Proc. Indian Acad. Sci. (Anim. Sci.), Vol. 93, No. 5, September 1984, pp. 455–461.

Haemolymph of female *Oxya hyla hyla* Serville (Orthoptera: Acrididae): A preliminary study

D GHOSH*, B GHOSH, R SAHA and S G PAL

Department of Zoology, University of Calcutta, 35 Ballygunge Circular Road, Calcutta 700 019, India

*Department of Life Science, Calcutta University, Post Graduate Centre, Agartala, Tripura 799 004, India

MS received 13 June 1983; revised 10 May 1984

Abstract. The preliminary light microscopic and biochemical study of the haemolymph of *Oxya hyla hyla* records the presence of five different types of haemocytes and gives a quantiative estimation of plasma proteins along with their gel electrophoretic band patterns. The higher number of total haemocyte count in adult female compared to that of immature one may be attributed to some kind of synthesis and transport of proteins or other yolk materials during vitellogenesis. High plasma protein concentration during monsoon may be associated with the reproductive maturity of these insects. The gel electrophoretic pattern of plasma proteins reveals the constant presence of two particular bands suggesting the occurrence of lipoprotein and juvenile hormone carrier protein.

Keywords. Haemolymph; haemocytes; plasma protein; vitellogenesis; total haemocyte count; gel electrophoresis; lipoprotein; juvenile hormone carrier protein.

1. Introduction

In insects the haemolymph comprising plasma and haemocytes have been characterised according to their structural, functional and developmental behaviours (Wigglesworth 1959; Kulkarni and Mehrotra 1970; Wigglesworth 1973; Wyatt and Pan 1978). The widely different role of plasma and haemocytes in insect metabolism is also well accepted. The plasma components range from salts, amino acids, proteins, lipids, carbohydrates, enzymes etc., (Wigglesworth 1973; Richards and Davies 1977). Wigglesworth (1959) summarized most of the earlier classifications of the insect haemocytes. Gupta (1979) put forward a classification and phylogenetic scheme of insect haemocytes based primarily on their morphology, function, stainability at the light microscopic and ultrastructural levels. In developing suitable light microscopic preparation of the insect haemocytes disodium EDTA (ethylene-diamine tetraacetic acid) has often been used along with the classical fixatives as anticoagulant (Shapiro 1979). But proper attention has not been paid to understand as to what extent EDTA itself effects the cellular morphology and nucleo-cytoplasmic stainability.

The plasma proteins of various insects have been characterized at the molecular level and categorized as glycoprotein, lipoprotein etc., (Wyatt and Pan 1978). Also the plasma proteins show both quantitative and qualitative variations depending on the age, nutritional state and/or particular metabolic state or condition of the insect body. In *Schistocerca gregaria* (Kulkarni and Mehrotra 1970) the total number of plasma

proteins described are twelve. Recent analysis by polyacrylamide gel electrophoresis has resolved 10–30 plasma proteins in an insect (Wyatt and Pan 1978). In female *Locusta migratoria* the juvenile hormone carrying lipoprotein has been identified (Emmeric and Hartmann 1973) and it is established that the concentration of the female protein (vitellogenin) increases just before oogenesis (Chen *et al* 1976). The haemolymph of acridid insects is being studied extensively to understand the role of haemolymph in molting process, flight, utilization in different synthetic processes, immune response etc. The present paper is concerned only with the characterization of different plasma protein components and haemocytes of female *Oxya hyla hyla*, one of the most abundant grasshoppers of Tripura.

2. Materials and methods

Adult female grasshoppers were collected during monsoon months from the vicinity of Agartala, and reared in the laboratory. The female insects were dissected and the haemolymph collected using a capillary tube to study the plasma protein and the haemocytes. To study the plasma protein content, the haemolymph in phosphate buffer (0·1 M, pH 8·5) was centrifuged. The polyacrilamide gel electrophoresis was employed for separating the proteins (Davis 1964). Total count of haemocytes was taken and was calculated as follows:

$$\text{cell number} = \frac{\text{number of cells counted} \times \text{dilution} \times 4000}{\text{number of small squares counted}}.$$

For *in vitro* studies hanging drop method in saline EDTA was followed (Gupta 1979). For light microscopic studies cells fixed by 1% formaldehyde with or without EDTA, Carnoy's fluid (6:3:1) or buffered glutaraldehyde, were stained with Haematoxylin-eosin or Giemsa or Leishman's stain.

