junctival sac.[3] It seems quite clear from the experiments with chimpanzees that the serum which splashed into the patient's eye actually contained dengue virus. As New Haven is not in an area in which dengue is endemic there appears to have been no other likely source of infection and none of the patient's family or associates were concurrently ill. The interval of 9 days between the accident and the development of symptoms is not too long for the incubation period of dengue and the clinical course of the illness was compatible with that of the naturally occurring disease. The serological finding that neutralizing antibodies against the mouse-adapted Hawaiian strain of dengue virus developed in the patient's serum in high titer within two weeks after the onset of illness is, of course, strong evidence in support of the diagnosis. The fact that the patient developed an acute febrile illness under the circumstances which have been presented may be regarded as additional evidence that the virus which produced inapparent infection in chimpanzees[2] as described in the preceding paper had retained its pathogenicity for man.

Summary. In the course of experiments with chimpanzees, human dengue virus was accidentally introduced on to the conjunctival surface of the eye of a laboratory worker. This was followed 9 days later by a febrile illness, clinically compatible with dengue. Neutralizing antibodies for the homologous mouse-adapted strain of dengue virus were absent in the patient's serum taken one day after onset of fever, but were present in high titer in specimens of serum obtained 13, 25, and 45 days after onset.

[3] Sabin, A. B., Unpublished studies.

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Serum Treatment of Western Equine Encephalitis in Mice Determined by the Course of Viral Infection.

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Certain features of serotherapy of experimental infections with neurotropic viruses are still under deliberation. A group of investigators maintain that animals prostrated by the infection can still be completely revived and restored to a normal-seeming state while other workers hold that once the virus multiplies actively or the host exhibits a definite syndrome reflecting the involvement of the central nervous system, *i.e.*, a destruction of tissue or neurons, then serotherapy is often ineffective. There is general agreement, however, on the successful prevention or treatment of the experimental disease if potent antiserum in sufficient amount is given early enough in its course, or, generally, before definite physical signs of infection of the central nervous system are manifest.

The problem was to determine at what stage during the course of an experimental infection antiserum was or was not effective, especially in relation to the a) time after exposure to the virus, b) first appearance of physical signs, and c) degree of multiplication of the virus in the brain. By means of the intracerebral route, 100% rate of lethal infection could be obtained with a sufficient dose of virus; and by the use of the Kelser strain of Western equine encephalitis virus which is only moderately active or invasive, the incubation period could be conveniently prolonged for serum treatment at different intervals after exposure to virus. Mice were

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TABLE I.
Multiplication of Western Equine Virus (Kelser Strain) in Mouse Brain After Intracerebral Inoculation of About 2700 LD_{50}.

Hr after inoculation	Virus titer of brain tissue removed from inoculated mice*	Hr after inoculation	Virus titer of brain tissue removed from inoculated mice*	
2	$<2\times10^{-1.0}$	50	$2\times10^{-6.2}$	
6	$2\times10^{-1.5}$	54	$2\times10^{-7.0}$	
10	$2\times10^{-2.0}$	57	$2\times10^{-7.4}$	
14	$2\times10^{-3.5}$	62	$2\times10^{-7.6}$	
18	$2\times10^{-4.0}$	66	$2\times10^{-7.2}$	
22	$2\times10^{-4.5}$	70	$2\times10^{-7.6}$	
26	$2\times10^{-4.5}$	74	$2\times10^{-8.2}$	
30	$2\times10^{-5.6}$	76	$2\times10^{-7.6}$	†
33	$2\times10^{-5.8}$	80	$2\times10^{-7.0}$	
38	$2\times10^{-6.5}$	86	$2\times10^{-7.2}$	
42	$2\times10^{-6.5}$	90	$2\times10^{-7.0}$	‡
46	$2\times10^{-5.4}$	94	$2\times10^{-7.0}$	

* Brain removed aseptically; diluted with 10% normal rabbit serum in saline solution and injected intracerebrally in test mice. The LD_{50} is given in the table.
† Signs of central nervous system involvement in this period.
‡ Death due to virus in this period.

the animals of choice since large numbers could be studied thus avoiding statistical pitfalls and yet permitting the conservation of a supply of the rabbit hyperimmune serum used in the present investigation. The blood volume of a mouse of about 17 g weight—the average weight of the test mice—is 1 cc; since 2 cc of the serum were given each animal, it was therefore the equivalent of twice the animal's blood volume.‡ More than 2 cc of this type of serum was toxic by itself, reaching a rate of 100% of deaths within a few hours after 5 cc were given.

