Eur. J. Entomol. **100**: 19–23, 2003
ISSN 1210-5759

Thin layer chromatographic analysis of pteridine-like pigments in the migratory locust, *Locusta migratoria migratorioides* (Orthoptera: Oedipodidae)

VÁCLAV NĚMEC[1*], MICHAEL BREUER[2] and ARNOLD DE LOOF[2]

[1]Institute of Entomology, Czech Academy of Sciences, Branišovská 31, České Budějovice, 370 05 Czech Republic
[2]Institute of Zoology, Catholic University of Leuven, B 3000 Naamsestraat 59, Leuven, Belgium

Key words. Pterines, phase-transition, juvenile hormone, precocene, cuticle, methoprene, neem, insect endocrinology

Abstract. Acidic methanolic extracts of haemolymph, cuticle and eyes of larvae and adults of the migratory locust, *Locusta migratoria migratorioides* (R. et L.), were subjected to thin layer chromatography. The Rf-values as well as the fluorescent colour of the spots under ultraviolet illumination at 350 nm, were compared to those of 12 pteridine standards. Most of the standards showed a major spot, as well as up to 3 additional minor spots, probably due to the presence of isomers and degradation products. The extract of the haemolymph of untreated larvae and adults yielded 6 different spots. Cuticle and eyes had their own specific pattern. Application of methoprene (analogue of juvenile hormone) induced solitary phase-like pigmentation of the cuticle and green-coloured haemolyph. The content of pterin, biopterin, leucopterin, isoxanthopterin, and probably also of lumazin, increased. The melanization of the cuticle nearly completely disappeared. Similar, but weak effects were observed in neem-oil treated locusts. Application of precocene caused a number of changes in the pigment pattern of all extracts. It also induced some melanization of the cuticle in particular in larvae. Many pigment spots were observed which did not correspond to any of the 12 standard pteridines. The classical concept about the pigment composition of locust haemolymph, namely that it is restricted to melanin, carotenes and biliverdin, needs modification.

INTRODUCTION

Pteridines or pterins (Ferre et al., 1991) represent one of the families of pigmentary colours of insect cuticle, but some of them are also important eye pigments (Chapman, 1969). They produce a variety of colours, ranging from white (leucopterin), or red (erythropterin) over yellow (xanthopterin) to fluorescent blue under ultraviolet light (biopterin). In addition, pteridines are important metabolically as co-factors of enzymes associated with growth and differentiation, and they may act as controlling agents in these processes (Chapman, 1969).

The biosynthetic pathway of pteridines starting from the purine guanosine–5-triphosphate has been well studied not only in insects, but also in species belonging to the majority of phyla of the animal kingdom (Ziegler & Harmsen, 1969). In insects, pteridines have been identified in some Holometabola (Fuzeau-Braesch, 1972), as well as in Hemimetabola. In the latter, in particular *Oncopeltus fasciatus* (Forrest et al., 1966), *Pyrrhocoris apterus* (Socha & Němec, 1992), *Rhodnius prolixus* (Viscontini & Schmid, 1963), and *Dysdercus cardinalis*, *D. intermedius* and *D. nigrifasciatus* (Melber & Schmidt, 1992, 1994, 1997) were intensively studied.

In locusts the presence of various other pigments such as mesobiliverdin, ß-carotene, astaxanthin and insecticyanine, has been frequently described (Goodwin & Srisukh, 1948; Fuzeau-Braesch, 1985; Kayser, 1985), but data on the occurrence of pteridines in locusts are very limited. To our knowledge, there are only reports from xanthopterin, isoxanthopterin and sepiapterin in the eyes, the integument or entire body of *Schistocerca gregaria* and *Locusta migratoria* (Busnel & Drilhon, 1942; Goodwin & Srisukh, 1951a; Bouthier, 1966; Harmsen, 1966; review by Ziegler & Harmsen, 1969) besides some findings in locust eggs (De Lerma 1951, 1952; Goodwin & Srisukh, 1951a, b). From the physiological point of view, locusts represent an interesting model because of the differences in colour between solitary and gregarious animals. Hence, the study of phase-related pigmentation will contribute to elucidating the nature of the hormonal factors controlling phase transition.

