VOLUME XXVIII FEBRUARY, 1929 No. 4

THE REACTION OF COMPLEMENT FIXATION IN FOOT-AND-MOUTH DISEASE AS A MEANS OF IDENTIFYING THE DIFFERENT TYPES OF VIRUS.

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NOTWITHSTANDING several efforts, a satisfactory method of serological diagnosis of foot-and-mouth disease has not been arrived at. This uncertainty of serological methods delayed the discovery of the plurality of type of the virus, although the observations of epizootics and the irregular results obtained by active and passive immunisation suggested multiplicity of type. Stimulated by such observations, Vallée and Carré (1922) experimented with two viruses of foot-and-mouth disease, one of which was of German and the other of French origin. They were able to show by experimental infection, confirmed by subsequent testing for active immunity, that cases of foot-and-mouth disease with the same clinical course and symptoms could be caused by two distinct types of virus. These they called A (Allemand) and O (Oise). The only difference between these two types was their inability to produce reciprocal immunity. The existence of more than one type of virus has been confirmed in England by Stockman and Minett (1926), and by Bedson, Maitland and Burbury (1927); in Germany, by Waldmann and Trautwein (1926), and Trautwein (1927); in France, by Lebailly (1926), Olitsky (1927), and in Sweden, by Magnusson and Hermansson (1926).

All these workers performed crossed immunity experiments, either obtaining active immunity by infection or passive immunity by means of serum. They confirmed the previous observations on the plurality of types of virus, using both naturally susceptible animals, *i.e.* cattle, sheep and swine and also guinea-pigs, which can only be infected experimentally. Waldmann and Trautwein (1926), who studied the disease during the epizootic of 1926 in Germany, ascertained that yet a third type of virus existed.

It is, therefore, now established by experiment that foot-and-mouth disease with the same symptoms and course can be produced by virus of three different types. These were called by Waldmann, A, B and C. They can only be distinguished by the specificity of their antigens, each of which is able to immunise an animal against itself only. It has been shown by Burbury (1928) that Waldmann's type A and B are identical with Vallée's "O" and "A" respectively. For this reason, animals can be infected with foot-and-mouth disease virus three times successively. According to Trautwein (1927) such

a consecutive series of infections is successful in 100 per cent. of guinea-pigs; in swine 84 per cent. can be infected when inoculated with a second virus and 72 per cent. with a third. In cattle 58 per cent. can be successfully infected twice and 37 per cent. three times. The same differences between different species of animals are found when immunisation is carried out passively with antiserum. Immune serum from animals after infection with one type is monovalent when derived from 100 per cent. of guinea-pigs, but heterologous antibodies are found 14 days after infection in 52 per cent. of cattle and 33 per cent. of swine.

Summing up these results:

(1) There are at least three different types of the virus of foot-and-mouth disease.

(2) An attack of the disease due to one type does not protect the animal against infection by the other types.

(3) The antibodies in the blood of animals after infection or after hyper-immunisation are specific.

Serological Tests in Foot-and-Mouth Disease.

Lourens (1909) used as antigen the virus passed through a Berkefeld filter and was able to show by complement fixation the presence of specific sensitising antibodies in the blood of hyperimmunised cattle. Ascoli (1910), however, using the same method, could not find sensitising antibodies in the serum of infected animals, but by the meiostigmin reaction obtained positive results, and he recommends this procedure as a good diagnostic method. Favero (1914), by testing the serum of guinea-pigs which had been repeatedly injected with large quantities of virus, found sensitising antibodies.

Ernst (1922) could not discover precipitating antibodies after the injection of lymph, virulent serum, or organic extracts of materials taken from sick animals; he found in some cases sensitising antibodies, but these were not specific for foot-and-mouth disease.

Titze (1922) vaccinated various animals with "cultures" of an ultra-microscopic germ which he claimed to have isolated from foot-and-mouth disease virus. He obtained complement fixation reactions with the blood of the animals 5 days after infection, using the same "cultures" as antigen.

Mezinescu, Baroni and Calinescu (1923), using vesicle fluid as antigen, failed to obtain complement fixation with the serum of convalescent cattle and swine, but succeeded with the serum of animals immunised by several injections with the same virus.

Waldmann and Trautwein (1923) could not obtain complement fixation either with the hyperimmune sera of cattle, swine or guinea-pigs.

Urbain (1927), who used as antigen emulsions of epithelium from lesions due to the disease, or vesicle fluid diluted 1 in 10 with saline, obtained a positive

amount of sensitising antibodies. All three sera gave a negative reaction in the presence of vaccinia lymph used as antigen.

Minett (1927) examined seven immune or hyperimmune sera from cattle and seven from convalescent guinea-pigs, with several antigens, viz. Bordet's antigen, both with and without cholesterol; alcoholic extract of the pads of infected or normal guinea-pigs; watery extracts of the same parts after 4 days' maceration in the ice chest; saline emulsion of the lesions; and diluted vesicle fluid. He did not, however, obtain a specific complement fixation reaction. Sachelarie, Boquet and Urbain (1928) attempted to differentiate between infections with the "A" and the "O" viruses in cattle by the method of fixation of alexine according to the technique of Calmette-Mussol, but obtained no definite results.

