STUDIES ON "HAEMIXODOVIN", THE PIGMENT IN THE EGGS OF THE CATTLE TICK *BOOPHILUS MICROPLUS* (ACARINA: IXODIDAE)

By K. C. BREMNER*

[*Manuscript received February* 24, 1959]

Summary

The pigment present in the eggs of the cattle tick *Boophilus microplus* is shown to be a chromoprotein made up of a water-insoluble globulin conjugated with the prosthetic group protohaem. The name "haemixodovin" is proposed for the pigment, and its spectral absorption characteristics have been described.

The physiological significance of this haemoprotein in the eggs is discussed, and evidence is presented which indicates that, whereas the protein moiety of the molecule is utilized as a food reserve by the developing larva, the haem grouping is probably a functionless inclusion derived directly from the bovine haemoglobin of the parent tick's diet.

I. INTRODUCTION

The first report on the nature of the pigment responsible for the brown colour of tick eggs was made by Wigglesworth (1942), who noted the presence of alkaline haematin in the eggs of *Ornithodorus moubata* (Murray) and *Ixodes ricinus* (L.). More recently, Riek (1959), in the course of studies on toxins present in the eggs of the cattle tick *Boophilus microplus* (Canestrini), observed that a pigmented protein component was present in aqueous and saline extracts of the eggs. Riek figures electrophoresis patterns of the egg extracts and refers to the pigment as fraction F_6.

Since Wigglesworth's (1942) studies gave no indication that the egg pigments he studied were protein-bound, the following investigations were made in an attempt to define the chemical nature of the pigment in the eggs of *B. microplus*, and to assess its metabolic fate.

II. EXPERIMENTAL

(*a*) *Solubility Characteristics of the Egg Pigment*

Aqueous extracts of eggs of *B. microplus*, which had been laid no longer than a week, were prepared by grinding eggs and distilled water in a mortar in the proportion of 1 g eggs to 2 ml water. The extract was centrifuged at 1200 *g* for 30 min, and 8 ml of the supernatant was subjected to zone electrophoresis in a starch-supporting medium as described by Kunkel and Slater (1952). Electrophoresis was carried out in an 0·05M phosphate buffer of pH 11·0, and a voltage of 10 V/cm was applied for 17 hr. The brown pigmented section was then cut from the starch block and eluted with phosphate buffer.

*Division of Animal Health and Production, C.S.I.R.O., Veterinary Parasitology Laboratory, Yeerongpilly, Qld.

This solution was then diluted to 200 ml with distilled water, and glacial acetic acid was added drop by drop with stirring until a dense brown precipitate settled out. The pH of the clear supernatant was then 5·1, and this was presumed to approximate the isoelectric point of the pigment. After allowing the mixture to stand for 30 min, most of the supernatant was decanted, and the remaining suspension centrifuged at 1200 *g* for 15 min. The supernatant was removed and the precipitate was shaken with 10 ml of distilled water and again centrifuged, but no pigment was found to have dissolved. After two washings with 10 ml of distilled water, the precipitate was shaken with 10 ml of 0·2 per cent. sodium chloride

TABLE 1

ABSORPTION SPECTRA OF CRYSTALLINE PROTOPORPHYRIN ESTER AND PORPHYRIN ESTER PREPARED FROM B. MICROPLUS EGG PIGMENT

Positions of maxima (mμ) and ratios of intensities (in parenthesis) of the visual absorption bands of samples of porphyrin in dioxane, the intensity of band IV being taken as 1·0

Pigment	Band IV	Band III	Band II	Band I
B. microplus porphyrin methyl ester	504 (1·0)	538 (0·80)	576 (0·465)	631 (0·359)
Crystalline protoporphyrin methyl ester	504 (1·0)	539 (0·784)	576 (0·441)	631 (0·374)
Protoporphyrin methyl ester*	503 (1·0)	537 (0·790)	575 (0·463)	630 (0·381)

*Stern and Wenderlein (1934).

solution, and complete solution of the pigment was obtained. Presumably sufficient inorganic ions to carry this protein into solution are present in the eggs when they are extracted with distilled water. The fact that dialysis of aqueous egg extracts against distilled water results in precipitation of the pigment supports this view.