In this paper, we report on the pteridine pattern of *Locusta* as well as on how it is affected by some biologically active substances like juvenoids, precocene, azadirachtine and metyrapone.

MATERIALS AND METHODS

Experimental animals

Locusta migratoria migratorioides (R. et L.) was reared at a constant temperature of 30°C under a 13/11 light/dark photoregime. The insects were fed on grass and oat flakes.

Chemicals

Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone from Fluka) was dissolved in redestilled water and applied to 4th instar larvae by injection of doses of 4 and 40 µg/animal. Precocene II (Sigma), methoprene (Zoecon) and neem oil (gift of Prof. S. Chakraworthy, India) were dissolved in acetone and topically applied in the same doses to 3rd and 4th instar hoppers (precocene and neem oil) or to 5th instar larvae (methoprene). The experimental animals were sampled approximately 8–11 days after treatment. Acetone-treated and water-

* Corresponding author. E-mail: nemecv@entu.cas.cz

TABLE 1. Rf-values and colours of fluorescent spots of pteridine standards, arranged according to increasing Rf-values. Spot 1 corresponds to the bulk of material present in the different standards. Spots 2, 3, and 4 probably represent isomers and degradation products. In the case of mixtures an asterisk indicates the colour of the major component.

	Spot 1		Spot 2		Spot 3		Spot 4	
	Rf	Colour	Rf	Colour	Rf	Colour	Rf	Colour
Sepiaptenine	0.12	B	0.47	I B*	0.56	W	0.67	I B
Biopterine	0.20	I B*	0.35	I B	0.82	I B		
Monapterine	0.22	B*	0.61	Y				
Leucopterine	0.23	B*	0.61	W				
Xanthopterine	0.23	Y	0.35	Y	0.39	Y*	0.79	Y
D.-neopterine	0.25	B*	0.32	B				
L-neopterine	0.26	B*						
Isoxanthopterine	0.32	Y*	0.56	V				
Pterine	0.32	I B	0.44	V	0.50	I B*	0.64	W
Lumazine	0.32	I B*	0.41	V	0.51	W	0.64	W
6-biopterine	0.34	I B	0.43	I B	0.54	I B*		
L-biopterine	0.43	I B*						

Abbreviations: I B – light blue; B – blue; Y – yellow; V – violet; W – white.
Visible yellow spot yielded sepiapterin and very faintly also xanthopterine with isoxanthopterine. The other pteridines were visible only under UV light.

injected hoppers and imagoes served as controls. The changes in pigmentation induced by the above chemicals appeared after the moult that followed the treatment of locust larvae. The sampling was performed within two days after the ecdysis of treated larvae.

Extraction of pteridines

Samples of haemolymph from five locusts, eyes (five pairs), or from 3 pronotums (samples of cuticle) were homogenized by means of a glass pestle in a mixture of methanol: acetic acid: water (4:1:5) and left overnight in darkness at room temperature. The homogenates were then centrifuged at 3000 rpm. for 10 min. The resulting supernatants were dried in a Speed-Vac concentrator and stored at –20°C until further analysis.

TABLE 2. Pteridines in haemolymph, cuticle, and eyes of untreated control locust larvae and adults.

Rf	Haem. larva	Haem. adult	Cuticle larva	Cuticle adult	Eye larva	Eye adult
0.08	-	–	Y	Y	–	
0.10	–	–	–	–	Y	Y
0.20	Y	Y	–	–	–	–
0.23	–	–	–	–	W	W
0.29	–	–	–	–	Y	Y
0.31	Y	Y	–	–	–	–
0.35	–	–	–	–	Y	–
0.36	W	W	–	–	–	–
0.41	–	–	–	–	B	B
0.41	O	O	–	–	–	–
0.43	–	–	B	B	–	–
0.46	I B	I B	–	–	–	–
0.50	–	–	Y	Y	–	–
0.53	–	V	–	–		–
0.54	–	–	–	–	–	V
0.55	–	–	B	B	–	–
0.61	–	–	W	W	–	–
0.67	–	–	–	–	Y	Y
0.70	Y	Y	–	–	–	–

Chromatographic analysis

The frozen extracts were redissolved in the extraction solvent mixture and applied to pre-washed TLC Silica-Gel G plates (Merck). The plates were developed twice with the solvent mixture n-butanol: 5N acetic acid (4:1) in darkness and at room temperature. Pteridines were tentatively identified according to their Rf-values and fluorescent colours that they display under UV-illumination at 350 nm, as compared with 12 standards which are listed, along with their Rf-values in Table 1. The spots were recorded on transparent sheets. The Rf-values were obtained by direct measurement. Most of the pteridine standards were a kind gift from Dr. L. Streinz (Czech Academy of Sciences, Prague) or were obtained from Aldrich.

