.A.B. and Hibitane, a mixture of Lubrol W (ethylene oxide condensate compound of aliphatic alcohols, I.C.I.) and egg-yolk extract (Davies, 1949; Davies, Francis, Martin, Rose & Swain, 1954). The egg-yolk extract, supplied by British Drug Houses, was designated as 'Lecithin' 33 %, and the combined medium is known as Lubrol–Lecithin medium; it should be noted however that Davies *et al.* (1954) reported that egg-yolk was an effective antagonist of Hibitane whereas Lecithin was not.

METHODS

Loading of blankets with bacteria

Cells from four cultures were collected by centrifugation, mixed thoroughly with 1 g. of dried skim-milk powder (not sterilized), and freeze dried. The crisp plug of milk and bacteria so formed was transferred to a sterilized ball mill half-filled with glass balls (8 mm. diam.). Five blanket pieces (unsterilized) weighted with

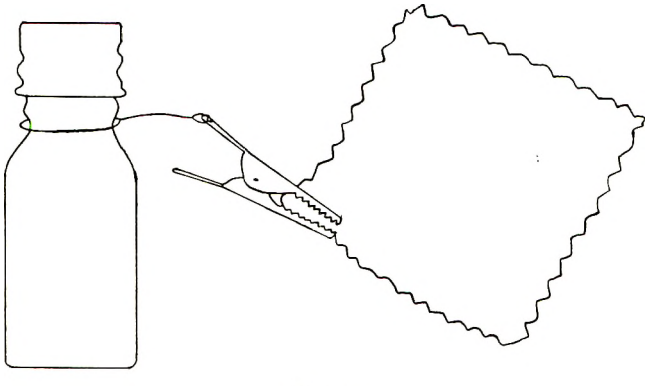

Fig. 1

sterile Macartney bottles (attached by wire and clips, see Fig. 1) were added to the ball mill, and the mill turned slowly for 6 hr. In this way the bacteria and milk formed a fine powder which impregnated the woollen fabric.

Preparation of wash liquors

Wash liquors contained 0·02 M sodium orthophosphate at pH 7·0, and detergents compatible with the bactericides. P.C.M.X. was maintained in solution with Alkanate D; it was dissolved in a minimal volume of ethanol, and added to solutions already containing Alkanate D at concentrations just sufficient to main-

tain solution of P.C.M.X. Lissapol N 450 was added with C.T.A.B. or Hibitane primarily as a washing agent, but its concentration was increased to dissolve Hibitane at high concentrations. For concentrations of agents see Table 1.

Table 1. *Concentrations of agents for pretreatment and washing of contaminated blanket*

	Concentrations of agents (mg./ml.)					
Treatment	PCMX	Alkanate D	CTAB	Lissapol N 450	Hibitane	Lissapol N 450
Pretreatments	4·0	8·0	4·0	0·5	3·2	0·1
Washes	0·5	0·5	0·5	0·5	0·1	0·5

Washing

All washing treatments were run under the following conditions: squares of blanket were immersed in 25 ml. of liquor contained in 100 ml. conical flasks, pre-heated to 50° C. and maintained at this temperature in a water-bath; preliminary tests showed that counts of survivors after washing with bactericides at 50° C. were lower than at 30° C. During washing the liquors and blanket pieces were shaken reciprocally at 130 r.p.m. with amplitude 4 cm. At completion of all washing treatments the blanket squares were rinsed in 25 ml. of water at room temperature (15–25° C.), and spin dried in a basket centrifuge (260 *g* for 90 sec.). In tests of bacterial inactivation care was taken to sterilize the neck of the flask after introduction of contaminated blanket, and operations after washing the blanket were performed to a rapid schedule in order to minimize post-washing kill.

Sampling bacterial populations on blanket

According to a modification of the method introduced by Frisby (1957), and elaborated by Jerram (1958), blanket pieces were cut aseptically to squares about 9 mm.^{2}, and disintegrated in 400 ml. of water by a top-drive macerator (Townson and Mercer, Croydon, England). Densities of bacteria in the supernatant liquor were determined by adding 1 ml. of appropriately diluted liquor to 15 ml. of the culture medium containing 1·5 % Difco agar and antagonist; for P.C.M.X., Tween 80, 1 % (w/v); for C.T.A.B. and Hibitane, Lubrol W, 1 % with egg-yolk extract ('Lecithin') 0·5 %.

Estimation of bactericides

For investigating the uptake of bactericides by the fabric the concentrations of P.C.M.X., C.T.A.B. and Hibitane remaining in the liquors were determined by adaptations of methods according to Folin & Ciocalteu (1927); Epton (1947) and Holbrook (1958) respectively.

EXPERIMENTS

Uptake of bactericides by blanket

Preliminary washes with bactericides at concentrations recommended for laundering indicated that reductions in counts of bacteria from 10^6 to 10^3 could be

expected for phenolic or quaternary ammonium compounds used under the present conditions. In trials with bacteria and bactericides, but without blanket, counts were reduced to zero, hence it seemed that the blanket was inhibiting the action of the bactericides. It is known that wool removes detergents from solution (Crewther, 1956; Goldsmith, Latlief, Friedl & Stuart, 1956); it was therefore considered probable that the bactericides were similarly being absorbed. Furthermore, it seemed possible that this supposed uptake could be inhibited by presaturation of the blanket with bactericides. Accordingly, pieces of blanket were

Table 2. *Loss of bactericides from solutions during pretreatment and subsequent washing of blanket*

		Concentration of bactericide after various times of treatment (mg./ml.)					
		Pretreatment liquor (min.)				Wash liquor (min.)	
Bactericide	Blanket piece no.	0	5	25	125	0	5
P.C.M.X.	1	0	0	0	0	0·5	0·00
	2	0·8	0·72	0·52	0·44	0·5	0·12
	3	1·6	1·4	1·2	0·88	0·5	0·24
	4	2·4	2·0	1·6	1·3	0·5	0·32
	5	3·2	—	2·3	1·5	0·5	0·44
	6	4·0	3·3	3·0	2·6	0·5	0·56
C.T.A.B.	7	0	0	0	0	0·5	0·12
	8	0·8	0·46	0·34	0·26	0·5	0·18
	9	1·6	1·0	0·76	0·65	0·5	0·26
	10	2·4	1·6	1·2	1·1	0·5	0·30
	11	3·2	2·1	1·8	1·5	0·5	0·35
	12	4·0	2·8	2·3	2·2	0·5	0·38
Hibitane	13	0	0	0	0	0·1	0·00
	14	0·8	0·04	0·03	0·00	0·1	0·00
	15	1·6	0·75	0·14	0·03	0·1	0·02
	16	2·4	1·4	0·73	0·44	0·1	0·09
	17	3·2	2·0	1·4	1·1	0·1	0·21
	18	4·0	2·5	2·9	2·9	0·1	0·30

washed in solutions containing bactericides at various concentrations for relatively long periods, and the concentrations of bactericides remaining in the wash liquors determined. The pretreated blanket samples were rinsed, and rewashed in solutions of bactericides at single concentrations for the standard time of 5 min. Bactericides remaining in the second wash liquors were also estimated. The data in Table 2 show that depletion of the bactericides from the wash liquors progressively decreased with increase in the concentrations of these agents in the pretreatments. In further tests with blanket pieces pretreated so that no bactericide was taken up in the second wash, if the second wash was repeated three times it was found that little if any bactericide was taken up in the repeated washes.

Washing of contaminated blanket

To determine whether the pretreatment of blanket with bactericide would lead to an increased bactericidal effect corresponding to the prevention of uptake of

bactericide from wash liquors, washes were run with contaminated blanket. Blanket pieces were pretreated (125 min.), rinsed, dried, and with untreated pieces as blanks, were contaminated with bacteria. Two sets of pretreated and untreated samples were tested; one set was sampled before washing to check the possibility that bacteria on the treated samples might have been inactivated. This 2^2 factorial design was employed in six separate tests of the three types of bactericide and two species of bacterium. The concentrations of agents were as given in Table 1. The data presented in Table 3 indicate that washing without pretreatment reduced counts from around 10^6 to 10^3, whereas with pretreatment the counts decreased to zero.

Table 3. *Washing of contaminated blanket*

		Bacteria in liquor from macerate of blanket (no./ml.)			
		Blanket not pretreated with bactericide		Blanket pretreated with bactericide	
Bactericide	Species of bacterium	Not washed	Washed	Not washed	Washed
P.C.M.X.	*S. aureus*	1×10^7	3×10	2×10^6	0
	E. coli	5×10^5	2×10^2	6×10^5	0
C.T.A.B.	*S. aureus*	4×10^5	0	7×10^5	0
	E. coli	1×10^5	3×10^2	1×10^5	5
Hibitane	*S. aureus*	9×10^4	3×10^2	2×10^4	0
	E. coli	3×10^5	3×10^3	4×10^5	0

In conjunction with the observation that the pretreatments of the fabric prevented removal of the bactericides from wash liquors, these data indicate that the dense populations of bacteria loaded on to the blanket were virtually destroyed. It was possible, however, that the lowering of the counts was due, at least in part, to bacteriostasis, in spite of the inclusion of antagonists in the plate medium. To check this possibility the amounts of the bactericides leached out during maceration of pretreated, washed and rinsed blanket were determined; the concentrations in the liquors were about 3, 10 and 20 μg./ml. for Hibitane, C.T.A.B. and P.C.M.X. respectively. In controls where antagonist, bacteria (at low densities) and bactericide were added in that order to the agar medium, these quantities of the bactericides did not cause bacteriostasis in the presence or absence of the antagonists. As a further check for bacteriostasis a duplicate set of plates was prepared in all tests, and to the plates were added suspensions of bacteria at calculable densities (1 ml. of the corresponding dilution of the bacterial suspension derived from the untreated, unwashed blanket). Expected counts were obtained except with blanket which had been pretreated and washed; for this treatment combination the counts varied from the expected value to about one-tenth of the expected value. These variations in counts might have been due to bacteriostasis, but were negligible in comparison with the effects attributable to the treatments.

DISCUSSION

There have been three outstanding difficulties in the problem of blanket hygiene in hospitals. Owing to the complexities of hospital epidemiology it has been difficult to assess the role of the blanket, and it has therefore not been possible to propose standards of laundering known definitely to be appropriate for minimizing cross-infection. Bound with this problem have been the related difficulties of estimating densities of micro-organisms held in fabrics, and the provision of cheap and efficient methods for disinfection.

Methods for sampling populations of micro-organisms on fabrics based on sweeping (Blowers & Wallace, 1955), percussion (McQuade & Sutherland, 1960), and contact (Rubbo & Dixson, 1960) all suffer the disadvantages that the permissible upper limit of count is about 10^3 colonies, and that the sampling fractions are not known. The finding by Frisby (1957) that the sweep-plate method samples 1 in 10^4 bacteria found by maceration points to the inadequacies of the percussion, sweeping and contact methods for analysing processes of disinfection. It follows that the reports that bactericides render blankets virtually sterile (Steingold *et al.* 1954; Blowers & Wallace, 1955) were not necessarily correct. It should be pointed out, too, that natural contaminants include spores, and because these were not distinguished from vegetative cells, the data of the above authors cannot be regarded as measures of the efficacy of the agents they examined, these agents (quaternary ammonium and non-ionic detergents) usually being regarded as virtually non-sporicidal.

The problem of determining the density of micro-organisms on blanket has not been completely resolved; at present no method can be claimed to provide unequivocal estimates of the absolute densities of micro-organisms held by a fabric where the organisms are initially applied as a powder. On the other hand, we were able to recover 100 % of bacteria applied as a broth culture, indicating that the cells were not adsorbed on the fibres, and giving reasonable ground for assuming that methods based on maceration of the fabric give fairly accurate estimates of the original densities of bacteria on woollen blanket.

In assessing the efficiency of disinfection by chemicals the maceration technique has the potential disadvantage that some bactericide may be leached from the treated fabric during maceration, and cause bacteriostasis in the cultures for counting. For this reason agents claimed to inhibit bacteriostasis were routinely added to the medium especially since Rubbo, Stratford & Dixson (1960) confirmed that bacteriostasis by Hibitane was reversed by Lubrol W and Lecithin added to broth cultures. However, under our conditions of culture none of the additives seemed to decrease bacteriostasis; the observed decrease in counts below the expected values may have been due to some cause other than bacteriostasis. It is interesting to note that impregnation of the blanket did not lead to appreciable lowering of recoveries of bacteria applied to the blanket as powder (compare columns 1 and 3 in Table 3) confirming the observation that under conditions of use no bactericidal effect can be expected against dry bacteria on woollen fabric impregnated with bactericide (Rubbo *et al.* 1960).

The demonstration that, in the presence of blanket, bactericides at conventional

concentrations are rapidly reduced to low levels, presumably by adsorption on the wool, provides an explanation for the low bactericidal efficiency of cetrimide found in laundering trials by Dickinson *et al.* (1962). The fact that three chemically different bactericides were adsorbed suggests that bactericides generally may be adsorbed on blankets freshly exposed to these agents. The further observation that pretreatment of the blankets lowers the rate of removal of bactericide from subsequent wash liquors points to the possibility of conserving the activity of bactericides in wash liquors by presoaking the fabrics in concentrated solutions of these agents. On introducing blankets to use, the presoaking could be delayed until immediately before the first wash and it might not be necessary to repeat the pretreatment. In unpublished tests we found also that repeated washing of blanket with bactericides at conventional concentrations led to a progressive increase of residual bactericides in sequential wash liquors; this observation may explain the improvement in cleaning noted by Frisby (1957) on repeated washing of blanket with a quaternary ammonium compound.

Sweep-plate counts for used hospital blankets are generally of the order of 10^3 for an area of blanket approximately 2500 cm.^{2}, and assuming the sweep-plate method samples 1 in 10^4 of the total population in the volume of blanket sampled, the densities would be around 10^5 per 25 cm.^{2}. The densities we achieved by artificial contamination, being of the order of 10^8 bacteria per 25 cm.^{2}, were much higher than would be expected in use; this observation in conjunction with the high kills observed suggest that bactericides properly used might prove very effective in the bacteriological cleaning of woollen fabrics. However, laundering trials would be necessary to determine the practibility of this suggestion because laboratory trials of bactericidal activity must be interpreted with caution (Sykes, 1962). Furthermore, the compatibility of the bactericides with the washing agents must be determined, and optimal conditions for the activity of the bactericides should be found. It is also preferable for the laundering of wool that the pH of the liquors should be kept below 7, and for frequent washing the fabric must be treated for shrink-resistance (Pressley & Morris, 1962).

SUMMARY

The efficiencies of bactericides in destroying bacteria on woollen blanket were investigated on a laboratory scale. The bactericides were not effective when tested with new blanket; this low efficiency was found to be related to the rapid adsorption of the bactericides by the wool. Pretreatment of the wool with concentrated solutions of bactericides depressed the rate of depletion of the bactericides from subsequent washing liquors with bactericides at customary concentrations, and led to more satisfactory rates of kill (inactivation factors about 10^6).

The test organisms, *Staphylococcus aureus* and *Escherichia coli*, were applied to the blanket as a powder, and the relative densities of bacteria on the blanket were determined using a procedure based on maceration of the fabric. The bactericides, 4-chloro-3,5-xylenol (P.C.M.X.), cetyl trimethyl ammonium bromide (C.T.A.B.) and bis-(4-chlorophenyl diguanido)-hexane diacetate (chlorhexidine diacetate) were tested in the presence of appropriate detergents.

Cultures were kindly supplied by Dr Hildred Butler of the Royal Women's Hospital, Melbourne.

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Replacement coliform flora in carriers of intestinal pathogens

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There are many antibacterial drugs now available, antibiotic and synthetic, which are strongly inhibitory to intestinal pathogens of the Salmonella and Shigella groups and to the *Escherichia coli* serotypes. Most of these drugs can be used singly or in combination with reasonable success in the alleviation of the symptoms and signs of infection, but none can be relied upon to eradicate the infecting organisms. In about 50 % of cases treated in our hospital, the pathogen disappears spontaneously, but in the remainder it persists for weeks despite intensive and even repeated courses of treatment. Of the various drugs now in use, neomycin, paromomycin and ampicillin are probably as active as any in killing these pathogens, but even these drugs used in combination or successively fail to clear a proportion of carriers (Coles & Stewart, 1961; Stewart, Coles, Nixon & Holt, 1961).

For some years past we have explored the possibility of preventing or treating the carrier state by giving bacterial suspensions concomitantly with antibacterial drugs. At first yoghourt was used, then suspensions of *Bacillus subtilis* and lactobacilli, without any success. More recently it was decided to try another approach based on the fact that during treatment with an antibacterial drug the patient's natural coliform flora is suppressed at least as strongly as is the pathogen. We therefore selected a number of cases immediately before treatment was instituted and assumed that some of them would relapse after standard courses of treatment. From the original faecal samples submitted from these patients we isolated the principal strain of *E. coli* and kept it in subculture. This organism was rendered resistant to a drug (either neomycin or paromomycin—see below). When in due course a proportion of these cases relapsed, the drug selected was then given together with the artificially resistant coliform to see if this organism would multiply during treatment, establish itself in the gut and 'crowd out' the pathogen. The present paper relates our experience with eight cases thus treated.

METHODS

Clinical

This trial was carried out in the Gastro-Enteritis Unit of this hospital. When patients were first admitted their stools or faecal swabs were submitted in accordance with routine practice to the laboratory. A number of cases from whom a definitive pathogen was isolated were selected for trial. Along with the pathogen, the principal strain of *E. coli* was isolated and made resistant to a drug as described

below. Meanwhile, pending the bacteriological result, all cases of gastro-enteritis when admitted were treated with either neomycin or paromomycin (each at 40 mg./lb. daily in four doses by mouth). The strains of *E. coli* isolated from patients receiving paromomycin were made resistant to neomycin, and the strains from those receiving neomycin were made resistant to paromomycin. The object here was to treat cases relapsing after paromomycin with neomycin, and vice versa, and to give the resistant organism as a replacement coliform flora fully resistant to the appropriate drug at the same time. Of twelve cases thus selected, eight relapsed and were given the replacement flora. Two of these cases (1 and 2) were well-established chronic carriers; the remainder (3–8) were convalescent carriers.

Bacteriological

Strains of *E. coli* isolated as described above were plated out to ensure purity, a number of colonies being sampled and tested for biochemical reactions. Several colonies were then subcultured into broth and passaged successively on gradient plates (Szybalski, 1952) of agar containing rising concentrations of neomycin or paromomycin from 20 to 200 μg./ml. Strain M.J. was made resistant to both drugs. Passage was ended when complete resistance to 200 μg./ml., confirmed by titration in liquid media, was attained, the original untrained strain being titrated at the same time. One strain (D.S.) attained this level of resistance in four passages; one (A.T.) required nine passages; the usual number was six or seven (Table 1). When passaged, each strain showed cross-resistance to other related drugs, each to almost the same level. At their final passage the trained strains usually grew more slowly into smaller colonies than the parent strain and showed some decrease or loss of certain biochemical reactions. After four subcultures on drug-free media, normal growth and biochemical activity returned and resistance to the drugs was maintained.

For administration to the patients the resistant strains were grown overnight in nutrient broth, the resulting cultures were centrifuged, twice washed in sterile saline, and then resuspended in sterile saline at densities of 2×10^7, 4×10^7 per ml. and upwards. These suspensions were checked for purity and viable counts.

During the period of administration of the drug together with the drug-resistant replacement coliform the faeces were examined as frequently as possible by plating on McConkey agar containing 20 μg./ml. of the appropriate drug. The replacement coliform was isolated selectively on this medium. At the same time, faeces were plated for identification of the pathogen and other organisms on drug-free McConkey agar, deoxycholate citrate, nutrient agar and Czapek-Dox agar, and in selenite or tetrathionate enrichment media. The anaerobic flora was not examined in this investigation.

Administration of replacement flora

Normally six doses were given, starting with 20 million resistant organisms, increasing in steps daily until 1000 million organisms were given on the 5th and 6th days. The organisms were added to milk or puree immediately before ingestion. The bacterial suspensions from which the doses were prepared were stored at

Table 1. *Replacement strains of* Escherichia coli *rendered resistant to neomycin or paromomycin* in vitro

Strain of *E. coli*	Rendered resistant to	Minimal inhibitory concentration (μg./ml.)		No. of passages	Cross resistance Minimum inhibitory concentration (μg./ml.)		
		Original	Final		Streptomycin	Paromomycin	Neomycin
S.W.	Neomycin	20	200	6	200	200	.
A.S.	Neomycin	20	200	6	50	200	.
J.F.	Paromomycin	20	200	7	200	.	150
A.T.	Neomycin	20	200	9	100	200	.
A.H.	Paromomycin	20	200	7	200	.	100
N.S.	Paromomycin	20	200	6	50	.	200
K.F.	Neomycin	20	200	6	100	200	.
D.S.	Paromomycin	40	200	4	100	.	200
C.F.	Paromomycin	20	200	7	50	.	100
M.J.	Neomycin and	20	200	8	100	.	.
	Paromomycin	20	200				
T.C.	Paromomycin	5	200	6	< 50	.	200
K.R.	Paromomycin	15	200	7	200	.	100

4° C. Each dose was checked by viable count at the time of administration. In all instances these viable counts showed good approximation to the estimated dose. In case 1 (S.W.) the replacement coliform was given for ten further daily doses of 1000 million organisms.

Drug treatment was started 1–3 days before administration of the replacement coliform, which was given for 3–4 days with the drug and then, for 2–3 days, alone, except in case 1.

RESULTS

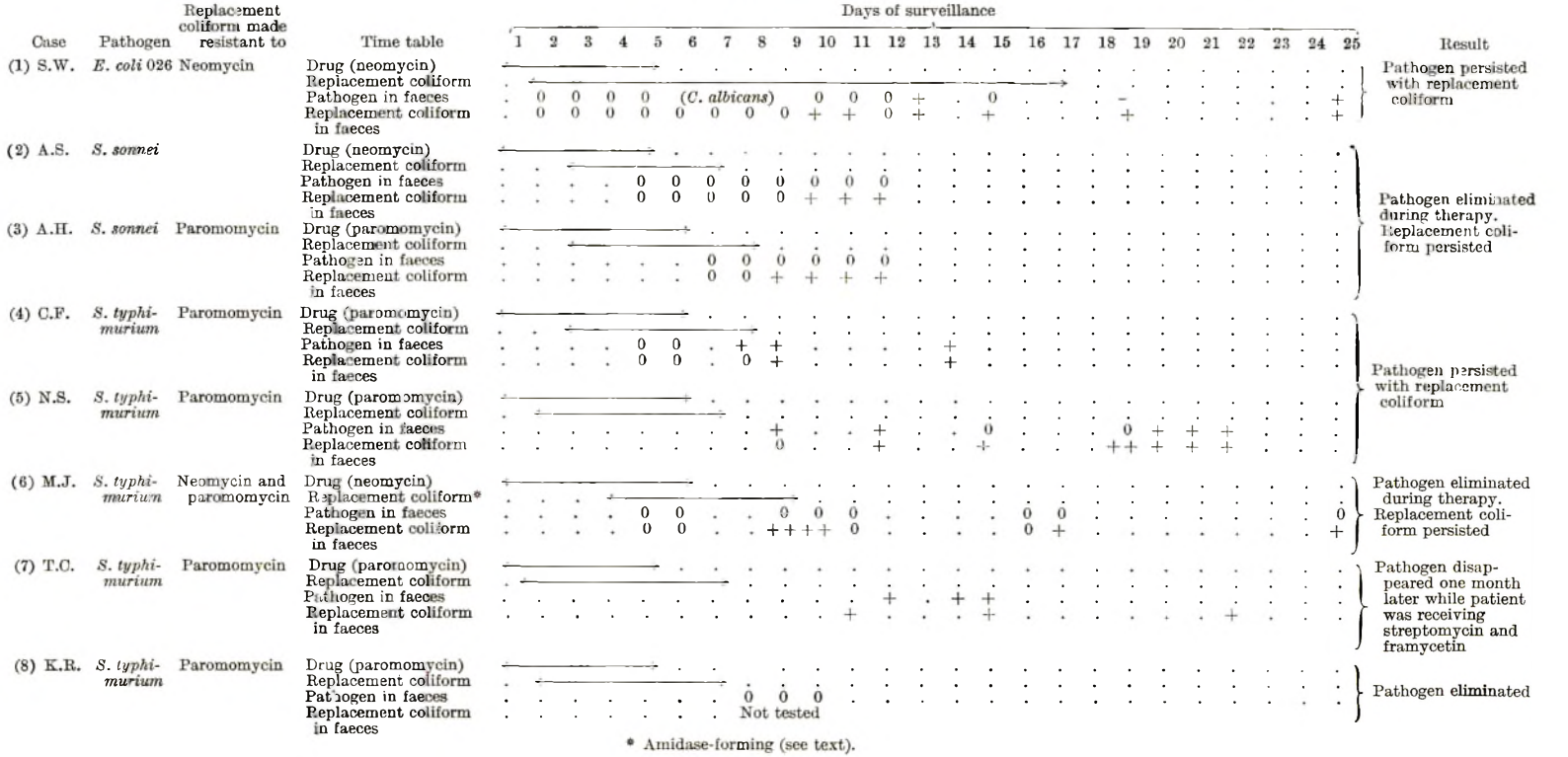
The replacement coliform organism began to appear in the faeces of cases 1–7 at or towards the end of the period during which it was administered (Table 2). In case 8, through an oversight, the faeces were plated only on selective media which precluded identification of the coliform. In cases 2, 3, 6 and 8 the pathogen disappeared during the period of therapy and failed to reappear in at least five specimens examined after the suppressive drug was no longer being excreted. These cases (2 *Shigella sonnei*, 2 *Salmonella typhi-murium* infections) were regarded as being cleared of infection and were discharged from hospital. In four others (3 *Salm. typhi-murium*, 1 *E. coli* 026) the pathogen disappeared, along with the remainder of the coliform flora, during the period of administration of the drug, but reappeared a day or two later and persisted for many days thereafter, along with the replacement coliform. On re-isolation these pathogens showed no drug-resistance.

Case 1 (S.W.) was given the replacement coliform for a total of 16 days. By the 10th day it was well established and it persisted until the 25th day, after which no further stools were examined. During this time the pathogen (*E. coli* 026) disappeared from the 2nd until the 12th day, reappeared on the 13th, was not isolated on the 15th, but reappeared again on the 19th and 25th days in increasing numbers. During this time *Candida albicans* also appeared in the stool, and for this reason nystatin (500,000 units twice daily) was given by mouth.

In case 6 (M.J.) the replacement coliform had a useful 'marker' property in that it formed amidase, which was identified by its power to break the carbon–nitrogen linkage in leucinamide, in penicillin G and other selected substrates. This property enabled the organism to be identified amid the natural coliforms in drug-free media and we were therefore able to estimate the degree of replacement semi-quantitatively. On the 9th and 10th days the replacement coliform accounted for the entire coliform flora. On the 11th day the natural coliforms returned in quantity, but on the 17th and 25th days the amidase-forming replacement coliform was still present in considerable quantity.

All the children were watched carefully for untoward effects during this procedure. The coliform suspension was accepted without complaint or distaste; there was no nausea or vomiting. Case 4 showed persistent mild diarrhoea, but this was present before the replacement coliform was given. None of the other five cases showed any diarrhoea during treatment, and cases 1, 5, 6, 7 and 8 were in fact constipated after the period of treatment.

Table 2. *Results of treatment with drugs and replacement flora of resistant coliforms in eight children carrying intestinal pathogens*



* Amidase-forming (see text).

DISCUSSION

It is difficult to explain the persistence of susceptible pathogens in the intestine after large doses of poorly absorbed, bactericidal drugs like neomycin and paromomycin. While the drugs are being administered, the entire aerobic flora is suppressed and the faeces may be sterile when cultured aerobically. Some pathogens (e.g. *Salmonella typhi* and *paratyphi* B) may linger in the biliary tract, but this is not known to occur with *Sh. sonnei* or *E. coli* serotypes, which may be equally persistent in the gut. It could be argued that the drugs act preferentially on the commensal flora, thereby entrenching the pathogen, but the results recorded above do not support this view, for the presence of a competitive drug-resistant flora was not conducive to elimination of the pathogen in four of the eight cases treated. In four other cases, including the two carriers of *Sh. sonnei*, the pathogen disappeared during this régime, but this might well have been due to chance.

It is clear therefore that a replacement flora of drug-resistant coliforms can be established in the gut, rapidly and comparatively easily, by giving a few doses of a suitably prepared bacterial suspension along with a selected drug. This flora persists after dosage ceases and does not prevent the return of part or all of the original flora.

As a therapeutic technique, the method described here may therefore be of use in maintaining a coliform flora during prolonged antibiotic therapy but cannot be relied upon to assist in the elimination of pathogenic intestinal bacteria.