The rate of multiplication of the Kelser strain had been studied[1] by sacrificing mice at 4-hour intervals from 2 to 94 hours after intracerebral inoculation of 2700 LD_{50} of the virus. As seen in Table I, the multiplication of the virus proceeded at a fairly regular rate between the 10th and 54th hour after inoculation. After about 57 hours, the virus reached its maximum titer which it maintained until the time of death. On the other hand, visible signs of illness were first noted 74 hours after inoculation and deaths occurred at the 86th to 96th hour, or later.

Thus a base line was constructed of a) the rate of multiplication of the virus in the brain of inoculated mice, b) the time of recognizable physical signs of infection and c) the time of death of the animal. The plan was to treat infected mice with antiserum at different periods of infection, for example, when no signs were visible and no multiplication of the virus occurred; when no signs were noted but multiplication was active, and when signs of illness were plainly seen. In this way a correlation of the effectiveness of the antiserum with the degree of multiplication of virus and of recognizable signs of infection could perhaps be obtained

Preparation of Antiserum. At first rabbits were immunized by intraperitoneal injection of mouse-brain virus in amounts representing at least $10^{7.5}$ LD_{50}. For example, after 6 such injections a rabbit antiserum when diluted 1:100 exhibited a neutralizing index of 500, as shown by the intracerebral mouse-neutralization test. After an added course of 3 inoculations of mouse-brain virus, 1:1000 dilution of the rabbit antiserum completely neutralized 65 LD_{50} of virus, and 1:5000 and 1:10,000 dilution of serum still showed neutralizing effect, although not completely (2 of 5 mice died) against the same amount

‡ The blood volume was calculated from the formula of Dreyer, G., and Ray, W., (*Philos. Trans. Roy. Soc.*, London, 1911, Series B, **201**, 133) *viz.*, B.V. $=$ $\frac{\text{Weight}^{2/3}}{6.70}$

[1] Schlesinger, R. S., unpublished observations made in this laboratory.

of virus. Beyond this point, further treatment failed to raise the level of neutralizing antibody. This is in good agreement with a prior conclusion that immunization prolonged over a period of months does not necessarily lead to antibody levels higher than those obtained by properly administered shorter courses.[2] However this may be, rabbit antiserum prepared by means of mouse-brain virus could not be used owing to the toxicity of such prodigious amounts as were given to test animals. *viz.*, twice the blood volume. Nontoxic rabbit serum was then prepared by employing as immunizing antigen embryonated hens' eggs infected via the yolk sac with the Kelser strain of Western equine encephalitis virus.‖ After 9 intraperitoneal injections of "egg" virus having a titer of $10^{-7.5}$ or higher, given over a period of a month, the pooled serum from 4-6 rabbits exhibited about the same degree of viral neutralization as antiserum prepared against mouse-brain virus just described. In a test, however, serum was used which in a dilution of 1:500 or higher could prevent infection of 5-25 lethal doses of virus injected intracerebrally into mice. This preliminary titration was made by inoculation of 1 cc of graded dilutions, up to 1:1000, of antiserum subcutaneously and followed 16 hours later by injection of a constant amount, $10^{-5.8}$–$10^{-6.0}$, of virus. A control titration of the virus by itself was included so that an exact computation of the number of doses used in the test could be obtained.