Lastly, Olitsky, Traum and Schoening, the Commissioners appointed by the U.S. Department of Agriculture (1928), explored the possibility of complement fixation as a diagnostic test for foot-and-mouth disease. They used as antigen, a variety of preparations of vesicle fluid and the epithelial coverings of vesicles from guinea-pigs, cattle and swine. The serum of convalescent and hyperimmunised animals was tested. They could not, however, discover a specific antibody in any of these sera.

Although the results of different experimenters are mostly negative, some of them indicate that sensitising antibodies may be found in the blood of cattle suffering from foot-and-mouth disease and in the blood of hyperimmunised animals. They do not, however, for the most part give detailed data concerning the techniques employed. It seemed worth while therefore to make a further attempt to find an antibody (sensibilisatrice) in the serum of animals attacked by this disease, for its discovery would have great importance now that it is known that the disease can be produced by several types of virus, and exact means for the identification of the type of virus present in an epizootic are needed in order to apply specific prophylaxis or treatment to the best advantage. For this purpose the reaction of fixation of complement would be of great value if it were found to be feasible. Detailed research with guinea-pigs has, accordingly, been carried out with the object of investigating:

(1) whether sensitising antibodies are present in the blood of animals infected with or hyperimmunised against each of the three known types of virus; and, if found,

(2) the time of their appearance and their duration in the blood;

(3) the specificity of the sensitising antibodies;

(4) and, lastly, the possible identification *in vitro* of the different types of virus by complement fixation.

MATERIALS USED IN THE REACTION: THEIR PREPARATION AND THE TECHNIQUE TO BE ADOPTED.

Red cells:—Sheep's red cells which had been washed four times were used in a dilution corresponding to 5 per cent. of blood. They were kept in the cold room but were not used after 6 days. Rabbit's haemolytic serum was used in twice the minimum quantity sufficient to give complete haemolysis.

As complement, the serum of guinea-pigs which had been bled by heart puncture was used: it was kept 24 to 48 hours and diluted 1 in 10 before use.

Specific antibodies:—The immune serum examined was either that of guinea-pigs which had been infected with foot-and-mouth disease, *i.e.* convalescent serum, or was derived from hyperimmunised guinea-pigs.

(*a*) *Convalescent serum.* Guinea-pigs recovered from intradermal infection with one type of virus were bled to death 4, 5, 8, 9, 10, 12, 18, 21, 23, 32, 37, 48, 50, 55, 75 and 120 days after infection. The blood was kept for 2 or 3 hours at room temperature and afterwards in the cold room. After 24 hours the serum was removed and kept until the red cells were deposited. It was then pipetted off and inactivated for half an hour at 55° C. and subsequently kept in the cold room. During the first 15 days of the disease the guinea-pigs' serum was apt to be red on account of the excessive fragility of the red blood corpuscles. To prevent the serum becoming coloured with haemoglobin the blood should be collected in test-tubes which have been sterilised with a small quantity of saline in them, so that the walls are moist when they receive the blood. The clot then separates readily from the walls and the serum contains much less haemoglobin.

(*b*) *Monovalent hyperimmune serum* was obtained from guinea-pigs which had been treated with one of the three known types of virus,

(i) type Vallée O (= Waldmann A),
(ii) type Vallée A (= Waldmann B),
(iii) type Waldmann C.

The technique of immunisation was as follows:

The guinea-pigs were infected intradermally with one of the types so that both local and generalised vesicles appeared. When complete recovery was established, *i.e.* 15 to 25 days after infection, further doses of the same virus were injected either intradermally, subcutaneously or intramuscularly. The same route was always used for the same animal.

In each procedure the hyperimmunisation was performed by three or five series of injections of virus. Each series consisted of three injections on successive days, and between each series there was an interval of 5 days. The dose of virus given each time was 0·25 c.c. in the first series, 0·5 c.c. in the second series and 1·0 c.c. in the third and subsequent series.

The virus used was the fluid from vesicles on the feet of guinea-pigs diluted 1 in 50 with phosphate solution of *p*H 7·6 for the first three series, but the dilution was 1 in 10 when fourth and fifth series were given. The titre of

the vesicle fluid used was 1 in 500,000 to 1 in 1,000,000 in the case of Vallée A type, and 1 in 2,000,000 to 5,000,000 when the other two types were used. The titre signifies that with such a dilution infection could be obtained by intradermal inoculation of a guinea-pig on the foot.

For making the intradermal injections it was found advisable to use a syringe with a very well-fitting metallic piston and very fine and sharp needles of 1·5 cm. in length.

The site of the injection.