Tests on the pigment in solution in 0·2 per cent. sodium chloride showed that it remained in solution after the addition of two volumes of 0·4N ammonia solution, but was precipitated by the addition of an equal volume of ethanol and by half saturation with ammonium sulphate. The pigment was coagulated by heating the solution to 100°C. From this evidence it was concluded that the protein component of the pigment was of the globulin type (Mitchell 1946).

(*b*) *Spectrophotometry of the Egg Pigment*

Since Wigglesworth (1942) has reported the presence of alkaline haematin in the eggs of the ticks *O. moubata* and *I. ricinus*, and in view of the marked red-brown pigmentation of clarified extracts of *B. microplus* eggs, it seemed likely that the prosthetic group of the egg pigment was of the haematin type. Accordingly, *Boophilus* eggs were extracted with ethyl acetate–acetic acid (3 : 1) and the extract was examined for haem and free porphyrins (cf. Dresel and Falk 1956). No free porphyrins were present, but spectroscopic examination indicated the presence of haem. A portion of the extracted haem was taken to dryness and converted

to pyridine haemochromogen; readings of the absorption spectrum in a Beckman spectrophotometer (model DU) showed maxima at 557 and 525 mμ, identical in position to those of a haemochromogen made from recrystallized protohaem under the same conditions. Thus there is little doubt that the egg pigment prosthetic group is protohaem.

To confirm this identification, a further portion of the extracted haem was converted to porphyrin as follows: The dried haem was dissolved in glacial acetic acid, and iron removed by the ferrous acetate–acetic acid method of Warburg and Negelein (1932). The porphyrin so formed was transferred to ether (which had been washed with ferrous sulphate and water to remove peroxides), washed with water, extracted into 4N hydrochloric acid, and returned to fresh ether. It was then dried and esterified by standing in methanol–5 per cent. sulphuric acid at 1°C for 20 hr. The porphyrin ester was then transferred to chloroform, washed with water and then with 2N ammonium hydroxide to remove any unesterified porphyrin, and finally washed with water.

The porphyrin ester was then taken to dryness and purified by chromatography in benzene on a column of Al_2O_3 (grade V) (Nicholas 1951). Benzene eluted most of the ester from the column and this was taken to dryness and dissolved in purified dioxane (Eigenberger 1931). The absorption spectrum of this solution was read on the Beckman spectrophotometer, and compared with the spectrum of a sample of known crystalline protoporphyrin methyl ester read under the same conditions. The correspondence of these two absorption spectra, shown in Table 1, confirms that the prosthetic group of the egg pigment was protohaem.

Studies on the spectrophotometry of the intact egg pigment were carried out on purified preparations obtained by eluting the pigmented band from the filter-paper strips with M/15 phosphate buffer, pH 7·8, after concentrated egg extracts had been subjected to electrophoresis on filter paper for 3 hr in the same buffer with an applied voltage of 10 V/cm (Kunkel and Tiselius 1951). Absorption curves of this preparation (Fig. 1) showed a maximum at 617 mμ, while less well-defined maxima occurred at about 530 and 500 mμ. Intense absorption was displayed with a maximum at 405 mμ in the region of the Soret maximum of pigments containing an intact porphyrin ring (Lemberg and Legge 1949). A further band of intense absorption was manifest in the ultraviolet with a maximum at 277 mμ.

Reduction of the pigment with sodium dithionite resulted in the bands at 617, 530, and 500 mμ being replaced by distinct absorption bands with maxima at 562 and 534 mμ, while the Soret maximum was displaced to about 420 mμ (Fig. 1). Accurate readings were not obtained at wavelengths below 400 mμ, because of interference by the reducing agent. Addition of a few drops of 2N sodium hydroxide to the reduced solution resulted in the immediate formation of a haemochrome with absorption maxima at 557 and 525 mμ (Fig. 2). Passage of carbon monoxide through a solution of the pigment previously reduced by dithionite resulted in the formation of a carboxy compound whose absorption curve (Fig. 2) is very similar to that of carboxyhaemoglobin, but with absorption maxima at 567 and 538 mμ.