Tests. Table II serves to demonstrate examples of the tests in which mice were injected with varying small multiples of lethal doses of the virus of Western equine encephalitis and given antiserum either before, along with, or after inoculation of the virus. Each mouse received .03 cc of the virus suspension intracerebrally and 2 cc of antiserum were administered intraperitoneally. A series of mice were also treated with similar injections of normal rabbit serum as controls but as the table shows, no influence of the normal serum was exerted on the course of the experimental infection. It will be noted that the number of infective units of virus used in the test was less than that employed for determining the rate of multiplication of the virus in the brain of virus-inoculated mice and the time of development of physical signs and death in them, the data of which are shown in Table I. This was purposely planned so that the reactions to the virus by the serum-treated animals would not be greater than those in these control mice, and furthermore, the smaller doses of virus used in the test animals would weigh in favor of any preventive or curative action of the antiserum. For it will be seen (Table II) that the antiserum even in the large quantities given became less pronounced in effect when the virus was increased from 5 to 25 LD_{50}, an outcome that was not unexpected. The increase of inoculated virus from 25 to 63 doses, however, exerted little if any influence on the action of the antiserum.

There was, in addition, variation in the effect of antiserum within the same series. This variation is found now and again in immunity tests of this sort and is perhaps an indicator of the varying but unpredictable individual factors of resistance that may be brought out by the action of hyperimmune serum, such as the rate of aging, physiological or constitutional elements, or other still unknown influences which would help certain animals.

It will be observed that the antiserum became less effective as the time after exposure to the virus lengthened. Thus, up to 16 hours after the virus was given, encephalitis could generally be prevented in treated mice. From 24 to 48 hours after the inoculation of virus, some but not all the treated animals remained apparently unaffected. At 72 hours, at a time when the mice were still free from signs of illness, only an occasional test animal receiv-

[2] Olitsky, P. K., Schlesinger, R. W., and Morgan, I. M., *J. Exp. Med.*, 1943, **77**, 359.

‖ In preparing the immunizing antigen in large amounts, contamination could be reduced or eliminated by placing harvested embryos in a saline solution containing 1000 units penicillin per cc. The embryos were kept in the solution until the required number was collected and were then transferred to a Waring Blendor.

TABLE II
Treatment with Hyperimmune and Normal Rabbit Serum of Mice Infected by Intracerebral Route with Varying Amounts of Virus.

	Hyperimmune rabbit serum				Normal rabbit serum†
Virus titer	$10^{-6.7}$	$10^{-7.2}$	$10^{-7.7}$	$10^{-7.6}$	
Dilution to infect	$10^{-6.0}$	$10^{-5.8}$	$10^{-6.0}$	$10^{-5.8}$	
LD_{50} used	5*	25	50	63	
Time when antiserum was given in relation to virus	Result Dead/No. used				Result Dead/No. used
2 hr before	—	0/6	—	—	5/5
1 " "	1/7	—	—	—	—
With virus	2/6	0/6	—	1/5	9/9
4 hr after	0/7	1/6	0/5	—	4/4
8 " "	—	2/6	—	—	5/5
16 " "	0/6	2/6	2/5	—	9/9
24 " "	3/7	5/6	—	4/6	9/9
30 " "	2/7	6/6	4/6	3/5	9/9
48 " "	4/6	6/6	3/6	6/6	9/9
72 " "	5/7	6/6	6/6	—	5/5

* Antilog of difference between lines 1 and 2 to nearest whole number.

† 2 cc of normal rabbit serum was given intraperitoneally to each of a series of animals receiving intracerebrally 25 and 63 LD_{50} of virus and the results of both tests are combined here.

