MULTIPLICATION AND SPREAD OF THE VIRUS OF ST. LOUIS ENCEPHALITIS IN MICE WITH SPECIAL EMPHASIS ON ITS FATE IN THE ALIMENTARY TRACT*

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The present study was undertaken in an attempt to learn more about the fate of certain neurotropic viruses from the moment they are inoculated into known parts of the body until the time when the animal succumbs to the infection. Such information is needed for a better understanding not only of the pathogenesis of these infections but also of the significance of certain findings postmortem in naturally infected hosts. Recent studies in this laboratory, for example, have focused attention on the presence of virus in the tissues of various levels of the alimentary tract in fatal cases of human poliomyelitis (1, 2), and certain experimental studies have been carried out with different strains of poliomyelitis virus in *rhesus* (3) and *cynomolgus* (2, 4) monkeys to aid in the proper interpretation of these findings. It is not yet feasible to utilize quantitative methods in such studies on experimental poliomyelitis with strains of recent human origin which are pathogenic only in monkeys, and it was believed that certain general, basic information might first be obtained with other viruses. Although we were especially interested to find out how other neurotropic viruses, entering the body by various routes, affected the alimentary tract, it was clear that for a proper interpretation of the results it was necessary to know what was happening to the virus in other parts of the body at the same time.

The results recorded in this communication were obtained with the virus of St. Louis encephalitis given to young mice either directly into the circulation, into the brain, or into the nose and mouth by nasal instillation. Beginning 4 hours after inoculation and continuing daily thereafter until the animals succumbed to the infection, the dissemination and multiplication of the virus was determined quantitatively by titration of suspensions of almost all the available tissues in the body.

Previous investigators have established that following nasal instillation in mice and occasionally also after intraperitoneal inoculation, the virus of St. Louis encephalitis invades the central nervous system along the olfactory pathway (5–7). Webster and Clow (5, 8) have recovered the virus from the blood as well as spleen of mice at various

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intervals after infection, but stated that the liver, lung, and kidney contained only negligible amounts of virus, and Brodie (6) reported that the parenchymatous organs were rarely positive in mice infected by the nasal route. However, Kudo and his associates (9) found the viruses of both St. Louis and Japanese B encephalitis in the heart's blood, lungs, liver, spleen, kidneys, and adrenal glands of mice during the terminal stages following infection by the intracerebral or intranasal routes. Evidence that the virus of St. Louis encephalitis may multiply outside the central nervous system of mice, was supplied by Webster and Clow (5) for the spleen and by Lennette and Smith for the testicles (10). In human beings, the virus of St. Louis encephalitis has been recovered only from the brain; there is no record of tests on other tissues, although negative results have been reported for blood, cerebrospinal fluid, and nasal washings (11, 12). Sulkin, Harford, and Bronfenbrenner (13), however, believed that nasopharyngeal washings from human cases might have contained some virus because some of their inoculated mice exhibited a certain amount of immunity. Cox, Philip, and Kilpatrick (14) recovered St. Louis encephalitis virus from the nasal washings of a horse that was inoculated intracerebrally 15 days earlier and had exhibited fever and encephalitic signs for 5 days, although daily tests for virus in the blood were negative. In another intracerebrally inoculated horse which had fever and encephalitic signs since the 9th day and was killed on the 12th day, virus was recovered from the brain and cord but not from the spinal fluid, heart, liver, kidneys, spleen, ileum, jejunum, colon, or feces.

Material and Methods

Mice.—White, Swiss mice obtained from The Rockland Farms, New City, New York, were used in this study. The animals were 2 to 3 weeks of age and weighed 7 to 9 gm. at the time of inoculation, unless otherwise indicated in the protocols. The virus titrations were carried out by intracerebral inoculation of 3 to 4 week old mice weighing 11 to 14 gm.

Virus.—The "Webster No. 3" strain of St. Louis encephalitis virus, obtained from the late Dr. L. T. Webster of The Rockefeller Institute, was used in this study. It had undergone numerous intracerebral passages in mice since its isolation in 1933, and after a series of rapid passages in this laboratory, intracerebral LD_{50} titers of 10^{-8} or more were obtained consistently. A 10 per cent suspension of infected mouse brain in undiluted, heated (56°C. for 30 minutes) rabbit serum, was stored in a box of dry ice. Separate ampoules from the same lot of virus were used in all the tests. The virus dilutions were made in saline solution containing 10 per cent of heated rabbit serum.

