THE ROLE OF THE SYMBIOTIC BACTERIA IN THE NUTRITION OF *RHODNIUS PROLIXUS* (HEMIPTERA)

By S. BAINES

Department of Bacteriology, University of Birmingham

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INTRODUCTION

The occurrence of supposedly symbiotic micro-organisms in insects is well known They may be found in the gut, in various other parts of the body and in special organs known as mycetomes. The micro-organisms are usually intracellular, and in many cases are difficult or impossible to grow in artificial culture. They appear to be highly specific to their host and may be transmitted from generation to generation by special and complex mechanisms. Among those previously described are forms considered to be yeasts, bacteria and rickettsiae (Buchner, 1953; Steinhaus, 1940, 1946, 1949; Paillot, 1933).

In some cases, the insect hosts have been shown to be adversely affected by the removal of the micro-organisms and the association is believed to be necessary for the normal development of the host (Brooks & Richards, 1955; Brues & Dunn, 1945; Blewett & Fraenkel, 1944; Brecher & Wigglesworth, 1944).

Blewett & Fraenkel (1944) have produced experimental evidence to show that the yeast-like symbiotes of the two beetles *Stegobium paniceum* and *Lasioderma serricorne* supply vitamins of the B group to their hosts. The symbiotes of the louse, *Pediculus vestimenti*, have a similar function (Puchta, 1955).

In the case of *Rhodnius*, a bacterium occurring in the lumen of the gut has been isolated in artificial culture. It was described as *Actinomyces rhodnii* by Brecher & Wigglesworth (1944), according to the classification of Erikson (1935), and has appeared in the lists of the National Collection of Type Cultures as *Nocardia rhodnii*. Brecher & Wigglesworth also showed that *Rhodnius* nymphs in which the symbiotic bacteria were absent failed to complete their development to the adult stage. Wigglesworth (1936) showed that this organism grown in culture could act as a source of B vitamins for sterile *Lucilia* larvae.

The present paper describes the symbiotic bacteria in the light of recent work on the classification of the filamentous bacteria (Bisset & Moore, 1949; Morris, 1951, 1952), and also provides evidence of the function of these bacteria in the nutrition of the host.

Rhodnius is a member of the Hemiptera and feeds on mammalian blood. There are five nymphal instars in the development from the egg to the adult, and the adult emerges from the fifth and final moult which constitutes metamorphosis.

METHODS

The isolation and examination of the symbiotic bacterium

The adult insects or nymphs were dissected from the dorsal surface and smears were prepared on glass slides from the contents of the midgut; glucose agar plates were inoculated with the same material. The inoculated plates were incubated for up to 7 days at 30° C. Growth of the symbiotic bacteria usually occurred within 3 days. The smears were heat-fixed and stained by Gram's method. It was found unnecessary to sterilize the outside of the insect before dissection because the proportion of contaminated plates when unsterilized insects were used was less than 0·5%.

The rearing of symbiote-free insects

Brecher & Wigglesworth (1944) showed that if the egg surface was sterilized with crystal violet solution the insects which hatched failed to acquire the usual infection with the bacterium. This procedure was adopted, but was discarded when it was found that equally good results could be obtained without sterilization if the eggs were isolated from sources of contamination. The eggs were removed from stocks of mated adults as soon as possible after oviposition and were transferred, in batches of about ten, to sterile tubes. Each insect was transferred to a fresh sterile tube after hatching and thereafter maintained in a sterile environment, being transferred to a fresh sterile tube after each feed and after each moult. Only two insects were found to be infected after such treatment from a total of over five hundred used in the course of the work.

Feeding of the insects

Rabbits and mice were used to provide blood for the feeding of the insects. In the former case, each insect was placed in a sterile test-tube with cotton gauze stretched over the mouth. The tube was inverted upon the ear of the rabbit and the insect was able to feed through the gauze. The mice were anaesthetized with nembutal (Abbots' Laboratories), and the insects in inverted tubes, as described above, were placed on the abdomen. The mice received 0·01 grain (=0·000648 g.) per 100 g. of body weight of nembutal by the intraperitoneal route.

