CULTIVATION OF RICKETTSIA-LIKE MICROÖRGANISMS FROM CERTAIN BLOOD-SUCKING PUPIPARA

I. J. KLIGLER AND M. ASCHNER

Department of Hygiene and Bacteriology, Hebrew University, Jerusalem

Received for publication, March 1, 1931

INTRODUCTION

Rickettsia or rickettsia-like organisms have been studied extensively during the last decade; but there is as yet no clarity as to the nature and precise definition of this group of microbes. At least five pathogenic and a large number of non-pathogenic species have been described in a large variety of blood-sucking as well as non-blood-sucking insects (Hertig and Wolbach (1924), Cowdry (1923), Weigel (1924)). These descriptions are, however, based entirely or chiefly on morphologic studies, and it is more than likely that at least part of the pleomorphism ascribed to rickettsia is due to a confusion of a variety of organisms, including so-called rickettsias as well as bacteria belonging to the group of insect symbionts.

Da Rocha Lima (1916) was the first to describe a peculiar organism which he found in the gut of lice fed on typhus patients. These organisms were elliptical, smaller than B. melitensis, and were stained reddish with Giemsa. They were named Rickettsia Prowazeki. The primary characteristic ascribed by Da Rocha Lima to these organisms was their power to penetrate the cells of the wall of the insect intestines and multiply, and consequently he maintained that only organisms having this property, in addition to the peculiar morphology and staining reaction, can be classed in this group.

The large amount of work published subsequently and summarized by Hertig and Wolbach (1924) and Cowdry (1923) made it abundantly clear that rickettsia-like organisms were found in a
large number of arthropods. Hertig and Wolbach, after a comprehensive critical review of the literature, suggest that the term Rickettsia be limited to intracellular pathogenic organisms in the sense originally defined by Da Rocha Lima. But this definition is too limited, since in almost every group of microorganisms there are pathogenic and non-pathogenic species in the same genus. These authors admit that such a limitation is at the moment difficult to make and state that "in the meantime 'rickettsia' will doubtless continue to be a loose but convenient group-name for certain minute microorganisms associated with arthropods."

The other characteristics are even more difficult to circumscribe. The minuteness of size, characteristic staining reaction with Giemsa and association with insect hosts are fairly constant characteristics. But wide divergence can be noted in the description of rickettsias found in the literature. One of the most confusing elements is the extreme pleomorphism ascribed to these organisms. The organism described by Arkwright, Atkin and Bacot (1921) in the bed bug as R. leticularia is extremely pleomorphic, and this pleomorphism has since been considered by them, as well as by other investigators, as a peculiar characteristic of the group. This pleomorphism also forms the basis of the proteus theory of the etiology of typhus which has been developed particularly by the researches of Kuczinsky (1922). Anigstein (1927) in his attempt to repeat the work of Noeller with R. melophagi also describes a variety of forms under the name Rickettsia, ranging from minute coccoid-like bodies to filamentous mould-like forms. He maintains with Kuczinsky that the rickettsia forms are only a stage in the complex life cycle of these organisms.

The extensive studies by Buchner (1930) and his pupils which showed that many insects harbour bacterial symbionts and that the same species may be regularly inhabited by more than one organism furnish an explanation of the confusion existing in the literature concerning the pleomorphic character of Rickettsia. It may well be that such symbionts, which in most cases differ widely in their morphology from rickettsia, have been responsible for the extreme pleomorphism ascribed to the latter. Thus, the
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organism described by Arkwright and his associates in the bed bug is most probably a symbiont and not a rickettsia. It has also been shown (Zacharias (1928), Aschner (1931)) that the pleomorphic organisms of the sheep ked pictured by Arkwright and Bacot (1921) and by Anigstein (1927) consist of two entirely distinct forms of bacteria which regularly inhabit this insect. Hertig and Wolbach (1924) were the first to attempt to differentiate between rickettsia and symbionts. They define rickettsias as Gram-negative, intracellular, minute, coccoid or diploccoccoid organisms, 0.3–0.5 μ; staining readily with Giemsa but poorly with other aniline stains, without any well defined contour and difficult to cultivate in vitro. These authors evidently do not consider extreme pleomorphism as a dominant characteristic of rickettsia. This definition is today the best available, except that it is doubtful whether we are justified in limiting the group to intracellular types only, since, as was pointed out above, a variety of accepted extracellular forms have been described (R. quintana, R. melophagi) which have the characteristics of the group. Cowdry also arrives at the conclusion that at present the only criterion for distinguishing the rickettsias from other insect organisms is their minute size.