Administration of Virus.—Although the intravenous injection of 10 per cent brain suspension causes almost immediate death of the majority of mice, a 1 per cent, centrifuged suspension could be given without harmful effects. Preliminary tests, shown in Table I, indicated that the younger mice were more susceptible, as after inoculation by other peripheral routes, and that in order to be certain that all inoculated animals would develop encephalitis, it was necessary to use 2 to 3 week old mice and the 10^{-2} dilution of virus. The intravenous dose, therefore, consisted of 0.3 cc. of the 10^{-2} dilution (about 10 million intracerebral LD_{50} of virus), the injection being given into the tail vein. To avoid leakage and contamination of the cage by virus, pressure was applied to the tail afterwards until there was no bleeding, the area was wiped off with a 3 per cent solution of hydrogen peroxide, and collodion was applied to the point of inoculation. The intracerebral dose, which was given under ether anesthesia, consisted of 0.03 cc. of the 10^{-6} dilution (about 100 intracerebral LD_{50}). The

intranasal dose was 0.03 cc. of the 10 per cent suspension (about 10 million intracerebral LD_{50}) and was dropped slowly on the external nares of the lightly anesthetized mouse. It should be noted that a good deal of this virus finds its way into the mouth not only through the nasopharynx but also from the external nares along the central labial fissure; it is also obvious that some is aspirated into the lungs and some swallowed, for 0.03 cc. is a relatively large volume for a very small mouse.

TABLE I

Effect of Intravenous Inoculation of St. Louis Encephalitis Virus in Mice

Experiment No.	Age of mice	Dilution of virus injected intravenously (dose, 0.3 cc.)					*Intracerebral* LD_{50} titer of same virus suspension (dose, 0.03 cc.)
		10^{-1}	10^{-2}	10^{-3},	10^{-4}	10^{-5}	
	wks.						
1	3–4	4*, 4*	4*, 4*, 4*, 4*, 0	6, 8, 8, 0, 0	0, 0, 0, 0		8.5‡
2	2–3		3*, 3*, 3*, 3, 3, 4, 4, 5, 5, 5				8.0
3	3–4		3*, 3*, 3*, 3*, 4, 5, 5, 6, 0, 0				9.0
4	8–12	4, 5, 5, 5	4, 5, 5, 6, 6				
5	2–3		3*, 3*, 3*, 4*, 4*, 4*, 4, 4, 5, 5, 5, 5, 5, 6, 7, 7	4, 5, 5, 7, 0 0	5, 5, 7, 2*, 0	6, 8*, 0, 0, 0	
	8–12		5, 5, 6, 7, 7	7, 9, 15, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	

* Each digit indicates one inoculated mouse; the numbers with asterisk indicate day on which mice were killed after exhibiting obvious evidence of involvement of the nervous system. Numbers without asterisk indicate day of death. "0" means that the mouse survived without exhibiting any signs of illness.

‡ The titers in this and subsequent tables are the reciprocals of the log of the LD_{50} end-point dilution; thus, the end-point of $10^{-8.5}$ is recorded as 8.5.

Collection and Preparation of Tissues and Other Specimens for Subinoculation.—The number of mice inoculated in each experiment included six to ten more than were needed, to make certain that the selected doses would have produced encephalitis at the expected times in the animals that were sacrificed for study. After intracerebral and intravenous inoculation some mice began to exhibit signs of encephalitis in about 72 hours, and at 96 hours the majority were either dead or prostrate, but the mice selected for study at 72 hours were as a rule the ones without encephalitic signs. After nasal instillation of the virus, the first encephalitic

signs were seen at 96 hours and the animals were prostrate or dead at 120 hours. During the period of observation, the mice were in cages with wire floors, which permitted the feces to fall through onto a paper towel or wire screen. Since a 10 per cent suspension of the stools was too concentrated to allow filtration, they were usually suspended in 20 to 50 parts of the heated rabbit serum; and after horizontal centrifugation, the supernatant liquid was put through a double-disc Seitz filter, previously saturated with broth, and a minimum of 8 to 10 cc. of filtrate was obtained. Urine was collected into a test tube by pressure on the abdomen, and the amount obtained from ten mice was pooled at each interval. Since intracerebral injection of undiluted urine is toxic for mice, it had to be diluted with two parts of distilled water.

At each period, indicated in the protocols, three mice were sacrificed and the blood or tissues were pooled for the tests. All procedures were carried out as aseptically as possible and separate instruments were used for each tissue. The animals were exsanguinated from the heart under ether anesthesia, and heparin (1:500) was used as the anticoagulant. Following this, the skin and inguinal lymph nodes were taken. The thoracic cage was next opened, the descending aorta was cannulated with a 25 gauge needle, and 10 cc. of sterile physiological salt solution was forced through to free the organs of most of the blood within their vessels. The heart and lungs were removed next. The peritoneal cavity was then opened and the abdominal organs were removed in the following order: liver, adrenals, kidneys, spleen, urinary bladder, aorta, and intestinal tract from the pylorus to the lower sigmoid. The tongue, pharynx, sciatic nerves, and leg muscles were obtained next. Finally the brain was removed and last of all the nasal mucosa. Needless to say, each organ was put in a separate dish in which it was further washed in sterile physiological salt solution. The intestinal tract was cut open and after the contents were lifted off or gently scraped away, the walls were washed in four changes of sterile physiological salt solution. All tissues, of which there was 0.05 gm. or more in the pool, were ground with enough undiluted, heated rabbit serum to make a 10 per cent suspension. The nasal mucosa (8 mg.), the adrenals (25 mg.), the lymph nodes (20 mg.), the sciatic nerves (5 mg.), and aorta were ground in 0.5 cc. The suspensions of the intestinal tract and contents were filtered in the same manner as the stools. The suspensions of the nasal mucosa, tongue, pharynx, and skin were spun in a Swedish angle centrifuge at about 3000 R. P. M. for 30 minutes, and this as a rule eliminated enough of the bacteria to prevent the majority of inoculated mice from dying of bacterial infection. The suspensions of all the other tissues were spun on the horizontal centrifuge just enough to sediment the large particles.

