# ENCEPHALOMYELITIS OF MICE

## I. Characteristics and Pathogenesis of the Virus

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The close resemblance, clinically and pathologically, between the virus of human poliomyelitis and that of spontaneous mouse encephalomyelitis (1) has added interest to the mouse disease. It was felt that a thorough understanding of the virus of mouse encephalomyelitis might indicate methods of approach for the study of the human disease. The study of human poliomyelitis was handicapped until recently because there was only one readily available susceptible animal, namely the *rhesus* monkey, which made work with this virus exceedingly expensive. Mice, however, are cheap and can be used in numbers large enough to make the results obtained statistically significant. Furthermore, in mice we are working with a virus in its natural host, and deductions from experimental procedures are consequently of greater significance. Information concerning the virus of human poliomyelitis obtained from experiments on *rhesus* monkeys is of doubtful value as it is highly improbable that the disease in monkeys is an exact image of this disease as it occurs in man.

Apart from the clinical and pathological similarities between human poliomyelitis and mouse encephalomyelitis, there are also certain epidemiological resemblances. Both viruses predominantly attack the young of their respective hosts, and to both there is a resistance developed with age. Evidence of contact infection with both is exceedingly difficult to establish.

Armstrong (2, 3) has recently shown that a species of rodent, the cotton rat, is susceptible to a strain of human poliomyelitis. This strain was also pathogenic for mice on intracerebral inoculation. Work in progress has shown that one of our strains of the mouse encephalomyelitis virus was also pathogenic for the cotton rat. In addition, we found that mice which had been infected with the virus of mouse encephalomyelitis were resistant to a subsequent intracerebral inoculation of Armstrong's Lansing strain of human poliomyelitis virus, suggesting further evidence of a relationship between the two viruses.

### *The Strains of Virus Studied*

On several occasions since the original discovery of the virus of encephalomyelitis of mice (1) similarly affected animals have been found among our stock mice. Virus recovered from these have, in the main, acted much like the strains first isolated. Work with these strains was rendered somewhat difficult and tedious on account of their low virulence. Recently, however, two highly virulent strains of virus have been found.

Both of the new strains were derived from mice becoming ill during experiments with the virus of yellow fever. The first, called GD VII, was obtained from the brain of a mouse inoculated 11 days previously with material suspected of containing yellow fever virus. To determine whether this mouse was suffering from yellow fever encephalitis, six normal mice were inoculated intracerebrally with a suspension of brain prepared from the sick mouse. Four of these mice developed a flaccid paralysis of the hind legs 14 to 16 days after inoculation. Since the clinical picture, as well as the incubation period, was not that characteristic of the strain of yellow fever virus used in the experiment, investigations were undertaken to determine its nature. Neutralization tests with immune yellow fever serum showed clearly that the virus was not that of yellow fever. The disease picture was different in many ways from that seen in mice artificially infected with known strains of the virus of spontaneous mouse encephalomyelitis; not only was the incubation period shorter but also the course of the disease. Most of the mice died within 24 to 48 hours following the onset of symptoms; very few recovered once they had developed signs of infection. Furthermore, the titer of infected brains was high. Following intracerebral inoculation the first sign was sometimes a hyperexcitable condition. Infected mice, as a rule, looked perfectly well. Symptoms referable to lesions of the cord predominated over those referable to lesions of the brain.

The second strain, named the FA strain, was so unusual in clinical manifestations that we at first believed we had discovered a new virus pathogenic for mice. Like the other, this virus was accidentally obtained during an experiment with yellow fever virus. In the serial passage of the French strain of yellow fever virus it was noted that the characteristics of the clinical picture altered suddenly after the 525th serial mouse brain passage. Whereas the average time of death of mice in this series had remained remarkably constant between 4 and 5 days, the average time of death of the 526th passage was more than 7 days. In addition, the deaths were very irregular, and the mice presented an entirely different clinical picture from that seen in mice suffering from yellow fever encephalitis. The outstanding clinical sign was an extreme hyperexcitability. The mice, huddled up with fur ruffled and looking obviously sick, would jump about excitedly at the slightest stimulus. Spasmodic movements of the fore legs were common, the mice sitting on their haunches performing rubbing movements of the face. Tonic convulsions with the hind legs extended and the fore limbs flexed were often observed. During such convulsions respiration might cease for several seconds and the animal appear to be dead. However, respiration often commenced again, at first slowly, gradually increasing in rate. At the same time the muscles would relax and the mouse gradually revive. Death might occur during one of these seizures. Pathological examination of mice infected with this strain of virus showed a marked encephalitis

with very little meningeal reaction. Sick mice might show some weakness of one of the limbs, but frank paralysis such as is seen with other strains of encephalomyelitis was comparatively rare, at least following intracerebral inoculation. As with GD VII, the titer of virus in infected mouse brains was high. By serial passage in mouse brains the incubation period gradually shortened until at present it is from 2 to 3 days after inoculation.

This virus proved to be non-pathogenic for *rhesus* monkeys in both intracerebral and intraperitoneal inoculation. Monkeys did not develop an immunity to a subsequent inoculation with the virus of yellow fever. Furthermore, yellow fever immune serum had no neutralizing action on this virus. These facts conclusively showed that in the serial passage of yellow fever virus a new virus had been picked up and the original yellow fever lost.