SUMMARY

Eight carriers of intestinal pathogens (5 *Salm. typhi-murium*, 2 *Sh. sonnei*, 1 *E. coli* 026) were given their own coliforms, rendered drug-resistant *in vitro* to neomycin or paromomycin, together with one of these drugs. In seven cases, the drug-resistant coliform flora established itself, while the natural coliform flora and the pathogen were suppressed. In four cases only (2 *Sh. sonnei*, 2 *Salm. typhi-murium*) the pathogen was eliminated in the course of this procedure.

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A survey of measles antibodies in different populations

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In previous studies of measles (Black, 1959; Bech, 1960; Snyder, McCrumb, Bigbee, Schluederberg & Togo, 1962) an interesting feature has been the presence of complement-fixing as well as neutralizing antibody in sera from populations of all ages. Both types of antibody persist long after the illness, which is essentially one of childhood. Further, measles is uncommon in the first 6 months of life mainly owing to passive immunity from maternal antibody. Mortality is highest in 6- to 11-month-old children (Langmuir, 1962), a very susceptible group, in whom maternal antibody no longer exerts a protective influence.

For this investigation, samples of serum obtained from persons of different ages were tested for measles complement-fixing antibody. The following groups were included:

(1) Twenty-two sera from infants under 1 year of age who had taken part in a study of live poliovirus vaccine (Report, 1961).

(2) 243 sera submitted to the Diagnostic Laboratory, Colindale, from patients of all ages in the London area, for anti-streptolysin 'O' (ASO) estimations.

(3) Twenty-seven pairs of sera from mothers shortly before childbirth and from the umbilical cord blood of their infants immediately after birth.

(4) Twenty-eight sera from mothers whose infants contracted measles before they were 10 months old.

(5) Eight sera from persons stating they had not had measles.

Additionally, measles virus neutralization tests were done on the sera from groups 3, 4 and 5.

MATERIALS AND METHODS

Measles antigen for the complement-fixation tests was prepared in HeLa cell cultures infected with the Edmonston strain of measles virus. The cells were grown in a medium of 0·5% lactalbumin hydrolysate in Hanks's balanced salt solution with 12% calf serum, 3% Hartley's infusion broth, 36·5 mg. $NaHCO_3$ per 100 ml. (0·83 ml. of a 4·4% $NaHCO_3$ solution), 200 units penicillin and 200 μg. streptomycin per ml. For maintenance, synthetic medium 199 (Morgan, Morton & Parker, 1950) with 2% inactivated horse serum, and 220 mg. $NaHCO_3$ per 100 ml. was used. The cells were inoculated with a dose of virus calculated to infect the entire cell sheet, which was harvested when the characteristic measles cytopathic changes were well advanced. The antigen consisted of the cells and tissue culture

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fluid harvested after freezing and thawing twice. This fluid was titrated as a chessboard in a WHO plastic plate with a serum from a hyperimmunized monkey and used at its optimal titre. The antigen was stored at $-70°$ C. Its specificity was checked against a measles-positive and measles-negative monkey serum.

The complement-fixation test was done by the method of Bradstreet & Taylor (1962) with two units of complement (2HC 50). Sera were inactivated at 60° C. for 20 min. immediately before use.

Virus suspensions for neutralization tests were prepared in a similar manner to the complement-fixing antigen. High titres were obtained by inoculating large amounts of virus and harvesting as soon as cytopathic changes were well evident in the HeLa cells. The harvested suspension was immediately dispensed in 3 ml. quantities in bijoux bottles and stored at $-70°$ C. It was titrated in serial half-log dilutions, duplicate HeLa tubes being inoculated with 0·1 ml. amounts. The highest dilution showing cytopathic change was the end point as calculated by the method of Reed & Muench (1938). Such a dilution represented one tissue culture dose (TCD 50) of virus per 0·1 ml. of suspension. In the neutralization tests, 500 TCD 50 of virus contained in this volume were employed.

Neutralization tests were done in roller cultures of HeLa cells used within 48 hr. because it was found that their sensitivity to measles virus decreased if older cultures were used. Sera were not inactivated. They were diluted in twofold steps in Hanks's balanced salt solution over a range from 1/10 to 1/1280. Equal volumes (0·3 ml.) of the serum dilutions and virus suspension were mixed and left at 4° C. for 1 hr. Each mixture was then inoculated in 0·2 ml. amounts into duplicate tubes. The cultures were incubated at 37° C. for 6 days when they were examined for cytopathic changes. The fluid in each tube was replaced with fresh maintenance medium and a final reading made after re-incubation for a further 24 hr. This was done because with measles and other syncytial viruses such as respiratory syncytial virus the cytopathic effect may be partly masked until the fluid is changed.

RESULTS

All results are presented in the form of histograms with the antibody titres in both the complement-fixation and the neutralization tests first subdivided broadly as shown in Table 1.

Table 1. *Range of antibody titres in complement-fixation and neutralization tests*

Antibody titre	Complement-fixation test	Neutralization test
Negative	< 8	$\leqslant 10$
Low	8–16	20–40
Intermediate	32–128	80–160
High	> 128	> 160

The figures given as titres are the reciprocals of the initial dilutions of serum. The sera composing the first two groups came from persons whose age varied from less than 1 year to more than 40 years. The results of investigations have been set

out in steps as follows: 1, 0–1 year—22 sera; 2(*a*) 1–4 years—45 sera; 2(*b*) 5–11 years—51 sera; 2(*c*) 12–20 years—48 sera; 2(*d*) 21–40 years—49 sera; 2(*e*) over 40 years—50 sera.

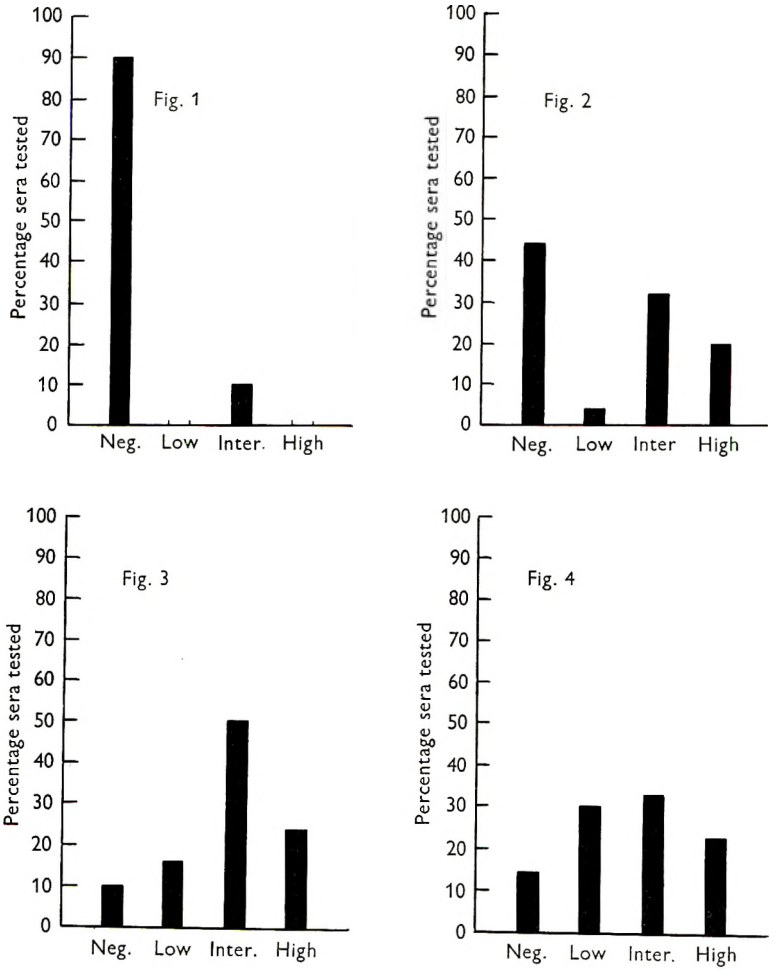

Fig. 1. Complement-fixing antibody titres in group 1.
Fig. 2. Complement-fixing antibody titres in group 2(*a*).
Fig. 3. Complement-fixing antibody titres in group 2(*b*).
Fig. 4. Complement-fixing antibody titres in group 2(*c*).

In group 1 complement-fixing antibody was present in only 10% of the sera, with the titres mainly in the intermediate range (Fig. 1). In group 2(*a*) the percentage of sera with complement-fixing antibody had risen to 56. Despite the fact that the sera were collected during a so-called non-epidemic period more than 50% showed intermediate or high titres (Fig. 2). In group 2(*b*) 90% of the sera had complement-fixing antibody. Those which were negative all came from 5-to 6-year-old children and those positive showed a scatter in their titres which was indicative of past infection at different times. The picture showed little further variation in antibody distribution for groups 2(*c–e*). Most persons had had measles

at some stage (Figs. 3–6). In those over 40 years of age, the antibody titres were intermediate or low suggesting little recent effective contact with the virus.

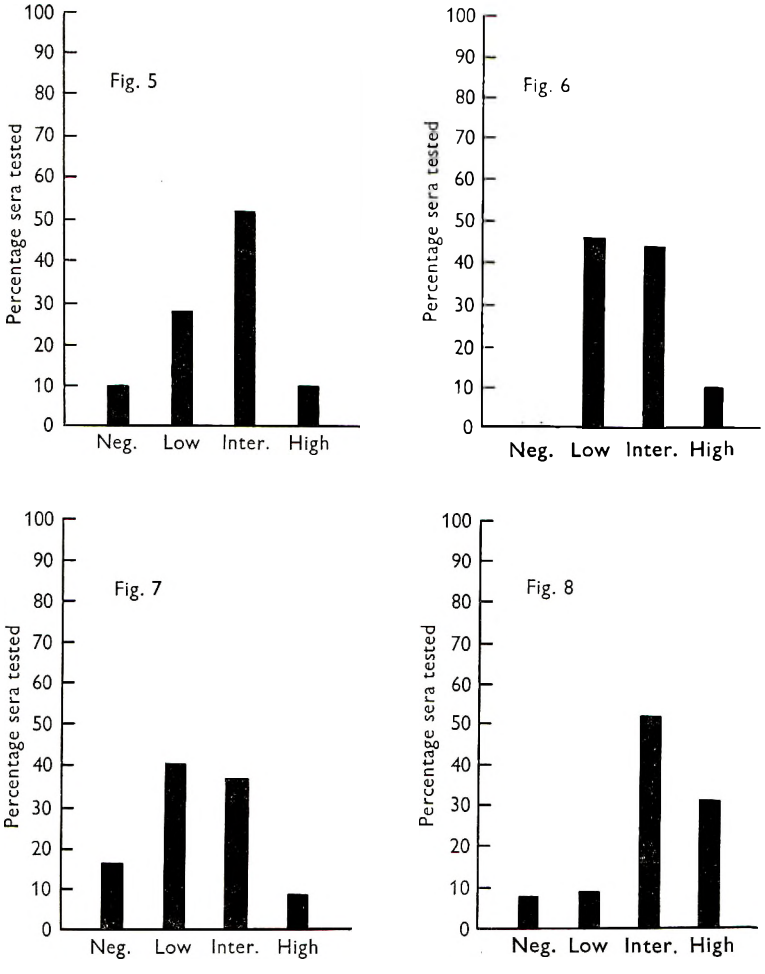

Fig. 5. Complement-fixing antibody titres in group 2 (*d*).
Fig. 6. Complement-fixing antibodyy titres in group 2 (*e*).
Fig. 7. Complement-fixing antibody titres in group 3 maternal sera.
Fig. 8. Complement-fixing antibody titres in group 3 cord sera.

In group 3—the study of maternal and cord sera—the antibody titres in the mothers' sera (Fig. 7) showed a similar pattern to that in group 2 (*d*) which was composed of persons of both sexes aged 21–40 years. When antibody was present in the maternal circulation, it was passively transferred to the infant across the placental barrier. It was further shown that in most instances a fourfold or occasionally greater concentration of antibody took place in the cord blood (Fig. 8). Neutralization tests (Figs. 9 and 10) with these sera showed the same pattern. Again the antibody in the cord sera was concentrated and in addition only two maternal sera were completely negative. This may have been due to non-specific inhibition of the formation of the antibody-antigen complex or to the amount of

specific antibody present in the maternal serum being too low to be measured easily.

In group 4, the distribution of antibody in mothers whose infants developed measles by the age of 10 months did not differ from the earlier groups (Figs. 11, 12). The passive antibody derived from the mothers disappears in 3–6 months and

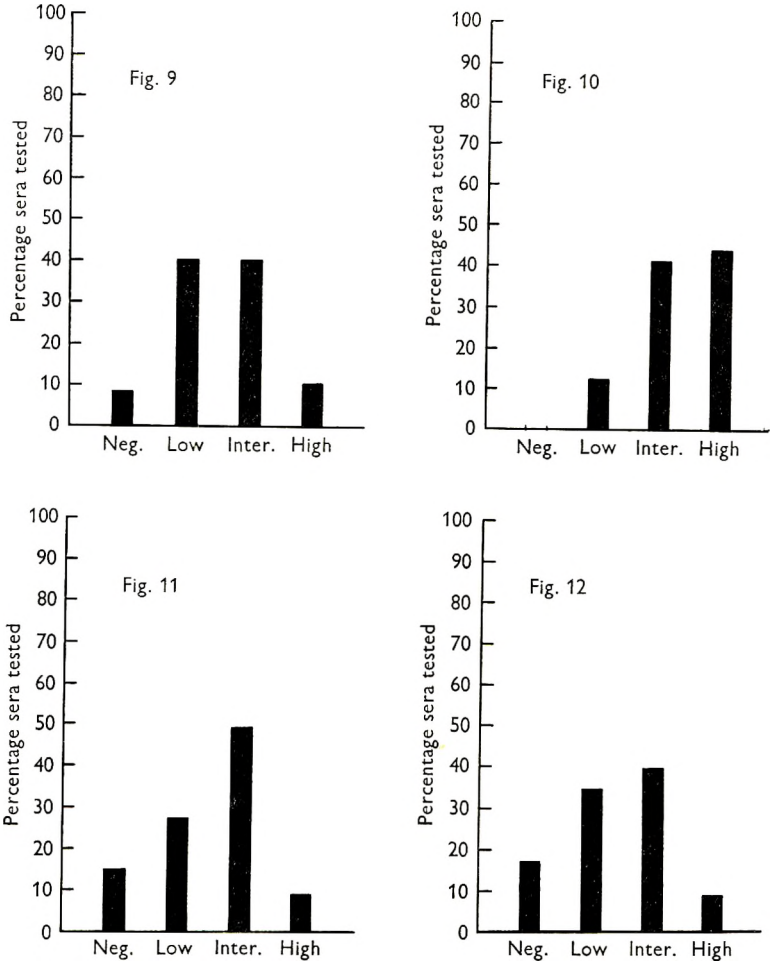

Fig. 9. Neutralizing antibody titres in group 3 maternal sera.
Fig. 10. Neutralizing antibody titres in group 3 cord sera.
Fig. 11. Complement-fixing antibody titres in group 4.
Fig. 12. Neutralizing antibody titres in group 4.

the development of measles in these infants is the result of their exposure to other patients with the disease.

In group 5, only two out of eight sera from persons with no history of measles failed to show the presence of both complement-fixing and neutralizing antibody.

DISCUSSION

The results of complement-fixation tests on the first two groups of sera showed the presence of antibody in 10% of the samples from children up to 1 year of age. Thereafter, the proportion positive increased rapidly to reach a plateau of about 90% in children between 5 and 11 years of age, and this proportion remained virtually unchanged throughout the age groups studied. Apart from indicating the widespread prevalence of measles, the results are noteworthy in that for most virus infections, complement-fixing antibody reaches its peak during the convalescent stage of illness and then often declines to an amount too small to detect within 9 to 12 months. With measles, however, after an initial reduction both complement-fixing and neutralizing antibody remain at a fairly constant titre after infection. It is uncertain whether this is due to frequent repeated exposure to measles virus or to the persistence of viral antigen in the body after the infection has subsided. No demonstration of latent virus has so far been made. Black (1962) showed that members of isolated communities might retain their complement-fixing antibody long after an epidemic had subsided. In Tahiti, for example, antibody titres decreased only twofold in $8\frac{1}{2}$ years during which time there was no measles in the community and no likelihood of any specific stimulus to measles antibody having occurred.

In the youngest infants studied, antibody titres were low although the infections must have been comparatively recent. This may be because in the first few months of life the antibody-producing mechanism is still immature. In the group of children under 5 years of age 44% were without antibody. These sera had been collected in an inter-epidemic period during which a sufficiently large number of susceptible children is built up to provide material for the next epidemic.

In group 3, twenty-four out of twenty-seven samples of cord serum contained an increased amount of antibody compared with their own maternal serum. This discrepancy, which was observed in both complement-fixation and neutralization tests, is in agreement with the report of Ruckle & Rogers (1957) who found a twofold concentration in five cord sera although, in the present series, the concentration for the most part appeared to be fourfold or even greater. The amount of γ-globulin in cord serum has been reported (Oberman, Gregory, Burke, Ross & Rice, 1956) to be the same as in the maternal serum but the serum albumins are considerably higher. It may be interesting to speculate whether any protein fraction in the cord serum other than γ-globulin contains measles antibody.

Of the twenty-eight sera tested in group 4, six were from mothers who were doubtful whether they had had measles but five of these contained antibody; three were from mothers who denied having had measles but two contained antibody. On the other hand, sera from two mothers who claimed that they had had measles contained no antibody. These, together with the additional results in group 5, are in keeping with the extensive morbidity of measles but suggest that in a few instances inapparent or very mild infection can occur.

SUMMARY

A survey of serum samples from all age groups in the population has shown a rapid acquisition of antibody between the ages of 1 and 6 years which reaches a plateau in concentration and does not disappear later.

The concentration of antibody in cord serum may be four or more times greater than that in the mother's serum.

Presence or absence of a record of past infection by measles is not necessarily reflected by the titre of antibody in the serum.

Low antibody titres in very young infants may be due to an imperfectly developed antibody producing mechanism.

The author gratefully acknowledges the assistance of all those who made this survey possible. In particular, she is indebted to Dr G. I. Watson of the College of General Practitioners and his colleagues (see list) for their help in the collection of so many of the serum samples, and to Mr D. A. Bamgboye for his excellent technical assistance.

Participating doctors

Dr J. Alexander
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Dr A. P. Barker
Dr F. V. A. Bose
Dr M. H. Butler
Dr M. P. Carter
Dr G. G. M. Edelsten
Dr S. Griffiths
Dr B. L. Hoskyns
Dr C. R. Kenchington
Dr J. D. E. Knox
Dr G. S. K. Little
Dr H. Z. Lewinska
Dr J. McAlister Williams
Dr J. O. Newman
Dr R. H. Sandiford
Dr E. Sawdon
Dr K. Scott
Dr H. Shire
Dr R. Simpson-White
Dr G. Stow
Dr W. Symonds
Dr J. Walker
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The epidemiology of louping ill in Ayrshire: the first year of studies in sheep

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Following an outbreak of louping ill in lambs on a hill farm (Camlarg) near Dalmellington, Ayrshire, a study was made of the epidemiology of the disease on three neighbouring farms (Mossdale, Dalcairnie, Knockgray—shaded in Fig. 1). The affected area was that in which Gordon, Brownlee, Wilson & Macleod (1932) and Gordon (1934) did much of the original investigation into louping ill infection of sheep. These workers developed a formalinized sheep brain vaccine which has been used prophylactically in louping ill areas of the British Isles ever since. Generally, lambs are vaccinated once during the second 6 months of their life and this is the only artificial protection they receive. In the area studied, lambs are born on the hills in April and remain there until weaned in September. The male lambs are then sold off the farm and the female lambs (ewe hoggs) are sent to a tick-free lowland pasture (the wintering) from October until the following April. Generally speaking they return to the hirsel (an area of the hills confined naturally by hill burns, ravines, etc.) on which they were born and where they remain until they are sold as cast ewes at the age of 5½–6 years. Black-face hoggs for Dalcairnie Farm are, however, born on Brownhill Farm some 7 miles away. Only cross-bred lambs are produced at Dalcairnie and all are sold in the autumn. The sheep on these farms are not mated (tupped) until November in their second year (as gimmers) after which they produce a lamb annually, usually for 4 subsequent years.

This paper reports the first year of work in a long-term study of which further reports will follow. The first year of work in sheep was mainly on Dalcairnie Farm which has two hirsels (Dalcairnie Hill and Barbeth) separated only by a stream and a farm road. Barbeth forms roughly the southern one-third of the farm.

MATERIALS AND METHODS

Virus. The Moredun strain of louping ill virus after 30–35 mouse passages was used.

Sera were stored at −20° C.

Haemagglutinin. After exsanguination the brains of sick infected mice were removed and ground up in 0·4 % bovine albumin in borate-buffered saline of pH 9.

After centrifugation at 3000 r.p.m. for 10 min., the supernatant was spun at 13,000 r.p.m. for 1 hr. in a Spinco angle head. The supernatant was stored at 4° C. and used as antigen. Optimal haemagglutination occurred at pH 6·3.

Haemagglutinin-inhibition (HI) tests. The sera were twice extracted with acetone in the cold (Clarke & Casals, 1958), then absorbed with goose cells. Using M.R.C. pattern Perspex plates, 8–16 units of haemagglutinin in pH 9 borate saline were added to two-fold dilutions of extracted serum starting at 1/10 in the same buffer. After standing at room temperature for 1 hr., 0·25 % goose cells were added in a phosphate buffer which adjusted the reaction to pH 6·3. The cells were allowed to sediment at room temperature. Titres are stated as the reciprocal of the highest dilution causing complete inhibition.

Neutralization tests. These were carried out in 3- to 4-week-old mice. Inactivated sera were mixed with an equal volume of about 100 LD 50 of virus diluted in equal parts of fresh guinea-pig serum and 10 % horse serum broth. The mixtures were incubated for 1 hr. at room temperature then inoculated intracerebrally into groups of six mice. The virus was titrated at $\sqrt{10}$-fold dilutions in 50 % normal sheep serum. The results were computed using survival times by the method of Smith & Westgarth (1957). Sera showing a significant difference from the controls at the 5 % level were recorded as positive.

Virus isolations. Brains of lambs were placed in 50 % glycerol saline and sent to London. The brains were then ground up as a 20 % suspension in borate-buffered 0·75 % bovine albumin (pH 9) and inoculated intracerebrally into two litters of baby mice. The brains of sick or dead mice were tested, after passage if necessary for complement-fixing antigen against a standardized mouse louping ill antiserum.

RESULTS

Preliminary study on Camlarg Farm

Heavy lamb losses occurred on some farms in the area in the spring of 1960. In September of that year, forty ewe hoggs were bled on Camlarg Farm (Fig. 1) where about 50 % of the lambs had died from louping ill in the spring. HI antibody was found in twenty-four (60 %) and neutralizing antibody in twenty-five (63 %): as the animals were then 5 months old this is unlikely to have been maternal in origin. In March 1961, thirty-eight of them were bled again. Examination of the March sera showed that six (25 %) of the animals with HI antibody in the previous September had lost it less than one year after infection and the median titre of the positive sera had fallen from 160 to 20. The pattern of the change corresponds with an eight-fold drop in the titre of all the animals in 6 months. The prevalence of neutralizing antibody, on the other hand, was unchanged between September and March (Fig. 2).

Main study on Dalcairnie Farm

In 1961 all the ewes on Dalcairnie Farm were ear-tagged and bled on 29/30 March and 5 June. At the first bleeding, approximately two-thirds of this flock (207 ewes) were given formalinized sheep brain vaccine* and the remainder (99) were kept as

* Vaccine supplied by Burroughs Wellcome and Co.

controls. This was their second dose of vaccine, the first having been given 1–5 years previously as hoggs.

A sample of each group was bled 3 weeks after vaccination. Post-mortem examinations were made and virus isolation attempted from brain of some of the

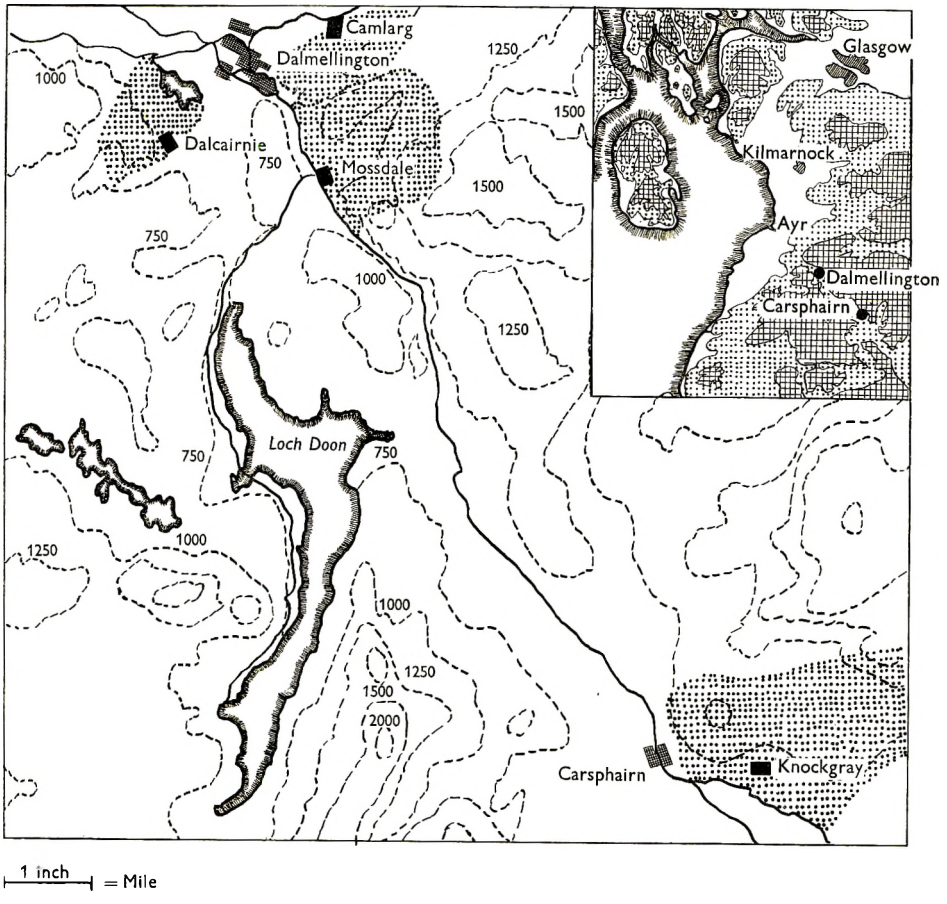

Fig. 1. Map showing the study areas in south-west Scotland.

lambs which died between March and June. Blood was also obtained from their dams as soon as possible after lamb death.

The distribution of ewes between the two hirsels of Dalcairnie Farm is shown in Table 1.

Haemagglutinin-inhibiting antibody in ewes

Of the Dalcairnie Hill ewes, 64 % had antibody in March compared with 35 % on Barbeth hirsel (Table 2). This difference is significant. On Barbeth hirsel the prevalence of antibody in both March and June was significantly higher in revaccinated (42 and 49 %) than control (20 and 18 %) animals. Of all the conversions (Table 3) from negative to positive antibody status, 67 % (10/15) at Dalcairnie Hill, and 20 % (1/5) at Barbeth, occurred in the 2-year-old ewes. Few conversions occurred at older ages. Two 2-year-old ewes from Dalcairnie Hill were

the only animals to show an increase in titre ⩾ 4-fold. Six ewes from the same hirsel (three of them 2 years old) and one from Barbeth lost antibody between March and June.

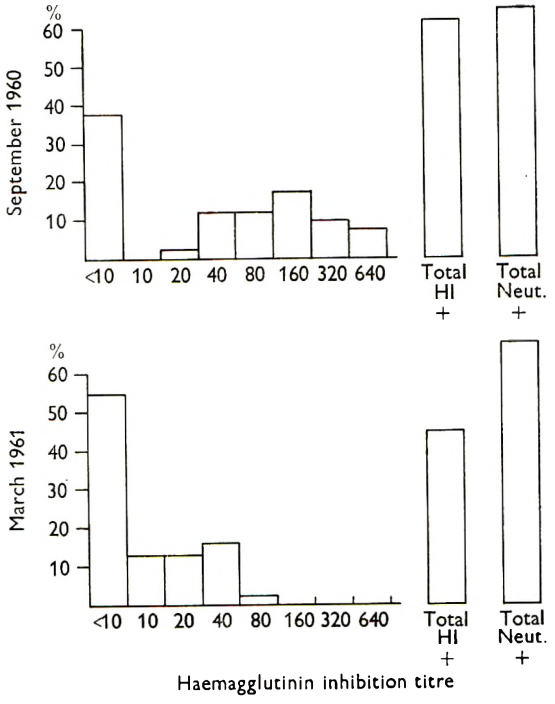

Fig. 2. The change in prevalence of antibody in the surviving lambs at Camlarg Farm where about 50 % died in May–June 1960.

