Review of literature:

Previous literature, with respect to the biochemicals in haemolymph, fat bodies and gonads of the lepidopteron insects, is reviewed in this chapter.

Haemolymph Biochemical's:

Haemolymph is the only extra cellular fluid in insects and it exists in an unbound, non-vascular state, in direct contact with tissue and organs. Wyatt, G.R. (1961) has done tremendous work on Biochemistry of Insect Haemolymph. He studied the general and physical properties of insect haemolymph as well as the inorganic and organic components of it.

The haemolymph proteins of insects have been investigated from various points (Chen and Levenbook, 1966 a and b). These include - 1) the mapping of protein parameters in various species for taxonomic purposes, 2) the identification of protein composition at successive developmental stages by both electrophoretic and immunological techniques, 3) proposal of possible functions of the protein components on the basis of enzymological and histological tests, and 4) the analysis of both site and mechanism of synthetic process by isotopic labeling.

Physical properties and chemical composition of insect blood is extensively reviewed by Buck, J.B. (1953); Wigglesworth (1965) and Florkin and
Jeuniaux (1974). According to these authors water is the major component of haemolymph and constitute about 84-92% of the total plasma.

**Proteins:**

The protein concentration in insect haemolymph is similar to that of the blood of man and other vertebrates, and generally higher than that of the internal fluids of other invertebrates. The average protein content is of 5gm/100ml in Hymenoptera and 3-4gm/100ml in Coleoptera, 2gm/100ml in Lepidoptera and 1gm/100ml in Orthoptera (Florkin, 1936a). Lue and Dixon, in 1967 studied that the number of different proteins is highly variable according to species and caste. Stephen and Steinhauer, (1957) and Kulkarni and Mehrotra,(1970) had presented the similar results with respect to sex of a species. Bodnaryk and Morison, in 1966, with respect to diet, Feir and Drazywda,(1969) with respect to starvation and Florkin and Jeuniaux, (1974) with respect to ontogenic stage.

Proteins (enzymes) play a central role in all metabolic process and in the structure and function of muscles and other tissues. Insect blood protein synthesis has been studied in several species during development and diapause (Telfer, 1954; Laufer, 1960; Wyatt, 1961 and Chen and Levenbook, 1966 a and b). Several possible roles of blood proteins have been postulated e.g. they may function as enzymes (Laufer, 1960); serve as amino acid reserves for adult tissues (Dinamarca and Levenbook, 1966) or
be used intact in constructing adult structures (Loughten and West, 1965 and Fox and Mils, 1969).

The synthesis of pupal blood proteins during diapause has been studied in *Cecropia* silkmoth (Patel, 1971) and blood protein synthesis in *Hyalophora cecropia* silkmoth (Ruh and Willis, 1974). Diapause associated proteins have been characterized by Turunen and Chippendale (1980). El-Ibarashy, (1965) has shown that during the pre-diapause, the total body contents of lipids, proteins, and carbohydrates increases. De Loof and De Wilde, (1970), have shown that diapausing proteins accumulate in haemolymph. Diapause is also characterized by the cessation of the haemolymph protein synthesis and Juvenile hormone was further proved by Dortland and De Kortein 1978. Changes in the concentration of metabolites in haemolymph during and after diapause in female Colorado potato beetles, *Leptinotarsa decemlineata* has been reported by Lefevere et al.