Boophilus eggs suspended in distilled water were placed in a spectrophotometer cuvette of 1 mm path length and the absorption spectrum of this preparation over the

wavelength range 450–650 mμ was found to be identical with that of the electrophoretically purified pigment extract at pH 7·8. Thus there is little doubt that the pigment does exist as a haemoprotein compound in the intact egg.

Finally, absorption curves were obtained of solutions of the pigment in phosphate and borate buffers covering the pH range 5·5–11·0, and it was found that the absorption spectrum remained unaltered over the pH range examined. This marked stability of the compound despite such changes in pH is very similar to that reported by Fairley (1941) for methaemalbumin.

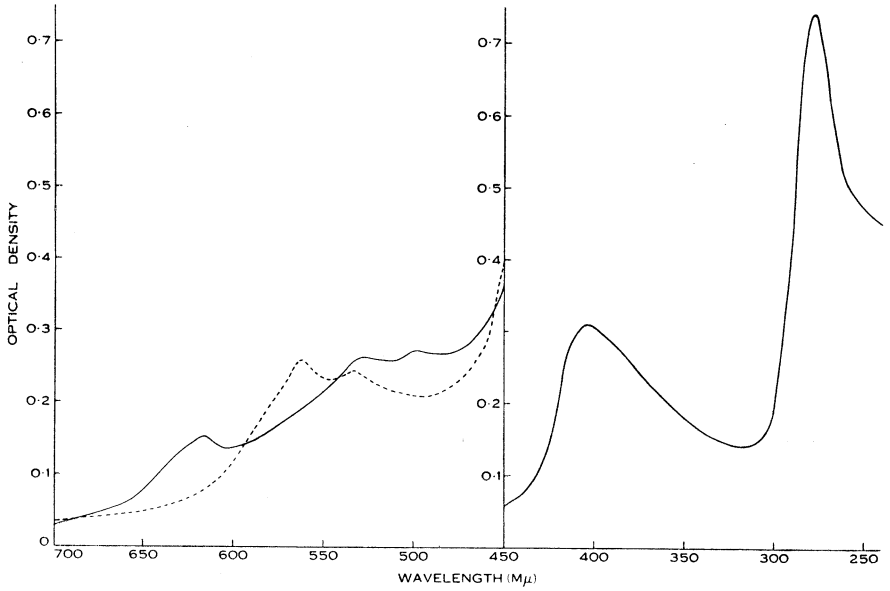

Fig. 1.—Absorption curves of *B. microplus* haemoprotein in phosphate buffer, pH 7·8, before (————) and after (– – – –) addition of sodium dithionite. Readings of absorption at wavelengths below 450 mμ were made on a 1 in 8 dilution of the pigment extract.

(*c*) *The Fate of Ingested Host Iron*

In view of the occurrence of an iron-containing protein in the eggs of *B. microplus*, and since the diet of the female tick includes large amounts of iron in the form of bovine haemoglobin, it was considered of interest to trace the fate of ingested host iron in female ticks. Sections of ovipositing ticks were cut and histochemical tests for the presence of free iron (Pearse 1953) were applied, but none could be demonstrated. Therefore, iron estimations on tick eggs and on engorged female ticks were made by the method of King (1948).

Forty fully engorged female ticks were collected from the same bovine host and were washed three times by shaking in distilled water. The iron content of 20 of these ticks was estimated immediately, and found to be 2·54 mg Fe. The remaining 20 ticks were maintained in an incubator at 30°C and 90 per cent. relative humidity until oviposition was completed 16 days later. The iron content of the

parent ticks was then found to be 2·40 mg Fe, while that of the 2·29 g of pooled eggs laid was 0·13 mg Fe. Thus, the total iron content of the eggs plus the parent ticks was 2·53 mg Fe, and 5·1 per cent. of the iron ingested by the adult female ticks had been transmitted to the eggs. Presumably the majority of this transmitted iron is incorporated in the haem of the egg pigment.

