

## EXPERIMENTS ON THE SURVIVAL OF THE FEBRILE HERPETIC AND ALLIED VIRUSES IN VITRO.

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(Received for publication, November 1, 1923.)

Much interest centers at present about the subject of the etiology of epidemic (lethargic) encephalitis and of febrile herpes. In both conditions a virus has been described as the incitant, but the properties of the two viruses are so nearly alike that as yet they cannot be separated by animal tests. There is a general agreement among investigators, however, regarding the qualities of the active agent, or the invisible infecting microorganism of febrile herpes associated with the experimental lesions characterizing the condition. In the case of the so called virus of encephalitis lethargica, on the other hand, different workers have isolated organisms varying widely in their morphological and cultural characteristics, each of which has been regarded as the incitant of the disease.

At the request of Dr. Flexner, I undertook to repeat certain experiments on the cultivation of the so called virus of epidemic encephalitis outside the body which were reported by Bradford, Bashford, and Wilson,<sup>1</sup> by Loewe and Strauss,<sup>2</sup> and by Thalhimer.<sup>3</sup> These observers claim to have isolated, by means of the Smith-Noguchi technique, organisms resembling the globoid bodies of poliomyelitis.

Although the results were negative and I was unable to confirm the work of the authors named, it seems desirable to report the experiments. Incidentally, the experimental data refute published reports of positive cultures of quite common bacterial species de-

<sup>1</sup> Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Quart. J. Med.*, 1918-19, xii, 88.

<sup>2</sup> Loewe, L., and Strauss, I., *J. Infect. Dis.*, 1920, xxvii, 250; *Proc. New York Path. Soc.*, 1920, xx, 18.

<sup>3</sup> Thalhimer, W., *Arch. Neurol. and Psychiat.*, 1921, v, 113.

scribed, among others, by Rosenow, Towne, and Wheeler,<sup>4</sup> von Wiesner,<sup>5</sup> Brasher, Caldwell, and Coombe,<sup>6</sup> Stafford,<sup>7</sup> Morse and Crump,<sup>8</sup> Bastai,<sup>9</sup> and Ottolenghi, d'Antona, and Tonietti.<sup>10</sup> On the other hand, some new facts have been secured which may be of value in any further cultivation tests which may be undertaken.

#### EXPERIMENTAL.

Specimens of the so called encephalitic virus were obtained from Dr. Flexner. They consisted of material kindly sent him by Dr. Levaditi and the Beckley strain isolated at The Rockefeller Institute.<sup>11</sup> In view of the similarity of the so called virus of encephalitis to that of febrile herpes, a sample of the latter was added, the H. F. strain, propagated through many rabbit generations at The Rockefeller Institute.<sup>11</sup>

The three strains of virus—one from a patient suffering from epidemic encephalitis (the Levaditi sample), the second from a case of neural syphilis (the Beckley specimen), and the third from a person suffering from febrile herpes (the H. F. strain)—comprised, then, the several materials studied. The specimens were inoculated intracerebrally into rabbits and the symptoms produced were minutely observed. As it is now well known from the many published reports,<sup>11</sup> these symptoms are characteristic; they show, as a rule, a quite regular evolution. Although they may be less striking at the first successful implantation of the virus, the symptom-complex becomes established when the virus is passed from rabbit to rabbit, thus permitting in most instances a diagnosis of the nature of the

<sup>4</sup> Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxxvii, 1202.

<sup>5</sup> von Wiesner, R., *Wien, klin. Woch.*, 1917, xxx, 933.

<sup>6</sup> Brasher, C. W. J., Caldwell, J. R., and Coombe, E. J., *Brit. Med. J.*, 1919, i, 733.

<sup>7</sup> Stafford, C. M., *J. Lab. and Clin. Med.*, 1918-19, iv, 691.

<sup>8</sup> Morse, P. F., and Crump, E. S., *J. Lab. and Clin. Med.*, 1919-20, v, 275.