(*a*) For intradermal immunisation the virus was injected into the dermis of the soles. The liquid penetrates with difficulty and must be injected slowly with considerable force until a wheal has formed occupying the whole of the sole. The injected region remains white for 1 or 2 hours and later becomes congested, warm and swollen and remains so for 3 or 4 days. After 5 days the foot again appears normal but the epidermis may peel off.

Throughout the different series the injections were given in the following order. The first injection of the series was given in the right hind foot, the second into the left hind foot, and the third into the right fore foot. In order to obtain as high a degree of immunity as possible the intradermal method was given a trial, in view of the researches of Levaditi, Nicolau and Galloway (1926), which led them to the conclusion that foot-and-mouth disease was an ectodermosis. Guinea-pigs immunised in this way stand the injections of virus very well and do not lose weight but may even increase a little.

(*b*) For subcutaneous immunisation injections were made under the skin on the inner side of the thigh. The first injection was made in the right thigh, the second in the left and the third in the right again in another place. These injections produced a very slight inflammation which passed away after 48 hours. This method also was very well borne.

(*c*) For intramuscular immunisation the virus was injected into the muscles of alternate hind limbs. There was slight weakening of the muscles and the animals lost a little weight, but soon recovered.

Method of bleeding. One or more guinea-pigs were bled to death from the heart on each of the following days after the last series of injections. The blood of one or more animals was obtained in this way on different days: 9, 10, 12, 14, 15, 17, 20, 29, 32, 33, 46, 50, 110, 140, after the last injection. The same technique was used for collecting and keeping the serum as in the case of the convalescent animals.

Antigen. Since the antigen is one of the decisive factors in the technique of complement fixation, various preparations of antigen were tested to find out which was the most sensitive and most stable. Three different methods of preparing antigen were tried in succession, (*a*) an alcoholic antigen, (*b*) diluted vesicular fluid, and (*c*) a watery antigen made by septic maceration of the skin lesions.

(*a*) The alcoholic antigen was prepared by Bordet's method in the usual way from vesicle epithelium removed from the feet of infected guinea-pigs.

It was diluted with saline 1 in 10 or 1 in 50 before use and tested for fixation of complement both with convalescent and hyperimmune sera. The results obtained were inconstant and this form of antigen was, therefore, abandoned with the intention of making renewed attempts later with different methods of preparation.

(*b*) Vesicular fluid for preparation of antigen was taken from vesicles on guinea-pigs' feet 24 hours after intradermal injection. It was then diluted 1 in 10 or 1 in 50 with phosphate solution *p*H 7·6, centrifuged and filtered through a Seitz filter. This material, when possible, was used after it had been kept for 15 to 60 days in the cold room in order to avoid the complication arising from the presence of complement in the vesicle fluid. It was found that this antigen could be used in the test in quantities of 0·4 to 0·5 c.c. when diluted 1 in 10, or of 0·5 to 0·7 c.c. when diluted 1 in 50. The anticomplementary action of the serum in the fluid was seen when doses of 0·8 to 1·2 c.c. were used. Almost all previous workers have made use exclusively of vesicle fluid as antigen. In the experiments here recorded it was found to be a sensitive but rather inconstant antigen when diluted 1 in 50, probably because the amount of virus present was not always sufficient. When used in a dilution of 1 in 10 the amount of serum which it contained was too great and often anticomplementary. The last inconvenience was especially difficult to overcome since the antibody used in the reaction was also guinea-pig serum. Nevertheless it was shown that vesicular fluid can be used as antigen if carefully prepared and titrated.

(*c*) Watery antigen obtained by septic maceration in saline solution. In view of the fact that in foot and mouth disease the epithelium covering the vesicles and their contents are the most virulent of the pathological products and that the virus has been shown to persist longest in the epithelium, and also having regard to the researches by Vallée, Carré and Rinjard (1925) on a vaccine for cattle in which they produced a highly effective vaccine with epithelium, it seemed possible that a more efficient antigen might be obtained from the vesicle epithelium of the guinea-pig than from the vesicular fluid. Epithelium taken from the lesions was, therefore, macerated in saline in order to liberate the infective germs and substances produced by the disintegration of the epithelial cells. It was hoped also so to modify, by autolysis, the colloidal state of the proteins in the infected tissues, that they would offer less hindrance to the reaction of fixation of complement than when in their unaltered condition. To obtain the infected tissues for the antigen the lesions on the feet of large guinea-pigs, 18 to 24 hours after inoculation at these sites, were washed with alcohol and the vesicle fluid removed. The epithelium was then cut off with scissors together with the whole of the dermis and underlying tissues down to the plantar aponeurosis. This was then weighed, cut into small pieces and ground with sand and nine times its weight of saline. It was then put in a sterilised bottle and allowed to macerate. For 8 to 10 hours each day the bottle was kept at the temperature of the laboratory and was placed in the cold

room at night. It was shaken several times daily. The resulting material had a smell like a culture of *B. coli* and proved on cultivation to contain either *B. proteus* and *B. coli* or *B. coli* alone. After maceration for 15 days, the emulsion was filtered first through paper and then a Seitz disc. Occasionally it was kept in the cold 30 or 40 days before filtration. The filtered fluid, after it had been kept in the cold room 3 to 6 months, was tested for virulence by intradermal inoculation of guinea-pigs and produced primary lesions after 30 to 48 hours and generalised lesions on the 4th or 5th day.

