STUDIES ON MEXICAN TYPHUS FEVER. I

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PLATE 27

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The following notes on experiments with the virus of typhus fever are presented in the briefest possible manner, since to a considerable extent they represent confirmations of previous work. Our results are published because, in a subject as difficult as this one, confirmation of important facts that have been observed a few times only and have in some cases been questioned, constitutes a necessary criterion for the planning of further investigation.

The Filtration of Typhus Virus

There are irregularities in reports of the filtration of this virus. Negative results have been reported by a number of workers (Ricketts and Wilder (1) Anderson and Goldberger (2), Olitsky (3)). Nicolle, Conor and Conseil (4), however, obtained doubtful results, and Nicolle and Lebailly (5) reported two positive filtrations through Chamberland L_2 filters which held back *Bacillus melitensis*.

We carried out a considerable number of filtration experiments through Berkefeld V candles with guinea pig brain proved infectious by control, using the method worked out and published by Ward and Tang (6), which gives almost 100 per cent filtration with brain material of typical filterable agents such as herpes and with the fresh pulp of vaccinia. This, therefore, established an excellent basis for comparison. *Prodigiosus* was used as control, and control inoculations of unfiltered material were always done. This understood, we omit protocols.

Twelve filtrations of brain material from European typhus gave only one filtration in which controls were satisfactory and the virus passed through. Of five

brain filtrations of Mexican typhus, only one positive filtration was obtained in an otherwise satisfactory experiment.

The brain having given us largely negative results, we passed on to the filtration through Berkefeld V candles of plasma from the defibrinated blood of Mexican typhus guinea pigs which, in the unfiltered state, was infectious in amounts from 0.05 to 0.2 cc. Plasma was ten times diluted with broth and filtered as before with suitable controls. Of ten Mexican typhus plasma filtrations, there were three experiments in which all the criteria of successful filtration with proper control were fulfilled. In all successful filtrations, the typhus nature of the disease was proved by transfer— in some by immunity test and in others by the demonstration of brain lesions.

Berkefeld N candles were used in six filtrations of plasma, these filters letting air through, under water, at pressures of from 10 to 14 pounds, as contrasted with about 6 pounds for the V candles. All these filtrations were negative.

In view of the regularity with which, with our present methods' we have been able to filter typical filterable agents like those of herpes and vaccinia virus, our results with the typhus material attain a particular significance. The comparison with herpes is especially important since, in both herpes and typhus, brain material was used under identical conditions. It would seem from these experiments that the agent of typhus did not, in regard to filtration, fall into the class of the so-called "filterable" agents. Its occasional passage through filters which held back bacteria in our own work as in the isolated experiments of Nicolle and his associates, can be taken to indicate that the typhus agent, while larger than the filterable viruses, yet was smaller than a typical small bacterium—a supposition which would be consistent with the observed size of the Mooser bodies (7) (*Rickettsia prowazeki* seen in the tunica of guinea pigs). Moreover, cultural studies on all the filtrates carried over periods of from 2 to 3 weeks after filtration would indicate that the typhus virus was not bacterial and had no relationship to the occasionally observed diphtheroid organisms that can be cultivated, from time to time, from typhus tunicas. The animals unsuccessfully inoculated with large amounts of filtrate were not immune.

Localization of the Typhus Virus in Guinea Pigs

Consistent with the results of other observers, the virus was found in blood, brain, spleen and scrapings from the tunica vaginalis. In regard to the blood, the cell-free blood plasma was virulent with considerable regularity in amounts of 0.1 cc., and on one occasion titrated down to 0.05 cc.

The question has been raised by Nicolle (8) as to whether the virus is present in the leucocytes. This was examined by producing aleuronat exudates in the peritoneal cavities of guinea pigs at a highly infectious stage of the disease, and washing these as free as possible of red blood cells. Guinea pigs injected with washed leucocytes either developed no temperature whatever or came down very much later than those injected with red cells exposed to the same delays and washing manipulation as the leucocytes from the same animal. Leucocyte injected animals occasionally reacted on the sixteenth or seventeenth day after inoculation, while heart's blood of the same animal gave a typical reaction with testicular swelling on the seventh and eighth days. Guinea pigs inoculated with twice washed red blood cells of the same animal reacted like the whole blood inoculated ones. We conclude from these results that the virus is not normally intra-leucocytic, the occasional reactions being probably due to phagocyted virus.[1]

In attempting to wash red blood cells free of virus, we encountered great difficulty. These experiments were carried out by washing with a sufficient amount of broth so that the last supernatant fluid from the red cell washings represented a dilution, in one case, of 1–7000 of the original plasma. It was apparent from these experiments that the virus was either attached to the red blood cells or actually in them, for the cells remained virulent even when four times washed with 50 cc. of broth.

