with comparable skill can tag from 1200 to 1500 flies per day without undue fatigue.

The mechanical unit consists of a fly-holding frame and glue dispenser, both made of aluminum, and an ordinary ¼-cc hypodermic syringe. The holding frame shown in Fig. 1 (top view) is a troughed bar divided into 25 compartments by means of notched ribs (D), and a foam-rubber-padded cover (L). A nylon thread (C in section A-A) is held in the bottom of the groove (F) by being stretched between jam cleats at both ends of the frame. Anesthetized flies are placed upside down in the trough (K) with their heads toward the holes (H) centered about 3/16 in. from the ribs. The frame is tilted slightly upward from the right end and tapped lightly causing the flies to slide down until their heads rest against the ribs. They are now in such a position that a conical hole (P and H), extending from the under side of the frame, is centered under the thorax of each fly. The cover (L) padded with sponge rubber (M) is snapped into place to hold the flies securely and the whole unit is inverted to expose the thoraces of the flies through the conical holes.

Glue is dispensed with a ¼-cc syringe held in the dispenser (Q). A micrometer screw (S), attached to the end of the dispenser barrel, moves the syringe plunger forward. The nose of the dispenser is placed in each hole and the knurled wheel (R) turned 1 notch clockwise. This procedure placed 1/1600 ml of Duco cement, thinned with 50% acetone, on each fly at the point where the thorax touches the nylon thread. The holding frame is now turned right side up, the cover removed, and the chain of 25 flies is taken out. The string is cut in front of the head of each fly and the separated flies are then placed in holding cages for later release tests.

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A Quantitative Analysis of the Factors Involved in the Variations in Virulence of Rickettsiae[1]

Winston H. Price[2]

Departments of Biochemistry and Epidemiology, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland

Previous investigators have observed that strains of *Rickettsia rickettsii,* the causative agent of Rocky Mountain spotted fever, recovered from ticks in nature differed widely in their apparent pathogenicity for guinea pigs (*1*). Furthermore, Spencer and Parker (*2*) found that, if *Dermacentor andersoni* ticks infected with virulent spotted fever were left in the icebox for several months, a ground suspension of these ticks injected into guinea pigs produced fewer, if any, spotted fever symptoms, yet immunized the animal against subsequent infection by the virulent agent. If such ticks were given a blood meal and then injected into guinea pigs, these animals came down with virulent spotted fever. This was termed a reactivation phenomenon. Unfortunately, the above observations are open to some criticism since little attention was paid to the quantitative relationships. However, this system suggested an intensive study of the factors involved in the variation in virulence of an intracellular microparasite.

The series of experiments here reported was undertaken to establish more precisely by quantitative methods the differences in virulence of the representative strains of *R. rickettsii,* the possibility of modifying strain virulence by animal passage and by tick passage, the nature of the reactivation phenomenon, and the identity of the enzyme systems principally involved in these changes of virulence.

Characterization of Strain Variation in Virulence.[3] The first series of experiments was planned to establish by quantitative methods the range in virulence of four representative strains of *R. rickettsii.* The following procedures were adopted. The strains were isolated from yolk sacs by a procedure similar to that of Wisseman *et al.* (*3*). The number of rickettsiae in the suspension was determined by the electron scope method described in Table 1. Each strain was titered in guinea pigs and chick embryos as described in Table 1. This experiment was repeated three times with similar findings. The more virulent organisms grow better in the guinea pig than the less virulent strains, the major differences being particularly noticeable in the heart, brain, and blood. Thus strain R reaches a maximum concentration of 300 and 100 LD_{50} in the brain and heart, respectively, and of 300 LD_{50} in the blood. Strains S and T show a maximum of only 1 to 3 LD_{50} in the brain and heart, and only about 10 LD_{50} in the blood.

Guinea pigs injected with 10,000 LD_{50} of one strain are immune to the three other strains. Furthermore, strains T and U will completely protect guinea pigs from strain R when given in 100 times the concentration (10^6 LD_{50} of T or U and 10^4 LD_{50} of R) with both weakly virulent and virulent strains injected intraperitoneally simultaneously.

