

EXPERIMENTAL INVESTIGATIONS ON THE
RESPIRATION AND PATHOGENESIS OF TYPHUS FEVER RICKETTSIAE

by

Theodore G. Metcalf
B.S., Massachusetts College of Pharmacy, 1940

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Advisory Committee

Redacted Signature

Chairman

Redacted Signature

Redacted Signature

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INTRODUCTION

The discovery of the rickettsiae by Ricketts and Wilder in 1910 revealed the presence of microorganisms whose morphology was similar to that observed for bacteria. Unlike the bacteria, however, the rickettsiae could not be cultivated in any of the known media acceptable for the propagation of bacteria.

The investigations of the rickettsiae from 1910 to 1938, while varied in nature, illustrated the difficulties which were encountered in the cultivation of these microorganisms. The use of the developing yolk sac of the chick embryo for the cultivation of the rickettsiae, which was reported by Gox in 1938, solved the troublesome problem of rickettsial propagation. This method was not only convenient but resulted in predictable yields of large numbers of rickettsiae.

The large scale cultivation of the rickettsiae made possible many scientific advances in the field of tropical medicine which contributed greatly to the successful war efforts of the United States in World War II. The large scale production of an improved vaccine for the protection of American troops against the louse-borne and murine forms of typhus fever resulted in a remarkable record of relative freedom from this disease. The development of improved laboratory methods for the diagnosis of the rickettsial

diseases ensured the rapid recognition of these diseases when they occurred.

The studies which are dedicated to the improvement of existing methods and technics continue to occupy the attention of many biologists engaged in rickettsial research. Of increasing interest and significance, however, is the appearance of studies that do not seek an immediate application of practical value, but instead are concerned with the biology of the rickettsiae. Among these studies are the investigations by Peterson and Fox on the mode of action of toluidin blue and the thionin dyes in experimental typhus infections of mice; the use of enzyme inhibitors and activators in experimental infections of mice and the chick embryo by Pinkerton, Greiff and Moragues; and the investigations of rickettsial metabolism by Bovarnick, Snyder and Miller. The study of the physiological aspects of the cultivation of the rickettsiae by Zinsser in 1936 antedated the above studies and represented the first serious endeavor in the investigations concerned with the microbiology of the rickettsiae.

The studies which form the basis for the research which is presented in this report were suggested by the early work of Zinsser on the physiology of the rickettsiae. The present studies seek to extend the knowledge of the physiology of the rickettsiae, and to relate their biochemical activities to the pathogenesis of experimental infections induced in an appropriate animal host.

LITERATURE.

Typhus fever is a disease which has been recognized by the medical profession since the eighteenth century. The pages of history are replete with accounts of ravaging epidemics which decimated human populations during the middle ages. There seems to be little doubt that many of these terrible epidemics were actually typhus fever. Gerhard in 1837 was credited with the scientific description of the clinical aspects of typhus fever which made possible the separation of typhus fever patients from those suffering with typhoid fever. Although the disease was well characterized clinically after 1837, the etiological agent was not disclosed until the year 1910, when Ricketts and Wilder, working in Mexico, observed short bacillus-like forms in the blood of typhus fever patients (1). These forms exhibited polar staining and appeared as small bacilli.

In 1910, von Prowazek, working in Serbia, described many small rod shaped bodies within leukocytes in the blood of typhus fever patients. Sergent, Foley and Vialatte in North Africa reported similar bodies in lice removed from typhus infected persons in 1914 (2). These workers infected three human volunteers with typhus, one by louse feeding, one by subcutaneous injection of louse emulsion, and one by scarification with crushed eggs of infected lice. It remained for da Rocha-Lima, an associate of von Prowazek, to

summarize the previous findings and in 1916 to give the name Rickettsia prowazeki to the etiological agent of typhus fever (3).

The European or louse-borne typhus was the only type of typhus which was recognized until 1917. The existence of another form of typhus which could be differentiated from the louse-borne type in male guinea pigs was indicated by Neil, who reported the development of scrotal swelling in male guinea pigs injected intraperitoneally with blood from typhus patients in Texas (4). In well developed male guinea pigs injected intraperitoneally with Mexican typhus, the temperature of the animal rose from 40.5 to 41° centigrade nine to fifteen days after injection, and a definite scrotal swelling was observed. Hemorrhages were found in the cremasteric fascia, just external to the parietal laminae of the tunica vaginalis, and immediately beneath the visceral laminae of the tunica vaginalis. The scrotal swelling subsided after the temperature returned to normal and the lesions cleared up rapidly. The findings of Neil were not fully appreciated until Mooser in 1928 showed that over 90 per cent of the male guinea pigs injected with Mexican typhus presented more or less pronounced swelling and reddening of the scrotum which was due to extensive specific lesions in the tunica cremasterica, tunica vaginalis and testicles (5). In addition Mooser demonstrated a minute

intracellular diplobacillus in the sections and smears of proliferated tunica vaginalis. The small organisms could not be cultured on bacteriological media, were always present when histological findings characteristic of Mexican typhus were demonstrated, and stained poorly with all but Romanowsky type stains.

An investigation of Brills disease by Maxcy in the southeastern United States resulted in findings which substantiated the studies of Neil and Mooser (6, 7, 8). Brills disease was reported by Dr. Nathan E. Brill of New York as a typhus-like disease occurring endemically in that city (9). The identity of Brills disease with typhus fever was shown by Anderson and Goldberger following isolation of the etiological agent in monkeys (10). Maxcy's investigations were based on epidemiological considerations and emphasized essential differences between old world typhus and the endemic typhus reported by Brill. The seasonal incidence for the endemic form reached a maximum in the summer and fall months. Outbreaks in cities showed no sharp localization. A focal center of the disease in cities seemed to be in the heart of the business district and persons attacked were those engaged in trade, especially those employed in food depots, groceries, feed stores and restaurants. The disease was not associated with lousiness. Maxcy concluded that a reservoir of infection existed apart from man, and that the reservoir was

in rodents, probably rats or mice, from which the disease was occasionally transmitted to man.

The concept that Brills disease and the endemic typhus reported by Maxcy were identical was challenged by Zinsser (11). Following a study of Brills disease in New York and Boston, Zinsser was forced to the conclusion that the disease was an imported form of the classical European typhus fever. The cases observed in New York and Boston were said to represent recrudescences of old infections originally acquired in European foci. Zinsser's views have received wide attention and the acceptance of many authorities on this subject. There are a few critics, however, who maintain that Brills disease is murine typhus.

In 1931 murine typhus rickettsiae were isolated from fleas taken from wild rats in Baltimore (12). In the same year murine typhus rickettsiae were isolated from wild rats (13). By the year 1931 it was clear that two types of typhus fever existed, the old world or louse-borne form and the new world or murine form.

The use of experimental animals for typhus fever investigations was greatly stimulated by the findings of Neil, Mooser and others. While the use of animals as an aid in detecting the type of typhus fever was encouraged around 1928, the application of animal inoculation to the isolation and cultivation of typhus rickettsiae dates back to 1909 when

Nicolle, Comte and Conseil reported the infection of monkeys with blood taken from a typhus fever patient (14). The transmission of typhus to guinea pigs was accomplished in 1911 (15). Nicolle and co-workers reported the use of white rats in experimental typhus infections (16, 17, 18). Although rats exhibited no fever, brain emulsions from injected rats proved infective upon passage. This led to the term "infection inapparente." Mooser in 1929 and Maxcy in the same year reported a sharp rise in the temperature of male white rats three to six days after intraperitoneal injection of murine typhus rickettsiae (19, 20). No scrotal swellings were noted, even though tunica scrapings demonstrated the presence of rickettsiae.

Other animals have been found which represent susceptible hosts. The susceptibility of rabbits, white mice and gerbils to louse-borne typhus was shown by Nicolle (21, 22). In a recent report by Snyder and co-workers the use of two species of gerbils in experimental typhus infections was studied (23). The rodents succumbed to large doses of either murine or louse-borne typhus rickettsiae by the intranasal, intravenous or intraperitoneal routes. White mice did not succumb to infection. Their response to louse-borne typhus assumed the form of an inapparent infection which could not be transmitted serially in mice. Similarly when white mice were infected with murine strains, the

infection was of the inapparent type, but could be transmitted serially in mice (24, 25, 26). Wohlrab not only confirmed the susceptibility of white mice to murine typhus but was able to produce infections which terminated in the death of the injected mice (27). Castaneda was able to produce a highly fatal pneumonitis in white mice following the intranasal injection of murine typhus rickettsiae (28). Lépine reported that the Macedonian spermophile (Citellus citellus) was susceptible to louse-borne but not murine typhus, while cats were shown to be susceptible to the murine strains (29, 30). Squirrels and dogs were successfully infected with murine strains by Blanc and Combiesco (31, 32). Dyer found woodchucks, house mice, meadow mice and white footed mice susceptible to murine typhus (33). Of this group, only the meadow mice died, the deaths occurring on the fourth day after infection. In another study Brigham and Dyer reported the following animals susceptible to murine typhus: opossum (Didelphis virginiana), field mouse (Peromyscus polionotus), cotton mouse (Peromyscus gossypinus gossypinus), golden mouse (Peromyscus nuttalli aureolus), wood rat (Neotoma floridana rubida), cotton rat (Sigmodon hispidus hispidus), rice rat (Oryzomys palustris palustris), and the flying squirrel (Glaucomys volans saturatus) (34).

The eastern cotton rat was shown to be as susceptible to infection with the murine strains as white

mice, and more susceptible to infection with the louse-borne strains than white mice (35). By subinoculation into the yolk sacs of fertile eggs, the rickettsiae were readily isolated from liver, brain, pericardial and peritoneal sources. Five strains representing both types of typhus were established in the cotton rats by serial passage, and in each case resulted in the production of infections terminated by death.

Fox in 1949 summarized the relative infectibility of laboratory animals and chick embryos with typhus rickettsiae (36). The cotton rat evinced maximum infectibility with murine and louse-borne strains. Swiss albino mice were as infectible with murine strains, but relatively insusceptible to infection with louse-borne strains. The guinea pig was consistently less infectible than the cotton rat with either variety of rickettsiae. The infectibility of the chick embryo was equal to that shown by the cotton rat for recently isolated murine strains. Infectibility with recently isolated louse-borne strains was usually lower. In contrast, louse-borne strains long passaged in eggs were equally infectious for both cotton rats and embryos.

The relative degree of susceptibility which an experimental host displays to typhus rickettsiae may be altered significantly by varying the treatment which the host receives either before or during infection. Zinsser and

Castaneda succeeded in producing massive invasion of white rats by murine strains when the rats were treated with a mixture of benzol and olive oil prior to injection (37). Rats could also be rendered more susceptible by roentgen irradiation (38). Rich suspensions of murine rickettsiae were obtained by repeated injections of large doses of guinea pig blood into the peritoneal cavity of typhus infected rats (39). A generalized peritoneal infection with murine strains was obtained in guinea pigs submitted to a deficiency diet (40). The resistance of rats to murine strains was shown to be lowered by a riboflavin deficiency (41). Topping showed that guinea pigs treated with sulfa drugs exhibited a lowered resistance to murine typhus (42). The production of a generalized rickettsial infection of the peritoneum in guinea pigs, rabbits, and sheep followed the lowering of body temperature (43). Pinkerton compared four strains of mice for susceptibility to murine typhus: dba, A-albino, brown agouti and swiss albino (44). He found the dba strain to be the most susceptible and demonstrated a variation in the morbidity and mortality of infections with changes in the environmental temperature (45).

The use of animals as the sole means of cultivating rickettsiae and maintaining stock cultures was based upon the absolute necessity of living tissues to support the growth of these organisms. In 1923, Wolbach, Pinkerton and

Schlesinger reported the use of tissue cultures for cultivating the louse-borne strains of rickettsiae (46). These cultures retained their virulence for guinea pigs for twenty days but could not be transferred serially in tissue culture. The murine typhus rickettsiae were cultivated in a tissue culture containing guinea pig tunica suspended in guinea pig plasma (47). While great numbers of rickettsiae were found in the tunica tissues, the cultures retained their virulence for guinea pigs for only ten days, and the rickettsiae could not be maintained by serial passage.

Nigg and Landsteiner were able to maintain murine typhus rickettsiae in tissue cultures by using minced guinea pig tunica suspended in a serum-tyrode medium (48, 49). Pinkerton and Hass showed that the rickettsiae retained their intra-cellular location in tissue culture and multiplication occurred exclusively in nonphagocytic cells believed to be of mesothelial origin (50). In 1936 Nigg reported successful transfers of louse-borne typhus in tissue cultures for a period of one and one half years. Similarly a murine strain was maintained for four years with no apparent loss in pathogenicity (51). The louse-borne strain produced fever and less severe scrotal reactions than the murine strain. The murine typhus rickettsiae appeared to show a predilection for tunica tissue. These findings were confirmed by Bengtson (52) and Plotz (53).

