REPORT OF EXPERIMENTAL WORK ON THE DILUTION METHOD OF IMMUNIZATION FROM RABIES.

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As is well known, the dried-cord method of immunizing human beings from the active principle of rabies was first worked out and used by Pasteur. Since his death (and even for a time before) little progress has been made in elucidating the still obscure pathology of the disease or toward the discovery of the special germ causing it. The dried-cord method of immunization is the one used in Paris and in other places where the treatment has been introduced and applied to human beings.

In examining the literature of the subject I was much interested in the investigations of Högyes* in Budapest, by whom a dilution method was employed in a series of experiments for the production of immunization from rabies. His hypothesis is that, contrary to the teaching of Pasteur and others, the dried cord contains a dilution pure and simple and not merely an attenuated virus. Therefore, if this supposition be true, a fresh dilution made, under aseptic precautions, from the medulla of a rabbit dead from rabies, would be more exact, require less time, and be less liable to produce infection by accidental contamination from extraneous organisms which in the older method might develop during the drying process. In Pasteur's method the

* Högyes, *Acad. des Sciences de Buda-Pest*, Oct. 17, 1887. *Centralbl. f. Bakter.*, 1887, ii, 579. Abstract with critical remarks by Roux in *Annales de l'Institut Pasteur*, 1888, ii, 94.

Pest. med.-chir. Presse, 1887, xxiii, 929.

Ann. de l'Inst. Pasteur, 1889, iii, 449.

Die experimentelle Basis der antirabischen Schutzimpfungen Pasteurs, etc., Stuttgart, 1889.

Trans. Seventh Internat. Congr. Hyg. and Demog., 1891, London, 1892, iii, 30.

Article "Lyssa," in Nothnagel's Spec. Path. u. Therap., v, 5. Th. 2. Abth. Wien., 1897.

cords are kept from three to fourteen days in glass jars or bottles at a temperature of 68° to 72° F. in dry air, and then emulsified before use. Emulsifying a cord is a long, tedious process, and considerable exposure of the material is necessary.

The dilution method does not rest upon a sufficiently satisfactory experimental basis and has not been developed enough to have secured its general adoption in the treatment of supposed infection by rabies in human beings. This method as used in Budapest is carried out in the following manner: A piece of the medulla weighing one gramme is taken from a rabbit just dead with rabies. To this is added 10 grammes of sterile broth and then with a glass rod it is beaten into an emulsion. This is the stock solution from which the dilutions are made, and is of the strength of one part of fresh medulla to ten parts of broth. The dilution used first is 1-10,000, then on successive days 1-8000, 1-6000, 1-5000, 1-2000, 1-1000, 1-500, 1-250, 1-200, 1-100, and finally the full strength (1-10) is given. The quantity of each solution injected subcutaneously is usually the same. The dilutions 1-10,000 to 1-6000 are supposed to correspond in strength to a cord dried by the Pasteur method from 14 to 8 days. Subdurally injected, they produce rabies only exceptionally; the dilution 1-5000 inoculated subdurally does not kill all rabbits and those killed succumb only after a protracted period of incubation; the dilutions 1-2000, 1-1000, 1-500, 1-250, always kill and after gradually lessening periods of incubation. The dilutions 1-100 and 1-10 act as quickly as the very thick emulsion made from the medulla used to produce rabies.

The advantages claimed for this method over the dried-cord method of Pasteur are, chiefly, much greater accuracy in giving a gradually increasing dose, and, secondarily, less chance of infection after removal of the cord from the body, as the stock solutions are made fresh each day and the dilutions are made from them, whereas in the Pasteur dried-cord method the cords are exposed to atmospheric changes for from 3 to 14 days before being used.

