

STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.*

I. DESCRIPTION OF THE STRAIN OF VIRUS USED. TITRATION AND CENTRIFUGATION EXPERIMENTS.

BY PETER K. OLITSKY, M.D., AND LOUIS BOËZ, M.D.

(From the Laboratories of the Commission to Study Foot-and-Mouth Disease at the
Institute of Hygiene and Bacteriology, Strasbourg, France.)

(Received for publication, December 16, 1926.)

The findings of the Commission to Study Foot-and-Mouth Disease will be presented in detail in a forthcoming publication by the United States Bureau of Animal Industry. A preliminary paper has already appeared¹ in which were reported comparative studies on vesicular stomatitis and foot-and-mouth disease. In a series of papers to be published in this *Journal*, of which this is the first, the writers will describe their experiments on the physical and chemical properties of the foot-and-mouth disease virus, and their attempts at its cultivation. Of the Commission's personnel, the writers were mainly interested in these particular problems. There was, however, a free interchange of ideas and suggestions, and a hearty cooperation among all the colleagues, so that all publications of the Commission may be regarded as the result of collaboration as a unit.

* The Commission to Study Foot-and-Mouth Disease was formed by the United States Department of Agriculture, under the direction of Dr. John R. Mohler, Chief of the Bureau of Animal Industry. It comprised Dr. Harry W. Schoening, of the Bureau of Animal Industry, Dr. Jacob Traum, of the Department of Veterinary Science, University of California, and, as Chairman, Dr. Peter K. Olitsky, of The Rockefeller Institute for Medical Research. Dr. Louis Boëz, of the Institute of Hygiene, joined the Commission later as a collaborator.

The writers wish to express their gratitude to the Doyen, Professor Weiss, to Professors Borrel, Vlès, and others, of the Institute and the University of Strasbourg, for their active and whole hearted cooperation.

¹ Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx (N. S. xxiii), 147.

In practically all of the experiments to be reported in the series of papers, we employed, except in a few instances, one strain of foot-and-mouth disease virus. This strain had the advantages of causing lesions promptly and regularly, of acting powerfully, of inducing in practically all instances well marked secondary lesions, and of bringing about relatively few deaths in the experimental animals, thus permitting a long period of observation. The animal used in these studies, unless otherwise stated, was the guinea pig.

Description of Strain of Virus.

On June 18, 1925, through the kindness of a local veterinarian, Dr. Fuchs, a herd of cattle with typical foot-and-mouth disease was placed at our disposal for study. The cattle were kept on a farm about 15 kilometers from Strasbourg. Of twelve animals in the same barn, all in different stages of the disease, one cow was selected which was the last to be infected, and in the 3rd day of illness. She drooled considerably, and had broken aphthæ about the lips, tongue, and buccal mucosa, but no obvious foot lesions, and her temperature was 41°C. It was difficult to aspirate the exudate in the vesicles so that in the end what was brought to the laboratory was an admixture of this cow's saliva and stable refuse. This material was diluted 1:3 with Tyrode solution and then injected, unfiltered, intradermally in the hairless skin of the posterior pads of five guinea pigs. Typical primary vesicles appeared at the sites of injection in 24 to 48 hours in all five. From that time until the investigations were terminated, about a year later, the virus (Strasbourg or "Str." strain) was propagated in the guinea pig for 261 consecutive passages in a pure state, except on two occasions in late passages, when staphylococci were found in the vesicular exudate. These latter were then removed by filtration, without harming the virus. It was found later, by failure of cross-immunity with Vallée's A type, that the Str. strain was probably identical with that of the Vallée "O."

It is to be noted from the effects of transferring the impure virus from the cow to the guinea pig that the virus, when admixed with contaminating microorganisms, could free itself of ordinary bacteria in the tissues of a susceptible animal. Thus a property common to

many filter-passing viruses is shown by the active agent of foot-and-mouth disease. Indeed, after the first series of transfers, no extraordinary care was taken for the cleanliness of the site injected; that this was a proper mode of procedure is gathered from the fact that only twice in 261 passages did the virus become contaminated.