Table 1. *The distribution of re-vaccinated and control ewes on the hirsels of Dalcairnie Farm*

	Dalcairnie Hill	Barbeth	Total
Revaccinated	143	64	207
Controls	69	30	99
Total	212	94	306

Neutralizing antibody in ewes

The preliminary study showed that HI antibody declines more rapidly than neutralizing antibody. The neutralization test was therefore mainly used to test sera without HI antibody. The distribution of the sera examined is shown in Table 4. The March and June sera from each animal were tested as far as possible in the same test.

No significant difference was found between the proportions with antibody in March (49 %) and June (67 %) on Barbeth (Table 5). However, there was a significant increase in prevalence between March (78 %) and June (97 %) on Dalcairnie Hill hirsel and there were significantly higher prevalences of antibody on Dalcairnie Hill than Barbeth in both March and June.

Table 2. *Haemagglutinin inhibiting-antibody in ewes on two neighbouring hirsels in* 1961

Hirsel	Age (years)	Control			Revaccinated			Total		
		March	June	% diff.	March	June	% diff.	March	June	% diff.
Dalcairnie Hill	2	7/15	11/15	+27	19/39	20/34	+10	26/54	31/49	+15
	3	17/21	16/21	−5	22/31	22/29	+5	39/52	38/50	+1
	4–6	26/33	24/30	+2	44/72	40/58	+8	70/105	64/88	+6
Total		50/69	51/66	.	85/142	82/121	.	135/211	133/187	.
%		73	77	+4	60	68	+8	64	71	+7
Barbeth	2	0/8	0/7	0	3/14	4/14	+7	3/22	4/21	+5
	3	2/9	1/9	−11	4/13	4/12	+2	6/22	5/21	−3
	4–6	4/13	4/12	+2	20/37	22/35	+9	24/50	26/47	+8
Total		6/30	5/28	.	27/64	30/61	.	33/94	35/89	.
%		20	18	−2	42	49	+7	35	39	+4

Table 3. *Haemagglutinin-inhibiting antibody conversions in paired sera from ewes on two neighbouring hirsels between March and June* 1961

Hirsel	Age (years)	No. tested	< 1 to ⩾ 1	⩾ Four-fold	Total	% conversion
Dalcarnie Hill	2	49	8	2	10	20
	3	50	2	0	2	4
	4–6	88	3	0	3	3
Total		187	13	2	15	8
Barbeth	2	21	1	0	1	5
	3	21	0	0	0	0
	4–6	47	4	0	4	8
Total		89	5	0	5	6

An estimate of the total cumulative infection rates can be obtained by adding to the number of sheep with HI antibody, a proportion derived from the sample tested for neutralizing antibody. This is calculated separately for each age group, place and for March and June.* The percentage figures which result give estimates of the proportion of the flock which has been naturally infected at some time during their lives (Table 6).

Table 4. *Sera from ewes examined for neutralizing antibody*

	Dalcairnie Hill	Barbeth	Totals
Revaccinated	42 (10)	31 (1)	73 (11)
Controls	45 (31)	14 (2)	59 (33)
Totals	87 (41)	45 (3)	132 (44)

Figures in parentheses = number with HI antibody.

Measure of agreement between HI and neutralization tests

The antibody status of 132 ewes bled in March and June and tested in both tests is shown in Table 7: 103 sera were positive and 58 negative in both tests. Thus, the two tests agreed in 61 % (161/262) of the sera examined. Neutralizing but not HI antibody was found in 39 % (101/262). Only two sera with HI antibody gave negative neutralization tests.

Antibody conversions between March and June

There was no change in 90 % of the sheep with both types of antibody (Table 8) in 73 % of those with neutralizing but not HI antibody, or in 35 % of those with neither antibody. 30 % of those without either type of antibody in March had both types by June, while 35 % developed neutralizing antibody only; thus altogether 65 % of these antibody-free sheep acquired neutralizing antibody by June. Of those with only neutralizing antibody in March, 19 % developed HI antibody by the summer.

In sera examined by both tests (Table 5) HI and neutralizing antibody responses followed a similar pattern. On Dalcairnie Hill 88 % (15/17) of the animals showing a neutralizing antibody response and 71 % (12/17) of those with a HI response were 2-year-old ewes. Six of the animals which had neutralizing antibody responses did not develop HI antibody. Nine animals showed both HI and neutralizing antibody responses. Another three animals with neutralizing antibody in March and June developed HI antibody. Three of the four Barbeth 2-year-old ewes which showed neutralizing antibody responses failed to show HI antibody conversions. In the older age groups on both hirsels, there were six

* E.g. Dalcairnie Hill, 3–6 years, June. Table 2: 36 of 138 had no HI antibody. Table 5: 16 of 17 without HI antibody had neutralizing antibody, i.e. 0·941. Therefore add 0·941 × 36 to the number with HI antibody in Table 2, i.e.

$$\frac{0{\cdot}941 \times 36 + 102}{138} = \frac{34 + 102}{138} = \frac{136}{138} = 99\,\%.$$

Table 5. *Neutralizing and haemagglutinin-inhibiting antibody and conversions in ewes tested by both tests on two neighbouring hirsels in* 1961

Hirsel	Age (years)	No. pairs of sera examined	Neutralizing antibody				HI antibody			
			March	June	Conversions of negative sera	(%)	March	June	Conversions of negative sera	(%)
Dalcairnie Hill	2	41	24	39	15	88	16	26*	12	48
	3	23	23	23	0	0	14	15†	2	22
	4–6	23	21	22‡	2	(100)	11	14	3	25
Total		87	68	84	17	.	41	55	17	.
%		.	78	97	89	.	47	63	37	.
Barbeth	2	16	3	7	4	31	0	1	1	6
	3	12	6	8‡	3	(50)	2	2	0	0
	4–6	17	13	15‡	3	(75)	1	3†	3	19
Total		45	22	30	10	.	3	6	4	.
%		.	49	67	43	.	5	12	10	.

* Two ewes lost haemagglutinin-inhibiting antibody between March and June.
† One ewe lost haemagglutinin–inhibiting antibody between March and June.
‡ One ewe lost neutralizing antibody between March and June.

Table 6. *Estimated cumulative percentage infection rates in ewes*

Age (years)	Dalcairnie Hill			Barbeth		
	March	June	Change	March	June	Change
2	67	98	+31	27	52	+25
3–6	98	99	+ 1	80	89	+ 9

animals with neutralizing antibody in March which developed HI antibody in June and four HI antibody-free animals which acquired neutralizing antibody only. One ewe from Dalcairnie Hill and two from Barbeth lost neutralizing antibody and four from both hirsels lost HI antibody (Table 5). There was a significantly higher proportion of HI and neutralizing antibody conversions on Dalcairnie Hill than on Barbeth.

Table 7. *Comparison of the prevalence of HI and neutralizing antibody in ewes*

		Neutralizing antibody				
		March		June		Total
		+	−	+	−	
HI antibody	+	42	2	61	0	105
	−	48	40	53	18	159
Total		90	42	114	18	264

Table 8. *Summary of antibody conversions*

		June							
			Gain						
March		No change	HI N	%	HI only	%	N only	%
HI+ N+	42	38	90	.	.	.	.	.	.
HI+ N−	2	0	0	.	.	.	.	2	100
HI− N+	48	35	73	.	.	9	19	.	.
HI− N−	40	14	35	12	30	.	.	14	35

Table 9. *The distributions of HI antibody titres before and after revaccination*

	Before revaccination			After revaccination	
	< 10	⩽ 10	Median	Four-fold increases	Median
Revaccinated	10	18	20	3	20
Control	5	12	40	1	40

Effects on revaccination

Seventeen control ewes and twenty-eight revaccinated ewes were bled 3 weeks after the vaccination of two-thirds of the flock. The distributions of HI antibody titres before and after revaccination are shown in Table 9. Clearly there was no difference between the two groups on this basis. The four-fold increases in antibody in both groups may have been due to natural infection.

Twelve of these pairs of sera (4 controls and 8 vaccinated) were tested for neutralizing antibody. Ten had neutralizing antibody in both specimens, two became positive and one lost antibody. Again, no difference between the groups was demonstrated, although the number tested was rather small.

Antibody in lambs

Lamb sera were obtained at the June bleeding and compared by the HI and neutralization tests with the corresponding maternal sera. HI antibody was present in sera from 16 % (12/76) of lambs from the control ewes and from 22 % (29/131) of lambs from the vaccinated ewes. There is no significant difference. There is, however, a barely significant difference on Barbeth between the lambs of control ewes (0/25) and of vaccinated ewes (6/43 : 14 %).

Table 10. *Haemagglutinin-inhibiting antibody in simultaneous samples from mothers and lambs in June* 1961 *at Dalcairnie Farm*

Mother's antibody	Antibody in lambs					
	< 1	1	2	3	4	5
< 1	**80**	**5**	1	.	.	.
1	**31**	**4**	**2**	.	.	.
2	40	**8**	**5**	**1**	.	1
3	18	5	**2**	.	.	.
4	2	.	1	.	**1**	.
5	1	.	1	1	.	.

Antibody titres: 1 = 1/10, 2 = 1/20, 3 = 1/40 etc.

Table 11. *Relationship of ewe and lamb haemagglutinin-inhibiting (HI) and neutralizing (N) antibody in June*

Ewe	Lamb			
	HI+ N+	HI− N+	HI− N−	Totals
HI+ N+	0	6	1	7
HI+ N−	0	0	1	1
HI− N+	4	10	5	19
HI− N−	0	0	6	6
Totals	4	16	13	33

When the titres of HI antibody in lambs were compared with those of their mothers, 7 % (6/86) of lambs from mothers without detectable antibody in June had antibody. Apart from these, only one lamb had a titre four-fold greater than its mother. On the other hand, sixty-nine had titres at least four-fold less than their mothers. Two-thirds (139/210) of lambs had similar amounts of antibody to their mother (Table 10). In only one case in ten pairs of lambs suckled by the same mother was there any disagreement between the HI antibody status of the twins: one was positive and the other negative.

Of these lamb sera, thirty-three were examined by the neutralization test and twenty-one had neutralizing antibody. In twenty instances where the lamb had neutralizing antibody, the June sample from the ewe was also found to have neutralizing antibody. Only four of the lamb sera had both types of antibody. The remainder had either the same neutralizing antibody status (48 %) or less than their mothers (39 %) (Table 11).

Lamb deaths

Three of the thirteen freshly dead lambs examined post mortem died from pyaemia. Brains from eight were inoculated intracerebrally into mice and louping ill virus was isolated from one Dalcairnie Hill lamb. Another Dalcairnie Hill lamb had louping ill but recovered. Tick pyaemia killed both the Barbeth lambs. Six of the dead lambs were born to gimmers (Table 12).

Sentinel hoggs

As part of a vaccination experiment to be reported later, twenty-two hoggs from tick-free land were released on Dalcairnie Farm on 25 April: ten on Barbeth and twelve on Dalcairnie Hill hirsels. On Barbeth, within 6 weeks, there was one fatal clinical case (unconfirmed in the laboratory) of louping ill and one was destroyed after falling over a cliff: a probable infection rate of 11 % (1/9). On Dalcairnie Hill, within the same period, there were two deaths from pneumonia, four confirmed louping ill deaths and one (or possibly two) with serological (HI and neutralizing antibody) evidence of louping ill infection: a probable infection rate of 50–60 % (5–6/9).

DISCUSSION

This paper sets the scene of a long-term study to elucidate the epidemiology of louping ill infection and hence to design improved control measures. Louping ill, like the other arthropod-borne encephalitides, is essentially a biphasic illness. Following tick-bite and virus multiplication in the cells initially infected, viraemia occurs and infection becomes widespread in the animal. This visceral phase causes only relatively mild symptoms, except possibly in young lambs. The visceral phase cannot, of course, be clinically diagnosed as louping ill. The virus may or may not succeed in reaching the cells of the central nervous system: if it does then the second phase of encephalitis follows: namely clinically recognizable louping ill. It is important to be clear about this because in preventing clinical and fatal louping ill we are essentially concerned in preventing the encephalitic phase (i.e. preventing spread of the virus to the central nervous system).

Infection of the central nervous system depends on the inability of the defence mechanisms to prevent the virus penetrating the blood-brain barrier. With the related West Nile virus, primary virus multiplication appears to occur in the walls of the blood vessels (Kundin, Liu, Hysell & Hamachige, 1963). The encephalitic phase therefore depends on the degree and duration of viraemia which is largely determined by the rate of development of circulating antibody. Factors which impair the defence mechanisms will increase the probability of encephalitis, for example:

(1) *Age.* Newborn animals (e.g. baby mice) are generally much more susceptible to arbovirus infections than older animals. Either higher virus concentrations are reached because of greater cell susceptibility or the defence mechanisms are incompletely developed.

(2) *Nutritional state.* Edward (1947) reported difficulty in producing encephalitis in sheep following the inoculation of virus subcutaneously during the summer

Table 12. *Disease in lambs at Dalcairnie Farm in* 1961

	Lamb			Mother				
					HI antibody			
Date of death	Post-mortem findings	Isolation of LI virus from brain	Age	Hirsel	Pre-vaccination	Post-vaccination	At lamb death	5. vi. 61
13. v.	NAD*	–	2	DH	< 1	.	1	< 1
13. v.	NAD	+	2	DH	< 1	< 1	< 1	< 1
23. v.	NAD	–	2	DH	< 1	.	< 1	1
24. v.	Pyaemia	–	2	DH	2	3	.	2
27. v.	NAD	ND†	2	DH	⩾ 6	.	.	3
31. v.	NAD	ND	2	DH	< 1	.	.	< 1
27. iv.	NAD	–	3	DH	< 1	.	< 1	1
10. v.	NAD	–	3	DH	< 1	.	< 1	< 1
13. v.	ND	–	3	DH	3	3	< 1	.
24. v.	Pyaemia	–	4	B	2	.	.	2
28. iv.	Pyaemia	ND	4	B	2	.	.	1
Survived	Clinical	HI:3 on 5. vi. 61	4	DH	< 1	.	< 1	.
							(24. v. 61)	.
14. v.	ND	ND	?	DH	< 1	.	1	.
14. v.	ND	ND	?	DH	< 1	.	1	< 1

* NAD = no abnormality detected. † ND = not done.

H1 antibody titres. 1 = 1/10, 2 = 1/20, 3 = 1/40, etc.

months when their general health and nutrition was good. In the vaccination experiment mentioned above (p. 62), fourteen susceptible hoggs were exposed on 25 March on Knockgray Farm at Carsphairn about 10 miles south of Dalmellington. At the end of June none showed any serological evidence of louping ill infection. They were then exposed on Camlarg Farm near Dalmellington and although none showed evidence of clinical disease, thirteen (93 %) had developed louping ill neutralizing antibody and nine HI antibody by the following September. In the group of similar hoggs exposed on Camlarg Farm from March to June, 100 % became infected and 50 % died of louping ill. Whether this difference between exposure in March and exposure in July was due to age or nutritional factors or to some other cause is unknown.

(3) *Concurrent disease.* Gordon *et al.* (1932) and MacLeod (1962) found that the simultaneous infection of sheep with tick-borne fever and louping ill significantly increased the incidence of encephalitis and the frequency of isolations of virus from their brains. Other diseases which occur during the same period, tick pyaemia, *Pasteurella* pneumonia, anaerobic infections, may also increase the risk of encephalitis.

(4) *Cold and increased blood carbon dioxide.* Sellars & Lavender (1962) showed in mice that increased carbon dioxide tension in the blood increased the permeability of the blood-brain barrier to virus and thus susceptibility to encephalitis. They also found that exposure to cold (4° C. for several days) increased the CO_2 tension in the blood. Muscular exercise is also known to increase the risk and degree of paralysis in poliomyelitis. Obviously both cold and exercise, and the concurrence of any disease causing an increase in blood CO_2 may be contributory factors in louping ill encephalitis.

Vaccination

In weaned animals there is evidence from laboratory studies (Edward, 1947), from field trials (Gordon, Brownlee, Wilson, & Macleod 1962) and from the consensus of opinion over the last 30 years, that vaccination with formalinized sheep brain vaccine prevents a high proportion of animals infected from developing encephalitis. Vaccination does not cause detectable antibody formation and as we have shown here, a second dose 1–4 years after the initial dose received as a hogg also does not give an antibody response. Wilson & Gordon (1948) inoculated sheep with 3–4 doses at 2-week intervals before detectable neutralizing antibody appeared. Young animals are vaccinated during their first winter and this appears to sensitize them so that when they are naturally infected their defence mechanisms are accelerated and the illness is limited to the visceral phase. Vaccination therefore does not prevent infection but does prevent encephalitis.

In this south-west part of Scotland, louping ill mainly affects young lambs, often in sharp epizootics such as that at Camlarg Farm described above. The problem, therefore, is in the unweaned lambs of vaccinated ewes.

The protection of young lambs does not depend directly on vaccination but on maternal antibody. A vaccinated ewe will not confer sensitization on her lamb so that unless she has had a natural infection, her lamb will be susceptible to infection

and probably death. Maternal antibody resulting from a natural infection should, however, protect a lamb throughout its first season of maximum tick biting activity.

In the preliminary investigation, an eight-fold drop in HI antibody was observed between 6 and 12 months after infection, 25 % of the animals thus becoming HI negative. In another experiment 33 % of vaccinated and experimentally infected hoggs lost HI antibody within 6 months of infection (O'Reilly, Smith & White, unpublished). Neither in the field investigation nor the laboratory experiment was there any reduction in the proportion with neutralizing antibody during the period of observation. Therefore, HI antibody is probably evidence of relatively recent infection, while neutralizing antibody indicates natural infection at any previous time. Maternal antibody of both types is transmitted to lambs.

Tick drags were made over the farms at intervals throughout the year and the main activity of nymphal *Ixodes ricinus* was found in April/May and the larval peak in May. Later studies suggest that the nymphal peak is a little earlier than the adult peak (Varma, *et al.* unpublished). Most, if not all of the infections of lambs and sheep must be attributed to the bites of adults or nymphs. Nymphal activity is maximal during or shortly after lambing and the peak of adult activity probably after the completion of lambing. The period of tick-biting activity varies from year to year according to the weather conditions. If infections occur 2–3 weeks before lambing then it is possible that previously non-infected ewes will be able to provide ample maternal immunity. In all probability (and certainly in 1962 and 1963) most of the tick activity is too late and protection of the lambs is dependent on infection in the ewes or hoggs in previous years.

The course of events in the latter situation is shown in Fig. 3; if the infection rate is too low for every hogg to be infected, some gimmers in the following year will have susceptible lambs, and with still lower infection rates, also older ewes. Thus with persistent annual infection rates of 40–60 %, the expectation of louping ill in lambs of gimmers would be about 25 % and in the whole flock about 12 %. With persistent infection rates over 90 % less than 2 % of all lambs would be expected to die of louping ill. This latter state is probably reached on some farms where louping ill is hyperendemic. When the tick-biting is early enough to immunize the ewes in the spring before lambing, the proportion of gimmers' lambs affected would be halved for infection rates of 40–60 % and reduced to nil for a 90 % rate.

Protective measures can be intelligently designed only with a knowledge of the prevailing infection rate and of changes which may occur in it. Where the infection rate is consistently very high (about 90 %) no clinical louping ill or almost none will be seen. Constant infection rates between 20 and 60 % can be calculated to give about the same overall lamb losses (11–13 %) and there are probably many farms with this level of morbidity. However, in those areas where louping ill occurs in periodic epizootics we have shown that the infection rate does not remain constant but fluctuates. The infection rate in sheep appears to fall after periods of high rate: later work on Dalcairnie Hill has shown that in 1962 there was an infection rate of about 35 % as compared with about 60 % in 1961.

There is little doubt that the present methods of vaccination are highly effective

in the presence of consistent high infection rates. With lower and fluctuating rates, vaccinated animals are well protected but a proportion of young lambs are not protected by maternal antibody.

Clearly in such situations any improved vaccine must be primarily designed to prevent disease in young lambs. Either it must itself produce such a level of antibody in the mother that all her subsequent lambs will be passively immune, or it must not prevent natural infection while protecting her from encephalitis. In the latter case it must be remembered that the bite of a tick probably inoculates virus directly into the bloodstream where it is immediately exposed to circulating antibody, and therefore, any such new vaccine must be tested by feeding infected ticks on vaccinated animals to determine whether natural infection can regularly occur causing a good antibody response.

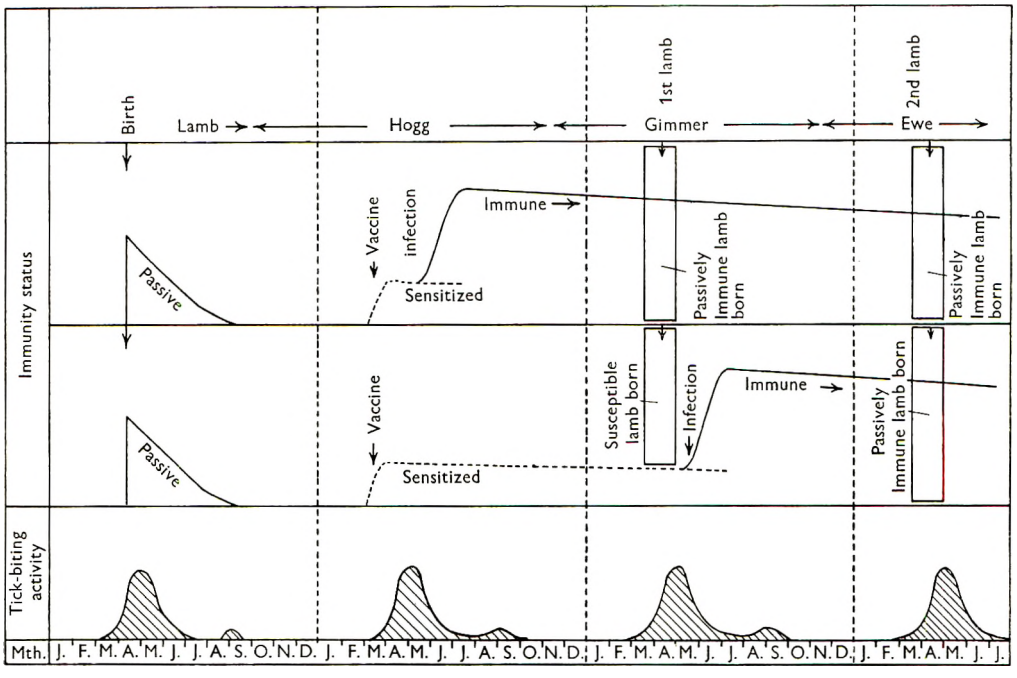

Fig. 3. Diagrammatic representation of the probable mechanism of vaccination in the control of louping ill.

Differences in infection rate between hirsels

Infection rates vary markedly between farms and between hirsels on an individual farm. The infection rate on Barbeth hirsel was significantly lower than on Dalcairnie Hill hirsel. The various estimates of the infection rates are shown in Table 13.

Barbeth is a sloping well-drained hirsel while Dalcairnie Hill has large areas of swampy ground with thick grass and rushes. Although comparative studies have not yet been made, the tick population on Dalcairnie Hill is probably higher than on Barbeth causing the difference in infection rate. It is notable that of the four farms on which we have studied tick populations, infection is absent only on Knockgray where the tick population seems to be lowest.

On the approximate basis of the means of these infection rates and the assumption that all lambs of mothers with antibody were protected, the expected numbers of lamb deaths from louping ill (2–3 on Barbeth and 1–9 on Dalcairnie Hill) were only a little higher than the observed numbers (0 on Barbeth and 1 on Dalcairnie Hill). The latter may well be underestimated because of failure to isolate virus from animals dead for some time before autopsy. Clearly even in the presence of a high louping ill infection rate there may be little or no clinical louping ill if the sheep population has been heavily infected in preceding years.

Table 13. *Estimates of the infection rates on the two hirsels on Dalcairnie Farm*

	Gimmers		
	HI conversions	Neut. conversions	Sentinel hoggs
Hirsel	(%)	(%)	(%)
Dalcairnie Hill	48	88	50–60
Barbeth	6	31	11

Twin lambs

Where there are twin lambs, one lamb sometimes acquires a lower level of passive immunity than the other. Batty, Thomson & Hepple (1954) have found a similar situation in anaerobic infections and Prydie (personal communication) with distemper antibodies in puppies. In this survey there was one of ten pairs of lambs in which at an age of 6 weeks one lamb had HI antibody and the other had not. In another experiment one of three pairs of lambs showed a similar discrepancy in neutralizing antibody at the same age, neither having HI antibody (O'Reilly, Smith and White, unpublished).

SUMMARY

1. Following an epizootic of louping ill on certain farms in south-west Ayrshire in 1960, a long-term study of several farms was initiated.

2. The flocks on two hirsels of one farm were studied during spring and early summer of 1961. Although only one lamb death was confirmed as due to louping ill, the infection rates in sentinel hoggs on the two hirsels were 50–60 % and 11 % respectively. The difference between the hirsels is probably attributable to the difference in the amount of tick habitat on them.

3. The ewes were bled in March and June and their lambs in June. Haemagglutinin inhibition (HI) and neutralization tests revealed that the HI antibody is much shorter lasting than neutralizing antibody. Many ewes, therefore, had neutralizing but not HI antibody. Otherwise agreement between the tests was good. In March almost all the ewes aged 3 years or more had antibody. Of the gimmers (2-year-olds) about two-thirds on one hirsel and one-third on the other had antibody in March: by June almost all the former and about half of the latter had antibody.

4. About two-thirds of the lambs had the same antibody status as their mothers in June and almost all the rest had less antibody than their mothers. Serological evidence suggestive of louping ill without recognizable clinical disease was found in six lambs and a further lamb recovered from clinical disease.

5. Revaccination of two-thirds of the flock failed to cause any detectable change in antibody status.

6. The epidemiology and pathogenesis are discussed in relation to immunity and infection rates, and to the design of control measures.

We are greatly indebted to the late Mr James Murdoch at Dalmellington, Mr John Murdoch at Dalcairnie Farm, and Mr David Murdoch at Knockgray Farm for permission to work on their farms and for all the help they gave us during the study.

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Observations on antigenic variation in a strain of *Trypanosoma brucei* growing in mice

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INTRODUCTION

The phenomenon of antigenic variation in trypanosomes has been known for many years. Earlier workers obtained variants in the form of relapse strains in infected experimental animals inadequately treated by chemotherapy (Ritz, 1914; Lowrie & O'Connor, 1937) and, although the existence of as many as twenty-two variants could be demonstrated by this means (Ritz, 1914), the experimental system made it difficult to investigate adequately the mechanism underlying variation.

Gray (1962) infected rabbits intravenously with *Trypanosoma brucei*, and at intervals injected infected blood from the rabbits into mice, in which strains of trypanosomes grew which were found by agglutination tests to be antigenically different from the strain originally injected into the rabbits. He studied five variants and observed some serological cross-reaction between them. It seemed possible that a modification of Gray's technique, in which variants would be produced in mice passively immunized with rabbit antisera, might clarify the problem further by enabling cross-protection tests to be carried out in addition to agglutination tests.

MATERIALS AND METHODS

Trypanosoma brucei

The strain used was supplied by Dr Hoare of the Wellcome Laboratories of Tropical Medicine, London. It was isolated in 1936 from a cow in Pong Tamale, Ghana, and had undergone more than 3000 passages in mice before being used in these experiments.

Preservation of trypanosomes

Mice under ether anaesthesia were bled from the axillary artery shortly before death. The blood was added to a drop of heparin (about 200 i.u.) and diluted 1 in 10 in fluid containing 10% (v/v) horse serum and 20% (v/v) glycerol in Hanks's balanced salts solution (BSS). The suspension was distributed in ampoules and kept at $-70°$ C. after rapid freezing in CO_2/alcohol. In these conditions trypanosomes remained viable for at least 3 months. Stock preparations contained about 10^8 trypanosomes per ml.

Counting of trypanosomes

Trypanosome suspensions were diluted in a white blood cell pipette in white blood cell counting fluid containing 0·1 % gentian violet, and counts were performed in a conventional counting chamber with an improved Neubauer ruling. As a routine, one side of the chamber was filled, and the trypanosomes in five of the central small squares (0·2 mm. × 0·2 mm.) were counted.

Rabbit antisera

Trypanosome suspension was diluted in 5 % horse serum in Hanks's BSS to contain 10^8 trypanosomes/ml. and 0·1 ml. was injected subcutaneously. The rabbits were bled out from the heart 7 days later and the serum was stored at −20° C. Sera were labelled AT 1, AT 2, AT 3 and so on (AT = anti-trypanosome; number = variant).