Experiments, reported later in this paper, showed that both GD VII and FA were immunologically related to each other and to the virus of mouse encephalomyelitis. By serial passage in mice the incubation period of both these strains gradually decreased. With both, a "fixed" strain was obtained. The average incubation period of both was found to be more constant than the average time of death. One of us (see following paper in this series) was able to devise a method for the estimation of the concentration of virus in a given suspension by the use of the average incubation period. Unless otherwise stated, all quantitative interpretations are based on this method.

## *Properties of the Virus*

*Size.*—The main difficulty encountered in filtration experiments with material prepared from brain or spinal cord is connected with the clarification of the suspension. In our earlier study on the virus of mouse encephalomyelitis (1) we met with the same problem. In the present study the most satisfactory results were obtained with the following simple procedure:—

Infected brains were ground thoroughly with sterile alundum and distilled water was added to make a 10 per cent suspension. This was centrifuged at 2500 R.P.M. for 45 to 60 minutes. The opalescent supernatant was diluted with an equal amount of plain broth and again centrifuged. This second centrifugation brought down a considerable additional sediment, the supernatant being practically water-clear. By the addition of broth and distilled water to the second supernatant the final concentrations were adjusted to 1 per cent brain and 15 per cent broth. This preparation was then passed successively through a Seitz filter, through which broth had been previously passed, and graded collodion membranes of 200 and 100 mμ A.P.D. The loss of activity in these preliminary filtrations usually did not exceed 30 per cent, and a suspension prepared in this way passed all grades of collodion membranes without clogging. For the determination of the filtration end point a series of graded collodion membranes was used. All filtrations were carried out under 20 pounds positive pressure of nitrogen. The filtrates were tested by intracerebral inoculation in mice.

In Table I the results of filtrations of the two strains, GD VII and FA, are shown. Both strains passed membranes of all grades down to 35 mμ

A.P.D. but failed to pass through a membrane of 27 mμ A.P.D. The loss of activity as a result of the filtration increased with decrease in pore size of the membranes. The 40 and 35 mμ membranes retained the virus to such an extent as to render impossible the demonstration of virus in the filtrate unless the strain used had an original titer of more than 1:1000. This fact alone is sufficient to explain the difference between the present and our previous results. According to Elford's (4) formula as applied to the ultrafiltration measurements of viruses, the virus particle equals one-third to one-half of the diameter of the largest retaining pore. Considering 27 mμ as the filtration end point, the particle size of the mouse encephalomyelitis virus would be 9 to 13 mμ.

TABLE I

*Ultrafiltration of Mouse Encephalomyelitis Virus through Graded Collodion Membranes*

| Average pore diameter of membranes | Results in mice inoculated with filtrates | |
|---|---|---|
| | Strain GD VII | Strain FA |
| mμ | | |
| 100 (control) | 6/6* | 6/6* |
| 60 | 6/6 | |
| 50 | 6/6 | 6/6 |
| 40 | 2/6 | 6/6 |
| 35 | 11/18 | 6/6 |
| 27 | 0/30 | 0/6 |

* The numerator represents the number of mice that developed infection; the denominator, the number of mice used in test.

It will be of interest to compare these results with those obtained with the virus of human poliomyelitis. The particle size of human poliomyelitis virus has been measured by means of ultrafiltration by Theiler and Bauer (5) and by Elford, Galloway, and Perdrau (6). Theiler and Bauer found that the virus passed membranes having an average pore diameter of 35 mμ, but was retained by 30 mμ membranes. By erroneously interpreting 35 mμ as the filtration end point, they considered the particle diameter to be 13 to 19 mμ. When their findings are corrected to comply with Elford's interpretation of the filtration end point, the diameter value of the particles becomes 10 to 15 mμ. Elford, Galloway, and Perdrau found that the poliomyelitis virus passed readily 40 mμ membranes, and in one instance a 27 mμ membrane filtrate apparently produced immunity in the test animal. From these findings they concluded that the filtration end point should be considered 25 mμ and, therefore, the virus particle diam-

eter 8 to 12 mμ. On the basis of these comparative measurements, it can be safely concluded that the particle size of the human poliomyelitis virus and of the mouse encephalomyelitis virus is of the same order of magnitude.

*Effect of Temperature.*—The virus of mouse encephalomyelitis is known to be rapidly destroyed at temperatures above 50°C. The determination of the "inactivation temperature" in the absence of pure preparations of virus was considered of minor importance and no attempts in this direction have been made. In a few experiments carried out at ice box temperature, as well as at 37°C., the rate of inactivation was found to be 40 to 80 times greater at the higher temperature.

*Desiccation.*—0.5 cc. amounts of an active brain suspension (GD VII) were frozen at −60°C. and then desiccated at −16°C. in the Bauer and Pickels (7) modification of the Flosdorf-Mudd apparatus. A set of control samples was likewise frozen at −60°C. and then simply kept at −16°C. Immediately after the conclusion of the desiccation the activity of both series of samples was tested. In the controls the titer was found to be $10^{3.4}$ M.I.D. per cc., whereas the desiccated material had a titer of only $10^{1.0}$ M.I.D. per cc. Thus, only 0.4 per cent of the activity was left after the desiccation. This activity, however, remained unchanged after a further 3 weeks of storage in the cold.