3. Observation

In the adult vitellogenic females of *Oxya hyla hyla* having eggs in different stages of maturity, the amount of total plasma proteins was about $9{\cdot}6 \pm 0{\cdot}3$ g/100 ml of haemolymph. Although the total protein content was similar in all the insects studied, a variability in the relative abundance of different protein species in gel electrophoretic pattern was apparent. A series of experiments with different degrees of freedom regarding acrylamide gel concentration, buffer strength current intensity and quantity of haemolymph revealed that the degree of separation was highest when the gel concentration was 7·5%. The maximum number of resolvable protein fractions was 10, according to their R_m values. The relative electrophoretic mobility (R_m) values of the separated protein bands were calculated as follows:

$$R_m = \left(\frac{\text{distance travelled by protein band from origin}}{\text{distance travelled by tracking dye from origin}}\right) 100$$

In 10% gel concentration the degree of separation was not sufficient enough and only three different zones were visible *viz* upper, zone I middle, zone II and the lower, zone III. Zone III was yellowish in colour. Again, when 5% gel concentration was used

the degree of separation was higher but often a diffused picture was got. However, the yellowish band was a constant feature having an R_m value of about 100. The use of 7.5% gel concentration revealed a better picture although minor variability remained regarding the exact R_m values of the discrete protein bands (figure 1).

The total haemocyte count (THC) in adult female of *Oxya hyla hyla* was 80200 ± 105·8, although variability persisted in relation to the length of the insects studied. Cells undergoing mitosis were also observed and the frequency was about 0·2%.

3.1 *Cell types*

Extensive light microscopical observations using different fixatives and staining procedures led us to recognize five different cell types—prohaemocytes, plasmatocytes, granulocytes, sphaerulocytes and coagulocytes.

3.2 *Prohaemocyte*

The prohaemocytes are small, round cells (8–16 μ, figure 2). The nucleus is large (8–12 μ), compact and occupies most of the cells volume; cytoplasm is very scanty and no granules are apparently discernable. The nucleus always stains more intensely than cytoplasm. The cell membrane appears to be smooth in both living and stained preparations (figure 5).

3.3 *Plasmatocyte*

This cell shows wide variations in shape, size and stainability. At least five morphologically different variant cells are encountered (figure 2). The cells (15–30 μ) are round, spindle-shaped vermiform, triangular or rectangular (figures 5 and 6) in outline. In hanging drop preparations, the large polymorphic cells showed pseudopodial out-

Figure 1. Schematic diagram of the protein bands obtained after polyacrylamide gel electrophoresis of vitellogenic female plasma of *Oxya hyla hyla*.