Additions to the blood diet of *Rhodnius* were made by injecting mixed solutions of vitamins intravenously into the rabbit or into the anaesthetized mouse immediately before the insects were fed. The vitamins were prepared in saline solution, stored at −20° C. and mixed immediately before use. The cholesterol content of the rabbits' blood was increased by adding cholesterol to their diet over a period of 2 or 3 weeks before they were required for use.

Rhodnius normally takes one complete feed during each nymphal instar, which results in a moult from which the next instar emerges.

RESULTS

The symbiotic bacteria

The cultures made by the methods described were examined at frequent intervals. Stained films showed that the bacteria were strongly Gram-positive and passed through a life cycle which included filamentous, rod-shaped and coccal forms. The filamentous forms which were multicellular soon fragmented into shorter filaments and rods, and finally into coccal forms consisting of one or two cells. These frequently remained in their original arrangement for some time before dispersing, and gave the appearance of chains of cocci. On transfer to a fresh medium the coccal forms germinated to produce filamentous forms. Branched forms were rare and were transient. In culture the complete cycle lasted about 24 hr. The bacterium produces a salmon pink non-diffusible pigment, which increases in intensity with age and exposure to light. The general morphology and life cycle and the appearance of the colonies confirmed that the bacterium is correctly classified as a member of the genus *Nocardia*, as defined by Bisset & Moore (1949) and Morris (1951, 1952). Further evidence of the systematic position of this organism will be provided in a separate communication elsewhere.

The life cycle of *N. rhodnii* was observed in the insect by the examination of smears made from the gut contents of adults and of nymphs in all stages of development, at daily intervals between successive feeds. The same life cycle occurs within the host insect as that described in artificial culture. The filamentous forms develop within a few days of the insect feeding, they gradually fragment and finally revert to the coccal form. The life cycle in the host occupies from 5 to 8 days. It was found that the bacteria were more numerous in the third, fourth and fifth nymphal instars (that is, at the stages in the insect's life history preceding metamorphosis) than either in the early stage nymphs or in the adult insects. Filamentous forms were rarely found in the adult insects even after they had recently fed. This is of importance in view of the fact, discussed later, that it is these later nymphal instars which are delayed in their development in the absence of the symbiotic bacteria.

Removal of the symbiote and its effect on the development of Rhodnius

Rhodnius nymphs, fed on rabbits' blood, were reared free from *Nocardia rhodnii* by isolating the eggs before hatching and keeping the nymphs in a sterile environment. Their development was compared with that of normal nymphs in which the symbiote was present; the rate of development was measured by the number of days elapsing between the insect feeding and the subsequent moult in each nymphal instar. All the nymphs were kept at 30° C. and the relative humidity was controlled at 75% by means of saturated sodium chloride solution. The development rates are compared in Table 1.

It is seen that in the absence of *N. rhodnii* the first and second instar nymphs developed at approximately the normal rate, whereas in the third instar some nymphs took much longer than normal to moult after feeding. In the fourth instar over

40% of the nymphs which fed failed to moult, and the remainder took up to 30 days more than the normal period before moulting to fifth-instar nymphs. Their further development, after feeding in the fifth and final instar, was arrested, and none of them completed metamorphosis.

Table 1. *The development of symbiote-free* Rhodnius *nymphs compared with that of normal nymphs*

Nymphal instar	*Nocardia rhodnii* absent				*Nocardia rhodnii* present
	No. of insects		No. of days from feed to moult		No. of days from feed to moult
	Fed	Moulted	Average	Range	Range
1	20	17*	8·25	7–10	7–9
2	32	32	9·80	9–12	8–9
3	29	29	18·00	10–51	9–10
4	23	10	28·10	20–40	10–12
5	10	0	—	—	16–20

* The three nymphs which failed to moult died within 2 days of feeding.