The site of the iron remaining in the tick after oviposition was then investigated. Two groups of 10 fully engorged ticks were collected as before, and both groups were allowed to complete their egg laying. The eggs were discarded, and the iron content of one group of ticks was estimated to be 1·24 mg Fe. Each of the

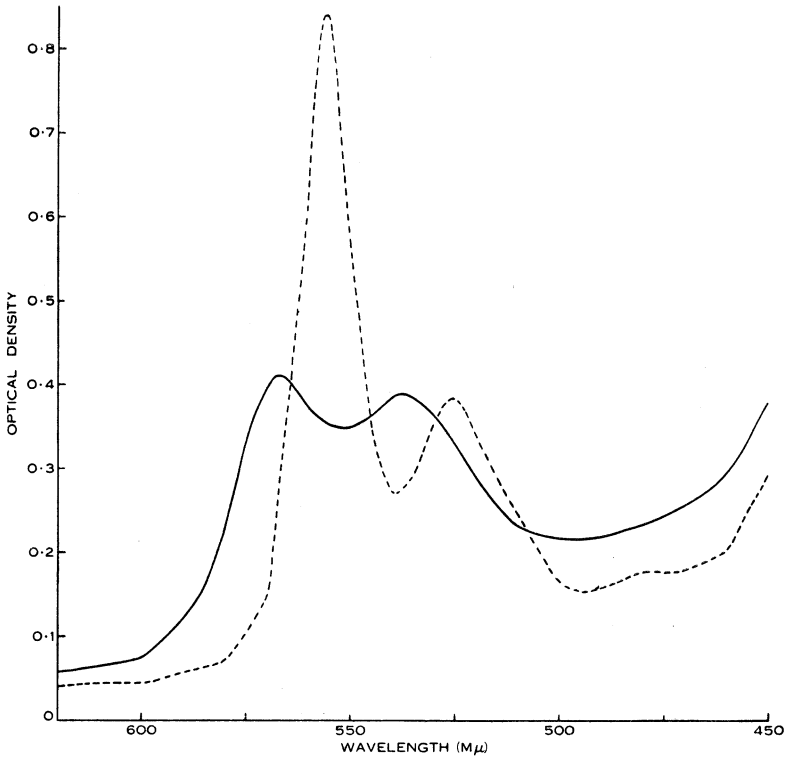

Fig. 2.—Absorption curves of *B. microplus* egg pigment haemochrome (– – – –) and the carboxy compound (————).

remaining 10 ticks was then injected subcutaneously with iron-free *n*-hexanol to expand the shrivelled cuticle and to harden the internal organs, thus allowing ease of dissection. The gut of each tick was then dissected out and the total iron content of the pooled guts was found to be 1·23 mg Fe.

Thus, it seems that the ingested iron which is not transmitted to the eggs is merely retained in the lumen of the gut, or in the gut-wall cells. This is in accord with the findings of Wigglesworth (1942) that in *I. ricinus* the contents of the gut and diverticula consist of a core of unaltered blood with a covering of granular black haematin, and the cells of the gut wall contain numerous granules of haematin.

(*d*) *Ultimate Fate of the Egg Pigment*

The high concentration of pigmented protein in the eggs of *Boophilus* suggests that the pigment may be a major food source for the developing larval tick. Microscopic examination of a newly hatched larva revealed that its sacculated gut was filled with reddish brown fluid. This fluid is undoubtedly entrapped yolk, around which the embryo has grown, and which has the function of sustaining the larva until it attaches to a host. As incubated larvae aged, increasing amounts of a black granular material were deposited around the walls of the larval gut. Extraction of incubated unfed larvae 4–5 weeks old with 0·9 per cent. sodium chloride solution yielded a pigmented extract which has absorption characteristics identical with that of solutions of the haemoprotein extracted from eggs. After extraction of the larval residues (two to three times) until no further pigment could be extracted by the salt solution, subsequent extraction with 0·05N sodium hydroxide solution readily dissolved the black granular material present in the larval residues, yielding a reddish purple supernatant. Passage of this alkaline extract through cellulose powder resulted in adsorption of a pink compound, and a greenish brown compound passed through unadsorbed. This brown solution displayed an absorption spectrum similar to that of alkaline haematin (King and Delory 1945), and readily formed a pyridine haemochrome with absorption maxima at 557 and 525 mμ after addition of sodium dithionite and pyridine. This suggests that the granular deposit in the larval gut contains a haematin-like substance.