Many references are available on protein titer of haemolymph. By using electrophoretic methods, identification of haemolymph proteins was done in many insects like *ostrinia sp.* (Beck and Hanec, 1960; Chippendale and Beck, 1966), *Hylophora cecropia* (Laufer, 1960, 1961) and *Periplanata americana* (Siaktos, 1960). The blood proteins of pupae of *Hyalophora cecropia* have also been studied by acrylamide gel electrophoresis (Ruh et al., 1972). The most striking features are haemolymph storage proteins in
holometabolous insects (Thomson, 1975; Wyatt and Pan, 1978). These proteins comprise the bulk of polypeptides in larval haemolymph and later in pupal fat body. Developmental stage-specific alterations in tissue localization have been reported for both Dipterans (Kinnear and Thomson, 1975; Thomson, 1975) and Lepidopterans (Chippendale and Kilby, 1969; Chippendale, 1970 a and b; Tojo et al., 1980; Krammer et al., 1978 a and b; Miller and Silhack, 1982). Haemolymph electrophoretic pattern of Ascia monuste orseis larvae (Lepidoptera: Pieridae) parasitized by Cotesia glomerata (Hymenoptera: Braconidae) was studied by M. Scaglia; M. R. Brochetto-Braga; J. Chaud-Netto; N. Gobbi. (2003)

In many holometabolous insects proteins accumulate during the last larval instar in preparation of non-feeding pupal stage (Telfer and Kunkel, 1991). These proteins are known as storage proteins, later these proteins are used for formation of adult structures (Koopmanscap et al., 1992). These haemolymph proteins are also reported in larval Manduca sexta (Beck et al., 1996); in last instar larvae of Hyphantria cunea (Jung Hee et al., 1996) antibacterial proteins in Hyalophora cecropia (Lockey and Ourth, 1996). Changes in haemolymph proteins during the metamorphosis of both sexes and castes of polygynous Formica rufa insecta: Hymenoptera has been well studied by Schmidt, G.H. and Schwankl. (1975)
Wyatt, G. and Pan, M.L. (1978) has provided an excellent comprehensive review on plasma proteins. They have done a comparative study of plasma proteins, Storage proteins, Vitellogenins, hemoglobin’s, Lipoproteins, Plasma proteins in defense and other plasma proteins, in various insects.

Haemolymph proteins have been reported in relation with vitellogenins and egg production in *Heliothis virescens* (Zeng et al, 1997), in greater wax moth, *Galleria mellonella* (Vileinakas et al., 1997) and in larva of *Argyrogramma agenta* (Shu et al., 1997). Chen, P, (1966); Price, G, (1973); Wyatt, G. and Pan, M. (1978); Keeley, L. (1078a) have provided various aspects of different proteins produced by fat body and their relationship to fat body.

Male specific proteins have been reported in *Galleria mellonella* (Lee et al., 1998) and lipoproteins have been identified during larval developmental of *Samia cynthia ricini* (Eri Silkworm) (Saito.1998). Changes in haemolymph proteins have been observed during moulting process by Steinhauer, And Stephen, W., in (1959), Fox, F.and Mills,R.,in(1969);Duhamel, R and Kunkel ,J., (1978). P. P. Srivastava , K. Thangavelu (1996) have identified the high molecular weight (680 KDa) glycolipoprotein from the haemolymph of male larvae of *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) as lipophorin by gradient KBr
Purification and characterization of an insect haemolymph protein promoting in vitro replication of the *Bombyx mori* nucleopolyhedrovirus was studied by Toshimichi Kanaya1 and Jun Kobayashi2 (2000). They have identified a novel protein that promotes *Bombyx mori* nucleopolyhedrovirus (BmNPV) replication *in vitro*. This protein was purified from heat-treated haemolymph of *B. mori* larvae by gel filtration and ion exchange chromatography, and designated as promoting protein (PP). The molecular mass of native PP estimated by column chromatography and that of denatured PP estimated by SDS–PAGE were 9600 Da and 15200 Da, respectively, suggesting that native PP is composed of a single polypeptide.