All strains shown in Table 1 possess a toxin[4] similar

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[2] I wish to express my sincere appreciation to Carl L. Larson, Director of the Rocky Mountain Laboratory, National Microbiological Institute, National Institutes of Health, Hamilton, Mont., for placing the facilities of the laboratory at my disposal. A few of the experiments reported in this paper were carried out at that laboratory. I wish also to thank David Lackman for carrying out many of the complement fixation determinations. Acknowledgment is also due to H. Sargent, L. Hughes, Mrs. M. Smith, Miss H. Emerson, C. H. Sawyer, and E. G. Pickens for valuable technical assistance in various phases of this work.

[3] All LD_{50} determinations refer to egg titrations. Using the R strain, 1 LD_{50} is equivalent to minimal infectious dose (ID_{50}) for guinea pigs. In all instances where eggs and tissues are titrated, the LD_{50} refers to the number of LD_{50} in 0.5 ml of a 10% suspension.

[4] E. J. Bell and E. G. Pickens of the Rocky Mountain Laboratory, in an independent series of investigations, have found that *R. rickettsii* produce a toxin.

TABLE 1*

A COMPARISON OF VARIOUS STRAINS OF SPOTTED FEVER†

Strain	Scrotal reaction	Days of fever	Av height of fever	Fatality	Minimum number of ricksettsiae		
					To infect		For 1 LD_{50} for eggs
					Eggs	Pigs (ID_{50})	
R	++++	8.1 ± 1.2	40.6 ± 0.16	12/20‡	1–10	50–200	50–200
S	++	4.1 ± 0.52	40.4 ± 0.12	0/20	1–10	50–200	50–200
T	—	4.3 ± 0.82	40.4 ± 0.14	0/20	1–10	50–200	50–200
U	—	0	—	0/20	1–10	—	50–200

* Twenty male guinea pigs weighing about 400 g were used for each strain. Each pig was injected intraperitoneally with 1000 ID_{50}'s of the appropriate strain. All strains before injection were isolated according to a procedure similar to that of Wisseman *et al.* (*3*). All parts of the experiment were carried out at the same time, and the same suspension was used for the guinea pig and egg titration of each strain. The number of organisms in a suspension was determined by electron microscopic count. A positive complement fixation reaction was used to determine the number of organisms infective for guinea pigs. In determining the number of rickettsiae necessary to infect eggs, the lowest number of organisms was selected that when injected into chick embryos gave positive microscopic rickettsial smears after two blind passages in chick embryos. Five-day-old chick embryos were used and inoculated into the yolk sac. ++++ indicates scrotal hemorrhage, necrosis, and sloughing off, and ++ indicates scrotal redness and swelling. Fever was taken as any temperature of 39.8° C or over.

† The rickettsiae in the suspensions have been counted under the electron microscope; a standardized suspension of latex particles was used. The absolute error of this counting method is 6% (*4*).

‡ Numerator indicates number of animals dying from spotted fever.

to that reported for *R. prowazeki.* Mice injected intravenously with the various strains begin to die in a few hours. The toxic effect is neutralized by specific immune serum. Purification experiments indicate that the toxin is associated with the viable rickettsiae. Mice titrations carried out with rickettsial suspensions prepared from yolk sacs, the number of organisms in the suspensions being determined by the electron microscope as described in Table 1, indicate that all strains have approximately the same amount of toxin. It is still possible, however, that in guinea pigs the virulent organisms have more toxin. Technical difficulties make the latter determination impossible.

All strains shown in Table 1, when grown in yolk sacs, have about the same amount of hemolytic factor, which is similar to that reported for epidemic and endemic typhus. The hemolytic factor can be neutralized by specific immune serum. As with the toxic factor, it is possible that the more virulent strains have more of the hemolytic factor when they multiply in the guinea pig than do the less virulent strains.

Within the experimental error of about 50 per cent, the minimum number of rickettsiae necessary to infect and kill chick embryos is the same irrespective of the virulence of the strain for the guinea pig (cf. Table 1). Therefore, within the error of the method, animals injected with the same LD_{50} dose of strains of varying virulence receive the same number of organisms.