The changes with time in the concentration of haemoprotein and haematin in eggs and larvae were followed in eggs laid by engorged ticks collected on the same day from an infested steer. These adults were incubated at 29°C and 90 per cent. relative humidity, and 1 week after the commencement of oviposition the pooled eggs were weighed into 1-g samples. One sample was extracted by grinding with 10 ml 0·9 per cent. sodium chloride solution and the resulting suspension was centrifuged at 9000 *g* for 10 min. The supernatant was removed and the residue stirred with a further 10 ml of salt solution and again centrifuged. This procedure was repeated with 5 ml of salt solution, and the supernatant after centrifugation then appeared colourless. The volume of the pooled supernatants was made up to 30 ml with salt solution, and this saline extract contained all the haemoprotein originally present in the 1-g sample of eggs.

The deposit remaining in the centrifuge tube was then extracted similarly with 0·05N sodium hydroxide solution. These supernatants were also pooled and made up to 30 ml, and the extract was filtered three times through Whatman No. 42 filter papers to remove by adsorption any of the unidentified pink compound present (it was not found in egg extracts, but appeared later in extracts of larvae). This alkaline extract contained all unbound haematin present in the original sample. An index of the amount of pigment present in each extract was obtained by reading the optical density at 530 mμ, a wavelength at which both *Boophilus* egg pigment and haematin absorb light strongly.

The remaining samples of eggs were returned to the incubator, and approximately on each succeeding week one sample was removed and extracted with saline

and alkaline solutions as before. The optical density of each extract was determined at 530 mμ.

Nineteen days after the commencement of oviposition larvae commenced to emerge from the eggs, so that extracts prepared 1 and 2 weeks after commencement of oviposition were of eggs, the third extract was of mixed eggs and larvae, and subsequent extracts were of larvae. Larvae began to die during the ninth week and no further extracts were prepared after this time. The amount of haemoprotein extracted from samples decreased rapidly during the first 4 weeks (Fig. 3), and was accompanied by a steady increase in the amount of haematin recovered. These

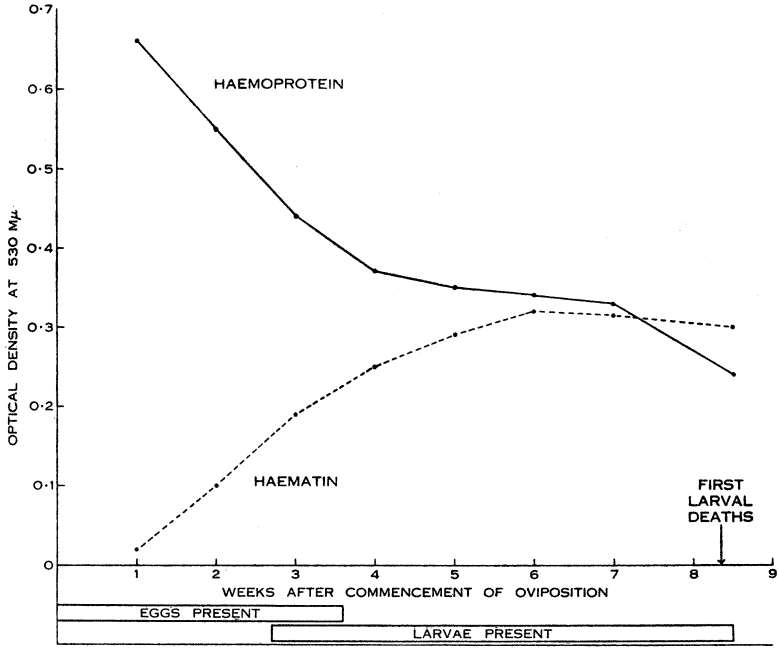

Fig. 3.—Age of sample plotted against the optical density of haemoprotein and haematin solutions extracted from standard samples of *B. microplus* eggs and larvae.

changes occurred during the period of development of the larvae within the eggs, and during hatching. From the fourth to the seventh week, when the larvae were relatively dormant, there was only a slight fall in the haemoprotein concentration of larval extracts, and a similarly slight increase in the concentration of haematin. The first larval deaths were observed on the 58th day of the experiment. The optical density at 530 mμ of the saline extract of larvae on the following day was 0·240, compared with 0·330 10 days earlier. This decrease in haemoprotein was accompanied by a very slight decrease in the corresponding haematin concentrations.