The blood and urine were always injected into mice on the same day. Whenever practicable the tissue suspensions were also inoculated on the same day. As a rule, however, it was necessary to freeze the suspensions in an insulated box containing solid carbon dioxide (dry ice) and carry out the quantitative determinations of the amount of virus in them at a later date. Previous tests in this laboratory established that when *undiluted* rabbit serum is used for the preparation of the suspensions (10 per cent rabbit serum saline is inadequate) the concentration of virus is practically the same after storage in the box of dry ice as in the fresh state. However, when the suspension is refrozen and thawed a second time, there is usually a drop in titer. For this reason, the titers shown in the protocols were obtained either with fresh material or suspensions that were frozen and thawed only once. The titrations were carried out by making tenfold, serial dilutions in saline containing 10 per cent of heated rabbit serum and inoculating 0.03 cc. intracerebrally into 3 to 4 week old mice. Five mice were inoculated with each dilution, except in the preliminary intravenous series, when three mice were used. The animals were observed for 14 days and no death that occurred during the first 3 days was considered as being due to the virus. The presence of virus in any tissue was confirmed by at least one positive, serial passage during the course of

the study. In addition to that the brain of any mouse considered of sufficient importance to warrant determining whether its death was caused by virus was passaged whether or not the mouse exhibited obvious signs of encephalitis. The urine always contained enough bacteria to kill some of the mice, but as a control in all animals dying after the 3rd day, filtered brain suspension was passaged. The LD_{50} titers indicated in the protocols were calculated by the method of Reed and Muench, the original blood being regarded as zero dilution, and the 10 per cent suspensions as the 10^{-1} dilutions. The calculation of the total amount of virus in a given organ was carried out as follows: if the LD_{50} titer of the spleen suspensions, for example, was 2.3 (*i.e.* $10^{-2.3}$), 0.03 cc. of the 10 per cent suspension (*i.e.* 10^{-1}) contained 20 LD_{50} of virus; since the entire spleen weighed 0.1 gm. (suspended in 1 cc. for the 10^{-1} dilution), the amount of virus in the spleen $= \frac{20 \times 1.0}{0.03} = 666$ LD_{50}.

RESULTS

The results of the quantitative determinations of the amounts of virus present throughout the body at various times after inoculation by different routes are summarized in Tables II, III, IV, and V.

Course of Events after Intravenous Inoculation.—4 hours after introduction of about 10 million LD_{50} of virus into the general circulation very little remained demonstrable, and these small amounts were found in the blood, spleen, liver, lungs, and heart, but not in the brain or peripheral nerves, not in any part of the alimentary tract or its contents, not in the adrenals, kidneys, bladder, or urine, and not in the skin, muscles, lymph nodes, nasal mucosa, or the walls of the large blood vessels such as the aorta. When one calculates the total amount of virus in the blood and organs from the titers in Table II, and the total volume of the blood and the weights of these tissues, one can account for only about 1,000 to 5,000 LD_{50} out of the 10 million LD_{50} that were inoculated. The bones and the marrow are not accounted for in this estimate, but it is doubtful that any more proportionally would be detected there than in the spleen or liver. One can estimate, therefore, that at the very most only about 1/1000 of the virus inoculated can be found in the body 4 hours after injection.

At 24 hours the blood and viscera, which contained the virus at 4 hours, were still positive with the exception of the liver which in one group of animals yielded a negative test and in another only a trace of virus. The changes in titer, either in the way of decrease or increase, did not seem to be very significant and there was no evidence from the data that the virus had multiplied at all or to any appreciable degree either in the blood vessels or in the viscera. It is noteworthy, however, that, in at least one of the two series of tests, traces of virus were now found in the brain (slightly less than 100 LD_{50}), intestinal tract, tongue, nasal mucosa, hamstring muscles, and inguinal lymph nodes; in the preliminary test the kidneys were positive and in the second series the urinary bladder. However, *at 48 hours* the kidneys, urinary bladder, nasal mucosa, hamstring muscles, inguinal lymph nodes, and tongue were again negative, and a progressive increase of virus was evident only in the brain (1.6 million LD_{50})