If the filtrate became again contaminated with bacteria it was re-filtered through a Seitz disc and sometimes a third filtration was found necessary, but its infectivity for guinea-pigs still remained after this treatment.

Antigen was prepared by this method monthly in large quantities from July 1927 to January 1928. From virus Vallée O, 700 c.c., from Vallée A, 400 c.c., and from Waldmann C, 300 c.c. have been prepared and always with similar results. On the average about 130 to 160 c.c. can be prepared from 30 guinea-pigs. More antigen was always obtained from the same number of guinea-pigs when virus Vallée O or Waldmann C were being used than from virus Vallée A, on account of the larger lesions obtained with the two former viruses.

The antigen obtained was very sensitive, and specific and constant reactions were obtained in the complement fixation reaction with different batches of antigen. One batch of antigen prepared with Vallée O and one with Vallée A were only slightly sensitive and these were not infective for guinea-pigs. On these occasions the emulsions of tissue had been made up in a dilution of 1 in 20 instead of 1 in 10. It was always found that the antigens prepared with Vallée O and Waldmann C were more active than those made with Vallée A.

This form of antigen was found to be anticomplementary in doses of 1·0 to 1·2 c.c. Numerous tests showed that as antigen, this preparation was very active in the complement fixation test in a dose of 0·4 c.c. when it was made with Vallée O and Waldmann C viruses, but Vallée A antigen had to be used in a dose of 0·5 c.c. These doses were found to be the most suitable throughout the tests.

The saline used in the reaction was a 0·9 per cent. solution of NaCl.

Technique of Complement Fixation Experiments.

The same method was used for testing all the sera, both those from convalescent and from hyperimmune animals.

The doses of antigen and complement were kept constant and the amount of antiserum was varied. Each test-tube contained 2·5 c.c. of liquid. Both systems, (1) antibody-antigen-complement, and (2) haemolysin-red corpuscles, were prepared and incubated at 37° C. for 1 hour separately. The two systems were then shaken and mixed, and the tubes kept at 37° for half an hour, during which time they were shaken and examined every 10 minutes. The results were

then noted and the final reading made after 16 hours at room temperature or in the cold room.

Table I shows the arrangement and contents of the tubes in all the experiments. The only variations were that the quantity of Vallée A antigen used was 0·5 c.c. instead of 0·4 c.c. and that, in testing the hyperimmune serum, the tube containing 0·15 c.c. of serum was not needed and the control tube

Table I. *Plan of the complement fixation experiments.*

	First system				Second system	
No. of tube	Antiserum	Antigen	Complement 1 in 10	Saline	Haemolytic serum	R.B.C. 5 %
1	0·05	0·4	0·3	0·75	0·5	0·5
2	0·1	0·4	0·3	0·70	0·5	0·5
3	0·15	0·4	0·3	0·65	0·5	0·5
4	0·3	0·0	0·3	0·90	0·5	0·5
5	0·0	0·8	0·3	0·40	0·5	0·5
6	0·0	0·0	0·3	1·2	0·5	0·5
7	0·0	0·0	0·0	1·5	0·5	0·5
8	0·0	0·0	0·0	2·0	0·0	0·5

without antigen contained 0·2 c.c. instead of 0·3 c.c. of immune serum. The actual amount of complement (diluted guinea-pig serum) used in the different experiments varied between 0·25 and 0·4 c.c. In each experiment four or sometimes six normal guinea-pigs' sera were used as controls to the immune sera.

Experiments with sera of guinea-pigs (1) *recovered from infection,* (2) *hyperimmunised with Vallée O virus* (= *Waldmann A*).

The largest number of experiments was made with the Vallée O type of virus because this type has been most used for other experimental researches and convalescent animals infected for other purposes were available.

Between July 1927 and May 1928 sera of 65 convalescent guinea-pigs were examined. In all these animals generalised lesions had appeared and blood had been taken between the 5th and the 72nd day after infection.

Each serum was tested three or four times at different intervals and on each occasion four or five positive hyperimmune sera and as many normal sera were tested at the same time. Both the normal and the convalescent sera were not pooled sera, but came from single animals. The serum was usually first tested about the 6th to the 10th day, and never less than 4 days after the bleeding, in order to avoid anticomplementary action of the fresh serum. Sera which were not contaminated or coloured with haemoglobin and after inactivation had been kept in the cold room could generally be employed for the reaction for 2 months. After that time some of them were found to have developed an anticomplementary action which was not removed by a second inactivation for 10 minutes at 55° C. When first tested about 8 per cent. of the convalescent sera and 6 per cent. of the hyperimmune sera had an anticomplementary action which disappeared after further heating for 10 minutes

at 55° C. or when tested again a week later. For this reason a larger quantity of antiserum than 0·15 c.c. has not been used.

