

Studies of the Rickettsias of the Typhus- Rocky - Mountain - Spotted - Fever Group in South Africa.

II.—Morphology and Cultivation.

By R. A. ALEXANDER and J. H. MASON, Section of
Protozoology and Virus Diseases, Onderstepoort.

IN the previous article (this journal) details were given of the source and method of isolation of five strains of rickettsia. These strains have been maintained either by serial passage in guinea-pigs, or by cultivation on the chorio-allantoic membrane of the chick embryo, or by both methods. In this paper the microscopic appearance of the scrotal exudate and method of egg-membrane cultivation will be described and the morphology of the rickettsias compared particularly in view of Pinkerton's classification of this group of organisms. (Pinkerton 1936.)

TECHNIQUE.

Smears of the exudate were made by scraping the surface of the retracted testicle, or the tunica vaginalis of the scrotum firmly but gently with a sharp scalpel, transferring the resulting fluid material to the edge of a clean glass slide and then drawing the film in the usual way. Similar preparations were made from the surface of the spleen and the parietal peritoneum.

Smears were fixed with May-Grünwald and stained with Giemsa (2 drops of Hollborn's Giemsa per c.c. of M/500 phosphate buffer pH 7.4 for 30 minutes). Differentiation in dilute acetic acid was not done but the stained films were washed very thoroughly in running water before being dried for examination. For the demonstration of intranuclear rickettsias it was found advantageous to differentiate overstained preparations by exposure to diffuse sunlight for several days. In our hands Lepine's or Castaneda's stains were found to be quite unsatisfactory, but later a staining method recommended by Pinkerton (personal communication) has been used with success. Since no reference to the method has been noticed in the literature up to the present time the technique is detailed with due

acknowledgement of the source. Smears are fixed by very gentle heat fixation or preferably only by thorough air drying. Stain with 0.25 per cent. aqueous solution of basic fuchsin for 3 to 5 minutes. Pour off the stain and without washing decolorize with 0.5 per cent. citric acid for about 5 seconds. Wash in water and counterstain with 1 per cent. aqueous methylene blue for 10 seconds. It is preferable to filter the basic fuchsin on to the slide through filter paper and to apply the citric acid from a pipette. The rickettsias stain bright red, the cellular material blue. This method has been of little value for morphological studies but it has been exceedingly useful for the rapid examination of preparations since it is quite remarkable with what rapidity rickettsias may be detected and an estimate of the degree of rickettsial infection obtained.

MICROSCOPIC APPEARANCE OF THE EXUDATE.

The general microscopic picture with all strains was practically identical. In the early stages there was a marked increase in the number of neutrophiles and eosinophiles followed by a relative increase in the number of large mononuclear cells other than serosa cells. Then serosa cells in various stages of mitosis made their appearance, and many were seen with a vacuolated cytoplasm. By the end of the second day after the commencement of the scrotal reaction cellular phagocytosis had commenced. This was always a prominent feature and by the time the scrotal swelling had begun to decrease it was common to find 4 or 5 cells per oil immersion field literally crammed with erythrocytes, neutrophiles or eosinophiles and even containing monocytes. As the reaction passed off the degree of promiscuous phagocytosis decreased, the number of polymorphonuclear cells, monocytes, mitotic and vacuolated serosa cells progressively diminished until the picture had returned to normal.

Rickettsias could be demonstrated regularly only in the early stages of the clinical scrotal reaction. Before this stage it was rare to find them, and later, when cellular phagocytosis was marked, a prolonged search was invariably fruitless. This observation is of interest in view of the high infectivity of testicular washings in the later stages of the reaction. Attention must be directed to one peculiarity which was observed constantly and that is the presence of a fairly fine, reddish-brown-staining, granular deposit which might be so voluminous as to obscure the general picture. The origin of this deposit and its nature is quite obscure, but careful control showed that it did not originate from either the fixative, the stain or the glass slides.

MORPHOLOGY OF THE RICKETTSIAS.

Strain "Hare".—Rickettsias were always exceedingly rare and could be demonstrated only in the initial stages of the scrotal reaction; even then it was frequently necessary to search through a well prepared and properly stained preparation for more than half-an-hour before finding 2 or 3 cells containing organisms. Typically the rickettsias are intracellular; extracellular forms have been found

but it seems probable that this location is due to rupture of cells during the process of drawing the film. The parasitized cells were of two different types:—

1. A large cell with a large light purple-staining, oval-shaped nucleus containing several well-defined nucleoli, i.e., a serosa cell.
2. A smaller cell with a more compact darker-staining and usually indented nucleus, i.e., a monocyte.

The exact nature of the cells was not determined either by supravital staining or by the injection of India ink.