Under the conditions shown in Table 1, 3.0 mg of cortisone injected subcutaneously daily for 96 hr before infection and daily for 72 hr after infection had no detectable effect on the virulence of strains R, S, T, and U. However, larger amounts of cortisone have a protective effect against *R. rickettsii.*

All strains, R, S, T, and U, grow to the same extent in chick embryo yolk sacs, which contain between 10^6 to 10^7 LD_{50}'s. It is of interest in this connection that in chick embryos *R. rickettsii* exhibit a multiplication pattern that is different from that of such other intracellular parasites as animal viruses. This statement is supported by the observation that *R. rickettsii* does not lose its infectious property before beginning to multiply, as do other intracellular parasites such as some animal viruses like influenza (*5*).

Animal and Tick Passage of R. rickettsii. Spotted fever strains isolated west of the Mississippi River from the rabbit tick, *Haemaphysalis leporis-palustris,* have given only very mild symptoms resulting in 2 to 3 days of fever (39.8–40.1° C) when injected into male guinea pigs (*1*). Our experiments so far have confirmed these results. Furthermore, egg titrations show that this very low virulence is not due to a low concentration of rickettsiae. These rickettsiae multiply very poorly in the guinea pig (1 LD_{50} or less in the spleen).[5]

Experiments were conducted to ascertain the effect of passage of strains through two different species of ticks, *Dermacentor andersoni* and *Haemaphysalis leporis-palustris.*

Passage of these weakly virulent strains through one complete life cycle in *D. andersoni,* from which species of tick many virulent strains have been isolated (*1*), does not increase their virulence for the guinea pig. Passage of virulent strains (similar to strain R in Table 1) through one complete life cycle in the rabbit tick does not decrease their virulence for the guinea pig. Three hundred and four *D. andersoni* and 295 rabbit ticks have been tested so far in these experiments. In all these studies the larvae were infected with the appropriate strains by feeding on infected guinea pigs or rabbits. The larvae were then fed on normal animals in the nymphal, adult, and next larval stage and tested at this stage. Careful attention was paid to the number of organisms injected in all experiments. Attempts to modify the strain virulence of *R. rickettsii* by passage in *D. vari-*

[5] After one egg passage the virulence of these strains is increased to that shown for strain S in Table 1.

abilis, A. maculatum, and *R. sanguineus* have been completely negative.

On the other hand, by continued spleen passage in cottontail rabbits, the virulence for the guinea pig of the virulent strain referred to above was decreased. By starting out with a strain similar to the R type, a strain similar to the S type was obtained after 8 to 13 spleen passages. This strain still caused the same fairly mild spotted fever symptoms in the cottontail rabbit as the original rickettsial suspensions before passage. If this weakly virulent strain was transferred back several times in guinea pigs by spleen passage, the original virulent strain was obtained. These experiments were repeated with two virulent strains. With another virulent strain, a strain similar to T was obtained. Careful attention was paid to quantitative relationships in all these experiments, i.e., the concentration of rickettsiae injected after the rabbit passages was compared to comparable concentrations of the rickettsial strain before passage. Other experiments indicate that the virulence of some strains of *R. rickettsii* can be increased by one passage in man. Our data would therefore indicate that the virulence of *R. rickettsii* for the guinea pig can be modified by animal passage but not by multiplication in their arthropod hosts.