As a result of the problems which were encountered in the preparation of tissue cultures, Zinsser became interested in the physiological aspects of the cultivation of rickettsiae (54, 55). He found that the oxygen consumption of uninoculated tissue cultures reached a maximum value at forty to forty-six hours. The potential of the system rose, reaching a maximum at forty hours. The pH fell from 7.8 to 7.0 at the end of six or seven days. The most active growth of rickettsiae did not seem to take place until the sixth or seventh day when tissue viability and metabolism came to a standstill.

The introduction of the use of embryonated hens eggs by Goodpasture and Woodruff in 1931 for the cultivation of ultramicroscopic forms, represented an important advance in virus research. Using the chorio-allantoic membrane technic of Goodpasture, Zia attempted the cultivation of louse-borne and murine strains in fertile eggs (56). Both strains could be cultivated, but large numbers of rickettsiae were not found. It remained for Cox to demonstrate the effectiveness of the chick embryo for the propagation of the rickettsiae (57). Inoculation of the yolk sac rather than the chorio-allantoic membrane resulted in abundant yields of rickettsiae.

The advance in methods of cultivation made it possible to study the action of antibiotics and chemo-

therapeutic agents upon the rickettsiae. Snyder and co-workers observed a rickettsiostatic effect of p-aminobenzoic acid in animals (58). The same finding was reported following the use of p-aminobenzoic acid in experimental infections of the chick embryo (59). Fitzpatrick found an analogue of DDT that resulted in a degree of survival for murine infected mice equal to that obtained when four times the amount of p-aminobenzoic acid was administered (60).

Chloromycetin, an antibiotic obtained from Streptomyces venezuelae, has been shown to exert a rickettsiostatic action against louse-borne and murine typhus (61). Aureomycin, an antibiotic obtained from Streptomyces aureofaciens Duggar, was shown to be effective in the treatment of typhus fever (62, 63).

The rickettsiostatic effect of toluidine blue in typhus-infected mice was demonstrated by Peterson (64). Andrewes, King and Walker showed that chemotherapeutic agents active against typhus infection in mice did not give evidence of activity against the same rickettsiae in vitro (65). Penicillin was found to be rickettsiostatic when used in experimental infections of the chick embryo and animals (66).

With the successful use of various antibiotic or chemotherapeutic agents, investigations were directed towards the mode of action. Fox and Peterson suggested that thionin

dyes exhibited a rickettsiostatic action by providing an alternate mechanism for intracellular respiration, thus sparing the host cells from the usual harmful effects produced by the rickettsiae (67). Greiff and Pinkerton investigated the metabolic interrelationship of the rickettsiae and their host cells through the action of enzyme inhibitors and activators (68, 69, 70). They found that a temperature of 40° centigrade suppressed rickettsial growth. The suppression which resulted from the temperature of 40° centigrade could be reversed by the addition of potassium cyanide. The reversal was interpreted to result from a depression of the high rate of metabolism observed at 40° centigrade. In general the rickettsial growth was inversely proportional to the respiratory rate of the host cells, regardless of the factors which determined that rate. The assumption was made that p-aminobenzoic acid owes its rickettsiostatic action to its ability to increase cellular respiration.

Bovarnick and Snyder studied the respiration of R. prowazeki and R. typhi in the conventional Warburg apparatus (71). Using casein hydrolysate and glutamate as the substrates, they were able to demonstrate an uptake of oxygen in the presence of viable rickettsiae. The respiratory activity was proportional to the concentration of the rickettsiae, as determined by the biologic activity of the rickettsial suspensions. In a recent report Bovarnick

and Miller extended the initial observations to a study of the oxidation and transamination of glutamate by rickettsiae (72). They showed the formation of aspartate and ammonia during the oxidation of glutamate. Transaminase was present and remained active after the loss of oxidative enzymes and viability. The authors have interpreted their results as suggestive of oxidation of glutamate by rickettsiae, proceeding through the citric acid cycle, with formation of aspartate as a by-product due to the presence of transaminase.

Additional information on the possible role of the rickettsiae in the pathogenesis of typhus fever was reported by Clark and Fox (73). Suspensions of yolk sacs infected with murine or louse-borne typhus were shown to possess the capacity to hemolyze the red cells of the rabbit or sheep in vitro. Under the same conditions such material did not cause the hemolysis of the cells from mice, cotton rats or guinea pigs. The toxicity of heavily infected yolk sacs for intravenously inoculated mice seemed to be related to a serious alteration in vascular permeability which caused a marked reduction in the blood volume. This was shown by an increased concentration of the red cells but not of the plasma proteins.

The toxicity of infective yolk sac suspensions for albino mice following intravenous or intraperitoneal injection

was reported by Gildemeister and Haagen in 1940 (74). The symptoms in the injected mice--prostration, convulsive movements, and labored respiration--occur promptly. The "toxic substance" seemed to be associated with the intact, living rickettsiae and could not be separated from the cells. The direct relation of toxicity to the concentration of viable rickettsiae has resulted in the application of toxicity titrations to the determination of the number of viable rickettsiae in rickettsial suspensions (75).

The demonstration of a toxic effect in mice after the intravenous injection of suspensions of viable rickettsiae was followed by the finding that the toxic action could be neutralized by immune serum (75). The resistance of experimental hosts to toxic death was greatly increased following infection or vaccination. These findings have been used in pathogenesis studies to measure the concentration of antibodies, the relative resistance of an experimental host, or the presence of an immunizing infection.

OBJECTIVES

The purpose of the studies which are presented here was to investigate the biochemical activities of the rickettsial cell and their relationship to the pathogenesis of rickettsial infections. The specific objectives were as follows:

1. A study of the respiratory activity exhibited by Rickettsia prowazeki.
2. A study of the conditions contributing to the respiratory activity of rickettsiae.
3. The evaluation of substrates used in respiratory studies by paper chromatography.
4. The pathogenesis of typhus fever in experimental hosts.
5. The comparative susceptibility of various experimental hosts for typhus fever rickettsiae.

MATERIALS AND METHODS

The Cultivation of Rickettsiae

Stock strains of typhus fever rickettsiae were obtained from the National Institutes of Health and the Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Montana.¹ The strains received included the Wilmington and Balboa Park of murine typhus; the Breinl and Madrid of louse-borne typhus.

The rickettsiae were cultivated in the developing yolk sac of embryonated hens eggs.² The eggs were inoculated when six days old with 0.5 ml of an infective yolk sac suspension. The yolk sac dilution was based upon the concentration of rickettsiae required to cause the death of the embryo on the sixth or seventh day following inoculation. The dilution factor was ascertained by titration of infective yolk sac stocks in eggs.

Infective yolk sacs were ground in tubes which consisted of 50 ml pyrex centrifuge tubes containing ground glass or alundum,³ and fitted with a glass rod which was held securely in the centrifuge tube by a cotton plug. After the

1 Obtained through the courtesy of Dr. David Lackman and Dr. Karl Gerloff.

2 White leghorns, English strain.

3 Crystalline alumina, 500 B. Norton Co., Worcester, Mass.

yolk sac was ground, a diluting fluid¹ was added to give the desired suspension.

The preparation of the chick embryo for inoculation began with the disinfection of the egg shell surface directly over the air sac with 7 per cent tincture of iodine. After an interval of a few minutes, a small opening was made through the egg shell with a dental drill. The opening received a second application of iodine and was then ready for inoculation. The injections were made with a 22 gauge, 2 inch needle directly into the yolk sac. The opening was sealed with flexible collodion and the eggs were incubated at 35 to 36° centigrade. At the end of three days the eggs were candled for evidence of traumatic deaths. All of the dead embryos were discarded. The candling thereafter was done three times daily until the embryos were moribund or dead. The eggs were harvested, the yolk sac removed, placed in a sterile container and immediately frozen in a dry ice-alcohol mixture. Each yolk sac was examined for bacterial contamination and rickettsial content. The sterility test was made by dropping a small portion of the yolk sac into Brewer's thioglycollate broth. A yolk sac smear was prepared

1	Dextrose	0.5 per cent
	NaCl	0.5 per cent
	Phosphate Buffer (pH 7.4)	
	Na ₂ HPO ₄ (0.1M)	-- 84 parts
	KH ₂ PO ₄ (0.1M)	--- 16 parts

for overnight staining in Giemsa. The solution was prepared from a stock solution which had been standardized against normal blood films and the dilution factor determined.

An arbitrary method of estimating the rickettsial concentration was adopted. If numerous rickettsiae could be demonstrated in every microscopic field examined, a 4 / rating was assigned. In a similar way, less numerous rickettsiae per field were designated as either 3 / or 2 /. If one to three rickettsiae were found in each field examined, or if they were irregular in occurrence, a 1 / value was assigned.

Preparation of Rickettsial Suspensions

When yolk sacs were shown to be free from bacterial contaminants and heavily laden with rickettsiae, they were thawed, pooled, and a 20 per cent suspension was prepared. The diluent used was Krebs-Ringer phosphate buffer¹ adjusted to a final pH of 7.4. The suspension was homogenized in a Waring blender and transferred to centrifuge tubes. All centrifuging was carried out in a refrigerated centrifuge set at 0° centigrade. The diluent, glassware, and blender bowl were chilled to ice box temperature prior to use.

-
- 1 0.90 % NaCl
 1.15 % KCl
 1.22 % CaCl₂
 2.11 % KH₂PO₄
 3.82 % MgSO₄·7H₂O
 0.1 M phosphate buffer, pH 7.4

Differential centrifugation was used to separate the rickettsiae from the yolk sac tissue.

The principle of differential centrifugation was to centrifuge the yolk sac suspensions at high speeds to sediment the rickettsiae, thus permitting a separation from the yolk and other soluble components, followed by low speed centrifugation to throw down the yolk sac tissues and leave the rickettsiae suspended in the supernate. Various centrifugal speeds were compared for their efficiency in separating the rickettsiae from the yolk sac tissues. In general, centrifuge speeds of 4000 to 5000 RPM for forty minutes sedimented about 90 per cent of the rickettsiae. For the separation of yolk sac tissues, speeds in excess of 1000 RPM for twenty minutes or longer resulted in a sedimentation of the rickettsiae as well as the yolk sac tissue. Consequently low speed centrifugation was never in excess of 1000 RPM for twenty to thirty minutes. The procedure employed was essentially the same as the differential centrifugation used in the preparation of the Cox type of typhus fever vaccine. The same procedure was employed by Bovarnick for the preparation of rickettsial suspensions used in respiration studies (71).

The use of various clarifying agents was considered. Topping reported the application of celite to facilitate the separation of rickettsiae from yolk sac tissues (75). The

celite preparation was responsible for a greater removal of yolk sac constituents, so that the final rickettsial suspensions were superior in appearance and rickettsial concentration to non-celite treated suspensions. Based upon the theoretical considerations of particle size and adsorptive capacity, the following substances were selected for trial: Celite-analytical filter aid, Celite 505, Filter-Gel, Hy-flo, light magnesium oxide, kieselguhr, barium sulfate, alundum, and activated charcoal. All of the powders were added at the rate of 1 gram for each 5 grams of yolk sac.

The use of the agents for the separation of the rickettsiae from the yolk sac tissues was studied to determine the procedure which gave the most effective results. The addition of an agent to the yolk sacs prior to their homogenization and followed by the differential centrifugation process, resulted in a final suspension that contained some yolk sac constituents. It was evident that the separation of the thick yolk sac cream was impaired in this procedure, and a small amount appeared in the final suspension. When an agent was added to the yolk sac suspension after homogenization and at the time of the second centrifugation, the final suspension which was obtained after the completion of the differential centrifugation process failed to exhibit the presence of yolk sac constituents. With this procedure the thick layer of yolk sac cream was found at the surface of the

suspension following the first centrifugation, and was easily removed by means of sterile applicator sticks.

The separation of rickettsiae from yolk sac constituents was attempted by means of various oils such as paraffin, turpentine and pine. The procedure followed was a modification of a previously reported technic (76). In each instance a 10 per cent yolk sac suspension was prepared in Krebs-Ringer phosphate buffer. The pH of the rickettsial suspension was varied from 7.4 to 5.5 in different experiments. Five ml of a 10 per cent suspension was mixed with an equal volume of the oil in a separatory funnel until an emulsion formed. The mixture stood at room temperature until a separation of the phases occurred. When the separation was complete, the aqueous phase, interface and oil phase were examined for rickettsiae.

Preparation of Toxic Test Substance

The chick embryo was inoculated via the yolk sac route with a dilution of rickettsiae calculated to cause the death of the embryo on the sixth to seventh day after inoculation. The eggs were candled three times daily and when moribund, harvested for yolk sacs. The yolk sacs were weighed and a 25 per cent suspension was prepared. Sterile skim milk (0.5 per cent) with a pH of 7.4 was used as the suspending vehicle. The yolk sacs were homogenized in a Waring blender and the suspension centrifuged for 5 minutes

at 1000 RPM to remove tissue fragments. The supernate was pooled, distributed in sterile containers, sealed and immediately shell frozen in a dry ice-alcohol mixture. Storage was in the dry ice box.

Preparation of Amino Acids

Amino acid¹ solutions were prepared in Krebs-Ringer phosphate buffer. Each solution had a final concentration of 5 milligrams per ml, and a pH of 7.4. All pH adjustments were made with potassium hydroxide. The solutions were passed through Seitz filters and stored at refrigerator temperature in sterile, sealed containers.