Norman *et al.* (1967), have reported, among the fourteen different protein fractions of the haemolymph of *Anthonomus grandis*, there is one fraction unique to the larval stage and another unique to the pupal stage. The last larval ecdysis, pupation and adult emergence of the large white butterfly, *Pieris brassicae*, were associated with notable changes in the pattern of haemolymph proteins and lipoproteins (ToltUrt E N, 1978). Slowly migrating lipoproteins were prominent in newly oviposited eggs and in fifth instar larvae, but were almost absent from molting fourth to fifth instar larvae and 2 day old pupae.
The protein analyses of whole blood of the lawn armyworm, *Spodoptera mauriti, S. acronyctoides* have revealed an increase in the quantity of haemolymph proteins and the appearance of some new proteins as the larva aged (Taxrl and Tamashir O, 1975). Chen and Levenbook (1966) also found changes in density of blood protein fractions in the blowfly, *Phormia regina*, as did Parker (1971) in the Colorado potato beetle, *Leptinotarsa decemlineata*. By means of immunoelectrophoretic methods L Ensky (1971) was able to identify in the honey bee, *Apis mellifera*, three main protein patterns, one present throughout the whole development, and one specific to the larval stage and one specific to the adult stage. Also, B Ounais (1975) has shown some modifications of the protein pattern which appear during the development.

In the present study attempts have been made to identify haemolymph proteins in different life stages of certain Rhophaloceran Lepidopterans. Using gel electrophoresis we report the detection and partial characterization of undegraded soluble blood protein fractions in the three developmental stages of Rhophaloceran Lepidopterans.

**Carbohydrates:**

It has been known for a long time that insect haemolymph generally contains only small amount of fermentable sugars, almost no saccharose, and little, if any, glycogen (Florkin and Jeuniaux, 1974).
Wyatt and Kalf (1956, 1957) reported existence of trehalose (α-glucose) in insects. The presence of trehalose in different insects’ species diverse orders has been compiled after Florkin and Jeuniaux, 1974). In most insects, the trehalose of the haemolymph is absorbed and used by the cells of most tissues due to an intracellular trehalose (Howden and Kilby, 1960, Clegg and Evans, 1961). In the fat body, an inverse relation exists in between glycogen and trehalose, the former disappearing at each moult, while trehalose remains at constant level (Saito, 1963).

Haemolymph glucose is known to be incorporated into the chitin of growing chitin of several insects’ orders (Bade and Wyatt, 1962).

High haemolymph sugar levels are reported in a number of species of Hymenoptera and Diptera (Florkin and Jeuniaux, 1974). In insects’ diapausing embryonic stages, sugars play a major role in diapause metabolism and there is a direct relationship between the cold hardiness and levels of polyoils (Salt, 1957 and 1959; Somme, 1967; Baust and Miller, 1970) Carbohydrates (Tanno, 1964; Somme, 1967) and unsaturated fatty acids have been reported for several hibernating insects.

Many diapausing species utilize stores of glycogen to generate cryoprotectants such as glycerol, sorbitol, or trehalose (Chino, 1957, 1958; Wyatt, 1967).
Changes in carbohydrates related to cryoprotection in *Pieris brassica* (Pullin, 1992); during pupal moult of *Manduca sexta* (Siegert, 1995); on metal effects on carbohydrate pH of *Lymantria dispar* (Ortel, 1995) are reported.

The haemolymph and total body composition viz. carbohydrates, proteins, lipids and free amino acids have been reported in Gypsy moth larvae *Lymantria dispar* (Bischof and Ortel, 1996).

P. Rajani Kanth, M. Sriramulu and Ch. Sreenivasa Rao (2005) conducted a laboratory experiment to study the effect of juvenile hormone III on total haemolymph protein and carbohydrate content of fifth instar *Bombyx mori* (L). The haemolymph carbohydrates content of 1 and 10µg treated larvae were found to be significantly lower compared to their control and the carbohydrate content due to JH- III treatment decreased with increase in concentration.

Annie John and D. Muraleedharan studied Hormonal modulation of glycogen reserves in fat body of castor semilooper *Achaea janata* Linn (Lepidoptera, Noctuidea). They elucidated the histochemical details of the fat body in the fifth instar larval stage, pupa and adult stage of this moth using light and electron microscopy in conjunction with glycogen storage patterns using polyacrylamide gel electrophoresy.
Lipids:

Lipids are transported by the haemolymph from the midgut to the fat body, where they are stored in more or less modified form and fat bodies to the tissues / organs. Role of lipids in post embryonic diapause have been described (Lees, 1955).