TABLE II

Distribution and Multiplication of the Virus of St. Louis Encephalitis in Selected Tissues at Intervals Following Intravenous Injection of about 10,000,000 Intracerebral LD_{50} Doses

Tissue	Preliminary orientation series*					Final series				
	Titer of virus per 0.03 cc. of tissue suspension at indicated times after inoculation					Titer of virus per 0.03 cc. of tissue suspension at indicated times after inoculation				
	4 hrs.	24 hrs.	48 hrs.	72 hrs.‡	96 hrs.§	4 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Nervous										
Brain	0	0	4.9	7.5	8.4+?	0	Trace (2/5)	5.2	7.6	8.0
‖Sciatic nerves	—	—	—	—	—	0	0	0	2.5+?	3.5
Alimentary tract										
Tongue	—	—	—	—	—	0	1.4	0	1.5	2.5
Pharynx	—	—	—	—	—	0	0	0	1.5+?	1.5+?
Intestinal tract	0	0	1.0	2.5	3.2	0	Trace (1/5)	1.3+?	3.2+?	4.4+?
Intestinal contents	0	0	0	0	Trace (1/3)	0	0	0	Trace (2/5)	1.4+?
‖Stool	0	0	0	0	0	0	0	0	0	0
Blood and viscera										
Blood (undiluted)	1.0	0.5	Trace (1/3)	0	0.2 (3/5)	0.3	0.6	0	0	Trace (1/5)
Spleen	2.2	2.8	1.0	Trace (2/5)	2.7	1.0	2.3	Trace (1/5)	0	1.8
Liver	2.3	Trace (1/3)	Trace (1/3)	0	2.2	1.2	0	0	0	0
Lungs	2.5	1.7	3.0	3.3	3.8	2.4	1.0	2.8	2.3	2.8
Heart	Trace (1/3)	Trace (1/3)	0	1.5	4.2+?	1.6	1.2	1.5	1.5	3.2
‖Adrenals	—	—	—	—	—	0	1.3	0	0	1.8
Urinary										
Kidneys	0	1.0	Trace (1/3)	1.7	3.7	0	0	0	Trace (2/5)	2.7
Bladder	0	0	0	3.4	—	0	1.2	0	0	2.6
Urine (1:3 dilution)	0	0	0	0	0	0	0	0	0	0
Miscellaneous										
Skin	—	—	—	—	—	0	0	0	Trace (2/5)	0
‖Nasal mucosa	0	0	0	Trace? (1?/3)	Trace (1/3)	0	Trace (1/5)	0	0	0
‖Inguinal lymph nodes	—	—	—	—	—	0	1.6	0	Trace (2/5)	0
Hamstring muscles	—	—	—	—	—	0	Trace (2/5)	0	1.5	2.2
‖Aorta	—	—	—	—	—	0	0	0	1.8+?	0

Explanation of titers: "0" means no virus detected in highest concentration tested which may be a 10 per cent suspension or less as indicated in the text. "Trace" (1/5) means that only one of the five mice inoculated with the highest concentration succumbed and that no titer could be calculated. In the case of blood, titers of less than 1 indicate that some or all of the mice inoculated with the undiluted portion (dilution 0) as well as some of those receiving the 10^{-1} dilution, etc., succumbed. The recorded titers are the reciprocals of the log of the LD_{50} end-point dilution; a "+?" signifies that no end-point was obtained in the titration.

* In the orientation series, several groups of mice inoculated at different times were used; in the final series, a single lot of mice was inoculated and groups of three were killed at the indicated intervals.

‡ The data for this group were derived from two lots of mice, one of which consisted of animals 3 to 4 weeks old at the time of inoculation.

§ All of these mice were 3 to 4 weeks old at the time of inoculation.

‖ Not used as 10 per cent suspension; for actual concentration see text.

and intestinal tract. In the viscera, there was evidence of disappearance of virus from the spleen in both series of tests; the lungs and heart had about as

TABLE III

Distribution and Multiplication of the Virus of St. Louis Encephalitis in Selected Tissues at Intervals Following Intracerebral Injection of 100 Intracerebral LD_{50} Doses

Tissue	Titer of virus per 0.03 cc. of tissue suspension at indicated times after inoculation				
	4 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Nervous					
Brain	0	1.8	5.6	7.6	8.5
Sciatic nerves	0	0	0	Trace (10^{-2}:1/5)	3.7
Alimentary tract					
Tongue	0	0	Trace (1/5)	1.5	3.5
Pharynx	0	0	0	1.8	1.5+?
Intestinal tract	0	0	0	0	2.6
Intestinal contents	—	—	—	—	0
Blood and viscera					
Blood (0 at 10 min.)	0	0	0	0	1.2
Spleen	0	Trace (2/5)	0	0	2.7
Liver	0	0	0	0	1.6
Lungs	0	0	0	0	3.2
Heart	0	0	0	0	3.3
Adrenals	0	0	0	2.9	1.8+?
Urinary					
Kidneys	0	0	0	0	2.4
Bladder	0	0	0	1.2	3.5
Miscellaneous					
Skin	0	0	0	0	Trace (1/5)
Nasal mucosa	0	0	0	0	Trace (2/5)
Hamstring muscles	0	0	Trace (2/5)	Trace (1/5)	3.8
Inguinal lymph nodes	—	—	—	—	0