The Reactivation Phenomenon. It has been thought that the reactivation phenomenon occurred because there were enough rickettsiae to immunize but not enough to infect. Our analysis of this phenomenon has shown that this is not true. Female *D. andersoni* were fed on guinea pigs infected with 1000 LD_{50}'s of the virulent R strain. After the eggs were laid, the resulting larvae and nymphs were also fed on infected guinea pigs. The adults were left 1 month at room temperature and were then stored at 6° C for 5 months.[6] Lots of ticks were removed at this time and ground into tick suspensions and titered in eggs. Such suspensions were found to contain 1000 LD_{50}'s per 0.5 ml. This is 1000 times the number of spotted fever organisms necessary for guinea pigs to come down with virulent spotted fever. Yet, when 20 guinea pigs were infected with the suspension containing the 1000 LD_{50}, 4 showed slight fever for 2 days (39.8°–40.0° C) and the other 16 remained normal (38.6°–39.6° C). All guinea pigs proved to be immune to 100 LD_{50} challenge doses of the virulent spotted fever. The rickettsiae could be reactivated, or made virulent by leaving the ticks at 37° C for 48 hr (*2*) or by passing them once through chick embryos. After reactivation, as little as 1 to 10 LD_{50} would cause typical virulent spotted fever, with the animals showing the typical symptoms and immune responses shown in Table 2. So far fifteen different experiments, involving 505 guinea pigs and over 5000 ticks, have been carried out. All data from these experiments are consistent with the above results. The avirulent phase was also found in eggs, larvae, and nymphs of ticks previously infected in the laboratory. Spotted fever rickettsiae in the avirulent phase can be isolated in nature from adult *D. andersoni* which have not fed.

TABLE 2

A COMPARISON OF THE VIRULENT AND AVIRULENT PHASES OF *R. rickettsii* FOR THE GUINEA PIG*

Avirulent phase	Virulent phase
Grows poorly (0 LD_{50} in spleen)	Grows well (300 LD_{50} in spleen)
Produces low, if any complement fixation titer (0–1: 20)	Produces high complement fixation titer (1: 254–1: 1024)
Produces toxin neutralizing antibody	Produces toxin neutralizing antibody
Cannot be passed serially from pig to pig	Can be passed from pig to pig
Produces immunity to virulent spotted fever	Produces immunity to virulent spotted fever
Produces no immunity to boutonneuse fever	Produces immunity to boutonneuse fever
Changed to highly virulent form by 1 egg passage	Virulence not changed on 1 egg passage
Changed to highly virulent state by keeping infected ticks at 37° C for 72 hr	Virulence not changed by keeping ticks infected with virulent form at 37° C for 72 hr
Produces little, if any, fever	Produces about 8 days of fever
Produces no scrotal reaction	Produces severe scrotal reaction
No fatality of guinea pigs	25–50% fatality

* Table based on 100 guinea pigs injected with 100 LD_{50} of avirulent phase and 100 guinea pigs injected with 100 LD_{50} of virulent phase.

All attempts to demonstrate multiplication of this avirulent phase of *R. rickettsii* in the guinea pigs have failed. It should be noted that the avirulent phase of *R. rickettsii* is different from the weakly virulent strains S, T, and U, since these strains cannot be activated by one egg passage or by a blood meal in ticks.

Table 2 gives a summary of the properties of the virulent and avirulent phases of *R. rickettsii* in the guinea pig. Other experiments have indicated that the reactivation phenomenon is not due to selection, spontaneous mutation, or to an inhibitor in the avirulent tick suspensions which prevent the guinea pigs from coming down with spotted fever.[7] Once reactivation occurs, the rickettsiae can be transferred by spleen passage from guinea pig to guinea pig for at least 16 transfers. The change from the avirulent to the virulent state thus appears to be hereditary. It is of interest that Q fever rickettsiae in adult *D. andersoni* have not been found to show the reactivation phenomenon (*6, 7*). It is clear that an understanding of this reactivation phenomenon in *R. rickettsii* will give us a much better understanding of the factors that are

[6] Some lots of infected ticks must be left as long as 12 months at 6° C. In carrying out the actual experiments, 10 to 30 ticks were ground and made up to 15.0 ml in a solution containing 0.22 *M* sucrose and 0.04 *M* potassium phosphate, pH 7.4; 0.5 ml of this suspension was then titered in eggs. Each egg also received 750 units of penicillin and 400 units of streptomycin.

[7] Recent experiments indicate that the avirulent phase is not due to the fact that the tick suspensions contain dead rickettsiae which interfere with the multiplication of living virulent rickettsiae. All evidence indicates that there is a change in the virulent organism which makes it avirulent.

concerned with the virulence of rickettsiae, and perhaps of other infectious agents, and may also give valuable clues as to how infectious agents persist in nature. It might be mentioned that the avirulent phase of *R. rickettsii* has properties similar to those of masked viruses (*8*), in that a non-infective state exists although the parasites can be made infective by the appropriate procedures. If this phenomenon turns out to be more common than is now thought, it must be taken into account in any study of the natural history of an infectious agent. In such studies the presence or absence of the agent is usually determined by infectivity measurements. However, in view of the above results the failure to find an infective agent by infectivity determinations would not necessarily mean that the agent is not present.