During the incubation of unfed larvae, deposits of ivory-coloured urinary excreta, predominantly guanine (Enigk and Grittner 1952), collected on the walls of the sample tubes. No pigment could be extracted from this solid excreta by sodium hydroxide solution.

When larvae 4–5 weeks old were mounted in 0·05N sodium hydroxide solution and examined microscopically, a reddish purple colour similar to that of the alkaline extracts was seen to develop in the gut caeca. This indicates that the unidentified pink compound and haematin present in alkaline extracts are in fact extracted from the gut contents or the gut wall. The association of the pink pigment with haematin suggests that it may be a breakdown product of haematin. Alkaline extracts of aged larvae prepared as before were passed through a chromatographic column of cellulose powder (length 4 cm, dia. 2 cm) in order to isolate this pigment.

Elution of the adsorbed pink material by 0·05N sodium hydroxide solution yielded a bright pink solution with absorption maxima at 541, 359, and 270 mμ. On addition of sodium dithionite the solution slowly decolorized, and no haemochrome was formed following the addition of pyridine to the reduced solution. During titration of a sample with 0·1N hydrochloric acid, the pink colour changed to violet below pH 7·2, the solution then exhibiting absorption maxima at 552 and 380 mμ, with a plateau in the curve between 340 and 360 mμ. This colour change was reversible.

No colour could be extracted from acid, neutral, or alkaline solutions of the pigment by ether, chloroform, or carbon tetrachloride, either before or after standing in boiling water for 1 hr. No colour change occurred in these solutions during heating. Neither acid nor alkaline solutions fluoresced in ultraviolet light.

On allowing an acid solution of the pigment to stand overnight, an amorphous blue-violet solid precipitated. Evaporation to dryness of an acid solution over a steam-bath also yielded an amorphous blue-violet solid. This was washed several times with distilled water, the washings were discarded, and the deposit was again dried. This solid proved insoluble in acetone, ether, chloroform, carbon tetrachloride, dioxane, methyl cyanide, and methanol, but readily dissolved in 0·05N sodium hydroxide solution to give a pink solution displaying the characteristics of the original eluate.

As the pigment was suspected of being a bile pigment, Van den Bergh's test and Gmelin's test (Lemberg and Legge 1949) were applied to neutral solutions. Both were negative. Similarly, an attempt to obtain a colour reaction by condensation with *p*-dimethylaminobenzaldehyde (Prunty 1945) yielded only a fine black precipitate.

III. Discussion

These findings suggest that the pigment responsible for the characteristic colour of the eggs of *B. microplus* is a conjugated protein with a water-insoluble globulin as its protein moiety, and protohaem as its pigmented prosthetic group. The rather flat appearance of the absorption curve of the pigment suggests that the iron atom in the complex is in the ferric state. A general property of ferriprotoporphyrin complexes is their less-marked absorption peaks compared with the corresponding ferroprotoporphyrin compounds (Lemberg and Legge 1949). The distinct peak in the red region of the spectrum also supports this assertion. The name "haemixodovin" is proposed for this pigment in order to indicate both the source of the protein

and the nature of its prosthetic group. It is quite probable that similar haemoproteins will be found in the eggs of other species of ixodid ticks.

