

## EXPERIMENTAL TYPHUS INFECTION IN THE EASTERN COTTON RAT (*SIGMODON HISPIDUS HISPIDUS*)

By CHARLES R. ANDERSON, M.D.

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

(Received for publication, July 27, 1944)

In a preliminary report (1) the susceptibility of the cotton rat to experimental infection with *Rickettsia prowazeki* of the Breinl strain and of 3 Madrid strains was described. A fatal infection in rats was found to result from the intraperitoneal, intracardial, or intranasal inoculation of heavily infected yolk sac suspensions, although susceptibility to intraperitoneal inoculation was slightly less than to inoculation by the other 2 routes. Further, a fatal infection with the Madrid-1 strain was carried through 5 passages by intracardial inoculation of liver material. The addition of specific immune serum to yolk sac suspensions was shown to prevent fatal outcome. Finally, it was noted that old rats resisted a dose which was lethal for younger, smaller animals.

Further studies with the cotton rat have since been carried out. Attempts to maintain passages resulting in fatal infection were unsuccessful beyond the 6th serial passage. Subsequent attempts, however, employing a modified technique have allowed us to maintain several typhus strains continuously in rats, with uniformly fatal infection. Additional observations have been made upon general aspects of the infection in cotton rats, including the problem of susceptibility and the development of a technique of serum neutralization in which cotton rat tissues are employed as the source of the infectious agent. In the following report the results of these studies are presented.

### *Materials and Methods*

*Cotton Rats.*—In most of the experiments cotton rats weighing from 20 to 30 gm. and between 3 and 4 weeks of age were used. The rats were from a colony that has been inbred for several years and were uniform in size. In the laboratory they grew well on a diet of wet whole wheat bread and fox chow.

*Strains of Rickettsiae.*—The Breinl and South American strains of louse-borne typhus and the Wilmington strain of murine typhus were used in these experiments. The Breinl and Wilmington strains were obtained some years ago from the National Institute of Health at Bethesda, Maryland. The South American strain was isolated in guinea pigs inoculated with the blood of a patient dying of louse-borne typhus in Bogota, Colombia. In this instance the guinea pigs of the 1st passage were sent directly from Bogota to this laboratory, where the strain was studied. Although the guinea pigs in the first few passages showed a high incidence of scrotal swelling, this phenomenon became infrequent in subsequent passages. The strain now shows all the characteristics at present associated with louse-borne typhus strains. Except as stated in the text, passage of all of these strains had been in guinea pigs prior to their use in the present experiments.

*Passages in Cotton Rats.*—Usually the livers of 2 rats, killed by ether, were pooled as the virus source for intracardial passages. These were removed as completely as possible, using sterile precautions and taking particular care to avoid perforating the alimentary tract. The livers, which averaged 1.5 gm. in 20 to 30 gm. rats, were ground with alundum in a mortar and suspended in 8 or 10 cc. of broth or distilled water. The resulting suspension was centrifuged at 1000 R.P.M. for 10 minutes. A smear of the supernatant, stained by Macchiavello's method (2), was examined microscopically to note the presence or absence of rickettsiae and bacteria. The succeeding passage was made into 4 rats which, under light ether anesthesia, were inoculated intracardially with 0.1 to 0.2 cc. of the supernatant.

For intracerebral passages the brain removed from an animal killed by ether was ground in a mortar with alundum, suspended in 5 to 6 cc. of plain broth, and centrifuged at 1000 R.P.M. for 10 minutes. The resulting supernatant was cultured and examined microscopically as described above and inoculated intracerebrally in volumes of 0.03 cc. into etherized rats of the succeeding passage.

For both intracardial and intracerebral inoculation a 0.25 cc. tuberculin syringe, fitted with a  $\frac{1}{8}$  inch 27 gauge needle, was employed.

*Neutralization Tests in Cotton Rats.*—Heavily infected cotton rat liver suspensions were used as antigen in all of the neutralization tests. Groups of from 15 to 20 rats weighing between 20 and 30 gm. were inoculated intracardially with a liver passage suspension of the desired strain. After from 48 to 72 hours, when a few animals were dead, the remaining sick animals were sacrificed and their livers were removed, weighed, and placed in a Waring blender together with enough broth to make a 20 per cent suspension. After homogenization the suspension was immediately frozen in sealed pyrex ampoules and stored at  $-76^{\circ}\text{C}$ . until used.