Protection tests and development of variants

Two groups of five Swiss albino mice received 0·2 ml. of diluted trypanosome suspension (10^6 trypanosomes) intra-peritoneally. One hour later five of the mice received 0·2 ml. of undiluted antiserum intra-peritoneally. The mice were examined twice a day, and deaths recorded. A mouse was assumed to have died half-way between the time it was last seen alive and the time at which it was found dead. The mean survival time (M.S.T.) was determined for each group and the protection conferred by the antiserum expressed as the difference in M.S.T. between the protected and unprotected groups. Variants were recovered by bleeding a mouse in the protected group which showed symptoms (lethargy, paralysis). Mice invariably died within 12 hr. of the onset of symptoms.

The parent strain (designated T 1) was derived from one of fifty mice which had received 0·1 ml. of a trypanosome suspension diluted to contain approximately 10 trypanosomes per ml. Forty-seven of the fifty mice died with an M.S.T. of 6·7 days. The mouse from which the parent strain was recovered was killed 6 days after inoculation. Since this was the sixth passage of *T. brucei* in mice in this laboratory, the parent strain was designated T 1/6. It was passed seven more times in mice, 10^7 trypanosomes being injected intra-peritoneally at each passage. The first variant, T 2/1, was derived from the thirteenth passage of the parent strain, T 1/13. For all variants except T 1 and T 2, agglutination and cross-protection tests were carried out on trypanosomes from the first passage.

Agglutination tests

Doubling dilutions of antisera were made in 0·5 ml. amounts in 5 % horse serum in Hanks's solution, from 1/20 to 1/10,240. Drops of 0·01 ml. of stock trypanosome suspension (10^6 trypanosomes) were delivered from an 'Agla' micro-syringe (Burroughs Wellcome) on to a Perspex plate 1 mm. thick and 0·05 ml. of antiserum dilution was placed on each drop of suspension, using a dropping pipette. The plate was kept at room temperature in a moist atmosphere for 1 hr. Drops were then examined for agglutination by dark-field microscopy with a 5 × objective and

20 × eyepieces (magnification 150 ×). The end-point was taken as the last dilution in which any clumps of trypanosomes were seen. Pl. 1 shows a large clump of agglutinated trypanosomes from a preparation which was dried, fixed with methyl alcohol and stained with May–Grünwald/Giemsa (George T. Gurr, London). The trypanosomes in the agglutinated masses remained actively motile for at least an hour.

Absorption of agglutinins

For the preparation of absorbing suspensions 0·1 ml. of stock suspension (about 10^7 trypanosomes) was injected intra-peritoneally into three mice. Two days later, when the blood contained more than 10^8 trypanosomes per ml., the mice were bled as described above. The trypanosomes in the heparinized blood were counted and the volume adjusted so that 0·5 ml. contained 10^8 trypanosomes. For absorption, 1 ml. of 5 % horse serum in Hanks's solution, 0·5 ml. of antiserum diluted 1 in 20, and 0·5 ml. of trypanosome suspension, 2000 μg. streptomycin, and 200 i.u. of heparin were placed in a 50 ml. conical flask. The flasks were stoppered and shaken gently overnight in a water-bath at 37° C. Next day the contents of the flask were centrifuged and the clear supernatant was stored at − 20° C. Agglutination tests with absorbed sera were carried out with dilutions from 1 in 2 to 1 in 128 (1/160 to 1/10,240 in terms of original serum).

RESULTS

Relationship of mean survival time to number of trypanosomes inoculated

Two experiments were performed in which tenfold dilutions of trypanosome suspension were inoculated intraperitoneally into groups of mice, and the mean survival times determined. Text-fig. 1 shows that the M.S.T. was inversely proportional to the number of trypanosomes injected. In one experiment all the mice were killed after receiving a 10^{-6} dilution (5·6 trypanosomes per dose) and in the other after receiving a 10^{-7} dilution (*c.* 1·3 trypanosomes per dose). The minimal lethal dose of this strain of *Trypanosoma brucei* was therefore probably one trypanosome.

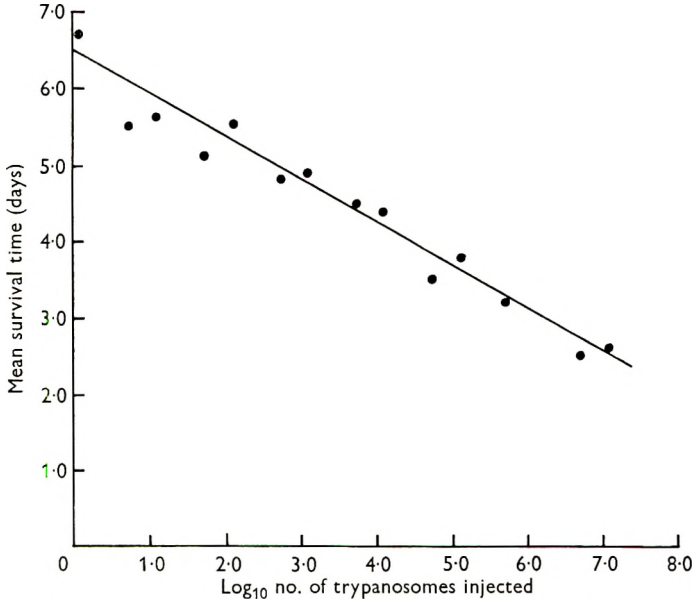
The best straight line drawn by eye through the points shows that a tenfold decrease in the number of trypanosomes inoculated extended the mean survival time by about half a day.

Cross-protection tests

Calculation of the standard errors of the mean survival times of eight randomly selected control groups of mice gave values ranging from 0·1 to 0·4 days. The means of these groups ranged from 3·2 to 4·1 days. A difference of 1 day or more between the mean survival times of the control group and the passively immunized group was therefore considered to have shown a highly significant level of protection by the antiserum.

Variant strains were derived sequentially from the parent strain. For example,

T2 was recovered from mice injected with T1 and protected by AT1; T3 from mice injected with T2 and protected by AT2, and so on. Sixty-seven cross-protection tests were carried out, in which antisera were tested against homologous and heterologous strains. Table 1 shows that the homologous serum conferred significant protection in every case. Protection against several strains was given only by the homologous serum, but some sera protected the mice against heterologous strains as well. The pattern of protection by antisera was different for every



Text-fig. 1. Regression of mean survival time of groups of five mice on $\log_{10}$ dose of *T. brucei* injected.

strain. With the exception of antiserum AT8, which gave 1 day's prolongation of M.S.T. against strain T9, antiserum against a given strain never gave protection against the next strain in the series, nor did it protect against the preceding strain in the series.

It is clear that in infected mice protected with a homologous serum death occurred 1–3 days later than in the unprotected controls. During this lag of 1–3 days the antigenic constitution of the protective antigen in the trypanosome population in the protected mice changed. This antigenic change could have arisen either by selection of an antigenically different mutant, or by adaptive alteration in the antigenic structure of the injected population. That the latter possibility is unlikely is shown by the following experiment:

Tenfold dilutions of trypanosomes were injected into groups of five mice, all of which received also 0·2 ml. of homologous antiserum. Table 2 shows that the serum gave complete protection against approximately 10,000 trypanosomes, or 10,000 minimal lethal doses, five of five surviving 14 days in one experiment and three of five surviving in the other. In the first experiment three of five mice were protected against about 100,000 trypanosomes, and in the second there were no

survivors after the same dose. The significance of these results is dealt with in the discussion section.

All the survivors of these experiments were challenged with the same strain 14 days after the original inoculation, each mouse receiving 10^6 trypanosomes. All the mice died at the same time as the controls, so that the mice had failed to develop immunity.

Table 1. *Cross-protection tests*

(Extension (days) of mean survival time by antiserum).

Anti-serum against strain	Trypanosome strain								
	1	2	3	4	5	6	7	8	9
1	2·0	0	0·18	0	1·0	0·8	1·2	3·1	1·5
2	0·5	1·8	0·6	0	1·0	0·4	0·4	0·7	0·1
3	2·0	0·3	1·4	0	1·1	0·3	0·6	0·4	0·5
4	0	–	0	1·3	0	0·3	0·2	0	0·4
5	0·8	–	0·6	0	2·0	0·3	0·6	0	0·2
6	0	–	–	0	0·2	2·4	0·7	0·1	0·2
7	0·2	–	–	0	0	0	1·4	0	0·2
8	0·3	–	–	0	1·3	0	0	1·9	1·0
9	0	–	–	–	–	0·4	–	–	1·1

– = not done.

Table 2. *Effect of dose of trypanosomes on protection by antiserum*

T 1/17		T 1/20	
Log_{10} dose	Survivors	Log_{10} dose	Survivors*
4·95	3/5	5·11	0/5
3·95	5/5	4·11	3/5
2·95	5/5	3·11	5/5
1·95	5/5	2·11	5/5
0·95	5/5	1·11	5/5

* All survivors challenged with 10^6 T 1/20 i.p. 2 weeks after first injection; all died at same time as normal controls.

Cross-agglutination tests

All strains were agglutinated to high titre by the homologous serum. An antiserum against a given strain generally failed to agglutinate the preceding and succeeding strains in the series. Cross-reactions were more frequent than in the protection tests (Table 3). Three strains (T 2, T 4 and T 6) gave rise to highly specific antisera.

Relation between agglutination and protection

The agglutinating and protective powers of the antisera are compared in the correlation diagram (Text-fig. 2) which is derived from Tables 1 and 3. In this diagram the agglutinating titre and extension of M.S.T. are charted for every combination of serum and strain on which both kinds of observation were made. The diagram shows that a serum with an agglutinating titre of less than 1/320 for a given strain failed to protect against that strain. Of thirty-six combinations of

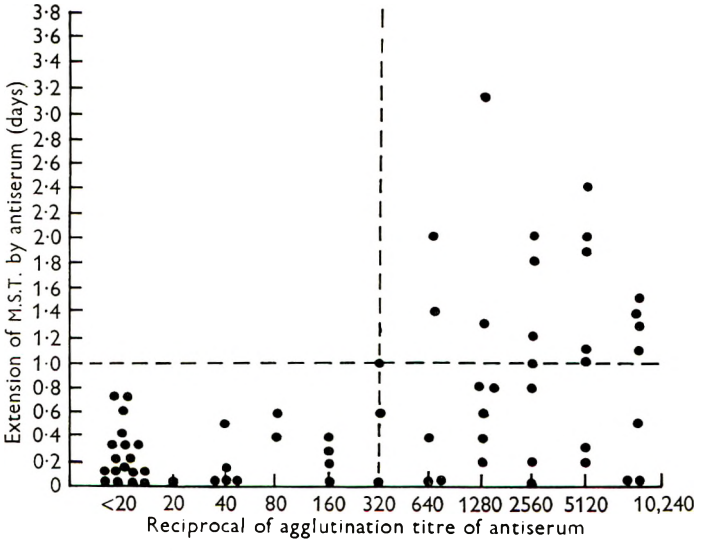
serum and trypanosome strain in which the agglutinating titre of the serum was 1/320 or greater, eighteen showed extension of the mean survival time of 1 day or more. Thus, all the sera which protected mice had high agglutination titres for the strain against which they protected, and none of the sera with low agglutination titres for a particular strain conferred protection against that strain.

Table 3. *Cross-agglutination by antisera*

Anti-serum against strain	Trypanosome strain										
	1	2	3	4	5	6	7	8	9	2a	9a
1	2,560	20	1,280	160	5,120	2,560	2,580	1,280	10,240	1,280	20
2	40	2,560	< 20	< 20	320	160	80	< 20	< 20	< 20	< 20
3	640	< 20	640	< 20	5,120	160	1,280	1,280	10,240	< 20	< 20
4	< 20	< 20	< 20	1,280	< 20	< 20	< 20	< 20	< 20	< 20	< 20
5	1,280	–	320	< 20	5,120	< 20	80	2,560	2,560	< 20	< 20
6	40	–	< 20	< 20	< 20	5,120	< 20	40	160	< 20	< 20
7	1,280	–	–	640	10,240	< 20	10,240	40	5,120	< 20	80
8	5,120	–	–	320	10,240	640	40	5,120	2,560	< 20	40
5	10,240	–	–	–	5,120	640	2,560	–	10,240	< 20	< 20
2a	< 20	–	–	–	< 20	–	–	–	–	10,240	–
9a	< 20	–	–	–	< 20	–	–	–	–	< 20	5,120

Note. Strain 2a from mice given T 1 and protected with AT 1. Strain 9a from mice given T 8 and protected with AT 1.

– = not done.



Text-fig. 2. Correlation diagram showing the relationship between the agglutinating titre of an antiserum for a given strain, and its ability to protect against that strain.

Cross-absorption tests

The results of these tests were erratic, in that a given strain sometimes failed to reduce the agglutination titre against itself. Some reactions in which an unequivocal result was obtained are set out in Table 4. It is clear that the antibodies in AT 1 which agglutinated T 1 and T 3 were removed by T 1 and T 3 and the

antibodies in AT3 which agglutinated T1 and T3 were removed by T3. The antibody in AT1 which agglutinated T1 is therefore identical with the antibody agglutinating T3. This would suggest that T1 and T3 were antigenically identical were it not for the fact that AT1 agglutinated strain T2a, while AT3 did not. Furthermore, T3 removed from AT3 the antibody agglutinating T9, but did not remove it from AT1, indicating that the antibody in AT1 which agglutinated T9 differed from the antibody in AT3 which agglutinated T9. It is also apparent that the antibodies agglutinating T1 and T3 differed from the antibodies agglutinating T5, T8 and T9. The cross-agglutination reactions were therefore not due to antigenic identity or even to the possession of common major (i.e. absorbing) antigens. Population T1, for example, must have contained several different antigens of which some were minor (i.e. giving sera of high agglutination titre but failing to absorb) and some were major (i.e. giving sera of high agglutination titre and absorbing).

Table 4. *Agglutination titres of absorbed antisera*

Antiserum	Absorbed with	Titrated against	Agglutination titre: Before absorption	Agglutination titre: After absorption
AT1	T1	T1	2,560	160
		T3	1,280	160
		T5	2,560	2,560
		T8	1,280	1,280
		T9	10,240	10,240
AT1	T3	T1	2,560	160
		T3	1,280	160
		T5	2,560	1,280
		T8	1,280	1,280
		T9	10,240	10,240
AT3	T3	T1	640	160
		T3	640	160
		T5	5,120	2,560
		T8	1,280	1,280
		T9	10,240	640

Antigenic variation in the absence of antiserum

Mice failed to develop immunity to *Trypanosoma brucei*. The development of new antigens in a trypanosome population growing in unprotected mice would thus provide evidence that the variation was caused by random mutation. In order to test this possibility a highly specific strain was used, designated T2a. This strain was isolated from mice protected with AT1 and challenged with T1, and differed from strain T2, which was derived from the same mixture of AT1 and T1 on another occasion. The antigenic structure was specified by the agglutination pattern, using all available antisera at a dilution of 1/320. The parent strain was agglutinated only by AT1 and AT2a (Table 5). Approximately 10^6 trypanosomes of this strain were injected into each of three mice. Three days later, when the mice were sick, blood from each mouse was diluted 1/10 in heparinized storage medium. The three mice had approximately 10^9 trypanosomes per ml. of blood at

this time. The agglutination reactions of each trypanosome population were then determined. One population ($T2a_1$) gave the same pattern as the parent. The second population ($T2a_2$) had developed a new antigen and was agglutinated by AT3. The third population ($T2a_3$) was agglutinated by every antiserum except AT8, so that it too had developed a new antigen, or new antigens. It should be noted that none of the three populations had lost their agglutinability with AT2a.

Table 5. *Development of new antigens in the absence of antiserum*

	Antiserum (1/320) against strains										
	1	2	2a	3	4	5	6	7	8	9	9a
Parent strain	+	−	+	−	−	−	−	−	−	−	−
Strains derived after a single passage in a mouse: 1	+	−	+	−	−	−	−	−	−	−	−
2	+	−	+	+	−	−	−	−	−	−	−
3	+	+	+	+	+	+	+	+	−	+	+

Note. + = agglutination; − = no agglutination.

Estimation of 'mutation rate' of antigenic structure

It has been shown that if mice are injected with a trypanosome strain and homologous antiserum the trypanosomes present in the mouse at death are antigenically different from the parent strain in that the original antiserum will no longer protect against them or agglutinate them. An attractive explanation for this would be that the original population contained a small proportion of trypanosomes carrying protective antigens (i.e. antigens giving rise to protecting antibodies) for which there was no corresponding antibody in the homologous antiserum. Suppression of the parent population by homologous antiserum would cause these variants to be selected to kill the mouse. The M.S.T. of a group of protected mice would, on this model, be determined by the number of variants present in the inoculum, and an estimate of that number could be obtained from the dose–response curve (Text-fig. 1). It will be argued in the discussion section that this model, of mutation and selection, is more likely to be correct than the alternative, of adaptational change in antigenic structure under the influence of antiserum.

An experiment was devised to give an estimate of the 'mutation rate per organism per division cycle' (Luria & Delbrück, 1943) according to the proposed model. The assumptions underlying the experiment were as follows:

(1) Mutants are selected by antiserum in protected mice and are responsible for death of these mice.

(2) Under the experimental conditions of development of clones, no back-mutation occurs in the mutant clones.

(3) The growth rates of parent and mutant trypanosomes are the same.

Armitage (1952), on a deterministic model of bacterial mutation, derived the following equation for the proportion of mutants present at time 't' in a population showing single mutation, in which the growth rates of mutant and parent are the same:

$$\Psi = 1 - \frac{x_0}{x_0 + y_0} e^{-\vartheta t}, \qquad (1)$$

where Ψ = proportion of mutants at time t, x_0 = number of parent organisms at time 0, y_0 = number of mutants at time 0, g = constant representing rate of mutation, t = time. If the probability of a mutation is evenly distributed throughout the division cycle the 'mutation rate per organism per division cycle' (Luria & Delbrück, 1943) is

$$\Lambda g = \frac{g \ln 2}{a+g} \quad \text{per organism (Armitage, 1952),} \tag{2}$$

where a = constant representing the division rate, Λ = generation time of the organisms.

In the experiment 50 mice were injected with 0·1 ml. of a trypanosome suspension (T 1/17) diluted to contain approximately 10 organisms per ml. Six days later, when most of the mice were sick or dead, ten sick mice were bled separately into tubes containing 200 i.u. of heparin. The trypanosomes in each of the ten samples of blood were counted, and an estimate of the total number of trypanosomes in the whole mouse was obtained by multiplying the count per ml. by an arbitrary factor of 4. The remaining 40 mice were allowed to die, and their M.S.T. was 6·3 days. This corresponds to a dose of about 3 trypanosomes in the dose–response curve (Text-fig. 1). The calculation of a was carried out on the assumption that each of the ten trypanosome populations was derived from a single organism, using the equation

$$x = x_0 e^{at}, \tag{3}$$

where x = number of trypanosomes at time t in the mouse in which the clone developed, $x_0 = 1$, $t = 144$ hr. (6 days). Each clone was then diluted so that 0·1 ml. contained approximately 10^6 trypanosomes, and 0·1 ml. of the diluted clone was injected into each of ten mice. Thirty minutes later, five mice in each group were passively immunized with 0·2 ml. of antiserum (AT 1). The M.S.T.'s of the protected and unprotected mice were determined, and the $\log_{10}$ doses corresponding to the M.S.T. determined from Text-fig. 1.

The proportion of mutants, Ψ, present in the original clone was calculated as shown in Table 6. The value of g was determined from equation (1), by taking $y_0 = 0$, and rearranging, and the mutation rate per trypanosome per division cycle was calculated from equation (2). The generation time, Λ, was also calculated, using equation (2), and the values obtained ranged from 4·4 to 5·4 hr. The mean value of the 'mutation rate' was found to be $10^{-5.17}$ mutations per trypanosome per division cycle, or about 1 in 150,000.

DISCUSSION

Two alternative explanations are possible for the change in protective antigen under the influence of antiserum. The first is mutation and selection, and the second adaptation. In the experiments summarized in Table 2, populations of 10^4, and, in three of five cases, of 10^5 trypanosomes, failed to modify their antigens so as to evade the protective action of the antiserum. Since the minimal lethal dose of trypanosomes was of the order of one trypanosome it is clear that, in the mice surviving the larger dose, less than 1 in 10^5 trypanosomes modified their

Table 6. *Estimation of 'mutation rate' of protective antigen in* Trypanosoma brucei

Clone no.	No. of trypanosomes/mouse in the parent population $= x$ ($= 4 \times$ number of tryps./ml. of blood)	Rate of division $= a = \frac{\log x}{144 \log e}$	M.S.T. of unprotected mice (days)	Log_{10} trypanosomes inoculated into unprotected mice (from Text-Fig. 2) $= p$	M.S.T. of protected mice (days)	Log_{10} 'mutants' inoculated into protected mice (from Text-fig. 2) $= q$	Log_{10} proportion of 'mutants' in original population $= q - p = \log \Psi$	Ψ	$= -\frac{g}{\log (1-\Psi)}{144 \log e}$	Mutation rate per trypanosome per division cycle $= \frac{g \text{ in } 2}{a+g}$	Generation time in hr. $= \Lambda$
1	10^9	0·144	3·1	6·1	4·8	3·1	−3·0	0·001	$6{\cdot}9 \times 10^{-6}$	$3{\cdot}3 \times 10^{-5}$	4·8
2	$4{\cdot}8 \times 10^9$	0·155	2·7	6·8	5·3	2·2	−4·6	0·00003	$3{\cdot}2 \times 10^{-7}$	$1{\cdot}4 \times 10^{-6}$	4·4
3	$3{\cdot}8 \times 10^9$	0·154	3·1	6·1	5·3	2·2	−3·9	0·00013	$9{\cdot}6 \times 10^{-7}$	$4{\cdot}3 \times 10^{-6}$	4·5
4	$9{\cdot}2 \times 10^8$	0·144	2·9	6·5	5·2	2·4	−4·1	0·00008	$6{\cdot}4 \times 10^{-7}$	$3{\cdot}1 \times 10^{-6}$	4·8
5	$3{\cdot}0 \times 10^9$	0·150	3·3	5·7	5·6	1·6	−4·1	0·00008	$6{\cdot}4 \times 10^{-7}$	$3{\cdot}1 \times 10^{-6}$	4·8
6	$3{\cdot}6 \times 10^8$	0·137	3·3	5·7	5·3	2·2	−3·5	0·00031	$2{\cdot}1 \times 10^{-6}$	$1{\cdot}1 \times 10^{-5}$	5·4
7	$4{\cdot}8 \times 10^9$	0·155	3·1	6·1	5·3	2·2	−3·9	0·00013	$9{\cdot}6 \times 10^{-7}$	$4{\cdot}3 \times 10^{-6}$	4·5
8	$2{\cdot}4 \times 10^9$	0·150	2·7	6·8	5·3	2·2	−4·6	0·00003	$3{\cdot}2 \times 10^{-7}$	$1{\cdot}4 \times 10^{-6}$	4·4
9	$5{\cdot}2 \times 10^9$	0·156	2·7	6·8	5·3	2·2	−4·6	0·00003	$3{\cdot}2 \times 10^{-7}$	$1{\cdot}4 \times 10^{-6}$	4·4
10	$2{\cdot}9 \times 10^9$	0·151	3·1	6·1	5·3	2·2	−3·9	0·00013	$9{\cdot}6 \times 10^{-7}$	$4{\cdot}3 \times 10^{-6}$	4·5

Mean value of mutation rate per trypanosome per division cycle = $10^{-5{\cdot}7}$

protective antigen. The frequency of 'adaptation', in these trypanosomes, was less than 10^{-5}. In Table 2, column 9, it is seen that, when about 10^6 trypanosomes were injected into mice, the frequency of adaptation (on the adaptational hypothesis) was of the order of 10^{-3} to 10^{-4}. Thus, if the hypothesis of adaptation is correct, the frequency of adaptation in a trypanosome population in the presence of antiserum is a function of the number of trypanosomes in the population, which means that the probability that a single trypanosome will adapt is not independent of adaptation in other trypanosomes. It seems unlikely that the process of adaptation in a single trypanosome could influence the ability of other trypanosomes to adapt. An 'adaptation-producing factor' would have to be postulated, which would be released as a result of adaptation, to confer the ability to adapt on other trypanosomes. The adaptation hypothesis therefore requires complex subsidiary hypotheses in order to explain the experimental findings. Furthermore, in Table 5 it is seen that variation in the agglutinating antigen can occur in the absence of antiserum. If the agglutinating antigen is the same as the protective antigen, as will be argued below, then variation in protective antigen occurs in the absence of antiserum. The balance of evidence therefore strongly favours the mutation and selection hypothesis.

Variation in the protective antigen was accompanied by variation in the agglutinating antigen, and the simplest explanation of this is that both antigens are the same. However, the correlation diagram (Text-fig. 2) shows that, while high protective capacity of an antiserum is associated with high agglutinating titre, the inverse correlation of high agglutinating titre with high protective capacity is less marked, in that only half the sera with agglutinating titres greater than 1/320 gave significant protection against the corresponding strain. Reference to Tables 1 and 3 will show that high agglutination titre and high protective capacity were associated in the nine paired reactions between a trypanosome strain and the homologous antiserum. A plausible explanation (assuming that protective and agglutinating antigens are the same) for the failure of some agglutinating antisera to protect in heterologous reactions can readily be devised. For successful performance of agglutination tests it was repeatedly observed that not much less than 10^6 trypanosomes were required for each drop of antiserum dilution; when 10^5 were used, no agglutination occurred because collisions were too infrequent. Suppose 90% of the members of a trypanosome population contained an antigen A, and 10% an antigen B, and the paired reaction was carried out with an antiserum containing only antibodies to A. Agglutination would occur, since very nearly 10^6 trypanosomes carrying antigen A would be present in the test. In the protected mice trypanosomes with antigen A would be destroyed and the effective dose of trypanosomes in these mice would have been reduced tenfold. Text-fig. 1 shows that a tenfold reduction of the inoculum extended the M.S.T. by about half a day. It is clear that in such a situation a serum would show high agglutinating power and low protective capacity. If the two antigens are not the same one would expect many sera to show low agglutinating power and high protective capacity, but in thirty-one tests this did not occur once. The results therefore support the view that the protective antigen is the same as the agglutinating antigen.

What is the mechanism underlying antigenic variation? The value of the mutation rate required to account for the experimental findings was $10^{-5.17}$ per trypanosome per division cycle, which is a high but not extraordinary rate. The number of possible variants is high, eleven having been produced in these experiments and twenty-two by Ritz (1914). It is reasonable to suppose that there must be a limit to the possible number of antigenic variants, but the determination of this number could be a thankless task. For example, proof of the existence of 100 different variants, using the methods described in this paper, would necessitate at least 10,000 cross-protection tests, using at least 100,000 mice. If to this is added the fact, deduced from the data in Table 4, that a given trypanosome population contains several different antigens, the prospect of the investigations required is sufficiently awesome to terrify even the most dedicated research worker. It should be borne in mind that a large number of different antigens could, in theory, be produced by a fairly simple underlying genetic mechanism. Suppose, for example, that in the gene locus determining the structure of the surface antigen there are n 'unstable' sites each of which determines a different portion of the antigen, and each of which may exist in one of two forms such that the two forms of the determinant at a given site differ from the two forms at all the other sites. Alternation between the two forms of determinant (i.e. mutation) at a site might occur randomly, with a frequency of about 10^{-5} mutations per trypanosome per division cycle, and independently of mutations at the other sites. The surface antigen of a single trypanosome could then exist in 2^n different forms. Thus, 1024 variants could be produced from as few as ten unstable determinants.

SUMMARY

Variation in agglutinating and protective antigens was studied in a strain of *Trypanosoma brucei* growing in mice protected with anti-trypanosomal rabbit serum. It was concluded that the agglutinating and protective antigens were the same, and that variation in the antigenic structure of trypanosome populations exposed to antiserum was due to mutation and selection rather than to adaptation. The mutation rate per organism per division cycle required to account for the experimental findings on the mutational hypothesis was found to have a mean value of $10^{-5.17}$.

