CULTIVATION EXPERIMENTS ON THE GLOBOID BODIES OF POLIOMYELITIS.*

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The microbic cause of poliomyelitis is a subject still in the foreground of interest with respect to the etiology of that disease. The present paper deals with the conditions surrounding the cultivation of the globoid bodies described by Flexner and Noguchi (1) from poliomyelitic tissues and is intended as a contribution to the technique of the method.

The technique of the cultivation is a complex and difficult procedure, even more difficult, perhaps, than the isolation of *Treponema pallidum*. With respect to the treponema, the experimenter is assisted by the fact that it is comparatively large, possesses clear-cut morphological characteristics, and is motile—properties which make its detection relatively easy.

The globoid bodies, on the other hand, are extremely minute and non-motile. They are not readily distinguished from tiny particles of detritus in the early generations of scantily growing cultures. Hence weeks, even a month or more, may elapse between the initial inoculations of the culture medium and the definite determination of successful cultivation of the organism; and it may happen that the growth then observed may fail to develop on transplantation, so that the opportunity for its identification may be lost. The difficulties surrounding the method of cultivation have resulted in the confirmation of Flexner and Noguchi's work by only a few bacteriologists, the larger part of those who have attempted to repeat it failing in their efforts.

The present study was undertaken in the hope of modifying the

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method by rendering it simpler and more certain of success. The original procedure is not only uncertain in its ultimate results, but also in the number of cultures yielded by a given lot of tubes inoculated with poliomyelitic tissue. Sometimes of 30 to 50 tubes thus inoculated, one or two only would yield cultures, the others remaining without demonstrable growth. Where the conditions all appear to be so similar, it would seem that a higher percentage of cultures should be obtained. Obviously the conditions within the tubes varied in an unaccountable manner; and it became also the purpose of this study to eliminate the factor of variation and thus to increase the proportion of cultures obtainable from a certain number of inoculated tubes.

Hitherto, the globoid bodies have been cultivated from the central nervous organs, which are the seat of the profounder effects of the disease. Now, according to present conceptions of the pathology of poliomyelitis, the disease partakes often of the nature of a general or systemic infection. Indeed, the non-paralytic or abortive cases, so called, pursue a course unattended by paralysis or even by marked symptomatic involvement of the central nervous system. Hence it became desirable to study the general viscera by culture methods.

The source of the tissues submitted to cultivation was *rhesus* or *cynomolgus* monkeys in which the inoculation form of the disease had been produced. The usual procedure was to permit the paralysis to develop to the point at which the inoculated animals became prostrate; they were then etherized. In certain instances animals were used which had been prostrate for several days or which had died some hours earlier. A comparison of the cultures from the two classes of animals—those recently paralyzed and still in good physical condition with those which have been *in extremis* for some time or have actually succumbed to the disease a number of hours before autopsy—is illuminating as regards the number and kind of cultures obtained.

The virus or emulsion of the nervous organs employed for inoculation was either an active virus which has been transmitted in the laboratories of The Rockefeller Institute for several years, or the less active M.A. virus (2), which has also passed through many monkeys. The mode of inoculation was usually intracerebral, but

sometimes it was by other routes—the nose and blood. A brief summary of the clinical data of the infected monkeys is given in Table I.

We propose to give the details of the technique of the successful cultivations of the globoid bodies, which will be followed by a description of the results obtained by the method, including both success and failure, and the probable causes of each.

TABLE I.

Summary of Clinical Data of Monkeys Infected with Poliomyelitic Virus.

Serial No.	Source of virus.	Amount inoculated.	Route of inoculation.	Duration of symptoms	Duration of prostration.	Length of illness.	Pathologic appearance.
				days	*days*	*days*	
1	Fresh mixed.	50.0 cc.	Intravenous.*	1½	½	7	Typical.
2	Glycerolated mixed.	2.0 cc suspension	Intracerebral.	4	1	13	"
3	Fresh mixed.		Nasal.	9	6	18	"
4	" "	0.2 cc. filtrate.	Intracerebral.	3	1	10	"
5	" "	50.0 cc.	Intravenous.*	2	0	7	"
6	" "	0.1 "	Intracerebral.	1½	¼	10	"
7	" "	0.1 "	"	1	0	11	"
8	" "		Nasal.	3	1	11	"
9	" "	0.1 cc.	Intracerebral.	2	1	7	"
10	" "	0.2 "	"	8	1	13	"†
11	" "	0.2 "	"	7	3	15	"†
12	M.A.	1.0 "	"	5	1½	9	"
13	Fresh mixed.		Nasal.	4	2	12	"
14	" "		"	2	½	10	"
15	" "	2.0 cc.	Intracerebral.	2	1	6	"
16	" "		"	2	½	6	"
17	" "		Nasal.	3	1	7	"
18	M.A. cultivated.	2.0 cc. mass culture.	Intracerebral.	5	0	12	"

* Intravenous inoculation of centrifuged emulsion following the injection of horse serum intraspinally.

† These animals had been dead 7 and 3 hours respectively when autopsied.

Cultivation Technique.

The methods used for the cultivation of the globoid bodies from the tissues of monkeys are essentially the same as those originally

described by Flexner and Noguchi (1). Certain variations of the technique will, however, be described. In part, the technique is a repetition of previously reported work, but is given as a convenient summary.

Test-tubes, 1.5 by 20 cm., should be used for all experiments, for the narrower tubes are much more likely to become contaminated. They should be plugged with cotton and placed in a surgical package containing 25 to 30 tubes, which should then be sterilized and not opened until the set of tubes is to be used.