<sup>9</sup> Bastai, P., *Riv. crit. clin. med.*, 1920, xxi, 139; *Boll. Ist. Sieroterap. milanese*, 1921, ii, 197.

<sup>10</sup> Ottolenghi, D., d'Antona, S., and Tonietti, F., *Policlinico*, Sec. Pract., 1920, xxvii, 1075.

<sup>11</sup> Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxii, 1688, 1785.

infection. The symptoms presented in order of appearance may be defined as fever, gnashing of teeth, muscular movements of various kinds and degrees, including convulsions, and paralyses, terminating finally in death.

The cultivation results here recorded differ from those of the investigators mentioned, and the disagreement may be ascribed to the fact that they failed to take into account what may be defined as the essentials for the establishment of the experimental disease in rabbits. In addition to the typical symptom-complex described, the action of the so called virus of encephalitis in these animals is characterized by (a) a definite incubation period, (b) absence of gross lesions in the organs, except for congestion of the brain, (c) absence of infection by ordinary bacteria, and (d) transmissibility of the experimental disease from rabbit to rabbit. Cerebral lesions in this animal cannot, by themselves, or to the exclusion of these criteria, be regarded as sufficient for a diagnosis, for changes indistinguishable from those described by previous investigators as the only condition necessary for determining the experimental disease have been found in a large number of control rabbits.<sup>11, 12</sup>

#### *Cultivation Tests.*

In order to obtain materials especially suited to cultivation tests, the rabbits were sacrificed when *in extremis*. The brain was immediately removed with strict aseptic precautions, fragments about 1 cm. square were cut for culture, and in addition a 5 per cent emulsion was made in saline solution for intracerebral inoculation into the rabbit, as a control for the presence of the virus. At the same time, fragments of the brain were added to broth and streaked on blood agar plates, incubated aerobically and anaerobically to determine any admixture with ordinary bacteria.

*Cultures in the Smith-Noguchi Medium.*—The Smith-Noguchi medium was prepared in accordance with the requirements of Loewe and Strauss.<sup>2</sup> The ascitic fluid was free from bile, clear, and had a high specific gravity. Cultures were examined every day, and those showing contamination were discarded. Since an accurate technique was used, it was only occasionally that such accidents occurred. When clouding occurred about the tissue, such as might be indicative of growth, it happened that a similar condition was noted in the uninoculated tubes. Film preparations were made from all tubes on the 5th, 7th, 10th, and 14th days

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<sup>12</sup> McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.

of incubation at 37°C. and stained by polychrome methylene blue, and by Gram's and Giemsa's methods. At the end of 10 days, subplants were made into fresh Smith-Noguchi medium, and again 10 days later. In other words, three subcultures were made before a final result was noted.

These experiments were repeated, variations of this medium being employed, such as substitution for the ascitic fluid of rabbit serum, undiluted or diluted with Ringer's solution or with dextrose broth.

The Levaditi and Beckley strains of the so called encephalitic virus and the H. F. strain of herpetic virus yielded no macroscopic or microscopic evidence of growth in the Smith-Noguchi medium even in the third subplant.

Since we failed to isolate the microorganisms described by Bradford, Bashford, and Wilson, Loewe and Strauss, and Thalhimer, it was thought that data regarding the persistence of the viruses in this medium would indicate whether they had multiplied or had merely survived, or had died within the cultures. The viability of the viruses was tested by inoculating rabbits intracerebrally with the fluid material surrounding the tissue and with the brain fragment itself. In this way we could determine not only the presence of the viruses or their multiplication in the surrounding medium, but also the length of time they survived in the brain fragment itself.

*Experiment 1.*—Virus in the form of pieces of brain from a rabbit killed when *in extremis* was inoculated into a series of tubes of Smith-Noguchi medium and incubated at 37°C. Every 2nd day a tube was examined for the presence of the virus. The ascitic fluid of the Smith-Noguchi culture was poured off, centrifuged at low speed to throw down particles of brain, and the supernatant fluid retained. The fragment of the brain tissue remaining in the culture was weighed in a sterile capsule and a 10 per cent emulsion made in normal saline.