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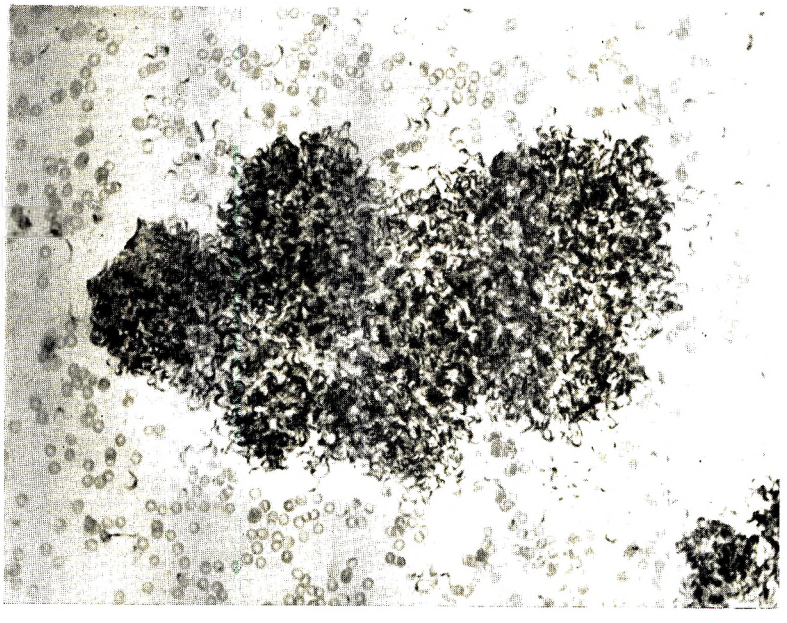
EXPLANATION OF PLATE

Clump of agglutinated trypanosomes. (May–Grünwald/Giemsa. × 200.)



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Studies on the pathogenesis of rinderpest in experimental cattle

I. Correlation of clinical signs, viraemia and virus excretion by various routes

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Very little information is available on the pathogenesis of rinderpest in any domestic animal or on the routes and duration of virus excretion; such data as exist (see Curasson, 1932, 1942) are usually fragmentary and qualitative. The recent development of tissue-culture techniques, for the quantitative recovery of virulent strains of rinderpest virus from animal tissues, has made it possible to re-investigate these problems in a systematic, though economical, manner. Studies of this type have a practical significance in indicating the optimal time for collection of materials for diagnostic purposes. They also assist in reaching sound conclusions on the epizootiology of the disease and have a comparative value for those interested in infections caused by the closely-related agents of human measles and canine distemper.

The first paper of this series describes the results of experiments designed to elucidate the relationships between viraemia, clinical signs and virus excretion in cattle infected with a single field isolate of moderately high virulence.

MATERIALS AND METHODS

(1) *Virus*

The virus strain, RGK/1, was isolated in primary calf kidney monolayers from the spleen and lymph nodes of a reticulated giraffe (*Giraffa reticulata*; de Winton) which was shot in the Northern Frontier District of Kenya in January 1962. This animal was one of many of the same species which succumbed to the infection, after showing typical clinical signs of rinderpest; cattle in the same area had been immunized by vaccination and did not have the disease but warthogs (*Phacochoerus aethiopicus* Pallas) died in large numbers, presumably from the same infection (W. Plowright, unpublished).

After a second passage in calf kidney cells the virus was inoculated into cattle to confirm its identity, re-isolated in tissue culture and then returned to three cattle. The spleen of one of these animals (no. 8186) was harvested on the 3rd day of fever and small pieces of the pulp were stored frozen at $-70°$ C. In a cattle

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titration of one sample of this material, sixteen of nineteen reacting cattle, i.e. 84 %, died. For use in these experiments a 10^{-1} or 10^{-2} (w/v) suspension of frozen spleen was prepared in culture maintenance medium and titrated by the technique described by Plowright & Ferris (1962), using primary calf kidney cells as the assay system. A few cattle were infected with virus in stored spleen fragments derived from another animal (no. 8994), which had been inoculated with spleen from ox no. 8186 and killed on the 4th day of pyrexia. The dose of virus administered to all animals was calculated from the results of simultaneous titrations in tissue cultures (see Table 1).

Table 1

Expt. no.	Ox no.	Route of infection	Dose of virus (TCD 50)	Incubation period (days)*	Duration of pyrexia*	Day of death
I	8361	Intranasal	$10^{5\cdot1}$	4	6	Recovered
	8362	Intranasal	$10^{5\cdot1}$	4	5	Recovered
	8363	Contact	N.D.†	8	6	Recovered
II	8918	Subcutaneous	$10^{3\cdot1}$	4	6	6/7
	8826	Subcutaneous	$10^{3\cdot1}$	4	5	6/7
III	8960	Intranasal	$10^{3\cdot0}$	4	6	Recovered
	8961	Intranasal	$10^{3\cdot0}$	4	5	7/8
	8920	Contact	N.D.	8	8	8/9
IV	8923	Subcutaneous	$10^{2\cdot1}$	5	4	Recovered
	8922	Contact	N.D.	9	9	11/12
	8985	Contact	N.D.	11	6	Recovered
V	8995	Subcutaneous	$10^{4\cdot7}$	3	6	Recovered
	8996	Subcutaneous	$10^{4\cdot7}$	3	5	8/9
	8997	Contact	N.D.	9	6	7/8
VI	9123	Subcutaneous	$10^{5\cdot1}$	3	4	6/7
	9124	Contact	N.D.	8	6	Recovered
VII	9153	Subcutaneous	$10^{5\cdot3}$	3	5	Recovered
	9154	Subcutaneous	$10^{5\cdot3}$	3	5	Recovered
	9134	Contact	N.D.	8	8	9/10
	9136	Contact	N.D.	8	6	7/8
VIII	9159	Intranasal	$10^{4\cdot9}$	3	6	6/7
	9162	Intranasal	$10^{4\cdot9}$	3	5	6/7
	9156	Contact	N.D.	8	24	Recovered
	9163	Contact	N.D.	9	6	Recovered
	9165	Contact	N.D.	9	9	Recovered

* Calculated from the day with first morning temperature > 102° F. For contact cases estimated from the time of first exposure to a viraemic donor.

† N.D. = Not determined.

(2) *Experimental animals and their infection*

The majority of the twenty-five cattle used were 2-year-old grade steers, with a varying though usually high percentage of the blood of such European breeds as the Ayrshire, Jersey, Guernsey and Friesian. All were housed in isolation units, which effectively prevented any transfer of infection from stall to stall. They were

fed on hay only, with water provided *ad lib.* No therapeutic treatment was administered except when *Babesia bigemina*, a common protozoal parasite, was detected in blood smears.

The sera of all cattle were screened for rinderpest-neutralizing antibody and found to be negative before they were placed on experiment (Plowright & Ferris, 1961). Rectal temperatures were recorded every morning before 9 a.m., beginning several days before virus exposure.

Eight separate experiments were performed, each being conducted in a single stall and utilizing two to five cattle (Table 1). Of twenty-five animals, eight were inoculated subcutaneously on the side of the neck, whilst six received 2 ml. of spleen suspension by the intranasal route. In the latter case the inoculum was dropped slowly into both nostrils with the head held in such a manner that the material flowed quickly over the turbinates towards the nasopharynx; great care was taken to avoid mechanical injury to any of the mucosae. The majority of cattle which were infected by stall contact with inoculated animals (Table 1), were first introduced 2 days after the latter had received virus. In this way accidental infection, by ingestion or inhalation of spleen virus, was avoided. In Expt. VIII, cattle nos. 9163 and 9165 acquired the disease by contact with the reacting ox, no. 9156; they therefore represented the 2nd contact passage.

All cattle were subjected to a careful clinical examination every day from at least the time of onset of pyrexia. Particular attention was paid to the oral and nasal mucosae, where necrosis and/or erosion are such characteristic features of rinderpest.

(3) *Collection of materials from cattle*

Collections of blood, nasal secretion, urine and faeces were made almost daily from all animals before, during and for some days after the time when it was expected that virus would be present. Blood was taken from the jugular vein into one-third the final volume of a 1·5 % solution of EDTA (ethylene-diamine-tetra-acetic acid: disodium salt) in 0·7 % NaCl (see Plowright & Ferris, 1962). Serum for antibody determinations was also collected at intervals of 1–4 days, depending on the stage of the disease; these samples were stored frozen at about -20° C. and the results of their examination will be described in a later communication.

Nasal secretion was obtained by inserting one sterile cotton swab about 7–8 cm. into each nostril; the amount of material adhering to the swab naturally varied with the type of discharge in various phases of the disease. The two swabs from each animal were broken off immediately into a single 1 oz. screw-cap bottle, containing 2 ml. of culture maintenance medium, in which antibiotics had been incorporated at the following concentrations per ml.: sodium penicillin, 1000 i.u.; streptomycin sulphate, 1000 μg.; nystatin, 500 units; neomycin sulphate, 500 units, later replaced by kanamycin sulphate, 500 μg.

Urine was collected, as voided by the animal, into an aluminium vessel about 20 cm. in diameter. Sometimes, in advanced stages of the disease, cattle became recumbent and severely dehydrated; in these cases urine could not be obtained easily and no sample was examined.

Faeces were also collected by the attendants into similar aluminium vessels, from which 0·5 to 1·5 g. was removed by a sterile tongue-depressor for transfer to weighed, empty screw-cap containers.

(4) *Further treatment of materials*

Samples of 10 ml. of blood-anticoagulant mixture were processed in the manner already described (Plowright & Ferris, 1962) to produce the same volume of a crude leucocyte suspension in maintenance medium. This was regarded as equivalent to undiluted blood and was distributed in a dose of 2 ml. to each of five tubes of primary calf kidney (BK) cells. Tenfold dilutions of blood-anticoagulant mixtures were prepared directly in maintenance medium and these were also inoculated in a dose of 2 ml. to each of five tubes.

All the excretions were treated by techniques designed to produce suspensions in which: (*a*) any toxicity for cultured cells had been eliminated or reduced, (*b*) bacterial, fungal or yeast contamination had been reduced to a minimum, and (*c*) the maximum possible quantities of virus were retained in a uniformly distributed state.

Nasal swabs were exposed for half an hour at room temperature to the concentrated antibiotic solution and then expressed with sterile forceps to yield about 1·5 ml. of crude extract. To this was added 10·5 ml. of normal maintenance medium, after which the mixture was pipetted vigorously and treated for 5 min. in an ultrasonic bath* containing iced water. This served to break up floccules of mucus and any desquamated cells. The resulting suspension was arbitrarily designated as a 10^{-1} (v/v) dilution of nasal secretion but the true dilution factor was probably considerably higher.

Of the urine samples, 1·2 ml. volumes were mixed with an equal volume of concentrated antibiotic solution, which contained phenol red and served to give an approximate indication of the pH of the mixture. Wide divergencies from neutrality were then corrected by the drop-wise addition of 1 N-NaOH or 1 N-HCl. Not infrequently two to three drops of acid were necessary to neutralize the normal pre-reaction alkalinity of urine and two to three drops of alkali to counteract the acidity observed during the middle to late course of the disease. The change of pH of the urine in rinderpest-infected cattle has been known for a very long time (Marcet, 1866; Curasson, 1932).

Faeces samples were suspended in maintenance medium with concentrated antibiotics to give a concentration of 10^{-1} (w/v). After vigorous shaking the suspensions were left for 0·5–1 hr. at room temperature and then subjected to 5 min. ultrasonic treatment, as already described for nasal secretions. Large particles were allowed to sediment for several minutes on the bench and the supernatant was finally diluted tenfold in normal maintenance medium to give a 10^{-2} (w/v) suspension of faeces. This was the most concentrated preparation which was ordinarily found to be of low toxicity for BK cells. It is important to note

* 'Soniclean' Transducer Tank, Type 1160/T24, 41.23 KC/S, Dawe Instruments, Ltd., London.

that centrifugation procedures could not be employed readily for faecal virus isolation, in view of the large size of the virus (Plowright, Cruickshank & Waterson, 1962) and its probable association with particles of mucus, blood or desquamated mucosal cells.

Further tenfold dilutions of the various excretions were prepared as necessary from the basic 10^{-1} or 10^{-2} suspensions and all were inoculated in a dose of 2 ml. into each of five tubes of BK cells from which all the previous medium had been discarded.

(5) *Preparation and maintenance of cell cultures*

Primary calf kidney cultures were prepared in tubes of 160 × 15 mm. dimensions. Details of the technique, the growth and maintenance media and the frequency of medium changes have already been given (Plowright & Ferris, 1962). The normal maintenance medium contained one-tenth the concentration of antibiotics mentioned in subsection (3); Kanamycin was found to be particularly useful in controlling the growth of otherwise resistant bacteria.

Cultures were inoculated at 4–11 days after seeding, the best results being obtained with cells not more than 1 week old. After inoculation all tubes were transferred to rollers, rotating at about 8 rev./hr. Debris from the inocula was removed after 24 or 48 hr. by three washes with 2–3 ml. of prewarmed P.B.S. (Dulbecco & Vogt, 1954). The shorter period of exposure was found to be an advantage, in that cytotoxicity and microbial contamination was thereby reduced in tubes inoculated with nasal secretion and faeces.

Microscopic examination was carried out at frequent intervals from the 3rd day post-inoculation and all cultures showing cytopathic changes typical for rinderpest (Plowright & Ferris, 1959, 1962) were discarded. Final observations were made on the 10th or 11th days and 50 % end-point titres were calculated by the method of Thompson (1947). In the case of blood these were expressed as $\log_{10}$ TCD50 per 2 ml., in all other instances as $\log_{10}$ TCD50 per ml. or per g.

(6) *Evaluation of the results*

Ultrasonic treatment probably had little or no effect on virus in the excretions, since it was known that culture virus, under identical conditions of exposure, had a half-life of 7·5 min. (Plowright, 1963*a*).

So far as completely negative results are concerned, it can be calculated in these instances that the virus was not detectable in: (*a*) 10 ml. of blood-EDTA mixture equivalent to 6·6 ml. of undiluted blood; (*b*) in 10 ml. of the basic dilution of nasal secretion arbitrarily designated as 10^{-1}; (*c*) in 1 ml. of urine, and (*d*) in 0·1 g. of faeces.

Where only one or two tubes became positive, out of five inoculated with the basic dilution of an excretion, the result was expressed as a 'trace'. This was also related to the same quantities of material as detailed above for completely negative results. Where three or more tubes were infected out of five used for the basic dilution, a titre was calculated on the assumption that all tubes would have become positive in the next lowest of a tenfold dilution series.

To minimize the effect of different incubation periods all viraemia and virus excretion data were related to the onset of pyrexia in the individual animal; for this purpose a morning temperature > 102° F. was regarded as abnormal.

RESULTS

Clinical signs

The incubation period, as determined by the first morning temperature of > 102° F., was 3–4 days in all cattle which were inoculated with $10^{3\cdot0}$ to $10^{5\cdot1}$ TCD 50 of virus; ox 8923, which received only $10^{2\cdot1}$ TCD 50, reacted on the 5th day (Table 1).

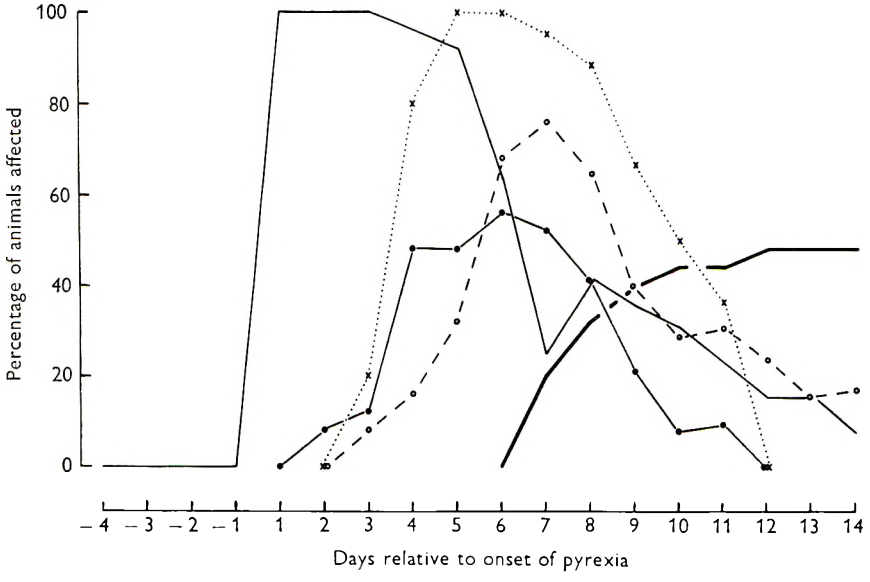

Fig. 1. Clinical signs in cattle infected experimentally with virulent rinderpest virus (strain RGK/1). —·—, Pyrexia (> 102·0° F.); × · · · ×, mouth lesions; ●—●, nasal lesions; ○– – –○, diarrhoea; ——, mortality.

The incubation periods for cattle infected by contact were calculated by assuming that the inoculated animals did not excrete virus before they were viraemic and that the time of exposure therefore began with the appearance of viraemia in any one of the donors. This assumption was justified by our data on virus excretion, to be described later; in fact, virus shedding was seldom detected before the first day of fever and the calculated incubation periods for contact infection can probably be regarded as maximal. As shown in Table 1 they ranged from 8 to 11 days, with a mean for eleven animals of 8·6 days.

The minimum duration of pyrexia was 4 days, the percentage of aninals with fever falling rapidly after the 5th day (Fig. 1); the mean rectal temperature reached a maximum of 104·8° F. on the 5th, falling to 102·8° F. on the 6th day. By the 7th day, eleven of thirteen cattle which eventually recovered had temperatures below 102° F. Nevertheless two of these eleven animals showed mild

relapses on the 8th and 9th days, and one had uninterrupted pyrexia for 24 days.

Nasal and ocular congestion with discharges of a serous or seromucoid type were commonly observed on the 2nd and 3rd days of the reaction, but these later became mucoid or mucopurulent. Necrosis and erosion of the visible parts of the nasal mucosae was first found on the 2nd day and the incidence of these lesions reached 50 % on the 4th day of pyrexia (Fig. 1 and Table 2). From the 7th day onwards regeneration of the epithelia occurred rapidly in cattle which survived and only one of thirteen animals showed nasal abnormalities as late as the 10th day of the disease. In general, therefore, nasal lesions appeared slightly before or simultaneously with those of the mouth cavity, but their incidence was reduced to about one-half and they disappeared more quickly.

Mouth lesions were usually preceded by 1 or 2 days of local or generalized hyperaemia of the mucosae. Characteristic raised, whitish foci of necrosis or small reddened erosions were first detected on the 3rd day and were present in all animals by the 5th day of the disease (Fig. 1). Predilection sites were the lips, gums and buccal papillae, especially near the corners of the mouth, together with the under-surface of the free part of the tongue; lesions of the hard palate often appeared 1–2 days later. Extensive confluent or semi-confluent necrosis was seen in twelve animals, beginning on the 6th day at the earliest. It is of interest to record here that seven of twelve cattle which died of the disease had numerous, rounded foci of yellowish papillary necrosis on the dorsal surface of the tongue. Such lesions were also beautifully depicted in the 1866 Cattle Plague report, and yet some recent publications (Maurer, Jones, Easterday & DeTray, 1955; Smith & Jones, 1957; Scott & Brown, 1961) state categorically that the anterior part of the dorsum of the tongue is never involved. Complete reconstitution of the mouth epithelia had occurred in all survivor cattle by the 12th day following onset of the disease.

'Diarrhoea' for the purpose of this paper was taken to mean the passage of fluid faeces containing varying quantities of mucus, blood or portions of desquamated intestinal mucosa. As shown in Fig. 1, this clinical sign was first recorded in a few animals on the 3rd or 4th days of pyrexia, but the proportion affected rose sharply on the 5th and 6th days, to reach 68 %. All the twelve cattle which died developed persistent diarrhoea, resulting in dehydration and, in the majority of cases, severe prostration during the 1 or 2 days before death.

In thirteen individuals diarrhoea developed 1–3 days (mean 1·5 days) after the first detection of oral lesions, but in one case it appeared on the day preceding and in three cases on the same day as oral necrosis and erosion. Hence, generally speaking, diarrhoea was the last of the major clinical signs of rinderpest to make its appearance (Fig. 1).

The first and heaviest mortality occurred on the 7th day but the peak figure of 48 % was reached on the 12th day following onset of the disease. There was no apparent relationship between the mortality rate and the route of infection; death occurred in five out of eleven animals infected by contact and in seven out of fourteen which were inoculated intranasally or subcutaneously.

Viraemia

Virus was first demonstrated in the blood of two animals on the 2nd day preceding the onset of fever. The time from inoculation to the detection of virus in the blood was 1–3 days (mean 2·5) in fourteen animals, whereas after contact exposure viraemia did not develop for 8–13 days (mean 9·2). On the day preceding first pyrexia, 76 % of the cattle had viraemia, with a mean titre of $10^{0·3}$ TCD 50 per ml. There was some indication that viraemia appeared later in animals infected by contact; thus, whereas all cattle infected by inoculation had viraemia on the 1st day of fever, four of eleven which acquired the disease naturally were found to be non-viraemic at this time. One further ox (no. 9124) did not have detectable quantities of virus in its blood until the 4th day of reaction.

As shown in Fig. 3, the peak mean titre for viraemia was $10^{2·3}$ TCD 50 per ml., attained on the 3rd day of the disease. It is necessary to point out, however, that the mean line, shown in Fig. 3, was depressed by the inclusion of results from many titrations in which, for various reasons, the end-point was not obtained. The highest blood titres recorded in individual animals were about $10^{4·0}$ TCD 50 per ml. (Fig. 3).

Of twelve animals which died, eight were viraemic until the day of death, i.e. 7–9 days after the onset of pyrexia; in three of them viraemia ceased 1, 2 and 5 days respectively prior to death, whilst in the remaining animal the test failed on the day preceding the fatal termination. In ten cattle which recovered and for which complete data were obtained, viraemia lasted 2–8 days, with a mean of 6·6 days.

Nasal excretion of virus

Virus was never recovered from nasal swabs taken on the day following inoculation, even in those animals which were infected by the intranasal route. Two animals showed viral excretion on the 2nd day preceding pyrexia, i.e. as early as circulating virus was detected, but the subsequent rate of development of nasal excretion was slower than the rate of increase of viraemia (Fig. 2). The nasal excretion rate rose to a maximum of 87·5 % on the 4th day and thereafter declined, steadily at first but later abruptly, so that by the 10th day virus was no longer demonstrable (Fig. 2). This latter time corresponded very well with the disappearance of viraemia, which was complete on the 9th day. It also recalled the disappearance of nasal erosion and necrosis, in all but one animal, by the 10th day. The exceptional animal was one which never excreted virus by the nasal route.

Fig. 4 shows that the greatest quantities of virus were present in nasal secretions on the 3rd to 7th days of the disease. Many swabs collected during this period yielded virus with a titre of $> 10^{4·0}$ TCD 50, and a few even exceeded $10^{5·0}$ TCD 50. The rapid decline in the nasal excretion rate after the 7th day was probably attributable, at least partially, to the death of severely affected animals. Thus, six of eight cattle which died on the 7th and 8th days, had a mean nasal-swab titre of $\geqslant 10^{4·7}$ on the day preceding death; but among fifteen of the seventeen survivors, which were successfully tested on the 8th day, only three showed nasal excretion.

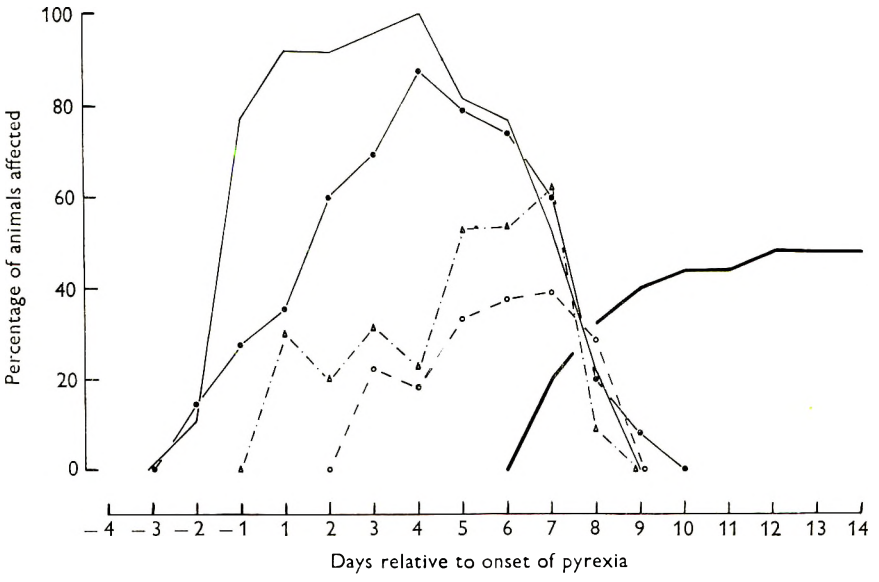

Fig. 2. Correlation of viraemia and virus excretion in cattle infected experimentally with virulent rinderpest virus (strain RGK/1). ——, Viraemia; ●—●, nasal excretion of virus; △—△, urinary excretion of virus; ○ – – ○, faecal excretion of virus; ▬▪▬, mortality.

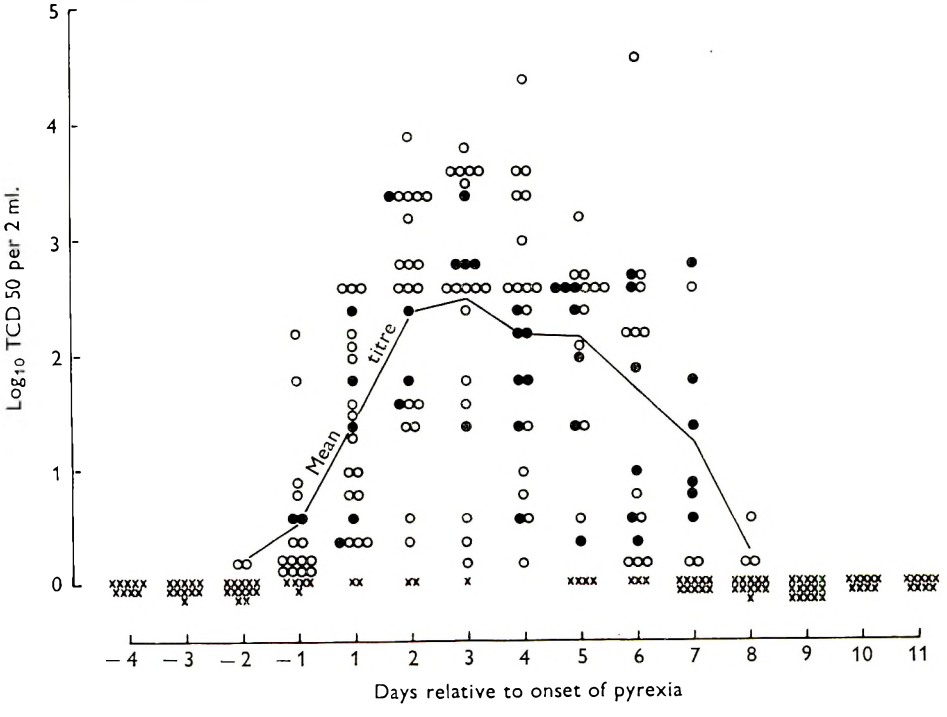

Fig. 3. Viraemia in cattle infected experimentally with virulent rinderpest virus (strain RGK/1). ●, End-point not obtained.

All but two of twenty-four animals (no. 8918 excluded) excreted virus at one time or another during the course of the infection; in four animals this was demonstrated on 1 day only, in eighteen animals on 2 or more days. Repeated sampling in the other two cattle (nos. 8922 and 9124) failed to reveal nasal excretion; both of these animals developed viraemia of short duration and low titre, although pyrexia was not less marked than in the remainder. One of them (no. 8922) died later, having developed diarrhoea and mouth lesions, the other (no. 9124) recovered after a mild disease course.

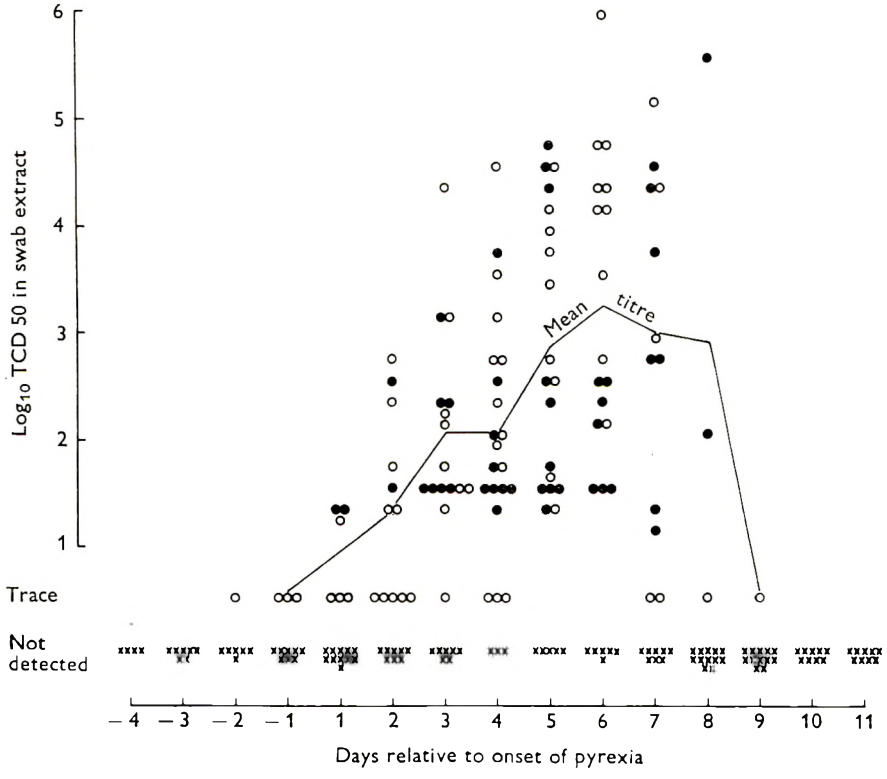

Fig. 4. Nasal excretion of virus by cattle infected with virulent rinderpest virus (strain RGK/1). ●, End-point not obtained.