Fresh sterile tissue is added to each tube. A healthy, fat, adult rabbit is chosen, one that has not even a scratch or other detectable defect upon any part of its body. The animal, which must be given no food for 24 hours, is lightly etherized and bled to death from the heart by means of a sterile needle and a large bulb pipette. All hair is then removed from the abdomen and anterior thoracic walls of the animal's body by sodium sulfide solution, and the skin is prepared with the usual surgical procedures, soap and water, bichloride solution, alcohol, and finally iodine. Instruments for obtaining the kidneys should not be boiled, but required instruments are made into a set and sterilized in a glass container in the hot air sterilizer. One set is used to open the skin, another set to open the peritoneal cavity, and a fresh pair of scissors and forceps are used to remove the kidneys. All procedures should be carried out in a dust-proof room, or under a hood, the walls of which have been wiped down with bichloride solution.

After removal to a large sterile Petri dish, the capsule is stripped from the kidney, each kidney is cut into 20 to 25 pieces, and one bit of tissue placed in each sterile test-tube. This procedure must also be carried out in a dust-proof room. While removing the capsule of the kidney and cutting it into pieces, the assistant should hold the cover of the Petri dish directly over the field of operation in order to avoid dust contamination.

The poliomyelitic material which is added to each tube consists of a bit of tissue of about the same size as the bit of rabbit kidney tissue. The infected monkey should not be allowed to die, nor, in fact, to become prostrate, but should be etherized at the height of the active symptoms. After etherization, the animal should be bled, and the visceral tissues, liver, spleen, kidney, thymus, etc., removed with exactly the same technique as was used in obtaining sterile rabbit kidney. It is important to use a separate pair of forceps and scissors for each organ.

The technique for obtaining uncontaminated poliomyelitic brain tissue is as follows: The calvarium is removed with aseptic precautions, great care being taken not to injure the dura. The dura over the hemisphere opposite the one that had been injected is then seared with a red hot knife blade, and the dura is opened with sterile instruments. A portion of the cerebrum is removed with a separate set of instruments and placed in a sterile Petri dish. This material is at once taken to a dust-proof room, when it may be cut into suitable pieces and placed immediately in the tissue test-tubes.

Methods for removing bits of sterile spinal cord are very unsatisfactory. The following method has proved the most useful. The entire cord is exposed under aseptic precautions, great care being taken not to injure the dura. A portion of the cord, 3 to 4 cm. long, is tightly tied at either end with a sterile thread and the portion removed. This bit of cord with intact dura is placed in bichloride alcohol for 60 seconds, then rapidly passed through five separate washings of sterile salt solution. The dura is wiped dry with sterile gauze, seared with a narrow hot knife blade, and is then opened with sterile instruments and the cord cut into 0.5 cm. segments, each portion being put into a tissue test-tube.

When the test-tubes have been inoculated with rabbit kidney and poliomyelitic material, ascitic fluid should be added, 15 or 18 cc. being sufficient. The ascitic fluid must, of course, have been tested carefully for sterility, both for aerobic and anaerobic organisms. It should have a specific gravity of at least 1,015, be clear, free from bile, not more than 3 months old, and should be stored in the refrigerator. It should not be heated above 50°C. or filtered. Chylous or bloody ascitic fluid is not suitable. *Before adding the ascitic fluid to the inoculated test-tubes, it should be warmed to 45°C. and placed under a vacuum to remove the air.*

Complete anaerobiosis must be obtained if one is to be successful in the cultivation of the globoid bodies. In our first experiments the vacuum jar was used, but the results were disappointing. The vacuum jars were either so poorly ground that they would not hold a vacuum, or, owing to defects in the glass, they cracked or exploded under the tremendous negative pressure. These difficulties were finally met by the use of the hydrogen-nitrogen jar, which utilizes the catalytic action of platinized asbestos upon hydrogen and oxygen, and removes all traces of oxygen from the jar. The details of this apparatus have already been published (3). Subsequent experiments with this jar have given most satisfactory results. The pyrogallic acid-sodium hydroxide indicator has remained unchanged in color for weeks, showing complete anaerobiosis; and the method is so rapid, clean, and simple that it seems to promise a simple satisfactory solution for the problem of anaerobic technique.

The inoculated ascitic fluid-tissue tubes, usually twenty-five to thirty tubes, are placed in the anaerobic jar and incubated for 11 to 12 days. Control tubes, inoculated with brain and other tissues, are incubated in the open air and examined daily in order to determine the presence of contaminating organisms.

After 12 days' incubation, the jar is opened, and 0.2 cc. of the ascitic fluid is removed from the bottom of each tube, without disturb-

ing the bits of brain or kidney tissue. The special pipettes devised by Gates (4) are very useful for all poliomyelitic culture work. The material is transferred directly into freshly prepared tissue tubes. 15 cc. of air-free, warmed ascitic fluid are added to each tube as before, the tubes placed in the anaerobic jar, and at once returned to the incubator. The original tubes may be placed in the refrigerator to be examined at leisure.