It is apparent from a review of the literature that there is a large group of microorganisms specifically adapted to insect hosts. Many of these have a definite relation to the biology of their host, as shown by the mutual adaptation of the microbes and the insect. Such forms are often highly pleomorphic, intracellular, and regularly transmitted from mother to offspring. These constitute the so-called symbionts. Of these symbionts no forms pathogenic to vertebrates are as yet known. There is another, more circumscribed and well defined group of organisms which is less definitely adapted to the insect host, and presumably plays no part in the normal biology of the insect. These correspond to the description usually given of rickettsia. Of this group some members are pathogenic for their host (R. Rocha-Liña, R. prowazeki), and others are pathogenic for mammals. A proper definition or differentiation of this latter group cannot be reached on the basis of morphologic characteristics alone. Progress in the study of
these organisms can be obtained only by a systematic study and
cultivation of the microbic flora of the parasitic insects. Such
studies would on the one hand extend our knowledge of the
biologic properties of the group as a whole, and on the other define
more specifically the characteristics of the genus Rickettsia.
The systematic study by Noguchi (1923) of the microbic flora of
the tick, *Dermacentor andersoni*, is illuminating in this connection.
Noguchi was able to cultivate three distinct species of bacteria,
one of which, however, proved to be the virus of spotted fever.

With this in view we have undertaken a study of the symbionts
and rickettsias of a number of species belonging to the pupipara.
The morphologic studies relating to symbionts and their biologic
significance will be published elsewhere (Aschner (1931)). At-
ttempts to cultivate the symbionts have not met with success.
However, we have succeeded in cultivating from several different
species of pupipara organisms resembling morphologically and
tinctorially the rickettsias present in these insects. The purpose
of this paper is to report the results of these experiments.

MATERIAL AND METHODS

Eight species of pupipara were studied, but the cultivation
experiments were confined to four. These were *Melophagus ovinus*, the common sheep ked, *Lipoptena caprina*, a parasite of
the goat, *Hippobosca equina* and *Hippobosca capensis* parasites
of the horse and dog respectively.

The pupipara are blood sucking diptera, characterized by the
fact that the larvae develop in the insects and pupate a few hours
after they are laid. They are extremely specific parasites and are
adapted only to a given host; seldom does it occur that an insect
can be transferred from one host to another. Well-defined
rickettsia-like organisms have been found in seven of the eight
species studied. These organisms are not present, always, in
every insect. In the morphological studies, both intracellular
and extracellular rickettsias have been noted. Intracellular
forms were found in *Nycteribosca kollari*, *Nycteribosca biarticulata*
and *blasii* (bat) and *Lynchia maura* (pigeon); they occurred
chiefly in the epithelial cells of the gut and in the cells of the mal-
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Pighian tubules, arranged singly or in small clumps. The extracellular forms were found in the lumen of the gut, usually massed on the epithelial cells. These forms were not equally abundant in all insects; they were most abundant in Lipoptena caprina and Melophagus ovinus, less so in Hippobosca capensis and only rarely in Hippobosca equina. In addition to the rickettsia-like organisms there were also noted large pleomorphic symbionts, and in some cases also small Gram-negative bacteria.

The rickettsia-like organisms noted in these insects resemble the symbionts in that they are transmitted to the larvae. Noeller has established this fact in the case of Melophagus, and one of us (Aschner (1931)) was able to demonstrate that this was also the case in five other species of those studied by us. As was first shown by Zacharias (1928) the extracellular rickettsias of M. ovinus do not invade the eggs but the larvae become infected from the milk glands. This seems to be true also for the other species. The number of rickettsias in the young adults is usually small, and they become particularly numerous after a blood feed. The intracellular rickettsias infest the eggs and are transmitted in that way.

Noeller was the first to report the successful cultivation of the rickettsia-like organisms from the sheep ked, Melophagus ovinus. He described on his blood agar plates gray, transparent colonies 0.4 to 0.6 mm. in diameter. The organisms were uniform in appearance and resembled those seen in the insect host. Jungmann (1918) and Hertig and Wolbach (1924) were subsequently able to confirm these findings. The important fact is that the culture forms described by these authors did not show wide morphological variations, and, only in older cultures, did Jungmann observe larger, more intensely stained, spherical or pear-shaped forms. These types have also been observed by us in our cultures, and probably represent degeneration forms. Anigstein (1927) who also attempted to confirm Noeller's work reports results which differ in many essentials from those of the other workers. He received at first dew drop colonies resembling those described by Noeller and by Hertig and Wolbach; later, however, these colonies lost their transparency and developed a greenish or yellow tint.
In contrast with the sparse growth obtained by the other authors he succeeded in getting an abundant growth. Furthermore, his cultures were pleomorphic, and he described a variety of strains which differed morphologically, culturally and serologically from one another. The relation of these findings to our own results will be discussed below.