In all, 20 control sera were used for these tests.

Table II. *Results of complement fixation experiments with the serum of guinea-pigs recovered from infection with Vallée O virus.*

Days after infection on which the animals were bled	No. of guinea-pigs bled	Results of test. Number of sera giving			
		Complete fixation	Partial fixation	Slight fixation	No fixation
5	5	—	—	—	5
6	6	—	—	—	6
8	3	—	—	—	3
9	12	—	3	5	4
10	3	—	3	—	—
18	2	—	2	—	—
21	7	7	—	—	—
22	3	3	—	—	—
23	2	2	—	—	—
27	2	2	—	—	—
30	4	4	—	—	—
32	3	3	—	—	—
37	2	2	—	—	—
48	2	2	—	—	—
50	4	4	—	—	—
55	1	1	—	—	—
72	4	—	—	—	4
Controls: normal guinea-pigs ...	20	—	—	—	20

The numbers of guinea-pigs whose sera gave complete, partial, slight, or no fixation are shown in the last four columns.

As can be seen in Table II the specific sensitising antibodies first appeared in the serum of convalescent animals on the 9th day; the quantity increased until a maximum was reached about the 21st day after infection. This maximum was maintained up to the 55th day. After 72 days the antibodies could no longer be detected. Under the same conditions positive reactions were obtained with 15 of the same sera when vesicle fluid of the same type of virus, diluted 1 in 10, was used as antigen. When a 1 in 50 dilution was used the reaction was less definite. The vesicle fluid used for this purpose had been collected 40 days before use.

Experiments with the sera of animals hyperimmunised with Vallée O virus.

The majority of the hyperimmune sera examined were taken from guinea-pigs treated with Vallée O type of virus. The sera of 81 guinea-pigs were tested. Of these, 26 were hyperimmunised intradermally by three series of injections and were bled between the 9th and 24th days after the last injection. Thirty-eight were hyperimmunised intradermally by five series of injections and were bled between the 16th and 140th days. Eleven guinea-pigs hyperimmunised intramuscularly by five series of injections were bled between the 28th and 33rd days, and 6 were hyperimmunised by the same number of injections given subcutaneously and were bled 23 days after the last injection. The immunisation was performed in three different ways in order to find out

which of the three methods produced the most active serum since it was hoped that if a serum of high activity could be obtained the reaction could be used for distinguishing the different types of virus. The animals were bled at different times after inoculation to obtain information as to the time at which the serum contains most antibody and how long the immune bodies remain after the injections have ceased. The complement fixation reaction was carried out as before. Each serum was examined on two or three occasions and several of them were again tested when they were used as controls in other experiments. The sera of 27 normal guinea-pigs were used as controls for the hyperimmune sera.

Table III. *Results of complement fixation experiments with the sera of guinea-pigs hyperimmunised with Vallée O virus.*

Hyperimmunised guinea-pigs						
	Days after last injection on which the animals were bled	No. of guinea-pigs bled on each day	Results of the test. Number of sera giving			
Injection			Complete fixation	Partial fixation	Slight fixation	No fixation
Hyperimmunised by three series of injections:						
Intradermal	9	3	3	—	—	—
,,	10	2	2	—	—	—
,,	12	4	4	—	—	—
,,	15	5	5	—	—	—
,,	17	3	3	—	—	—
,,	20	6	6	—	—	—
,,	21	2	2	—	—	—
,,	24	1	1	—	—	—
Hyperimmunised by five series of injections:						
Intradermal	16	6	6	—	—	—
,,	29	8	8	—	—	—
,,	32	5	5	—	—	—
,,	50	7	7	—	—	—
,,	110	6	4	1	—	1
,,	140	6	3	2	—	1
Subcutaneous	23	6	3	1	—	2
Intramuscular	28	6	4	2	—	—
,,	33	5	2	1	—	2
Controls: normal guinea-pigs		... 27	—	—	—	27

The results given in Table III show that a strongly positive complement fixation was obtained with all the sera tested. Hyperimmune serum in so small a quantity as 0·05 c.c. gave a positive reaction as early as 9 days after the last injection. The antibodies persisted in the serum in some cases for as long as the 140th day. After 110 days, of 6 guinea-pigs examined, 4 gave a strongly positive reaction, 1 a partial fixation, and 1 a negative reaction. Only 3 of the same guinea-pigs bled after 140 days gave a strongly positive reaction, 2 a partly positive, and 1 gave a negative reaction as before.