*Influence of the pH.*—In order to maintain a constant ionic medium, as well as constant ionic strength, throughout the whole pH range to be studied, we decided to use the phosphate-acetate-glycine buffer of Northrop and De Kruif (8). All test samples consisted of 1.0 cc. of a 10 per cent brain suspension, 2.5 cc. of buffer, and 6.5 cc. of N/10 NaCl solution. The experiments were carried out at 37°C. under aerobic conditions.

In a number of experiments the effect of different H-ion concentrations within the range from pH 2.7 to 8.75 was tested. The activity was determined at the beginning of the experiment and again after 6 to 24 hours. From these data the rate of inactivation was calculated on the assumption that it had remained constant throughout the observation period. The results of these experiments are shown in Chart 1, giving the pH inactivation curve for the FA strain. The actual course of the curve in the range below pH 5.5 can only be roughly estimated as the 6-hour experiments in the range from 2.7 to 4.1 (recorded separately in the chart) were carried out under conditions that make a direct comparison impossible. However, there exist obviously two different optima of stability, one in the vicinity of pH 8, the other at about pH 3.3.

The intermediate stability minimum coincides with the isoelectric point

of the brain proteins as shown by the flocculation curve at the bottom of the chart. There is reason to believe that a connection between the two phenomena exists. In one experiment an infective brain suspension was flocculated at pH 4.5. The precipitate and the clear supernatant were tested separately for virus activity in mice. About 90 per cent of the total activity was found in the precipitate. In the stability tests every

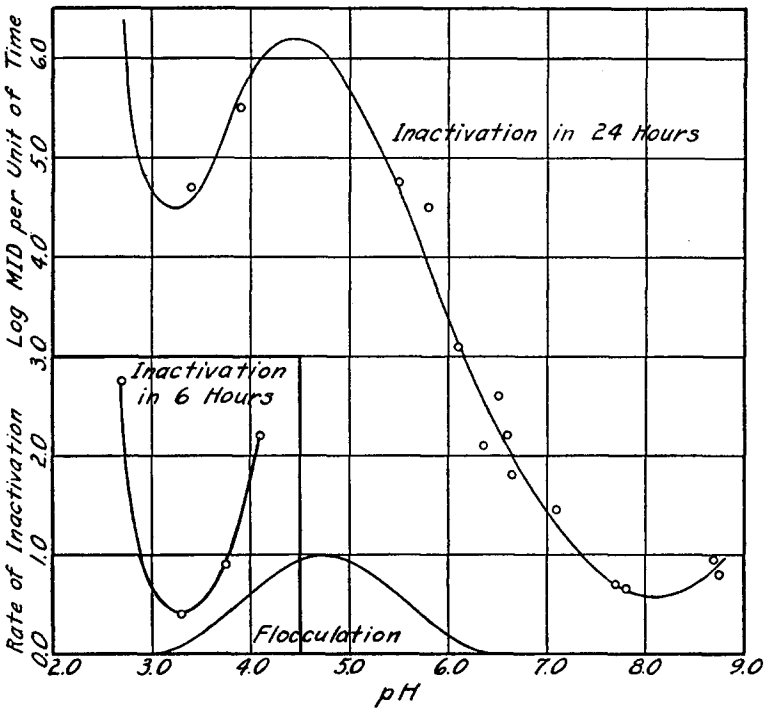


CHART 1. Inactivation of mouse encephalomyelitis virus at different H-ion concentrations.

precaution was taken to obtain an even distribution of the floccules when the test samples were drawn, and we feel that the differences observed could not be explained as due merely to inhomogeneous distribution of the virus. However, no attempt at an explanation can be made at present.

*Oxidation.*—Exposure to the air seems to have little influence on the stability of the virus. In one experiment, for instance, where the rate of inactivation under anaerobic and aerobic conditions was determined over a period of 5 days it was found to be 0.36 and 0.37 log M.I.D. per day, respectively, the difference observed being well within the limits of error of the methods used.

The virus is, however, sensitive to the action of oxygen in the nascent state. Exposure at 37°C. for 2 hours to 0.01 per cent $H_2O_2$ had no demonstrable effect, whereas 0.1 per cent caused a significant decrease in activity, and after treatment with 1 per cent $H_2O_2$ almost complete inactivation had occurred. When the experiment was carried out at ice box temperature, even 1 per cent $H_2O_2$ failed to give a demonstrable effect.

*Organic Solvents.*—After being shaken with ether, an aqueous brain suspension separates into three distinct layers, an etherous clear top layer, an intermediate disk of water and other insoluble material, and an aqueous bottom layer. All of the original activity is present in the bottom layer. Treatment with ether seems to have no effect at all on the virus. A brain suspension was kept with an equal volume of ether in the ice box and tested at intervals during a period of 100 days. No apparent loss of activity could be demonstrated.

Ethyl alcohol has a definite inactivating effect.