Measurement of Amino Acid Concentration

Paper chromatography was used to determine the concentration of the amino acid before and after respiration measurements. The technic is a modification of other published methods (77, 78, 79, 80).

Whatman No. 1 filter paper was cut into strips one and one-half by twenty-two inches. The amino acid solution was pipetted onto the paper three inches from the bottom, a total volume of 0.02 ml being used. The paper was allowed to dry, and then suspended in a closed bell jar so that the bottom portion was beneath the surface of the solvent. The

¹ H. M. Chemical Co. Ltd., Los Angeles, California.

solvent used in all the studies was an 82 per cent solution of phenol. The solution was prepared with redistilled phenol and distilled water. The chromatogram was allowed to develop for twenty-four hours. At the end of this interval, the strips were removed, dried at 100° centigrade and sprayed with 0.05 per cent ninhydrin in butanol saturated with water. The strips were dried at 100° centigrade and the amino acids could then be located on the paper. The spots of amino acids were cut into small pieces and placed in test tubes. Three ml of 2 per cent ninhydrin in ethyl cellosolve-citrate buffer¹ was added to each tube, and the tubes placed in a boiling water bath for twenty minutes. The tubes were cooled, 17 ml of propanol-water solution² added, and the color intensity read on a Coleman spectrophotometer at 570 mu. A blank containing filter paper alone and a standard of 50 micrograms of the respective amino acid was run with each set of samples.

Measurement of Oxygen Consumption

The reaction mixture was composed of 2.0 ml of rickettsial suspension, 1.0 ml of amino acid solution, 0.2 ml

1 20.0 grams of citric acid, 8.0 grams of sodium hydroxide, 0.8 grams $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in a total volume of 500 ml. pH adjusted to 5.0. Ninhydrin, 2 per cent, in mixture of equal volumes of ethyl cellosolve and citrate buffer.

2 Mixture of equal volumes of N-propyl alcohol and distilled water.

of Krebs-Ringer phosphate buffer and 0.2 ml of 10 per cent potassium hydroxide in the center well. The measurements were made by the Warburg method at 34.4° centigrade. The manometer readings were made at intervals for two to three hours. Normal yolk sac preparations received treatment identical to the infected yolk sacs and the final suspension was used in the same manner as the rickettsial suspension to control the results for each amino acid investigated. The pH of the reaction mixture was varied from 7.4 to 6.7 in different experiments.

Pathogenesis Studies. Injection of Animals

1. Guinea Pigs

Male guinea pigs weighing from 400 to 800 grams were injected intraperitoneally with 2 to 5 ml of a 10 per cent yolk sac suspension. A daily temperature chart was maintained for each pig, the temperature being taken rectally. A temperature of 39.6° centigrade or higher constituted a fever. The febrile period may or may not be accompanied by a scrotal swelling. Passage of the infectious tissues was usually attempted on the third or fourth day of fever for louse-borne typhus, and on the second or third day of fever for the murine strains. In the absence of fever, the scrotal reaction was used as an index of infection. In the latter circumstance, passage of louse-borne typhus was attempted on the third day of scrotal swelling, and on the

second day of marked swelling for murine strains. Passage of louse-borne typhus was made with 10 per cent suspensions of either brain or spleen. The murine strains were passaged with 10 per cent suspensions of brain, spleen or tunica vaginalis. Rickettsiae of either strain could be passed serially from guinea pig to guinea pig, guinea pig to embryonated eggs, or guinea pig to other susceptible hosts.

The production of antibodies was measured by a mouse neutralization test (75). Serial two-fold dilutions of pooled guinea pig sera were made in sterile nutrient broth. An equal volume of toxic test substance, containing $4LD_{50}$ per 0.5 ml and prepared from the homologous strain, was added to the serum dilutions. The mixtures were allowed to stand at room temperature (26° centigrade) for two hours. The extent of neutralization of the toxic test substance was determined by the intravenous injection of albino mice. The pooled sera from normal guinea pigs was used in the test as a control serum.

2. White Rats

Adult white rats¹ were injected intravenously with the Balboa Park or Wilmington strains of murine typhus. The inoculum was 0.5 to 1.0 ml of a 10 per cent yolk sac suspension. Serial passage was carried out with suspensions

1 Obtained from Sprague-Dawley Co., Madison, Wis.

of pooled brain, liver and spleen. Each passage was made by the intravenous route with inocula of 1.0 ml. The rats were either sacrificed and the tissues examined microscopically for the presence of rickettsiae, or blood was withdrawn, the serum separated and examined for the presence of complement fixing antibodies.

3. Cotton Rats

Cotton rats² weighing 40 to 50 grams each were injected with 10 per cent yolk sac suspensions via the intraperitoneal, intracerebral and intracardial routes. One ml was given intraperitoneally, 0.03 ml intracerebrally and 0.4 ml intracardially. Passage of the Breinl and Wilmington strains was made with 10 per cent suspensions of pooled brain, liver and spleen inocula from moribund rats via the intracardial and intraperitoneal routes. The tissues were examined microscopically for the presence of rickettsiae.

4. Dbu Mice

Dbu mice² were injected with 10 per cent yolk sac suspensions by the intravenous, intracerebral and intraperitoneal routes. Mice injected intravenously received 0.5

1 Eastern cotton rat (*Sigmodon hispidus hispidus*) obtained from Tumblebrook Farms, Brant Lake, N. Y.

2 Dbu strains obtained from following sources:
(1) Jackson Memorial Laboratories, Bar Harbor, Maine
(2) Rockland Farms, New City, N.Y.

ml, while the intracerebral inoculum was 0.03 ml and the intraperitoneal inoculum was 0.5 to 1.0 ml.

The mice were sacrificed while moribund, and 10 per cent suspensions of pooled brain, spleen and liver inocula were passaged. Each passage was made with 0.5 to 1.0 ml volumes injected intraperitoneally. The mice were either sacrificed and the tissues examined microscopically for the presence of rickettsiae, or they were subjected to toxic challenge to determine the presence of an immunizing infection.

Titration of Toxic Test Substance

Toxic test substance (T.T.S.) was prepared for the Breinl and Wilmington strains. Albino¹ and dba mice were injected intravenously with 0.5 ml volumes of two-fold dilutions prepared in sterile nutrient broth. Control mice of both species were inoculated with 0.5 ml of sterile nutrient broth. Deaths occurring within eight hours were considered to be toxic, and the LD₅₀ value was calculated (81). All of the mice surviving toxic death were challenged intravenously with approximately 4LD₅₀ doses of T.T.S. after an interval of fourteen days to determine the presence of an immunizing infection. The toxicity of the challenge was controlled by a simultaneous titration in albino mice.

1 White mice obtained from Maple Grove Rabbitry, Springfield, Missouri.

Infectivity Determinations

Albino and dba mice were injected intraperitoneally with 0.25 ml volumes of ten-fold dilutions prepared in sterile nutrient broth. After an interval of twenty-one days the mice were challenged intravenously with approximately $4LD_{50}$ doses of T.T.S. The toxicity of the challenge was controlled by a simultaneous titration in albino mice. All of the mice surviving a toxic challenge were considered to have developed an immunizing infection. The infective dose was expressed as the $1D_{50}$ value (81).

Determination of Biological Activity of Rickettsial Suspensions

The biological activity of each rickettsial suspension used in the respiration studies was measured in terms of its toxic value in albino mice, and its infectivity value in dba mice.

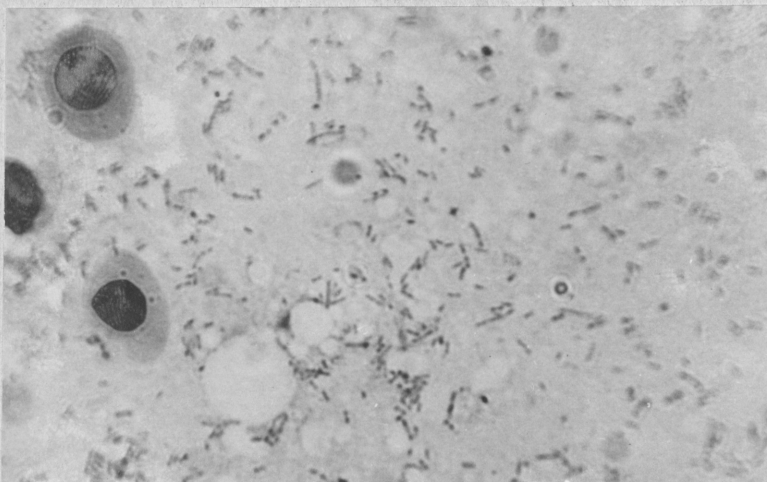
RESULTS

The Cultivation of Rickettsiae

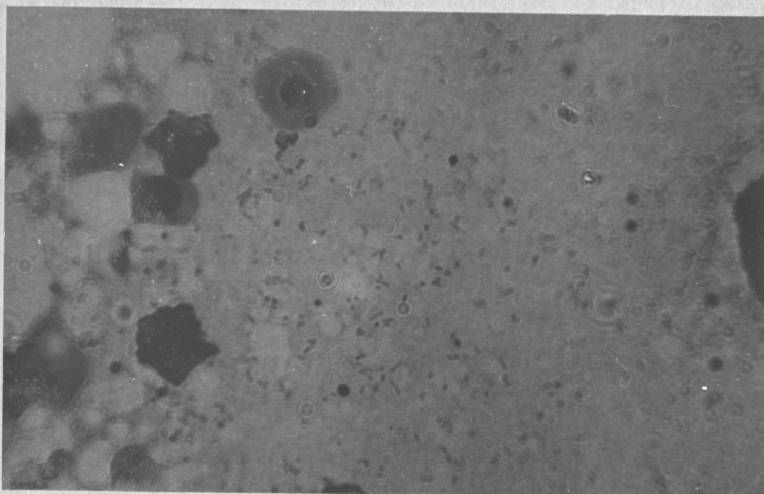
The establishment of the louse-borne and murine typhus strains in the chick embryo was accomplished by serial passage of infective yolk sac suspensions. The rickettsiae seen in the smears prepared from infective yolk sacs and stained by Giemsa, appeared as small bacilli, red to purple in color, and arranged in a characteristic diploid position. The rickettsiae were observed as intracellular forms lying within the cytoplasm of the yolk sac entodermal cells, and extracellular forms lying free among the yolk sac tissue cells. The extracellular forms were a result of the rupturing of yolk sac cells during the preparation of the smears, at which time innumerable rickettsiae were released from their initial intracellular position. An illustration of the rickettsiae seen in the yolk sac smears is given in Plate 1.

The results of the serial passage of the Breinl and Wilmington strains in chick embryos are given in Table 1. The average day of death (ADD) for the chick embryos was directly proportional to the number of the viable rickettsiae which were present in the inoculum as shown in Breinl passage #3. While it was possible to show the effect of the dilution of the inoculum upon the ADD for the embryos which were inoculated in the same passage, the ADD which followed the

PLATE 1

A. *Rickettsia prowazeki* in a yolk sac smear

Magnification -
Stain -- Giemsa
Strain - Breinl

B. *Rickettsia typhi* in a yolk sac smear

Magnification -
Stain -- Giemsa
Strain - Wilmington

inoculation of eggs with a given dilution in one passage could not be compared with the ADD which followed the inoculation of other eggs with the same dilution of a different inoculum in a different passage. This difference in the ADD was attributed to the discrepancy in the number of viable rickettsiae which existed in the different yolk sac inocula.

The number of rickettsiae which were found in the smears prepared from the harvested yolk sacs of the same passage was directly proportional to the ADD as shown in Breinl passage #3. Like the ADD which was found to vary in the different passages following the inoculation of embryos with the same dilution of different inocula, the number of rickettsiae in the yolk sacs harvested from different passages could not be compared on the basis of the dilution of the inocula.

The inoculation of large numbers of rickettsiae resulted in the early death of the embryo, and a low concentration of rickettsiae in the harvested yolk sacs. On the other hand, the inoculation of small numbers of rickettsiae allowed the embryo to survive longer and resulted in large numbers of rickettsiae in the harvested yolk sacs. By adjusting the dilution of the inoculum, the approximate time of embryo death could be estimated, and a predictable yield of rickettsiae resulted.

TABLE 1

The serial passage of typhus fever rickettsiae
in the chick embryo

Passage	Rickettsiae	Inoculum (log of dilution)	Average Day of Death	*Rickettsial Concentration (average)
1	Breinl	10 ⁻²	5.50	
		10 ⁻³	6.50	
2	Breinl	10 ⁻²	4.50	
		10 ⁻³	4.80	
3	Breinl	10 ⁻²	5.50	1.50 /
		10 ⁻³	6.33	3.66 /
		10 ⁻⁴	9.25	4.00 /
		10 ⁻⁵	11.33	4.00 /
4	Breinl	10 ⁻²	5.00	3.25 /
		10 ⁻³	6.00	4.00 /
5	Breinl	10 ⁻³	6.25	3.83 /
6	Breinl	10 ⁻³	5.33	3.33 /
		10 ^{-3.69}	5.85	3.42 /
7	Breinl	10 ⁻²	5.82	3.39 /
1	Wilmington	10 ⁻²	6.25	
		10 ⁻³	6.25	
2	Wilmington	10 ⁻²	5.80	
		10 ⁻³	6.40	
3	Wilmington	10 ⁻²	5.75	2.50 /
		10 ⁻³	6.50	2.66 /
4	Wilmington	10 ⁻²	7.00	3.54 /
5	Wilmington	10 ⁻³	6.33	2.16 /
		10 ^{-3.60}	6.64	2.15 /

Legend: * Estimated concentration based upon examination
of yolk sac smears.