Sera for testing were inactivated at  $56^{\circ}\text{C}$ . for 30 minutes. Fivefold dilutions of each serum were prepared in saline and pipetted into small test tubes. In each test there were included known normal and immune serum controls prepared in a similar manner. The stored liver suspension to be used as antigen was then thawed rapidly and centrifuged for 10 minutes at 1000 R.P.M. to remove large particles and fibrin shreds. The resulting supernatant was diluted in broth to twice the concentration desired for the final test and distributed so that all tubes contained equal volumes of antigen and serum dilutions. In addition, serial dilutions (usually fivefold) of the antigen in broth were prepared for the titration of its 50 per cent mortality end point (3). The concentration of antigen employed in the test was determined on the basis of previous titrations and was that which, when mixed with an equal volume of normal serum, would contain just enough rickettsiae in the volume inoculated to kill all rats. With the Breinl strain from 10 to 20 M.L.D. usually were sufficient, while with the Wilmington murine strain from 40 to 60 M.L.D. were required per rat.

After the antigen had been distributed, each tube was shaken individually to mix thoroughly the serum and antigen. All tubes were then placed in ice water where they were allowed to remain for an "incubation" period of 1 hour and also during the time required for inoculation. From each tube 4 rats were inoculated intracardially, each receiving 0.1 cc. To insure obtaining reproducible results great care was employed in this inoculation. If any doubt existed as to the introduction of the inoculum into the heart, the animal was discarded.

The animals were observed daily for 8 days after inoculation in tests with Breinl antigen and for 12 days when Wilmington antigen was employed. Rats dying within the first 2 days were excluded from the test. The final results were expressed in terms of the death or survival of the remaining animals.

*Toxin-Neutralization Tests in Mice.*—The procedure followed in the neutralization tests with the mouse was essentially that described by Bengston, Topping, and Henderson (4). The only differences were that before inoculations the serum-antigen mixtures were incubated for 1 hour in ice water instead of at room temperature, and that the amount inoculated into the tail vein was 0.2 cc. instead of 0.5 cc.

*Calculation of Titration End Points.*—All titers were calculated by the 50 per cent end point method of Reed and Muench (3). In all cases the figures presented refer to the final dilution of the original material employed and bear no relationship to the volumes inoculated.

EXPERIMENTAL

*The Course of Typhus Infection in Cotton Rats*

*Signs of the Disease.*—Typhus infection in cotton rats inoculated intracardially presented a typical course.

TABLE I  
*Titration of Liver Suspensions Infected with Different Strains of Typhus*

Breinl		Murine	
Dilution	Days after inoculation	Dilution	Days after inoculation
	0 1 2 3 4 5 6 7 8		0 1 2 3 4 5 6 7 8 9 10 11
1:10	D	1:10	D
	D		D
	D		D
	X used for passage, dying		
1:50	D	1:40	D
	D		D
	D		D
1:250	D	1:160	D
	D		D
	D		D
	X		D
1:1250	D	1:640	D
	S		D
	S		S
1:6250	S	1:2560	D
	S		S
	S		S
	S		S

D = death from typhus  
X = death not due to typhus  
S = survival

The first sign usually noted was irritability, which was manifested by exaggerated movements when the rats were stimulated, and a hunched, tense habitus. The following day the rats failed to eat well, and their fur was ruffled. The disease then progressed rather

rapidly. The rats became sluggish and developed conjunctivitis with a purulent exudate. Within the next 24 to 48 hours the animals were completely prostrated and usually had generalized or localized convulsions. The rate of respiration in the moribund animals was almost always slower than normal, and the respirations were often of the Cheyne-Stokes type. With large doses the entire course of the disease was shortened, and often the rats succumbed during a severe convulsion within a few hours after the onset of apparent illness. The signs presented by infection with the murine strain were the same as those in animals infected with the Breinl or South American epidemic strains, the only difference being that the course of the murine disease was more prolonged. Cotton rats that received just sublethal doses usually became obviously sick about the 6th or 7th day with the Breinl strain and about the 8th or 9th day with the Wilmington murine strain.

*Relation of Dose to the Time of Death.*—In Table I are presented the results of titrations of 2 typical liver suspensions, one infected with the Breinl epidemic strain and the other with the Wilmington murine strain. In each the inoculum was 0.1 cc. given intracardially. The data reveal several interesting differences between the murine and the epidemic type of infection in the cotton rat. Rats infected with murine rickettsia died, on the average, several days later than the animals that received a similar dose of the Breinl strain. This difference, with minor variation, held true for the other epidemic and murine strains that were studied. Although not shown in Table I, it should be stated that it was possible to prepare liver suspensions infected with the murine strain which would kill cotton rats within 2 or 3 days after inoculation.

Also, the susceptibility of the cotton rat to the Breinl strain apparently was more uniform than its susceptibility to the murine strain. This was manifested by the greater uniformity of the time of death within each dilution group of Breinl-infected rats and also by the sharper demarcation of the lethal end point.

#### *Pathology of Typhus in Cotton Rats*

Although a systematic pathological study has not yet been carried out, a number of observations have been made.