It seems almost certain that the haem of haemixodovin is derived from the bovine haemoglobin ingested by the parent female tick. As no free iron could be demonstrated in any tick tissue it would seem that bovine haematin is not broken down in the gut of the tick to provide free iron for incorporation into the haemixodovin of eggs. Rather, it is probable that haem is split free from the ingested bovine haemoglobin and some proportion of it absorbed as such from the gut into the haemolymph, and transferred in combination with a protein (possibly as haemixodovin) to the ovaries where it is incorporated into the yolk of developing eggs. The results of iron analyses suggest that approximately 5 per cent. of the ingested bovine haem could be transferred into eggs as haemixodovin. Wigglesworth (1942) has drawn attention to the presence of a pigment in the haemolymph of the engorged sheep tick *I. ricinus* which exhibits absorption bands similar to those of ferrihaem-albumin, and also to those of *B. microplus* haemixodovin. Later work by Lees and Beament (1948) with *O. moubata* has shown that large haemoprotein molecules can readily pass from the haemolymph through the shell layer of ovarian eggs into the yolk.

It has not been possible to assign any function to the intact pigment. As the iron atom is in the ferric state it is unlikely that the pigment can act as an oxygen carrier, nor is any oxygen liberated from egg extracts by the addition of potassium ferricyanide. Wigglesworth (1942) investigated a haemoprotein occurring in the eggs of the blood-sucking hemipteran *Rhodnius prolixus*, but was unable to demonstrate any function for the pigment. He suggested that such pigments are merely functionless inclusions derived from the blood of the host. However, Lemberg and Legge (1949) have suggested that perhaps haematin compounds in the eggs of blood-sucking insects serve as reserve food for the construction of haematin enzymes before the larvae receive blood. In this connection, it is interesting to note that Riek (personal communication) found that larvae of *B. microplus* feeding on bovines do not ingest red cells, but appear to obtain their nutritional requirements from tissue fluids. Also, Frick (1936), quoted by Schulze (1955), suggested that the pigments of the cuticles of ixodids (reddish brown to very dark brown, more rarely green) are probably derivatives of host blood pigment.

However, extraction of aging eggs and larvae has shown that as the concentration of haemixodovin decreases with time, increasing amounts of a haematin-like compound can be recovered. Concomitantly, increasing amounts of a black pigment are deposited in the larval gut. Therefore, while a small proportion of the egg haematin may be used in the construction of larval haematin enzymes, or in cuticle pigmentation, circumstantial evidence indicates that, in the main, only the protein portion of the haemixodovin molecule is utilized by the larva as a reserve food, the discarded haematin being left as a deposit in the larval gut. None of this deposit is excreted as faeces while the larva remains unfed, but Enigk and Grittner (1952) have described the evacuation of reddish black faeces by ixodid larvae after they had attached to a host and commenced blood sucking. These authors explain this delayed evacuation of faeces on anatomical grounds.

The rapid decline in vitelline reserves of haemoprotein in developing eggs is in accord with the assumption that metabolic activity in the growing embryo would be proceeding rapidly, with consequent rapid utilization of reserves. After emergence of the larvae, followed by hardening of their cuticles for a few days, larval metabolism would probably be reduced to a minimum, the larvae then lying dormant. It is notable that during this resting period only minor changes occurred in the amounts of haemixodovin and haematin extracted from the larvae. The drop in concentration of haemoprotein recorded from larvae between the seventh week and the time that the final extracts were made is not readily explained. As there was an interval of 10 days between these last two extractions, the greatest fall may have occurred during the last day or two before the final extraction.

Hitchcock (1955) has found that, after the first deaths occur in a batch of incubated unfed *Boophilus* larvae, almost all the larvae in the batch die within a few days. Thus the majority of living larvae in the final sample extracted would be virtually moribund, and one would expect larval metabolism to have altered from the optimum. If there was an increased utilization of haemoprotein after the seventh week due perhaps to exhaustion of other vitelline reserves, increased larval haematin deposits would have been expected. No such increase occurred. Possible effects on larval metabolism by the rickettsial-like organisms reported from *B. microplus* larvae by Pierce and Pierce (1956), and seen by the author in the larvae he extracted, also should be borne in mind.