The rickettsias were never clumped together nor aggregated in the form of large masses of organisms to distend the cytoplasm or displace the nucleus. On the contrary they were irregularly scattered throughout the cytoplasm in numbers which permitted easy counting, the usual number being 6 to 14 though 30 or more have been found. (Cf. Plate I, fig. 1 and 1A.) In spite of a very careful search, intranuclear organisms have never been seen in preparations from the guinea-pig. The morphology of individual organisms is typical. In stained smears the colour tends to the bluish side of purple and there is a characteristic "soft" appearance in contrast to the "hard" metallic lustre of granules of the polymorphonuclear cells. Usually the shape is lanceolate but all forms varying from paired cocci to elongated bacilli have been seen. No tendency to chain formation was observed at any time. Attention must be directed to the observation that, surrounding the large majority of individual rickettsias, there is a clear unstained halo of variable width; recognition of this halo is frequently of great assistance in identifying the organisms though it must be stated that whenever a typical rickettsia is seen there is seldom any doubt as to the identity.

Rickettsias have been found more constantly in preparations from the testis but they have been present in peritoneum and spleen surface films.

Strains "Appleton", "Robertson" and Fièvre boutonneuse.—As regards the frequency of the organisms, their localization and morphology, these strains were identical with strain "Hare" in all respects.

Strain Rat Typhus.—In striking contrast to the scarcity of the organisms found in the exudate of guinea-pigs infected with strain "Hare", with the rat typhus strain, infected cells were frequently found in enormous numbers. Although the best preparations have been obtained during the early stages of the scrotal reaction infected cells have been found up to the 7th and 8th day. The typical location is intracytoplasmic although numerous organisms were extracellular, again undoubtedly due to mechanical factors. Only one type of cell has been found parasitized, viz., serosa cells, and rickettsias have not been found in monocytes or neutrophils except under conditions certainly related to mechanical and traumatic factors. Within the serosa cells the rickettsias show a decided tendency to be aggregated in clusters or colonies; this

tendency is exemplified in plate 2, fig. 3, which illustrates a cell containing a limited number of organisms yet the clumping is well-defined. Typically an infected cell is crammed with rickettsias (plate 2, fig. 5) so much so that the outline of the cell and the nucleus is distorted. Such cells may be picked out easily under low magnification since the distended dark blue cytoplasm is conspicuous. The rickettsias stain a delicate bluish purple and morphologically give the impression of being longer and finer than rickettsias of strain "Hare". In the early stages of the reaction when individual cells contain only a limited number of parasites there is a definite tendency to chain formation so that one may pick up cells containing a thread-like network of rickettsias. As proliferation advances the organisms tend to become smaller so that when they are most numerous they take on a diplococcal or cocco-bacillary form. The morphology of individual organisms can be studied best by observing the extracellular forms. Usually these are in pairs consisting of two delicate rods separated by an unstained space of variable size, though frequently there is no intervening space so that a single bacillary form is found. The ends are usually rounded though they may be lanceolate. In the majority of cases the pairs are in a straight line but they may be bent at any angle and occasionally crescent forms are seen. Never have any intranuclear rickettsias been observed although particular attention has been paid to this point.

Again rickettsias were found in greatest numbers and with greatest regularity in preparations from the tunica vaginalis of the testes; usually they could be demonstrated on the testes before being seen in films from the peritoneum or spleen surface. It may be stated generally that the order of their appearance was first on the testes, then on the peritoneum and then on the spleen and that they disappeared in the same rotation.

CULTIVATION ON THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO.

Apart entirely from reports on the application of tissue culture methods to the cultivation of the rickettsias of the typhus—Rocky Mountain spotted fever groups of disease, the literature contains several references to the use of the chorio-allantoic membrane of the developing chick embryo as a culture medium. Zia (1934) reported the cultivation of European and Mexican typhus rickettsias through 3 generations and came to the conclusion that there was very little hope of applying the method to mass production for vaccine purposes. Da Cunha (1934) cultivated the Sao Paulo typhus rickettsia, demonstrated the presence of organisms in membranes of the second generation, and showed their infectivity for guinea-pigs. Bengtson and Dyer (1935) cultivated the virus of Rocky Mountain spotted fever on the chorio-allantois through 20 passages without diminution in virulence for either the embryo or guinea-pig and described the lesions produced in the membrane and the morphology of the rickettsias. On the other hand, Pijper and Crocker (1938), failed to obtain any multiplication of tick-bite fever rickettsias on egg membranes. They point out that no serial transfers from egg to egg were made, and it will be seen below that this omission was probably

the cause of their failure. The technique of Barykine, Kompanesy, Botcharowa and Bayer (1938) who recommend injecting infective material into the yolk sac of 3 to 4 day old embryos, has not been investigated up to the present time. In the present report details are given of the cultivation of 4 strains of rickettsias.

