STUDIES ON THE CULTIVATION OF THE VIRUS OF VACCINIA.*†

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Many investigators have shown that the virus of vaccinia, except in a dried form, retains its virulence but a short time at 37° C. The virus is usually found inactive after 9 to 13 days' incubation, altho under special conditions it has been kept as long as 16 to 21 days. No definite subcultures, however, have been obtainable.

We wish to refer in some detail, in this connection, to the work of Williams and Flournoy.[1] They inoculated with vaccine virus the corneas of rabbits, which were removed 24 hours after inoculation and placed in tubes of ascitic broth. The tubes were incubated at 36° C. After 24 hours' incubation the loosened epithelial cells formed an emulsion in the fluid. The inoculation of this emulsion on the skin of a rabbit gave a typical eruption. In one experiment, in which transfers to fresh broth were made on alternate days, the inoculations of the second and third transfers on the cornea of a rabbit produced typical ulcers. Inoculations from the fifth and sixth transfers on the skin of a calf gave a few scattered pustules; inoculations of subsequent transfers were negative. The inoculations of the fourth, fifth, and sixth transfers on the skin of rabbits were negative. The authors attribute the activity of the transfers to a carrying over of the original virus, since they saw no evidence of multiplication. The total period of incubation of the virus in their experiments was from 10 to 12 days.

EXPERIMENTAL WORK.

In previous work with the virus of rabies, done in conjunction with D. W. Poor,[2] we applied Harrison's method of growing

* Received for publication June 9, 1913.

† Read at the annual meeting of the American Association of Pathologists and Bacteriologists, Washington, May, 1913.

[1] *Studies from the Rockefeller Institute*, 1905, 3, p. 146. [2] *Jour. Infect. Dis.*, 1912, 11, p. 459.

tissue *in vitro* to brain tissue and were able to produce inclusions in normal ganglion cells closely resembling certain small forms of Negri bodies, but were not able to cause any multiplication of the virus of rabies. After these results with rabies, we decided to continue the work with Harrison's method, applying it in the present experiments to the virus of vaccinia incubated with corneal tissue and plasma, again, with two objects in view: the possible production of vaccine bodies *in vitro*, and the possible cultivation of the virus of vaccinia outside of the body.

The virus was taken from the stock of glycerinated, carbolized, calf virus as distributed by the Board of Health of New York City. It was dialyzed through collodion sacs by the method of Poor and Steinhardt.[1] It was usually allowed to stand in the icebox over night, thus permitting the coarse particles to settle. The supernatant fluid was drawn off and diluted with Ringer's or physiological salt solution. By this means a fairly uniform emulsion is obtained.

Technic.—Small pieces of rabbit or guinea-pig cornea were placed for a few minutes in this weak emulsion of virus. The pieces were then transferred with a small quantity of the virus to cover-glasses to which drops of rabbit or guinea-pig blood plasma were added. The cover-glasses were immediately inverted and sealed over hollow-ground slides, which were incubated at 37° C. To control the microscopic work, similar preparations were put up without virus, and to control the necessity of the cornea in the animal inoculation work, pieces of paraffin were substituted for cornea in a series of preparations.

Microscopic studies.—Approximately 100 hanging-drop preparations upon five series of experiments were studied histologically, using several methods of fixation and staining. Examinations were also made on fresh unstained preparations with light and dark field illumination. The preparations were stained after varying periods of incubation. As a rule, four slides were taken out at once and studied in several ways. To fix a specimen, the cover-glass with the attached clot was dropped directly into a dish of the fixing fluid unless the fixitive was bichlorid, when, according to the method of

[1] *Jour. Infect. Dis.*, 1913, 12, p. 202.

Lambert,[1] it was first soaked for 1–2 hours in salt or Ringer's solution to prevent the precipitation of proteins giving subsequently a cloudy specimen. The subsequent staining was carried out as tho the specimen were a paraffin or celloidin section attached to a slide, except that on account of the thickness of the hanging drop a longer time is required in the process.

Briefly stated, the methods employed were: (1) Zenker fixation, followed by eosin-methylene blue stain; (2) formalin fixation, Sudan III iron hematoxylin stain; (3) Giemsa's stain with and without previous fixation; (4) eosin-methylene blue without previous fixation; (5) unstained preparations with direct and dark field illumination.

The results of these studies may be given in a few words. The corneal epithelium shows an active lateral spreading through the clot, forming sheets or groups of cells in the plasma. The cells early show an accumulation of fat in their cytoplasm, but may retain their form for several weeks even when not transferred to fresh plasma. Careful studies have failed to reveal any specific vaccine bodies in the preparations; only smaller, undifferentiated forms have been seen and these have been found in both the controls without virus and the virus preparations after incubation.

We have also studied the corneas of guinea-pigs, inoculated with virus, which were removed 24 hours later and put up in hanging drops as described above. Only the smaller forms of the vaccine bodies were found in the beginning, and no further developments were seen. These negative findings are of interest on account of the results of the animal inoculations of the incubated preparations as given below.