In view of the presence of an unidentified pink compound in alkaline extracts of old larvae, it is possible that haematin is not the sole end-product of haemixodovin breakdown. Figure 3 indicates that there was a slight fall in larval haematin between the sixth and ninth weeks of incubation, and as no haematin was recovered from larval excreta, it may well be that some haematin is catabolized to form the pink unknown. Haemoprotein catabolism in mammals leads to the formation of bile pigments or porphyrins, but the pigment did not display the characteristic four-banded spectrum of porphyrins and showed none of the solubility or reactions characteristic of any of the known bile pigments (Lemberg and Legge 1949). However, this does not preclude the possibility of the unknown being a tetrapyrrole or dipyrrole pigment of a type hitherto undescribed. Whatever the nature of this pigment, the fact remains that at the time of death large deposits of haematin are present in the larval gut, and it would seem that haematin is the main end-point of haemixodovin catabolism in these larvae.

One further set of observations remains to be considered. Hunt and Collins (1896) cite Wynne's observations that *B. microplus* collected from cattle with severe anaemia due to heavy tick infestation have a translucent appearance due to the lack of haemoglobin in their diet. Hitchcock (unpublished data) showed that translucent ticks laid fewer and lighter-coloured eggs than did ticks collected from healthy cattle. Furthermore, fewer larvae hatched from eggs of translucent ticks than from "normal" eggs. Although this lowered fertility of translucent ticks may be due to factors other than a reduced haemoglobin intake, the possibility remains that the presence of a minimum level of protein-bound haematin may be essential for the normal development of the eggs of *B. microplus*.

IV. Acknowledgments

The author is indebted to Dr. J. E. Falk, Division of Plant Industry, C.S.I.R.O., and Mr. J. E. O'Hagan, Red Cross Blood Transfusion Service, for their advice and assistance in the identification of haematin compounds. Mr. R. F. Riek, Division of Animal Health and Production, C.S.I.R.O., kindly advised on the preparation of the manuscript. The histochemical tests for free iron in sections of ticks were carried out by Mr. J. R. Saal.

V. References

Dresel, E. I. B., and Falk, J. E. (1956).—*Biochem. J.* **63**: 72.

Eigenberger, E. (1931).—*J. prakt. Chem.* **130**: 75.

Enigk, K., and Grittner, I. (1952).—*Z. Tropenmed. u. Parasit.* **4**: 77.

Fairley, N. H. (1941).—*Quart. J. Med.* **10**: 115.

Frick, G. (1936).—*Z. Morph. Ökol. Tiere* **31**: 142.

Hitchcock, L. F. (1955).—*Aust. J. Zool.* **3**: 295.

Hunt, J. S., and Collins, W. (1896).—"Report on Tick Fever." pp. 40–2. (Govt. Printer: Brisbane.)

King, E. J. (1948).—*Lancet* **1**: 478.

King, E. J., and Delory, G. E. (1945).—*Biochem. J.* **39**: 111.

Kunkel, H., and Slater, R. (1952).—*Proc. Soc. Exp. Biol., N.Y.* **80**: 42.

Kunkel, H., and Tiselius, A. (1951).—*J. Gen. Physiol.* **35**: 89.

Lees, A. D., and Beament, J. W. L. (1948).—*Quart. J. Micr. Sci.* **89**: 291.

Lemberg, R., and Legge, J. W. (1949).—"Haematin Compounds and Bile Pigments." (Interscience Publishers Inc.: New York.)

Mitchell, P. H. (1946).—"A Textbook of Biochemistry." (McGraw Hill Book Co. Inc.: New York.)

Nicholas, R. E. H. (1951).—*Biochem. J.* **48**: 309.

Pearse, A. G. E. (1953).—"Histochemistry." (Churchill & Co.: London.)

Pierce, A. E., and Pierce, M. H. (1956).—*Aust. Vet. J.* **32**: 144.

Prunty, F. I. G. (1945).—*Biochem. J.* **39**: 446.

Riek, R. F. (1959).—*Aust. J. Agric. Res.* **10**: 604.

Schulze, P. (1955).—*Z. Parasitenk.* **17**: 217.

Stern, A., and Wenderlein, H. (1934).—*Z. physik. Chem.* A **170**: 345.

Warburg, O., and Negelein, E. (1932).—*Biochem. Z.* **244**: 9.

Wigglesworth, V. B. (1942).—*Proc. Roy. Soc.* B **131**: 313.