RESULTS

Purity of the standards

Only two of the pteridine standards proved to yield only a single spot on the TLC plates, namely D-neopterin and L-biopterin. All others were less chemically homogeneous and yielded, in addition to a bulk component (designated as spot 1), 2 or 3 more compounds. Their nature is not known. However, it is likely that they represent either isomers or metabolites of the major component. This evidently complicates the interpretation of the results of the treatments. On the other hand, when an extract contained a violet coloured spot, it was clear that this could not be due to the presence of the main components of any of the 12 standards, because none of them displayed this colour.

Pigments present in control

The three control groups were compared: Namely a) untreated locusts, b) acetone-treated and c) water-injected locusts. Differences in pteridine patterns were found only in the hemolymph samples (Tables 2a, 3a, b.). The Rf of a yellow spot in an eye extract of hoppers corresponded with that of xanthopterin.

TABLE 3. Rf-values and colours of fluorescent spots in extracts of haemolymph of larvae and adults of water-injected control locusts, of metyrapone-injected animals, and of larvae and adults topically treated with neem oil.

Rf	Haem. larva control injected	Haem. adult control injected	Haem. larva metyra-pone injected	Haem. adult metyra-pone injected	Haem. larva neem-oil top. applied	Haem. adult neem-oil top. applied
0.13	-	–	–	–	Y	–
0.20	–	–	–	–	Y	–
0.27	–	–	–	–	–	I B
0.27	–	–	Y	Y	–	–
0.28	Y	Y	–	–	–	–
0.29	–	–	–	–	Y	–
0.32	–	–	–	–	–	I B
0.36	Y	Y	–	–	–	–
0.39	–	–	Y	Y	Y	Y
0.40	Y	Y	–	–	–	–
0.44	–	–	W	W	–	–
0.45	W	W	–	–	W	W
0.49	–	–	Y	Y	–	–
0.50	–	–	–	–	Y	Y
0.51	O	O	–	–	–	–
0.54	–	–	I B	I B	I B	–
0.55	I B	I B	–	–	–	–
0.63	–	–	–	–	I B	–
0.64	I B	I B	I B	I B	–	–
0.77	Y	Y	Y	Y	Y	–
0.78	Y	Y	–	–	–	–

Abbreviations: I B – light blue; B – blue; O – orange; Y – yellow; V – violet; W – white.

TABLE 4. Rf-values and colours of fluorescent spots in extracts of haemolymph, cuticle and eyes of larvae and adults of methoprene-treated locusts with solitary pigmentation, minute melanin scale and green haemolymph.

Rf	Haem. larva methopr-treated	Haem. adult methopr-treated	Cutic. larva methopr-treated	Cutic. adult methopr-treated	Eye larva ethopr-treated	Eye adult ethopr-treated
0.10	–	–	W	W	–	–
0.12	–	–	–	–	Y	Y
0.13	W	W	–	–	–	–
0.22	Y	Y	–	–	–	–
0.30	Y	–	–	–	–	–
0.31	–	–	Y	Y	–	–
0.32	W	W	–	–	W	W
0.37	W	W	–	–	–	–
0.39	–	–	–	–	V	–
0.42	–	–	–	–	–	B
0.42	–	Y	Y	Y	–	–
0.44	–	–	–	–	W	–
0.46	–	–	–	–	–	Y
0.47	–	–	Y	–	–	–
0.48	I B	I B	–	–	–	–
0.51	–	–	Y	Y	–	–
0.52	–	–	–	–	–	W
0.53	Y	–	–	–	–	Y
0.57	–	–	B	B	–	–
0.57	–	–	–	–	I B	I B
0.57	Y	Y	–	–	–	–
0.71	O	-	O	O	–	-

Abbreviations: I B – light blue; B – blue; O – orange; Y – yellow; V – violet; W – white.