To a series of five samples of active brain suspension alcohol was added to make a final concentration of 5, 7.5, 10, 15, and 20 per cent, respectively. A precipitate was formed in all samples, gradually increasing in volume with increasing concentration of alcohol. After 45 minutes in the ice box the samples were centrifuged and supernatant and sediment tested separately for presence of virus. The amount of virus recovered from the precipitate was only about 1 per cent of the original virus content in all samples. In the supernatant the loss of activity was proportional to the concentration of alcohol. Thus, in samples containing 5 per cent of alcohol, only 10 per cent of the virus was inactivated, whereas in those with 20 per cent of alcohol, the loss of virus activity exceeded 95 per cent.

The addition of acetone to an aqueous suspension of mouse brain resulted in a precipitate formation when the concentration of the acetone reached about 25 per cent, and the virus was found to be present in both the precipitate and the supernatant. However, when the concentration of the acetone was increased proportionately, more virus appeared in the precipitate. When the concentration of the acetone reached 50 per cent, much of the virus became inactivated. Inasmuch as the acetone precipitation is to a certain extent a desiccation process, the virus inactivation can probably be explained on this basis.

*Precipitation with Ammonium Sulfate.*—To a crude brain suspension one volume of saturated ammonium sulfate solution was added. A heavy flocculation occurred. The precipitate was collected, extracted twice with distilled water, and the insoluble residue discarded. The original supernatant was dialyzed against distilled water. Activity tests were made on the original brain suspension, the supernatant after precipitation,

and the extract of the precipitate. There was no demonstrable difference in activity between the original suspension and the extract. 0.1 per cent of the virus was found in the supernatant.

### *Routes of Inoculation*

In the original description of the virus of mouse encephalomyelitis it was found that mice could be infected most readily by intracerebral inoculation, less easily by the intranasal route, and not at all by intraperitoneal inoculation. Suggestive evidence was obtained that successful intranasal inoculation depended on the virulence and quantity of virus administered. Furthermore, it had been found that mice after an intranasal instillation

TABLE II

*Results of Intraperitoneal and Intranasal Inoculation of FA Strain in Mice*

| Route of inoculation | Amount of virus injected | | No. of mice | Morbidity rate | Mortality rate | Average incubation period |
|---|---|---|---|---|---|---|
| | *cc.* | *dilution* | | *per cent* | *per cent* | *days* |
| Intraperitoneal | 0.5 | $10^{-1}$ | 37 | 70 | 49 | 9.4 |
| " | 0.5 | $10^{-2}$ | 36 | 17 | 11 | 10.3 |
| " | 0.5 | $10^{-3}$ | 38 | 5.5 | 2.8 | 10.0 |
| Intranasal | 0.05 | $10^{-1}$ | 40 | 67.5 | 62.5 | 15.5 |
| " | 0.05 | $10^{-2}$ | 41 | 59 | 59 | 14.7 |
| " | 0.05 | $10^{-3}$ | 36 | 11 | 11 | 15.0 |

of virus, although they remained perfectly well, developed a relative resistance to a subsequent intracerebral inoculation. Work designed to elucidate the factors involved in this phenomenon was rendered extremely difficult on account of the low virulence of virus of the strains available at the time. Consequently, on the discovery of two strains of virus, both of which were highly virulent and therefore could be used in higher concentration, the study of the effects of the various routes of inoculation was undertaken using chiefly the FA strain of virus.

Three groups of mice 6 weeks of age were inoculated intraperitoneally with 0.5 cc. of three decimal dilutions of an active brain suspension. Three more groups were inoculated by intranasal instillation of 0.05 cc. of the same material. The results are summarized in Table II. It will be observed that by both routes of inoculation, mice became infected.

*Attack Rate.*—Approximately $0.3 \times 10^{-1}$ gm. of wet infected brain given intraperitoneally, and $0.03 \times 10^{-2}$ gm. when given by the intranasal route,

resulted in the 50 per cent attack rate end point of Reed and Muench (9). As the end point by intracerebral inoculation was usually about $0.03 \times 10^{-7}$ gm., the susceptibility of the mouse to the infection by this route was about 100,000 times as high as by the intranasal and as much as 10 million times as high as by the intraperitoneal route.

*Incubation Period.*—In contrast to conditions after intracerebral inoculation, the amount of virus introduced by the intraperitoneal or intranasal route seemed to have no influence whatever on the length of the incubation. Furthermore, the incubation time depended upon the mode of inoculation. The difference in this respect between the intranasal and the intraperitoneal routes amounted to $15.2 \pm 0.41 - 9.6 \pm 0.62 = 5.6 \pm 0.75$ days and was statistically significant.

*Clinical Picture.*—The disease following intranasal instillation of the FA strain had in all essential points the clinical characteristics of the encephalitis observed after intracerebral inoculation. Most of the mice died in that state, but the few survivors usually later developed paralysis. After intraperitoneal inoculation the clinical picture was quite different. Practically all mice becoming sick developed a flaccid paralysis of the limbs and only occasionally showed signs of encephalitis. The mortality was definitely lower. This was not due to a change in the properties of the virus, as intracerebral inoculation of material derived from such paralyzed mice consistently caused a disease with a clinical picture typical of the FA strain.

The observations mentioned above indicated that after intraperitoneal inoculation the virus did not, as has been suggested in the case of experimental poliomyelitis in monkeys, enter the circulation and after penetration through the nasal mucosa reach the central nervous system by the olfactory pathway.