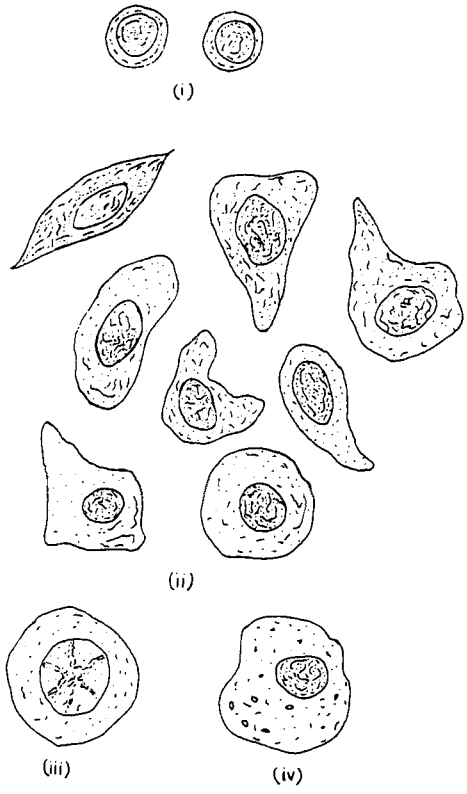
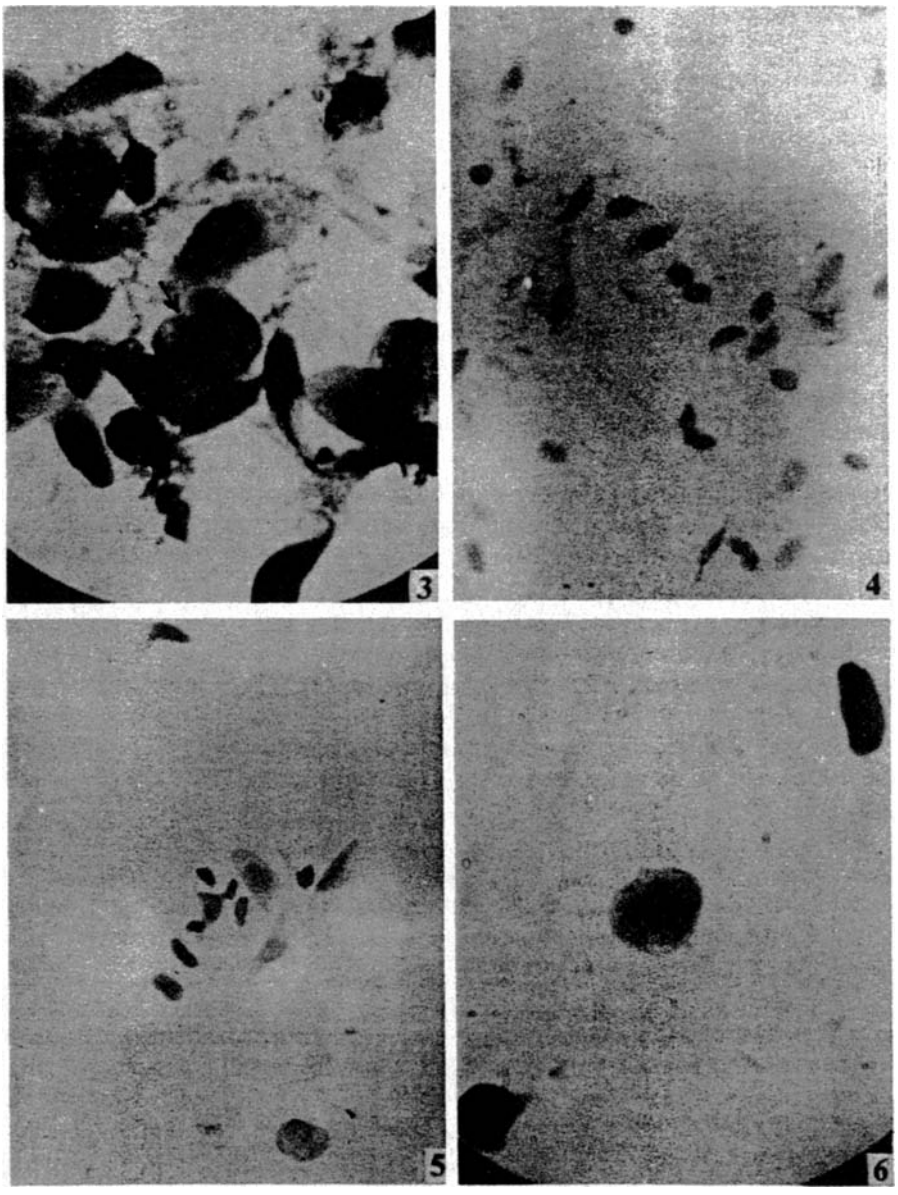


Figure 2. Semidiagrammatic representation of the different cell types of female *Oxya hyla hyla*. (i) prohaemocytes (ii) plasmatocyte of different types (iii) coagulocyte and (iv) granulocyte.

growths which are often large and threadlike (figure 5). The length and breadth of the spindle-shaped cells (figure 4) varies from 7–12 μ and 4–6 μ. In vermiform cells, the cell length may be much longer. In all cases the nucleus is slightly eccentric in position. In spindle-shaped or vermiform cells the nucleus is compact, elliptical and occupies the central position. But in all the other cell types, the nucleus is less compact and the size varies from 7–18 μ. The cytoplasm shows a fine granular consistency. No deeply stained granules are observed.