Wide variations of the lipids concentration are observed during muscular activity (flight), development or metamorphosis (Nowosielky and Patton, 1965; Nelson et al., 1967; Mayer and Candy, 1969).

Proportional quantification of phospholipids, total lipids, sterols, un-sterified fatty acids and natural glycerides have been reported in *Galleria mellonella* (Wlodawer and Wisniewska, 1965) and *Acheta domesticus* (Wang and Patton, 1969).

Metabolic relation between lipid transportation by haemolymph and place of storage has been reported in Wax moth (Wlodawer and Wisniewska, 1965), and in *Hylophora cecropia* (Martin, 1969). Whole body lipid content of *Platypena scabra* have been reported on the basis of daily and seasonal changes by Mason *et al*, 1990.

Comparison of fatty acid composition in lipids of diapause and non- diapause eggs of *Bombyx mori* has been observed by Shimizu, 1992.
Reduction in lipids contents in *Lymantria dispar* has been observed due to metal intoxification by Ortel, 1995.

Effect of parasitism on lipid content in *Lymantria dispar* has also been reported by Bischof and Ortel in 1996.

Using an analysis of fatty acid composition of seven insect's orders, Thompson .M.J. and Saoboda J.A (1975) Svoboda, J.A., J.N. Kaplanis, W. E. Robbins, have identified possible phylogenetic trends. These trends have provided insights into potential relationships between insect orders. In the order Lepidoptera, they found a high content of linolenic acid in most of species that they analyzed.

Yingming Wang, William Connor and Dos S Lin. (2006) reported a high content of polyunsaturated fatty acids in both larva and adult butterfly species. They studied the effects of diet and metamorphosis upon the sterol composition of the butterfly *Morpho peleides*. They also studied the predominance of poly unsaturated fatty acids in the same species before and after metamorphosis.

**Uric acid:**

Haemolymph generally contains excretory end products in the form of uric acid (Florkin and Jeuniaux, 1974). Its concentration depends upon feeding
(Ramkrishna and Pawar, 1975), photoperiodicity (Hillard and Butz, 1969), Parasitisation (Condon and Gordon, 1977) and development (Buckner and Caldwell, 1980).

Uric acid is generally regarded as a major nitrogenous end-product, which is produced in the fat body and is eliminated after transported through haemolymph (Mullin, 1985).

Uric acid in insects is also found as uric acid ribosides in G. mellonella (Krazyzanowska and Niemierko, 1979, 1980), which facilitates transport of citric acid.

**Haemocytes:**
The blood or haemolymph of insects is present in the general body cavity and consists of liquid plasma and numerous blood cells or haemocytes (Jones, 1962; Richards and Davies, 1977; Gupta, 1979a and b). The haemocytes originate from the mesodermal bands in the embryo (Jones, 1970).

In insects several types of haemocytes are observed in haemolymph (Arnold, 1979a and, Jones 1979). There is a disagreement among haemologists about the type of haemocytes in insects, i.e. from one or a few to as many as nine or more types (Ravindranath, 1978 and Gupta, 1979a and b). According to Millara (1947), Cuenot (1896) was the first to classify the
haemocytes into four categories, which was further followed by Hollande (1909 and 1911). Wigglesworth (1939) after summarizing most of the earlier classifications presented a classification that was widely accepted most useful system of classifying insects haemocytes in the one developed by Jones (1962, 1964 and 1970), though there is much confusion regarding haemocytes classification (Wigglesworth, 1959, Jones in 1962 and 1964, Gupta, 1969, Arnold, 1972). Our present knowledge of insect haemocytes is limited to studies of not more than 200 insect species in about 100 genera (Arnold, 1979). Haemocytes have been studied mostly in Lepidoptera, Hymenoptera, Coleoptera, and Diptera (Gupta, 1985). The free hemocytes of lepidopterous insects have been studied in a wide range of species (Yeager, 1945; Munson, 1953; Shapiro, 1979; Boiteau & Perron, 1977; Akai and Sato, 1978, 1979; Arnold, 1982; Ashhurst, 1982; Beeman et al., 1983; Essawy et al., 1985; Saxena et al., 1988; Butt & Humber, 1989; Andrade et al., 2003).