When several of the fourth- and fifth-instar nymphs which failed to moult were fed again, moulting occurred in two out of five fourth-instar nymphs and in one out of five fifth-instar nymphs, though only after periods ranging from 39 to 94 days. One of seven nymphs fed for a third time moulted 94 days later.

Moulting was also induced when *N. rhodnii* was introduced into sterile fourth- and fifth-instar nymphs. Infection was produced by transferring these nymphs either to tubes seeded with *N. rhodnii* from cultures, to tubes containing fresh faeces of normal *Rhodnius*; or to stocks of *Rhodnius* nymphs in which *Nocardia rhodnii* was present.

All these nymphs developed normally after they were infected, and the period between infection and moulting was in every case only 2 or 3 days longer than that between feed and moult in normal nymphs at the corresponding stage in development, irrespective of the method of infection, or of the time which had elapsed between feeding and the introduction of *N. rhodnii*.

The next series of experiments was devised to discover if the symbiotic bacteria were supplying some essential B group vitamins to the host insect. Accordingly, symbiote-free nymphs were fed on anaesthetized mice, previously injected by the intravenous route with mixtures of various B vitamins in solution. The concentrations of the vitamins in the various mixtures are shown in Table 2.

Mice were injected with 1 ml. of a mixture for each 10 g. of body weight. The actual quantities of the vitamins injected (μg./g. of blood) are given in Table 3, assuming that the weight of the blood is *c.* 10% of the total body weight of the mouse.

Riboflavin was found to be toxic to mice when injected at the rate of either 100 or 50 μg./g. of blood, and was therefore excluded from the experiments.

Table 2. *Mixtures of B group vitamins for injection into mice*

Vitamin	Source	Final concentration in mixture (mg./ml.)							
		M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8
Thiamine	L. Light and Co. Ltd.	1	0	1	1	1	1	1	1
Nicotinamide	L. Light and Co. Ltd.	1	1	0	1	1	1	1	1
Pyridoxin	L. Light and Co. Ltd.	1	1	1	0	1	1	1	1
Ca pantothenate	Roche Products	1	1	1	1	0	1	1	1
Folic acid	L. Light and Co. Ltd.	1	1	1	1	1	0	1	1
Biotin	L. Light and Co. Ltd.	0·01	0·01	0·01	0·01	0·01	0·01	0	0·01
Cyanocobalamin	Roche Products	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0

The development of the nymphs fed on mouse blood with the addition of various vitamins was observed and compared with that of nymphs fed on mouse blood without additions. Control groups of nymphs with *N. rhodnii* in the gut were also fed on mice and their development compared with that of the symbiote-free nymphs.

Table 3. *Quantities of vitamins added to the mouse blood diet of the nymphs by the injection of the vitamin mixtures*

Vitamin	Quantities added by injection of the mixtures (μg./g. of blood)							
	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8
Thiamine	100	0	100	100	100	100	100	100
Nicotinamide	100	100	0	100	100	100	100	100
Pyridoxin	100	100	100	0	100	100	100	100
Ca pantothenate	100	100	100	100	0	100	100	100
Folic acid	100	100	100	100	100	0	100	100
Biotin	1	1	1	1	1	1	0	1
Cyanocobalamin	1	1	1	1	1	1	1	0

In the first four nymphal instars the nymphs in all the groups developed at the same rate, irrespective of the addition of vitamins or of the presence of *N. rhodnii*; the comparative rates of development in the fifth and final instar are shown in Table 4.

