THE QUANTITATIVE STUDY

OF

FOOT-AND-MOUTH DISEASE VIRUS

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The first attempt at a quantitative study of the virus content of the tissues of an animal affected with foot-and-mouth disease was made by Loeffler and Frosch (1898). They described an experiment designed to de-:termine the quantity of bovine vesicle lymph necessary to infect calves. Their unsuccessful search for a small experimental animal susceptible to the virus of foot-and-mouth disease forced them to use cattle and swine for their work which naturally introduced certain limitations.

It was not until 1920, when Waldmann and Pape re-:ported the successful transmission of the disease to the guinea-pig, that greater opportunity was afforded of studying the properties of the virus in the labor-:atory. Their observations were readily confirmed by other workers and numerous descriptions of the disease in the guinea-pig were published [Hobmaier (1921); Uhlenhuth (1921); Gins and Krause (1924); and Bedson, Burbury and Maitland (1925)]. Since that time the guinea-pig has been extensively and almost solely used for any quantitative studies of the virus that have been made. During recent years, however, work has been proceeding independently in Britain and in Germany with a view to devising a more reliable method/

method of titration of virus suspensions involving the use of cattle instead of guinea-pigs.

The scope of this work is to discuss the necessity for the quantitative study of a filterable virus, the methods available for such a study and those applicable to foot-and-mouth disease virus; to discuss the use of the guinea-pig and its limitations, with the description of the evolution of a method of study using cattle, with a discussion of the effectiveness of the method as at present devised; and to present the information gained as a result of these quantitative studies.

THE/

THE NECESSITY FOR THE QUANTITATIVE STUDY OF A FILTERABLE VIRUS.

Quantitative studies of a virus may be conveniently divided into two parts: (i) those dealing with the virus as it occurs in the body of the affected organism, and (ii) those dealing with the properties of the virus as they are investigated in the laboratory.

A quantitative study of the virus as it occurs in the body of the affected organism is essential for the elucidation of the progress of the disease, for the determination of the sites of multiplication and the tissues affected in the host. The quantitative study of the virus content of the tissues and fluids of the host is particularly important in virus diseases as in many cases these tissues and fluids are the sole source of virus. When the virus is used as an antigen in the production of a vaccine, it is essential to know which is the richest source and at what stage in the course of the disease the virus can be collected in the greatest quantity.

Quantitative determinations are equally important in the investigation of the properties of a virus. The studies in which these are especially valuable may be/

be summarised as follows:

Multiplication in tissue culture.

Survival under different conditions.

Rate of inactivation or neutralization following different treatments.

Centrifugation studies.

Filtration studies.

Tests of the immunising properties of vaccines or sera.

Determination of the potency of virus suspensions used for inoculation of experimental animals.

Purification methods.

Comparison of counts of virus particles with the effect produced on the host.

In vitro tests involving the use of virus suspensions.

METHODS/

METHODS AVAILABLE FOR THE QUANTITATIVE STUDY OF A FILTERABLE VIRUS.

In considering the methods available for work with filterable viruses, it is perhaps relevant to review those available for the estimation of the number of bacteria per unit volume of a bacterial suspension. Briefly these methods are as follows:-

(1) Direct count of suitably stained organisms in a counting chamber.

(2) Comparison of the opacity of the suspension with suitable standards.

(3) Counting the colonies that develop when a suitable medium is inoculated with a measured volume of bacterial suspension.

(4) Progressive dilution of the suspension with a test for the presence of viable bacteria in each dilution by inoculation of a suitable medium or by inoculation of a susceptible animal.

With certain modifications all these methods are applicable to the study of filterable viruses. For example, Parker and Rivers (1936) describe the direct enumeration of the elementary bodies of vaccinia virus by means of a Petroff-Hausser counting chamber and the estimation/

estimation of the number of elementary bodies in a suspension by means of a Gates densitometer. The method of counting bacterial colonies on a suitable medium is essentially the same as that of counting the pocks caused by vaccinia virus on a rabbit's skin or rabbit's cornea, of counting the individual lesions pro-:duced by many viruses on the chorio-allantoic membrane of the developing chick embryo, or of counting the necrotic spots produced by tobacco mosaic virus on the leaves of susceptible plants, although in the case of the filterable viruses the result indicates the number of infectious units and not necessarily the number of particles. The most generally used methods in virus work are those of inoculating susceptible animals with a series of dilutions or, if the virus can be propagat-:ed in the developing egg, of inoculating the chorio-allantoic membrane. The results obtained by either of these methods enable an estimation to be made of the relative concentration of virus in suspensions.

METHODS/

METHODS APPLICABLE TO THE QUANTITATIVE STUDY OF FOOT-AND-MOUTH DISEASE VIRUS.

The small size of the virus particle, 8 to 12 mμ, precludes the use of any method of direct count or comparison of opacities.[1] The most suitable medium for the counting of "virus colonies" is undoubtedly the chorio-allantoic membrane of the developing chick embryo, but efforts to propagate the virus of foot-and-mouth disease by the routine methods of inoculation of the developing egg of the hen or the duck have failed [Galloway and Elford (1933, 1935); Galloway (1937)], although Frenkel and van Waveren (1934) found evidence of multiplication. Richter (1939) was unable to confirm Peragallo's report (1937) that the virus could be propagated if 15-day embryos were used. The only method at present available, therefore, for the routine quantitative/

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[1] Recent advances in microscopy suggest that the particles may be rendered visible, but present difficulties of technique make it unlikely that this will be of immediate practical importance in quantitative studies of the virus.

quantitative study of the virus is that of inoculation of susceptible animals with a series of dilutions of the virus suspension. As already stated, Loeffler and Frosch (1898) used calves as the susceptible animals in their titration of bovine vesicle lymph, but since Waldmann and Pape (1920) reported the successful transmission of the disease to the guinea-pig this animal has been extensively used for titration of virus suspensions. Owing to certain limitations in the employment of the guinea-pig for titration of strains recovered from other species, it has once more been found expedient to use cattle.

THE/

THE USE OF THE GUINEA-PIG FOR THE TITRATION OF SUSPENSIONS OF FOOT-AND-MOUTH DISEASE VIRUS.

Descriptions of the disease as seen in the guinea-pig are too numerous to warrant repetition: all that need be described is the technique as used in this laboratory.

Choice of guinea-pig.

For satisfactory results with foot-and-mouth dis-:ease virus the guinea-pigs used must be in good condition and, in the writer's opinion, should weigh at least 450 gm. Guinea-pigs whose feet have unpigmented pads should be chosen, as black pigmentation of the pads makes it difficult for small lesions to be seen. Since 1940 all the guinea-pigs used at this Research Station have been supplied from a specially selected stock bred and maintained for this purpose in a build-:ing separate from the premises used for experimental work. Such an arrangement ensures that the animals used for experiments are much more uniform than is possible when the requirements are met by various deal-:ers. In addition, the risk of interference by the presence of intercurrent disease is practically ex-:cluded.

Housing/

Housing of the guinea-pigs.

While the guinea-pigs are under experiment they are kept in galvanized metal buckets with straight sides. Three sizes of bucket are used; a small one for three guinea-pigs, 12.5 ins. high and 12 ins. in diameter; one for four guinea-pigs, 14 ins. high and 17 ins. in diameter; and a larger one for six guinea-pigs, 12 ins. high and 24 ins. in diameter. The guinea-pigs are bedded on sawdust in these buckets which are not cleaned out while the animals are under experi-:ment, usually a period of seven days. The buckets are kept on metal racks in rooms with a minimum temp-:erature of 70° F. At the completion of an experiment the guinea-pigs are killed and the carcases burned; the litter from the tins is also burned and the tins, after being washed in a 5 per cent. solution of sodium carbonate, are sterilised in a steam cabinet.

Feeding of the guinea-pigs.

The guinea-pigs are fed twice daily, one meal consisting of freshly cut kale, the other meal con-:sisting of crushed oats and bran. These items may be supplemented with freshly cut grass, chicory or chopped beetroot, according to the season of the year.

Route/

Route of inoculation of the guinea-pigs.

The site chosen for inoculation is the large hair-:less pad of the hind foot. These pads are inoculated intradermally by making tracks in the thickness of the skin by means of an ordinary hypodermic needle, 0.7 by 15 mm., which has had the sharp point filed off (Fig.1). A series of these tracks is made and the inoculum is injected at the same time.

Rate of development of the lesions following intra-:dermal inoculation of the hind pads.

The rate of development of the lesions depends on the potency of the virus suspension and the extent to which the virus strain is adapted to the guinea-pig. Undiluted vesicle lymph of a strain that has had fre-:quent passages in the guinea-pig will produce ex-:tensive lesions on the hind pads within 24 hours and secondary lesions on the pads of the fore feet and on the tongue within 48 hours. With a high dilution of such a strain the lesions will be about 12 to 24 hours later in developing. Undiluted material freshly re-:covered from cattle does not as a rule produce such good reactions as a guinea-pig adapted strain. Some cattle strains may only produce slight lesions after a lapse of 3 or 4 days and secondary lesions may not oc-:cur until the strain has been given a number of passages in the guinea-pig. This question of adapt-:ation/

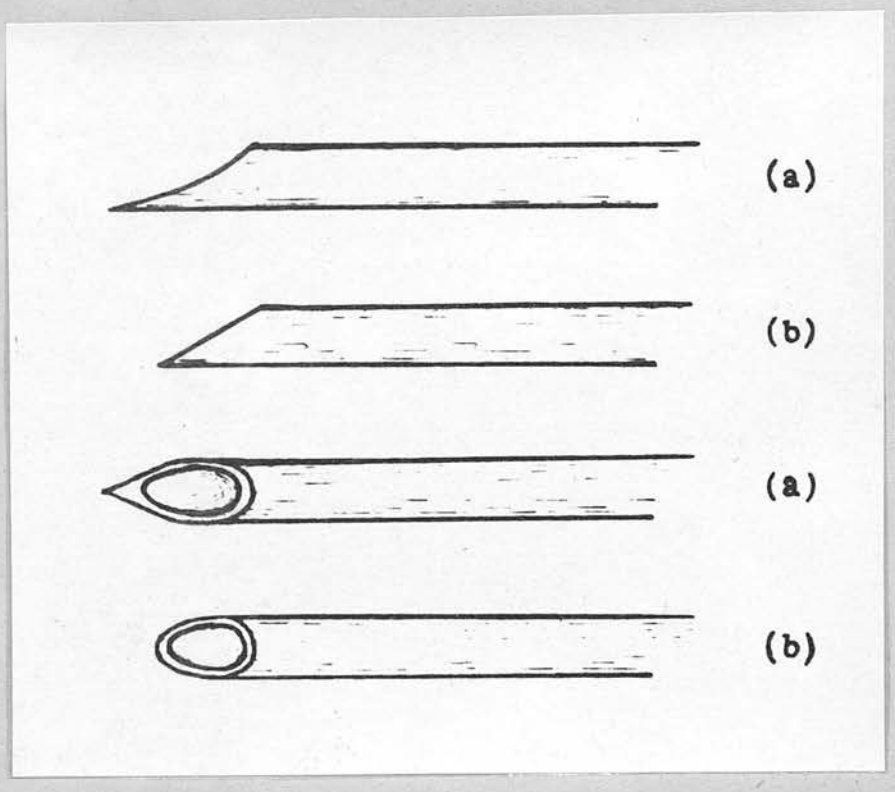


Fig. 1. Conversion of hypodermic needle for intradermal inoculations.

(a) Hypodermic needle.

(b) Same needle with filed-off point.

adaptation is of great importance in considering the usefulness of the guinea-pig for titration of virus suspensions.

Preparation of a series of dilutions of a virus suspension,

The diluent used in all these experiments was either M/25 buffered phosphate saline solution, pH 7.6 (Douglas, Eyre, Laidlaw and Wolf, 1927), digest broth, pH 7.6 (Hartley, 1922), or a mixture of phosphate and broth. For routine titrations in the guinea-pig a tenfold dilution series is usually chosen. This is prepared by adding 0.5 c.c. of the undiluted suspension to 4.5 c.c. of the M/25 buffered phosphate saline solution to make a 1/10 dilution, 0.5 c.c. of this 1/10 dilution is added to another 4.5 c.c. of phosphate solution to make a 1/100 dilution and so on. The dilutions are made up in small test-tubes, 15 by 50 mm., the 4.5 c.c. of phosphate solution being run in from a burette and the 0.5 c.c. volumes measured with 1 c.c. all-glass syringes with needles attached, using a fresh syringe for each dilution. The contents of each tube are mixed by filling and emptying the syringe a number of times.

Titration of a dilution series by intradermal inoculation of guinea-pigs.

For routine determinations of the potency of a suspension where no great accuracy is required, it is customary/

customary to use three guinea-pigs for each dilution of a tenfold series, the three animals for each dilution being kept in a separate tin. More reliable results can be obtained by increasing the number of guinea-pigs used for each dilution and by decreasing the dilution-factor, but in many cases improvement of the experiment in this way is unnecessary and is often restricted by the need for economy in the use of experimental animals. Starting with the highest dilution, both hind pads of each of the three guinea-pigs are inoculated intra-:dermally by making a number of tracks and using a port-:ion of the dilution, the exact quantity of which is not measured as the liquid runs out of the tracks and the volume remaining in situ cannot be estimated, but it is generally found that 1 to 1.5 c.c. is sufficient for each group of three guinea-pigs. The next group of three guinea-pigs is then inoculated with the next lower dilution using the same syringe, and so on until all the dilutions chosen have been inoculated.

The guinea-pig may respond in three ways: (1) it may show no reaction; (2) it may develop prim-:ary lesions at the sites of inoculation; or (3) it may develop primary lesions followed by secondary lesions in the mouth and on the fore feet, usually referred to as "generalization". It is convenient to denote these/

these results by means of symbols, i.e.:-

00	-	no reaction.
+0	-	primary lesions only.
++	-	primary lesions followed by generalization.

The type of result obtained is illustrated by the following titrations of vesicle lymph of stock guinea-pig strains:-

13.3.39.

Vallée O Type guinea-pig adapted strain, titration of filtered vesicle lymph, stored 19 days at 4° c.

10^{-3}	++	++	++
10^{-4}	++	++	++
10^{-5}	++	++	+0
10^{-6}	00	00	00

10.7.39.

Vallée A Type guinea-pig adapted strain, titration of filtered vesicle lymph, stored 24 days at 4° C.

10^{-3}	++	++	++
10^{-4}	++	++	++
10^{-5}	++	++	+0
10^{-6}	++	00	00

11.4.39./

11.4.39.

Waldmann C Type guinea-pig adapted strain, titration of filtered vesicle lymph, stored 34 days at 4° C.

10^{-3}	++	++	++
10^{-4}	++	++	+0
10^{-5}	++	00	00
10^{-6}	00	00	00

These examples are given only to show the type of result that may be obtained when titrating strains that are well adapted to the guinea-pig. They should not be regarded as being indicative of the relative potenc-:ies of the three virus strains: this will be dealt with more fully in a later section.

With these well adapted strains, using tenfold dil-:utions, a fair degree of regularity may be expected, provided the animals used are in good condition and weigh at least 450 gm. The importance of condition and weight has already been stressed by many workers (Bedson, Burbury and Maitland, 1925; Bedson, Maitland and Burbury, 1927; Waldmann and Trautwein, 1929; and Edwards, 1937). All are not in agreement regarding a minimum weight of 450 gm. For example, Waldmann and Trautwein recommend 350 to 450 gm., but in the writer's opinion the heavier weight should be taken as a minimum standard, especially if strains of poor adaptation to the guinea-pig are being used.

The adaptation of strains of foot-and-mouth disease virus to the guinea-pig and its influence on the reliability of the use of the guinea-pig for quant-:itative studies.

It is well known that a strain of virus recovered from cattle or pigs may not necessarily produce good lesions when first inoculated into guinea-pigs and that a number of serial passages in the guinea-pig may be necessary before good primary vesicles occur which are regularly followed by secondary lesions (Stockman et al., 1927; Burbury, 1928; and Andrews et al., 1931, 1937). In the writer's own experience with tests for determin-:ation of the immunological type of strains recovered from field outbreaks it was found that of 25 strains of bovine origin the number of passages required for guinea-pig adaptation were as follows.

Passages	Strains
2	6
3	4
4	4
5	3
6	1
7	1
8/	

Passages (continued)	Strains (continued)
8	1
9	2
12	1
14	1

One strain showed no reaction in the guinea-pig even after seven passages.

The difficulty of producing the typical disease in the guinea-pig is not altogether a question of the low virus content of the original material, as material which may produce only a poor lesion in the guinea-pig may produce a severe reaction when inoculated into cattle. On the other hand, there is no doubt that the state of the bovine lesions at the time when the material is collected is of importance. With a "refractory strain" it has sometimes been found that adaptation to the guinea-pig is more easily and rapidly attained if the original bovine material as received from the field is given one passage in cattle by intradermal inoculation of the tongue, followed by collection of epithelium from fresh unruptured vesicles (Andrews et al., 1937; and Henderson, unpublished work).

In Great Britain, where the present policy for the control of foot-and-mouth disease involves the immediate slaughter of all the animals in the affected herd, material for the recovery of virus has to be collected regardless/

regardless of the stage of the disease. The animals cannot be spared until one of them shows fresh unruptur-:ed tongue lesions which would provide the best material for the laboratory. The position is different where the slaughter policy is not applied. In a circular issued by the Reichs Minister of the Interior to the veterinary staff in the field in Germany (1944), the im-:portance is stressed of the collection of suitable material for the laboratory: examination of all the animals in the affected herd must be continued until one is found with lesions at a suitable stage of development.

Nevertheless, as will be clearly demonstrated lat-:er, a suspension of freshly collected material from one species of animal will not necessarily produce dis-:ease of the same severity or have the same limiting infective dilution when inoculated into animals of an-:other species. As a general rule, it is necessary to give the strain a number of serial passages before ad-:aptation to the heterologous species is complete.

As a corollary to this, it will be appreciated that if, for example, freshly collected bovine material is titrated in guinea-pigs in an attempt to determine its virus content, then the amount of virus as detected by the reaction in guinea-pigs will be much less than that actually present, or would be detected, if the homolog-:ous species of animal was used for the titration.

This/

This lower titre of a virus suspension as indicated by the results of tests in guinea-pigs would not be a great disadvantage if the end-point obtained in guinea-pigs was always lower to the same degree. The guinea-pig could then be usefully employed for comparison of the relative virus content of different suspensions. It has been found from a considerable number of observ-:ations that consistent results are not to be expected. Not only may there be individual differences between strains as regards the relative titres recorded in guinea-pigs and cattle respectively, but there appears to be appreciable variation in the behaviour, from time to time, of the same strain when tested in guinea-pigs. Another disadvantage is that, with many cattle strains, adaptation to the guinea-pig at the first passage is so poor that any dilution of the suspension will prevent reactions occurring, thus imposing a severe limitation on comparison by titration. Trautwein (1929), in re-:viewing the results obtained by different workers in their titrations of foot-and-mouth disease virus sus-:pensions in the guinea-pig, comments that one factor which must be considered in explaining the variation in the titres recorded is the degree of adaptation of the strain to the guinea-pig.

There is no doubt that the guinea-pig is an extreme-:ly/

extremely useful small experimental animal for work with foot-and-mouth disease virus when a guinea-pig adapted strain is being used, but there are severe limitations to its use for any quantitative work with strains that are not so adapted. It will be shown later that the converse is true, namely that the end-point in a titration of a well adapted guinea-pig strain may be lower in cattle than in guinea-pigs.

The necessity for quantitative studies of bovine strains in a programme of immunity experiments in cattle was the main reason for the investigation of the pos-:sibility of the economical use of cattle for such work. A satisfactory method has been evolved and its evolut-:ion will now be described.

THE/

THE USE OF CATTLE FOR THE QUANTITATIVE STUDY OF FOOT-AND-MOUTH DISEASE VIRUS.

As has already been mentioned, the first quantit-:ative study of foot-and-mouth disease virus was made by Loeffler and Frosch who used calves for their work. They prepared dilutions of bovine vesicle lymph in boiled tap-water and inoculated calves with these dil-:utions. In the section of their report (1898) dealing with this experiment they do not mention the route of inoculation, but from the description of their other work it is probable that the method employed was to rub in a small drop of lymph on to the lightly scarified mucous membrane of the upper and lower lips.

They found that 1/5,000 c.c. of lymph gave positive results, that 1/10,000 to 1/20,000 c.c. gave uncertain results, and that 1/50,000 to 1/100,000 c.c. gave neg-:ative results. The purpose of their experiment was, not to estimate the virus content of the particular sample of lymph that they were using, but to find out the quantity of lymph necessary to infect calves.

In 1938, in a private communication to a represent-:ative of the British Foot-and-Mouth Disease Research Committee, Waldmann, Director of the German Foot-and-Mouth Disease Research Institute, stated that he and his colleagues/

colleagues were using cattle for the titration of virus suspensions by scarification of the mucous membrane of the mouth with more than one dilution, but no details of the technique or of the results obtained were given. Waldmann and Nagel (1939) state that by far the most certain method of demonstrating the presence of foot-and-mouth disease virus is by introduction of the virul-:ent material on to the scarified mucous membrane of the mouth of cattle. No mention is made of the use of this route in titration experiments, but they cite Wolf as titrating virus suspensions by the intradermal in-:jection of the tongue of healthy cattle: no details of the technique were given.

It was never in doubt that virus suspensions could be titrated by using cattle as test animals, but some uncertainty was felt as to the possibility of inoculat-:ing more than one dilution on the same tongue. If a separate animal was used for each dilution, it is obvious that the number of cattle required would make their use quite uneconomical.

Before describing the experiments in which cattle were used for quantitative determinations, details will be given of the source of supply and housing of these animals, the method of inoculation and the development of the disease following inoculation.

The/

The supply and housing of the cattle used in these experiments.

All the cattle used in these experiments were Devon steers, about 18 months old. When required for experiment they were brought to the Research Station from the premises of a cattle dealer who had obtained them previously from various parts of southern England. Because of the rigid control of foot-and-mouth disease in England, Wales and Scotland by the Veterinary Staff of the Animal Health Division of the Ministry of Agriculture and Fisheries, it is certain that the animals had not previously suffered from the disease, and, apart from the remote possibility of having encountered a sub-infective dose of virus or possessing some inherent natural immunity, they can all be considered to be fully susceptible at the time of reaching Pirbright.

Every precaution is taken to prevent the accidental spread of infection amongst the cattle while under experiment. They are housed in loose boxes which are so designed that the animals can be watered and fed without the attendant having to enter the box (see Plans, First Progress Report, Foot-and-Mouth Disease Research Committee, 1925). These loose boxes are arranged in units forming separate compounds, before entering any of which each person dons rubber clothing. This rubber clothing may readily be sprayed with a disinfectant/

disinfectant solution and when this is done before entering or leaving the loose boxes in addition to disinfection of the hands, the disease can be kept strictly confined to each box. Portions of this rubber clothing are shown in Fig.5. (See page 34b.)

The method of inoculation of the cattle.

All the inoculations in these experiments are by the intradermal route and the site chosen is the animal's tongue. The same type of needle is used as has already been described for the intradermal inoculation of guinea-pigs.

The tongue is grasped near the tip with a piece of rough cloth such as Turkish towelling: this enables it to be pulled sufficiently far out of the mouth to make the free portion of the tongue between the tip and the dorsum readily accessible for inoculation (Fig.5). The needle attached to the syringe is used to make tracks through the superficial layers of the thick epidermal covering of the tongue, the inoculum being injected at the same time as these tracks are made, and in this way weals may occasionally be raised (Fig.2). The liquid runs easily out of the tracks, therefore the actual volume of the inoculum that remains *in situ* cannot be estimated with accuracy, unless each site of inoculation receives no more than about 0.2c.c., but

even/

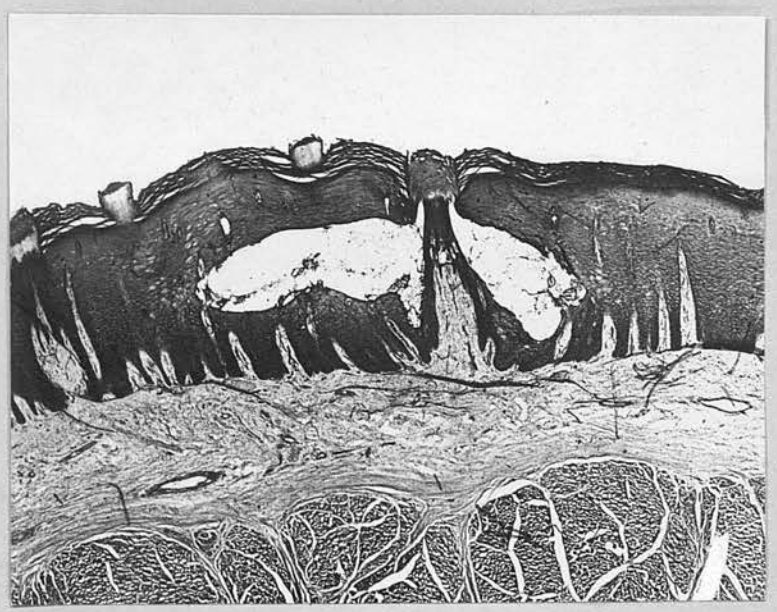


Fig. 2. Section of the mucous membrane of a steer's tongue inoculated intra-:dermally with indian ink. Animal killed 20 minutes after inoculation. The cavity in the epithelial layer indicates the position of the bleb of inoculum which has been lost during the preparation of the section.