Technique.—The technique of inoculating the eggs was that described for similar studies on the propagation of the neurotropic virus of horsesickness (Alexander 1938). The inoculum used for seeding was a saline emulsion of scrapings from the tunica vaginalis of guinea-pigs sacrificed at a stage slightly before the height of the scrotal reaction. In some cases this reaction was marked, in other cases barely detectable, and on two occasions the inoculum was prepared at a time when a reaction, had it occurred, could reasonably have been anticipated; yet in every instance a successful series of cultures was initiated without undue difficulty. The eggs containing 8 to 10 day old embryos were incubated at approximately 33° C. (91.4° F.) for from 4 to 6 days. A lower temperature could not be used because, particularly in the summer months, the laboratory temperature rises to 33° C. or higher. An incubation temperature lower than that usually employed for bacteriological purposes was used because of the experience of other workers on cultivation by *in vitro* methods and also because it was early apparent that better results were obtained. The low temperature may account for some mortality of the embryos but the procedure was entirely practical. Material for transfers was prepared by emulsifying pieces of membrane about 1.5 cm. square selected from two eggs from the site of inoculation. The pieces of membrane were minced finely with scissors, then ground up with the roughened end of a glass rod without the addition of sand and before adding 25 c.c. of sterile saline. The emulsion was not clarified by centrifugation because the particles of membrane quickly disperse either to the surface of the fluid or the bottom of the tube so that it was possible to remove turbid fluid free from any conspicuous particulate matter from the middle portion. The sterility of each inoculum was tested by seeding 0.5 c.c. on to ordinary agar and incubating for 2 days at 37° C. Tests for rickettsial infectivity were carried out by inoculating guinea-pigs intraperitoneally. Usually 6 eggs were seeded with each inoculum (dose 0.2 c.c.) at every transfer and it was found essential to run a series in duplicate to minimize the risk of total loss from bacterial contamination. It is necessary to emphasize that it is far easier to carry on a series of cultures where the embryo itself is used as a source of virus. A slight bacterial contamination in the majority of cases appears to remain localized on the membrane, absorption of the virus is not interfered with and a sterile embryo emulsion is the outcome; on the other hand, it is apparent that the bacteria contained in a contaminated seeding fluid find the membrane a suitable medium (for propagation) and in this work many cultures had to be abandoned.

The technique of preparation of impression smears of the membranes was that described by Bengtson and Dyer (1935). A portion of the membrane at the site of the inoculation was excised, placed ectodermal side down on a clean glass slide, and covered with a strip of blotting paper over which was placed another slide. The

two slides were clamped together by means of a stout binder clip and gently flamed. The blotting paper was then removed leaving an impression of ectodermal cells behind and a second glass slide was placed in apposition to the remnant of membrane adhering to the blotting paper. In this way it was possible to make 3 preparations from each membrane.

The Lesion on the Membrane.—Since the gross macroscopical lesion appeared to be identical in the case of all the strains of rickettsias cultivated, a single description will suffice. It was not possible to follow the development of the lesion in any particular egg as the window method of seeding was not used but during the course of the work eggs were opened at different stages so that an accurate conception of the typical picture has been obtained.

In the early stage of infection (2nd day) the membranes lose their smooth, glistening, and transparent appearance and show the presence of a variable number of opaque areas, pin-point in size. It was found that the number of opaque spots was quite inconstant in different eggs seeded at the same time with the same emulsion and opened simultaneously, although it is admitted that had considerably greater care been taken to prepare a uniformly disperse inoculum, this discrepancy might not have occurred. However, the impression has been gained that in spite of refinement of technique, it would be exceedingly difficult to obtain uniform and constant multiplication of rickettsias in different eggs, and consequently the number of lesions per membrane would be variable. Later (3rd and 4th day), the opaque spots have increased in size but there appears to be little uniformity, minute pin points and areas of opacity being evident at one and the same time. Still later (the 4th and 5th day) the opacity has become confluent so that after removing the egg shell and shell membrane it is not possible to see the structure below. At this stage the membrane is thick (fully 2 mm.), moist and oedematous and when removed may have the appearance of a piece of greyish-white opaque jelly. In about 10 per cent. of cases it was noticed that a white amorphous powdery material accumulated on the ectodermal surface; its nature or significance is not known but it bore no connection with bacterial contamination and was not correlated with abundance of demonstrable rickettsias. After the death of the embryo the membrane takes on a dry, greyish, crinkled appearance.

Infection with the rickettsias studied destroys the embryo. In the case of the rat typhus strain approximately 40 per cent. of the embryos were dead on the 6th day after seeding, the majority were dead on the 7th day and it was rare for any to survive 8 days. It may be argued that at least the late deaths was the result of incubation at an unfavourably low temperature. Control eggs, however, seeded with either saline or non-infective emulsion frequently survived for longer than 8 days, although in some experiments there was a mortality of 30 per cent. on the 8th day. Further, in the case of the "Hare", "Robertson" and *fièvre boutonneuse* strains, embryos commenced to die on the 4th day and it was rare for any to survive 6 days. Therefore it is apparent that in the case of these strains at least, the organisms and not the unfavourable hatching conditions were the cause of death.