*Gross Findings.*—Gross pathological examinations of dead or dying cotton rats revealed little. After intracerebral inoculation of infected yolk sac the organs of the dead animals showed no grossly abnormal lesions except in the cranial cavity. In addition to slight hemorrhage at the site of inoculation, there was sometimes dilation of the surface blood vessels and usually a thin exudate covering the brain. Following intraperitoneal inoculation of heavily infected liver suspensions only the abdominal cavity showed gross evidence of the disease. The contained viscera were covered by a thick, pearly gray, fibrinous exudate and the intestines often were firmly matted together by adhesions. In animals which died following intracardial inoculation of heavily infected liver suspensions, a pericarditis developed. The exudate varied greatly in its appearance but was usually thin and not so fibrinous as the peritoneal exudate. There was also a yellowish necrotic area in the heart wall at the site of the inoculation. The spleen was usually of normal size but at times seemed slightly enlarged. The liver ordinarily was darker than normal and sometimes presented a marked "nutmeg" appearance. In addition to a usual vascular congestion the intestines at times presented multiple punctate hemorrhages. This finding was particularly frequent in animals infected

with one Breinl substrain. The lungs usually showed no gross lesions, but from time to time small areas of consolidation were observed.

*Microscopic Findings.*—Rickettsiae were best demonstrated in impression smears of the various organs or in smears of suspensions of these organs stained by the Macchiavello technique (2). In sections the rickettsiae were much more difficult to detect. The greatest number of rickettsiae were found in the liver, and the next most heavily infected organ was the brain. Smaller numbers were found in all of the organs examined, including spleen, lung, kidney, heart, and muscle. The exudates resulting from intracardial and intraperitoneal inoculation contained enormous numbers of rickettsiae, both lying free and in cells. However, the exudate around the brain after intracerebral inoculation contained a smaller number of organisms.

TABLE II  
*Lethal Titers of Several Suspensions of Liver from Cotton Rats Infected with Different Strains of Typhus*

Breinl strain		South American strain		Wilmington murine strain	
Pool No.	Lethal titer*	Pool No.	Lethal titer*	Pool No.	Lethal titer*
485	160	421	63	495	1250
	160		230‡		
494	710	450	80	544	970
			80		
543	125	461	160		
		465	63		
			50		
			63		

\* All titers are recorded as the dilution which should cause a 50 per cent mortality.

‡ This titer calculated from tenfold dilutions.

Sections were prepared by a technique similar to that described by Wolbach *et al.* (5), from the organs of animals dying of typhus following intracardial inoculation of a suspension of liver infected with the Breinl strain. They were fixed in Regaud and stained with Giemsa. Sections of the spleen revealed sinusoids engorged with blood and very conspicuous lymphoid follicles giving the impression of increased activity. The kidneys showed small areas of tubular necrosis and occasional tubules containing red cells. In sections of the left ventricle areas of infiltration by mononuclear and lymphoid cells, similar to those described by Wolbach *et al.* in humans (5), were observed. However, necrosis of the muscle fibers as described by these authors was not detected with certainty. Microscopic changes were more evident in the liver than in any of the other organs. The portal vessels appeared to be distended with blood, and in the surrounding area there were fairly large numbers of mononuclear lymphoid cells. The reaction decreased in intensity as one moved from the portal vessels toward the central vein. The Kupfer cells were very conspicuous and appeared swollen, often filling the liver sinusoids. Their cytoplasm was granular and contained a large amount of dark staining material. Rickettsiae could be found in cells lining the sinusoids but were not easy to demonstrate. Sections of the lungs and brains failed to reveal any notable changes.

*Recovery of Rickettsiae from Infected Cotton Rats.*—Attempts to recover rickettsiae from the blood, brain, liver, and peritoneal washings without bacterial contamination were relatively successful. Even in suspensions of liver used for routine passages bacteria were demonstrated only on a single occasion. In eggs inoculated into the yolk sac (6) with a suspension of liver or brain from rats which had been intracardially inoculated, heavy infections were obtained in the first passage. Isolation of rickettsiae in eggs from blood was less often successful in that the yolk sac suspensions of the first passage were much less heavily infected.

#### *The Relative Susceptibility of Cotton Rats to Typhus Infection*

*The Minimal Lethal Dose and the Minimal Infectious Dose.*—The determination of the minimal lethal dose, as already described, represented a rapid and fairly satisfactory method of estimating the relative infectivity of preparations heavily infected with rickettsiae of either the louse-borne or the murine type. Representative examples of the 50 per cent mortality end points obtained in titrations of liver suspension pools made with different strains of rickettsiae are presented in Table II. In most cases the volume inoculated intracardially was 0.1 cc., and the dilutions employed were four- or fivefold. The infected organ suspensions had all been stored at  $-76^{\circ}\text{C}.$  for varying periods of time up to several months after preparation. Several duplicate and triplicate titration end points were included to demonstrate the reproducibility that may be expected.