Freedom from Ordinary Bacteria.—Aside from the fact that the virus could purify itself in the guinea pig tissues, deliberate cultivation tests were made when it was first acquired to determine whether there might be any constant bacteria associated with this strain. Repeated cultures were attempted with the blood, with the fluid aspirated from the vesicles (the so called "lymph"), with ground infected pads and the latter filtered—all obtained on the 1st day of illness.

No constant visible microorganisms were revealed by the different methods of culture—the details of which will be described in another paper on experiments on cultivation. Hence this strain was pure, and its effects could not be ascribed to any agent cultivable by ordinary means.

The Disease in the Guinea Pig.—The clinical picture of the experimental disease in the guinea pig corresponded in the main with the comprehensive descriptions given by Waldmann and Pape, by Gins and Krause,² and by the members of the British Foot-and-Mouth Disease Research Committee.³⁻⁵ Certain of the more important points are, however, worthy of recapitulation.

Method of Injection.—After a long trial with various methods of injection of the virus, we found that the method of choice, and the one by which practically all guinea pigs were infected, is the intradermal. The procedure consists in running a fine needle in the skin along the length of the hairless pads of the posterior

² For a general historical and descriptive account of the experimental disease in the guinea pig, see Waldmann, O., and Pape, J., *Berl. tierärztl. Woch.*, 1920, xxxvi, 519; and Gins, H. A., and Krause, C., *Ergebn. allg. Path. u. path. Anat.*, 1923-24, xx, pt. 2, 805.

³ First Progress Report of the Foot-and-Mouth Research Committee, Ministry of Agriculture and Fisheries, London, 1925.

⁴ Arkwright, J. A., Burbury, M., Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

⁵ Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

extremities. Attached to this needle is a syringe containing the test fluid, and with pressure on the piston the skin tunnel thus made is filled as the needle is withdrawn. Three or four such injections are made on each posterior pad, and besides, the thin, loose skin along the outer and inner margins of the pad is filled until it distends to a distinct bleb-like formation. If the virus is weak or diluted, an additional inoculation is made subcutaneously in these pads. Inoculations by means of skin tunnels were also found advantageous by the workers of the British Commission.^{4,6}

Injection of the virus in sites other than the pad skin was followed by specific lesions, but certainly not as regularly as those occurring after the intradermal inoculation,⁶ as the following experiment illustrates.

Protocol 1.—All animals were injected with the same sample of virus taken from a guinea pig in the 1st day of illness. The infected pads of the latter were removed, ground in sand, and suspended in phosphate buffer solution (pH 7.6) to a dilution of 1:25. $\frac{1}{2}$ cc. was inoculated in each guinea pig.

(a) Intramuscularly in the thigh. Of six animals only two showed the typical apthæ: in one, in all four pads; in the second, in all pads and in the tongue. Incubation period 48 to 72 hours.

(b) Intraperitoneally. Of five animals three exhibited vesicles in all pads and in the mouth. Incubation period 3 to 4 days.

(c) Subcutaneous, under the abdominal skin. Of five guinea pigs, two showed apthæ in all pads and in the tongue, one in two pads, and one in one pad. Incubation period 3 to 6 days.

(d) Intracutaneous in abdominal skin. Of five animals, four showed lesions in all pads and in the tongue. Incubation period 3 to 5 days.

In none of these were any lesions noted at the site of the injection.

When these methods of injection are compared with the intradermal one, already described, by which practically all of over 2000 guinea pigs were infected, it will be noted that the latter was more reliable. This test also illustrates the epitheliotropic nature of the infection, for irrespective of the site of injection, the lesions appeared only in the epithelium of the pads or of the tongue or of the mouth. Furthermore, if a proper injection of the virus was given, no natural immunity to foot-and-mouth disease could be found in guinea pigs.

⁶ In this place, as in all others in which reference is made to "intradermal" inoculation, we mean to imply the particular method of injection described in the preceding paragraph.