3.4 *Granulocyte*

The granulocytes are round oval cells (19 ± 3 μ dia.), larger than the prohaemocytes (figure 2). The nucleus ($15 + 2$ μ dia.) is centrally placed, compact and round or slight elongated in outline. These cells do not show pseudopodial outgrowths in hanging drop preparation. The cytoplasm is packed with large eosinophilic granules. The size of the granules is not fixed and the degree of eosinophilia varies (figure 3).



Figures 3–6. (Giemsa preparation × 900). **3.** Different cell types present in a cluster. Prohaemocytes, plasmatocytes are visible. The arrow indicate the granulocyte along with granules. **4.** Prohaemocyte (arrow) and different types of plasmatocytes present in the haemolymph of *Oxya hyla hyla*. **5.** Detailed view of the different types of plasmatocytes. The pseudopodial outgrowths are clearly visible. **6.** Coagulocyte of *Oxya hyla hyla*.

3.5 *Sphaerulocyte*

The sphaerulocytes are slightly larger than the granulocytes and engorged with spherical granules which makes the nucleus almost obscure. The granules are sometimes released out of the cell.

3.6 *Coagulocyte*

These cells are large in size (16·7–18 μ), round or oval. The plasma membrane is smooth. The deeply stained nucleus is large (9–11 μ) with sharp outlines and some rod like structures are noted. No granular structures are found in the cytoplasm (figure 6).

4. Discussion

The present study shows that the plasma protein concentration is very high during the monsoon months but its maximum value does not exceed 10 g/100 ml of haemolymph. This high protein concentration could be related with the reproductive maturity of the insects, as this also supports the fact that the female grasshoppers studied were in pre-vitellogenic or just vitellogenic stages of reproductive cycle. It is also clear that the degree of separation of the protein components varies with the acrylamide gel concentration and only 7·5% gel concentration separates these 10 fractions distinctly.

Using different gel concentrations (3·5, 4·5 and 7%), Patel (1971) showed that a particular protein fraction which appears as a single band in 7% gel concentration could be separated into 3 sub-fractions when electrophoresed in 3·5%, 4·5% gel concentrations.

Regarding the presence or absence of the different bands it is apparent that the yellowish band having R_m value 96 ± 2 is a constant feature in all situations. Such a band has also been reported in *Locusta migratoria* and *Schistocerca gregaria* (Kulkarni and Mehrotra 1970; Emmerich and Hartmann 1973). Again, the protein band with R_m value 100 is also a constant feature, such a situation has also been reported in *Hyalophora cecropia, Schistocerca gregaria* and *Locusta migratoria* (Thomas and Gilbert 1968; Kulkarni and Mehrotra 1970; Emmerich and Hartmann 1973). From the present findings, it may be suggested that in *Oxya hyla hyla*, like *Locusta migratoria* the lipoprotein fraction serves as a carrier of juvenile hormone. The most plausible hypothesis explaining the findings is that these protein fractions in females is related with the reproductive maturity.

In the present study of female *Oxya hyla hyla* the total THC is $80200 \pm 105{\cdot}8$. The insects were all pre-vitellogenic or early vitellogenic in nature. It could be assumed that this rise in THC may be related with the process of vitellogenesis and most probably the haemocytes are engaged in some synthesis and transport role of protein or other yolk materials. The haemocytes are highly pleomorphic and the particular form they present at any one time depends on the age, developmental stages as well as on the methods of collection and examination. The hanging drop preparation reveals many details regarding the haemocyte structure and alterations. The cells undergo regular changes of form pseudopodial outgrowths. EDTA has already been recommended as an anticoagulant (Shapiro 1979). Regarding the use of EDTA, the present study demonstrated that the stainability is altered to some extent, causing blackening of both the cytoplasmic and nuclear materials.