From the relatively low lethal end points obtained for suspensions rich in rickettsiae it is evident that massive doses are required to produce a fatal infection in cotton rats. Actually, studies of the subsequent immunity of animals surviving the inoculation of non-lethal doses of infectious material indicated that the cotton rat was much more susceptible to typhus infection than the mortality end points suggested. Determination of the immunizing end points (based upon resistance to challenge with a known lethal dose) revealed that, with both the Wilmington and the Breinl strain, a lethal dose consisted of 10,000 to 100,000 immunizing doses.

The relation between the minimal infecting dose and the minimal immunizing dose was investigated in experiments with both the Breinl and the Wilmington strain.

Serial dilutions of infectious preparations were inoculated intraperitoneally into groups of 5 rats. After 1 week the brains and livers of 2 rats in each dilution group were removed and suspended as a pool in 15 cc. of broth. After centrifugation the suspensions from each pair of animals were passed to new groups of rats, each of which received 2 cc. of the supernatant intraperitoneally. Three weeks later intracardial challenge inocula were administered to the remaining rats in the original series, to the passage animals, and to suitable normal controls.

It was found that, if the suspended livers from a pair of sacrificed rats had produced an immunizing infection in the passage animals, the remaining members of the same dilution group also resisted the challenge inoculum. Hence it was concluded that an immunizing dose and an infecting dose were the same.

*The Influence of Age upon Susceptibility.*—In the earlier report (1) it was stated that as cotton rats became older their susceptibility decreased very strikingly, and the data implied that old rats became totally refractory. The original work was done employing an infected yolk sac suspension of an epidemic strain. However, further work using similar material showed a less striking relation between age and susceptibility. Although rats of 2 to 3 weeks

TABLE III  
*Influence of Age on Susceptibility to Typhus*

Strain	Lethal titers* in 3 to 4 wk. old rats (20 to 30 gm.)	Lethal titers* in 6 to 7 wk. old rats (35 to 50 gm.)
South American.....	230	63
South American.....	80	80
Breinl.....	160	160
Breinl.....	127	320
Wilmington.....	970	475
		145

\* Results recorded as the dilution of liver containing one 50 per cent mortality dose in the volume inoculated.

of age (weighing from 15 to 20 gm.) often proved more susceptible than older rats, the individual variability of the latter was so great that they have not been extensively used. Within the age range more commonly employed (from 3 to 7 weeks) little difference in susceptibility could be detected. This is illustrated in Table III in which are presented the results of several intracardial titrations of infected liver suspensions in 3 to 4 week old rats (from 20 to 30 gm.) and in rats of 6 to 7 weeks (from 35 to 50 gm.). Only in the case of the Wilmington strain, with which our experience has been considerably less, did it appear that the older rats developed increased resistance to infection. For most experimental procedures, however, this has proved unimportant, since it has always been possible to administer a fatal dose.

*The Susceptibility of Cotton Rats as Compared with That of White Mice and Guinea Pigs.*—Although this problem was not investigated extensively, the observations made indicate that of the strains employed in these studies only the Breinl strain differed significantly in its infectiousness for mice as com-

pared with that for cotton rats. With this strain rats could be immunized against a lethal challenge dose by a dilution more than a thousand times greater than the maximum dilution capable of immunizing mice against a fatal toxic dose of the homologous strain. Using Breinl-infected guinea pig brain suspensions, it was found that the minimal immunizing dose for guinea pigs and cotton rats was the same. The Wilmington and South American strains have not as yet been compared in guinea pigs and cotton rats, but there has been no evidence thus far to indicate that there would be much difference in the susceptibility of the two animals.

*Relationship of Typhus "Toxin" to the Disease in Cotton Rats.*—Gildemeister and Haagen (7) were able to kill mice in a few hours by the intraperitoneal inoculation of yolk sac suspensions heavily infected with a murine strain of typhus. Bengston, Topping, and Henderson (4) have reported that yolk sac suspensions of epidemic strains of typhus also contain a toxic factor. Though doses 30 to 50 times greater than those that would kill mice were inoculated into cotton rats, it was not possible to kill them within 24 hours. Rats given such doses often died during the 2nd day after inoculation, but death was very probably due to infection. Whether or not "toxin" plays a rôle in the cotton rat disease is still uncertain. It is of interest, however, that infected cotton rat liver suspensions were shown to be essentially as toxic for mice as infected yolk sac suspensions of the same strains.

The exact relationship between the 50 per cent mortality toxic dose for mice and the 50 per cent mortality infective dose for cotton rats varied with the strain but in general the lethal infective dose for rats was considerably less than the lethal toxic dose for mice. The number of M.L.D. for rats contained in 1 M.L.D. (toxic) for mice was approximately 4 for the Breinl strain, 10 for the Wilmington murine strain, and 20 for the South American strain.