*Distribution of the Virus after Intracerebral Inoculation.*—

Twelve mice were inoculated intracerebrally with 0.03 cc. of a 0.1 per cent active brain suspension (strain FA). Each day one of the mice was killed and the virus content of different organs was determined, namely, brain, spinal cord, pool of lungs and heart, pool of liver, spleen, kidneys, and suprarenals, stomach with contents, gut with contents. To avoid contamination from the central nervous system, all the viscera were removed before the vertebral canal and the skull were opened. Table III shows the results of the titrations.

On the 1st day after inoculation a large amount of virus was found in the intestines, probably originating from leakage at the site of inoculation and being subsequently licked off and swallowed by the mouse. On several

occasions there was isolated from the gut virus distinctly different from the FA strain and obviously having the characteristics of the native fecal virus to be described in an accompanying paper. Apart from this there were no significant differences in the concentration of FA virus in the various thoracic and abdominal viscera tested. In Table III, therefore, the column "Viscera" contains the averages of these figures.

Theiler (1) was unable to demonstrate the virus in the blood either during the pre- or the postparalytic stage. Neither could he find it in the

TABLE III

*Concentration of Virus in Terms of the Logarithm of Minimum Infective Dose (M.I.D.) per 0.03 Gm. of Various Organs at Different Time Intervals after Intracerebral Inoculation with the FA Strain of Virus*

| Interval after inoculation | Log M.I.D. per 0.03 gm. in following organs | | |
|---|---|---|---|
| | Brain | Cord | Viscera |
| *days* | | | |
| 0 | 1.43 | — | — |
| 1 | 2.25 | 0.15 | <0 |
| 2 | 4.95 | 0.4 | 0.1 |
| 3 | 3.85 | 3.80 | <0 |
| 4 | 5.65 | 2.2 | 0.35 |
| 5 | 5.65 | 2.25 | 0.5 |
| 6 | 6.35 | 3.2 | 0.85 |
| 7 | 6.05 | 4.4 | 0.9 |
| 8 | 6.1 | 4.8 | 0.75 |
| 9 | 5.6 | 4.6 | 0.3 |
| 10 | 4.6 | 4.3 | 0.25 |

abdominal viscera. The sciatic nerve, however, proved infective on one occasion.

Our present study on the FA strain showed that virus regularly appeared in the viscera at the time when its concentration in the central nervous system was at its maximum. The discrepancy could be explained entirely by the difference in virulence between the strains used. It is more than likely that the presence of such small amounts of virus as we have just shown could not have been demonstrated unless the virulence were high.

### *Distribution and Persistence of the FA Strain in Infected Mice*

#### *Spread of Virus Following Intranasal Inoculation.—*

To determine the spread of virus, twenty-four mice were inoculated intranasally with 0.05 cc. of a 20 per cent infective brain suspension. Another lot of mice,

similarly treated, was kept as a control. At intervals three animals were killed. After the skin had been removed from the head, the nose was cut about 2 mm. behind the nostrils, and the lateral and frontal walls of the nasal cavity, together with the septum, were removed. The spinal cord was then taken out and finally the brain and olfactory bulbs were removed separately. Organs from the three animals were pooled, ground in a mortar with alundum, and emulsified with distilled water. The activity of the suspensions was tested by intracerebral inoculation of mice. The attack rate among the controls was 100 per cent with an average incubation time of 12.2 days. In Table IV are shown the results of the activity tests. The concentration of nasal mucosa in the different suspensions could, of course, only be roughly estimated, and the figures on the activity were, therefore, perhaps not directly comparable to the rest.

TABLE IV

*Concentration of Virus in Terms of the Logarithm of Minimum Infective Dose (M.I.D.) per 0.03 Gm. of Various Organs at Different Time Intervals after Intranasal Inoculation with the FA Strain of Virus*

| Interval after inoculation | Log M.I.D. per 0.03 gm. in following organs | | | |
|---|---|---|---|---|
| | Nasal mucosa | Olfactory bulb | Brain | Cord |
| *days* | | | | |
| 1 | 5.1 | 1.3 | <0 | |
| 3 | 3.0 | 3.1 | <0 | |
| 5 | 4.5 | 4.0 | 1.9 | <0 |
| 7 | 3.5 | 4.4 | 3.3 | <0 |
| 11 | 2.0 | 4.5 | 6.1 | 4.9 |
| 14 | <0 | 3.4 | 5.0 | 3.7 |

The following points about the distribution of virus after intranasal inoculation may be mentioned:—

*Nasal Mucosa.*—Virus was consistently present in the mucosa, although in gradually decreasing amount, up to the 11th day but could not be demonstrated on the 14th day.

*Olfactory Bulb.*—Even after 1 day virus was shown to be present in the olfactory bulbs. The maximum concentration was reached about the 5th day and remained at a comparatively low level until the 11th day, after which a decrease in titer was observed.

*Brain.*—Virus could not be demonstrated in the brain until the 5th day. The concentration then rapidly increased to reach a maximum on the 11th day, simultaneously with the onset of symptoms, then began to decrease again.

*Cord.*—Virus could not be demonstrated on the 5th and 7th days. However, on the 11th day the maximum concentration, less than 1/10 of that

in the brain, was recorded. The decrease from then on was at about the same rate as in the brain.