However, in view of the observations reported with Rocky Mountain spotted fever by Spencer and Parker (9), we carried out an experiment in which normal red cells were exposed to infectious plasma and, after standing for 10 minutes, separated and twice washed with broth sufficient to give a final dilution of the infectious plasma of 1–130. The guinea pig injected with the normal cells so treated came down typically with testicular swelling and a temperature of 105°C. on the ninth day, and the virus could be carried on from this animal in passage.

We conclude from these experiments that in Mexican typhus fever, the virus is not normally present in the circulating leucocytes except perhaps occasionally by phagocytosis; that it is not easily separable from the red cells, though it is probably not within them, but adheres firmly to their surfaces; and that it is present in the plasma in a concentration too low to permit the reasonable hope of seeing *Rickettsia* or Mooser bodies in the plasma preparations.

[1] In experiments going on at present we have been able, by the use of a new stain which has been perfected by our associate Dr. Castaneda and is to be described elsewhere, to observe actual phagocytosis of Mooser bodies in polynuclear neutrophile leucocytes.

Comparative Virulence of Blood Plasma and Tunica Scrapings of the Same Animal

In a number of experiments of this kind we had irregular results, but on two occasions the tunica material was virulent in much smaller amounts than the blood plasma. On one occasion a dilution of 1–1000 of the material from a whole tunica vaginalis, ground in a mortar with broth, produced the typical disease when the plasma of the same guinea pig did not titrate below 0.1 cc. We have never found plasma infectious in amounts lower than 0.05 cc. A peculiarity of this experiment which we cannot explain is the fact that in the case of the tunica just mentioned guinea pigs receiving 0.1 cc., 0.05 cc. and 0.01 cc. remained negative, whereas the one receiving 0.001 cc. came down. We believe that the comparative titrations indicate that the virus is more concentrated in the tunica where the *Rickettsia* bodies are found. (See also Mooser (7).)

Tissue Cultures

Tissue cultures were carried out by the usual manner in guinea pig plasma with tunica cells which had shown the presence of the Mooser bodies. They were incubated at 37.5°, which we believe was a mistake, and the cultures will be repeated at lower temperatures. On the other hand, we were able to observe what was either an increase of the bodies or a plentiful discharge from bursting cells as late as the eleventh day after planting. We were never able to grow these bodies in the second generation, and they disappeared in all tissue cultures when the cells ceased growing.

One week old material from tissue cultures was still infectious. The injection of tissue cultures from which the organisms had disappeared was not infectious.

Our tissue cultures, as far as they have gone, indicate that the *Rickettsia* remain visible only so long as the tissue is alive and growing. The difficulty of transferring to a second generation may be due to the fact that the organisms die out with the cells containing them. Extracellular multiplication could not be proved, because its suggested success—as in the accompanying plate—may easily have been due to a discharge from bursting cells. The tissue cultures seem to indicate again that the observed Mooser bodies or *Rickettsia* are not bacterial in nature, and that the infectiousness of the tissue cultures is roughly parallel with the presence of these appearances.

We were never able to demonstrate the bodies in tissue cultures of spleen or brain.

Intraperitoneal Capsules

Glass capsules, with a hole left in them, were filled with normal plasma and tunica scrapings of infected animals. They were left in the guinea pigs' peritoneal cavities for varying periods. A capsule taken out of the peritoneum of a guinea pig so treated was removed on the sixth day, before the animal had developed any symptoms of disease, and was proved to be still infectious, giving a typical reaction, but no Mooser bodies could be found in smears of the contents. Another guinea pig, into which a similar capsule had been put, showed a temperature of 105° on the twelfth day, but no tunica swelling. The contents of the capsule taken out at this time consisted of clear fluid. There was a fibrinous plug in the mouth of the tube. The smears from these materials were entirely negative for bodies, but the contents injected into another animal gave a typical swelling on the eighth day and a temperature of 105° on the tenth, with typical bodies in the tunica on examination.

Whole infectious blood placed into capsules and similarly observed caused the typical disease, but never showed Mooser bodies when removed and examined.