On the basis of the results shown in Table 1, a 1-500 dilution of infective yolk sac suspension was used routinely for the cultivation of the Breinl strain in the developing yolk sac of the chick embryo.

The Wilmington strain did not appear to be as well adapted to the yolk sac method of cultivation. A 1-100 dilution of infective yolk sac suspension was used for the cultivation of murine rickettsiae. The time of embryo death could be predicted, but the rickettsial concentration in the harvested yolk sacs showed a greater variation than the day of death.

Judging from the ADD and the number of rickettsiae which were found in smears of the harvested yolk sacs, the results of the yolk sac cultivation of the murine strain were less consistent than those which were obtained for the louse-borne strain.

Purification and Concentration of Rickettsiae

Differential centrifugation without the aid of a clarifying agent gave a satisfactory concentration of rickettsiae, but the final suspensions also contained considerable amounts of yolk sac constituents. Yolk sac tissue was found microscopically. Macroscopically the suspensions exhibited a definite yellow color resembling yolk sac fluid. The egg impurities could be removed by increasing the number of separations in the centrifuge. However, the

number of viable rickettsiae, as determined by the decreased infectibility of such suspensions for the developing yolk sac of the chick embryo, decreased with the added separations.

The use of clarifying agents made it possible to achieve a good separation of rickettsiae with a minimal number of centrifugations. Light magnesium oxide gave uniformly satisfactory results. The concentration of rickettsiae was excellent, and no tissue fragments were observed microscopically. However, the pH of the yolk sac suspensions into which the magnesium oxide was incorporated was 9.6. All attempts to buffer the magnesium oxide-yolk sac suspensions at a pH of 7.5 to 8.0 led to an unsatisfactory product. No viability studies were made on rickettsial suspensions prepared with the aid of magnesium oxide. Due to the alkalinity of these suspensions the use of magnesium oxide was discontinued.

Kieselguhr did not give satisfactory results. The degree of rickettsial concentration was approximately equivalent to that obtained with magnesium oxide, but yolk sac material was present in the final suspensions. A yellow coloration was imparted to the suspensions and tissue debris was demonstrated.

Barium sulfate, alundum and activated charcoal treatment failed to give final rickettsial suspensions free from yolk sac tissue and debris.

The addition of celite preparations to the yolk sac suspensions during the process of differential centrifugation facilitated the separation of the rickettsiae from the yolk sac tissues. The purification and concentration of the rickettsiae which followed the use of celite was approximately equivalent to the results which were obtained after twice as many separations in the centrifuge without the benefit of celite. The use of celite products to facilitate the preparation of rickettsial suspensions permitted a good separation of rickettsiae from yolk sac constituents with a minimal number of manipulations. Of even more critical importance was the increased number of viable cells which were obtained.

Celite-analytical filter aid was selected as the preparation which seemed to give the most desirable rickettsial suspension. While all of the celite products were found to be difficult to remove from the yolk sac suspensions during differential centrifugation, the celite-analytical filter aid product was found in the lowest concentration in the final suspension.

The use of oils to separate the rickettsiae from the yolk sac tissues depended upon the formation of three phases following the initial mixture of yolk sac suspension and oil in a separatory funnel. A relatively clear supernate was found which was separated from an opalescent solution by a thin layer of yolk sac tissue fragments. The time required

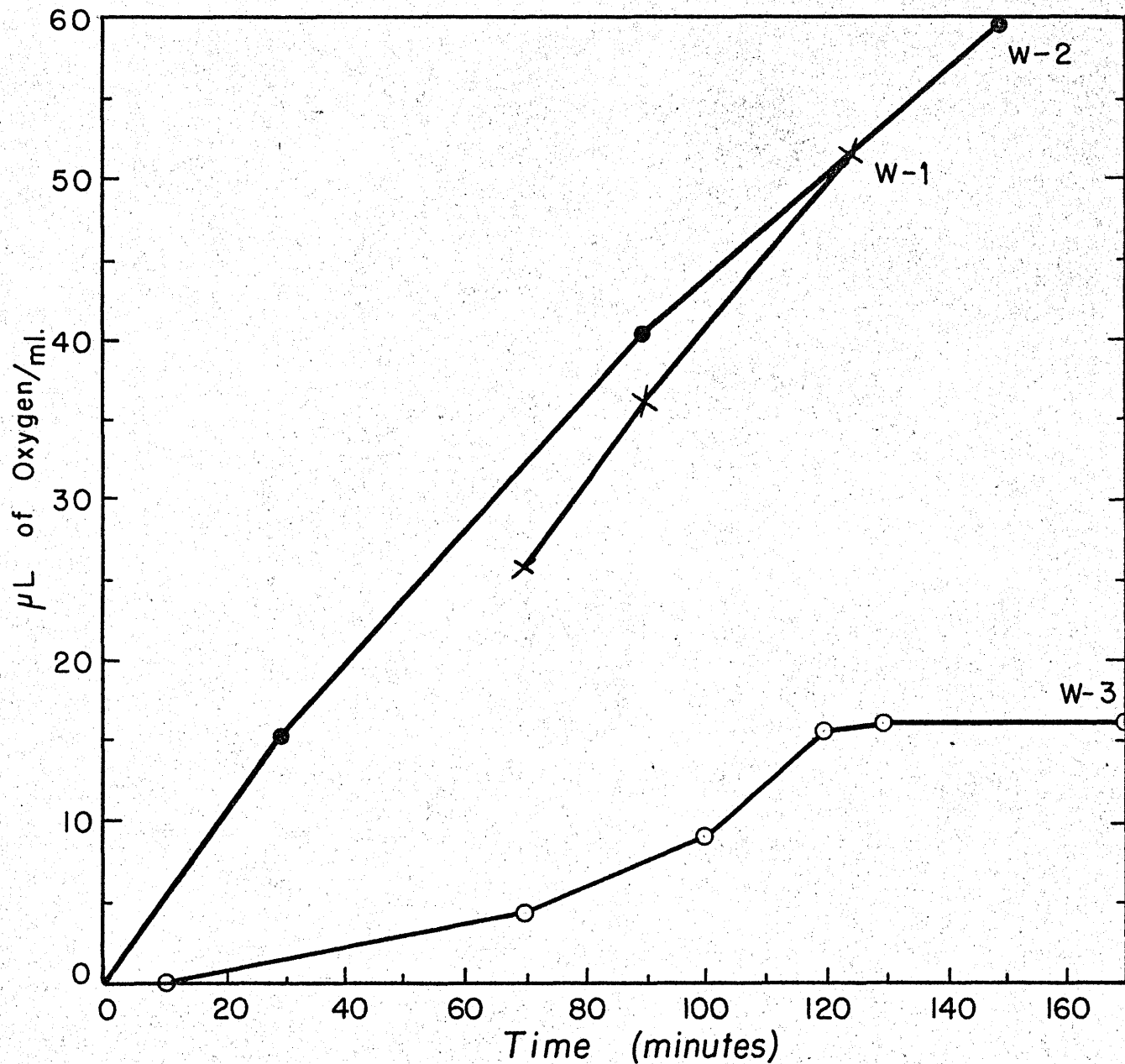
for separation depended somewhat on the emulsion formed, but usually occurred within twenty to thirty minutes.

Rickettsiae were demonstrated in the aqueous phase and at the interface. The greatest number of rickettsiae were found at the interface. The aqueous phase was withdrawn from the separatory funnel and the approximate number of rickettsiae in this suspension were determined by means of a microscopic examination. When the rickettsial concentration of the aqueous phase was compared to the number of rickettsiae in the suspensions prepared by differential centrifugation it was evident that the aqueous phase contained very few rickettsiae and did not represent a satisfactory concentration of the rickettsiae for the respiration studies. Furthermore the aqueous phase contained a fair amount of yolk as evidenced by the definite yellow coloration. When the extraction was carried out at a pH of 5.5 the separation of yolk and tissue debris was greatly increased but the results were not comparable to those obtained by differential centrifugation. Because the more acid pH value failed to give satisfactory separation of the rickettsiae, no attempts were made to evaluate the viability of the rickettsiae in these suspensions and the method was discontinued.

Respiration

The respiration of Rickettsia prowazeki is given in Table 2 and Figure 1. The results of the biological tests

The Respiration of Typhus Rickettsiae, Breinl Strain



Legend

××× (W-1) Glutamic Acid

●●● (W-2) Cysteine

○-○-○ (W-3) Glutamic Acid

TABLE 2

The respiration and biologic activity of typhus rickettsiae, Breinl strain

Amino Acids (5 mgm/1 ml)	pH	Oxygen Uptake (ul / hr / 1.0 ml) Experiment					Toxicity † Experiment					Biologic Activity Infectivity †† Experiment				
		W-1	W-2	W-3	W-4	W-5	W-1	W-2	W-3	W-4	W-5	W-1	W-2	W-3	W-4	W-5
		DL-Alanine	7.4	0			0									
L-Arginine	7.4	0			0											
L-Aspartic	7.4	0			0											
L-Cysteine HCL	7.4		41.7*					14					10 ⁻⁵			
L-Glutamic	7.4	26.5		5.3	0	0	14	12	0	0	10 ⁻³		10 ⁻⁴	10 ^{-1.2}	10 ⁻⁴	
Glycine	7.4	0			0											
L-Histidine	7.4	0			0											
DL-Isoleucine	7.4		0	0		0										
L-Leucine	7.4		0	0		0										
L-Lysine HCL	7.4		0	0		0										
L-Phenylalanine	7.4	0														
L-Tryptophane	7.4		0	0		0										
DL-Valine	7.4		0	0		0										

Legend † Dilution of test suspension lethal for albino mice when administered intravenously in 0.5 ml volume. Calculated as LD₅₀ by method of Reed and Muench. (81)

†† Dilution of test suspension infective for dba mice, calculated as ID₅₀.

* Rate of oxygen uptake for 1.5 hours.

are also summarized in Table 2. In the four experiments cited only L-glutamic acid and L-cysteine hydrochloride demonstrated an uptake of oxygen in the presence of the rickettsiae. No attempt was made to determine the respiratory quotient. The respiration measurements were made over a period of three hours, a progressive uptake of oxygen occurring during this time. The rate of uptake seemed to be greater for cysteine than for glutamic acid. However the values shown for respiration were obtained from experimentation conducted at different times. The difference therefore may have been due to the effective concentration of viable rickettsiae. The details of the toxicity and infectivity tests which are given in Tables 3 and 4 indicate that a difference in rickettsial concentration did exist. The rickettsial suspensions used in the various tests are identified by the letter W and the appropriate number. The toxicity test failed to show a difference in the number of rickettsiae which were used in W-1 and W-2. The infectivity test, however, indicated a greater number of rickettsiae were present in the suspension designated as W-2. The increased respiratory activity shown for cysteine could, therefore, have been attributed to the greater number of rickettsiae.

The complete failure of the normal yolk sac suspensions to show any evidence of an uptake of oxygen in

TABLE 3

Toxic death in albino mice following intravenous injection
of partially purified rickettsial suspensions
used in respiration studies

Preparation	Rickettsiae	Dilutions of Rickettsial Suspension				LD ₅₀ **
		1:10	1:20	1:40	1:80	
W-1	Breisl	4/4*	0/4	0/4	0/4	14
W-2	Breisl	4/4	0/4	0/4	0/4	14
W-3	Breisl	3/4	0/4	0/4	0/4	12
W-4	Breisl	0/4	0/4	0/4	0/4	0
W-5	Breisl	0/4	0/4	0/4	0/4	0

* Numerator represents the number dead.

Denominator represents the number tested.