Excretion of virus in urine

Traces of virus were first detected in the urine of two animals on the first day of fever, hence excretion by this route commenced 2 days after viraemia first became demonstrable. In this connexion, however, it is necessary to remember that the technique adopted utilized the equivalent of only 0·2 ml. of undiluted urine, whereas the method used to demonstrate virus in blood made use of leucocytes from 6·6 ml. of blood (see Materials and Methods).

The urinary excretion rate was in the region of 20–30 % during the first 4 days of the disease, during which time the mean titre did not change greatly (Figs. 2 and 5). On the 5th day, however, at a time when the amount of virus in the circulating blood was already beginning to decline, there was a sudden increase in

the proportion of animals (53 %) shedding virus in the urine and, on the 7th day of the illness, the excretion rate rose still further, to a peak of 62·5 %. The highest mean titre, of $10^{1.7}$ TCD 50 per ml., was attained on the 6th day. The abrupt decline of urinary excretion on the 8th day coincided with the rapidly mounting figures for mortality and the decline in those for viraemia and nasal excretion.

Of twenty-four animals for which satisfactory results were obtained, twelve shed virus in the urine on 2 or more days; in four animals virus was only detected on one day, while in the remaining eight cattle it was not demonstrated at all. To some extent this may have been due to the difficulties, already mentioned, in collecting urine from very sick, recumbent animals. Nevertheless, three animals which were tested regularly during the critical period and all of which recovered (nos. 8923, 8985 and 8995) were never found to excrete virus by this route; one of them (no. 8995) showed a moderately severe clinical course, including extensive mouth and nasal lesions together with diarrhoea.

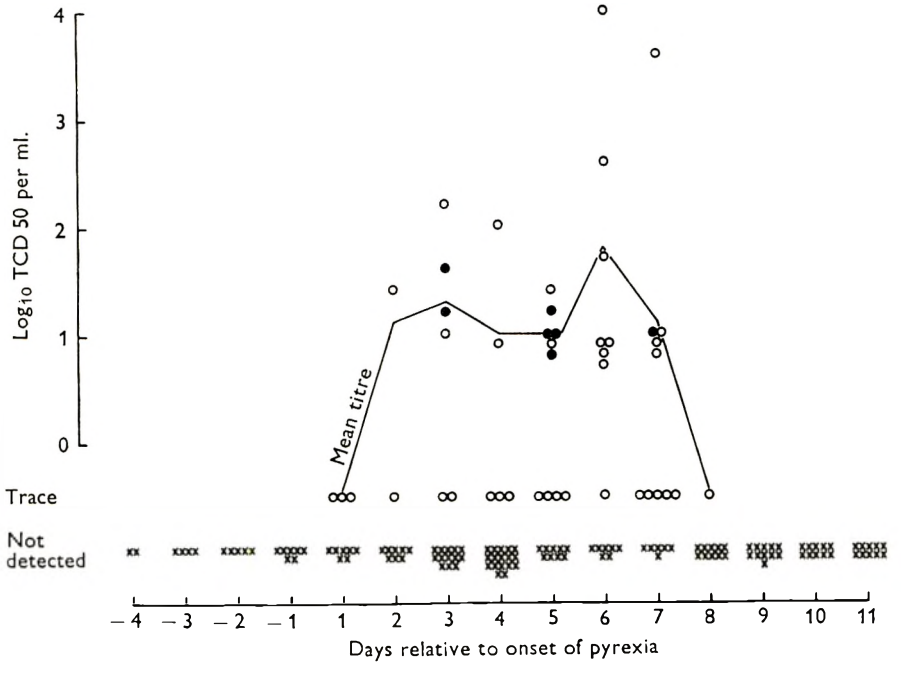

Fig. 5. Urinary excretion of virus by cattle infected with virulent rinderpest virus (strain RGK/1). ●, End-point not obtained.

Excretion of virus in faeces

Virus was first detected in faeces on the 3rd day of fever in two of nine cattle (nos. 8362 and 8363). Neither of these animals had diarrhoea at the time, although one of them (no. 8362) did develop it 2 days later. As shown in Fig. 1, diarrhoea was first seen in two animals on the 3rd day of pyrexia; in one of them (no. 8826) no virus was demonstrated in faeces taken on the same day, while in the other case (ox, no. 9123) cultures became contaminated. The latter animal did, however, excrete considerable quantities of virus in its faeces on the following day ($\geqslant 10^{3.2}$

TCD 50 per g.) and the high level was maintained until death on the 6th–7th days of the disease. Similarly, virus was found in the faeces of ox no. 8826, on the 5th, 6th and 7th days, the titre shortly after its death being $\geq 10^{4.2}$ TCD 50/g.

The faecal excretion rate rose to a maximum of 40% (7/18) on the 7th day of the disease but this was still considerably less than the maximal proportion (76%) of animals with diarrhoea (Fig. 1). The rapid decline on the 8th day in both the number of animals with diarrhoea, as also those showing faecal excretion of virus, was undoubtedly associated with the death at this time of many severely affected animals. On the 9th day four survivors still had diarrhoea but virus was no longer detectable; it was not recovered later from animals with prolonged enteric signs, which were tested continuously for as long as 16 days.

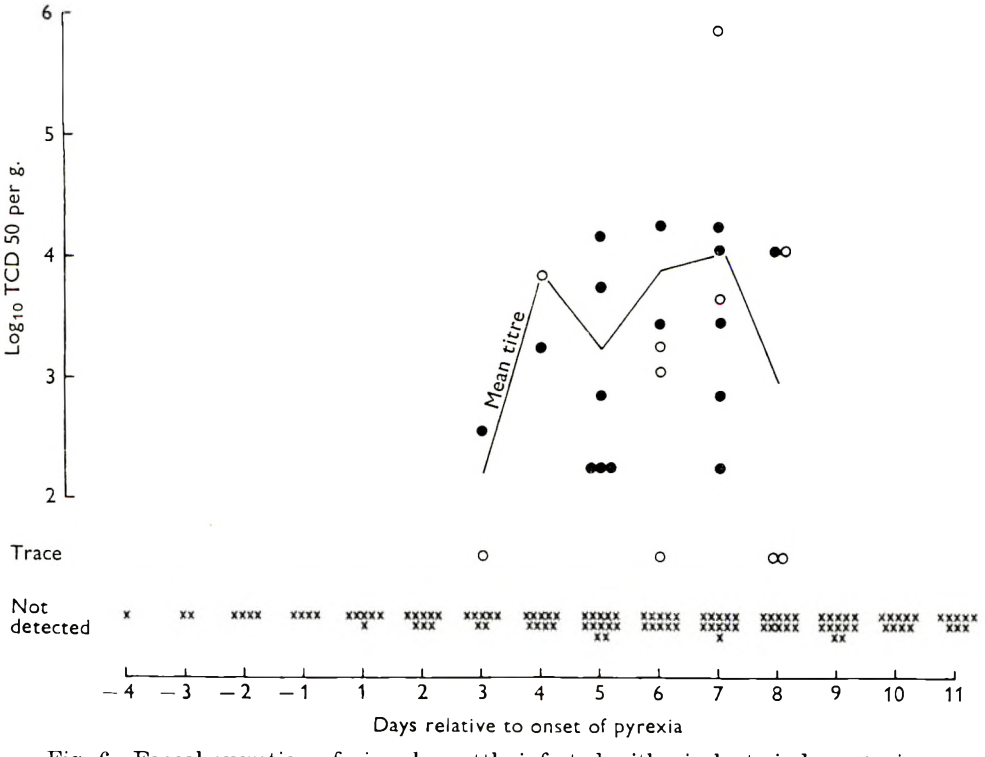

Fig. 6. Faecal excretion of virus by cattle infected with virulent rinderpest virus (strain RGK/1). ●, End-point not obtained.

Of nineteen cattle which exhibited diarrhoea fourteen were shown to excrete virus in the faeces at some time or another between the 3rd and 8th days of the disease. The results for many animals were irregular, since cytotoxicity of faeces or microbial contamination of the cultures terminated many faecal tests prematurely; in addition, the total quantity of faeces tested (0·1 g.) was perhaps too small to exclude low-level excretion. Nevertheless, several animals which developed diarrhoea were successfully tested virtually every day during the time when the clinical sign persisted and yet no virus was recoverable. It must be concluded

that, in these individuals, the total amount of virus excreted in the faeces could not have been great. The behaviour of these animals should be compared with others for which faecal titres were recorded of $> 10^4$ TCD 50, rising to $10^{6.2}$ TCD 50/g. (Fig. 6).

DISCUSSION

Much of the information on the disease course and clinical signs of rinderpest was acquired several decades or even more than a century ago. It has often been repeated in text-books without any reference to the original sources or the experimental methods employed. In the past, the necessity for using cattle as the only satisfactory virus-susceptible species, carried with it the danger of prior immunity in some of the experimental animals, and also the risk of contact infections amongst those which had been inoculated. Furthermore, the vast majority of these older observations were usually qualitative and at best semi-quantitative, whereas tissue culture techniques allow a quantitative approach throughout.

The virus strain used in our study can probably be regarded as typical of the more virulent field strains at present current in East Africa. It had undergone a maximum of two culture and three cattle passages following first isolation and had been given, therefore, very little opportunity for modification. Its virulence for East African grade cattle was considerably greater than that of many of the strains isolated in this laboratory during the past 3–4 years, from cattle or game animals (Plowright, 1963*b*; Plowright, unpublished observations). The difference between the 84 % mortality, observed in a titration of the spleen of ox no. 8186 (see Materials and Methods) and the 48 % death-rate in these experiments, may be attributable to variations in the genetic resistance of the comparatively small groups of animals which were employed. It was, in fact, fortunate that the clinical picture varied so markedly, since we were thereby enabled to study cases of different degrees of severity, ranging from very mild to rapidly fatal.

The use of three different routes of infection—i.e. subcutaneous and intranasal inoculation, or close contact with infected animals—made it possible to compare the course of events in the disease acquired by artificial or natural means. Although numbers were small the figures for mortality and death time did not suggest that there was any material difference between infection by inoculation and by contact. There was simply an elongation of the incubation period from a mean of 3·57 to 8·63 days, which may have been due to a smaller virus uptake on susceptible mucosae.

It is commonly asserted in the literature that natural infection of cattle usually takes place by the oral route (Edmonds & Walker, 1929; Curasson, 1932; Hutyra, Marek & Manninger, 1946; Hagan & Bruner, 1957) and yet there are numerous reports that the virus easily invades the body from the nasal mucosae (see, for example, Hornby, 1926; Hall, 1933) whilst drenching with virulent material frequently fails to set up the disease (Schein & Jacotot, 1925; Hornby, 1926). Our results with intranasal installation certainly agree with those of Hornby and Hall and suggest that this is the common natural route of infection with rinderpest.

It has been known for nearly 100 years that rinderpest virus appears in the blood at the time of the first rise of temperature to 102° F. or above (*Third Cattle Plague Report*, 1866, p. iv) and it is commonly held to persist throughout the period of early clinical reaction and diarrhoea. Plowright (1963*b*), in a study of recently isolated strains of low cattle pathogenicity, found that viraemia was detectable from the 2nd day before the onset of fever to the 3rd day after its subsidence, but in the majority of animals (82 %) virus was no longer demonstrable in the blood on the 2nd day of normal temperature. These results were essentially in agreement with those for survivor cattle in the experiments here described, but differ somewhat from those of Hall (1933) who found that viraemia in some animals persisted until the 4th or 5th days of normal temperature. They also conflict with the data provided by MacOwan (1956); he reported that the eclipse phase following a large parenteral dose of virulent, Kabete 'O' virus was only 1 day and that the duration of this phase was inversely proportional to the dose. The duration of viraemia was 13–14 days and independent of the size of the inoculum. Some of the apparent discrepancies may be related to the strains of virus employed and to the sensitivity of the techniques adopted for the detection of virus. Hall (1933), for example, inoculated 2 ml. quantities of blood subcutaneously into cattle; this is, quite possibly, a more sensitive technique than cultural isolation.

Curasson (1932), in a discussion of some earlier reports on the possibility of prolonged retention of virus by convalescent cattle, described how he had infected an ox with the blood of an animal whose temperature had returned to normal 37 days previously. This and other incidents he regarded as evidence for the occasional existence of virus 'carriers'. We obtained no indications from our viraemia or excretion studies that such a carrier state was established with the RGK/1 strain of virus in East African grade cattle.

Hornby (1926) stated that nasal discharge was infective as early as the second day of fever and within 5 days of inoculation; he further noted that a mucopurulent discharge was generally obvious at that time and suggested that the leucocytes contained therein were carriers of the virus. In addition, he reported that infectivity was no longer detectable 2–3 days after complete remission of the fever. Hall (1933) asserted that the nasal secretions were nearly always virulent on either the 2nd or 3rd days of thermal reaction and continued to be so until the end of pyrexia or even afterwards in severe clinical cases. He also found that the infectivity of nasal discharges could exceed that of blood collected simultaneously—in one experiment, for example, $10^{-2.7}$ ml. of blood was infective compared with $10^{-3.7}$ ml. of nasal exudate.

These observations are in close agreement with our own, although in a few animals we were able to recover virus from nasal swabs taken on the 1st and 2nd days preceding pyrexia. The constant disappearance of virus from the nasal secretions on the 2 days following its intranasal instillation, suggested that there was no primary local proliferation but that it was rapidly removed to the regional lymphoid tissues. The hypothesis has received support from further studies in this laboratory on the pathogenesis of rinderpest in the tissues of cattle (Plowright & Liess—to be published); it also does not conflict with the facts that nasal excretion

ılways followed viraemia, accompanied the emergence of nasal abnormalities (in the form of mucosal congestion, necrosis or erosion) and finally, in our experience, ceased with the disappearance of nasal lesions.

Although Hornby (1926) stated that urine in small quantities was not infective before the 3rd day and had lost its virulence on the 3rd to 5th days following the subsidence of fever, many text-books still emphasize the importance of urine in disseminating the infection (Hutyra *et al.* 1946; Hagan & Bruner, 1957). Our experiments showed that virus became detectable on the first day of pyrexia and was demonstrable up to the 9th day following its onset. It is of interest to note here that Curasson (1932) established the limits of urinary excretion as the day preceding fever to the 9th day following, whereas Hall (1933) smeared urine in the nostrils of susceptible cattle and established by this means that viruria did not appear until the 2nd day of pyrexia and was no longer demonstrable 2 days after return to normal temperature.

The infrequency with which urinary excretion was detected in many animals, considered together with the low titres recorded, cannot be regarded as lending support to the idea of the importance of urinary contamination in transmitting the RGK/1 strain of rinderpest virus. The very high nasal excretion rate and frequent elevated titres for nasal discharges leave little room for doubt about the relative significance of these two routes of excretion.

The origin of the virus in the urine is not at present established; we failed to find in fatal cases any evidence for an acute cystitis such as was mentioned by Hornby (1926), neither were there macroscopic signs of a nephritis.

According to the data reported here, faecal excretion began, at the earliest, on the 3rd day of temperature reaction and, on occasion, somewhat before the onset of diarrhoea (e.g. ox no. 8362) or in the absence of dysenteric signs (e.g. ox no. 8363). The limits of faecal excretion were given by Hornby (1926) as the 6th to the 12th days following onset of fever, but he accepted that animals with severe diarrhoea might shed virus for 'several days' longer. Curasson (1932) asserted that virus was present in the faeces from the first day of pyrexia and for 2–3 days after its subsidence in cattle with diarrhoea. Neither of these authors gave details of their experimental protocols. Hall (1933) found that the faeces became infective after the appearance of diarrhoea and at some time between the 2nd and 7th days of temperature reaction. Faecal excretion, in cases with severe diarrhoea, was not demonstrable 4 days after the subsidence of fever, whilst in other instances no virus was detected on the 2nd or 3rd days after the end of pyrexia.

From our accumulated data on rinderpest virus excretion it is now possible to answer the problem posed in the 1866 Cattle Plague Report, viz. 'how soon after the poison is put into the blood the animal becomes capable of giving the disease by natural infection to other animals'. Virus may be excreted from the time of onset of viraemia, at least by the nasal route; infected cattle may, therefore, contaminate their environment 2 days before the first clinical manifestation of disease, i.e. fever, and at least 5 days before diagnostic lesions become detectable in the mouth cavity. These factors have an obvious significance in formulating disease-control regulations for enzootic or rinderpest-free countries.

This discussion would not be complete without some comparison of the rinderpest data with those available for canine distemper and human measles. In distemper-infected dogs, Bindrich (1954) established that virus circulated in the blood on the first day of the temperature reaction, whilst Rockborn (1957*a*, *b*) detected viraemia on the 4th and 6th days following experimental or contact exposure. After intramuscular inoculation of virus, viraemia was not detectable after the 6th day, whereas in dogs infected by contact it persisted for at least 14–19 days (Rockborn, 1957*b*, 1958). Liu & Coffin (1957) demonstrated by the immunofluorescent technique that virus-specific antigens appeared in the circulating leucocytes of ferrets 3–4 days after inoculation of virulent distemper virus and before the onset of pyrexia.

Clinically recognizable changes occur in the nasal mucosae of infected dogs as early as the 2nd day of fever; they are accompanied by a serous discharge (Bindrich, 1950). It has been known for several decades that nasal exudates are infectious and it has been reported that mink, infected by aerosols, excreted virus in the nasal discharges from the 5th to 46th day post-exposure (see Gorham (1960) for a review).

So far as urinary excretion of distemper virus is concerned, Bindrich (1950) stated that he had demonstrated viruria in experimentally-infected dogs from the 3rd to the 17th days following temperature reaction and thereafter irregularly to the 8th week post-inoculation. Hence urinary excretion began at least 2 days after the appearance of viraemia. Bindrich unfortunately restricted his observations on the first 2 days of fever to only 4 dogs; if he had been able to investigate a larger number of cases, his results may well have been comparable to ours with rinderpest, in which three of ten cattle had viruria on the first day of fever.

Mucoid or blood-streaked diarrhoea is common in the later course of virulent distemper infection (Hutyra *et al.* 1946) but data on faecal viral excretion have not been published either for the dog, the ferret or the mink. Gorham & Brandley (1953), however, failed to find virus in the colon contents of infected ferrets.

Systematic investigations of measles viraemia and virus excretion in the natural host present considerable difficulties; nevertheless, some information has recently become available, particularly through the use of tissue culture techniques. The natural incubation period of 10–11 days (Robbins, 1962) or 5–9 days (Rake, 1959) corresponds quite closely with the contact incubation periods in rinderpest. It is followed by a prodromal phase of 1–5 days (Rake, 1959) or 2–4 days (Robbins, 1962) which is characterized by fever, conjunctivitis, rhinitis and tracheobronchitis. Koplik's spots appear during this stage on the mucosae of the cheeks, soft palate and lower lips. The prodromal period is terminated by the appearance of the rash, at first a focal, macular or maculopapular exanthem, which may later become confluent (Rake, 1959; Robbins, 1962). Virus is probably present in the blood and nasopharyngeal secretions during the whole of the prodromal period and up to 32 hr. after the appearance of the rash (Ruckle & Rogers, 1957). According to Papp (1937) it could also be demonstrated in the blood during the incubation period, by the subinoculation of leucocytes into susceptible children; Enders,

Katz & Medearis (1959) found that monkeys developed measles viraemia 5–7 days after inoculation and 3–4 days before the first clinical signs.

There are obvious similarities between the course of the infection in rinderpest and measles. In both of them viraemia becomes detectable during the incubation period; the latter is followed by a prodromal phase of 3–5 days duration, accompanied by non-specific, general clinical signs, with continuous viraemia. In rinderpest the prodromal period is terminated by the appearance of a pathognomonic enanthem, after which the viraemia declines rapidly. In measles the oral enanthem appears *during* the phase which is designated prodromal, and this phase is ended by the appearance of the skin exanthem. Stated in this manner the differences between measles and rinderpest are quite artificial, especially when it is known that the skin eruption of rinderpest, if it occurs, appears on the 4th or 5th days of clinical reaction (Burdon-Sanderson, 1866) at the same time as diarrhoea and stomatitis (Curasson, 1932). Although Robbins (1962) claimed that there was no direct evidence that Koplik's spots are the primary site of viral proliferation in the mucous membrane, multinucleate syncytia have been observed in the buccal or tongue epithelia and these may well indicate localization of virus in these situations (for example, Masugi & Minami, 1938; Semsroth, 1939; Roberts & Bain, 1958). Similarly, Grist (1950) stated: 'The thinness of buccal mucosa may explain why Koplik's spots are visible before the skin rash, though both types of lesion are initiated at the same time.'

While measles in many countries is now a relatively mild disease, in which enteric signs are often not considered worthy of mention (Robbins, 1962) or receive very brief consideration (Rake, 1959), this is certainly not true of certain African countries or of nineteenth-century England and France (Morley, Woodland & Martin, 1963). In Nigeria, the last-named authors found that diarrhoea or dysentery was 'second only in importance to bronchopneumonia as a feature of measles'. It most commonly appeared after the rash but sometimes began during the prodromal period or at the end of the latter; the weight loss and dehydration associated with it was a major cause of the mortality, which was estimated to reach an over-all 5% in Nigeria. Thus measles virus may, in some highly susceptible populations, cause a clinical syndrome which is comparable in all important respects to rinderpest in cattle.

Little is known about the urinary and faecal excretion of the measles agent, but Gresser & Katz (1960) were able to isolate virus from the urine of eight out of eleven patients tested; in one instance virus was still present 4 days after appearance of the rash. Enders (1962) regarded the question of faecal excretion of measles virus as still unanswered, while Ruckle & Rogers (1957) could not recover virus from the faeces of patients.

SUMMARY

A total of twenty-five grade cattle were infected experimentally with a strain of rinderpest virus of moderately high virulence (RGK/1). Three methods of introduction of the virus were employed, i.e. subcutaneous or intranasal inoculation (14 animals) and housing in contact with reacting cattle (11 animals).

A quantitative study of the viraemia and of virus excretion by the nasal, urinary and faecal routes, was made by the inoculation of primary calf kidney cultures. All virological data were related to the onset of pyrexia, the incubation period in inoculated animals being 3–5 days, while in those infected by contact it was 8–11 days. Viraemia preceded the first rise of temperature by as much as 2 days in inoculated animals and 1 day in the contact cases. All animals had viraemia by the 4th day of the disease, but thereafter the percentage of positives declined rapidly, reaching nil by the 9th day.

The case mortality rate was 48% with the majority of animals dying on the 7th to 10th days after reaction.

Virus was detected in the nasal secretions of some animals on the 2nd day preceding pyrexia. The proportion of nasal excretors rose slowly to reach a maximum of 87·5% on the 4th day and no positives were detected after the 9th day of fever. The titre of virus in nasal discharges reached high levels, often probably in the region of $10^{5.0}$ to $10^{6.0}$ TCD50/ml.

Urinary excretion of virus began on the 1st day of fever in about 25% of animals; reaching a maximum of 62·5% on the 7th day. No virus was recovered from the urine after the 8th day of the disease, the rapid decline in the excretor rate being only partially attributable to the death of severe cases. Urinary titres were relatively low; they seldom exceeded $10^{2.0}$ TCD50/ml.

Virus was first recovered from the faeces on the 3rd day of pyrexia. Excretion by this route was not constantly associated with the development of diarrhoea, but the rapid decline which occurred in the excretor rate on the 8th and 9th days was undoubtedly attributable to the death of diarrhoeic individuals which took place at that time. The majority of faecal titres were in the approximate range of $10^{3.0}$ to $10^{4.0}$ TCD50/g. but occasional samples attained *ca.* $10^{6.0}$ TCD50/g.

It was calculated, from the clinical and virological data that cattle can excrete virus, at least by the nasal route, as much as 6 days before the appearance of pathognomonic clinical signs, i.e. oral necrosis and erosion.

Our findings for rinderpest were compared with those of other investigators of this disease and also with the available information on canine distemper and human measles.

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Factors affecting the growth of virus of foot-and-mouth disease on surviving explants of bovine tongue epithelium

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INTRODUCTION

The large-scale cultivation of the virus of foot-and-mouth disease on explants of bovine tongue epithelium is limited, almost exclusively, to the method devised by Frenkel (1947) and modified by Frenkel & Ribelin (1956). Experience of this method led the authors to believe that the full potential of the system as a source of virus was not being realized. The yield of infective material often varied from culture to culture because many of the physical variables were neither clearly defined nor controlled.

Examination of published data shows that, in developing this method for production, small vessels were used which in no way resembled those subsequently employed for large-scale cultivation, so it could be argued that some reservation should have been made before applying the results of one to the other. Since a stirred system was in use at Pirbright for large-scale cultivation of the virus, attempts were made to develop a laboratory-sized experimental apparatus having a similar system of agitation and, proportionally, of the same dimensions so that it might be easier to simulate the conditions of the larger vessels.

Some years have elapsed since the following experiments were attempted. The findings were subsequently successfully applied to large-scale procedures and are now in regular use for preparation of inactivated vaccine.

APPARATUS AND MATERIALS

Culture equipment

A 2 lb. size straight-sided domestic fruit bottling jar* (Kilner Jar Improved Pattern—United Glass Ltd., London, S.W. 1) was used as the culture vessel. The vessel had a total volume of 850 c.c. and was of such dimensions that the ratio of the depth of liquid to the diameter of vessels was approximately unity (Cooper, Fernstrom & Miller, 1944) when the jar held 400 ml. of medium.

A $\frac{3}{8}$ in. hole was drilled in the bottom of the jar to admit the shank of a metal-mounted $\frac{5}{8}$ in. × $\frac{5}{8}$ in. Kieselguhr filter candle (British Berkefeld Ltd., Kent. Size 9a). The jars were annealed at 500° C. to eliminate stresses set up in the glass by drilling.

The glass cap was replaced by one of stainless steel, or cadmium plated brass,

* The Improved Pattern Kilner Jar has been superseded by the 'Dual Purpose' jar which is of slightly different shape. The alterations in dimensions are such as not to change significantly the ratio of depth of liquid/diameter when used as described in this paper.

machined so as to locate it centrally within the neck of the jar and fitted with a $\frac{1}{16}$ in. rubber gasket. The stirrer was flat bladed, a stainless steel strip 3 in. × $\frac{3}{8}$ in., fitted to a bush screwed to the end of a $\frac{1}{4}$ in. shaft. The shaft passed first through a graphite impregnated Ferrobestos journal bearing (J. W. Roberts, Lostock, Bolton), thence through a 3 in. × $\frac{1}{2}$ in. i.d. tube tightly packed with superfine glass wool (Fibreglass Ltd., St Helens, Lancs) and finally through a second graphite impregnated bearing, which was a press fit in the top of the glass-wool packed tube. The whole was located and held in position by a distance piece assembly. Two $\frac{1}{4}$ in. internal diameter stainless steel Schrader nipples were screwed into the lid to provide exhaust and inoculating or sampling ports (Pl. 1*a*).

A metal framework held three such bottles immersed in a thermostatically controlled water-bath 18 in. × 18$\frac{1}{2}$ in. × 8 in. (Grant Instruments Ltd., Cambridge). The bath was set in a simple scaffold constructed from slotted angle framework (Messrs Dexion Ltd., London, N.W. 6) on which was mounted a single-phase fractional horse-power motor geared to 60 r.p.m. (Hillman Electric Motors Ltd., London, N. 7), clips to locate small sterile glass-wool packed filters and a manifold to conduct away the exhaust gases to a pair of acid traps (Pl. 1*b*).

The assembled culture vessel together with the gas inlet filter and vent tubing was sterilized in an autoclave at 15 p.s.i. for 15 min.

Culture medium

The standard medium was Tyrode solution modified by increasing the sodium bicarbonate concentration from 0·1 % (w/v) to 0·2 % (w/v). Lactalbumin hydrolysate was added to a final concentration of 0·5 % (w/v). The medium was sterilized by E. K. filtration. Penicillin 450 i.u./ml., Neomycin 45 i.u./ml. and Nystatin 20 i.u./ml. were added immediately before use.

When aeration was in excess of 60 cm.^{3}/l./min., 5 ml. of 10% (v/v) suspension of antifoam (MS Antifoam R.D., Midland Silicones Ltd.) in sterile saline was added per 400 ml. of culture medium to suppress foaming.