It is necessary to mention that when initiating a series of egg cultures the results obtained with the first few generations are somewhat disappointing. In the majority of cases the control guinea-pigs inoculated with membrane emulsions comprising the first, second, or even the third generation either failed to react and later were found to be susceptible or showed an indefinite thermal or scrotal reaction and later were found to be immune or susceptible. Although the early generations might be non-infective for guinea-pigs and rickettsias not demonstrable in the impression preparations, later generations of the same series might be virulent and yield smears showing many organisms. Therefore it was always necessary to continue subinoculations for at least 3 generations before deciding if the attempted cultivation had been successful or not. After a strain had been "adapted" to eggs it was simple to alternate from guinea-pig to egg, the first generation on membranes always proving fully infective.

MORPHOLOGY AND CHARACTERISTICS OF THE RICKETTSIAS.

Strain "Hare".—Typical rickettsias were found in all generations from the 4th onwards, though the number of rickettsias in preparations from different eggs varied within very wide limits. Moreover, in many smears organisms were localized in definite small areas as if they had been present in isolated nodules.

Morphologically there was no difference between the organisms seen in membrane smears and those found in tunica smears from guinea-pigs except that the greater number present emphasized the extreme pleomorphism. No difficulty was ever experienced in identifying the bacillary forms but care has to be exercised in differentiating coccal, diplococcal, and cocco-bacillary forms from the granules of the immature polymorphonuclear cells which appear to aggregate at the site of multiplication. However, the rickettsias stain a delicate bluish purple and invariably are surrounded by a more or less distinct halo while cell granules stain reddish in colour and have a metallic lustre. In spite of these criteria many granules were seen about which there exists a definite doubt; the large organisms referred to by Bengtson and Dyer (1935) were encountered but no decided opinion as to their identity or significance can be expressed at present.

In some preparations rickettsias were exceedingly numerous (many hundreds per field) but this was the exception. Typically they were not particularly frequent and a good preparation was considered one that contained 5 or 6 infected cells per field over a number of separate areas each limited to about 20 fields in extent. In at least 20 per cent. of preparations it was not possible to demonstrate rickettsias at all, yet an emulsion would prove highly infective for guinea-pigs. The reason for the variation in the number of organisms in smears from different membranes is not known; it is unlikely to be associated with the somewhat crude method of preparing the impression.

The majority of rickettsias are intracellular or are seen in the vicinity of a ruptured cell; on the other hand, in a good preparation, large numbers may be found scattered amongst intact cells. In the cytoplasm of infected cells they were rather more numerous than in

guinea-pig preparations (up to 100 per cell) but they were always scattered in a disorderly manner and there was no tendency for them to be aggregated into clumps (plate I, figs. 1 and 1A). However the important feature is that intranuclear forms were common (plate I, fig. 2A), although the intracytoplasmic forms were by far the more frequent. The number of rickettsias within the nucleus varied from a single individual to a crowded mass which distended and distorted the nucleus. Morphologically they did not differ from the extranuclear forms though it will be appreciated that recognition of the smaller forms was exceedingly difficult. Unless the staining is good and carried out at a pH not more acid than 7.4, identification within the nucleus is difficult and great care must be taken with the examination. Later the use of Pinkerton's stain greatly facilitated determination of the intranuclear habitat. When the number of intranuclear parasites is limited, a well-defined halo is frequently evident thus giving the appearance of a small bacillus lying in an unstained hole in the nuclear chromatin; small numbers may lie in apposition to one another in a manner suggesting division by binary fission; when large numbers are crowded together the halo is obscured and the general picture is that of bluish-purple rods lying between or embedded in the reddish chromatin. The only tendency to the formation of groups or clusters of rickettsias has been in the case of the intranuclear forms; these infected cells may be picked out under low magnification owing to the decided bluish colour of the nucleus. This intranuclear localization was a striking feature.

Three attempts have been made to determine the effect of long continued passage upon the virus but unfortunately the procedure has been abandoned on each occasion owing to bacterial contamination, once at the 9th, once at the 11th, and once at the 16th subculture. All generations from the 5th to the 16th were fully virulent for guinea-pigs and rickettsias were demonstrable in the impression smears. It is worthy of note that on many occasions an emulsion of contaminated membranes has produced the normal febrile and scrotal reaction on intraperitoneal injection into guinea-pigs, and that smears showed rickettsias intermingled with the easily recognizable cocci or bacilli. All attempts to separate rickettsias from contaminants by dilution methods either in the guinea-pig or on eggs have proved unsuccessful.