** Calculated according to Reed and Muench (13).

TABLE 4

Infectivity determinations in dba mice following intraperitoneal injection of partially purified rickettsial suspensions used in respiration studies

Experiment	Rickettsiae	Previous treatment*		Challenge		ID ₅₀
				21 days after infective dose		
				Toxic dose ID ₅₀ **	Dead Tested	
W-1	Breinl	10 ⁻³	0.25 ml	6	4/4	10 ^{-3.0}
		10 ⁻⁵	0.25 ml		4/4	
		10 ⁻⁷	0.25 ml		4/4	
		Sterile broth "	4/4			
W-2	Breinl	10 ⁻¹	0.25 ml	4.7	0/4	10 ^{-5.0}
		10 ⁻²	0.25 ml		1/4	
		10 ⁻³	0.25 ml		0/4	
		10 ⁻⁴	0.25 ml		1/4	
		10 ⁻⁵	0.25 ml		1/4	
		Sterile broth "	4/4			
W-3	Breinl	10 ⁻¹	0.25 ml	3.0	0/4	10 ^{-4.0}
		10 ⁻²	0.25 ml		0/4	
		10 ⁻³	0.25 ml		0/4	
		10 ⁻⁴	0.25 ml		0/4	
		Sterile broth "	4/4			
W-4	Breinl	10 ⁻¹	0.25 ml	4.0	2/4	10 ^{-1.23}
		10 ⁻²	0.25 ml		3/4	
		10 ⁻³	0.25 ml		4/4	
		Sterile broth "	4/4			
W-5	Breinl	10 ⁻²	0.25 ml	3.0	0/4	10 ^{-4.0}
		10 ⁻³	0.25 ml		0/4	
		10 ⁻⁴	0.25 ml		2/4	
		10 ⁻⁵	0.25 ml		4/4	
		Sterile broth "	4/4			

* The various dilutions of yolk sac material were injected intraperitoneally.

** LD₅₀ value received by mice as determined by a control toxicity titration at the time of challenge.

the presence of the amino acid substrates offered a striking comparison to the results obtained with the rickettsial suspensions. Consequently the respiration shown by suspensions prepared from infective yolk sac pools was presumably a function of the rickettsial cell.

The respiratory activity seemed to be directly proportional to the concentration of viable cells. In the case of W-4, judging from the results of the toxicity and infectivity tests, an insufficient number of rickettsiae were present to give evidence of respiratory activity.

The relationship between the respiratory activity and the rickettsial concentration was also shown by the number of infective yolk sacs required for the preparation of the rickettsial suspensions. A comparison of the different suspensions is given in Table 5. Suspensions which were prepared from less than nine yolk sacs failed to demonstrate an uptake of oxygen in the presence of glutamic acid or cysteine. The failure of the suspensions designated as W-4 and W-5 to demonstrate respiratory activity was shown by the toxic and infective determinations to result from a low concentration of viable cells. The measurement of the rickettsial concentration by microscopic examination of smears prepared from the suspensions was shown to be unreliable.

TABLE 5

The preparation of suspensions used in respiration studies

Suspension	Yolk Sacs		Rickettsial Conc. (average)	*Toxicity (LD ₅₀)	**Infectivity (ID ₅₀)	Activity in Respiration Studies (ul / hour / 1.0 ml)	
	Number	Weight (gms)				Glutamic	Cysteine
W-1	15	20.5	3.8 f	14	10 ⁻³	26.5	
W-2	12	16.5	3.08 f	14	10 ⁻⁵		41.7#
W-3	9	11.7	3.44 f	12	10 ⁻⁴	5.3	
W-4	6	8.3	3.3 f	0	10 ^{-1.23}	0	0
W-5	2	3.0	3.8 f	0	10 ⁻⁴	0	0

- Legend * Dilution of test suspension lethal for albino mice when administered intravenously in 0.5 ml volume. Calculated as ID₅₀ by method of Reed and Muench. (81)
- ** Dilution of test suspension infective for dba mice when administered intraperitoneally in 0.25 ml volume. Calculated as ID₅₀ by method of Reed and Muench.
- # Rate of oxygen uptake for 1.5 hours.

The Evaluation of Toxic Preparations

The comparative toxicity of the yolk sac suspensions is given in Table 6. The number of lethal doses per gram of yolk sac weight compared favorably with the toxicity standard established by the National Institutes of Health, USPHS (72). According to these standards, toxic preparations must possess a minimum toxicity of 80 MLD per gram of yolk sac weight. Wilmington TTS, Lot #2, representing the lowest toxic value obtained, was two and a half times as potent as the minimum value allowed. Breinl TTS, Lots #2 and #3, represented the highest toxic values obtained. These values were eight times as potent as the minimum value allowed.

Measurement of Amino Acid Concentration

The efficiency of paper chromatography which was used to evaluate the concentration of amino acids before use in the respiration studies was found to be satisfactory. Glutamic acid dissolved in distilled water and Krebs-Ringer phosphate buffer (5 mgm per ml) gave recovery values as shown in Table 7. The recovery from phosphate buffered solutions seemed to show a greater variation than from the distilled water solutions. An examination of phosphate buffered solutions of glutamic acid was made in which the concentration of amino acid was varied, and the ninhydrin-developed solutions were filtered prior to the evaluation

TABLE 6

The preparation and comparative toxicity of several
toxic test substances

Toxic test Substance	Yolk Sacs Number	Sacs Weight (gms)	Skim Milk (ml)	Suspension (per cent)	Toxicity	
					*LD ₅₀	LD ₅₀ /1.0gm Y.S.
Breinl						
Lot#1	3	6.1	24.4	20	31.6	316
Lot#2	13	18.3	54.0	25	80.0	640
Lot#3	8	17.6	52.8	25	80.0	640
Wilmington						
Lot#1	6	12.1	48.4	20	31.6	316
Lot#2	12	20.2	60.6	25	20.0	200

* Final dilution of rickettsial suspension, 0.5 ml of which equals LD₅₀.

TABLE 7

The recovery of glutamic acid from aqueous solutions by paper chromatography

Trial	Distilled Water ¹				Krebs-Ringer Phosphate ¹			
	Blank ²	Standard ³	Sample	% Recov.	Blank ²	Standard ³	Sample	% Recov.
1	.615 ⁴	.683	.680	97.01	.562	.620	.616	93.1
2	.605	.678	.675	95.8	.570	.618	.620	104.1
3	.625	.685	.682	95.0	.404	.458	.462	107.4

1 pH of both solutions adjusted to 7.4.

2 Blank = Whatman No. 1 filter paper.

3 Standard = Whatman No. 1 filter paper with 0.02 ml representing 10 gamma of Glutamic acid.

4 Optical density reading.

of the optical densities. The results are shown in Table 8. The use of samples containing from 25 to 50 micrograms of glutamic acid was followed by a recovery of 99 to 102 per cent. When solutions containing glutamic acid in excess of 50 or less than 25 micrograms were measured, the recovery was considerably less. The use of filtered solutions for estimating optical densities had the advantage of freedom from paper fibers and resulted in measurements consistent with greater accuracy.

The analysis of the reaction mixtures at the termination of the respiration studies was restricted to an evaluation of the glutamic acid concentration. The results of these analyses indicated a lowered concentration of the substrate for the rickettsial suspension as well as for the normal yolk sac suspension. In experiment W-1 a loss of 97 micrograms was recorded for the infective yolk sac suspension, while a loss of 35 micrograms occurred with the normal yolk sac suspension. In experiment W-3 a loss of 46 micrograms occurred with the infective yolk sac suspension, while a loss of 23 micrograms was recorded with the normal yolk sac suspension.

Although a loss in the concentration of glutamic acid was found in both the test and the control systems, the test system was responsible for the greatest loss. Thus an actual loss of 62 micrograms was attributed to the action of

TABLE 8

The chromatographic analysis of glutamic acid solutions

Solution	Concentration (micrograms)	Blank	Measurement by Spectrophotometer				Recovery (per cent)	
			*Standard Filtered	Not Filtered	**Sample Filtered	Not Filtered	Filtered	Not Filtered
1	100	0	/.645	.71	.73	.77	113.1	108.0
2	50	0	.465	.49	.462	.48	99.3	97.9
3	25	0	.27	.258	.275	.285	101.8	110.4
4	12	0	.31	.315	.227	.215	73.2	68.2

Legend * Control containing same concentration of glutamic acid as sample. The control was not developed in solvent.

** Solution developed overnight in solvent.

/ Optical density measurement.

the rickettsial suspension in W-1, and a loss of 23 micrograms in W-3.

The greater loss of glutamic acid which was found in experiment W-1, compared to the loss found in W-3, indicated that the loss in substrate concentration was related to the increased respiratory activity found in W-1, as evidenced by the greater uptake of oxygen recorded for experiment W-1. The greater uptake of oxygen which was found in W-1 was attributed to the presence of greater numbers of rickettsiae contained in the test suspension of W-1 compared to the test suspension of W-3, as shown by the biologic activity of the rickettsial suspensions.

The evaluation of substrate concentration by paper chromatography methods indicated that the use of glutamic acid in the respiration studies conducted with R. prowazeki was followed by a quantitative reduction in concentration, and the loss could be related to the degree of respiratory activity exhibited by viable rickettsiae.

Pathogenesis Studies

The use of animals for the production of experimental typhus infections had a two-fold purpose: The quest for an experimental host which would serve as a reliable index of the biologic activity exhibited by R. prowazeki and R. typhi, and a host in whose tissues the rickettsiae could be cultivated in numbers sufficient for use in the preparation

of the test suspensions employed in the respiration studies.

The evaluation of host susceptibility was determined by the response to injections of infective yolk sac suspensions. The more susceptible hosts could be infected with fewer rickettsiae, and in some cases these infections terminated in the death of the host. Other hosts proved more resistant to infection following the injection of infective yolk sac suspensions and the only evidence of infection which could be demonstrated was the production of antibodies, or the presence of a resistant state to the intravenous injection of a toxic challenge.

The animals which were used in these studies included guinea pigs, white rats, cotton rats, and dba mice.

1. Guinea Pigs

Guinea pigs were used to study the multiplication of the rickettsiae in the tissues. Male pigs were employed in order to compare the scrotal reaction for the louse-borne and murine strains. Three serial passages were made for each of four strains: The Breinl and Madrid strains of louse-borne typhus; the Wilmington and Balboa Park strains of murine typhus. Two guinea pigs were used for each passage. Typical results of guinea pig injection and passage are given in Figures 2 and 3. Several observations of interest were made. All of the strains caused a scrotal swelling in the pigs. It was evident that the swelling might occur in the

absence of fever. Once a strain was established in pigs, a regular fever curve could generally be demonstrated. No deaths occurred at any time with any of the strains. Microscopic examination of the brain, spleen and tunica vaginalis from a pig infected with louse-borne typhus usually revealed the presence of rickettsiae in the spleen and occasionally in the brain and tunica vaginalis. Upon passage of suspensions prepared from the brain, spleen or tunica tissues of an infected pig, infections were routinely produced in the injected pigs. The microscopic examination of the same tissues removed from a pig infected with murine strains revealed a greater degree of tunica involvement than that encountered with the louse-borne strains. Rickettsiae could be demonstrated in the spleen and occasionally in the brain. Suspensions of the tunica and the spleen were regularly infective upon passage.

When the various tissues were examined and the rickettsial concentration determined, it was apparent that the guinea pig tissues did not compare favorably with the yolk sac tissues for the cultivation of rickettsiae in large numbers.

Experimental typhus infection in the guinea pig resulted in the formation of antibodies which could be demonstrated twenty-one days after injection. The results of a mouse neutralization test in which the pooled sera from

The Infection of Guinea Pigs with Typhus Fever Rickettsiae, Breinl Strain

Passage 1

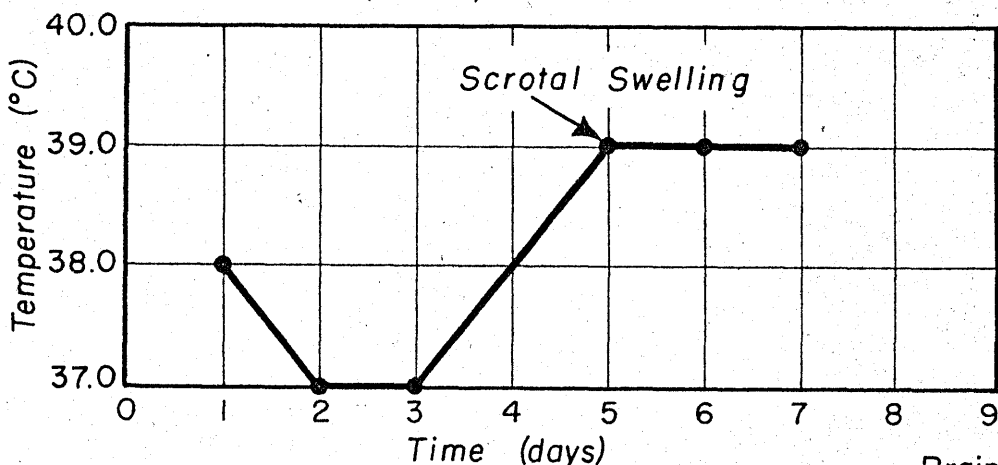
Inoculation:

Source —
Yolk Sac

Volume —
2.0 ml

Route —
1 P

Temperature Chart



Disposition: Sacrificed on 7th day. Brain removed and passed.