Altho we have observed numerous granules in the incubated preparations, these have not been sufficiently definite in character with the methods employed thus far in our studies to allow us as yet to make any positive statements in regard to them.

We wish here to record our indebtedness to Dr. A. W. Williams for her very helpful criticisms and suggestions in the microscopic work just described.

[1] *Bull. 4*, International Association of Medical Museums, 1913.

ANIMAL INOCULATIONS.

To demonstrate the activity of the virus, we have adopted the method of Calmette and Guerin.[1] They have shown that the virus

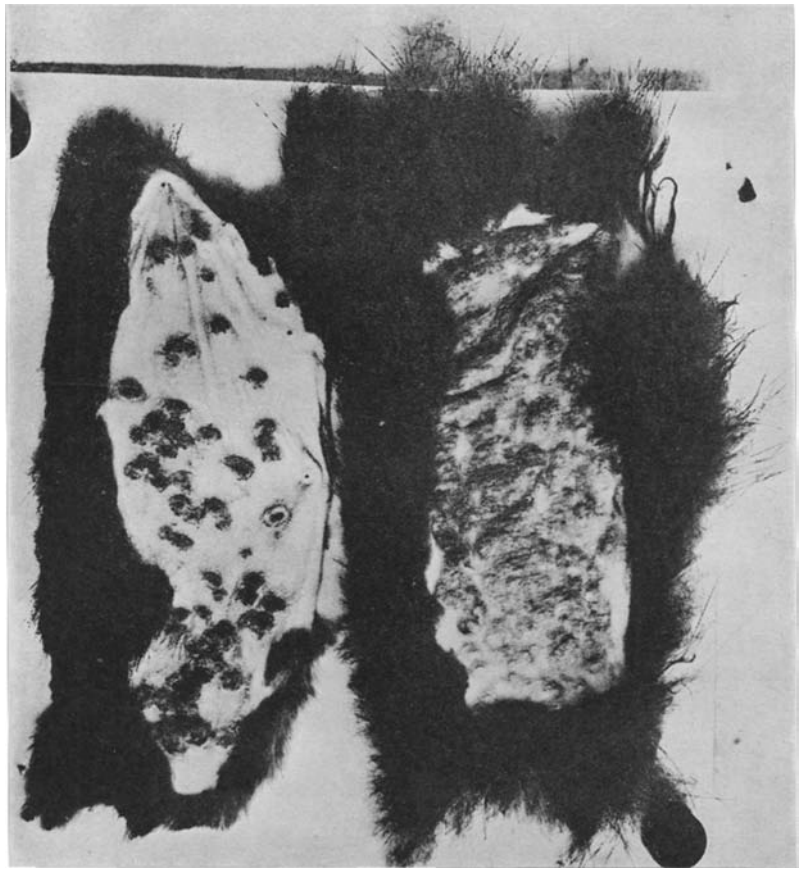

FIG. 1. FIG. 2.

FIG. 1.—Right side of rabbit inoculated with 9 unincubated cornea preparations. Result: 50–55 pustules.

FIG. 2.—Left side of rabbit inoculated with 9 cornea preparations, same series, incubated 14 days. Result: confluent raised eruption.

rubbed on the freshly shaven skin of the rabbit produces a typical vaccinia eruption, and, furthermore, by methods of dilution, that the

[1] *Ann. de l'Inst. Pasteur*, 1905, 19, p. 317.

number of vesicles in the eruption indicates approximately the quantity of virus present. This method is generally used in commercial laboratories for standardizing the virus.

In our experiments, the skin of a rabbit was inoculated at once with a small number of *unincubated* preparations, and similar inoculations were made later with *incubated* preparations to determine whether or not there was an increase in the virus.

The "takes" with unincubated preparations, using eight different viruses, varied from 10–50 vesicles, whereas a similar number of preparations incubated 7–18 days gave in every instance an extensive confluent eruption, showing that a definite increase had taken place. Repetitions of these experiments with the different viruses have in all cases given the same results. As yet we have not found a virus that has not shown increased activity under these conditions of incubation. Preparations containing small pieces of paraffin showed, on the other hand, no increase in activity upon incubation.

The following protocols are examples of the results obtained.

Experiment R. 490.—A series of hanging drop plasma preparations were made with rabbit cornea and Virus A. A second series were made using small pieces of paraffin in place of cornea, care being taken to make the quantity of virus the same for both series.

A rabbit inoculated on the skin of the back with 12 unincubated preparations presented on the third to fifth day 9–10 vesicles. No later lesions appeared.

A rabbit inoculated on the left side with 9 cornea preparations incubated 7 days gave an extensive, raised, confluent take. We estimated that at least 200 pustules must have been present to have given a confluent eruption over the area involved (Fig. 4). The same rabbit was inoculated simultaneously on the right side with 9 paraffin preparations also incubated 7 days. Five pustules were observed on the fifth day, and there were no further developments. (See Fig. 3.)