The methods employed in this investigation were briefly as follows:

Morphological examination of insect. For sections, the insects were fixed in Regaud or Carnoy fixative. For demonstration of rickettsia-like organisms the former fixative is the most satisfactory. The sections were stained with Giemsa by the Noguchi method. Smears of guts or feces were fixed in 95 per cent alcohol, stained for thirty minutes with Giemsa and washed with distilled water. The organisms take the stain readily. When there were large masses of organisms, the ordinary Gram stain could be used.

Methods of cultivation. For the cultivation experiments the first problem was to free the insects of external bacteria. The following procedure proved entirely satisfactory. The insects were dipped in a 5 per cent tincture of iodine for five to ten seconds, then washed in 95 per cent alcohol for the same length of time, and then rinsed in sterile saline. The insects were then placed on sterile slides under the dissecting microscope, the abdomen cut off and the contents of the intestines pressed out into a drop of sterile saline solution. The abdomen was then transferred to a second sterile slide, the intestines teased out and separated from the sex organs. All, or parts, of the intestines were then placed on Noeller blood agar plates or into the fluid medium. The cultures were incubated at 26°C. and observed for a period of at least two weeks.

A check of the satisfactory character of the sterilizing procedure was furnished by the peristaltic movements of the gut as well as by the crithidia of the goat trypanosome present in the goat parasite. These crithidia grew readily in some of the media tested, and consequently served as an index of the toxic effect of the sterilizing procedure. It appeared that the iodine-alcohol
treatment sterilized the exterior of the insects without in any way affecting the viability of the organisms present in the gut.

It was our object in these experiments to grow only those organisms found in the insects which appeared tinctorially and morphologically to correspond to the rickettsia-like forms. We did not attempt, therefore, to classify other types of bacteria which from time to time grew out on one or another of the media used.

Media employed. A large variety of media were tested. Among these were Noeller's blood agar plates and Noguchi's media, as well as many others. We shall here confine ourselves to a description of those media which proved most useful in the cultivation of those rickettsia-like organisms which failed to grow on any other culture media. On these media closely related organisms were cultivated from all of the insects studied, but repeated effort failed to bring about their adaptation to the more common culture media.

Peptone-gelatine-blood medium. This medium consisted of a solution of salts, peptone and gelatine. A stock solution was prepared consisting of 10 grams peptone, 10 grams gelatine and 100 cc. water. One cubic centimeter of this solution was diluted in 10 cc. Locke solution, saline or glucosal. At first these mixtures were adjusted to pH 7.3, and sterilized in the autoclave; later it was found that a reaction of pH 6.6 was more favourable. After autoclaving, the media were divided into specially cleaned neutral test tubes, 2 cc. per tube. A day or two before use 0.25 cc. of defibrinated sheep or goat blood was added to each tube.

This medium proved more satisfactory than blood broth, particularly for primary cultures. On the one hand it is not favourable for the growth of trypanosomes which overgrow the rickettsia in blood-broth cultures; on the other the red cells lake less readily than in the broth.

Locke-semisolid. This medium consists of a mixture of 1 part nutrient agar, 2 parts defibrinated rabbit blood and 6 parts Locke solution. This medium is suitable for maintenance of strains, but is not satisfactory for primary cultures.

Noeller-blood agar. This medium consists of two parts agar
and one part blood. The blood is added while the agar is at 80°C, giving the medium a chocolate-brown appearance. Noeller first cultivated the R. melophagis of the sheep ked on this medium. We were able to obtain growth on plates around the bits of insect intestine. Sub-cultures on the solid medium were, however, difficult and only rarely successful, except in the case of the horse strains.

**CULTIVATION EXPERIMENTS**

As stated above, cultivation experiments were made with four species of insects. From all these species an organism was cultivated which had characteristic cultural and morphological properties and which resembled morphologically and tinctorially those seen in the guts of the insects.