The reaction produced by membrane virus in guinea-pigs differed from that produced by infective guinea-pig brain in the course of routine passage only by the significantly shorter incubation period and the more constant incidence of a well marked scrotal reaction (practically 100 per cent. in sexually mature guinea-pigs). This is almost certainly due to the large number of infecting doses contained in the membrane emulsions; in serial dilution experiments to determine the infective titre, the guinea-pigs which received the higher dilutions reacted in a manner identical with that of the guinea-pig passage animals. On several occasions it was found that injection of a dilution representing 1 in 12,000 of the original membrane was infective. It should be mentioned that guinea-pigs which receive higher dilutions may show no clinical reaction yet on immunity test may or may not be found to be immune (subclinical or inapparent infection).

The effect of passage on eggs appeared to result in an increase of virulence for the embryo. In the early generations subculture could be delayed until the 6th day after seeding; later it became necessary to subculture on the 5th day and the reason why at least one of the late generation cultures was lost was that all embryos were found dead on the morning of the 5th day though they had appeared normally active on candling the previous afternoon.

Portions of membrane some distance from the site of inoculation, i.e., membrane not included in the artificial air sac, were found to be infective though the reaction produced indicated that a large quantity of virus was not present. Similarly an emulsion of an embryo produced a mild reaction without scrotal involvement in 2 guinea-pigs which were found to be immune 4 weeks later.

Strain "Robertson".—The behaviour of this strain on eggs was practically identical with strain "Hare". Morphologically the 2 rickettsias in impression smears are indistinguishable and some beautiful preparations demonstrating the intra-nuclear forms were obtained.

It has been shown in another publication (Part III, this journal) that this strain of rickettsia is maintained in guinea-pigs with some difficulty, that the reactions produced by infective brain material are sometimes indefinite with a low percentage involvement of the scrotum and testes, and that inapparent infections are frequent. On the other hand egg-membrane virus proved highly virulent for guinea-pigs, produced a well-marked febrile reaction with a short incubation period (3 days) and a high percentage of scrotal lesions (about 70 per cent. in mature guinea-pigs). A noteworthy feature was the rapidity with which the scrotal lesion developed. Slight redness, swelling and oedema only might be noticed in the morning and yet by midday or the early afternoon the reaction would be pronounced. If anything strain "Robertson" proved more virulent for the embryos than strain "Hare" so that sub-culture on the 4th day was necessary on many occasions.

Strain "Appleton".—This strain had been abandoned before the egg-membrane culture technique had been perfected.

Strain "Fièvre boutonneuse".—The behaviour of this strain was identical in all respects to strain "Robertson".

Strain Rat Typhus.—The murine strain of rickettsia was found to multiply prolifically on the chorio-allantoic membrane. The initiation of cultures appeared to be somewhat more difficult than in the case of other strains. On two occasions testicular washings which were shown to be highly infective for guinea-pigs failed to establish infection on eggs. Further, in successful series before rickettsias could be demonstrated and before control guinea-pigs showed well-defined reactions it was necessary to carry out at least three egg-to-egg transfers.

The morphology of the rickettsias did not differ from that seen in preparations from guinea-pig tests, but again the rather more numerous organisms emphasized the extreme pleomorphism. Coccal and diplo-coccal forms, some so small as to be just within the range of

visibility, were common but the bacillary forms were also numerous. A consideration of the photo-micrographs indicates the intracytoplasmic habitat but, what is more important, shows very clearly the formation of masses of innumerable intracellular organisms in marked contrast to the irregular scattering of the other strains. Further it is of the utmost importance to emphasize that in spite of a very careful search through hundreds of preparations intranuclear forms were never observed. Individual organisms were seen lying on top of a nucleus but in every case it was abundantly clear that the rickettsias had been superimposed mechanically.