For control, similar pieces of brain from the same rabbit were placed in tubes of 10 cc. of 1 per cent dextrose veal infusion broth (pH 7.4) and incubated aerobically. The supernatant broth and the remaining brain tissue were treated in the same manner as the Smith-Noguchi medium series.

A uniform amount, 0.25 cc., was injected intracerebrally into young rabbits of similar size and weight (1,000 gm.). The tests were made several times with each of the three strains with like results. In Tables I and II are presented the details of one series of experiments (Beckley strain), the results of which are typical of those obtained equally with the Levaditi and H. F. strains.

From the foregoing protocols, the fact is evident that the so called virus of encephalitis lethargica and that of febrile herpes contained

TABLE I.  
*Survival of Encephalitic Virus, Strain Beckley, in Smith-Noguchi Medium.*

Incubation at 37°C.	Material.	Result of rabbit inoculation.
<i>days</i>		
2	Centrifuged fluid.	No reaction after 21 days.
2	Brain.	Typical symptoms. Died in 5 days.
4	Centrifuged fluid.	No reaction.
4	Brain.	Typical symptoms. Died in 6 days.
6	Centrifuged fluid.	No reaction.
6	Brain.	Typical symptoms. Died in 7 days.
8	Centrifuged fluid.	No reaction.
8	Brain.	" "
10	Centrifuged fluid.	" "
10	Brain.	" "
12	Centrifuged fluid.	" "
12	Brain.	" "
14	Centrifuged fluid.	" "
14	Brain.	" "

TABLE II.  
*Survival of Encephalitic Virus, Strain Beckley, in Aerobic 1 Per Cent Dextrose Broth.*

Incubation at 37°C.	Material.	Result of rabbit inoculation.
<i>days</i>		
2	Centrifuged broth.	Typical symptoms. Died in 5 days.
2	Brain.	" " " " 5 "
4	Centrifuged broth.	" " " " 5 "
4	Brain.	" " " " 5 "
6	Centrifuged broth.	" " " " 6 "
6	Brain.	" " " " 5 "
8	Centrifuged broth.	No reaction.
8	Brain.	Typical symptoms. Died in 6 days.
10	Centrifuged broth.	No reaction.
10	Brain.	Typical symptoms. Died in 6 days.
12	Centrifuged broth.	No reaction.
12	Brain.	Typical symptoms. Died in 5 days.
14	Centrifuged broth.	No reaction.
14	Brain.	" "

in the rabbit brain and incubated in the Smith-Noguchi medium had not multiplied or even diffused into the surrounding medium after 2 days. They were, however, still viable in the brain itself up to the

6th and occasionally the 8th days, the larger the piece of brain the longer being the survival. On the other hand, when placed in dextrose broth and incubated under aerobic conditions, they were found in the surrounding fluid up to the 6th day, and 0.25 cc. of the supernatant broth produced symptoms as severe as those induced by the fresh viruses. The brain fragments in this medium were still infective on the 10th to the 12th days.

It is clear that the three samples of viruses did not multiply in the Smith-Noguchi medium. They survived in aerobic broth, in which they were demonstrated both in the supernatant fluid and in the brain fragment long after they had become inactivated in the Smith-Noguchi medium, which, therefore, should be regarded as exerting an unfavorable effect.

A further confirmation of this conclusion is afforded by a succeeding experiment in which virus was added in the form of an emulsion of ground brain tissue to the Smith-Noguchi medium and to aerobic broth, in order that a wide surface of the inoculum might be exposed to the media. Three sets of tests were made with each strain with practically uniform results.

*Experiment 2.*—Rabbit brains containing respectively the fresh active virus of the Levaditi, Beckley, and H. F. strains were ground up in a sterile mortar with sand. Saline solution was added to form a 20 per cent emulsion. After centrifuging at low speed to deposit the particles of brain, 1 cc. of the emulsion was added to 15 cc. of Smith-Noguchi medium, and 1 cc. to a like amount of dextrose broth.