*Morphology and staining.* All the organisms isolated were minute bacilli, often coccaloidal in shape, discrete or in large masses. Although of somewhat variable morphology the range of size was not greater than 0.5 to 0.3. They were all Gram-negative and stained uniformly with Giemsa, taking a violet tint. No bipolar staining was noted. In older cultures the staining was irregular, the tints being violet, pink and blue; the same clump contained also larger pear-shaped or coccus-like intensely stained organisms. Young cultures were, however, morphologically and tinctorially homogenous.

*Biological characters.* Successful results were obtained only in liquid media, the most uniform results being obtained in the peptone-gelatine-blood medium. The cultures were incubated at 26 to 28°C. The growth was localized on the surface of the red cells at the bottom of the tube, while the supernatent fluid remained entirely clear and unchanged. When growth was abundant there could be noted a greyish-white net-like deposit or else small discrete whitish pin point colonies on top of the red cells. This whitish film resembled a layer of white cells, and could easily be mistaken for it. Red blood cells were essential for growth. Hemoglobin as such was not sufficient, and if the cells hemolized too quickly no growth occurred. Probably for this reason sheep or goat cells which we found less subject to lysis than rabbit cells
proved more satisfactory. The amount of blood did not appear to be of great importance, provided that there was enough to form a layer of cells at the bottom of the tube which could serve as a matrix for the growth of the organisms.

Growth was at the best very slow. Usually, seven to ten days were required for growth to appear, and good growth could be noted only after two weeks.

On solid media growth was scarcely visible, and often the colonies were so minute that growth could only be demonstrated by staining. None of the strains, except the ones from Hippobosca equina, could be maintained on solid or semi-solid media for more than two generations. The horse strain was the only one that grew on glucose-blood-agar slants, but the growth developed slowly and never became abundant, even after many sub-cultures.

Transfers had to be made fairly frequently. The best interval for sub-culture was two weeks. Often, however, sub-cultures failed without any reason at all, although every care was taken to keep the composition of the media constant and uniform.

Efforts to determine the fermentative characters of these organisms were not successful. At best the growth was relatively so scanty as to produce no measurable change in the pH of the media. Glucose and glycerol appeared to have a favourable effect on the growth, but no fermentative action could be detected.

None of the organisms had any pathogenic effect on test animals. Large amounts of culture were inoculated into mice and guinea pigs without any noticeable effect, either on the temperature or blood picture. Attempts to recover the organisms from the peritoneal fluid or blood of the inoculated animals at various intervals after the inoculation yielded negative results.

The relation of these organisms to those cultivated by other investigators, notably Noeller (1923), Jungmann (1918), Hertig and Wolbach (1924), and Anigstein (1927) cannot be stated with certainty. Morphologically and tinctorially they resemble Rickettsia and correspond with Noeller's description of R. melophagi. Unlike Noeller's strain the organisms cultivated by use from the sheep ked failed to grow on solid media. They differed, however, both culturally and serologically from two of the strains culti-
vated by Anigstein, which he was kind enough to send us. These cultures grew readily on blood agar, giving a heavy yellowish growth; they were also Gram-negative, but on the whole larger than those grown by us. Whether this difference in size is a real one or due to the difference in media it is difficult to say; but the gross cultural differences were striking. Serologically, too, they proved to be entirely unrelated. Immune sera produced by our strain isolated from Melophagus ovinus had no effect on Anigstein's cultures isolated from the same species of insect.

The relation of the organisms cultivated by us to those found in the insect must also for the present remain a matter of conjecture. The morphologic and tinctorial resemblances were striking, as can be seen from plates 1 and 2. Serologic comparisons did not yield any clear cut results. Moreover, in the case of Lipoptena it was possible to recover the same organism in 30 out of 40 insects cultured at different times and on the different media described. This is a reasonable indication that we were dealing with the same organism as that present in the insect.

But whether the organisms cultivated were identical with, or only one of, those noted in the insect it is clear that we have cultivated from a variety of pupipara insects organisms which appear closely related to one another culturally, morphologically and tinctorially, and bear a close resemblance to the rickettsias found in the insects. It is our purpose to extend these methods to the study of the microbiic flora of insect vectors of pathogenic rickettsias.