For explanation of titers, etc., see footnotes to Table II.

much as at 4 hours after inoculation, and the amount in the blood was negative or negligible. *At 72 hours* there was evidence of still greater increase of virus in the central nervous system (400 million LD_{50}) and intestinal tract and the

first appearance of virus in tissues which had been negative hitherto. That at this stage the virus is most likely spreading centrifugally from the central

TABLE IV

Distribution and Multiplication of the Virus of St. Louis Encephalitis in Selected Tissues at Intervals Following Nasal Instillation of 10,000,000 Intracerebral LD_{50} Doses

Tissue	Titer of virus per 0.03 cc. of tissue suspension at indicated times after inoculation.					
	4 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.
Nervous						
Brain	0	0	2.7	3.0	7.6	8.5
Sciatic nerves	0	0	0	0	0	2.5
Alimentary tract						
Tongue	0	0	0	0	3.2+?	1.8
Pharynx	0	0	0	0	1.9	1.4
Intestinal tract	0	0	0	0	0	1.8
Stool	0	0	0	0	0	0
Blood and viscera						
Blood (0 at 10 min.)	Trace (1/5)	0	0	0	0	Trace (1/5)
Spleen	0	0	0	0	0	1.6
Liver	0	0	0	0	0	1.6
Lungs	4.5+?	3.7	1.5	1.5	2.5	2.7
Heart	0	0	0	0	1.3	2.2
Aorta	—	—	—	—	—	0
Adrenals	0	0	0	0	1.5	2.1
Urinary						
Kidneys	Trace (1/4)	0	0	0	0	1.5
Bladder	0	0	0	0	0	2.0
Miscellaneous						
Skin	0	0	0	0	0	1.4
Nasal mucosa	0	0	Trace (2/5)	0	0	0
Hamstring muscles	0	0	0	0	Trace (1/5)	3.4
Inguinal lymph nodes	—	—	—	—	—	0

For explanation of titers, etc., see footnotes to Table II.

nervous system along the peripheral nerves is indicated by the fact that it was found in appreciable concentration in the sciatic nerves and hamstring muscles, while none was detected (as in the second series) in the blood, spleen, or liver. *At 96 hours*, when the concentration of virus in the entire brain was 1 billion

LD_{50} or more and the animals were almost prostrate, the dissemination of virus was so widespread, that on inspecting the data one's attention is attracted rather to the negative sites. Among these it is especially noteworthy that the blood had only a trace and that unlike the pharynx and tongue which contained appreciable concentrations of virus, the nasal mucosa yielded none or only a trace.

TABLE V

Distribution of the Virus of St. Louis Encephalitis in Selected Tissues of Two Mice with Central Nervous System Involvement Developing Late Following Intravenous Injection

Tissue	Mouse with encephalitic signs on 8th day*	Mouse with posterior paralysis on 12th day‡
Brain	7.8	1.5+? (less than 2.6)
Tongue	—	Trace (1/5)
Pharynx	—	Trace (2/5)
Intestinal tract	1.2	0
Intestinal contents	0	0
Blood	0	0
Spleen	Trace (2/5)	0
Liver	1.3	Trace (1/5)
Lungs	1.8	0
Kidneys	2.3	0
Urinary bladder	—	0
Nasal mucosa	Trace (1/5)	0
Hamstring muscles	—	2.4

For explanation of titers, etc., see footnotes to Table II.

* Dose of virus intravenously: 10,000 intracerebral LD_{50} doses.

‡ Dose of virus intravenously: 100,000 intracerebral LD_{50} doses.

To determine whether or not the picture was different in mice that succumbed after a longer incubation period, the tissues of two additional young mice of the same age were studied. The results are shown in Table V. One of these mice received 10,000 intracerebral LD_{50} of virus intravenously and first exhibited generalized signs of involvement of the nervous system 7 days after inoculation; it was killed for study on the 8th day when it was prostrate. In general the distribution and quantities of virus found in this mouse are not different from the results obtained in the mice that succumbed 4 days after inoculation. The second mouse, however, exhibited different signs and also a different distribution of virus. This mouse received 100,000 intracerebral

LD_{50} of virus intravenously and first exhibited paralysis of the posterior extremities 12 days after inoculation when it was killed for study. Although the spinal cord was not tested, the lumbar portion was the main seat of the infection as was apparent from the signs manifested and the pathological changes found in the histological sections. The entire brain, however, had not less than 320 LD_{50} but not more than 3,200 LD_{50} of virus. It is, therefore, notworthy that while the leg muscles had as much or more virus per milligram of tissue than the brain, there was little evidence of widespread centrifugal spread of virus, although traces were already present in the tongue, pharynx, and liver. The absence of virus in the intestinal tract of this mouse may be due to the fact that virus does not localize here after intravenous injection of smaller doses or that it had already multiplied and disappeared. At any rate it is apparent that localization of virus in a level of spinal cord which leads to paralysis of the posterior extremities and centrifugal spread of the virus to the leg muscles, is not followed by any appreciable centrifugal spread to the intestinal tract.