Enzyme Studies. In the final analysis, the difference in the virulence of various strains is probably due to a difference in their enzyme systems. Experiments were therefore carried out on strains R, S, T, and U described in Table 1, to see if any enzyme difference could be found. Because Bovarnick and Miller (*9*) and Wisseman *et al.* (*10*) have found part of the Krebs cycle to be functioning in *R. mooseri,* this system was studied first.

No differences have been found so far in any of the enzyme systems of the various strains. All strains of purified rickettsiae (*cf.* Table 1 for procedure) oxidize glutamate, pyruvate, α-ketoglutarate, succinate, fumarate, malate, and oxalacetate. The oxidation of pyruvate and glutamate is greatly stimulated by the addition of inorganic phosphate. In three experiments of seven using carefully washed rickettsiae, a definite phosphorus uptake was observed when glutamate was oxidized. No phosphorus was taken up anaerobically. It would seem that in *R. rickettsii* the oxidation of substrates is coupled to phosphorylation, as it is in other types of cells. When glutamate is oxidized, succinate accumulates in the presence of malonate. Although no oxidation occurred with added citrate, isocitrate, or *cis*-aconitate, preliminary evidence indicates that the addition of pyruvate and oxalacetate leads to the accumulation of citrate in the presence of fluoroacetate, coenzyme A, adenosine triphosphate coenzyme 1, α-lipoic acid, and glutathione. If this result is substantiated by future experiments, the indication is that a tricarboxylic acid is concerned in rickettsial metabolism. None of the reactions listed above was found in normal yolk sacs treated by the procedure used to isolate the rickettsial preparations.

Although rickettsial preparations purified by the celite and albumin procedure have been found to contain phosphatase and other enzymatic activities, treatment of such preparations with antisera prepared to normal yolk sac material gets rid of these enzymes activities, leaving only the Krebs cycle reactions mentioned above. Rickettsial preparations purified to such a degree that no host material can be detected serologically contain both pentosenucleic acid and deoxyribonucleic acid.

Details of all this work will be published in future papers.

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Inductive Epithelio-mesenchymal Interaction in Cultured Organ Rudiments of the Mouse

Clifford Grobstein[1]

National Cancer Institute,
National Institutes of Health, Bethesda, Maryland

The importance of inductive interaction between tissues of the developing embryo has been amply demonstrated but the mechanisms remain obscure (*1*). Studies of inductive processes in the mammalian embryo have been extremely limited due to technical difficulties. Recently it has been found that very early rudiments of the mouse submandibular salivary gland (*2*), kidney, and lung (*3*), continue surprisingly normal morphogenesis at the glass-clot interface in Carrel flasks, and that each of these rudiments can be separated into viable epithelial and mesenchymal components by exposure to trypsin—as has been described for chick rudiments by Moscona and Moscona (*4*). These procedures have allowed study of epithelio-mesenchymal interaction by observing the behavior *in vitro* of heterogeneous combinations, e.g., submandibular epithelium and kidney mesenchyme (nephrogenic cord), or kidney epithelium (ureteric bud) and submandibular capsular mesenchyme. The results of one such experiment are reported here to indicate that: (1) the method affords approaches to problems of inductive interaction of embryonic tissues; (2) as has been inferred on indirect evidence (*5*), inductive processes similar to those described in lower vertebrates also occur in mammals; (3) even in the relatively simple epithelio-mesenchymal systems considered here, at least two types of inductive processes appear to be operating.

All embryos were BALB/C × C3H F_1 hybrids. Under a dissecting microscope, the submandibular rudiments were isolated on the 13th day of pregnancy (observation of a vaginal plug = 0 day), kidney rudi-

[1] With the technical assistance of Derrell Freese, George Parker and Edward J. Soban.