Our capsule experiments indicate that the virus may remain alive in the peritoneal cavity of a guinea pig in a glass capsule for as long as 12 days, but that the Mooser bodies are too few to be found unless one wishes to assume that all negative investigations of infectious tissues and blood can be explained by the fact that a mutation of form of some kind takes place. This we are loth to assume, largely because of our filtration experiments.

Strains of Mexican Typhus Fever without Swelling of the Testicles

Guinea pigs injected either subcutaneously or intracutaneously do not develop tunica lesions. In such animals a temperature resembling the European typhus temperature develops between the tenth and fifteenth day. If blood from such animals is, at the proper time, injected intraperitoneally into full-grown guinea pigs, these again will often develop swelling. This of course is no proof that we have not carried Mooser bodies along through the negative animals, but it does indicate that the Mooser bodies can remain alive in typical typhus guinea pigs without being detectable in any of the organs, or in the blood, and quite capable of again arousing the characteristic tunica lesion on subsequent proper inoculation.

Whenever, as on three occasions, we have obtained a strain without swelling which has run along for five to seven inoculation generations through adult, intraperitoneally inoculated male guinea pigs, we have found that we were dealing with intercurrent contamination. In our first strain of this kind, which ran for

four generations without swelling, there was no contaminating infection, but the virulence of the strain died down in these animals and was completely gone on the fifth trans-inoculation. The same thing happened in one other strain, and in a third one we found that we were carrying along a low grade bacterial infection, which ended in death in the fourth generation.

In these three cases, the surviving animals were all tested for immunity, and were found to be susceptible.

While such evidence is indirect, it seems to us to support the view that the tunica swelling with Mooser bodies is an integral part of the Mexican typhus infection in guinea pigs, and not an accidental occurrence.

TABLE I

Guinea pig	No. of days between drop in temperature and taking of blood	Protection
1	1	Typical temperature, no tunica lesion*
2	1	Complete protection
3	5	Complete protection
4	5	Incomplete protection typical course**
5	9	Complete protection
6	12	Modified, mild course. No tunica lesion
7	25	No protection
8	34	Typical temperature. No tunica lesion
9	39	No protection

* This serum, taken on the day the temperature dropped, exerted a very definite modification on the course of the fever, which was late in development and never showed orchitis; but the blood of this guinea pig was still virulent, as proved by inoculation of another animal. There was thus the peculiar condition of a blood already containing protective bodies, but still containing virus.

** This serum was kept about 36 hours in the ice chest before use.

Protective Power of Convalescent Serum of Mexican Typhus Guinea Pigs

Table I shows our results.

Two of these guinea pigs, one completely protected by the 1-day serum, the other completely protected by the 5-day serum, were re-inoculated with virus on the thirty-sixth and fortieth day respectively after the primary mixture had been administered. These animals developed no reaction whatever, while controls were characteristic. It appeared as though the mixture had conferred an active immunity.

These experiments indicate that convalescent serum mixed with virus before injection will protect if the serum is taken between the second and the tenth day after defervescence; that after the tenth day such protective action is doubtful, and that after the twentieth day it is negative. The two animals cited also indicate that guinea pigs so treated may be immune for at least 3 weeks, and that probably—since the injection of serum separately from the virus does not protect—they represent an active rather than a passive immunity.

The rapidity with which the protective bodies disappear from the blood would suggest that they are not of the nature of ordinary bacterial antibodies.

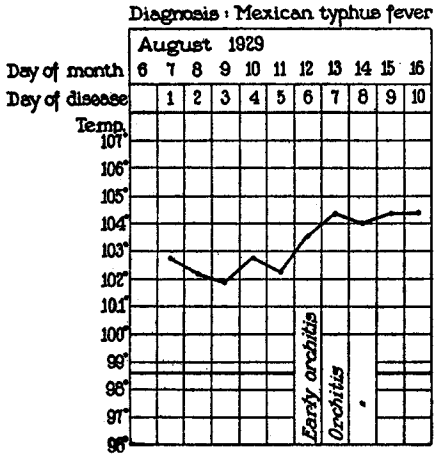

CHART 1.[2] Guinea pig 2–03. Source of virus, simple transfer from 2–02.

Complement fixations were carried out, using virulent plasma as the antigen and protective serum as the antibody, and by such a technique absolutely no indication of complement fixation was obtained.[3]

[2] The word orchitis is used for purposes of convenience; the lesions are almost entirely limited to the tunica and surface of the testicle.