The work in which I have been engaged consists in modifying the dilution method by keeping one stock solution, from which the dilu-

tions are made daily, throughout the whole course of treatment, instead of making a new stock solution each day as is done in Budapest. The advantages of this procedure over the Budapest method I believe to be the following: (1) Avoidance of the tedious process of making an emulsion daily; (2) ability to test before use the stock solution for extraneous organisms, which may produce serious symptoms; (3) and most important (if this method eventually proves successful), the possibility of sending out from one centre, where the material is prepared, the stock solution, with directions regarding dilutions and doses. The physician in charge can then treat cases which perhaps are not able to travel a long way from home for treatment.

TECHNIQUE EMPLOYED IN PREPARING THE EMULSIONS AND GIVING THE SUBDURAL INOCULATIONS.

As the result of considerable experimental work carried on during the last eight months (the tables of a part of which are given below), I now make and preserve a stock solution in the following way: The brain of a rabbit dead from laboratory rabies (fixed virus) is beaten into an emulsion composed of 8 parts of brain and 80 parts of sterile glycerine and water. The amount of glycerine used is $\frac{1}{5}$ part of the whole emulsion; at first $\frac{2}{3}$ glycerine was employed, then $\frac{1}{2}$, then $\frac{1}{3}$, and finally $\frac{1}{5}$, which last has been entirely satisfactory. As Table I (p. 185) shows, larger amounts of glycerine than $\frac{1}{5}$ part diminish the virulence of the virus, increase the incubation period, and prolong the lives of the animals inoculated. The brain, after being thoroughly beaten up and emulsified by the aid of a glass rod, is then poured into a sterile cheese-cloth bag and filtered, in this way making a smooth mixture with no visible particles. To this emulsion $\frac{1}{5}$ part of glycerine is then added and thoroughly mixed. The mixture is then placed in a sterile flask. During these manipulations every possible precaution is taken to avoid bacterial contamination. The flask is placed in an ice-chest. This, then, is the stock glycerine solution from which all dilutions are made. Before being used, the solution is tested for extraneous germs. Experiments have shown that a stock solution may be kept in this way seven weeks with no diminution in its virulence. As a course of immunization treatment lasts about 2 to 4

weeks, there can be no question of the virulence of the stock solution remaining in full strength for that length of time.

In nearly all subdural operations chloroform was used as an anæsthetic with perhaps 2 deaths in 50 animals. A collodion dressing has been found serviceable as a protection to the wound, and since using it no abscess of the brain has formed, as had previously occurred in a few cases. Instead of holding the animal during subdural inoculations by tying it down on a board by each leg, I have substituted an apparatus which has been used with satisfaction. The reason for the change was that tying a rabbit or a guinea-pig to a board causes the animal to struggle and become frightened and prolongs the operation. The holder which I have devised is a round tin cylinder 10 inches long and 6 inches in diameter. Into one end of this fits a block of wood hollowed out on the inner end to fit the hind part of the animal. This block is pushed into the cylinder, according to the size of the animal. At the other end, fitting around the cylinder, is a movable bag-like arrangement which is provided on the free end with a drawstring. On each side of the bag-like arrangement are straps which fasten to the block of wood by nails. These straps are provided with a number of holes to provide for the size of the animal. The drawstring which fits around the animal's neck, is pulled fairly tight, and the ends are fastened on two nails on each side of the cradle which holds the cylinder in place. In this way the animal is held securely in a telescope-like apparatus. A smaller size is preferable for guinea-pigs.

The virus used in the laboratory came from Dr. Ruhräh of Baltimore, and has been passed through a series of about 180 animals until now the rabbits show beginning paralysis on the 5th to 6th day; marked paralysis on the 8th day; complete paralysis on the 9th day, and death on the 10th day. It has become, in other words, a " fixed virus." In guinea-pigs inoculated with this fresh virus, rabies appears on the 6th day and death on the 7th day after inoculation. These periods of incubation are very constant when this virus is used.

The experiments detailed in Table I were undertaken to test the effect of different proportions of glycerine on the incubation period

of this virus kept for 1-7 weeks in a glycerine solution. At first $\frac{2}{3}$ part of sterile glycerine was added to an emulsion of the fresh virus, then $\frac{1}{2}$ part, then $\frac{1}{3}$, and finally $\frac{1}{5}$. Rabbits and guinea-pigs were the animals used for the experiments.