The second generation of ascitic fluid-tissue tubes is incubated for 12 days and the anaerobic jar is then opened. As before, immediate transfer is carried out, 0.2 cc. of the ascitic fluid near the bottom of the tube being transferred to freshly prepared tissue tubes. To a second set of tissue tubes is added 0.1 cc. of the material from the bottom of the tubes of the second generation. To Set 1, containing 0.2 cc. of transferred material, is added warmed air-free ascitic fluid as before. To Set 2, containing 0.1 cc. of the material, are added 15 cc. of semisolid medium which has been prepared as follows:

Veal infusion agar, 2 per cent, with a reaction of +0.5 to phenolphthalein, is melted, boiled, and then rapidly cooled to 50°C. Ascitic fluid, clear, straw-colored, free from bile, with a specific gravity of 1,015 or higher, is warmed to 45°C. and placed under a vacuum to remove the air. 1 part of the melted agar, cooled to 50°C., is added to 2 parts of warmed ascitic fluid, and 15 cc. of the mixture are added to each inoculated test-tube of Set 2.

The two sets of tubes are at once placed in separate anaerobic jars and incubated. The second generation of ascitic fluid tubes from which the transfer was made is placed in the refrigerator and examined on subsequent days for the presence of the globoid bodies.

The anaerobic jar containing the set of semisolid tubes (Lot 2) is incubated for 8 days, and the jar is then opened and the tubes are examined. If any of the tubes contain suspected colonies in the depths of the semisolid medium, these tiny colonies are removed by means of a capillary pipette and examined microscopically. If positive, fresh semisolid tissue tubes are made from each colony, and these tubes are placed in the anaerobic jar and incubated. If none of the tubes show visible colonies, they are all replaced in the anaerobic jar and reincubated for 8 days. If at the end of this reincubation there is no growth in the semisolid tubes, the set is discarded.

The anaerobic jar containing the ascitic fluid tubes of the third

generation, Lot 1, is incubated for 12 days, then opened, and fresh sets of ascitic fluid and semisolid tissue tubes are made exactly as described for the second generation. This process of making ascitic fluid and semisolid tubes is continued for at least five generations, after which time, if no growth has been obtained, all tubes are discarded. Every original tube inoculated is therefore a separate experiment and must be given its own number, and a detailed record must be kept of each subsequent generation of that culture. Each lot of ascitic fluid tubes, after their period of incubation, is kept in the refrigerator, so that one may subsequently return to the earlier generation in case it is necessary to pick up a positive culture that may have died out in subsequent generations.

For example, Culture 6, brain tube No. 1, after growing for two generations in semisolid medium, refused to grow further in this medium, so that it was necessary to return to the ascitic fluid tube, brain tube No. 1, third generation, from which the strain had originally been obtained, and though the growth was very scanty, and the tube had been in the refrigerator for more than a month, nevertheless a positive permanent culture was obtained.

Criteria for the Determination of the Globoid Bodies.

It is necessary to have definite criteria for the determination of the globoid bodies. These have been described in the original publication by Flexner and Noguchi (1) and in later publications and need simply be summarized for convenience.

Morphology and Staining Characteristics.—Morphology and staining characteristics are somewhat variable factors, the variation depending not so much upon the organism as upon the observer, and, though confirmatory, they are insufficient indices in the identification of an organism.

Characteristic Growth in Semisolid Medium.—It is not enough to determine the presence of an organism in the ascitic fluid medium which seems to the observer to possess the characteristic morphology of the globoid bodies. Semisolid medium should be inoculated and observed daily. There should be no apparent growth in the medium for 3 or 4 days. At the end of 72 hours or later, the characteristic minute colonies begin to appear, being definitely established only at the end of 6 to 7 days. In the anaerobic jar, the line of demarcation of growth is about 1 cc. from the surface of the medium. If the semisolid tube has been incubated in the open air, however, only a few colonies will be seen about the tissue in the bottom of the tube and extending upward about 1 cc.

Action upon Carbohydrates.—An additional factor for the identification of the globoid bodies depends upon their inability to attack carbohydrates. Tissue tubes of ascitic dextrose broth are inoculated with a small fragment of a semisolid culture. The broth should be sugar-free veal infusion, +0.2 to +0.3 acid to phenolphthalein. To each 5 cc. tube of broth is added an equal amount of unheated sterile ascitic fluid plus sufficient 10 per cent sterile sugar solution to give a concentration of 0.5 per cent. The hydrogen ion concentration of the medium should be approximately that of blood neutrality; namely, 7.4. Control tubes should be made both of uninoculated tubes and of tubes inoculated with various types of streptococci. The tubes should be incubated in the anaerobic jar for 7 days and the hydrogen ion concentration determined. The globoid bodies will not attack the simple sugar, whereas the control tubes of streptococci will show a marked increase in acidity.

Results Obtained in the Cultivation of the Globoid Bodies.

Material from eighteen poliomyelitic monkeys has been used in the attempted cultivation of the globoid bodies. For the first four experiments, the anaerobic technique used was the vacuum jar, and in each instance the experiment was unsuccessful. For all the later experiments, the platinized asbestos method was used, and far more satisfactory results were obtained.

The material from three of the monkeys was contaminated with a pure culture of streptococcus in the first generation in many or all of the tubes. This contamination was not due to faulty technique, because all control tubes were negative, but was the result of a secondary invading streptococcus which is commonly found in the blood and tissues of animals and human beings who have been prostrate and moribund for hours or days before death finally occurred.

Of the remaining eleven monkeys, the typical globoid bodies were cultivated from seven, and a total of twenty-two separate cultures or strains was obtained. The largest number of strains obtained from a single monkey was six, whereas in two instances only one strain was obtained. Besides the twenty-two strains, eleven other strains were cultivated in the ascitic fluid medium, but I was unable to transfer them to semisolid medium, and they are, therefore, not included in the total number of cultivations. The eleven strains, incompletely isolated, were derived in part from monkeys yielding completely isolated strains. Table II summarizes the successful and partially successful results of the cultivations.