[3] We do not attach much negative value to the complement fixations because of the surely extreme dilution of the antigen in the virulent plasma.

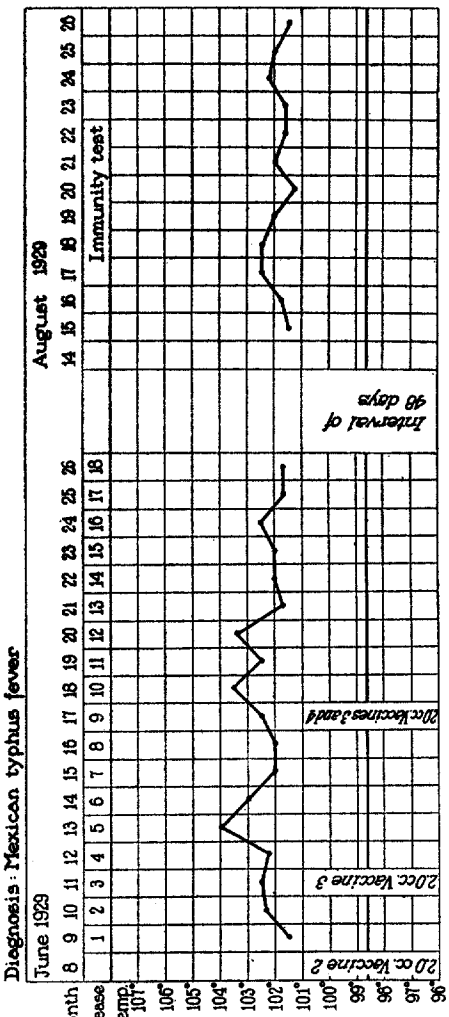

CHART 2. Guinea pig 1–68. Tunic vaccine 2.

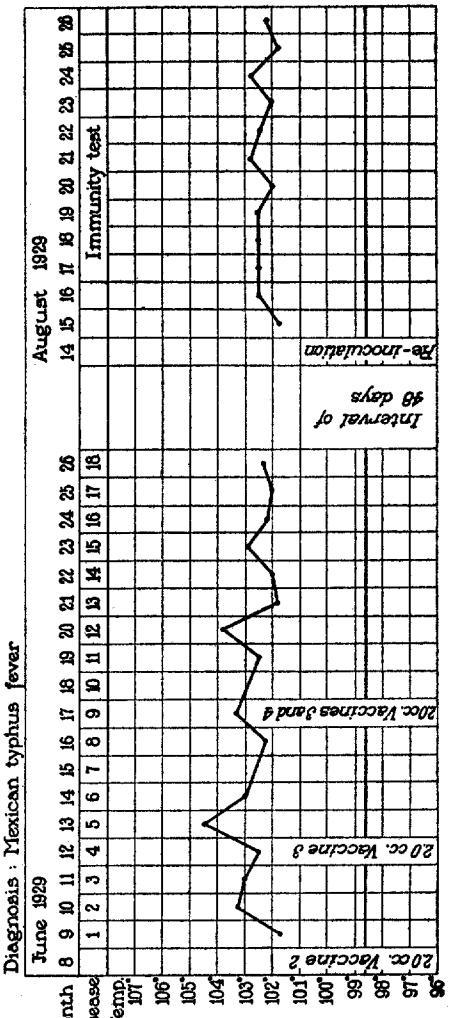

CHART 3. Guinea pig 1–67. Tunic vaccine 2.

Protection of Animals with Formalinized Tunica Material

Both from the experiments of Spencer and Parker (9) and of Conner (10) with spotted fever, it appeared that protection with dead infectious material might be feasible in *Rickettsia* diseases, provided that a sufficient concentration of virus could be attained. Having found that the tunica scrapings, containing many of the Mooser bodies, were much more highly infectious than the blood plasma of the same guinea pig, we thought it advisable to attempt active immunization with such material killed with weak formalin solutions by more or less the same method as that which has been successful for distemper in the hands of Laidlaw and Dunkin (11). Charts 1 to 4 represent a few experiments which gave encouraging