To summarize the events after intravenous inoculation of 10 million intracerebral LD_{50} of virus, it would appear that there is evidence of primary multiplication only in the central nervous system and probably also to a lesser extent in the intestinal tract. There is no evidence from these data that multiplication occurs in the viscera or blood vessels, although one cannot be certain that persistence of the virus might not have been due to low grade multiplication. These tests were not designed to indicate how the virus reached the central nervous system from the blood. It is noteworthy, however, that the rate of multiplication of the virus in the brain after intravenous inoculation of the large doses is more like that which occurred after intracerebral injection (Table III) than after nasal instillation (Table IV). It is not improbable, however, that in mice which succumb 6 or 7 days or longer after intravenous inoculation of smaller doses, the nervous system may be invaded in a different manner, as was evident for example in the mouse which developed primary paralysis of the posterior extremities. At any rate, we have no evidence here of any extensive multiplication in the vascular tissues, and the time relationships are such as to indicate that small amounts of virus can find their way into the brain in less than 24 hours after it is introduced into the general circulation. Partial serial sections of the entire brain and spinal cord of three mice were studied histologically—all three animals succumbed with signs of encephalitis 4 days after intravenous inoculation, two receiving 100 million intracerebral LD_{50} and one, 10 million LD_{50}. The lesions were widespread and diffuse, and their distribution, particularly throughout the cerebral cortex, was unlike that seen in sections of mice succumbing with encephalitis after intracerebral or intransal inoculation, or those developing paralysis after inoculation into the muscles of the posterior extremities. The histological evidence indicates

that following intravenous injection of large amounts, the virus may localize simultaneously in many foci in the central nervous system. Occasionally, however, particularly after smaller doses, there can be primary localization in the spinal cord with resulting flaccid paralysis of the posterior extremities as the primary manifestation, and extensive lesions in the lumbar region of the spinal cord of the same severity that may be seen after injection of the virus in the muscles of the posterior extremities. (Studies on the effect of intramuscular injection of St. Louis virus in mice were previously made by one of us in association with Dr. Robert Ward.)

Course of Events after Intracerebral Inoculation.—The results shown in Table III indicate that *4 hours* after the introduction directly into the brain of at least 100 intracerebral LD_{50} of virus, none could be detected in any of the tissues tested. If all the injected virus had remained in the brain, one should have been able to detect only traces after the dilution it had undergone in the preparation of the material for inoculation. *At 24 hours* there is evidence of beginning multiplication in the brain and the presence of a trace of virus in the spleen (not more than about 20 LD_{50} for the entire spleen) indicates that some of the virus has found its way into the general circulation in this particular group of mice. At this time the total amount of virus demonstrable in the brain is about 630 LD_{50}. *At 48 hours* the total amount of virus in the brain had reached about 4 million LD_{50}, and the presence of traces of virus in the tongue and hamstring muscles (taken together with its absence in all the other tissues) suggests beginning centrifugal spread along some of the peripheral nerves. *At 72 hours* the amount of virus in the brain had reached about 400 million LD_{50} and further evidence of centrifugal spread by peripheral nerves is now evident from the presence of small amounts in the sciatic nerves, tongue, pharynx, adrenals, urinary bladder, and leg muscles—but still none in the blood, any of the viscera, or the intestinal tract. *At 96 hours* (with the animals moribund) the virus concentration in the brain was up to 3.2 billion LD_{50}, and the only place where virus could not be found was in the intestinal contents and inguinal lymph nodes. Although it is obvious that at this stage virus had spilled over into the circulating blood, whether it be from the brain or the viscera, it is evident that the presence of appreciable amounts of virus in such sites as the intestinal tract, tongue, pharynx, kidneys, urinary bladder, leg muscles, and sciatic nerves, is probably due to centrifugal spread from the brain. This assumption gains added weight from the fact that no virus could be detected in any of these tissues 4 hours after the intravenous injection of about 10 million LD_{50} of virus.