TABLE II.

Successful and Partially Successful Cultivation of the Globoid Bodies.

Serial No.	Generation carried before discarding.	Virus cultivations in semisolid medium.			Virus cultivations in ascitic fluid medium but not transferred to semisolid medium.		
		No. of strains obtained.	Source.	Generation in which culture was obtained.	No. of strains obtained.	Source.	Generation in which culture was obtained.
1	3rd	None.			None.		
2	4th	"			"		
3	12th	"			1	Mesenteric node.	4th
4	9th	"			1	Kidney.	4th
5	10th	1	Brain.	3rd	1	Brain.	4th
6	12th	2	" 1, cord 1.	5th 6th	1	"	4th
7	10th	4	Brain.	4th	None.		
8	11th	3	"	4th	"		
9	3rd	None.			"		
10	2nd*	"			"		
11	1st*	"			"		
12	12th	5	Brain.	1, 3rd 1, 4th	"		
13	8th	6	" 4, spleen 2.	4th	1	Adrenal.	4th
14	7th	None.			3	Brain 2, spleen 1.	3rd
15	4th	"			1	Brain.	3rd
16	4th	"			2	"	3rd
17	1st*	"			None.		
18	4th	1	Brain.	3rd	"		
Total.....		22			11		

* All tubes contaminated with a streptococcus.

Nineteen of the completely isolated strains were obtained from brain substance, one strain was cultivated from the cervical portion of the spinal cord, and two were cultivated from the spleen. Of the eleven incompletely isolated strains, seven were cultivated from the brain, one from the kidney, one from a mesenteric node, and one from the spleen. The shortest period of time between the making of the cultures at autopsy of the monkey and definite establishment of a positive culture was 28 days, and in the third generation. The longest

period required for the definite establishment of a strain in semisolid medium was 54 days, although the organism in this case had been found in the ascitic fluid tube in the third generation on the 30th day.

In no instance was a definitely positive culture found in the first generation of tubes, but usually the organisms were present in sufficient numbers in the second generation to suggest at least that the result would eventually be positive. The globoid bodies are so small and may so readily be confused with detritus, that even a presumptive decision should not be reached unless characteristic forms are found under at least five different microscopic fields. The organisms are so few in number in the second generation that a presumptive test, though possible, is a long and tedious process. It is much simpler and more satisfactory to make subcultures of all tubes of the second generation into the semisolid medium and thus reach a positive conclusion in the succeeding generation by means of the typical growth in colony form.

Occurrence of Streptococci and Other Organisms.

The occurrence of streptococci in certain series of the cultures has been noted. More rarely other microorganisms than streptococci were cultivated instead of or with the globoid bodies. We should consider the sources and endeavor to estimate the significance of these classes of organisms. It is obvious that culture tubes which require so much manipulation will sometimes become contaminated. This condition will account for the miscellaneous bacteria sometimes encountered in the tubes, but probably not for the streptococci which have come to occupy a position of prominence, even if not of importance, with regard to the vexed question of the etiology of poliomyelitis.

The streptococci, when present in the cultures, have not, as a rule certainly, entered with the ascitic fluid or the kidney fragment or from the air. There is no reason to doubt that they have been introduced with the fragment of nervous or other tissue with which cultivation was attempted. Whether they are also to be regarded as contaminations in the broad sense is the question at issue.

A summary of all the extraneous microorganisms encountered (Table III) reveals that a large proportion was streptococci. It is of interest to have found that the tubes prepared from the liver and

kidney of the monkeys were more frequently attended by growth of streptococci, and other extraneous organisms, than those of the spleen, and the tubes of the spleen more often showed extraneous organisms than those of the brain.

In three monkeys there was a rich growth of a pure culture of streptococci in all the tubes of the first generation, the control tubes remaining sterile. In all these instances the animal had been prostrate 24 hours or longer, and in two instances had been dead several hours when the autopsy was performed. This finding is in such definite contrast with the results of the cultures prepared from the corresponding tissues of monkeys severely paralyzed but not yet prostrate and moribund or dead that it would seem to throw considerable

TABLE III.

Summary of Contaminations with Streptococci and Other Organisms in Poliomyelitic Cultivation Experiments.

Tissue.	No. of original culture tubes made.	No. of tubes contaminated with streptococci in 1st generation.	No. of tubes contaminated with streptococci in all later generations.	No. of tubes contaminated with organisms other than streptococci in all generations.
Brain	137	25	11	10
Spinal cord	14	0	7	0
Kidney	31	7	5	0
Liver	31	5	2	3
Spleen	30	6	1	2
Adrenal	17	3	1	1
Mesenteric node	19	2	0	0
Thymus	10	2	0	0
Pancreas	4	0	0	0
Total	293	50*	27†	16

* Forty-six of the fifty tubes that were contaminated with streptococci in the first generation were obtained from four monkeys that had been prostrate and moribund for a considerable time before autopsy. The remaining four streptococcic contaminations in the first generation of tubes were from fourteen other monkeys, autopsied under more favorable conditions.

† An average of more than five subcultures from each original tube was made so that the total number of contaminations with streptococci in all subsequent generations (27) represents the extraneous contaminations in over 1,000 tissue culture tubes.

light on the nature of the streptococcal invasion, detected by Mathers (5), Rosenow, Towne, and Wheeler (6), and Nuzum and Herzog (7) in human beings and monkeys who have succumbed to poliomyelitic infection.