Bovine tongue epithelium

Whole cattle tongues were obtained from local abattoirs within 2 hr. of slaughter. Preparation of the tongues and method of removal of the epithelial tissue was as described by Frenkel (1954).

Keratinous tissue was removed in a slice approximately 100 μ thick and was discarded. The underlying tissue was collected in slices 75–100 μ thick and stored in normal Tyrode solution at 4° C. until required for use.

Using this technique, 20–25 g. of epithelial tissue slices was obtained from each tongue.

Virus

Except where otherwise stated, the strain of virus used was 'Dutch O' type O, which originated from the Dutch State Veterinary Laboratories where it is used as a strain for vaccine production. It was well adapted to tongue epithelial culture, having been passaged serially over 32 passages when received and a further 40 times during the current work.

Virus assay

Tissue/liquid extracts from cultures were diluted by fivefold steps in 0·04 M phosphate buffer at pH 7·6. Each dilution was inoculated intraperitoneally (Skinner, 1951) into groups of six unweaned Pirbright strain mice (Subak-Sharpe, 1961). The Spearman–Kärber method of computation was used to calculate the 50 % end-point dilution. The fiducial limits of the test described above are $\pm 10^{0.36}$ (Subak-Sharpe, 1961).

Standard culture procedure

For comparative experiments, epithelial tissue slices from several tongues were pooled, finely minced with scissors and weighed out into equal portions of 32 g. Each culture vessel was charged with 400 ml. of the standard culture medium containing antibiotics, with or without antifoam, followed by the minced tissue. The vessels were immersed in the water-bath at 37° C., stirred at 60 r.p.m. and aerated with a mixture of 5 % carbon dioxide in oxygen at a rate of 50 cm.^{3}/min. (125 cm.^{3}/l./min.) for 30 min. A measured volume of seed virus of known infectivity was added to each vessel through the inoculation port. The cultures were usually incubated for 16–18 hr.

Tissue harvested from a culture was ground with sand in a mortar, resuspended in its own culture medium, centrifuged, E. K. filtered and diluted for assay of infectivity.

Estimation of glucose

Harding's modification (Harding, Nicholson, Grant, Hern & Dowes, 1932; Harding & Downs, 1933) of the Schaffer–Hartmann method was used. It consists of re-oxidation of the glucose reduced alkaline copper with iodine and estimating the amount of iodine used with sodium thiosulphate.

Two ml. of a centrifuged sample of the culture medium was used for estimation. A 'blank' test was always included.

RESULTS

Hydrogen-ion concentration

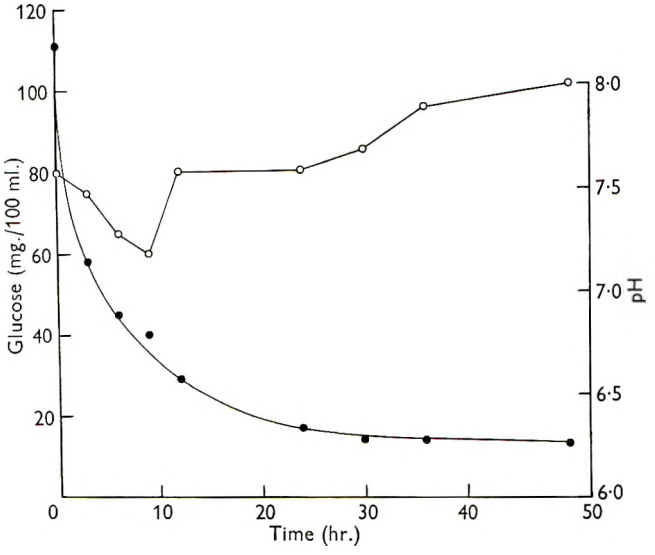
Using the culture technique as described by Frenkel & Ribelin (1956), considerable variation of the reaction of the medium was possible. Values as low as pH 6·8 have been recorded (Frenkel & Ribelin, 1956), pH 7·0 has been reported by Henderson (unpublished data) and pH 7·0–7·2 has been found by us. It is, however, recognized that the pH range for maximal survival of infective virus is as narrow as pH 7·5–7·7 and that, particularly at 37° C., relatively small changes in hydrogen-ion concentration are of great importance.

When oxygen was substituted for 5 % carbon dioxide/oxygen, the pH of the cultures at 18 hr. was increased from approximately pH 7·0 to pH 7·2–7·3, resulting in slightly better yields of infective material; but, if the flow rate of oxygen were increased beyond 40 cm.^{3}/l./min., the pH value rose above pH 8·0 and the yield of infective material was low.

Examination of a system aerated with oxygen at 25 cm.^{3}/l./min. (Text-Fig. 1) showed a rapid fall in pH during the first 9 hr. of cultivation followed by a rise to

pH 7·6 in 24 hr., thence to pH 8·0 in 48 hr. Simultaneously, there was rapid utilization of glucose during the early part of culture—55 % utilized during the first 5 hr., whereas only a further 10 % was used during the next 5 hr. After 24 hr. the amount of glucose remaining was 17 % of the original value.

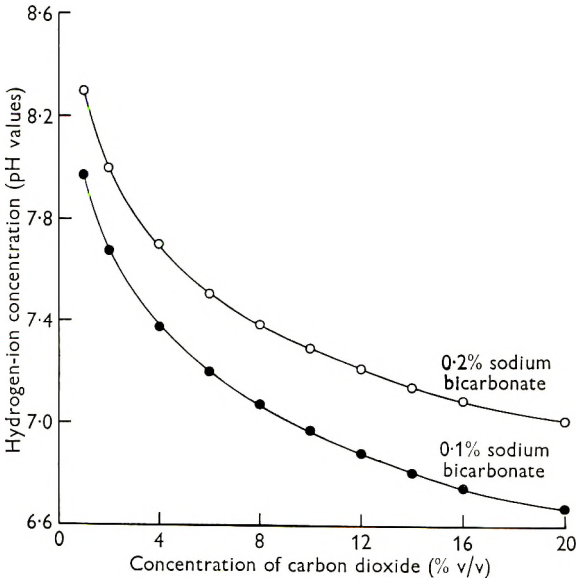
The initial rapid fall in pH was attributed to rapid evolution of carbon dioxide by respiring cells. Agitation and aeration at this stage was apparently insufficient to control the reaction of the medium.



Text-fig. 1. Rate of glucose utilization and pH change in culture medium containing 0·1 % sodium bicarbonate aerated with oxygen. pH, O—O; glucose, ●—●.

The hydrogen-ion concentration of a bicarbonate-carbon dioxide buffered system such as Tyrode solution at stated temperature and pressure is dependent on the relative concentration of bicarbonate and carbon dioxide. A 0·1 % solution of sodium bicarbonate ($1{\cdot}19 \times 10^{-2}$M) aerated with 5 % carbon dioxide at 37° C., as defined by the Henderson–Hasselbalch equation, is pH 7·3, and the reaction of the system is fixed so long as these conditions are maintained. Since a value within the range pH 7·5–7·7 was required, the concentration of sodium bicarbonate only was adjusted to 0·2 % (w/v) ($2{\cdot}38 \times 10^{-2}$M), thus giving a smaller swing of pH in the face of increased concentrations of carbon dioxide (Text-fig. 2). It was also decided to aerate vigorously with 5 % carbon dioxide in oxygen to attempt to carry off carbon dioxide evolved in the medium and maintain values close to 5 %, particularly when glucose was being most actively metabolized.

When the standard culture medium containing 0·2 % (w/v) sodium bicarbonate and 8 % (w/v) tongue epithelial fragments was aerated with 5 % carbon dioxide in oxygen at 250 cm.^{3}/l./min. at 37° C., a hydrogen-ion concentration of pH 7·6 was maintained throughout the 7 hr. of incubation (Table 1), indicating that the concentration of carbon dioxide may be controlled within acceptable limits without major alteration to the existing culture technique.



Text-fig. 2. Hydrogen-ion concentration of solutions of bicarbonate at 37° C. aerated with varied concentrations of carbon dioxide.

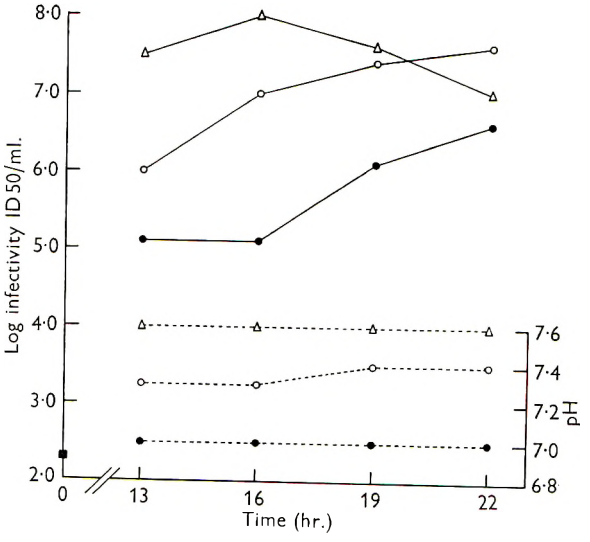
Table 1. *Utilization of glucose by tongue epithelial fragments* 8 % (*w/v*)

Time (hr.)	pH	Conc. glucose (mg./100 ml.)	Corrected glucose values (mg./100 ml.)
0	7·6	95	100
0·5	7·6	96	96
1·0	7·6	83	89
1·5	7·6	89	85
2·0	7·6	79	80
2·5	7·6	78	74
3·0	7·6	72	69
3·5	7·6	70	64
4·0	7·6	60	62
4·5	7·6	64	58
5·0	7·6	52	54
5·5	7·6	55	50
6·0	7·6	44	47
6·5	7·6	41	45
7·0	7·6	38	42

Aeration

Efficient aeration will depend upon the geometry of the vessel, degree of agitation and gas flow rate (Cooper *et al.* 1944). In a vessel of standard design increased gas solution rates are obtained by shear action of a high-speed impeller in a baffled vessel or by use of a device to reduce bubble size when slow stirrer speeds are involved (Finn, 1954). Equipment already available was of the second type so that aerators examined included single holed spargers of approximately 1·0 mm.

orifices, sintered stainless steel gauze 8–10 μ porosity (Hixon & Gaden, 1950) and standard grade Berkefeld bacteriological filter candles. The latter proved to be superior, providing a sulphite oxidation rate of 30 mmoles O_2/l./hr. at 20° C. from an oxygen flow rate of 125 cm.³/l./min. Both of these conditions were found to be consistent with control of hydrogen-ion concentration and increased virus production.



Text-fig. 3. Effect of bicarbonate concentration and aeration upon the growth of Dutch 'O' virus. △—△, 0·2 % sodium bicarbonate, 5 % CO_2/O_2 125 cm.³/l./min. Berkefeld candle aerator; ○—○, 0·2 % sodium bicarbonate, 5 % CO_2/O_2 17·5 cm.³/l./min., 0·5 mm. sparger; ●—●, 0·1 % sodium bicarbonate, oxygen 17·5 cm.³/l./min. 0·5 mm. sparger; ---, pH values; ■, infectivity of starting material.

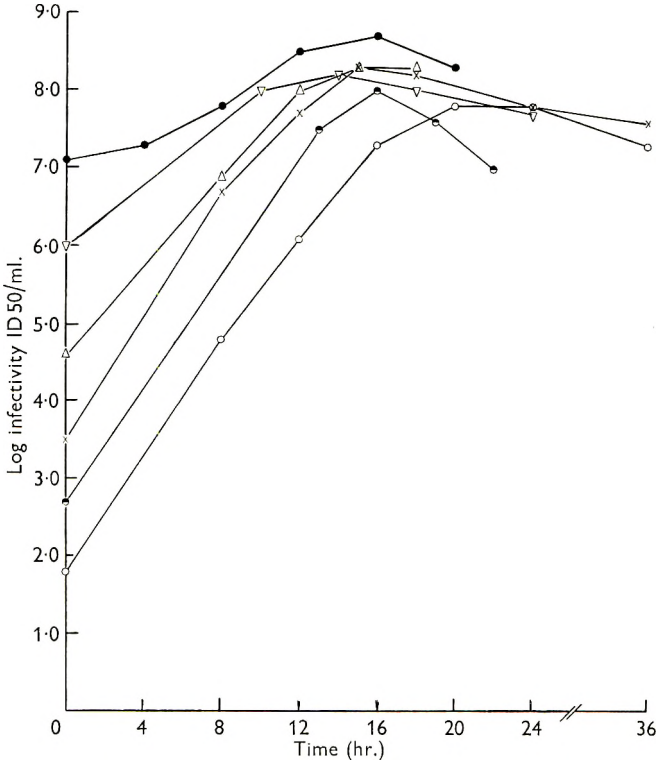
The effects of hydrogen-ion concentration and aeration upon infective virus yield is shown in a series of cultures inoculated with the same amount of seed virus (Text-fig. 3) contrasting the older method of cultivation and the modified technique with higher gas solution rate. As may be expected, the yield of virus is enhanced when the hydrogen-ion concentration is maintained at a level more conducive to its survival. It is also seen that the peak titre appears sooner under improved conditions of aeration and pH control.

It is difficult to separate the effects due to pH control and supply of oxygen but it is likely that the rate of oxygen utilization is independent of the rate of supply, since the yield of infective material may be maintained by substitution of 5% carbon dioxide in air. When nitrogen is substituted for air or oxygen in the gas mixture, virus does not multiply and the hydrogen-ion concentration is not controlled to the same extent as in aerobic cultures.

The difference may be due to fermentation on the one hand and respiration on the other, with predominant production of lactic acid and carbon dioxide respectively (Dickens, 1959). This avenue has not been investigated but rather used as a case for good aerobic conditions within the culture system (Baron, Porterfield & Isaacs, 1961).

Table 2. *Anaerobic cultivation of the virus of foot-and-mouth disease*

Time (hr.)	Glucose (mg./100 ml.)	pH	Infectivity (ID 50/ml.)
0	100	7·6	$10^{4·6}$
16	17	7·3	$10^{3·5}$
24	13	7·2	$10^{3·8}$
40	12	7·3	$10^{3·5}$



Text-fig. 4. Effect of inoculum upon rate of growth of Dutch 'O' virus.

Inoculum

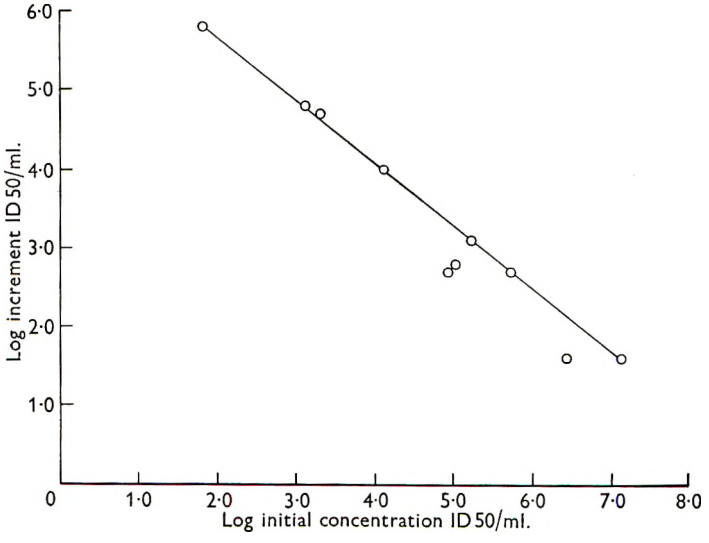
The peak of infectivity has been reported as occurring after 18–24 hr. of culture (Henderson, 1953); Frenkel reports a mean incubation period of 19·2 hr. (Frenkel & Ribelin, 1956). After this point the infectivity titre wanes at a rate depending upon the strain of virus (Henderson, 1953). In our experience the infectivity approximates closely to its peak value after 16–18 hr. incubation (Text-fig. 4), although the range may be as wide as 14–20 hr. in cultures originating from inocula at the higher and lower extremities of the inoculum scale, respectively.

Under standard conditions of culture and time of harvest, the relationship between the infectivity of the starting and final materials is quantitative (Text-fig. 5).

This relation between titres of inoculum and harvested material applies to other strains of virus adapted to epithelial culture. Table 3 shows a number of stock

strains passaged routinely for use in the laboratory. Although, with one exception, the initial concentrations of infective material are of a similar order, the amount actually harvested is closely related in each case to the predicted amount calculated from Text-fig. 5.

The ability to be able to forecast the amount of infective material which could be obtained from a particular culture was used to indicate whether a virus had been adapted to this form of cultivation. Any disparity suggested that the virus strain was not so modified.



Text-fig. 5. Relationship between inoculum and material harvested after 16–18 hr. incubation at 37° C.

Table 3. *Relationship between inoculum and harvest infectivity titres*

Virus type	Titre at 0 hr. (ID 50/ml.)	Culture period (hr.)	Titre of harvest (ID 50/ml.)
O ('Dutch O')	$10^{6\cdot1}$	16	$10^{8\cdot3}$
C (C 997)	$10^{6\cdot4}$	16·5	$10^{8\cdot3}$
C (C 997)	$10^{5\cdot7}$	16	$10^{8\cdot1}$
C (U/C/54)	$10^{6\cdot0}$	18	$10^{8\cdot2}$
SAT 1 (RV. 11)	$10^{6\cdot1}$	15	$10^{8\cdot1}$
SAT 1 (Bec. 19/60)	$10^{6\cdot0}$	16·5	$10^{8\cdot0}$
SAT 2 (Ken. 3/57)	$10^{6\cdot8}$	17	$10^{8\cdot5}$
ASIA 1 (Pak 1)	$10^{5\cdot8}$	15	$10^{8\cdot3}$
ASIA 1 (Pak 1)	$10^{6\cdot1}$	16	$10^{8\cdot3}$

The volume of inoculum to produce a high initial infectivity is relatively easily obtained for small-scale cultures but in serial large-scale cultures the same proportional volume would involve material which might represent a large amount of inactivated vaccine. In the latter case, experience has shown that the most economical starting virus titre is approximately $10^{6\cdot0}$ID 50/ml., which will yield

$10^{8\cdot2}$ID 50/ml. in 16–18 hr. of culture. Assuming serial batch cultures, this would mean reservation of 1 % of the harvested material in each successive passage or 1 l. of seed at $10^{8\cdot0}$ID 50/ml. to a culture of 100 l.

Tissue

Slices of epithelium removed from bovine tongues by means of a Berkel bacon slicer vary in the number and size of susceptible cells, depending upon the level in the epithelial layer from which they are removed. Tissue immediately above the connective tissue layer is most susceptible (Brooksby & Wardle, 1954) and keratinous tissue not at all. It has therefore been general practice to discard a slice of keratinous tissue some 100 μ thick and to collect the underlying tissue in slices 75–100 μ thick down to, but not including, the musculature.

In small-scale experiments the effect of thickness is not always evident, since the epithelial slices are finely minced with scissors to make them more manageable in culture (Table 4).

Table 4. *Cultivation of virus of foot-and-mouth disease in finely clipped tissue*

Average thickness (μ)	Titre at 0 hr. (ID 50/ml.)	pH	Titre of harvest (ID 50/ml.)
Thin, 100	$10^{3\cdot4}$	7·6	$10^{7\cdot7}$
Medium, 500	—	7·6	$10^{7\cdot9}$
Thick, 1000	—	7·6	$10^{7\cdot7}$

Table 5. *Cultivation of the virus of foot-and-mouth disease in coarsely clipped tissue*

Average thickness (μ)	Titre at 0 hr. (ID 50/ml.)	pH	Titre of harvest (ID 50/ml.)
Thin, 100	$10^{3\cdot4}$	7·6	$10^{7\cdot7}$
Medium, 500	—	7·5	$10^{7\cdot3}$
Thick, 1000	—	7·5	$10^{7\cdot1}$

But when the sliced tissue is coarsely minced, approximately $\frac{1}{4}$ in. squares, the difference is more apparent (Table 5).

In large-scale cultivation it is not easy to reduce the tissue slices to small pieces by clipping because a machine of special design (Frenkel, 1951) is required. For cultures of the order of 100 l., considerable time is consumed in clipping a batch of tissue slices and added to this there is the risk of bacterial contamination during the process. It has been shown (Frenkel, 1954) that thinly sliced tissue, in long strips as removed from bovine tongue, is as effective in promoting virus growth as thicker slices when clipped. Strips of tissue, despite their thinness, may clog exit ports of large-scale culture vessels during harvesting procedures. Because of this we examined the effect of mincing epithelial slices by means of a domestic mincing machine. In Table 6 the virus yield from tissue in various states of division is shown; in these experiments tissue was sliced and then bulked before

further manipulation. Despite probable additional cell damage caused by compression as well as cutting, the relative yields of virus justify the method. Mincing tissue through a sterile, electrically operated butcher's mincing machine (Hobart Ltd., London N.) has been adopted here, using a $\frac{5}{16}$ in. cutting die. Tissue treated by this method will not block the outlet port and is still of such a size as to be separated from the culture medium during harvesting by means of a stainless steel gauze sieve (18-mesh).

Table 6. *Cultivation of virus of foot-and-mouth disease in tissue of various states of division*

(Infectivity titres of tissue/liquid extracts after cultivation for 16 hr. expressed in ID 50/ml.)

	Whole slices	Clipped slices	Minced slices
1	$10^{8\cdot2}$	$10^{8\cdot9}$	—
2	—	$10^{8\cdot1}$	$10^{7\cdot9}$
3	$10^{7\cdot8}$	$10^{6\cdot9}$	$10^{7\cdot8}$
4	$10^{7\cdot9}$	—	$10^{7\cdot8}$
5	$10^{7\cdot7}$	$10^{8\cdot0}$	$10^{8\cdot0}$

The time which elapses between removal of the epithelium from cattle tongues and its use in culture may be of importance in yielding material of high infectivity, particularly in large-scale culture. Tissue for small-scale cultures can be kept for 24 hr. or so under favourable conditions with respect to its concentration in the suspending fluid and reaction of the medium (Table 7).

Table 7. *Effect of storage of bovine tongue epithelial tissue (small scale) upon virus yield, 8 % suspension (w/v) in normal Tyrode solution*

(Infectivity titres of tissue/liquid extracts after cultivation for 16 hr. expressed in ID50/ml.)

Temp. of storage (° C.)	Length of time stored (hr.)			
	0	18	24	72
37	$10^{8\cdot3}$	—	$10^{7\cdot3}$	$10^{6\cdot4}$
37	$10^{8\cdot0}$	$10^{8\cdot3}$	—	—
4	$10^{8\cdot0}$	$10^{7\cdot8}$	—	—

In the case of tissue for large-scale cultivation where considerable distance may separate supplier and user (Frenkel, 1953) and where, for economy of space and transport, the tissue is collected in jars of Tyrode's solution at many times the concentration it would be used at during cultivation of virus, there is evidence of reduced viability after 24 hr. storage at 4° C. (Table 8).

Table 8. *Effects of storage of bovine tongue epithelial tissue (large scale) upon virus yield, 50–60 % suspension (w/v) in normal Tyrode solution*

(Temperature of storage 4° C.)

Virus type and strain	Age of tissue (hr.)	Initial virus titre (ID 50/ml.)	Titre of harvest (ID 50/ml.)
SAT 1 (Bec. 19/60)	24	$10^{5\cdot8}$	$10^{7\cdot3}$
SAT 1 (Bec. 19/60)	3–8	$10^{6\cdot0}$	$10^{8\cdot1}$
SAT 1 (RV. 11)	24	$10^{5\cdot8}$	$10^{7\cdot5}$
SAT 1 (RV. 11)	4–12	$10^{6\cdot0}$	$10^{8\cdot3}$

Adaptation

Adaptation of virus from a field sample is not a difficult problem if a sizeable sample of infected tissue is available. More commonly, field material may consist of a very small quantity of material of low infectivity.

The procedure adopted with such a sample is to prepare an EK filtrate of a $\frac{1}{2}$–$\frac{1}{5}$ (w/v) suspension in 0·04 M phosphate buffer, pH 7·6. The filtrate is inoculated intraperitoneally into unweaned mice 5–7 days old and also used as seed for a tongue tissue culture. The virus is taken from one or the other and is passaged serially in culture. If after 4–6 passages material of $10^{7.5}$–$10^{8.0}$ ID 50/ml. has not been obtained, the culture material should be passaged once again through mice and thence to culture. Normally one mouse passage is sufficient to render the virus amenable to culture but in a few cases a second may be found necessary.

DISCUSSION

Using a laboratory-sized culture equipment of particular design to improve gas solution rates and gas 'hold up' (Finn, 1954) in an examination of the bicarbonate–carbon dioxide buffer system, it has been possible to control the hydrogen-ion concentration throughout the period of culture and, by suitable adjustment in conjunction with other defined variables, to increase the output of infective material approximately tenfold.

There was no evidence of major variation between batches of tissue when freshly collected since it was possible to predict, fairly accurately, the expected yield of virus under defined conditions of culture and within experimental error. Although it was possible to store tissue for a considerable length of time without apparent deterioration in susceptibility, when large amounts of tissue are transported over long distances it is difficult to arrange optimum conditions. Reduction of the time interval between collection and use may be necessary to compensate for these difficulties.

The higher concentration of bicarbonate in conjunction with 5 % carbon dioxide in oxygen provided good pH control during cultivation of the virus, in addition to damping the pH swing at higher concentrations of carbon dioxide. But it is seen that the slope of the curve increases steeply as the values of carbon dioxide fall below 5 %. The increase towards the alkaline side (i.e. > pH 7·8) is not desirable when harvesting large-scale cultures because of the length of time the virus may be exposed to unsuitable pH conditions. We have minimized such a rise in routine large-scale procedure by emptying the culture vessel under pressure of 5 % carbon dioxide in oxygen and by bulking with the filtrate the filter washings, consisting of about one-third of its volume of 0·04 M phosphate buffer, pH 7·6. The pH of harvested medium and the virus filtrate is thus maintained at approximately pH 7·8.

Aerobic conditions of culture appear to be necessary for virus multiplication. This has been shown in the case of other viruses (Baron *et al.* 1961; Taylor-Robinson, Zwartow & Westwood, 1961). The oxygen gradient within the thicker slices (Minami, 1923) may be partly responsible when such slices are employed.

The concentration of glucose had little effect on virus production but did indicate cell viability. Current experiments indicate that similar virus yields may be obtained, under certain conditions, in the absence of glucose.

In trying to assess whether epithelial tissue produced virus to maximal capacity, it was estimated, by repeated trypsinization of sliced and minced tissue, that there were $10^{8\cdot0}$ cells/g. Under optimal culture conditions the virus output was $10^{9\cdot6}$ ID 50/g. of tissue which, if attributable to the whole tissue mass, amounted to 40ID 50/cell. This figure is lower than that found when cultivating the virus in other cell systems (Sellers, 1959; Patty, Bachrach & Hess, 1960). If it is assumed that most of the virus is produced by cells immediately above the musculature (Brooksby & Wardle, 1954), which is about one-fifth of the total weight used in this technique, a value of similar order to that obtained in the case of cell cultures is achieved. The technique may thus be considered to be no less efficient than others and the inclusion of so much relatively inert matter is justified only by the simplicity and rapidity with which the whole may be collected and the uncertainty of how much epithelial matter may be discarded without detriment to the virus yield.

The standard culture procedure outlined earlier in this text has been applied to large-scale culture procedures for production of inactivated vaccines. It was possible to prepare two million doses of SAT 1 vaccine during the winter of 1961 for an outbreak of foot-and-mouth disease in South-West Africa (Galloway, 1962).

SUMMARY

A simple, laboratory scale, stirred system has been developed to examine conditions of culture necessary for optimum return of infective virus from explants of surviving bovine tongue epithelium, with particular bias towards large-scale procedures.

Control of hydrogen-ion concentration and aeration of the culture are emphasized.