TABLE I.

EFFECTS OF DIFFERENT PROPORTIONS OF GLYCERINE ON DURATION OF INCUBATION PERIOD.

Proportion of Glycerine.	No. of Experiments.	Kind of animal used.	Date of preparation of virus.	Time solution was kept.	Effect on incubation period.
$\frac{2}{3}$ part.	3	Guinea-pigs.	July 7.	9 days.	Increased 2 days.
$\frac{1}{2}$ part.	3	Guinea-pigs.	July 15.	8 days.	Increased 1 day.
$\frac{1}{3}$ part.	Not sufficient to tabulate.				
$\frac{1}{5}$ part.	1	Guinea-pig.	Oct. 5.	7 days.	Not increased.
$\frac{1}{5}$ part.	1	Rabbit.	Oct. 5.	10 days.	Not increased.
$\frac{1}{5}$ part.	4	Rabbits.	Oct. 13.	7 weeks.	Not increased.
$\frac{1}{5}$ part.	2	Rabbits.	Oct. 25.	5 weeks.	Not increased.

Examination of Table I shows plainly that the proportion of $\frac{1}{5}$ sterile glycerine in the stock solution does not diminish the virulence of the solution during a period of seven weeks, rabies being produced at the end of this time with the same incubation period as that produced by an emulsion freshly made. This is an important point, because it shows that one stock solution may be kept through a whole course of treatment, which never lasts longer than 2 to 4 weeks. Many more experiments were made to test the virulence of solutions kept in this way, and made with dilutions from a stock solution; but the results simply confirmed those given and they need not therefore be repeated.

EXPERIMENTS IN IMMUNIZING ANIMALS FROM RABIES.

The principle of the dilution method is that inoculations of a very small amount of virulent material do not produce rabies, and that the gradual increase of the amounts injected accustom the animal to the most virulent material. As the stock solution is represented by one

part of medulla to 10 parts of sterile water and glycerine, we should in the immunization treatment take a small part of this and dilute it to 1-10,000 for the first treatment, giving part of a cubic centimetre subcutaneously to each animal as a first dose. For the second injection, the dilution is diminished to 1-9000, then to 1-8000, and so on, lessening the dilution each day. In Budapest the injections are begun with a dilution of 1-10,000 and the strength rapidly increased within the period of two weeks to a dilution of 1-10. In the first few series of experiments on immunization of animals I followed the scheme of dilutions and doses as described by the investigators in Budapest; but I now believe that the dilutions should be as follows: 1st day, 1-10,000; then upon successive days 1-9000, 1-8000, 1-7000, 1-6000, 1-5000, 1-4000, 1-3000, 1-2000, 1-1000, 1-900, 1-800, 1-700, 1-600, 1-500, 1-400, 1-300, 1-200, and then this last dilution employed until treatment has been continued 2 to 4 weeks. Any dilution below 1-200 is likely to produce rabies. The dose of these various dilutions should be proportioned to the size of the animal. It should be noted that guinea-pigs are very susceptible to the virus of rabies, apparently much more so than rabbits. After finishing the immunization treatment in each series of animals, a subdural inoculation of fresh virus has been made in nearly all the cases, as a test of immunity. These inoculations have been made in some animals immediately at the end of immunization and in others within 2 weeks of its cessation. A guinea-pig or rabbit subdurally inoculated with fresh laboratory virus invariably dies, unless previously immunized, as has been proven in a large number of experiments. If, therefore, after a subdural inoculation with fresh laboratory virus the animal does not die within 10 days, we may feel sure that immunity has been secured by the treatment given. The subdural inoculation test is the most severe we can employ for testing the immunity of an animal.