Before incubation at 37°C., material was removed from each tube and inoculated intracerebrally into control rabbits which developed typical symptoms and died after 5 days. Fluid was withdrawn from the tubes on the 2nd and 4th days of incubation at 37°C., and the test dose, 0.25 cc., was injected intracerebrally into a series of animals.

The material from the broth medium caused typical symptoms, and death occurred as early as in the controls, whereas similar material from the Smith-Noguchi medium was entirely without effect.

This experiment shows that in the Smith-Noguchi medium at 37°C., the viruses as contained in the ground brain tissue are either killed or rendered inactive within 2 days, while in aerobic broth at 37°C., they still remain active after 4 days incubation.

*Oxygen Tension in Relation to the Viability of the Viruses.*—In view of the fact that under the experimental conditions the viruses survive in broth under aerobic conditions, the question arises as to the part played by oxygen tension in preserving or destroying activity.

*Experiment 3.*—Twelve tubes of dextrose broth were inoculated with each of the viruses in the form of pieces of fresh brain tissue, all from the same rabbit. The tubes were divided into three sets, and four tubes were incubated aerobically, four anaerobically in the Brown jar,<sup>13</sup> and the remainder under a petrolatum seal.<sup>14</sup>

On the 2nd and 4th days of incubation, the broth from two tubes of each set was centrifuged, and 0.25 cc. of supernatant clear fluid from each tube was inoculated intracerebrally into rabbits. All twelve animals thus inoculated showed typical symptoms, and death occurred within the same time as in the control animals similarly inoculated with fresh virus.

In all, three series of twelve tubes were used, each of the three viruses being employed for inoculation.

From this experiment it appears that oxygen tension as such does not play a material part in deciding either survival or destruction of the viruses.

*Acidity of the Medium in Relation to the Destruction of the Viruses.*—To ascertain the effect of the acidity of the medium as a factor in the destruction of the viruses, two different experiments were made. In one the Smith-Noguchi medium was buffered at pH 7.4. This is the hydrogen ion concentration of the dextrose broth in which the viruses survive. In the other was measured<sup>15</sup> the pH of the broth to which was added a fragment of brain. Finally, a comparison was made with the pH of the 50 per cent glycerol in which, as is known, the viruses survive for long periods of time.

*Experiment 4.*—Uninoculated ascitic fluid-kidney tissue medium with a petrolatum seal after 7 days incubation showed a pH of 7.2 in the upper part of the medium, and a pH of 7.1 in the fluid surrounding the kidney tissue. Smith-Noguchi medium to which brain tissue was added exhibited a pH of 7.24 at the upper and 7.2 at the lowest part of the fluid. Varying amounts of sodium phosphate buffer solution were added to this medium containing brain tissue. It

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<sup>13</sup> Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

<sup>14</sup> Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

<sup>15</sup> The determinations of hydrogen ion concentrations were made by Dr. J. H. Northrop.

was found that two parts of ascitic fluid and one part of sodium phosphate solution (pH 8.0) added to fresh kidney and brain tissue revealed, after 7 days incubation at 37°C., a pH of 7.4 in the fluid at the bottom of the tube.

A series of tests was carried out with this buffered ascitic fluid-kidney tissue medium, with each of the viruses. In no case, on intracerebral inoculation into rabbits, was the fluid in proximity to the brain and kidney tissues active.

The pH of the dextrose broth to which a fragment of brain from a normal rabbit was added and then incubated for varying periods, compared with the pH of the glycerol, is shown in Table III.

TABLE III.

*Determination of pH of Dextrose Broth and Brain, Incubated Aerobically at 37°C.*

Day.	Upper layer of medium.	Lowest layer of medium.
	<i>pH</i>	<i>pH</i>
2nd	6.7	6.6
4th	6.22	6.22
6th	6.42	6.42

pH of 50 per cent glycerol 5.54.