*Spread of Virus Following Intraperitoneal Inoculation.*—This experiment was carried out along the same general lines as the previous one. The attack rate among the control animals was 37.5 per cent and the average incubation time 8.8 days. Organs tested were: brain, spinal cord, and abdominal viscera. The results are shown in Table V.

The virus content in the viscera was at the beginning rather high, decreased gradually, and had on the 8th day reached the same level as after intracerebral inoculation. Virus was demonstrated both in the brain and in the cord on the 2nd day. The concentration reached a maximum be-

TABLE V

*Concentration of Virus in Terms of the Logarithm of Minimum Infective Dose (M.I.D.) per 0.03 Gm. of Various Organs at Different Time Intervals after Intraperitoneal Inoculation with the FA Strain of Virus*

| Interval after inoculation | Log M.I.D. per 0.03 gm. in following organs | | |
|---|---|---|---|
| | Brain | Cord | Abdominal viscera |
| *days* | | | |
| 1 | — | — | 4.47 |
| 2 | 0.75 | 0.9 | 2.2 |
| 4 | 1.5 | <0 | <0 |
| 6 | 5.3 | 4.9 | 1.1 |
| 8 | 5.7 | 6.0 | 0.55 |
| 13 | 2.3 | 2.4 | <0 |

tween the 6th and 8th days, and then decreased rapidly. The concentration in the cord was generally slightly higher than in the brain. The onset of symptoms coincided with maximal virus concentration.

The findings concerning the spread of virus following different routes of inoculation may be summarized as follows: The FA strain of virus has specific affinity for certain cortical and subcortical centers, as well as for motor centers in the spinal cord. When introduced into these centers the virus rapidly multiplies. After its concentration has reached a certain level, disintegration of cellular elements and dysfunction occur. Multiplication then comes to a standstill and a destruction of virus follows. No evidence of an excretion of virus from the central nervous system has been obtained. When it multiplies in one part of the central nervous system and another center becomes infected later, the development of the infection in this second place seems to be somewhat inhibited. Thus, after intra-

cerebral inoculation the rate of multiplication of the virus is considerably lower in the spinal cord than in the brain. On the other hand, after intraperitoneal inoculation when the virus seems to appear almost simultaneously in the brain and the cord, no such retardation is observed.

### *Immunity*

*Influence of Age.*—In attempting to assess the immunity produced by some experimental procedure the resistance developing with age had to be kept in mind. When two groups of normal mice of the same strain but differing in age were inoculated intracerebrally with a relatively avirulent

TABLE VI

*Influence of Age on the Susceptibility of Mice from Different Sources to Intracerebral Inoculation with GD VII Strain*

| Designation of source of mice | Age of mice at time of inoculation | No. inoculated | Average incubation period | No. of mice died |
|---|---|---|---|---|
| | *wks.* | | *days* | |
| C | 13 | 18 | 12.6 | 17 |
| C | 9 | 16 | 11.5 | 16 |
| C | 4 | 38 | 9.3 | 38 |
| CFG | 8 | 15 | 10.5 | 14 |
| CFG | 6 | 24 | 10.2 | 24 |
| CFG | 4 | 24 | 9.5 | 24 |
| CF 1 | 8 | 23 | 9.4 | 22 |
| CF 1 | 7 | 25 | 9.4 | 25 |
| CF 1 | 4 | 24 | 8.25 | 24 |

virus, the difference in the resulting morbidity and mortality was often striking. The resistance to experimental infection was developed rapidly in the younger age groups, *i.e.*, from birth up to 6 or 7 weeks of age, but more slowly in the older ones. The difference in morbidity between two groups of adult mice was often insignificant, but invariably the older mice showed more resistance than the younger.

Using the two strains of most highly virulent virus available, a test was carried out with each to determine the influence of age of mice on the disease picture following experimental inoculation. In the first of these experiments nine groups of Swiss mice supplied by three different dealers were inoculated intracerebrally with a 1:10 dilution of the GD VII strain of virus. Three different age groups were represented in each lot of mice. The results are shown in Table VI. In each instance the youngest mice

had the shortest average incubation period. The only survivors, three in all, were in the oldest age group of each breed of mice. A similar experiment to determine the influence of age on the experimental disease was done with the FA strain of virus, and the results were essentially the same as in the first experiment with GD VII strain.

The results of both of these experiments with highly virulent strains of virus thus confirmed the earlier findings with relatively avirulent strains. In all experiments the resistance had been tested by the intracerebral inoculation of virus, the most severe test available in that the virus is brought into direct contact with the nervous system. It seemed of interest to determine whether the same phenomenon could be shown using the intranasal route of inoculation. This was tested with FA strain of virus, which is the most virulent of all our strains when inoculated by the nasal route.

Two groups of mice, A and B, 6 and 12 weeks of age, respectively, were given an intranasal inoculation of 1 per cent suspension of FA virus. Of the 50 mice in the younger group A, twenty-eight died and twenty-two remained well, whereas of the forty-eight mice in the older group B, only six died and forty-two remained well. The increased resistance developing with age demonstrable by the intracerebral inoculation of virus was also clearly shown by the intranasal route of inoculation.