H. and E. x 17.

even then some liquid may be observed to seep out of the end of the track.

In most cases, sufficient restraint of the animal can be obtained for these inoculations if the beast is secured by a halter to a ring in the wall of the loose box, with an assistant holding the animal's head by the nostrils and one horn. For more delicate work, when a large number of carefully arranged injections have to be made, this method of restraint is insufficient. Instead, the animal is narcotized by an intravenous dose of the barbiturate Pentothal sodium and the injections are made while the animal is under the influence of this drug. A description of the use of this drug as a narcotic in cattle has already been published (Henderson, 1944).

Development of the disease in cattle following intradermal inoculation of the tongue. The intradermal inoculation of the tongue with a suspension of active virus is followed, usually within 24 hours, by the development of a vesicle at the site of inoculation. The first sign of this reaction is a small area of blanched epithelium which gradually extends, and at the same time the epithelium at this spot becomes raised up in the form of a vesicle containing clear, straw-coloured lymph. This vesicle denotes a site of active multiplication of the virus and within a few hours/

hours the virus gains the blood stream and so reaches the other sites of predilection where secondary lesions then develop. These secondary lesions may be observed on the tongue at sites other than those already occup-:ied by the primary lesions as well as on the lips and feet and usually develop within twelve to twenty-four hours later. In the normal course of the disease, the development of the lesions is accompanied by a febrile reaction which may be noted slightly before, or coin-:cident with, the appearance of the lesions. It has been found, however, that when a weak dilution of a virus suspension is used for the inoculum the temperat-:ure does not rise until some hours after the develop-:ment of the primary lesions and the febrile reaction is more an indication of approaching generalization.

INOCULATION/

INOCULATION OF ONE DILUTION.

The first of the experiments in this present work in which cattle were used was to determine whether there was any difference in the end-point of a titration of infective bovine blood when titrated in cattle and in guinea-pigs. Tenfold dilutions of the blood were made and, although only one animal was used for each dilution in the cattle experiment, a very interesting result was obtained in comparison with the result using guinea-pigs.

2.7.41.

Titration of pooled samples of defibrinated bovine blood, stored 8 to 20 days at +4° C. Virus strain No. 336, Vallée O type.

Titration using guinea-pigs.

Undiluted	++	++	+0
10^{-1}	++	00	00
10^{-2}	00	00	00
10^{-3}	00	00	00

Titration using cattle.

10^{-1}	++	(C/W 37)
10^{-2}	++	(C/W 39)
10^{-3}	++	(C/W 40)
10^{-4}	00	(C/W 38)

It was immediately apparent that the result obtain-

:ed/

obtained by using guinea-pigs was no indication of the virus content of the blood as shown by the titration in cattle, thus demonstrating the necessity for using cattle for this particular strain if an accurate result was required.

REPEATED/

REPEATED INOCULATION OF ONE DILUTION.

In an effort to economise in the number of cattle required, the following method of inoculation was tried:

A series of dilutions was made of the virus suspension and a dilution that would probably be non-infective was selected. This dilution was then inoculated into the tongue of one animal, and if no reaction had appeared within two days the next lower dilution was inoculated into another portion of the tongue, and so on until a dilution was reached that gave a positive result. For example:-

15.11.41.

Repeated inoculation of dilutions of pooled samples of defibrinated bovine blood stored 2 to 4 days at +4° C. Virus strain No. 39, Vallée O type.

Steer No. C/X 87 (Fig. 3).

15.11.41.
Inoculated with the 10^{-5} dilution -

Negative.

17.11.41.
Inoculated with the 10^{-4} dilution -

Negative.

20.11.41.
Inoculated with the 10^{-3} dilution -

Reacted.

The/

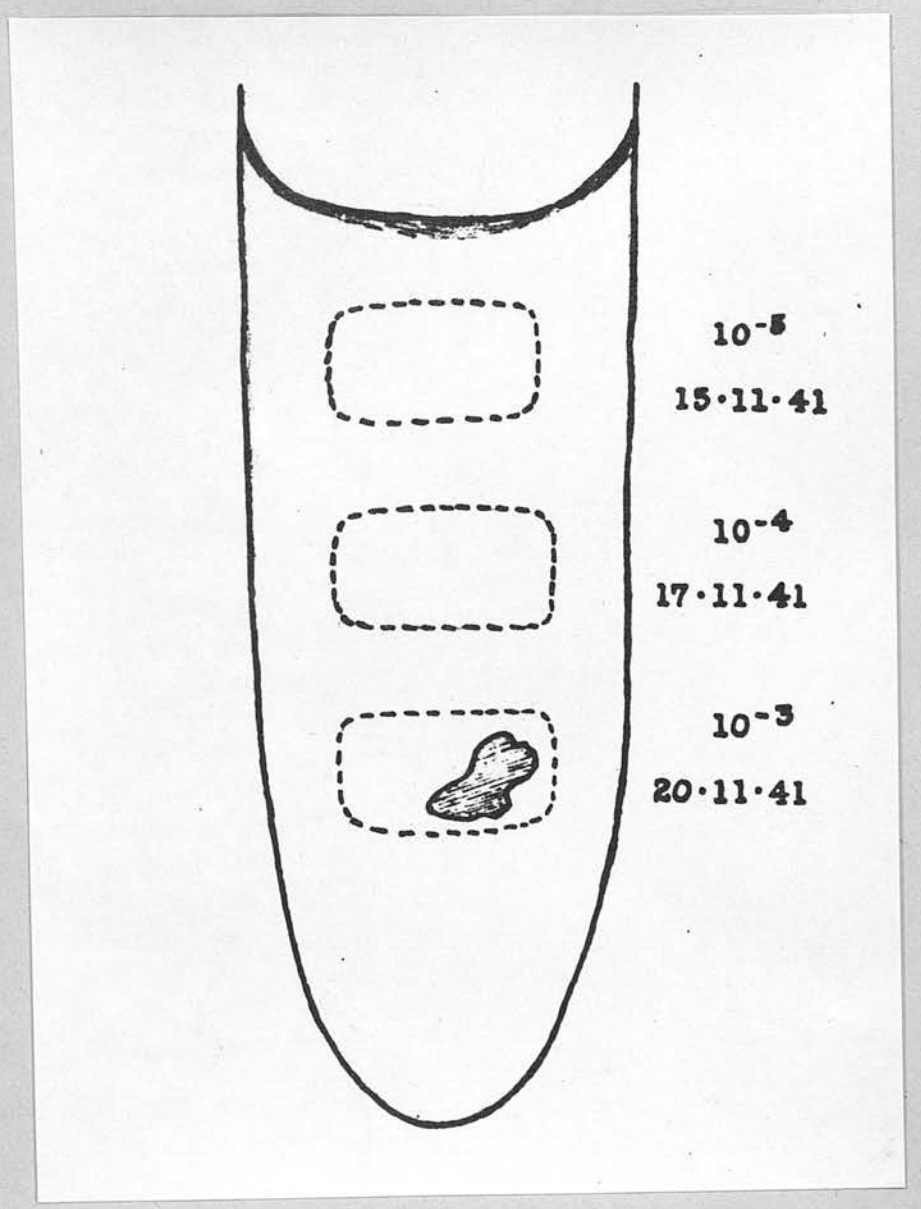


Fig. 3. C/X 87. Repeated inoculation of one dilution.

In this and subsequent diagrams the dotted lines indicate the sites of inoculation and the shaded areas represent lesions.

The titration in guinea-pigs of the same blood sample is shown below.

10^{-1}	++	+0	+0
10^{-2}	00	00	00
10^{-3}	00	00	00

This method has several disadvantages.

(1) With a virus suspension of unknown strength the first dilution to be chosen may be infective, in which case, unless a fresh start be made, no end-point is obtained.

(2) When one or more dilutions have to be inoculat-:ed before an infective one is reached, then, either the dilutions prepared originally must be stored, or else a fresh dilution series must be made up each time from the stored suspension. In both cases the result of the titration may be vitiated by loss of virus pot-:ency during such storage. If a number of dilutions have to be tried before one is found to be infective, a considerable delay is caused, as at least two days must be allowed to elapse before the result of an inoculation can be regarded as negative.

(3) In inoculating an animal with progressively lower dilutions until one is reached which proves in-:fective/

infective, it is possible that a dilution which proves non-infective may nevertheless contain sufficient virus to stimulate an immunological response and so render the animal less susceptible to a subsequent dose of virus which might have produced the disease in a fresh susceptible animal.

(4) A satisfactory result could never be obtained with this method by using only one animal. A positive result with a certain dilution might mean that the concentration of virus particles was such that this dilution would always prove infective, or it might mean that the concentration of virus particles was minimal and that there would be little probability of a sample of this dilution causing infection, the result being more likely to be affected by chance.

Eight virus suspensions comprising different strains were titrated in guinea-pigs and in each case one steer was inoculated in the manner just described. In three of the tests an end-point was not obtained, but in all the other five tests the steer reacted to a dilution at least tenfold higher than the highest dilution to produce a reaction in a group of three guinea-pigs: on two occasions the difference was one hundredfold. Even considering the question of chance reactions to dilutions containing a small amount of virus/

virus, these experiments seem to indicate that the end-point of a titration of a bovine strain in cattle is likely to be higher than that obtained using guinea-pigs.

SIMULTANEOUS/

SIMULTANEOUS INOCULATION OF DIFFERENT DILUTIONS.

Following the trial of repeated inoculations of different dilutions, a method was tried of inoculating the various dilutions at the same time. This proced-:ure is similar to that used for the routine titration of vaccinia virus by the simultaneous inoculation of the shaved skin of the rabbit with a number of differ-:ent dilutions. A certain amount of doubt was felt before attempting to adapt this technique to the intra-:dermal inoculation of the bovine tongue with a sus-:pension of foot-and-mouth disease virus as it was realized that it would be impossible to be certain that no inoculum escaped from the site of inoculation and spread over the tongue. If this should happen there would be a risk that the sites of inoculation of the higher dilutions would be contaminated with, and might react to, the virus in a lower dilution. It was soon found, however, that such contamination did not occur, even although excess liquid did escape from the inoc-:ulated areas, and there was every reason to believe that a primary reaction at a site of inoculation was due to the injection made at that site. The inference of the emphasis on the word 'primary' will be more ob-:vious when a description has been given of the method.

Method/

Method adopted for the simultaneous inoculation of different dilutions.

In the first experiment in which this method was used the cattle were inoculated in the following manner. The tongue was drawn out of the mouth and a number of circles were drawn on it with an indelible pencil between the dorsum and the tip, corresponding to the number of dilutions to be inoculated which could be any number up to six. Each circle was between 2 and 3 cm. in diameter, and the circles were arranged as shown in Fig. 4. To minimize contamination of a higher dilution with a low dilution, the highest dilution was inoculated first by making a number of needle tracks within the circle nearest the dorsum. The lower dilutions were then inoculated in series until the lowest was inoculated last within the circle nearest the tip of the tongue: about 1 c.c. of inoculum was used while making the tracks at each site. This method of inoculation is illustrated in Fig. 5.

The type of reaction obtained is shown in the following example.

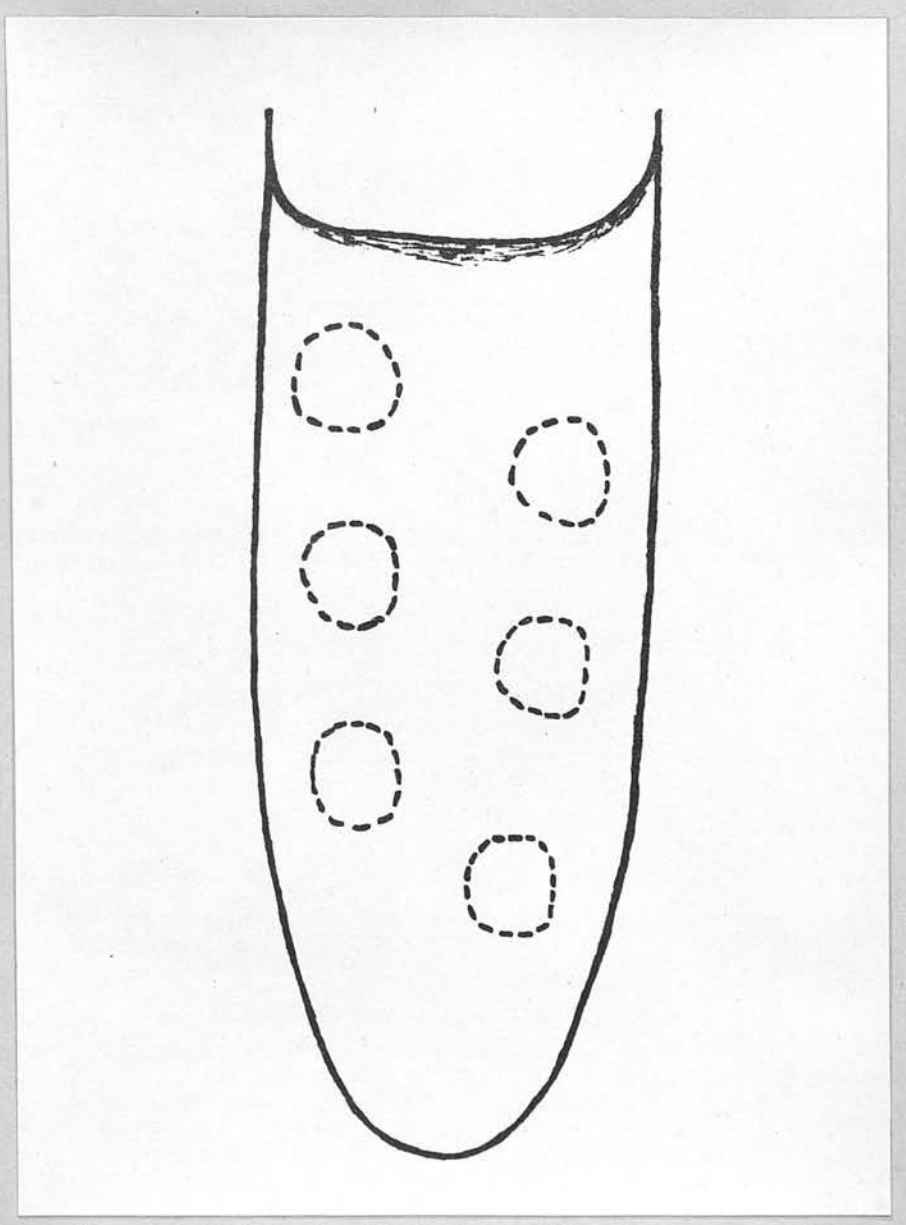


Fig. 4. Simultaneous inoculation of dif-:ferent dilutions, arrangement of six inoculation sites.

Fig. 5. Simultaneous inoculation of four dif-:ferent dilutions. The circles drawn on the tongue mark the inoc-:ulation sites, one to each dilution. The first site to be inoculated is not visible, being just inside the steer's mouth.

22.6.42.

Simultaneous inoculation of different dilutions of pooled samples of defibrinated bovine blood, stored 4 to 10 days at 4°C.
Virus strain No. 39, Vallée O type.

Steer No. C/Z 94 (Fig. 6).

Date	Time	Temp-:erat-:ure °F.	Remarks
22.6.42	12 noon	102	Inoculated i/d with the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions, 10^{-4} first nearest the dorsum of the tongue.
23.6.42.	10 am.	101	Lesions developing at the 10^{-1} and the 10^{-2} sites of inocul-:ation. Small blanched areas at the 10^{-3} site.
	4 p.m.	102.8	Unruptured vesicles at the 10^{-1} 10^{-2} and 10^{-3} sites. 10^{-4} site negative.
24.6.42	10 a.m.	105	The three vesicles had now coalesced to form one large lesion with considerable ex-:tensions from the original sites. 10^{-4} negative. Ex-:tensive lesions on both lips. Developing lesions on all the feet.

In this case the highest dilution to produce a reaction was 10^{-3}; the same dilutions were inoculated into groups of three guinea-pigs and in these animals the 10^{-1} dilution was the highest to produce a reaction.

With/

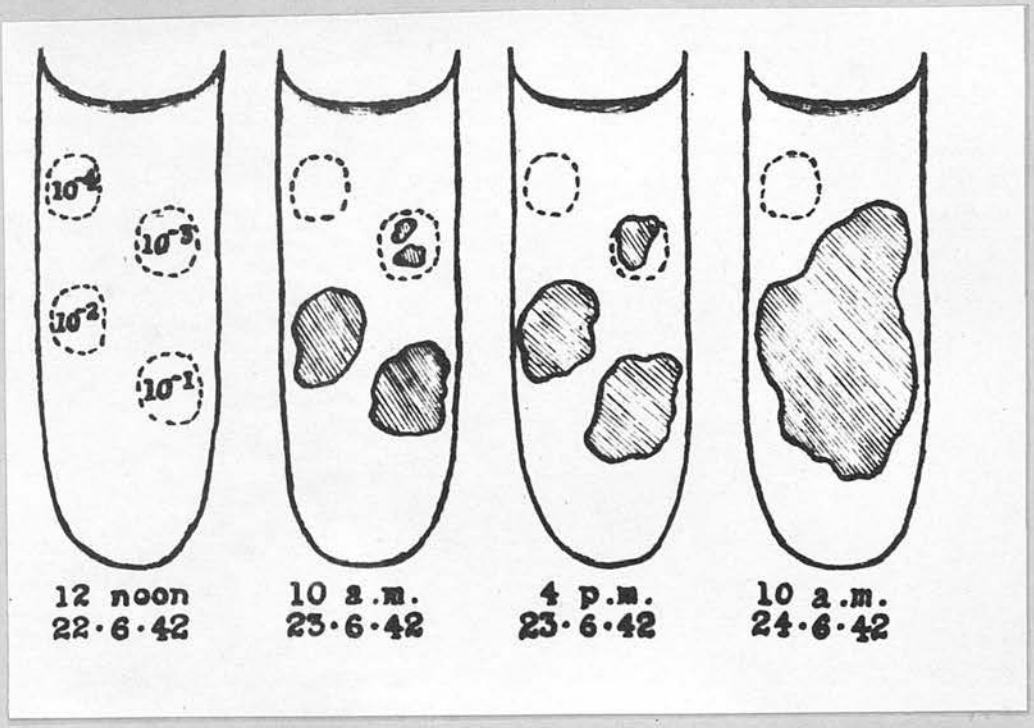


Fig. 6. C/Z 94. Simultaneous inoculation of four dilutions of a tenfold series.

With this method in cattle there is a limit to the length of time during which the sites of inoculation should be examined for developing lesions, as whenever a secondary lesion is detected, either in the mouth or on the feet, then there can be no certainty that a lesion developing at a site of inoculation is due to the inoc-:ulum or, on the contrary, is the result of generaliz-:ation. For this reason examinations should be frequent, if possible, every four to six hours after the first eighteen to twenty hours until secondary lesions are detected, which will probably not be before about the thirty-sixth hour. It has been found that a con-:venient routine is to inoculate the cattle at 3 p.m., then examine them at 10 a.m., 4 p.m. and 10 p.m. on the following day. By 10 p.m. on the second day the temp-:erature of the animal may be about 104 or 105° F. which, from experience, is an indication that secondary lesions will soon be appearing. A further examination is made the following morning by which time generaliz-:ation has usually occurred. This routine, however, must be adapted to the behaviour of the strain which is being used, as a very virulent strain may require earlier examination owing to the quicker development of primary and secondary lesions.

A disadvantage of this method of inoculation with the/

the highest dilution nearest the dorsum is that the lower dilutions, containing more virus, react more quickly, therefore the sites near the tip of the tongue become covered with lesions at a time when most attent-:ion has to be paid to the sites further back. These lesions near the tip make it very difficult to grasp the tongue in order to draw it far enough out of the mouth to allow inspection to be made of the sites nearer the dorsum. For this reason the arrangement of the dilut-:ions was changed so that the lowest were nearest the dorsum, thus leaving the last sites to react near the tip where they can be easily examined. The first animal inoculated using this modified arrangement of the dilutions was done starting with the highest dilut-:ion and working back the tongue to the lowest dilution: this sequence was not nearly so easy to follow as that of starting near the dorsum and working to the tip. A further modification was therefore made by inoculating the lowest dilution first nearest the dorsum and, taking a fresh syringe for each dilution, the inoculations were made progressively nearer the tip of the tongue. By this time the practice of marking a circle as a guide to the inoculation had been discontinued as it was found that the arrangement of the dilutions could be made just as well without this aid.

This/

This modified arrangement and sequence of inoculation is illustrated by the following example.

17.9.42.

Simultaneous inoculation of different dilutions of pooled samples of defibrinated bovine blood, stored 7 to 12 days at 4° C.
Virus strain No. 39, Vallée O type.

Steer No. C/28 B (Fig. 7).

Date	Time	Temperature °F.	Remarks
17.9.42.	12 noon	102	Inoculated i/d with the undiluted blood, the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions, the undiluted blood being inoculated first near the dorsum of the tongue.
	10 p.m.	102	Examined: negative.
18.9.42	10 a.m.	102.4	Unruptures vesicles at the undiluted, 10^{-1} and 10^{-2} sites. Developing lesions at 10^{-3}.
	4 p.m.	102.4	Extensions of the undiluted 10^{-1}, 10^{-2}, and 10^{-3} lesions so that they almost form one large vesicle; the undiluted lesions ruptured; 10^{-4} and 10^{-5} sites negative. No secondary lesions observed.
19.9.42	10 a.m.	104.4	The whole of the surface of the tongue, from the dorsum to the tip, involved in one extensive lesion. Ruptured lesion lower lip. Developing lesions on all the feet.

In/

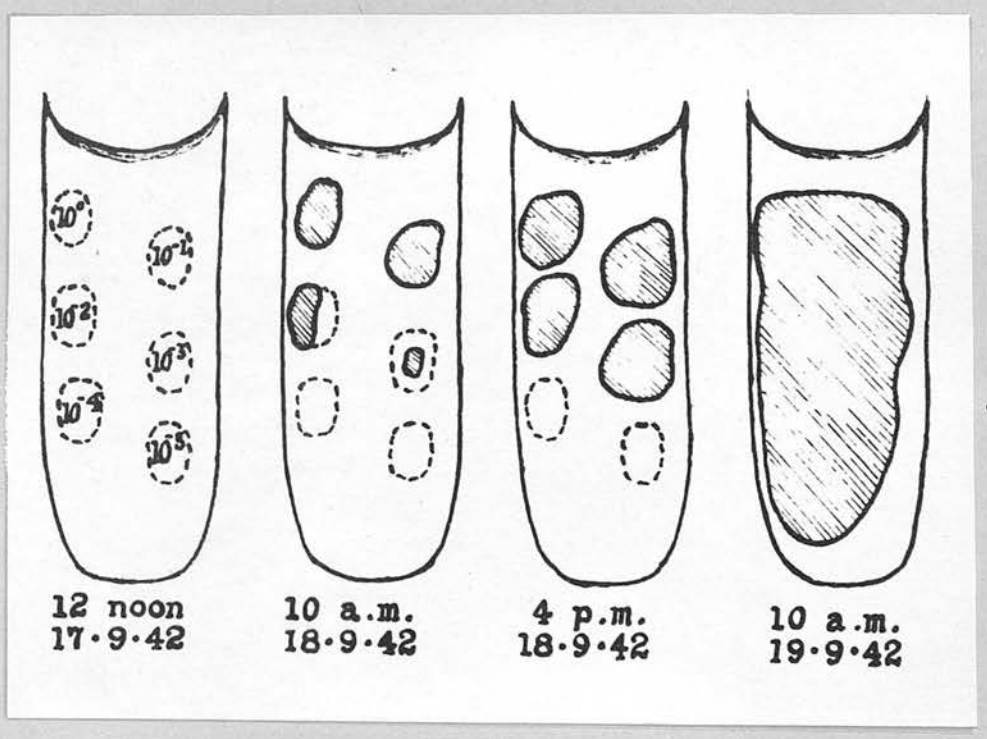


Fig. 7. C/28 B. Simultaneous inoculation of six dilutions of a tenfold series.