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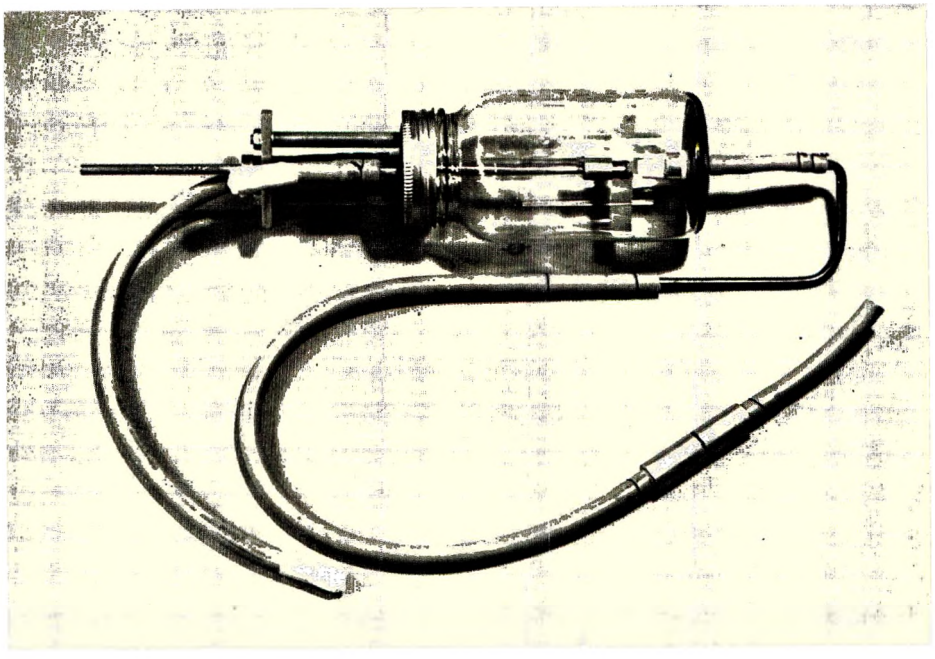

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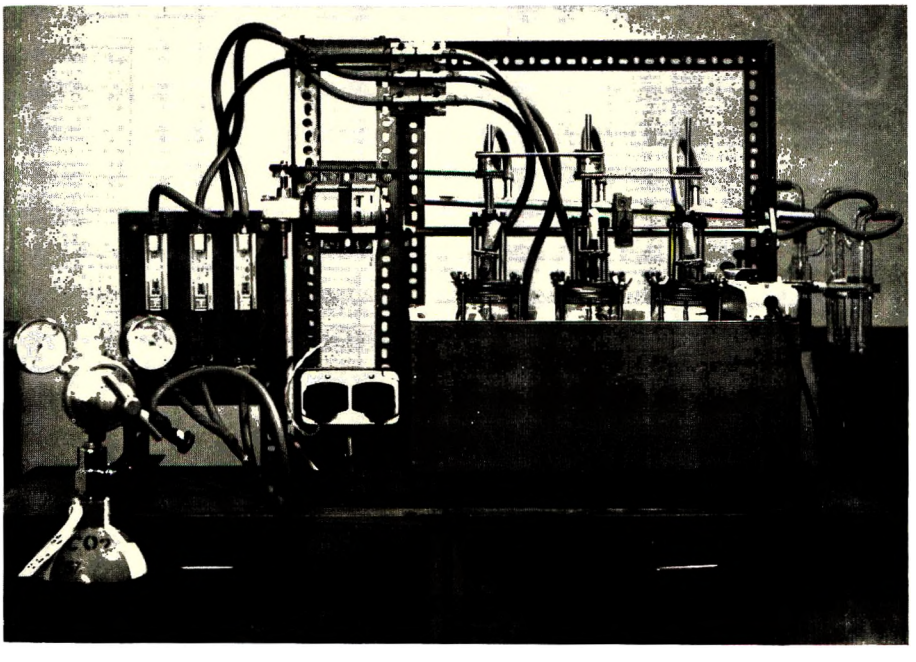

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EXPLANATION OF PLATE

(*a*) The 400 ml. Kilner jar culture vessel assembled ready for sterilization. Note the gas inlet filter fitted to the open-ended rubber tube.

(*b*) Three 400 ml. culture vessels housed in the complete culture assembly. The individual gas metering system is seen on the left and the acid traps for dealing with the exhaust gas on the right.

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The specificity of the antibody responses of human volunteers to certain respiratory viruses

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Minor upper respiratory infections are due to a great many viruses, belonging to several biological groups, and one of the most important of these is the picornavirus group (Report, 1963). This includes, on the one hand, the enteroviruses, polioviruses, echoviruses and coxsackieviruses, and on the other, the rhinoviruses which are classified as M rhinoviruses or H rhinoviruses according to whether they can grow in both monkey and human cells (M) or in human cells only (H).* Enteroviruses often invade the upper respiratory tract and may cause illness with symptoms such as sore throat in addition to constitutional upset. Rhinoviruses cause common colds (Tyrrell & Chanock, 1963). It seems probable that the average man is infected with a succession of enteroviruses of different serotypes, particularly in childhood, and with a similar succession of rhinoviruses, in childhood, adolescence and in later life. Following infection, antibodies against the invading virus are usually produced. Generally speaking the possession of neutralizing antibody is correlated with resistance to infection by the corresponding enterovirus or rhinovirus.

Almost all picornaviruses seem to be antigenically quite distinct from each other when studied by cross-neutralization tests with sera from experimentally inoculated animals, but when studied by complement fixation (CF) cross-reactions between enteroviruses may be detected. When paired sera from subjects who are naturally infected with enteroviruses are tested by CF, antibody responses to several viruses may be found; these are less commonly observed when sera are tested by neutralization.

Mogabgab (1962*a*) has recently stressed the fact that, using neutralization and haemagglutination-inhibition as well as complement-fixation tests, he detected in the sera of patients suffering from acute respiratory infections simultaneous antibody rises against a number of enteroviruses and rhinoviruses. He draws the conclusion that the viruses are so interrelated that a specific serological diagnosis is impossible. He also believes that vaccination with one or two viruses of this group might protect against a large number of the viruses which cause minor upper respiratory infections.

* The rhinoviruses have not yet been allocated to numbered serotypes as have the enteroviruses and so they are referred to by initials or laboratory numbers. In addition, one rhinovirus was originally designated as ECHO 28, and the name will be printed thus, with capital letters, in order to distinguish between it and typical echoviruses printed with small letters in accordance with the new convention.

This paper reports the antibody responses of volunteers inoculated with picornaviruses which are known to infect the respiratory tract. Sera kept from experiments performed during the past few years have been tested against a variety of viruses; the results bear on the specificity of antibody rises, the mechanism by which immunity is built up in natural conditions and the likelihood of enhancing general immunity by means of a vaccine containing a few picornaviruses.

MATERIALS AND METHODS

All volunteers were aged between 18 and 50 years and most were kept in isolation. One group was inoculated intranasally with echovirus 11 (strain U) passed in tissue culture (Buckland, Bynoe, Philipson & Tyrrell, 1959) and another with a strain of coxsackievirus A 21 which had been passed in tissue cultures of human amnion cells (see Parsons, Bynoe, Pereira & Tyrrell, 1960), back to man and then once more into tissue culture, this time of human embryo kidney. Other volunteers were inoculated with an H rhinovirus, D.C., which had been passed in tissue cultures and again in man (Tyrrell, Bynoe, Buckland & Hayflick, 1962). The pairs of sera studied all showed an antibody rise against the infecting virus. Other volunteers received active M rhinovirus, P.K., passed 8 times in human embryo tissue cultures and administered as a single intramuscular injection of 1 ml. of undiluted tissue culture fluid. Still others were given a formalin-inactivated M rhinovirus, ECHO 28, as another experimental vaccine, but this last group of volunteers was not kept in isolation. These intramuscular injections produced particularly high antibody responses against the homologous virus (Doggett, Bynoe & Tyrrell, 1963) and therefore these sera were chosen in preference to those of volunteers who were given virus by the intranasal route.

The sera were collected between 2 and 3 weeks after the administration of virus and had all been kept at -20° C. after being collected; although rather small volumes remained of some of these it was possible to test them by using (1) the micromethod of Takátsy & Furesz (1954) as modified by Sever (1962) for the haemagglutination-inhibition tests (HI) and (2) the microplaque reduction test (Taylor-Robinson & Tyrrell, 1962*a*) for measuring neutralizing activity (K). Previous experiments had shown that very similar results were obtained with the HI test and neutralization tests using either echovirus 11 or coxsackievirus A 21, so the HI test was used because it was simple and economical.

RESULTS

Heterotypic antibody responses were rarely found. Their distribution can be seen from Table 1. The detailed results in typical cases in which no heterotypic rise occurred and in those in which it did are shown in Table 2. Earlier studies had shown that antibody rises against M rhinovirus, B 632, occurred in volunteers vaccinated with ECHO 28 virus (Doggett *et al.* 1963; Mogabgab, 1962*b*). The further experiments reported here show that heterotypic responses did not occur against picornaviruses in general. The rise in antibody against the serologically distinct M rhinovirus H.G.P. occurred in a volunteer who was inoculated with ECHO 28 virus before coming to the Unit and who was therefore not isolated at

Table 1. *Summary of antibody responses to two enteroviruses and three rhinoviruses*

Virus strain given	Type	Administration	Geometric mean rise against virus given	4-fold or greater antibody* rises against			
				Coe	U	H.G.P.	ECHO 28
Coe	Coxsackievirus A21	Nasal drops	16·3	6/6†	1/6	0/5	0/5
U	Echovirus 11	Nasal drops	13·1	1/9	9/9	0/9	0/9
D.C.	H Rhinovirus	Nasal drops	8·1	0/7	0/7	0/7	0/7
P.K.	M Rhinovirus	Intramuscular (isolated)	17·2	0/6	0/6	6/6	0/6
ECHO 28	M Rhinovirus	Intramuscular (not isolated)	6·8	0/5	0/5	1/5	5/5

* Antibody against Coe and ECHO 11 was measured by HI and against the rhinoviruses by neutralization. Only the sera from volunteers given D.C. were titrated with D.C.

† Numerator = number showing rise. Denominator = number tested. P.K. and H.G.P. are antigenically identical.

Table 2. *The usual, specific antibody responses and three unusual heterotypic responses*

Virus strain given	Volunteer	Antibody titre against			
		U*	Coe*	H.G.P.†	ECHO 28†
U	Tu.	<4/32	<4/<4	0·21/0·35	0·23/0·23
U	Ja.	<4/32	<4/8	2·5/2·5	2·7/1·9
Coe	Sm.	<4/<4	16/256	1·5/2·4	>54/35
Coe	Ga.	<4/8	4/256	—	—
ECHO 28	Jo.	<4/<4	<4/<4	5·7/6·1	0·41/4·9
ECHO 28	Ho.	<4/<4	4/<4	0·3/>7·4	<0·1/1·2

* HI antibody titre—numerator = titre of first specimen and denominator = titre of second specimen.

† Serum neutralizing activity (K value).

the time; he developed a cold between the time of inoculation and the collection of serum after vaccination. It was not possible to test his nasal secretions for the presence of a virus, but he might have been infected with an agent antigenically related to H.G.P. Additional experiments were done on the sera of two volunteers who had been infected by intranasal inoculation with B632 and two with ECHO 28. These showed that although there was an antibody rise against ECHO 28 in one subject infected with B632, and a rise against B632 in one subject infected with ECHO 28, there was no rise in antibody against Coe or H.G.P.

Inspection of the original data shows that there were no regular small rises in antibody titre against heterotypic viruses which might have been significant in total although too small individually to be so regarded. In fact, small falls were about as frequent as small rises, and probably both reflected random errors as a result of the method of titration used.

All in all, heterotypic antibody rises were found in three tests whereas they might have been found in 104 if heterotypic responses had occurred generally.

DISCUSSION

In earlier experiments it was shown that when adults were injected intramuscularly with ECHO 28 virus there were frequent heterotypic responses to M rhinoviruses strain B632 (Doggett *et al.* 1963) or K779 (Mogabgab, 1962*b*); the latter two strains are very similar antigenically (Tyrrell unpublished) and can be shown to be related to ECHO 28 by neutralization tests on sera prepared in rabbits (Taylor-Robinson & Tyrrell, 1962*b*). In our present studies we can find little evidence that infection or vaccination with one serotype of the viruses used in this study induced antibody responses against the others to any important degree although there appears to be a slight cross-reaction between coxsackievirus A21 and echovirus 11. Ketler, Hamparian & Hilleman (1962) measured neutralizing antibody rises in patients infected naturally with antigenically distinct strains of rhinovirus; the responses seemed to be completely specific, but this may have been partly because only low homologous titres were detected.

Mogabgab showed a high frequency of heterotypic antibody responses; this may have been (1) because his subjects were not isolated, and therefore could have had double infections, (2) because they had previous experience of a wider variety of picornavirus than our volunteers, (3) because the infecting viruses stimulated more antibodies than did ours, or (4) because the viruses used in his serological tests were different; in this connexion he himself believed that the strains he used, which had been passed in KB cells, had been made thereby better detectors of heterotypic antibody (Mogabgab, 1962*a*). Possibility (1) above suggests that our results may be more significant than Mogabgab's. Although (2) cannot be tested we think that the groups are likely to be of similar socioeconomic background and therefore to have had similar experiences. Regarding (3) the illnesses he studied may have been more severe than ours, but the antigenic stimulus of an intramuscular injection of H.G.P. and ECHO 28 appears to be greater than that of a nasal infection. Regarding (4) we know that antibody measurements of the type we made can be well correlated with the immunity of the subject (Bynoe, Hobson, Horner, Kipps, Schild & Tyrrell, 1961; Tyrrell, 1963). We therefore feel that although the technique used by Mogabgab might have broadened the activity of the antigen in a way which had some advantages for a diagnostic test, our results can be more readily interpreted in terms of the probable changes in the immunity of the subjects.

In view of all the evidence we believe that antibodies to the viruses studied are usually acquired one-by-one following infection with each new serotype. In addition we would not expect a broad immunity to follow vaccination with any one of the strains used in these studies.

SUMMARY

Experiments reported here were performed on the sera of thirty-three volunteers who were infected or inoculated intranasally or intramuscularly in groups of five to nine with one of two enteroviruses, namely, echovirus 11 and coxsackievirus A21 or with one of three rhinoviruses, namely, M viruses P.K. and ECHO

28, and H virus D.C. Antibody responses occurred against the virus administered, and rarely against the other viruses used. It was concluded that although these viruses are related biologically the antibody responses in the volunteers were largely specific.

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The site of specific substances in capsulated organisms

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The considerable advances made in electron microscopy have afforded means of investigating the minutest structures of bacterial cells. However, it has not been possible to produce electron micrographs of the bacterial capsule. Both in a recently published treatise and in a subsequent paper of Salton (1960, 1961) the structure of the capsule is illustrated by diagrams. According to the definitions adopted, 'capsules are apparently formed by the accumulation of various types of polymeric substances of high viscosity around the bacterial cell wall'. Electron microscopy has established that, in general, the capsule is less electron-dense than the cell wall. In opposition to this finding, the viscous extracellular material (the 'slime'), formed of the same substances as are supposed to be contained in the capsule, is fairly opaque to the electron beam and has been demonstrated in electron micrographs by Knaysi (1950). Although it is detectable in the light microscope only, and only in particular circumstances, the capsule is considered to be a well-defined morphological entity of the cell and its presence is often thought to be connected with the existence of specific substances in mucoid germs. As regards the staining properties of capsules, Winne & Volkman (1963) showed that by using two classic staining methods apparent capsular patterns, similar to those recorded in capsulated germs, could be demonstrated in nine organisms generally not thought to be encapsulated. The failure of attempts made in this field is summarized by Brieger (1963) as follows: 'When reviewing literature one can hardly suppress the feeling that disproportionately more effort has been spent upon the staining of the bacterial capsule than upon the elucidation of its nature.'

In this department we have for some time been concerned with attempts to elucidate the nature of the bacterial capsule. The specific polymeric material assumed to form the capsule cannot possibly be less electron-dense when it is contained in the capsule than when it is present outside it in the shape of 'slime'. It was precisely due to the electron density of this material that in former investigations Barber, Petrovici, Zilisteaunu & Nafta (1960) and Barber, Stamatescu-Eustatziou, Tulpan & Petrovici (1960) could demonstrate the presence of polymeric specific substances within the cells of mucoid bacteria. Tomcsik's (1956) attempt to account for the failure of capsules to appear in electron micrographs by the occurrence of 'shrinkage by drying' of the material is not supported by factual evidence, since under the same circumstances of drying both the 'slime' and the material secreted by the germs are clearly apparent in electron micrographs. Barber *et al.* (1960) showed that the highly polymerized polysaccharides

isolated from mucoid *Klebsiella* strains and the polypeptide of *B. anthracis* were both situated within the cells. The electron micrographs of a solution of the polymeric polysaccharide could likewise be produced.

As stated by Tomcsik (1956) at the Symposium of Bacterial Anatomy the 'specific capsular reaction' used as a test for the demonstration of capsules consists in swelling of the bacterial body put in contact with antibody. In point of fact this reaction, which by mere contrast results in a more clear-cut image of the 'halo' representing the capsule, affords evidence of the presence of specific substances within the bacterial cell and in the cell wall, and not of their existence beyond the border line of this wall. Reliance upon the specific capsular reaction or 'swelling of the capsule', as it is sometimes called, has always led the respective investigators to puzzling results. Both the more ancient investigations of Goslings (1935), subsequently followed by those of Read, Keller & Cabelli (1957), concerning *Klebsiella*, and more recent ones of Sahab (1960), concerning colon bacilli are full of discrepancies as regards the relationship between the presence of capsules, as apparent in the light microscope, and their reaction with the corresponding immune sera. In staphylococci so-called 'swelling of the capsules', which is due to antibody precipitation on the bacteria, could be recorded by Price & Kneeland (1956) and by Lenhart, Li, DeCourcy & Mudd (1962) both with capsulated and non-capsulated strains according to the amount of antibody contained in the antisera used.

In their first investigations concerning *Klebsiella*, Barber, Stamatescu & Wisner (1958), Ciucă, Stamatescu-Eustatziou, Barber, Voinea & Tulpan (1959) and Eustatziou, Barber, Voinea & Tulpan (1960), recorded the existence of a parallelism between the size of the capsules (as apparent in indian ink) and the amount of antigenic material obtained by extraction with 90 % phenol. This caused them to assume at the time that this antigenic material was of capsular origin. However, as regards the specific polysaccharides which could subsequently be extracted in a highly polymerized state and in very large amounts (20–25 %), irrespective of the size of the capsules, the authors stated that these could hardly be located elsewhere than in the cells themselves. Subsequent immunochemical and electron-microscopic investigations confirmed the latter assumption.

In the present work the intracellular site of the specific substance in a strain of pneumococci belonging to Type II is demonstrated by applying the method of selective separation of the specific polymers, as used in former investigations. The results of immunochemical investigations concerning the same strain of pneumococci were reported by Barber, Baldovin-Agapi, Beloiu & Caffé (1959) in another paper.

MATERIAL AND METHODS

Cells of the Copenhagen strain of pneumococcus Type II, washed by centrifugation and dried with acetone, were used for the extraction of antigenic material. Before being subjected to electron micrography the intact cells were hydrated with bi-distilled water and immediately centrifuged (Pl. 1, fig. 1).

The cells were suspended in 90 % phenol and maintained for 24 hr. at 37° C. with frequent stirring, then centrifuged and resuspended in 90 % phenol. After

removal of the phenol layer the bacterial sediment was washed with acetone until any trace of phenol had disappeared.

At this stage (Pl. 1, fig. 2) the pneumococci have lost the material soluble in 90 % phenol amounting to 40–50 % of their weight. The change in electron density of the cells parallels this loss of material.

The polymeric complex insoluble in phenol and formed of specific polysaccharide bound to ribonucleic acid is contained within the pneumococcus cells. In order to obtain adequate electron micrographs in this stage, the organisms must be washed with bi-distilled water and immediately centrifuged, since the material is extremely soluble and passes very rapidly into the water used for washing.

Extraction of the total amount of polymeric specific substance was performed by suspending the organisms in isotonic saline containing 20 % methanol and maintaining them at 37° C. for several hours. After centrifugation 2 vol. of 96 % ethanol were added to the supernatant and a material of fibrous constitution was precipitated which after drying proved highly soluble in water. Exhaustive extraction of this water-soluble material was performed by repeating the operation, and the cells were then subjected to morphological examination (Pl. 1, fig. 3).

After isolation of the polymeric complex in this stage, Heidelberger's soluble specific substance (SSS) was extracted with hot 1 % HCl from the cell walls of the pneumococci. The yield amounted to 7–8 % and the material obtained displayed strong serological activity to a titre of 1 in 5 million.

RESULTS AND DISCUSSION

Examination in the electron microscope of the organisms before and after extraction of the specific substances revealed the occurrence of changes in electron density corresponding to the stages of the extraction.

In the case of the Type II pneumococcus investigated, treatment with 90% phenol dissolves 40–50 % of the dry weight of the organisms. However, the high polymers are not soluble in phenol. The polymeric complex consisting of polysaccharide and ribonucleic acid and corresponding to the 'cellular carbohydrate' of Wadsworth & Brown (1933) is still contained in the cells (Pl. 1, fig. 2).

This material, like the soluble specific substance isolated from the cell wall, displays strong serological activity and is precipitated in 1/5 million dilution by pneumococcus Type II antisera: in absorption reactions the serum saturated with the specific soluble substance still precipitates a large quantity of antibody corresponding to the nucleic acid and the carbohydrate of the complex.

The yield of this material, as isolated from various batches of pneumococci, ranged from 8 to 15 %. The difference in electron density between the stage in which the polymer is still contained in the cell and the stage in which the cell has been depleted of this material is clearly apparent. (It should be taken into account that however rapidly the washed cells are centrifuged at this stage, some of the polymer will be lost owing to its extremely high solubility in water.) At any rate the polymeric specific material isolated from mucoid organisms displays a degree of electron density which permits its demonstration in electron micrographs. If

any such material were present beyond the border marked by the cell wall, it should be demonstrable as well.

The very few electron micrographs of capsules which have been published are of questionable accuracy. In a recent paper of Smith & Metzger (1962) electron micrographs are produced showing a capsular reaction around an organism which is considered to be non-capsulated, i.e. *Listeria monocytogenes*. In this instance it is clearly evident from the description of the method used that precipitation by immune serum was carried out with a raw material including the culture medium, the cultivated germs and the substance excreted into the fluid. Such raw material is by no means adequate for the demonstration of the existence of capsules.

In the course of investigations we are carrying out in staphylococci we were able to obtain electron micrographs of a non-capsulated staphylococcus containing large amounts of polysaccharide, in a stage in which in our conditions of shadow casting the secreted polysaccharide was apparent without the addition of serum in all the preparations (Pl. 2, fig. 4). Moreover, an electron micrograph could be made of the polymeric polysaccharide diffused into distilled water from a highly mucoid strain of *Klebsiella* (Pl. 2, fig. 5).

From these results, which show that polymers are demonstrable in electron micrographs wherever they are present, we consider it justifiable to infer that the failure to demonstrate by electron microscopy the capsules detectable in the light microscope is likely to be due to the absence of polymeric specific material in the respective area. The presence of capsules, as shown by common optical means, is as a rule associated with a marked mucoid character of the germs. This mucoid character is due to the high degree of polymerization and to the large amount of the soluble material contained in the cells. From seven mucoid *Klebsiella* strains displaying capsules of varying sizes in the light microscope, Barber *et al.* (1958), Barber, Eustatziou & Andreica (1961) and Ciucă *et al.* (1959), were able to extract highly polymerized polysaccharide material in amounts ranging from 20 to 25 %, as against only 5–6 % specific substance isolated from non-capsulated mutants. In investigations concerning staphylococci and their mutants which had become capsulated following passages in guinea-pig leukocytes, Barber & Taga (to be published) record differences of a similar type.

The immunological homogeneity of the polysaccharides isolated from strains possessing capsules (as shown by common optical means) and from their mutants has been demonstrated by absorption tests with the corresponding sera.

The appearance of capsules in the light microscope may possibly be due to some particular physical state of the specific substances contained in the cell wall. As a consequence of these physical conditions the density of the area surrounding the bacterial cell may be altered. The polymers are known to be highly hydrophilic and the surface of the bacteria displays a strong negative charge due to the uronic acids contained in these polymers. This may cause changes of refractive index in the medium around the cell, resulting in the appearance of that 'halo' which is the capsule. Even enzymic decapsulation of the bacteria may be accounted for. Depolymerases acting on the specific substances of the bacteria cause depolymerization of these substances and may determine in this way the physical changes

which result in disappearance of the capsule without any impairment of the viability of the germs.

To conclude, additional investigations are required in order to account for the appearance of capsules, as demonstrable in the common light microscope. However, it has been demonstrated that the specific polymeric material assumed to form the capsule is situated within the bacterial cell and that the electron density of this material is sufficiently high to permit its detection. The possibility of obtaining electron micrographs of the polymeric specific substances, as contained in the cells or after their diffusion into the medium, or when secreted in the shape of 'slime' or as *in vitro* solutions, and the fact that the 'specific capsular reaction' is a precipitation of antibody on the bacterial cell, suggest that the current concept of the capsule as the site of the polymeric specific substances may have to be revised.

SUMMARY

Applying a method of selective separation of the specific high polymers the intracellular site of the specific substance in the Copenhagen strain of Pneumococcus Type II was demonstrated, by electron microscopy; the specific polymeric material assumed to form the capsule is situated within the bacterial cell and the electron density of this material is sufficiently high to permit its detection.

The possibility of obtaining electron micrographs of the polymeric substances as contained in the cells or after their diffusion into the medium or when secreted as slime, suggests that the current concept of the capsules as the site of the specific substances may have to be reviewed.

The electron micrographs were made by Dr A. Petrovici to whom I express my thanks.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Pneumococcus Type II. Acetone-dried organisms resuspended in distilled water. (× 15,000.)

Fig. 2. Pneumococcus Type II. Organism treated with 90% phenol, washed with acetone, dried and resuspended in water. (× 15,000.) The polymerized polysaccharide–nucleic acid complex is still contained in the cells.

Fig. 3. Pneumococcus Type II. Organisms from which the polymerized polysaccharide–nucleic acid complex has been extracted. Cell walls. (× 15,000.)

PLATE 2

Fig. 4. Non-capsulated (in the light microscope) strain of *Staphylococcus albus*. The secreted polysaccharide is apparent in electron micrographs.

Fig. 5. A highly mucoid *Klebsiella* strain suspended in water for 24 hr. Diffusion of the polysaccharide is apparent.

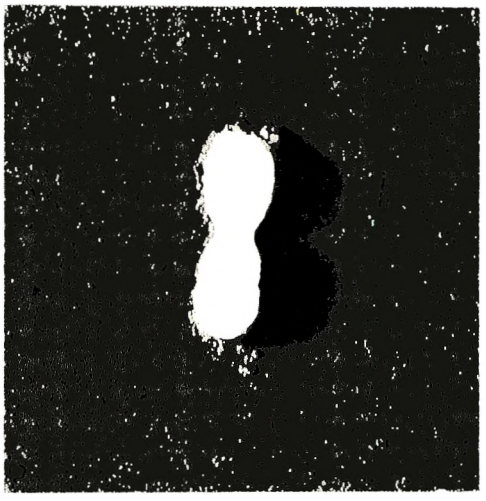

Fig. 1

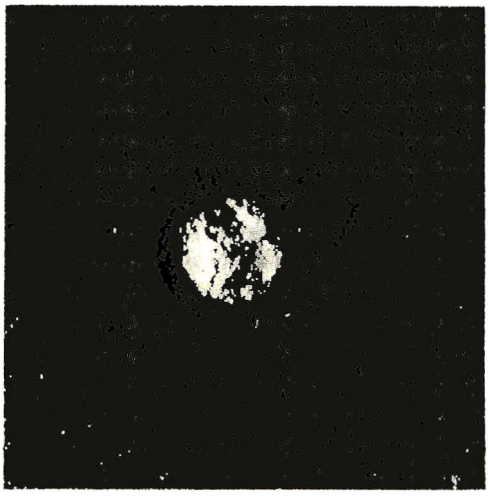

Fig. 2

Fig. 3

Fig. 4

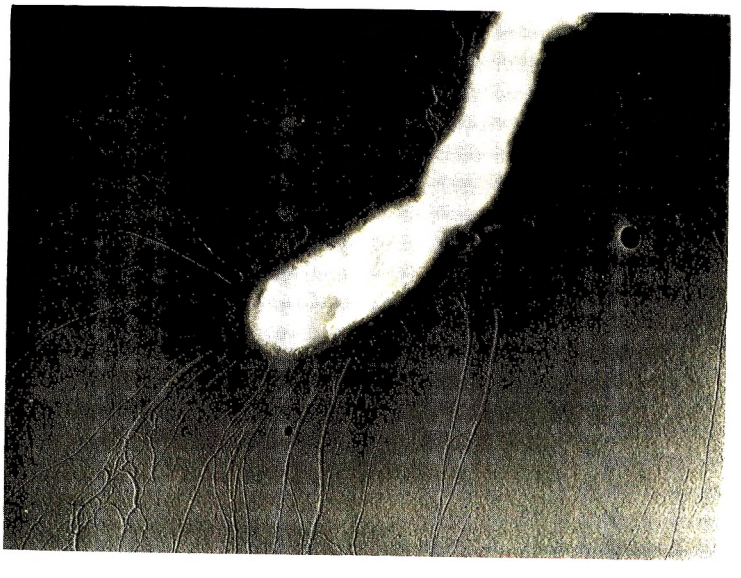

Fig. 5