*Immunity Produced by Intraperitoneal and Intranasal Inoculation of Virus.*—In attempting to find methods for determining whether or not the recently isolated strains GD VII and FA were related to the virus of mouse encephalomyelitis, the effect of inoculating mice by various routes with these two strains was determined. Although it was known that mice which had survived following the intracerebral inoculation of strain I were immune to a subsequent intracerebral inoculation of GD VII and FA, this was not accepted as unequivocal evidence of immunological similarity. Such an effect can quite easily be explained by the interference phenomenon for it is known that mice surviving an intracerebral inoculation of virus, as a rule, harbor the virus in their central nervous systems for long periods of time.

In the first experiment an attempt was made to determine whether an intranasal or intraperitoneal inoculation of the GD VII strain of virus resulted in immunity to a subsequent intracerebral inoculation of strain I.

A group of twenty-five mice was given an intraperitoneal injection of 0.4 cc. of a 10 per cent suspension of mouse brain infected with the GD VII strain. A second group of twenty-nine mice received an intranasal instillation of 0.05 cc. of the same virus sus-

pension. Ten mice were inoculated intracerebrally as virus controls. A fourth group consisting of twenty-nine mice of the same strain and age was kept uninoculated as controls for the age resistance factors. The results are shown in Table VII. 24 days after the original inoculation the immunity of all the surviving mice was tested by the intracerebral inoculation of strain I. The virus suspension used for the immunity test was prepared from infected mouse brains representing the 83rd consecutive serial mouse brain passage.

The results of this experiment illustrated several points. First, following intraperitoneal inoculation five of the twenty-five inoculated mice developed infection of the central nervous system and seven died. Of the eighteen survivors sixteen, or 89 per cent, remained perfectly well

TABLE VII

*Cross Immunity Test with Two Strains of Mouse Encephalomyelitis Virus**

| Immunizing inoculations with GD VII strain | | | | Immunity test with strain I given intracerebrally | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Route of inoculation | No. of mice inoculated | No. of mice paralyzed | No. of mice died | No. of mice in immunity test | No. paralyzed | No. died | Survivors | | | | | |
| | | | | | | | Total | | Paralyzed | | Well | |
| | | | | | | | No. | Per cent | No. | Per cent | No. | Per cent |
| Intraperitoneal | 25 | 5 | 7 | 18 | 2 | 1 | 17 | 94.5 | 1 | 5.5 | 16 | 89 |
| Intranasal | 29 | 4 | 4 | 23 | 9 | 6 | 17 | 74 | 4 | 17 | 13 | 56 |
| Uninoculated controls | 29 | 0 | 0 | 29 | 26 | 20 | 9 | 31 | 6 | 21 | 3 | 10 |

* The immunizing injections were given by the intraperitoneal and intranasal routes, and the mice were tested for immunity by intracerebral inoculation with a heterologous strain.

following the immunity test with the heterologous strain I, whereas of the twenty-nine normal controls only three, or 10 per cent, remained well. Such finding following intraperitoneal inoculation was quite at variance with our previous results from which we had concluded that no infection of the central nervous system followed this route of inoculation nor was any immunity developed as a result of such inoculation. These differing results, however, were probably due to the much higher titer obtainable with the more virulent strain GD VII, and consequently mice were inoculated with larger amounts of virus.

This experiment likewise confirmed the original observation that mice which remained well following an intranasal instillation of virus developed a relative immunity to a subsequent intracerebral inoculation of virus. Thus, of twenty-three mice which were alive and well following the intra-

nasal inoculation of GD VII and whose immunity was tested by the intracerebral inoculation of strain I (Theiler, 1), thirteen, or 56 per cent, remained perfectly well. This experiment also showed that the highly virulent strain GD VII was immunologically related to strain I, the first strain of the virus of mouse encephalomyelitis studied.

In order to ascertain whether the encephalitogenic FA strain of virus was immunologically related to the more typical strains of mouse encephalomyelitis virus, it was necessary first to determine whether any immunity was produced in mice when they were inoculated by the peritoneal and nasal routes.

TABLE VIII

*Cross Immunity Test between GD VII and FA Strains of Mouse Encephalomyelitis Virus*

| Strain of virus used to test immunity | Immunizing inoculation with FA strain of virus | | No. of mice inoculated | No. of mice developed infection | No. of mice died | Percentage of mice developed infection | Percentage of mice died |
|---|---|---|---|---|---|---|---|
| | Amount and route | Dilution of virus | | | | | |
| FA | 0.4 cc. | $10^{-1}$ | 6 | 3 | 2 | | |
| FA | Intraperitoneal | $10^{-2}$ | 13 | 4 | 2 | 50 | 8 |
| FA | | $10^{-3}$ | 19 | 12 | 4 | | |
| FA | 0.05 cc. | $10^{-1}$ | 7 | 5 | 3 | | |
| FA | Intranasal | $10^{-2}$ | 7 | 4 | 1 | 79 | 55 |
| FA | | $10^{-3}$ | 15 | 14 | 12 | | |
| | Normal controls | — | 34 | 34 | 29 | 100 | 85 |
| GD VII | 0.4 cc. | $10^{-1}$ | 4 | 0 | 0 | | |
| GD VII | Intraperitoneal | $10^{-2}$ | 13 | 11 | 3 | 72 | 31 |
| GD VII | | $10^{-3}$ | 15 | 12 | 7 | | |
| GD VII | 0.05 cc. | $10^{-1}$ | 5 | 3 | 2 | | |
| GD VII | Intranasal | $10^{-2}$ | 9 | 7 | 5 | 85 | 59 |
| GD VII | | $10^{-3}$ | 13 | 13 | 9 | | |
| | Normal controls | — | 35 | 35 | 27 | 100 | 77 |