In the absence of reactions at the 10^{-4} and 10^{-5} sites prior to the appearance of secondary lesions, it must be taken that the highest dilution to produce a reaction was 10^{-3}.

Inoculation of groups of guinea-pigs with the same dilutions gave the following result.

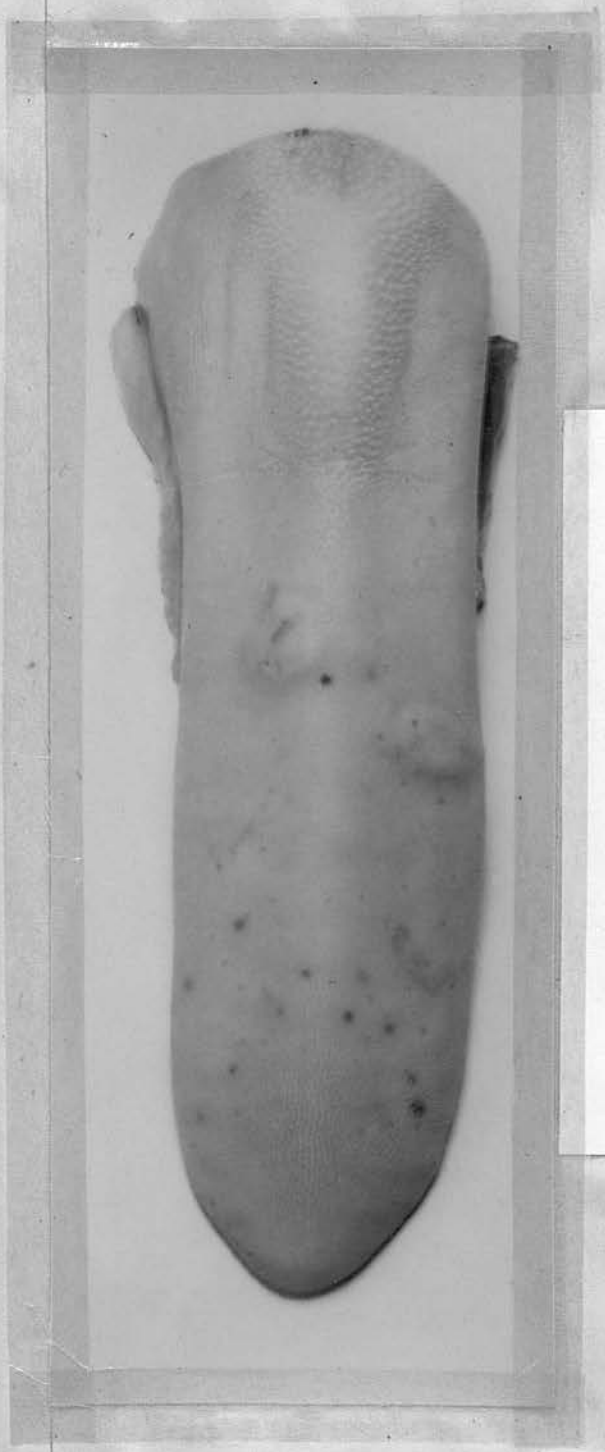
Undiluted	++	++	++
10^{-1}	++	++	00
10^{-2}	00	00	00
10^{-3}	00	00	00

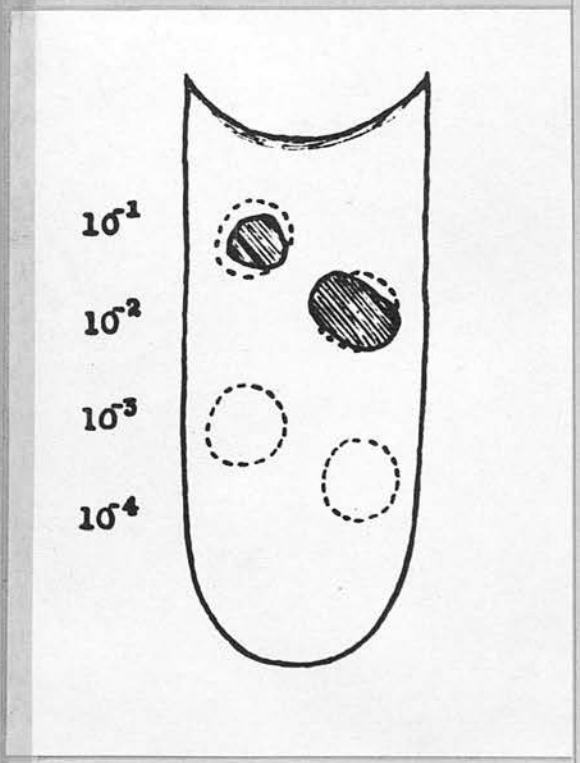
The type of reaction that is obtained with this method when four inoculation sites are used is shown in Fig. 8.

In five preliminary trials, two of which have just been described, only one steer was used in each case. As it was now apparent that this method of inoculation was likely to prove useful, consideration had to be given to the number of animals required for a titration and to the manner in which the results should be inter-:preted.

When each dilution is inoculated an area of at least 5 sq.cm. is subjected to numerous needle tracks and about 1 c.c. of the inoculum is run into these tracks under slight pressure which occasionally raises

a/





Key to Fig. 8.

Fig. 8. Bovine tongue, 20 hours after the simultaneous inoculation of four dilutions of a tenfold series, one site for each dilution.

a small bleb (Fig. 2). From the tracks and blebs the inoculum penetrates between the cells of the surrounding tissue (Fig. 9). In this way it is brought into con-:tact with a considerable amount of susceptible tissue and, if the concentration of virus particles is great enough to constitute an infectious unit in the sample of the dilution that is used, a reaction may be expected to occur. An "infectious unit" has been defined by Parker (1938) for vaccinia virus as being "the smallest amount of the active agent capable of initiating infect-:ion when inoculated under certain conditions into a specified host": the same definition is also applicable to foot-and-mouth disease virus. By using a tenfold dilution series the difference in virus concentration between one dilution and the next is comparatively great so that if one dilution just contains sufficient virus particles to constitute one infectious unit, correspond-:ing to this method of inoculation, there is little probability of the dilution tenfold higher containing sufficient virus particles to provoke a reaction. If a dilution has a sufficient concentration of virus particles to constitute a number of infectious units, then the tenfold higher dilution may or may not have a sufficient number to cause a reaction, but it is highly probable that the hundredfold weaker dilution will give a negative result. In other words, to

use/

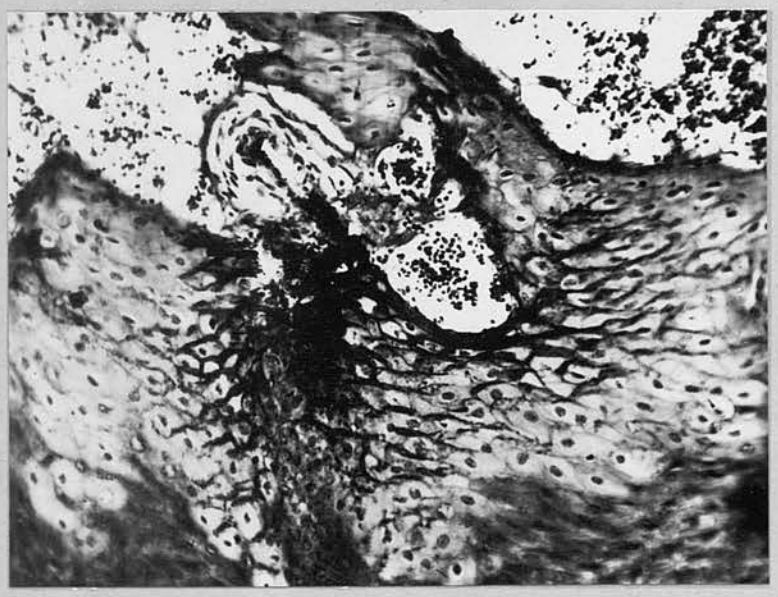


Fig. 9. Section of the mucous membrane of a steer's tongue inoculated intra-:dermally with indian ink. A higher magnification of a portion of Fig. 2 showing the tissue ad-:jacent to the inoculation bleb. The lower half of the photograph shows penetration of the indian ink between the epithelium-cells.

H. and E. x 180.

use the phrase of Doerr and Seidenberg (1933), a zone of inconstant result ("Zone der inkonstanten Resultate") is not likely to occur if a tenfold dilution series is used for inoculation, by one of the most certain routes of artificial infection, of animals of high susceptibil-:ity with a virulent virus: in this particular case, the intradermal inoculation of the tongue of cattle with unattenuated bovine strains of foot-and-mouth disease virus.

A total of fifty-three cattle has been used for this simultaneous method of inoculation of from four to six tenfold dilutions of bovine virus suspensions, and in every case there has been a clear cut demarcation between positive and negative results asfar as the indiv-:idual animals were concerned. This does not take into account, however, the question of the individual susceptibility of the cattle, and, although there may be no zone of inconstant result, there may not be agree-:ment as to the highest dilution to cause a reaction if more than one animal is used. Neither does it take into account the fact that only one sample of the dil-:ution is being used for each inoculation. Thus, if the total volume of the dilution does not contain many virus particles, then the chance variation of the number of particles in each sample withdrawn from the total volume/

volume of the dilution will influence the result. As neither of these two factors can be controlled, it is possibly as much a matter of chance that the end-point in two animals should be the same as that it should be dif-:ferent.

Sixteen titrations of bovine material have been made using more than one animal. In six instances there was agreement; in eight instances there was a ten-:fold difference; and in the remaining two instances the difference was one hundredfold: the details of these results are given in Table I.

TABLE/

TABLE I.

SIMULTANEOUS INOCULATION OF DIFFERENT DILUTIONS. SUMMARY OF TITRATION RESULTS IN INDIVIDUAL ANIMALS.

Date	Material	Animal No.	Highest Dilution To React
Strain No. 39, Vallée O Type.			
27.7.42	Blood	C/27A	10^{-3}
		C/28A	(1) 10^{-4} (2) 10^{-3}
3.9.42.	1/5 Suspension Epithelium	C/94A	10^{-3}
		C/95A	10^{-5}
23.9.42	1/5 Suspension Epithelium	C/29B	10^{-6}
		C/30B	10^{-6}
30.11.42	Blood	C/82B	Undiluted
		C/47B (Type A Immune)	10^{-1}
12.12.42 / 15.12.42	Blood	C/16C	10^{-2}
		C/20C	10^{-3}
Strain No. 119, Vallée A Type.			
22.10.42	1/5 Suspension Epithelium	C/49B	10^{-5}
		C/50B	10^{-6}
2.11.42	Blood	C/57B	10^{-3}
		C/58B	10^{-2}
10.5.43	Blood	C/50D	10^{-3}
		C/51D	10^{-3}
28.7.43	Blood	C/52E	10^{-3}
		C/53E	10^{-4}
7.1.44	Blood	C/60H	10^{-3}
		C/61H	10^{-3}
1.3.44/			

TABLE I. (contd.)

Date	Material	Animal No.	Highest Dilution To React
Strain No. 119 , Vallée A Type(contd.).			
1.3.44.	Blood	C/21J	10^{-3}
		C/22J	10^{-3}
15.3.44.	Filtrate of a 1/100 Suspension Epithelium	C/30C (O Type Immune)	10^{-3}
		C/76D (C Type Immune)	10^{-4}
		C/25J	10^{-4}
		C/36J	10^{-3}
17.5.44.	1/5 Suspension Epith-:elium	C/2 L	10^{-5}
		C/3 L	10^{-5}
30.5.44	Filtrate of a 1/10 Suspension Epithelium	C/29L	10^{-6}
		C/30L	10^{-6}
Strain No. 149, Waldmann C Type.			
3.3.43.	Blood	C/0 D	10^{-2}
		C/2 D	10^{-4}
		C/3 D (All O Type Immune)	10^{-3}
25.3.43.	Blood	C/11D	10^{-3}
		C/12D	10^{-4}

With/

With regard to the usefulness of this method it would appear that if no great degree of accuracy is re-:quired the titre can be expressed as the highest dil-:ution that produces a reaction, but if only one animal is used the result may be misleading and an underestim-:ate may be made of the virus content of the suspension. This conclusion is based on experience gained by the use of many hundreds of Devon steers for experimental work with virus of foot-and-mouth disease, from which it is apparent that the great majority of these cattle are susceptible to a high degree, with a small minority that are more resistant. In this way, the more obvious results of individual variation in susceptibility tend towards underestimation of virus potency. The result obtained in one experiment emphasizes this point. A sample of blood was titrated in one animal and the titre was unexpectedly low, so the titration was re-:peated in a second animal. In addition, a third animal was inoculated with the same dilution as produced the original end-point reaction, and a fourth and fifth beast were inoculated with the next higher dilution using four sites in each case. The result can best be shown by reference to Fig. 10.

There was no doubt from the second series of in-:oculations that the titre of the blood was certainly 10^{-3}/

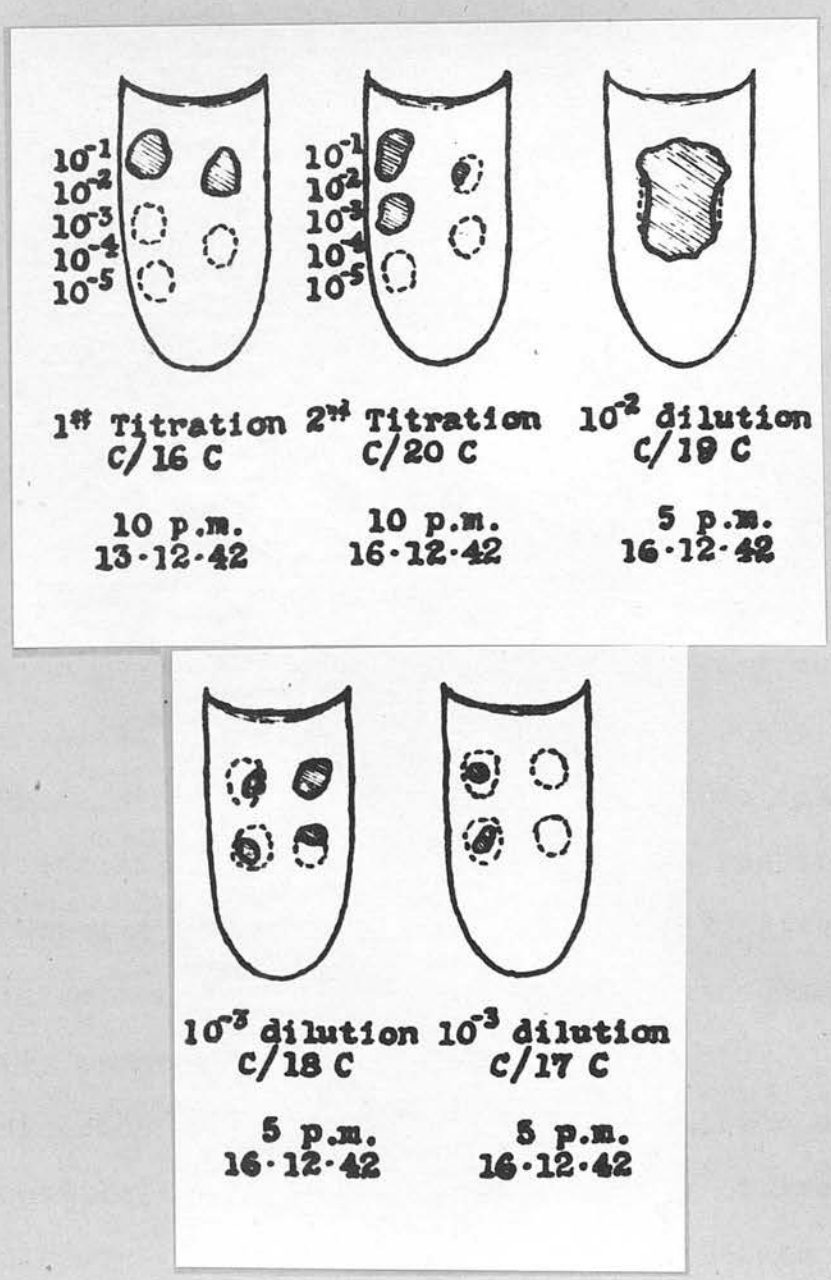


Fig. 10. Result of the inoculation of various dilutions of defibrinated bovine blood, strain No. 39, Vallée O type.
C/16 C inoculated 1 p.m., 12.12.42.
C/17, 18, 19 and 20 C inoculated 11 am., 15.12.42.

10^{-3} instead of 10^{-2} as shown by the first animal. The same dilution series when inoculated into groups of guinea-pigs again showed the difference in titre to be expected between results obtained from the two species.

Undiluted	++	+0	+0
10^{-1}	++	++	++
10^{-2}	00	00	00
10^{-3}	00	00	00

The conclusion reached is that this is a valuable method of inoculation as the reaction produced by up to six dilutions may be observed at the same time on the one animal and the result is obtained very quickly, usually within thirty hours. By using only one animal the method may be accompanied by considerable inaccur-:acy if the titre of a virus suspension is required, but by using a number of animals in order to give sever-:al observations for each dilution, the method should give very accurate results.

Reference has already been made to reports of the use of cattle in titrations of suspensions of foot-and-mouth disease virus by the workers at the German Foot-and-Mouth Disease Research Institute, and two papers have recently been published showing that the investig-:ations on this point have been proceeding on the same lines as those just described. Möhlmann and Stohr (1943)/

(1943), seeking a rapid and sensitive test for the rate of inactivation of the virus during the preparation of vaccine relied on the intradermal inoculation of the bovine tongue with the test material. Their technique consists of the simultaneous inoculation of four ten-:fold dilutions of the vaccine, thus combining a titrat-:ion with the infectivity test. A plate in their paper shows the arrangement of the dilutions to be the highest at the back of the tongue and the lowest near the tip, one animal being used for each test. The disadvantages of this arrangement of the dilutions has already been discussed as well as the inaccuracy to be expected if only one observation is made for each dilution.

Möhlmann (1944), in a paper on the complement-fixation test in foot-and-mouth disease, mentions this method of titration for determination of the virus content of the suspension used as the antigen in the complement-fixat-:ion test. The technique of simultaneous inoculation of four tenfold dilutions using only one animal does not appear to have been improved, but the method of interpretation of the results has been modified in an effort to detect smaller quantitative differences.

The size of the vesicle produced by the highest dilution to cause a reaction is graded and indicated by a series of positive signs, thus, + to ++++. The titre of a

virus/

virus suspension being then given as, say, 10^{-6}+++, indicates the presence of more virus than if the titre had been 10^{-6}+.

It is true that, in general, the size of the lesion is proportional to the strength of the dilution inoculated, but experience at Pirbright has shown that this is not a hard and fast rule and that there may be a difference in the rate of development of lesions in different animals and between different strains. Although grading of the size of the lesion may give an indication of a quantitative difference, in our view the only way to increase the accuracy and sensitivity of the method is to increase the number of observations for each dilution and if possible to decrease the dilution-ratio employed.

By the simultaneous inoculation of different dilutions at one site for each, increased accuracy can only be obtained if a considerable number of cattle are employed for each test: this would make the method uneconomical and so a further modification of the technique has been devised to overcome this limitation.

SIMULTANEOUS/

SIMULTANEOUS MULTIPLE INOCULATION OF DIFFERENT DILUTIONS

The object of this modification was to determine whether the number of observations could be increased for each dilution by inoculation of a number of sites instead of confining the inoculation of any one dilut-:ion to one site. It was found possible to arrange five small sites of inoculation in a row across the tongue from side to side, there being sufficient room between the dorsum and the horny epithelium of the tip of the tongue to fit in four such rows of five sites each. On the tongue of one animal, therefore, five observations may be obtained for each of four dilutions. It was realised that more restraint of the animal would be required for this more exacting technique. The re-:quired measure of restraint was obtained by narcotizing the cattle by the intravenous injection of Pentothal sodium.

From the first trial of this method two cattle have been used, thus providing ten observations for each dilution. The results have been interpreted by the method described by Parker and Rivers (1936) and Reed and Muench (1938). By this method the dilution which theoretically gives an equal number of positive and negative results is calculated. This dilution is regarded/

regarded as the best to take for the end-point of a suspension, being less affected by chance variation in the distribution of virus particles than any other. This choice of the fifty per cent. positive dilution is based on the same thesis as propounded for giving the best indication of the potency of biological products by choosing the LD50 dose, namely the dose which kills fifty per cent. of a large group of animals (Trevan, 1927; Gaddum, 1933).

Technique of inoculation of five sites for each dilution

When the animal is under the narcotic influence of the Pentothal sodium, the tongue is drawn out of the mouth and the lowest dilution is inoculated first at five sites in a row across the tongue just in front of the dorsum. Using a fresh syringe, the next higher dilution is inoculated nearer the tip of the tongue, and so on until the four dilutions have been done (Fig. 11). Each site is inoculated by making one track about 1.5 to 2 cm. long, parallel to the long axis of the tongue, and then running the needle up and down the original track two or three times; about 0.1 to 0.2 c.c. is injected, but, as already mentioned, the actual amount remaining *in situ* cannot be accurately estimated. The arrangement of the inoculations is shown/

Fig. 11. Simultaneous multiple inoculation of four different dilutions, five sites for each dilution. Animal narcotized by the intravenous injection of Pento-:thal sodium. The lines drawn on the tongue show the position of the inoc-:ulation sites.

shown in Fig. 12 and the appearance of the reactions produced is illustrated in Fig. 13. The type of result obtained is shown by the following example.

17.5.44.

Simultaneous multiple inoculation of different dilutions of a 1/5 suspension of bovine vesicle epithelium, centrifuged for 5 minutes at 3000 r.p.m. Epithelium stored for 25 days in equal parts glycerine and buffered phosphate saline solution, pH 7.6, at 4° C.
Stain No. 119, Vallée A type.

Steer No. C/O L - 2 gm. Pentothal sodium i/v prior to inoculation (Fig. 12).

Date	Time	Temp-:erat-:ure °F.	Remarks
17.5.44.	4 p.m.		Inoculated i/d 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions, five sites for each dilution, numbered 1 to 5.
18.5.44.	10 a.m.	101.2	10^{-4}, reactions at sites 1, 2, 3, and 4. 10^{-5}, reaction at site 1.
	4 p.m.	104.8	10^{-4}, reactions at sites 1, 2,3,4, and 5. 10^{-5}, reactions at sites 1, 2, 3 and 5 with a blanched line at 4. 10^{-6}, reactions at sites 1 and 3.
	10 p.m.	104	10^{-5}, definite reaction at site 4. 10^{-7}, negative.
19.5.44/			

Date	Time	Temp-:erat-:ure °F.	Remarks
19.5.44.	10 a.m.	102.6	Extensions of lesions; sites that were negative on 18.5.44 now involved, also some inoculated areas.
20.5.44.	10 a.m.	102.8	Unruptured lesions on two feet. Slaughtered.
	Steer No. C/I L -		2 gm. Pentothal sodium i/v prior to inoculation (Fig. 12).
17.5.44.	4 p.m.		Inoculated i/d 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.
18.5.44.	10 a.m.	100.8	10^{-4}, reactions at sites 3 and 5.
	4 p.m.	102.6	10^{-4}, reactions at sites 1, 2, 3, 4 and 5. 10^{-5}, reactions at sites 1, 2, 3, 4 and 5. 10^{-6}, reaction at site 2.
	10 p.m.	104.8	10^{-6}, reactions at sites 1 and 2. 10^{-7}, negative.
19.5.44.	10 a.m.	103.8	Extensions of all sites in-:volving much of the tongue.
20.5.44.	10 a.m.	102.6	Unruptured lesion, one foot. Slaughtered.