While considering the presence of different cell types, it is noted that the plasmatocytes are the most abundant variety among the haemocytes showing wide range of structural variability. We have noted only 5 types of haemocytes—prohaemocytes, plasmatocytes, granulocytes, sphaerulocytes, and coagulocytes. This finding differs from the observations of Hoffman *et al* (1969), on *Locusta migratoria*.

At present altogether seven haemocyte types have been recognized and described in different insect orders. The previously named haemocytes are amoebocytes and/or plasmatocytes, sphaerulocytes, granulocytes etc. The morphologically variable cell types have been correlated with their phylogeny and functional variation. The presence of dividing haemocytes in circulation is similar to earlier observations and suggestions (Jones 1956; Shapiro 1979). The low frequency may be due to the fact that only metaphase plates were counted. Finally the presence of all sorts of structures, intermediate cells between prohaemocytes and plasmatocytes supports the main haemocyte differentiation pathway suggested by Arnold (1974) and Gupta (1979).

Acknowledgements

The authors are grateful to the late Prof. D N Ray-Choudhuri and Prof. Y D Pande for facilities and encouragement.

References

Arnold J W 1974 The haemocytes of insects; in M Rockstein (ed) *The Physiology of Insecta.* Vol. 5. 2nd ed. (New York: Academic Press)

Chen T T Couble P DeLucca F L and Wyatt G R 1976 Juvenile hormone control of vitellogenin synthesis in *Licusta migratoria*; In Gilbert L I (ed) *The Juvenile Hormones* (New York: Plenum publishing corporation)

Davis B J 1964 Disc Electrophoresis—11. Method and application to human serum proteins; *Ann. N.Y. Acad. Sci.* **121** 404–427

Emmerich H and Hartmann R 1973 A carrier lipoprotein for juvenile hormone in the haemolymph of *Locusta migratoria*; *J. Insect. Physiol.* **19** 1663–1675

Gupta A P 1979 Arthropod haemocytes and phylogeny in *Arthropod phylogeny* (ed.) A P Gupta (Reihold, New York: Van Nostrand) pp. 669–735

Hoffmann J A Stoekel M E and Joly P 1969 Ultrastructure des hemocytes de *Locusta migratoria* (orthoptere); *C.R. Acad. Sci. Paris* **266** 503–505

Jones J C 1964 The circulatory system of insects in *The physiology of insecta* (ed.) M Rockstein (New York: Academic Press) Vol. 3, 1st edn, pp. 1–107

Kulkarni A P and Mehrotra K N 1970 Amino acid, nitrogen and protein in the haemolymph of adult desert locusts, *Schistocerca gregaria*; *J. Insect. Physiol.* **16** 2181–2199

Patel N G 1971 Protein synthesis during insect development; *J. Insect. Biochem.* 1 391–427

Richards O W and Davies R G 1977 Imms A General Textbook of Entomology. ELBS

Shapiro M 1979 *Techniques for total and differential haemocyte counts and blood volume, and mitotic index determinations on insect haemocytes* (Cambridge: Univeristy Press)

Thomas K K and Gilbert L I 1968 Isolation and characterization of the heamolymph lipoproteins of the American silkmoth, *Hyalophora cecropia. Arehs Biochem. Biophys.* **127** 512–521

Wigglesworth V B 1959 Insect blood cells; *Annu. Rev. Entomol.* **4** 1–16

Wigglesworth V B 1973 Haemocytes and basement membrane formation in Rhodnius; *J. Insect. Physiol.* **19** 831–844

Wyatt G R and Pan M L 1978 Insect plasma proteins; *Annu. Rev. Biochem.* **47** 779–817