Secondary Lesions.—As already mentioned, the Str. strain induced secondary lesions regularly. These appeared in the pads or mouth or tongue, in sites other than those injected, as early as 36 hours and as late as 5 days, but as a rule from 48 to 72 hours after inoculation. For an unequivocal determination of experimental foot-and-mouth disease in the guinea pig, the presence of these secondary lesions was essential. In doubtful instances, such as when the primary vesicles were obscured by necrosis—a condition encountered frequently in experiments with antiseptics, to be mentioned later—recourse was had to transfer to normal animals for observation of primary and secondary signs before a conclusive diagnosis could be made.

Period of Incubation.—In about 95 per cent of the cases the period of incubation was from 18 to 48 hours. In one instance, the first symptoms were noted after 12 days and in three cases, after 10 days. In the remainder, however, the primary aphthæ appeared from 3 to 6 days after injection. For these reasons, observations were prolonged to 10 to 12 days before an exact definition was obtained; and when necessary, an injection of active material was made in animals which did not react, to determine the presence of immunity. A noteworthy fact is that the period of incubation is shortened and the severity of the disease is increased as the dose or concentration of the virus is augmented.

Time of Greatest Infectivity.—Tests showed that the blood of the guinea pig was active, when withdrawn 24 and 48 hours after intradermal inoculation. On the other hand, if this blood, in a defibrinated condition, was kept at 37°C. *in vitro*, it was active for 24, but not for 48 hours. The period of greatest activity of the virus in lymph aspirated from aphthæ or in ground infected pads was obtained from lesions up to 24 hours in age. From this time to 72 hours there was a gradual diminution in virulence and after 3 days such virus was only exceptionally active. At about this latter time, the active agent, conforming to the tendency of filter-passing viruses in general, invited sometimes invasion by secondary microorganisms, followed by purulent inflammation.

Mortality.—The mortality rate from infection of guinea pigs with the Str. strain was about 1 per cent. As this rate corresponded to the normal death rate among stock animals, it may be stated that

the experimental disease induced by this strain was practically non-lethal.

Transfer to Cattle.—Finally, this strain, after propagation in the guinea pig, could induce, with ease and at will, in cattle and hogs, typical foot-and-mouth disease indistinguishable from the natural affection. The material used for such transfers was derived from guinea pigs either in the early or last passages.

Titration of the Virus.

It was essential to determine quantitatively the activity of the virus, for this estimation was important from the point of view of controlling survival or preservation, as opposed to multiplication, of the virus in subplants of cultivation tests. This information also served as a guide for interpretation of the cataphoresis tests to be reported later, and gave an idea of the relative size of the active agent.

The virus was found to be active in remarkably high dilutions as the following experiments will show:

Protocol 2.—Summary of fifteen experiments. The virus employed for these tests consisted of the aspirated lymph from 24 hour old vesicles. The diluent consisted of phosphate buffer solution at pH = 7.5.⁷ Dilutions were made with pipettes. The diluted virus was injected by the intradermal method, already described, in both posterior pads of guinea pigs.

Of fifteen tests, the virus was found active in a dilution of 1:10,000,000, twice; 1:2,000,000, thrice; and 1:1,000,000, twice. In the foregoing trials, these were the final dilutions. In four additional tests where dilutions were made higher than that of the active range, in one case 1:4,000,000 was positive, but 1:8 to 32,000,000, negative; in another, 1:2,000,000 was active, but not 1:4 to 80,000,000; in a third, 1:1,000,000 was positive, but 1:2,000,000 negative; in the last case, 1:400,000 was active, but not 1:1,000,000.

All these experiments were made with the virus cleared of particles by filtration through filter paper. In the next four tests filtrations were made through Berkefeld V candles. Activity was shown in all four instances in a dilution of 1:2,000,000, the highest made.

⁷ Phosphate buffer, as mentioned here and elsewhere in these articles, is made by adding 2.5 gm. potassium acid phosphate (KH_2PO_4) to a liter of distilled water. The solution is then adjusted to the desired hydrogen ion concentration by means of potassium or sodium hydroxide. It is of utmost importance to readjust the material immediately before use, for sterilization renders it acid.