#### *Serial Passage of Typhus Infection in Cotton Rats*

*The Initiation and Maintenance of Fatal Typhus Infection by Intracardial Inoculation.*—During the study of the course of typhus infection in cotton rats inoculated intracardially with heavily infected yolk sac suspensions it was noted that: (a) typhus infection was not highly virulent for the cotton rat since large numbers of rickettsiae were contained in a minimal lethal dose; (b) fewer rickettsiae occurred in smears of organs of rats dying after inoculation of a minimal lethal dose than in smears from rats dying after a somewhat larger dose; (c) intraperitoneal inoculation of yolk sac suspensions was followed during the first few days by an increase in leucocytes in the peritoneal cavity which later in the disease were largely replaced by mononuclear cells; (d) passages from liver of animals succumbing to a minimal dose often failed to produce a lethal infection in the passage rats; (e) there existed a very close relationship between the time of death after inoculation and the size of the infecting dose, even though the range was small.

On the basis of these observations it was postulated that rickettsial multiplication in the cotton rat is limited, possibly by the early onset of an immune reaction suggested by the development of the mononuclear cell response; that a large total number of rickettsiae are necessary to cause an amount of damage sufficient to be fatal; and that, because of the limits imposed upon rickettsial multiplication, this necessary total number of rickettsiae is achieved only when relatively large numbers are contained in the original inoculum. In the light of this hypothesis, the early attempts to maintain a serially passed lethal infection in rats failed because the liver suspensions employed after the 6th transfer contained insufficient virus. A review of the records suggested that the initial failure was due to the use of an unduly dilute suspension of liver and that the subsequent failures were attributable to too great delay in harvesting the livers, which by the 6th or 7th day no longer contained a high concentration of rickettsiae. This delay resulted from waiting, usually in vain, for the rats to develop signs of serious illness before sacrifice for passage.

A new attempt to maintain a fatal infection in passage was begun with a yolk sac preparation of the South American strain and employing the techniques described under Materials and Methods. The passages were made on the 2nd or 3rd day from animals that were obviously sick. Except for those sacrificed for passage, all of the animals in a series of more than 50 transfers, including those of the first passage, succumbed to the infection within 2 to 5 days after inoculation, with most of the deaths occurring on the 3rd and 4th days.

An attempt was then made to determine whether, by making passages at a time when the rickettsiae had multiplied to a maximum level, the number of organisms might be increased sufficiently so that an originally non-fatal infection would become fatal after a few transfers. It was reasoned that the peak of rickettsial multiplication preceded the onset of any immune response which, as possibly indicated by the development of the mononuclear cell response, might be taken to occur from the 4th to the 6th day after inoculation. Accordingly, a 4 day passage interval was decided upon. Using this interval and the intracardial passage technique described under Materials and Methods, a passage was initiated from a 10 per cent suspension of the brain of a cotton rat inoculated in the 55th intracerebral transfer of the Breinl strain. During this long passage series no rats had died except those in the 1st passage. The liver suspension of the 3rd passage of this new series contained recognizable rickettsiae. In the 7th passage one fatal infection occurred, and since the 8th passage all rats have succumbed except those sacrificed to make transfers. A second Breinl series was then begun using a suspension of guinea pig brain containing about 100 guinea pig infectious doses, or approximately the amount encountered in blood from human typhus cases. Following the technique described above, the presence of rickettsiae in the smears of liver suspensions was noted on and

after the 2nd passage, and from the 7th passage on all animals died. This series was stopped after the 15th passage.

The serial passages of the Wilmington murine strain were started by inoculating a suspension of infected guinea pig tunica intracardially into cotton rats. The liver passages were made on the 6th and 7th days following inoculation because the course of the murine infection was more prolonged than that of the epidemic type of infection. Rickettsiae were seen in smears of the liver suspension of the 3rd passage, and all but 2 of the animals in the subsequent passages died. These failures were in all probability the result of technical errors at the time of inoculation. This strain was carried through more than 60 passages.

*Effect of Prolonged Intracardial Passage on the Virulence of Typhus Strains for Cotton Rats.*—One change that has been found to occur frequently during continuous passage of an infection in a particular animal species has been an increase in virulence for that animal. In general this has been demonstrated by comparing the effect of infectious material from early and late passages of the same series. Because material from the early passages of typhus strains in cotton rats did not contain sufficient rickettsiae to produce a fatal infection, it was necessary to compare the virulence of late passage material with that of homologous rickettsiae obtained from a different host.

A comparison was made, therefore, of the virulence of the following two preparations of Breinl rickettsiae: (a) a yolk sac suspension of a substrain which, after more than 90 passages in eggs, was carried through 3 passages in cotton rats and then returned to eggs for an additional 3 passages; and (b) a cotton rat liver suspension representing the 47th passage in the Breinl intracardial series described above.