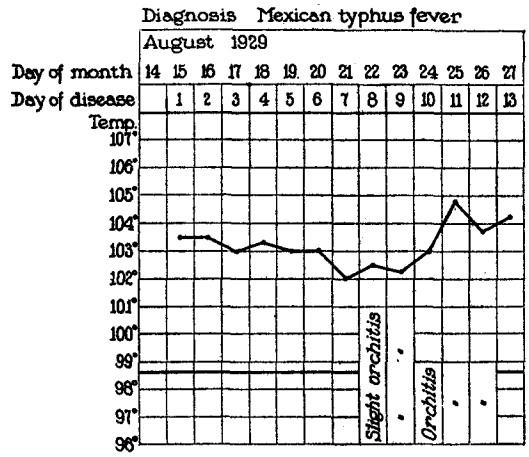

CHART 4. Guinea pig 2–06. Control of reinoculation, simple transfer from 2–03.

results, and which are merely preliminary to a continuation along the same lines with which we are now occupied. The vaccine used was guinea pig tunica taken at a time when it contained Mooser bodies, ground with sand, emulsified in 0.2 per cent formalin, left one night at room temperature and after that put in the ice box. It will be noticed that in the first two animals there was a short rise to 104° after one of the vaccine injections, but that on re-inoculation the animal showed no reaction whatever, although the control came down characteristically.

In the other two, the disease was modified, in that there was swelling of the testes without temperature. This is less significant, because it occasionally—though not often—happens in ordinary Mexican passage animals.

We had one other experiment which was more striking than the first one, but which cannot be included because one of the re-inoculation controls did not react.

Subsequent experiments with formalinized tunics have rendered it uncertain whether we were dealing with dead or attenuated tunic material. This will have to be worked out in the series of experiments with which we are occupied at present, but we believe it worth while to report the above at the present time as an indication that a modification of the disease can be obtained by active immunization with tunic material in which there is a minimum of blood plasma and a maximum of Mooser bodies—a fact which further suggests the etiological importance of these appearances.

Human Infection in the Laboratory

A single case of human infection has occurred in the laboratory. The origin of this case cannot be easily proved, but since the individual was extensively engaged in tunica scraping within the incubation time, and had made only a few blood transfers by heart puncture, which he had been doing for two years without relaxing precautions and without accident, the conclusion is almost forced that this infection originated in tunica material, a small amount of which was probably deposited upon the recently shaven skin of the face.

SUMMARY AND CONCLUSIONS

The preceding studies on typhus fever, chiefly done with a Mexican strain obtained from Dr. Mooser, concern themselves largely with reinvestigations of some of the fundamental problems of this disease.

Filtration experiments carried out with methods almost regularly successful with true filterable viruses, in regard to material, suspension fluid, reaction, nature of filters and pressure employed for filtration, indicate that the virus is not filterable in the ordinary sense in which this expression is employed. It is probably smaller than bacteria and the results of filtration experiments suggest that its magnitude is consistent with the tunica bodies observed by Mooser.

Negative filtrates did not immunize, a result consistent with the previous work of Olitsky.

The virus is present in blood plasma, hardly if at all in leucocytes, and becomes closely associated with the red blood cells, though we do not believe that it is contained in them. It becomes firmly associated

with normal red blood cells when these are exposed to infectious plasma, a result similar to that obtained in Rocky Mountain spotted fever by Spencer and Parker.

In tissue culture, tunica material with Mooser bodies remains alive and virulent for about 10 days, but so far we have not been able to determine that it can keep alive without the presence of living cells. These results do not carry this subject any further than it has been carried for European typhus in tissue cultures with the same method by Wolbach, Schlesinger and Pinkerton (12).

Within glass capsules in the peritoneum of guinea pigs, the virus may remain alive for about the same length of time as in the tissue cultures.

Rough comparative virulence estimations between blood plasma in which it would be hardly possible to find a limited number of Mooser bodies, even though they were present, showed the blood plasma to be less infectious than the tunica material, in which considerable numbers of Mooser bodies were visible.

The testicular swelling characteristic of Mexican typhus and showing the above mentioned bodies—probably *Rickettsia*—may be absent in individual guinea pigs under ordinary conditions and in guinea pigs inoculated by other than the intraperitoneal route. On re-inoculation into the peritoneum after non-orchitic passages, the swelling reappears. Whenever it did not so reappear, we found that the strain had either degenerated in virulence or it had been contaminated by intercurrent infection. Though we can not prove it at the present time, we believe that the tunica lesion is an integral part of this disease in guinea pigs, and not an accidental accompaniment.