Brain = +
Spleen = +
Tunica = -

Passage 2

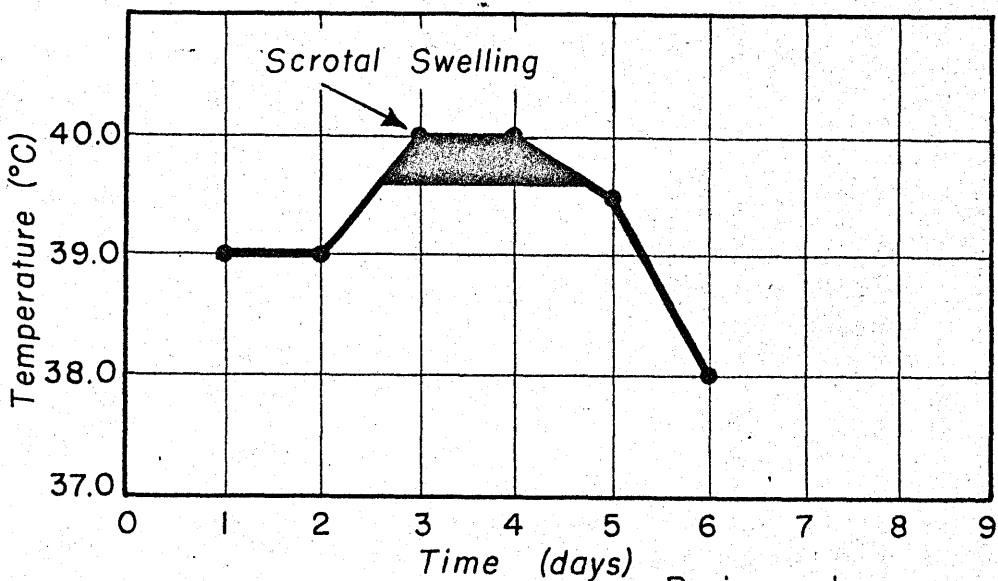
Temperature Chart

Inoculation:

Source —
Brain

Volume —
2.0 ml

Route —
1 P



Disposition: Sacrificed on 6th day.

Brain = +
Spleen = +
Tunica = +

The Infection of Guinea Pigs with Typhus Fever Rickettsiae, Wilmington Strain

Passage 1

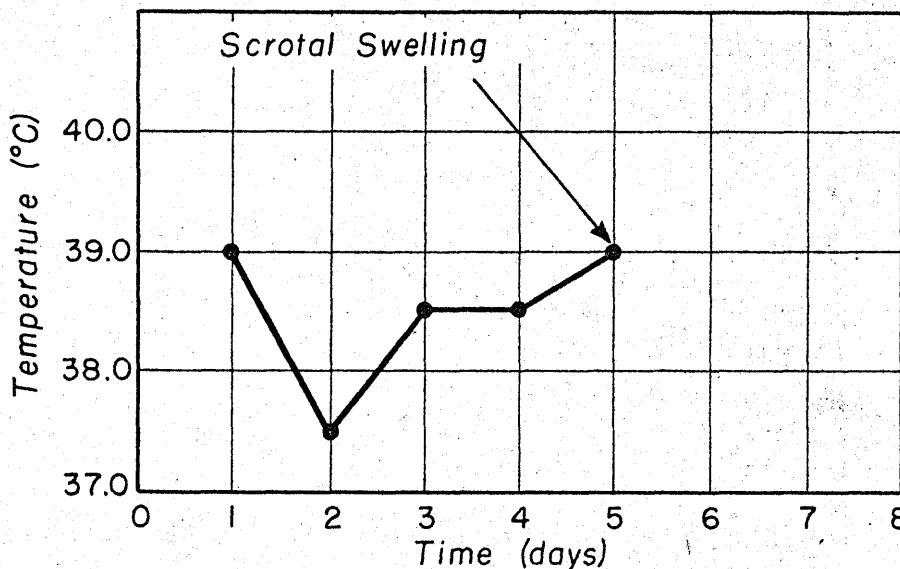
Inoculation:

Source -
Yolk Sac

Volume -
2.0 ml

Route -
1 P

Temperature Chart



Brain = -
Spleen = +
Tunica = +

Disposition: Sacrificed on 6th day. Tunica removed and passed.

Passage 2

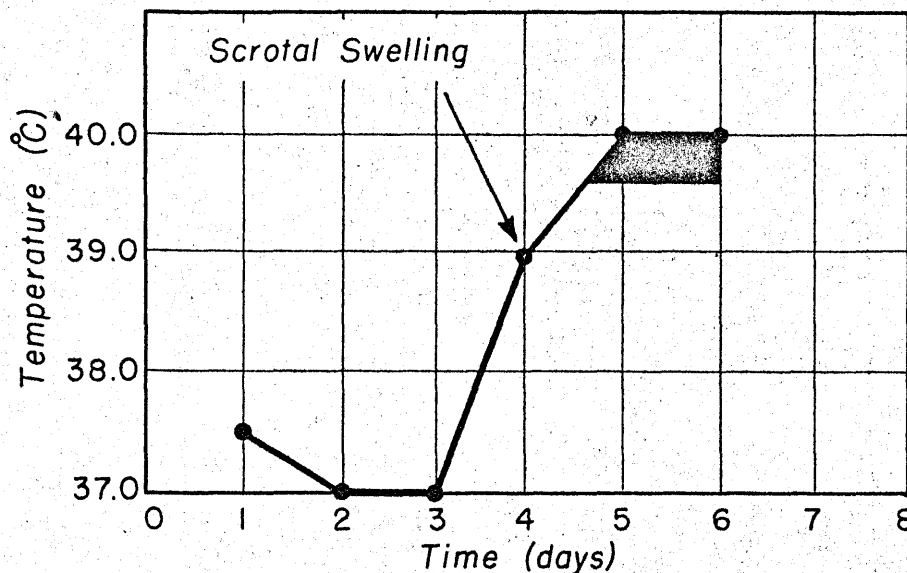
Inoculation:

Source -
Tunica

Volume -
2.0 ml

Route -
1 P

Temperature Chart



Disposition: Sacrificed on 6th day.

Brain = +
Spleen = +
Tunica = +

five infected guinea pigs was used, are given in Table 9. The serum which was obtained from the infected guinea pigs showed an ability, in a 1-90 dilution, to protect albino mice from a toxic challenge.

Albino mice that were injected with the normal guinea pig serum in a 1-4 dilution, succumbed to the toxic challenge. The results of a toxicity titration which was conducted simultaneously with the neutralization test showed that the dilution of the toxic test substance used in the test contained 3LD₅₀ doses.

2. White Rats

White rats were examined for evidence of infection following the intravenous injections of murine strains. Three serial passages were made in which four rats were used for each passage. The rats gave no visible indication of illness, and the microscopic examinations of brain, liver, spleen and peritoneal scrapings failed to demonstrate the presence of rickettsiae. One rat from the second passage was bled on the twenty-second day after injection, and the serum was examined for the presence of complement fixing antibodies.¹ A 4 / fixation of complement was found in a 1-16 dilution of the serum when antigen prepared from murine

¹ The complement fixation tests were performed through the courtesy of Mr. J. Holper, Department of Bacteriology, University of Kansas.

TABLE 9

The neutralization of toxic test substance by the serum of a guinea pig infected with louse-borne typhus

Titration of toxic test substance (Breinl)			Neutralizing power of guinea pig sera							Sera control	
Dilution of rickettsial suspension			LD ₅₀	Dilutions of sera #				LD ₅₀ used in test	ID ₅₀	Dilution of Sera #	
1-20	1-40	1-80		1-32	1-64	1-128	1-256	1-512		1-4	
* 4/4	4/4	1/4	**63.4	0/4	1/4	3/4	4/4	4/4	3	##90.5	4/4

Legend * Numerator represents the number dead.
Denominator represents the number tested.

** Dilution of toxic test substance, 0.5 ml of which represents 1 LD₅₀. Calculated according to Reed and Muench (81).

Represents final serum dilution.

Dilution of guinea pig sera which will protect fifty per cent of the albino mice injected against 3 LD₅₀ of toxic test substance. Calculated according to Reed and Muench (81).

strains was used. A $\frac{1}{4}$ fixation of complement was found in a $\frac{1}{4}$ dilution of the same serum when antigen prepared from louse-borne strains was used. Serum obtained from normal rats failed to demonstrate any fixation of complement in the presence of the above antigens.

The identification of complement fixing antibodies in the serum of an injected rat for antigens prepared from the murine and louse-borne strains was the only evidence of infection which could be shown.

Since the white rat suffered only an inapparent form of infection and possessed a relatively high degree of resistance to experimental typhus, further studies were discontinued.

3. Cotton Rats

Cotton rats were injected via the intracardial, intracerebral and intraperitoneal routes with infective yolk sac suspensions of the Breinl and Wilmington strains. Two rats were used for each route of injection and two series of two passages each were carried out for the two strains.

The intracardial injection of infective yolk sac suspensions resulted in visible symptoms of illness with the two strains of rickettsiae. The injected rats were listless and refused food and water. Of the two rats injected with the Breinl strain in the first passage, one died on the fourth day following injection, and the other became moribund

and was sacrificed on the seventh day. The brain, spleen and liver were removed from the rat which died on the fourth day. A microscopic examination of these tissues revealed the presence of rickettsiae in small numbers. An examination of the tissues from the other rat revealed the presence of rickettsiae in small numbers in the liver and spleen. No rickettsiae were found in the brain.

In the second passage, a suspension of brain, spleen and liver from the moribund rat was injected intracardially. One rat was sacrificed on the ninth day after injection, and the other was sacrificed on the twenty-second day. An examination of material from the brain, spleen and liver failed to demonstrate the presence of rickettsiae in the rats.

The intracardial injection of murine strains was followed by visible signs of illness in the rats. In the first passage, one rat died on the fifth day after injection and the second rat became moribund on the seventh day and was sacrificed. The brain, spleen and liver were removed from the dead rat and examined microscopically for the presence of rickettsiae. Rickettsiae were found in small numbers in each of the three tissues. A microscopic examination of the brain, spleen and liver from the moribund rat revealed the presence of rickettsiae in the liver and spleen. No rickettsiae were found in the brain. A suspension of liver and spleen was prepared and used as the inoculum for the second passage. The

rats were observed for a period of twenty-one days following the injection. No signs of illness were detected. The rats were sacrificed on the twenty-second day after injection and the brain, spleen and liver were examined microscopically for the presence of rickettsiae. No rickettsiae were found in the tissues of either rat.

The intracerebral injection of rats with infective yolk sac suspensions of the Breinl and Wilmington strains was followed by visible symptoms of illness. Of the rats injected with the Breinl strain, one died on the fourth day after injection. The second rat became moribund on the sixth day after injection and was sacrificed. The rat which died on the fourth day was autopsied and a microscopic examination was made of the brain, liver and spleen. Rickettsiae in small numbers were found in these tissues. The brain, spleen and liver were removed from the rat which was sacrificed and a microscopic examination of the tissues was made. Rickettsiae were found in small numbers in each tissue.

A suspension of brain was prepared from the rat which was sacrificed, and rats of the second passage were injected intracerebrally. No symptoms of illness were noted during a period of twenty-one days. The rats were sacrificed on the twenty-second day and the brain, liver and spleen were examined microscopically. No rickettsiae were found in these tissues.

The intracerebral injection of rats with infective yolk sac suspensions of the Wilmington strain was followed by visible evidence of infection. One rat died on the fourth day after injection. The second rat became moribund on the fifth day after injection, and was sacrificed on the sixth day. A microscopic examination of the brain, liver and spleen of the rat which died revealed the presence of rickettsiae in small numbers in these tissues. A microscopic examination of the brain, liver and spleen of the rat which was sacrificed showed the presence of rickettsiae in small numbers in the brain. No rickettsiae were found in the liver or spleen.

A suspension was prepared from the brain of the rat which was sacrificed, and injected intracerebrally into rats of the second passage. The rats were observed for a period of twenty-one days. No symptoms of illness were noted. The rats were sacrificed on the twenty-second day after injection and the brain, spleen and liver were examined microscopically. No rickettsiae were found in these tissues.

The intraperitoneal injection of rats with infective yolk sac suspensions of the Breinl and Wilmington strains was not followed by visible symptoms of illness. The microscopic examinations of the brain, spleen and liver failed to show the presence of rickettsiae. The passage in rats of suspensions prepared from the brain, spleen and liver resulted

in no deaths, and no symptoms of illness. The microscopic examinations of the brain, spleen and liver failed to reveal the presence of rickettsiae.

The production of experimental typhus infections in cotton rats of several months age followed the intracardial and intracerebral injections of infective yolk sac suspensions of the Breinl and Wilmington strains. The presence of infection was determined by the occurrence of symptoms of illness in the injected animals, followed in some cases by death. The presence of rickettsiae in the tissues of these animals was demonstrated by microscopic examination at the time of autopsy. Both strains failed to give demonstrable evidence of infection upon passage in cotton rats. The second passage rats survived injections by all routes with no symptoms of illness, and the microscopic examination of the tissues at the time of autopsy failed to reveal the presence of rickettsiae.

The inability to establish experimental typhus infections in the cotton rats, and the small numbers of rickettsiae which were found in the tissues of the first passage animals indicated that the cotton rats which were used in these studies were not suitable for the production of experimental infections.