Taking the results of all ten observations together, the dilution that should give 50 per cent. positive re-:sults/

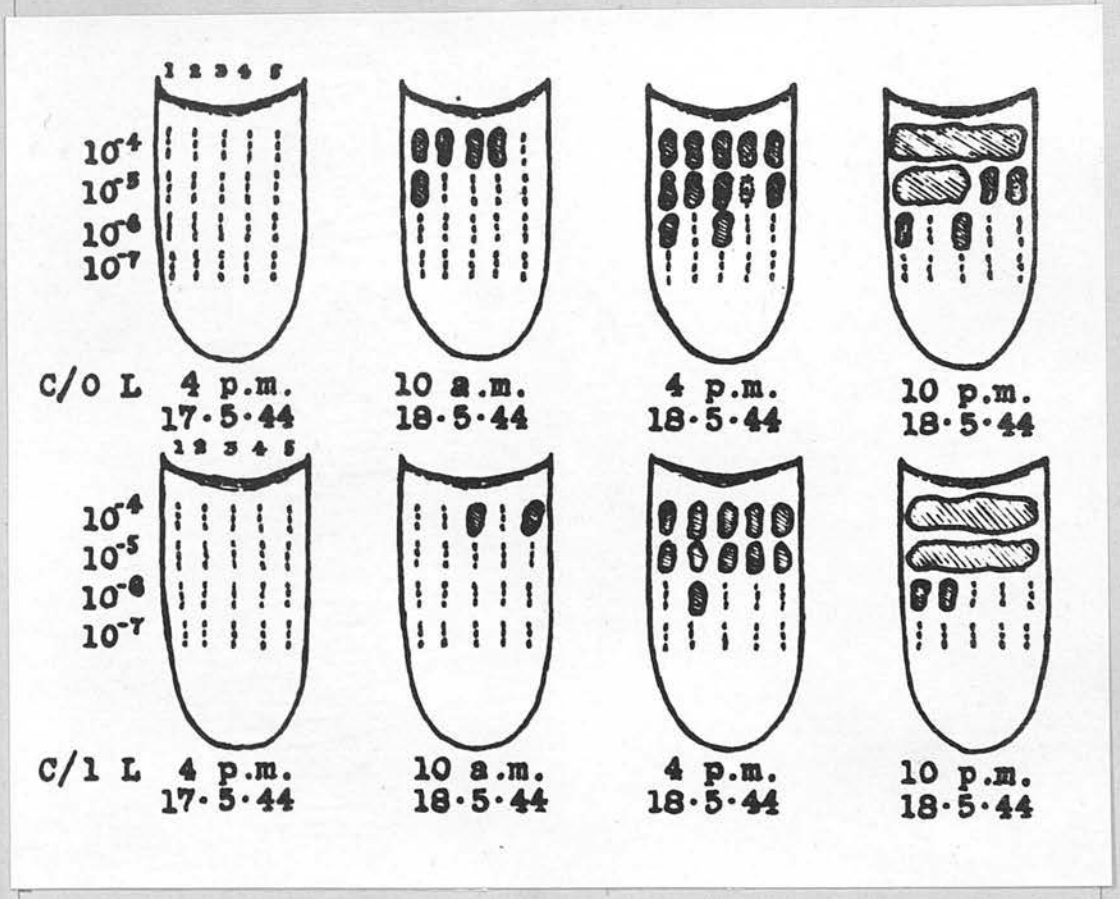
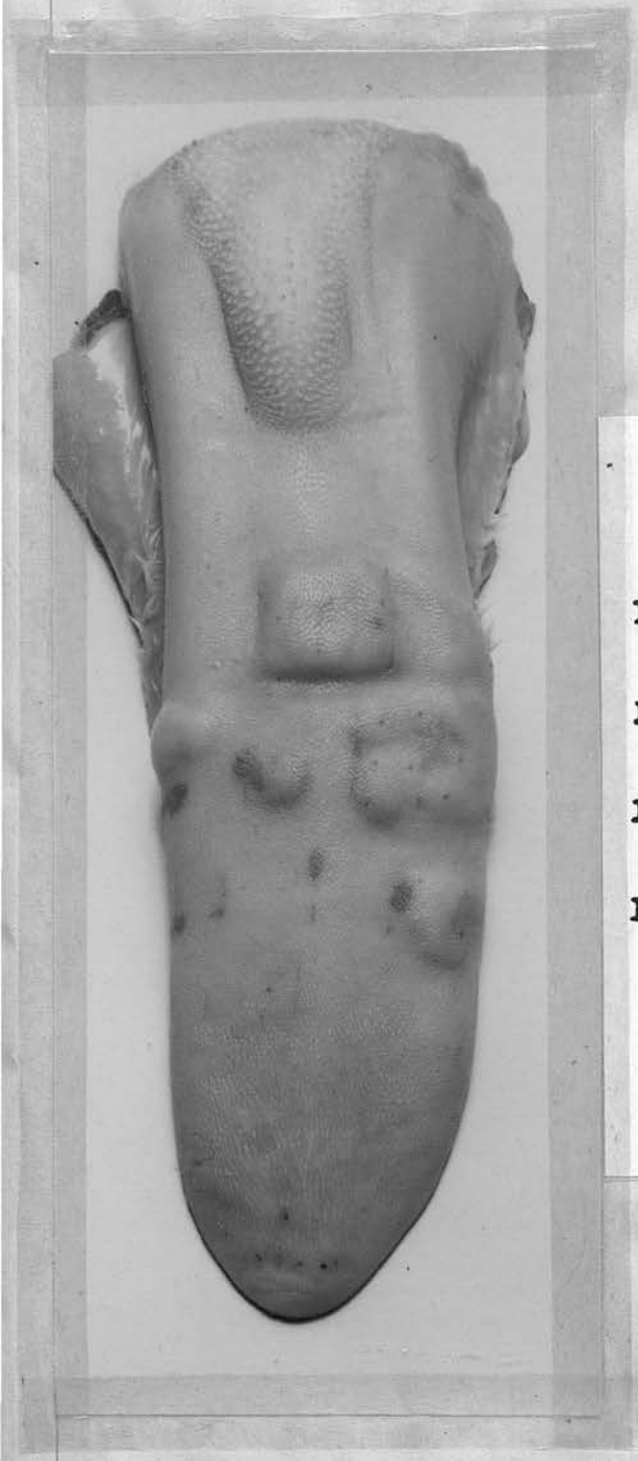
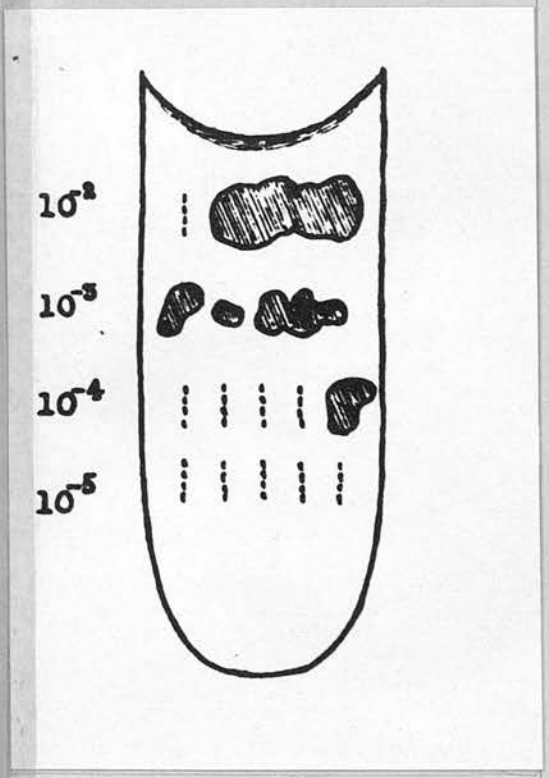


Fig. 12. Simultaneous multiple inoculation of four dilutions of a tenfold series, five sites for each dil-:ution.





Key to Fig. 13.

Fig. 13. Bovine tongue, 22 hours after the simultaneous multiple inoculation of four dilutions of a tenfold series, five sites for each dilution.

results may then be calculated by first noting the number of positive and negative results for each dil-:ution and then accumulating the number of positive and negative results for each dilution. This is done by taking the number of positive results at that and all higher dilutions and by taking the number of negative results at that and all lower dilutions. The percent-:age of positives is then calculated from the accumul-:ated results for each dilution (see Table II).

TABLE/

TABLE II.

RESULT OF TITRATION OF 17.5.44.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	Positive
10^{-4}	10	0	24	0	100
10^{-5}	10	0	14	0	100
10^{-6}	4	6	4	6	40
10^{-7}	0	10	0	16	0

In this case the fifty per cent. positive dilution must lie between 10^{-5} and 10^{-6}. The proportional distance is given by the following formula.

$$\frac{\text{(Percentage of positives at lower dilution)} - 50}{\text{(Percentage of positives at lower dilution)} - \text{(Percentage of positives at higher dilution)}}$$

that is,

$$\frac{100 - 50}{100 - 40} = \frac{5}{6} = 0.8 \text{ of a tenfold interval}$$

therefore the 50 per cent. positive dilution is $10^{-5.8}$.

Arrangement/

Arrangement of the dilution sites with regard to possible variation of susceptibility of different areas of the tongue.

It will be noticed that the same arrangement of the dilutions is used as with the method of simultaneous inoculation of different dilutions with one site for each, namely that the lowest is at the back of the tongue. This immediately raises the question of whether there is any variation in the susceptibility of different areas of the epithelium and whether the technique would not be better if a random selection of sites was adopted. There is no doubt that a random selection of sites would be preferable in order to minimise the effect of any variation in susceptibility that might exist, but whenever an irregular arrangement of the dilution sites has been tried it has proved to be very difficult to read since the reaction of low dilutions near the tip has hindered the examination of the back of the tongue and extension of the reaction to a low dilution has overrun the site of a higher dilution before that has had time to react. When all the low dilutions are together they can only extend from one to another, and as they usually react about the same time, the individual vesicles may be seen before they coalesce.

The disadvantages of randomisation in this type of experiment are best illustrated by some hypothetical cases/

cases in which the four dilutions are arranged in five strips down the tongue (Fig. 14). It will be seen in each example that some of the sites of the highest dil-:utions are in danger of being overrun by the reaction from neighbouring low dilutions, and in each case low dilution sites are near the front of the tongue, the reactions from which would make it difficult to examine the back of the tongue.

Since the limitations of the technique do not allow for the use of randomisation, it is necessary to consider the effect of using a systematic arrangement.

Histological examination of cross sections of the portion of the tongue used for inoculation show no significant difference in structure (Figs. 15 to 25), therefore one important factor that might affect the susceptibility of different areas may be dismissed. The response of the susceptible cells cannot be sub-:jected to such particular study but an analysis of the particular sites that reacted in a number of different titrations is useful in assessing whether there is any significant variation in the susceptibility of one portion of the tongue compared with another.

In nine titrations with the systematic arrangement of a tenfold dilution series, 28 cattle[1] were used.

A/

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[1] As a rule two cattle were used for each titration, but this number of 28 contained some larger groups used in connection with other work.

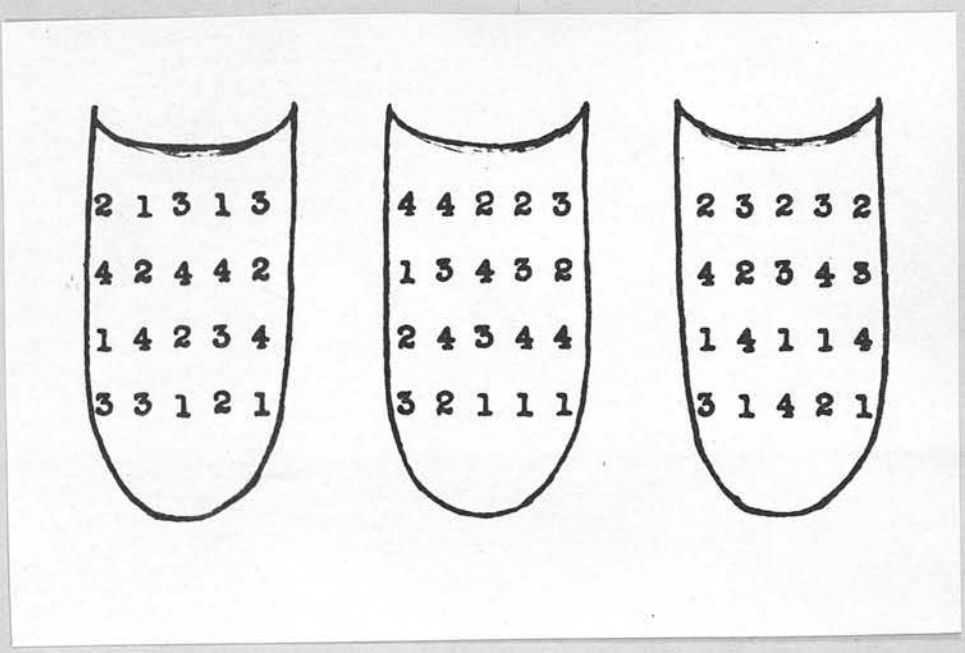

Fig. 14. Simultaneous multiple inoculation. Three examples of a random arrange-:ment of the inoculation sites of four dilutions numbered 1 to 4.

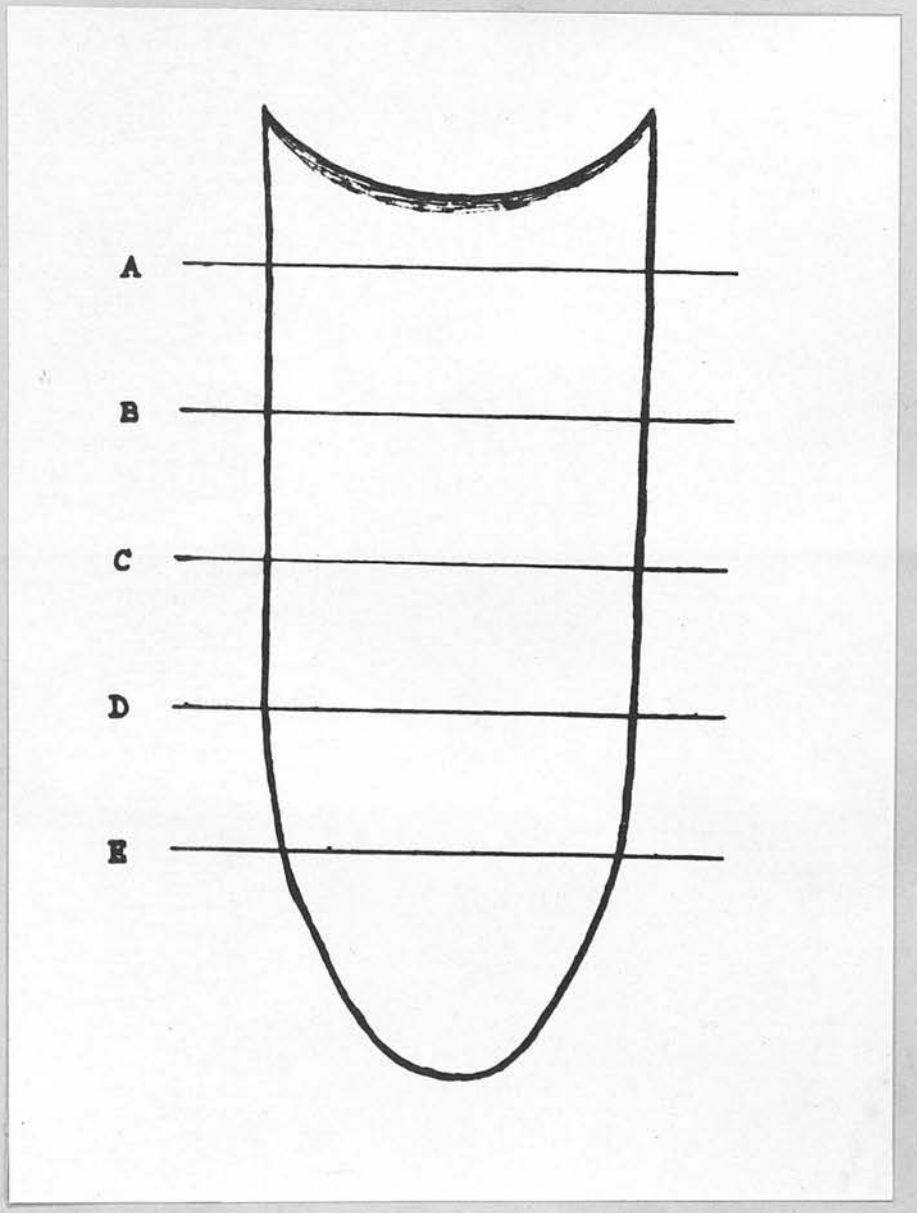

Fig. 15. Sites of cross-sections of the bovine tongue illustrated in Figs. 16 to 25.

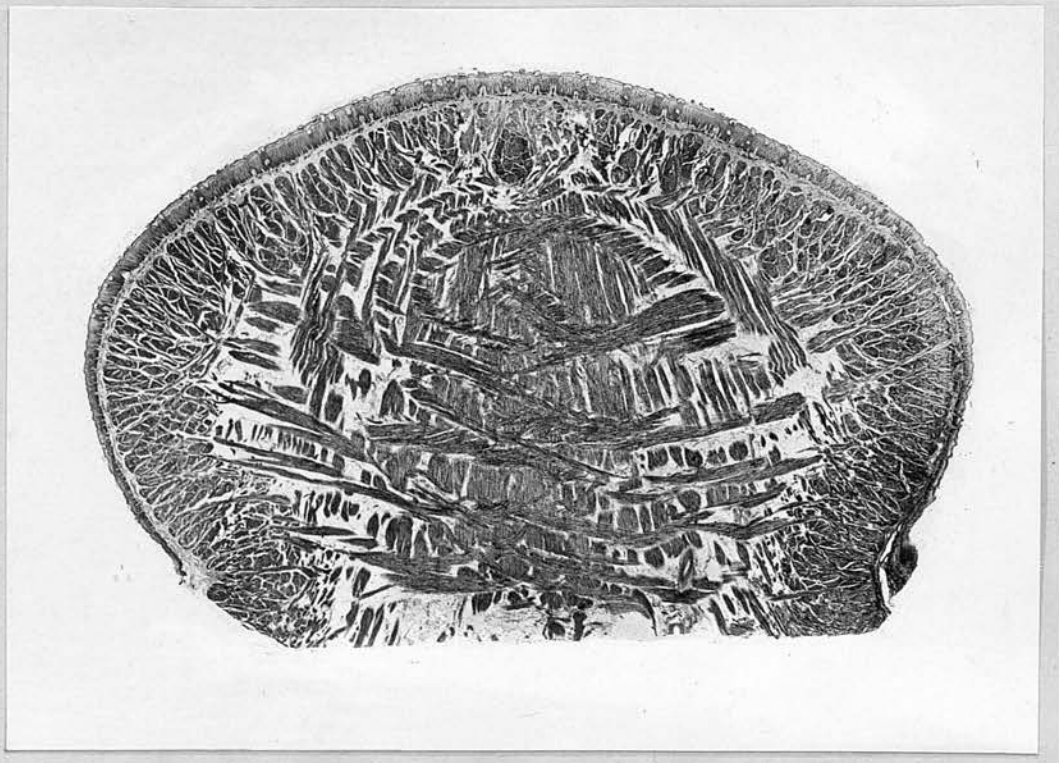

Fig. 16. Cross-section A. Area usually inoculated with the lowest dilution.
H and E. x 2.

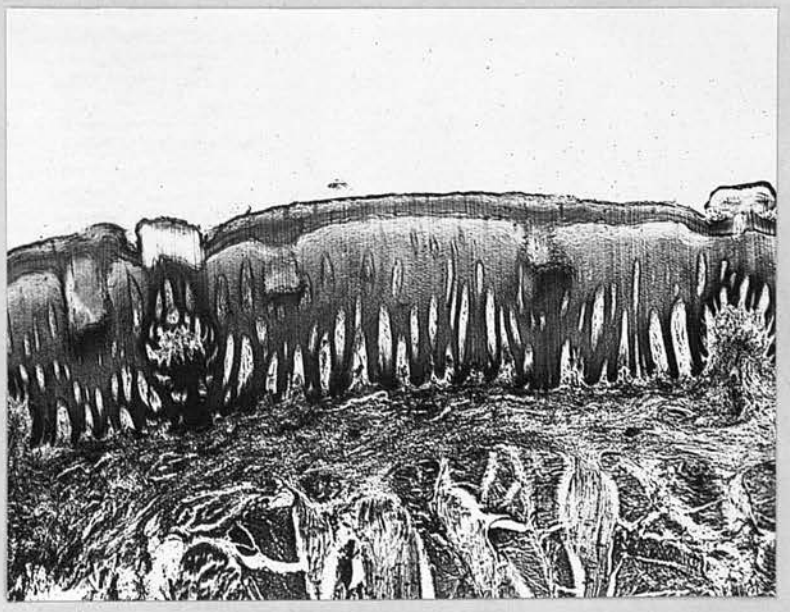

Fig. 17. Cross-section A. Portion of the mucous membrane.
H and E. x 17.

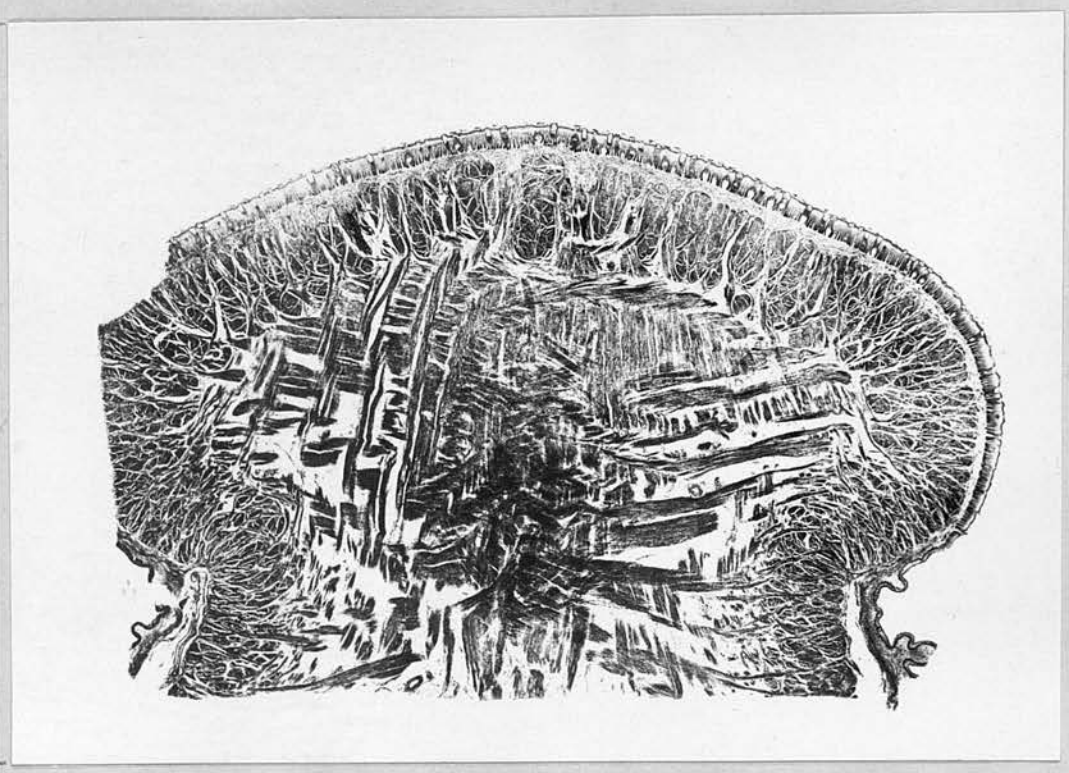

Fig. 18. Cross-section B. Area usually inoculated with the second lowest dilution.
H. and E. x 2.

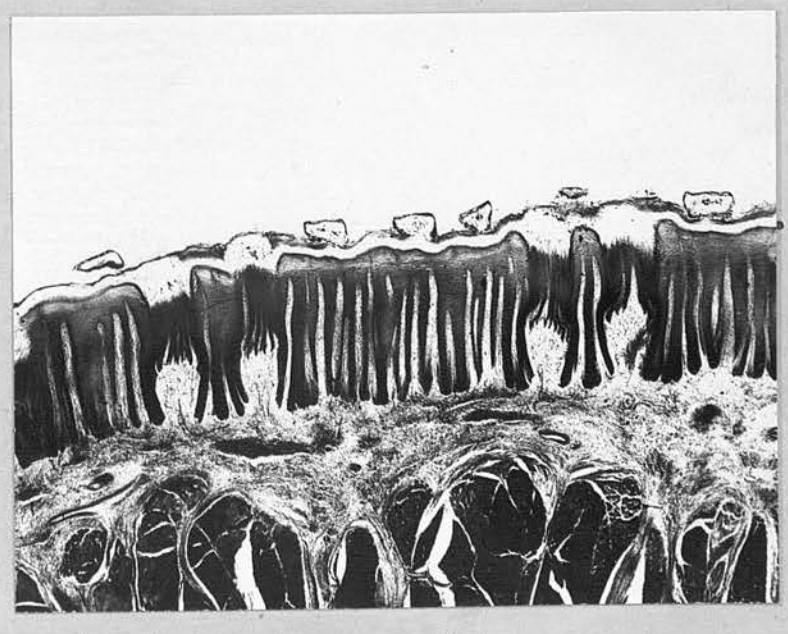

Fig. 19. Cross-section B. Portion of the mucous membrane.
H. and E. x 17.