Table II shows the results in different series of animals treated by the glycerine dilution method to produce immunity from rabies. In the first experiment, two to three stock solutions were used during the course of the treatment and dilutions made from them. In the last two series, one stock solution has been kept throughout and the

daily dilutions made from it, beginning with a dilution of 1-10,000, and then gradually increasing the strength as described above until 1-200 was reached in the case of the guinea-pigs and 1-100 in the case of rabbits.

TABLE II.

RESULTS OF IMMUNIZING TREATMENT BY DILUTION METHOD.

Number of animals in series.	Duration of treatment.	Highest and lowest dilutions used.	Animals died rabid during treatment.	Died from other causes.	Immune from effects of subdural inoculations after treatment.
A. 4 guinea-pigs	14 days	1–10,000 1–10	None	None.	None.
B. 6 rabbits	4 weeks	1–10,000 1–10	None	5 *	1
C. 9 guinea-pigs	3 weeks	1–10,000 1–10	5	1	1 †
D. 40 guinea-pigs	20, 3 weeks 20, 4 weeks	1–6,000 1–10	20	2 1 lost	17 ‡
E. 6 rabbits	3, 3 weeks 1, 4 weeks	1–10,000 1–100	None	1 injured 1 lost	1
F. 50 guinea-pigs	20, 3½ weeks 30, 4 weeks	1–10,000 1–200	None	3 suddenly 2 injured	20 §

In the final inoculations to test immunity control animals were inoculated in the same way as the treated animals, and all died from rabies in 7 to 10 days. It will readily be seen from Table II that, while immunity can be produced by this method of treatment, in several instances rabies was produced by the treatment. This result, in my opinion, annuls any advantages this method may otherwise have over the dried-cord method of Pasteur.

The foregoing results were obtained during the year 1897 and the investigations were continued during 1898, with both the dilution and the dried-cord methods. I have had good opportunity to test the dried-cord method of Pasteur during the past eleven months.

The Health Department of New York City has adopted the Pas-

* This series was treated during the heat of the summer when unused animals in the laboratory died in large numbers from no apparent cause. Inoculations made from these animals showed no evidence of rabies.

† The other two animals in this series received subcutaneous inoculations with fresh virus, and lived.

‡ All the animals which lived through the treatment were immune from effects of subdural inoculations.

§ 25 animals were not immune from effects of subdural inoculation.

teur antirabic treatment and during the past eleven months I have treated thirteen cases by the Pasteur method, the first six in conjunction with Dr. Robert J. Wilson. Besides the treatment of human beings experiments upon animals have been conducted in order to test the Pasteur method and various modifications of it. The results have been in general confirmatory of those reported from the Pasteur Institute in Paris. In order to make sure of the absence of contamination from the inoculated material we have not only tested the cords before using, but have also tested the emulsion of the cord after it had been prepared and have awaited for 18 to 24 hours the results of the bacteriological test, the emulsions during this period being kept on ice, a procedure which can be followed without any impairment of the virulence of the emulsion, as we have proven experimentally. Besides these precautions, animals can be simultaneously treated with the same material as that given to the persons under treatment. Under these precautions we have observed no local or general disturbance whatever after the injections. In four instances the emulsions of the dried cords made each day were sent by a special messenger out of town packed in ice and were used with perfectly satisfactory results. We have thus secured by the dried-cord method the special advantages claimed for the dilution method without the dangers of the latter.

CONCLUSIONS.

1. I have simplified the dilution method by using a stock glycerine emulsion of the virulent cord, from which the desired dilutions can be readily prepared. The proportion of glycerine should not exceed $\frac{1}{5}$ part, if it is desired to retain the full virulence of the emulsion.

2. There is some danger of giving rabies to animals in the dilution immunization treatment, a danger which is not present in the Pasteur method.

3. The dried-cord method does not rest solely upon the principle of dilution, but is based also upon attenuation of the virus.

4. The Pasteur method being entirely free from the element of danger which pertains to the glycerine dilution method and resting upon a sounder experimental basis is the one to be preferred.