Experiment G. 422.—Hanging-drop specimens were prepared with guinea-pig cornea and Virus B. Nine unincubated preparations used for immediate skin inoculation gave 20–30 lesions. (See Fig. 1.) An inoculation with a similar number of preparations incubated 14 days gave an extensive confluent take. (See Fig. 2.)

Experiment G.—Hanging-drop plasma preparations were made using guinea-pig cornea and Virus C and were incubated at 37° C. On the ninth day of incubation nine preparations were used for a rabbit skin vaccination. A typical rash appeared before the fifth day, in which 40–50 pustules were counted and on the seventh day, 55 pustules. Nine preparations incubated 18 days were used for vaccinating a second rabbit. An extensive, elevated, confluent rash resulted composed of at least 200–250 lesions. (See Fig. 2.)

Subcultures of the preparations have been made and successful skin inoculations have been obtained from the third transfer. The

virus has remained active after 34 days' incubation, and this obviously does not represent the limit of activity under such cultural

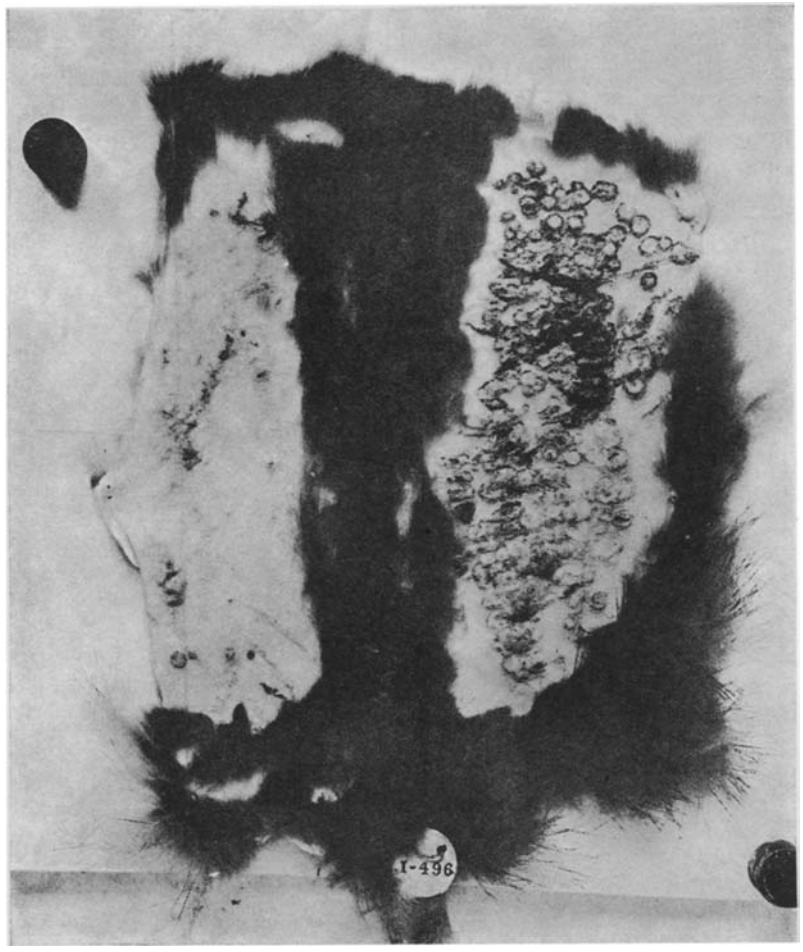

FIG. 3. FIG. 4.

FIG. 3.—Right side of rabbit inoculated with 9 paraffin preparations incubated 7 days. Result: 5 pustules (other marks are crusts from shaving wounds).

FIG. 4.—Left side of rabbit inoculated with 9 cornea preparations incubated 7 days (same series as paraffin preparations). Result: confluent raised eruption. (Inoculation with 12 similar cornea preparations, same series, unincubated, was followed by a take of 10 pustules.)

conditions. In control experiments it was found that the virus alone was greatly weakened after a week's incubation, and that after

three weeks' incubation, inoculations on rabbits were negative altho much larger quantities of the virus were used than with the tissue preparations. Experiments undertaken with the object of obtaining a growth of the virus in test tubes have thus far given interesting and encouraging results.

Work on this point, with further experiments on subcultures, on the use of other organs and the necessity of living tissue in the culture experiments, methods of obtaining a sterile virus, immunity reactions *in vitro*, and the results of further microscopic studies will be described shortly in a later communication.

CONCLUSION.

From our work thus far on the application of Harrison's method to the cultivation of tissue *in vitro* to corneal tissue plus the virus of vaccinia, we are able to state that there is a multiplication of the virus of vaccinia altho no specific vaccine bodies are found in the preparations.