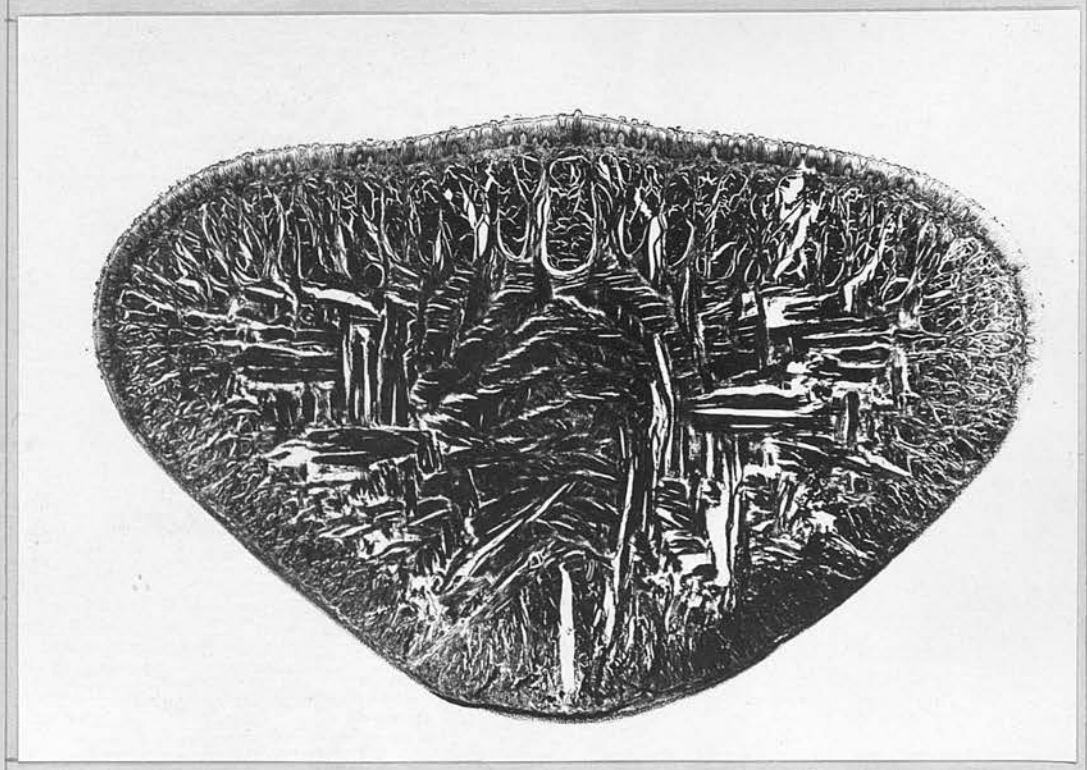

Fig. 20. Cross-section C. Area usually inoculated with the third lowest dilution.
H. and E. x 2.

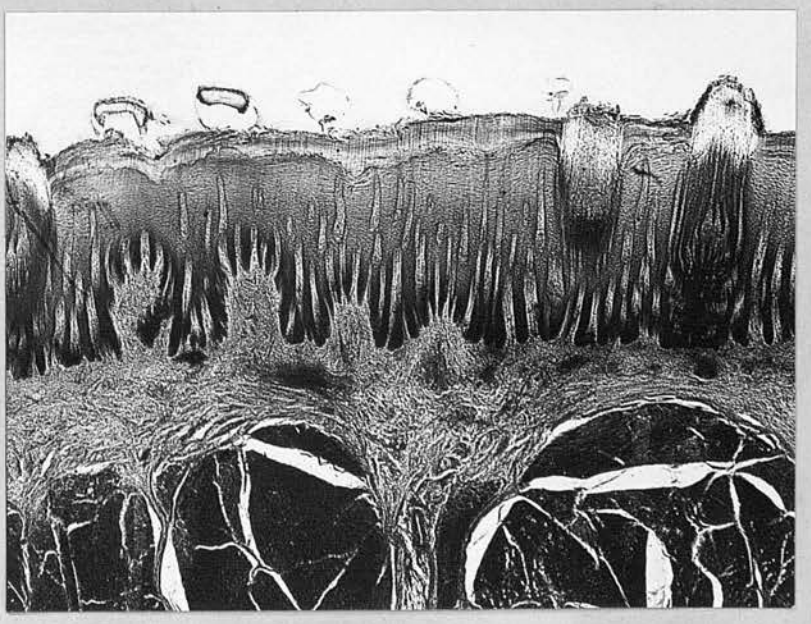

Fig. 21. Cross-section C. Portion of the mucous membrane.
H. and E. x 17.

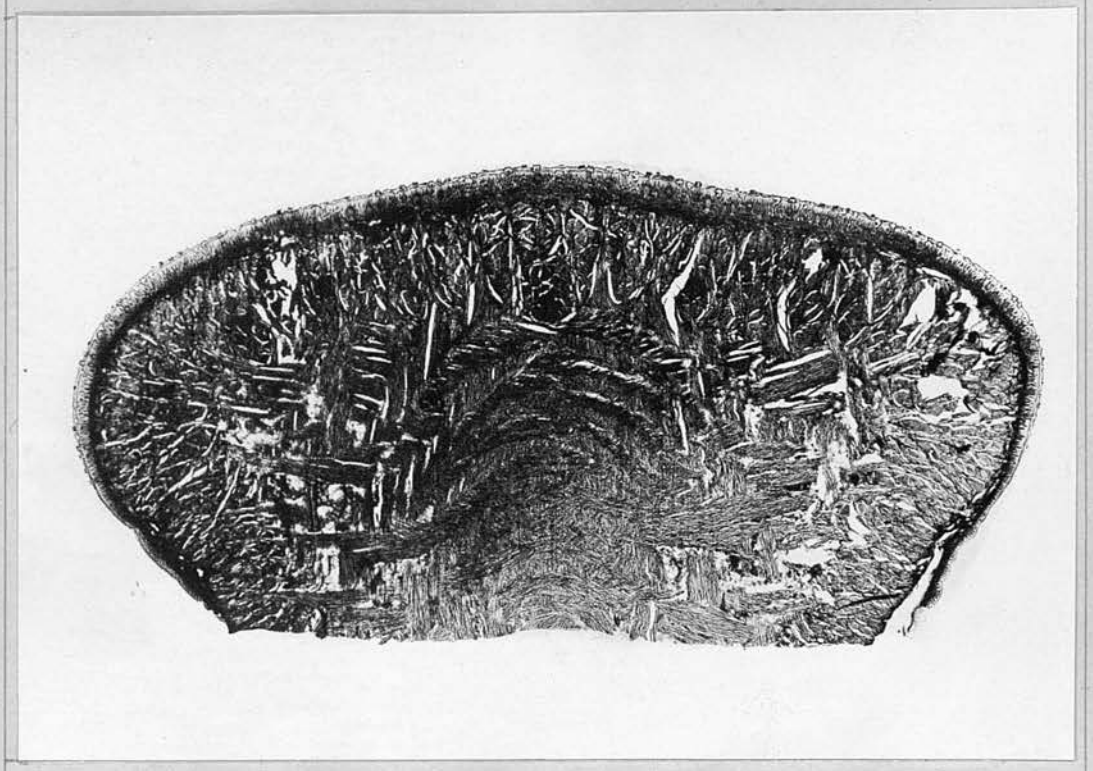

Fig. 22. Cross-section D. Area usually inoculated with the highest dilution.
H. and E. x 2.

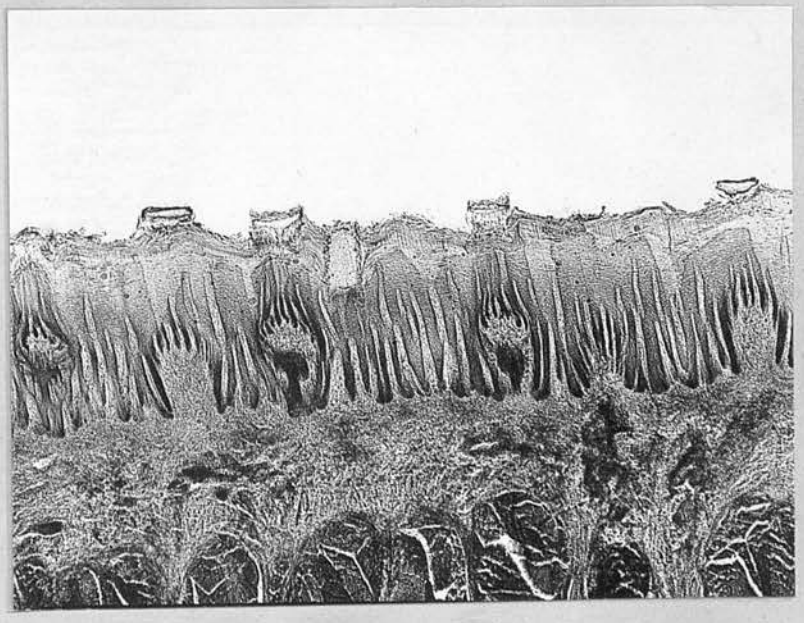

Fig. 23. Cross-section D. Portion of the mucous membrane.
H. and E. x 17.

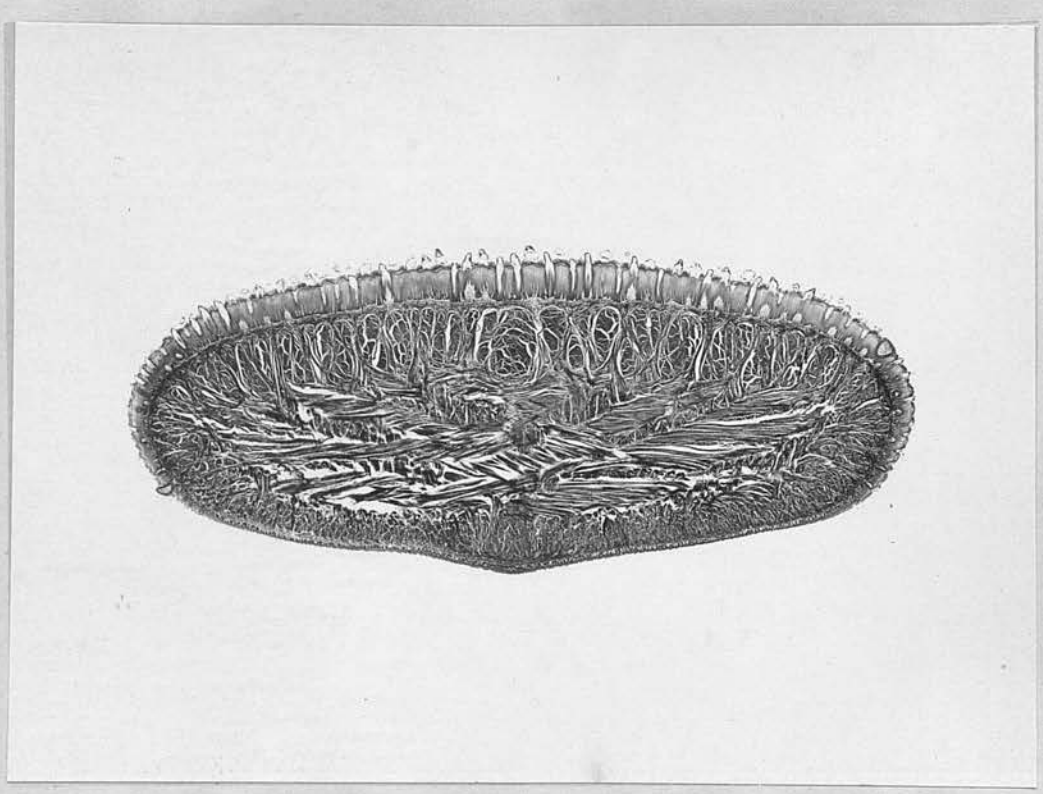

Fig. 24. Cross-section E. Tip of the tongue with numerous large conical papillae. This area is not usually inoculated.
H. and E. x2.

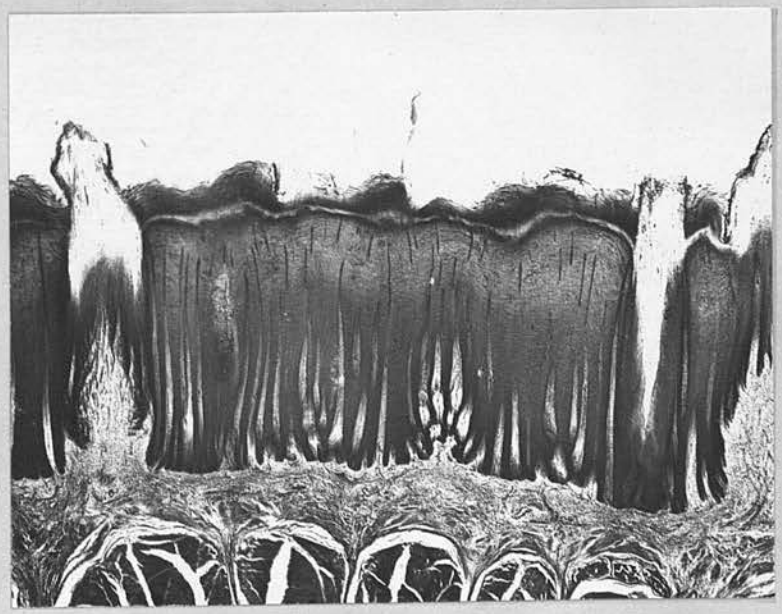

Fig. 25. Cross-section E. Portion of the mucous membrane.
H. and E. x 17.

A count was made of the number of times that each particular site reacted, and the result shown in Fig.26 was obtained.

The fifty per cent. positive end-point for each titration did not occupy the same place in the range of dilutions selected and thus the decrease in the number of positive observations from row to row does not correspond exactly with the falling dilution series. When the end-point lay between rows B and C there were no positive results in row D: the positive results in row D belong to titrations for which the end-point lay between rows C and D. Where the majority of the observations for any one site are positive it is unlikely that any variation in susceptibility could be detected because the amount of virus inoculated at those sites would be great enough to cause a reaction, unless the site was completely non-susceptible. Nevertheless, there is no indication of any significant variation in the susceptibility between sites in the same row, and there is no indication of variation from row to row as the drop in the proportion of positive results from row A to row D would appear to be consistent with the falling dilution series even although the end-point of each titration did not occur at the same place in each case.

By/

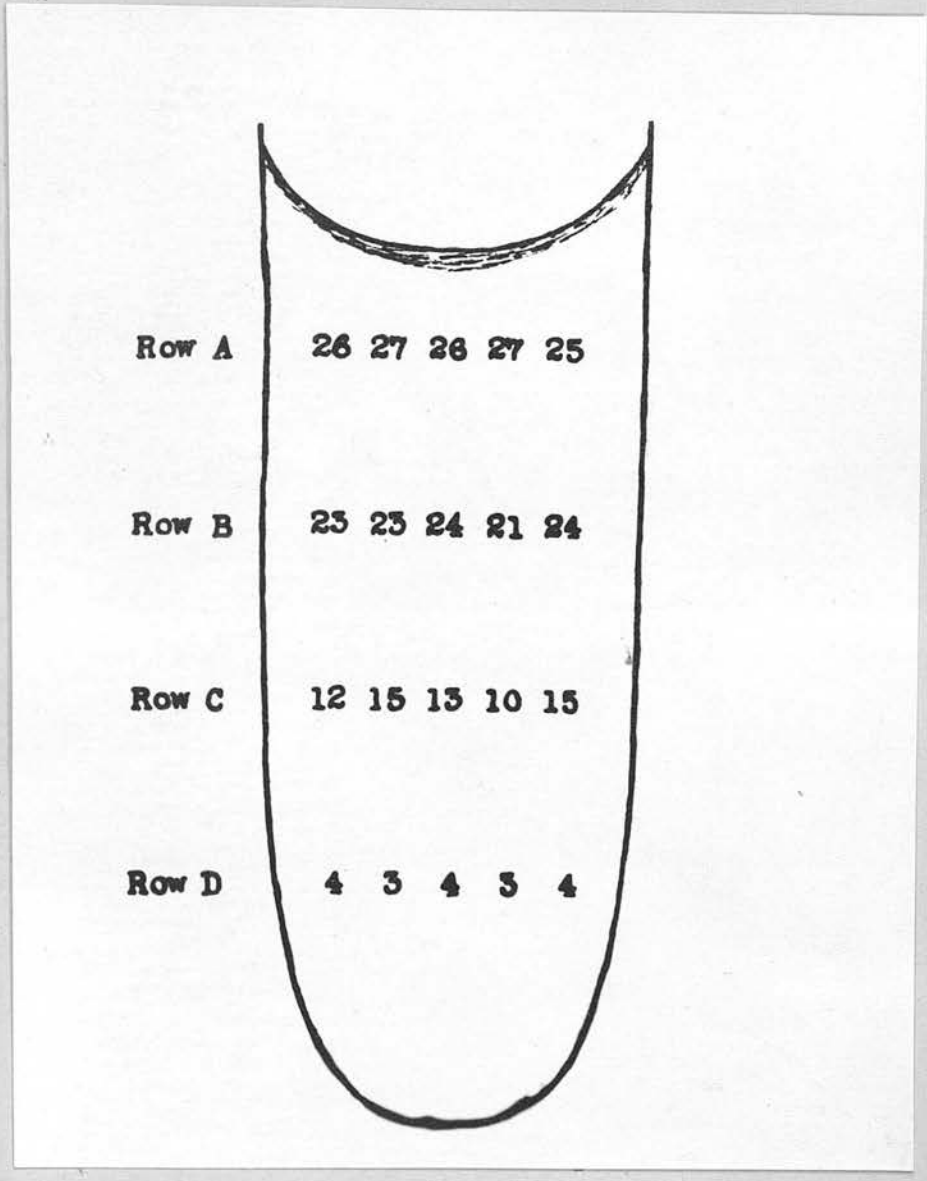

Fig. 26. Simultaneous multiple inoculation. Frequency with which each site re-:acted in a series of 28 cattle in nine titrations using tenfold dilut-:ions.

By a consideration of the frequency with which the positive results occurred at each site in a series of titrations, the conclusion is reached that any vari-:ation in tissue susceptibility that may occur is not sufficiently great to warrant substituting randomis-:ation for the systematic arrangement of the dilution sites since the practical advantages of the latter arrangement far outweigh any improvement that might result from a diminution of a possible cause of error.

The choice of the dilution-factor.

The successful results obtained by means of the technique just described, in which the number of ob-:servations for each dilution was substantially in-:creased while still using only two cattle, made it worth while considering the employment of a dilution-factor of less than 10. The size of the animal's tongue does not permit of more than four rows of inoculation sites and if five sites are required for each dilution, the range of any series is limited to four dilutions. The range that is thus available, using some of the dilution-factors between 2 and 10, is shown in Table III.

TABLE/

TABLE III.

RANGE COVERED BY FOUR DILUTIONS
USING VARIOUS DILUTION FACTORS

Dilution-factor	Approximate Logarithmic Interval	Logarithmic Range
2	0.30	0.9
3	0.48	1.4
4	0.60	1.8
5	0.70	2.0
6	0.78	2.3
10	1.00	3.0

If/

If the limitations of the technique do not have to be considered, the choice of the dilution-ratio depends on the knowledge possessed of the approximate end-point of the virus suspension to be titrated. This knowledge may be obtained by a preliminary titration using a high dilution-factor, or it may be possessed by virtue of experience of the probable virus content of a particular suspension. When the test animals are cattle their high cost prohibits the conduct of pre-:liminary trials in routine titrations and so, if there is little knowledge of the probable position of the end-point, a tenfold dilution series is the best to employ. Fig. 27 shows a modification of the arrangement of the dilution sites that has proved useful when it has been necessary to cover the possibility of loss of infectiv-:ity, no great accuracy being required if such a loss has occurred.

When the probable end-point has been known, usually from experience of the virus content of the type of suspension being used, routine titrations with two cattle have been carried out using a fourfold or a six-:fold dilution series. The limited range of a two-:fold series, when confined to four dilutions, makes the choice of this dilution-factor unsuitable unless a preliminary titration is made to determine the approx-:imate position of the end-point. One experiment in

which/

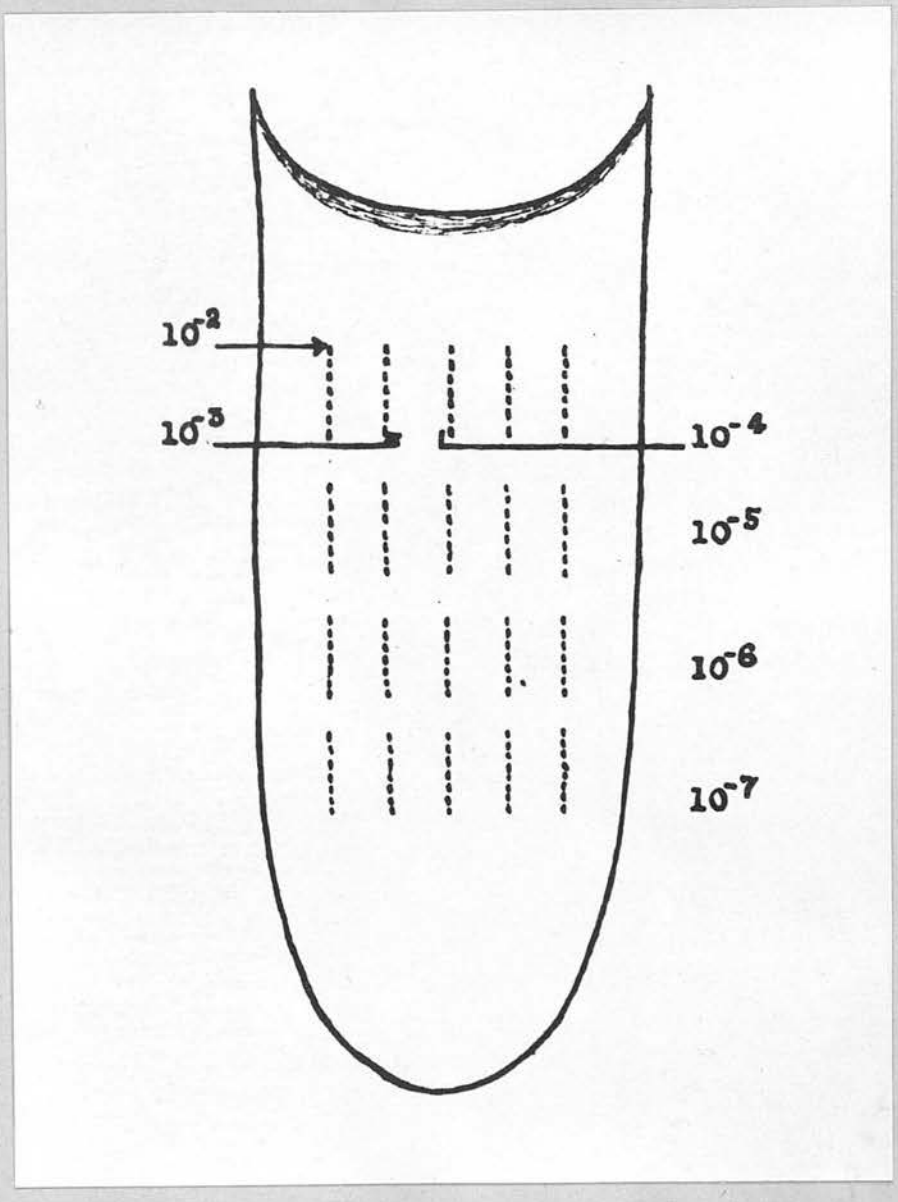

Fig. 27. Simultaneous multiple inoculation. Arrangement of the inoculation sites for six dilutions when most accuracy is required in the neighbourhood of the 10^{-6} dilution.

which this series was used is described later.

The sixfold dilution series is particularly useful for the titration of infective blood. If the blood has been collected at the optimum time the end-point is usually about 10^{-3}, and with four dilutions of a sixfold series the range can extend from 10^{-2} to $10^{-4.3}$.

The following examples illustrate the type of result obtained using a fourfold and sixfold dilution series.

Titration using a fourfold dilution series.

10.8.44.

Titration of a 1/10 suspension of bovine vesicle epithelium, centrifuged for 15 minutes at 3000 r.p.m. No storage of the epithelium. Strain No. 39, Vallée O type.

Steer No. C/72 L - 3 gm. Pentothal sodium i/v prior to inoculation (Fig. 28)

Date	Time	Temperature °F.	Remarks
10.8.44	3 p.m.		Inoculated i/d 10^{-5}, $10^{-5.6}$, $10^{-6.2}$ and $10^{-6.8}$ dilutions.
11.8.44	10 a.m.	101.4	10^{-5}, good reactions at sites 1,2,3,4 and 5. $10^{-5.6}$, reactions at sites 1,2,3,4 and 5. $10^{-6.2}$, reactions at sites 2, 3, and 4.
	4 p.m.	101.6	$10^{-6.2}$, reactions at sites 1,2,3,4 and 5. $10^{-6.8}$, reactions at sites 2, 3, and 5.