In order to obtain more light on this subject, we cultivated, by means of the technique employed for the globoid bodies, the tissues of monkeys which had succumbed in the laboratory to tuberculosis, dysentery, and other diseases, and isolated, in several instances, streptococci from the liver, kidney, spleen, and nervous tissues.

The streptococci yielded by the tissues of the moribund and dead poliomyelitic monkeys were transplanted into carbohydrate media and injected into rabbits. The fermentation reactions were those of streptococci in general and sharply distinguished them from the globoid bodies. The inoculations gave results so closely in accordance with those described by Bull (8) as not to call for restatement here.

Typical Cultivation Experiments.

Experiment A.—February 23. *Macacus cynomolgus.* Inoculated into left cerebral hemisphere with 0.1 cc. of an N Berkefeld filtrate of a centrifuged 5 per cent suspension of glycerolated brain and spinal cord carrying mixed virus. March 4. Tremor of head, slow movements, weakness of arms. March 5. Extensive paralysis; animal unable to rise. Etherized and bled to death from the heart. The autopsy showed the presence of typical lesions of poliomyelitis.

Cultures were made as follows: brain, 8 tubes; spinal cord, 4 tubes; intervertebral ganglion, 1 tube; kidney, 3 tubes; liver, spleen, thymus, 2 tubes each. Rabbit kidney fragments from a single animal were employed, but two specimens of ascitic fluid were used. The even-numbered tubes received ascitic fluid, Lot 7, the odd-numbered tubes Lot 10. The control tubes consisted of ascitic fluid plus kidney, ascitic fluid alone, and a kidney tissue-ascitic fluid inoculated with stock culture of globoid bodies No. 973. All the tubes were placed in the hydrogen-nitrogen jar and incubated, and in addition three tubes of ascitic fluid plus kidney and brain were incubated in the open air and examined every other day. The latter series remained sterile and was discarded on the 8th day.

March 15. Hydrogen jar opened. Sodium pyrogallate solution was colorless; hence the jar had been oxygen-free. Fresh kidney tissue-ascitic tubes were reinoculated from each of the original tubes except the thymus tube. The fragment of thymus had floated to the top, and the tube was discarded. Rabbit kidney No. 9 was used for the new tubes; and for the even-numbered tubes ascitic fluid No. 7 and in the odd-numbered tubes ascitic fluid No. 6 were used. The usual controls were added and all placed as before in the anaerobic jar.

March 17. The microscopic examination of the first generation tubes was negative throughout.

March 26. The second anaerobic jar had remained oxygen-free. In removing the tubes, spleen tube No. 20 and brain tubes Nos. 7 and 8 were broken. Microscopic examination of the remaining intact tubes gave the following result:

Brain Tube No. 1.—Organisms suggestive of globoid bodies.

Spinal Cord Tubes Nos. 10 and 12, Ganglion Tube No. 13, and Spleen Tube No. 19.—Indefinite bodies, somewhat suggestive of the globoid bodies.

Cord Tube No. 11 and Liver Tube No. 17.—Grossly contaminated.

March 26. Ascitic fluid-kidney tissue tubes were inoculated from the second generation, using ascitic fluid No. 13 for the odd-numbered and No. 6 for the even-numbered tubes. Anaerobic cultivation.

April 4. The jar had remained free of oxygen. Microscopic examination of the tubes gave the following result:

Brain Tube No. 1.—No growth.

Brain Tube No. 5.—Same as No. 1.

Other tubes of the third generation suggestive of globoid bodies were: brain No. 6, cord No. 12, and kidney No. 16.

April 4. Tubes of semisolid medium were inoculated and placed in the anaerobic jar.

April 10. Jar opened. All the cultures were negative except brain No. 6, cord No. 12, intervertebral ganglion No. 13, and spleen No. 19, which were merely suggestive, and cord No. 10, which contained a growth of the globoid bodies. Transplantation from the tubes which were suggestive and the one positive to fresh tubes of semisolid medium gave no result. Thus far the cultivation experiments with this specimen of virus, which had been carried through 7 or 8 weeks, resulted in three cultures of the globoid bodies which could not be developed in the fourth generation.

The ascitic fluid-kidney tissue culture of March 26 was preserved in the refrigerator for 20 days. Transplantation from it was again made into fluid and semisolid media. Brain tube No. 1 gave a positive growth which again failed to grow in the next transplantation. But by returning again to the fluid culture of March 26, a strain of brain No. 1 culture was secured which continued to grow in semisolid medium. Similarly the ascitic fluid-tissue tube of cord No. 12, made on April 14 from the mother tube of March 26, was positive and yielded a culture capable of growing in subcultures.

Thus from a total of more than 100 tubes carried over a period of more than 2 months and through five or six generations, two strains of the globoid bodies which bore subculturing were finally obtained. In each instance the organism was detected in the second generation; but patience and persistence were needed to obtain strains which would continue to grow in artificial media. All the cultivation ex-

periments were not so long, tedious, and difficult as this one; but it has been given in detail to illustrate the intricacy of the problem of the cultivation of the globoid bodies.