It will be noted that broth in which the viruses survive is considerably more acid than the Smith-Noguchi medium in which they rapidly become inactivated; moreover, the 50 per cent glycerol in which they may survive for long periods is still more acid.

From these results it appears that the destruction of the viruses in the Smith-Noguchi medium cannot be ascribed to acid production.

#### DISCUSSION.

This paper is concerned with the study of the survival and multiplication of the so called virus of epidemic encephalitis and the virus of febrile herpes in artificial cultures.

Two major sets of experiments were carried out. In the first, an attempt was made to repeat the experiments reported by Bradford, Bashford, and Wilson,<sup>1</sup> by Loewe and Strauss,<sup>2</sup> and by Thalhimer.<sup>3</sup> These observers believe they have succeeded, by the use of the Smith-Noguchi anaerobic tissue method of cultivation, in obtaining from the so called virus of encephalitis lethargica growths of a microorganism which is visible under the microscope, and active when inoculated into rabbits.

We have been unable to confirm the published reports; on the contrary, our experiments have taught us that the Smith-Noguchi medium is not favorable even for the survival of the Levaditi and Beckley strains of the viruses, much less for their multiplication or growth. The same fact is true for the virus of febrile herpes. It has been shown, moreover, that the viruses diffusing into the fluid part of the Smith-Noguchi medium at 37°C. rapidly lose their activity; that is to say, in less than 2 days. Within the brain fragment itself, on the other hand, they remain active up to the 6th day. In no instance could any formed bodies be detected in this medium which could be regarded as microorganisms, although detritus of very fine type is always present, which might possibly be so construed.

While we found that the Smith-Noguchi medium was unfavorable to the survival of the viruses, it was ascertained that aerobic broth is less destructive to the active material. Under the experimental conditions, it was determined that the viruses diffusing into the surrounding broth remained active for 6 days, but within the brain fragment itself they survived for 12 days.

In connection with these studies an attempt was made to determine the relation of oxygen tension and of hydrogen ion concentration to the destruction of the viruses in the Smith-Noguchi medium and to their survival in aerobic broth. Changes in oxygen tension, and variations in hydrogen ion concentration between the limits of pH 7.4 and 6.2, do not exert in this relation any appreciable effect. The precise cause of the injurious action of the Smith-Noguchi medium has not been determined.

Not only do these results fail to uphold Bradford, Bashford, and Wilson, Loewe and Strauss, and Thalhimer, but they contradict also the published reports of von Wiesner, Rosenow, Towne, and Wheeler, and others, who would identify the encephalitis virus with certain common bacteria. It can be stated unequivocally that with an accurate technique the so called virus of encephalitis lethargica and the virus of febrile herpes may be passed from brain to brain in the rabbit, under conditions in which no visible microorganisms can be made to appear in films or to grow in aerobic or anaerobic culture media.

## SUMMARY.

These studies fail to confirm the statements previously made that microorganisms of the class of the globoid bodies of poliomyelitis may be cultivated in the Smith-Noguchi medium from the so called virus of encephalitis lethargica. They show equally that the herpes virus does not multiply in this medium. The experiments indicate, moreover, that the medium is unfavorable to the survival of the virus, while ordinary broth under aerobic conditions is more favorable for maintaining the activity of both the encephalitic and the herpes viruses. Probably no multiplication of either takes place in the latter medium but merely a survival, and for a maximum period of 6 days in the broth itself, and 12 days in the fragment of brain tissue immersed in the broth. Finally, it has been shown that with a suitable technique the viruses can be passed from the brain of one rabbit to that of another through a long series without contamination with cocci or other common bacterial forms. Hence we regard all reports of the finding of ordinary bacteria in the brain of cases of epidemic or lethargic encephalitis as instances of mixed or secondary infection arising during life, or examples of postmortem invasion of the body, or of faulty technique at the autopsy.