Steer/

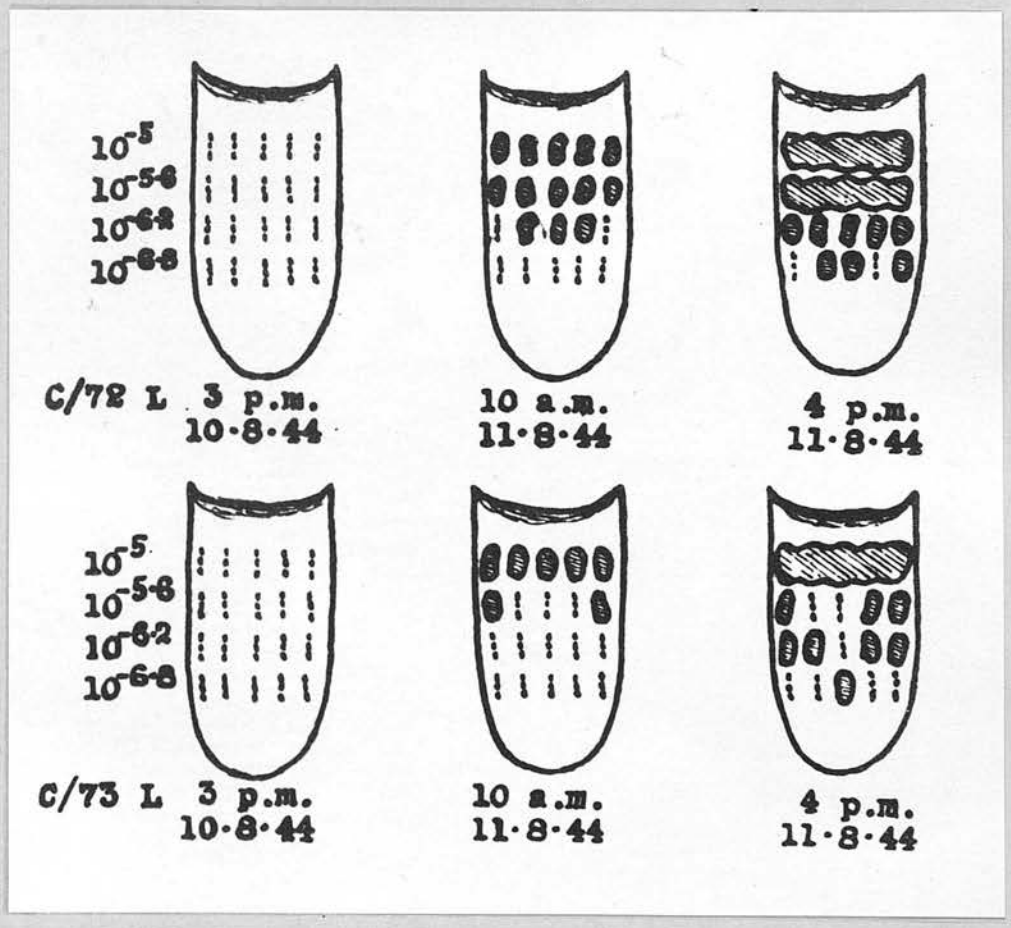

Fig. 28. Simultaneous multiple inoculation of four dilutions of a fourfold series.

Steer No. C/73 L - 3 gm. Pentothal sodium prior to inoculation (Fig. 28).

Date	Time	Temp-:erat-:ure °F.	Remarks
10.8.44	3 p.m.		Inoculated i/d 10^{-5}, $10^{-5.6}$, $10^{-6.2}$ and $10^{-6.8}$ dilutions.
11.8.44	10 a.m.	102.4	10^{-5}, reactions at sites 1, 2, 3, 4, and 5. $10^{-5.6}$, reactions at sites 1 and 5.
	4 p.m.	102.5	$10^{-5.6}$, reactions at sites 1, 4 and 5. $10^{-6.2}$, reactions at sites 1, 2, 4, and 5. $10^{-6.8}$, reaction at site 3.

Table IV shows the percentage positive for each dilution, from which it may be calculated that the fifty per cent. positive dilution is $10^{-6.6}$.

TABLE/

TABLE IV.

RESULT OF TITRATION OF 10.8.44.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	Positive
10^{-5}	10	0	31	0	100
$10^{-5.6}$	8	2	21	2	91
$10^{-6.2}$	9	1	13	3	81
$10^{-6.8}$	4	6	4	9	31

Titration/

Titration using a sixfold dilution series.

12.3.45.

Titration of a filtrate of a 1/25 suspension of bovine vesicle epithelium. Suspension prepared in M/25 buffered phosphate saline and Hartley's digest broth, centrifuged for 10 minutes at 2000 r.p.m., supernatant passed through a sand and paper pulp filter then a gradocol membrane of 0.6μ A.P.D. Epithelium stored without preservative for 1 day at -20° C. No storage of the filtrate.
Strain No. 149, Waldmann C type.

Steer No. C/76 P - 3 gm. Pentothal sodium i/v prior to inoculation (Fig. 29).

Date	Time	Temp-:erat-:ure °F.	Remarks
12.3.45.	3 pm.		Inoculated i/d $10^{-4.8}$, $10^{-5.6}$, $10^{-6.4}$ and $10^{-7.2}$ dilutions.
13.3.45.	3 p.m.	102.2	$10^{-4.8}$, reactions at sites 2, 3, and 5. $10^{-5.6}$, reaction at site 5.
	9 p.m.	104.8	$10^{-4.8}$, reactions at sites 1, 2, 3, 4 and 5. $10^{-5.6}$, reaction at site 5.
	Steer No. C/77 P -		4. gm. Pentothal sodium i/v prior to inoculation (Fig. 29).
12.3.45.	3.15 p.m.		Inoculated i/d $10^{-4.8}$, $10^{-5.6}$, $10^{-6.4}$ and $10^{-7.2}$ dilutions.
13.3.45	3 p.m.	101.6	$10^{-4.8}$, reactions at sites 1, 3, and 4. $10^{-5.6}$, reaction at site 5.
	9 p.m.	105.2	No further development.

Table/

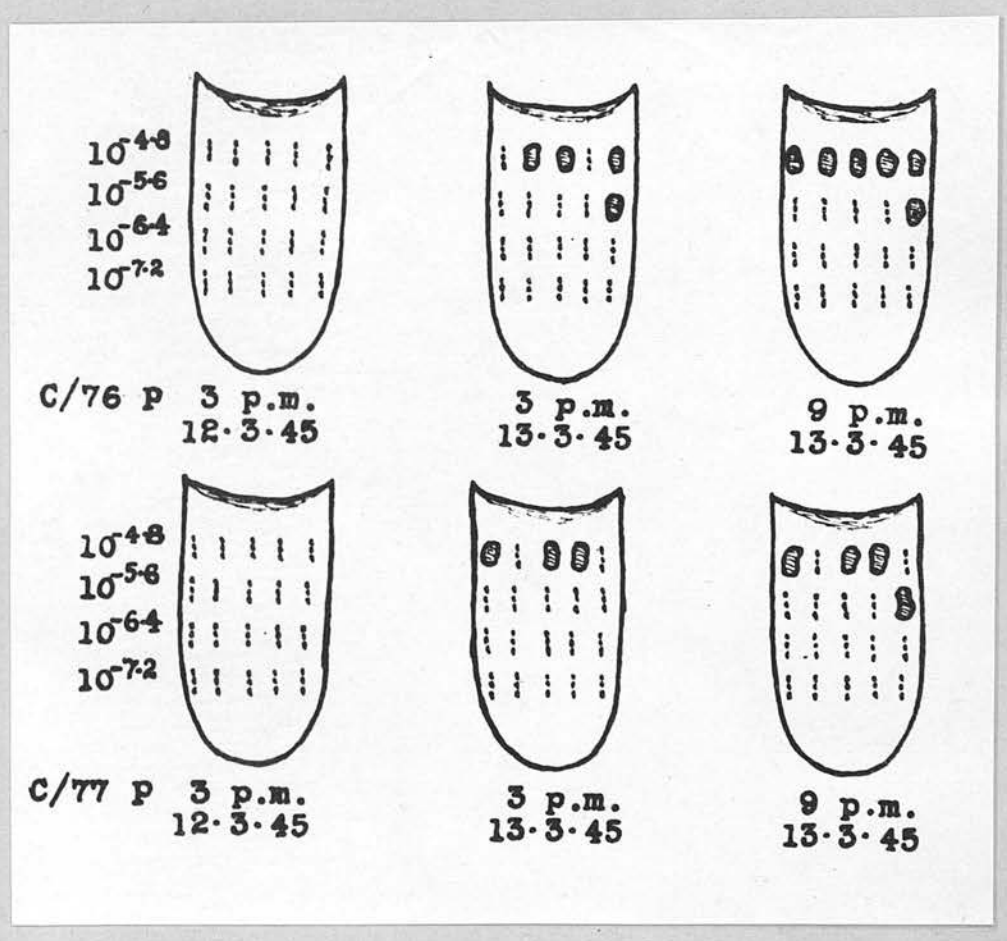

Fig. 29. Simultaneous multiple inoculation of four dilutions of a sixfold series.

Table V shows the percentage positive for each dilution, from which it may be calculated that the fifty per cent. positive dilution is $10^{-5.2}$.

TABLE V.

RESULT OF TITRATION OF 12.3.45.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	Positive
$10^{-4.8}$	8	2	10	2	83
$10^{-5.6}$	2	8	2	10	17
$10^{-6.4}$	0	10	0	20	0
$10^{-7.2}$	0	10	0	30	0

These two examples are representative of the titrations that have been done when a fourfold or a sixfold dilution series has been selected. This comprises work with five different virus strains of the Vallée O type and one strain of the Waldmann C type. From the results obtained it is obviously quite practicable, with this technique, to decrease the dilution-ratio and thus increase the accuracy and reliability of the quantitative study.

The/

The degree of reliability that may be expected by using the simultaneous multiple inoculation technique and the described method of interpretation of the results.

Two experiments have been performed to determine whether this technique and the method of interpretation of the results were reliable enough to assign a particular frequency ratio to any chosen dilution.

30.5.44.

Titration of a Seitz filtrate of a 1/10 suspension of bovine vesicle epithelium.
Epithelium stored for 11 to 12 days in equal parts glycerine and buffered phosphate saline solution, pH 7.6 at 4°C.
Strain No. 119, Vallée A type.

A preliminary titration using a tenfold dilution series gave the result shown in Table VI, from which it may be calculated that the fifty per cent. positive dilution is $10^{-5.8}$.

TABLE/

TABLE VI.

RESULT OF TITRATION OF 30.5.44.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	Positive
10^{-3}	10	0	34	0	100
10^{-4}	10	0	24	0	100
10^{-5}	10	0	14	0	100
10^{-6}	4	6	4	6	40

As soon as this result was obtained three dilutions were selected and freshly prepared. Each of these dilutions was then inoculated into two cattle, 20 sites on each tongue, making a total of forty observations. The dilutions selected were:

(1) 10^{-5}, estimated as being the highest dilution to give 100 per cent. positive results;

(2) $10^{-5.8}$, estimated to give 50 per cent. positive results; and

(3) 10^{-7}, estimated as being the lowest dilution to give 100 per cent. negative results.

(1)/

(1) 10^{-5} dilution.

Steer No. C/35 L - 3gm. Pentothal sodium i/v prior to inoculation (Fig. 30).

Date	Time	Temp-:erat-:ure °F.	Remarks
1.6.44	1.30p.m.		Inoculated i/d 10^{-5} dilution, 20 sites.
2.6.44.	11.30a.m.	103.2	Reactions at 17 sites.
	3 p.m.	104.8	Considerable extension of lesions.
	10 p.m.	103.4	3 sites seen to be negative.

Steer No. C/36 L - 3gm. Pentothal sodium i/v prior to inoculation (Fig. 30).

1.6.44.	1.30p.m.		Inoculated i/d 10^{-5} dilution. 20 sites.
2.6.44.)	11.30a.m.	101.2	Reactions at 14 sites.
	3 p.m.	101	Reactions at 17 sites, ex-:tension of lesions.
	10 p.m.	101.8	3 sites seen to be negative.

Result: 34 positive sites and

6 negative sites.

(2)/

(2) $10^{-5.8}$ dilution.

Steer No. C/37 L - 3gm. Pentothal sodium i/v prior to inoculation. (Fig. 30).

Date	Time	Temp-erature °F.	Remarks
1.6.44	1 p.m.		Inoculated i/d $10^{-5.8}$ dilution, 20 sites.
2.6.44.	11.30a.m.	100.2	Reactions at 13 sites.
	3 p.m.	102.6	Extension of lesions.
	10 p.m.	104	7 sites still negative.
	Steer No. C/38 L		- 3gm. Pentothal sodium i/v prior to inoculation (Fig. 30).
1.6.44	1.p.m.		Inoculated i/d $10^{-5.8}$ dilution, 20 sites.
2.6.44.	11.30a.m.	102.8	Reactions at 9 sites.
	3 p.m.	102	Reactions at 10 sites.
	10 p.m.	103	10 sites still negative.

Result: 23 positive sites and

17 negative sites.

(3)/

(3) 10^{-7} dilution.

Steer No. C/39 L - 3 gm. Pentothal sodium i/v prior to inoculation. (Fig. 30)

Date	Time	Temp-:erat-:ure °F.	Remarks
1.6.44.	12.30p.m.		Inoculated i/d 10^{-7} dilution, 20 sites.
2.6.44	11.30a.m.	101.4	Reaction at 1 site.
	3 p.m.	101.6	Small reaction at another site.
	10 p.m.	105	Only 2 reactions.

Steer No. C/40 L - 3gm. Pentothal sodium i/v prior to inoculation (Fig. 30).

1.6.44.	12.30p.m.		Inoculated i/d 10^{-7} dilution, 20 sites.
2.6.44.	11.30a.m.	101.2	Reactions at 2 sites.
	3 p.m.	101	Slight extension of lesions.
	10 p.m.	101.8	Only 2 reactions.

Result: 4 positive sites and

36 negative sites.

A comparison of the observed and estimated fre-:quencies is shown in Table VII.

TABLE/

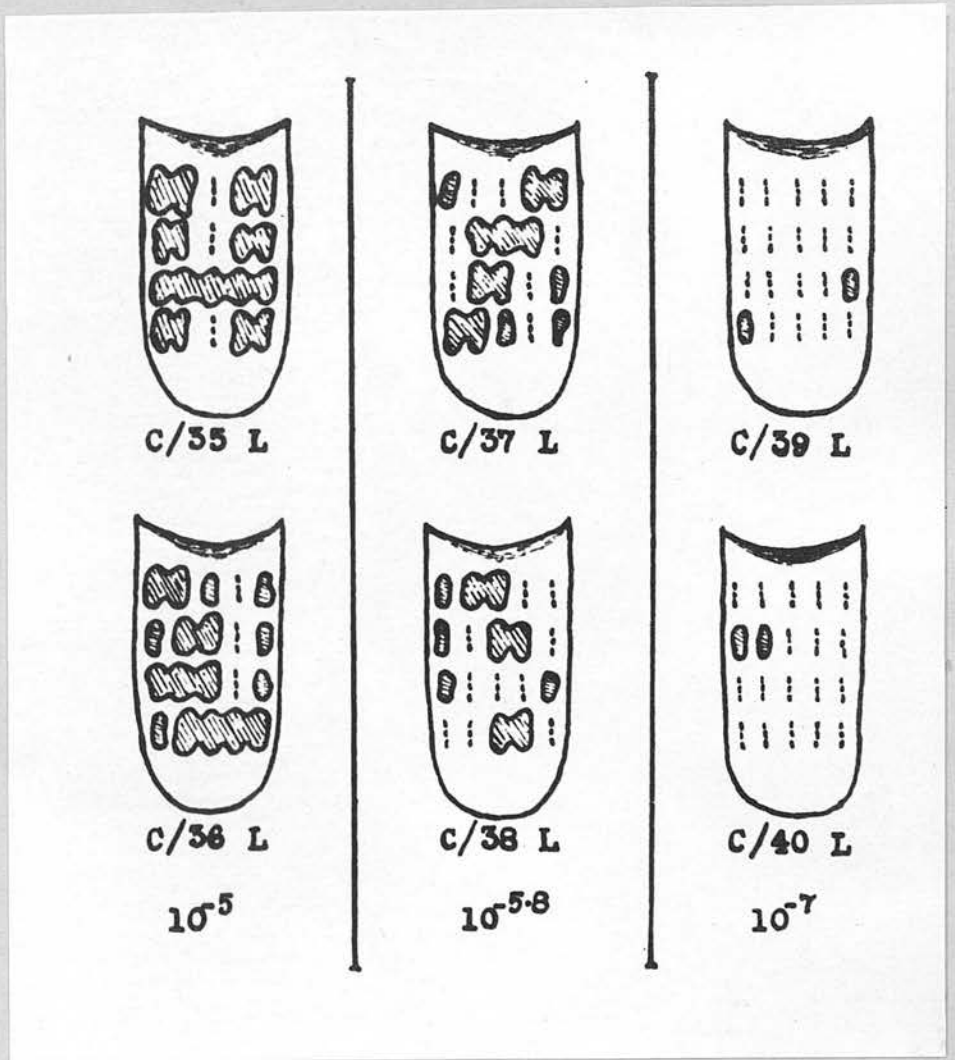

Fig. 30. Tongues of cattle inoculated at 20 sites with one dilution. Reactions observed at 10 p.m., 2.6.44.

TABLE VII.

COMPARISON OF THE OBSERVED AND ESTIMATED FREQUENCIES OF REACTION FOR THE DILUTIONS INOCULATED AT 40 SITES ON TWO TONGUES.

Dilution	Observed Frequency		Estimated Frequency	
	Positive	Negative	Positive	Negative
10^{-5}	34	6	40	0
$10^{-5.8}$	23	17	20	20
10^{-7}	4	36	0	40

The difference between the observed and estimated frequencies for the 10^{-5} and 10^{-7} dilutions is not unduly large considering the greater effect that is produced by chance variation in the distribution of virus particles in the neighbourhood of the completely positive and completely negative points. The most important result to consider is that provided by the $10^{-5.8}$ dilution where the observed frequency corresponds closely to that estimated from the result of the previous titration.

The/

The result of this experiment showed that a certain degree of reliability could be placed on this method of titration and that a more severe test was justified. By covering the maximum range on both sides of the 50 per cent. point, that is, by taking the nearest 100 and 0 per cent. positive points, the difference on the logarithmic scale of the dilutions was 0.8 and 1.2 re-:spectively. The same type of experiment was therefore repeated, choosing the dilutions estimated to give 75, 50 and 25 per cent. positive results. The differ-:ence on the logarithmic scale in this case proved to be 0.26 and 0.27 respectively, slightly less than a twofold difference in concentration.

12.3.45.

Titration of a filtrate of a 1/25 suspension of bovine vesicle epithelium.
Strain No. 149, Waldmann C type.

Details of the preparation of this filtrate and preliminary titration have just been given as an example of the use of a sixfold dilution series, see page 64. From the result of this titration it was estimated that the $10^{-4.93}$ dilution would give 75 per cent. positive results, $10^{-5.2}$ 50 per cent. and $10^{-5.46}$ 25 per cent. positive results (see page 85 and Fig.33). A total of 40 inoculations of each dilution was made using two cattle in each case. The result is shown in Table VIII.

TABLE/

TABLE VIII.

COMPARISON OF THE OBSERVED AND ESTIMATED FREQUENCIES OF REACTION FOR THE DILUTIONS INOCULATED AT 40 SITES ON TWO TONGUES.

Dilutions	Animal No.	Observed Frequency		Estimated Frequency	
		Positive	Negative	Positive	Negative
$10^{-4.93}$	C/82 P	11	9	15	5
	C/83 P	16	4	15	5
		27	13	30	10
$10^{-5.2}$	C/80 P	5	15	10	10
	C/81 P	9	11	10	10
		14	26	20	20
$10^{-5.46}$	C/78 P	11	9	5	15
	C/79 P	4	16	5	15
		15	25	10	30

In/

In this experiment the reactions of the individual animals are of interest. It will be seen that in each pair of animals there was one that showed very close agreement between the observed and estimated frequency, whereas the other showed some divergence which in the most extreme case (C/82 P and C/78 P) corresponded to a threefold difference in titre. It is unlikely that the distribution of virus particles in the suspensions would influence the result to such an extent and the explanation would appear to be simply one of individual variation in susceptibility. This was more obvious in the case of C/80 P which showed no reaction until the second day after inoculation, whereas the other five animals all reacted on the first day after inoculation. This demonstration of variation in individual susceptibility emphasises the necessity of using more than one animal for each titration. It will be seen from Table VIII that the result provided by two animals may show no significant difference for approximately a twofold difference in dilutions, i.e., $10^{-5.2}$ and $10^{-5.46}$.

It might be argued that if this variation in individual susceptibility exists then the problem is no nearer resolution by increasing the number of observations per dilution if the actual number of animals is not increased. This would be true if the variation in susceptibility was very marked. In an occasional instance/

instance one animal, compared with its fellow, has shown only a mild reaction after some delay. This re-:sistance has been noted in 4 cattle out of a total of 187 used in titration experiments. When such an animal is found, the best procedure is to discard its con-:tribution to the result. Such variation as existed in the remainder of the cattle appeared to be small enough to warrant the use of the refinement in technique made by having ten inoculation sites for each dilution.

An essential concomitant to the description of this technique of titration is an attempt to determine the accuracy with which these estimates are made. It has already been mentioned that a twofold difference in con-:centration was not detected by using two cattle. One method of determining the significant difference between results would be to perform a large number of titrat-:ions of the same virus suspension at the same time by the routine method and calculate the standard deviation. Under ordinary circumstances such an undertaking is hardly practicable when cattle are the test animals.

The error of the test is composed of two main com-:ponents, the error "within each tongue" and the error "between each tongue". The error within each tongue is probably not very great. The evidence for this as-:sumption is that already put forward when considering the possibility of variation in susceptibility of different/

different areas of the tongue. For the purposes of this discussion this error will be ignored.

The error between each tongue is mainly the con-:tribution of the variation in individual susceptibility and may be determined approximately by an analysis of the variance of the fifty per cent. positive end-point as calculated from the observations provided by the individual animals of each pair. Thus, in 26 titrat-:ions in which 2 animals were used, we have the follow-:ing result.

TABLE/

TABLE IX.

THE NEGATIVE POWERS OF 10 OF THE 50 PER CENT. POSITIVE END-POINTS CALCULATED FROM THE OBSERVATIONS PROVIDED BY THE INDIVIDUAL ANIMALS OF EACH PAIR USED FOR 26 TITRATIONS BY THE METHOD OF SIMULTANEOUS MULTIPLE INOCULATION.

Steer 1 a	Steer 2 a′	a^2	a^{-2}	$(a+a')$	$(a + a')^2$
5.8	5.8	33.64	33.64	11.6	134.56
5.6	6.2	31.36	38.44	11.8	139.24
5.6	5.6	31.36	31.36	11.2	125.44
5.8	6.7	33.64	44.89	12.5	156.25
6.0	5.8	36.00	33.64	11.8	139.24
5.5	6.5	30.25	42.25	12.0	144.00
3.5	2.8	12.25	7.84	6.3	39.69
5.8	5.2	33.64	27.04	11.0	121.00
5.5	6.5	30.25	42.25	12.0	144.00
6.5	6.4	42.25	40.96	12.9	166.41
6.9	6.4	47.61	40.96	13.3	176.89
5.4	5.0	29.16	25.00	10.4	108.16
5.8	5.1	33.64	26.01	10.9	118.81
3.8	3.4	14.44	11.56	7.2	51.84
2.9	3.1	8.41	9.61	6.0	36.00
3.8	2.5	14.44	6.25	6.3	39.69
5.3	5.1	28.09	26.01	10.4	108.16
1.4	0.9	1.96	0.81	2.3	5.29
3.9	2.1	15.21	4.41	6.0	36.00
1.6/					

TABLE IX (continued).

Steer 1 a	Steer 2 a′	a^2	a^{-2}	(a+a′)	$(a + a')^2$
1.6	2.6	2.56	6.76	4.2	17.64
4.2	4.4	17.64	19.36	8.6	73.96
4.4	4.4	19.36	19.36	8.8	77.44
3.2	3.1	10.24	9.61	6.3	39.69
2.1	2.8	4.41	7.84	4.9	24.01
3.0	2.9	9.0	8.41	5.9	34.81
3.9	3.7	15.21	13.69	7.6	57.76
		586.02	577.96	232.2	2315.98

$S(a^2 + a^{-2}) = 1163.98$ $\quad \frac{S(a + a')^2}{2} = 1157.99$

Correction factor for considering the mean as zero

$$= \frac{232.2^2}{52} = 1036.86$$

Corrected: $S(a^2 + a^{-2}) = 1163.98 - 1036.86$

$= 127.12$;

and corrected:

$S(a + a')^2 = 1157.99 - 1036.86$

$= 121.13.$

Consequently, the analysis of variance is as follows.