Prior to its maintenance in intracardial passage the substrain had been carried through 55 intracerebral passages, so the material tested represented the 102nd passage in cotton rats. Both preparations were frozen and stored at  $-76^{\circ}\text{C}$ . To determine the immunizing or infectivity end points, a series of rats was inoculated intraperitoneally with falling tenfold dilutions of each preparation. Three weeks later the rats received as a challenge a lethal dose of the same preparation with which they had been originally inoculated, administered intracardially. At this time the respective preparations were titrated for their lethal end points by intracardial inoculation of serial fourfold dilutions into normal rats of the same age. From the latter titrations it also was determined that the challenge inocula had contained 16 and 32 M.L.D. of the yolk sac and cotton rat liver preparations, respectively.

The results of this experiment are presented in Table IV and indicate that the difference between the lethal and immunizing end points of each suspension was the same. To supplement this experiment a broth suspension of the brain of a cotton rat of the 98th passage of the same strain was inoculated into the yolk sac of several 6 day old chick embryos. With the yolk sac suspension prepared from these embryos the mortality and infectivity end points

were determined as described above. The same relationship between the lethal and infective end points was found to exist.

Similar data obtained in reference to the other strains of typhus, together with the constancy of the day of death in routine passage animals and the relatively slight variations in the numbers of rickettsiae seen in smears of the particular strain, tend to coincide with the view that the virulence of none of the strains has so far been markedly affected by prolonged passage in cotton rats.

TABLE IV  
*Relationship of the Infectivity and Lethal End Points of Liver and Yolk Sac Suspensions of the Breinl Strain of Typhus*

Infectivity titration*			Lethal titration†		
Dilution	Yolk sac	Liver	Dilution	Yolk sac	Liver
10 <sup>-5</sup>	5/5	4/4	1:10		4/4
10 <sup>-6</sup>	5/5	5/5	1:20	4/4	
10 <sup>-7</sup>	5/5	5/5	1:40		4/4
10 <sup>-8</sup>	5/5	3/4	1:80	4/4	
10 <sup>-9</sup>	0/5	1/4	1:160		3/4
10 <sup>-10</sup>	0/4		1:320	2/4	
			1:640		1/4
			1:1280	0/4	
Titers.....	320,000,000	320,000,000		320	320

\* The results recorded in the infectivity titration are those obtained after administering the challenge dose. The numerator indicates the number of animals surviving the challenge and hence immunized by the previous inoculation; the denominator indicates the number of rats challenged.

† In the lethal titration the results are expressed as the ratio of the number of rats succumbing to infection to that inoculated.

*Intracerebral Passage in Cotton Rats.*—A lethal infection has been maintained in serial passage in cotton rats by only one route of inoculation other than the intracardial and with only one strain of rickettsiae.

The Wilmington murine strain has been passed as a lethal infection by intracerebral inoculation of infected brain suspensions. The series was started with infected cotton rat liver, and the passages were made with a 10 per cent dilution of brain in broth. A volume of 0.03 cc. was inoculated intracerebrally under light ether anesthesia. The infection was carried through 37 passages in young rats; during the first 10 passages very few animals died, but in the subsequent passages most succumbed.

The rats usually showed signs of encephalitis on the 3rd or 4th day after inoculation. The first sign was irritability, followed by loss of appetite, convulsions, prostration, and death. The picture was very similar to that seen in animals infected intracardially except that convulsions played a more prominent rôle and often were followed by death. Rickettsiae could

be seen in smears of the brain suspensions, but only on rare occasions were organisms found in smears from the other organs. Death in most cases occurred between the 5th and 7th days after inoculation although the total range of occurrence was from the 4th to the 11th day. Fatal infections, furthermore, could be produced with near uniformity only in young cotton rats between 2 and 3 weeks of age. On reaching 3 or 4 weeks of age the rats became very much more resistant to intracerebral infection, and repeated attempts to maintain serial passages resulting in fatal infection in such rats failed. This increased resistance was only relative, however, since these older rats would succumb to intracerebral injection of heavily infected yolk sac suspensions and also to intracardial injection of the brain passage material.

It has not been found possible to kill cotton rats in serial passages by the intracerebral route with any of the louse-borne strains, but suspensions of heavily infected yolk sac inoculated intracerebrally regularly killed most of them. The Breinl strain was passed over 50 times and the South American strain over 30 times without killing any of the animals except those in the 1st passage. When a 10 per cent brain passage suspension was inoculated intraperitoneally into guinea pigs, their temperature rose to 40°C. or over within 48 hours, indicating the presence of large numbers of organisms, and these suspensions were found to immunize cotton rats in dilutions as high as 10<sup>-5</sup>.

It was possible to alternate a yolk sac passage with an intracerebral passage and to maintain a fatal infection in this manner with the Breinl strain. However, subsequent brain to brain transfers in cotton rats failed to produce a lethal infection for more than 1 passage. Thirteen such alternate passages were made with no evidence of increase in virulence.

Animals inoculated intracerebrally were immune to challenge by the intracardial route.