Experiment B.—April 15. *Macacus rhesus.* A cotton plug, containing 1 gm. of fresh mixed virus, was placed in the left nares, where it was allowed to remain for 16 hours. April 22. Right arm was paralyzed. April 25. Paralysis has slowly progressed; animal was almost prostrate but was bright. April 27. Extensive paralysis; complete prostration. Etherized and bled from the heart. Autopsy showed the presence of typical lesions of poliomyelitis in the cord and brain.

Cultures were made as follows: Brain, 8 tubes; kidney, 2 tubes; spleen, 3 tubes; adrenal, 2 tubes; liver, 3 tubes; mesenteric nodes, 2 tubes; and pancreas, 2 tubes; a total of 22 tubes. Three lots of ascitic fluid were used and kidney tissue from two rabbits, the same ascitic fluid being placed in every third tube and the same rabbit kidney tissue in every alternate tube. Controls were made as usual. The hydrogen-nitrogen jar was used and was satisfactory throughout the experiment.

May 8. The anaerobic jar was opened and all tubes were found to be in good condition except one liver tube, which was broken. A complete set of tissue tubes was made up, one rabbit kidney and two fresh ascitic fluids being used. These were placed in the hydrogen-nitrogen jar as before. Microscopic examination of the tubes of the first generation showed no growth in any tube.

May 20. The anaerobic jar of May 8 was opened. Examination of the tubes revealed a typical microscopic picture of the globoid bodies in brain tubes Nos. 1 and 5. One spleen tube and one liver tube were contaminated with a large Gram-positive bacillus and were discarded. Semisolid tissue culture tubes were made from each of the remaining tissue culture tubes of the second generation and placed in a hydrogen-nitrogen jar.

May 29. The anaerobic jar of May 20 was opened. A few tiny colonies were to be seen in the bottom of the following tubes: brain No. 4, brain No. 5, brain No. 6, spleen No. 12, spleen No. 13, and adrenal No. 11. Microscopic examination confirmed the presence of the globoid bodies. There was no growth in the other tubes, and they were discarded.

Fresh semisolid tissue tubes were at once made of all the suggestive semisolid cultures of the third generation and also from the ascitic fluid tubes of the second generation, which had been kept in the ice box since May 20. These tubes were all placed in the anaerobic jar as usual.

The fourth generation tubes resulted in the definite establishment of four brain strains, three from the semisolid media of May 29 and one from the ascitic fluid media of May 20. The two spleen strains were also definitely established from the spleen semisolid tubes of May 29. Adrenal tube No. 11 yielded a typical growth in the third generation, but it refused to grow in all subsequent generations and therefore cannot be included in the series.

In this experiment, therefore, a total of six positive cultures was obtained, four from the brain and two from the spleen, out of a total of twenty-two original tubes. There were two contaminations in the series, both occurring in the second generation.

Carbohydrate Reactions of the Globoid Bodies.

In their original publication Flexner and Noguchi (1) state that the globoid bodies have no ability to split the polysaccharides, alcohols, or even the simple hexoses. Since it is obvious that if an organ-

TABLE IV.

Reaction of the Globoid Bodies upon Simple Sugars.

Strains.	Strain No.	Dextrose.		Lactose.	
		Titration to phenol-phthalein in the cold.	pH	Titration to phenol-phthalein in the cold.	pH
New strains.	5	0.60	7.3	0.72	7.3
	6 (Brain No. 1).	0.56	7.3	0.60	7.2
	6 (Cord " 12).	0.62	7.3	0.62	7.3
	7	0.52	7.25	0.50	7.3
	8	0.50	7.3	0.64	7.2
	12	0.64	7.2	0.68	7.2
	4 (Brain No. 3).	0.46	7.3	0.64	7.2
	4 (Spleen " 13).	0.56	7.3	0.58	7.3
Stock strains.	1281	0.56	7.2	0.48	7.3
	1328	0.52	7.2	0.60	7.2
	973	0.56	7.3	0.70	7.3
Controls. Streptococci.*	1623-2	6.6	5.3	5.6	5.6
	1556	4.56	5.8	3.1	6.0
Controls.					
Kidney + ascitic broth.		0.60	7.1	0.62	7.2
Ascitic broth alone.		0.54	7.25	0.54	7.3

Titration results are expressed in the number of cc. of 0.1 N sodium hydroxide which would be required to neutralize 100 cc. of the medium.

The hydrogen ion concentrations were done by the Henderson-Palmer colorimetric method (9).

* Both strains of streptococci were obtained from the first generation of brain tissue tubes from poliomyelitic monkeys.

ism is unable to affect simple sugars, it will also be unable to affect higher ones, the first necessary experiment is to determine the reaction of the organism upon simple sugars.

In our experiments dextrose and lactose only were used. The medium employed is described in the paragraphs upon technique. There are so many buffer salts in an ascitic bouillon medium that the determination of acid production by titration with 0.05 N sodium hydroxide is not satisfactory. Instead, one should use one of the various methods for the determination of the hydrogen ion concentration.

Eleven strains of the globoid bodies were inoculated into the sugar media. Eight of them had been isolated within the past 3 months, and three were stock cultures that had been cultivated in the laboratory for one or more years. Controls were added to the series of two strains of streptococci which, as contaminations, had been isolated from poliomyelitic monkeys during the previous 3 months. Controls also were added of tubes containing ascitic broth plus rabbit kidney tissue and ascitic broth alone. The tubes were incubated in an anaerobic jar for 7 days, at the end of which time there was a good growth in each tube. The results of the experiment are summarized in Table IV. Thus none of the globoid bodies were able to attack the simple sugars, while the two strains of streptococci attacked them vigorously.