This conclusion was confirmed by the results of two passages of murine typhus in baby cotton rats. The

observations were confined to the intracerebral route of administration. The rats were less than four weeks of age and due to the very limited number which were available, only two were used for each passage. These cotton rats died on the fourth to the sixth day after the intracerebral injection of a 10 per cent yolk sac suspension. A microscopic examination of the brain, spleen and liver tissues revealed the presence of rickettsiae in these tissues in large numbers. The intracerebral injection of other baby cotton rats with a 10 per cent suspension of brain removed from one of the dead rats at the time of autopsy, was followed by the death of the injected rats on the fourth to the sixth day after injection. A microscopic examination of the brain, spleen and liver tissues of the dead rats again revealed the presence of rickettsiae in large numbers.

4. DbA Mice

DbA mice of ten to fifteen gram weight were injected with infective yolk sac suspensions of the Breinl and Wilmington strains via the intracerebral, intravenous and intraperitoneal routes. Four mice were used for each route of injection and two series of two passages each were carried out for the two strains. The mice were kept at an environmental temperature of 65° to 77° centigrade.

The intracerebral injection of four dba mice with an infective yolk sac suspension of the Wilmington strain

resulted in the death of two mice on the third day after injection. Of the two remaining mice, one was sacrificed on the seventh day after injection and the other on the eleventh day. The brain, spleen and liver tissues were examined microscopically for the presence of rickettsiae. The rickettsiae were found in small numbers in the tissues of the mice that died. No rickettsiae were found in the tissues of those mice that were sacrificed.

A suspension was prepared from the brain and spleen of one of the dead mice and injected intracerebrally into the mice of the second passage. Two mice died on the fourth day after injection, and two survived a twenty-one day period of observation. A microscopic examination was made of the brain, spleen and liver of the dead mice. Rickettsiae were found in small numbers in the tissues of the dead mice. The surviving mice were examined for evidence of infection by means of their response to a toxic challenge of rickettsiae. When compared to non-injected mice, the resistance of the injected mice was found to be greatly increased. The control mice succumbed to toxic death following the intravenous injection of a rickettsial suspension containing $4LD_{50}$. The injected mice survived this toxic challenge. The resistance to the toxic challenge which was shown by the mice of the second passage indicated the presence of an inapparent infection which could be

recognized by the lack of reaction to the challenge as compared to the normal controls.

The intracerebral injection of four dba mice with an infective yolk sac suspension of the Breinl strain resulted in the death of one mouse on the fourth day after injection. The three remaining mice survived an observation period of twenty-one days. A microscopic examination was made of the brain, liver and spleen of the dead mouse. Rickettsiae were found in the brain, but could not be demonstrated in the liver or spleen. The surviving mice were challenged with $4LD_{50}$ of a rickettsial suspension on the twenty-second day. All of the mice survived the challenge.

A suspension was prepared from the brain and spleen of the dead mouse and injected intracerebrally into the mice of the second passage. Two mice died on the fifth day after injection and two survived an observation period of twenty-one days. The survivors were examined for the presence of an inapparent infection. Both mice survived $4LD_{50}$, while two control mice succumbed to toxic death.

The intravenous injection of dba mice with infective yolk sac suspensions of the Wilmington and Breinl strains rich with rickettsiae was followed by the death of the mice within twenty hours. The microscopic examinations of the brain, spleen and liver failed to show the presence of rickettsiae in these tissues.

When the yolk sac suspensions were diluted beyond their ability to cause toxic death, the intravenous injection of four dba mice with a 1-100 dilution of the Breinl strain resulted in the survival of the mice during an observation period of twenty-one days. The intravenous injection of four dba mice with a 1-100 dilution of the Wilmington strain was followed by the death of two mice on the fourth day after the injection. The microscopic examinations of the brain, liver and spleen of the dead mice showed the presence of rickettsiae in small numbers in the spleen and liver tissues. No rickettsiae were found in the brain tissues. The mice that survived the intravenous injections of the Breinl and Wilmington strains were challenged twenty-one days later with $4LD_{50}$ of the appropriate T.T.S. All of the mice survived.

The intraperitoneal injection of dba mice with 10 per cent yolk sac suspensions of the Breinl and Wilmington strains usually failed to kill the mice. When yolk sac suspensions rich with rickettsiae were used, however, the injected mice succumbed to toxic death. The intraperitoneal injections were made with four mice for each of the two strains. No deaths were recorded following the injection of the mice with either strain. One mouse from each passage was sacrificed on the eleventh day after injection and a 10 per cent suspension was prepared from the brain, spleen and liver. Each suspension was used for the intraperitoneal

injection of four mice of the second passage. No deaths occurred with either strain, and the mice were challenged twenty-one days after the injections with $4LD_{50}$ of the appropriate T.T.S. All of the mice survived.

The three remaining mice of the first passage for each strain were challenged twenty-one days after the injections with $4LD_{50}$ of the appropriate T.T.S. All of these mice survived.

The production of experimental typhus in the dba mice followed the injection of infective yolk sac suspensions by all of the routes examined. The intracerebral, intravenous and intraperitoneal injection of dba mice resulted, in some cases, in the death of the mice. The microscopic examinations of the brain, liver and spleen which were taken from the dead animals at the time of autopsy revealed the presence of rickettsiae in small numbers in these tissues. Those mice that did not die demonstrated the presence of inapparent infections by their ability to resist a toxic challenge containing $4LD_{50}$. The intraperitoneal injection of dba mice resulted in an inapparent infection which produced immunity as shown by the resistance of the mice to toxic challenge. Since the numbers of rickettsiae which were found in the tissues of the dead mice were small, this host was not used for the large scale production of rickettsiae.

The susceptibility of the dba mice to infections caused by R. prowazeki and R. typhi was shown by the results

of the pathogenesis studies. This susceptibility was found to be of value in estimating the biologic activity of the rickettsial suspensions used in the respiration studies. The details of the estimation of the biologic activity of the rickettsial suspensions will be presented as a part of the results for the investigations upon the toxicity and infectivity of the rickettsiae.

Toxicity Titrations

The intravenous or intraperitoneal administration of yolk sac suspensions containing large numbers of rickettsiae resulted in the toxic death of the dba and albino mice. The cotton rats and white rats which were injected intracardially with yolk sac suspensions containing large numbers of rickettsiae did not succumb to toxic death. The intraperitoneal injection of guinea pigs was not followed by toxic death. No attempt was made to study the effect of intracardial injections of yolk sac suspensions in guinea pigs.

A characteristic sequence of events followed the injection of toxic suspensions into mice. In the course of a few minutes they became lethargic, moving about with apparent difficulty and finally lying prostrate. They suffered from dyspnoea. Occasionally some of the mice were seized with convulsive movements, jumping rigidly and finally falling on their side, unable to rise. A post mortem

examination of the mice revealed no gross changes in the tissues. There were no areas of hemorrhage, and the internal organs gave no evidence of being engorged with blood. The deaths occurred within eight hours, and in some instances within forty-five minutes following an intravenous injection. The toxic deaths resulting from intraperitoneal injection were more delayed, the shortest time observed being two hours. Regardless of the route of administration, the deaths which occurred after eight hours were not considered as toxic. The direct relationship of rickettsial concentration to the toxic effect of the yolk sac suspensions could be demonstrated by dilution of the suspensions. When the numbers of rickettsiae in a yolk sac suspension were reduced by dilution, a suspension was eventually attained in which the numbers of rickettsiae that remained were too few to result in toxic death. The results of the toxicity titrations are given in Table 10.

The relation of the rickettsial concentration to the occurrence of toxic death following the intravenous injection of albino mice was applied to the biologic measurement of the rickettsial concentration of the test suspensions used in the respiration studies. By calculating the LD₅₀ of a test suspension, a threshold value was determined which served as a point of reference for the comparison of the rickettsial concentration of other test

TABLE 10

Toxic death in dba and albino mice following intravenous or intraperitoneal injection of rickettsiae

Experiment	Rickettsiae	Dilutions of Toxic Test Substance									LD ₅₀	
		1:10	1:20	Intravenous			Intraperitoneal			IV*	IP**	
				1:40	1:80	1:160	1:4	1:8	1:16	1:32		
1-albino	Breind TTS No. 2A	4/4 ¹	4/4	4/4	3/4	0/4					100 ²	
1-dba	Breind TTS No. 2A	4/4	4/4	4/4	2/4	0/4					80	
2-albino	Breind TTS No. 3A	4/4	4/4	4/4	2/4	0/4	—	4/4	4/4	0/4	80	22.6
3-albino	Wilmington TTS No. 2B	4/4	2/4	0/4	0/4	0/4	2/4	1/4	0/4	0/4	20	4.7
3-dba	Wilmington TTS No. 2B	4/4	3/4	0/4	0/4	0/4					25	

1 Numerator represents number dead.
Denominator represents number tested.

2 Final dilution of rickettsial suspension, 0.5 ml of which equals 1 LD₅₀.

* IV = Intravenous

** IP = Intraperitoneal

suspensions. Thus it was possible to show that a test suspension with an LD₅₀ value of fourteen contained twice as many viable rickettsiae as a test suspension with an LD₅₀ value of seven.

There were no significant differences between the dba and albino mice in susceptibility to toxic death. The intraperitoneal route of administration, when compared to the intravenous in albino mice, resulted in lower LD₅₀ values. The difference in the toxicity of the Breinl and Wilmington strains for the experimental hosts was more apparent than real. Microscopically the yolk sacs used in the preparation of the Wilmington T.T.S. did not contain as many rickettsiae as the yolk sacs used for the Breinl preparation. The difference in the toxicity was attributed to the relative rickettsial concentration of the respective preparations.

The albino mice were selected as the test animal in which the toxicity titrations were carried out. Their selection was based upon the ease and convenience with which intravenous injections were made when compared to the use of dba mice for the same purpose.

Infectivity Determinations

The results of the pathogenesis studies with all of the experimental hosts examined indicated the need for a method which would permit the recognition of inapparent infections, and with some degree of accuracy, allow the

evaluation of host susceptibility of R. prowazeki and R. typhi. The investigations of host susceptibility to the toxic preparations of the rickettsiae indicated that the dba and albino mice were the most satisfactory hosts for the measurement of the toxic action of rickettsiae. The demonstration of toxic death in these mice following the injection of large numbers of rickettsiae, and their resistance to toxic death after inapparent infections, suggested the use of these animals as experimental hosts for the determination of the presence of small numbers of viable rickettsiae.

The studies on the infectivity of rickettsiae for the dba and albino mice were based upon the host resistance to toxic challenge which could be demonstrated after the injection of rickettsiae. The susceptibility of the two strains of mice was compared by determining the degree of resistance to toxic challenge which followed the intraperitoneal injection of dilutions of rickettsial suspensions. The host which showed the greater resistance to toxic challenge following the intraperitoneal injection of the smallest number of rickettsiae was considered to be more susceptible.

The mice that survived toxic death following the intravenous injection of dilutions of the infective yolk sac suspensions used in the toxicity titrations were shown to exhibit different degrees of resistance to a toxic challenge

administered twenty-one days later. The results are given in Table 11.

The survival of the dba mice that were treated with the Breinl strain varied from a value of 83 to 100 per cent, while the albino mice showed a survival value of 80 per cent to the toxic challenge. The survival of the dba mice that were treated with the Wilmington strain had a value of 100 per cent, compared to no survivals of the albino mice that received identical treatment. The untreated control mice showed no resistance to the toxic challenge. The results of the intraperitoneal injection of the dba and albino mice with one hundred-fold dilutions of infective yolk sac suspensions of the Breinl and Wilmington strains is given in Table 12.

The susceptibility of the dba and albino mice to the Breinl strain (TTS #1A), as determined by the ID₅₀, was shown to be the same. With the Breinl TTS #2A, the ID₅₀ was considerably less for the dba than for the albino mice. A comparison of the susceptibility of dba and albino mice to the Wilmington strain, as determined by the ID₅₀, indicated that the dba mice were more susceptible. The ID₅₀ was ten thousand times less for the dba than for the albino mice.

The infectivity studies indicated that the dba mice were more susceptible to R. typhi than the albino mice, and at least as susceptible to R. prowazeki. Because the dba mice were considered to be more suitable for studies involving both

TABLE 11

The development of an immunizing infection in survivors
of toxic death titrations

Experiment	Rickettsiae	Fraction of Toxic Dose Originally Received	Challenge ^{**} LD ₅₀	Survivors Number Per Cent
1-albino	Breind TTS No. 2A	0.937	4	1/5* 80.0
1-dba	Breind TTS No. 2A	0.75	4	1/6 83.3
1-control		none received	4	4/4 0.0
2-albino	Breind TTS No. 3A	0.75	4	0/6 100.0
2-control	Breind TTS No. 3A	none received	4	4/4 0.0
3-albino	Wilmington TTS No. 2B	0.564	2	12/12 0.0
3-dba	Wilmington TTS No. 2B	0.390	2	0/4 100.0
3-control		none received	2	4/4 0.0

** LD₅₀ value received by mice as determined by a control toxicity titration at the time of challenge.

* Numerator represents number dead.

Denominator represents number tested.