#### *Immunological Studies in Cotton Rats*

The immunological studies thus far have been restricted to an exploration of the possible uses of the cotton rat as a test animal in studies of the antigenic structure of *Rickettsia prowazeki*.

*Postinfection Immunity.*—It already has been mentioned that sublethal but infectious doses of typhus rickettsiae confer upon rats a solid immunity to large and certainly lethal challenge doses of homologous virus. Cross-immunity between louse-borne and murine strains was also demonstrated. In experiments in which cotton rat liver suspensions were employed, this cross-immunity appeared to be complete. However, in one experiment in which animals were immunized with yolk sac material an indication was obtained that small doses of Breinl virus may not confer complete immunity to virus of the murine strain, although minimal infectious doses of murine rickettsiae produced a solid resistance to subsequent Breinl infection.

Although the sera of convalescent cotton rats were not studied exhaustively, a number of pertinent observations were made. By means of the neutraliza-

tion test in mice such sera were shown to possess antitoxic antibodies in high titer. Also demonstrated were complement-fixing antibodies, opsonins, and antibodies capable of neutralizing typhus infection in cotton rats. The reactivity of these antibodies with heterologous antigen has not as yet been sufficiently studied. Agglutinins for proteus OX19 could not be demonstrated.

*A Neutralization Test in Cotton Rats.*—One of the most obvious applications of the susceptibility of cotton rats to typhus infection was the utilization of these animals for the demonstration of protective antibodies. Since the previous

TABLE V  
*Cotton Rat Protection Test Titers Obtained with Convalescent Sera*

Source of serum	Type of infection	Titer with Breinl antigen	Titer with Wilmington murine antigen
Cotton rat.....	Epidemic (Breinl)	559*	5, 8
Cotton rat.....	Epidemic (South American)	559, >250	46
Cotton rat.....	Murine (Wilmington)	30	22, 35, 110
Human.....	Epidemic	250	10
Human.....	Epidemic	>250	34, 50
Human.....	Epidemic	>250	10

\* Titers shown represent the dilution of serum which should protect 50 per cent of the animals.

> The titer was greater than the figure shown, which represents the highest dilution of serum which was tested.

report in which the neutralization of infected yolk sac suspensions was described (1), it was found possible to use as the test antigen suspensions of infected cotton rat livers, thus eliminating from the reaction possible complications arising from the presence of heterologous tissue proteins.

The technique at present employed is described under Materials and Methods. Numerous tests indicated that normal sera from human beings, cotton rats, guinea pigs, and rabbits possessed no protective capacity. Conversely, convalescent and other postinfection sera of similar origin were shown to contain easily demonstrable neutralizing antibodies.

In Table V are presented some of the titers obtained in the neutralization tests using the Breinl epidemic and Wilmington murine strains as antigens.

It was found that the titers of epidemic convalescent sera were higher when tested with an epidemic strain than were the titers obtained with murine sera when tested with the Wilmington murine strain. This difference in titer is probably explained by the fact that, to insure killing all of the controls, it was necessary to employ a test dose of murine antigen containing 3 or 4 times the number of lethal doses required in the case of the Breinl antigen. Although definite cross-neutralization was ordinarily demonstrable, it was not complete. Sera tested against antigens of homologous immunologic character revealed higher titers than when tested against a heterologous strain.

Cotton rats that survived as a result of the neutralization of the test dose were found to have developed an active immunity when challenged 3 weeks later by intracardial inoculation of a lethal dose of infected liver.

#### DISCUSSION

While this report was being prepared, Dr. O. L. Peterson of this laboratory, using the technique described above, established a lethal infection in cotton rats with 2 more strains of typhus. In both instances serial passages were begun with the intracardial inoculation of sublethal doses of infected yolk sac suspensions. One of the strains was of the louse-borne type and was isolated in Madrid, Spain, in 1941 by Dr. J. C. Snyder. The other was a typical murine strain isolated several years ago in South Africa by Dr. J. H. S. Gear. Thus, 5 strains, representing 4 continents and both types of typhus, have been established as a uniformly fatal infection in cotton rats by serial passages. It seems probable, therefore, that most other strains of typhus will prove similarly pathogenic when employed in a sufficiently large dose. To the writer's knowledge only one other animal has been described in which both types of typhus will produce a fatal infection, and at the present time there are not sufficient data to make a comparison of the two. This animal is the South African gerbil, genus *Tatera*, described by Gear *et al.* (8, 9).

Although the strains so far studied have been only moderately virulent for cotton rats, even after prolonged passage, it is possible that strains may exist which possess a higher virulence. The susceptibility of the cotton rat to infection, even with strains of moderate virulence, was found to be very great. This observation suggests that cotton rats could be used for isolation of new strains. However, in attempting to develop a newly isolated strain into a fatal infection as rapidly as possible, the cardinal points to be borne in mind are the necessity of inoculating as large a dose as possible and of making passages as soon as maximum rickettsial multiplication shall have occurred. It also should be emphasized that new strains may differ from the laboratory strains in respect to their rate of multiplication.