Among the 81 hyperimmune sera examined, two from animals which had been immunised intradermally showed a persistently anticomplementary action in spite of being twice heated at 55° C. Experiments were made in the hope of obtaining a precipitating reaction with hyperimmune sera diluted

1 in 10, and antigen consisting either of vesicle fluid diluted 1 in 10 or 1 in 50, but all attempts in this direction failed.

Experiments with the sera of guinea-pigs (1) *recovered from infection with virus of the type of Vallée A* (= *Waldmann B*), (2) *hyperimmunised with the same virus.*

The sera of 52 *convalescent* guinea-pigs was obtained by bleeding the animals to death on the 5th to the 52nd day from infection.

The experiments were made in exactly the same way as when the infection was with Vallée O virus except that the antigen was used in a dose of 0·5, instead of 0·4 c.c., on account of its lesser activity.

Table IV. *Results of complement fixation experiments with the serum of guinea-pigs after treatment with Vallée A virus. Convalescent and hyperimmunised animals.*

	Days on which animals were bled after last injection	No. of guinea-pigs bled	Results of the test. Number of sera which gave the reaction: Complete fixation	Partial fixation	Slight fixation	No fixation
Guinea-pigs convalescent after simple infection:						
	5	5	—	—	—	5
	7	7	—	—	3	4
	12	4	2	—	1	1
	15	5	3	—	1	1
	21	6	6	—	—	—
	30	5	5	—	—	—
	35	7	7	—	—	—
	40	5	5	—	—	—
	50	8	8	—	—	—
Guinea-pigs hyperimmunised by intradermal injections:						
Three series of injections	20	4	4	—	—	—
Five series of injections	23	6	4	2	—	—
	32	5	5	—	—	—
	46	6	5	1	—	—
	140	1	1	—	—	—
Controls: normal guinea-pigs ...		16	—	—	—	16

Table IV shows that the sensitising antibodies appeared in the blood on the 7th day and that the number of sera giving positive reactions increased from the 12th day onwards. As in the case of the animals infected with Vallée O all the sera gave positive reactions in as small a dose as 0·05 c.c. from the 12th day onwards, and the antibodies were still present in high concentration after 50 days from infection. Under the same conditions 12 convalescent sera and 8 hyperimmune sera gave intensely positive reactions when the antigen used was diluted 1 in 10 vesicle fluid from guinea-pigs infected with Vallée A virus. Much less clear results were obtained when the vesicle fluid used as antigen was diluted 1 in 50. As controls for the sera of animals immune to Vallée A virus the sera of 16 normal guinea-pigs were used.

The sera of 22 guinea-pigs *hyperimmunised* with Vallée A were also examined. Of these 4 received three and 18 received five series of injections. These animals were bled between the 20th and 140th days after the last injection.

https://doi.org/10.1017/S0022172400009657 Published online by Cambridge University Press

Table IV shows that the sera of 19 animals gave a strongly positive, and 3 only a partial reaction of complement fixation. The immunity produced with this type of virus is quite definite and the reactions obtained well marked, and the fact that 3 animals failed to produce antibodies was probably due to individual peculiarities.

Experiments with the sera of guinea-pigs (1) *recovered from infection with the Waldmann C type of virus, and* (2) *after hyperimmunisation with this virus.*

The sera of 11 guinea-pigs which had *recovered* from infection with virus Waldmann C were examined. Of these 8 were bled on successive occasions on the 6th, 12th, 21st or 35th days, and 3 were bled on the 43rd and 103rd days.

Table V. *Results of complement fixation experiments with the sera of guinea-pigs after treatment with Waldmann C virus. Convalescent and hyperimmunised animals.*

Days on which animals were bled after last injection	No. of guinea-pigs bled	Results of the test. Number of sera giving the reaction			
		Complete fixation	Partial fixation	Slight fixation	No fixation
Guinea-pigs convalescent after infection:					
6	8	—	—	1	7
12	8	—	—	3	5
21	8	8	—	—	—
35	8	8	—	—	—
43	3	3	—	—	—
103	3	0	—	—	0
Guinea-pigs hyperimmunised intradermally:					
21	8	8	—	—	—
60	6	6	—	—	—
Controls: normal guinea-pigs ...	10	—	—	—	10

Table V shows that antibodies could be recognised by complement fixation on the 6th and 12th days in small amounts and had greatly increased by the 21st day, after which they also gave a strong reaction on the 35th and 43rd days. By the 103rd day after infection, however, antibodies could no longer be recognised.

Eight guinea-pigs which had been *hyperimmunised* with Waldmann C virus by the intradermal method were bled on the 21st day, and 6 of these which were still alive were bled on the 60th day after the last inoculation.

Experiments using as antigen vesicle fluid diluted 1 in 10 from animals infected with Waldmann C virus, gave strongly positive reactions with all these hyperimmune sera. Vesicle fluid diluted 1 in 50 gave a much weaker reaction. In this series of tests the sera of 10 normal guinea-pigs were used as controls to the hyperimmune sera.

Experiments showing the strict specificity of the sensitising antibodies and the antigens of the three types of virus.