TABLE/

TABLE X.

Variance	Degrees of Freedom	Sum of Squares
Total	51	127.12
Between each tongue	25	121.13
Error between animals in same test	26	5.99
	$\delta^2 = 0.2304$	
	$\delta = 0.48$	

That is to say, the standard error of the end-point as determined with one animal is ± 0.48 of the logarithmic scale. As the standard error of the ob-:servations is inversely proportional to the square root of the number of animals, this figure is reduced to ±0.34 when two animals are used for the test.

In comparing the results of two titrations obtain-:ed by the method of simultaneous multiple inoculation of two cattle the same standard deviation applies to each test, namely ± 0.34. The standard error of the difference is therefore

$$\pm \sqrt{0.34^2 + 0.34^2}$$ (Fisher, 1941, Section 23)

$$= \pm 0.48$$

To be significant the difference between two results must be at least twice this standard error, in other words the difference between the negative powers of 10 must exceed 0.96.

The/

The fall in the frequency of reaction obtained by the progressive twofold dilution of a virus suspension.

Progressive dilution of a virus suspension event-:ually produces a progressive diminution in the fre-:quency with which a reaction is obtained under the conditions of the biological test. Routine titrations with a large dilution ratio and a comparatively small number of observations give only a general indication of this progressive decrease in the proportion of re-:actions with dilution. A more exact determination may be made by increasing the number of observations and decreasing the dilution-ratio. One experiment in cattle with a bovine strain has been performed on these lines.

By using twelve animals a total of thirty observ-:ations was obtained for each of eight dilutions of a twofold series. A preliminary titration was performed to determine the approximate dilution giving 50 per cent. positive results so that an appropriate starting point for the twofold series could be selected.

Titration/

Titration of a filtrate of a 1/25 suspension of bovine vesicle epithelium.
Strain No. 149, Waldmann C type.

Details of the preparation of this filtrate are given in the section describing the use of a six-:fold dilution series (see page 64).

12.3.45.

Preliminary titration: 50 per cent. positive dilution estimated to be $10^{-5.2}$.

15.3.45.

Titration using eight dilutions of a twofold series, thirty observations to each dilution. Dilutions selected: $10^{-4.3}$, $10^{-4.6}$, $10^{-4.9}$, $10^{-5.2}$, $10^{-5.5}$, $10^{-5.8}$, $10^{-6.1}$, and $10^{-6.4}$.

Each of the twelve steers received four dilutions by the ordinary technique of simultaneous multiple inoculation while narcotized by the intravenous inject-:ion of Pentothal sodium. The four dilutions for each animal were chosen at random with the restriction that each dilution should not occur more than once on each tongue. These four dilutions were then arranged systematically with the lowest dilution at the back of the tongue.

This method of selection resulted in the following plan of inoculation, the dilutions being numbered 1 to 8 from $10^{-4.3}$ to $10^{-6.4}$.

TABLE/

TABLE XI.

TITRATION OF 15.3.45.
PLAN OF INOCULATION

C/84 P	C/85 P	C/86 P	C/87 P
1	1	1	2
4	2	2	4
5	4	3	7
7	5	4	8
C/88 P	**C/89 P**	**C/90 P**	**C/91 P**
2	1	1	2
6	3	5	3
7	6	7	4
8	8	8	8
C/92 P	**C/93 P**	**C/94 P**	**C/95 P**
4	1	3	3
5	2	5	5
6	3	6	6
7	6	7	8

The cattle were inoculated at approximately ten minute intervals from 10 a.m. to 12 noon. The rate of development of primary lesions was uniform throughout the group. Readings were terminated at 9 p.m.,16.3.45. Complete generalization followed in every case.

The/

The result of this titration is shown in Table XII.

TABLE XII.

RESULT OF TITRATION OF 15.3.45.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	Positive
$10^{-4.3}$	29	1	48	1	98
$10^{-4.6}$	17	13	69	14	83
$10^{-4.9}$	14	16	52	30	63
$10^{-5.2}$	12	18	36	48	44
$10^{-5.5}$	8	22	26	70	27
$10^{-5.8}$	8	22	18	92	16
$10^{-6.1}$	5	25	10	119	8
$10^{-6.4}$	5	25	5	142	3

The 50 per cent. positive dilution as calculated from these accumulated results is $10^{-5.1}$. The preliminary sixfold titration using two cattle, it will be remembered gave an end-point of $10^{-5.2}$.

GRAPHICAL/

GRAPHICAL REPRESENTATION OF TITRATION EXPERIMENTS.

The simplest method of graphical representation of a titration experiment is to plot the percentage of re-:actions against the logarithmic scale of the dilutions. The result of the titration given in Table XII gives a typical S-shaped curve when plotted in this way (Fig.31). The observations on which these quantitative studies are based are simply the facts of whether a reaction did or did not occur at an inoculated site. In other words, the titration depends on a "quantal response" as de-:fined by Gaddum (1933): 'the term"quantal response" is used for any "all-or-none" biological reaction, i.e., a reaction of such a type or observed under such condit-:ions, that only the bare fact of its presence or absence in each animal is recorded.'

Another function which may be calculated from the observed results is, therefore, the normal deviation, y, equivalent to the probability, p, that an inoculated site will give a positive response. If this normal equivalent deviation, y, is plotted instead of the percentages, the points usually lie on an approximately straight line. Bliss (1935) describes the use of "probits" (probability units) devised by adding 5 to each value of 'y' to facilitate calculations by making all values positive. Tables of "probits" and other relationships/

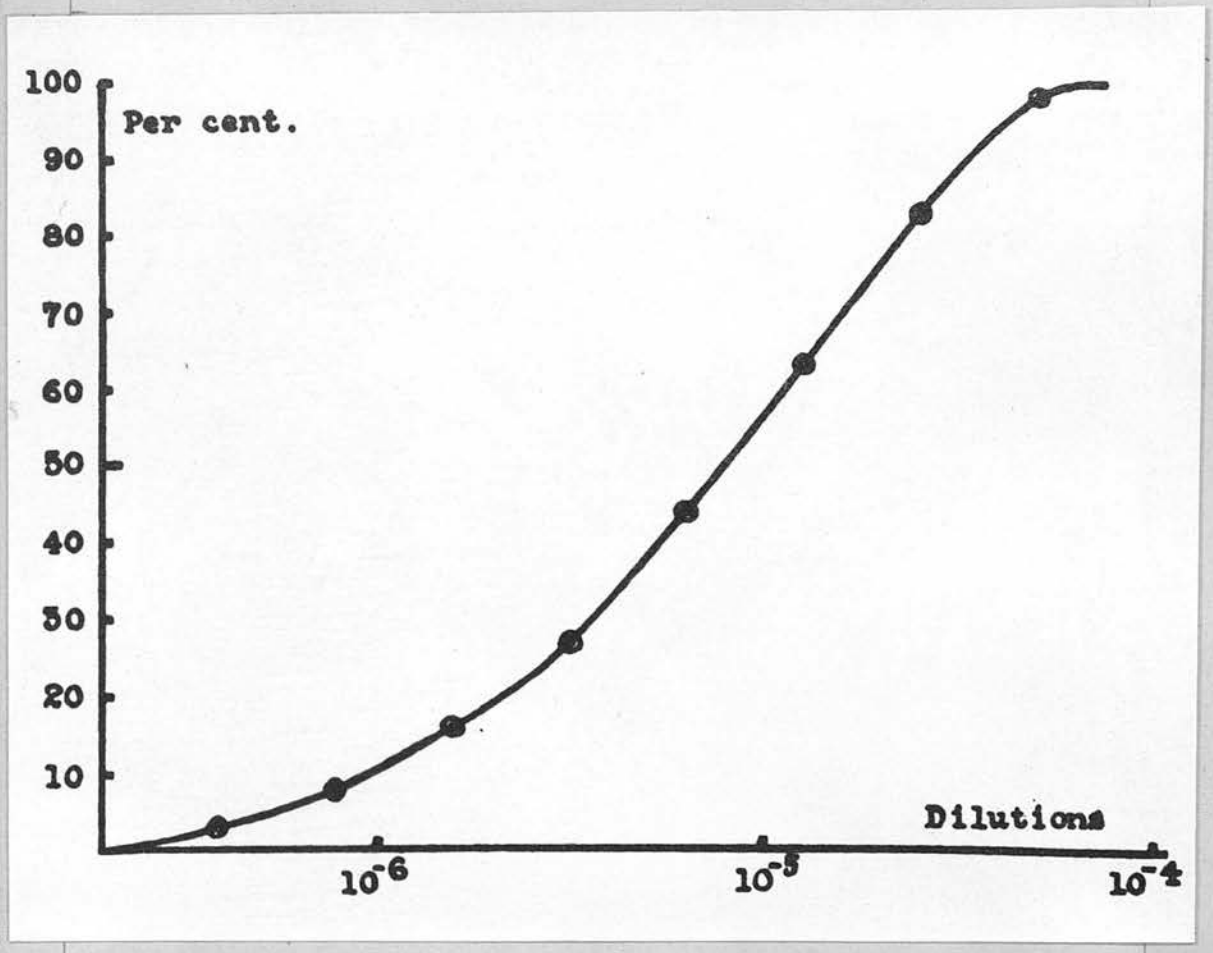

Fig. 31. Graphical representation of the result of the titration of 15.3.45. Percentage of positive results plotted against the dilutions on a logarithmic scale.

relationships are given by Fisher and Yates (1943).

Although this method of presentation of results is largely used by toxicologists, there is no reason why it should not be adapted to other biological tests of a suitable nature and advantage taken of mathematical advances in the handling of data.

Treatment of the result given in Table XII by this method shows that the curve of Fig. 31 becomes trans-:formed into a straight line (Fig. 32). By means of this straight line the theoretical dilution necessary to give any specified result can be quickly estimated. The dilutions selected in the experiment described on page 72 were estimated in this way (Fig. 33).

In most cases it has been sufficient to draw the provisional straight line graphically, but if its pos-:ition and slope is required with more exactitude then the regression line may be fitted as described by Gaddum (1933), Bliss (1935), and Fisher and Yates (1943).

Fitting of the regression line in Fig. 32 gives a value of 1.6557 for "b", the regression coefficient, this being the amount by which the probit of reaction is increased for every unit increase of the logarithmic scale of the dilutions. This determination of the slope of the regression line for the titration of foot-and-mouth disease virus in cattle is useful for the estimation of the 50 per cent. positive end-point in cases/

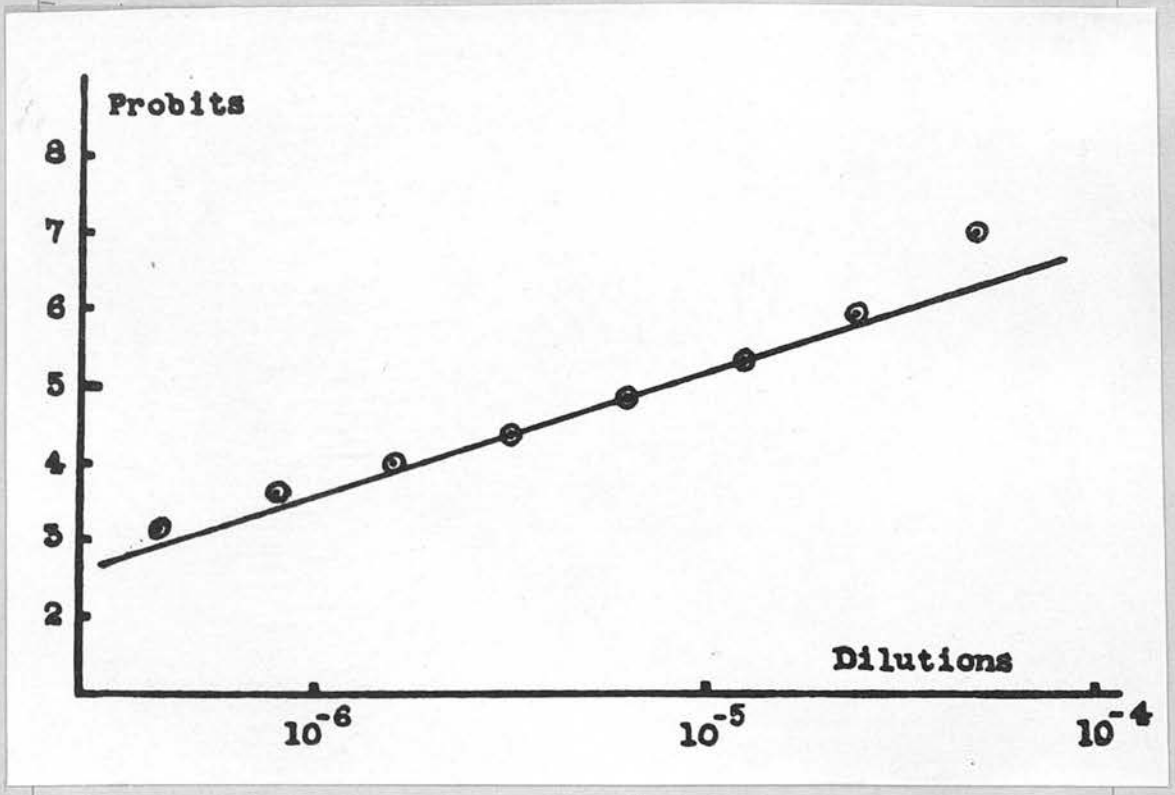

Fig. 32. Graphical representation of the result of the titration of 15.3.45. Transformation of the S-shaped curve of Fig. 31 to a straight line by plotting probits instead of percent-:ages. The scale of the abscissa is five times the scale of the ordinate.

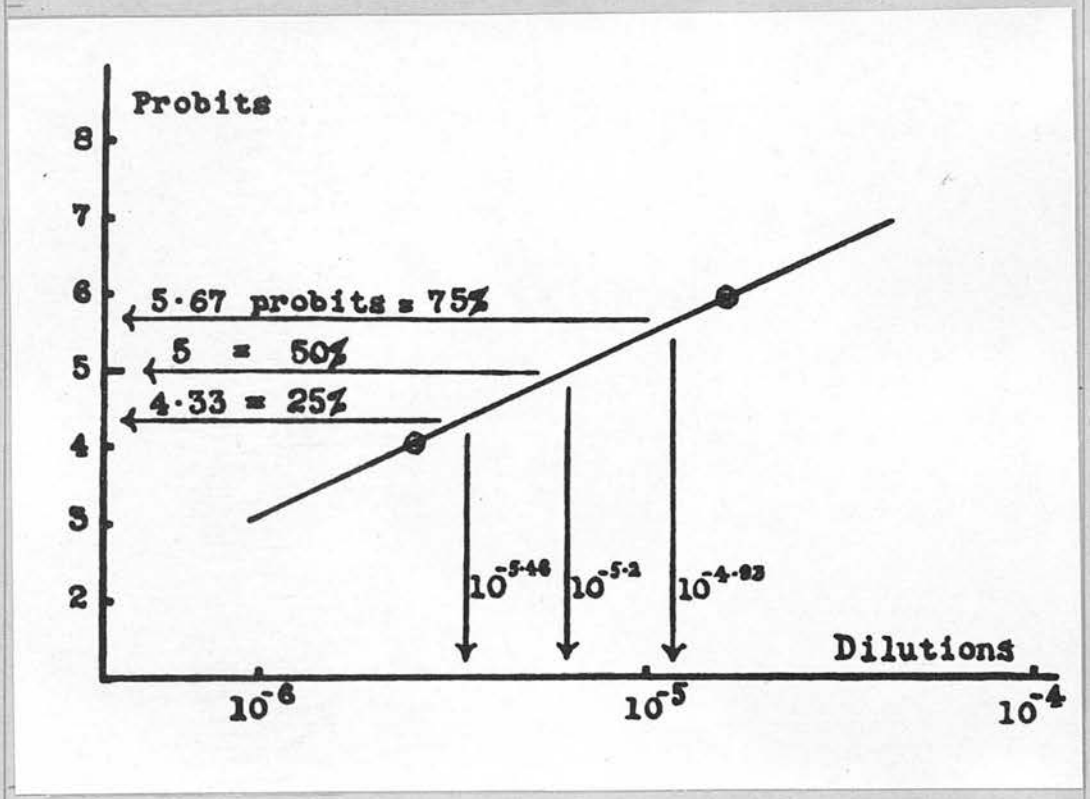

Fig. 33. Graphical representation of the result of the titration of 12.3.45. Method used to estimate the dilutions required to produce certain specified results.

cases where the probit of reaction is available for only one dilution. For example, suppose the 10^{-3} dilution of a suspension produced 20 per cent. positive results, 4.2 probits, then the dilution theoretically producing 50 per cent. positive results, 5 probits, would be $10^{-3.5}$ as

$5 - 4.2 = 0.8 = b/2$

b corresponds to 1 unit of the logarithmic scale of the dilutions

therefore,

b/2 corresponds to 0.5 units

therefore,

5 probits correspond to $10^{-3.5}$.

This result could, of course, be read direct from a graph made by drawing a regression line of slope b = 1.66 through the plotted point of the one available observation.

ESTIMATION/

ESTIMATION BY THE DILUTION METHOD OF THE NUMBER OF INFECTIOUS UNITS PRESENT IN A VIRUS SUSPENSION.

Greenwood and Yule (1917), by using the Poisson series, evolved the dilution method for estimating the number of bacteria in a sample of water where the organ-:isms could not be counted but where their presence or absence could be detected by inoculation of a suitable medium. This type of estimation is discussed by Fisher (1941, 1942), and a full account of its applic-:ation, with the appropriate tables, is given by Fisher and Yates (1943).

If one assumes that one infectious unit of virus corresponds to one bacterium, that each inoculation site of the test animal corresponds to a tube of culture medium, and that a known volume of the virus suspension may be inoculated at such a site, then this method can be used to estimate the number of infectious units in a suspension of virus particles.

For most purposes, however, it is convenient to take the reciprocal of the end-point dilution as an estimate of the number of infectious units in the un-:diluted suspension per volume inoculated at each site. For example, the end-point of the titration of 15.3.45 was $10^{-5.1}$: if it was assumed that 0.2 c.c. of each dil-:ution/

dilution was inoculated at each site, then the undiluted filtrate contained $10^{5.1}$ infectious units per 0.2 c.c., or $10^{5.8}$ per c.c. The filtrate was prepared from a 1/25 suspension of bovine vesicle epithelium so that this material must have contained approximately $10^{7.2}$ infectious units per gramme.

In performing these titrations not all the inoculum remains at each site, thus, when it can only be stated that not more than a specified volume was injected, then the calculations based on this value will tend towards underestimation owing to the escape of a portion of the specified volume.

BRIEF/

BRIEF REPORT OF UNSUCCESSFUL ATTEMPTS TO IMPROVE THE TECHNIQUE USED IN QUANTITATIVE STUDIES OF FOOT-AND-MOUTH DISEASE VIRUS.

Titration using guinea-pigs.

For the routine titration of virus suspensions it is customary to make numerous tracks by intradermal inoculation on both hind pads of each of a group of three guinea-pigs per dilution (Fig. 34). The res-:ponse of the guinea-pig is then considered and more note is taken of the result if the primary lesions are followed by generalization: this means that each guinea-pig provides only one observation. Following the success of the method of increasing the number of observations for each dilution by making a series of inoculations on the bovine tongue, an attempt was made to increase the number of observations available from each guinea-pig.

Four separate sites were obtained on each guinea-pig by making two separate tracks on each hind foot (Fig. 34). The results were then read on the appear-:ance of primary lesions only. Thus, by using three guinea-pigs for each dilution, a total of twelve ob-:servations was available.

No advantage was gained by this modification as it was found that fewer guinea-pigs reacted when inoculat-:ed by making four tracks compared with numerous tracks, and/

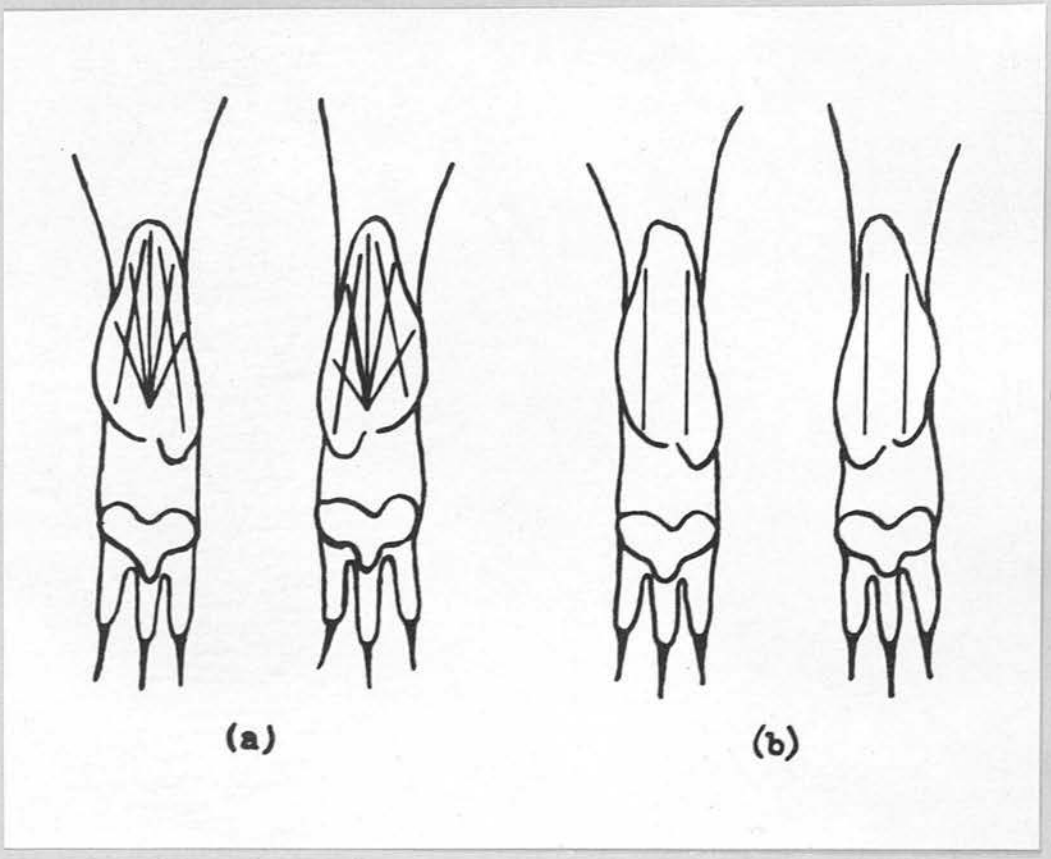

Fig. 34. Inoculation of the hind pads of the guinea-pig.

(a) Routine method.

(b) Modified method in an attempt to get four observations from each animal.

and those that did react had lesions at all four sites. This is well illustrated by the following example.

9.5.44.

Titration of a Seitz filtrate of 1/50 guinea-pig lymph.
Guinea-pig adapted strain GB, Vallée A type.

(1) Routine method:

10^{-3}	++	++	++
10^{-4}	++	++	++
10^{-5}	++	++	++
10^{-6}	++	++	++
10^{-7}	00	00	00

(2) Modified method, primary lesions only:

10^{-3}	++++	++++	++++
10^{-4}	++++	++++	++++
10^{-5}	++++	++++	0000
10^{-6}	++++	0000	0000

This titration was repeated using a larger number of guinea-pigs in order to accentuate any difference that might exist.

(1) Routine method:

10^{-5}		++	++	++		
10^{-6}	++ 00	++ 00	++ 00	++ 00	++ 00	++ 00

(2)/

(2) Modified method, primary lesions only:

10^{-5}	++++	++++	0000	
10^{-6}	++++	++++	0000	0000
	0000	0000	0000	0000
	0000	0000	0000	0000

When, with the routine method of inoculation, the dose of virus has not been sufficient to cause a reaction in all the guinea-pigs of one group, the frequency has been determined with which those that did react have had lesions on one or both hind feet. Out of a total of 529 guinea-pigs, 79 had primary lesions on one foot, the remaining 450 having reactions on both feet. This means that only 1 in 7 guinea-pigs may be expected to show any gradation between a complete primary response and no response, thus showing unequivocally that no benefit could be expected from the modification just described.

Titration using cattle.