Table 4. *The development of symbiote-free nymphs in the fifth instar with the addition of B group vitamins to the mouse blood diet*

Vitamin supplement	No. of insects		No. of days from feed to moult		
	Fed	Moulted	Average	Range	
M-1	7	6	20	19–22	
M-2	6	5	21·5	16–26	
M-3	7	7	22	19–27	
M-4	5	4	29	21–33	
M-5	7	6	23	18–28	*N. rhodnii* absent
M-6	6	6	22	19–27	
M-7	7	7	19·5	19–20	
M-8	6	4	19·5	19–21	
None	7	4	47	27–68	
None	5	5	17·5	16–19	*N. rhodnii* present

When all the vitamins used in the experiment were added simultaneously to mouse blood, all but one of the nymphs fed on the supplemented diet (M-1) were enabled to complete their development. Furthermore, those nymphs which moulted after feeding did so after approximately the same period of time as normal nymphs in which the symbiote was present. The omission from the mixture of either thiamine (M-2), pyridoxin (M-4), Ca pantothenate (M-5) or cyanocobalamin (M-8) resulted in one or two nymphs in each case failing to moult, but when either nicotinamide (M-3), folic acid (M-6) or biotin (M-7) was omitted all the nymphs moulted. The nymphs fed on blood from which biotin (M-7) or cyanocobalamin (M-8) was omitted moulted after approximately the same interval as did normal nymphs, but in the other groups of nymphs some took longer to moult. This delay in moulting was most pronounced when pyridoxin was omitted, and less so when Ca pantothenate, nicotinamide, folic acid or thiamine was omitted. The nymphs which received no vitamin supplement took a very much longer time than normal nymphs to moult after they were fed on mouse blood, and a high proportion of them failed to moult. Similar symbiote-free fifth-instar nymphs fed on rabbits' blood always failed to moult (Table 1) and the period between feed and moult in the third and fourth instars was also longer than that in normal nymphs. This fact, and the increase in the development rate brought about by the addition of B group vitamins to mouse blood, suggested that the difference in the development rate of nymphs fed on mouse blood on the one hand and on rabbit blood on the other may be due to a higher concentration of some of the B vitamins in normal mouse blood. Symbiote-free nymphs were therefore fed on rabbits previously injected with a mixture of the B group vitamins and their development was compared with that of similar nymphs fed on normal rabbits. Riboflavin was again omitted from the mixture because of its toxicity at the desired concentration. Inositol (L. Light and Co. Ltd.) and choline (L. Light and Co. Ltd.) were included in these experiments. The rates of development of all these nymphs are shown in Table 5.

The addition of the vitamins caused an increase in the rate of development of the fourth-instar symbiote-free nymphs fed on rabbit blood, but none of them completed their development to the adult stage. Furthermore, these nymphs developed less rapidly than similar nymphs fed on mouse blood without vitamin supplement, even though the concentration of the added vitamins was undoubtedly greater in the supplemented rabbit blood than in the normal mouse blood. The addition of inositol and choline chloride had little effect on the rate of development. It therefore appears that some other factor is supplied by *N. rhodnii* to its host, that this factor is essential to the normal development of *Rhodnius* and is present in a greater concentration in mouse blood than in rabbit blood.

This factor could not be identified with cholesterol because symbiote-free nymphs fed on rabbits with a high cholesterol level in their blood developed no more rapidly than those on normal rabbits, and less rapidly than those on normal mice. The cholesterol levels were: normal rabbit, *c.* 750 μg./g. of blood; high cholesterol rabbit, *c.* 3000 μg./g. of blood; normal mice, *c.* 1000 μg./g. of blood.

Table 5. *The development of symbiote-free* Rhodnius *nymphs fed on rabbit blood supplemented with B group vitamins*

Nymphal instar	Vitamin* supplement	No. of insects		No. of days from feed to moult	
		Fed	Moulted	Average	Range
3	R-1	9	9	11·5	11–13
	R-2	1	1	13	13
	R-3	1	1	11	11
	None	4	4	14	11–16
4	R-1	9	9	20	12–31
	R-2	4	4	14	10–18
	R-3	3	3	19	13–27
	None	9	1	17	17
5	R-1	9	0	—	—
	R-2	6	0	—	—
	R-3	3	0	—	—
	None	1	0	—	—

* Vitamin supplements:
R-1 contained thiamine, nicotinamide, pyridoxin, Ca pantothenate, folic acid, biotin and cyanocobalamin but no inositol or choline chloride.
R-2 contained all the above vitamins and inositol (final concentration = *c.* 500 g./g. of blood).
R-3 contained all the above vitamins and inositol and choline chloride (500 g./g. of blood).