Convalescent blood from Mexican typhus guinea pigs mixed in the test tube with virus affords protection if the blood is taken between the first to the tenth day after defervescence. After the third week, the blood no longer contains protective bodies although the guinea pigs may still be immune.

In one case a serum was obtained which was both protective in such a test but at the same time seemed still to contain virus, a result which we cannot explain.

No complement-fixing antibodies were found when virus serum was used as antigen and convalescent serum as antibody. The low concentration of the virus in the serum may account for this.

In a limited number of observations guinea pigs which were negatively inoculated with virus-serum mixtures proved on re-inoculation to be immune. In one of these cases the protective serum mixture with the virus was taken 1 day, in the other 5 days after temperature had returned to normal and the re-inoculations were done 36 and 40 days after the primary injection. This recalls similar experiences of Nicolle and encourages further immunological study in this direction.

In a number of experiments active immunization with formalinized tunica material containing large numbers of the Mooser bodies seems to have modified the course of subsequent inoculations in the direction of protection.

A single accidental human infection seemed particularly associated with tunica material, although this cannot be positively asserted.

All that part of our work which has bearing on the infectious agent is consistent with the assumption that the small, Giemsa-staining bodies observed by Mooser in the tunica of Mexican typhus guinea pigs represent the virus of the disease.

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EXPLANATION OF PLATE 27

FIG. 1. Mooser bodies (*Rickettsia*) in an 8 day tunica tissue culture.

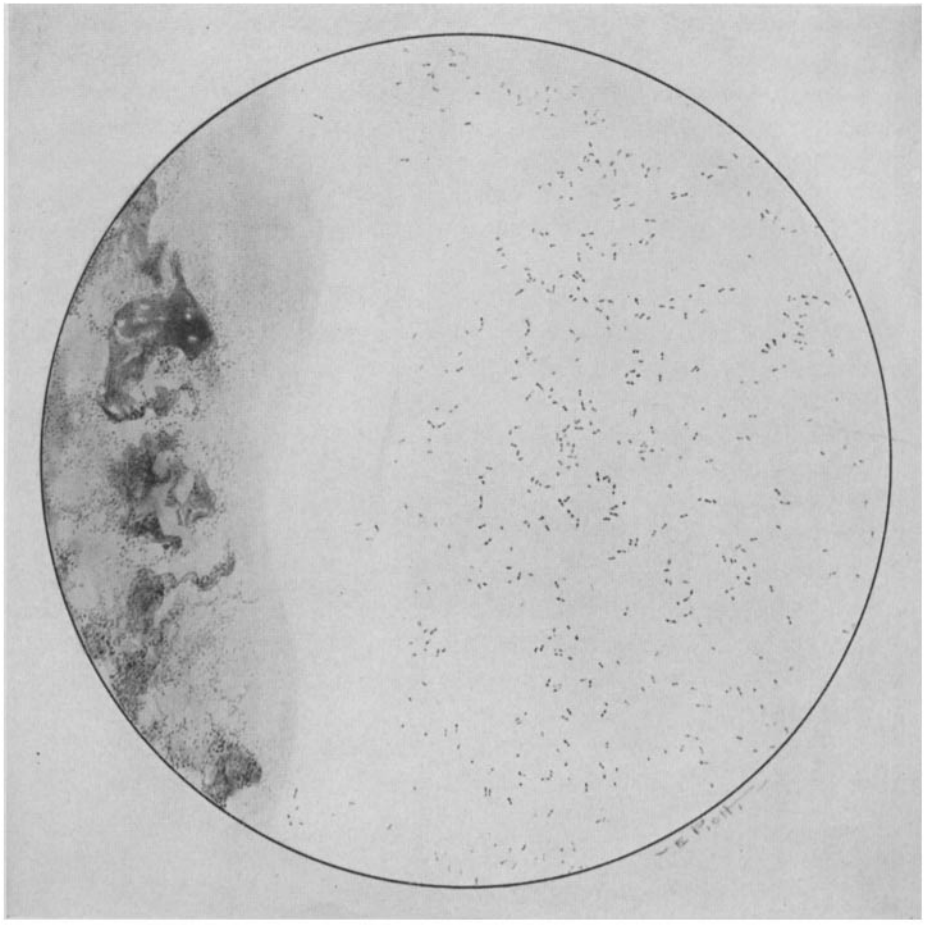

FIG. 1

(Zinsser and Batchelder: Mexican typhus fever. I)