Many of the problems which could be studied with the aid of cotton rats are too obvious to be expanded. These are mostly of fundamental investigative

nature. Cotton rats should, for example, lend themselves well to studies of the pathological physiology of typhus infections, both fatal and non-fatal. A problem of current importance is the comparison of various methods of detecting antibodies. The use of the cotton rat in such a study is peculiarly appropriate since its convalescent serum contains protective, antitoxic, and complement-fixing antibodies and its infected tissues can provide the necessary antigens for all these tests. The preparation from cotton rat peritoneum of rich suspensions of rickettsiae relatively free of cells and tissue debris has just been described (10). The same tissue has also been used as a source of complement-fixing antigen.

The use of the neutralization test in cotton rats as a general diagnostic procedure is probably precluded by the data obtained by the United States of America Typhus Commission as to the accuracy of the complement fixation test (11). However, should it be found that neutralizing antibody persists longer than other antibodies, its demonstration would have a valuable application as a more sensitive indicator of previous infection. Some indication of this can be found in long-term studies of infected cotton rats. The applicability of the neutralization technique would be broadened if a strain of rickettsia could be found which could be neutralized in high titer by sera of both murine and epidemic origin. That such a strain may exist is suggested by the fact that of the strains already investigated some appear to cross more than others when used as antigen in the neutralization test.

#### SUMMARY

1. The course of typhus infection in cotton rats has been described, and a relationship between the dose administered and the time of death has been demonstrated.

2. Rather incomplete pathological studies indicated that typhus in the cotton rat is an acute infection primarily involving mesothelial tissue and tissues of mesothelial origin. Depending somewhat upon the route of infection, rickettsiae were most easily demonstrated in smears from liver, brain, and pericardial and peritoneal exudates. By subinoculation into the yolk sacs of fertile eggs, rickettsiae were readily isolated from these sources and, with rather less facility, from blood.

3. Although relatively large numbers of rickettsiae were found necessary to produce lethal infections, the susceptibility of the cotton rat as measured by the development of an immunizing infection proved to be very high. The supporting data suggest that cotton rats might be suitable for the isolation of new strains.

4. Contrary to the earlier report (1), age was not found to influence markedly the susceptibility of the cotton rat to lethal infection with any of the strains studied.

5. Comparative studies indicated that cotton rats were just as susceptible to murine infection as white mice and more susceptible to Breinl infection than the latter animal. The susceptibility of cotton rats and of guinea pigs to Breinl infection was found to be about the same. Comparative studies in these animals were not made with other strains.

6. No toxic activity of rickettsial suspensions could be demonstrated for cotton rats, although suspensions of infected cotton rat livers were shown to be toxic for mice.

7. A method has been described by which three strains of *Rickettsia prowazeki* were carried in serial intracardial passage as fatal infections for cotton rats. No evidence was obtained that prolongation of such passage increased the virulence of the strains for the rats.

8. Cotton rats surviving typhus infection were shown to be solidly immune to reinfection with homologous virus and to possess nearly complete immunity to rickettsiae of heterologous immunologic character. Sera from recovered rats, furthermore, were shown to possess neutralizing, complement-fixing, and antitoxic antibodies but no agglutinins for proteus OX19.

9. The technique of a serum neutralization test in cotton rats has been described in which the test antigen consists of suspensions of infected cotton rat livers. By this technique neutralizing antibody was demonstrated in human, rabbit, guinea pig, and cotton rat sera. Although some degree of cross-neutralization could be shown, serum titers against homologous antigen were uniformly greater than against comparable doses of antigen of heterologous immune nature.

The writer is indebted to Dr. O. L. Peterson for the performance of the complement fixation tests referred to in this paper.

#### BIBLIOGRAPHY

1. Snyder, J. C., and Anderson, C. R., *Science*, 1942, **95**, 23.
2. Zinsser, H., Fitzpatrick, F., and Wei, H., *J. Exp. Med.*, 1939, **69**, 179.
3. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
4. Bengston, I. A., Topping, N. H., and Henderson, R. C., confidential report.
5. Wolbach, S. B., Todd, J. L., and Palfrey, F. W., *The etiology and pathology of typhus*, The League of Red Cross Societies, Cambridge, Harvard University Press, 1922.
6. Cox, H. R., *Pub. Health Rep., U. S. P. H. S.*, 1938, **53**, 2241; 1940, **55**, 110.
7. Gildemeister, E., and Haagen, E., *Deutsch. med. Woch.*, 1940, **66**, 878.
8. Gear, J., and Davis, D. H. S., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1942, **36**, 1.
9. Gear, J., Harris, L. C., and Saner, R. G., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1942, **36**, 95.
10. Anderson, C. R., *J. Bact.*, 1944, **47**, 519.
11. Plotz, H., personal communication.