Inoculation of Monkeys with Cultures of the Globoid Bodies.

That the inoculation of pure cultures of the globoid bodies, even in a remote generation, will sometimes produce infection in monkeys, attended by the symptoms and specific lesions of experimental poliomyelitis, has been shown by the reports of Flexner and Noguchi (1), and Flexner, Noguchi, and Amoss (10). On the other hand, their experiments indicate that it is exceptional for the cultivated globoid bodies to exhibit definite pathogenic properties. However, the observation was made that in some animals in which a single inoculation failed to cause any symptoms, a subsequent one was followed by typical paralysis attended by the specific lesions in the central nervous organs of the infected monkeys.

The question naturally arose whether any of the cultures of the globoid bodies isolated by me possessed pathogenic properties. Eight different strains were inoculated into *Macacus rhesus* monkeys. In some instances a single injection, in others several injections were given. Three of the inoculated animals developed some degree of paralysis following intracranial or intraspinal inoculation. In one instance the culture was in the fourth, in the remaining two in the fifth generation. The other five animals did not develop suspicious symptoms. A brief recapitulation of the protocols of the three monkeys showing paralysis is given, followed by a discussion of the significance of the experiments.

Experiment C.—Macacus rhesus. May 19. Left intracerebral inoculation of 2 cc. of an ascitic fluid culture M.A. in the fifth generation of the globoid bodies derived from the brain of a monkey. May 25. Animal had a convulsion, following which it was ataxic and dazed. Lumbar puncture yielded a turbid fluid under pressure containing 1,400 cells, chiefly lymphocytes, and + + globulin with 0.1 cc. May 26. Left arm protected; slight left facial palsy; ataxia. May 28. Facial paralysis marked. Following lumbar puncture brief convulsion. Cerebrospinal fluid clearing; excess of lymphocytes. May 31. Condition improved; probably will recover; etherized for cultures and histology. The autopsy revealed a cyst at the point of inoculation, containing yellow fluid. A filtrate of the local site and an emulsion of the spinal cord and medulla were inoculated into two *rhesus* monkeys, respectively; neither developed symptoms.

Cultures were prepared from the brain, kidney, liver, spleen, mesenteric node, and adrenals. All tubes were carried through three generations without contamination. The globoid bodies were isolated from one tube only, brain tube No. 2.

Sections were prepared from the medulla, cervical and lumbar regions of the spinal cord, and intervertebral ganglia. In none were any lesions characteristic of poliomyelitis found.

The globoid bodies employed for inoculation were derived from the M.A. virus, which, in its present condition, is of low virulence for monkeys. The inoculation gave rise to a local cyst, which rarely results from the intracerebral inoculations, attended by a distinct left facial palsy and weakness (?) of the left arm. It is doubtful whether the symptoms denoted experimental poliomyelitis. Probably the facial palsy was related to the cyst formation at the inoculation site; as definite paralysis did not appear in the arm, the appearance of

weakness may have been deceptive. The complete absence of histological lesions is inconsistent with the production of typical experimental poliomyelitis; although since the only developed paralysis was facial, it is always possible that exhaustive microscopic study might have revealed poliomyelitic lesions in the nucleus of the facial nerve. However, the failure of the filtrate and tissue emulsion to transmit the disease to other monkeys also speaks against the condition having been experimental poliomyelitis.

On the other hand, the injection of the globoid bodies gave rise to a marked cellular reaction in the cerebrospinal fluid, in which the predominating cells were lymphocytic. Ordinary bacteria which set up a meningitis usually produce polymorphonuclear cells. But the chief point of importance is the great difficulty and slight success attending the recovery, by cultivation, of the globoid bodies, even from the inoculated nervous tissues. Once these cultivated microorganisms readapt themselves to the condition of growth within the living body, they resist artificial cultivation as do the original tissue parasites—a point noted in Flexner and Noguchi's first communication.

Experiment D.—Macacus rhesus. June 9. Inoculated into the left cerebral hemisphere with 2 cc. of a lightly centrifuged unwashed sediment of a 5 day old mass culture of the globoid bodies isolated from the spleen of a monkey. The strain of the globoid bodies had passed through five generations and been under cultivation for 55 days. This animal developed convulsions, ataxia, and weakness of arms and legs. A lumbar puncture performed on June 14 yielded fluid not under pressure, containing ++ globulin 0.1 cc. and 110 mononuclear cells per c. mm. June 24. The symptoms had all diminished. The animal was given 2 cc. of a culture of the globoid bodies from the spleen intraspinally and 4 cc. intraperitoneally. The only result of this injection was to bring about a temporary increase of the ataxia. Recovery finally became complete.

While a definite reaction was obtained in this instance, there is doubt whether the symptoms really indicated the production of experimental poliomyelitis.

Experiment E.—Macacus rhesus. June 2. An intraspinal injection was made of 2 cc. of a mass M.A. culture in the fourth generation. June 7. The animal was somewhat ataxic and excitable, and the right arm was protected. June 18. The animal was distinctly weak and tended to fall to the left side. Etherized. The autopsy revealed a generalized tuberculosis of the viscera. A few tubercles were detected in the meninges.

The histological examination of the spinal cord, medulla, brain, and intervertebral ganglia revealed no poliomyelitic lesions. A miliary tubercle was present in the intima of a small vein in one of the ganglia.

In other words, aside from the symptoms suggestive of experimental poliomyelitis, no pathologic basis for the diagnosis could be obtained. The experiment should be regarded as negative.