Treatment with metyrapone

This compound, 2-methyl-1,2-di-3-pyridyl-1-propanone, is used in both vertebrate and invertebrate endocrinology as a blocker of the activity of steroid monooxygenase. In insects, this hydroxylating enzyme plays a key role in the biosynthesis of ecdysteroids, in particular of 20-OH-ecdysone.

Metyrapon- and neem oil-treatments did not induce any externally visible change in the pigmentation of the eyes and cuticle. Hence only extracts of haemolymph were prepared. As is seen in Table 3, the pigment pattern of the haemolymph extract of metyrapone-treated locusts was very similar to that of the injected controls. Only the yellow fluorescent spot with an Rf of 0.36 was absent.

Treatment with Neem oil

The major active substance of neem oil is azadirachtin. This compound affects many different functions in the body, with uncertainty about its exact mode of action as a result. A major effect is inhibition of ecdysone biosynthesis.

As was the case with the metyrapone treatment, there was no difference in pigmentation of the cuticle and the eye between experimental and control animals. Only the haemolymph was analysed. Not less than 6 spots were visible in the haemolymph of larvae (Table 3), while 2 others were restricted to adults. The experimental animals lacked the yellow fluorescent pigment with an Rf 0.36 that was present in the controls. However, the Neem oil - treated animals had more pigments than the controls. The light blue fluorescent pteridine with an Rf 0.54 in larvae (Table 3) corresponded to lumazine or biopterin standards (Table 1).

Methoprene treatment

This compound mimics the effects of juvenile hormone. This hormone shifts the colour of locusts towards that which is usual for the solitary phase. The juvenilized larvae and imagoes both have a light-green pigmented cuticle and hemolymph. As this was also the case in other treatments, some pigments could not be identified by comparing their Rf and fluorescent colour with the standards (see Table 1). The pigment pattern of larval haemolymph, and to a lesser extent of the cuticle as well, is more complex than that of adults (Table 4). The pigment pattern of the haemolymph differed from the control in three spots, namely in the white-fluorescent one with an Rf 0.13, and in the yellow-fluorescent ones with Rf 0.53 and 0.57, respectively. In the cuticle, 3 pigments were identical with the controls, 3 are specific for the juvenilized locusts, and one was absent. The pattern from eyes contained 8 pteridines. Only two were identical with

TABLE 5. Rf-values and colours of fluorescent spots in extracts of haemolymph, cuticle and eyes of larvae and adults of locusts treated with precocene II.

Rf	Haem. larva precocene -treated	Haem. adult precocene -treated	Cutic. larva precocene -treated	Cutic. adult precocene -treated	Eye larva precocene -treated	Eye adult precocene-treated
0.13	–	–	W	W	W	–
0.23	–	–	W	–	–	–
0.24	–	–	–	–	W	–
0.25	Y	–	–	–	–	–
0.30	–	–	–	–	O	–
0.32	–	W	W	W	W	W
0.39	–	–	–	–	–	B
0.40	–	–	B	–	–	–
0.41	Y	–	–	–	–	–
0.44	W	W	W	W	W	–
0.48	–	–	–	–	–	Y
0.49	Y	Y	Y	Y	–	–
0.50	–	–	–	–	B	–
0.56	–	–	V	V	V	V
0.62	–	–	–	–	–	V
0.63	V	V	–	–	–	–
0.65	Y	Y	–	–	Y	–
0.91	–	–	–	–	–	I B
0.94	–	I B	–	–	–	I B

Abbreviations: I B – light blue; B – blue; O – orange; Y – yellow; V – violet; W – white.

those in the control, 3 were the same in larvae and adults, and 3 were specific for adults.

Precocene treatment

This compound destroys the corpora allata, but because of its high degree of general toxicity, some side effects cannot be excluded. The precocene-treated animals displayed some slight morphological deformations that resembled some effects of juvenoids, in spite of the fact that they are thought to be anti-JH compounds. Although precocene treatment did not cause clear changes in pigmentation of the cuticle, five pigment spots differed from the control. In the haemolymph extract, two spots were different as compared to the controls. Precocene treatment strongly affected the pigment pattern of the eyes, with marked differences between larvae and adults (Table 5). Six spots were seen in larvae only, 5 were specific for the adults, and two occurred in both larvae and adults. The pigment pattern of the eyes was the richest of all samples studied.