The experiments described above show that there is a well-marked development of sensitising antibodies to the three types of virus in the blood

of animals immune to these types; and, that with the homologous antigens the reaction of complement fixation can be obtained. In order to test the specificity of the antibodies and antigens for the several types of virus, and the possibility of distinguishing the types by this means, a comparative series of tests was performed with 3 convalescent sera of each of the types Vallée O and A, 4 of type Waldmann C and 4 normal sera, using the separate antigens of the three types. In another experiment made under the same conditions, 11 hyperimmune sera of the Vallée O type, 11 of type Vallée A, 4 of Waldmann C and 4 normal sera were examined. The only antigens used in this series were made by maceration in saline solution of material from Vallée O and Waldmann C infections. The sera used for these comparative tests were selected from among those which had given very strong positive reactions with the homologous antigen, when the smallest amount of serum used, 0·05 c.c., was sufficient to cause complete fixation.

Table VI. *Results of experiments comparing the reaction of sera of different types with homologous and heterologous antigens. Showing the specificity of the antisera and antigens of the three types of virus.*

Sera of convalescent and hyperimmunised guinea-pigs	No. of sera tested	Results of the reaction		
		Antigen Vallée O	Antigen Vallée A	Antigen Waldmann C
Convalescent sera:				
Vallée O	3	+ + +	o o o	o o o
Vallée A	3	o o o	+ + +	o o o
Waldmann C	4	o o o	o o o	+ + +
Control: normal serum ...	4	o o o	o o o	o o o
Hyperimmunised sera:				
Vallée O	11	+ + +	o o o	o o o
Vallée A	11	o o o	+ + +	o o o
Waldmann C	4	o o o	o o o	+ + +
Control: normal serum ...	4	o o o	o o o	o o o

+ + + signifies complete fixation of complement.
o o o signifies no fixation of complement.

The results given in Table VI show that the complement fixation reaction with serum from an animal recently recovered from infection, or further hyperimmunised against any one of the three known types of virus, is strictly specific. Each immune serum gives a strongly positive reaction with its homologous antigen, but a completely negative one with the heterologous antigens, and a normal serum gives a completely negative reaction with each antigen. In these tests there was no group reaction between the three viruses and the type of antigen or immune sera from animals infected with each of the three viruses can be clearly identified.

CONCLUSIONS.

From a consideration of the experiments recorded above with the three known types of virus of foot-and-mouth disease and a large number of sera from guinea-pigs, including the sera from 155 animals recently recovered from

the disease, from 117 hyperimmunised animals, and from 73 which were normal, the following conclusions can be drawn.

1. By complement fixation in the presence of a suitable antigen it is always possible to demonstrate sensitising antibodies in the serum of guinea-pigs which are either convalescent after an infection with foot-and-mouth disease or have in addition been hyperimmunised by successive injections of virus.

2. By septic maceration of the diseased tissues taken from the pads of guinea-pigs 24 hours after infection a very sensitive antigen can regularly be obtained, which is strictly specific and only fixes complement in the presence of the antibodies for the homologous type of virus.

3. The fluid from vesicles on animals infected with any one of the three types of virus, diluted 1 in 10, has also been found to be active as antigen when used with the homologous antibodies. The same vesicle fluid diluted 1 in 50 is less sensitive and reliable.

4. Antibodies can be demonstrated in the serum of convalescent guinea-pigs on the 7th day after infection, but they increase later and attain a maximum for all three types about the 21st day in 100 per cent. of animals. They then remain constant up to the 55th day, but can no longer be detected after 72 days.

5. In hyperimmunised guinea-pigs the antibodies can be demonstrated from the 9th to the 140th day after the last dose of virus; when the animals have been hyperimmunised by the intradermal route this occurs in 100 per cent.; when the intramuscular route is used in 99 per cent., and when the subcutaneous, in 70 per cent.

6. The antibodies and the watery antigens of the three viruses Vallée O, Vallée A and Waldmann C are strictly specific: (*a*) the antibodies can only be demonstrated in the presence of homologous antigens, and (*b*) complement fixation is never obtained with the serum of normal guinea-pigs in the presence of these antigens under corresponding conditions.

7. The results of these researches with guinea-pigs encourage the hope that it will be possible to use the method of complement fixation for determining the type of foot-and-mouth disease virus in other animals.

8. The reaction of precipitation with hyperimmune serum against Vallée O virus and the homologous watery antigen or vesicle fluid has given negative results even when the antiserum has been used in a dilution of 1 in 10.

I should like to take this opportunity of thanking Sir Charles Martin, Director of the Lister Institute and Chairman of the Foot-and-Mouth Disease Research Committee, for putting at my disposal the material necessary for carrying out this research and thus enabling me to bring this work to a successful conclusion, and I should also like to thank Dr J. A. Arkwright for his advice and interest in my work.

REFERENCES.