Course of Events after Nasal Instillation of Virus.—When 0.03 cc. of suspension is put drop by drop on the nostrils of lightly anesthetized mice, it is aspirated through the nose, comes out through the mouth, some is very likely swallowed, and a good bit reaches the lungs. Thus, *4 hours* after nasal instilla-

tion of at least 10 million intracerebral LD_{50} of virus, a great deal was found in the lungs (no end-point at a dilution of $10^{-4.5}$) and with the exception of a trace in the blood (and in this instance some also in the kidneys) none could be detected in the nasal mucosa, tongue, pharynx, intestinal tract, or in any of the other tissues that were tested (Table IV). This rapid disappearance of nasally instilled virus from the nasal mucosa of animals that without doubt would have succumbed to the infection if they had not been killed for the experiment, has previously been noted with vesicular stomatitis virus in mice (15) and guinea pigs (16) and poliomyelitis virus in monkeys (17). *At 24 hours* the virus could be found only in the lungs and, although it was present there in appreciable concentration, the amount was less than at 4 hours. *At 48 hours* there was evidence of beginning multiplication in the brain with a trace in the nasal mucosa, and further decrease in the amount present in the lungs. This appearance of small amounts of virus in the nasal mucosa at a time when there is the greatest multiplication in the olfactory bulbs has also been observed with the virus of poliomyelitis in monkeys (17). That it probably represents only a slight overflow from the olfactory bulbs is suggested by the fact that it fails to multiply and invariably disappears within about 24 hours. *At 72 hours* there has occurred very little increase in the amount of virus in the brain, and with the exception that none was detected in the nasal mucosa, the picture is identical with that found at 48 hours. Multiplication in the brain thus progressed more slowly than in the intracerebral or intravenous groups. *At 96 hours* the virus in the brain had reached a concentration of 400 million LD_{50} and the distribution of virus is now almost identical with that found in the intracerebral group at 72 hours (Table III). It would appear that centrifugal spread begins when the virus reaches this concentration in the brain, but it should be noted that some tissues such as the tongue and pharynx are reached sooner than others. *At 120 hours* with about 3.2 billion LD_{50} of virus in the brain, the distribution is practically identical with that found in the intracerebral group at 96 hours with the interesting exception that in the intranasal group the nasal mucosa did not have even a trace of virus.

Presence of Virus in Intestinal Tract and Its Absence in Stools.—It has already been pointed out that in the mice which received the virus directly into the brain or into the nose (and mouth), the intestinal tract showed the presence of virus only at the final stage of the infectious process when the concentration of virus was at the highest level in the central nervous system and there was evidence of widespread centrifugal spread to almost all the other tissues of the body. However, after intravenous inoculation of large amounts, the virus made its appearance 24 hours later in traces simultaneously in the brain and intestinal tract, and there was evidence of progressive multiplication in the intestinal tract before the concentration of virus in the brain had reached the level of 400 million LD_{50} at which centrifugal spread began in the intracerebral

and intranasal groups. This is especially noteworthy since, unlike such viscera as the spleen, liver, and lungs, which contained appreciable amounts of virus 4 hours after intravenous inoculation and in which there was no evidence of progressive increase, the intestinal tract like the brain had no demonstrable virus at 4 hours. It is, therefore, quite probable that the virus actually multiplied in the intestinal tract following early localization from the blood stream. Since the intestinal contents invariably contained bits of mucosa, it is not surprising that small amounts of virus were demonstrable on three occasions. However, the stools which were passed by the mice and allowed to fall through the floor of the cage on to a paper towel, had no demonstrable virus at any time even when the concentration in the walls of the intestine was quite high. Al-

TABLE VI

Identification of Virus Recovered from Intestinal Tract by Neutralization Test

Material tested	Final dilution of virus in mixtures*						LD_{50} titer	Neutralization index
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
St. Louis virus + normal rabbit serum	—	—	4/5	4/5	0/5	0/4	7.3	—
St. Louis virus + St. Louis immune hamster serum	4/5	1/5	0/5	0/4	—	—	4.5	630
Intestinal tract virus + normal rabbit serum	—	—	5/5	1/5	0/5	0/5	6.6	—
Intestinal tract virus + St. Louis immune hamster serum	3/5	0/5	0/5	0/5	—	—	4.2−?	250+?

Fractions: Numerator = number died; denominator = number inoculated.

* Mixtures incubated at 37°C. in water bath for 2 hours.

though the general manifestations are distinctly different and the incubation period very much longer with the virus of spontaneous mouse poliomyelitis (Theiler's disease (18)), rendering confusion with the St. Louis encephalitis virus unlikely, a neutralization test was, nevertheless, carried out with the virus recovered from the intestinal tract of these mice. The results shown in Table VI clearly identify the virus as that of St. Louis encephalitis. Another test was carried out to determine whether or not the stools of mice contained an inhibitory factor for the St. Louis virus, but none was found (see Table VII). It is, furthermore, noteworthy that two of the stool specimens produced flaccid paralysis in the inoculated mice after incubation periods of 14 to 15 days and the results of further passage indicated that we were dealing with Theiler's virus. It would appear, therefore, that unlike Theiler's virus in mice (19, 20) or poliomyelitis virus in monkeys (2, 4) and human beings (1, 2), the St. Louis virus can be present in high concentration in the walls of the intestinal tract of mice without being liberated in the stools.