From these titrations it is to be noted that the virus diluted to 10 million times its volume may still be effective. This fact leads to several considerations, the first of which is with reference to the probable size of the active agent. Since the material is not solid with virus, but contains the diluent and various products usually found in an inflammatory exudate, the active agent is only a part of this quantity. Hence the agent should be quite minute. That the size of the virus is less than 100 m μ has also been confirmed in the study on "molecular" filtration, to be described in another paper.

Secondly, great care should be exercised in measuring or transferring virus in glass receptacles in tests for comparative activity, such as the determination of the killing effect of antiseptics, the relative value of different media in cultivation tests, etc. Spattering of the virus along the glass walls may give rise to infections which may have no bearing on the problem in hand. We have therefore substituted for test-tubes, whenever practical, chemical mixing glasses or conical beakers.

A third point is that in our experiences with cultivation of the virus, the third subplant showed activity in five different media. In this third subplant the virus was diluted by our method of seeding about 1:1,000,000. But in the fourth subplant the active agent was diluted 1:15,000,000. In this latter, however, the virus was without action. Apart from this, we had other and ample evidence to show that these media were not suitable for growth. Hence the conclusion derived from such experiments is that preservation or survival, but not multiplication is involved. Therefore, the work of Titze⁸ and of others who maintained virulence only in the first few "subcultures" should be interpreted as probably indicating a mere dilution of the active agent.

Another consideration is that since one sample of the virus is active in a dilution of 1:400,000, and another in a dilution of 1:10,000,000, a factor of difference in activity between one specimen and another is 25. The investigators of the British Committee⁸ have found a still greater factor to exist; one sample was inactive at 1:5000, and another active at 1:500,000. Hence, in comparative tests, such as

⁸ Titze, C., *Z. Infektionskrankh. Haustiere*, 1924, xxvi, 107.

in the study of the survival of the virus in different media, trials should be made with the same specimen of virus.

Finally, the severity of the disease and the incubation period depended on the concentration of the virus: the more concentrated the virus, the more severe was the infection and the shorter the period of incubation. For example, with one sample of a 24 hour aspirated lymph virus, a dilution of 1:1000 induced the experimental disease of severe type in 24 hours in all of five guinea pigs; of 1:200,000, after 3 days, in one of two animals; of 1:2,000,000, after 4 days, mildly in one of two animals; and of 1:4,000,000, after 9 days, one vesicle was noted on the tongue of one guinea pig, of two inoculated. Higher dilutions were negative.

The rate and energy of action, therefore, are proportional to the concentration of the active agent.

Centrifugation.

Centrifugation tests were made with two objects in view: (1) to determine the centrifugability of the virus, and (2) if the virus were not centrifugable, to attempt to free, by this process, the supernatant fluid from a hypothetical body which may be the inhibiting factor in cultures, for example such bodies as fragments of tissue or cells.

As for the first object, it was determined by repeated tests that with the active guinea pig blood, aspirated lymph, or suspensions of ground infected pad tissue, centrifugation at 2500 to 3000 revolutions per minute for 2 hours did not cause the deposition of the virus: the lowest portion of the specimen was as active as the topmost layer. Thus we have confirmed the prior findings of others, notably the workers of the British Committee,³⁻⁵ Abe,⁹ and Gins.¹⁰ However, we could not confirm thereby Frosch and Dahmen's¹¹ first step in cultivation, namely a concentration or sedimentation of the virus by centrifugation at 3000 revolutions per minute for $\frac{1}{2}$ to $1\frac{1}{2}$ hours.

The inability to sediment the virus may indicate that the active agent is very minute, but not necessarily that it is of the nature of a

⁹ Abe, T., *Z. Infektionskrankh. Haustiere*, 1925, xxviii, 111.

¹⁰ Gins, H. A., *Berl. tierärztl. Woch.*, 1924, xl, 661.