TABLE III.
Serum Treatment of Mice at the Time of First Definite Signs of Illness.*

Mouse No.	LD_{50} of virus injected	First sign shown after virus inoculation	Signs shown after virus inoculated in hours
1	*ca.* 6	Leg weakness	73
2	"	Tail spastic	73
3	"	Ruffled fur; slow	90
4	"	" " tremors	90
5	"	" " slow	90
6	"	Circling	93
7	"	Biting (itch)	93
8	"	Ruffled fur; slow	93
9	"	" " "	93
10	"	" " "	96
11	5	" " "	90
12	5	" " "	93

* All animals died after administration of 2 cc of antiserum given iper. at the time of the appearance of physical signs.

ing the minimal amount of virus survived. When the same amount of antiserum was given to animals when definite physical signs of illness occurred, at a time corresponding to 73 or more hours after exposure to minimal amounts, *i.e.*, about 5 or 6 LD_{50} virus, the treatment proved valueless since all treated mice died (Table III).

Discussion. There is no expediency for the acceptance of the pattern laid here for serum treatment of mice as a model for that of human beings. The infection was induced in mice by intracerebral inoculation of the virus of Western equine encephalitis which is not the way of infection in nature. The effect of serotherapy in the human disease is at present not definitely known since no controlled series of observations are as yet available; Hammon[3] states that convalescent serum therapy is probably ineffective.

In the present study mice and the intracerebral route of infection have been employed for the purpose of providing sufficiently large numbers of conveniently handled animals for several tests, and secondly, to

[3] Hammon, W. M., *Clinics*, 1945, **4**, 485.

have a more solid foundation on which to discern real and not apparent results since the intracerebral route could be counted upon to induce lethal encephalitis in every instance among controls. It therefore follows that what was concluded from the experiments with mice and the intracerebral route of inoculation might not correspond with the results of tests on larger laboratory animals, such as guinea pigs and monkeys, and on the use of peripheral (nonneural) route of inoculation of virus. Then, of necessity, smaller numbers of animals have to be used and the peripheral route of viral exposure is not always disease-producing in the control animals.

What is significant is that in the present investigation a certain dosage of antiserum which could prevent infection at one particular stage during the course of infection, when the virus was even actively multiplying, was ineffective in another, when virus was increasing, or remained stationary at a higher level, Either the antiserum was incapable of neutralizing the virus at its place of multiplication[4] or an amount of serum might have been needed that in terms of blood volume of the host was impractical to use, or changes in the tissues progressed to a degree which could no longer be influenced by serotherapy. It should be stressed, however, that the stage during which antiserum was ineffective was one in which the treated animals could still appear to be normal to the observer.

Summary. If 2 cc of rabbit hyperimmune antiserum of high titer were given intraperitoneally to mice of about 17 g weight—a prodigious amount since it represents twice the total blood volume of the host—encephalitis was prevented from developing in animals receiving intracerebrally ordinarily lethal doses of the virus of Western equine encephalitis. The preventive effect was noted when the virus was multiplying but had not as yet reached its maximal titer. Thus, if animals were treated with antiserum 2 hours before, at the same time, or from 4-16 hours after the virus was inoculated, it was effective in preventing an attack of encephalitis. Even if serum was administered 24, 36 or 48 hours after the virus certain but not all mice survived without recognizable signs of the disease. At 72 hours, however, only an exceptional mouse survived after serum treatment. Finally, at 73 or more hours after virus inoculation, when definite manifestations of experimental Western equine encephalitis could be observed, injection of antiserum was found to have no influence on the course of the lethal infection.

[4] Rivers, T. M., *J. Am. Med. Assn.*, 1948, **136**, 291.

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Penicillin Aerosol in the Treatment of Experimental Pneumococcus Pneumonia.*

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Inhalational therapy with penicillin mist, or aerosol, in acute and chronic respiratory infections in man has recently been receiving the attention of clinical investigators both here and abroad.[1-10] In fact, the clinical

* Work done under a grant from the Medical Division of the Chemical Warfare Service, Edgewood Arsenal, Md.

[1] Garthwaite, B., and Barach, A. L., *Am. J. Med.*, 1947, **3**, 261.

[2] Olsen, A. M., *Proc. Mayo Clinic*, 1945, **20**, 184.

[3] Segal, M. S., and Ryder, C. M., *New Eng. J. Med.*, 1945, **233**, 747.