There is an inherent variability of haemocytes within a species as well as among closely related species (Arnold 1974; Gupta 1979). Haemocyte picture of various types have been investigated in the German cockroach *Blatella germanica* both in the nymphs and adults (Hazarika and Gupta 1987; Chiang et al 1988). Little work has been carried out in Rhopaloceran Lepidopterans notably the butterflies. Information on Haemocyte population within an insect is essential for many types of
physiological studies. The haemogram is a statement of the haemocytes population picture in an insect at a given time. Total haemocytes counts (THC) in a standard quantity of blood (usually mm3) or in the specific area (Arvy et al., 1948, Lee, 1961) together with an estimate of the relative number of haemocytes in different categories (Differential haemocyte count or DHC) in a random sample (Jones, 1962 and Arnold, 1972) give haemogram picture of the blood.

Haemogram also includes blood volume which has bearings of THC and DHC and meaningful way of expressing them is in absolute number (Wheeler, 1963; Jones, 1967a). This represents the total population as divided from total and differential count in relation to blood volume. Tauber and Yeager (1934) were the first to study the Differential haemocyte count in several insects and later (1935) in several orders. There are large differences in number of haemocytes in different insects’ species. (Jhoes. 1962, Arnold. 1972). The volume of haemoglobin varies widely according to age and development at different stages. It has been reported that most insects seem to have average of 20,000 to 30,000 cells mm3 but with a wide deviation (Laigo and Paschke, 1966; Gupta and Sutherland, 1968, Shapiro, 1968, Hoffmann, 1969).
Haemopoietic organs and effect of their ablation on total haemocyte count in lemon-butterfly, Papilio demoleus L. has been studied by Tiwari RK, Pandey JP and Salehi R (2002).

Effect of certain stresses and 20-hydroxyecdysone injection on total haemocyte count in lemon butterfly, Papilio demoleus L. (Lepidoptera), was studied by Tiwari and Shukla (2000).

Effect of Ganglionectomy of the larval body form and metamorphosis of lemon butterfly, Papilio demoleus L. (Lepidoptera), was studied by Shukla, Tiwari and Agrawal (1993).

Pandey, et al., have studied the haemocytes of Papilio demoleus, under certain stress conditions. They have also studied the effect of repeated haemolymph withdrawals on haemocyte counts and moulting in, Papilio demoleus L. Similar studies have been done by Pandey, J.P. and R.K. Tiwari, in plain tiger-butterfly, Danais chrysippus (2008).

Pandey, Upadhyay and Tiwari, have also studied the effect of some plant extracts on haemocyte count and moulting of Danais chrysippus larvae.

Reduction in haemocyte mediated immune response in Danais chrysippus following treatment with neem-based insecticides was reported by Pandey, Tiwari and Kumar, (2008). They have also shown that, Temperature and

A study of the hemoglobin of the silkworm *Hylophora cecropia* has been done (Lee, 1964).

Total haemocyte count in the *Halys dentate* has been observed (Bhadun and Pathak, 1971). Modification of haemogram in *Locusta migratonia* after bacterial infection has been reported (Hoffmann et al., 1977).

Arnold (1979a) has given a comparative study of the haemocytes of 16 species of cockroaches in relation with the size of haemocytes and their number. Total and differential haemocyte counts in the larvae of noctuid *Euxoa declarata* have been reported by Arnold and Hinks (1976).