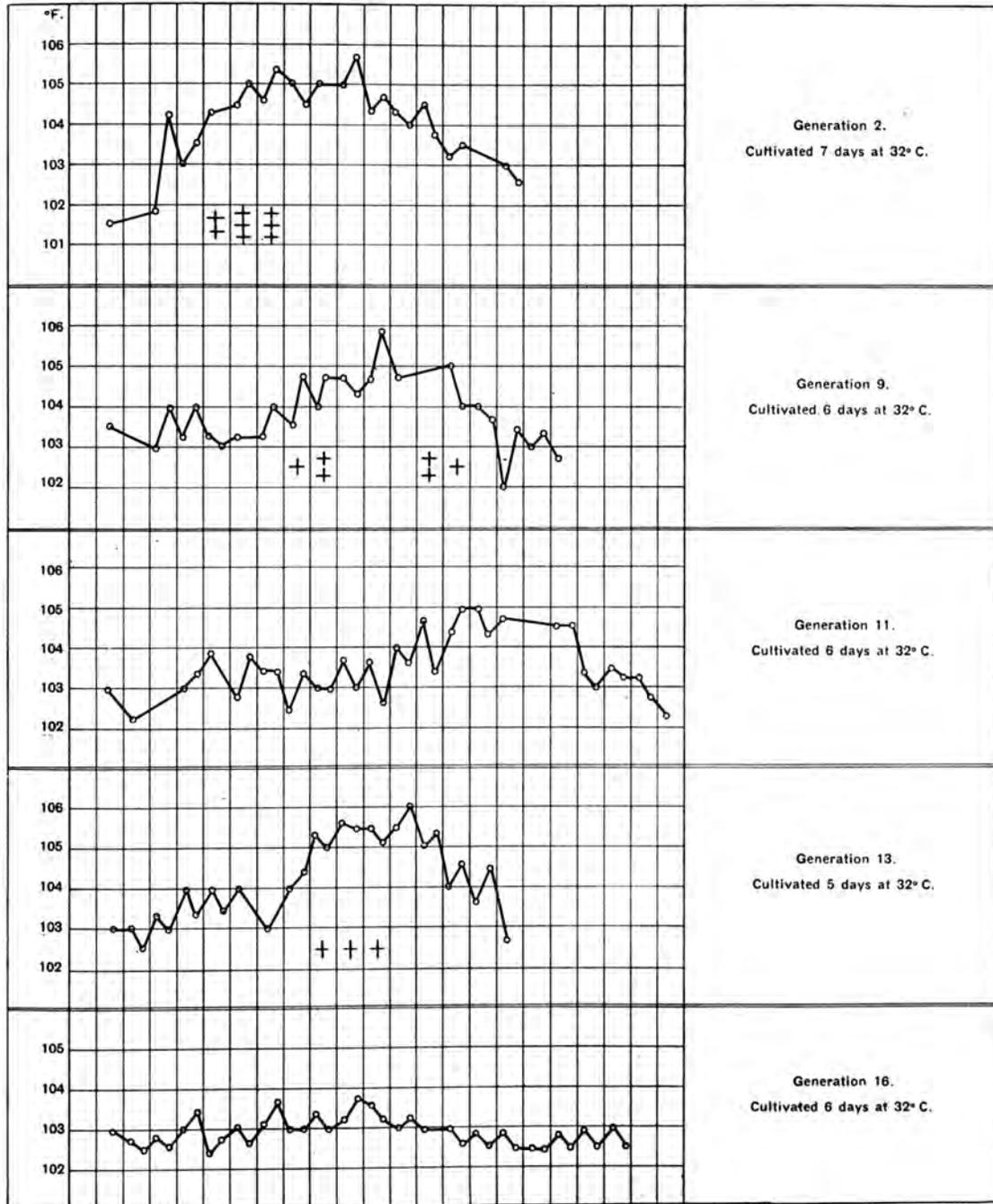
Four attempts were made to determine the effect of continued serial passage. A series of temperature curves is shown (Chart I) which illustrates the tendency to die out on repeated sub-culture. These charts indicate the reactions produced in guinea-pigs by a strain which, first having been adapted to the chorio-allantoic membrane, was then passed through one generation in guinea-pigs before being transferred back to eggs. A consideration of the charts shows that the second generation on membranes produced a severe reaction characterised by a short incubation period and a marked scrotal reaction. The 9th generation was infective but the incubation period was considerably lengthened and the degree of scrotal swelling was less. The 11th generation culture produced a slight febrile reaction which was delayed, and no scrotal lesion. While generation 13 was again infective, generation 16 was avirulent. Generation 18 has not been reproduced in the chart but it was non-infective. On immunity test applied 6 weeks after the test injection all guinea-pigs up to generation 13 were solidly immune; the guinea-pigs of generation 16 and 18 were fully susceptible. In addition to the loss of infectivity for guinea-pigs, impression smears up to generation 9 consistently showed the presence of many rickettsias, though in the latter passages there appeared to be a preponderance of minute coccal and cocco-bacillary forms. From the 10th sub-culture onwards rickettsias became progressively scarce and could not be found in passages 13 to 18 when the experiment was abandoned.

Out of 4 attempts at serial passage one was abandoned at generation 6 owing to bacterial contamination and, in the other three, infection died out after the 13th sub-culture. Sufficient work has not been carried out to determine with certainty whether this tendency to die out on serial passage through eggs is a definite characteristic of the murine strain or whether it represents either an inexplicable coincidence or some deficiency in technique which has not been appreciated. The point is being subjected to further investigation, but for the purposes of the experimental work involved in this study it has been found advisable never to continue a series for more than 9 generations, and then to continue with tunica washings after 1 passage through the guinea-pig. Moreover, for microscopic purposes, impression smears were made from cultures not less than generation 3 and not more than generation 9.

In connection with the effect of the rat typhus strain on the embryo itself it must be stated that it appeared to be less virulent than the other strains. Subcultures are usually carried out on the 6th day after seeding and survival of the embryos for more than 7 days was not uncommon.

CHART I.

Temperature Reactions in Guinea-pigs Produced by Egg-membrane Cultures of Rat Typhus.



+ - +++ = Degree of Scrotal Reaction.