Presence of Virus in the Urinary Bladder and Its Absence in the Urine.—It has been shown that while the virus regularly appeared in the urinary bladder after spread from the central nervous system had begun, none was found in the urine. However, the test shown in Table VII indicates that 0.03 cc. of urine can inactivate at least 630 LD_{50} of virus contained in 10 per cent rabbit serum saline. The mouse urine originally had a pH of 5.58 and after mixture with an equal volume of physiological salt solution containing 10 per cent of rabbit serum the pH was raised only slightly to 5.72. One cannot tell, therefore, whether the virus fails to leave the bladder wall, or whether it is liberated and then destroyed by the urine.

Significance of Relative Concentration of Virus in Various Tissues at the Terminal Stage of the Infection.—The titers recorded in Tables II, III, IV, and V indicate the concentration of virus per unit of weight of a given tissue (*i.e.*,

TABLE VII

Action of Mouse Urine and Stool Filtrate on St. Louis Encephalitis Virus

Material added to indicated dilutions of virus in 10 per cent rabbit serum saline	Final dilution of virus in mixtures*					LD_{50} titer	Inactivation index
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Undiluted rabbit serum	—	4/5	4/5	0/5	0/4	7.3	—
Filtrate of stool in undiluted rabbit serum	5/5	5/5	2/5	0/5	—	6.8	3
Physiologic salt solution	5/5	5/5	1/5	1/4	—	6.7	4
Urine	0/5	0/5	0/5	0/5	—	4.5—?	630+?

Fractions: Numerator = number died; denominator = number inoculated.

* Mixtures incubated at 37°C. in water bath for 2 hours.

roughly 30 mg.), and it is, therefore, noteworthy that at the terminal stage of the infection tissues such as the leg muscles, heart, lungs, spleen, tongue, and urinary bladder may contain as much or more virus per unit of weight as the sciatic nerves. It would appear, therefore, that the virus content of those tissues cannot be accounted for on the basis of that which may be present in the nerve fibers within the substance of those tissues, if, for example, the sciatic nerves can be taken as in index to what may be present in the smaller nerve fibers within the leg muscles. Thus in the mice which were tested when they were moribund after intracerebral inoculation (96 hours, Table III), the virus content per milligram of tissue was about the same in the sciatic nerves and the leg muscles, urinary bladder, heart, lungs, and tongue. Since the amount in the blood was found to be negligible, this would suggest that the virus may either accumulate in these tissues by diffusion from the nerve fibers along which centrifugal spread from the central nervous system is occurring, or that it is multiplying in some other constituent of these tissues. The presence of nerve

cells of the autonomic system in some of these tissues (such as the heart and bladder as well as the intestinal tract) might be a factor in further multiplication of the virus, but would not explain what happens in other tissues such as the muscles. In a previous study carried out by one of us in association with Dr. Robert Ward, it was found that following intramuscular injection in young mice the St. Louis virus could produce extensive, necrotic lesions in the muscle fibers and their sheaths, suggesting a direct action of the virus on the muscles.

SUMMARY

1. Beginning at 24 hours after intravenous injection of about 10 million intracerebral LD_{50} of virus there was evidence of simultaneous, progressive multiplication in the brain and intestinal tract.

2. When the virus was introduced directly into the brain or the nasal cavities and mouth, none was found in the intestinal tract until there was general centrifugal spread from the central nervous system during the last stages of the infection at 96 or 120 hours after inoculation when the virus in the entire brain had reached a concentration of about 3 billion LD_{50}.

3. Centrifugal spread began when the virus in the brain reached a concentration of about 400 million LD_{50} and virus appeared in the pharynx, tongue, and adrenals before it was demonstrable in the intestinal tract, blood, or viscera such as the spleen, liver, and kidneys.

4. Despite the high concentrations of virus which developed in the intestinal tract following intravenous inoculation, it was not demonstrable in the stools, differing in this respect from Theiler's virus in mice and poliomyelitis virus in human beings and monkeys.

5. No antiviral agent was found in the stools, but the urine of normal mice having a pH of 5.6, inactivated large amounts of St. Louis encephalitis virus.

6. There was no evidence of multiplication in the nasal mucosa of mice which succumbed with encephalitis following nasal instillation of the virus, the course of events being comparable in this respect to the behavior of the M.V. poliomyelitis virus in *rhesus* monkeys.

7. At the terminal stage of infection the virus content per milligram of tissue was as great in the leg muscles as in the sciatic nerves. Since this was a lso true for the urinary bladder, heart, lungs, and tongue among other tissues, and since the amount in the blood was too negligible to account for it, it would appear that the virus either accumulated in these tissues by diffusion from the nerve fibers, along which it was spreading from the central nervous system, or that it multiplied in some constituent other than the nerve fibers.

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