It has already been mentioned that a lesion at a site of inoculation on the tongue may only be regarded as being caused by the inoculum provided no secondary lesions have developed. It is evident that this proviso may mean that a true primary lesion that develops later than usual will be disregarded. An attempt was made to avoid this possibility by giving the cattle

a/

a dose of convalescent serum in an effort to retard or prevent the development of secondary lesions. Earlier work had shown that such serum did not prevent the development of primary lesions following the intradermal inoculation of the tongue. Only one experiment was performed on these lines in which a sample of defibrinated bovine blood (Strain No. 149, Waldmann C type) was titrated by the simultaneous inoculation of different dilutions of a tenfold series. Four cattle were inoculated using one site for each dilution, two having received a subcutaneous dose of C type convalescent serum at the rate of 50 c.c. per 112 lb. body weight, twenty-four hours before inoculation.

The highest dilution to cause a reaction in the two untreated cattle was 10^{-4} and 10^{-3} respectively, followed by complete generalization. In the two serum-treated animals the highest dilution was 10^{-2} in both cases with no development of secondary lesions. The lesions in the serum-treated animals were less extensive and not so vesicular as the lesions in the untreated animals. This fact, combined with the lower end-point, suggested that although a subcutaneous dose of convalescent serum could not prevent the development of primary lesions following the intradermal inoculation of the tongue with a potent virus suspension, the serum, when given twenty-four hours earlier, could render/

render the animal less sensitive to the inoculation of small doses of virus. For this reason the investigat-:ion was not proceeded with, as it would appear that any advantage that might be gained by preventing the devel-:opment of secondary lesions would not justify the use of the number of cattle that would be required to com-:plete the work by studying the effect of altering the dose and the time of administration of the serum.

INFORMATION/

INFORMATION GAINED BY THE QUANTITATIVE STUDY OF FOOT-AND-MOUTH DISEASE VIRUS.

Titration of well-adapted guinea-pig strains in guinea-pigs.

Work has been done with three well-adapted guinea-pig strains, namely strain no. 1 of Vallée O type, strain GB of Vallée A type and strain GC of Waldmann C type.

The material usually selected for work in guinea-pigs is filtered vesicle lymph. For collection of lymph, a number of guinea-pigs are inoculated intra-:dermally on both hind pads. Twenty-four hours later, when good primary lesions should have developed, the contents of the vesicles are aspirated with a Pasteur pipette. The lymph thus collected is diluted immed-:iately with nine parts of buffered phosphate saline solution. After centrifugation at a slow speed to remove clot and cellular debris, the supernatant is further diluted to 1/25 or 1/50 with phosphate saline and Hartley's broth. This diluted lymph is now passed through a Seitz EK filter disc or a gradocol membrane of 0.6μ A.P.D.

The titre of this filtered lymph will be influenc-:ed by the condition and response of the donor guinea-pigs, the storage period of the material used for the inoculum/

inoculum, the time of collection and the period that elapses between collection and titration. There is no appreciable loss of titre, however, during the first month if the filtrate is stored at about 4°C.

These three guinea-pig strains have been passaged at regular intervals, lymph collected at the optimum time and a routine titration performed.[1] Massing all the observations for each titration gives a mean titre for the various samples of lymph. It is felt that this presentation of the experience gained during the time the work has been in progress is more useful than cit-:ing the highest "take" that has been recorded.

The mean fifty per cent. positive dilution for each strain is : Strain No. 1, $10^{-6.4}$; Strain GB, $10^{-6.3}$; and Strain GC, 10^{-6}. As these end-points are within a comparatively small range, the total re-:sults for the three strains have been taken together to give a mean titre for guinea-pig lymph of a well-adapted strain: the fifty per cent. positive dilution in this case is $10^{-6.26}$. The percentage positive for each dilution is shown in Table XIII.

TABLE/

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[1] Some of these collections and titrations of guinea-pig lymph have been made by my colleague, Mr J.B. Brooksby, to whom I am indebted for the data recorded by him.

TABLE XIII.

COLLECTED OBSERVATIONS FROM TITRATIONS IN GUINEA-PIGS OF LYMPH FROM THREE WELL-ADAPTED GUINEA-PIG STRAINS.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	Positive
10^{-2}	54	0	420	0	100
10^{-3}	70	0	366	0	100
10^{-4}	105	0	296	0	100
10^{-5}	104	10	191	10	95
10^{-6}	71	44	87	54	62
10^{-7}	16	31	16	85	16
10^{-8}	0	6	0	91	0

To get more accurate information about the re-:gression line characteristic of the titration of guinea-pig lymph in guinea-pigs, a twofold dilution series was prepared and ten guinea-pigs were inoculated with each dilution.

30.4.45/

30.4.45.

Titration of guinea-pig lymph. Passed through a 0.6μ A.P.D. Gradocol membrane in a dilution of 1/50. Dilutions for test prepared in terms of the undiluted lymph.
Strain GC, Waldmann C type.

10^{-6}	++	++	++	++	++	++	++	++	++	+0
$10^{-6.3}$	++	++	++	++	++	++	+0	+0	00	00
$10^{-6.6}$	++	++	++	++	+0	00	00	00	00	00
$10^{-6.9}$	++	++	++	++	00	00	00	00	00	00
$10^{-7.2}$	++	++	+0	00	00	00	00	00	00	00
$10^{-7.5}$	++	++	00	00	00	00	00	00	00	00

Considering the primary reactions, only the per-:centage positive for each dilution is shown in Table XIV.

TABLE/

TABLE XIV.

RESULT OF TITRATION OF 30.4.45.

Dilutions	Observations		Accumulations		Per cent.
	Positive	Negative	Positive	Negative	
10^{-6}	10	0	32	0	100
$10^{-6.3}$	8	2	22	2	92
$10^{-6.6}$	5	5	14	7	67
$10^{-6.9}$	4	6	9	13	41
$10^{-7.2}$	3	7	5	20	20
$10^{-7.5}$	2	8	2	28	7

The graphical representation of these results is shown in Fig. 35.

The/

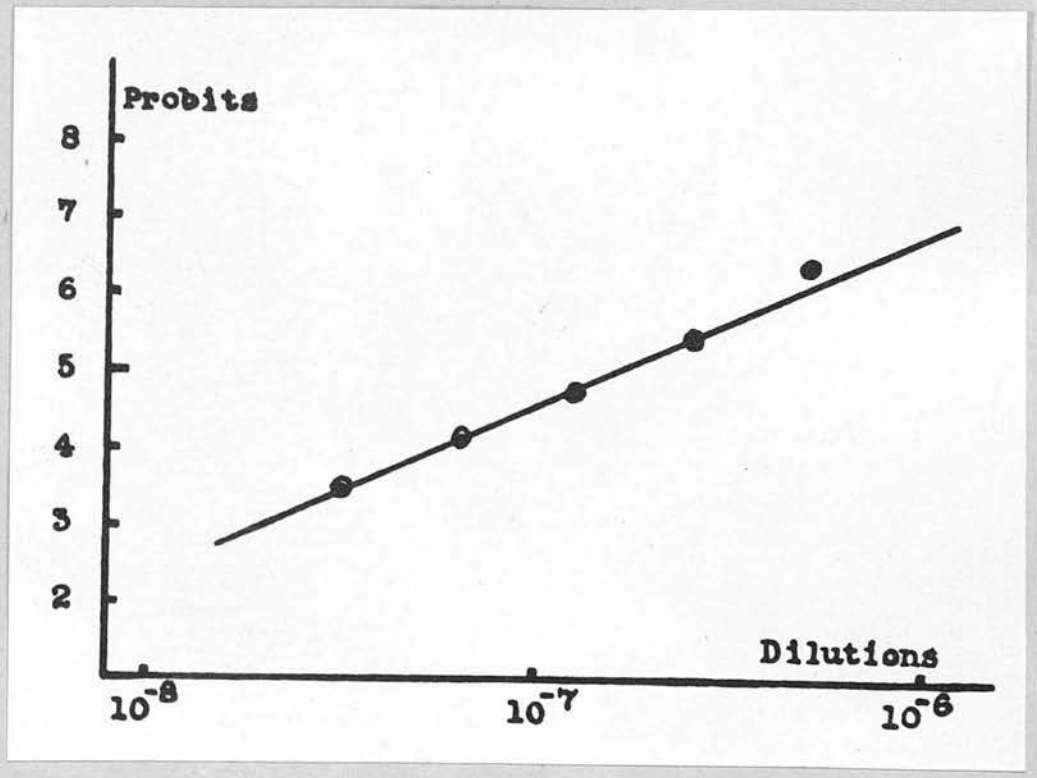

Fig. 35. Graphical representation of the result of the titration of 30.4.45.

The regression line of this titration shows that a tenfold difference in concentration corresponds to 2.3 probits, whereas it has frequently been noted in titrations with only three guinea-pigs for each dilution that a tenfold difference covers the whole range of probability from 3/3 to 0/3 positive, more than 6 probits. No titration in cattle or guinea-pigs in which ten or more observations have been available for each dilution has so far yielded a regression line steep enough to fit such a result. The conclusion reached is that three guinea-pigs per dilution are inadequate for the estimation of the 50 per cent. positive end-point and, although the information obtained may none the less be useful, the result must be interpreted by citing the highest dilution to cause a reaction.

Titration of well-adapted guinea-pig strains in cattle and in guinea-pigs.

In discussing the question of the adaptation of strains of foot-and-mouth disease virus to the guinea-pig, it was mentioned that as a converse to the low titre of a bovine strain as shown by titration in guinea-pigs, a guinea-pig strain appeared to have a lower titre when titrated in cattle. The following experiments illustrate this point.

29.12.43./

29.12.43.

Titration of vesicle lymph of a well-adapted guinea-pig strain. Lymph filtered and stored 7 days at 4°C.
Strain No. 1, vallée O type.

(1) Titration using guinea-pigs.

10^{-5}	++	++	++
10^{-6}	++	++	++
10^{-7}	++	OO	OO

(2) Titration using cattle; simultaneous inoculation of different dilutions, one site for each.

Steer No. C/48 H — Inoculated intradermally 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.

Reactions at the 10^{-3} and 10^{-4} sites: no generalization.

Steer No. C/49 H — Inoculated intradermally 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.

Reactions at the 10^{-3} and 10^{-4} sites: no generalization.

29.12.43/

2912.43.

Titration of vesicle lymph of a well-adapted guinea-pig strain. Lymph filtered and stored 7 days at 4°C.
Strain No.GB, Vallée A type.

(1) Titration using guinea-pigs.

10^{-5}	++	++	++
10^{-6}	++	++	++
10^{-7}	++	++	00

(2) Titration using cattle; simultaneous inoculation of different dilutions, one site for each.

Steer No. C/50 H — Inoculated intradermally 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.

Reactions at the 10^{-3}, 10^{-4} and 10^{-5} sites; no generalization.

Steer No. C/51 H — Inoculated intradermally 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.

Reactions at the 10^{-3}, 10^{-4} and 10^{-5} sites; no generalization.

29.12.43.

Titration of vesicle lymph of a well-adapted guinea-pig strain. Lymph filtered and stored 7 days at 4°C.
Strain GC, Waldmann C type.

(1) Titration using guinea-pigs.

10^{-5}	++	++	++
10^{-6}	++	++	+0
10^{-7}	00	00	00

(2)/

(2) Titration using cattle; simultaneous inoculation of different dilutions, one site for each.

Steer No. C/52 H	Inoculated intradermally 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions.
	No reaction at any of these sites.
1.1.44	Inoculated intradermally 1/50 lymph (undiluted filtrate).
	Reacted: no generalization.
Steer No. C/53 H	Inoculated intradermally 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions.
	Reactions at the 10^{-3} and 10^{-4} sites, secondary lesions on three feet.

The results of these three experiments are summarised in Table XV.

TABLE/

TABLE XV.

TITRATION OF WELL-ADAPTED GUINEA-PIG STRAINS IN CATTLE AND IN GUINEA-PIGS.

Guinea-pig adapted strain	Highest dilution to react	
	Guinea-pigs	Cattle
No. 1, O type	10^{-7}	10^{-4}
GB, A type	10^{-7}	10^{-5}
GC, C type	10^{-6}	10^{-4}

It is of interest to observe how long serial passage in guinea-pigs of a strain recovered from cattle decreases the virulence of the strain for its original host. These results also emphasize the point that if accuracy is required in quantitative studies of foot-and-mouth disease virus, then suspensions of the virus must be titrated using the same species of animal as that for which the strain is adapted.

Titration/

Titration of bovine strains in cattle and in guinea-pigs.

Virus strains of bovine origin representing all three immunological types have been titrated using cattle and guinea-pigs. Various infected tissues and fluids have been studied, most work having been done, however, with defibrinated blood, vesicle epithelium and vesicle lymph.

The results of separate titrations have been ac-:cumulated to give the mean fifty per cent. positive end-point for each tissue and strain examined. The number of observations from which each mean end-point was calculated has been noted to give an indication of the weight that can be attached to each determination. Comparison of the values obtained using cattle with those obtained using guinea-pigs provides ample evid-:ence of the limitations of the use of the latter species for this work.

Accumulated results of titrations of defibrinated bovine blood. The various samples of blood used in these tests were taken from the jugular vein as near as possible to what appears to be the optimum time for collection of blood of high titre, namely during the process of generalization when secondary lesions have developed at some, but not all, of the sites of pre-:dilection. Table XVI gives the mean fifty per cent. end-point provided by six O type, one A type and one C type strain.

TABLE/

TABLE XIV.

MEAN FIFTY PER CENT. POSITIVE END-POINT CALCULATED FROM ACCUMULATED RESULTS OF TITRATIONS OF DEFIBRINATED BOVINE BLOOD.

Virus Strain and Type	Titration using			
	Cattle		Guinea-pigs	
	Mean end-Point	No. of observations	Mean end-Point	No. of observations
No. 39, O	$10^{-3.3}$	116	$10^{-1.1}$	500
No. 336, O	$10^{-3.4}$	10	$10^{-0.8}$	222
No. 476, O	$10^{-3.5}$	44	$10^{-0.8}$	48
ASJ, O	$10^{-3.3}$	100	-	-
FC, O	10^{-3}	40	$10^{-1.8}$	12
WA, O	10^{-4}	120	$10^{-1.5}$	12
No. 119, A	$10^{-3.5}$	42	$10^{-2.2}$	307
No. 149, C	10^{-4}	16	$10^{-2.1}$	144

Accumulated/

Accumulated results of titrations of bovine vesicle epithelium. Suspensions of epithelium or filtrates of suspensions were used for these studies, but as various strengths of suspension were used in different experiments, the dilutions have been converted into terms of epithelium in accumulating these results. The epithelium was collected from fresh unruptured tongue lesions, minced, ground with sand and M/25 buffered phosphate saline solution, centrifuged for up to 30 minutes at 2000 r.p.m. and the supernatant used as the virus suspension. If a filtrate was required, the suspension was diluted with an equal volume of Hartley's digest broth and passed through a Seitz EK disc or a sand and paper pulp filter, then a gradocol membrane.

Table XVII gives the mean fifty per cent. positive end-point provided by a strain of each type.

TABLE/

TABLE XVII.

MEAN FIFTY PER CENT. POSITIVE END-POINT CALCULATED FROM ACCUMULATED RESULTS OF TITRATIONS OF BOVINE VESICLE EPITHELIUM.

Virus Strain and Type	Titration using			
	Cattle		Guinea-pigs	
	Mean end-Point	No. of observations	Mean end-Point	No. of observations
No. 39, O	$10^{-6.8}$	413	$10^{-4.7}$	276
No. 119, A	$10^{-6.8}$	423	$10^{-3.9}$	243
No. 149, C	$10^{-6.5}$	420	$10^{-4.2}$	93

Accumulated results of titrations of bovine vesicle lymph. Lymph was collected from unruptured tongue lesions and a 1/10 dilution immediately prepared using equal parts M/25 buffered phosphate saline solution and Hartley's digest broth as diluent. This diluted lymph was then centrifuged for up to 30 minutes at 2000 r.p.m. and the supernatant passed through a sand and paper pulp filter then a gradocol membrane of 0.6μ A.P.D.

Table/

Table 18 gives the mean fifty per cent. positive end-point provided by two O type strains: the dilutions are in terms of the undiluted lymph.

TABLE XVIII.

MEAN FIFTY PER CENT. POSITIVE END-POINT CALCULATED FROM ACCUMULATED RESULTS OF TITRATIONS OF BOVINE VESICLE LYMPH.

Virus Strain and Type	Titration using			
	Cattle		Guinea-pigs	
	Mean end-Point	No. of observations	Mean end-Point	No. of observations
ASJ, O	$10^{-7.9}$	80	-	-
WA, O	$10^{-7.7}$	40	-	-

These results are of interest in showing the relative concentration of virus in the three main sources available. So far as this work goes there is no indication of any great difference in the relative virus contents of the tissues of an animal infected with one strain compared with those of an animal infected with another strain, but clinical observations show that this quantitative similarity is not necessarily linked with virulence.

DISCUSSION/

DISCUSSION.

The quantitative study of the virus of foot-and-mouth disease depends on the production of the disease in a susceptible animal, therefore the success of such a study depends on the degree of susceptibility of the test animal. This degree of susceptibility can only be estimated in relation to a characteristic of the particular virus strain with which it is proposed to work. This characteristic is its degree of adaptation to different host species, and this may be modified by continued serial passage. The test animal for a quantitative determination must be chosen, therefore, in relation to the current species adaptation of the strain.

Having decided on the species of test animal for any particular experiment, the next difficulty is met in connection with the variation in individual susceptibility that will probably exist amongst the animals available for the test. If the strain is well adapted to the guinea-pig, the guinea-pig is the animal of choice, and variation in individual susceptibility is largely influenced by age and condition. If a bovine strain is being used, then, to get satisfactory results, cattle will probably have to be chosen. In

a/

a country free from the disease or where the disease is strictly controlled, as in Britain, by the slaughter of all ruminants and swine infected or exposed to the risk of infection, this difficulty is simply that due to variation in natural susceptibility and is not sufficiently great to prevent advantage being taken of the refinements in method described in this work. Where the disease is endemic, however, as in many parts of Europe, Asia, Africa and South America, there may be no knowledge of an individual animal's history and so a test animal, having already suffered from the disease, may prove to be completely resistant to strains of a particular immunological type. Under these circumstances, a number of cattle may have to be used for each titration in the hope of finding two that appear to react as if they were fully susceptible. It would probably be sufficient in this case to use the method of one inoculation site for each dilution, further improvement of the technique being a waste of effort in view of such a large potential source of error.

The cattle used in these experiments have all been of the same age and breed, namely Devon steers from 1½ to two years old. No observations are available for the relative susceptibility of cattle of other breeds and ages. In countries such as India and Africa, where there/

there are indigenous breeds of cattle as well as im-:ported European stocks, a difference in natural sus-:ceptibility may exist between these two groups. Comparative quantitative tests would provide one of the best ways of investigating this point and so enable the most susceptible type of animal to be selected. By using two Devon steers and by obtaining ten observations for each dilution, the standard error of each test has been estimated to be ± 0.34 of the logarithmic scale of the dilutions. This error would be substantially reduced if it were possible to titrate a standard pre-:paration of stable potency at the same time as the unknown virus suspension. By using one half of the tongue for titration of the standard and the other half of the tongue for titration of the unknown then the error due to variation in individual susceptibility could be largely eliminated. No attempt has yet been made to reduce the error of the test in this way as insufficient work has been done on the survival of the virus under conditions of storage likely to produce a stable preparation.

One advantage of using cattle instead of guinea-pigs has become apparent and that is the speed with which the result of a titration is obtained with cattle. In this case the result is known within 24

to/

to 36 hours after inoculation, whereas with guinea-pigs some bovine material is not capable of producing a primary reaction in less than 4 to 5 days.

SUMMARY/

SUMMARY.

A review is given of the methods available for the quantitative study of filterable viruses and those methods applicable to the virus of foot-and-mouth dis-:ease.

The quantitative study of fo ot-and-mouth disease virus depends on the detection of the infective agent in progressive dilutions of the virus suspension by inoculation of susceptible animals.

Since the discovery of the susceptibility of the guinea-pig to the virus, this animal has been largely used in tests for its detection.

The usefulness of the guinea-pig for this work is limited by the fact that strains of foot-and-mouth disease virus possess a strong species adaptation.

This adaptation to a particular species may us-:ually be modified by serial passage in another species.

Until a virus strain has become adapted to the guinea-pig, this animal cannot be used for the detection of small amountsof the virus of that particular strain. For example, the limiting infective dilution of bovine strains is from ten to a thousandfold lower when de-:termined by using guinea-pigs compared with cattle.

For/

For the quantitative study of bovine strains of foot-and-mouth disease virus methods of titration using cattle have been evolved.

The most successful of these methods consists of the simultaneous inoculation of the bovine tongue with different dilutions of the virus suspension. The routine method of titration eventually adopted consists of the simultaneous inoculation of the tongues of two cattle with four dilutions using five inoculation sites on each tongue for each dilution, thus providing ten observations from two animals for each of four dilutions.

The end-point of such a titration is expressed as the theoretical dilution that should give an equal number of positive and negative results when inoculated into the same host species under the same conditions, the fifty per cent. positive end-point.

Using two Devon steers, 1½ to 2 years old, the estimated error of such a test is ± 0.34 of the logarithmic scale of the dilutions.

A method is described of the graphical representation of the result of a titration experiment by plotting the dilutions on a logarithmic scale against the units of probability, or "probits".

A brief report is given of unsuccessful attempts to/

to improve the technique used in quantitative studies of foot-and-mouth disease virus.

The performing of these methods of titration using cattle is discussed in connection with the difficulty of obtaining susceptible animals in countries where the disease is endemic.

The mean fifty per cent. positive end-point of vesicle lymph of well-adapted guinea-pig strains titrated in guinea-pigs was found to be $10^{-6.26}$.

The mean fifty per cent. positive end-points of bovine defibrinated blood, bovine vesicle epithelium and bovine vesicle lymph titrated in cattle were found to be $10^{-3.5}$, $10^{-6.7}$ and $10^{-7.8}$ respectively.

This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have kindly given permission for its publication. It is a pleasure to acknowledge the co-operation of the Director and staff of the Committee's Research Station during the time this work has been in progress, in particular, the able assistance of W.J. Brownsea. Histological preparations are by R.H. Treadwell. Photo-micrographs are by F.E. Welch, and the photographical reproduction of the diagrams by C. Sutton of the National Institute for Medical Research, Hampstead. Colour photographs are by P.F. Hennell of the Metal Box Co., Ltd., London.

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APPENDIX/

APPENDIX.

PARTICULARS OF THE STRAINS OF FOOT-AND-MOUTH DISEASE VIRUS USED IN THIS WORK.

Well-adapted guinea-pig strains.

Strain No. 1, Vallée O type. Isolated from an outbreak in cattle in Great Britain in 1924. Passaged regularly in guinea-pigs since that date and used as the stock O type guinea-pig strain.

Strain GB, Vallée A type. Received from the German Foot-and-Mouth Disease Research Institute, Insel Riems, in 1929. Described as their stock guinea-pig strain, passaged regularly in guinea-pigs at Pirbright since receipt and used as the stock A type guinea-pig strain.

Strain GC, Waldmann C type. Received from the German Foot-and-Mouth Disease Research Institute, Insel Riems, in 1933. Described as their stock guinea-pig strain, passaged regularly in guinea-pigs at Pirbright since receipt and used as the stock C type guinea-pig strain.

Bovine strains.

Strain No. 39, Vallée O type. Isolated from an outbreak in cattle in Great Britain in 1928. Used as the stock O type bovine strain.

Strain/

Strain No. 336, Vallée O type. Isolated from an outbreak in cattle in Great Britain in 1939.

Strain No. 476, Vallée O type. Isolated from an outbreak in cattle in Great Britain in 1942.

Strain ASJ, Vallée O type. An Argentine strain received from Drs. Schang and Rossi in 1944. Isolated from an outbreak in cattle in 1935 on the "San Justino" ranch, Buenos Aires.

Strain FC, Vallée O type. A French strain isol-:ated from an outbreak in cattle in 1937 at Critot, Seine Inférieure.

Strain WA, Vallée O type. A German strain re-:ceived from the German Foot-and-Mouth Disease Research Institute, Insel Riems, in 1939. Described as the stock O type strain used in the preparation of Waldmann and Kübe's aluminium hydroxide adsorbed vaccine.

Strain No. 119, Vallée A type. Isolated from an outbreak in cattle in Great Britain in 1932. Used as the stock A type bovine strain.

Strain No. 149, Waldmann C type. Isolated from an outbreak in cattle in Great Britain in 1934. Used as the stock C type bovine strain.

In the intervals between passage these strains are/

are maintained by storage of vesicle epithelium at + 4° C. in equal parts of glycerine and M/25 buffered phosphate saline solution, pH 7.6. Periodic cross-immunity tests have shown that their type specificity has been retained.