DISCUSSION.

Apart entirely from the demonstration that the chorio-allantoic membrane of the chick embryo constitutes a suitable medium for the propagation of rickettsias, the results of the experimental work detailed above bring out several points of great interest.

At first it was believed that the large numbers of organisms found in smears of infected membranes, indicated the application of the method not only to vaccine production, but also to the production of antigens for such purposes as agglutination, complement-fixation, and *in vitro* neutralization tests. It was early apparent however that multiplication was too irregular and too inconstant for these purposes. It has been shown that the rickettsias studied fall into two main groups:—

- I. A group where the organisms in the guinea-pig are easily demonstrated as masses within the cytoplasm of serosa cells only. On artificial cultivation this tendency to intracytoplasmic aggregation persists but an intranuclear habitat is not assumed.
- II. A group where the organisms in the guinea-pig are not only extremely rare but when found are sparsely distributed throughout the cytoplasm of both serosa cells and monocytes without any tendency to aggregation into clumps. On adaptation to multiplication on egg membranes these rickettsias, although present in much larger numbers, again are dispersed throughout the cellular cytoplasm but in addition also have an intranuclear habitat. Within the nuclei they may be present in aggregations of uncountable numbers of organisms. This group includes all the strains other than rat typhus.

Minor morphological differences appear to exist between the rickettsias of the two groups, e.g. as a rule those of rat typhus appear to be rather finer and more delicate in contrast to the rather more squat, plumper forms of the other group. These differences in morphology, however, cannot be regarded as of equal significance to the criteria enumerated above from the point of view of differentiation.

In conformity with the views of Pinkerton (1936) it would appear that some evidence has been brought forward to place the above group II [strains "Hare", "Appleton", "Robertson" (tick-bite fever) and fièvre boutonneuse] into his Rocky Mountain spotted fever group and group I (rat typhus) into his typhus group. It is obvious that even this broad classification could not be attempted without due consideration being paid to other essential properties of the virus strains so that a full discussion will be delayed until consideration of part III of these studies.

SUMMARY.

1. The microscopic appearance of the scrotal exudate in guinea-pigs infected with each of five strains of rickettsia is described.
2. The technique of cultivation on the chorio-allantoic membrane of the chick embryo is described.

PLATE I.

3. The morphology of the rickettsias as they appear in the scrotal exudate of the guinea-pig and on the chorio-allantois is described and compared.

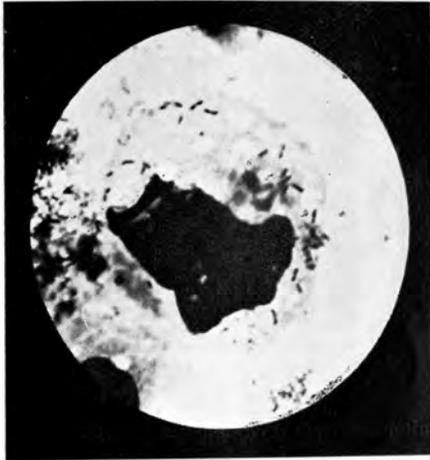


Fig. 1.

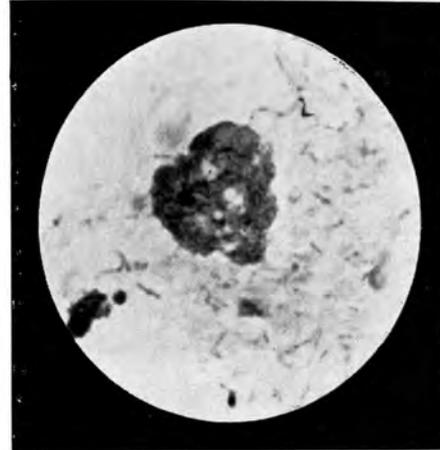


Fig. 2.

Fig. 1.—Strain "Hare" guinea-pig tunica preparation.

Fig. 2.—Strain "Hare". Chorio-allantoic membrane preparation.



Fig. 1A.

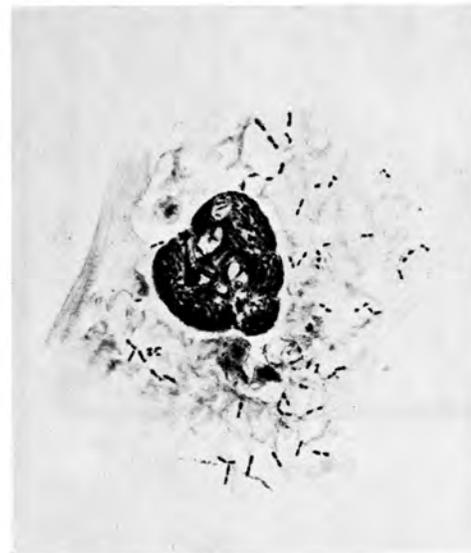


Fig. 2A.