¹¹ Frosch, P., and Dahmen, H., *Berl. tierärztl. Woch.*, 1924, xl, 185, 273, 341; *Arch. wissenschaft. u. prakt. Thierheilk.*, 1924, li, 99.

contagium vivum fluidum. Duclaux¹² has shown that centrifugation is not an adequate means for deposition of minute particles. For theoretically with a centrifugal force 40,000 times as great as the gravity force of a particle (such as is obtained in large centrifuges) a particle measuring 10 $m\mu$ will settle only 1 cm. in 4 hours. But practically, in addition to this slow deposition due to size, the whirling current and that due to convection, since all machines develop a certain amount of heat, operate to prevent deposition. In the example cited the variations due to these currents should be multiplied by 40,000 to obtain the actual reading of the forces preventing sedimentation (Duclaux).

In respect to the removal of a hypothetical inhibiting body by centrifugation, our repeated experiences showed that the active guinea pig blood kept at 37°C. contained no virus either in the sediment or supernatant fluid, when tested 3 days after centrifugation. But at room temperature similar ground pad virus suspended in phosphate buffer at pH = 7.5, remained alive for 9 days in the sediment and for 12 days in the supernatant fluid. With aspirated lymph under the same conditions, however, both the sediment and supernatant fluid were active for at least 14 days. No further tests were made in this instance.

To complete these experiments, three additional trials were made with the sediment after showing inactivity by standing for 4 days at room temperature. This sediment was added to either fresh, or old, active supernatant fluids, and the mixture kept for from 18 hours to 3 days at 20°C. But no inhibition of the virus in the supernatant fluid was observable.

We conclude from these experiments that the hypothetical virucidal bodies cannot be deposited from suspensions of ground infected pad tissues, or of aspirated lymph, by centrifugation.

At this point, brief reference may be made to attempts to remove substances which inhibit the viability or multiplication of the virus in artificial media, by precipitation of the globulins, or inactivation of complement. For this purpose carbon dioxide was employed,

¹² Duclaux, J., *Les colloïdes*, Paris, 2nd edition, 1925, 84 ff.

and prolonged passage of the gas was allowed through material containing the active agent. But these tests likewise ended in failure.

SUMMARY AND CONCLUSIONS.

A strain of foot-and-mouth disease virus was recovered from a cow at the height of the disease, and was propagated through at least 261 passages in the guinea pig. Considerably over 2000 animals proved susceptible to the virus, and the virus could be transferred at will back to cattle and hogs, and then again returned to guinea pigs. No natural immunity was discovered in guinea pigs. Secondary lesions were easily and regularly induced, thus making this strain particularly favorable to experimental purposes. In general, the guinea pig may therefore be regarded as the animal of choice for laboratory studies.

The guinea pig could be infected by different methods of injection in different sites, but constant and regular production of primary and secondary lesions—or generalization of the disease—followed intradermal “tunneling” in the manner described, combined with subcutaneous inoculation of the posterior hairless pads of full grown animals. As we have indicated, the virus was peculiarly epitheliotropic, which in turn gives support to the opinion that its portal of entry may be limited.

The active agent could purify itself of chance concomitant bacteria in the first passages, in a susceptible animal—a character possessed by filter-passing viruses in general.

The virus was active in dilutions of 1:10,000,000. This shows not only the minuteness of the active agent, but also the necessity for a change of technique from that employed with larger sized infectious agents. Apart from this, the dilution factor is important in interpreting mere preservation of the virus rather than multiplication, when only early successive subplants in culture experiments are positive. Furthermore, some samples of virus were not so active—a factor of twenty-five existed between the weakest and strongest samples among fifteen titrated. This indicates that comparative tests, as, for example, of survival in different media, should be made with the same specimen. In any case, the rate and energy of action of the virus were proportional to its concentration, thus differing from the behavior of certain enzymes.

The incitant is not sedimented by centrifugation. Non-centrifugability, a property of some other filter-passing, infectious agents, is not an indication of the fluid character of the virus, as we have already explained. In view of the evidence presented and other tests to be reported later, failure of deposition is related to the minute size of the incitant. The method of centrifugation has also failed to remove “virucidal bodies” in the meaning of Frosch and Dahmen.