If we review the results of the inoculation of monkeys with cultures of the globoid bodies, we must conclude that, while certain symptoms suggestive of poliomyelitis were sometimes produced, in no instance was the experimental disease, as determined by the presence of typical lesions in the nervous organs, actually set up. Our experiments confirm, therefore, the conclusion arrived at by Flexner and Noguchi, that it is the very exceptional cultures only which retain pathogenic power sufficient to cause infection in monkeys.

DISCUSSION.

The presentation of this work on the cultivation of the globoid bodies may be considered from several points of view. Perhaps the first point that should be discussed is that relating to the ultimate results achieved. The experiments show clearly, I think, that if the experimenter has suitable poliomyelitic tissues to work on and suitable samples of ascitic fluid, and if the inoculated tubes are kept under strict anaerobic conditions, and transferred at proper intervals, successful results, in some degree, will almost surely follow. In fact, the decision arrived at was to the effect that it may be possible to cultivate the globoid bodies from practically all cases from which suitable material is available. If, for example, the ascitic fluid available during the period of May 15 to June 1 had been suitable, the globoid bodies present in the fluid medium could probably have been grown in the semisolid medium. Because of this failure, I can report the cultures merely as suggestive instead of as positive. Similarly, in other experiments (Nos. 3 and 4, Table II), it is highly probable that growth in the semisolid medium would have taken place had the hydrogen-nitrogen jar been employed instead of the vacuum jar.

However, the introduction of the hydrogen-nitrogen jar has not removed the chief drawbacks of the method; namely, the personal factor of painstaking care and perseverance. At best the methods

are long, tedious, often discouraging, requiring now and again months of time, abundance of suitable material, and an exact technique in order to succeed even in one experiment. I do not consider that I have modified fundamentally the original Noguchi technique. I have devised an alternative method which seems to possess certain advantages. And yet I never succeeded in identifying the globoid bodies in the first generation, although the original investigators did so.

The chief difficulty encountered is the establishment of the strain. It would appear either that the more pathogenic of the organisms do not develop in the artificial cultures, or that when they develop they do so at the expense, as a rule, of their power to produce infection. In this respect they may be said to resemble *Treponema pallidum*, with which they share so many cultural requirements and immunological reactions, as has previously been pointed out by others (1, 11).

After having once become established and accustomed to the artificial media, the globoid bodies grow more readily, may be more easily transferred, and will survive at refrigerator temperature for months. There are certain limitations, however, beyond which they will not go. Body fluids, preferably ascitic fluid, are required for their development, and strict anaerobiosis is also essential. Furthermore, the reaction of the culture media must be at approximately blood neutrality, and indicators, such as litmus, neutral red, Andrade's, etc., inhibit their growth.

The results obtained from the cultures of organs other than nervous tissues were unsatisfactory. The great obstacles to success with them appear to be contaminating organisms, particularly the streptococcus. This common microorganism appears to be more frequently present in the general viscera than in the central nervous organs; and the cultures prepared from the kidney and liver are the ones most often developing streptococci. Apparently also the globoid bodies are present less constantly or in smaller numbers in the general viscera. Finally, despite the exsanguination of the animal, the visceral tissues always contain a certain amount of blood, which probably interferes with the development of the globoid bodies in the initial culture tubes.

Despite these adverse factors, the successful cultivation of the organ-

ism from the spleen shows that it is contained outside the nervous and in the lymphatic visceral organs. Moreover, in four other instances, the globoid bodies were cultivated from the visceral tissues in the fluid medium, although they could not be developed in the semisolid medium. These strains included one from the adrenal of the animal yielding the spleen culture, another spleen culture, one from the kidney, and one from the mesenteric lymph node of two other monkeys. The experiments are the first reported in which the globoid bodies have been cultivated from tissues other than those of the central nervous system.

The inoculation of monkeys with the cultures should be regarded as having failed to produce the experimental poliomyelitis. The circumstances surrounding the failures are in themselves instructive. Had the deductions been based merely on the clinical symptoms, they would have pointed to the induction of the infection. The ultimate criteria of experimental poliomyelitis are (1) the typical histological lesions and (2) recommunicability of the disease by inoculation of the nervous tissues of the suspected case. Since neither of these could be satisfied in the experiments, I regarded them as negative.

CONCLUSIONS.

The globoid bodies, identical in morphological and cultural characteristics with the organisms described by Flexner and Noguchi, have been obtained in twenty-two cultures from the tissues of seven monkeys suffering from experimental poliomyelitis.

Twenty of the strains were cultivated from the central nervous organs, all being obtained from the cerebrum except one, which was cultivated from the cervical portion of the spinal cord.

Two strains were cultivated from the spleen.

None of the cultivated strains inoculated produced typical poliomyelitis in monkeys.

The recovery of a strain of the globoid bodies from the inoculated monkey is as difficult as is the original cultivation of the organisms from animals inoculated with the ordinary virus of poliomyelitis.

Nothing in this study has served to implicate the streptococcus in the pathology of the poliomyelitic process; the streptococcus is, how-

ever, encountered as a common contaminant or secondary invader, especially in animals which have been etherized while moribund, or which had died some hours previous to the autopsy. When the infected and paralyzed animals are killed while still strong, secondary invading bacteria, including the streptococcus, tend to be absent from the tissues.

A modified, perhaps improved, but alternative method has been devised for the cultivation of the globoid bodies and other microorganisms demanding a high degree of anaerobiosis.

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