Camera lucida drawings of above.

PLATE II.



Fig. 3.

Fig. 3.—Rat Typhus. Guinea-pig tunica. Small number of intracytoplasmic organisms showing tendency to clumping.

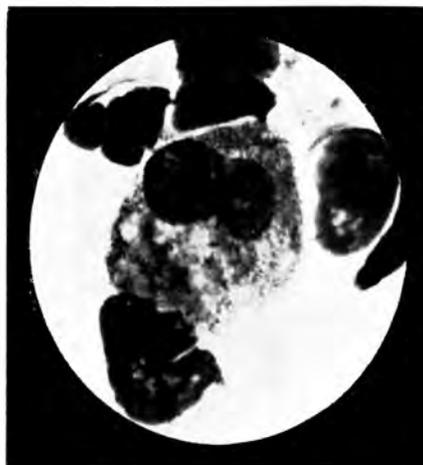


Fig. 4.

Fig. 4.—Rat Typhus. Guinea-pig tunica. Intracytoplasmic mass of rickettsias.

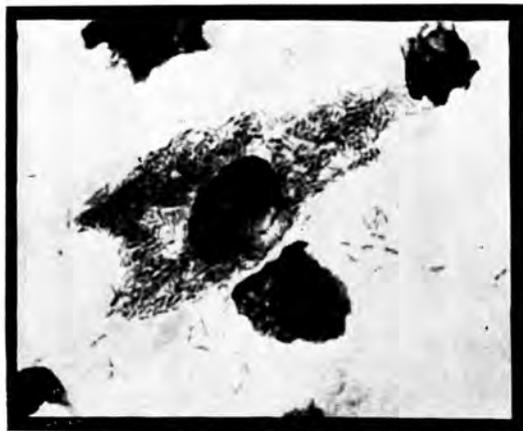


Fig. 5.

Fig. 5.—Rat Typhus. Membrane culture showing mass of intracytoplasmic rickettsias.

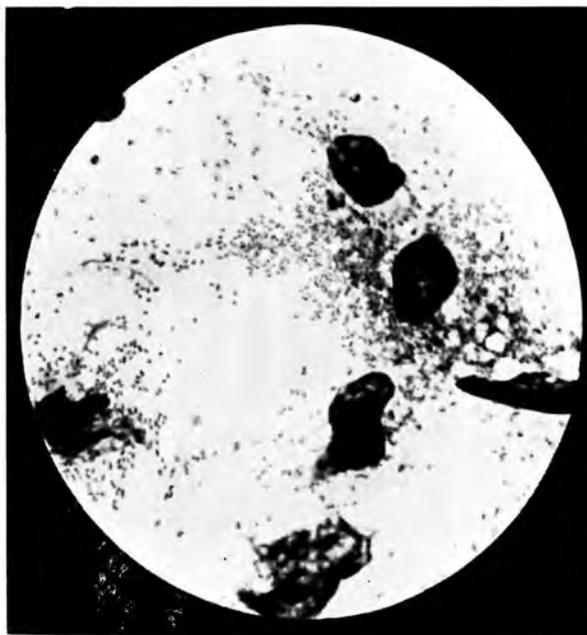


Fig. 6.

Fig. 6.—Rat Typhus. Membrane culture showing mass of coecal and cocco-bacillary forms. Cell ruptured.

PLATE II (*continued*).

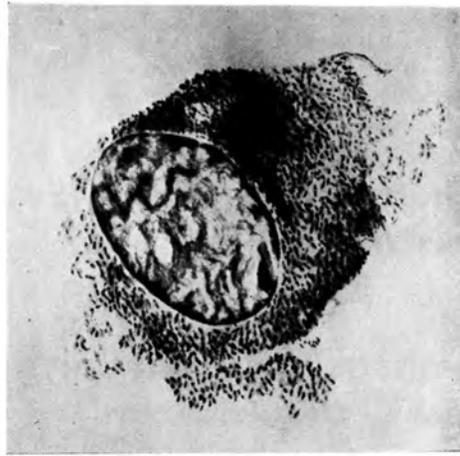


Fig. 7.—Camera lucida drawing of cell similar to Fig. 5.

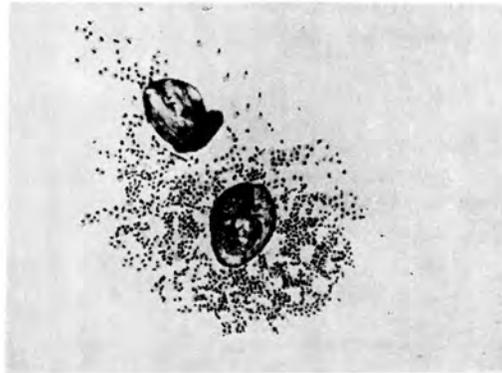


Fig. 8.—Camera lucida drawing of portion of Fig. 6.