**DISCUSSION**

Our studies have now extended over a period of two years. During this time many insects have been examined and a large number of cultures made. The work is extremely difficult, but our observations thus far have convinced us that further advances in our knowledge of this group of microbes depends on a greater familiarity with the flora of insect parasites in general, and, more particularly, with the group of organisms having the general characteristics of Rickettsia. Knowledge of the cultural properties of the non-pathogenic members of this group of microbes may aid in the study of the pathogenic members of the group.
It is essential at the outset to differentiate between the symbionts and rickettsia-like organisms, both of which are adapted to the insect host; and between these and chance invaders. Furthermore, it should be emphasized that the symbionts differ essentially from Rickettsia. The former represent a wide variety of forms some of which definitely belong to the bacteria, and others to the higher bacteria and moulds and like them show extreme pleomorphism and irregular staining reactions. The bacillus cultivated by Glaser (1930a and b) from the American and German cockroach, for example, belongs to this group. It is a Gram-positive pleomorphic diphtheroid resembling morphologically and tinctorially the intracellular organisms seen in the insect. The mere fact that they are found in the insect cells is in our opinion no justification for classing them with the entirely distinctive group of Rickettsia.

The rickettsias have a much more uniform morphology and size, and are all Gram-negative. The work of Anigstein stands unconfirmed, and our own observations have convinced us that this investigator obtained a variety of organisms which he unjustifiably considered different aspects of the same species. Unless future studies prove the contrary, the present indication is that the more conservative view that the group is fairly stable within narrow limits appears to be more in accord with the facts. Ascribing extreme pleomorphism to an organism on the basis of appearances in insects known to harbour other organisms leads to confusion and not to a precise delimitation of the group.

**SUMMARY**

Experiments are reported dealing with the cultivation of extracellular non-pathogenic rickettsias from a number of pupipara. Methods are described by means of which cultures of Rickettsia were obtained repeatedly from the parasitic pupipara of the sheep, goat, horse and dog. The organisms obtained in culture were minute, Gram-negative, cocccoidal rods corresponding to the usual description of Rickettsia and resembling the forms seen in the guts of the insects.
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PLATE 1

Fig. 1. Section through the intestine of Hippobosca capensis Olfers, showing intracellular symbionts at the bases of epithelial cells as well as a few rickettsia on the surface of the middle cell. Fixation, Regaud; stain, Giemsa; ca. 700×.

Fig. 2. Section through the intestine of Lipoptena caprina Austen showing a thick layer of rickettsia covering the epithelial cells. Fixation, Regaud; stain, Giemsa; ca. 700×.

Fig. 3. Smear of faeces of Hippobosca capensis Olfers with numerous rickettsia. The filamentous organism is a symbiont. Stain, Giemsa; ca. 700×.

Fig. 4. Rickettsia from Hippobosca capensis Olfers in a Locke-peptone-gelatine-sheep-blood culture; note irregular staining of the clump. Stain, Giemsa; ca. 700×.

Fig. 5. Rickettsia and symbionts of Lipoptena caprina Austen in a faeces preparation. Stain, Giemsa; ca. 600×.

Fig. 6. Rickettsia from Lipoptena caprina Austen from a blood agar culture. Stain, Giemsa; ca. 700×.

Fig. 7. Rickettsia melophagi from the intestine of Melophagus ovinus. Stain, Giemsa; ca. 700×.

Fig. 8. Rickettsia melophagi in a Locke-peptone-gelatine-sheep-blood culture. Stain, Giemsa; ca. 700×.
(G. J. Kligler and M. Aschen: Rickettsia-like microorganisms.)
PLATE 2

Fig. 1. Rickettsia from *Hippobosca equina* in a Locke-peptone-gelatine culture, showing the formation of large intensely stained bodies described by Jungmann in *R. Melophagi* cultures. Stain, Giemsa; ca. 1000X.

Fig. 2. The same in a blood culture of *Rickettsia melophagi*. Stain, Giemsa; ca. 1000X.

Fig. 3. *Hippobosca capensis* strain in a sheep-blood-bouillon culture. Uniform growth. Stain, Giemsa; ca. 1200X.

Fig. 4. *Leptotena caprina* strain in a goat-blood-bouillon culture. Streptococcus-like chain-forming growth. Stain, Giemsa; ca. 1200X.

Fig. 5. *Hippobosca equina* strain on glucose-blood-agar. Stain, Giemsa; ca. 1200X.

Fig. 6. *Melophagus* strain in a sheep-blood-bouillon culture. Stain, Giemsa; ca. 1200X.

Fig. 7. Augstein's strain C. 17 from *Melophagus orinus* on blood agar; contrast with the various strains of rickettsia. Stain, Giemsa; ca. 1200X.
(I. J. Kligler and M. Aschner: Rickettsia-like microorganisms.)