DISCUSSION

The major result from this preliminary study is that the pigment pattern of locusts, in particular with respect to pteridine-like molecules, is much more complex than has been reported in the literature. The commonly accepted idea is that the major pigments of locusts are melanin (black), carotene (yellow), and biliverdin (green). Mahmat et al. (1997) stated that the haemolymph of solitary *Schistocerca gregaria* contains only carotenes and biliverdin. They determined these substances only by measurement of absorbance at 460 nm (maximum absorbancy of carotenoids) and at 600 nm (maximum absorbancy of biliverdin). They did not do any other analyses.

When we sampled the organs from locusts in the same way as we did previously with the fire bug, *Pyrrhocoris apterus* (Němec & Socha, 1988; Socha & Němec, 1992) and compared the patterns with standards (see Table 1), we found in the body of locust quite a number of extractable pteridine-like pigments, as shown in the Results section.

It is remarkable indeed that with exception of a few authors the occurrence of pteridines in locusts has never been reported. Pteridines were mentioned for the first time by Hopkins in 1895. These pyrazino-pyrimidine derivatives were named "pteridines" by Wieland in 1925 according to their occurrence in nature: e.g., in butterfly wings (Pfleiderer, 1992). Pteridines also occur in fish and amphibians (Ziegler & Harmsen, 1969). Pteridines play several important physiological roles:

1. They are co-factors for hydroxylation of phenylalanine to 3,4-dihydroxyphenylalanine (DOPA), which is the precursor of melanin, a major cuticular pigment in locusts. They are also cofactors of the enzymes involved in ommochrome synthesis. In *Drosophila*, five pterins have been isolated from the eye (Chapman, 1969).
2. The auto-chelating potency of simple pteridines enable the formation, under physiological conditions, of products of very low solubility. These products are a part of the general nitrogen pool and are a means of inactivation and deposition of toxic products of nitrogen metabolism. They can be deposited in the cuticle and play a role as well in signalling (Harmsen, 1966; Melber & Schmidt, 1997).
3. The redox potential of some pteridines indicates that they play a role in cellular electron transport. (Rembold, 1975).

Changes in locust pigmentation due to the changes in hormonal milieu has already been described decades ago (for a literature survey, see Fuzeau-Braesch 1972, 1985; Kruse-Pedersen, 1978; Pener, 1991). Nobody mentioned a role for pteridines in locust pigmentation.

Very recently, Wybrandt & Anderson (2001) isolated and sequenced a yellow protein from the cuticle of sexually mature gregarious male adult *Schistocerca*. The yellow pigment is absent in females and in solitary males as well. Goodwin and Srisukh (1948) thought that this yellow pigment was beta-carotene. Our results suggest that it might be worthwhile to investigate whether the yellow protein is indeed a carrier of carotene only or, perhaps, of some other yellow pigment as well.

Application of the JH-analogue methoprene not only suppressed the synthesis of melanin, but it also increased the concentrations of xanthopterin and particularly of leucopterin as compared to the control (compare Table 2 with Table 4). This is in agreement with the observation of Isaka (1952), who found that transformation of xanthopterin into leucopterin is coupled to the transformation

of DOPA into phenylalanine-quinone, which prevents the polymerization of cyclic compounds, capable of forming melanin.

The large number of spots with Rf-values and colour that do not match those of the pteridine standards illustrates that it might be worthwhile to reopen this chapter in insect physiology. It may perhaps shed new light on the still incompletely solved problem of the primary physiological causes of phase transition in locusts.

The identification of pteridine-like pigments in *Locusta* will require more sophisticated methods, namely HPLC coupled with mass spectrometry, which we hope to utilize in the near future.

ACKNOWLEDGEMENTS. This project was supported by GOA-Research project No. 2000/04 from the research council of the Katholieke Universiteit Leuven.

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Received January 15, 2002; revised June 4, 2002; accepted August 29, 2002