For this purpose groups of mice were inoculated by the two routes with $10^{-1}$, $10^{-2}$, and $10^{-3}$ dilutions of mouse brains infected with the FA strain. One month after the original inoculation the surviving mice in each group were divided into two new groups. One of these was then inoculated intracerebrally with a 1 per cent suspension of the homologous FA strain and the other with a similar dose of the GD VII strain of virus. As controls two groups of thirty-four and thirty-five mice of the same age and strain were also inoculated with the two virus suspensions and kept under the same conditions as the experimental mice. The results are shown in Table VIII.

Immunization of mice with the FA strain of virus definitely produced a relative immunity to a subsequent intracerebral inoculation of the homologous strain, as well as to the heterologous GD VII. The two strains were consequently immunologically related, thus producing evidence that the encephalitogenic FA strain was an unusually virulent strain of the virus of mouse encephalomyelitis. This experiment, however, did not prove the identity of the two viruses. The immunity produced in mice appeared to be greater when tested with the homologous FA virus than when tested with the heterologous strain. However, no definite conclusions could be drawn on this point as it was not known whether the amounts of virus in the test doses were the same, although both were 1 per cent suspensions of infective mouse brains. However, subsequent experiments performed in a similar manner tended to confirm the above finding, *viz.*, that the two strains, FA and GD VII, though related, were not identical immunologically.

As the number of mice in the groups was small, no conclusion could be drawn as to the relative efficacy of the two routes of inoculation in producing immunity. In evaluating the results it must be borne in mind that mice inoculated intranasally with a given dilution received approximately ten times less than mice inoculated intraperitoneally with the same dilutions because by the first route only 0.05 cc. was inoculated, whereas by the second 0.5 cc. was given. There did not seem to be any difference in efficiency between the two routes of immunization.

*Production of Immunity by Infection of Central Nervous System.*—Previously it has been noted that some of the mice which had remained well following an intracerebral inoculation of a relatively avirulent strain of virus were rendered immune to a second inoculation by the same route. In fact, the immunity produced by an actual infection of the central nervous system was of a more substantial nature than that produced by other means of immunization. When more highly virulent strains of the virus of mouse encephalomyelitis became available it seemed of importance to restudy this question. The method of procedure was to infect mice by intracerebral inoculation with an avirulent strain and to test their immunity subsequently by the inoculation by the same route of the highly virulent GD VII strain.

Strain XIV was chosen for the first inoculation as this was the least virulent available. In the first experiment forty-two mice were given an intracerebral inoculation of strain XIV. During the month following, nineteen became paralyzed and six died. One or 2 months after the primary inoculation, groups of ten mice which had remained well were

given an intracerebral inoculation of GD VII. At the same time groups of normal mice of the same breed and age were also inoculated with the same test virus. All control animals became paralyzed, whereas none of the mice which had been inoculated previously with strain XIV became paralytic.

In a second experiment the time intervals between the first and second inoculation were ½ hour, 3, 7, 14, and 21 days, and the results showed that the immunity to reinoculation was well established 1 week after the primary inoculation.

## SUMMARY

1. The two strains of virus named GD VII and FA, respectively, accidentally discovered during experiments with yellow fever, have been shown to be immunologically related to each other, as well as to the virus of mouse encephalomyelitis.

2. Infection of the central nervous system can be produced with both strains by intracerebral, intranasal, or intraperitoneal inoculations. The cardinal symptom produced by the GD VII strain of virus by all three methods of inoculation is a flaccid paralysis of the limbs. The symptoms produced by the FA strain are referable to lesions of the brain when infection is produced by intracerebral and intranasal inoculation. Following intraperitoneal inoculation of the FA strain of virus, however, a flaccid paralysis is usually produced.

3. By the use of graded collodion membranes the particle size of the virus of mouse encephalomyelitis has been shown to be from 9 to 13 mμ.

4. The stability of the virus at different hydrogen ion concentrations has been tested. It has been found that there are two optima of stability, one at about pH 8.0 and the other at pH 3.3.

5. The virus is readily inactivated at 37°C. by 1 per cent hydrogen peroxide.

6. Of organic solvents tested, ether had no action, whereas ethyl alcohol in 20 per cent concentration almost completely inactivated the virus after 45 minutes in the cold.

7. The virus can be precipitated by means of ammonium sulfate.

8. With increasing age mice acquire a relative resistance to the virus.

9. Immunity to a subsequent intracerebral inoculation can be produced by intraperitoneal, as well as intranasal, administrations of relatively large amounts of virus.

10. Mice infected by the intracerebral inoculation of a relatively avirulent virus acquire a high degree of immunity to a subsequent inoculation of a highly virulent strain.

11. The course of infection in mice following intracerebral, intranasal, and intraperitoneal inoculation of the FA strain of virus has been studied.

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