ASCOLI, A. (1910). Über die Meiostagminreaction bei der Maul- und Klauenseuche. *Zeitschr. f. Infektionskr. der Haustiere*, **8**, 308.

—— (1910). La reazione Meiostagmica nell' afta epizootica. *Clin. Veter.* p. 206.

BEDSON, S. P., MAITLAND, H. B. and BURBURY, Y. M. (1927). *Foot-and-Mouth Disease Research Committee, Second Progress Report*, London, p. 97.

—— —— —— (1927). Further Observations on Foot-and-Mouth Disease. *J. of Compar. Path. and Therap.* **40**, 79.

BURBURY, Y. M. (1928). Determination of the Immunological Types to which different Strains of Virus belong. *Third Report of the Foot and Mouth Disease Research Committee*, Appendix II, p. 65.

ERNST, W. (1922). Weitere Mitteilungen zur Maul- und Klauenseuchefrage. *München. tierärztl. Wochenschr.* p. 550.

FAVERO, F. (1914). Sulla presenza di una Sensibilizzatrice antiaftosa. *Clin. Veter.* p. 327.

LEBAILLY, CH. (1925). *Rapport à l'Institut des recherches agronomiques*, Paris. (Publication du Ministère de l'Agriculture.)

—— (1926). Expériences concernant le virus aphteux. *C.R. Acad. Sci.* **183**, 578.

LEVADITI, NICOLAU and GALLOWAY (1926). Les affinités tissulaires du virus aphteux. *C.R. Soc. Biol.* **95**, 1230.

LOURENS, L. (1909). La Sérothérapie, la Sérophylaxie et la Vaccination de la fièvre aphteuse et sa Valeur au Point de Vue de la Police Sanitaire. *IX Congrès Internat. de Méd. Vétér.* Haye, **3**.

MAGNUSSON, H. and HERMANSSON, K. A. (1926). Einige Beobachtungen über die Maul- und Klauenseuche in Schweden. *Acta Pathol. u. Microbiol. Scandinav.* **3**, 737.

MEZINESCU, O., BARONI, V. and CALINESCU, J. (1923). Recherches expérimentales sur la fièvre aphteuse. *Ann. Inst. Pasteur*, **37**, 1057.

MINETT (1927). *J. of Compar. Path. and Therap.* **40**, 173.

OLITSKY, P. K. (1927). Summary of Observations of the Commission to Study Foot-and-Mouth Disease. *J. Amer. Vet. Assoc.* **70**, 926.

OLITSKY, TRAUM and SCHOENING (1928). *Report of the Foot-and-Mouth Disease Commission of the U.S. Department of Agriculture, Technical Bulletin* No. 76, p. 98.

SACHELARIE, BOQUET and URBAIN (1928). Sur la pluralité du virus aphteux. *Rapport de l'Office International des Épizooties.* Paris, 1928, Rapport 1., p. 5.

STOCKMAN, S. and MINETT, F. (1926). Researches on the Virus of Foot-and-Mouth Disease. *J. of Compar. Path. and Therap.* **39**, 1.

—— —— (1926). Experiments on Foot-and-Mouth Disease. *Ibid.* **39**, 231.

—— —— (1925). *Foot-and-Mouth Disease Research Committee, First Progress Report*, H.M. Stationery Office, London, p. 19.

TITZE, C. (1922). Die Züchtung des Erregers der Maul- und Klauenseuche. *Berlin. tierärztl. Wochenschr.* p. 44.

TRAUTWEIN, K. (1927). Die Pluralität des Maul- und Klauenseuchevirus. *Arch. f. wiss. u. prakt. Tierheilk.* **56**, 505.

URBAIN, ACH. (1927). La réaction de fixation appliquée au diagnostic de certaines maladies microbiennes ou parasitaires communes à l'homme et aux animaux. *Traité. Édition de la Revue de Pathol. Comparée et d'Hygiène Générale.* Paris, 1927, p. 174.

VALLÉE, H. and CARRÉ, H. (1922). Sur l'Immunité anti-aphteuse. *C.R. Acad. Sci.* **174**, 207.

—— —— (1922). Sur la pluralité du virus aphteux. *Ibid.* **174**, 1498.

—— —— (1928). Sur la pluralité du virus aphteux. *Rapport de l'Office International des Épizooties.* Paris, R. 1.

VALLÉE, H., CARRÉ, H. and RINJARD (1925). Sur l'Immunisation antiaphteuse. *Bull. Soc. Centrale de Méd. Vétér.* p. 297.

WALDMANN, O. and TRAUTWEIN, K. (1926). Experimentelle Untersuchungen über die Pluralität des Maul- und Klauenseuchevirus. *Berlin. tierärztl. Wochenschr.* **42**, 569.

—— —— (1923). Versuche zur aktiven Immunisierung gegen Maul- und Klauenseuche. *Arch. f. wiss. u. prakt. Tierheilk.* **50**, 229.

(*MS. received for publication* 18. IX. 1928.—Ed.)