CONCLUSIONS

The results presented in the first part of this paper confirm those of Brecher & Wigglesworth (1944) who found that the bacterium is extracellular, in the gut lumen, and no special mechanism exists for the transfer of the bacterium from generation to generation of the host. *Nocardia rhodnii* is transmitted in the faeces of the host, and the infection is acquired from the egg surface only when it is contaminated by excrement from infected nymphs and adults. In their natural environment these insects are gregarious, living in the burrows of the small mammals on which they feed and hiding in crevices in the burrows between feeds. Under these conditions the method of transmission of the bacterium is undoubtedly effective, because of the close contact of the insects for long periods. However, laboratory cultures of the insects may at times die out because of the failure of the transmission mechanism, particularly if the insects are kept in small numbers and under relatively clean conditions. *N. rhodnii* is therefore essential to the normal development of the host insect, and in its absence the insect fails to reach the adult stage.

The evidence presented in the second part of the present work shows that the function of the bacterium is the supply of certain B vitamins in which the normal blood diet of *Rhodnius* is apparently deficient. The insect is dependent on the symbiotic bacteria for its supply of pyridoxin, Ca pantothenate, nicotinamide and thiamine when it is fed on mouse blood. Biotin and folic acid are, however, present in adequate concentration in the blood to meet the insect's requirements. The position of cyanocobalamin is not clear because the results were somewhat conflicting, one-third of the nymphs tested appeared to be dependent on the bacterium

for the supply of this vitamin whereas the rest obtained adequate quantities for their normal development from mouse blood. Another factor is also required by *Rhodnius* for normal development and is normally supplied by the bacterium. This has not been identified but is present in greater concentration in mouse blood than in rabbit's blood. It cannot be identified with any of the B vitamins investigated, nor with choline chloride, inositol or cholesterol because rabbit's blood with the addition of any or all of these factors was less adequate as a diet for the symbiote-free *Rhodnius* nymphs than normal mouse blood, even though the supplemented rabbit's blood contained greater concentrations of the vitamins than the normal mouse blood. *Rhodnius* develops equally well and at the same rate on the blood of either species when *Nocardia rhodnii* is present, proving that the unidentified factor is supplied by the bacterium. Riboflavin had to be excluded from these experiments because it was not tolerated by mice or rabbits at the dosage level required, and it is possible that it could be the unidentified factor. Variations in the experiments such as that mentioned in connexion with cyanocobalamin may be caused by the limiting influence of the unidentified factor. Variations of this kind are to be expected when it is realized that the blood used in these experiments as the basic diet was not, of course, free from vitamins, and furthermore the concentrations of the vitamins would vary considerably in different animal species, in different individuals of the same species and in an individual at different times.

Further work on the identification of the unknown factor is in progress, and it is hoped to clarify the results further by the use of a basic diet which can be standardized and fed to the insects through a membrane. However, it is clear that the role of the symbiotic bacterium in *Rhodnius* is similar to that of other symbiotic micro-organisms in other insect species so far studied.

Symbiote-free *Rhodnius* nymphs are bacteriologically sterile, and it appears possible that they could be usefully employed in the investigation of certain nutritional problems which are normally complicated by the presence of bacteria in the gut of the experimental animals employed.

SUMMARY

1. Earlier views of the nature of the association between *Rhodnius* and its symbiotic bacteria are confirmed.

2. The bacterium is described as *Nocardia rhodnii* in accordance with more recent views of the classification of the Gram-positive filamentous bacteria.

3. Experimental evidence is provided to show that the symbiotic bacterium is essential to *Rhodnius* because of its function in supplying certain B group vitamins, in which the normal blood diet of the host is deficient.

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