The total and differential haemocyte count and their morphometry were also studied in *Catamiarus brevipennis* (Ambrose and George, 1994).

Effect of juvenile hormone on haemocytes counts have been reported in *Battella germanica* (Hazarika and Gupta, 1997).

Total and differential haemocyte counts (THC and DHC) were made during various larval instars of the saturnid moth *Antheraea assama* and gradual increase in THC was recorded with the increased number of instars, (Bardoloi and Hazarika, 1995).
Haemocyte count in the larvae of *Pericallia ricini* have been reported, relating it with nutrient quality of two castor varieties (Jey Kumar *et al.*, 1995).

An increasing haemogram pattern from early instar to adult has been reported in *Rhynocoris marginatus*.

Baruco *et al.*, (1988) did not observe large variation in Total haemocyte count of different stages of Larva of *Diatraea saccharalis*.

There was no difference in differential haemocyte count in between parasitized and non-parasitized *Pseudoplusia includens* (Strand and Noda, 1991).

Changes in number of few haemocytes during metamorphosis of *Lymantria disper* have been reported (Kim *et al.*, 1990 a).

Effect of endocrine extracts on the blood volume and population of haemocytes in *Halya dentate* had no effect on blood volume, but it changed population of haemocytes (Pathak, 1991). A decrease in number of haemocytes of the noctuid *Mythimna separata* larva treated with *Bacillus thuringiensis* was observed (Sha and Xie, 1992). Instar dependant changes in THC and DHC in *Pieris brassica* have been reported after Parasitisation (Bauer *et al.*, 1998).

Studies on the total haemocyte count and haemolymph volume *Periplanata americana* in relation to moultng have been done (Wheeler,
1961, 1963). He reported that the absolute number of circulating haemocytes in the entire *Periplanata americana* does not increase prior to ecdysis even though the number of haemocytes per cubic millimeter increases. Further, he also reported that, the absolute number of circulating haemocytes significantly decreases 24 hr after ecdysis, but there is no change in the number of haemocytes per cubic millimeter at this time because the haemolymph volume has returned to normal.

Multiplication and increase in haemogram with instar has been reported in *Hyalophora cecropia* (Lee, 1964; Lea and Gilbert, 1966) and also *Galleria mellonella* at various stages of development (Shapiro, 1968).

The role of the Haemocytes in the growth and molting of an insect is studied by Wigglesworth, V.B. (1955)

The haemocyte types, differential and total count in *Papilio demoleus L.* (Lepidoptera: Papilionidae) during post-embryonic development was studied by Jalal Jalali and Rasol Salehi. They recognized six types of haemocytes in *P. demoleus* on the basis of morphological and cytological features, revealed by light (LM), phase (PCM) and scanning electron microscopy (SEM). They also reported that the total haemocyte count steadily decreased during the developmental stages, attained its peak in the late 5th instar and steeply declined again in the pupa.
Thus, it is seen that there are different types of haemocytes in various insect species, which may or may not be specific to an insect order and changes in blood volume, haemogram, THC and DHC often occur with the different developmental stages of insects’ sexes and diapausing and non-diapausing state. This may be true in case of butterflies, in its larval instars, pupae of different age groups and in adults of both the sexes. Therefore this study has been taken up to collect first hand information on this physiological aspect of haemolymph of different species of rhophaloceran lepidopterans i.e. butterflies. The present study is primarily concerned with the haemocyte profile particularly total haemocyte count of the four species of butterflies belonging to families Nymphalidae, Papilionidae and with emphasis on the changes in the total population.

Review of Literature reveals that a tremendous research has been carried out on haemolymph of various insects and on different aspects of Rophaloceran- Lepidopterans yet detailed studies in biochemical, molecular, immunological and genetical field need to be done. Hence the present work has been undertaken with respect to the biochemical studies of these lepidopterans.