TABLE 12

Infectivity determinations in dba and albino mice

Rickettsias	Previous Treatment*		Challenge		ID ₅₀		
			21 d. after Toxic Dose** LD ₅₀	infective dose Dead/Tested dba albino	dba	albino	
Brein1	10 ⁻⁴	0.25 ml	4	1/4	1/4	10 ^{-4.6}	10 ^{-4.6}
TTS No. 1A	10 ⁻⁶	0.25 ml		4/4	4/4		
	10 ⁻⁸	0.25 ml		4/4	4/4		
	Sterile broth "			4/4	4/4		
Brein1	10 ⁻³	0.25 ml	4	0/4	4/4	10 ^{-6.3}	10 ^{-3.0}
TTS No. 2A	10 ⁻⁵	0.25 ml		0/4	4/4		
	10 ⁻⁷	0.25 ml		3/4	4/4		
	10 ⁻⁹	0.25 ml		4/4	4/4		
	Sterile broth "			4/4	4/4		
Wilmington	10 ⁻³	0.25 ml	2	0/4	0/3	10 ^{-8.0}	10 ^{-4.0}
TTS No. 2B	10 ⁻⁵	0.25 ml		0/4	4/4		
	10 ⁻⁷	0.25 ml		0/4	4/4		
	10 ⁻⁹	0.25 ml		4/4	4/4		
	Sterile broth "			4/4	4/4		

* The various dilutions of yolk sac material were injected intraperitoneally.

** ID₅₀ value received by mice as determined by a control toxicity titration at the time of challenge.

R. typhi and R. prowazeki, they were selected for use in the infectivity determinations.

Where the LD₅₀ and ID₅₀ for the Breinl strain were compared in dba mice, the ID₅₀ was found to be approximately ten thousand times less. A similar comparison of the LD₅₀ and ID₅₀ for the Wilmington strain in dba mice indicated that the ID₅₀ was approximately one million times less. This finding indicated that with the Breinl strain, the smallest number of rickettsiae capable of causing the toxic death of the mice was approximately ten thousand times the smallest number capable of initiating an immunizing infection. With the Wilmington strain, the smallest number of rickettsiae capable of causing the toxic death of the mice was approximately one million times the smallest number capable of initiating an immunizing infection.

The difference between the LD₅₀ and the ID₅₀ suggested at first that the use of the LD₅₀ could be replaced by the ID₅₀ because the latter made it possible to determine the presence of smaller numbers of rickettsiae in the test suspensions used in the respiration studies, and thus afforded the investigator a more delicate measurement of the rickettsial concentration. Although the use of the ID₅₀ did make it possible to measure smaller numbers of rickettsiae in a test suspension, this procedure did not ensure the measurement of viable rickettsiae alone. The ID₅₀ was considered to be the result of the total effect of both viable

and non-viable rickettsiae, while the LD₅₀ represented the exclusive measurement of viable rickettsiae. Because the results of the respiration studies indicated that a direct relation existed between the uptake of oxygen and the number of viable rickettsiae present, the final procedure which was selected for the biologic measurement of the rickettsial concentration included the determination of the LD₅₀ in addition to the ID₅₀.

DISCUSSION

The study of the cultivation of typhus fever rickettsiae in several hosts was undertaken to determine the most suitable source of rickettsiae for the respiration studies. The developing yolk sac of the chick embryo was selected for its convenience and predictable yields of large numbers of rickettsiae. The examination of the tissues from infected guinea pigs, cotton rats, white rats and mice revealed the presence of rickettsiae in concentrations.

The preparation of the rickettsial suspensions used in the respiration studies depended upon the separation of large numbers of viable rickettsiae from the yolk sac tissues. The respective efficiencies of differential centrifugation and oil-water emulsions for the separation of the rickettsiae were compared. The use of turpentine, pine and paraffin oils which was reported by Giroud and Meunier (76) for the separation of rickettsiae from animal tissues did not prove applicable to the separation of rickettsiae from yolk sac tissues. The differential centrifugation method which was reported by Bovarnick and Snyder (71) was found to be the most satisfactory for the routine preparation of the rickettsial suspensions.

The need for large numbers of viable rickettsiae in the respiration studies was made clear in the early stages of the investigations. In order to prepare rickettsial

suspensions which contained a sufficient concentration of viable rickettsiae, it was necessary to use at the outset at least nine yolk sacs containing numerous rickettsiae and to use a clarifying agent in the process of differential centrifugation. Several agents were selected for a comparative study of their ability to increase the efficiency of separation in the differential centrifugation procedure. Of the agents studied, magnesium oxide and celite-analytical filter aid were found to give the most satisfactory results. The magnesium oxide resulted in pH values considered to be incompatible with viability of the rickettsiae.

Because the alkalinity of the magnesium oxide preparations was 9.6 or higher, the celite-analytical filter aid was selected for routine use. Bovarnick and Snyder have also reported the use of celite in the preparation of rickettsial suspensions employed for respiration studies (71). These authors prepared their rickettsial suspensions with the aid of a phosphate buffer to which glutamate was added. The glutamate was present in all of the centrifugations except the last washing. Because the use of glutamate in the diluent was considered to represent a possible source of error in the interpretation of the respiration studies, the diluent employed in the present studies was Krebs-Ringer phosphate buffer which did not contain glutamate.

The results of the respiration studies demonstrated an uptake of oxygen with L-glutamic acid and L-cysteine

hydrochloride in the presence of R. prowazeki. Bovarnick and Snyder and Bovarnick and Miller reported similar results for L-glutamic acid but failed to obtain an uptake of oxygen with any of the other naturally occurring amino acids (71, 72).

In the present studies the respiration was shown to be affected by pH, as there was no uptake of oxygen at 6.7. The uptake of oxygen in the presence of glutamate and cysteine was proportional to the number of viable rickettsiae in the test suspension, as evidenced by the respective biologic activity of these suspensions when tested for their toxicity and infectivity in animals. The use of suspensions with relatively small numbers of viable rickettsiae, as determined by their biologic activity, failed to show an uptake of oxygen in the presence of glutamate or cysteine.

The glutamic acid used in the respiration studies was measured by means of paper chromatography. The measurement of cysteine by paper chromatography resulted in recovery values of less than fifty per cent. Other investigators have been unable to obtain quantitative recoveries of cysteine by paper chromatography methods (80). Because cysteine could not be measured with accuracy, the evaluation of the substrates used in the respiration studies was restricted to glutamic acid. The glutamic acid concentration of the test and control systems was shown to decrease during respiration.

The greatest diminution of the glutamic acid content occurred with the test suspension of viable rickettsiae. The decrease in the glutamic acid concentration was accompanied by a corresponding uptake of oxygen in the test system. The control system gave no significant uptake of oxygen. Bovarnick and Miller reported similar results for their studies on the transaminase activity of R. prowazeki (72). In these studies the authors removed a transaminase, which was found in normal yolk sac preparations, by agglutination in 6 per cent serum albumin. With rickettsial preparations that were treated with 6 per cent serum albumin, the yolk sac transaminase was removed and it was possible to show the existence of a rickettsial transaminase that remained active after the loss of oxidative enzymes and viability.

The pathogenesis studies in animals showed the guinea pig, cotton rats and dba mice to be the most susceptible hosts for R. prowazeki and R. typhi. The production of experimental typhus in the white rat was followed by an inapparent form of infection which could be detected only by the appearance of complement fixing antibodies. Infection in the guinea pig, cotton rat and dba mice was accompanied by symptoms of illness. Infection in the cotton rats and mice terminated in some instances in death. No deaths occurred in the infected guinea pigs. The microscopic

examination of the brain, spleen and liver tissues of these animals revealed the presence of rickettsiae in small numbers. The serial passage of rickettsiae was accomplished in the guinea pigs and dba mice, but serial passage of rickettsiae in cotton rats was not successful. The cotton rats were taken from a stock which was several months old as indicated by the large size of the animals and their average weight of 40 to 50 grams. The results of Snyder and Anderson who reported the successful adaptation of the Breinl and Wilmington strains to the eastern cotton rat, indicated that rats of more than two months of age did not exhibit the same degree of susceptibility as younger rats (82). In a later report Anderson stated that rats of two months of age did not differ significantly in susceptibility from younger rats (35). The results of the present studies indicated that cotton rats of approximately three months of age cannot be substituted for cotton rats of less than two months age. The findings also indicated that the cotton rats should not be more than six weeks of age for the most satisfactory use of this host in rickettsial research.

The use of dba mice as an experimental host for the evaluation of the biologic activity manifested by the rickettsiae followed the demonstration of their greater susceptibility when compared to guinea pigs, cotton rats and albino mice. The intraperitoneal injection of dilutions

of infective yolk sac suspensions was followed by inapparent infections in the albino and dba strains of mice. The occurrence of these infections was determined by the resistance of the mice to an intravenous challenge consisting of $4LD_{50}$ which resulted in the toxic death of the untreated control mice. The dba mice were found to be susceptible to smaller numbers of rickettsiae than the albino mice, and as they represented a host in which small numbers of rickettsiae could be measured, they were used for the determinations of the infectivity of rickettsial suspensions. These results confirm the findings of Moragues and Pinkerton who showed that a greater number of dba mice died from infection with R. typhi than albino mice (44). These authors used death as their criterion of susceptibility, while the present studies made use of the immunizing infection. The principle of the immunizing infection was based upon the resistance to toxic challenge which was displayed by mice following the injection of rickettsiae. The mice exhibited no evidence of an infection at any time. The inapparent infections in the present studies followed the injection of small numbers of rickettsiae. The recognition of these infections enabled the investigator to detect the presence of rickettsiae in the inoculum.

The intravenous injection of dba and albino mice with infective yolk sac suspensions was followed by the death of both strains of mice. The deaths occurred within eight

hours after the injections. Upon dilution of the infective yolk sac suspensions a point was eventually reached where the mice survived toxic death. The presence of an inapparent infection in the survivors was shown by their ability to resist a toxic challenge containing $4LD_{50}$. No significant difference in the susceptibility to toxic death was shown for the dba and albino mice. Because the intravenous injection of the albino mice could be accomplished with greater ease in a shorter time interval, this strain was used for the determinations of the toxicity of rickettsial suspensions.

The comparison of the LD_{50} and ID_{50} values for the same yolk sac suspension indicated that in the case of the Breinl strain, the ID_{50} was approximately ten thousand times less than the LD_{50} . The ID_{50} value for the Wilmington strain was one million times less than the LD_{50} . The LD_{50} was considered to result from the action of viable rickettsiae alone, while it was possible for the ID_{50} to represent the action of both viable and non-viable rickettsiae. Thus the ID_{50} may have represented the effect of small numbers of rickettsiae, an indeterminate number of which may have been non viable.

The evaluation of the biologic activity of the rickettsial suspensions was based upon the determination of both the LD_{50} and ID_{50} . Through the use of these measurements two points of reference were established for each

suspension, and it was possible not only to compare their activity in the respiration studies, but to relate this activity to the approximate number of rickettsiae in the test suspensions.

The differences in the ratios of the LD₅₀ to the ID₅₀ which were observed for the Breinl and Wilmington strains were not attributed to a greater toxicity on the part of the Breinl strain. While this possibility could not be eliminated, consideration was also given to the greater numbers of rickettsiae which were found in the Breinl preparations, and the appearance of a state of resistance to toxic challenge in dba mice following the injection of small numbers of rickettsiae of the Wilmington strain. These findings indicated that the observed difference in the ratio of the LD₅₀ to the ID₅₀ for the Breinl and Wilmington strains could be ascribed to one or more of the following factors: a greater toxicity of the Breinl strain for mice; a greater concentration of rickettsiae in the Breinl preparations; a greater susceptibility of dba mice for the Wilmington strain.

SUMMARY

1. The cultivation of typhus fever rickettsiae in the developing yolk sac of the chick embryo resulted in a satisfactory yield of viable rickettsiae for the respiration studies.
2. Differential centrifugation was used to separate the rickettsiae from yolk sac tissue.
3. The use of clarifying agents to increase the efficiency of differential centrifugation was studied.
4. Celite-analytical filter aid was selected as the clarifying agent to increase the yield of viable rickettsiae obtained by differential centrifugation.
5. The Breinl strain of louse-borne typhus demonstrated an uptake of oxygen in the presence of L-cysteine hydrochloride and L-glutamic acid.
6. The use of paper chromatography indicated that a quantitative reduction in the concentration of glutamic acid occurred during respiration.
7. The uptake of oxygen in the presence of L-cysteine HCl and L-glutamic acid was proportional to the concentration of viable rickettsiae present.

8. The biologic activity of the rickettsial suspensions was measured by their toxicity for albino mice and infectivity for dba mice.
9. No significant difference in susceptibility to toxic death was demonstrated for the dba and albino mice.
10. Dba mice were more susceptible to the Wilmington strain of murine typhus than albino mice. With the Breinl strain of louse-borne typhus the dba strain was at least as susceptible as albino mice.
11. Cotton rats, three months old, exhibited a high order of resistance to experimental typhus. Cotton rats six weeks old were shown to be very susceptible to experimental typhus.
12. White rats were shown to possess a high level of resistance for the rickettsiae of murine typhus.
13. The production of experimental typhus in male guinea pigs was accompanied by a scrotal swelling which was observed for both the louse-borne and murine strains.

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